A Role for Estrogen-related Receptor α in the Control of Mitochondrial Fatty Acid β-Oxidation during Brown Adipocyte Differentiation*

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Little is known about the factors involved in the brown adipocyte gene regulatory program. In contrast to the white adipocyte, the brown adipocyte is characterized by abundant mitochondria and high expression of mitochondrial fatty acid β-oxidation enzymes. Previous studies in transgenic mice have shown that the brown adipose-enriched expression of a key β-oxidation enzyme, medium chain acyl-coenzyme A dehydrogenase (MCAD), requires cis-acting elements located within the proximal promoter region of the MCAD gene. The levels of mRNA encoding MCAD and several other β-oxidation cycle enzymes were coordinately induced during differentiation of brown adipocytes in culture. Expression of transgenes comprised of MCAD gene promoter fragments fused to chloramphenicol acetyletransferase reporters in differentiating brown adipocytes revealed that a known nuclear receptor response element (NRRE-1) was required for the transcriptional induction of the MCAD gene during brown adipocyte differentiation. Electrophoretic mobility shift assays and antibody recognition studies identified distinct brown adipocyte differentiation stage-specific, NRRE-1–protein complexes; the orphan nuclear receptors, chicken ovalbumin upstream promoter transcription factors I and II, were identified as major the NRRE-1 binding proteins in the pre-adipocyte, whereas the estrogen-related receptor α (ERRα) bound NRRE-1 in extracts prepared from differentiated brown adipocytes. DNA binding studies performed with a series of NRRE-1 mutant probes indicated that ERRα was capable of binding two distinct sites within NRRE-1, each of which conform to the known ERRα monomeric binding consensus. The expression of ERRα paralleled NRRE-1 binding activities and MCAD expression during brown adipocyte differentiation, cardiac development, and among a variety of adult mouse tissues. These results identify a new class of ERRα target genes and implicate ERRα and chicken ovalbumin upstream promoter transcription factor in the control of a pivotal metabolic pathway during brown adipocyte differentiation.

In contrast to the white adipocyte, which functions mainly as a lipid storage cell, the brown adipocyte is a specialized thermogenic cell characterized by the presence of the mitochondrial uncoupling protein (UCP).1 UCP functions to disrupt the proton gradient across the inner mitochondrial membrane resulting in the “uncoupling” of oxidative phosphorylation and dissipation of energy as heat. Evidence is emerging that in addition to thermogenesis, brown adipose tissue (BAT) plays a pivotal role in mammalian whole body energy homeostasis and control of body weight (1–4). Fatty acid β-oxidation provides the reducing equivalents for the mitochondrial uncoupling reaction. Thus, flux through the β-oxidation cycle is one important determinant of the rate of uncoupling of oxidative phosphorylation in a given tissue. The majority of cellular fatty acid oxidation occurs in the mitochondria via a four-step cycle catalyzed by nuclear-encoded enzymes (5). The expression of mitochondrial fatty acid oxidation enzymes is very high in BAT and other tissues with high oxidative energy demands, such as heart. Accordingly, in contrast to the white adipocyte, the brown adipocyte has abundant mitochondria and a high capacity for fatty acid oxidation.

Little is known about the brown adipocyte differentiation program or about the molecular regulatory factors that distinguish BAT from white adipose tissue. Recently, several factors involved in the general adipocyte program have been elucidated. The peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor transcription factor superfamily, plays a pivotal role in adipocyte differentiation. In the presence of activators of PPARγ, ectopic overexpression of this nuclear receptor has been shown to trigger the adipocyte program in a variety of cells, including non-adipocytes (6, 7). Members of the C/EBP family of transcription factors have also been shown to be critical components of the adipogenic differentiation program (8, 9). These factors, however, are common to the brown and white adipocyte and, thus, are unlikely to account for the biochemical and structural differences between the two cell types.

