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COMPARISON OF THE ESTROGEN RESPONSIVENESS OF THE RAT AND BOVINE OXYTOCIN GENE PROMOTERS

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DNA sequences in the 5'-flanking region of rat and bovine oxytocin genes were examined for their capacity to confer estrogen responsiveness to their homologous promoters. In contrast to the 5'-flanking region of the rat oxytocin gene, upstream promoter sequences up to 3200 bp of the bovine gene linked to the chloramphenicol acetyltransferase (CAT) reporter gene which were transfected in estrogen receptor expressing MCF-7 cells did not respond to estrogen. Testing 5'-deletion mutants of the rat upstream region linked to the luciferase gene in P19 embryocarcinoma cells co-transfected with an estrogen receptor expression plasmid showed that two regions each associated with approximately 15-fold stimulation of promoter activity were located between nucleotides -172 and -149 and between -148 and +16 in the rat gene. The former region contains the imperfect palindrome GGTGACCTTGACC which differs in one nucleotide from the estrogen response element (ERE) consensus. It is concluded that the corresponding motive CATAACCTTGACC of the bovine gene is not a functional ERE. Thus, the estrogen responsiveness of oxytocin genes is species-dependent.

Oxytocin (OT) is a hormone and neuropeptide mainly involved in the control of reproductive functions (1). The OT gene is expressed in a limited number of hypothalamic cell groups as well as in peripheral organs including the gonads and the adrenal gland (2,3). Estrogens are thought to play a role in the regulation of OT-producing systems (4,5). Recently, the stimulation of promoter activity of the human and rat OT genes has been demonstrated (6,7) and an estrogen response element (ERE) has been located in the upstream region of the human gene (6). Here it is shown that unlike the rat OT gene the bovine gene is not responsive to estrogens. The ERE localized in the rat OT gene differs in three nucleotides from the corresponding motif in the bovine gene.

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MATERIALS AND METHODS

Plasmids

The -363/+16 rat OT upstream region (8) was cloned into p19LUC (9) as a HindIII-Sau3A fragment to construct p-363ROLUC. p-172ROLUC and p-148ROLUC are 5'-deletion mutants of p-363ROLUC made by PCR. p-188ROSCAT was constructed by cloning the -188/+16 upstream region of the rat OT promoter as a SacI-Sau3A fragment in front of the chloramphenicol acetyl transferase (CAT) gene in pSCAT. pSCAT is a pSVO-CAT derivative (10). For construction of CAT expression vectors containing bovine OT promoter sequences an artificial HindIII site was introduced at position +17 of the bovine OT gene by exonuclease digestion and ligation to a blunted HindIII site. An AvaII/HindIII fragment encompassing the region from -148 to +17, an EcoRI/HindIII fragment encompassing the region from about -600 to +17, and a PstI/HindIII fragment encompassing the region from about -3200 to +17 were cloned into the polylinker site in front of the CAT gene in pUMSCAT yielding p-184BOPCAT, p-600BOPCAT, and p-3200BOPCAT. The promoterless CAT plasmid pUMSCAT contains the mouse c-mos gene known to inhibit read-through transcription from upstream promoter sequences (11). pHEO is a plasmid having the human estrogen receptor coding region cloned in the eukaryotic expression vector pSG5 (12). pGEM4 is a commercially available cloning vector (Promega Corporation, Madison, WI, USA).

Transfection and reporter gene assays

P19 embryocarcinoma (EC) cells (13) and MCF-7 breast tumor cells (14) were cultured in DMEM without phenol red and supplemented with charcoal-treated fetal calf serum. The cells were transfected overnight with the calcium phosphate method (15).

P19 EC cells received 10 μg OT promoter-reporter gene plasmid and 3 μg pHEO or pGEM4. MCF-7 cells received 10 μg OT promoter-reporter gene plasmid only. Transfected cells were treated with 100 nM 17,β-estradiol in ethanol for 24 h, as used by Kumar and Chambon and Seiler-Tuyns et al. (16,17). Controls received ethanol only. Luciferase and CAT activities were determined as described (18). Further experimental details have been described (7).

RESULTS

After transfection of the rat OT promoter-reporter gene construct p-188ROSCAT in MCF-7 cells which have endogenous estrogen receptors 17,β-estradiol caused a significant increase in CAT activity (Fig 1). The result was in agreement with a previous observation (7). When p-184BOPCAT which has the corresponding upstream region of the bovine OT gene was used no stimulation of basal activity by 17,β-estradiol was found (Fig 1). Including further upstream regions in p-600BOPCAT and p-3200BOPCAT did not reveal any responsiveness to estrogen. In the latter construct basal activity was very low, while in the promoterless vector pUMSCAT basal activity was absent (Fig 2). The element responsible for estrogen responsiveness of the OT promoter was than further defined by testing several 5'-deletion mutants linked to the luciferase reporter gene.In P19 EC cells co-transfected with the human estrogen receptor expression vector pHEO and p-363ROLUC luciferase activity was strongly stimulated by 17,β-estradiol treatment (Fig 2). The average stimulation over three independent experiments in this system was more than 300-fold. The estrogen stimulation obtained with p-172ROLUC, in which the region upstream of the putative ERE was removed, was about 250-fold. When nucleotides -172 to -149 were deleted the estrogen stimulation dropped to about 16-fold for p-148ROLUC (Fig 2). The resulting estrogen responsiveness of p-148ROLUC was still

