MyoD Is Indispensable for Muscle-specific Alternative Splicing in Mouse Mitochondrial ATP Synthase γ-Subunit Pre-mRNA

(Received for publication, October 3, 1997, and in revised form, December 12, 1997)

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Muscle-specific alternative RNA splicing is an essential step during myogenesis. In this paper, we report that a muscle-specific transcription factor, MyoD, plays a central role in the induction of muscle-specific alternative splicing during myogenesis. Recently, we reported that muscle and nonmuscle isoforms of the mitochondrial ATP synthase γ-subunit (F1γ) were generated by alternative splicing and that acidic stimulation promoted this muscle-specific alternative splicing (Endo, H., Matsuda, C., and Kagawa, Y. (1994) J. Biol. Chem. 269, 12488–12493). In this report, mouse myoblasts are shown to express the muscle-specific isoform of F1γ after induction with low-serum medium (differentiation medium) or acidic medium, although myotube formation was not detected after acidic induction. RNA blot analysis revealed that the expression levels of both MEF2 and myogenin were increased by low-serum induction, but not by acidic induction. High expression of MyoD mRNA was observed after both types of induction. Overexpression of exogenous MyoD in fibroblasts showed that MyoD was necessary for muscle-specific alternative splicing in both types of induction. Exogenous Id, a negative regulator of MyoD, blocked muscle-specific alternative splicing of F1γ pre-mRNA by both types of induction. In addition, MyoD induced several muscle-specific alternative splicings, including structural protein pre-mRNAs such as β-tropomyosin and neural-cell adhesion molecule and transcriptional protein pre-mRNAs such as MEF2A and MEF2D. Our analysis of the two induction systems shows a common MyoD-dependent mechanism of muscle-specific alternative splicing in several genes, independent of MEF2 and myogenin.

Alternative pre-mRNA splicing is a fundamental process in eukaryotes and is regulated by cell-specific, tissue-specific, and developmental stage-specific pathways to generate mRNAs that differ in protein-coding potential, stability, and translation efficacy (1, 2). In vertebrates, cis-regulatory elements or trans-acting factors that affect tissue-specific alternative splicing have been identified (1–4). Several factors are responsible for alternative splicings, such as serine/arginine-rich (SR)1 proteins, U2 auxiliary factor, polypyrimidine tract-binding protein, and exon recognition factors (1, 2, 5). Understanding the regulatory cascade for tissue-specific alternative splicing during terminal differentiation would elucidate a direct trigger for the alternative splicing mechanism.

Myogenic differentiation involves both muscle-specific RNA processing and transcriptional activation of muscle-specific genes, e.g. the basic helix-loop-helix (bHLH) proteins of the MyoD family including MyoD, Myf5, MRF4, and myogenin, which promote skeletal muscle-specific gene expression (6–17) and are built up to a positive autoregulatory loop (6–12). On the other hand, Id, a helix-loop-helix protein, is a negative regulator of bHLH proteins through direct protein-protein interaction. Id is expressed at various levels in some stem cell lines, and a decrease in Id mRNA levels triggers terminal differentiation (18). Myocyte-specific enhancer factor 2 (MEF2) was originally identified as a muscle-specific DNA-binding factor induced when skeletal myoblasts differentiate into myotubes (19). There are four members of the MEF2 family of proteins (MEF2A through MEF2D), and all share a region of homology, the MADS box and the MEF2 domain (20–25). A recent study shows that the MEF2 and MyoD families act within a regulatory network that establishes differentiated phenotypes of skeletal muscle and that MEF2 factors act as coregulators to potentiate the myogenic activities of myogenic bHLH proteins (26).

