Retinoid-dependent in vitro transcription mediated by the RXR/RAR heterodimer

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The effects of retinoids on gene regulation are mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Here, we provide the first biochemical evidence that, in vitro, ligand governs the transcriptional activity of RXR α /RAR α by inducing conformational changes in the ligand-binding domains. Using limited proteolytic digestion we show that binding of the cognate ligand causes a conformational change in the carboxy-terminal part of the receptor. We also show that recombinant RXR α /RAR α is partially active in the absence of exogenously added ligand. *Trans*-activation depends critically on the ligand-dependent transcriptional activation function AF-2 of RAR α . Full activation by recombinant RXR α /RAR α , however, requires the addition of either all-*trans* RA, 9-*cis* RA, or other RAR-specific agonists, whereas an RAR α -specific antagonist abolishes *trans*-activation. Intriguingly, the ligand-dependent AF-2 of RXR does not contribute to the level of transcription from the RAR β_2 promoter in vitro even when the cognate ligand (9-*cis* RA) is bound. Thus, the major role of RXR in *trans*-activation of the RAR β_2 promoter is to serve as an auxiliary factor required for the binding of RAR which, in turn, is directly responsible for transcriptional activity.

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Retinoids play an important role in development, differentiation, and homeostasis (De Luca 1991; Leid et al. 1992a). Two classes of nuclear retinoid-binding proteins have been implicated: retinoic acid and retinoid X receptors (RAR and RXR, respectively), which belong to the nuclear receptor superfamily of ligand-inducible transcription factors. RAR and RXR (each consisting of three subtypes α , β , and γ) belong to the type II subfamily, which includes the receptors for thyroid hormone (T₃R), vitamin D₃ (VD₃R), and the peroxisome proliferator-activated receptor (PPAR) (for review, see Stunnenberg 1993). With the possible exception of RXR, type II receptors do not bind to their cognate binding sites as homodimers but require RXR for high-affinity binding (Yu et al. 1991; Bugge et al. 1992; Kliewer et al. 1992a,b; Leid et al. 1992b; Marks et al. 1992; Zhang et al. 1992). Type II receptors activate transcription through binding sites composed of closely related motifs arranged as direct repeats spaced by 1–5 nucleotides, termed DR+1 to DR+5 elements. RXR/RAR bind and *trans*-activate preferentially through DR+5, DR+2 and, to a much lesser extent, DR+1 elements (for review, see Leid et al. 1992a; Stunnenberg 1993, and references therein). RXR homodimers have been reported to bind and *trans*-activate through the DR+1 element (Mangelsdorf et al. 1991; Zhang et al. 1992).

Similar to other members of the nuclear receptor superfamily, RAR and RXR exhibit a modular structure composed of three distinct domains: an amino-terminal domain containing a constitutive *trans*-activation function (AF-1) (Folkers et al. 1993; Nagpal et al. 1993), a central DNA-binding domain that also encompasses determinants for polarity in binding of the heterodimer to direct repeat elements (Kurokawa et al. 1993; Perlmann et al. 1993; Zechel et al. 1994a,b), and a carboxy-terminal domain comprising a ligand-binding dimerization and ligand-dependent *trans*-activation function (AF-2) (for review, see Leid et al. 1992a; Stunnenberg 1993). The relative contributions of the individual AFs of RAR and RXR in *trans*-activation vary according to the nature of the RA response element (RARE) and its promoter context (Durand et al. 1992; Nagpal et al. 1992).

The transcriptional activity of the ligand-dependent AF-2 in the carboxy-terminal part of RAR and RXR is thought to be governed by the ligand. All-*trans* and 9-*cis* retinoic acid (T-RA and 9C-RA) bind to RAR and induce a conformational change in its ligand-binding domain (LBD), whereas RXR only binds 9C-RA (Heyman et al. 1992; Levin et al. 1992) and likewise induces a conformational change (this study; Keidel et al. 1994; Leid 1994). An RAR α -specific antagonist has been shown to induce a conformational change in RAR α (Keidel et al. 1994) different from that obtained with agonist, probably resulting in an impaired ability of the antagonist-loaded

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receptor to interact with other molecules, such as cofactors or components of the basal transcription machinery.

The dissection of regulatory circuits by cotransfer experiments is complicated by the presence of multiple retinoid receptor isoforms (Leid et al. 1992a), some of which appear to be expressed ubiquitously, and the utilization of different response elements. Furthermore, RXR can heterodimerize with a number of different receptors other than RAR, such as T_3R , VD_3R , and PPAR, and as such can bind and *trans*-activate through response elements that are identical or very similar to RAREs. Finally, there is a high degree of redundancy in the retinoid signaling pathway, as revealed by gene-targeting experiments (Chambon 1994). Hence, it is extremely difficult to attribute a particular effect to a distinct receptor isoform.

Therefore, a ligand-dependent *in vitro* transcription assay would be extremely useful, not only in studying the molecular mechanisms underlying ligand-induced *trans*-activation but also in identifying cofactors that are required for gene activation. Here, we describe an *in vitro* transcription system that supports retinoid-dependent *trans*-activation from a promoter fragment derived from the human $RAR\beta_2$ gene. *In vivo*, the expression of this gene is up-regulated in response to RA through a RARE (DR + 5) located in close proximity to the TATA box (De Thé et al. 1990; Sucov et al. 1990; Vivanco Ruiz et al. 1991). We have assessed the *trans*-activating abilities of recombinant $RAR\alpha$ and $RXR\alpha$ coexpressed and copurified as a heterodimer. Finally, we have analyzed the effects of various retinoids and synthetic agonists and antagonists in this *trans*-activation assay.

