Two Receptor Interacting Domains in the Nuclear Hormone Receptor Corepressor RIP13/N-CoR

Wongi Seol, Matthew J. Mahon, Yoon-Kwang Lee, and David D. Moore

Department of Molecular Biology
Massachusetts General Hospital
Boston, Massachusetts 02114

The thyroid hormone receptor (TR) and the retinoic acid receptor (RAR) act as transcriptional repressors when they are not occupied by their cognate ligands. This repressor function is mediated by proteins called corepressors. One of the nuclear hormone receptor corepressors, N-CoR, was originally isolated as a retinoid X receptor-interacting protein called RIP13. We have isolated a new potential variant of RIP13/N-CoR that is missing previously described transcriptional repressor domains but is similar in structure to the related corepressor termed SMRT or TRAC-2. Detailed analysis of the interaction with TR and RAR demonstrates that RIP13/N-CoR contains a new receptor interaction domain, termed ID-II, in addition to the previously described domain, referred to here as ID-I. Both ID-I and ID-II are capable of interacting independently with either TR or RAR, as assessed by the yeast two-hybrid system, by a mammalian two-hybrid system, or by direct *in vitro* binding. Results with all three approaches confirm that RIP13/N-CoR also interacts with retinoid X receptor, but this interaction is weaker than that with TR or RAR. Together, these results demonstrate that RIP13/N-CoR can interact with several different nuclear hormone receptors via two separate receptor interaction domains. Differences between the interactions observed in the different systems suggest that corepressor function may be modified by additional factors present in various cell types. (Molecular Endocrinology 10: 1646–1655, 1996)

INTRODUCTION

The nuclear hormone receptor superfamily is a group of transcription factors that regulate target genes in response to steroids, thyroid hormone, retinoids, vitamin D, and other small hydrophobic compounds (1–5). Thyroid hormone receptor (TR) and retinoic acid receptor (RAR) belong to a subfamily of the hormone receptor superfamily that also contains retinoid X receptor (RXR), vitamin D receptor (VDR), and a number of orphan receptors whose ligands are unknown (2, 3, 6–13). Although these proteins generally transactivate transcription through their specific DNA-binding sites, both TR and RAR have also been reported to silence transcriptional activity of the target genes in the absence of their cognate ligands (14–18). This silencing activity of the receptors has been shown to require specific cellular cofactors called corepressors, which contact receptors physically (19–23).

Recently, two distinct proteins, N-CoR and SMRT/TRAC, have been characterized as such corepressors (24–26). Both are reported to interact with unliganded TR and RAR but are released from the receptors upon ligand binding. N-CoR contains two repressor domains at its amino terminus and one receptor interaction domain at the carboxyl terminus (25). SMRT/TRAC has a generally similar arrangement, with sequences required for repression and the amino terminus and sequences required for receptor interaction at the carboxyl terminus (24, 26).

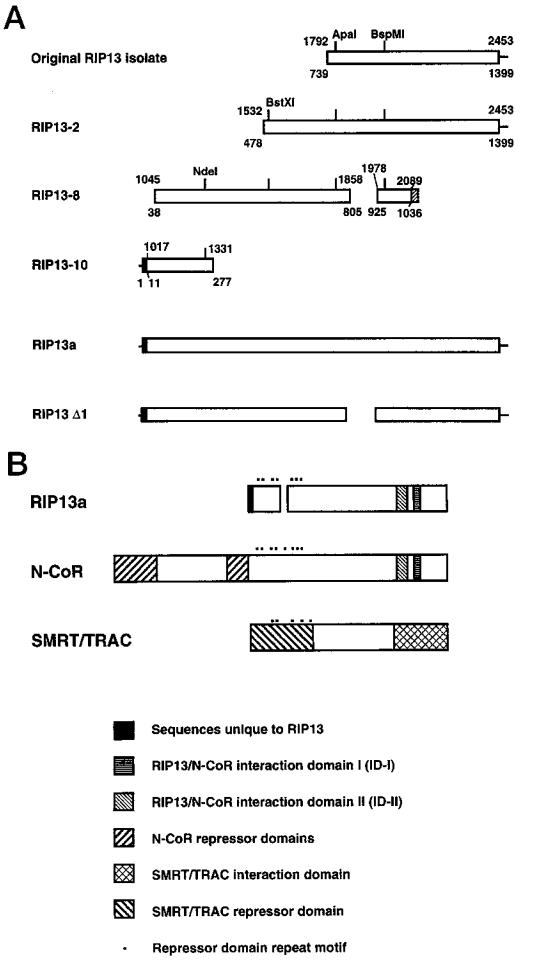
RIP13, one of a number of RXR-interacting proteins isolated using the yeast two-hybrid system (11), is identical to the carboxyl-terminal portion of N-CoR (25). Additional cDNA clones extending beyond the original RIP13 isolate were obtained by conventional screening, leading to the identification of a variant form of N-CoR referred to as RIP13a. The interaction of this protein with various receptors was characterized genetically using the two-hybrid system in both yeast and mammalian cells and also biochemically using glutathione-S-transferase (GST) fusion proteins. These studies define a new RIP13/N-CoR receptor interaction domain, termed ID-II, which is distinct from the carboxyl-terminal domain described previously. Each of these domains can interact independently with either TR or RAR.

RESULTS

Sequence Comparison of RIP13 and N-CoR

To obtain RIP13 sequences beyond those present in the original yeast two-hybrid isolate (11), that original

