Gonadal steroids affect brain function primarily by altering the expression of specific genes, yet the specific mechanisms by which neuronal target genes undergo such regulation are unknown. Recent evidence suggests that the expression of the neuropeptide gene for oxytocin (OT) is modulated by estrogens. We therefore examined the possibility that this regulation occurred via a direct interaction of the estrogen-receptor complex with cis-acting elements flanking the OT gene. DNA-mediated gene transfer experiments were performed using Neuro-2a neuroblastoma cells and chimeric plasmids containing portions of the human OT gene 5'-flanking region linked to the chloramphenicol acetyltransferase gene. We identified a 19-base pair region located at -164 to -146 upstream of the OT gene 5'-flanking region linked to the chloramphenicol acetyltransferase gene. The identified region contains a novel imperfect palindrome (GTTGACCTTGCACC) with sequence similarity to other estrogen response elements (EREs). To define cis-acting elements that function in synergism with the ERE, sequences 3' to the ERE were deleted, including the CCAAT box, two additional motifs corresponding to the right half of the ERE palindrom (TGACC), as well as a CTGCTAA heptamer similar to the "elegans box" found in Caenorhabditis elegans. Interestingly, optimal function of the identified ERE was fully independent of the presence of these elements and only required a short promoter region (~49 to +36). Our studies define a molecular mechanism by which estrogens can directly modulate OT gene expression. However, only a subset of OT neurons are capable of binding estrogens, therefore, direct action of estrogens on the OT gene may be restricted to a subpopulation of OT neurons.

Steroid hormones are among the most potent and specific extrinsic chemical signals affecting brain function. In particular, actions of estrogens on nerve cells have been extensively studied and were shown to induce changes at the morphological, chemical and electrophysiological levels (1). Most of these changes are thought to be mediated by regulation of specific neuronal genes. Estrogens, like other steroid hormones, bind to their cognate intracellular receptors to form steroid-receptor complexes. These complexes modulate gene transcription by interacting with specific DNA regions, referred to as estrogen-response elements (EREs) (2-10). Gene transfer studies have identified functional EREs in the upstream regulatory regions of several estrogen responsive genes including the Xenopus and chicken vitellogenin genes (2, 4); the chicken ovalbumin (5) and apoVLDL II (6) genes; the rat prolactin (7-8) and β-luteotropin genes (9); and the human pS2 gene (10). In spite of the pronounced effects of estrogens in the central nervous system, no EREs have so far been identified in any neuronal expressed genes. This report shows the identification and characterization of an ERE that regulates the human gene encoding the hypothalamic neuropeptide oxytocin, a neuropeptide that is largely involved in regulation of reproductive functions.

Oxytocin (OT) acts as both a circulating hormone and neurotransmitter. Circulating OT is implicated in uterine contraction and milk ejection during labor and lactation (11-12), whereas central actions include induction of specific sexual and maternal behaviors (13). Ovarian steroids have long been known to have marked effects on OT physiology. Estrogens, in particular, stimulate OT release into the circulation (14-15) and affect morphology and electrophysiology of oxytocinergic neurons (16-19). Recent studies provided evidence that estrogens are also capable of modulating OT mRNA accumulation (20-21). The precise mechanisms mediating these changes so far remained unknown. In the present study, we investigated the possibility that estrogens exert their effect via direct action on the OT gene promoter.

**MATERIALS AND METHODS**

Plasmid Construction—The plasmid pCAT was constructed as follows: the structural gene for CAT (chloramphenicol acetyltransferase) along with the SV40 splicing and polyadenylation signal was excised from the vector pSV2-CAT (22) by digestion with HindIII and BamHI and inserted into Bluescript KS* (Strategene) between the HindIII and BamHI sites present in the polylinker region. The plasmid pOT-381CAT was obtained by inserting a BamHI/XbaI fragment of the human OT gene (23) at the HindIII site of pCAT, using HindIII linkers. The inserted fragment contained 381 base pairs (bp) of the 5'-flanking region and 36 bp of the 5'-untranslated region of the human OT gene.

