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Homo- and heterodimers of the retinoid X receptor (RXR) activate transcription in yeast

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ABSTRACT

The polymorphic nature of sequences which act as retinoic acid response elements (RAREs and RXREs) in transactivation assays in mammalian cells, suggests that elements consisting of a direct repetition of a half site motif, separated by 1 to 5 base pairs (DR1 to DR5), are targets for retinoic acid (RA) signalling. In a previous report we showed that in yeast cells, heterodimers of the retinoic acid receptors RARα and RXRα were required for efficient transcription of a reporter gene containing a DR5 element [Heery *et al.*, (1993); *Proc. Natl. Acad. Sci. USA*, 90: 4281–4285]. Here we report that DR1 to DR5 elements containing a direct repeat of the 5′-AGGTCA-3′ motif, and an inverted repeat of the same sequence with no spacer (IR0), behave as RAREs in yeast cells coexpressing RARα and RXRα, albeit with different efficacies. Heterodimer activity was strongest on a DR5 reporter gene, and the strength of activation of the reporter series (DR5 > DR1 > DR3 > DR2 = IR0 = DR4) correlated with the ability of the heterodimer to bind to the corresponding sequences *in vitro*. Significantly, a reporter containing a DR1 element was selectively and efficiently activated in yeast cells expressing only RXRα. This activity was dependent on the induction by *9-cis* retinoic acid of an activation function (AF-2) located in the RXRα ligand binding domain. In addition, a strong synergistic activity of RXRα was observed on a reporter containing the putative RXR element (RXRE) from the rat CRBPII gene promoter. Thus, RXRα can function independently as a transcription factor, in the absence of RARs or other heteromeric partners. Similarly, homodimers of RARα selectively stimulated the transcription of a DR5 reporter in a ligand-dependent manner, but less efficiently than RARα/RXRα heterodimers.

INTRODUCTION

Retinoic acid (RA) signalling involves at least two classes of proteins, the retinoic acid receptors (RARα, RARβ, RARγ) and retinoid X receptors (RXRα, RXRβ, RXRγ) (reviewed in 1–5). RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily, and exhibit the modular protein structure typical to this group, including domains which function in DNA binding, dimerisation, ligand binding and transactivation. Ligand competition experiments and binding studies revealed that, while the three RAR types show strong affinities for both the *all-trans* and *9-cis* isoforms of RA, the RXRs showed a marked specificity for the latter molecule (6–8). Ligand binding appears to be required to induce transactivation functions (AFs) which overlap the N-terminal and ligand binding domains of these proteins (9–11). It has recently been shown that the affinities of RARs for their target sequences is strongly increased when they are complexed as heterodimers with RXRs (12–18), and a series of heptad repeats present in the E domains of both receptors, are largely responsible for heterodimer formation in solution (Ref. 19 for review). RXRs exhibit promiscuous heterodimerisation properties *in vitro*, forming complexes with other factors including the thyroid receptor (TR), Vitamin D receptor (VDR), peroxisome proliferator activated receptor (PPAR), the COUP-TF receptor, which stimulates their cooperative and selective binding to cognate hormone response elements *in vitro* (Refs. 5, 20 and references therein).

Naturally occurring RA response elements (RAREs) have been identified in the promoters of a number of genes, and generally consist of direct repetitions (DR) of one or more copies of a moderately conserved hexamer sequence, 5′-PuG(G/T)TCA-3′. The length of the spacer sequence between the hexameric motifs is variable, being 5 bp in RAREs found in the genes encoding RARα2 (21), RARβ2 (22, 23), RARγ2 (24) and alcohol dehydrogenase 3 (ADH3; 25), 4 bp in the Laminin B1 gene (26), 2 bp as in the cellular retinol binding protein (CRBPI; 27) and cellular retinoic acid binding protein (CRABPII; 28) genes, or 1 bp as found in the genes encoding CRABPII (28), phosphoenolpyruvate carboxykinase (PEPCK; 29), apolipoprotein A1 (ApoA1; 30) and medium chain acyl-CoA dehydrogenase (MCAD; 31). A systematic study by Mader *et al.*, (32) showed that the sequences of the half sites, the length and sequence of the spacer between them and the flanking sequences, all influence the capacity of these elements to function as RAREs in transient transfection experiments. In addition, it has been proposed that

