Cyclic AMP-induced Forkhead Transcription Factor, FKHR, Cooperates with CCAAT/Enhancer-binding Protein β in Differentiating Human Endometrial Stromal Cells*

Mark Christian‡‡, Xiaohui Zhang‡‡, Tanja Schneider-Merkel‡, Terry G. Unterman‡, Birgit Gellersen‡, John O. White‡, and Jan J. Brosens‡‡‡

From the ‡Institute of Reproductive and Developmental Biology, Wolfson & Weston Research Centre for Family Health, Imperial College Faculty of Medicine, Hammersmith Hospital, London W12 0NN, United Kingdom, the §University of Illinois College of Medicine and the Chicago Area Veterans Healthcare System (West Side Division), Chicago, Illinois 60612, and the ¶Institute for Hormone and Fertility Research, University of Hamburg, Hamburg 22529, Germany

Received for publication, January 30, 2002, and in revised form, March 7, 2002

Decidual transformation of human endometrial stromal (ES) cells requires sustained activation of the protein kinase A (PKA) pathway. In a search for novel transcriptional mediators of this process, we used differential display PCR analysis of undifferentiated primary ES cells and cells stimulated with 8-bromocAMP (8-Br-cAMP). We now report on the role of forkhead homologue in rhabdomyosarcoma (FKHR), a recently described member of the forkhead-winged-helix transcription factor family, as a mediator of endometrial differentiation. Sustained 8-Br-cAMP stimulation resulted in the induction and nuclear accumulation of FKHR in differentiating ES cells. Immunohistochemical studies revealed that endometrial stromal expression of FKHR in vivo is confined to decidualizing cells during the late secretory phase of the cycle and coincides with the expression of CCAAT/enhancer-binding protein β (C/EBPβ). Reporter gene studies showed that FKHR potently enhances PKA-dependent activation of the tissue-specific decidual prolactin (dPRL) promoter, a major differentiation marker in human ES cells. Transcriptional augmentation by FKHR was effected through functional cooperation with C/EBPβ and binding to a composite FKHR-C/EBPβ response unit in the proximal promoter region. Furthermore, FKHR and C/EBPβ were shown to interact directly in a glutathione S-transferase pull-down assay. These results provide the first evidence of regulated expression of FKHR and demonstrate that FKHR has an integral role in PKA-dependent endometrial differentiation through its ability to bind and functionally cooperate with C/EBPβ.

During the menstrual cycle, ovarian estradiol and progesterone stimulate the ordered growth and differentiation of endometrial tissue compartments. In the human, this includes synchronous growth and coiling of the spiral arteries, secretory transformation of glandular epithelium, migration of bone marrow-derived cells, and decidualization of the stroma, which is thought to be essential for blastocyst implantation and subsequent formation of a hemochorial placenta. Decidualization of human endometrial stromal (ES)1 cells represents a process of morphological differentiation accompanied by distinct biochemical phenotypic changes. Decidual transformation is first apparent in stromal cells surrounding the spiral arteries approximately 10 days after the postovulatory rise in ovarian progesterone levels, indicating that the expression of decidual-specific genes is unlikely to be under direct transcriptional control of activated steroid hormone receptors.

There is compelling evidence to suggest that initiation of the decidual process requires elevated intracellular cAMP levels and sustained activation of the protein kinase A (PKA) pathway (1–5). Expression of PRL, under control of the tissue-specific decidual PRL (dPRL) promoter (3, 6), by ES cells coincides with decidual differentiation and is widely used as a biochemical marker of this process (2–5, 7). Previous studies have shown that CCAAT/enhancer-binding protein β (C/EBPβ), a member of the C/EBP subfamily of basic region/leucine zipper transcription factors, is induced during ES cell differentiation (4). Furthermore, C/EBPβ participates in the formation of a nucleoprotein complex that binds the proximal dPRL promoter region upon PKA activation (4). Although other members of the C/EBP family are expressed in cultured human ES cells, including C/EBPα and δ, C/EBPβ is the major and cAMP-inducible form (4).

To establish the identity of additional factors relevant to the decidual process, we used differential display PCR and isolated FKHR (forkhead homologue in rhabdomyosarcoma) as a cAMP-inducible gene in differentiating human ES cells. Forkhead or “winged helix” transcription factors have been shown to play important roles in cell differentiation, embryogenesis, and oncogenesis (8, 9). FKHR was first identified as a transcription factor involved in a translocation with PAX3 in alveolar rhabd...
domyosarcoma (10–12). It also has an important role in apoptosis, glucose homeostasis, cell cycle regulation, and as a nuclear receptor cofactor (13–18). We now report that FKHR is induced in decidualizing endometrium and participates in PKA signal transduction through its ability to interact and transcriptionally cooperate with C/EBPβ.

