Muscle-specific isoform of the mitochondrial ATP synthase γ subunit (F1γ) was generated by alternative splicing, and exon 9 of the gene was found to be lacking particularly in skeletal muscle and heart tissue. Recently, we reported that alternative splicing of exon 9 was induced by low serum or acidic media in mouse myoblasts, and that this splicing required de novo protein synthesis of a negative regulatory factor (Ichida, M., Endo, H., Ikeda, U., Matsuda, C., Ueno, E., Shimada, K., and Kagawa, Y. (1998) J. Biol. Chem. 273, 8492–8501; Hayakawa, M., Endo, H., Hamamoto, T., and Kagawa, Y. (1998) Biochem. Biophys. Res. Commun. 251, 603–608). In the present report, we identified a cis-acting element on the muscle-specific alternatively spliced exon of F1γ gene by an in vivo splicing system using cultured cells and transgenic mice. We constructed a F1γ wild-type minigene, containing the full-length gene from exon 8 to exon 10, and two mutants; one mutant involved a pyrimidine-rich substitution on exon 9, whereas the other was a purine-rich substitution, abbreviated as F1γ Pu-del and F1γ Pu-rich mutants, respectively. Based on an in vivo splicing assay using low serum- or acid-stimulated splicing induction system in mouse myoblasts, Pu-del mutation inhibited exon inclusion, indicating that a Pu-del mutation would disrupt an exon splicing enhancer. On the other hand, the Pu-rich mutation blocked muscle-specific exon exclusion following both inductions. Next, we produced transgenic mice bearing both mutant minigenes and analyzed their splicing patterns in tissues. Based on an analysis of F1γ Pu-del minigene transgenic mice, the purine nucleotide of this element was shown to be necessary for exon inclusion in non-muscle tissue. In contrast, analysis of F1γ Pu-rich minigene transgenic mice revealed that the F1γ Pu-rich mutant exon had been excluded from heart and skeletal muscle of these transgenic mice, despite the fact mutation of the exon inhibited muscle-specific exon exclusion in myotubes of early embryonic stage. These results suggested that the splicing regulatory mechanism underlying F1γ pre-mRNA differed between myotubes and myofibers during myogenesis and cardiogenesis.

Alternative pre-mRNA splicing is a fundamental process in eukaryotes that contributes to tissue-specific and developmentally regulated patterns of gene expression at the posttranscriptional level. Recently, both cis-acting elements and trans-acting factors have been reported to varying degrees (1–3). Significant progress has been made in identifying the alternative splicing mechanism involved in the Drosophila sex determination pathway (4–7).

Exonic and intronic cis-acting regulatory elements for RNA splicing have been reported in a number of mammalian genes located near weak 5′ and 3′ splice sites, and these elements appear to be involved in the control of stage- or tissue-specific splicing events (5, 8–13). For example, a majority of exonic splicing enhancer (ESE)1 elements have been reported to be abundant in purine nucleotides (14, 15), and these elements have been shown to bind the splicing factor for control of alternative splicing. Serine/arginine-rich (SR) proteins, a group of splicing factors, play important roles both in ESE-independent and ESE-dependent splicing (8, 9, 16, 17). SR proteins show RNA binding activity to each cis-acting element, as well as protein-protein interaction activity in the formation of a commitment complex of spliceosome (16). SF2/ASF and SC35, two SR proteins, differ in their ability to commit different pre-mRNAs to the splicing pathway (18). SF2/ASF, but not SC35, recognized sequences very similar to purine-rich elements found in various natural splicing enhancers (9, 19). SRp40, another member of the SR protein group, binds specific ESE, and one study showed that phosphorylation of the arginine/serine-rich domain is necessary for sequence-specific binding (20). Research has shown that tissue-specific distribution of SR proteins varies to a certain degree and that overexpression of SR proteins in vivo alters the splicing pattern. Hence, the likelihood is that either tissue-specific or development stage-specific alternative splicing is controlled by changes in the amount of SR proteins or by covalent modification of these SR proteins, as in phosphorylation control (16).

Previously, we cloned the mitochondrial ATP synthase γ-subunit (F1γ) gene and reported that its tissue-specific isoforms were generated by alternative splicing in the human, cow, and mouse (21–23). RNA transcripts excluding exon 9 are specifically expressed in heart and skeletal muscle tissue (21, 22). F1γ mRNA contains exon 9 in mouse myoblast C2C12 cells, but F1γ mRNA showed exclusion of exon 9 in myotubes (23). This muscle-type mRNA is cell-specifically and reversibly induced by acidic stimulation in human fibrosarcoma, human rhabdomyosarcoma, and mouse myoblast cells (23, 24).
thermore, this process of muscle-specific exon selection was inhibited by cycloheximide, a protein synthesis inhibitor, and the protein kinase C inhibitor H-7 in these cell lines (23, 24).

