GENES: STRUCTURE AND REGULATION:

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Cloning of Human Acetyl-CoA Carboxylase β Promoter and Its Regulation by Muscle Regulatory Factors*

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The 280-kDa β-isoform of acetyl-CoA carboxylase (ACCβ) is predominantly expressed in heart and skeletal muscle, whereas the 265-kDa α-isoform (ACCα) is the major ACC in lipogenic tissues. The ACCβ promoter showed myoblast-specific promoter activity and was strongly induced by MyoD in NIH3T3 cells. Serial deletions of the promoter revealed that MyoD acts on the E-boxes located at positions −498 to −403 and on the proximal region including the 5′-untranslated region. Destruction of the E-boxes at positions −498 to −403 by site-directed mutagenesis resulted in a significant decrease of MyoD responsiveness. The "TGAAA" at −32 to −28 and the region around the transcription start site play important roles in basal transcription, probably as a TATA box and an Inr element, respectively. Mutations of another E-box at −14 to −9 and a "GCCTGTCA" sequence at +17 to +24 drastically decreased the MyoD responsiveness. The novel *cis*-element GCCTGTCA was preferentially bound by MyoD homodimer in EMSA and conferred MyoD responsiveness to a luciferase reporter, which was repressed by the overexpression of E12. This finding is unique since activation via E-boxes is mediated by heterodimers of MyoD and E-proteins. We screened a human skeletal muscle cDNA library to isolate clones expressing proteins that bind to the region around the GCCTGTCA (+8 to +27) sequence, and isolated Myf4 and Myf6 cDNAs. Electrophoretic mobility shift assay showed that recombinant Myf4 and Myf6 bind to this novel *cis*-element. Moreover, transient expression of Myf6 induced significant activation on the ACCβ promoter or an artificial promoter harboring this novel *cis*-element. These findings suggest that muscle regulatory factors, such as MyoD, Myf4, and Myf6, contribute to the muscle-specific expression of ACCβ *via* E-boxes and the novel *cis*-element GCCTGTCA.

Acetyl-CoA carboxylase (ACC)[1] is a key enzyme in fatty acid biosynthesis catalyzing the transfer of a carboxyl group to acetyl-CoA to form malonyl-CoA. Malonyl-CoA serves as the active donor of two carbon atoms in fatty acid synthesis and strongly inhibits fatty acid β-oxidation by acting as a potent inhibitor of carnitine palmitoyltransferase I (1, 2). Carnitine palmitoyltransferase I resides on the surface of the mitochondrial membrane and generates palmitoylcarnitine from palmitoyl-CoA. This step is critical for fatty acid β-oxidation because cytosolic fatty acyl-CoA cannot translocate into the mitochondria where fatty acid β-oxidation occurs. Palmitoylcarnitine is transported into mitochondra by a carrier system and then reconverted into palmitoyl-CoA by carnitine palmitoyltransferase II. This shuttle system is the first step of fatty acid β-oxidation, which supplies tissues with energy.

Mammals have two major isoforms of acetyl-CoA carboxylase. The 265-kDa isoform, designated as ACCα, is highly expressed in liver and adipose tissues, and the 280-kDa isoform, designated as ACCβ, is predominantly expressed in heart and skeletal muscle (3–5). Human ACCα and ACCβ have about 80% amino acid sequence homology and both produce malonyl-CoA. They are, however, encoded by separate genes, mapped to chromosome 17q21 and 12q23.1, respectively, and show distinct tissue distribution and nutritional regulation (6–8). The presence of the β-isoform in tissues that do not actively synthesize fatty acid *de novo* has not been clearly explained. It has been suggested that ACCβ may play a role in mitochondrial fatty acid β-oxidation in muscle tissues. Its product malonyl-CoA might be a critical regulatory tool for mitochondrial fatty acid uptake and metabolism (9, 10).

Compared with ACCα, little is known about the regulation of ACCβ. To study the transcriptional regulation of ACCβ we cloned the 17-kb human ACCβ promoter and analyzed its *cis*-elements and *trans*-acting factors. Transcriptional regulation of muscle-specific genes is commonly studied by investigating the effects of muscle-specific and stage-specific transcription factors (11–14). One of the sequence motifs characterized as a critical regulatory component in muscle gene expression is the E-box (CANNTG) (15–17). Muscle-specific genes, such as muscle creatine kinase, myosin light chain, and myogenin genes, have multiple E-boxes in their enhancers or promoters that act cooperatively to regulate gene transcription (18–20). E-boxes are binding sites for ubiquitously expressed bHLH family pro-

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The nucleotide sequences of PIβ *and PIIβ have been submitted to the GenBank™/EBI Data Bank with accession numbers AF268378 and AF268379, respectively.*

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[1] The abbreviations used are: ACC, acetyl-CoA carboxylase; MRF, muscle regulatory factor; bHLH, basic helix-loop-helix; 5′-UTR, 5′-untranslated region; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid.

teins, known as E-proteins. In muscle cells, E-proteins dimerize with muscle-related bHLH transcription factors, called muscle regulatory factors (MRFs), enabling the MRFs to act on muscle-specific regulatory elements (21, 22). The four muscle-specific MRFs, MyoD, myogenin, Myf5, and MRF4, have been characterized in terms of their significance in development through stage-specific utilization of those factors (23–25).

