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Transcriptional Control of a Nuclear Gene Encoding a Mitochondrial Fatty Acid Oxidation Enzyme in Transgenic Mice: Role for Nuclear Receptors in Cardiac and Brown Adipose Expression

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Expression of the gene encoding medium-chain acyl coenzyme A dehydrogenase (MCAD), a nuclearly encoded mitochondrial fatty acid β-oxidation enzyme, is regulated in parallel with fatty acid oxidation rates among tissues and during development. We have shown previously that the human MCAD gene promoter contains a pleiotropic element (nuclear receptor response element [NRRE-1]) that confers transcriptional activation or repression by members of the nuclear receptor superfamily. Mice transgenic for human MCAD gene promoter fragments fused to a chloramphenicol acetyltransferase gene reporter were produced and characterized to evaluate the role of NRRE-1 and other promoter elements in the transcriptional control of the MCAD gene in vivo. Expression of the full-length MCAD promoter-chloramphenicol acetyltransferase transgene (MCADCAT.371) paralleled the known tissue-specific differences in mitochondrial β-oxidation rates and MCAD expression. MCADCAT.371 transcripts were abundant in heart tissue and brown adipose tissue, tissues with high-level MCAD expression. During perinatal cardiac developmental stages, expression of the MCADCAT.371 transgene paralleled mouse MCAD mRNA levels. In contrast, expression of a mutant MCADCAT transgene, which lacked NRRE-1 (MCADCATΔNRRE-1), was not enriched in heart or brown adipose tissue and did not exhibit appropriate postnatal induction in the developing heart. Transient-transfection studies with MCAD promoter-luciferase constructs containing normal or mutant NRRE-1 sequences demonstrated that the nuclear receptor binding sequences within NRRE-1 are necessary for high-level transcriptional activity in primary rat cardiocytes. Electrophoretic mobility shift assays demonstrated that NRRE-1 was bound by several cardiac and brown adipose nuclear proteins and that these interactions required the NRRE-1 receptor binding hexamer sequences. Antibody supershift studies identified the orphan nuclear receptor COUP-TF as one of the endogenous cardiac proteins which bound NRRE-1. These results dictate an important role for nuclear receptors in the transcriptional control of a nuclear gene encoding a mitochondrial fatty acid oxidation enzyme and identify a gene regulatory pathway involved in cardiac energy metabolism.

Medium-chain acyl coenzyme A dehydrogenase (MCAD; EC 1.3.99.3) is one of four distinct, nuclearly encoded, chain-length-specific enzymes that catalyze the initial reaction in the mitochondrial fatty acid β-oxidation cycle (3, 31). The substrate for MCAD is derived from (i) products of long-chain saturated and unsaturated acyl-thioester mitochondrial β-oxidation, (ii) dietary medium-chain fatty acid thioesters, and (iii) intermediates of peroxisomal long-chain fatty acid oxidation. Because these diverse pathways of lipid metabolism converge at this point, MCAD catalyzes a pivotal step in cellular fatty acid oxidation. The importance of MCAD in cellular energy transduction is underscored by the severe and frequently fatal clinical consequences of inherited MCAD deficiency, a common inborn error in human metabolism (11, 32).

Expression of the MCAD gene is tightly regulated among tissues and during development in accordance with substrate availability and cellular energy demands. MCAD mRNA and enzyme are expressed abundantly in tissues, such as the heart, that rely on oxidation of fatty acids for energy (4, 31). Expression of MCAD is also enriched in brown adipose tissue, consistent with the high β-oxidative capacity necessary to provide reducing equivalents for the uncoupling of oxidative phosphorylation in this specialized cell. During cardiac development, MCAD gene expression is induced in concert with the perinatal switch from glucose to fatty acids as the chief energy substrate (6, 15, 19, 28). The gene regulatory pathways involved in the tissue and developmental expression of the nuclear genes encoding MCAD and other mitochondrial β-oxidation enzymes are unknown. Characterization of these gene regulatory mechanisms should allow the delineation of signals involved in the control of cellular fatty acid oxidative capacity and in the coordinate expression of nuclear genes encoding mitochondrial proteins. To this end, we have isolated and characterized the human MCAD gene promoter region. The MCAD gene promoter comprises a series of Sp1 binding sites and nuclear hormone receptor response elements (23, 38). Previous mammalian cell line transfection experiments and DNA-protein binding studies have identified one of the MCAD promoter nuclear receptor response elements, nuclear receptor response element 1 (NRRE-1), as a novel, pleiotropic element that interacts with several retinoid and orphan nuclear receptor

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dimers to confer transcriptional activation (retinoid X receptor [RXR]-retinoic acid receptor [RAR], RXR-peroxisome proliferator activated receptor [PPAR], and HNF-4) or repression (COUP-TF and ARP-1) (7, 8, 14, 29).

To examine the role of NRRE-1 and other MCAD gene promoter elements in the control of MCAD gene expression in vivo, we have produced mice transgenic for human MCAD gene promoter fragments fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. Herein, we demonstrate that the proximal MCAD promoter region is sufficient to direct appropriate transcription among most adult mouse tissues and during cardiac postnatal development. Moreover, the altered expression pattern of a mutated MCADCAT transgene dictates a pivotal role for NRRE-1 in high-level expression of the MCAD gene in the heart and brown adipose tissue. DNA-protein binding studies demonstrate that COUP-TF and additional transcription factors bind hexameric receptor binding sites within NRRE-1 in the heart and brown adipose tissue. These findings implicate members of the nuclear receptor superfamily in tissue-specific transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes and expand the role of these transcription factors to the regulation of cardiac energy metabolism.

MATERIALS AND METHODS

Production and characterization of the MCADCAT.371 and MCAD CATΔNRRE-1 transgenic lines. The MCADCAT.371 and MCADCAT ΔNRRE-1 DNA fragments were isolated as *Hin*dIII-*Pvu*I restriction fragments from plasmids described previously (23, 38). The DNA fragments were microinjected into the pronuclei of one-cell (C57BL/6J × SJL/J)F_2 mouse zygotes to produce transgenic mice. Founders and positive offspring were bred with (C57BL/6J × SJL/J)F_1 nontransgenic mice to generate hemizygous offspring. Transgenic mice were identified by amplification of the CAT gene from mouse tail DNA by PCR using CAT gene-specific oligonucleotide primers. Isolation of tail DNA and genomic Southern blot analysis were performed by standard protocols. Animal care and euthanasia protocols were in accordance with the Institutional Animal Care and Use Committees of Washington University School of Medicine and the University of Alabama at Birmingham.

Southern blot analyses were performed by standard protocol. A 600-bp *Nco*I-*Xba*I bacterial CAT gene probe, labeled to high specific activity with [α-^{32}P]dCTP, was used as a probe in the Southern blot studies. To determine transgene copy number, slot blot analysis was performed with tail DNA. The signal obtained with the CAT probe for each line was compared with that of standards prepared by spiking known amounts of linearized MCADCAT.371 or MCADCATΔNRRE-1 fragments (from 1 to 100 transgene copies per haploid genome) into 10 μg of tail genomic DNA isolated from a nontransgenic littermate. The signal intensity of the major *Bam*HI fragment was quantified by laser densitometry, and the copy number was determined by comparison with a plot of the intensity of standard signals versus copy number. This approach was performed with at least two animals per line to establish the final transgene copy numbers shown in Table 1.

