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Identification and Expression of a New Sulfhydryl Oxidase SOx-3 during the Cell Cycle and the Estrus Cycle in Uterine Cells

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Using differential hybridization of a guinea pig endometrial cell cDNA library, a potentially negatively estrogen-regulated gene, *SOX-3,* was isolated. According to the nucleotide and protein sequence similarities, SOx-3 belonged to the FAD-linked sulfhydryl oxidase family containing the egg white sulfhydryl oxidase, the rat seminal vesicle sulfhydryl oxidase-2 SOx-2, the quiescence-inducible protein hQ6. The *SOX-3* transcript in the guinea pig as well as 5 different mRNAs in human tissues appeared differentially expressed in the tissues studied. In secondary endometrial cell culture, the *SOX-3* mRNA level increased during a serum depletion-induced quiescence, decreased when cells enter the G1 phase after serum stimulation, and was restored during the S and G2/M phases. Thus, *SOX-3* could be implicated in the negative cell cycle control. The SOx-3 protein appeared to be specific of epithelial cells in the uterus. Its expression level varied during the estrus cycle in the guinea pig, suggesting a regulation by steroid hormones. © 2001 Academic Press

Key Words: **cell cycle; quiescence; endometrium; estrogen; molecular cloning.**

Cell proliferation is a complex process that requires specific mechanisms of positive and negative controls (1). These controls are altered in cancer cells. The transition from the quiescent phase of the cell cycle (G_0) to the prereplicative G_1 phase is a major point in control of cell proliferation (2). During this transition, some genes are induced by mitogens while others are negatively regulated. Among this latter category of genes, there are tumor suppressor genes and genes which regulate the reversible entry into a quiescence state (e.g., *decorin* (3), *BTG1* (4), *gas-1* (5)).

Estrogens regulate cell proliferation by regulating gene expression in target tissues (6). The identification of cell cycle-specific genes that are regulated by estrogens during stimulation of cell proliferation is an essential step for the understanding of the mitogenic activity of these hormones. Among estrogen-regulated genes, the inducible genes have been studied the most. Based upon the kinetics of the response and the binding of estrogen receptors to the target genes, a classification into three groups has been proposed: primary response genes, secondary response genes and delayed primary response genes (7, 8). A well known example of a primary response gene is the *c-fos* gene which is rapidly and transiently induced after estradiol-17β (E_2) treatment (9–11). The two other groups of estrogen-inducible genes are still not completely identified. There is little information concerning the negative estrogen action on gene regulation. However, it has been demonstrated that, in the rat uterus, E_2 represses the expression of *gas-1* which belongs to the growth arrest-specific gene family (12). This study clearly established that estrogens can affect the uterine growth state by down-regulating the expression of negatively acting genes.

Our laboratory has developed an *in vitro* model of guinea pig endometrial glandular epithelial cells (GEC). It has been demonstrated that: (i) the cultured cells are estrogen-responsive (13); (ii) they can be made quiescent by serum depletion (14); (iii) quiescent cells are able to enter again the cell cycle under unspecific

The nucleotide *SOX-3* sequence reported in this paper has been deposited in the EMBL/GenBank data bank with Accession No. U82982 under the name *gec3*.

Abbreviations used: *gas* genes, growth arrest specific genes; GEC, glandular epithelial cells; SC, stromal cells; E_2, estradiol-17β; Chx, cycloheximide; RACE, rapid amplification of cDNA ends; dNTP, deoxyribonucleoside triphosphate; GAPD, glyceraldehyde-3-phosphate dehydrogenase; SOx, sulfhydryl oxidase.

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mitogen stimulation (i.e., fetal calf serum) (15); (iv) E_2 has no mitogenic effect but, in association with a protein synthesis inhibitor such as cycloheximide (Chx), it induces *c-fos* gene expression (14, 16). In an effort to identify genes that may be estrogen-regulated in the same conditions as *c-fos*, a cDNA library has been constructed from GEC stimulated for 2 h with E_2 plus Chx. By differential screening, an early estrogen-induced gene, called *gec1*, has been identified (17, 18).

In the present study, the cDNA library screening was continued and led to the identification of a putative estrogen-regulated gene, called *SOX-3*. Sequencing of the cDNA revealed identities with the rat sulfhydryl oxidase-2 (*SOX-2*) suggesting that SOx-3 shares the same enzymatic activity. SOx-3 appears to be a member of a new family of FAD-linked sulfhydryl oxidases together with rat SOx-2, human BPGF-1 and quiescin Q6. The differential tissue expression of *SOX-3* mRNAs suggested a regulation of the expression and led to the investigation of the stimuli able to modulate the mRNA and protein level in GEC and in the uterus during the estrus cycle. *SOX-3* appears to be implicated in the negative control of the cell cycle and could contribute to uterine differentiation.

MATERIALS AND METHODS

Cell culture. The method for the isolation of glandular organoids from guinea pig endometrium and the epithelial cell culture procedure have been previously described (13). Briefly, the endometria were dissociated by collagenase. After dissociation, the epithelial glands were separated from the stromal cell suspension by sedimentation. The epithelial glands and stromal cells were plated separately in growth medium (GM) consisting of Ham–F12 supplemented with 5% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone. Glandular epithelial cells (GEC) and stromal endometrial cells (SC) were cultured at 37°C in an atmosphere of 5% CO_2. Subconfluent GEC were obtained within 6–7 days and were either made quiescent by serum depletion over a 60-h period (14) or subcultured.

