BIOLOGY OF REPRODUCTION **66,** 322–332 (2002)

Ovarian Steroid-Regulated Synthesis and Secretion of Complement C3 and Factor B in Mouse Endometrium During the Natural Estrous Cycle and Pregnancy Period[1]

Sheng-Hsiang Li,[2,4] Hsien-Lu Huang,[2] and Yee-Hsiung Chen[2,3,4]

Institute of Biochemical Sciences,[3] College of Science, National Taiwan University, Taipei, Taiwan
Institute of Biological Chemistry,[4] Academia Sinica, Taipei, Taiwan

ABSTRACT

We demonstrate the presence of complement factor B (Bf) and complement C3 in uterine luminal fluid collected from estrogen-stimulated immature and adult female mice. We examined the synthesis and secretion of these two proteins in mouse endometrium at various stages of the natural estrous cycle and during the pregnancy period. The mRNA levels of these two proteins increased markedly in proestrus and estrus and declined sharply in metestrus to an undetectable level. The Bf mRNA remained undetectable, whereas a readily detectable C3 mRNA level reappeared, in diestrus. Meanwhile, these two proteins were immunolocalized to the apical cytoplasm of glandular and luminal epithelial cells of the endometrium during the estrous cycle. Administration of an estrogenic steroid to immature or ovariectomized adult mice markedly stimulated the expression of Bf, C3, and their RNA messages in the endometrium, whereas injection of progesterone alone to ovariectomized animals did not stimulate their expression. Expression of C3 was remarkably enhanced, whereas that of Bf changed only slightly, after injection of combined estrogen and progesterone to ovariectomized animals. In pregnant mice (Day [D] 1 = day of vaginal plug), Bf mRNA was at a high level on D1 and D2, dropped to an almost undetectable level from D3 to D8, and then increased to a low level thereafter until delivery. The C3 mRNA was at a high level on D1, dropped on D2 to an almost undetectable level from D3 to D9, increased to a very high level from D10 to D18, and then declined sharply before delivery. Immunohistochemical patterns of both proteins in the endometrium during preimplantation were positively correlated with changes in their mRNA levels.

estradiol, ovulatory cycle, pregnancy, progesterone, uterus

INTRODUCTION

Ovarian steroids induce a profound alteration in the biochemical and cytological characteristics of the reproductive tract of female mammals. These hormone-regulated events include secretion of various proteins that may serve as molecular markers for examining the steroid regulation of gene expression in a mammalian reproductive system. The rodent uterus is a well-studied target tissue for this purpose. Estrogen-stimulated growth of the mouse uterus is accompanied by increases in the expression of mRNAs coding for secretory proteins such as lactoferrin [1, 2] and 24p3 [3, 4]. Active complement proteins have been identified in human ovarian follicular fluid and in mouse uterine luminal fluid (ULF) before implantation [5, 6]. Attempts have been made to elucidate how ovarian steroids regulate the synthesis and secretion of complement proteins in the genital tract. In this regard, C3 and complement factor B (Bf) have been immunolocalized to human endometrium in the luteal phase [7–10], and secretion of C3 has been demonstrated in organ culture of rat uteri during the estrous cycle [11, 12]. We conducted this study to extend previously published work, which is rather fragmentary, to gain a better understanding regarding ovarian steroid regulation of the synthesis and secretion of C3 and Bf in the endometrium. We examined the production of C3 and Bf in mouse endometrium and their secretion to the uterine lumen through the estrous cycle and the pregnancy period, and we investigated the effects of an exogenous steroid on expression of these factors. Our results indicate that stimulation of C3 and Bf expression in endometrial epithelial cells is positively correlated with serum 17β-estradiol (E_2) concentrations during the natural estrous cycle and the period of pregnancy. Unlike the inhibitory effect of progesterone (P_4) on E_2-stimulated C3 expression in rat uterus [12], our results show that a high level of serum P_4 concentration produces a slight effect on E_2-stimulated Bf expression but potentiates E_2-stimulated C3 expression.

MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources: Sephadex G-100 and Full Range Rainbow molecular-weight markers (Amersham Pharmacia Biotech, Uppsala, Sweden); CM-Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA); Protein PAK SP 5PW column (Waters, Milford, MA); Nucleosil C_{18} column (Macherey-Nagel GmbH & Co., Düren, Germany); BCA protein assay kit (Pierce, Rockford, IL); E_2, P_4, diethylstilbestrol dipropionate (DES), nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), PMSF, and silanated glass slides (Sigma Chemical Co., St Louis, MO); goat anti-human Bf (Calbiochem, La Jolla, CA); goat anti-mouse C3 (ICN Biomedicals, Inc., Irvine, CA); biotin-conjugated rabbit anti-goat IgG, alkaline phosphatase-conjugated streptavidin, and horse radish peroxidase-conjugated rabbit anti-goat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD); nuclear fast red (Vector Laboratories, Burlingame, CA); enhanced chemiluminescent (ECL) substrate and [α-^{32}P]dATP (NEN Life Science Products, Boston, MA); Prime-a-Gene kit and pGEM-T-easy vector (Promega, Madison, WI); Ultraspec-II RNA isolation kit (Biotecx Laboratories, Inc., Houston, TX); ZAP cDNA synthesis kit (Stratagene, La Jolla, CA); and Oligotex mRNA kit (Qiagen, Chatsworth, CA). Mouse uterus stage blots designed to observe gene expression during the pregnancy period were purchased from Seegene, Inc. (Seoul, Korea). All other chemicals were reagent grade.

Animals and Steroid Hormone Treatment

Outbred ICR mice (Charles River Laboratories, Wilmington, MA) were supplied by the Animal Center, College of Medicine, National Taiwan University. Animals were kept under conditions following the institutional guidelines for the care and use of experimental animals. They were housed under controlled lighting (14L:10D), and both immature (21-day-old) and sexually mature (6- to 8-wk-old) female mice were used for the study.

[1]Supported in part by grants NSC 89-2311-B-001-193 and NSC 89-2311-B-002-104 from the National Science Council, Taiwan.
[2]Correspondence: Yee-Hsiung Chen, Institute of Biochemical Sciences, College of Science, National Taiwan University, P.O. Box 23-106, Taipei 106, Taiwan, Republic of China. FAX: 886 2 2363 5038; e-mail: bc304@gate.sinica.edu.tw

Received: 10 May 2001.
First decision: 4 June 2001.
Accepted: 17 September 2001.

ISSN: 0006-3363. http://www.biolreprod.org

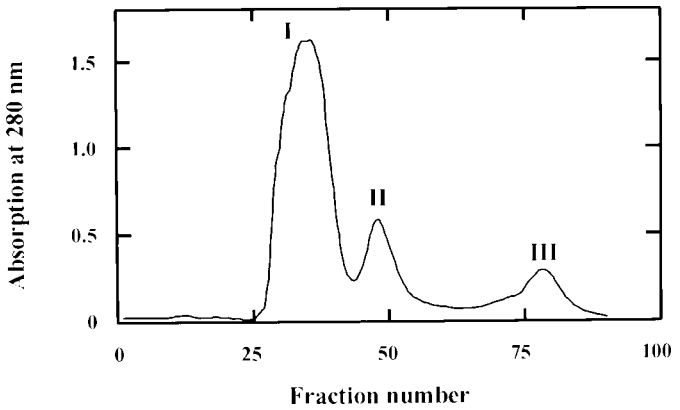

FIG. 1. Fractionation of the protein components in mouse ULF by gel filtration. The ULF collected from DES-stimulated immature mice was subjected to gel chromatography on a Sephadex G-100 column (2.6 × 80 cm) pre-equilibrated in PBS containing 1 mM EDTA at pH 7.4. The column was washed with the buffer at a flow rate of 12 ml/h. Fractions (2 ml) were collected, and their absorbance at 280 nm was recorded. The fraction II sample was passed through a CM-Affi-Gel Blue column to remove serum albumin.

The estrous cycle was staged by examining vaginal smears. The presence of a vaginal plug after mating was designated as Day (D) 1 of pregnancy.

To investigate the effect of ovarian steroids, immature mice received a daily s.c. injection of DES in corn oil (30 ng/g body weight) for 3 consecutive days, and adult female mice, in which the ovaries had been removed by bilateral oophorectomy 1 wk before initiation of steroid treatment, received a daily s.c. injection of E_2 (30 ng/g body weight) and/or P_4 (150 μg/g body weight) in corn oil for 3 consecutive days. Control animals received corn oil only. Uteri were removed from animals 12 h after the last injection.

