Identification of a Retinoic Acid Response Element in the Human Oxytocin Promoter*

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Retinoids are known to have profound effects on cellular differentiation and embryo pattern formation. In the adult organism, retinoid acid (RA) receptors are present in a large variety of tissues, including brain. However, little is known of the precise roles of RA at these different sites. In the present study, we have identified a novel potential target of RA action by identifying an RA response element (RARE) in the human oxytocin (OT) gene promoter. We have used DNA-mediated gene transfer techniques to introduce various portions of the OT 5′-flanking sequences next to the chloramphenicol acetyltransferase (CAT) gene in neuroblastoma cells. RA elicited a marked stimulation of the transcriptional activity of the OT promoter in cells cotransfected with either the human RA receptor α, β, or γ. In cells cotransfected with the RA receptor α, the ED₅₀ of this response was 5 × 10⁻¹⁰ M. The RA response could also be conferred to a heterologous promoter independent of orientation. 5′-Deletions as well as site-directed mutations demonstrated that four TGACC motifs, located at −162, −156, −103, and −83 in the OT promoter, are necessary for optimal RA induction. Mutation or deletion of any of these elements reduces significantly the RA response. Interestingly, the first two TGACC motifs overlap with the estrogen response element that we have previously characterized in this gene. Furthermore, the TGACC motif located at −83 overlaps with the CCAAT box. We further demonstrate that in neuroblastoma cells transfected with an RARα expression vector, expression of the endogenous OT gene is stimulated >4-fold in response to RA. Our studies constitute the first report of a RARE in a neuropeptide gene and define a mechanism by which OT gene expression can be modulated by retinoic acid.

The retinoids form a group of related compounds which exert profound effects on cell growth and differentiation. In the developing chicken limb, a concentration gradient of retinoic acid (RA) conveys positional information to individual cells along the anterior-posterior axis (for review, see Summerbell and Maden, 1990; Brockes, 1990). In addition, RA induces differentiation of epithelial cells (Ruberte et al., 1990) and teratocarcinoma stem cells (Strickland, 1980). In mammals, RA has powerful teratogenic effects if administered during pregnancy (Lammer et al., 1985).

Transduction of the RA signal at the level of gene expression involves a growing family of nuclear receptors which are members of the steroid/thyroid hormone receptor superfamily. In mouse and human, three types of RA receptors (denoted RARα, -β, and -γ) have been discovered (Petkovich et al., 1987; Giguere et al., 1987; Brand et al., 1988; Benbrook et al., 1988; Krust et al., 1989; Zelent et al., 1989; Kastner et al., 1990). Furthermore, an RARγ-related RAR (termed RARγ) has been identified in the urodel blastema (Ragsdale et al., 1989). A novel RAR with a substantially different ligand specificity (termed RXRα) has been isolated from a human liver cDNA library (Mangelsdorf et al., 1990). Each member of the RAR family displays a unique pattern of tissue-specific expression in the developing as well as in the adult organism (Ruberte et al., 1990; Kastner et al., 1990). The presence of RARs in various adult tissues is one of the indications that the role of RA extends beyond the developmental period. Specifically, the high amounts of RARα and β expression in adult brain (Giguere et al., 1987; Benbrook et al., 1988; Zelent et al., 1989) may suggest hitherto unrecognized roles of RA in brain function.

Like other nuclear receptors of the steroid/thyroid hormone superfamily, RA receptors function as ligand-induced transactivators and modulate the expression of specific genes by binding as a ligand/receptor complex to specific DNA sequences termed RA response elements (RARE). Despite the large spectrum of RA-mediated actions, naturally occurring RAREs have only been identified in a small number of genes. These include the RARβ gene itself (de Thé et al., 1989; Sucof et al., 1990; Hoffmann et al., 1990), the laminin B1 gene (Vasios et al., 1989), the osteocalcin gene (Schule et al., 1990), and the gene for complement H factor (Munoz-Canoves et al., 1990). Furthermore, in two cases, a thyroid hormone response element was also shown to impart RA responsiveness (Umesono et al., 1988; Bedo et al., 1989). However, no unequivocal RARE consensus sequence could be derived so far. Whereas DNA elements mediating responses to estrogens and glucocorticoids are typically palindromic, several naturally occurring RAREs identified thus far contain two to four direct repeats of the pentamers TGACC, TCACC, or variations thereof, separated by 3–15 nucleotides. The pentamer motif itself is related to estrogen and thyroid hormone response elements, since TGACC is found in the right half of most estrogen or thyroid hormone response elements (Beato, 1989).

