Uncoupling Protein-3 (UCP3) mRNA Expression in Reconstituted Human Muscle after Myoblast Transplantation in RAG2⁻/⁻/F⁻c/C5⁻ Immunodeficient Mice*

Received for publication, August 7, 2002, and in revised form, September 25, 2002
Published, JBC Papers in Press, September 25, 2002, DOI 10.1074/jbc.M208048200

Nolwen Guigal‡, Marianne Rodriguez‡, Raquel N. Cooper§, Sandra Dromaint‡, James P. Di Santo¶, Vincent Moully§, Jean A. Boutin¶, and Jean-Pierre Galizzi‡

From the ¶Institut de Recherches Servier, Division de Pharmacologie Moléculaire et Cellulaire, 125 Chemin de Ronde, 78290 Croissy-sur-Seine, France, §CNRS UMR 7000, Faculté de Médecine Pitié-Salpêtrière, 105 Boulevard de l’Hôpital, F-75634 Paris cedex 13, France, and the ‡Institut Pasteur, Unité des Cytokines et Développement Lymphoïde, 25 rue du Docteur Roux, 75724 Paris cedex 15, France

Uncoupling protein-3 (UCP3), which is expressed abundantly in skeletal muscle, is one of the carrier proteins dissipating the transmitochondrial electrochemical gradient as heat and has therefore been implicated in the regulation of energy metabolism. Myoblasts or differentiated muscle cells in vitro expressed little if any UCP3, compared with the levels detected in biopsies of skeletal muscle. In the present report, we sought to investigate UCP3 mRNA expression in human muscle generated by myoblast transplantation in the skeletal muscle of an immunodeficient mouse model. Time course experiments demonstrated that 7–8 weeks following transplantation fully differentiated human muscle fibers were formed. The presence of differentiated human muscle fibers was assessed by quantitative PCR measurement of the human α-actin mRNA together with immunochemical staining using specific antibodies for spectrin and the slow adult myosin heavy chain. Interestingly, we found that the expression of UCP3 mRNA was dependent on human muscle differentiation and that the UCP3 mRNA level was comparable with that found in human muscle biopsies. Moreover, the human UCP3 (hUCP3) promoter seems to be fully functional, since triiodothyronine treatment of the mice not only stimulated the mouse UCP3 (mUCP3) mRNA expression but also strongly stimulated the hUCP3 mRNA expression in human fibers formed after myoblast transplantation. To our knowledge, this is the first time that primary myoblasts could be induced to express the UCP3 gene at a level comparable of that found in human muscle fibers.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 33-1-55722748; Fax: 33-1-55722810; E-mail: jean.boutin@fr.netgrs.com.

§ The abbreviations used are: UCP3, uncoupling protein-3; hUCP3, human UCP3; T3, triiodothyronine; TA, tibialis anterior; RT, reverse transcriptase; MHC, myosin heavy chain; TRE, thyroid responsive element.
acterizing this promoter is due to the fact that virtually all cultured muscle cells express little if any UCP3. These data suggest that specific transcriptional programs generating full muscle phenotype are exclusively activated in vivo but not in cultured muscle cells. Alternatively, in vivo muscle fibers might receive external signals triggering UCP3 gene expression (16).

To study the mechanisms regulating hUCP3 gene transcription and identify cis-/trans-acting DNA elements, we searched for an experimental model that would express a hUCP3 mRNA at levels similar to that found in human skeletal muscle biopsies. In the present work, we have injected human myoblasts into the tibialis anterior (TA) of immunodeficient mice RAG2−/−/c/C5−/− (21). In this in vivo environment, injected cells proliferate and fuse to form muscle fibers that become innervated and contract. In these reconstituted muscles, we were able to detect normal levels of hUCP3 gene expression. Moreover, the hUCP3 promoter appears to be fully functional, since the effect of T3 on hUCP3 mRNA expression was similar to that found on mouse UCP3 (mUCP3) mRNA expression. To our knowledge, this is the first model that permits both the characterization of the hUCP3 promoter and the physiological function of the putative uncoupling protein UCP3.