On the other hand, post-transcriptional control is shown during myogenesis, e.g. the transcripts of β-tropomyosin (β-TM) (3, 27–30), neural-cell adhesion molecule (N-CAM) (31–33), MEF2A (21), and MEF2D (24, 25), which are regulated by alternative splicing in substantially different splicing patterns. The gene of β-TM contains two sets of alternatively spliced, mutually exclusive exons whose utilization is tissue-dependent and developmentally regulated (3, 27–30); exon 6a is shared by mRNAs expressed in mouse smooth muscle and nonmuscle tissues, whereas exon 6b is present only in skeletal muscle-specific transcripts (27, 28). N-CAM mRNA includes the muscle-specific sequence domain (MSD) located between exons 12 and 13 in a cassette mode. Whereas β-TM mRNA contains exon 6a and N-CAM mRNA does not include the MSD in mouse myoblasts, exon 6b is selected in β-TM mRNA and the MSD is included in N-CAM mRNA when myoblasts convert to myotubes (27, 28, 32, 33). MEF2A transcripts are ubiquitous, but accumulate preferentially in skeletal muscle, heart, and brain.
Recently, we cloned the mitochondrial ATP synthase \(\gamma\)-subunit (F\(_{\gamma}\)F\(_{\gamma}\)) gene and showed that its heart and skeletal muscle-specific isoforms are generated by alternative splicing in the human and the cow (34, 35). As muscle tissues require a rapid energy supply, the expression of muscle-type F\(_{\gamma}\) is thought to be an adaptation to the tissue-specific energy requirement. We also showed that this muscle-type mRNA excluding exon 9 in a cassette mode was induced cell-specifically by intracellular acidosis in human fibrosarcoma and rhabdomyosarcoma cells (36). This acidic induction of alternative splicing is a reversible system, indicating that the nonmuscle-type mRNA containing exon 9 is a default type and that de novo protein synthesis is required for muscle-specific alternative splicing. The alternative splicing of F\(_{\gamma}\) is very useful as a marker for studying muscle-specific splicing because the splicing is controlled in a simple cassette mode and the expression levels before and after induction do not change significantly.

In this report, we show the new muscle-specific alternative splicing induction system using mouse myoblasts, in which acidic stimulation induces muscle-specific splicing without expression of MEF2 and myogenin and finally without myotube formation. We identified a muscle-specific transcriptional regulatory factor involved in splicing regulation and investigated its influence on many genes. A common MyoD-dependent regulatory cascade for muscle-specific alternative splicing is described, and Id is shown to inhibit muscle-specific alternative splicing via blocking the induction of MyoD.

**MATERIALS AND METHODS**

cDNA Cloning and Amplification of a Portion of the Mouse F\(_{\gamma}\) Genomic DNA—The cDNA encoding mouse F\(_{\gamma}\) was isolated from a BALB/c 3T3 cDNA library by using the \(5'\) and \(3'\) primers described above and the cloned PCR product of human F\(_{\gamma}\) cDNA as a probe (35). For preparation of the probe, sense and antisense strand oligonucleotides (5'-TTG-GAT-CTG-CTC-TCG-TTT-GT-3' and 5'-AGT-GTC-TTC-CCC-AGA-GAT-CCC-3') were synthesized, and 5'-end labeled by T4 nucleotide kinase. RNA blot hybridization was performed as described above by heating the \(X\) fragment of pcDEB SR, encompassing nucleotides 61–851 (18), mouse myogenin cDNA encompassing nucleotides 611–766 (7), and human MEF2A cDNA encompassing nucleotides 1234–1427 (21) were constructed by RT-PCR. First-strand DNA was synthesized with SuperScript II™ (Life Technologies, Inc.) and primed with oligo(dT)\(_{18}\) primer. PCR was carried out for 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min in a total volume of 100 \(\mu\)l containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\(_2\), 200 \(\mu\)M each dNTP, 10 pmol each of oligonucleotide primer, 1:20 (v/v) synthesized first-strand cDNA, and 2.5 units of recombinant Taq DNA polymerase (Takara Shuzo Co.). These cloned cDNAs were subcloned into the EcoRV site of pBluescript II SK" and subsequently sequenced according to the dideoxy termination method (37). Primer sequences used for amplification were as follows: mouse Id, 5'-CTC-TTG-GAT-CTG-CTG-TCT-TCC-3' (sense strand) and 5'-ACT-GTC-TTC-CCC-AGA-GAT-CCC-3' (antisense strand); MEF2A, 5'-CGA-GCC-GAT-TCC-ATC-TCA-3' (sense strand, primer G) and 5'-GGT-TAG-GCA-GCC-ATG-GTG-3' (antisense strand, primer H); and myogenin, 5'-CCG-CTA-CAG-GCC-TTG-CTG-3' (sense strand) and 5'-GCA-ACA-GAT-ATC-AGC-3' (antisense strand).