Because there are several E-boxes on the ACCβ promoter, we expected that the muscle-specific expression of ACCβ might be explained by the responsiveness of its promoter to MRFs. In this study, we report the fact that ACCβ is transcriptionally activated by MyoD through the E-boxes on its promoter. We also report a novel *cis*-acting element which serves as an MRF-binding site and is critically required for the MyoD-mediated ACCβ promoter activation. Elaboration upon the transcriptional regulation of ACCβ in the context of myogenesis-related factors might provide further understanding of the muscle-specific expression of ACCβ.

MATERIALS AND METHODS

Cloning of ACCβ Promoter—Previous isolation of the ACCβ cDNA from a human skeletal muscle cDNA library (7) yielded two cDNA clones with different 5′-untranslated regions (5′-UTR) of 300 and 42 bp, respectively.[2] In the presence of the sense primer (5′-ATGTAACCCTGAATGCACGGTGGGGAGGACAT-3′) of the 300 bp 5′-UTR and the antisense primer (5′-TCACTGGGGATGCAGCCACCAGCTCCATT-3′) of the 42-bp 5′-UTR, PCR using human genomic DNA as a template produced a 14.5-kb DNA fragment spanning these two 5′-UTRs. The PCR reaction was performed using Expand™ Long Template PCR system (Roche Molecular Biochemicals). The PCR was set at 94 °C for 10 s for denaturation, at 60 °C for 30 s for annealing, and at 68 °C for 8 min for extension and repeated for 30 cycles. After the final cycle, the elongation was carried out at 68 °C for 7 min. The amplified DNA fragment was subcloned into pGEM4Z (Promega) and its restriction map was determined (Fig. 1*A*). The 5′-flanking region of the 300-bp 5′-UTR was obtained by inverse PCR. Ten μg of human genomic DNA was digested with 50 units of *Eco*RI and followed by phenol/chloroform extraction and ethanol precipitation. The precipitated DNA was self-ligated in the volume of 1 ml using T4 DNA ligase (100 units) at 16 °C for 14 h. Self-ligated DNA was precipitated by ethanol after phenol/chloroform extraction, and was dissolved in 20 μl of TE buffer (pH 8.0). The flanking sequence of 300 bp 5′-UTR was amplified by PCR using 5′ antisense primer (5′-CCTGGAGCACTTAACCTTAACCTTCA-3′), and 3′ sense primer (5′-TAAGCAGCAAGCAGGCTTAG-3′), and 0.5 μg of self-ligated DNA. The amplified DNA was subcloned into pGEM4Z and sequenced.

Primer Extension—An oligonucleotide (5′-CAGGAAAAGGTCAGACAGACAGGA-3′), complementary to the region +37 to +56 from the ATG codon of ACCβ was labeled with [γ-^{32}P]ATP by polynucleotide kinase. The labeled oligonucleotide (10^5 cpm) was mixed with 1 μg of human skeletal muscle poly(A)$^+$ RNA (CLONTECH) in 10 μl of 25 mM PIPES (pH 6.8), 400 mM NaCl, 1 mM EDTA. The mixture was incubated at 70 °C for 3 min, then slowly cooled down to 37 °C and incubated overnight at 37 °C. Annealed primer/RNA was precipitated by adding 25 μl of ethanol and used for extension reaction. This primer was extended with 200 units of SuperScript II reverse transcriptase (Life Technologies, Inc.) at 42 °C for 1 h under the buffer conditions the manufacturer recommended. After phenol/chloroform (1:1, v/v) extraction and ethanol precipitation, the size of primer extension product was determined by 8% denaturing polyacrylamide gel electrophoresis. The nucleotide numbering of the ACCβ promoter was based on the transcription start site proved by primer extension analysis. To confirm that the primer extension product contains the 5′-UTR of the ACCβ cDNA, the primer extension product was isolated from the denaturing polyacrylamide gel and used as a template in following PCR. Primers used in the PCR were the 5′ sense primer corresponding to the region −72 to −53, or −55 to −34 from the ATG codon and the 3′ antisense primer used in primer extension analysis. The size of the PCR products was determined on an 8% native polyacrylamide gel.

RT-PCR—Total RNAs were extracted from Alexander cells, HepG2 cells, and human skeletal muscle, using the TRIzol (Life Technologies, Inc.) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 2 μg of total RNA in 20 μl of reaction volume using SuperScript II reverse transcriptase (Life Technologies, Inc.). Each reverse transcription mixture (0.5 μl) was used as the template for amplifying ACCβ cDNA. The PCR primers for the 5′ terminal region of ACCβ transcript, spanning exon 1b and exon 2, were the sense primer corresponding to the region −72 to −53 from the ATG codon and the antisense primer same as that used in the primer extension analysis. The ACCβ cDNA corresponding to the region +6001 to +7000 from the ATG codon and glyceraldehyde-3-phosphate dehydrogenase cDNA were amplified using following primers: ACCβ sense, 5′-TCCAACAACCAGCTGGGTGGCGTTC-3′; ACCβ antisense, 5′-CCAGCATCCGGCCGGGTGTGTCATG-3′; glyceraldehyde-3-phosphate dehydrogenase sense, 5′-ACCACAGTCCATGCCATCAC-3′; and glyceraldehyde-3-phosphate dehydrogenase antisense, 5′-GGAGACCTTCTGCTCAGTCGACG-3′. The sizes of the PCR products were determined on an 8% native polyacrylamide gel or on 1.0% agarose gels.

