Differential Nuclear Receptor Signalling From DR4-Type Response Elements

Marcus Quack,1 Christian Frank,2 and Carsten Carlberg1,2*

1Institute for Physiological Chemistry I, Heinrich-Heine-University, D-40001 Düsseldorf, Germany
2Department of Biochemistry, University of Kuopio, FIN-70211 Kuopio, Finland

Abstract

Nuclear receptors form a large family of highly related transcription factors that transform an incoming signal in the form of a lipophilic hormone into an activation of the basal transcriptional machinery. The specific recognition of nuclear receptor DNA binding sites, referred to as response elements (REs), determines the genes that can be regulated by nuclear hormones. In this study, it was shown that the complexes of the retinoid X receptor (RXR) with either the vitamin D3 receptor (VDR), the thyroid hormone receptor (T3R) or the liver X receptor (LXR) have comparable functionality on a RE of the rat pit-1 gene that is formed by a direct repeat of two hexameric binding motifs spaced by 4 nucleotides (DR4). The sequence of two nucleotides 5'-flanking the downstream binding motif of this DR4-type RE and, interestingly, also those flanking the upstream motif were shown to have in part rather drastic and receptor-specific effects on heterodimer complex formation on DNA. In particular, a downstream substitution into GA reduced the complex formation for LXR specifically, while upstream substitutions into AA or TA increase complex formation for LXR and, to a lesser extent, T3R. The preference of this in vitro complex formation was shown to correlate well with the functional activity of the nuclear receptors in living cells. The results of this study allow (i) a more detailed understanding of known REs, (ii) a more straightforward search for putative REs in newly identified promoter sequences, for example, of the whole human genome, and (iii) a more precise prediction of the hormone responsiveness of the respective genes.


Key words: transcriptional regulation; vitamin D receptor; thyroid hormone receptor; liver X receptor; retinoid X receptor; heterodimeric nuclear receptor; complexes; response elements

Nuclear receptors form a large family of transcription factors (48 human members) that have critical roles in nearly all aspects of vertebrate development and adult physiology by transducing the effects of small, lipophilic compounds into transcriptional responses [Mangelsdorf et al., 1995]. Two domains, the central, highly conserved DNA-binding domain (DBD) of 66 amino acids and the carboxy-terminal, structurally conserved ligand-binding domain (LBD) of approximately 250 amino acids, define the family [Glass, 1994; Moras and Gronemeyer, 1998]. The LBD serves as a molecular switch that interacts in its agonistic conformation with coactivator proteins and activates in this way target gene transcription [Rachez and Freedman, 2000]. The receptors for the steroids estrogen, progesterone, testosterone, cortisol and aldosterone and those for the biologically active form of vitamin D3, 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3), vitamin D3 receptor (VDR) or NR1I1, for the thyroid hormone 3,5,3'-triiodothyronine (T3R), thyroid hormone receptor (T3R) or NR1A1-2, and for the vitamin A derivative all-trans retinoic acid, RAR or NR1B1-3, are classical endocrine receptors [Carlberg, 1999]. However, the vast majority of the
superfamily members have been cloned before their specific ligands were known; these nuclear receptors are called orphans [Blumberg and Evans, 1998]. Some of these orphans are referred to as adopted orphan nuclear receptors [Chawla et al., 2001], since for them interesting ligands have been identified during the last years, such as 9-cis retinoic acid for the retinoid X receptor (RXR or NR2B1-3) [Levin et al., 1992] and 22(R)-hydroxycholesterol (22-HC) and other oxysterols for the liver X receptor (LXR or NR1H2-3) [Janowski et al., 1996].

