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Transactivation of the proximal promoter of human oxytocin gene by TR4 orphan receptor

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Abstract

The human testicular receptor 4 (TR4) shares structural homology with members of the nuclear receptor superfamily. Some other members of this superfamily were able to regulate the transcriptional activity of the human oxytocin (OXT) promoter by binding to the first DR0 regulatory site. However, little investigation was conducted systematically in the study of the second dDR4 site of OXT proximal promoter, and the relationship between the first and the second sites of OXT promoter. Here, we demonstrated for the first time that TR4 could increase the proximal promoter activity of the human *OXT* gene via DR0, dDR4, and OXT (both DR0 and dDR4) elements, respectively. TR4 might induce OXT gene expression through the OXT element in a dose-dependent manner. However, there is no synergistic effect between DR0 and dDR4 elements during TR4 transactivation. Taken together, these results suggested that TR4 should be one of important regulators of OXT gene expression.

Keywords: Oxytocin; Nuclear receptor superfamily; Orphan receptor; Gene regulation

The function of steroid and thyroid hormones is mediated through the action of specific nuclear receptors [1]. Members of the nuclear receptor superfamily are ligand-dependent transcription factors that regulate the expression of target genes by binding to their hormone response elements (HREs) [2]. Orphan receptors comprise a huge group of nuclear receptor-like proteins whose cognate ligands remain to be identified. The human testicular receptor 4 (TR4) (classified as NR2C2) is an orphan member of the nuclear receptor superfamily [3], that was initially isolated from human prostate and testis cDNA libraries [4]. TR4 directly modulates target gene expression through binding to HRE with consensus AGGTCA direct-repeat (DR). In general, the expression of TR4 transcripts occurs in many mouse tissues. TR4 transcripts were detected as early as embryonic day 9 (E9) in mouse embryos [5]. TR4 transcripts were expressed highly in the central nervous system at E14.5–19.5 in rat [6], and in a stage-dependent and androgen-inductive manner in testis of rat [7]. Study of TR4-knockout (TR4KO) mice further revealed that TR4 might function as a master regulator in many signaling pathways, including female reproductive function and maternal behavior [8].

Oxytocin (OXT) is a multifunctional hormone that mediates uterine contractions, milk ejection, specific behavioral patterns, and natriuresis [9]. This peptide hormone contains nine amino-acid residues and is synthesized *in vivo* as part of a long polypeptide precursor. The *OXT* gene has a characteristic promoter, including overlapping elements for several members of the nuclear receptor superfamily [10]. Basically, there are two regulatory footprints among the proximal OXT promoter, the first site is −164 to −145 upstream from the transcription initiation site, and the second one is −110 to −79 [9]. Estrogen receptors (ERα and ERβ) have been shown to bind to a palindromic estrogen response element (ERE) at

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the first site of the proximal promoter of the human *OXT* gene. This palindromic ERE overlaps with a direct AGGTCA repeat, corresponding to a natural variant of a so-called DR0 (direct-repeat spaced by 0 nucleotide). The second regulatory site contains three copies of AGGTCA repeat spaced by four nucleotides each, corresponding to a double-DR4 (dDR4).

Materials and methods

Plasmid constructions. pCMX-TR4 and pET14b-TR4 were described previously [11,12]. pGL3-OXT-DR0 plasmid containing one copy of the DR0 element was generated by insertion of two annealing primers OXT-DR0U (5′-GATCGGTGACCTTGACCCCG-3′) and OXT-DR0D (5′-GATCCGGGGTCAAGGTCACC-3′) at *Bgl*II site of pGL3-Promoter vector (Promega). pGL3-OXT-dDR4 plasmid consisting of one copy of the dDR4 element was constructed by insertion of two annealing primers OXT-dDR4U (5′-GATCCCTGACCCACGGCGACCCTCTGTGACC AAT-3′) and OXT-dDR4D (5′-GATCATTGGTCACAGAGGGTCG CCGTGGGTCAGG-3′) at *Bgl*II site of pGL3-Promoter. pGL3-OXT plasmid containing one copy of the OXT element (including both DR0 and dDR4 elements) was generated by insertion of two annealing long primers OXT-U (5′-GATCTGACCTTGACCCCGCCTGACCCACG GCGACCCTCTGTGACCA-3′) and OXT-D (5′-GATCTGGTCACA GAGGGTCGCCGTGGGTCAGGCGGGGTCAAGGTCA-3′) at *Bgl*II site of pGL3-Promoter. All plasmids were sequenced by the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). DNA plasmid was purified with the Nucleobond AX-100 Kit (Macherey-Nagel) according to the manufacturer's protocol.

