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Biochemistry

Reconstitution of retinoid X receptor function and combinatorial regulation of other nuclear hormone receptors in the yeast *Saccharomyces cerevisiae*

(DNA binding/dimerization/retinoic acid/thyroid hormone)

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ABSTRACT **The nuclear hormone receptor family of transcription factors regulates gene expression via a complex combinatorial network of interactions. Of particular interest is the ability of retinoid X receptors (RXRs) to form heterodimers with retinoic acid receptors (RARs) and thyroid hormone receptors (TRs), thereby modifying their activities. We report here that RXR, RAR, and TR function can be reconstituted in the yeast *Saccharomyces cerevisiae* and demonstrate that the combinatorial regulation seen in vertebrate cells can be reproduced in the yeast background. Using this system, we have shown that RARs respond to a wide variety of retinoid ligands but that RXRs are specific for the 9-*cis* isomer of retinoic acid. RXR enhanced the activity of RARs and TRs on a variety of hormone response elements without demonstrably altering their DNA specificity. Interestingly, the ability of RXR to potentiate gene activation by RARs and by TRs varied for different receptor isoforms.**

The nuclear hormone receptors, a family of interrelated transcription factors, play key roles in vertebrate development, differentiation, and regulation of cell growth (refs. 1 and 2 and references therein). These receptors modulate gene expression in response to hormone by binding to specific "hormone response elements" (HREs) in the promoter regions of target genes (1, 2). The nuclear hormone receptor family includes the steroid receptors, thyroid hormone receptors (TRs), retinoic acid receptors (RARs), and retinoid X receptors (RXRs). RARs respond to all-*trans* and 9-*cis* retinoic acid (RA), whereas RXRs have been reported as specific for the 9-*cis* isomer (3, 4). Although it was once believed that each nuclear hormone receptor functioned autonomously, it is now recognized that these receptors interact with one another and participate in a complex network of combinatorial control (5–9). Of particular significance is the observation that RXRs can form heterodimers with TRs and RARs, thereby enhancing their DNA binding and transcriptional activation properties.

Investigation of these RXR interactions by transfection studies of vertebrate cells has been complicated by the unavoidable background of endogenous nuclear hormone receptors present in these cells and by the metabolic interconversion of different retinoid isomers (3–9). It has been particularly difficult to separate the autonomous functions of the RXR moiety from its effects on other nuclear hormone receptors in the cell or to resolve which retinoid isomers are actually involved in receptor activation. To circumvent the complexity of a vertebrate cell system, we investigated the usefulness of the yeast *Saccharomyces cerevisiae* for studying RXRs, RARs, TRs, and their interactions. *S. cerevisiae* lack known nuclear hormone receptors and it is likely that they lack much of the metabolic machinery for creating or modifying the corresponding hormone ligands. Nonetheless, a variety of vertebrate receptors function when expressed ectopically in *S. cerevisiae* (e.g., refs. 10–12 and references therein). We report here the reconstitution of the transcriptional activities of RXR and RAR in *S. cerevisiae* and demonstrate that the combinatorial interactions between these receptors seen in vertebrate cells can be reproduced in the yeast.

MATERIALS AND METHODS

Plasmids and Oligonucleotides. The pΔSS reporter, pG1-c-*erb*Aα (TRα), pG1-c-*erb*Aβ (TRβ), and pG1-RARβ constructs have been described (12, 13). Chicken RXRγ (the generous gift of Paul Brickell, University College, London) was introduced into the pG1 vector by subcloning the chicken RXRγ-containing *Eco*RI fragment from the pR2 plasmid (14) into pBluescriptI, modifying the *Eco*RV site in the polylinker to a *Bgl* II site, and then transferring a *Bgl* II–*Bam*HI (partial digest) fragment into the *Bam*HI site of pG1. The pG1-RARγ plasmid has the RARγ-containing *Bam*HI fragment from pSG5-RARγ (15) inserted at the *Bam*HI site of pG1. The *Bam*HI fragments from pG1-c-erbAβ (12) and pSG5-RARβ (15) containing the receptor coding domains were inserted into the *Bam*HI site of the p2HG vector (16) to create the plasmids p2HG-c-erbAβ and p2HG-RARβ, respectively.

