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The Receptor-DNA Complex Determines the Retinoid Response: a Mechanism for the Diversification of the Ligand Signal

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To obtain insights into the principles governing the complex biological responses to retinoids, we have analyzed the ligand sensitivities of various retinoid receptor-DNA complexes. We find that different retinoid receptor heterodimers show distinct activation patterns with various response elements while a given heterodimer can be activated at different retinoic acid concentrations on different response elements. In vitro binding experiments suggest that the same retinoic acid receptor-retinoid X receptor (RAR-RXR) heterodimer can have different ligand affinities, depending on the response element it is bound to. The differential responses of a particular receptor heterodimer with various retinoic acid responsive elements can be enhanced through the use of conformationally restricted retinoids. RAR- and RXR-selective retinoids can also synergistically activate the receptor heterodimers, indicating that both partners in the heterodimer can contribute to ligand-induced transcriptional activation. However, the relative influence of the RAR or RXR partner is specific for each response element. Together, our data demonstrate that it is the receptor-DNA complex and not the receptor alone that determines the ligand response. This flexibility allows for a highly pleiotropic retinoid response. Furthermore, conformationally restricted retinoids can accentuate the differential responses and exhibit a certain degree of gene selectivity by differentially activating the RAR or RXR component in the context of a given response element.

Vitamin A and its active natural and synthetic analogs and derivatives, the retinoids, regulate a diversity of important biological processes, including morphogenesis, growth, differentiation and development, metabolism, and homeostasis (for reviews, see references 22, 48, and 68). Clinically, retinoids serve as the most effective remedy for several skin diseases as well as in the treatment of promyelocytic leukemia (8, 12, 29, 65). In addition, numerous reports suggest that retinoids could serve as preventive and therapeutic agents for a number of other cancers (reviewed in references 9, 26, 28, 34, 35, 46, and 67). The molecular signal mechanism underlying the pleiotropic actions of retinoids has therefore been of great interest. In addition, several thousand synthetic retinoids have been synthesized in the last two decades, with the aim of obtaining derivatives with optimal therapeutic indices (7, 64). Since the first cloning of a specific receptor for the most common, natural active retinoid, all-*trans*-retinoic acid (tRA) (5, 10, 20, 57), it has become apparent that with the exception of those for the vision process, retinoid signals are mediated by hormone-like nuclear receptors. These receptors are part of a complex network of interacting nuclear receptors: the retinoid-thyroid hormone receptor subfamily (13, 21, 52, 59).

Two types of nuclear retinoid receptors, the RA receptors (RARs) and the retinoid X receptors (RXRs), have been identified. Both types are coded for by three genes (α, β, and γ) from which multiple receptor isoforms can be generated by differential splicing and the use of alternative promoters (for recent reviews, see references 13, 21, 52, and 59). RARs bind and are activated by tRA and 9-*cis*-RA, while RXRs only interact with 9-*cis*-RA (1, 25, 45, 51). In addition, several other natural RA derivatives that may play specific roles during development and/or in certain tissues have been identified (60, 70). The receptors mediate retinoid signals either by binding to specific DNA sequences or RA responsive elements (RAREs) as RAR-RXR heterodimers or RXR homodimers (13, 52, 59, 72, 76, 78) or by interacting with other transcriptional regulators, notably AP-1 (19, 58, 63, 66, 73). The RXRs not only are critical for allowing effective binding of the RARs to DNA but also enhance the DNA binding of other receptors that interact with different hormones or vitamin derivatives such as the thyroid hormone receptors, the vitamin D_3 receptor, the peroxisome proliferator activator receptors, and several orphan receptors (2, 3, 11, 24, 32, 33, 43, 54, 74, 78). The RXRs thus play a central role in mediating a variety of hormonal signals in the nucleus. In addition, they can allow cross talk between retinoids and other ligands (41). This diversity of retinoid receptor action and the central roles of retinoid receptors in the vitamin and hormonal signalling pathways are consistent with the profound and pleiotropic effects of retinoids on biological processes.

Vitamin A and its natural acid and aldehyde derivatives are also unique among the fat-soluble hormones in that they are quite flexible molecules that can convert into distinct isomers with specific conformations and activities, examples of which are tRA and 9-*cis*-RA, two molecules with different receptor activation profiles (1, 25, 45). Since RARs predominantly function as heterodimers with RXR, the various RAR-RXR heterodimers could have distinct ligand response profiles which together with the observed tissue- and cell type-specific expression patterns of the various RAR and RXR subtypes and isoforms (reviewed in references 13, 21, 44, 52, 59) would allow for a highly fine-tuned pleiotropic response mechanism. Possible evidence for the existence of such a complex mechanism is the large variety of structurally different RAREs that have now been defined (16, 27, 30, 38, 42, 53, 62, 69, 71).

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In contrast to steroid hormone response elements (4), RAREs are very diverse, since they include palindromes and direct repeats with various spacers as well as inverted palindromes (see Fig. 1). Although no significant differences in RARE binding have been observed so far for different RAR-RXR heterodimers (24), it has been observed that RARγ1 is a poor activator of DR-5 RAREs (a direct repeat of the half-site with a 5-bp spacer) (27, 31, 42) and that the DR-1 from the cellular retinol-binding protein type II (CRBPII-RARE) is mostly activated by RXR homodimers (40, 53, 77). In addition, a comparison of various RA-responsive synthetic genes showed that activations by different receptors can vary (56). Furthermore, it has been shown that the ligand binding pockets of RARα, -β, and -γ are distinct, since synthetic retinoids that preferentially bind and activate these receptors could be defined (6, 23, 39).

We have now investigated the possibility that the activity of a ligand is not a simple function determined by its affinity for a given retinoid receptor; rather, the activity is determined by the receptor heterodimer-DNA complex. We were particularly interested in examining whether interactions of various RAR-RXR heterodimers with the different RAREs could affect the sensitivity of the ligand response and how this change in the ligand response could be exploited with synthetic retinoids having structurally restricted configurations. We observed that the various retinoid receptor heterodimers show distinct activation patterns with various response elements while a given heterodimer can be activated at different tRA or 9-*cis*-RA concentrations on various RAREs, thus allowing for differential retinoid responses of various RAREs and their associated genes. This response profile differs, in addition, for different heterodimers. In vitro binding experiments indicate that the differential activities of a ligand with the same heterodimer on different response elements could be due to differential affinities of a receptor for the ligand when the receptor is present in different heterodimer-RARE complexes. Certain synthetic retinoids with restricted conformations could be shown to further enhance this preferential activation of RAR-RXR heterodimers on selective response elements. In addition, various heterodimers allowed distinct sequential and/or synergistic activation by RAR- and RXR-selective retinoids. Our observations define new principles for ligand-nuclear receptor interactions and show that the ligand response is not determined by the receptor alone but by the receptor heterodimer-DNA complex.