**RT-PCR Analysis and Southern Blot Analysis of the Alternative Exon**—To identify the possible alternative exons of F\(_{\gamma}\), \(\beta\)-TM from exons 5 to 7, N-CAM from exons 12 and 13, MEF2A, and MEF2D (10 \(\mu\)g of total RNA) were used to synthesize first-strand cDNAs as described above. PCR amplifications of F\(_{\gamma}\), \(\beta\)-TM, N-CAM, and MEF2A were performed in a total volume of 100 \(\mu\)l as described above by heating the DNA at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min in 25, 25, 30 cycles, respectively. PCR of MEF2D was performed (stored at 94 °C for 9 min; subjected to 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and then stored at 72 °C for 7 min) in a total volume 100 \(\mu\)l containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\(_2\), 200 \(\mu\)M each dNTP, 10 pmol each of oligonucleotide primer, 1:20 (v/v) synthesized first-strand cDNA, and 2.5 units of recombinant Taq DNA polymerase (AmpliTaq Gold™, Perkin-Elmer). The oligonucleotides were subcloned into the M13-\(\scriptstyle{m}X\)-174 EcoRI site and sequenced.

**Cell Culture**—Mouse C3H10T1/2 clone 8 fibroblast cells (10T1/2 cells) were obtained from the Japanese Cancer Research Resources Bank. Mouse C2C12 cells (a myoblast cell line) and BC3H1 cells (a myoblastoid cell line) were obtained from the American Type Culture Collection. The C2C12 and 10T1/2 cells were grown in growth medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Flow Laboratories) at 37 °C under 5% \(\text{CO}_2\). The cells were plated on 10-cm diameter tissue culture dishes. When the C2C12 cells grew to semiconfluence, the growth medium was replaced with two different media. Differentiation (low-serum) medium contained Dulbecco's modified Eagle's medium with 2% heat-inactivated horse serum (Irvine Scientific) and 2 mM L-glutamine (Irvine Scientific), and acidic (low-pH) medium contained Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 2.7 mM NaHCO\(_3\) for C2C12 and 10T1/2 cells or 5.4 mM NaHCO\(_3\) for BC3H1 cells. BC3H1 cells are less viable in acidic medium. Cells were harvested after 3 days.

**RNA Preparation and RNA Blot Analysis**—Total RNA was prepared from C2C12 and 10T1/2 cells using the acid guanidine method (39). genomic DNA gel for RNA electrophoresis was labeled previously (38). cDNA for probes were labeled with \(\alpha\)-32PdCTP (Amersham Pharmacia Biotech) using a random priming kit (Amersham Pharmacia Biotech). RNA blot hybridization was performed as described previously (38). Full-length mouse MyoD cDNA (provided by Dr. H. Weintraub, Hutchinson Cancer Research Center) (6) was digested with XhoI (cDNA encompassing codons 1–1785). Mouse Id cDNA encompassing nucleotides 61–851 (18), mouse myogenin cDNA encompassing nucleotides 511–766 (7), and human MEF2A cDNA encompassing nucleotides 1234–1427 (21) were constructed by RT-PCR.
MyoD Required for Muscle-specific $F_1\gamma$ Pre-mRNA Splicing

