Expression of an Alternative Dnmt1 Isoform during Muscle Differentiation[1]

Ana M. Aguirre-Arteta, Ingrid Grunewald, M. Cristina Cardoso, and Heinrich Leonhardt[2]

Max Delbrück Center for Molecular Medicine, D-13125 Berlin, Germany

Abstract

The methylation pattern of genomic DNA undergoes dramatic changes during mammalian development, with extensive *de novo* methylation occurring during gametogenesis and after implantation. We identified an alternative *Dnmt1* transcript in skeletal muscle by Northern blot analysis and cloned the corresponding cDNA by rapid amplification of cDNA ends and reverse transcription-PCR. Using an *in vitro* skeletal muscle differentiation system, we show that this alternative Dnmt1 isoform is specifically expressed in differentiated myotubes, whereas the ubiquitously expressed isoform is down-regulated during myogenesis. Sequence analysis showed that this skeletal Dnmt1 isoform is identical to the one present in testis, which had been described as untranslatable. Here we present evidence that this alternative *Dnmt1* transcript present in testis and skeletal muscle is translated despite the presence of several out-of-frame upstream ATGs and gives rise to a shorter Dnmt1 isoform, which could play an active role in the change of DNA methylation patterns during gametogenesis and myogenesis.

Introduction

In vertebrates, genomic DNA is often methylated at the 5 position of cytosine to form 5mC[3] at the dinucleotide sequence CpG, and this methylation is thought to affect gene expression (1). In mammals, levels of 5mC increase mainly during gametogenesis and after implantation, when tissue-specific patterns are established (2). After fertilization, the gametic methylation patterns undergo major changes, with an overall decrease in 5mC levels that reaches its lowest point at the blastocyst stage (3), with the exception of imprinted loci that retain specific methylation patterns throughout development (4, 5). During differentiation, when tissue-specific genes begin to be expressed, the promoter regions of many of these genes are demethylated, whereas the ones from inactive genes are methylated (1, 6). Until now, very little has been known about the regulation of DNA methylation during differentiation.

The predominant DNA MTase in mammals is Dnmt1 (7), which contains a large NH_2-terminal domain with regulatory sequences for nuclear localization targeting replication foci during S phase (8) and cytoplasmic localization during early development (9). ES cells carrying a null mutation in the *Dnmt1* gene lack maintenance methylation activity but retain the ability to methylate integrated provirus DNA *de novo*, suggesting the presence of at least one additional DNA MTase in mammalian cells (10, 11). Recently, three additional DNA MTases were cloned, Dnmt2 (12–14), Dnmt3 α, and Dnmt3 β (15) and the latter two MTases are indeed essential for *de novo* methylation and mammalian development (16). Three alternative 5′ exons have recently been identified in the *Dnmt1* gene, one specific to the oocyte, one specific to the pachytene spermatocyte (17), and one found in somatic cells (18, 19). At the translational level, the *Dnmt1* gene contains at least four in-frame ATGs that could potentially produce multiple MTase isoforms. A longer isoform, whose translation starts at ATG3 located in the first exon, is present in ES cells and somatic tissues (20). Another shorter isoform has been identified in oocytes and preimplantation embryos and starts at ATG4 located in the fourth exon (17, 20). This shorter isoform is active *in vivo* and can restore wild-type DNA methylation levels as well as the differentiation capacity of *Dnmt1* null ES cells (20). The presence of several out-of-frame ATGs in the spermatocyte-specific 5′ exon was proposed to prevent translation of this mRNA (17).

Skeletal myogenesis is one of the best-characterized systems for studying the role and regulation of DNA methylation during differentiation and in the establishment of tissue-specific gene expression. The formation of skeletal muscle during embryogenesis involves commitment of mesodermal progenitors to the myogenic lineage and subsequent differentiation of skeletal MBs into terminally differentiated MTs that express muscle-specific genes (21). Treatment of C3H10T1/2 fibroblasts with 5-azacytidine (22) as well as expression of an antisense strand of *Dnmt1* (23) was shown to induce MT formation, suggesting that genome-wide demethylation might play a role in myogenic differentiation. Indeed, demethylation activity has been detected in differentiating chicken MBs (24). Although overall DNA hypomethylation seems to promote MB differentiation, methylation of specific loci including inactive genes takes place concomitantly. In fact, ectopic overexpression of a shorter Dnmt1 isoform that starts at ATG4 and corresponds to the oocyte isoform did not lead to inhibition of myogenic differentiation as predicted by the 5-azacytidine treatments but

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[2] To whom requests for reprints should be addressed, at Franz Volhard Clinic, Wiltbergstrasse 50, 13125 Berlin. Phone: (030) 9417-2341; Fax: (030) 9417-2336; E-mail: leonhardt@fvk-berlin.de.

[3] The abbreviations used are: 5mC, 5-methylcytosine; MTase, (cytosine-5) methyltransferase; RACE, rapid amplification of cDNA ends; RT, reverse transcription; MB, myoblast; MT, myotube; ES, embryonic stem; poly(A) RNA, polyadenylated RNA; ORF, open reading frame.

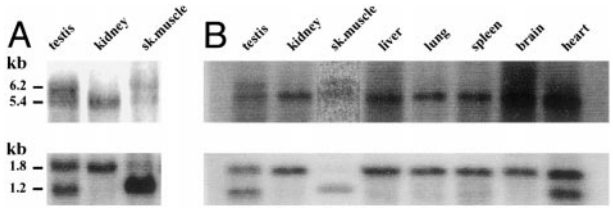

Fig. 1. Expression of *Dnmt1* mRNA in different tissues. Northern blots containing poly(A) RNA from different mouse tissues are shown. The blots were hybridized with two different Dnmt1 probes derived from the NH_2-terminal (*A*) and COOH-terminal (*B*) domains, as described in "Materials and Methods." All blots were reprobed with a mouse β-actin probe (*bottom panels*) as a loading control.

rather to an acceleration of MT formation (25). Overexpression of this shorter protein in MBs was accompanied by high *de novo* methylation activity that could be positively correlated with the methylation of CpG sites in the myogenic determination factor *MyoD1* gene and its increased transcription (25). These results prompted us to search for the presence of novel Dnmt1 isoforms during myogenic differentiation.