Protein Isolation and Characterization

The ULF was immediately collected from animals after they were killed between 0900 and 1000 h on a specified day by cervical dislocation. The ULF was mixed in a final concentration of 1.0 mM EDTA and 1.0 mM PMSF. The solution was centrifuged at 14 000 × *g* for 10 min to remove insoluble materials and stored at −70°C until use. According to the previously described procedure [2], ULF collected from uteri of DES-stimulated immature mice was resolved into 3 fractions by gel filtration (denoted as fractions I-III in Fig. 1). The fraction II sample was passed through a CM-Affi-Gel Blue column (1.5 × 10 cm) to remove serum albumin.

The protein solution was concentrated using a Centricon (Millipore, Bedford, MA). The amount of protein was determined using a BCA protein assay kit. A modification of the method of Van der Geer et al. [13] was generally followed to cleave a protein with 2.36 mM CNBr in 70% formic acid at room temperature (RT) for 18 h. Automated Edman degradation for the determination of protein sequence was carried out by a gas-phase sequenator (Applied Biosystems, Foster City, CA).

Western Blot Analysis and Immunohistochemical Staining

Protein components in ULF were resolved by SDS-PAGE according to the method of Laemmli [14]. Proteins were transferred from the gel slab to a nitrocellulose membrane (Micron Separations, Inc., Westborough, MA) using an electroblotting method, which was conducted at 35 V and 4°C for 18 h in a solution containing 25 mM Tris-HCl, 197 mM glycine, and 13.3% methanol. Filters were blocked with 5.0% (w/v) skim milk and 0.1% (v/v) Tween 20 in PBS (blocking solution) for 2 h, then incubated with polyclonal goat anti-human Bf or goat anti-mouse C3 diluted to 1: 15 000 (v/v) in the blocking solution for 1 h at RT. After filters were gently agitated in 4 changes of PBS containing 0.1% Tween 20 (washing solution) for 15 min each, they were immunoreacted with horse radish peroxidase-conjugated rabbit anti-goat IgG diluted to 1:15 000 (v/v) in the blocking solution for 1 h at RT. Immunoreactive bands were revealed using an ECL substrate according to the manufacturer's instructions.

Mouse uteri were fixed in Bouin solution and embedded in paraffin. Then, 8-μm serial cross-sections were mounted on silanated glass slides. Deparaffinized sections were blocked in the blocking solution for 1 h at RT, then incubated with polyclonal goat anti-human Bf or goat anti-mouse C3 diluted to 1:1000 in the blocking solution for 1 h at RT. After slides were gently agitated in 3 changes of the washing solution for 10 min each, they were treated with biotin-conjugated rabbit anti-goat IgG (1 μg/ml) in the blocking solution for 1 h at RT. Slides were washed as mentioned, then incubated with alkaline phosphatase-conjugated streptavidin (1 μg/ml) in the blocking solution for 1 h at RT. Protein signals on specimens were observed after slides were incubated for 8 min with 0.0375% NBT and 0.0188% BCIP in a solution containing 100 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$ at pH 9.5. Slides were washed in 3 changes of water for 3 min each and counterstained with nuclear fast red for 3 min. Finally, slides were briefly washed with water and photographed under bright-field illumination using a microscope (AH3-RFCA; Olympus, Tokyo, Japan).

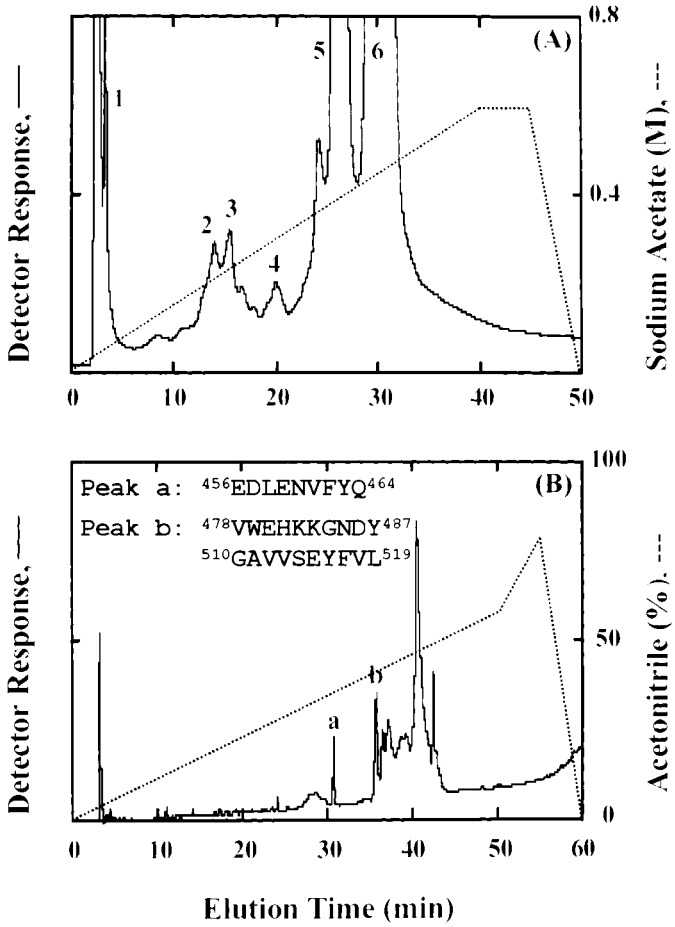

FIG. 2. Identification of Bf in mouse ULF. **A**) The fraction II sample from Figure 1 was subjected to HPLC on a Waters Protein Pak SP 5PW column pre-equilibrated with 20 mM phosphate buffer at pH 7.4. The column was washed with a linear gradient of 0–0.6 M sodium acetate at a flow rate of 1.0 ml/min for 50 min. **B**) The peak 4 sample of **A** was cleaved with CNBr, and the reaction mixture was resolved by HPLC on a reverse-phase C_{18} column (4.6 × 250 mm) by a linear gradient of 0%–60% acetonitrile at a flow rate of 1.0 ml/min. The peptide sequences determined from automated Edman degradation of peaks a and b are listed.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from uterine homogenates of mice using an Ultraspec-II RNA isolation kit. Following the standard procedures recommended for use of the ZAP cDNA synthesis kit, double-stranded cDNAs were prepared from the polyadenylated fraction, which was isolated from the total RNA using an Oligotex mRNA mini kit according to the manufacturer's instructions. We synthesized two primer pairs that were employed for polymerase chain reaction to amplify cDNAs to obtain DNA fragments of nucleotides 989–2064 in Bf cDNA [15] and nucleotides 4141 to 4586 in C3 cDNA [16]. One primer pair included the oligonucleotide 5′-TGATTGAGAAGGTGGCGAGTTACG-3′, which represents 989–1012

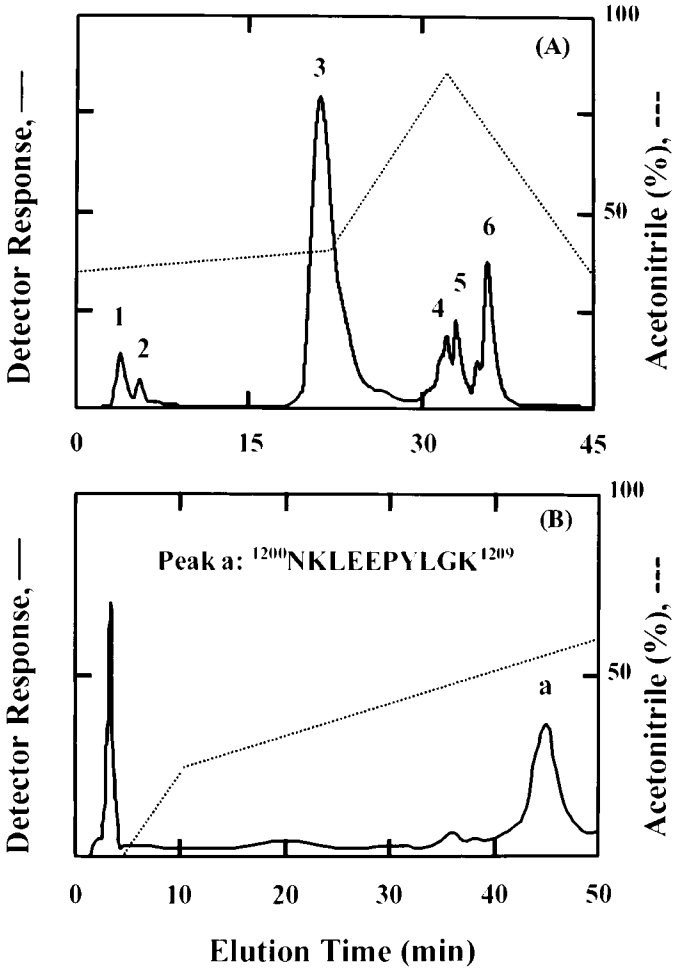

FIG. 3. Identification of C3 in mouse ULF. **A**) The protein samples collected from fraction III from Figure 1 were subjected to HPLC on a reverse-phase C_{18} column using the indicated gradient of acetonitrile at a flow rate of 1.0 ml/min. **B**) The peak 6 sample was cleaved with CNBr, and the reaction mixture was resolved by the same HPLC method described in **A** at a different gradient of acetonitrile. The peptide sequences determined from automated Edman degradation of peak a are listed.

