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(Received for publication, November 18, 2008, accepted, July 6, 2009 and published online, July 18, 2009)

THE JOURNAL OF GENE MEDICINE
J Gene Med 2008; **10**: 217–224.
Published online 12 December 2007 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/jgm.1140

RESEARCH ARTICLE

Drug-induced readthrough of premature stop codons leads to the stabilization of laminin α2 chain mRNA in CMD myotubes

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Received: 6 July 2007
Revised: 17 September 2007
Accepted: 19 October 2007

Abstract

Background The most common form of congenital muscular dystrophy is caused by a deficiency in the α2 chain of laminin-211, a protein of the extracellular matrix. A wide variety of mutations, including 20 to 30% of nonsense mutations, have been identified in the corresponding gene, *LAMA2*. A promising approach for the treatment of genetic disorders due to premature termination codons (PTCs) is the use of drugs to force stop codon readthrough.

Methods Here, we analyzed the effects of two compounds on a PTC in the *LAMA2* gene that targets the mRNA to nonsense-mediated RNA decay, *in vitro* using a dual reporter assay, as well as *ex vivo* in patient-derived myotubes.

Results We first showed that both gentamicin and negamycin promote significant readthrough of this PTC. We then demonstrated that the mutant mRNAs were strongly stabilized in patient-derived myotubes after administration of negamycin, but not gentamicin. Nevertheless, neither treatment allowed re-expression of the laminin α2-chain protein, pointing to problems that may have arisen at the translational or post-translational levels.

Conclusions Taken together, our results emphasize that achievement of a clinical benefit upon treatment with novel readthrough-inducing agents would require several favourable conditions including PTC nucleotide context, intrinsic and induced stability of mRNA and correct synthesis of a full-length active protein. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords congenital muscular dystrophy; laminin α2 chain; premature termination codon; antibiotic-mediated readthrough; nonsense-mediated mRNA decay

Introduction

Congenital muscular dystrophy (CMD) is a clinically and genetically heterogeneous group of neuromuscular disorders with autosomal recessive inheritance. The 'classic' (or occidental) form of CMD is characterized by clinical manifestations mainly affecting skeletal muscle [1–4]. A specific deficiency of the α2 chain of laminin-211 is responsible for about 30 to 40% of these cases (MDC1A, MIM#607855). Numerous mutations have now been identified in the *LAMA2* gene encoding the α2 chain of laminin, leading to either complete or partial protein deficiency [5–9]. Notably, 20 to 30% of the mutations identified in the *LAMA2* gene are nonsense mutations leading to a premature termination codon (PTC).

Thus, up to 15% of 'classical' CMD patients would potentially benefit from a pharmacological strategy designed to promote translation of the endogenous *LAMA2* mRNA interrupted by a PTC. From early work in *Escherichia coli*, it has been demonstrated that aminoglycoside antibiotics such as gentamicin can specifically act on ribosomal RNA to suppress codon-anticodon recognition by aminoacyl tRNAs, thereby interfering with the translation termination process and suppressing stop codons [10–14]. Aminoglycoside-mediated translational readthrough of PTCs involved in several human genetic disorders, including Duchenne muscular dystrophy, has now been reported with variable degrees of success [15–26]. It has also become increasingly evident that only a small subset of stop codon mutations would benefit from gentamicin treatment, depending on their nucleotide context [21,27–30]. In addition, strong side effects of gentamicin, the most commonly used aminoglycoside antibiotic, have been well documented [31,32] and hamper its utilization as a potential therapeutic agent especially if long-term treatment is required. Furthermore, gentamicin-induced readthrough is dependent on its chemical composition and its reproducibility is debatable [33].

To address these problems, we tested two other compounds with antibiotic activities, negamycin and amikacin [34], known to provide effective suppression of nonsense mutations in cellular and animal models and to present a lower toxicity than gentamicin [20,34–38]. Unlike gentamicin and amikacin, that belong to the aminoglycoside family, negamycin is a dipeptide antibiotic, which also interacts with the ribosomal A site to mediate readthrough [38].

Due to the absence of an adequate animal model for MDC1A, we chose an *ex vivo* approach based on muscle explants obtained from a patient presenting a complete merosin deficiency due to a homozygous nonsense mutation (c.C4687A) in exon 31 of the *LAMA2* gene causing a PTC (C1546X) [39]. We treated myotubes derived from this patient with either gentamicin or negamycin. Since readthrough efficiency depends on the nature of the PTC and its surrounding sequences [21,28,40–43], in parallel we measured termination readthrough for this specific *LAMA2* mutation, in its nucleotidic context, in a sensitive and reproducible dual reporter assay [40,43]. In addition, the presence of PTCs often results in the rapid degradation of the mutant mRNA by the nonsense-mediated mRNA decay (NMD) pathway (for a review, see [44]). It has been suggested that gentamicin, by allowing some translational readthrough, may result in reduced levels of NMD [14,45].

In this study, using a dual reporter assay, we first demonstrated, *ex vivo* as well as *in vivo*, that negamycin is as effective as gentamicin in inducing PTC readthrough. Interestingly, negamycin-induced readthrough of the *LAMA2* PTC studied here was among the highest levels reached using this assay. Moreover, by quantitative reverse-transcription polymerase chain reaction (RT-PCR) we demonstrated that negamycin treatment, in contrast to gentamicin, strongly stabilized the patient's laminin α2-chain mRNA levels. Indeed, mutant mRNA levels were significantly decreased in the untreated patient's cells, likely due to the NMD pathway. However, re-expression of the laminin α2-chain protein could not be detected in our experimental conditions.

In conclusion, our data demonstrate that for some mutations, stabilization of the mutant mRNA can be obtained through a sufficient level of readthrough, which, in some cases, could allow a synergistic effect on PTC suppression. Nonetheless, this may not be sufficient to allow re-expression of a functional full-length protein, suggesting that numerous steps need to be fulfilled to obtain a clinical benefit through PTC-induced suppression.

Materials and methods

Cell culture and transfection

NIH3T3 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Gibco, Cergy, France) supplemented with 7% fetal calf serum (Gibco BRL), and incubated at 37 °C in humidity saturated 6.5% CO_2. Cells were electroporated with 18 µg of each pAC(lacZ-luc) plasmid construct, as described [21], and plated immediately in culture medium without antibiotic. After 20 h and 28 h the medium was replaced with fresh medium complemented or not with gentamicin (Invitrogen-Gibco, Cergy, France), negamycin (both used at 600 µg/ml) or amikacin (GIBCO BRL; at 2mg/ml). Two days after transfection, cells were harvested, spun for 5 min at 4 °C and lysed by repeated pipetting in 150 µl of cold luciferase assay buffer. For each construct, at least five independent transfection experiments were performed.

Reporter plasmid cloning and stop mutation targets

Complementary oligonucleotides corresponding to the sequence of each stop mutation embedded in its natural nucleotide context were annealed and cloned in a dual gene reporter system between the lacZ and luc coding sequences as previously described [21]. The nucleotidic context around the C1546X stop mutation sequence is: 5′ GTC ACA GGA TTC TGA ACG TGC CGA CCT 3′. For the other stop targets oligonucleotides sequences were previously published [21].

Enzyme assays

Luciferase and β-galactosidase activities were measured from the same crude extract as described [43]. Stop codon readthrough was calculated by dividing the ratio of luciferase to β-galactosidase activity obtained with a given test construct by the ratio obtained from the control

construct where the stop codon was replaced by a sense codon. Each value of readthrough efficiency corresponds to the mean of five to six independent experiments. Standard deviation errors did not exceed 20%.

Cell culture and immunohistochemistry

Primary myoblasts obtained from a control foetus (12 weeks of gestation) and a merosin-deficient CMD foetus (15 weeks of gestation), presenting a homozygous nonsense mutation in exon 31 of the *LAMA2* gene, were grown in F10 medium + 20% SVF + penicillin/streptomycin (10 U/ml and 10 μg/ml, respectively; Invitrogen-Gibco). Muscle cells were obtained in accordance with the French legislation on ethical rules. Myoblasts were allowed to fuse for 7 days on 0.5% gelatin-coated coverslips in antibiotic-free or negamycin-containing (300 μg/ml; Institute of Microbiology, Tokyo, Japan) differentiation medium (DMEM 4.5 g glucose + 2% horse serum + 10^{-6} M insulin + 2.5×10^{-6} M dexamethasone). Immunohistochemical analyses were performed on methanol-fixed cells (10 min at 4 °C) as follows: non-specific sites were blocked for 30 min in 5% bovine serum albumin (BSA)/1X phosphate-buffered saline (PBS), and cells were then incubated overnight at 4 °C with primary antibodies against myosin (MF20, a gift from Dr. Denis Furling, Inserm-UMR S 787, Institut de Myologie, Paris, France) and laminin α2 chain (4H8-2, Alexis Biochemicals, Lausen, Switzerland) diluted into 1% BSA/1X PBS. Following three washes in 1% BSA/1X PBS, cells were incubated for 90 min in appropriate FITC or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). After three additional washes, cells were mounted with Mowiol containing DAPI (0.05 μg/μl final) to stain all of the nuclei and observed under a Zeiss microscope. Images were captured under identical conditions using the Metaview software (Ropper Scientific GmbH, Germany).

RNA extraction and quantitative RT-PCR

Total RNAs were extracted using conventional TRIzol™ reagent (Life Technologies, Inc.) extraction protocol as recommended by the manufacturer. RNA quality control and quantification was performed using the Bioanalyzer apparatus (Agilent). First strand cDNA was synthesized from 1 μg of total RNA using oligo$(dT)_{18}$ and Superscript reverse transcriptase II (Invitrogen-Gibco, Cergy, France) as recommended by the manufacturer. Quantitative PCR was then carried out on equal amounts of cDNAs, using the LightCycler real-time PCR machine (Roche Diagnostics, Germany), and *LAMA2* mRNA levels were quantified relative to the mRNA of the troponin T1 (*TPNT1*) gene. In each experiment, results were expressed relative to control myotubes normalized to 100. The oligonucleotide pairs used for the amplifications are as follows: *TPNT1* forward: 5′ TGGAGCTGCAGACACTCATC 3′ and reverse: 5′ GCTTCTGTTCTGCCTTGACC 3′; *LAMA2* forward: 5′ TGTGCTGCAGAATCAGAACC 3′ and reverse: 5′ ATTGATTTTGGTGGGGATCA 3′. Mean values of several quantitative RT-PCRs (n = 4) are presented + standard deviation to mean. Statistical significance was determined by a t-test ($^*p < 0.001$).