To prepare the probes for differential hybridization, GEC in primary culture made quiescent by serum depletion were stimulated either with basal medium (17) supplemented with Chx (10 μg/ml) or with basal medium supplemented with E_2 (10^{-8} M) plus Chx (10 μg/ml).

To study *SOX-3* gene expression, GEC or SC from the primary cultures were subcultured at a cell density of 4000 cells/cm^2, grown in GM for 3 days, were then made quiescent by serum depletion and, when this occurred, were stimulated with 10% FCS.

Construction and screening of cDNA library. A cDNA library had been previously constructed (17) from poly(A)$^+$ RNAs extracted from GEC stimulated with E_2 (10^{-8} M) plus Chx (10 μg/ml) for 2 h using the λ-gt 10 cloning system (Amersham). Differential screening of the library was performed as previously described (17). Poly(A$^+$) RNAs were prepared from either E_2 plus Chx-treated cells or Chx-treated cells (for 2 h) and used as templates for the synthesis of digoxigenin-labeled single strand cDNA probes by reverse transcriptase, as described by the manufacturer (Boehringer). Duplicate nylon filter blots (Hybond N$^+$, Amersham) were prepared from 130 cm^2 dishes (each of which contained 5×10^2 phage plaques) and hybridized with the two kinds of probes. Prehybridization, hybridization to digoxigenin-labeled probes and chemiluminescence detection were performed as described in the DIG system user's guide for filter hybridization. After hybridization, final washing steps was in 0.1× SSC, 0.1% SDS at 65°C. Positive plaques were purified by secondary and tertiary plating and screening.

cDNA sequence determination and analysis. The insert of phage recombinants (corresponding to the E_2-regulated sequence) was sequenced using the dideoxy chain termination method. Sequencing of both cDNA strands was performed five times by going along the sequence, using synthetic oligonucleotides as primers.

For an E_2-regulated sequence, called *SOX-3*, the 5′-rapid amplification of cDNA ends (5′-RACE) was performed using an Amplifinder kit (Clontech). The amplified cDNAs were submitted to direct sequencing (dideoxy method). To confirm the 5′ RACE sequence, the 5′ end of the *SOX-3* mRNA was reverse transcribed and amplified. A reverse transcription was carried out on 1 μg GEC total RNAs by Thermoscript reverse transcriptase (Life Technologies) with an oligo(dT) (17 mer) as reverse primer according to the manufacturer's instructions. The cDNA was then amplified by PCR with reverse and sense primers corresponding respectively to bases 915–934 and bases 1–17 of the complete sequence. The reverse primer was chosen from the initial cDNA identified by screening and the sense primer was the 5′end of the 5′RACE product. Addition of 5% DMSO in the PCR buffer was needed to disrupt the cDNA template secondary structures. After 40 cycles, a 934-bp unique product was obtained, subcloned in pGEMT plasmid (Promega), and submitted to sequencing.

Probes. A 1035 bp *SOX-3*-specific probe (nucleotide 885 to 1919 of the cDNA) was generated by PCR. For the PCR, *SOX-3* cDNA inserted in λ-gt 10 was used as a template. The glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) cDNA probe, the *β-actin* cDNA probe and the histone 2B (*H2B*) cDNA probe were from Clontech. The 18S ribosomal RNA probe was from Ambion. The cDNA probes were labeled with [^{32}P]-dCTP by nick-translation. A 30-b oligonucleotide complementary to the sequence 188–216 of *SOX-3* cDNA was used to probe the human Northern blots. Twenty picomoles of DNA were labeled by 5′ phosphorylation in the presence of [γ-^{32}P]ATP.

RNA extraction and Northern blot analysis. To study the expression of mRNAs in guinea pig tissues, a pool of each tissue was prepared from four guinea pigs and RNAs were extracted using the cesium chloride gradient method (19). Poly(A)$^+$ mRNAs were selected by two passages over oligo(dT)-cellulose columns. 5 μg of poly(A)$^+$ mRNAs were denatured (20), electrophoresed in 1.1% agarose gels and blotted to nylon filters (Zeta-probe, Bio-Rad) according to the vaccugene method (Pharmacia). The filters were baked (80°C, 1 h), prehybridized, hybridized with [^{32}P]-labeled the *SOX-3* cDNA probe and washed as previously described (14, 21). They were then exposed to X-ray films with an intensifying screen at −80°C, dehybridized and hybridized again with the probe used as control.

To study *SOX-3* gene expression in GEC or SC during serum depletion and after serum stimulation, total RNAs were isolated from secondary endometrial cell cultures (22). 20 μg samples were submitted to Northern blot analysis according to the procedure described for guinea pig tissue poly(A)$^+$ mRNAs.

Two human multiple tissue Northern blots were purchased from Clontech. The blots were prehybridised and hybridized with either the cDNA probe or the oligonucleotide probe in a "Expresshyb solution" (Clontech) according to the manufacturer's recommendations. When the cDNA probes were used, the washing conditions were the following: twice in 2× SSC, 0.1% SDS at room temperature for 15 min, four times in 0.5× SSC, 0.1% SDS at 50°C for 15 min and four times in 0.1× SSC, 0.1% SDS at 50°C for 15 min. When the oligonucleotide probe was used, the filters were washed at room temperature once in 2× SSC–0.1% SDS and once in 0.5× SSC–0.5% SDS.