0888-8809/96/$3.00/0
Molecular Endocrinology



C

```
RIP13/N-CoR  1 .........................MCASCSSVPP..VLQPAPHQ... 18/1025
                                        :: . .,|||  |:.:.||.
      SMRT   1 MEAWDAHPDKEAFAAEAQKLPGDPPCWTSGLPFPVPPREVIKASPHAPDP 50

            19 .VITNLPEGVRLP.....TTRPTRPPPPLIPSSKTTVASEKPSFIMG... 59/1066
               .:.  |.| .||     |.||. |.|| |..... :.|.|.. :::
            51 SAFSYAPPGHPLPLGLHDTARPVLPRPPTISNPPPLISSAKHPSVLERQI 100
               1          .          .  2       .          .
            60 GSISQGTPGTYLSSHNQAYPQEAPKPSVGSISLGLPRQQESTKAAPLTYI 109/1116
               |.||||     :| : :.   | :|:.||.:.:||| . :..| ||:. :
           101 GAISQG.....MSVQLHVPYSEHAKAPVGPVTMGLPLPMDPKKLAPFSGV 145
                                                  .          3
           110 KQEEFSPRSQNSQPEGLLVR.AQHEGVVRGTA.GAVQEGSITRGTPASKI 157/1164
               |||::|||:|.:.||:| |. ||..:|:|||| |.|.:||||:|.|..::
           146 KQEQLSPRGQAGPPESLGVPTAQEASVLRGTALGSVPGGSITKGIPSTRV 195
                        4          .          .          .
           158 SVETISSLRGSITQGTPALPQAGIPTEALVKGPVSRMPIEESSP..EKVR 205/1212
               .:.  ..|||||:|||       :.| ||:.:|:  |:|..  ::.|
           196 PSDSAITYRGSITHGTPA.....DVLYKGTITRIIGEDSPSRLDRGR 237
                                                  .          .
           206 EEAASKGHVIYEGKSGHILSY..................................... 226/1233
               |:. .|||||||||.||:|||
           238 EDSLPKGHVIYEGKKGHVLSYEGGMSVTQCSKEDGRSSSGPPHETAAPKR 287
                                                  .          5
           227 ..................DSLICRALP..RGSPHSDLKERTVLSGSTMQG 256/1310
                                  ::|:.||:|  | ||| .|||.  :.||| ||
           288 TYDMMEGRVGRAISSASIEGLMGRAIPPERHSPH.HLKEQHHIRGSITQG 336
                                                  .          6 .
           257 IPRATAESFEDGL.KYPKQIKRESPP.............IRAFEGAITK 291/1345
               .||. .|. || | : :| ::|||.|             ..|::.  |
           337 IPRSYVEAQEDYLRREAKLLKREGTPPPPPPSRDLTEAYKTQALGPLKLK 386
                                                  .          7 .
           292 GKPYDGITTIKEMGRSIHEIPRQDILTQESRKTPEVVQSTRPIIEGSISQ 341/1395
               . : :.|:|| ||||||||:::     |.|||:. ..||: ||||.|
           387 PAHEGLVATVKEAGRSIHEIPREEL.....RHTPELPLAPRPLKEGSITQ 431
                                                  .          .
           342 GTPIKFD.NNSGQSAIKHNVKSLITGPSK.LPRGMLEIVPENIKVVERGK 389/1443
               |||:|:| ..|. :. ||:|:|||.:|:: :|..    | .: :.:||:
           432 GTPLKYDTGASTTGSKKHDVRSLIGSPGRTFPPVHPLDVMADARALERAC 481
                                                  .          .
           390 YEDVKAGEPVRARHTSVVSSGPSVLRST..LHEAPKAQLSPGLYDDSSA 436/1490
               ||     |.::.|.... |||.|: |:.   :.| .|:. || |:| :|
           482 YE.....ESLKSRPGTASSSGGSIARGAPVIVPELGKPRQSPLTYEDHGA 526
```

Fig. 1. Isolation of RIP13 Clones

A, Results of RIP13 screening. The RIP13-2, 13–8, and 13–10 clones were isolated successively from a mouse liver cDNA library constructed in λ gt11, using as probes the clone (Genbank accession number: U22016) was used as a probe to screen a conventional mouse liver cDNA library. Three clones, RIP13-2, 13–8, and 13–10 were isolated from three successive screenings (Fig. 1A) and used to reconstruct a putative full-length clone, referred to as RIP13a (Genbank accession number: L78294) to differentiate from the previously reported sequence of the original RIP13 isolate. Since the RIP13-8 clone has an internal deletion of 357 bp (base number 2453–2809 in RIP13a and 5689–6045 in N-CoR) relative to the other clones and to N-CoR, a variant form of RIP13 termed RIP13Δ1 was also constructed.

There are two major differences between RIP13a and N-CoR (Fig. 1B). One is that the first 3166 bp of N-CoR are replaced by 68 unique base pairs at the 5′ end of RIP13a, resulting in the substitution of 10 unique amino acids for the amino-terminal 1016 amino acids of N-CoR. This replacement removes two repressor domains of N-CoR (25). However, RIP13a retains at its amino terminus a domain that includes seven copies of a repeated motif, G–s–l–s/t–q–G–t–P (capitalized residues present in all repeats, small letters in most), that is observed in comparisons of RIP13/N-CoR and SMRT (Fig. 1C). This amino-terminal region is associated with repressor function in SMRT (24) and is likely to play a similar role in RIP13/N-CoR. Another difference is that RIP13a is missing 141 bp of N-CoR (base number 3821–3961), resulting

original RIP13 isolate or the clone obtained from the previous screening. *Numbers* indicate positions of the start and end of the coding regions of each clone relative to the N-CoR (*above bar*) or RIP13a (*below bar*) amino acid sequence. Unique sequences present in RIP13 and not in N-CoR are indicated by a *solid box*. Although the RIP13-10 clone contains the most 5′-sequences isolated, the first initiator codon in that sequence is not preceded by an in-frame termination codon, and the RIP13a variant may include additional amino-terminal sequences. The RIP13-8 clone has a 49-bp substitution (indicated by *hatched box*) relative to other clones after base number 3152 (N-CoR base number 6388), suggesting the existence of an additional RIP13/N-CoR variant. The restriction endonucleases used for construction of RIP13a and RIP13Δ1 are indicated as *vertical lines* and 5′- and 3′-untranslated region as *horizontal lines*. B, Alignment of RIP13, N-CoR, and SMRT/TRAC. Repressor and receptor interaction domains are indicated, based on previous reports (24–26, 31). The amino-terminal extension unique to RIP13a is indicated. *Dots* indicate a repeated motif in the SMRT/TRAC repressor domain that is also present in N-CoR/RIP13. C, Sequence comparison of amino termini of RIP13a and SMRT. RIP13a (from amino acid 1–436) and SMRT (amino acid from 1–526) were compared using the GAP program in the GCG package (38). Positions in the N-CoR sequence are also indicated. Overall identity is 43%. *Underlines* indicate repeated motifs, G–s–l–s/t–q–G–t–P (*capitalized* residues conserved in all repeats, *small letters* in most). One repeat of SMRT not present in RIP13 is also *underlined*. Although RIP13 has seven repeats, only four (3, 4, 5, 7) of them are present at analogous positions in SMRT.

in replacement of 48 amino acids of N-CoR (amino acid number 1235–1282) with a serine in RIP13a (amino acid number 228). The amino-terminal variation between RIP13a and N-CoR may be due to either alternative promoter utilization or alternative splicing, while the internal deletion is likely to be a consequence of the latter mechanism.

There are also seven minor differences relative to N-CoR: an alanine addition in RIP13a (amino acid number 534), a deletion of serine in RIP13a (N-CoR amino acid number 2145), and five differences of a single base, two of which change amino acids (isoleucine to valine at RIP13a amino acid 494, and alanine to proline at RIP13a amino acid 1037). The first and second minor changes were not observed in all cDNA clones isolated and are presumably due to polymorphism. The additional variations may represent either polymorphisms or sequencing errors.

Yeast Two-Hybrid Results Identify a New Receptor Interaction Domain in RIP13a/N-CoR

To study the interaction of RIP13/N-CoR and receptors in detail, a number of deletion mutants of RIP13 were constructed as fusions to the B42 transactivation domain (Fig. 2) and tested for their ability to interact with previously described LexA fusions containing the hinge and ligand-binding domains (LBD) of various receptors (11, 27) in the yeast two-hybrid system (28). Table 1 summarizes results with the RIP13 deletion mutants in tests of interaction with TR, RAR, and RXR. A very similar pattern of interaction was observed in a quantitative analysis of β-galactosidase activity (data not shown).

