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Isolation of Complementary Deoxyribonucleic Acids Encoding Putative Secreted and Membrane-Bound Folate Binding Proteins from Endometrium of Swine[1]

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ABSTRACT

Two distinct forms of endometrial folate binding protein (FBP) cDNAs were isolated using reverse transcription-polymerase chain reaction and 3′ and 5′ rapid amplification of cDNA ends (RACE) procedures. On the basis of the absence or presence of an intact glycophosphatidylinositol linkage site in the C terminus of the predicted amino acid sequences, the two forms appear to encode secreted and membrane-bound forms of FBP. The cDNAs for the putative secreted and membrane forms encoded 252- and 249-amino acid proteins, respectively, that were 73% identical with each other and were 66–82% identical with other known FBPs. However, the nucleotide sequences within the 5′ untranslated region and from codons 224 and 223 of the secreted and membrane forms, respectively, to the 3′ ends of each RNA, were divergent. The divergence in the 3′ ends of the two cDNAs was exploited to determine changes in concentrations of each mRNA in the endometrium during the estrous cycle and early pregnancy. Northern blots of endometrial total RNA probed with a putative secreted FBP specific probe indicated that mRNA concentrations do not change during early pregnancy. In contrast, blots probed with a putative membrane FBP specific probe indicated that mRNA concentrations increase dramatically from Day 15 to Day 24 of pregnancy. Finally, N-terminal amino acid sequencing of FBP purified from Day 15 pregnant uterine flushings matched the secreted form of FBP mRNA. These data are consistent with a role for putative secreted and membrane-bound forms of FBPs in the transport of folate to the developing swine conceptus during early pregnancy.

INTRODUCTION

Folic acid is a vitamin that is a required cofactor in the transfer of methyl groups between substrates [1]. Synthesis of the purine ring, methionine, and thymidine all require metabolites of folic acid, and these compounds are all essential for continued cell division and growth [2]. Deficiencies in folic acid have the greatest deleterious effect on those tissues undergoing rapid cell division, such as the erythron [3] and the developing conceptus [4] during pregnancy.

One component of the mechanism controlling folate delivery to these and other tissues is a group of folate binding proteins (FBPs)/folate receptors (FRs) [5]. The soluble FBPs are thought to transport and protect folic acid in extracellular fluids. Membrane-bound FRs are thought to be involved in specific uptake of folic acid by cells. In mice and humans, cDNAs for two different types of folate receptor (FR-α and -β) have been characterized [6–8]. In humans, a third, secreted form (FR-γ) has also been reported [9]. Receptor forms are anchored to the cell membrane by a glycophosphatidylinositol (GPI) linkage site present in the C-terminal end of the protein [10, 11]. The secreted form does not have a glycophosphatidylinositol linkage site [12]. Receptor forms can be liberated from cell membranes either by proteolysis [13] or by cleavage of the phosphatidylinositol linkage site by phospholipase C [10]. Thus, all forms are potentially capable of being released from cells. Serum [14], bovine milk [15], and pig uterine flushings [16, 17] contain FBPs, but their origins (receptor or secreted form), and therefore mechanisms governing control of their concentrations, have not been investigated.

Our laboratory is interested in factors influencing conceptus development and fetal erythropoiesis during early pregnancy in swine. It has been demonstrated by Western blotting that the amount of FBP increases dramatically from Day 10 to 15 of pregnancy in uterine flushings of swine [17]. The objective of the current study was to clone the cDNA for this protein to begin to understand the mechanisms governing intrauterine concentrations of FBP and therefore folate delivery to the conceptus during pregnancy in pigs.

MATERIALS AND METHODS

To generate an initial partial cDNA for endometrial FBP, four forward (1–4, Table 1) and four reverse (1–4, Table 1) primers were designed on the basis of the mouse FR-β cDNA. Primers were designed to be within the coding region of the mature protein, have a melting temperature of 70°C, and be nonoverlapping. Total RNA was isolated from endometrial tissue collected from a Day 30 pregnant pig at slaughter using the RNeasy kit (Qiagen, Santa Clarita, CA). Total RNA was reverse-transcribed using oligo 18-dT, and then 1 μl of the resultant product was amplified with each of the 16 combinations of the above primers. To determine which combinations resulted in the correct product, products were electrophoresed, blotted onto Hybond membrane (Amersham Pharmacia Biotech, Uppsala, Sweden), and probed with mouse FR-β cDNA (kindly donated by K.E. Brigle, Virginia Commonwealth University, MCV Hospitals, Richmond, VA) labeled with [^{32}P]dATP using the Megaprime DNA labeling system (Amersham, Arlington Heights, IL). Blots were hybridized using Rapid-Hyb (Amersham, Arlington Heights, IL) with 1×10^6 cpm per milliliter of radiolabeled probe. Blots were washed twice in double-strength SSC (single-strength SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 0.1% SDS at room temperature, and then twice in 0.5-strength SSC, 0.1% SDS at 50°C. The product generating the greatest signal by Southern blotting (forward 3-reverse 4, not shown) was

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[1]Mention of product names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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TABLE 1. Primers used at each stage in the characterization of the FBP cDNAs.

