

# The human uncoupling protein-3 gene promoter requires MyoD and is induced by retinoic acid in muscle cells<sup>1</sup>

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## SPECIFIC AIMS

The expression of the uncoupling protein-3 (UCP-3) gene in skeletal muscle is under a strict transcriptional regulation. However, the molecular mechanisms controlling the human UCP-3 gene promoter in relation to muscle cell-specific expression and hormonal regulation have not been established. Here we report that the UCP-3 gene is substantially expressed in muscle cells only when differentiated and that UCP-3 promoter activity is largely dependent on MyoD. A novel regulatory pathway for UCP-3 gene expression is established by the identification of retinoic acid (RA) as a major inducer of UCP-3 gene transcription and an RA-responsive element is identified in the proximal region of the human UCP-3 gene promoter.

## PRINCIPAL FINDINGS

### 1. Retinoic acid induces UCP-3 mRNA expression in differentiated C2C12 and L6E9 muscle cells

Northern blot analysis revealed that neither C2C12 nor L6E9 (mouse and rat muscle cell lines) expressed detectable levels of UCP-3 mRNA in the myoblastic stage. Nevertheless, when cells were differentiated and acquired a myotube phenotype, low but detectable levels of UCP-3 mRNA were present. When the cells were nondifferentiated, treatment with all-*trans* RA had no effect on UCP-3 expression. Nevertheless, treatment of myotubes with all-*trans* RA induced UCP-3 mRNA expression by 8- to 10-fold over basal levels in both cell types. When 9-*cis* RA was added to 4, day differentiated L6E9 cells induction of UCP-3 mRNA expression was about 50% of the induction observed with all-*trans* RA.

### 2. Retinoic acid activates the expression of the human UCP-3 gene promoter in L6E9 cells MyoD is required for the basal promoter activity and RA responsiveness

We have cloned and sequenced a 3 kb fragment of the human UCP-3 promoter and this was fused to lucif-

erase reporter gene. The promoter-reporter constructs -2903hUCP3-Luc and -1588hUCP3-Luc were used in transient transfection studies in L6E9 cells. Results with both constructs showed very low basal luciferase activity. To test the hypothesis that a myogenic factor could be involved in UCP-3 promoter regulation, we cotransfected the -2903hUCP3-Luc and -1588hUCP3-Luc with the MyoD expression plasmid, and a 7-fold and 10-fold induction of the activity over the basal values were obtained, respectively (Fig. 1).

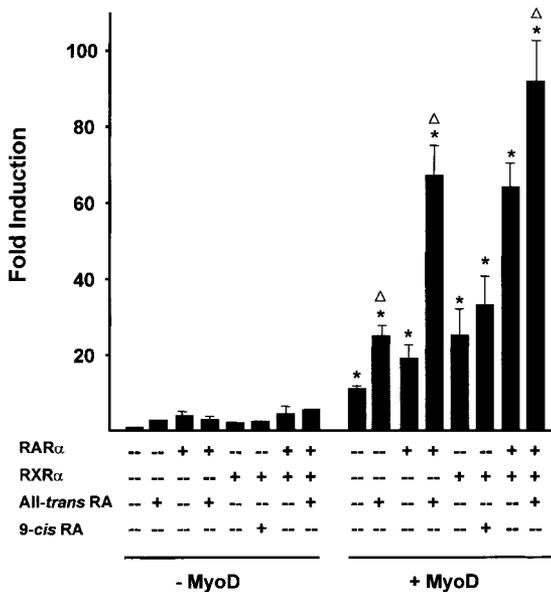
To analyze the role of retinoids in UCP-3 promoter activity, cotransfection experiments with expression vectors for RAR and RXR were performed. Results showed that when -1588hUCP3-Luc was transfected in the absence of cotransfected MyoD, no significant differences over the basal level were obtained either by cotransfection with RAR $\alpha$  or RXR $\alpha$  alone or together or by the addition of their agonists, all-*trans* RA or 9-*cis* RA. When MyoD was cotransfected, RA caused a significant induction of the promoter. Furthermore, when a RAR $\alpha$  expression vector was cotransfected, induction by all-*trans* RA was much higher (Fig. 1). In contrast, exposure to 9-*cis* RA in the presence of cotransfected RXR $\alpha$  had no significant effect on UCP-3 promoter activity. Experiments performed with both types of receptor together showed a higher induction due to cotransfection, which was significantly increased by exposure to all-*trans* RA.

### 3. Deletion and point mutation analysis of the human UCP-3 promoter reveals an RA-responsive element in the proximal promoter region

To search for regulatory elements responsible for the effects of RA on the hUCP3 promoter, a set of

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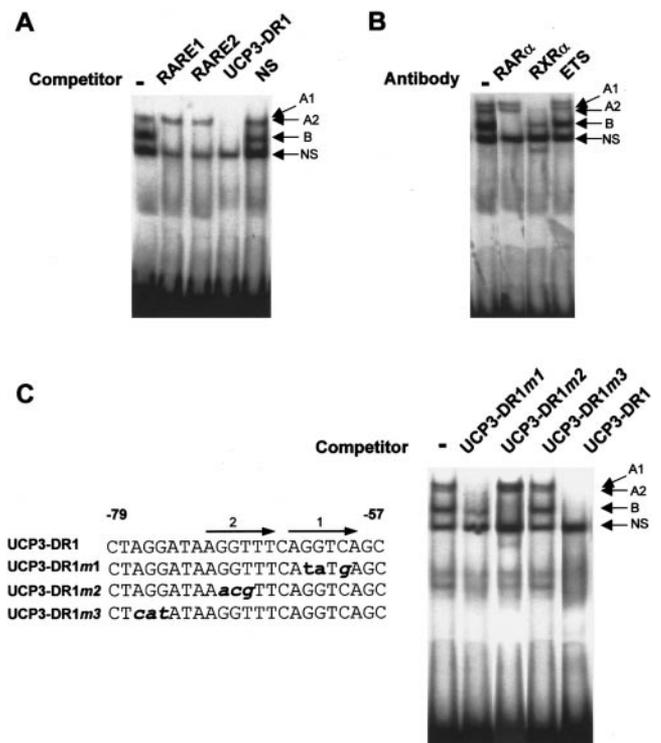
**Figure 1.** Expression of  $-1588\text{hUCP3-Luc}$  in L6E9 cells. Effects of MyoD, RAR or RXR and effects of RA. L6E9 cells were cotransfected with  $-1588\text{hUCP3-Luc}$  either with or without MyoD plus or minus RAR $\alpha$ - and RXR $\alpha$ -encoding expression vectors. Statistically significant differences ( $P < 0.05$ ) due to the cotransfection with MyoD are shown by an asterisk; those due to the presence of a ligand (all-*trans* RA or 9-*cis* RA) with respect to its control are shown by a  $\Delta$ .

deletion mutants was created. A major deletion that contained only the proximal 5' region of the gene,  $-165\text{hUCP3-Luc}$ , retained the same basal activity as the  $-1588\text{hUCP3-Luc}$  and was activated eightfold by MyoD. All-*trans* RA in the presence of cotransfected MyoD and RAR $\alpha$  induced 30-fold the activity of  $-165\text{hUCP3-Luc}$ . An additional deletion mutant,  $-61\text{hUCP3-Luc}$ , still responded to MyoD but no induction was observed after RA treatment, indicating that elements for RA responsiveness are present in the human UCP-3 promoter region between  $-165$  and  $-61$  and are distinct to those mediating MyoD-dependent activity. Point mutations in a proximal DR1-like element (see Fig. 2C) generated on the  $-165\text{hUCP3-Luc}$  or in the  $-1588\text{hUCP3-Luc}$  revealed that none of these mutations affect the ability of MyoD to activate the expression of the gene. In the presence of MyoD and RAR $\alpha$ , *mut1*UCP3-Luc lost around 80% of the induction by all-*trans* RA when compared to  $-165\text{hUCP3-Luc}$ , whereas *mut2*UCP3-Luc lost around 75%. Mutation of both sites in the same construct (*mut1/mut2*hUCP3-Luc) abolished the activation of the promoter by RA, demonstrating the involvement of these sites in the regulation of hUCP3 promoter by all-*trans* RA.