Here we report the presence of a second transcript in skeletal muscle that corresponds to the previously reported spermatocyte-specific cDNA. This isoform is specifically expressed in differentiated skeletal muscle and was not detected in proliferating MBs. Despite the presence of several out-of-frame ATGs in this transcript, a protein starting at ATG4 in exon 4 could be produced in transfected cells. Furthermore, a shorter Dnmt1 protein could be detected in extracts from MT cultures. This shorter isoform of Dnmt1 could account for the *de novo* methylation of specific loci and could thus induce muscle differentiation.

Results

An Alternative Dnmt1 Transcript Is Present in Skeletal Muscle. Because ectopic expression of a truncated Dnmt1 protein in MBs leads to changes in gene expression and induction of myogenic differentiation (25), we speculated that such a Dnmt1 isoform could be present in skeletal muscle. To test this hypothesis, we probed Northern blots for the presence of novel transcripts of the *Dnmt1* gene. Poly(A) RNA from several mouse tissues was hybridized with probes spanning most of the COOH-terminal domain as well as a part of the NH_2-terminal domain of the *Dnmt1* cDNA (Fig. 1). A *Dnmt1* mRNA transcript of 5.4 kb was found in all tissues examined, and it was most abundant in mouse brain and heart and less abundant in skeletal muscle. Examination of the blot after a longer exposure showed the presence of a slower migrating mRNA transcript of about 6.2 kb that was present in testis and in skeletal muscle. A longer *Dnmt1* mRNA generated by a testis-specific alternative transcriptional start has been described previously (26). The presence of a second longer transcript in skeletal muscle was confirmed using two different cDNA probes derived from the NH_2- and the COOH-terminal domain of *Dnmt1* (Fig. 1) and clearly demonstrates the existence of an alternative isoform in this tissue.

Cloning of the Alternative Dnmt1 Isoform from Skeletal Muscle. To clone the cDNA corresponding to the new *Dnmt1* skeletal muscle transcript, we used a combination of RT-PCR and RACE protocols and scanned the entire cDNA for the presence of alternatively spliced exons and/or novel 5′ and 3′ ends. The *Dnmt1* gene comprises 39 exons and spans over 56 kb (27). We compared the RT-PCR products from skeletal muscle RNA with the previously described somatic isoform of *Dnmt1* (18) from exons 5–37 and found no evidence for the presence of alternatively spliced exons (Fig. 2*B*). We next tested whether new 3′ or 5′ exons are present in this isoform by performing RACE reactions using gene-specific primers located in exon 37 and exon 4, respectively. The products obtained in both reactions were cloned and sequenced. The sequences obtained from the 3′ RACE reaction (Fig. 2*A*) contained exons 37–39 from the previously described somatic isoform of *Dnmt1* and utilized the first polyadenylation site (Fig. 2*A*, *red box*; Ref. 27), which could not account for the longer mRNA found by Northern blot analysis in the skeletal muscle RNA. However, two different size products were obtained from the 5′ RACE reactions, and the respective sequences contained either exons 1–4 of the previously described ubiquitous isoform (18) or the testis-specific exon followed by exons 2–4 (Ref. 17; Fig. 2*C*). Because no longer RACE products were obtained, we performed RT-PCR reactions using primers located at different positions in the genomic region upstream of the 5′ end of the RACE product corresponding to the testis-specific isoform to map the beginning of this exon in the skeletal muscle isoform. Some of the primer combinations and reaction products are shown in Fig. 2*C*. The sequence of the longest 5′ extension product (GenBank accession number AF175432) amplified from skeletal muscle RNA is shown at the *bottom* of Fig. 2*C*. This sequence is identical to the one previously thought to be exclusively expressed in pachytene spermatocytes (17).

The Muscle Isoform Is Expressed Specifically in Differentiated MTs. The identification of this alternative *Dnmt1* isoform in skeletal muscle raises the question of when it is expressed during myogenic differentiation. To answer this question, we made use of a very well-characterized *in vitro* differentiation system that is based on the ability of C2C12 mouse MBs to spontaneously differentiate into MTs on mitogen withdrawal (Fig. 3*A*). We isolated RNA from proliferating MBs as well as from differentiated MTs and used RT-PCR to analyze the expression of the two *Dnmt1* isoforms using upstream primers located either in exon 1 or in the testis/skeletal muscle-specific exon (Fig. 3*B*). We found that the ubiquitously expressed isoform containing exon 1 was present in both MBs and MTs, albeit at a lower level in differentiated cells (Fig. 3*C*). This is in agreement with a previous report showing a similar transcription rate but a shorter half-life of the mRNA for *Dnmt1* in differentiated mouse MTs using a different MB cell line (28). The new isoform, however, was not detectable in undifferentiated MBs and was only expressed in differentiated MTs (Fig. 3*C*). Equal amounts of input cDNA were present in both cases, as shown by the similar amounts of glyceraldehyde-3-phosphate dehydrogenase-specific PCR fragment obtained using the same cDNA

