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# Isolation and Characterization of a 30-kDa Endometrial Glycoprotein Synthesized during the Estrous Cycle and Early Pregnancy of the Pig[1]

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## ABSTRACT

Endometrial polypeptide synthesis, which is regulated through ovarian steroid secretion and steroid production by the developing conceptus, not only provides the necessary secretory components vital to conceptus development but also presents the adhesive changes in the epithelial surface essential for conceptus attachment. In the present study, a 30-kDa, basic endometrial glycoprotein (pGP30) was isolated and characterized during the estrous cycle and early pregnancy of the pig. Uterine flushings and endometrial culture media were obtained from gilts on Days 0, 5, 10, 12, 15, and 18 of the estrous cycle and Days 10, 12, 15, and 18 of pregnancy. A polyclonal antibody was generated to pGP30 after isolation of medium from Day 15 pregnant endometrial cultures separated by gel filtration and PAGE. Western blot analysis indicated that the antiserum reacted with isoforms of pGP30 and cross-reacted with a 90-kDa component in serum that was not removed after cleavage of the oligosaccharide chains from the 90-kDa glycoprotein. Antiserum did not detect a 30-kDa band in media from cultures of kidney, fat, heart, muscle, liver, or serum; however, heart and muscle did contain bands of different molecular masses that cross-reacted with the antiserum. Multiple bands of higher molecular mass (35–40 kDa) were detected in the endometrial cultures from gilts on Days 0 through 10 of the estrous cycle. Treatment of ovariectomized gilts with estradiol-17β stimulated a similar response. During the mid- to late luteal phase of the estrous cycle (Days 12–18), the 30-kDa band as well as an additional 32-kDa band was present on Western blots. Administration of progesterone for 14 days stimulated the synthesis of both the 30- and 32-kDa products in ovariectomized gilts. However, only the pGP30 was detected on Days 12–18 of pregnancy. Immunocytochemical localization with antiserum to pGP30 indicated that the glycoprotein is present in the endometrial epithelium, with the surface epithelium demonstrating the strongest reaction product. Discrete changes in staining and cellular localization were observed during the early stages of the estrous cycle (Days 0–5) and the midluteal (Day 10) phase. A similar response was achieved with administration of steroids to ovariectomized gilts. Data indicate that discrete changes in epithelial synthesis of the endometrial glycoprotein occur at the time of conceptus trophoblastic elongation and placental attachment in the pig.

## INTRODUCTION

Conceptus attachment to the endometrium in the mouse occurs via cell surface glycoproteins that compose the glycocalyx present on both the trophoblast and uterine apical border of the epithelial surface [1, 2]. Several studies [3, 4] have indicated that significant alterations occur in the composition of endometrial cell surface carbohydrates/glycocalyx that serves to anchor the conceptus prior to implantation in many rodent species. Specific changes in the binding of carbohydrate-selective lectins to endometrial glycoproteins during early pregnancy have been reported in the pig [5, 6]. In the pig, previous work has demonstrated that conceptus attachment requires binding between cell surface glycocalyx present on the trophoblast and the maternal epithelium [7]. Thus, adhesion between the glycocalyx components of the conceptus and epithelium is the first physical step in conceptus attachment and placentation. Interactions between glycocalyx of the conceptus trophoblast and apical surface of the uterine epithelium are essential for the diffuse type of placentation and nutrient exchange that occurs between the maternal endometrium and fetal placenta of the pig.

Cell adhesion between the maternal endometrium and conceptus is influenced by endocrine changes in steroids during early pregnancy [8, 9]. Conceptus secretion of estrogen in the gilt is involved not only with maintaining functional CL throughout pregnancy [10] but also with regulating changes in the uterine epithelial glycocalyx that has been shown to be essential for conceptus attachment [7, 11, 12]. The porcine conceptus punctually secretes estrogen on Days 11 and 12 of pregnancy [10]. Administration of estrogen to cyclic gilts during this period induces changes in the uterine epithelial glycocalyx that are indistinguishable from those on Days 11 to 12 of pregnancy [11–13]. However, estrogen administration on Days 9 and 10 of pregnancy reduces the number of conceptuses undergoing attachment and increases conceptus mortality [12, 14, 15]. The adverse effects of precocious estrogen stimulation coexist with sloughing of the uterine epithelial glycocalyx [12]. The sloughing of the glycocalyx and resultant failure of conceptus attachment may represent a mechanism of estrogen-induced embryonic mortality in the pig.

Porcine endometrial explants synthesize and secrete a group of 30-kDa, basic (pI 7.9–8.4) glycoproteins termed

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pGP30 [12, 15]. In vivo estrogen treatment on Days 9 and 10 was found not only to increase conceptus mortality but also to reduce endometrial production of the 30-kDa, basic glycoproteins [15]. We have hypothesized that pGP30 may be an important uterine component of conceptus development and as such may be subject to regulation by sex steroids and conceptus secretory products. The objectives of this work, therefore, were 1) to determine the time-dependent expression of pGP30 by the endometrium before, during, and after the critical period of conceptus attachment; 2) to determine the cell-specific localization of pGP30; and 3) to determine conceptus-specific and steroid-dependent effects on uterine expression of pGP30 in the endometrium of the porcine uterus.

## MATERIALS AND METHODS

### *Uterine Expression of pGP30 in Cyclic and Pregnant Gilts*

Mature, cyclic, crossbred gilts were observed for estrous behavior daily in the presence of intact boars. After displaying two estrous cycles of normal duration (17–22 days), gilts were assigned to either nonpregnant (cyclic) or pregnant treatment groups. Gilts assigned to the pregnant group were mated to fertile boars at the onset of estrus (Day 0) and 12 and 24 h later. Endometrium was obtained from gilts (n = 3/day) on Days 0, 5, 10, 12, 15, and 18 of the estrous cycle and Days 10, 12, 15, and 18 of pregnancy. Gilts were hysterectomized as previously described [15]. A surgical plane of anesthesia was induced with a 5% solution of thiopentone sodium (Abbott Laboratories, North Chicago, IL) administered i.v. Anesthesia was maintained on a closed-circuit system of halothane (2–5% Fluothane; Aveco Company, Inc., Fort Dodge, IA) and oxygen (2.0 L/min). After exposure following midventral laparotomy, the uterine horns and ovaries were surgically removed. One excised uterine horn was flushed with 20 ml of sterile saline (0.9%), and the flushing was examined for the presence of conceptuses to confirm pregnancy in mated gilts. The second uterine horn was transported on ice to a sterile horizontal flow hood. The surgical incision was closed in a routine fashion, and gilts were treated i.m. with procaine penicillin G (20 000 IU/kg BW).

Immediately upon removal of the uterine horns, a 7–10-cm section anterior to the uterine body was excised and opened along its antimesometrial border. Small segments (2–3 cm) of endometrium were removed from the underlying myometrium with scissors and immersed in Bouin's for 3–4 h at room temperature.

Uterine flushings were centrifuged at 12 000 × *g* for 15 min at 4°C. The supernatant was decanted and stored at −20°C until utilized for Western blot analysis. Endometrial tissue from the nonflushed side was dissected from the myometrium, cut into 3–4-mm explants, and placed into Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY). Antibiotic and antimycotic solution (Gibco) containing penicillin (100 000 U/ml) and streptomycin (10 mg/ml) was added to media prior to culture. To remove blood from tissue, the endometrial explants (500 mg) were cultured in 15 ml of MEM for 3 h at 37°C in an atmosphere of 5% $CO_2$:45% $N_2$:50% $O_2$. After the initial 3-h preincubation period, the medium was removed and replaced with fresh MEM, and incubation was performed for an additional 24 h. Upon completion of the 24-h incubation, the culture medium was separated from tissue and centrifuged at 12 000 × *g* for 15 min. The supernatant was decanted and stored at −20°C until utilized for Western blot analysis. Protein content of the uterine flushings and the endometrial culture medium was determined by the method of Lowry [16].

Samples of porcine liver, lung, muscle, heart, kidney, and fat were obtained after slaughter of nonpregnant gilts at a local abattoir. Samples were placed in MEM and transported (15 min) on ice to the laboratory for explant culture as previously described for the endometrium. Tissue sections (2–3 cm) were fixed in Bouin's as previously described.

