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The Transcription Factor SOX17 is Involved in the Transcriptional Control of the Uteroglobin Gene in Rabbit Endometrium

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Abstract The transcription of the uteroglobin gene (*ug*) is induced by progesterone in the rabbit endometrium, primarily through the binding of the progesterone receptor to the distal region of the *ug* promoter. However, other transcription factors participate in the progesterone action. The proximal *ug* promoter contains several putative consensus sequences for the binding of various progesterone-dependent endometrial nuclear factors (Perez Martinez et al. [1996] Arch Biochem Biophys 333: 12–18), suggesting that several transcription factors might be implicated in the hormonal induction of *ug*. We report here that one of these progesterone-dependent factors specifically binds to the sequence CACAATG (−183/−177) of the rabbit *ug* promoter. This sequence (hereafter called element G′) is very similar to the consensus sequence for binding of the SOX family of transcription factors. Mutation of the element G′ reduced transcription from the *ug* promoter in transient expression experiments. The endometrial factor was purified and analyzed by nano-liquid chromatography and ion trap coupled mass spectrometry yielding two partial amino acid sequences corresponding to a region of SOX17 that is highly conserved inter-species. This identification was confirmed by immunological techniques using a specific anti-SOX17 antibody. In agreement with the above findings, overexpression of SOX17 in transfected endometrial cells increased transcription from the *ug* promoter. SOX17 gradually accumulated in the nucleus in vivo concomitant with the induction of *ug* expression by progesterone in the endometrium. Thus, these findings implicate, for the first time, SOX17 in the transcriptional control of rabbit *ug*. J. Cell. Biochem. 102: 665–679, 2007.

Key words: Uteroglobin; uterus; transcription; progesterone; mass spectrometry; SOX17

The molecular mechanism of gene regulation by steroid hormones has been studied for several decades as one of the main paradigms of cellular differentiation. Thus, steroid hormones produce in many cases deep modifications in the expression of some specific genes, making these experimental systems relatively simple models for the study of cellular differentiation. As is well known, the basic molecular mechanism of gene regulation by steroid hormones [Beato and Klug, 2000] relies on the interaction of these hormones with specific protein receptors (the steroid receptors, or SRs) which, upon binding of the hormone, become activated and interact with short palindromic DNA sequences of 13–15 bp called hormone response elements (HREs). The HREs are often located within the promoter of steroid-regulated genes at variable distances from the

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Abbreviations used: SR, steroid receptor; HRE, hormone response element; UG, uteroglobin; HMG, high mobility group; EMSA, electromobility shift assay; TBE, Tris-borate/EDTA electrophoresis buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's Medium; ABC, ammonium bicarbonate; MSDB, Mass Spectrometry DataBase; HCG, human choriogonadotropin; NLS, nuclear localization signal; NES, nuclear export signal.

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transcriptional start site. Binding of the activated SRs to the HREs leads to a still partially known mechanism that seems to involve the interaction of bound SRs with components of the transcriptional machinery including other transcription factors and co-activators/co-repressors possessing histone acetylase/deacetylase activities, respectively [Lonard and O'Malley, 2005]. These events appear to results in local alterations of the chromatin structure [Beato and Klug, 2000; Hebbar and Archer, 2003] and, in turn, in the regulation of the transcription of the target genes.

The uteroglobin gene (*ug*) codes for a small secretory protein first discovered in the uterine secretions of the early pregnant rabbits [Krishnan and Daniel, 1967; Beier, 1968]. Uteroglobin (UG) was later characterized in other mammalian species [Miele et al., 1994; Macias et al., 2004] and other names (CCSP or CC10 protein) were then coined for the protein. A recent meeting on related proteins established that UG belongs to the so-called secretoglobin family and the name Secretoglobin 1A1 (SCGB1A1) was accepted for the protein [Klug et al., 2000]. However, the trivial (and most commonly known) name will be retained in this work.

UG and its gene have received considerable attention since the synthesis of the protein is regulated by several steroid hormones in different tissues. Thus, UG is induced by estrogens in the oviduct [Kay and Feigelson, 1972], by androgens in the epididymis [Lopez de Haro et al., 1988], by glucocorticoids in the lung [Fernandez-Renau et al., 1984], and by progesterone in the endometrium [see Miele et al. (1994) for review]. Particularly, the progesterone-induced transcription of *ug* in the endometrium has been a subject of great interest since it appears to be a good model for the molecular mechanisms of action of progesterone in mammals. The hormonal induction of *ug* in the endometrium occurs progressively during early pregnancy (or after progesterone-induced pseudopregnancy) reaching a maximum at day 5 [Krishnan and Daniel, 1967; Nieto and Beato, 1980]. The action of progesterone appears to be mediated through a region of the *ug* promoter, far from the transcriptional start site (about 2.7–2.3 kb), and that contains progesterone/glucocorticoid response elements. This region binds the progesterone receptor in vitro [Bailly et al., 1986] and seems to be functional in vivo as judged by both transient expression experiments in transfected cells [Scholz et al., 1999] and expression in transgenic mice [Gomez Lahoz et al., 1992].

Although the sole presence of a HRE appears to be sufficient to confer hormonal regulation to minimal promoters [Strahle et al., 1987], the involvement of different transcription factors in the gene regulation by steroid hormones is a well-known fact [Schule et al., 1988]. The promoters of the steroid-regulated genes usually contain consensus sequences for the binding of different transcription factors that can help to modulate (or even be indispensable for) the action of the steroid receptor [Lucas and Granner, 1992; Beato and Klug, 2000]. In the case of *ug*, some transcription factors have been shown to be implicated in both its basal transcription and its progesterone-mediated regulation in the rabbit endometrium. These transcription factors include YY1 [Klug and Beato, 1996], members of the Sp family [Dennig et al., 1995; Scholz et al., 1998], NF-Y [Scholz et al., 1999], and RUSH/SMARCA3 [Hayward-Lester et al., 1996]. Other studies, using deletions or mutations of the promoter as well as in vitro analysis of DNAase protection [Misseyanni et al., 1991; Suske et al., 1992] suggested than other not yet identified transcription factors might be implicated in the progesterone-regulated transcription of rabbit *ug* in the endometrium.

Among the numerous transcription factors described so far, the SOX family is a recently characterized group of structurally related proteins which play essential roles in different processes of cellular differentiation. The SOX proteins form a family of more than 20 transcription factors all of them containing a well conserved HMG box through which they bind to DNA. This HMG domain is very similar to that found in the first characterized member of the family, that is, Sry (for sex-determining region of the Y chromosome) [Wegner, 1999]. The HMG box of the different SOX proteins binds to the same consensus sequence 5′-(A/T)(A/T)CAA(A/T)G-3′ through the minor groove of the DNA [Wegner, 1999].