EXPERIMENTAL PROCEDURES

Human Myoblasts Origin and Culture—A biopsy from the quadriceps muscles of a 5-day-old infant was obtained during autopsy in accordance with the French legislation on ethical rules. From this biopsy, satellite cell populations were isolated from biopsies as described previously (22, 23). These cells were expanded in growth medium, which consists of Ham’s F-10 (Invitrogen) supplemented with 50 μg/ml of gentamycin and 20% fetal calf serum (Biomedia), and were called CHQ5B. The cells displayed a myogenic purity of 80%, as assessed by desmin staining (21, 24). To induce myotube formation, confluent cultures were cultivated for 10 days in Dulbecco’s modified Eagle’s medium supplemented with 10 μg/ml of insulin (Sigma) and 100 μg/ml of transferrin (Invitrogen).

Animals—Fifty-five immunodeficient RAG2−/−/c− mice, 2–3 months old, were used in this study as recipients for human myoblast implantation. All experiments were carried out in the specific pathogen-free animal facilities at the Pasteur Institute.

Cell Preparation and Myoblast Transplantation—The cells used for transplantation (CHQ5B) were expanded to 20 population doubling levels. Prior to injection the cells were trypsinized, centrifuged (380 × g) and resuspended in growth medium. Sufficient aliquots of cells were prepared in siliconized Eppendorf tubes. Following additional centrifugation, the supernatant was aspirated leaving a pellet containing 5 × 10⁶ cells per injection. For implantation, the mice were anesthetized with hypnorm/hynovel. The TA muscles of both hindlimbs were dissected and 10s muscle fibers were harvested from each section. The muscle fibers were then closed with fine sutures. Mice were sacrificed at 24 h later, and the TA muscles were dissected and stored frozen for mRNA extraction.

RT-PCR Experiments—Total RNA was isolated from the TA of mice using the RNAse kit (Eurbio, Paris, France). One microgram of total RNA was reverse-transcribed using the Moloney murine leukemia virus reverse transcriptase in 20 μl of its own buffer (Invitrogen) and oligo(dT) at 37 °C for 1 h. Quantitative real-time PCR was performed with a LightCycler using FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals). The cDNA product was amplified in a total volume of 20 μl with 0.5 μM of each primer, 4 μl MgCl₂ (final concentration). All the primers were designed to anneal and amplify only mRNA. Moreover, primers were chosen to distinguish between human from mice mRNA.

RESULTS

Comparative Study of UCP3 mRNA Expression in Cultivated Human Myoblasts and Skeletal Muscle Biopsies—UCP3 mRNA levels were evaluated in cultures of human myoblasts and myotubes as well as in human muscle biopsies using real-time RT-PCR. As reported in Table II, the levels of UCP3 expressed in both myoblasts and myotubes were very much lower than that expressed in muscle biopsies. Indeed, in human muscle biopsies, the level of UCP3 mRNA was 500- and 423-fold greater than that measured either in myoblasts or myotubes. There was virtually no difference between undifferentiated and differentiated myoblast. This would suggest that the in vitro culture conditions do not allow sufficient maturation to induce UCP3 expression to the level measured in mature skeletal muscle fibers.

Human Muscle Regeneration in Immunodeficient RAG2−/−/c−/C5−/− Mice after Myoblast Transplantation—Human myoblasts were injected into the TA muscle of an immunodeficient RAG2−/−/c−C5− mouse. The presence of human muscle fibers in regenerated TA muscle was verified by immunohistochem-

### Table I

Primers sequence designed for and used in the quantitative PCR experiments for the measurement of the expression of mouse and human uncoupling protein-3 and control mouse a-actin.

| Primers | Forward | Reverse |
|---------|---------|---------|
| Human β2-microglobulin | 5'-GTG-TCT-GGG-TTT-CAT-CCA-TC-3' | 5'-TAC-AAA-CAT-CAC-GTT-CC-3' |
| Mouse β2-microglobulin | 5'-TCA-GTA-GCA-CTA-CTC-GAC-GTC-3' | 5'-TCA-GTA-ACA-CAG-TTC-CAC-CC-3' |
| Human UCP3 | 5'-TCA-CCT-CCA-GGC-CAG-TAC-TT-3' | 5'-TCA-GTA-ACA-CAG-TTC-CAC-CC-3' |
| Mouse UCP3 | 5'-CCG-GGA-ATA-CTC-GGT-GTG-GA-3' | 5'-CCC-CCT-CAT-TGA-GAA-3' |
| Human a-actin | 5'-GGG-ATC-GGA-TGC-3' | 5'-TAA-CAA-GAG-TTC-GAT-3' |