Previous results identified a receptor interaction domain between amino acids 2239 and 2300 of N-CoR (25), which corresponds to amino acids from 1185 to 1246 of RIP13a. As expected, B42-RIP13a, B42-RIP13Δ1, and deletion mutants containing this N-CoR interaction domain, referred to here as ID-I (Interaction Domain I), show strong interaction with LexA-TRβ and -RARβ. However, several B42-RIP13 fusion proteins that do not contain ID-I still display a strong interaction with TR and RAR. Specifically, the RIP13 deletion mutants ΔI2 and ΔI5 exhibit a strong interaction with TR and RAR, suggesting the existence of a new interaction domain, referred to as ID-II, located between amino acids 806-1089 (N-CoR amino acids 1859–2142). To map this domain further, additional deletion mutants were constructed and tested for interaction. The smallest deletion that retains interaction is ΔI8, minimally mapping ID-II to RIP13 amino acids 1010–1089 (N-CoR amino acids 2063–2142). Interestingly, the minimal ID-II in ΔI8 did not show significant interaction with TR or RAR in the presence of ligand, while other mutants with additional amino-terminal residues (ΔI2 & ΔI5) showed a weaker ligand effect with RAR and no significant effect with TR (Table 1).

As expected from the fact that the initial isolate of RIP13 was obtained using a LexA-RXR fusion as bait

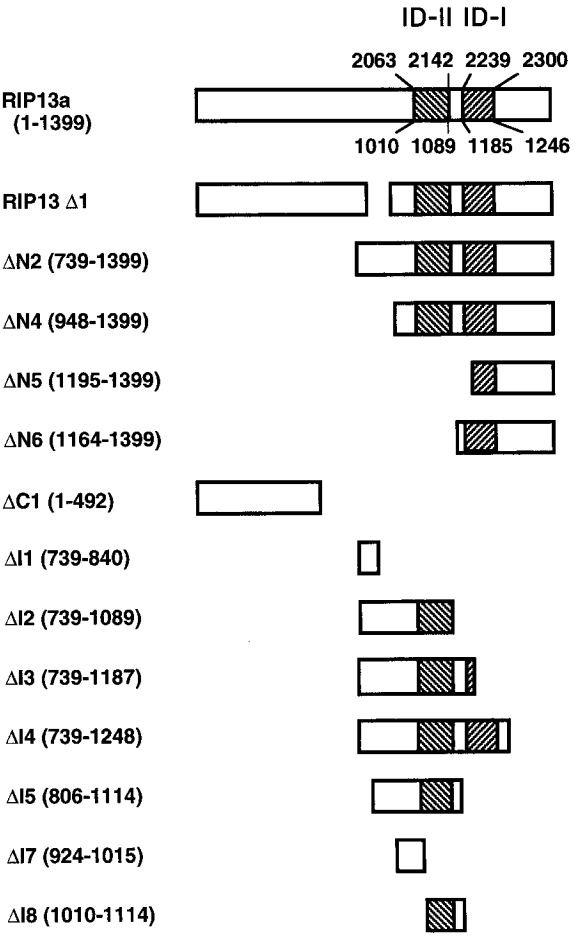


Fig. 2. Various Deletion Mutants of RIP13

The interaction domains ID-I (25) and ID-II are indicated, and their positions in the amino acid sequences of both N-CoR (*top*) and RIP13a (*bottom*) are included. For ID-II, only the minimal sequences for interaction are shown, as identified by results with ΔI2 and ΔI8 in yeast. However, extension of this minimal region modifies results obtained with ID-II constructs. Deletion constructs were generated as described in *Materials and Methods*, and their start and end points are shown relative to the RIP13a sequence.

(11), the B42-RIP13a fusion and a number of the deletion constructs show a pattern of ligand-independent interaction with LexA-RXR similar to that observed with the TR and RAR fusions. Both this RXR interaction and the lack of an inhibitory effect of ligand on interaction with TR and RAR interaction contrast with previously described results using similar LexA-receptor fusions and a B42-N-CoR fusion (25). The basis for these discrepancies is unclear, but they could be a consequence of the specific region of N-CoR used in the prior work or the fact that the previous results were obtained with TRα and RXRγ, while TRβ and RXRα were used here. However, the lack of ligand effect is not due to problems with uptake or metabolism, since numerous other receptor-interacting proteins show ligand- stimulated interactions in these yeast strains (11, 27).

ID-I and ID-II Function in Mammalian Cells

To characterize the interaction of RIP13/N-CoR with the receptors in mammalian cells, a mammalian two-hybrid system was used (30). This system, which is analogous to those used in yeast, is based on hybrids containing either the GAL4 DNA-binding domain or the VP16 transcriptional activation domain. RIP13a and various deletion mutants were fused to GAL4 DNA-binding domain. These hybrids were tested for activation of a reporter gene containing three copies of a GAL4 response element in cotransfections with VP16-receptor hybrids containing receptor hinge and LBD. VP16 fusions including full-length receptor proteins were also used.

Table 1. Interaction of RIP13 with Various Receptors in Yeast

	LexA Fusion						
	LexA-	-RAR		-TR		-RXR	
Ligand:		−	+	−	+	−	+
B42 fusion:							
B42-	W	W	W	W	W	W	W
−RIP13a	W	B	B	B	B	B	B
−Δ1	W	B	B	B	B	B	B
−ΔN2	W	B	b	B	B	B	B
−ΔN5	W	B	b	B	B	B	B
−ΔI1	W	W	W	W	W	W	W
−ΔI2	W	B	b	B	B	B	B
−ΔI3	W	B	b	B	B	B	B
−ΔI4	W	B	b	B	B	B	B
−ΔI5	W	B	b	B	B	B	B
−ΔI7	W	W	W	W	W	W	W
−ΔI8	W	B	W	B	b	B	W

The indicated B42-RIP13 and LexA-receptor plasmids were transformed into a yeast strain containing an appropriate β-galactosidase reporter gene. At least three separate transformants from each transformation were transferred to indicator plates containing X-gal, and reproducible results were obtained using colonies from a separate transformation. W, White colonies (no interaction); B, blue colonies after 1 day (strong interaction); b, blue colonies after 3 days (weak interaction).