Western blot analysis. TR4 protein was overexpressed from pET14b-TR4-transformed *Escherichia coli* and purified with His-Bind column as previously described [13–15]. Western blot analysis was employed as described previously [16]. Briefly, 1 μg of TR4 protein was separated by SDS–PAGE on a 12% polyacrylamide gel and electroblotted onto a PVDF membrane (Amersham) according to the manufacturer's protocol. After nonspecific blocking, the membrane was treated with rabbit anti-6his antibody (Bethyl), followed by HRP-conjugated goat anti-rabbit Ig antibody (MDBio, Taiwan), and then visualized by an enhanced chemiluminescence detection kit (ECL Plus Western Blotting Detection System, Amersham).

Electrophoretic mobility shift assay (EMSA). This assay was conducted mainly as previously described [17]. TR4 protein was generated from a coupled *in vitro* transcription and translation system as previously described [11,17]. A 42 bp double-stranded oligonucleotide OXT probe was generated by annealing of primers OXT-U and OXT-D and digoxigenin (DIG)-labeled (Roche) according to the manufacturer's protocol.

Transient transfection and dual-luciferase assays. Human lung cancer A549 cells (American Type Culture Collection, CCL-185) were cultured as previously described [14]. Transient transfection and dual-luciferase assays were conducted as previously described [12,18,19].

Results

Evolutionary sequence conservation among promoters of OXT genes

Two regulatory footprints of the human OXT promoter are located between −167 and −149 (the first site, corresponding to nucleotide numbers 215 and 233 of Accession No. M11186, GenBank), and between −105 and −76 (the second site) (Fig. 1A). In the first site, a DR0 element is between nucleotide numbers 220 and 231, overlapping with a palindromic ERE. Consensus sequences of the DR0 element in rat, mouse, and bovine showed 100, 100, and 91.7% homologies with that of human, respectively. In the second site, a dDR4 element contains three copies of AGGTCA consensus motif spaced by four nucleotides each in human (nucleotide numbers 279 and 304) and bovine promoters, while one nucleotide deleted in a space region in rat and mouse promoters. Consensus sequences of dDR4 in rat, mouse, and bovine displayed 88.9, 83.3, and 88.9% homologies with that of human, respectively. These results suggested that OXT elements (including DR0 and dDR4 elements) were evolutionally conserved among these species.

TR4 orphan receptor specifically binds to the human OXT proximal promoter

TR4 was purified from cellular extracts isolated from *E. coli* BL21(DE3) transformed with pET14b-TR4 expressing plasmid. TR4 protein was confirmed on a 12% SDS–PAGE, followed by Western blot analysis (Fig. 1B). TR4 protein corresponding to the expected molecular mass of 67.3 kDa could be detected in both isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cellular lysates (lane 2) and eluent after purification (lane 3), but not in non-induced lysates (lane 1).

A 42 bp double-stranded oligonucleotide OXT probe was generated by annealing two primers OXT-U and OXT-D, and DIG-labeled. TR4 protein was generated from a coupled *in vitro* transcription and translation system with pET14b-TR4 plasmid. A specific DNA–protein complex was revealed when TR4 was incubated with the OXT element probe (Fig. 1C, lane 3). This DNA–protein complex could be abolished in the presence of 100-fold molar excess of unlabeled oligonucleotides (lane 4). Nonspecific DNA–protein complexes appeared at the position right below the specific complex in the mock-translated product (lane 2). These data indicated that TR4 could specifically bind to the OXT element of the human OXT proximal promoter. In addition, TR4 might also form specific DNA–protein complexes with DR0 and dDR4 elements in the EMSA, respectively (data not shown).

