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Transcriptional Repression of Peri-Implantation EMX2 Expression in Mammalian Reproduction by HOXA10

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HOXA10 is necessary for mammalian reproduction; however, its transcriptional targets are not completely defined. EMX2, a divergent homeobox gene, is necessary for urogenital tract development. In these studies we identify and characterize the regulation of EMX2 by HOXA10. By using Northern analysis and in situ hybridization, we found that EMX2 is expressed in the adult urogenital tract in an inverse temporal pattern from HOXA10, suggestive of a negative regulatory relationship. Constitutive expression of HOXA10 diminished EMX2 mRNA, whereas blocking HOXA10 through the use of antisense resulted in high EMX2 mRNA expression. Deletional analysis of the EMX2 5′ regulatory region revealed that a 150-bp element mediated transcriptional repression when cotransfected with pcDNA3.1/HOXA10 in transient-transfection assays. Binding of HOXA10 protein to this element was demonstrated by electrophoretic mobility shift assay and further localized to a consensus HOXA10 binding site within this element by DNase I footprinting. Site-directed mutagenesis abolished binding, as well as the negative transcriptional regulation. Transcriptional activation of *empty spiracles*, the *Drosophila* ortholog of EMX2, by *Abdominal-B* (HOXA10 ortholog) has been previously demonstrated. These findings demonstrate conservation of the transcription factor-target gene relationship, although the direction of regulation is reversed with possible evolutionary implications.

HOX genes encode homeodomain transcription factors that are essential to the establishment of the metazoan body plan (1, 19, 35, 37). Homeodomain transcription factors are pleiotropic transcriptional modulators. They regulate conserved hierarchical pathways, resulting in tissue differentiation (20, 35). Few HOX target genes have been described, and none have been implicated in reproductive tract development.

Differentiation of the reproductive tract occurs in two phases: the organogenic and functional phases (29). Organogenic differentiation confers organ specificity to the Müllerian duct. Organ specification occurs only once during development and is irreversible. Functional differentiation describes cyclic growth and differentiation in response to sex steroids enabling receptivity to embryo implantation and pregnancy. Sex steroids are necessary and sufficient for functional development in the reproductive tract. The molecular pathways resulting in these events are not well defined and are likely to involve HOX genes. HOXA10 and HOXA11 are expressed in both the embryonic and the adult reproductive tracts, where HOXA10 is predominantly expressed in the uterus (55). Uterine expression of HOXA10 is regulated by 17β-estradiol and progesterone; HOXA10 expression varies throughout the reproductive cycle, reaching high levels at the time of embryo implantation (54). HOXA10 is necessary for proper uterine development and for receptivity to embryo implantation in both murine and human endometrium (4, 5, 7, 8, 46).

The essential role of HOXA10 during reproductive tract organogenesis has been demonstrated by anomalous uterine development associated with altered HOXA10 expression (8). This altered uterine development has been shown to result in reproductive wastage (45). In addition, persistent adult expression of HOXA10 is necessary for functional endometrial maturation, resulting in receptivity to embryo implantation. Mice with a targeted Hoxa10 gene mutation exhibit failure of implantation resulting in infertility (7, 46). Further, in adult mice, blockade or overexpression of Hoxa10 by using either HOXA10 antisense or a HOXA10 expression vector results in a corresponding decrease or increase in litter size, respectively (5).

Although the functional derangements resulting from altered HOXA10 expression in the reproductive tract have been previously reported, the transcriptional targets that underlie these defects have not yet been identified. EMX2 is a ho-

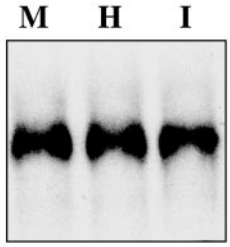

FIG. 1. EMX2 is expressed in the adult reproductive tract. Persistent expression of Emx2/EMX2 is seen in adult mouse and human endometrium and in Ishikawa cells. Total RNA from estrus cycle day 1 adult mouse uteri or adult human proliferative-phase endometrium was analyzed by Northern blotting with a 3′-UTR EMX2 riboprobe. Northern blot results of representative samples are shown (M, mouse; H, human). Ishikawa (I) cells are a well-differentiated endometrial adenocarcinoma cell line (39). Expression of estrogen and progesterone receptors, as well as HOXA10, in this cell line has been previously well characterized (23, 24, 30, 54). Expression of EMX2 mRNA in this cell line is also shown.

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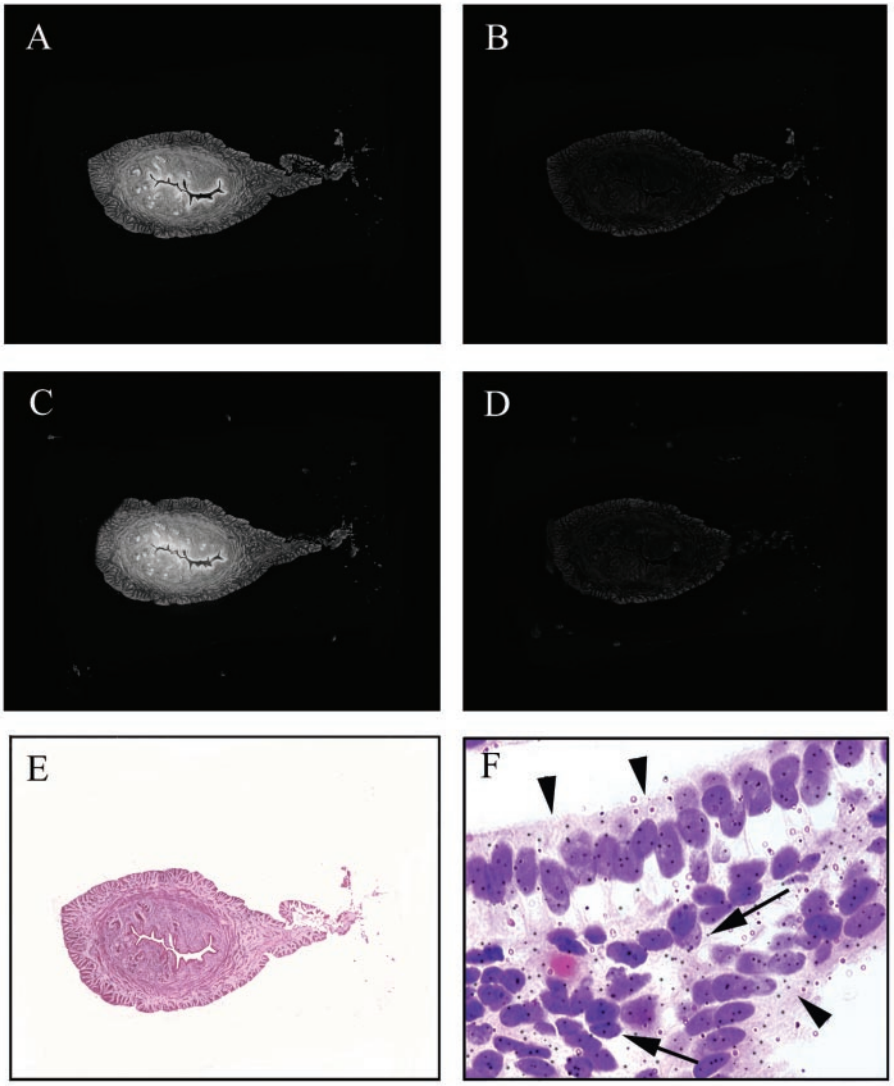

FIG. 2. Localization of adult uterine Emx2 and Hoxa10 expression. Both Emx2 and Hoxa10 expression are evident throughout the uterus, with the highest expression in the endometrium. To localize Emx2 and Hoxa10 expression within the adult uterus, in situ hybridization was performed with ^{33}P-labeled EMX2 and HOXA10 riboprobes, respectively. Representative photomicrographs of results are shown (×100 magnification). (A) Dark-field photomicrograph of mouse uterus hybridized to the EMX2 riboprobe. (B) Lack of hybridization to a labeled EMX2 sense probe. (C) Dark-field photomicrograph of mouse uterus hybridized to a HOXA10 riboprobe. (D) Lack of hybridization to a labeled HOXA10 sense probe. (E) Hematoxylin-eosin-stained section revealing the layers of a mouse uterus on a transverse section. The endometrium adjacent to the lumen is comprised of surface epithelium immediately lining the uterine cavity and of glands. The interglandular tissue comprises the stroma. The endometrium is surrounded by a few layers of circular smooth muscle (myometrium) and finally the external layer (serosa), comprised of loose connective tissue. (F) High-power photomicrograph (×400) showing results of in situ hybridization of human endometrium with silver grains over both the glandular epithelium and endometrial stroma. Arrow, endometrial stroma; arrowhead: endometrial glands.

meobox gene located outside of the HOX cluster (26). Mammalian Emx2/EMX2 are orthologues of the *Drosophila empty spiracles* (*ems*) gene (16, 21). The *Drosophila* HOXA10 ortholog, *Abdominal-B*, has been previously shown to directly regulate *ems* (25, 53). Emx2/EMX2 are essential for dorsal telencephalon development and are also expressed in the epithelial components of the developing urogenital system (9, 41, 50, 51, 56, 57). Emx2 mutant mice exhibit Müllerian duct agenesis and die in utero from renal anomalies (36). The embryo lethal phenotype has precluded investigation of the function of Emx2 in the adult reproductive tract of Emx2 mutant mice. Since extensive cyclic growth and development continue to occur in the adult reproductive tract, we used this tissue to investigate the possibility that EMX2 expression is directly regulated by HOXA10.