Construction of Transfection Plasmids—The PIIβ promoter-reporter construct of pPIIβ−1317 was generated by subcloning the 3′ 1.4-kb *Xba*I fragment (−1317 to +65) of the 14.5-kb PCR product into *Sma*I site of pGL3-Basic (Promega). The constructs designated as pPIIβ−1090, pPIIβ−569, pPIIβ−349, and pPIIβ−203 were produced by self-ligation of pPIIβ−1317 after digestion with *Kpn*I, *Kpn*I/*Apa*I, *Kpn*I/*Dra*I, and *Kpn*I/*Hin*dIII, respectively. The constructs of pPIIβ−800, pPIIβ−93, pPIIβ−38, and pPIIβ+7 were generated by insertion of the DNA fragments amplified by PCR into *Sma*I site of pGL3-Basic. The construct of pPIIβ−1317/+17 was produced by ligation of the 4.5-kb *Nar*I-flushed/*Xba*I fragment of pPIIβ-1317 and 1.7-kb *Sma*I/*Xba*I luciferase gene of pGL3-Basic. The construct, pPIIβ−1317/+3 was prepared by subcloning the DNA fragment from −1317 to +3 of PIIβ amplified by PCR into *Sma*I site of pGL3-Basic. Mutant constructs were generated employing QuikChange™ site-directed mutagenesis kit (Stratagen) using the mutagenic oligonucleotides. Every mutated sequence is explained in the legends to Figs. 5 and 6, and the sequences of all mutant constructs were confirmed by DNA sequencing. The construction of the pE-box-tk-luc and the p6×(+8/+27)-tk-luc was performed by inserting −569 to −204 of PIIβ and six copies of the fragment from +8 to +27 of PIIβ, respectively, into the upstream of the tk promoter of ptk-luc, a tk-promoter-luciferase reporter construct.

Cell Culture and Transient Transfection Assay—The established cell lines, obtained from American Type Culture Collection (Rockville, MD), were cultured in the recommended media (H9C2 in DMEM/F12; Alexander in MEM; HeLa in MEM; NIH3T3 in Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate at 37 °C under 5% CO_2, 95% air. All cell culture materials were purchased from Life Technologies, Inc. For the transient transfection assay, cells were plated at a density of 2×10^5 cells/35-mm dish. On the next day, transfection was performed with 0.4 μg of the indicated pPIIβ-luciferase constructs, 0.2 μg of the pCMV β-galactosidase plasmid (CLONTECH) and the indicated amounts of pcDNA3 (Invitrogen), pcMyoD, or pcE12. The pcMyoD and pcE12 plasmids were kindly provided by Stephan F. Konieczny (Purdue University) and express MyoD and E12, respectively, under the control of the cytomegalovirus IE promoter. Transfection was performed using LipofectAMINE Plus transfection reagent (Life Technologies, Inc.) for 3 h according to the manufacturer's instructions. After 2 days, cells were washed with phosphate-buffered saline (Life Technologies, Inc.) and lysed in 200 μl of reporter lysis buffer (Promega). Luciferase activities were measured using the Luciferase Assay System (Promega) and normalized by the β-galactosidase activities to correct for the transfection efficiency. All plasmid DNA for transfections was purified by Qiagen Plasmid Midi kit (Qiagen Inc.)

Yeast One-hybrid Screening—A human skeletal muscle cDNA library was screened employing the Matchmaker one-hybrid system (CLONTECH). All procedures were performed according to the manufacturer's instructions. For the construction of a target-reporter yeast strain, 3 copies of the 20-bp fragment corresponding to nucleotides from −42/−23, −10/+10, and +8/+27 was inserted into the *Sac*I site of pHisi-1 and pLacZi. The resulting plasmids were linearized and transformed into *Saccharomyces cerevisiae* strain YM4271 by the lithium acetate method, and the transformants were selected on SD-His,Ura solid medium for integration of the target-reporter construct into the *HIS3* locus. To screen the clones expressing the proteins that bind to the target sequences *in vivo*, a human skeletal muscle cDNA library constructed in pGAD10 was introduced into the above transformed YM4271. pGAD10, the expression vector of the Matchmaker yeast two-hybrid system produces the inserted gene product as a C terminus

[2] J-J. Lee, Y-A. Moon, J-H. Ha, D-J. Yoon, Y-H. Ahn, and K-S. Kim, unpublished data.