TR4 activates OXT gene expression via DR0 and dDR4 elements

To investigate, whether TR4 could regulate the human OXT gene expression through the interaction with proximal promoter, we performed dual-luciferase assay in human A549 cells. Cells were co-transfected with various luciferase reporter plasmids (Fig. 2) in the presence or absence of pCMX-TR4 expression plasmid. In the case of DR0 and dDR4 elements, TR4 was able to activate luciferase reporter gene activities of the pGL3-OXT-DR0 (Fig. 3A) and pGL3-OXT-dDR4 (Fig. 3B) plasmids, respectively. These results suggested that TR4 might induce OXT gene expression through DR0 and dDR4 elements in proximal promoter, respectively.

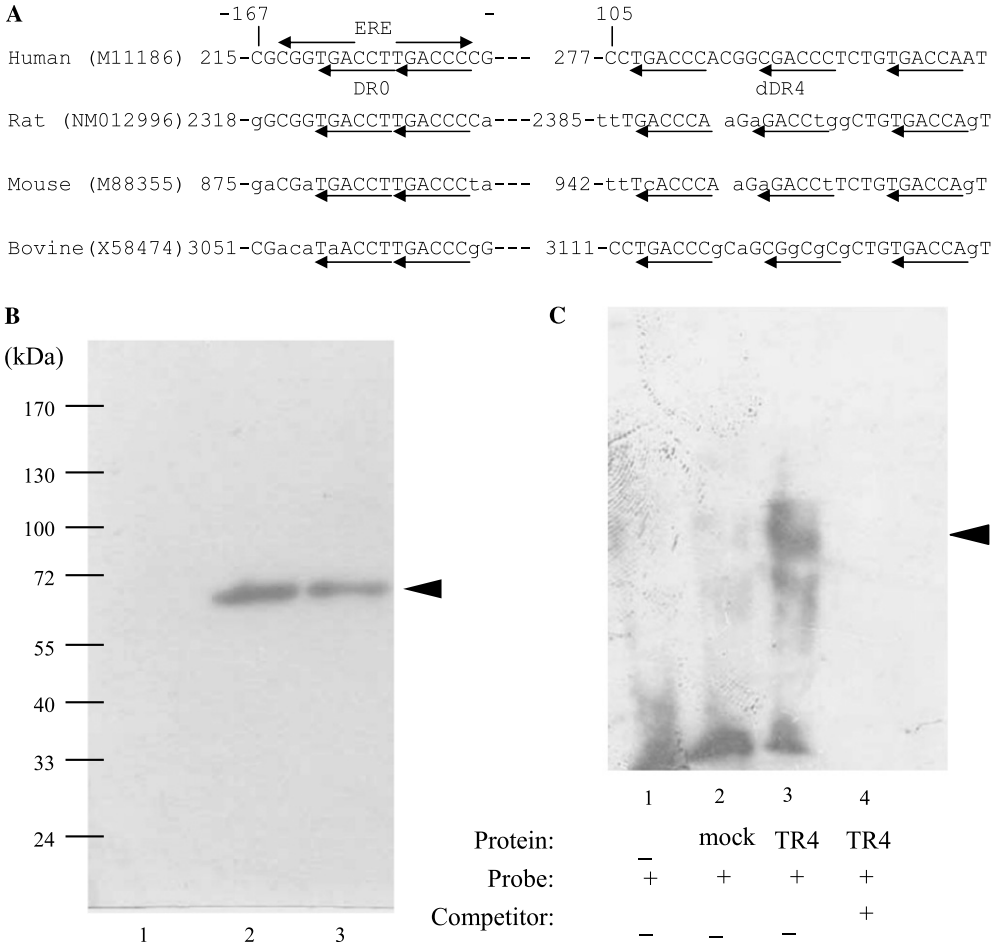

Fig. 1. Interaction between TR4 and OXT promoter. (A) Sequences of the proximal OXT promoters. Identical sequence is shown in capital, but difference from the human sequence is indicated in lower-case. Nucleotide numbers from different species with the Accession Numbers (GenBank) are indicated, while minus numbers denote nucleotides before the transcription initiation site in human. Potential AGGTCA repeats are indicated by arrows under sequences, and an ERE is shown by arrows above sequences. (B) Western blot analysis of TR4. Protein samples were extracted either from non-induced *E. coli* strain BL21 (DE3) (lane 1), IPTG-induced *E. coli* (lane 2), or after purification (lane 3). Standard protein molecular masses are displayed on the left. TR4 protein, corresponding to the expected molecular mass of 67.3 kDa is indicated by an arrowhead. (C) Binding of the *in vitro* expressed TR4 to the OXT element. Lane 1 displays the DIG-labeled OXT probe alone. Binding reaction mixtures were incubated with the probe and either the mock-translated product (lane 2) or the *in vitro* synthesized TR4 (lanes 3 and 4) in the presence of 100-fold molar excesses of unlabeled OXT (lane 4). The retarded complexes are indicated by an arrowhead for specific DNA–protein complexes, whereas nonspecific complexes appear below.

