Functional Relationship between MyoD and Peroxisome Proliferator-Activated Receptor-Dependent Regulatory Pathways in the Control of the Human Uncoupling Protein-3 Gene Transcription

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Uncoupling protein-3 (UCP3) gene is a member of the mitochondrial carrier superfamily preferentially expressed in skeletal muscle and up-regulated by fatty acids. Peroxisome proliferator-activated receptor (PPAR)α and PPARδ (also known as PPARβ) mediate human UCP3 gene regulation by fatty acids through a direct-repeat (DR-1) element in the promoter. DR-1 mutation renders UCP3 promoter unresponsive to PPAR ligand *in vitro* and consistently blocks gene induction by fatty acids *in vivo*. Although they act through separate sites in the promoter, MyoD and PPAR-dependent regulatory pathways are functionally connected: only in the presence of MyoD, does UCP3 become sensitive to PPAR ligand-dependent regulation. MyoD controls UCP3 promoter activity through a noncanonical Ebox site located in the proximal region, close to transcription initiation site. Moreover, acetylation processes play a crucial role in the control of UCP3 gene regulation. The coactivator p300 protein enhances PPAR ligand-mediated regulation whereas a mutant form devoid of histone acetylase activity blocks the response of the promoter to fatty acids. Conversely, histone deacetylase-1 blunts MyoD-dependent expression of the UCP3 promoter and reduces PPAR-dependent responsiveness. A mutated form of MyoD unable to be acetylated has a lower transactivation capacity on the human UCP3 promoter with respect to wild-type MyoD. It is concluded that MyoD and PPAR-dependent pathways mediate human UCP3 gene regulation and that acetylase activity elicited by coregulators is implicated in the functional interaction between these regulatory pathways. Therefore the convergence of MyoD and PPAR-dependent pathways provides a molecular mechanism for skeletal muscle specificity and fatty acid regulation of human UCP3 gene. (*Molecular Endocrinology* **17**: 1944–1958, 2003)

UNCOUPLING PROTEIN-3 (UCP3) is a member of the mitochondrial carrier superfamily, which shares 56% and 73% identity to UCP1 and UCP2, respectively (1, 2). UCP3 differs from the other two mainly because of its predominant expression in skeletal muscle in humans (3) and in skeletal muscle and brown fat in rodents, whereas UCP1 is present only in brown fat and UCP2 is almost ubiquitous. Several studies have shown that UCP3, when overexpressed in yeast or mammalian muscle cells, causes a reduction in the mitochondrial membrane potential that is consistent with a potential role as an uncoupler (4, 5). Thus, when UCP3 is overexpressed in skeletal muscle of transgenic mice, it increases metabolic rate, mitochondrial protein leak, and lipid oxidation (6). However, it is not clear whether this effect is a specific result of UCP3 activity or nonspecific owing to overexpression of a mitochondrial membrane protein (7). Moreover, mitochondria from mice with a targeted disruption of the UCP3 gene show reduced mitochondrial proton leak (8, 9) and increased efficiency in ATP production (10). However, no major impact in overall metabolism at the whole-body level is observed.

Accumulating evidences indicate that UCP3 contributes to lipid handling by the skeletal muscle rather than uncoupling mitochondrial oxidative phosphorylation. There is preferential fatty acid oxidation with respect to glucose in human myotubes overexpressing UCP3 (11) and a tendency to an impaired fatty acid oxidation in UCP3 knockout mice (12). In humans, a splice-site mutation in the UCP3 gene that prevents the synthesis of the full-length UCP3 protein resulted in impaired fat oxidation (13), although other studies did not confirm these findings (14). On the other hand, a single nucleotide (T to C) polymorphism in the 5′-proximal noncoding region of the human UCP3 gene has been associated with disturbances in fat accumulation in several human populations (15, 16).

Abbreviations: C/EBPα, CCAAT enhancer binding protein; ChIP, chromatin immunoprecipitation; DR, direct repeat; HAT, histone acetyltransferase; HDAC1, histone deacetylase 1; PPAR, peroxisome proliferator-activated receptor; PLB, passive lysis buffer; PPRE, PPAR-response element; p300, p300 protein; SDS, sodium dodecyl sulfate; TSA, trichostatin A; UCP, uncoupling protein.

The preferential expression of UCP3 in the skeletal muscle is of interest because this tissue is an important site of energy homeostasis in mammals. UCP3 gene transcription in skeletal muscle is highly regulated in relation to cell differentiation. In cultured muscle cells, either from myogenic cell lines or primary cultures of human myotubes, differentiated *in vitro* UCP3 mRNA expression is induced in association with differentiation although at much lower levels than in muscle *in vivo* (17). UCP3 gene transcription is highly sensitive to several metabolic and hormonal signals, especially those related to fatty acid availability. In rodents, situations in which circulating free fatty acid levels are up-regulated, such as starvation, postnatal development, exercise, and high-fat diets (18), are associated with up-regulation of UCP3 mRNA expression whereas, when free fatty acid levels decrease, as in lactation, UCP3 mRNA levels are low (19). In this sense, lipid infusion to human volunteers causes a significant up-regulation of the UCP3 gene (20). There is a strong interaction between myogenic and fatty acid-dependent regulation of UCP3 gene expression. This interaction is highlighted during mouse development when the UCP3 gene transcription is switched on specifically in skeletal muscle only after birth and under the stimulus of the initiation of suckling and the appearance of high levels of free fatty acids in the circulation (21).

Accumulating evidence indicates that fatty acids may act through peroxisome proliferator-activated receptors (PPARs) to induce UCP3 gene expression (21) although other events such as activation of AMP-activated protein kinase may also contribute to this regulation (22). Single injections of PPARα or PPARα + δ activators into mice increase UCP3 mRNA levels in skeletal muscle, especially in physiological situations in which basal levels of circulating free fatty acids are low (starved neonates, lactating dams) (19, 21). In contrast, the capacity of PPARγ activators to induce UCP3 gene expression *in vivo* depends on the physiological situation, thus suggesting an indirect mechanism of action (19, 23, 24). In cell cultures, PPARδ activation has been claimed to be the main inducer of UCP3 mRNA expression, probably due to the lack of expression of PPARα in myogenic cells differentiated in culture (25). However, the molecular mechanisms driving UCP3 gene transcription in skeletal muscle and in response to fatty acids have not been established.

In the present work, we report that fatty acids induce the UCP3 gene transcription through a PPAR-responsive site, we define a novel site for the action of MyoD, which is required for UCP3 promoter activity and PPAR-dependent responsiveness, and we propose a molecular mechanism for functional interaction of fatty acid- and MyoD-dependent activation based on the action of p300 protein (p300) coactivation and histone acetylation activity.

RESULTS

Fatty Acid Activation of the Human UCP3 Promoter through PPARα and PPARδ; MyoD Is Required for PPAR-Dependent Activation