The SOX proteins have been classified into nine subgroups, on the basis of both the homology between the HMG boxes and the presence of different motifs outside this domain [Wegner, 1999; Bowles et al., 2000]. The subgroup F consists of three related proteins called SOX7, SOX17, and SOX18. SOX17 seems

to act as an early inducer of the endodermal tissue [Hudson et al., 1997] and it may also be involved in the pre-meiotic stage of spermatogenesis [Kanai et al., 1996]. Other members of the family are implicated in several processes of cellular differentiation [Bowles et al., 2000]. Thus, the SOX transcription factors appear to be implicated in broad aspects of the development and might also be important for the regulation of a variety of genes activated during the processes of cellular differentiation.

We have previously described footprinting experiments indicating that several progesterone-dependent endometrial nuclear factors bound in vitro to a 404 bp fragment of the rabbit *ug* promoter [Perez Martinez et al., 1996]. In the present study, we have focused on one of those footprints (footprint or element G′). We show here that the element G′ participates in the transcriptional control of rabbit *ug* and that its sequence is recognized by a member of the SOX family of transcription factors, namely SOX17. Thus, SOX17 seems to be a transcription factor implicated in the hormonal induction of rabbit *ug*.

MATERIALS AND METHODS

Animals

New Zealand female rabbits (8–12 months-old) were made pseudopregnant by daily intramuscular injections of progesterone as described previously [Nieto and Beato, 1980]. In some experiments, pseudopregnancy was induced by an intravenous injection of human corionic gonadotrophin (HCG) (Farma-Lepori, Spain) (50 i.u.). Animals were housed and handled according to the guideline 86/609 for animal research, approved by The European Community Council to fulfill the ethical conditions of animal experimentation.

Preparation of Nuclear Extracts

Uteri were surgically removed, placed on an ice-cooled glass plate and opened longitudinally. The endometrium was obtained by scrapping with a micro slide. The endometrial tissue was homogenized in 5 vol. of buffer H [Perez Martinez et al., 1996] containing 50 μM sodium orthovanadate. The homogenate was filtered through four layers of cheese-cloth and centrifuged at 3,000× g for 10 min. The pellet of the 3,000× g centrifugation (crude nuclei) was resuspended in 1.9 M sucrose in buffer H and processed for obtention of purified nuclei and nuclear extracts as described previously [Perez Martinez et al., 1996]. All the above steps were carried out at 0–4°C. Purity of the nuclei was preliminarily assessed by phase contrast microscopy. As an additional control, the purified nuclei were shown to be completely free of cytoplasmic contamination as judged by the total absence of the cytoplasmic marker α-tubulin in Western blot analysis of nuclear extracts.

Electrophoretic Mobility Shift Assays (EMSA)

A synthetic oligonucleotide (Isogen Biosciences, Maarssen, Holland) (oligo G′wt), containing the sequence of the element G′ and flanking sequences (−192/−169) of the rabbit *ug* promoter (see Fig. 1B), was labeled at the 5′ end with [γ-^{32}P] ATP (GE Healthcare, UK) and polynucleotide kinase (Roche Applied Science, Spain) [Sambrook et al., 1989]. The labeled oligonucleotide was annealed to its complementary synthetic strand and purified by ion-exchange chromatography on DEAE-cellulose. For the EMSA, the nuclear extracts (5 μg of protein) were pre-incubated for 10 min at 4°C with 1–2 μg of poly(dG-dC) (Roche Applied Science) in a final volume of 15–20 μl of buffer F [Perez Martinez et al., 1996] containing 2% (w/v) CHAPS and 2mg/ml of BSA. After pre-incubation ,the labeled oligonucleotide was added either alone or together with the unlabeled competitor oligonucleotides (25-fold in molar excess) and the binding reaction was allowed to proceed for 20 min at 25°C. The samples were analyzed by electrophoresis in 4% or 6% polyacrylamide gels in 0.5× TBE buffer. Gels were dried and autoradiographied at −70°C with intensifying screens. When working with purified proteins, the unspecific competitor poly(dG-dC) was either omitted or its amount lowered to 0.1 μg. For the super-shift mobility assays, the antibodies (Santa Cruz Biotechnology, CA) were added at the pre-incubation step at concentrations ranging from 0.05 to 0.2 mg/ml.

Purification of Proteins that Bound to the Element G′

Endometrial nuclear extracts from 5-day pseudopregnant rabbits were precipitated for 1 h at 0°C with ammonium sulfate at a final concentration of 50%. This step precipitated about 70% of the proteins including most of