The plasmid ptkCAT was obtained as follows: a BamHI fragment containing 109 bp of the herpes simplex thymidine kinase (tk) promoter linked to the structural gene for CAT was excised from the plasmid pTE1 (24) and inserted into the BamHI site of Bluescript KS*. pOTtkCAT and ptkCAT were obtained by inserting a BamHI/SacI fragment of the human OT genomic subclone (~381 to ~49) in either orientation into the HindIII site of ptkCAT using HindIII linkers.

The abbreviations used are: ERE, estrogen response element; OT, oxytocin; CAT, chloramphenicol acetyltransferase; bp, base pair(s).
Plasmid pOT-164CAT was obtained by digesting pOT-381CAT with BstEII (at position -164 relative to the transcription start site) and ClaI (in the Bluescript polylinker, 5' to the inserted OT gene fragment) followed by religation of the blunted ends. By this strategy, the original BstEII site was reconstructed. The mutant pOTdAaGCAT was obtained by the same approach, but using Xhol instead of ClaI. By this method, the first G in the original BstEII site was replaced by an A following religation. The mutants pOTdAaGCAT and pOTdAaGACAT were obtained by the same protocol and are most likely the result of incomplete fill-in reactions at the BstEII and the Xhol sites, respectively. Plasmid pOT-155CAT was obtained by restricting pOT-149CAT with BstEII followed by S1 nuclease treatment, blunt ending, and religation. Plasmid pOT-116CAT was prepared by using the Real site at position -116 in pOT-381CAT. Since this site is not unique in this vector, a fragment spanning from the Real site to the HindIII site (in front of the CAT gene) was isolated and inserted between the HindIII and the cloned ClaI site of pCAT. Plasmid pOT-49CAT was obtained by the same strategy but using the SacI site (located at -49 in pOT-381CAT) instead of the Rsal site.

Plasmid pOTdAaGCAT was constructed from pOT-164CAT by mutating the heptamer CTGCTAA (-139 to -133) into AGATCT (a BgIII site) by site-directed mutagenesis using the Amer sham Corp. in vitro h. Mutagenesis System (version 2) and an appropriate mutagenic primer (3'mer) produced on a Cyclone DNA synthesizer (Milligen). Plasmid pOT49+eCAT corresponds to pOT-49CAT, but contains sequences -164 to -146 of the OT gene at their original positions. The plasmid was produced by inserting the HindIII site of pCAT a 299-bp fragment containing the ERE palindrome (-164 to -160) of the OT gene by sequence-specific cleavage of pOT49CAT (23). Oligonucleotide sequences from -145 to -50 and OT sequences from -49 to +36). This fragment was produced with the aid of the polymerase chain reaction technique (25 cycles; 94°C, 1 min; 37°C, 2 min; 72°C, 3 min; final extension: 72°C, 10 min) using pOT-49CAT as target DNA and the DNA Thermal Cycler as well as the GeneAmp DNA Amplification Reagent Kit (both by Perkin-Elmer Cetus). The forward primer (48-mer) contained a HindIII site followed by sequences -164 to -146 of the OT gene. The rest of the primer (20 nucleotides) corresponded to the region -145 to -126 of the target DNA (pOT-49CAT). The reverse primer was complementary to a 20-bp region of the CAT gene downstream of the HindIII site. The amplified fragment was initially 229-bp long and was reduced to 269-bp by digestion at the two HindIII sites prior to vector insertion. All constructs were verified by dideoxynucleotide sequencing.