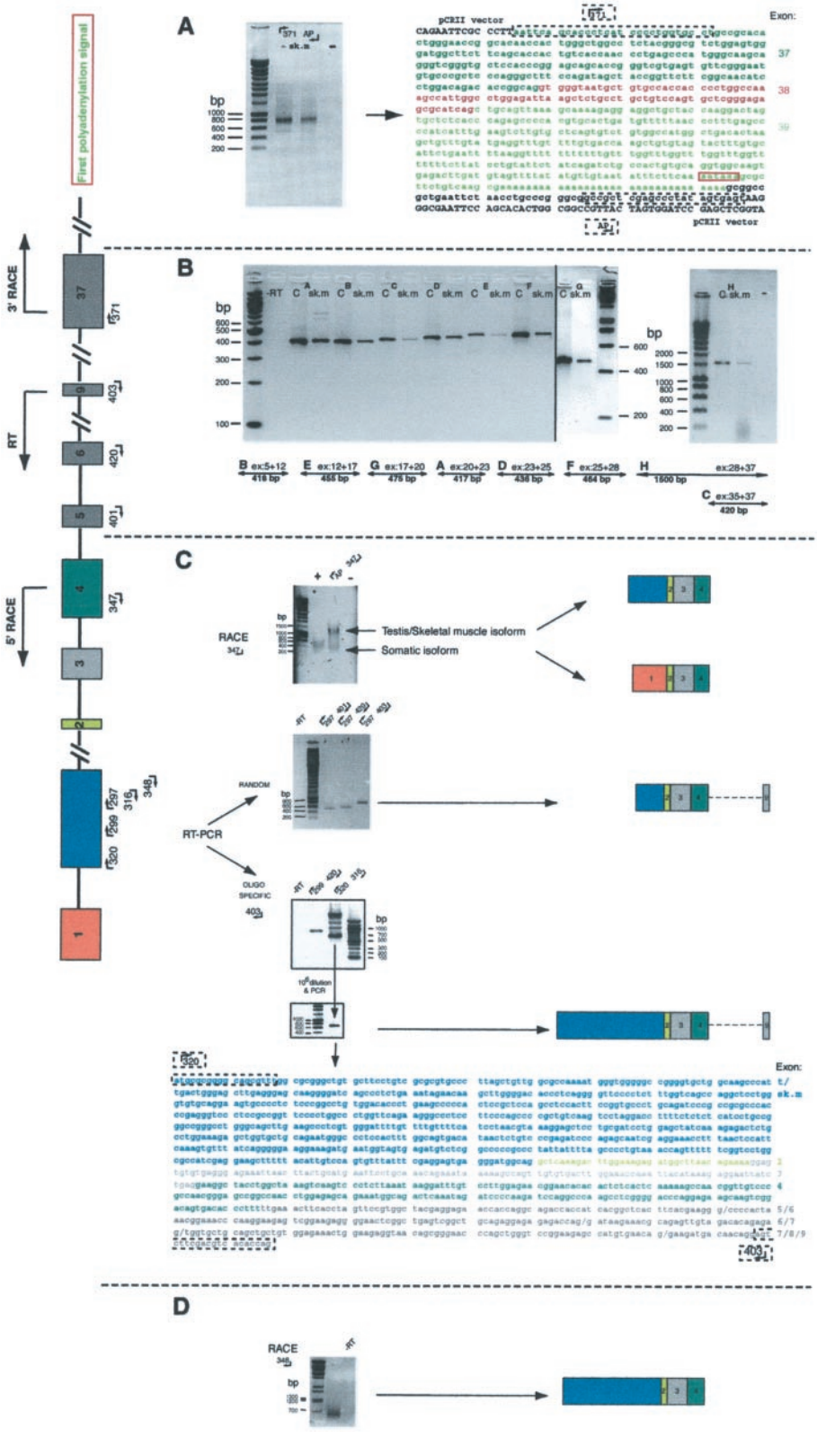

Fig. 2. Cloning of the skeletal muscle *Dnmt1* isoform. *A*, 3′ RACE was used to screen for differences at the 3′ end that could account for the novel *Dnmt1* transcript in skeletal muscle. A forward oligonucleotide in exon 37 (*371*) was used in combination with the adaptor primer (*AP*). The PCR reaction was carried out in duplicate, and the agarose gel shows the PCR products obtained whose size corresponds to approximately 800 bp. The PCR products were then cloned into pCRII vector (the sequence in *capital letters*) and sequenced. *sk.m*, the PCR product from skeletal muscle cDNA; −, the minus cDNA control PCR. The sequence obtained shows no new exons (from exons 37–39) and uses the first polyadenylation signal (*red box*; Ref. 27). *B*, screening for the presence of alternative exons by comparing RT-PCR products from skeletal muscle cDNA with a plasmid containing the ubiquitously expressed *Dnmt1* cDNA. Skeletal muscle RNA was reverse transcribed using random primers. Different PCR reactions were done using oligonucleotide pairs as indicated, spanning the known *Dnmt1* cDNA from exon 5 to exon 37. −*RT*, the negative control without reverse transcriptase; −, the negative control for the PCR reaction without cDNA template; *C*, PCR product from the plasmid; *sk.m*, PCR product from skeletal muscle cDNA. *C*, to screen for new 5′ end and/or alternative exons, a combination of oligonucleotides was used in 5′ RACE and RT-PCR reactions using skeletal muscle mRNA. The corresponding location of the primers used and the 5′ genomic structure (Ref. 27; see also Fig. 4) of the ubiquitously expressed *Dnmt1* (exons 1–9) and the testis-specific isoform (indicated in *blue*) are shown. Reverse transcriptase reactions were done both with random and with an oligonucleotide (403) located in exon 9, and the RACE reaction was performed with an oligonucleotide positioned in exon 4. −*RT*, the negative control without reverse transcriptase, as in *B*; −, minus cDNA; +, positive PCR control. All of the PCR products were cloned into pCRII and sequenced. Two isoforms were obtained multiple times from the RACE reaction that contain either exon 1 or the isoform described as testis specific. RT-PCR analysis using primers at different positions of the testis-specific exon further confirmed the presence of this isoform in skeletal muscle mRNA. The sequence of the longest PCR product is shown *below*, and the different exons are color-coded as in the genomic structure. *D*, reactions were run in parallel on testis mRNA. A first 5′ RACE reaction was done with an oligonucleotide located in exon 4, followed by a second internal 5′ RACE reaction with an oligonucleotide (*348*) located in the previously reported testis-specific exon (depicted in *blue*). −*RT*, the negative control without reverse transcriptase, as in *B*. The band obtained with the internal 5′ RACE reaction was sequenced, and it was found to be identical to the skeletal muscle isoform (data not shown).

