MOLECULAR AND CELLULAR BIOLOGY, Sept. 1997, p. 5400–5409 Vol. 17, No. 9
0270-7306/97/$04.00+0

The Orphan Nuclear Receptor Estrogen-Related Receptor α Is a Transcriptional Regulator of the Human Medium-Chain Acyl Coenzyme A Dehydrogenase Gene

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Received 30 April 1997/Returned for modification 5 June 1997/Accepted 13 June 1997

Estrogen-related receptor α (ERRα) is an orphan member of the superfamily of nuclear hormone receptors. ERRα was initially isolated based on its sequence homology to the estrogen receptor but is not activated by classic estrogens. To identify possible physiologic functions for this orphan receptor, we cloned the mouse ERRα cDNA and used it to characterize the expression of ERRα transcripts and to identify potential ERRα target genes. RNA in situ hybridization studies detect ERRα transcripts in an organ-specific manner through mid- to late embryonic development, with persistent high-level expression in brown adipose tissue and intestinal mucosa. In the adult mouse, ERRα is most highly expressed in kidney, heart, and brown adipocytes, tissues which preferentially metabolize fatty acids. Binding site selection experiments show that ERRα preferentially binds to an ERRα response element (ERRE) containing a single consensus half-site, TNAAGGTCA. An ERRE is present in the 5′-flanking region of the gene encoding medium-chain acyl coenzyme A dehydrogenase (MCAD), a key enzyme involved in the mitochondrial β-oxidation of fat. The MCAD nuclear receptor response element 1 (NRRE-1) interacts in vitro with ERRα expressed in COS-7 cells. Supershift experiments show that endogenous ERRα present in nuclear extracts obtained from a brown fat tumor cell line (HIB) interacts with NRRE-1. In the absence of its putative ligand, ERRα does not activate the MCAD promoter in transient transfection studies; however, a VP16-ERRα chimera activates natural and synthetic promoters containing NRRE-1. In addition, ERRα efficiently represses retinoic acid induction mediated by NRRE-1. These results demonstrate that ERRα can control the expression of MCAD through the NRRE-1 and thus may play an important role in regulating cellular energy balance in vivo.

The orphan nuclear receptor estrogen-related receptor α (ERRα) was initially cloned by low-stringency screening of a human kidney library with an estrogen receptor DNA binding domain probe (12). Subsequently, protein micropurification and microsequencing techniques identified ERRα as a repressor of the simian virus 40 major late promoter and implicated the receptor as a key regulator of the early-to-late switch of simian virus 40 gene expression (35). ERRα has also been shown to accentuate estrogen-dependent induction of the complex lactoferrin estrogen response element (ERE), possibly by forming heterodimers with the estrogen receptor (39). While ERRα displays significant homology to the estrogen receptor, it does not bind estrogens in vitro, nor is its transcriptional activity modulated by estrogens (12, 39). Like many other members of the nuclear receptor superfamily, ERRα has no known ligand and is therefore considered an orphan receptor. In the absence of an associated ligand, one approach to uncovering potential physiologic roles for ERRα is to identify possible target genes.

Medium-chain acyl coenzyme A dehydrogenase (MCAD) is one of three nuclearly encoded proteins mediating the initial step in mitochondrial β-oxidation of fatty acids (2, 26, 29). Modulation of MCAD gene expression is an important control of the rate of tissue fatty acid β-oxidation (23) and is tightly regulated by tissue energy demands during organ development (4, 15), as well as by energy substrate supply (23). The highest MCAD levels are found in organs that preferentially utilize lipids as a source of cellular energy, such as the heart, kidneys, and brown adipose tissue (2, 15, 17, 23). In humans, MCAD deficiency may cause childhood nonketotic hypoglycemia, coma, or sudden death, often in association with prolonged fasting or intercurrent illness (8, 26, 31).

Initial characterization of the MCAD promoter by DNase I footprinting has identified six protected regions (19). Three protected sites bind the ubiquitous transcription factor Sp1. The remaining protected sites, designated NRRE-1 (nuclear receptor response element 1), NRRE-2, and NRRE-3, contain consensus binding sites for members of the nuclear receptor superfamily (19). Transgenic studies have suggested that the response element NRRE-1 is responsible for much of the tissue-specific and developmental regulation of the MCAD gene (7). Several members of the nuclear receptor superfamily have been shown to interact specifically with NRRE-1, including COUP (5), PPAR (14), RAR/RXR (24), and ARP-1 and HNF-4 (6). Despite the identification of multiple potential regulators of the MCAD promoter, it remains unclear which of these nuclear receptors are important for regulating the tissue-specific and developmental patterns of gene expression in vivo.

In this paper, we report the cloning and functional characterization of the murine homolog of ERRα. Using the selected and amplified binding (SAAB) technique (1), we demonstrate that ERRα binds to an ERRα response element (ERRE) containing a single core motif, AGGTCA, preceded by a 3-bp TNA flank. Potential ERREs are present in the 5′-flanking regions of several genes regulating cellular energy metabolism,

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including the gene encoding MCAD. Because adult mice express high levels of ERRα in tissues with a high β-oxidative capacity, we studied the interaction between ERRα and regulatory regions of the MCAD promoter. Our results demonstrate that ERRα can bind the MCAD response element NRRE-1 in vitro. In addition, nuclear extracts obtained from a brown fat tumor cell line form multiple complexes with the NRRE-1 element when studied by electrophoretic mobility shift assay (EMSA); one of these complexes is abolished by polyclonal antiserum directed against the ERRα N terminus. Transient transfection studies show that a constitutively active VP16-ERRα chimeric receptor can increase transactivation mediated by the MCAD promoter and that ERRα can modulate the promoter's retinoic acid responsiveness. Taken together, these results suggest that ERRα may be an important regulator of the MCAD promoter in vivo.

MATERIALS AND METHODS

Molecular cloning of mERRα. The 2.2-kb cDNA clone λmERRα-3.2 was isolated from a λgt11 10.5-day-old BALB/c mouse embryo library (a gift of A. Joyner, Skirball Institute, New York, N.Y.) screened with a probe obtained from the DNA binding domain of human ERRα (hERRα) (GenBank accession no. X51416). Duplicate filters were hybridized overnight at 42°C in a solution of 35% formamide, 5× SSPE (1× SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA), 0.3% sodium dodecyl sulfate (SDS), 10% (wt/vol) dextran sulfate, 100 mg of denatured salmon sperm DNA ml^{-1}, 1× Denhardt's solution, and 10^6 cpm of ^{32}P-labeled probe ml^{-1}. Membranes were washed twice for 30 min each at 55°C in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM trisodium citrate)–1% SDS and then autoradiographed at −70°C. The cDNA was subcloned into pBluescript(KS) (Stratagene), and DNA sequencing was performed according to standard protocols (28).

Preparation of cell extracts. Whole-cell extracts were prepared from monolayer cell cultures grown to 80 to 90% confluence on 10-cm-diameter tissue culture plates. The cells were washed twice with cold phosphate-buffered saline prior to harvesting by scraping and were resuspended in 200 μl of cold lysis buffer (20 mM HEPES [pH 7.9], 20% [vol/vol] glycerol, 400 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, 1.0 mM dithiothreitol [DTT], 1.0 mM phenylmethylsulfonyl fluoride [PMSF]). Lysis was performed by three freeze-thaw cycles (dry ice-ethanol bath for 3 min followed by incubation at 37°C for 3 min). After lysis, the reaction mixture was centrifuged in a refrigerated microcentrifuge for 10 min; the supernatant was frozen in a dry ice-ethanol bath and stored at −70°C. Nuclear extracts were prepared by harvesting monolayer cultures by scraping and lysing the cells by three freeze-thaw cycles in a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF. A crude nuclear pellet was obtained by centrifuging the whole-cell extract in a microcentrifuge for 20 s. The supernatant was discarded, and the pellet was resuspended in two-thirds volume nuclear extraction buffer (20 mM HEPES [pH 7.9], 25% [vol/vol] glycerol, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). The reaction mixture was incubated on ice for 30 min with occasional agitation, and the nuclear debris was pelleted by centrifugation for 5 min in a cold microcentrifuge. Protein concentrations were measured with the Bradford assay (Bio-Rad).