To understand the molecular regulatory mechanisms involved in the control of the mitochondrial β-oxidation pathway in BAT and as an initial step toward the elucidation of the factors involved in the brown adipocyte differentiation program, we have begun to dissect the regulatory pathway controlling expression of genes encoding mitochondrial fatty acid oxidation enzymes in BAT. To this end, we have focused on the nuclear gene encoding, medium chain acyl-coenzyme A dehydrogenase (MCAD) (2,3-oxidoreductase, EC 1.3.99.3), a pivotal

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* The abbreviations used are: UCP, uncoupling protein; MCAD, medium chain acyl-coenzyme A dehydrogenase; EMSA, electrophoretic mobility shift assays; NRRE, nuclear receptor response element; ERRα, estrogen-related receptor α; BAT, brown adipose tissue; PPARγ, peroxisome proliferator-activated receptor γ; RXR, retinoic X receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; CAT, chloramphenicol acetylttransferase.

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enzy me in the mitochondrial fatty acid β-oxidation cycle. These studies revealed that a complex nuclear receptor response element (NRRE-1), located in the proximal region of the human MCAD gene promoter, is required for high level transcription of a reporter gene in BAT and heart in adult transgenic mice (10). Studies performed in vitro have shown that NRRE-1 is a pleiotropic element capable of conferring transcriptional activation via several nuclear receptors including the retinoid X receptor (RXR) and PPARα (11) or repression by the orphan receptors chicken ovalbumin upstream promoter transcription factors I and II (COUP-TF I and II; Ref. 12). However, the endogenous nuclear receptors that interact with NRRE-1 in the brown adipocyte in vivo have not been identified.

In this report we demonstrate that NRRE-1 is necessary for the transcriptional induction of the MCAD gene during brown adipocyte differentiation in culture and describe the identification of two endogenous nuclear receptors that bind NRRE-1. NRRE-1 is shown to interact with COUP-TF I and II in the pre-brown adipocyte, whereas the orphan receptor estrogen-related receptor α or ERRα binds NRRE-1 in the differentiated brown adipocyte. Moreover, we show that the expression of ERRα during brown adipocyte differentiation and among mouse tissues parallels MCAD expression and the known tissue-specific differences in β-oxidation rates. These results implicate members of the nuclear receptor superfamily in a pivotal brown adipocyte metabolic gene regulatory program and identify a potential role for ERRα in the control of mitochondrial fatty acid oxidation.

MATERIALS AND METHODS

Primary Brown Adipocyte Isolation and Culture—Brown pre-adipocytes were isolated from the interscapular brown fat pads of 3–4-week-old C57Bl/6/tg SJL mice as described (13). In brief, the fat pads were removed and digested with collagenase. The digested tissue was sequentially passed through 250- and 60-μm nylon filters. The filtrate was centrifuged at 800 g to pellet the stromal vascular cell fraction. The cells were grown in Dulbecco’s modified Eagle’s medium/F-12 (Cellgro, Mediatech) plus 10% fetal calf serum. On day 3 of culture, one-half of the media was removed and replaced with differentiation medium composed of 20% insulin and 2% triiodothyronine. On day 9, the media was replaced with fresh differentiation medium. The cells were originally plated at a density such that confluence was reached by day 6. Using this approach, greater than 75% of the cells were differentiated by day 10. The cells were judged to be differentiated based on the appearance of multilocular lipid droplets and a marked increase in mitochondrial size and number as judged by light and electron microscopy.

Northern Blot Analysis—Total RNA was isolated from brown adipocytes in culture using the RNAzol (Tel-Test, Inc.) method. Northern blot analysis was performed as described (14) using cDNA probes labeled to high specific activity via the random-primer labeling technique. Probes used included a mouse MCAD cDNA (10), a rat long chain acyl-CoA dehydrogenase cDNA (a gift from Dr. Bryan Hainline, Indiana University), a DNA encoding human β subunit of 3-OH long chain acyl-CoA dehydrogenase, (a gift from Dr. Arnold Strauss, Washington University), and a PPARγ cDNA (a gift from Dr. Jeff Gimble, Oklahoma Medical Research Foundation). An 18 S rRNA probe signal was used as a control for loading amounts.

