Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity.

Beatrice Claude Durand, M Saunders, C Gaudon, B Roy, R. Losson, P. Chambon

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Activation function 2 (AF-2) of retinoic acid receptor and 9-*cis* retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity

Béatrice Durand[1], Michael Saunders[2], Claudine Gaudon, Bidyut Roy, Régine Losson and Pierre Chambon[3]

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP/Collège de France, BP 163, 67404 Illkirch-cedex, CU de Strasbourg, France
[1]Present address: MRC Laboratory for Molecular Cell Biology, University College London, Gower Street, London WC1E 6BT, UK
[2]Present address: GLAXO, 25 avenue du Québec, 91951 Les Ulis, France

[3]Corresponding author

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A motif essential for the transcriptional activation function 2 (AF-2) present in the E region of retinoic acid receptor (RAR) α and 9-*cis* retinoic acid receptor (RXR) α has been characterized as an amphipathic α-helix whose main features are conserved between transcriptionally active members of the nuclear receptor superfamily. This conserved motif, which can activate autonomously in the absence of ligand in animal and yeast cells, can be swapped between nuclear receptors without affecting the ligand dependency for activation of transcription, thus indicating that a ligand-dependent conformational change is necessary to reveal the AF-2 activation potential within the E region of the nuclear receptor. Interestingly, we show that the precise nature of the direct repeat response element to which RAR/RXR heterodimers are bound can affect the activity of the AF-2s of the heterodimeric partners, as well as the relative efficiency with which *all-trans* and 9-*cis* retinoic acids activate the RAR partner.

Key words: dominant negative mutant/ligand-inducible transactivation function/nuclear receptors/response element/retinoid receptors

Introduction

Nuclear receptors represent a superfamily of transcriptional regulatory proteins that includes the ligand-inducible steroid, thyroid, vitamin D3 and retinoic acid (RA) receptors (Evans, 1988; Green and Chambon, 1988; Gronemeyer, 1991; Laudet *et al.*, 1992) and the so-called orphan receptors (O'Malley and Conneely, 1992). Six regions (A–F; see Figure 1) exhibit different degrees of evolutionary conservation. Region C, the DNA binding domain, is responsible for specific binding to *cis*-acting response elements (REs). The E region contains the ligand binding domain, a ligand-dependent transcriptional activation function 2 (AF-2) and a dimerization surface composed of conserved heptad repeats (for reviews and references see Forman and Samuels, 1990; Gronemeyer, 1991; Leid *et al.*, 1992a). An autonomous activation function (AF-1) is present in the N-terminal A/B region. Neither of these AFs appears to contain conventional transcriptional activation motifs (for references see Cress and Triezenberg, 1991; Tjian and Maniatis, 1994).

Retinoids are signalling molecules exerting profound effects on vertebrate development, cellular differentiation and homeostasis (for reviews and references see Blomhoff, 1994; Chambon, 1994; Sporn *et al.*, 1994). The *all-trans* (T-RA) and 9-*cis* (9C-RA) RA signals are transduced by two families of receptors. Retinoic acid receptor (RAR) α, β and γ (and their isoforms) are activated by both T-RA and 9C-RA, whereas 9-*cis* retinoic acid receptor (RXR) α, β and γ are specifically activated by 9C-RA (for reviews see Leid *et al.*, 1992a; Petkovich, 1992; Kastner *et al.*, 1994; Mangelsdorf *et al.*, 1994). These receptors modulate the expression of their target genes by interacting with RA REs (RAREs). Whereas steroid receptors bind their REs as homodimers (Gronemeyer, 1991, and references therein), RAR/RXR heterodimers bind much more efficiently *in vitro* than homodimers of either receptor to a number of RAREs (for reviews see Leid *et al.*, 1992a; Stunnenberg, 1993; Kastner *et al.*, 1994; Mangelsdorf *et al.*, 1994). Most of the naturally occurring RAREs consist of the direct repetition (DR) of two core motifs [5′-PuG(G/T)TCA-3′] separated by 5 (DR5), 2 (DR2) or 1 bp (DR1). In addition, RXRs preferentially bind as homodimers to DR1 elements (for references see Giguère, 1994; Zechel *et al.*, 1994). RAR/RXR heterodimers are also formed in solution both *in vitro* and *in vivo* (for references see Nagpal *et al.*, 1993), and were shown to bind and transactivate from different RAREs in cultured cells (Durand *et al.*, 1992). RXRs also heterodimerize with other receptors like thyroid hormone receptors (TRs), vitamin D3 receptor and orphan receptors (reviewed in Green, 1993; Mangelsdorf *et al.*, 1994). The chicken ovalbumin upstream promoter transcription factor (COUP-TF) family includes orphan receptors such as chicken COUP-TF1 (Wang *et al.*, 1991) and its human homologue ear3 (Miyajima *et al.*, 1988), human ear2 (Miyajima *et al.*, 1988), COUP-TFII (Wang *et al.*, 1991) and its human homologue apolipoprotein regulatory protein number 1 (ARP-1; Ladias *et al.*, 1992, and references therein), and *seven-up* (dSvp), the *Drosophila* homologue of COUP-TF1 (Segraves, 1994, and references therein). COUP-TF receptors, which bind DNA as homodimers or as heterodimers with RXR, recognize a large panel of REs (Cooney *et al.*, 1993) and have been shown to repress the expression of target genes responding to RARs (Tran *et al.*, 1992; Widom *et al.*, 1992), TRs, vitamin D3 receptor (Cooney *et al.*, 1993), hepatocyte nuclear factor-4 (Ladias *et al.*, 1992; Mietus-Snyter *et al.*, 1992; Nakshatri and

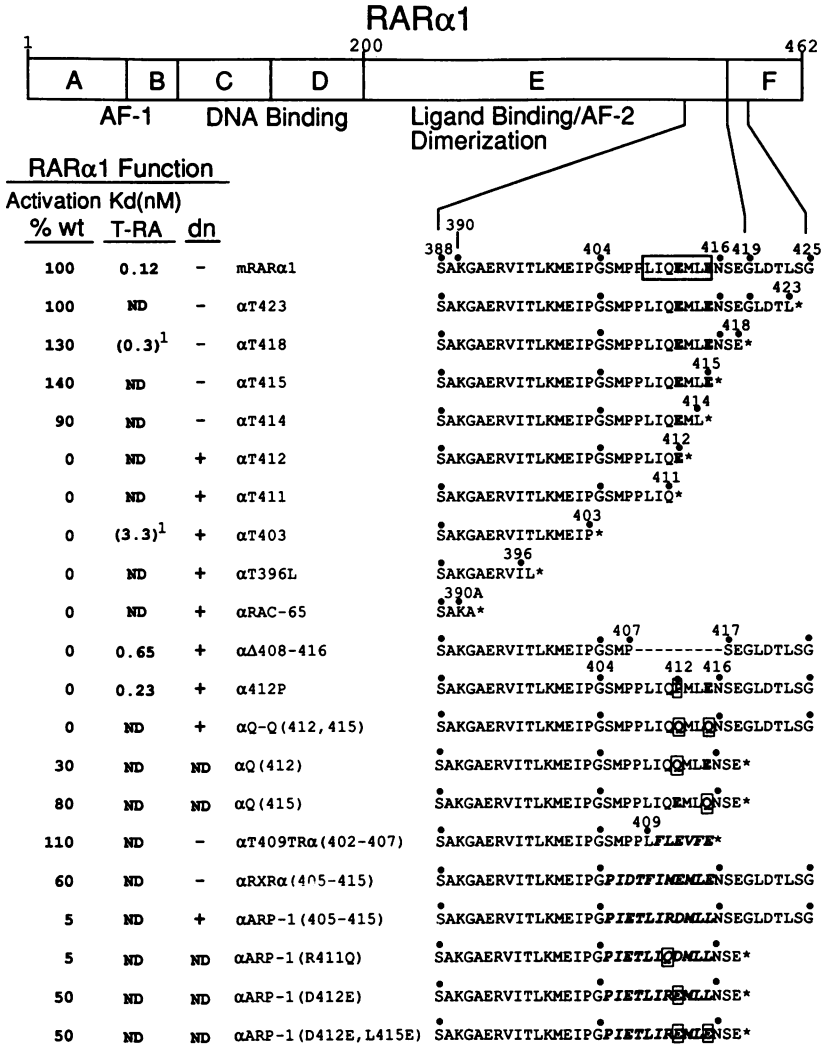

Fig. 1. Structure, dissociation constant for *all-trans* RA (T-RA), transcriptional and dominant negative activity of C-terminal mutants of mRARα1. The amino acid sequence (one-letter code) surrounding the border between regions E and F of RARα1 (amino acids 388–425) is shown and the sequences of individual mutants are indicated (see text and Materials and methods). The endpoints of the truncated mutants are indicated by a star, and the position of the last amino acid residue originated from RARα1 by a dark point and the corresponding number. The conserved motif (residues 409–415) is boxed, and glutamic acid residues 412 and 415 are indicated in bold. The nine amino acid deletion in αΔ408–416 is indicated by a dashed line; the amino acids derived from the sequences of TRα in αT409TRα(402–407), from mRXRα in αRXRα(405–415) and from ARP-1 in αARP-1(405–415) are in bold. The boundaries prior to and after the deletion or the insertion of a 'foreign' sequence are indicated by a dark point. For each mutant (where appropriate) the percentage of T-RA-dependent activation [compared with wild-type (wt) mRARα1] is indicated, as well as the dominant negative properties of the receptor (+, dominant negative mutant; ND, non determined). The dissociation constant for T-RA is given in nM. K_ds were measured by charcoal binding assays (see Materials and methods), except for αT418 and αT403 which were taken from Tate *et al.* (1994).

