Muscle-specific Exonic Splicing Silencer for Exon Exclusion in Human ATP Synthase γ-Subunit Pre-mRNA* $$\text{S}\text{S}$$

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Mitochondrial ATP synthase γ-subunit (F1γ) pre-mRNA undergoes alternative splicing in a tissue- or cell type-specific manner. Exon 9 of F1γ pre-mRNA is specifically excluded in heart and skeletal muscle tissues and in acid-stimulated human fibrosarcoma HT1080 cells, rhabdomyosarcoma KYM-1 cells, and mouse myoblast C2C12 cells. Recently, we found a purine-rich exonic splicing enhancer (ESE) element on exon 9 via transgenic mice bearing F1γ mutant minigenes and demonstrated that this ESE function ubiquitously with exclusion of muscle tissue (Ichida, M., Hakamata, Y., Hayakawa, M., Ueno E., Ikeda, U., Shimada, K., Hamamoto, T., Kagawa, Y., Endo, H. (2000) J. Biol. Chem. 275, 15992–16001). Here, we identified an exonic negative regulatory element responsible for muscle-specific exclusion of exon 9 using both in vitro and in vivo splicing systems. A supplementation assay with nuclear extracts from HeLa cells and acid-stimulated HT1080 cells was performed for an in vitro reaction of muscle-specific alternative splicing of F1γ minigene and revealed that the splicing reaction between exons 8 and 9 was the key step for regulation of muscle-specific exon exclusion. Poly(ADP-ribose) polymerase in intron 8 requires ESE on exon 9 for constitutive splice site selection. Mutation analyses on the F1γ Ex8-9 minigene using a supplementation assay demonstrated that the muscle-specific negative regulatory element is positioned in the middle region of exon 9, immediately downstream from ESE. Detailed mutation analyses identified seven nucleotides (5'-AGUUCCA-3') as a negative regulatory element responsible for muscle-specific exon exclusion. This element was shown to cause exon skipping in in vivo splicing systems using acid-stimulated HT1080 cells after transient transfection of several mutant F1γ Ex8-9-10 minigenes. These results demonstrated that the 5'-AGUUCCA-3' immediately downstream from ESE is a muscle-specific exonic splicing silencer (MS-ESS) responsible for exclusion of exon 9 in vivo and in vitro.

Alternate RNA splicing is an important process that regulates developmental stage- and/or tissue-specific gene expression in higher eukaryotes (1–3). This process can generate multiple mRNAs from a single primary transcript. The pattern of alternative splicing is classified by the combination of splice sites. The patterns are categorized as retained intron, selecting internal donor or acceptor sites, mutually exclusive exon, and cassette exon. The determination of an alternative splice site is regulated by interaction between cis-acting regulatory elements and trans-acting regulatory factors. The Drosoptera sex determination pathway mediated by Sex-lethal (Sxl), Transformer (Tra), and Transformer-2 (Tra-2) proteins is an example of regulation by alternative splicing (4). However, the molecular mechanism of alternative splicing in many mammalian genes is unknown. Therefore, the mechanism of splice site recognition should be studied to better understand gene expression in higher eukaryotes.

Exonic cis-acting regulatory elements (e.g. exonic splicing enhancers (ESEs)† and exonic splicing silencers (ESSs)) play a key role in splice site selection in alternative splicing. ESEs act as positive regulatory elements for exon inclusion and promote the use of nearby weak 3’ splice sites. The ESE was originally identified in mouse immunoglobulin M (IgM) exon M2 (5). Subsequently, ESEs have been found within the exons of various genes (6–15). The most prominent feature of these ESEs is a purine-rich sequence. A number of serine/arginine-rich (SR) proteins can recognize purine-rich ESEs through RNA-protein interaction and activate splicing (8, 15–18). On the other hand, ESSs act as negative regulatory elements for exon exclusion. ESSs have been identified in only a small number of pre-mRNAs (namely human fibronectin EDA exon 11, human immunodeficiency virus type 1 (HIV-1) tat exon 2 and tat-rev exon 3 (19, 20), fibroblast growth factor receptor 2 K-SAM exon (21), bovine papillomavirus type 1 (BPV-1) exon 2 (22), cell surface molecule CD44 exon 5 (23), and rat β-tropomyosin (β-TM) exon 7 (24). No nucleotide sequences common to ESSs have as yet been found, although ESS elements generally seem to be located near ESE elements in the genes listed above. These ESSs are also recognized by specific regulatory factors such as SR protein (25) and heterogeneous nuclear ribonucleoprotein (hnRNP) (24, 26).

We have used the human and mouse ATP synthase γ-subunit (F1γ) genes as a model for studying the regulatory mech-

† The abbreviations used are: ESE, exonic splicing enhancer; F1γ, mitochondrial ATP synthase γ-subunit; DMEM, Dulbecco’s modified Eagle’s medium; S-MEM, minimum essential medium; FBS, fetal bovine serum; RT, reverse transcription; ESS, exonic splicing silencer; SR protein, serine/arginine-rich protein; NE, nuclear extract; MS-ESS, muscle-specific exonic splicing silencer; hnRNP, heterogeneous nuclear ribonucleoprotein; β-TM, β-tropomyosin; PTB, poly(A) tract-binding protein; WT, wild type.