Transgenic Mice and CAT Assays—The MCADCAT-371 and MCADCATANRRE-1 transgenic mice have been described (10). CAT activity assays were performed with protein extracts prepared from primary brown adipocytes isolated from pooled litters of 21-day-old MCADCAT-371 (line 10–1) and MCADCATANRRE-1 mice (line 11–3) as described (10). In brief, cells were harvested at the indicated time points using a commercial lysis buffer per manufacturer’s (Promega) instructions. Protein concentrations of the lysates were determined by the Lowry method. Fifty micrograms of protein was used for each assay. CAT activity assays were performed using n-butylryl coenzyme A and [14C]chloramphenicol as substrates. Butyrylated [14C]chloramphenicol was separated from free chloramphenicol by xylene extraction. Labeled chloramphenicol was quantified by scintillation counting on a Beckman LS 6000 IC scintillation counter.

Electrophoretic Mobility Shift Assays (EMSA)—Preparation of crude nuclear protein extracts from cells and tissues and EMSA were performed as described (10, 15) using the double-stranded oligonucleotide probes shown in Fig. 5B. The SF-1 probe sense strand sequence is 5’-GAGTTTTCTAAGGTCTATGCTCAATTT-3’. The probes were incubated with 10 μg of total nuclear protein or 2 μl of in vitro transcribed/translated ERRα. In vitro transcription and translation was performed with a human ERRα cDNA template generously provided by Dr. Christine Teng (National Institute of Environmental Health Sciences). Double-stranded oligonucleotides were 5’-P-labeled by Klenow ‘fill-in’ of a 5’-GATC overhang. Recombinant human ERRα was produced using the TNT-coupled reticulocyte lysate system (Promega) per the manufacturer’s instructions. Competition EMSA experiments were performed by addition of a molar excess of unlabeled, unrelated, size-matched human ERRα cDNA and unlabeled NRRE-1 or NRRE-1 mutant oligonucleotides. Antibody supershift experiments were performed with polyclonal antibodies directed against human ERRα (a gift from Dr. Vincent Giugue, McGill University), COUP-TF I and II (MPS3) and COUP-TF 1 (MPS1) (gifts from Dr. M. G. Parker, Imperial Cancer Research Fund), and a mixture of monoclonal antibodies directed against RXRα, β, γ (a gift from Dr. Pierre Chambon, Institut National de la Santé et de la Recherche Médicale). Antibody supershift studies were carried out by incubating the protein extracts with preimmune serum or specific antibodies on ice for 15 min. The complete binding reaction mix including buffer, poly(dI:dC), and labeled probe were then added to the extract and sera and incubated at room temperature for 20 min before resolving on 5% non-denaturing polyacrylamide gel.

Western Blot Studies—Analysis was performed with 5 μg of total nuclear protein lysate prepared from pre- and post-brown adipocytes or mouse heart (embryonic day 20 and adult). For the tissue expression panel, 10 μg of whole cell extract was used. Tissue protein lysates were prepared by homogenization of the tissue in lysis buffer (10 mM Tris, 5 mM EGTA, 0.1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 0.125 mg of leupeptin, and 0.5% SDS) followed by removal of cellular debris by centrifugation at 15,000 × g. A modification of the protein immunoblot analysis by Burnett (16) was performed using the enhanced chemiluminescence detection system (Amersham Corp.). The anti-ERRα and anti-COUP-TF antibodies described above were used. The anti-MCAD antibody has been described (17).