### *Steroid Regulation of pGP30*

Twelve mature, cyclic gilts were ovariectomized through midventral laparotomy and allowed a 14-day recovery period before administration of steroid treatments was begun. Ovariectomized gilts were randomly assigned (n = 3 per treatment) to one of the following treatment groups: 1) VEH—i.m. injection (2 ml) of corn oil from Days 0 to 13; 2) EB—i.m. injection (400 μg) of estradiol benzoate once daily from Days 0 to 3; 3) PR—i.m. injection (100 mg) of progesterone once daily from Days 0 to 13; or 4) PRE—i.m. injection (400 μg) of estradiol benzoate from Days 0 to 3 and progesterone (100 mg) from Days 4 to 13. Blood samples were collected into heparinized tubes via venipuncture on Days 0, 3, 7, 10, and 13. Samples were placed on ice and centrifuged at 2600 × *g* for 15 min. Plasma was collected and stored at −20°C until analyzed for progesterone and estradiol-17β by RIA. Gilts were hysterectomized on Day 14 with the exception of EB gilts, on which surgery was performed on Day 4. The uterine horns were removed, processed, and analyzed as described above.

### *Steroid RIAs*

RIA reagents were obtained from Diagnostic Products Incorporated (Los Angles, CA). Progesterone in porcine plasma was quantified without solvent extraction by solid-phase RIA using progesterone standards in human plasma. [$^{125}$I]-progesterone in buffer (1.0 ml) was incubated with 0.1 ml of standards, ovine plasma pools, or porcine plasma samples in antibody-coated polypropylene tubes for 3 h at room temperature. Tubes were decanted and the antibody-

bound counts quantified in a gamma counter (MicroMedic, Horsham, PA). RIA was validated for quantification of progesterone in porcine samples as follows. Progesterone amounts of 0.5, 1, 5, 10, 25, and 50 ng were added (0.05 ml) to aliquots (0.95 ml) of porcine plasma (n = 4) to assess accuracy. Recovery of the added progesterone was linear and averaged 100%. Serial dilutions of porcine plasma (n = 2) inhibited binding of [$^{125}$I]-progesterone to antibody in a manner parallel to that for progesterone standards in progesterone-free human plasma. The sensitivity of the assay was 0.05 ng/ml. The intraassay and interassay coefficients of variation were 6% and 10%, respectively.

Estradiol-17β was quantified without solvent extraction by double-antibody RIA using standards prepared in charcoal-absorbed porcine plasma. Two hundred microliters of standards, plasma pools, or porcine plasma samples was added to polypropylene tubes and incubated (4°C) with 0.1 ml antiserum for 12–16 h before 0.75 ml [$^{125}$I]-estradiol-17β was added. After further incubation (4°C) for 2 h, 1.0 ml cold (4°C) precipitating solution was added, and the incubation was continued for 30 min at 4°C. Tubes were centrifuged (3000 × *g*) at 4°C for 30 min and decanted. Antibody-bound [$^{125}$I]-estradiol-17β in the precipitate of tubes was quantified in a gamma counter. The RIA was validated for quantification of estradiol in porcine plasma as follows. Estradiol-17β amounts of 12.5, 25, 50, and 100 pg were added (0.05 ml) to aliquots (0.95 ml) of porcine plasma (n = 4 pigs) to determine assay accuracy. Essentially 100% of the added estradiol-17β was measured by the RIA. Inhibition of [$^{125}$I]-estradiol-17β binding to the antibody by serial dilutions of porcine plasma (n = 2) paralleled the inhibition of binding caused by estradiol standards prepared in steroid-depleted porcine plasma. The sensitivity of the assay was 1.5 pg/ml. The charcoal-treated porcine plasma used to prepare the standards for the direct RIA procedure contained 0–2 pg/ml immunoreactive estradiol when it was extracted with diethyl ether, and the reconstituted extract was assayed repeatedly in RIA employing estradiol standards in buffer (pH 7.2) containing 0.1 M phosphate, 0.15 M NaCl, 0.01% sodium azide, and 0.1% BSA.

### *Purification of pGP30*

Media collected from short-term explant cultures of Day 14 pregnant pig endometrium were pooled and concentrated by ultrafiltration at 4°C in an Amicon Model 8400 stirred cell (Amicon Corp., Danvers, MA) over a YM10 membrane (10 000 $M_r$ cutoff).

All chromatographic procedures were conducted at 4°C. The concentrated culture medium containing uterine secretory protein was fractionated by gel filtration on a 5 × 92-cm bed of Ultrogel AcA-44 (Pharmacia Biotech Inc., Piscataway, NJ) packed in a Pharmacia K50/100 column. The column was eluted at 40 ml/h with 0.01 M $NaPO_4$ and 0.15 M NaCl (pH 7.2), and 20-ml fractions were collected. This column had previously been calibrated using IgG and carbonic anhydrase ($M_r$ 31 000) as molecular weight markers. Fractions 65–75 (corresponding to the elution position of carbonic anhydrase) obtained from gel filtration of the uterine proteins were pooled and concentrated by ultrafiltration over an Amicon YM10 membrane.

The partially purified uterine proteins from several gel filtration separations were combined and further fractionated by chromatofocusing. The concentrated fractions 65–75 were applied to a 0.9 × 27-cm bed of Polybuffer Exchanger 94 (Pharmacia Biotech Inc.) packed in a Pharmacia K9/30 column and equilibrated with 25 mM ethanolamine-acetate buffer, pH 9.4. The sample was washed onto the column with 25 ml of equilibration buffer; elution was then carried out with 300 ml of elution buffer consisting of Polybuffer 96 (Pharmacia Biotech Inc.) diluted 1:10 and adjusted to pH 6.0 with glacial acetic acid. The column was eluted at 12 ml/h, and 4-ml fractions were collected. The pH of the fractions from chromatofocusing was determined, and fractions within the pH range 8.5–6.0 (fractions 21–69) were analyzed by one-dimensional (1D) SDS-PAGE as described by Laemmli [17] to determine the elution position of pGP30.

Final purification of pGP30 was accomplished by preparative gel electrophoresis. Material from fraction 33 of the chromatofocusing separation was loaded onto a 12.5% 1D SDS-PAGE gel. After electrophoresis and staining, the band corresponding to pGP30 was cut from the gel and used for preparation of antiserum.

### *Preparation of Antiserum to pGP30*

Polyacrylamide gel from the preparative electrophoresis step, containing approximately 60 mg protein, was finely minced in PBS and emulsified with an equal volume of Freund's complete adjuvant. A female New Zealand white rabbit was anesthetized with ketamine/xylazine [18], and the emulsion was injected at multiple intradermal sites dorsal to the scapulae. Two booster injections consisting of 60 mg of protein prepared with Freund's incomplete adjuvant were given at 2-wk intervals. Two weeks after the second booster, a blood sample was taken for initial evaluation of immune response. Blood was collected from the central ear artery into a 50-ml centrifuge tube and allowed to clot overnight. After centrifugation, the serum was collected by aspiration and stored at 4°C. The antiserum utilized in this study was from serum collected at 36 wk after the initial immunization.

### *Western Blot Analysis*

All uterine flushings and culture media were analyzed by Western blotting for the presence of immunoreactivity to antiserum against pGP30. Polypeptides in samples were separated by 1D SDS-PAGE and immediately transferred to Millipore Immobilon-P transfer membranes (Millipore Corp., Bedford, MA) at 300 mA constant current for 35 min.

After electroblotting, the membrane was washed in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) and incubated for 1 h with the first blocking solution of 3% gelatin in TBS. After being washed in TBS, the membrane was incubated for an additional 1 h with a second blocking solution containing 2.5 mg goat IgG (Sigma Chemical Co., St. Louis, MO) in 25 ml of TBS. The membrane was washed with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) and incubated for 1 h with primary rabbit antiserum (1:300 dilution) in 1% gelatin TTBS. After washing in TTBS, the immunoreactive polypeptides were detected through use of the Bio-Rad Immun-Blot kit (Bio-Rad Laboratories, Richmond, CA) according to manufacturer's specifications.