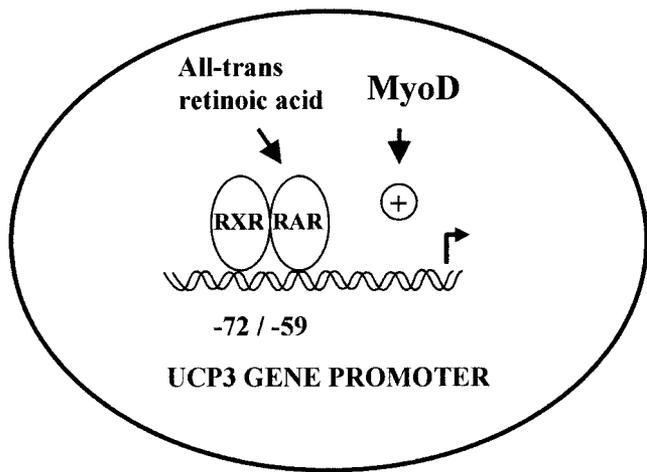
#### 4. A DR1-like region in the human UCP-3 gene binds RAR and RXR from L6E9 nuclear extracts

To search for the proteins that bind the RA-responsive DR1-like sequence, we performed electro-

phoretic mobility shift assays with L6E9 nuclei extracts. We used the UCP3-DR1 oligonucleotide, corresponding to the  $-79/-57$  region of the UCP-3 gene, as a labeled probe (Fig. 2). Four retarded bands appeared: A1, A2, B, and NS. Competition assays using the nonlabeled UCP3-DR1 probe revealed that the NS band is nonspecific and the other bands (A1, A2, and B) were specific. The B band disappeared when either DR5-RARE1 or DR2-RARE2 was added to the incubation buffer. An excess of an unlabeled unrelated oligonucleotide (NS) was used as a negative control and, as expected, no competition effect was detected (Fig. 2A). The presence of RAR $\alpha$  antibody in the incubation media prevented the formation of the retarded band B, and a partial reduction in the intensity of the band B was also observed with the RXR $\alpha$  antibody. In addition, the RXR $\alpha$  antibody prevented the formation of bands A1 and A2 whereas the ETS antibody had no



**Figure 2.** Electrophoretic mobility shift assays of the nuclear proteins that interact with the DR1-like element in the human UCP-3 gene. A double-stranded oligonucleotide corresponding to the  $-79/-57$  region of the hUCP3 gene (UCP3-DR1) was used as a labeled probe. A) Competitors were added at a 100-fold molar excess relative to probe concentration. RARE1 and RARE2 are double-stranded oligonucleotides corresponding to the DR5 RARE from the RAR $\beta$  gene and to the DR2 RARE from the mouse cellular retinol binding protein type I. NS is a GA-rich oligonucleotide used as a negative control. B) Effects of RAR $\alpha$ , RXR $\alpha$ , and ETS antibodies C) Competition analysis between the UCP3-DR1 and UCP3-DR1m1, UCP3-DR1m2 and UCP3-DR1m3 (right) and sequence of the UCP3-DR1 oligonucleotide and point mutation derivatives (UCP3-DR1m1, UCP3-DR1m2, and UCP3-DR1m3) (left).



### SKELETAL MUSCLE CELL

**Figure 3.** Schematic diagram of the regulation of the human UCP-3 gene promoter by MyoD and retinoic acid.

effect (Fig. 2B). Therefore, it is concluded that band B is formed by a complex containing RAR and RXR whereas bands A contain RXR but not RAR. Competition analysis with an excess of unlabeled oligonucleotides that carry the mutations assayed in the functional promoter analysis (UCP3-DR1 *m1* and UCP3-DR1 *m2*, Fig. 2C) were performed to identify the sites involved in the DNA-protein binding. The unlabeled UCP3-DR1 *m1* oligonucleotide competed for proteins that form the complexes A1 and A2. The UCP3-DR1 *m2* oligonucleotide competed for the formation of the RAR-containing complex leading to band B. The oligonucleotide UCP3-DR1 *m3* had no competition effect. Thus, this result strongly indicates that site 1 is required for binding RAR/RXR complexes whereas site 2 is also involved in the formation of other RXR binding complexes that do not include RAR.

### CONCLUSIONS

Here we have established that the UCP-3 gene is expressed in cultured muscle cells only when differentiated. Moreover, RA has been identified as a powerful stimulator of UCP-3 gene expression, although expression of the UCP-3 gene was sensitive to RA only when cells were in the myotube stage. These findings are consistent with the behavior of the human UCP-3 gene promoter when transfected into myoblasts. Basal expression of the promoter was extremely low and there was no sensitivity to RA. However, MyoD conferred to the UCP-3 gene promoter a substantial expression as well as sensitivity to RA. MyoD is a master regulator of the differentiation program of muscle cell, and a

requirement of the UCP-3 gene promoter for MyoD may be responsible for the preferential expression of the gene in differentiated muscle cells and its skeletal muscle-specific expression *in vivo* (Fig. 3).

The finding that RAR enhanced the responsiveness of the UCP-3 gene promoter to all-trans RA whereas RXR and 9-cis RA were much less effective indicates the presence of a RARE in the UCP-3 gene. In most of the RA-responsive genes studied so far, RXR has an auxiliary role of providing the heterodimerization partner for RAR and we observed that cotransfection of RAR plus RXR was maximally effective in inducing the UCP-3 gene promoter.

A major RARE of the human UCP-3 gene is identified in the proximal promoter region, between -71 and -59. It consists of a DR1-like motif containing an AGGTCA sequence separated by one base pair from another imperfect half site. This DR1 element was required for RA responsiveness and it bound a nuclear protein complex from L6E9 muscle cells that contains RAR and RXR. We have observed that the DR1 sequence in the UCP-3 promoter also binds other RXR-containing complexes from cell muscle nuclei. The expression of the UCP-3 gene is sensitive *in vivo* to several hormonal signals other than RA that also act through nuclear receptors, such as, for instance, fatty acid derivatives (via PPARs) or thyroid hormones (via thyroid hormone receptors). Further research will be needed to establish whether the UCP-3 gene promoter is sensitive to activation by other ligands and whether the DR1 RARE identified here acts as a multihormonal cis-acting element in the UCP-3 gene.

The presence of deletion and point mutation constructs that are responsive to MyoD but unresponsive to RA indicated that the RARE in the UCP-3 gene promoter is distinct and physically separated in the DNA from the *cis*-acting elements eliciting MyoD action. Mapping the precise site for MyoD action in the UCP-3 gene promoter is beyond the scope of the present study, but it appears that MyoD acts through the minimal promoter region of the UCP-3 gene in the absence of a conventional E-box binding element. If, as described in other muscle-specific genes, MyoD activates gene expression by interaction with the basal transcription machinery, such a mechanism would provide a consistent explanation for the dramatic requirement of UCP-3 promoter activity, both basal or RA-stimulated, for MyoD.