Results

Expression and purification of $RAR\alpha$ and $RXR\alpha$

Human $RAR\alpha$ and $RXR\alpha$ and truncated versions thereof were expressed in HeLa cells using recombinant vaccinia

virus encoding either the authentic receptor or a fusion protein containing a stretch of six histidines fused to the amino terminus (abbreviated 6H). $RXR\alpha/6H-RAR\alpha$ heterodimer prepared from HeLa cells infected with a mixture of two viruses was purified through Ni^{2+} -NTA column chromatography as described (Janknecht et al. 1991; Schmitt et al. 1993). The receptor purifications were analyzed by silver staining, Western blot analysis, and bandshift assays. Shown in Figure 1 is a silver-staining and Western blot analysis of $RXR\alpha/6H-RAR\alpha$ purified over Ni^{2+} -NTA. 6H- $RAR\alpha$ and $RXR\alpha$ are present in roughly stoichiometric amounts in the heterodimer preparation, as revealed by bandshift analysis using a $RARE\beta_2$ oligonucleotide (data not shown). The polyclonal antibodies were raised against the D and E domains of $RAR\alpha$ and $RXR\alpha$ expressed in *Escherichia coli*. The two polypeptides revealed by the RAR-specific antibody (Fig. 1, lanes 6,7) represent different phosphorylation forms of 6H- $RAR\alpha$ (C.G. Jiménez and G. de Martiño, unpubl.).

Ligand induces a conformational change in $RAR\alpha$ and $RXR\alpha$

To analyze the ligand-binding properties of vaccinia virus-expressed $RAR\alpha$ and $RXR\alpha$, we performed protease clipping experiments. Binding of the cognate ligand induces a conformational change in the LBD that can be monitored by limited protease digestion (Allan et al. 1992; Keidel et al. 1994). The proteolytic fragmentation patterns of NTA-purified receptors were revealed by Western blotting using polyclonal antibodies raised against the respective LBDs. Digestion of coexpressed and copurified $RXR\alpha/6H-RAR\alpha$ (Fig. 2A, lanes 2–6) or 6H- $RAR\alpha$ alone (data not shown) yielded a small amount of an ~30-kD $RAR\alpha$ -derived polypeptide that is resistant to trypsin digestion in the absence of exogenously added ligand. This trypsin-resistant fragment could not be de-

Figure 1. Analysis of the Ni^{2+} -NTA-purified $RXR\alpha/6H-RAR\alpha$. Silver staining (lanes 1–4) and Western blot analysis (lanes 5–10) of the Ni^{2+} -NTA purified heterodimer are shown. (Lanes 1,5,8) Molecular mass markers (M). The positions at which $RXR\alpha$ and 6H- $RAR\alpha$ migrate and the molecular mass of the markers are indicated. The amount of heterodimer is indicated in nanograms. Lanes 5–7 were developed with an $RAR\alpha$ antibody (raised against the $RAR\alpha$ DE domain); lanes 8–10 were developed with an $RXR\alpha$ antibody (raised against the $RXR\alpha$ DE domain), using alkaline phosphatase-linked anti-rabbit immunoglobulin as the second antibody.

Figure 2. Retinoids induce a conformational change in the ligand-binding domain of RAR α and RXR α . The amount of heterodimer used was 1.2 μ g/reaction. The amount of trypsin (μ g/ml) and the concentration of the ligands (μ M) are indicated. The sizes of molecular mass markers are indicated at *right*. (A) RXR α /6H-RAR α was incubated with trypsin at the indicated concentrations, in the presence of T-RA, 9C-RA, or solvent alone (2.5% ethanol), and the proteolytic products were analyzed by Western blotting with a polyclonal antibody raised against the ligand-binding domain of RAR α . The peptide resistant to trypsin cleavage is indicated with an arrow. (B) 6H-RXR α was incubated with trypsin in the presence of 9C-RA or solvent alone (2.5% DMSO), and the proteolytic products were analyzed by Western blotting with a polyclonal antibody raised against the ligand-binding domain of RXR α . The bracket at *right* indicates resistant peptides.

tected with RXR α /6H-RAR α or 6H-RAR α produced in reticulocyte lysates in the absence of ligand (Keidel et al. 1994; data not shown). Addition of increasing amounts of either T-RA or 9C-RA resulted in proportional increases in the amount of the 30-kD polypeptide refractory to trypsin digestion (Fig. 2A, lanes 8–13, 15–20). Virtually quantitative protection could be obtained at 10 μ M T-RA and 1 μ M 9C-RA (Fig. 2A, cf. lanes 7 and 14 with lanes 13 and 20).

