Identification of a Novel DNA Binding Site for Nuclear Orphan Receptor OR1*

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The nuclear orphan receptor OR1 has been shown to bind as a heterodimer with retinoid X receptor (RXR) to direct repeat 4 (DR4) response elements. It remained unclear, however, whether this represents the only or the optimal binding site for this receptor. Therefore, we performed a DNA binding site selection assay that allows the identification of novel DNA binding sites for OR1 in an unbiased manner. While OR1 alone was not able to select a specific sequence from the pool of oligonucleotides, the OR1/RXR heterodimer selected a highly conserved DR1 element, termed DR1s, with two AGGTCA motifs spaced by one adenosine. The functional activity of the consensus binding site was verified in transient transfection assays and corroborated by in vitro studies. Based on the sequence of the consensus DR1s, we located putative natural binding sites in the 5′-promoter flanking regions of the rat S14 gene and the rat cholecystokinin type A receptor gene. Furthermore, we could show that although the OR1/RXR heterodimer has a distinct binding orientation on a DR4 element, it is able to bind in both orientations to the DR1s element. The OR1 paralog LXRα does not bind as a heterodimer with RXR to the DR1s element, indicating that these receptors, despite their homology, are involved in the regulation of different sets of genes.

Nuclear receptors constitute a superfamily of ligand-activated transcription factors that link extracellular signals directly to transcriptional responses and are involved in the regulation of many diverse processes, including development, homeostasis, differentiation, and oncogenesis (reviewed in Refs. 1–4). Important members of this family are the steroid hormone receptors, the thyroid hormone receptor (TR),1 the retinoic acid receptor (RAR), the retinoid X receptor (RXR), the vitamin D receptor (VDR), and an increasing number of orphan receptors for which a ligand has not yet been identified. All members of this family share a common modular structure consisting of four domains (5). A highly conserved DNA binding domain (DBD) forms a two zinc finger structure, which is involved in specific DNA binding and protein-protein interactions (6, 7). The C-terminal ligand binding domain, which is moderately conserved among the members of the family, is involved in ligand binding and receptor dimerization and contains transactivation and/or repression functions (8–10). The N-terminal parts of these proteins vary highly both in length and sequence and often contain a transactivation domain (11, 12). The function of the hinge region that separates the DNA binding and the ligand binding domain is not yet fully understood but might involve interactions with regulatory proteins (13).

Nuclear receptors control transcription of their target genes by interacting with specific DNA sequences termed hormone response elements (6, 14). All nuclear receptors recognize a minimal 6-base pair sequence (AGGTCA or AGAACA) referred to as a consensus half site motif. The primary determinant to discriminate between these two motifs is the P-box (15), a 6-amino acid sequence at the C-terminal base of the first zinc finger. To achieve high affinity binding and specificity for target genes, most receptors bind as dimers to DNA. The steroid hormone receptors interact as homodimers with their cognate DNA binding sites, usually palindromic repeats spaced by 3 nucleotides. In contrast, members of the thyroid/retinoid receptor subgroup bind most efficiently as heterodimers with RXR to direct or inverted repeat elements of the AGGTCA type. Both spacing of the half sites and surrounding nucleotides are crucial determinants for the specificity of binding. Thus, elements spaced by 1–5 nucleotides are bound by different subsets of heterodimers (16–18). Since direct repeats are asymmetric elements, binding of RXR heterodimers occurs in an asymmetric arrangement in which RXR usually occupies the 5′-half site (19, 20). Interestingly, this polarity of binding is reversed in heterodimers binding to DR1 elements (21, 22).

Some receptors that bind as monomers to DNA display an increased binding affinity for a single half site caused by an C-terminal extension, also referred to as A-box, at the end of the second conserved zinc finger. This motif contacts base pairs 5′ of the upstream half site, usually an A/T rich sequence, which thus can form part of the recognition sequence (23, 24). Another subgroup of receptors is able to bind as homodimers to direct or inverted repeats (25–27). RXR binds to direct repeats spaced by 1 base pair utilizing a third helix, also known as the T-box, that is located immediately downstream of the second zinc finger (28). Several receptors are able to bind to more than one DNA binding site and in variable configurations, often displaying differential activation properties with the differential binding behavior (18, 20, 26, 29, 30).