RNA isolation and Northern (RNA) blot analyses. Upon harvest, tissues were snap frozen in liquid N_2 and stored at −80°C. Heart samples contained only ventricular tissue. Brown adipose tissue was isolated from the intrascapular region. White adipose tissue was obtained from the retroperitoneal region of the abdominal cavity. Total RNA isolation and Northern blot analyses were performed as described elsewhere (19) using the CAT gene probe and an [α-^{32}P]dCTP-labeled 1,700-bp *Eco*RI mouse MCAD cDNA fragment (34). Twenty-five micrograms of total RNA was loaded in each lane for Northern blot analysis. The blots were washed under stringent conditions of 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate at 42°C and subjected to autoradiography for 10 to 15 h (MCAD) or 18 to 24 h (CAT). The signals were quantified by laser densitometric analysis within the linear range of film sensitivity. The densitometric values shown were normalized to the signal obtained with an 18S ribosomal probe to control for any minor differences in RNA sample loading or RNA integrity.

For the developmental studies, transgenic animals were identified within each litter by PCR and Southern blot analysis. All mice were weaned on postnatal day 18. Samples were obtained from transgenic animals at each of the following time points: postcoital day 18 (day −3) and postnatal days 1, 7, 14, 21, and 70 (adulthood). Pools were made from samples from two to five littermates at a single time point. In the case of samples taken on days −3, 1, 7, and 14, the pools were used for preparation of total cellular protein extract for CAT activity (and, when possible, isolation of total RNA). The pooled samples from the nontransgenic littermates were used for isolation of RNA for determination of mouse MCAD mRNA levels. Samples representing days 21 and 70 (adult) were taken from at least four individual littermates, and each sample was divided for preparation of protein extract and RNA isolation. CAT activities were determined for at least three samples from at least two litters per time point for MCADCAT.371 (line 10-4) and at least two litters per time point from each of MCAD CATΔNRRE-1 lines 11-3 and 11-2. Samples for MCAD mRNA levels represent combined data from all MCADCAT.371 and MCADCATΔNRRE-1 litters characterized. In addition, both CAT mRNA and MCAD mRNA levels were determined for the same sample at least once for each time point for the MCAD CAT.371 line (see inset, Fig. 5). RNA blotting was as described above. A universal actin cDNA probe was used in the RNA blot analyses to control for loading differences.

Tissue CAT assays. Protein extracts for CAT assays were prepared by snap freezing tissues in liquid N_2 immediately after harvest and then sonicating them in 0.25 M Tris buffer. The suspension was cleared by centrifugation, and the total protein content of the supernatant was determined by the Lowry method. CAT assays were performed with 100 μg of total protein as described previously (29) with *n*-butyryl coenzyme A and ^{14}C-chloramphenicol substrates. Butyrylated chloramphenicol was separated from free chloramphenicol by xylene extraction. Samples were counted in duplicate on a Beckman LS 6000IC scintillation counter.

Plasmid constructs. The pNRRE.MCAD.Luc and pNRREmut.MCAD.Luc plasmids were made as follows. A *Bam*HI-*Hin*dIII fragment of the human MCAD gene promoter containing 306 bp upstream and 190 bp downstream of the transcription start site was subcloned into pGEM-3Z (Promega). A double-stranded NRRE-1 oligonucleotide containing the MCAD promoter sequence from −345 to −307 (sense strand sequence, 5′-gatcCGGGTTTGACCTTTCTC TCCGGGTAAAGGTGAAGGCTGACt-3′) with a *Bam*HI overhang at the 5′ end and *Xba*I overhang at the 3′ end (overhangs indicated by lowercase letters) was subcloned immediately 5′ to the MCAD promoter fragment in pGEM-3Z and was termed pMCAD.NRRE.3Z. A second oligonucleotide which was identical to the NRRE-1 oligonucleotide, except for single-base substitutions in each of the known nuclear receptor binding half-sites (sense strand sequence, 5′-gat cCGGGTTTGACGTTTCTCTCCGCGTAAACGTGAAGGCTGACt-3′; mutations are underlined), was also subcloned into the *Bam*HI-*Xba*I site upstream of the MCAD promoter in pGEM-3Z for the construction of pMCAD.NRREmut.3Z. The *Bam*HI-*Hin*dIII fragments were removed from the pMCAD.NRRE.pG and pMCAD.NRREmut.pG plasmids and subcloned into pGL2-basic (Promega) to create pNRRE.MCAD.Luc and pNRREmut.MCAD-.Luc, respectively (schematics shown in Fig. 6).

Cell culture. Primary rat neonatal cardiocytes were prepared from 1- to 2-day-old rats as previously described (12). Briefly, myocytes were prepared from the hearts of 1- to 2-day-old Sprague-Dawley rats by enzymatic digestion with 0.2% collagenase (Wako-Chemicals, Richmond, Va.). Cells were pooled in PC-1 medium (Hycor, Irvine, Calif.) with 0.10 mM 5-bromo-2′-deoxyuridine (Sigma, St. Louis, Mo.) in the absence of serum supplement and subjected to a 15-min period of differential plating to reduce fibroblast population contamination. Nonadherent cells (enhanced cardiocyte fraction) were plated at a density of 2 × 10^6 cells per cm^2 on 60-mm-diameter dishes pretreated with collagen (Sigma). After 24 h, the cell medium was switched to a 2:1 combination of PC-1 and Dulbecco's modified Eagle's medium with Ham's F-12 nutrient mixture, also in the absence of serum supplement. 5-Bromo-2′-deoxyuridine was again added at a concentration of 0.10 mM in order to reduce the noncardiocyte cell fraction. Greater than 95% of the cells were beating by 36 h following the medium change.

Cardiocyte transient transfections were performed with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate) (Boehringer Mannheim) in 12-well dishes. For each transfection, 4 μg of reporter DNA and 2 μg of a plasmid containing a β-galactosidase gene downstream of the Rous sarcoma virus promoter (RSV.βgal) were added to 15 μl of DOTAP–20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) solution and incubated for 15 min at room temperature. The mixture was subsequently added to the primary cardiocytes prior to initial plating. The cells were washed and refed the following morning. The cells were harvested 48 h after plating. Luciferase activity was measured by the standard luciferin-ATP assay, and β-galactosidase activity was measured by the Galacto-Light chemiluminescence assay (Tropix) in an Analytical Luminescence Monolight 2010 luminometer.

The brown adipocyte cell line used, HIB 1B, was provided as a generous gift by Bruce Spiegelman (Harvard Medical School). This cell line was maintained as described previously (20) in Dulbecco's modified Eagle's medium–Ham's F-12 nutritive medium (1:1, vol/vol), supplemented with 10% fetal calf serum. At confluence, cells were refed and insulin (17 nM) and thyroid hormone (T3) (1 nM) were added. Seven to ten days following addition of insulin and T3, the cells were fully differentiated on the basis of morphologic criteria. At this differentiated stage, the cells were harvested and nuclear protein extracts were prepared as described elsewhere (23).

