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ANALYSIS OF OESTROGEN RECEPTOR DIMERISATION USING CHIMERIC PROTEINS

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Summary—Sequences essential for dimerisation have been identified in the hormone binding domain of the mouse oestrogen receptor by insertional and point mutagenesis and sequence comparisons reveal that equivalent residues may be conserved in other members of the nuclear hormone receptor superfamily. To assess functional compatibility of this region between members of the receptor superfamily, peptide sequences corresponding to the equivalent regions of the human androgen receptor and retinoic acid receptor have been substituted for the dimerisation domain of the mouse oestrogen receptor. The resulting chimeric proteins were analysed for high affinity DNA binding using a gel retardation assay and shown to bind with reduced affinity compared to the wild type oestrogen receptor. The reduction in DNA binding observed may result from the intramolecular incompatibility of functional elements within the hormone binding domain of nuclear hormone receptors.

INTRODUCTION

Steroid hormone receptors are ligand inducible transcription factors which regulate gene expression by binding to specific sequences in responsive genes [1, 2]. The binding sites, termed response elements, have been characterised and shown to contain inverted repeats of the sequence TGACC for the oestrogen receptor and TGTTCT for the glucocorticoid receptor [3, 4]. It has subsequently been shown that the receptors for androgen and progestin can also act through a glucocorticoid response element (GRE) [5]. Response elements for retinoic acid and thyroid hormone receptors have been identified and appear to resemble an oestrogen response element (ERE), consisting of an inverted repeat of the TGACC sequence but lacking the 3 base pair gap in the centre of the repeat [6]. Recent evidence however suggests that a direct repeat of this sequence may also function as a response element for retinoic acid receptor [7].

It has been demonstrated that the human oestrogen receptor [8] and the rat glucocorticoid receptor [9] bind to their response elements as dimers and that a dimerisation function is contained within the hormone binding domain [8].

A series of deletion mutants has been used to define the C-terminal boundary for dimerisation of the mouse oestrogen receptor (MOR) and has demonstrated that sequences between residues 507 and 538 are required for both dimerisation and steroid binding [10]. A conserved heptad repeat of hydrophobic residues was identified within this region that resembled the leucine zipper or coiled coil structure implicated in the dimerisation of a number of DNA binding proteins. However subsequent point mutagenesis has shown that critical residues for receptor dimerisation and high affinity DNA binding are restricted to the N-terminal half of the repeat and include the arginine at residue 507 (R-507), the leucine at residue 511 (L-511) and the isoleucine at 518 (I-518), implying that dimerisation of the oestrogen receptor may be mediated through a novel structural motif. The importance of this region has also been demonstrated by analysing the activity of fusion proteins which contain a 22 amino acid peptide corresponding to residues 501 to 522. This was sufficient to partially restore DNA binding activity to a truncated non-DNA binding form of the MOR [11]. The region 507–518 in the MOR includes sequences which are conserved between all members of the nuclear hormone receptor superfamily and sequences homologous to this region of the MOR have been implicated in the heterodimerisation of the human thyroid hormone receptor with the human retinoic acid receptor enabling the cooperative binding of the

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α retinoic acid receptor to a subset of thyroid hormone response elements [12].

The experiments described here extend the analysis of sequences required for dimerisation, high affinity DNA binding, and steroid binding of the MOR. We have attempted to investigate the role of these sequences in the specificity of receptor dimerisation by the transfer of the corresponding region from other members of the nuclear hormone receptor superfamily into the equivalent position in the MOR and the analysis of these chimeric receptors in a gel retardation assay.

EXPERIMENTAL

Receptor mutants

The isolation of a mouse oestrogen receptor cDNA clone, the construction of a series of point mutants and their transfer into SP64 and SP65 has been described previously [13]. Point mutations are described by the amino acid and position in the receptor followed by the substituting residue. The insertion mutants L-501V,Q-504,Q H-505 and L-515,A,S-516 were constructed by oligonucleotide directed mutagenesis [14] and the insertion of oligonucleotide cassettes, respectively. The chimeric mutants MOR[507–536→hAR 854–883], MOR [507–521→hAR 854–868] and MOR[503–537→ hRARα 339–373] were constructed by inserting oligonucleotide cassettes encoding peptide sequences derived from the human androgen receptor and human retinoic acid receptor between the *Cel* II site at 1716 and the *Kpn* I site at 1805 in the MOR [10].