The orphan receptor OR1 (also referred to as LXRβ, RIP15, UR, and NER (31–34)) and its paralog LXRs (also referred to as RLD-1 (35, 36)) have been isolated on the basis of their homol-
ology to sequences in the DNA binding domain of known receptors. In situ hybridization on fetal and adult rat tissues revealed an almost ubiquitous but differentially regulated expression of OR1 (31, 37) indicative of a general role for this receptor. In contrast, LXRα shows a more restricted expression pattern, with the highest expression levels in metabolically active organs, such as liver, intestine, and adrenals (35, 36). An important step toward the understanding of the physiological functions of OR1 and LXRα was the observation that both receptors are activated by various oxysterols (38, 39). These compounds have important functions in cholesterol homeostasis and constitute precursors in the biosynthesis of steroid hormones and bile acids (40–42). These findings led to the suggestion that at least LXRα acts as a sensor for cholesterol derivatives and directly connects cholesterol homeostasis and gene regulation (38, 39). This hypothesis has recently been corroborated in a study on LRα-deficient mice, which were no longer able to dispose excess cholesterol from the diet via bile acid excretion (43). The rationale given for these results was the failure of LRα mediated induction of the 7α hydroxylase (CYP7A) gene, which encodes the enzyme responsible for the rate-limiting step in the conversion of cholesterol to bile acids (39, 43).

The P-box sequence of both OR1 and LRα identifies them as members of the TR/RAR subgroup of nuclear receptors. The receptors have been shown to form heterodimers with RXR on AGGTCA half sites spaced by 4 nucleotides, but also by 2 or 5 nucleotides, in gel shift analysis (31–34, 44). Interestingly, both OR1/RXR and LRα/RXR heterodimers confer not only constitutive activation on a DR4 element but are also inducible by the respective ligands, oxysterols, and 9-cis-retinoic acid, the ligand for RXR, enabling these receptors to simultaneously respond to a whole subset of different signals (31, 32, 44, 45).

In an effort to identify new target genes for OR1, we decided to determine the optimal DNA binding site for OR1 without the bias introduced by known or consensus elements. Therefore we used a selection and amplification technique that allows identification of high affinity binding sites from a pool of random oligonucleotides. Interestingly, we found that the OR1/RXR heterodimer preferentially binds to a DR1 type element, termed DR1s, and provide evidence that the selected consensus sequence is able to mediate transactivation of a reporter gene upon coexpression of the respective receptors. A data base search based on the selected DNA sequence located putative response elements in the 5′-promoter flanking regions of several genes. Oligonucleotides containing these potential targets efficiently compete with binding of the OR1/RXR heterodimer to the selected DR1s. Moreover, we report the first example of an RXR-containing heterodimer that is able to bind in both orientations to a direct repeat. These results suggest that OR1 might play a central role in multiple hormonal signaling pathways by responding to various signals and binding to different subsets of genes.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

**Plasmids for Overexpression in Escherichia coli and in Vitro Translation—**His_{6}-tagged proteins were expressed in the pET3a vector (Novagen) modified as follows: pET3a-OR1 expresses full-length OR1 fused to the amino acid sequence MHHHHHHEIHR, including 6 histidines and a factor Xa cleavage site. The vector pET3a-ΔNOR1 is an analogous construct that lacks codons 7–74 of OR1. Full-length RXR (pET3a-RXR) is preceded by the same sequence as in pET3a-OR1, and the analogous construct pET3a-ΔNRXR was obtained by deleting codons 11–111 of RXR. Vector sequences obtained by PCR were confirmed by DNA sequencing or replaced by template material. The same plasmids were used for in vitro transcription/translation from the T7 promoter in the pET3a-vector.

**Vectors for Transfection Studies—**2DR1s-thymidine kinase (TK)-LUC contains two copies of the sequence TAAAGGTCAAGGTGCAAGT spaced by 5 nucleotides in front of the TK-LUC vector. This reporter vector is a derivative of pGL3-Basic (Promega) in which a BglII/HindIII fragment of the TK promoter has been inserted. For expression of the receptors in CHO-K1 cells, DNA fragments encoding full-length proteins were subcloned in the multiple-vector (kindly provided by D. Mangelsdorf) under control of the CMV promoter. The empty vector was used as a control. pCMV5-5RC1 was derived from a gift of B. W. O’Malley and as described by Onate et al. (46) but in vector pCMV5.