nucleotide sequences of Bf cDNA, and the oligonucleotide 5′-GGCTTTCTTGTCCCCATTCTTGAT-3′, which is complementary to nucleotides 2041 to 2064 of Bf cDNA. The other primer pair was the oligonucleotide 5′-ACCTCAGGGTCAGCATA-3′, which represents 4141–4157 nucleotide sequences of C3 cDNA, and the oligonucleotide 5′-GCTGTGCCACAGTGAAATG-3′, which is complementary to nucleotides 4568 to 4586 of C3 cDNA sequence. Polymerase chain reaction with *Taq* polymerase was carried out for 30 cycles as follows: 94°C for 1 min, 55°C for 30 sec, and 68°C for 3 min. These two amplified DNA fragments were separately ligated to pGEM-T-easy vector via TA cloning, and recombinant plasmids were introduced into *Escherichia coli* JM109 strain using a transformation technique [17]. Positive clones containing the Bf cDNA fragment (1076 base pairs [bp]) and the C3 cDNA fragment (446 bp) were sequenced to confirm 99% identify to their parental cDNA.

The DNA fragments inserted in pGEM-T-easy and a cDNA fragment (1233 bp) of the mouse glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) inserted in pGEM3 vector were used as a template to prepare the ^{32}P-labeled cDNA probe using a Promega random-priming kit. The RNA samples (20 μg) were subjected to denaturing 1.0% agarose-formaldehyde gel electrophoresis, then blotted onto nylon membranes by capillary transfer as previously described [18]. After incubation with the prehybridization buffer (50% deionized formamide, 6× SSC [single strength: 0.15 M sodium chloride and 0.015 M sodium citrate], 5× Denhardt solution, 1.0% SDS, and 100 μg/ml of sheared salmon sperm DNA) for 2 h at 60°C, membranes were hybridized with 1 labeled probe overnight at 60°C. Following hybridization, membranes were washed using standard procedures. The RNA messages on 1 filter membrane were observed after

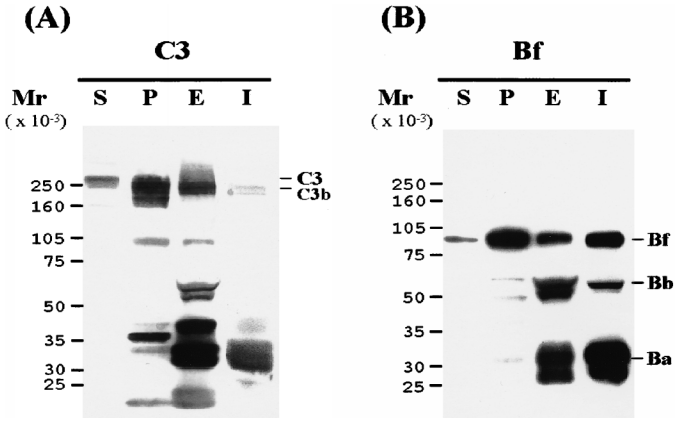

FIG. 4. Immunodetection of Bf, C3 and their fragments in mouse ULF. Approximately 20 μg of protein in normal serum (S) or in ULF collected either from adult mice during proestrus (P) and estrus (E) or from DES-stimulated immature mice (I) was subjected to SDS-PAGE on an 8%–16% gradient polyacrylamide slab gel (8.0 × 7.0 × 0.1 cm). The proteins in the gel were transferred to a nitrocellulose membrane and immunodetected by Western blot using antiserum against mouse C3 (**A**) or antiserum against human Bf (**B**) in the blocking solution (see *Materials and Methods* for details). According to the molecular size of the proteins involved in the alternative pathway of the complement system, the immunoreactive bands corresponding to C3, Bf, and their fragments, such as C3b, Bb, and Ba, are denoted. The smaller bands on the C3 Western blot may be the unidentified degradation products.

autoradiography, and probes were removed from membranes as previously described [18]. The same membrane was then hybridized with another labeled probe. Thus, hybridization with Bf, C3, and GAPDH probes was performed on the same filter membrane.

Statistical Analysis

Bonferroni post-hoc test was followed by one-way ANOVA using Instat software (Graph Pad, San Diego, CA). Data are presented as mean ± SD. A P value <0.05 was considered to be significant.

RESULTS

Demonstration of Bf and C3 in Mouse ULF

The ULF appears only in the postpubertal period in proestrus and estrus, and immature mice can be induced to secrete ULF by estrogen stimulation. As shown in Figure 1, the ULF collected from DES-stimulated immature mice was resolved into three fractions by gel filtration chromatography. The fraction II sample was further resolved by

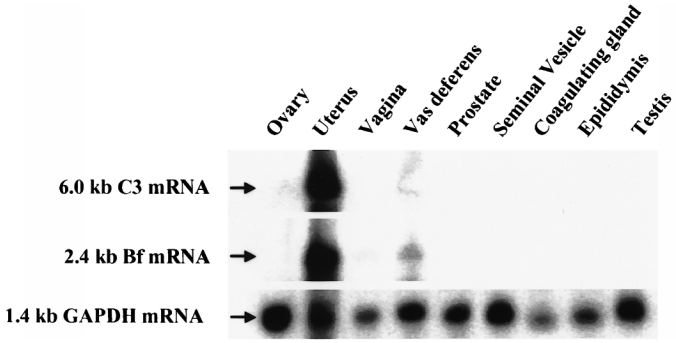

FIG. 5. Northern blot analysis for 2.4-kb Bf mRNA and 6.0-kb C3 mRNA in various tissues. Total RNA (20 μg) prepared from tissues of adult mice was run on a 1.0% formaldehyde-agarose gel and transferred to a nylon membrane. The same filter was separately hybridized with ^{32}P-labeled, random-primed DNA to the protein cDNA or GAPDH as a housekeeping gene (see *Materials and Methods* for details).