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a distinct signalling pathway governed by RXR homodimers mediated through RXR-specific response elements (RXREs) exists *in vivo* (33). Evidence to support this hypothesis came from transient transfection studies in animal cells where transfection of plasmids expressing RXRs, but not RARs, resulted in RA-dependent activation of reporter genes driven by putative RXREs from the rat CRBPII (33), human ApoA1 (30) and mouse CRBPII (34) promoters, and a synthetic RXRE (32). These elements share a common arrangement of one or more half sites repeated at single base pair intervals (i.e. DR1-type elements). Additionally, homodimers of RXRs show a preference for binding to DR1-type elements *in vitro* (32, 34, 35). However, transient transfection studies (even in insect cell-lines) are prone to interference from endogenous factors which may contribute to the apparent RXR-specific activity. For this reason, we have chosen the yeast system to address the questions on the apparent polymorphic nature of RAREs and the possible independent function of RXR *in vivo*.

MATERIALS AND METHODS

Yeast strains and expression plasmids

S.cerevisiae strain YPH250 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) (36) was used for all yeast experiments and was a gift from P.Hieter. The human RARα1 (aa 1−462), mouse RXRα (aa 1−467), and mouse dnRXRα (aa 1−448) cDNAs (10) and the cDNA encoding the mouse RXRαΔAB (aa 140−467) (11) were cloned into the unique EcoR1 site of the yeast PGK expression cassette present in the yeast expression vectors YEp90, YCp90, YEp10 or YCp10 as required. The construction of the 2μ containing expression vector YEp90, which contains the yeast HIS3 gene as a selectable marker, has been described previously (37). The plasmid YEp10 (a gift from D.Metzger) is identical to YEp90 except that the HIS3 marker is replaced by a *Bgl*II fragment containing the yeast TRP1 gene. YCp90 and YCp10 are ARS CEN containing vectors which carry HIS3 or TRP1 markers respectively, and which were derived from the plasmids pRS313 and pRS314 (36) as follows. First the unique *Eco*RI sites in the polylinker sequences of the plasmids pRS313 and pRS314 were destroyed by *Eco*RI digestion, treatment with Klenow fragment and religation. The yeast PGK expression cassette from the plasmid pTG848 (38) was then cloned as a *Cla*I fragment into the unique *Cla*I site in the polylinkers of both plasmids.

Reporter plasmids

The URA3 reporter system and the construction of the pRS315-DR5-URA3 reporter plasmid have been described previously (10, 37). To construct a URA3 reporter series driven by different response elements (DR1−DR4 and IR0), we synthesised a set of adaptors containing single response element sequences as shown in Fig. 1A. All elements were flanked by identical sequences on their 5′ (5′-GATCC-3′) and 3′ (5′-G-3′) sides which were designed to generate ends compatible with *Nhe*I and *Bgl*II. One copy of each adaptor was inserted into the unique *Nhe*I and *Bgl*II restriction sites present in an engineered URA3 promoter, carried in pBluescript as a *Hin*DIII−*Pst*I fragment (10). The DRn-URA3 and IR0-URA3 promoters were then excised as *Hin*DIII−*Pst*I fragments and used to replace the corresponding fragment in the pRS315-DR5-URA3 reporter plasmid, to create the new series of URA3 reporter genes. The CRBPII-URA3 reporter was constructed in an identical fashion using an adaptor containing the RXRE sequence from the rat CRBPII gene promoter (33).