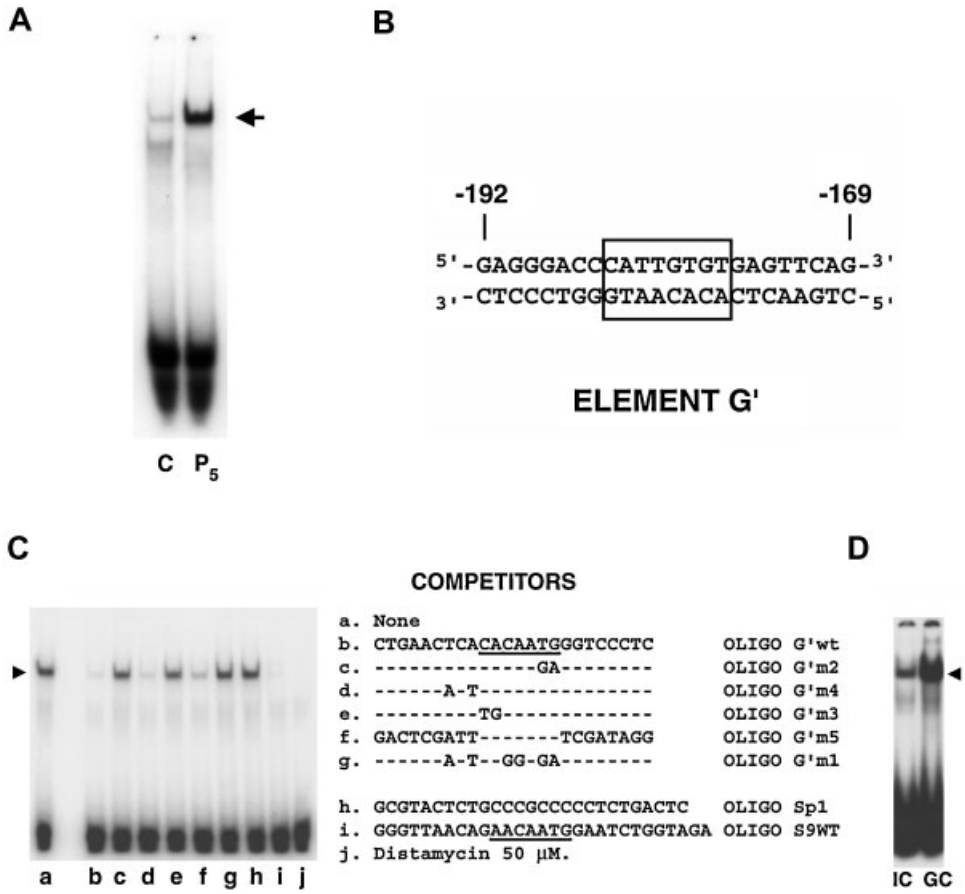

Fig. 1. A progesterone-dependent endometrial nuclear factor binds to a sequence of the rabbit *ug* promoter (element G′) that is similar to the consensus binding sequence for the SOX transcription factors. **A**: EMSA of rabbit endometrial nuclear extracts from either control animals (C) or rabbits treated with progesterone for 5 days (P_5). The arrow points to the retarded band increased by the progesterone treatment. **B**: nucleotide sequence of the oligonucleotide G′wt used as the radioactive probe. The sequence corresponds to a region of the rabbit *ug* promoter numbered from the transcription start site. The element G′ is boxed. Note that the sequence 5′-ACACAATG-3′ is in the lower strand. **C**: competitive EMSA experiments using the oligo G′wt labeled probe and endometrial nuclear extracts from rabbits treated for 5 days with progesterone. The arrowhead points to the shifted band. The unlabeled competitors used in each lane are indicated on the right. Dashed lines indicate nucleotide sequences identical to the G′wt probe whereas the mutated bases are indicated by their corresponding letters. The oligonucleotide Sp1, containing a consensus-binding motif for the Sp1 transcription factor, was used as an unspecific competitor. The oligo S9WT contained a perfect consensus sequence for the binding of the SOX9 transcription factor. The sequences for the binding of the SOX family of proteins are underlined. **D**: comparison of the effects of the unspecific competitors poly(dI-dC) and poly(dG-dC) on the binding of the endometrial nuclear factor to the labeled G′wt probe.

those binding to the element G′. The pellet of this precipitation was resuspended in buffer F and a 10th volume of 10× sample buffer [Laemmli, 1970] was added. After incubation for 10 min at 37°C [Ossipow et al., 1993] the samples were loaded and run in 8% polyacrylamide slab gels [Laemmli, 1970] with pre-stained molecular mass markers in adjacent lanes. After electrophoresis the gels were cut into fractions which were each extracted for 3 h at 37°C in 10 vol of buffer E (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1 M NaCl, 2 mM DTT, 0.5 mM PMSF, 10 μg/ml of each aprotinin and leupeptin, 50 μM sodium orthovanadate, and 10% (v/v) glycerol). The protein of the extracts were concentrated and equilibrated in buffer F by three successive centrifugations in Ultrafree-MC microfilters (Millipore) with the addition of buffer F before each centrifugation. The extracts were assayed by EMSA to identify the gel fraction(s) containing the binding activity to the element G′. As estimated by EMSA, recovery of binding activity at this step was about 60–70% of the activity initially applied to the gel.

The extracts containing binding activity were applied to a column containing concatemers of the oligonucleotide G′wt covalently linked to an

agarose matrix [Kadonaga and Tjian, 1986] and equilibrated in buffer F. The sample was allowed to interact with the immobilized oligonucleotide during 3 h at 4°C. The column was then washed with 10 vol of buffer F and the bound proteins were eluted with this buffer containing 0.5 M NaCl. The eluted material was subjected to SDS–PAGE and protein bands were stained with a silver-staining method (PlusOne Silver Staining Kit, GE Healthcare, Amersham Biosciences).

Plasmid Constructions, Cell Culture and Assays of Promoter Activity

The construct pUG400/luc-wt, derived from pGL3-Enhancer/luc plasmid, and bearing the luciferase reporter gene under the control of a 404 bp fragment of the rabbit *ug* promoter, has been described previously [Garcia and Nieto, 1999]. This construct served as template for site-directed mutagenesis of the element G′ sequence. Mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used to construct pUG400/luc-m1 were: 5′-CCCGAGGGACCTCTCCTGAGTGTTCAGTTTCAATAGG-3′ and 5′-CCTATTGAAACTGAACACTCAGGAGAGGTCCCTCGGG-3′. Primers for the construct pUG400/luc-m4 were: 5′-GAGGGACCCATTGTGAGTGTTCAG-3′ and 5′-CTGAACACTCACAATGGGTCCCTC-3′. Underlined bases denote changes with respect to the sequence of the element G′. The mutated sequences were identical to that present in the synthetic oligonucleotides G′m1 and G′m4 described in the Fig. 1C. The mutations were confirmed by automatic DNA sequencing. Plasmid pHRE-UG79/luc was prepared in our laboratory by Dr. K. Solorzano (to be published elsewhere). It contained the region from the rabbit *ug* promoter bearing the HREs for progesterone (−2777/−2587) immediately upstream of a minimal rabbit *ug* promoter (−79/+8). These elements were also inserted in the pGL3-Enhancer/luc plasmid.

The HEC-1A endometrial cell line (ATCC, Rockville, MD) was grown in Dulbecco's Minimal Essential Medium (DMEM) containing 10% (v/v) fetal calf serum. For assays of promoter activity, cells were plated on P35 dishes at half of maximal density and then transfected with 1 μg of the plasmids using Lipofectamins (Invitrogen Life Technologies, Spain) and following the protocol suggested by the supplier. Parallel dishes were transfected with the promoterless pGL3-Enhancer plasmid (Promega, Madison, WT), to serve as control of background activity. In assays designed to observe the effect of SOX17 on the activity of the *ug* promoter, cells were co-transfected with the expression vector pCMV-Tag2B/SOX17 [Murakami et al., 2004]. Parallel dishes were co-transfected with the pCMV-Tag2B plasmid (Stratagene) to observe the background activity. In all cases, the cells were co-transfected with 0.5 μg of the pSVβGal plasmid (Promega) to correct for differences in transfection efficiency. After 48–72 h of transfection, cells were lysed and the luciferase and β-galactosidase activities were determined as described previously [Sagal and Nieto, 1998].

In-Gel Tryptic Digestion of Monodimensional SDS–PAGE Gel Bands

Proteins bands were excised from the gel and incubated for several minutes in ultra-pure water and digested as described previously [Schevchenko et al., 1996] with some minor variations. Gel pieces were equilibrated in 50 mM ammonium bicarbonate (ABC) prior to reduction with 10 mM DTT and alkylation with 0.1 M iodoacetamide, both in 50 mM ABC. Modified porcine trypsin (sequencing grade, Promega) was added to dried bands at a final concentration of 0.4 μg per reaction in 50 mM ABC and the digestion was allowed to proceed at 37 °C overnight. Finally, tryptic peptides were extracted with 0.5% trifluoroacetic acid. The total digestion solution was vacuum-dried and re-dissolved in 20 μl of a solution containing 5% acetonitrile and 0.5% acetic acid in water.