Yeast Assays. Double transformants of the BJ2168 strain of *S. cerevisiae* were isolated and maintained on S medium lacking tryptophan and uracil; triple transformants of the YPH 399 strain were isolated and maintained on S medium lacking histidine, tryptophan, and uracil (12, 13, 16). The BJ2168 yeast strain is deficient in three proteases and reporter gene activity is measurably higher in this strain than in the YPH399 background. Trans-activation assays were performed as described (12, 13, 16). All of the data presented represent results obtained from at least two independent yeast transformants.

Synthesis of Retinoid Mixtures. A 5 mM solution of all-*trans* RA, retinal, or retinol in 100% ethanol was photoisomerized by fluorescent irradiation for 1.5 hr. The presence of 9-*cis* RA in the RA mixture was confirmed by HPLC analysis as described (3). Purified 9-*cis* RA was generously provided by Richard Heyman and Ligand Pharmaceutical (San Diego).

RESULTS

RXR and RARs Function in Yeast. We first asked if RXRs and RARs could each activate the expression of a suitable reporter gene in a hormone-specific fashion in *S. cerevisiae*.

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Abbreviations: RXR, retinoid X receptor; RAR, retinoic acid receptor; TR, thyroid hormone receptor; RA, retinoic acid; DR, direct repeat; HRE, hormone response element.

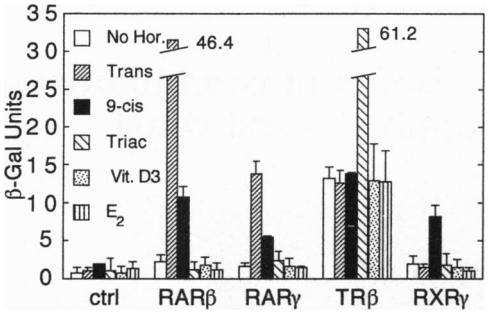

FIG. 1. Activity and hormone specificity of RARs, RXR, and TR. Yeast strain BJ2168 isolates containing the pΔSS-TRE_{pal} reporter plasmid (bearing an inverted repeat of AGGTCA, Fig. 5*A*) and expressing RARβ, RARγ, TRβ, or RXRγ were grown in the absence of hormone or the presence of 10 μM all-*trans* RA (Trans), 9-*cis* RA (9-cis), triac, vitamin D3 (Vit. D3), or estradiol (E_2) as indicated. The cultures were then harvested and were assayed for β-galactosidase (β-Gal) production. Control yeast isolates containing the pG1 vector without an insert were also analyzed (ctrl). The values for RARβ plus all-*trans* RA and TRβ plus triac were 46.4 ± 4.0 and 61.2 ± 12.6, respectively.

We cotransformed yeast with two plasmids, one expressing the receptor of interest and the other expressing a reporter plasmid containing a yeast promoter, an HRE for the receptor, and the easily assayed *lacZ* gene. In this manner, receptor function in the resulting transformants was coupled to β-galactosidase expression. The reporter in our initial experiments contained an inverted repeat sequence (AGGTCATGACCT) that functions as a HRE for RARs, RXRs, and TRs in mammalian cells (5–9). RARβ was able to strongly activate expression of this reporter gene in the yeast in response to either all-*trans* or 9-*cis* RA; none of the nonretinoid hormones tested could substitute in this regard (Fig. 1). The RARγ isoform demonstrated lower, but otherwise parallel, activities (Fig. 1). In contrast, RXRγ was a more modest activator of reporter gene expression in the yeast and was specific for 9-*cis* RA (Fig. 1). As previously reported, TRβ was also able to activate expression of this reporter gene (12, 16). Although exhibiting some hormone-independent activity, TRβ activity was further stimulated by triac, a thyroid hormone derivative, and was insensitive to the retinoids, vitamin D3, or estradiol (Fig. 1).