Generation of polyclonal ERRα antibody. A 300-bp *Sty*I-*Stu*I fragment encoding the mERRα N terminus was cloned into the *Sma*I site of the prokaryotic glutathione *S*-transferase (GST) expression vector pGEX-2T (Pharmacia Biotech). The GST fusion protein was expressed in *Escherichia coli* DH5α cells and batch purified on glutathione-Sepharose 4B (Pharmacia Biotech). Bound proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie staining. The Sepharose-bound proteins were used to inoculate rabbits (JK Antibodies, Castro Valley, Calif.). Western blotting of programmed reticulocyte lysates and transfected COS-7 cell extracts expressing mERRα, mERRβ, and mERα were used to demonstrate the binding specificity of the resulting polyclonal rabbit serum.

Western blots. Protein samples were separated on SDS-PAGE gels and transferred to nitrocellulose membranes (Hybond C Extra; Amersham). The membranes were processed with the BM chemiluminescence Western blotting kit (Boehringer Mannheim) according to the instructions of the manufacturer. The ERRα polyclonal antiserum was used at a dilution of 1:1,000 in maleic acid buffer containing 1% blocking reagent. Acid phosphatase treatment was performed by incubating 1 μg of cell extract together with 1 μl of potato acid phosphatase (Boehringer Mannheim) in gel shift buffer for 30 min at room temperature.

Northern blots. Total RNA was collected from animal tissues or cultured cells and processed with TRIZOL reagent (Life Technologies). RNA samples (10 to 20 μg of total RNA) were separated by electrophoresis in a 1% agarose–0.4% formaldehyde–1× MOPS (morpholinepropanesulfonic acid) gel and transferred to a nylon membrane (Hybond N; Amersham) in 20× SSC buffer. The RNA was UV cross-linked to the membrane and then prehybridized at 42°C for 1 to 4 h in a buffer containing 50% formamide, 5× SSPE, 5× Denhardt's solution, 1% glycine, and 100 mg of denatured salmon sperm DNA ml^{-1}. After prehybridization, the membranes were hybridized overnight at 42°C in a solution of 50% formamide, 5× SSPE, 1× Denhardt's solution, 0.3% SDS, 10% (wt/vol) dextran sulfate, 100 mg of denatured salmon sperm DNA ml^{-1}, and 10^6 cpm of ^{32}P-labeled probe ml^{-1}. Membranes were washed twice for 30 min each at 65°C in 0.1× SSC–1% SDS and then autoradiographed at −70°C. PPARγ2 and UCP cDNA probes were a gift of E. Silva (Lady Davis Research Institute, Montreal, Quebec, Canada). All other cDNA probes were purchased from Research Genetics; their identity was verified by restriction mapping and end sequencing.

RNA in situ hybridization. Embryos and extraembryonic tissues from heterozygous (C57BL/6J × 129sv) intercrosses were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections (7 μm thick) were processed for in situ hybridization according to published protocols (36) with a ^{33}P-labeled riboprobe obtained from the 3′-untranslated region of mouse ERRα (bp 1730 to 2027).

Adipocyte differentiation. NIH 3T3-L1 cells (obtained from American Type Culture Collection) and HIB cells (a gift of B. Spiegelman, Dana Farber Cancer Institute, Boston, Mass.) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). 3T3-L1 cells were induced to differentiate according to published protocols (38). HIB cells were differentiated by allowing them to grow to full confluence and then maintaining them without changing the medium for 1 week. The cells were then treated for 72 h with differentiation medium (DMEM containing 10% FBS, 0.125 mM indomethacin, 0.5 mM 1-isobutyl-3-methylxanthine, 20 nM insulin, and 0.5 μM hydrocortisone). The differentiation medium was replaced with DMEM supplemented with 10% FBS, and the cells were harvested 4 days later.

Plasmid construction. Reporter plasmid TKLUC contains bp −105 to +51 of the thymidine kinase (TK) promoter linked to the firefly luciferase gene. Reporter plasmids ERRE-TKLUC and ERRE-S1-TKLUC contain single copies of ERRE (5′-TCGACGCTTTCAAGGTCATATGCG-3′) or ERRE-S1 (see Fig. 5) cloned into the *Sal*I and *Bam*HI sites of TKLUC; plasmid IR3-TKLUC contains a single copy of IR3 (5′-AGCTTGAAGAGGTCACTGTGACCTAC AACG-3′) cloned into the *Sal*I and *Hin*dIII sites of TKLUC. Reporter plasmids MCAD-TKLUC and $MCAD_3$-TKLUC contain one and three copies of the MCAD NRRE-1 cloned into the *Sal*I and *Hin*dIII sites of TKLUC. Plasmids NRRE-MCAD-LUC and NRREmut-MCAD-LUC (7) were generous gifts of D. P. Kelly (Washington University School of Medicine, St. Louis, Mo.). NRRE-MCAD-LUC contains an NRRE-1 oligonucleotide (5′-gatcCGGGTTTGACCT TTCTCTCCGGGTAAAGGTGAAGGCTGACt-3′) cloned upstream of bp −306 to +191 of the MCAD promoter. NRREmut-MCAD-LUC contains a mutant NRRE-1 oligonucleotide with point mutations (underlined) in each half-site (5′-gatcCGGGTTTGACGTTTCTCTCCGCGTAAACGTGAAGGCT GACt-3′) cloned upstream of the MCAD promoter fragment. pCMXmERRα contains a 1.8-kb *Sty*I fragment of mouse ERRα cloned into the *Sma*I site of pCMX (34). Expression vectors pCMXhRARα and pCMXhRXRα have been described previously (33).

Binding site selection protocol. mERRα was synthesized in vitro with the TNT-T7 reticulocyte lysate kit (Promega). A pool of radiolabelled oligonucleotides containing 30 random bp flanked by two 20-base primers was synthesized by PCR. Three cycles of denaturation for 1 min at 95°C, annealing for 2 min at 55°C, and extension for 2 min at 72°C were performed under standard conditions in a 50-μl reaction volume containing 20 pmol of the random oligomer 5′-CCG GATCCTGCAGCTCGAG-N_{30}-GTCGACAAGCTTCTAGAGCA-3′ with 400 pmol each of ^{32}P-labelled forward (5′-CGCGGATCCTGCAGCTCGAG-3′) and reverse (5′-TGCTCTAGAAGCTTGTCGAC-3′) primers (gifts of T. Look, St. Jude's Children's Hospital, Memphis Tenn.). Five microliters of the PCR product was used for the initial selection round. Binding reactions were performed by incubating the amplified oligonucleotides with 5 μl of programmed or nonprogrammed reticulocyte lysate in a 20-μl reaction mixture containing 10 mM Tris (pH 8.0), 40 mM KCl, 1 mM DTT, 0.05% Nonidet P-40, 2 mg of poly(dI-dC), 0.1 μg of single-stranded DNA, 0.1 μg of acetylated bovine serum albumin, and 6% glycerol. Bound probe and free probe were separated on a 5% nondenaturing polyacrylamide gel and detected by autoradiography. A parallel binding reaction with a ^{32}P-labelled consensus DR2 probe (5′-AGCTTGAAGA GGTCAAAAGGTCAACACG-3′) was used to estimate the position of the shifted band for the initial round of selection. On subsequent selection rounds, the amplified random oligonucleotides produced a retarded band that was clearly visible on autoradiography. The section of dried gel containing the bound fragments was eluted at 37°C for 2 h in 500 μl of elution buffer (500 mM ammonium acetate, 1 mM EDTA). The DNA was extracted with phenol-chloroform and precipitated with ethanol. The selected fragments were resuspended in 20 μl of TE: 2 μl was amplified by 12 cycles of PCR, and 1/10 of the PCR mixture was used as the input for subsequent rounds of selection. After eight purification cycles, the DNA fragments were digested with *Sal*I and *Bam*HI, subcloned into pBluescript(KS) (Stratagene), and sequenced.