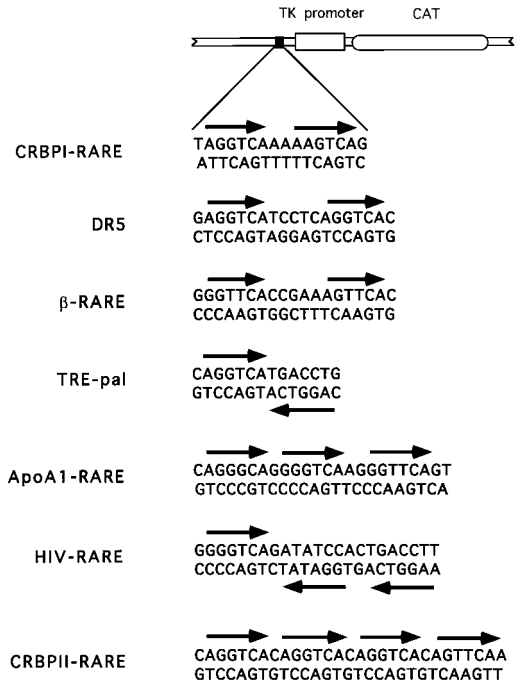

FIG. 1. Structurally distinct RAREs. Chloramphenicol acetyltransferase reporter constructs containing structurally distinct RAREs were used. The arrows indicate the orientations of the half-sites. The putative center half-site in the HIV-RARE is not essential for retinoid receptors. TK, thymidine kinase.

MATERIALS AND METHODS

Plasmids. Plasmids pECE-RAR(α,β,γ) and pECE-RXRα have been described previously (42). A 2.2-kb *Eco*RI cDNA containing the entire coding phase of murine RXRβ was inserted into the *Eco*RI site of the polylinker of expression vector pECE (18) to obtain expression vector pECE-RXRβ. Similarly, a 1.67-kb *Eco*RI cDNA fragment containing the entire coding phase of murine RXRγ was inserted in the *Eco*RI fragment of the pECE vector to yield pECE-RXRγ. The same coding phases were inserted in the pBluescript vector (Stratagene) and used for in vitro translation of the corresponding proteins.

Transfection assays. Green monkey kidney cells (CV-1) grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum were used for all transfection assays. Transient transfections were performed by a modified calcium-phosphate precipitation procedure as described previously (24, 30, 76). Briefly, 5×10^5 cells were transfected with a total of 1 μg of DNA containing 50 ng of each receptor expression vector, 100 ng of reporter gene, and 150 ng of β-galactosidase expression plasmid pCH110. After 15 h, the cells were washed twice in phosphate-buffered saline and fresh medium containing charcoal-treated serum, and the appropriate retinoids were added. Following 24 h of further incubation, the cells were lysed by three cycles of freeze-thawing and analyzed for chloramphenicol acetyltransferase and β-galactosidase activity.

Retinoids. The following designations for the retinoids were used: SR11237, 2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-(4-carboxyphenyl)-1,3-dioxalane; SR11234, 2-(5,6,7,8-tetramethyl-2-naphthalenyl)-2-(4-carboxyphenyl)-1,3-dithiolane; SR11217, 4-[1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-methylpropenyl]benzoic acid; SR11223, 4-[1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)ethyl]benzoic acid; SR11221, 4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-oxoethyl]benzoic acid; SR11146, 4-(1-amino-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid; R18 (SR3907), 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtho[2,3-b]thienyl)benzoic acid; R16 (TTAB or SR3961), 4-(1-amino-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl) benzoic acid; Am80, 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)aminocarbonyl]benzoic acid; and Am580, 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid.

DNA-dependent ligand binding assay. Double-stranded, biotinylated oligonucleotides 5′-bioGAT CCT GAC TTT TTG ACC TG (CRBPI) and 5′-bioGAT CCA GGT CAT CCT CAG GTC AG (DR-5) (synthesized by GIBCO BRL) were bound to magnetic beads coated with streptavidin (Dynabeads) according to the protocol given by the manufacturer (Dynal).

RARα and RXRα full-length cDNAs cloned into pBluescript (Stratagene) were transcribed in vitro with T3 or T7 polymerase. To generate mRNAs, the receptor proteins were prepared by in vitro translation reactions with rabbit reticulocyte lysate (Promega).

Six-microliter portions of each translation product were incubated for 15 min at room temperature with 3 μl of DR-5 or CRBPI Dynabeads in a 50-μl final volume of 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.91) containing 250 mM KCl, 5 mM dithiothreitol, 12.5 mM $MgCl_2$, 50% glycerol, and 0.05 μg of poly(dI-dC) per μl (1× HEPES buffer).

[^{3}H]RA (Dupont, NEN) with a specific radioactivity of 49.6 Ci/mmol was then added at a concentration of either 60 or 100 nM, and the volume was adjusted to 100 μl. Nonspecific binding was measured by incubation in the presence of a 200-fold molar excess of unlabelled ligand. After 15 min of incubation at room temperature, the ligand-receptor-beads complexes were concentrated with a magnetic particle concentrator (Dynal MPC-E) and subjected to four cycles of washing in 1× HEPES buffer. The beads were transferred into scintillation vials, and the radioactivity bound to the beads was measured with a scintillation counter.