cRNA synthesis and in vitro translation

Mutant clones in SP64 or SP65 were linearised using *Hin*d III. Capped RNA was synthesised using SP6 polymerase essentially as described by Melton *et al.* [15] with the following modifications. Templates were transcribed with 0.5 mM ATP, UTP, and CTP; 50 μM GTP and 0.5 mM RNA cap structure analogue [m7 G(5′)ppp(5′)G]. Approx. 15–30 ng/μl cRNA was used to prime synthesis of protein in a rabbit reticulocyte lysate (Promega Biotec.) containing 0.1 mM ZnCl2, methionine-free amino acid mix and either 0.1 mM methionine or 1 μCi/μl[^{35}S]-methionine (Amersham, SA1000 Ci/mmol) as indicated. Translations were carried out at 30°C for 60 min. [^{35}S]Methionine labelled receptor mutants were analysed by SDS–polyacrylamide gel electrophoresis and these data were used to normalise input of labelled receptor in DNA binding and ligand binding assays.

DNA and ligand binding assays

DNA binding was assayed using an electrophoretic mobility shift assay as described previously [10]. Briefly, 1–5 μl of *in vitro* translated receptor was pre-incubated for 15 min in 20 μl of binding buffer (10 mM HEPES pH 7.4, 50 mM KCl, 1 mM MgCl2, 1 mM 2-mercaptoethanol, 20% glycerol) containing 1 μg poly dI.dC:poly dI.dC and 100 μg BSA. After addition of 0.5 ng of a radiolabelled double stranded oligonucleotide probe containing a consensus ERE sequence, the samples were incubated for a further 30 min at room temperature followed by 30 min at 4°C. In experiments involving MP16, the antiserum was added to the samples 15 min after the addition of the ERE probe. Samples were applied directly onto pre-run 6.0% polyacrylamide (30% acrylamide:0.8% bisacrylamide stock solution) 0.5X TBE gels, and electrophoresed in 0.5X TBE at 250 V for 60 min. Gels were fixed for 15 min in 10% acetic acid, 30% methanol dried and autoradiographed. Ligand binding was assayed using 1 nM [^{125}I]iodoestradiol (Amersham SA 2000 Ci/mmol) or [^{3}H]5α-dihydrotestosterone (Amersham SA 125 Ci/mmol) and dextran-coated charcoal as described previously [16].

Antibody production

The peptide NH_2-CQQVPYYLENEPSA-COOH corresponding to residues 130–142, respectively of the MOR, was synthesised using standard techniques and coupled to thyroglobulin via the added N-terminal cysteine. The peptide conjugate was used to immunise rabbits [17]. The resulting antiserum MP16 was tested by Western blotting and found to specifically recognise mouse and human estrogen receptors (SEF unpublished).

RESULTS

Identification of sequences required for dimerisation, DNA-binding and steroid binding

The analysis of dimerisation, DNA-binding and steroid binding following the introduction of specific mutations at key positions within the region of the MOR from Leu 501 to Val 537 are summarised in (Fig. 1). Mutagenesis of residues in the N-terminal part of the sequence primarily

	Sequence (heptad positions ● 511, 518, 525, 532)	DNA BINDING / DIMERISATION	STEROID BINDING
wt MOR	L T **L** Q Q Q H R **R** L A Q **L** L L I L S H **I** R H M S N K **G** M E H L Y N **M** K C K N V	+	+
L-511R	.... **R**	–	+
I-518R	.... **R**	–	–
G-525R	.... **R**	+	–
M-532R	.... **R**	+	+
H-520,A,M-521	.... R H **A** M S N K	+	+
ΔM-521,S-522	.... R H N K	+	–
L-515,A,S-516	.... L L I L **A** S H	–	–
R-507A	.... **A**	–	+
L-501V,Q-504,Q,H-505	...... **V** Q Q Q **Q** H R R	+	+

DIMERISATION

STEROID BINDING

Fig. 1. Summary of mutagenesis of the MOR between residues 499 and 537. The complete sequence of the wild type MOR between amino acids 499 and 537 is shown. Residues in **bold** type correspond to the positions of mutations with the mutated sequence shown below. Black circles above the sequence mark the positions of the heptad repeat. The analysis of DNA binding, dimerisation and steroid binding of the mutant receptors is indicated in the columns on the right-hand side. The nomenclature of the mutants is as follows: L-511R describes a point mutation of leucine at residue 511 to arginine; L515,A,S516 indicates the insertion of one amino acid (alanine) between leucine 515 and serine 516: Δ indicates a deletion of the residues shown.

