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A Pleiotropic Element in the Medium-Chain Acyl Coenzyme A Dehydrogenase Gene Promoter Mediates Transcriptional Regulation by Multiple Nuclear Receptor Transcription Factors and Defines Novel Receptor-DNA Binding Motifs

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We previously identified a complex regulatory element in the medium-chain acyl coenzyme A dehydrogenase gene promoter that confers transcriptional regulation by the retinoid receptors RAR and RXR and the orphan nuclear receptor HNF-4. In this study we demonstrate a *trans*-repressing regulatory function for the orphan receptor COUP-TF at this same nuclear receptor response element (NRRE-1). The transcriptional regulatory properties and receptor binding sequences of each nuclear receptor response element within NRRE-1 are also characterized. NRRE-1 consists of four potential nuclear hormone receptor hexamer binding sites, arranged as [←1-$(n)_8$-2→-3→$(n)_4$←4-], three of which are used in alternative pairwise binding by COUP-TF and HNF-4 homodimers and by RAR-RXR heterodimers, as demonstrated by mobility shift assays and methylation interference analysis. Binding and transactivation studies with mutant NRRE-1 elements confirmed the existence of distinct retinoid, COUP-TF, and HNF-4 response elements that define novel receptor binding motifs: COUP-TF homodimers bound sites 1 and 3 (two hexamer repeat sequences arranged as an everted imperfect repeat separated by 14 bp or ER14), RAR-RXR heterodimers bound sites 1 and 2 (ER8), and HNF-4 homodimers bound sites 2 and 3 (imperfect DR0). Mixing cotransfection experiments demonstrated that the nuclear receptor dimers compete at NRRE-1 to modulate constitutive and ligand-mediated transcriptional activity. These data suggest a mechanism for the transcriptional modulation of genes encoding enzymes involved in cellular metabolism.

Medium-chain acyl coenzyme A dehydrogenase (MCAD; EC 1.3.99.3) is a mitochondrial flavoenzyme encoded by a nuclear gene that catalyzes the initial step in β-oxidation of medium-chain-length (C6 to C12) fatty acids (6). The enzyme catalyzes oxidative desaturation of acyl coenzyme A thioesters derived from multiple metabolic pathways, including products of longer-chain fatty acid mitochondrial β-oxidation, incompletely oxidized fatty acids derived from peroxisomal β-oxidation, and unsaturated fatty acid intermediates. Accordingly, MCAD is a pivotal enzyme in cellular fatty acid oxidative metabolism. This fact is underscored by the severe consequences resulting from inherited MCAD deficiency, including hypoglycemia and sudden death (15, 51).

As an initial step in determining the molecular mechanisms involved in transcriptional regulation of nuclear genes involved in mitochondrial metabolic pathways, we have begun to delineate *cis*-acting regulatory elements in the MCAD gene promoter (10, 46, 65). In this pursuit, we identified a retinoic acid (RA) response element (RARE) that confers retinoid-mediated activation of the MCAD gene promoter (46). In cotransfection experiments, both the all-*trans* RA receptors (RARs) and the 9-*cis* RA receptors (RXRs) conferred RA-mediated induction of the MCAD promoter through interactions with this response element. The element also functioned in a heterologous promoter context and bound RARs and RXRs in nuclear extracts derived from cells infected with recombinant vaccinia viruses that express these proteins.

RARs and RXRs are members of the TR/RAR/VDR subgroup of the steroid/thyroid nuclear hormone receptor superfamily that share a highly conserved DNA-binding domain segment called the proximal (P) box that dictates recognition of a consensus hexamer DNA sequence RG$^{G}_{T}$TCA (18, 34–36, 43, 56, 61). Response elements for these receptors generally consist of paired hexamer sites that bind receptor homo- or heterodimers, with specificity determined, at least in part, by the relative orientation and spacing of hexamers (16, 27, 32, 36, 40, 56, 62–64). Retinoid ligands regulate transcription through interactions with both RARs and RXRs (21). Previously reported RAREs consist of direct repeat paired hexamers with 1 (DR1)-, 2 (DR2)-, or 5 (DR5)-bp spacing or inverted repeat adjacent hexamers (IR0), all of which bind RAR-RXR heterodimers with high affinity (16, 36, 40). Reported RXREs consist of DR1 or IR0 elements that bind RXR homodimers (64). In our original report, we demonstrated that the MCAD RARE contained two hexamer receptor binding consensus sequences arranged as an everted imperfect repeat with 8-bp separation (ER8) and was thus a novel RARE (46). Subsequently, an ER8 RARE has been identified in the γF-crystallin gene (54).

The MCAD promoter region surrounding the RARE actually contains four potential nuclear receptor hexamer sites arranged as an everted repeat with 8-bp separation (ER8), an

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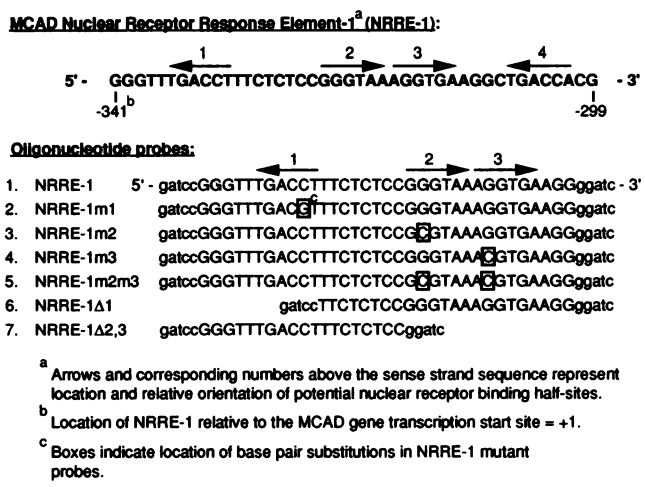

FIG. 1. DNA sequences of MCAD NRRE-1 and oligonucleotides used in this study.

imperfect adjacent direct repeat (DR0), and an inverted repeat with 4 intervening bp (IR4) (Fig. 1). The complex architecture of this element suggested that other receptor transcription factors might regulate MCAD expression as well. In fact, we have recently reported that the orphan receptor hepatocyte nuclear factor 4 (HNF-4) (50), confers transactivation of MCAD through this element (10). Because this unique element is capable of interacting with multiple nuclear receptors, its designation is NRRE-1, for nuclear receptor response element 1.

In the present report we show that NRRE-1 also interacts with chicken ovalbumin upstream promoter transcription factor (COUP-TF), another orphan receptor previously reported to regulate expression of several genes involved in lipid metabolism (30, 38, 47, 57). COUP-TF produces potent repression of transcription from homologous or heterologous promoters via NRRE-1. Thus, MCAD NRRE-1 confers orphan receptor-mediated activation (HNF-4) and repression (COUP-TF), as well as retinoid-dependent transactivation. Further, since none of the potential paired hexamer site arrangements in MCAD NRRE-1 conforms to existing recognized response element architectures for these transcription factors, a detailed dissection of the NRRE-1-receptor binding interactions is presented. Novel response elements that consist of alternatively paired hexamers within the complex element NRRE-1 are identified for each receptor. Our findings suggest a mechanism whereby multiple nuclear receptors can activate or repress transcription via a single pleiotropic regulatory element.

MATERIALS AND METHODS

Reporter plasmids. Construction of MCADCAT (−361/+189) (with reference to the transcription start site as +1) has been described (65). pTKCAT is a pUC-based plasmid constructed by replacing the herpes simplex virus tk1 gene/chloramphenicol acetyltransferase (CAT) gene hybrid of pUT KAT3 (45) with a Kozak consensus sequence upstream of the CAT open reading frame, followed by the simian virus 40 early region splice and polyadenylation signals. pTKLUC was constructed by replacing the CAT open reading frame fragment of pTKCAT with a firefly luciferase (14) open reading frame PCR product. pTKCAT(NRRE-1), pTKCAT(NRRE-1)$_4$, pTKCAT(NRRE-1m1), pTKCAT(NRRE-1m2), and pTKCAT(NRRE-1m3) were each constructed by ligating the corresponding double-stranded oligonucleotide fragment(s) (sequences shown in Fig. 1) into the *Bam*HI site of pTKCAT. DNA sequencing by the dideoxy method confirmed the locations and orientations of the DNA inserts for all constructs.

Eukaryotic expression vectors. Plasmids for expression of human RARβ (pCDM-RAR), human RXRα (pCDM-RXR), and hamster COUP-TF (pCDM-COUP) in tissue culture transfection experiments were constructed by inserting fragments containing appropriate receptor coding sequences into the CDM expression plasmid (2). pCDM-RXRα$_{DEF}$ was constructed by removing a fragment of pCDM-RXR to leave an expression cassette for amino acids 198 to 462 of the receptor, corresponding to RXR domains D, E, and F. Vector insert sequences were confirmed by the dideoxy method.