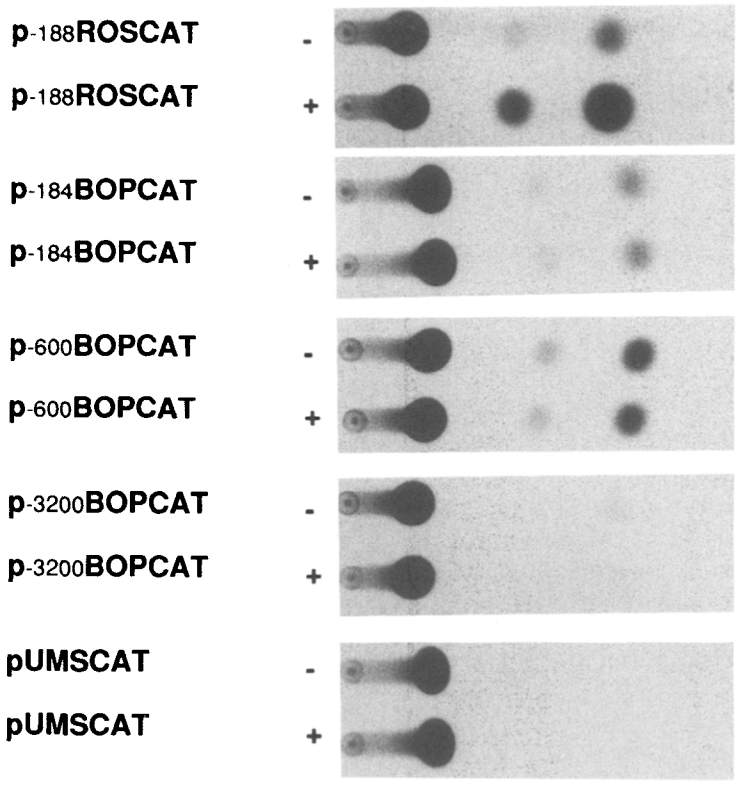

Figure 1
Comparison of the effect of estrogen on the 5'-flanking regions of the bovine and rat OT genes. 5'-Deletion mutants of the bovine OT gene(-3200/+17, -1600/+17, and -184/+17) and the -188/+16 fragment of the rat OT gene were cloned in front of the CAT gene and tested for estrogen responsiveness in MCF-7 cells. pUMSCAT is the promoterless vector. CAT activity was determined in extracts of 17,β-estradiol- (+) and vehicle-treated cells (-) and visualized by autoradiography of thin-layer plates.

markedly high. No stimulation was found when co-transfection with pHEO was omitted in P19 EC cells, nor any estrogen effect on the promoterless vector p19LUC or pRSVLUC was found. The basal activity of the three plasmids was always several fold over the background of p19LUC.

DISCUSSION

The data show that the 5'-flanking region of the rat OT gene promoter harbours two domains which confer estrogen responsiveness. The estrogen-responsiveness accounting for a 15-fold stimulation is situated in the sequence between nucleotides -172 and -149. Additional responsiveness accounting for a similar stimulation was associated with the -148 to +16 fragment. The former region contains the sequence GGTGACCTTGACC which has one mismatch with the perfectly palindromic consensus sequence of the ERE GGTCAnnnTGACC (19) (Fig 3). The element of the rat OT gene is identical to a motive in the human OT upstream region which has been shown to be a functional ERE (6). It has one mismatch in

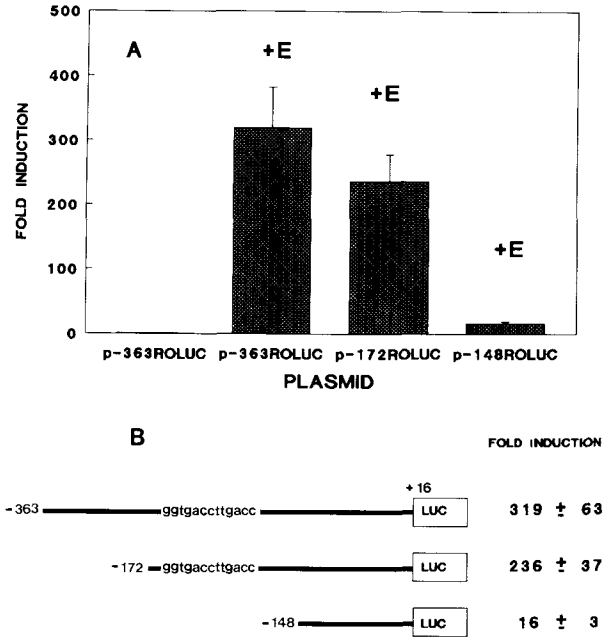

Figure 2
Localization of an ERE in the 5'-flanking region of the rat OT gene. 5'-Deletion mutants (-363/+16, -172/+16 and -148/+16) were made just upstream and downstream of an ERE-like motive, cloned in front of the luciferase gene and tested for estrogen responsiveness in P19 EC cells co-transfected with the estrogen receptor expression plasmid pHEO. Luciferase activity per μg protein in cells treated with 17,β-estradiol or with vehicle alone was determined. The results are expressed as fold induction by taking the ratio of activity in 17,β-estradiol- and vehicle-treated cells. Panel A shows the effect of 17,β-estradiol on the different constructs. Panel B outlines the constructs and displays the ERE-like motive. Data are the average ± SEM; n=6, 11 and 9 for p-363ROLUC, p-172ROLUC and p-148ROLUC, respectively.