MATERIALS AND METHODS

Cell culture. All cell lines were of human origin. Ishikawa cells were maintained in charcoal stripped, phenol-red free minimal essential medium (Invitrogen, Carlsbad, Calif.) containing 2.0 mM L-glutamine and Earle's salts and supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% penicillin-streptomycin. BT-20 cells were maintained in charcoal-stripped, phenol

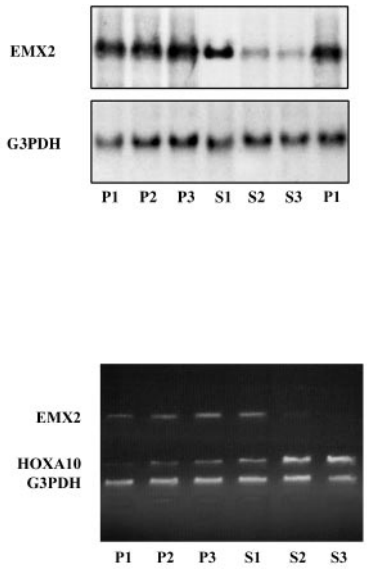

FIG. 3. EMX2 expression varies throughout the human reproductive cycle. EMX2 is expressed in the endometrium throughout the reproductive cycle. Endometrium from 32 subjects representing each developmental phase of the reproductive cycle was analyzed by Northern blotting and RT-PCR. (Top panel) Northern analysis was performed with a riboprobe complementary to the 3′-UTR of EMX2. Hybridization with a G3PDH probe was used as control. EMX2 mRNA expression rises significantly throughout the proliferative phase of the reproductive cycle (days 1 to 14 or 28) and peaks in the early secretory phase. This is rapidly followed by an ca. 50% decrease. Representative results of the Northern blot are shown. P1, P2, and P3 correspond to the early (days 1 to 5), middle (days 6 to 10), and late (days 9 to 14) proliferative phases, respectively. Similarly, S1, S2, and S3 correspond to the early (days 15 to 18), middle (days 19 to 23), and late (days 24 to 28) secretory phases, respectively, of the human reproductive cycle. (Bottom panel) Semiquantitative RT-PCR was performed simultaneously with primers that specifically amplify HOXA10 and EMX2. Amplification of G3PDH was performed as a control. EMX2 expression increases throughout the proliferative phase of the reproductive cycle (days 1 to 14 of 28) to peak in the late proliferative and early secretory phases. Expression declined by >50% (normalized to G3PDH) in the mid-secretory phase (S2), with continued decline in the late secretory phase (S3). Simultaneously, HOXA10 was expressed at low levels throughout the proliferative phase (P1, P2, and P3) and increased by the middle and late secretory phases (S2 and S3).

red-free RPMI 1640 (Invitrogen) containing 2.0 mM L-glutamine and Earle's salts supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% penicillin-streptomycin.

Tissue collection. Endometrium samples were collected from normal cycling human subjects by Pipelle endometrial biopsy under an approved human investigations committee protocol. The tissue was immediately placed in liquid nitrogen and stored at −72°C. Endometrial dating was determined from cycle history and confirmed histologically based upon the criteria of Noyes et al. (40).

Northern blot analysis. A 203-bp element of the EMX2 3′-untranslated region (3′-UTR) was amplified by PCR. The product was phenol-chloroform purified, ethanol precipitated, and cloned into the *Srf*I site in PCR Script-SK(+) plasmid (Stratagene, La Jolla, Calif.). The vector was linearized with *Eco*RI, ethanol precipitated, and used as a template for riboprobe synthesis. For HOXA10, pGEM plasmid containing 103 bp of the HOXA10 3′-UTR was used as previously described (54). RNA probes were generated by in vitro transcription by using the riboprobe system kit (Promega, Madison, Wis.). T3 polymerase and SP6 were used for synthesis of the EMX2 and HOXA10 riboprobes, respectively. Total cellular RNA was isolated from Ishikawa cells by using Trizol (Invitrogen) according to the manufacturer's guidelines. Total RNA (40 to 50 μg) was size fractionated on 1% agarose–0.66 M formaldehyde gel and transferred to nylon membranes. The membrane was hybridized to the EMX2 riboprobe.

Western blot analysis. Ishikawa cells were lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM $NaSO_4$, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 12,000 × *g* for 2 min at 4°C, and the supernatant was collected. The protein content was quantified by the Bradford method by using a protein assay kit (Bio-Rad Laboratories, Richmond, Va.). Then, 30-μg aliquots were loaded onto a sodium dodecyl sulfate–6% polyacrylamide gel, size fractionated, and transferred to a nitrocellulose membrane by using a Transblot apparatus (Bio-Rad) at 100 V for 2 h at 4°C. The membrane was immersed in a 3% gelatin–Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl) blocking solution for 30 min at room temperature, washed for 10 min in TBS (20 mM Tris, 500 mM NaCl, 0.05% Tween 20; pH 7.5), and then incubated for 1 h with a 1:1,000 dilution of HOXA10 polyclonal antibody (Covance, Richmond, Calif.). The membrane was washed with TBS for 5 min at room temperature and incubated for 1 h with a 1:200 dilution of goat anti-mouse immunoglobulin G-horseradish peroxidase (Bio-Rad). The membrane was then washed twice in TBS for 5 min at room temperature and then immersed in a horseradish peroxidase color developer buffer (Bio-Rad) for 30 min. Photographs were taken immediately after color development.

HOXA10 fusion protein and in vitro translation or isolation. HOXA10 cDNA was a gift of C. Largman. The fusion protein was constructed by cloning HOXA10 cDNA into the *Eco*RI site of the pcDNA3.1(+) plasmid (Invitrogen). Subsequently, oligodeoxyribonucleotides encoding the FLAG tag were cloned in frame into an *Nhe*I site in the polylinker immediately 5′ to the pcDNA3.1-HOXA10. The FLAG Tag sequence was 5′-TCTGCTAGCCCCATGGACTA CGA AGGACGACGATGACAACGATCTCCCGCCCGCGCTAGCCTCCTC T-3′. Ishikawa cells, grown to 70% confluence in 25-cm^2 flasks, were transfected for 5 h with 6 μg of HOXA10FLAG-pcDNA3.1 by using Lipofectamine {Invitrogen; 3:1 [wt/wt] formulation of 2,3-dioleyloxy-*N*- [2(sperminecarboxamido) ethyl]-*N*,*N*-dimethyl-1-propanium trifluoroacetate [DOPSA] and dioleoylphosphatidyl ethanolamine [DOPE]}. After transfection, cells were washed with phosphate-buffered saline (PBS) and allowed to grow for an additional 72 h. HOXA10-Flag protein was isolated from the cellular lysate by using an anti-Flag M1 affinity gel (Sigma, St. Louis, Mo.).

Reporter constructs and expression plasmids. To test the function of putative HOXA10 binding sites in the region from bp 0 to −2,000 5′ of the EMX2 translation start site, specific deletions were constructed. Four elements of 400 to 500 bp were amplified and analyzed in transient-transfection assays. Deletional analysis revealed that a single 450-bp region termed EMXE encoding the region from bp −700 to −250 (GenBank accession no. AY116142) drove HOXA10-mediated reporter gene repression. A PCR product corresponding to EMXE was synthesized by using *Taq* polymerase (Promega) and cloned into the *Kpn*I/*Sac*I sites of the pGL3 promoter vector (Promega) to generate pGL3/EMXE. A 150-bp oligonucleotide, EMXA (corresponding to nucleotides [nt] −400 to −250 of the EMX2 5′ region), was synthesized. The oligonucleotide was digested with *Kpn*I and *Sac*I and inserted into the corresponding sites of the pGL3 promoter vector (pGL3/EMXA). The oligonucleotide corresponding to the region from bp −549 to −401, adjacent to EMXA described above, was termed EMXB and was inserted into the *Sac*I/*Sma*I sites of pGL3 promoter to create pGL3/EMXB. The region corresponding to bp −700 to −550 was termed EMXC and was cloned into the *Sma*I/*Xho*I sites of pGL3 promoter to form pGL3-EMXC. The EMXC insert was cloned into pGL3-EMXB at *Eco*RI/*Xho*I sites to form pGL3-EMXD, a 300-bp insert. In other experiments, five copies of a 40-bp sequence encompassing the HOXA10 binding site in either EMXB (nt −467 to −506) or EMXC (nt −566 to −605) was synthesized as described above and inserted into the *Kpn*I/*Xho*I sites of the pGL3 Control vector (Promega) to generate either pGL3/5XEMXB or pGL3/5XEMXC. The identity of the inserts was confirmed by sequencing.