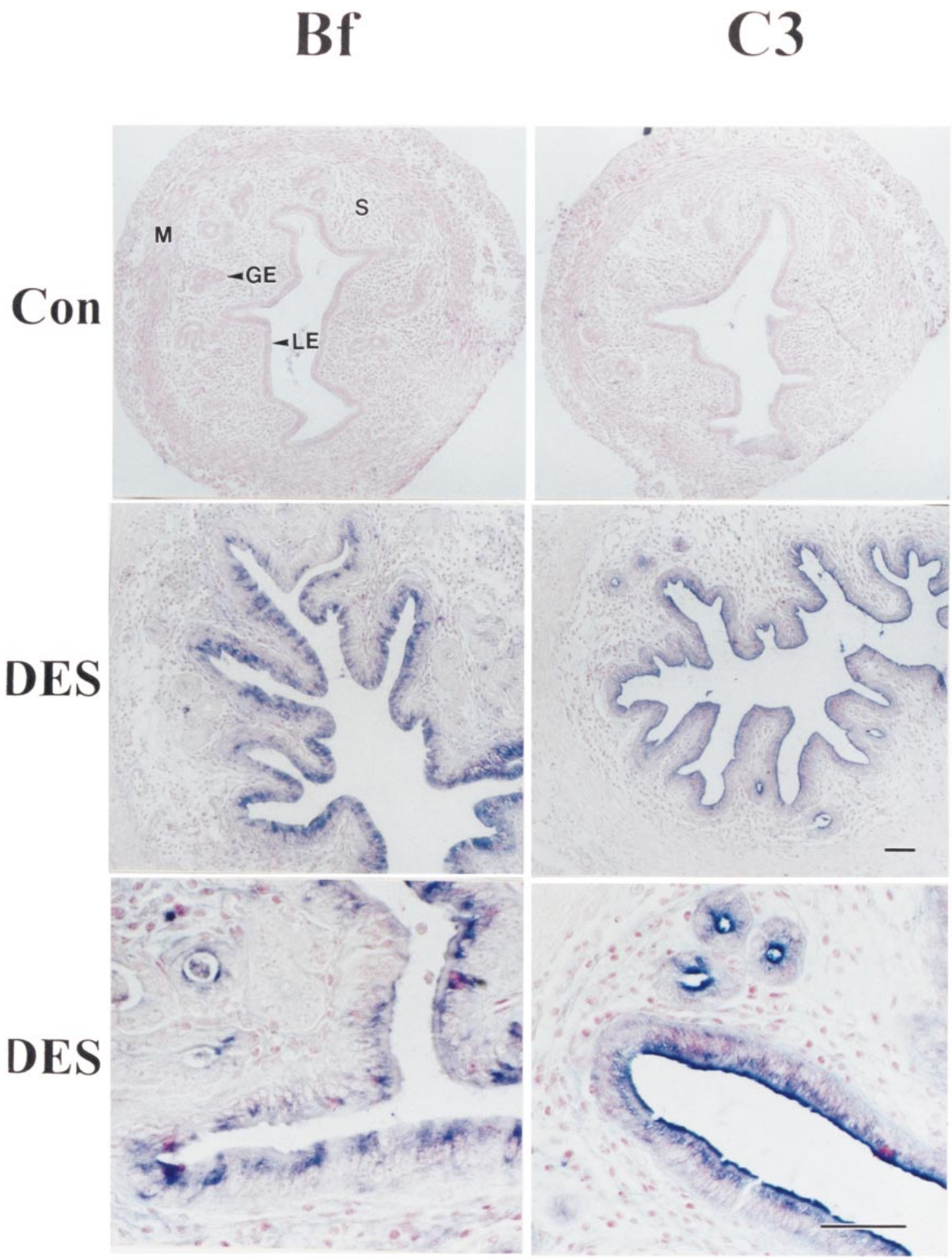

FIG. 6. Histochemical staining patterns of Bf and C3 from uterine sections of immature mice. Tissue sections of uteri from control animals (Con) and DES-stimulated animals were histochemically stained for Bf or C3 (see *Materials and Methods* for details). The staining of nuclear fast red is in pink, and the protein signals of Bf/C3, represented by alkaline phosphatase activity staining, are in dark blue. Photographs were taken with bright-field illumination. GE, Glandular epithelium; LE, luminal epithelium; M, myometrium; S, stroma. Bar = 50 μm, ×330 or ×1000.

ion-exchange high-performance liquid chromatography (HPLC) into peaks 1–6 as shown in Figure 2A. To identify Bf in mouse ULF, the peak 4 sample was cleaved with CNBr, and the reaction mixture was resolved by reverse-phase HPLC (Fig. 2B). Automated Edman degradation of either the peak a or peak b sample in Figure 2B for 10 cycles gave reliable data. A peptide sequence of EDLE-NVFYQ, which is identical to residues 456–464 of mouse Bf [15] in all positions, was determined from peak a. Two predominant amino acids could be detected at each cycle

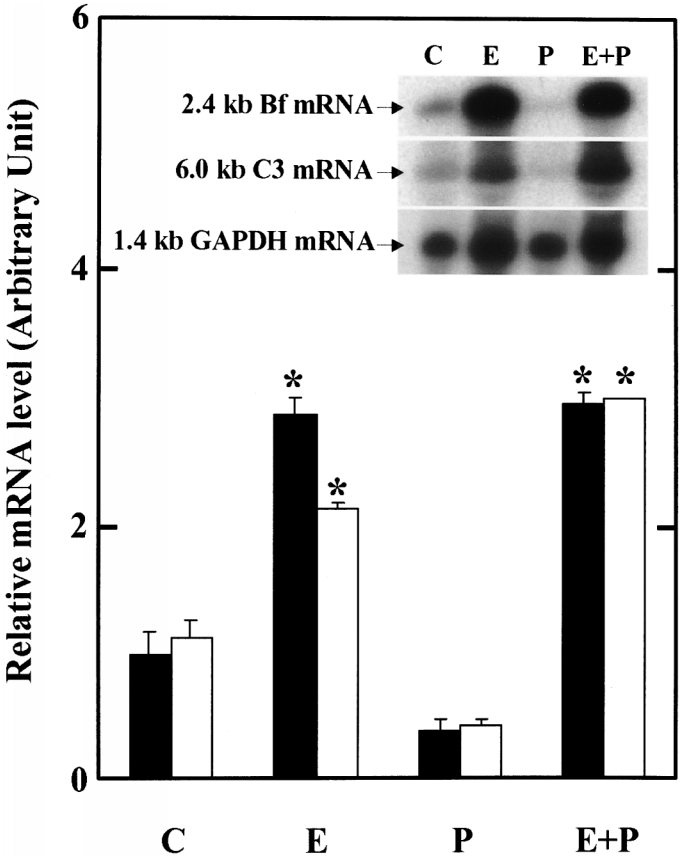

FIG. 7. The uterine levels of Bf mRNA and C3 mRNA in ovariectomized adult mice after hormonal treatment. Total RNA was prepared from the uteri of ovariectomized adults without hormonal treatment (C) or with the injection of estradiol (E_2) and/or progesterone (P_4). Detection of 2.4-kb mRNA and 6.0-kb C3 mRNA in the RNA samples followed the procedures described in Figure 5. The relative amounts of Bf mRNA (solid bars) and C3 mRNA (open bars) were determined by densitometric scanning of the autoradiogram and were adjusted with respect to the GAPDH mRNA level. Data represent the means of three individual experiments, and error bars represent the SD. *, $P < 0.001$ in the paired statistical comparison with the control as described in *Materials and Methods.*

in the analysis of peak b. The actual yields of these two sequences in an individual cycle were such that both components appeared to be present in nearly equal amounts. Matching these sequences with the protein sequences of mouse Bf were interpreted as representing residues 478–487 and residues 510–519 of the protein. To identify C3 in mouse ULF, the fraction III sample of Figure 1 was concentrated and resolved into six main peaks by reverse-phase HPLC (Fig. 3A). The peak 6 sample was cleaved with CNBr, and the reaction mixture was subjected to reverse-phase HPLC (Fig. 3B). Automated Edman degradation of peak a as shown in Figure 3B gave a peptide sequence of NKLEEPYLGK, which is identical to residues 1200–1209 in the α chain of mouse C3 [16]. Apparently, Bf and C3 coexisted in the ULF of the DES-stimulated immature mice.

Mouse Bf is a single polypeptide with a molecular mass of 95 kDa. Mouse C3 has a molecular mass of 180 kDa, comprising two polypeptide chains, an α chain cross-linked with a β chain by one disulfide bond [19]. In complement activation through the alternative pathway, C3 cleaves to C3b, and Bf cleaves to Ba and Bb, to form a C3bBb complex that serves as a C3 convertase. According to SDS-PAGE under nonreducing conditions, Bf, Ba, and Bb were identified as a protein band of 95, 63, and 33 kDa, and C3 and C3b were indistinguishable as a broad band of approximately 180–250 kDa. We detected these protein species by Western blot analysis (Fig. 4). Both C3 and Bf, but none of their degradative products, were immunodetected in normal serum. The C3/C3b and several bands of smaller molecular weight, which might be the unidentified degradation products of C3/C3b, were immunodetected in the ULF accumulated in either DES-stimulated immature mice or adult females in proestrus and estrus. The Bf, Ba, and Bb appeared in the ULF of DES-stimulated immature mice. In the ULF of adults in proestrus, Bf was predominant and Ba and Bb were in a trace, whereas in the ULF of adults in estrus, Bf, Ba, and Bb were all prominent. The simultaneous expression of Bf and C3 as well as the presence of their fragments suggested activation via the alternative pathway in the uterine lumen.

Uterine Expression of Bf and C3 after Ovarian Steroid Administration to Immature or Ovariectomized Mice

We examined the distribution of Bf mRNA and C3 mRNA in tissue homogenates of the genital tracts of adult mice using Northern blot analysis. The Bf mRNA appeared as a single RNA species of approximately 2.4 kilobases (kb). Consistent with a previous report [20], C3 mRNAs included a predominant species of 6.0 kb and three minor species of approximately 4.3, 2.3, and 1.9 kb. As shown in Figure 5, both the 2.4-kb Bf mRNA and the 6.0-kb C3 mRNA were abundant in uterine homogenates and in a trace in the vas deferens, but they were barely detectable in homogenates of other sexual glands, such as the ovary, vagina, testis, epididymis, prostate, seminal vesicle, and coagulating gland. Apparently, transcription of both RNA messages in the mouse reproductive tract was tissue specific. This prompted us to study how ovarian steroids regulate the expression of Bf, C3, and their RNA messages in mouse uteri.

