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RARs and RXRs: evidence for two autonomous transactivation functions (AF-1 and AF-2) and heterodimerization *in vivo*

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We have previously reported that the AB regions of retinoic acid receptors (RARs and RXRs) contain a transcriptional activation function capable of modulating the activity of the ligand-dependent activation function present in the C-terminal DE regions of these receptors. However, we could not demonstrate that these AB regions possess an autonomous activation function similar to the AF-1s found in the AB regions of steroid hormone receptors. Using the mouse CRBPII promoter as a reporter gene, we now report that the AB regions of RARα, β and γ, as well as those of RXRα and γ, contain an autonomous, ligand-independent activation function, AF-1, which can efficiently synergize with AF-2s. Moreover, AF-1s account for the ligand-independent, constitutive activation of transcription by RXRα and γ. We also show that RARs and RXRs preferentially heterodimerize in solution in cultured cells *in vivo*, through the dimerization interface present in their E region, irrespective of the presence of all-*trans* or 9-*cis* retinoic acid. Furthermore, our results indicate that homodimeric interactions are not observed in cultured cells *in vivo* under conditions where heterodimeric interactions readily occur, which is in agreement with previous observations showing the preferential binding of RAR-RXR heterodimers to RA response elements *in vitro*.

Key words: AF-1/AF-2/autonomous transactivation functions/heterodimerization/retinoic acid receptors

Introduction

Nuclear receptors are members of a superfamily of ligand-inducible transcriptional regulatory factors that include steroid hormone, thyroid hormone, vitamin D3 and retinoid receptors (for a review, see Evans, 1988; Green and Chambon, 1988, 1991; Beato, 1989; De Luca, 1991; Gronemeyer, 1991; Leid *et al.*, 1992a; Linney, 1992; Yu *et al.*, 1992). Like other transcriptional regulators (see Ptashne, 1992), nuclear receptors exhibit a modular structure which reflects the existence of several autonomous functional domains. Based originally on amino acid sequence similarities between the chicken oestrogen receptor, the human oestrogen and glucocorticoid receptors, and the v-erbA oncogene, Krust *et al.* (1986) defined six regions, A, B, C, D, E and F (see Figure 1B), which display different degrees of evolutionary conservation amongst the various members of the nuclear receptor superfamily and were shown to correspond to functional domains. The highly conserved 66 amino acid region C contains two zinc fingers and corresponds to the core of the DNA-binding domain (DBD), which is responsible for specific recognition of cognate response elements. Region E is functionally complex, since in addition to the ligand-binding domain (LBD), it contains a ligand-dependent activation function (AF-2) and a dimerization interface. An autonomous transcriptional activation function (AF-1) is present in the non-conserved N-terminal A/B regions of the oestrogen, progesterone and glucocorticoid receptors. Interestingly, both AF-1 and AF-2 of steroid receptors exhibit differential transcriptional activation properties which appear to be both cell type and promoter context specific (for a review and references, see Green and Chambon, 1988, 1991; Gronemeyer, 1991).

The all-*trans* (T-RA) and 9-*cis* (9C-RA) retinoic acid signals are transduced by two families of nuclear receptors. RARα, β and γ (and their isoforms) are activated by both T-RA and 9C-RA, whereas RXRα, β and γ are exclusively activated by 9C-RA (for reviews and references, see De Luca, 1991; Leid *et al.*, 1992a; Linney, 1992; Yu *et al.*, 1992; Allenby *et al.*, 1993; Kastner *et al.*, 1993). The three RAR types differ in their B regions, and their main isoforms (α1 and α2, β1-β4, and γ1 and γ2) have different N-terminal A regions (reviewed in Leid *et al.*, 1992a). Similarly, the three RXRs characterized up to now differ in their AB regions (Leid *et al.*, 1992a,b; Mangelsdorf *et al.*, 1992). It was thus assumed that specific activation functions, similar to the AF-1s of steroid receptors, could be present in the A/B regions of the RARs and RXRs. In agreement with this hypothesis, we recently demonstrated the existence of promoter context-specific transcriptional functions in the N-terminal A/B regions of RARs and RXRs which could synergize with and modulate the autonomous ligand-dependent AF-2 activation function associated with Region E (Nagpal *et al.*, 1992). However, we could not demonstrate that the AF-1s of RAR and RXR possess an autonomous activation function, as is the case for steroid receptors.

The E region of RARs and RXRs has also been shown to contain a dimerization interface (reviewed in Laudet and Stehelin, 1992; Leid *et al.*, 1992a; Yu *et al.*, 1992). Most interestingly, it was demonstrated that RAR/RXR heterodimers bind much more efficiently *in vitro* than homodimers of either receptor to a number of RA response elements (RAREs) (Yu *et al.*, 1991; Berrodin *et al.*, 1992; Bugge *et al.*, 1992; Hall *et al.*, 1992; Hallenbeck *et al.*, 1992; Husmann *et al.*, 1992; Kliewer *et al.*, 1992b; Leid *et al.*, 1992b; Marks *et al.*, 1992; Zhang *et al.*, 1992a; for reviews, see Laudet and Stehelin, 1992; Leid *et al.*, 1992a; Yu *et al.*, 1992). RAR and RXR heterodimers are also preferentially formed in solution *in vitro* (Yu *et al.*, 1991; Leid *et al.*, 1992b; Marks *et al.*, 1992), although the addition of 9C-RA

appears to enhance the formation of RXR homodimers *in vitro* (Lehmann *et al.*, 1992; Zhang *et al.*, 1992b). That RAR−RXR heterodimers, rather than the corresponding homodimers, could also preferentially bind to RAREs in cultured cells *in vivo* has been strongly supported by a recent report from our laboratory (Durand *et al.*, 1992), although the possible formation of RXR homodimers, particularly in the presence of 9C-RA, could not be excluded.

The present study was undertaken: (i) to demonstrate that the A/B regions of RARs and RXRs contain transcriptional activation functions similar to AF-1s of steroid receptors, i.e. capable of functioning autonomously in the absence of the AF-2s which are co-localized with the ligand-binding and dimerization functions in the E region; (ii) to investigate the potential of RARs and RXRs to homodimerize and to heterodimerize in cultured cells *in vivo*, both in the absence and in the presence of retinoic acids. We report that the N-terminal A/B region of RARα, β and γ, as well as those of RXRα and γ, contain an autonomous, ligand-independent AF-1 which can efficiently synergize with AF-2s, and also accounts for RXR ligand-independent activation of transcription. Furthermore, we show that RARs and RXRs preferentially heterodimerize in solution in cultured cells *in vivo*, through the dimerization interface present in their E region, irrespective of the presence of either T-RA or 9C-RA ligand.

Results

Experimental design

Two types of hybrid proteins were used to provide direct experimental evidence that the N-terminal AB regions of RARs and RXRs contain independent activation functions (AF-1s), and to investigate whether these activation functions could synergize with the ligand-dependent activation functions (AF-2s) associated with the C-terminal DE regions. In (AB)-ER(C) expression vectors, the AB regions of RARα1, RARβ2, RARγ1, RXRα, RXRβ and RXRγ were associated with the DNA-binding domain (DBD, region C) of the human oestrogen receptor (ER); in Gal4-RAR(DEF) and Gal4-RXR(DE) receptor expression vectors, the DEF and DE regions of the various RARs and RXRs were associated with the DBD of the yeast transactivator Gal4[Gal(1-147)] (see Figure 1B). The activation potential of these hybrid proteins was tested in transient transfections in Cos-1 cells using the chloramphenicol acetyl transferase (CAT) reporter construct mCRBPII(17m-ERE)/CAT, in which the putative RA response elements (RARE2 and RARE3) of the mouse CRBPII (mCRBPII) promoter (H.Nakshatri and P.Chambon, in preparation) were replaced with a synthetic oligonucleotide containing a GAL4 DNA-binding site (17m) and an oestrogen response element (ERE) (see Figure 1A).

The possible intracellular formation of RAR and RXR homo- and heterodimers through the dimerization interfaces, which are located in their DE regions (for references, see Introduction and Laudet and Stehelin, 1992; Leid *et al.*, 1992a; Yu *et al.*, 1992) was investigated by transfecting Gal4-RAR or Gal4-RXR with either RAR or RXR expression vectors, together with the same mCRBPII(17m-ERE)/CAT reporter gene. Since RAR and RXR cannot bind on their own to either the 17m GAL4 binding site or the ERE, any increase in CAT activity above that observed with Gal4-RAR or Gal4-RXR should reflect, under these conditions, the binding of RAR or RXR to the dimerization interface of Gal4-RAR or Gal4-RXR.

In all experiments, the RA ligands were used at 50 nM to discriminate between RAR and RXR transactivation, since at this concentration T-RA activates RARs, but not RXRs, while 9C-RA activates both RARs and RXRs (Durand *et al.*, 1992; Allenby *et al.*, 1993). Using the expression vector pSG5, all natural receptors and their derivatives (Figure 1B) were produced in transfected Cos-1 cells at similar levels (within a factor of 2−3), as judged by Western immunoblotting and (or) gel shift/retardation analysis. Variations in transfection efficiencies were normalized before CAT activity measurements, by determining the β-galactosidase activity generated from the co-transfected *Escherichia coli* β-galactosidase expression vector pCH110. All results were expressed as fold stimulation in CAT activity, taking as one the activity in the absence of co-transfected transactivator expression vector, but in the presence of ligand when applicable. All data given in the figures were reproduced (±20%) in at least three independent transfection experiments.