Nuclear hormone responsive genes are defined through the presence and specific recognition of nuclear receptor binding sites, referred to as response elements (REs), in their promoter regions [Glass, 1994; Carlberg, 1995]. Most members of the nuclear receptor superfamily, such as VDR, T3R, and LXR, preferentially form heterodimeric complexes with RXR on REs that are formed by two hexameric binding motifs of the consensus sequence RGKTCA (R = A or G, K = G or T) [Mangelsdorf and Evans, 1995]. These binding motifs are mainly in a directly repeated (DR) arrangement with a critical number of spacing nucleotides [Umesono et al., 1991]. The number of different RE types is clearly lower than the number of different nuclear receptor complexes, so that each RE type is recognised by multiple receptor complexes. The crystal structure of DNA-bound T3R-RXR heterodimers [Rastinejad et al., 1995] indicated that REs, which are formed by a direct repeat spaced by 4 nucleotides (DR4-type REs), may represent ideal nuclear receptor binding sites. At this binding motif distance both receptor DBDs face the same side of the DNA, which then favours their interaction. In fact, several nuclear receptor complexes, such as T3R-RXR and LXR-RXR heterodimers, are known to preferentially bind to DR4-type REs and recently also a high affinity binding of VDR-RXR heterodimers to the DR4-type RE of the rat pit-1 gene has been shown [Quack and Carlberg, 2000]. The heterodimer crystal structure demonstrated a head-to-tail arrangement of the receptors with the T3R DBD binding to the downstream motif and the RXR DBD binding to the upstream motif [Rastinejad et al., 1995]. It is generally assumed that also other nuclear receptor complexes, such as VDR-RXR and LXR-RXR heterodimers, show the same type of polarity, i.e., RXR binds in all these cases to the upstream motif [Gronemeyer and Moras, 1995].

The high level of conservation both on the level of the DBD structure as well as on the level of the hexameric binding motif caused the question, how nuclear receptors can achieve target gene specificity. One idea is that a region within the less conserved carboxy-terminal extension of the DBD, referred to as GRIP box [Zhao et al., 1998], provides receptor-specific interfaces for an interaction with nucleotides that are 5′-flanking to the hexameric binding motif. The GRIP box has been suggested for nuclear receptors that preferentially bind as monomers to DNA, such as NGFI-B and RZR/ROR, but may also play a role for receptors that preferentially (e.g., T3R) or exclusively (e.g., VDR and LXR) bind as dimers to DNA. The DNA binding affinity of T3R monomers has been shown to be strongly modulated by the two nucleotides that directly flank the hexameric binding motif [Schräder et al., 1994a], which appears to influence also the binding affinity and selectivity of heterodimeric nuclear receptor complexes [Schräder et al., 1994b, 1995]. The crystal structure of DNA-bound T3R-RXR heterodimers [Rastinejad et al., 1995] confirmed the contact of amino acids of the carboxy-terminal extension of the T3R-DBD with two 5′-flanking nucleotides.

In this study, the heterodimeric complexes of the ubiquitously expressed nuclear receptors VDR, T3R and LXR with RXR were chosen for a comparison of their specificity and functionality on DR4-type REs. The two 5′-flanking nucleotides to both the downstream and the upstream binding motif of the rat pit-1 gene DR4-type model RE were investigated concerning their influence on heterodimer complex formation and functional activity. For all three nuclear receptor complexes in vitro complex formation was shown to correlate well with their functional activity in living cells.

**MATERIALS AND METHODS**

**Compounds**

1α,25(OH)2D3 was kindly provided by L. Binderup, Leo Pharmaceutical Products (Ballerup, Denmark) and T3 and 22-HC were from Sigma (Deisenhofen, Germany). 1α,25(OH)2D3 was dissolved in 2-propanol, whereas the other compounds were dissolved in dimethyl sulfoxide (DMSO); further dilutions were made in DMSO (for in vitro experiments) or in ethanol (for cell culture experiments).
DNA Constructs

The cDNA for human VDR (NR1I1) [Carlberg et al., 1993], chicken T₃Rα (NR1A1) [Sap et al., 1986], human LXRβ (NR1H2) [Teboul et al., 1995], and human RXRα (NR2B1) [Levin et al., 1992] were subcloned into the SV40 promoter-driven expression vector pSG5 (Stratagene, Heidelberg, Germany). Each copy of the wild type DR4-type RE from the rat pit-1 gene [Rhodes et al., 1993] (for core sequence see Fig. 1), the 15 variations of the 5-flanking sequence of its downstream motif (see Fig. 2), and the 15 variations of the 5-flanking sequence of its upstream motif (see Fig. 3) were fused with the thymidine kinase (tk) promoter driving the luciferase reporter gene. All 31 reporter gene constructs were verified by sequencing.