Production of anti SOx-3 polyclonal antibodies and Western blot analysis. A full-length recombinant polypeptide was produced as a fusion protein between SOx-3 and a polyhistidine tag (Qiagen). The fusion protein was purified by affinity chromatography on nickel-agarose according to the manufacturer's instructions and used to immunize a rabbit.

The proteins were extracted from endometrial tissue in PBS containing 1% Nonidet NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors. Proteins were first separated by SDS–PAGE according to Laemmli and then transferred to PVDF membrane using a Bio-Rad transblot apparatus. The membrane was incubated with anti SOx-3 polyclonal antibodies (1:1000), followed by incubation with a horseradish peroxidase conjugated anti rabbit IgG antibodies (1:16,000). The bound antibodies were detected by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham).

Immunofluorescence analysis of the SOx-3 protein expression. On the day of vaginal opening or 10 days after the vaginal opening, the animal was anesthetized and perfused with 1% formaldehyde in PBS. The uterus was removed, embedded in Tissutech, deep frozen and sliced by a cryomicrotome. Slices were subjected to the following incubations: 1 h in PBS–0.1% Tween 20, 2 h in rabbit anti SOx-3 polyclonal antibodies diluted 1:100 in PBS–20% FCS and 30 min with FITC conjugated anti rabbit IgG antibody (Dako).

Primary cultured endometrial cells were fixed by 20 min incubation in methanol at −20°C. After a 30-min saturation step in PBS–20% FCS, the cells were incubated 2 h in the primary antibody. They were then treated with the FITC conjugated anti rabbit IgG for 30 min. The preparations were examined under a Nikon inverted epifluorescence microscope.

RESULTS

Isolation of a Putative Estrogen-Regulated mRNA

Screening of 7000 phage plaques led to the identification of three putative E_2-regulated sequences. Two sequences hybridized more strongly with the E_2-stimulated probe and another sequence, called *SOX-3* hybridized less strongly with this probe (not shown). Following second and third screenings, it was confirmed that the *SOX-3* phage plaque contained a potentially negatively E_2-regulated sequence. This *SOX-3* clone was selected for further analysis.

Sequence Analysis of SOX-3 cDNA and SOx-3 Protein

The size of the insert from the *SOX-3* recombinant phage was estimated at 2300 bp on agarose gel. After purification, both strands were sequenced. The *SOX-3* insert corresponded to the 3′ end of mRNA with a polyadenylation signal located 16 nucleotides upstream from the poly(A) tail.

As the *SOX-3* insert was not a full length cDNA, the 5′-RACE method was used to further identify the *SOX-3* sequence. By this method, a fragment of about 390 bp was isolated (not shown). Direct sequencing of this 390 bp fragment gave, as expected, the 5′ part of the known *SOX-3* cDNA sequence and supplied an extension of 298 nucleotides. This sequence was confirmed by sequencing the 934 bp RT-PCR product obtained with the primers defined under Material and Methods. The full sequence has been deposited in GenBank under the Accession No. U82982.

The 2492 bp *SOX-3* cDNA shares sequence identities with the 3298 pb Quiescin h*Q6* cDNA (23), with the 3228 bp h*BPGF-1* cDNA and the rat sulfhydryl oxidase-2 (*SOX-2*) (24). Indeed, a long part of the 5′ *SOX-3* sequence (nt 149 to 1683) shares more than an 80% identity with the 5′ h*Q6, rat SOx-2 or* h*BPGF-1* sequences (not shown).

The *SOX-3* complete cDNA encodes a protein of 613 amino-acid residues having a 68.6 kDa molecular weight and a p*I* estimated at 8.25.