Autonomous activation functions (AF-1s) in the N-terminal AB regions of RXRα and γ

Both RXRα(AB)-ER(C) and RXRγ(AB)-ER(C) activated the expression of mCRBPII(17m-ERE)/CAT (Figure 2A, lanes 9 and 16), thus demonstrating the presence of AF-1 autonomous activation functions in the AB regions of RXRα and RXRγ. In contrast, no AF-1 activity could be detected in the case of RXRβ(AB)-ER(C) (lane 23), consistent with the previously reported weak activation of the natural mCRBPII promoter construct mCRBPII/CAT1 by RXRβ (Nagpal *et al.*, 1992). The autonomous 9C-RA-dependent activation functions AF-2, which were previously characterized in all three RXRs (Nagpal *et al.*, 1992; Allenby *et al.*, 1993), also activated the mCRBPII(17m-ERE)/CAT reporter (Figure 2A, lanes 1−8). Furthermore, AF-1s of either RXRα or γ efficiently synergized with AF-2s of either RXRα, β or γ (lanes 11, 13 and 15, and lanes 18, 20 and 22, respectively; note that two scales are used in the figure). In contrast, no synergism was observed in the case of the RXRβ AB region (lanes 23−29).

The existence of an autonomous ligand-independent activation function AF-1 in RXRα and γ was confirmed by experiments which showed that the mCRBPII promoter (Figure 3A, and data not shown), the mCRABPII promoter (Figure 3B, and data not shown), and the osteocalcin promoter (Figure 3B, and data not shown) could be activated by RXRα and RXRγ, but not by RXRβ, in the absence of 9C-RA. In all three cases, the activation by RXRα and RXRγ was lost when the AB region was deleted (ΔAB constructs in Figure 3), but not when the AF-2 activation function was inactivated by deletion of the receptor C-terminal region (as shown for dnRXRα, which has lost the AF-2 function, but can still efficiently dimerize and bind DNA; see Durand *et al.*, 1992). In contrast, the expression of the DR1G-tk-CAT reporter gene was not stimulated by RXRα (or dnRXRα) or RXRγ in the absence of 9C-RA (data not shown), which is in agreement with the lack of stimulation of the tk promoter by AF-1s of RXRα and γ (see Nagpal *et al.*, 1992).

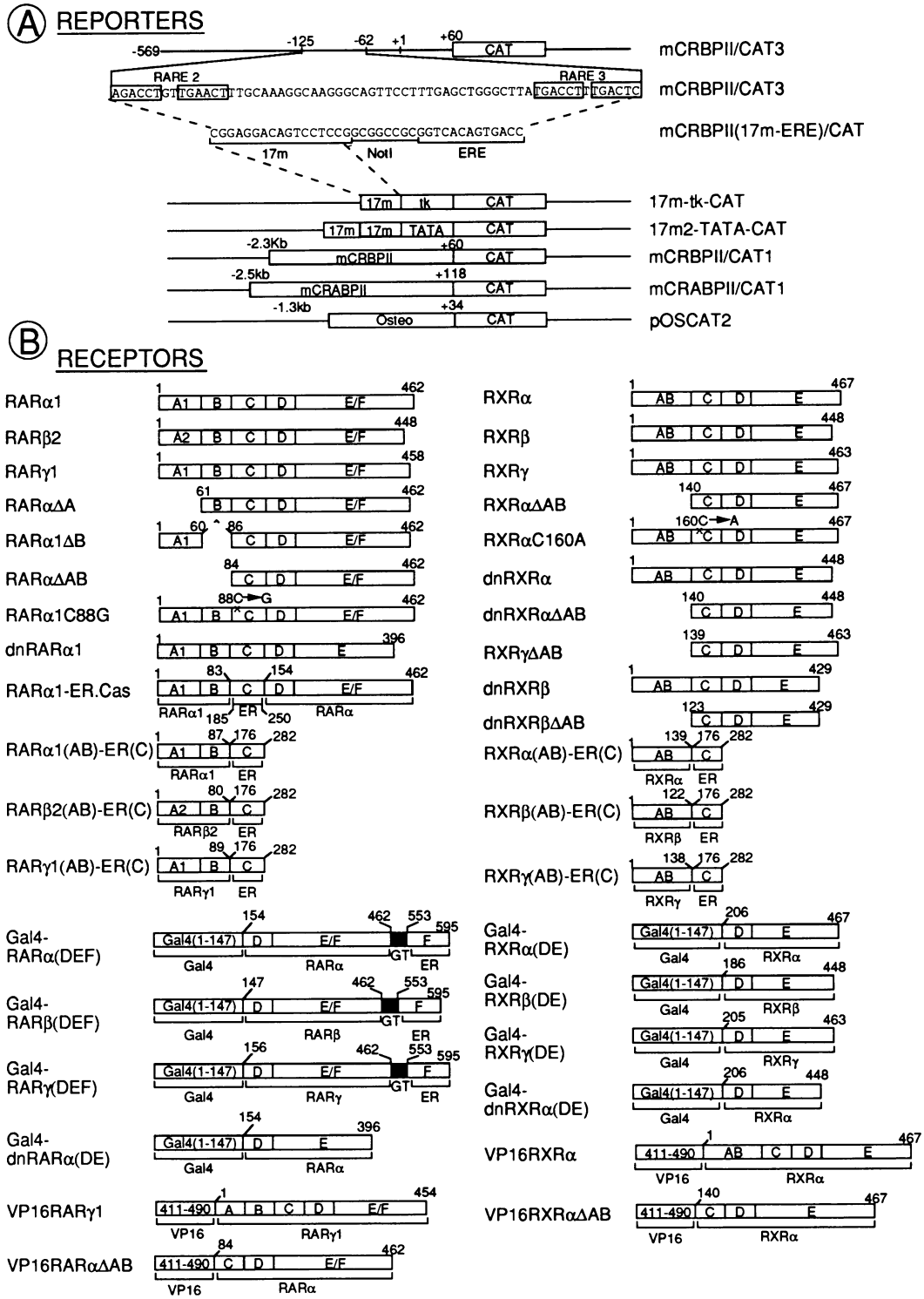

Fig. 1. Schematic representation of reporter genes (**A**) and receptor expression vectors (**B**) (see Materials and methods, and the text). Minus and plus numbers are with respect to the RNA start site (+1). In B, the various regions (A–F) of wild-type RARs and RXRs, as well as their truncation mutants, substitution mutants and chimeric receptor constructs are schematically represented (not to scale) (see Zelent *et al.*, 1993; Leid *et al.*, 1992a,b; Nagpal *et al.*, 1992; Allenby *et al.*, 1993). Numbers indicate the amino acid positions in the wild-type receptor. The positions of amino acid substitutions are indicated with an arrow.

Autonomous activation functions (AF-1s) in the N-terminal AB regions of RARα, β and γ

RARα1(AB)-ER(C), RARβ2(AB)-ER(C) and RARγ1(AB)-ER(C) (Figure 1B) activated the expression of mCRBPII(17m-ERE)/CAT by ~10-fold (Figure 2B, lanes 9, 16 and 23), thus indicating that RARα1, β2 and γ1 each contains an autonomous activation function in its N-terminal AB region. In agreement with our previous report showing that the C-terminal region of all three RARs contains an independent AF-2 activation function (Nagpal *et al.*, 1992;

