BIOLOGY OF REPRODUCTION **68,** 24–30 (2003)
Published online before print 14 October 2002.
DOI 10.1095/biolreprod.102.009316

# Regulation of Insulin-Like Growth Factor Binding Protein-1 Promoter Activity by FKHR and HOXA10 in Primate Endometrial Cells[1]

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## ABSTRACT

**Insulin-like growth factor binding protein-1 (IGFBP-1) is abundantly expressed in the liver and decidualized endometrium. FKHR, a FOXO forkhead transcription factor, stimulates IGFBP-1 promoter activity in liver cells through the insulin response sequences (IRSs). HOXA10, a homeobox transcription factor, is important in the decidualization process. Here we show that FKHR and HOXA10 are expressed in baboon endometrium during the menstrual cycle and pregnancy. Levels are lowest during the follicular phase and highest in pregnancy. Reporter gene studies reveal that FKHR stimulates both baboon and human IGFBP-1 promoter activity, whereas HOXA10 alone has a relatively weak effect. When FKHR and HOXA10 are expressed together, promoter activity is markedly up-regulated, which is indicative of cooperativity. A DNA binding-deficient FKHR mutant fails to stimulate promoter activity, even in the presence of HOXA10, and deletion or mutation of IRSs also disrupts the effect of FKHR and cooperativity with HOXA10. Conversely, the IRS region placed upstream of the 31 base pair IGFBP-1 minimal promoter is sufficient to mediate effects of FKHR and cooperativity with HOXA10. Pull-down studies reveal physical association between GST-FKHR and $^{35}$S-HOXA10. These studies show that FKHR and HOXA10 interact directly and can function cooperatively to stimulate IGFBP-1 promoter activity in endometrial cells and perhaps in other settings.**

*decidua, female reproductive tract, gene regulation, implantation, uterus*

## INTRODUCTION

Insulin-like growth factor binding protein-1 (IGFBP-1) is expressed primarily in the liver, kidney, decidualized endometrium, and luteinizing granulosa cells [1]. Its proposed roles in reproductive physiology and pathology are numerous ([1], reviewed in [2]). During pregnancy, IGFBP-1 is abundantly expressed in decidualized endometrial stromal cells where it is believed to play an important role during blastocyst implantation, either as an insulin-like growth factor (IGF)-binding protein or via IGF-independent effects at the maternal-fetal interface [3]. We have previously shown that stromal cells isolated from the baboon endometrium decidualize and produce high levels of IGFBP-1 after treatment with estradiol, medroxyprogesterone acetate, relaxin, and dibutyryl cAMP [4]. A similar response was shown with human fibroblasts (HuFs) isolated from decidua parietalis from term pregnancies [5].

Multiple factors contribute to the regulation of IGFBP-1 gene expression, including insulin, glucocorticoids, progesterone, cytokines, and hypoxia [1, 6–8]. Recent studies have revealed that FKHR, a member of the FOXO subfamily of forkhead/winged-helix family of transcription factors, plays an important role in mediating effects of insulin on IGFBP-1 promoter activity in liver-derived cells [9–12]. FKHR binds directly to insulin response sequences (IRSs) in the proximal IGFBP-1 promoter, whereas phosphorylation of FKHR by protein kinase B (PKB) and other phosphatidylinositol-3′ kinase-dependent kinases suppress transactivation by FKHR, thereby inhibiting IGFBP-1 expression [9]. Recent studies have revealed that FKHR is induced in differentiating human endometrial stromal cells [13]. Based on these findings, we asked whether FKHR might contribute to the regulation of IGFBP-1 in uterine cells.

Homeobox (HOX) proteins are developmentally regulated transcription factors that are important in spatial identity and differentiation of tissues in the developing embryo. These genes contain a common sequence of 183 base pairs (bp), known as the homeobox, which encodes for a highly conserved 61-amino acid homeodomain. Homeodomain proteins can activate or repress the expression of target genes [14]. In the developing reproductive tract, four genes of the HOXA cluster (HOXA9, HOXA10, HOXA11, and HOXA13) are expressed [15]. HOXA10 is expressed in the developing uterus, specifically in the endometrial glands and stroma of the endometrium, where its expression is dependent on the stage of the menstrual cycle, which dramatically increases at the time of implantation [15–17]. HOXA10-deficient mice exhibit uterine factor infertility due to implantation defects. Specifically, decidualization of the endometrium is severely compromised during blastocyst implantation [18]. It is interesting that the IGFBP-1 gene is located in close proximity to the HOXA gene cluster on chromosome 7, suggesting evolutionary linkage of the IGFBP and HOX gene families.

Recent studies have suggested that overexpression of HOXA5 in transgenic mice up-regulates IGFBP-1 expression in the liver [19]. However, the role of HOX proteins

[1]These studies were supported in part by National Institutes of Health grants HD36759 to A.T.F. and HD36887 to H.S.T., and by grant DK41430 to T.G.U. from the Department of Veterans Affairs Merit Review Program.
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Received: 11 July 2002.
First decision: 7 August 2002.
Accepted: 12 August 2002.