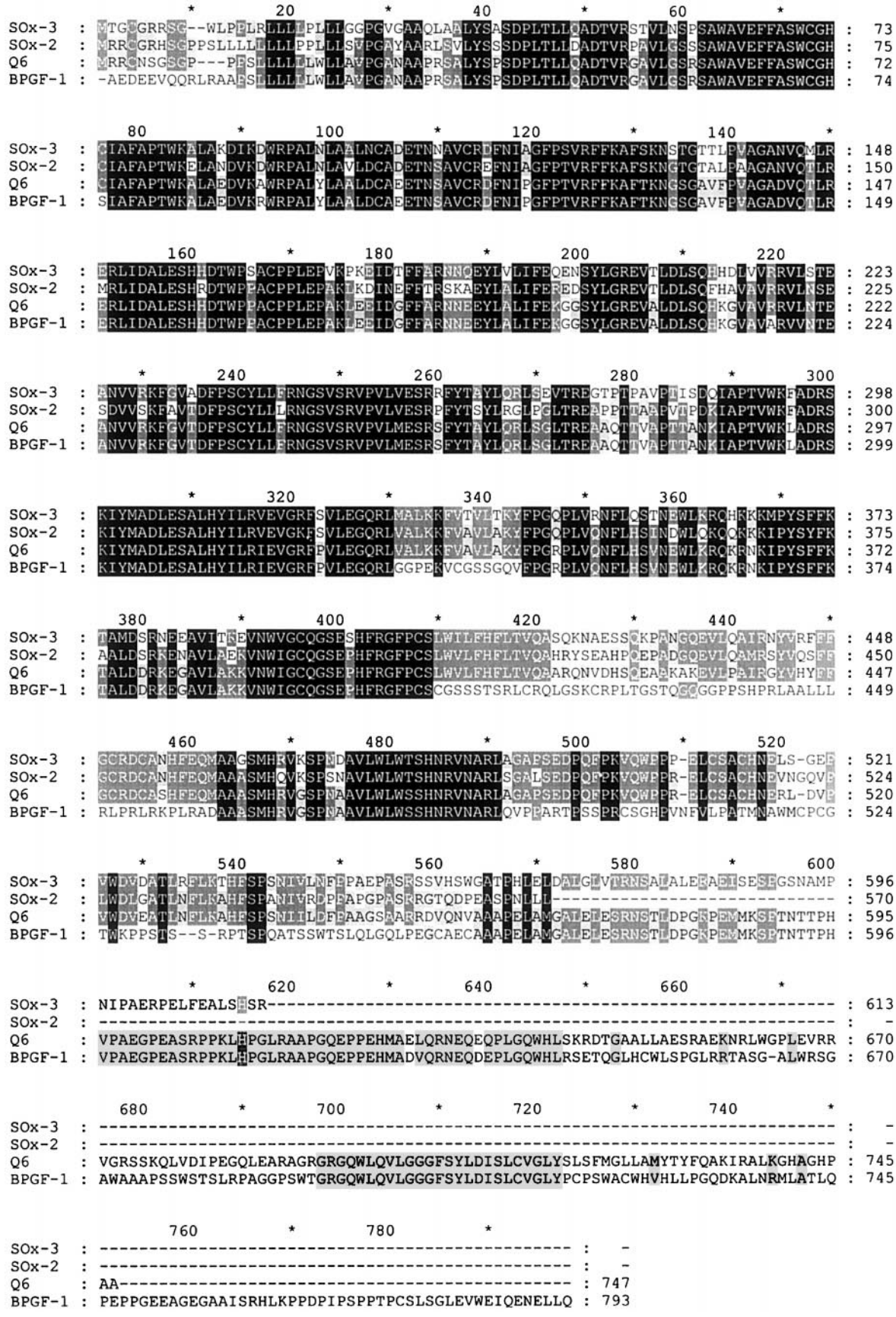
SOX-3, h*Q6* and rat *SOx-2* showed an ATG translation initiation codon. Although the initiation codon was missing for *BPGF-1*, a single putative open reading frame could be determined. As shown in Fig. 1, the inner 540 amino acids of the proteins were well conserved, whereas the N and C-terminus displayed the greatest dissimilarities.

Tissue-Specific Expression of Guinea Pig SOX-3 and Its Human Counterpart

A 1035 nucleotide *SOX-3*-specific probe hybridized with human genomic DNA (not shown). Thus, it was used to investigate not only expression of guinea pig *SOX-3*, but also an expression of its *SOX-3* human counterpart.

The results of a representative experiment with guinea pig poly(A)$^+$ mRNAs are reported in Fig. 2A. A 2.7-kb transcript was detected in lung, ovary and endometrium, but not in brain, liver and kidney tissue. The lack of *SOX-3*-specific mRNAs in these latter three tissues was not due to mRNA degradation as attested by hybridization with the control *GAPD* probe. The expression of *SOX-3* was also studied in the two endometrium cell types, i.e., GEC or SC in secondary cultures. The results presented in Fig. 2B show that the expression of the 2.7-kb transcript was stronger in the epithelial than in the stromal cells. In experiments made on cultured endometrial cells like the one presented in Fig. 4B, a minor band at 3.4 kb was also observed.

The hybridization of human Northern blots with the 30-b oligonucleotide or the cDNA probes is presented in Fig. 3. High level of two mRNA species of 3.2 and 2.5 kb were detected in testis, placenta and pancreas tissue. The expression of these mRNAs was lower in the other tissues examined and undetectable in brain tissue. A large mRNA of 4.8 kb was strongly expressed in placenta and pancreas, weakly present in prostate, testis, small intestine and colon tissue and almost undetectable in the other tissues. In placenta, a mRNA of 2.9 kb seemed to be specifically over expressed. The hybridization of the human Northern blots with the cDNA probe revealed an additional 1.2 kb transcript that was expressed in heart, skeletal muscle and barely detectable in brain tissue. Finally, the 1.8 kb transcript detected by the oligonucleotide probe and not confirmed by the cDNA probe probably corresponds to a non specific binding of the oligonucleotide. As the *ß actin* mRNA level