Vaccinia virus-expressed 6H-RXR α was sensitive to trypsin digestion in the absence of 9C-RA or in the presence of T-RA (Fig. 2B, lanes 1–3; data not shown) but refractory to digestion in the presence 9C-RA. A number of resistant polypeptides ranging in molecular mass between 27 and 35 kD (Fig. 2B, lanes 4–6) could be detected. However, RXR α produced in reticulocyte lysates is refractory to trypsin digestion even in the absence of ligand (Keidel et al. 1994; Leid 1994). Chymotrypsin digestion of vaccinia virus-expressed RXR α /6H-RAR α or 6H-RXR α alone yielded results similar to those reported previously using RXR α produced in reticulocyte lysates, that is, RXR α became refractory to protease digestion proportional to the amount of added ligand reaching maximum levels at 1 μ M 9C-RA (Keidel et al. 1994; data not shown). In contrast, a recent study reported that the 9C-RA-induced conformational changes of *in vitro*-translated RXR α could not be monitored with chymotrypsin but only with subtilisin (Leid 1994).

We conclude that vaccinia virus-expressed 6H-RAR α as well as 6H-RXR α are able to bind T-RA (RAR) and 9C-RA (RAR and RXR) because they undergo ligand-dependent conformational changes. Our data also show that a small percentage of recombinant RAR α had already undergone this conformational change.

RXR/RAR-dependent transcription

The natural, RA-responsive RAR β_2 promoter from –124 to –21, including the RARE and the TATA-box (De Thé et al. 1990) fused to a G-less cassette of 380 nucleotides, was used as the reporter (Berkenstam et al. 1992). As an internal control, a template comprising the adenovirus major late TATA box initiator fused to a G-less cassette of 320 nucleotides (TI) (Pugh and Tjian 1990) was used. After completion of the transcription reaction a labeled DNA fragment was added to monitor the recovery during sample preparation. These controls, internal control plasmid and recovery spike, allow us to draw conclusions from the results of an experiment without having to rely on cross-experiment comparisons. Addition of increasing amounts of coexpressed and copurified RXR α /6H-RAR α resulted in a >10-fold enhancement of the level of transcription from the RAR β_2 reporter, without affecting transcription from the TI template (Fig. 3A). The enhancement in the level of transcription was not attributable to contamination in the receptor prepara-

Figure 3. RXRα/6H-RARα-dependent *trans*-activation *in vitro*. The amount of heterodimer is indicated in nanograms. TI and RARβ₂ indicate the positions of transcripts from the internal control and test template, respectively. Fifty ng of TI and 25 ng of RARβ₂ plasmid were used per reaction. The recovery control is marked with an asterisk (*). (A) Addition of the NTA-purified heterodimer enhances the level of transcription *in vitro*. (B) *Trans*-activation is RARE dependent. A schematic diagram of the different test templates is shown. (C) Effect of different TATA box mutations on the level of receptor-dependent *trans*-activation.

tion because control extracts derived from wild-type vaccinia virus-infected or mock-infected cells did not boost the level of transcription, either from the RARβ₂ promoter or from the TI template (data not shown).

To analyze whether the receptor-dependent *trans*-activation was mediated by the RARE, a minimal promoter fragment spanning the region from -59 to -21, containing only the consensus RARE and TATA box, was constructed. This minimal promoter mediated RXR/RAR-dependent *trans*-activation, albeit at a reduced level (Fig. 3B, cf. lanes 2 and 4). Mutations introduced in either one of the consensus half-site motifs in the context of this minimal promoter fragment (m7-ups and m3-ups) reduced the RXR/RAR-dependent *trans*-activation even further (Fig. 3B, lane 8; data not shown). In the context of

the longer promoter fragment a mutation introduced in the proposed RAR-specific half-site (Perlmann et al. 1993; Zechel et al. 1994a,b) of the consensus RARE at position -53 to -37 (reporter m7) did not affect the level of transcription. Introducing a mutation in the RXR-specific half-site (reporter m3) caused a diminution but not abolition of the level of transcription (Fig. 3B, cf. lanes 2, 6, and 10). Inspection of the DNA sequence revealed a potential, nonconsensus RARE located farther upstream (position -83 to -67). Mutations introduced into either one or both of the half-sites of this upstream potential RARE reduced the level of transcription to that obtained with the minimal promoter fragment, indicating that this imperfect RARE contributed to, but was not essential for, RXR/RAR-dependent transcription *in vitro*.

(data not shown). Transient transfection experiments showed that this element was not essential for, but potentiated, the RA response, and it was demonstrated to bind RXR α /RAR α in vitro (B. Leblanc and H.G. Stunnenberg, in prep.).

We also tested the importance of the TATA box in receptor-dependent in vitro transcription. Mutations in the wild-type RAR β_2 TATA box -TATATTA- that are known to abolish or diminish TBP/TFIID binding, namely -TGTA AAA- (Strubin and Struhl 1992) and -TAGAGAA- (Berkenstam et al. 1992), abolished in vitro transcription (Fig. 3C, lanes 3–6). Replacement of the RAR β_2 TATA box with the TATA box from the adenovirus major late promoter (AdMLP) -TATAAAA- resulted in a marked increase in the basal level of transcription, whereas receptor-dependent enhancement of transcription was minimal (Fig. 3C, lanes 7,8). We surmise that in vitro binding of TFIID to the strong AdMLP TATA box is sufficient to yield high levels of transcription. In contrast, binding of the receptor is required for efficient preinitiation complex formation on the RAR β_2 TATA box both in vitro (Fig. 3) and in vivo (Berkenstam et al. 1992). Taken together, these experiments document bona fide receptor-dependent *trans*-activation from the RAR β_2 promoter.