Both Bf mRNA and C3 mRNA were detected in uterine homogenates of immature animals, indicating a basal transcription of both protein genes before animal maturity. Compared with the corresponding basal transcription, DES enhanced Bf and C3 mRNA expression 3.6- and 3.7-fold, respectively (data not shown). These mRNA levels were positively correlated with the immunohistochemical results shown in Figure 6. Neither Bf nor C3 was immunodetected in uterine cells of immature mice. However, DES stimulated the uterine growth of immature animals, and uterine cells, particularly the luminal epithelium, became hypertrophied compared with the cell morphology of control animals. An intensive immunohistochemical staining of both proteins appeared around the apical region in the cytoplasm of luminal and glandular epithelia. Meanwhile, a much lower immunoactivity in the stroma toward the luminal side was detected, but almost no immunoreactivity was noted in the stroma on the other side or in the myometrium. No immunoreactivity of Bf and C3 appeared on the luminal surface of the endometrium, suggesting no deposition of Bf and C3 on the cell surface. These data support the idea that, among uterine cells, DES-stimulated uterine expression of Bf mRNA and C3 mRNA and their translated proteins mainly occurs in the endometrial epithelia. Apparently, Bf and C3 in the ULF are a primary result of their secretion from the luminal epithelium.

Removal of ovaries by bilateral oophorectomy caused atrophy of adult uteri. Administration of E_2 (30 ng/g body

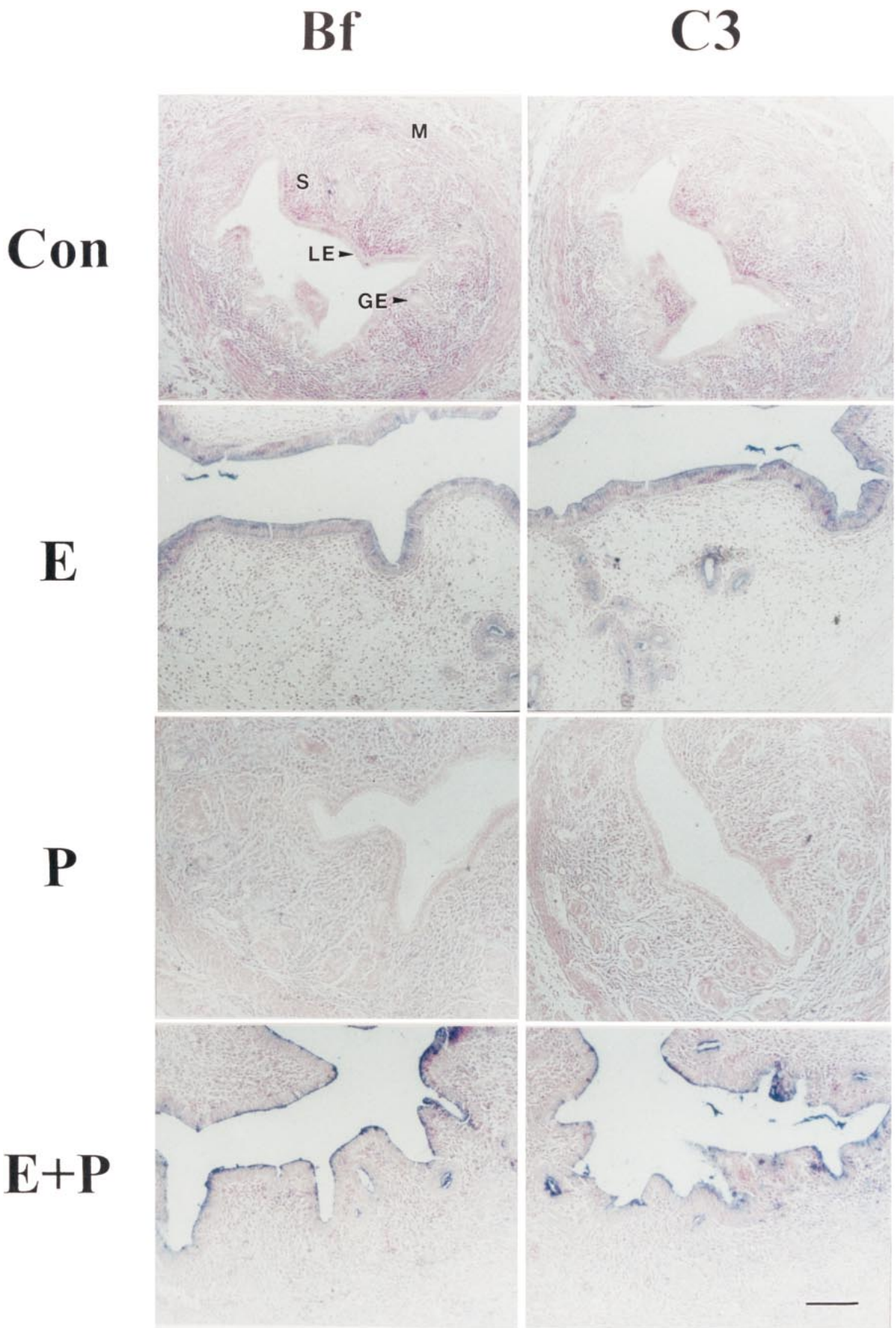

FIG. 8. Histochemical staining patterns of Bf and C3 on the uterine sections of ovariectomized adult mice. The uterine sections prepared from ovariectomized female mice without hormonal administration (Con) or after injection of estradiol (E_2) and/or progesterone (P_4) were histochemically stained as described in Figure 6. Bar = 100 μm, ×330.

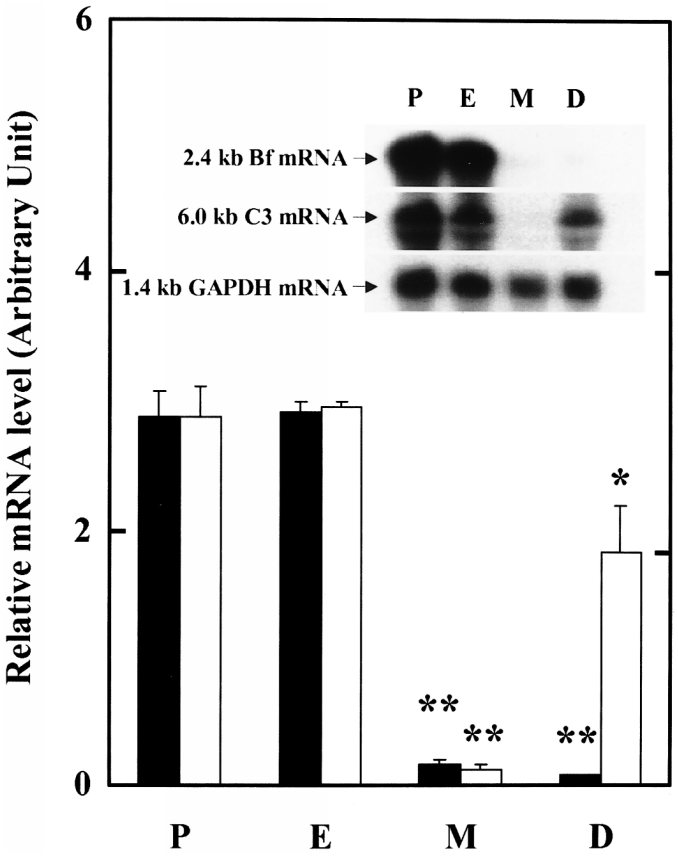

FIG. 9. Levels of Bf mRNA and C3 mRNA in the mouse uterus at different stages of the estrous cycle. For each experiment, the uteri of 5 mature mice during proestrus (P), estrus (E), metestrus (M), or diestrus (D) were homogenated. The relative amounts of Bf mRNA (solid bars) and C3 mRNA (open bars) in the total RNA (20 μg) prepared from the tissue homogenates were determined by the same procedures as described in Figure 7. Data represent the means of three individual experiments, and error bars represent the SD. *, $P < 0.01$ and **, $P < 0.001$ in the pairwise comparison relative to the mRNA level of proestrus as described in *Materials and Methods.*

weight) alone to ovariectomized adults for 3 consecutive days restored uterine cells to a hypertrophied stage, whereas cells remained atrophied after injection of P_4 alone (150 μg/g body weight) to animals. We examined how the hormonal treatment of ovariectomized adults affected the uterine expression of both Bf and C3. The levels of both Bf mRNA and C3 mRNA increased remarkably in E_2-treated animals but tended to decrease in P_4-treated animals compared with the basal transcription in controls. The E_2-stimulated Bf mRNA expression was slightly enhanced, whereas the E_2-stimulated C3 mRNA expression was considerably potentiated, as the animals were injected with E_2 and P_4 together (Fig. 7). Parallel to the change in mRNA level, immunohistochemical patterns of uterine sections (Fig. 8) revealed that the intensive immunoreactivity of both proteins in animals treated with E_2 alone or with E_2 and P_4 together was similar to that in DES-stimulated immature mice, compared with no immunoreactivity in control and P_4-treated animals.