To study the basis for fatty acid-dependent regulation of the muscle-specific UCP3 gene, transient transfection experiments were performed using a construct containing 1.5 kb of the 5′-region of the human UCP3 gene fused to luciferase and the L6 myogenic cell line. These cells do not express endogenous MyoD and can undergo myogenic differentiation, thanks to the redundant role of other myogenic factors such as Myf-5 (26). Basal expression of UCP3 is very low in L6 cells, even when differentiated, and they are highly responsive to transfected MyoD (17). As the human UCP3 promoter has been demonstrated to be highly sensitive to MyoD (17), the effects of fatty acids were determined in the presence of cotransfected MyoD. We also studied the influence of PPAR subtype cotransfection in UCP3 transcriptional regulation. In the absence of cotransfected MyoD, the human UCP3 promoter was not sensitive either to oleic acid or to other PPAR activators (Fig. 1A). MyoD cotransfection increased UCP3 promoter activity, but oleic acid alone had no significant effect. However, when either PPARα or PPARδ (but not PPARγ) was cotransfected, the UCP3 promoter was induced by oleic acid. Similar results were found for linoleic acid (not shown). Cells were then cotransfected with PPAR subtypes in the presence of MyoD and exposed to agonists with a range of PPAR-subtype specificities. Then, 10 μM Wy14,643, which is specific for PPARα activation, stimulated the UCP3 gene promoter activity only when PPARα was cotransfected. Bezafibrate, which activates both PPARα and PPARδ, induced the UCP3 promoter when either of the two receptors was cotransfected. Even in the presence of the specific PPARγ activator rosiglitazone and the cotransfected PPARγ receptor, the UCP3 promoter was not induced. To ensure that the lack of response to PPARγ-dependent activation was not due to impaired expression of the receptor, the three PPARs were transfected to L6 cells, and a specific antibody against each PPAR subtype was used to detect the protein. PPARγ as well as PPARα and -δ were highly expressed in the cells after transfection (Fig. 1B). Moreover, a construct containing the PPAR-response element (PPRE) from the apolipoprotein AII gene upstream from the basal thymidine kinase promoter was sensitive to ligand-dependent activation of the three PPAR subtypes, including PPARγ (Fig. 1C). Therefore, it is concluded that fatty acids can activate the UCP3 gene promoter through PPARα or PPARδ depending on MyoD. It is worth noting that the response to oleate through PPARα or PPARδ and the lack of responsiveness to PPARγ activation was similar when a construct containing a longer sequence of the 5′ region (-2903 hUCP3-Luc) was tested (data not shown).
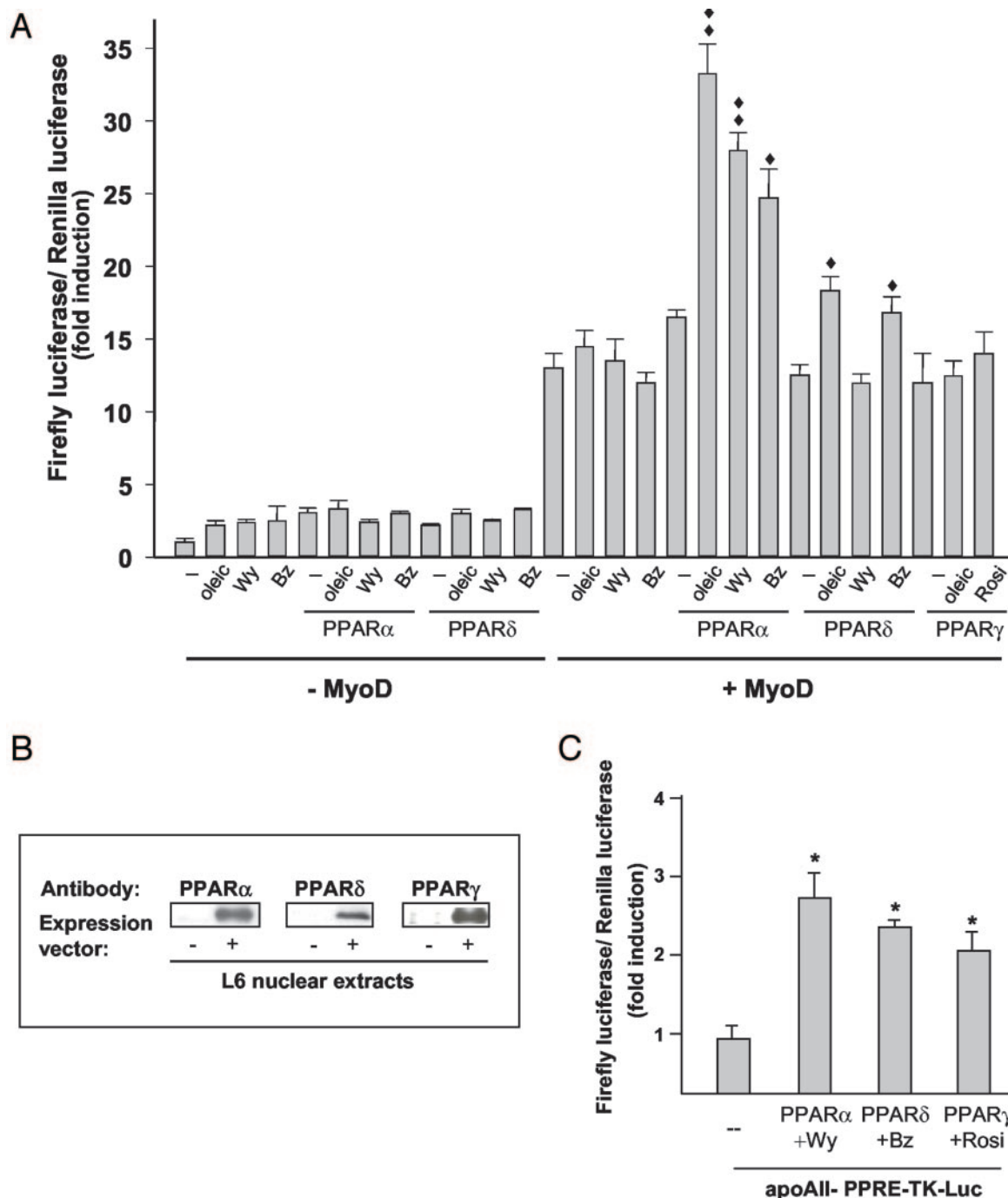
Fig. 1. Effects of Oleic Acid, PPAR Agonists, and MyoD on Human UCP3 Promoter Activity

A, L6 cells were cotransfected with −1588 hUCP3-Luc either with or without MyoD plus or minus PPARα, PPARδ, or PPARγ-expression vectors. When indicated, cells were treated for 24 h with 500 μM oleic acid (oleic), 10 μM Wy14,643 (Wy), 100 μM bezafibrate (Bz), or 10 μM Rosiglitazone (Rosi). Basal expression of −1588 hUCP3-Luc is set to 1. Results are the mean ± SEM of at least three independent experiments performed in triplicate. All samples cotransfected with MyoD-expression vectors show significantly higher differences than noncotransfected ones ($P < 0.001$). Significant differences due to addition of the agonists with respect to their relative controls are shown as ♦, $P < 0.05$; or ♦ ♦, $P < 0.01$. B, Western blot analysis of the expression of PPARs in L6 cells after transfection. PPARα, PPARδ, or PPARγ expression vectors were transfected when indicated as + and 20 μg of nuclear protein extracts were separated in a polyacrylamide gel as described in *Materials and Methods*. Specific antibodies against each PPAR subtype were used to detect the protein. C, Functional analysis of the overexpressed receptors. The plasmid containing three copies of the PPRE from mouse apolipoprotein AII gene upstream TK-Luciferase (250 ng) were cotransfected with 50 ng of the PPARα, -δ, or - γ expression vectors to L6 cells. Cells were treated as in panel A. Significant differences due to addition of the agonists (at doses indicated above) with respect to the control are shown as *, $P < 0.05$.

To characterize the effects of MyoD on PPAR-dependent activation on the UCP3 promoter, dose-response experiments were performed. Effects of different amounts of MyoD over the −1588 hUCP3-Luc construct and the influence on PPAR-dependent responsiveness were determined (Fig. 2). The UCP3 promoter showed a dose-dependent activation by MyoD and the cotransfection with 0.3 μg of PPARα per plate did not affect significantly the dose-response curve for MyoD. Although low amounts of MyoD were already permissive for PPARα ligand-mediated activation by Wy14,643, PPARα ligand-mediated activation of the UCP3 promoter increased with the amount of MyoD.

A Direct Repeat (DR)-1 Site in the Proximal Region of Human UCP3 Promoter Is Responsible for PPAR-Dependent Activation of the UCP3 Gene Promoter

Previous work in our laboratory described a DR-1 site at the position −71/−59 of the 5′-flanking region of the human UCP3 gene (Fig. 3A) as the element responsible for the transcriptional activation of the UCP3 promoter by retinoic acid (17). Because DR-1 elements may act as promiscuous sites of binding nuclear receptors, we investigated the role of this DR-1 on PPAR-dependent activation. Promoter constructs that contain point mutations in either one half-site or both half-sites of the DR-1 were cotransfected with MyoD and PPARα-expression vectors and treated with Wy14,643 (Fig. 3B). The sensitivity of −1588*mut1DR1*hUCP3-Luc and −165*mut1DR1*hUCP3-Luc (mutant constructs with one mutated half-site) to

Wy14,643 decreased by 70% when compared with the wild-type construct. Mutation of both half-sites of the DR-1 (−165*mutDR1*hUCP3-Luc), or complete deletion of the DR-1 (−60 hUCP3-Luc), caused even greater reduction of PPARα-dependent activation. The same was observed for PPARδ-mediated responsiveness to bezafibrate (data not shown). Therefore, it is concluded that this DR-1 at position −71/−59 mediates the response of the promoter to PPAR agonists.

The T-to-C polymorphism in the UCP3 gene is placed in the adjacent region (4 bp 3′ from the second half-site) to the DR-1 (see *asterisk* in Fig. 3A). A mutated version of our parental UCP3 promoter construct in which T was changed to C was designed and studied for PPAR-dependent responsiveness. There was no difference in the behavior of the UCP3 promoter in response to Wy14,643 in the presence of cotransfected PPARα. The same results were found for PPARδ-dependent activation (not shown).