Nano-Liquid Chromatography and Ion-Trap Tandem Mass Spectrometric Analysis of Tryptic Peptide

The tryptic peptides were online injected onto a C-18 reversed-phase nano-column (Discovery® BIO Wide pore, Supelco, Bellafonte, PA) and analyzed in a continous acetonitrile gradient consisting of 0–50% B in 45 min, 50–90% B in 1 min. (B: 95% acetonitrile, 0.5% acetic acid). A flow rate of ca. 300 nl/min was used to elute peptides from the reversed-phase nano-column to a PicoTip™ emitter nano-spray needle (New Objective, Woburn, MA) for real-time ionization and peptide fragmentation on an Esquire HCT ion-trap (Bruker-Daltoniks, Bremen, Germany) mass spectrometer. Every 1 s, the instrument

cycled through acquisition of a full-scan mass spectrum and one MS/MS spectrum. A 3 Da window (precursor m/z ± 1.5), an MS/MS fragmentation amplitude of 0.90 V and a dynamic exclusion time of 0.30 min were used for peptide fragmentation. Nano-liquid chromatography was automaticaly performed on an advanced nano-gradient generator (Ultimate nano-HPLC, LC Packings, Amsterdam, The Netherlands) coupled to an autosampler (Famos, LC Packings). The software Hystar 2.3 was used to control the whole analytical process.

Database Analysis

MS/MS spectra were batch-processed by using DataAnalysis 5.1 SR1 and BioTools 2.0 software packages and searched against the MSDB protein database using Mascot software (Matrix Science, London, UK).

UG and UG-mRNA Determinations

The amount of UG in the uterine luminal secretion was determined by a specific radioimmunoassay [Nieto and Beato, 1980]. The relative amount of UG mRNA in the endometrium was evaluated by a quantitative dot-blot hybridization method, as desdribed previously [Fernandez-Renau et al., 1984].

RESULTS

A Progesterone-Dependent Endometrial Nuclear Factor Binds to a Sequence (Element G′) of the Rabbit *ug* Promoter

Previous studies from our laboratory indicated that nuclear factors from the endometrium of rabbits treated for 5 days with progesterone (i.e., pseudopregnant) bound several specific sequences along a 400 bp proximal fragment of the *ug* promoter, as judged by in vitro footprinting analysis [Perez Martinez et al., 1996]. One of these prominent footprints ocurred over a short sequence of eight nucleotides (ACACAATG, −176/−183) located in the lower strand of the DNA and hereafter called element G′ [see Perez Martinez et al. (1996) and Fig. 1B]. To further characterize the binding of the nuclear factor to this element, we performed EMSA experiments using a [^{32}P]-synthetic oligonucleotide probe and nuclear extracts from either controls or progesterone-stimulated rabbit endometrium. The probe contained the element G′ as well as 5′ and 3′ flanking sequences from the *ug* promoter. The results of these experiments indicated that a nuclear factor from control rabbit endometrium bound the probe, producing a faint retarded band (Fig. 1A, lane C, arrow). This band reached a great intensity with nuclear extracts from animals stimulated for 5 days with the hormone (lane P_5), a time around which the transcription of *ug* in vivo has been described to reach maximum levels [Kumar et al., 1982; Miele et al., 1994]. Similar results were obtained using endometrial nuclear extracts from rabbits made pseudopregnant by treatment with HCG as described in Materials and Methods (not shown).

Thus, we concluded that the rabbit endometrium contained a nuclear factor that bound to the element G′ and whose amount (or binding activity) was increased by progesterone, either exogenously administered or endogenously produced by animals treated with HCG.

The Element G′ Contains a Putative Consensus Binding Sequence for the SOX Transcription Factors Family

To further identify the sequence responsible for the binding of the nuclear factor as well as to sustain the specificity of the binding, oligonucleotides containing mutations, either in the core or in the flanking sequences of the element G′, were tested as competitors for the binding activity in EMSA experiments.

As shown in Fig. 1C, lane g, extensive mutations in the core sequence (oligo G′m1) completely destroyed the ability to compete for the binding to the natural sequence of the probe. Two point mutations either on the right or the left sides of the core sequence (oligos G′m2 and G′m3, respectively) led to a considerable or complete loss of competition. The single mutation of the first nucleotide of element G′ (oligo G′m4) did not affect the ability to compete for binding to the nuclear factor. Moreover, complete changes in the sequences of the 5′ and 3′ flanking regions did not at all affected the binding of the nuclear factor (oligo G′m5, lane f). On the other hand, an oligonucleotide of nonrelated sequence (oligo Sp1, lane h) was unable to compete for binding to the nuclear factor. Thus, these experiments indicated that the sequence CACAATG, encompassing the footprint G′, was necessary for binding to the nuclear factor. A computer-based comparison of this sequence with those already described for the binding of different transcription factors,

indicated that it was very similar to the consensus sequence (A/T)(A/T)CAA(A/T)G, described for the SOX family of transcription factors [Pevny and Lovell-Badge, 1997; Wegner, 1999] and identical to one described for the binding of SOX17 [Kanai et al., 1996]. Indeed, the oligonucleotide S9WT (containing a perfect consensus sequence for the binding of the SOX family) was an excellent competitor (Fig. 1C, lane i).

Taking into account this first indication, we performed experiments that supported the idea that the endometrial nuclear factor might be a member of the SOX family. Thus, it is known that some polynucleotides (used as general competitors to reduce background) can differentially enhance the shifts produced by different transcription factors in EMSA experiments [Lee and Schwartz, 1992]. In the case of the SOX family, it has been described that the use of the competitor poly(dG-dC) is better than the generally used poly(dI-dC) for revealing the shifts produced by these transcription factors in EMSA experiments [Dailey et al., 1994]. In agreement with this observation, Figure 1D shows that the shift generated by the endometrial nuclear factor was considerably enhanced when poly(dG-dC), instead of poly(dI-dC), was used as unspecific competitor.

Furthermore, most of the transcription factors characterized so far appear to bind to the major groove of DNA. In contrast, the SOX proteins contain a conserved HMG-like domain through which they bind to the minor groove of DNA [Wegner, 1999]. Dystamicin, a drug that binds to the minor groove, competitively inhibits the interaction of DNA with proteins that attach to this groove. Therefore, we tested the effect of distamycin on the in vitro binding of the endometrial factor to the oligonucleotide probe. As shown in Figure 1C, lane j, the drug completely inhibited this binding, suggesting that the factor bound to the minor groove of the DNA.

In summary, the above results collectively suggested that the endometrial nuclear factor might belong to the SOX family of transcription factors.