Stage	Primer	Sequence
Initial clone	Forward	
	1	ACCTACTCAACGTTTGCATGGATGC
	2	AAACACCATAAGACAAAGCCGGGCC
	3	CCGAGGACAAGCTGCATGACCAGT
	4	TCCATGGAAGAAAAATGCCTGTTGCT
	Reverse	
	1	AGCCAGGGAGCCATAATGACAGCAC
	2	GGCCAGGCTGGGCACAAGAAGTAC
	3	GGGGCACAGTCCCAGATGTCATAAA
	4	ATTGGGATTGCCCTGGGTTGAGTC
3′-RACE	Reverse transcription	CACACACACACAGGATCGAATTCAAGC$(T)_{18}$
	Forward	
	1	GAGTGCTCGCCCAACCTG
	2	CGCCACTTCATTCAAGACACC
	Reverse	
	1	CACAGGATCCGAATTCAAGC
5′-RACE	Reverse transcription	
	1	TGAGCTCCAGTTCCAGCC
	Ligation oligonucleotides	
	1	P-GAATTCGATTACGGAAGACGCTCAA-NH_2
	2	P-TCTAGAGTCAAGTCAGGCAATGTCGTT-NH_2
	3	P-GCGGCCGCCCGTGATTGCCACTAGATGA-NH_2
	4	P-GAATTCCTACTGACTACGGGCACTGGA-NH_2
	Forward	
	1	TTGAGTGCGTCTTCCGTAATC
	2	AACGACATTGCCTGACTTGAC
	3	TCTATCTACTGGCAATCACGG
	4	TCCAGTGCCCGTAGTCAGTAG
	Reverse	
	1	ACGTTCAGGATCCGCTCTC
	2	AGGAGCACGAAGAGCGTC
	3	TCATGCAGGCCATCTTCC
Northern blotting	Forward	
	1	GATCGGGCCTCTCCTGAC
	2	GCCTGGCCTCTCCAGTTCG
	Reverse	
	1	ATTGGAAGAGGCCATCAGAG
	2	GGTTTATTCAGAGTCCCCCC

subcloned using the PCR-II vector (Invitrogen, Carlsbad, CA). Positive clones were sequenced in both directions.

The 3′ and 5′ ends of the cDNA for FBP were isolated using rapid amplification of cDNA ends (RACE). For 3′-RACE, total RNA isolated from a Day 30 pregnant gilt was reverse-transcribed (Table 1), and the resulting product was then amplified with the forward 1 and reverse 1 primers indicated in Table 1. This product was then reamplified using the above reverse primer and forward 2 as the forward primer, and the product was then subcloned into PCR-II vector and sequenced in both directions.

For 5′-RACE, a modification of the procedure of Apte and Siebert [18] was used. Briefly, total RNA from endometrium of a Day 30 pregnant gilt was reverse-transcribed using the primer in Table 1. RNA was degraded, and cDNA was separated from free nucleotides using glass milk as described, lyophilized, and redissolved in 10 μl sterile water. One-microliter aliquots of this solution were then used in a ligation reaction using T4 RNA ligase and the four different linker oligonucleotides listed in Table 1. The resulting products were then amplified using the forward primers 1–4 (Table 1), respectively, as forward 5′-RACE primers, and the reverse primer 1 as the reverse primer. Resultant products were electrophoresed and Southern-blotted as previously described (not shown), and the products from the two forward primers generating the best signal (forward 2 and 4) were subcloned into pNoTA/T7 vector (5 Prime→3 Prime, Boulder, CO) and sequenced. To further evaluate possible divergence in the 5′ untranslated region (UTR) of each mRNA, the above polymerase chain reaction (PCR) product was reamplified using the appropriate forward 5′-RACE primer and reverse primers 2 and 3, which were specific to each cDNA. These products were subcloned into PCR-II vector and sequenced. To confirm the cDNA sequences generated, primers designed to span the coding regions were generated and used to amplify each mRNA. Products were subcloned into PCR-II vector and sequenced in both directions. At least three separate clones for each cDNA were completely sequenced. The sequence reported is the majority sequence obtained.

Northern Blotting

Total RNA was isolated, using RNeasy, from endometrium of gilts (3 per day) from Days 10, 13, and 15 of the estrous cycle or pregnancy and Days 24, 30, and 40 of pregnancy. Total RNA was also isolated from spleen, lung, kidney, liver, and placenta from two additional Day 30 pregnant gilts. Five micrograms total RNA was electrophoresed in MOPS (3-[*N*-morpholino] propane-sulfonic acid)/formaldehyde gels, and the gels were then electroblotted onto Hybond membranes. Membranes were probed sequen-

tially with probes specific for the 3′ ends of the cDNAs for putative secreted and membrane-bound forms of endometrial FBP. As a control for RNA loading, blots were then probed with a glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA (corresponding to nucleotides 641–964, Genbank Accession No. AF017079). Probes were generated by PCR in the presence of [^{32}P]CTP using the 3′-RACE clones for each FBP cDNA type as template and the oligonucleotides forward 1 and reverse 1 (Table 1) for secreted FBP (sFBP) and forward 2 and reverse 2 for membrane-bound FBP (mFBP). Blots were hybridized with Rapid Hyb along with 1×10^6 cpm radiolabeled probe per milliliter at 65°C for 2 h and then washed twice with double-strength SSC, 0.1% SDS at room temperature and twice again with 0.5-strength SSC, 0.1% SDS at 55°C (secreted form) or 60°C (membrane form). The GAPDH cDNA was obtained by reverse-transcribing 1 μg Day 30 pregnant endometrium using the primer GAAGCAGGGATGATGTTCTGG followed by PCR using the same primer and the forward primer CACGACCATGGAGAAGGC. The resulting cDNA corresponding to the correct size was cloned using PCR-II vector and sequenced in both directions. A probe was generated using the above primers, and blots were processed as described for the secreted form probe. To quantitate changes in each mRNA, autoradiographs were subjected to densitometry using a laser densitometer (Ultrascan XL; Pharmacia LKB, Piscataway, NJ).