position 4 as compared to the perfect ERE. Another natural mutant of the ERE with a mismatch in position 4 (T) is found in the vitellogenin B1 gene of Xenopus laevis (20). This element interacts with the estrogen receptor but is not functional (21). Our data and those of Richard and Zingg (6) show that a G in position 4 retains the functional properties of the ERE.

The data show that the corresponding element in the bovine upstream region (CATAACCTTGACC, residues -164 to -152 (22)) does not function as an ERE. It has

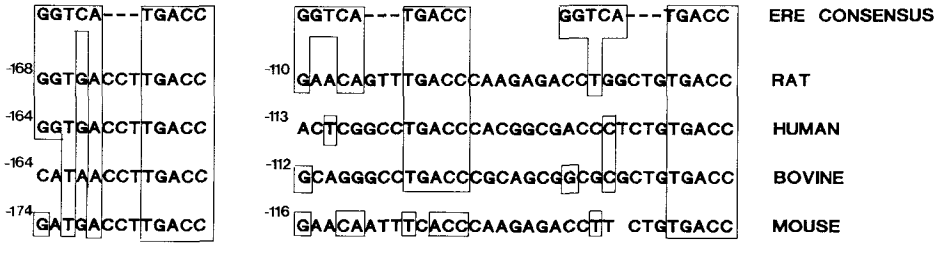

Figure 3
Comparison of the ERE consensus and the related motives and half palindromes in the 5'-flanking regions of the rat, human, bovine and mouse genes. Sequences are from references (8,22,24,25).

mismatches in position 1, 2 and 4 by comparison with the rat/human element (Fig 3). From the results obtained with constructs having extended upstream regions up to -3200 nucleotides it can be concluded that there are no other sequence elements in this region of the bovine OT gene which confer estrogen responsiveness to the bovine OT promoter. Thus, the bovine OT gene, in contrast to the human and rat OT genes, may be unresponsive to estrogens. There are no in vivo data yet to evaluate the role of estrogens in the hypothalamic bovine OT systems. However, in bovine luteal cells which highly express the OT gene, estradiol has been shown to be without effect on the levels of OT mRNA (23). It is of interest to note that the ERE-like sequence of the recently cloned mouse OT gene, GATGACCTTGACC (24), which differs in one nucleotide (position 2) from the rat and human sequence, is a functional, but weaker ERE than the rat/human element (6).

Significant estrogen responsiveness was also associated with the -148 to +16 promoter fragment of the rat OT gene. The half ERE palindrome motive TGACC is present twice in this sequence at nucleotides -102 to -98 and at -82 to -78 (8). In particular the left-half site of the motive -102 to -98 in the rat gene has only two mismatches with the perfect ERE, while in the human and bovine sequences only one correct base is present (22,25). Richard and Zingg reported that constructs containing these two elements of the human OT gene conferred a 1.8-fold response to estrogen (6). Notably, the bovine OT gene also contains these two TGACC elements, but no response to estrogens was found in the three constructs which carried these elements in addition to the ERE-like motive, while the same reporter gene was used as by Richard and Zingg (6). The rat gene displays several differences from the human gene in and around these two elements, which may be responsible for the strikingly different response (Fig 3). Studies with imperfect tandem EREs have shown that paired imperfect ERE-like motives mediate synergistic stimulation of transcription by the estrogen receptor (26-28). This synergism may occur in the rat gene and not, or to a lesser extent in the human and bovine genes. Furthermore, the greater sensitivity and range of the luciferase system by comparison with the CAT system, in particular to quantitate basal expression of the constructs, may have contributed partly to the apparently large difference in responsiveness of the proximal rat and human sequences. It does not explain the difference between the bovine and human responsiveness which was tested under similar conditions (6).

It is concluded that the estrogen responsiveness of the known OT genes is restricted to the human and the rat through a common, functional ERE. Whether the ERE is employed under physiological conditions by OT gene expressing cells further depends on the presence of estrogen receptors. So far we have found that this potential to respond to estrogens is not used in the hypothalamic magnocellular nuclei (7). Other neuronal systems may employ this potential (5,29). Similarly, the estrogen responsiveness may operate in some peripheral organs and in other species. The present results question whether the proposed role of estrogens for the OT system (4,5) is universal to all mammalian species. The further characterization of the 5'-flanking region of the OT gene of other species will therefore be of interest.

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