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Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors

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The oestrogen receptor stimulates transcription by means of at least two distinct transcriptional activation domains, TAF-1 in the N-terminal domain and TAF-2 in the hormone binding domain. Here we show that TAF-2 activity requires a region in the C-terminus of the hormone binding domain between residues 538 and 552 in the mouse oestrogen receptor which is conserved among many nuclear hormone receptors. Point mutagenesis of conserved hydrophobic and charged residues significantly reduced ligand dependent transcriptional activation but had no effect on steroid or DNA binding. Mutation of the corresponding residues in the glucocorticoid receptor also abolished transcriptional activation. We therefore propose that the conserved region may be essential for ligand dependent transcriptional activation by other members of the nuclear receptor family.

Key words: oestrogen receptor/glucocorticoid receptor/transcriptional activation

Introduction

Steroid receptors belong to a family of nuclear hormone receptors which act as ligand inducible transcription factors by interacting with specific *cis*-acting enhancer sequences called hormone response elements (Green and Chambon, 1988; Evans, 1988; Beato, 1989; Ham and Parker, 1989). The binding of steroid causes dissociation of receptor from an inactive complex containing hsp90 allowing subsequent receptor dimerization, high affinity DNA binding (Catelli *et al.*, 1985; Sanchez *et al.*, 1985; Denis *et al.*, 1988; Kumar and Chambon, 1988; Tsai *et al.*, 1988, 1989) and transcriptional activation (Kumar *et al.*, 1987; Godowski *et al.*, 1988; Webster *et al.*, 1988). The regions of the receptor responsible for a number of these functions are conserved at both the structural and functional levels within the members of the nuclear receptor family. For example the DNA binding domain, which is located between the N-terminal domain and the hormone binding domain, is not only highly conserved in sequence but its structures, at least in the glucocorticoid and oestrogen receptors, is also similar (Hard *et al.*, 1990; Schwabe *et al.*, 1990; Luisi *et al.*, 1991). Other regions that are conserved in sequence include the hormone binding domain (Evans, 1988) which also contains residues for receptor dimerization (Fawell *et al.*, 1990a) and a nuclear localization signal near the DNA binding domain (Picard and Yamamoto, 1987; Guiochon-Mantel *et al.*, 1989).

In the oestrogen receptor two transcriptional activation functions (TAFs) have been defined using transient transfection experiments. TAF-1, in the N-terminal domain, is constitutively active while the activity of TAF-2, in the hormone binding domain, requires the binding of hormone (Kumar *et al.*, 1987; Webster *et al.*, 1988, 1989; Lees *et al.*, 1989; Tora *et al.*, 1989). The activities of TAF-1 and TAF-2 vary depending upon the responsive promoter and cell type and, in some cases, both are required for full transcriptional stimulation (Tora *et al.*, 1989). Neither of these TAFs appear to contain transcriptional activation motifs composed of acidic, or glutamine residues (see Mitchell and Tjian, 1989). The glucocorticoid receptor also contains transcriptional activation regions located within the N-terminal and the hormone binding domains as well as an activating region in the DNA binding domain (Danielsen *et al.*, 1987; Hollenberg *et al.*, 1987; Miesfeld *et al.*, 1987; Godowski *et al.*, 1988; Hollenberg and Evans, 1988; Webster *et al.*, 1988; Tasset *et al.*, 1990).

In view of the degree of conservation in a number of receptor functions such as DNA and hormone binding (Evans, 1988), we considered the possibility that certain transcriptional activation functions might also be conserved. In this paper we have identified amino acids near the C-terminus of the mouse oestrogen and glucocorticoid receptors which are essential for hormone dependent stimulation of transcription. The conservation of this region suggests that it participates in ligand dependent transcriptional activation by other members of the nuclear receptor family.

Results

A region required for hormone dependent stimulation of transcription by the mouse oestrogen receptor is conserved in other nuclear hormone receptors

Our previous deletion analysis showed that the C-terminal boundary of TAF-2 in the mouse oestrogen receptor (MOR) is located between residues 538 and 552 (Lees *et al.*, 1989). An alignment of sequences in this part of the hormone binding domain of members of the nuclear receptor family revealed a conserved region. The major features include an invariant glutamic acid residue flanked by two pairs of hydrophobic residues (Figure 1). The sequence is located in a similar position at the C-terminus of these proteins and is preceded by proline(s) in each case. The region was absent from v-erbA and some nuclear receptor related proteins including COUP, nur77/N10 and a number of *Drosophila* proteins (see Discussion). In the oestrogen receptor analysis of secondary structure predictions indicates that this region may form an α-helix, the relative positions of three

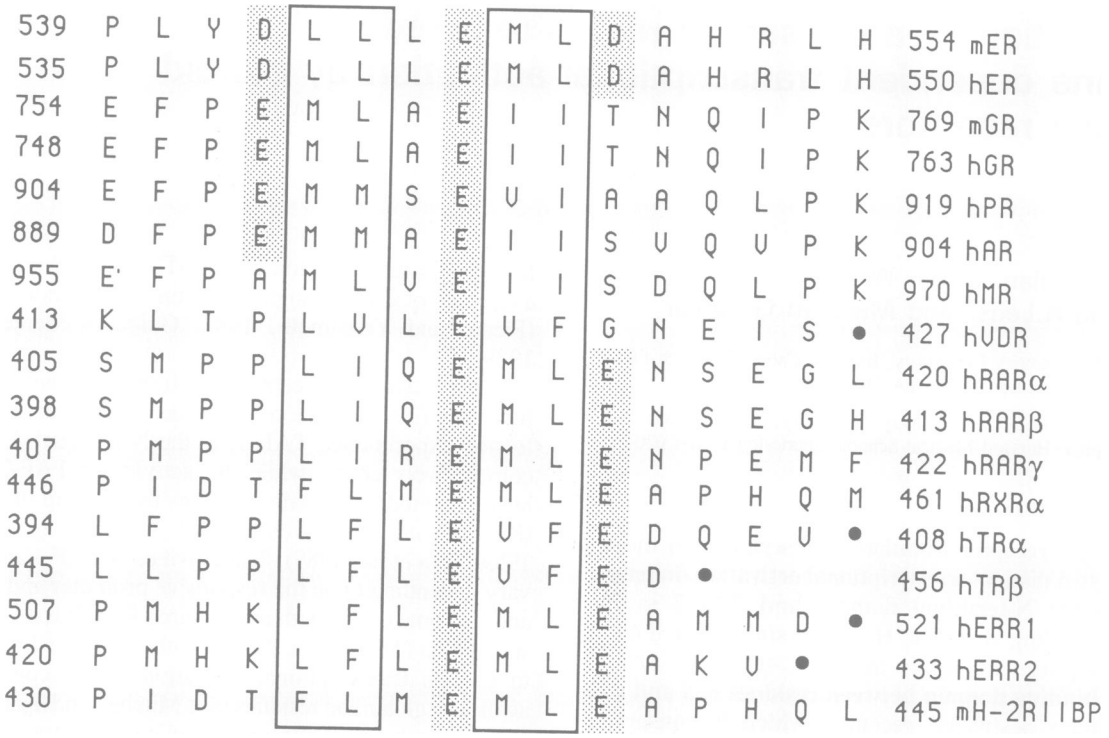

Fig. 1. Sequence alignment of nuclear receptor proteins. The sequences of mouse (m) or human (h) proteins are presented (White *et al.*, 1987; Green *et al.*, 1986; Hollenberg *et al.*, 1985; Misrahi *et al.*, 1987; Chang *et al.*, 1988; Arriza *et al.*, 1987; Baker *et al.*, 1988; Giguére *et al.*, 1987; Benbrook *et al.*, 1988; Krust *et al.*, 1989; Mangelsdorf *et al.*, 1990; Sap *et al.*, 1986; Weinberger *et al.*, 1986; Giguére *et al.*, 1988; Hamada *et al.*, 1989) with the conserved hydrophobic residues boxed and negatively charged residues shaded. The residues are conserved in the listed receptors from other species. Amino acid numbers are indicated and solid circles represent the C-terminus of the protein.