Electrophoretic mobility shift assays (EMSAs). Complementary single-stranded oligodeoxyribonucleotides designed to incorporate the HOXA10 binding site in oligonucleotide EMXC (described above) and its flanking sequence (nt −588 to −565) in the EMX2 5′ regulatory region were synthesized. Corresponding oligonucleotides containing the mutated HOXA10 binding site were similarly synthesized. The oligonucleotide probes had the following sequences: EMXC probe, 5′-AGGAAGCTGTTTATGTGATCCCCG-3′; and mutant EMXC probe, 5′-AGGAAGCTGTGCATGTGATCCCCG-3′. The oligonucleotide probes were end labeled with [^{32}P]dATP (Amersham, Arlington Heights, Ill.) by using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) and purified with QuickSpin columns (Roche, Indianapolis, Ind.). Nuclei were isolated, and nuclear extracts prepared from either Ishikawa or primary endometrial epithelial cells growing at log phase by the method described by Dignam et al. (17). Binding reactions were performed on ice for 30 min with either 0.5 to

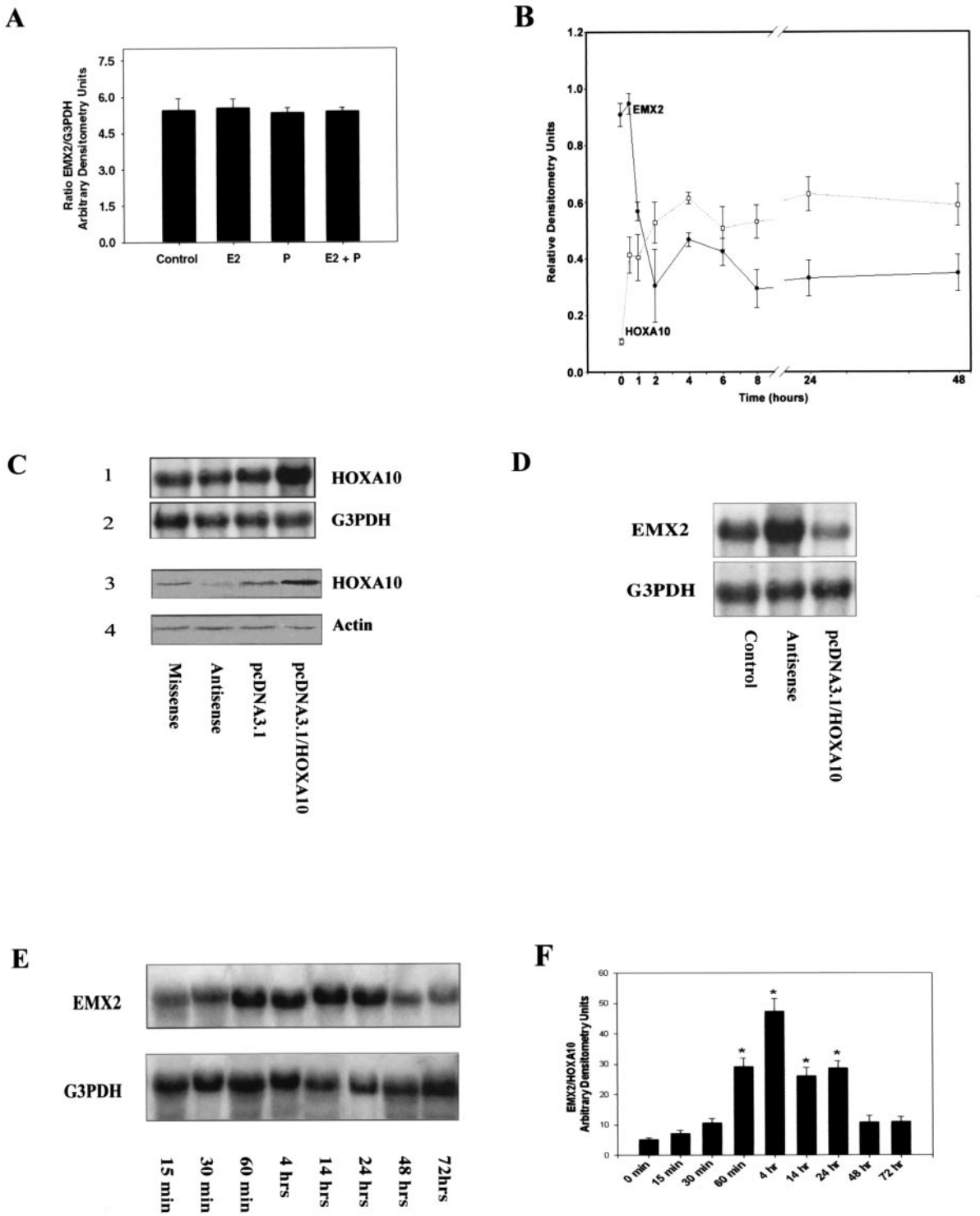

FIG. 4. Alteration of HOXA10 expression affects EMX2 expression. (A) The sex steroids estrogen and progesterone do not directly regulate EMX2 expression. Ishikawa cells were pretreated with cycloheximide (to block the effects of sex steroids on HOXA10) and then treated with 5×10^{-8} M 17β-estradiol and/or 10^{-7} M progesterone. Control cells were treated with cycloheximide only. Analysis by Northern blotting with a 3′-UTR EMX2 riboprobe showed no significant change in EMX2 expression in response to sex steroids. The results were quantified by densitometry and normalized to G3PDH expression. (B) To evaluate the temporal effect of sex steroid treatment on HOXA10 and EMX2 expression, Northern analysis with ^{32}P-labeled 3′-UTR EMX2 and HOXA10 riboprobes was performed in Ishikawa cells treated with 5×10^{-8} M 17β-estradiol and 10^{-7} M progesterone. RNA for Northern analysis was extracted at specified time points after sex steroid treatment over a 48-h period. G3PDH was used as a control. Expression of HOXA10 (normalized to G3PDH) is shown. Expression of HOXA10 increased from basal (0 h) levels by 30 min and continued to increase for 4 h. EMX2 mRNA expression declined to 30% of basal levels after 1 h, with a further decline by 2 h. The expression stabilized thereafter at 50% basal expression levels. EMX2 repression occurred subsequent to HOXA10 activation

1.0 μg of purified recombinant FLAG/HOXA10 protein or 0.1 to 5 μg of nuclear extract (Ishikawa or primary endometrial epithelial cell) and 80,000 cpm of labeled DNA in a final volume of 25 μl containing 25 mM HEPES (pH 7.6), 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 5 mM $MgCl_2$, 10 μg of salmon sperm DNA/ml, and 10% glycerol. Antiserum to HOXA10 was obtained from Covance. Anti-Pbx1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). For antibody binding assays, 1 μg of the corresponding antibody was added to the reaction mixture described above followed by incubation on ice for 15 min prior to the addition of ^{32}P-labeled oligonucleotide probe.

All samples were fractionated for 3 h at 200 V in a 4% nondenaturing polyacrylamide gel containing 1× Tris-borate-EDTA at 4°C. The gel was dried under a vacuum at 80°C for 45 min, exposed overnight on X-OMAT film (Kodak), and then developed.

In vitro mutagenesis. The EMXD insert from pGL3-EMXD was cloned into the *Kpn*I/*Hin*dIII sites of the pALTER vector (Altered Sites II in vitro mutagenesis system [Promega]) to create pALTER/EMXD. The HOXA10 binding site located at bp −578 was mutated by using a 36-mer oligonucleotide, 5′-CTTCG GAGGAAGCTGTGCATGTGATCCCCGCACTAA-3′. The mutagenesis reaction was carried out according to the manufacturer's protocol. For pGL3/MUTD2, the second HOXA10 binding site located at bp −483 was additionally mutated by using a 40-mer oligonucleotide, 5′-CTTCCATGGATTGTCCTTG GACGCCTGGCGCCCTGCCCGC-3′. Mutants were selected and cloned back into the *Kpn*I/*Xho*I site of the pGL3 plasmid to create the pGL3/MUTD and pGL3/MUTD2 constructs. pGL3/MUTC was generated by restriction digestion of pGL3/MUTD by using *Eco*RI/*Xho*I, followed by religation of the plasmid. The authenticity of the mutation was confirmed by sequencing.

Transfection and luciferase assays. Preconfluent (75 to 80%) BT-20 cells, in 25-cm^2 flasks were transfected with Lipofectamine (Invitrogen). The cells were transfected with either 4 μg of pcDNA3.1-HOXA10 or empty pcDNA3.1 and one of the following: pGL3-EMXA, -B, -C, or -D; pGL3-EMXMUTC or -D; or empty pGL3 plasmid, along with 4 μg of LacZ-pcDNA3.1 (to normalize for transfection efficiency). In other experiments, BT-20 cells were similarly transfected with 4 μg of either pGL3Control/5XEMXB or pGL3Control/5XEMXC. Transfectants were incubated for 5 h at 37°C in 5% CO_2, washed with 1× PBS, and grown to confluence for an additional 48 h. After the 48 h, the cells were washed with cold PBS and lysed with 1× reporter lysis buffer (Promega), and the lysate was collected. The lysate was snap-frozen in a dry ice-ethanol bath and microcentrifuged at maximum speed for 2 min. The luciferase activity was determined in the supernatant by using the Luciferase assay kit (Promega) and a luminometer. The β-galactosidase activity was determined by using the B-Galactosidase Kit (Tropix, Bedford, Mass.) and a luminometer. β-Galactosidase was used to normalize luciferase values.