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

in regulating the IGFBP-1 gene in the differentiating endometrium is unknown. Since HOXA10 [17] and FKHR [13] are expressed in human endometrial stromal cells, we asked whether HOXA10 and FKHR contribute to IGFBP-1 regulation in primate endometrial stromal cells. Here, we show that FKHR and HOXA10 follow similar patterns of expression during different stages of the baboon menstrual cycle and pregnancy. Reporter gene studies indicate that FKHR and HOXA10 function cooperatively to stimulate baboon and human IGFBP-1 promoter activity, and direct physical association between FKHR and HOXA10 is observed in glutathione S-transferase (GST) pull-down studies. Together, these findings indicate that FKHR and HOXA10 may play an important role and function cooperatively in regulating the expression of IGFBP-1 and perhaps other genes in the primate endometrium.

## MATERIALS AND METHODS

### *Isolation of Primary Baboon Stromal Cells and Human Uterine Fibroblasts*

Midluteal phase (9–11 days postovulation [PO]) endometrial tissue was obtained from adult female baboons (*Papio anubis*) by endometriectomy or hysterectomy. All animal studies were approved by the Animal Care Committee at the University of Illinois at Chicago. Stromal cells were isolated from baboon endometrial tissues as described previously [4].

HuF cells were isolated from decidua parietalis dissected from the placental membranes after normal vaginal delivery at term, as previously described [5]. Decidualized uterine endometrium maintains a proliferating population of predecidual fibroblastic cells, which closely resemble stromal cells [20, 21]. These cells were passaged as needed up to a maximum of seven passages.

### *RNA Isolation and Analysis*

Tissue was homogenized in TriReagent (Molecular Research Center Inc., Cincinnati, OH) and RNA was extracted using the protocol provided by the manufacturer. One microgram of total RNA was reverse transcribed and polymerase chain reaction (PCR) amplification was performed for 33 cycles using FKHR primers and 24 cycles using H3.3 primers under conditions previously described [4, 9]. PCR products were electrophoresed in 1% agarose gel containing ethidium bromide. Densitometric analysis of the PCR products was performed using the EDAS 290 Imaging System (Kodak, New York, NY). The densitometric values were normalized to H3.3. For Northern blotting, total RNA (40–50 μg) was size fractionated on 1% agarose/0.66 M formaldehyde by gel electrophoresis and transferred to nylon membranes. Membranes were hybridized at 68°C in Perfecthyb buffer (Sigma, St. Louis, MO), 0.2% tRNA, and $^{32}$P-labeled riboprobe at $2 \times 10^6$ cpm/ml. The filter was washed twice at 60°C for 20 min in 0.1× saline-sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS). X-Omat AR (Kodak) was exposed overnight at −70°C. The autoradiographic bands were quantified using a laser densitometer. Each HOXA10 band was normalized to the value obtained from the same lane hybridized to GAPDH.

### *Cloning and Sequencing of the Baboon IGFBP-1 Promoter Region*

Colony lifts of an amplified male baboon kidney tissue genomic DNA Lambda Dash II library were screened with a randomly labeled 1.2-kilobase (kb) human IGFBP-1 cDNA probe [22]. Positive clones were subjected to two further rounds of screening. The insert was isolated on agarose gels and cloned into the *Not*I site of pBluescriptII SK−. A *Hin*dIII subfragment containing approximately 4 kb of the 5′-flanking region through the start of transcription was subcloned into the same vector. The 4-kb fragment was sequenced by dideoxy sequencing at the University of Illinois at Chicago Sequence Center. This gene sequence was deposited in GenBank under accession number AY095345.

### *Reporter Gene Constructs and Expression Vectors*

A region spanning −358 to +75 relative to the transcription start site was isolated by PCR from both human and baboon IGFBP-1 promoters using as templates, the human p3.6BP1.CAT plasmid (a gift from D. Powell, Lexicon Genetics Inc., The Woodlands, TX) and the cloned 4-kb baboon promoter fragment, respectively. The 5′ primer corresponded to bases −358 to −339 of the human sequence (accession number M59316) and additional bases were added to the 5′ end of the primer to create an *Xho*I site. The 3′ primer corresponded to bases +58 to +75, including additional bases at the 3′ end to create a *Hin*dIII site. The PCR product was subcloned into the *Xho*I/*Hin*dIII sites of the promoterless pGL3-basic vector (Promega, Madison, WI). The promoter fragments were verified by dideoxy sequencing. The human pHbp1-358.Luc and baboon pBbp1-358.Luc constructs were then used for transient transfections. For construction of mutated IRS constructs, the pHbp1-358.Luc was first truncated to the region spanning −224 to +75 (pHbp1-224.Luc) by PCR. IRSA (−118 to −111) and IRSB (−108 to −101) in the pHbp1-224.Luc construct were mutated (pHbp1-224/IRSABmut.Luc) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) (Fig. 5). For the construct in which only the IRSAB region (−118 to −101) remained, sequences flanking the IRS region (−358 through −119 and −100 through −32) were deleted as shown in Figure 5 using the QuikChange kit, resulting in the plasmid pHbp1-31/IRS.Luc. The IGFBP-1 minimal promoter (−31 to +75) was isolated by PCR using pHbp1-358.Luc as the template and subcloned into the *Xho*I/*Hin*dIII sites of the pGL3-basic vector. All sequences were verified by dideoxy sequencing. The human FKHR expression vector used in these studies is the mutant form in which the three consensus PKB phosphorylation sites, Thr-24, Ser-256, and Ser-319 were mutated to alanines to create a constitutively active form [9]. The DNA binding-defective FKHR expression vector (FKHR-Helix 3.2M) containing a mutation of Trp-209 to Gly and His-215 to Pro has been previously described [23]. The HOXA10 cDNA was a gift from C. Largman (University of California VA Medical Center, San Francisco, CA), which was subsequently inserted into the *Eco*RI site of pcDNA3.1 (+) (Invitrogen, Carlsbad, CA) by H. Taylor.