Other muscle-specific alternative splicing in the neural cell-adhesion molecules, β-tropomyosin, MEF2A, and MEF2D genes was induced by this acidic stimulation (23). The above muscle-specific alternative splicing likely requires de novo protein synthesis of a splicing regulatory factor. In fact, we found evidence using an in vitro splicing system that a negative regulatory factor for muscle-specific exon exclusion of human Fgγ pre-mRNA existed in nuclear extracts from acid-stimulated human fibrosarcoma cells (25). However, we were unable to determine the exact regulatory mechanism underlying muscle-specific alternative splicing in muscle tissue.

In the present study, we identified the cis-acting regulatory element for alternative splicing of Fgγ pre-mRNA and showed that the action of this element in myotubes and mature muscle fibers differed. Using in vivo splicing analysis of mouse Fgγ minigenes, we determined that a cis-acting regulatory element on the alternatively spliced exon was important for both exon inclusion and exclusion in culture cells. Purine-rich mutation on the exon of Fgγ minigene, which likely destroys the negative regulatory element, blocked muscle-specific exon exclusion in myotubes. In contrast, the purine-rich mutation of the Fgγ minigene failed to block exon exclusion in skeletal and heart muscle tissues in transgenic mice. This difference between the splicing regulation that occurs during myogenesis and cardiogenesis will be discussed.

MATERIALS AND METHODS

Cell Culture—C2C12, mouse myoblast cells, were obtained from the American Type Culture Collection. L929, mouse fibrosarcoma cells, were provided by Dr. T. Kirikae (Jichi Medical School, Tochigi, Japan). The C2C12 cells and L929 cells were grown in DMEM (Dulbecco’s modified Eagle’s medium) containing 10% fetal bovine serum (Life Technologies, Inc.) as growth medium at 37 °C under 5% CO2. The cells were plated on 10-cm diameter tissue culture dishes. Once the C2C12 transgenic mice was plated at 106/60-mm diameter tissue culture dish.

Construction of Expression Plasmids of Fgγ Minigene—A mouse Fgγ genomic fragment from exon 8 to exon 10 was obtained from plasmid pBluescript II (SK+)-Fgγ (pBS-Fgγ) (23) and subcloned into pcDEBSmin-mammalian expression vector (28) (provided by Dr. H. Hayakawa, Kyushu University, Fukuoka, Japan) as Fgγ wild-type minigene (pcDEBS-Fgγ, wild-type minigene) for in vivo splicing assay.

Mutations on the alternatively spliced exon of pcDEBS-Fgγ, wild-type minigene was created using two-piece PCR cloning strategy (13) using pBS-Fgγ plasmid DNA. Mutant overlapping primer pairs (MU1 and MU2, MU3 and MU4) were generated for purine-rich and pyrimidine-rich mutants on exon 9, respectively. Primers M1 and M2, M3 and M4, and pBluescript II were used as the common outside oligonucleotides. MU1 with M13RV and MU2 with M13 were used to generate individual mutated ends of the clone of interest. The two PCR products were then separated on 1% agarose gel and eluted. A third round of PCR using M13 and M13RV with the two PCR products was used to generate the full-length mutated insert. Two full-length inserts were reassembled into following the procedure of Bluescript II and confirmed sequencing on both strands around the mutated regions. Finally, these Fgγ mutants were subcloned into pcDEBSmin as described above. Pyrimidine-rich Fgγ mutant minigene and purine-rich Fgγ mutant minigenes were termed Fgγ Pu-del and Fgγ Pu-rich minigenes, respectively. Sequences of the oligonucleotides were as follows: 5′-CCT-AAT-ATA-ATA-ACC-AGG-GAT-TTT-TTT-TTA-AGT-TGG-TG-3′ (sense strand; MU1) and 3′-TGG-TCC-CTA-AAA-AAA-AAA-AGT-AGC-TAG-G-3′ (antisense strand; MU2) for Fgγ Pu-rich mutant minigene.

DNA Transfection to Cells—After C2C12 myoblasts and L929 fibrosarcoma cells had been cultured with growth medium to semiconfluence, the cells were transfected with 5 μg each of pcDEBS-Fgγ wild-type and two mutant minigenes using the polycationic liposome method (DMRIE-C, Life Technologies, Inc.). After transfection, the cells were cultured with growth medium, acidic medium, and differentiation medium for 3 days, and then harvested.