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¶ The on-line version of this article (available at http://www.jbc.org) contains a description of methods of plasmid construction and one table.

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FIG. 1. \( F_1 \gamma \) exon 9 is specifically excluded under acidic conditions in HT1080 cells. A, schematic representation of alternative splicing in \( F_1 \gamma \) pre-mRNA. Boxes and horizontal lines represent exons and introns, respectively. The gray box shows an alternatively spliced exon. B, a reversible induction system in HT1080 and HeLa S3 cells. \( a \) and \( b \), time course and effect of cycloheximide (CHX) treatment on acidic stimulation in HT1080 and HeLa S3 cells, respectively. \( c \) and \( d \), time course and effect of cycloheximide treatment on induction with normal medium (pH 7.4) in HT1080 cells and HeLa S3 cells, respectively. HT1080 and HeLa S3 cells were cultured in normal media (pH 7.4) and acidic media (pH 6.6) as described under "Experimental Procedures." In panels \( a \) and \( b \), normal media were replaced with acidic media. In panels \( c \) and \( d \), acidic media were replaced with normal media. Cells were cultured in the absence or presence of 10 μg/ml cycloheximide. Total RNA from cells was analyzed by RT-PCR using a sense primer in exon 8 and an antisense primer in exon 10. PCR products were separated on 3% agarose gels. Splicing patterns are indicated at the right. Lane M, molecular size markers (\( \phi X 174/HaeIII \)).

Experimental Procedures

Cell Culture—HT1080 cells, human fibrosarcoma cells, were obtained from the Japanese Cancer Research Resources Bank. HeLa S3 cells were provided by Dr. H. Sakamoto (Kobe University, Hyogo, Japan). All cells were cultured at 37 °C in a humidified atmosphere under 5% CO2. HT1080 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (final pH 7.4 under 5% CO2) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Acidic stimulation was performed as follows. Once HT1080 cells were grown to semiconfluence, culture medium was replaced with acidic medium (final pH 6.6 under 5% CO2) supplemented with 10% FBS and 2.7 mM NaHCO3. The cells then underwent further culturing for 48 h under acidic stimulation. Cycloheximide was added to create a final concentration of 10 μg/ml in the medium. HeLa S3 cells were cultured under two different conditions. One condition utilized DMEM supplemented with 10% FBS in a similar manner to the culture conditions for HT1080 cells. HeLa S3 cells adhered to culture plates under these conditions. The cells were stimulated with an acidic medium as described above. The other condition was utilized for the preparation of nuclear extracts. HeLa S3 cells were cultured in spinner flasks in minimum essential medium (S-MEM) supplemented with 5% horse serum (Invitrogen).
Splicing between exons 8 and 9 is the key reaction for exon exclusion. A, schematic representation of $\beta$-globin, $F_1\gamma\text{Ex8-9}$, $F_1\gamma\text{Ex9-10}$, and $F_1\gamma\text{Ex8-10}$ substrates. The radiolabeled RNA substrates were synthesized in vitro using linearized plasmid DNAs with SP6 RNA polymerase.
**Plasmid Construction**—All human F\(\gamma\) minigene constructs were introduced into the pCMV-SPORT vector (Invitrogen) and confirmed by sequence analysis. The pSP64H36 containing human \(\beta\)-globin minigene was kindly donated by Dr. A. Watakabe (National Institute for Basic Biology, Aichi, Japan) (5).

These human F\(\gamma\) minigene, p\(\text{F}^\gamma\)E9x9-10 and p\(\text{F}^\gamma\)E8x10, were derived from the phage clone HATPG21, a human genomic clone of the F\(\gamma\) gene (28). The p\(\text{F}^\gamma\)E9x9-9 was constructed previously (32). The p\(\text{F}^\gamma\)E8x9-9 (In 9) was constructed from p\(\text{F}^\gamma\)E9x9-10 and p\(\text{F}^\gamma\)E9x9-9. The p\(\text{M}^\gamma\)1-2 and p\(\text{M}^\gamma\)3 were cloned into the pCMV-SPORT vector. The p\(\text{M}^\gamma\)E9x9-3, p\(\text{M}^\gamma\)E9x9-9 and p\(\text{M}^\gamma\)E9x9-13 were derived from a part of exon 9 of the F\(\gamma\) gene. The p\(\text{F}^\gamma\)E8x9-9-10 was constructed from p\(\text{F}^\gamma\)E8x9-9 and p\(\text{F}^\gamma\)E8x9-10. Mutant minigene constructs from p\(\text{F}^\gamma\)E9x9-9 (Ex9-MU1, Ex9-MU2, Ex9-MU3, Ex9-MU4, mut.1, mut.2, mut.3, mut.4, mut.5, mut.6, mut.7, mut.8 and mut.9) and other mutants from p\(\text{F}^\gamma\)E9x9-9-10 (mut.a, mut.b, mut.c, mut.d, mut.e, mut.f, and mut.g) were created by PCR mutagenesis (33). For detailed methods of plasmid construction described above, see the Supplemental Material.