N-Terminal Amino Acid Sequencing

Day 15 pregnant gilts were slaughtered, and the uterus was recovered. Each uterine horn was flushed with 20 ml saline. Approximately 100 ml of pooled Day 15 uterine flushings were then passed through a 5-ml column of Folate Sepharose (Sigma Chemical Co., St. Louis, MO) equilibrated in 50 mM Tris, 1 M NaCl, pH 8.2, and washed extensively using the equilibration buffer. Bound FBP was eluted using 50 mM glycine, 1 M NaCl, pH 2.8. Fractions containing protein (measured by absorbance at 280 nm) were dialyzed against distilled water and lyophilized. Protein was redissolved in distilled water at a concentration of 10 mg/ml (by absorbance at 280 nm), and 10 μl was subjected to SDS-PAGE. Gels were blotted onto polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) using the procedure of Matsudaira [19]. A single molecular-weight band was present on the blot after staining with Coomassie blue dye (not shown). This band was excised from the blot and sequenced at the Protein Sequencing Core Facility, University of Nebraska, Lincoln, NE.

Statistical Analysis

Densitometric values for putative sFBP and mFBP were analyzed by ANOVA using the General Linear Models procedure of the statistical analysis system (SAS Institute, Inc., Cary, NC). The model included effects of status, day of the cycle or pregnancy, and the status-by-day interaction, with the densitometric values for GAPDH used as a covariate in each analysis. In both cases, no effect of status or status-by-day interaction was obtained, and these effects were dropped from the model. Day effects were then more fully evaluated using a set of orthogonal contrasts. Contrasts were 1) Day 10 versus Day 13; 2) Day 10 and 13 combined versus Day 15; 3) Day 24 versus Day 30; 4) Day 24 and 30 combined versus Day 40; and 5) Days 10, 13, and 15 combined versus Days 24, 30, and 40 combined.

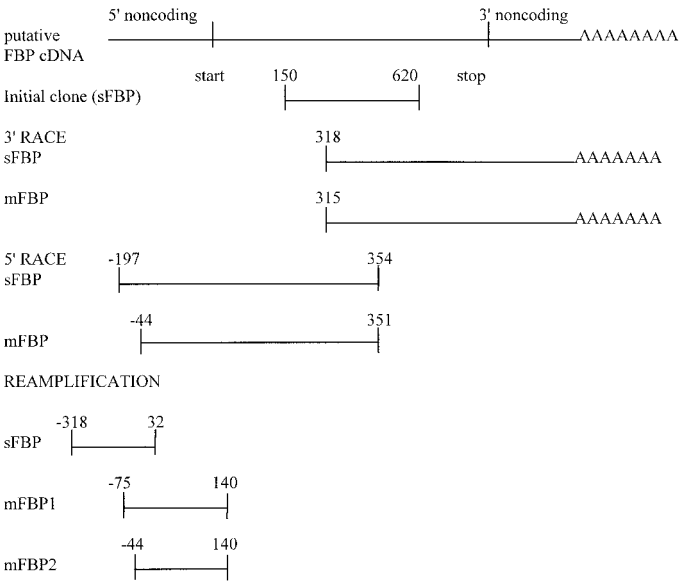

FIG. 1. The cloning strategy used in this experiment and the clones obtained are illustrated schematically. An initial clone spanning the region from bases 150–620 from the start site was obtained. Then 3′-RACE was used to obtain the 3′ ends of the cDNAs for putative sFBP and mFBP beginning from base 318 and base 315, respectively. Next, 5′-RACE was used to obtain the 5′ end of the cDNAs. Initially, 5′-RACE resulted in clones containing 197 (secreted form) and 44 (membrane form) bases upstream of the start codon. Reamplification of each resulted in a clone containing 318 bases upstream of the start codon for sFBP and two different 5′-UTR for mFBP (75 and 44 bases upstream of the start sequence; see Fig. 2 for details of the sequence).

RESULTS

Figure 1 illustrates the cloning strategy used and the clones obtained at each stage of the experiment. Two distinct types of FBP/FR cDNAs were obtained from endometrium (Fig. 2; GenBank accession numbers AF137373 and AF137374, respectively). Substantial amino acid sequence identity was found between the two forms and between both forms and other known FBP/FR from swine and other species (Fig. 3, Table 2). The predicted amino acid sequence of the first type had three charged residues and a putative *N*-glycosylation site within the C-terminal region in which the glycophosphatidylinositol linkage site [10, 11] was expected. Ten to 20 uninterrupted hydrophobic residues in this region are required for glycophosphatidylinositol linkage to occur [20]. Thus, this protein was tentatively identified as a putative sFBP. The predicted amino acid sequence of the other cDNA type encoded 11 uninterrupted hydrophobic residues among the 12 C-terminal residues. Thus this protein was identified as a putative mFBP.

