

# A mutation mimicking ligand-induced conformational change yields a constitutive RXR that senses allosteric effects in heterodimers

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**Mutations of a single residue in the retinoid X receptor  $\alpha$  (RXR $\alpha$ ) ligand-binding pocket (LBP) generate constitutive, ligand-binding-competent mutants with structural and functional characteristics similar to those of agonist-bound wild-type RXR. Modelling of the mouse RXR $\alpha$ F318A LBP suggests that, like agonist binding, the mutation disrupts a cluster of van der Waals interactions that maintains helix H11 in the apo-receptor location, thereby shifting the thermodynamic equilibrium to the holo form. Heterodimerization with some apo-receptors (retinoic acid, thyroid hormone and vitamin D<sub>3</sub> receptors) results in ‘silencing’ of RXR $\alpha$ F318A constitutive activity, which, on the other hand, efficiently contributes to synergistic transactivation within NGFI-B–RXR heterodimers. RAR mutants disabled for corepressor binding and/or lacking a functional AF-2 activation domain, do not relieve RXR ‘silencing’. Not only RAR agonists, but also the RAR antagonist BMS614 induce conformational changes allowing RXR to exert constitutive (RXR $\alpha$ F318A) or agonist-induced (wild-type RXR) activity in heterodimers. Interestingly, the RXR $\alpha$ F318A constitutive activity generated within heterodimers in the presence of BMS614 requires the integrity of both RXR and RAR AF-2 domains. These observations suggest that, within RXR–RAR heterodimers, RAR can adopt a structure distinct from that of the active holo-RAR, thus allowing RXR to become transcriptionally responsive to agonists.**

**Keywords:** constitutively transcriptionally active RXR mutant/ligand-binding pocket/retinoid receptors/RXR–RAR heterodimers/RXR subordination

## Introduction

Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are members of the nuclear receptor superfamily and act as ligand-inducible transregulators of gene net-

works. They mediate the pleiotropic effects of retinoids on morphogenesis, homeostasis, cell growth, differentiation and apoptosis by directly interacting with DNA response elements of target genes, as well as by ‘crosstalk-ing’ to other signalling pathways (see Beato *et al.*, 1995; Gronemeyer and Laudet, 1995; Kastner *et al.*, 1995; Keaveny and Stunnenberg, 1995; Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995; Chambon, 1996 and references therein). RARs bind, and are activated by, all-*trans* (tRA) and 9-*cis* (9c-RA) retinoic acids, while RXRs respond only to 9c-RA. In addition, synthetic retinoids have been described which act as RAR (isotype)- and pan-RXR-specific agonists or antagonists (see Lehmann *et al.*, 1992; Apfel *et al.*, 1995; Chen *et al.*, 1995, 1996; Klein *et al.*, 1996; Lala *et al.*, 1996; Taneja *et al.*, 1996; and references therein). RXRs not only form homodimeric DNA complexes, but can promiscuously heterodimerize with various nuclear receptors, such as RAR and the thyroid hormone (TR), vitamin D<sub>3</sub> (VDR) and orphan receptors (e.g. NGFI-B). The response element repertoires of these homodimers and heterodimers are dictated by dimerization surfaces in the corresponding DNA-binding domains (Gronemeyer and Laudet, 1995; Gronemeyer and Moras, 1995; and references therein).

All receptors with known ligands contain two autonomous activation functions (AFs), one in the N-terminal region, termed AF-1, which is constitutively active when taken out of the context of the receptor, and a ligand-inducible AF-2 in the ligand-binding domain (LBD). The activity of AF-2 in animal cells is entirely dependent on the integrity of a sequence in the C-terminal part of the LBD, referred to as AF-2 AD core, whose features are conserved among all AF-2-containing receptors (Danielian *et al.*, 1992; Saatcioglu *et al.*, 1993; Baretino *et al.*, 1994; Durand *et al.*, 1994). The AF-2 of the promiscuous heterodimerization partner RXR is unique in that, in RAR or TR heterodimers, its activity is controlled not only by its own ligand, but also by the nature and ligand status of its dimerization partner. For example, in heterodimers with non-liganded (apo-)RAR or TR, but not with the orphan nuclear receptor NGFI-B, RXR could not activate transcription in response to its cognate ligand (Forman *et al.*, 1995; Perlmann and Jansson, 1995). Although it was initially concluded that RXR was unable to bind its ligand in heterodimers with apo-RAR (Forman *et al.*, 1995; Kurokawa *et al.*, 1994), subsequent studies showed very clearly that RXR is fully competent for ligand binding in such heterodimers (Apfel *et al.*, 1995; Chen *et al.*, 1996; Kersten *et al.*, 1996; Li *et al.*, 1997; Z.P.Chen, J.Iyer, W.Bourguet, P.Held, C.Mioskowski, L.Lebeau, N.Noy, P.Chambon, and H.Gronemeyer, submitted).

Based on transcriptional interference/squelching studies, we proposed the existence of transcriptional intermediary factors (TIFs, mediators) which transduce the activity of

nuclear receptor AF-2s to the transcriptional machinery (Bocquel *et al.*, 1989; Meyer *et al.*, 1989; Tasset *et al.*, 1990). Several putative TIFs/coactivators have been identified recently by their ability to interact with nuclear receptor LBDs in an agonist- and AF-2 integrity-dependent manner (for review, see Chambon, 1996; Glass *et al.*, 1997). Among those, SRC-1 (Onate *et al.*, 1995), TIF-2 (Voegel *et al.*, 1996) and CBP/p300 (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996; Smith *et al.*, 1996) behave as bona fide coactivators of nuclear receptor AF-2s according to several criteria (Voegel *et al.*, 1996). CBP/p300 interacts not only with nuclear receptors, but also with SRC-1 (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996; Smith *et al.*, 1996; Yao *et al.*, 1996) and TIF-2 (J.J.Voegel, M.J.S.Heine, M.Tini, P.Chambon, and H.Gronemeyer, unpublished) and is assumed to play an 'integrator' role for several signalling pathways (Janknecht and Hunter, 1996; Glass *et al.*, 1997; and references therein). Corepressors (N-CoR and SMRT) have also been identified. They efficiently interact with apo-RAR and apo-TR, and are released in the presence of agonists; the 'silencing' of cognate promoters by these apo-receptors appears to result from corepressor binding (Chen and Evans, 1995; Hörlein *et al.*, 1995).

Elucidation of the crystal structures of the apo-LBD of RXR $\alpha$ , and of the holo-LBDs of RAR $\gamma$  and TR $\alpha$  (Bourguet *et al.*, 1995a; Renaud *et al.*, 1995; Wagner *et al.*, 1995), revealed that ligand binding induces a major structural change; the most striking (but not only) difference between the apo and holo structures is a change in conformation of the 'activation' helix H12 which contains the AF-2 AD core. We have proposed a mechanism for generating active nuclear receptor AF-2s according to which the change in conformation of the LBD upon ligand binding creates the surface(s) for coactivator binding and, for some nuclear receptors, concomitantly destroys the corepressor-apo-receptor interface, thus resulting in a transcriptionally competent nuclear receptor (Renaud *et al.*, 1995; Wurtz *et al.*, 1996). It has remained unclear, however, how the ligand induces this change in LBD conformation. Similarly, the mechanism(s) leading to RXR silencing in heterodimers with certain apo nuclear receptors, an essential point to understand the role of RXR ligands in the thyroid, vitamin D<sub>3</sub> and PPAR ligand signalling pathways, has remained elusive.

Here we report the identification of constitutively active RXR mutants, which allow us to (i) propose a sequence of molecular events by which the ligand induces the LBD transconformation and (ii) study the basis of RXR silencing in certain heterodimers.

## Results

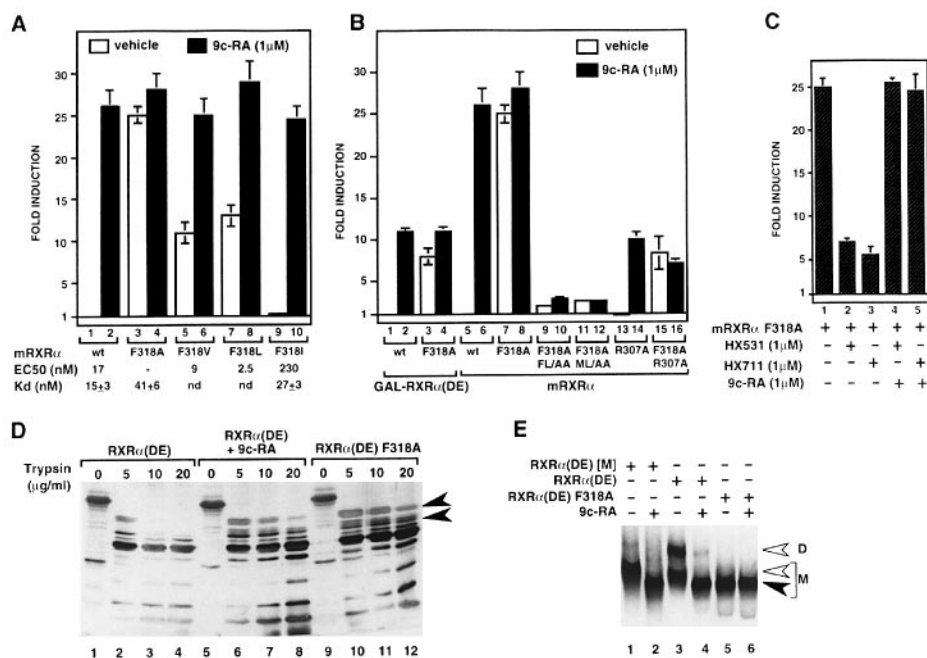
### **Mutations of a single residue in the ligand-binding pocket (LBP) of RXR $\alpha$ generate a transcriptionally constitutively active receptor homodimer**