To elucidate the differences in splicing mechanisms in mouse myoblasts between low-serum induction with differentiation medium and acidic stimulation with low-pH medium, we treated each medium with TGF-$\beta_1$. TGF-$\beta_1$ plays an important role in myogenic differentiation. Treatment of skeletal myoblast cell lines or primary muscle cells with differentiation medium containing TGF-$\beta_1$ inhibits terminal differentiation (41–44). As shown in Fig. 2, C2C12 cells were cultured with differentiation medium and acidic medium containing various concentrations of recombinant human TGF-$\beta_1$ (Austrial Biological). In differentiation medium, both myotube formation and the induction of muscle-type $F_1\gamma$ were inhibited by treatment with $>2$ ng/ml TGF-$\beta_1$ (Fig. 2, a, lanes 5 and 6; and b, lanes 3–6). Conversely, in acidic medium supplemented with TGF-$\beta_1$, induction of the muscle-type isoform was not inhibited (Fig. 2c, lanes 2–6). These results suggest that low-serum stimulation and acidic stimulation trigger different gateways to induce alternative splicing. Specifically, acidic stimulation primed the splicing regulatory cascade downstream from the TGF-$\beta_1$-blocking site. Additionally, the mechanism for muscle-specific alternative splicing of $F_1\gamma$ pre-mRNA was suggested to be different from that for myotube formation.

Expression of Endogenous MyoD Is Necessary for Induction of Muscle-type $F_1\gamma$—RNA blot analyses in myoblasts in differentiation medium and acidic medium were performed on muscle-specific transcription factors such as MyoD, MEF2A, myogenin, and Id. Initially, when C2C12 myoblasts were cultured in differentiation medium, the expression level of MyoD mRNA was increased steadily until 72 h (Fig. 3a, lanes 1–6). MyoD

RESULTS

TGF-$\beta_1$ Inhibits the Induction of Muscle-specific Splicing in Differentiation Medium, but Not in Acidic Medium—We cloned muscle and nonmuscle types of mouse $F_1\gamma$ cDNA and part of the genomic gene as described under “Materials and Methods.” Two isoforms were produced by alternative splicing of a cassette exon corresponding to exon 9 of the human $F_1\gamma$ gene (Fig. 1). In mouse C2C12 myoblasts, we induced muscle-specific alternative splicing in $F_1\gamma$ pre-mRNA during myogenesis using differentiation medium (Fig. 2a, lane 1). This splicing was detected 48 h after induction (Fig. 2a, lane 1). At this time, the myotube had not yet formed (Fig. 2b, lane 1). Acidic stimulation also induced the muscle type of $F_1\gamma$ in the myoblasts (Fig. 2c, lane 1), but myotubes did not form for more than 3 days. In addition, acidic induction of muscle-specific alternative splicing in $F_1\gamma$ pre-mRNA appeared to be reversible in mouse myoblasts (see below), as in human cells (36).

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mRNA was also induced in acidic medium (Fig. 3b, lanes 1–6). Next, the expression level of Id mRNA rapidly decreased by placement in differentiation medium, but did not significantly decrease in acidic medium (Fig. 3, c, lanes 1–6; and d, lanes 1–6). Expression of myogenin and MEF2 mRNAs was induced in differentiation medium, whereas neither of these mRNAs was expressed in acidic medium (Fig. 3, e and f). The inhibition of myogenic differentiation by TGF-β1 treatment is thought to be due to suppression of the gene expression and function of MyoD and myogenin (42–44). The expression of MyoD mRNA increased moderately in differentiation medium and acidic medium containing TGF-β1 (Fig. 3, a, lanes 7–12; and b, lanes 7–12). Id expression rapidly decreased in differentiation medium, but was steadily expressed in acidic medium, even in the presence of TGF-β1 in both media, as shown in Fig. 3 (c and d).

In the C2C12 myoblasts we used, the initial expression level of
MyoD mRNA (lane 1 of Fig. 3, a and b) was slightly lower than that previously reported (6), perhaps owing to variation in the cell line. However, our results indicate that an increase in MyoD expression is necessary for the induction of muscle-type F1γ, and the expression levels of Id, myogenin, and MEF2 appear not to be important.