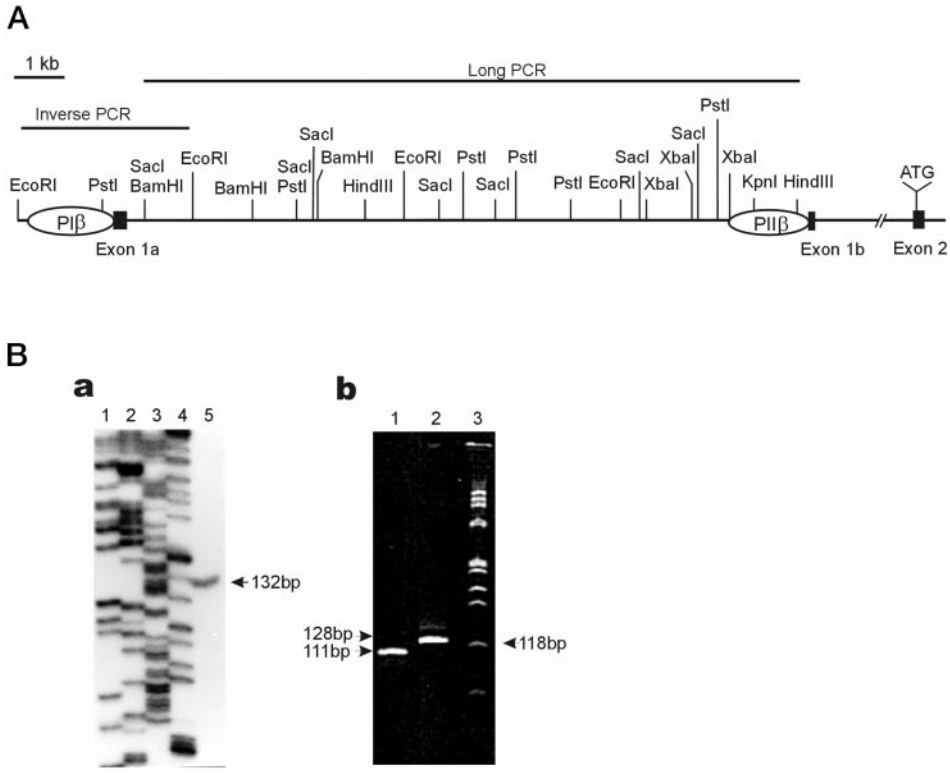

FIG. 1. **Cloning of the upstream 17-kb region of ACCβ gene and determination of its transcription initiation site.** *A,* describes the cloning strategy used to obtain the 17-kb 5′-flanking region of the ACCβ gene and the restriction sites of the cloned fragment. The regions of two 5′-UTRs (300 and 42 bp described in text) are indicated as *exon 1a* and *exon 1b*, and their 5′-flanking regions are designated as PIβ and PIIβ, respectively. The ATG start codon in exon 2 is illustrated. *B,* shows the primer extension product resolved on an 8% denaturing polyacrylamide gel (*a, lane 5*). A dideoxy sequence ladder (*a, lanes 1, 2, 3,* and *4*) is shown for size determination of the primer extension product. The primer extension product (132 bp) was eluted from gel slice and used for the nested PCR to amplify the region −55 to +56 (*b, lane 1*) or −72 to +56 (*b, lane 2*) from the ATG codon. The size of nested PCR products was measured on a 8% native polyacrylamide gel in 1 × TBE by comparing with the ϕX174 DNA/*Hae*III marker (*b, lane 3*)

of GAL4 activation domain-fusion protein. Clones were selected on SD-His,Ura,Leu solid medium containing 40 mM 3-amino-1,2,4-triazole (Sigma). Plasmids isolated from the selected yeast clones were transformed into the *Escherichia coli* strain DH5α and subjected to sequencing for identification.

Preparation of Recombinant MyoD, E47, Myf4, and Myf6—Recombinant MyoD and E47 were expressed in *E. coli* SG13009[pREP4] containing pQT-MyoD or pQT-E47, kindly provided by Stephan F. Konieczny. Expression plasmids of pET-Myf6 and pGST-Myf4 were prepared by inserting Myf6 or Myf4 cDNA into *Bam*HI/*Hin*dIII sites of pET-21a (Novagene) or *Eco*RI/*Xho*I sites of pGEX4T3 (Amersham Pharmacia Biotech), respectively. Myf6 and GST-Myf4 were prepared in *E. coli* BL21(DE3) and *E. coli* DH5α, respectively. The bacteria freshly transformed with each expression vector were grown to mid-log phase and proteins were induced for 4 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The bacteria were harvested by centrifugation and disrupted by sonication. The recombinant proteins containing histidine-tag were purified to homogeneity by Ni-NTA-agarose (Qiagen) chromatography. GST-Myf4 was purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech) chromatography according to the protocol provided by the manufacturer. The purity and the concentration of the recombinant proteins were verified by SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining.

Electrophoretic Mobility Shift Assay—The EMSA probes used are as follows (sequence of sense oligomers are presented here); E1 (−418/−394), 5′-CCCTTGGAACATCTGTCGATGCTG-3′; E2 (−435/−412), 5′-GGCGTCTGACAGATGAACCCCTTG-3′; E3 (−512/−489), 5′-CTGTGTGTCCAGCTGGCCATTCGT-3′; Pr1 (−43/−15) 5′-CAGCCTCCCGCTGAAAGGTGACACTCTGCC-3′; Pr2 (−33/−15), 5′-CTGAAAGGTGACACTCTGCC-3′; Pr3 (−24/+4), 5′-GACACTCTGCCAGCTGGGTTCCCTTAGT-3′; Pr4 (−10/+10), 5′-TGGGTTCCCTTAGTCACCCT-3′; Pr5 (+4/+29), 5′-TCACCCTGTGGGCGCCTGTCAGCCTC-3′. Pr5 mutants used for competition assay are shown at Fig. 7*A*.

Each sense oligonucleotide was labeled with polynucleotide kinase (Takara) using [γ-^{32}P]ATP as substrate and annealed with 5-fold excess of its complementary, unlabeled oligonucleotide by heating at 90 °C for 5 min and allowing it to slowly cool to room temperature in 0.1 M Tris (pH 8.0), 50 mM NaCl. The annealed probe was purified by spun column chromatography and probes of 100,000 cpm were used for each reaction. Recombinant MyoD, GST-Myf4, Myf6, or MyoD/E47 heterodimer were incubated with the prepared probes in a final volume of 20 μl containing 10 mM HEPES (pH 7.9), 75 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol, 1 μg of poly(dI-dC) (Amersham Pharmacia Biotech), and 0.5% fetal calf serum. After 20 min incubation at room temperature, samples were subjected to electrophoresis on 5% polyacrylamide gel in 1 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) at 250 V for ~1 h at room temperature. For the competition assays, competitors prepared by annealing the unlabeled sense and antisense oligonucleotides were added at ~50–200-fold molar excess for each competition assay.