Generation of Transgenic Mice—Transgenic mice were established following the standard protocol described previously (23). To construct the expression plasmid for transgenic mouse, two Fgγ mutant minigenes were subcloned into the pCAGGS expression vector (28) provided by Dr. J. Miyazaki (Osaka University, Osaka, Japan). pCAGGS-Fgγ mutant mice were digested with SsII and BamHI. The DNA fragments were separated on 1% agarose gel, eluted, and extracted by phenol/chloroform/isooamylic alcohol. The purified DNA fragment was dissolved to a final concentration of 500 copies/μl with 10 μM Tris-HCl (pH 7.5), 0.25 mM EDTA for microinjection. C57BL/6J mice were used for microinjection, and ICR mice were used as foster mothers.

We simultaneously co-injected green fluorescent protein (GFP) gene constructed in the same pCAGGS expression vector, pMG2 (29) provided by Dr. K. Kohno, Nara Institute of Science and Technology, Nara, Japan. pMG2 were digested with SsII and BamHI. The purified GFP DNA fragments were mixed with the Fgγ mutant minigene DNA fragments (ratio = 1:1, the final concentration 500 copies/μl) subjected to microinjection.

RNA Preparation and RT-PCR Analysis of Alternatively Spliced Exon—Total RNAs were prepared from C2C12 cells, L929 cells, and the tissue of transgenic mice following the acid guanidine method (30), and then each 10 μg of total RNA was subjected to RT-PCR as described previously (23). To analyze splicing patterns of Fgγ mRNA, PCR was performed at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min in a total volume of 100 μl, which contained 10 μl MinTaq (Biolase Ltd, RIKEN, Japan). Thirty PCR cycles were used. The PCR primers sets were as follows; for endogenous Fgγ mRNA, 5′-GTC-ATC-ACC-AAA-GAG-TTG-ATT-G-3′ (sense strand, corresponding to a portion of exon 8 of human Fgγ) and 5′-TAA-TGG-AGG-AAG-AGT-TTC-TTC-G-3′ (antisense strand, corresponding to a portion of exon 10 of human Fgγ); for exogenous Fgγ minigene mRNA, 5′-GTC-ATC-ACC-AAA-GAG-TTG-ATT-G-3′ (sense strand, corresponding to a portion of exon 8 of human Fgγ) and 5′-TAA-TGG-AGG-AAG-AGT-TTC-TTC-G-3′ (antisense strand, corresponding to a portion of Fgγ mRNA) were used.

RESULTS

In Vivo Alternative Splicing System of Fgγ Minigene in C2C12 Myoblasts—To investigate the role of exon recognition for muscle-specific alternative splicing, we constructed a mouse Fgγ minigene and developed an in vivo alternative splicing system by inducing differentiation in C2C12 mouse myoblasts (23). The Fgγ wild-type minigene was constructed in pcDEBSmin-mammalian expression vector (26) (pcDEBSmin-Fgγ, wild-type minigene), which contained a full-length mouse Fgγ genomic gene corresponding to human Fgγ gene from exon 8 to exon 10 (GenBankTM/EMBL/DDJB Data Bank accession no. U43893) (Fig. 1a). Muscle-specific alternative splicing of endogenous Fgγ pre-mRNA in C2C12 mouse myoblasts is induced in two different ways; one is replacement from growth medium to
contains all genomic sequences from exon 8 to exon 10 of mouse F_{1γ} gene, and the spliced transcripts of the minigene was independently regulated by both induction systems in a similar manner as endogenous induction. These results indicated that the F_{1γ} wild-type minigene contained all cis-acting regulatory elements involved in muscle-specific alternative splicing.

**cis-Acting Element Exists on the Alternatively Spliced Exon in Culture Cells—** Based on a comparison of the genomic sequences around exon 9 of F_{1γ} gene in the human, cow, and mouse, a relatively purine-rich sequence was found at 5' region of exon 9 in all three animal models (Fig. 2). Previously, exonic splicing enhancer containing purine-rich sequences was reported to play an important role in splice site selection (10). To determine whether this sequence is a cis-acting element involved in the regulation of alternative splicing of F_{1γ}, we constructed two types of mutant minigenes derived from the pcDEBSR-F_{1γ} wild-type minigene (Fig. 2b). One type was a F_{1γ} Pu-del mutant, made by introducing an 11-nucleotide stretch of poly(U) into the exon of wild-type minigene in place of the relatively purine-rich sequence at the 5' region of the exon. The other was a F_{1γ} Pu-rich mutant, made by introducing a purine-rich sequence, AAAGAGAAGAGA, into the middle region of the exon. This purine-rich sequence was derived from the 5' region of exon 10 of F_{1γ} gene and resembled the relatively purine-rich sequence at the 5' region of exon 9 (Fig. 2b).