Exogenous MyoD Induces Muscle-type Splicing of F1γ pre-mRNA—To investigate whether MyoD expression can induce muscle-specific alternative splicing of F1γ pre-mRNA, we analyzed the pattern of F1γ mRNA associated with MyoD expression in mouse fibroblasts (10T1/2 cells) in which MyoD was not expressed. The mouse MyoD cDNAs were subcloned into a mammalian expression vector (pcDEBSRα) (Fig. 4a). As a control, we used human hMGMT cDNA subcloned into pcDEBSRα (40). MyoD- and hMGMT-expressing permanent transformants from 10T1/2 cells were selected, cloned, and named 10T-MyoD and 10T-hMGMT cells, respectively. After they had grown to semiconfluence in growth medium, 10T-MyoD cells, 10T-hMGMT cells, and wild-type 10T1/2 cells were exposed to differentiation medium or acidic medium for 72 h. Exogenous MyoD mRNA was expressed in 10T-MyoD cells cultured in differentiation medium or acidic medium (Fig. 4b, lanes 7–9). Exogenous MyoD mRNA was not expressed in wild-type 10T1/2 cells or in 10T-hMGMT cells (Fig. 4b, lanes 1–6). When 10T-MyoD cells were cultured in differentiation medium or acidic medium, muscle-type F1γ mRNA was induced (Fig. 4c, lanes 8 and 9). 10T-MyoD cells converted into multinucleate myotubes in differentiation medium, but not in acidic medium (data not shown). In wild-type 10T1/2 cells and in 10T-hMGMT cells, muscle-type F1γ mRNA was undetectable or appeared only as faint bands, and myotubes were not induced.

Next, to investigate whether Id expression blocks muscle-specific alternative splicing of F1γ pre-mRNA, we transfected mouse Id cDNA subcloned into pcDEBSRα into mouse C2C12 myoblasts and cloned several permanent transfectants. We
used hMGMT cDNA in pcDEBSR<sub>a</sub> as a control DNA. When C2C12 cells transfected with Id (C2-Id cells) were cultured in differentiation medium, muscle-specific splicing was inhibited, and myotube formation was not detected (Fig. 5a, lane 8). In addition, induction of the muscle-specific isoform was mostly suppressed in acidic medium (Fig. 5a, lane 8), and myotube formation was not detected (Fig. 5a, lane 8). Muscle-type splicing of F<sub>1γ</sub> pre-mRNA was observed in untransfected C2C12 cells and C2-hMGMT cells cultured in differentiation medium and acidic medium (Fig. 5a, lanes 2, 3, 5, and 6). These data indicate that MyoD directly induces muscle-type F<sub>1γ</sub> mRNA in fibroblasts and that overexpression of Id inhibits this splicing in C2C12 myoblasts by suppressing the increase in endogenous MyoD expression (Fig. 5b). Thus, MyoD is an indispensable factor that plays an important role in the initial stage of muscle-specific alternative splicing, which is induced in artificial acidic medium at the same time as myogenic differentiation.

_Muscle-specific Exon Selection Requires de Novo Protein Synthesis and Activated Protein Kinase C—_Alternative splicing in the F<sub>1γ</sub> pre-mRNA would require a common intracellular signal cascade in the two induction systems at the late stage of muscle-specific alternative splicing. As acidic induction is a reversible splicing regulation system (Fig. 6), we can identify which course requires a regulatory factor for alternative splicing in mouse C2C12 myoblasts and BC3H1 cells. When these cells were cultured with acidic medium, muscle-specific F<sub>1γ</sub> mRNA was induced. This induction was inhibited by cycloheximide, a protein synthesis inhibitor (Fig. 6, a and c). On the other hand, the reverse switch from muscle-type to nonmuscle-type F<sub>1γ</sub> mRNA was not inhibited by cycloheximide (Fig. 6, b and c). In these cells, muscle-specific exon exclusion of F<sub>1γ</sub> pre-mRNA required de novo protein synthesis of such a regulatory factor. Next, we tested the protein kinase C inhibitor H-7 and HA1004 in the system. As shown in Fig. 6 (e and f), H-7 inhibited muscle-specific exon exclusion in BC3H1 cells in acid medium, but did not inhibit the reverse course. HA1004, an analogue of H-7, was used as a control. These results suggest that activated protein kinase C is directly involved in muscle-specific exon selection. Considering that both the protein synthesis inhibitor and the protein kinase C inhibitor suppress only the induction of exon exclusion, it is likely that activated protein kinase C is involved in the regulation of alternative splicing via activation or expression of a trans-acting protein factor.