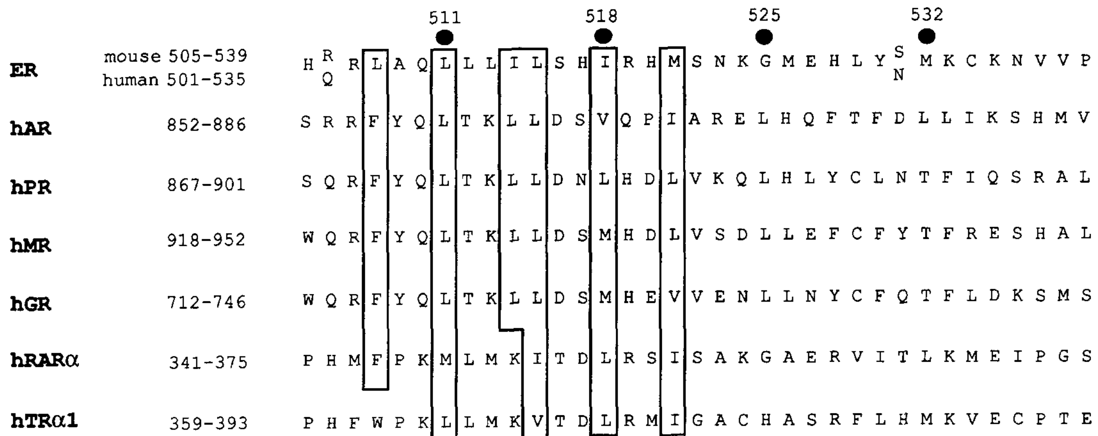

Fig. 2. Sequence comparison of the dimerisation domain of the MOR with other members of the steroid hormone receptor superfamily. The list of sequences is as follows: ER, mouse [13], human [18]; hAR [19]; hPR [20]; hMR [21]; hGR [22]; hRARα [23]; hTRα1 [24]. Boxed regions indicate conserved hydrophobic residues.

affect dimerisation and high affinity DNA-binding as determined by the gel retardation assay, while residues in the centre of the region are required for steroid binding. Mutations introduced into the C-terminal half of the sequence have no effect on dimerisation or DNA-binding. The extent of the sequences in this region involved in dimersation is demonstrated by the three insertion mutants H-520,A,M-521; L-515,A,S-516 and L-501-V,Q-504,Q,H-505 (Fig. 1). Of these three mutants, only L-515,A,S-516 disrupts high affinity DNA-binding, the other mutations show the same phenotype as the wild type MOR. The L-515,A,S-516 insertion mutation also fails to bind oestradiol, demonstrating the colocalisation of the steroid binding and dimerisation activities in the MOR.

The region of the MOR implicated in dimerisation and high affinity DNA-binding may be aligned with homologous sequences in other members of the nuclear hormone receptor superfamily (Fig. 2). The most highly conserved region corresponds to residues 507–518 in the MOR, which encompasses the sequences shown to be required for dimerisation. This region is predominantly hydrophobic in character and the critical residues involved in dimerisation, R-507 and L-511, are completely conserved throughout the steroid hormone receptors. In view of this conservation of primary sequence, the corresponding regions from the human androgen receptor and human retinoic acid receptor were introduced into the MOR to analyse the specificity of monomer to monomer interactions in this region of the receptor.

Analysis of chimeric receptors

The corresponding sequences from other members of the receptor superfamily were inserted into the MOR using oligonucleotide cassettes so as to maintain the relative spacing and character of the predicted sequence conservation between the receptors. The chimeric receptors constructed are shown diagrammatically in (Fig. 3). These were then compared to the wild type MOR and the dimerisation deficient mutant I-518R for the ability to bind to a concensus ERE in the gel shift assay (Fig. 4). Two mutants were generated using sequences from the human androgen receptor (hAR), one containing a 30 amino acid peptide encompassing the heptad repeat of hydrophobic residues and a second containing a 15 amino acid peptide corresponding to the sequences in the MOR demonstrated to be required for dimerisation. These chimeric receptors (Fig. 4, lanes 3 and 4, respectively) bind to DNA better than the dimerisation deficient mutant I-518R (Fig. 4, lane 2) but weakly when compared with the wild type MOR (Fig. 4, lane 1). When analysed for ligand binding these receptors fail to bind either oestradiol or testosterone. A receptor containing a 35 amino acid sequence from the human retinoic acid receptor (hRARα) generates a retarded complex (Fig. 4, lane 5) which fails to produce a discrete band and migrates more slowly than the wild type MOR DNA complex in the gel shift assay. This MOR–hRARα fusion is also negative for oestradiol binding in a ligand binding assay.