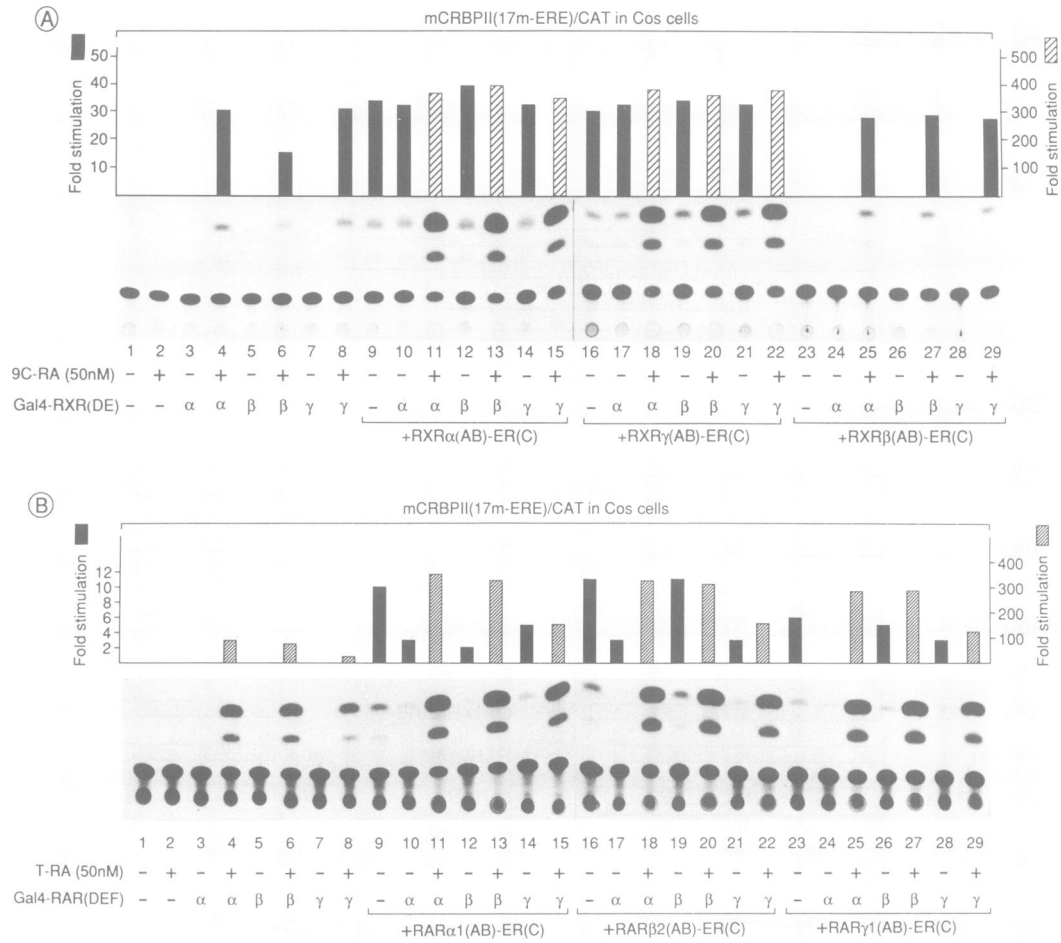

Fig. 2. AF-1 and AF-2 transcriptional activation functions of RXRs and RARs. (**A**) Cos-1 cells were transfected as indicated with the reporter gene mCRBPII(17m-ERE)/CAT (10 μg) along with either 250 ng each of Gal4-RXRα(DE) (lanes 3, 4, 10, 11, 17, 18, 24 and 25), Gal4-RXRβ(DE) (lanes 5, 6, 12, 13, 19, 20, 26 and 27) or Gal4-RXRγ(DE) (lanes 7, 8, 14, 15, 21, 22, 28 and 29), and/or 1 μg each of RXRα(AB)-ER(C) (lanes 9–15), RXRγ(AB)-ER(C) (lanes 16–22) and RXRβ(AB)-ER(C) (lanes 23–29) receptor expression vectors. The transfections were performed in the absence (–) or presence (+) of 50 nM 9C-RA, and the resulting CAT activities obtained from 10 β-galactosidase units of Cos-1 cell extract are shown along with their quantitative representation in the histogram. (**B**) Transient transfections were performed in Cos-1 cells as indicated with mCRBPII(17m-ERE)/CAT (10 μg) and either 50 ng each of Gal4-RARα(DEF) (lanes 3, 4, 10, 11, 17, 18, 24 and 25), Gal4-RARβ(DEF) (lanes 5, 6, 12, 13, 19, 20, 26 and 27) or Gal4-RARγ(DEF) (lanes 7, 8, 14, 15, 21, 22, 28 and 29), and/or 1 μg each of RARα1(AB)-ER(C) (lanes 9–15), RARβ2(AB)-ER(C) (lanes 16–22) and RARγ1(AB)-ER(C) (lanes 23–29) in the absence (–) or presence (+) of T-RA (50 nM). The resulting CAT activities obtained from 40 β-galactosidase units of Cos-1 cell extract are shown along with their quantitative representation in the histogram.

Allenby *et al.*, 1993), Gal4-RARα, β or γ(DEF) activated the expression of mCRBPII(17m-ERE)/CAT in the presence of T-RA (Figure 2B, lanes 1–8). Furthermore, the three RAR(AB)-ER(C) hybrid proteins activated synergistically with either one of the three Gal4-RAR(DEF) chimeric receptors, indicating that the AF-1s of RARα1, β2 or γ1 can synergize with either one of the AF-2s of these receptors, to stimulate transcription from the mCRBPII(17m-ERE) promoter (Figure 2B, lanes 10–15, 17–22 and 24–29).

It is important to note that RAR AF-1s appear to be ~150-fold less efficient than RAR AF-2s at activating transcription from the CRBPII promoter, taking into account the amount of expression vector DNA which was transfected and the amount of β-galactosidase units used for the CAT assays (see the legend to Figure 2B). Moreover, RAR AF-1s also appear to be weaker than RXR AF-1s (~10-fold). Note also that RARα and β AF-2s were ~3 times more efficient at activating the expression of mCRBPII(17m-ERE)/CAT than RXR AF-2s (compare Figure 2A and B, and see their legends). This relative 'weakness' of RAR AF-1s probably accounts for the lack of stimulation of the mCRBPII promoter by RARα1, β2 and γ1 in the absence of T-RA (data not shown), under conditions where a stimulation could be observed with RXRα and γ in the absence of 9C-RA (Figure 3A). However, it cannot be excluded that the RAR DEF regions could act negatively on transcription in the absence of T-RA, as suggested by the decrease in CAT activity when RAR(AB)-ER(C) (either α1, β2 or γ1) was co-transfected in the absence of T-RA with Gal4-RAR(DEF) constructs, particularly with Gal4-RARα(DEF) (lanes 10, 17 and 24, in Figure 2B).

Heterodimerization in vivo between RARs and DNA-bound Gal4-RXRα(DE)

As expected, the expression of mCRBPII(17m-ERE)/CAT, in which there is no RARE, could not be activated by either RARα1, RARβ2 or RARγ1 (Figure 4A, lanes 3, 6 and 8, lanes 12, 15 and 17, and lanes 21, 24 and 26) and in the presence of T-RA, Gal4-RXRα(DE) also did not activate (lane 4). However, when both Gal4-RXRα(DE) and either RARα1, RARβ2 or RARγ1 were co-transfected, a strong stimulation was observed in the presence of T-RA (lanes 5,

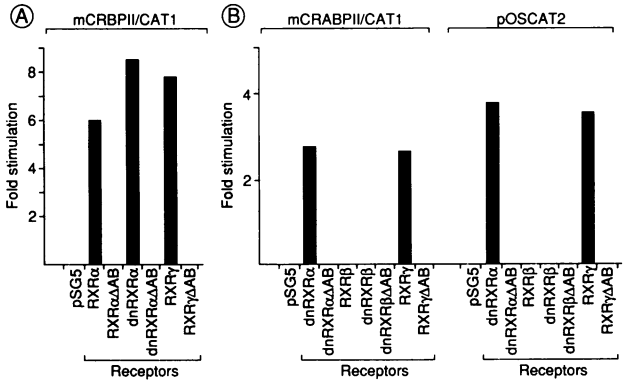

Fig. 3. Ligand-independent, AB region-dependent transcriptional activation by RXRα and RXRγ. The schematic organization of receptors and reporter genes is shown in Figure 1. (**A**) Quantitative representation of RXRα and RXRγ AB region-mediated induction of the mCRBPII promoter. Cos-1 cells were transfected with the reporter gene mCRBPII/CAT1 (10 μg) along with 1 μg of the indicated receptor expression vector in the absence of the RA ligand. All the results are expressed as fold-stimulation, taking the basal level expression of the reporter gene in the absence of ligand as one. (**B**) RXRα and RXRγ AB regions mediate the ligand-independent activation of mCRABPII and human osteocalcin gene promoters. Cos-1 cells were co-transfected with either mCRABPII/CAT1 (10 μg) or pOSCAT2 (5 μg) along with 1 μg of the receptor expression vector as indicated, in the absence of RA ligand.

7 and 9). Interestingly, the stimulation achieved with RARγ1 was much lower than that observed with RARα1 and RARβ2, consistent with our previous data obtained with a reporter (mCRBPII/CAT1) containing the natural mCRBPII promoter (Nagpal *et al.*, 1992). We interpret these results as evidence for the formation of heterodimers between 17m-bound Gal4-RXRα(DE) and either RARα1, β2 or γ1 present in solution. In the case of RARα1 and RARβ2 activated by T-RA, a further stimulation of mCRBPII(17m-ERE)/CAT activity was observed in the presence of either 9C-RA, or 9C-RA and T-RA (lanes 14 and 16, and lanes 23 and 25, respectively), due to concomitant activation of transcription by the AF-2 of Gal4-RXRα(DE) (lanes 13 and 22). In contrast, in the case of RARγ1, there was little or no increase in the reporter expression in the presence of 9C-RA or 9C-RA and T-RA (lanes 18 and 27), presumably because RARγ1 is less efficiently activated by 9C-RA than by T-RA (Allenby *et al.*, 1993). Similar results were obtained using a 17m-tk-CAT reporter (see Figure 1A) and 17m-bound Gal4-RXRα(DE) together with RARα1 or β2 in solution (data not shown).

No activation of the reporter gene could be detected when Gal4-RXRα(DE) was co-transfected with either one of the RARs in the absence of ligand, consistent with the observations that AF-1s of RARs are weak (see above). However, a fraction of the stimulation observed in the presence of T-RA or 9C-RA resulted from AF-1 activity, as illustrated in Figure 4C in the case of RARα1, which shows that deletion of region A (RARαΔA), but not of region B (RARα1ΔB), resulted in a decrease of the stimulation (compare lanes 5, 7, 9 and 11). This observation is in agreement with our previous data which indicated that RARαΔA had lost the capacity to transactivate a reporter (mCRBPII/CAT1) containing the mCRBPII promoter (Nagpal *et al.*, 1992), whereas deletion of the B region (RARα1ΔB) had no effect (S.Nagpal, S.Friant and P.Chambon, unpublished results).

It is noteworthy that the integrity of the RAR DNA-binding domain was not required for the formation of heterodimers between the 17m-bound Gal4-RXRα(DE) and RARs, as shown by using the RARα1C88G mutant in which the cysteine residue 88 of the first zinc finger of the DBD was mutated to a glycine residue. Indeed, this mutant was efficient at stimulating transcription when co-transfected with Gal4-RXRα(DE) (Figure 4C, lanes 12 and 13), even though it has totally lost its capacity to bind to a RARE *in vitro*, as either a homodimer or a heterodimer with RXR (data not shown; see Leid *et al.*, 1992b).