In summary, RA action constitutes a novel pathway of regulation of UCP-3 gene expression that, in addition to MyoD action, may be critical for the differentiation and development-dependent regulation of the UCP-3 gene expression in skeletal muscle. EJ

## The human uncoupling protein-3 gene promoter requires myod and is induced by retinoic acid in muscle cells

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### ABSTRACT

The uncoupling protein-3 (UCP-3) gene encodes for a mitochondrial protein expressed preferentially in skeletal muscle. UCP-3 mRNA is expressed in cultured muscle cells (C2C12 or L6E9) only when differentiated, at which stage UCP-3 is highly induced by all-*trans* retinoic acid (RA). Here we report that human UCP-3 promoter activity is dependent on MyoD and inducible by all *trans*-RA. The action of all *trans*-RA is increased by co-transfection with RA receptor (RAR). We have characterized the RA response element that controls the induction by RA in the 5' noncoding region of the UCP-3 gene. Deletion and point-mutation analysis of the hUCP-3 promoter led us to identify a direct-repeat element with one base-pair spacing (DR1) at position -71/-59 responsible for the induction by RA of the activity of the promoter. This DR1 element bound a nuclear protein complex from muscle cells that contain RAR and retinoid X receptor (RXR). In the absence of this element, the promoter became unresponsive to RA, but it was still dependent on MyoD. In conclusion, it has been established that UCP-3 gene promoter activity is dependent on MyoD, and the first regulatory pathway for UCP-3 gene promoter regulation has been recognized by identifying RA as a transcriptional activator of the gene.

**Key words:** UCP-3 • mitochondria • skeletal muscle differentiation

**T**he uncoupling protein-3 (UCP-3) gene encodes for a protein 60% similar to the brown fat-specific mitochondrial uncoupling protein UCP-1 (1, 2). It is located in chromosome 11 on humans and chromosome 7 in mice (3). UCP-3 gene expression is predominant in skeletal muscle of humans and in brown fat and skeletal muscle of rodents. UCP-3 acts as an uncoupler of

oxidative phosphorylation when over-expressed in yeast, and, by analogy with UCP-1, it has been proposed to mediate nonshivering thermogenesis in skeletal muscle, although its physiological function has not been established (for review see ref 4). Impaired UCP-3 gene expression in muscle has been reported in NIDDM and obese patients (5), although other reports did not confirm these findings (6).

UCP-3 gene expression in muscle is highly sensitive to the metabolic and hormonal status. The gene is up-regulated in physiological situations associated with high free fatty acid levels such as starvation (7), postnatal development (8), or high-fat diets (9), regardless of the thermogenic activity of muscle. Fatty acids themselves induce UCP-3 gene expression when administered *in vivo* (10, 11), which has led to the hypothesis that UCP-3 function may be more related to fatty acid metabolism in muscle rather than to nonshivering thermogenesis. The recently obtained mice with targeted disruption of the UCP-3 gene did not show a major impairment in regulatory thermogenesis despite altered mitochondrial proton leak and enhanced reactive oxygen production in muscle mitochondria (12, 13). Multiple hormonal treatments *in vivo*, such as leptin, thyroid hormones, glucocorticoids, and especially activators of PPAR, cause dramatic changes in UCP-3 gene expression in muscle (7, 11, 14). However, the hormonal signals and elements in the UCP-3 gene responsible for transcriptional regulation are unknown.

Retinoic acid (RA), a natural derivative of vitamin A, affects many biological processes including embryonic pattern formation, cell growth, and differentiation, mainly by influencing gene transcription. RA acts through two kinds of receptors, the RA receptor (RAR) and the retinoid-X receptor (RXR), members of the nuclear hormone receptor superfamily. Moreover, two active

isomers of RA, all-*trans* RA and 9-*cis* RA, affect gene transcription through distinct pathways. All-*trans* RA activates gene expression mainly through RAR/RXR heterodimers that bind to RA response elements (RARE) and stimulate transcription as a result of the binding of all-*trans* RA to RAR (for review see ref 15). 9-*cis* RA activates the RAR component of RAR/RXR heterodimers, but it also activates gene expression by binding to RXR (16). 9-*cis* RA activates transcription of genes containing regulatory elements capable of binding RXR homodimers or heterodimers of RXR and other receptors of the thyroid/retinoid receptor family such as PPARs or orphan receptors (17, 18).

Skeletal muscle, which contains RAR and RXR receptors, is a target of RA effects (15), and RA promotes myogenic differentiation of cultured muscle cells (19–21). Thus, RA has been reported to induce basic helix-loop-helix (b-HLH) myogenic regulatory proteins such as MyoD or myogenin (21, 22). These muscle-specific b-HLH factors dimerize with a ubiquitous class of b-HLH E-proteins that bind the E-box elements in gene promoters and activate muscle-specific genes (23).

Here we report that UCP-3 gene is expressed in muscle cells only when differentiated, and UCP-3 promoter activity is largely dependent on MyoD. A novel regulatory pathway for UCP-3 gene expression is established by the identification of RA as a major inducer of UCP-3 gene transcription and an RA-responsive element in the proximal region of the human UCP-3 gene promoter.

## METHODS

### Cell culture, RNA isolation, and Northern blot analysis

L6E9 and C2C12 cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS). To induce differentiation, L6E9 cells at 80% confluence were changed to DMEM containing 2% FBS and C2C12 to DMEM containing 2% horse serum (HS). L6E9 were then maintained for 4 days and C2C12 for 10 days in these conditions of culture to acquire myotube morphology. Total RNA was extracted from cultured L6E9 or C2C12 cells by using Tripure Isolation Reagent (Boehringer) and 20 µg of total RNA was loaded. Ethidium bromide staining was used to confirm equal loading of RNA. Northern blot analyses were performed using standard methods. Blots were hybridized with full-length rat UCP-3 cDNA (1). Autoradiographs were quantified by densitometric scanning (LKB Instruments).

### Polymerase chain reaction amplification of human UCP-3 gene promoter

A fragment of the human UCP-3 gene was amplified by PCR using 200 ng of human genomic DNA. The complementary (3') primer corresponded to bases from +47 to +24 downstream the transcription start site, according to Schrauwen et al. (24) and GenBank/EMBL data (Accession# AF032871). The complementary (5') primer corresponded to -2903 to -2872. The 3' and 5' complementary primers included six base pair noncomplementary extensions capable of generating *Sma*I and *Xho*I restriction sites. Reaction was performed in a 50 µl final volume containing 25 pm of each primer, 1.5 mM each dNTP, 10 mM MgCl<sub>2</sub>, and 4 units of Taq DNA polymerase. Thirty cycles were performed at 92°C for 30 sec, 58°C for 1 min, and 72°C for 3 min. The resulting DNA product of ~3 kb was purified, digested with *Xho*I and *Sma*I, and cloned in pGL3-basic plasmid (Promega) (opened by *Bgl*II, blunt-ended and digested by *Xho*I), which contains the cDNA for firefly (*Photinus pyralis*) luciferase as a reporter gene. The whole fragment was sequenced by the dideoxy method using flanking and internal synthetic oligonucleotides. The sequence for the cloned human UCP-3 gene fragment from -2903 to +47 was identical to that previously shown in the GenBank/EMBL data base (Accession #AF032871) and differed from that in ref 25 by the presence of the C to T polymorphism in the proximal region of the promoter, 6 bp upstream the putative TATA box (24).

### Plasmid constructions

The insertion of the fragment from -2903 to +47 of the human UCP-3 gene into pGL3-basic generates -2903hUCP3-Luc. Plasmids containing 5' deletion mutants of this hUCP3-Luc were generated by restriction enzymes. A *Sac*I restriction and religation generates the -588hUCP3-Luc construct. A further deletion was generated from this last construct by restriction with *Sac*I and *Bgl*II, blunt ending and religation, thus generating -165hUCP3-Luc.