RESULTS

The Expression of Nuclear Genes Encoding Mitochondrial Fatty Acid Oxidation Enzymes Is Induced during Brown Adipocyte Differentiation—To examine the expression of nuclear genes involved in mitochondrial fatty acid β-oxidation during brown adipocyte differentiation, levels of mRNAs encoding enzymes catalyzing the first (medium chain acyl-CoA dehydrogenase (MCAD) and long chain acyl-CoA dehydrogenase) and third (3-hydroxy long chain acyl-CoA dehydrogenase) steps of the mitochondrial β-oxidation cycle were delineated by RNA blot analysis. Expression of long chain fatty-acyl-CoA synthase, which catalyzes the thioesterification of free fatty acids following cellular import was also analyzed. For these experiments, primary pre-brown adipocytes were cultured following isolation from the interscapular brown fat pad of 3–4-week-old C57Bl/6/tg SJL mice. The pre-adipocytes were grown to confluence and induced to differentiate using an established protocol described previously (see Ref. 18 and “Materials and Methods”). Total RNA was isolated from subconfluent pre-adipocytes and differentiated brown adipocytes. The differentiated cells exhibited the morphological characteristics typical of brown adipocytes including the appearance of intracellular, multilocular lipid droplets and abundant, large mitochondria. The levels of mRNA encoding long chain fatty-acyl-CoA synthase and the β-oxidation cycle enzymes were markedly higher in the differentiated brown adipocytes compared with that of the pre-adipocytes (Fig. 1). Expression of PPARγ, a known marker for adipocyte differentiation (19), was also induced confirming that the adipocyte program was activated in this cell culture system.

Transcriptional Activation of the MCAD Gene during Brown Adipocyte Differentiation Requires a Nuclear Receptor Response Element—We have shown previously that a 560-base
pair human MCAD promoter fragment fused to a chloramphenicol acetyltransferase reporter (MCADCAT.371) is expressed at high levels in BAT in parallel with the endogenous MCAD gene in adult transgenic mice (10). In contrast, expression of a transgene (MCADCATΔNRRE-1) that differs only in a 54-base pair deletion of a region containing a known nuclear receptor response element (NRRE-1) is not BAT-enriched (10). To determine whether the induction of MCAD gene expression during brown adipocyte differentiation occurs at the level of transcription and to define the role of NRRE-1 in this regulation, transgene expression was characterized during differentiation of pre-brown adipocytes isolated from MCADCAT.371 and MCADCATΔNRRE-1 mice. Transgene expression was determined by measurement of CAT activity in lysates prepared from the cultured cells at different time points following a switch to differentiation media (see "Materials and Methods"). Day 0 represents the point at which the pre-adipocytes reached confluency. By day 4 greater than 75% of the cells exhibited the mature brown adipocyte phenotype as judged by the morphological characteristics described under "Materials and Methods" and gene markers shown in Fig. 1. CAT activity in the MCADCAT.371 cells increased markedly (12–13 fold) upon differentiation (Fig. 2). This dramatic induction of CAT activity corresponded with the appearance of immunodetectable endogenous MCAD protein (Fig. 2). In contrast, expression of the MCADCATΔNRRE-1 transgene, which lacks NRRE-1, was unchanged during differentiation of the cultured brown adipocytes (Fig. 2). These results indicate that the induction of MCAD gene expression during brown adipocyte differentiation occurs at the level of transcription and requires the nuclear receptor response element, NRRE-1.

Identification of Brown Adipocyte Differentiation Stage-specific NRRE-1 Binding Activities—Electrophoretic mobility shift assays (EMSA) were performed to begin to characterize the transcription factors involved in the induction of MCAD gene transcription during brown adipocyte differentiation. For these experiments, an NRRE-1 probe was incubated with nuclear protein extracts isolated from pre- and post-brown adipocytes. As shown in Fig. 3A, distinct NRRE-1 binding patterns were obtained with the two types of nuclear extracts. Two complexes (pI and pII) of similar but distinct mobilities formed with nuclear protein extracts prepared from pre-brown adipocytes. When the EMSA were performed with extracts prepared from the differentiated brown adipocytes, neither complex pI or pII was observed; rather two new NRRE-1-protein complexes formed (dI and dII; Fig. 3A). dI was a faint, low mobility complex, and complex dII was a prominent complex migrating slightly faster than pI and pII. We have shown previously that NRRE-1-protein complexes of identical mobilities to that of dI and dII also form with nuclear protein extracts prepared from adult mouse heart and the brown adipocyte cell line HIB-1B (Ref. 10 and data not shown). Competition experiments performed with a molar excess of specific (NRRE-1) or an unrelated, size-matched, double-stranded oligonucleotide confirmed that all four complexes represented specific NRRE-1/protein interactions (Fig. 3B). These data identify brown adipocyte differentiation stage-specific NRRE-1 binding activities and suggest that distinct transcription factors interact with NRRE-1 during different stages of brown adipocyte differentiation.