**EXPERIMENTAL PROCEDURES**

**Primary ES Cell Culture**—ES cells were isolated from normal proliferative endometrial tissues obtained from cycling women by endometrial biopsy at the time of diagnostic laparoscopy and hysterectomy (13, 20). Ham Smith and Queen Charlotte’s Hospital Research and Ethics Committee approved the study, and patient consent was obtained before biopsy. Samples were collected in Earle’s buffered saline containing 100 units/ml penicillin and 100 μg/ml streptomycin. After enzymatic digestion, the stromal cells were separated from epithelial cells and passed into culture as described previously (2, 7). Frustrating ES cells were cultured in maintenance medium of Dulbecco’s modified Eagle’s medium/F-12 containing 10% dextran-coated charcoal-treated FBS (DCC-FBS) and 1% antibiotic-antimycotic solution. Confluent monolayers were treated in Dulbecco’s modified Eagle’s medium/F-12 containing 2% DCC-FBS with 0.5 mM 8-Br-cAMP to induce a differentiated phenotype. All experiments were carried out before the fourth cell passage.

**RNA Isolation and Analysis**—Total RNA was extracted, using RNAzol B (Biogenesis), from primary cultures of ES cells after 12-h treatment with or without 0.5 mM 8-Br-cAMP. The experiment was carried out in duplicate using different biopsy samples. The differential display technique was performed on total RNA (100 ng) using RNAImage (GenHunter Corp., Nashville, TN), according to the manufacturer’s instructions. The radioactive RT-PCR products were size-fractionated on denaturing 6% polyacrylamide gels and visualized by autoradiography. The resulting autoradiographs were examined to locate cDNA bands corresponding to bases 270 of the dPRL promoter, with or without specific mutations (Table II), corresponding to bases 270FHmut)/BglII-dPRL/32/luc3 were generated previously (4, 5, 7). The resultant construct retains both C/EBP binding sites D and B. The BglII fragment was subcloned in-frame with a C-terminal 6x(His)-tag in pET-21b (Novagen). BL21-DE3 cells (Stratagene) were transformed with the expression vector, and protein expression was induced at mid-logarithmic growth by addition of 1 μM isopropyl-1-thio-

FKHR and C/EBPβ interact and cooperate in vivo. Immunofluorescence Microscopy and Immunohistochemistry—ES cells cultured on chamber slides (LabTek) were ﬁxed in methanol and permeabilized in 0.5% Triton. Cells were immunostained using anti-human FKHR antibody (N-18) diluted 1:100, followed by fluorescein isothiocyanate-conjugated rabbit anti-goat immunoglobulins (Dako) diluted 1:50. Parafﬁn-embedded, formalin-ﬁxed endometrial specimens were examined for in vivo FKHR and C/EBPβ immunoreactivity. All specimens were obtained from cycling premenopausal women and were free of intruterine disease such as endometrial hyperplasia or polyps. Using standard criteria, endometria were allocated to proliferative phase (n = 9), mid-secretory phase (n = 9), and late secretory phase (n = 6). Upon sections, placed on 1% w/v poly-lysine slides, were deparafﬁnized, dehydrated, exposed to 0.3% v/v H2O2 for 15 min, and subsequently microwaved in 0.01 M citrate buffer, pH 6.0. Immunostaining was carried out using anti-human FKHR antibody (N-18) diluted 1:100; biotinylated horse anti-goat Ig, diluted 1:500; and peroxidase-conjugated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200. In control slides, the primary antibody was replaced with normal rabbit IgG.

**Recombinant Protein and Gel Shift Assay**—The cDNA coding for amino acids 180–266 of FKHR, encompassing the DNA binding domain and the C-terminal 10 amino acids, was ampliﬁed, and NdeI and XhoI sites were created at the 5′- and 3′-ends, respectively, by PCR using the full-length FKHR cDNA as template and sense (5′-AGTTGTCACATAGTTGCTTTCCGGG-3′) and antisense (5′-GGCACGCAGCTGAGCGCTATTACAGAA-3′) primers. The ampliﬁed fragment was cloned into pcRS2.1 (Invitrogen) and sequenced. The NdeI-XhoI fragment was subcloned in-frame with a C-terminal 6xHis-tag in pET-21b (Novagen). BL21-DE3 cells (Stratagene) were transformed with the expression vector, and protein expression was induced at mid-logarithmic growth by addition of 1 μM isopropyl-1-thio-

β-d-galactopyranoside. Washed cells were lysed with 8 m urea, pH 4.5, and recombinant protein was puriﬁed by nickel-agarose chromatography and renatured folding. Recombinant protein was quantitated by Bradford dye-binding assay (Bio-Rad) and stored at −70 °C in 10% glycerol, 0.1 mM sodium phosphate buffer, pH 6.5.