### *Deglycosylation of Endometrial Cultures, Porcine Serum, and Heart Muscle*

Since our pGP30 antiserum cross-reacted with products in porcine serum and culture media from heart muscle, samples of Day 5 cyclic and Day 15 pregnant endometrial culture, porcine serum, and heart muscle culture media were incubated with *O*-glycanase. Endometrial culture media, serum, and heart muscle were also incubated with *N*-glycanase and *O*-glycanase. Approximately 20 μg of total protein was incubated with the enzymes according to the manufacturer's specifications (Genzyme Corporation, Cambridge, MA). The reaction products were then resolved by 1D PAGE analysis and Western blotting as described above.

### *Immunohistochemistry*

Fixed endometrial tissue sections were dehydrated in graded ethanol changes, cleared with toluene, and embedded in paraffin. Sections (5 mm) were cut with an AO model 820 rotary microtome (American Optical, Buffalo, NY), placed on poly-L-lysine (Sigma Chemical Co., St. Louis, MO)-coated slides, deparaffinized, and rehydrated for immunostaining with a Biomeda Biostain Super ABC kit (Biomeda Corp., Foster City, CA). Primary rabbit antiserum (1:1000 dilution) against pGP30 was incubated with serial tissue sections. Tissue sections incubated with preimmune rabbit serum instead of primary antiserum, or with primary antiserum preabsorbed with pGP30, served as negative controls. Immunostaining was observed and photographed with an Olympus BH-2 photomicroscope (Olympus Corp., Lake Success, NY).

### *Statistical Analysis*

Data for analysis of plasma estradiol-17β concentrations between VEH- and PR-treated ovariectomized gilts were examined by least squares analyses of variance using the General Linear Models procedures of SAS [19]. Estradiol-17β concentrations were analyzed for differences between treatment, day, and treatment-by-day interaction.

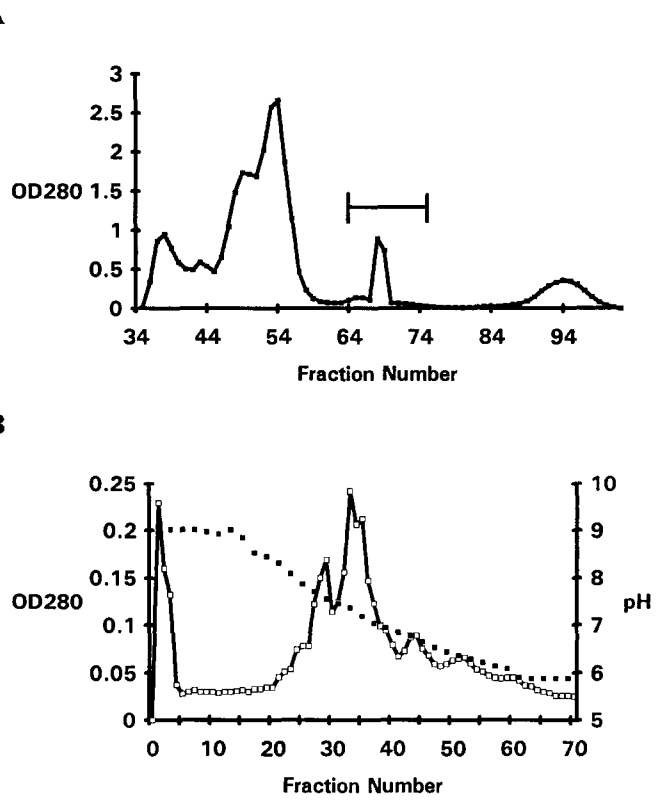



FIG. 1. Gel filtration of culture medium from Day 14 pregnant pig endometrial explants. **A)** Protein profile of concentrated uterine secretory proteins separated on an Ultrogel AcA-44 preparative column. Twenty-milliliter fractions were collected and fractions 65–75 were pooled, concentrated, and applied to a chromatofocusing column. **B)** Elutions from a chromatofocusing column of uterine secretory proteins in fractions 65–75. Open squares represent the optical density 280 readings and closed squares the pH of the 4-ml fractions collected. After 1D SDS-PAGE analysis, fraction 33 was utilized for preparative 1D SDS-PAGE analysis.

## RESULTS

### *Purification of pGP30*

The protein profile of concentrated porcine endometrial culture medium, separated on the Ultrogel AcA-44 preparative column and chromatofocusing column, is presented in Figure 1. Gel filtration of the endometrial culture medium removed many of the large serum contaminants, and chromatofocusing removed endometrial proteins such as uteroferrin, which has a molecular mass similar to that to pGP30 but a more basic pI [20]. On the basis of its molecular mass, its concentration, and its contamination with the fewest proteins, fraction 33 was utilized for final purification through 1D PAGE (Fig. 2).

### *Characterization of Antiserum to pGP30*

Western analysis (Fig. 3A) of Day 15 endometrial explant culture medium, separated by two-dimensional (2D) PAGE (Fig. 3B), indicated that the pGP30 antiserum recognized the isoforms of pGP30 with similar molecular mass but dif-


FIG. 2. Preparative 1D SDS-PAGE of fraction 33 from chromatofocusing column of Day 14 pregnant endometrial culture medium. Arrow depicts the Coomassie-stained protein band that was removed and utilized for immunization of a rabbit. ST = molecular weight standards.

fering pI previously described by our laboratory [15]. However, the antiserum also detected an approximately 90-kDa protein. It appears that pGP30 is specific for the reproductive tract of the gilt, as Western blot analysis did not detect positive staining in culture media from the lung, liver, kidney, and adipose tissue incubated with pGP30 antiserum (Fig. 4). However, Western blot analysis of culture media from muscle, heart, and serum indicated that the antiserum reacted with polypeptides that were not the same in molecular mass as the endometrial glycoprotein. Both heart and muscle contained an approximately 40-kDa reaction product, and two additional bands of approximately 25–27 kDa were also present in heart. A higher-molecular-mass polypeptide of approximately 90 kDa (pI 6.0–6.5), similar to that observed in endometrial culture medium (see Fig. 3A), was detected in serum. Although staining was detected with some of the tissues tested, the bands did not correspond to the same molecular mass or pI as pGP30 in the pig endometrium. Given that our polyclonal antiserum was raised against a glycoprotein, it is possible that the antiserum recognizes similar oligosaccharides within different proteins. Treatment of porcine serum with *O*-glycanase resulted in a decrease in the molecular mass of the band (87 kDa) but did not abolish reactivity with pGP30 antiserum (Fig. 5A). The serum 90-kDa band contained both *O*- and *N*-linked oligosaccharides, as incubation with both *O*- and *N*-glycanase resulted in a further decrease in molecular mass as seen in endometrial culture medium (Fig. 5B). Serum was present in the endometrial culture medium (see Fig. 3A), and the change in molecular mass following deglycosylation is evident in Figure 5, A and B. Reactive bands in heart culture medium were not changed following *O*-glycanase treatment (Fig. 5A). Although it is possible that the heart reactive products are resistant to our enzyme procedures, it appears that serum, muscle, and heart contain one or more proteins that share some protein homology to pGP30. Although a cross-reactive product was present in muscle and heart, we have not detected products with these molecular masses in either endometrial culture medium or uterine flushings; and no other tissue except oviduct expresses pGP30. Oviductal explant culture medium contained pGP30; this indicates that pGP30 is specific not only to the endometrium of the porcine reproductive tract (data not presented). Deglycosylation of pGP30 indicated that the glycoprotein contained mostly *O*-linked oligosaccharides, as there was a large shift in the pGP30 molecular mass following reaction with *O*-glycanase with little change when *N*-glycanase was also added (see Fig. 5, A and B). Deglycosylation of pGP30 resulted in a protein of approximately 28 kDa.