Fig. 1. Heterodimer complex formation and functional activity of VDR-RXR, T₃R-RXR and LXR-RXR heterodimers on a DR4-type RE. (A) Ligand-dependent gel shift experiments were performed with the indicated combinations of in vitro translated RXR, VDR, T₃R, and LXR in the presence of 10 μM 1α,25(OH)₂D₃, 10 μM T₃ or 100 μM 22-HC (or solvent as a control) on the [³²P]-labelled rat pit-1 gene DR4-type RE (core sequence indicated on top). The protein-DNA complexes were separated from free probe on 8% non-denaturing polyacrylamide gels. Representative experiments are shown. The amount of specific protein-complexes was quantified on a Bioimager in relation to free probe. (B) Reporter gene assays were performed with extracts from MCF-7 cells that were transiently transfected with a luciferase reporter construct containing the rat pit-1 gene DR4-type RE and the indicated expression vectors for RXR and VDR, T₃R or LXR. The cells were treated for 16 h with 100 nM 1α,25(OH)₂D₃, 1 μM T₃ or 100 μM 22-HC (or solvent as control) and relative luciferase activities were measured. Columns represent means from three experiments and bars indicate standard deviations.
Gel Shift Assays

Linearised DNAs of the pSG5-based constructs of VDR, T3R, LXR and RXR were transcribed with T7 RNA polymerase and the respective RNAs were translated using rabbit reticulocyte lysate as recommended by the supplier (Promega, Mannheim, Germany). RXR protein was mixed with equal amounts of VDR, T3R and LXR (please note that protein amounts were always normalised to each other) and incubated in the presence of indicated ligand concentrations (or solvent as control) for 15 min at room temperature in a total volume of 20 µl binding buffer (10 mM HEPES [pH 7.9], 1 mM DTT, 0.2 µg/µl poly(dI-C) and 5% glycerol). The buffer had been adjusted to 150 mM of monovalent cations by the addition of KCl. Partially double-stranded oligonucleotides carrying the 31 different REs were labelled by a fill-in reaction using [γ-32P]-dCTP and the Klenow fragment of DNA polymerase I (Promega) and normalised to each other. Approximately 1 ng of labelled probe (50,000 cpm) was added to the receptor-ligand mixture and incubation was continued for 20 min. Protein-DNA complexes were resolved from free probe on 8% non-denaturing polyacrylamide gels. For each RE the specific heterodimer complex formation was quantified on a Bioimager in relation to free probe and normalised to the strength of the wild type rat pit-1 gene DR4-type RE (AG). Columns represent means from three experiments and bars indicate standard deviations.
Transfection and Luciferase Assays

MCF-7 human breast cancer cells (10^5 cells/ml) were seeded into 6-well plates and grown overnight in phenol red-free DMEM supplemented with 5% charcoal-treated fetal bovine serum (FBS). Liposomes were formed by incubating 1 μg of the DR4-type reporter construct, 1 μg of pSG5-based receptor expression vectors for VDR, T3R or LXR (each together with 1 μg of expression vector for RXR) and 15 μg N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Roth, Karlsruhe) for 15 min at room temperature in a total volume of 100 μl. The liposomes were added to the cells, after dilution with 0.9 ml phenol red-free DMEM. DMEM supplemented with 15% charcoal-treated FBS (500 μl) was added 4 h after transfection. At this time, 100 nM 1α,25(OH)2D3, 1 μM T3 or 100 μM 22-HC or solvent (0.1%) were also added. The cells were lysed 16 h after onset of stimulation using the reporter gene lysis buffer (Roche Diagnostics, Mannheim, Germany) and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Canberra-Packard, Dreieich, Germany). The luciferase activities were normalised with respect to protein concentration and relative reporter gene activities were related to that of the wild type RE without receptor overexpression.
RESULTS

