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To cite this version:

Sunil Nagpal, Michael Saunders, Philippe Kastner, Beatrice Claude Durand, Harikrishna Nakshatri, et al.. Promoter context- and response element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. Cell, 1992, 70 (6), pp.1007-1019. 10.1016/0092-8674(92)90250-g . hal-02561148

HAL Id: hal-02561148

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Cell, Vol. 70, 1007–1019, September 18, 1992, Copyright © 1992 by Cell Press

Promoter Context– and Response Element–Dependent Specificity of the Transcriptional Activation and Modulating Functions of Retinoic Acid Receptors

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Summary

Using several naturally occurring and synthetic retinoic acid (RA)–responsive reporter genes, we show that the patterns of transcriptional activation by various retinoic acid receptor (RAR) and retinoid X receptor (RXR) forms vary according to the nature of the RA response element and the context of the stimulated promoter. We demonstrate the presence of autonomous, ligand-inducible, and promoter context–dependent transactivation functions (AF-2s) located in the C-terminal region of all RARs and RXRs. In addition, promoter context–specific modulating transactivation functions are associated with the N-terminal A and B regions of RARs and RXRs. We also show that these transactivation and modulating functions exhibit response-element specificity. The modulating functions display a marked specificity in their cooperation with the AF-2 transactivation functions, cooperation that depends on the receptor origin of the modulating and transactivation functions and the promoter context of the RA-responsive gene, thus accounting for the specific transactivation properties of RAR and RXR types.

Introduction

The highly pleiotropic effects of retinoids during vertebrate embryogenesis, cytodifferentiation, and in homeostasis (for reviews and references see Brockes, 1989, 1990; Lotan, 1980; Mendelsohn et al., 1992; Saurat, 1990; Sherman, 1986; Summerbell and Maden, 1990; Tabin, 1991) appear to be mediated by two families of receptors that belong to the superfamily of nuclear receptors acting as ligand-dependent transcriptional regulators (Beato, 1989; Evans, 1988; Green and Chambon, 1988). The first family is represented by three different retinoic acid receptor (RAR) types, designated as RARα, β, and γ, which have been identified in both humans and mice and have been shown to mediate transcriptional activation by all-trans retinoic acid (RA) (Benbrook et al., 1988; Brand et al., 1988; Giguère et al., 1987; Krust et al., 1989; Mattei et al., 1991; Petkovich et al., 1987; Zelent et al., 1989). Each RAR gene is expressed as several isoforms that differ only in their N-terminal A region and that are generated by differential usage of two promoters and/or alternative splicing (for review see Chambon et al., 1991). RARα is expressed as two main isoforms (α1 and α2) (Leroy et al., 1991a, 1991b), RARβ as four isoforms (β1, β2, β3, and β4) (Nagpal et al., 1992; Zelent et al., 1991), and RARγ as two main isoforms (γ1 and γ2) (Giguère et al., 1990; Kastner et al., 1990a; Krust et al., 1989) (Figure 1A). The second family corresponds to the three retinoid X receptor (RXR) types, RXRα, β, and γ (Hamada et al., 1989; Leid et al., 1992; Mangelsdorf et al., 1990, 1992; Rowe et al., 1991; Blumberg et al., 1992), whose specific ligand has been recently identified as 9-cis RA (Heyman et al., 1992; Levin et al., 1992; Mangelsdorf et al., 1992). However, owing to the conversion of all-trans RA to 9-cis RA in cultured animal cells, all three RXRs can also activate transcription in transfected cells treated with high concentrations (10^{-6}–10^{-5} M) of all-trans RA (Mangelsdorf et al., 1990, 1992; M. T. Bocquel et al., unpublished data; S. N., M. S., P. K., B. D., H. N., and P. C., unpublished data).

A comparison of the amino acid sequences of RAR and RXR types and isoforms has revealed a striking interspecies conservation of a given RAR or RXR type or isoform that is higher than the similarity existing between either the three RARs or the three RXR types (α, β, and γ) within a given species. The interspecies conservation of given RAR and RXR types (or isoforms) is particularly noteworthy in the N-terminal A regions that do not exhibit a significant conservation when either RARα, β, and γ or RXRα, β, and γ are compared within a given species. Furthermore, in situ hybridization studies on embryo sections have shown specific spatio-temporal patterns of expression for each mouse RAR gene (mRARα, β, and γ; Dollé et al., 1989, 1990; Ruberte et al., 1990, 1991a, 1991b) and each mouse RXR gene (mRXRα, β, and γ; Mangelsdorf et al., 1992; P. K., P. Dollé, and P. C., unpublished data). These observations have suggested that each RAR type (and isoform) and RXR type may specifically control the expression of subsets of RA target genes (Chambon et al., 1991; Kastner et al., 1990a).

Two autonomous transcriptional activation functions (AFs) have been characterized in the estrogen receptor (ER), progesterone receptor, and glucocorticoid receptor (for reviews see Green and Chambon, 1988; Gronemeyer, 1991). A constitutive AF and a hormone-dependent AF are located within the N-terminal A/B region (AF-1, formerly called TAF-1) and the ligand-binding domain (region E, AF-2, formerly called TAF-2), respectively (Meyer et al., 1990; Tora et al., 1989; Tasset et al., 1990; Webster et al., 1988). Both AF-1 and AF-2 of a given receptor exhibit distinct cell type and promoter context specificity, and AF-1s of the three steroid hormone receptors, as well as their AF-2s, have specific transactivation characteristics (Berry et al., 1990; Bocquel et al., 1989; Tora et al., 1989; Metzger et al., 1988; White et al., 1988). Moreover, the two

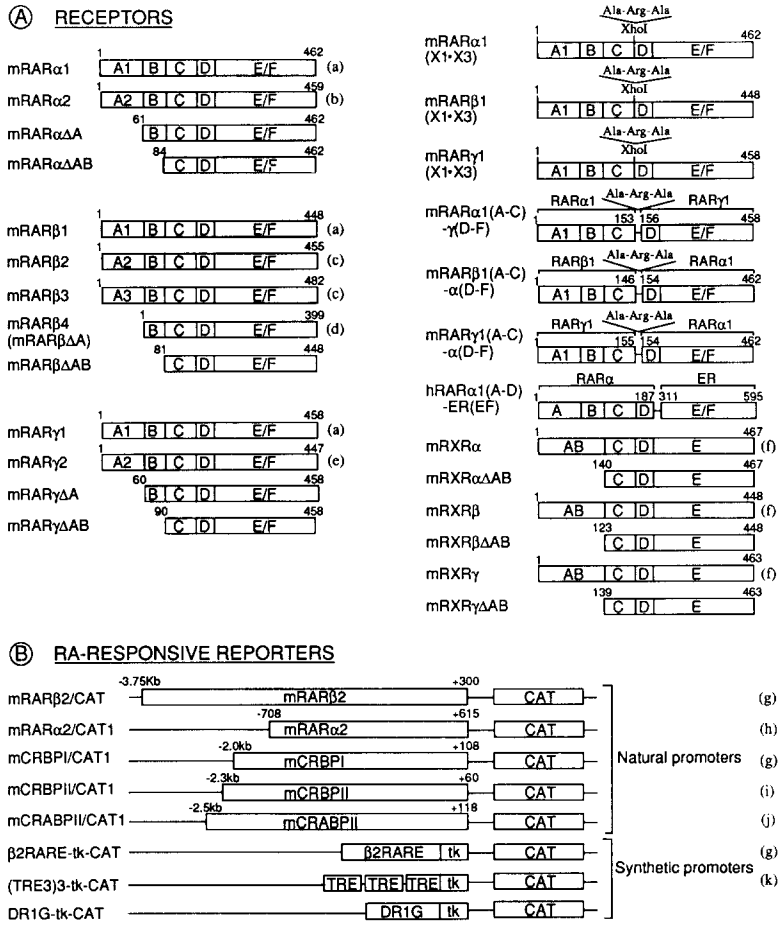

Figure 1. Schematic Structure of Receptors and RA-Responsive Reporter Genes

(A) Wild-type RAR and RXR types, as well as their N-terminal truncation mutants (ΔA and ΔAB) and chimeric receptor constructs, are schematically represented (not to scale). The A–F regions of the receptors (Chambon et al., 1991; Leid et al., 1992) have been aligned. Numbers indicate amino acid positions in the wild-type receptors. Addition of three extra amino acids as a result of insertion of an XhoI restriction site has been indicated (see Experimental Procedures).

(B) The length of the reporter constructs (not to scale) containing naturally occurring RA-responsive promoter fragments, as well as the diagrammatic representation of synthetic RA-responsive promoter constructs, is depicted. Minus and plus numbers are with respect to the RNA start site (+1).