### *Changes in Endometrial pGP30 during the Estrous Cycle and Early Pregnancy*

Analysis of uterine flushings and endometrial culture media with pGP30 antiserum showed consistent bands within each sample day. Representative Western blot analyses, after 1D SDS-PAGE of endometrial tissue culture medium and uterine flushings from gilts during the estrous cycle and early pregnancy, are presented in Figures 6 and 7. The appearance of pGP30 in medium from endometrial tissue cultures varied with stage of the estrous cycle and was affected by the presence of conceptuses in the uterine lumen (Fig. 6). On the day of estrus (Day 0), the antiserum detected two immunoreactive bands, a major band that corresponded to pGP30 and a second band of slightly higher molecular mass (32 kDa). The 30-kDa band was absent from endometrial culture media on Days 5 and 10 of the estrous cycle, but three to four bands of higher molecular mass (35–40 kDa) were detected. The two bands corresponding to 30- and 32-kDa polypeptides observed at estrus were present on Day 12 and continued to be present on Days 15 and 18 of the estrous cycle. The multiple bands between 35 and 40 kDa were also detected on Day 10 of pregnancy, but in contrast to samples from nonpregnant gilts, samples from pregnant gilts also exhibited the 30-kDa band. After Day 10, only the single band representing the 30-kDa glycoprotein immunostained throughout the early stages of pregnancy (Days 12–18).

The multiple, higher-molecular-mass bands detected on Days 5 and 10 of the estrous cycle appear to represent glycosylated forms of a single protein, as treatment with *O*-glycanase and *N*-glycanase resulted in detection of a single 32-kDa band following Western blot analysis (Fig. 5B). Although deglycosylation of the multiple bands present on Days 5 and 10 resulted in a shift to a single molecular mass, the molecular mass was still greater than in the bands detected on Day 12 of the estrous cycle and Day 15 of pregnancy. Western blot analysis of Day 12 cyclic endometrial culture medium treated with *O*-glycanase and *N*-glycanase

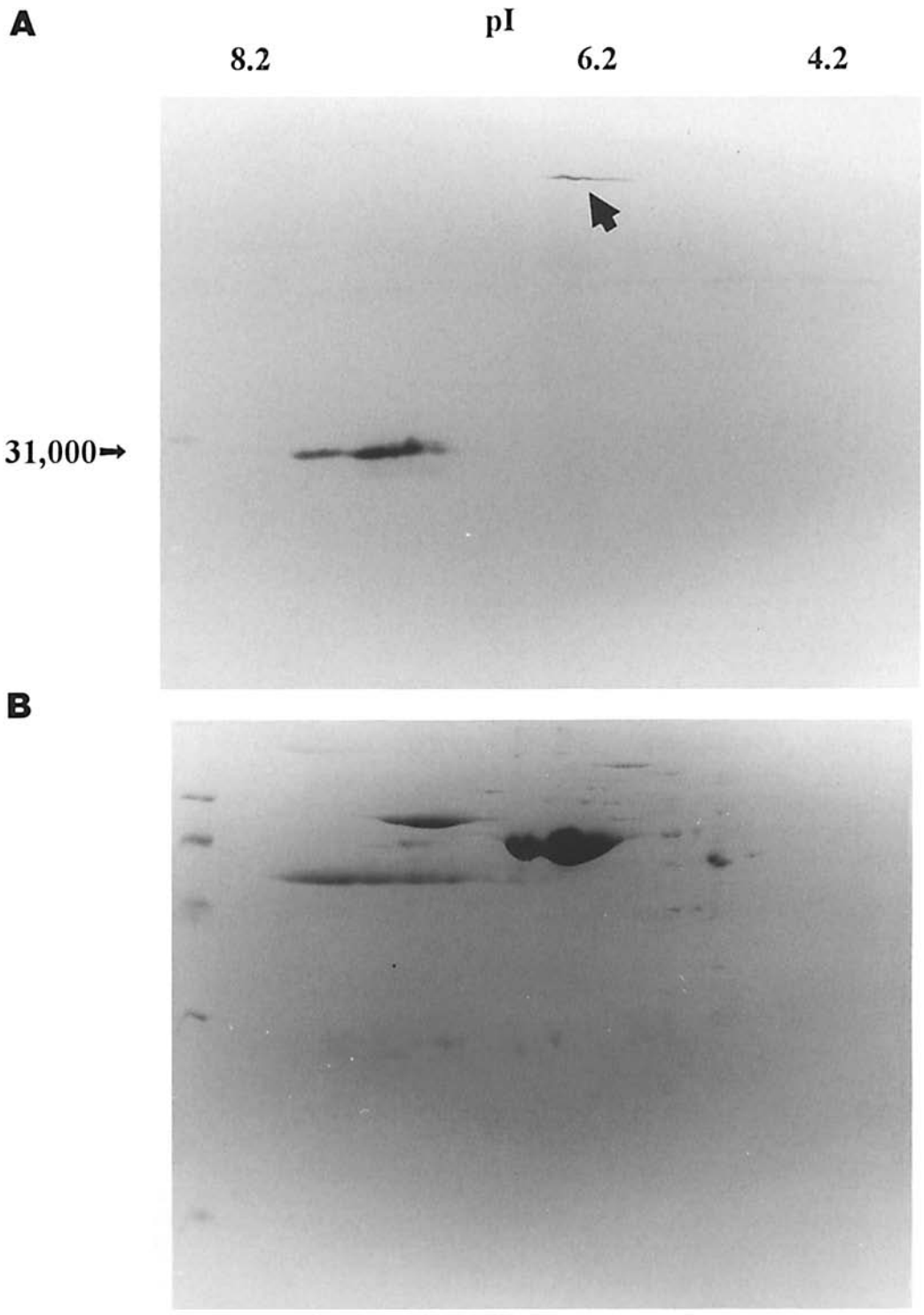



FIG. 3. **A**) Western blot analysis of an aliquot (200 μg of protein) of Day 15 endometrial culture medium following separation by 2D SDS-PAGE (12.5% slab gel). The polyclonal antibody reacted with a group of basic antigens with molecular weight of 30 000 and slightly acidic antigens of approximately 90 000 molecular weight (arrow). **B**) Coomassie blue stain of 2D SDS-PAGE gel representative of proteins present in Day 15 endometrial culture media.

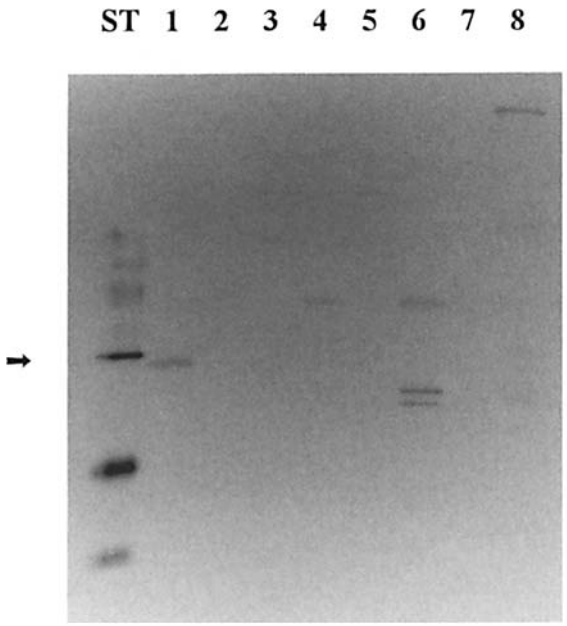



FIG. 4. Western blot analysis of tissue explant media from porcine Day 15 pregnant endometrium (lane 1), lung (lane 2), kidney (lane 3), muscle (lane 4), liver (lane 5), heart (lane 6), adipose tissue (lane 7), and serum (lane 8). Media samples (50 μg) were separated by 1D SDS-PAGE (12.5% gel) and transferred to Immobilon-P transfer membranes. ST = biotinylated standards. Note the 90-kDa band detected in serum (lane 8). Arrow on left indicates 31 000 molecular weight standard.