### *Cell Transfection and Reporter Gene Studies*

Transient transfection of baboon endometrial stromal cells and HuF cells grown in 12-well plates was performed using Lipofectamine 2000 (Invitrogen). Cells were transfected in Dulbecco modified Eagle medium with 1 μg/well of the IGFBP-1 promoter constructs with or without 0.5 μg/well FKHR, 0.5 μg/well HOXA10 expression vectors (or both) and pcDNA3.1(+) as an empty vector. After 4 h, the media was changed to RPMI-1640 (Invitrogen) and cells were incubated for an additional 20 h. Cell extracts were harvested and luciferase activity was measured with the luciferase reagent kit (Promega). Transfections were performed in triplicate and experiments were repeated at least three times.

### *GST-Pulldown*

Bacterially expressed GST and GST-FKHR recombinant proteins were purchased (Upstate Biotechnology, Waltham, MA). $^{35}$S-Labeled HOXA10 protein was prepared by in vitro transcription-translation using the TNT T7 Coupled Reticulocyte Lysate System (Promega) and $^{35}$S-methionine (Amersham Pharmacia Biotech, Piscataway, NJ). Glutathione-sepharose beads (Amersham Pharmacia Biotech) were washed three times with binding buffer (50 mM Hepes, 100 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.1% Tween-20, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.3 mM sodium vanadate, 1 mM NaF, 5 μg/ml aprotinin [24]). Equimolar amounts of GST-FKHR or GST were preincubated with washed glutathione-sepharose beads in binding buffer for 1 h at 4°C on a rocker platform. Beads were washed twice with fresh binding buffer. $^{35}$S-labeled HOXA10 was added to the beads and incubated on a rocker for 3 h at 4°C. Beads were washed three times with binding buffer containing 0.5% IGEPAL (Sigma), resuspended in SDS-polyacrylamide gel electrophoresis buffer, boiled for 5 min, and resolved by 10% SDS-polyacrylamide gel electrophoresis. The input $^{35}$S-HOXA10 protein was diluted 1:10 before loading onto the gel. The presence of $^{35}$S-HOXA10 was detected by autoradiography.

### *Statistical Analysis*

A paired *t*-test was performed to compare the effects of FKHR, HOXA10, or both on IGFBP-1 promoter activity compared to control (basal IGFBP-1 promoter activity). The mean values obtained for FKHR alone or HOXA10 alone were also compared to the effects of FKHR + HOXA10 using the paired *t*-test.

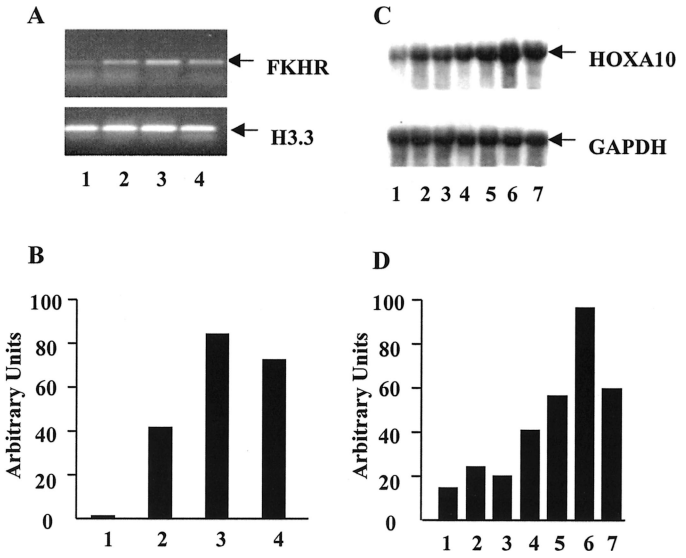


FIG. 1. Expression of FKHR and HOXA10 mRNAs in baboon endometrial tissue during the menstrual cycle and pregnancy. **A**) FKHR mRNA expression was detected by RT-PCR in baboon endometrium at different stages of the menstrual cycle including 1) late follicular, 2) Day 10 postovulation, 3) pregnancy at 39 days, and 4) term decidua. Densitometric values normalized to H3.3 are shown in **B**. **C**) HOXA10 mRNA expression was detected by Northern blot analysis in baboon endometrium from the menstrual cycle at 1) late follicular, 2) Day 8 PO, 3) Day 10 PO, 4) Day 12 PO, 5) Day 14 PO, 6) at pregnancy, 51 days, and 7) term decidua. Densitometric values normalized to GAPDH are shown in **D**.