The Element G′ is Implicated in the Transcription of the Rabbit *ug*

Since the element G′ seemed to bind a nuclear factor that, like the UG gene, was induced by progesterone, we wondered whether this element might be involved in the transcriptional regulation of *ug*. To investigate this point, we performed transient expression experiments with genetic constructs transfected in HEC-1A cells. In these experiments, we compared three constructs bearing the 400 bp fragment of the *ug* promoter either with its intact sequence (i.e., with the intact element G′) or with mutated versions of this element. These promoters controlled the transcription of the luciferase reporter gene. The results of these experiments (Fig. 2) indicated that a mutation of the element G′ (pUG400/luc-m1) that abolished the binding of the nuclear factor (see Fig. 1C), decreased the transcriptional activity of the *ug* promoter to about half of that observed using the wild type promoter bearing the intact element G′. As expected, the construct pUG400/luc-m4 (bearing a mutation that did not affect the binding of the factor, see Fig. 1C) conserved the same

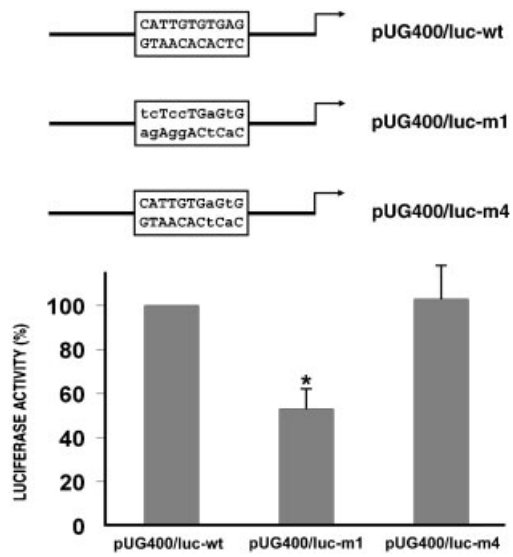

Fig. 2. The element G′ is involved in the transcriptional activity of the *ug* promoter. Endometrial HEC-1A cells cultured in vitro were transfected with the three constructs shown in the upper part of the figure. The plasmid pUG400/luc-wt bore a 404 bp proximal fragment of the *ug* promoter containing the natural sequence of the element G′ (boxed). This promoter drove the transcription of the luciferase reporter gene. The arrow indicates the transcription initiation point. The plasmids pUG400/luc-m1 and pUG400/luc-m4 were identical to the aforementioned but contained mutated versions of the element G′ (mutated bases are shown in lower case). These mutated sequences were similar to those of the oligonucleotides G′m1 and G′m4 (see Fig. 1C). Following transient expression, the luciferase activity was measured and expressed as percentage respect to the pUG400/luc-wt. The vertical bar indicates the standard deviation (SD) from the mean obtained from five different experiments each made in duplicate. *, significantly different from pUG400/luc-wt ($P < 0.01$).

activity as that observed for the wild type promoter.

The above experiments indicated that the element G′ bound a progesterone-dependent endometrial nuclear factor and that changes in the sequence of this element, that abolished the binding of the factor, concomitantly led to a decreased transcriptional activity of the *ug* promoter.

Thus, the element G′ appeared to act as a positive regulator of the transcription from the *ug* promoter.

Purification of the Endometrial Nuclear Factor

To identify the endometrial factor, it was purified by ammonium sulfate fractionation of nuclear extracts followed by semi-preparative SDS–PAGE and DNA sequence-recognition affinity chromatography (see "Materials and Methods" section). Fractionation with ammonium sulfate yielded a very heterogeneous precipitate, as judged by SDS–PAGE (Fig. 3A, lane 1), which contained most of the binding activity and about 70% of the initial protein content (data not shown). The precipitated material was analyzed by SDS–PAGE and the gels were cut into fractions which were extracted (see "Materials and Methods" section) for EMSA analysis of the binding activity. These analyses indicated that the activity was recovered from a zone of the gels corresponding to proteins with molecular mass between 50 and 65 kDa (not shown). Based on these findings, the ammonium sulfate precipitate was subjected to semi-preparative SDS–PAGE and the gel zone containing the binding activity was cut out and extracted as described above. The extracted material contained about 50% of the initial binding activity and several protein bands (Fig. 3A, lane 2). The extract was applied to a column containing the oligonucleotide G′wt immobilized on a Sepharose matrix. The flow-through of the column contained most of the initially applied proteins (Fig. 3A, lane 3) but only small amounts of binding activity (Fig. 3B, lane 2) whereas the high-salt eluate contained almost all of the recovered binding activity (Fig. 3B, lane 3) and was enriched in several proteins, including two closely migrating bands with molecular mass of 58 and 56 kDa, respectively. These two bands, barely observed in the material before affinity chromatography, were named S1 and S2 (Fig. 3A, lane 4). A portion of the high-salt eluate was run in a parallel lane which was fractionated for determination of binding activity by the EMSA technique. These determinations indicated that the binding activity was associated with bands S1 and S2 whereas other bands did not contain activity (not shown).

The Endometrial Nuclear Factor is the Transcription Factor SOX17

In order to identify the purified nuclear factor(s), we used ion-trap tandem mass spectrometry analysis. The bands S1 and S2, obtained by SDS–PAGE, were individually excised and in-gel digested with trypsin. The resulting tryptic peptides were analyzed by reversed-phase nanoliquid chromatography that was online coupled to an ion-trap mass spectrometer for ion fragmentation and protein identification.

Several proteins were identified within each band, including two heterogeneous nuclear ribonucleoproteins (K and L), and the HMG-box transcription factor SOX17. MS/MS spectra from triply charged ion at m/z 599.8 Da and doubly charged ion at m/z 566.7 Da, corresponding, respectively, to sequences LAQQNPDLHNAELSK and RPFVEEAER, were used to unambiguously identify the HMG-Box transcription factor SOX17 (see Supplementary material: Figs. S1A and S1B). Both ions had exactly the same chromatographic properties in the two bands (S1 and S2) and in two independent experiments (Fig. S1C).

Since the mass spectrometry analysis indicated that the endometrial factor was SOX17, we further supported this finding by inmunochemical techniques such as detection of the factor by Western blot analysis and super-shift assays in EMSA experiments. Western blot analysis of the purification steps (see above) showed that crude endometrial nuclear extracts from progesterone-treated rabbits contained two protein bands that reacted with the antibody against SOX17 (Fig. 3C, lane 2). These inmunoreactive bands had electrophoretic mobilities (i.e., apparent molecular mass) identical to those observed for the bands S1 and S2 obtained in the affinity-purified material. The two-inmunoreactive bands were scarcely detectable in endometrial nuclear extracts from control rabbits (Fig. 3C, lane 1) in agreement with the determinations made by EMSA experiments (see Fig. 1). The flowthrough of the affinity column did not contain appreciable