RESULTS

Activation of RAREs is receptor specific. To analyze whether structurally distinct RAREs require specific dimeric retinoid receptor complexes for optimal activation, we carried out a systematic analysis with a series of different RAREs and all RAR-RXR subtype combinations. The sequences of the

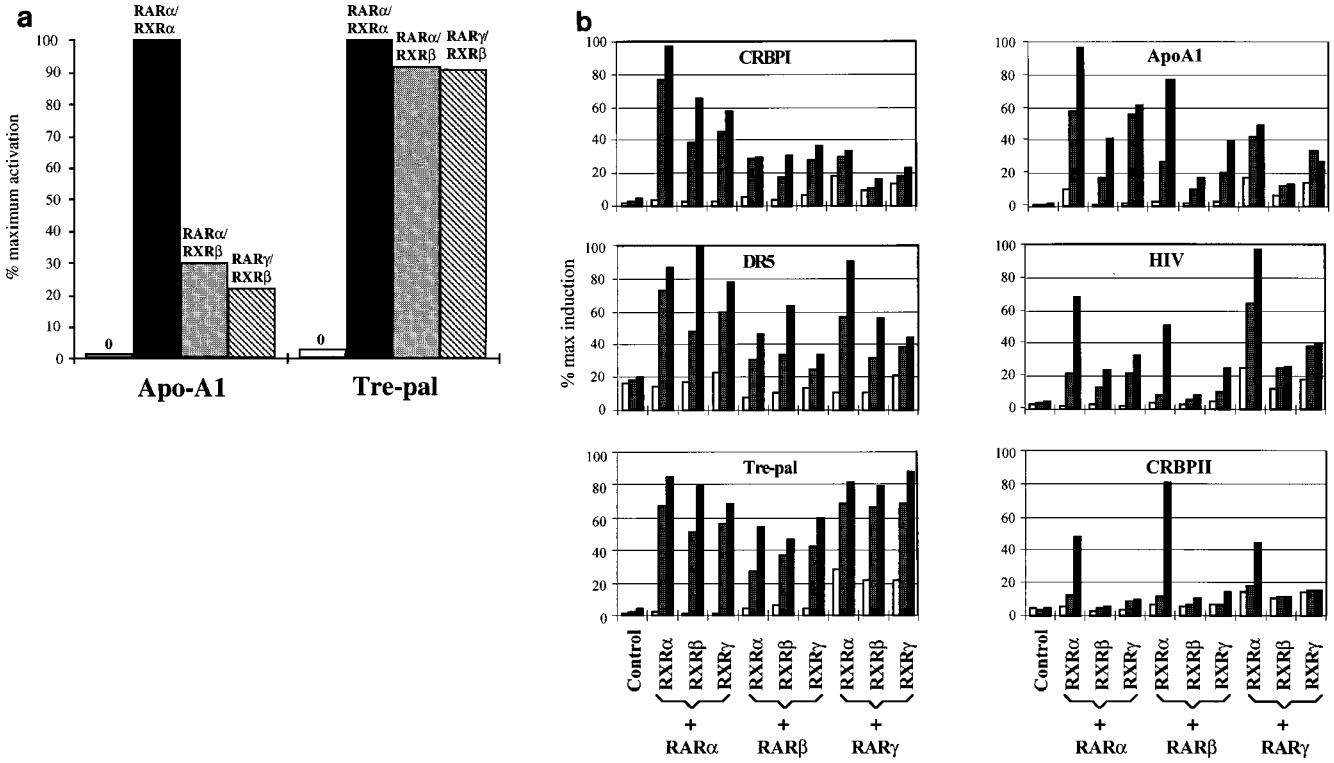

FIG. 2. Differential receptor activities on various response elements. (a) Activation of ApoA1-RARE and TREpal by the RARα-RXRα, RARα-RXRβ, and RARγ-RXRβ heterodimers in the presence of 10^{-7} M tRA. (b) Relative activations of ApoA1-RARE, HIV-RARE, β-RARE, CRBPII-RARE, CRBPI-RARE, and TREpal in the presence of various RAR-RXR heterodimers and in the absence of ligand (open bar) or upon induction by 10^{-7} M tRA (stippled bar) and 10^{-7} M 9-*cis*-RA (solid bar). Results are expressed as percent maximum induction obtained for each response element. Results are the means of at least three different experiments differing by less than 20%. Chloramphenicol acetyltransferase activity was normalized to β-galactosidase activity.

different RAREs are shown in Fig. 1. CRBPI-RARE and ApoA1 represent different natural DR-2s (direct repeats of half-sites with a 2-bp spacer); β-RARE and DR-5 represent examples of response elements in which the two half-sites are separated by 5-bp spacers, while CRBPII-RARE is a DR-1. In TREpal and human immunodeficiency virus type 1 (HIV-1)–RARE, the half-sites are arranged as palindromes with 0- or 9-bp spacers, respectively. Chloramphenicol acetyltransferase reporter constructs carrying a particular RARE were cotransfected with expression vectors for the various RAR and RXR subtypes into CV-1 cells, and reporter gene activity was determined in the presence of 10^{-7} M tRA or 9-*cis*-RA. For each response element, we essentially observed specific activation patterns with various receptor combinations. Figure 2a demonstrates that RARα-RXRα is a much better activator of ApoA1-RARE than is RARα-RXRβ or RARγ-RXRβ and that RARα-RXRβ and RARγ-RXRβ are equally good activators of the TREpal element. A summary of the data obtained with six RAREs is given in Fig. 2b. In the presence of 9-*cis*-RA, which is known to activate both RARs as well as RXRs (1, 25, 45) and to induce RXR homodimer binding on a DR-1 (77), a higher activation than that with tRA was observed in most cases, in particular with CRBPII-RARE (Fig. 2b), which was previously shown to be activated mostly by RXR homodimers (40, 53, 77). However, heterodimers containing RARγ often showed little difference in their responses to 9-*cis*-RA and tRA. Certain heterodimers, for instance, RARα-RXRα, were highly active on all the response elements investigated, with the exception of CRBPII-RARE, on which RARα-RXRα activity was lower than that of the RARβ-RXRα heterodimer (Fig. 2b) and that of the RXRα homodimer (data not shown), which is consistent with previous results (40, 53). Dose-response curves carried out with retinoid receptor expression vectors demonstrated that 50 ng of the expression vectors was sufficient to obtain maximal activity (data not shown). In addition, as can be seen from a comparison of all the data presented in Fig. 2b, the activities of the different heterodimers can vary dramatically from response element to response element, with the least degree of difference seen for TREpal. Therefore, the data in Fig. 2b clearly show that different RAREs require different heterodimers for optimal activation at this ligand concentration. In addition, the activations induced by tRA and 9-*cis*-RA can differ very significantly or hardly at all, depending on the response element and the receptors present. This result implies that the cellular retinoid receptor expression pattern is important in determining which RARE can be activated to what extent. In addition, the levels of activation can depend on the RA isomer present in the cell.