DNase I footprinting. DNase I footprinting experiments were carried out as described by Heberlein et al. (22) except that 7 U of DNase I was used per reaction. FLAG-HOXA10 protein, produced in Ishikawa cells as described above, was used for footprinting.

RT-PCR. Ishikawa cells, a well-differentiated human endometrial adenocarcinoma cell line, and BT-20 cells, a breast carcinoma cell line, were grown to 80% confluence as described above. Total RNA was extracted from 25-cm^2 cellular monolayers by using Trizol reagent in accordance with the manufacturer's guidelines (Life Technologies, Inc., Gaithersburg, Md.). The reverse transcription (RT) step was done by using the bulk first-strand reaction mix (Amersham/Pharmacia Biotech, Piscataway, N.J.) according to the manufacturer's directions. Briefly, 1 μg of extracted RNA was diluted in 20 μl of RNase-free water, heated to 65°C for 10 min, and then chilled on ice. For first-strand cDNA synthesis, heat-denatured RNA solution, along with deoxynucleoside triphosphates and 200 mM dithiothreitol, was added to 11 μl of reagent, followed by incubation at 37°C for 1 h, and then heated to 90°C for 5 min and chilled on ice.

In order to amplify the 500-bp region (nt 47176 to 47632; GenBank accession no. AC004080) of HOXA10, the following primers were designed: sense, 5′-GCTCTCTTCTTTGATGTTCTGCG-3′; antisense, 5′-CCACAACAATGTCATGCTCGGA-3′. PCR was performed as follows: 2 min at 95°C; followed by 35 cycles of 95°C for 1 min, 52°C for 30 s, and 72°C for 2 min; followed by an 8-min extension at 72°C. For quantitative PCR, the linear amplification range was determined empirically. EMX2 (primers: sense, ACTAGCCCCGAGAGTTTCATTTTG; antisense, CTCCAGCTTCTGCCTTTTGAACTTT), HOXA10, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were amplified simultaneously. The expected PCR product sizes were 231, 500, and 750 bp for EMX2, HOXA10, and G3PDH, respectively. PCR was performed as follows: 95°C for 5 min; followed by 25 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 90 s; followed by a 7-min extension at 72°C.

Nucleotide sequence accession numbers. The GenBank accession number for the EMX2 5′ regulatory region is AY116142.

RESULTS

EMX2 is expressed in the adult reproductive tract. Emx2 expression has been previously demonstrated in the developing reproductive tract in embryonic mice (51). To determine whether Emx2/EMX2 is persistently expressed in the reproductive tract of adult mice and humans, Northern analysis was performed with a riboprobe complementary to the 3′-UTR of the Emx2/EMX2 gene. Total RNA was extracted from adult mouse uteri obtained on estrus cycle day 1 or from adult human proliferative-phase endometrium from normal fertile subjects. Northern blot results of representative samples are shown in Fig. 1. Both mouse and human endometria demonstrated persistent Emx2/EMX2 expression in adults.

Ishikawa cells are a well-differentiated endometrial adenocarcinoma cell line (39). The expression of estrogen and progesterone receptors, as well as HOXA10, in this cell line has been well characterized (23, 24, 30, 54). To determine whether EMX2 was expressed in Ishikawa cells, Northern analysis was performed. The cells were grown to 80% confluence, and total RNA was extracted, purified, and separated on a 0.8% formaldehyde gel. The gel was blotted and hybridized with a ^{32}P-labeled EMX2 riboprobe. Expression of EMX2 in this cell line is also shown in Fig. 1.

To localize Emx2 and Hoxa10 expression within the adult uterus, in situ hybridization was performed with a ^{33}P-labeled EMX2 or HOXA10 riboprobe. Representative photomicrographs of sections of mouse uterus hybridized to either the EMX2 or HOXA10 riboprobe are shown in Fig. 2A and C. Both EMX2 and HOXA10 expression was evident throughout the uterus, with the highest expression in the endometrium.

by sex steroids. (C) To further characterize the effect of HOXA10 on EMX2 in endometrial cells, Ishikawa cells were treated with constructs that alter HOXA10 expression and corresponding changes in EMX2 mRNA were determined by Northern blotting. HOXA10 mRNA and protein expression were determined by Northern and Western blotting, respectively, in transfectants treated with either HOXA10 antisense or the HOXA10 expression vector pcDNA3.1/HOXA10. The HOXA10 antisense construct was a 30-bp antisense phosphothiorate-modified oligodeoxynucleotide complementary to the HOXA10 translation start site. Corresponding results from controls treated with either a missense oligonucleotide (of the same length and nucleotide composition, but in random order) or with pcDNA3.1 were used for comparison. G3PDH or actin were used as loading controls in Northern or Western blots, respectively. Representative results of the Northern (panels 1 and 2) and Western (panels 3 and 4) blots are shown. Antisense treatment did not alter HOXA10 mRNA expression but decreased HOXA10 protein levels by ca. 50%. HOXA10 mRNA and protein levels were increased after transfection with pcDNA3.1/HOXA10, which constitutively expresses HOXA10 cDNA. (D) Treatment with HOXA10 antisense results in simultaneously increased EMX2 mRNA expression. In cells treated with pcDNA3.1/HOXA10, EMX2 mRNA levels correspondingly decrease. (E) In transfectants treated with HOXA10 antisense, EMX2 mRNA levels increase significantly 30 min after treatment and remain elevated at 72 h. (F) Results of densitometric analysis of EMX2 mRNA levels normalized to G3PDH expression over a time course of 72 h since treatment with HOXA10 antisense are shown. The asterisk indicates a statistically significant difference from the control of $P \leq 0.001$ (as determined by analysis of variance). The results are an average of four experiments ± the standard error of the mean.