_Muscle-specific Alternative Splicing Is Induced Cooperatively by MyoD_—Some genes have transcripts produced by muscle-specific alternative splicing. Structural proteins such as β-TM and N-CAM and transcription factors such as MEF2A and MEF2D show alternative splicing accompanied by myotube formation and transcriptional activation of muscle-specific genes, although the modes of these splicings are different. β-TM and MEF2D mRNAs are regulated in mutually exclusive modes, and F<sub>1γ</sub>, N-CAM, and MEF2A mRNA are regulated in cassette modes. We proved that these muscle-specific alternative splicings were simultaneously induced in C2C12 myoblasts cultured with acidic medium (data not shown) as well as during myogenesis (20, 21, 24, 25, 27, 28, 32, 33). Then, using RT-PCR in 10T-MyoD cells, we investigated whether these alternative splicings require MyoD expression in the early stage of muscle-specific splicing induction. At first, RT-PCR fragments around exons 6a and 6b of β-TM were amplified and subjected to Southern blot analysis using oligonucleotides corresponding to exons 6a and 6b as hybridization probes. Untransfected 10T1/2 cells, 10T-hMGMT cells, and 10T-MyoD cells cultured in growth medium showed predominant use of exon 6a; the ratio of exon 6b to exon 6a was below 1 (Fig. 7a). In contrast, 10T-MyoD cells cultured in differentiation medium and acidic medium preferentially se-
FIG. 6. Muscle-specific exon selection of F1γ in myogenic differentiation requires protein synthesis and activated protein kinase C. a, time course and effect of cycloheximide treatment on induction by acidic stimulation in C2C12 cells. Cells prepared to express nonmuscle-type transcripts were cultured in acidic (low-pH) medium and harvested at the indicated times to obtain total RNAs, which were subjected to RT-PCR. Cells were grown in the absence or presence of 10 μg/ml cycloheximide (CHX). b, time course and effect of cycloheximide treatment on induction at normal pH. Using C2C12 cells carrying only muscle-type transcripts, the time dependence of induction of alternative splicing under normal conditions in the presence or absence of cycloheximide was examined by RT-PCR. c, time course and effect of cycloheximide treatment on acidic (low-pH) induction in BC3H1 cells. d, time course and effect of cycloheximide treatment on normal-pH induction in BC3H1 cells. e and f, time course and effect of HA1007 and H-7 on muscle-specific alternative splicing in BC3H1 cells. The protein kinase inhibitor H-7 (40 μM; Seikagaku Kogyo Co., Hitachi, Japan) was used to inhibit protein kinase C, and the protein kinase inhibitor HA1004 (40 μM; Seikagaku Kogyo Co.), an analogue of H-7, was used as a control. The time courses and RNA pattern of alteration from liver type to muscle type (e) and from muscle type to nonmuscle type (f) were determined by RT-PCR. The sizes of the PCR fragments are indicated in base pairs (bp).
lected exon 6b (Fig. 7a, lanes 8 and 9); the ratios of exon 6b to exon 6a were 7.4 with differentiation medium and 6.7 with acidic medium (lanes 8 and 9).

Fig. 7b shows the splicing patterns of the N-CAM pre-mRNA in the same system. 10T-MyoD cells cultured in differentiation medium and acidic medium selected MSD1, which was included between exons 12 and 13 specifically in muscle (Fig. 7b, lane 8 and 9) (32, 33). When the medium was changed to differentiation medium, inclusion of MSD1 was observed prior to myotube formation (data not shown).

Next, we tested cassette-type alternative splicing in the MEF2A pre-mRNA (21) and mutually exclusive splicing in the MEF2D pre-mRNA (24). Fig. 7 (lanes 8 and 9 in c and d) shows that muscle-specific alternative splicings in MEF2A and MEF2D pre-mRNAs were induced in 10T1/2-MyoD cells grown in differentiation medium and acidic medium. In addition,
MEF2A gene expression increased in differentiation medium, but not in acidic medium in C2C12 cells (Fig. 3e). Considering that muscle-specific splicing of MEF2A was shown in 10T1/2 MyoD cells after acidic stimulation even at low expression levels, the regulatory mechanisms of alternative splicing and gene expression are thought to be different.