The Orphan Nuclear Receptors, COUP-TF and ERRs, Bind NRRE-1 in a Brown Adipocyte Differentiation Stage-specific Manner—Antibody recognition studies were performed to identify the proteins bound to NRRE-1 in the complexes shown Fig. 3. A panel of antibodies raised against various members of the nuclear receptor superfamily were used in these studies. The initial studies focused on the protein-DNA complexes (pI and pII) formed with the pre-adipocyte nuclear protein extracts. Antibodies to RXRα, PPARα, PPARγ, thyroid receptor α, and the orphan receptor, ERRα, did not recognize proteins in complex pI or pII (Fig. 4 and data not shown). However, an anti-COUP-TF antibody that recognizes both COUP-TF I and II (MP33; Ref. 20) abolished the formation of NRRE-1 complexes (Fig. 4 and data not shown). Addition of a COUP-TF II-specific antibody (MP31) prevented the formation of complex pI but not complex pII. Accordingly, complex pI and pII contain...
COUP-TF I and II or closely related proteins. These data are consistent with the results of our previous characterization of NRRE-1 demonstrating that COUP-TF I, overproduced in bacteria, binds NRRE-1 (12). We have also shown that overexpression of COUP-TF I in mammalian cell lines in culture represses transcription of an MCAD promoter-reporter construct via NRRE-1 (12).

The antibody EMSA studies were repeated with nuclear protein extracts prepared from differentiated brown adipocytes. Initial studies focused on the prominent NRRE-1-protein complex dII shown in Fig. 3. Previous EMSA and cell cotransfection studies have demonstrated that RXRα/PPARα heterodimers bind NRRE-1 to activate transcription from heterologous and homologous promoters (11). These results together with the known role of PPARγ in the adipocyte differentiation program suggested that complex dII may contain PPAR/RXR heterodimers bound to NRRE-1. Surprisingly, however, complex dII was not recognized by anti-RXRα, anti-PPARα, or anti-PPARγ antibodies (data not shown). The anti-COUP antibodies, MP31 and MP33, also failed to influence the formation or mobility of complex dII (Fig. 4, lane 8). However, an antibody to the orphan nuclear receptor ERRα abolished the formation of complex dII (Fig. 4, lane 9). Taken together with the results shown above, these data strongly suggest that the known transcriptional repressors COUP-TF I and II bind NRRE-1 to silence MCAD gene expression in the pre-brown adipocyte but that during brown adipocyte differentiation the orphan receptor ERRα becomes the predominant NRRE-1 binding protein.

Regarding the faint complex dI, none of the antibodies used above, including the anti-COUP-TF antibodies, affected its formation or mobility (data not shown). However, we have shown previously that NRRE-1-protein complexes with mobilities identical to that of dI formed with nuclear extracts prepared from adult mouse heart or H1B-1B cells were recognized by a separate anti-COUP-TF antiserum raised to COUP-TF I purified from HeLa cells (21). This antibody, which also prevented the formation of complex dI (data not shown), has been shown to recognize at least two other higher molecular weight proteins in addition to COUP-TF I (21). Thus, we conclude that complex dI does not contain COUP-TF I or COUP-TF II but may contain a structurally related protein.