Electrophoretic mobility shift assays (EMSA). EMSA were performed as described previously (30) using the normal and mutant double-stranded oligonucleotides encoding NRRE-1 (sequences shown in "Plasmid constructs" above). Crude rat tissue nuclear extracts were prepared from adult rat ventricle, intrascapular brown adipose, and HIB 1B cells as described (8, 14). Antibody supershift experiments were performed with a polyclonal antibody to human COUP-TF (kindly provided by Ming-Jer Tsai, Baylor College of Medicine).

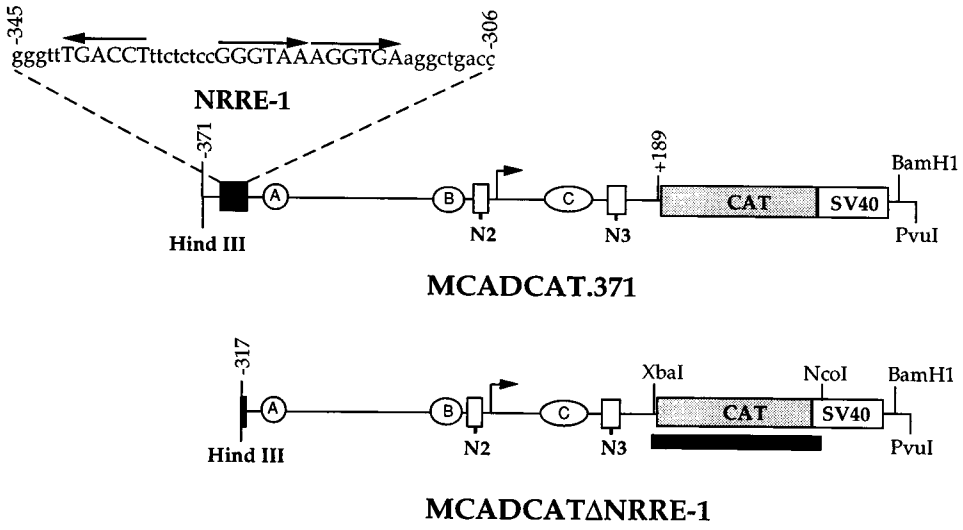

FIG. 1. DNA fragments used for microinjection of single-cell mouse embryos to produce the MCADCAT transgenic lines. MCADCAT.371 (top) contains a human MCAD gene promoter fragment (horizontal line) containing 371 bp upstream to 189 bp downstream of the transcription start site (arrow) fused to a bacterial CAT gene-simian virus 40 (SV40) intron-polyadenylation signal sequence. The NRRE-1 sequence from −345 to −306 is expanded at the top. The arrows and uppercase letters denote the sequences, locations, and relative orientations of the hexameric receptor binding half-sites within NRRE-1 (7). The positions of additional MCAD gene promoter elements defined previously (23) are represented by the symbols labeled A to C (Sp1 binding sites) and N2 and N3 (putative nuclear receptor binding sites). The *Bam*HI site used to linearize the transgene concatemers for Southern blot analyses is also shown. A schematic of the second DNA fragment used for microinjection, MCADCATΔNRRE-1, is shown below MCADCAT.371. This fragment is identical to the MCADCAT.371 fragment except that it lacks 54 bp at the 5′ end which contain the three NRRE-1 receptor binding sites. The shaded band beneath the CAT gene schematic represents the *Xba*I-*Nco*I fragment used as a probe for the Southern blot analyses described in the legend to Fig. 2.

Additional antibody supershifts (data not shown) were performed with a mixture of monoclonal antibodies to the D or E domain of RXRα, RXRβ, and RXRγ (generously provided by Pierre Chambon, Institut National de la Santé et de la Recherche Médicale, Strasbourg, France); a polyclonal antibody directed to PPARα (αFP_2; a gift from Michael Arand, University of Mainz); a polyclonal antibody to HNF-4 (provided by Frances Sladek, University of California, Riverside); and a polyclonal anti-$TR\beta_1$ antibody (Affinity Bioreagents, Inc.).

Statistical analysis. All values shown represent the mean ± standard error of the mean (SE). *P* values were determined by unpaired *t* test analyses. A statistically significant value was defined as a *P* value of <0.05.

RESULTS

The MCAD gene promoter region between bp −371 and +189 directs appropriate transcription of a reporter gene in most adult mouse tissues: a role for NRRE-1. Transgenic mice were produced to determine whether the human MCAD gene promoter region, defined by previous studies with cell cultures (23, 38), was capable of directing appropriate transcription among tissues in vivo. Two MCADCAT constructs (MCADCAT.371 and MCADCATΔNRRE-1) were microinjected into one-cell fertilized mouse embryos (Fig. 1). MCADCAT.371 contains the entire functional promoter from bp −371 upstream to bp +189 downstream of the transcription start site (+1) (23). This region contains three Sp1 binding sites (A to C) and three nuclear receptor response elements (NRRE-1, N2, and N3). MCADCATΔNRRE-1 is identical to MCADCAT.371 except that 54 bp (from −371 to −318) have been deleted from the 5′ end, resulting in removal or disruption of the three known receptor binding sites within NRRE-1 (Fig. 1). Previous DNase I protection studies have shown that within this deleted region, only the NRRE-1 sequence binds nuclear proteins isolated from mammalian cell lines or mouse tissues (10, 23). MCADCAT.371 microinjections produced seven transgenic founder mice, four of which expressed the transgene and were further characterized. MCADCATΔNRRE-1 microinjections produced four transgenic founder mice, all of which were characterized. Genomic Southern blot analysis using a labeled CAT gene probe confirmed that each of the eight transgenic lines contained a single independent genomic integration site (Fig. 2). Quantitative DNA slot blot analysis (data not shown; protocol described in Materials and Methods) demonstrated that each transgenic line contained a distinct number of integrated MCADCAT fragments ranging from 2 to 55 copies per haploid genome.

The adult tissue expression pattern of CAT mRNA in all four MCADCAT.371 lines paralleled that of the endogenous

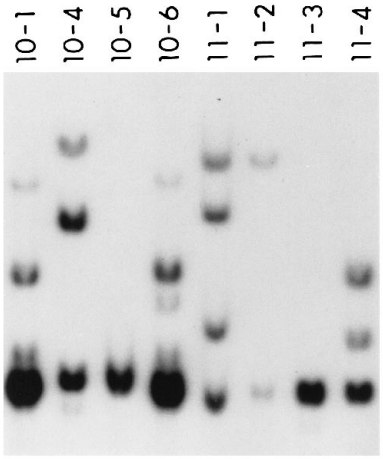

FIG. 2. Characterization of the MCADCAT transgene integration sites by genomic Southern blot analysis. An autoradiogram of a genomic Southern blot performed with DNA isolated from each of the MCADCAT.371 (10-3, 10-4, 10-5, and 10-6) and MCADCATΔNRRE-1 (11-1, 11-2, 11-3, and 11-4) lines. Each lane contains 10 μg of tail DNA digested with *Bam*HI (the location of this site within the transgene is depicted in Fig. 1). The blot was hybridized with a ^{32}P-labeled CAT gene probe. The intense lower band present in most lanes represents an internal *Bam*HI fragment released from a tandem array of integrated transgene DNA.