The crystal structure (Bourguet *et al.*, 1995a) and structure-based nuclear receptor sequence alignments (Renaud *et al.*, 1995; Wurtz *et al.*, 1996) suggested that three mouse RXR $\alpha$  phenylalanine residues (mRXR $\alpha$ F318, F442 and F443) could have critical roles in shaping the LBP and in the change of LBD conformation triggered by ligand binding. We mutated each of these residues to alanine and characterized the resulting mutants. mRXR $\alpha$ F442A bound

9-*cis*-RA with 15-fold reduced affinity and mRXR $\alpha$ F443A exhibited wild-type characteristics (not shown). In contrast, mRXR $\alpha$ F318A was constitutively transcriptionally active on a DR1 reporter gene and could also bind 9-*cis*-RA with affinity 3-fold lower than wild-type (Figure 1A, lanes 3 and 4). Increasing the size of the aliphatic side chain of residue 318 decreased the constitutive activity, such that, similarly to wild-type mRXR $\alpha$ , mRXR $\alpha$ F318I was transcriptionally active in the presence of 9c-RA only (Figure 1A, lanes 9 and 10). The constitutive activity of mRXR $\alpha$ F318A was observed in both HeLa and Cos-1 cells with reporter genes harbouring four different promoters (Table I), and did not depend on its activation function 1 (AF-1; Nagpal *et al.*, 1992, 1993), as GAL4-mRXR $\alpha$ (DE)F318A was also constitutively active on (17m) $\times$ 5-G-CAT (Figure 1B, lane 3).

### **The functional and structural features of mRXR $\alpha$ F318A are similar to those of wild-type holo-RXR**

Several lines of evidence strongly suggest that the conformation of the F318A mutant LBD is similar to that of holo-mRXR $\alpha$ : (i) the same mutations [mRXR $\alpha$ (F318A, FL/AA) and mRXR $\alpha$ (F318A, ML/AA); see Figure 3B] in the AF-2 AD core within the activation helix H12 abolish the transcriptional activity of the wild-type (not shown, see Le Douarin *et al.*, 1995) and mutant mRXR $\alpha$  (Figure 1B, lanes 9–12); (ii) the mutation mRXR $\alpha$ R307A, homologous to hRAR $\gamma$ K264A which disrupts a salt bridge (illustrated in Figure 3A) important for the interaction between helices H12 and H4 in the crystal structure of holo-RAR $\gamma$  (Renaud *et al.*, 1995), similarly decreases the ligand-induced and constitutive activities of wild-type RXR and mRXR $\alpha$ F318A, respectively (Figure 1B, lanes 14–16; see discussions in Renaud *et al.*, 1995 and Wurtz *et al.*, 1996); (iii) the RXR pan-antagonists HX531 and HX711 (our unpublished results) also inhibit the constitutive activity of mRXR $\alpha$ F318A, and this inhibition is relieved by an excess of 9c-RA (Figure 1C); (iv) partial proteolysis of purified *Escherichia coli*-expressed LBDs of mRXR $\alpha$ F318A (Figure 1D, lanes 9–12) and holo-mRXR $\alpha$  (lanes 5–8) generates indistinguishable peptide maps which differ significantly from that of the apo-mRXR $\alpha$  LBD (lanes 1–4); (v) in the absence or presence of 9c-RA, the mRXR $\alpha$ F318A LBD (Figure 1E, lanes 5 and 6) exists exclusively as monomers in solution which migrate with an electrophoretic mobility distinct from that of the wild-type apo-LBD monomer (lanes 1), but identical to that of the wild-type holo-LBD monomer (lanes 2 and 4); note that the wild-type apo-mRXR $\alpha$  LBD exists in solution as a mixture of monomers and dimers (lane 3), and that exposure to 9c-RA causes dissociation of the LBD dimers (lane 4); (vi) in contrast to mRXR $\alpha$ , the interaction of the mRXR $\alpha$ F318A LBD with TIFs (a property of the holo-LBD conformation; see Le Douarin *et al.*, 1995, 1996; Onate *et al.*, 1995; Voegel *et al.*, 1996; for reviews see Gronemeyer and Laudet, 1995; Chambon, 1996) is ligand-independent (see below); and (vii) the decreased affinity of mRXR $\alpha$ F318A for 9c-RA is in keeping with a holo-RXR conformation in which the helix H12 'lid' laid on the entrance of the LBP (Renaud *et al.*, 1995; Wurtz *et al.*, 1996) is expected to decrease ligand association kinetics.



**Fig. 1.** Mutation of mRXR $\alpha$  residue F318 generates constitutively active receptors. (A–C) Transcriptional activity of full-length wild-type and mutant mRXR $\alpha$ , and of a chimera comprising the mRXR $\alpha$  LBD fused to the GAL4 DNA-binding domain [GAL-RXR $\alpha$ (DE)], analysed by transient transactivation assays in Cos1 cells with the indicated mRXR $\alpha$  (mutant) expression vectors and the DR1-tk-CAT reporter gene (panels A, B and C, lanes 5–16) or GAL-RXR $\alpha$ (DE) together with the cognate (17m) $\times$ 5-G-CAT reporter (panel B, lanes 1–4). Cells were exposed to the indicated ligands or vehicle (ethanol) for 24 h. CAT levels were determined by ELISA. Results are expressed as fold induction of basal reporter gene activity and represent the mean  $\pm$  SEM of three to six independent experiments. In (A),  $K_{dS}$  for 9c-RA were determined by Scatchard analysis; EC<sub>50</sub> values correspond to the ligand concentration giving half-maximal DR1 reporter gene transactivation (for the ligand-dependent activity component); nd, not determined. (C) The RXR antagonists HX531 and HX711 inhibit the ligand-independent activity of mRXR $\alpha$ F318A (compare lane 1 with lanes 2 and 3). Note that, due to its much higher affinity, 9c-RA can efficiently compete with the HX compounds at equimolar ratios (lanes 4 and 5). (D) Tryptic partial proteolysis maps of purified *E. coli*-expressed apo (lanes 1–4) and holo wild-type RXR $\alpha$  (lanes 5–8) and apo-RXR $\alpha$ F318A (lanes 9–12) LBDs. Digestion products were separated by SDS-PAGE and visualized by silver staining. Arrowheads point to major differences between the apo and holo patterns. (E) Non-denaturing gel electrophoresis revealing that both apo- and holo-mRXR $\alpha$ F318A monomers migrate with a mobility similar to that of wild-type holo- but not apo- mRXR $\alpha$  LBD monomers. Mobilities were revealed by Coomassie-staining of non-denaturing 6% polyacrylamide gels loaded with purified apo- (lane 5) and holo- (lane 6) mRXR $\alpha$ F318A monomers, purified wild-type mRXR $\alpha$  apo- (lane 1) and holo-LBD monomers (lane 2) and a partially purified preparation containing both mRXR $\alpha$  LBD dimers (D) and monomers (M) in the absence (lane 3) and presence (lane 4) of 9-*cis*-RA. Note that the purified wild-type RXR apo-LBD exists in solution as monomers and dimers which dissociate into monomers upon ligand exposure (compare lanes 3 and 4), while only monomers, migrating with wild-type mRXR $\alpha$  holo-LBD mobility, were observed for the mRXR $\alpha$ F318A LBD. D, dimers; M, monomers (open triangles, apo-LBDs; closed triangles, holo-LBDs). The figures in (D) and (E) are representative of three separate experiments.