Overexpression of receptors in a vaccinia virus-HeLa cell system. Receptor coding sequences in the CDM expression vectors were excised and cloned into the replaceable fragment of the vaccinia virus transfer vector TKgSLP (modified from a gift of Brian Seed and Martine Amiot, Massachusetts General Hospital). This plasmid contains the vaccinia virus thymidine kinase (TK) gene (for homologous recombination) interrupted by a cassette for heterologous gene insertion. The TKgSLP cassette contains the *Escherichia coli gpt* gene driven by the vaccinia virus 7.5-kDa protein promoter that is used to select for recombinants and a synthetic idealized vaccinia virus late promoter (13) upstream of insert cloning sites.

Recombinant vaccinia viruses were generated by infection of CV-1 cells with wild-type (WT) WR strain virus, transfection of the appropriate transfer vector by the calcium phosphate coprecipitation method, and subsequent isolation of plaques in cell monolayers grown in selection medium (4). Appropriate recombination in viral isolates was confirmed by amplification of the insert DNA by PCR with a sense-strand primer homologous to the synthetic vaccinia virus promoter and an antisense primer corresponding to the appropriate receptor insert and by electrophoretic mobility shift assay (EMSA) with nuclear extracts and oligonucleotide probes containing previously reported receptor response element sequences. For receptor overproduction, HeLa S3 cells infected with amplified recombinant vaccinia virus were harvested after 24 h. Nuclei were isolated by centrifugation after cellular homogenization in lysis buffer (100 mM Tris [pH 7.8], 0.5% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 10 mg of leupeptin per ml, 0.2 mM phenylmethylsulfonyl fluoride) by repeated passage through a 25-gauge needle. Nuclear extracts were prepared in buffer consisting of 50 mM Tris (pH 7.8), 400 mM KCl, 2 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 10 mg of leupeptin per ml, and 0.2 mM phenylmethylsulfonyl fluoride and stored in aliquots at −80°C.

Overexpression of receptors in bacterial cells. The pT7flu bacterial expression plasmid was constructed by replacing the *Ssp*I-to-*Eco*RI fragment of pGEX-2T with a cassette including the T7 polymerase binding site, a consensus *lac* operator, the epsilon translational enhancer sequence (41), sequence encoding the influenza virus hemagglutinin (flu) epitope (19), and a T7 transcriptional termination signal. For expression of native receptors, coding sequences in the CDM or TKgSLP vectors were cloned into pT7flu. Receptors with an amino-terminal hemagglutinin epitope tag were constructed by cloning receptor cassettes into pT7flu, providing the appropriate in-frame fusion with the sequence MVYPYDVPDYAVD (epitope underlined).

E. coli BL21/DE3, in which the T7 polymerase gene exists as a genomic integrate under *lac*-inducible control (52), served as

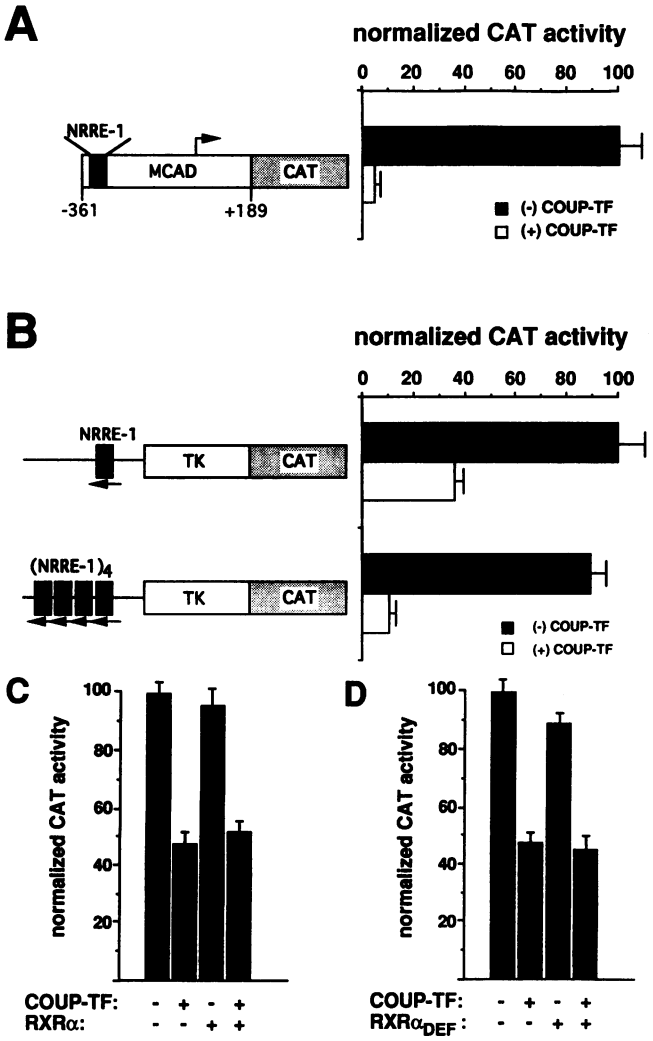

FIG. 2. COUP-TF suppresses transcription via NRRE-1 by an RXR-independent mechanism. (A) The homologous promoter reporter MCADCAT(−361/+189) was cotransfected with 1.0 μg of either pCDM (shaded bar) or pCDM-COUP (open bar) into CV-1 cells. The bars represent mean (± standard error of the mean) CAT activity/β-galactosidase activity normalized (=100) to the activity of MCADCAT(−361/+189) cotransfected with pCDM. (B) Transcriptional activity of TKCAT(NRRE-1) and TKCAT(NRRE-1)$_4$ cotransfected with 1.0 μg of pCDM (shaded bars) or pCDM-COUP (open bars). The bars represent mean CAT activity/luciferase activity normalized (=100) to the activity of pTKCAT(NRRE-1) cotransfected with pCDM. Schematics of MCADCAT(−361/+189) and pTKCAT-(NRRE-1) constructs are shown to the left of the corresponding data. (C) The results of cotransfection of pCDM-RXR on COUP-TF-mediated repression of pTKCAT(NRRE-1) transcription. pTKCAT-(NRRE-1) was cotransfected with equivalent amounts (250 ng) of pCDM-RXR, pCDM-COUP, both, or pCDM as indicated below the abscissa. The ordinate represents CAT activity/luciferase activity normalized (=100) to the activity of pTKCAT(NRRE-1) cotransfected with pCDM. (D) pTKCAT(NRRE-1) was cotransfected with a 10-fold molar excess of the dominant negative RXR expression plasmid pCDMRXRα$_{DEF}$ or an equivalent amount of pCDM. The data shown in each panel represent the mean results of at least three independent experiments.

the host for the pT7flu derivative plasmids for receptor overexpression. After growth to an optical density at 600 nm of 0.5, cells were diluted and were grown further. At an optical density at 600 nm of 0.6, 1 ml of 500 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 50 mg of ampicillin were added to cultures. After 2 to 3 h, cells were harvested by centrifugation, resuspended in A500 buffer (50 mM Tris [pH 7.5], 500 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM EDTA, 10 mg of leupeptin per ml, 0.2 mM phenylmethylsulfonyl fluoride) and subjected to three freeze-thaw cycles followed by 10 10-s sonications on ice. Bacterial extracts were prepared as previously described (44).

Mammalian cell transfections. Simian CV-1 cells were employed for all transfection experiments. Cells were maintained at 37°C in Dulbecco's modified Eagle medium supplemented with 10% charcoal-stripped fetal calf serum (46) in a 5% CO_2 environment. Transient cotransfections were performed as previously described (10, 46) by the calcium phosphate coprecipitation technique in 60-mm-diameter dishes with 10 to 15 μg of MCADCAT(−361/+189) or pTKCAT derivative plasmids and 0.1 to 2.5 μg (as indicated in the figure legends) receptor expression plasmid or an equivalent amount of pCDM lacking a DNA insert (pCDM). One microgram of a Rous sarcoma virus β-galactosidase expression vector (RSVβgal) or pTKLUC (in all experiments involving pCDM-COUP cotransfection) was included to correct for transfection efficiency. Cells were harvested 48 h after transfection. In experiments involving retinoid stimulation, parallel dishes were incubated in the presence or absence of all-*trans* (Sigma Chemical Co.) or 9-*cis* (Ligand Pharmaceuticals) RA at the indicated concentrations during the 36 h prior to harvesting. CAT assays were performed as previously described (46) by the xylene extraction method. β-Galactosidase activity was measured by using the Galacto-Light chemiluminescence assay (Promega), and luciferase activity was measured by using the standard luciferin-ATP assay (14), both in an Analytical Luminescence Monolight 2010 luminometer.