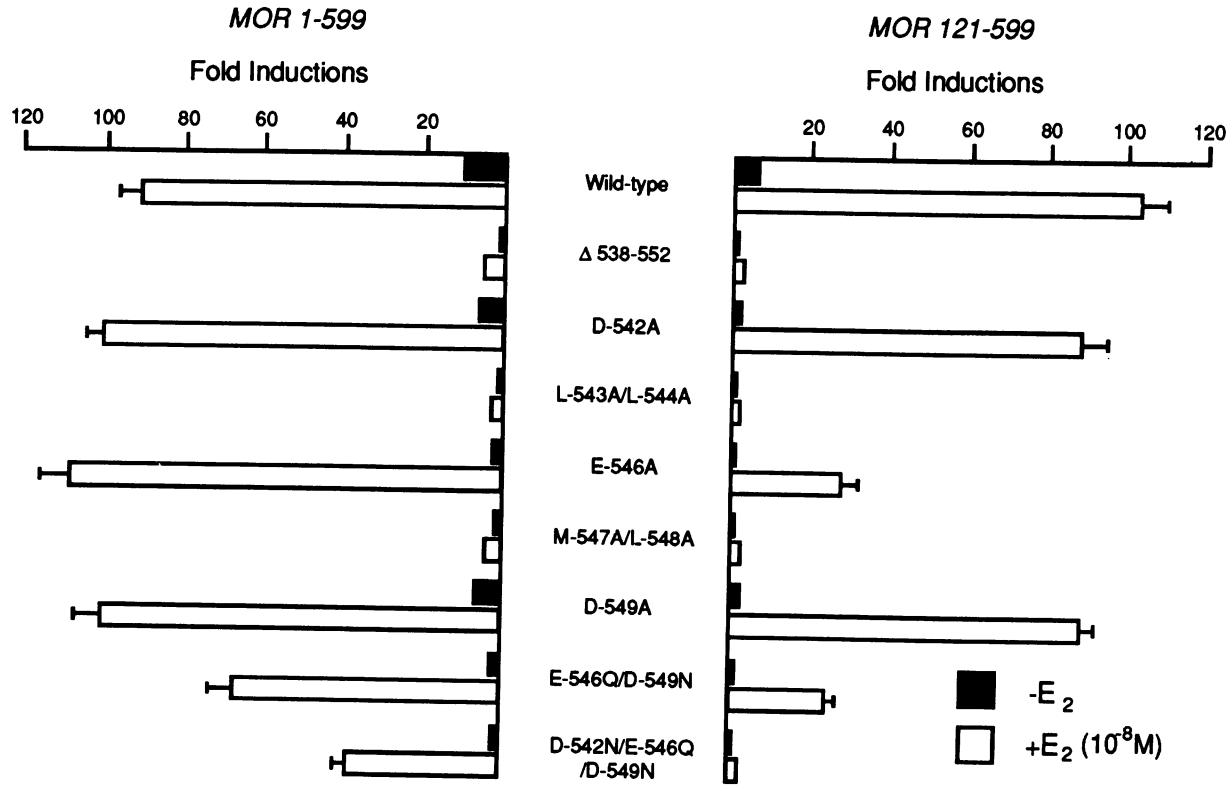

Fig. 2. Transcriptional activation by mutant oestrogen receptors. The ability of a series of mutant mouse oestrogen receptors (MORs) to stimulate transcription of a reporter gene pEREBLCAT was tested by transient transfection in NIH3T3 cells (see Materials and methods). Transcriptional activation is expressed as total induction over the reporter alone in the absence and presence of 10^{-8} M oestradiol (E_2). The mutations were introduced into the full-length receptor MOR1−599 and into MOR121−599 lacking TAF-1 (see Figure 3A). The error bars (which are too small to be visible in some cases) represent standard errors determined from at least three transfection experiments each carried out in duplicate.

negatively charged residues and the hydrophobic residues suggest that it may be amphipathic in nature.

Transcriptional activation by mutant mouse oestrogen receptors

To confirm the importance of the conserved region we examined the effect of deleting residues 538–552 from the wild-type receptor on its ability to stimulate transcription from pEREBLCAT in a transient transfection assay. This deletion reduced the hormone dependent transcriptional activation from ~90-fold to <5-fold (Figure 2) whilst not significantly affecting DNA and steroid binding functions (see later and Table I). To test if this region itself was capable of stimulating transcription we fused residues 538–552 to the DNA binding domain of GAL4 but found that it was unable to stimulate transcription in transient transfection assays (data not shown). The contribution of the conserved residues to transcriptional activation was determined by examining the effects of replacing the conserved negatively charged and hydrophobic residues with other amino acids. The point mutations were introduced either into the full length receptor (MOR1–599) or into an N-terminal deletion mutant (MOR121–599) which lacks TAF-1 (see Figure 3A). This allowed us to determine the effects of mutations upon TAF-2 activity in the absence or presence of TAF-1.

We first examined the effects of the mutations upon the full-length receptor. Analysis of transcriptional activation by these mutants showed that the most disruptive mutations were those involving the hydrophobic amino acids flanking the conserved glutamic acid E-546 (Figure 2). Both the mutants L-543A/L-544A and M-547A/L-548A had negligible transcriptional activity (4-fold or less). Replacement of these residues with aspartic acid also had a similar effect (data not shown). In contrast replacement of either of the aspartic acid residues (D-542A or D-549A) or the highly conserved glutamic acid residue with an alanine residue (E-546A) had little effect upon the level of transcriptional activation. To examine the contribution of the charged residues further we replaced two (E-546Q/D-549N) or all three (D-542N/E-546Q/D-549N) of the negatively charged residues in the putative amphipathic α-helix and found that transcriptional activity was still stimulated 40- to 70-fold.

The effect of the point mutations was also tested in MOR121–599 to determine their effects upon the activity of TAF-2 in the absence of TAF-1. Mutation of the pairs of hydrophobic residues in L-543A/L-544A and M-547A/L-548A, as expected, reduced the hormone dependent transcriptional activation to <3-fold (Figure 2). The result of mutating either of the aspartic acid residues also was similar to that in the full-length receptor having little effect upon transcriptional activation. However, mutation of the glutamic acid residue (E-546A) reduced transcriptional activation to ~25% that of the wild-type receptor and replacement of all three charged residues (D-542N/E-546/Q/D-549N) further reduced transcriptional activation to only ~3-fold. Of the three charged residues it appeared that the conserved glutamic acid (E-546) made the greatest contribution to TAF-2 whilst some TAF-2 activity was retained when only one aspartic acid residue was present (compare E-546Q/D-549N and D-542N/E-546Q/D-549N).