**RNA Preparation and RT-PCR Analysis**—Total RNAs were prepared from HT1080 and HeLa S3 cells by the acid guanidium method (35). Five micrograms of total RNA were denatured at 65 °C for 10 min and immediately chilled on ice. First-strand cDNA was synthesized at 42 °C for 1 h in a total volume of 20 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl\(_2\), 10 mM dithiothreitol, 1 mM dNTPs, 0.1 mM oligo(dT)\(_{18}\) primer, and 50 units of Superscript II (Life Technologies, Inc.). PCR amplification was performed in a total volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\(_2\), 200 mM dNTPs, 0.2 μM each of the forward and reverse primers, 1 unit of Taq DNA polymerase (Takara Shuzo Co.), and 1 μl of first strand cDNA. PCR was carried out for 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The endogenous F\(\gamma\) cDNA was amplified with a sense primer in exon 8 (5′-GTCACTCAAAAGAGTGGTG-3′) and an antisense primer in exon 10 (5′-TAATGGAGGAACTTCTTC-3′) and an antisense primer in exon 10. Ten microliters of the PCR products were separated by electrophoresis on a 3% agarose gel and then stained by ethidium bromide.

**Muscle Preparation and in vitro Splicing Assay**—Nuclear extracts were prepared from HeLa S3 (1.2 × 10\(^6\) cells), unstimulated HT1080 cells (7.5 × 10\(^6\) cells), and acid-stimulated HT1080 cells (5.4 × 10\(^6\) cells) by Dignam’s method (36). Immediately, nuclear extracts were dialyzed against buffer D (20 mM Hepes, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). For in vitro transcription, plasmid DNAs were linearized by XhoI or BamHI. Capped human minigene mRNAs were synthesized in vitro for 1 h in a total volume of 10 μl containing 40 mM Tris-HCl (pH 7.5), 6 mM MgCl\(_2\), 2 mM spermidine, 5 mM dithiothreitol, 1 mM m\(^{32}\)P-UTP, dNTPs (0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, and 0.1 mM UTP), 5 mM [α-\(^{32}\)P]UTP (PerkinElmer Life Sciences), 10 units of Ribonuclease Inhibitor (Takara Shuzo Co.), 22.5 units of SP6 RNA polymerase (Amersham Biosciences, Inc.), and 2 μg of linearized plasmid DNA. Full-length transcripts were separated on 4% polyacrylamide-7% urea gels and eluted from the gel slices in elution buffer (1% SDS and 2 mM ammonium acetate) at 37 °C for 3 h. Standard in vitro splicing reactions were carried out at 30 °C for the indicated periods in a total volume of 25 μl containing 60% (v/v) nuclear extracts, 2.6% (w/v) polyvinyl alcohol, 3.0 mM MgCl\(_2\), 0.5 mM ATP, 20 mM creatine phosphate, 20,000 cpn or 32P-labeled RNA substrate, and 10 units of ribonuclease inhibitor. Supplementation assays were performed by adding nuclear extracts from HT1080 cells (40 or 80 μg of total protein) to those from HeLa S3 cells (80 μg of total protein) (Fig. 2B). After incubation, the reactions were terminated by treatment with protease K (Roche Molecular Biochemicals) at 30 °C for 30 min. The splicing products were separated and detected by electrophoresis on 5% or 6% polyacrylamide-7% urea gels and then detected by autoradiography with x-ray film (KX-U, Fuji Photo Film Co.).

**Muscle Splicing Assay**—HT1080 cells were cultured with DME (final pH 7.4 under 5% CO\(_2\)) supplemented with 10% FBS. About 5 × 10\(^5\) cells were transfected with 10 μg of each wild type and mutant p\(\text{F}^\gamma\)E8x9-10 minigene by the calcium phosphate precipitation method (37). After transfection, the cells were cultured for 24 h in DME (final pH 7.4 under 5% CO\(_2\)) supplemented with 10% FBS. Unstimulated cells were cultured further for 24 h in normal medium (pH 7.4). Acidic stimulation was performed as described above. After stimulation, the muscle-specific and acid-stimulated minigenes were harvested, total RNAs were prepared by the acid guanidium method. First strand cDNA synthesis and RT-PCR analysis were performed as described above. The exogenous F\(\gamma\) transcripts were amplified with a sense primer in the SP6 promoter and an antisense primer in exon 10. The endogenous F\(\gamma\) transcripts were amplified with a sense primer in exon 7 (5′-GCCAGATCCCATCCTACTACT-3′) and an antisense primer in exon 10. Ten microliters of the PCR products were resolved by electrophoresis on 3% agarose gels and then stained by ethidium bromide.