Letters shown in parentheses correspond to the following references: (a) Zelent et al., 1989; (b) Leroy et al., 1991a, 1991b; (c) Zelent et al., 1991; (d) Nagpal et al., 1992; (e) Kastner et al., 1990a; (f) Leid et al., 1992; (g) Smith et al., 1991; (h) Leroy et al., 1991b; (i) H. N. et al., unpublished data; (j) B. D., M. S., P. Leroy, M. Leid, and P. C., unpublished data; (k) de Verneuil and Metzger, 1990.

chicken and human progesterone receptor isoforms A and B, which differ from one another only in their N-terminal region, differentially activate target genes (Kastner et al., 1990b; Meyer et al., 1992; Tora et al., 1988; Turcotte et al., 1991).

The differential transcriptional activation properties exhibited by AF-1 and AF-2 of steroid hormone receptors clearly suggest that the three RAR types (and their isoforms) and the three RXR types could also possess AFs characterized by specific transactivation properties. Using several reporter constructs containing either naturally occurring RA-responsive promoter regions or synthetic RA response elements (RAREs), we show here that an autonomous AF is associated with the ligand-binding domain of each RAR and RXR type, whereas a transcriptional modulating function is associated with the N-terminal A/B region. Moreover, we show that these activation and modulating functions exhibit specific patterns of activity that are dependent on both promoter context and response element.

Results

Experimental Design

The possible transactivation specificity of the various RAR and RXR types and isoforms (Figure 1A) was investigated in COS-1 cells cotransfected with reporter constructs containing the Escherichia coli chloramphenicol acetyltransferase (CAT) gene under the control of either naturally occurring or synthetic RA-responsive promoter regions (Figure 1B). A synthetic trimeric palindromic thyroid hormone response element ((TRE3)3) and the RARE of the RARβ2 gene are inserted upstream of the herpes simplex virus thymidine kinase (TK) promoter of pBLCAT8+ (Klein-Hitpass et al., 1988) to form (TRE3)3–TK-CAT (de Verneuil and Metzger, 1990) and β2RARE–TK-CAT (Smith et al., 1991), respectively. DR1G–TK-CAT, which contains a synthetic RARE whose directly repeated core motifs 5′-PuGGTCA-3′ (Pu stands for a purine residue) are separated by 1 bp, was used to provide a synthetic promoter preferentially activated by RXRs (Kliewer et al., 1992b; Mader et al., 1992; Mangelsdorf et al., 1991). The "natural" promoter–CAT constructs contain the RA-responsive promoter region of the mRARβ2 (mRARβ2–CAT; Mendelsohn et al., 1991; Smith et al., 1991; Sucov et al., 1990; de Thé et al., 1990; Zelent et al., 1991), mRARα2 (mRARα2–CAT1; Leroy et al., 1991b), mouse cellular retinol binding protein (CRBP) I (mCRBPI–CAT1; Smith et al., 1991), mouse CRBPII (mCRBPII–CAT1; H. N. et al., unpublished data), and mouse cellular retinoic acid-binding protein (CRABP) II (mCRABPII–CAT1; B. D., M. S., P. Leroy, M. Leid, and P. C., unpublished data) genes.

Table 1. Relative Transcriptional Activity of RARs and RXRs

RA-Responsive Promoters							
Receptors	mRARβ2	mRARα2	mCRBPI	mCRBPII	mCRABPII	(TRE3)3-TK	DR1G-TK
RARα1	9.2	3.6	4.2	15.0	16.0	31.0	10.7
RARα2	9.7	3.4	3.5	18.0	11.0	30.0	9.4
RARαΔA	6.0	3.4	2.5	1.3	2.0	20.0	5.3
RARαΔAB	2.0	1.0	1.0	1.0	3.0	7.0	5.9
RARβ1	5.2	4.0	6.0	17.0	76.0	23.0	11.3
RARβ2	5.9	3.3	6.0	7.0	44.0	33.0	14.2
RARβ3	5.0	3.3	5.7	12.0	68.0	25.0	11.0
RARβ4 (RARβΔA)	5.6	3.2	9.3	1.0	83.0	31.0	11.1
RARβΔAB	2.0	2.5	8.5	1.0	50.0	33.0	21.8
RARγ1	1.4	1.3	2.6	2.5	45.0	19.0	4.3
RARγ2	1.3	1.4	3.0	2.5	25.0	17.0	3.1
RARγΔA	0.7	1.2	1.0	1.0	17.3	13.0	2.8
RARγΔAB	1.6	0.6	1.0	1.0	1.0	5.0	2.2
RARα1(A–C)–γ(D–F)	7.5	3.3	2.0	1.0	52.0	20.0	7.4
RARβ1(A–C)–α(D–F)	11.0	4.1	3.8	2.4	11.0	35.0	12.9
RARγ1(A–C)–α(D–F)	1.1	1.3	1.0	1.0	7.0	33.0	2.0
RXRα	6.3	0.9	2.0	24.0	96	54	50
RXRαΔAB	4.3	ND	1.0	2.3	16	54	75
RXRβ	2.9	1.1	1.5	3.0	93	36	58
RXRβΔAB	2.5	ND	1.0	2.3	78	27	60
RXRγ	3.2	1.0	1.0	30.0	88	46	58
RXRγΔAB	2.2	ND	1.0	2.3	14	46	51

Differential activation of a variety of natural and synthetic RA-responsive promoters by RARs, RXRs, their N-terminally truncated mutants, and chimeric RARs. For each promoter, numbers correspond to relative CAT activity, taking the increase in expression of the reporter gene in the presence of ligand, but in the absence of transfected receptor expression vector, as 1. COS-1 cells were transfected with one of the reported constructs (10 μg of either mRARβ2–CAT, mRARα2–CAT1, mCRBPI–CAT1, mCRBPII–CAT1, or mCRABPII–CAT1; or 5 μg of DR1G-TK–CAT; or 2 μg of (TRE3)3–TK–CAT) along with 1 μg of the wild-type, deletion mutant, or chimeric receptor expression vector. The values, which were taken from the various figures and data not shown, correspond to the average (±20%) of at least three independent experiments.

The transcriptional activities of the eight main mRAR isoforms (mRARα1 and α2; mRARβ1, β2, β3, and β4; mRARγ1 and γ2) and of the recently cloned mRXRα, β, and γ (Leid et al., 1992) were tested. In the case of mRARβ4, we used the mutant form in which the natural initiator codon CTG has been replaced by ATG, since it is more efficiently expressed in transfected cells (mRARβ4–ATG, called hereafter mRARβ4; see Nagpal et al., 1992). In each case, the amount of receptor produced in transfected COS-1 cells by the corresponding pSG5-based expression vector (Green et al., 1988) was tested by Western blotting analysis using specific antibodies (Gaub et al., 1992; Rochette-Egly et al., 1991; S. N. et al., unpublished data) and by a DNA-binding gel shift assay with a ^{32}P-labeled RARE probe (see Experimental Procedures).

Using these criteria, all RAR isoforms and RXR types, as well as their mutants truncated for the N-terminal A (ΔA) or A/B (ΔAB) regions, were similarly expressed within a factor of 2, when identical amounts of expression vector DNA were transfected in COS-1 cells (data not shown). In all transfection experiments, a plasmid expressing E. coli β-galactosidase (pCH110) was cotransfected, and β-galactosidase activity was used as an internal control to normalize for variations in transfection efficiency. The transactivation efficiency of the various receptors in the presence of 10^{-6} M all-trans RA was expressed as relative CAT activity, taking the RA-dependent increase in CAT activity in the absence of cotransfected receptor expression vector, as 1. All results given in the figures and in the table correspond to the average (±20%) of at least three independent transfection experiments.

mRARα1 and α2 Isoforms

Transcription from all reporter constructs, whether synthetic or natural, was stimulated by mRARα1 and α2 (see Figures 2–6 and Table 1; data not shown). For all reporter constructs, RARα1 or α2 activated similarly. However, the transactivation efficiency of the two RARα isoforms relative to those of other RAR forms varied with the different reporter constructs. These variations are particularly clear when transactivation by RARα and RARβ isoforms are compared. RARα and β isoforms stimulated to roughly similar extents the synthetic (TRE3)3–TK-CAT, DR1G–TK-CAT, and β2RARE–TK-CAT reporters and the natural mRARα2–CAT1 and mCRBPI–CAT1 reporters (Figure 2; see Figure 5 and Table 1; data not shown). However, the RARα isoforms were more efficient than their RARβ counterparts with the mRARβ2–CAT and mCRBPII–CAT1 (with the exception of mRARβ1) reporters (Figure 3; see Figure 5 and Table 1). In contrast, both RARα1 and α2 were much less efficient than any of the mRARβ isoforms at transactivating the mCRABPII promoter (see Figure 6; Table 1). Interestingly, this promoter was stimulated to a similar extent by all three RARs in P19 embryonal carcinoma cells, indicating that differential RAR transcriptional activity may be not only promoter dependent but also cell type dependent (B. D. and S. N., unpublished data).