Chambon, 1994), peroxisome proliferation activated receptors (PPARs) (Miyata *et al.*, 1993) and oestrogen receptor (ER; Liu *et al.*, 1993), most probably by competing with these nuclear receptors for their DNA binding sites.

RA induces the differentiation of embryonal carcinoma (EC) cell lines. A P19-derived EC cell line, RAC-65, that fails to differentiate in response to T-RA, has been isolated (Pratt *et al.*, 1990); further studies revealed that RAC-65 cells contain a C-terminally truncated 391 amino acid-long RARα protein (Figure 1, αRAC-65) which behaves as a dominant negative (dn) receptor (Damm *et al.*, 1993, and references therein). We have identified here an amino acid sequence in the C-terminal region of RARα whose alteration abolishes AF-2 activity in cultured animal cells and yields dominant negative mutant receptors. We demonstrate that dominant negative activity requires the integrity of both the DNA binding and heterodimerization functions of RARα. An activating domain of AF-2 has been characterized and shown to possess an autonomous transcriptional activity and to be structurally and functionally conserved amongst many members of the nuclear receptor superfamily. We also show that the transactivation potential of the AF-2s of the RAR and RXR heterodimeric partners is dependent on the nature of the RARE to which they are bound.

Results

Alterations in the C-terminal region of RARα result in dominant negative mutants

To delineate the region responsible for the dominant negative activity of the RAC-65 mouse RARα1 (mRARα;

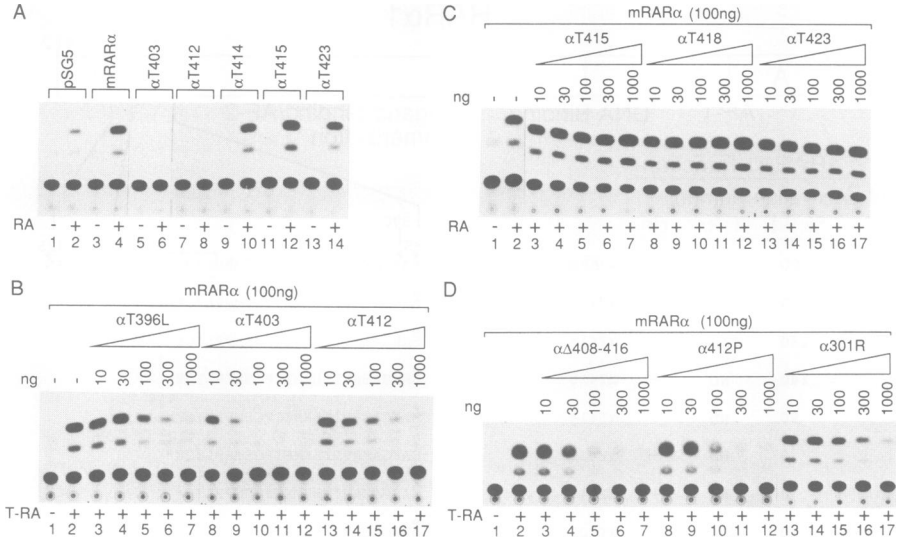

Fig. 2. Transcriptional activation and dominant negative properties of mRARα1 mutants. (**A**) T-RA-dependent activation. COS-1 cells were transfected with mRARβ2/CAT (5 μg) together with 100 ng of the receptor expression vectors as indicated. (**B**–**D**) Dominant negative activity. COS-1 cells were transfected with mRARβ2/CAT (5 μg), 100 ng of mRARα1 and increasing amounts (0–1000 ng) of the indicated mRARα1 mutants. (A–D) 100 nM RA were added for 12–15 h as indicated (+). Each experiment has been performed at least three times.

Figure 1, αRAC-65), a series of C-terminally truncated mutants (αT mutants) were constructed and assayed for activation and dominant negative activity in COS-1 cells (Figures 1 and 2, and data not shown; mouse and human RARα1 E regions are identical in the sequences considered here). COS-1 cells were transfected with a RA responsive reporter containing the mouse RARβ2 promoter (mRARβ2/CAT; Smith *et al.*, 1991), and the parental vector pSG5, the mRARα1 expression vector (designated mRARα) or a given αT expression vector. T-RA treatment (100 nM) of cells transfected with pSG5 resulted in a 2.5-fold activation (Figure 2A, lanes 1 and 2) due to endogenous RARs. Cells transfected with mRARα (100 ng) showed a 15- to 30-fold T-RA-dependent activation (Figure 2A, lanes 3 and 4). The mutants αT423, αT418, αT415 and αT414 similarly activated (Figures 1 and 2A, lanes 9–14, and data not shown). In contrast, no activation was seen with αT412, αT411, αT403, and αT396L (Figures 1 and 2A, lanes 5–8, and data not shown).

The dominant negative activity of these αT mutants was then investigated, as indicated in Figure 2B. Any receptor mutant which inhibited activation mediated by mRARα, when present in a 10-fold excess, was defined as dominant negative. αT396L, αT403, αT412 (Figure 2B) and αT411 (data not shown) all acted as dominant negative mutants (Figure 2B, lanes 3–17, compared with lane 2). As observed previously by Damm *et al.* (1993), αT403 was much more efficient at inhibiting mRARα activity than the other αT mutants. As expected (Figure 2A), αT414, αT415, αT418 and αT423 (Figure 2C, lanes 3–17, and data not shown) were not inhibitory.

The region localized between Leu409 and Glu415 of mRARα (boxed in Figure 1) is well conserved in most members of the receptor family (see below and Figure 3D). To investigate the importance of this region in RARα, we either deleted amino acids 408–416 (αΔ408–416, Figure 1), or altered the structure of this region by replacing Glu412 by a proline residue (α412P, Figure 1). These two mutants did not activate (data not shown), and acted as dominant negative mutants in cotransfection assays (Figure 2D, lanes 3–7 for αΔ408–416, and lanes 8–12 for α412P).

Efficient dominant negative activity requires both DNA binding and heterodimerization, but not ligand binding

Using an electrophoretic mobility shift assay and in agreement with previous reports from this and other laboratories (Yu *et al.*, 1991; Leid *et al.*, 1992b; see Introduction), neither mRARα, αΔ408–416, α412P nor any of the αT mutants translated *in vitro* efficiently bound the β2RARE probe in the absence of RXR (Figure 4A, lanes 1–6, and data not shown). In contrast, in the presence of mRXRα, equal amounts of mRARα or of any of the mutated RARs yielded specific shifted complexes of equal intensity corresponding to binding of heterodimers (Figure 4A, lanes 7–11, and data not shown). Thus, none of these dominant negative mutations affect the DNA binding and heterodimerization functions of the mutated proteins.

Three additional mutations were then introduced in either mRARα (αM series) or αT403 (αTM series) to investigate further whether DNA binding and heterodimerization were required for dominant negative activity. Mutation M1 (αM1 and αTM1), which corresponds to a Cys to Gly substitution at position 88 [Cys residue of the first zinc finger (CI) of the DNA binding domain], has been shown to abolish DNA binding in the case of the glucocorticoid receptor (GR; Freedman *et al.*, 1988). Mutation M2 (αM2 and αTM2) corresponds to a Leu to Pro substitution at position 328 (Figure 3B, boxed). Leu328 is one of the hydrophobic residues present in heptad repeat 5, which has been proposed to be part of the dimerization domain (Forman and Samuels, 1990). Mutation M3 (αM3 and αTM3) corresponds to the substitution of Met377 (the hydrophobic amino acid present at position 1 of heptad repeat 9) by an arginine residue (Figure 3C, boxed). The corresponding mutation in the mouse oestrogen receptor (mER; Fawell *et al.*, 1990) has