The ligand-dependent AF-2 of RAR α is essential for *trans*-activation

The above documented activation of transcription by the heterodimer was obtained in the absence of exogenously added ligands. This raised the possibility that the enhancement of the level of transcription was attributable to the constitutive, ligand-independent amino-terminal AF-1 of RAR and/or RXR. Therefore, RXR α and RAR α mutants with truncated carboxy-terminal domains that abolish ligand binding were generated to assess whether

the observed *trans*-activation was mediated by the AF1 or AF-2, or both (Fig. 4). Full-length RXR α coexpressed and copurified with 6H-RAR α truncated at amino acid position 419 deleting the F-domain (6H-RAR α 419), resulted in a level of transcription comparable to RXR α /6H-RAR α (Fig. 4, cf. lanes 2 and 3). However, a RAR α truncation up to position 390 (6H-RAR α 390) deleting the putative AF-2 completely abolished *trans*-activation (lane 4). In contrast, deleting the putative AF-2 of RXR α (RXR α 445) had no effect on the level of transcription (cf. lanes 2 and 5). Bandshift assays revealed that the truncated receptors were not affected in their ability to bind to the RARE β_2 (data not shown). Thus, the ligand-dependent *trans*-activation domain of RAR α , but not RXR α , is essential for *trans*-activation of the RAR β_2 promoter in vitro.

Ligand-dependent activation

The above results demonstrate that the ligand-dependent AF-2 of RAR α is required for *trans*-activation and that recombinant 6H-RAR α and 6H-RXR α are able to bind their cognate ligand. The experiments depicted in Figure 2 showed that a small proportion of vaccinia virus-expressed RAR α was resistant to limited proteolytic digestion, suggesting that this receptor subpopulation had already undergone the conformational change and, consequently, may have been active transcriptionally. Therefore, we undertook experiments to determine whether we could either reverse the activated state or load the remainder presumably unliganded, inactive receptor population with ligand in vitro. NTA-purified RXR α /6H-RAR α was incubated in the presence of solvent or ligand at room temperature, and subsequently, the pretreated heterodimer was added to the transcription reaction. Pretreatment of the heterodimer in the presence of 10 μ M T-RA strongly boosted the level of

Figure 4. Receptor domain requirements for *trans*-activation in vitro. A schematic diagram of the RXR α and RAR α deletion mutants is shown. DNA affinity-purified receptors were used in this assay (see Materials and methods). The amount of heterodimer added to the reactions was normalized by Western blotting and bandshift assays, and 200 ng of heterodimer was added to each reaction. Amounts of template were 10 ng of TI and 25 ng of RAR β_2 per reaction.

transcription as compared with untreated heterodimer or pretreated in the presence of 10% dimethylsulfoxide (DMSO) (Fig. 5A, cf. lanes 1–6 with 7–9). Bandshift assays revealed that the ligand or solvent pretreatment did not affect the ability of the heterodimer to bind the RARE β_2 oligonucleotide (Fig. 5B). We conclude from this that T-RA governs the transcriptional activity of RXR α /6H-RAR α .

The experiments described above (see Fig. 4) suggest that only the ligand-dependent AF-2 domain of RAR α is required for *trans*-activation of the RAR β_2 promoter and that the AF-2 of RXR α is not involved in *trans*-activation. A possible caveat is that the initial experiments were performed in the absence of the ligand for RXR α . Furthermore, the LBD of RXR α was sensitive to limited protease digestion, suggesting that RXR α was not in an activated status as observed with RAR α . Therefore, we tested whether RXR α might contribute to the level of transcription when loaded with its respective ligand 9C-RA (Heyman et al. 1992; Levin et al. 1992). RXR α /6H-RAR α pretreated with 9C-RA elicited a level of *trans*-activation indistinguishable from that obtained with T-RA pretreatment but significantly higher than that observed with untreated or DMSO-treated heterodimer (Fig. 6, lanes 2–12). A 10-fold lower concentration of 9C-RA as compared with T-RA was required to obtain maximal *trans*-activation of the RAR β_2 promoter in line with the results obtained with limited protease digestion experiments (see Fig. 2A). Further experiments clearly showed that the contribution of ligand-activated RXR α

AF-2 was not simply masked in our *in vitro* transcription experiments by a strong AF-2 of RAR α . The RXR α /6H-RAR α 390 heterodimer lacking the AF-2 of RAR α was unable to elicit *trans*-activation even after pretreatment with 9C-RA (data not shown). Furthermore, specific activation of the RAR α AF-2 in the heterodimer by the RAR α -subtype-specific or RAR-specific synthetic agonists Ro 40-6055 and Ro 13-7410, respectively, elicited levels of transcription indistinguishable from those obtained with 9C-RA and T-RA (Fig. 6, lanes 13–19). The maximal level of *trans*-activation was already achieved at 400 nM of the synthetic agonist Ro 13-7410, although RAR α has a threefold lower affinity for this analog as compared with T-RA or 9C-RA (Apfel et al. 1992).