The MCAD ERRα Recognition Site Requires Two Receptor Binding Half-site Sequences Arranged as an Imperfect Everted Repeat—To confirm that ERRα binds NRRE-1, EMSA were performed with recombinant ERRα protein produced in an in
NRRE-1 is a novel, pleiotropic element composed of three potential nuclear receptor binding half-site sequences (shown in Fig. 5B). We have shown previously (12) that the unique arrangement of the hexameric receptor binding half-sites within NRRE-1 dictates three potential receptor dimer binding elements; an everted imperfect repeat separated by 13 bases (ER-13, sites 1 and 3), an ER-8 (sites 1 and 2), and an imperfect direct repeat (DR-O, sites 2 and 3). To define the ERR binding site requirements within NRRE-1, EMSA competition studies were performed. Recombinant ERR protein was used for these studies. As expected, a molar excess of unlabeled NRRE-1 prevented formation of the NRRE-1-ERR complex, whereas an identical molar excess of a nonspecific, size-matched probe had no effect on its formation (Fig. 5C, lanes 1–3). Previous studies by others (22) have demonstrated that ERRs bind with high affinity to the extended half-site sequence, 5′-TCAAGGTCA-3′, present in the steroidogenic factor 1 (SF-1) consensus binding site. A probe containing the SF-1 site was also efficient at preventing the ERR-NRRE-1 complex formation (Fig. 5B, lane 4). To define the NRRE-1 binding sites necessary for ERR binding, competition studies were performed with mutated NRRE-1 oligonucleotide fragments containing G to C substitutions at the invariant second position within each of the three potential receptor binding half-sites (sequences are shown in Fig. 5B). Although the NRRE-1 site 2 mutant (M2) was capable of competing with NRRE-1, the site 1 (M1) and site 3 (M3) mutants did not prevent complex formation (Fig. 5C, lanes 5–7). These results suggest that ERR interacts with sites 1 and 3 within NRRE-1. Finally, to determine whether NRRE-1 sites 1 and 3 were required for the interaction of NRRE-1 with endogenous ERR, nuclear protein extracts prepared from differentiated brown adipocytes were incubated with radiolabeled NRRE-1, M1, or M3 probes. The ERR-NRRE-1 complex did not form with the M1 probe and was markedly diminished with the M3 probe (Fig. 5A, lanes 12-14) Taken together, these data indicate that ERRs bind NRRE-1 sites 1 and 3, possibly as a dimer. Of note, we have shown previously that recombinant COUP-TF I homodimers bind NRRE-1 at sites 1 and 3 (12) and that this complex co-migrates with complex pII (data not shown).

Expression of ERR Parallels Expression of the MCAD Gene during BAT Differentiation and among Murine Tissues—The results shown above implicate ERRα in the control of nuclear genes encoding MCAD and other mitochondrial fatty acid oxidation enzymes during brown adipocyte differentiation. To determine whether the nuclear expression of ERRα parallels its NRRE-1 binding activity and MCAD expression during differentiation of the brown adipocyte, Western blot studies were performed with nuclear protein extracts prepared from pre- and post-adipocytes. Steady-state nuclear levels of ERRα were markedly induced during the transition from pre- to post-

**Fig. 5. ERRα binds NRRE-1 at sites 1 and 3.** A, using EMSA, in vitro transcribed/translated ERRα (ERRα lysate) was tested for its ability to bind NRRE-1 and compared with the results with nuclear protein extracts (NE) prepared from BAT and unprogrammed reticulocyte lysate (Unprog. Lysate). ERRα antibody (ERR Ab) was added to lane 4 to confirm the presence of ERRα in the complex. B, sense-strand sequences of the oligonucleotide probes used in the EMSA shown in C. The location and relative orientation of the three potential hexameric receptor binding half-sites within NRRE-1 are denoted by the arrows. The single base pair substitutions in mutant probes M1, M2, and M3 are underlined. C, cross-competition (lanes 1–7) with 100-fold molar excess of NRRE-1 (Sp), unrelated (NS), a known ERRα recognition site (SF1), or NRRE-1 mutants (M1, M2, and M3). In lanes 8–10, 32P-labeled wild-type (wt) M1, and M3 probes were incubated with BAT nuclear protein extract prepared from differentiated brown adipocytes.
brown adipocyte (Fig. 6A). The induction of ERRα expression during brown adipocyte differentiation paralleled MCAD protein levels (Fig. 6A), the transcriptional activity of the MCAD-CAT.371 transgene (Fig. 2), and NRRE-1 binding activities (Fig. 3). In contrast, COUP-TF was expressed in a reciprocal pattern during adipocyte differentiation as predicted by NRRE-1 binding activity (Fig. 3) and the known role of COUP-TF as a transcriptional repressor of the MCAD gene (12).