**RESULTS**

**Exon 9 Is Not Excluded by Acidic Stimulation in HeLa Cells but Is Excluded in HT1080 Cells**—The pattern of alternative splicing in F\(\gamma\) pre-mRNA represented in Fig. 1A shows that exon 9 is specifically excluded in heart and skeletal muscle tissues. In cultured cells, we previously reported that muscle-specific exclusion of exon 9 was reversibly induced by cultivation under acidic medium in human fibrosarcoma HT1080 cells and mouse myoblast C2C12 cells (29, 31). Here, prior to using nuclear extracts from HeLa cells for an in vitro splicing assay, we examined the effects of acidic stimulation on HeLa cells (Fig. 1B). In HT1080 cells, the muscle-specific exclusion of exon 9 was observed after treatment with acidic medium (pH 6.6) (Fig. 1B, lanes 1–5 in panel a). Replacement of acidic medium (pH 6.6) with normal medium (pH 7.4) then shifted the splicing pattern from muscle to nonmuscle type for F\(\gamma\) mRNA (Fig. 1B, lanes 1–5 in panel c). Treatment with the protein synthesis inhibitor cycloheximide on both methods of induction revealed that inclusion of exon 9 was not inhibited (Fig. 1B, lanes 6–10 in panel b), but exclusion of exon 9 was specifically inhibited in HT1080 cells (Fig. 1B, lanes 6–10 in panel a). On the other hand, in HeLa S3 cells, muscle-specific exon exclusion was not observed under any culture conditions (Fig. 1B, lanes 1–5 in both panels b and d) and was not influenced by treatment with cycloheximide (Fig. 1B, lanes 6–10 in both panels b and d). These results indicated that the splicing process of muscle-specific exon exclusion requires a newly synthesized protein factor and that this splicing regulatory factor is not induced in HeLa cells, but induced in acid-stimulated HT1080 cells. Considering the above, we will be able to construct an in vitro splicing system for muscle-specific alternative splicing in F\(\gamma\)
Splicing between Exons 8 and 9 Is the Key Reaction for Exon Exclusion in Supplementation Assay

To investigate the key reaction for muscle-specific exon skipping, at first, we constructed the three types of human F1γ/H9253 minigenes for an in vitro splicing assay (Fig. 2A): F1γ/H9253Ex8-9, a minigene containing exon 8, intron 8, and exon 9; F1γ/H9253Ex9-10, containing exon 9, intron 9, and exon 10; and F1γ/H9253Ex8-10, containing exon 8, donor site of intron 8, acceptor site of intron 9, and exon 10. The minigene of /H9252-globin was used as a control in splicing reactions. An in vitro splicing assay using HeLa cell NEs was performed as described previously (32). Fig. 2C shows that the in vitro splicing reactions of the F1γ/H9253Ex8-9, F1γ/H9253Ex9-10, and F1γ/H9253Ex8-10 substrates proceeded smoothly, as did that of the β-globin substrate. Comparing splicing efficiencies of these three F1γ minigene substrates, the reaction of F1γ/H9253Ex9-10 substrate proceeded the most smoothly. A final spliced product of this substrate was already detected with a lariat structure containing exon 10 at 1 h of incubation, and the lariat structure containing exon 10 disappeared in proportion to the increase of a lariat structure without exon 10 during the incubation period (Fig. 2C, lanes 9, 4, 7, and 8). Electrophoresis proceeded as described in the legend to Fig. 3B. Open and shaded squares represent HT1080 NE (N) and HT1080 NE (A).

**FIG. 3.** The acceptor site in intron 8 is required for exon exclusion. A, schematic representation of wild type and mutant F1γ/H9253 substrates. In the F1γ/H9253Ex8-9 (In 9), white and gray lines indicate introns 8 and 9, respectively. Double lines in introns indicate junctions of 5’ and 3’ splice sites. Lengths of each region are indicated in nucleotides (nt), and each exon contains linker sequences derived from pCMV-SPORT. B, in vitro splicing analysis of F1γ/H9253Ex8-9 (WT) and F1γ/H9253Ex8-9 (In 9) substrates. Substrates were incubated at 30 °C in HeLa cell nuclear extracts (160 μg of total protein), for periods indicated above each lane. Electrophoresis proceeded on a 6% polyacrylamide gel containing 7 M urea. Intermediates and splicing products are indicated at the right. Lane M, molecular size markers (6X174/HinfI). C, supplementation assay of F1γ/H9253Ex8-9 (WT) and F1γ/H9253Ex8-9 (In 9) substrates. Substrates were incubated at 30 °C for 4 h in HeLa NE supplemented with HT1080 NE. The protein amount of HeLa NE was 160 μg in lanes 2 and 6 and 80 μg in lanes 3, 4, 7, and 8. The protein amount of HT1080 NE (N) and HT1080 NE (A) was 80 μg in lanes 3, 4, 7, and 8. Electrophoresis proceeded as described in the legend to Fig. 3B. Open and shaded squares represent HT1080 NE (N) and HT1080 NE (A).
and 10 using a supplementation assay (Fig. 2B). The supplementation assay is performed as an in vitro splicing assay with the addition of NEs from acid-stimulated HT1080 cells, HT1080 NE (A). The assay presents muscle-specific alternative splicing in F1γ pre-mRNA (32). Here, we examined the splicing reactions of the three minigene substrates under muscle-specific conditions using the supplementation assay (Fig. 2D). All splicing products were detected after 4 h of reaction when supplementation assay was performed using NEs from unstimulated HT1080 cells, HT1080 NE (N). The splicing reactions of β-globin and F1γEx9-8 substrates were not affected (Fig. 2D, lanes 2, 3, 7, and 8), and those of F1γEx9-10 and F1γEx9-10 substrates were slightly enhanced (Fig. 2D, lanes 12, 13, 17, and 18). On the other hand, supplementation assay with HT1080 NE (A) indicated different influences on the splicing reactions between these substrates. HT1080 NE (A) specifically inhibited the splicing reaction of F1γEx9-8 substrate in a dose-dependent manner (Fig. 2D, lanes 9 and 10). On the other hand, the splicing reactions of β-globin, F1γEx9-10, and F1γEx9-10 substrates were not inhibited by the addition of HT1080 NE (A) (Fig. 2D, lanes 4, 5, 14, 15, 19, and 20). Since the splicing reaction between exons 8 and 9 was specifically inhibited by supplementation of HT1080 NE (A), this splicing reaction is considered to be the key reaction for muscle-specific exon skipping. It is likely that HT1080 NE (A) contains a negative regulatory factor for splicing between exons 8 and 9. These results indicated that a negative regulatory cis-acting element affected by such a negative regulatory factor should exist in the F1γEx9-8 substrates, probably within the acceptor site of intron 8 or in the region of exon 9.