The complex formation of equal molar amounts of VDR-RXR, T3R-RXR and LXR-RXR heterodimers on the rat pit-1 gene DR4-type RE [Rhodes et al., 1993] was analysed by ligand-dependent gel shift assays (Fig. 1A). VDR-RXR and LXR-RXR heterodimers showed ligand-independent DNA binding that was approximately 35 and 10%, respectively, of that of T3R-RXR heterodimers. Interestingly, a ligand-dependent complex formation (approximately twofold increase) could be observed only with VDR-RXR heterodimers, but not with T3R-RXR and LXR-RXR heterodimers. No formation of RXR, VDR, T3R or LXR homodimers could be detected on any of the DR4-type REs (Fig. 1A).

The functional activity mediated by VDR-RXR, T3R-RXR and LXR-RXR heterodimers was analysed by reporter gene assays in MCF-7 human breast cancer cells (Fig. 1B). MCF-7 is an established model cell line for various nuclear receptor-signalling pathways [Carlberg et al., 1993]. The cells were transiently transfected with a luciferase gene driven by one copy of the rat pit-1 gene DR4-type RE and indicated receptor expression vectors. Equal overexpression of receptor proteins was verified by Western blotting (data not shown). In the absence of overexpressed receptors, no significant stimulation of reporter gene activity to the receptor agonists 1α,25(OH)2D3, T3 or 22-HC could be detected. Interestingly, the overexpression of VDR-RXR and T3R-RXR heterodimers reduced the basal reporter gene activity by a factor of approximately 2, whereas the overexpression of LXR-RXR heterodimers showed no significant effect on basal activity. This could be explained by the known repressing effect of non-liganded VDR and T3R [Polly et al., 2000] and suggests that in contrast to VDR and T3R LXR does not efficiently recruit corepressor proteins. Stimulation with 1α,25(OH)2D3 and T3 resulted in an 8.9- and 2.8-fold induction of VDR-RXR and T3R-RXR heterodimer-driven reporter gene activity, respectively, whereas in MCF-7 cells LXR-RXR heterodimers did not show any significant response to 22-HC.

For testing the influence of two 5′-flanking nucleotides of the downstream motif of the rat pit-1 gene DR4-type RE (binding RXR [Rastinejad et al., 1995]) were analysed by ligand-dependent gel shift assays for their effect on binding of VDR-RXR, T3R-RXR and LXR-RXR heterodimers (Fig. 2). T3R-RXR and LXR-RXR heterodimers did not display any ligand-dependent complex formation (compare Fig. 1A) and were therefore tested only in the presence of their respective agonists. Interestingly, the AG flanking sequence of the wild type rat pit-1 RE appeared to be optimal for all three heterodimeric complexes, since no flanking sequence variation resulted in an increased amount of complex formation (Fig. 2A–C). However, the sequences GA and TA also provided maximal amount of complex formation for VDR-RXR and T3R-RXR heterodimers, respectively (Fig. 2A,B).

Moreover, for VDR-RXR heterodimers the sequences GC and TA (Fig. 2A) and for T3R-RXR heterodimers the sequences GA and GC (Fig. 2B) provided approximately 80% of maximal complex formation. For VDR-RXR heterodimers the 11 remaining 5′-flanking sequence variations resulted in less than 30% of maximal binding, but heterodimer complex formation was found to be ligand-dependent on all DR4-type elements (Fig. 2A). T3R-RXR heterodimers showed on the elements with the 5′-flanking sequences CA, CC, CG, CT, GG, TC and TG approximately 60% of maximal binding and only on those that carry the sequences AA, AC, AT, GT and TT 40% or less of maximal complex formation (Fig. 2B). LXR-RXR heterodimers showed to be most selective for the 5′-flanking sequence of the downstream motif, since only the sequence AA provided 60% of maximal binding, whereas the remaining 14 downstream motif variations displayed 40% or less of maximal complex formation (Fig. 2C).