EMSAs were performed to determine whether PPARα present in muscle nuclear extracts binds to the DR-1 sequence (Fig. 3C). As a labeled probe we used a oligonucleotide corresponding to the −79 to −50 region of the human UCP3 gene. After incubation of the labeled double-stranded probe with nuclear protein extracts, a characteristic pattern of DNA-protein binding complexes was formed. NS was a nonspecific band as determined by the lack of competition when incubated with a 100-fold excess of cold oligonucleotide. The antibody against PPARα prevented the formation of bands a and b, which otherwise were unaltered when the antibody against CCAAT enhancer binding protein (C/EBPα) (negative control) was used. Incubation of the extracts in the presence of 100 μM Wy14,643 did not strengthen the intensity of bands a and b (data not shown). In conclusion, both bands a and b are formed by protein-DNA complexes containing PPARα.

In Vivo Gene Transfer Indicates a Major Role for the DR-1 PPAR-Responsive Region of UCP3 Gene Promoter in Mediating Fatty Acid Responsiveness

To investigate the relevance of this DR-1 in the responsiveness to fatty acids *in vivo*, somatic gene transfer to skeletal muscle was performed as reported in *Materials and Methods*. The plasmid constructs depicted on the *left* of Fig. 4 (wild type, DR-1-mutated and empty plasmid) were analyzed. The experiments were performed in neonatal mice, a situation in which mouse UCP3 gene expression is known to be dramatically up-regulated due to the rise in circulating fatty acids as a consequence of milk intake (21). The injected promoterless plasmid (pGL3-basic) showed negligible luciferase activity. The wild-type construct showed a significant activity, and the point-mutated version in the DR-1 showed a dramatic reduction in activity with respect to the parental construct. These findings indicate that the DR-1 PPAR-responsive ele-
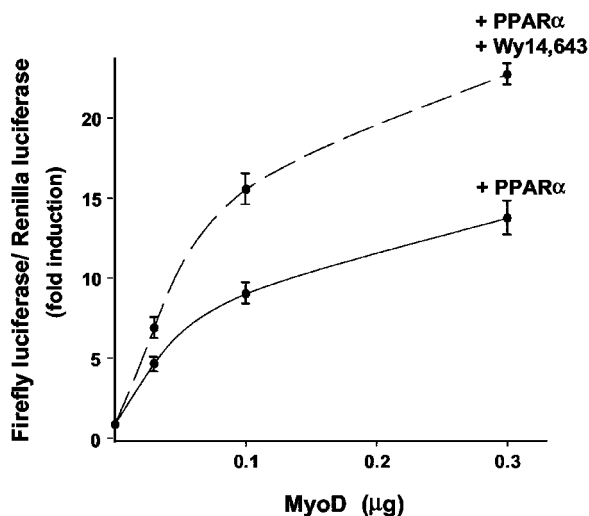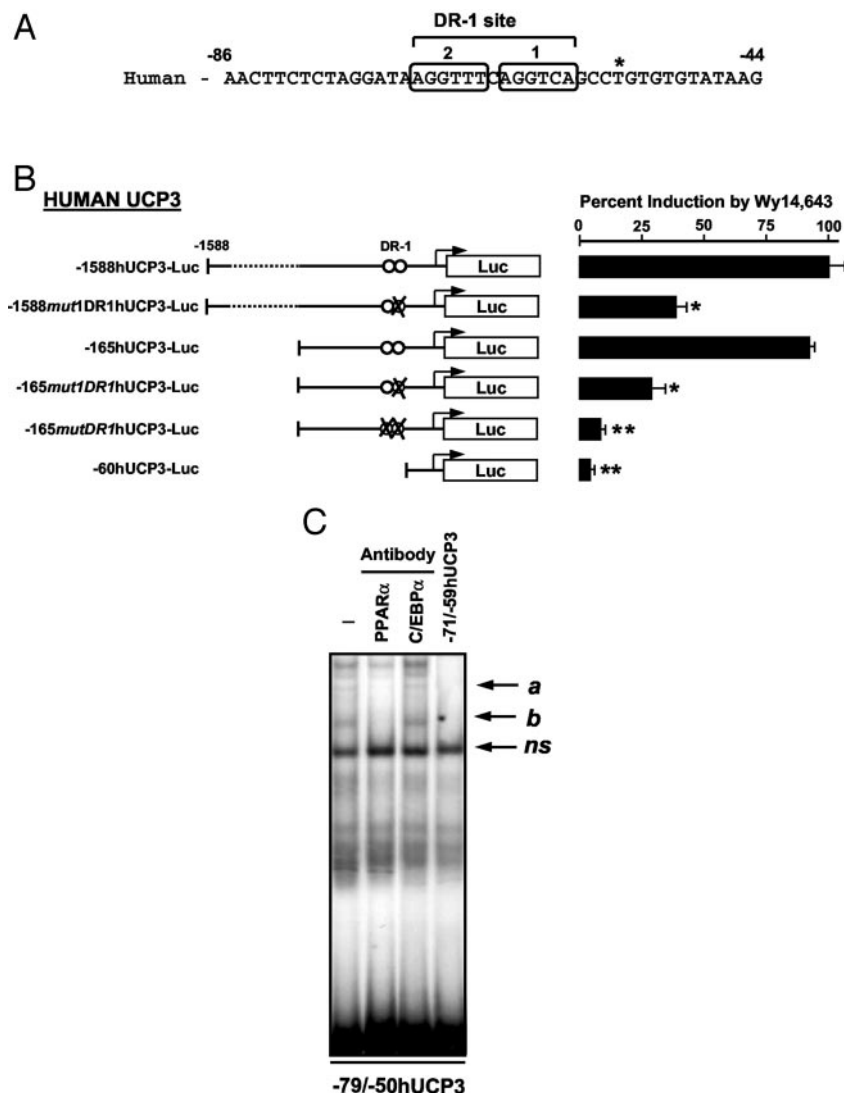
Fig. 2. Responsiveness of Human UCP3 Promoter to PPARα-Dependent Activation with Increasing Concentration of MyoD

−1588 hUCP3-Luc was cotransfected with MyoD and PPARα expression vector plus or minus 10 μM of Wy14,643 for 24 h. Basal expression of −1588 hUCP3-Luc is set to 1. Results are the mean ± SEM of three independent experiments done in triplicate.

Fig. 3. A DR-1 Site on UCP3 Gene Promoter Is Responsible for PPARα-Dependent Activation of the Human UCP3 Gene Promoter

A, Sequence of the proximal region of human UCP3 promoter. *Squares* show an alignment of half-sites for a DR-1. *Asterisk* indicates a potential change from T to C corresponding to the polymorphism described in the UCP3 promoter in the human (15, 16). B, Deletion and point mutation analysis of the human UCP3 promoter in response to the PPARα agonist Wy14,643 (10 μM) in the presence of cotransfected MyoD and PPARα expression vectors. Results represent means ± SEM of three to five independent experiments, and they are percentages with respect to the maximum effect achieved on the −1588 hUCP3-Luc (100%). C, EMSA to study the interaction of PPARα on the DR-1 at position −71 to −59. A double-stranded oligonucleotide corresponding to −79/−50 region of the human UCP3 promoter was used as a labeled probe. Nuclear protein extracts (5 μg) from mouse skeletal muscle were incubated as described in *Materials and Methods* and, when indicated, antibodies against PPARα and C/EBPα (negative control) were added. *a*, *b*, and *ns* denote the retarded complexes formed.

ment, in the proximal region of the human UCP3 promoter, is involved in fatty acid-dependent regulation *in vivo*.

MyoD Activates the Human UCP3 Promoter through a Proximal Region Next to a Transcription Initiation Site

To further characterize the molecular mechanisms responsible for transcriptional regulation of UCP3 gene promoter, we searched for elements mediating MyoD-dependent activation (Fig. 5). In agreement with previous findings (17), results obtained from constructs containing 1588 bp of the promoter (−1588 hUCP3-Luc) or 165 bp (−165 hUCP3-Luc) showed similar levels of induction when cotransfected with MyoD expression vector, thus indicating that the sequence responsible for MyoD-dependent activation is within −165 bp of the promoter. Computer-based analysis of the sequence from −165 to the first exon of human
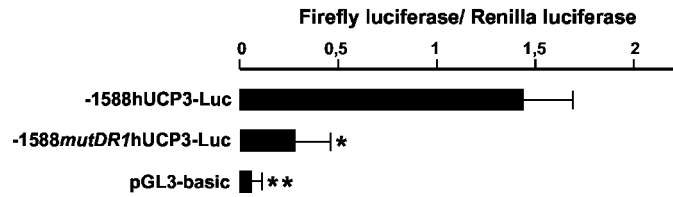
Fig. 4. Expression of UCP3 Promoter Constructs Transferred *in Vivo* to Skeletal Muscle of Neonatal Mice