**Protein Expression and Purification**

_E. coli_ strain BL21(DE3)/pLYS8, freshly transformed with one of the expression vectors, was grown at 30 °C in M9 minimal medium supplemented with 0.6% casamino acids (1 mg/ml ammonium chloride, 72 μg/ml ampicillin) and 200 mM imidazole in Buffer A. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis; pure fractions were combined, and the protein was precipitated with ammonium sulfate, dialyzed against 20 mM Tris-HCl, pH 8.0, 10%, glycerol, 1 mM phenylmethylsulfonyl fluoride, and stored at −80 °C.

_In vitro_ transcription and translation was performed with the TNT-T7 rabbit reticulocyte lysate kit (Promega, Madison, WI) as recommended by the manufacturer. The integrity of all proteins was analyzed by SDS-polyacrylamide gel electrophoresis using [35S]methionine in the protocol.

**Binding Site Selection Assay**

The amplification and binding selection method was modified as described by Alex et al. (Ref. 47 and references therein).

The oligonucleotide pools N20 or 2N10 consist of the following sequences: GGCTACGGAATTGGTCGTACAGGATGTGAC or GGCTAGCGGAATTGGTCGTACAGGATGTGAC. The flanking sequences contain an EcoRI and BamHI restriction site used for subcloning and primer binding sites for PCR amplification. Single stranded oligonucleotides were made double strand by PCR. 2 ng of either pool of oligonucleotides was incubated with 200 ng of total protein in a binding reaction mix containing 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 50% glycerol, 100 ng poly(dI-dC)-poly(dI-dC), 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. After 20 min of incubation at room temperature, the protein volume was increased to 100 μl, and the mixture was filtered through cellulose nitrate filters (0.45-μm pore size, Schleicher & Schuell). Filters were washed five times with 200 μl of binding buffer, dried, and boiled in 300 μl of water to elute bound oligonucleotides. 30 μl of this solution was used as a template for PCR amplification and approximately 2 ng of the amplified product was used for the next round of selection. After six rounds of filter binding and PCR amplification the selected oligonucleotides were labeled in a standard PCR containing 5 ng of template and [α-32P]dCTP and subsequently used as a probe in electrophoretic mobility shift assays (EMSA). The observed retarded DNA-protein complexes were cut out of the dried gel, and the DNA was eluted overnight in elution buffer (300 μM NaCl, 30 mM Tris-HCl, pH 8.0, 3 mM EDTA, pH 8.0). The eluted DNA was reamplified by PCR and either subcloned directly in a T-vector (pCR™ 2.1, Invitrogen) or cut with EcoRI/BamHI prior to subcloning in pBluescript SK (Strategene). The subcloned oligonucleotides were then sequenced with the dyeoxy chain termination method using the T7 sequencing kit from Amersham Pharmacia Biotech.

**Electrophoretic Mobility Shift Assay (EMSA)**

Synthetic oligonucleotide probes for EMSAs were end-labeled with [α-32P]dCTP by Klenow enzyme and purified from a 10% nondenaturing 5% polyacrylamide gel. Approximately 5 pmol (0–200 fmol) of the probe was used in a binding reaction with 2–5 μl of programmed reticulocyte lysate in a buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% glycerol, 100 ng poly(dI-dC)-poly(dI-dC) and 100 ng of single strand DNA as unspecific competitor, 1 mM dithiothreitol, 0.1% bovine serum albumin, and specific competitor or antibodies as indicated. The reactions were incubated for 20 min at room temperature and subsequently...
run on a 4.5% nondenaturing polyacrylamide gel in 1× TBE (Tris-borate-EDTA) at 4 °C.

**RESULTS**

**Selection of OR1 Binding Sites**—To select specific binding sites for the nuclear receptor OR1 in an unbiased way, we chose a strategy that combined several rounds of selection via filter binding and subsequent amplification via PCR with a final selection step using EMSA (Fig. 1 and see under “Experimental Procedures”). It had already been established that OR1 binds as a heterodimer with RXR to DR4, like response elements that contain the consensus core motif AGGTCA. On this basis, we screened a mixture of oligonucleotides that contained one AGGTCA core motif flanked by 10 random nucleotides on each side (2N10), as well as a pool of oligonucleotides containing 20 random nucleotides (N20), giving room for two half sites spaced by, at most, 6 nucleotides.