ERRα and COUP-TF expression was also examined in the perinatal developing heart. The expression of mitochondrial fatty acid β-oxidation enzymes is known to be markedly induced in heart following birth as the chief myocardial energy substrate switches from glucose to fatty acids. Levels of ERRα were induced from the fetal (embryonic day 20) to adult mouse heart in parallel with MCAD protein levels, whereas COUP-TF expression followed a reciprocal pattern (Fig. 6A). These results implicate ERRα and COUP-TF in the differential transcriptional control of nuclear genes involved in mitochondrial β-oxidation during brown adipocyte differentiation and perinatal cardiac development.

The expression of ERRα was also delineated in a variety of adult mouse tissues with distinct capacities for mitochondrial fatty acid oxidation. The tissue expression pattern of ERRα was compared with that of MCAD. ERRα expression was greatest in tissues with high capacity for fatty acid oxidation and abundant expression of MCAD, such as BAT and heart (Fig. 6B). In contrast, ERRα expression was low in white adipose tissue, brain, and lung, tissues with low β-oxidation rates. These data suggest that in addition to the control of β-oxidation enzyme expression during adipocyte differentiation and perinatal cardiac development, ERRα plays a role in the expression of mitochondrial β-oxidation enzymes among adult mammalian tissues.

**DISCUSSION**

The molecular regulatory mechanisms involved in the commitment to separate brown and white adipocyte differentiation programs are presently unknown. One of the major biochemical differences between white and brown adipose tissue is the level of mitochondrial fatty acid β-oxidation. Although the white adipocyte is a storage depot for fatty acids, the brown adipocyte actively metabolizes fatty acids to produce reducing equivalents for the mitochondrial uncoupling reaction. Differentiation of the brown adipocyte leads to a marked increase in the expression of nuclear genes encoding mitochondrial fatty acid β-oxidation enzymes coincident with a dramatic increase in the size and number of mitochondria. We sought to define the transcriptional regulatory pathway responsible for high level expression of fatty acid oxidation enzyme genes in BAT as an initial step in the elucidation of the brown adipocyte gene regulatory program. In this report, we provide evidence for the role of two orphan nuclear receptors, COUP-TF and ERRα, in the transcriptional control of a pivotal β-oxidation enzyme gene during brown adipocyte differentiation.

The mitochondrial UCP, a distinctive and specific marker for the brown adipocyte, has been a major focus of investigation of BAT gene expression. Characterization of the UCP promoter has implicated several transcription factors that appear to interact in a complex manner to direct high level UCP gene expression. Among these factors are CREBP (23), RXR/TR (24), Ets1 (24), and RXR/PParγ (25). To our knowledge, none of these transcription factors exhibit BAT-enriched expression. Recently, we have identified a region of human MCAD gene promoter that is necessary for high level BAT expression in transgenic mice (10). The nuclear receptor response element, NRRE-1, is located within this MCAD gene promoter region. Previous studies performed in cell culture have shown that NRRE-1 is a pleiotropic nuclear receptor responsive element capable of conferring transcriptional activation by RXR/PParα heterodimers or repression by COUP-TF homodimers (11, 12). Surprisingly, we did not detect RXR or PPAR in the NRRE-1-protein complexes formed with crude nuclear protein extracts prepared from pre- or post-differentiated brown adipocytes. Rather, a switch in NRRE-1 binding from COUP-TF to ERRα was identified during the transition from pre- to post-differentiated brown adipocyte. In contrast to other factors shown previously to interact with the MCAD and UCP promoters, ERRα is expressed in a BAT-enriched pattern. Nuclear levels of ERRα increase markedly during brown adipocyte differentiation. Moreover, ERRα expression is significantly higher in BAT compared with white adipose tissue. Accordingly, we propose that ERRα is a candidate regulator of the brown adipocyte mitochondrial β-oxidation pathway.