Point mutation constructs were generated using a Quick-change site-directed mutagenesis kit (Stratagene). 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 -165hUCP3-Luc were incubated with 2.5 units of *Pfu*Turbo DNA polymerase. PCR reaction was done for 17 cycles at 95°C for 30 sec, 55°C for 1 min, and 68°C for 14 min. Then, 10 units of *Dpn*I restriction enzyme was added to each PCR reaction 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 the mutations, the

oligonucleotides used were: for the E box mutation (-165mutHUCP3-Luc) 5' GCCAGCCTCTTGTCATGG ATCAGGCTGTC 3' (introducing *NcoI* restriction site); for *mut1* (*mut1hUCP3-Luc*), 5' GGATAAGGTTTCATATGAGCCCCGTGTGTATAAGA CCA GTGCC 3' (introducing a *NdeI* restriction site); for *mut2* (*mut2hUCP3-Luc*), 5' CCAACTTCTCTAGGATAAAC GTTCAGGTCAGCCCCGTGTG 3' (introducing *AcI* restriction site). The plasmid -61hUCP3-Luc was generated from *mut1hUCP3-Luc* by digestion with *KpnI* and *NdeI*, blunt-ending, and ligation.

### DNA transfection and determination of luciferase activity

Transfection experiments were carried out in L6E9 cells at 50% confluence by using FuGene6 Transfection Reagent (Boehringer) and were performed according to manufacturer's instructions. Each transfection point was done in triplicate in a 6-well plate and contained 1.5 µg of luciferase reporter vector, 0.2 µg of MyoD, pRSV-RAR $\alpha$  or pRSV-RXR $\alpha$  expression vectors, and 3 ng of pRL-CMV, 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 treated with or without 2 µM all-*trans* RA or 2 µM 9-*cis* RA for 24 h before harvest. Firefly luciferase and Renilla luciferase activities were measured in a luminometer using the Dual Luciferase Reporter assay system kit (Promega). Cells were lysed in a 500 µl of PLB (passive lysis buffer) in agitation for 15 min and 20 µl was used for measurement. Luciferase activity elicited by UCP-3 promoter constructs was normalized for variation in transfection efficiency using Renilla luciferase as an internal standard.

### Electrophoretic mobility shift assays

Nuclear protein extracts from L6E9 muscle cells were isolated as reported previously (26), and protein concentration was determined by the micromethod of Bio-Rad (Richmond, CA) using BSA as standard. For gel retardation assays, the double-stranded UCP3-DR1 oligonucleotide corresponding to the -79 to -57 sequence of the UCP-3 gene was end-labeled using [ $\alpha$ <sup>32</sup>P]dCTP and klenow enzyme. The DNA probe (25,000 cpm) was incubated for 30 min at 25°C with 5 µg of L6E9 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 0.5 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 0.5X TBE. In the competition experiments, 100-fold molar excess of unlabeled double-stranded oligonucleotides was included in each respective binding reaction. RARE1 and RARE2 are 24-base pair double-stranded oligonucleotides corresponding to the DR5 RA responsive element in the RAR $\beta$  gene (27) and to the DR2 RA responsive element in the mouse cellular retinol-binding protein type I gene (28), respectively. ETS is an oligonucleotide corresponding to the GA-rich region in the stromelysin promoter used as negative control (29). When indicated, 2 µl of antisera against RAR $\alpha$  (C20), RXR $\alpha$  (D20) or ETS (C275) (Santa Cruz) was added to the incubation media for 15 min.

### Statistical analysis

Where appropriate, statistical analysis was performed by Student's *t* test; significance is indicated in the text.

## RESULTS

### Retinoic acid induces UCP-3 mRNA expression in differentiated C2C12 and L6E9 muscle cells

Northern blot analysis revealed that neither C2C12 nor L6E9 (mouse and rat muscle cell lines) expressed detectable levels of UCP-3 mRNA in the myoblastic stage (Fig. 1A, B). Nevertheless, when cells were differentiated and acquired a myotube phenotype, low but detectable levels of UCP-3 mRNA were present. As C2C12 need longer time of culture than L6E9 cells to acquire myotube morphology, detectable levels of UCP-3 mRNA appeared later. When the cells were nondifferentiated, treatment with all-*trans* RA had no effect on UCP-3 expression. However, treatment of myotubes with all-*trans* RA induced UCP-3 mRNA expression by 8- to tenfold over basal levels in both cell types (Fig. 1A, B). UCP-3 mRNA levels in differentiated cells after RA treatment were 87 ( $\pm$ 15% of those in mouse gastrocnemius muscle. 9-*cis* RA was added to 4-day differentiated L6E9 cells and induction of UCP-3 mRNA expression was ~50% of the induction observed with all-*trans* RA (Fig. 1C). Therefore, expression of UCP-3 mRNA in C2C12 and L6E9 is inducible by RA only when they are differentiated.

### Retinoic acid activates the expression of the human UCP-3 gene promoter in L6E9 cells—MyoD is required for the basal promoter activity and RA responsiveness.

We have cloned and sequenced a 3-kb fragment of the human UCP-3 promoter, and this was fused to luciferase reporter gene to study its regulation. The promoter-reporter constructs -2903hUCP3-Luc and -1588hUCP3-

Luc were used in transient transfection studies in L6E9 cells to identify the fragment of the promoter that contained the elements necessary for expression in muscle cells. Results with both constructs showed low basal luciferase activity. This indicated that either some regulatory elements could be absent in the 3-kb fragment upstream of transcription start site of the UCP-3 gene or that the UCP-3 gene promoter is poorly expressed in these cells because they are myoblasts. This last possibility would be in agreement with the undetectable levels of expression of UCP-3 mRNA in myoblasts shown before. To test the hypothesis that a myogenic factor could be involved in UCP-3 promoter regulation, we co-transfected the -2903hUCP3-Luc and -1588hUCP3-Luc with the MyoD expression plasmid and a 7- and 10-fold induction of the activity over the basal values was obtained respectively (Fig. 2 and data not shown). In contrast, no effect was observed when an expression vector for Mef2C, a myogenic factor from the MADS family, was co-transfected.

To analyze the role of retinoids in UCP-3 promoter activity, co-transfection experiments with expression vectors for RAR and RXR were performed. Results showed that when -1588hUCP3-Luc was transfected in the absence of co-transfected MyoD, no significant differences over the basal level were obtained either by co-transfection with RAR $\alpha$  or RXR $\alpha$  alone or together, or by the addition of their agonists, all-*trans* RA or 9-*cis* RA. When MyoD was co-transfected, RA caused a significant induction of the promoter. Furthermore, when a RAR $\alpha$  expression vector was co-transfected, induction by all-*trans* RA was much higher. In fact, co-transfection of MyoD plus RAR $\alpha$  and treatment with all-*trans* RA caused ~70-fold induction of the UCP-3 promoter respect to basal activity (Fig. 2). In contrast, exposure to 9-*cis* RA in the presence of co-transfected RXR $\alpha$  had no significant effect on UCP-3 promoter activity. Experiments performed with both types of receptor together showed a higher induction as a result of co-transfection, which was significantly increased by exposure to all-*trans* RA (Fig. 2). Similar results were obtained with the -2903hUCP3-Luc construct (data not shown). All these results together indicate that MyoD is essential for the activity of the human UCP-3 gene promoter and for RA responsiveness, which is mediated mostly by RAR.