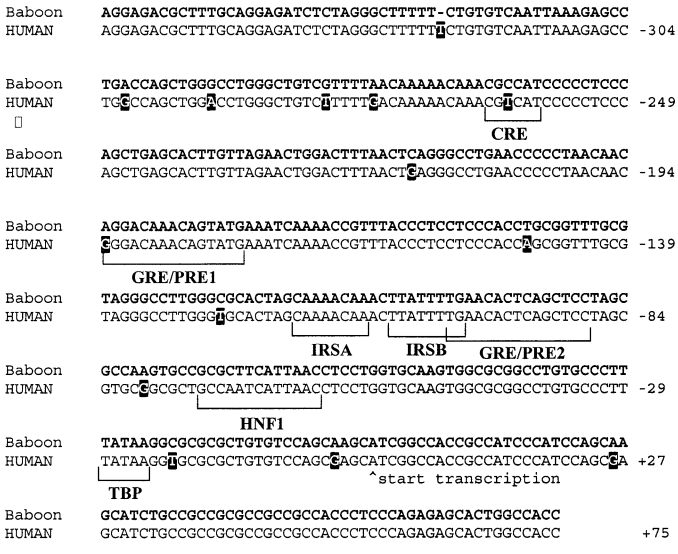


FIG. 2. Alignment of the baboon and human IGFBP-1 promoter sequences (−358 to +75). The baboon IGFBP-1 promoter region (~4 kb) was cloned from a baboon kidney tissue genomic DNA Lambda Dash II library. The sequence spanning the region −358 to +75, which was used for transient transfection studies, was compared to the human sequence (accession number M59316) and found to be 96% identical. The CRE, IRSA, IRSB, and GRE/PRE response elements and the HNF-1 binding site are conserved. Shading indicates base pairs that are not conserved. TBP, TATA binding protein.

## RESULTS

### *Expression of FKHR and HOXA10 mRNA in Baboon Endometrium*

FKHR mRNA levels were examined in the baboon endometrium at various stages of the menstrual cycle and pregnancy by reverse transcriptase-PCR (Fig. 1A). Late follicular phase endometrium exhibited the lowest level of FKHR mRNA. By midluteal phase of the menstrual cycle (Day 10 PO), FKHR mRNA abundance increased. Levels of FKHR mRNA levels were highest in pregnant endometrium and remained high until term (term decidua; Fig 1B). As shown in Figure 1C, HOXA10 mRNA levels were examined in baboon endometrium by Northern blotting. Follicular phase endometrial tissue expressed the lowest amount of HOXA10 mRNA, and HOXA10 mRNA levels were increased in luteal phase tissues (Days 8–14 PO). HOXA10 mRNA abundance was highest in pregnant endometrium and remained high at term (Fig. 1D) following a similar pattern to FKHR expression (Fig 1, A and B).

### *Cloning of the Baboon IGFBP-1 Promoter*

In order to determine whether FKHR and HOXA10 might stimulate the expression of IGFBP-1 in baboon endometrium, a ~4-kb fragment of the 5′-upstream region of the baboon IGFBP-1 gene was cloned and sequenced and deposited in GenBank under accession number AY095345. A smaller fragment (−358 to +75) was cloned into PGL3-basic vector (Bbp1-358.Luc) for transient transfection studies (Fig. 2). Alignment with the human IGFBP-1 promoter shows that this region is highly conserved, exhibiting 96% identity between baboon and human sequences (Fig. 2). Several hormone response elements including a cAMP responsive element (CRE), two insulin response sequences (IRSA and IRSB), two glucocorticoid/progesterone response elements (GRE/PRE1, GRE/PRE2) [8, 25, 26], and an HNF-1 binding site [27] are conserved in human and baboon IGFBP-1 promoters.

### *Regulation of the IGFBP-1 Promoter by FKHR and HOXA10*

To examine the effects of FKHR and HOXA10 on IGFBP-1 promoter activity, we first performed transient cotransfection studies in baboon endometrial stromal cells using the baboon IGFBP-1 promoter reporter gene construct (Bbp1-358.Luc). As shown in Figure 3A, cotransfection with the FKHR expression vector stimulated the activity of the baboon IGFBP-1 promoter ~8-fold ($P < 0.05$), consistent with previous studies indicating that FKHR can stimulate IGFBP-1 promoter function [9, 12]. Cotransfection with a HOXA10 expression vector had limited effect on promoter activity (1.3-fold increase), which was not statistically different from the control. However, coexpression of HOXA10 with FKHR markedly stimulated promoter activity (32-fold increase vs. control; $P < 0.05$). This stimulation exceeded the sum of the effects observed with FKHR and HOXA10 alone, suggesting that these transcription factors may function cooperatively to stimulate IGFBP-1 promoter activity. The increase observed with FKHR + HOXA10 compared to either FKHR alone or HOXA10 alone was statistically significant ($P < 0.05$).