Transfections and Chloramphenicol Acetyltransferase Assays—Neuro-2a cells were obtained from the American Type Culture Collection, Rockville, MD (Catalog No. ATCC CCL 131) and were maintained in Earle's minimal essential medium supplemented with 10% fetal bovine serum. Twenty-four hours prior to transfection, the cells were replated on 10-cm plastic petri dishes at a density of 10^6 cells/plate. The cells were transfected by the calcium phosphate procedure (25). Unless stated otherwise, 5 µg of a given CAT construct, 5 µg of the estrogen receptor expression vector (pCH110, Pharmacia LKB Biotechnology, Inc.), and 7 µg of the estrogen receptor expression plasmid HEO (26), or 7 µg of carrier salmon sperm DNA, were used per dish. For experiments involving dexamethasone treatment, 7 µg of the glucocorticoid receptor expression plasmid HGO (27) was also included. The plasmids HEO and HGO contained the coding sequences for the human estrogen receptor (HEO) or the human glucocorticoid receptor (HGO) inserted into the eukaryotic expression vector pCR2 downstream from the SV40 early promoter (26-27). The plasmid pCH110 contained the structural gene for ß-galactosidase under the control of the SV40 early promoter was also co-transfected. Cell extracts (10 µg of protein) from either control (lanes 1 and 3) or 17b-estradiol (E2) treated cultures (10^-7 M, lanes 2 and 4) were assayed for CAT activity using thin layer chromatography followed by autoradiography (22). The band with the lowest mobility at the bottom part of the autoradiogram corresponds to the nonacetylated [3H]chloramphenicol, whereas the two upper bands represent monoaecetylated forms. C, quantitation of CAT activity (filled bars) and ß-galactosidase (ß-GAL) activity (stipped bars) from extracts in control (E2) or estradiol treated (E2) cultures transfected either with carrier DNA (HEO-) or with the expression vector for the estrogen receptor (HEO+). The experiment shown is representative of three independent transfections.

was determined as described (29). In short, cells were homogenized in 5 mM sodium phosphate, 10% glycerol, 12 mM monochloroacetic acid, 20 mM sodium molybdate, pH 7.5. Following centrifugation for 1 h at 105,000 x g, 0.4 ml of the supernatant was incubated on ice with 50 nM [3H]dexamethasone for 18 h in presence or absence of 20 μM unlabeled dexamethasone. Free dexamethasone was separated from bound by fractionation on Sephadex LH-20 columns.

RESULTS AND DISCUSSION

In order to determine whether the estrogen-receptor complex could directly influence OT gene promoter activity, 381 bp of the 5'-flanking region of the human OT gene (23) were linked to the structural gene for CAT (22). B, CAT activities resulting from transient transfection of pOT-381CAT in Neuro-2a cells. Lanes 1 and 2 represent pOT-381CAT co-transfected with carrier DNA (salmon sperm DNA), whereas lanes 3 and 4 represent co-transfections with the expression vector containing the estrogen receptor cDNA (HEO, Ref. 26). Neuro-2a cells were maintained and transiently transfected as described under "Materials and Methods." As an internal control for transfection efficiency, a plasmid (pCH110) containing the structural gene for ß-galactosidase under the control of the SV40 early promoter was also co-transfected. Cell extracts (10 µg of protein) from either control (lanes 1 and 3) or 17b-estradiol (E2) treated cultures (10^-7 M, lanes 2 and 4) were assayed for CAT activity using thin layer chromatography followed by autoradiography (22). The band with the lowest mobility at the bottom part of the autoradiogram corresponds to the nonacetylated [3H]chloramphenicol, whereas the two upper bands represent monoaecetylated forms. C, quantitation of CAT activity (filled bars) and ß-galactosidase (ß-GAL) activity (stipped bars) from extracts in control (E2) or estradiol treated (E2) cultures transfected either with carrier DNA (HEO-) or with the expression vector for the estrogen receptor (HEO+). The experiment shown is representative of three independent transfections.