We repeated these experiments using a range of concentrations of purified 9-*cis* or all-*trans* RA (Fig. 2). RARβ (Fig. 2*A*) and RARγ (Fig. 2*B*) responded to both isomers and exhibited similar dose–responses, with a peak of activity at 1 μM in the culture medium. RXRγ responded only to the 9-*cis* isomer and exhibited no response to all-*trans* RA even at quite high hormone concentrations (Fig. 2*C*). We next tested a variety of other retinoid ligands (Fig. 3). RARβ and RARγ were able to respond to a wide range of retinoids, including multiple isomers of RA and of the aldehyde, retinal. In contrast, RXRγ retained a rigid specificity for the 9-*cis* RA ligand (and for a photoisomerized RA mix containing 9-*cis* RA) and did not respond to any other retinoid tested or to photoisomerized mixtures generated from retinal or retinol (Fig. 3).

RXR Synergism with RAR and TR Occurs in *S. cerevisiae* and Does Not Require the 9-*cis* Ligand. We next addressed whether the synergistic effects seen between RXRs and RARs or RXRs and TRs in vertebrates could also be reproduced in yeast cells. We created yeast YPH399 triple transformants containing the reporter plasmid, a p2HG vector to express the TR or RAR, and a pG1 vector to express the RXR. We first repeated our prior experiments by expressing the receptors individually, but in the triple plasmid strain. If an individual receptor was to be tested alone, the corresponding "empty" vector was introduced into the YPH399 strain to maintain a three-plasmid system. Perhaps as a result of the additional plasmid load or a strain-related difference, the activity of any individual receptor was lower when expressed in these triple transformants than that seen previously in the BJ2168 strain (Fig. 4). Nevertheless, significant stimulation was seen when these receptors were coexpressed with one another. For example, the RXR response to 9-*cis* RA was enhanced 10- to 20-fold by coexpression of TRβ (Fig. 4*B*). Reciprocally, the TRβ response to triac was also reproducibly enhanced in the RXRγ cotransformant, although in this case by a more modest 1.5- to 2-fold (Fig. 4*A*; also see Fig. 7). This mutual enhancement of activity is similar to that previously reported in animal cells and is generally attributed to the formation of RXR/TR heterodimers (5–9). Similar results were obtained using a direct repeat (DR) response element with a four base spacer (data not shown).

RXRγ and RARs exhibited equally dramatic synergistic interactions in the yeast. Coexpression of RXRγ enhanced the RARβ response to all-*trans* RA 6- to 7-fold over that seen for RARβ alone (Fig. 4*D*), whereas RXRγ exhibited no response to all-*trans* RA on its own (Fig. 1). Response to 9-*cis* RA was also greater in RXRγ/RARβ cotransformants than in transformants expressing only one of the receptors (Fig. 4*E*). Similarly, RARγ together with RXRγ resulted in a 5-fold enhancement of reporter gene transcription over that seen in independent assays (data not shown). RAR and RXR interactions were also seen on a DR-5 response element (data not shown).

In yeast coexpressing TRβ and RXRγ, addition of triac and retinoid hormones together had a slightly less than additive effect on the reporter response compared to either ligand alone (compare Fig. 4*C* with the sum of RXRγ plus TRβ in Fig. 4 *A* and *B*). The ligand requirement of the RAR/RXRγ interaction was more difficult to dissect, given that RARs respond to all-*trans* and 9-*cis* RA. Notably, however, RXRγ enhanced the RARβ response to a mixture of 9-*cis* and all-*trans* RA to the same extent (≈6-fold) as to all-*trans* RA alone (Fig. 4, compare *D* and *F* in the absence or presence of RXRγ). These results suggest that the hormone response of