As a next step, the 15 variations of two 5′-flanking nucleotides of the upstream motif of the rat pit-1 gene DR4-type RE (binding RXR [Rastinejad et al., 1995]) were analysed by ligand-dependent gel shift assays for their effect on the binding of VDR-RXR, T3R-RXR and LXR-RXR heterodimers (Fig. 3). VDR-RXR heterodimers showed not to be very much affected by sequence variations, since most of the 5′-flanking nucleotide variations resulted in 80–100% of maximal complex formation and only the sequences CT, GC, GG and GT provided an activity reduction by approximately 50% (Fig. 3A). Importantly, VDR-RXR heterodimer complex formation stayed inducible by 1α,25(OH)2D3 on all 16 REs. T3R-RXR heterodimers were found to be influenced more effective by the 5′-flanking
nucleotides of the upstream motif than VDR-RXR heterodimers (Fig. 3B). Compared with the wild type rat pit-1 RE (sequence GA), the sequence AA provided an increase of T3R-RXR heterodimer complex formation by approximately 50%, whereas the sequences AC, AT, CA, CC and TA showed between 80 and 120% of wild type element binding. The remaining nine 5′-flanking nucleotide variations resulted in 50% or less of T3R-RXR heterodimer binding compared with the wild type rat pit-1 RE. LXR-RXR heterodimers showed an interesting response to variations of the 5′-flanking nucleotide of the upstream motif, since the elements with the sequences AA and TA provided an increase of complex formation of this heterodimer type by approximately 300 and 200%, respectively (Fig. 3C). Moreover, also REs with the 5′-flanking nucleotide variations AC, AT, CA and TG resulted in an increase of complex formation by 100% and elements with the variations AG, CG, CT, GC, TT and TG were found to be at least as potent as the natural pit-1 RE. Only REs with the 5′-flanking nucleotide variations GC, GG and GT showed to be less potent binding sites for LXR-RXR heterodimers than the wild type RE.

Another critical question was, whether the in vitro binding patterns of VDR-RXR, T3R-RXR and LXR-RXR heterodimers reflect their functional activities in living cells. Therefore, reporter gene assays were performed with the 15 variations of the downstream motif of the DR4-type RE (Fig. 4) and also with the 15 variations of its upstream motif (Fig. 5). The 30 different assays were designed to be comparable to each other and were each related to the activity of the wild type rat pit-1 RE without receptor over-expression (Fig. 1B). Constant observation in all assays was a 7- to 10-fold induction of VDR-RXR-driven reporter gene activity after 1α,25(OH)2D3 treatment and a 2- to fourfold induction of T3R-RXR-driven luciferase activity after T3 stimulation, which fits well with the induction of the wild type element (Fig. 1B). Moreover, on none of the 30 variations 22-HC was able to stimulate LXR-RXR-driven reporter gene activity more than 1.4-fold. However, as predicted by the in vitro binding analysis (Figs. 2 and 3), the three receptor complexes showed individual preferences for the 30 different DR4-type REs. Like on the wild type DR4-type RE (Fig. 1B), also on the downstream 5′-flanking sequence variations AA, AT, GA, GC, GT and TA and on most of the upstream 5′-flanking sequence variations (AC, AG, AG, CA, CC, CG, CT, GC, GG, GT, TC, TG and TT) VDR-RXR-mediated 1α,25(OH)2D3 signalling showed to be more prominent than the activity of the two other receptors. On the downstream variations TC, TG and TT, but on none of the upstream variations, T3R-RXR-mediated T3 signalling was found to be dominant over the other two receptors. On the downstream variations AC, CA, CC CG, CT and GG VDR-RXR and T3R-RXR heterodimers were found to be equal in their functional activity, whereas on the upstream variations AA and TA VDR-RXR and LXR-RXR heterodimers showed comparable activity. The low ligand responsiveness of LXR explains why in the presence of ligand LXR-RXR heterodimers were not found to dominate any of the 31 DR4-type REs. However, in the absence of ligand the relative high constitutive activity of LXR made this receptor dominant on most REs (on the downstream variations AA, AC, AT and on all upstream variations with the exception of GC).