**Table I.** The mRXR $\alpha$ F318A mutant exhibits ligand-independent activity with several RXR-responsive promoters

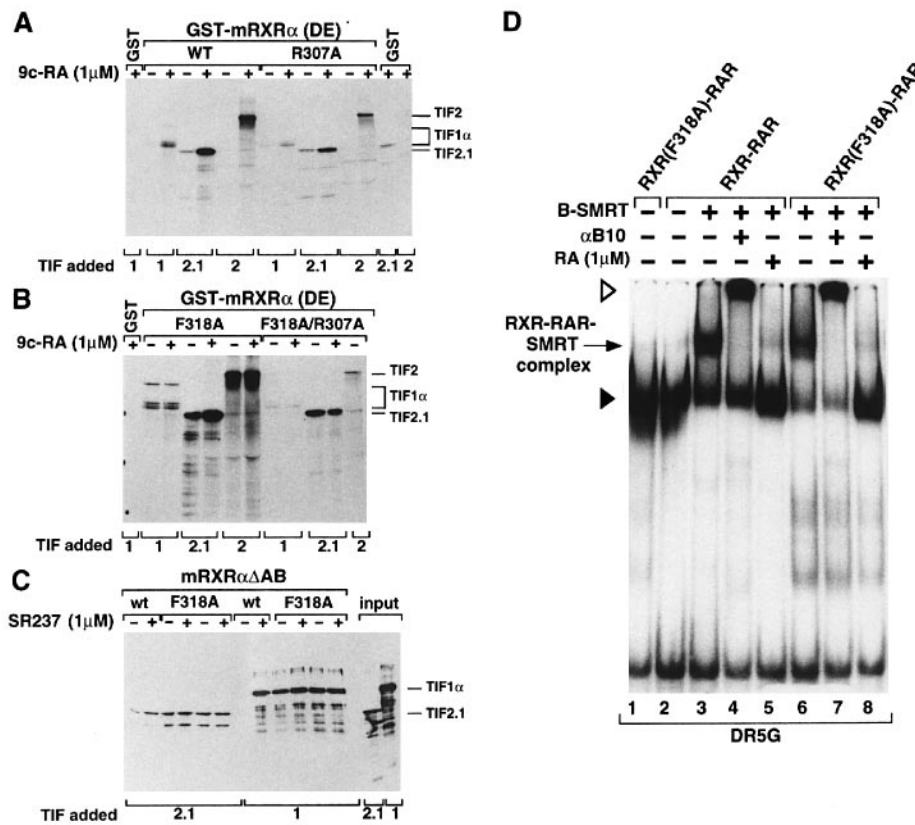
| Receptor               | Fold induction |                   |              |             |                   |               |                   |               |              |
|------------------------|----------------|-------------------|--------------|-------------|-------------------|---------------|-------------------|---------------|--------------|
|                        | DR1-tk         |                   |              | CRABPII     |                   | (TRE)3-tk     |                   | DR5-tk        |              |
|                        | –              | 9- <i>cis</i> -RA | SR237        | –           | 9- <i>cis</i> -RA | –             | 9- <i>cis</i> -RA | –             | SR237        |
| RXR $\alpha$ wild-type | 1 $\pm$ 0.1    | 26 $\pm$ 2        | 26 $\pm$ 0.5 | 1 $\pm$ 0.1 | 40 $\pm$ 3        | 1 $\pm$ 0.1   | 11 $\pm$ 1        | 0.8 $\pm$ 0.1 | 13 $\pm$ 1.5 |
| RXR $\alpha$ F318A     | 25 $\pm$ 1     | 28 $\pm$ 2        | 43 $\pm$ 4.5 | 27 $\pm$ 2  | 28 $\pm$ 2        | 7.4 $\pm$ 1.6 | 13 $\pm$ 2        | 10 $\pm$ 0.3  | 27 $\pm$ 2   |

The transient transactivation assays were performed in Cos1 cells; results are expressed as fold induction of reporter gene basal activity. Data are the mean  $\pm$  SEM of three to six independent experiments.

**In contrast to wild-type RXR, the constitutively active mRXR $\alpha$ F318A interacts with coactivators also in the absence of ligand**

Both TIF1 $\alpha$  (Le Douarin *et al.*, 1995, 1996) and TIF2 (Voegel *et al.*, 1996) interact with the wild-type RXR LBD in an agonist- and AF-2-integrity-dependent manner (Figure 2A). As reported for RAR $\gamma$  (Renaud *et al.*, 1995; Wurtz *et al.*, 1996), the equivalent mRXR $\alpha$ R307A mutation which destabilizes the helix H4–H12 interaction (see Figure 3A) that is important for generating an ‘efficient’ AF-2, significantly weakened both TIF1 $\alpha$ - and

TIF2–RXR LBD interactions in glutathione-*S*-transferase (GST) pull-down assays (Figure 2A). Interestingly, both TIFs constitutively interacted with the mRXR $\alpha$ F318A LBD, and the additional mutation R307A weakened this interaction as in the case of wild-type mRXR $\alpha$  (Figure 2B). Both the ligand-dependent and ligand-independent interactions of TIF1 $\alpha$  and TIF2 with wild-type mRXR $\alpha$  and mRXR $\alpha$ F318A, respectively, were also seen with the corresponding receptor homodimers bound to the cognate DR1 response elements in ABCD assays (Figure 2C). Together the above data suggest that the mRXR $\alpha$ F318A



**Fig. 2.** The constitutively active mRXR $\alpha$ F318A interacts *in vitro* in a ligand-independent manner with the putative transcriptional mediators TIF1 $\alpha$  and TIF2. (**A** and **B**), GST pull-down assays: *In vitro* translated [<sup>35</sup>S]methionine-labelled TIF1, TIF2 and TIF2.1 were incubated with GST-fused LBDs of mRXR $\alpha$  wild-type, R307A, F318A and F318A/R307A mutants, in the absence (-) or presence (+) of 1  $\mu$ M 9c-RA. After elution, bound proteins were separated on a 10% SDS-polyacrylamide gel and revealed by autoradiography. Note that TIF1 $\alpha$  is partially proteolysed. (**C**) ABCD assays: purified wild-type and F318A mRXR $\alpha$  $\Delta$ AB bound to biotinylated DR1-containing oligonucleotides were mixed with purified TIF1 and TIF2.1 in the absence (-) or presence (+) of 1  $\mu$ M SR237. The complexes were separated on a 10% SDS-polyacrylamide gel and the coactivators were revealed by Western blotting. (**D**) The ligand-sensitive interaction of the corepressor SMRT with RXR-RAR-DNA complexes is not modified by the F318A mutation as shown by EMSA. Mutant (lane 1) or wild-type (lane 2) RXR-RAR heterodimer bound to <sup>32</sup>P-labelled DR5G oligonucleotides were mixed without (-) or with (+) B epitope-tagged SMRT in the absence (-) or presence (+) of 1  $\mu$ M RA. The complexes, resolved on 5% polyacrylamide gels, were revealed by autoradiography. The mobility of the ternary RXR-RAR-DR5G complex is indicated by a filled triangle; the presence of B-SMRT in the quaternary complex (lanes 3 and 6) is revealed by a further supershift (open triangle; lanes 4 and 7) with anti-B-epitope antibodies. All these figures are representative of at least three independent experiments.

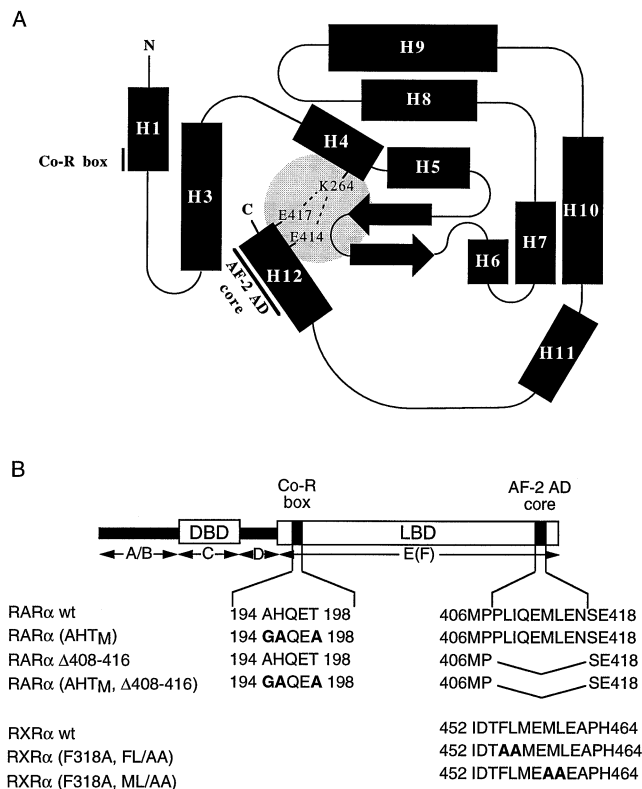
apo-LBD adopts a structure very similar, if not identical, to the ligand-induced wild-type RXR holo-LBD, including the cognate interaction surfaces for at least two distinct TIFs.

#### **The constitutively active AF-2 of mRXR $\alpha$ F318A is silenced in heterodimers with apo-RAR, TR and VDR**

The availability of a constitutively active RXR offers a unique opportunity to investigate the contribution of the RXR partner to the transcriptional activity exerted by heterodimers in the absence of an RXR ligand (for which it is difficult to rule out the possibility that it could also interact with RAR within an RXR-RAR heterodimer). Heterodimerization with apo-RAR $\alpha$  decreased the constitutive mRXR $\alpha$ F318A homodimer activity seen with DR5 (~10-fold activation) and DR1 (~30-fold activation) reporter genes by a factor of 5 and 15, respectively (Figure 4A, compare lanes 1 and 7; DR1 data not shown). Similarly, very little mRXR $\alpha$ F318A constitutive activity was seen when it was associated with either apo-TR on DR4 or apo-VDR on DR3 elements (not shown). The RXR-specific ligand SR237 (SR11237 in Lehmann *et al.*,

1992), which in contrast to 9c-RA further stimulates the constitutive activity of mRXR $\alpha$ F318A homodimers (Table I and Figure 4A, lanes 1 and 2), could not efficiently relieve the silencing effect exerted by these apo-receptors on the constitutive activity of mRXR $\alpha$ F318A (Figure 4A, lane 8). The RAR-specific agonist Am80 strongly activated the mRXR $\alpha$ F318A-RAR heterodimer (Figure 4A, lane 9) in an RAR AF-2 AD integrity-dependent manner, as no activity was observed with RAR $\alpha$  $\Delta$ 408-416 in which the AF-2 AD core is deleted (Figure 4A, lane 23; for a description of the mutant see Figure 3B and Durand *et al.*, 1994). Mutating the mRXR $\alpha$ F318A AF-2 AD core (in mutant mRXR $\alpha$ F318A, ML/AA; see Figure 3B) reduced the Am80-induced heterodimer activity (lanes 9 and 19) to levels seen in the presence of the RXR antagonist HX531 (lane 10) or with the wild-type RXR-RAR heterodimer (lane 14), and abrogated the additional stimulation seen in the presence of the RXR agonist SR237 (compare lanes 11 and 20).