EMSA. EMSAs were performed as previously described (10). Complementary single-stranded oligonucleotides were synthesized on an Applied Biosystems PCRmate synthesizer, annealed, and gel purified before being labeled with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP. Oligonucleotide sequences are shown in Fig. 1. Receptor proteins produced in either the vaccinia virus-HeLa or bacterial expression systems were used in these experiments. Antibody supershifting experiments were performed with an amino-terminally epitope-tagged COUP-TF overproduced in bacteria (flu-COUP) with the monoclonal antibody 12CA5 (19) directed to the epitope (gift of Brian Seed). The monoclonal antibody 9E10 (Oncogene Science) directed to an epitope in the c-Myc protein (17) served as a negative control in these experiments.

Methylation interference assays. Methylation interference was performed by standard methods (3). In brief, the sense and antisense strands of an NRRE-1 oligonucleotide were γ labeled with [γ-^{32}P]dATP in separate reactions, annealed with the unlabeled complementary DNA strand, gel purified, and partially methylated with dimethyl sulfate prior to EMSA with receptors produced in the bacterial expression system. Bound and unbound probe bands were eluted from EMSA gels and cleaved with 10% piperidine prior to separation of products on 9% denaturing polyacrylamide gels and visualization by autoradiography.

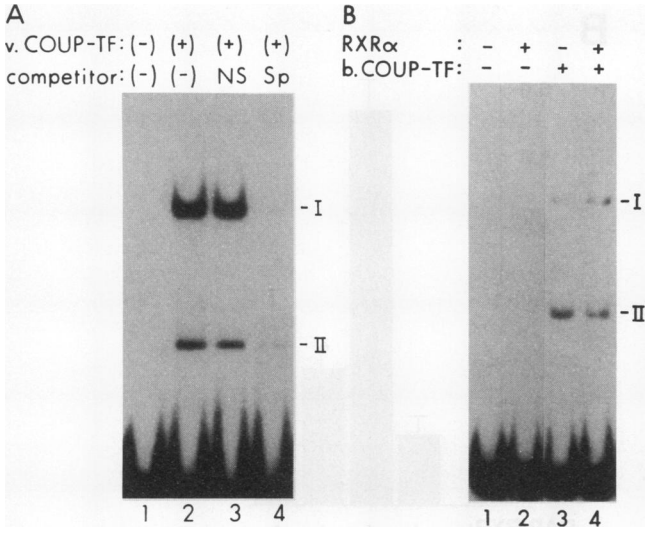

FIG. 3. Binding of NRRE-1 by COUP-TF. (A) Autoradiograph of EMSA performed with a ^{32}P-labeled NRRE-1 oligonucleotide probe (sequence shown in Fig. 1) and COUP-TF overproduced in vaccinia virus-infected HeLa cells (v. COUP-TF). A 50-fold molar excess of unlabeled, size-matched unrelated competitor oligonucleotide DNA (NS) was added for the incubation shown in lane 3 and a 50-fold molar excess of unlabeled NRRE-1 DNA (Sp) was added for the incubation shown in lane 4. Complexes exhibiting low and high mobilities are labeled I and II, respectively. (B) EMSA performed with the NRRE-1 probe and COUP-TF and RXRα overproduced in bacteria. The specific receptor-containing protein extract added to the incubation is indicated above each lane. The amount of protein extract used in this experiment was titrated down to the lower limits of detection by EMSA, as described in the text. Equivalent amounts of RXR- and COUP-TF-containing extract were used.

RESULTS

COUP-TF represses transcription through NRRE-1. COUP-TF (also known as erbA-related protein 3 or EAR-3 [39]) is a transcription factor originally identified as regulating transcription by binding an element in the chicken ovalbumin gene promoter (58). It was subsequently shown to be an orphan nuclear receptor and to regulate expression of several genes through response elements that consist of paired GGTCA sequences (12, 57). Because the recognized target genes for COUP-TF (and its isoform, ARP-1) are largely those which encode products involved in lipid metabolism (29, 30, 38, 47) and because of the overlap of RXREs, HNF-4 response elements, and COUP-TF response elements in several other genes (30, 37, 38, 47, 55), we investigated the potential regulation of MCAD expression by COUP-TF. CV-1 cells were cotransfected with a CAT reporter plasmid containing MCAD promoter sequence extending from −361 to +189 (relative to the transcription start site of +1) fused to CAT [MCADCAT(−361/+189)] with pCDM-COUP, an expression plasmid for COUP-TF, or with pCDM, which lacks a cDNA insert. As shown in Fig. 2A, COUP-TF cotransfection resulted in repression of MCAD promoter transcription to less than 10% of that seen in control cells. Additional experiments with an MCADCAT 5′ deletion series demonstrated that most of the COUP-TF-mediated repressor activity mapped to NRRE-1 (data not shown). The MCAD promoter sequence downstream of NRRE-1 contains two unpaired receptor consensus hexamers that appear to mediate the residual repressive effect (65).

To confirm the function of NRRE-1 in mediating transcriptional repression by COUP-TF, cotransfection experiments were performed with reporters containing one or four copies of NRRE-1 cloned upstream of a minimal herpes simplex virus TK promoter driving CAT expression [pTKCAT(NRRE-1) and pTKCAT(NRRE-1)$_4$, respectively]. COUP-TF has modest repressive effects on basal herpes simplex virus TK promoter activity, so in these experiments an analogous TK-luciferase reporter lacking the NRRE-1 insert(s) (pTKLUC) was used to normalize for this effect. Transcription of pTKCAT(NRRE-1) was repressed approximately 65% by COUP-TF in these experiments, while pTKCAT(NRRE-1)$_4$ transcription was virtually abolished to background levels (Fig. 2B). Transcriptional activity of the two reporters in the absence of receptor cotransfection did not differ significantly, confirming that the level of COUP-TF-mediated transrepression was attributable to NRRE-1 copy number. Similar results were obtained with a reporter containing a single copy of NRRE-1 cloned upstream of the simian virus 40 early promoter (data not shown). Thus, MCAD NRRE-1 confers potent COUP-TF-mediated *trans*-repression to homologous and heterologous promoters.

COUP-TF-mediated repression via NRRE-1 is RXR independent. The mechanism of COUP-TF-mediated transcriptional repression is unknown. Results of previous studies have suggested that COUP-TF binds and regulates from cognate response elements as a homodimer (11, 55) or a RXR-COUP heterodimer (26). Thus, we investigated the combinatorial effects of COUP-TF and RXR on NRRE-1-mediated transcriptional regulation by using pTKCAT(NRRE-1), pCDM-COUP, and expression plasmids for full-length RXRα (pCDM-RXR). In these experiments, all transfected expression plasmid amounts were reduced (to 250 ng) to maximize the opportunity to detect an RXR-mediated potentiation effect. Furthermore, transfected cells were incubated in charcoal-stripped serum to eliminate RA-mediated transactivation. As shown in Fig. 2C, COUP-TF repressed transcription of the reporter to 50% of the control level in these experiments. The transcriptional activity of pTKCAT(NRRE-1) in cells cotransfected with equivalent amounts of pCDM-COUP and pCDM-RXR did not differ significantly from that in cells transfected with pCDM-COUP alone. The absence of potentiation of COUP-TF-mediated repression by RXR is most consistent with an RXR-independent COUP-TF interaction with NRRE-1.

Further support for an RXR-independent NRRE-1–COUP-TF interaction was derived from experiments using an expression plasmid for a mutant RXRα (pCDM-RXRα$_{DEF}$) deleted for the DNA-binding domain. The protein expressed by this vector functions as a dominant negative for all receptors with which RXR can heterodimerize. Cotransfection of this construct produces dose-dependent abolition of thyroid hormone (T_3) or RA responsiveness at T_3 response elements (5) and RAREs (20), respectively, as well as elimination of the constitutive activation of a subset of RAREs produced by the new orphan receptor and RXR partner MB67 (5). Cells cotransfected with a concentration gradient of pCDM-RXRα$_{DEF}$ (up to 10-fold molar excess over pCDM-RXR) failed to exhibit any change in COUP-TF-mediated transrepression compared with control levels (Fig. 2D, and data not shown). Taken together, these results strongly suggest that the COUP-TF-mediated repression of transcription via NRRE-1 occurs independently of RXR.

