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Genetics

Efficient transactivation by retinoic acid receptors in yeast requires retinoid X receptors

(heterodimer/transcription factor)

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ABSTRACT **All-*trans* and 9-*cis* retinoic acids are natural derivatives of vitamin A that modulate gene expression as a consequence of binding to nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RXRs form heterodimers with RARs *in vitro*, and such complexes display enhanced binding affinities for cognate DNA response elements. As yeast is devoid of endogenous RARs and RXRs, we used this organism to investigate whether transactivation *in vivo* requires RAR/RXR heterodimers. Using a domain-swapping approach, we demonstrate that chimeric RARα1 and RXRα containing the DNA-binding domain of the estrogen receptor activate transcription of a cognate reporter gene in yeast, independently of each other. These activities result from an inducible transcription activation function located in the ligand-binding domains of RARα1 and RXRα and a constitutive activation function located in the A/B region of RARα1. The inducible activation function of RXRα is induced exclusively by 9-*cis*-retinoic acid in this system. Transactivation of a reporter gene containing a retinoic acid response element by RARα was considerably increased by RXRα, even in the absence of ligand. Optimal induction was achieved with 9-*cis*-retinoic acid, which stimulates the activity of both receptors. This study illustrates the utility of yeast to investigate signal transduction by retinoids in the absence of endogenous RARs, RXRs, and detectable retinoic acid isomerization.**

The pleiotropic action of retinoids, which appear to play a major role in embryogenesis, cell growth and differentiation, and homeostasis (for reviews see refs. 1–7), is mediated by multiple receptors which belong to the superfamily of nuclear receptors (8–10) and act as ligand-inducible transcriptional activators that regulate target gene transcription upon binding to cognate enhancer sequences, referred to as retinoic acid (RA) response elements (RAREs). Natural RAREs appear to consist of a direct repeat of the core motif 5′-RG(G or T)TCA-3′ separated by a spacer of variable length. Three RA receptors (RARα, -β, and -γ) can bind and activate transcription in response to all-*trans*-RA and 9-*cis*-RA, while retinoid X receptors (RXRα, -β, and -γ) bind and respond to 9-*cis*-RA, but not all-*trans*-RA (refs. 11–13; for review see ref. 14). RARs and RXRs bind cooperatively as heterodimers to RAREs *in vitro*, and their efficiency of DNA binding is much higher than that of the corresponding homodimers (14–20). While the formation of heteromeric complexes *in vitro* is well established, the contributions of the individual receptors within these complexes to DNA binding, ligand binding, and transcriptional enhancement *in vivo* are unclear.

Much of what is known about the transcriptional properties of RARs and RXRs in living cells has been derived from studies of reporter gene activation in transiently transfected mammalian or insect cell lines. However, due to some inherent disadvantages of these systems, several important questions remain unanswered. For example, the presence of endogenous RARs and RXRs in the cells of higher eukaryotes makes it difficult to study the individual receptor types in isolation and limits the use of mutant receptor derivatives. In addition, interactions with other proteins capable of forming heterodimers with RARs or RXRs (see ref. 14) may complicate the interpretation of transactivation studies in these systems. Further, it is likely that retinoid metabolism pathways exist in insect and mammalian cells which may interfere with the assessment of the agonist or antagonist properties of retinoid derivatives. In this study we use a eukaryotic organism, the yeast *Saccharomyces cerevisiae*, in which several mammalian hormone receptors, including steroid (21–26), thyroid hormone (27), and vitamin D receptors (28), are known to be functional and which is devoid of endogenous retinoid receptors, to assess transcriptional activation by RARs and RXRs expressed singly or in combination. Our results demonstrate that RAR/RXR heterodimer formation is required for efficient transactivation in yeast and that both receptors contain intrinsic transactivation functions which cooperate within the complex.

MATERIALS AND METHODS

Strains and Media. The *S. cerevisiae* strains used were PL3 (*MATα ura3-Δ1 his3-Δ200 leu2-Δ1 trp1::3ERE-URA3*) (25) and YPH250 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) (29). Yeast was grown in YEPD or selective medium (30). Yeast cells were transformed by electroporation (31).