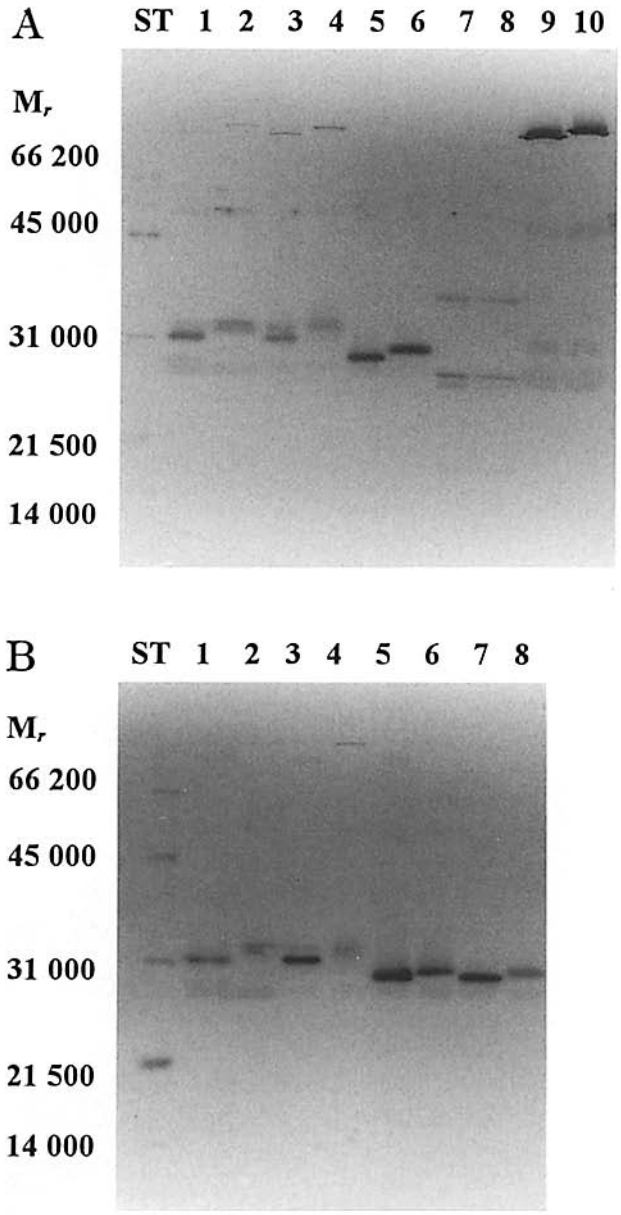



FIG. 5. **A)** Western blot analysis with pGP30 antiserum of SDS 1D-PAGE from untreated Day 5 cyclic (lane 2), Day 10 cyclic (lane 4), and Day 15 pregnant (lane 6) endometrial culture media as well as culture media from heart (lane 8) and serum (lane 10). Culture media from Day 5 cyclic endometrium (lane 1), Day 10 cyclic endometrium (lane 3), Day 15 pregnant endometrium (lane 5), and heart (lane 7) and porcine serum (lane 9) following treatment with neuraminidase and *O*-glycanase. Lanes contain approximately 20 μg of initial protein. ST = biotinylated standards. **B)** Western blot analysis with pGP30 antiserum of SDS 1D-PAGE from untreated Day 5 cyclic (lane 2), Day 10 cyclic (lane 4), Day 12 cyclic (lane 6), and Day 15 pregnant (lane 8) endometrial culture media. Endometrial culture media from Day 5 cyclic (lane 1), Day 10 cyclic (lane 3), Day 12 cyclic (lane 5), and Day 15 pregnant gilts following treatment with neuraminidase, *N*-glycanase, and *O*-glycanase. Lanes contain approximately 20 μg of initial protein. ST = biotinylated standards.

resulted in the detection of a single 28-kDa reactive product similar to the band detected after enzyme treatment of Day 15 endometrial culture medium (Fig. 5).

Although the staining intensity was greatly diminished compared to that in culture medium, pGP30 was faintly to lightly detected in a number of uterine flushings throughout the estrous cycle and early pregnancy (Fig. 7). However, an immunoreactive band of approximately 90 kDa was present in uterine flushings obtained on Days 10 and 12 of either the estrous cycle or pregnancy. The 90-kDa immunoreactive band, which is similar to the immunoreactive product in serum, was not detected in uterine flushing from any of the other days of the estrous cycle or pregnancy analyzed.

Detection and localization of pGP30 in endometrial tissue during the estrous cycle and early pregnancy was performed through immunocytochemical analysis (Fig. 8, B-L). Immunocytochemical analysis of endometrial tissue samples after preabsorption of the antiserum with purified pGP30 resulted in the removal of positive staining observed in the endometrial tissue (Fig. 8A). No positive immunostaining was revealed in lung, heart, muscle, kidney, liver, or adipose tissue (data not presented). Light staining was detected in the uterine surface epithelial cells during estrus (Fig. 8B), with the majority of immunoreactive product present within the apical region of the cells. Nonspecific staining was also detected in the numerous peroxidase-containing immunocytes that were present at the basal lamina and between the surface uterine epithelial cells. These cells were also stained in the absence of primary antiserum (data not shown). Faint to no staining was present within the glandular epithelium on Day 0 (Fig. 8K). Immunostaining was specifically localized to the uterine epithelial cells

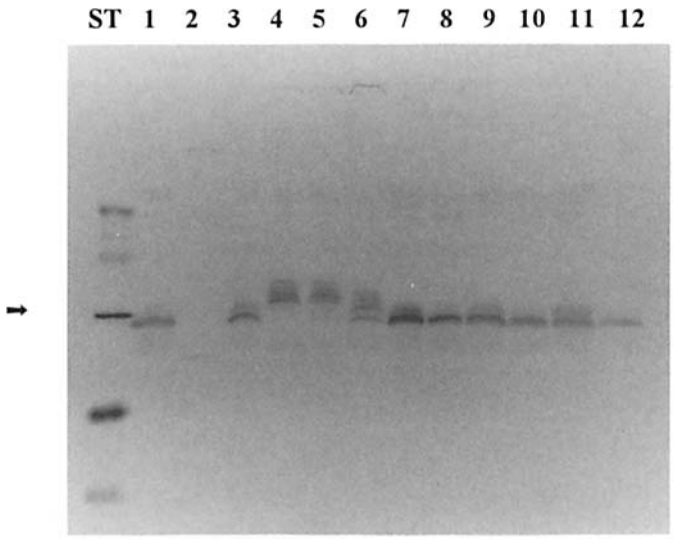



FIG. 6. Western blot analysis of polypeptides (200 μg protein) in media taken from endometrial cultures of gilts during Day 0 (lane 3), 5 (lane 4), 10 (lane 5), 12 (lane 7), 15 (lane 9), and 18 (lane 11) of the estrous cycle and Day 10 (lane 6), 12 (lane 8), 15 (lane 10), and 18 (lane 12) of pregnancy following separation by 1D SDS-PAGE (12.5% gel). Lane 1 represents a positive control of pregnant Day 15 endometrial culture medium, and lane 2 is negative control of lung tissue culture medium. ST = biotinylated standards. Note the multiple, higher-molecular-mass bands in the endometrial cultures during the early phase of the estrous cycle and pregnancy. Two bands of lower molecular mass are evident in endometrial cultures prepared from gilts on Days 12, 15, and 18 of the estrous cycle. However, only a single 30-kDa band is present after Day 10 in pregnant gilts. Arrow on left indicates 31 000 molecular weight standard.