Protein extraction and immunodetection

Total proteins were extracted in SDS buffer (80 mM Tris-HCl, pH6.8, 10% SDS, 0.12 M sucrose, 10 mM EDTA, 1 mM PMSF, 1 mM benzamidine). Concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with BSA as a standard. Proteins were subsequently resolved under reducing conditions by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred to PVDF membranes. Detection of the laminin α2 chain was performed by using an affinity purified polyclonal antibody (rabbit 180, a gift from Dr. Kevin P. Campbell, Molecular Physiology and Biophysics, Howard Hughes Medical Institute, Iowa City, USA). HRP-conjugated secondary antibody anti-rabbit IgG (DakoCytomation, Glostrup, Denmark) was used at a dilution of 1 : 2000. Immunoblots were developed using enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate; Pierce, Rockford, IL). In order to control the amounts of proteins which had been loaded, the gel was stained with Coomassie blue following transfer.

In vivo studies

All animal studies conformed to the guidelines on animal use procedures approved by Inserm. Mice were anesthetized by intraperitoneal injection of 10 mg/ml ketamine (Clorketam 1000; Vétoquinols SA, Lure, France) and 5 mg/ml xylazine (Rompun 2%; Bayer Pharma, Puteaux, France). Plasmid DNA (35 μg) was injected percutaneously into the tibialis anterior muscle. Two minutes after the injection, transcutaneous electric pulses were applied through external stainless steel plates (200 V/cm, 8 pulses of 20 ms each, 2 Hz) on a ECM 830 BTX electroporator (San Diego, USA).

Animals were subsequently treated with gentamicin (34 mg/kg; Invitrogen-Gibco) or PBS once daily for 3 days, by percutaneous injections. On the last day, mice were euthanized and the injected muscles were harvested.

Results

Negamycin promotes readthrough in a dual reporter assay in NIH3T3 cells

The C1546X mutation in the *LAMA2* gene leads to a UGA premature termination codon (PTC) which has been

reported to be the most sensitive codon to aminoglycoside-induced readthrough [28,41,42]. Using a previously published dual reporter assay [40], we demonstrated that this PTC is responsive to amikacin and gentamicin (0.4% and 0.6%, respectively, which correspond to 2.5- and 3.8-fold increases in readthrough; Figure 1). Interestingly, readthrough was increased up to 3% (19-fold) following negamycin treatment which is among the highest levels we have found using this assay [21]. To examine more precisely the effect of negamycin on other stop targets, five other nonsense mutations leading to PTCs in either the human or murine dystrophin genes (UGA/319d and UAA/mdx, respectively) and the *LAMA2* gene (UGA/1326c, UAG/1437c, UAA/1240c), as well as the natural termination codon of the *LAMA2* gene (UGA/STOP LAM), were tested under the same conditions. These targets have previously been analyzed for gentamicin sensitivity [21]. For six of the seven stop targets presented in Figure 1, negamycin is equally or more effective than gentamicin in promoting readthrough. Nevertheless, in five of these targets the efficiency remained modest (0.4%; 2.5-fold). Only one stop mutation (UGA/319d) was more sensitive to gentamicin (2.6%, 44-fold) than to negamycin (1.2%, 20-fold). Thus, among six mutations only two can be highly bypassed by antibiotic treatment: C1546X by negamycin and UGA/319d by gentamicin; the latter also being moderately bypassed by negamycin.

Negamycin allows readthrough *in vivo*

Since the availability and metabolism of negamycin might be modulated *in vivo*, the results in cultured cells might not reflect exactly the situation in muscles.

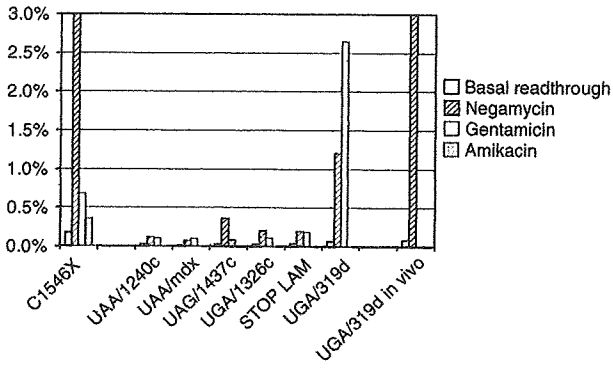

Figure 1. Induction of readthrough in NIH3T3 cells. Sequences corresponding to the C1546X stop mutation as well as other stop targets corresponding to DMC (c) or DMD (d) stop mutations or to the natural termination codon of the *LAMA2* gene (UGA/STOP LAM) were cloned in a dual gene reporter system between lacZ and luc coding sequences. NIH3T3 cells were treated with gentamicin, negamycin or amikacin. Termination readthrough is expressed as the luciferase/β-galactosidase ratio of the test construct normalized to an in-frame control where stop codons have been replaced by a sense codon. Each value of readthrough efficiency corresponds to the mean of four to six independent experiments. Standard deviation errors did not exceed 20%

As previously shown for gentamicin [21], we assessed the effect of negamycin *in vivo*, in C57BL/6 mice. The vector carrying the UGA/319d mutation was injected into skeletal muscle with subsequent electrotransfer and thereafter mice were treated intramuscularly once daily with negamycin (34 mg/kg) or PBS for the following 3 days. Results showed that both basal and negamycin-induced readthrough efficiency were similar in NIH3T3 cells and *in vivo*, increasing from 0.08% in the absence to 3% (38-fold) in the presence of the drug (Figure 1).

Stabilization of *LAMA2* mRNA in negamycin-treated myotubes

The mutation in exon 31 of the *LAMA2* gene leads to the replacement of a cysteine residue by a premature UGA stop (C1546X) that is placed more than 50 nucleotides upstream of the 3′ most exon-exon junction and is therefore in a favourable condition for the degradation of the mutant mRNA by the NMD pathway [44]. Indeed, quantitative RT-PCR analysis demonstrated a significant decrease in the level of *LAMA2* transcripts which were reduced to 6.25% of control values (Figure 2). We hypothesized that, by promoting translational readthrough of the mutant mRNA, gentamicin and negamycin would prevent its rapid degradation by the NMD pathway. Treatment of the patient's myotubes by two different batches of gentamicin (GIBCO BRL or American Pharmaceutical Partners, Inc.) did not trigger a significant increase in the level of transcripts. However, negamycin treatment strongly sustained the stabilization of the *LAMA2* transcripts up to 77% of control levels (Figure 2). These results are in agreement with those obtained in *S. cerevisiae* where previous studies indicated an inverse relationship between readthrough level and NMD-dependent decrease in mRNA abundance [20,46]. Moreover, the mRNA quantification data can be correlated to those obtained in our cell culture expression system in which negamycin was more efficient than gentamicin in promoting translational readthrough of the *LAMA2* nonsense mutation (Figure 1).

Neither gentamicin nor negamycin enabled re-expression of laminin α2 chain in MDC1A myotubes

In parallel to the mRNA studies, we investigated whether gentamicin or negamycin treatment induced re-expression of the laminin α2 chain in the patients' myotubes at the protein level. Control and MDC1A myoblasts were allowed to differentiate *ex vivo* for 7 days and subsequently myotubes were treated with gentamicin (GIBCO BRL or American Pharmaceutical Partners, Inc.) or negamycin for 7 or 15 days. Expression of myosin and the laminin α2 chain was then assessed on fixed myotubes by immunohistochemistry (Figure 3A). First, we observed that MDC1A myoblasts were indeed able to efficiently differentiate into myotubes as indicated by

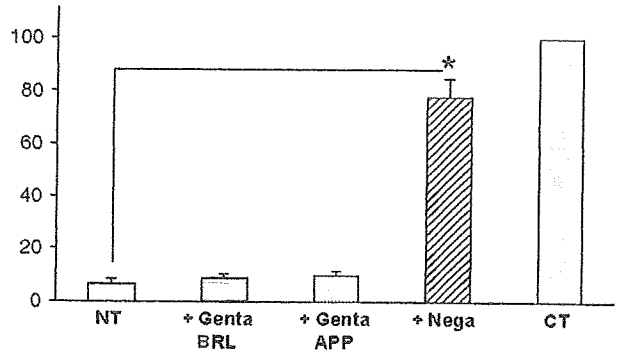

Figure 2. *LAMA2* mRNA levels in cultured MDC1A myotubes treated with gentamicin and negamycin. Quantitative PCR were carried out on equal amounts of cDNAs, using the LightCycler real-time PCR machine (Roche Diagnostics, Germany). *LAMA2* mRNA levels were quantified relative to the mRNA of the troponin T1 (*TPNT1*) gene. In each experiment, results were expressed relative to control myotubes normalized to 100. Mean values are presented + standard deviation to mean. Statistical significance was determined by a t-test ($^*p < 0.001$)

the strong expression of myosin (panel d) which was comparable to that observed in the control cells (panel a). However, immunohistochemical analyses did not allow detection of the laminin $\alpha 2$ chain following either a 7- or 15-day-long treatment with doses as high as 300 μg/ml of gentamicin or negamycin (panels h, i and data not shown). Biochemical analyses confirmed these results for all three compounds tested (Figure 3B), and even though the detection threshold of our experiment was as low as 1% (Figure 3B and data not shown).

Discussion

Here we present *in vitro* and *in vivo* data that address important questions regarding the potential use of drugs able to induce readthrough of stop codon mutations, to treat genetic disorders due to the presence of a PTC. Indeed, re-expression of a functional protein, which could provide a clinical benefit, is dependent on the correct realization of several steps. We first analyzed the efficacy of drug-mediated readthrough of premature termination codons by different compounds with antibiotic activities. We showed that negamycin, a dipeptide antibiotic, showed a higher suppression activity than gentamicin on several PTCs. In particular, the C1546X *LAMA2* mutation that we analyzed in more detail is especially responsive to negamycin as compared to gentamicin and amikacin. This illustrates the importance of testing the efficiency of treatments in sensitive, rapid, and reproducible systems such as the one presented here. Since there are no hot spots of mutation within the *LAMA2* gene, all mutations are 'private' and the consequences of the treatment may vary considerably between patients. The variable response to readthrough drugs has been recently illustrated in cystic fibrosis patients treated with gentamicin [30]. In that report, it was demonstrated, using the same dual reporter system as in the present work, that suppression of stop mutations in the *CFTR* gene with parenteral gentamicin can be predicted *in vitro*. The study established that in a small subgroup of patients with the Y122X mutation, gentamicin treatment was associated with clinical benefit and significant modification of the CFTR-mediated Cl^- transport in nasal and sweat gland epithelium [30].

Although readthrough-inducing compounds primarily affect translation, it is becoming clear that mutant mRNA stability is a significant factor in the process of termination readthrough. Importantly, we demonstrated that negamycin has a dual action since it enables readthrough levels high enough to strongly counteract mRNA degradation by the NMD pathway. Indeed, it has been shown that termination readthrough above the threshold value of 0.5% antagonizes NMD, leading to mRNA stabilization [46]. This likely explains the striking stabilization of *LAMA2* mRNA observed following negamycin treatment, since we detected 3% of readthrough. On the other hand, the level of gentamicin-induced readthrough (0.6%) being just above the threshold value was probably not sufficient for mRNA stabilization. This would have important consequences for predicting the efficiency of pharmacological readthrough on patients' mutations before envisioning clinical trials, and again stresses the necessity to test *in vitro* the response of individual mutations to pharmacological treatment. Accordingly, gentamicin response was shown to depend on the efficiency of NMD in cystic fibrosis which varied greatly between patients and cell types [47]. In addition, in that study, the authors did not observe stabilization of the nonsense *CFTR* transcripts after gentamicin treatment in patients and cell lines [47], whereas it had been shown that G418, a more potent readthrough drug than gentamicin, could restore CFTR mRNA levels [16].