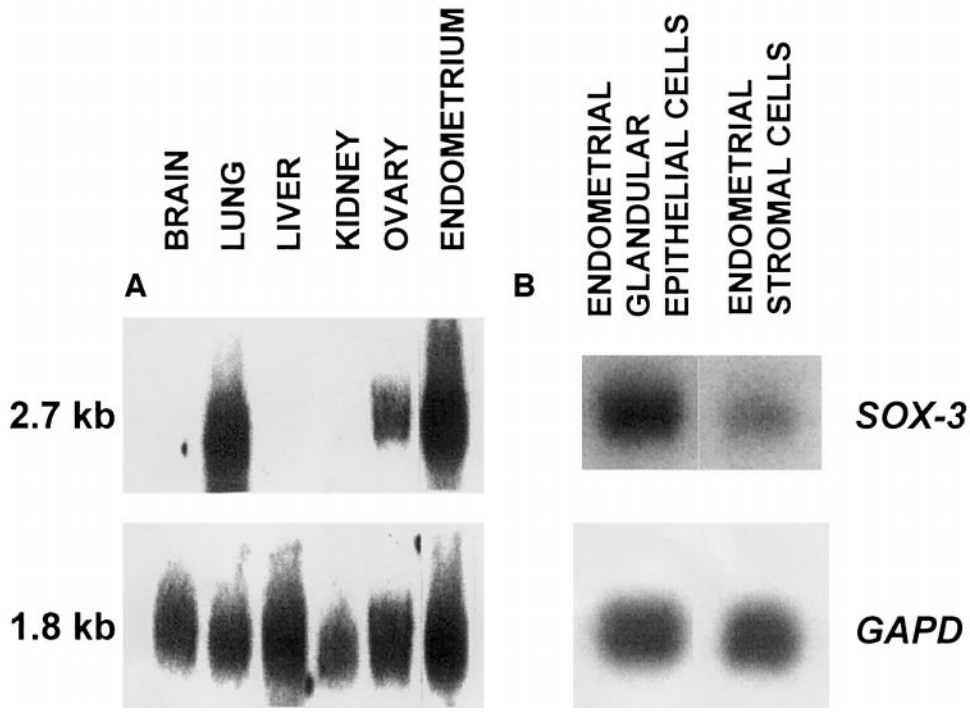


FIG. 2. Tissue-specific expression of guinea pig *SOX-3*. (A) A Northern blot of 5 μg poly(A)$^+$mRNAs isolated from guinea pig tissues was hybridized sequentially with [^{32}P]-labeled guinea pig *SOX-3* probe and human *GAPD* probe. (B) Northern blot of 20 μg total RNA extracted from GEC or SC in secondary culture after 60 h serum depletion.

varied from one lane to another, the expression of transcripts might be underestimated in some tissues such as that of lung.

Regulation of SOX-3 Gene Expression

As *SOX-3* shares identities with quiescence h*Q6* gene, this would suggest that it could be part of a program of quiescence that is tied to growth arrest by serum depletion. It has been previously reported that the h*Q6* mRNA level was up-regulated by either cell contact inhibition due to confluence, cell trypsination or serum depletion (25). It was therefore decided to investigate the experimental conditions to study the serum depletion effect independently of the two other factors. Maximal cell density was sought as well as the time following trypsination that was required for the *SOX-3* mRNA to decrease to basal level (results not shown). Then, the cells were seeded at a maximal density of 4000 cells/cm^2 and cultivated for 3 days in growth medium before being serum depleted. As shown in Fig. 4A, *SOX-3* mRNAs were at a very low basal level in exponential growth (time 0) and suddenly accumulated with a maximum increase 60 h after the beginning of serum depletion. At this time, there was a 10-fold increase of *SOX-3* mRNAs, compared with the level in growing cells. Thus, *SOX-3* expression appears to be linked to the growth-arrested state in GEC.

Since the *SOX-3* mRNA accumulated when epithelial cell growth was arrested, it can be supposed that the transcript level would decrease once the cells enter the cell cycle again. Therefore, after a serum depletion of 60 h, GEC were stimulated with 10% FCS for 72 h. As shown in Fig. 4B, the *SOX-3* mRNA level went down after 8 h of mitogenic stimulation, but, the *SOX-3* transcript started to accumulate after 24 h of serum stimulation, reached a maximum after 48 h and decreased again after 72 h. As indicated by the *H2B* probe, during this time course, the cells went through a complete cycle and started a second cycle. Previous GEC proliferation studies (15) have indicated that the S phase is maximal after 16 h

FIG. 1. Protein sequence alignment. The clustal multiple alignment sequence program was used to align the protein sequences. The darkness of the shadowing increases with the level of amino-acid conservation in the different sequences. SOx-3: guinea pig SOx-3 protein, cDNA (recorded in the data bank under the name *gec3*) GenBank Accession No. U82982; SOx-2: rat sulfhydryl oxidase-2, cDNA GenBank Accession No. AF285078; Q6: human quiescin Q6, cDNA GenBank Accession No. U97276; BPGF-1: human Bone Derived Growth Factor-1, cDNA GenBank Accession No. L42379.