These data show that muscle-specific alternative splicing in mouse F_{1,γ}, β-TM, N-CAM, MEF2A, and MEF2D pre-mRNAs is coordinately regulated in mouse myoblasts not only in differentiation medium, but also in acidic medium. In both inductions, an increase in MyoD expression is an indispensable step at the early stage of muscle-specific alternative splicing in many genes. Previously, the muscle-specific splicing mechanism could not be separated from the myogenic differentiation pathway, which was induced in differentiation medium. This study shows that acidic stimulation only induces muscle-specific alternative splicing, far from expression of MEF2 and myogenin mRNAs (Fig. 8), suggesting the presence of a common cascade to stimulate splicing regulatory factors for muscle-specific alternative splicing.

**DISCUSSION**

In this paper, we characterized an *in vivo* model system for inducing muscle-specific alternative splicing in mouse myoblasts employing mouse F_{1,γ} pre-mRNA as a useful marker during muscle differentiation (Fig. 1). We showed that acidic stimulation induces muscle-specific alternative splicing in F_{1,γ} pre-mRNA and in other muscle-specific pre-mRNAs in mouse myoblasts (C2C12 cells) and does not induce gene expression of MEF2 and myogenin and myotube formation. Analyzing the difference between the two induction systems (one is a low-serum induction system, and the other is an acidic one), we showed that MyoD is an essential factor for muscle-specific alternative splicing in several mouse genes.

Muscle-specific alternative splicing events were shown to occur prior to myotube formation (Fig. 2). Myogenin is indispensable for myotube formation (45, 46), and MEF2 is important in the action of MyoD (26) and in the expression of myogenin (47, 48). Thus, the absence of myotube formation after acidic induction can be attributed to low expression levels of myogenin and MEF2 in mouse myoblasts (Fig. 3). On the other hand, actin, a member of the cytoskeletal protein family, does not polymerize at low pH (49). Because the pH value of the acidic medium was 6.6, myoblasts could not form myotubes, even if myogenin was expressed in the cells. In addition, acidic stimulation was shown to induce muscle-specific alternative splicings in mouse N-CAM, β-TM, MEF2A, and MEF2D pre-mRNAs (Fig. 7), with different patterns of alternative splicing: those of β-TM and MEF2D were mutually exclusive types, and those of F_{1,γ}, N-CAM, and MEF2A were cassette types. Therefore, it is likely that a common regulatory cascade for muscle-specific alternative splicing exists after acidic stimulation. We showed that the mechanism of muscle-specific alternative splicing in many genes is independent of the gene expression of MEF2 and myogenin and is different from the cascade for myotube formation.

The other striking difference between these two induction systems was in their response to TGF-β1 treatment. TGF-β1 inhibited muscle-specific alternative splicing in C2C12 cells cultured in differentiation medium, but not in acidic medium (Fig. 2). TGF-β1 can block myogenic differentiation by inhibiting the increase in expression of MyoD and myogenin (42–44), so it is likely that acidic induction skips the action point of TGF-β1 or stimulates a signal cascade downstream of the action points of MyoD and TGF-β1.

To induce and maintain terminal differentiation in mouse myoblasts, a certain trigger is needed to promote the autoregulation of MyoD, MEF2, and myogenin, and the expression and activities of MyoD and myogenin must overcome threshold values (14). MyoD induces MEF2 gene expression (50), and then both cooperatively act to increase myogenin gene expression (47, 48, 51) in myogenic differentiation. Under acidic conditions, the induced expression of MyoD prepares the splicing regulatory apparatus beyond the threshold, but does not induce transcriptional activation of the MEF2 gene. Functions of MyoD include 1) promotion of muscle-specific gene expression, such as structural proteins and transcriptional factors; 2) permanent cell cycle arrest via gene expression of the cyclin-dependent kinase inhibitor p21; and 3) preparation of a splicing regulatory apparatus. The thresholds of MyoD action in these functions vary.