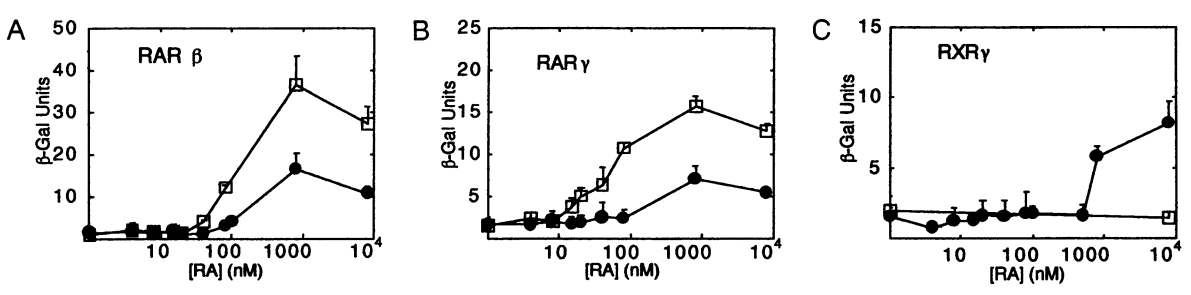

FIG. 2. Dose–response of retinoid receptors to hormone ligand. Yeast strain BJ2168 isolates containing the pΔSS-TRE_{pal} reporter plasmid and expressing RARβ (*A*), RARγ (*B*), or RXRγ (*C*) were grown in the presence of all-*trans* (open squares) or purified 9-*cis* (closed circles) RA, ranging in concentration from 0 to 10 μM, and assayed for β-galactosidase production.

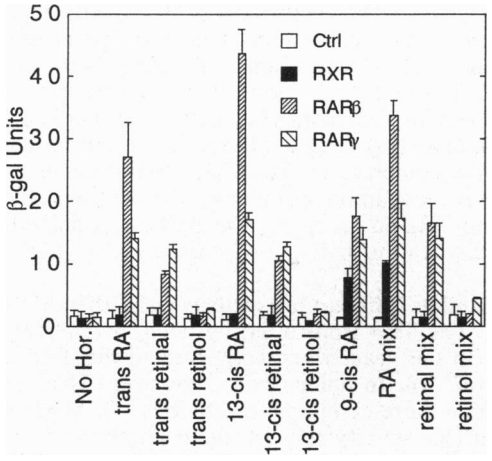

FIG. 3. Activation of retinoid receptors by various retinoids. Yeast strain BJ2168 isolates containing the pΔSS-TRE$_{pal}$ reporter vector and expressing RARβ, RARγ, or RXRγ were grown in either the absence of hormone or the presence of 10 μM purified retinoid hormone, either all-*trans* RA, all-*trans* retinal, all-*trans* retinol, 13-*cis* RA, 13-*cis* retinal, 13-*cis* retinol, or 9-*cis* RA, as indicated. Alternatively, the yeast were cultured in the presence of 40 μM photoisomerized RA mixture, retinal mixture, or retinol mixture. The yeast were then harvested and assayed for β-galactosidase production. β-Galactosidase expression in yeast containing the pG1 vector without an insert was <2.0 units for all hormones tested.

one partner in the RXR heterodimer was not further stimulated by hormone occupancy of the other partner.

RXR Enhances the Activities of RAR and TR on Several Different Response Elements Without Significantly Altering Their DNA Specificity. It has been proposed that RXR interaction with other receptors might influence their DNA binding specificity. HREs consist of repeated hexanucleotide sequences denoted "half-sites;" the ability of a receptor to recognize a given HRE depends on the sequence and the orientation of the half-sites that comprise the element. RARs