RESULTS

Cloning of Human ACCβ Promoter—Two ACCβ cDNA clones containing different 5′-UTRs of 300 and 42 bp, respectively, were previously isolated from a human skeletal muscle cDNA library (7). A sense primer based on the 300-bp 5′-UTR and an antisense primer based on the 42-bp 5′-UTR were used to amplify a 14.5-kb product. To clone the upstream sequence of the 300-bp 5′-UTR, inverse PCR was performed using circular genomic DNA self-ligated after *Eco*RI digestion as template. The resulting 3.5-kb DNA fragment contained 2.3-kb upstream and 1.2-kb downstream sequences of the 300-bp 5′-UTR. The cloning strategy used to obtain the 17-kb 5′-flanking sequence and its restriction map are shown in Fig. 1*A*. The regions of 300- and 42-bp 5′-UTRs were named as exon 1a and exon 1b, respectively, and their 5′-flanking sequences were designated as PIβ and PIIβ.

To determine the transcription initiation site of ACCβ, a ^{32}P-labeled primer complementary to the region +37 to +56

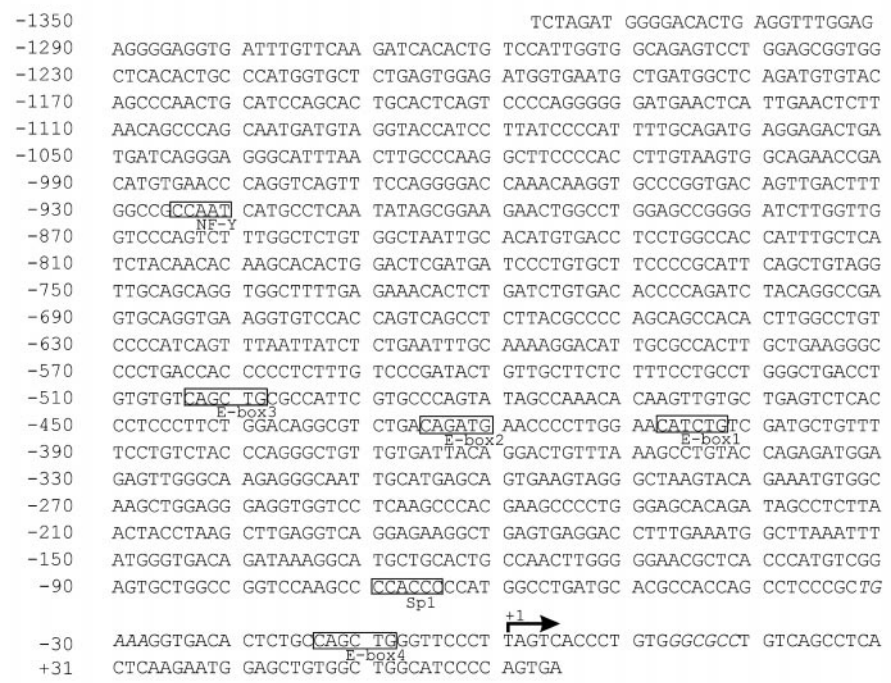

FIG. 2. **Sequence analysis of the −1317 to +65 fragment of the PIIβ promoter.** The E-boxes and other putative *cis*-elements recognized by computer-based analysis (MatInspector V2.2 based on Transfac 4.0) are *boxed* on the sequence of the PIIβ promoter (GenBank™ accession number AF268379). An *arrow* indicates the transcription start site.

from the ATG codon of ACCβ cDNA was extended using human skeletal muscle poly(A)$^+$ RNA as template. The products were analyzed on a sequencing gel and a single 132-bp fragment was observed (Fig. 1*B, a*). Because the primer-extension gave only one product smaller than the 300-bp 5′-UTR, we performed PCRs to confirm whether it contained the sequence of the 42-bp 5′-UTR. The 132-bp fragment was isolated from the sequencing gel slice and used as a template, and two 5′ sense primers corresponding to the 42-bp 5′-UTR and one common 3′ antisense primer of the previous primer extension, were used. Those primers amplified two PCR products of our expecting sizes, 111 bp (−55/+56) and 128 bp (−72/+56), respectively. This result suggests that the ACCβ mRNA in muscle originates from PIIβ (Fig. 1*B, b*). The first transcribed nucleotide of PIIβ, determined by the primer extension, was designated as +1 for numbering the nucleotides in the ACCβ gene.

We tested the promoter activities of the 5′-flanking fragments of those two 5′-UTRs. The PIIβ region from −1317 to +65 showed high promoter activity in H9C2 myoblast cells, while the 2.3-kb PIβ region upstream of the 300-bp 5′-UTR did not have transcription activity at all in various cell lines. Computer-based analysis (MatInspector V2.2 based on Transfac 4.0) of PIIβ revealed several transcription factor-binding sites including multiple E-boxes (Fig. 2). Therefore, in this study we investigated the *cis*-elements and *trans*-acting factors involved in the transcription regulation of PIIβ promoter.