To examine interactions between the FKHR DBD and by two-step dialysis. This process was repeated to ensure proper DNA targets. Synthetic double-stranded oligonucleotide probes (Table I) were end-labeled with 32P by T4 kinase and incubated with 0–30 ng of protein for 20 min at 4 °C in 20 μl of binding buffer (40 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol, 50 μg KCl, 1% glycerol, 1 mg/ml bovine serum albumin, and 50 ng/ml poly[dG-dC]-poly[dG-dC]), then loaded for 10% polyacrylamide non-denaturing gel electrophoresis. Autoradiograms were examined to locate cDNA bands that exhibited differential intensity in treated and untreated cells. Satisfactory cDNA bands were cut from the dried polyacrylamide gels, re-ampliﬁed by PCR, cloned into the plasmid vector pGEM-T Easy (Promega), and sequenced. Clone identities were determined by performing BLAST searches against the GenBankTM database. A single tube duplex reverse transcribe (RT)-PCR strategy was used to examine FKHR mRNA expression in differentiating ES cells. Briefly, 1 μg of total RNA, obtained from untreated cultures and cells stimulated with 8-Br-cAMP for 24 h, was reverse-transcribed and ampliﬁed in a single reaction using the Access RT-PCR System (Promega) according to the supplier’s instructions. Simultaneous ampliﬁcation of FKHR and β-actin was performed by adding 50 pmol each of the following oligonucleotides to each reaction: FKHR-sense (5′-AAGAGCCTTGGCCCTACTTCCA-3′), FKHR-antisense (5′-AACCTGTCGACAGCCTGCTGTG-3′), act-in-sense (5′-GGAGCAATGCTACCTGTCCT-3′), and act-in-sense (5′-CCCTCTGGCTGGACGTCC-3′). The β-actin cDNA, representing a non-regulated gene, served as an internal control. Ampliﬁcation efﬁciencies were monitored by the exponential phase of the ampliﬁcation reaction as determined by cycle proﬁling. Southern blots of the PCR products were successively hybridized with a 32P-labeled FKHR PCR product, ampliﬁed from the expression vector pCMV-FKHR using the oligonucleotides FKHR-sense and FKHR-antisense, and a 32P-labeled β-actin PCR product, ampliﬁed from RNA extracted from ES cells using the actin sense and FKHR antisense, and a 32P-labeled FKHR antisense (5′-AACTGTCGACAGCCTGCTGTG-3′), act-in-sense (5′-GGAGCAATGCTACCTGTCCT-3′), and act-in-sense (5′-CCCTCTGGCTGGACGTCC-3′). The β-actin cDNA, representing a non-regulated gene, served as an internal control. Ampliﬁcation efﬁciencies were monitored by the exponential phase of the ampliﬁcation reaction as determined by cycle proﬁling. Southern blots of the PCR products were successively hybridized with a 32P-labeled FKHR PCR product, ampliﬁed from the expression vector pCMV-FKHR using the oligonucleotides FKHR-sense and FKHR-antisense, and a 32P-labeled β-actin PCR product, ampliﬁed from RNA extracted from ES cells using the actin sense and FKHR antisense, and a 32P-labeled β-actin PCR product, ampliﬁed from RNA extracted from ES cells using the actin sense and FKHR antisense oligos.

**SDS-PAGE, Western Blotting, and Immunodetection**—A modiﬁed method of Rittenhouse and Marcus (19) was used for protein analysis. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories). Equal amounts of nuclear and cytosolic proteins (20 μg) were separated on a 10% SDS-polyacrylamide gel before electrotransfer at 80 V onto a polyvinylidene diﬂuoride membrane (Hybond P, Amersham Biosciences, Inc.). Even loading and transfer efﬁciency were conﬁrmed by Ponceau S staining. Non-speciﬁc binding sites were blocked by overnight incubation with 5% dried skimmed milk in Tris-buffered saline (TBS, 130 mM NaCl, 20 mM Tris, pH 7.6). For FKHR immuno-detection, blots were exposed to a primary rabbit polyclonal anti-FKHR antibody (N-18, Santa Cruz Biotechnology), diluted 1:1000 in TBS with 5% skimmed milk, for 1 h at room temperature. Blots were incubated with secondary peroxidase-conjugated rabbit anti-goat IgG (Sigma Chemical Co.), also for 1 h at room temperature. The primary antibody for C/EBPβ immuno-detection was a rabbit polyclonal anti-C/EBPβ antisera (C-19, Santa Cruz). Protein bands were visualized by enhanced chemiluminescence (ECL Western blotting detection, Amersham Biosciences, Inc.).
been reported (14, 17). Selective expression vectors for the activating or inhibiting isoform of C/EBPβ, pSG/LAP, and pSG/LIP, respectively, have also been described previously (4). Plasmid pRSV-C, encoding the PKA catalytic subunit, Cα, was a gift from Dr. Richard Maurer (Portland, OR).

**Transfection Studies**—Transient transfections of ES cells plated at a density of 2.5 \times 10^5 \text{ cells/well} in 24-well plates were performed by calcium phosphate precipitation in medium supplemented with 2% DCC-FBS. Promoter-reporter constructs and expression constructs were transfected at concentrations of 0.5 \mu g/well and 125 ng/well, respectively. The empty expression vectors pcDNA or pALTER were included as filler constructs when required. Cell extracts were harvested, and luciferase activity was measured with the luciferase reagent kit (Promega) and expressed as relative light units. Transfections were performed in triplicate and repeated at least three times. Representative experiments are shown (means \pm S.D.).