#### **Deletion analysis of the human UCP-3 promoter reveals a RA responsive element in the proximal promoter region**

To search for regulatory elements responsible for the effects of RA on the hUCP3 promoter, a set of deletion mutants was created. A major deletion that contained only

the proximal 5' region of the gene, -165hUCP3-Luc, retained the same basal activity as the -1588hUCP3-Luc (data not shown) and was activated 8-fold by MyoD. All-*trans* RA in the presence of co-transfected MyoD and RAR $\alpha$  induced 30-fold the activity of -165hUCP3-Luc (Fig. 3). Moreover, it significantly raised the expression of the construct respect to the MyoD plus RAR $\alpha$ -co-transfected in the absence of added ligand. This activation depended on the presence of MyoD (data not shown). Although the level of induction by RA treatment in the -165hUCP3-Luc was somewhat lower than in -1588hUCP3-Luc and, therefore, it was not possible to rule out the presence of RA-responsive elements upstream of -165, it appeared that this proximal fragment should contain the regulatory elements responsible for MyoD and RA action.

Searching for consensus regulatory sequences within this -165 region of the human UCP-3 promoter, an Ebox sequence and one direct repeat (DR1)-like element were located (from -106 to -101 and from -71 to -59, respectively) close upstream from the putative TATA box. A point mutation in the Ebox (-165*mut*hUCP3-Luc), known to disrupt binding of b-HLH proteins (30, 31), was performed, and no differences were observed regarding MyoD or RA-dependent activation when compared with the wild-type construct. An additional deletion mutant, -61hUCP3-Luc, which lacks both putative sites (Ebox and DR1), still responded to MyoD. Nevertheless, no induction was observed after RA treatment (Fig. 3), indicating that elements for RA responsiveness are present in the human UCP-3 promoter region between -165 and -61 and are distinct to those mediating MyoD-dependent activity of the promoter.

#### **Point mutation analysis identifies a RARE element in the DR1-like sequence of the proximal UCP-3 gene promoter region**

To identify sites responsible for RA action, two different point mutations in the DR1-like element were generated on the -165hUCP3-Luc as described in Methods. As expected, transfection experiments revealed that none of these mutations affect the ability of MyoD to activate the expression of the gene (data not shown). In the presence of MyoD and RAR $\alpha$ , *mut1*UCP3-Luc lost ~80% of the induction by all-*trans* RA when compared with -165hUCP3-Luc, whereas *mut2*UCP3-Luc lost ~75% (Fig. 4B). Mutation of both sites in the same construct (*mut1/mut2*hUCP3-Luc) abolished the activation of the promoter by RA, demonstrating the involvement of these sites in the regulation of hUCP3 promoter by all-*trans* RA. To explore the possible presence of additional RA-responsive elements upstream -165, the same point-

mutation analysis was performed on the -1588hUCP3-Luc constructs (Fig. 4C). Results were basically identical to those with the -165hUCP3-Luc: either mutation of the site 1 or of the site 2 suppressed most of RA responsiveness, and mutation of both sites abolished it. These results established that RA responsiveness in the UCP-3 gene promoter is mediated by the DR1 proximal element.

### **The DR1-like region in the human UCP-3 gene binds RAR and RXR from L6E9 nuclear extracts**

To search for the proteins that bind the RA-responsive DR1-like sequence, we performed electrophoretic mobility shift assays with L6E9 nuclei extracts. We used the UCP3-DR1 oligonucleotide, corresponding to the -79/-57 region of the UCP-3 gene, as a labeled probe (Fig. 5C). A characteristic pattern of DNA-protein binding complexes was formed, as shown in Figure 5A. Four retarded bands appeared: A1, A2, B, and NS. The appearance of A1 and A2 as separate bands was evident only in long electrophoretic runs (Fig. 5B). Competition assays using the nonlabeled UCP3-DR1 probe revealed that the NS band is nonspecific, because unlabeled oligonucleotide could not compete for the complex. The other bands, A1, A2, and B, were specific because they disappeared in these conditions. The B band disappeared when either DR5-RARE1 (27) or DR2-RARE2 (28) was added to the incubation buffer. An excess of an unlabeled unrelated oligonucleotide (the GA-rich sequence from the stromelysin ETS binding site; see Methods) was used as a negative control and, as expected, no competition effect was detected.

The effects of including specific antibodies against RAR $\alpha$ , RXR $\alpha$ , or ETS in the electrophoretic mobility shift assays were determined to confirm the identity of the proteins forming the specific retarded complexes (Fig. 5B). The presence of RAR $\alpha$  antibody in the incubation media prevented the formation of the retarded band B. A partial reduction in the intensity of the band B was also observed when RXR $\alpha$  antibody was present in the assay. In addition, RXR $\alpha$  antibody prevented the formation of bands A1 and A2, whereas the ETS antibody did not affect the pattern of retarded bands. Therefore, we conclude that band B is formed by a complex containing RAR and RXR, whereas band A contains RXR but not RAR.

Competition analysis with an excess of unlabeled oligonucleotides that carry the mutations assayed in the functional promoter analysis (UCP3-DR1m1 and UCP3-DR1m2, shown in Figure 5C) were performed to identify the sites involved in the DNA-protein binding (Fig. 5C). The unlabeled UCP3-DR1m1 oligonucleotide competed for proteins that form the complexes A1 and A2, thus

preventing the formation of these two bands. The UCP3-DR1m2 oligonucleotide competed for the formation of the RAR-containing complex leading to band B. The oligonucleotide UCP3-DR1m3, which carries a mutation outside the putative RARE element, had no competition effect for the formation of any of the bands. Thus, this result strongly indicates that site 1 is the major site required for binding RAR/RXR complexes, whereas site 2 is also involved in the formation of other RXR-binding complexes that do not include RAR.

### **DISCUSSION**

Here we have established that the UCP-3 gene is expressed in cultured muscle cells only when differentiated. This agrees with the late appearance of UCP-3 gene expression during skeletal muscle ontogeny (11) and indicates that the UCP-3 gene could be considered as a maker of the differentiated stage of muscle cells. Moreover, RA has been identified as a powerful stimulator of UCP-3 gene expression, although the expression of the UCP-3 gene was sensitive to RA only when cells were in the myotube stage. This is similar to the recent report (32) describing a positive effect of 9-*cis* RA on UCP-3 mRNA in myotubes. These findings are consistent with the behavior of the human UCP-3 gene promoter when transfected into myoblasts. Basal expression of the promoter was negligible and there was no sensitivity to RA. However, MyoD conferred to the UCP-3 gene promoter a substantial expression as well as sensitivity to RA. MyoD is a master regulator of the differentiation program of muscle cell (23), and the requirement of the UCP-3 gene promoter for MyoD may be responsible for the preferential expression of the gene in differentiated muscle cells and its skeletal muscle-specific expression *in vivo*.

The finding that RAR enhanced the responsiveness of the UCP-3 gene promoter to all-*trans* RA, whereas RXR and 9-*cis* RA were much less effective, indicates the presence of a RARE in the UCP-3 gene. In most of the RA-responsive genes studied so far RXR has an auxiliary role of providing the heterodimerization partner for RAR (15), and we observed that co-transfection of RAR plus RXR was maximally effective in inducing the UCP-3 gene promoter. Although ligand-dependent activation of RXR, as a partner of either RAR or other nuclear receptors, cannot be excluded, transfection experiments indicated a major involvement for RAR in mediating responsiveness to RA.