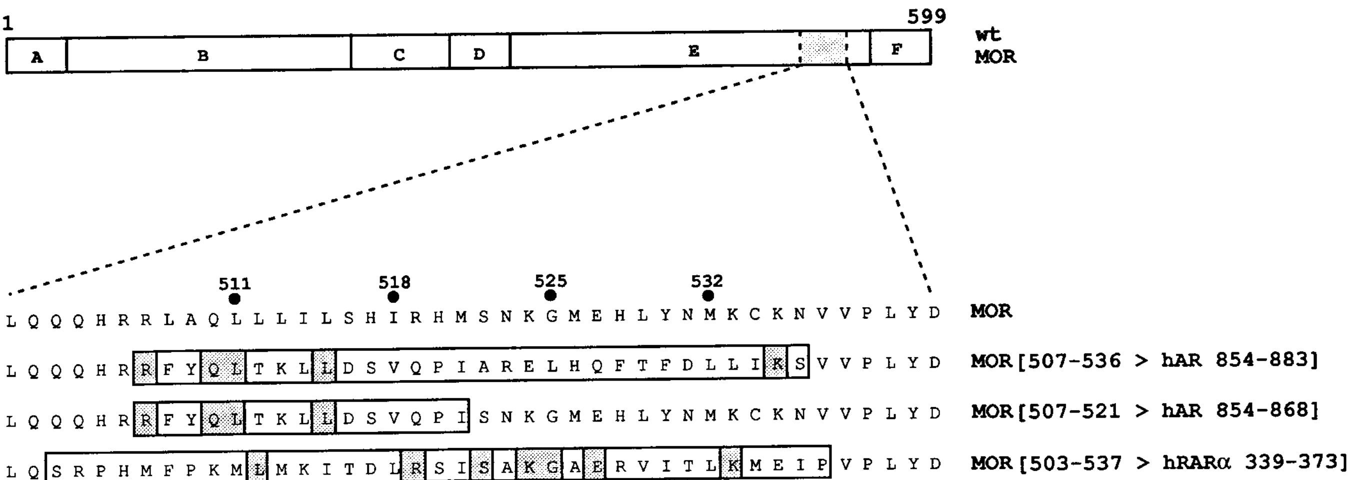

Fig. 3. Organisation of chimeric receptor mutants. The position of the sequences introduced into the MOR is indicated in the schematic diagram at the top of the figure. The regions exchanged are boxed, with the amino acid numbers and the source of the sequence shown on the right-hand side. The shaded amino acids indicate residues completely conserved between the wild type MOR and the chimeric receptors.

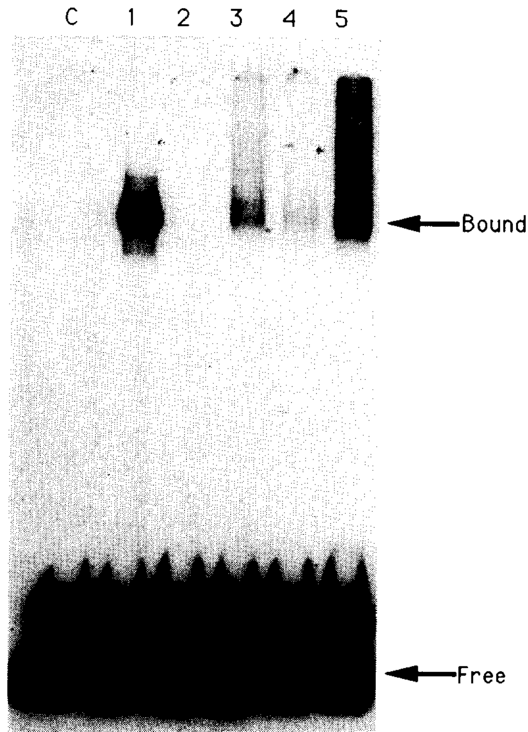

Fig. 4. Gel retardation analysis of chimeric receptor mutants. The position of the free probe and the bound probe are indicated on the right-hand side. C, control rabbit reticulocyte lysate; Lane 1, MOR; Lane 2, I-518R; Lane 3, MOR[507–536→hAR 854–883]; Lane 4, MOR[507–521→hAR 854–868]; Lane 5 MOR[503–537→hRARα 339–373].