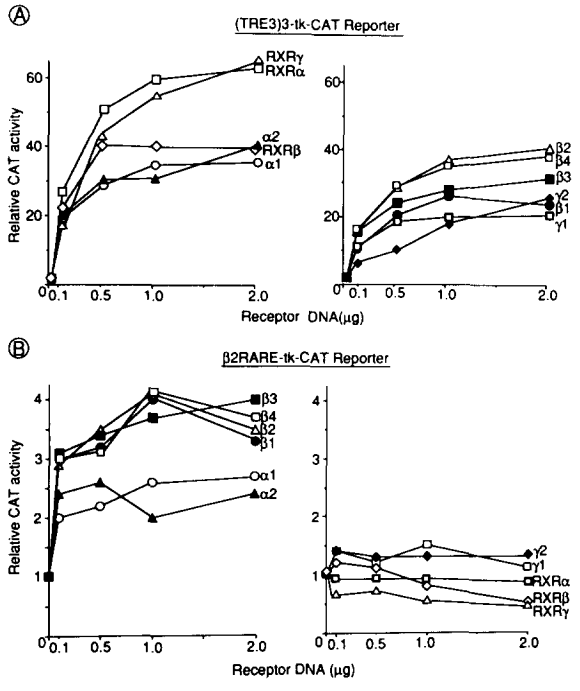

Figure 2. Transactivation of Synthetic RA-Responsive Reporters by RARs and RXRs

(A) All mRARs and mRXRs activate the expression of the synthetic promoter construct (TRE3)3-TK-CAT. COS-1 cells were transfected with (TRE3)3-TK-CAT (2 μg) along with varying concentrations (0.1–2.0 μg) of the receptor expression vectors as indicated. All the results are expressed as relative CAT activity, taking the increase in expression of the reporter gene in the presence of ligand (10^{-6} M RA), but in the absence of receptor expression vector, as 1. mRAR isoforms are denoted by appropriate Greek letters and numbers.

(B) Differential activation of β2RARE-TK-CAT by RARs and RXRs. Transient transfection assays were performed in COS-1 cells using β2RARE-TK-CAT (1 μg) and varying concentrations of the receptor expression vectors as indicated. Same symbols and expression of CAT activity as in (A).

mRARβ1, β2, β3, and β4 Isoforms

All mRARβ isoforms similarly stimulated transcription from the synthetic (TRE3)3-TK-CAT (see Figure 2A), DR1G-TK-CAT (Table 1 and data not shown), and β2RARE-TK-CAT (see Figure 2B) reporters, as well as from the natural promoters of mRARβ2 (Figure 3), mRARα2 (Figure 4), mCRBPI (Table 1 and data not shown), and mCRABPII (see Figure 6 and Table 1; data not shown) genes (mRARβ2, however, may be less efficient in the case of the latter gene). However, the relative extent of these stimulations varied with the different promoters when a comparison was made with stimulation brought about by the RARα isoforms (see previous section). Strikingly, mRARβ4 did not transactivate the mCRBPII promoter reporter construct under conditions when mRARβ1, β2, and β3 cotransfection resulted in efficient stimulation (Figure 5). Since the mRARβ4 isoform can be considered as a mRARβ isoform lacking the N-terminal A regions present in mRARβ1, β2, and β3 (Nagpal et al., 1992), the above observation suggests that the presence of these A regions is mandatory for transactivation of the CRBPII promoter but not for any of the other synthetic or natural promoters tested in the present study. Interestingly, the same CRBPII promoter appears also to be differentially activated by the mRARβ1, β2, and β3 isoforms.

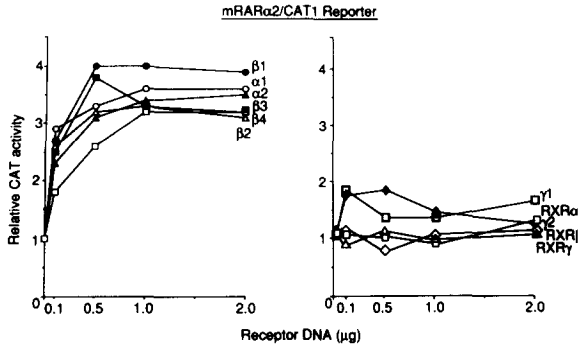

Figure 4. Differential Activation of the mRARα2 Promoter by RXRs and RARs

COS-1 cells were cotransfected with the reporter gene mRARα2-CAT1 (10 μg) along with receptor expression vector as indicated. Same symbols and expression of CAT activity as in Figure 2A.

mRARγ1 and γ2 Isoforms

The pattern of transactivation by the RARγ isoforms was

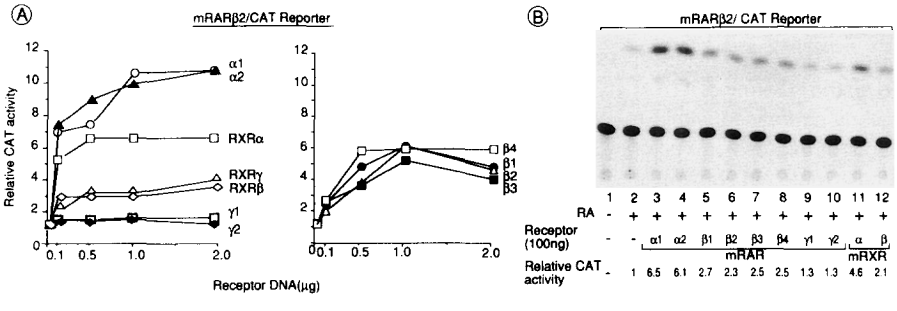

Figure 3. The mRARβ2 Promoter Responds Differentially to RARs and RXRs

(A) Quantitative representation of RAR- and RXR-mediated induction of mRARβ2-CAT in COS-1 cells. Cells were transfected with the reporter gene mRARβ2-CAT (10 μg) and increasing concentrations (0.1–2.0 μg) of receptor expression vectors as indicated. Same symbols and expression of CAT activity as in Figure 2A.

(B) Representative CAT assays. COS-1 cells were transfected with mRARβ2-CAT (10 μg) along with 100 ng of receptor expression vectors in the presence of RA (10^{-6} M).

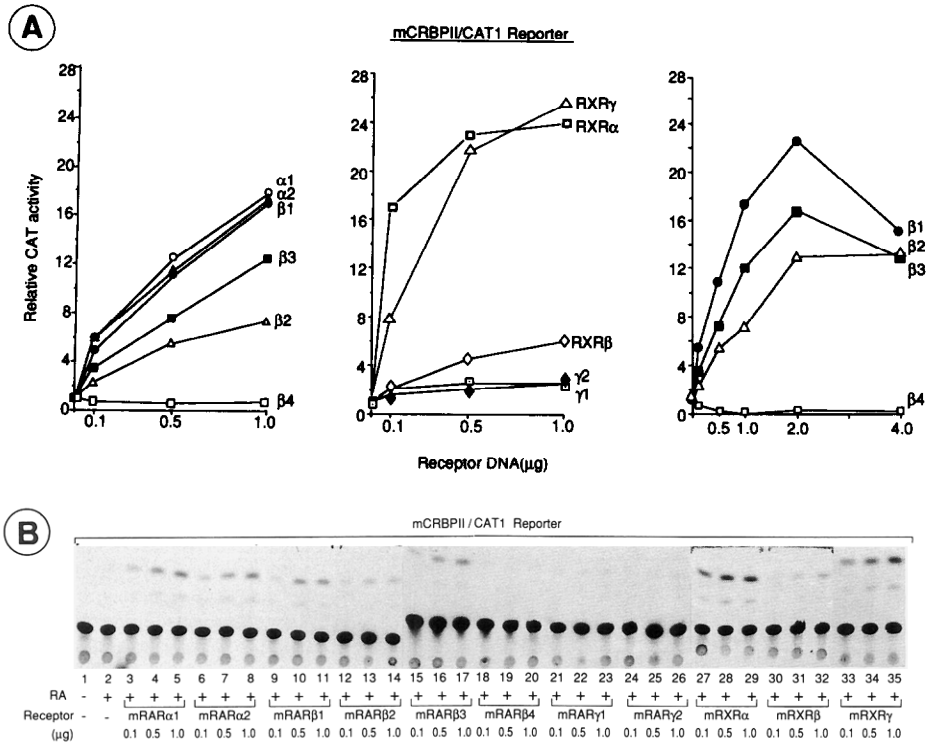

Figure 5. The Various RARs and RXRs Differentially Activate CRBPII Promoter

(A) Quantitative representation of mRAR- and mRXR-mediated activation of expression of mCRPBII–CAT1. Transient transfections were performed in COS-1 cells using mCRBPII–CAT1 (10 μg) along with increasing concentrations of mRAR or mRXR expression vectors as indicated. Symbols and expression of CAT activity are as in Figure 2A.