Our results show that a major RARE of the human UCP-3 gene is placed in the proximal promoter region, between -71 and -59. It consists of a DR1-like motif containing an AGGTCA sequence separated by one base pair from

another imperfect half site, and the whole sequence is identical in the UCP-3 gene promoter from mouse (33). This DR1 element was required for RA responsiveness, and it bound a nuclear protein complex from L6E9 muscle cells that contain RAR and RXR. A DR1, which is a 1-bp-separated alignment of direct repeats, has been observed as a characteristic alignment of several RAREs (34, 35), although it is a less frequent mediator of RAR-dependent responsiveness than other structures of RAREs such as DR5 or DR2 (15). However, in contrast to these last alignments, which are very restrictive for binding RAR/RXR heterodimers, DR1 elements are highly promiscuous in binding, and they can mediate biological responsiveness to other RXR-containing heterodimers, such as PPAR/RXR, RXR homodimers, and HNF-4 or COUP-TF (36). Subtle differences in the half-site and flanking sequences of DR1 and promoter context have been reported to mediate preferential binding or responsiveness to different members of the nuclear receptor superfamily (37, 38). We have observed that the DR1 sequence in the UCP-3 promoter, in addition to RAR/RXR, also binds other RXR-containing complexes from cell muscle nuclei. The expression of the UCP-3 gene is sensitive *in vivo* to several hormonal signals other than RA that also act through nuclear receptors, as for instance fatty acid derivatives (via PPARs) (11) or thyroid hormones (via thyroid hormone receptors) (7). Further research will be needed to establish whether the UCP-3 gene promoter is sensitive to activation by other ligands and whether the DR1 RARE identified here acts as a multihormonal *cis*-acting element in the UCP-3 gene.

Recent evidence has shown that RAR and RXR may functionally and physically interact with MyoD when bound to DNA (22). The requirement of MyoD for UCP-3 gene promoter activity in response to RA could be compatible with such an interaction. However, the presence of deletion and point-mutation constructs that are responsive to MyoD but unresponsive to RA indicated that the RARE in the UCP-3 gene promoter is distinct and physically separated in the DNA from the *cis*-acting elements eliciting MyoD action. Mapping the precise site for MyoD action in the UCP-3 gene promoter is beyond the scope of the present study, but it appears that MyoD acts through the minimal promoter region of the UCP-3 gene, in the absence of a conventional E-box binding element. This is similar to the E-box independent action of MyoD on the myosin heavy chain IIB promoter, another skeletal muscle-specific gene, in which MyoD activates gene expression by interaction with the basal transcription machinery (39). Such a mechanism, if confirmed, would provide a consistent explanation for the dramatic requirement of UCP-3 promoter activity, basal or RA-stimulated, for MyoD.

In summary, RA action constitutes a novel pathway of regulation of UCP-3 gene expression that, in addition to MyoD action, may be critical for the differentiation and development-dependent regulation of the UCP-3 gene expression in skeletal muscle. In 1995, RA was identified as a major inducer of the expression of the UCP-1 gene, as powerful as the classical noradrenergic pathway of regulation of this gene (40), although the exact physiological meaning of this effect has not been established yet. Recently, we described that 9-*cis* RA is a major hormonal regulator of UCP-2 gene expression in brown adipocytes (41), and here we show that transcription of UCP-3 is extremely sensitive to RA. The three UCP proteins are known to uncouple oxidative phosphorylation, and, although their respective physiological functions in relation to thermogenesis and mitochondrial metabolism appear to be distinct, it is remarkable that they are all highly sensitive to retinoids. This may be added to the recent observation that the uncoupling activities of at least UCP1 and UCP2 are affected directly by RA (42). Recent evidence from studies on transgenic mice bearing a targeted disruption of the UCP-3 gene suggests that UCP-3 may be involved in the regulation of reactive oxygen species production rather than nonshivering thermogenesis (13). Vitamin A derivatives are essential for the development and differentiation of several tissues, including skeletal muscle, during ontogeny, and for the regular physiological functions of most cell types in the adult. Further research will be needed to define the physiological significance of the action of RA on the expression of UCP-3 gene reported here and, in general, on the function of UCP-3 as part of the mitochondrial energy-producing machinery.