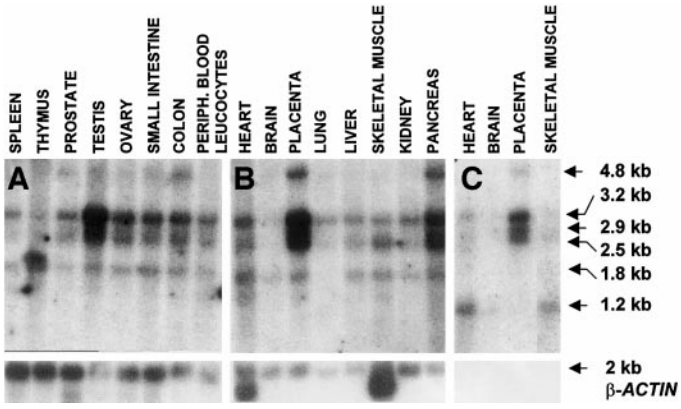


FIG. 3. Tissue-specific expression of *SOX-3* human counterpart. (A and B) Two Northern blots with 2 μg of poly(A)$^+$ mRNAs from various human tissues were hybridized sequentially with the [^{32}P]-labeled *SOX-3* oligonucleotide probe and *β-actin* cDNA probe. (C) Part of the blot presented in B and hybridized with the [^{32}P]-labeled *SOX-3* cDNA probe.

of serum stimulation and the cells complete a cycle within 48 h.

Despite several attempts, and differently from serum stimulation, no decrease of *SOX-3* mRNA could be

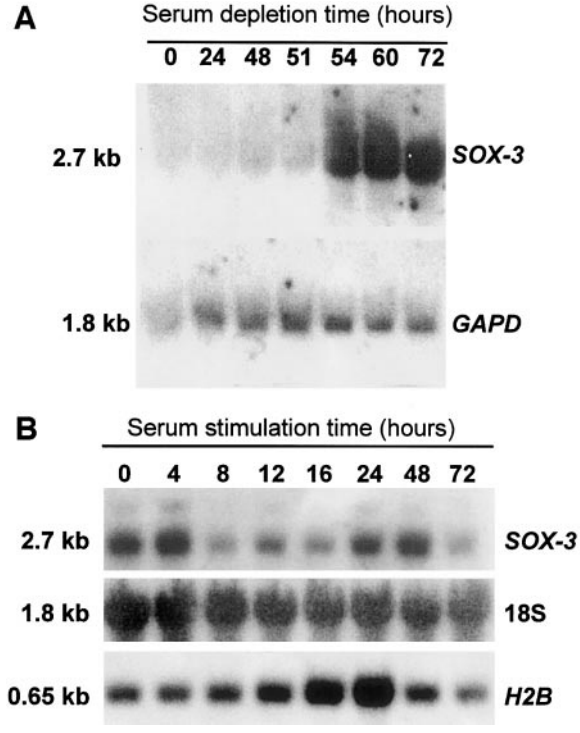


FIG. 4. Effect of serum depletion and serum stimulation on *SOX-3* gene expression. (A) After a growth period (3 days), GEC were submitted, at time 0, to serum depletion for 60 h. (B) After 60 h serum depletion, GEC were submitted to serum stimulation during 72 h. In A and B, total RNAs were extracted at the indicated times. A Northern blot with 20 μg total RNAs was hybridized sequentially with [^{32}P]-labeled guinea pig *SOX-3* probe, 18S rRNA probe and H2B probe.

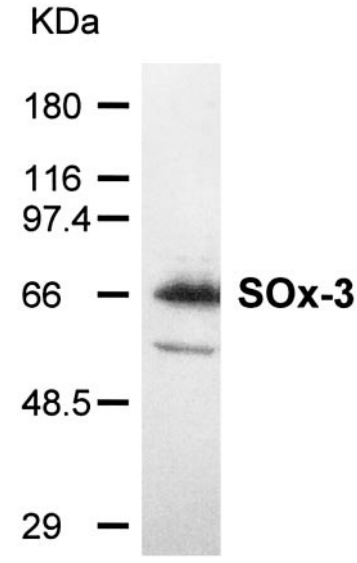


FIG. 5. Specificity of anti SOx-3 polyclonal antibodies. Fifty micrograms (lane 1) or 100 μg (lane 2) of total endometrial proteins was separated by SDS–PAGE on a 10% acrylamide gel and transferred onto PVDF membrane. After incubation with anti SOx-3 antibodies, the SOx-3 was detected by enhanced chemiluminescence system.

observed when serum depleted GEC were stimulated by E_2 alone or in association with cycloheximide.

SOx-3 Protein Expression in Guinea Pig Endometrium

The specificity of the polyclonal anti SOx-3 serum was controlled by Western blot analysis on total guinea pig endometrial proteins. As shown in Fig. 5, a major band was revealed with a size evaluated at 68 kDa, consistent with the predicted SOx-3 molecular weight. A weaker 57-kDa band could be either a proteolytic degradation product of SOx-3 or the translation product from the weakly expressed 3.4 kb mRNA.

Immunofluorescence experiments were carried on guinea pig uterus slices. The uteri were taken from animals on the first day and the 10th day of the estrus cycle. At the first day of the estrus cycle, the estrogen plasma level is very high when the progesterone level is at its lowest. After 10 days, the estrogen concentration is low when the progesterone concentration increases and is already high (26). On the 10th day of the estrus cycle, a strong fluorescence was detected in glandular epithelial cells whereas the fluorescence staining was at the level of the background on the stroma (Fig. 6B). In the glandular epithelial cells, a much weaker signal could be observed when the uterus was taken from an animal at the 1st day of the estrus cycle, indicating that the level of SOx-3 expression varies during to the estrus cycle (Fig. 6C). The incubation of the antiserum with