COUP-TF homodimers and monomers bind MCAD NRRE-1. In order to directly examine the interaction of COUP-TF with MCAD NRRE-1, we used EMSA with an NRRE-1 oligonucleotide probe (Fig. 1) and COUP-TF over-

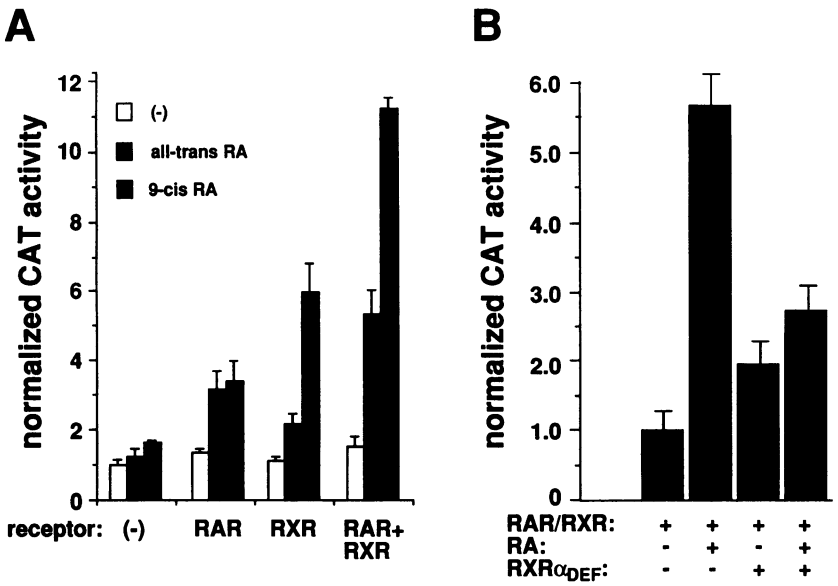

FIG. 4. 9-*cis* and all-*trans* RA-mediated transcriptional activation via NRRE-1 is RXR dependent. (A) Transcriptional activity of pTKCAT-(NRRE-1) transfected into CV-1 cells with 2.5 μg of pCDM-RAR, pCDM-RXR, both, or pCDM [denoted (−) in the abscissa] in the presence of all-*trans* RA (10^{-7} M), 9-*cis* RA (10^{-7} M), or vehicle (−) (as indicated by the shading key). The ordinate represents normalized mean CAT activity/β-galactosidase activity. (B) Effects of cotransfection of a 10-fold molar excess of the dominant negative expression plasmid pCDMRXRα$_{DEF}$ on all-*trans* RA-mediated activation of pTKCAT(NRRE-1). Equivalent amounts (1 μg) of pCDM-RXR and pCDM-RAR were cotransfected in each experiment. Addition of all-*trans* RA (10^{-7} M) and pCDMRXRα$_{DEF}$ is indicated below the abscissa. The data shown in both panels represent the mean results of at least three independent experiments.

produced in cells infected with recombinant vaccinia virus that express this receptor. As shown in Fig. 3A, two protein-DNA complexes formed in these assays: a prominent complex with low mobility (I), and a minor complex with higher mobility (II). Formation of both complexes was inhibited by a 50-fold molar excess of unlabeled NRRE-1 oligonucleotide but not by a size-matched unrelated competitor oligonucleotide (Fig. 3A, lanes 3 and 4). These results demonstrate specific NRRE-1–COUP-1 interactions.

Because the HeLa cell nuclei in which COUP-TF was overproduced contain significant concentrations of RXRs (32), the foregoing experiments could not exclude the possibility that RXR participated in the formation of COUP-TF–NRRE-1 EMSA complexes. Thus, we next utilized receptor proteins overexpressed in bacteria to avoid potential contaminating endogenous RXR. Here, COUP-TF and RXRα protein concentrations in binding reaction mixtures were titrated down in order to detect any potentiation of COUP-TF binding conferred by RXR. As before, COUP-TF reactions produced two specific bands that comigrated with those seen with vaccinia virus-HeLa cell COUP-TF–NRRE-1 EMSA (Fig. 3B). However, in contrast to the previously observed predominance of the more slowly migrating COUP-TF–NRRE-1 complex I, in these studies complex II was prominent. The two bands represent monomer and dimer receptor-DNA complexes, respectively (see below). There was no appreciable enhancement or diminution of either complex with RXRα coincubation (Fig. 3B, lanes 3 and 4). Similar results were obtained in studies using a wide concentration range of RXRα and with receptors overproduced in bacteria, by in vitro transcription and translation, or in recombinant vaccinia virus-infected HeLa cells (data not shown). Taken together, these EMSAs and the cotransfection studies confirm that the COUP-TF–NRRE-1 interaction occurs independently of RXR.

All-*trans* RA- and 9-*cis* RA-mediated transcriptional activation via NRRE-1 is RXR dependent. Previously reported retinoid responsive elements activate transcription by interacting with RAR-RXR heterodimers or RXR-RXR homodimers in the presence of the retinoid ligands all-*trans* RA and its isomer 9-*cis* RA (16, 27, 33, 64). Our initial studies of NRRE-1 as a retinoid responsive element showed a preferential activation by RXRs over RARs and binding of both receptors to the element (46). However, these studies used receptor proteins prepared in a vaccinia virus-HeLa cell expression system such that strict discrimination of homodimer versus heterodimer interactions was not possible. Further, it had not yet been established that 9-*cis* RA was the cognate ligand for RXRs at the time of this work. Thus, we examined the function and binding of these receptors on NRRE-1 in greater detail. Cotransfection experiments were performed by using pTKCAT(NRRE-1) with pCDM-RAR and pCDM-RXR alone or in combination. Transfected CV-1 cells were then incubated in the presence or absence of all-*trans* RA or 9-*cis* RA at 10^{-7} M. Addition of either ligand to the medium of cells transfected with pTKCAT(NRRE-1) without cotransfected receptor (Fig. 4A) resulted in a minimal increase in transcription, consistent with the known low levels of retinoid receptors in CV-1 cells. Cotransfection of RARβ resulted in a 3.0- to 3.5-fold activation of TKCAT(NRRE-1) by either 9-*cis* RA or all-*trans* RA. In contrast, with cotransfection of RXRα alone, activation was significantly greater with 9-*cis* RA (6- to 7-fold) than with all-*trans* RA (2- to 2.2-fold), consistent with activation of RXR by its cognate ligand 9-*cis* RA. Transcriptional activation of TKCAT(NRRE-1) by either all-*trans* RA or 9-*cis* RA was greatest with cotransfection of both RARβ and RXRα. Furthermore, in the presence of both RXR and RAR, 9-*cis* RA activated transcription to a greater extent than all-*trans* RA (11- to 12-fold versus 5- to 6-fold). In parallel experiments with higher concentrations of all-*trans* RA (10^{-6}

M), the level of RXR-mediated activation of pTKCAT-(NRRE-1) was virtually identical to that of 9-*cis* RA. These data are consistent with a cooperative interaction of RXR and RAR at NRRE-1, although additive homodimer RAR- and RXR-mediated effects cannot be excluded. Moreover, although the architecture of NRRE-1 is unique compared with those of other reported retinoid responsive elements, this element is capable of conferring both 9-*cis* and all-*trans* RA-mediated transcriptional activation in mammalian cells in a pattern similar to that of several other reported direct repeat elements that interact with RAR-RXR heterodimers (64).

To further elucidate the functional interactions of RAR and RXR on NRRE-1, cotransfection experiments using the dominant negative RXR expression plasmid pCDM-RXRα_{DEF} were carried out. CV-1 cells were transfected with the pTKCAT(NRRE-1) reporter, subsaturating concentrations of pCDM-RXRα and pCDM-RARβ, and either pCDM-RXRα_{DEF} or an equivalent amount of pCDM. Cells were then incubated in the presence or absence of 10^{-7} M RA to examine the influence of dominant negative RXR mutant receptor expression on basal and RA-mediated transactivation. A 10-fold molar excess of pCDM-RXRα_{DEF} expression vector virtually abolished the 5.5-fold RA-mediated reporter induction in these experiments (Fig. 4B). Both an increase in basal transcriptional activities and a marked diminution in RA-activated transcriptional activities were seen. The results of these experiments mimic those seen with other RXR heterodimer partners on cognate response elements (5, 20) and confirm that the retinoid response conferred by NRRE-1 requires RXR.