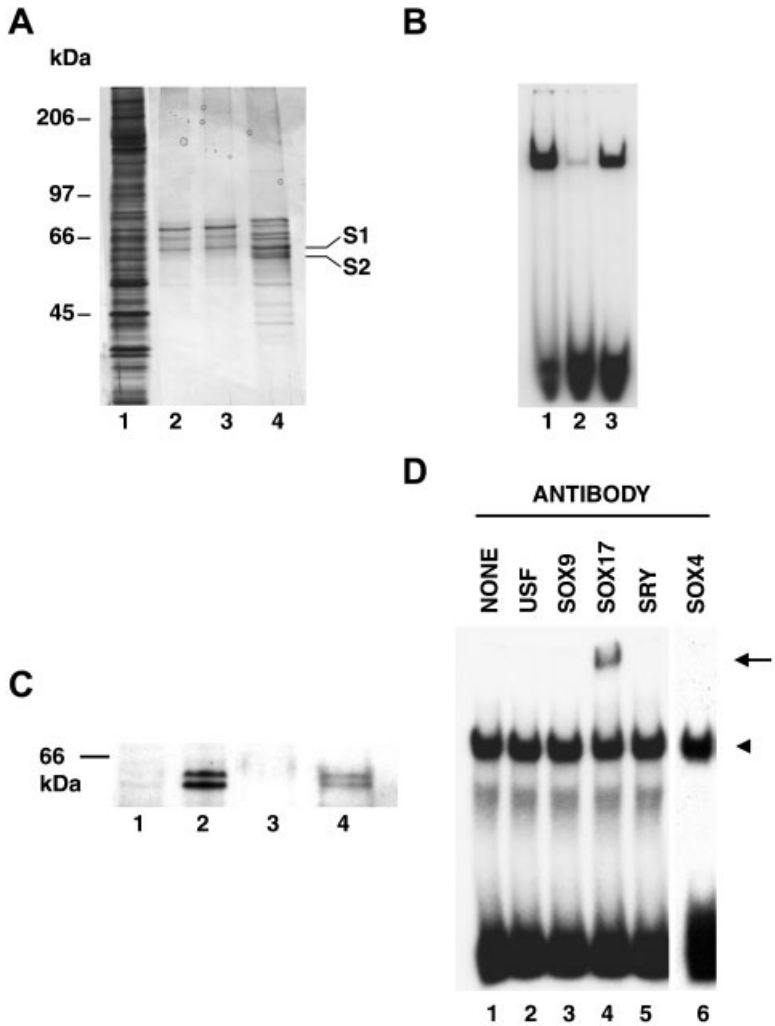

Fig. 3. Purification of the progesterone-dependent nuclear factor from rabbit endometrium and its identification as SOX17 by immunological techniques. **A**: silver-stained SDS-PAGE of the materials from the purification steps. **Lane 1**: starting material (ammonium sulfate precipitate of nuclear extracts); **lane 2**: proteins extracted from the selected zone of a semi-preparative SDS-PAGE of the starting material; **lane 3**: material from the flowthrough of the oligonucleotide-affinity chromatography; **lane 4**: proteins from the high-salt eluate of this chromatography. **S1** and **S2** denote the protein bands with binding activity as analyzed by EMSA. Note that this lane contained material corresponding to about 100-times to that applied to lanes 2 and 3. **B**: EMSA of the materials obtained in the purification steps. **Lane 1**: material from the semi-preparative SDS-PAGE (corresponding to lane 2 in **A**); **lanes 2** and **3**: materials from the flowthrough and eluate of the affinity chromatography, respectively (corresponding to lanes 3 and 4 in A, respectively). **C**: Western blot analysis of different rabbit endometrial materials. **Lanes 1** and **2** correspond to equivalent amounts of endometrial nuclear extract from control and 5 days pseudopregnant rabbits, respectively. **Lanes 3** and **4** correspond, respectively, to equivalent amounts of the flowthrough and the high-salt eluate from the affinity chromatography step. The position of the protein marker of known molecular mass is indicated to the left. Note that the two immunoreactive bands migrated at identical positions as the S1 and S2 bands in A. The anti-SOX17 used in the experiment was described to recognize both the human and the mouse proteins. **D**: EMSA of endometrial nuclear extracts from pseudopregnant rabbits pre-incubated with the antibodies indicated in each lane. The band generated by the progesterone-dependent endometrial factor was indicated by an arrowhead whereas the super-shifted band produced by the SOX17 antibody was indicated by an arrow.

immunoreactive bands whereas the purified material eluted from the column contained most of the inmunoreactive bands (Fig. 3C, lanes 3 and 4, respectively). These results were also in agreement with those obtained by EMSA determinations using the corresponding materials from the purification steps (see Fig. 3B). The observation of two SOX17-related immunoreactive bands will be commented in the Discussion.

We also performed super-shift assays with anti-SOX17 using the EMSA technique to assess whether the shifted band observed with this technique was generated by SOX17. As shown in the Fig. 3D (lane 4, arrow), anti-SOX17 produced a super-shifted band using either crude nuclear extracts or affinity-purified material. Furthermore, densitometric analysis of autoradiographies at different exposure times demonstrated that the radioactivity appearing in the super-shifted band exactly correlated with that disappearing in the band produced by the endometrial nuclear factor (data not shown). The specificity of the experiment was indicated by the fact that neither anti-SOX9 nor anti-SRY and anti-SOX4 (three other members of the SOX family) were unable to produce any super-shift (Fig. 3D, lanes 3, 5, and 6). Neither an unrelated antibody (anti-USF) was able to produce any change of the band generated by the endometrial factor (Fig. 3D, lane 2).

SOX17 Stimulates Transcription from the *ug* Promoter

All the above experiments indicated that the element G′ of the *ug* promoter was recognized by SOX17 and that mutation of this element reduced the transcription of a reporter gene driven by the *ug* promoter. Therefore, it might be expected that overexpression of SOX17 in cells would lead to a stimulation of the transcription from this promoter. To test this hypothesis, we transfected uterine HEC-1A cells with the pUG400/luc-wt construct either alone or co-transfected with the expression vector pCMV-Tag-SOX17, coding for SOX17 [Murakami et al., 2004]. The void pGL3 plasmid was used as a control. The results of these experiments (Fig. 4A) showed that overexpression of SOX17 stimulated the transcription from the 400 bp *ug* promoter about two to threefold. More importantly, overexpression of SOX17 did not at all stimulate the transcription from the