For the selection, we used modified proteins for the following reasons: full-length RXR expressed in *E. coli* is unstable and often results in specific degradation products due to degradation signals in the amino terminus of the protein (48). Similar problems arose when we expressed full-length OR1 in *E. coli*. Therefore, we constructed expression vectors for both proteins that lack the corresponding sequences in the N terminus, resulting in more stable and soluble products (ΔNOR1 and ΔNRXR, Fig. 1B).

In EMSAs, we could detect a specific complex already after two rounds of subsequent filter binding and PCR amplification using the pool of 2N10 oligonucleotides that contain one AGGTCA motif (Fig. 2, lanes 4–6). This complex increased in strength in the following selection cycles, whereas a second, unspecific complex diminished with every round of selection. A similar specific complex was also observed using the pool of N20 oligonucleotides bearing a random sequence, although its appearance was delayed by two rounds of selection (data not shown). After six rounds of selection, a strong complex was formed in EMSAs (Fig. 2, lanes 7–9) from which the DNA was recovered by elution and then subcloned and sequenced as described under “Experimental Procedures.”

**Sequence Analysis of Selected Sites**—We sequenced 50 and...
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30 subcloned binding sites derived from the pool of AGGTCAG-containing and random oligonucleotides, respectively. Only independent, unique clones, as judged by the unselected and thus random sequences surrounding the binding site, were further analyzed. About 20% of the sequenced 2N10 oligonucleotides did not contain any recognizable binding site, three contained a DR4-like element, and two contained three half sites spaced by 1 and 2 base pairs, respectively. The majority of these sequences, however, contained a well conserved direct repeat spaced by one nucleotide. Alignment of the DR1 like binding sites allowed us to derive a consensus OR1/RXR binding element, from the frequency of A, C, G, and T at each position (Fig. 3). Variation from the consensus occurred basically only in the first nucleotide of the 5’ binding site, where adenosine was replaced by guanosine in about 30% of the cases. The 1-nucleotide spacer is almost exclusively an adenosine, indicating the functional importance of this nucleotide. The surrounding sequence is in some positions (data not shown).

The DR1 Elements Are Binding Sites for the OR1/RXR Heterodimer—As the selected binding site resembles the DR1 type response element of RXR homodimers, we examined whether the sequence was in fact selected by the OR1/RXR heterodimer and not by RXR homodimers. For that purpose, we used different size receptor constructs that allowed us to discriminate between the different dimers. As shown in Fig. 4, OR1 and RXR formed a strong complex with the selected oligonucleotides regardless of which protein combination was used: full-length (lane 2), ΔN constructs (lane 5), or a mixture of both types of proteins (lanes 3 and 4). Whereas OR1 alone was unable to bind to the selected sequences (lanes 6 and 8), both RXR and ΔNRXR were able to form homodimers with these elements (lanes 7 and 9). Both complexes show a different migration behavior in the gel, distinguishing them unequivocally from the heterodimeric complexes. However, weak homodimeric complexes are also visible above and below the respective heterodimeric complexes (lanes 2 and 4).

OR1 Efficiently Inhibits RXR Homodimer Formation on the DR1s—Based on the sequence analysis, we synthesized the consensus DR1s element TAAAGGTCAAAGGTCAACG and analyzed its binding to the OR1/RXR heterodimer in EMSAs. As expected, the heterodimer formed a strong complex with the synthesized oligonucleotide (Fig. 4B, lane 1), but RXR no longer formed a homodimeric complex in the presence of OR1. The presence of both proteins in the complex was verified by supershifts using a polyclonal antibody directed against OR1 and a monoclonal antibody directed against RXR. A third antibody, directed against the glucocorticoid receptor, was used as an unspecific control and did not affect migration of the complex.