RESULTS

Direct repeats of the motif 5′-AGGTCA-3′ with different spacings and an inverted repeat of the same motif function as RAREs in yeast

In a previous study, we demonstrated that efficient RA induction of a reporter gene driven by a RARE sequence (DR5-URA3) in yeast required heterodimers of RARs and RXRs (10), as observed in animal cells (28). As considerable polymorphism exists in the interval lengths found in sequences which function as RAREs in animal cells (20−31), a logical extension of this work was to test the effect of varying the distance between the half site motifs in the response element on responsiveness to RARs and RXRs, in yeast. The RARE used previously (10) consisted of a direct repetition of the motif 5′-AGGTCA-3′ separated by a 5 bp sequence identical to that found in the RARE from the RARα2 promoter (21, 39). Reporters consisting of the yeast URA3 gene preceded by direct repeats of the same motif separated by spacers of 1 to 4 base pairs (DR1-URA3 through DR4-URA3), and also a reporter containing an inverted repeat of the same motif with no spacing (IR0-URA3), were constructed (See Materials and Methods). The sequences of these response elements and the DR5 element are indicated in Fig. 1A. The reporters were cloned into a centromeric plasmid, as previously described (10), and introduced into a yeast strain expressing RARα, RXRα or both receptors from multicopy (2μ) plasmid vectors (See Materials and Methods). Reporter (orotidine-5′-monophosphate decarboxylase; OMPdecase) activity in cell-free extracts of cultures grown to exponential phase in the presence or absence of 500nM *all-trans* RA or *9-cis* RA, was determined as previously described (40). As shown in Fig.1B, little or no reporter activities were observed in the absence of RA receptors (control), indicating that endogenous yeast factors were incapable of significant activation of these reporters. In the absence of ligand, coexpression of RARα and RXRα led to a 3-fold constitutive increase in the activities of the DR2, DR3 and DR4 reporters, and 10-fold and 16-fold increases in the activities of the DR1 and DR5 reporters, respectively. Addition of *all-trans* RA (500 nM) to the growth medium further induced the activities of all reporters (5- to 6-fold above the control for DR2, DR3 and DR4, 18-fold for DR1 and 27-fold for DR5). Addition of *9-cis* RA (500 nM) to the growth medium induced the DR2, DR3 and DR4 reporters approximately 10-fold, the DR1 reporter 36-fold and the DR5 reporter 60-fold above their respective control activities. Thus, direct repetitions of the motif 5′-AGGTCA-3′ with spacings of 1 to 5 base pairs render a reporter gene inducible by RA in yeast, albeit with different efficacies, which is consistent with similar observations in animal cells (5, 21−32). Coexpression of RARα and RXRα was also found to increase the activity of a reporter containing an inverted repeat of 5′-AGGTCA-3′. The basal activity in the absence of receptors (control) of the IR0-URA3 reporter was increased 15-fold by the heterodimer in the absence of ligand, and induced 20-fold and 40-fold above the control activity in the presence of *all-trans* RA and *9-cis* RA, respectively (Fig. 1B). However, note that despite the apparently high activity in terms of fold stimulation, the actual levels of activity for this reporter are quite low when compared to the DR5 reporter. Other groups have reported RAR/RXR