In vivo splicing assay using F_{1γ} Pu-del mutant minigene transfected to C2C12 myoblasts revealed that no inclusion of the Pu-del mutant exon had occurred in the C2C12 cells cultured in growth medium, despite the fact that the wild-type exon of endogenous F_{1γ} pre-mRNA was included (Fig. 2c). In addition, the Pu-del mutant exon was also excluded from C2C12 cells cultured with differentiation and acidic media (Fig. 2d). A similar experiment revealed that, as in the myoblasts, the Pu-del mutant exon was not included in mouse fibrosarcoma L929 cells (Fig. 3b). The transcripts of F_{1γ} wild-type minigene in L929 cells included exon 9 in both growth and acidic media (Fig. 3b), as observed in endogenous F_{1γ} transcripts in the cells (data not shown). These results indicated that the presence of purine nucleotides on the wild-type exon was very important in splice site selection of this alternatively spliced exon.

The other mutant minigene, F_{1γ} Pu-rich minigene constructed in pcDEBSR-Pu, was also tested. The alternative exon of F_{1γ} Pu-rich minigene pre-mRNA was selected in C2C12 cells cultured not only in growth medium but also in differentiation and acidic media (Fig. 3a, lanes 1–3). In addition, the Pu-rich exon was primarily included in L929 cells cultured in growth and acidic media, whereas the wild-type exon of F_{1γ} minigene was partially excluded from cells with acidic medium (Fig. 3b, lanes 3 and 4). These data suggested that the purine-rich mutant sequence enhanced the inclusion of an alternative exon of F_{1γ} pre-mRNA in myoblast, myotubes, and non-muscle cells.

In summary, the moderately purine-rich sequence at the 5' region of wild-type exon played a role in the selection of exon 9 as an exonic splicing enhancer, and the Pu-del mutant sequence disrupted this putative enhancer sequence and its activity. On the other hand, muscle-specific exon exclusion was inhibited in Pu-rich mutant minigene. Based on these results, we constructed two types of mutant minigenes; one was a Pu-del mutant minigene, the exon of which is constitutively excluded, while the other was a Pu-rich mutant minigene, the exon of which is included in every culture condition.

**Gene Expression Pattern of CAG Promoter in Various Organs of Transgenic Mice Using GFP as a Useful Coinjection Marker—** In order to determine whether this exonic cis-acting element was important for alternative splicing of F_{1γ} pre-mRNA.
in various tissues in vivo, we produced transgenic mice carrying either a Pu-del or Pu-rich mutant minigene, both of which were re-subcloned into a pCAGGS mammalian expression vector (28) (Fig. 4a). Both of the minigenes in the pCAGGS vector which had transfected into myoblasts were confirmed to work well, in the same way as minigenes in the pcDEBSRα vector (data not shown). Three lines of transgenic mice containing F1γ Pu-del mutant minigenes were produced from the 42 eggs surviving after microinjection, while four lines of transgenic mice containing F1γ Pu-rich mutant minigene were produced from 77 eggs (Table I).

At the same time, we co-injected a modified green fluorescent protein (GFP) gene in the same expression vector, pMG2 (29), in order to allow for: 1) selection of transgenic mice using GFP and 2) detection of expression patterns of the promoter activity in various tissues of transgenic mice. GFP has been shown to be useful as a marker in transgenic mice given its lack of toxicity, high sensitivity, and reproducibility (31, 32). We co-injected two types of DNA fragments, F1γ mutant minigene and GFP cDNA. The GFP used in this study was a double mutant (S65T/S147P) that is strongly fluorescent and heat-stable (29). The majority of GFP-carrying F0 transgenic pups, which were detected by UV irradiation (wavelength 360 nm), carried both minigenes and expressed their mRNA (Table I). F1 transgenic mice were obtained from all F0 mice expressing GFP and F1γ mutant minigene, using simple selection of transgenic pups by GFP (Fig. 4b).

The pCAGGS mammalian expression vector has a cytomegalovirus enhancer and chicken β-actin promoter (28). The fluorescent signals of GFP expressed in various tissues of transgenic mice were observed using a CCD camera under 488 nm excitation light (fluorescent microscopy MZ FL III, Leica). As shown in Fig. 4c, skeletal muscle, heart, liver, and brain were visualized as strongly green under blue light. On the other hand, very weak fluorescence was observed in the spleen. These results indicated that the chicken β-actin promoter showed activity in various tissues of transgenic mice. Thus, we demonstrated that GFP is a useful marker for selection of living transgenic mice and for simple detection of promoter activity.