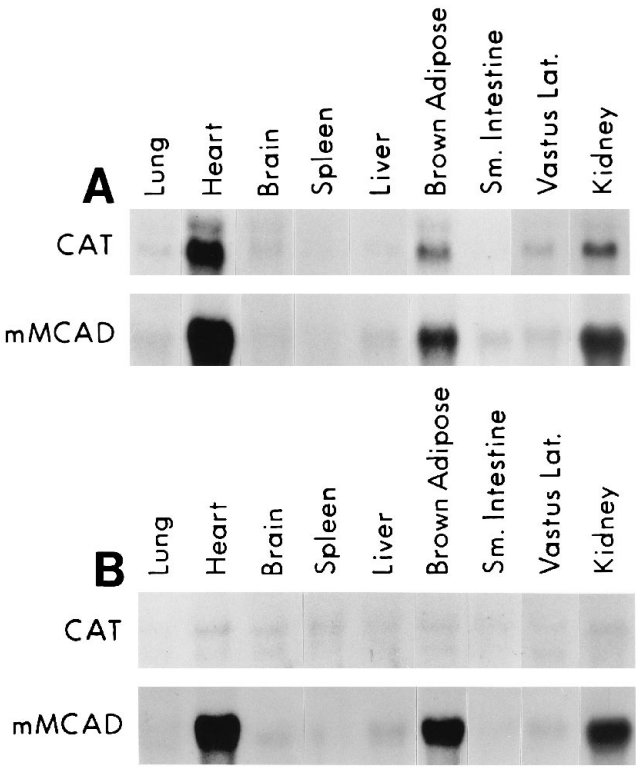

FIG. 3. Tissue expression patterns of the MCADCAT.371 and MCADCATΔNRRE-1 transgenes. (A) Representative Northern blot analysis performed on total RNA isolated from tissues of an adult male mouse hemizygous for MCADCAT.371 (line 10-4). The blot was sequentially hybridized with radiolabeled CAT gene and mouse MCAD cDNA probes. The signal obtained with an 18S rRNA probe confirmed equivalent loading among lanes (not shown). In contrast to the other tissues, the relative signal intensities for the vastus lateralis (Vastus Lat.) and kidney varied markedly among the four MCADCAT.371 lines, and therefore, the results shown for these two tissues are not representative of all lines. (B) Tissue expression pattern of the MCADCATΔNRRE-1 transgene. A representative Northern blot analysis performed with total RNA isolated from an adult male mouse hemizygous for MCADCATΔNRRE-1 (line 11-2) is shown.

MCAD gene in most tissues (representative Northern blot shown in Fig. 3A). Steady-state CAT and mouse MCAD mRNA levels were high in tissues with known high mitochondrial fatty acid oxidation rates (heart, brown adipose tissue, and kidney) compared with those in tissues with low rates (brain, lung, small intestine, and spleen). Tissue CAT activities in each of the lines correlated with CAT mRNA expression (Table 1). The absolute level of transgene expression correlated directly with transgene copy number in most tissues in each of the MCADCAT.371 lines (Table 1). However, transgene expression in the kidney and skeletal muscle (vastus lateralis) was not copy number dependent and varied markedly among lines, suggesting that additional *cis*-acting elements not present in the MCADCAT fragment are necessary for appropriate transcription in these tissues.

To test the hypothesis that NRRE-1 mediates transcriptional control of the MCAD gene in vivo, the tissue expression pattern of the MCADCATΔNRRE-1 transgene was evaluated (Fig. 3B). As with the MCADCAT.371 lines, expression of the MCADCATΔNRRE-1 transgene in all four lines correlated with transgene copy number and was significantly higher than background levels in all tissues examined. The tissue expression pattern of MCADCATΔNRRE-1 among the four lines was identical. In contrast to MCADCAT.371, the tissue expression pattern of the MCADCATΔNRRE-1 transgene was markedly different from that of the endogenous MCAD gene. Although the transgene copy number range for the MCADCATΔNRRE-1 was lower than that of the MCADCAT.371 lines, comparison of the tissue expression patterns of the transgenes corrected for copy number and comparison of lines with similar copy numbers (e.g., 11-2 and 11-1 versus 10-4) revealed striking differences. The differences were most notable in the heart and brown adipose tissue, tissues with high fatty acid oxidative capacities and abundant MCAD expression (Table 1 and Fig. 3B). Comparison of mean tissue CAT activities (corrected for transgene copy number) in the MCADCAT.371 lines with those in MCADCATΔNRRE-1 lines revealed that removal of NRRE-1 resulted in a dramatic reduction in mean CAT activities in the heart (99.2% decrease; $P < 0.01$) and brown adipose tissue (88.1% decrease; $P < 0.01$) (Fig. 4). Deletion of NRRE-1 resulted in a significant but more modest reduction in transgene expression in liver (77.8%; $P = 0.04$) and white adipose (75.7%; $P = 0.05$) tissue. In tissues with known low β-oxidation rates and low levels of MCAD

TABLE 1. Tissue CAT activities in the MCADCAT transgenic lines

Transgenic line (copy no.[a])	CAT activity (10^1 cpm/μg of total protein)[b] in:							
	Heart	Brown adipose tissue	Lung	Liver	Spleen	Brain	Small intestine	White adipose tissue
MCADCAT.371								
10-1 (55)	2,036.7 ± 308.8	988.2 ± 38.9	10.1 ± 0.9	17.9 ± 0.9	7.9 ± 0.3	0.3	4.2 ± 1.2	78.0 ± 0.7
10-4 (14)	111.2 ± 33.7	259.3 ± 54.5	2.6 ± 0.8	0.6	0.8	2.6 ± 0.4	0.2	5.8 ± 0.2
10-5 (2)	2.8 ± 1.2	12.9 ± 5.9	0.5 ± 0.2	0.1	0.7	1.3 ± 0.5	0.1	0.3
10-6 (50)	2,540.0 ± 721.9	1,055.8 ± 65.1	21.4 ± 11.4	33.1 ± 11.1	7.9 ± 1.4	1.1 ± 0.2	1.7 ± 0.3	49.1 ± 7.4
MCADCATΔNRRE-1								
11-1 (13)	2.6 ± 0.3	23.9 ± 2.2	2.4 ± 0.2	0.6	2.3 ± 0.2	4.8 ± 0.2	0.3	3.5
11-2 (4)	0.7 ± 0.3	2.8 ± 0.7	1.0 ± 0.3	0.1	0.5	0.6	0.2	0.4
11-3 (16)	3.3 ± 0.4	44.2 ± 2.7	3.3 ± 0.2	0.2	1.8	3.4	0.5 ± 0.2	1.6
11-4 (11)	2.6 ± 0.3	25.6 ± 0.3	2.8 ± 0.2	0.5	2.5 ± 0.3	5.5 ± 0.3	0.2	3.5 ± 0.2

[a] The transgene copy number per haploid genome was determined by quantitative slot blot analysis of tail DNA.