RAR and RXR bind cooperatively to MCAD NRRE-1 as a heterodimer. To directly examine the interaction of RAR and RXR with NRRE-1, we performed EMSAs with receptor proteins prepared in bacteria, thereby eliminating the potential contribution of contaminating mammalian nuclear proteins to receptor binding. As shown in Fig. 5, at concentrations of RARβ and RXRβ that failed to produce a retarded complex with the NRRE-1 oligonucleotide probe when incubated in binding reaction mixtures alone, coincubation resulted in formation of a prominent retarded band. In parallel experiments this band was inhibited by excess unlabeled NRE-1, as well as by an oligonucleotide containing sequence from the potent DR5 RARE of the RARβ gene promoter (53) that also binds RAR-RXR heterodimers (data not shown). Addition of all-*trans* RA (10^{-6} M) or 9-*cis* RA (10^{-7} M) to the incubation mixtures did not induce RXR homodimer binding or enhance RAR-RXR heterodimer binding to the NRRE-1 probe (data not shown). These data are consistent with the cotransfection results and indicate that RAR and RXR interact in a cooperative and synergistic manner in binding to NRRE-1. Accordingly, the retinoid response conferred by MCAD NRRE-1 is likely attributable to ligand-mediated effects via RAR-RXR heterodimers at this element.

RAR-RXR heterodimers and HNF-4 and COUP-TF homodimers bind distinct MCAD NRRE-1 half-site pairs and define novel DNA-nuclear receptor binding sites. The receptor-DNA binding and cotransfection data shown here indicate that COUP-TF interacts with NRRE-1 to repress transcription by an RXR-independent mechanism, whereas retinoid-mediated activation of transcription through NRRE-1 is RXR dependent. We have also shown previously that the orphan receptor HNF-4 confers constitutive NRRE-1-mediated transcriptional activation independently of RXR (10). Thus, we have identified three distinct nuclear receptor dimer interactions with NRRE-1 that confer bidirectional and ligand-dependent or -independent regulation of MCAD gene transcription. The architecture of NRRE-1 is unique compared with those of other previously identified TR/RAR/VDR response elements. Specifically, the 5′ hexamer (site 1) is in the antisense orientation 8 bp upstream of an imperfect sense repeat (sites 2 and 3) (Fig. 1). A fourth potential hexamer site (TGGTCA) exists in the antisense orientation 4 bp downstream of site 3 but has not been shown to contribute to any of the functional or DNA-binding activities of the receptors studied to date. The potential pairwise receptor hexamer binding sites within this element do not correspond to prototypical receptor response elements such that sequences contributing to receptor function were not predictable from previous reports. Accordingly, we next directly explored the receptor-DNA interactions in detail.

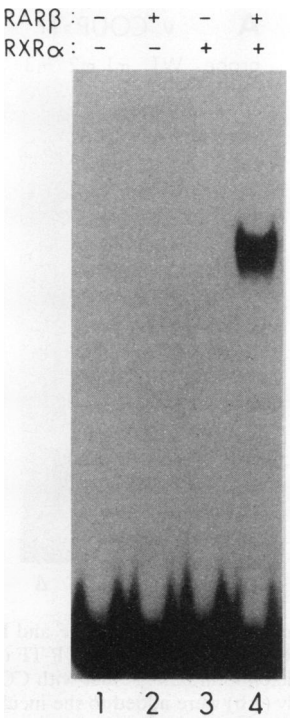

FIG. 5. RARβ and RXRα bind to NRRE-1 cooperatively as a heterodimer. An EMSA performed with the NRRE-1 probe and equivalent amounts of protein extract containing RARβ and RARα overproduced in bacteria. The receptor(s) added to each incubation is indicated above each lane.

EMSAs with mutant NRRE-1 oligonucleotide probes were utilized as a first step in delineation of receptor binding sites in this complex element. In addition to the WT NRRE-1 probe, mutant oligonucleotide probes (Fig. 1) containing G-to-C substitutions at the invariant second-position guanine of NRRE-1 binding site 1 (NRRE-1m1), site 2 (NRRE-1m2), site 3 (NRRE-1m3), or both sites 2 and 3 (NRRE-1m2m3) were employed in EMSA with overexpressed receptors. In some studies, oligonucleotide probes containing sequences deleted for either site 1 (NRRE-1Δ1) or sites 2 and 3 (NRRE-1Δ2,3) were also used. Assays of COUP-TF, RAR-RXR, and HNF-4 binding to these probes were comprehensively explored and compared.

Figure 6A shows an EMSA in which the WT and substitution mutation NRRE-1 probes were used in reaction mixtures with nuclear extracts from HeLa cells infected with COUP-TF-producing recombinant vaccinia virus. As before, complex I was predominant in reactions with the WT NRRE-1 probe.

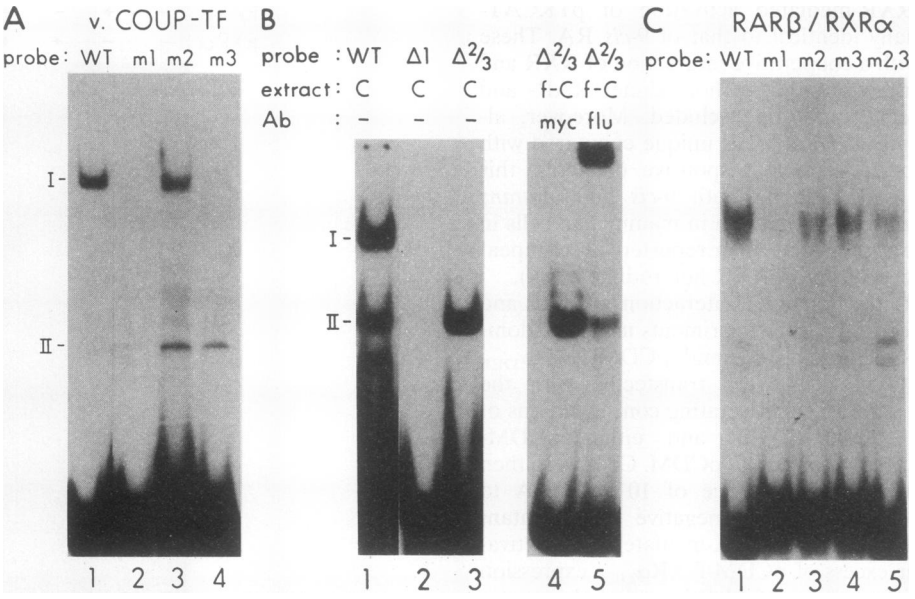

FIG. 6. Mutational analysis of COUP-TF and RAR-RXR binding sites within NRRE-1. (A) EMSA performed with an NRRE-1 probe and a series of mutant NRRE-1 probes with COUP-TF overproduced in vaccinia virus-infected HeLa cells. (B) Mobility shift and supershift assays with NRRE-1 WT and deletion mutant probes with COUP-TF (C) and flu-COUP (f-C [see text for description]) overproduced in bacteria. Anti-Myc and anti-flu antibody (Ab) were added to the incubations in lanes 4 and 5, respectively. (C) EMSA performed with the NRRE-1 WT and mutant probes with RARβ and RXRα overproduced in bacteria. Equivalent amounts of RARβ- and RXRα-containing protein extract were added to each lane. Probe abbreviations (oligonucleotide sequences shown in Fig. 1): WT, NRRE-1; m1, NRRE-1m1; m2, NRRE-1m2; m3, NRRE-1m3; m2,3, NRRE-1m2m3; Δ1, NRRE-1Δ1; Δ2/3, NRRE-1Δ2,3.

Binding to the NRRE-1m1 probe was not detectable. The formation of complex I with the NRRE-1m2 probe was not significantly different from that observed with the WT probe, but complex II appeared slightly more prominent. The NRRE-1m3 probe incubations gave only a complex II band. Taken together, these findings indicate that both sites 1 and 3 are required for complex I formation, that site 2 is irrelevant to COUP-TF binding to NRRE-1, and that site 1 alone may be responsible for complex II. Furthermore, the relative migration of the complexes as well as their pattern with these NRRE-1 mutant probes supported the hypothesis that complexes I and II represented COUP-TF homodimer and monomer–NRRE-1 complexes, respectively.

To confirm the hypothesis that complex II in Fig. 6A represented an interaction of NRRE-1 site 1 with a COUP-TF monomer, both native COUP-TF and COUP-TF tagged with an amino-terminal flu epitope (flu-COUP; see Materials and Methods) were overproduced in bacteria and used in mobility shift experiments with the WT, NRRE-1Δ1, and NRRE-1Δ2,3 probes. Complexes I and II were evident in incubations of native COUP-TF with the WT NRRE-1 probe, while neither was seen with NRRE-1Δ1, and only complex II was seen with NRRE-1Δ2,3 probe reactions (Fig. 6B). Reaction mixtures with flu-COUP permitted confirmation that complex II contained COUP-TF. Specifically, the complex II formed with the NRRE-1Δ2,3 probe was supershifted in coincubation reactions with antibody to the flu epitope but not with an unrelated antibody (anti-Myc) (Fig. 6B, lanes 4 and 5). Thus, monomer COUP-TF can bind site 1, while homodimer COUP-TF binds sites 1 and 3. This remarkable COUP-TF response element consists of hexamer sites arranged as an everted repeat with 14 intervening bp. That dimerization is present on the ER14 element, as distinct from dual monomer cooccupation, is evidenced by the fact that site 3 is occupied only in the context of an intact site 1. Thus, COUP-TF monomer binds exclusively to site 1, and dimer binding is necessary for simultaneous occupation of half-sites 1 and 3.