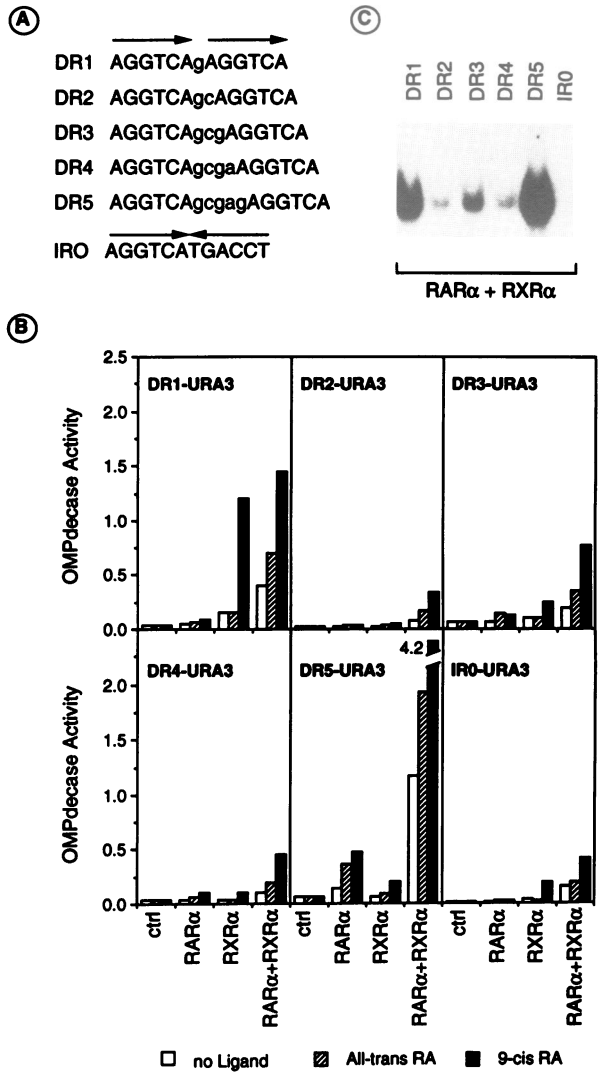

Figure 1. Direct repeats of a half site motif with different spacings and an inverted repeat of the same sequence with no spacing, function as RAREs in yeast. **A.** Sequences of the response elements used in this study consisting of a direct repeat of a hexamer sequence spaced by one to five base pairs (DR1−DR5), or an inverted repeat of the same hexamer with no spacing (IR0). The hexamer repeats are indicated by arrows and the spacer sequences are presented in lower case. **B.** Transactivation of URA3 reporter genes containing the different elements by homodimers and heterodimers of RARα and RXRα. The sequences represented in Fig. 1A were cloned into the promoter of a URA3 reporter gene carried on a centromeric plasmid (See Materials and Methods) to generate the DRn-URA3 reporter series and IR0-URA3. The reporters were introduced into a yeast strain containing multicopy vectors expressing RARα, RXRα or no receptor. Reporter activities in the presence or absence of ligand (500nM) were determined by measuring the OMPdecase activity (38). 'Control' indicates the basal reporter activity in the absence of receptors (using 'empty' expression vectors), and the experiments using only one receptor were performed in the presence of the corresponding 'empty' vector. The white, hatched and black columns indicate the reporter activity in the presence of no ligand, *all-trans* RA and *9-cis* RA, respectively. The reporter activities are given as units of OMPdecase activity per minute per mg protein, and the values represent the average of at least 2 experiments using at least 2 different clones per experiment. Deviation of values was less than 10%. **C.** Gel retardation assays using radiolabelled probes (containing the sequences described in 1A) and cell-free extracts from yeast coexpressing RARα and RXRα were performed as described previously (10). Similar amounts of each probe (50,000 cpm) were used in the assays.