**GST Pull-down Assays**—GST pull-down assays were performed as described previously (7). \textsuperscript{35}S-Labeled proteins were prepared by the in vitro transcription-translation method, using the TnT T7 Coupled Reticulocyte Lysate System following the supplier’s protocol (Promega). The presence of \textsuperscript{35}S)methionine (>1000 Ci/mmol, Amersham Biosciences, Inc.) in the incubation mixture was used to produce labeled FKHR protein from the plasmid pcDNA/FKHR.

**RESULTS AND DISCUSSION**

Cyclic AMP Induces FKHR Expression in Differentiating Human ES Cells—Differential display PCR analysis of mRNA obtained from untreated primary ES cell cultures and cells treated with the cell-permeable cAMP analogue 8-Br-cAMP for 12 h yielded 19 apparently differentially expressed cDNAs. One clone was found to be 99% homologous to the reported sequence for the FKHR cDNA between nucleotides 1834 and 2078 relative to its start codon (20). The regulated expression of FKHR mRNA during cAMP-induced ES cell differentiation was confirmed by simultaneously amplified FKHR and \beta-actin mRNAs (Fig. 1A). Induction of FKHR protein upon 8-Br-cAMP treatment was apparent by Western blotting after 24 to 48 h of stimulation, with some variation between cultures, and its expression was sustained even after 8 days of treatment (Fig. 1B). To our knowledge, this is the first example of regulated expression of FKHR in mammalian cells.

FKHR and related members of the FOXO subgroup of the forkhead/winged helix family, including FKHRL1 and AFX (21, 22), have been previously identified as targets of protein kinase B (PKB/Akt), a serine/threonine kinase located downstream of phosphatidylinositol 3-kinase (13, 23–26). FKHR has three putative PKB/Akt phosphorylation sites (Thr-24, Ser-256, Ser-319), which are also conserved in DAF16, the nematode Caenorhabditis elegans homologue. Upon PKB/Akt phosphorylation, DAF16 and its human counterparts are retained in the cytoplasm, and their exclusion from the nucleus is associated with reduced transcriptional activity (13, 14, 23, 24). Hence, we determined the subcellular localization of FKHR in untreated and 8-Br-cAMP-stimulated human ES cell cultures. Immunofluorescence microscopy studies demonstrated that, upon 8-Br-cAMP treatment, FKHR accumulated predominantly in the nucleus (Fig. 1C). The absence of discernible cytoplasmic translocation suggests that FKHR is transcriptionally active in differentiating ES cells.

**Cycle-dependent Expression of FKHR and C/EBPβ in Human Endometrium**—Previous studies have shown that C/EBPβ is induced during cAMP-dependent differentiation of ES cells in
culture in a similar manner to FKHR (4). To delineate a potential role for these transcription factors in vivo, we investigated if FKHR and C/EBPβ are expressed in human endometrium in a cycle-dependent manner. Endometrial biopsies obtained at different phases of the menstrual cycle were immunohistochemically stained for either FKHR or C/EBPβ. Fig. 2 demonstrates weak immunoreactivity for FKHR but not C/EBPβ in the glandular compartment during the proliferative phase of the cycle. The glandular expression of both factors increased in the early secretory phase and was most intense toward the end of the cycle. In contrast, stromal expression of FKHR and C/EBPβ was confined to the late secretory phase of the cycle and most apparent in the decidualizing perivascular stroma. The distinct spatio-temporal expression of FKHR and C/EBPβ in differentiating human endometrial stroma suggests a role for these transcription factors in the regulation of the expression of decidua-specific genes in vivo.

There are two isoforms of C/EBPβ, the full-length liver-enriched activating protein (LAP) and the truncated liver-enriched inhibitory protein (LIP). The latter lacks the N-terminal transactivation domains of LAP and acts as a potent repressor of C/EBP-dependent transcription (27). Additional Western blot analysis studies showed the presence of LAP (1033 and 36 kDa), but not LIP (1156 kDa) in normal non-pregnant human endometrium (data not shown). This allowed us to conclude that the immunoreactive C/EBPβ in vivo represents the activating isoform LAP but not the transcriptional repressor LIP.