Reporter gene constructs depicted on the *left* (100 μg) were combined with 10 μg of pRL-CMV and directly injected into hindlimb leg muscle of neonatal mice just after birth (before initiation of suckling). Pups were kept with their mothers and 72 h later muscles were collected and homogenized in PLB buffer and analyzed for Firefly and *Renilla* luciferase activities. Results are the mean ± SEM of four to six different mice. The significance is *, $P < 0.05$; **, $P < 0.01$.
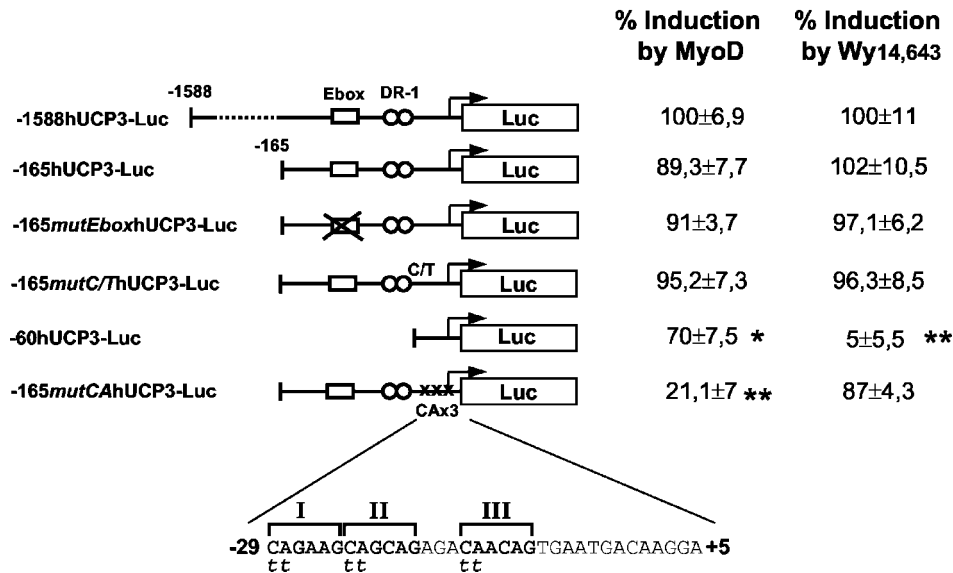

Fig. 5. MyoD Action on Human UCP3 Promoter

Transcriptional activity of mutant constructs in response to MyoD. Cells were transfected with the reporter gene constructs shown on the *left* and with MyoD-expression vector as described in *Materials and Methods*. Results are expressed as percentages with respect to the activation achieved with −1588 hUCP3-Luc and are the mean ± SEM of three to six different experiments. Statistical differences are shown as *, $P < 0.05$; and **, $P < 0.01$. The sequence represents the fragment from −29 to +5 human UCP3 promoter containing the three CAnnAG sites. The *tt* are the point mutations performed in the −165*mut*CAhUCP3-Luc.

UCP3 promoter for sequence elements potentially capable of binding MyoD showed a unique canonical Ebox (CANNTG), found at position −106 to −101. Mutation analysis of this Ebox site (−165*mutEbox*-hUCP3-Luc) demonstrated no reduction in luciferase activity in response to MyoD when compared with its control (−165 hUCP3-Luc).

Further deletions in the promoter indicated that the sequence responsible for the MyoD action was between −60 to +47 because −60 hUCP3-Luc retained most of MyoD-dependent responsiveness although it was completely insensitive to the PPAR-dependent pathway. Sequence analysis of the proximal region of the human UCP3 promoter indicated that the sequence surrounding the C-to-T polymorphism (*GCCTGT*GT) was similar to a recently reported noncanonical element in the human acetyl-CoA carboxylase β promoter (*GCCTGT*CA) mediating MyoD responsiveness (27). Only when the

T was present, did the sequence of the UCP3 promoter fit the MyoD-responsive element in the acetyl-CoA carboxylase β promoter. However, results indicated that the capacity of MyoD to transactivate the C or T versions of the UCP3 promoter did not show statistically significant differences (Fig. 5).

Further sequence analysis of the proximal UCP3 promoter region for potential sites of MyoD responsiveness was performed. This led to the identification, at position −29 to −9, close to the transcriptional start site, of two consecutive noncanonical Eboxes (CANNAG sequence) and an identical third box separated from the other two by three bases. Site-directed mutagenesis changed three CAs to TTs on the three Ebox-like sequences, and transcriptional induction by MyoD was assessed. Results with this mutant construct, −165*mut*CAhUCP3-Luc, showed 80% reduction when cotransfected with MyoD compared with its control (−165 hUCP3-Luc). This result indicates that

most of the effect of MyoD on the UCP3 gene promoter takes place through proximal noncanonical sites located close to the transcription initiation.

Binding of MyoD to the Proximal Region of the UCP3 Gene Promoter

The interaction of MyoD with the region of the UCP3 gene, which contains the three Ebox-like sequences, was established by EMSA (Fig. 6B). Three double-stranded DNA probes were generated from the human UCP3 promoter sequence (Fig. 6A). The first two are wild-type oligonucleotides of different sizes, and the third contains the three CAs changed to TTs (the same point-mutation tested functionally, Fig. 5). The −29/+5 hUCP3 labeled probe, which includes the three Ebox-like sites plus the surrounding sequences to transcription initiation, was incubated with L6 nuclear extracts, which are devoid of MyoD (28). This led to the appearance of two retarded complexes, *a1* and *a2*. The in-

cubation of this oligonucleotide with recombinant MyoD alone generated two complexes, *b1* and *b2,* of differing mobility. When MyoD was added to the incubation buffer together with the nuclear extracts *b1* and *b2,* complexes remained and a novel low-mobility band, indicated as *c*, appeared. It appears, therefore, that complex *c* is formed as a consequence of the interaction of nuclear proteins from L6 with MyoD upon this DNA region. When the shorter oligonucleotide −29/−9 hUCP3, in which the Ebox-like elements remain but sequences surrounding the transcription start site are absent, was incubated with L6 nuclear protein extracts, the retarded complexes *a1* and *a2* did not form. Therefore, bands *a1* and *a2* contain proteins that interact with the DNA sequence between −9 and +5. When MyoD alone was incubated, complexes *b1* and *b2* were formed, similarly to those observed with the full-length probe. However, in this case the incubation of MyoD plus nuclear extracts caused only the appearance of the *b1* and *b2* complexes, but *c* did not
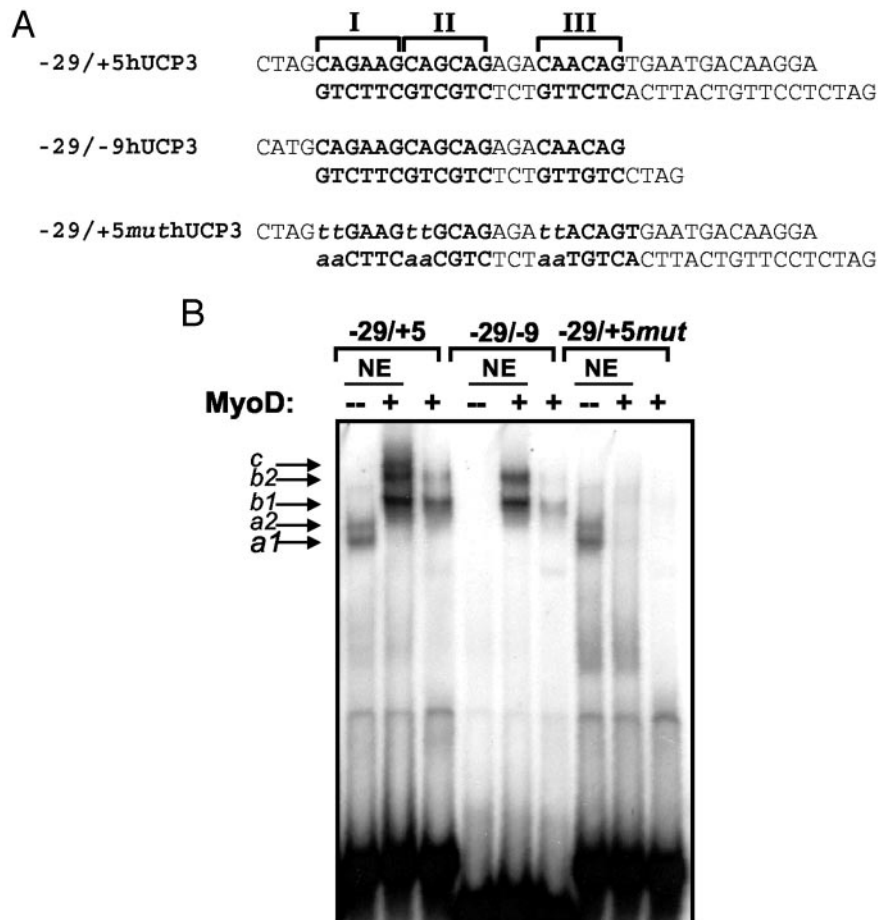

Fig. 6. MyoD Binds to Noncanonical Eboxes on Human UCP3 Promoter
A, Sequence of the oligonucleotides corresponding to the proximal region of the human UCP3 gene promoter used for EMSAs. I, II, and III indicate the noncanonical Eboxes present in the sequence. *Italic letters* represent the point mutations generated on the sequence. B, EMSA of MyoD binding to the proximal region of the hUCP3 promoter. L6 nuclear extracts (NE) and MyoD recombinant protein were incubated as described in *Materials and Methods* with the three double-stranded labeled probes shown in panel A. Different retarded bands are pointed out with *arrows*.

appear. When −29/+5*mut*hUCP3 was incubated with nuclear L6 protein extracts, retarded bands *a1* and *a2* were formed as with the non-Ebox-mutated version. No retarded bands *b1, b2,* or *c* were observed when MyoD was added to the incubation media either alone or in combination with extracts. These findings indicate that the Ebox-like sequences are required for MyoD binding to the human UCP3 promoter sequence, that other nuclear proteins bind sites around the transcription initiation site, and that they interact with MyoD when bound to DNA.