Construction of Receptor Expression Vectors. The human RARα1 (aa 1–462) (32–34), mouse RXRα (aa 1–467) (17) and mouse dnRXRα (aa 1–448) (35) cDNAs have been described. Human dnRARαΔAB (aa 88–396) and mouse dnRXRαΔAB (aa 140–448) cDNAs were gifts from S. Nagpal (this laboratory). To express receptors in yeast, the 5′ noncoding sequences were removed. The 5′ flank of each construct contains a mammalian Kozak sequence sandwiched between an *Eco*RI site and the translation start codon (5′-GAATTCCACCATG-3′). The receptor cDNAs were subcloned as *Eco*RI fragments from the plasmid pSG5 (36) into the *Eco*RI sites of the yeast expression plasmids YCp10,

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Abbreviations: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; RARE, RA response element; ER, estrogen receptor; ERE, estrogen response element; DBD, DNA-binding domain; OMP-decase, orotidine-5′-monophosphate decarboxylase; AF-1, constitutive activation function; AF-2, inducible, ligand-dependent activation function.

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YCp90, YEp10, and YEp90, which contain an expression cassette under control of the *PGK* promoter (25). Details of the construction of chimeric receptors containing a DNA-binding-domain (DBD) cassette (ER.CAS; aa 185–250) (34) or the complete DBD of the human estrogen receptor [ER(C); aa 176–282) (plasmid pSG5-HE81; S. Mader and P.C., unpublished work)] are available on request.

Construction of Reporter Plasmids. To construct the RAR reporter gene, a *Hin*dIII–*Pst* I fragment from pFL39-1ERE-URA3 (25) containing a single estrogen response element (ERE) and part of the *URA3* promoter was cloned into pBluescript SK(−) (Stratagene). By site-directed mutagenesis, the ERE sequence at position −139 relative to the translational start site was removed and restriction sites for *Bgl* II and *Nhe* I were generated; these sites were used to insert an adaptor containing the DR5 sequence 5′-AGG-TCAgcgagAGGTCA-3′. The *Hin*dIII–*Pst* I DR5–*URA3* promoter fragment was recloned into the parent vector to yield pFL39-DR5-URA3. The complete URA3 coding sequence with its DR5–*URA3* promoter was excised by *Xma* I digestion and recloned into the *Xma* I site of the *LEU2*-containing centromeric plasmid pRS315 (29).

Transactivation Assay. Yeast extracts were prepared (37) from transformants grown exponentially for about five generations in selective medium in the presence or absence of ligand, in diffuse light. Orotidine-5′-monophosphate decarboxylase (OMPdecase) activity in cell-free extracts was measured (38) and is expressed as nanomoles of substrate transformed per milligram of total protein per minute.

DNA Binding Assay. Yeast transformants expressing the receptors were grown overnight in selective medium (20 ml), washed once, and lysed in 0.15 ml of high-salt buffer [20 mM Tris·HCl, pH 7.5/0.4 M KCl/2 mM dithiothreitol/20% (vol/vol) glycerol, containing protease inhibitors] by the glass-bead disruption method. After clearing at 10,000 × *g* for 15 min, 20 μg (≈2.5 μl) of the supernatant protein was diluted with 7 μl of low-salt buffer (same as high-salt buffer but containing 0.05 M KCl). Poly(dI-dC)·poly(dI-dC) (1 μl at 10 mg/ml) was added and the mixture was incubated for 15 min at 4°C. Gel retardation (using 50,000 cpm of labeled probe) and antibody supershift assays were performed as described (39).