'Silencing' of the constitutive mRXR $\alpha$ F318A AF-2 by apo-RAR did not require the formation of a DNA-bound RXR-RAR heterodimer, since GAL-mRXR $\alpha$ F318A activity (Figure 4B, lane 1) was impaired by coexpressed



**Fig. 3.** Illustration of helix H4, corepressor box (Co-R box) and AF-2 AD box mutants in the LBD of RAR and RXR. (A) Topological representation of the general fold of nuclear receptor holo-LBDs (Renaud *et al.*, 1995; Wurtz *et al.*, 1996), illustrating the salt bridge observed for RAR $\gamma$  (Renaud *et al.*, 1995) between K264 (helix 4) and E414 and E417 in the AF-2 AD core-containing helix 12. Note that mRXR $\alpha$ R307 corresponds to hRAR $\gamma$ K264. (B) Schematic representation of the modular structure of nuclear receptors, detailing the corepressor box and AF-2 AD core wild-type and mutant sequences. AF-2 AD corresponds to the amphipathic  $\alpha$ -helix conserved in all transcriptionally active nuclear receptors and which was defined as critical for the AF-2 function. Mutations in the corepressor and AF-2 AD boxes are in bold.

RAR (lane 5). That this is a consequence of heterodimerization between RAR and the ligand-binding domain of GAL-mRXR $\alpha$ F318A is apparent from the efficient stimulation of the 17m-tk-CAT GAL4 reporter by the RAR-specific retinoid Am80 (lane 7). In the presence of RAR, the Am80-induced transactivation seen with the constitutive GAL-mRXR $\alpha$ F318A was greater than that seen with its wild-type homologue (compare lanes 7 and 12), and could not be further stimulated by the RXR agonist SR237 (lane 9), whereas it was reduced (lanes 7 and 8), unlike that of the wild-type homologue (lanes 12 and 13), by the RXR-selective antagonists HX531. In contrast, the RXR-selective agonist SR237 and the RAR-selective agonist Am80 synergistically activated the wild-type heterodimer (lane 14). These results demonstrate that agonist binding to RAR not only relieves the silencing on RXR, but also that the AF-2 activation function of RXR contributes to transactivation by RXR-RAR heterodimers.

**In contrast to apo-RAR, TR and VDR, the orphan receptor NGFI-B synergizes with the activity of the constitutive mRXR $\alpha$ F318A**

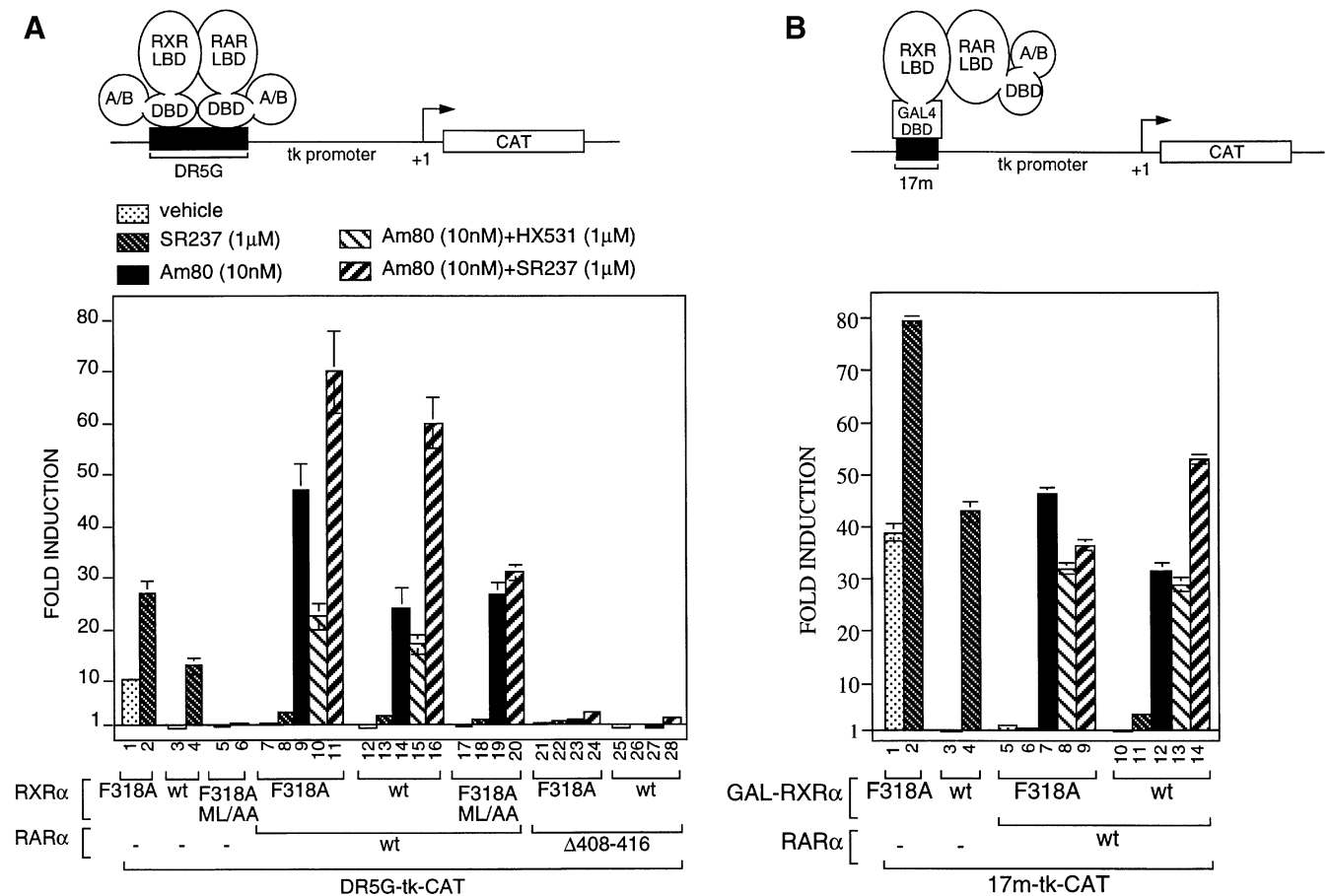
The 'silencing' of the constitutive activity of mRXR $\alpha$ F318A was dimerization partner-specific, since the con-

stitutive activities of isolated NGFI-B and mRXR $\alpha$ F318A (2- and 3-fold induction, respectively; Figure 5, lanes 1 and 6) strongly synergized within heterodimers to yield a 33-fold activation of an RAR $\beta$ 2 promoter-based reporter (lane 8). The activity of the mRXR $\alpha$ F318A-NGFI-B heterodimer was similar to that seen with the wild-type RXR-NGFI-B heterodimer in the presence of the RXR ligand (lane 5) and could be further enhanced with the RXR ligand SR237 (lane 9) or 9c-RA (not shown). The RXR antagonist HX531 decreased mRXR $\alpha$ F318A-NGFI-B transactivation, indicating the contribution of mRXR $\alpha$ F318A AF-2 to this activity (compare lanes 8 and 10). Thus, the ability of the constitutive RXR $\alpha$ F318A mutant to activate transcription in various heterodimeric complexes parallels that reported for holo-RXR $\alpha$  (Forman *et al.*, 1995; Perlmann and Jansson, 1995).

**The silencing of constitutive RXR activity by unliganded RAR is not relieved by preventing corepressor binding to RAR and is not caused by the apo conformation of RAR helix H12 per se**

The 'silencing' of the constitutive AF-2 of mRXR $\alpha$ F318A (and of the wild-type holo-RXR) in heterodimers could result from an inhibition by apo-RAR-bound transcriptional corepressors, such as N-CoR (Hörlein *et al.*, 1995) or SMRT (Chen and Evans, 1995). Electrophoretic mobility shift assays (EMSA) showed that the mRXR $\alpha$ F318A-RAR-DR5 complex efficiently formed quaternary complexes with SMRT *in vitro* (Figure 2D, compare lane 6 with lane 3; similar results were obtained with DR1, DR2 and IR0 response elements, data not shown), and exposure to RA quantitatively dissociated the corepressor (lane 8), as in the case of wild-type heterodimers (lane 5), concomitantly inducing coactivator association (see above; and data not shown). However, an RAR triple mutant [hRAR $\alpha$ (A194G,H195A,T198A), termed RAR $\alpha$ (AHT<sub>M</sub>); see Figure 3B], incapable of efficient N-CoR (Hörlein *et al.*, 1995) and SMRT (Schulman *et al.*, 1997, and our unpublished data) binding, also silenced the constitutive activity of mRXR $\alpha$ F318A in the corresponding heterodimer (Figure 6; compare lanes 1 and 3, and lanes 2 and 4), as well as that of GAL-mRXR $\alpha$ F318A (data not shown), thus arguing against corepressor binding to the apo-RAR-mRXR $\alpha$ F318A complex as the sole cause of mRXR $\alpha$ F318A silencing.