**FKHR Enhances dPRL Promoter Activity in Response to cAMP**—The coordinated expression of FKHR in the endometrial stroma during the late secretory phase of the cycle suggested a putative role in decidualization. Expression of PRL, a cardinal phenotypical marker of decidualization, is detectable in culture after ~48 h of 8-Br-cAMP treatment (2). The pattern of induction and nuclear retention of FKHR upon 8-Br-cAMP treatment in vitro suggested the dPRL promoter is a potential target for FKHR action. To test this hypothesis, primary cultures were transiently transfected with a luciferase reporter gene construct under control of either 3 kb of the dPRL promoter region (dPRL-3000/luc3) or the minimal cAMP-responsive promoter region (dPRL-332/luc3). Cotransfection of a FKHR expression vector minimally stimulated the basal activity of these promoter-reporter constructs (Fig. 3A). However, FKHR markedly enhanced induction of dPRL-3000/luc3 activity upon cAMP stimulation or in response to coexpressed catalytic subunit, Co, of the PKA holoenzyme (Fig. 3A, left panel). FKHR also enhanced cAMP- or Co-dependent activation of the dPRL-332/luc3 construct, and, qualitatively, this response was indistinguishable to that observed with the dPRL-3000/luc3 construct (Fig. 3A, right panel). These observations indicated that the minimal cAMP-responsive promoter region could be a target for FKHR. Additional transfection studies with a series of truncated promoter-reporter constructs identified the region between positions ~332 to ~270 as critical for FKHR-mediated enhancement of dPRL promoter activity (Fig. 3B).

Dependent upon the cellular context, cAMP or its effector PKA have been suggested to either stimulate or inhibit the PKB/Akt signaling pathway (28–30). This raised the possibility that cAMP could enhance the trans-activation potential of FKHR in differentiating ES cells by reducing PKB/Akt activity and thereby facilitating nuclear targeting of FKHR. However, overexpression of FKHR-(T/S/S)-A, a constitutively active mutant in which the three PKB/Akt phosphorylation sites (Thr-24, Ser-256, and Ser-319) are changed to alanines ((T/S/S)-A), only elicited a 3-fold increase in basal dPRL promoter activity (Fig. 3C). Furthermore, this FKHR mutant was still capable of enhancing promoter activity in response to 8-Br-cAMP treatment, indicating that phosphorylation of FKHR by PKB/Akt is not required for this effect. Overexpression of a DNA binding-deficient FKHR mutant (FKHR-Helix3.2M), in which critical residues within helix 3 of the DNA binding domain are mutated, failed to augment luciferase activity (Fig. 3C). This observation provides further evidence for a transcriptional role.
for FKHR in regulating dPRL gene expression and indicates a requirement for direct interaction with the dPRL promoter.

To determine whether the −332−270 region of the dPRL promoter contains specific binding sites for FKHR, we performed gel shift assays with [32P]-labeled oligonucleotide probes (Table I) and a bacterially expressed recombinant protein, and the formation of this nucleoprotein complex increased with the addition of more protein in a dose-dependent fashion. Studies with an excess of the unlabeled dPRL(332/270) competitor confirmed that this binding is competitive (data not shown).

It has been previously reported that the −332−270 dPRL promoter region contains two response elements (C/EBP D and B; Table I) that can form nucleoprotein complexes containing C/EBPβ in combination with nuclear proteins prepared from differentiated ES cells (4). Additional gel shift studies with truncated oligonucleotide probes revealed that residues between −301 and −270 are sufficient to interact with the FKHR DBD (Fig. 4B). Interestingly, a mutation that disrupts both D and B sites (probe dPRL(310/281).HMut) also disrupts FKHR DBD binding to dPRL(332/270) dPRL promoter region, and contribute to cAMP-stimu-
labeled promoter activity, suggested possible functional cooperation between these distinct transcription factors. To test this hypothesis, primary human ES cells were transiently transfected with a luciferase construct under control of the −332/−270 region fused to the dPRL proximal promoter region and for transcriptional synergy with C/EBPβ.

We previously reported that overexpression of C/EBPβ modestly activates the control reporter construct dPRL-32/luc3 (5-fold) as well as the promoterless construct pGL3-Basic (7). Activation of dPRL(−332/−270Dmut)/−32/luc3, which does not have functional C/EBP or FKHR binding sites, in the presence of C/EBPβ was identical to that observed with the dPRL-32/luc3 construct. Furthermore, coexpression of FKHR and C/EBPβ had no additional effect upon reporter activity. Selective ablation of the distal C/EBPβ binding site (dPRL(−332/−270Dmut)/−32/luc3) also markedly blunted C/EBPβ-dependent trans-activation of the −332/−270 region (7-fold). However, transcriptional cooperation between C/EBPβ and FKHR was still apparent, because coexpression of both factors elicited a 16-fold induction in dPRL(−332/−270Dmut)/−32/luc3 activity (Fig. 5A). In contrast, targeted deletion of the FKHR binding sites (dPRL(−332/−270FHmut)/−32/luc3) not only impaired C/EBPβ trans-activation of the −332/−270 promoter region but also abolished its cooperation with FKHR. Together, these results indicate that the proximal composite FKHR/C/EBPβ binding site is essential for C/EBPβ trans-activation of the −332/−270 promoter region but for transcriptional synergy with FKHR. In contrast, the distal C/EBPβ binding site is also nec-

![Diagram](https://via.placeholder.com/150)