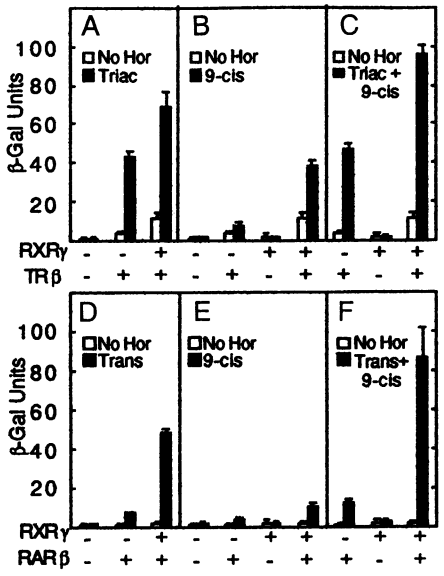

FIG. 4. Reporter gene activation by coexpression of RXR with RAR or TR. Yeast strain YPH399 isolates containing the pΔSS-TRE$_{pal}$ reporter plasmid and containing an empty p2HG plasmid, an empty pG1 vector, or expressing TRβ (*A–C*) or RARβ (*D–F*) in the presence or absence of RXRγ were isolated. The yeast were then grown in the absence of hormone or in the presence of 10 μM triac (*A*), 10 μM 9-*cis* RA (*B* and *E*), 10 μM triac plus 10 μM 9-*cis* RA (*C*), 10 μM all-*trans* RA (*D*), or 10 μM all-*trans* RA plus 10 μM 9-*cis* RA (*F*) and were assayed for β-galactosidase production.

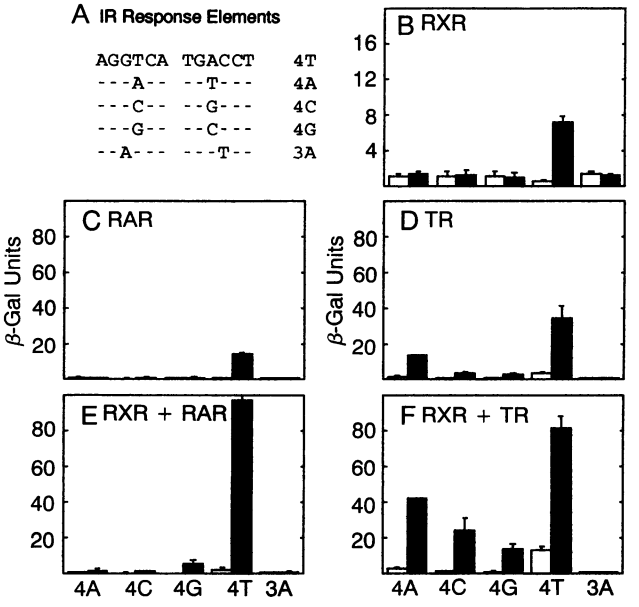

FIG. 5. Half-site specificity of RAR and TR, with or without RXR, on inverted repeat elements. Yeast strain YPH399 transformants containing a reporter plasmid linked to various inverted repeat (IR) response elements (*A*) and expressing RARβ (*C*), TRβ (*D*), RARβ plus RXRγ (*E*), or TRβ plus RXRγ (*F*) were grown in the absence of hormone (open bars) or in the presence (solid bars) of a 40 μM RA mixture (*B*, *C*, and *E*) or 10 μM triac plus a 40 μM RA mixture (*D* and *F*) and were assayed for β-galactosidase production. The activity of RXRγ in BJ2168 was similarly evaluated (*B*); RXRγ activity alone in YPH399 was <2.0 units on all elements tested. Control yeast isolates containing the pG1 and p2HG vectors without inserts were also assayed and exhibited <2.0 units of activity for all elements tested.

and TRs can bind and activate transcription at AGGTCA half-sites displayed as inverted repeats or DRs (see Fig. 5*A*), but only TRs can also recognize AGGACA, and more modestly AGGCCA and AGGGCA half-sites (13). These specificites were reproduced in the yeast (Fig. 5 *C* and *D*; ref. 13). RXRγ, like RARβ, exhibited a strong preference for the AGGTCA half-site element (most easily visualized in the stronger BJ2168 background, Fig. 5*B*). Coexpression of RXRγ and RARβ resulted in enhanced activation on the AGGTCA element but no significant change in specificity from that of RARβ alone (compare Fig. 5 *C* and *E*). Similarly, coexpression of RXRγ and TRβ enhanced the absolute levels of reporter gene expression without changing the relative order of recognition of the different half-sites (AGGTCA > AGGACA > AGGCCA > AGGGCA) from that seen for TRβ alone (Fig. 5 *D* and *F*).