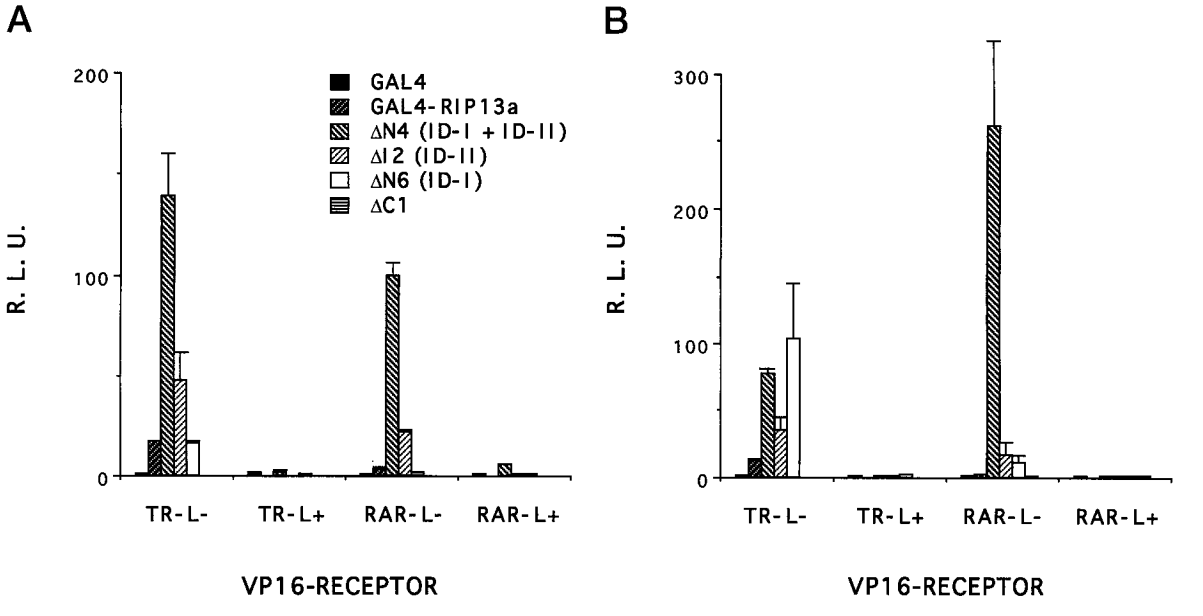


Fig. 3. Mammalian Two-Hybrid Interaction of GAL4 Fusions of RIP13a and Deletions with VP16 Fusions to the Hinge and LBD of Human TRβ and Human RARα

GAL4-RIP13a and deletion mutants and VP16-TR-hinge/LBD (TR-L) and VP16-RAR-hinge/LBD (RAR-L) were cotransfected with TKGH internal control and GAL4/TKLuc reporter plasmids into HepG2 (panel A) or JEG-3 cells (panel B). In transfections denoted TR-L+ and RAR-L+, ligands (1 μM T_3 or 1 μM all-*trans*-retinoic acid (t-RA)) were present. In transfections denoted TR-L- and RAR-L-, no ligand was added. ΔN4 contains both ID-I and ID-II, ΔN6 contains ID-I, and ΔI2 contains ID-II. Normalized luciferase expression from duplicate samples is presented relative to the transactivation observed in transfections of vector alone (GAL4) with each VP16-receptor in each cell type. Activation of the reporter gene is expressed as relative luciferase units (R.L.U.).

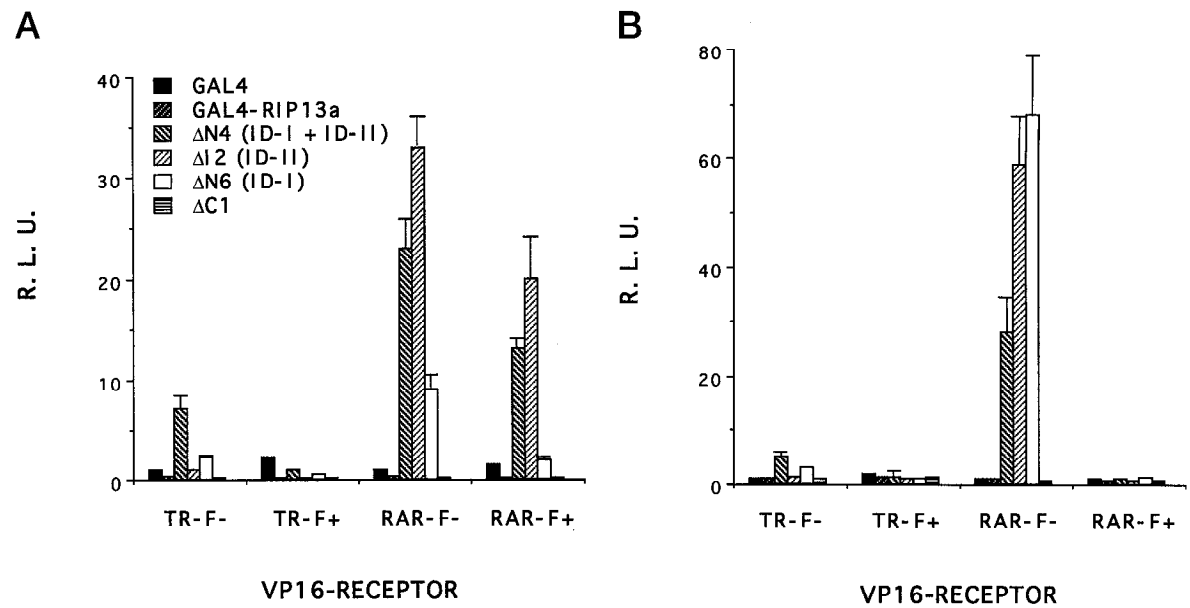


Fig. 4. Mammalian Two-Hybrid Interaction of GAL4 Fusions of RIP13a and Deletions with VP16 Fusions to Full-Length Human TRβ and Human RARα

GAL4-RIP13a and deletion mutants and VP16 full-length TR (TR-F) and VP16 full-length RAR (RAR-F) were cotransfected with TKGH internal control and GAL4/TKLuc reporter plasmids into HepG2 (panel A) or JEG-3 cells (panel B). In transfections denoted TR-L+ and RAR-L+, ligands (1 μM T_3 or 1 μM all-*trans*-retinoic acid (t-RA)) were present. In transfections denoted TR-L- and RAR-L-, no ligand was added. ΔN4 contains both ID-I and ID-II, ΔN6 contains ID-I, and ΔI2 contains ID-II. Normalized luciferase expression from duplicate samples is presented relative to the transactivation observed in transfections of vector alone (GAL4) with each VP16 receptor in each cell type. Activation of the reporter gene is expressed as relative luciferase units (R.L.U.).

When cotransfected with VP-16 fused to the hinge/LBD of either TR or RAR, all of the GAL4-RIP13 constructs tested except ΔC1 activate the luciferase reporter gene in either hepatoma-derived HepG2 (Fig. 3A) or choriocarcinoma-derived JEG-3 (Fig. 3B) cells. These data demonstrate that RIP13 interacts with TR and RAR within mammalian cells, as expected. For both the TR and RAR fusions in HepG2 cells (Fig. 3A), the observed interaction is strongest with GAL4-ΔN4, which contains both ID-1 and ID-II, but significant interaction is also observed with ΔI2 (ID-II alone) and ΔN6 (ID-I alone). The pattern of interactions in JEG-3 cells is quite similar, except that the TR hinge/LBD fusion interacted most strongly with ΔN6 (ID-I alone, Fig. 3B). In contrast to the results in yeast, interaction in both cell lines is essentially completely lost in the presence of ligand, with only the combination of ΔN4 and the RAR hinge/LBD fusions showing residual ligand-resistant interaction (Fig. 3, A and B).

Unexpectedly, the results with the full-length TR and RAR fusions differ substantially from those with the hinge and LBD constructs (Fig. 4). In the case of TR, only ΔN4 (ID-I and ID-II) and ΔN6 (ID-I) showed a relatively weak interaction (note the difference of scales in Figs. 3 and 4). This interaction was significantly decreased in the presence of ligand in both HepG2 and JEG-3 cells (Fig. 4). In contrast, full-length RAR showed significant interaction with ΔN4 (ID-I and ID-II), ΔN6 (ID-I), and ΔI2 (ID-II) (Fig. 4), and interaction was also retained with ΔI8 (minimal ID-II) (data not shown). As observed with the RAR hinge and LBD, these interactions were strongly inhibited by all-*trans*-retinoic acid in JEG-3 cells (Fig. 4B). In HepG2 cells (Fig. 4A), however, ligand decreased interaction approximately 4-fold with ΔN6 but had a less than 2-fold effect with ΔN4 and ΔI2.