To examine the effects of FKHR and HOXA10 on the human IGFBP-1 promoter, we next performed transfection studies with a luciferase reporter gene construct containing the corresponding region of the human IGFBP-1 promoter (Hbp1-358.Luc) in human uterine predecidual fibroblast (HuF) cells. As shown in Figure 3B, expression of FKHR or HOXA10 alone stimulated the activity of the human IGFBP-1 promoter 6-fold ($P < 0.05$) and 4-fold ($P < 0.05$), respectively. Coexpression of FKHR and HOXA10 together resulted in synergistic stimulation of promoter activity

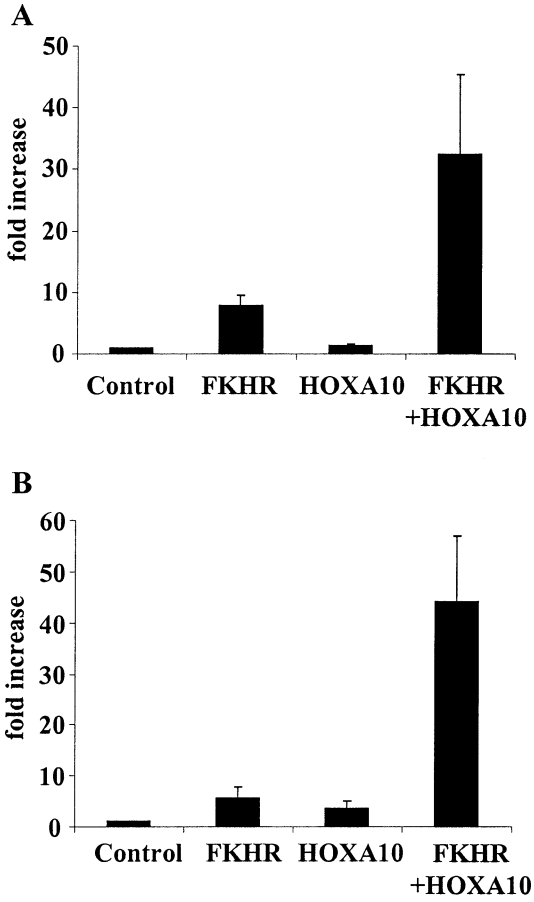


FIG. 3. Effect of FKHR and HOXA10 on IGFBP-1 promoter activity. **A**) Baboon endometrial stromal cells and **B**) human fibroblasts (HuF) were transfected with Bbp1-358.Luc or Hbp1-358.Luc reporter gene constructs, respectively, along with the FKHR expression vector, the HOXA10 expression vector, or both together with appropriate amounts of empty vector. Levels of reporter gene activity are expressed as fold increases relative to control without FKHR or HOXA10. Twelve independent determinants were made for each construct by performing triplicates in four separate experiments. The mean (n = 4) ± SEM is shown.

(44-fold; $P < 0.05$), similar to results obtained with the baboon promoter in baboon stromal cells. All subsequent studies were performed using constructs containing the human IGFBP-1 promoter transfected into HuF cells.

### *Stimulation by FKHR and Cooperativity with HOXA10 Requires Binding to IRSs*

In order to determine whether FKHR binding to DNA is necessary for cooperative interactions with HOXA10, we performed transient transfection studies with a DNA-binding-deficient FKHR mutant (FKHR-Helix3.2M; [23]). As shown in Figure 4, cotransfection with the FKHR-Helix3.2M expression vector failed to stimulate Hbp1-358.Luc activity in HuF cells. Coexpression of both HOXA10 and FKHR-Helix3.2M also failed to stimulate Hbp1-358.Luc activity (Fig. 4), indicating that DNA binding by FKHR is required for cooperativity with HOXA10.

FKHR can interact with either IRSA or IRSB in the IGFBP-1 promoter and stimulate promoter activity [9]. Mutation of both IRSA and IRSB (see Fig. 5 for mutated sequences) disrupted the ability of FKHR to stimulate the activity of a reporter gene construct containing 224 bp of the human IGFBP-1 promoter (Hbp1-224.Luc; Fig. 6A), which is consistent with previous studies in liver-derived