Heterodimerization in vivo between RXRs and the DNA-bound DEF region of RARα

As expected, RXRα, β or γ, which cannot bind to the mCRBPII(17m-ERE)/CAT reporter, did not stimulate its expression in the presence of T-RA, 9C-RA, or T-RA and 9C-RA (Figure 4B, lanes 3, 6 and 8, lanes 12, 15 and 17, and lanes 21, 24 and 26), whereas a ligand-dependent activation was observed with Gal4-RARα(DEF) (lanes 4, 13 and 22). Interestingly, a further stimulation of the reporter activity was seen when either RXRα or RXRγ was co-transfected with Gal4-RARα(DEF) in the presence of T-RA (lanes 5 and 9). A slight additional increase was achieved in the presence of 9C-RA (lanes 14 and 18), or T-RA and 9C-RA (lanes 23 and 27). On the other hand, no stimulation over that seen with Gal4-RARα(DEF) was observed when Gal4-RARα(DEF) and RXRβ were transfected in the presence of T-RA (lane 7), and only a weak stimulation was observed when 9C-RA (lane 16), or T-RA and 9C-RA (lane 25) were added. These results are in agreement with the formation of heterodimers between the 17m-bound Gal4-RARα(DEF) and either RXRα, β or γ present in solution. Indeed, the further activation observed when RXRα or γ was co-transfected in the presence of T-RA (which cannot activate RXR AF-2s) can be readily accounted for by the AF-1 activation function present in the AB regions of RXRα and RXRγ (see Figure 2A), whereas the lack of activation by the co-transfected RXRβ reflects the absence of an active AF-1 in this receptor (see above, Figure 2A). As was the case for RAR, the integrity of the RXR DBD was not required for heterodimerization with 17m-bound Gal4-RARα(DEF), since the further stimulation seen upon RXR co-transfection was not affected (see Figure 4B, lanes 28 and 29) by a mutation in the first zinc finger of the RXRα DBD (Cys160 → Ala, see Figure 1B), which prevents RXR binding to a RARE *in vitro* as either a homodimer or RAR−RXR heterodimer (Leid *et al.*, 1992b, and data not shown).

To further support the conclusion that RXR in solution can functionally heterodimerize with a DNA-bound RAR, we used RARα1-ER.Cas (Figure 1B) in which the RARα region C has been replaced by the core of the ER DBD (ER.Cas). As expected, transfection of RARα1-ER.Cas with mCRBPII(17m-ERE)/CAT resulted in a T-RA- or 9C-RA-dependent stimulation of CAT activity (Figure 4D, lanes 2 and 12), whereas RXRα could not stimulate the expression of the reporter gene even in the presence of 9C-RA (lanes 3 and 13). However, a further stimulation of CAT activity was observed when RARα1-ER.Cas and RXRα were co-transfected (lanes 4 and 14). As observed above in the Gal4-RARα(DEF)/RXRα co-transfection, this additional stimulation was essentially due to RXRα AF-1 activity, since: (i) almost the same stimulation was achieved in the

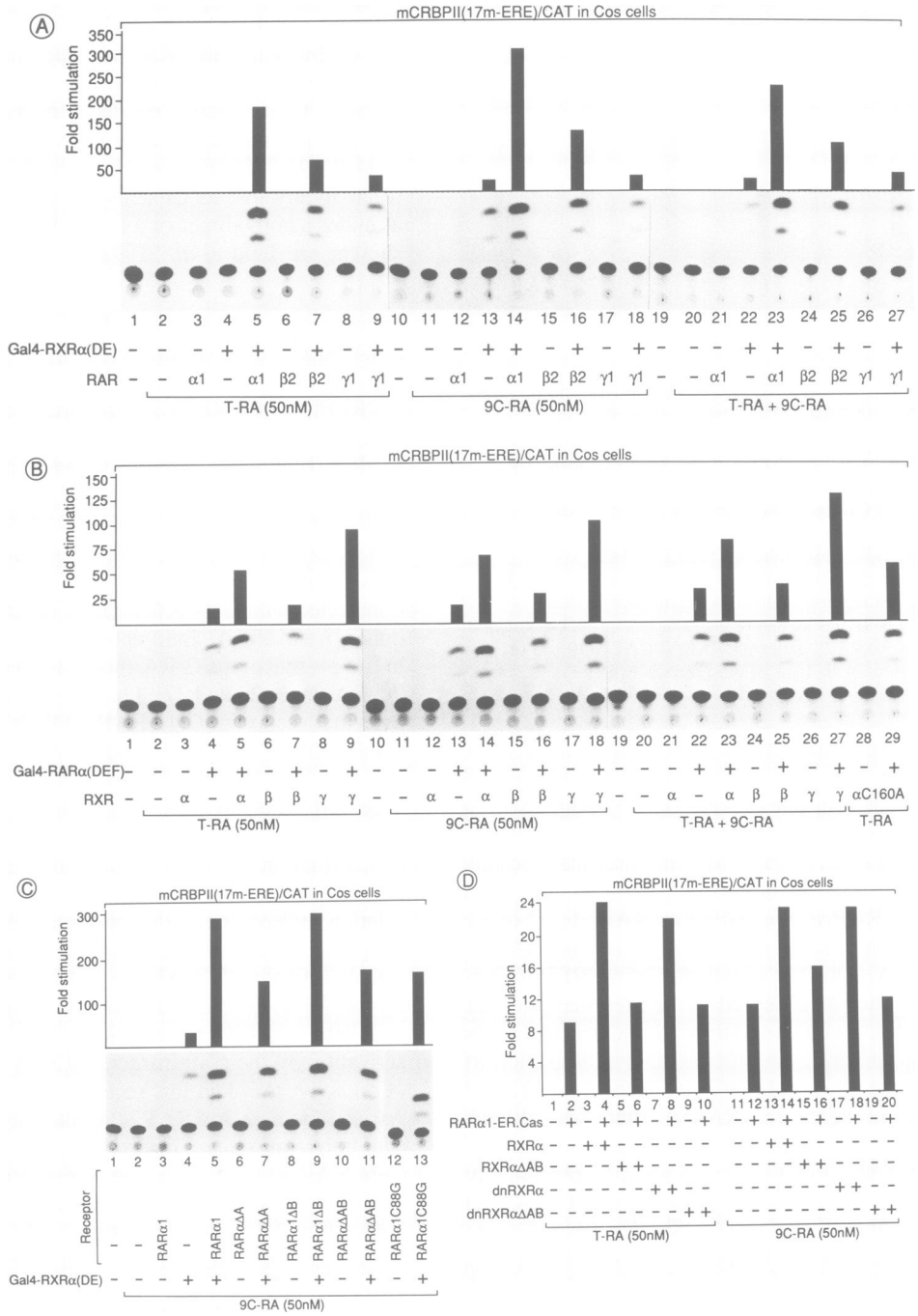

Fig. 4. Formation of RAR−RXR heterodimers *in vivo*. (**A**) Transcriptional activation by RAR and Gal4-RXRα(DE) heterodimers. Cos-1 cells were transfected with the reporter mCRBPII(17m-ERE)/CAT (10 μg), 250 ng of Gal4-RXRα(DE) expression vector (lanes 4, 5, 7, 9, 13, 14, 16, 18, 22, 23, 25 and 27) along with 250 ng of expression vector for RARα1 (lanes 3, 5, 12, 14, 21 and 23), RARβ2 (lanes 6, 7, 15, 16, 24 and 25) or RARγ1 (lanes 8, 9, 17, 18, 26 and 27), as indicated. T-RA (lanes 2−9), 9C-RA (lanes 11−18) or both (lanes 20−27) were added at a final concentration of 50 nM. (**B**) Transcriptional activation by RXR and Gal4-RARα(DEF) heterodimers. Cells were transfected with the reporter mCRBPII(17m-ERE)/CAT (10 μg), 50 ng of Gal4-RARα(DEF) expression vector (lanes 4, 5, 7, 9, 13, 14, 16, 18, 22, 23, 25, 27 and 29), along with 250 ng of either RXRα (lanes 3, 5, 12, 14, 21 and 23), RXRβ (lanes 6, 7, 15, 16, 24 and 25), RXRγ (lanes 8, 9, 17, 18, 26 and 27) or RXRαC160A (lanes 28 and 29), as indicated. T-RA (50 nM) (lanes 2−9 and 20−29) and 9C-RA (lanes 11−18 and 20−27) was added to each transfection assay. (**C**) Transcriptional activation by heterodimers between Gal4-RXRα(DE) and RARα1 mutants. Cos-1 cells were transfected as indicated with the reporter mCRBPII(17m-ERE)/CAT (10 μg) and 250 ng of Gal4-RXRα(DE) expression vector (lanes 4, 5, 7, 9, 11 and 13), along with either 250 ng of RARα1 (lanes 3 and 5), RARαΔA (lanes 6 and 7), RARα1ΔB (lanes 8 and 9), RARαΔAB (lanes 10 and 11) or RARα1C88G (lanes 12 and 13) in the presence of 9C-RA (50 nM). (**D**) Transactivation by RXRα and RARα1-ER.Cas heterodimers. Cos-1 cells were transfected as indicated with mCRBPII(17m-ERE)/CAT (10 μg), RARα1-ER.Cas (50 ng) expression vector (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20), along with either 250 ng of RXRα (lanes 3, 4, 13 and 14), RXRαΔAB (lanes 5, 6, 15 and 16), dnRXRα (lanes 7, 8, 17 and 18) or dnRXRαΔAB (lanes 9, 10, 19 and 20). T-RA (50 nM) (lanes 1−10) or 9C-RA (lanes 11−20) was added as indicated.