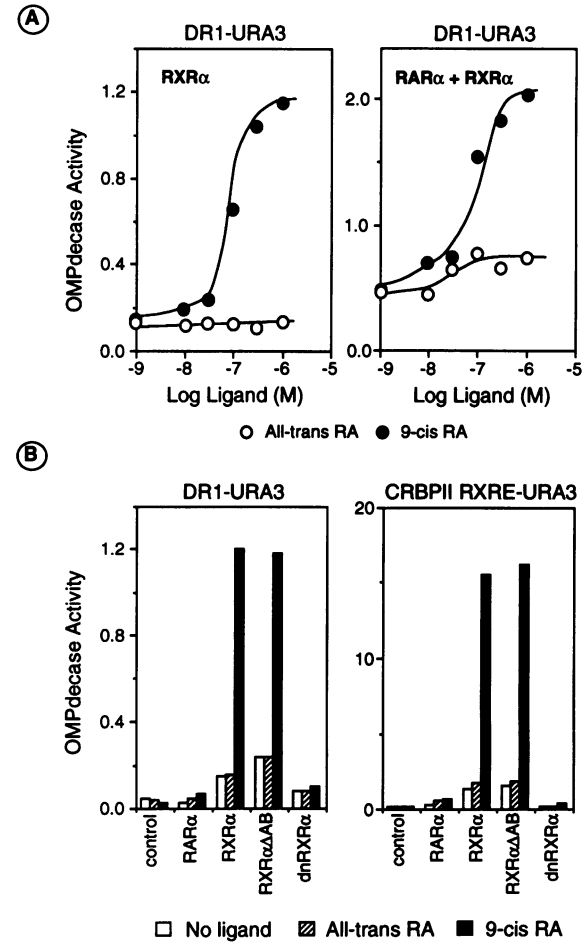

Figure 2. Transactivation of the DR1-URA3 reporter gene by RXRα and RARα/RXRα heterodimers, synergistic activation of the CRBPII RXRE-URA3 by RXRα, and effect of deletions in RXRα on reporter activation. **A.** Ligand dose responses of DR1 reporter activity in the presence of RXRα and RARα/RXRα heterodimers. Activation of the DR1-URA3 reporter measured as OMPdecase activities in cell-free extracts of yeast expressing RXRα, or RARα and RXRα together, grown in the presence of *all-trans* RA (empty circles) and *9-cis* RA (filled circles) at the indicated concentrations, as described previously (10). **B.** Activation of DR1-URA3 and CRBPII RXRE-URA3 reporters in yeast expressing RARα, RXRα, RXRαΔAB and dnRXRα or no receptor (control) in the presence or absence of ligand.

activity on a similar response element (TREpal) in animal cells (33, 41) and in yeast (42).

To determine how the observed transactivation data correlated with the ability of the receptors to bind to the corresponding sequences, we performed *in vitro* DNA binding assays. Extracts from yeast cells expressing both receptors efficiently retarded the mobility of the DR1 and DR5 probes, and bound more weakly to the DR2, DR3, DR4 probes under identical conditions (Fig. 1C, lanes 1−5). A weak band shift was also observed for the IR0 element (lane 6) after prolonged autoradiography. The presence of RARα and RXRα in these complexes was verified by supershift assays using specific monoclonal antibodies against the receptors (not shown). Thus, the efficiency of reporter activation correlates closely with the capacity of the heterodimer to bind to the corresponding element *in vitro*. Note that the relatively low affinity of RAR/RXR heterodimers for the DR2

and DR4 probes *in vitro*, and the weak activation of the corresponding reporter genes in yeast, is a consequence of the spacer and flanking sequences of the elements used in this study. For example, a DR2 probe with different spacer and flanking sequences was bound 6-fold more efficiently by RARs and RXRs produced in yeast, or *E.coli* (Z.P.Chen, personal communication). This is consistent with the observations that changes in the spacer, half site motif or the flanking sequences can significantly affect binding and activation by RARs and RXRs produced in mammalian cells (21, 32) and in yeast (our unpublished results).

The ability of the isolated receptors to activate the reporter series was also tested. As previously reported (10) expression of RARα alone in yeast resulted in a 5- to 7-fold activation of DR5 reporter in the presence of either *all-trans* RA or *9-cis* RA (Fig. 1B). However, RARα alone showed little if any ability to stimulate the activity of the DR1, DR2, DR3, DR4 and IR0 reporters, in the presence or absence of ligand. Similarly RXRα had little effect on the DR2, DR3, DR4 and DR5 reporter activities (2−3 fold *9-cis* RA-dependent stimulation), but stimulated the activity of the DR1 reporter at least 30-fold in response to *9-cis* RA, but not *all-trans* RA (Fig. 1B). Western blot experiments using specific monoclonal antibodies (gifts from C.Egly and M.P.Gaub) detected similar levels of RARα or RXRα in extracts from cells expressing either one or both receptors (data not shown), thus eliminating the possibility that the observed increases in DNA binding and transactivation by heterodimers were due to altered levels of receptor proteins. RXRα also activated the IR0 reporter 20-fold above background in a ligand dependent manner (Fig. 1B), although as stated above, the absolute value of this activity was low (6-fold lower than the DR1 reporter). Cell-free extracts from transformants expressing no receptor, or either of the receptors alone, failed to retard the DR5 probe (10) or any of the other DR probes in gel shift experiments under conditions identical to those used in the experiment shown in Fig. 1C (data not shown). However, after prolonged autoradiography, a weak band shift was detected for RXRα homodimers which was specific for the DR1 probe. These results are consistent with the lack of significant constitutive activation of the reporter gene series by homodimers. In our hands, the inclusion of *all-trans* RA or *9-cis* RA (500 nM) in the growth medium, extraction buffer and assay buffer did not further stimulate the binding of either homodimers or heterodimers to any of the probes (data not shown), although we did not determine if the conditions used in the gel shift experiments were optimal for the binding of ligand to the receptors.