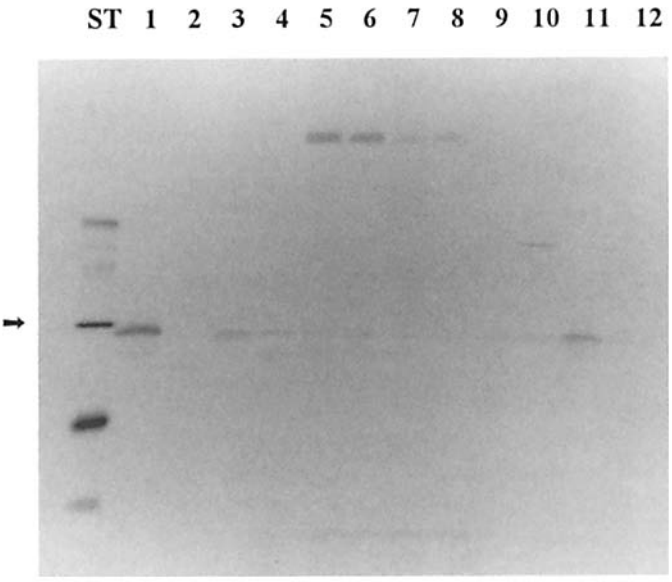



FIG. 7. Western blot analysis of polypeptides (200 μg protein) in uterine flushings from gilts during Day 0 (lane 3), 5 (lane 4), 10 (lane 5), 12 (lane 7), 15 (lane 9), and 18 (lane 11) of the estrous cycle and Day 10 (lane 6), 12 (lane 8), 15 (lane 10), and 18 (lane 12) of pregnancy following separation by 1D SDS-PAGE (12.5% gel). Lane 1 represents a positive control of pregnant Day 15 endometrial culture medium, and lane 2 is negative control of lung tissue culture medium. ST = biotinylated standards. Note the presence of a 90-kDa band on Days 10 and 12 of the estrous cycle and pregnancy. Arrow on left indicates 31 000 molecular weight standard.

throughout the estrous cycle and pregnancy, as staining was not evident in the stroma. Intensity of immunostaining within the surface epithelium was light and appeared in the apical portion of the cells on Day 5 of the estrous cycle (Fig. 8C) but was clearly present and intense throughout the uterine surface epithelium on Day 12 of the estrous cycle (Fig. 8D). There was also an increase in the presence of immunostaining in glandular epithelium, but the intensity was usually less than in the surface epithelium (Fig. 8L). Immunostaining for pGP30 remained similar throughout the remainder of the estrous cycle (Fig. 8, D-F). Strong immunostaining was evident within the uterine surface epithelium from Day 10 to 18 of pregnancy (Fig. 8, G-J). Positive immunostaining was also detected within conceptus tissue attached to the uterine surface epithelium on Day 18 of pregnancy (Fig. 8J).

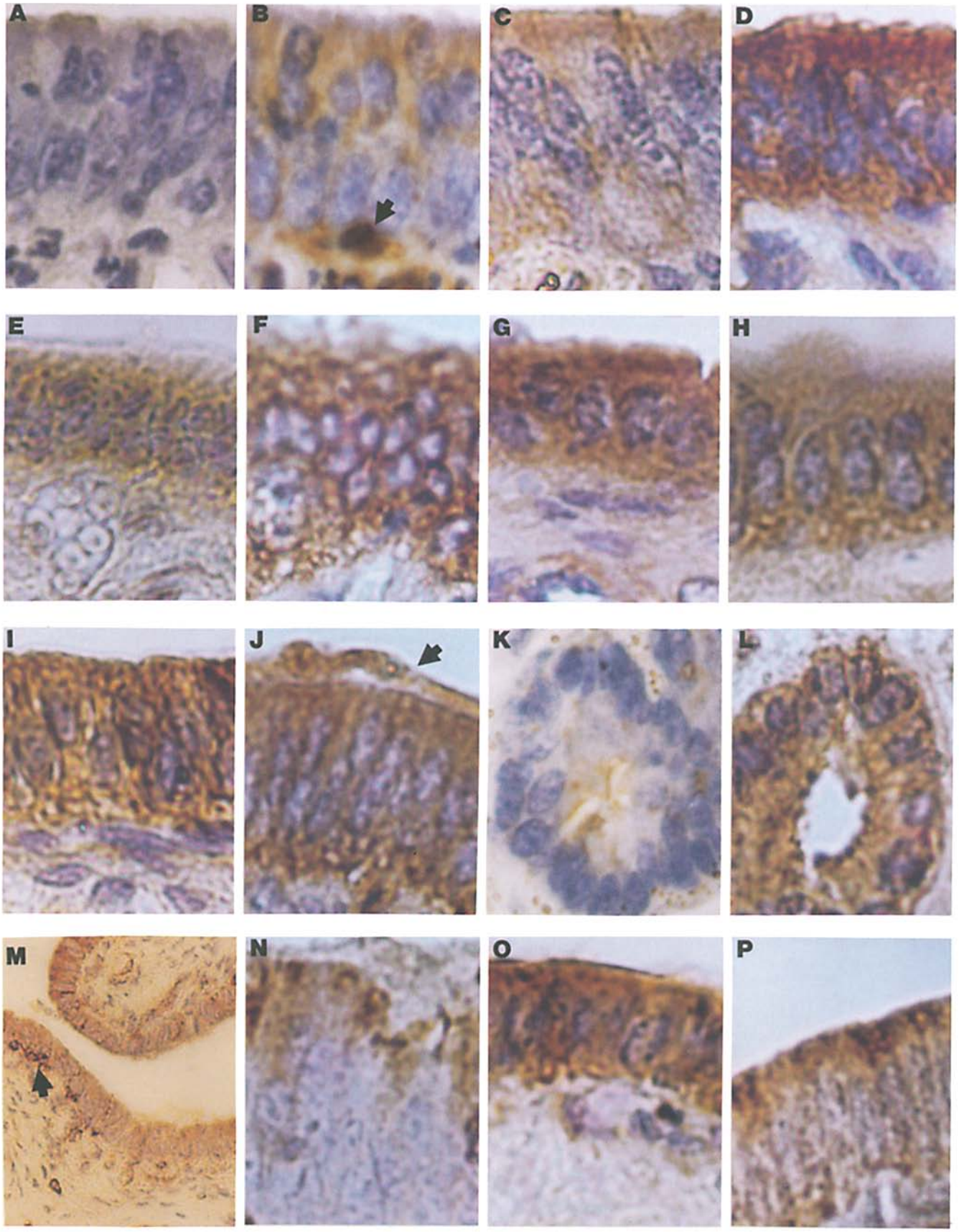
### *Steroid Modulation of pGP30*

The plasma content of estradiol-17β and progesterone after steroid replacement therapy in ovariectomized gilts is presented in Table 1. Concentrations of plasma progesterone in ovariectomized VEH-treated females were negligible (< 0.05 ng/ml). However, not only were plasma estradiol-17β concentrations detectable in VEH-treated ovariectomized gilts (12.8 pg/ml), but they were two-fold greater ($p <$

TABLE 1. Plasma concentration of estradiol-17β (pg/ml) and progesterone (ng/ml) in ovariectomized gilts that received steroid replacement therapy.

| | VEH | | EB | | PR | | PRE | |
|---|---|---|---|---|---|---|---|---|
| Treatments[a] | E2 | P4[b] | E2 | P4[b] | E2 | P4[b] | E2 | P4[b] |
| Day 0 | 20.7 ± 6.6 | .05 | 5.9 ± 0.5 | .05 | 6.1 ± 2.1 | .05 | 5.6 ± 2.3 | .05 |
| Day 1 | 12.0 ± 4.5 | .05 | 16.0 ± 1.8 | .05 | 5.9 ± 2.3 | 8.9 ± 3.9 | 20.6 ± 8.0 | .05 |
| Day 4 | 11.4 ± 3.5 | .05 | 61.7 ± 8.8 | .05 | 4.5 ± 2.1 | 26.5 ± 1.7 | 57.5 ± 11.6 | .5 ± .05 |
| Day 7 | 10.5 ± 3.4 | .05 | | | 6.6 ± 1.0 | 23.8 ± 4.6 | 42.9 ± 8.9 | 21.8 ± 3.8 |
| Day 10 | 10.3 ± 2.8 | .05 | | | 6.8 ± 1.5 | 18.5 ± 4.2 | 27.4 ± 2.3 | 42.9 ± 14.3 |
| Day 14 | 12.0 ± 1.0 | .05 | | | 7.3 ± 1.0 | 21.4 ± 3.4 | 19.7 ± 2.2 | 14.9 ± 2.1 |

[a] Treatments: ovariectomized gilts treated with oil (VEH), estradiol benzoate (EB), progesterone (PR), or estradiol benzoate followed by progesterone (PRE). E2 = estradiol-17β and P4 = progesterone.
[b] Progesterone concentrations below sensitivity (0.05 ng/ml) of the assay were assigned a value of 0.05 ng/ml.