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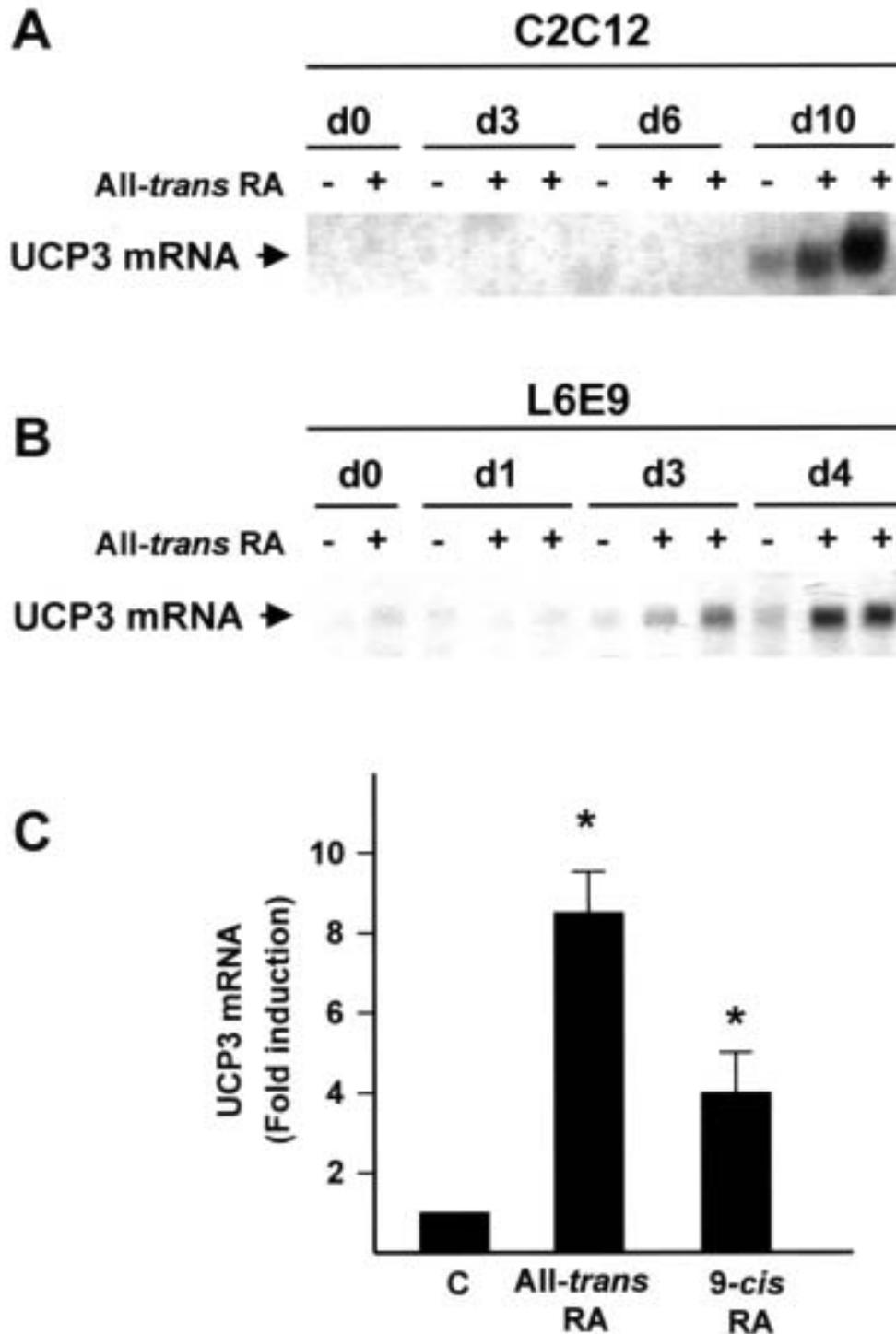
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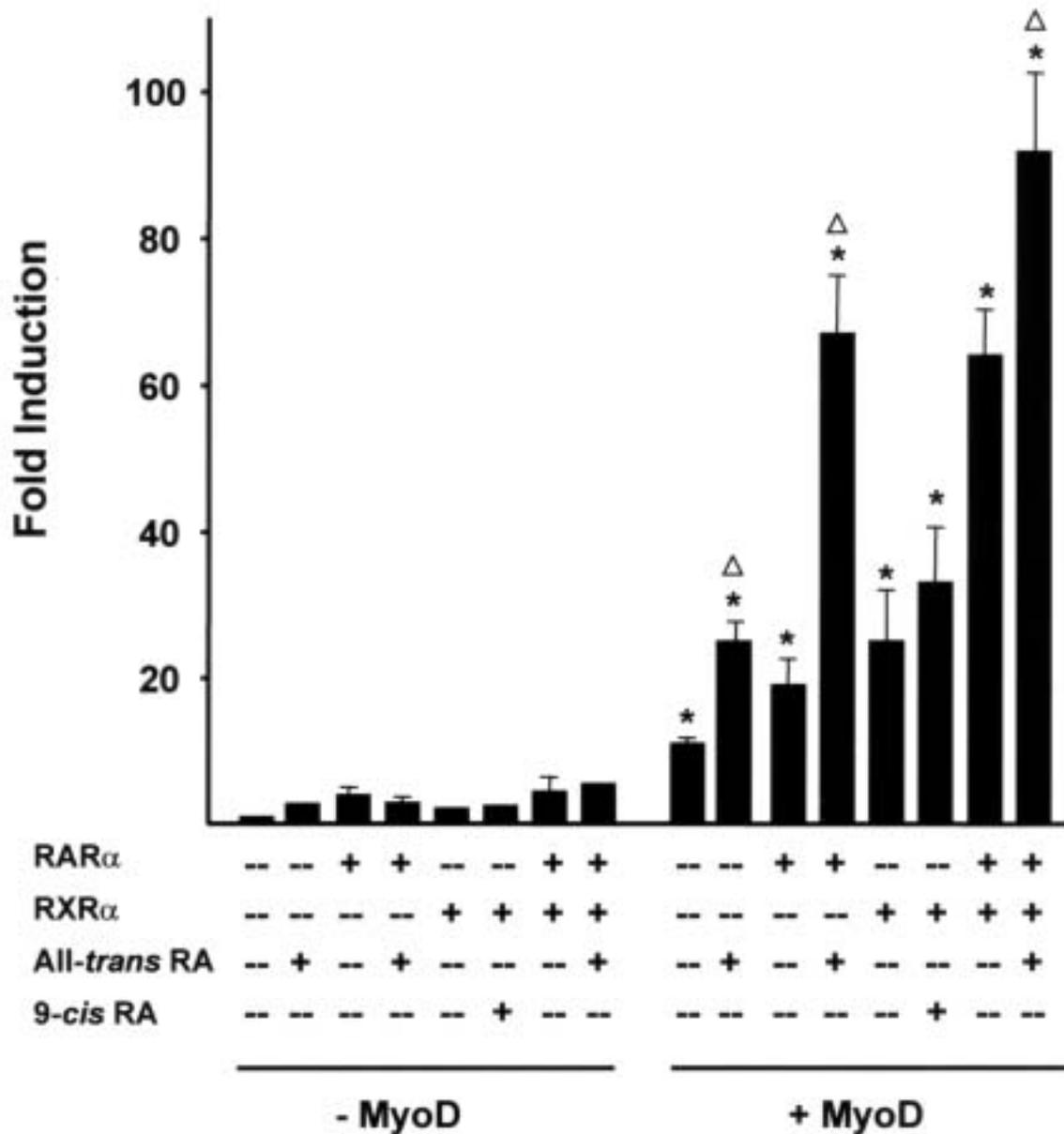
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Fig. 1



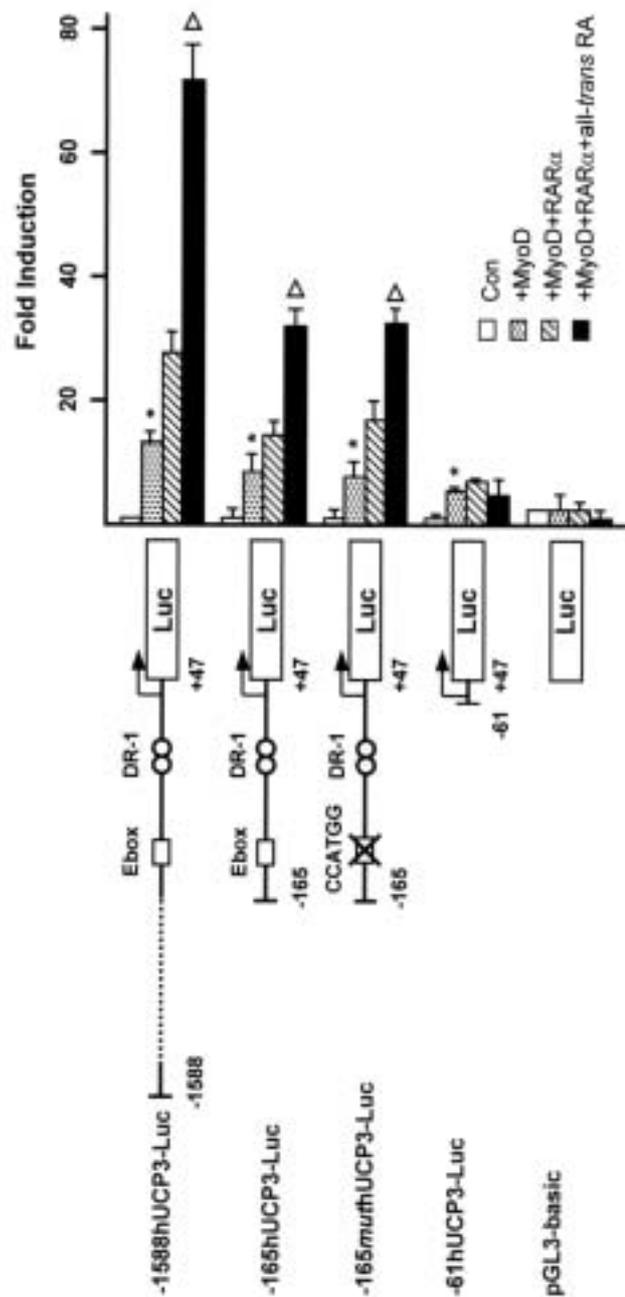
**Figure 1. UCP-3 mRNA expression in differentiating muscle cell lines. Effects of RA.** A, B, Northern Blot analysis of UCP-3 mRNA expression during L6E9 (A) and C2C12 (B) myogenic differentiation. L6E9 and C2C12 cells were cultured in media with 10% FBS until 80% confluence. To induce differentiation 10% FBS was replaced with 2% FBS (L6E9) or 2 % HS (C2C12) respectively (d0). Either vehicle (-) or all-*trans* RA (2  $\mu$ M) (+) was added to cells at the indicated days of differentiation. Total RNA was isolated after 24 h and probed with the rat UCP -3 cDNA. C, Quantification analysis of the induction of UCP-3 mRNA levels in L6E9 cells after 4 days of differentiation and treatment with vehicle, all-*trans* RA (2  $\mu$ M) or 9-*cis* RA (2  $\mu$ M). Results represent the mean  $\pm$  SE of four different samples.