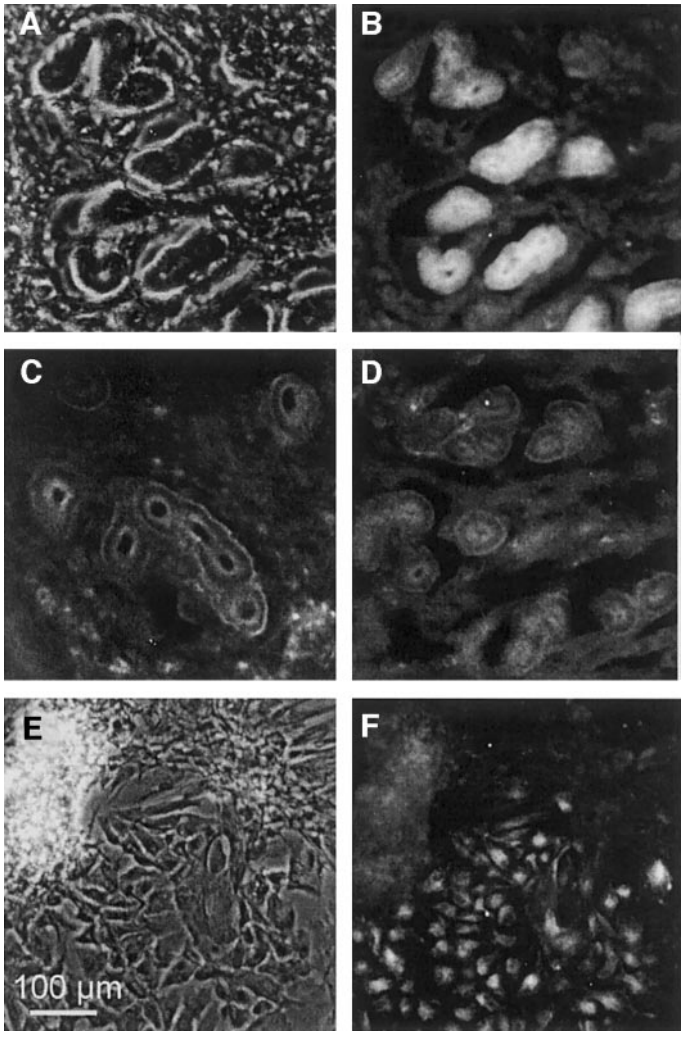


FIG. 6. Immunofluorescence analysis of SOx-3 expression in guinea pig endometrium. (A to D) Cryosections of guinea pig uterus: In A, B, and D, the uterus was taken at the 10th day of the estrus cycle (low estrogen and high progesterone) and in C at the 1st day of the estrus cycle (high estrogen and low progesterone). A is the phase contrast picture of B. In B and C, the sections were incubated with anti SOx-3 polyclonal antiserum (1:100) and with secondary FITC-anti rabbit IgG antibodies. In D, the SOx-3 antibody dilution had been previously incubated with 2 μg of recombinant SOx-3 protein. (E and F) Primary culture of GEC and SC. E is the phase contrast of F. In F, the cells were incubated with the anti SOx-3 antibodies (1:100). In the upper left corner, the rest of the epithelial gland can be seen. SC are in the upper right corner, whereas GEC are in the lower half of the picture. The final magnification ($\times$100) is equal for all the pictures.

the soluble SOx-3 protein strongly extinguished the fluorescent labeling of the epithelium in the endometrium (Fig. 6D) when no signal decrease could be observed in the stroma. According to these results, it would appear that the SOx-3 was strongly expressed in the epithelium and that there was no expression or an undetectable SOx-3 expression in the stroma. The cell type specificity was confirmed by the staining of GEC (Fig. 6F) and the absence of staining of SC in primary culture.

DISCUSSION

Using GEC, the guinea pig *SOX-3* incomplete cDNA was identified and appeared putatively negatively regulated by E_2. Using a 5′-RACE method, the initiation codon and the 5′ untranslated region were obtained. The mRNA size (including the 3′ poly(A) tail) was estimated at 2.7 kb. The nucleotide sequence comparison showed that *SOX-3* had a high sequence identity with the human quiescence-inducible Quiescin h*Q6* gene, a human sequence called *BPGF-1* for which no further information was published, and the rat seminal vesicles sulfhydryl oxidase *SOX-2*. The latter nucleotide sequence was obtained after a partial protein sequencing of the purified rat sulfhydryl oxidase-2 (24). Furthermore, the partial sequencing of the egg white FAD-linked sulfhydryl oxidase (27) pointed out similarities between this oxidase and the proteins deduced from the quiescence inducible h*Q6* cDNA or *BPGF-1* cDNA.

The SOx-3 protein shares many structural features with the egg white FAD-linked sulfhydryl oxidase, the rat SOx-2 and h*Q6*. The proteins SOx-3, rat SOx-2 and hQ6 appear to be formed by the fusion of at least two domains i.e., an N-terminus domain that contains a thioredoxin motif and a C-terminal domain related to an ERV1 domain. The ERV1 gene in *Saccharomyces cerevisiae* encodes a FAD-linked sulfhydryl oxidase (28) which is necessary for mitochondrial biogenesis and survival. The ERV1 domain seems to be needed for the FAD binding as well as for the catalysis since a CRDCA redox disulfide motif is located there (29).