RESULTS AND DISCUSSION

Activation Domains of RAR and RXR Are Functional in Yeast. To investigate whether the human RARα1 and mouse RXRα receptors (hereafter referred to as RARα and RXRα) could activate transcription in yeast, we constructed chimeric receptors in which the native DBDs were replaced with the DBD of the human ER. These chimeric receptors (designated RARα1–ER.CAS and RXRα–ER(C); Fig. 1*A*) were expressed in a yeast reporter strain containing a chromosomally integrated *URA3* reporter gene regulated by three EREs (25). Induction of the reporter was determined by measuring the specific activity of the *URA3* gene product, OMPdecase. Ligand dose–response experiments (Fig. 1*B*) showed that both RARα1–ER.CAS and RXRα–ER(C) activate transcription in a ligand-inducible and dose-dependent manner in yeast. Transactivation by RARα1–ER.CAS was comparably stimulated by all-*trans*-retinoic acid or 9-*cis*-retinoic acid (Fig. 1*B*) or by several other derivatives of RA (Fig. 1*C*), while activation by RXRα–ER(C) was induced only by 9-*cis*-RA or 9-*cis*-3,4-didehydro-RA at the concentrations used (Fig. 1 *B* and *C*). This is consistent with recent data on the binding affinities of RARs and RXRs for RA

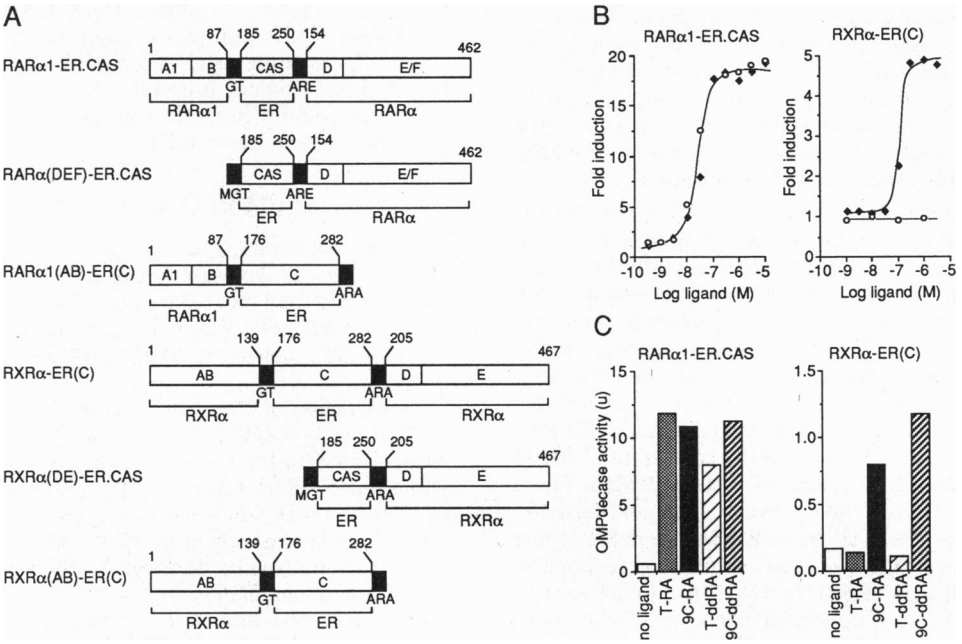

FIG. 1. Chimeric RARα and RXRα activate transcription autonomously in yeast. (*A*) RARα1–ER.CAS and RXRα–ER(C) chimeras and their truncated derivatives. Numbers refer to the amino acid boundaries of the receptor fragments specified below each illustration. Nonreceptor sequences present in the chimeras are indicated as black boxes; the additional amino acids are given immediately below the boxes. The chimeric receptors contain the DBD of human ER (cassette, CAS, or complete, C) and were expressed from the constitutive yeast phosphoglycerate kinase gene (*PGK*) promoter in the 2μ-derived yeast multicopy plasmids YEp10 and YEp90. (*B*) Dose–responses of RARα–ER.CAS and RXRα–ER(C) to all-*trans*-RA and 9-*cis*-RA. The chimeric receptors RARα1–ER.CAS and RXRα–ER(C) were expressed in the reporter strain PL3, which contains a chromosomally integrated 3ERE–*URA3* reporter gene, in the presence of all-*trans*-RA (○) or 9-*cis*-RA (◆) at the concentrations indicated. Transcription of the reporter gene was determined by measuring the specific activity of the *URA3* gene product, OMPdecase, and is represented as fold induction above the level of OMPdecase activity observed in the absence of ligand. (*C*) RA derivatives differentially induce transactivation by RARα–ER.CAS and RXRα–ER(C). Induction of reporter activity in the yeast strain PL3 expressing RARα–ER.CAS or RXRα–ER(C) was determined in the presence of all-*trans*-RA (T-RA), 9-*cis*-RA (9C-RA), all-*trans*-3,4-didehydro-RA (T-ddRA), or 9-*cis*-3,4-didehydro-RA (9C-ddRA) at 1 μM. Transactivation is given as units (u) of OMPdecase activity.