EMSA. For EMSAs, proteins were obtained from transfected COS-7 cells or from HIB nuclear extracts. DNA probes were labelled by end filling with Klenow fragment to a specific activity of 10^8 cpm μg^{-1}. The binding reactions were performed by incubating 1 μg of whole-cell lysate or 10 to 20 μg of nuclear extract in a 20-μl reaction mixture containing 10 mM HEPES (pH 7.9), 100 mM

KCl, 1 mM DTT, 0.05% Nonidet P-40, 2 μg of poly(dI-dC), and 10% glycerol for 20 min on ice. The reaction mixtures were then incubated for 10 min at room temperature with 0.25 ng of labelled probe with or without 200-fold molar excess of cold competitor. Oligonucleotide NS (5′-AGCTTGCGAAAATTGTCACT TCCTGTGTACACCGA-3′) was used as a nonspecific competitor. Reactions were analyzed on 4 to 5% polyacrylamide gels with 0.5× TBE running buffer. Antibody supershift assays were performed by incubating the DNA binding reaction mixture with 2 μl of polyclonal antiserum or preimmune serum for 10 min immediately prior to electrophoresis.

Cell culture and transient transfection. HeLa and HIB cells were maintained in DMEM-F12 medium, and COS cells were maintained in alpha-minimal essential medium, both supplemented with 10% fetal bovine serum (FBS) (Life Technologies). Cells were transfected by the calcium phosphate coprecipitation technique in 35-mm-diameter plates. Typically, 1 μg of reporter plasmid was transfected together with 100 ng to 2 μg of expression vector (as indicated in the figure legends). Transfection efficiency was assessed with the control plasmids pRSVβGal (COS and HIB cells) or pCMVβGal (HeLa cells). Luciferase and β-galactosidase assays were performed as previously described (33).

RESULTS

Molecular cloning of mERRα. The murine homolog of hERRα was cloned by screening a 10.5-day BALB/c mouse embryo library under low-stringency conditions with a probe obtained from the DNA binding domain of hERRα. A total of six independent clones were obtained, the longest of which was a 2.2-kb cDNA encoding a 1.26-kb open reading frame. Shorter overlapping clones were obtained by screening an adult mouse brain library (Strategene) under high-stringency conditions using mERRα as a probe (data not shown). The predicted N terminus of mERRα is shorter than that originally reported for hERRα (12); however, the common amino-terminal coding regions of mERRα, hERRα, and the recently reported isoform hERRα1 (39) share 94% predicted amino acid homology, while the DNA binding domain and C-terminal regions have 100 and 95% predicted amino acid sequence homology, respectively. The high degree of homology between the human and murine predicted amino acid sequences suggests that ERRα may perform a highly conserved function in mammalian species. The sequence has been deposited in GenBank, and its accession number is U85259.

Based on the cDNA sequence, the mouse translation start site occurs at bp 171; however, this start site is not preceded by an in-frame stop codon. In addition, significant homology exists between hERRα and mERRα upstream of the putative start site. Western blotting was performed to determine whether translation initiates at bp 171 or at an alternate start codon (Fig. 1A). Endogenous ERRα, obtained from HIB and HeLa nuclear extracts (Fig. 1A, lanes 3 and 4), was compared in size to mouse ERRα overexpressed in COS-7 cells transiently transfected with pCMXmERRα (Fig. 1A, lane 2). The transfected COS-7 whole-cell extract contains two bands with apparent molecular masses of 48 and 62 kDa. Neither band is present in the untransfected COS-7 control extract. In contrast, HIB and HeLa nuclear extracts contain a single band with an apparent molecular mass of 62 kDa. If translation initiates at bp 171 of the transfected ERRα cDNA, it should result in a translated protein with an apparent mass of 47 kDa. The ERRα expression plasmid contains no additional start codons in the cDNA fragment used to generate the ERRα polyclonal antiserum. This suggests that the 62-kDa band could result either from posttranslational modification of ERRα or from induction in transfected cells of a protein containing ERRα idiotypes. Sequence analysis of the ERRα coding region demonstrated the presence of several potential phosphorylation sites. In order to determine if ERRα was phosphorylated in vivo, we incubated the COS-7 extracts with potato acid phosphatase (Fig. 1B); phosphatase treatment results in the disappearance of the higher band (compare lanes 2 and 3), suggesting that the receptor is posttranslationally phosphorylated.

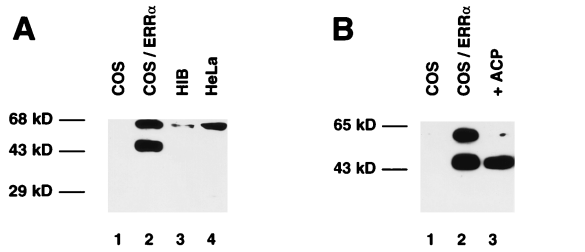

FIG. 1. mERRα encodes peptides with apparent molecular masses of 48 and 65 kDa. (A) Whole-cell extracts obtained from COS-7 cells transfected with the expression vectors pCMX (lane 1) and pCMXmERRα (lane 2) as well as crude nuclear extracts obtained from HIB (lane 3) and HeLa cells (lane 4) were separated by SDS-PAGE (10% polyacrylamide). The gel was blotted onto nitrocellulose, probed with polyclonal antiserum directed against the ERRα N terminus, and detected with the BM chemiluminescence kit. (B) Whole-cell extracts were obtained from COS-7 cells transfected with the expression vectors pCMX (lanes 1) and pCMXmERRα (lanes 2 and 3). Extracts were diluted in EMSA buffer and incubated for 30 min at room temperature with (lane 3) or without (lanes 1 and 2) potato acid phosphatase (ACP). Reaction products were separated on an SDS-PAGE (10% polyacrylamide) gel, blotted onto nitrocellulose, and detected as described above.

Developmental and ontologic expression of mERRα. In situ hybridization studies were used to assess the developmental expression of mERRα. A 300-bp riboprobe hybridizing to the ERRα 3′-untranslated region was used to study receptor expression in embryos between 5.5 and 17.5 days postcoitum (dpc). While low-level ERRα expression was detected in extraembryonic structures starting at 9.5 dpc, consistent embryonic ERRα expression was not detected prior to 13.5 dpc (data not shown). At 14.5 dpc, low-level ERRα expression is detected in the heart (data not shown). By 15.5 dpc, ERRα expression is detected in the developing dorsal brown fat pad, dorsal nerve roots, the intestine, and the heart (Fig. 2A to F). At this stage, ERRα expression in brown fat is highly specific: other mesodermal derivatives such as trunk muscles and rib cartilage primordia do not express ERRα (Fig. 2A and B). At later developmental stages, ERRα becomes more widely expressed. By 16.5 dpc, high-level ERRα expression is detected in brown fat and intestine (Fig. 2G to L). In addition, expression is present throughout the spinal cord and in many regions of the brain (data not shown). At 17.5 dpc, ERRα expression is present in most tissues, with the highest level of expression in brown fat, intestinal mucosa, and developing thyroid, as well as a lower level of expression in the nervous system (Fig. 2O to T). In summary, prominent ERRα expression is present in brown adipose tissue and intestine throughout mid- and late-embryonic development. Lower-level ERRα expression is found in many tissues, including the heart and nervous system. Murine ERRα expression is not restricted to a particular germ cell lineage, but rather its expression appears to be regulated in an organ-specific manner.