Thus the mutation of the charged residues had different effects upon the transcriptional activity of the full-length and N-terminally deleted receptors. This was most marked in the case of the triple mutant D-542N/E-546Q/D-549N where transcription was stimulated 3-fold when the receptor contained TAF-2 alone (MOR121–599) but ~40-fold in

Table I. Oestrogen binding activity of wild-type and mutant receptors

Receptor	Steroid binding kDa (nM)
Wild-type	0.13
D-542A	0.20
L-543A/L-544A	0.28
E-546A	0.20
M-547A/L-548A	0.39
D-549A	0.64
E-546Q/D-549N	0.16
D-542N/E-546Q/D-549N	0.21

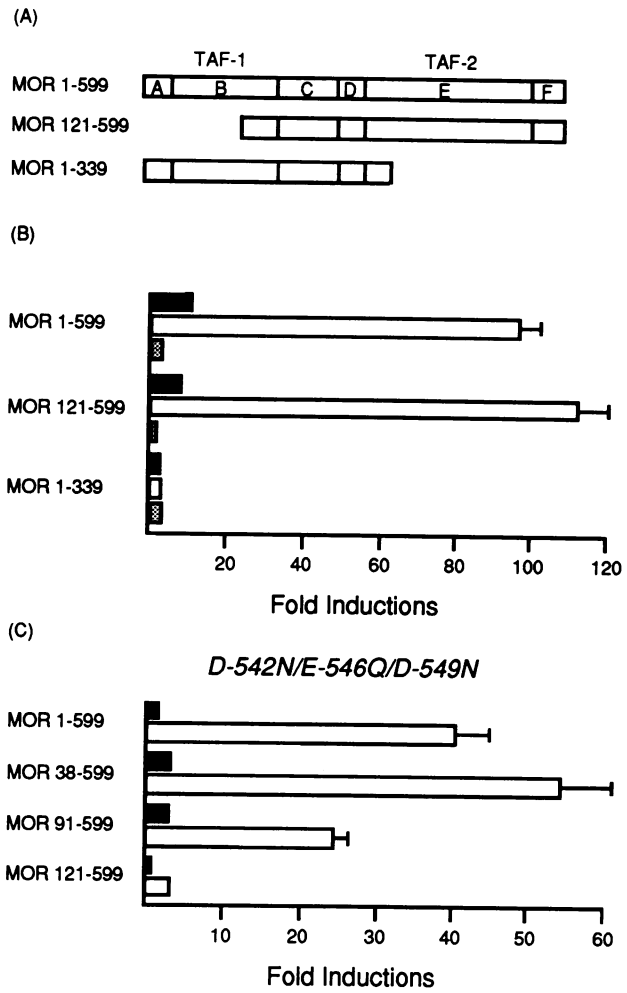

Fig. 3. Co-operation between the N- and C-terminal domains in transcriptional activation. (**A**) Schematic organization of the MOR deletion mutants. The positions of TAF-1 and TAF-2 are indicated together with the regions denoted A–F (Krust *et al.*, 1986). (**B**) The full-length receptor MOR1–599 and the mutant receptors MOR1–339 containing TAF-1 and MOR121–599 containing TAF-2 were tested for their transcriptional activity in the absence (solid bars) or presence of 10^{-8} M oestradiol (open bars) and 10^{-6} M tamoxifen (shaded bars). (**C**) The triple mutation D-542N/E-546Q/D-549N was introduced into MOR1–599, MOR38–599, MOR91–599 and MOR121–599 and tested for transcriptional activation. The error bars represent standard errors from at least three transfection experiments each carried out in duplicate.

MOR1−599 when TAF-1 was present. The transcriptional activity of TAF-1 alone was 3- to 5-fold (Figure 3B) when tested either as a deletion mutant MOR1−339 (Figure 3A) or in the full length receptor in the presence of tamoxifen to inhibit TAF-2 activity (Lees *et al.*, 1989; Berry *et al.*, 1990). Thus, in the case of D-542N/E-546Q/D-549N, TAF-1 virtually rescues wild-type levels of transcriptional activity. The mutants E-546A and E-546Q/D-549N showed a similar result. It is unclear from these results whether TAF-1 and TAF-2 are able to interact in the wild-type receptor since deletion of TAF-1 had little effect on the level of transcriptional activation using the reporter pEREBLCAT (Figure 2). We therefore performed similar experiments using a reporter containing a single ERE placed directly upstream from a TATA box, pEREMLTCAT, where TAF-1 and TAF-2 are both required for full transcriptional activity (Figure 4). Deletion of TAF-1 or TAF-2 resulted in 17% or 2% transcriptional activity respectively in the presence of oestradiol compared with that of the wild-type receptor. Replacement of the negatively charged residues in the absence of TAF-1 reduced transcriptional activity to 7% in the case of E-546A and to 1% for D-542N/E-546Q/D-549N. The presence of TAF-1, which in isolation has little activity, rescued transcriptional activity to nearly wild-type levels (40%) for E-546A and to 9% for D-542N/E-546Q/D-549N. When the conserved hydrophobic residues were replaced with alanines (M-547A/L-548A) TAF-1 was unable to rescue transcriptional activity; this is similar to the results observed with pEREBLCAT.

The region of the N-terminus of the protein responsible for rescuing transcriptional activity was mapped by analysing the activity of a series of deletion mutants of D-542N/E-546Q/D-549N on pEREBLCAT. The N-terminus of the oestrogen receptor has been divided into two regions A and B on the basis of amino acid homology between different receptors (Krust *et al.*, 1986). Deletion of region A (MOR38−599), which is highly conserved among oestrogen receptors from different species, did not reduce the transcriptional activity compared with the full-length receptor (Figure 3C). The fact that the majority of the activity of the full-length receptor was also retained by MOR91−599 suggests that residues between 91 and 121 are important in the interaction with TAF-2.

Mutations within the conserved region which abolished transcriptional activation did not significantly affect steroid or DNA binding

To establish whether the conserved reigon was directly involved in transcriptional activation we investigated whether the mutations affected the expression of the receptor protein or its ability to bind oestradiol or DNA. Expression of the wild-type and mutant receptor proteins in transfected NIH3T3 cells was studied by immunofluorescence using the monoclonal antibody H222 raised against the human oestrogen receptor (Greene *et al.*, 1984). The transfected cells were shown to contain wild-type and mutant receptor proteins in similar amounts and all receptor proteins were located to the nucleus (data not shown). The receptor proteins made in NIH3T3 cells were difficult to detect by other methods so we additionally evaluated the relative levels of wild-type and mutant receptor proteins in transfected COS-1 cells. Gel-shift assays using whole cell extracts of transfected cells confirmed that the receptors retained high affinity DNA