Recently, PTC124, a new molecule, has been shown to induce ribosomal readthrough of PTCs [48]. Interestingly, the levels of a nonsense-containing mRNA were unaffected in PTC124-treated cells. More generally, the synthesis and stability of few, if any, cellular mRNAs are altered in response to levels of PTC124. Although an off-target effect might be detrimental, the lack of stabilization of PTC-containing mRNAs by PTC124 may limit its use to mutations which do not lead to mRNA degradation by the NMD pathway.

In the present study, since gentamicin treatment did not allow stabilization of the mutant *LAMA2* mRNA, it seems coherent that no re-expression of the protein occurred. On the other hand, considering the extent of mRNA stabilization that we detected following treatment with negamycin, the lack of laminin $\alpha 2$-chain re-expression may appear surprising. Several hypotheses, which are not mutually exclusive, may explain our results: (1) a longer treatment may be needed in order to be able to detect this large extracellular matrix protein; however, it was not possible to treat the cells for longer periods of time since the myotubes started detaching from the gelatine-coated dishes after 16 to 17 days of differentiation. (2) It is thought that PTCs would be misread by near-cognate aminoacyl-tRNAs bearing anti-codons with one

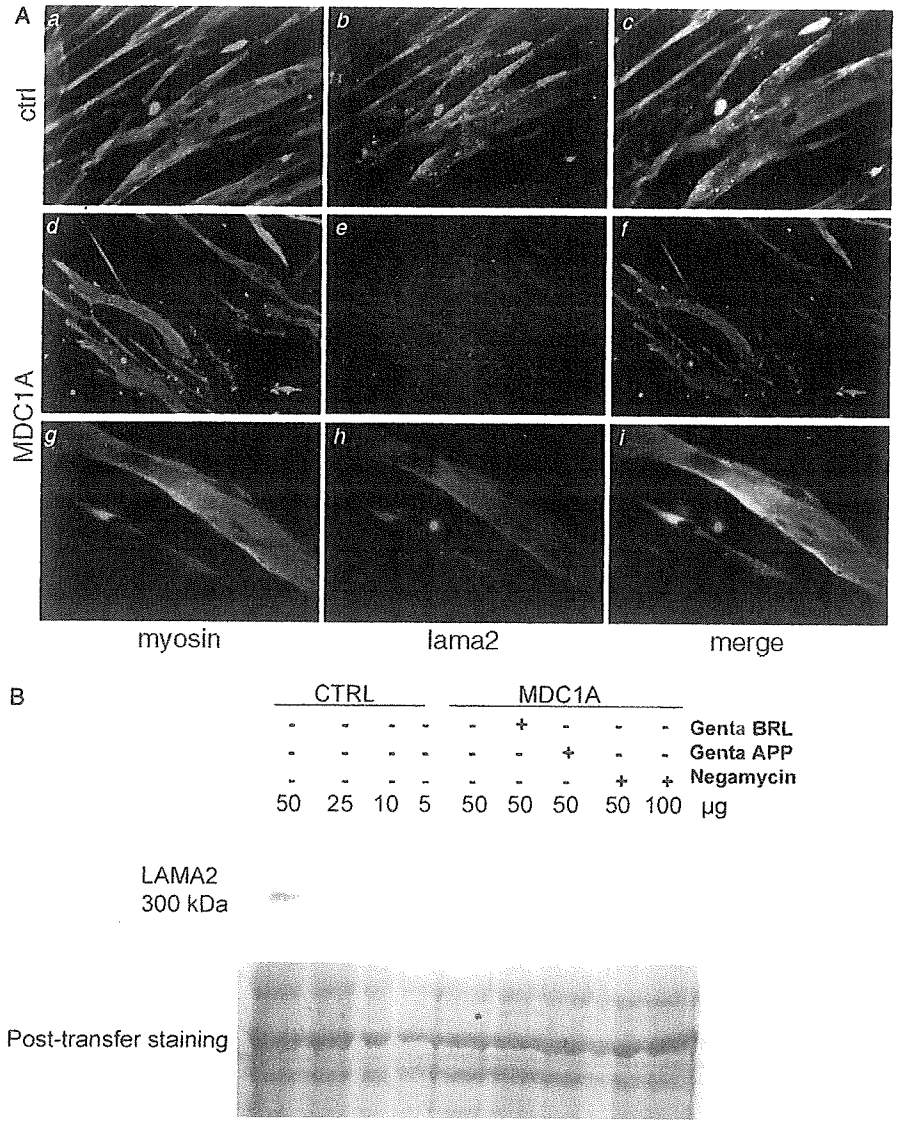

Figure 3. (A) Myosin and laminin α2-chain expression in control and MDC1A cultured myotubes after a 7-day negamycin treatment. Immunohistochemical analyses of primary myotubes from a control foetus (a–c) and a merosin-deficient CMD foetus (d–i) presenting a premature termination codon (C1546X) in the laminin α2 chain with antibodies against myosin (a, d, g) and the laminin α2 chain (b, e, h). Magnification 60× (a–c, g–i). Magnification 25× (d–f). c, f and i are merged images. (B)Western blot analysis in negamycin-treated and untreated control and MDC1A samples. Detection of laminin α2 chain on total proteins using an affinity purified polyclonal antibody (rabbit 180, a gift from Dr. Kevin P. Campbell). In order to control the amounts of proteins which had been loaded, the gel was stained with Coomassie blue following transfer

base difference. In *E. coli*, Nilsson and Ryden-Aulin [49] showed that stop codons UAG and UAA are replaced by glutamine whereas UGA would be substituted by tryptophan. Following this hypothesis, readthrough of the *LAMA2* UGA PTC would thus lead to the replacement of the normal cysteine by a tryptophan or an arginine residue. This residue is localized close to the junction site where the α2 chain of laminin-211 assembles with the β1 and γ1 chains to form the heterotrimeric molecule which will then be secreted. It seems likely that the insertion of a tryptophan residue would perturb this assembly and destabilize the protein which might thereafter be degraded. It should be noted that replacement of the cysteine residue by a tryptophan was predicted to be deleterious by using the Web server PolyPhen [50] for polymorphism phenotyping [51]. It should be emphasized that treatments aiming at re-expressing a full-length protein by suppression of a PTC will be faced with this general problem. Although some structural proteins, like dystrophin, might tolerate large modifications of their coding sequences, enzymatic proteins, which are involved in most genetic disorders, will be very sensitive to the specific residue inserted in place of the PTC. This would necessitate a precise analysis of the site of the mutation in the context of the three-dimensional and functional structure of the protein, and of the potential near-cognate tRNA involved in decoding the stop codon upon drug treatment.

In conclusion, our study demonstrates that negamycin, a compound less toxic and more efficient for premature termination readthrough than gentamicin, appears an attractive alternative for treatment of patients carrying a PTC mutation. Its striking effect on mRNA stabilization suggests that, even for patients with a low level of mutant mRNA, treatment might be effective through readthrough-induced mRNA stabilization. However, although sufficient levels of mRNA are likely to be a preliminary requirement, correct synthesis of the corresponding full-length protein also depends on the effect of the insertion of a novel amino acid in place of the premature stop codon, which should be carefully considered prior to treatment.

Overall, our results emphasize that numerous steps need to be fulfilled to achieve a clinical benefit by inducing suppression of nonsense mutations.

Acknowledgments

We gratefully acknowledge Gaëlle Piron (Inserm UMR-S787, Institut de Myologie, Paris, France) for expert technical advice for the primary cultures of myoblasts. We are grateful to Denis Furling (Inserm UMR-S787, Institut de Myologie, Paris, France) and Kevin P. Campbell (Molecular Physiology and Biophysics, Howard Hughes Medical Institute, Iowa City, USA) for the gift of antibodies MF20 and R180, respectively. We are grateful to Séga N'Diaye (IGM, Université Paris-Sud, Orsay, France) for expert technical assistance with readthrough determination. VA was supported by a grant from the Fondation pour la Recherche Médicale. This work was funded by the Association Française contre les Myopathies (to VA and JPR), Association pour la Recherche sur le Cancer (grant N°3849 to JPR), INSERM and Université Paris 6 Pierre et Marie Curie, and the Japan Foundation for Aging and Health (to RM and PG). MPJ, GSBB and VM are supported by CNRS. This work was supported by a Research Grant (18A-1, to RM) for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare and by a Grant for Research in Brain Science from the Ministry of Health, Labor and Welfare, Japan (to RM).

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Efficient total synthesis of (+)-negamycin, a potential chemotherapeutic agent for genetic diseases†

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Received (in Cambridge, UK) 28th January 2008, Accepted 25th February 2008
First published as an Advance Article on the web 18th March 2008
DOI: 10.1039/b801498a

Herein, we describe an efficient strategy for the total synthesis of (+)-negamycin using commercially available achiral *N*-Boc-2-aminoacetaldehyde as starting material with 42% overall yield for a limited number of steps.

(+)-Negamycin (**1**, Scheme 1), an unusual antibiotic containing a hydrazine peptide bond was isolated for the first time by Umezawa *et al.* in 1970 from culture filtrates of three strains related to *Streptomyces purperfuscus*. This natural product exhibits very low acute toxicity and strong inhibitory activity against multiple drug-resistant enteric Gram-negative bacteria including *Pseudomonas aerginosa*.[1] (+)-**1**'s anti-microbial activity is derived from a genetic miscoding on bacterial ribosomal systems, and thereby leading to a specific inhibition of protein biosynthesis.[2] Because this miscoding causes read-through of termination signals, considerable attention is focused on (+)-**1** as a potential therapeutic agent against genetic diseases. Indeed, the aminoglycoside antibiotic gentamicin and the less toxic negamycin both restore dystrophin expression in skeletal and cardiac muscles of mdx mice, an animal model of Duchenne muscular dystrophy (DMD) with a nonsense mutation in the dystrophin gene.[3] Therefore, an efficient shortened synthetic route of (+)-**1** and its derivatives appears significant to develop promising new therapeutic candidates for DMD and other diseases caused by nonsense mutations. The first total synthesis of (+)-**1** from D-galacturonic acid was reported in 1972 and confirmed the assigned structure of the natural product.[4] Over three decades, numerous total syntheses have been reported on both racemic and optically active (+)-**1** but with moderate overall yield.[5]

Our fast and efficient route consists first on an asymmetric allylboration of *N*-Boc-glycinal **5** using the established Brown's procedure for preparation of chiral allylic alcohols[6] that led to a corresponding chiral intermediate (Scheme 1). This resulting chiral amino alcohol was directly engaged without further purification to form the target oxazolidine **4** by treatment with 2,2-dimethoxypropane (DMP) in the presence of boron trifluoride diethyl ether complex ($BF_3 \cdot Et_2O$) in acetone. As a result, **4** was generated in high yield after purification by silica gel column chromatography (90%). To prepare the key intermediate **3**, a cross-metathesis (CM) reaction between **4** and *tert*-butyl acrylate **6** was investigated. Although the efficiency of ruthenium-based catalysts for ring-opening metathesis polymerization (ROMP) and ring-closing metathesis (RCM) is now well established, most alkene CM variants have fewer successful applications because of the multiple possible side reactions that cause relatively low synthetic yields.[7] Because our substrates are categorized as rapid and slow homodimerizable compounds according to Grubbs *et al.*'s empirical model for predicting the outcome of CM reactions,[8] we screened different reaction conditions to avoid forming unwanted dimers and selectively provide the target compound **3** by varying catalysts (Grubbs first [Ru-I] and second-generation [Ru-II] catalysts), amount of reactant (1 or 5 equiv. of **6**), duration of reaction as well as heating method (conventional or microwave-assisted heating).