Similar studies were undertaken with this panel of WT and mutant NRRE-1 probes in examination of RAR-RXR receptor binding. The specific complex formed with the WT NRRE-1 probe was not present in NRRE-1m1 probe reactions (Fig. 6C), indicating that site 1 is essential for retinoid receptor dimer binding. Binding reactions with the other probes did not permit clear definition of a second RAR–RXR–NRRE-1 contact site. Each of the NRRE-1m2, NRRE-1m3, and NRRE-1m2m3 probes formed a complex with the NRRE-1 probe that was less prominent compared with that formed on the WT probe, with the NRRE-1m3 probe exhibiting the least diminution. These results do not permit distinction between several explanations for the maintenance of binding to the site 2 and site 3 mutant NRRE-1 probes. On the basis of these results it is possible that in the context of a mutated site 2, site 3 can serve as the second dimer binding site with site 1 or that the RAR-RXR heterodimer requires only site 1 for binding. Alternatively, a more trivial explanation is that the hexamer single-point mutations, in a context of an intact adjacent site 1 consensus hexamer, create permissive binding sites that allow weak binding.

To confirm the results of the nuclear receptor-DNA binding studies and to clarify the RAR-RXR–NRRE-1 interactions within this complex element, methylation interference assays with partially methylated sense and antisense NRRE-1 oligonucleotide probes end labeled with ^{32}P and receptor proteins produced in bacteria were performed (Fig. 7A and B). The COUP-TF–NRRE-1 interaction within complex I involved guanine residues in sites 1 and 3 but not site 2. Complex II exhibited methylation interference only at site 1, indicating that this was the exclusive contact site for monomer COUP-

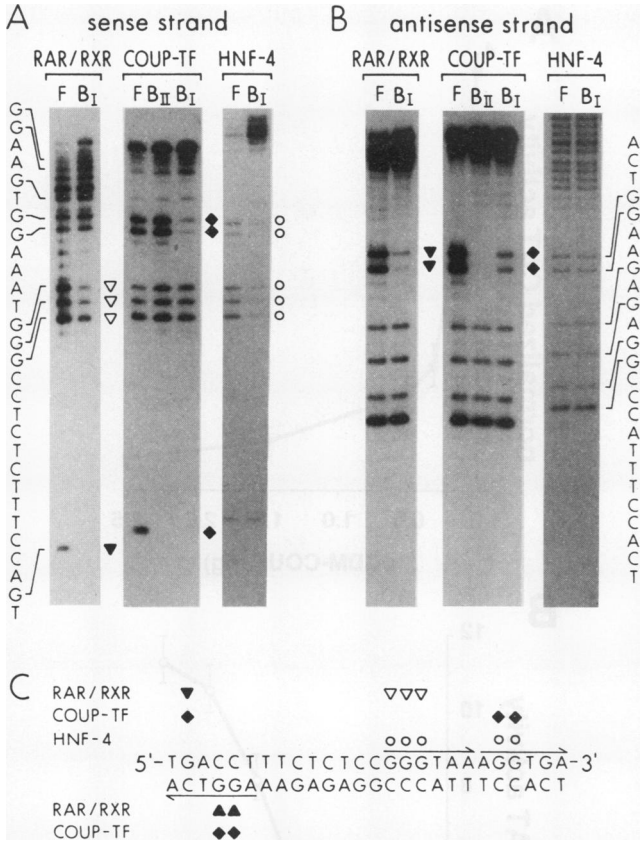

FIG. 7. Delineation of NRRE-1 receptor binding sites by methylation interference assays. (A and B) Results of methylation interference assays with end-labeled sense (A) and antisense (B) NRRE-1 oligonucleotide probes and receptors overproduced in bacteria. The total counts per minute applied to each lane were equivalent. The guanine residues (G) within free (F) probe and receptor dimer (B_I)- and receptor monomer (B_{II})-bound complexes are denoted. Triangles, diamonds, and circles denote methylated guanines that interfered with RAR-RXR, COUP-TF, and HNF-4, respectively. Closed symbols represent a strong interaction, and open symbols represent a weak-to-moderate interaction, as determined by comparison with the corresponding F lane. (C) Schematic representation comparing guanine contact points of each receptor dimer with both strands of the NRRE-1 sequence. The arrows indicate the location and relative orientation of the hexamer binding sites within NRRE-1.

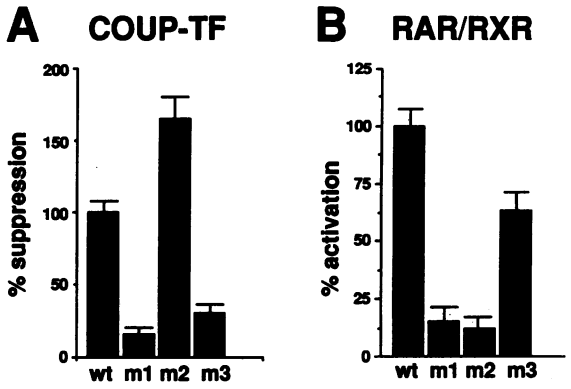

FIG. 8. Retinoid- and COUP-TF-mediated transcriptional regulation via mutant NRRE-1 elements. (A) COUP-TF-mediated suppression of TKCAT(NRRE-1) and corresponding NRRE-1 mutant constructs. The cotransfected mutant construct is indicated along the abscissa. WT, pTKCAT(NRRE-1); m1, pTKCAT(NRRE-1m1); m2, pTKCAT(NRRE-1m2); m3, pTKCAT(NRRE-1m3). The ordinate represents mean (± standard error of the mean) COUP-TF-mediated suppression as a percentage of that observed with WT TKCAT-(NRRE-1). (B) Retinoid-mediated transactivation of TKCAT (NRRE-1) and NRRE-1 mutant constructs. TKCAT(NRRE-1) plasmid constructs were cotransfected with pCDM-RAR (1.5 μg) and pCDM-RXR (1.5 μg) into CV-1 cells in the absence (vehicle only) and presence of all-*trans* RA (10^{-7} M). The ordinate represents mean (± standard error of the mean) RA-mediated activation as a percentage of that observed with wild-type TKCAT(NRRE-1). Data shown in both panels represent the mean results of at least three independent experiments.

TF–NRRE-1 interactions. These observations confirmed the results provided by the NRRE-1 mutant oligonucleotide EMSA studies.

The methylation interference assays clarified the interaction of RAR-RXR with NRRE-1 (Fig. 7A and B). Contacted guanine residues in this complex were restricted to those in sites 1 and 2. Specifically, there was no evidence for receptor contact with residues located in the spacer region between sites 1 and 2 or with those in site 3. Thus, in the context of a WT NRRE-1, the retinoid receptor heterodimer binds an element consisting of everted repeat hexamer sites separated by 8 bp (ER8).

Our previous studies have shown that HNF-4 binds and transactivates through the other possible combination of paired sites in NRRE-1, sites 2 and 3. Methylation interference assays corroborated the lack of involvement of site 1 in HNF-4–NRRE-1 interactions because contacted guanine residues were restricted to sites 2 and 3 (Fig. 7A and B). Taken together, these biochemical studies indicate the recognition of three distinct hexamer pair combinations within MCAD NRRE-1 by three different nuclear receptor dimers (Fig. 7C).

Distinct NRRE-1 hexamer pairs are necessary and sufficient for differential transcriptional regulation by COUP-TF, RAR-RXR, and HNF-4. To examine functional correlates of the preceding biochemical data and to confirm that the ER14, ER8, and DR0 elements functioned differentially as COUP-TF, retinoid, and HNF-4 response elements, respectively, cotransfection studies were performed with receptors containing single copies of the NRRE-1 mutant oligonucleotides (Fig. 1) inserted in pTKCAT. Figure 8A depicts results of experiments in which COUP-TF-mediated *trans*-repression was examined with this series of reporters. COUP-TF activity was virtually abolished at the NRRE-1 m1 and m3 elements, whereas the site 2 mutation resulted in approximately 50% greater activity compared with that of the WT response element. These results indicate that, despite strong COUP-TF monomer binding to site 1, in vitro repressor function requires intact sites 1 and 3. The reason for augmented suppression of pTKCAT(NRRE-1m2) is not clear but may represent the loss of competitive activation by endogenous nuclear hormone receptor transcription factors that require site 2 for activity.