### Table II

Evaluation of UCP3 mRNA level in myoblast, myotubes, CHQ5B cells, and in human muscle biopsies by real-time RT-PCR

| Sample Type          | REL  | Standard deviation |
|----------------------|------|--------------------|
| Human skeletal muscle | 0.22 | 0.0015             |
| CHQ5B                | 0.0044 | 0.00025            |
| Differentiated CHQ5B | 0.00052 | 0.00013            |
istry using a species-specific (human, non-mouse) antibody against spectrin that specifically stains the basal lamina fibers (Fig. 1A). Antibodies against spectrin, which specifically stains the basal lamina of human fibers, (A, red; Alexa Fluor 594) and slow adult MHC (B, green; Alexa Fluor 488) were used to demonstrate the presence of mature, differentiated fibers of human origin. The asterisks in A and B show an example of a mature spectrin-positive human fiber that also expresses the slow MHC. The arrows indicate an immature human fiber that is negative for slow MHC. Bar = 60 μm.

FIG. 1. Immunofluorescence staining was performed on 5-μm serial transverse sections of the RAG2/c5 mouse tibialis anterior muscle, 8 weeks following implantation of human myoblast. Antibodies against spectrin, which specifically stains the basal lamina of human fibers, (A, red; Alexa Fluor 594) and slow adult MHC (B, green; Alexa Fluor 488) were used to demonstrate the presence of mature, differentiated fibers of human origin. The asterisks in A and B show an example of a mature spectrin-positive human fiber that also expresses the slow MHC. The arrows indicate an immature human fiber that is negative for slow MHC. Bar = 60 μm.

Fig. 2. α-Actin mRNA expression was measured in RAG2−/−/c5− mouse tibialis muscle 8 weeks following CHQ5B myoblast transplantation. α-Actin mRNA expression was measured by real-time RT-PCR. Column 1, human muscle; column 2, muscle 8 weeks after transplantation; column 3, CHQ5B cells; column 4, differentiated CHQ5B cells. βm2, β2-microglobulin.

Fig. 3. hUCP3 mRNA expression in muscle fibers after transplantation in of RAG2−/−/c5− by real-time RT-PCR. UCP3 mRNA levels were measured 1, 2, 4, 6, 8, 9, and 10 weeks later. mRNA levels in human muscle biopsies are also presented. hβm2, human β2-microglobulin.

Fig. 4. Effect of T3 on UCP3 mRNA level. T3 was injected intraperitoneally into RAG2−/−/c5− mice, and 24 h later transplanted tibialis muscles were removed. Mice received a single T3 injection at 2.5, 10, and 100 μg/100g body mass (in 100 μl), whereas the other received vehicle (5 mM NaOH). UCP3 mRNA expression was measured by real-time RT-PCR. mβm2, mouse β2-microglobulin; hβm2, human β2-microglobulin.

FIG. 2 . hUCP3 mRNA expression was measured in RAG2−/−/c5− mouse tibialis muscle 8 weeks following CHQ5B myoblast transplantation. α-Actin mRNA expression was measured by real-time RT-PCR. Column 1, human muscle; column 2, muscle 8 weeks after transplantation; column 3, CHQ5B cells; column 4, differentiated CHQ5B cells. βm2, β2-microglobulin.

FIG. 3 . hUCP3 mRNA expression in muscle fibers after transplantation in of RAG2−/−/c5− by real-time RT-PCR. UCP3 mRNA levels were measured 1, 2, 4, 6, 8, 9, and 10 weeks later. mRNA levels in human muscle biopsies are also presented. hβm2, human β2-microglobulin.