To investigate whether the presence/positioning in the apo conformation of RAR helix H12, which encompasses the AF-2 AD core (Renaud *et al.*, 1995), could be responsible for RXR silencing, the activity of mRXR $\alpha$ F318A was studied in heterodimers containing the RAR mutant RAR $\alpha$  $\Delta$ 408-416 which lacks H12 (see Figure 3A and B). No ligand-independent activity could be detected (Figure 4A, lane 21), thus excluding the possibility that the apo conformation of RAR helix H12 *per se* could impair the activity of the constitutive RXR. Furthermore, mRXR $\alpha$ F318A silencing was not relieved in heterodimers containing RAR mutants [RAR $\alpha$ (AHT<sub>M</sub>,  $\Delta$ 408-416); illustrated in Figure 3B] lacking both the AF-2 AD core and the ability to interact with corepressors in the absence or presence of SR237 (Figure 6, lanes 17 and 18), thus excluding a direct implication of the apo-RAR H12 conformation in RXR silencing, even in the absence of bound corepressor. This conclusion was further supported



**Fig. 4.** The constitutive activity of RXR $\alpha$ F318A is silenced upon heterodimerization with RAR and can be relieved by RAR agonists. Transcriptional activity of either wild-type or mutant RXR–RAR (A) and GAL–RXR–RAR (B) heterodimers on DR5- and 17m-based reporter genes, respectively, cotransfected in Cos1 cells. After transfection, cells were treated for 24 h with ethanol or the indicated ligand [note that the key to shading of the bars in (A) applies also to (B)]. CAT levels were quantified by ELISA, normalized according to  $\beta$ -galactosidase coexpressed from the internal control vector. Results are expressed as fold induction of the basal reporter gene activity. Data represent the mean  $\pm$  SEM of at least three independent experiments. A cartoon illustrating the experimental design is shown at the top of each figure.

by the observation that the constitutive GAL-mRXR $\alpha$ -F318A and SR237-induced GAL-RXR $\alpha$  activity were completely silenced, even in the presence of RAR agonists, when RAR mutants lacking the AF-2 AD core or double mutants bearing in addition the AHT<sub>M</sub> mutation were co-overexpressed to generate LBD heterodimers (data not shown).

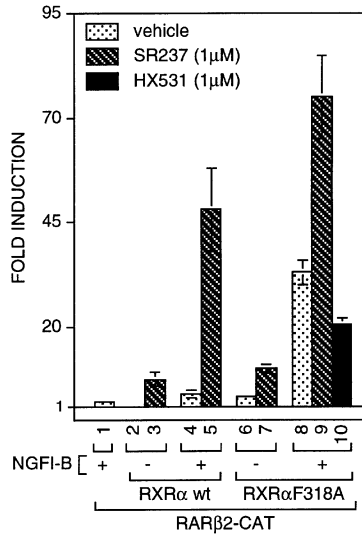
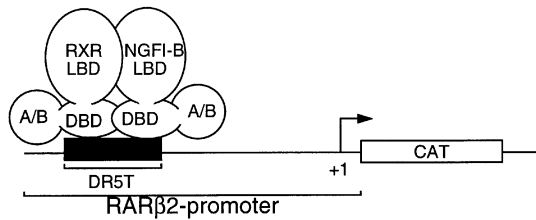
**The integrity of the RAR AF-2 AD is essential for agonist-induced activity of an RXR–RAR heterodimer, while heterodimers lacking a functional RXR AF-2 AD are activated by RAR agonists**

As discussed above, mutation of the mRXR $\alpha$ F318A AF-2 AD core decreased, but did not abrogate, the Am80-induced activity of the corresponding heterodimer with RAR (Figure 4A, lane 19), indicating that RAR agonists can autonomously activate their cognate receptors in a heterodimeric setting. In contrast, RXR lost its autonomy in RAR heterodimers, since a deletion of the RAR AF-2 AD core fully abrogated the Am80-induced transactivation originating from the mRXR $\alpha$ F318A–RAR heterodimer (Figure 4A, lane 23), as well as that seen in the case of wild-type RXR in the presence of Am80 and SR237 (lane 28), even though the RXR antagonist data indicate a

significant contribution of the constitutive RXR AF-2 to the heterodimer activity (see above; note that RAR $\alpha$  $\Delta$ 408–416 still binds RA; Durand *et al.*, 1994, and data not shown). The absence of activity was not due to a stabilization of RAR–corepressor interaction upon H12 deletion (Chen and Evans, 1995), since essentially identical results were obtained with RAR $\alpha$ (AHT<sub>M</sub>,  $\Delta$ 408–416) (Figure 6, lanes 17–24). Thus, RXR requires the presence of an intact RAR helix H12 to exert its activity in a heterodimer. In keeping with the above results, the constitutive activity of GAL-mRXR $\alpha$ F318A (or the SR237-induced activity of wild-type GAL-RXR) remained silenced when the AF-2-disabled RAR was co-overexpressed, even when Am80 was present, irrespective of whether RAR could or could not (AHT<sub>M</sub> mutant) interact with corepressors (data not shown).

**An agonist- or antagonist-induced structural change of the RAR LBD is required to relieve RXR silencing in RXR–RAR heterodimers**

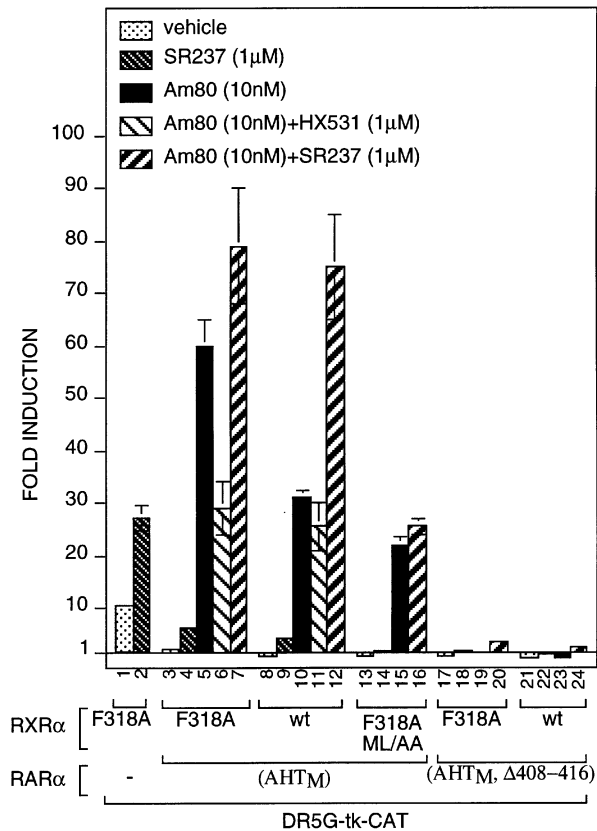
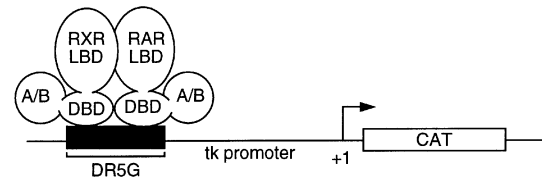
Using a panel of synthetic retinoids, we studied the characteristics of RAR ligands which could relieve RXR silencing by apo-RAR. Interestingly, not only RAR agonists but also the RAR $\alpha$  antagonist BMS614 (Chen *et al.*, 1996) relieved the mRXR $\alpha$ F318A silencing (Figure



**Fig. 5.** NGFI-B and RXRαF318A synergistically activate the RARβ2 promoter. The figure shows the transcriptional activity in the presence of the indicated ligands of wild-type and mutant RXR–NGFI-B heterodimers transiently expressed in Cos1 cells together with an RARβ2–CAT reporter gene. Presentation of the results and illustration of the experimental design are analogous to those in Figure 4.

7A; compare lanes 4 and 5, and lanes 13 and 14). Moreover, the constitutive activity of GAL-mRXRα-F318A, but not the wild-type GAL-RXRα, was efficiently ‘derepressed’ when BMS614 was bound to co-overexpressed RAR (Figure 7B, compare lanes 3 and 4 with lanes 6 and 7). Note that BMS614 acted efficiently as an RAR antagonist also in the context of the RXRα–RARα heterodimer (Figure 7A, lane 8, and data not shown). Thus, it appears that BMS614 induces a change in RARα conformation which is insufficient to form an ‘active’ holo-RARα AF-2, but allows the constitutive mRXRαF318A to exert its activity in the context of the heterodimer. In keeping with this notion, mutation of the mRXRαF318A AF-2 AD core inhibited the BMS614-relieved activity (mutant F318A, ML/AA in Figure 7A; compare lanes 5 and 11 and lanes 14 and 20). Moreover, if this BMS614-induced activity were due to a relief of RXR silencing upon BMS614 binding to RAR, the RXR-selective ligand SR237 should also activate the wild-type RXR–RAR in the presence of this retinoid, which was indeed the case (Figure 7A, lanes 9 and 18; Figure 7B, lane 8).