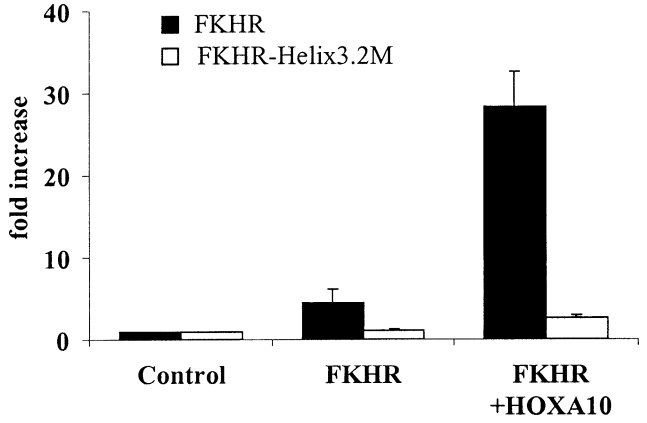


FIG. 4. Effect of FKHR-Helix 3.2M and HOXA10 on IGFBP-1 promoter activity. HuFs were transfected with Hbp1-358.Luc and either the wild-type FKHR (black bars) or the DNA binding-defective FKHR-Helix3.2M expression vector (white bars) as well as with HOXA10 expression vector and the appropriate amounts of empty vector. Levels of reporter gene activity are expressed as fold increases relative to control without FKHR or HOXA10. Nine independent determinants were made for each construct by performing triplicates in three separate experiments. The mean (n = 3) ± SEM is shown.

cell lines. It is interesting that cotransfection with the HOXA10 expression vector had a modest effect on the activity of the human IGFBP-1 promoter, and this effect was also diminished by mutation of the IRSs, suggesting that HOXA10 may interact with the IRS or with endogenous FKHR or related forkhead proteins in HuF cells to enhance promoter activity in an IRS-dependent fashion. Coexpression studies demonstrated that HOXA10 enhances the effect of FKHR on promoter function and mutation of the IRSs disrupts this effect. These results together indicate that interaction with IRSs in the IGFBP-1 promoter is required for the transactivation by FKHR and for HOXA10 to enhance the effect of FKHR on IGFBP-1 promoter activity.

To further characterize the role of IRSs in mediating the effects of FKHR and HOXA10 on IGFBP-1 promoter activity, we created a series of deletions in the human IGFBP-1 promoter (Fig. 5). As shown in Figure 6B, deletion of all

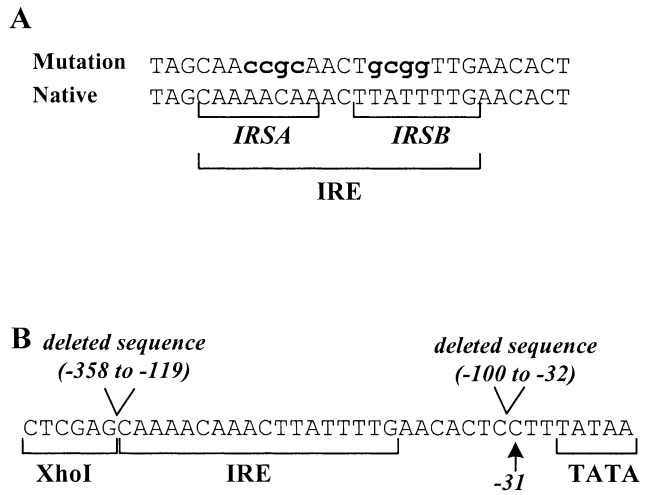


FIG. 5. Mutations and deletions of the IGFBP-1 promoter. **A**) IRSA and IRSB of the Hbp1-224.Luc construct was mutated as shown in highlighted lower case letters to create Hbp1-224/IRSABmut.Luc. **B**) Sequences flanking the IRE (−358 through −119 and −100 through −32) were deleted, placing the region (−118 to −101) containing the IRSs adjacent to the 31-bp minimal IGFBP-1 promoter region (Hbp1-31.Luc).