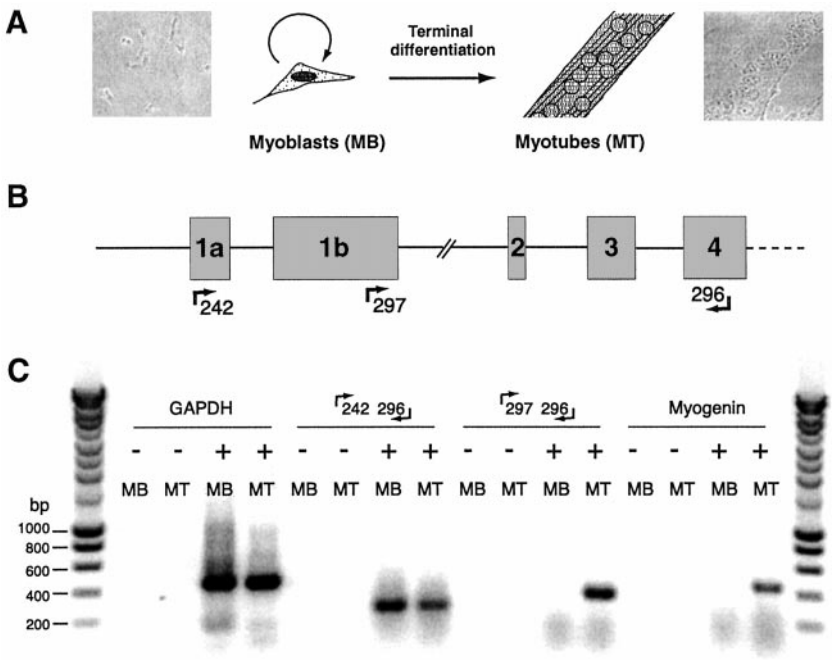

Fig. 3. Dnmt1 skeletal muscle isoform is expressed specifically in terminally differentiated muscle cells. *A*, the images and diagram illustrate the myogenic differentiation system. On serum deprivation, MBs fuse to form multinucleated MTs that express several muscle-specific proteins such as the basic helix-loop-helix transcription factor myogenin. *B*, location of the oligonucleotides used for RT-PCR. The nomenclature of the exons is as described in the Fig. 4 legend. *C*, RT-PCR products (with and without reverse transcriptase, + and −) were analyzed in agarose gels. A 343-bp fragment was obtained in MTs but not in MBs using oligonucleotides located in the testis/skeletal muscle exon (1b) and in exon 4. For the ubiquitously expressed isoform, oligonucleotides located in exon 1a and exon 4 were used in the RT-PCR reaction, and the expected fragment of 264 bp was obtained in MBs and at a lower level in MTs. RT-PCR with glyceraldehyde-3-phosphate dehydrogenase-specific oligonucleotides was used as a loading control giving rise to the 463-bp product seen at equal amounts in MBs and MTs. As a control for myogenic differentiation, oligonucleotides that amplify a 450-bp fragment of the myogenin transcription factor were used, and, as expected, a product was obtained in MTs and not in MBs.

samples (Fig. 3*C*). As a differentiation control, we used primers specific for mouse myogenin cDNA, a skeletal muscle-specific transcription factor expressed only after the onset of terminal differentiation (29). As expected, myogenin could only be amplified from MT RNA (Fig. 3*C*). These results show that this alternative Dnmt1 isoform is specifically expressed in differentiated MTs.

Genomic Organization of the Dnmt1 Isoforms. The most prominent Dnmt1 isoforms seem to be generated by alternative transcriptional start sites (17, 18). We therefore sequenced the 5′ end of the *Dnmt1* gene (GenBank accession number AF175410; about 26 kb from the oocyte-specific exon to exon 9). In Fig. 4, we show the structure of the 5′ end of the three *Dnmt1* isoforms identified thus far, together with their genomic localization and tissue distribution. Because these three alternative exons are not expressed in single tissues and to avoid confusion with exons identified in the future, we propose to clarify the nomenclature and simply name them as follows according to the chronological order of their identification: (*a*) 1a, exon 1 (18); (*b*) 1b, spermatocyte (17) and skeletal muscle exon (this work); and (*c*) 1c, oocyte exon (17).

To identify CpG islands in the complex 5′ end of the *Dnmt1* gene, we plotted the CpG *versus* GpC sites (Fig. 4). The only CpG island identified between exon 1c and exon 3 is located around exon 1a, the start of the ubiquitous isoform, and continues until the start of tissue-specific exon 1b. In comparison, no CpG island could be identified around exon 3, which had previously been described as transcriptional start site of the ubiquitous isoform (30). Because transcriptional start sites of housekeeping genes are typically associated with CpG islands, these results further support a transcriptional start of the ubiquitous isoform with exon 1a.

The Alternative Dnmt1 Transcript in Skeletal Muscle Produces a Truncated Protein. Sequence analysis of the alternative *Dnmt1* transcript expressed in skeletal muscle and in spermatocytes revealed several short ORFs (see Fig. 5). The first ATG that is in-frame with the Dnmt1 ORF is located in exon 4 and corresponds to ATG4 of the ubiquitously expressed isoform.