Our observation that COUP-TF interacts with NRRE-1 in nuclear extracts prepared from the pre-brown adipocyte is consistent with its known role as a transcriptional repressor. We have shown previously that COUP-TF is a potent repressor of MCAD gene transcription and is capable of silencing retinoid-mediated transcriptional activation via NRRE-1 (12, 26). In this report we demonstrate that COUP-TF expression is highest in the pre-adipocyte, falling during differentiation. Taken together, these data strongly suggest that COUP-TF silences the expression of the MCAD gene in the pre-adipocyte.

ERRα was originally identified in a screen of a testis cDNA library with the estrogen receptor DNA binding domain (27). To date, few true targets for ERRα have been identified. ERRα response elements have been identified within the SV40 late promoter (28) and the human lactoferrin promoter (29). More recently, ERRα has been shown to activate transcription from the bone-specific osteopontin promoter in cell culture studies (30). The expression pattern of ERRα is consistent with a role for the receptor in bone development (30). Our ERRα expression studies are consistent with the NRRE-1 binding data and strongly suggest that genes involved in mitochondrial fatty acid oxidation are targets for ERRα. ERRα expression is induced during brown adipocyte differentiation and following birth in heart, in parallel with fatty acid oxidation rates and expression of β-oxidation enzymes (10, 14, 31). Furthermore,
the expression of ERRα among adult murine tissues parallels MCAD expression with abundant levels in tissues with high fatty acid utilization rates (BAT and heart) and low expression in less oxidative tissues such as white adipose and brain. The actual role of ERRs in the transcriptional control of MCAD and other β-oxidation enzymes in vivo remains unknown. We have shown previously in cell culture studies that MCAD gene transcription is activated by PPARα in the presence of fatty acids or inhibitors of carnitine palmitoyltransferase I, known ligands for this nuclear receptor (11, 32, 33). Our results do not exclude the possibility that PPAR is a regulator of MCAD gene expression under certain circumstances in vivo. It is also tempting to speculate that a metabolite ligand exists for ERR. The precise function of PPARα and ERRα in the expression of genes encoding MCAD and other mitochondrial β-oxidation enzymes in vivo will require further investigation.

All ERRα recognition sites identified to date are extended half-site sequences similar to the SF-1 response element, 5’-TCAAGGTCA-3’, to which ERRα binds as a monomer (22). Our results demonstrate that NRRE-1 binding sites 1 and 3 (Fig. 5B) are required for the NRRE-1/ERRα interaction. Indeed, the extended sequences of sites 1 (5’-GAAAGGTCA-3’) and 3 (5’-TAAAGGTGA-3’) are similar to the known ERRα binding consensus. In contrast, NRRE-1 site 2 (5’-TCCGGGTAA-3’), which is not required for ERRα binding, does not conform to the consensus. Our mutational EMSA studies indicate that both sites 1 and 3 are necessary for the NRRE-1/ERRα interaction. These results suggest that in contrast to previously defined ERRα targets, ERRα may bind NRRE-1 as a homodimer to the everted imperfect repeat comprised of sites 1 and 3. However, our data do not exclude the possibility that independent ERRα monomers bind NRRE-1 sites 1 and 3 in a cooperative manner.

In summary, we have demonstrated that the transcriptional induction of a nuclear gene encoding a key mitochondrial fatty acid β-oxidation cycle enzyme during brown adipocyte differentiation requires the pleiotropic nuclear receptor response element, NRRE-1. The orphan nuclear receptors, COUP-TF and ERRα, were shown to bind NRRE-1 in a brown adipocyte differentiation stage-specific manner. These results implicate ERRα in the brown adipocyte gene regulatory program and identify genes involved in mitochondrial fatty acid oxidation as potential ERRα targets.

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Note Added in Proof—During review of this manuscript, Sladek et al. (34) described the regulation of MCAD gene expression by ERRα.