The induction activity of OXT promoter by TR4 in a dose-dependent fashion

To determine any synergistic effect between DR0 and dDR4 elements in the human OXT proximal promoter, A549 cells were transiently co-transfected with either pGL3-Promoter or pGL3-OXT reporter plasmid in the presence or absence of pCMX-TR4 plasmid. In the case of the OXT element, TR4 could regularly activate luciferase reporter gene activity of the pGL3-OXT plasmid (Fig. 4A). These data indicated that TR4 might induce OXT gene expression through the OXT element without synergistic effect between DR0 and dDR4 elements.

To further reveal this transactivation of OXT proximal promoter by TR4, cells were co-transfected with pGL3-OXT reporter plasmid and increasing amounts of pCMX-TR4 plasmid (Fig. 4B). Results showed that TR4 could trigger the luciferase reporter activity via OXT proximal promoter in a dose-dependent manner.

Discussion

In recent years, several reports have focused on the study of the multiple HREs at the first site of the proximal promoters of human or rat *OXT* genes. These studies revealed that this multiple HRE is responsive to estrogen, thyroid hormone, or retinoids [10]. In addition, previous study indicated that two orphan receptors, steroidogenic factor 1 (SF-1) and chicken ovalbumin upstream promoter transcription factor (COUP-TF), preferentially bound to this DR0 element in the bovine OXT promoter [20]. These two orphan receptors appeared to compete for binding to this DR0 element, since SF-1 was associated with OXT gene activation and COUP-TF with its repression. Subsequently,