The presence of these short upstream ORFs has been previously proposed to prevent translation of this isoform in spermatocytes (17). The expression of an untranslatable mRNA, however, could hardly play an active role in the regulation of DNA methylation. Also, the 5′ end of the *Dnmt1* transcript found in oocytes contains several short upstream ORFs (17) but is highly expressed *in vivo*. Even the ubiquitous isoform includes three ATGs in the first exon (exon 1a), and only the third one is used *in vivo* (20). Therefore, we decided to test whether the skeletal muscle and spermatocyte *Dnmt1* transcript could be translated *in vivo*. We generated a set of mammalian expression constructs containing the entire exon 1b as well as 5′-truncated forms that contain only some of the short upstream ORFs. All of these constructs include

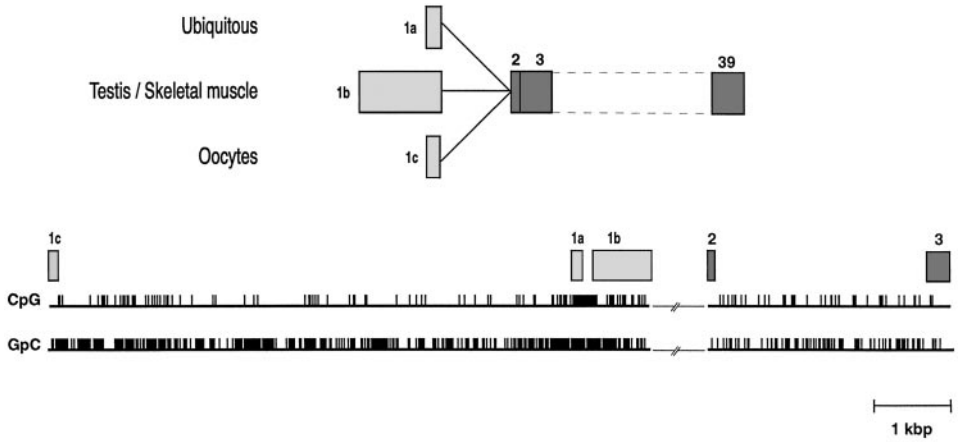

Fig. 4. The mouse *Dnmt1* gene contains at least three alternative exons at the 5′ end. The diagram depicts the genomic structure of the 5′ end of murine *Dnmt1*. Three alternative 5′ exons have been identified: one is specific to the oocyte, one is specific to the testis and skeletal muscle, and one is specific to somatic cells. The exons of *Dnmt1* are represented to scale with respect to their size and relative position in the gene (GenBank accession number AF175410). The exons present in each isoform are indicated and have been renamed as follows according to the chronological order of identification: (*a*) 1a, exon 1 (18); (*b*) 1b, spermatocyte (17) and skeletal muscle exon (this work); and (c) 1c, oocyte exon (17). CpG and GpC incidence diagrams are plotted to scale at the *bottom* of the figure. The presence of a CpG island around exon 1a is a typical feature of housekeeping genes and fits well with the ubiquitous expression of this isoform.

exons 1b—9 followed by the FLAG epitope sequence that is in frame with the Dnmt1 ORF, and transcription is controlled by the cytomegalovirus promoter (Fig. 5). In these constructs, the expression of the FLAG epitope is only possible when translation initiation takes place at ATG4 in exon 4. As a positive control, we generated a construct containing most of exon 1a of the ubiquitously expressed isoform. Plasmid DNA was prepared from these constructs and used for transfection of COS-7 cells. Three days after transfection, expression of the FLAG-tagged Dnmt1 protein was analyzed by immunofluorescence staining and Western blotting using an anti-FLAG monoclonal antibody. All of the constructs were able to generate tagged Dnmt1 truncated protein in COS-7 cells as indicated by the positive FLAG signal in the immunofluorescence images shown in Fig. 5. The nonspecific background fluorescence under the same conditions is shown in the image of the stained mock-transfected COS-7 cells.

Protein extracts from transfection experiments with a construct containing exon 1b and a control containing instead exon 1a were compared by Western blot analysis with antibodies against the FLAG epitope tag. Both constructs gave rise to single bands on the Western blot (Fig. 5). ATG4 is the only possible translation initiation site in the exon 1b constructs that is in frame with the FLAG epitope. These results clearly show that transcripts starting with exon 1b are translatable.

To test whether this truncated Dnmt1 isoform can be detected in differentiated muscle cells, we prepared extracts from MTs and probed them with anti-Dnmt1 antibodies. As shown in Fig. 6, a faint but clear signal is detectable below the ubiquitous Dnmt1 protein band (*right panel*). Because MB extracts contain much higher levels of the ubiquitous Dnmt1 isoform, we loaded lower amounts of these extracts for comparison (*middle panel*). Under these comparable conditions, similar amounts of the major isoform are visible, but the shorter isoform is present only in MTs. We cannot rule out that this shorter Dnmt1 isoform is generated by protein degradation. However, these Western blot results are in good agreement with the mRNA expression data described above. Therefore, these results further support the conclusion that a shorter Dnmt1 isoform is expressed during development that could play an active role in the change of DNA methylation patterns during oogenesis, gametogenesis, and myogenesis.

Discussion

DNA methylation clearly plays an important role in the regulation of gene expression and during development but very little is known about its regulation. Conflicting data have been published concerning the exact role of DNA methylation during differentiation. Thus, on one hand, artificially induced demethylation seems to stimulate myogenic differentiation (22), which is in accordance with the observation that the ubiquitously expressed Dnmt1 isoform is down-regulated during myogenesis (28). On the other hand, forced overexpression of Dnmt1 causing *de novo* methylation in the *MyoD1* gene induced its expression and also stimulated myogenesis (25).

We have identified a longer, alternative *Dnmt1* transcript in skeletal muscle (Fig. 1). We show that this mRNA (starting with exon 1b) is specifically up-regulated during myogenesis, whereas the shorter, ubiquitous transcript (starting with exon 1a) is down-regulated (Fig. 3). These results could reconcile the above-mentioned contradiction in the sense that the major Dnmt1 isoform responsible for maintenance of DNA methylation patterns is indeed down-regulated, but an alternative isoform with potentially different properties is up-regulated.