[b] All values represent a mean ± SE from at least three independent assays. The SE is ≤0.1 if not indicated. Mean CAT activities based on analysis of at least two adult male animals from each line are shown. CAT activities for the kidney and vastus lateralis were highly variable and did not correlate with CAT mRNA levels in each of the eight lines and are therefore not shown. Background CAT activity was 0.05×10^1 to 0.06×10^1 cpm/μg of total protein.

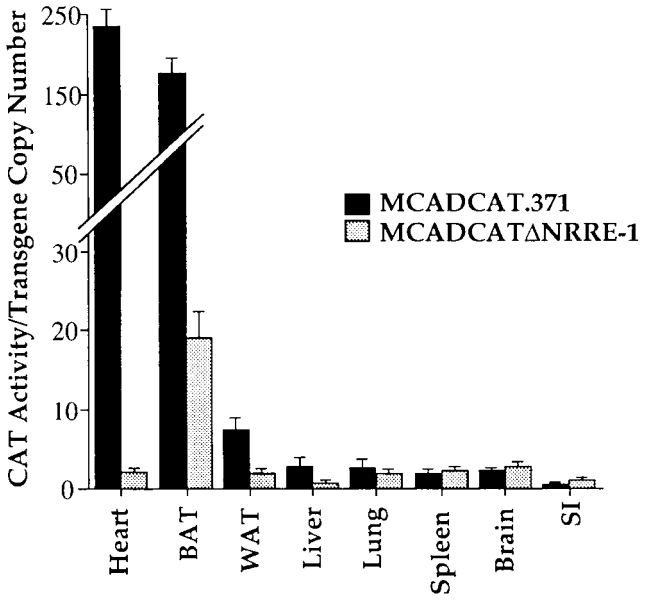

FIG. 4. Comparison of mean tissue CAT activities among adult tissues in the MCADCAT.371 and MCADCATΔNRRE-1 transgenic lines. Values represent the mean (± SE) counts per minute (10^2/μg of total protein) divided by transgene copy number for at least three independent assays performed on protein extracts prepared from adult male tissues harvested from at least two animals from each of the MCADCAT.371 (n = 4) and MCADCATΔNRRE-1 (n = 4) lines. Transgene copy number (Table 1) was determined by quantitative DNA slot blot analysis. The double line across the ordinate denotes a change in scale. Abbreviations: BAT, brown adipose tissue; WAT, white adipose tissue; SI, small intestine.

expression (brain, spleen, lung, and small intestine), the corrected MCADCATΔNRRE-1 expression was not significantly different from that of MCADCAT.371. These data suggest that NRRE-1 confers transcriptional regulation of the MCAD gene in parallel with tissue-specific mitochondrial β-oxidation requirements and that this element is necessary for high-level expression in the heart and brown adipose tissue.

To determine whether additional elements involved in the tissue-specific control of MCAD gene transcription existed upstream of the promoter fragment used to produce the MCADCAT.371 lines, mice transgenic for a third MCADCAT construct (MCADCAT.1197), identical to MCADCAT.371 except for an additional 826 bp of contiguous upstream MCAD gene 5′ flanking DNA (from bp −372 to −1197), were produced. The adult tissue expression pattern of two independent MCADCAT.1197 lines was similar to that of MCADCAT.371 (data not shown). Notably, as with the MCADCAT.371 lines, kidney expression did not correlate with transgene copy number in either of the MCADCAT.1197 lines. Accordingly, additional transcriptional regulatory elements involved in tissue expression of the MCAD gene were not detected in this proximal 5′ flanking region.

NRRE-1 is required for proper cardiac developmental-stage-specific transcription of the MCADCAT gene chimera. The expression of nuclear genes encoding mitochondrial energy-producing enzymes is coordinately upregulated in the heart following birth, in parallel with the postnatal biogenesis of mitochondria and known developmental energy substrate switch from glucose to fatty acids in this organ (2, 5, 16, 19, 25, 37). During the postnatal period, an increase in mitochondrial fatty acid β-oxidation capacity is necessary to meet the increased energy demands imposed on the left ventricle following birth. To test the hypothesis that NRRE-1 mediates transcriptional control of the MCAD gene in parallel with increased myocardial oxidative energy demands following birth, expression of the MCADCAT.371 and MCADCATΔNRRE-1 transgenes was examined in a variety of perinatal cardiac developmental stages and compared with that of the endogenous MCAD gene. As described previously in the developing rat (15, 19, 28), accumulation of endogenous mouse heart MCAD mRNA increased over 10-fold between gestational day 18 (day −3) and adulthood (day 70) (Fig. 5A). During the postnatal period, distinct peaks in MCAD mRNA expression occurred on postnatal day 1, between postnatal days 7 and 14, and again during the transition from the late postnatal period to adulthood (day 70). CAT activities (Fig. 5A) and CAT mRNA levels (representative Northern blots shown in the Fig. 5A inset) in developing hearts from the MCADCAT.371 transgenic mice paralleled those of the endogenous MCAD gene throughout all developmental time points.

In marked contrast to the cardiac developmental expression pattern of MCADCAT.371, the expression of MCADCATΔ NRRE-1 was not induced following birth in the heart (Fig. 5B). Except for a modest peak at postnatal day 7, MCADC ATΔNRRE-1 expression remained at prenatal levels throughout all postnatal developmental stages and in the adult. This pattern was identical in two different MCADCATΔNRRE-1 lines (lines 11-2 and 11-3; combined data shown in Fig. 5B). These data indicate that NRRE-1 is required for the postnatal induction of MCAD gene expression in the heart during the transition from reliance on glucose to reliance on fatty acids as the chief energy substrate.

The nuclear receptor binding sequences within NRRE-1 are necessary for high-level transcriptional activity of the MCAD gene promoter in primary cardiocytes and for binding cardiac and brown adipose nuclear proteins. The MCADCAT transgene expression data shown above demonstrated that a 54-bp human MCAD gene promoter fragment containing sequence from −371 to −318 was required for high-level transcription in the heart. Previous DNase I footprinting assays performed with this region of the MCAD promoter have demonstrated that a sequence from −345 to −306, which comprises NRRE-1, bound nuclear proteins prepared from a variety of mammalian cell lines and tissues, including rat heart (23, 24). We next sought to determine whether the known nuclear receptor binding site sequences within NRRE-1 were necessary for the high-level activity of the MCAD gene promoter in cardiocytes. Transient-transfection studies were performed with primary rat neonatal cardiocytes in culture using luciferase reporter constructs containing the MCAD gene promoter region from bp −306 to +191, downstream of either an oligonucleotide containing NRRE-1 (pNRRE.MCAD.Luc) or a mutant NRRE-1 containing single-base substitutions in each of the three known NRRE-1 nuclear receptor binding half-site sequences (pNRREmut.MCAD.Luc), mutations shown previously to abolish nuclear receptor binding to this element (7). As shown in Fig. 6, the transcriptional activity of pNRRE.MCAD.Luc in cardiocytes was markedly higher than background levels, consistent with the known enriched expression of the MCAD gene in heart. In contrast, the transcriptional activity of pNRREmut.MCAD.Luc was approximately 90% lower than that of pNRRE.MCAD.Luc, a difference similar to that observed in comparison of the MCADCAT.371 and MCADCATΔNRRE-1 transgene expression levels in the mouse heart. Accordingly, as predicted by the results of previous DNase I footprint analyses (23, 24) and the transgene expression data presented here, the nuclear receptor binding