We have previously analyzed the steroid hormone regulation of the gene encoding the hypothalamic neuropeptide oxytocin (OT) and identified an imperfect palindrome starting at position −164 that mediates estrogen responsiveness.
constructs were verified by dideoxynucleotide sequencing. Each plate was transfected with a total of 19 pg of DNA using the calcium phosphate procedure (Graham and Van der Eb, 1973). Unless otherwise stated, cells were transfected with 7 pg of the appropriate plasmid linked to the tk promoter and the CAT gene.

The plasmid pOT-164 CAT consists of the BamHI/NcoI fragment (-381 to +36) of the human OT gene (Saussville et al., 1985) inserted with HindIII linkers at the HindIII site of the CAT gene in pCAT. The plasmid pOT-381CAT consists of the BamHI/SstI fragment of the human OT genomic clone inserted into the HindIII site of pTKCAT using HindIII linkers. The plasmid pOT-49/-381tkCAT is similar to the previous one, but with the insert in the opposite orientation. The plasmid pOT-381tkCAT was created using a self-complementary oligonucleotide with the sequence 5'-AGC TTC AGG TCA TGA CCT GA-3'. The oligonucleotide was designed such that following self-annealing the double-stranded oligonucleotide contained HindIII-compatible ends on either side. Using these sites, the oligonucleotide was inserted into the HindIII site of pTKCAT. The plasmid used contained a single insertion of the oligonucleotide. The plasmid RARE-RAREtkCAT (a gift from Dr. Pierre Chambon, Unité 184 de l'INSERM, Strasbourg, France) contained the RARE gene linked to the tk promoter and the CAT gene.

Plasmid Construction.—Plasmid pCAT contains the coding sequences for chloramphenicol acetyltransferase (CAT) (Gorman et al., 1982) inserted into vector Bluescript KS+ (Stratagene). The plasmid pOT-381CAT consists of the BamHI/NcoI fragment of the human OT gene (Saussville et al., 1985) inserted with HindIII linkers at the HindIII site of the CAT gene in pCAT. The plasmid pOT-381CAT was constructed by removing a 109-base pair segment containing the herpes simplex thymidine kinase (tk) promoter linked to the CAT gene from the plasmid pTK1 (Edlund et al., 1985) and ligating it into the BamHI site of Bluescript KS+. The plasmid pOT-381/-49tkCAT consists of the BamHI/SstI fragment of the human OT genomic clone inserted into the HindIII site of pTKCAT using HindIII linkers. The plasmid pOT-49/-381tkCAT is similar to the previous one, but with the insert in the opposite orientation. The plasmid TREpalktkCAT was created using a self-complementary oligonucleotide with the sequence 5'-AGC TTC AGG TCA TGA CCT GA-3'. The oligonucleotide was designed such that following self-annealing the double-stranded oligonucleotide contained HindIII-compatible ends on either side. Using these sites, the oligonucleotide was inserted into the HindIII site of pTKCAT. The plasmid used contained a single insertion of the oligonucleotide. The plasmid RARE-RAREtkCAT (a gift from Dr. Pierre Chambon, Unité 184 de l'INSERM, Strasbourg, France) contained the RARE gene linked to the tk promoter and the CAT gene.