Exon Exclusion Requires the Acceptor Site of Intron 8—The polypyrimidine tracts of splicing acceptor sites are very important for exon selection (5). Compared with the acceptor site of intron 9, the polypyrimidine tract of intron 8 is considered to be relatively “weak” because the region contains many purine nucleotides. To test the influence of the polypyrimidine tract of intron 8 on muscle-specific exon skipping, we constructed the novel chimeric minigene F1γEx9-8 (In 9), in which the acceptor site was replaced by that of intron 9 (Fig. 3A). The wild type F1γEx9-8 (WT) and chimeric F1γEx9-8 (In 9) substrate reactions proceeded smoothly during in vitro splicing assay using HeLa NE (Fig. 3B). In the supplementation assay, although the splicing of F1γEx9-8 (WT) substrate was inhibited by addition with HT1080 NE (A), the splicing reaction of F1γEx9-8 (In 9) substrate appeared to be resistant to supplementation with HT1080 NE (A) (Fig. 3C, lane 8). These results indicate that the acceptor site including the polypyrimidine tract of intron 8 is necessary for muscle-specific exon skipping. If this were not the case, the polypyrimidine tract of intron 9 would be stronger than that of intron 8 and would not require any splicing enhancer element in exon 9 (see below).

Utilization of Acceptor Site in Intron 8 Requires Exonic Elements—In general, weak polypyrimidine tracts require splicing enhancer elements such as ESEs. Previously, we found an ESE element on exon 9 in in vivo splicing systems using cultured cells and transgenic mice (33). This element is a purine-rich sequence and responsible for the inclusion of exon 9 in non-muscle tissues. To analyze the positive and negative splicing regulatory elements on the exon, we first demonstrated ESE activity using an in vitro splicing assay. Next, we introduced mutation in every region of the exon and scanned the exon region responsible for negative regulatory activity using the supplementation assay.

To examine the ESE activity of exon 9 in an in vitro splicing assay, we constructed three kinds of chimeric minigenes in the mouse immunoglobulin μ (μM1–2) minigene (Fig. 4, A and B). The natural ESE on M2 exon of μM1–2 minigene was substituted for each of three tandem repeats of the 5‘, middle, or 3‘ regions of exon 9, named E9a, E9b, and E9c, respectively.
These minigenes were subjected to an in vitro splicing assay using HeLa NE. The splicing reaction for μM1–2 containing natural ESE proceeded smoothly, while that for μΔM substrate lacking ESE did not proceed at all, as described previously (5) (Fig. 4C). Splicing for μMΔE9aX3 substrate was strongly enhanced in the same manner as μM1–2 substrate. The final spliced product was detected at low levels after 30 min and thereafter gradually accumulated during incubation (Fig. 4C, lanes 9–12). Although the splicing of μMΔE9cX3 substrate was also restored (Fig. 4C, lanes 17–20), the activation of the E9c region was less than that of the E9a region. The μMΔE9bX3 substrate was not spliced throughout the incubation period (Fig. 4C, lanes 13–16). From these results, the 5′ region (E9a) of the exon is shown to possess ESE activity instead of wild type ESE from the μM1–2 minigene. In addition, E9a is strongly suggested to function as the ESE in FγEx8-9 substrates lacking ESE. Next, to examine whether this ESE element is responsible for the activity of the FγEx8-9 minigene, we constructed an MU1 mutant minigene carrying a purine-to-pyrimidine substitution in the E9a region (Fig. 5A). As shown in Fig. 5B, the splicing of Ex9-MU1 substrate was completely inhibited, and a final spliced product was not detected during incubation (Fig. 5B, lanes 6–10). This result demonstrates that the purine-rich element in the E9a region is an ESE element required for the constitutive inclusion of exon 9. These observations were consistent with the results of analyses using in vivo splicing systems and transgenic mice (33).