An RAR α -specific antagonist abolishes *trans*-activation

To strengthen our conclusion that *in vitro* transcription of the RAR β_2 promoter is ligand dependent, we used the RAR α -specific antagonist Ro 41-5253 (Apfel et al. 1992). This compound has also been shown to induce a conformation distinct from that obtained with agonists (Keidel et al. 1994; data not shown). Pretreatment of the heterodimer with Ro 41-5253 abolished receptor-dependent *trans*-activation at 1 μ M Ro 41-5253 (Fig. 7A, cf. lane 2 with lanes 4–6). The concentrations of the antagonist used in these experiments did not affect the ability of the heterodimer to bind to RARE β_2 (Fig. 7B). We conclude that the antagonist induced a conformational change in the LBD of RAR α distinct from that obtained with ago-

Figure 5. T-RA increases the *trans*-activation mediated by the heterodimer. (A) T-RA effect on *trans*-activation *in vitro*. Amounts of template were 50 ng of TI and 25 ng of RAR β_2 per reaction. The amount of heterodimer is indicated in nanograms. (B) Effect of the T-RA in DNA binding. A 32 P-labeled RARE β_2 oligonucleotide was used as a probe. The amount of heterodimer used is indicated in nanograms.

nists and that this altered conformation of RAR α prevented *trans*-activation of the RAR β_2 promoter by the RXR α /6H-RAR α heterodimer in vitro.

Discussion

We have reconstituted ligand-dependent transcription by

RXR α /6H-RAR α from the natural RAR β_2 promoter in a cell-free system. The extracts used in our *in vitro* transcription assays consist of HeLa nuclear extract devoid of the phosphocellulose D-fraction complemented with a TFIID fraction from 293 cells (Graham and van der Eb 1973). RXR α /6H-RAR α heterodimers are expressed in vaccinia virus-infected HeLa cells and highly enriched

by Ni^{2+} -NTA chromatography. At present, it cannot be excluded that the heterodimer preparation is "contaminated" with a putative coactivator as the heterodimer is produced in mammalian cells and not in bacteria. This could be attributable to sequestration of such a factor by the heterodimer. Alternatively, several factors have been cloned that contain a stretch of histidines and have an intrinsic ability to bind to the Ni^{2+} -NTA column [e.g., TFIIA α/β (DeJong and Roeder 1993; Ma et al. 1993) and YY1 (Shi et al. 1991)]. However, such putative coactivators would be present in our HeLa nuclear extracts. Furthermore, control extracts derived from wild-type vaccinia virus-infected or mock-infected cells did not boost the level of transcription either from the RAR β_2 promoter or from the TI template.

Receptor-dependent *trans*-activation could be obtained with a promoter fragment comprising only the proximal RARE and TATA box. A cryptic, hitherto unidentified, RARE in the RAR β_2 promoter (position -83 to -67) boosted the level of transcription *in vitro* (Fig. 3B) and *in vivo* (B. Leblanc and H.G. Stunnenberg, in prep.). Pretreatment of the heterodimer with T-RA or 9C-RA boosted *trans*-activation to a level higher than that obtained with untreated or DMSO-treated heterodimer. Using truncated receptors, we could show that *trans*-activation by RXR α /6H-RAR α was fully dependent on an intact carboxy-terminal, ligand-dependent AF-2 of RAR α . This notion is corroborated and extended by the finding that maximal levels of transcription were obtained by pretreatment with RAR-specific agonists only (i.e., in the absence of a ligand-activated RXR). The RAR-specific agonists Ro 40-6055 and Ro 13-7410 boosted *trans*-activation of the RAR β_2 promoter to a level equivalent to that obtained with T-RA. Preincubation of the heterodimer with 9C-RA, which binds both RAR α and RXR α , did not yield a higher level of transcription as compared with T-RA or the RAR-specific agonists. The increased transcriptional activity of the heterodimer in response to agonist treatment parallels the increased resistance of RAR α to limited proteolysis (Fig. 2; Keidel et al. 1994), strongly suggesting that the ligand-induced conformational change in the LBD of RAR α exposes the transcriptional activation domain. Finally, incubation of the heterodimer with the RAR α -specific antagonist resulted in a complete loss of transcriptional activity. One possible caveat could be that agonist treatment would promote solubilization (renaturation) of a partially aggregated heterodimer resulting in an apparent increase in the level of transcription. Extending this argument, antagonist treatment would then promote aggregation (denaturation). If this is the case, agonist/antagonist treatment should result in an increased/decreased ability of the heterodimer to bind to the RARE β_2 oligonucleotide, which could not be observed. Furthermore, the conformational changes induced by both the agonist and the antagonist parallel the transcriptional activity of the receptors. Therefore, we conclude that ligand governs the transcriptional activity of recombinant RXR α /RAR α .