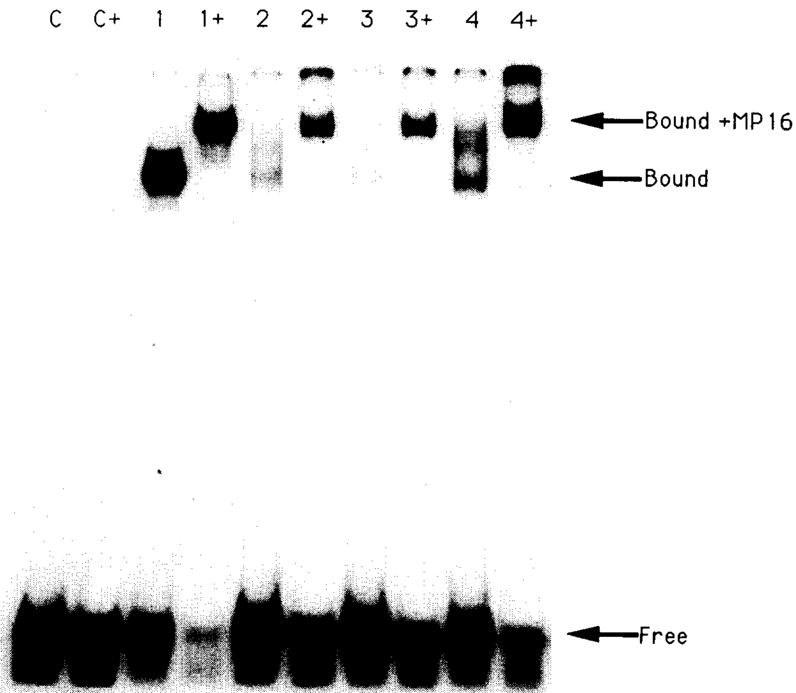

Fig. 5. Confirmation of DNA binding activity of chimeric mutants using MP16 antiserum. The position of free, bound and supershifted probe are indicated on the right-hand side. A + above a lane indicates samples incubated in the presence of MP16. C and C +, control lysate; Lanes 1 and 1 +, MOR; Lanes 2 and 2 +, MOR[507–536→hAR 854–883]; Lanes 3 and 3 +, MOR[507–521→hAR 854–868]; Lanes 4 and 4 +, MOR[503–537→hRARα 339–373].

The ability of the chimeric receptors to bind to DNA was confirmed using the MP16 antibody which has been shown to be capable of restoring DNA-binding to dimerisation deficient mutants of the MOR presumably as a result of the bivalent character of the antibody (Fig. 5) [25]. In the presence of the MP16 antiserum the retarded wild type receptor/DNA complex is "supershifted" due to the addition of the IgG molecule. A similar effect is demonstrated with the three chimeric receptors indicating that the DNA-binding domains of these mutants are fully functional.

DISCUSSION

The analysis of specific point mutations and insertion or deletion mutations in the MOR between amino acids 507 and 538 has demonstrated the importance of a short region of predominantly hydrophobic residues in receptor dimerisation [10]. Alteration of single residues in this sequence generates forms of the receptor which fail to dimerise and bind DNA with high affinity. The extent of the sequences in this region required for dimerisation has been determined using insertion mutations. The critical residues implicated in dimer formation are therefore defined to extend between R-507 and I-518. This region is conserved both in relative position and primary sequence throughout the nuclear hormone receptor superfamily, and therefore may represent a conserved functional domain. Analogous sequences have been implicated in the heterodimerisation of the retinoic acid and thyroid hormone receptors. Although mutation of hydrophobic residues in this region of these receptors to the different hydrophobic amino acid isoleucine had no effect, insertion of four amino acids between the first and second conserved hydrophobic positions in the thyroid hormone receptor, which was predicted to destroy α-helical structure, abolished the ability of the receptors to interact cooperatively [12].