0.01) than in PR gilts (5.8 pg/ml). The difference in plasma estradiol-17β was the result of elevated concentrations in one VEH-treated gilt. Treatment of ovariectomized gilts with EB for 4 days produced plasma estradiol-17β concentrations of approximately 61 pg/ml while progesterone concentrations remained below 0.05 ng/ml. In ovariectomized gilts treated with PR alone for 14 days, plasma progesterone concentrations were increased from 8.9 ng/ml on Day 1 to 21.4 ng/ml on Day 14. Ovariectomized gilts treated with estradiol benzoate for 4 days and then with progesterone for 10 days had plasma estradiol-17β concentrations of 19.7 pg/ml on Day 14 with corresponding plasma progesterone concentrations of 14.9 ng/ml.

Western blot analysis of endometrial culture medium and uterine flushes from ovariectomized steroid-treated gilts is presented in Figures 9 and 10, respectively. The multiple higher-molecular-mass bands observed on Days 5 and 10 of the estrous cycle (Fig. 6) were evident in endometrial culture media (Fig. 9) of VEH- and EB-treated gilts. Endometrial culture media from PR and PRE gilts contained two bands of 32 and 30 kDa as previously observed on Day 12 of the estrous cycle (Fig. 6). Intensity of staining was greater in PR than in PRE gilts. The 90-kDa serum immunoreactive product was detected in all endometrial cultures. Immunoreactive bands similar to those in the endometrial cultures were present in uterine flushings of VEH- and EB-treated gilts (Fig. 10), but the 30-kDa band was evident in uterine flushing only of the PR- and PRE-treated gilts.

Immunocytochemical staining of VEH-treated ovariectomized gilts showed slight immunostaining in the uterine surface epithelium (Fig. 8M). No immunostaining was apparent in small, poorly developed glandular epithelium. Treatment of gilts with EB resulted in localization of immunostaining for pGP30 in the apical region of the surface epithelium (Fig. 8N). Staining of surface epithelial cells was variable, the intensity appearing as light to dark within individual cells. Peroxidase-positive immunocytes were also present beneath the basal lamina of the surface epithelium.

FIG. 8. Light microscopy of avidin-biotin staining of endometrial tissue using antibody to porcine endometrial pGP30 or preabsorption of antiserum with pGP30 (**A**). Immunoreactive product was restricted to the apical region of the uterine surface epithelium on Day 0 (**B**) as well as immunocytes (arrow) along the basement membrane. Immunostaining was light on Day 0 and Day 5 (**C**) but became intense throughout the entire cytoplasm of the surface epithelium on Day 12 of the estrous cycle (**D**). Staining intensity was maintained in the uterine surface epithelium from Day 15 (**E**) to Day 18 (**F**) of the estrous cycle. Strong immunostaining was evident within the uterine surface epithelium on Day 10 (**G**), 12 (**H**), 15 (**I**), and 18 (**J**). Note that positive immunostaining was also observed in an attached conceptus (**J**), indicated by the arrowhead. Light immunostaining was present in the uterine glands on Day 0 (**K**), but staining became intense on Day 12 (**L**). No staining was present in the uterine stroma during the estrous cycle or early pregnancy. Light staining was present in the uterine surface epithelium of ovariectomized gilts (**M**). Treatment of ovariectomized gilts with EB resulted in immunostaining localized only in the apical region of the surface epithelium (**N**). Intensive staining was apparent throughout the uterine surface epithelium following treatment of ovariectomized gilts with either progesterone (**O**) or estradiol benzoate followed by progesterone (**P**).

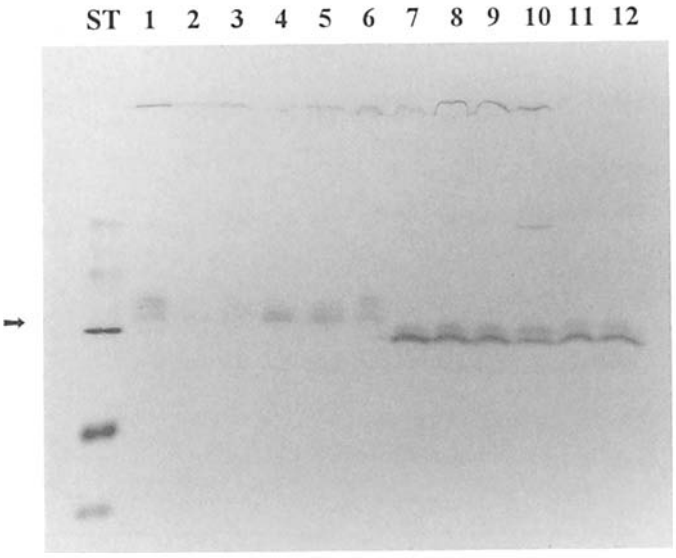



FIG. 9. Western blot analysis of endometrial culture medium, separated by 1D SDS-PAGE, from ovariectomized gilts (n = 3 per group) treated with either VEH (lanes 1–3), EB (lanes 4–6), PR (lanes 7–9), or PRE (lanes 10–12). Note the multiple higher-molecular-mass bands present in VEH- and estrogen-treated gilts as compared to the two lower-molecular-mass bands in gilts treated with progesterone. ST = biotinylated standards. Arrow on left indicates 31 000 molecular weight standard.

No immunostaining was apparent in the glandular epithelium. The surface and glandular epithelium of PR (Fig. 8O) and PRE (Fig. 8P) gilts was strongly positive to the antiserum against pGP30. Stromal immunostaining was not apparent in any of the treatment groups.

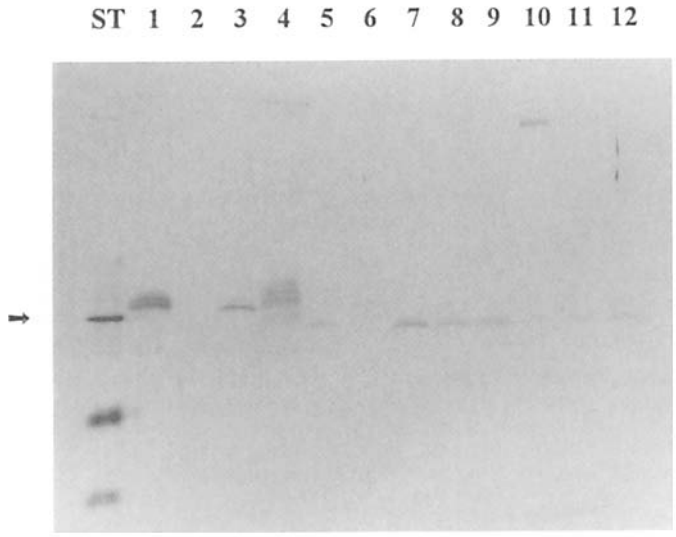



FIG. 10. Western blot analysis of uterine flushings, separated by 1D SDS-PAGE, taken from ovariectomized gilts treated (n = 3 per group) with either VEH (lanes 1–3), EB (lanes 4–6), PR (lanes 7–9), or PRE (lanes 10–12). Note the multiple higher-molecular-mass bands present in VEH- and estrogen-treated gilts compared to the one 30-kDa band in gilts treated with progesterone and with estrogen followed by progesterone. ST = biotinylated standards. Arrow on left indicates 31 000 molecular weight standard.