Taken together, the data suggest that *trans*-activation

of the RAR β_2 promoter by RXR α /6H-RAR α in our reconstituted cell-free system required the presence of the AF-2 activation function of RAR α and that the AF-2 of RXR α did not contribute detectably to the level of transcription *in vitro*. These findings are in keeping with our *in vivo* cotransfection data showing that RAR α 390, but not RXR α 445, acted as a dominant-negative receptor interfering with activation of the RAR β_2 promoter (D. Barretto, unpubl.). In line with our conclusions, it has been reported recently that *in vivo* a RXR-specific ligand had no effect on the transcriptional activation elicited by RXR α /RAR α , using a synthetic promoter that contains two DR5 elements, similar to the natural RAR β_2 promoter used in our study (Kurokawa et al. 1994). Thus, the major role of RXR α in *trans*-activation of the RAR β_2 promoter *in vitro* and *in vivo* is to serve as an auxiliary factor required for efficient DNA binding of RAR α to the RARE. The question arises whether RXR can act as a ligand-inducible transcription factor. If binding of the biologically active ligand 9C-RA to RXR yields a transcriptionally competent receptor, how can this be reconciled with its promiscuous role as an auxiliary factor required for *trans*-activation by RAR, thyroid hormone, vitamin D $_3$, and other type II receptors? Transcriptional activation of RXR would inevitably elicit a pleiotropic but devastating effect because of the simultaneous activation of signaling pathways controlled by ligands that are physiologically unrelated to 9C-RA such as thyroid hormone and vitamin D $_3$. In the case of the RA-inducible RAR β_2 promoter, a contribution of 9C-RA-activated RXR to the level of transcription could have been envisioned easily, yet *trans*-activation appears to depend solely on ligand-activated RAR.

To explain the supraphysiological concentrations of ligands required in this *in vitro* system, two points should be considered: First, the concentration of heterodimer during the loading of the ligand was about 3 μM . Although moderate *trans*-activation could be obtained after pretreatment at concentrations as low as 10 nM 9C-RA and 100 nM T-RA, maximal activation could only be achieved at 1 and 10 μM , respectively. Note that in the transcription reactions the final concentrations of the ligands required to obtain maximal activation were 30 and 300 nM for 9C-RA and T-RA, respectively. Second, *in vivo*, transport and delivery of retinoids are protein-mediated phenomena (Blomhoff et al. 1990) that may not have been mimicked in our *in vitro* system and that different ligands could have different accessibility to the receptors *in vitro*.

It should also be noted that the concentration of T-RA, 9C-RA, and the synthetic agonists required in the pretreatment to yield fully active receptor were not inversely proportional to the affinity of RAR α for that particular ligand. For example, although the dissociation constants for the binding of T-RA and 9C-RA to RAR α are equal (0.2 and 0.24 nM, respectively; Allenby et al. 1993), a 10-fold higher concentration of T-RA as compared with 9C-RA was required for full activation in our *in vitro* transcription assays. Using high pressure liquid chromatography separation of retinoids, we can exclude

that T-RA becomes substantially isomerized during the pretreatment step (T. Houthaeve and C.G. Jiménez, unpubl.). More striking is the fact that as little as 400 nM of the synthetic agonist Ro 13-7410 was sufficient to obtain full *trans*-activation, although Ro 13-7410 has been reported to bind with a lower affinity to RAR α as compared with T-RA (Keidel et al. 1994). The antagonist Ro 41-5253 is more effective in inhibiting *trans*-activation in our in vitro transcription system than in preventing activation of RA-responsive genes in vivo as compared with the other ligands (Keidel et al. 1994). Taken together, these data indicate that the binding affinity is not the only parameter determining the potency of a given agonist or antagonist in exerting its function.

We provide the first biochemical evidence that the transcriptional activity of RXR α /RAR α is governed by ligand and that this parallels with conformational changes in the receptors. Attempts to show similar effects with type I receptors in the past have yielded conflicting results. In vitro transcription assays using progesterone (PR) yielded ambiguous data. Receptor preparations from natural sources and loaded with the cognate ligand before extraction appeared to be somewhat more active in transcription assays than unliganded or anti-hormone-loaded receptors (Kalfi et al. 1990; Bagchi et al. 1992), and recombinant PR receptor produced from a baculovirus vector was reported to activate transcription in vitro in a ligand-dependent fashion (Elliston et al. 1992). It should be noted that PR requires ligand to bind DNA in vitro efficiently, which complicates an unambiguous assessment of the role of ligand in *trans*-activation (Bagchi et al. 1992). The level of transcription elicited by recombinant PR, glucocorticoid (GR), estrogen (ER), and androgen (AR) was shown to be unaffected by addition of the cognate agonist or antagonist (Klein-Hitpass et al. 1990; Tsai et al. 1990; Shemschedini et al. 1992; Brou et al. 1993; Schmitt and Stunnenberg 1993; De Vos et al. 1994).

Ligand effects have been reported for type II receptors. Bacterially expressed T₃R α was shown to repress transcription from the growth hormone gene and other thyroid hormone response element (TRE)-containing promoters in vitro, and this repression could be alleviated by addition of T₃ (Fondell et al. 1993). The level of transcription elicited by baculovirus-expressed T₃R α -RXR β heterodimer was reported to be mostly ligand independent, although a modest activation could be obtained with addition of T₃ that could not be further enhanced by 9-C RA (Lee et al. 1994). The latter result seems to be in line with our conclusion that the major role of RXR is to serve as an auxiliary factor.