ACCβ Promoter Was Highly Active in Myoblast Cells and Was Strongly Activated by MyoD—According to previous reports of the tissue-specific expression of ACCβ (6, 7, 26), we tested the promoter activity of the −1317 to +65 fragment in various cell lines. A transiently transfected luciferase reporter construct (pPIIβ-1317) showed the highest promoter activity in H9C2, a rat cardiomyoblast cell line. However, its promoter activity was very low or hardly detectable in Alexander, a human hepatoma cell line, in HeLa, a human cervical carcinoma cell line, or in NIH3T3, a mouse embryonic fibroblast cell line (Fig. 3*A*). The extremely low activity of pPIIβ−1317 in Alexander cells was rather intriguing, because ACCβ expression has been observed in human liver and HepG2, another human hepatoma cell line (6, 26). The inactiveness of this promoter in Alexander cells suggested that liver might employ another promoter for ACCβ expression. To explore whether a different promoter from PIIβ drives ACCβ expression in liver, we examined if the ACCβ mRNA in liver cell lines has different 5′-UTR from that of muscle tissue. We prepared cDNAs from total RNA of Alexander cells, and HepG2 cells, and human skeletal muscle tissue to perform RT-PCR. Only the muscle cDNA generated the 128-bp PCR product containing 5′-UTR (exon 1b) sequences, while those three cDNAs produced a 1-kb product for the C terminus coding region (Fig. 3*B*). Both muscle and liver definitely express the ACCβ mRNAs but the mRNAs in those two tissues have different 5′-UTRs. These results strongly imply the presence of liver-type ACCβ promoter, and we concluded that PIIβ is the muscle-type ACCβ promoter.

The multiple E-boxes picked up by the computer-based analysis suggested MRFs might act on the PIIβ (Fig. 2). Overexpression of MyoD, a typical MRF, indeed activated the transiently transfected ACCβ promoter effectively in every cell line except in HeLa cells (Fig. 3*A*). Its relative activation by MyoD in H9C2 cells is lower than in NIH3T3 cells or Alexander cells due to high basal transcription activity in H9C2 cells, probably caused by the endogenous myogenic transcription factors. We therefore used NIH3T3 cells for further experiments instead of myoblast cells to exclude the effect of endogenous MyoD and other muscle related factors.

To identify the MyoD-responsive regions in this promoter, we constructed a group of deletion reporters containing the PIIβ promoter serially deleted from the 5′ or 3′ end of the −1317 to +65 fragment (Fig. 4). The deletion of −569 to −349 and −38 to −19, among the 5′ deletion constructs, most drastically decreased the MyoD responsiveness. Deletion of the 220 bp from −569 to −349, containing a cluster of three E-boxes, decreased the MyoD responsiveness by about 50%. The reduced responsiveness was recovered in further deletions up to −39, suggesting the presence of inhibitory elements for MyoD responsiveness in the region from −340 to −39. The deletion from −93 to −39, where Sp1 can bind, most outstandingly enhanced the MyoD responsiveness. Although we cannot reveal the prescise mechanisms involved in this inhibitory action in the present study, the Sp1-binding site in this inhibitory

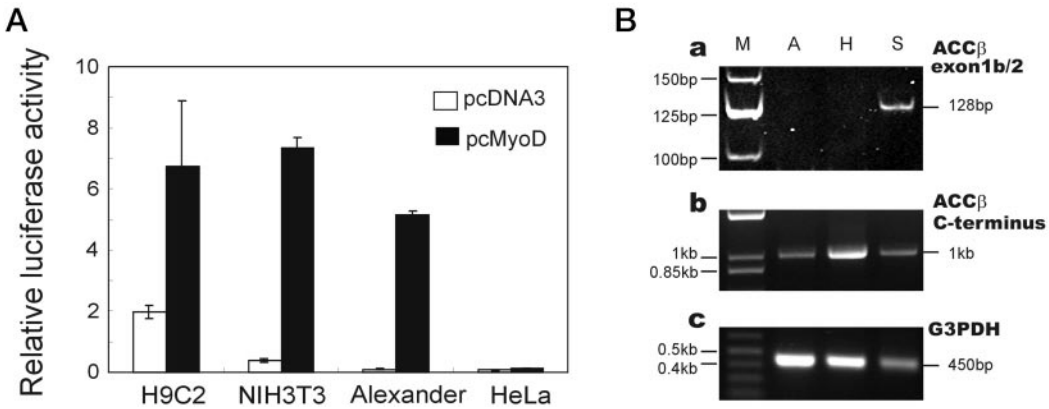

FIG. 3. **Muscle-specific promoter activity of PIIβ and its MyoD responsiveness in various cell lines.** *A,* pPIIβ-1317 was transiently transfected into H9C2, NIH3T3, Alexander, and HeLa cells. The basal activity and the MyoD stimulated activity of the reporter in each tested cell line are presented as *white bars* and *black bars*, respectively. Reporter (0.4 μg) and pCMV β-galactosidase (0.2 μg) were transfected into each cell line with pcMyoD or pcDNA3 (0.4 μg each). The reporter activities are shown as the relative luciferase activity normalized by the β-galactosidase activity. The data represents the mean ± S.D. of three independent experiments performed in triplicates. *B,* RT-PCR to compare the ACCβ transcripts of Alexander cells (*A*), HepG2 cells (*H*), and human muscle tissue (*S*). The ACCβ cDNA fragments, containing the sequence of exon 1b and exon 2 (*a*), or +6001 to +7000 from the ATG codon (*b*), or glyceraldehyde-3-phosphate dehydrogenase cDNA as an internal control for the quality of cDNAs (*c*) were PCR-amplified and the product sizes were measured on a 8% polyacrylamide gel (*a*) or 1% agarose gel (*b* and *c*). For the size marker (M), the 25-bp DNA ladder or 1-kb DNA ladder (Life Technologies, Inc.) was used.

