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THE COUP TRANSCRIPTION FACTOR (COUP-TF) IS DIRECTLY INVOLVED IN THE REGULATION OF OXYTOCIN GENE EXPRESSION IN LUTEINIZING BOVINE GRANULOSA CELLS

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Competition with specific oligonucleotides in DNA-binding experiments, polyacrylamide gel electrophoresis, and recognition by specific antibodies have identified the ubiquitous transcription factor COUP as one of the nuclear proteins binding to the promoter region of the bovine oxytocin gene in endogenously expressing bovine granulosa cells. PCR cloning of partial cDNA sequences for bovine COUP-TF I and II and development of RNase protection assays demonstrated the up-regulation of COUP-TF in bovine granulosa cells and corpus luteum under conditions where the oxytocin gene is switched off. These experimental results from *in vitro* and *in vivo* studies point to the direct involvement of COUP-TF in oxytocin gene down-regulation during luteinization of bovine granulosa cells.

The neurohypophyseal nonapeptide hormone oxytocin is the product of a single copy gene (1), but studies on the regulation of oxytocin gene expression have been hampered by the absence of oxytocin-producing cell cultures. Consequently little progress has been made either in the elucidation of the signal transduction pathways or in identifying the regulatory elements on the gene and their binding factors. Recently oxytocin gene constructs have been transfected into cell lines which do not endogenously express the oxytocin gene (2-5). Such heterologous systems, though useful, do not convey information necessarily relevant to the in vivo situation.

In cows the granulosa cells of the ovarian preovulatory follicle produce large quantities of oxytocin *in vivo* and *in vitro*, accompanying the differentiation change associated with ovulation. cDNA cloning and primer extension analysis showed that the oxytocin gene in these cells was transcribed from the same initiation site as in the hypothalamus, presumably being controlled by the same promoter (1). Using serum-free cultures of these cells *in vitro* it could be shown

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that up-regulation of the oxytocin gene was dependent on the synergistic activity of gonadotropins and either insulin or IGF-I (6,7). Computer analysis of the bovine oxytocin gene upstream promoter region and a comparison with the promoter regions from the oxytocin genes of other species highlighted several conserved regions (8). One of these shows similarity with the binding sites for the receptors for estrogen, thyroid hormone, and vitamin D. *In vitro* protein-DNA binding studies, using nuclear protein extracts from early bovine corpora lutea where the oxytocin gene is endogenously up-regulated, also emphasized the importance of this cis element in the control of the oxytocin gene (8). However, in a heterologous system it could be shown that this site on the bovine oxytocin gene does not function as an estrogen responsive element (9). Using specific competition with several different oligonucleotides in protein-DNA binding studies, we were able to demonstrate that this element is a potential binding site for the COUP transcription factor (COUP-TF) (8), a member of the steroid hormone receptor superfamily for which there is no known ligand (10). The COUP-like complex that is formed with the bovine oxytocin gene promoter appears to be inversely expressed during up-regulation of the gene in the bovine ovary, and appears to be ousted during up-regulation by another transcription factor complex competing for the same cis element (8). This reciprocal appearance of two factors binding at the same site on the gene would suggest that the COUP-like complex may be acting *in vivo* as a down-regulator of gene transcription, as has recently been postulated in another gene system (11).

We have now applied a more extensive analysis of the COUP-like complex formed with the bovine oxytocin gene promoter, and show that it indeed comprises the bovine equivalent of the COUP-TF. Additionally, we have isolated two closely related COUP-TF cDNA clones from the bovine ovary and show that these are expressed in the granulosa cell system in a manner that is consistent with their role as down-regulators of oxytocin gene expression.

MATERIALS AND METHODS

Characterization of specific protein-DNA complexes by gel retardation assays and SDS-PAGE

Preparation of nuclear extracts and gel retardation assays were carried out as described previously (8). For characterization of the proteins complexed with DNA the gel was briefly exposed to X-ray film and the band corresponding to complex B was excised from the gel. The protein was electroeluted and the eluate (ca 50 ng protein) was electrophoresed on a 12 % SDS-PAGE gel and silver-stained (12).

Identification of COUP-TF by a specific antiserum to COUP-TF

Two µl of rabbit polyclonal antiserum to COUP-TF (a kind gift from Dr M.-J. Tsai, Houston, USA) or an equivalent amount of control rabbit serum was preincubated for 20 minutes a 22°C with 1 µg of crude nuclear extract from bovine corpus luteum under the conditions described for gel retardation assays, then incubated for 30 minutes at 22°C in the presence of 1 fmol of [^{32}P]-labelled 60 bp fragment from the bovine oxytocin promoter and run on a non-denaturing

polyacrylamide gel. The gel was exposed to X-ray film using an intensifying screen.