9-cis retinoic acid induced activity of RXRα on putative RXRE sequences in yeast

Ligand dose response curves (Fig. 2A) revealed that the concentration of *9-cis* RA required for half maximal activity of RXRα on the DR1 reporter in yeast was approximately 100nM. In contrast, no induction was observed in the presence of *all-trans* RA, even at a concentration of 1μM. This is in agreement with our previous data for the chimeric receptors RXRα-ER(C) and RXRα(DEF)-ER.Cas, whose activities were induced exclusively by *9-cis* stereoisomers of RA in yeast (10). The dose response curves in the presence of both RARα and RXRα confirmed the modest stimulation of the DR1 reporter activity by the heterodimer in the presence of *all-trans* RA (Fig. 2A). In addition, experiments using a transcriptionally inactive dominant negative RXRα (dnRXRαΔAB; refs. 10, 28) confirmed that RARα is responsible for this stimulation by *all-trans* RA (D.M.H. and B.P., unpublished results). Thus, the DR1 element appears to bind both RAR/RXR heterodimers and RXR alone (presumably as homodimers). This result is in contrast to a recent report by Hall *et al.*, (42) who were unable to demonstrate significant activity of RXR on a DR1 element. Note that these authors used another receptor isoform (RXRγ), a different reporter (based on the yeast CYC1 promoter), and a different spacer nucleotide in the DR1 sequence as compared to this study. To confirm that the RXRα activity we observed on DR1 was not simply due to high level expression of the receptor in yeast, we expressed RXRα from a centromeric expression vector. Significant *9-cis* RA induced activation was maintained on the DR1 and IR0 reporters (10- to 15-fold), despite a strong decrease (30- to 50-fold) in the level of receptor detectable in western blot analyses (data not shown).

The rat CRBPII gene promoter contains a sequence consisting of four almost perfect repeats of the hexamer motif 5′-AGGTCA -3′ with 1bp spacers, which has been reported to act as a RXRE in animal cells (33, 34). We determined if this element would also function as a target for transactivation by RXRα in yeast. As shown in Fig. 2B, the rat CRBPII RXRE reporter was very efficiently activated (116-fold over the control) by RXRα in the presence of *9-cis* RA in yeast, i.e. almost 20-fold higher than that of the DR1 reporter in the same system. This result indicates a synergistic activation by RXRα molecules bound to the multiple binding sites in this promoter. As observed with the DR1 reporter, the activity of the rat CRBPII RXRE reporter was also significantly induced (12-fold) in the presence of 9-*cis* RA, when RXRα was expressed from a centromeric vector (data not shown). These results indicate a synergistic activation by RXRα molecules bound to the multiple binding sites in this promoter. Coexpression of RARα and RXRα in yeast increased the activity of the rat CRBPII RXRE reporter 14-fold above the control in the absence of ligand, and 21-fold and 70-fold above the control in the presence of *all-trans* RA and *9-cis* RA, respectively (data not shown). Accordingly, gel shift experiments showed that RARα strongly increased the binding of RXRα to the rat CRBPII RXRE element *in vitro* (not shown), and thus coexpression of both receptors in yeast did not have the same dramatic negative effect on activation of the rat CRBPII RXRE reporter as previously observed in some (33), but not all (34), animal cells.