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RESULTS AND DISCUSSION

In order to determine whether the estrogen-receptor complex could directly influence OT gene promoter activity, 381 bp of the 5'-flanking region of the human OT gene (23) were linked to the bacterial reporter gene, CAT (Ref. 22). Since there are no OT-producing cell lines, the resulting construct (pOT-381CAT, Fig. 1A) was transfected into the neuroblastoma derived cell line Neuro-2a (30). Although this cell line does not express the endogenous OT gene, the transfected promoter elements displayed a readily detectable base-line activity in the absence of external stimulation (Fig. 1B). When these cells were endowed with estrogen receptors by co-transfection with an expression vector containing the estrogen receptor cDNA (HEO, Ref. 26), an addition of 10^-7 M
17β-estradiol elicited a 12-fold increase in expression of the chimeric gene pOT-381CAT (Fig. 1B). This effect was specific since the expression of a co-transfected plasmid containing the Lac Z gene, placed under control of the SV40 early promoter, remained unaffected (Fig. 1C). Moreover, this effect was estrogen receptor-dependent, since estrogen induction could only be observed in host cells co-transfected with the estrogen receptor expression plasmid HEO (Fig. 1).

We next characterized the dose dependence of this hormonal induction. A dose-response relationship was established with respect to both the dose of added hormone and the dose of co-transfected estrogen receptor plasmid (Fig. 2). The minimal estradiol concentration capable of eliciting a statistically significant response was $10^{-8}$ M (Fig. 2A). On the other hand, the minimal amount of co-transfected estrogen receptor plasmid necessary to produce a significant transcriptional effect with $10^{-8}$ M estradiol was as low as 200 ng (Fig. 2B). The low doses capable of induction attested to the specificity of the observed response. As a further test for specificity, we assessed the response to glucocorticoids in cells co-transfected with both estrogen and glucocorticoid receptor expression plasmids. The results depicted in Fig. 3 indicate that the response is specific for estrogens and does not occur after dexamethasone addition, although transfected cells exhibited a high dexamethasone binding capacity ($334 \pm 29$ fmol/mg protein). Moreover, addition of both steroids to cells co-transfected with both steroid receptor plasmids induced a response that did not differ significantly from the response induced by estrogens alone.

In order to test whether the observed increase in CAT activity was indeed the result of trans-activation of transcription, S1 analysis was performed. This analysis indicated, furthermore, the position at which initiation of CAT mRNA transcription occurred. As shown in Fig. 4 (lanes 1 and 2), estradiol treatment of Neuro-2a cells co-transfected with pOT-381CAT and the expression plasmid HEO resulted in a marked rise in correctly initiated CAT mRNA. Densitometric scanning of the autoradiogram indicated a 14-fold increase in band intensity.

To determine whether the 5'-flanking region of the OT gene could also confer estrogen responsiveness to a heterologous promoter in an orientation independent manner, a restriction fragment, encompassing bases $-381$ to $-49$ of the OT gene, was inserted in front of the herpes simplex thymidine kinase (tk) promoter linked to the CAT gene (p74kCAT, Ref. 24). In both orientations, the inserted fragment was able...
to induce a 7-fold increase in the transcriptional activity of the promoter. By contrast, ptkCAT, a plasmid devoid of any OT sequences, was unaffected by the estradiol treatment (Fig. 5).