derivatives (12, 13) and demonstrates the specificity of RXRα for 9-*cis* derivatives of RA in living cells. In addition, our results show that all-*trans*-RA and all-*trans*-3,4-didehydro-RA are not significantly converted to 9-*cis* stereoisomers in yeast. In contrast, studies in mammalian and *Drosophila* cells which showed stimulation of RXR activity by high concentrations of all-*trans*-RA (11–13, 40) suggest that a RA isomerase activity exists in higher eukaryotes; this activity is apparently absent in yeast.

To identify transcription activation functions in RARα and RXRα, we constructed chimeras containing either the A/B or D/E/F regions of RAR and RXR linked to the DBD of the ER. RARα(DEF)–ER.CAS (Fig. 1*A*) increased the OMPdecase activity 10-fold in the presence of 9-*cis*- or all-*trans*-RA (Table 1), indicating that RARα contains an inducible activation function (AF-2) in the ligand binding domain which is functional in yeast, as has been found for steroid receptors (25, 41, 42). RARα1(AB)–ER(C) (Fig. 1*A*) increased the activity of the reporter (150-fold above background) in a ligand-independent manner (Table 1). Thus we conclude that the A/B region of human RARα1 contains an autonomous activation function (AF-1), in keeping with recent results from our laboratory showing that the transactivation properties of RARs and RXRs in mammalian cells are modulated by their A/B domains, depending on the promoter environment (43). Interestingly, RARα1–ER.CAS exhibited some ligand-independent activation (Table 1), in contrast to the ER expressed in the same reporter strain (25), which indicates that this chimeric RARα does not require ligand to bind EREs. RXRα was also found to contain an AF-2 in its ligand-binding domain, as RXRα(DE)–ER.CAS stimulated the reporter activity 5-fold in the presence of 9-*cis*-RA (Table 1). In contrast to the corresponding RARα chimeric receptors, RXRα–ER(C) and RXRα(DE)–ER.CAS displayed similar degrees of induction (5-fold) in response to 9-*cis*-RA (Table 1), suggesting that the A/B domain of RXRα does not contribute significantly to transcriptional activation of this reporter. Accordingly, we did not observe any *URA3*-reporter activation by RXRα(AB)–ER(C) in yeast (Table 1). However, the possibility that the A/B region of RXRα contains an AF-1 which may activate transcription from other promoters is not excluded. It is unclear why the constitutive and induced activities of RXRα(DE)–ER.CAS were 10-fold higher than those observed for RXRα–ER(C) in these experiments (Table 1). Note, however, that RXRα–ER(C) was expressed at a much lower level than RXRα(DE)–ER.CAS (as determined by immunoblots), whereas RARα1–ER.CAS and RARα(DEF)–ER.CAS were expressed at similar levels (data not shown). In summary, the above results demonstrate the presence of AFs in RARα1 (AF-1 and AF-2) and RXRα (AF-2) which can stimulate transcription autonomously in yeast cells.

Table 1. Characterization of autonomous activation functions in RARα and RXRα

Receptor	OMPdecase activity, units (fold induction)		
	No RA	all-*trans*-RA	9-*cis*-RA
None	0.02	0.02 (1)	0.02 (1)
RARα1–ER.CAS	0.65	14.70 (23)	12.90 (20)
RARα(DEF)–ER.CAS	0.09	0.82 (9)	0.94 (10)
RARα1(AB)–ER(C)	3.12	3.03 (1)	3.09 (1)
RXRα–ER(C)	0.22	0.20 (1)	1.05 (5)
RXRα(DE)–ER.CAS	2.01	1.85 (1)	9.92 (5)
RXRα(AB)–ER(C)	0.02	0.02 (1)	0.02 (1)

The chimeric receptors illustrated in Fig. 1*A* were tested for their ability to activate the 3ERE–*URA3* reporter gene in yeast strain PL3 in the presence or absence of all-*trans*-RA or 9-*cis*-RA. Vector without insert was used as a control ("None"). Transactivation data are expressed as OMPdecase activities; the mean values shown in the table were determined from duplicate assays with three individual transformants for each clone. Numbers in parentheses indicate the fold induction over the activity observed for each receptor in the absence of ligand.