(B) Representative CAT assays for the activation of mCRBPII promoter by mRARs and mRXRs as indicated.

very different from those obtained for the RARα and β isoforms (see Figures 2–6 and Table 1). Both mRARγ1 and γ2 stimulated transcription from the synthetic (TRE3)3–TK-CAT and DR1G–TK-CAT reporters with an efficiency not much lower than that achieved with RARα and β isoforms (see Figure 2A and Table 1; data not shown). Furthermore, both RARγ isoforms were more efficient than the RARα isoforms at transactivating the mCRABPII promoter reporter (Figure 6; Table 1). Thus, both RARγ1 and RARγ2 contain a transactivation function(s). However, no significant transactivation was observed by either of the two isoforms with mRARβ2 and mRARα2 promoter constructs (see Figures 3 and 4), whereas a weak stimulation was seen with the mCRBPI and mCRBPII promoters (see Figure 5; Table 1). These results clearly show the role of the promoter context in transactivation by the RARγ isoforms, since no significant difference in the binding of the three RAR types to the β2RARE was ever observed in vitro (Vasios et al., 1991; S. N., M. S., P. K., B. D., H. N., and P. C., unpublished data).

Transactivation by mRXRα, β, and γ

In the presence of 10^{-6} M all-trans RA, mRXRα, β, and γ all stimulated transcription from the synthetic (TRE3)3–TK-CAT reporter construct with efficiencies similar to or higher than those achieved with the various RAR isoforms (see Figure 2A; Table 1). The natural RA-responsive mRARβ2 promoter was also stimulated by RXRα as efficiently as by RARα and β isoforms, whereas lower activations were seen with RXRβ and γ (see Figure 3; Table 1). Interestingly, no or very little stimulation was seen using the reporter constructs β2RARE–TK-CAT, mRARα2–CAT1, and mCRBPI–CAT1 and either RXRα, β, or γ, even though the RARE of two of these promoters is identical

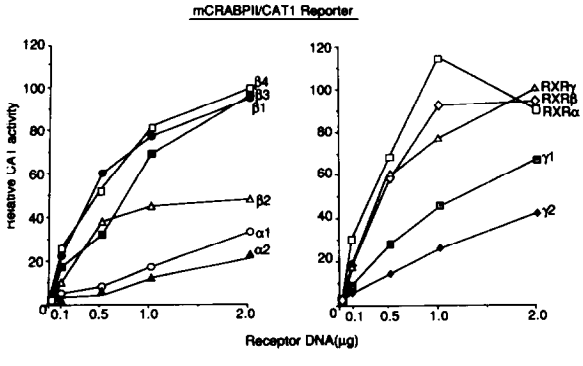

Figure 6. Transactivation of the mCRABPII Promoter by mRARs and mRXRs

COS-1 cells were transfected with mCRABPII–CAT1 (10 μg) together with varying concentrations of the receptor expression vector as indicated. Symbols and expression of CAT activity are as in Figure 2A.

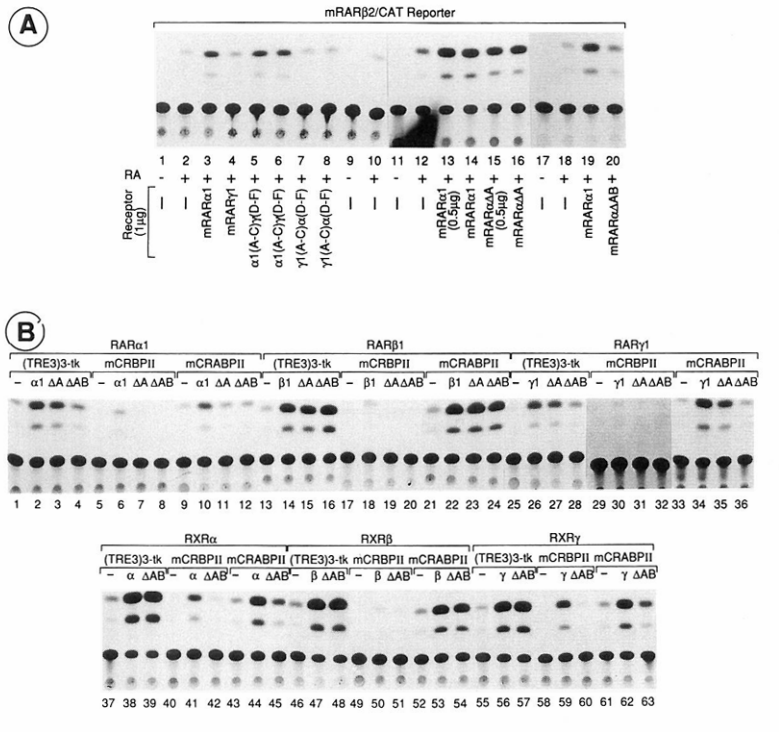

Figure 7. Characterization of Activation and Modulating Functions of RARs and RXRs

(A) Characterization of mRARα1 regions involved in the transactivation of the mRARβ2 promoter. COS-1 cells were transiently transfected as indicated with mRARβ2–CAT (10 μg) along with 1 μg (or 0.5 μg, where indicated) of the receptor expression vector, its deletion mutants (ΔA or ΔAB), or chimeric receptor constructs. Lanes 1, 9, 11, and 17 represent the basal level expression of mRARβ2–CAT in the absence of RA. The addition of RA (10^{-6} M) to transfected cells resulted in an increase in the basal level activity of the reporter construct (lanes 2, 10, 12, and 18). CAT activity resulting from cotransfection with mRARα1 (lanes 3, 13, 14, and 19), mRARγ1 (lane 4), chimeric receptor constructs mRARα1(A–C)–γ(D–F) (lanes 5 and 6), and mRARγ1(A–C)–α(D–F) (lanes 7 and 8), as well as deletion mutants mRARαΔA (lanes 15 and 16) and mRARαΔAB (lane 20), are shown.

(B) Transactivation of (TRE3)3–TK, mCRBPII, and mCRABPII promoters by RARs, RXRs, and their ΔA and ΔAB truncation mutants. COS-1 cells were transfected as indicated with either (TRE3)3–TK-CAT (2 μg), mCRBPII–CAT1 (10 μg), or mCRABPII–CAT1 (10 μg), along with 1 μg each of the indicated receptor constructs or their N-terminal truncation mutants. RA (10^{-6} M) was added to all the transfected cells.

(β2RARE–TK-CAT) or very closely related (mRARα2–CAT1; see Leroy et al., 1991b) to that of the RARβ2 promoter, thus indicating a promoter context dependency of the RXR AF(s) (see Figures 2B and 4; Table 1). The highest stimulations were observed with the mCRABPII promoter that was similarly activated by all three RXRs, to extents that were clearly higher than those achieved with RARα and γ isoforms and nearly identical to those obtained with the RARβ isoforms (Figure 6; Table 1). RXRα and γ also stimulated the mCRBPII promoter better than any of the RAR isoforms, but, in contrast with the results obtained with the CRABPII promoter, RXRβ was clearly much less efficient than the two other RXR types (see Figure 5; Table 1), which also support the promoter context specificity of the transactivation functions of the three RXRs. This specificity was also supported by the results obtained with the synthetic DR1G–TK-CAT reporter construct that was similarly and efficiently transactivated by all three RXRs (Table 1 and data not shown).

Promoter Context–Dependent Transactivation Functions Are Associated with the C-Terminal Region of RARs and RXRs

To investigate whether transcriptional AFs analogous to those that are contained within the ligand-binding domain (region E) of steroid hormone receptors (see Introduction) are also present in the three RAR and RXR types, we tested the transactivational properties of RARs and RXRs truncated for the N-terminal A/B region, using the same promoter reporter constructs as above (ΔAB constructs in Figure 7; see Figure 1 and Table 1; data not shown). All A/B region–truncated RARs and RXRs activated transcription from the synthetic (TRE3)3–TK-CAT and DR1G–TK-CAT reporter constructs in the presence of 10^{-6} M RA, indicating the existence of an autonomous AF(s) in the C-terminal half of all of these receptors. Since this AF is ligand dependent and, therefore, is likely to be the counterpart of the steroid hormone receptor AF-2 (see Introduction for references), we call it AF-2 hereafter for the sake of simplicity (see Discussion).