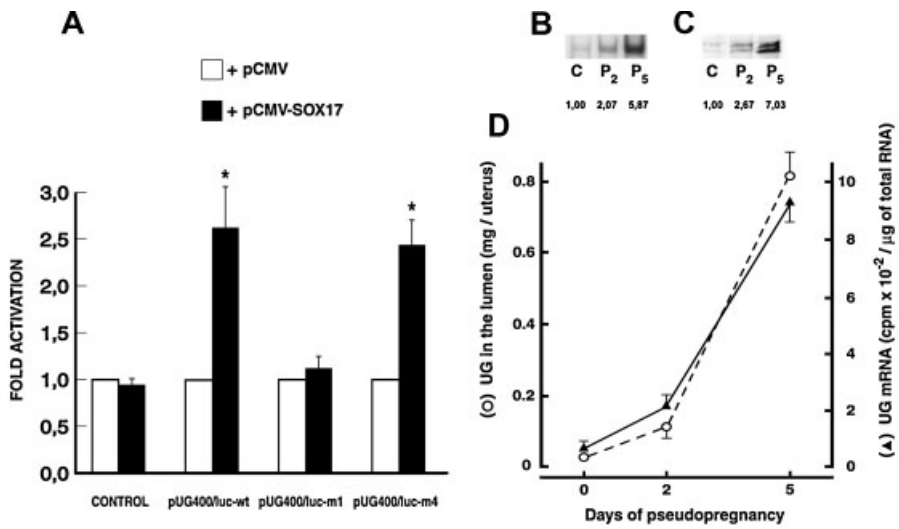

Fig. 4. SOX17 increases the transcription from the rabbit *ug* promoter. **A**: endometrial HEC-1A cells were co-transfected with the plasmids indicated below the histogram and with the expression vectors pCMV (void histogram) or pCMV-SOX17 (black histogram). The control plasmid was the promoterless pGL3 Enhancer/luc used to construct the pUG400/luc series. After 48 h, the cells were lysed and the luciferase activity was determined. This activity was expressed as fold activation of each construct (one for constructs co-transfected with the void expression plasmid pCMV). Vertical bars denote the SD. *, significantly different from its respective couple by Student's *t*-test, $P < 0.01$. The difference between the values for pUG400/luc-wt and pUG400/luc-m4 were not statistically significant. SOX17 increases in the nucleus of endometrial cells in parallel to the hormone-induced expression of the rabbit *ug*. **B**: EMSA determination of the SOX17 content in the nuclear extracts of rabbit endometrial cells at 0 (control, C), 2 (P_2), and 5 days (P_5) of progesterone treatment. Equivalent amounts of each nuclear extract were used for the assay. The relative amounts of SOX17 ($C = 1$) were estimated from densitometric analysis of the band generated by SOX17 and shown below each lane. **C**: Western blot determination of SOX17 in nuclear extracts of endometrial cells along the progesterone treatment. The two isoforms of SOX17 were identified in electrophoretic blots with a specific antibody and their relative amounts (determined by densitometry of the autoradiographies) are shown below each corresponding lane as in B. **D**: time-course of the expression of *ug* in the endometrium of progesterone-treated (pseudopregnant) rabbits. The absolute amount of the protein UG secreted into the uterine lumen was measured by radioimmunoassay. The relative amount of UG mRNA was evaluated by dot-blot hybridization with a specific radioactive UG cDNA probe. Vertical bars denote the standard deviations from at least three different experiments.

pUG400/luc-m1 construct that was identical to the 400 bp *ug* promoter but whose element G′ was mutated to a sequence that is not recognized by SOX17. Also, in accordance with the results described above, a mutation of the element G′ (pUG400/luc-m4) that affected neither the binding of SOX17 nor the basal transcriptional activity (see Figs. 1C and 2) was stimulated at the same extent as the wild type pUG400/luc-wt.

Thus, these findings, together with others above-mentioned results, collectively indicated that SOX17 positively regulated the transcription from the *ug* promoter through binding to the DNA sequence of the element G′.

SOX17 Increases in the Nucleus Concomitantly with the Progesterone-Mediated Induction of *ug*

The results shown in Fig. 1 indicated that the levels of SOX17 in the endometrial nucleus strongly increased at day 5 of pseudopregnancy. However, they did not allow us to conclude whether this increment occurred quickly at an early time or gradually like the hormonal induction of *ug*.

To answer this question, we determined the levels of SOX17 in the nucleus of endometrial cells during the time-course of the hormonal induction of *ug*. SOX17 was measured both by EMSA and Western blot analysis. At the same time, we measured the uterine luminal amounts of UG as well as the levels of UG-mRNA, both of which have been shown to reflect the progesterone-induced transcription of *ug* [Kumar et al., 1982; Miele et al., 1994].

The results obtained by EMSA determinations (Fig. 4B) indicated that the concentration of SOX17 in the endometrial nucleus increased progressively during the pseudopregnancy. Densitometric analysis of the band generated by SOX17 showed that at day 2 of the progesterone treatment the nuclear concentration of SOX17 was increased twofold with respect to the untreated control and that at day 5 of pseudopregnancy this concentration reached about six times that found in the controls. Similar conclusions were obtained from Western blot analysis (Fig. 4C), which unequivocally demonstrated that the absolute amount of SOX17 increased in the nucleus and that the increased band observed in the EMSA was not a progesterone-induced "activation" of a preexisting fixed amount of the protein. On the other hand, Fig. 4D shows the time-course of the progesterone-induced accumulation of the protein UG in the uterine lumen as well as of that its mRNA in the endometrium. The concentration of UG in the uterine luminal secretion as well as the amount of UG-mRNA in the endometrial cells were very low in control animals (day 0) and then progressively increased during the progesterone treatment, reaching high levels at day 5. These results are in agreement with previously published observations [Nieto and Beato, 1980; Kumar et al., 1982; Miele et al., 1994].

In summary, these experiments indicated that the amount of SOX17 increased in the nucleus of the endometrium during the progesterone treatment and that the time-course of this increase correlated with that of the induction of *ug* by the hormone.

DISCUSSION

In this study, we describe for the first time the involvement of the transcription factor SOX17 in the transcriptional control of the rabbit UG gene. More importantly, this control by SOX17 appeared to be concomitant with the transcriptional induction of rabbit *ug* by progesterone, suggesting a role of SOX17 in the hormone-induced transcription of this gene. The rabbit *ug* promoter seems to be rather complex encompassing at least 2.7 kb upstream of the transcriptional initiation site. Within this promoter exist HREs for progesterone which appear to be functional both in cultured cells [Scholz et al., 1999] and in transgenic animals [Gomez Lahoz et al., 1992]. In addition to these HREs (essential for progesterone action), the *ug* promoter has been shown to be modulated by several transcription factors, including Sp1 [Dennig et al., 1995; Scholz et al., 1998], RUSH [Hayward-Lester et al., 1996], and YY1 [Klug and Beato, 1996]. The results of this study add SOX17 to the list of transcription factors that modulate *ug* expression, and strengthen the idea that the *ug* promoter is modulated by the combinatorial action of various transcription factors [Dennig et al., 1995]. This complex modulation might be necessary for the cell type-specific and hormone-regulated expression of *ug* observed in various tissues [Miele et al., 1994].

Experiments of in vitro footprinting suggested the involvement of numerous nuclear factors in the control of the *ug* promoter [Perez

Martinez et al., 1996]. Some of the footprints observed in those experiments were later confirmed to be produced by transcription factors actually regulating the transcription from the *ug* promoter [Klug and Beato, 1996; Garcia and Nieto, 1999], which strongly suggests that these footprints truly reflect the binding of different transcription factors rather than being merely artefactual findings. One of these progesterone-dependent footprints, called footprint G′ (or element G′) was shown in this study to be important for the transcription of rabbit *ug*. Experiments of EMSA further demonstrated that a nuclear factor, strongly induced by progesterone in the rabbit endometrium, specifically bound to the element G′. Purification and characterization of the nuclear factor binding to the element G′ demonstrated that it was SOX17. In agreement with the identification of SOX17 as the factor that bound the element G′, overexpression of SOX17 in co-transfection experiments demonstrated that it stimulated the transcription from the intact *ug* promoter but not from the same promoter with a mutated element G′ (Fig. 4A). As mentioned above, mutation of this element reduced the transcription for the *ug* promoter to half of the normal activity. On the other hand, overexpression of SOX17 stimulated transcription about 2.5-fold. These latter results would suggest that the element G′ is a positive, but not indispensable, helper of the transcriptional control of rabbit *ug*.