In order to delineate more precisely the area necessary for estrogen responsiveness, 5' deletion mutants were constructed. Deletions extending beyond nucleotide -164 resulted in a significant decrease in the estrogen response (Fig. 6). Inspection of the sequence at this breakpoint revealed the presence of the sequence element 5'-GGTGACCTTGACC-3', an imperfect palindrome closely resembling the palindromic ERE consensus sequence 5'-GGTCANNNTGACC-3' (31) (difference with consensus sequence is underlined). The present palindrome differs from the consensus ERE palindrome at position 4, where the C is replaced by a G. Previous studies (32-34) as well as our own data (Fig. 6) show that certain point mutations involving the first 3 bases of the palindrome can abolish or significantly reduce estrogen inducibility. Position 4, however, has not yet been subjected to mutational analysis. Furthermore, the naturally occurring functional EREs identified so far all contain a C in position 4. A notable exception is the first of the two ERE palindromes in the Xenopus vitellogenin gene B1 which contains a T at this position (and an A in position 1). Yet this latter palindrome is not functional on its own (35). Data from methylation interference analysis suggest that position 4 in the palindrome is indeed of functional importance, since the G which base-pairs with the C in this position forms a contact site with the estrogen receptor molecule (35). Thus, the present ERE represents a novel version of a fully functional ERE which differs at a relevant position from the consensus palindrome. It is also worthy of note that the rat OT gene 5'-flanking region contains an identical sequence element at a similar position (36-37). By contrast, the corresponding sequence element in the bovine OT gene flanking region is CATACCTTGACC (differences underlined) (38). It would be highly interesting to determine whether this mutated element is involved in estrogen regulation or whether another sequence element fulfills this role in the cow.

Inspection of the sequence of the human OT 5'-flanking region reveals the presence of two additional sequence elements that correspond to the right half of the ERE palindrome (TGACC). These elements are present at positions -103 and -83 (indicated by black vertical bars in Fig. 6) and have been conserved in the rat and bovine OT gene promoters where they are present at similar positions (36-38). Interestingly, an identical element, but in the opposite orientation (GGTCA), functions as an ERE in the chicken ovalbumin gene promoter (5). Either of the two constructs that contained the two TGACC elements, but not the palindromic ERE, exhibited a small but consistent 1.8-fold response to estrogen (Fig. 6, constructs pOT-155CAT and pOT-116CAT). Although

**Fig. 5.** The human OT gene upstream regulatory sequence confers estrogen inducibility to a heterologous promoter. Left panel, schematic representation of the transfected constructs. Right panel, Neuro-2a cells were co-transfected with the chimeric CAT plasmids indicated on the left, along with the plasmid HEO. The bars represent CAT activity present in extracts of control cells (stippled bars) or cells treated with 10^-7 M estradiol (filled bars). Each bar represents the mean ± S.E. of three experiments.

**Fig. 6.** Localization of the ERE in the human OT gene. Left panel, chimeric constructs containing various portions of the 5'-flanking region and 36 bp of the 5'-untranslated region of the human OT gene inserted in front of the coding sequences for CAT. The palindromic region (−164 to −152) is indicated as a filled box. Wild type sequences are indicated in capital letters and mutations are indicated in small letters. The location of the two half-palindromic sequences (TGACC) located at −103 and −83 are indicated by vertical black bars, whereas the thin horizontal lines represent Bluescript KS+ vector sequences. Right panel, Neuro-2a cells were transfected with the plasmids shown in the left panel, along with the plasmid HEO. CAT activity present in extracts of control cells (stippled bars) or cells treated with 10^-7 M estradiol (filled bars) was determined as described under “Materials and Methods.” Each bar represents the mean ± S.E. of six transfections.
these responses were statistically significant ($p < 0.01$, $n = 3$, for either construct), they were weak compared to the 12-fold increase mediated by the palindromic ERE at −164. Moreover, the experiments discussed in the following paragraph do not lend any support to the concept that these elements may act in synergy with the functional ERE at −164. At present, a functional significance of these elements cannot be fully excluded, since, as demonstrated by Tora et al. (5), the function of this half-palindrome may depend on the presence of additional cell-specific transcription factors. A definite answer has thus to await the availability of a cell line expressing the endogenous OT gene.