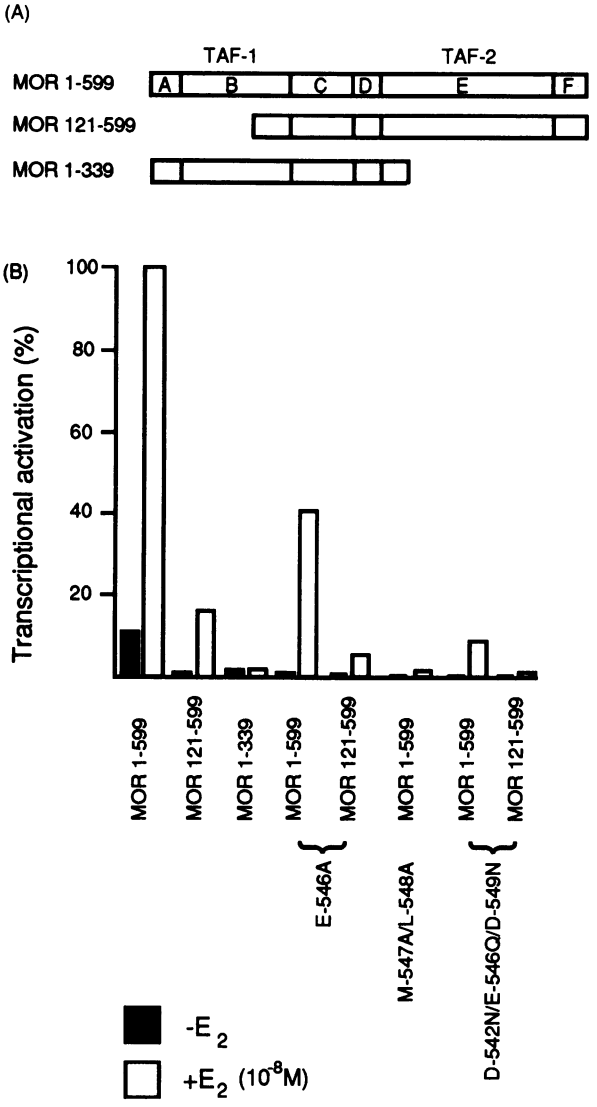

Fig. 4. Co-operation between the N- and C-terminal domains in transcriptional activation. (**A**) Schematic organization of the MOR deletion mutants. The positions of TAF-1 and TAF-2 are indicated together with the regions denoted A−F (Krust *et al.*, 1986). (**B**) The full-length receptor MOR1−599 and the mutant receptors MOR1−339 containing TAF-1 and MOR121−599 containing TAF-2 were tested for their ability to stimulate transcription from pEREMLTCAT in the absence (solid bars) or presence of 10^{-8} M oestradiol (open bars). Transcriptional activation is expressed as a percentage of the activation achieved by the wild-type receptor in the presence of 10^{-8} M oestradiol. The results were obtained from two transfection experiments each carried out in duplicate.

binding and were expressed in comparable amounts with insufficient variations to account for the alterations in transcriptional activity (Figure 5). Since the transcriptional activity of TAF-2 is dependent upon oestradiol binding we next tested the ability of the mutant receptors to bind oestradiol. The wild-type and mutant receptor proteins were synthesized *in vitro* and their affinity for oestradiol determined by Scatchard analysis. The dissociation constants of the mutant receptors were in the range 0.16−0.64 nM (Table I) compared with 0.13 nM for the wild-type receptor. Thus all the receptors would be expected to be saturated with hormone at the concentration used in the transfection experiments, namely 10^{-8} M.

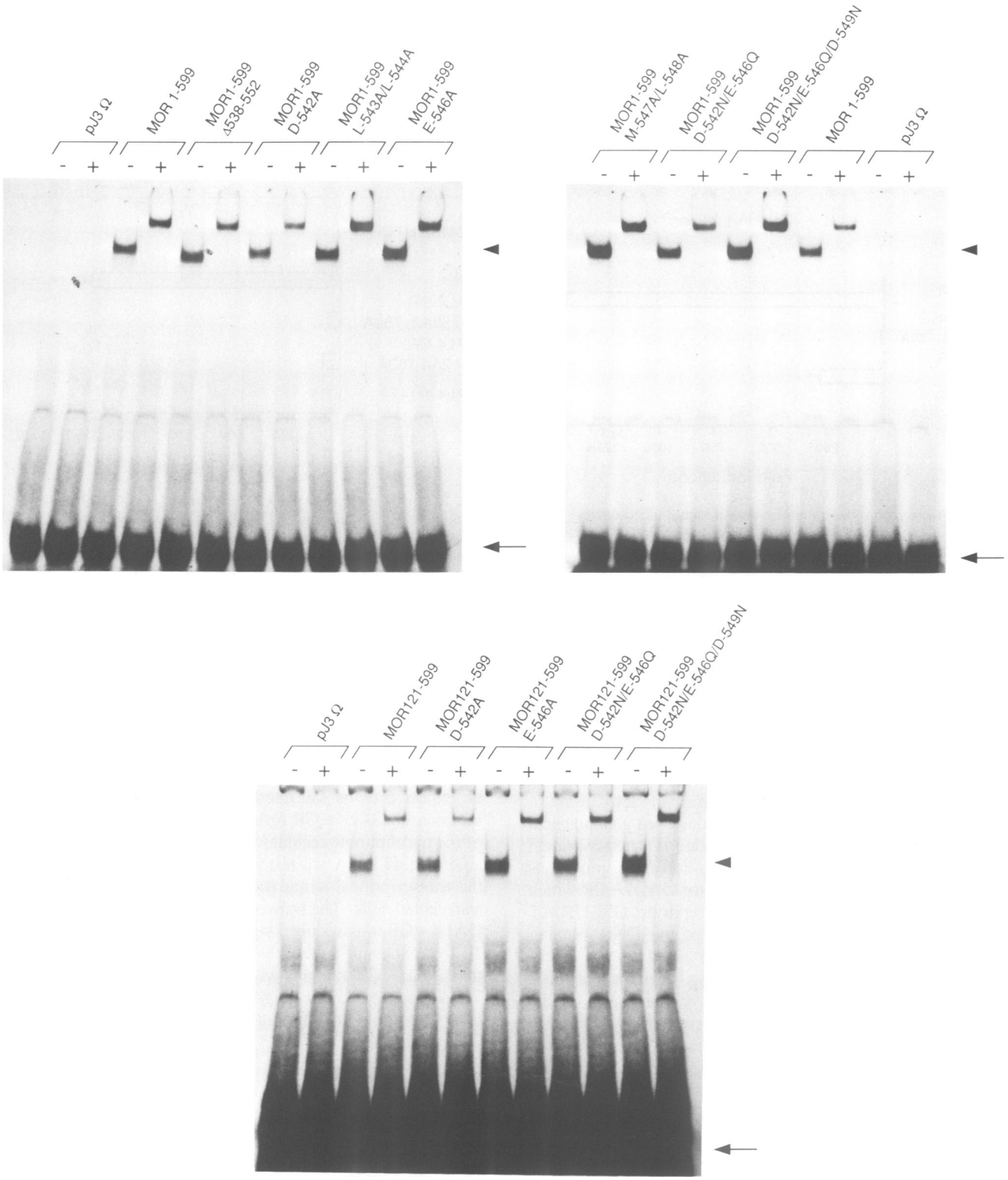

Fig. 5. DNA binding activity of wild-type and mutant oestrogen receptors expressed in COS-1 cells. The amounts of wild-type and mutant receptors that were transiently expressed were determined by examining their DNA binding activity. Equal amounts of protein extract from transfected COS-1 cells were tested in a gel-shift assay using a DNA probe consisting of a ^{32}P-labeled oligonucleotide containing a consensus ERE sequence (Materials and methods). The oestrogen receptor–DNA complexes were identified by using pre-immune serum (−) or a specific oestrogen receptor antiserum MP16 (+). The arrow and arrowhead indicate free and retarded probe, respectively.