RESULTS

Expression in Neuro-2a Cells.—RNA was extracted from Neuro-2a cells treated with or without RA and quantitatively and qualitatively assessed by spectrophotometry at A360 and agarose gel electrophoresis. One μg of total RNA was reverse transcribed in "PCR buffer" (50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM MgCl2, 100 μg/ml gelatin) in the presence of 1 μl RNasin (28 units/μl; Promega), 1 mM dNTPs, 0.5 μg oligo(dT) primers, and 10 units of reverse transcriptase (Life Sciences, St. Petersburg, FL). The first strand reaction proceeded at 37°C for 10 min and then at 42°C for 60 min in a total volume of 20 μl. The reaction was stopped by heating to 94°C for 5 min. Specific amplification of OT cDNA was achieved by the use of two primers corresponding to parts of exon A and exon C of the mouse OT gene. The exon A primer (forward primer) corresponded to nucleotides 99–115 and exon C primer (reverse primer) corresponded to nucleotides 773–798 of the mouse OT gene (Hara et al., 1991). One hundred pmol of each primer was added to the reaction mixture along with 2.5 units of Taq polymerase (Amplitaq, Perkin-Elmer Cetus Instruments) and 0.01% final concentration of PCR buffer. As an internal control for amplification efficiency, an additional primer pair corresponding to exons 3 and 5 of the mouse β-actin gene was added to the reaction. The forward primer corresponded to nucleotides 1501–1521, and the reverse primer was complementary to nucleotides 2860–2880 of the β-actin gene (Nudel et al., 1983). Based on the distance between the priming sites on a β-actin cDNA, the predicted size of the amplified band was 766 bp. The reaction conditions for the PCR amplification were: 25 cycles, 94°C, 45 s; 55°C, 45 s; 72°C, 45 s; final extension: 72°C for 10 min. One-tenth of a PCR reaction was loaded on a 1.5% agarose gel and, following electrophoresis and denaturation by 0.5 N NaOH, transferred to a nylon membrane (Amersham Corp.) and hybridized to a 5′-specific oligonucleotide probe corresponding to nucleotides 615–624 of the mouse OT gene (Hara et al., 1991). Hybridization occurred at 42°C in 0.8 M NaCl and 50% formamide. The washing solution contained 12.5 M NaCl and 0.125% sodium dodecyl sulfate (5 × 10 min at 56°C).

RESULTS

Co-transfection of RARα, β, or γ Expression Vectors with OT Promoter/CAT Constructs

In order to determine whether the transcriptional activity of the human OT gene promoter can be modulated by RA, a segment containing 381 bp of the OT gene 5′-flanking region was inserted upstream of the chloramphenicol acetyltransferase gene (pOT-381CAT). This construct was transiently expressed in mouse neuroblastoma cells, with or without co-transfection with the human RAR expression vectors RARα, RARβ, or RARγ.
RAβ, or RAγ (Petkovich et al., 1987; Giguere et al., 1987; Brand et al., 1988; Benbrook et al., 1988; Krust et al., 1989; Zelent et al., 1989; Kastner et al., 1990). As shown in Fig. 1A, in the absence of any co-transfected RAR expression vectors, pOT-381 CAT exhibited a readily detectable level of transcriptional activity, with no significant change after RA addition. By contrast, if either RAα, RAβ, or RAγ was co-transfected with pOT-381 CAT, addition of $10^{-7}$ M RA led to a strong and reproducible increase in CAT activity. The maximal increase (RA-treated RAR co-transfected cells versus untreated, non-RAR co-transfected cells) was 40-fold, using RAα.

In all cases, co-transfection with any of the RAR expression vectors led to a consistent increase of CAT expression in the absence of added ligand. The same phenomenon was observed with some other RARE/CAT constructs (Vasios et al., 1989) and may be due to the presence of low levels of retinoids remaining in charcoal-stripped serum and/or to some ligand-independent transactivation activity of RA receptors. Although marked activation was observed with any of the three RA receptor types tested, the strongest effect was observed with RAα. Subsequent experiments were, therefore, only carried out with RAα.

In order to quantitate more precisely RA action, a dose-response curve was established. As depicted in Fig. 1B, the OT RARE was highly sensitive and responded to RA with an ED50 of approximately $5 \times 10^{-10}$ M. A dose as low as $10^{-10}$ M was able to elicit a statistically significant response ($p < 0.01$) and doses of $10^{-7}$ M RA caused a maximal response. We next determined the dose-response relationship with respect to the amount of transfected RAR expression vector. Fig. 1C demonstrates that a maximal response was obtained with 15 μg of transfected expression plasmid, but as little as 200 ng was sufficient to elicit a statistically significant response to $10^{-7}$ M RA.