FIG. 4 . Effect of T3 on UCP3 mRNA level. T3 was injected intraperitoneally into RAG2−/−/c5− mice, and 24 h later transplanted tibialis muscles were removed. Mice received a single T3 injection at 2.5, 10, and 100 μg/100g body mass (in 100 μl), whereas the other received vehicle (5 mM NaOH). UCP3 mRNA expression was measured by real-time RT-PCR. mβm2, mouse β2-microglobulin; hβm2, human β2-microglobulin.

skeletal muscle sections could be clearly seen 8 week after transplantation (Fig. 1B). The human α-actin was amplified by real-time RT-PCR using human-specific primers that did not cross-react with the mouse α-actin. Fig. 2 shows that 8 weeks after transplantation, the α-actin mRNA level reached a level similar to that measured in the human muscle biopsies, whereas in myoblast and myotubes CHQ5B cultured in vitro, the α-actin mRNA level was, respectively, 1272- and 315-fold less than in human muscle biopsies. PCR amplification of the α-actin product was not due to genomic contamination, since PCR amplification without reverse transcriptase did not produce any fluorescent signal. The specificity of the PCR products was assessed by Southern blotting with an internal oligonucleotide probe.

Together these results demonstrate that transplantation of human myoblasts into RAG2−/−/c5− mouse muscles produces fully differentiated human muscle fibers that express UCP3.

Recovery of hUCP3 mRNA Level in Implanted Human Myoblasts—The hUCP3 mRNA level was measured immediately after myoblast transplantation and at 1-, 2-, 4-, 6-, 7-, 8-, 9-, and 10-week intervals (Fig. 3). These results show that the hUCP3
mRNA level increased between 2 and 7 weeks after transplantation and then reached a plateau at 7–8 weeks, at which time hUCP3 mRNA levels in human implants and human muscle biopsies were comparable (Fig. 3).

T3 Has a Similar Effect on hUCP3 and mUCP3 mRNA Expression—Eight weeks after transplantation, mice were injected intraperitoneally with T3, and 24 h later the TA muscles were removed. The in vivo effect of T3 on both human and mouse UCP3 mRNA expression is presented in Fig. 4. T3 induced a dose-dependent increase in hUCP3 mRNA levels with 2-, 6.7-, and 8.5-fold induction at 0.1, 2.5, and 10 mg/kg T3, respectively (Fig. 4A). Similarly, T3 induced a dose-dependent increase in mUCP3 mRNA levels with 3.6-, 4.6-, and 6.9-fold induction at 0.1, 2.5, and 10 mg/kg T3, respectively (Fig. 4B).

DISCUSSION

In this study we described for the first time an efficient model study UCP3 gene expression in human myoblasts. In this model, following transplantation of human CHQ5B myoblasts into the TA muscle of immunodeficient mice, RAG2-/-/c/c5 mice, human muscle fibers were formed. When human myoblasts were cultured using standard in vitro culture conditions, UCP3 gene expression was hardly detectable compared with its expression in vivo. In this study, we first confirmed that transplantation of human myoblasts into regenerating TA muscle of adult MHC 8 weeks post-transplantation, confirmed the presence of regenerated mature human muscle fibers in the TA of RAG2-/-/c/c5 mice produced fully differentiated human muscle fibers. The expression of human spectrin and differentiation markers, including the human α-actin and the slow adult MHC 8 weeks post-transplantation, confirmed the presence of regenerated mature human muscle fibers in the TA of RAG2-/-/c/c5 mice. Second, we showed that transplantation of human myoblasts restores the expression of the human UCP3 gene from a very low level in myoblast to a level similar to that found in human muscle biopsies. In the reconstructed human skeletal muscle UCP3 gene expression followed a kinetic pattern that reached a plateau at 7–8 weeks post-transplantation. Taken together these results indicate that an increase in UCP3 gene expression at 7–8 weeks following human myoblast transplantation was associated with in vivo human muscle cell differentiation and maturation. The results also suggest that an additional specific in vivo transcriptional program is required to trigger UCP3 gene expression. Alternatively, in vivo biological signals absent in vitro might participate in UCP3 gene expression. Numerous studies have shown that T3 treatment strongly stimulates UCP3 gene expression in rodent and human skeletal muscles. As expected, we found that T3 treatment increased UCP3 mRNA in a dose-dependent manner in human implants, thus confirming the stimulatory effect of T3 on UCP3 gene expression. In addition, UCP3 gene regulation by T3 in humans and mice likely shares common features, as T3 treatment increases both human and mouse UCP3 transcripts to a similar level. Several mechanisms could account for the effect of T3 on UCP3 gene expression. The UCP3 promoter contains a sequence that varies by a single base from the canonical thyroid response element (TRE) (20), but it is not known whether this putative TRE is functional. Interestingly, the mUCP3 promoter might also contain a similar TRE motif. Therefore, the transplant of human myoblasts stably expressing the UCP3 promoter region should help to functionally characterize the putative TRE-responsive element.