Three mutant FγEx8-9 minigenes (MU2-MU4) were subjected to supplementation assays for locating muscle-specific negative regulatory region in exon 9 (Fig. 5A). Mutations of MU2 and MU3 are generated by pyrimidine-to-purine nucleotide substitutions in the E9a and E9b regions, respectively. These purine-rich sequences are predicted to act as ESE elements. The mutation of MU4 was generated by purine-to-pyrimidine substitution in the E9c region with the aim of destroying the weak ESE activity of the region. In vitro splicing reactions of these minigenes proceeded smoothly in HeLa NEs (Fig. 5C). These results indicated that the purine-rich sequence at the E9a region of MU2 possesses splicing enhancer activity for constitutive exon inclusion, although MU2 mutation is predicted to generate another ESE element. In addition, these results also indicated that the purine-rich sequence of the E9c region is not required for exon inclusion, because the splicing reaction of the MU4 minigene proceeded efficiently. Considering the above, an ESE element for constitutive exon inclusion is located in the E9a region, and the ESE element acted with the acceptor site of intron 8. Next, these minigenes were subjected to supplementation assay. The splicing of MU2 substrate was dose-dependently inhibited in the same manner as that of wild type substrate (Fig. 5C, lanes 9 and 10), indicating that muscle-specific negative regulatory activity still functioned even in the MU2 minigene containing another ESE sequence. We then examined the regions downstream from ESE on the exon using MU3 and MU4 mutants. Although the MU4 mutation did not
influence the splicing reaction (Fig. 5C, lanes 19 and 20), MU3 mutation inhibited the negative regulatory activity (Fig. 5C, lanes 14 and 15). From these results, the middle region of the exon in the E9b region would be responsible for muscle-specific exon skipping. However, since the MU3 mutation is speculated to generate another purine-rich ESE element, the possibility that the double ESE elements on the MU3 exon simply enhance splicing reactions and thus overcome negative regulatory activity must be excluded. We therefore shifted the focus of this study to detailed examinations of the middle region of exon 9.

**Supplementation Assay Reveals the Muscle-specific Negative Regulatory Element**—To address a cis-acting negative regulatory element for the exclusion of exon 9, we designed nine mutant FγEx8-9 substrates (Fig. 6A). These mutations were introduced into a wide range of areas in exon 9 except for the purine-rich ESE element, and nucleotides were substituted so
as not to generate any known ESE sequences. The wild type and mutant FγEx8-9 substrates were subjected to supplementation assay with HT1080 NE (A). All mutant substrates were smoothly spliced within HeLa NE, and the splicing patterns of mutant substrates were identical to those of wild type substrate (Fig. 6B, compare lane 1 with lanes 4, 7, 10, 13, 16, 19, 22, 25, and 28). When HT1080 NE (A) was supplemented to HeLa NE in the splicing reactions of these substrates, mutations in mut.3–6 minigenes resulted in varying degrees of cancellation of the splicing inhibition (Fig. 6B, lanes 11, 12, 14, 15, 17, 18, 20, and 21). However, the splicing reactions of other mutant substrates were inhibited in a dose-dependent manner by the addition of HT1080 NE (A) to the same degree as wild type substrate (Fig. 6B, lanes 5, 6, 8, 9, 23, 24, 26, 27, 29, and 30). In particular, the splicing of mut.6 substrates resulted in obvious cancellation of splicing inhibition (Fig. 6A, lanes 20 and 21). These results showed that mutations at the 5’-AGUUCCA-3’ sequence in the E9b region prevented splicing inhibition of FγEx8-9 substrate by the supplementation of HT1080 NE (A). These mutations are not similar to any known ESE element, and we can therefore exclude the possibility of double ESEs overcoming negative regulatory activity. We therefore indicated that this seven-nucleotide sequence in the middle region of exon 9 acted as a negative regulatory element for muscle-specific exon skipping in FγEx8-9 substrate. In addition, this muscle-specific negative regulatory element is located immediately downstream from ESE and functions well with other ESE elements such as the M52E2 sequence.