Figure 8B shows the results of analogous experiments conducted with the mutant NRRE-1 reporter series in cotransfection experiments with pCDM-RAR and pCDM-RXR. All-*trans* RA-mediated activation of both the m1 and m2 NRRE-1 reporters was virtually abolished, consistent with a requirement for both sites 1 and 2 for a functional RARE. Transactivation of pTKCAT(NRRE-1m3) was slightly diminished compared with that of the WT NRRE-1 reporter. This result is compatible with a minor role for site 3 in binding receptor heterodimers directly or an indirect effect on the affinity of

receptor binding to the adjacent site 2. In the case of several different complex response elements studied by others, including the rat growth hormone receptor gene T_3RE (7) and the crystallin gene RARE (54), a similar potentiating role for a third hexamer site has been demonstrated. Thus, the retinoid response element within NRRE-1 is composed of sites 1 and 2 arranged as an everted repeat with 8-bp spacing (ER8).

Regarding HNF-4 function at NRRE-1, we have previously reported that mutation or removal of site 1 has no effect on HNF-4-mediated transactivation (10). Taken together with results of the HNF-4–NRRE-1 methylation interference studies shown here, these data confirm the role of the imperfect repeat composed of sites 2 and 3 as the HNF-4 responsive element within NRRE-1.

Nuclear receptors compete at MCAD NRRE-1 to modulate constitutive and ligand-mediated transcriptional activity. The methylation interference experiments, mobility shift assays with WT and substitution mutant NRRE-1 oligonucleotides, and the analysis of nuclear receptor-mediated transcriptional regulation via NRRE-1 and its mutants have demonstrated that the complex architecture of NRRE-1 allows interaction with multiple receptor dimers. Further, these dimers recognize sequence motifs within NRRE-1 that are novel compared with previously reported response elements and represent all possible pairwise combinations of three hexamer sites. These results suggest that basal and ligand-mediated MCAD gene transcription is modulated in vivo by the relative nuclear concentrations of endogenous receptor transcription factors that compete for binding to NRRE-1. In fact, we have shown recently that NRRE-1 interacts with multiple endogenous proteins present in crude rat liver nuclear protein extracts including HNF-4 and COUP-TF (8, 10). To examine this hypothesis further, we performed a series of cotransfection experiments with CV-1 cells to explore the functional consequences of these interactions.

To evaluate the influence of COUP-TF on HNF-4-mediated activation of NRRE-1, increasing amounts of pCDM-COUP were cotransfected with a fixed amount of pCDM-HNF-4 and pTKCAT(NRRE-1). COUP-TF produced a dose-dependent inhibition of the fourfold HNF-4-mediated activation and at higher amounts repressed transcription of TKCAT(NRRE-1) below the level of basal activity observed in the absence of cotransfected receptors (Fig. 9A). These data demonstrate that a competitive interaction of HNF-4 and COUP-TF at NRRE-1 results in bidirectional modulation of transcription over a 20-fold range.

Similar cotransfection mixing experiments were performed to examine the effect of COUP-TF on retinoid-mediated activation of NRRE-1. Increasing amounts of pCDM-COUP (0, 0.5, or 2.5 μg) were cotransfected with pCDM-RXR and pCDM-RAR in the presence of a concentration range of all-*trans* RA. The RA-mediated activation curve was shifted downward by cotransfection of 0.5 μg of pCDM-COUP (Fig. 9B). At the higher amount (2.5 μg) of cotransfected pCDM-COUP, the retinoid responsiveness was virtually abolished. These results reveal a mechanism by which the magnitude of ligand-mediated receptor transactivation through NRRE-1 can be modulated over a wide range by the competitive interaction of RAR-RXR heterodimers and COUP-TF homodimers.

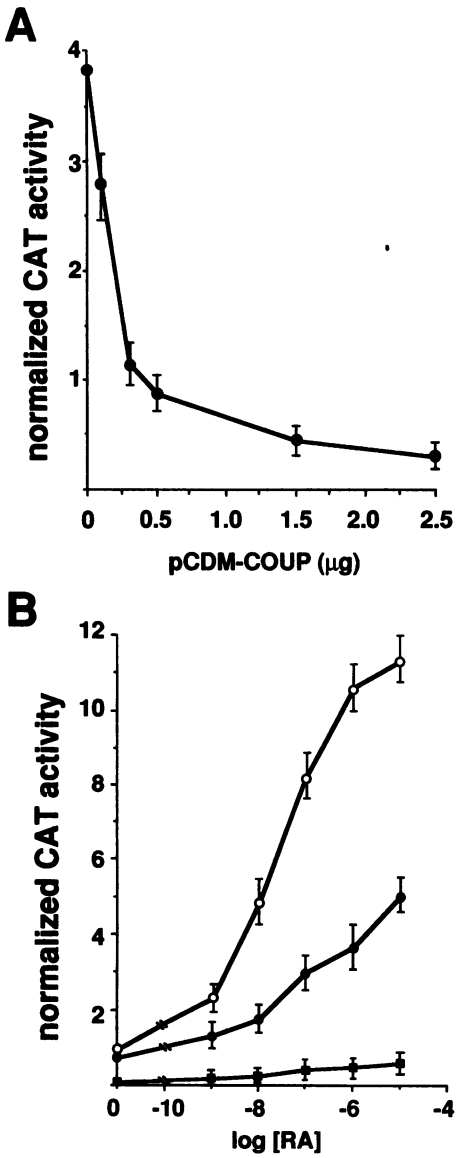

FIG. 9. Modulation of HNF-4 and retinoid-mediated activation of NRRE-1 by COUP-TF. (A) TKCAT(NRRE-1) was cotransfected with a fixed amount of pCDM-HNF-4 (2.5 μg) and increasing amounts of pCDM-COUP (0 to 2.5 μg) into CV-1 cells. The ordinate represents mean CAT activity/luciferase activity normalized (=1.0) to the activity of TKCAT(NRRE-1) cotransfected with pCDM. The data represent the mean results of at least three experiments. (B) The all-*trans* RA-mediated activation of TKCAT(NRRE-1) over a range of all-*trans* RA concentrations in the absence (pCDM only [open circles]) and presence (0.5 μg [closed circles] and 2.5 μg [closed squares]) of cotransfected pCDM-COUP is shown. Cells were cotransfected with equivalent amounts of pCDM-RAR and pCDM-RXR in all experiments. The ordinate represents CAT activity/β-galactosidase activity in the presence of RA normalized (=1.0) to the activity of TKCAT-(NRRE-1) cotransfected with pCDM-RAR and pCDM-RXR in the absence of RA (vehicle only).

DISCUSSION

In this report we describe the characterization of a complex transcriptional regulatory element in the MCAD gene promoter, NRRE-1, and present evidence that it contains three distinct nuclear receptor response elements for the retinoid receptors and the orphan receptors COUP-TF and HNF-4. Each element is novel compared with established prototypical cognate elements. The versatility of NRRE-1 is underscored by its response to these ligand-activated and constitutively repressing and activating transcription factors.

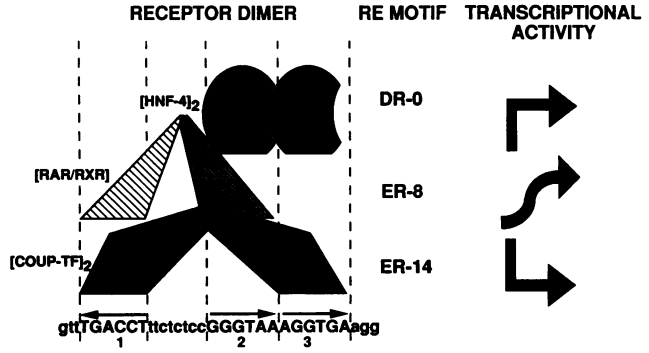

FIG. 10. Bidirectional transcriptional regulation by multiple receptor transcription factor dimers via MCAD NRRE-1. Schematic representation of the interaction of multiple nuclear receptor dimers with NRRE-1. The NRRE-1 sense-strand DNA sequence and location and relative orientation of hexamer binding sites 1 to 3 is shown at the bottom. The receptor dimers are depicted above the corresponding response element. The response element motif and effect of the dimer on transcriptional activity are shown on the right.