Despite the increasing number of reports on UCP3, the molecular and/or cellular mechanism controlling UCP3 gene expression in human skeletal muscle remains poorly understood. This might be due to the fact that all studies aiming at characterizing UCP3 promoter were carried out in vitro where the UCP3 gene is poorly expressed, thus making it difficult to identify the cis-regulatory elements controlling UCP3 gene transcription. The means to restore UCP3 gene expression in differentiated human myoblast in vitro has not yet been found, despite considerable research on this topic. Therefore, myoblast transplantation into immunodeficient mouse skeletal muscles appears to be the model of choice to study human UCP3 gene regulation and its promoter. Screening assays based on promoter knowledge should help to select compounds that stimulate hUCP3 gene expression with a view to potential application in obesity therapy.

Acknowledgments—We are extremely grateful to D. Thissen and E. Ecocruff for in vivo expertise. We also thank J. Richard and C. De Montrion for kind interest and support.

REFERENCES

1. Solanes, G., Vidal-Puig, A., Grujic, D., Flier, J. S., and Lowell, B. B. (1997) J. Biol. Chem. 272, 52433–52436
2. Vidal-Puig, A., Solanes, G., Dru, J. S., and Lowell, B. B. (1997) Biochem. Biophys. Res. Commun. 235, 79–82
3. Ross, O., Samec, S., Paolini-Giacobino, A., Rossier, C., Dullo, A., Seydoux, J., Muzin, P., and Giacobino, J. P. (1997) FEBS Lett. 408, 39–42
4. Zhang, C. Y., Hagen, T., Mootha, V. K., Sieker, L. J., and Lowell, B. B. (1999) FEBS Lett. 449, 129–134
5. Bloch, P. (1999) Int. J. Obes. Relat. Metab. Disord. 23, S19–S23
6. Vidal-Puig, A., Grujic, D., Zhang, C. Y., Hagen, T., Boss, O., Ito, Y., Szczezapuka, A., Wadie, J., Mootha, V., Cartwright, B., Musio, M., and Berridge, M. J. (1999) J. Biol. Chem. 274, 16258–16266
7. Gong, D. W., Monjemdou, S., Gavrilova, O., Leon, L. R., Marcus-Samuels, B., Cuz, C. J., Everett, E. C., Konak, L. P., Li, C., Deng, C., Harper, M. E., and Reitman, M. L. (2000) J. Biol. Chem. 275, 16251–16257
8. Clapham, J. C., Arch, J. R., Chapman, H., Haynes, A., Lister, C., Moore, G. B., Frier, V., Carter, S. A., Yemm, W. F., Smith, S. A., Booley, L. J., Godden, R. J., Herrity, N., Skel, M., Changani, K. K., Hockings, P. D., Reid, D. G., Squires, S. M., Hatcher, J., Trail, B., Latcham, J., Bastian, S., Harper, A. J., Rademachers, S., Buckingham, J. A., Brand, M. D., and Aubin, A. (2000) Nature 406, 415–418
9. Cadenas, S., Echtay, K. S., Harper, J. A., Jakobson, M. B., Buckingham, J. A., Grau, E., Aubin, A., Chapman, J. C., and Brand, M. D. (2002) J. Biol. Chem. 277, 3773–3778
10. Harper, J. A., Stuart, J. A., Jakobson, M. B., Roussel, D., Brindle, K. M., Dickinson, K., Jones, R. B., and Brand, M. D. (2002) Biochem. J. 361, 49–56
11. Argyropoulos, G., Brown, A. M., Willi, S. M., Zhu, H., Y. Reitman, M., Geva, S. M., Spruijl, I., and Garvey, W. T. (1998) J. Clin. Invest. 102, 1345–1351
12. Ogata, S., Clement, K., Dubois, S., Lapretre, P., Folloux, V., Leibl, R., Chung, W., Boutil, P., Fugroed, P., and Vasseur, F. (1999) Diabetes 48, 206–208
13. Ogata, S., Clement, K., Dina, C., Folloux, V., Guy-Grand, B., Fugroed, P., and Vasseur, F. (2000) Diabetologia 43, 245–249
14. Gong, D. W., He, Y., Karas, M., and Reitman, M. (1997) J. Biol. Chem. 272, 24129–24132
15. Millet, L., Vidal, H., Airelle, F., Larrouy, D., Roussel, D., Brindle, K. M., Dickinson, K., Jones, R. B., and Brand, M. D. (2002) Biochem. J. 361, 49–56
16. Brun, S., Carmona, M. C., Mampel, T., Vinas, O., Giralt, M., and Villarroya, F. (1999) FEBS Lett. 453, 205–209
17. Matsuda, J., Hosada, K., Itoh, H., Son, C., Doi, K., Tanaka, T., Fukunaga, Y., Inose, G., Nishimura, H., Yokomasa, Y., Yamori, Y., and Nakao, K. (1997) FEBS Lett. 418, 200–204
18. Brun, S., Carmona, M. C., Mampel, T., Vinas, O., Giralt, M., and Villarroya, F. (1999) Diabetes 48, 1217–1222
19. Khalfallah, Y., Fage, S., Lavelle, M., Langin, D., and Vidal, H. (2000) Diabetes 49, 25–31
20. Acín, A., Rodríguez, M., Rique, H., Canet, E., Boutsin, J. A., and Galiliz, J. P. (1999) Biochem. Biophys. Res. Commun. 258, 278–283
Human UCP3 Expression in Immunodeficient Mice