Northern blots performed with RNA extracted from adult mouse tissues demonstrated a single 2.4-kb ERRα mRNA, which, although present in all tissues studied, was most highly expressed in heart and kidney (Fig. 3A) as well as in brown adipose tissue (Fig. 3B). This pattern of expression agreed with the results of the in situ hybridization studies, with the exception of the kidney, in which no ERRα expression was detected during development (data not shown). Analysis of RNA obtained from immature animals shows that renal ERRα expression is markedly higher in adult mice than in 18.5-dpc embryos and 1-week-old animals; in addition, cardiac ERRα expression

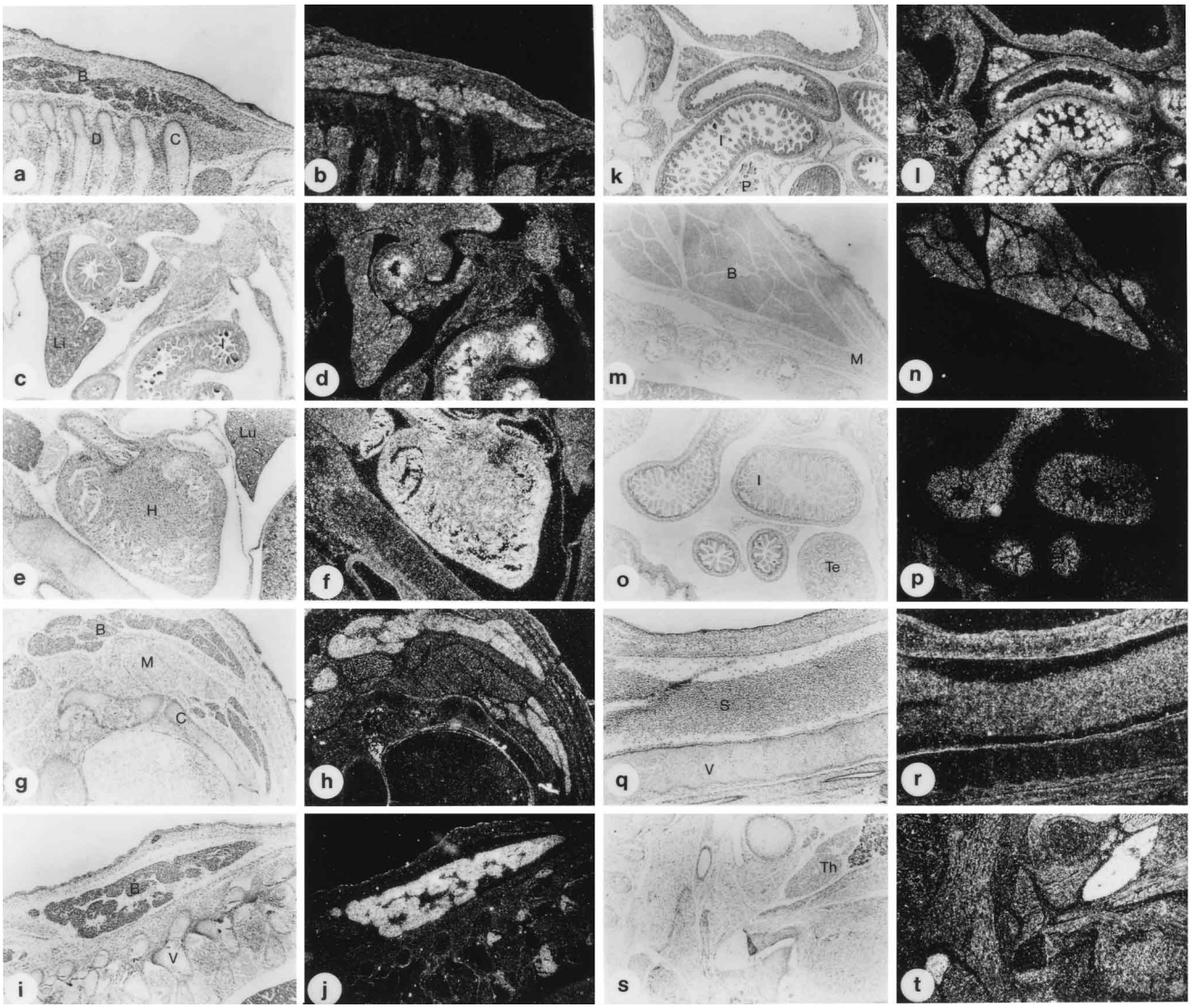

FIG. 2. Tissue-specific mERRα expression during murine development. ERRα expression in mouse embryos was studied by in situ hybridization with a 300-bp riboprobe hybridizing to the 3′-untranslated region. Bright-field and dark-field photographs of a 15.5-dpc embryo show ERRα expression in the developing dorsal brown fat pad (a and b), the heart (c and d), and the intestine (e and f). At 16.5 dpc, high-level ERRα expression is detected in brown fat (g to j) and intestine (k and l). By 17.5 dpc, ERRα expression is present in most tissues, with prominent expression in brown fat (m and n), intestinal mucosa (o and p), spinal cord (q and r) and thyroid (s and t). B, brown fat; C, cartilage primordia; D, dorsal nerve root; H, heart; I, intestinal mucosa; Li, liver; Lu, lung; M, muscle; P, pancreas; S, spinal cord; Te, testis; Th, thyroid; V, vertebral primordia.

is slightly higher in adults (Fig. 3C). These data suggest that ERRα may play a role in the postnatal maturation or physiology of these organs.

ERRα expression is regulated during adipocyte differentiation. In situ hybridization studies suggested that ERRα expression was regulated during brown adipocyte development. This hypothesis was tested by studying ERRα expression in differentiating adipocyte cell lines (Fig. 4). Two cell lines were studied. NIH 3T3-L1 cells are a classic model of white adipocyte differentiation (13), while HIB cells—derived from a simian virus 40-induced brown fat tumor (27)—model brown adipocyte differentiation. Morphologic changes, as well as increased expression of the adipocyte markers PPARγ2 and aP2 and the brown adipocyte marker UCP, were used to confirm differentiation (Fig. 4). A six- to eightfold increase in ERRα expression accompanies differentiation of NIH 3T3-L1 preadipocytes (Fig. 4, top panel). In contrast, differentiation of HIB cells is associated with a two- to threefold increase in ERRα expression (Fig. 4, top panel). The difference in fold induction between the two adipocyte lines is in part explained by higher basal levels of ERRα in HIB cells: undifferentiated HIB preadipocytes also express moderate levels of PPARγ2, a marker of terminal adipocyte differentiation. This experiment demonstrates that ERRα expression is regulated in two in vitro models of adipocyte differentiation and suggests that ERRα may regulate genes important for adipocyte differentiation or metabolism.