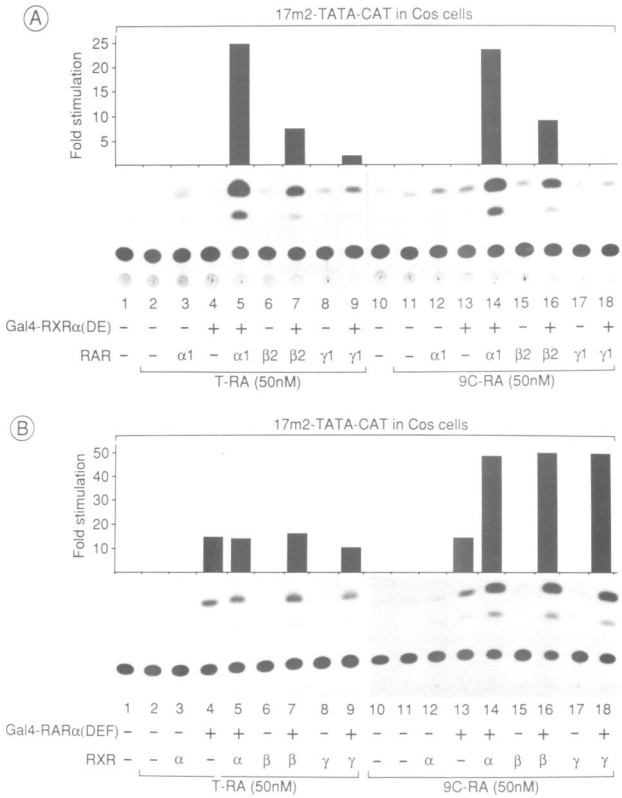

Fig. 5. Transcriptional activation of a minimal promoter by RAR–RXR heterodimers. (**A**) Formation of transcriptionally active heterodimers between RARs and Gal4-RXRα(DE). Cells were transfected as indicated with 17m2-TATA-CAT (3 μg) and 250 ng of Gal4-RXRα(DE) expression vector (lanes 4, 5, 7, 9, 13, 14, 16 and 18), along with either 250 ng of RARα1 (lanes 3, 5, 12 and 14), RARβ2 (lanes 6, 7, 15 and 16) or RARγ1 (lanes 8, 9, 17 and 18) in the presence of 50 nM T-RA (lanes 2–9) or 9C-RA (lanes 11–18). (**B**) Formation of transcriptionally active heterodimers between RXRs and Gal4-RARα(DEF). Transfections were performed as indicated with 17m2-TATA-CAT (3 μg) and 50 ng of Gal4-RARα(DEF) expression vector (lanes 4, 5, 7, 9, 13, 14, 16 and 18), along with either 250 ng of RXRα (lanes 3, 5, 12 and 14), RXRβ (lanes 6, 7, 15 and 16) or RXRγ (lanes 8, 9, 17 and 18) in the presence of 50 nM T-RA (lanes 2–9) or 9C-RA (lanes 11–18).

presence of either T-RA or 9C-RA (lanes 4 and 14); (ii) no significant additional stimulation was observed with RXRαΔAB (lanes 6 and 16); (iii) dnRXRα, which lacks the AF-2 activation function, was almost as active as RXRα (compare lanes 4 and 8, and lanes 14 and 18); (iv) the additional stimulation brought about by dnRXRα was lost when the RXR AB region was also deleted (dnRXRαΔAB, lanes 10 and 20).

Activation of a minimal promoter by RAR–RXR heterodimers

To demonstrate that the formation of transcriptionally active heterodimers did not require the presence of factor(s) which could possibly be bound to the mCRBPII promoter region, we used the Gal4-responsive minimal promoter 17m2-TATA-CAT as a reporter gene in which a 17m tandem repeat is located immediately upstream of the TATA box of the adenovirus-2 major late promoter (see Figure 1A, and Tora *et al.*, 1989). This reporter gene was not significantly activated by RARα1, β2 or γ1 (Figure 5A, lanes 3, 6, 8, 12, 15 and 17) and only very weakly, if at all, by Gal4-RXRα(DE) (lanes 4 and 13) in the presence of T-RA or 9C-RA. However, a stimulation was seen when Gal4-RXRα(DE) was co-transfected with either RARα1 or RARβ2 (lanes 5, 7, 14 and 16). In agreement with the data shown in Figure 4A, a much weaker stimulation was observed upon co-transfection of RARγ1 in the presence of T-RA (lane 9), indicating that RARγ1 is also a much less efficient activator on this minimal promoter. In addition, no significant stimulation occurred in the presence of 9C-RA, which is less efficient than T-RA at inducing the activity of RARγ AF-2 (see above, and Allenby *et al.*, 1993).

Similarly, the 17m2-TATA-CAT reporter was not activated by RXRα, β or γ (Figure 5B, lanes 3, 6, 8, 12, 15 and 17). However, a stimulation was seen when Gal4-RARα(DEF) was transfected (lanes 4 and 13), showing that unlike RXRα AF-2, RARα AF-2 can activate a minimal promoter on its own. No further stimulation was observed when Gal4-RARα(DEF) was co-transfected with either RXRα, β or γ in the presence of T-RA (Figure 5B, lanes 5, 7 and 9), whereas a significant increase in CAT activity occurred in all three cases in the presence of 9C-RA (lanes 14, 16 and 18). From this data, we conclude that RXRs can form functional heterodimers in transfected Cos-1 cells with the DNA-bound DEF region of RARα, and furthermore that RXR AF-2s can synergize with RARα AF-2 to activate a minimal promoter. In contrast, RXRα and γ AF-1s, which could activate the more complex CRBPII promoter, appear to be inactive on a minimal promoter (compare lanes 1–9 in Figures 4B and 5B).

RAR–RXR heterodimerization in vivo in the absence of ligand

To investigate whether heterodimers are formed in the absence of the RA ligands, we used two chimeric receptors (VP16RXRα and VP16RARγ1) in which RXRα and RARγ1 were fused with the acidic activating domain of the herpes simplex virus transcriptional activator VP16 (see Figure 1B). The mCRBPII(17m-ERE)/CAT reporter was not activated when transfected with either Gal4-RARα(DEF), Gal4-RXRα(DE), VP16RXRα or VP16RARγ1. However, co-transfection of either Gal4-RARα(DEF) and VP16RXRα, or Gal4-RXRα(DE) and VP16RARγ1, resulted in a marked stimulation of CAT activity, indicating that RAR and RXR heterodimerize *in vivo* in the absence of T-RA or 9C-RA (Figure 6). Furthermore, the CAT activity was still increased when either VP16RXRαΔAB and Gal4-RARα(DEF), VP16-RARαΔAB and Gal4-RXRα(DE), or VP16-RXRαΔAB and Gal4-dnRARα(DE) (in which the RARα AF-2, but not the dimerization domain is lacking, see Figure 1B), were co-transfected in the absence of ligand, showing in addition that the VP16 moiety of the chimeric receptor on its own can stimulate transcription of the mCRBPII(17m-ERE)/CAT reporter, in the absence of RAR or RXR AF-1 or AF-2 activity (Figure 6).

RAR–RXR heterodimerization is preferred in vivo over homodimerization, both in the absence and presence of RA

The above results revealed that a DNA-bound RAR and a RXR present in solution (and reciprocally) could readily heterodimerize *in vivo* in the absence of ligand. However, similar co-transfection experiments failed to reveal the formation of either RAR or RXR homodimers. Indeed,

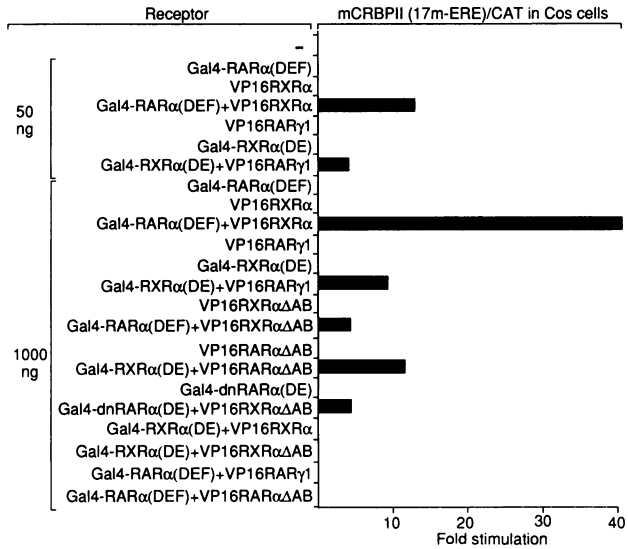

Fig. 6. Ligand-independent formation of transcriptionally active RAR–RXR heterodimers *in vivo*. Transient transfections were performed in Cos-1 cells with mCRBPII(17m-ERE)/CAT (10 μg), along with either 50 ng or 1 μg of each receptor expression vector, as indicated, in the absence of RA ligand. The schematic organization of the various Gal4- and VP16-chimeric receptor constructs is shown in Figure 1B. Quantitative representation of the resulting CAT activities is shown in the histogram.