Uterine Expression of Bf and C3 in Adult Mice

We compared levels of Bf and C3 RNA messages in uterine homogenates and examined the distribution of Bf and C3 in tissue sections at various stages of the estrous cycle and during the pregnancy period. As shown in Figure 9, both RNA messages were abundant during proestrus and estrus but declined rapidly during metestrus. It was of interest to note that Bf mRNA remained at an undetectable level, but that C3 mRNA increased, during diestrus. This characteristic change in mRNA levels was positively correlated with the immunohistochemical staining patterns of both proteins in tissue sections (Fig. 10). The immunoreactivity of both Bf and C3 was strong in glandular and luminal epithelial cells of the endometrium during proestrus and estrus, compared to no immunoreactivity in the stroma and myometrium. Immunostaining intensity for both Bf and C3 in metestrus was weakly positive. During diestrus, almost no cells immunoreactive to Bf antiserum were seen, but an intensive immunoreactivity to C3 appeared in glandular and luminal epithelia.

Figure 11 shows the levels of Bf mRNA and C3 mRNA in uterine homogenates from D1 to D19 after copulatory plug formation. The postimplantation uterine samples did not contain the placenta or the conceptus tissues, but some decidual samples may be contaminated during sampling of the uterus. The Bf mRNA was abundant on D1, increased to a slightly higher level on D2, and declined to an almost undetectable level on D3. From D10 on, Bf mRNA increased to a fairly constant, low level until delivery. The C3 mRNA was at a high level on D1, declined rapidly from D2 to D9 to a low level, remarkably increased from D10 to reach a maximum level on D18, and quickly dropped near delivery. The change in mRNA levels during the preimplantation period was in accord with the immunoreactivity of Bf and C3 as detected in the uterine section (Fig. 12). Immunohistochemical staining of either protein was intensive in glandular and luminal epithelia on D1. Very weakly immunoreactive cells were seen on D3 and D4. Immunoreactivity of Bf appeared in the luminal and glandular epithelium, whereas C3 was immunodetected only in the glandular epithelium on D2.

DISCUSSION

Among the sexual organs of adult female mice, Bf and C3 are exclusively expressed in the uterus (Fig. 5). Because Bf and C3 were not detected in the glandular or luminal epithelia of endometria in either immature or ovariectomized adult mice, basal levels of Bf and C3 mRNAs in uterine homogenates of these animals may be attributed to their expression in cells other than luminal epithelial cells. Estrogen administration to these animals remarkably increases the Bf and C3 mRNA levels as well as the immunoreactivity of Bf and C3 in glandular and luminal epithelial cells of the endometrium (Figs. 6–8), indicating that the simultaneous syntheses of these two proteins are estrogen stimulated in mouse endometrium. Moreover, estrogen-stimulated C3 gene expression is significantly enhanced, but estrogen-stimulated Bf gene expression is only slightly affected, by administration of P_4 to E_2-treated animals, despite P_4 alone tending to suppress their expression, suggesting that P_4 enhances C3 gene expression in the estrogen-primed uterus.

Because the response of endometrial epithelial cells to E_2 and P_4 is rarely maintained under in vitro conditions [21, 22], our analyses of Bf and C3 gene expression in mouse uterus during the natural estrous cycle and the pregnancy period remain essential for investigating how ovarian steroids regulate their expression in the uterus in vivo. In response to physiological changes in the reproductive tract, serum ovarian steroid concentrations in mouse and rat dur-

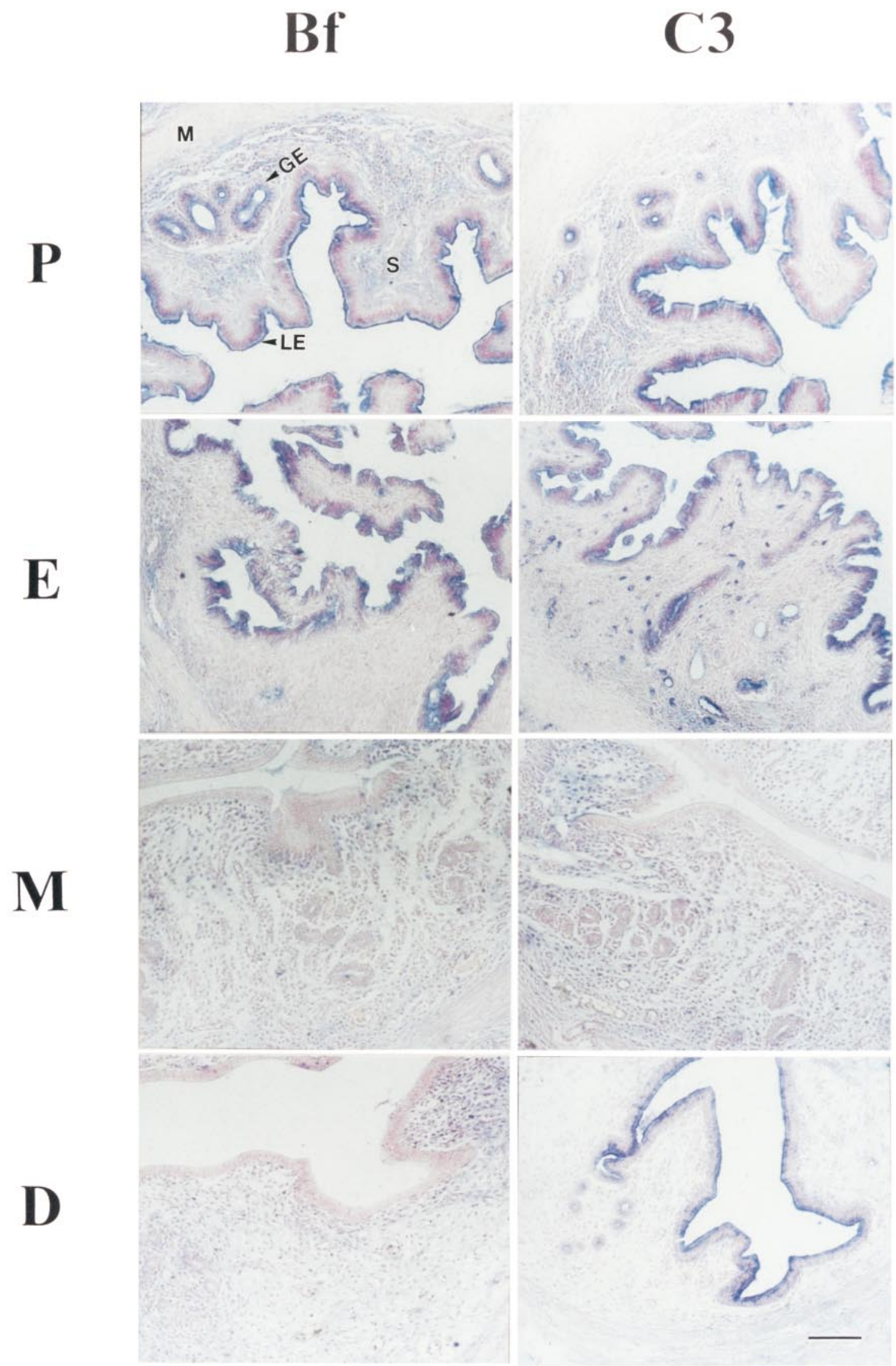

FIG. 10. Immunolocalization of Bf and C3 in mouse uterus at different stages of the estrous cycle. The uterine sections of animals during proestrus (P), estrus (E), metestrus (M), or diestrus (D) were histochemically stained according to the procedures described in Figure 6. Bar = 100 μm, ×330.

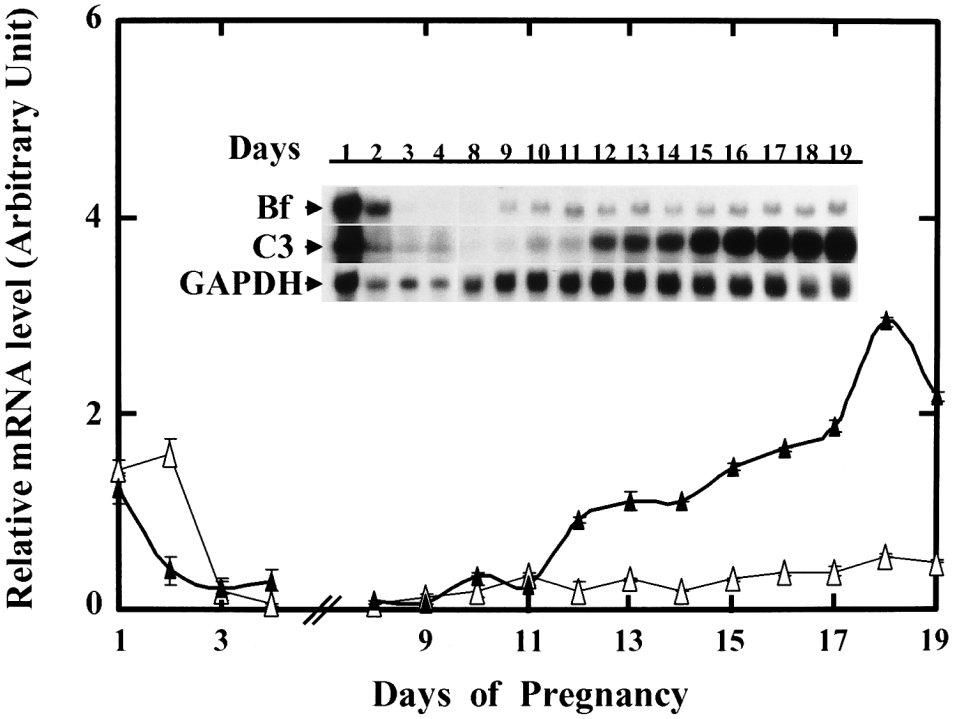

FIG. 11. Changes in Bf mRNA and C3 mRNA levels during the pregnancy period. The uteri of 5 pregnant mice on the specified day after vaginal plug formation were homogenated. The relative amounts of Bf mRNA (△) and C3 mRNA (▲) in the total RNA (20 μg) prepared from the tissue homogenates were determined by the same procedures as described in Figure 7. Data represent the means of three individual experiments.