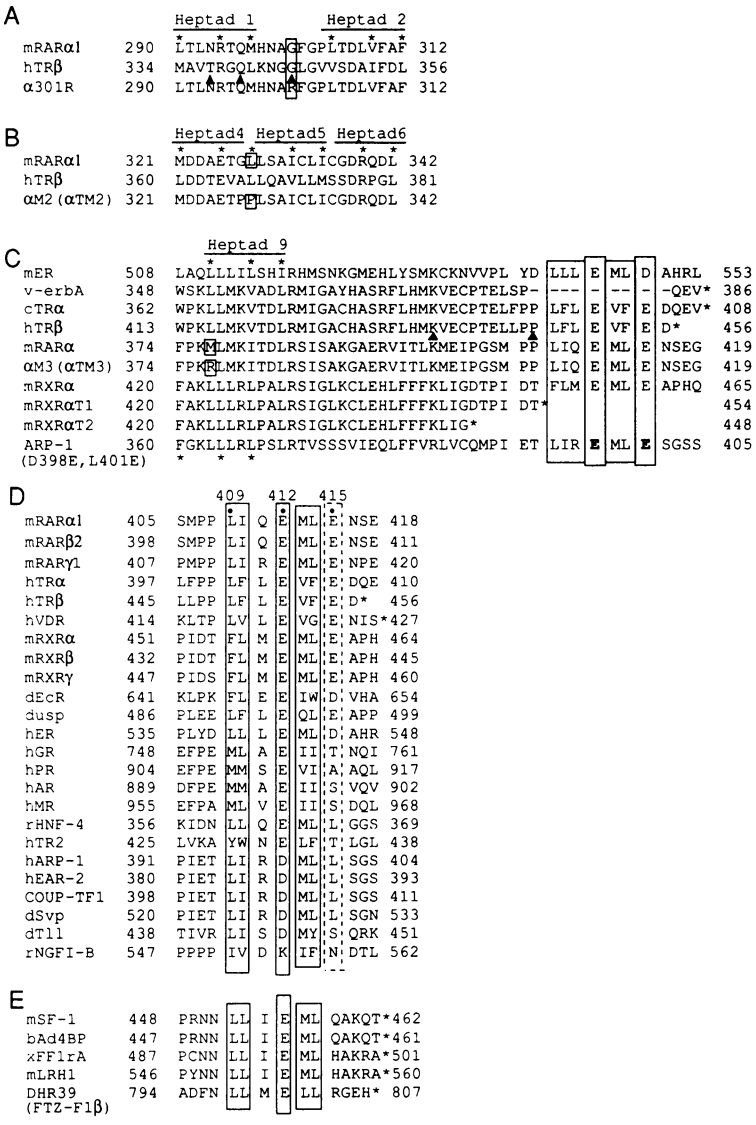

Fig. 3. Sequence alignments of members of the nuclear receptor superfamily. (**A**) Alignment of amino acid sequences (single-letter code) of heptad repeats 1 and 2 of mRARα1, hTRβ and α301R mutant. The amino acids at positions 1, 5 and 8 of the heptad repeats are indicated by stars. hTRβ G345, that is mutated to R in a naturally occurring mutation that causes GRTH (Refetoff *et al.*, 1993), and the analogous Gly301 of mRARα1, that is substituted for an arginine in the dominant negative mutant α301R, are boxed. The amino acid positions of naturally occurring mutations found in various GRTH kindreds are indicated by arrowheads (ΔT337, Q340H, G345R; Refetoff *et al.*, 1993). (**B**) Alignment of amino acid sequences of heptad repeats 4–6 of mRARα1, hTRβ, αM2 and αTM2 mutants. Leu328 that is mutated to Pro in mutants αM2 and αTM2 is boxed. (**C**) Alignment of a number of nuclear receptors showing the amino acid sequences of heptad repeat 9 and of the conserved putative amphipathic α-helical region. Note that the amino acid residues at positions 1, 5 and 8 of the heptad repeat 9, indicated by a star above or below the sequences, are different between steroid/RAR/TR families and members of the RXR and ARP-1 families. Met377 that is mutated to Arg in mutants αM3 and αTM3 is boxed, as well as the central Glu residue and the two blocks of hydrophobic residues. Terminal amino acids are indicated by stars, where appropriate. The positions of mutated amino acids in GRTH kindreds are indicated by arrowheads (K443E, P453H; Refetoff *et al.*, 1993). The deletion in v-erbA is indicated by a dashed line. The sequences of mRXRαT1, mRXRαT2 and ARP-1(D398E,L401E) (where the two mutated amino acids are in bold) are given. (**D**) Sequence alignment of the putative amphipathic helices of several members of the nuclear receptor superfamily. The central conserved acidic amino acid and the two pairs of hydrophobic amino acids are boxed, whereas the second less conserved acidic amino acid is boxed in a dashed line. Leu409, Glu412 and Glu415 correspond to mRARα1. Terminal amino acids are indicated by a star, where appropriate. m, mouse; h, human; d, *D.melanogaster*; r, rat; b, bovine; x, *Xenopus*. RARs (retinoic acid receptors), TRα (thyroid hormone receptor α), TRβ (thyroid hormone receptor β), VDR (vitamin D3 receptor), RXRs (9-*cis* retinoic acid receptors; Leid *et al.*, 1992b), EcR (ecdysone receptor; Koelle *et al.*, 1991), usp (ultraspiracle), ER (oestrogen receptor), GR (glucocorticoid receptor), PR (progesterone receptor), AR (androgen receptor), MR (mineralocorticoid receptor), HNF-4 (hepatocyte nuclear factor-4), hTR2 (thyroid receptor 2), ARP-1 (apolipoprotein regulatory protein number 1), EAR-2 (erbA-related 2; Miyajima *et al.*, 1988), COUP-TF1 (Miyajima *et al.*, 1988), Svp (seven-up), Tll (tailless), NGFI-B (nur77/N10; Paulsen *et al.*, 1992; Davis *et al.*, 1993, and references therein). (**E**) Sequence alignment of the AF-2 activating domain of members of the FTZ-F1 receptor family. The endpoint of the receptor is indicated by a star. mSF-1 (mouse steroidogenic factor 1; Ikeda *et al.*, 1993), bAd4BP (bovine Ad4 binding protein), xFF1rA (*Xenopus* FTZ-F1-related receptor 1; Ellinger-Ziegelbauer *et al.*, 1994), mLRH1 (mouse liver receptor homologous protein 1; Genbank accession number M81385), DHR39 [*D.melanogaster* hormone receptor 39, also called FTZ-F1β (Fushi tarazu type 1β)]. (For references see Gronemeyer, 1991; Lazar, 1993; Chambon, 1994; Segraves, 1994.)

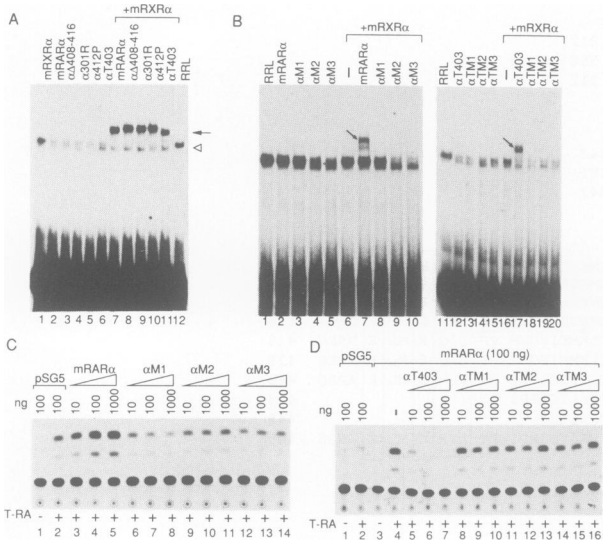

Fig. 4. DNA binding of mutated RARs is required for strong dominant negative activity. (**A** and **B**) Gel retardation assays are shown where *in vitro*-translated mRARα1 and indicated receptor mutants are incubated alone (A, lanes 1–6; B, lanes 2–5 and 12–15) or coincubated with *in vitro*-translated mRXRα (A, lanes 7–11; B, lanes 6–10 and 16–20) in the presence of a ^{32}P-labelled β2RARE oligonucleotide probe (upper strand, 5′-TCGAGGGTAG**GGTTCA**CCGAA**AGTTCA**-CTCG-3′). Specific complexes are marked with an arrow, non-specific complexes by an open arrowhead. RRL (lane 12), control assay with an unprogrammed rabbit reticulocyte lysate. (**C** and **D**) Transactivation by DNA binding-deficient mutants αM1, αM2 and αM3, and dominant negative properties of DNA binding-deficient mutants αTM1, αTM2 and αTM3. COS-1 cells were transfected with mRARβ2/CAT (5 μg) and either (C) the indicated amounts (in ng) of pSG5, mRARα1 and the αM series of mutant receptors or (D) mRARα1 (100 ng) and increasing amounts (0–1000 ng) of either αT403, αTM1, αTM2 or αTM3. 100 nM T-RA were added for 15 h where indicated (+).

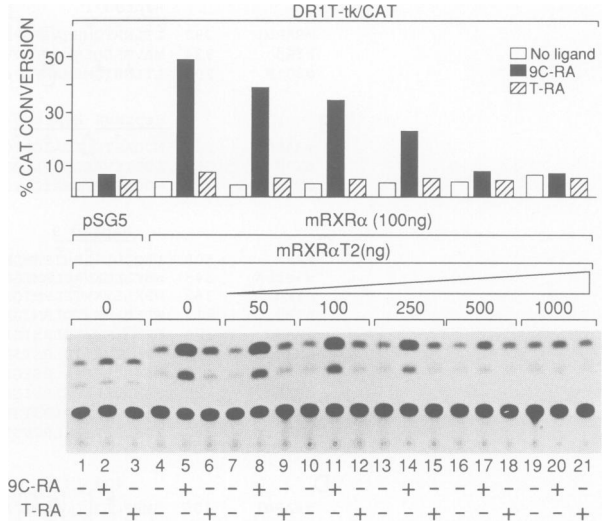

Fig. 5. Characterization of a dominant negative mutation of mRXRα. COS-1 cells were transfected with DR1T-tk/CAT (2 μg; see text) and either pSG5 or the mRXRα expression vector (100 ng). Increasing amounts (0–1000 ng) of mRXRdn (corresponding to mRXRαT2 of Figure 3C) were cotransfected with mRXRα (100 ng). Cells were treated with vehicle (ethanol) (−), T-RA (50 nM) or 9C-RA (50 nM) as indicated. A representative CAT assay and graphs corresponding to average values measured by scintillation counting from three independent experiments are shown.