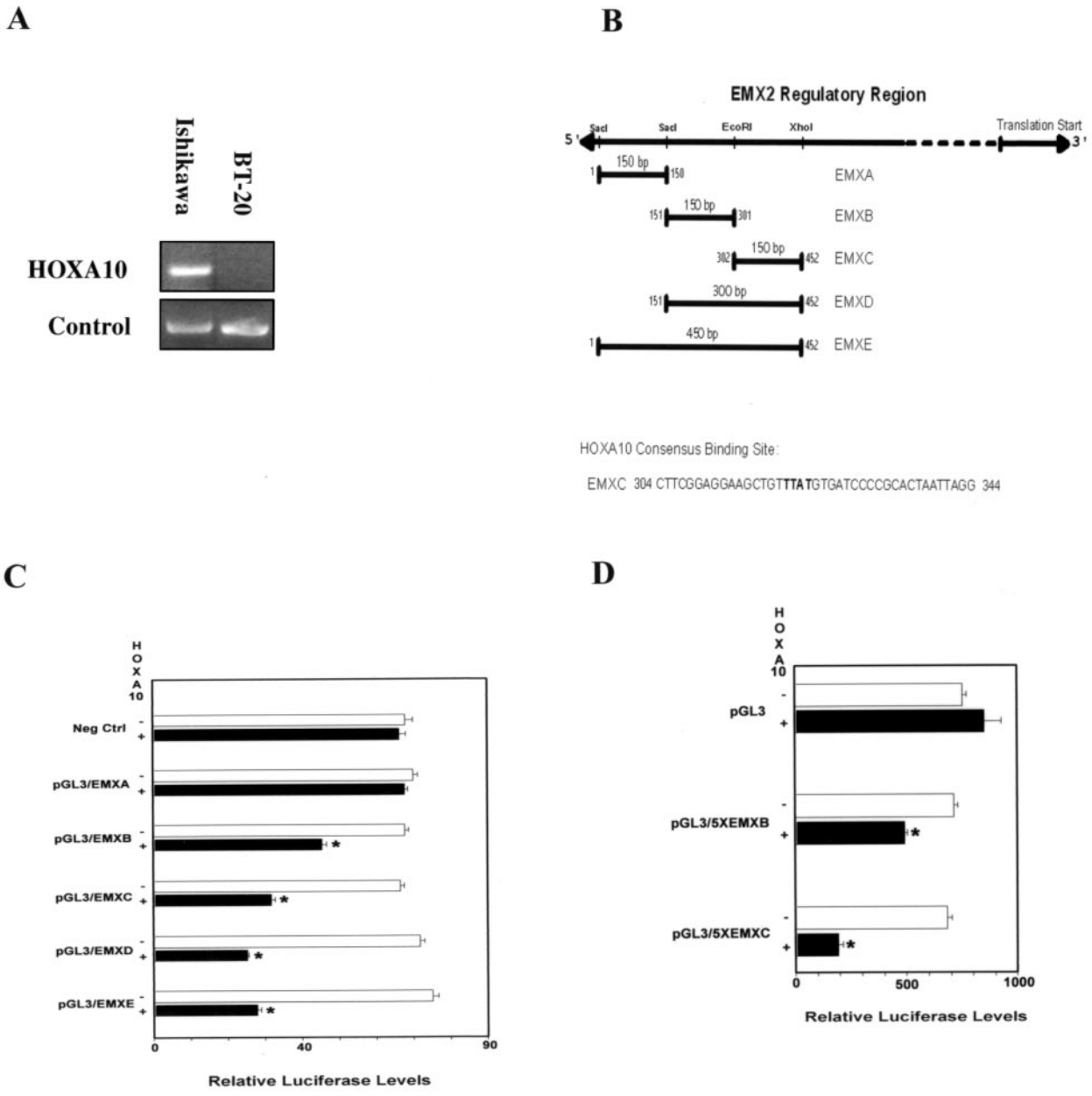

FIG. 5. HOXA10 regulates EMX2 expression via two 150-bp negative regulatory elements. The effect of HOXA10 on EMX2 enhancer-luciferase reporter gene constructs (pGL3promoter/EMX2) was tested in BT-20 cells. (A) BT-20 cells are a breast adenocarcinoma cell line that we demonstrated by RT-PCR not to express HOXA10. (B) Schematic representation of the EMX2 gene regulatory region. Transcriptional suppression by HOXA10 was demonstrated in constructs containing the oligonucleotide termed EMXE (450 bp; nt −700 to −250). This region was further subdivided into oligonucleotides termed EMXA (150 bp; nt −250 to −400) and EMXD (300 bp; nt −250 to −550). EMXD was then divided to EMXB and EMXC (both 150 bp; nt −401 to −549 and −550 to −700, respectively). These oligonucleotides were tested in reporter constructs. The portion of the 150-bp EMXC sequence containing the consensus HOXA10 binding site is shown. (C) Overexpression of HOXA10 in BT-20 cells repressed reporter gene expression from artificial promoter constructs containing elements from the EMX2 5′ regulatory region. BT-20 cells were transfected with 4 μg of an artificial promoter construct that contained a single copy of an EMX2 regulatory element (EMXA-D), a minimal promoter and a luciferase reporter. Additionally, the cells were cotransfected with either 4 μg of a HOXA10 expression vector (pcDNA3.1/HOXA10) or control vector (pcDNA3.1) and 4 μg of pcDNA3.1/LacZ as a control for transfection efficiency. Luciferase and β-galactosidase activity were measured in the cellular lysate. Results are reported as luciferase activity normalized to β-galactosidase activity and each experiment was repeated at least three times. pGL3 promoter, cotransfected with either pcDNA3.1 or with pCDNA3.1/HOXA10 is the negative control. As a positive control we used a construct (pGL3/β3INTS) that, when cotransfected with HOXA10, has been previously demonstrated to drive high luciferase expression (15). Overexpression of HOXA10 did not cause transcriptional repression of the artificial reporter construct pGL3/EMXA, which contains the 150-bp EMXA region. Transcriptional repression of reporter gene expression by HOXA10 was seen with constructs pGL3promoter/EMXB, -C, -D, and E. pGL3/EMXB demonstrated a slight but significant reduction in luciferase activity when cotransfected with HOXA10. Overexpression of HOXA10 with pGL3promoter/EMXC reduced luciferase activity expression to ca. 50% of levels in the absence of HOXA10. Cotransfection of HOXA10 with either pGL3promoter/EMXD or pGL3promoter/EMXE resulted in an equivalent (ca. 65%) reduction in luciferase activity compared to cotransfection with pcDNA3.1. The asterisk indicates statistically significant differences from

EMX2 expression colocalized to the same cells expressing HOXA10 (Fig. 2A and C). A labeled sense probe showed a lack of hybridization (Fig. 2B and D). High-power photomicrographs of human endometrium hybridized to the EMX2 probe showed silver grains over the glandular epithelium, as well as endometrial stroma (Fig. 2F).

Expression of EMX2 varies with the developmental stage of the reproductive cycle. To further investigate the role of EMX2 in functional development of the endometrium, reproductive cycle stage-specific expression was determined by Northern analysis. Endometrium samples were obtained from 32 subjects. The samples represent each developmental phase of the reproductive cycle and were categorized by the criteria of Noyes et al. (40). Approximately equal groups corresponding to the early and late proliferative phases and the early, middle, and late secretory phases of the reproductive cycle, respectively, were analyzed by Northern blot as described earlier. Hybridization with G3PDH was performed for normalization. Figure 3 demonstrates representative results of the Northern blot with samples from each developmental phase of the reproductive cycle. EMX2 is expressed in the endometrium throughout the cycle. Densitometry of the Northern blot revealed that normalized EMX2 mRNA expression rises significantly throughout the proliferative phase of the reproductive cycle (days 1 to 14 of 28) and peaks in the early secretory phase. This is followed by an ~50% decrease during the mid-secretory phase, at the time of embryo implantation, corresponding temporally with the previously reported rapid increase in levels of endometrial HOXA10 expression (54).

Similar results were obtained by semiquantitative RT-PCR with endometrial samples obtained from each developmental phase of the reproductive cycle (Fig. 3B). For semiquantitative RT-PCR, primers specific for HOXA10 and EMX2 were simultaneously used. Amplification of G3PDH was performed as a control. The resultant PCR product sizes were sufficiently discrete so as to enable simultaneous determination. EMX2 expression increased throughout the proliferative phase of the reproductive cycle (days 1 to 14 of 28) to peak in the early secretory phase. In the mid-secretory phase (S2), EMX2 expression (normalized to G3PDH) declined to ca. 50% of that in the proliferative phase. Expression continued to decline further in the late secretory phase (S3). Simultaneously, HOXA10 was expressed at low levels throughout the proliferative phase (i.e., P1, P2, and P3), with increased expression in the middle and late secretory phases (S2 and S3).

Alteration of HOXA10 expression affects EMX2 expression. To determine the mechanism by which EMX2 expression is regulated, we first investigated the role of sex steroids in EMX2 expression. Sex steroids are necessary and sufficient to induce complete endometrial development and embryo receptivity. To determine whether sex steroids directly regulate endometrial EMX2 expression, Ishikawa cells were pretreated with cycloheximide (to block the effects of sex steroids on HOXA10) and then treated with 5×10^{-8} M 17β-estradiol and/or 10^{-7} M progesterone. Analysis by Northern blotting with a 3′-UTR EMX2 riboprobe showed no significant change in EMX2 expression in response to sex steroids. The results were quantified by densitometry and normalized to G3PDH expression (Fig. 4A).

To evaluate the temporal effect of sex steroids on HOXA10 and EMX2 expression, Ishikawa cells were treated with 5×10^{-8} M 17β-estradiol and 10^{-7} M progesterone. Northern analysis was performed with ^{32}P-labeled 3′-UTR EMX2 and HOXA10 riboprobes. G3PDH was used as a control. Expression of HOXA10 and EMX2 (normalized to G3PDH) at specified time points over a period of 48 h after treatment with sex steroids is shown (Fig. 4B). Expression of HOXA10 increased from basal (0 h) levels by 30 min and continued to rise for 4 h. EMX2 mRNA expression declined to 30% of basal levels in 1 h. This decline in EMX2 mRNA expression occurred after the initial increase in HOXA10 expression. EMX2 mRNA expression declined further at 2 h and stabilized thereafter at 50% basal expression levels in the presence of continued, elevated HOXA10 expression.

To determine the effect of HOXA10 on EMX2 expression, Ishikawa cells were treated with constructs that alter HOXA10 expression. A 30-bp antisense phosphothiorate-modified oligodeoxynucleotide was designed complementary to the HOXA10 translation start site. A 25-cm^2 cellular monolayer was transfected with this antisense construct; HOXA10 mRNA and protein expression were determined and compared to corresponding results with both untreated cells and control cells (treated with a missense oligonucleotide of the same length and nucleotide composition, but in random order). As shown in Fig. 4C, antisense treatment does not alter HOXA10 mRNA expression but decreases HOXA10 protein levels ca. 50%, a finding consistent with blockade of translation.

Treatment with HOXA10 antisense results in simultaneously increased EMX2 mRNA expression (Fig. 4D). EMX2 mRNA levels increase significantly 30 min after treatment with HOXA10 antisense and remain elevated at 72 h (Fig. 4E).

Similarly, we transfected Ishikawa cells with pcDNA3.1/HOXA10, which constitutively expresses HOXA10 cDNA. As shown in Fig. 4C, HOXA10 mRNA and protein levels are increased after transfection with this construct. Increased HOXA10 expression in turn leads to a decrease in EMX2 mRNA (Fig. 4D), a finding consistent with the proposed negative regulatory relationship.

150-bp regulatory elements mediate transcriptional repression of EMX2 by HOXA10. To test the function of individual consensus HOXA10 binding sites in the region from bp 0 to −2000 5′ of the EMX2 gene, specific deletions were con-

the control of $P \leq 0.001$ for EMXB, $P \leq 0.004$ for EMXC, and $P \leq 0.002$ for EMXD and EMXE as determined by using the Mann-Whitney rank sum test. (D) In order to further characterize HOXA10-mediated transcriptional repression, BT-20 cells were cotransfected with pcDNA3.1/HOXA10 and the artificial promoter constructs pGL3control/5XEMXB and pGL3control/5XEMXC, containing five copies of either EMXB and EMXC, respectively, linked to a simian virus 40 promoter and enhancer expressing high basal luciferase activity. The luciferase activity was significantly repressed in transfectants treated with pcDNA3.1/HOXA10 compared to controls (treated with pcDNA3.1). Luciferase repression was significantly greater with pGL3/5XEMXC than with pGL3/5XEMXB ($P < 0.0003$). Each experiment was done in triplicate and was repeated three times. An asterisk indicates a statistically significant difference from the control of $P \leq 0.001$ as determined by the Student t test.