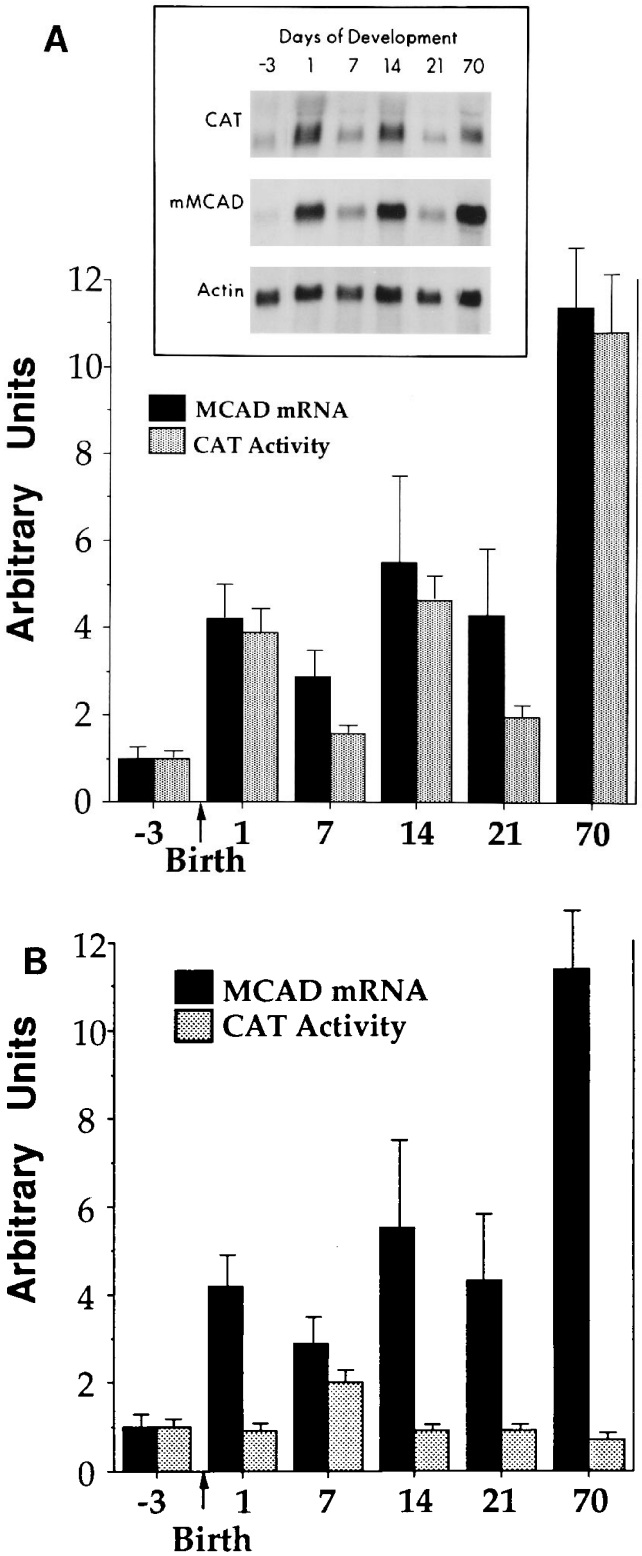

FIG. 5. Expression of the MCADCAT.371 (A) and MCADCATΔNRRE-1 (B) transgenes during perinatal cardiac developmental stages. (A) Mean (± SE) steady-state murine heart MCAD mRNA levels were determined by quantitative laser densitometric analysis of Northern blots hybridized with a mouse MCAD cDNA probe. In comparison, mean heart tissue CAT activities are shown for each developmental stage. The six time points (days) included in the developmental analyses are indicated on the abscissa (day −3 equals gestational day 18). All values are normalized (=1.0) to that of day −3 to allow comparison of the patterns of expression of MCAD mRNA and CAT activity. Total protein for CAT activities was prepared from at least three pooled or individual heart samples from at least two MCADCAT.371 (10-4 line) litters as described in Materials and Methods. MCAD mRNA levels were determined in samples from the nontransgenic littermates of the transgenic animals used to analyze the CAT activities (days −3, 1, 7, and 14) or from transgenic animals (days 21 and 70) from all of the litters used to generate the CAT data in both panels. Thus, MCAD mRNA levels are composite data from at least six different samples from at least four litters of three different lines (10-4, 11-3, and 11-2). A representative comparison of MCAD mRNA and CAT mRNA levels within the same sample at each developmental time point is depicted in the Northern blot autoradiographs (inset). The blots were hybridized sequentially with mouse MCAD, CAT, and β/γ actin probes. (B) Comparison of MCADCATΔNRRE-1 expression with endogenous MCAD mRNA levels during perinatal cardiac development. CAT activity data represent at least three different samples from two or more litters from both the 11-2 and 11-3 lines. Values shown were normalized as described for panel A. Note that the absolute heart CAT activity values in the MCADCATΔNRRE-1 mice are approximately 1% of the corresponding adult MCADCAT.371 values (Fig. 3B). Representative CAT mRNA levels are not shown because expression of the MCADCATΔNRRE-1 was too low for detection by Northern blot analysis.

sites within NRRE-1 are required for high-level transcriptional activity of the MCAD promoter in cardiocytes.

EMSA were performed to examine the interaction of NRRE-1 with endogenous cardiac and brown adipose nuclear proteins. EMSA were performed with a ^{32}P-labeled NRRE-1 probe and crude nuclear protein extracts prepared from adult rat heart or brown adipose tissue. NRRE-1 formed three complexes with cardiac nuclear proteins (Fig. 7A, lane 2). An identical mobility shift pattern was observed with the brown adipocyte protein extract (Fig. 7A, lane 3). Competition studies performed with a molar excess of unlabeled specific and unrelated double-stranded oligonucleotides revealed that two of the complexes (complexes I and II [Fig. 7A]) represented specific NRRE-1–protein interactions. These results suggest that several endogenous tissue nuclear proteins bind NRRE-1 and that these factors are similar or identical in heart and brown adipose tissues with known high-level MCAD expression.