GAL4-ΔN2, which contains additional amino-terminal residues relative to ΔN4, showed a pattern of interaction that was very similar to ΔN4, although the apparent interaction of ΔN2 with the VP16-RAR-hinge/LBD was somewhat weaker in both HepG2 and JEG-3 cells (data not shown).

Overall, these results confirm the yeast results demonstrating that both ID-I and ID-II are capable of interacting independently with the hinge/LBD regions of TR and RAR. However, the substantial difference in ligand dependence between yeast and mammalian cells and the more subtle differences between mammalian cell types suggest that additional factors may complicate such interactions.

ID-I and ID-II Bind Receptors *in Vitro*

To further confirm the presence of ID-II in RIP13/N-CoR, an *in vitro* interaction assay based on GST fusion proteins was used. GST-RIP13 fusions containing ID-I (GST-ΔN6), ID-II (GST-ΔI5), and both ID-I and ID-II (GST-ΔN2) were tested for interaction with full-length ^{35}S-labeled RARβ or TRβ (Fig. 5).

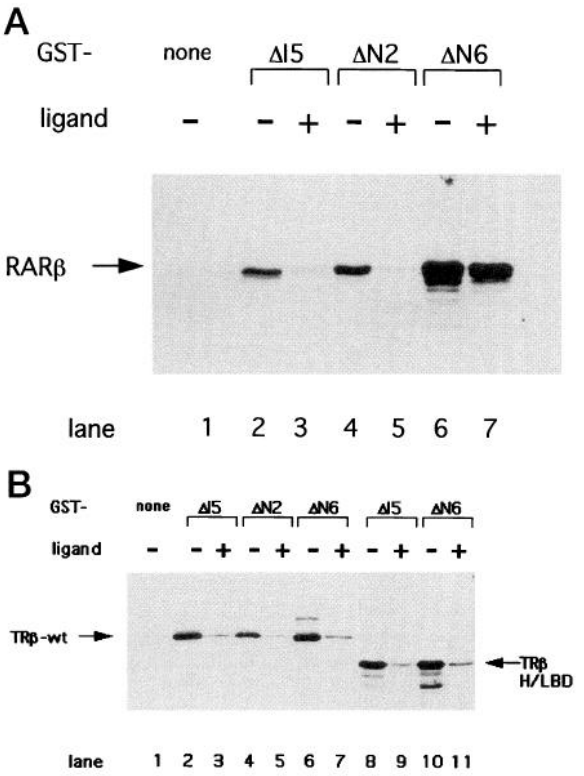


Fig. 5. *In Vitro* Interaction of GST-RIP13 Mutants with TR and RAR

Rat RARβ (panel A) and rat TRβ (panel B) were radiolabeled with [^{35}S]methionine by *in vitro* translation and tested for interaction with GST alone (lane A1 and B1), and GST-ΔI5 (ID-II, lanes A2 and A3 and B2, B3, B8, and B9), -ΔN2 (ID-I and ID-II, lanes A4 and A5 and B4 and B5) and -ΔN6 (ID-I, lanes A6 and A7 and B6, B7, B10, and B11) in the absence (*even numbered lanes* and lane 1) and presence (*odd numbered lanes* except lane 1) of their ligands (1 μM t-RA and T_3). For TR, both full-length (TRβ-wt, lanes B1-B7) and a carboxyl-terminal fragment including the hinge and LBD (TRβ-H/LBD, lanes B8-B11) are also shown. Film was exposed overnight.

All three GST fusions showed substantial, ligand-inhibited interaction with RAR, demonstrating that both ID-I and ID-II are capable of interacting independently with RAR *in vitro* (Fig. 5A). GST-ΔN6, which includes only ID-I, showed a somewhat stronger and less ligand-sensitive interaction (Fig. 5A, lanes 6 and 7).

For TR, both full-length and hinge/LBD proteins were tested for interaction. Full-length TR interacted with all three GST-RIP13 fusion proteins (Fig. 5B, lanes 2–7). The hinge/LBD portion of TR also showed interaction with ID-I and with ID-II that was similar to that of the full- length TR (Fig. 5B, lanes 8–11). Both forms of TR were released from GST-RIP13 fusion proteins in the presence of ligand. Interaction of either ID-I or ID-II with a truncated TR LBD protein that did not contain the hinge domain was much weaker than the larger TR proteins, as expected from previous results mapping corepressor interaction to the hinge region (data not shown).

Taken together, these results demonstrate that both ID-I and ID-II can interact independently with TR or RAR.

RIP13/N-CoR Interacts with RXR

Although RIP13 was originally isolated by interaction with RXR in the yeast two-hybrid system and this interaction was confirmed by additional studies in yeast described above, N-CoR failed to interact with RXR in a previous report (25). To further examine this discrepancy, we tested the interaction of RIP13/N-CoR with RXR in the mammalian two-hybrid system and *in vitro*.

In contrast to the results with TR and RAR, only the ΔN4 mutant showed interaction with RXR in HepG2 cells. With the full-length RXRα fusion to VP16 and the ΔN4 (ID-I and ID-II) fusion to GAL4, a modest 5-fold transactivation relative to GAL4 alone was observed in the absence, but not the presence, of *9-cis*-RA (Fig. 6A). No significant interaction was observed with the VP16-RXR ligand-binding domain fusion. GAL4 fusions containing the RIP13/N-CoR domain related to the SMRT repressor domain showed significantly reduced expression relative to GAL4 alone in either the presence of the RXR fusions (Fig. 6A) or in the absence of any VP16 fusions (data not shown), suggesting that this is an active repressor domain in both proteins. Essentially identical results were obtained with JEG-3 cells.

To confirm their physical interaction *in vitro*, ^{35}S-labeled human RXRα was incubated with GST-ΔI5 (ID-II alone), -ΔN2 (ID-I and ID-II), and -ΔN6 (ID-I alone). RXRα showed significant interaction with GST-ΔN6 (Fig. 6B, lanes 6 and 7). This interaction is approximately 10- fold weaker than that observed with TR and RAR, however, as assessed by the relative amount of input radioactivity retained. RXR also showed an even weaker interaction with GST-ΔI5 (Fig. 6B, lanes 2 and 3) and -ΔN2 (Fig. 6B, lanes 4 and 5). The interaction with ΔN6 was not affected by ligand, but that with ΔI5 was inhibited in the presence of ligand. To further confirm these interactions, a GST fusion to the full-length RXR (GST-RXR) was incubated with ^{35}S-labeled RIP13a and ΔN4. Specific, but somewhat weak interaction was observed with both RIP13 proteins, regardless of the presence or absence of 9-*cis*-retinoic acid (data not shown).

Overall, these results confirm that RIP13/N-CoR, like SMRT/TRAC (26, 31), can interact with RXR. Particularly in the mammalian system and *in vitro*, however, this interaction is weaker than that observed with TR and RAR.