To further examine the interaction of MyoD with the proximal region of the human UCP3 gene promoter *in vivo*, chromatin immunoprecipitation (ChIP) experiments were performed using human skeletal muscle cells in culture (Fig. 7). Immunoprecipitation of protein-DNA complexes with the MyoD antibody caused a specific enrichment of the 191-bp PCR product corresponding to the −166 to +26 region of the endogenous hUCP3 gene. Transfection with the MyoD expression vector increased this effect (Fig. 7A), which is consistent with the expression of endogenous MyoD in human skeletal muscle cells and the increase in MyoD elicited by transfection with the MyoD expression vector (Fig. 7B). To establish the involvement of the three Ebox-like sequences in the MyoD binding *in vivo*, ChIP experiments were performed. The −165 hUCP3-Luc or −165 *mutCA*hUCP3-Luc constructs were cotransfected with MyoD expres-

sion vector in L6 cells (Fig. 7C). When cells were transfected with −165 hUCP3-Luc and immunoprecipitated with MyoD antibody, PCR amplification detected a specific enrichment in the fragment of DNA region containing the three noncanonical Eboxes. Conversely, when −165*mutCA*hUCP3-Luc was transfected, immunoprecipitation of that DNA fragment with MyoD antibody was not enriched compared with a similarly handled sample in the absence of this antibody. Identical results were found when −1588 hUCP3-Luc and −1588*mutCA*hUCP3-Luc (a version with the mutated Ebox-like sites) were compared (data not shown). These results confirm the interaction of MyoD with the Ebox-like elements in the proximal region of the human UCP3 promoter.

p300 Histone Acetylase Activity Enhances PPAR-Dependent Ligand Activation of the UCP3 Gene Promoter

Considering the strong functional interaction between PPAR- and MyoD-dependent pathways and the separate placement of their respective response elements in the UCP3 gene promoter, several coactivators, previously described to interact with nuclear receptors and/or MyoD, were checked for their capacity to influence MyoD and PPAR-dependent effects. When p300, P/CAF, steroid receptor coactivator-1, and Tat-
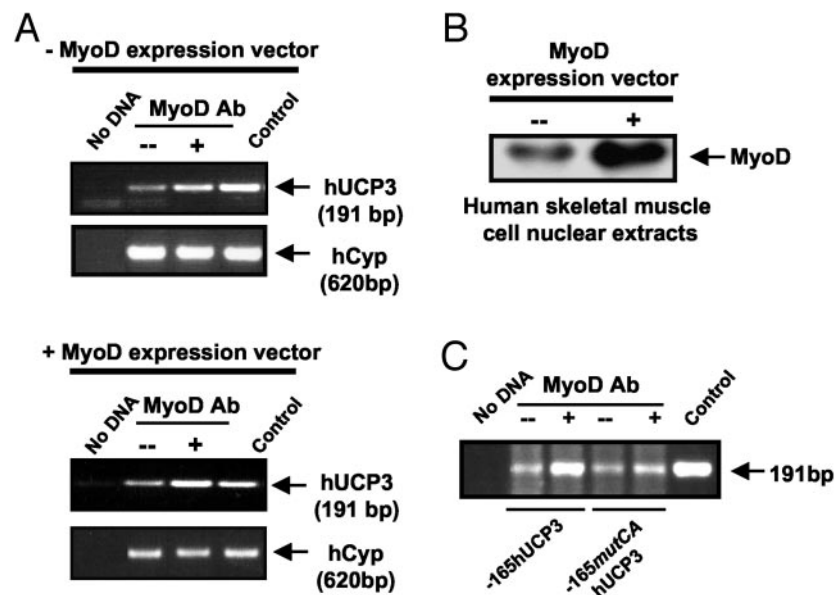

Fig. 7. ChIP Analysis of MyoD Binding to the Human UCP3 Promoter
   A, ChIP analysis of MyoD binding to the endogenous hUCP3 gene promoter in human skeletal muscle cells in culture. ChIP was performed in cells transfected (*bottom*) or not transfected (*top*) with MyoD expression vector. MyoD antibody (+) or an unrelated Ig (−) (20 μg) was used to immunoprecipitate the protein-DNA complexes (see *Materials and Methods*). *Arrows* indicate the 191-bp hUCP3 and 620-bp cyclophilin A (hCyp) PCR products. No DNA and Control represent negative and positive PCR controls, respectively. Results shown are representative of three independent experiments. B, Western blot analysis of MyoD protein expression in human skeletal muscle cells in cultures transfected or not transfected with the MyoD expression vector. Each lane corresponds to 15 μg nuclear extract protein. C, L6 cells were transfected with −165 hUCP3-Luc or with −165*mutCA*hUCP3-Luc always in the presence of MyoD expression vector. Immunoprecipitation and PCR amplification were performed as in panel A.

interactive protein-60 kDa expression vectors were cotransfected with human UCP3 promoter reporter constructs, only p300 caused a significant effect on the behavior of the UCP3 promoter: it did not modify the effects due to transactivation by MyoD, but it enhanced the ligand-dependent activation through

PPARα (Fig. 8A). p300 is a nuclear hormone receptor coactivator, and it has been shown to interact with PPARα (29) and to acetylate both histones and MyoD (30). To study the role of the acetylation activity of p300 on UCP3 promoter regulation, cotransfection experiments using a mutant form of p300 with no histone
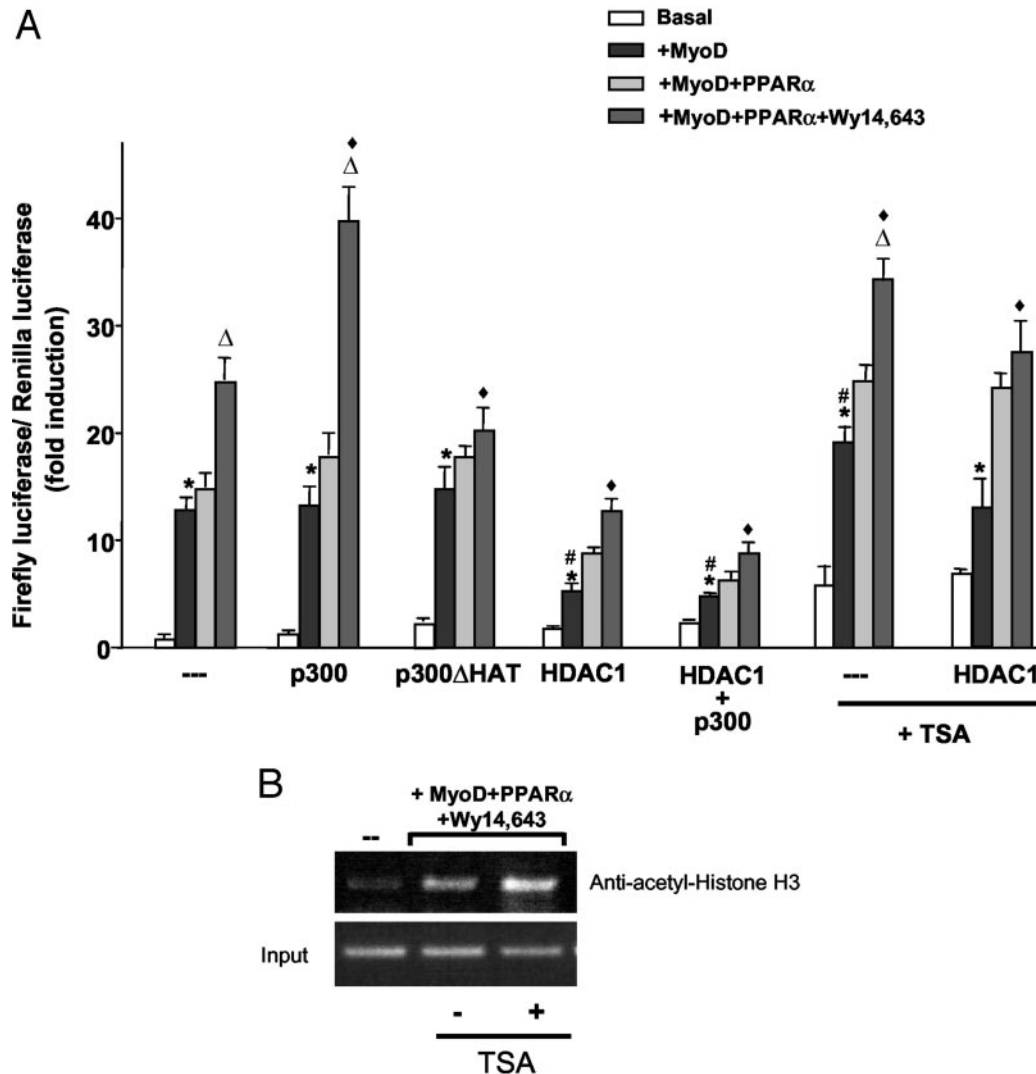