The 5′-RACE analysis indicated that both cDNA types were heterogeneous within their 5′-UTRs. For sFBP, 5′-RACE clones were obtained that lacked an 86-base pair (bp) portion of the 5′-UTR present in other clones. The sequences at the beginning and end of this deletion were consistent with removal of the 86-bp portion by RNA splicing. For mFBP, clones were isolated that diverged after the tenth base 5′ to the putative translation start site. This divergence may also be due to differential RNA splicing.

Representative Northern blots for sFBP, mFBP, and GAPDH mRNAs are illustrated in Figure 4. Statistical analysis of densitometry data after correction using GAPDH results indicated that no status or status-by-day effects were present for either sFBP or mFBP mRNA (Fig. 5). Analysis

A.

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gacactgcttccgggtgggcctccaggagggccgaggcagaggagcctctgcctgtgggtgaagcactggctggcgaa
ctccggaaggggaggtccggagaggtggtgcctccccccgcagcaaagctcagactgcactgtcctcaggtggcagtg
gtgtcctaccacttggcacagacctccacgggcccttcatcgcttggctccactgtgctgtgggGTaagcggcgcg
gggagggacgacgatctgggcttggaagggaaacaggaaatctggccaagaagcttacggcagctt
tctggcAGaagtggatcaac

ATG GCC TGG CGG CTG ACG CTC TTC GTG CTC CTG GGT TTG GTG GCT GCT GTG GGG GGC
M   A   W   R   L   T   L   F   V   L   L   G   L   V   A   A   V   G   G

GCC CGG GCC AAG TCG GAC ATG CTC AAT GTC TGC ATG GAT GCC AAG CAC CAC AAG CCA
A   R   A   K   S   D   M   L   N   V   C   M   D   A   K   H   H   K   P

AAG CCA AGC CCG GAG GAC AAG CTG CAC GAC CAG TGC AGC CCC TGG AGG AAG AAC TCC
K   P   S   P   E   D   K   L   H   D   Q   C   S   P   W   R   K   N   S

TGC TGC TCA GTC AAC ACC AGC CTA GAA GCC CAT AAA GAC ATC TCC TAC CTG TAC AGA
C   C   S   V   N   T   S   L   E   A   H   K   D   I   S   Y   L   Y   R

TTC AAC TGG GAC CAC TGC GGC AAG ATG GAG CCG GCC TGC AAG CGC CAC TTC ATT CAA
F   N   W   D   H   C   G   K   M   E   P   A   C   K   R   H   F   I   Q

GAC ACC TGT CTC TAT GAG TGC TCG CCC AAC CTG GGG CCC TGG ATC CAG GAG GTG AAC
D   T   C   L   Y   E   C   S   P   N   L   G   P   W   I   Q   E   V   N

CAG AAG TGG CGC AGA GAG CGG ATC CTG AAC GTG CCC CTC TGC AAA GAG GAC TGT CAG
Q   K   W   R   R   E   R   I   L   N   V   P   L   C   K   E   D   C   Q

ATC TGG TGG GAA GAC TGC CGT ACC TCC TAC ACC TGC AAG AGC AAC TGG CAC AAG GGC
I   W   W   E   D   C   R   T   S   Y   T   C   K   S   N   W   H   K   G

TGG AAC TGG ACC TCA GGG TAT AAC CAG TGC CCA GTG AGC GCC GCC TGC CAC CGC TTC
W   N   W   T   S   G   Y   N   Q   C   P   V   S   A   A   C   H   R   F

GAC TTC TAC TTC CCC ACG CCC GCT GCC CTG TGC AAC GAG ATC TGG AGC CAC TCC TTT
D   F   Y   F   P   T   P   A   A   L   C   N   E   I   W   S   H   S   F

GAA GTC AGC AGC TAC AGC CGG GGC AGC GGC CGC TGC ATC CAG ATG TGG TTC GAC CCG
E   V   S   S   Y   S   R   G   S   G   R   C   I   Q   M   W   F   D   P

GCC CAG GGC AAC CCC AAC GAG GCG GTG GCG AGA TAC TAT GCA GAG AAT GGG GAT GCT
A   Q   G   N   P   N   E   A   V   A   R   Y   Y   A   E   N   G   D   A

GGG GCC GTG GCC CAG GGG ATC GGG CCT CTC CTG ACC AAC TTG ACG GAG ATG GTG AAA
G   A   V   A   Q   G   I   G   P   L   L   T   N   L   T   E   M   V   K

CAC TGG GTC ACC GGC TAA
H   W   V   T   G   Stop

gctgttcccccgccgacccctgctttccgcccacacccccctgggttactctccgggtggcctcagcaccccggtcatt
ggctcctgatctaagatccgatggggagcctctgatggcctcttccAATACAatatccacgtg
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B.