**Splicing Patterns of Pu-del or Pu-rich Mutant Minigenes in**

![Fig. 2. Sequence comparison and mutation of mouse F1γ minigene. a, overview of homologous regions of F1γ genomic gene from exons 8–10. Underline indicates relatively high homologous sequences of F1γ gene from exons 8–10 among the cow, human, and mouse (GenBank/EMBL/DDJB Data Bank accession nos. M22463 and D16564, D16561, and U43893, respectively). b, upper figure shows a sequence comparison of exon 9 and exon 10 in cow, human, and mouse. Open boxes indicate the same bases between exon 9 and exon 10. Black bar indicates wild-type moderately purine nucleotide-rich sequence of exon 9. Slanting bar indicates purine nucleotide-rich sequence of exon 10. Lower figure shows two mutations introduced into exon 9 of mouse F1γ minigene: Open bar and slanting bar indicate the region of purine deletion at 5’ portion of exon 9 and the region of purine-rich mutation at the middle portion, respectively. BV, cow; HS, human; MM, mouse.](https://example.com/fig2.png)

![Fig. 3. Splicing patterns of F1γ mutant minigenes in C2C12 myoblasts and L929 cells. Mouse F1γ mutant minigene was ligated into pcDEBSRα mammalian expression vector. C2C12 myoblasts and L929 cells were transiently transfected with pcDEBSRα including F1γ mutant minigene and were cultured in growth medium (G). After cells had grown to semiconfluence, medium was changed to differentiation medium (D) and acidic medium (A), and the cells were then cultured for 72 h. A 10-μg aliquot of total RNAs from these cells was used for RT-PCR. Primers for PCR were described in the legend for Fig. 1. The size of the PCR products was determined by 3% agarose gel electrophoresis, and the products were stained with ethidium bromide. a, splicing pattern in C2C12 myoblasts; b, splicing pattern in L929 cells. wt represents F1γ wild-type minigene.](https://example.com/fig3.png)
Various Organs of Transgenic Mice—As the promoter of pCAGGS was shown to be expressed in various tissues, we investigated the splicing pattern of F1 mutant minigenes in the organs of transgenic mice. Total RNA was prepared from several organs of 4-week-old transgenic mice and then subjected to RT-PCR analysis (Fig. 5). Splicing patterns of endogenous mouse F1 pre-mRNA in various tissues revealed that exclusion of exon 9 was specific to heart and skeletal muscle tissues (Fig. 5a). The alternative exon of F1 Pu-del pre-mRNA was excluded not only in skeletal and heart muscle tissues, but also in non-muscle organs such as the liver, spleen, and kidney (Fig. 5b). These data indicated that purine nucleotides at the 5' region of wild-type exon were necessary for exon inclusion to occur in cultured myoblast cells and non-muscle organs, suggesting that this region is an exonic splicing enhancer in non-muscle organs as same as in culture cells.

FIG. 4. Generation of transgenic mice and expression patterns of transgenes in various tissues. a, construction of pCAGGS-F1 γ mutant minigenes and pMG2. Mouse F1 γ Pu-del and Pu-rich mutant minigenes were ligated into pUC-CAGGS mammalian expression vector. pMG2, GFP with double mutants (S65T/S147P) constructed in the same vector, was provided by Dr. Kohno (28). pCAGGS-F1 γ mutants and pMG2 were digested with SalI and BamHI. Equivalent amounts of two different purified DNA fragments, F1 γ mutant minigene and GFP cDNA, were co-injected to generate transgenic mice. b, ease of selection of transgenic mice using GFP. F1 transgenic pups expressing GFP and F1 γ mutant minigene were detected under UV irradiation (wavelength 360 nm) (right panel). GFP and F1 γ mutant minigenes were not separated in every pup, and thus these two transgenes were likely located on the same chromosome. c, gene expression pattern of CAG promoter in various tissues of transgenic mice. The fluorescent signals of GFP expressed in various tissues of transgenic mice and wild mouse were observed using a CCD camera under 488 nm excitation light (fluorescent microscopy; MZ FL III, Leica). Each organ on the right was obtained from transgenic mice, whereas those on the left were from control mice.
Next, we analyzed the splicing patterns of Pu-rich mutant minigene transcripts in various tissues. As shown in Fig. 5c, interestingly, the Pu-rich mutant exon of exogenous F₁γ minigene was excluded in skeletal and heart muscle tissues, although this exon was included even in myotubes (Fig. 3a). The difference between the splicing patterns in cultured myotubes and those in mature muscle fibers suggested that differential regulation is involved in the muscle-specific alternative splicing for each stage of muscle development.