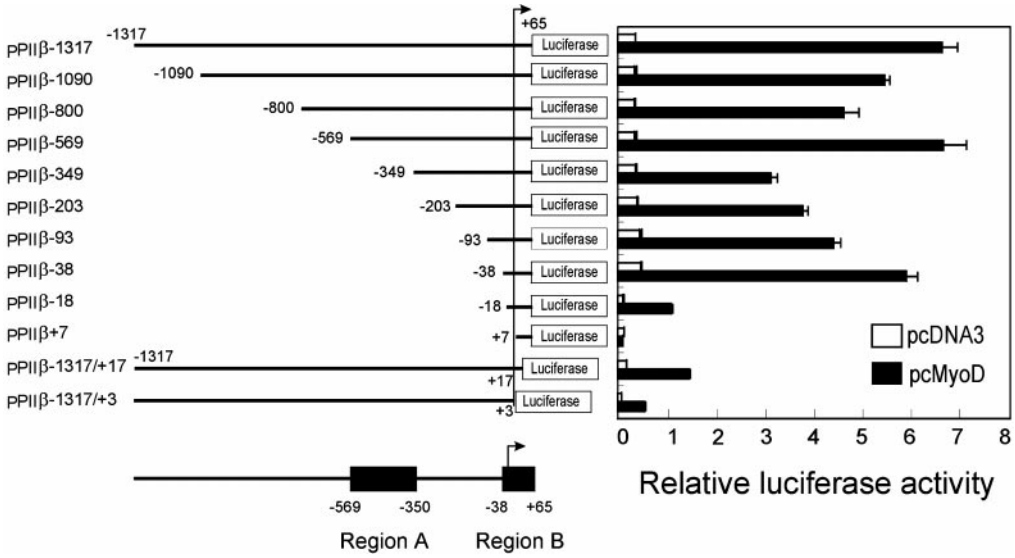

FIG. 4. **MyoD-responsive regions of PIIβ have been narrowed down to two main regions by transient transfection analysis of serially deleted reporters.** Schematic diagram of the PIIβ serial deletion reporter constructs and their MyoD responsiveness are presented. The two putative regions on which MyoD acts, designated as *region A* and *region B*, are presented at the *bottom*. Reporter (0.4 μg) and pCMV β-galactosidase (0.2 μg) were transfected into NIH3T3 cells with pcMyoD or pcDNA3 (0.4 μg each). The reporter activities are shown as the relative luciferase activity normalized by the β-galactosidase activity. The data represents the mean ± S.D. of four independent experiments performed in triplicate.

region is considered to be noteworthy since Sp1 expression markedly decreases during myogenesis (36, 37). Deletion from −38 to −19 resulted in a significant decrease in basal transcription as well as the decrease in MyoD responsiveness, and the further deletion to +7 completely abolished MyoD responsiveness of PIIβ. Deletions of the 5′-UTR from pPIIβ−1317 also significantly diminished the MyoD responsiveness and the basal transcription level. The regions of −569 to −349 and −39 to +65, which play important roles in MyoD responsiveness and/or basal transcription, are hence designated as regions A and B, respectively (Fig. 4).

MyoD Acts on the E-boxes Located in Region A—MyoD functions as a heterodimer with E-proteins such as E12 and E47 (21). Although MyoD/MyoD homodimers can bind to E-boxes *in vitro*, the MyoD/E-protein heterodimers recognize E-boxes on DNA strands *in vivo* (26, 27). There exist three E-boxes in the −569 to −349 region designated as E-box 1, E-box 2, and E-box 3 (Fig. 5*A*). E-box 1 and 2 are juxtaposed with intervening 12 nucleotides, and E-box 3 is located about 60 bp upstream from E-box 2. Interestingly, strikingly similar distributions of three E-boxes are found in various muscle-specific promoters and enhancers, such as the myosin light chain enhancer, muscle creatine kinase promoter, and acetylcholine receptor α and δ subunit enhancers (26). We presumed the cluster of three E-boxes on the ACCβ promoter might contribute to its MyoD responsiveness and examined their role in MyoD responsiveness by introducing a site-specific mutation (TG → AA) in each (Fig. 5*B*). Each single E-box mutation such as M(E1), M(E2), and M(E3) reduced the MyoD responsiveness to different extents. Mutation on E-box 1 most significantly decreased the responsiveness. Interestingly, the influences of the E-box destructions were neither synergistic nor additive. Double mutant (M(E1+2)) or triple mutant (M(E1+2+3)) showed a similar extent of MyoD responsiveness to that of the single mutant M(E1). To confirm the MyoD binding to these E-boxes *in vitro*, we performed EMSA with recombinant MyoD and recombinant