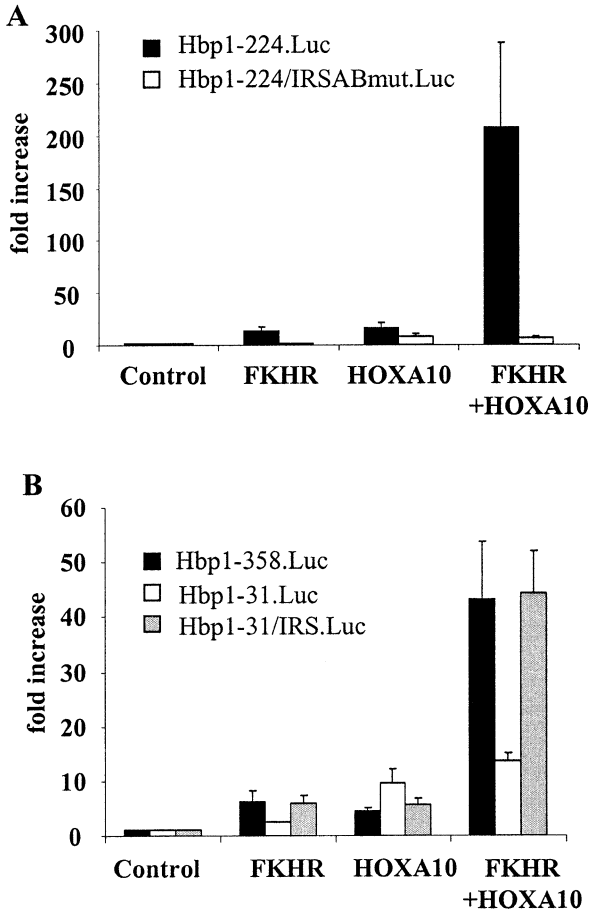


FIG. 6. Requirement of the IRS for cooperative up-regulation of the IGFBP-1 promoter by FKHR and HOXA10. **A**) HuFs were transfected with Hbp1-224.Luc (black bars) or the mutant Hbp1-224/IRSABmut.Luc (white bars) with FKHR, HOXA10, or both expression vectors. **B**) HuFs were transfected with Hbp1-358.Luc (black bars), Hbp1-31.Luc (white bars) or Hbp1-31/IRS.Luc (gray bars) constructs with FKHR, HOXA10, or both expression vectors. Levels of reporter gene activity are expressed as fold increases relative to control without FKHR or HOXA10. Nine independent determinants were made for each construct by performing triplicates in three separate experiments. The mean (n = 3) ± SEM is shown.

residues located upstream of the 31-bp minimal IGFBP-1 promoter disrupted the ability of FKHR to stimulate promoter activity and to cooperate with HOXA10. Introducing the IRSs and their flanking sequence immediately upstream of the 31-bp promoter restored FKHR stimulation of promoter function ($P < 0.05$) and the ability of HOXA10 to enhance this effect ($P < 0.05$). This result indicates that together with the minimal IGFBP-1 promoter and flanking sequences, IRSs are sufficient to confer the ability of FKHR to stimulate promoter activity and to cooperate with HOXA10.

### *GST-Pulldown*

Because FKHR and HOXA10 can act in a cooperative manner, we considered the possibility that there may be a physical association between these transcription factors. As shown in Figure 7, $^{35}$S-labeled recombinant HOXA10 prepared by in vitro transcription/translation interacted with the bacterially expressed fusion protein containing GST in frame with FKHR, but not with GST alone. $^{35}$S-Labeled luciferase was run in parallel, and no interaction was observed between this protein and either GST-FKHR or GST alone (results not shown).

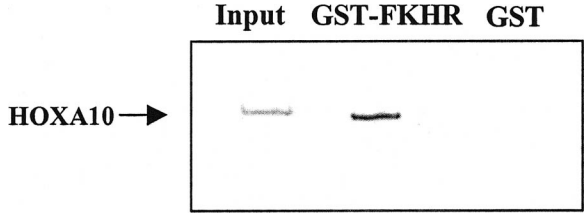


FIG. 7. Physical association of FKHR and HOXA10. Bacterially expressed GST and GST-FKHR recombinant proteins were bound to glutathione-sepharose beads, then incubated with $^{35}$S-labeled HOXA10 synthesized by in vitro transcription/translation. Bound proteins were eluted and loaded for SDS/PAGE and autoradiography. $^{35}$S-HOXA10 protein is identified by an arrow. The input $^{35}$S-HOXA10 protein was diluted 1:10 before loading onto the gel.

## DISCUSSION

In the present study, we found that FKHR and HOXA10 are expressed in parallel during the menstrual cycle and pregnancy in the baboon endometrium, similar to recent results obtained in human endometrium [13, 15]. The observation that the expression of both FKHR and HOXA10 are highest during pregnancy in the baboon suggests an important role for these factors during the establishment and maintenance of pregnancy. HOXA10 is expressed in the human endometrium in the endometrial glands and stroma throughout the menstrual cycle, with highest expression being observed at the time implantation would take place [16, 17]. Furthermore, HOXA10 is regulated by progesterone in human endometrium [17]. In HOXA10-deficient mice, decidualization is severely compromised resulting in infertility, demonstrating a requirement for HOXA10 in the peri-implantation uterus [18, 28]. The requirement for maternal HOXA10 is further supported by a study showing that HOXA10 antisense oligodeoxyribonucleotide is able to block implantation [29]. It has been demonstrated that HOXA10-deficient mice exhibit impaired stromal cell responsiveness to progesterone, specifically in stromal cell proliferation and expression of the $PGE_2$ receptor subtypes, EP3 and EP4 [30]. The mechanisms by which HOXA10 regulates these events remain unclear.

FKHR is expressed in a variety of tissues, including the brain, placenta, testes, and liver [31]. The expression of FKHR in baboon endometrium is shown in this report. The factors that induce FKHR expression, however, remain unclear. Christian et al. [13] have identified FKHR using differential display as one of the major transcripts induced in stromal cells of human endometrium by treatment with 8-bromo-cAMP. 8-Bromo-cAMP treatment causes these stromal cells to decidualize and produce IGFBP-1. This would be consistent with FKHR acting as a transcription factor that regulates IGFBP-1 gene expression in stromal cells during decidualization. It has recently been shown that cAMP mediates the effects of FSH on FKHR expression in ovarian granulosa cells [32]. Interestingly, estradiol enhances FKHR gene expression in granulosa cells [32], suggesting that sex steroids also may contribute to the regulation of FKHR.