21. Cooper, R. N., Irintchev, A., Di Santo, J. P., Zweyer, M., Morgan, J. E., Partridge, T. A., Butler-Browne, G. S., Mouly, V., and Wernig, A. (2001) Hum. Gene Ther. 12, 823–831
22. Decary, S., Mouly, V., and Butler-Browne, G. S. (1996) Hum. Gene Ther. 7, 1347–1350
23. Edom-Vovard, F., Mouly, V., Barbet, J. P., and Butler-Browne, G. S. (1999) J. Cell Sci. 112, 191–199
24. Kaufman, S. J., and Foster, R. F. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9606–9610
25. Irintchev, A., Langer, M., Zweyer, M., Theisen, R., and Wernig, A. (1997) J. Physiol. (Lond.) 500, 775–785
26. Nagase, I., Yoshida, S., Canas, X., Irie, Y., Kimura, K., Yoshida, T., and Saito, M. (1999) FEBS Lett. 461, 319–322
27. Hwang, C. S., and Lane, M. D. (1999) Biochem. Biophys. Res. Commun. 258, 464–469
28. Michael, L. F., Wu, Z., Cheatham, R. B., Puigserver, P., Adelmant, G., Lehman, J. J., Kelly, D. P., and Spiegelman, B. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3820–3825
Uncoupling Protein-3 (UCP3) mRNA Expression in Reconstituted Human Muscle after Myoblast Transplantation in RAG2−/−/c/c5−/− Immunodeficient Mice

Nolwen Guigal, Marianne Rodriguez, Raquel N. Cooper, Sandra Dromaint, James P. Di Santo, Vincent Moully, Jean A. Boutin and Jean-Pierre Galizzi

J. Biol. Chem. 2002, 277:47407-47411.
doi: 10.1074/jbc.M208048200 originally published online September 25, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208048200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 10 of which can be accessed free at
http://www.jbc.org/content/277/49/47407.full.html#ref-list-1