Mutations of the conserved residues in the mouse glucocorticoid receptor have similar effects

The importance of the conserved region for hormone dependent stimulation of transcription was investigated further by making corresponding mutations in the mouse glucocorticoid receptor. We first examined the full-length receptor which stimulated transcription from the reporter plasmid MMTV-CAT ~25-fold in the presence of 10^{-6} M

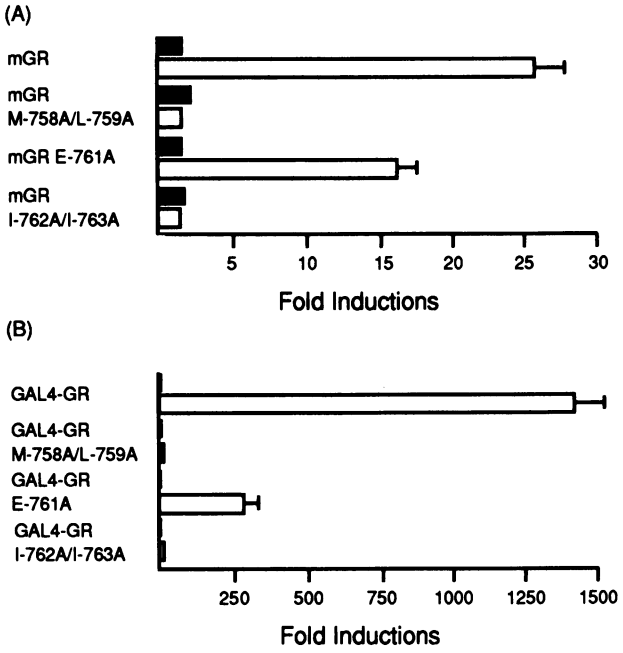

Fig. 6. Transcriptional activation by mutant mouse glucocorticoid receptors. The ability of wild-type and mutant glucocorticoid receptors to stimulate transcription was tested by transient transfection. (**A**) The full-length receptors were cotransfected with the MMTV−CAT reporter and transcriptional activation is expressed as total induction over the reporter alone in the absence (solid bars) or presence of 10^{-6} M dexamethasone (open bars). (**B**) The chimeric receptors, GAL4−GR, were cotransfected with the G5E1BCAT reporter and transcriptional activation determined as in (A). The error bars (which are not always apparent) represent standard errors determined from at least three experiments, each carried out in duplicate.

Table II. Dexamethasone binding in transfected COS-1 cells

	Dexamethasone binding (fmol/mg protein at 10^{-7} M)
Wild-type	3806 ± 260
M-758A/L-759A	10212 ± 7
E-761A	3992 ± 645
I-762A/I-763A	1568 ± 360
GAL41−147	145 ± 22

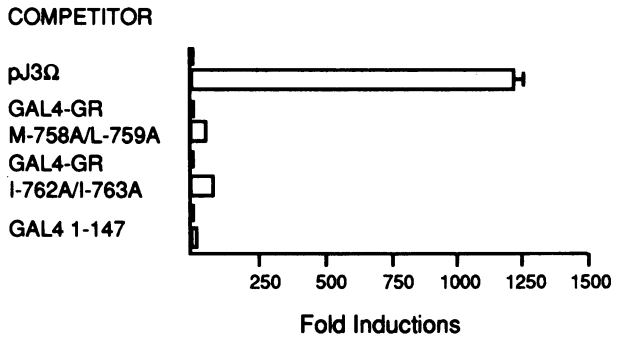

Fig. 7. *In vivo* interference assay. The ability of GAL4−GR mutants to interfere with the activity of GAL4−GR was examined by cotransfection with the reporter plasmid G5E1BCAT in COS-1 cells. Transcriptional activation is expressed as the fold induction achieved in the absence (solid bars) or the presence of 10^{-6} M dexamethasone (open bars). Transcription was inhibited 95% by GAL4−GR M-758ATL-759A, 93% by GAL4−GR I-762A/I-763A and 99% by GAL4 1−147. pJ3Ω indicates a control vector. The error bars (which are not always apparent) represent standard errors determined from at least three experiments, each carried out in duplicate.

dexamethasone (Figure 6A). Mutation of the pairs of conserved hydrophobic residues (M-758A/L-759A or I-762A/I-763A) reduced transcriptional activation to <2-fold. Replacement of the highly conserved glutamic acid residue (E-761A) with alanine reduced transcriptional activation slightly to 15-fold. In addition these mutations were introduced into a chimeric receptor GAL4−GR which stimulated transcription from the reporter G5E1BCAT >1000-fold. GAL4−GR consisted of the DNA binding domain of GAL4 (residues 1−147) fused to the glucocorticoid receptor hormone binding domain (residues 506−783) and G5E1BCAT contained five copies of the GAL4 DNA binding site (Lillie and Green, 1989). Mutation of the conserved hydrophobic residues again resulted in a receptor with negligible transcriptional activity (Figure 6B). When the glutamic acid residue was replaced with alanine (E-761A) transcriptional activation was reduced by 80−85%. To determine if these mutations specifically affected transcriptional activation the expression of the wild-type and mutant full-length receptor proteins in transfected cells was studied by immunofluorescence using the monoclonal antibody 250 raised against the rat glucocorticoid receptor. The transfected cells were shown to contain wild-type and mutant receptor proteins in similar amounts and all receptor proteins were located to the nucleus in the presence of dexamethasone (data not shown). We next tested the ability of the mutant receptor proteins to bind hormone using a whole cell binding assay (Danielsen *et al.*, 1986). All the mutants receptor proteins bound dexamethasone at 10^{-7} M (Table II). The binding capacity of E-761A was similar to that of the wild-type receptor and that of the hydrophobic mutants was 25−40% that of the wild-type receptor. Since the transient transfections were carried out at 10^{-6} M dexamethasone all the mutant receptors would be expected to be saturated with hormone. To determine whether the GAL4−GR fusion proteins containing mutations of the hydrophobic residues were located in the nucleus and able to bind DNA we tested their ability to interfere with the transcriptional stimulation achieved by GAL4−GR in transfected cells. The transcriptional activation achieved by GAL4−GR was reduced significantly in the presence of a 10-fold excess of either the GAL4 DNA binding domain or the GAL4−GR mutants M-758A/L-759A and L-762A/L-763A (Figure 7) indicating that the mutant receptors were competing for and binding to the GAL4 binding site in transfected cells.

Discussion

Steroid receptors stimulate gene transcription by means of multiple transcriptional activation regions which function either constitutively or depend on hormone binding for their activity. A hormone dependent activation function TAF-2 has been characterized in the oestrogen receptor (Kumar *et al.*, 1987; Webster *et al.*, 1988, 1989; Lees *et al.*, 1989; Tora *et al.*, 1989) and analogous functions have also been identified in the receptors for glucocorticoids (Webster *et al.*, 1988; Tasset *et al.*, 1990) and progestins (Meyer *et al.*, 1990). Previous studies of TAF-2 have indicated that it is comprised of a number of dispersed elements throughout the hormone binding domain brought together upon oestrogen binding (Webster *et al.*, 1989).

Here we have shown that an essential element of TAF-2 is located between residues 538 and 552 in the mouse oestrogen receptor. Point mutagenesis of this conserved region showed that hydrophobic residues flanking the conserved glutamic acid are essential for transcriptional activation. Replacement of either pair of hydrophobic residues with alanines abolished transcriptional activation without significantly affecting steroid and DNA binding functions, indicating that these residues are specifically involved in transcriptional activation. The negatively charged residues within the putative amphipathic α-helix are important for TAF-2 activity in the absence of TAF-1 since the replacement of all three residues with the corresponding amide (D-542N/E-546Q/D-549N) significantly reduced TAF-2 activity whilst retaining steroid and DNA binding functions.

Previous work has demonstrated that TAF-1 and TAF-2 are able to synergize on certain promoters (Tora *et al.*, 1989; Tasset *et al.*, 1990). We have found that the ability of TAF-1 to synergize with TAF-2 on pEREMLTCAT was not observed when we replaced the hydrophobic residues but was retained when the negatively charged residues were mutated. These mutants showed similar effects on pEREBLCAT although in this case the deletion of TAF-1 had little effect on the activity of the wild-type receptor. Thus our results with the mutant receptors suggest that TAF-1 and TAF-2 might interact although synergistic interactions between them may not be apparent in the wild-type receptor. The conserved element we have identified here therefore may be essential not only for TAF-2 activity but also its ability to synergize with TAF-1. Part of the region in the N-terminus which co-operates with TAF-2 was mapped to between residues 91 and 121 in the MOR.