The response element determines the ligand response of a heterodimer. One way to explain the various activities of various dimeric receptor complexes on different RAREs would be to assume that the receptor ligand binding domains in the heterodimer-response element complex can be in various configurations so that the ligand (tRA or 9-*cis*-RA) can interact more or less optimally with it. Alternatively, another explanation could be that the ligand-induced conformational change of one or both of the receptors required for transcriptional activation is inhibited or favored after the receptor dimer interacts with a particular RARE. To examine these hypotheses further, we compared in detail the ligand responses of two heterodimers—RARβ-RXRα and RARα-RXRα—on six RAREs.

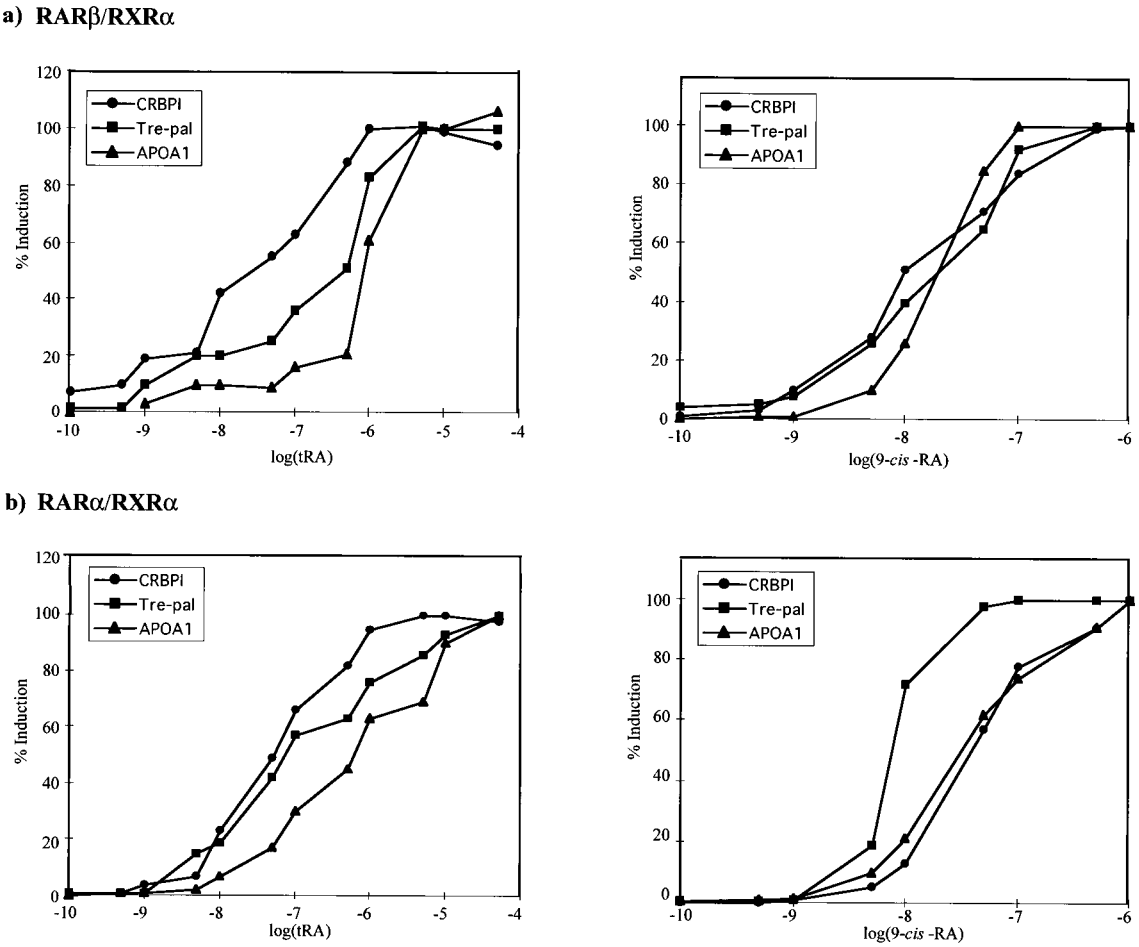

FIG. 3. Differential RA responses are dependent on the response element. Dose-response curves for tRA and 9-*cis*-RA with RARβ-RXRα (a) and RARα-RXRα (b) on CRBPI-RARE, TREpal, and ApoA1-RARE are shown. Each point represents the average of two or three experiments differing by less than 20%. Results are expressed as percent induction obtained with 5×10^{-5} M tRA and 10^{-6} M 9-*cis*-RA.

We observed marked differences in the ligand response curves for a given heterodimer on individual response elements (Fig. 3). For one given heterodimer, different ligand sensitivities were observed on the different response elements. The data are summarized in Table 1, in which the retinoid concentrations required for half-maximum activation of the various heterodimer-RARE complexes (50% effective concentrations [EC_{50}s]) are given. For RARβ-RXRα, CRBPI-RARE and DR-5 allowed the most sensitive responses to tRA, giving EC_{50}s of 40 and 50 nM, respectively, and they were followed by β-RARE (EC_{50} = 300 nM) and TREpal (EC_{50} = 500 nM). ApoA1-RARE, HIV-RARE, and CRBPII-RARE were the least sensitive elements, requiring at least 1,000 nM tRA for 50% maximal stimulation. The 9-*cis*-RA dose-response curves showed increased sensitivities compared with those for tRA, and in general, fewer differences among the RAREs were observed (EC_{50}s = 10 to 50 nM) (Table 1). Interestingly, the RARα-RXRα–TREpal complex showed the highest sensitivity (Fig. 3b). We should point out that the values given in Table 1 for β-RARE are also likely to reflect endogenous receptor activities, since β-RARE, in contrast to the other response elements, shows high activity in the absence of cotransfected receptors.

The observation that a particular heterodimer, such as RARβ-RXRα, shows different ligand sensitivities on various

TABLE 1. Responses to tRA and 9-*cis*-RA are determined by the heterodimer-RARE complex

Heterodimer	Ligand	EC_{50}[a]						
		CRBPI-RARE	DR-5	β-RARE	TREpal	ApoA1-RARE	HIV-RARE	CRBPII-RARE
RARβ-RXRα	tRA	40	50	300	500	1,000	>1,000	>1,000
	9-*cis*-RA	10	5	20	16	25	40	50
RARα-RXRα	tRA	60	1,000	1,000	100	600	>1,000	>1,000
	9-*cis*-RA	40	20	80	25	40	20	20

[a] The EC_{50} is the concentration of ligand (nanomolar) required for half-maximal activation of a reporter gene carrying a defined RARE in the presence of cotransfected retinoid receptors.