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1-gatgagggagtccaggagttccagcaagctcgacctgcttaacactcccagacggtcacaggattcag
2-                                ggattcctgctgcttttgaccacagttctttctgcag

gacaagc

ATG GCC CTT GGG AGA GCA CGG CTG CTG CTG CTC TTG GTG TGT GTG GCT GTC ACA TGG
M   A   L   G   R   A   R   L   L   L   L   V   C   V   A   V   T   W

GCG GCC CGG CCT GAT CTC CTC AAC ATC TGC ATG GAC GCC AAG CAC CAC AAG ACC AAG
A   A   R   P   D   L   L   N   I   C   M   D   A   K   H   H   K   T   K

CCC GGC CCG GAA GAT GGC CTG CAT GAG CAG TGC AGC CCC TGG GAG ATG AAC GCC TGC
P   G   P   E   D   G   L   H   E   Q   C   S   P   W   E   M   N   A   C

TGC TCC GTC AAC ACC AGC CAA GAA GCC CAT AAC GAC ATC TCC TAC CTG TAC AAA TTC
C   S   V   N   T   S   Q   E   A   H   N   D   I   S   Y   L   Y   K   F

AAC TGG GAG CAC TGC GGC AAG ATG AAG CCG GCC TGC AAG CGC CAC TTC ATT CAA GAC
N   W   E   H   C   G   K   M   K   P   A   C   K   R   H   F   I   Q   D

ACC TGT CTC TAT GAG TGC TCG CCC AAC CTG GGG CCC TGG ATC CAG GAG GTG AAC CAG
T   C   L   Y   E   C   S   P   N   L   G   P   W   I   Q   E   V   N   Q

AAG TGG CGC AGA GAG CGG ATC CTG AAC GTG CCC CTC TGC AAA GAG GAC TGT CAG AAC
K   W   R   R   E   R   I   L   N   V   P   L   C   K   E   D   C   Q   N

TGG TGG GAA GAC TGC CGC ACC TCC TAC ACC TGC AAG AGC AAC TGG CAC GAG GGC TGG
W   W   E   D   C   R   T   S   Y   T   C   K   S   N   W   H   E   G   W

AAC TGG AGC TCA GGG TAT AAC CGG TGC CCC GCG AAC GCC GCC TGC CAC CCC TTC GAC
N   W   S   S   G   Y   N   R   C   P   A   N   A   A   C   H   P   F   D

TTC TAC TTC CCC ACG CCT GCT GCC CTG TGC AGC CAG ATC TGG AGC AAC TCC TAC AAA
F   Y   F   P   T   P   A   A   L   C   S   Q   I   W   S   N   S   Y   K

CAA AGC AAC TAC AGC CGG GGC AGC GGC CGC TGC ATC CAG ATG TGG TTC GAC CCG GAA
Q   S   N   Y   S   R   G   S   G   R   C   I   Q   M   W   F   D   P   E

CAG GGC AAC CCC AAC GAG GTG GTG GCG AGA TAC TAC GCC CAG ATC ATG AGT GGC GCT
Q   G   N   P   N   E   V   V   A   R   Y   Y   A   Q   I   M   S   G   A

GGG CTC TCC GAG GCC TGG CCT CTC CAG TTC GGC CTG GCC CTG ACG CTG CTC TGG CTG
G   L   S   E   A   W   P   L   Q   F   G   L   A   L   T   L   L   W   L

CTG AGC TGA
L   S   Stop

gcttctgtcttcggagagctggacagccctcccctgttcggccccacagcacccagctcgtcagtgcctcag
tggtggtggtagtggtggtggtggcggcgggggggactctgAATAAAccagtcaccccac
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FIG. 2. Nucleotide sequences and predicted amino acid sequences for putative sFBP (**A**) and mFBP (**B**) are shown. Translated and untranslated regions are in capital and small letters, respectively. In **A,** the part of the 5′-UTR that was deleted in some clones is indicated in bold letters. In **B,** sequences 1 and 2 indicate divergent 5′-UTR sequences obtained by 5′-RACE. A triplet repeat region is underlined. The capital letters in the 3′-UTRs of both cDNAs indicate the putative polyadenylation signal sequence for each message.

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sFBP   MAWRLT.LFVLLGLVAAVGGARAKSDMLNVCMDAKHHKPKPSPEDKLHDQCSPWRKN
mFBP   --LGRA.RLL--LVCV--TW-.-RP-L--I--------T--G---G--E-----EM-
plFBP  -P-K--A-LLF-AG-VS-CR---RT-L-----------VE-G---E-----V--K--

       SCCSVNTSLEAHKDISYLYRFNWDHCGKMEPACKRHFIQDTCLYECSPNLGPWIQEV
       A--------Q---N------K---E-----K--------------------------
       A---ARV-H-L-R-K-S--N-S-E---R-----------NN-------------F---

       NQKWRRERILNVPLCKEDCQNWWEDCRTSYTCKSNWHEGWNWSSGYNQCPVSAACHR
       ----------------------------------------R--AN----P
       -----K--F----------LD-------------S--K-------S----TGTT-DT

       FDFYFPTPAALCNEIWSHSFEVSSYSRGSGRCIQMWFDPAQGNPNEAVARYYAENGD
       ------------SQ---N-YKQ-N---------------E------V------QIMS
       -ESF--------EG--N-DYKFTN----------------A-E-----E---F--LALS

                 *   **
       AGAVAQGIGPLLTNLTEMVKHWVTG
       GAGLSEAW.--QFG-ALTLLWLLS.
       --TMSL-T----LSAAL-LPLGLLD
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FIG. 3. The similarity in sequence between the two cDNAs isolated in this study (putative sFBP and mFBP), compared with the sequence of pig liver folate receptor (plFR) isolated previously [21] is illustrated. Potential *N*-glycosylation sites are indicated in bold letters. Sequences corresponding to the *N*-terminal sequences reported previously [17] for Day 60 allantoic fluid (first box), Day 60 pseudopregnant uterine flushings (second box), and Day 15 pregnant uterine flushings (this study; first box) are boxed. The putative GPI linkage sites are underlined. The charged amino acids that disrupt this sequence for sFBP are marked with asterisks.