**Different Alternative Splicing Patterns of F₁γ Pu-rich Pre-mRNA Were Observed during Myogenesis and Cardiogenesis in Uteri**—To confirm the possibility of differential regulation of alternative splicing in F₁γ pre-mRNA during myogenesis and cardiogenesis, we analyzed the splicing pattern of F₁γ Pu-rich and endogenous F₁γ pre-mRNAs in the heart and forelimbs of transgenic mice at each embryonic day. Heart and skeletal muscle tissues of 4-week-old transgenic mice were used as controls. Fetal hearts from embryonic day (E) 9–15 were prepared under a stereoscopic microscope. The E9 heart had already begun to beat at that point. As shown in Fig. 6a, the endogenous exon 9 of F₁γ pre-mRNA was primarily excluded in heart from E9 to adult. In contrast, the Pu-rich mutant exon was shown to be partially excluded in fetal hearts; the ratio was approximately 1:1. This Pu-rich mutant exon was mostly excluded in adult heart, and thus a differential regulation of muscle-specific splicing likely exists between fetal and adult heart muscles.

Forelimbs were prepared from E10 to E15 transgenic mice containing both muscle and non-muscle tissues. In general, myoblasts appeared in forelimb from E10 to E11 and myotubes from E11 to E12 (33, 34). RT-PCR analysis revealed that E10 forelimb expressed the non-muscle type of both endogenous and exogenous F₁γ pre-mRNAs (Fig. 6b). Exclusion of the endogenous wild-type exon was detectable in E11 and E12 forelimbs, whereas no exclusion of Pu-rich mutant exon was detected in the same samples. Exogenous Pu-rich mutant exon of F₁γ minigene was shown to be excluded from E13 forelimbs. At the early stage of myotube formation in forelimbs, exon exclusion was inhibited in Pu-rich mutant minigene transcripts. These data indicated that muscle-specific alternative splicing of F₁γ pre-mRNA was differentially regulated during myogenesis and cardiogenesis.

**Primary Cultures of Myoblasts from Transgenic Mice**

**Fig. 5. Splicing patterns of F₁γ mutant minigene in transgenic mice.** A 10-μg aliquot of total RNAs from tissues of transgenic mice was subjected to RT-PCR. The two pairs of PCR primers for endogenous and exogenous transcripts were described in the legend for Fig. 1. The size of the PCR products was determined by 3% agarose gel electrophoresis. A, tissue distribution of splicing pattern of endogenous F₁γ mRNA. B, splicing pattern in transgenic mice with F₁γ Pu-del mutant minigene. C, splicing pattern in transgenic mice with F₁γ Pu-rich mutant minigene. H, heart; M, skeletal muscle; L, liver; B, brain; S, spleen; K, kidney; C1, muscle-type F₁γ cDNA as a control; C2, nonmuscle-type F₁γ cDNA as another control.

**Table I**

| F₀ transgenic mice | Total | F₁γ | GFP | F₁γ and GFP |
|-------------------|------|-----|-----|-------------|
| Pu-del            | 42   | 3   | 2   | 2           |
| Pu-rich           | 77   | 4   | 3   | 3           |

**Primary Cultures of Myoblasts from Transgenic Mice**

—to establish that a difference exists between the alternative splicing regulations of F₁γ pre-mRNA in myotubes and that in mature muscle fibers, we analyzed primary cultures of myoblasts and myotubes from skeletal muscle of transgenic mice using a F₁γ Pu-rich mutant minigene. Primary cultures were contaminated with a number of different fibroblasts, although myotube formation was observed after treatment with differentiation medium (Fig. 7a). From RT-PCR analysis of primary culture cells, muscle-specific exon exclusion of endogenous F₁γ pre-mRNA was observed after treatment with acidic or differentiation medium (Fig. 7b, lanes 5 and 6). In contrast, no exclusion of Pu-rich mutant exon was observed after treatment with either medium (Fig. 7b, lanes 2 and 3). In these cells, the non-muscle type of F₁γ...
splicing pattern was predominant, so that an abundance of non-myoblast cells, such as fibroblasts, was found in the primary cultures. Although they were very faint, bands of muscle-type endogenous F1γ transcripts were detected after induction (Fig. 7b, lanes 5 and 6). These results from primary cultures of myoblasts and myotubes supported the finding that muscle-specific alternative splicing of F1γ pre-mRNA was differentially regulated between myotubes and mature muscle tissues.