To determine whether OT gene 5′-flanking regions could confer RA responsiveness to a heterologous promoter, OT gene sequences spanning from −381 to −49 were inserted in front of the herpes simplex thymidine kinase (tk) promoter, linked to the CAT gene. Independent of orientation, the OT gene sequences were able to confer a 7-fold RA-dependent induction of CAT activity (Table I). This effect was specific and was not observed with the parent plasmid ptk-CAT, which was devoid of any OT gene sequences (Table I).

**Comparison with Other RAREs**

To judge the effectiveness of the OT gene RARE in comparison with other known RAREs, the RA induction of the tk promoter by the OT RARE was compared with RA induction of the same heterologous promoter by the RARE present in the RAβ gene (de Thé et al., 1990) and a synthetic RARE (TREpal) described by Umesono et al. (1988). As shown in Table I, the -fold induction of both the synthetic RARE as well as the RAβ RARE was relatively weak in Neuro-2a.

![Fig. 1. Retinoic acid and retinoic acid receptor-dependent transactivation of the human oxytocin gene promoter](image)

**Table I**

**The 5′-flanking region of the human OT gene confers retinoic acid responsiveness to a heterologous promoter and comparison with known RAREs**

| Construct         | CAT activity (% acetylation) |
|-------------------|-----------------------------|
|                   | −RA | +RA | fold stimulation |
| ptkCAT            | 2.6 | 2.7 | 1.0 ± 0.2         | NS               |
| pOT-381/-49tkCAT  | 2.3 | 15.4| 6.7 ± 1.1         | <0.01            |
| pOT-49/-381tkCAT  | 2.5 | 17.7| 7.1 ± 1.4         | <0.01            |
| TREpal-tkCAT      | 1.8 | 3.8 | 2.1 ± 0.2         | <0.01            |
| RARβ-RAREtkCAT    | 3.2 | 6.7 | 2.1 ± 0.5         | <0.01            |

Plasmid ptkCAT contains 109 bp of the 5′-flanking region of the herpes simplex thymidine kinase (tk) promoter linked to the structural gene for CAT in Bluescript (KS7). pOT-381/-49tkCAT and pOT-49/-381tkCAT contain the human OT sequences from −381 to −49 in front of the tk promoter in forward or reverse orientation, respectively. TREpal-tkCAT contains the rat growth hormone gene thyroid hormone response element linked to tkCAT. This element constitutes the first sequence element shown to act as a RARE (Umesono et al., 1988). RAβ-RAREtkCAT contains the RARE of the RAβ gene linked to tkCAT. (For sequences, see Fig. 3B). Neuro-2a cells were co-transfected with the above constructs along with 7 μg of RAα. CAT activity is shown as percent acetylation in the presence (+RA) and in the absence (−RA) of $10^{-7}$ M RA and as RA-induced fold stimulation (means ± S.E. of five independent transfections). Statistical analysis was as in Fig. 1B. NS, not significant.
cells and notably weaker than the RA induction of the OT RARE under the same conditions.

**Delineation of Sequences Necessary for RA Induction**

5'-Deletion Mutants—To determine more precisely the regions mediating the observed RA induction, 5'-deletion mutants were tested for their ability to respond to RA. Deletion of sequences upstream of position -164 (pOT-164 CAT) did not significantly affect the RA response. However, deletions extending to -155 or -116 (pOT-155CAT and pOT-116CAT, respectively) significantly reduced, but did not abolish, the RA response (Fig. 2A). With the latter two constructs, RA induced a low, but reproducible, 2-fold stimulation. Further removal of OT sequences down to position -49 (pOT-49CAT) or complete removal of OT sequences (pCAT) led to a complete loss of RA responsiveness.

The region mediating RA responsiveness comprises four direct repeat of the pentamer TGACC (as indicated by black boxes in Fig. 2A). These pentamers are present at positions at -162, -156, -103, and -83. Constructs containing all four repeats (pOT-164CAT) exhibited strong RA induction (5-6-fold), but removal of the two upstream repeats (pOT-155CAT) led to a marked reduction in RA responsiveness (2-fold).

Although these results clearly indicated that the two upstream TGACC repeats were necessary, but not sufficient for full RA responsiveness, the importance of the two downstream pentamer repeats remained unclear. Therefore, in order to delineate more precisely the role of the two downstream repeats, we next analyzed the RA responsiveness of constructs containing the two upstream repeats but carrying specific point mutations in either of the two downstream repeats.