To analyze whether RXR was still able to bind to the derived consensus oligonucleotide as a homodimer, we performed an EMSA with RXR alone and with increasing amounts of OR1. Fig. 5 shows that RXR formed a weak complex in the absence of OR1 (lane 1). However, RXR homodimerization was abolished in the presence of only small amounts of OR1, and heterodimers were formed instead. In the presence of equimolar amounts of protein (Fig. 5, lane 4) RXR homodimers were no longer visible. This observation was in contrast to the results obtained with the originally selected oligonucleotides, indicating that most could contain a mixture of binding sites selected by both the heterodimer and the RXR homodimer.

Mutational Analysis and Native DR1s Elements—To define more precisely the requirements for high affinity binding, we asked the question of whether an exchange of the spacer nucleotide would be tolerated, considering that the main characteristic of the selected binding site is its high conservation of a consensus DR1. For that purpose, we synthesized DR1 elements with the spacer nucleotide consisting of any one of all four nucleotides and tested their ability to compete with the selected binding site for binding to the OR1/RXR heterodimer (Fig. 6, lanes 1–5). Using equal amounts of competitor in each lane, it is evident that by exchanging the spacer nucleotide the ability of these oligonucleotides to compete with the DR1s was greatly diminished. Thus, the A as a spacer is important for high affinity binding.

Competition experiments with naturally occurring DR1 elements that serve as binding sites for e.g. RXR homodimers or PPAR/RXR heterodimers and often are composed of less well conserved half sites indicated that exchanges in the half sites reduce the ability to compete for OR1/RXR binding (data not shown). Therefore, we performed a data base search to identify natural promoter elements that match the consensus precisely and found several candidates. We chose two interesting examples: one was found in the 5’-upstream region of the rat cholecystokinin type A receptor gene (cckar) in the vicinity of additional hormone regulatory elements, and the other one was present in the 5’-upstream region of the rat S14 protein gene. The putative binding sites were synthesized and analyzed for OR1/RXR binding in EMSA. As shown in Fig. 6, the cckar and S14 oligonucleotides could compete with binding to the labeled.
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Figure 4. Evidence for a heterodimeric complex formed on the pool of selected binding sites and the derived consensus sequence. A, the presence of both OR1 and RXR in the observed complex was confirmed by using constructs that yielded complexes of different size in EMSAs. All combinations of OR1 and RXR (lanes 2, full-length proteins; lanes 3 and 4, a mixture of full-length and ΔN-proteins; lane 5, ΔN-proteins) formed a complex with the labeled pool of selected oligonucleotides. Neither of the OR1 proteins was able to bind on its own (lane 6 and 8), whereas both RXR proteins formed a weak homodimeric complex (lanes 7 and 9). B, OR1 and RXR formed a strong complex on the labeled DR1s consensus sequence (lane 1) that was upshifted by an antibody specific for OR1 (lane 2) or an antibody specific for RXR (lane 3), but not by an unrelated antibody directed against the glucocorticoid receptor (lane 4).

Figure 5. OR1 inhibits homodimerization of RXR on the DR1s. Gel shift analysis with His6-OR1 and His6-RXR bound to the labeled DR1s oligonucleotide. Each lane contained the same amount of total protein. Lane 1 contained 100% RXR, and lanes 2–6 contained increasing amounts of OR1 protein and decreasing amounts of RXR protein, with equal amounts of both proteins in lane 4. Lane 7 contained 100% OR1. RXR was able to form a homodimeric complex on the DR1s, but the presence of even small amounts of OR1 inhibited homodimer formation.

The DR1s is a Functional Element in Transfection Assays—To analyze whether the selected DR1s element represents not only the most effective binding site for the OR1/RXR heterodimer but also a functional element that confers transactivation, we subcloned two copies of the element in front of a luciferase reporter construct driven by the TK promoter (2DR1s-TK-LUC). The reporter gene and receptor expression vectors were cotransfected into CHO K1 cells and assayed for luciferase activity (Fig. 7). No increase in reporter gene activity was measured due to insertion of the DR1s elements alone when compared with the TK-LUC reporter gene (Fig. 7A). Cotransfection of OR1, however, led to a significant induction (7–8-fold) of luciferase activity, possibly due to the presence of endogenous RXR, that was even exceeded when exogenous RXR was added: cotransfection of both OR1 and RXR expression vectors led to a 17-fold induction of luciferase activity in the absence of ligand (Fig. 7A). Cotransfection of RXR alone likewise resulted in an induction of the reporter gene activity (5-fold). In contrast, the peroxisome proliferator-activated receptor PPAR was not able to mediate activation of the reporter through the DR1s elements in the absence of ligand (Fig. 7A).