**Fig. 4. Identification of FKHR binding sites in the dPRL promoter.** A, dose-dependent binding. A double-stranded 32P-labeled probe spanning residues −332 to −270 of the dPRL promoter was incubated with various amounts of recombinant protein (0–30 ng) containing the DBD of FKHR (amino acids 160–266), then loaded for non-denaturing polyacrylamide gel electrophoresis. Free and bound probe were identified by autoradiography of dried gels. B, localization of FKHR binding sites within the dPRL promoter. Gel shift studies were performed using probes containing smaller portions of the −332/−270 dPRL promoter region with or without targeted mutations as described in Table I. C, relative activity of the FKHR binding sites in the dPRL promoter. 32P-Labeled oligonucleotide probes (20,000 cpm/lane) containing FKHR-1 (dPRL(310/281).FHmut2) or FKHR-2 (dPRL(310/281).FHmut1) sites from the dPRL promoter, or the insulin response sequence from the IGFBP-1 promoter were incubated with increasing amounts of recombinant protein containing the FKHR DBD prior to gel electrophoresis. Free and bound probes were identified by autoradiography. D, quantification of the binding activities for probes containing mutations of FKHR-1 (dPRL(310/281).FHmut1) or FKHR-2 (dPRL(310/281).FHmut2) sites from the dPRL promoter or IRS-1 (ΔIRS-1) from the IGFBP-1 promoter. Free and bound radioactivity, in the presence of various concentrations of recombinant protein, was quantified by phosphorimagery, and binding activity was defined as the percentage of bound probe.

![Diagram](https://via.placeholder.com/150)

**Fig. 5. Functional and physical interaction between FKHR and C/EBPβ.** A, the composite FKHR/C/EBPβ binding site in the dPRL promoter is required for transcriptional cooperation between FKHR and C/EBPβ. Primary cultures were transfected with one of the following reporter constructs, described in Table II, as indicated: dPRL(−332/−270wt)/−32/luc3, dPRL(−332/−270Dmut)/−32/luc3, dPRL(−332/−270FHmut)/−32/luc3, dPRL(−332/−270DBmut)/−32/luc3, and dPRL(−332/−270FHmut)/−32/luc3. Expression vectors for FKHR-WT or the LAP isoform of C/EBPβ were cotransfected as indicated. Cellular extracts were harvested after 40 h, and the results show mean luciferase activity ± S.D. of triplicate measurements. B, FKHR binds the C-terminal region of C/EBPβ. In vitro translated 35S-labeled FKHR was incubated with GST-fused full-length C/EBPβ (GST-LAP), GST-tagged truncated C/EBPβ (GST-LIP), or GST alone immobilized on glutathione-agarose beads as indicated. The bound proteins were resolved on 10% SDS-polyacrylamide gels and visualized by autoradiography.

C/EBPβ trans-activation of the −332/−270 promoter region. The results indicate that the proximal composite FKHR/C/EBPβ binding site is essential for C/EBPβ trans-activation of the −332/−270 promoter region but also abolished its cooperation with FKHR. Together, these results indicate that the proximal composite FKHR/C/EBPβ binding site is essential for C/EBPβ trans-activation of the −332/−270 promoter region and for transcriptional synergy with FKHR. In contrast, the distal C/EBPβ binding site is also nec-
cAMP-induced FKHR Cooperates with C/EBPβ

Sequence of the dPRL -332/-270 wild-type (wt) element and mutations in the C/EBP binding site D (Dmut), and the FKHR binding sites D and B (DBmut), and the FKHR sites 1 and 2 (FHmut). The targeted binding regions are underlined, and mutated bases are given in boldface lowercase letters. The elements were inserted into the minimal dPRL promoter/luciferase reporter gene plasmid dPRL -32/luc3 to yield dPRL -332/-270wt/ -32/luc3, dPRL -332/-270Dmut/ -32/luc3, dPRL -332/-270DBmut/ -32/luc3, and dPRL -332/-270FHmut/ -32/luc3, respectively.

| Table II  |
|------------|
| Mutations of the dPRL -332/-270 promoter region for transient transfection studies |
| Sequence of the dPRL -332/-270 promoter region for transient transfection studies |
| dPRL -332/-270wt ATTATGTTCTGAGGCGTCTCTGCTGTTGTTGTTGTTAAGATGTAGGACACATGCTCTGTCTCTGCTC |
| dPRL -332/-270Dmut ATTATGTTCTGAGGCGTCTCTGCTGTTGTTGTTGTTAAGATGTAGTGACACATGCTCTGTCTCTGCTC |
| dPRL -332/-270DBmut ATTATGTTCTGAGGCGTCTCTGCTGTTGTTGTTGTTAAGATGTAGTGACACATGCTCTGTCTCTGCTC |
| dPRL -332/-270FHmut ATTATGTTCTGAGGCGTCTCTGCTGTTGTTGTTGTTAAGATGTAGTGACACATGCTCTGTCTCTGCTC |

The ability of FKHR and C/EBPβ to regulate transcriptional activation of the -332/-270 promoter region through interaction with a composite response element raised the possibility of physical association between these distinct transcription factors. This was confirmed by in vitro protein binding studies demonstrating specific interactions between FKHR and the glutathione S-transferase (GST)-tagged full-length C/EBPβ (GST-LAP). FKHR also interacted with the GST-fused truncated C/EBPβ isoform (GST-LIP) (Fig. SB), indicating that the N-terminal trans-activation domains of C/EBPβ are not required for physical association with FKHR. This is in agreement with other studies demonstrating that the C terminal domain of C/EBPβ mediates binding to other nuclear factors, including the phosphoprotein Nopp140, members of the nuclear factor-x family, Ets-1, and various members of the nuclear receptor superfamily (7, 32–35).