The residues we identified as being important for transcriptional activation by the mouse oestrogen receptor were also found to be essential for transcriptional activation by the mouse glucocorticoid receptor. The mutation of the corresponding hydrophobic residues and conserved glutamic acid affected transcriptional activation in a similar fashion to that for the oestrogen receptor indicating that the function of these residues is conserved. This is in agreement with studies which have suggested that in addition to the relatively weak acidic activating regions in the glucocorticoid receptor there are transcriptional activation functions analogous to TAF-1 and TAF-2 characterized in the oestrogen receptor (Tasset *et al.*, 1990). Moreover the N- and C-terminal transcriptional activation functions of the glucocorticoid receptor may also co-operate with one another to stimulate transcription (Godowski *et al.*, 1988; Tasset *et al.*, 1990).

It has been proposed that transcription factors interact with basic transcription factors and/or coactivators (bridging proteins) to stimulate gene transcription (Ptashne, 1988; Ptashne and Gann, 1990; Lewin, 1990 and references therein). It is possible that the region we have identified may interact directly with these target proteins or might be involved in maintaining the structure of the interacting surface. Similarly, the synergy between the N- and C-terminal domains may reflect intramolecular interactions within the receptor dimer involved in the formation of an interacting surface. One approach to study the target proteins of transcription factors has been to examine the ability of transcription factors to co-operate with (synergize) or interfere with (squelch) one another. By this method the activities of TAF-1 and TAF-2 in the oestrogen receptor have been compared with each other, with those in the glucocorticoid receptor and with the acidic activation domain of VP16 (Bocquel *et al.*, 1989; Meyer *et al.*, 1989; Tora *et al.*, 1989; Tasset *et al.*, 1990). These experiments suggest that TAF-2 from both receptors was able to interact with similar target proteins that were distinct from those with which TAF-1 and the acidic activation domain interacted. Recent work, in which it was shown that the oestrogen receptor is able to synergize with CTF/NF-1, suggests that TAF-1, TAF-2 and the proline-rich activation region of CTF/NF-1 interact with at least one common target protein (Martinez *et al.*, 1991). It is conceivable that mutation of the hydrophobic residues in the conserved region would abolish this synergistic transcriptional activation.

The importance of the conserved region in hormone dependent transcriptional stimulation is consistent with the observation that its deletion is responsible for the lack of transcriptional activation by v-erbA (Damm *et al.*, 1989; Zenke *et al.*, 1990). V-erbA is a mutant version of the thyroid hormone type α receptor (TRα) (Sap *et al.*, 1986; Weinberger *et al.*, 1986) and the deletion of the conserved region may contribute to its oncogenic activity (Zenke *et al.*, 1990). The conserved region is also absent from a number of hormone receptor related proteins including COUP (Wang *et al.*, 1987), nur77/N10 (Hazel *et al.*, 1988; Ryseck *et al.*, 1989), HNF-4 (Sladek *et al.*, 1990), ARP-1 (Ladias and Karathanasis, 1991) and several *Drosophila* proteins (Nauber *et al.*, 1988; Oro *et al.*, 1988; Feigl *et al.*, 1989; Rothe *et al.*, 1989; Lavorgna *et al.*, 1990; Mlodzik *et al.*, 1990; Pignoni *et al.*, 1990; Segraves and Hogness, 1990). It is interesting to note that none of these proteins have been shown to have a ligand which suggests that the conserved region may be involved specifically in ligand inducible transcriptional activation. Members of the nuclear hormone receptor family lacking the conserved region may therefore stimulate transcription by a different mechanism.

Transcriptional activation functions have yet to be identified in many nuclear hormone receptors. However, the receptors for retinoic acid (Kakizuka *et al.*, 1991; de Thé *et al.*, 1991) and thyroid hormone (Thompson and Evans, 1989) appear to lack a constitutive TAF-1 activity and this may also be the case for receptors lacking appreciable N-terminal sequences, such as the vitamin D receptor (Baker *et al.*, 1988). As a consequence they may rely entirely on sequences within their hormone binding domains for transcriptional activation (O'Donnell and Koenig, 1990; Kakizuka *et al.*, 1991; de Thé *et al.*, 1991). Since the C-terminal region we have characterized is present in these receptors as well as in all other steroid receptors, we suggest that it may play an essential role in ligand dependent transcriptional activation.

Materials and methods

Receptor expression vectors

Oligonucleotide-directed mutagenesis was used to introduce mutations into the mouse oestrogen receptor (MOR) cDNA in the vectors pSP64 or pGEM3 (Promega Limited, Southampton, UK) and these were transferred into pJ3MOR (White *et al.*, 1987) for transient transfection. pJ3MOR91−599 (previously named pJ3MOR89−599) and pJ3MOR121−599 have been described previously (Lees *et al.*, 1989) and pJ3MOR38−599 was generated by oligonucleotide-directed mutagenesis in pGEM3MOR and then transferred into the expression vector pJ3Ω (Morgenstern and Land, 1990). Oligonucleotide-directed mutagenesis was also used to introduce mutations into the mouse glucocorticoid receptor cDNA in the expression vector pSV2Wrec (Danielsen *et al.*, 1986). The chimeric glucocorticoid receptor,

GAL4−GR, encoding residues 1−147 of the GAL4 DNA binding domain fused to residues 506−783 of the mouse glucocorticoid receptor, was constructed by transferring a fragment from pSV2Wrec into pSG424 (Sadowski and Ptashne, 1989) resulting in a phenylalanine residue at the fusion point. All mutations were verified by DNA sequencing. The point mutants are described as the amino acid, its position and the amino acid with which it was replaced, e.g. D-542A indicates that the aspartic acid at position 542 in the wild type receptor was replaced with an alanine residue, a Δ indicates that these residues were deleted from the wild-type receptor.

Reporter constructs

pEREBLCAT (Lees *et al.*, 1989) contains on oestrogen response element (ERE) derived from the vitellogenin A2 promoter upstream of the promoter sequences of the herpes simplex viral thymidine kinase gene linked to the reporter gene chloramphenicol acetyl transferase (CAT). pEREMLTCAT contains an oestrogen response element (identical to that in pEREBLCAT) upstream of the adenovirus major late promoter sequences (−44 to +11) (Hu and Manley, 1981) linked to the reporter gene chloramphenicol acetyl transferase (CAT). The glucocorticoid reporter plasmid MMTV−CAT contains sequences from −1225 to +268 of the mouse mammary tumour virus LTR fused to the CAT gene (Parker *et al.*, 1987). The GAL4 reporter G5E1BCAT contained five GAL4 DNA binding sites upstream of the E1b TATA box linked to CAT sequences (Lillie and Green, 1989).