AF-2s of RARα, β, and γ, as well as those of RXRα, β, and γ, exhibited a clear promoter context specificity (Table 1 and Figure 7; data not shown). mRARα2, mCRBPI, and mCRABPII promoters were not activated by RARαΔAB, RARβΔAB did not activate the mCRBPII promoter, and RARγΔAB stimulated only (TRE3)3–TK-CAT and DR1G–TK-CAT. A promoter context dependency of AF-2 of RXRs was also observed (compare, for instance, the transactivations of mCRABPII, mCRBPII, DR1G–TK, and (TRE3)-3–TK promoters in Figure 7B and Table 1).

Modulating Transactivation Functions Are Associated with the A and B Regions of RARs and RXRs

In the case of steroid hormone receptors, a second independent transactivation function, AF-1, which was shown to be autonomously active, is associated with the N-terminal A/B region (see Introduction). We have not yet been able to demonstrate the existence of a similar autonomous AF-1 in the A/B region of the various RAR and RXR types. In all cases, RARs and RXRs truncated for the E/F region failed to stimulate transcription from any one of the pro-

moter reporter constructs used in the present study. However, the use of RARs and RXRs truncated for either the A (ΔA) or A/B (ΔAB) regions revealed that these regions contain at least a modulating transactivation function(s) that can cooperate with AF-2 in a promoter context-dependent manner to achieve the stimulation observed with the complete receptors.

In the case of RARα, there is clearly a modulating function associated with the A and/or B region that is required for maximal stimulation of transcription from (TRE3)3-TK, DR1G-TK, mRARβ2, and mCRBPI promoters (compare RARα, RARαΔA, and RARαΔAB in Figure 7 and Table 1). In the case of mCRBPII and mCRABPII promoters, the modulating functions are associated with both the A1 and A2 regions of RARα1 and α2, whereas they appear to be located mostly in the B region in the case of the mRARα2 promoter. Thus, the A1, A2, and B regions of both RARα1 and RARα2 contain modulating transactivation functions that act together with the C-terminal AF-2 in a promoter context-dependent fashion.

Similar observations were made using RARβ isoforms truncated for either the A (ΔA = mRARβ4) or A/B (ΔAB) regions. However, in contrast with what was observed in the case of RARα, the A and B regions of the RARβ isoforms do not appear to contain a modulating function capable of acting together with RARβ AF-2s on the mCRBPI, mCRABPII, (TRE3)3-TK, and DR1G-TK promoters (Figure 7B and Table 1). Furthermore, the modulating functions present in the A regions of RARβ1, β2, and β3 appear to be dispensable for transactivation of the mRARβ2 but not of the mCRBPII promoter (Figure 7B and Table 1).

Promoter context-dependent modulating functions also appear to be associated with both the A and B regions of both RARγ isoforms (see mCRABPII, mCRBPI, mCRBPII, and (TRE3)3-TK promoter constructs in Figure 7B and Table 1).

Similarly, promoter context-dependent modulating functions are clearly present in the A/B regions of RXRα and γ (Figure 7B and Table 1). However, the A/B region of RXRβ does not appear to contain a modulating function when tested with the present promoter constructs (Figure 7B and Table 1).

Specificity of Cooperation between the AF-2 Transactivation Functions and the N-Terminal Modulating Functions

Chimeric receptors were constructed to investigate the possibility that the cooperation observed above could exhibit a specificity with respect to both the origin of the modulating and transactivation functions and the nature of the transactivated promoter. In RARγ1(A-C)-α(D-F), the A to C regions of RARγ1 are fused to the D to F regions of RARα (see Figure 1A). The A/B modulating region of RARγ1 cooperated with RARα1 AF-2 on the mCRABPII promoter and (TRE3)3-TK-CAT reporter constructs but not on mRARβ2, mRARα2, and mCRBPII promoters (Table 1, Figure 7A, and data not shown), whereas the A/B modulating region of RARα1 readily cooperated with RARα1 AF-2 on the same promoters (see RARα1). On the other hand, the A/B modulating region of RARβ1 cooperated with RARα1 AF-2 on all reporter constructs except on the mCRBPII promoter (Table 1 and data not shown). These results indicate that the A/B modulating regions of RARα1, RARβ1, and RARγ1 are different from one another (compare the activation patterns of RARα1 with those of RARβ1(A-C)-α(D-F) and RARγ1(A-C)-α(D-F) in Table 1). Furthermore, the comparison of the activation patterns of RARβ1 and RARβ1(A-C)-α(D-F) shows that RARα and RARβ AF-2s are different (see Table 1, comparing in particular the activation of mCRBPII and mCRABPII promoters).

The A/B modulating region of RARα1 cooperated with RARγ AF-2 on all reporter constructs with the exception of the mCRBPII promoter (RARα1(A-C)-γ(D-F); see Figure 1, Table 1, and Figure 7A; data not shown; note that this synergism is particularly efficient on the mCRABPII promoter). These results also indicate that RARα and RARγ AF-2s are different from one another. Furthermore, it appears that the inactivity of RARγ1 on the mRARβ2 and mRARα2 promoters is due both to a complete lack of activity of its A/B region modulating function within the context of these promoters and to a low activity of RARγ AF-2 on its own on these promoters.

In an attempt to demonstrate the existence of an autonomous transactivation function in the N-terminal A/B region of RARα1, we constructed a chimeric receptor RARα1(A-D)-ER(EF) (see Figure 1) containing the A to D regions of RARα1 fused to the E/F region of the ER that contains both the ligand-binding domain and the AF-2 transcriptional activation function (Tora et al., 1989). This chimeric receptor stimulated transcription from the synthetic (TRE3)3-TK-CAT reporter in the presence of estradiol (E2) (Figure 8B). However, no stimulation was observed in the presence of the antagonist 4-hydroxytamoxifen that inhibits ER AF-2, but not ER AF-1, activity (Berry et al., 1990), indicating that the modulating function present in the A/B region of RARα1 has no transactivating activity on its own on the (TRE3)3-TK-CAT reporter.

The chimeric RARα1(A-D)-ER(EF) receptor was also transfected with the mRARβ2 and mCRBPII promoter reporter genes whose efficient transactivation by RARα1 requires the presence of the A/B region (see above and Table 1). No stimulation of transcription was observed in the presence of estradiol (Figure 8A and data not shown), even though this chimeric receptor bound specifically to a RARE-β2 oligonucleotide both in vitro (Figure 8C and data not shown) and in vivo as judged by its inhibitory effect on RA-dependent transactivation of the mRAR-β2-CAT reporter by mRARβ2 in the presence of either estradiol or hydroxy-tamoxifen (data not shown). It appears therefore that, within the context of the mRARβ2 and mCRBPII promoters, the modulating function present in the A/B region of RARα1 cannot cooperate with ER AF-2, whereas it cooperates efficiently with RARα AF-2 (see above), indicating that AF-2s of RARα and ER correspond to different promoter context-dependent AFs.

Discussion

We have investigated the transcriptional activation proper-

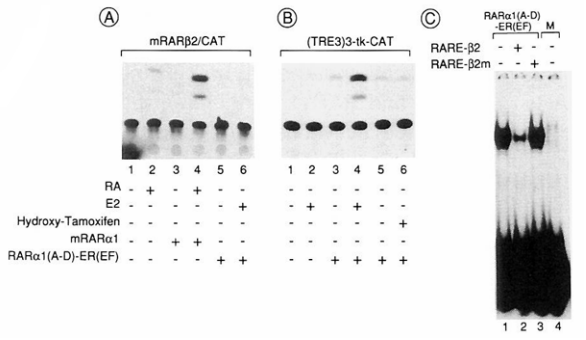

Figure 8. No Evidence for an Autonomous Transactivation Function in RARα A/B Region

(A) The mRARα A/B region does not activate the expression of the mRARβ2 promoter. COS-1 cells were transiently transfected with 10 μg of mRARβ2–CAT (lanes 1–6), along with 1 μg of either mRARα1 (lanes 3 and 4) or RARα1(A–D)–ER(EF) (lanes 5 and 6), in the absence of any ligand (lanes 1, 3, and 5) or in the presence of either 10^{-6} M RA (lanes 2 and 4) or 10^{-7} M estradiol (E2) (lane 6).

(B) RARα1(A–D)–ER(EF) activates the expression of (TRE3)3–TK-CAT through the ER EF regions. COS-1 cells were transfected with 2 μg of (TRE3)3–TK-CAT along with 1 μg of mRARα1(A–D)–ER(EF) (lanes 3–6) in the absence of any ligand (lanes 1, 3, and 5) or in the presence of either 10^{-7} M E2 (lanes 2 and 4) or 10^{-6} M anti-estrogen hydroxytamoxifen (lane 6).