been shown to prevent its homodimerization. Upon the addition of RXRα, only mRARα and αT403 bound the DNA probe (Figure 4B, lanes 7 and 17, arrows), whereas αM1, αM2 and αM3 (Figure 4B, lanes 8–10) or αTM1, αTM2 and αTM3 (lanes 18–20) did not bind, indicating that the integrity of heptads 5 and 9 is required for RAR/RXR heterodimerization. Cotransfection of increasing amounts of mRARα caused a clear increase in ligand-dependent activation of mRARβ2/CAT (Figure 4C, lanes 3–5), whereas cotransfection of the αM1, αM2 and αM3 did not result in any increase, in agreement with the inability of these mutants to bind DNA (Figure 4C, lanes 6–14). In contrast, Au-Fliegner *et al.* (1993) reported that a human RARα mutant, apparently identical to our αM3 mutant (M377R), stimulated transcription in cotransfection assays. The reason for this discrepancy is unknown, but may be related to differences in assay conditions [10^{-6} M T-RA for 40 h in the case of Au-Fliegner *et al.* (1993), under conditions where T-RA is known to be converted to 9C-RA (Levin *et al.*, 1992), instead of 10^{-7} M RA for 12–15 h in our case]. In this respect we also note that Au-Fliegner *et al.* (1993) reported that their M377R mutant heterodimerized *in vitro* with RXR in the presence, but not in the absence, of 10^{-6} M T-RA. Interestingly, activation by endogenous RARs was decreased upon cotransfection of 100 and 1000 ng of αM1, the mutant which carries the CI zinc finger mutation (Figure 4C, compare lanes 7 and 8 with lane 2). Cotransfection of increasing amounts of cotransfected αT403 progressively extinguished the activation by mRARα (Figure 4D, lanes 5–7, compare with lane 4), whereas no decrease was observed when αTM1, αTM2 or αTM3 were cotransfected (Figure 4D, lanes 8–16), even at the highest level (1000 ng). However, similar to the αM1 mutant (see above), high levels (1000 ng) of αTM1 transfected alone slightly inhibited activation by endogenous RARs, whereas αTM2 and αTM3 did not (data not shown). Thus, the integrities of both the DNA binding domain and the dimerization surface are required for efficient dominant negative activity.

We next investigated whether ligand binding was mandatory for dominant negative activity. In the dominantly inherited syndrome of generalized resistance to thyroid hormones (GRTH syndrome; reviewed in Refetoff *et al.*, 1993), the TRβ dominant negative mutants are mutated in either the N- (Figure 3A; ΔT337, Q340H, G345R) or C-terminal (Figure 3C; K443E, P453H) part of region E (Refetoff *et al.*, 1993, and references therein). By analogy with the hTRβ G345R dominant negative mutant which has lost its ability to bind T3 (Refetoff *et al.*, 1993), we substituted Gly301 in mRARα with an arginine residue (Figure 3A, α301R). α301R behaved as a dominant negative mutant (Figure 2D, lanes 13–17). RA binding to bacterially expressed mRARα, and to α412P, αΔ408–416 and α301R dominant negative mutants, was determined using a dextran-coated charcoal absorption assay. mRARα, α412P and αΔ408–416 bound [^{3}H]RA, whereas α301R did not (Figure 1 and data not shown); α412P and αΔ408–416 reproducibly had a 2- and a 5- to 6-fold reduced affinity for T-RA, respectively (Figure 1). From all of these results, we conclude that DNA binding and heterodimerization, but not ligand binding, are required for dominant negative activity.

RXR dominant negative mutations

We tested two C-terminal deletion mutants of mouse RXRα, mRXRαΔ455–466 (mRXRαT1) and mRXRαΔ 449–466 (mRXRαT2) (Figure 3C; Leid *et al.*, 1992b). These mutants, which are known to bind DNA as homodimers or as heterodimers with RARs (Leid *et al.*, 1992b), were cotransfected with DR1T-tk/CAT which contains a DR1T RE (see below) and has been shown to be transactivated upon RXRα cotransfection (Figure 5; Mader *et al.*, 1993b). Transfection of COS-1 cells with 100 ng mRXRα yielded a strong activation in the presence of 50 nM 9C-RA, with very little activation with 50 nM T-RA (Figure 5, lanes 4–6), in agreement with previous results showing that RXRs are not activated at 50 nM T-RA (Durand *et al.*, 1992). Cotransfection of mRXRα and increasing amounts of mRXRαT2 (50–1000 ng; Figure 5, lanes 7–21) reduced activation of the reporter (Figure 5, lanes 8, 11, 14, 17 and 20), indicating that mRXRαT2 is a dominant negative mutant, and that mRXRα was responsible for the observed activation. mRXRαT1 exhibited similar dominant negative properties (data not shown).

Characterization of an activating domain in the C-terminal part of the E region

The mRARα1 motif located between amino acids 409 and 415 is conserved in many members of the nuclear receptor family including several insect receptors (Zenke *et al.*, 1990; Koelle *et al.*, 1991; Danielan *et al.*, 1992; Figure 3D and E). The main features of this motif are a central acidic amino acid residue [Glu, with the exceptions of Asp in the case of the COUP-TF family (including dSvp) and *Drosophila* tailless (dTll), and Lys in the case of nur77/N10 (NGFI-B); Figure 3D], flanked by two pairs of hydrophobic residues (Figure 3D). In the case of the RAR and TR families, two proline residues precede this motif, which can be predicted to form an amphipathic α-helical structure (Zenke *et al.*, 1990). That the Glu residues 412 and 415 in mRARα1 are the only amino acids of this helix to be conserved in all members of the RAR, RXR and TR families (Figure 3D), suggested to us that they could be important for transactivation. The RARα1 mutant αQ-Q(412,415) (Figure 1), in which these Glu residues were replaced by Gln residues, did not transactivate mRARβ2/CAT (Figure 6A, lanes 11 and 12, and C, lanes 7 and 8) and behaved as a dominant negative mutant (Figure 6B, lanes 8–12). The replacement of Glu412 by a Gln residue decreased transactivation by ~70% [αQ(412); Figures 1 and 6C, lane 6], whereas mutant αQ(415) transactivated with ~80% of RARα wild-type efficiency, indicating that both Glu residues are required for optimal activation. Chimeric receptors between RAR and either TR, RXR or ARP-1 were constructed (Figure 1). Amino acids 410–415 of αT415 were replaced by the corresponding TRα residues [αT409TRα(402–407)], and residues 405–415 of RARα1 were replaced by those of either RXRα [αRXRα(405–415)] or ARP-1 [αARP-1(405–415)] (Figure 1). αT409TRα(402–407) transactivated in the presence of T-RA at least as efficiently as RARα (Figure 6A, compare lane 6 with lane 4), whereas αRXRα(405–415) was slightly less efficient than RARα [60% of mRARα activation; Figure 6A, compare lane 8 with lane 4; similar data (not shown) were obtained in the presence of 9C-RA]. In marked contrast, αARP-1(405–415) did not efficiently stimulate (Figure 6A, lanes 9 and 10, and C, lanes 9 and 10) and behaved as a dominant negative mutant (Figure 6B, lanes 3–7); note, however, that a residual T-RA-dependent activation (~5% of wild-type RARα1) was repeatedly observed in the presence of αARP-1(405–415) (Figure 6B, compare lanes 5–7 with lanes 10–12), suggesting that this chimera possesses a weak activation function. The conversion of ARP-1 Asp398, or of both Asp398 and Leu401 (Figure 3D), into Glu residues in the αARP-1(405–415) chimera generated receptors [αARP-1(D412E) and αARP-1(D412E,L415E), respectively; see Figure 1], which activated almost as efficiently as the αRXRα(405–415) chimera (Figures 1 and 6C, lanes 14 and 16 compare with lane 18), thus confirming the critical role of RARα Glu412 in activation. Note also that conversion of Arg411 into a Gln residue (as it is in RARα) in the αARP-1(405–415) chimera had no effect on transactivation efficiency [Figures 1, αARP-1(R411Q), and 6C, lane 12].

The same D398E and L401E mutations were also made in full-length ARP-1 [Figure 3C, ARP-1(D398E,L401E)] to investigate whether ARP-1 could be transformed from a repressor into an activator. COS-1 cells were transfected with ARP-1(D398E,L401E) with mCRBPII/CAT or DR1G-tk/CAT, which possess either the promoter sequence of the mouse CRBPII gene (Nagpal *et al.*, 1993; Nakshatri and Chambon, 1994) or a DR1G-tk promoter (Mader *et al.*, 1993a; see below). Both have been shown previously to contain DNA elements which can efficiently bind ARP-1 or RXRs (Ladias *et al.*, 1992; Nakshatri and Chambon, 1994). Interestingly, these reporters were not activated by ARP-1(D398E,L401E) under conditions where RXRα efficiently activated (data not shown). It should be stressed that ARP-1(D398E,L401E) contains in the ARP-1 context exactly the same sequence (PIETLIREMLE) which, in the RARα context [αARP-1(D412E,L415E)], results in an efficient activator (Figure 1).