Amplification of COUP-TF cDNA sequence by Polymerase Chain Reaction
Total RNA from bovine corpus luteum was prepared according to a previously described method (13). The RNA was reverse transcribed into cDNA using oligo(dT) as primer and AMV reverse transcriptase (Boehringer Mannheim, Germany). A set of four synthetic oligonucleotides (5'-CTGGCGAGATCCGCAG-GACGACG-3', 5'-TCCTCTTGAAGAAACTTTTGCAGCCCTCGCAG-GTGAATTG-3', 5'-CAGAGCCAGCAGCACATCGAGTGCG-3', 5'-CGAGATGTAGCCGGACAGGTAGCAGTG-3'), based on the human COUP-TF I sequence (14), was used to amplify the N-terminal- and the DNA-binding domains. DNA fragments were PCR amplified as previously described (15), with an annealing temperature of 50°C. The PCR products were analyzed on agarose gels, purified and subcloned into pBluescript KS (Stratagene, USA) using standard procedures.

RNase Protection Assay
RNase protection assays were performed using the RPA^{II}-kit (Ambion, USA). To make the probes, the PCR-fragments for COUP-TF I and COUP-TF II in pBluescript KS were linearized with SmaI (COUP-TF I) or Bam HI (COUP-TF II), and transcribed *in vitro* with T3 polymerase (Stratagene, USA). The length of the riboprobes was 253 bp (COUP-TF I) and 408 bp (COUP-TF II) with specific activities in excess of 10^8cpm/µg. At least 10^6cpm/lane were used in the assays. The expected sizes of the protected fragments are 174 bp (COUP-TF I) and 312 bp (COUP-TF II).

RESULTS

Interaction of COUP-TF with the bovine oxytocin promoter

Figure 1A illustrates gel retardation experiments performed using nuclear proteins derived from early bovine corpora lutea where the endogenous oxytocin gene is maximally up-regulated. Two discrete complexes A and B are formed (Fig. 1A, lane 2). Whereas the lower complex A is competed specifically by oligonucleotides corresponding to the cis element of the bovine oxytocin gene included within the radiolabelled DNA fragment used as probe (Fig. 1A, lane 3), the upper complex B is preferentially competed by the cis element derived from the chicken ovalbumin gene (Fig. 1A, lane 4), considered to be specific for the COUP-TF. Neither a mutated variant of the oxytocin gene specific oligonucleotide, nor an estrogen response element can compete for binding to the luteal nuclear proteins (8).

Nuclear protein extracts obtained from later corpora lutea wherein the oxytocin gene is already down-regulated *in vivo* indicated almost exclusively complex B with only negligble amounts of complex A (Fig. 1B, lane 5). To test whether this complex B indeed contained the COUP-TF, two experiments were performed. Firstly, gel retardation experiments were performed as in Fig. 1A, except that antibodies specifically recognizing the human COUP-TF were included (Fig. 1B, lane 8). Specific DNA binding was clearly disturbed. No effect was seen when control antibodies were added (Fig. 1B, lane 9). Thus the addition of COUP-specific antibodies destabilized the protein-DNA complex B, thus identifying the COUP-TF as a constituent of this complex.

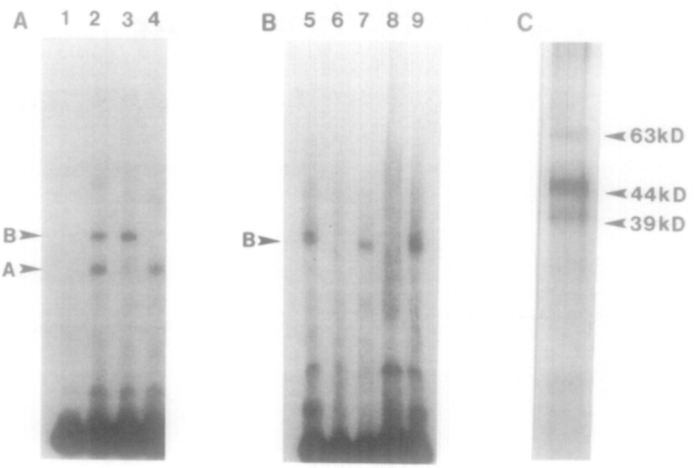

Figure 1.
Gel retardation assay with crude nuclear extract from early (**A**) and late (**B**) bovine corpus luteum. Lane **1**: BSA; Lanes **2** and **5**: 2 µg crude corpus luteum extract; Lane **3**: as lane 2 with 1 ng of double-stranded oligonucleotide competitor (-146 to -165 of the bovine oxytocin gene); Lanes **4** and **6**: as lane 2 with 1 ng of double-stranded oligonucleotide competitor (COUP binding site); Lane **7**: as lane 1 with 1 ng of double-stranded oligonucleotide competitor (mutation of -146 to -165 fragment of the bovine oxytocin gene, see (8)); Lane **8**: as lane 1 with 2 µl of rabbit antiserum to COUP-TF; Lane **9**: as lane 1 with 2 µl of control serum. (**C**) Silver stained SDS-PAGE analysis of proteins from complex B. The major protein bands of the complex are indicated by arrowheads.