The C-terminal boundary for steroid binding by the MOR has been mapped approximately to residue 538 [16]. Analysis of steroid binding by receptors containing mutations between 507 and 538 demonstrated the importance of sequences in the centre of this region. The mutant receptors I-518R, G-525R and the deletion ΔM-521,S522 were all negative for hormone binding. The insertion mutant L-515,A,S-516 which alters the relative spacing between the key hydrophobic residues implicated in dimerisation at positions 511 and 518 in the MOR also fails to bind oestradiol. This is in agreement with the proposal that the dimerisation and steroid binding functions in this region of the protein are overlapping but not coincident.

To further analyse the extent of this region and to determine the function of sequences shown to be important for dimerisation in the specificity of receptor interaction, the corresponding sequences from either the hAR or hRARα were introduced into the MOR. The mutant MOR[507-521→hAR854–868] contains the 15 amino acids from the hAR which correspond to the dimerisation domain of the MOR. The critical residues R-507 and L-511 determined by point mutagenesis are both conserved between the MOR and hAR, while I-518 is replaced with a different hydrophobic amino acid, valine. This residue has been shown to be essential for androgen receptor function [26], since the mutation V-866M in the hAR reduces the affinity of the receptor for dihydrotestosterone three-fold and creates a completely androgen insensitivity phenotype *in vivo*. The intermediate hydrophobic residue L-515 is also conserved in this switch between the two receptors. However with both the 15 amino acid and a 30 amino acid switch MOR[507–536→hAR854–883] the binding of the chimeric receptors to a concensus ERE is significantly reduced compared to the wild type MOR. Similarly the insertion of these sequences destroys the ability of the receptors to bind oestradiol. The 35 amino acid switch MOR[503–537→hRARα339–373] which encompasses the region involved in heterodimerisation between the thyroid hormone and retinoic acid receptors also shows reduced DNA-binding compared to the wild type MOR in the gel shift assay and fails to bind oestradiol. This mutant does retain significantly more DNA-binding activity than the MOR-hAR mutants and the dimerisation deficient mutant I-518R, however the diffuse nature of the protein–DNA complex may represent a reduction in either stability of the dimeric complex or a lower affinity for DNA.

The reason for the reduction in DNA-binding activity of the chimeric receptors is unknown. Although the region between 501 and 522 of the MOR has been shown to encode a major part of the dimer interface [11], it is unlikely to be sufficient to maintain the wild type protein in the form of a dimer. Therefore the reduced DNA-binding activity of these mutants may result from the incompatibility of the inserted

sequence with other parts of the MOR protein. This may explain the correlation between increase in length of exchanged sequence with increased DNA-binding, the short 15 amino acid human androgen receptor sequence may be less compatible than the longer hAR and hRARα sequences. It has been shown that chimeric mutants consisting of either the human oestrogen receptor (hER) containing a glucocorticoid receptor DNA-binding domain or the human RARα containing the hER DNA-binding domain are fully functional as trans-activators of promoters containing a GRE or ERE, respectively [23, 27] and therefore can presumably dimerise and bind to DNA *in vivo*. This demonstrates that the DNA binding domains and hormone binding domains of different receptors are functionally compatible. The reduction in DNA binding of the MOR chimeric receptors compared to the wild type MOR may therefore result from the loss of sequence compatibility within the hormone binding domain. The loss of steroid binding may also account in part for the reduction in DNA-binding, since it has been shown that mutants of the MOR which are deficient in dimerisation and high affinity DNA-binding, but retain steroid binding, show restored DNA, binding activity in the presence of oestradiol (P. Danielian, personal communication). However steroid binding is not an essential prerequisite for dimerisation and DNA binding in the gel shift assay [10].

In conclusion, sequences have been identified in the hormone binding domain of the MOR which are essential for dimerisation and high affinity DNA, binding and a sequence comparison reveals that equivalent residues may be conserved in other members of the nuclear hormone receptor superfamily. The introduction of corresponding sequences from the hAR and HRARα into the MOR generates chimeric mutants with reduced DNA-binding activity compared to the wild type receptor. This implies that dimerisation function of the wild type receptor is not restricted solely to the sequence which forms a major part of the dimer interface, and may therefore involve a number of intramolecular interactions throughout the hormone binding domain.

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