DISCUSSION

RIP13/N-CoR and SMRT/TRAC Isoforms

RIP13a and N-CoR are essentially identical for large stretches, but RIP13a has a distinct amino terminus

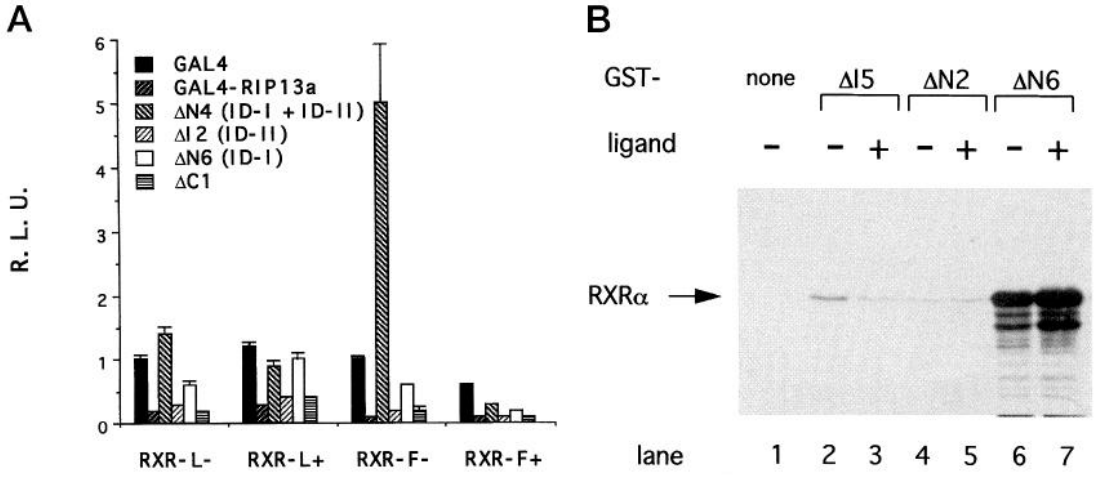


Fig. 6. Interaction of RIP13 with RXR

A, Cotransfection assay using mammalian two-hybrid system. GAL4-RIP13a and deletion mutants and VP16-human RXRα-hinge/LBD (RXR-L) and -full length (RXR-F) fusions were cotransfected into HepG2 cells. RXR-L+ and RXR-F+ indicate that ligand (1 μM 9-*cis*-RA) was added, and RXR-L- and RXR-F- indicate that no ligand was added. ΔN4 contains both ID-I and ID-II, ΔN6 contains ID-I, and ΔI2 contains ID-II. Normalized luciferase expression from duplicate samples is presented relative to the transactivation observed in transfection of vector alone (GAL4) and each VP16 receptor in each cell type. Activation of the reporter gene is expressed as relative luciferase units (R.L.U.). Similar results were obtained with JEG-3 cells (data not shown). B, *In vitro* interaction of RIP13 mutants with RXR. Human RXRα was radiolabeled with [^{35}S]methionine by *in vitro* translation and tested for interaction with GST alone (lane 1) and GST-ΔI5 (ID-II, lanes 2 and 3), -ΔN2 (ID-I and ID-II, lanes 4 and 5) and -ΔN6 (ID-I, lanes 6 and 7) in the absence (*even numbered lanes* and lane 1) and presence (*odd numbered lanes* except lane 1) of its ligand (1 μM *9-cis*-RA). Film was exposed for 1 week, approximately 12-fold longer than in Fig. 5, A and B.

and is lacking a short internal stretch of amino acids relative to N-CoR (Fig. 1B). The unique 68 bp at the 5′-end of the RIP13 clone could be derived by use of an alternative promoter or by alternative splicing of a primary transcript identical to that of N-CoR. Although the clones identified to date represent the most extensive 5′-sequences obtained after several rounds of cDNA screening, the lack of an in- frame translational terminator upstream of the predicted RIP13a-coding region leaves open the possibility that additional amino-terminal sequences exist in this variant. The internal deletion of N-CoR amino acids 1235–1282 is presumably a simple consequence of alternative splicing. The existence of a second internal alternatively spliced variant missing RIP13a residues 806–924 (N-CoR amino acids 1859–1977) is suggested by the structure of the cDNA isolate RIP13-8, and a third potential variant lacking RIP13a residues 1279–1317 (N-CoR amino acids 2333–2371) was noted in the original description of N-CoR cDNA clones (25). The related corepressor, termed SMRT (24) or TRAC (26), also has isoforms, termed TRAC-1 and TRAC-2, with distinct amino-terminal sequences (26). It is apparent that both the RIP13/N-CoR and SMRT/TRAC genes encode distinct proteins that may have divergent functions.

Although the RIP13a protein is missing two repressor domains of N-CoR, its overall arrangement is quite similar to that of the corepressor SMRT/TRAC-2, which is also about 1000 amino acids shorter than N-CoR at its amino terminus (Fig. 1B). As previously noted (24, 26), based on the initially reported partial RIP13 sequence, the carboxyl termini of these proteins share an approximately 260-amino acid region of relatively extensive sequence identity (>45%). This region includes the previously described interaction domain of N-CoR (ID-I), as well as sequences associated with receptor interaction in the TRAC proteins (26).

Interestingly, the amino termini of RIP13 and SMRT also share significant, although somewhat lesser identity over a relatively large amino-terminal region. This region has been demonstrated to include a transcriptional repressor domain in SMRT/TRAC-2. As expected for a repressor function for this region of RIP13/N-CoR, both the GAL4-RIP13a fusion and the shorter fusion including the first 492 amino acids of RIP13a (ΔC1) consistently show 10-fold repressor function relative to GAL4 alone in HepG2 cell transfections. The sequence similarity in this amino-terminal region is focused on several copies of a repeated motif, G–s–I–s/t–q–G–t–P (Fig. 1C). This conservation suggests an important role for this motif in repressor function.

The repressor function of this amino-terminal domain may explain the apparently lower interaction of the full-length RIP13a protein with TR and RAR in the mammalian two-hybrid system. Thus, it is possible that the true interaction of the full-length GAL4-RIP13a is comparable to that of the shorter products (*e.g.* GAL4-RIP13ΔN4), but that inclusion of the repressor domain in the former construct decreases the tran-

scriptional activation that is indicative of interaction in this system.

Two Independent Receptor Interaction Domains in RIP13/N-CoR

Both *in vivo* and *in vitro* results demonstrate the existence of two receptor-interacting domains of RIP13/N-CoR, each of which can interact independently with TR or RAR. The new interaction domain described here, ID-II, does not include obvious sequence similarity to the previously described ID-I interaction domain. However, it does include a relatively short stretch of sequence similarity with SMRT/TRAC within one of at least two regions of TRAC associated with receptor interaction (26).

Although the two interaction domains of RIP13/N-CoR are each able to interact independently with TR or RAR in a variety of circumstances, they do not function equivalently. In the *in vitro* interactions, for example, binding of either ID-I alone or ID-II alone to TR is strongly inhibited by ligand, but binding of ID-I to RAR is relatively unaffected. In the mammalian two-hybrid system, ID-II interacts more strongly with the RAR hinge and LBD than ID-I, and the combination of the two domains shows synergistic interaction. With the analogous TR construct, however, ID-I appears to dominate the interaction, at least in JEG-3 cells. Interestingly, preliminary results in both the yeast and mammalian two-hybrid systems suggest that murine PPARα interacts with ID-I, but not with ID-II. SMRT/TRAC has also been reported to interact with PPAR (26), although the absence of apparent repressor function of PPAR leaves the significance of such interactions uncertain.