of day effects indicated that sFBP mRNA concentrations did not change between Days 10 and 15 of the cycle and pregnancy, nor did they change from Day 24 to Day 40 of pregnancy. However, there was a decrease ($p < 0.05$) in sFBP mRNA from early (Days 10–15) to later (24–40) days. Similar to sFBP, mFBP mRNA concentrations did not differ from Day 10–15 or from Day 24–30. In contrast to sFBP, mFBP mRNA dramatically increased ($p < 0.01$) between early (Days 10–15) and later (Days 24–40) time periods. Northern analysis also indicated that the two mRNAs differed in expression patterns among different tissues. All tissues appeared to express low levels of sFBP mRNA, and all tissues but liver expressed mFBP. However, mRNA concentrations for sFBP appeared to be greatest in Day 10–15 endometrium and spleen, while concentrations for mFBP appeared to be greater in Day 24–40 endometrium and Day 30 placenta. The GAPDH mRNA levels could not be used to correct mRNA levels between tissues, because of differences in expression for each tissue (e.g., liver versus kidney).

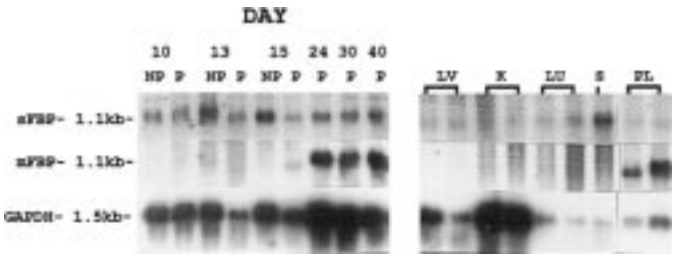

FIG. 4. Representative Northern blots of total cellular RNA (5 μg) from endometrium collected from nonpregnant (NP) and pregnant (P) gilts on Days 10, 13, and 15, and from pregnant gilts on Days 24, 30, and 40. Also shown are representative Northern blots of total cellular RNA (5 μg) collected from liver (LV), kidney (K), lung (LU), spleen (S), and Day 30 placenta (PL). Blots were probed sequentially with probes specific for the 3′ end of the putative sFBP and mFBP, cDNAs, and then GAPDH. See Figure 5 for densitometric evaluation of putative sFBP and mFBP mRNA in endometrium. Of the tissues examined, spleen and Day 10–15 endometrium had the highest levels of mRNA for putative sFBP, whereas Days 24–40 endometrium and Day 30 placenta had the highest levels of mRNA for putative mFBP.

TABLE 2. Percent identities between amino acid sequences of the secreted (psFBP) and membrane (mFBP) endometrial cDNAs reported here and bovine milk folate binding protein (bFBP) [21], mouse α and β folate receptors (mαFR, mβFR) [8], human α, β and γ folate receptors (hαFR, hβFR, hγFR) [6, 7, 9] and porcine liver folate receptor (plFR) [20].

	bFBP	mβFR	mαFR	hγFR	hβFR	hαFR	plFR	pmFBP
sFBP	82	66	72	72	69	72	66	73
mFBP	76	66	72	68	72	75	66	
plFR	70	66	65	72	74	67		
hαFR	79	70	79	73	74			
hβFR	74	78	71	82				
hγFR	75	75	70					
mαFR	76	71						
mβFR	72							

The N-terminal sequence of FBP obtained from pooled Day 15 pregnant uterine flushings was ARAKSDMLNVX-MDAKHHKPKPS, which was 100% identical to the inferred sequence for sFBP beginning at amino acid 20. This sequence differed from the inferred sequence for mFBP at 8 amino acids out of 20. These results indicate that the FBP found in intrauterine flushings corresponds to sFBP.

DISCUSSION

Results indicate that mRNA encoding two different FBP/FR-related proteins are present in porcine endometrium. Both of these mRNAs are different from the only other known FBP/FR sequence that has been described in swine [21]. The amino acid sequence of the two forms reported in this study are most similar to that of bovine milk FBP [22] compared to the other known sequences. Northern analysis combined with densitometry indicated that FBP mRNA concentrations did not change during early pregnancy (Days 10–15), despite previous reports indicating that FBP in uterine flushings increased dramatically during this period [16, 17] and despite the identification of this protein by N-terminal sequencing as corresponding to sFBP mRNA. In contrast, mFBP mRNA increased dramatically between Days 15 and 24 of pregnancy, and was also present in Day 30 placenta. These results are consistent with a role for both proteins in the transport of folate to the developing swine conceptus during pregnancy.

The sFBP cDNA and amino acid sequence reported here is only the second sequence reported coding for a protein that is likely to be directly secreted rather than linked to the cell membrane. The other reported amino acid sequence, human γ-FR [9], is truncated before the glycophosphatidylinositol linkage site. In contrast, porcine endometrial sFBP is not truncated, but the glycophosphatidylinositol linkage site is disrupted by the presence of an *N*-linked glycosylation site and 3 charged amino acid residues. These modifications make the protein an unlikely substrate for glycophosphatidylinositol linkage, and the identification of the putative secreted form as the form present in the intrauterine environment by N-terminal sequencing is consistent with this protein's being an sFBP.