As detailed in the ESI,† the conversion and chemoselectivity enhancements are definitely more pronounced for [Ru]-**II** than [Ru]-**I**. Furthermore, we observed that microwave irradiation drastically shortened the CM reaction time by 20-fold. As it pertains to microwave-assisted synthesis, this acceleration is commonly attributed to the very high local temperatures and the ease to which microwave irradiation reaches such conditions but the scientific community is still divided in opinion on the involvement of a specific non-thermal effect induced by the dielectric heating produced using microwaves.[9] Interestingly, although our observations provide a new example for the thermal effect, the involvement of such "specific effect" is neither confirmed nor disproved in these reaction conditions. Bargiggia *et al.* arrived to similar conclusions while studying CM reactions[10] and Garbaccia *et al.* have described similar observations for RCM reactions in 2003.[11] As a result, the desired product **3** was isolated with 83% yield. NOE experiments revealed that the stereochemistry of the olefin moiety in **3** was an *E* configuration ($J_{\text{vinylic protons}}$ = 15.7 Hz). Thus, the desired chiral intermediate **3** was obtained with 75% yield after two steps from achiral *N*-Boc-glycinal **5**.

With intermediate **3** in hand, our focus shifted toward the asymmetric Michael addition reaction. Recently, Node *et al.*

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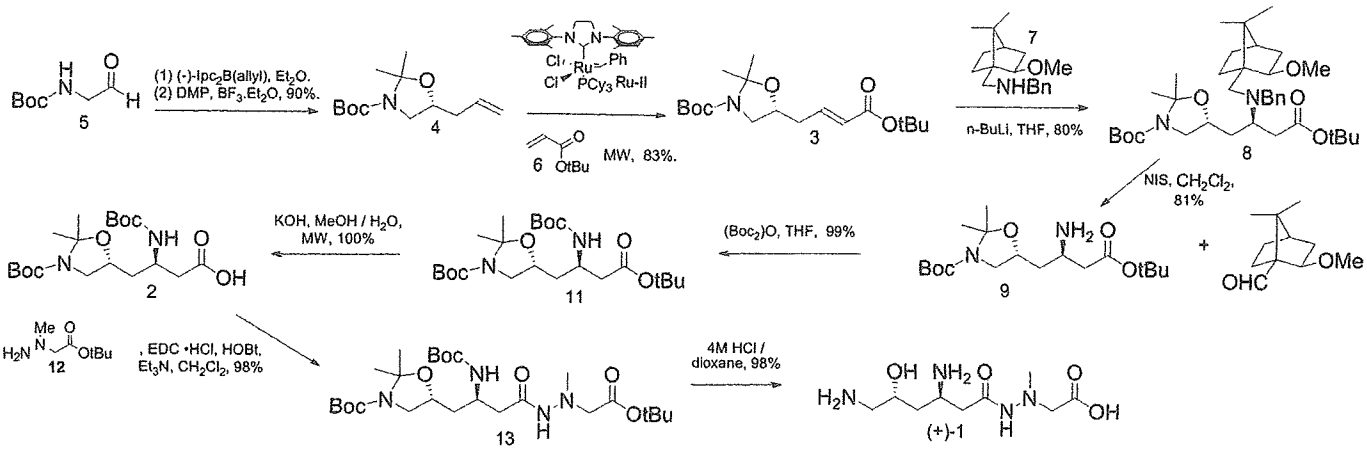
† Electronic supplementary information (ESI) available: Analytical data. See DOI: 10.1039/b801498a



Scheme 1 Total synthesis of (+)-negamycin.

reported a highly stereoselective asymmetric Michael addition toward *tert*-butyl α,β-unsaturated carbonyl compounds using chiral amine **7**.[12] This approach, when applied to the α,β-unsaturated *tert*-butyl ester **3**, allowed the introduction of the amine moiety with an excellent enantiomeric excess.[13] The chiral reagent **7** was prepared from the corresponding keto-pinic acid[12] and reacted with **3** in the presence of *n*-BuLi in THF at −78 °C to afford compound **8** as a single diastereomer (de >99%) in 80% yield after purification. Removal of both benzyl and 2-methoxybornyl protecting groups located on the same amine moiety could be achieved efficiently using 4 equiv. of *N*-iodosuccinimide (NIS) in dichloromethane to obtain free amino compound **9** in 81% yield. This deprotection proceeded by oxidation with NIS to imine and subsequent spontaneous hydrolysis to afford *tert*-butyl esters of β-amino acids and 2-methoxy-D-bornylaldehyde.[12] No epimerization was observed during this reaction. Furthermore, one of the advantages of the protocol is that the initial chiral inducer **7** can be easily regenerated from 2-methoxy-D-bornaldehyde, generated during the cleavage by reductive amination, using benzylamine in the presence of sodium cyanoborohydride (data not shown).

The last part of the synthesis of (+)-**1** consisted of introducing a hydrazine unit, prior to a final deprotection. A Boc-protection of **9** using standard procedures was first quantitatively performed to afford *N*-protected *tert*-butyl ester **11**, that was then efficiently converted to acid **2** by a microwave-assisted saponification with 2M KOH in MeOH, and coupling with hydrazine unit **12** was then performed using the classical EDC·HCl–HOBt method. The synthesis of hydrazine **12** was achieved by reacting *N*-methyl hydrazine with *tert*-butyl bromoacetate with 40% yield after purification. Deprotection of compound **13** and purification by ion exchange chromatography on Amberlite CG50 (NH_4^+ form) afforded the target compound (+)-**1** in 98% yield, $[\alpha]_D^{25.2}$ +2.4° (*c* 0.36, H_2O), lit. $[\alpha]_D^{29.0}$ + 2.5° (*c* 2.00, H_2O). The final compound was fully characterized and compared with the published data for the natural product to confirm the success of this new total synthesis of (+)-**1** (*e.g.* ^1H NMR data for natural and synthesized (+)-**1**, available in ESI†). Furthermore, the *in vivo* read-through activity of termination codons during protein biosynthesis[3] of the synthesized (+)-**1** in mice was very similar to that of the native (+)-**1** (data not shown). Further derivatization of the **1** structure using the above synthetic methodology will contribute to a better understanding of the structure–activity relationship of **1** and the development of more potent compounds with efficient read-through activity. Studies in this regard are currently in progress and details pertaining to the biological activity will soon be published elsewhere.

In conclusion, the proposed synthetic route for the total synthesis of optically active (+)-negamycin starting from *N*-Boc-glycinal **5**, led to the desired product with a total yield of 42% over only eight steps. To our knowledge, this study represents the most efficient strategy to prepare (+)-**1**. Current efforts with this new synthetic approach are now expanding into medicinal chemistry to discover new drug candidates with potent read-through activity for Duchenne muscular dystrophy. The chemical biology of negamycin is also now being investigated to better understand its read-through mechanism.

This research was supported by the Research Grant (17A-10) for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare. T. R. is grateful for the Postdoctoral Fellowship of JSPS. We thank Dr J.-T. Nguyen for valuable help during manuscript preparation.

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ZOOLOGICAL SCIENCE 25: 1066–1074 (2008)

The Expression of Myogenin, but Not of MyoD, is Temperature-Sensitive in Mouse Skeletal Muscle Cells

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Homothermal animals need to keep their body temperature within a narrow range. Only a few degrees Celsius change in temperature has a dynamic influence on many physiological processes. To investigate the effect of the body temperature on muscle cell differentiation, we cultured the mouse myoblast cell lines C2C12 and Sol8 at lower temperatures than mouse body temperature. At 38°C, the cells fused into multinucleated myotubes within 4 days after the induction of differentiation. However, myotube formation was blocked at 30°C, whereas it was delayed but relatively normal at 35°C. The myoblasts expressed MyoD, but not myogenin, at 30°C. Id3, which acts as a negative regulator of myogenic regulatory factors (MRFs), was expressed at a higher level at 30°C than at 38°C, whereas the expression level of E2A, which acts as a positive regulator of MRF expression, exhibited no difference between these temperatures. We also found that the expression of muscle-enriched microRNAs decreased at 30°C. In addition, we investigated the expressions of MyoD and myogenin during mouse satellite-cell activation in single-fiber culture as an in-vivo model, and found that the expression of myogenin, but not of MyoD, was inhibited. These results suggest that skeletal muscle formation can be regulated by temperature, and that the physiological body temperature plays a crucial role in the myogenesis of homothermal animals.

Key words: myogenesis, MyoD, myogenin, temperature, differentiation, skeletal muscle, transcriptional regulation, mouse

INTRODUCTION

The emergence of homothermal animals is one of the most significant events in vertebrate evolution (Colbert, 1980). Homothermal animals are able to keep their body temperatures constant in spite of variation in environmental temperatures. Body temperature has recently been shown to play an important role in various physiological processes in mammals, including insulin secretion (Togashi et al., 2006) and hippocampal neural activity (Shibasaki et al., 2007). However, the effect of body temperature on cell differentiation has not yet been sufficiently elucidated. We investigated the effect of temperature on myogenic differentiation, of which the molecular cascade is relatively well understood compared with other cell types.

The myogenic regulatory factors (MRFs) MyoD, myf-5, myogenin, and MRF4 are a family of basic helix-loop-helix (bHLH) transcriptional factors responsible for the induction and maintenance of muscle differentiation (Berkes and Tapscott, 2005). MyoD and myf-5 are required for the initial determination of a myogenic lineage, whereas myogenin and MRF4 are responsible for the commitment to myogenesis. MyoD or myf-5 single-knockout mice show no morphological abnormalities in the skeletal muscles (Braun et al., 1992; Rudnicki et al., 1992), but MyoD/myf-5 double-knockout mice fail to generate myoblasts (Rudnicki et al., 1993). That is, MyoD and myf-5 have overlapping, or redundant, functions (Braun and Arnold, 1994). In myogenin-knockout mice, myoblasts are formed in the correct place but do not fuse into mature myofibers (Hasty et al., 1993; Nabeshima et al., 1993).