Identification of a consensus ERRα binding site. In order to identify potential ERRα target genes, a PCR-based site selection strategy (1) was used to identify a consensus ERRα binding site. Candidate binding sites generated from a pool of radiolabelled oligonucleotides containing 30 random base

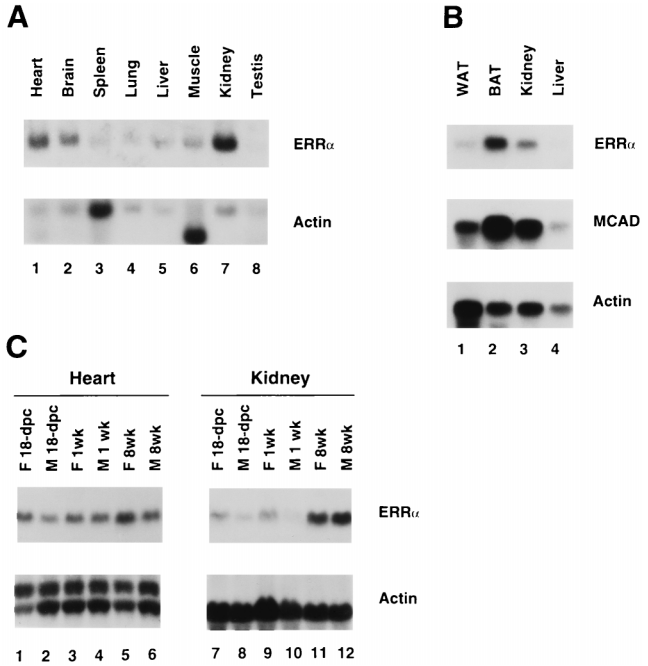

FIG. 3. Expression of mERRα in adult and immature mice. (A) A full-length mERRα cDNA probe was hybridized to a multiple-tissue Northern Blot (Clontech) containing approximately 2 μg of poly(A)$^+$ RNA from the indicated adult mouse tissues (upper panel). A single 2.4-kb mRNA species was detected in each tissue. (B) A full-length mERRα cDNA probe was hybridized to 10 μg of total RNA obtained from the indicated adult mouse tissues (upper panel). The blot was reprobed with a 1-kb MCAD cDNA fragment (middle panel). WAT, white adipose tissue; BAT, brown adipose tissue. (C) RNA was obtained from the heart (lanes 1 to 6) and kidneys (lanes 7 to 12) of 18.5-dpc mouse embryos (lanes 1, 2, 7, and 8), 1-week-old mice (lanes 3, 4, 9, and 10), and adult mice (lanes 5, 6, 11, and 12). The mice were pooled according to their sex, and 10 μg of total RNA was loaded per lane (females [F] in odd-numbered lanes and males [M] in even-numbered lanes). The blot was probed with a full-length mERRα cDNA probe (upper panel). In each experiment, a 1-kb β-actin cDNA probe was used to monitor RNA integrity.

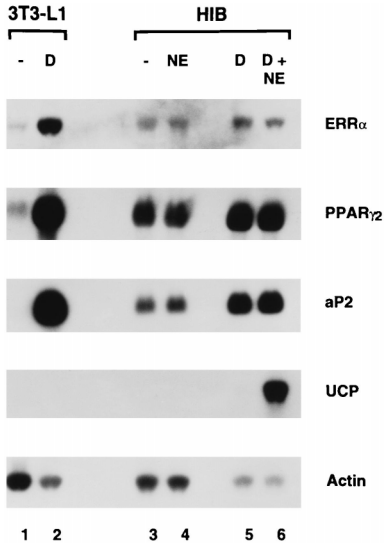

FIG. 4. mERRα is upregulated during adipocyte differentiation. RNA was obtained from undifferentiated NIH 3T3-L1 preadipocytes (lane 1) and HIB cells (lanes 3 and 4) as well as their differentiated (D) counterparts (lanes 2, 5, and 6). The blot was hybridized to a 2.2-kb mERRα cDNA probe (top panel) as well as to probes specific for the adipocyte differentiation markers PPAR$_{\gamma 2}$, aP2, and UCP. UCP expression was induced by treating HIB cells with 10^{-7} M norepinephrine (NE) for 4 h prior to harvesting RNA (lanes 4 and 6).

pairs were incubated with ERRα-programmed rabbit reticulocyte lysate. Bound probe and free probe were separated by nondenaturing PAGE and detected by autoradiography. The bound fragments were eluted and used as input for subsequent rounds of purification. Sequence convergence was monitored by sequencing small numbers of fragments after four and six cycles of selection (data not shown). After eight purification cycles, sequences were obtained for a total of 25 fragments (Fig. 5).

The DNA binding domains of mERRα and mERα are highly conserved, and both share critical P and D box residues, suggesting that they should bind to common response elements (34). Surprisingly, examination of the selected binding sites suggested that ERRα would bind efficiently to a monomeric response element with consensus TNAAGGTCA. This hypothesis was tested in vivo by cotransfecting HeLa cells with TKLUC-based reporter constructs containing a consensus ERRα response element (ERRE), a single-copy estrogen response element (IR3), or a fragment obtained from the site selection procedure (ERRE-S1). The transfections were performed with mERRα, as well as a chimeric receptor in which the VP-16 activation domain was fused to the N terminus of ERRα. This constitutively active chimera allows assessment of receptor-DNA interactions without interference caused by the absence of potential ligands or cell-specific coactivators. Under these conditions, ERRα does not significantly alter transactivation of the reporter construct; however, the ERRα-VP16 chimera efficiently increases transactivation by each reporter (Fig. 6). This experiment shows that ERRα can recognize both dimeric and monomeric binding sites in vivo. The lack of transactivation mediated by ERRα may be due to a weak activation

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S1    CTTGAAGGGTTGTTTTCGCCTCA AGGTCA Cctc
S2              CTTCACTAGCGA AGGTCA AGGTCTTCTCAG
S3          CTTATCTGGTTTAGCA AGGTCA TGTCAACC
S4        CTTCTAGTTAAGCGGTAG AGGTCA CCTAAC
S5      CACTGGACACGGCAGTGACA AGGTCA GACG
S6                GGGGGTTAG CGGTCA TTCCTTAGTTTCAAG
S7           CCCTGGGACAAATAA AGGTCA TTCGTTATC
S8                CTAACTACA AGGTCA CGTTCATTGAAGGTT
S9     CTTGAGTGATCAGTAGGATGA AGGTCA GACc
S10               gCTTGAGAA AGGTGC CATCAAGGTTGTGTTG
S11  TTCATGTCCAAGGTAATCCTGA AGGTCA TTgg
S12             CTTGAAAGGGC AGGACA AACTCCCCGTTTG
S13       CAATCCAAGGGACTTTAG AGGTCA CAGCAA
S14               GTGACATCT AGGACA TTTGAAGGTTGAAAC
S15      AGGATAATGTCATTGTACA AGGTCA AACGC
S16    CTTAAGGAGTCATCCCCATGA AGGTGA TACc
S17               gagATCACA AGGTCA GAATCCCGTGAAGTCAAG
S18               ctgagATAT TGGTCA TCGGCCGTTGCTTCTATTTG
S19         TACCTGCTCTTCATAG AGGTCT TCCACAAG
S20         TTATCATCGTTTCTCA AGGTCA GAATGA
S21                 cagctcg agGTCA CTTTGTAAGTCAAAGTGTGCCGCGT
S22        CGCTAAGTTAAATAGA AGGTCA AACGCCTA
S23      TGACTGAAGGGTATTCCAA AGGACA GGAAG
S24               cagctcg agGTCA TTTGCAAGGTTACTCTCGATTTAGC
S25               cagctgTTA AGGTCA AATGCGAGGTGTCCTTCGAAC

Consensus:

     -6  -5  -4  -3  -2  -1   1   2   3   4   5   6   7   8

A     7   5   8   5   8  16  22   0   0   3   0  22   5  11
C     3   6   8   2  10   1   1   0   0   0  22   1   6   3
G     5   8   4   3   6   6   0  24  24   0   2   0   5   5
T    10   6   5  15   1   2   1   0   0  21   0   1   9   6