**Mutations in the -145/-50 Region**—The experiments in this section were designed to test for each of the two downstream repeats their roles in the context of entire -164/+36 region. In plasmids pOTA-103CAT only two nucleotides of the third TGACC repeat (at -103) were mutated, resulting in the sequence gAT CAT. Mutant pOTA-83CAT differs from the wild type with respect to three nucleotides in the fourth TGACC repeat (at -83), changing it into gagCC. In both cases, these point mutations led to a marked reduction in RA inducibility, i.e. from 5-fold to 1.5- and 2.5-fold, respectively. Mutation of the entire stretch from -145 to -50, leaving only the two upstream repeats at their original position, led equally to a drastic decrease of RA responsiveness (1.5-fold induction). Taken together, these data indicate that all four TGACC repeats are required for the full RA response exhibited by the wild type promoter.

**Sequence Comparisons**—If the corresponding sequences in the human, rat, and bovine genome are compared, it is readily apparent that all four TGACC motives are conserved (Fig. 3A). There is only one exception in the bovine genome, where sequences mediating retinoic acid response in

**Fig. 2. Localization of the RARE in the human OT gene. A**: left panel, schematic representation of the chimeric constructs containing various portions of the 5' flanking region of the human OT gene inserted in front of the structural gene for CAT. The black boxes at -162, -152, -103, and -83 represent the pentamer repeat elements TGACC. Thin horizontal lines indicate Bluescript vector sequences. Right panel, Neuro-2a cells were transfected with the construct indicated on the left along with RARα and assayed for CAT activity. CAT activities are shown as -fold stimulation upon retinoic acid treatment (10^{-7} M). Each bar represents the mean ± S.E. of at least three transfections. B, effects of mutating sequences downstream of -164. The left panel illustrates schematically the mutated plasmids. The black boxes at -162, -156, -103, and -83 represent the TGACC motives. White boxes represent mutated sequences introduced without changing the relative distance of the upstream sequences. Wild type sequences are indicated in **uppercase letters**, and mutations are represented by **lowercase letters**. The horizontal lines indicate Bluescript vector sequences. Right panel, the constructs indicated on the left were cotransfected with the RARα in Neuro-2a cells and assayed for CAT activity. CAT activities are shown as -fold stimulation upon retinoic acid treatment (10^{-7} M). Each bar represents the mean ± S.E. of at least three transfections.

**Fig. 3. Organization of retinoic acid response elements in the OT gene and other retinoic acid responsive genes. A**: comparison of sequences from -167 to -70 of the human OT gene with the corresponding sequences from the rat and bovine genes. The arrow marks the location of the TGACC motifs. Asterisks denote sequence identities. B, sequences mediating retinoic acid response in other genes. The numbers represent the location of the retinoic acid response elements in the corresponding genes. TGACC and TGACC-related motives are indicated by arrows.
the first pentamer is TAACC instead of TGACC. The conservation of the four pentamer repeats is all the more striking since there is considerable sequence diversion in areas surrounding the pentamer repeats.

RA Regulation of the Endogenous OT Gene in Neuro-2a Cells

To assess further the physiological relevance of these findings in a biological context, we examined the endogenous expression of the OT gene by Neuro-2a cells and its regulation by RA. By PCR analysis we were able to demonstrate that Neuro-2a cells express the endogenous OT gene, albeit at a lower level than hypothalamic neurons in vivo (Fig. 4B). In order to determine whether expression of the endogenous OT gene could also be stimulated by the action of ligand activated RAR, Neuro-2a cell were transfected with an expression vector for the RARα and treated with 10⁻⁷ M RA. As shown in Figs. 4, A and B, this treatment led to a marked increase of endogenous OT gene expression without a notable effect on the β-actin gene.

**DISCUSSION**

We have delineated an RARE between nucleotides -164 and -49 of the 5'-flanking region of the human OT gene. Specific point mutations and 5'-deletions indicate that four direct repeats of the pentamer TGACC are necessary for mediating full RA induction. The strong conservation of these pentamer motifs in the human, rat, and bovine genome are a further indication that these elements are of biological importance. Our contention that these elements form a RARE is based on their functional capacity to confer RA responsiveness to the homologous as well as a heterologous promoter in a DNA transfection assay. In analogy to other hormone response elements, it is, therefore, likely that they serve as binding sites for the corresponding receptor/ligand complexes. However, direct demonstration of their capacity to bind RAR/RA complexes will require an in vitro DNA binding assay.