Cell culture, transient transfection and CAT assays

NIH3T3 and COS-1 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco, Paisley, Scotland). For transient transfection experiments the NIH3T3 cells were plated out at 2×10^5 cells/6 cm dish in phenol red-free DMEM, 10% dextran-charcoal treated fetal calf serum and transfected using the calcium phosphate coprecipitation method. The transfected DNA included a reporter plasmid pEREBLCAT (5 μg), pEREMLTCAT (5 μg), MMTV−CAT (5 μg) or G5E1BCAT (1 μg); 1 μg of an internal control plasmid (pJ3 luciferase); the appropriate receptor expression vector MOR (0.5 μg), pSV2Wrec (4 μg) or GAL4−GR (1.2 μg) and pJ3Ω (Morgenstern and Land, 1990) to a total of 10 μg/dish. Following transfection, cells were maintained in the absence or presence of 10^{-8} M oestradiol, 10^{-6} M tamoxifen or 10^{-6} M dexamethasone as indicated. After 48 h the cells were harvested and extracts assayed for luciferase (De Wet *et al.*, 1987) and CAT activity (Sleigh, 1986). Luciferase activity was used to correct for differences in transfection efficiency in all experiments. COS-1 cells were transfected by electroporation. Cells were harvested when 70% confluent, resuspended in phosphate buffered saline at 6×10^6/ml, and 20 μg of receptor expression vector plus 1 μg of pJ3 luciferase added. The cells were then electroporated at 450 V and 250 μF using Bio-Rad Gene Pulser and plated out in phenol red-free DMEM containing 10% dextran-charcoal treated fetal calf serum. After 50 h the transfected cells were used for whole cell binding assays or harvested for either gel-shift assays (Fawell *et al.*, 1990b) or luciferase assays (De Wet *et al.*, 1987). For the *in vivo* interference experiments 1 μg of G5E1BCAT and 1 μg of pJ3Luciferase were cotransfected into COS-1 cells with either pJ3Ω (reporter alone) or 1 μg of GAL4−GR plus 10 μg of one of the following pJ3Ω, GAL4−GR M-758A/L-759A, GAL4−GR I-762/I-763A or GAL4 1−147.

Gel shift assays

COS-1 cells expressing wild-type and mutant MORs were harvested, collected by centrifugation and the pellet frozen at −70°C. Whole cell extracts were prepared in a high salt buffer (Fawell *et al.*, 1990b) and the protein concentration was determined using Bio-Rad protein assay reagent. The DNA binding activity of receptor protein was determined in a gel-shift assay using 2 μg of whole cell extract and a ^{32}P-labelled oligonucleotide corresponding to a 32 bp fragment of the vitellogenin A2 gene containing a consensus ERE (Lees *et al.*, 1989). Preimmune or specific oestrogen receptor antiserum MP16 (Fawell *et al.*, 1990b) was added to the binding reaction as indicated.

cRNA synthesis and in vitro translation

Recombinant MOR cDNAs in pSP64 or pGEM3 were linearized as appropriate, transcribed with SP6 RNA polymerase (Promega Biotec, Southampton, UK) and the capped mRNA used to programme synthesis of protein in rabbit reticulocyte lysate (Promega Biotec, Southampton, UK) as described previously (Fawell *et al.*, 1990a). [^{35}S]methionine labelled receptors translated in parallel were analysed by SDS−PAGE and these data used to normalize the input of unlabelled receptor in steroid binding assays.

Ligand binding assays

Ligand binding by wild-type and mutant MOR was analysed as described previously using [^{125}I]16α-iodoestradiol (NEN Research Products, Stevenage, UK) (Fawell *et al.*, 1989). Ligand binding by wild-type and mutant glucocorticoid receptor proteins was assayed in transfected COS-1 cells using a whole cell binding assay with the GAL4 DNA binding domain as a control (Danielsen *et al.*, 1986). Transfected cells were washed three times in phenol red-free DMEM and incubated with 100 nM [^{3}H]dexamethasone (Amersham, UK) in phenol red-free DMEM with or without a 500-fold excess of unlabelled dexamethasone for 1 h. Cells were then washed four times with ice-cold phosphate buffered saline, lysed in 1 M NaOH for 1 h and the retained ligand determined by scintillation counting. In each assay all points were performed in duplicate and the specifically bound counts were corrected for protein levels.

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References

Arriza,J.L., Weinberger,C., Cerelli,G., Glaser,T.M., Handelin,B.L., Housman,D.E. and Evans,R.D. (1987) *Science*, **237**, 268−275.

Baker,A.R., McDonnell,D.P., Hughes,M., Crisp,T.M., Mangelsdorf,D.J., Haussler,M.R., Pike,W.J., Shine,J. and O'Malley,B.W. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3294−3298.

Beato,M. (1989) *Cell*, **56**, 335−344.

Benbrook,D., Lernhardt,E. and Pfahl,M. (1988) *Nature*, **333**, 669−672.

Berry,M., Metzger,D. and Chambon,P. (1990) *EMBO J.*, **9**, 2811−2818.

Bocquel,M.T., Kumar,V., Soricker,C., Chambon,P. and Gronemeyer,H. (1989) *Nucleic Acids Res.*, **17**, 2581−2595.

Catelli,M.G., Binart,N., Jung-Testas,I., Renoir,J.M., Baulieu,E.-E., Fermisco,J.R. and Welch,W.J. (1985) *EMBO J.*, **4**, 3131−3135.

Chang,C., Kokontis,J. and Liao,S. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7211−7215.

Damm,K., Thompson,C.C. and Evans,R.M. (1989) *Nature*, **339**, 593−597.

Danielsen,M., Northrop,J.P. and Ringold,G.M. (1986) *EMBO J.*, **5**, 2513−2522.

Danielsen,M., Northrop,J.P., Jonklass,J. and Ringold,G.M. (1987) *Mol. Endocrinol.*, **1**, 816−822.

Denis,M., Poellinger,L., Wikström,A. and Gustafsson,J. (1988) *Nature*, **333**, 686−688.

de Thé,H., Lavau,C., Marchio,A., Chomienne,C., Degos,L. and Dejean,A. (1991) *Cell*, **66**, 675−684.

De Wet,J.R., Wood,K.V., Pehuea,M., Helinski,P.R. and Subromani,S. (1987) *Mol. Cell. Biol.*, **7**, 725−737.

Evans,R.M. (1988) *Science*, **240**, 889−895.

Fawell,S.E., Lees,J.A. and Parker,M.G. (1989) *Mol. Endocrinol.*, **3**, 1002−1005.

Fawell,S.E., Lees,J.A., White,R. and Parker,M.G. (1990a) *Cell*, **60**, 953−962.

Fawell,S.E., White,R., Hoare,S.M., Sydenham,M., Page,M. and Parker,M.G. (1990b) *Proc. Natl. Acad. Sci. USA*, **87**, 6883−6887.

Feigl,G., Gram,M. and Pongs,O. (1989) *Nucleic Acids Res.*, **18**, 7167−7177.

Giguére,V., Ong,E.S., Segui,P. and Evans,R.M. (1987) *Nature*, **330**, 624−629.

Giguére,V., Yang,N., Segui,P. and Evans,R.M. (1988) *Nature*, **331**, 91−94.

Godowski,P.J., Picard,D. and Yamamoto,K.R. (1988) *Science*, **241**, 812−816.

Green,S. and Chambon,P. (1988) *Trends Genet.*, **4**, 309−314.

Green,S., Walter,P., Kumar,V., Krust,A., Bornert,J.-M., Argos,P. and Chambon,P. (1986) *Nature*, **320**, 134−139.

Greene,G.L., Sobel,N.B., King,W.J. and Jensen,E.V. (1984) *J. Steroid Biochem.*, **20**, 51−56.

Guiochon-Mantel,A., Loosfelt,H., Lescop,P., Sar,S., Atger,M., Perrot-Applanat,M. and Milgrom,E. (1989) *Cell*, **57**, 1147−1154.