                  T   N   A   A   G   G   T   C   A
```

FIG. 5. Consensus sequences binding to ERRα. The sequences of fragments S1 to S25 are shown at the top. The core half-site residues are highlighted in boldface.

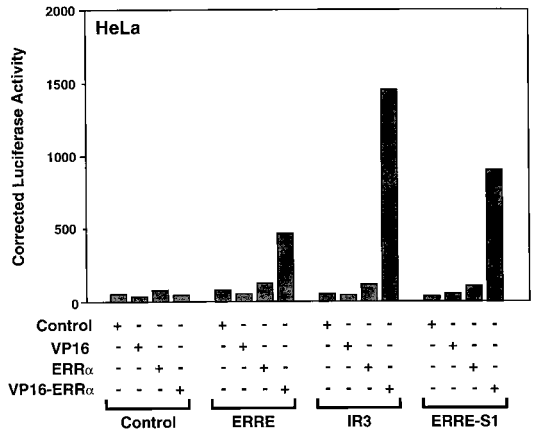

FIG. 6. The VP16-mERRα chimeric receptor modulates transactivation mediated through consensus ERRE and IR3 response elements. HeLa cells were cotransfected with 1 μg (per plate) of the reporter constructs TKLUC (Control), ERRE-TKLUC (ERRE), IR3-TKLUC (IR3), or ERRE-S1-TKLUC (ERRE-S1), as well as pCMX-based expression vectors encoding the VP16 activation domain, mERRα, or the VP16-mERRα chimera (200 ng per plate). pCMXβgal (1 μg per plate) was used as an internal control. The cells were harvested 24 h after transfection and assayed for luciferase and β-galactosidase activity.

domain, the absence of an appropriate ligand, or inhibitory interactions with the TK minimal promoter.

ERRα binds to a regulatory element in the MCAD promoter. The consensus ERRE can be found in the 5′-flanking and promoter regions of many families of genes, including estrogen-regulated proteins (16), as well as enzymes involved in steroid synthesis (18), glycolysis, and oxidative metabolism. Because ERRα is highly expressed in adult tissues with a large capacity for fatty acid β-oxidation, we chose to determine whether ERRα could regulate the expression of MCAD, a gene which encodes an early regulatory step in this pathway. In adult mouse tissues, the expression of MCAD RNA roughly parallels ERRα expression (Fig. 3B). The MCAD promoter contains six regions which are protected from DNase I digestion. Of these, the consensus ERRE is contained in response element NRRE-1.

The interaction between ERRα and NRRE-1 was first studied with receptor protein synthesized in transfected COS-7 cells (Fig. 7A). A single retarded complex containing a predominant high-mobility band and a fainter lower-mobility side band is observed when the NRRE-1 oligonucleotide probe is incubated with whole-cell extracts obtained from COS-7 cells transiently transfected with the expression vector pCMXmERRα (Fig. 7A, lane 2). The interaction is specific (Fig. 7A, lanes 3 and 4) and is not observed with COS-7 cells transfected with the pCMX plasmid backbone (Fig. 7A, lane 1). EMSA failed to detect specific interaction between ERRα and either NRRE-2 or NRRE-3 (data not shown).

We next wished to determine whether endogenous ERRα present in an MCAD-expressing cell line could interact with the NRRE-1 response elements. EMSA performed with crude nuclear extracts obtained from differentiated HIB cells demonstrated two specific retarded complexes (Fig. 7B, lanes 1 to 5 [complexes C1 and C2]). Competition studies performed with an ERRE oligonucleotide partially obliterated complex C1, suggesting that it may contain an ERR isoform (Fig. 7B, lanes 6 to 8). Previous studies of the interaction between NRRE-1 and brown fat nuclear extracts suggest that complex C2 is formed by COUP-TF. In support of this hypothesis, competition with a consensus DR1 obliterates complex C2 (Fig. 7B, lanes 9 to 11). In addition, the DR1 oligonucleotide competitor significantly reduces the intensity of complex C1; this could occur if the C1 complex was formed by isoforms of receptors such as COUP, PPAR, or ERR. These data suggest that an endogenous ERR in HIB nuclear extracts may interact with the MCAD NRRE-1 response element.

EMSA was performed with ERRα-specific antibodies to confirm that ERRα interacts with NRRE-1. Antibody raised against the N terminus of ERRα completely obliterated the retarded complex seen in EMSA performed with COS-7 cells overexpressing ERRα (Fig. 7A, lane 7). In addition, the ERRα antibody produced a band with reduced electrophoretic mobility which is also weakly present when preimmune serum is added to the EMSA binding reaction mixture (Fig. 7C, lanes 7 and 8). When the ERRα antibody is incubated with binding reaction mixtures containing HIB nuclear extracts, it partially obliterates retarded complex C1 (Fig. 7C, lane 4). By altering the EMSA electrophoresis conditions, complex C1 can be resolved into two distinct bands (Fig. 7D). Addition of the mERRα antibody completely titrates complex C1a (Fig. 7D, lane 4); this band has the same mobility as ERRα overexpressed in COS-7 whole-cell extracts (Fig. 7D, lane 1). Taken together, these experiments demonstrate that ERRα binds specifically to the MCAD response element NRRE-1 and that the interaction is detected with both overexpressed and endogenous receptor.

A VP16-ERRα chimera modulates transactivation mediated by MCAD NRRE-1. Transient transfection assays were used to determine whether ERRα could alter the rate of transcription mediated by the MCAD promoter. Because ERRα does not alter transactivation mediated by its consensus response element, transfections were performed with both mERRα and the constitutively active chimera VP16-ERRα. Reporter plasmid NRRE-MCAD-LUC (7) contains an NRRE-1 oligonucleotide cloned upstream of bp −306 to +191 of the MCAD promoter, while NRRE-MCAD-LUC contains the MCAD promoter cloned downstream of a mutant NRRE-1 oligonucleotide containing point mutations in each of three consensus half-sites. HIB transfection shows no induction of NRRE-MCAD-LUC with ERRα and a 2.5-fold induction with VP16-ERRα (Fig. 8A). In COS cells, VP16-ERRα induces NRRE-MCAD-LUC fivefold (Fig. 8B). The chimeric receptor does not induce NRREmut-MCAD-LUC in either cell line. Interaction of ERRα and NRRE-1 was also demonstrated with a synthetic promoter containing a single copy of the NRRE-1 oligonucleotide cloned upstream of the TK minimal promoter (Fig. 8C and D). VP16-ERRα induces this construct sevenfold in HIB cells and fivefold in COS cells. ERRα does not alter transactivation by this construct, nor is the control plasmid TKLUC induced by VP16-ERRα.