All proteins possess a putative signal peptide indicating the extracellular destination of these proteins. Indeed, h*Q6* has been shown to be secreted into the cell medium (25). Two potential N-glycosylation sites are located in the N part of the proteins and are well conserved. It has been shown that the rat-SOx-2 protein is glycosylated (24).

Egg white FAD-linked sulfhydryl oxidases or rat SOx-2 protein catalyze the generation of disulfide bridges with the reduction of molecular oxygen to H_2O_2. Kinetic studies of the egg white sulfhydryl oxidase indicated that the enzyme is able to introduce disulfide bonds into a wide range of proteins and peptides (30). The catalytic efficiency was higher than that for free cysteine or glutathione. Furthermore, the sulfhydryl oxidase and the protein disulfide isomerase were able to work together to generate and rearrange the disulfide bonds and fully restore the enzymatic activity of RNase A (30). Nucleotide or amino-acids comparisons between *SOX-3* and these sulfhydryl oxidases suggest that SOx-3 is another member of a growing family of FAD-dependent sulfhydryl oxidases.

A major *SOX-3* transcript was detected in guinea pig tissues when five different transcripts were detected with the *SOX-3* probe in human tissues. The size of the two major human transcripts (3.2 and 2.5 kb) that we identified fitted well with the 2 mRNAs (3.2 and 2.5 kb) detected with the h*Q6* probe (23). However, the 3 other mRNAs of 4.8 kb, 2.9 and 1.2 have not been recorded so far.

A computer comparison between the guinea pig *SOX-3* nucleotide sequence or h*Q6* nucleotide sequence and the human genome revealed the existence of only one gene located on the chromosome 1 that aligned over the whole *SOX-3 or* h*Q6* sequences. Moreover, a FISH experiment made with an h*Q6* probe on normal human cells localized h*Q6* on 1q24 (29). Thus, the existence of only one gene in the human genome indicates that the five human transcripts arise from an alternative splicing. It remains to be determined which human transcript is the counterpart of guinea pig 2.7 kb *SOX-3* mRNA.

The level of *SOX-3* expression would appear to be dependent on the tissue since no mRNA was detected in guinea pig brain or liver by Northern blotting. The different levels of the 5 transcripts in human tissues suggest a complex tissue-specific regulation of the mRNA expression and splicing. Brain seemed to be the organ expressing the least *SOX-3* in both species. However, the *SOX-3* expression in brain tissue could be localized in specific areas. The rat *SOX-2* mRNA was also ubiquitously present but a high level has only been detected in seminal vesicles and epididymis (24).

It has been previously reported that GEC enter the quiescence state under serum depletion (14). The present study has demonstrated that *SOX-3* gene expression is induced by serum depletion. Although it has been observed that the h*Q6* gene is strongly induced in the transition from exponential growth to quiescence (23, 25), no induction kinetic during serum depletion has been reported. On the other hand, this kinetic has been investigated for the growth arrest-specific gene *gas1.* This same gene is also involved in the negative control of the cell cycle (5, 31) but has no identity with the *SOX-3* gene. After a low serum addition to the exponentially growing NIH 3T3 cells, the *gas1* mRNA level started to increase after 12 h and reached its maximal value after 48 h (5). The *SOX-3* gene induction occurred later and more suddenly.

Although *SOX-3* was overexpressed when the cells were in the G_0 state, it appeared to be repressed when GEC went through the G_1 phase. The increase in *SOX-3* mRNA after 24 h of serum stimulation seems to indicate that *SOX-3* mRNA was expressed again during the S phase, remained stable during G2/M and decreased during the next G_1. According to these results, *SOX-3* appears to be more like a gene whose expression decreases in the G_1 phase rather than like a gene whose expression is strictly connected to growth arrest. *SOX-3* could be a gene involved in the negative control of the cell cycle. The lack of E_2 effect on *SOX-3* expression could be explained by our previous demonstration that E_2 alone is not a mitogen for endometrial epithelial cells in culture (14).

The polyclonal antibodies raised against a recombinant protein showed that the SOx-3 protein is a marker of the epithelial cell population in uterus tissue. Despite the presence of mRNA in the cultured SC, it would appear that this sub-population does not express the protein, suggesting that *SOX-3* expression could be regulated at the translational level. The difference of SOx-3 concentration between the 1st and the 10th day of the estrus cycle suggests for the first time that a sulfhydryl oxidase could be regulated by E_2 and/or progesterone either alone or in conjunction with a growth factor in the uterine epithelial cells. Experiments are now underway to study the role of steroid hormones on *SOX-3* transcription as well as translation. Furthermore, thioredoxin which is involved in the oxido/reduction of disulfide bonds is also a protein whose expression and secretion are controlled by steroid hormones in the endometrium and occur concomitantly with the onset of *in vitro* decidualisation (32). Thus, the regulation of proteins implicated in the redox state modification appears to be implicated in the decidualisation process.

If SOx-3 is indeed an exported sulfhydryl oxidase, it appears likely that this protein is involved in extracellular matrix modification during uterus differentiation and blastocyst implantation. This expression during a differentiation process is in good agreement with the role of SOx-3 in the negative control of the cell cycle. It is now important to elucidate the proteins that can be a substrate of SOx-3 and the consequences of this thiol modification on the physiology of the endometrium.

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