The above results indicate that the conserved motif is not sufficient to bring about activation within the ARP-1 context, but do not exclude that it could constitute an activating domain on its own. Thus, Gal4-RARα(404–418)$_3$, containing a trimeric repeat of the conserved motif of RARα1 fused to the DNA binding domain of the yeast transactivator Gal4 [Gal(1–147)], was constructed and transfected with 17M5-G/CAT containing five repeats of the DNA binding site of Gal4 (17mer) inserted upstream of the β-globin promoter and the CAT gene (Webster *et al.*, 1988). A reproducible ~4-fold ligand-independent activation of the reporter was observed (Figure 6D, compare lane 1 with lane 2). Two additional chimeric proteins containing the DNA binding domain of the ER [amino acid residues 176–282, ER(C)], which fused to either residues 390–462 of mRARα [ER(C)RARα(390–462)] or residues 436–467 of mRXRα [ER(C)RXRα(436–467)], were transfected with (ERE)$_2$-tk/CAT containing two EREs in front of the tk/CAT reporter. As controls, we used either ER(C)RAR(−) (see Materials and methods) or a derivative of ER(C)RARα(390–462) harbouring the two mutations E412Q and E415Q in the conserved motif [referred to in Figure 6E as ER(C)RARα(E412Q,E415Q)]. A reproducible ~4-fold stimulation of (ERE)$_2$-tk/CAT activity was observed upon cotransfection of ER(C)RARα(390–462)

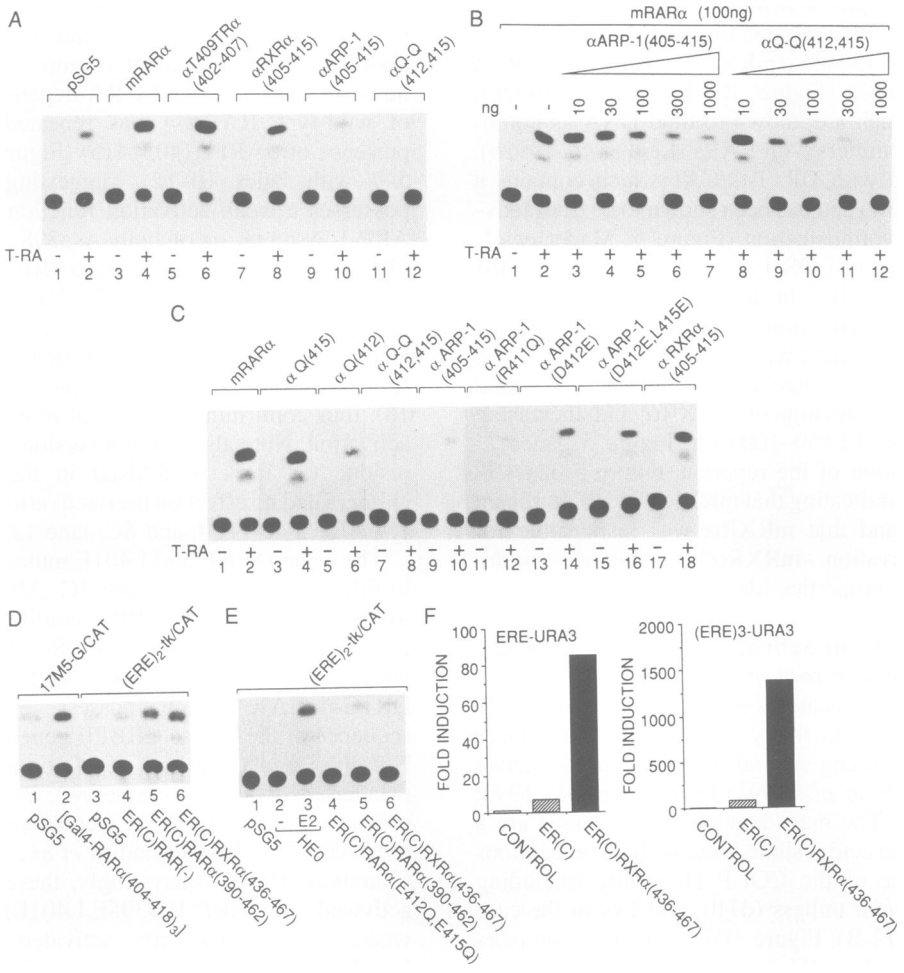

Fig. 6. Transcriptional activation and dominant negative properties of mutant and chimeric receptors derived from αT409TRα(402–407), αRXRα(405–415), αARP-1(405–415), αQ-Q(412,415) and AF-2 activating domain on its own. (**A**–**C**) T-RA-dependent activation properties of αT409TRα(402–407), αRXRα(405–415), αARP-1(405–415), αQ-Q(412,415) and receptor mutants derived from αARP-1(405–415) and αQ-Q(412,415) and dominant negative properties of αARP-1(405–415) and αQ-Q(412,415) receptors. COS-1 cells were transfected with mRARβ2/CAT (5 μg), together with 100 ng of receptor expression vector as indicated (A and C), or 100 ng of mRARα1 expression vector and increasing concentrations (0–1000 ng) of either αARP-1(405–415) or αQ-Q(412,415) (B). Cells were treated with T-RA at 100 nM for 15 h as indicated. (**D** and **E**) Transactivation capacity of AF-2 activating domain on its own. COS-1 cells were transfected with either the 17M5-G/CAT (1 μg) or the $(ERE)_2$-tk/CAT (1 μg) reporter gene together with either pSG5, Gal4-RAR(404–416)$_3$ (500 ng), ER(C)RAR(−) (500 ng), ER(C)RAR(390–462) (500 ng), ER(C)RXR(436–467) (500 ng) (D), or hER (HEO) [in the absence or presence of 10^{-8} M oestradiol (E2) for 15 h], ER(C)RAR(E412Q, E415Q) (500 ng) (E) [the difference in spot intensity between (D) lanes 5 and 6 and (E) lanes 5 and 6 is due to a difference in time exposure]. (**F**) A 2mer-derived yeast multicopy vector YEp90 expressing ER(C), ER(C)RXRα(436–467) or no protein (control) was introduced into the yeast reporter strains PL1 and PL3, containing a chromosomally integrated URA3 reporter gene regulated by one or three oestrogen response elements, respectively. Reporter gene activities were determined by measuring the specific activity of the URA3 gene product orotidine-5′-monophosphate decarboxilase (OMPdecase). Activities in cell-free extracts were measured as described in Pierrat *et al.* (1992) and are represented as fold induction over the basal reporter gene expression level exhibited by the control. At least three individual yeast transformants were assayed for each sample.

or ER(C)RXRα(436–467) (Figure 6D, lanes 3–6 and E, compare lane 4 with lanes 5 and 6; for comparison, note in Figure 6E that the full-length ER expression vector HEO resulted in an ~5-fold stronger activation).

The ability of the conserved motif to function as an autonomous activating domain was also investigated in yeast. Yeast cells which contain one or three EREs in front of the URA3 reporter gene [ERE-URA3 and (ERE)3-URA3; Pierrat *et al.*, 1992] were transformed with vectors encoding ER(C) or ER(C)RXRα(436–467). ER(C) RXRα(436–467), but not ER(C), strongly activated expression of the URA3 reporter genes (80- and 1300-fold, Figure 6F). A chimeric protein LexA-RXRα(436–467), containing the conserved motif of RXRα fused to the LexA DNA binding domain (amino acids 1–211), was also a strong activator in yeast (data not shown).

Response element and promoter context dependence of the activation potential of the AF-2 function of RAR and RXR

We have shown (Durand *et al.*, 1992) that RARαT412 and RXRαT1 (designated in our previous study and below as mRARdn and mRXRdn, respectively) act by competing with wild-type RAR and RXR, respectively, during the formation of RAR/RXR heterodimers. Since the ligand-dependent AF-2 is abrogated in these dominant negative mutants, we used them here as tools to investigate whether the activities of RAR and RXR AF-2s could depend on