Role of ligand

In light of the observation that *trans*-activation by RXR α /6H-RAR α is fully dependent on the ligand-dependent AF-2 domain of RAR α , it was surprising to find that recombinant heterodimer was partially active in the absence of exogenously added ligand. Because a small proportion of the recombinant receptor appeared to be resis-

tant to protease digestion, it is possible that the recombinant receptors are partially loaded with ligand notwithstanding the fact that virus-infected cells were incubated in the absence of serum to minimize exposure to retinoids. Relatively high amounts of ligand could, however, be present within the cell bound to cellular retinoic acid-binding proteins (CRABPs) (Blomhoff et al. 1990). Although this possibility cannot be excluded at present, we suggest the following model: In vivo, the ligand-binding domain of RAR is highly disordered in the absence of ligand and, hence, very sensitive to protease digestion. The *trans*-activation function is either not exposed on the surface of the ligand-binding domain or is masked by a repressor. At present, it cannot be ruled out that in vivo the RAR and RXR are monomeric in the absence of ligand and that ligand plays a role in dimerization and, hence, affects partner selection (Zhang et al. 1992; Thomas et al. 1993; Yao et al. 1993). In line with such a scenario is the finding by Ozato and co-workers that RXR/RAR is not occupying the RARE in the RAR β ₂ promoter in the absence of ligand as revealed by in vivo footprint experiments (Minucci et al. 1994). In vaccinia virus-infected cells, where recombinant RXR and RAR are made, the receptors might be in a "transition" state that is characterized by a more compact folding of the LBD and, hence, RXR and RAR form "stable" heterodimers. In this state, the AF-2 can display low transcriptional activity although the ligand pocket is still disordered in the absence of ligand. This transition state might be attributable to a viral infection-linked modification of the receptor such as phosphorylation.

Agonist treatment of vaccinia virus-expressed heterodimer induces a very compact folding by ordering residues in the ligand pocket and, consequently, the LBD is insensitive to protease digestion. Thus, the AF-2 in the LBD is in a fully transcriptionally active state, optimally displaying the surface for interaction with the basal transcriptional machinery or presumed coactivators. Addition of DMSO or ethanol in the pretreatment step might be important to obtain efficient ligand binding. These solvents may allow exposure of hydrophobic residues, thereby facilitating entry of the ligand into a hydrophobic pocket. Presumably, ligand-receptor interactions are of the "induced fit" type, as postulated for avidin-biotin, where residues that are disordered in the free protein are ordered with binding of biotin enclosing this ligand in a "hydrophobic box" (Livnah et al. 1993).

In previous studies we have postulated that RAR/RXR can activate the RAR β ₂ promoter by two different signaling routes: a "default" pathway used in differentiated cells and a "super-activation" pathway that seems to be restricted to embryonal cells. Superactivation can be mimicked in non-embryonic carcinoma (EC) cells by cotransfection of the TATA-binding protein (TBP), along with the adenovirus E1A₁₃₅ protein (Berkenstam et al. 1992; Keaveney et al. 1993). The extracts used in our in vitro transcription assays consist of a HeLa extract devoid of the phosphocellulose D-fraction complemented with a TFIID fraction from 293 cells that are transformed with E1A and E1B (Graham and van der Eb 1973). At

present, it is unclear whether *trans*-activation signaling in our cell-free system occurs by the default or superactivation pathway. Experiments are in progress to assess the potential role of E1A or the cellular E1A-like activity in the activation of the RAR β_2 promoter.

Materials and methods

Recombinant vaccinia viruses

Recombinant vaccinia viruses expressing 6H-RAR α , 6H-RXR α , and RXR α were prepared as described [Stunnenberg et al. 1988; Bugge et al. 1992; Schmitt and Stunnenberg 1993]. 6H-RAR α 419 and 6H-RAR α 390 were constructed by introducing stop codons at positions 420 and 391, respectively, using PCR. 6H-RXR α was obtained by subcloning the human RXR α cDNA into pMS56 [Janknecht et al. 1991]. The amino acids -MSHHH-HHHGEF- precede the second amino acid of human RXR α . RXR α 445 was constructed by inserting a PCR fragment with a stop codon at amino acid position 446 into pSG5. A fragment of RXR α 445 was then subcloned into a pATA-18-derived plasmid [Stunnenberg et al. 1988] to obtain pATA-RXR α 445.

Transcription templates

The RAR β_2 [380] G-less construct contains the RAR β_2 promoter from sequence -124 to -21 as described [Berkenstam et al. 1992] in front of a G-less cassette [Sawadogo and Sentenac 1990]. The Tl[320] G-less construct used as an internal control is described in Pugh and Tjian [1990]. The G-less reporter plasmids containing the TATA box mutations -TGTA AAA-, -TATAAAA-, and -TATATTA- were obtained by subcloning *Xba*I fragments, including the promoter from the M1, M2, and M4 mutants [Berkenstam et al. 1992; Keaveney et al. 1993] into the *EcoRV* site of the p(C₂AT)[380]-G-less vector [Monaci et al. 1988]. The promoter fragment differs from the natural RAR β_2 promoter also at position -32 (C $\rightarrow$ A). The m3, m7 [Vivanco Ruiz et al. 1991], and TAGAGAA [Berkenstam et al. 1992] mutants were used to obtain PCR fragments that were inserted subsequently into the *EcoRV* site of p(C₂AT)[380]-G-less vector to generate the m3-, m7-, and TAGAGAA-G-less templates. Mutations m3 and m7 affect the proximal RARE of the RAR β_2 promoter GGTTCACCGAAAGTTCA [position -53 to -51 to AAC and -41 to -39 to TCG, respectively]. The RAR β_2 Δ ups-m3-ups-, and m7-ups-G-less mutants were obtained by deleting the fragment from -124 to -59 corresponding to the natural RAR β_2 promoter.