The ranking of the VDR-RXR heterodimer-mediated reporter gene activity on the 15 downstream variations (Fig. 4) was found to be very comparable to that of their in vitro complex formation (Fig. 2): the wild type dinucleotide AG mediated highest activity as well as the variations GA, GC and TA, whereas AC, TC, TG and TT were found to be nearly 10-fold weaker. A similar fitting between functional data and in vitro binding were observed for the upstream variations (compare Figs. 3 and 5): concerning 1α,25(OH)2D3 signalling the dinucleotide variations CT, GC, GG and GT gave approximately twofold weaker results than the other 12 variations. The ranking of in vitro binding activity of T3R-RXR and LXR-RXR heterodimers on all 31 DR4-type REs (Figs. 2 and 3) were also found to correlate well with that of their relative functional activity in MCF-7 cells (Figs. 4 and 5). Taken together, the functional activities of the three heterodimers reflected quite well the ability of the receptor pairs for complex formation on DNA.

**DISCUSSION**

Nuclear receptors are complex regulators of transcription and important drug targets, which makes them interesting both in basic research on gene regulation as well as in the therapy of various diseases. Classical nuclear receptor are
the genomic mediators of the endocrine pathways, for which nuclear hormones were known since long time, whereas orphan nuclear receptors are the critical nuclear switches for newly identified endocrine systems that are probably equally important as the established systems [Chawla et al., 2001]. Nearly all tissues express several members of the nuclear receptor family at the same time, which in part recognize the same type of REs. In this study, DR4-type REs were chosen as representative REs that can be bound by a reasonable proportion of the members of the nuclear receptor superfamily. The receptors VDR, T₃R₂ and LXRβ were selected,
because on one hand they represent different subfamilies of RXR-interacting nuclear receptors [Wiebel and Gustafsson, 1997; Quack and Carlberg, 2000] that show a different functionality and on the other hand their ubiquitous expression makes it likely that in the physiological context they are competing for the same REs.

It is known since long time that nuclear receptors that can bind as monomers to DNA, such as RZR/ROR or T3R, have a clear preference for certain 5'-flanking sequences [Carlberg et al.,
the comparison of the in vitro binding data (Figs. 1A, 4 and 5) indicates that the prediction of the relative functionality of a several nuclear receptors for the same RE is more complex. VDR, T3R and LXR have their characteristic profile in recruitment of corepressor and coactivator proteins, which translates into a different intrinsic repression in their non-liganded state and ligand-dependent activation in their active state (Fig. 1B) [Herdick and Carlberg, 2000]. However, the individual cofactor interaction profiles of the investigated nuclear receptors seem not to be affected significantly by sequence variations of the bound RE, since on all 31 tested REs the three heterodimers showed the same profile of receptor-mediated repression and activation (compare Figs. 1B, 4 and 5). Therefore, the resulting functional activity of a nuclear receptor complex can be considered to be the product of its RE binding preference and its cofactor recruitment profile. This seems to be the explanation for the good correlation between the amount of heterodimer complex formation on the different DR4-type REs in vitro and the functional activity of the respective receptor on these elements in living cells. This concept is also an important basis for future studies that evaluate the functionality of REs purely on the basis of in vitro binding profile.