ing the estrous cycle and pregnancy period have been measured [23–25]. Correlating the endometrial expression of Bf and C3 to serum concentrations of ovarian steroids may shed some light on the in vivo hormonal regulation of uterine Bf and C3 synthesis and secretion. The stimulation of Bf and C3 gene expression occurs in proestrus and estrus, during which time a large E_2 surge is accompanied by a basal P_4 concentration. Their expressions sharply decline in metestrus, when both serum E_2 and P_4 are at a basal level, suggesting that the concentration of ovarian steroids in endometrial cells might not reach a threshold sufficient to exert their effects on the expression of these two genes. In diestrus, when serum E_2 is at a low level and serum P_4 increases to a high level, C3 gene, but not Bf gene, is expressed, suggesting that the low serum E_2 level is enough for C3 gene expression but is insufficient for Bf gene expression; furthermore, E_2-stimulated C3 gene expression is enhanced by P_4. The endometrial expression of Bf and C3 during D1 and D2 of the preimplantation period may reflect preovulatory estrogen stimulation. Both Bf and C3 gene are barely expressed from D3 to D9, during which time serum E_2 is maintained at a basal level and serum P_4 increases. During late pregnancy, from D10 to D20, serum E_2 rises to a level as high as that in proestrus and estrus, and serum P_4 increases to a level much higher than that in the estrous cycle. Expression of C3 gene increases remarkably, whereas expression of Bf gene is not as prominent as that in proestrus and estrus. Again, P_4 potentiates E_2-stimulated C3 gene expression, but some unknown factor other than P_4 may suppress the stimulation of Bf gene expression by E_2. Apparently, the hormone-regulated endometrial expression of these two genes during the estrous cycle and the pregnancy period is congruent with the characteristics of their expression in the uteri of ovariectomized adult mice after hormonal treatments (Figs. 7 and 8).

Hasty et al. [8] immunodetected relatively large amounts of C3 and Bf in the glandular epithelium of human endometrium in the P_4-dominant luteal phase, but only small amounts of these two proteins in tissue in the E_2-dominant proliferative phase, during the menstrual cycle. They raised the possibility that synthesis of these two proteins in human endometrium may be regulated by P_4. This concept seems to be supported by an increase in their syntheses in patients receiving exogenous P_4 treatments [7]. On the other hand, a summary of our data in this work clearly illustrates that P_4 alone produces almost no effect, but that E_2 stimulates the gene expression of Bf and C3 in mouse endometrium. A similar situation has been previously illustrated for C3 mRNA expression in the uterus of immature rats receiving exogenous E_2 treatment [11, 12]. The apparent P_4 regulation in human endometrium and E_2 regulation in rodent endometrium may suggest different molecular machinery between orders of mammals in the control of endometrial C3 and Bf gene expression. Even in the same family, P_4 produces an inhibitory effect on E_2-stimulated C3 gene expression in rat endometrium [12], but it potentiates E_2-stimulated C3 gene expression in mouse endometrium (Figs. 7 and 8). Many lines of evidence indicate that the primary action of steroid hormones at the level of transcription involves the participation of a complex formed by a steroid and its receptor. Such a receptor complex modulates cooperative interactions of hormone response elements, such as estrogen response element (ERE), progesterone response element (PRE), and glucocorticoid response element (GRE), and other accessory response elements in the steroid-responsive genes. The C3 gene promoter has been used as a standard promoter in studying estrogen response. Fan et al. [26] has identified three functional EREs in the human C3 gene promoter. Vollmer and Schneider [27] have assayed the expression of C3 as an E_2-induced protein to assess the estrogenic and antiestrogenic potency of antiestrogens. As yet, no functional steroid hormone response elements have been demonstrated in the mouse/rat C3 and Bf gene promoters. We applied the method of Quandt et al. [28] to predict consensus matches in the mouse C3 and Bf gene promoter [29, 30]: No potential PRE/GRE, but three and five potential EREs, are respectively predicted in the mouse C3 and Bf gene promoter. Possibly, P_4 regulation

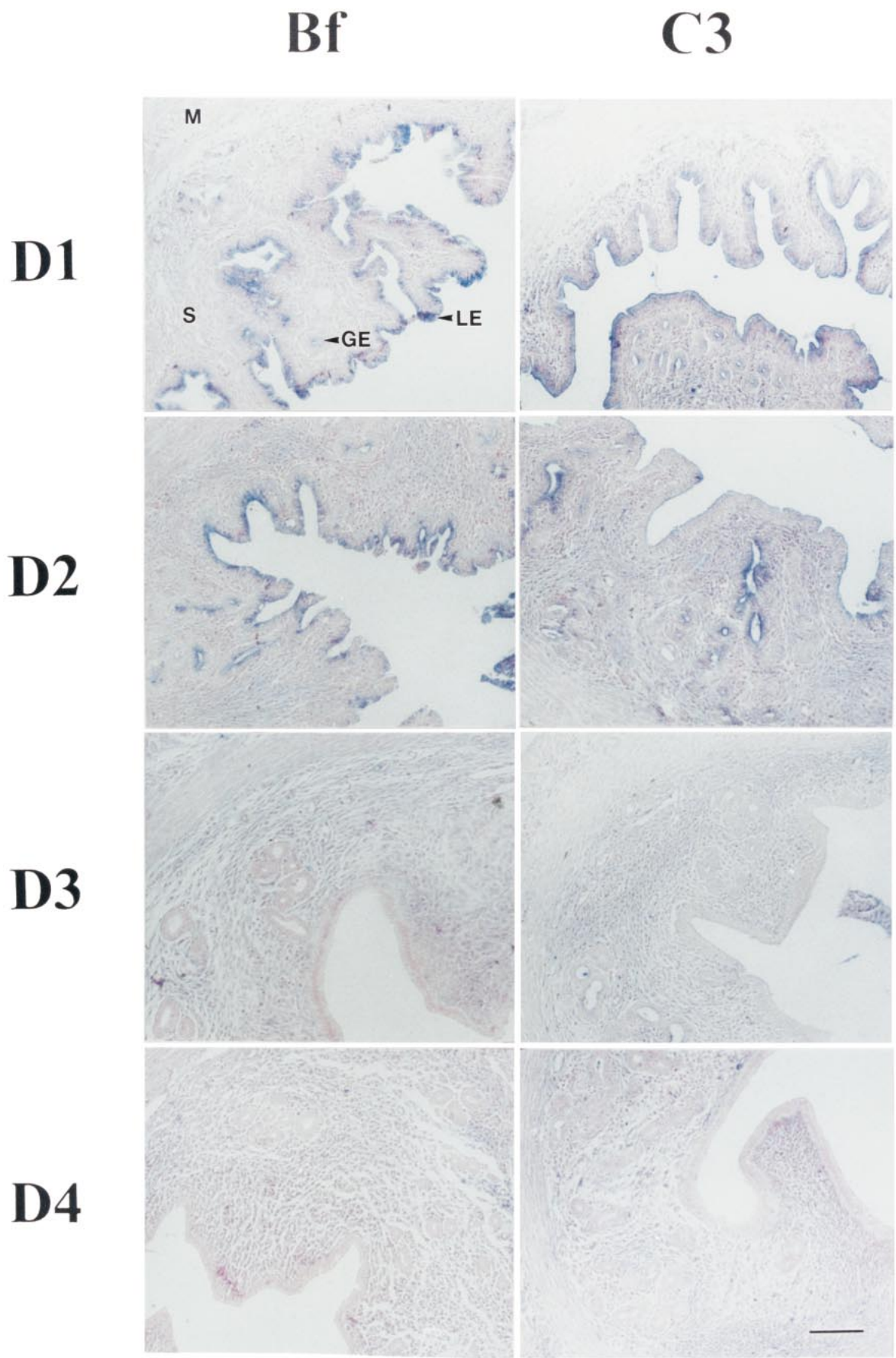

FIG. 12. Immunolocalization of Bf and C3 in mouse uterus during the preimplantation period. The uterine sections were prepared from animals during the first 4 days of pregnancy (D1–D4) and were histochemically stained as described in Figure 6. Bar = 100 μm, ×330.