RAR/RXR Heterodimers, But Not Homodimers, Efficiently Activate Transcription of a DR5 Reporter Gene in Yeast. The formation of heterodimers in solution results in cooperative DNA binding of RARs and RXRs to RAREs *in vitro* (14–18). Gel retardation experiments confirmed that only extracts from yeast expressing both receptors could efficiently retard the mobility of a synthetic DR5 RARE probe (Fig. 2, lane 7; see below for a description of a DR5 RARE). The presence of both receptors in the retarded complex was verified by supershifting the complex with anti-RARα (Fig. 2, lane 8) or anti-RXRα (data not shown) monoclonal antibodies. Thus, RARs and RXRs produced in yeast form heterodimers *in vitro*, as has been observed for receptors produced in other systems.

To investigate whether the activation functions of RAR and RXR were sufficient to achieve RA-enhanced transcription with the natural receptor(s) in yeast, we constructed a *URA3* reporter gene containing a synthetic RARE element (Fig. 3*A*). This response element is a direct repeat of the motif 5′-AGGTCA-3′ separated by a spacer of 5 bp (DR5), as in the RARE of the RARβ$_2$ gene previously shown to function as a RA-responsive enhancer in mammalian cells (45–47). Receptors were expressed in an appropriate yeast strain carrying the DR5–*URA3* reporter gene on a single-copy vector. In transformants expressing either of the receptors alone, no increase was observed in reporter basal activity (i.e., control without receptors) in the absence of ligand (measured as OMPdecase activity; Fig. 3*B*). In clones expressing RARα alone, all-*trans*-RA or 9-*cis*-RA (0.5 μM) induced the OMPdecase activity 5-fold, showing that RARα can activate transcription of a DR5 element in yeast, albeit weakly, in a ligand-dependent manner in the absence of RXR. However,

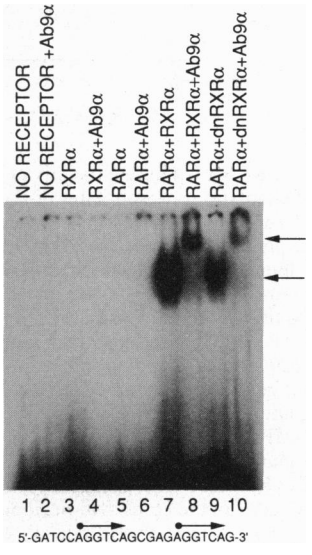

FIG. 2. RARα and RXRα produced in yeast cooperate for DNA binding to a RARE *in vitro*. Gel retardation assays were performed with a labeled DR5 probe (whose sequence is given at the bottom) and cell-free extracts prepared from yeast transformants expressing no receptor (lane 1), RXRα (mouse RXRα) (lane 3), RARα (human RARα1) (lane 5), RARα and RXRα (lane 7), or RARα and dnRXRα (mouse dnRXRα) (lane 9). Specificity of binding was verified by supershifting retarded complexes with the RARα-specific monoclonal antibody Ab9α (44). Lanes 2, 4, 6, 8, and 10: samples identical to those in lanes 1, 3, 5, 7, and 9, respectively, but incubated with the antibody immediately before electrophoresis. Arrows indicate the specific and supershifted complexes.