As previously indicated, the second type of FBP cDNA isolated from porcine endometrium was identified as a putative mFBP because the C-terminal end of the inferred amino acid sequence appears to contain an intact glycophosphatidylinositol (GPI) site. The signal sequence required for GPI linkage consists of 10–20 hydrophobic amino acids in the C-terminal end of the protein [20]. The

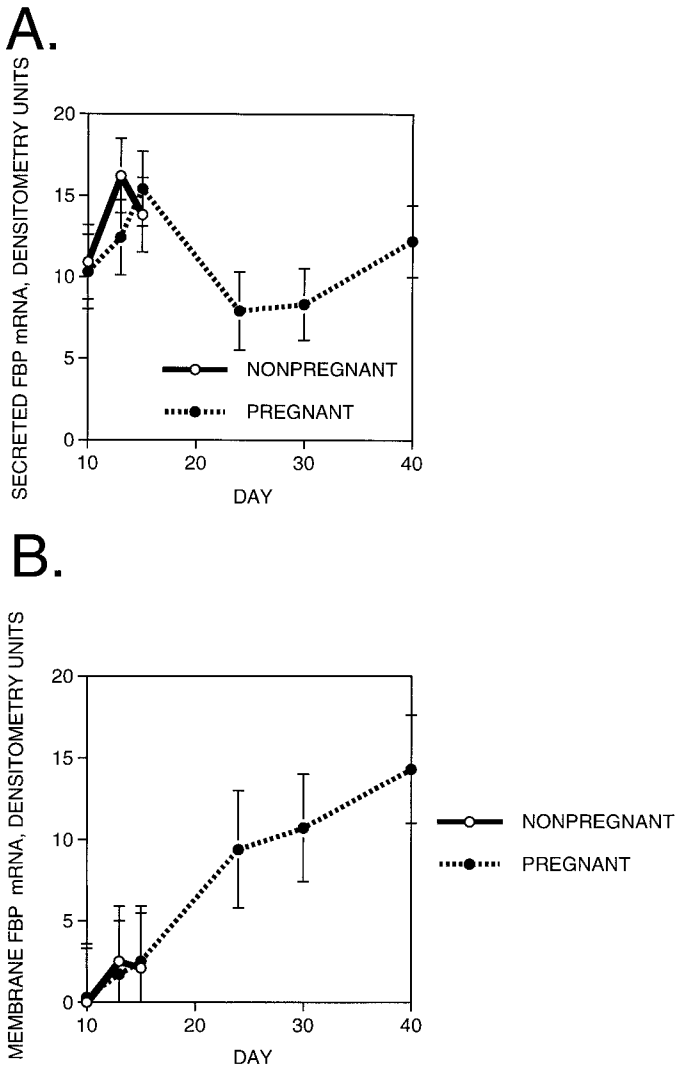

FIG. 5. Least-squares mean (± SEM) densitometric values (after correction using GAPDH mRNA values) for putative sFBP (**A**) and mFBP (**B**) in endometrium during the estrous cycle and pregnancy are illustrated. For both putative sFBP and mFBP, mRNA concentrations did not differ from Days 10 to 15 of the cycle or pregnancy, nor were they different from Days 24 to 40 of pregnancy. Values for putative sFBP from Days 24 to 40 combined were less ($p < 0.05$) than for Days 10–15 combined. Values for putative mFBP were greater ($p < 0.01$) for Days 24–40 combined than for Days 10–15 combined.

inferred mFBP amino acid sequence has 11 hydrophobic residues. During GPI linkage, the protein is cleaved and the new C-terminus is covalently linked to GPI. The linkage site is between 8 and 12 amino acids from the hydrophobic region. Previous studies indicate that the amino acids serine, glycine, alanine, aspartate, asparagine, and cysteine can all support GPI linkage [23, 24]. It has further been reported that the two amino acids C-terminal to the GPI attachment site must have small side chains [24, 25]. Putative mFBP has a serine and a glycine residue 12 and 11 amino acids, respectively, from the hydrophobic region. The serine is followed by glycine and alanine, the glycine by alanine and glycine. Thus, either site is suitable for GPI linkage. Nevertheless, determination of whether mFBP is a GPI-linked folate receptor requires confirmation using other methods.

The presence of two highly related but distinct forms of FBP/FR proteins within the endometrium and placenta of swine suggests a mechanism for transport of folates to the developing conceptus during pregnancy. The high homology of these cDNAs with bovine milk FBP suggests that the function of these and the bovine milk proteins may be to transport relatively large quantities of folate from one extracellular compartment to another. The FBP in milk is probably involved in transport of folate from the maternal blood stream to the milk. During pregnancy in swine, folates also must get from the maternal circulating blood to the extracellular environment and/or circulating blood of the developing conceptus. Because folates are negatively charged, they would not be expected to diffuse easily through biological membranes. Both the uterine luminal epithelium and the trophectoderm/chorion of the developing conceptus therefore present barriers to the efficient transport of folates to fetal tissues. The mFBP in endometrium may be responsible for taking up folates from the maternal blood stream. Once inside the endometrial epithelial cell, the folate would be combined with sFBP and the complex released into the intrauterine lumen. The presence of mFBP in placenta suggests that the placenta may then take up folates via competition with sFBP within the intrauterine lumen. Binding and uptake of folate by the cell via membrane FR would most likely be favored because of intracellular enzymes that polyglutamate folates and prevent them from diffusing back out of the cell [1]. Given this scenario, the efficiency of folate transport to the developing conceptus may be governed by the concentrations of folate within the maternal blood, the concentrations of mFBP on both the endometrium and placenta, the production rate of sFBP by the endometrium, and the activity of the polyglutamating enzymes. Which of these components primarily controls the rate of folate delivery to the conceptus at various stages of pregnancy will require further investigation.