For several steroid inducible genes, a synergistic action between steroid receptors and other trans acting factors has been demonstrated (7, 39–42). Therefore, we asked whether neighboring sequences on the promoter might serve as binding sites for synergistically acting transcription factors. Since removal of 217 bases upstream of the ERE palindromic did not affect estrogen inducibility (Fig. 6), we focused next on sequences downstream of this element. In addition to the two half-palindromes mentioned previously, this promoter area includes the following sequence elements which display considerable sequence conservation through species evolution: (i) a TATA box equivalent (CATAAAA) beginning at −28; (ii) a CCAAT box at −80; and (iii) the motif CTGCTAA at −139 that is reminiscent of the heptamer CTGATAA. This latter element, termed “elegans box,” is thought to play a supportive role in estrogen control (2) and is closely associated with functional ER Es in the vitellogenin genes of several species including the nematode Caenorhabditis elegans (43). However, either removal of the elegans box alone (pOTeCRAAT) or replacement of sequences −145 to −50 by unrelated spacer sequences (pOT-49+eCAT) did not significantly affect estrogen induced expression of the CAT reporter gene (Fig. 7). In the latter construct, the elegans box, as well as the CCAAT box and the two ERE half-palindromes, were all replaced by vector sequences. S1 analysis revealed that even in the truncated 5′-flanking region of pOT-49+eCAT, initiation occurred faithfully at the correct transcription start site (Fig. 4, lane 3).

It is of note that, in studies by Strähle et al. (39), a glucocorticoid response element was not functional on its own when placed 351 bp upstream of a tyrosine aminotransferase promoter. However, inducibility could be obtained either by duplication of the glucocorticoid response element or by introduction of a CCAAT box (39). The latter suggests functional cooperativity between the CCAAT box binding factor and the steroid receptor complex. In the present study, the ERE, located 164 bp from the transcription start site, is fully active in the absence of a CCAAT motif and in the absence of the two ERE half-palindromes that could conceivably substitute for an additional ERE (pOT 49+eCAT, Fig. 7). The only sequence requirements for full inducibility are present in a short promoter fragment that contains the TATA box (−49 to +36). It remains to be determined whether this small region contains other specific sequence elements that act in synergy with the ERE. By contrast, any cooperative action of the elements located between −145 and −50 may require cell specific trans-acting factors that are absent from Neuro-2a cells.

In conclusion, the present data defines a molecular mechanism by which estrogens can directly modulate OT gene expression. The existence of such a mechanism has been suggested by a series of in vivo observations which established a relationship between estrogen levels, on the one hand, and neuronal OT mRNA or peptide accumulation on the other (20–21, 44–47). This concept has been supported further by the demonstration that oxytocinergic neurons are capable of concentrating exogenously added labeled estrogens (48–49). However, since the ability to concentrate estrogens is apparently limited to only a subset of oxytocinergic neurons (48–49), direct action of estrogens on the OT gene may be restricted to a subpopulation of OT neurons. Continued investigation at the molecular level of steroid regulation of neuronal gene expression should shed further light on the complex actions of steroids on brain function.

**Fig. 7. Effect of deletions 3′ to the ERE.** Left panel, schematic representation of mutant plasmids. The palindromic estrogen response element at −164 (ERE), the heptamer CTGCTAA at −139 resembling the elegans box (EB, Ref. 2), the CCAAT box at −80 (CAAT), and the TATA box equivalent at −28 (CAT A) are indicated. The half-palindromes TGACC (not shown) are at −103 and −83. Thin horizontal lines indicate Bluescript KS+ vector sequences. Note that the distance of the ERE (if present) to the TATA box equivalent is identical in all constructs. The plasmid pOTΔebCAT was derived from pOT-164CAT by site-directed mutagenesis, and pOT-49+eCAT was constructed from pOT-49CAT using the polymerase chain reaction technique. All constructs were verified by dideoxynucleotide sequencing. Right panel, Neuro-2a cells were transfected with the constructs indicated on the left together with the plasmid HEOD. CAT activity present in extracts of control cells (stippled bars) or cells treated with 10−10 M estradiol (filled bars) was determined as described under “Materials and Methods.” Each bar represents the mean ± S.E. of three transfections.
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