Receptor purifications

Preparation of nuclear extracts from virus-infected cells was performed as described [Vivanco Ruiz et al. 1991]. Receptor purifications by Ni²⁺-NTA chromatography were done essentially as described [Schmitt et al. 1993], using buffer D [20 mM HEPES (pH 7.9), 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10 mM NaF, 0.1 mM PMSF, 20% glycerol, and 0.1% Triton X-100] supplemented with 100 mM NaCl. Western blotting using polyclonal antibodies directed against the LBD of RAR α and RXR α and mobility-shift assays, which were carried out as described previously [Bugge et al. 1992], were used to follow the purification of the receptors.

DNA affinity purification was performed as follows: A double-stranded DNA fragment containing the RARE (DR + 5) from the RAR β_2 promoter (position -60 to -32) extended with two biotinylated carboxyl residues was bound to streptavidin-coated paramagnetic beads as recommended by the manufacturer

(Dyna). Ni²⁺-NTA-purified receptors were added to the magnetic beads and incubated for 30 min at 4°C. The beads were washed twice with five volumes of buffer D50 (D with 50 mM NaCl) and twice with D250. Bound receptors were eluted with five bead volumes of D1000. Western blotting, mobility-shift assays, and silver nitrate staining were used to determine their purity and quantity. The purity of the various receptor preparations was estimated by silver nitrate staining. Figure 1 shows an example with Ni²⁺-NTA-purified heterodimer and >80% purity was achieved when DNA affinity chromatography was carried out as well (data not shown). DNA affinity-purified receptors were used for the experiments presented in Figure 4 and the rest of the experiments were performed using Ni²⁺-NTA purified receptors. All purified receptors were quick-frozen in liquid nitrogen and stored at -80°C.

In vitro transcription assays

Nuclear extracts were prepared from HeLa S3 and 293 cells and chromatographed on phosphocellulose columns as described [Berkenstam et al. 1992]. Thirty liters of HeLa cells was used for preparing the nuclear extract that was dialyzed to adjust the KCl concentration to 500 mM and loaded on a phosphocellulose column as described [Berkenstam et al. 1992]. The 500 mM KCl HeLa fraction was used as a source of general factors (with a protein concentration of 8 mg/ml as determined by the Bradford method). One hundred liters of 293 cells was used to prepare a nuclear extract that was adjusted to a KCl concentration of 100 mM and loaded on a Whatman P11 phosphocellulose column (1 ml of packed cell volume per milliliter of packed phosphocellulose) equilibrated in 20 mM HEPES-KOH (pH 7.8), 20% glycerol, 10 μ M ZnCl₂, 10 mM 2-mercaptoethanol, and 100 mM KCl. The fraction eluted at 750 mM KCl (with a protein concentration of 1.5 mg/ml as determined by the Bradford method), containing TFIID, was used for complementing the 500 mM KCl HeLa fraction in the *in vitro* transcription reactions.

Transcription assays contained 20 mM HEPES (pH 7.9), 6 mM MgCl₂, 70 mM KCl, 0.2 mM EDTA, 1 mM DTT, 7.5% glycerol, 2.5% polyethylene glycol 6000, 10 mM creatine phosphate, 1 μ g of calf thymus DNA, 70 units of RNase T1 (Sigma), 10–50 ng of each template (~10–50 fmoles), 0.6 mM ATP, 0.6 mM CTP, 20 μ M UTP, 0.5 mM 3'-O methyl GTP (Pharmacia), and 0.4 μ M [α -³²P]UTP (800 Ci/mmol; Amersham) in a final volume of 30 μ l. Fifteen to 30 μ g of the 500 mM KCl HeLa fraction and 2 to 4 μ g of the 750 mM KCl 293 fraction were used per reaction. Pretreatment of receptors with ligands or solvent alone (10% DMSO final concentration) involved incubation of aliquots of receptor (3 μ M final concentration) with 0.1 volume of ligand (in 100% DMSO) for 20 min at room temperature in siliconized tubes.

Transcription reaction mixtures were preincubated at 30°C for 30 min in the absence of nucleotides. Transcription was initiated by the addition of nucleotides, followed by an incubation for 1 hr at 30°C. Reactions were terminated by the addition of 180 μ l of stop mix [0.1 M sodium acetate (pH 5.2), 10 mM EDTA, 0.1% SDS, 60 μ g/ml of tRNA] containing ~300 cpm of a 600-bp ³²P-labeled DNA fragment, followed by phenol-chloroform-isoamyl alcohol (25:25:1) extraction and isopropanol precipitation. RNA products were separated on 6% polyacrylamide sequencing gels, dried, and autoradiographed for 24–48 hr at -80°C with an intensifying screen.

Limited proteolytic digestion

Receptors (1.2 μ g) were incubated with the proteases and retinoids in siliconized tubes in a final volume of 10 μ l as described

(Keidel et al. 1994). Proteolytic products were analyzed by Western blotting using polyclonal antibodies directed against the human RAR α LBD (amino acids 152 to 419) and RXR α LBD (amino acids 204–462) (J. Pohl and H.G. Stunnenberg, unpubl.). Horseradish peroxidase-linked anti-rabbit immunoglobulin was used as the second antibody, and protein–antibody complexes were visualized by an enhanced chemiluminescence Western blotting detection system according to the recommendations of the manufacturer (Amersham).

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