Hamada,K., Gleason,S.L., Levi,B.-Z., Hirschfeld,S., Appella,E. and Ozato,K. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8289–8293.
Ham,J. and Parker,M.G. (1989) *Current Opinion in Cell Biology*, **1**, 503–511.
Hard,T., Kellenbach,E., Boelens,R., Maler,B.A., Dahlman,K., Freedman,L.P., Carlstedt-Duke,J., Yamamoto,K.R., Gustaffson,J.A. and Kaptein,R. (1990) *Science*, **249**, 157–160.
Hazel,T.G., Nathans,D. and Lau,L.F. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8444–8448.
Hollenberg,S.M. and Evans,R.M. (1988) *Cell*, **55**, 899–906.
Hollenberg,S.M., Weinberger,C., Ong,E.S., Cerelli,G., Oro,A., Lebo,R., Thompson,E.B., Rosenfeld,M.G. and Evans,R.M. (1985) *Nature*, **318**, 635–641.
Hollenberg,S.M, Giguere,V., Segui,P. and Evans,R.M. (1987) *Cell*, **49**, 39–46.
Hu,S.-L. and Manley,J.L. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 820–824.
Kakizuka,A., Miller,Jr,W.H., Umesono,K., Warrell,Jr,R.P., Frankel,S.R., Murty,V.V.V.S., Dmitrovsky,E. and Evans,R.M. (1991) *Cell*, **66**, 663–674.
Krust,A., Green,S., Argos,P., Kumar,V., Walter,P., Bornert,J.-M. and Chambon,P. (1986) *EMBO J.*, **5**, 891–897.
Krust,A., Kastner,P.H., Petkovich,M., Zelent,A. and Chambon,P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5310–5314.
Kumar,V. and Chambon,P. (1988) *Cell*, **55**, 145–156.
Kumar,V., Green,S., Stack,G., Berry,M., Jin,J.R. and Chambon,P. (1987) *Cell*, **51**, 941–951.
Ladias,J.A.A. and Karathanasis,S.K. (1991) *Science*, **251**, 561–565.
Lavornga,G., Ueda,H., Clos,J. and Wu,C. (1990) *Science*, **252**, 848–851.
Lees,J.A., Fawell,S.E. and Parker,M.G. (1989) *Nucleic Acids Res.*, **17**, 5477–5488.
Lewin,B. (1990) *Cell*, **61**, 1161–1164.
Lillie,J.W. and Green,M.P. (1989) *Nature*, **338**, 39–44.
Luisi,B.F., Xu,W.X., Otwinowski,Z., Freedman,L.P., Yamamoto,K.R. and Sigler,P.B. (1991) *Nature*, **352**, 497–505.
Mangelsdorf,D.J., Ong,S.E., Dyck,J.A. and Evans,R.M. (1990) *Nature*, **345**, 224–229.
Martinez,E., Dusserre,Y., Wahli,W. and Mermod,N. (1991) *Mol. Cell. Biol.*, **11**, 2937–2945.
Meyer,M.-E., Gronemeyer,H., Turcotte,B., Bocquel,M.-T., Tasset,D. and Chambon,P. (1989) *Cell*, **57**, 433–442.
Meyer,M.-E., Pornon,A., Ji,J., Bocquel,M.-T., Chambon,P. and Gronemeyer,H. (1990) *EMBO J.*, **9**, 3923–3932.
Miesfeld,R., Godowski,P.J., Maler,B.A. and Yamamoto,K.R. (1987) *Science*, **236**, 423–427.
Misrahi,N., Atger,M., D'Auriol,L., Loosfeld,H., Meriel,C., Fridlansky,F., Guiochon-Mantel,A., Galibert,F. and Milgrom,E. (1987) *Biochem. Biophys. Res. Commun.*, **143**, 740–748.
Mitchell,P.J. and Tjian,R. (1989) *Science*, **245**, 371–378.
Mlodzik,M., Hiromi,Y., Weber,U., Goodman,C.S. and Rubin,G.M. (1990) *Cell*, **60**, 211–224.
Morgenstern,J.P. and Land,H. (1990) *Nucleic Acids Res.*, **18**, 1068.
Nauber,Y., Pankratz,M.J., Kienlin,A., Seifert,E., Klemm,U. and Jäckle,H. (1988) *Nature*, **336**, 489–492.
O'Donnell,A.L. and Koenig,R.J. (1990) *Mol. Endocrinol.*, **4**, 715–720.
Oro,A.E., Ong,E.S., Margolis,J.S., Posakony,J.W., McKeown,M. and Evans,R.M. (1988) *Nature*, **336**, 493–496.
Parker,M.G., Webb,P., Needham,M., White,R. and Ham,J. (1987) *J. Cell. Biochem.*, **35**, 285–292.
Picard,D. and Yamamoto,K.R. (1987) *EMBO J.*, **6**, 3333–3340.
Pignoni,F., Baldarelli,R.M., Steingrimsson,E., Diaz,R.J., Patapoutian,A., Merriam,J.R. and Lengyel,J.A. (1990) *Cell*, **62**, 151–163.
Ptashne,M. (1988) *Nature*, **335**, 683–689.
Ptashne,M. and Gann,A.A.F. (1990) *Nature*, **346**, 329–331.
Rothe,M., Nauber,U. and Jäckle,H. (1989) *EMBO J.*, **8**, 3087–3094.
Ryseck,R.-P., Macdonald-Bravo,H., Mattéi,M.-G., Ruppert,S. and Bravo,R. (1989) *EMBO J.*, **8**, 3327–3335.
Sadowski,I. and Ptashne,M. (1989) *Nucleic Acids Res.*, **17**, 7539.
Sanchez,E.R., Toft,D.O., Schlesinger,M.J. and Pratt,W.B. (1985) *J. Biol. Chem.*, **260**, 12398–12401.
Sap,J., Munoz,A., Damm,K., Goldberg,Y., Ghysdael,J., Leutz,A., Beug,H. and Vennstrom,B. (1986) *Nature*, **324**, 635–640.
Schwabe,J.W.R., Neuhaus,P. and Rhodes,D. (1990) *Nature*, **348**, 458–461.
Segraves,W.A. and Hogness,D.S. (1990) *Genes Dev.*, **4**, 204–219.
Sladek,F.M., Zhong,W., Lai,E. and Darnell,Jr,J.E. (1990) *Genes Dev.*, **4**, 2353–2365.
Sleigh,M.J. (1986) *Anal. Biochem.*, **156**, 251–256.
Tasset,D., Tora,L., Fromental,C., Scheer,E. and Chambon,P. (1990) *Cell*, **62**, 1177–1187.
Thompson,C.C. and Evans,R.M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 3494–3498.
Tora,L., White,J., Brou,C., Tasset,D., Webster,N., Scheer,E. and Chambon,P. (1989) *Cell*, **59**, 477–487.
Tsai,S.Y., Carlstedt-Duke,J., Weigel,N.L., Dahlman,K., Gustafsson,J.-A., Tsai,M.-J., and O'Malley,B.W. (1988) *Cell*, **55**, 361–369.
Tsai,S.Y., Tasi,M.-J. and O'Malley,B.W. (1989) *Cell*, **57**, 443–448.
Wang,L.M., Tsai,S.Y., Cook,R.G., Beattie,W.G., Tsai,M.J. and O'Malley,B.W. (1987) *Nature*, **340**, 163–166.
Webster,N.J.G., Green,S., Jin,J.R. and Chambon,P. (1988) *Cell*, **54**, 199–207.
Webster,N.J.G., Green,S., Tasset,D., Ponglikitmongkol,M. and Chambon,P. (1989) *EMBO J.*, **8**, 1441–1446.
Weinberger,C., Thompson,C.C., Ong,E.S., Lebo,R., Gruol,D.J. and Evans,R.M. (1986) *Nature*, **324**, 641–646.
White,R., Lees,J.A., Needham,M., Ham,J. and Parker,M. (1987) *Mol. Endocrinol.*, **1**, 735–744.
Zenke,M., Muñoz,A., Sap,J., Vennström,B. and Beug,H. (1990) *Cell*, **61**, 1035–1049.

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