Fig. 8. Effects of p300 and Role of Acetylation Processes on the MyoD and PPAR-Ligand Dependent Activity of the hUCP3 Promoter

A, Effects of p300, p300ΔHAT and HDAC1 on the MyoD and PPAR-ligand dependent responsiveness of −1588 hUCP3-Luc. Cotransfection experiments were performed as described in *Materials and Methods* and when indicated 0.3 μg of p300, p300ΔHAT and/or HDAC-1 expression vectors per plate were included into the experiment. Basal expression of −1588 hUCP3-Luc is set to 1. When indicated 10 μM of Wy14,643 or 100 ng/ml TSA was added to the cells for 24 h Results show the mean ± SEM of at least three independent experiments done in triplicate. Statistical significance of comparison between basal levels and MyoD cotransfected cells is shown by *, $P < 0.001$. Comparison between cells treated with 10 μM Wy14,643 with respect to its nontreated control is shown by Δ, $P < 0.01$. Comparison of the effect of Wy14,643 or MyoD between different cotransfected groups is shown by ♦, $P < 0.01$; and #, $P < 0.01$, respectively. B, ChIP analysis of the histone H3 acetylation status at hUCP3 promoter upon activation with MyoD and PPARα. Chromatin cross-linking was performed using human skeletal muscle cells that were transfected or not with the MyoD and PPARα expression vectors and exposed to 10 μM Wy14,643 with (+) or without (−) 100 ng/ml TSA for 24 h. Immunoprecipitation was performed using a specific antibody for acetylated histone H3. PCR amplification product corresponds to the 191 bp of the hUCP3 gene promoter (see *Materials and Methods*). Input corresponds to the 191-bp product obtained by PCR of cleared chromatin before immunoprecipitation for each experimental point. Results shown are representative of three independent experiments.

acetylase activity (p300ΔHAT) were performed. As shown in Fig. 8A, p300ΔHAT did not significantly modify transactivation of the promoter by MyoD but completely blocked the induction by PPAR ligands, thus indicating that acetylation by p300 is crucial for PPAR-dependent activation of human UCP3.

Role of Acetylation Processes on the Control of Human UCP3 Gene Transcription

To further study the role of acetylation on the control of UCP3 gene transcription, cells were transfected with an expression vector driving histone deacetylase 1 (HDAC1), an enzyme recently implicated in silencing mammalian genes by deacetylation of histones as well as of MyoD (31, 32). HDAC1 inhibited the capacity of MyoD to induce UCP3 promoter activity and reduced the activity of the promoter in the presence of PPARα and its ligand (Fig. 8A). The positive effects of p300 on PPAR ligand-dependent activation of the UCP3 promoter were completely suppressed when HDAC1 expression vector was cotransfected. The effects of transfected HDAC1 were due to its deacetylase activity, as treatment of cells with trichostatin A (TSA), a histone deacetylase inhibitor, eliminated the repressive effects of HDAC1. It is concluded that histone acetylase activity participates in the MyoD- and PPAR-dependent regulation of human UCP3 promoter.

To check whether the acetylation status of histones in the proximal region of the endogenous hUCP3 gene promoter was affected by gene activation elicited by MyoD and the PPARα-dependent pathway, ChIP assays for acetylated histone H3 were performed using human skeletal muscle cells. As shown in Fig. 8B, cotransfection with MyoD and PPARα expression vectors in the presence of Wy14,643 enhanced the levels of acetylated histone H3 bound at the hUCP3 promoter. In addition, TSA treatment led to a further increase in the levels of acetylated histone H3 on the promoter.

MyoD Acetylation Is Necessary for the Full Activation of the Human UCP3 Promoter

Because p300 can acetylate not only histones but also MyoD and other transcription factors, we next tested whether the specific acetylation of MyoD was functionally relevant in the activity of the human UCP3 promoter. We performed transient transfections using a nonacetylable form of MyoD generated by direct mutagenesis from Lys-to-Arg substitutions at positions 99, 102, and 104 (MyoD-RRR), as reported previously (33). As shown in Fig. 9, the human UCP3 promoter activity achieved by MyoD-RRR cotransfection was 50% of that observed by wild-type MyoD. In the presence of MyoD-RRR, the activity elicited by PPARα plus Wy14,643 was lower than in the presence of MyoD, although sensitivity to ligand remained. This result indicates that acetylation of MyoD is necessary for the full activity of the promoter.
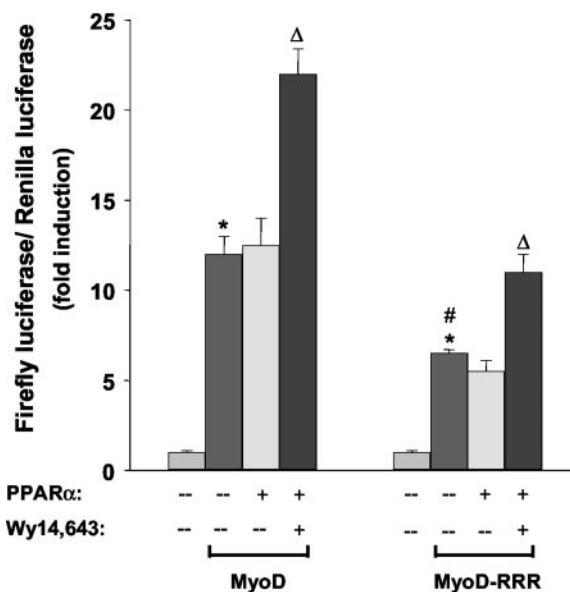

Fig. 9. Effects of Nonacetylable Form of MyoD on the Transactivation of UCP3 Promoter

−1588 hUCP3-Luc was transfected either alone (basal) or with PPARα expression vector (+PPAR) in the presence or absence of cotransfected MyoD and MyoD-RRR. When indicated 10 μM Wy14,643 was added to the cells for 24 h. Basal expression of −1588 hUCP3-Luc is set to 1. *Bars* indicate the mean ± SEM of three independent experiments done in triplicate. Significant differences between basal and MyoD-transfected cells are shown by *, $P < 0.001$. Comparison between PPAR transfected with and without treatment with 10 μM Wy14,643 is shown by Δ, $P < 0.05$; and comparison between cotransfection with MyoD-RRR with respect to MyoD by #, $P < 0.05$.

DISCUSSION

Major features of UCP3 gene regulation in humans are skeletal muscle specificity and fatty acid-dependent regulation. Here we show that the human UCP3 promoter is regulated by two mechanisms consistent with this behavior: the myogenic factor MyoD is required for substantial promoter activity, and fatty acids activate the promoter. PPARα or PPARδ are necessary to mediate fatty acid-dependent regulation. Moreover, there is a functional interaction between myogenic differentiation-dependent and fatty acid activation of the UCP3 gene, which is also evidenced by UCP3 promoter regulation: MyoD is required not only for a high UCP3 promoter activity but also for sensitivity to ligand-dependent activation of PPARs.

The sites mediating PPAR and MyoD-dependent activation were physically separated in the promoter. PPAR action occurs through a DR-1 element in the proximal promoter region that we previously reported to act also as a retinoic acid-responsive element (17). This site was critical for fatty acid induction of the promoter *in vivo*, as demonstrated here by the lack of responsiveness of point-mutated constructs of the promoter directly injected to the hind limb of newborn

mice under the stimulus of fatty acids coming from milk intake (21).

The structure of this element as a DR-1 is in agreement with the characteristic alignment of PPAR-responsive elements. The presence of this sequence in the UCP3 promoter was also described previously by Acin *et al.* (34). However, the role of this DR-1 in mediating retinoic acid-dependent effects, not only through activation of the retinoid X receptor moiety of the PPAR/retinoid X receptor heterodimer, but also through retinoic acid receptor (17), indicates that it behaves as a multihormonal-regulatory element in the human UCP3 gene and agrees with the DR-1 structure which, is highly permissive for multiple nuclear receptor-mediated responses.