Deletion of the AF-2 AD core of RAR (mutant RARαΔ408–416; Figure 3B) also abrogated the BMS614-relieved activity of the constitutive mRXRαF318A and GAL-mRXRαF318A, as well as the BMS614+SR237-induced activity of the wild-type RXR–RAR heterodimer (Figure 7A, lanes 22–27, and data not shown). Thus, the integrity of both the RAR and RXR AF-2 AD cores (helix 12) is requisite for signalling through the RXR partner of



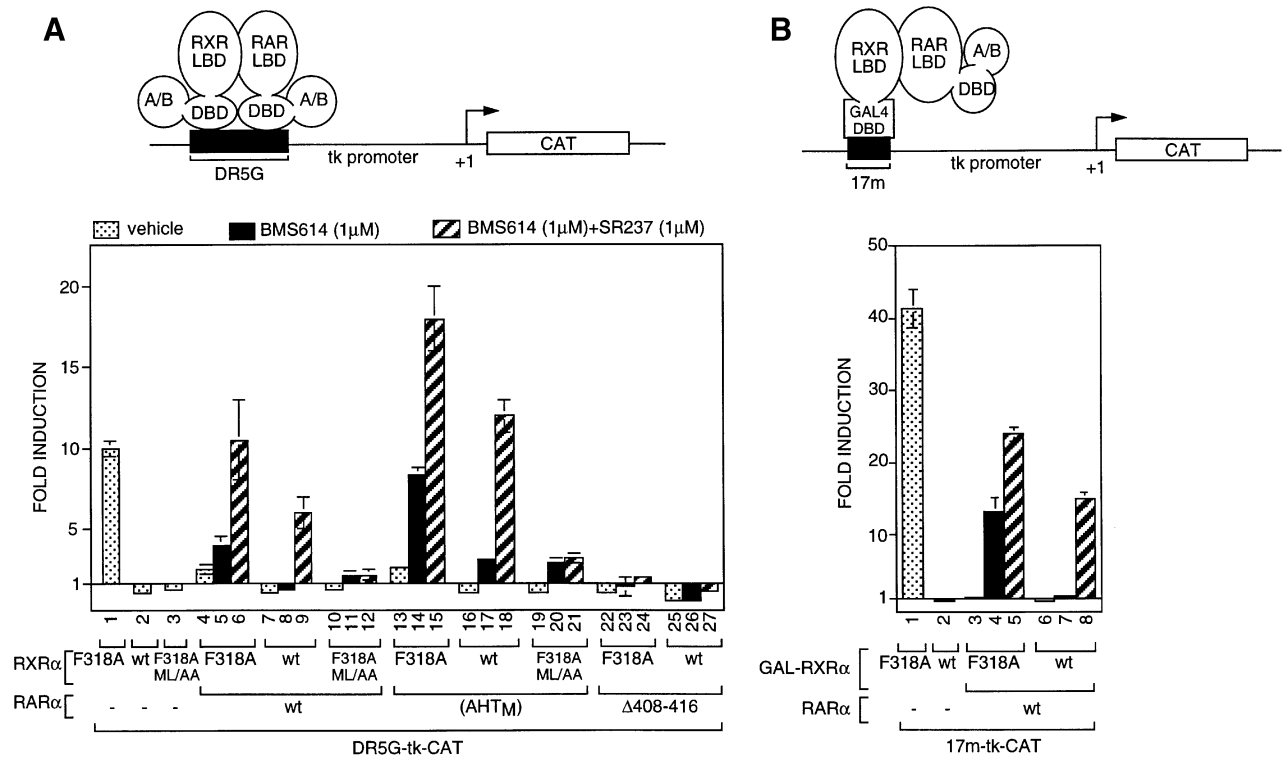
**Fig. 6.** Corepressor binding to apo-RAR is not the cause of RXR silencing. Transcriptional activity in the presence of the indicated ligands of either wild-type or mutant RXR–RAR heterodimers on DR5G-based reporter gene. Transient transactivation in Cos1 cells of the DR5G-tk-CAT reporter by wild-type mRXRα or mRXRαF318A, coexpressed with RARα(AHT<sub>M</sub>) or RARα(AHT<sub>M</sub>, Δ408–416). Presentation of the results and illustration of the experimental design are analogous to those in Figure 4.

the heterodimer, implying that, in contrast to the RAR subunit which responds autonomously to its ligand, the RXR activity depends critically on RAR, since it requires an RAR ligand-induced structural change of the heterodimer, which necessitates an intact RAR helix 12, but not the transconformation which generates the transcriptionally active AF-2 of holo-RAR.

## Discussion

### Modelling of the ligand-binding pocket of the constitutively active RXR suggests how the ligand triggers the LBD transconformation

In the crystal structure of the apo-hRXRα LBD (Bourguet *et al.*, 1995a), residue F313 of H5 (homologue of mRXRαF318) anchors a cluster of van der Waals interactions with H5, H7, the β-turn and H11 residues, thus generating a strongly hydrophobic environment and maintaining the position of H11 relative to the conserved LBD



**Fig. 7.** RXR silencing in RXR–RAR heterodimers is relieved by the RAR $\alpha$ -selective antagonist BMS614. Transcriptional activity in the presence of the indicated ligands of wild-type or mutant RXR–RAR (**A**) or GAL–RXR–RAR (**B**) heterodimers, transiently transfected in Cos1 cells, on DR5G- or 17m-tk-CAT as reporter genes. Presentation of the results and illustration of the experimental design are analogous to those in Figure 4 [note that the key to shading of the bars in (A) applies also to (B)].

core (Wurtz *et al.*, 1996) (Figure 8A). A disruption of these interactions by mutating the anchor residue (hRXR $\alpha$ F313) or by ligand binding to wild-type RXR (Figure 8B and C shows the ligand in the mutant LBP) releases H11. Note that for clarity not all interactions constituting this network are depicted in Figure 8 and that residues other than F313 are not necessarily critical for maintaining the integrity of the network, as, for example, the mutation F438A generates a mutant with wild-type ligand binding and transactivation characteristics (see Results; F438 corresponds to F443 in the mouse homologue). As previously proposed (Renaud *et al.*, 1995; Wurtz *et al.*, 1996), it is reasonable to assume that the release of H11 results in a disruption of the contacts of loop 11–12 and H12 with H3, such that H11 and H12 can rearrange according to an equilibrium which is determined by novel interactions including an important salt bridge apparently established between H4 and H12 in both mRXRF318A (this report) and the holo-RXR $\alpha$  (Renaud *et al.*, 1995; Wurtz *et al.*, 1996). In addition, solvation forces imposed on the modified surfaces of H11 and H12 will contribute to this new equilibrium, as, for example, the buried apo-hRXR H11 residues F437 and F438 are on the exterior in the holoreceptor, while exterior residues of the apo form (e.g. hRXR F439) become part of the hydrophobic pocket in the holoreceptor.

Together, the above considerations lead to a dynamic model for the LBD structure which, upon ligand binding and dissociation, would preferentially adopt the holo and apo conformations, respectively, interconversion of which follows a defined path of structural alterations. According to this model, the mRXR $\alpha$ F318A mutation would shift

the thermodynamic equilibrium to the holo form by destabilizing the apo form, thus increasing the lifetime of the alternative H11–H12 conformation.

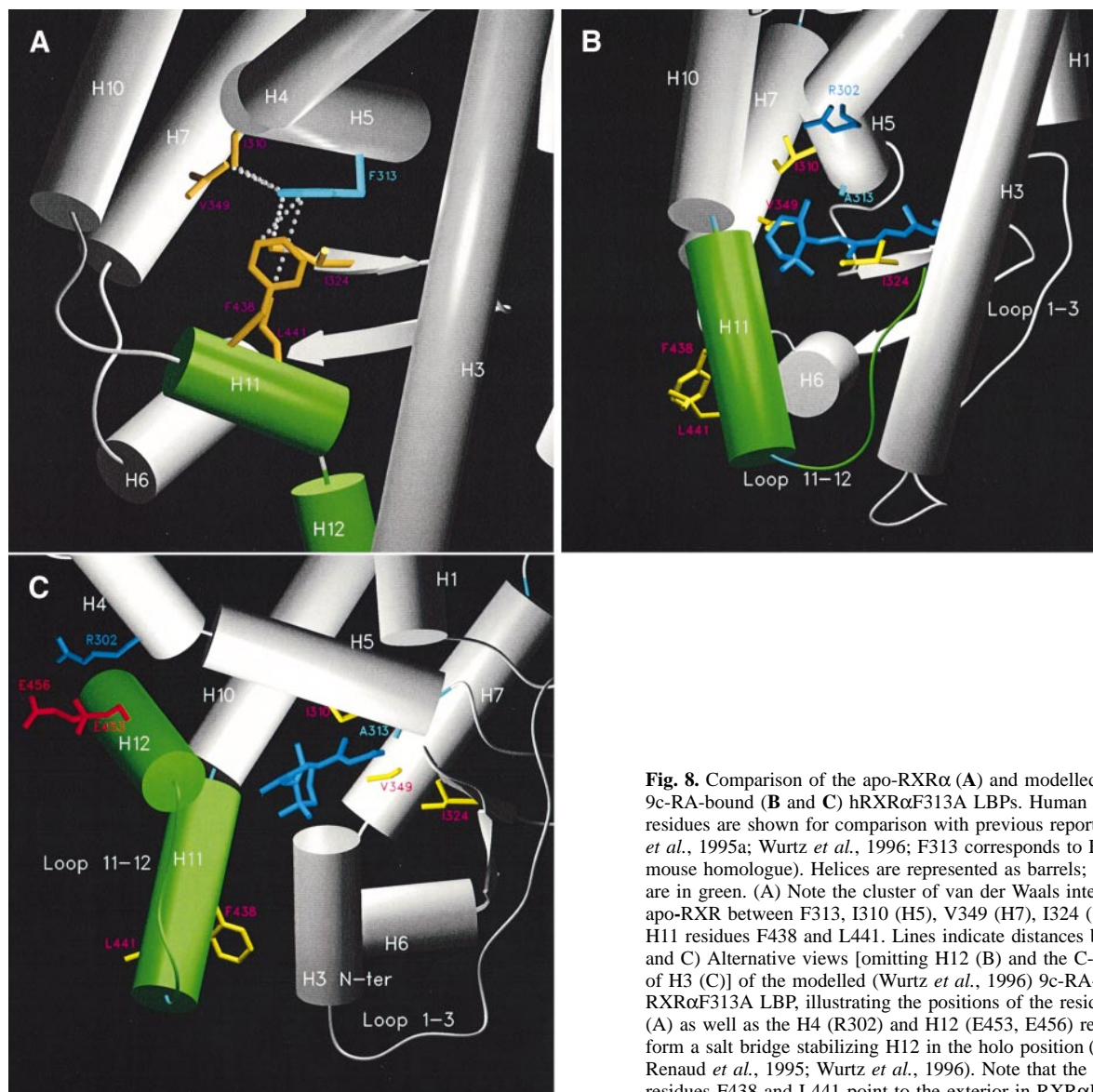
#### **Implications for ligand-independent activity of other nuclear receptors**

Mouse RXR $\alpha$ F318 (F313 in hRXR $\alpha$ ) is not conserved in other nuclear receptors, and neither mutation of homologous residues nor mutations disrupting clusters of interactions similar to those in the apo-RXR LBP generate constitutively active RAR, PR or oestrogen receptor mutants (details available on request). Different and/or additional interactions may stabilize the apo (and holo) form of these receptors. Interestingly, the generation of constitutively active retinoid X and oestrogen (Weis *et al.*, 1996; White *et al.*, 1997) receptors by single amino acid residue mutations within and outside of their LBPs, respectively, suggests that post-translational modifications of nuclear receptor LBD residues (e.g. by phosphorylation) could also shift the equilibrium between the apo and holo nuclear receptor LBD structures in the absence of ligand (see discussion in Weis *et al.*, 1996; White *et al.*, 1997), or alter this equilibrium in the presence of agonistic or antagonistic ligands. In this way, signals from other pathways could be transduced through nuclear receptors in the absence of cognate ligands or alter the activity of ligand-bound nuclear receptors.