In addition, OR1 was not able to induce activation of a reporter gene containing a PPAR-responsive element (aox, see Fig. 6) instead of the DR1s (data not shown).

The constitutive activation property of the OR1/RXR heterodimer has already been established on a DR4 element and is thus confirmed for the DR1s. We next asked the question of whether the addition of activators for OR1 (22(R))-hydroxycholesterol) or RXR (9-cis-retinoic acid) could increase the observed constitutive activation. When OR1 was cotransfected alone, no significant increase in reporter gene activity was observed when either of the activators was added (Fig. 7B, left). In contrast, expression of RXR alone induced luciferase activity 5-fold when its cognate ligand 9-cis-retinoic acid was added but remained unaffected by addition of 22(R)-hydroxycholesterol (Fig. 7B, middle). However, the high constitutive activation that was observed when both receptors were cotransfected was only moderately affected by either compound (Fig. 7B, right).

The observed activation pattern was supported by EMSAs, including the coactivator SRC1, which has been identified as a mediator of transactivation by both OR1 and RXR (Fig. 7C). Whereas the DR1s bound heterodimer interacted with SRC1 in the absence as well as in the presence of ligand (Fig. 7C, lanes 1).
interaction with the RXR homodimer basically occurred only in the presence of its ligand 9-cis-retinoic acid (Fig. 7C, lanes 4–6).

**Polarity of Binding**—Previous studies have shown that the OR1 paralog LXRα is able to bind to a DR4 element as a heterodimer with RXR and that in this configuration RXR...
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In this study, we characterized in an unbiased manner the optimal consensus binding site for OR1 by an EMSA/PCR based strategy that selects oligonucleotides with highest affinity for OR1 from a pool of degenerate oligonucleotides. OR1 alone was not able to select a binding site with this approach, indicating that the protein is not able to bind DNA as a monomer or homodimer. Because the selection was carried out in the absence of cognate ligands, the possibility remains that ligand-activated receptor might have changed DNA binding properties. However, none of our data, including EMSAs in the presence of the activator 22(R)-hydroxycholesterol, provide evidence in favor of such an assumption (data not shown).

The majority of oligonucleotides selected by the OR1/RXR heterodimer in this approach contained a direct repeat of the sequence AGGTCA spaced by one nucleotide. This result was somewhat unexpected, because so far, mainly DR4 elements have been described for the receptor dimer. Therefore, we performed competition studies with both binding sites, in which we demonstrated that a DR4 oligonucleotide was able to compete with the DR1s for OR1/RXR binding but obviously with lower affinity. A possible reason why DR1 elements have not been positively identified as targets for the heterodimer might be the fact that all previous studies used DR1 elements spaced by C or G but not A (31–34). We could show that these elements display a significantly lower binding affinity when compared with the DR1s.

The DR1s selected in this study was preferentially a target site for OR1/RXR heterodimers and not RXR homodimers, as confirmed by EMSAs employing differently sized receptor proteins or specific antibodies to either receptor. In a similar study, performed with RXRα alone, Castelein et al. (49) found, in addition to DR2 and PALO elements, several DR1 elements as binding sites for the RXR homodimer. The consensus sequence selected in that study resembles the DR1s in so far that it contains an A as a spacer and two rather conserved half sites. However, the 5′-half site was more often a GG/GCTCA sequence and also the first upstream position next to this half site showed a clear preference for G and not A as in the DR1s, indicating that the binding specificities of OR1/RXR heterodimers and RXR homodimers are subtly distinct.

DR1 elements have been described as DNA binding targets not only for RXR homodimers, but also for RXR heterodimers with RAR, PPAR, COUP-TF, and TR and homodimers of HNF4 and COUP-TF (25, 30, 50–54). As OR1 also some of these receptors bind to more than one DNA binding site, thereby often displaying differential activation properties. Regulation of gene expression by RXR heterodimers at promoters containing DR1 elements provides a paradigm for positive and negative transcriptional control by nuclear receptors. The response to regulating ligands is dependent upon the RXR partner, the DNA binding site and its context. Therefore, it is necessary to carefully analyze not only the exact binding specificities/requirements of each heterodimer and the RXR homodimer but also the activation properties of the different dimers on the respective element.