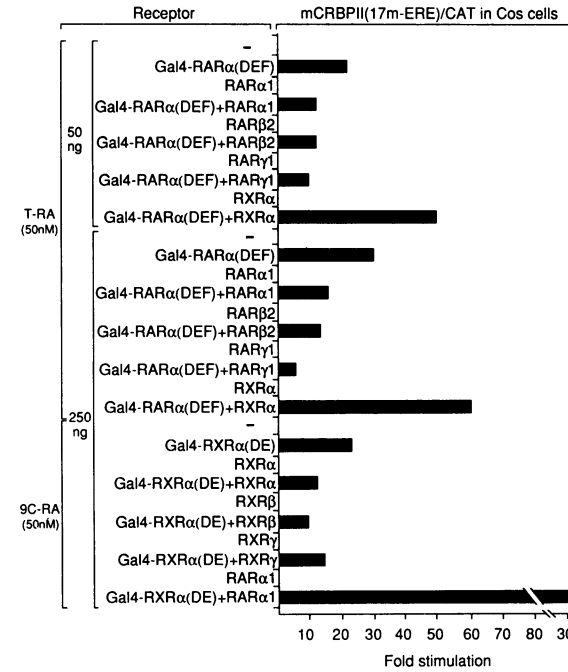

Fig. 7. RARs and RXRs preferentially heterodimerize *in vivo*. Cos-1 cells were transfected with mCRBPII(17m-ERE)/CAT (10 μg), and either 50 or 250 ng of each receptor expression vector (see Figure 1B) in the presence of 50 nM T-RA or 9C-RA, as indicated. Quantitative representation of the resulting CAT activities is shown in the histogram.

co-transfections of either Gal4-RXRα(DE) and VP16RXRα (or VP16RXRαΔAB), or Gal4-RARα(DEF) and VP16-RARγ1 (or VP16RARαΔAB), did not result in any detectable increase in CAT activity generated from the mCRBPII-(17m-ERE)/CAT reporter (Figure 6).

Several lines of evidence indicate that heterodimers were also formed much more efficiently than homodimers in the presence of RA ligands. Co-transfection of Gal4-RARα(DEF) and either RARα1, RARβ2 or RARγ1 in the presence of T-RA resulted in a decrease in the activation brought about by Gal4-RARα(DEF) on its own, instead of the increase which was observed when RXRα was co-transfected (Figure 7). Since RXR AF-1s can synergize with RAR AF-2s (data not shown), a likely interpretation of this decrease [which was also observed upon co-transfection of Gal4-RARα(DEF) with either RARαΔAB or VP16RARγ1, data not shown] is that a fraction of the original activation brought about by Gal4-RARα(DEF) was due to the formation of heterodimers with Cos-1 cell endogenous RXRs, which would now preferentially form heterodimers with the overexpressed RARs. A similar interpretation could be proposed to account for the decrease in activation by Gal4-RXRα(DE) when co-transfected with either RXRα, β or γ in the presence of 9C-RA (Figure 7; similar decreases were observed upon co-transfection with either RXRαΔAB or VP16RXRα, data not shown).

Additional experiments confirmed the inefficiency of homodimer formation in the presence of 9C-RA, under conditions where activation of the reporter gene readily reflected the formation of heterodimers (Figure 8). Co-transfection of either Gal4-dnRARα(DE) (which has no AF-2 activity) and VP16RXRαΔAB, or Gal4-dnRXRα(DE) (which also has no AF-2 activity) and VP16RARαΔAB, strongly stimulated the expression of the reporter gene in the presence of 9C-RA (lanes 5 and 9). In contrast, no stimulation could be detected when either Gal4-dn-RXRα(DE) and VP16RXRαΔAB, or Gal4-dnRARα(DE) and VP16RARαΔAB, were co-transfected in the presence of 9C-RA (lanes 7 and 10). These results, and similar results which were obtained with the minimal promoter reporter 17m2-TATA-CAT (data not shown), indicated that the presence of 9C-RA did not promote the formation of either RAR or RXR homodimers in Cos-1 cells. This conclusion was further supported by experiments in which either Gal4-RXRα(DE) and VP16RXRαΔAB, or Gal4-RARα(DEF) and VP16RARαΔAB, were co-transfected (Figure 8, lanes 17 and 20, respectively). These co-transfections resulted in a reduction of the activity brought about by either Gal4-RARα(DEF) or Gal4-RXRα(DE) on its own (lane 13 and 16). These decreases which as above could be accounted for by the formation of heterodimers between endogenous RARs or RXRs and overexpressed VP16RXR-αΔAB or VP16RARαΔAB, are particularly striking in view of the fact that any homodimer formation should have resulted in an increase of CAT activity. VP16 on its own can indeed stimulate the expression of the mCRBPII(17m-ERE)/CAT reporter gene (see above, Figure 6) and, furthermore, ER(C)-VP16 (Tasset *et al.*, 1990) can efficiently synergize with either Gal4-RARα(DEF) or Gal4-RXRα(DE) to stimulate expression of the mCRBPII(17m-ERE)/CAT reporter gene (data not shown).

Discussion

The N-terminal regions of RARs and RXRs contain an AF-1 autonomous ligand-independent activation function

In a previous study, we concluded that the AB regions of RARα (α1 and α2), RARβ (β1, β2 and β3), RARγ (γ1 and

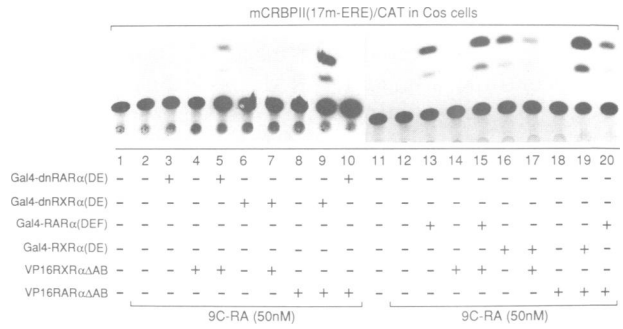

Fig. 8. Preferential formation of RAR−RXR heterodimers *in vivo*. Cos-1 cells were transfected with mCRBPII(17m-ERE)/CAT (10 μg) and the following receptor expression vectors (1 μg) in the presence of 50 nM 9C-RA, as indicated: Gal4-dnRARα(DE) (**lanes 3**, **5** and **10**); Gal4-dnRXRα(DE) (**lanes 6**, **7** and **9**); Gal4-RARα(DEF) (**lanes 13**, **15** and **20**); Gal4-RXRα(DE) (**lanes 16**, **17** and **19**); VP16RXRαΔAB (**lanes 4**, **5**, **7**, **14**, **15** and **17**); VP16RARαΔAB (**lanes 8**, **9**, **10**, **18**, **19** and **20**).

γ2), RXRα and RXRγ contain a transcriptional function capable of modulating the activity of the ligand-dependent activation function AF-2 present in the DE regions (ligand-binding domain). However, we could not demonstrate that these AB regions possess an autonomous activation function similar to the AF-1 found in the N-terminal AB region of other members of the nuclear receptor superfamily, most notably steroid receptors, e.g. the oestrogen receptor (Green and Chambon, 1988, 1991; Gronemeyer, 1991). In the present study, we have employed the mouse CRBPII promoter as a reporter gene, since its efficient transcriptional activation by RARs and RXRs requires the modulating action of the AB regions (Nagpal *et al.*, 1992), but we replaced its putative RAREs by binding sites for the oestrogen receptor and the yeast GAL4 transactivator. By co-transfection of Cos-1 cells with this reporter gene and vectors expressing hybrid proteins associating the ER DBD and the AB region of either RARα1, RARβ2, RARγ1, RXRα, RXRβ or RXRγ, we have demonstrated the existence of an autonomous ligand-independent activation function, AF-1, in all of these receptors, except RXRβ, which also failed to show a modulating function with all promoters used in our previous study, including the mCRBPII promoter (Nagpal *et al.*, 1992). Thus, the AB region of RXRβ may possibly contain an AF-1 which exhibits a much narrower promoter specificity than those of the other RXRs and RARs. Note, in this respect, that none of the RXR AF-1s could activate a minimal promoter (see Figure 5B).

Interestingly, AF-1s of RXRα and RXRγ appear to be ~10-fold stronger at stimulating the CRBPII promoter than those of RARα1, RARβ2 and RARγ1 (Figure 2A and B; and see their legends). This difference may account for the observation that RXRα and RXRγ could activate transcription in the absence of ligand under conditions where no activation was seen with RARs (Figure 3, data not shown). These results, which also show that RXRs can bind to a response element in the absence of ligand, raise the interesting possibility that RXRα and RXRγ could act as physiological constitutive transactivators in the absence of 9C-RA. It is, of course, not unlikely that the stimulation brought about by AF-1s of RARs could be critically dependent on their synergism with other promoter-bound specific factors and, therefore, much higher in a different promoter context, resulting in constitutive transactivation.