ERRα modulates RA-induced transactivation mediated by NRRE-1. The preceding transfection experiments suggest that ERRα can bind the NRRE-1 element in vivo. The low fold induction seen with the VP16-ERRα chimera might be caused by weak binding to the response element or by attenuation of the VP16-mediated activation by repression domains contained in ERRα. In order to demonstrate direct interaction between ERRα and NRRE-1, we studied the receptor's ability to modulate retinoic acid-induced transactivation mediated by the response element. Reporter plasmid $pNRRE_3$-TKLUC contains three copies of NRRE-1 cloned upstream of the TK minimal promoter. While ERRα demonstrates no activity on this construct, it efficiently represses retinoid induction of the synthetic promoter (Fig. 8E). Taken together, these transfection experiments demonstrate that ERRα can interact directly with the MCAD promoter response element NRRE-1 in vivo.

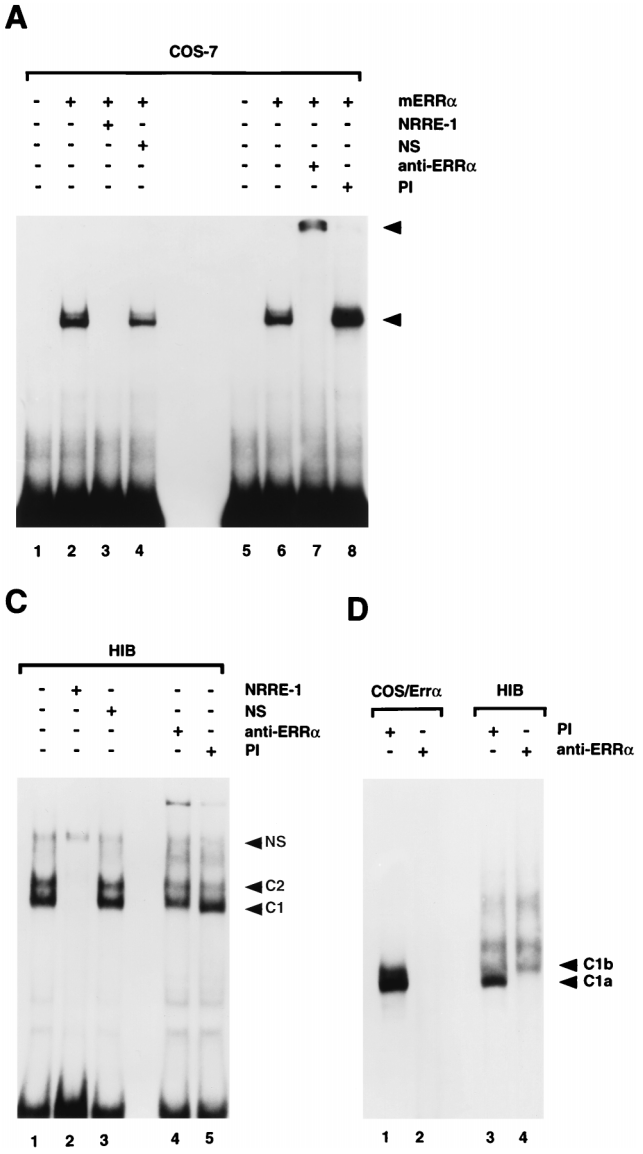

FIG. 7. mERRα binds the MCAD promoter response element NRRE-1. (A) Radiolabelled MCAD NRRE-1 was incubated with 1 μg of COS extract transfected with pCMX (lanes 1 and 5) or pCMXmERRα (remaining lanes). A retarded complex (lower arrowhead) is seen after electrophoresis on a 5% acrylamide–0.5× Tris-borate-EDTA (TBE) gel. The complex is competed by 200-fold molar excess NRRE-1 (lane 3), but not by an equivalent amount of nonspecific competitor oligonucleotide (NS). Incubation of the binding reaction mixture with 3 μl of polyclonal ERRα antiserum supershifts (upper arrow) the retarded complex (anti-ERRα; lane 7); preimmune serum minimally supershifts the retarded band (PI; lane 8). (B) HIB nuclear extracts (10 μg per lane) were incubated with radiolabelled NRRE-1 and analyzed by electrophoresis on a 5% acrylamide–0.5× TBE gel. Competition studies were performed with unlabelled nonspecific competitor (lane 2), NRRE-1 (lanes 3 to 5), ERRE (lanes 6 to 8), and consensus DR1 (lanes 9 to 11) at the indicated molar ratios. (C) Crude nuclear extracts from differentiated HIB cells (10 μg per lane) were incubated with radiolabelled NRRE-1 and analyzed by electrophoresis on a 5% acrylamide–0.5× TBE gel. Competition studies were performed with unlabelled NRRE-1 (lane 2) and nonspecific competitor (lane 3) at 200-fold molar excess. Supershift studies were performed by incubating the binding reaction mixture with 3 μl of polyclonal ERRα antiserum (lane 4) or preimmune serum (lane 5) prior to electrophoresis. (D) COS whole-cell extracts (1 μg per lane, lanes 1 and 2) or HIB nuclear extracts (10 μg per lane, lanes 3 and 4) were incubated with radiolabelled NRRE-1 and analyzed by electrophoresis on a 4.5% acrylamide–0.5× TBE gel. Supershift studies were performed by incubating the binding reaction mixture with 3 μl of preimmune serum (lanes 1 and 3) or polyclonal ERRα antiserum (lanes 2 and 4) prior to electrophoresis. In this experiment, the unbound DNA probe was run off the gel in order to obtain better resolution of the DNA-protein complexes.

DISCUSSION

In this paper, we have shown that the orphan nuclear receptor mERRα binds to a key regulatory element of the MCAD gene promoter and can modulate transcription mediated by this element. Data obtained from EMSA binding site competition studies and antibody supershift experiments show that mERRα forms part of the high-mobility complex that has been implicated in the tissue-specific and developmental expression of the MCAD gene (7). In addition, the spatial and temporal patterns of expression of mERRα suggest that it might contribute significantly to regulating levels of MCAD expression in the adult mouse. These results suggest that mERRα may play an important role in regulating cellular energy metabolism.

hERRα was first identified as a result of its cDNA sequence similarity to ERα (12). To further study potential physiologic and developmental roles for ERRα, we have cloned its murine homolog. mERRα is encoded by a 2.2-kb cDNA which is expressed at various levels in all adult tissues. The mERRα cDNA is shorter than that initially described for hERRα (12); however, it shares significant homology with the recently described hERRα1 isoform in both the coding and 5′-untranslated regions (39). Recently, a 42-kDa ERRα isoform has been identified in human endometrial (RL95-2) and breast (HBL 100) carcinoma lines (39); nuclear extracts from RL95-2 and HeLa cells also contained a less-abundant 53-kDa isoform. In contrast, other studies have demonstrated 53- and 58-kDa ERRα isoforms in HeLa cells and seven other mammalian cell lines (16). In our experiments, the mouse ERRα cDNA encodes peptides with apparent masses of 48 and 62 kDa when transfected in COS cells. The 62-kDa protein is not present in untransfected COS cells, suggesting that it could result from posttranslational modification of ERRα. Acid phosphatase treatment of the COS-7 whole-cell extract ablates the higher protein band, suggesting that the receptor is phosphorylated in

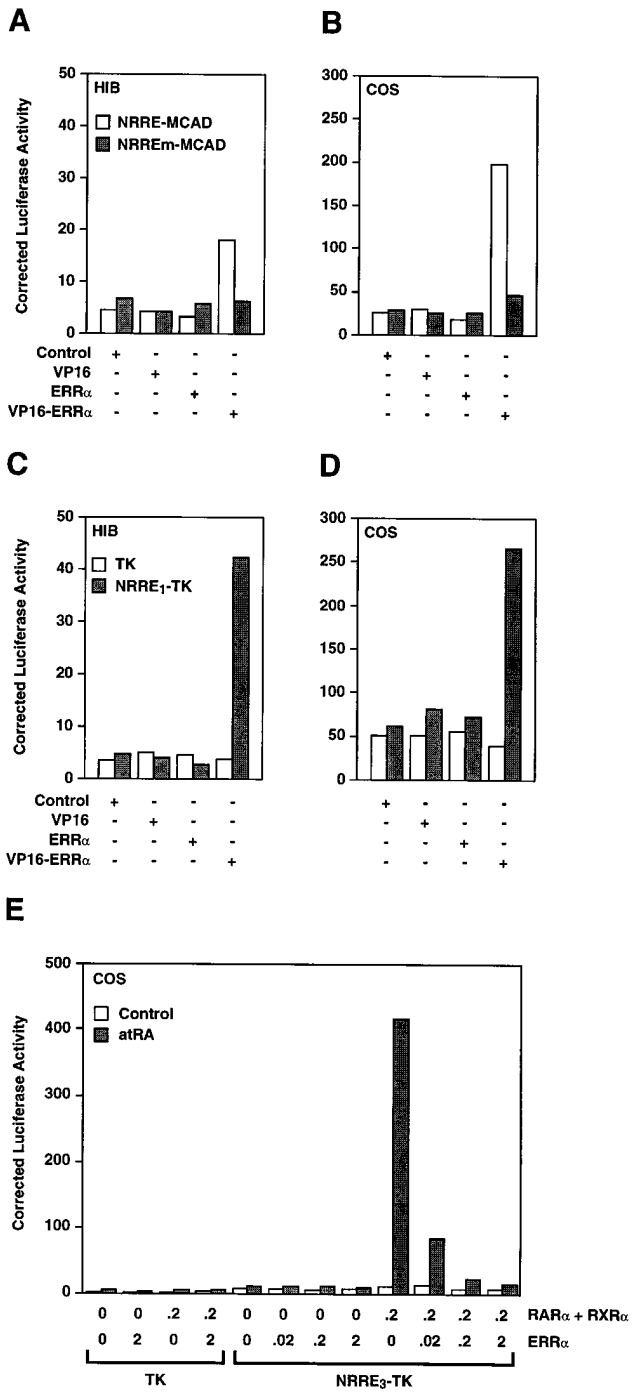

FIG. 8. mERRα and the constitutively active VP16-mERRα chimera modulate transactivation mediated by the MCAD promoter response element NRRE-1. (A to D) HIB and COS cells were cotransfected with 1 μg of the indicated reporter construct per plate as well as pCMX-based expression vectors for the VP16 activation domain, ERRα, and the VP16-ERRα chimera (100 ng per plate). pRSV-βgal (1 μg per plate) was used as an internal control. After overnight incubation with the DNA precipitate, the HIB cells were treated with 10% glycerol for 3 min to improve transfection efficiency. The cells were harvested 24 h after transfection and assayed for luciferase and β-galactosidase activity. (E) COS cells were cotransfected with 1 μg of the reporter plasmid MCAD-TKLUC or $MCAD_3$-TKLUC per plate as well as the indicated amounts of expression vectors pCMX, pCMXmERRα, pCMXhRARα, and pCMX hRXRα. pRSV-βgal (1 μg per plate) was used as an internal control. After overnight incubation with the DNA coprecipitate, the cells were treated for 24 h with 10^{-7} M all-*trans*-retinoic acid (atRA) or vehicle alone. The cells were harvested and assayed for luciferase and β-galactosidase activity.

vivo. In both HIB and HeLa cells, only the 62-kDa protein is observed; this discrepancy from previously published results could be caused by differences in cell culture conditions leading to distinct posttranslational modification of the protein.