In addition to TR, RAR, RXR, and PPAR, RIP13/N-CoR may also interact with other members of the nuclear hormone receptor superfamily. Our results in yeast suggest that it interacts with several orphans (Ref. 11 and data not shown), and recent reports from other groups suggest that it may mediate the repressor function of the orphans Rev-Erb (M. Lazar, personal communication) and COUP-TFII (G. E. O. Muscat, personal communication).

The existence of two independent domains capable of interacting with several members of the receptor superfamily substantially complicates models for RIP13/N-CoR function. The two domains could independently contact individual components of a heterodimer, for example, or could link two receptor complexes bound to distinct hormone response elements.

In this context, however, it should be emphasized that the apparent interactions observed with the two domains either alone or in combination are quite dependent on the experimental system used. Thus, a variety of RIP13/N-CoR constructs maintain strong interaction with both TR and RAR in the yeast two-hybrid system, even when their cognate ligands are present. In the mammalian two-hybrid system and *in vitro*, however, essentially all such interactions with TR and most with RAR are strongly inhibited by ligand. Similarly, RXR shows relatively strong interactions with numerous RIP13 constructs in yeast, and similar results have been reported for TRAC (26), but interactions with RXR in mammalian cells and *in vitro* appear weaker.

In general, such differences highlight the importance of using more than a single approach to characterize protein-protein interactions. More specifically, they also raise the possibility that the functions of RIP13/N-CoR are modulated by different factors present in the various conditions used to assess interactions with receptors. It is possible, for example, that the relatively promiscuous interactions observed in yeast reflect the absence of factors required for corepressor release in mammalian cells. Similarly, the significantly different results obtained with the full-length receptor fusions and the hinge/LBD fusions in the mammalian two-hybrid system suggest that the interactions of RIP13/N-CoR with the receptors may be inhibited directly by the receptor amino-terminal or DNA-binding domains or by a cellular factor that interacts with them. Since these results rely directly on transcriptional activation as an assessment of interaction, it is also possible that the apparent decrease in interaction with the full-length receptors is a consequence of increased corepressor activity of the RIP13/N-CoR fusion recruited to the reporter.

Over the last several years it has become clear that the functions of the members of the nuclear hormone receptor superfamily are much more complex than those originally envisaged for simple ligand-dependent transcriptional activators. It is likely that the relatively simple models for RIP13/N-CoR and SMRT/TRAC as TR and RAR corepressors will become more complex as more details of their structures and functions become clear.

MATERIALS AND METHODS

Plasmids

The RIP13-2, 13–8, and 13–10 clones were obtained by conventional screening of a mouse liver cDNA library using standard methods (32). For construction of RIP13a, RIP13-2, 13–8, and 13–10 clones were joined using the unique restriction sites *Nde*I and *Bst*XI. RIP13a fragment was cut out by *Eco*RI, a cloning site of the original library vector, and inserted into a modified CDM8 mammalian expression vector (33). RIP13Δ1 was constructed by replacing an *Apa*I/*Bsp*MI fragment of RIP13a with the *Apa*I/*Bsp*MI fragment of the RIP13-8 clone. For B42-RIP13a and mutant constructions, an *Eco*RI/*Xho*I fragment of RIP13a was inserted into cgatrp2 (11), a B42 expression vector. To make ΔN4, *Bsp*EI, derived from an adapter in the original cDNA library, and the insert at its 5′-end and *Bsp*MI were used, and fragment containing vector and RIP13 region was fused intramolecularly. For ΔN5, an *Eco*RI site present just after 5′-end of the insert and an internal *Bgl*II site were used. ΔN2 is the clone isolated from the original yeast screening. For construction of ΔI1, I2, I3, and I4, a *Pac*I site present just after 3′-end of the insert in cgatrp2 vector was used with *Bgl*II, *Sma*I, *Pvu*II, and *Pfl*MI,

respectively, and fragments containing vector and desirable RIP13 region were joined intramolecularly. For construction of ΔI5, ΔI7, and ΔI8, corresponding fragments were generated by PCR and inserted into the vector using *Eco*RI and *Xho*I sites.

GAL4-RIP13a was constructed by insertion of *Eco*RI/*Eco*RI RIP13a fragment into *Eco*RI site of CMX-GAL4N vector and GAL4-ΔN and ΔI deletion mutants were constructed by insertion of *Eco*RI/*Xho*I fragments of the corresponding cgatrp2-RIP13 constructs into *Eco*RI/*Sal*I of GAL4 vector, except GAL4-ΔN6 (GAL4-mN-CoR), which was a gift from Dr. G. E. O. Muscat (University of Queensland, St Lucia, Australia). GAL4-ΔC1 was constructed by intramolecular ligation of GAL4-RIP13a after *Bam*HI digestion, deleting 2.8 kb of 3′-end of the RIP13 gene.

GST-ΔN2 and -ΔI2 were constructed by insertion of *Eco*RI/*Xho*I fragments of corresponding cgatrp2-RIP13 constructs into *Eco*RI/*Xho*I of pGEX4T-1 vector (Pharmacia, Piscataway, NJ) and GST-ΔN6 was constructed by intramolecular ligation of GST-ΔI2 after *Eco*RI/*Nco*I digestion.

All constructs were confirmed by sequencing of appropriate fusion junctions or of the entire inserted fragment when PCR products were used. Both hinge/LBD and full-length human TRβ, RARα, and RXRα and murine PPARα VP16 fusions were obtained from Drs. B. Forman and R. Evans (Salk Institute, San Diego, CA)

Yeast Two-Hybrid Test

For the yeast two-hybrid system (28, 34, 35), LexA-receptor fusions and B42-RIP13 fusion plasmids were cotransformed into *Saccharomyces cerevisiae* EGY48 strain containing the β-gal reporter plasmid, 8H18-34. All LexA-receptor fusions contain sequences extending from the carboxyl-terminal portion of the DNA-binding domains to the carboxyl termini of the receptors (11, 27). Plate and liquid assays of β-gal expression were carried out as described (11, 27). Similar results were obtained in more than two similar experiments.

Cell Culture and Transfections

HepG2 and JEG-3 cells were propagated in DMEM plus 10% FBS. Cells were grown in 12-well plates with medium supplemented with 10% charcoal-stripped serum for 24 h and transfected as described (32) using the diethylaminoethyl-dextran/chloroquine method followed by dimethylsulfoxide shock, with 1 μg of both TKGH (36) and GAL4-TKLuc (30) and 0.5 μg of each GAL4 and VP16 fusion plasmids per well. Luciferase was assayed with a kit as described by the manufacturer (Promega, Madison, WI), and the results were normalized by using GH expression from the internal TKGH control. Similar results were obtained in more than two similar experiments.