MS-ESS Causes Exon Skipping in Vivo—The middle region of exon 9, which has been determined as a muscle-specific negative regulatory element for splicing reactions between exons 8 and 9 in the supplementation assay, had to be proven to function for muscle-specific exon skipping during in vivo splicing. Seven mutant minigenes were generated from the wild type FγEx8-9-10 minigene constructed in a mammalian expression vector (Fig. 7A), and then subjected to transient transfection into HT1080 cells. Transfected HT1080 cells were cultured in normal medium (pH 7.4) or acidic medium (pH 6.6). Total RNAs were then isolated and subjected to RT-PCR analysis (Fig. 7B). The splicing pattern of endogenous Fγ pre-mRNA was analyzed for detection of acid stimulation performance in each transfected cell (Fig. 7B, panel b). When the cells were cultured on acidic media, the transcripts of mut.a, mut.f, and mut.g minigenes were shown to include exon 9 in the same manner as WT minigene (Fig. 7B, lanes 2, 4, 14, and 16 in panel a). On the other hand, the mutated exon 9 in mut.b, mut.c, mut.d, and mut.e minigenes did not appear to be excluded even under acidic conditions (Fig. 7B, lanes 6, 8, 10, and 12 in panel a). Mutant nucleotide sequences in exon 9 of mut.b, mut.c, mut.d, and mut.e minigenes were identical to those of mut.3–6 (Fig. 6A), and these observations are consistent with results from the supplementation assay (Fig. 6B). These results provided evidence that the seven-nucleotide sequence, 5’-AGUUCCA-3’, in the middle region of exon 9 was sensitive to acidic stimulation and essential for muscle-specific negative selection of exon 9 in the FγEx8-9-10 minigene. We therefore concluded that the 5’-AGUUCCA-3’ element on exon 9 is a cis-acting negative regulatory element for exon exclusion and functions as an MS-ESS in splicing regulation of Fγ pre-mRNA.

**DISCUSSION**

The present study demonstrated that an exonic cis-acting negative regulatory element participated in alternative splicing regulation of human Fγ pre-mRNA. Analyses of the in vitro splicing system coupled with supplementation assay, which reflected the conditions for muscle-specific alternative splicing in Fγ pre-mRNA, revealed that splicing between exons 8 and 9 was key to muscle-specific exclusion of exon 9. From the detailed mutation analyses of Fγ minigenes using in vitro systems, the muscle-specific negative regulatory element was found to exist in the middle region downstream from the purine-rich ESE element within exon 9. Furthermore, the 5’-AGUUCCA-3’ element was shown to be essential for muscle-specific exon exclusion using an in vivo splicing assay. From these results, this seven-nucleotide sequence is considered an essential cis-acting negative regulatory element for muscle-specific exon exclusion.
MS-ESS for exon exclusion. This MS-ESS requires both an acceptor site in intron 8 and an ESE in exon 9. These structures are necessary for ubiquitous exon selection in nonmuscle tissues. The MS-ESS interferes with this ubiquitous exon selection mechanism in a muscle-specific manner (Fig. 8) and plays an important role in tissue-specific alternative splicing of human F1γ pre-mRNA.

The negative regulatory element (5′-AGUUCCA-3′) on exon 9, which here we identified as MS-ESS, has three notable features. First, this element specifically functions under in vivo and in vitro conditions to reflect muscle-specific alternative splicing. In the supplementation assay, the splicing of F1γEx8-9 substrate was not inhibited by adding NEs from normal pH HT1080 but was inhibited by adding NEs from acid-stimulated HT1080 (Fig. 2D). Mutations in this element blocked splicing inhibition of F1γEx8-9 substrate in supplementation assay with NEs from acid-stimulated HT1080 (Fig. 6B). During the in vivo splicing assay, exon 9 of the F1γEx8-9-10 minigene was specifically excluded under acidic conditions in HT1080 cells. However, mutations in this ESS element inhibited muscle-specific exon skipping (Fig. 7B). These results caused us to label this element the “muscle-specific exonic splicing silencer.” Second, the MS-ESS activity of this element couples with a 3′ acceptor site in intron 8. Supplementation with NEs from acid-stimulated HT1080 during the in vitro splicing assay inhibited selection of exon 9 in wild type F1γEx8-9 substrate. However, selection was not inhibited in either the F1γEx8-10 substrate (Fig. 2D) or the mutant F1γEx8-9 (In 9) substrate, in which the acceptor site was replaced with that of intron 9 (Fig. 3C). From these results, it was concluded that the acceptor site in intron 8, containing a “weak” polypyrimidine tract, is required for proper function of the MS-ESS element. The effect upon utilization of 3′ splice sites has been demonstrated in ESSs from HIV-1 tat exon 2, tat-rev exon 3 (19, 20), BPV-1 exon 2 (22), and rat β-TM exon 7 (24). Third, this ESS element is located immediately downstream from the ESE element on exon 9. Positional correlation between ESE and ESS elements has been reported in several exons (e.g. human fibronectin EDA exon 11, HIV-1 tat exon 2, and tat-rev exon 3 (19, 20) and BPV-1 exon 2 (22)). The ESE element is located downstream from the ESS element in exon 7 of the β-TM gene, which is specifically selected in muscle. The ESE and ESS elements are located close to one another in a number of studies, and the proximity in all cases would interfere with function.