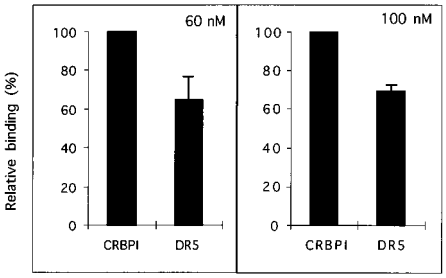

FIG. 4. Ligand binding of the RARα-RXRα heterodimer in the presence of different response elements. Biotinylated oligonucleotides containing the CRBPI or DR-5 response element were bound to Dynabeads and incubated with in vitro-translated RARα and RXRα in the presence of 60 and 100 nM [^{3}H]tRA. The RARα-RXRα-[^{3}H]tRA complexes were separated from unbound radioactivity with a magnetic particle concentrator, and the bound radioactivity was quantitated with a scintillation counter. Nonspecific binding was determined by incubation in the presence of a 200-fold molar excess of unlabelled ligand. Data are the averages of two experiments carried out in triplicate. The results are plotted as percent maximum binding. The binding obtained for CRBPI-RARE was defined as 100%.

RAREs (Fig. 3a) suggests that the ligand binding pockets of RARβ and RXRα or their accessibilities could vary, depending on the response element with which the heterodimer interacts. Furthermore, the differential ligand responses on the various RAREs were not identical after we compared the RARβ-RXRα and RARα-RXRα heterodimers. For the RARα-RXRα heterodimer, in general, smaller differences were observed, and the order of sensitivity to tRA was different from the one observed with RARβ-RXRα (Fig. 3 and Table 1). While CRBPI-RARE still gave the most sensitive response (an EC_{50} of 60 nM with tRA), β-RARE required 10-fold more tRA than did TREpal for 50% maximal activation. Overall, our results suggest that the sensitivity of a given retinoid-responsive gene is dependent on the type of response element it contains as well as on the particular heterodimer present.

The affinity for the RAR ligand is influenced by the response element. In order to obtain some insights into the mechanism responsible for the differences in ligand sensitivity observed in the presence of different response elements, we investigated the possibility that RAR ligand affinity in the RARα-RXRα heterodimer was altered by the nature of the response element. In vitro-translated RARα and RXRα were allowed to form a complex with either the CRBPI element or the DR-5 element in the presence of 60 or 100 nM tritiated tRA that could be precipitated by magnetic beads (see Materials and Methods). A quantitation of the amount of specifically bound tRA shows that complex RARα-RXRα-DR-5 binds only 64% of the amount [^{3}H]tRA bound by RARα-RXRα–CRBPI at 60 nM. At 100 nM, this ratio is 69% (Fig. 4). Because of the relatively low specific activity of [^{3}H]tRA, measurements at lower ligand concentrations could not be carried out. However, the results obtained suggest that alteration of receptor ligand affinity in the various heterodimer-DNA complexes contributes to the difference in EC_{50}s observed with DR-5 (EC_{50} of tRA, 1,000 nM) and CRBPI (EC_{50} of tRA, 60 nM). Additional mechanisms, such as response element-dependent activation changes, could further enhance the differences.

Conformationally restricted retinoids display response element specificities. We next investigated whether the differential responses of a particular heterodimer to tRA and 9-*cis*-RA that were observed with various RAREs could be enhanced when synthetic, conformationally restricted retinoids were used instead of the flexible natural retinoids tRA and 9-*cis*-RA. More than 50 different synthetic retinoids were compared on six response elements in the presence of the RARβ-RXRα heterodimer. Activation in the presence of 10^{-6} M 9-*cis*-RA was taken as a reference point, since this retinoid is a panactivator. Examples of retinoids with different response element selectivities with RARβ-RXRα are shown in Fig. 5a. R16 is a good activator of CRBPI-RARE, TREpal, and β-RARE but does not activate the other RAREs, while SR11234 does not activate β-RARE and CRBPI-RARE but does activate all other RAREs. While each of the retinoids (Fig. 5a) has its own specific response element activation pattern, the patterns overall fall into three groups. Retinoids that show preferential activation on CRBPI-RARE and β-RARE usually showed little activity on ApoA1-RARE, HIV-RARE, and CRBPII-RARE. Although this pattern of activation is similar to the one obtained with tRA, a clearly increased selectivity against ApoA1-RARE is observed. The second group of retinoids activates mostly ApoA1-RARE, HIV-RARE, and CRBPII-RARE, with no significant activity on CRBPI-RARE and β-RARE. TREpal represents a separate group, since it allows the most versatile responses, i.e., it is sensitive to most of the retinoids tested.

The first group of retinoids includes compounds SR11146, SR11221, R16, and R18. These retinoids have a 2-carbon spacer between the lipophilic and benzoic acid termini, and most of them have been found to be RAR-selective activators (1, 39). The second group of retinoids contains such compounds as SR11234, SR11237, SR11217, and SR11223, which have a 1-carbon spacer and have been found to be selective for RXR (14, 40). This result suggests that depending on the response element, activation of the RARβ-RXRα heterodimer preferentially occurs through the interaction of the ligand with either the RAR partner or the RXR partner.

With the RARα-RXRα heterodimer, the RAR-selective compounds were also found to preferentially activate (Am580 and Am80) the CRBPI, β-RARE, and TREpal elements. However, activation in the presence of TREpal, ApoA1, and HIV-RARE was clearly increased compared with that with the RARβ-RXRα heterodimer (Fig. 5b). In addition, in the presence of RARα-RXRα, RXR-selective retinoids SR11234 and SR11237 were completely inactive on CRBPI-RARE and β-RARE and showed very reduced activities on ApoA1-RARE, HIV-RARE, and CRBPII-RARE. Thus, in the case of the RARα-RXRα heterodimer, activation via RXR was not possible or very inefficient on most of the response elements. However, the RARβ-RXRα heterodimer allowed strong activation by RXR-selective compounds on some RAREs investigated (Fig. 5a). These results demonstrate that the two different heterodimers analyzed here allow distinct ligand responses on certain RAREs, i.e., the RAR subtype present in the heterodimer can be very critical for the activation pattern observed. In addition, depending on the response element, RAR or RXR can be either active or silent. It therefore appears that, depending on the response element, either RAR or RXR can dominate the response.