#### **Multiple ligand-controlled allosteric events are required to relieve RXR 'silencing' in heterodimers with some apo nuclear receptors**

The recognition of RXR as a promiscuous dimerization partner for receptors mediating non-retinoid signals such as





**Fig. 8.** Comparison of the apo-RXR $\alpha$  (A) and modelled 9c-RA-bound (B and C) hRXR $\alpha$ F313A LBPs. Human receptor residues are shown for comparison with previous reports (Bourguet *et al.*, 1995a; Wurtz *et al.*, 1996; F313 corresponds to F318 in the mouse homologue). Helices are represented as barrels; H11 and H12 are in green. (A) Note the cluster of van der Waals interactions in the apo-RXR between F313, I310 (H5), V349 (H7), I324 ( $\beta$ -turn) and the H11 residues F438 and L441. Lines indicate distances below 4 Å. (B and C) Alternative views [omitting H12 (B) and the C-terminal region of H3 (C)] of the modelled (Wurtz *et al.*, 1996) 9c-RA-bound RXR $\alpha$ F313A LBP, illustrating the positions of the residues shown in (A) as well as the H4 (R302) and H12 (E453, E456) residues that form a salt bridge stabilizing H12 in the holo position (see Figure 3A; Renaud *et al.*, 1995; Wurtz *et al.*, 1996). Note that the apo-LBP residues F438 and L441 point to the exterior in RXR $\alpha$ F318A LBP.

thyroid hormone (T3) or vitamin D3 created a conundrum, since retinoids were not known to affect these signalling pathways *in vivo* and RA-deprived animals do not exhibit abnormalities that could be readily related to impaired thyroid hormone or vitamin D<sub>3</sub> signalling. Moreover, RXR-selective ligands on their own could not trigger RXR–RAR heterodimer-mediated RA-induced events in various cell systems (Roy *et al.*, 1995; Chen *et al.*, 1996; Clifford *et al.*, 1996; Taneja *et al.*, 1996; Chiba *et al.*, 1997). This is, however, not due to an inability of the RXR partner to bind its cognate ligand in DNA-bound heterodimers, as has been previously suggested (Kurokawa *et al.*, 1994), because RXR ligand binding has been demonstrated to occur in such complexes in several studies *in vitro*, and synergistic transactivation induced by RAR- and RXR-selective ligands has been observed *in vivo* (Apfel *et al.*, 1995; Roy *et al.*, 1995; Chen *et al.*, 1996, 1997; Clifford *et al.*, 1996; Kersten *et al.*, 1996; Taneja *et al.*, 1996; Chiba *et al.*, 1997; Li *et al.*, 1997; Minucci *et al.*, 1997). Together with our present results, which further support the conclusion that RXR can be transcrip-

tionally active within an RAR–RXR heterodimer provided the RAR partner is adequately liganded, all of these observations rule out the initial concept that RXR is *a priori* a transcriptionally ‘silent’ partner in RAR–RXR heterodimers (Forman *et al.*, 1995; Kurokawa *et al.*, 1994). Rather, RAR apparently ‘controls’ the activity of RXR–RAR heterodimers in two ways: it induces transcription in response to its own ligand and it silences RXR activity in the absence of an RAR ligand. Consequently, the only way for RXR to affect transactivation in response to its ligand in RXR–RAR heterodimers is through synergy with RAR ligands. This concept of RXR silencing may not apply to all nuclear receptor partners, as the ligand-induced RXR activity was fully permissive in heterodimers with NGFI-B, leading even to a synergistic response (Figure 5; Forman *et al.*, 1995; Perlmann and Jansson, 1995). However, neither the existence of an endogenous NGFI-B ligand nor a weak constitutive activity of the NGFI-B AF-2 can be excluded; both of these scenarios would readily explain RXR activity and NGFI-B–RXR synergy due to the absence of RXR silencing. Note that

the same argument would apply to the seemingly RXR ligand-induced activity of RXR–PPAR heterodimers (Mukherjee *et al.*, 1997).

The observation that the characteristics of mRXR $\alpha$ -F318A are similar to those of holo-RXR provides us with an excellent tool for investigating the molecular basis for RXR silencing in RXR–RAR heterodimers without the need to use a ligand in order to induce RXR activity. In this respect, note that, throughout this study, we did not observe any systematic differences between the constitutive activity of mRXR $\alpha$ F318A and the RXR-agonist-induced activity of the wild-type RXR in heterodimers with RAR, TR or VDR. Our results can be summarized as follows: (i) LBD heterodimerization is sufficient, and apo-RAR-mediated silencing of the constitutive RXR AF-2 activity does not require binding of both heterodimer subunits to DNA, since apo-RAR can inhibit the (constitutive or RXR agonist-induced) GAL-RXR activity on a GAL4 reporter, apparently through the formation of LBD heterodimers; (ii) this silencing occurs independently of corepressor interaction with apo-RAR, since mutants unable to bind corepressors efficiently still repress the constitutive AF-2 of RXR and (iii) it is not the apo conformation of RAR helix H12 which could account for the silencing effect, either on its own or in combination with corepressor binding. Thus, the inactivity of the constitutive RXR AF-2 is probably linked to a structural constraint within the LBD heterodimer which is incompatible with the formation of an ‘active’ surface for transcriptional coactivator interaction. In this respect, we have recently hypothesized that ligand-induced activation of RXR–RAR heterodimers may comprise two events, one of which is characterized by the ability of RAR antagonists, which themselves do not induce activity, to generate a structure that allows RXR agonists to generate a transcriptionally active RXR within the heterodimer, while the second event corresponds to the generation of an ‘active’ RAR AF-2 in response to RAR agonist binding (Chen *et al.*, 1996). Indeed, we show here that BMS614, an RAR $\alpha$ -specific antagonist which does not interact with RAR $\beta$  or RAR $\gamma$  (Chen *et al.*, 1996; M.Gehin, V.Vivat, J.M.Würtz, D.Moras, H.Gronemeyer and P.Chambon, unpublished and data not shown), generates such a ‘permissive’ heterodimer structure in which the silencing of RXR by RAR is relieved. Taken together, and in view of the fact that all conclusions drawn from experiments with the constitutive RXR apply also to agonist-bound wild-type RXR, our results establish RXR silencing as a mechanism through which a heterodimer-mediated signalling cascade has to be initiated in target cells by the binding of the signalling ligand, while the efficiency of the response can be modulated by ligand binding to the promiscuous RXR heterodimerization partner.

#### **A mechanistic view of allosteric events governing RXR–RAR heterodimer activity**

At least two mechanisms may in principle account for RXR ‘silencing’ in RXR–RAR heterodimers. (i) The ‘subordination’ model implies that the structure of apo-RAR–RXR heterodimers is incompatible with the existence of an ‘active’ RXR AF-2 interaction surface for coactivators. This ‘subordination’ is intraheterodimeric and occurs independently of corepressor binding. Binding

of the RAR ligand then induces an allosteric change in conformation allowing the generation of an ‘active’ RXR AF-2 interaction surface for coactivators (see Renaud *et al.*, 1995; Wurtz *et al.*, 1996). (ii) The ‘RAR AF-2 only’ model implies that the ligand-induced transcriptional activity of RXR AF-2 is exerted entirely through the AF-2 function of the RAR partner whose activity is controlled negatively by corepressors and positively by coactivators. The function of agonist-bound RXR would be limited to an allosteric increase of the affinity of coactivators for the RAR AF-2 interaction surface, thus accounting for the synergistic effects of RAR and RXR agonists. This ‘RAR AF2 only’ model is based mainly on that recently proposed for RXR–LXR heterodimers (Willy and Mangelsdorf, 1997) and a particular RXR ligand (Schulman *et al.*, 1997), in which the integrity of the AF-2 of the partner (LXR, RAR) is requisite for RXR ligand-mediated activity (Schulman *et al.*, 1997; Willy and Mangelsdorf, 1997), while the RXR AF-2 AD is not required. Note, however, that the integrity of RXR AF2 is indispensable for the present RXR constitutive activity in RAR-liganded heterodimers, which necessarily implies different allosteric transitions.