## DISCUSSION

Purification of pGP30 based on the known biochemical characteristics of the glycoprotein provided material of suitable purity and quality for preparation of antiserum. Polyclonal antiserum to the 30-kDa glycoprotein was successfully generated, and it recognized the isoforms of the pGP30 synthesized by the endometrium in vitro as previously described by Gries et al. [15] and Blair et al. [12]. It appears that pGP30 is specific to the reproductive tract, as Western blot and immunocytochemical analysis of lung, muscle, kidney, fat, heart, and liver were negative for the 30-kDa glycoprotein. However, the antiserum did detect immunoreactive products of different molecular masses in the culture media of muscle, heart, and serum. Given that our polyclonal antiserum was made against a glycoprotein, we investigated whether or not the oligosaccharide chains caused the cross-reactivity with our pGP30 antiserum. Deglycosylation of the porcine serum and heart culture media did not remove the recognition by pGP30 antiserum, although enzyme treatment did shift the molecular mass of the serum reactive product. It is possible that heart and muscle are resistant to enzymatic treatment and that the serum band is not completely devoid of carbohydrate after treatment. However, results would tend to suggest that the polyclonal antiserum does recognize the protein core of these products. The relevance of these different molecular mass proteins as compared to pGP30 synthesized in the porcine endometrium is unknown at this time. Although we acknowledge that the cross-reactivity of the heart muscle-secreted proteins and 90-kDa serum glycoprotein indicates that the antiserum is not absolutely specific to pGP30, this does not invalidate results obtained concerning the porcine endometrium. The cross-reacting proteins present in heart muscle culture medium were of a different molecular mass and were not detected in the endometrium. The serum glycoprotein was detected in flushings as well as in endometrial cultures due to leaching of serum from the tissue. However, we can separate this cross-reactivity through 1D PAGE analysis. Immunocytochemical localization of pGP30 demonstrated that the glycoprotein changes in intensity during the estrous cycle while no specific staining was evident in heart muscle, which would also contain serum (data not shown). The 30-kDa glycoprotein is not specific to the endometrium of the reproductive tract, as a positive reaction product of the same molecular mass was also detected in culture medium from luteal phase oviductal tissue explants (data not presented). Recognition of a 30-kDa band in the porcine oviduct with our antiserum is consistent with the secretion of a glycoprotein of similar molecular mass and pI from the porcine isthmus [21]. Antiserum also detected an additional 90-kDa immunoreactive product in porcine endometrial cultures. The 90-kDa product was detected in serum and therefore indicates the presence of serum proteins within the tissue and uterine flushings. Our results indicate that a 90-kDa immunoproduct appears in uterine flushings on Days 10 and 12 of either the estrous cycle or pregnancy. There is an increase in vascular permeability during trophoblast attachment in the pig [22, 23]. However, the presence of the 90-kDa immunoproduct was detected before conceptus estrogen secretion occurred (Day 10) and was clearly evident at the same time of the estrous cycle. Why there is a specific increase in the 90-kDa immunoproduct on Days 10 and 12 is not known.

Results of the present study indicate that endometrial synthesis of pGP30 is time- and steroid-dependent. Western blot analyses of endometrial culture medium revealed distinct alterations in molecular mass and in the number of bands of pGP30 detected during the estrous cycle and early pregnancy. Multiple, higher-molecular-mass bands were expressed during the early stages of the estrous cycle; this was followed by detection of the 30-kDa band on Day 12 of the estrous cycle plus an additional 32-kDa protein throughout the remainder of the estrous cycle. The pattern and synthesis of the endometrial proteins that reacted with the antiserum are under steroid regulation as demonstrated by the steroid replacement therapy with ovariectomized gilts. Estrogen stimulated detection of multiple, higher-molecular-mass forms of glycoproteins recognized by the pGP30 antiserum, while PR alone for 14 days induced the synthesis of the 30- and 32-kDa bands. Steroid administration to ovariectomized gilts provided plasma steroid concentrations similar to those present during the normal estrous cycle [24]. The antiserum detected the multiple, higher-molecular-mass bands in VEH-treated gilts; this was unexpected. However, plasma concentrations of estradiol-17β in VEH-treated gilts not only were detectable but, at the concentrations detected, could stimulate the endometrium in the absence of progesterone. It is possible that the adrenal glands contribute as a source of estrogen, which, at 7–15 pg/ml, could still induce production of the endometrial protein in ovariectomized gilts. The changes in the proteins during the estrous cycle and in PRE-treated ovariectomized gilts indicate that induction of the 30- and 32-kDa proteins occurs after approximately 10 days of progesterone stimulation. The alteration in the glycoprotein not only corresponds to plasma steroid concentrations but is most likely directly affected by the changes in the steroid receptors that occur on Days 10–12. Uterine epithelial progesterone receptors are specifically down-regulated on Days 10–12 of the estrous cycle of the gilt, while stromal receptors are maintained [25]. Coincidentally, there is a dramatic increase in uterine epithelial estrogen receptors on Days 10 and 12 [26]. The multiple, higher-molecular-mass forms of glycoproteins that appear during the early phase of the estrous cycle are induced by estrogen and appear to be inhibited following loss of epithelial progesterone receptors on Day 10. Deglycosylation of Days 5 and 10 cyclic

endometrial culture media decreased the molecular mass of the higher-molecular-mass forms recognized with our pGP30 antiserum to a single 32-kDa band. Results would suggest that the Day 5 reactive products have a protein core similar to that of pGP30. However, the 32-kDa deglycosylated band on Day 5 is larger than both the native (30 kDa) and the deglycosylated (28 kDa) form of pGP30 on Day 12 or 15. Antisera to the protein core only are necessary to clearly determine whether or not there is a similar protein core between the glycoproteins present on Days 5 and 12. It is evident that there is a distinct change in the glycoproteins between Days 10 and 12 of the porcine estrous cycle. The alteration in glycoproteins would therefore correspond with the changes in progesterone and the cellular location of its receptor within the porcine endometrium [25]. These changes may precisely time the uterine changes necessary for conceptus development and trophoblastic elongation. However, further studies are necessary to determine the identification and the relationship of the higher-molecular-mass glycoproteins with the initiation of pGP30 synthesis on Day 12.

The modifications in glycoprotein synthesis throughout the estrous cycle suggest that it may serve an important function in physiological events during early pregnancy of the pig. The conversion to the 30-kDa form on Days 10–12 of pregnancy corresponds to the time of rapid trophoblastic elongation and initiation of placental attachment (see [10, 11, 27]). The presence of the conceptus also appears to alter the forms of the glycoprotein, as only the 30-kDa band was detected in culture medium of pregnant gilts. Conceptus estrogen synthesis appears to regulate the change in the glycoprotein's secretion from endometrial cultures, as administration of estrogen to gilts on Day 12 of the estrous cycle results in detection of only the 30-kDa band (Geisert et al., unpublished results). The 32-kDa band appears to be a more glycosylated form of pGP30, since deglycosylation of Day 12 cyclic endometrial culture medium resulted in the same single 28-kDa band as Day 15 pregnant culture medium. Regulation of the glycoprotein by progesterone, its presence and increase in staining intensity within the uterine surface epithelium on Day 10, its absence in stroma, and its modification by the conceptus provide evidence that the glycoprotein could possibly constitute an important physiological process for the establishment of pregnancy in the pig. Further studies are necessary to determine whether the glycoprotein has any possible function in conceptus attachment to the surface epithelium. Detection of the glycoprotein was much greater in the endometrial culture medium than in uterine flushings. In fact, the glycoprotein was originally discovered through evaluation of secretory changes following in vitro labeling of endometrial explants [15]. The glycoprotein was not observed in 2D PAGE analysis of uterine flushings. The localization of the glycoprotein throughout the cytoplasm of the epithelium rather than localization specifically at the apical border would tend to argue that it functions as an intracellular protein. However, positive staining within the attaching trophoblast indicates that the glycoprotein is presented to the conceptus at the uterine luminal interface. Nevertheless, we cannot exclude the possibility that pGP30 antiserum recognizes polysaccharides present on the conceptus surface as well. Unlike the major progesterone-responsive endometrial secretory proteins released into the uterine lumen of the pig (see [20]) such as uteroferrin [28], proteins belonging to the Kunitz family of inhibitors [29], and retinol-binding protein [30], the 30-kDa glycoprotein is not easily detected in uterine flushings through 2D SDS-PAGE without the use of Western blot analysis.

At present, we can only speculate on the function of pGP30 in conceptus development from the information obtained. Amino terminal acid microsequencing and cDNA sequencing of pGP30 have as yet not revealed homology with any known protein (Geisert, unpublished results). The alteration of the multiple forms and the higher molecular mass from early phases of the estrous cycle to the double band on Day 10 would suggest that the glycoprotein could be involved with the rapid elongation of the porcine conceptus that occurs between Days 10 and 12 of pregnancy or possibly be involved with trophoblastic attachment initiated on Day 13 of gestation (see [10]). Our results also indicate that the conceptus appears to have an effect on the glycoprotein, as only the 30-kDa glycoprotein band was detected after Day 12 of pregnancy. This result would suggest that either conceptus secretion of estrogen or possibly other secretory products (see [10]) influences posttranscriptional modification of the glycoprotein.

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