(C) RARα1(A–D)–ER(EF) binds in vitro to the RARβ2 gene RARE (RARE-β2). A gel shift assay was performed using ^{32}P-labeled RARE-β2 probe and either RARα1(A–D)–ER(EF) (lanes 1–3) or pSG5 (mock transfected, or M; lane 4) transfected COS-1 cell extracts (3 μg of protein). Unlabeled wild-type (RARE-β2) and mutated RARE-β2 (RARE-β2m) probes (100-fold excess) were added as competitors to lanes 2 and 3, respectively.

ties of the three types of RAR (α, β, and γ) and of their major isoforms, as well as those of the three mRXR types (α, β, and γ) that have been more recently characterized. Using several synthetic or natural RA-responsive reporter genes, we show that the various RAR and RXR receptors exhibit differential transcriptional activation potential, which is dependent on both the nature of the RARE and the promoter context of these reporter genes. We also show that for each receptor these differences result from both the stimulatory specificity of an AF that is located in the C-terminal region and a modulating function present in the N-terminal region.

The Different Transactivation Patterns of the Multiple RARs and RXRs Are Dependent on Both the Nature of the RARE and the Promoter Context of RA-Responsive Reporter Genes

Our results are summarized in Table 1, where the relative transcriptional activity of the various RARs and RXRs corresponds to the transfection of 1 μg of receptor vector DNA in the presence of 10^{-6} M all-trans RA (see figures). It has recently been shown that at 50 nM ligand, RAR activity is induced by both all-trans RA and 9-cis RA, whereas RXR activity is induced specifically by 9-cis RA (Heyman et al., 1992; Levin et al., 1992; Mangelsdorf et al., 1992; M. T. Bocquel et al., unpublished data; S. N., M. S., P. K., B. D., H. N., and P. C., unpublished data). However, since all-trans RA can be converted into 9-cis RA in cultured cells (Heyman et al., 1992; Levin et al., 1992), RXRs are also activated when transfected into cells maintained at elevated concentrations of all-trans RA. In control experiments (data not shown), we found that with the present reporter genes RARs were similarly activated by 9-cis RA or all-trans RA at 10^{-6} M, whereas 10^{-6} M 9-cis RA was two to three times more efficient than 10^{-6} M all-trans RA at activating RXRs.

All RAR isoforms and RXR types transactivate the TK promoter placed under the control of the synthetic RARE (TRE3)3 (de Verneuil and Metzger, 1990), indicating that each receptor possesses at least one transactivation function. However, there are some significant variations in the magnitude of this transcriptional stimulation, which suggests that the activation properties of the various receptors are not identical. This is clearly demonstrated when the transactivation patterns of RARs and RXRs are compared using a variety of reporter genes corresponding to either natural (mRARβ2, mRARα2, mCRBPI, mCRBPII, and mCRABPII) or synthetic (β2RARE–TK and DR1G–TK) RA-responsive genes. mRARγ1 and γ2 represent the most dramatic cases, since the mCRABPII promoter is the only natural promoter to be highly activated by these RAR isoforms. These observations are in agreement with the previous report of Husmann et al. (1991), who showed that RARγ1 did not activate transcription from the RARβ2 and the β2RARE–TK promoters, whereas the TK promoter controlled by a synthetic RARE was activated. In contrast, Husmann et al. (1991) reported that RARγ2 could activate the RARβ2 promoter. The reason for this discrepancy with our results is unknown and may possibly be due to the use of CV1 cells and/or a shorter RARβ2 promoter fragment in the Husmann et al. study. The RARα and β activation properties also appear to be different from each other and from those of the RXRs when their transactivation patterns are compared (see Results, Figures 2–6, and Table 1). On the other hand, the isoforms of a given RAR type (either α, β, or γ) exhibited similar activation patterns, with the exception of mRARβ4, which, in contrast with the other mRARβ isoforms, did not activate the mCRBPII promoter, thus supporting the proposal that RAR isoforms could specifically transactivate different RA target genes. This possibility is further supported by the observation that RARβ1, β2, and β3 stimulated the mCRBPII promoter to significantly different levels.

Two main factors appear to be involved in the specificity of transactivation by the various RARs and RXRs. That the nature of the response element plays some role is clearly apparent in the case of both RARs and RXRs when the results obtained with the three reporter genes containing the TK promoter are compared (see (TRE3)3–TK-CAT, β2RARE–TK-CAT, and DR1G–TK-CAT in Figure 2 and Table 1). In the case of RXRs, this may be related to different binding efficiencies of the receptors for the β2RARE, (TRE3)3, and DR1G elements (see Leid et al., 1992). However, RXRs do activate, albeit less efficiently, the mRARβ2–CAT reporter that contains the same β2RARE (Figure 3; Table 1). A difference in binding efficiency is unlikely to account for the lack of activation of the β2RARE–TK-CAT reporter by RARγ1 and γ2, since RARγ

isoforms appear to bind as efficiently to the β2RARE in vitro as do the RARα and RARβ isoforms (Vasios et al., 1991; S. N. et al., unpublished data). However, in view of the recent findings that RARs and RXRs may bind to and transactivate from their response elements as heterodimers (Kliewer et al., 1992a; Leid et al., 1992; Yu et al., 1991; Zhang et al., 1992), one cannot exclude the possibility that the transactivation specificity that is related to the nature of the response element reflects the availability of a COS-1 cell endogenous partner for heterodimerization. Alternatively, this partner may block the activation function of the receptor on some response elements. In this regard, it is important to stress that the present transcriptional activations could not be generated by replacing the various RARs and RXRs with their C-terminally truncated dominant negative mutants, which bind RAREs as either homodimers or heterodimers as efficiently as their wild-type counterparts but have lost their transactivation function (M. S. et al., unpublished data).

The promoter context also appears to be an important factor in determining the specificity of activation by the multiple RARs and RXRs. The β2RARE–TK-CAT and mRARβ2–CAT reporter genes have the same RARE, although within different promoter contexts. The mRARβ2 promoter is activated more efficiently by RARα than by RARβ isoforms, whereas the reverse is observed with the β2RARE–TK-CAT reporter. Similarly, the three RXR types are totally inactive on the β2RARE–TK-CAT reporter, whereas they activate the mRARβ2 promoter. This promoter context dependence is likely to reflect a specificity in the cooperation between the activation functions of the receptors (see below) and other transactivators specifically bound to a given promoter region.

A Promoter Context–Specific Activation Function (AF-2) Is Present in the C-Terminal Region of All RARs and RXRs

All RARs and RXRs truncated for the N-terminal A/B region are still capable of activating transcription from the synthetic (TRE3)3–TK-CAT reporter gene, albeit with a reduced efficiency in the case of RARα and RARγ. It is most probable that the corresponding AF is located in the E region, since chimeric receptors comprised of the GAL4 DNA-binding domain tethered to the E regions of the various RARs and RXRs are efficient transactivators of promoters containing the 17-mer GAL4 response element (M. T. Bocquel et al., unpublished data; M. S. et al., unpublished data). A similarly located ligand-dependent transcriptional activation function (AF-2) has been previously identified in the corresponding region of steroid hormone receptors (Tora et al., 1989). Therefore, the activation function present in the C-terminal region of RARs and RXRs is referred to as AF-2.

The activities of the AF-2s of the various RARs and RXRs are clearly promoter context dependent. On the basis of these differential activities, it is most probable that RARγ AF-2 is different from the AF-2s of all the other RARs and RXRs. In addition, it is likely that AF-2s of RARα and β are different from one another and from those of RXRα, β, and γ and that AF-2 of RXRβ is different from those of RXRα and γ, since RXRβΔAB, but neither RXRαΔAB nor RXRγ-ΔAB, stimulated transcription from the mCRABPII promoter. We note in this respect that Nagata et al. (1992) also found that mRXRβ truncated for the A/B region transactivates a major histocompatibility complex class I promoter. That AF-2s of RARα and RARγ are different is further supported by the comparison of the activation patterns of RARα1 and of the chimeric receptor RARα1(A–C)–γ(D–F): the D to F region of RARγ can efficiently replace that of RARα with the promoters (TRE3)3–TK, mRARβ2, mRARα2, and mCRABPII (where it is even much better), but it is totally inactive with the mCRBPII promoter. Comparison of the activations of CRBPII and CRABPII promoters by RARβ1 and by the chimeric receptor RARβ1(A–C)–α(D–F) also shows that AF-2s of RARβ and RARα are different (Table 1). It appears, therefore, that the ligand-dependent AF-2s of all RARs and RXRs may be different from one another. In this regard, it should be possible to identify the amino acids responsible for these differences, since the E region sequences of the three RARs are ~90% similar and those of the RXRs are also conserved to a similar extent (Chambon et al., 1991; Leid et al., 1992). Finally, the results obtained with the chimeric RAR–ER receptor RARα1(A–D)–ER(EF) indicate that RARα AF-2 is also different from ER AF-2 (Figure 8).