So far only a few other RAREs have been characterized in detail (Fig. 3B). Initial observations by Umesono et al. (1989) indicated that the RARα is able to bind to a synthetic thyroid hormone response element (TGACC GGTCA). Analysis of a naturally occurring RARE in the laminin B1 gene revealed the presence of three direct repeats of TGACC-related elements (TGACC, TAACC, and TCACC) (Vasios et al., 1989). It is of interest to note, that the second element (TAACC) corresponds to the version of the first repeat element present in the bovine OT gene (Fig. 3A). The RARE present in the RARβ gene contains a direct repeat of the element GTTCA (corresponding to TGACC on the opposite strand). Mutation studies demonstrated that both these elements are necessary for RA responsiveness (Sucov et al., 1990). The RARE of the osteocalcin gene contains three occurrences of the TCACC element (one on the sense strand and two on the antisense strand) (Schule et al., 1990). As shown in the present study, the repeats mediating RA responsiveness in the OT gene consist uniformly of the sequences TGACC (with one exception [TAACC] in the bovine gene). From the information available so far, it can be concluded that positions 1, 3, and 5 of the repeat pentamers are consistently occupied by T, A, and C, respectively, whereas positions 2 and 4 are more variable.

In the previously identified RAREs, the space between the repeat elements varies from 3 to 15 nucleotides. In the present case, the region necessary for maximal responsiveness extends over 83 base pairs and can be considered as consisting of two RAREs, each containing two repeats. The distance between repeats 3 and 4 is 20 bp, which corresponds approximately to two turns of the double helix, assuming a B-DNA conformation. Therefore, the structures are likely to be located on the same face of the double helix. By contrast, repeats 1 and 2 are much more closely spaced and are assumed to lay on opposing faces of the DNA. To what extent each of these pentamer pairs serves as a binding site for a RAR dimer remains to be investigated. Since our deletion and mutation experiments indicated that all four pentamer repeats are necessary for RA action, the two pentamer pairs are likely to act in synergism over the 53-bp distance. Cooperativity of two cis-linked nuclear receptor binding sites has been shown to occur at the level of DNA binding in the case of two tandemly arranged progesterone response elements (Klein-Hitpass et al., 1990; Ponglikitmongkol et al., 1990) as well as at the level of transactivation of transcription in the case of two consecutive estrogen response elements (Ponglikitmongkol et al., 1990). It is tempting to speculate that a similar interaction may take place between the two pairs of pentamer repeats, at the level of receptor/DNA interaction, at the level of transactivation, or both.

In addition to the growing list of demonstrated interactions between transcription factors that bind to neighboring cis-linked DNA elements (Schule et al., 1988), there are also several recent examples of direct overlaps of binding sites for distinct transcription factors. These include an overlap between a CAMP response element and a glucocorticoid response element in the pituitary glycoprotein hormone α-subunit gene (Ackerblom et al., 1988) and an overlap between an AP1 site and a RARE in the case of the osteocalcin gene (Schule et al., 1990). Interestingly, the RARE identified in this study represents an example of a dual overlap (Fig. 3A). First, the last two Cs of the TGACC repeat 4 coincide with the first two Cs of the CCAAT box located at -80 in the human OT gene. Being aware of this overlap and in order to avoid interference with the CCAAT box function, our mutation of repeat 4 (pOTα-83 CAT) was designed in such a way that it did not alter the CCAAT box consensus sequence (Lewin, 1983). Second, repeats 1 and 2 form part of the previously identified palindromic estrogen response element (Fig. 3A) (Richard and Zingg, 1990). These overlaps may provide further examples where different regulatory factors modulate expression by binding to common or overlapping sequences (Schule et al., 1990).

At a molecular level, the model system presented in this paper should serve as a useful tool for further investigations of the precise mechanisms of RA receptor/DNA interactions.
and the mechanisms linking this event to transcriptional activation. Moreover, the present data may serve as an impetus for widening our current concepts of RA physiology and point towards a possible role of RA in the modulation of neuropeptide gene expression and brain function.

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