Fig. 2



**Figure 2. Expression of -1588hUCP3-Luc in L6E9 cells. Effects of MyoD, RAR or RXR and effects of RA.** L6E9 cells were co-transfected with -1588hUCP3-Luc either with or without MyoD plus or minus RAR  $\alpha$ - and RXR $\alpha$ -encoding expression vectors, as indicated in methods. Following transfection, the cells were cultured for 48h in presence or absence of either 2  $\mu$ M all-trans RA or 2  $\mu$  -cis RA for 24h. The -1588hUCP3-driven luciferase activity levels were standardized to pRL-CMV-driven Renilla luciferase as transfection internal control. The basal activity of the -1588hUCP3-Luc plasmid alone was set to 1. Means  $\pm$  SE of at least three independent triplicate experiments are shown. Statistically significant differences ( $p < 0.05$ ) due to the co-transfection with MyoD for each experimental group are shown by \*, and those due to the presence of a ligand (all-trans RA or 9-cis RA) respect to its control are shown by  $\Delta$ .

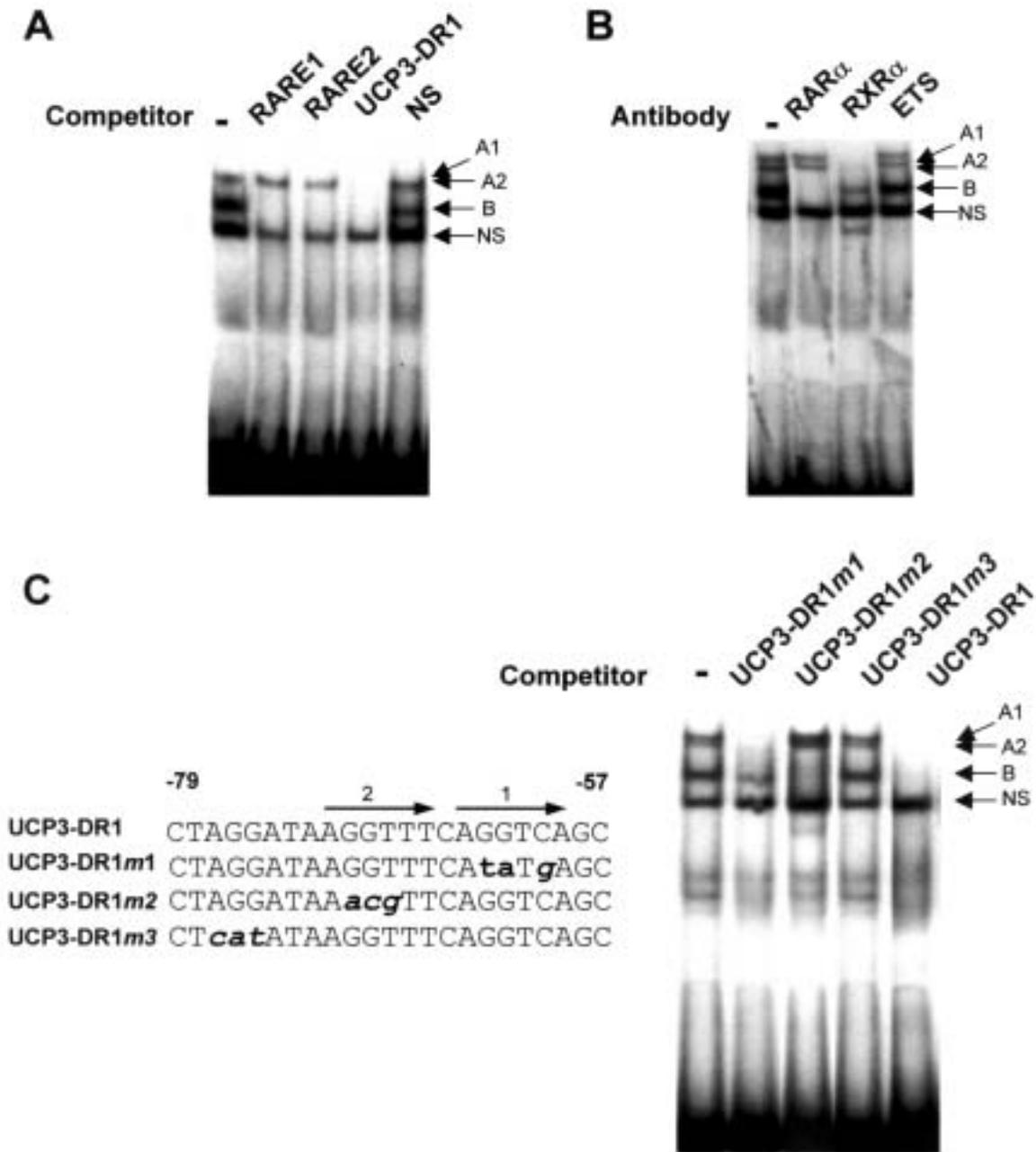
Fig. 3



**Figure 3. Deletion analysis of the effects of MyoD, RAR and all-*trans* RA on the UCP-3 gene promoter. Effects of a point-mutation in the proximal E-box sequence.** L6E9 cells were co-transfected with the luciferase reporter constructs depicted on the left and either with or without MyoD plus or minus RAR  $\alpha$  expression vectors. The boxes indicate the position of an E-box sequence (-106/-101) and DR1-like element (-71/-59). Following transfection, the cells were cultured for 48h in presence or absence of 2  $\mu$ M all-*trans* RA for 24h. The luciferase activity levels were standardized to pRL-CMV-driven Renilla luciferase as transfection internal control. The basal activity of the -1588hUCP3-Luc plasmid alone was set to 1. Means  $\pm$  SE of at least three independent triplicate experiments are shown. Statistically significant differences ( $p < 0.05$ ) due to the co-transfection with MyoD for each experimental group are shown by \*, and those due to the presence of all-*trans* RA respect to its control are shown by  $\Delta$ .



Fig. 5



**Figure 5. Electrophoretic mobility shift assays of the nuclear proteins that interact with the DR1-like element in the human UCP-3 gene.** A double-stranded oligonucleotide corresponding to the -79/-57 region of the hUCP3 gene (named as UCP3-DR1) was used as a labeled probe. All the assays were performed in the presence of 5  $\mu$ g of L6E9 nuclear protein extracts. **A**, The probe was incubated with L6E9 nuclei extracts and competitors were added at a 100-fold molar excess relative to probe concentration. RARE1 and RARE2 are double-stranded oligonucleotides corresponding to the DR5 RARE from the RAR $\beta$  gene (27) and to the DR2 RARE from the mouse cellular retinol binding protein type I (28). ETS is a GA-rich oligonucleotide used as a negative control. **B**, Effects of RAR $\alpha$ , RXR $\alpha$  and ETS antibodies on the formation of DNA-protein complexes between the UCP3-DR1 oligonucleotide and nuclei extracts. 2  $\mu$ l (2  $\mu$ g/ $\mu$ l) of each antibody were added to the assay as described in Methods section. **C**, Competition analysis between the UCP3-DR1 and UCP3-DR1m1 (3 base mutation in site 1), UCP3-DR1m2 (3 base mutation in site 2) and UCP3-DR1m3 (3 base mutation outside sites 1 and 2) oligonucleotides for the binding with the proteins involved in the formation of the retarded bands A and B (right) and sequence of the UCP3-DR1 oligonucleotide and point-mutation derivatives (UCP3-DR1m1, UCP3-DR1m2 and UCP3-DR1m3) in which the three changed bases are shown in lowercase (left).