Promoter Context–Specific Modulating Functions Are Present in the N-Terminal A and B Regions of RARs and RXRs

The N-terminal A/B regions of steroid hormone receptors possess a second promoter context–dependent activation function, AF-1 (see Introduction for references). We therefore looked for the presence of similar AFs in the A and B regions of RARs and RXRs, with the assumption that the A regions of the different RAR types and isoforms contain distinct AFs that could differentially activate the transcription of RA target genes.

The A/B regions of mRARα1, mRARβ2, mRARγ1, mRXRα, mRXRβ, and mRXRγ were linked to the GAL4 (1-147) DNA-binding domain. None of these chimeric transcripts activated transcription from the GAL4 reporter genes that were previously used to reveal AF-1s of steroid hormone receptors (Tora et al., 1989; M. S. et al., unpublished data). Similarly, we were not able to reveal any independent AFs in the A/B region of RARα1 using the chimeric construct RARα1(A–D)–ER(EF) (Figure 8). These results could mean that there are no independent AFs in the N-terminal regions of RARs and RXRs or, alternatively, that these activities are more promoter context dependent than those of steroid hormone receptor AF-1s.

We therefore investigated the effect of deleting the A and the A/B regions of RARs and RXRs on their ability to activate the various natural and synthetic RA-responsive promoters used in the present study. Our results (summarized in Table 1) show that both the A and B regions of RARs contain functions that modulate the activity of AF-2 in a promoter context–dependent manner. For instance, the A regions of RARα1 and α2 are absolutely required for efficient transcription from the mCRBPII and mCRABPII promoters but not from the mRARβ2 and (TRE3)3–TK pro-

moters. However, RARα region B is required for efficient transcription from the latter two promoters. Similar modulating functions, which cooperate in a promoter context-specific manner, are present in the A and B regions of RARβ and RARγ and in the A/B regions of RXRs. The similarity in the transactivation properties of RXRα and RXRγ, and the differences between these RXR types and mRXRβ, can be correlated with the observation that the A/B regions of RXRα and γ are more related to each other than to that of RXRβ (see Leid et al., 1992, for an alignment of RXRs).

Using the present reporter genes, we do not find any dramatic differential effect of the modulating functions present in the different A regions of the isoforms of a given RAR type (α, β, or γ). The A1 and A2 regions of RARα1 and α2, the A1, A2, and A3 regions of RARβ1, β2, and β3 (with the possible exception of the mCRBPII promoter), as well as the A1 and A2 regions of RARγ1 and γ2, appear to have similar modulating functions. That, however, the modulating function present in the A1 region of RARα1 is different from those present in the A1 region of both RARβ1 and RARγ1 is clearly shown by comparing the activation properties of RARα1 and the chimeric constructs between RARα1 and either RARβ1 or RARγ1 on the various promoter reporters (see Table 1, comparing RARα1 with RARβ1(A–C)–α(D–F) and RARγ1(A–C)–α-(D–F)).

Conclusions and Perspectives

The present results support our initial suggestion (Chambon et al., 1991; Kastner et al., 1990a) that the three α, β, and γ members of the RAR family may preferentially control the transcription of different subsets of RA-responsive genes and indicate that the RXR family could behave similarly. These transcriptional specificities result from the cooperation between autonomous activation functions (AF-2s) and modulating functions, which appear to be receptor specific and whose activities are promoter context dependent. It remains to be seen whether the modulating function is truly modulating in nature or, rather, corresponds to an AF similar to steroid hormone receptor AF-1 but more restricted in its promoter context and cell type specificity.

It has been recently demonstrated that, when compared with the corresponding homodimers, RAR–RXR heterodimers bind more strongly, albeit with variable efficiencies, to different RAREs present in naturally occurring and synthetic RA-responsive promoters (B. D., M. S., P. Leroy, M. Leid, and P. C., unpublished data; Kliewer et al., 1992a; Leid et al., 1992; Yu et al., 1991; Zhang et al., 1992; S. N., M. S., P. K., B. D., H. N., and P. C., unpublished data). Furthermore, these RAREs generally consist of the direct repetition of two core motifs (5′-PuG[G/T]TCA-3′ or degenerate derivatives thereof) that are asymmetric in nature, thus generating additional possibilities for increased diversity in RAR–RXR heterodimer binding and promoter transactivation. In addition, it has been found that RARs can be activated by both all-trans RA and 9-cis RA, whereas RXRs are activated by 9-cis RA only (see above for references). Taken together with these findings, the existence of transcriptional activation and modulating functions, whose activities in each RAR and RXR could be promoter context specific (i.e., cooperating in a specific fashion with the other factors that are bound to a given target gene promoter), may generate the numerous combinatorial possibilities that ultimately must exist to account for the highly pleiotropic effects of RA during embryogenesis and cytodifferentiation and in the adult vertebrate. It is, however, important to stress that our present results indicate that a certain degree of redundancy may exist, since some of the natural promoters that have been tested could be activated, albeit to different extents, by more than one RAR or RXR form. Genetic evidence is obviously required to prove that such highly evolved combinatorial processes are in fact responsible for the enormous diversity of effects generated by the simple RA signal.

Experimental Procedures

Plasmid Constructions

The expression vectors for mRARα1, α2 (Zelent et al., 1989; Leroy et al., 1991a), β1, β2, β3 (Zelent et al., 1989, 1991), β4 (Nagpal et al., 1992), γ1, and γ2 (Zelent et al., 1989; Kastner et al., 1990a), and mRXRα, β, and γ (Leid et al., 1992) in pSG5 (Green et al., 1988) have been described (Figure 1). For the construction of mRARα1–mRARγ1 and mRARα1–RARβ1 chimeric expression vectors, XhoI restriction sites were introduced by site-directed mutagenesis in mRARα1, mRARβ1, and mRARγ1, first upstream of the initiator ATG (generating mRARα1(X1), mRARβ1(X1), and mRARγ1(X1)) and then at the end of the C region, yielding mRARα1 (X1.X3), mRARβ1(X1.X3), and mRARγ1 (X1.X3), which contain three additional amino acids (Ala-Arg-Ala) immediately after the C region. mRARα1(X1.X3), mRARβ1 (X1.X3), and mRARγ1(X1.X3) A–C region XhoI–XhoI fragments were reciprocally swapped for the construction of mRARα1(A–C)–γ(D–F), mRARβ1(A–C)–α(D–F), and mRARγ1(A–C)–α(D–F) expression vectors. mRARα1(A–C)–γ(D–F) contains amino acids 1–153 of mRARα1 and amino acids 156–458 of mRARγ1; mRARβ1(A–C)–α(D–F) contains amino acids 1–146 of mRARβ1 and amino acids 154–462 of mRARα1; and mRARγ1(A–C)–α(D–F) contains amino acids 1–155 of mRARγ1 and amino acids 154–462 of mRARα1. For the construction of mRARαΔA and mRARαΔAB, mRARα1(X1) was digested with XhoI and PstI, and the larger plasmid fragment containing the deletion of nucleotides 191–624 was gel purified using diethylaminoethyl paper (Schleicher & Schuell). mRARαΔA was prepared by polymerase chain reaction (PCR) amplification of nucleotides 371–624 of mRARα1 by using the primer pairs 5′-TTCTCGAGCTTGCATGGCCATCGAGACC-CAGAGCAGT and 5′-CGAAACATTTCTGCAGCCGGC and subsequently subcloning the PCR-amplified product into XhoI–PstI sites of mRARα1(X1). Thus, mRARαΔA contained Met and Ala as its first two amino acids, followed by amino acids 61–462 of the mRARα1. The mRARαΔAB expression vector contained a deletion of both A and B regions of the receptor, except for the last four amino acids of the B region, in addition to two extra amino acids, Met and Gly, at the N-terminus. mRARαΔAB was prepared by PCR amplification of nucleotides 441–624 of mRARα1 using oligonucleotide primers 5′-TT-CTCGAGCTTGCATGGGCATCTACAAGCCTTGCTTT and 5′-CGA-AACATTTCTGCAGCCGGC and subcloning the amplified product into XhoI–PstI–digested mRARα1(X1). Thus, it contained amino acids 84–462 of mRARα1. The mRARγΔA expression vector was constructed by PCR amplification of the sequence encoding the B to F regions (amino acids 60–458) of mRARγ and subcloning into EcoRI–BglII sites of pSG5-Cas. (pSG5-Cas is identical to pSG5 [Green et al., 1988] but contains an EcoRI–KpnI–XmaIII–NaeI–BalI–XhoI–BglII polylinker sequence in tandem with the in-frame Kozak sequence and ATG initiator codon.) mRARγ1 B–F regions were PCR amplified using the primer pair 5′-AGCTCGAGTCGGTGGAGACACAGAGC and 5′-AGCTCGAG-GGGCCCCTGGTCAGGTTG. The mRARγΔAB expression vector was constructed by PCR amplification of the C–F regions (amino acids 90–458) of mRARγ1 using primer pairs 5′-AGGGTACCTGCTTTGTATG-CAATGACAAG and 5′-AAGCTGCAATAAACAAGTTC and subcloning