**Fig. 6.** Splicing pattern of F1γ Pu-rich minigene in transgenic mouse embryo. A 10-μg aliquot of total RNAs from forelimbs and hearts of transgenic mice embryo and those of 4 week-old transgenic mice were subjected to RT-PCR. The two pairs of PCR primers for endogenous and exogenous transcripts are indicated in panel c and under “Materials and Methods.” The size of the PCR products was determined by 3% agarose gel electrophoresis. a, splicing pattern in hearts of transgenic mice carrying the F1γ Pu-rich mutant minigene. AH, adult hearts of 4-week-old transgenic mice. b, splicing pattern in forelimbs of transgenic mice carrying the F1γ Pu-rich mutant minigene. AM, adult skeletal muscle of 4-week-old transgenic mice. c, arrows indicate the location of primers for endogenous and exogenous transcripts. Primer sequences were described under “Materials and Methods.”
The present study demonstrated that the cis-acting elements play an essential role in exonic alternative splicing of \( F_{1\gamma} \) pre-mRNA both during myogenesis and cardiogenesis. First, in vivo splicing analyses in mouse myoblasts and in a transgenic mouse system using \( F_{1\gamma} \) mutant minigenes revealed that purine nucleotides at the 5' region of the alternatively spliced exon were important for exon inclusion in non-muscle cells and tissues. Second, a Pu-rich mutation at the middle region of the exon inhibited muscle-specific exon exclusion in myotubes, but did not inhibit in heart and skeletal muscle tissues of transgenic mice. This discrepancy in the splice patterns of myoblasts from transgenic mice. Primary cultures of myoblasts were grown under the three types of medium: growth medium (G), differentiation medium (D), and acidic medium (A). The cells were cultured for 72 h. Total RNAs from the cells were used for RT-PCR analysis. The size of the PCR products was determined by 3% agarose gel electrophoresis. Primer sequences were described in the legend of Fig. 6c.

The exonic cis-acting positive regulatory element of \( F_{1\gamma} \) we demonstrated here, located on the 5' region of the alternatively spliced exon. In general, splicing of eukaryotic pre-mRNAs requires the consensus sequences in 5' and 3' splice sites, branching point, and polypyrimidine tract. In cases where the polypyrimidine tract is weak, splicing enhancer sequences are needed, such as ESEs (9, 10). ESE-dependent splicing reaction requires SR proteins, for example SF2/ASF and SC35, which are needed at an early stage of spliceosome assembly. These SR proteins are able to bind to each specific RNA cis-element (9, 19), and these elements have been primarily reported to be purine nucleotide-rich sequences. An exon inclusion activity of the enhancer element was clearly proved by in vivo splicing assay using a Pu-del mutant minigene in myoblasts and in non-muscle tissues of transgenic mice (Figs. 3 and 5b). The enhancer element was rich in purine nucleotide and was not identical to well known ESE sequences. This exonic enhancer sequence would require a sequence-specific splicing factor such as SR proteins. Previously, we reported on a reversible induction system of muscle-specific alternative splicing of \( F_{1\gamma} \) using human fibrosarcoma and mouse myoblast cells and revealed that the non-muscle type of exon inclusion is a default type and that the muscle-specific exon exclusion required de novo protein synthesis of an intracellular protein factor (23, 24). Considering above, it is likely that this exonic enhancer element is necessary for constitutive splicing of \( F_{1\gamma} \) in non-muscle tissues, and that a splicing factor for this enhancer element, such as SR proteins, is also required (Fig. 8, left column).

The negative regulation for selection of exon 9 in the \( F_{1\gamma} \) gene was induced in low serum- or acid-stimulated cells (23, 24). We also established that the nuclear extracts from post-induced cells contained a sequence-specific negative regulatory factor of exon selection by in vitro splicing assay (25). Based on these findings, a negative regulatory factor was induced, which in turn inhibited the constitutive splicing in \( F_{1\gamma}-wt \) minigene in both myotubes and post-induced cells (Fig. 1c). When the Pu-rich mutation was introduced at the middle region of the exon inhibited muscle-specific exon exclusion in myotubes, but did not inhibit in heart and skeletal muscle tissues of transgenic mice. This discrepancy in the splice patterns of the mutant exon between myotubes and mature muscle fibers suggests that there is a difference of the alternative splicing of \( F_{1\gamma} \) in each step of muscle development.

The exonic cis-acting positive regulatory element of \( F_{1\gamma} \) was clearly proved by in vivo splicing assay using a Pu-del mutant minigene in myoblasts and in non-muscle tissues of transgenic mice (Figs. 3 and 5b). The enhancer element was rich in purine nucleotide and was not identical to well known ESE sequences. This exonic enhancer sequence would require a sequence-specific splicing factor such as SR proteins. Previously, we reported on a reversible induction system of muscle-specific alternative splicing of \( F_{1\gamma} \) using human fibrosarcoma and mouse myoblast cells and revealed that the non-muscle type of exon inclusion is a default type and that the muscle-specific exon exclusion required de novo protein synthesis of an intracellular protein factor (23, 24). Considering above, it is likely that this exonic enhancer element is necessary for constitutive splicing of \( F_{1\gamma} \) in non-muscle tissues, and that a splicing factor for this enhancer element, such as SR proteins, is also required (Fig. 8, left column).