The effects of RXR on recognition of the topology of the half-sites that comprise a response element were tested by comparing similar half-sites, but oriented as direct repeats with different spacings. We used either the "consensus" AGGTCA half-site with a 1-, 3-, 4-, or 5-base pair spacer (denoted DR-1, DR-3, DR-4, and DR-5, respectively) or the TR-specific AGGACA half-site oriented as a DR-4 (denoted 4A-DR-4) (Fig. 6*A*). Interestingly, RXRγ alone failed to activate transcription of the reporter gene on any DR element in either strain of *S. cerevisiae* (Fig. 6*B* and data not shown). When expressed alone, RARβ exhibited a preference toward a DR-5 element, although it could also activate from a DR-3 or DR-4 element (shown for the YPH399 strain in Fig. 6*C*; activation of DR-3 and DR-4 elements by RARβ alone is more clearly seen with the higher levels of reporter gene expression exhibited by the BJ2168 yeast strain; ref. 13). When RXRγ and RARβ were present, an enhanced activation of all AGGTCA DR elements was seen, including a slight response

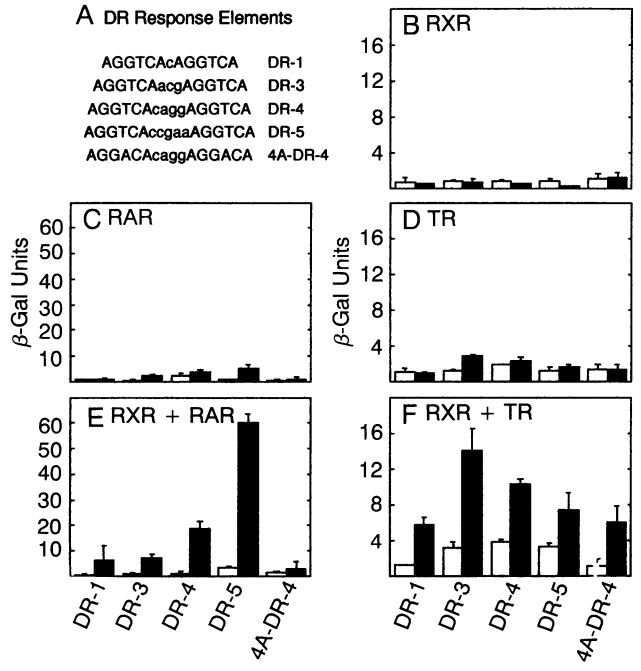

FIG. 6. Response element specificity of RXR with RAR and TR on direct repeat elements. Yeast strain YPH399 transformants containing a reporter plasmid linked to various DR response elements (*A*) and expressing RXRγ (*B*), RARβ (*C*), TRβ (*D*), RARβ plus RXRγ (*E*), or TRβ plus RXRγ (*F*) were grown in the absence of hormone (open bars) or the presence (solid bars) of a 40 μM RA mixture (*B*, *C*, and *E*) or 10 μM triac plus a 40 μM RA mixture (*D* and *F*) and were assayed for β-galactosidase production. Control yeast isolates containing the pG1 and p2HG vectors without inserts were also assayed and exhibited <2.0 units of β-galactosidase activity for all elements tested.

on a DR-1 element not detectable in the absence of RXR. However, the relative pattern of RARβ activation in the presence of RXRγ generally paralleled that of RARβ alone, with strongest activity on a DR-5 and weaker, but easily detectable, function on a DR-4 and a DR-3.