may represent an indirect action through stimulation of various cytokines. In fact, functional response elements of interleukin 1 and interferon-γ are present in the mouse and human Bf gene [29], and functional response elements of interleukin 1 and interleukin 6 are present in the mouse C3 gene [30]. The study of De et al. [31] indicates that interleukin 1, interleukin 6, and tumor necrosis factor-α are produced in the mouse uterus during the estrous cycle and are induced by estrogen and P_4. The RNA messages of these cytokines are also present in the mouse uterus at early preimplantation [32, 33]. Dissimilar to the C3 and Bf gene expressions described, E_2-stimulated expressions of lactoferrin, a major ULF protein [2, 34], and of 24p3 protein, a lipocalin [3, 4, 35], in mouse endometrium are inhibited by P_4. Further studies are needed to unravel the molecular mechanism underlying how P_4 functions as an enhancer or silencer to regulate E_2-stimulated gene expression.

Once C3 and Bf are simultaneously synthesized in the endometrium, they secrete to the uterine lumen to participate in the initiation and amplification of alternative pathways (Fig. 4). In addition to the inflammatory host defense against the invasion of viruses and bacteria, an exact role, if any, for these complement proteins in reproductive processes has yet to be determined.

REFERENCES

1. Pentecost BT, Teng CT. Lactotransferrin is the major estrogen inducible protein of mouse uterine secretions. J Biol Chem 1987; 262: 10134–10139.
2. Teng CT, Pentecost BT, Chen YH, Newbold EM, Eddy EM, McLachlan JA. Lactotransferrin gene expression in the mouse uterus and mammary gland. Endocrinology 1989; 124:992–999.
3. Chu ST, Huang HL, Chen JM, Chen YH. Demonstration of a glycoprotein derived from the 24p3 gene in mouse uterine luminal fluid. Biochem J 1996; 316:545–550.
4. Huang HL, Chu ST, Chen YH. Ovarian steroids regulate 24p3 expression in mouse uterus during the natural estrous cycle and the preimplantation period. J Endocrinol 1999; 162:11–19.
5. Perricone R, Pasetto N, De Carolis C, Vaquero E, Piccione E, Baschieri L, Fontana L. Functionally active complement is present in human ovarian follicular fluid and can be activated by seminal plasma. Clin Exp Immunol 1992; 89:154–157.
6. Jin M, Larsson A, Ove Nilsson B. A functionally active complement system is present in uterine secretion of the mouse prior to implantation. Am J Reprod Immunol 1991; 26:53–57.
7. Hasty LA, Brockman WW, Lambris JD, Lyttle CR. Hormonal regulation of complement factor b in human endometrium. Am J Reprod Immunol 1993; 30:63–67.
8. Hasty LA, Lambris JD, Lessey BA, Pruksananonda K, Lyttle CR. Hormonal regulation of complement components and receptor throughout the menstrual cycle. Am J Obstet Gynecol 1994; 170:168–175.
9. Bischof P, Planas-Basset D, Meisser A, Campana A. Investigations on the cell type responsible for the endometrial secretion of complement component 3 (C3). Hum Reprod 1994; 9:1652–1659.
10. Sayegh RA, Tao XJ, Awwad JT, Isaacson KB. Localization of the expression of complement component 3 in the human endometrium by in situ hybridization. J Clin Endocrinol Metab 1996; 81:1641–1649.
11. Sundstrom SA, Komm BS, Ponce-de-Leon H, Yi Z, Teuscher C, Lyttle CR. Estrogen regulation of tissue-specific expression of complement C3. J Biol Chem 1989; 246:16941–16947.
12. Brown EO, Sundstrom SA, Komm BS, Yi Z, Teuscher C, Lyttle CR. Progesterone regulation of estradiol-induced rat uterine secretory protein, complement C3. Biol Reprod 1990; 42:713–719.
13. Van der Geer P, Luo K, Sefton BM, Hunter T. Phosphopeptide mapping and phosphoamino acid analysis on cellulose thin-layer plates. In: Hardie DG (eds.), Protein Phosphorylation: A Practical Approach. Oxford: IRL Press, 1993: 40–41.
14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.
15. Ishikawa N, Nonaka M, Wetsel RA, Colten HR. Murine complement C2 and factor B genomic and cDNA cloning reveals different mechanisms for multiple transcripts of C2 and B. J Biol Chem 1990; 265: 19040–19046.
16. Fey GH, Lundwall A, Wetsel RA, Tack BF, de Bruijn MH, Domdey H. Nucleotide sequence of complementary DNA and derived amino acid sequence of murine complement protein C3. Philos Trans R Soc Lond B Biol Sci 1984; 306:333–344.
17. Hanahan D, Jessee J, Bloom FR. Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol 1991; 204:63–113.
18. Maniatis T, Fritsch EF, Sambrook J. Molecular Cloning—A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989: 7.46, 9.58.
19. Bednarczyk JL, Capra JD. Posttranslational processing of the murine third component of complement. Scand J Immunol 1988; 27:83–95.
20. Hong MH, Jin CH, Sato T, Ishimi Y, Abe E, Suda T. Transcriptional regulation of the production of the third component of complement (C3) by 1α,25-dihydroxyvitamin D_3 in mouse marrow-derived stromal cells (ST2) and primary osteoblastic cells. Endocrinology 1991; 129: 2774–2779.
21. Tomooka Y, DiAugustine RP, McLachlan JA. Proliferation of mouse uterine epithelial cells in vitro. Endocrinology 1986; 118:1011–1018.
22. Julian J, Carson DD, Glasser SR. Polarized rat uterine epithelium in vitro: constitutive expression of estrogen-induced proteins. Endocrinology 1992; 130:79–87.
23. Walmer DK, Wrona MA, Hughes CL, Nelson KG. Lactoferrin expression in the mouse reproductive tract during the natural estrous cycle: correlation with circulating estradiol and progesterone. Endocrinology 1992; 131:1458–1466.
24. Smith MS, Freeman ME, Neill JD. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 1975; 96:219–226.
25. Bridges RS. A quantitative analysis of the roles of dosage, sequence and duration of estradiol and progesterone exposure in the regulation of maternal behavior in the rat. Endocrinology 1984; 114:930–940.
26. Fan JD, Wagner BL, McDonnell DP. Identification of the sequences within the human complement 3 promoter required for estrogen responsiveness provides insight into the mechanism of tamoxifen mixed agonist activity. Mol Endocrinol 1996; 10:1605–1616.
27. Vollmer G, Schneider MR. The rat endometrial adenocarcinoma cell line RUCA-I: a novel hormone-responsive in vivo/in vitro tumor model. J Steroid Biochem Mol Biol 1996; 58:103–115.
28. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new, fast, and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 1995; 23: 4878–4884.
29. Nonaka M, Gitlin JD, Colten HR. Regulation of human and murine complement: comparison of 5′ structural and functional elements regulating human and murine complement factor B gene expression. Mol Cell Biochem 1989; 89:1–14.
30. Kawamura N, Singer L, Wetsel RA, Colten HR. *cis*- And *trans*-acting elements required for constitutive and cytokine-regulated expression of the mouse complement C3 gene. Biochem J 1992; 283:705–712.
31. De M, Sanford TR, Wood GW. Interleukin 1, interleukin 6, and tumor necrosis factor alpha are produced in the mouse uterus during the estrous cycle and are induced by estrogen and progesterone. Dev Biol 1992; 151:297–305.
32. De M, Sanford TR, Wood GW. Expression of interleukin 1, interleukin 6 and tumor necrosis factor alpha in mouse uterus during the periimplantation period of pregnancy. J Reprod Fertil 1993; 97:83–89.
33. McMaster MT, Newton RC, Dey SK, Andrews GK. Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. J Immunol 1992; 148:1699–1705.
34. McMaster MT, Teng CT, Dey SK, Andrews GK. Lactoferrin in the mouse uterus: analyses of the preimplantation period and regulation by ovarian steroids. Mol Endocrinol 1992; 6:101–111.
35. Chu ST, Lin HJ, Huang HL, Chen YH. The hydrophobic pocket of 24p3 protein from mouse uterine luminal fluid: fatty acid and retinol binding activity and predicted structural similarity to lipocalins. J Pept Res 1998; 52:390–397.