The sequence analysis of this alternative *Dnmt1* isoform from skeletal muscle (Fig. 2) showed that it is actually iden-

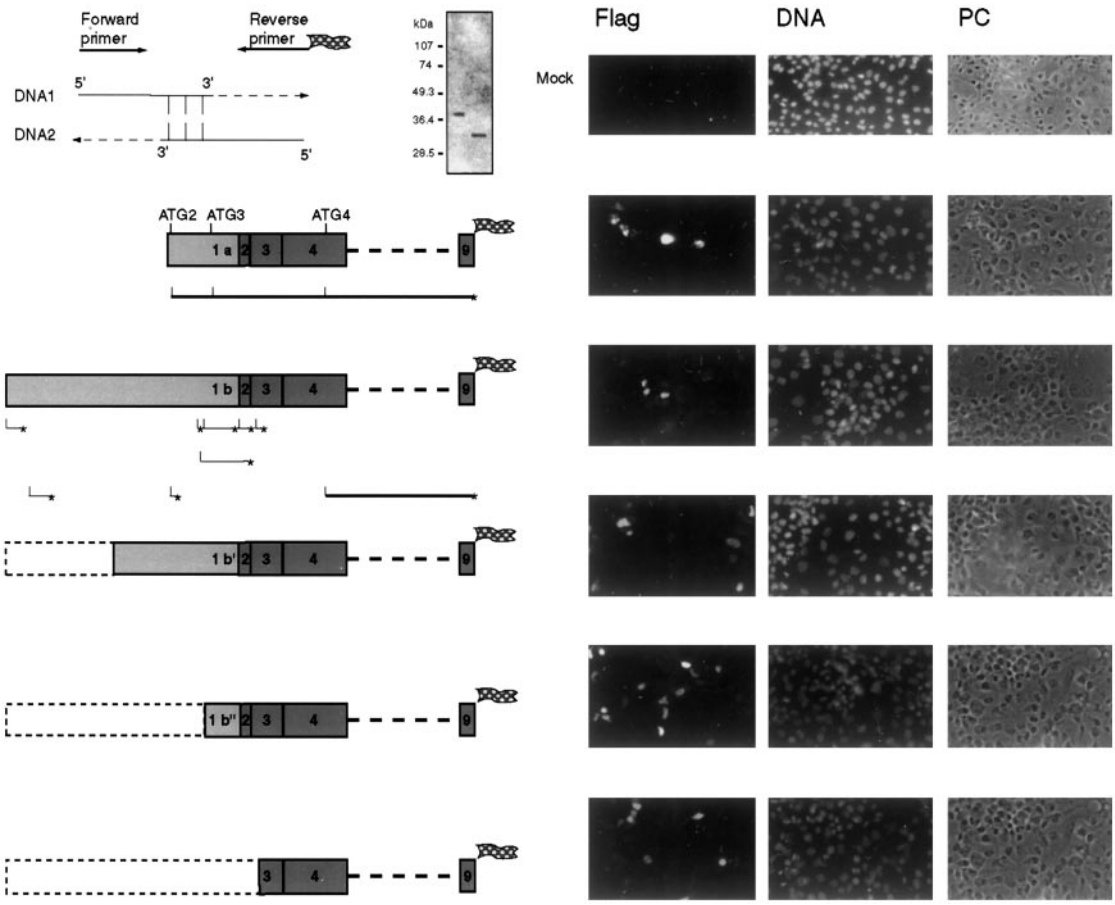

Fig. 5. The skeletal muscle Dnmt1 isoform produces a protein. Different expression constructs were designed to test whether the skeletal muscle isoform could generate a truncated Dnmt1 protein. The strategy followed to generate some of the Dnmt1 expression constructs is depicted at the *top left* of the figure. In the first PCR cycle, all of the reagents necessary for a PCR reaction except the flanking oligonucleotides were included. The first cycle allows the annealing and extension of the overlapping area between the two denatured DNAs (*DNA1* and *DNA2*). In the next cycles, the oligonucleotides were added, and amplification of the desired area occurs. The different epitope-tagged Dnmt1 expression constructs used to transfect COS-7 cells [1a-9, 1b-9, 3-9, 1b′-9, and 1b″-9 (b′ and b″-9 correspond to nucleotides 651 and 831 to 1418, respectively, from GenBank sequence accession number AF175432)] are shown *below* this scheme. The exons included in each of the constructs are indicated (see the Fig. 4 legend for definition of the exon nomenclature). The *dotted lines* in exon 1b, which is found in testis and skeletal muscle cDNA, represent the areas that were deleted in the specific construct. The *small flags* at the end of each construct represent the 8-amino acid FLAG coding sequence that was added to each of them. ORFs starting at ATG2 and ATG4 in exons 1a and 4, respectively, of the ubiquitously expressed Dnmt1 isoform cDNA (20) and ending in the FLAG tag are represented by *thick lines*, and the short ORFs 5′ of the Dnmt1 ORF are represented by *thin lines*. The *short vertical lines* correspond to ATGs in the particular reading frame, and the *stars* represent stop codons. The different expression plasmids were used for transient transfection of COS-7 cells, and expression of the FLAG-tagged Dnmt1 protein (*thick lines*) was analyzed by immunofluorescence staining with the anti-Flag M2 monoclonal antibody. The DNA counterstaining was performed with Hoechst 33258, and the corresponding phase-contrast (*PC*) images are also shown. The mock transfection is depicted at the *top* using similar exposure conditions. In the Western blot at the *top* of the figure, the two types of Dnmt1 protein bands obtained by probing with the anti-FLAG M2 monoclonal antibody using the Dnmt1 constructs 1a-9 and 1b″-9 are shown.