TRβ alone exhibited a fairly broad specificity for different half-site spacings, activating from a DR-3, DR-4, or DR-5 element (Fig. 6*D* and seen more clearly at the higher reporter expression levels exhibited by the BJ2168 strain background; ref. 13). Coexpression of RXRγ enhanced TRβ reporter gene

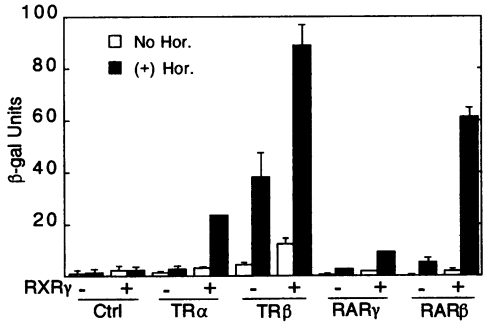

FIG. 7. RXR interactions with different receptor isoforms. Yeast strain YPH399 isolates containing the pΔSS-TRE$_{pal}$ (for TRα and TRβ) or the pΔSS-DR-5 (for RARβ and RARγ) reporter plasmids and expressing RXRγ alone (Ctrl +), TRα alone, TRβ alone, RARγ alone, RARβ alone, or these receptors plus RXRγ (as indicated) were grown in the absence of hormone (open bars) or the presence of 10 μM triac plus 40 μM RA mixture (TRα and TRβ, solid bars) or 40 μM RA mixture (RARβ and RARγ, solid bars) and were assayed for β-galactosidase production. Control yeast isolates containing the pG1 and p2HG vectors (Ctrl −) without inserts were also assayed.

expression on all DR elements (Fig. 6*F*), with the relative pattern on each reporter very similar to that observed for TRβ alone (13). Coexpression of RXRγ with TRβ also produced detectable activation with a DR-1 element that was not observed for TRβ alone (Fig. 6*F*); we do not know if this activation of a DR-1 simply reflects the enhanced sensitivity of detection conferred by TR/RXR coexpression or a truly novel DNA recognition specificity. The different abilities of RARβ and TRβ to recognize the AGGΔCA half-site were preserved when this half-site was displayed as a DR (Fig. 6 *E* and *F*).

RXR Exhibits Different Interactions with Different Receptor Isoforms. The TRα isoform is, by itself, a relatively weak activator in the yeast when compared to the TRβ isoform (Fig. 7; ref. 16). In animal cells, however, TRα and TRβ demonstrate more comparable activities (1). Might the difference in TRα activity in yeast and in vertebrate cells reflect a greater dependence of the TRα isoform on RXR heterodimer formation? Consistent with this hypothesis, coexpression of TRα and RXRγ in the yeast was able to confer significant reporter gene expression in response to triac, and the enhancement of the TRα response by RXRγ was greater than that seen for TRβ (≈5-fold for TRα vs. 2- to 2.5-fold for TRβ; Fig. 7). The isoform specificity of the RXR interaction extended to the RAR receptors; on a DR-5 element, for example, RXRγ enhanced RARβ activity 9-fold but enhanced RARγ activity only 3-fold (Fig. 7). Intriguingly, this difference between RAR isoforms was not as pronounced when an inverted repeat element was used (data not shown).

DISCUSSION

RXRs can interact with many other nuclear hormone receptors by forming heterodimers, and these interactions may play a key role in the regulation of transcription (5–9). It has been difficult, however, to definitively characterize these interactions in vertebrate cells. We have demonstrated here that TRs, RXRs, and RARs are able to mediate hormone-dependent activation of appropriate reporter genes in *S. cerevisiae* and that many of the synergistic receptor interactions described in vertebrate cells can be duplicated in the yeast. The *S. cerevisiae* system also appears to lack many of the problems arising in animal cells due to the presence of endogenous hormones and endogeneous nuclear hormone receptors.