Cooperativity between RAR and RXR ligands. tRA and the synthetic RAR- and RXR-specific compounds show a response element-specific activity pattern and reduced activity compared with those with 9-*cis*-RA. This outcome could be due to the fact that 9-*cis*-RA can adopt the optimal conformation necessary to interact with and maximally activate both the RAR partner and the RXR partner in the heterodimer-RARE complex. In contrast, tRA and the conformationally restricted retinoids lacking this flexibility should only be able to activate one of the heterodimer partners. To test this hypothesis, we investigated whether RAR- and RXR-selective compounds could cooperate in the activation of an RARE.

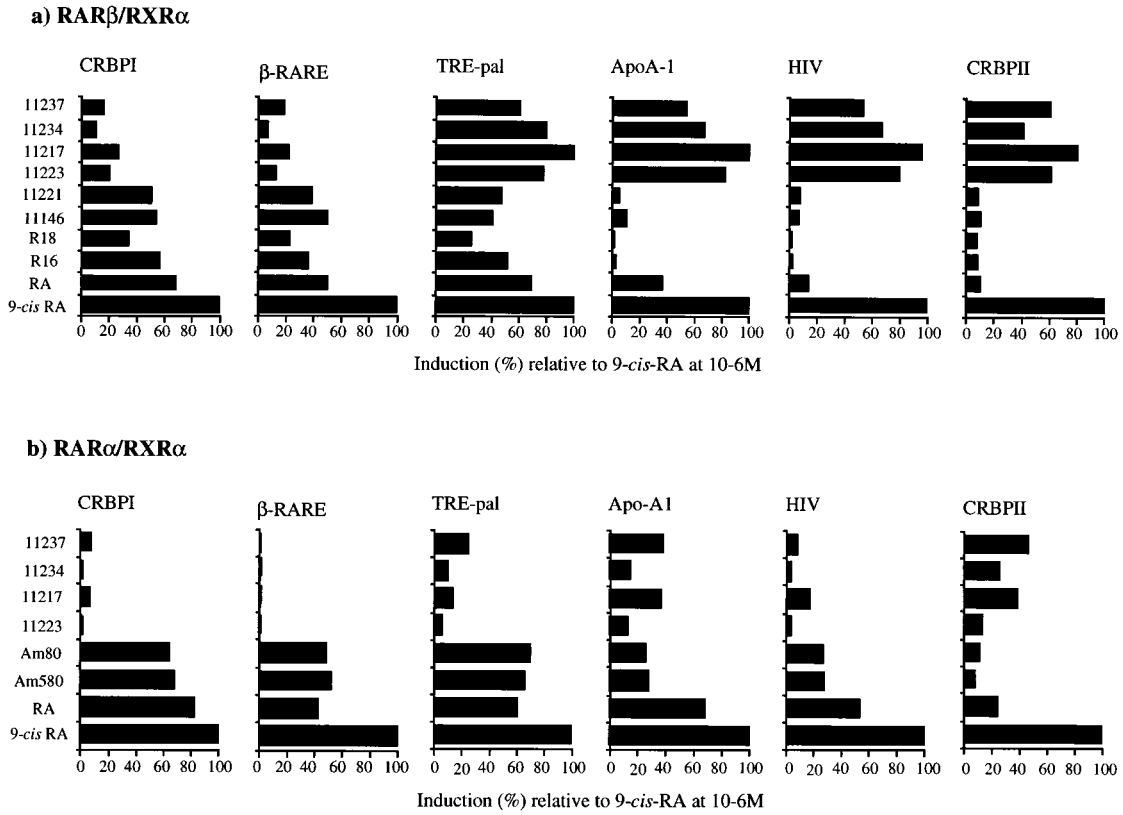

FIG. 5. Differential responses to conformationally restricted retinoids. Transcriptional responses obtained in the presence of RARβ-RXRα (a) and RARα-RXRα (b) with the indicated synthetic retinoids (at 10^{-6} M) on CRBPI-RARE, β-RARE, TREpal, ApoA1-RARE, HIV-RARE, and CRBPII-RARE are shown. Retinoid-induced reporter activation is expressed as a percentage of maximal activation obtained with 10^{-6} M 9-*cis*-RA for each response element after constitutive receptor activity was subtracted. The data represent the averages of two or three experiments with a standard deviation of less than 20%.

Clear, increased effects were observed on the RARβ-RXR as well as the RARα-RXRα heterodimers after RAR-selective compounds were combined with RXR-selective retinoids. For example, RAR-selective compound R18 strongly enhances the effect of RXR-selective retinoid SR11237 on the RARβ-RXRα heterodimer so that the activity was comparable (88%) to that of 9-*cis*-RA on TREpal (Fig. 6). A synergistic effect between Am580 and SR11237 on the RARα-RXRα heterodimer was also observed. In contrast, combining two similar compounds—such as Am580 and Am80, SR11237 and SR11234, or R18 and R16—produced no significant increase in activity on either one of the two heterodimers investigated (Fig. 6). When we analyzed the cooperativity of RAR- and RXR-selective compounds on different RAREs, we observed that the degree of cooperativity depended on the response elements as well as on the receptor heterodimer (Fig. 7). Our results therefore demonstrate that both RAR and RXR contribute to transcriptional activation, although binding of the ligand to either RAR or RXR may be the primary requirement, depending on the response element and the receptor subtypes.

DISCUSSION

We analyzed in detail several molecular components that could determine the retinoid signal response. We were particularly interested in whether the various receptor subtypes, in combination with structurally distinct response elements, could constitute a system that enhances differential ligand responsiveness. Our data point to novel concepts for signal transduction by nuclear receptors.

It is well established that tRA and related natural and synthetic retinoids induce pleiotropic biological responses. The specific responses can vary widely, depending on the concentration of the retinoid used. A striking example of this, for instance, is the observation that the concentration of tRA can determine the differentiated cell types formed by a teratocarcinoma cell line (17). This clearly implies that different cellular programs are activated at different RA concentrations. It has previously been shown that activation of retinoid-responsive promoters by different receptors can vary (27, 31, 56). It has also become apparent that although the amino acid sequences of the ligand binding domains of RARα, -β, and -γ are highly homologous, synthetic retinoids that distinguish among these receptors can be defined (6, 15, 23, 39, 47).