The expressions of MRFs are regulated by various chemical environmental factors, such as growth factors and extracellular matrices. For instance, IGF-I induces the elevation of myogenin expression and stimulates myogenic differentiation (Florini et al., 1996). The inhibition of collagen synthesis prevents muscle cell differentiation (de la Haba and Bricker, 1981) by blocking the expression of MyoD and myogenin (Saitoh et al., 1992). In addition, some physical environmental factors, such as mechanical and electrical stimulation, were shown to influence skeletal muscle growth and/or differentiation in vivo (Goldspink et al., 1991) and in vitro (Vandenburgh et al., 1991). Heat stress was also shown to induce muscular hypertrophy due to an increase in heat-shock protein (HSP) 72 (Uehara et al., 2004).

In this study, we cultured mouse myoblast cell line C2C12, which is a well-established model for studying myogenesis in vitro, at lower temperature than the mouse body temperature (38°C). We report here that mouse myoblast cells cultured at 30°C did not undergo terminal differentiation due to the inhibition of myogenin expression.

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doi:10.2108/zsj.25.1066

MATERIALS AND METHODS

Cell culture

A C2C12 cell line (Blau et al., 1983), a subclone of the C2 cell line isolated from the thigh muscle of an adult C3H mouse (Yaffe and Saxel, 1977), was kindly provided by Dr. Yoichi Nabeshima (Kyoto University, Kyoto, Japan). A Sol8 cell line (Daubas et al., 1988) was kindly provided by Dr. Muthu Periasamy (Ohio State University, OH, USA). The cells were grown in DMEM (high-glucose) (Invitrogen, Carlsbad, CA, USA) containing 20% FBS (SAFC Biosciences, Lenexa, KS, USA), 50 U/ml penicillin, and 50 mg/ml streptomycin in plastic dishes (AGC Techno Glass, Chiba, Japan) coated with 1% bovine skin gelatin (Sigma-Aldrich, St. Louis, MO, USA) at 38°C in 5% CO_2. At a point near confluence, the medium was replaced with MEM (Invitrogen) containing 10% horse serum (Invitrogen), 50 U/ml penicillin, and 50 mg/ml streptomycin to induce differentiation, and the cells were then cultured at 30°C, 35°C, or 38°C in 5% CO_2 for 4 days (C2C12 cells) or 3 days (Sol8 cells). The medium was changed every other day.

Single-fiber culture

Single muscle fibers were prepared as described previously (Nagata et al., 2006). Briefly, the extensor digitorum longus (EDL) muscles were removed from adult C3H/HeNJc1 mice (CLEA Japan, Tokyo, Japan) by excising the tendons, and treated with 0.1% collagenase Type I (Sigma-Aldrich) in DMEM at 38°C for 2 h. Single fibers were liberated from the EDL muscles by gentle pipetting and cultured in DMEM (high-glucose) containing 20% FBS, 50 U/ml penicillin and 50 mg/ml streptomycin. Experimental fibers were cultured at 38°C for 24 h and then at 30°C for 2 days, while the control fibers were cultured at 38°C for 3 days. Animal experimentation was carried out according to the guidelines of the animal committee of The University of Tokyo.

Immunofluorescence

Cells or mouse muscle fibers were fixed with 10% formalin in PBS for 20 min at room temperature, followed by treatment with 100% methanol for 15 min at room temperature. Non-specific protein binding was blocked by incubation with 10% HS, 3% BSA in PBS. The samples were incubated with primary antibodies [mouse anti-myosin heavy chain, MF20 (1:2; Developmental Studies Hybridoma Bank, Iowa City, LA, USA); mouse anti-MyoD (1:50; Dako Cytomation, Glostrup, Denmark); rabbit anti-MyoD (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-myogenin, F5D (1:1; a gift from Dr. Woodring E. Wright of the University of Texas, Southwestern Medical Center, TX, USA); and rabbit anti-E2A.E12 (1:50; Santa Cruz Biotechnology)] for 1 h at room temperature, followed by Alexa Fluor dye-conjugated secondary antibodies (1:400; Invitrogen) for 1 h at room temperature. The antibodies were diluted with 3% BSA in PBS. The cell nuclei were stained with Hoechst 33258 (Sigma-Aldrich). The samples were observed by using a fluorescence microscope (Axioplan, Carl Zeiss, Oberkochen, Germany), and images were taken with a digital camera system (VB-7000, Keyence, Osaka, Japan).

mRNA isolation and RT-PCR

Four days after the induction of differentiation (day 4), total RNA was isolated from the C2C12 cells using RNAZol B reagent (Tel-Test, Friendswood, TX, USA) in accordance with the manufacturer's protocol. Reverse transcription of the isolated mRNA and amplification of cDNA were performed with 100 ng RNA by using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen). PCR conditions were 94°C for 15 s, 60°C for 30 s, and 55°C for 45 s for 23 cycles (Ids, E2A) or 25 cycles (MyoD, myogenin). Primer sequences for MyoD, myogenin, E2A, Id1, Id2, Id3, and β-actin were as follows: MyoD (Shi et al., 2005), 5'-AGG CTC TGC TGC GCG ACC-3' and 5'-TGC AGT CGA TCT CTC AAA GCA CC-3'; myogenin (Kadota et al., 2000), 5'-GAG CTG TAT GAG ACA TCC CC-3' and 5'-GTA AGG GAG TGC AGA TTG TG-3'; E2A (Sun et al., 2007), 5'-TAC CCC TCC GCC AAG ACC-3' and 5'-TTG GGG GAT AAG GCA CTG-3'; Id1 (Sakuma et al., 2005), 5'-GTC CTG CTC TAC GAC ATG AAC G-3' and 5'-GAT CAA ACC CTC TAC CCA CTG G-3'; Id2 (Saika et al., 2006), 5'-AAA ACA GCC TGT CGG ACC AC-3' and 5'-CTG GGC ACC AGT TCC TTG AG-3'; Id3 (Riechmann et al., 1994), 5'-AAG GCG CTG AGC CCG GTG C-3' and 5'-TCG GGA GGT GCC AGG ACG-3'; β-actin (Riechmann et al., 1994; Shi et al., 2005), 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3' (349 bp), or 5'-AGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' and 5'-CTA GAA GCA CTT GCG GTG CAC GAT GGA AGG G-3' (661 bp). PCR products were resolved by electrophoresis on 2% agarose gels and stained with 1 μM SYTO60 Red Fluorescent Nucleic Acid Stain (Invitrogen) for 20 min. The band intensity was measured by using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and normalized to the β-actin band intensity at each temperature.

MicroRNA isolation and RT-PCR

Small RNAs were isolated from the C2C12 cells on day 4 by using a *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. Reverse transcription of the isolated small RNAs and amplification of cDNA were performed with 10 ng RNA by using a *mir*Vana qRT-PCR miRNA Detection Kit (Ambion) and *mir*Vana qRT-PCR Primer Sets (Ambion). The primers for microRNA(miR)-1, miR-133a, miR-181a, miR-206, and 5S rRNA were hsa-miR-1, hsa-miR-133a, hsa-miR-181a, hsa-miR-206, and 5s, respectively. PCR was performed for 20 cycles. The PCR products were resolved by electrophoresis on 3.5% agarose gels and stained with 1 μM SYTO60 dye for 20 min. The band intensity was normalized to the 5S rRNA band intensity at each temperature.

Statistical analysis

The cell nuclei expressing MyoD or myogenin in the C2C12 cells or Sol8 cells were counted in more than three different views for each sample, and the ratio of MyoD- or myogenin-positive nuclei to total nuclei (more than 150 nuclei per view) was calculated. For the single-fiber culture analysis, the satellite cells of 51 myofibers (38°C) or 31 myofibers (30°C) were counted and the ratios of myogenin-positive satellite cells to MyoD-positive satellite cells per myofiber were calculated. The ratios are expressed as mean percentages (%) ±SD. The *t*-test was used to evaluate the significance of differences between ratios.

RESULTS

Myotube formation in C2C12 myoblasts is retarded at 35°C and blocked at 30°C

To investigate the effect of low culture temperature on the differentiation of mouse myoblasts, C2C12 cells were grown in 20% FBS/DMEM at 38°C and differentiated in 10% horse serum/MEM at 35°C or at 30°C. As a control, C2C12 cells were differentiated at 38°C. The C2C12 myoblasts in the control cultures fused into multinucleated myotubes within 4 days after the induction of differentiation (day 4), whereas the myoblasts cultured at 35°C formed myotubes on day 5. Myotube formation was delayed for a day at 35°C, but the myotubes showed no significant changes in appearance. At 30°C, however, no myotubes were observed even when the myoblasts were cultured in differentiation medium for 10 days. When the myoblasts were returned to 38°C after being cultured at 30°C for 4 days, they formed myotubes again within 4 days. That is, the myogenic

differentiation of the C2C12 cells was reversibly blocked at 30°C.

To examine muscle-specific protein synthesis in the C2C12 cells cultured at 30°C, the cells were stained on day 4 with monoclonal antibody against sarcomeric myosin heavy chain (MyHC), MF20 (Bader et al., 1982). Immunofluorescence staining revealed that the myoblasts did not express MyHC at all at 30°C, whereas many large multinucleated myotubes were formed and expressed MyHC at 38°C (Fig. 1).

To determine whether apoptosis was induced by the change in temperature from 38°C to 30°C, we performed a TUNEL assay using C2C12 cells 6 h, 12 h, 24 h or 4 days after the induction of differentiation. No significant apoptosis occurred at any time point of the analysis (data not shown).

C2C12 cells express MyoD, but not myogenin, at 30°C

C2C12 cells were fixed on day 4 and immunostained for MyoD, which is required for myogenic determination, and myogenin, which mediates terminal differentiation. The C2C12 cells in the control cultures expressed both MyoD and myogenin. However, myogenin expression at 30°C was significantly decreased compared with the expression at 38°C, while there was no significant difference in MyoD expression between the two conditions (Fig. 2). The nuclei appeared to be spread out and flattened at 30°C, for a reason not yet elucidated.

Sol8 cells also do not express myogenin at 30°C

To demonstrate that the inhibition of myogenesis at 30°C occurred not only in the C2C12 cell line but also occurs in mouse skeletal muscle cells generally, we also cultured Sol8 cells, which were derived from primary cultures of the soleus muscle isolated from a C3H mouse (Daubas et al., 1988). Sol8 cells were grown in 20% FBS/DMEM at 38°C and differentiated in 10% horse serum/MEM at 30°C or at 38°C in the same way as the C2C12 cells. The Sol8 cells fused into multinucleated myotubes within 3 days after the induction of differentiation, which was earlier than the C2C12 cells. Sol8 cells were fixed on day 3 and immunostained for MyoD and myogenin. The Sol8 cells cultured at 30°C showed the same immunostaining patterns as the C2C12 cells cultured at 30°C; that is, they expressed MyoD, but not myogenin (Fig. 3).