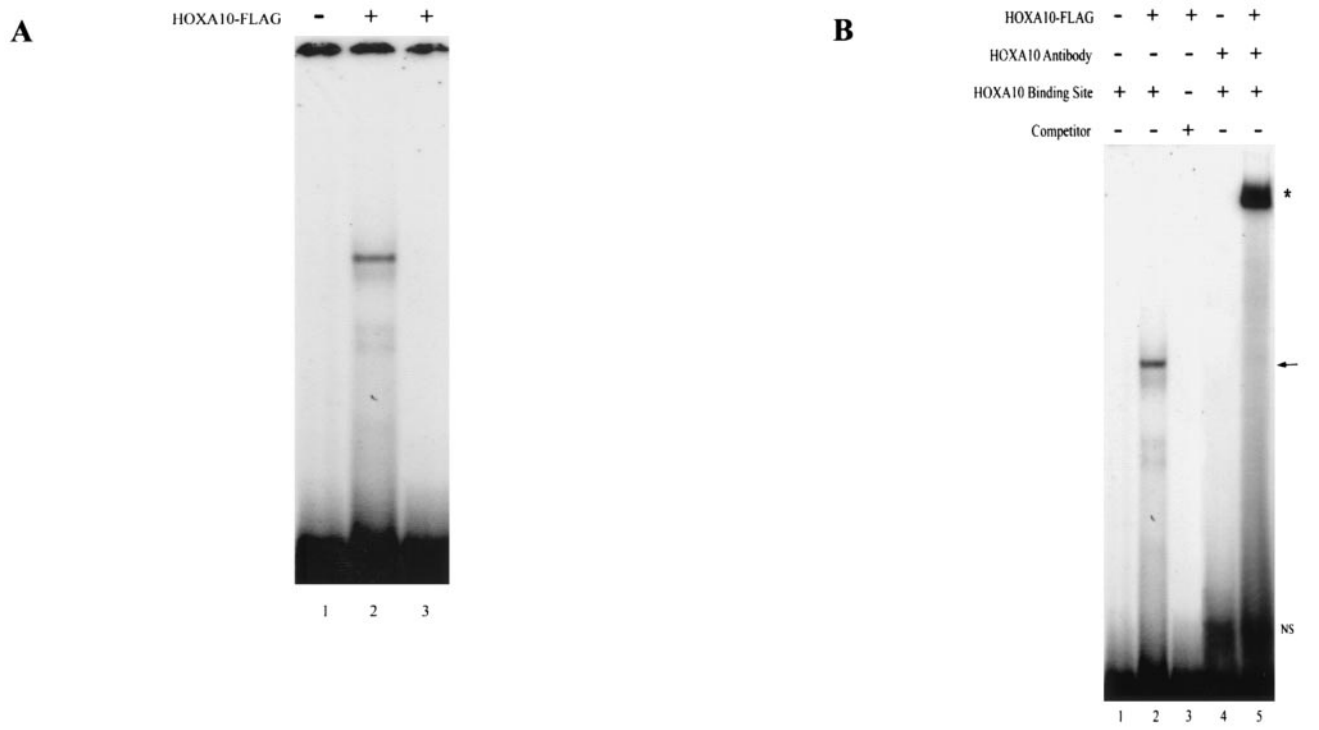

FIG. 6. EMX2 transcriptional repression by HOXA10 is mediated by HOXA10 binding to the 150-bp EMX2 regulatory element. To evaluate HOXA10 protein binding, Ishikawa nuclear extract, primary endometrial epithelial cell nuclear extract, or Flag-HOXA10 protein was used. Ishikawa cells were transfected with a Flag-pcDNA3.1/HOXA10 construct. Flag-HOXA10 protein was isolated and used. (A) EMSA of Flag-HOXA10 protein and ^{32}P-labeled oligonucleotides EMXC encompassing the HOXA10 binding site *TTAT* or MUTC containing the mutated HOXA10 binding site *GCAT*. Lane 1, no protein; lane 2, Flag-HOXA10 protein and ^{32}P-labeled EMXC demonstrate a shift; lane 3, Flag-HOXA10 protein and ^{32}P-labeled EMX/MUTC demonstrate absent binding. (B) Flag-HOXA10 protein binding to ^{32}P-labeled EMXC (lanes 1 to 5) is competed for by excess unlabeled competitor and supershifted with antibody directed against HOXA10. Lane 1, no protein; lane 2, Flag-HOXA10 protein; lane 3, a 160-fold molar excess of unlabeled oligonucleotide EMXC; lane 4, HOXA10 antibody, no protein; lane 5, Flag-HOXA10 protein and HOXA10 antibody. The arrow indicates the shift; an asterisk indicates a supershift, and "NS" indicates nonspecific binding. (C) EMSA of Ishikawa cell nuclear extract binding to ^{32}P-labeled EMXC (lanes 1 to 6) is dose responsive. Lane 1, no nuclear extract; lanes 2 to 6, increasing concentrations of nuclear extract (0.1 to 5 μg). The arrow and arrowhead indicate two specific shifts, and "NS" indicates nonspecific binding. (D) EMSA of Ishikawa cell nuclear extract and ^{32}P-labeled EMXC (lanes 1 to 5) further demonstrates that binding is competed for by excess unlabeled competitor and unaffected by unlabeled oligonucleotide EMXMUTC containing the mutated HOXA10 binding site. Lane 1, no nuclear extract; lane 2, Ishikawa cell nuclear extract; lanes 3 and 4, 160- and 320-fold molar excesses of unlabeled EMXC; lane 5, a 160-fold molar excess unlabeled oligonucleotide EMXMUTC. The arrow and arrowhead indicate two specific shifts, and "NS" indicates nonspecific binding. (E) EMSA of Ishikawa cell nuclear extract and ^{32}P-labeled EMXC (lanes 1 to 5). Lane 1, two specific shifts with nuclear extract indicated by the arrow and arrowhead; lane 2, a 160-fold excess of unlabeled EMXC effectively competed away detectable protein–^{32}P-labeled EMXC complexes; lane 3, complex resulting from Flag-HOXA10 binding to EMXC comigrates with the nuclear extract–^{32}P-labeled EMXC complex (lane 1, indicated by arrow). In lane 4, nuclear extracts were supershifted with an antibody directed against HOXA10 (indicated by the asterisk). Lane 5, HOXA10 antibody with no added protein; lane 6, absence of supershift in lane containing nuclear extract and antibody to Pbx1. (F) EMSA of primary endometrial epithelial cell nuclear extract and ^{32}P-labeled EMXC demonstrates binding of a single complex in a dose-responsive manner. Lane 1, no nuclear extract; lanes 2 to 6, increasing concentrations of nuclear extract (0.1 to 5 μg).

structed and analyzed in transient-transfection assays. The effect of HOXA10 on EMX2 enhancer-luciferase reporter gene constructs (pGL3promoter/EMX2) was tested in BT-20 cells. BT-20 cells are a breast adenocarcinoma cell line that we showed by RT-PCR does not express HOXA10 (Fig. 5A). EMX2 enhancer-luciferase reporter gene constructs were cotransfected with either a HOXA10 expression vector (pcDNA3.1/HOXA10) or a control vector (pcDNA3.1). Additionally, the cells were also transfected with pcDNA3.1/LacZ as a control for transfection efficiency. Luciferase and β-galactosidase activity were measured in the cellular lysate. Luciferase activity was normalized to β-galactosidase activity.

A single 450-bp segment termed EMXE repressed luciferase gene activity in the presence of HOXA10 (Fig. 5B and C). This region was subdivided into two regions, termed EMXA (150 bp) and EMXD (300 bp), cloned into the polylinker of the pGL3 promoter vector, and the luciferase activity was determined in response to HOXA10 in BT-20 cells. Cotransfection with pGL3/EMXA, which corresponds to the 150-bp EMXA region, and pcDNA-HOXA10 failed to show a reduction in the luciferase activity. Cotransfection of pcDNA3.1/HOXA10 with pGL3promoter/EMXD resulted in a 65% reduction in luciferase activity compared to cotransfection with pcDNA3.1. Transcriptional repression of reporter gene with this construct was similar to that obtained with pGL3promoter/EMXE (Fig. 5C).