tical to the one present in testis, which was proposed to be untranslatable (17). This transcript was nevertheless shown in testis to be weakly associated with ribosomes (26), which would be compatible with a low level of translation of this isoform. Here, we show that this alternative transcript that starts with exon 1b is indeed translatable, giving rise to a truncated Dnmt1 isoform starting at ATG4 (Fig. 5, exon 4). Interestingly, this translation product is identical to the one obtained from the oocyte-specific transcript, which starts with exon 1c located 7.2 kb further upstream and is transcribed from an independent promoter (Fig. 4). Thus, the same truncated, shorter Dnmt1 isoform could be expressed during oogenesis, spermatogenesis, and myogenesis. The expression of this shorter Dnmt1 isoform has clearly been shown in mature oocytes, where it is the only form present (17, 20). We have obtained evidence for a shorter Dnmt1 protein present in differentiated MTs, but we cannot rule out that it is a degradation product of the ubiquitously expressed, longer Dnmt1 isoform (Fig. 6).

These results, however, still do not prove any specific role for the shorter Dnmt1 isoform because both isoforms have very similar biochemical properties *in vitro* (31). The shorter isoform seems to be able to substitute for the longer isoform during differentiation and to restore DNA methylation patterns in *Dnmt1* null ES cells (20). Thus, a specific role of the shorter isoform would require tissue-specific, interacting fac-

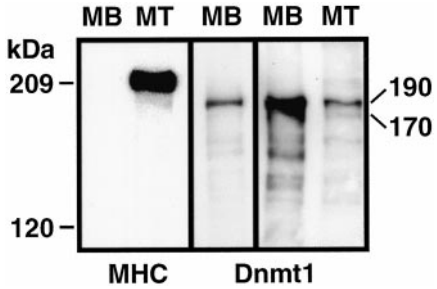

Fig. 6. A shorter Dnmt1 protein can be detected in differentiated C2C12 MTs. Whole cell extracts from MBs and MTs (see Fig. 3) were subjected to Western blot analysis. The blot was first incubated with anti-Dnmt1 antibody (*right panel*). MT extracts showed a low abundance of the shorter Dnmt1 isoform (M_r 170,000) in addition to the ubiquitous Dnmt1 isoform (M_r 190,000). Because MBs express Dnmt1 at much higher levels, we show, for comparison, a lower amount of MB extract in which this shorter band is not detectable (*middle panel*). As an additional control for differentiation, the blot shown in the *right panel* was sequentially probed with anti-myosin heavy chain antibody, which is a late marker and a major constituent of differentiated muscle and is therefore only visible in the MT extract (*left panel*).

tors that could differentially interact with the Dnmt1 isoform and thus generate specificity. The proof that the shorter Dnmt1 isoform we identified in skeletal muscle can indeed stimulate myogenic differentiation has already been provided by serendipity. Since the complete *Dnmt1* cDNA for the ubiquitously expressed isoform became known and available in 1996 (18, 19), a partial cDNA was used for the overexpression in MBs, giving rise to a truncated Dnmt1 starting at ATG4, which is identical to the shorter isoform described here. The forced expression of this Dnmt1 isoform in MBs was shown to correlate with a high level of *de novo* methylation activity and to induce myogenic differentiation (25). These experiments clearly show that the shorter Dnmt1 isoform from skeletal muscle cells can indeed induce myogenic differentiation. However, additional experiments are necessary to elucidate the exact role of Dnmt1 isoforms during development.

Database Access. The sequences reported in this study have been deposited in the GenBank database under accession numbers AF175432 and AF175410.

Materials and Methods

Northern Blot Hybridization Analysis. Mouse multiple tissue Northern blots (Clontech) containing ~2 μg poly(A)+ RNA/lane were probed with a 377-bp fragment (nucleotides 674-1050) and a 950-bp (nucleotides 2946–3895) fragment located in the NH_2-terminal and COOH-terminal region of the methyltransferase cDNA, respectively (GenBank accession number AF162282; Ref. 27). The probes were labeled with [α-^{32}P]dCTP and [α-^{32}P]dATP by random priming (Amersham). The blots were washed at high stringency (twice for 20 min each with 0.1× SSC and 0.1% SDS at 65°C) and exposed to a phosphorimager screen overnight. The blots were stripped by boiling with SDS solution (0.5% SDS) twice for 15 min before being rehybridized with a mouse β-actin probe for RNA loading control.

RT-PCR and RACE Analysis. Total RNA was prepared from skeletal muscle from the legs of C57BL6 mice. RNA was isolated by adsorption to a silica gel-based membrane in a high-salt buffer system according to the manufacturer's instructions (Qiagen). Total RNA (~1 μg) was then subjected to random or oligonucleotide-specific RT. PCRs were performed with the appropriate oligonucleotides and cycling conditions with respect to the melting and annealing temperatures of the primers. The fresh PCR products were cloned into pCRII vector (Invitrogen). Positive clones were sequenced with the dye terminator method and analyzed on an automated DNA sequencer (Applied Biosystems).

To identify potentially new 5′ or 3′ end sequences, RACE reactions were performed using cDNA from skeletal muscle (Marathon-Ready cDNA; Clontech). An antisense primer was designed (347-R; located in exon 4) for 5′ RACE PCR and a sense primer (371-F; located in exon 37) was designed for 3′ RACE PCR. The products from the RACE reactions were cloned into pCRII vector (Invitrogen) and sequenced as described above.