Myogenin expression is inhibited at the transcriptional level at 30°C

To determine whether myogenin expression was inhibited at the transcriptional level at 30°C, total RNA was isolated on day 4 from C2C12 cells cultured at 30°C or at 38°C, and RT-PCR for MyoD and myogenin was performed. The myogenin band intensity at 30°C was less than 15% of that at 38°C (Fig. 4), which showed that the inhibition of myogenin expression in C2C12 cells cultured at 30°C occurred at the transcriptional level.

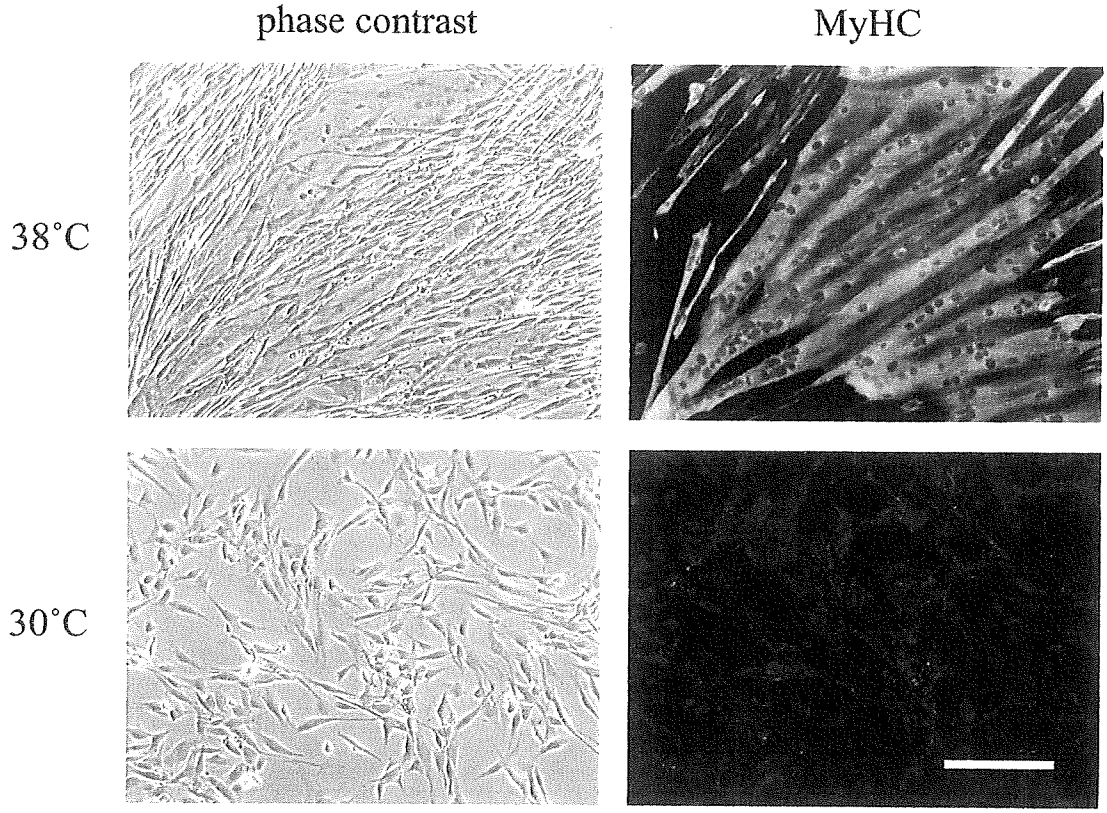

Fig. 1. MyHC expression in C2C12 cells cultured at 30°C or at 38°C. The cells were cultured in differentiation medium at 30°C or at 38°C for 4 days and immunostained with MF20, an anti-MyHC antibody. Many large multinucleated myotubes expressing MyHC were formed at 38°C. The myoblasts did not express MyHC, and myoblast fusion was blocked, at 30°C, suggesting that myogenic differentiation was inhibited at 30°C. Scale bar, 200 μm.

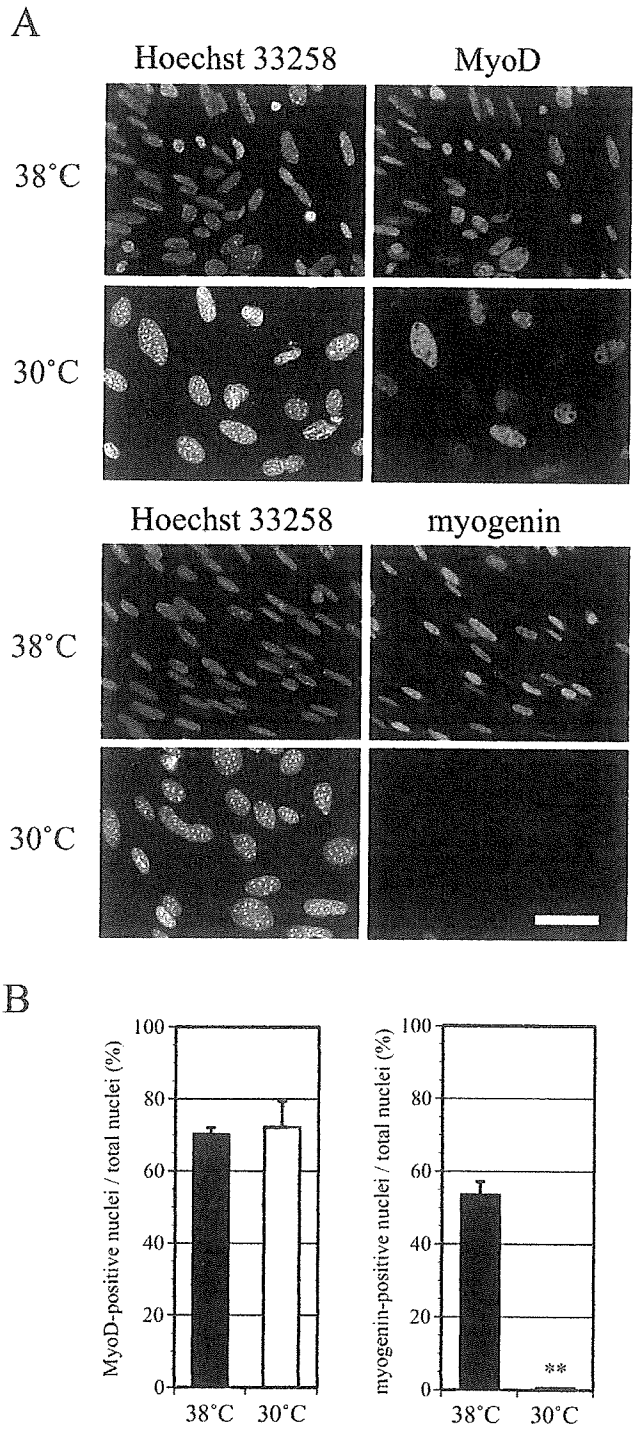

Fig. 2. Immunofluorescence analysis of MyoD and myogenin in C2C12 cells cultured at 30°C or at 38°C. **(A)** Cells were fixed on day 4 and immunostained with anti-MyoD and anti-myogenin antibodies, and also stained with Hoechst 33258. Scale bar, 50 μm. **(B)** Ratios of MyoD- or myogenin-positive nuclei to total nuclei are given as mean percentages±SD. Myogenin expression was significantly lower at 30°C than at 38°C (**p<0.01; *t*-test), whereas there was no significant difference in MyoD expression.

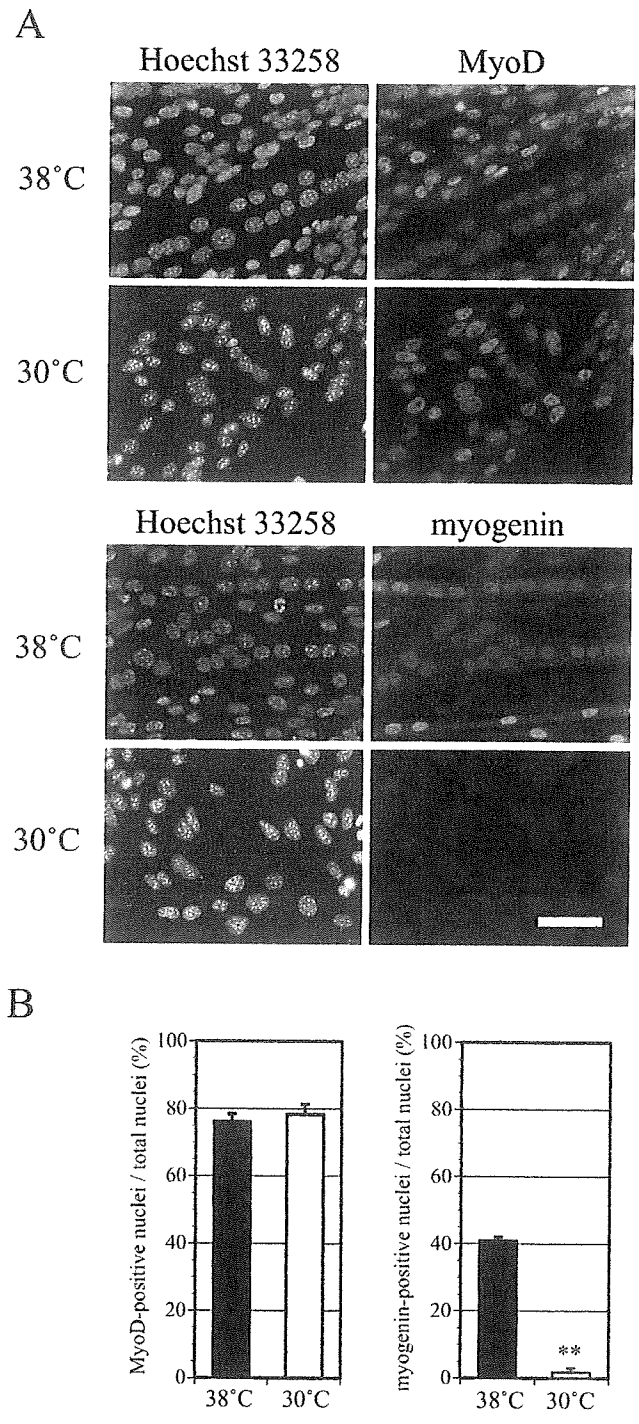

Fig. 3. Expression of MyoD and myogenin in Sol8 cells cultured at 30°C or at 38°C. **(A)** Cells were fixed on day 3 and immunostained with anti-MyoD and anti-myogenin antibodies, and also stained with Hoechst 33258. Scale bar, 50 μm. **(B)** Ratios of MyoD- or myogenin-positive nuclei to total nuclei are given as mean percentages±SD. There was a significant difference in myogenin expression between the cells cultured at 38°C and those cultured at 30°C (**p<0.01; *t*-test), but not in MyoD expression, as was observed in C2C12 cells (Fig. 2).