A previous report showed that myogenin can induce muscle-specific alternative splicing of α- and β-tropomyosin pre-mRNAs in differentiation medium in fibroblasts (52). However, overexpression of myogenin leads to activation of MyoD expression because of its positive autoregulatory loop (51, 52). In contrast, the acidic induction described here induced only MyoD expression, but did not induce myogenin and MEF2 expression. Considering the previous report and our results, overexpression of
myogenin induced MyoD, and then muscle-specific alternative splicings of α- and β-tropomyosins would be induced.

In addition, Id, a negative regulator of MyoD that is highly expressed under high-serum conditions, can block the MyoD-dependent gene expression and the activation of the positive loop of muscle-specific transcription factors by preventing the formation of MyoD heterodimer containing E12/E47, other bHLH proteins. Therefore, when MyoD is overexpressed in fibroblasts under high-serum conditions, the transfected cells cannot convert to myotubes. On the other hand, overexpression of Id in myoblasts prevents muscle-specific alternative splicings of F$_1 \gamma$ pre-mRNA and other pre-mRNAs under acidic or low-serum conditions by blocking the positive feedback of MyoD expression (Fig. 6). These data indicated that MyoD is necessary for muscle-specific alternative splicing.

The MEF2 family is differentially controlled at the transcriptional and post-transcriptional levels during muscle differentiation. As shown in Figs. 3 and 7, the gene expression of MEF2A is not induced, but muscle-specific splicings of MEF2A and MEF2D are induced in acidic stimulation. MEF2A and MEF2D accumulate preferentially in skeletal muscle, heart, and brain (20, 21, 24, 25), and these tissue-specific isoforms correlate exactly with the presence of endogenous MEF2A activity. This finding indicates that tissue-specific alternative domains of MEF2A and MEF2D play a key role in the regulation of MEF2 activities in vivo (21, 24, 25). The regulatory system of muscle-specific alternative splicing should be prepared prior to the transactivation of MEF2 function, so it is likely that post-transcriptional control of alternative RNA splicing contributes to muscle differentiation via the regulation of MEF2A and MEF2D function.

Acidic stimulation appears to trigger a common mechanism for muscle-specific alternative splicing in the presence of MyoD. As a result of the inhibition of muscle-specific exclusion of an alternatively spliced exon in F$_1 \gamma$ pre-mRNA by cycloheximide and the protein kinase inhibitor H-7, it is suggested that de novo protein synthesis of an intracellular protein factor and activated protein kinase C directly regulates this splicing. The activated protein kinase C directly regulates this splicing. The activated protein kinase C directly regulates this splicing. The activated protein kinase C directly regulates this splicing.

We compared the acidic induction system for muscle-specific alternative splicing in mouse myoblasts and MyoD-transfected fibroblasts with the usual low-serum stimulation system induced in differentiation medium. The striking difference is that myogenin and MEF2 are not required for muscle-specific splicing regulation. MyoD is required for muscle-specific alternative splicings in many genes induced by either low-serum or acidic stimulation at an early stage of myogenesis. Thus, we identified a common signal cascade for muscle-specific alternative splicing different from that of myotube formation (Fig. 8). In future investigations, we must find a direct effector for the formation of muscle-specific splicingosomes during myogenesis.

**Acknowledgments**—We thank Dr. H. Weintraub for providing mouse MyoD cDNA, Dr. S. Tomina for the mouse BALB/c 3T3 cDNA library, Dr. H. Hayakawa for pDEB-hyg and human 6-α-methylguanine-DNA methyltransferase, and Dr. Y. Takebe (National Institute of Infectious Disease) for the Sφo promoter.
MyoD Is Indispensable for Muscle-specific Alternative Splicing in Mouse Mitochondrial ATP Synthase γ-Subunit Pre-mRNA
Masaru Ichida, Hitoshi Endo, Uichi Ikeda, Chie Matsuda, Eriko Ueno, Kazuyuki Shimada and Yasuo Kagawa

J. Biol. Chem. 1998, 273:8492-8501. doi: 10.1074/jbc.273.14.8492

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