Our present data, obtained with chimeric receptors which associate the GAL4 DBD with either RAR or RXR DE regions, further support the previous conclusion that all six RARs and RXRs possess an autonomous ligand-dependent activation function AF-2 (Nagpal *et al.*, 1992; Allenby *et al.*, 1993). In addition, our present observations indicate that AF-1s of either RARα1, RARβ2 or RARγ1 can synergize with AF-2s of either RARα, RARβ or RARγ and that AF-1s of either RXRα or RXRγ can synergize with AF-2s of either RXRα, RXRβ or RXRγ. At first sight, these results are surprising in view of our previous data which indicated a specificity of cooperation between the AF-2 activation functions of the various RARs and their N-terminal modulating functions for the activation of the CRBPII promoter (Nagpal *et al.*, 1992). Note, however, that the two assay systems are different. In our previous study, the reporter promoter was the 'natural' mCRBPII promoter, and the cooperation between N-terminal modulating functions and AF-2s was tested using chimeric receptors associating the ABC regions of one RAR type with the DEF region of another one (Nagpal *et al.*, 1992), which may better reflect a possible physiological specificity of AF-1s and AF-2s.

Heterodimeric interactions between RARs and RXRs in vivo

Using gel shift/retardation assays, several recent studies have demonstrated that RARs and RXRs bind cooperatively much more efficiently *in vitro* to a variety of DNA response elements (RAREs) as heterodimers than as homodimers of either RARs or RXRs (for references, see Introduction). Furthermore, cross-linking and co-immunoprecipitation experiments employing RAR- or RXR-specific antibodies have revealed that heterodimeric interactions also preferentially occur in solution *in vitro*, and that these interactions are strengthened by binding to RAREs (for references, see Introduction). That RAR−RXR heterodimers may bind to a variety of RAREs *in vivo* more efficiently than either RAR or RXR homodimers has been suggested in several reports which have shown that transactivation by co-transfected RARs and RXRs was more efficient than could be expected from the simple additivity of the transactivations brought about by either RAR and RXR when transfected alone (Husmann *et al.*, 1992; Marks *et al.*, 1992; Zhang *et al.*, 1992a). In a recent study, we have co-transfected reporter genes containing the mCRABPII RAREs and either a RAR and a C-terminally truncated RXR (dnRXR, which can still bind cooperatively with RAR to DNA *in vitro*, but cannot transactivate due to the loss of AF-2), or the reciprocal RXR−dnRAR combination, to prove that RAR and RXR also bind more efficiently *in vivo* to RAREs as heterodimers than as homodimers (Durand *et al.*, 1992).

However, none of the above studies answered the question as to whether productive RAR−RXR heterodimeric interactions can occur *in vivo*, in the absence of a DNA response element on which RAR and RXR are known to bind cooperatively. Our present results show conclusively that, in transfected cells, heterodimeric interactions occur between the C-terminal DEF region of a RAR bound to DNA [Gal4-RARα(DEF)] and RXRs for which there is no binding site in the promoter of the reporter gene and, reciprocally, that similar heterodimeric interactions can occur between a

DNA-bound C-terminal DE region of RXR [Gal4-RXRα(DE)] and RARs in solution. That these heterodimeric interactions do not require the binding of the RARs or RXRs to DNA is further supported by the observation that they still occur with RARs and RXRs which have been mutated in their DBD and cannot bind to RAREs *in vitro*. Thus, the previously identified dimerization interfaces (Yu *et al.*, 1991; Leid *et al.*, 1992b) which are present within the E region of RARs and RXRs, which also contains the ligand-binding domain and AF-2, and may involve the nine heptad repeats of hydrophobic amino acids pointed out by Forman and Samuels (1990), are most probably responsible for these heterodimeric interactions. Furthermore, our results show that these heterodimeric interactions can also occur in the context of a minimal promoter and therefore do not require the presence of additional factors which may be bound to upstream elements of the responsive naturally occurring promoter (Figure 5). In addition, these heterodimeric interactions can efficiently take place in the absence of any ligand including the RXR-specific ligand 9C-RA (see Figures 4 and 6). In this respect, we note that the heterodimeric binding of RAR and RXR to RAREs *in vitro* is apparently not affected by the presence of T-RA, 9C-RA or both ligands (M.Leid, B.Durand and P.Chambon, unpublished results).

It has previously been shown that efficient binding of RAR and RXR as heterodimers to a RARE *in vitro* requires the integrity of both of the two directly repeated motifs 5′PuG(G/T)TCA3′ which constitute that RARE (Mader *et al.*, 1993). However, binding of RAR−RXR heterodimers can occur at high receptor concentration on a DNA element containing a single motif (S.Mader, unpublished results). Thus, our present data lead to the prediction that RAR−RXR heterodimers could also bind and transactivate *in vivo* from a response element consisting of a single motif, provided that DNA binding could be further stabilized, for instance by additional interactions within the context of other promoter-bound factor(s). Whether such 'single motif' elements in fact exist remains to be seen.

Homodimeric versus heterodimeric interactions in vivo

All of our results (see Figures 6−8) indicate that productive functional homodimeric interactions do not occur in transfected cells under conditions where heterodimeric interactions can be readily detected (see Figures 4, 6−8). On the contrary, the transactivation brought about by Gal4-RARα(DEF) and Gal4-RXRα(DE) was decreased upon RAR and RXR co-transfection, respectively (see Figure 7). These observations strongly suggest that, in fact, some of the transactivation brought about by Gal4-RARα(DEF) and Gal4-RXRα(DE) is due to the heterodimeric binding of endogenous RXRs and RARs, respectively, which would be titrated out as soluble RAR−RXR heterodimers upon overexpression of RAR and RXR in the transfected cells.

Zhang *et al.* (1992b) have reported that the presence of 9C-RA strongly stimulates the formation of homodimers of RXR translated *in vitro*. Our present data do not provide supporting evidence indicating that 9C-RA may act similarly *in vivo* (see Figures 6 and 8). Nevertheless, we cannot exclude the possibility that the presence of 9C-RA may enhance RXR homodimeric interactions *in vivo* which, however, would remain weaker than RAR−RXR heterodimeric interactions, and therefore could not be detected under the present conditions. Thus, transactivation by RXR homodimers would occur only in cells in which RXR levels are much higher than those of RARs (Marks *et al.*, 1992), and/or on specific response elements which have a much higher intrinsic affinity for RXR homodimers than for RAR−RXR heterodimers, or to which the binding of RXR homodimers, but not RAR−RXR heterodimers, is enhanced by additional factors. Whether the putative rat CRBPII RXRE (Mangelsdorf *et al.*, 1991; H.Nakshatri and P.Chambon, unpublished) and DR1 elements (Nagpal *et al.*, 1991; Durand *et al.*, 1992; Kliewer *et al.*, 1992a) could correspond to such elements remains to be seen. Note, however, that even these elements bind RAR−RXR heterodimers more efficiently than RXR homodimers *in vitro* (H.Nakshatri and P.Chambon, in preparation). Genetic evidence is clearly required to establish whether RXR homodimers can mediate some of the 9C-RA effects *in vivo*.

Materials and methods

Plasmid constructions

All expression vectors [in pSG5 (Green *et al.*, 1988) or pSG5.Cas (Nagpal *et al.*, 1992)] for retinoic acid receptors (RARs and RXRs) and their derivatives correspond to mouse RARs (Zelent *et al.*, 1989) and RXRs (Leid *et al.*, 1992b), except where indicated. The expression vectors for RARα1, β2 and γ1 (Zelent *et al.*, 1989), RXRα, β and γ (Leid *et al.*, 1992b), RARαΔA, RARαΔAB, RXRαΔAB, RXRγΔAB (Nagpal *et al.*, 1992), Gal4-RARα(DEF), Gal4-RARβ(DEF), Gal4-RARγ(DEF), Gal4-RXRα(DE), Gal4-RXRβ(DE) and Gal4-RXRγ(DE) (Allenby *et al.*, 1993) in pSG5 have been described. RARα1ΔB was constructed by deleting region B (amino acids 61−85) of mRARα1 by site-directed mutagenesis using the oligonucleotide 5′-CCGTCCCCAGCCACCAAGCTTAAGCCTTGC-TTTGTT-3′. It contained two amino acids (lysine and leucine, *Hin*dIII restriction site) in place of region B, followed by the two last amino acids of region B. In RARα1C88G, cysteine 88 is replaced by glycine. dnRARα1 (dn, dominant negative mutant) is a C-terminally truncated RARα1 which is 396 amino acids long (Saunders *et al.*, in preparation). For the construction of RARα1-ER.Cas, a construct RARα1(X3.K1) was prepared by site-directed mutagenesis by creating a *Kpn*I and a *Xho*I site, respectively, at the beginning and at the end of the C region of RARα1, resulting in two additional amino acids (Gly-Thr) at the C-terminal end of RAR B region and in three additional amino acids (Ala-Arg-Ala) at the N-terminal end of RAR D region (see Petkovich *et al.*, 1987). The *Kpn*I−*Xho*I RARα C-region fragment of RARα(X3.K1) was replaced with *Kpn*I−*Xho*I fragment (core of the ER region C) from HE28 (Green and Chambon, 1987) to construct RARα1-ER.Cas in which the additional amino acids Gly-Thr and Ala-Arg-Ala are flanking the core of the ER region C on the N-terminal and C-terminal side, respectively. RARα1(AB)-ER(C), which has been described elsewhere (Heery *et al.*, 1993), contains the AB region (amino acids 1−87) of human RARα1 (Brand *et al.*, 1988) separated by two amino acids (Gly-Thr) from the ER(C) region (amino acids 176−282). RARβ2(AB)-ER(C), which contains the first 80 amino acids of mouse RARβ2 separated by the amino acids Gly-Thr from the ER(C) region, was prepared by polymerase chain reaction (PCR) amplification of the RARβ2 AB region using the primer pairs 5′-GTAATACGACTCACTATAGG-3′ and 5′-AGGGTACCGGGCTTGTACACCCGAGG-3′, and ligating the *Eco*RI−*Kpn*I-digested amplified product into the *Eco*RI−*Kpn*I sites of pSG5.Cas-HE81 (a gift of S.Mader). RARγ1(AB)-ER(C) contains the first 89 amino acids of human RARγ1 (Krust *et al.*, 1989) separated by the amino acids Gly-Thr from the ER(C) region (gift of T.Zacharewski). For VP16RARγ1 (a gift of M.Leid), a plasmid NVP16 was constructed by placing in tandem the nuclear localization sequence and VP16 amino acids 411−490 from the *Eco*RI−*Kpn*I fragment of VP16(N) (Tasset *et al.*, 1990) into the *Eco*RI−*Kpn*I sites of pTL1 (Leid *et al.*, 1992b). The *Bam*HI fragment of human RARγ1 was inserted into the *Bgl*II site of NVP16 to produce VP16RARγ1 in which the VP16 sequence is separated by amino acids Gly-Thr-Arg-Ser-Thr from the A1 region of RARγ1. VP16RARαΔAB was prepared by ligating the RARα(CDEF) *Kpn*I fragment of RARα1(X3.K1) into the *Kpn*I site of NVP16, so that the amino acids Gly-Thr are separating the VP16 sequence from the RARα C region. Gal4-dnRARα(DE) was prepared by swapping the *Eco*RV−*Xba*I fragment of Gal4-RARα(DEF) with the *Eco*RV−*Xba*I RARα(DE) fragment of dnRARα1, which resulted in the additional amino acids Ile-Gly-Arg-Pro-