The E2A expression level does not change at 30°C

We investigated the expression of other transcriptional factors that interact with MRFs as positive or negative regulators, because it was quite possible that MyoD did not function as a transcriptional activator at 30°C. First, we examined the expression of the E2A gene product, which includes E12 and E47 as splice variants (Sun and Baltimore, 1991). E2A is a ubiquitous basic helix-loop-helix (bHLH)

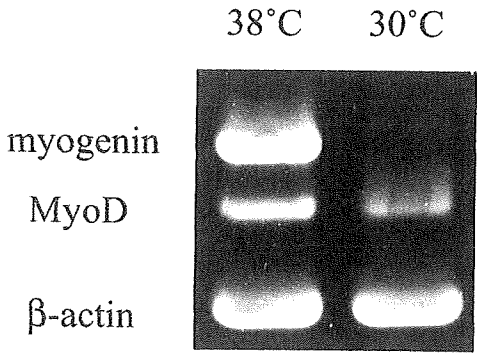

Fig. 4. RT-PCR of MyoD and myogenin in C2C12 cells. Total RNA was isolated from C2C12 cells cultured at 30°C or at 38°C on day 4. The gel was stained with SYTO60 dye and scanned with an infrared imaging system. The expression level of β-actin mRNA, which was almost the same in the cells cultured at 30°C and at 38°C, was used as a positive control. At 30°C, myogenin mRNA expression was extremely low, whereas MyoD mRNA was strongly expressed.

protein forming a heterodimer with MRFs. The MRF/E2A heterodimer binds to a consensus DNA sequence (CANNTG) called an E-box, which is present in promoters or enhancers of muscle-specific genes (Lassar et al., 1989) and activates the transcription of its target genes. We examined by RT-PCR E2A expression in the C2C12 cells cultured at 30°C for 4 days. As controls, cells which were cultured in the growth medium and in which differentiation was not induced (day 0) and cells differentiated at 38°C for 4 days were examined. As shown in Fig. 5A, E2A mRNA expression was low in all samples. The level was the same in myoblasts at day 0 and myotubes differentiated at 38°C, which coincided with the results of a previous study (Sun et al., 2007). Additionally, that previous study showed that the E2A protein level was regulated post-translationally and that its localization changed from the nuclei to both the nuclei and the cytoplasm in C2C12 cells as differentiation progressed from myoblasts to myotubes (Sun et al., 2007). Therefore, we also performed an immunofluorescence

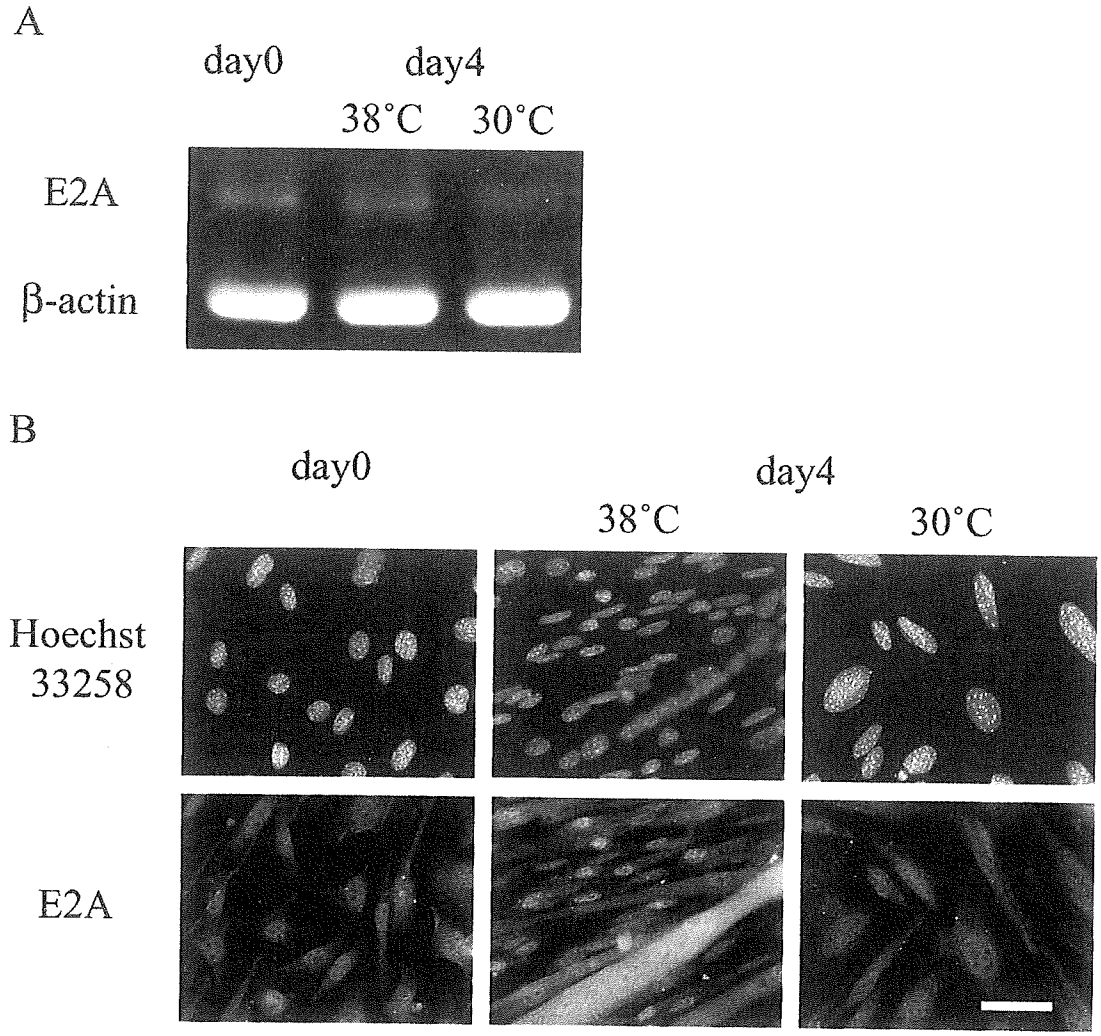

Fig. 5. E2A expression in C2C12 cells. **(A)** Total RNA was isolated from cells before the induction of differentiation (day 0) and from cells cultured for 4 days at 30°C or at 38°C, and RT-PCR was performed. E2A mRNA expression was low in all samples, and its level remained unchanged before and after the induction of differentiation. **(B)** Cells were fixed on day 0 or day 4 and immunostained with anti-E2A antibody, and also stained with Hoechst 33258. E2A proteins were expressed in the nuclei of the myoblasts on day 0 and in both the nuclei and the cytoplasm of the cells differentiated at 38°C. In the cells cultured at 30°C, E2A proteins were also expressed and localized in the nuclei. Scale bar, 50 μm.

analysis to investigate E2A protein expression and its localization. We confirmed that E2A proteins were expressed in the nuclei of myoblasts at day 0 and in both the nuclei and the cytoplasm of cells differentiated at 38°C. In cells cultured at 30°C, E2A proteins were also expressed and localized in the nuclei (Fig. 5B), suggesting that the expression pattern of E2A was not influenced by the low temperature.

Id3 is expressed at higher levels and accumulates in the nuclei at 30°C

We investigated the expression of Ids as negative regulators of MRFs. Ids are a family of dominant negative HLH proteins that contain HLH dimerization domains but lack DNA-binding basic regions (Benezra et al., 1990). Ids disturb MRF/E2A heterodimer formation by binding to E2A with high affinity (Berkes and Tapscott, 2005). The Id family includes Id1, Id2, Id3, and Id4, in which Id1, Id2, and Id3 have been shown to negatively regulate myogenesis (Jen et al., 1992; Melnikova and Christy, 1996; Melnikova et al., 1999). We examined by RT-PCR the expression of Id1, Id2, and Id3 in C2C12 cells, isolating total RNA from the cells before the induction of differentiation (day 0) and from cells differentiated at 30°C or 38°C for 4 days. As shown in Fig. 6, the cells on day 0 expressed all Ids at the highest level, which coincided with the results of a previous study showing that Ids were highly expressed in myoblasts, but decreased upon the initiation of differentiation (Benezra et al., 1990). Compared to the cells differentiated at 38°C, the cells cultured at 30°C expressed Id3 mRNA at higher levels, while the Id1 mRNA expression level was lower. The Id3 band intensity at 30°C was 1.4 times that at 38°C. Id2 mRNA expression was low at both 30°C and 38°C. These results suggest that Id3 might prevent MRFs from heterodimerizing with E2A and thus inhibit myogenic differentiation at 30°C.

MicroRNA expression decreases at 30°C

We investigated the microRNA (miRNA) expression patterns in C2C12 cells cultured at 30°C as one of the indexes of myogenic differentiation. MiRNAs are a class of small non-coding RNAs that negatively regulate gene expression and are involved in many biological processes, such as cell proliferation, differentiation, and apoptosis (Bartel, 2004). It has been shown that several miRNAs are specifically expressed or highly enriched in skeletal muscles and regulate myogenesis (Callis et al., 2007). We examined the expressions of miRNA-1, -133a, -181a, and -206 by RT-PCR, isolating small RNAs from C2C12 cells cultured for 4 days at 30°C or at 38°C. We found that miR-1, -133a, and -206 were regularly expressed at 38°C, as shown in a previous study (Kim et al., 2006), whereas they were hardly detected at 30°C; miR-181a was slightly expressed even at 30°C, but the expression level was lower than that at 38°C (Fig. 7).

Myogenin expression in activated satellite cells is also inhibited at 30°C

We investigated the expressions of MyoD and myogenin during satellite-cell activation as an in-vivo model. Satellite cells are myogenic stem cells (Mauro, 1961) that are mitotically quiescent in adult muscles (Schultz et al., 1978), but can be activated by signals released from crushed myofibers (Chargé and Rudnicki, 2004). The activated satellite cells enter the cell cycle and produce myogenic precursor cells, which differentiate into multinucleated myotubes (Snow, 1977) and then contribute to muscle repair (Zammit and Beauchamp, 2001). We isolated myofibers from C3H mice and incubated them in a medium containing serum which activates satellite cells (Yablonka-Reuveni and Rivera, 1994). After incubating the cells for 24 h at 38°C, we performed an immunofluorescence analysis and found that the satellite cells expressed MyoD but not yet myogenin. The cells were then cultured at 30°C for 2 more days. On day 3, the satellite cells cultured at 38°C for 3 days as a control expressed both MyoD and myogenin, whereas the cells cultured at 30°C for the last 2 days expressed MyoD, but not myogenin (Fig. 8).