Competition experiments revealed that the OR1/RXR heterodimer has a very restricted binding behavior and tolerates only few exchanges in the consensus DR1s. Since the majority of natural response elements for nuclear receptors described so far are composed of half sites that differ from the consensus, we performed a database search to find putative natural targets for the OR1/RXR heterodimer. Two candidates displaying highest homology to the consensus were analyzed in EMSAs and identified as strong competitors for DR1s binding. One binding site was derived from the 5′-upstream region of the rat chole-

Fig. 8. Polarity of the OR1/RXR heterodimer on DNA. The orientation of the OR1/RXR heterodimer when bound to a DR4 or to the DR1s element was assessed with the help of an OR1 construct in which the amino acid sequence of the P-box in the DBD was mutated to that of a glucocorticoid receptor P-box (upper box). An altered specificity mutant that recognizes GRE half sites was incubated together with RXR on labeled binding sites in which either the 5′- or the 3′-half site was changed to the binding site of the glucocorticoid receptor P-box (Fig. 8, upper right box). Thus, this receptor has gained an altered DNA binding specificity and will recognize an AGAACA half-site instead. Corresponding oligonucleotides, designated D4G and G4D (or D1G and G1D, respectively) were synthesized in which either the 5′- or the 3′-half site was changed to the binding site of the glucocorticoid receptor (Fig. 8, lower right box). EMSAs with these oligonucleotides as labeled probes showed that the mutated OR1 protein as a heterodimer with wild type RXR was able to bind only to the D4G element on which RXR is allowed to bind to the 5′-position, and OR1 consequently binds to the 3′-position, but not to the G4D element (Fig. 8, lanes 1 and 2). In contrast, on the binding sites spaced by one nucleotide, the heterodimer was able to bind in both orientations because a complex was formed with both the D1G and the G1D oligonucleotide (Fig. 8, lanes 3 and 4). A somewhat higher affinity for the D1G was observed when nonsaturating amounts of protein were used (Fig. 8, lanes 5 and 6). Heterodimer formation is a prerequisite for binding to these artificial elements because we could not detect any complex formation when using mutated OR1 or wild type RXR individually (data not shown). To our knowledge, this is the first reported example of a receptor occupying the 5′-position in a heterodimer with RXR formed on a DR1 element, in which orientation of binding usually is reversed.
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cysteokinin type A receptor gene (ckcar) (55). The cholecystokinin family of peptide hormones was originally isolated from the mammalian gastrointestinal tract and subsequently also discovered in brain. The cck type A receptor (CCKAR) mediates the physiological functions of these peptide hormones that, among other functions, play a significant role in the nervous system control of satiety (56–58). The presence of an OR1/RXR binding site in this gene and colocalization of CCKAR and OR1 in many tissues, especially in brain, as well as its involvement in feeding disorders render this gene a likely target for OR1 action. In this context, it is also interesting to note that a putative ligand for OR1, 24(S)-hydroxycholesterol, is present in high concentrations in brain and has therefore been called “cerebrosterol” (41).

A second putative target was localized in the 5'-upstream region of the rat S14 gene in near proximity to several hypersensitive sites and a thyroid hormone (T3)-responsive element (58). S14 is a nuclear protein that is mainly expressed in liver and adipose tissue and its expression is regulated by polyunsaturated fatty acids, hormones and carbohydrates (59, 60). It is thought to have a significant role in lipid metabolism in liver based on its tissue distribution and correlation with lipogenesis. Interestingly, PPARs are not involved in the fatty acid regulation of this gene (61). The presence of both a conserved DR1s element and a thyroid hormone-responsive element, which is a DR4 type element, might render this gene a dual target for the OR1/RXR heterodimer. Extended studies on these genes might help to reveal a functional connection between food intake, lipid metabolism, and cholesterol homeostasis.