EMXD was further subdivided into EMXB and -C, each of which consisted of 150 bp. EMXB demonstrated a slight

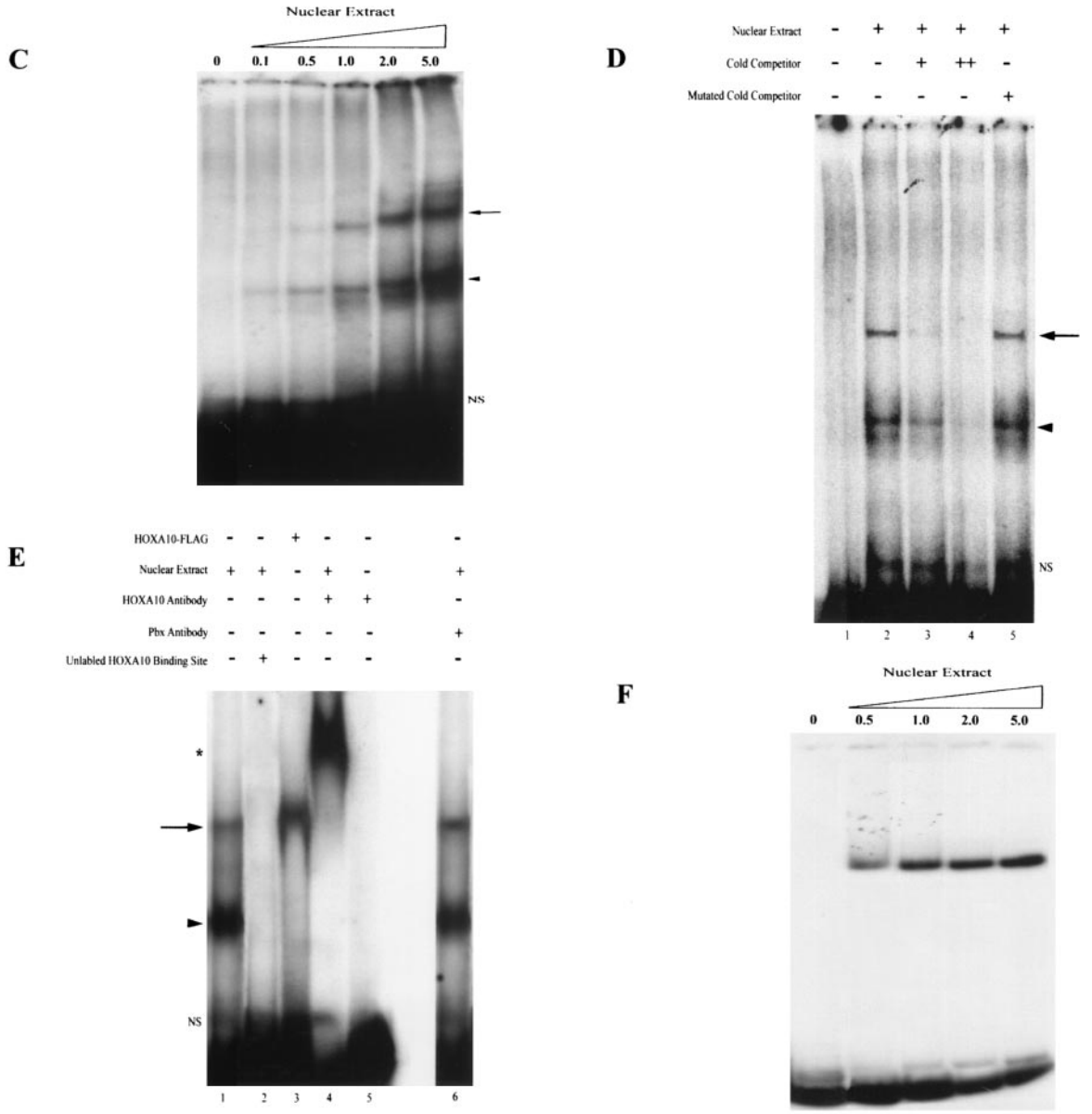

FIG. 6—*Continued*.

but significant reduction in luciferase activity when cotransfected with pcDNA3.1/HOXA10 (Fig. 5B and C). The majority of the negative repressive effect of HOXA10 on the EMX2 upstream regulatory element is modulated by EMXC, a 150-bp element. When cotransfected with pcDNA3.1/HOXA10, pGL3promoter/EMXC reduced luciferase activity to ca. 50% of the levels obtained in the absence of HOXA10. This result is of similar magnitude to the decrease in EMX2 mRNA expression seen in vivo at the time of increased HOXA10 expression (Fig. 5C).

In order to further characterize the transcriptional repression of EMX2, we used an artificial promoter-enhancer construct (pGL3control), containing a simian virus 40 promoter and enhancer, with high basal luciferase expression. Five copies of either the EMXB or the EMXC element were cloned into the polylinker of the vector, and BT-20 cells were cotransfected with either pcDNA3.1/HOXA10 or pcDNA3.1. Luciferase activity in transfectants treated with pGL3/5XEMXC and pcDNA3.1/HOXA10 was repressed to 30% of the activity in controls treated with pcDNA3.1. Luciferase expression was also suppressed to a significant, but lesser extent in cells cotransfected with pGL3/5XEMXB and pcDNA3.1/HOXA10 (ca. 70% of luciferase activity compared to control cells treated with pcDNA3.1) (Fig. 5D). HOXA10 repressed reporter gene expression mediated by pGL3/5XEMXC to a greater extent than that mediated by pGL3/5XEMXB ($P < 0.0003$).

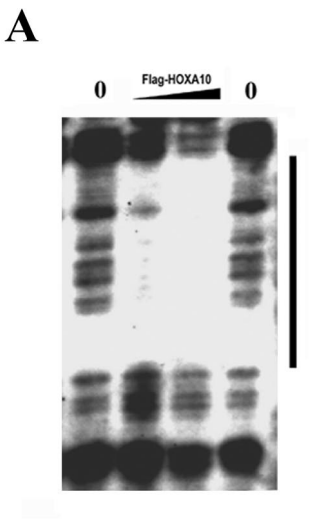

FIG. 7. HOXA10 binding is localized to two HOXA10 binding sites located within EMXC and EMXB. By using DNase I footprinting analysis, HOXA10 transcription factor binding within the 150-bp regions EMXC (A) and EMXB (B) was localized to a 10-bp region (EMXC) or a 6-bp region (EMXB), respectively. The EMXC footprint contained one HOXA10 consensus site. The EMXB footprint demonstrates weak protection of a nonconsensus site. The solid bar on the right indicates the extent of the footprint. No Flag-HOXA10 protein was added to lanes labeled "0." The triangle above indicates serially increasing Flag-HOXA10 protein concentrations. The oligonucleotide sequences containing the nucleotides protected by DNase I footprinting are displayed. The consensus HOXA10 binding site in EMXC is underlined.

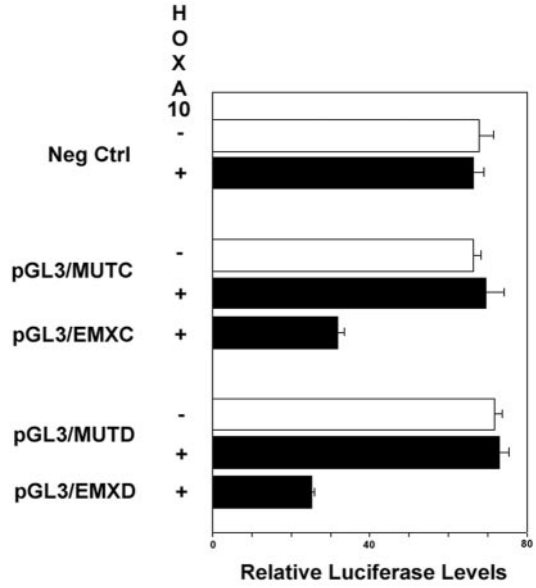

FIG. 8. Mutation of the HOXA10 binding site results in loss of negative regulation. Mutation of the HOXA10 binding site in the EMX2 regulatory region resulted in loss of HOXA10-mediated transcriptional repression. BT-20 cells were cotransfected as described earlier (Fig. 5) with either 4 μg of pcDNA3.1/HOXA10 or pcDNA3.1 and with 4 μg of reporter constructs containing mutated EMX2 elements (pGL3/MUTC and pGL3/MUTD). In vitro mutagenesis was used to alter the HOXA10 binding site from TTAT to GCAT. Results demonstrating the relative luciferase activity normalized to the β-galactosidase activity are shown. The effect of HOXA10 on the corresponding nonmutated elements pGL3/EMXC and -D described earlier (Fig. 5) are shown here for comparison only. Each experiment was repeated at least three times.

EMX2 transcriptional repression by HOXA10 is mediated by HOXA10 binding to the 150-bp EMX2 regulatory element. To evaluate HOXA10 binding to the consensus HOXA10 binding sites identified above, EMSAs were performed. A Flag-HOXA10 construct was designed which substituted the Flag tag for five N-terminal amino acids of HOXA10. Flag-HOXA10 was cloned into pcDNA3.1, and Ishikawa cells were transfected. Flag-HOXA10 protein was isolated and used for EMSA. Each oligonucleotide (EMXA, -B, and -C) containing putative binding sites was tested for its ability to bind Flag-HOXA10 protein. ^{32}P-labeled EMXC demonstrated the highest affinity binding to HOXA10 protein (Fig. 6A, lane 2). In vitro mutagenesis was used to mutate the single consensus HOXA10 binding site TTAT to GCAT. When Flag-HOXA10 was added to mutated EMXC, no shift was obtained (Fig. 6A, lane 3).