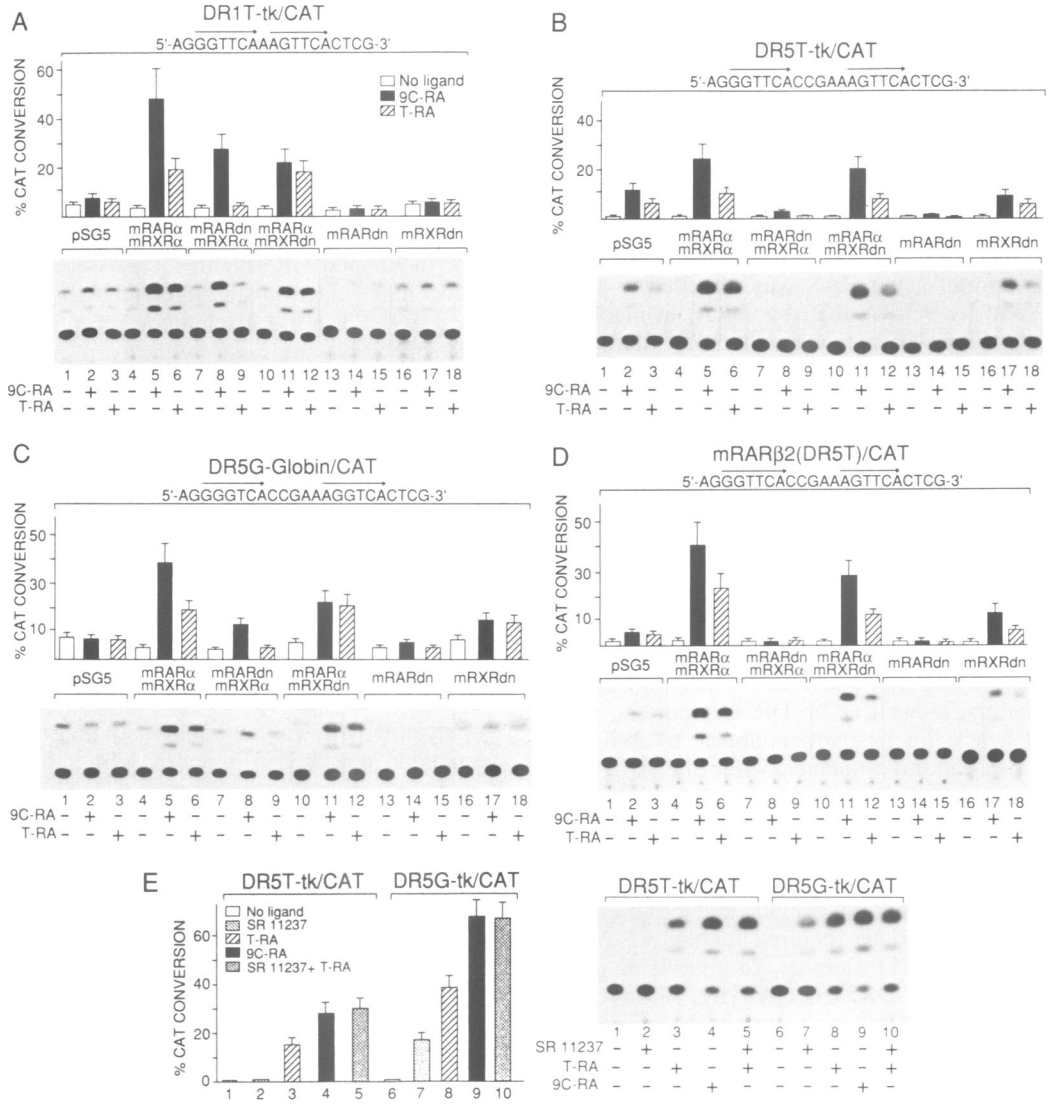

Fig. 7. The AF-2s of RAR and RXR heterodimeric partners have different transactivation potentials depending on the response element. (**A**–**D**) COS-1 cells were transfected with 2 μg of DR1T-tk/CAT (A), 2 μg of DR5T-tk/CAT (B), 2 μg of DR5G-globin/CAT (C) or 5 μg of mRARβ2/CAT (D) with 250 ng of mRARα, mRXRα, mRARdn (αT412) and mRXRdn (mRXRαT2) as indicated. In all cases the upper strand of the responsive element is indicated. Cells were treated with either ethanol vehicle (−), 50 nM 9-*cis* RA (9C-RA), or 50 nM *all-trans* RA (T-RA) for 12–15 h; CAT activity was determined. (**E**) HeLa cells were transfected with 5 μg of DR1T-tk/CAT or 5 μg of DR5G-tk/CAT, as indicated. Cells were treated with either EtOH (−), 100 nM SR11237 (SR11237), 50 nM T-RA (T-RA), 50 nM 9C-RA(9C-RA), or 100 nM SR11237 and 50 nM T-RA (SR11237 + T-RA) for 12–15 h; CAT activity was determined. (A–E) Representative CAT assays and graphs corresponding to average values of several independent experiments are shown.

the nature or the spacing of the response elements on which they are bound and on the promoter context (Durand *et al.*, 1992). COS-1 cells were transfected with DR1T-tk/CAT (Figure 7A, DR1T), DR1G-tk/CAT (DR1G, 5′-AG**GGGTCAAAGGTCA**CTCG-3′; data not shown), DR5T-tk/CAT (Figure 7B, DR5T), DR5G-globin/CAT (Figure 7C, DR5G), DR5G-tk/CAT (data not shown) or mRARβ2(DR5T)/CAT (Figure 7D), together with vectors encoding mRARα1, mRXRα, mRARdn or mRXRdn alone or in various combinations. A total of 50 nM 9C-RA or 50 nM T-RA were added for 12 h, under conditions where RAR AF-2 is activated by both T-RA and 9C-RA, whereas RXR AF-2 is activated by 9C-RA alone (Durand *et al.*, 1992, and references therein). With DR1T-tk/CAT, T-RA activates transcription to the same extent in the presence of mRAR and mRXRdn, as in the presence of mRAR and mRXR wild-type (Figure 7A, compare lane 12 with lane 6); furthermore, cotransfection of mRXR and mRARdn resulted in a 9C-RA-dependent RXR activity which corresponded to the difference between the T-RA- and 9C-RA-dependent activities observed when mRAR and mRXR were transfected together (Figure 7A, compare lanes 7–9 with lanes 4–6). Since similar results were obtained with DR1G-tk/CAT (data not shown), we conclude that, in agreement with our previous results (Durand *et al.*, 1992), the AF-2s of RAR and RXR bound as heterodimeric partners to DR1 elements may act independently of one another and in an additive fashion. Furthermore, RAR was as efficiently activated by T-RA as by 9C-RA (Figure 7A, lanes 11 and 12), whereas as expected RXR was selectively activated by 9C-RA (Figure 7A, lanes 8 and 9).

Similar transfection experiments were then performed

using DR5T-tk/CAT and mRARβ2(DR5T)/CAT (the natural mouse RARβ2 promoter; Figure 7B and D). Cotransfection of mRARdn with mRXRα decreased the expression of DR5T-tk/CAT and mRARβ2/CAT to levels even lower than those seen with endogenous receptors (Figure 7B and D, compare lanes 7–9 with lanes 1–3; see also mRARdn on its own in lanes 13–15). In contrast, transfection of RAR with RXRdn instead of RXR only moderately decreased activation by either T-RA or 9C-RA (compare lanes 4–6 with lanes 10–12). Most interestingly, on both DR5T reporter genes RAR was activated to a 2-fold greater extent by 9C-RA than by T-RA, in marked contrast with the observation made with DR1 elements (Figure 7A, B and D, compare lanes 10–12). To investigate whether these differences between DR1 and DR5 reporters were dependent on the spacing of the directly repeated motifs, on the sequence of the response element and/or on the promoter context, additional transfections were carried out with either DR5G-tk/CAT (data not shown) or DR5G-globin/CAT (Figure 7C). Strikingly, the activation profiles were very similar to those obtained with DR1T or DR1G reporters (compare Figure 7A with C), indicating that with both of these DR5G reporters, AF-2s of RAR and RXR were active and additive, and that RAR was similarly activated by 9C-RA and T-RA. Thus, on DR1T and DR1G reporters, as well as on DR5G reporters, the RAR and RXR heterodimeric partners appear to activate independently in an additive manner, while the AF-2 of RAR may be preferentially responsible for the activity of the heterodimer on DR5T reporters (note that this accounts for the complete repression by RARdn mutants which was observed in experiments reported in Figure 2).

The above experiments did not exclude that a transcriptionally active RAR would be required for the AF-2 of RXR to be active on a DR5T element. To investigate this possibility, activation of DR5T-tk/CAT by HeLa cell endogenous RARs and RXRs was tested in the presence of either a specific RXR ligand (SR11237; Lehmann *et al.*, 1992), T-RA, 9C-RA or T-RA, together with SR11237 (Figure 7E). As expected from the above results, no activation was seen in the presence of SR11237, while DR5T-tk/CAT expression was induced by T-RA (50 nM) addition (Figure 7E, lanes 2 and 3). However, the simultaneous addition of SR11237 and T-RA resulted in a stronger activation, similar to that achieved with 9C-RA (Figure 7E, lanes 4 and 5). Under similar conditions DR5G-tk/CAT, as well as DR1T-tk/CAT (data not shown), were efficiently activated by the RXR-specific ligand (Figure 7E, lane 7) and, as expected from the results shown in Figure 7A and C, these activations were additive with those induced by T-RA (Figure 7E, compare lanes 7 and 8 with lanes 9 and 10). These results confirm that the AF-2s of RAR/RXR heterodimers bound to DR1 and DR5G elements activate independently and additively, and most interestingly reveal that on DR5T elements the presence of a transcriptionally active RAR partner is required for the AF-2 of the RXR partner to participate in activation of transcription.

Discussion

An autonomous constitutive AF-2 activating domain and ligand dependency of AF-2

We have identified here a motif located in the C-terminal part of the E region (boxed in Figure 1) whose integrity is required for the activity of the AF-2 of RARα or RXRα. Mutagenesis in this motif, which is highly conserved in many members of the nuclear receptor family (Figure 3D and E), can selectively abrogate AF-2 activity without significantly altering DNA binding, heterodimerization and T-RA binding by RARα. Interestingly, the corresponding motif is absent in v-erbA which has no AF-2 activity (Zenke *et al.*, 1990; Saatcioglu *et al.*, 1993); point mutagenesis of the hydrophobic or charged residues of this motif in ER and GR (Figure 3D) reduces AF-2 activity, but has little effect on steroid or DNA binding (Danielan *et al.*, 1992). The use of chimeric receptors in which the RARα motif was replaced by the corresponding motif of TRα or RXRα (Figures 1 and 3) actually demonstrates that these motifs (which have the potential to form an amphipathic α-helical structure; Zenke *et al.*, 1990) are functionally interchangeable. Considering the first hydrophobic amino acid residue (L409 in the case of mRARα1) as position 1 of the helix, an acidic residue is present at position 4 in many members of the nuclear receptor superfamily (Figure 3D), while another acidic residue is frequently found at position 7. In fact, both Glu412 and Glu415 appear to be required for optimal RARα AF-2 activity, and mutagenesis of both of them to glutamine abrogates this activity. Peptides containing the above amphipathic helix can autonomously transactivate in both animal and yeast cells (Figure 6D–F), indicating that this helix is an activating domain of AF-2. This conclusion is strongly supported (i) by the observation that mutating the conserved Glu residues in these peptides abrogates their activation function (Figure 6D and E), and (ii) by Barettino *et al.* (1994) who have reported after completion of the present paper that the same region of the thyroid hormone receptor also possesses an autonomous activation domain.