Protein Expression and Interaction Analysis

GST fusion proteins were expressed in *E. coli* and purified using glutathione-Sepharose affinity chromatography essentially as described (13, 32). The TNT-coupled transcription-translation system was used for *in vitro* translation, with conditions as described by the manufacturer (Promega, Madison, WI). For protein-protein interactions, GST and GST fusion proteins were bound to glutathione-Sepharose 4B beads (Pharmacia, Piscataway, NJ) in PBS, followed by 2 × 1 ml washing with PBS. The beads were washed once with binding buffer (25 mM HEPES, pH 7.6, 2 mM dithiothreitol, 20 mM NaCl, 20% glycerol, 2 mg/ml BSA, 10 mg/ml *E. coli* extract, and protease inhibitors: 0.3 mM phenylmethylsulfonylfluoride, 1.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, 0.5 μg/ml pepstatin A, 100 μg/ml chymostatin), followed by mixing with 250 μl of bead-binding buffer overnight at 4 C. Equivalent amounts of [^{35}S]methionine-labeled, *in vitro*-translated proteins, as determined by phosphor-imaging (Molecular Dynamics, Sunnyvale, CA), were mixed with the GST fusion protein-bound beads for 3 h at 4 C. After the interaction, the beads were washed 2 × 1 ml with binding buffer + 0.1% Triton X-100 and with 1 ml 50 mM Tris (pH 8.0) buffer. Specifically bound proteins were eluted with freshly prepared 15 mM reduced glutathione in 50 mM Tris buffer. Care was taken not to include any of the beads when removing the supernatant. Eluted proteins were resolved by SDS-PAGE and visualized by fluorography.

Acknowledgments

We thank Drs. B. Forman and R. Evans for Gal4 and VP16-receptor plasmids and G. Muscat for the GAL4 plasmid containing N-CoR interaction domain I (GAL4-ΔN6).

Received July 17, 1996. Revision received August 26, 1996. Accepted September 5, 1996.

Address requests for reprints to: David D. Moore, Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114.

This work was supported by NIH Grant R01 DK-43382. Dr. Seol is a recipient of a Helen Hay Whitney postdoctoral fellowship and Dr. Mahon is a recipient of an NIH postdoctoral fellowship (DK-09438).

REFERENCES

1. Beato M, Herrlich P, Schutz G 1995 Steroid hormone receptors: many actors in search of plot. Cell 83:851–857
2. Mangelsdorf D, Evans RM 1995 The RXR heterodimers and orphan receptors. Cell 83:841–850
3. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835–839
4. Thummel CS 1995 From embryogenesis to metamorphosis: the regulation and function of Drosophila nuclear receptor superfamily members. Cell 83:871–877
5. Kastner P, Mark M, Chambon P 1995 Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? Cell 83:859–869
6. Lazar MA 1993 Thyroid hormone receptors: multiple forms, multiple possibilities. Endocr Rev 14:184–193
7. Lebalanc BP, Stunnenberg HG 1995 9-cis retinoic acid signaling: changing partners causes some excitement. Genes Dev 9:1811–1816
8. Ribeiro RC, Kushner PJ, Baxter JD 1995 The nuclear hormone receptor gene superfamily. Annu Rev Med 46:443–453
9. Leid M, Kastner P, Chambon P 1992 Multiplicity generates diversity in the retinoic acid signalling pathways. Trends Biochem Sci 17:427–433
10. Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM 1990 Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 345:224–229
11. Seol W, Choi H-S, Moore DD 1995 Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. Mol Endocrinol 9:72–85
12. Baes M, Gulick T, Choi H-S, Martinoli MG, Simha D, Moore DD 1994 A new orphan member of the nuclear receptor superfamily that interacts with a subset of retinoic acid response elements. Mol Cell Biol 14:1544–1552
13. Seol W, Choi H-S, Moore DD 1996 An orphan nuclear

hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. Science 272:1336–1339
14. Sharif M, Privalsky ML 1991 V-erbA oncogene function in neoplasia correlates with its ability to repress retinoic acid receptor action. Cell 66:885–893
15. Damm K, Thompson CC, Evans RM 1989 Protein encoded by v-erbA functions as a thyroid hormone receptor antagonist. Nature 339:593–597
16. Brent GA, Dunn MK, Harney JW, Gulick T, Larsen PR, Moore DD 1989 Thyroid hormone aporeceptor represses T3-inducible promoters and blocks activity of the retinoic acid receptor. New Biol 1:329–336
17. Baniahmad A, Steiner C, Kohne AC, Renkawitz R 1990 Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. Cell 61:505–514
18. Tsai M, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486
19. Casanova J, Helmer E, Selmi-Ruby S, Qi J, Au-Fliegner M, Desai-Yajnik V, Koudinova N, Yarm F, Raaka BM, Samuels HH 1994 Functional evidence for ligand-dependent dissociation of thyroid hormone and retinoic acid receptors from an inhibitory cellular factor. Mol Cell Biol 14:5756–5765
20. Baniahmad A, Leng X, Burris TP, Tsai SY, Tsai M, O'Malley BW 1995 The τ4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing. Mol Cell Biol 15:76–86
21. Qi J, Desai-Yajnik V, Greene ME, Raaka B, Samuels HH 1995 The ligand-binding domain of the thyroid hormone/retinoid receptor gene superfamily function *in vivo* to mediate heterodimerization, gene silencing, and transactivation. Mol Cell Biol 15:1817–1825
22. Tong G, Jeyakumar M, Tahen MR, Bagchi MK 1996 Transcriptional silencing by unliganded thyroid hormone receptor β requires a soluble corepressor that interacts with the ligand-binding domain of the receptor. Mol Cell Biol 16:1909–1920
23. Baniahmad A, Ha I, Reinberg D, Tsai S, Tsai M-J, O'Malley BW 1993 Interaction of human thyroid hormone receptor beta with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone. Proc Natl Acad Sci USA 90:8832–8836
24. Chen JD, Evans RM 1995 A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457
25. Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK, Rosenfeld MG 1995 Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397–404
26. Sande S, Privalsky ML 1996 Identification of TRACs (T3 receptor-associating cofactors), a family of cofactors that associate with, and modulate the activity of, nuclear hormone receptors. Mol Endocrinol 16:813–825
27. Lee JW, Choi H-S, Gyuris J, Brent R, Moore DD 1995 Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. Mol Endocrinol 9:243–254
28. Finley R, Brent R 1995 Interaction trap cloning with yeast. In: Glover DM, Hanes BD (eds) DNA Cloning 2 — A Practical Approach: Expression Systems. IP Press, Oxford, U.K., pp 169–254
29. Deleted in proof
30. Forman BM, Umesono K, Chen J, Evans RM 1995 Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell 81:541–550
31. Chen JD, Umesono K, Evans RM 1996 SMRT isoforms mediate repression and anti-repression of nuclear receptor heterodimers. Proc Natl Acad Sci USA 93:7567–7571
32. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K 1995 Current Protocols in Molecular Biology, Greene Assoc, New York
33. Seed B 1987 An LFA-3 cDNA encodes a phospholipid linked membrane protein homologous to its receptor, CD2. Nature 329:840–842
34. Gyuris J, Golemis E, Chertkov H, Brent R 1993 Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell 75:791–803
35. Zervos AS, Gyuris J, Brent R 1993 Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell 72:223–232
36. Selden RF, Howie KB, Rowe ME, Goodman HM, Moore DD 1986 Human growth hormone as a reporter gene in regulation studies employing transient gene expression. Mol Cell Biol 6:3173–3179
37. Deleted in proof
38. Devereux J, Haeberli P, Smithies O 1984 A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12:387–395