In order to confirm the specificity of binding of Flag-HOXA10 to EMXC, EMSAs were performed in the presence of excess unlabeled oligonucleotide EMXC or HOXA10 antibody. In the presence of a 150-fold molar excess of unlabeled oligonucleotide, no shift was obtained (Fig. 6B, lane 3). The addition of a HOXA10 polyclonal antibody resulted in a supershift (Fig. 6B, lane 5).

To assess the binding of HOXA10 protein to EMXC ex vivo, EMSAs with Ishikawa cell nuclear extracts were performed. Two specific shifts were obtained when nuclear extract was added to ^{32}P-labeled EMXC (Fig. 6C). Further, the binding affinity of nuclear extracts to ^{32}P-labeled EMXC was dose responsive (Fig. 6C). When excess unlabeled EMXC was added, the shifts were abolished (Fig. 6D, lanes 3 and 4). Addition of excess unlabeled mutated EMXC did not affect binding of nuclear extract to ^{32}P-labeled EMXC (Fig. 6D, lane 5). To confirm that the shifted complex contained HOXA10

protein, EMSA was performed in the presence of HOXA10 antibody, resulting in a supershift (Fig. 6E, lane 4). Additionally, no supershift was obtained on EMSA with nuclear extract and Pbx1 antibody, confirming that Pbx1 was not a constituent of the shifted protein complex, as predicted by the lack of a consensus Pbx site adjacent to the HOXA10 binding site (Fig. 6E, lane 6).

To determine whether the second shift obtained with Ishikawa nuclear extract was restricted to the adenocarcinoma cell line, EMSA was performed by using primary endometrial epithelial cell nuclear extract. A single dose-responsive shift was obtained (Fig. 6F). An additional truncated form of HOXA10 exists in the endometrial adenocarcinoma cell line, but not in vivo, which binds to the HOXA10 site, causing the second specific shift, and is supershifted by HOXA10 antibody.

To localize HOXA10 binding within the 150-bp elements EMXB and EMXC, DNase I footprinting was performed with Flag-HOXA10 protein. HOXA10 binding was confined to a 10-bp region that contained a consensus HOXA10 binding site. Figure 7 demonstrates the footprints, along with the sequences of the protected fragments.

Mutation of the HOXA10 binding site results in loss of negative regulation. To determine that transcriptional repression of EMX2 by HOXA10 in endometrial cells was mediated by the binding site defined above, the HOXA10 binding site in EMXC was altered from TTAT to GCAT by in vitro mutagenesis. BT-20 cells were cotransfected with pcDNA3.1/HOXA10 and reporter constructs containing the mutated EMX2 element (pGL3promoter/EMXCMUT and pGL3/EMXMUTD). There was no significant difference in relative luciferase activity between transfectants cotransfected with pcDNA3.1/HOXA10 and controls cotransfected with pcDNA3.1 (Fig. 8). Additionally, no transcriptional repression compared to controls was observed in cells transfected with another construct (pGL3promoter/EMXMUTD2) containing mutations in both HOXA10 binding sites (in EMXB and EMXC [data not shown]). Mutation of the HOXA10 binding site in EMXC alone was sufficient for the loss of negative regulation of EMX2.

DISCUSSION

In these studies we have linked two developmental genes, HOXA10 and EMX2, in a regulatory relationship that has evolutionary and functional implications. Emx2/EMX2 was expressed in endometrial cells in both the murine and human adult reproductive tract. EMX2 displayed a dynamic expression pattern that varied with the developmental phase of the human reproductive cycle. In contrast to HOXA10, the expression of EMX2 mRNA was lowest in the secretory phase. The sex steroids estrogen and progesterone did not directly regulate EMX2 expression in endometrial cells. In order to investigate the potential of HOXA10 to regulate EMX2, we designed constructs that would alter HOXA10 transcription factor levels and tested their effect on EMX2 mRNA expression in endometrial cells. Blockade of HOXA10 expression with HOXA10 antisense was associated with an increase in EMX2 mRNA. EMX2 mRNA levels significantly increased at 1 h and remained elevated 72 h after treatment with HOXA10 antisense. Conversely, diminished EMX2 mRNA expression was found when HOXA10 was overexpressed. These findings suggest an inverse relationship between HOXA10 and EMX2 in the adult endometrium. Transcriptional repression was observed when HOXA10 was cotransfected with EMX2 promoter-luciferase reporter gene constructs. Maximal repression was observed with a 150-bp *cis* element (EMXC) in the EMX2 5′ regulatory region. Significant but less repression also occurred with the EMXB element. In vitro binding of Flag-HOXA10 protein to EMXC was observed by using EMSA. Ex vivo binding was observed in EMSA by using nuclear extracts from either Ishikawa cells (an endometrial adenocarcinoma cell line) or primary endometrial epithelial cells. DNase I footprinting localized the binding of the HOXA10 transcription factor to a consensus HOXA10 binding site within the EMXC element and weak binding to the consensus HOXA10 site in EMXB. The HOXA10 binding site in EMXC was then mutated and evaluated in a transient-transfection assay. The negative regulation of EMXC observed with HOXA10 was abolished in the presence of mutated EMXC, further confirming that HOXA10 caused transcriptional repression of EMX2 primarily through a single HOXA10 binding site.

Empty spiracles (*ems*), the *Drosophila* ortholog of mammalian Emx2/EMX2, is regulated by *Abdominal-B*, which is the ortholog of mammalian Hoxa10/HOXA10 (25, 53). The direct regulation of EMX2 by HOXA10 demonstrates the evolutionary conservation of a gene regulatory relationship. However, in contrast to *Drosophila*, in which *Abdominal-B* positively regulates *ems*, the direction of regulation is reversed in human endometrium. In *Drosophila*, *Abdominal-B* regulates *ems* via a 1.2-kb element composed of three *Abdominal-B* binding subelements (53). The vertebrate EMX2 regulatory region contains a single subelement (EMXC) with no apparent homology to the *Drosophila ems* regulatory element. Therefore, although the transcription factor-target gene correspondence is conserved, the direction of regulation is not.

Cofactor binding has been shown to affect the affinity and specificity of HOX-DNA interactions leading to alterations in target gene expression (11, 18). A switch in target gene regulation from repression to activation has been shown to occur when a monomeric instead of a heterodimeric Hox transcription factor binds a target gene (52). Further, differential cofactor binding can also switch the function of the HOX transcription factor from activation to repression at different stages of development (42, 44). HOX proteins bind DNA as heterodimeric complexes with proteins from the *exd*/Pbx homeodomain family (43). Specifically, consensus Pbx-Hox binding sites have been described (49). Cooperative Pbx-Hox DNA binding occurs through such consensus sites (13, 28, 31, 49). Target gene repression occurs when corepressors such as SMRT, NCoR, mSIN3B, or HDAC1 and -3 are additionally bound to the Pbx/Hox heterodimer (2, 44). Conversely, binding of CREB-binding protein to the heterodimer results in target gene activation (2). Pbx has also been shown to be necessary to enable the formation of heterotrimeric DNA-binding complexes involving Meis proteins (10, 12, 38, 47, 48). Specifically, Hoxa10 binds DNA as a heterodimeric complex with Pbx in myeloid cells, leading to transcriptional repression of the CYBB gene (18).

In the present study the functionally active HOXA10 binding site and the adjacent DNA sequence in the EMX2 regu-

latory region did not contain a Pbx binding site. As expected, Pbx did not bind to this element in EMSA. Pbx is therefore unlikely to participate in HOXA10-mediated transcriptional repression at this site. Meis-1 binds Pbx in higher-order complexes with Hox genes; therefore, in the absence of Pbx, Meis-1 is not likely to be involved in transcription repression of EMX2 by HOXA10 at this site. It is possible that non-DNA-binding cofactors heterodimerize with HOXA10 or that other, as-yet-unidentified cofactors bind and modulate HOXA10-mediated target gene expression.

Changes in enhancer elements that affect the cellular localization, timing, or level of gene expression are one way by which species have evolved a high degree of diversity in structure and function. Although the spatial domains of expression of homeotic genes have been conserved, changes in the regulation of Hox genes have been demonstrated to occur. These changes correlate with species-specific identity. For example, the expansion of Hox genes specifying thoracic identity corresponds with extended thoracic vertebrae in snakes (14). The Hoxc8 early enhancer is differentially activated in the mouse and chicken (6). A 4-bp deletion in one element of the Hoxc8 early enhancer underlies differential gene expression between baleen whales and mice (6). Although the function of Hox genes and their *cis*-regulatory elements is conserved to a high degree across species (3, 27, 32–34), subtle differences such as these have been demonstrated. The EMX2 element in vertebrates bears no apparent sequence homology to the *Drosophila ems* element. It is possible that transcription factor target gene relationships are conserved; however, differences in the enhancer sequence may result in altered cofactor selection or cofactor-Hox interaction, thereby resulting in differential gene regulation between species. Such variations in homeobox gene enhancer function may contribute to our understanding of how distinct structures evolve in different species despite highly conserved genetic signaling pathways.

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