Transcriptional activators appear to interact with basal transcription factors, TBP-associated factors (TAFs) or transcriptional intermediary factors (TIFs) to stimulate gene transcription (Tasset *et al.*, 1990; Tjian and Maniatis, 1994, and references therein). Replacing a Glu by an Asp in AF-2 activating domain [compare αARP-1(D412E) and αARP-1(405–415); Figure 1] greatly decreases transactivation, strongly arguing for a mechanism whereby the activity of AF-2 is transduced through protein interactions involving specific interfaces. It also indicates that the AF-2 activating domain motif is distinct from other acidic 'VP16-like' activating domains (Cress and Triezenberg, 1991), in agreement with our previous squelching data (Tasset *et al.*, 1990).

The presence of an autonomous transcriptional activity in RAR and RXR AF-2 activating domains raises the question as to why the activity of AF-2 is dependent on ligand binding and abrogated in mutants (e.g. α301R) unable to bind T-RA. When embedded in region E, the AF-2 activating domain helix may either be in a transcriptionally active form but masked in some way, or exist in a 'deformed' inactive form. The ligand might be required to induce a conformational change in region E which would either unmask or generate an active AF-2 activating domain. In this respect, we note that binding of the ligand has been shown to induce conformational changes in several nuclear receptors (e.g. Keidel *et al.*, 1994; Tate *et al.*, 1994). The hypothesis that an otherwise

active AF-2 activating domain is unmasked seems the more likely, since it does not appear that induction by the ligand requires a precise match between the ligand binding domain and AF-2 activating domain; indeed, RARα chimeric receptors in which the RAR AF-2 activating domain has been replaced by that of a heterologous receptor (cTRα or mutated ARP-1) are efficiently activated by T-RA (Figure 1). The fact that the chimeric receptor αARP-1(D412E) is readily inducible by T-RA also indicates that the binding pocket for T-RA is largely independent of the AF-2 activating domain. This conclusion is in agreement with the finding (Tate *et al.*, 1994) that RARα sequences C-terminal to amino acid 404 are not required for efficient binding of T-RA (see also our mutant αΔ408–416 in Figure 1); note also that the identity of the amino acids located C-terminal to position 404 are unlikely to be crucial for the binding of 9C-RA, since αT409TRα(402–407) and αARP-1(D412E) were both stimulated by T-RA and 9C-RA (Figure 1 and data not shown). The inability of mutations D398E and L401E to generate an activator in the context of the full-length ARP-1 (Figure 1) may then reflect either the requirement for a putative ARP-1 ligand or the absence of a ligand binding domain in the COUP-TF family. Thus, the ARP-1(D398E,L401E) protein (and similar proteins for other orphan receptors; see below) could be used in searches for putative cognate ligands.

In the case of steroid receptors, ligand binding may result in (i) release of the heat shock protein hsp90 which may preclude efficient DNA binding (reviewed in Pratt and Welsh, 1994), and (ii) induction of AF-2 activity (Green and Chambon, 1988). This dual role is strongly supported by the fact that the oestrogen antagonist, tamoxifen, can efficiently promote DNA binding of the ER in animal and yeast cells, but not induction of AF-2 activity (Berry *et al.*, 1990; Metzger *et al.*, 1992). Since AF-2 activating domains of RAR and RXR are inactive within the unliganded E region, even though these receptors are not associated with hsp90 (Dalman *et al.*, 1991), we propose that the second strictly agonist-dependent activation step of steroid receptor corresponds to a conformational change similar to that which is necessary to expose an active RAR AF-2 activating domain. This conformational change would not be achieved by steroid antagonists functionally similar to tamoxifen which, however, would induce the structural transition required for hsp90 dissociation.

Are all orphan receptors possible ligand-inducible activators?

Orphan receptors are members of the nuclear receptor superfamily which have not yet been matched with any ligand (Laudet *et al.*, 1992; O'Malley and Conneely, 1992). C-terminal sequence comparisons show that, in addition to the members of the COUP-TF family (see Introduction), a number of other orphan receptors [e.g. nur 77/NGFI-B/N10, hTR2, EAR-1/rev-erbA, and several *Drosophila* proteins like *tailless* (dTll), *seven up* (dSvp) and E75] completely lack the conserved motif, lack an acidic residue at position 4 of the motif or possess an Asp instead of a Glu residue at this position (Figure 3D). Interestingly, most of the orphan receptors which possess an Asp at position 4 may belong to a family which may have evolved from NGFI-B as a common ancestor (Laudet *et al.*, 1992). Our results indicate that even if they would bind ligands not yet discovered, all of the AF-2 activating domain-lacking orphan receptors would most probably lack an activation function similar to the present AF-2s. Thus, these orphan receptors may either prevent activation by other ligand-inducible receptors (e.g. the members of the COUP-TF/ARP-1 family which bind promiscuously to a variety of REs) or transactivate through a constitutive AF-1 located in the A/B region (e.g. NGF1-B; Paulsen *et al.*, 1992; Davis *et al.*, 1993; Yoon and Lau, 1993). In contrast, orphan receptors which contain an AF-2 activating domain-like amphipathic α-helix in their E region may well possess AF-2s inducible by ligands which remain to be discovered.

The Fushi tarazu type 1 (FTZ-F1) family includes *Drosophila* FTZ-F1α (Lavorgna *et al.*, 1991), mouse steroidogenic factor 1 (mSF-1) and its splice variant mELP, bovine Ad4 binding protein (bAd4BP), *Drosophila melanogaster* hormone receptor 39 (DHR39, also called FTZ-F1β), *Xenopus* FTZ-F1-related receptor 1 (xFF1rA) and xFF1rB and their mouse homologue liver receptor homologous protein 1 (mLRH1; Figure 3E; Segraves, 1994, and references therein). Most of these receptors contain a Glu residue at position 4 of the conserved motif, with the exception of dFTZ-F1α and mELP which completely lack this motif. Interestingly, mSF-1 and mELP, which are derived from the same gene by alternative splicing, have been shown to be a constitutive transactivator (mSF-1, which possesses an AF-2 activating domain) and a constitutive repressor (mELP, which lacks an AF-2 activating domain; Ikeda *et al.*, 1993). This observation, together with the observation that xFF1rA and bAd4BP behave as activators (Ellinger-Ziegelbauer *et al.*, 1994, and references therein), support the notion that the AF-2 activating domain is critical for the function of all members of the nuclear receptor family which act as activators. Moreover, changing an activator into a repressor by elimination of the AF-2 activating domain through a splicing event (as in the case of mELP) offers a simple mechanism to control, either positively or negatively, the expression of the same set of target genes.

Different class of dominant negative receptors

In the light of our results, dominant negative receptors can be classified into two groups: (i) potent dominant negative receptors which have lost their transactivation capacity but have kept their DNA binding and heterodimerization functions intact, and (ii) weak dominant negative receptors which have lost their DNA binding ability.

Potent dominant negative receptors, which have silent AF-2s and compete in a molar ratio with wild-type receptors for DNA binding upon heterodimerization, can be divided further. Receptors belonging to a first class have lost their AF-2 activating domain, by either deletion (e.g. RAC-65 and v-erbA; Saatcioglu *et al.*, 1993) or point mutation (our point mutants and various dominant negative forms of RXR, TR or ER which are mutated in or near the AF-2 activating domain; Danielan *et al.*, 1992; Ince *et al.*, 1993; Refetoff *et al.*, 1993; Tate *et al.*, 1994). Potent dominant negative receptors of the second class

have lost the capacity to bind their ligand which prevents activation of their AF-2 activating domain [e.g. our α301R mutant and TRα2 (an isoform of TRα; Lazar, 1993, and references therein)]. A third class of dominant negative mutant receptors might possibly exist which would be mutated in amino acids involved in the ligand-induced conformational change necessary to activate the AF-2 activating domain (see above).

A second group of dominant negative RAR mutants, which decreased transactivation mediated by the endogenous receptor only when produced at high concentration (e.g. αM1 and αTM1; Figure 4D), is similar to other dominant negative receptors which are active only at high concentrations and do not bind DNA (Wang *et al.*, 1991; Bigler *et al.*, 1992; Barettino *et al.*, 1993). We propose that dominant negative receptors belonging to this second group act by heterodimerizing in solution with RXR (Nagpal *et al.*, 1993), preventing it heterodimerizing with RAR (see also Barettino *et al.*, 1993). Transcriptional squelching is unlikely to provide an alternative inhibitory mechanism, since AF-2 activity is abrogated in our αTM RAR mutants. Thus, natural isoforms of TR (Bigler *et al.*, 1992; Barettino *et al.*, 1993; Lazar, 1993), RARα, β and γ (Kastner *et al.*, 1990; Leroy *et al.*, 1991; Zelent *et al.*, 1991) and ER (Wang *et al.*, 1991), which all cannot bind to DNA, could possibly participate in the control of the transcriptional activity of nuclear receptors through sequestration of a dimeric partner.