Although ERRα and ERα share significant (68%) homology in the DNA binding domain, the SAAB technique shows that ERRα preferentially binds a response element containing a single consensus half-site, 5′-TNAAGGTCA-3′ (ERRE). The SAAB data confirm previous experiments in which ERRα was shown to bind a monomeric response element (16). This result is intriguing because the homology between the two receptors includes single conserved substitutions in the P and D boxes of the DNA binding domain. The P and D box motifs have been shown to determine the specific half-site sequences recognized by other members of the nuclear receptor superfamily as well as the half-site spacing in dimeric response elements (34). It is possible that residues outside the DNA binding domain stabilize ERRα interactions with monomeric response elements. Alternatively, binding of a putative ERRα ligand may facilitate receptor interaction with either monomeric or dimeric response elements.

Monomeric response elements are recognized by several other members of the nuclear receptor superfamily. For example, the receptor SF-1 also binds preferentially to the ERRE (18), while the receptors ROR and NGFIB bind as monomers to distinct sites (11, 22, 37). In each case, the specificity of receptor binding to the monomeric response element is determined by the base composition of the site's 5′ flank. Interestingly, while the SAAB consensus ERRα 5′ flank was 5′-TNA-3′, the most commonly selected 5′ flanks contained the sequences 5′-ACA-3′ and 5′-TAG-3′ (Fig. 5). This suggests that ERRα may be able to differentially modulate promoter elements containing a wide range of half-sites. In addition, mERRα can bind to the palindromic ERE as well as complex response elements containing multiple half-sites lacking the consensus 5′-flanking sequence (16). In some promoters, such as those regulating expression of the oxytocin (25), prolactin (30), and lactoferrin (39) genes, a putative ERRE overlaps a known ERE: a potential physiologic role of ERRα may be to modulate the hormone responsiveness of a subset of estrogen receptor target genes (16, 39).

Consensus ERREs are also present in the 5′-flanking regions of several genes involved in cellular metabolism, including the MCAD promoter element NRRE-1 (19), type 2 hexokinase (21), enolase (10), triosephosphate isomerase (3), lactate dehydrogenase (9, 32), and bifunctional enzyme (40). The MCAD NRRE-1 element has previously been shown to bind several members of the nuclear receptor superfamily, including the ligand-dependent activators RAR and PPAR (14, 24). Transgenic studies have shown that deletion of NRRE-1 in the context of the MCAD promoter blocks the physiologic postnatal induction of MCAD gene expression and significantly decreases basal MCAD expression in heart, brown adipose tissue, and kidney (7). The transcriptional effects of ERRα on the MCAD promoter were studied with transient transfection assays. While a constitutively active VP16-ERRα chimera efficiently induced transactivation mediated by a synthetic promoter containing a single copy of NRRE-1, ERRα neither activated nor repressed promoters containing this response element. In cotransfection studies, ERRα efficiently ablated the retinoic acid responsiveness of the MCAD gene, demonstrating that the receptor was expressed at levels adequate to alter NRRE-1 activity in vivo. Previous studies have demonstrated that transfection of hERRα represses (16, 35, 41) or does not alter (39) promoter activity. In addition, cotransfection of hERRα increased estrogen-dependent activa-

tion mediated by the lactoferrin promoter but not by a palindromic consensus ERE (39). A chimeric receptor containing the progesterone receptor DNA binding domain and the putative hERRα ligand binding domain efficiently activated progesterone response element-containing promoters in yeast and CV-1 cells (20). These data suggest that lack of ERRα transcriptional activity in our experiments may not be due solely to the lack of an appropriate ligand for the receptor. Rather, ERRα may play a role as a transcriptional modulator whose contribution to promoter activity could be determined both by the context of the ERRE within a complex hormone response element and by the potential interactions between ERRα and other nuclear hormone receptors allowed by a specific response element.

When previously studied by EMSA, brown adipose tissue was found to form two complexes with the MCAD NRRE-1 (7). A low-mobility complex (designated complex II) was present in all tissues regardless of their level of MCAD expression; this complex is supershifted with antibody directed against the COUP transcription factor. A higher-mobility complex (designated complex I) was present only in tissues which express high levels of MCAD. Although PPARs are abundantly expressed in brown adipocytes and had been thought to play a significant role in MCAD induction in this tissue, supershift experiments failed to detect these receptors in the high-mobility complex. Similarly, the composition of complex I could not be determined by supershift experiments performed with antibodies directed against a panel of orphan nuclear receptors (7). The results presented have clearly shown that ERRα forms part of this high-mobility complex. In addition, ERRα is highly expressed in tissues with a high β-oxidative capacity, suggesting that this receptor may play a role in determining the metabolic potential of these tissues. In this context, it would be interesting to determine what physiologic signals are transduced by altered ERRα expression levels and whether ERRα activity can be modulated by a yet undiscovered ligand.

ACKNOWLEDGMENTS

We thank D. Kelly, B. Spiegelman, T. Look, A. Joyner, and E. Silva for cell lines and reagents; Annie Matthyssen and Savita Amin for technical assistance; and Rogerio Rabelo, Alain Nepveu, and members of the Giguère laboratory for helpful discussions.

V.G. is a Medical Research Council of Canada (MRC) Scientist, and R.S. was supported by an National Cancer Institute of Canada Terry Fox Clinician-Scientist Fellowship. This work was supported by grants from the MRC.

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