We analyzed here additional parameters that can influence the retinoid response. Taking into consideration that the individual retinoid receptors are most likely to function in the context of RAR-RXR heterodimers bound to various RAREs, we compared the degrees of responsiveness of the various possible heterodimers in the presence of structurally distinct RAREs. We observed that at a given tRA or 9-*cis*-RA concentration, the various RAR-RXR heterodimer complexes showed distinct activation patterns with the different RAREs. After we analyzed in detail the levels of responsiveness of two heterodimers—RARα-RXRα and RARβ-RXRα—on a series of different RAREs, we found that the amount of ligand re-

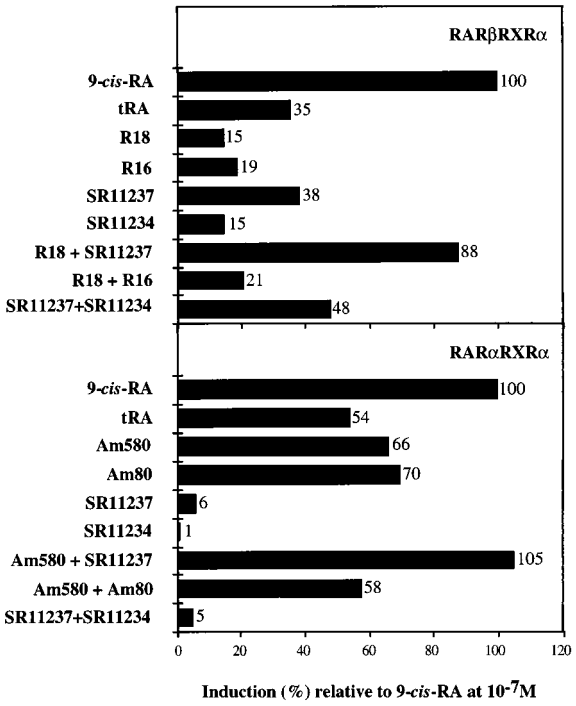

FIG. 6. Cooperation of RAR- and RXR-selective retinoids in the activation of heterodimers. RARβ-RXRα and RARα-RXRα activities on the TREpal reporter in the presence of RAR-selective (R18 and R16) and RXR-selective (SR11237 and SR11234) retinoids (at 10^{-7} M each) and combinations of both were determined by transient transfection assays. Results are expressed as percentages of the inductions relative to that obtained with 10^{-7} M 9-*cis*-RA for each RARE.

quired to observe half-maximum activation of a specific heterodimer can vary greatly.

An alteration of the affinity for the receptor ligand in the RAR-RXR heterodimer response element complex appears to be at least partially responsible for the differences of sensitivity observed among RAREs in the presence of the same concentrations of ligand. Therefore, the precise structure of the response element appears to determine the conformation of the receptor complex and modulate the affinity of the ligand binding domain for a retinoid. We cannot exclude the contribution of other events, such as the conformational changes of the domains involved in the interaction of activators or inhibitors of RAR such as the nuclear corepressor N-CoR or the p140 and p160 proteins, which were recently proposed to play a role in the modulation of RAR and RXR activity (37).

Furthermore (in particular, in the case of tRA), the response curves of the heterodimer-RARE combinations stretch over 3 orders of magnitude. For instance, in the case of the RARβ-RXRα heterodimer–CRBPI-RARE complex, clear activation is seen at 10^{-9} M tRA, while full activation is only seen at 10^{-6} M. The response curves are therefore unlikely to result from simple bimolecular interactions but rather from a multiple-component system, most likely the heterodimer-DNA complex. In the absence of DNA, RARs and RXRs are known to have largely different affinities for tRA. The response curves for tRA appear to reflect this fact and suggest that in particular in the presence of some response elements—for instance, the RARβ-RXRα–ApoA1-RARE complex—the RXR partner contributes very significantly to the total activation seen at elevated tRA concentrations (10^{-6} M and above) (Fig. 3a). It is known that tRA can isomerize to 9-*cis*-RA, the high-affinity ligand for RXR (1, 25, 45). Whether sufficient conversion of tRA to 9-*cis*-RA occurs under our assay conditions or whether tRA at high concentrations can itself be an activator of the RXR component in the heterodimer has not been determined.

Because of their long polyene side chains, natural retinoids tRA and 9-*cis*-RA are very flexible molecules that can potentially adopt various conformations to suit the geometrical requirements of a ligand binding pocket. Using conformationally restricted synthetic retinoids that either activate only RARs or RXRs, we were able to demonstrate that both the RAR and RXR components in the complex are, at least in some cases, required for a full response. Certain heterodimers, particularly RARβ-RXRα, as shown here, display broader differences in sensitivity to natural ligands after being bound to structurally distinct RAREs than other heterodimers. Conformationally restricted retinoids can be used to further specify the retinoid responses of particular genes, since these retinoids, in contrast to tRA and 9-*cis*-RA, activate a given heterodimer with an increased RARE selectivity. Interestingly, activation of a given RARE by a particular RAR-RXR heterodimer can be more sensitive to the activation of the RAR or RXR partner. Such response elements as CRBPI-RARE and β-RARE allow activation by RAR, while such response elements as ApoA1-RARE, HIV-RARE, and CRBPII-RARE require mostly activation through the RXR component. Although these latter RAREs have been proposed to represent RXR response elements (53, 62), it is unlikely that the activities observed here in the presence of both RAR and RXR are due to RXR homodimer activity (with the possible exception of that on CRBPII-RARE [Fig. 2]). Indeed, it has been shown that in the presence of both receptor species, formation of heterodimers occurs preferentially in vivo (55) and in vitro (36), even in the presence of 9-*cis*-RA, which induces RXR homodimers (40, 77).