Takeda et al. have shown in vivo and in vitro results indicate that FKHR is an important effector of the decidual response in the late secretory phase of the menstrual cycle. Interestingly, our results indicate that FKHR interacts with the PKA signal transduction pathway in at least two ways to contribute to the coordinated expression of decidua-specific genes in differentiating human ES cells. First, differential display studies revealed that FKHR mRNA is induced in ES cells after stimulation with cAMP, and subsequent studies confirmed that levels of FKHR protein also are increased during ES cell differentiation in vivo and in vitro. Previous studies have shown that the expression of FKHR and other members of the FOXO subfamily of forkhead/winged-helix transcription factors is tissue-specific (21, 23, 31). To our knowledge, the observation that FKHR expression is regulated through a CAMP-dependent pathway provides the first report indicating that FOXO proteins can be regulated in response to activation of a discrete signaling pathway. Studies are in progress to examine specific mechanisms mediating this effect of CAMP on FKHR expression and to determine whether FKHR gene expression is responsive to activation of the PKA pathway in different cells.

We also found that expression of FKHR enhances dPRL promoter activity in the presence of activated PKA. This result indicates that, once expressed, FKHR interacts with and enhances the function of other cellular factors mediating effects of CAMP on promoter activity. We find that FKHR functions cooperatively with C/EBPβ to stimulate dPRL promoter function through a previously identified C/EBP response element in the proximal promoter region. Direct interaction between FKHR and C/EBPβ may contribute to their ability to function cooperatively but does not exclude other potential mechanisms, including the recruitment of shared coactivators such as p300/CREB (36, 37). Previous in vitro binding studies indicate that C/EBP and forkhead proteins interact with overlapping elements in the phosphoenolpyruvate carboxyl kinase promoter (38, 39). We and others have reported that a nucleoprotein complex containing C/EBPβ interacts with a known FKHR binding site (IRSA) in the IGBP-1 promoter (40), and recent studies indicate that FOXO forkhead proteins may contribute to the formation of this complex. These observations indicate that the functional interaction between FOXO forkhead family members and C/EBP transcription factors may be important for transcriptional activation of diverse genes.

Acknowledgments—We thank Xiau Feng Li and Fred Barker for their technical assistance and Philip Cohen and Richard Maurer for providing reagents. We are indebted to Yvonne Pohnke for promoter constructs and critical discussion. We also thank the clinicians at Hamersmith Hospital for providing endometrial biopsies.

REFERENCES

1. Brar, A. K., Frank, G. R., Kessler, C. A., Cedars, M. I., and Handwerger, S. (1997) Endocrine 6, 301–307
2. Brosens, J. J., Hayashi, N., and White, J. O. (1999) Endocrinology 140, 4809–4820
3. Gellersen, B., Kempf, R., Telgmann, R., and DiMattia, G. E. (1994) Mol. Endocrinol. 8, 356–373
4. Pohnke, Y., Kempf, R., and Gellersen, B. (1999) J. Biol. Chem. 274, 24808–24818
5. Telgmann, R., Maronde, E., Tasken, K., and Gellersen, B. (1997) Endocrinology 138, 929–937
6. Berwaer, M., Martial, J. A., and Davis, J. R. (1994) Mol. Endocrinol. 8, 635–642
7. Christian, M., Pohnke, Y., Kempf, R., Gellersen, B., and Brosens, J. J. (2002) Mol. Endocrinol. 16, 141–154
8. Kaufmann, E., and Knochel, W. (1996) Mech. Dev. 57, 3–20
9. Lai, E., Clark, K. L., Burley, S. K., and Darnell, J. E., Jr. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10421–10423
10. Davis, R. J., D’Cruz, C. M., Lovell, M. A., Biegl, J. A., and Barr, F. G. (1994) Cancer Res. 54, 2869–2872
11. Fredericks, W. J., Galli, N., Mukhopadhyay, S., Rovera, G., Bennicelli, J., Barr, F. G., and Rauscher, F. J., 3rd (1995) Mol. Cell. Biol. 15, 1522–1535
12. Shapiro, D. N., Sublett, J. E., Li, B., Downing, J. R., and Naeve, C. W. (1993) J. Biol. Chem. 268, 17164–17170
13. Kettenring, A., Bohn, A., Zignoli, M. J., Liu, M. Z., Joo, P. H., Lu, S. K., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) Cell 96, 857–868
14. Guo, S., Rena, G., Cichy, S., He, X., Cohen, P., and Unterman, T. (1999) J. Biol. Chem. 274, 17184–17192
15. Nakamura, N., Rama, B., Vazquez, F., Signoretto, S., Loda, M., and Sellers, W. R. (2000) Mol. Cell. Biol. 20, 8969–8982
16. Schuur, E. R., Loken, A. V., Sharma, M., Sun, Z., Roth, R. A., and Weigel, R. J. (2001) J. Biol. Chem. 276, 33524–33530
17. Yeagle, D., Guo, S., Unterman, T., and Quinn, P. G. (2001) J. Biol. Chem. 276, 35705–35710
18. Zhao, H. H., Herrera, R. E., Coronado-Heinsohn, E., Yang, M. C., Ludescher, D., Meier, B., and Meyer, J. H. (2001) J. Biol. Chem. 276, 37907–37912
19. Rittenhouse, J., and Marcus, F. (1984) Anal. Biochem. 138, 442–448
20. Galili, N., Davis, R. J., Fredericks, W. J., Mukhopadhyay, S., Rauscher, F. J., 3rd, Emanuel, B. S., Rovera, G., and Barr, F. G. (1995) Nat. Genet. 9, 230–235
21. Anderson, M. J., Viars, C. S., Czekay, S., Cavenee, W. K., and Arden, K. C. 22. X. Zhang and T. Unterman, unpublished observations.
cAMP-induced FKHR Cooperates with C/EBPβ