Because the rate of delivery of folate to the developing swine conceptus is probably influenced by the production of FBP/FR by the endometrium and placenta, the way in which production of each protein is controlled is of interest. Previous results indicated that FBP in the intrauterine lumen increases dramatically during early pregnancy [16, 17]. Results of the current experiment confirm that this protein corresponds to sFBP mRNA and that the increase in this protein occurs in the absence of changes in mRNA levels. Several explanations for this result are possible. One possibility is that sFBP is synthesized and stored on Day 10 and that secretion of the stored sFBP is triggered from Days 11 to 13 of the cycle and pregnancy. It has been reported that during this period of the cycle or pregnancy, secretory vesicles within the endometrium disappear [26]. Another possibility is that the increase in sFBP occurs by changes in the translation rate of the mRNA. The heterogeneous nature of the 5′-UTR suggests the possibility that differences in the 5′-UTR could cause differences in translation efficiency. In support of this possibility, Sun and Anthony [27] reported that the 5′-UTR of the human FR-α mRNA contains an 18-base *cis* element that is critical for translation. The human FR-α gene is known to be controlled by two promoters [28], and the 18-base *cis* element is located in exon 1, which would be incorporated into cDNAs transcribed from the first promoter. The mRNAs transcribed from the second promoter lack this sequence and therefore presumably would be translated less efficiently. However, no sequence corresponding to the *cis* element described in humans is found in the deleted portion of sFBP mRNA 5′-UTR of pigs. To search for other possible *cis* elements within the deleted portion of the sFBP cDNA 5′-UTR, the

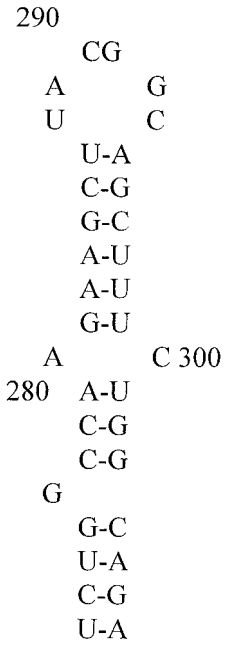

FIG. 6. Stem and loop diagram determined by FOLDRNA of part of the sequence within the 86-base deleted region of the 5′-UTR of the putative sFBP gene. The free energy of the structure was −13.8 kcal.

cDNA sequence with and without the deleted region was processed by the FOLDRNA program [29] of the GCG computer package (Genetics Computer Group, Inc., Madison, WI). The 86-base region contains a possible stem loop structure (Fig. 6) that is within 20 bases of the putative start site of translation. The free energy of the structure (−13.8 kcal) is unlikely to be great enough to directly interfere with translation [30], but this or other sites could bind proteins that may enhance or interfere with translation. Further studies are needed to determine whether the discrepancy between protein levels and mRNA levels is caused by changes in secretion, changes in translation, or an alternative mechanism.

In contrast to sFBP mRNA, mRNA concentrations for mFBP increased dramatically between Days 15 and 24 of pregnancy. The increase in mRNA concentration coincides with the onset of fetal liver erythropoiesis and a dramatic increase in the red blood cell concentrations in the blood supply of the developing swine fetus [31]. The temporal association of the two phenomena along with the known requirement for folate in efficient erythropoiesis suggests a role for endometrial mFBP in delivery of folate to the conceptus during a critical period of fetal development. Factors that influence production of this protein in the endometrium could therefore influence the efficiency of erythropoiesis in the swine fetus. Results indicate that at least one aspect of the control of production of this protein is via changes in mRNA concentrations, either by increased transcription of the gene, or decreased degradation of the mRNA. The heterogeneity of the 5′-untranslated region of the cDNA could be involved in either mechanism. The heterogeneity observed here could be consistent with transcription initiation at two different promoters, which has been described for FR-α from humans [28]. Alternatively, the different 5′-untranslated regions may influence degradation of the mRNA. Further experiments, including the isolation and characterization of the gene, are required to distinguish between these possibilities.

Northern analysis indicated that spleen had high concentrations of mRNA for sFBP. The function of sFBP secreted by the spleen is unknown. However, because the spleen is a site of erythropoiesis in adults and folates are required for efficient erythropoiesis to occur, sFBP may be present in the spleen to increase the local concentration of folates in order to improve the efficiency of erythropoiesis. The actual function of sFBP in spleen, endometrium, and placenta probably depends on which cells produce FBP. In situ hybridization studies are currently underway to investigate this question.

In conclusion, we have isolated and characterized cDNAs corresponding to two different FBP/FR proteins from the endometrium of swine. One was identified as a putative sFBP, on the basis of lack of an intact glycophosphatidylinositol linkage site. The other was identified as a putative mFBP, because it appeared to have an intact glycophosphatidylinositol linkage site. The sFBP corresponded to the folate binding activity found in uterine flushings. Concentrations of endometrial sFBP mRNA did not change during early pregnancy (Days 1–15). Concentrations of endometrial mFBP mRNA increased from Days 15 to 24 of early pregnancy. These results indicate a role for both proteins in the delivery of folate to the developing swine conceptus during early pregnancy.

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