Secondly, protein-DNA complexes were prepared as in Fig. 1B where predominantly complex B was obtained. These complexes were excised from the agarose gel used for band retardation and after denaturation subjected to SDS-polyacrylamide gel electrophoresis, followed by silver staining (Fig. 1C). The protein pattern obtained corresponded closely with that reported for COUP-TF by Wang et al. (14), with two protein bands at 39 and 44 kD and a larger band migrating at 63 kD. The significance of the minor bands of lower molecular weight is not clear. Control experiments showed that the DNA probe itself did not stain in this gel system.

PCR cloning of partial cDNA sequences of bovine COUP-TF I and COUP-TF II

For the analysis of COUP-TF expression in bovine granulosa cells and corpus luteum, nucleic acid probes had to be developed which were (a) bovine specific and therefore suitable also for use in RNase protection experiments, and (b) able to discriminate between the two known gene transcripts encoding COUP-TF in humans, namely COUP-TF I and COUP-TF II (14, 16). RNA from bovine corpora lutea was therefore reverse transcribed and COUP-specific sequences amplified by PCR using oligonucleotide primers designed to amplify the N-terminal and the DNA-binding domain (see Materials and Methods). The specific PCR

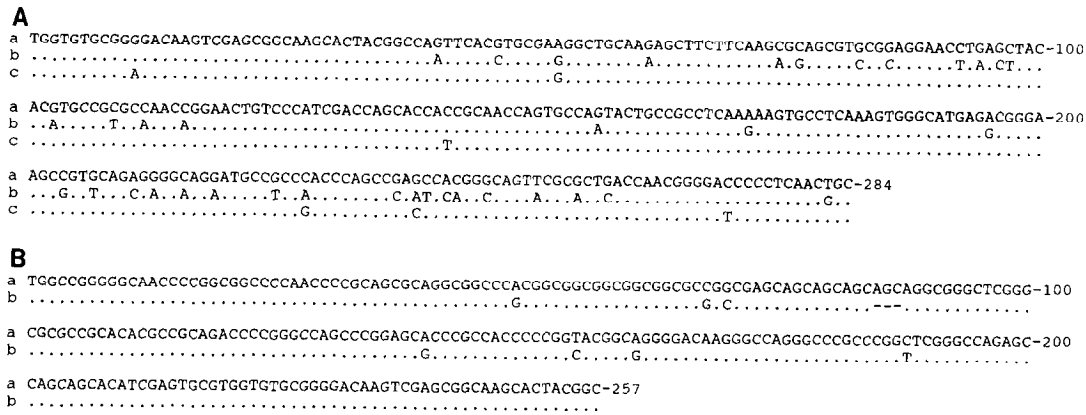

Figure 2.
Alignment of the PCR-amplified sequences from the DNA-binding domain (**A**) and the N-terminal domain (**B**) of bovine COUP-TF (**a**) with the published sequences for human COUP-TF I (**b**) and COUP-TF II (**c**). Between the bovine COUP-TF N-terminal sequence and the human COUP-TF II sequence no homology sufficient for alignment could be observed. The amplified DNA-binding domain appears to represent the homologue of COUP-TF II, and the N-terminal domain the homologue of COUP-TF I.

products were cloned into pBluescript KS plasmid vectors and sequenced. Clones containing the N-terminal domain of COUP-TF I and the DNA-binding domain of COUP-TF II were obtained, the sequences are shown in Fig. 2. Comparison of these sequences with their human counterparts shows as expected a very good conservation between species. The sequences have been submitted to the EMBL database.

Expression of COUP-TF I and COUP-TF II in bovine granulosa cells and corpus luteum

In order to measure the COUP-TF expression with high sensitivity as well as high specificity, RNase protection analyses were performed using the cloned bovine COUP-TF sequences as probes. The intensity of the bands representing the protected fragments is a direct measure of the level of the specific transcript in the investigated tissue (Fig. 3). Expression of COUP-TF I is not detectable in freshly prepared granulosa cells, but is up-regulated by culture of these cells in the presence of fetal calf serum, conditions known to be inimical to the expression of the oxytocin gene (17). In serum-free cultures in the presence of insulin or insulin and forskolin, conditions favouring endogenous up-regulation of the oxytocin gene (6, 7), transcription of COUP-TF I remains below the limit of detection for this assay. Within the corpus luteum, mRNA for COUP-TF I increases to relatively high levels. This up-regulation of COUP-TF I transcription coincides with the down-regulation of oxytocin gene expression during luteinization.