The negative regulation for selection of exon 9 in the \( F_{1\gamma} \) gene was induced in low serum- or acid-stimulated cells (23, 24). We also established that the nuclear extracts from post-induced cells contained a sequence-specific negative regulatory factor of exon selection by in vitro splicing assay (25). Based on these findings, a negative regulatory factor was induced, which in turn inhibited the constitutive splicing in \( F_{1\gamma}-wt \) minigene in both myotubes and post-induced cells (Fig. 1c). When the Pu-rich mutation was introduced at the middle region of the wild-type exon 9, induction of exon exclusion by acidic stimulation or differentiation medium was blocked (Fig. 3). From the result, this mutation likely affected the negative regulatory element on the exon. Alternatively, the Pu-rich mutation would make a new exonic splicing enhancer sequence. However, the Pu-rich mutant sequence resembles, but is not identical to, SF2/ASF-binding consensus element (41). In addition, this mutant sequence did not inactivate the enhancer element on the alternatively spliced exon, but inhibit the silencer activity in myotubes. Considering this, it is likely that the negative regulatory element located on the middle region of the exon (Fig. 8, middle column).

This Pu-rich mutation on the exon did not work in skeletal and heart muscle tissues of the transgenic mice (Fig. 5c). However, this mutation was observed to inhibit the exon exclusion not only in primary cultures of myoblasts from the transgenic mice (Fig. 7) but also in premature heart and skeletal muscle...
during fetal development in uteri (Fig. 6). These data indicated that there was a differential regulation of muscle-specific alternative splicing between myotubes and mature muscle fibers. When the Pu-rich mutation made the exonic silencer element inactivated, there were two possibilities to explain the difference of the splicing regulation between myotubes and mature muscle fibers. First, a negative regulatory factor that acts to the negative regulatory element would be different quantitatively or qualitatively between myotubes and myofibers. Second, a positive regulatory factor for the enhancer element would differ quantitatively or qualitatively. In fact, the amount of SR protein has been reported to differ among various tissues (38, 39). For example, tissue distribution of rat SF2/ASF and hnRNP A1 proteins have been reported (40). The above study found that the rat heart muscle contained only trace amounts of SF2/ASF. It is then likely that the protein level of a constitutive splicing factor is only decreased in mature muscle fibers, which in turn results in exon exclusion, even when the silencer element is inactivated (right column). See text details.

There are many muscle-specific alternative splicings in several genes, e.g. α- or β-tropomyosin, neural cell-adhesion molecule, myocyte-specific enhancer proteins, and F$_1$. Although the development stage of myotubes is earlier rather than that of myofibers, most of muscle-specific alternative splicings were reported to appear at myotubes (23). Our Pu-rich mutant minigene succeeded in clearly demonstrating the difference in splicing regulation between myotubes and mature muscle fibers.

We have identified the splicing enhancer sequence of alternative splicing of F$_1$γ pre-mRNA in vivo. This enhancer element is rich in purine nucleotides, exists on the alternatively spliced exon, and works to include this exon in myoblasts and non-muscle tissues. Muscle-type F$_1$γ mRNA was created by the induction of a negative regulatory factor (25), whereas a differential regulation of muscle-specific exon exclusion was present between myotubes and mature muscle fibers. To elucidate the exact mechanism underlying alternative splicing during myogenesis, muscle-specific splicing regulatory factors must be identified.

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FIG. 8. Model for muscle-specific splicing in ATP synthase γ-subunit pre-mRNA in myoblasts, in myotubes, and in mature muscle fibers. The exonic cis-acting positive regulatory element (black box) located on the 5′ region of exon 9. This enhancer element is necessary for constitutive splicing in non-muscle tissues and myoblasts, and a constitutive splicing factor is also required. The Pu-del mutation (gray box) at the 5′ region affected exon inclusion in any tissue and cell (left column). A negative regulatory factor was induced and then inhibited the constitutive splicing in myotubes. The Pu-rich mutation (horizontal box) at the middle region of exon 9 inhibited induction of exon exclusion (middle column). The Pu-rich mutation (horizontal box) did not work in skeletal and heart muscle tissues. In this model, the protein level of a constitutive splicing factor is decreased in mature muscle fibers, which in turn results in exon exclusion, even when the silencer element is inactivated (right column). See text details.
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