The ESE on exon 9 of F1γ gene has been identified by in vivo splicing systems using cultured cells and transgenic mice (33). Utilizing in vitro splicing assay, we proved that this element (5′-AUUAUGAAAA-3′) is located in the 5′ region of exon 9 and possesses splicing enhancer activity in the μMΔ minigene (Figs. 4C and 5B). Generally, such ESEs help exon selection by enhancing utilization of weak polypyrimidine tracts in acceptor sites. For selection of exon 9, utilization of the polypyrimidine tract in intron 8 requires the ESE on exon 9. Interestingly, this natural ESE can be replaced by other purine-rich elements, such as 5′-AAGAAGAAAAA-3′ (Fig. 5C). These results indicate that the ESE element required for selection of exon 9 is not exclusive. On the other hand, splice site selection via the combination of weak polypyrimidine tract and ESE is inhibited by MS-ESS under conditions reflecting muscle-specific alternative splicing (Fig. 8). From detailed mutation analyses, the MS-ESS element seems to possess a very rigidly defined nucleotide sequence (Figs. 6 and 7). MS-ESS activity still remains even with the use of other ESE elements such as the MU2 mutation (Fig. 5C). From these results, it seems likely that the positional correlation between MS-ESS and ESE is very important for performance of MS-ESS in exon skipping.

In general, trans-acting regulatory factors recognize cis-acting regulatory elements and regulate exon selection by constitutive or alternative splicing. A number of serine/arginine-rich (SR) proteins can recognize ESE elements and activate splicing (8, 15–18). For instance, SF2/ASF (38, 39) binds to ESE within the last exon of bovine growth hormone pre-mRNA and promotes inclusion of the exon in vitro (38). In the same fashion, ESSs have also been reported to be recognized and controlled by specific trans-acting regulatory factors. For example, the 35- and 54–55-kDa SR proteins bind to the ESS within BPV-1 and inhibit spliceosome assembly (25). The protein hnRNP A1 binds to HIV-1 tat exon 2 and fibroblast growth factor receptor 2 K-SAM exon (26), and hnRNP H binds to the ESS within exon 7 of β-TM to cause exclusion of the exon (24). Such ESS elements do not resemble the MS-ESS element of F1γ gene. On the other hand, intronic splicing suppressors have also reportedly been recognized by a kind of RNA-binding protein. The consensus sequences of these intronic splicing suppressors are 5'-UUCUCU-3', 5'-UCCCUU-3', and 5'-CUUCUU-3' (40), similar to the core sequence of F1γ MS-ESS, 5'-UUCC-3'. Polypyrimidine tract-binding protein (PTB) (41) binds to intronic splicing repressors such as these, which are found in α- and β-TM (42, 43), fibronectin (44), c-src (45), and the γ2 subunit of GABAγ receptor pre-mRNAs (40). It is plausible that these well known RNA-binding proteins function for constitutive exon selection. However, it is unlikely that such an RNA-binding protein is the trigger factor for tissue-specific alternative splicing, since these proteins are largely ubiquitous. For example, hnRNP H binds to ESS of β-TM exon 7 and represses exon selection. β-TM exon 7 is specifically selected in muscle tissue, although hnRNP H is expressed within muscle tissue (24). It is therefore necessary to identify the direct trigger for tissue-specific alternative splicing.

The muscle-specific exon skipping of F1γ gene is reversibly induced by acidic stimulation in HT1080 cells and C2C12 cells.
From investigations utilizing protein synthesis inhibitors, acidic stimulation was shown to induce a newly synthesized protein for muscle-specific alternative splicing in a cell type-specific manner (Fig. 1). Nuclear extracts from acid-stimulated HT1080 cells contain a trans-acting regulatory factor for exon skipping. Such a protein factor is a candidate for the direct trigger for muscle-specific alternative splicing. In addition, such a protein would bind directly to the MS-ESS element. The core sequence of MS-ESS resembles an intronic splicing enhancer (29, 31). From investigations utilizing protein synthesis inhibitors, acidic stimulation can induce muscle-specific exon skipping. Such a protein factor is a candidate for the direct trigger for muscle-specific alternative splicing. In any case, since mutations on MS-ESS blocked the levels of PTB protein in HT1080 cells before and after acidic stimulation, and no differences in amounts of PTB were observed between the two conditions (32). It is therefore unlikely that PTB is a direct trigger for muscle-specific alternative splicing. In any case, since mutations on MS-ESS blocked the levels of PTB protein in HT1080 cells before and after acidic stimulation, and no differences in amounts of PTB were observed between the two conditions (32). It is therefore unlikely that PTB is a direct trigger for muscle-specific alternative splicing. In any case, since mutations on MS-ESS blocked the levels of PTB protein in HT1080 cells before and after acidic stimulation, and no differences in amounts of PTB were observed between the two conditions (32). It is therefore unlikely that PTB is a direct trigger for muscle-specific alternative splicing. In any case, since mutations on MS-ESS blocked the levels of PTB protein in HT1080 cells before and after acidic stimulation, and no differences in amounts of PTB were observed between the two conditions (32). It is therefore unlikely that PTB is a direct trigger for muscle-specific alternative splicing.

In summary, we presented MS-ESS as an element involved in muscle-specific exon exclusion in human F$_1^\gamma$ pre-mRNA. This element is located immediately downstream from the ESE element on the alternatively spliced exon and functions under in vivo and in vitro conditions to enable muscle-specific alternative splicing. This event is accompanied by de novo protein synthesis. However, no trans-acting regulatory factors recognizing this element have been identified. The negative trans-acting regulatory factor must be isolated and characterized before the molecular mechanisms of alternative splicing in human F$_1^\gamma$ pre-mRNA can be fully understood.

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