The specific responsiveness of the UCP3 gene promoter to PPARα or PPARδ and their respective ligands, but the lack of sensitivity to PPARγ, is consistent with the responsiveness of the gene to physiological situations associated with increased circulating free fatty acids. A broad array of fatty acids of different lengths and saturations behave as natural activators of PPARα and PPARδ subtypes, but PPARγ is less sensitive to them (35). PPAR-response elements in mammalian genes are often highly promiscuous in response to the different PPAR subtypes, and their activation by the different PPAR subtype-dependent pathways is due to the action of specific ligands or to the expression of specific PPAR subtypes in target cells (35, 36). However, there are also several examples of partially PPAR subtype-specific responsiveness (37), such as the present findings on the human UCP3 gene promoter. Further research will be needed to assess whether the specific sequence of the UCP3 DR-1 dictates the differential responsiveness to PPARα and -δ subtypes with respect to PPARγ or this is due to the particular placement of the DR-1 in the context of the UCP3 promoter structure.

Although several consensus sequences (Eboxes) have been described as putative sites for MyoD binding within the human UCP3 promoter (34), our results demonstrate that MyoD did not act in the UCP3 gene promoter through canonical Ebox elements but through a proximal region, close to the transcription initiation site, which contains degenerate Ebox elements. A similar atypical site of action for MyoD through interaction with elements close to transcription initiation has been reported for other skeletal muscle-specific genes such as skeletal myosin heavy chain IIB or acetyl CoA carboxylase-β (27, 38). MyoD can interact with this region either alone or by forming complexes with other nuclear proteins in L6 cells, which are devoid of endogenous MyoD (28). Whereas degenerate Ebox elements are required for MyoD binding and MyoD-dependent transactivation, the presence of adjacent regions close to the transcription initiation site leads to the formation of ternary complexes also containing MyoD. Thus, the MyoD is likely to interact with proteins associated with basal transcription machinery. The location of MyoD binding close to transcription initiation and the interaction with other proteins

associated with this region are consistent with the high dependency of the human UCP3 promoter activity on MyoD.

Despite the separate location of PPAR and MyoD sites in the UCP3 promoter, the functional interaction between the two pathways of UCP3 promoter regulation was explored. When the effects of several coactivators known to interact with nuclear hormone receptors was determined, only p300 potentiated PPAR-ligand dependent activation. P300 has been reported to interact with and coactivate PPARα (29), and it also acetylates histones as well as MyoD, thus promoting MyoD transcriptional activity (39, 40). The dramatic silencing of PPAR-dependent activation mediated by the nonacetylating mutant form of p300 provides evidence for the involvement of histone acetylase activity driven by p300 in the regulation of human UCP3 promoter by PPAR.

The involvement of acetylation processes in UCP3 promoter regulation was confirmed by the repressing effects of cotransfection with an expression vector for HDAC-1, which has recently been reported to modulate transcriptional activity by deacetylation, not only of histones but also MyoD (31). Moreover, transcriptional activation of the hUCP3 gene by MyoD and PPARα was associated with increased acetylation status of histones bound to the promoter. These findings suggest that the transcriptionally active complex formed when PPAR interacts with ligands in the presence of MyoD involves p300 as a bridging molecule and that acetylation of histones and MyoD are important events to provide active transcription and ligand-dependent sensitivity to the system. The direct effect of MyoD on the promoter without adding any further acetylating coactivator is also compatible with this proposal since most of MyoD is known to be acetylated when transfected to myoblastic cells (33). The specific role of MyoD acetylation in the UCP3 promoter activity was evidenced by the reduced transactivation capacity of a nonacetylable mutant form of MyoD. The finding that the nonacetylable form of MyoD remains partially permissive to PPAR-dependent activation can indicate either that other factors or histones are acetylated by p300 or that MyoD retains some activity and still binds to the same or other transcription factors despite the mutation.

In summary, we propose a tentative model for the transcriptional control of the UCP3 gene by MyoD and PPAR-dependent pathways (see Fig. 10). Coactivators such as p300 may provide a bridging mechanism between PPAR-dependent activation and the basal transcription machinery, which includes MyoD. Acetylation elicited by p300 or other acetylases, on MyoD and histones, would be associated with the transcriptionally active status of the gene, both in basal conditions and especially in response to PPAR-dependent activation. Present findings suggest that these mechanisms provide the molecular basis for the transcriptional regulation of the UCP3 gene by fatty acids in human skeletal muscle.
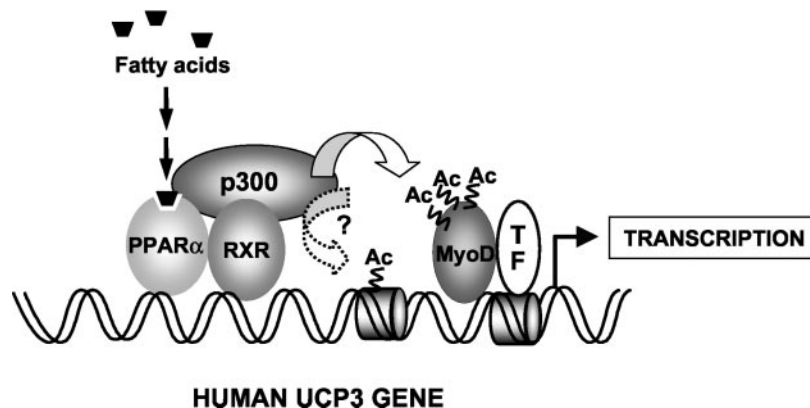
Fig. 10. Schematic Representation of the Possible Mechanism for the Regulation of Human UCP3 Gene by MyoD and PPAR
   A schematic overview of the major events in the regulation of the human UCP3 promoter is proposed on the basis of the present findings.

MATERIALS AND METHODS

Materials

Oleic and linoleic acids, Wy14,643 (pirinixic acid), bezafibrate, and trichostatin A were obtained from Sigma (St. Louis, MO). Rosiglitazone was a kind gift from Dr. L. Casteilla (University of Toulouse, Toulouse, France). [α-^{32}P]dCTP was from Amersham Pharmacia Biotech (Arlington Heights, IL).

Construction of Transfection Plasmids

The insertion of the fragment from −2903 to +47 of the human UCP3 gene into pGL3-basic generates −2903 hUCP3-Luc and a *Sac*I restriction, and religation generates the −1588 hUCP3-Luc construct, according to a previous report (17). A further deletion was generated from this last construct by restriction with *Sac*I and *Bgl*II, blunt ending, and religation, thus generating −165 hUCP3-Luc.
   Point mutation constructs were generated using a QuikChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA). In summary, two complementary oligonucleotides containing the desired mutations, flanked by unmodified nucleotide sequence, were synthesized. Of each oligonucleotide, 125 ng and 50 ng of double-stranded −165 hUCP3-Luc were incubated with 2.5 U of *Pfu*Turbo DNA polymerase. PCR was done for 17 cycles at 95 C for 30 sec, 55 C for 1 min, and 68 C for 14 min. Then, 10 U of *Dpn*I restriction enzyme were added to each PCR and incubated at 37 C for at least 1 h to digest parental DNA. One microliter of the *Dpn*I-treated DNA was then transformed. To generate point mutations, the oligonucleotides used were: for the Ebox mutation (−165*mutEbox*hUCP3-Luc) 5′-GCCAGCCTCTTGTC*cAtg*-GATCAGGCTGTC-3′ (introducing a *Nco*I restriction site); for mutation of one half-site of the DR-1 (−1588*mut*1DR1 hUCP3-Luc and −165*mut*1DR1 hUCP3-Luc) 5′-GGATAAGGTTTCA*taTg*AGCCCGTGTGTATAAG-ACCAGTGCC-3′ (introducing a *Nde*I restriction site); for the mutation of the other half-site of the DR-1 (−165*mut*DR1 hUCP3-Luc) 5′-CCAACTTCTCTAGGATAA*acg*TTCAGGT-CAGCCCGTGTG-3′ (introducing an *Acl*I restriction site); to study the presence of C instead of T according to the polymorphism described in the promoter, (−165*mutC/T*: 5′-GGTTTCAGGTCACC*c*GTGTGTATAAGACCAGTGC-3′; to mutate the three *CA* close to the transcription start site, 5′ CCAGTGCCAAGC*ttt*GCAGAG*att*ACAGTG-AATGACAAGGAGGG-3′ (introducing a *Hin*dIII restriction site). To generate pCMV-MyoD-RRR (change of Lys 99, 102,

and 104 to Arg) the oligo used was 5′-GGGCCTG-CA*g*GGCGTGCA*g*GCGCA*g*GACCACCAACG-3′. Point-mutated constructs were checked by direct DNA sequencing. The plasmid −60 hUCP3-Luc was generated from *mut*1DR1 hUCP3-Luc by digestion with *Kpn*I and *Nde*I, blunt-ending, and ligation.