Our previous study (10) established that a *9-cis* RA-inducible activation function (AF-2) present in the ligand binding domain is largely responsible for the activity of RXRα in yeast, as we failed to detect a strong activation function in the N-terminal (AB) region of RXRα by linking it to a heterologous DBD. Accordingly, deletion of the A/B region of RXRα (RXRαΔAB) did not adversely affect the levels of activation achieved on either the DR1 or rat CRBPII RXRE reporters, in the presence or absence of ligand (Fig. 2B). In contrast, a truncated receptor in which the 19 most carboxy-terminal amino acids were deleted (dnRXRα), which behaves as a dominant negative receptor in mammalian cells (28), failed to mediate a response of either the DR1 or the rat CRBPII RXRE reporters to *9-cis* RA in yeast (Fig. 2B), despite being expressed to a similar level as the full length receptor (not shown). This result indicates that the carboxyl terminal region is required for ligand-induced transcription by RXRα. It is also of note that unliganded RXRα stimulated the rat CRBPII RXRE reporter activity 8- to 10-fold over the activity of the control (Fig. 2B). This constitutive activity was observed

even when the A/B domain was deleted, and thus may indicate a weak constitutive activity of AF-2.

DISCUSSION

We have demonstrated that RXRα activates transcription efficiently in a biological system devoid of other members of the nuclear receptor superfamily. This activity of RXRα was found to be specific for elements having a DR1-type structure, so-called 'RXREs', and to a lesser extent, an inverted repeat similar to TRE-pal. DR1-type elements have been found in the promoters of genes including the rat CRBPII (33), human ApoA1 (30) and mouse CRABPII (28) genes, which are involved in vitamin A metabolism, and it has been suggested that this may signify the existence of a *9-cis* RA autoregulatory network which operates through RXR (43). However, DR1 elements are also recognised by other members of the steroid receptor superfamily, including PPAR, COUP-TF, Arp-1, HNF4, and ear2 (20, and references therein). Thus it was important to test the ability of RXR to activate transcription from a DR1 element in a system which has no known homologues of nuclear receptors. Transactivation was very potent on the rat CRBPII RXRE reporter, which contains multiple DR1 repeats, suggesting synergistic activation by RXRα molecules bound to the multiple target sites. Our findings that RXRα strongly activates reporter genes driven by DR1 elements in yeast are consistent with the hypothesis that, in addition to their role as heterodimeric partners of RAR, VDR, PPAR and TR, RXRs may also function independently as ligand-dependent transcription factors, in the more classical fashion of steroid receptors, i.e. as homodimers. Similarly, homodimers of RARα were found to activate transcription in yeast (albeit less efficiently than heterodimers) in a ligand-dependent manner, but only on a DR5 reporter. As with steroid receptors such as the ER, transactivation by homodimers of RARs and RXRs appears to be largely ligand-dependent, and in the case of RXRα is due to the presence of an activation function (AF-2) in the ligand-binding domain which is specifically induced by 9-*cis* stereoisomers of RA. In addition AF-2 may also be responsible for the weak constitutive activity of RXRα observed on the rat CRBPII RXRE and DR1 reporters. In contrast, the A/B region of RXRα appears to be dispensable for the transcriptional activity of this receptor in our experiments.

RA-dependent transactivation in animal cells appears to be mediated through DRs consisting of half-site motifs spaced by 1 to 5 base pairs. Our results in yeast corroborate this observation, showing a strong correlation between the affinity of the heterodimer complex for a sequence and the strength of activation of the corresponding reporter gene in yeast. Although in this report we have only considered the effect of spacer length on DNA binding and activation, additional experiments have confirmed that the sequences of the half sites, spacers and flanking DNA all clearly influence the strength of binding and activation by heterodimers in yeast (our unpublished results), in agreement with the findings of Mader *et al.*, (32).

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