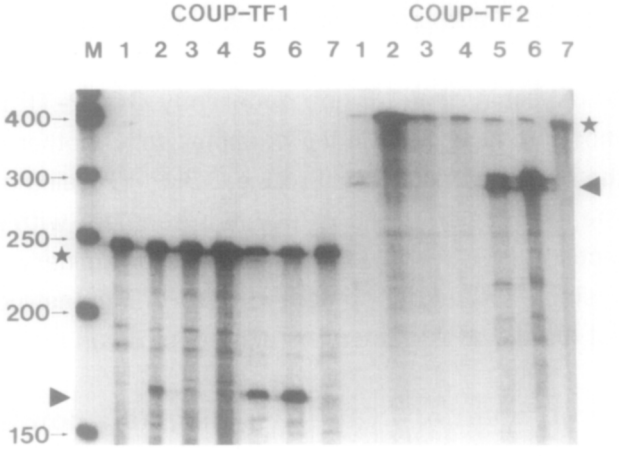

Figure 3.
RNase protection assay with RNA from bovine granulosa and luteal cells. The positions of the riboprobes are indicated with *, the protected fragments are indicated with ▶. Lane **M**: Radiolabelled PhiX174 HinfI DNA Marker (Promega, USA); Lane **1**: Primary granulosa cells (10 µg RNA); Lane **2**: Granulosa cells cultured with 10% FCS (7 µg RNA); Lane **3**: Granulosa cells cultured with 5 µg/ml insulin (2 µg RNA); Lane **4**: Granulosa cells cultured with 5 µg/ml insulin and 10 µM forskolin (3 µg RNA); Lane **5**: Corpus luteum day 1 - 4 of the cycle (10 µg RNA); Lane **6**: Corpus luteum day 12 of the cycle (10 µg RNA); Lane **7**: Control RNA yeast (10 µg RNA).

The expression pattern of COUP-TF II is somewhat different. COUP-TF II is transcribed in freshly prepared granulosa cells, at a time before the oxytocin gene becomes up-regulated. COUP-TF II mRNA, however decreases under conditions where the oxytocin gene is up- or down-regulated. During development of the corpus luteum COUP-TF II expression is up-regulated to relatively high levels following the same pattern as COUP-TF I, and again shows a good correlation to the down-regulation of the oxytocin gene. However, COUP-TF II differs from COUP-TF I in that it is not up-regulated in granulosa cells cultivated in the presence of serum, conditions when there is no oxytocin gene transcription.

DISCUSSION

Three different criteria (specific oligonucleotide competition for the DNA-binding site, recognition by specific antibodies, correct apparent molecular weight on SDS-PAGE) identify a major component of the DNA-protein complex B formed by interaction with the bovine oxytocin gene promoter as the COUP transcription

factor. As in the human, two genes appear to encode two closely related transcription factors COUP-TF I and COUP-TF II, both of which are expressed in the oxytocin-producing cells of the bovine ovary. Cloning of fragments of the bovine genes for these two transcription factors allowed the establishment of sensitive and specific RNase protection assays, which confirmed the supposition based on the gel retardation experiments, that COUP-TF is inversely expressed in regard to the oxytocin gene. Since apparently COUP-TF is competing for binding to the most prominent site in the promoter with another as yet undefined complex (8), the results strongly point to the COUP-TF being a down-regulator of oxytocin gene expression. This is fully in agreement with a recent *in vitro* study of the function of COUP-TF (11), as well as preliminary experiments in a heterologous system where co-transfected COUP-TF constructs were able to negate the effect of other transfected steroid receptors on the rat oxytocin gene *in vitro* (18).

This is the first report of COUP-TF acting as a down-regulator in a homologous system corresponding to an *in vivo* situation. It will be extremely interesting to define the other factor(s) with which COUP-TF is competing for binding to the oxytocin promoter. Since the signal transduction systems identified in the whole cell studies proved to be activators of tyrosine kinases as well as protein kinase A, it seems likely that regulation of the oxytocin gene in bovine granulosa cells involves a complex kinase cascade associated with the differentiation switch referred to as luteinization. The finding that at least one of the factors involved in oxytocin gene regulation is an orphan member of the steroid receptor superfamily further supports this idea.

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