The mechanism underlying the nature of the RAR or RXR sensitivity of a given response element is not clear. It may be related to the binding polarity of the heterodimer on the RARE. It is now well established that the binding of an RAR-RXR heterodimer occurs with a given polarity, with RXR usually being positioned on the 5′ half-site and RAR being positioned on the 3′ half-site (37a, 50, 61, 75). Our data are consistent with these reports in the cases of β-RARE and CRBPI-RARE, since the 3′-located receptor, RARα, controls the ligand response. Activation through the 5′-located receptor (RXR) does not lead to a significant activation or requires higher concentrations of ligands. The fact that the RARβ-RXRα heterodimer allows activation from ApoA1-RARE, HIV-RARE, and CRBPII-RARE in the presence of such RXR-specific ligands as SR11237 and SR11234 could indicate that the orientations of the two receptors on these RAREs are inverted, with RXR being bound to the 3′ half-site and RAR being bound to the 5′ half-site. Indeed, it has been suggested that an inverted polarity may occur on DR-1 and DR-2 elements (36, 75). However, much less activation on these RAREs was observed in the presence of the RARα-RXRα heterodimer. Therefore, RARα and RARβ differ significantly in their control of heterodimer activity. RARα inhibits activation of the heterodimer by RXR-selective compounds, while RARβ is more permissive and allows activation of the heterodimer by RXR-selective compounds on several of the RAREs. Thus, only some of our data are consistent with the published observations that an RAR-RXR heterodimer binds in the predicted inverse polarity on the DR-1 CRBPII-RARE and that binding of RAR to the 5′ half-site prevents interaction of the RXRα partner with its ligand (37a, 75). Our data also show that the parameters that allow RARα to inhibit the interaction between RXRα and its ligand are specific to RARα

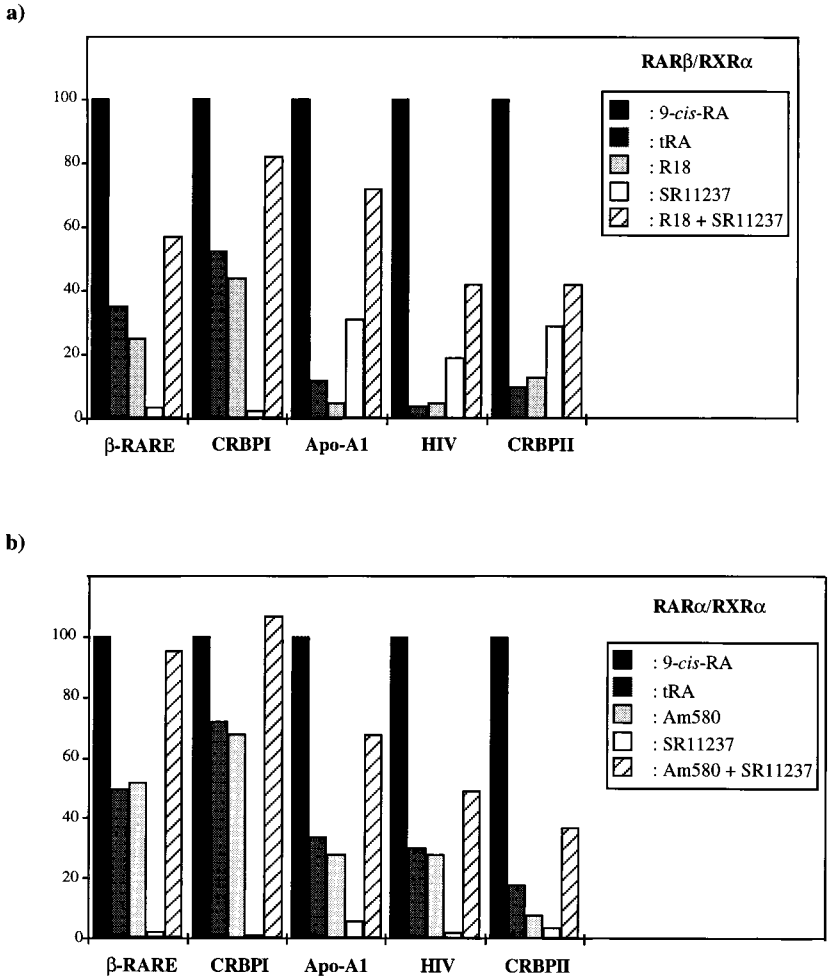

FIG. 7. Comparison of synergistic ligand responses on various RAREs. Activations of the RARβ-RXRα (a) and RARα-RXRα (b) heterodimers on β-RARE, CRBPI-RARE, ApoA1-RARE, HIV-RARE, and CRBPII-RARE in the presence of RAR- and/or RXR-selective retinoids (at 10^{-7} M each) were compared by transient transfection assays. R18 and Am580 are RAR-selective retinoids, while SR11237 is an RXR-selective compound.

and do not apply to RARβ. The molecular basis for this difference between the two RAR subtypes and the diverse ligand sensitivities of the different RAREs remains to be established. It is very likely that the ligand binding domains of the receptors contribute to most of the selective effects observed here, since these domains couple ligand binding to dimerization and transactivation. However, we have previously observed that the two heterodimers induce different DNA bending in the presence and absence of a ligand (49). This observation clearly suggests not only that the conformation of the ligand binding domains can differ but that the structure of the overall receptor-DNA complex may be distinct for each heterodimer-DNA complex. The role of the ligand binding domains is further emphasized by our observation that strong synergism can occur between RAR- and RXR-selective ligands, which demonstrates also that for the full activation of most heterodimers, both of the partners require ligand binding. This cooperative effect is observed for both the RARα-RXRα heterodimer and the RARβ-RXRα heterodimer. This cooperativity, which can be negatively dominated by the RAR or RXR partner, is likely to result in a gradual induction system that does not overeact to small changes in ligand concentrations.

Limiting the large variety of retinoid responses to specific targets has been a major goal in the development of retinoid as therapeutica in the last two decades. We show here that synthetic retinoids with restricted conformations can be highly selective for certain receptor-RARE combinations and could serve as gene-selective activators. Because of their limited biological activity, such compounds could have many fewer side effects than the flexible natural retinoids with their broad-spectrum activities. Such gene-selective retinoids could therefore result in the activation of new or different combinations of genes and thereby evoke very different biological responses that may increase activities against certain cancer cells and/or have fewer side effects, as has been observed with certain synthetic retinoids. It is likely that natural retinoids with highly selective activities also exist. A number of active natural retinoids associated with specific tissues and/or developmental stages have been reported in recent years (60, 70). A more detailed analysis of their activities in the context of specific receptor-DNA complexes may reveal more data on their selective roles.

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