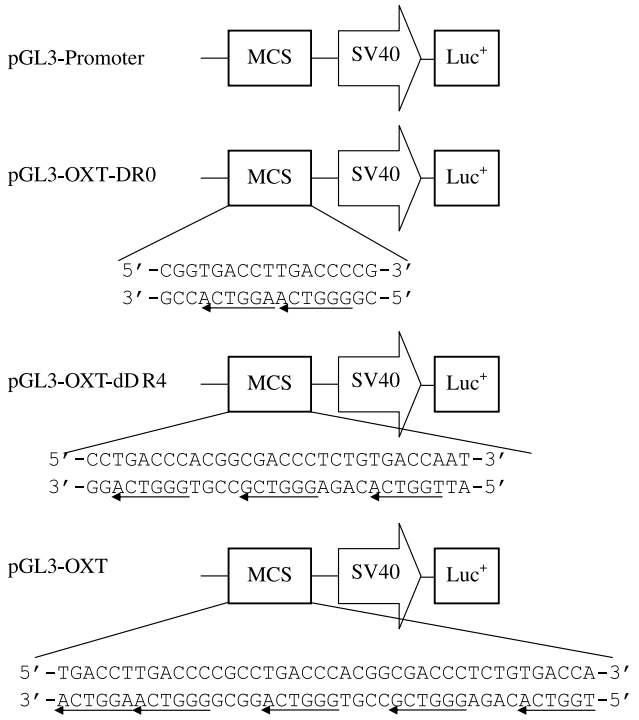

Fig. 2. Schematic structure of reporter gene plasmids. pGL3-OXT-DR0, pGL3-OXT-dDR4, and pGL3-OXT plasmids contain one copy of DR0, dDR4, and the full-length regulatory OXT elements in the sense orientation at the multiple cloning site (MCS) in front of the simian virus 40 (SV40) promoter and the modified coding region of luciferase reporter gene (Luc^{+}) in the parent pGL3-Promoter vector, respectively.

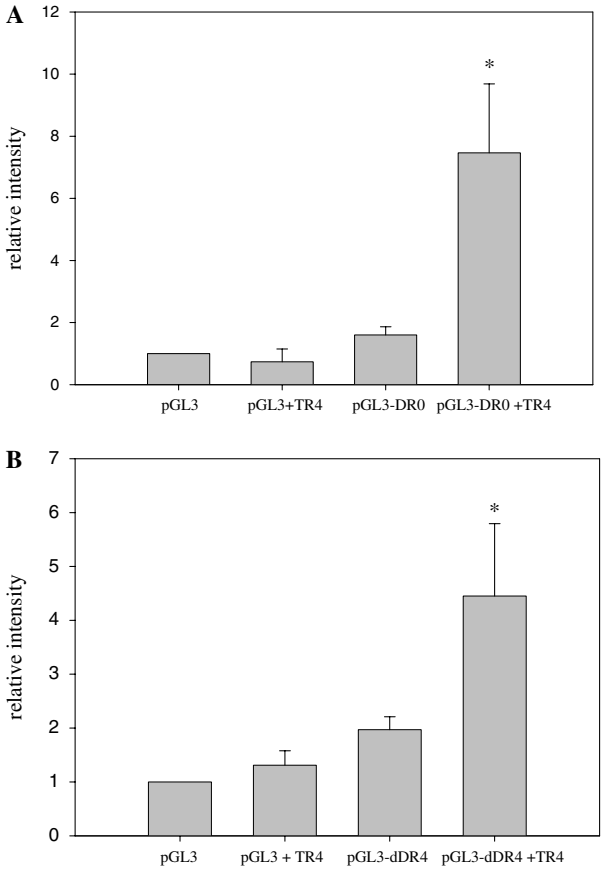

Fig. 3. TR4 induction of luciferase reporter gene activity of the OXT proximal promoter via DR0 and dDR4 elements, respectively. (A) Functional assay with pGL3-OXT-DR0 plasmid. (B) Functional assay with pGL3-OXT-dDR4 plasmid. Human A549 cells were transiently co-transfected with different pGL3 reporter plasmids (2 μg of each) and 1 μg phRL-TK internal control plasmid in the presence or absence of 2.5 μg pCMX-TR4 expressing plasmid. All firefly luciferase activities were normalized with *Renilla* luciferase activities. Data represent means ± SD of three independent experiments performed in duplicate. Significant difference ($P < 0.05$) between control and experimental groups is marked with an asterisk.

two COUP members (COUP-TFII and Ear-2) were identified to suppress basal OXT promoter activity via DR0 and dDR4 elements [9]. Especially, the dDR4 element was essential for this silencing effect. The specific binding preference to the DR0 element was in the order of COUP-TF = TRα > SF-1 > RAR > ER [10]. Nevertheless, the complex formed between the breast cell nuclear extracts and the DR0 element exposed that an unknown transcription factor remained to be identified. This factor was none of identified nuclear receptors, but was likely to be another member of the nuclear receptor superfamily [10].