Mass spectrometry (Fig. S1) as well as immunoblot analysis (Fig. 3C) demonstrated that rabbit SOX17 exists in the endometrium as two isoforms of close but different molecular mass, an observation that has also been described for the mouse SOX17 [Kanai et al., 1996]. The mouse isoforms seem to be produced by the translation starting from different methionines within the open reading frame of the mRNA [Kanai et al., 1996]. It remains to be established whether the two rabbit isoforms are produced by a similar mechanism.

Our results suggest that the effect of SOX17 on the transcription from the *ug* promoter occurs after the increment of this factor in the nucleus of the endometrial cells. There, SOX17 would bind to the element G′ to co-operate in the activation of transcription of *ug*. Expression of *ug* in the rabbit endometrium is transient both during the physiological and the progesterone-induced pseudopregnancy. Thus, *ug* is progressively activated in these conditions, reaching maximum levels of expression around day 5, and then progressively declining so that at day 10 its expression is minimal again [Kumar et al., 1982; Miele et al., 1994]. The apparent correlation between the increase of SOX17 in the nucleus and the activation of rabbit *ug* in the endometrium was further strengthened by the fact that at day 10 of progesterone treatment the nuclear amount of SOX17 was also minimal again (data not shown).

Regarding the mechanism(s) of the increase of SOX17 in the endometrial nucleus, two possibilities appear the most likely: (a) it might be a simple consequence of an increased intracellular concentration of the protein due to a progesterone-stimulated de novo synthesis of SOX17; (b) a pre-existing cytoplasmic pool of SOX17 translocates into the nucleus upon stimulation by progesterone. Experiments from our laboratory suggest that both possibilities occur in fact. The progesterone treatment certainly gradually increased the total intracellular amount of endometrial SOX17 but, at the same time, a specific and gradual translocation of the protein from the cytoplasm to the nucleus also occurs (our unpublished results). The traffic of proteins between the cytoplasm and the nucleus is mainly mediated by short amino acid sequences called nuclear localization signals (NLS) and nuclear export signals (NES) that regulate the shuttling of these proteins between both cellular compartiments. Both signals have been characterized in SOX9, SOX10, and SRY [reviewed in [Smith and Koopman, 2004] and appear to be conserved in other members of the SOX family, including SOX17 [Argentaro et al., 2003]. Thus, SOX17 is most likely able to translocate to the nucleus after a specific stimulus.

It is worth to mention the slow rate of appearance of SOX17 in the nucleus of rabbit endometrial cells. We observed a gradual increase of SOX17 along a period of 5 days, while the time of appearance in the nucleus (translocation) of many transcription factors, after being activated by a specific stimulus, is usually within the range of minutes or a few hours. However, there are also examples of nuclear translocation needing times of the same order as that observed for SOX17 in the endometrial cells under the stimulus of progesterone. One of these examples is the involvement of SOX9 in the developing mammalian

gonad, where translocation of SOX9 occurs progressively over several days [Morais da Silva et al., 1996; Barbara et al., 2000]. Other similar kinetics of nuclear accumulation has been described for the transcription factor FKHR that is involved in the formation of the skeletal muscle. In this case, the nuclear increase of FKHR occurs gradually during the 3 days period of differentiation [Nishiyama et al., 2004].

As mentioned above, SOX17 gradually increased in the nucleus with kinetics similar to the progesterone induction of rabbit *ug* in the endometrium. Therefore, one can wonder whether this hormone could actually be the primary stimulus for this increment. We assessed this possibility by treating HEC-1A cells, over-expressing SOX17, with progesterone. Then, the kinetics of the possible progesterone-triggered translocation of SOX17 was monitored by Western blot analysis. Unfortunately, we found that the expressed SOX17 was quickly localized exclusively in the nucleus, even in the absence of progesterone (data not shown). A similar quick and exclusive nuclear localization of SOX17 has also been observed in COS cells expressing the protein by transfection with a plasmid for SOX17 [Kanai et al., 1996]. It should be stressed that we obtained evidence for translocation of SOX17 directly from progesterone-treated animals. It would therefore probably be necessary to develop an experimental system reproducing the intracellular conditions of the rabbit endometrial cells in vivo. Perhaps, a solution to this problem would be the use of primary cultures of rabbit endometrial cells but, in our hands, this system was very difficult to handle as these cells appear to lose after 1 of 2 days of culture part of their phenotype, including the expression of uteroglobin (our unpublished observations).

It is important to know whether there are others sequences in the distal *ug* promoter that might bind SOX17 and, hence, be functional. This possibility seems to be of relevance, particularly in the region around −2.7 kb, since this region appears to be very important for the progesterone control of *ug* [Scholz et al., 1999]. A computer-based search of the distal *ug* promoter (−2.8–0.4 kb) indicated that two non-canonical sequences (CACAAAG, located at −2715 and −2434) might be putative SOX17-binding elements. To test the functional character of these sequences, we performed experiments similar to that shown in Figure 4A but using the plasmid pHRE-79UG/luc (see Materials and Methods section) bearing a distal fragment of the *ug* promoter that contained one of these putative sequences. These experiments showed that overexpression of SOX17 did not at all increase the transcription of the reporter gene (data not shown). Therefore, only the element G′ seems to be functional in the context of the *ug* promoter.

Regarding the mechanism(s) of action of SOX17 on the transcriptional activation of the rabbit *ug*, one can only speculate. It has been postulated that the SOX proteins could function as coupling factors that would interact with others transcription factors bound to different elements of a promoter, even at sites far from the SOX-binding elements. This mechanism would be based, in part, on the well-demonstrated ability of SOX proteins to bend the DNA following their binding to the minor groove [Wegner, 1999]. Bending of DNA has been associated with transcriptional regulation of different genes [Perez-Martin and de Lorenzo, 1997; Alvarez et al., 2003]. On the other hand, it has been shown that the interaction of the steroid hormone receptors with their cognate HREs initiates changes in the chromatin structure which allow the subsequent recruitment of other transcription factors to specific binding sites in the promoters of the steroid hormone-regulated genes [Truss et al., 1995; Scholz et al., 1998, 1999; Vicent et al., 2002]. In the case of the transcriptional regulation of the rabbit *ug* by steroid hormones, this mechanism was observed for the recruitment of transcription factors such as Sp1 [Scholz et al., 1998] and NF-Y [Scholz et al., 1999]. Thus, the gradual decondensation of the chromatin structure of the rabbit *ug*, produced by the binding of the progesterone receptor to the HRE of the promoter, might allow the concomitant and gradual access of SOX17 to the element G′ described in this study. Then, binding of SOX17 to this sequence might help to activate the transcription from the *ug* promoter.

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