The results of this study suggest that not only monomeric nuclear receptors but also VDR, T3R and LXR have critical amino acids in the carboxy-terminal extension of their DBDs that have an individual preference for the contact of 5'-flanking nucleotides. This preference should be independent of the type of RE, so that the results of this study can be extended, for example, in the case of VDR-RXR heterodimers to direct repeats spaced by three nucleotides (DR3) or inverted palindromes with nine intervening nucleotides (IP9) [Carlberg and Polly, 1998]. This suggests that more likely octameric motifs instead of hexameric sequences should be considered as specific nuclear receptor binding sites. Moreover, already characterised REs should be reanalysed for their 5'-flanking sequences, which will provide a better understanding of their relative strength. Even more important is the possibility of a most accurate prediction of putative nuclear receptor binding sites within newly identified promoter sequences, for example, from the human genome sequence. However, it has to be noted that without a more detailed understanding of the rules of chromatin formation an in silico analysis of promoter sequences has its limits. The DR4-type RE of the rat pit-1 gene, which was used in this study as a model RE, represents a rather optimal nuclear receptor binding site. However, in the adult the region of this RE within the pit-1 gene promoter seems to be
covered by nucleosomes, so that the responsiveness of the pit-1 gene to 1α,25(OH)₂D₃ or T₃ is lower than expected [Castillo et al., 1999]. Although it was not experimentally proven for the DR-4 type RE studied here, there should be no doubt that in all three heterodimeric complexes RXR binds to the upstream motif of the RE. This makes the observation quite surprising that also variations of the 5′-flanking sequence of the upstream motif have a reasonable influence on the complex formation and functional activity of the heterodimers and would suggest that also RXR specifically contacts 5′-flanking nucleotides to its binding motif. Moreover, the three tested heterodimeric complexes show individual preferences for the variations of the flanking sequence of the upstream motif. This suggests that the specificity of the DNA contacts of RXR can be modulated by its heterodimeric binding partner. In addition, the observation that LXR-RXR heterodimers are much more affected by variations of the 5′-flanking nucleotides to the upstream motif than VDR-RXR heterodimers indicates that within the latter complex RXR contributes less to the overall DNA binding capacity than in LXR-RXR heterodimers.

In conclusion, this study demonstrated that DR4-type REs can be effective binding sites for VDR-RXR, T₃-R-RXR and LXR-RXR heterodimers and that 5′-flanking sequences to the downstream and the upstream motif of DR4-type REs can have a rather drastic modulatory effect on the strength and responsiveness of dimeric nuclear receptor binding sites. This strongly suggests that 5′-flanking sequences should be considered as an integral part of a RE. In turn, the recognition of 5′-flanking sequences will allow a more accurate evaluation and prediction of the functionality of nuclear receptor binding sites.

Acknowledgments

We thank M. Hiltunen for skilled technical assistance, L. Binderup for 1α,25(OH)₂D₃ and D. Mangelsdorf for the LXRβ expression vector. This work was supported by the Academy of Finland and the Medical Faculty of the Heinrich-Heine-University Düsseldorf (all to C.C).

References


Morphological analysis of the pit-1 gene to 1α,25(OH)₂D₃ or T₃ is lower than expected [Castillo et al., 1999]. Although it was not experimentally proven for the DR-4 type RE studied here, there should be no doubt that in all three heterodimeric complexes RXR binds to the upstream motif of the RE. This makes the observation quite surprising that also variations of the 5′-flanking sequence of the upstream motif have a reasonable influence on the complex formation and functional activity of the heterodimers and would suggest that also RXR specifically contacts 5′-flanking nucleotides to its binding motif. Moreover, the three tested heterodimeric complexes show individual preferences for the variations of the flanking sequence of the upstream motif. This suggests that the specificity of the DNA contacts of RXR can be modulated by its heterodimeric binding partner. In addition, the observation that LXR-RXR heterodimers are much more affected by variations of the 5′-flanking nucleotides to the upstream motif than VDR-RXR heterodimers indicates that within the latter complex RXR contributes less to the overall DNA binding capacity than in LXR-RXR heterodimers.

In conclusion, this study demonstrated that DR4-type REs can be effective binding sites for VDR-RXR, T₃-R-RXR and LXR-RXR heterodimers and that 5′-flanking sequences to the downstream and the upstream motif of DR4-type REs can have a rather drastic modulatory effect on the strength and responsiveness of dimeric nuclear receptor binding sites. This strongly suggests that 5′-flanking sequences should be considered as an integral part of a RE. In turn, the recognition of 5′-flanking sequences will allow a more accurate evaluation and prediction of the functionality of nuclear receptor binding sites.

ACKNOWLEDGMENTS

We thank M. Hiltunen for skilled technical assistance, L. Binderup for 1α,25(OH)₂D₃ and D. Mangelsdorf for the LXRβ expression vector. This work was supported by the Academy of Finland and the Medical Faculty of the Heinrich-Heine-University Düsseldorf (all to C.C).