Our previous DNA binding and mammalian cell transfection studies have demonstrated that NRRE-1 is capable of interacting with multiple nuclear receptor dimers to regulate transcription (7, 8, 14, 29). The pleiotropic binding properties of this element are consistent with its novel architecture, which includes three potential hexameric binding half-sites (7). Nuclear receptors known to bind NRRE-1 in vitro include RXR-containing heterodimers (RXRα-RARβ and RXRα-PPARα) and homodimers of the orphan receptors COUP-TF and HNF-4. To determine whether any of these receptors known to bind NRRE-1 in vitro were present in complex I or II, antibody supershift experiments were performed. A panel of antibodies directed to nuclear receptors known to bind NRRE-1 in vitro (anti-RXRα, -RXRβ, and -RXRγ; anti-TRβ; anti-PPARα; anti-HNF-4; and anti-COUP-TF) were used to identify endogenous nuclear receptors present in complex I or II. Anti-COUP-TF resulted in a partial supershift and loss of complex I but not complex II (Fig. 7B, lanes 1 to 3). Two light bands with distinct mobilities were supershifted with anti-COUP-TF, suggesting that complex I contained two types of COUP-TF–NRRE-1 complexes or that the antibody–COUP-TF interaction occurred through two different stoichiometric combinations. None of the other nuclear receptor antibodies altered the formation of complex I or II (data not shown). To determine whether formation of complex II required the known nuclear receptor binding sites within NRRE-1 and to correlate NRRE-1–cardiac protein binding properties with the results of the cardiocyte transfection studies, EMSA were repeated with a labeled NRREmut probe which contains a single nucleotide

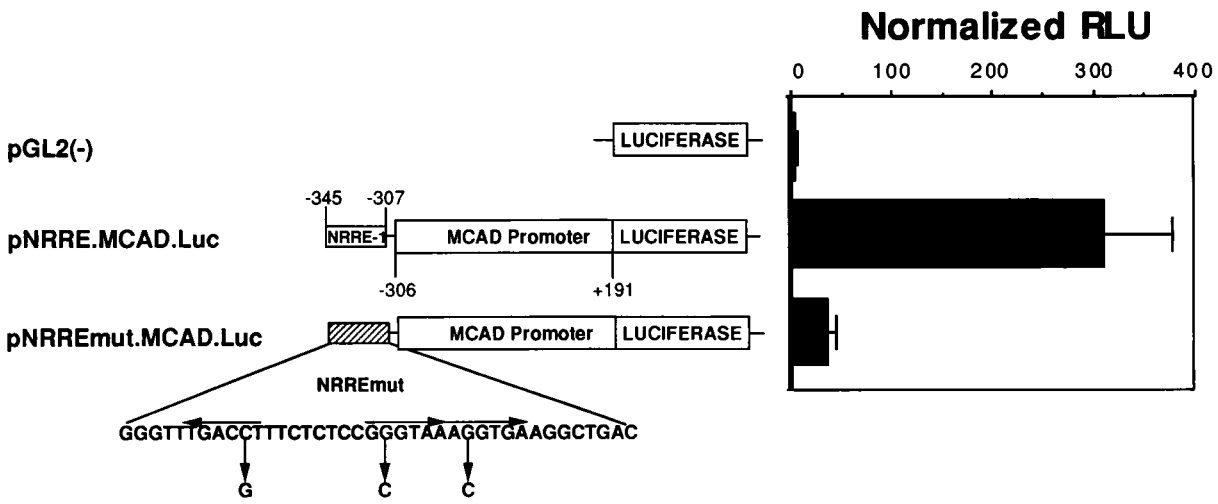

FIG. 6. Role of NRRE-1 in the high-level transcriptional activity of the human MCAD gene promoter in rat neonatal cardiocytes. The results of transient transfections of the luciferase reporter plasmids depicted on the left in primary rat neonatal cardiocytes in culture. The bars represent mean (± SE) luciferase activity in relative light units (RLU) normalized (=1.0) to the activity of the promoterless luciferase plasmid, pGL2(−), following correction for transfection efficiency based on the activity of cotransfected RSV.βgal (see Materials and Methods). The pNRRE.MCAD.Luc construct contains the MCAD promoter fragment (from −306 to +191) downstream of an NRRE-1 oligonucleotide. pNRREmut.MCAD.Luc is identical to pNRRE.MCAD.Luc except for the three receptor binding site mutations indicated. The arrows denote the hexameric binding half-sites defined previously (7).

substitution in each of the three receptor binding sites within NRRE-1 (mutated sequence shown in Fig. 6). The formation of both complex I and complex II was nearly abolished with the NRREmut probe (Fig. 7B, lane 4). Identical supershift and mutational results were obtained with brown adipose tissue-derived nuclear protein extracts (data not shown). Thus, as predicted by the results of previous studies performed in vitro and by the transgene expression data presented here, endogenous nuclear receptors bind NRRE-1.

The lack of an interaction between the anti-RXR antibody and either complex I or II was surprising given the results of our previous cell cotransfection studies, demonstrating that

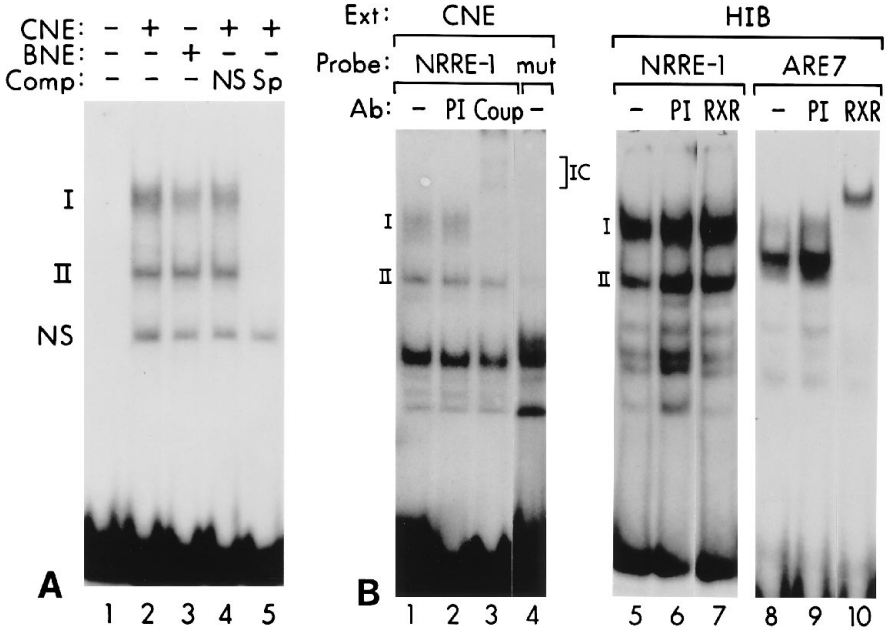

FIG. 7. Interaction of NRRE-1 with endogenous rat heart and brown adipose nuclear proteins as defined by EMSA and antibody supershift assays. (A) Autoradiograph of EMSA performed with a ^{32}P-labeled NRRE-1 oligonucleotide and 20 μg of crude nuclear protein extract prepared from adult rat cardiac tissue (CNE) or brown adipose tissue (BNE). The key at the top indicates the lanes in which a 50-fold molar excess of unlabeled NRRE-1 DNA (Sp) or an unrelated unlabeled DNA (NS) was included. The specific complexes I and II are indicated. The lower band represents a nonspecific (NS) interaction based on the competition results. (B) Evaluation of the protein binding properties of the NRRE probe by EMSA and antibody supershift studies. Studies were performed with nuclear protein extracts (Ext) prepared from rat heart (CNE) or differentiated brown adipocyte (HIB 1B) cells. Probes used include NRRE-1, NRREmut (mut), and the known PPAR-RE, ARE7. Lanes containing antibody (Ab) to COUP-TF or RXR or the preimmune serum control (PI) are indicated at the top. The position of the supershifted immune complexes (IC) in lane 3 is indicated.