into the KpnI–BglII sites of pSG5-Cas. mRARβΔAB plasmid was prepared by PCR amplification of the sequence containing C–F regions (amino acids 81–448) of mRARβ2, using the primers 5′-TTGGATCCATCATGTTCCCCTGCTTCGTTTGCCAGGACAAG and 5′-TAGGATCCTCACTGCAGCAGTGGTGACTG and subcloning the amplified product into the BamHI sites of pSG5. RARα1(A–D)–ER(EF) contains A–D regions of human RARα (hRARα) and EF regions of human ER and harbors amino acids 1–187 of hRARα1 in tandem with amino acids 311–595 of human ER (S. Mader, unpublished data). mRXRαΔAB was constructed by PCR amplification of the C–E regions (amino acids 140–467) of mRXRα, using primers 5′-GCGGTACCTGTGCTATCTGTGGGGACCGC and 5′-GCGGTACCCTAGGTGGCTTGATGTGGTGC and subcloning into the KpnI site of pSG5-Cas. Similarly, mRXRβΔAB and mRXRγΔAB were prepared by subcloning PCR-amplified products of C–E regions of mRXRβ (amino acids 123–448) and mRXRγ (amino acids 139–463), respectively, into the KpnI site of pSG5-Cas. Oligo primer pairs used for PCR amplification for mRXRβ C–E regions were 5′-GCGGTACCCGGCTCTGTGCAATCTGCGGG and 5′-GCGGTACCTCAGGCTAGCTGGTGGGGAGC, and those used for amplification of C–E regions of mRXRγ were 5′-GCGGTACCTGTGCCATCTGTGGGGACAGA and 5′-GCGGTACCTCAGGTGATCTGCAGTGGGGT.

The reporter genes mRARβ2–CAT (Smith et al., 1991), mRARα2–CAT1 (Leroy et al., 1991b), mCRBPI–CAT1 (Smith et al., 1991), mCRBPII–CAT1 (H. N. et al., unpublished data), mCRABPII–CAT1 (B. D., M. S., P. Leroy, M. Leid, and P. C., unpublished data), (TRE3)3–TK-CAT (de Verneuil and Metzger, 1990), and β2RARE–TK-CAT (Smith et al., 1991) have been described. DR1G–TK-CAT was constructed by annealing oligonucleotides 5′-TCGAGGGTAGGGGTCAGAGGTCACTCG and 5′-TCGACGAGTGACCTCTGACCCCTACCC and ligating them with SalI-digested pBLCAT8$^+$ (Klein-Hitpass et al., 1988). Thus, it contains a direct repeat of the 5′-PuGGTCA-3′ core motif separated by 1 nt.

Transient Transfection of Cells and CAT Expression Analysis

COS-1 cells maintained in Dulbecco's modified Eagle's medium plus 5% delipidated fetal calf serum (stripped of hormones) was plated at 40%–50% confluency for transfection using the calcium phosphate procedure (Kumar et al., 1986). The amount of reporter constructs as well as receptor expression vectors used in transfection experiments have been indicated in the figure legends. All the assays were performed in the presence of 2 μg of pCH110 (Pharmacia), a β-galactosidase expression plasmid used as an internal control to normalize for variations in transfection efficiency. The total amount of DNA in each transfection was standardized to 20 μg using carrier DNA (Bluescript). After 20 hr incubation with calcium phosphate–precipitated DNA, the cells were washed with the medium before incubation for an additional 20–24 hr along with the ligand (RA at a 10^{-6} M or E2 at a 10^{-7} M concentration), as indicated in the figure legends.

Cellular extracts were prepared and normalized for β-galactosidase activity as described (Petkovich et al., 1987). Generally, CAT assays contained extracts corresponding to 30 U of β-galactosidase for mRARβ2–CAT and mCRABPII–CAT1; 50 U for mRARα2–CAT1, mCRBPI–CAT1, and mCRBPII–CAT1; 10 U for (TRE3)3–TK-CAT; 5 U for DR1G–TK-CAT; and 2 U for β2RARE–TK-CAT. The CAT activity was quantified by scintillation counting.

Receptor expression vectors were overexpressed by transfection of COS-1 cells with 10 μg of the wild-type or mutant receptor plasmids. Whole-cell extracts were prepared by lysing the cells in 100 μl of high salt extraction buffer (0.4 mM KCl, 20 mM Tris–HCl [pH 8.0], 2 mM dithiothreitol, 20% v/v glycerol, and protease inhibitors) by 3 cycles of freeze–thaw. The extracts were centrifuged at 10,000 × g for 15 min at 4°C to remove cell debris.

Quantification of Receptors in Transfected COS-1 Cells by Gel Retardation/Shift Assay and Western Blotting

The amount of the various receptors was quantified both by Western blotting and by using both gel retardation assays in the presence of an excess of either hRXRβ (in the case of RARs) or bacterially produced hRARγ (in the case of RXRs) to ensure efficient binding of the receptors as heterodimers. For gel retardation, protein extracts from COS-1 cells transfected with the various expression vectors were preincubated in 10 mM Tris–HCl (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM $MgCl_2$, 5% glycerol, and 4 μg of poly(dI–dC) for 15 min on ice. Subsequently, 50,000 cpm of ^{32}P-labeled oligonucleotide probe (~14 fmol) was added in a total volume of 20 μl and incubated for an additional 15 min on ice. For quantitative gel retardation analysis of various mRAR isoforms, COS-1 cells were transfected with 2 μg of the receptor expression vector. Cell extract (3 μg of protein) was preincubated with excess hRXRβ (2 μg of semipurified RXRβ fraction; Leid et al., 1992) in gel retardation buffer on ice for 15 min. For quantitative gel retardation analysis of mRXRα, β, and γ, transfected COS-1 cell extracts were incubated with an excess of bacterially produced hRARγ (Leid et al., 1992). No binding was observed with either hRXRβ or bacterially produced hRARγ alone. Protein–DNA complexes were resolved by electrophoresis (25 mA for 1 hr) on a 5% polyacrylamide gel prepared in 0.5 × TBE (0.09 M Tris–borate, 0.09 M boric acid, and 0.002 M EDTA). The gels were dried and autoradiographed with intensifying screen at −80°C. The specificity of the RAR–RXR complexes was confirmed either by supershift assay, using mouse monoclonal antibodies against mRARs (Rochette-Egly et al., 1991; Gaub et al., 1992; S. N. et al., unpublished data), or by using excess (100-fold) of unlabeled wild-type or mutant RARE from the RARβ2 gene (Nicholson et al., 1990) as competitors during the first incubation. Western blotting of RARs was as described (Rochette-Egly et al., 1991; Gaub et al., 1992; S. N. et al., unpublished data).

Acknowledgments

We are grateful to Drs. S. Mader, P. Leroy, C. Mendelsohn, and D. Metzger for their generous gifts of pSG5-Cas and RARα1(A–D)–ER(EF), mRARα2–CAT1, mRARβ2–CAT, and (TRE3)3–TK-CAT plasmids, respectively. We thank Drs. H. Gronemeyer, M. Leid, and P. Leroy for critically reading the manuscript, all of the members of the receptor group for their advice and useful discussion, A. Staub and F. Ruffenach for synthesizing the oligonucleotides, S. Friant and the cell culture group for technical assistance, C. Werlé, B. Boulay, and J. M. Lafontaine for preparing the figures, and the secretarial staff for typing the manuscript. S. N. was supported by a fellowship from the Institut National de la Santé et de la Recherche Médicale (INSERM), M. S. was a recipient of a European Molecular Biology Organization fellowship, and H. N. was supported by a fellowship from the Université Louis Pasteur. This work was supported by the Centre National de la Recherche Scientifique, INSERM, the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur le Cancer, the Ligue Nationale Française contre le Cancer, the Fondation pour la Recherche Médicale Française, and the Human Science Frontier Program.

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Received May 1, 1992; revised June 26, 1992.

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Note Added in Proof

The data by M. T. Bocquel et al. listed as unpublished is now in press (Allenby, G., Bocquel, M. T., Saunders, M., Kazmer, S., Speck, J., Rosenberger, M., Lovey, A., Kastner, P., Grippo, J. F., Chambon, P., and Levin, A. A. [1992]. Proc. Natl. Acad. Sci. USA *89*, in press).

The data by B. D., M. S., P. Leroy, M. Leid, and P. C. listed as unpublished is now in press (Durand, B., Saunders, M., Leroy, P., Leid, M., and Chambon, P. [1992]. All-trans and 9-cis retinoic acid induction of CRABPII transcription is mediated by RAR–RXR heterodimers bound to DR1 and DR2 repeated motifs. Cell *71*, in press).