According to the ‘subordination’ model, the silencing of the constitutive (or ligand-bound) RXR in heterodimers with some apo nuclear receptors would correspond to partner-specific RXR AF-2 subordination, while the ‘RAR AF-2 only’ model would explain this silencing as the inability of RXR AF-2 to act through apo nuclear receptors which form ‘non-permissive’ heterodimers. The observation that an RAR antagonist induces a structure which is insufficient for RAR AF-2 activity within the heterodimer, while relieving the silencing of RXR AF-2 activity, indicates that the ‘permissive’ RAR structure is not necessarily the same as that required for RAR AF-2 activity, as would be predicted from the ‘RAR AF-2 only’ model; thus, we currently favour the RXR ‘subordination’ model. Two experimental approaches will ultimately allow the two models to be distinguished: (i) the experimentally demanding demonstration that RXR can or cannot associate with coactivators in liganded heterodimers and/or (ii) the crystallization of mRXR $\alpha$ F318A–RAR heterodimers in the absence or presence of BMS614 and an RAR agonist, which should also reveal the structural events underlying the multiple allosteric phenomena that govern heterodimer activity.

## **Materials and methods**

### **Ligands**

All-*trans*-RA was from Sigma and 9-*cis*-RA was a gift of Michael Sporn. The RXR antagonists used were HX531: 4-[5H-2,3-(2,5-dimethyl-2,5-hexano)-5-methyl-8-nitro]benzo-[b,e][1,4]diazepin-11-yl]benzoic acid and HX711: 4-[5H-10,11-dihydro-5,10-dimethyl-2,3-(2,5-dimethyl-2,5-hexano)-dibenzo[b,e][1,4]diazepin-11-yl]benzoic acid; their synthesis and characterization will be reported separately.

### **Mutant expression vectors**

Mutants were constructed by PCR-assisted site-directed mutagenesis, using Deep Vent DNA polymerase (Biolabs) and the following plasmids as templates: pSG5-mRXR $\alpha$  (Nagpal *et al.*, 1993) for substitution of F318 by A, V, L or I; pSG5-mRXR $\alpha$ F318A for additional R307A, F455A/L456A or M459A/L460A mutations; pSG5-mRAR $\alpha$  (Durand *et al.*, 1994) for the  $\Delta$ 408–416 deletion; pSG5-hRAR $\alpha$  for mutations of A194, H195 and T198 to G, A and A respectively [called RAR $\alpha$ (AHT<sub>M</sub>)].

and pSG5-RAR $\alpha$ (AHT<sub>M</sub>) for the additional deletion of amino acid residues 408–416 yielding pSG5-RAR $\alpha$ (AHT<sub>M</sub>,  $\Delta$ 408–416). GAL-mRXR $\alpha$ F318A was obtained by subcloning the *Bam*HI–*Bgl*III fragment (containing the F318A mutation) from pSG5-mRXR $\alpha$ F318A into the *Bam*HI and *Bgl*III sites of pG4MpolyII-mRXR $\alpha$ (DE). Sequences of the PCR primers are available on request. All constructs were verified by automated DNA sequencing. The expression and size of the mutants were verified by Western blotting.

#### Cell culture and transfection

Cos1 cells plated at a density of  $1.5 \times 10^6$  cells per dish were transfected as described (Bocquel *et al.*, 1989) using a calcium phosphate method. Precipitates contained, for RXR or GAL-RXR homodimer activity studies, 0.2  $\mu$ g of receptor expression vector, 2  $\mu$ g of reporter gene [DR1G-tk-CAT for full-length RXRs, (17m) $\times$ 5-G-CAT (Chen *et al.*, 1995) or 17m-tk-CAT for the RXR LBD fused to GAL4 DNA-binding domain] and 0.5  $\mu$ g of CMV- $\beta$ GAL (used as internal control to normalize for transfection efficiency). For RXR–RAR heterodimer activity studies, 0.2  $\mu$ g of pSG5-mRXR $\alpha$  (wild-type or mutant) was cotransfected with 0.2  $\mu$ g pSG5-RAR $\alpha$  wild-type or AHT mutant vectors, or 1  $\mu$ g of the ( $\Delta$ 408–416) or (AHT<sub>M</sub>,  $\Delta$ 408–416) mutant RAR expression vectors, or 2  $\mu$ g of pCMV-NGFI-B; 0.2  $\mu$ g of GAL-RXR $\alpha$ (DE) was cotransfected with 1  $\mu$ g of RAR expression vectors; and 0.2  $\mu$ g of pSG5-RAR $\alpha$  was cotransfected with 1  $\mu$ g pSG5-mRXR $\alpha$ (F318A, ML/AA) (see Figure 3B). Two micrograms of reporter genes [DR5-tk-CAT (Chen *et al.*, 1995) for RXR–RAR heterodimers, 17m-tk-CAT for GAL-RXR–RAR heterodimers or RAR $\beta$ 2-CAT (Nagpal *et al.*, 1993) for RXR–NGFI-B heterodimers] and 0.5  $\mu$ g of CMV- $\beta$ GAL were added as indicated in the corresponding figures. The CAT was quantified by ELISA (Boehringer Mannheim) according to the manufacturer's recommendations. Results are given as fold induction of reporter gene basal activity. Data represent the mean  $\pm$  SEM of three to six independent experiments.

#### Limited proteolysis

Two micrograms of *E. coli*-expressed, immobilized metal affinity chromatography (IMAC)-purified (using Ni<sup>2+</sup>; for purification see Bourguet *et al.*, 1995b; Rochel *et al.*, 1997) histidine-tagged wild-type or RXR $\alpha$ F318A LBD in the presence or absence of a saturating concentration of 9c-RA, were incubated as described (Leid, 1994) with 0, 5, 10 or 20  $\mu$ g/ml trypsin for 10 min at room temperature in a final volume of 10  $\mu$ l. Proteolysis was terminated by adding 1 vol. of Laemmli sample buffer. The proteolytic fragments were separated on a 15% SDS–polyacrylamide gel and visualized by silver staining.

#### Non-denaturing gel electrophoresis

Fifty micrograms of IMAC-purified histidine-tagged RXR $\alpha$ F318A LBD and additionally gel filtration-separated RXR LBD monomer and dimer in the presence or absence of a saturating concentration of 9c-RA were run on a 6% non-denaturing polyacrylamide gel (McLellan, 1982) in 300 mM Tris base, 100 mM CAPS, pH 9.4, and visualized by Coomassie blue staining.

#### In vitro binding assays

*GST-pull down assays.* Glutathione–Sepharose loaded with *E. coli*-expressed RXR LBDs fused to GST (GST–RXR $\alpha$  wild-type and GST–RXR $\alpha$ F318A) was incubated as previously described (Le Douarin *et al.*, 1995; Voegel *et al.*, 1996) in the presence or absence of 1  $\mu$ M 9c-RA with *in vitro* translated TIF1, TIF2 and TIF2.1 labelled with [<sup>35</sup>S]methionine. Complexes separated on a 10% SDS–polyacrylamide gel were revealed by autoradiography.

*Avidin–biotin-complexed DNA (ABCD) assays.* Two micrograms of *E. coli*-expressed IMAC- and gel filtration-purified histidine-tagged mRXR $\alpha$  $\Delta$ AB (residues 133–467) and mRXR $\alpha$  $\Delta$ AB(F318A), assembled on DR1-containing oligonucleotides and immobilized on an avidin–biotin matrix, were incubated in the presence or absence of 1  $\mu$ M 9c-RA with *E. coli*-expressed histidine-tagged TIF2.1 (0.5  $\mu$ g) and baculovirus-expressed TIF1 (1  $\mu$ g) and separated on a 10% SDS–polyacrylamide gel; the coactivators were revealed by Western blotting using monoclonal TIF1 and TIF2 antibodies (Le Douarin *et al.*, 1995; Voegel *et al.*, 1996).

#### Electrophoretic mobility shift assay

EMSA were done as described by Zechel *et al.* (1994a,b). Recombinant proteins used for EMSAs were *E. coli*-expressed histidine-tagged mRXR $\alpha$  $\Delta$ AB (residues 133–467), mRAR $\alpha$  $\Delta$ AB (residues 81–462), mRXR $\alpha$ F318A $\Delta$ AB (residues 133–467); histidine-tagged B-SMRT carries the oestrogen receptor B-epitope (Shemshedini *et al.*, 1992; Ali

*et al.*, 1993) in front of SMRT residues 982–1495 [corresponding to C-SMRT (Chen and Evans, 1995)].

## Acknowledgements

We thank Jeffrey Milbrand for the CMV-NGFI-B expression vector, Thomas Perlmann for anti-NGFI-B antiserum, Michael Sporn for a generous gift of 9c-RA, Bertrand Le Douarin and Johannes Voegel for TIF1 $\alpha$  and TIF2, Thierry Lerouge for cloning and providing the SMRT corepressor cDNA, Cathie Erb, Jean-Marie Garnier and Thierry Lerouge for help in the construction of recombinants, Jaya Iyer, Dominique Bonnier, Virginie Andry and Florence Granger for gifts of purified retinoid receptor (complexes), and the IGBMC services for cell culture, DNA sequencing, oligonucleotide synthesis and computer-assisted design. SR11237 (BMS188,649) and BMS614 (BMS195,614) were kindly provided by Peter Reczek, Bristol-Myers-Squibb. This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Ministère de la Recherche et de la Technologie, the Collège de France, the EC Biomed Programme (BMH4-CT96-0181) and Bristol-Myers-Squibb.

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*Received on May 2, 1997; revised on July 1, 1997*