By exploiting the yeast system, we have been able to more clearly define the hormone specificity of RXR and RAR. In animal cells, high levels of all-*trans* RA can stimulate RXR activity, and this has been attributed to metabolic interconversion of the all-*trans* isomer to 9-*cis* RA (3, 4). We confirmed this hypothesis by demonstrating that in *S. cerevisiae* RXRγ only responded to 9-*cis* RA and not to any other retinoid tested. RARβ and RARγ, on the other hand, responded to a much wider range of RAs and retinals, including the 9-*cis* and 13-*cis* isomers. This pattern is very similar to that seen in vertebrate cells with one exception: in the yeast all-*trans* RA was a stronger activator of RARs than was 9-*cis* RA, whereas in mammalian cells the 9-*cis* isomer has been reported as the more active isomer (3). This apparent difference in the response of RARs in yeast and in mammalian cells may be due to differences in permeability, stability, or interconversion of the two RA isomers. Alternatively, the glucocorticoid receptor expressed in yeast displays an altered hormone specificity from that seen in vertebrate cells (10), and our results may be a reflection of a similar phenomenon.

Given their predisposition for strong interactions with other nuclear hormone receptors, the autonomous functions of RXRs have been particularly difficult to elucidate in animal cells. One controversy has centered on the relative contribution of RXR homodimers and RXR/RAR heterodimers to

the transcriptional activation of DR-1 and DR-5 response elements (17–19). RXRγ by itself could not function with any of the DR elements we tested, including a DR-1. In contrast, RXR/RARβ cotransformants did demonstrate low but detectable activation at a DR-1 element as well as strong activation of DR-5 elements. Our results appear to support the concept that RXRs best recognize DR-1 and DR-5 response elements when in a heterodimer with another receptor, such as an RAR. However, we cannot fully exclude that the RXR response on a DR-1 is simply too weak to detect in our experiments, or that some other entity, absent in *S. cerevisiae*, regulates RXR recognition of DR-1 elements.

We could mimic in the yeast many of the synergistic RXR/RAR and RXR/TR interactions observed in animal cells. Therefore, these interactions must largely reflect autonomous properties of these receptors and must not require additional factors unique to metazoan cells. These receptor interactions appear to be largely reciprocal. For example, the response of the RXR/TR heterodimer to either 9-*cis* RA or to thyroid hormone was elevated relative to that seen with the RXR and TR assayed separately. Notably, occupancy of one receptor by cognate ligand was not necessary for enhancement of the activity of the other receptor partner. However, the greatest overall stimulation of reporter gene expression was seen when ligands for both heterodimer partners were present. We propose that the presence of both ligands serves to recruit both receptors in the heterodimer as active transcription factors, resulting in a combined activity approximately equal to the sum of that seen in the presence of each ligand separately. An alternative suggestion, that the presence of both ligands might actually alter the nature of the interaction between the two partners of the heterodimer, would predict a nonadditive effect, which we do not see.

It has been postulated that heterodimer formation of other receptors with RXRs may modify their actions not just by enhancing their transcriptional response but also by altering their DNA binding specificity. This did not appear to be the case for the range of response elements examined here. Intriguingly, however, we found that RXRγ did not interact equally with different isoforms of the TRs and RARs. It will be interesting to examine the interactions of the different RAR and TR isoforms with RXRα and RXRβ. We anticipate that use of yeast for studying these receptor interactions, particularly when coupled to the genetics available in this organism, will provide a powerful system for the elucidation of combinatorial regulation by nuclear hormone receptors.

B.L.H. and Z.S.-M. contributed equally to this work. We thank Paul Brickell for supplying the avian RXRγ clone, Richard Heyman and Ligand Pharmaceutical for the purified 9-*cis* RA, Matthew Terry and J. Clark Lagarias for assistance with the HPLC, and Pierre Chambon for providing the RARβ and RARγ clones. We also thank Cathy Judelson for her expert technical assistance. This work was funded by Public Health Service Grant CA53394 from the National Cancer Institute. B.L.H. was supported, in part, by the Floyd and Mary Schwall Fellowship Fund and by a National Institutes of Health predoctoral training grant. Z.S.-M. was supported by National Research Service Award Fellowship F32 CA09050.

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