Although FKHR has been associated with the regulation of the IGFBP-1 gene in hepatic cells, its role in IGFBP-1 gene regulation in uterine cells has not been previously reported. We find that FKHR stimulates IGFBP-1 promoter activity in endometrial cells and that this effect requires interaction with IRSs in the IGFBP-1 promoter. It has been well documented in HepG2 cells that FKHR stimulates the IGFBP-1 promoter through IRSs [9, 10, 12, 33]. Phos-

phorylation of FKHR by insulin causes this transcription factor to be retained in the cytoplasm and its exclusion from the nucleus is associated with a loss of target gene expression [34, 35]. Studies with human decidualized cells indicate that FKHR is targeted to the nucleus where it can direct effects on gene expression [13]. Here, we used a mutant FKHR, which is not susceptible to phosphorylation by PKB or related phosphatidylinositol-3′kinase-dependent kinases, allowing for constitutive expression of FKHR in the nucleus. Whether factors associated with decidualization and IGFBP-1 expression regulate the distribution of endogenous FKHR to the nucleus, or its interaction with other nuclear factors, including HOXA10, is under investigation.

Recent studies have shown that FKHR can interact with other nuclear transcription factors, including the estrogen receptor, retinoic acid receptor, and thyroid hormone receptor, and either repress or stimulate transactivation mediated by the different nuclear hormone receptors [24, 36]. In this report we find that HOXA10 enhances the ability of FKHR to stimulate the IGFBP-1 promoter activity, and that these two transcription factors can physically associate with one another. Related studies suggest that HOXA5 can interact directly with FKHR, and we have found that HOXA5 also can function cooperatively with FKHR to stimulate the IGFBP-1 promoter in HuF cells [19]. This suggests that multiple HOX transcription factors might interact with FKHR, and perhaps with other FOXO forkhead proteins. Studies are in progress to identify specific domains and motifs that may mediate interactions between FKHR and HOX family members.

Further studies are required to determine the precise mechanism or mechanisms by which FKHR and HOXA10 function cooperatively to enhance IGFBP-1 promoter activity. It is unclear whether there are separate DNA binding sites for FKHR and HOXA10 in the IGFBP-1 promoter, or whether just one site is required for an FKHR-HOXA10 complex to bind. The present study demonstrates that DNA binding of FKHR is critical for FKHR and HOXA10 to act cooperatively on IGFBP-1 promoter activity. Previous studies have shown that FKHR interacts with IRSs in the IGFBP-1 promoter in a sequence-specific fashion [9, 12]. In the present study we found that interaction with IRS is required for functional cooperation between FKHR and HOXA10, and that the IRS region, together with the minimal IGFBP-1 promoter, is sufficient to mediate these effects. Because FKHR can interact directly with HOXA10, it is possible that this interaction may enhance either the binding of FKHR to IRSs or the recruitment of coactivating factors to this site. Alternatively, HOXA10 may interact weakly with *cis*-acting sequences in the region of the IRSs or the minimal IGFBP-1 promoter, and interaction with FKHR may help to stabilize HOXA10 binding. DNA binding by HOX proteins is mediated by the homeodomain, a conserved 61-amino acid sequence [37]. Homeodomains often interact with (A+T)-rich DNA sequences [14, 38–41], similar to those found in the region of the IRSs and minimal IGFBP-1 promoter. Electrophoresis mobility shift and supershift studies would help to characterize potential HOXA10 binding sites on the IGFBP-1 promoter and the possibility of a FKHR-HOXA10 complex. However, due to the lack of appropriate HOXA10 antibodies, experiments to test this possibility are not currently feasible.

Previous studies have suggested that DNA-binding by HOX family members may involve interactions with cofactors such as PBX, the mammalian homolog of *Drosophila* extradenticle [42–44]. PBX proteins bind to DNA cooperatively with mammalian HOX proteins and it is believed that interactions with PBX cofactors may contribute to the regulatory control and refinement of HOX protein function. In the present study we found that HOXA10 by itself has a limited effect on IGFBP-1 promoter activity, but functions effectively in combination with FKHR to enhance promoter activity. It is interesting to speculate that interactions with FKHR may modify the ability of HOXA10 to bind to (A+T)-rich target sites in the IGFBP-1 promoter and stimulate transcription.

Forkhead and HOX transcription factors may play an important role not only in the regulation of the IGFBP-1 gene but other genes as well in the endometrium. Greater expression of FKHR and HOXA10 during late luteal phase and in pregnancy suggests that these transcription factors play a role in regulating the expression of genes associated with implantation and the maintenance of pregnancy. Because FOXO forkhead and HOX proteins are found in a number of other tissues, it is interesting to speculate that members of these transcription factor families also may act coordinately to regulate gene expression in other settings.

## ACKNOWLEDGMENT

We thank David Powell for generously donating the human IGFBP-1 promoter construct.

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