The nature of the response element influences the activity of the AF-2s of RAR and RXR heterodimeric partners

Using various reporter genes containing RAREs of either DR1T, DR1G or DR5G and RAR/RXRdn or RARdn/RXR combinations, we have shown here that transcriptional activation results from the additive effect of the AF-2 activities of the RAR and RXR heterodimeric partners, acting independently irrespective of the promoter context. This conclusion, which was confirmed by using an RXR-specific ligand, is in agreement with our previous observations which indicated similar additive effects for activation mediated by either the DR1 or the DR2 RAREs of the mouse CRABPII gene (Durand *et al.*, 1992). In contrast, using reporter genes containing a DR5T-type RARE (e.g. that of the RARβ2 gene) and the same RAR/RXRdn and RARdn/RXR combinations, transactivation appears to result only from the AF-2 of the RAR heterodimeric partner, RXR behaving as a silent (non-activating) partner irrespective of the promoter context. Most interestingly, the use of an RXR-specific ligand revealed that in fact activation by the AF-2 of RXR requires the presence of a transcriptionally active RAR partner. Our results also show that the AF-2 activity of the RAR partner of RAR/RXRdn heterodimers bound to either a DR1T, DR1G or DR5G-type RARE was similarly induced by T-RA and 9C-RA, whereas 9C-RA was twice as efficient as T-RA at activating the RAR AF-2 when the heterodimers were bound to a DR5T-type RARE.

Since the above differential effects are independent of the precise nature of the responsive promoters, it is likely that they result from conformational modifications of the heterodimers which depend on the nature (sequence and spacing of the repeated motifs) of the DR elements. As a strong dimerization function, the ligand binding domain and the AF-2 overlap in RAR and RXR regions E (Leid *et al.*, 1992a,b); binding to different RAREs may result in modifications of ligand binding and/or AF-2 activity. Such a possibility is supported by the observation that the *Drosophila* ecdysone nuclear receptor (EcR) cannot bind its cognate ligand unless heterodimerized with ultraspiracle (usp), the *Drosophila* RXR homologue (Yao *et al.*, 1993; D.Hogness, personal communication). When compared with RAR/RXR heterodimers bound to DR1, DR2 or DR5G elements, the binding of RXR/RAR heterodimers to DR5T elements may change both the conformation of the E region (resulting in a silent RXR AF-2 in the absence of a transcriptionally active RAR partner) and the relative efficiency of T-RA and 9C-RA at inducing the AF-2 activity of the RAR partner (9C-RA being more efficient than T-RA). In this respect, note that the RAR binding pocket for 9C-RA may be partly different from that for T-RA (Tate *et al.*, 1994). In any event, and irrespective of the possible underlying mechanism, our data clearly establish that the activity of the AF-2s of RXR/RAR heterodimers and their ligand response can vary depending on the nature of the response elements, irrespective of the context of the activated promoter. We have reported previously that the activity of the AF-2s of the different RARs and RXRs are promoter context-dependent (Nagpal *et al.*, 1992, 1993). Thus, the transcriptional activity of a given heterodimeric combination of RAR (α, β or γ) and RXR (α, β or γ) appears to be dependent on the nature of both the bound RARE and the factors which are bound to the responsive promoter. This further expands the combinatorial possibilities (reviewed in Leid *et al.*, 1992a; Chambon, 1994) which are necessary to generate the highly pleiotropic effects of the simple retinoid signal.

Materials and methods

Construction of receptor mutants

The C-terminal truncations of mRARα1 (αT mutants) were constructed by exchanging the *Eco*RV−*Bam*HI fragment of the mRARα1 expression vector (encoding amino acids 267–462) with *Eco*RV−*Bam*HI- or *Eco*RV−*Bgl*II-digested PCR-amplified fragments corresponding to mRARα1 (encoding amino acid 267 to the chosen C-terminal amino acid). αT409TRα(402–407) was similarly constructed using a 3′ PCR primer containing antisense information for mRARα1 amino acids 404–409, for the TR amino acids FLEVFE and for a stop codon. αΔ408-416, α412P, αQ-Q(412,415), αRXRα(405–415) and αARP-1(405–415) were constructed by double PCR amplification (Ho *et al.*, 1989) to generate *Eco*RV−*Xma*I or *Eco*RV−*Bgl*II fragments containing the appropriate mutations. αQ(412) and αQ(415) were constructed by exchanging the *Eco*RV−*Bam*HI fragment of the mRARα1 expression vector, with *Eco*RV−*Bgl*II-digested PCR fragments corresponding to αQ-Q(412–415) amplified using a 3′ PCR primer encompassing the stop codon and *Bgl*II restriction site, and encoding the E412Q or E415Q substitutions. αARP-1(R411Q), αARP-1(D412E) and αARP-1(D412E,L415E) were similarly constructed using PCR fragments corresponding to αARP-1(405–415) amplified using a 3′ oligonucleotide encompassing the stop and *Bgl*II restriction sites and encoding for respectively R411Q, D412E or D412E and L415E substitutions. ARP-1(D398E,L401E) was constructed by a double PCR amplification procedure on an ARP-1 expression vector (gift from T.Lerouge) to generate a *Bam*HI−*Eco*RI fragment containing the appropriate mutations. αM1, αM2 and αM3 and the corresponding mutations in αT403 (αTM1, αTM2 and αTM3), as well as α301R, were similarly constructed. PCR-amplified fragments containing mutation M1 were cloned into *Kpn*I−*Sst*I-digested mRARα1 or αT403 expression vectors; PCR-amplified fragments containing mutations M2 and M3 were subcloned into mRARα1

as *Eco*RV−*Xma*I fragments and into αT403 as *Eco*RV−*Bam*HI fragments. The *Eco*RV−*Xma*I PCR-amplified fragment containing mutation G301R was subcloned into *Eco*RV−*Xma*I-digested mRARα1. The C-terminal deletion mutants of mRXRα, which are dominant negative mutations [mRXRαT1 truncated at amino acid 454 and mRXRαT2 truncated at amino acid 448 (also referred to as mRXRdn)] were constructed by PCR amplification of *Eco*RV−*Bgl*II fragments encoding the new termination point and subcloned into *Eco*RV−*Bgl*II-digested mRXRα expression vector (Leid *et al.*, 1992b).

The chimeric protein Gal4-RARα(404–418)$_3$ was constructed by cloning a trimer of the oligonucleotide 5′-GTACGTCCATGCCACCGC-TGATCCAGGAAATGCTGGAGAACTCTGAGC-3′ in triplicate in the *Kpn*I site of pG4M (Nagpal *et al.*, 1993). The chimeric proteins ER(C)RAR(390–462) and ER(C)RXR(436–467) were created by cloning in pSG5 an *Eco*RI fragment encoding 5′ to 3′ the F region (residues 553–595), the DNA binding domain (residues 176–282) of the hER [ER(C)], the residues Pro-Gly-Gly and either amino acids 390–462 of mRARα1 or 436–462 of mRXR. ER(C)RAR(−) was constructed by inserting the *Eco*RI fragment in the reverse orientation. ER(C) and ER(C)RXRα(436–467) were also cloned into the *Eco*RI site of the yeast expression plasmids YEp90 (Pierrat *et al.*, 1992).

The mRARβ2/CAT reporter gene has been described previously (Smith *et al.*, 1991), as well as DR1T-tk/CAT, DR1G-tk/CAT, DR5T-tk/CAT and DR5G-tk/CAT (Mader *et al.*, 1993a). DR5G-globin/CAT was generously provided by S.Kato. (ERE)$_2$-tk/CAT was constructed by twice inserting an oligonucleotide corresponding to a consensus ERE into the *Sal*I site of pBLCAT2$^+$. 17M5-G/CAT was derived from the 17M2-G/CAT vector by inserting three additional 17mer repeats into the *Bam*HI site (Webster *et al.*, 1988). The *Saccharomyces cerevisiae* reporter strains used were PL1 and PL3 (Pierrat *et al.*, 1992).

Additional information and all oligonucleotide sequences are available upon request.

Transient transfection of COS-1 cells, CAT expression analysis and gel retardation/shift assays

COS-1 cell transfections, CAT assays and gel retardation/shift experiments were performed as described previously (Smith *et al.*, 1991; Durand *et al.*, 1992; Leid *et al.*, 1992b).

Bacterial expression of RAR proteins and RA binding assays

mRARα1, α301R, αΔ408–416 and α412P were each inserted into the *Nco*I−*Bam*HI sites of pET3d (Novagen). Bacterial extracts containing the mRARα1 or mutant mRARα proteins were prepared as described previously (Leid *et al.*, 1992b). Protein integrity was controlled by Western blot analysis (data not shown). The charcoal binding assay was used to determine RA binding of mRARα1 and the selected mutants α301R, αΔ408–416 and α412P. A selected amount of [^{3}H]RA (Dupont de Nemours) was dried so that the final ethanol concentration did not exceed 2%, and mixed with 1 ml of bacterial lysate/RA binding buffer mix [generally 1 μl RAR-containing bacterial extract, 79 μl BL21(DE$_3$)plysS extract in 920 μl of RA binding buffer (0.12 M KCl, 8 mM Tris−HCl pH 7.5, 8% glycerol, 4 mM DTT, 0.24 mM phenylmethylsulfonyl fluoride)]. Determination of non-specific binding was performed by adding a 500-fold molar excess of unlabelled T-RA to the tritiated T-RA prior to the addition of bacterial extract. Incubation was carried out on ice for 16 h in the dark. 500 μl of dextran-coated charcoal (3% DCC, w/v) in G-P buffer (150 mM NaCl, 100 mM Na_2HPO_4, 0.1% gelatin, 15 mM NaN_3) were added, mixed and incubated on ice for 15 min. Charcoal-absorbed RA was separated from receptor-bound RA by centrifugation; RA in the supernatant was quantitated by scintillation counting. Analysis was performed according to Scatchard.

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