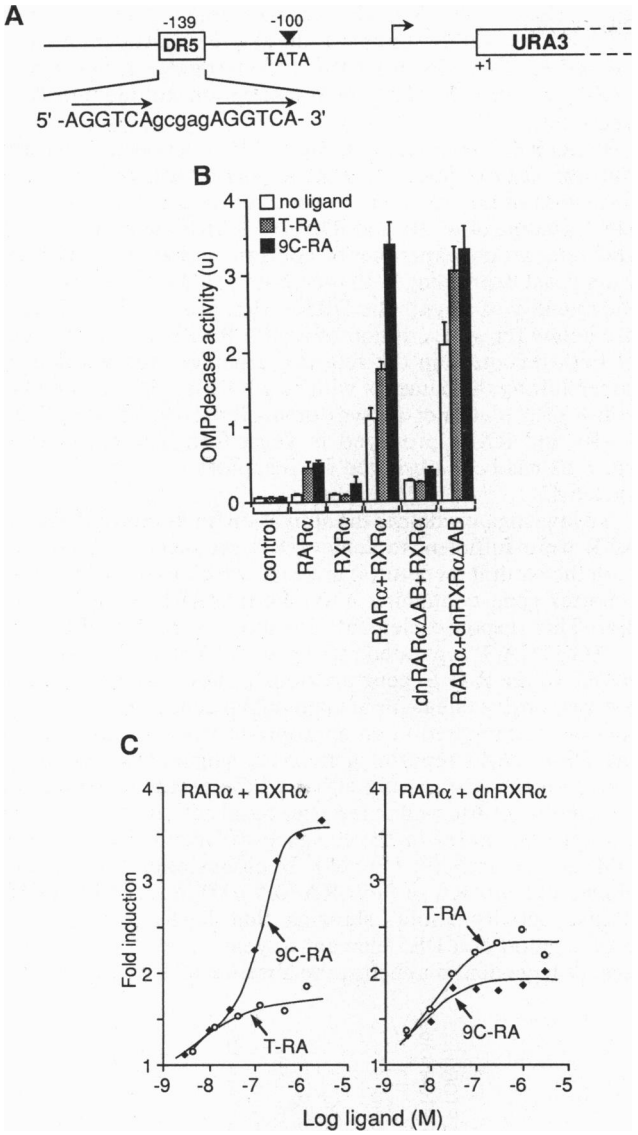

FIG. 3. RARα and RXRα cooperate to activate a RARE reporter gene in yeast. (*A*) Schematic representation of the promoter region of the DR5–*URA3* reporter used in transactivation experiments. *URA3* promoter sequences required for both basal and activated transcription were deleted and replaced with a RARE. This element consists of a direct repeat of the motif 5′-AGGTCA-3′ separated by 5 bp (DR5). The positions of the DR5 and the TATA box relative to the ATG start codon (+1) are also shown, and the bent arrow indicates the approximate site of initiation of transcription of *URA3* mRNA. The reporter gene was maintained in yeast strain YPH250 on a centromeric plasmid. (*B*) RXRα enhances RARα activity on a DR5 element in yeast. OMPdecase activities (u, units) were measured in extracts of transformants containing the DR5–*URA3* reporter plasmid and multicopy plasmids expressing RARα, dnRARαΔAB, RXRα, and dnRXRαΔAB in the combinations indicated, or parental vectors (control), in the presence or absence of ligand [all-*trans*-RA (T-RA) or 9-*cis*-RA (9C-RA) at 0.5 μM]. In dnRARαΔAB and dnRXRαΔAB, the A/B regions and part of the C terminus of RARα and RXRα, respectively, have been deleted, resulting in transcriptionally inactive mutant derivatives (data not shown). The mean values and standard deviations presented are derived from at least two experiments using three separate transformants for each clone. (*C*) 9C-RA induces RXRα activity on a DR5 reporter gene in yeast. Dose–responses to T-RA (○) and 9C-RA (◆) in transformants containing the DR5–*URA3* reporter plasmid and coexpressing RARα with RXRα or dnRXRα are shown. dnRXRα is a dominant negative receptor which contains a C-terminal deletion (35). Transactivation is represented as fold induction of reporter activity above the value obtained in the absence of ligand.