Mammalian Expression Constructs. The different *Dnmt1* pCR3.1 expression plasmids are designated 1a-9, 1b-9, 1b′-9, 1b″-9 and 3-9 (numbers correspond to the exons present; see Fig. 4 for nomenclature of exons). To generate these constructs, paired fragments of double-stranded DNA containing a common region that can be annealed after denaturation were used (see the diagram in Fig. 5). Briefly, for each construct, two paired PCR products of double-stranded DNA were mixed with all of the reagents necessary for a PCR reaction except the oligonucleotides. The oligonucleotide sequence and exon localization are indicated in Table 1. The oligonucleotide pairs were as follows: (*a*) 584–582 and 583–578 for 1a-9; (*b*) 320–316 and 579–578 for

Table 1 Nucleotide sequences of DNA primers

Oligonucleotide (exon)	Sequences
584-F (exon 1a)	5′TAGCCAGGAGGTGTGGGTGCCTCCGTTGCGC3′
582-R (exon 3)	5′CTGTTTGCAGGAATTCATGCAGTAAGTTTAATTTCTCC3′
583-F (exon 3)	5′GCTCAAAGACTTGGAAAGAGATGGCTTAACAGAAAAG3′
578-R (exon 9)	5′CTT**CTTGTCATCGTCGTCCTTGTAGTC**TCTGGTGTGA3′[a]
320-F (exon 1b)	5′ATGCGCGGGGCAGCGTTT3′
316-R (exon 1b)	5′TAAAATAATAGGGCGGGGGC3′
579-F (exon 1b)	5′GCCCGGCTGTCAAGTCCTAGGACCTTTTCTCTCTCAT3′
581-R (exon 1b)	5′TTCCCCTCTTCCGACTCTTCCTTGGGTTTCCGTTTAGT3′
577-F (exon 1b)	5′GCCCCCGCCCTATTATTTTAGCCCCTGTAAACCAGT3′
616-R (FLAG)	5′CTT**CTTGTCATCGTCGTCCTTGTAGTC**3′[a]

[a] The FLAG sequence is indicated by bold letters.

1b-9; and (*c*) 579–581 and 577–578 for 1b′-9. The mixture was then allowed to denature, anneal, and extend for one cycle on a PCR machine (Biometra). After this initial cycle, oligonucleotides flanking the newly formed double-stranded DNA were added to the reaction, and the PCR was carried out for another 30 cycles. The reverse oligonucleotide used in the latter reaction was the same in all of the cases (616-R, which contains the FLAG sequence indicated in bold letters in Table 1). For the constructs 1b″-9 and 3-9, PCR reactions with oligonucleotides 577–578 and 325–616, respectively, were carried out. The resulting PCR products were placed under the control of the cytomegalovirus promoter by cloning into the pCR3.1 mammalian expression vector (Invitrogen).

Cell Culture and Transfections. COS-7 cells were grown in a humidified atmosphere of 5% CO_2 at 37°C and maintained at subconfluent density in DMEM supplemented with 10% fetal bovine serum. Cells were plated at 3×10^5 cells/p100 and transfected 1 day later with the corresponding FLAG-tagged *Dnmt1* expression plasmid using the GenePorter transfection reagent according to the manufacturer's instructions (Gene Therapy Systems, Inc.). Three days after transfection, cells were harvested for Western blot analysis or fixed for immunofluorescence analysis.

C2C12 cells were grown and differentiated as described previously (32). Proliferating MBs and differentiated MTs were harvested for Western blot analysis and RNA preparation.

Immunofluorescence. Cells were washed three times with PBS and fixed with 3.7% formaldehyde in PBS for 10 min. Immunostaining was performed by first blocking the sample with 0.2% fish skin gelatin for 30 min, followed by a 2-h incubation with the anti-Flag M2 mouse monoclonal antibody (Kodak) diluted 1:2000 in 0.2% Tween 20 in PBS and a 1-h incubation with FITC-conjugated antimouse IgG antibody (Boehringer Mannheim) diluted 1:100 in 0.2% Tween 20 in PBS. Nuclear DNA counterstaining was performed with Hoechst 33258 at a concentration of 1 μg/ml, and samples were mounted in Mowiol containing 2.5% DABCO. Samples were analyzed on a Zeiss Axioplan 2 microscope equipped with phase-contrast and epifluorescence optics using a $\times$20 PlanNeofluar objective, and images were acquired with a cooled charge-coupled device camera (Sensicam; 1280 $\times$ 1024 pixels; 6.7-μm pixel size) using Axiovision software (Zeiss).

Cell Extracts and Western Blotting. Cells were harvested by centrifugation, and cell pellets were resuspended in ice-cold radioimmunoprecipitation assay buffer [500 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS] with protease inhibitors (leupeptin, aprotinin, and pepstatin) and incubated on ice for 5 min. Whole cell extracts were recovered from the supernatant after centrifugation for 5 min at 4°C. Protein extracts were then separated on a 10–20% gradient SDS-PAGE (COS-7 cell extracts) or 6% SDS-PAGE (C2C12 cell extracts) and transferred to polyvinylidene difluoride membranes using a semidry blotting apparatus. Transfected COS-7 cell extracts were probed sequentially with anti-FLAG antibody M2 (Kodak; 1:5,000), antimouse IgG-biotin (Amersham; 1:10,000), and streptavidin-horseradish peroxidase (Amersham; 1:500) and washed extensively with 0.2% Tween 20 in PBS between the different incubations. C2C12 cell extracts were probed first with affinity-purified rabbit polyclonal anti-Dnmt1 antibody (1:500), antirabbit IgG-biotin (Amersham; 1:10,000), and streptavidin-horseradish peroxidase (Amersham; 1:500) and sequentially reprobed with anti-myosin heavy chain mouse monoclonal antibody (Ref. 33; MF-20 hybridoma tissue culture supernatant diluted 1:2), followed by antimouse IgG-horseradish peroxidase (Amersham; 1:10,000). The signals were detected with enhanced chemiluminescence (ECL) or ECL+ reagents (Amersham) and recorded with a luminescent image reader machine using ImageReader software (Fuji).

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