Pro-Arg-Ala between Gal4 and RARα region D. The construction of RXRαC16OA has been described previously (mRXR-αΔC4; Leid *et al.*, 1992b). dnRXRα (dn, dominant negative mutant) corresponds to a 448 amino acid long C-terminally truncated RXRα (Durand *et al.*, 1992). dnRXRαΔAB was generated by PCR amplification of amino acids 140–448 of RXRα using the primer pairs 5′-GCGGTACCTGTGCTATCTGTGGGGACCGC-3′ and 5′-TCAAGATCTACCCGATGAGCTTGAAGAAGAACAG-3′, and cloning the amplified product into *Kpn*I–*Bgl*II sites of pSG5.Cas (Nagpal *et al.*, 1992), which resulted in two additional N-terminal amino acids (Gly-Thr). dnRXRβ was constructed by PCR amplification of amino acids 1–429 of RXRβ using the primer pairs 5′-AGGGTACCATGGGACCGGATTCCCGAAGC-3′ and 5′-CGGGTACCGCCAATGAGCTTGAAGAAGAA-3′, and ligating the PCR product into the *Kpn*I site of pSG5.Cas. dnRXRβΔAB contains amino acids 123–429 of RXRβ and was constructed by PCR amplification using the primer pairs 5′-GCGGTACCCGGCTCTGTGCAATCTGCGGG-3′ and 5′-CGGGTACCGCCAATGAGCTTGAAGAAGAA-3′, and ligating the PCR product into the *Kpn*I site of pSG5.Cas, which resulted in two additional N-terminal amino acids (Gly-Thr). RXRα(AB)-γ(C-E) was prepared by ligating the PCR-amplified RXRα(AB) *Eco*RI–*Xho*I and RXRγ(C-E) *Xho*I–*Eco*RI products into the *Eco*RI site of pSG5. The RXRαAB region was amplified using primer pairs 5′-AGCTCGAGGATGTGCTTGGTGAAGGAGGC-3′ and 5′-GTAATACGACTCACTATAGG-3′, and RXRγC-E region was PCR amplified using the primer pairs 5′-GCCTCGAGTGTGCCATCTGTGGGGACAGA-3′ and 5′-AAGCTGCAATAAACAAGTTC-3′. RXRα(AB) region was separated from the RXRγ(CE) regions by two amino acids (Leu-Glu). For the construction of RXRα(AB)-ER(C), the ER(C) region was PCR amplified using the primer pairs 5′-AGCTCGAGATGGAATCTGCCAAGGAG-3′ and 5′-AAGCTGCAATAAACAAGTTC-3′ from pSG5.Cas-HE81 (see above), and the resulting fragment (after digestion with *Xho*I and *Bgl*II) was cloned into the *Xho*I–*Bgl*II sites of RXRα(AB)-γ(C-E) after elimination of the RXRγ(C-E) *Xho*I–*Bgl*II fragment, which resulted in the additional amino acids Leu-Glu between the RXRα(AB) and ER(C) regions. RXRβ(AB)-ER.C was prepared by PCR amplification of the RXRβAB region (amino acids 1–122) using oligonucleotides 5′-GTAATACGACTCACTATAGG-3′ and 5′-AGGGTACCTTTGCCAGCCCCAGGACCACC-3′, and the resulting amplified product was cloned into the *Eco*RI–*Kpn*I sites of pSG5.Cas-HE81, which resulted in the additional amino acids Gly-Thr between the RXRβ(AB) and ER(C) regions. Similarly, RXRγ(AB)-ER(C) was constructed by amplifying the RXRγ AB region (amino acids 1–138), and cloning the amplified products into *Eco*RI–*Kpn*I-digested pSG5.Cas-HE81, which resulted in the same additional amino acids. The primer pairs used for amplification were 5′-GTAATACGACTCACTATAGG-3′ and 5′-AGGGTACCGATGTGTTTCACCAGAGACC-3′. VP16RXRα (a gift of M.Leid) was constructed by inserting a *Bgl*II site immediately before the ATG codon in mRXRα by site-directed mutagenesis using the oligonucleotide 5′-GGGCATGAGTTAGTCAGATCTATGGACACCAAACAT-3′, excising the *Bgl*II RXRα fragment and ligating into the *Bgl*II site of NVP16, which resulted in the four additional amino acids (Gly-Thr-Arg-Ser) between VP16 and RXRα(AB) sequences. VP16RXRαΔAB was prepared by ligating the RXRα(CDE) *Kpn*I fragment of RXRαΔAB into the *Kpn*I site of NVP16, which resulted in the additional amino acids Gly-Thr between the VP16 and RXRα region C. Gal4-dnRXRα(DE) was prepared by swapping the *Bam*HI–*Xba*I fragment of Gal4-RXRα(DE) with the *Bam*HI–*Xba*I fragment of dnRXRα.

To construct the mCRBPII(17m-ERE)/CAT reporter gene, the −125 to −62 mouse CRBPII promoter sequence was deleted by 'PCR-cloning' and replaced by one DNA-binding site each for Gal4 and human ER (see Figure 1A). The −62 to +60 base pair fragment (fragment 1) of the mCRBPII promoter was PCR amplified using the primers 5′-GCTTGCATGCCTGCAGGTCG-3′ and 5′-AGGCGGCCGCGGTCACAGTGACCTATTATCTTTATATACCTGGTCCA-3′. Similarly, the −569 to −125 base pair fragment (fragment 2) was PCR amplified using primer pairs 5′CGATGCCATTGGGATATATC-3′ and 5′-AGGCGGCCGCCGGAGGACTGTCCTCCGGTGGGCAGTCCAGGCAGAAAG-3′. Fragments 1 and 2 were then digested with *Not*I–*Xho*I and *Sph*I–*Not*I, respectively, and ligated with *Sph*I–*Xho*I-digested pBLCAT3+ (Luckow and Schütz, 1987). 17m-tk-CAT (Webster *et al.*, 1988), 17m2-TATA-CAT (Tora *et al.*, 1989), mCRBPII/CAT1 (H.Nakshatri and P.Chambon, in preparation; Nagpal *et al.*, 1992), mCRABPII/CAT1 (Durand *et al.*, 1992; Nagpal *et al.*, 1992), pOSCAT2 (Morrison *et al.*, 1989) and DR1G-tk-CAT (Nagpal *et al.*, 1992) were previously described.

Transient transfections and CAT expression analysis

Cos-1 cells maintained in Dulbecco's modified Eagle's medium containing 5% delipidated fetal calf serum (stripped of retinoids) were plated at 40–50% confluency and transfected using the calcium phosphate procedure (Kumar *et al.*, 1986). A total of 2 μg of pCH110 (Pharmacia, a β-galactosidase expression vector) was used in all transfections as internal control to normalize for variations in transfection efficiency (Petkovich *et al.*, 1987). The total amount of DNA in each transfection was standardized to 20 μg using carrier DNA (Bluescript). After 20 h incubation with calcium phosphate-precipitated DNA, the cells were washed before incubating for another 20–24 h in the presence of the ligand (T-RA and/or 9C-RA), as indicated in the figure legends. mCRBPII(17m-ERE)/CAT, 17m2-TATA-CAT, mCRBPII/CAT1, mCRABPII/CAT1, pOSCAT2, 17m-tk-CAT and DR1G-tk-CAT reporter constructs were used at 10, 3, 10, 10, 5, 2 and 5 μg plasmid DNA, respectively, and receptor expression vectors were as indicated in the legends to the figures. Generally, CAT assays were carried out with cell extracts corresponding to 10 U of β-galactosidase for mCRBPII(17m-ERE)/CAT, 17m2-TATA-CAT and 17m-tk-CAT, 30 U for mCRABPII/CAT1, 100 U for mCRBPII/CAT1 and 50 U for pOSCAT2 and DR1G-tk-CAT. The CAT activity was quantified by scintillation counting.

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