no transactivation was observed when the gene dosage of RARα was reduced by expressing it from a single-copy plasmid (data not shown). Clones expressing RXRα from single- or high-copy-number plasmids showed a weak (2-fold) increase in reporter activity in response to 9-*cis*-RA, but not all-*trans*-RA. Remarkably, however, coexpression of RARα and RXRα strongly increased both the constitutive and induced levels of reporter gene activity. The constitutive activity was 18-fold above that of the control, while the all-*trans*-RA- and 9-*cis*-RA-induced activities increased to 30-fold and 60-fold above the control, respectively, when both receptors were expressed from high-copy-number plasmids (Fig. 3*B*). The constitutive and induced activities were conserved (but 4-fold lower) when both receptors were expressed from single-copy plasmids (data not shown). These results show that RARα and RXRα cooperate to transactivate a DR5 element in yeast, even in the absence of ligand. Dose–responses to all-*trans*-RA and 9-*cis*-RA in transformants expressing both RARα and RXRα confirmed that 9-*cis*-RA is the more potent inducer in this system (Fig. 3*C*).

While the above results support the idea that RAR and RXR cooperate in DNA binding and transactivation, they do not reveal the origin of the differential responses to 9-*cis*- and all-*trans*-RA (Fig. 3*C*). To investigate the individual contributions of RARα and RXRα to heterodimer-mediated transcription, we used a dominant negative receptor mutant, dnRXRα (35), which has lost its ligand-inducible activity, but retains its ability to enhance the DNA binding of RARα *in vitro* through heterodimerization (Fig. 2, lanes 9 and 10). Transactivation experiments showed that coexpression of RARα and dnRXRα resulted in a loss of the differential response to all-*trans*- and 9-*cis*-RA (Fig. 3*C*). This indicates that RXRα not only enhances the binding of RARα to a DR5 element but also mediates, at least in part, the 9-*cis*-RA stimulation of the DR5 reporter. To investigate the origin of the ligand-independent activity of the heterodimer, we constructed transcriptionally compromised mutants of RARα (dnRARαΔAB) and RXRα (dnRXRαΔAB) with deletions of their entire A/B regions, in addition to the C-terminal truncations present in dnRARα and dnRXRα. Note that deletion of the A/B region does not affect the ability of these receptors to bind DNA cooperatively *in vitro* (unpublished results). Coexpression of dnRARαΔAB and RXRα strongly reduced the constitutive activity and abolished the response to all-*trans*-RA observed with full-length receptors, while some induction occurred in the presence of 9-*cis*-RA (Fig. 3*B*). In contrast, no reduction in the constitutive activity was observed when RARα and dnRXRαΔAB were coexpressed, indicating that RARα is largely responsible for the ligand-independent activation. This is consistent with the presence of a strong AF-1 in RARα1, but not RXRα, as shown above. Further, as observed for RARα/dnRXRα (Fig. 3*C*), the differential response to all-*trans*-RA and 9-*cis*-RA was lost when RARα and dnRXRαΔAB were coexpressed. In summary, our results show that RAR/RXR heterodimers possess a constitutive activity which is essentially due to the strong AF-1 present in the N-terminal A/B region of hRARα1. In the presence of ligand, the induced activity is due to both the RARα AF-2, which is similarly activated by all-*trans*-RA and 9-*cis*-RA, and the weaker RXRα AF-2, which is exclusively activated by 9-*cis*-RA.

Conclusion. This study provides compelling evidence that heterodimers of RARα and RXRα are required for efficient activation of a DR5-regulated reporter gene *in vivo*. Moreover, it demonstrates that heterodimers of RARs and RXRs can bind to a RARE in living cells and stimulate gene transcription in the absence of ligand. This distinguishes RARs and RXRs from steroid receptors, whose activities appear to be entirely dependent on ligand (ref. 10 and references therein). The experimental advantages over mam-

malian systems—namely, the absence of endogenous RARs, RXRs, and RA isomerase activities—make yeast an excellent model to investigate the molecular mechanisms underlying retinoid signal transduction. It remains to be seen whether promoters containing RAREs with different spacer lengths between the directly repeated motifs (DR1–DR4), or inverted repeats, will also be preferentially activated in yeast by heterodimers of RARs (or other nuclear receptors) and RXRs in response to their cognate ligands.

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