Cell Culture and Transient Transfection Assays

Myoblastic L6 cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in DMEM containing 10% fetal bovine serum (FBS). Human skeletal muscle cells obtained from normal human tissue were purchased from Cambrex (Verviers, Belgium) and were grown in the culture medium provided by the supplier (Skeletal Muscle Cell Growth Medium Bullet Kit). Transfection experiments were carried out in L6 or human skeletal muscle cells at 50% confluence by using FuGene6 Transfection Reagent (Roche Diagnostics Applied Science, Barcelona, Spain) and were performed according to the manufacturer's instructions. For L6 transfection each point was assayed (unless indicated) in triplicate in a six-well plate and contained 1.5 μg of luciferase reporter vector, 0.3 μg of the mammalian expression vectors pCMV-MyoD (41), pSG5-PPARα, pSG5-PPARδ, pSG5-PPARγ (42–44), pCMV-p300 and pCMV-p300ΔHAT (45), pcM/HDAC-1 (31), pcx-P/CAF (46), Tat-interactive protein-60 kDa (47) and pcDNA3-SRC1 (48) expression vectors, and 3 ng of pRL-CMV (Promega Corp., Madison, WI), an expression vector for the sea pansy (*Renilla reniformis*) luciferase used as an internal transfection control. Cells were incubated for 48 h after transfection and, when indicated, were treated for 24 h before harvest with or without 10 μM Wy14,643, 100 μM bezafibrate, 10 μM Rosiglitazone, 500 μM oleic acid, and 500 μM linoleic acid. TSA treatment was performed at 100 ng/ml for 24 h. For assays of the activity of apoAII-PPRE-TKpGL3 (a gift of Dr. L. Fajas), the same conditions were used but including only 0.25 μg luciferase reporter vector.
   Firefly luciferase and *Renilla* luciferase activities were measured in a Turner Designs Luminometer (model TD20/20) using the Dual Luciferase Reporter assay system kit (Promega Corp.). Homogenates from cells were prepared with 500 μl of PLB (passive lysis buffer, Promega Corp.). Cells were lysed in agitation for 15 min and homogenate (20 μl) was used for measurement. Luciferase activity elicited by UCP3 promoter constructs was normalized for variation in transfection efficiency using *Renilla* luciferase as an internal standard.

Western Blot Analysis

Nuclear extracts were obtained as described (49). Protein (20 μg) was mixed with 2× sodium dodecyl sulfate (SDS) loading buffer, incubated at 90 C for 5 min, and subjected to SDS-PAGE (12% wt/vol). Proteins were transferred to polyvinylidine difluoride membranes and immunological detection was performed with specific antibodies against PPARα, PPARδ, or PPARγ (Santa Cruz Biotechnology, Inc; no. sc-9000; no. sc-1987; and no. sc-7196, respectively). Detection was achieved by the enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech).

Somatic Gene Transfer via im DNA Injections

Direct injection of plasmid DNA to neonatal hind limb muscles was performed essentially as reported previously (50). All plasmids were purified using QIAGEN (Chatsworth, CA) columns and resuspended in NaCl (0.9% vol/vol). DNA concentrations were quantified by spectrophotometry at 260 nm. Each firefly luciferase reporter plasmid (100 μg) was mixed with 10 μg of pRL-CMV and directly injected into the hind limb leg muscle of neonatal Swiss mice before initiation of suckling. Wild-type construct ($-$1588 hUCP3-Luc) was injected to one hind limb and the mutated version ($-$1588*mut*DR1 hUCP3-Luc) to the other. Neonates were kept with their mothers for 72 h and total muscle from every hind limb was collected and homogenized with a Polytron in 500 μl of PLB buffer and analyzed for firefly and *Renilla* luciferase activity as described previously.

EMSAs

Nuclear protein extracts from L6 cells and from Swiss mice gastrocnemius muscle were isolated as reported previously (51, 52), and protein concentration was determined by the micromethod of Bio-Rad Laboratories, Inc. (Richmond, CA) using BSA as standard. For gel retardation assays, the double-stranded UCP3-DR1 oligonucleotide corresponding to the $-$79 to $-$50 sequence of the UCP3 gene was end-labeled using [α^{32}P]dCTP and Klenow enzyme. The DNA probe (25,000 cpm) was incubated for 30 min at 25 C with 5 μg of nuclear protein extract. Reactions were carried out in a final volume of 25 μl containing 20 mM HEPES (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 10% glycerol, and 2.5 μg of poly dI.dC (deoxyinosinic-deoxycytidylic acid). Samples were analyzed by electrophoresis at 4 C for 60–80 min in nondenaturing 5% polyacrylamide gels in 0.5× Tris-borate-EDTA. In the competition experiments, 100-fold molar excess of unlabeled double-stranded oligonucleotides was included in each respective binding reaction. When indicated 2 μl of antisera against PPARα (Santa Cruz no. sc-9000) or C/EBPα (Santa Cruz no. sc-9314) was added to the incubation media for 15 min. The reactions including recombinant MyoD (Santa Cruz, no. sc-4080) were carried out in 25 mM HEPES (pH 7.5), 0.5 mM dithiothreitol, 50 mM KCl, 5% glycerol, 12.5 μM ZnSO$_4$, 0.1% Nonidet P-40, 1 mg/ml BSA, 50 μg/ml poly dI.dC, and 300 ng MyoD.

ChIP Assay

Human skeletal muscle cells were transfected or not with MyoD expression vector. L6 cells were transfected with $-$165 hUCP3-Luc or $-$165*mutCA*hUCP3-Luc in the presence of MyoD expression vector. Protein-DNA cross-linking was achieved by adding formaldehyde (final concentration 1%) to the culture medium for 10 min at 37 C. The cells were washed twice with cold PBS containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A (protease inhibitors). Cells were then scraped in PBS, centrifuged, and resuspended in 0.2 ml cell lysis buffer [5 mM PIPES (KOH) (pH 8.0), 85 mM KCl, 0.5% (vol/vol) Nonidet P-40 plus the pro-

tease inhibitors]. Samples were incubated on ice for 10 min and centrifuged for 10 min at 4 C. Pellets were resuspended in 0.2 ml SDS buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), plus the protease inhibitors] and incubated on ice for 10 min. The samples were sonicated and centrifuged for 10 min at 4 C, and supernatants were diluted 10-fold in immunoprecipitation dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl plus the protease inhibitors]. To reduce nonspecific binding, samples were incubated at 4 C for 1 h, with 80 μl of protein G-sepharose slurry as 50% suspension Tris-EDTA, which was pretreated with salmon sperm DNA.

Precleared chromatin solutions were incubated overnight at 4 C with 20 μg of anti-MyoD antibody (Santa Cruz, no. sc-760) or an equal amount of an unrelated immunoglobulin (Santa Cruz no. sc-9314). The immunocomplex was then collected by binding to 60 μl protein G-sepharose slurry as before. After incubation for 1 h at 4 C, the sepharose beads were collected by centrifugation, subsequently washed with 1 ml low-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], high-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl], LiCl buffer [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)], and twice with Tris-EDTA. The complexes were eluted by two successive 15-min incubations with 250 μl of elution buffer (1% SDS, 0.1 M NaHCO$_3$) at room temperature. The pooled eluates were heated to 65 C for 4 h in the presence of 20 μl NaCl (5 M), to reverse the formaldehyde cross-links and then treated with proteinase K for 1 h at 45 C. After phenol-chloroform extraction, DNA was used for PCR analysis. ChIP analysis of acetyl-histone H3 was performed using the reagents (Acetyl-Histone H3 Immunoprecipitation, ChIP, Assay Kit) from Upstate Biotechnology, Inc. (Lake Placid, NY) and following the specific instruction from the supplier. Input sample corresponds to cleared chromatin before immunoprecipitation with the antiacetylated histone H3 antibody.

Primers for amplifying a 191-bp fragment encompassing the noncanonical Eboxes of the human UCP3 promoter were forward 5′-GATCTGGAACTCACTCACC-3′ and reverse 5′-CAGCAGGGATTGGATGGC-3′ and those amplifying a 620-bp fragment of the human cyclophilin A gene, used as a control, were forward 5′-GTGTACTATTAGCCATGGTCAA-CCC-3′ and reverse 5′-CTGCAGCGAGAGCACAAAGAT-TCT-3′. After 35 cycles of amplification, PCR products were electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis

Where appropriate, statistical analysis was performed by Mann-Whitney nonparametric *U* test; significance is indicated in the text.

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