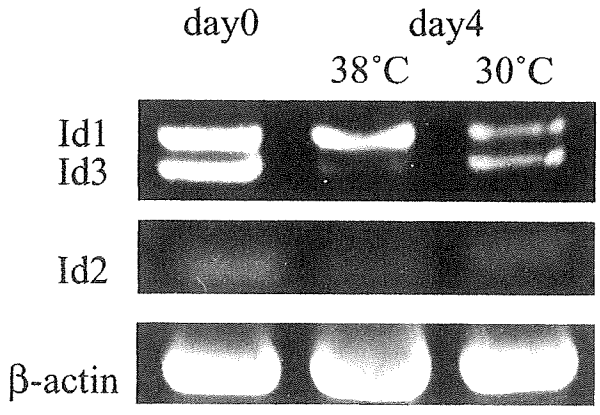

Fig. 6. Id expression in C2C12 cells. Total RNA was isolated from cells before the induction of differentiation (day 0) and from cells cultured for 4 days at 30°C or at 38°C, and RT-PCR was performed for Id1, Id2, and Id3. All Ids were expressed at the highest level on day 0, and their expression levels decreased on day 4 when the cells were cultured at 38°C, but cells cultured at 30°C still expressed Id3 mRNA at higher levels than at 38°C.

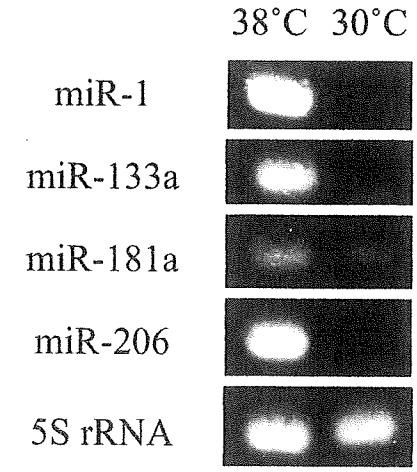

Fig. 7. MicroRNA (miR) expression in C2C12 cells. Small RNAs were isolated on day 4 from C2C12 cells cultured at 30°C or 38°C, and RT-PCR was performed for miR-1, -133a, -181a, and -206. As a positive control, 5S rRNA expression was examined. MiR-1, -133a, and -206 were regularly expressed at 38°C, but were hardly detected at 30°C. MiR-181a was expressed at both 30°C and 38°C, but was decreased in cells cultured at 30°C.

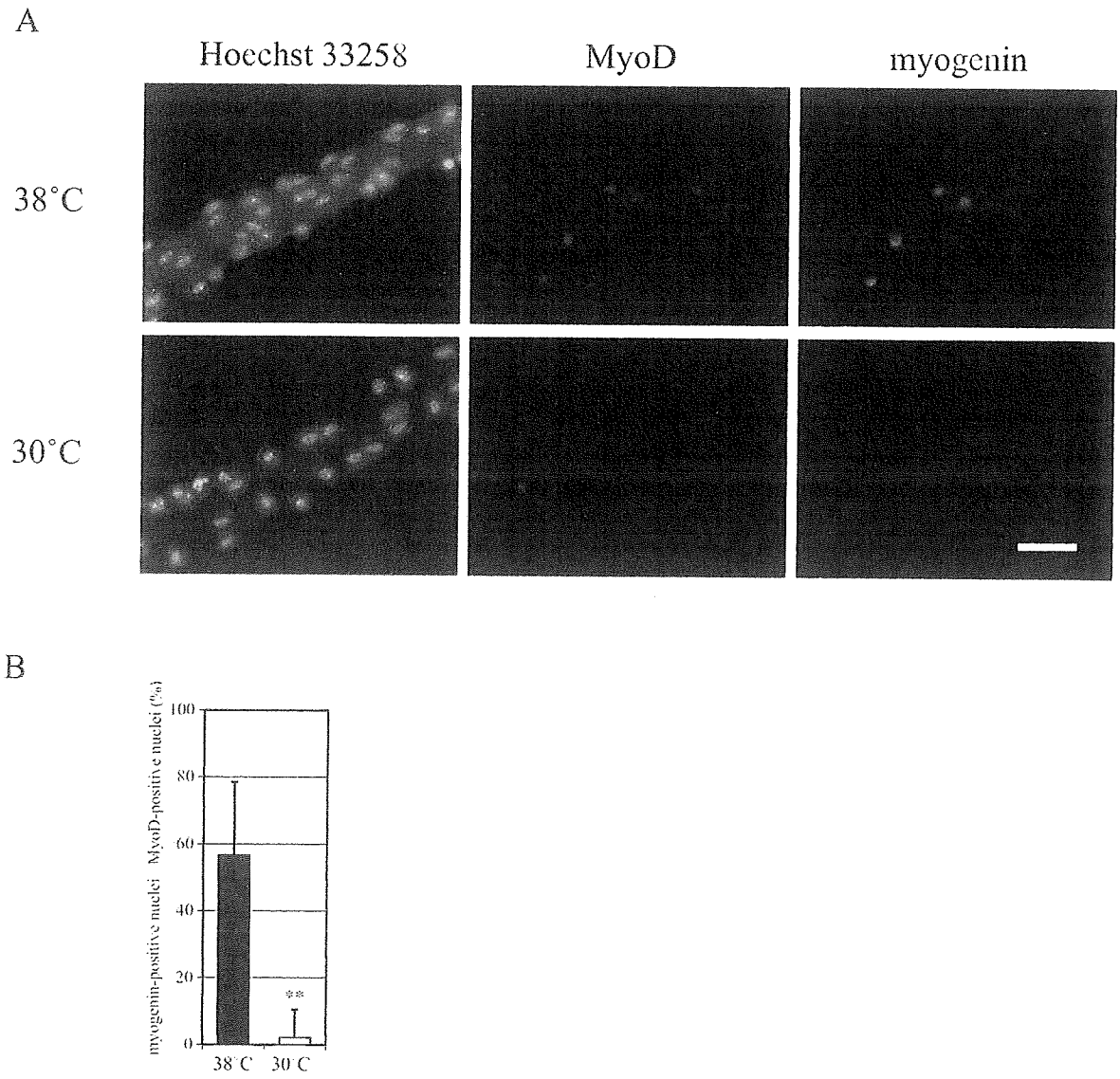

Fig. 8. MyoD and myogenin expression in the activated satellite cells of mouse myofibers. Single muscle fibers were isolated from the EDL muscles of C3H mice and incubated in 20% FBS/DMEM to activate the satellite cells. After 24 h of incubation at 38°C, the myofibers were cultured at 30°C for 2 more days. As a control, the fibers were cultured at 38°C for 3 days. **(A)** Myofibers were fixed and immunostained with anti-MyoD and anti-myogenin antibodies, and also stained with Hoechst 33258. Scale bar, 50 μm. **(B)** Ratios of myogenin-positive nuclei to MyoD-positive nuclei are expressed as mean percentages±SD. Satellite cells cultured at 38°C expressed both MyoD and myogenin, whereas cells cultured at 30°C for the last 2 days expressed MyoD, but not myogenin. There was a significant difference between the ratios (**$p<0.01$; *t*-test).

These results indicate that the inhibition of myogenin expression at 30°C occurs not only in muscle cell lines such as C2C12 and Sol8, but also in the satellite cells of mouse myofibers.

DISCUSSION

To elucidate the inhibitory mechanism of myogenin expression at 30°C, we investigated the expression of E2A and Ids. Myogenin expression is directly activated by MyoD (Hollenberg et al., 1993). So, why was myogenin not expressed at 30°C, even though MyoD was expressed and localized in the nuclei? It might be because MyoD did not attain its transcriptional activation potential at 30°C. E2A proteins, which form a heterodimer with MRFs and act as positive regulators of MRF expression, were expressed at 30°C as well as at 38°C. However, Id3, which disturbs MRF/E2A heterodimer formation and acts as a negative regulator of myogenesis, was expressed at higher levels at 30°C than at 38°C. This suggests that Id3 might prevent MyoD from attaining its transcriptional activation potential at 30°C.

We also investigated by RT-PCR the miRNA expression patterns as indices of myogenic differentiation. In C2C12 cells cultured at 30°C, miR-1, -133a, and -206 were hardly detected, but miR-181a was slightly expressed. MiR-1, -133, and -206 have been shown to have upstream MyoD- and myogenin-binding sites and, therefore, are likely to be regulated by MyoD and/or myogenin (Rao et al., 2006; Rosenberg et al., 2006). On the other hand, miR-181 is expressed upstream of MyoD and induces MyoD expression (Naguibneva et al., 2006). Thus, these results, in which an miRNA acting upstream of MyoD was expressed, but miRNAs acting downstream of MyoD were not, also suggest that

MyoD might not attain its transcriptional activation potential at 30°C.

Maltby et al. (2004) found that myogenin expression in turkey embryos incubated at 35.5°C for 5 to 8 embryonic days was lower than in control embryos incubated at 37.5°C, and that the peak of myogenin expression appeared later in temperature-manipulated embryos. Together with our data, these findings imply that myogenin expression is inhibited at low temperature in birds and mammals.

While only a few studies on the effect of temperature on myogenesis have been carried out in homothermal animals, many studies have been performed using fish, which can adapt to a wide range of water temperatures. Whether or not the expression patterns of MRFs change depending on the water temperature has also been examined in the embryos of many different fish species. No changes have been observed in any developmental stage in the herring (Temple et al., 2001), Atlantic cod (Hall et al., 2003), carp (Cole et al., 2004), and Atlantic halibut (Galloway et al., 2006). On the other hand, in the Atlantic salmon, the expression of myf-5 and MRF4, but not of MyoD and myogenin, is retarded with respect to the somite stage at 2°C compared to that at 8°C (Macqueen et al., 2007). In the rainbow trout, the myogenin expression is delayed but prolonged at 4°C compared to 12°C (Xie et al., 2001). Although a slight delay in the expression of MRFs has been observed in some fish species, the intensive inhibition of myogenesis shown in the present study has not been observed at low temperature in fish. This is probably because temperature has a greater effect on homothermal animals than on poikilothermal ones. Our data on mouse skeletal muscle cells revealed several interesting new phenomena that have not been observed in fish. We speculate that the inhibition of myogenesis at low temperature might explain why homothermal animals keep their body temperature around 38°C in spite of the high energy consumption. This is of interest in comparing the temperature sensitivity of myogenesis between homothermal and poikilothermal animals.

Here we showed that the differentiation of mouse skeletal muscle cells may be regulated by temperature. We speculate that the regulation of cell differentiation by body temperature is a key to understanding the evolution of homothermal animals. In addition, manipulating temperature in living organisms is less invasive than administering chemical factors. Therefore, the regulation of myogenesis by temperature might be widely applicable in various fields, such as athletic training, stock raising, and the treatment of intractable muscular diseases such as muscular dystrophy.

ACKNOWLEDGMENTS

This work was supported by grants 18A-1 for Nervous and Mental Disorders and H19-kokoro-020 for Research in Brain Science from the Ministry of Health, Labour and Welfare, Japan and 17570058 and 14654174 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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