NRRE-1 is capable of mediating transcriptional activation by RXR-RAR and RXR-PPAR heterodimers (14, 29). To explore this negative result further and to control for the ability of the anti-RXR antibody to recognize DNA-protein complexes containing RXR-PPAR heterodimers, the EMSA supershift experiments were repeated using nuclear protein extracts prepared from differentiated HIB 1B cells, a brown adipocyte cell line known to express both RXR and PPAR (35). A probe containing a known DR-1-type PPAR response element (the adipocyte P2 gene promoter PPAR-RE, ARE7 [35]) was used as a positive control in these experiments. NRRE-1 formed two complexes (I and II) with HIB 1B cell nuclear proteins, the mobilities of which were identical to those formed with cardiac cell- and brown adipose cell-derived nuclear proteins (Fig. 7B, lane 5). The formation and mobilities of complexes I and II were unaffected by addition of the anti-RXR antiserum (Fig. 7B, lanes 5 to 7). In contrast, the ARE7 probe formed a single complex with the HIB 1B nuclear protein extract (Fig. 7B, lane 8). The ARE7-protein complex was completely supershifted by the anti-RXR antiserum but not by a preimmune control (Fig. 7B, lanes 8 to 10). These results confirm that the anti-RXR is capable of recognizing and supershifting endogenous RXR-containing DNA-protein complexes and strongly suggest that the NRRE-1–protein complexes do not contain RXR.

DISCUSSION

This report demonstrates that approximately 500 bp of the human MCAD gene promoter region is sufficient to direct appropriate transcription in vivo among most murine tissues and during perinatal cardiac development. Moreover, our results demonstrate an important role for nuclear receptor transcription factors in directing high-level expression of the MCAD gene in the heart and brown adipose tissue. In support of this latter conclusion, (i) deletion of a 54-bp region of MCAD gene promoter, containing NRRE-1, results in a dramatic reduction in expression of the MCADCAT transgene in the heart and brown adipose tissue, (ii) single-base-pair mutations in each of the three known hexameric nuclear receptor binding half-sites within NRRE-1 result in a marked reduction in MCAD promoter activity in primary rat cardiocytes and abolish all specific cardiac and brown adipose nuclear protein binding to this element, and (iii) antibody supershift studies demonstrate that at least one known endogenous nuclear receptor (COUP-TF) binds NRRE-1.

Abundant expression of enzymes involved in the mitochondrial fatty acid oxidation cycle is necessary to meet the remarkably high energy requirements of the mammalian heart. Little is known about the mechanisms involved in the transcriptional control of genes involved in cardiac energy transduction or about the cardiac gene program in general. Several transcription factors involved in the cardiac tissue-specific expression of genes encoding sarcomeric proteins have been identified (17, 27, 39, 40). Recently, mouse gene disruption studies have dictated an important role for retinoid receptors in cardiac morphogenesis (18, 33). Our data suggest that orphan members of the nuclear receptor superfamily play a role in the transcriptional control of a subset of genes involved in energy metabolism during postnatal cardiac development and in the adult heart. One mechanism for the control of flux through the cardiac mitochondrial fatty acid oxidation cycle could involve the interaction of ligand-activated nuclear receptor transcription factors with target genes encoding enzymes catalyzing rate-limiting steps in this pathway, such as MCAD.

High-level expression of the MCAD gene in brown adipose tissue is another example of the regulation of this gene in parallel with tissue-specific fatty acid β-oxidation requirements. Previous studies also indicate that in addition to thermogenesis, the brown adipocyte mitochondrial uncoupling reaction is a mechanism for regulating total body energy expenditure in rodents and possibly humans (13, 26). Abundant mitochondria and a high-capacity mitochondrial β-oxidation pathway are required to generate reducing equivalents for uncoupled oxidative phosphorylation in the brown adipocyte. Our data indicate that as in the heart, NRRE-1 is required for high-level expression of the MCAD gene in brown adipose tissue. Moreover, our DNA-protein binding studies strongly suggest that the NRRE-1-binding proteins in brown adipose tissue and the heart are similar or identical. Recently, several nuclear receptor response elements have been identified in the promoter region of the gene encoding the brown adipocyte mitochondrial uncoupling protein (1, 9). Accordingly, nuclear receptor-mediated pathways may be involved in directing coordinate expression of the nuclear genes encoding MCAD and the uncoupling protein in the brown adipocyte.

Studies performed in vitro have identified several nuclear receptor dimers which interact with distinct but overlapping binding site pairs within the MCAD gene promoter element, NRRE-1, to activate or repress transcription (7, 8, 14, 23, 29). RXRα-PPARα heterodimers activate MCAD gene transcription in the presence of either the RXR ligand, 9-*cis* retinoic acid, or known activators of PPAR, including fibrates and long-chain fatty acids (14). Our previous studies (7) have also shown that members of the COUP-TF/ARP-1 orphan receptor subfamily (22, 36) repress transcription via NRRE-1. Antibody supershift studies performed with nuclear proteins prepared from NIH 3T3 fibroblasts, cells with known low MCAD expression, have confirmed that endogenous COUP-TF binds NRRE-1 (23). In fact, COUP-TF binds NRRE-1 in all cell lines and tissues that we have studied to date (10, 23, 24). We show here that one of the two NRRE-1–protein complexes formed with cardiac and brown adipose nuclear protein extracts contains the transcriptional repressor, COUP-TF, or a structurally related protein. Surprisingly, our supershift studies did not identify RXR within the NRRE-1–cardiac nuclear protein complexes. Accordingly, the putative cardiac and brown adipose NRRE-1 activator in complex II remains unidentified. The novel arrangement and spacing of the potential binding sites within NRRE-1 suggest the intriguing possibility that this element interacts with a novel nuclear receptor. Moreover, the existence of three potential receptor binding sites within NRRE-1 may allow multiple nuclear receptor dimers, including RXR heterodimers, to regulate transcription via this complex element, depending on the cellular and physiologic context. In support of this model, our previous characterization of NRRE-1 using nuclear receptor cotransfection studies and EMSA demonstrated that multiple nuclear receptor dimers [RXR-RAR, $(HNF\text{-}4)_2$, and $(COUP\text{-}TF)_2$] interact, in a competitive manner, with alternative pairwise hexamer sites within this pleiotropic element (7). Because of the potential insensitivity of EMSA supershift approaches and the increasing recognition of nuclear receptor isoforms, we have not completely excluded the possibility that the endogenous cardiac NRRE-1-binding proteins are members of the RXR and PPAR subfamilies of nuclear receptors. Isolation and characterization of the endogenous nuclear proteins that bind NRRE-1 will be necessary to fully define this transcriptional regulatory mechanism and to delineate the precise upstream regulatory pathways involved in the control of cardiac fatty acid oxidation enzyme expression.

In summary, we have shown that the MCAD gene promoter

region from bp −371 to +189 directs appropriate expression of a reporter gene among most mouse tissues and during perinatal cardiac development. Moreover, our data implicate nuclear receptors in the transcriptional control of a gene encoding a mitochondrial β-oxidation enzyme in parallel with tissue-specific fatty acid oxidation requirements in vivo. These results identify a group of transcription factors involved in the cardiac and brown adipose metabolic gene regulatory programs and expand the biological role of nuclear receptors to include the regulation of genes involved in mitochondrial energy transducing pathways.

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