(1998) Genomics 47, 187–199
22. Borkhardt, A., Repp, R., Haas, O. A., Leis, T., Harbott, J., Kreuder, J., Hammann, J., Henn, T., and Lampert, F. (1997) Oncogene 14, 195–202
23. Biggs, W. H., 3rd, Meisenhelder, J., Hunter, T., Cavenee, W. K., and Arden, K. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7421–7426
24. del Peso, L., Gonzalez, V. M., Hernandez, R., Barr, F. G., and Nunez, G. (1999) Oncogene 18, 7328–7333
25. Rena, G., Guo, S., Cichy, S. C., Unterman, T. G., and Cohen, P. (1999) J. Biol. Chem. 274, 17179–17183
26. Tang, E. D., Nunez, G., Barr, F. G., and Guan, K. L. (1999) J. Biol. Chem. 274, 16741–16746
27. Descombes, P., and Schibler, U. (1991) Cell 67, 569–579
28. Cass, L. A., Summers, S. A., Prendergast, G. V., Bassler, J. M., Birnbaum, M. J., and Meinkoth, J. L. (1999) Mol. Cell. Biol. 19, 5882–5891
29. Tsygankova, O. M., Saavedra, A., Rehuhn, J. F., Quilliam, L. A., and Meinkoth, J. L. (2001) Mol. Cell. Biol. 21, 1921–1929
30. Wang, L., Liu, F., and Adame, M. L. (2001) J. Biol. Chem. 276, 37242–37249
31. Furuyama, T., Nakazawa, T., Nakano, I., and Mori, N. (2000) Biochem. J. 349, 629–634
32. Lee, Y. M., Miau, L. H., Chang, C. J., and Lee, S. C. (1996) Mol. Cell. Biol. 16, 4257–4263
33. McNagny, K. M., Sieweke, M. H., Doderlein, G., Graf, T., and Nerlov, C. (1998) EMBO J. 17, 3669–3680
34. Miau, L. H., Chang, C. J., Tsai, W. H., and Lee, S. C. (1997) Mol. Cell. Biol. 17, 239–239
35. Stein, B., Cogswell, P. C., and Baldwin, A. S., Jr. (1993) Mol. Cell. Biol. 13, 3964–3974
36. Mink, S., Haenig, B., and Klempnauer, K. H. (1997) Mol. Cell. Biol. 17, 6609–6617
37. Nasrin, N., Ogg, S., Cahill, C. M., Biggs, W., Nui, S., Dore, J., Calvo, D., Shi, Y., Ruvkun, G., and Alexander-Bridges, M. C. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10412–10417
38. O’Brien, R. M., Noisin, E. L., Suwanichkul, A., Yamasaki, T., Lucas, P. C., Wang, J. C., Powell, D. R., and Granner, D. K. (1995) Mol. Cell. Biol. 15, 1747–1758
39. O’Brien, R. M., Lucas, P. C., Yamasaki, T., Noisin, E. L., and Granner, D. K. (1994) J. Biol. Chem. 269, 30419–30428
40. Durham, S. K., Suwanichkul, A., Scheimann, A. O., Yee, D., Jackson, J. G., Barr, F. G., and Powell, D. R. (1999) Endocrinology 140, 3140–3146
41. Ghosh, A. K., Lacson, R., Liu, P., Cichy, S. B., Danilkovich, A., Guo, S., and Unterman, T. G. (2001) J. Biol. Chem. 276, 8507–8515