In the present study, we found that TR4 could directly bind to DR0, dDR4, and OXT elements, respectively. The TR4-DR0 binding complex could be abolished in the presence of 100-fold molar excesses of unlabeled oligonucleotides, while the TR4-dDR4 complex might be impaired in the presence of only 30-fold molar excesses of competitors (data not shown). These data suggested that the DR0 element could be essential for mediating TR4 transactivation among two regulatory sites. That is probably true due to the consequence that TR4 possesses higher affinity to the DR0 element than the dDR4 element. Characterization of the binding preference of TR4 to synthetic DRs indicated in the order of DR6 > DR5 > DR1 > DR3 > DR2 > DR4 [12]. It is interesting to note that the dDR4 element of the human OXT promoter may represent another type of two direct-repeats spaced by 14 nucleotides apart (DR14). The medium-chain ethyl coenzyme A dehydrogenase gene promoter contains a complex regulatory element with four hexamer binding sites of nuclear receptors [21]. This regulatory element previously conferred transcriptional regulation by ARA/RXR and HNF-4 orphan receptor. COUP family homodimers were able to interact and function with an everted repeat with the same spacing of 14 nucleotides (ER14) of this gene promoter.

OXT is produced in neurons of the paraventricular and supraoptic nuclei of the hypothalamus, as well as in specific tissues and cell types peripherally [22]. Physiological functions of OXT have been well studied, and this peptide hormone is known to affect both central and peripheral systems, as well as behavior. In order to prove OXT might be a TR4 target gene, more direct physiological evidence may come from the study of TR4KO mice [8]. Results demonstrated high rates of early postnatal mortality and significant growth retardation in TR4KO mice. TR4KO females further showed defects in reproduction and maternal behavior. Especially, the reduced OXT expression in the

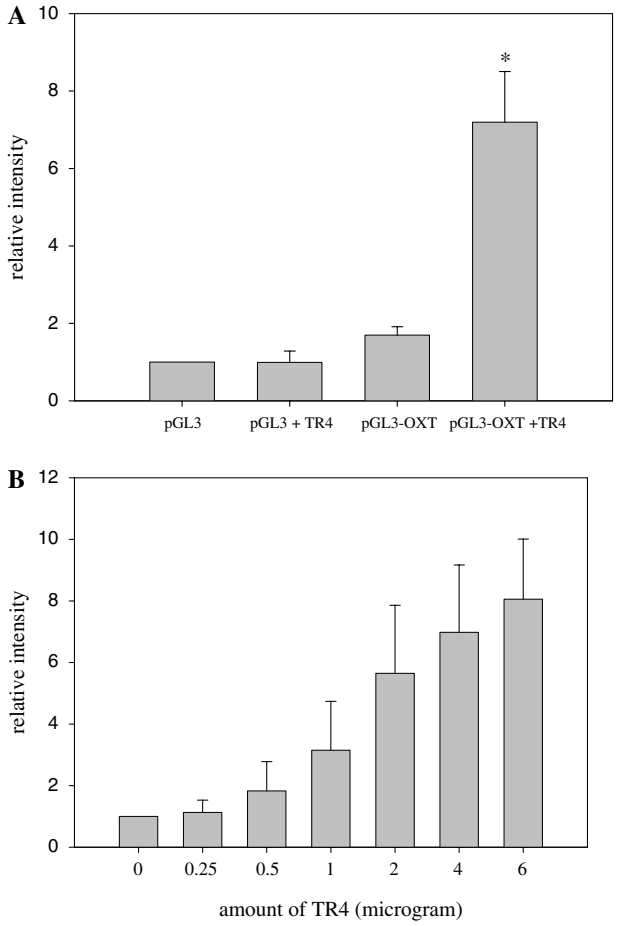

Fig. 4. TR4 Induction of luciferase reporter gene activity of the OXT proximal promoter via the OXT element in a dose-dependent manner. (A) Functional assay with pGL3-OXT plasmid. (B) Functional assay of dosage effect with pGL3-OXT plasmid. A549 cells were co-transfected with 2 μg pGL3-OXT reporter plasmid and 1 μg phRL-TK internal control plasmid in the presence of serially increasing amounts of pCMX-TR4 (0.25–6 μg) expressing plasmid.

hypothalamic regions of TR4KO mice [8] suggested that the defect in regulatory levels of this peptide hormone, resulting from the disruption of TR4 expression.

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