NRRE-1 is composed of three functionally relevant sequences that conform to the RG$^{G}_{T}$TCA consensus hexamer for binding to members of the TR/RAR/VDR subgroup of the nuclear receptor superfamily. Functional response elements for orphan receptor COUP-TF and HNF-4 homodimers and for RAR-RXR heterodimers are composed of each possible pairwise combination of these three sites (a proposed scheme is shown in Fig. 10). Thus, COUP-TF binds and represses from the pair of sites within NRRE-1 that are everted with respect to each other and separated by 14 bp (ER14). In contrast, the HNF-4 element consists of the two adjacent sites that are oriented in the same direction (DR0). Finally, the retinoid receptor heterodimer activates from the two sites that are everted and separated by 8 bp (ER8). Although *cis*-acting elements in the promoters of the genes encoding apoAI, apoCIII, apoB (30, 37, 38), ornithine transcarbamylase (25) and intestinal fatty acid binding protein (47) each interact with multiple nuclear receptors, including those studied in this report, all contain shared, overlapping COUP-TF, HNF-4, and RXR recognition sequences. The contrasting alternative pairwise binding of the three sites in NRRE-1 by these receptors (Fig. 10) is unique and predicts differential transcriptional effects of retinoids and the orphan receptors via NRRE-1 compared with those of other reported pleiotropic elements.

The COUP-TF element within NRRE-1 is particularly remarkable in that it is composed of hexamers separated by 14 bp, an interval that, to our knowledge, is the longest found in any naturally occurring nuclear receptor response element reported to date. Several lines of evidence indicate that COUP-TF binds this ER14 element as a homodimer rather than as two noncooperative monomers. First, mutation of site 1 abolished all COUP-TF binding, including the COUP-TF monomer band on EMSA, whereas mutations of site 3 or even deletion of sites 2 and 3 resulted in selective loss of the dimer but not monomer complex. Since COUP-TF monomers bound site 1 exclusively and binding of site 3 was dependent on the presence and occupation of site 1, COUP-TF binding to the ER14 element is necessarily cooperative. Second, our cotransfection studies with COUP-TF and mutant NRRE-1 elements demonstrated that both site 1 and site 3 must be intact for repressor function. Taken together, these data as well as the highly selective methylation interference pattern confirm the ER14 structure of the COUP-TF responsive element within NRRE-1.

The intervening 14 bp in the COUP-TF element of NRRE-1 dictate that the center-to-center interval between bound hexamers is 20 bp, or approximately two integral turns of helical DNA. Such integral spacing has been previously shown to be important in the cooperative binding of DNA by pairs of lambda repressor dimers (23) and by pairs of steroid hormone receptors (49) but has never been noted for a single receptor dimer. While this appears to represent a logical explanation for the cooperative binding of COUP-TF at the ER14 sites of NRRE-1, preliminary studies with various NRRE-1 mutant oligonucleotides deleted for residues between sites 1 and 3 have shown maintenance of COUP-TF dimer binding when residues immediately flanking the hexamers are maintained (9). Thus, it appears that the specific hexamer sequences or immediate flanking residues, rather than spacing, dictate COUP-TF dimer binding to NRRE-1.

Comparison of the DNA sequences and arrangement of NRRE-1 binding sites with the list of other known naturally occurring and artificial COUP-TF response elements compiled by Cooney et al. (12) demonstrates that only site 1 matches the proposed consensus binding sequence GGTCA, whereas site 2 (GGTAA) and site 3 (GGTGA) contain a mismatch in the penultimate residue. From our data, this suggests that in the context of an ER element, a cytosine in this position is optimal for COUP-TF binding, a guanine is permissive, and an adenine is prohibitive. Alternatively, nucleotides flanking the hexamer sequences may play a role in selective binding of sites in NRRE-1 by this receptor. In this regard, both sites 1 and 3 have two 5′ flanking adenine residues, while site 2 has flanking cytosines (Fig. 1). Other orphan (59, 60) and conventional (24) receptors have been shown to exhibit strong binding preferences for hexamer sites with AT-rich 5′ flanking sequences. Further studies to more precisely define COUP-TF response element sequence and hexamer spacing requirements are under way. However, our data and this comparative analysis demonstrate that this receptor may interact with elements possessing a wide variety of architectures, a phenomenon consistent with the hypothesis that it acts as a general suppressor of many genes (11, 12, 55).

In view of recent interest in the role of monomer nuclear receptor-mediated transcriptional regulation (24, 59), it is interesting that COUP-TF binds with high affinity to site 1 of NRRE-1 but functions only in the context of an appropriately oriented paired hexamer, implying a dimerization requirement for function. Several explanations for this phenomenon exist. First, the repressor function of this receptor may require a shared domain. Second, it is possible that some COUP-TF monomer sites exhibit function while others do not. For example, a functional element might exist when an optimal COUP-TF monomer binding site overlaps a suboptimal transactivating factor element. Because COUP-TF represses both the herpes simplex virus TK promoter and the proximal MCAD promoter (downstream of NRRE-1) to some degree, neither of which appears to possess potential paired hexamer sites, we favor this latter hypothesis. It is unclear whether such a mechanism for monomer receptor function would generalize to other members of the superfamily. Finally, although COUP-TF does not function via an isolated NRRE-1 site 1, it is possible that COUP-TF monomer binding at this site modulates the function of receptor dimers occupying sites 2 and 3 in vivo.

We have previously demonstrated by cotransfection studies that sites 2 and 3 of NRRE-1, which comprise an imperfect adjacent direct repeat (DR0), function as an HNF-4 response

element (10). In the present study, we confirm by methylation interference that HNF-4-contacted residues within NRRE-1 are confined to these sites. Unlike other characterized elements for this receptor, wherein COUP-TF, RAR-RXR, and HNF-4 dimers contact virtually identical residues, in NRRE-1 HNF-4 contacts just one of two hexamers that define the COUP-TF or RAR-RXR response elements. Accordingly, HNF-4 is predicted to exhibit a different pattern of transactivation via NRRE-1 compared with other prototypical elements in the context of a nuclear environment where receptors may be present at varying concentrations.

That sites 1 and 2 within NRRE-1 function as an all-*trans* RARE was demonstrated by us previously (46) and was, at that time, the first demonstration of an ER8 RARE. Since our description, a second RARE of this type has been identified in the γF-crystallin gene (54), suggesting that in a subgroup of genes, this may be a preferred motif for retinoid responsive elements. In the present study we have extended our previous findings by demonstrating that this element interacts with RAR-RXR heterodimers and confers activation by either all-*trans* or 9-*cis* RA. Thus, despite its unusual architecture, the activation and receptor binding properties of the ER8 RARE in NRRE-1 are similar to previously reported DR2 and DR5 RAREs, both of which also preferentially bind retinoid receptor heterodimers. Heterodimer formation on this ER element is particularly interesting in light of recent work that implies a role for residues within the DNA-binding domain first zinc finger loop (the direct repeat box or DR box) of receptors that dimerize with RXR (28, 42). The location of the DR box in the DNA-bound RAR would be expected to be constrained in such a way as to be prohibitive for interaction with the recently identified RXR dimerization domain located within the hinge or D region (28, 31, 42, 60) on ER elements such as NRRE-1. Thus, RAR (and RXR) domains critical for heterodimerization on DR and ER elements are likely to differ.

Evidence is emerging that expression of numerous genes whose products participate in metabolic pathways, and particularly lipid metabolism (1, 10, 22, 25, 29, 30, 37, 38, 46, 47, 48), is modulated by multiple nuclear receptors, often via complex transcriptional regulatory elements. The expression of many of these genes is regulated by COUP-TF, HNF-4, and RAR-RXR. Our cotransfection studies with NRRE-1 shown here and in a previous report (10) confirm that basal and ligand-activated transcription can be modulated by the competitive interaction of activator and repressor nuclear receptors over a 400-fold range. Furthermore, we have shown recently that NRRE-1 interacts with several proteins present in rat liver nuclear extract, including HNF-4 and COUP-TF (8, 10). We concur with the proposal of others (30, 38) that these competitive interactions occur in vivo to modulate transcription of target genes, with net activity determined by the relative intranuclear concentrations of the receptors and, where present, their cognate ligand activators.

In summary, we have shown that the complex regulatory element NRRE-1, present in the MCAD gene promoter, contains three distinct nuclear receptor response elements that mediate transcriptional activation or repression and define novel receptor binding motifs. The element enables modulation of MCAD gene transcription perhaps in response to a variety of metabolic and physiological signals and in a pattern that is predicted to differ in different cell types as a function of nuclear receptor expression and ligand availability.

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M.E.C. and T.G. contributed equally to this work.

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