On most direct repeat elements studied so far, heterodimerized RXR occupies the 5'-position of a given binding site. RXR acts either as a silent, not ligand-induced partner, when heterodimerized with RAR, VDR, or TR, or as an active, ligand-responsive receptor, as exemplified by the heterodimer LXRα/RXR (reviewed in Ref. 4). This polarity is reversed, however, in heterodimers binding to a DR1 element, such as in the PPAR/RXR heterodimer, in which RXR occupies the 3'-half site of a DR1 element whereas PPAR binds to the upstream 5'-half site. RXR remains a ligand-inducible partner in this configuration (21). Another example is the RAR/RXR heterodimer; in this case, however, the consequence of the reversed polarity is that RXR is no longer ligand-responsive, and the heterodimer acts as a repressor by competing with the ligand-activated RXR homodimer (22).

An analysis of the binding polarity of the OR1 paralog LXRα bound to a DR4 element with RXR revealed that LXRα bound as expected to the 3'-half site (44). Therefore, we analyzed the polarity of binding of the OR1/RXR heterodimer both on a DR4 and on the DR1s element because we suspected that, as with RAR/RXR and PPAR/RXR heterodimers, the polarity of binding might be reversed on the DR1s. We found that the heterodimer binds to a DR4 element only when OR1 was allowed to occupy the 3'-half site similar to other RXR heterodimers. In contrast, binding to the DR1s was possible in both orientations although with a slightly higher affinity when OR1 was allowed to bind to the 3'-half site. RXR is able to occupy both half sites of a DR1 element since it can form homodimers on this element. Modeling of the dimerization interface of the RXR homodimer predicted that the molecule occupying the 5'-half site provides part of the CII finger and the 3'-molecule the T-box as a dimerization motif in the DBD. The same type of dimerization interface was also predicted for RXR heterodimers on narrow spaced elements with RXR in the 5'-position (20). The clear polarity of binding that was observed in most heterodimers, however, is abolished in the OR1/RXR heterodimer. This mode of binding is reflected by the symmetry of the consensus sequence in which both half sites are preceded by the same nucleotides. Since the polarity of binding can result in different activation properties, it will be interesting to analyze whether only one or both configurations could be transcriptionally active and/or inducible.

It has previously been shown that the OR1/RXR heterodimer is able to transactivate a reporter gene both constitutively and in response to ligands for both partners. Interaction between the RXR and OR1 ligand binding domains leads to dimerization induced and thus constitutive activation for which we proposed a novel mechanism of nuclear receptor activation (45). We now found that the DR1s element confers constitutive activation to a reporter gene when OR1 and RXR are coexpressed in CHO-K1 cells. However, the dimer was no longer significantly responsive to 9-cis-retinoic acid or 22(R)-hydroxycholesterol in this system. Thus, the mode of activation of the receptor heterodimer might be response element-dependent. The presence of OR1 inhibits the 9-cis-retinoic acid inducibility of the RXR homodimer on this element, although the absolute values of the reporter gene activity remain high. These results cannot, however, rule out the possibility that the heterodimer might be responsive to an as yet unidentified ligand for OR1. As an additional mode of action, a ligand could alter or influence the binding affinity for different elements or, in the case of the DR1s, affect the polarity of binding to this element.

In contrast to OR1, LXRα is not able to bind to the DR1s as a heterodimer with RXR. The DBDs of both proteins share about 76% sequence identity. However, while the first Zn-fingers, including the P-box, are almost 100% identical, the second Zn-fingers are much less well conserved (64%). Since the second Zn-finger provides part of the dimerization interface of the DBDs, these amino acid exchanges might be decisive for the different ability of the two receptors to form RXR heterodimeric complexes on DNA. Additionally, interactions between the ligand binding domains of the respective partners might contribute to the stabilization of the complexes with different DNA binding sites (8–10, 62, 63).

The broader DNA binding specificity of OR1 is paralleled by its ubiquitous expression pattern, indicating a more general role for this receptor. LXRα, on the other hand, might have a more specific role in agreement with its restricted expression pattern. Due to their different ligand and DNA binding specificities, these receptors offer the possibility of simultaneously regulating a whole subset of genes both in a ligand-dependent and ligand-independent way. In conclusion, the identification of the preferred binding site for the OR1/RXR heterodimer will be helpful in predicting whether nuclear receptor binding sites in natural promoters can be targets for OR1 action. Additional studies will be necessary to elucidate the precise DNA binding properties of these receptors and to establish their role in the complex network of nuclear receptor interactions.

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