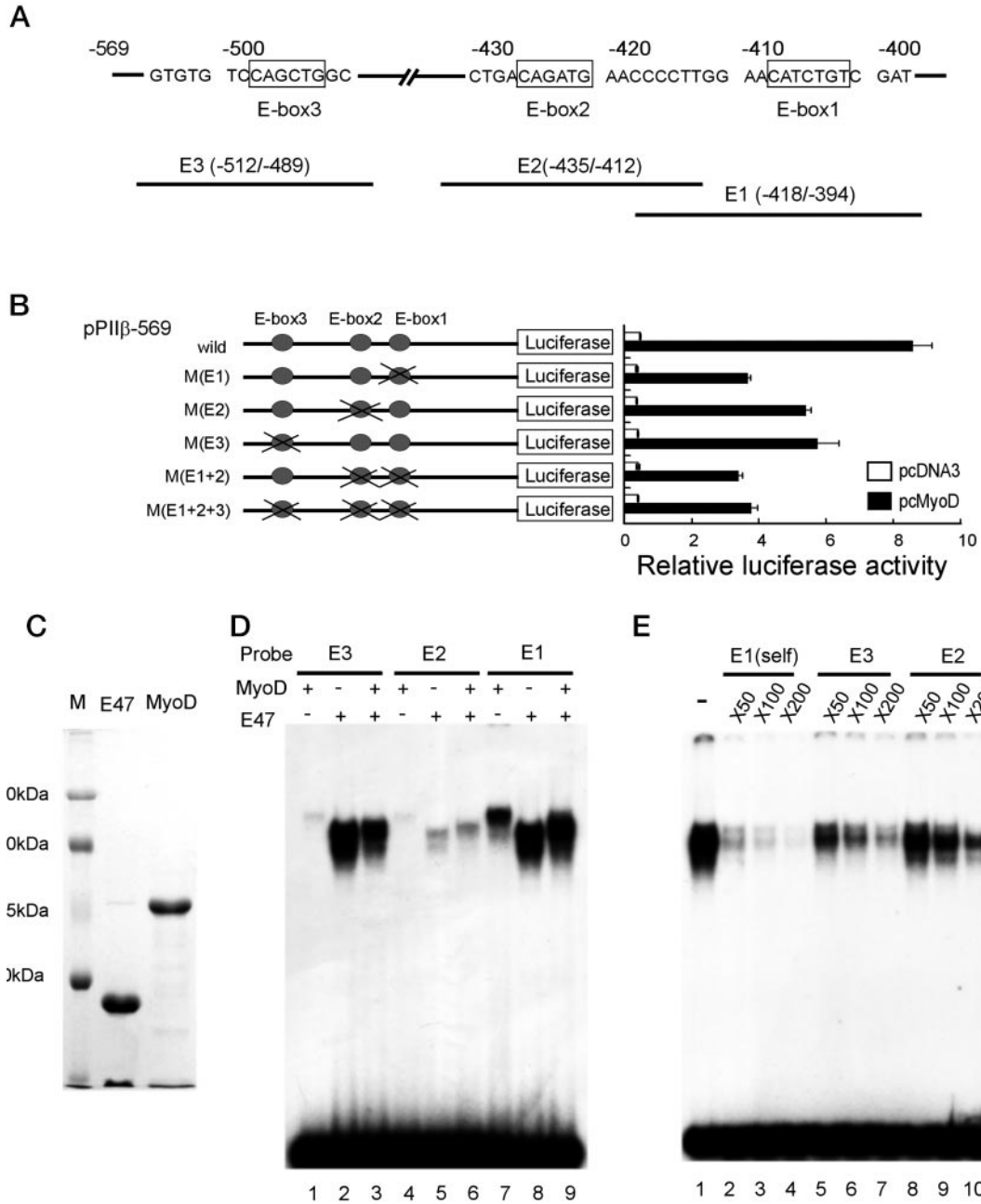

FIG. 5. **MyoD acts on the three E-boxes of region A.** *A* shows the sequences of the E-boxes in region A and the location of the probes E1, E2, and E3 used in *D* and *E*. *B*, single, double or triple E-box mutants (mE1, mE2, mE3, mE1+2, and mE1+2+3), schematically shown at the *left side*, were prepared by site-directed mutagenesis substituting TG of CANNTG with AA. Wild type (pPIIα−569) or E-box-mutant reporters (0.4 μg) and pCMV β-galactosidase (0.2 μg) were transfected into NIH3T3 cells with pcMyoD or pcDNA3 (0.4 μg each). The reporter activities are shown as the relative luciferase activity normalized by the β-galactosidase activity. The data represents the mean ± S.D. of three independent experiments performed in triplicate. *C,* SDS-PAGE of the recombinant E47 and MyoD. Those proteins are bacterially expressed and purified for EMSA. *M* is the molecular weight standard. *D,* EMSA of the E-boxes with recombinant MyoD and/or E47. ^{32}P-Labeled probes (E1, E2, and E3) were incubated with 300 ng of MyoD (*lanes 1, 4*, and *7*) or 30 ng of E47 (*lanes 2, 5*, and *8*) or 150 ng/15 ng of MyoD/E47 mixture (*lanes 3, 6*, and *9*). *E,* competition assays. The E1 band shifted by MyoD/E47 (150 ng/15 ng) was competed by 50–200-fold molar excess amount of unlabeled competitors, such as E1 (*lanes 2–4*), E2 (*lanes 8–10*), and E3 (*lanes 5–7*).

E47 (Fig. 5, *C* and *D*). The probe containing E-box 1 (E1) was well shifted by MyoD, E47, or the mixture of MyoD and E47 (MyoD/E47). The probes containing E-box 2 (E2) or E-box 3 (E3) were not effectively bound by MyoD, but were shifted by E47 or MyoD/E47. All three probes were bound by MyoD/E47, even though the intensity of their shifted bands was significantly different from one another, E1 showed the strongest band shift, E3 the next, and E2 the weakest. To determine whether the differences result from differences in their binding affinity, we performed competition assays (Fig. 5*E*). The complex formation between E1 and MyoD/E47 was effectively competed by cold E1, the self-competitor, moderately by cold E3, and slightly by excess amount of cold E2. Thus, E-box 1 has the strongest affinity to MyoD/E47 among the three E-boxes in region A.

Region B Has Multiple cis-Elements Crucial for Basal Transcription and MyoD Responsiveness—To investigate the *cis*-elements for the MyoD responsiveness located around −38 to +65 (region B), we first introduced a mutation on the single E-box (−14 to −9), designated as E-box 4, of pPIIβ−93 (Fig. 6*A*). The mutation of TG to AA (M(E4)) in E-box 4 reduced the MyoD responsiveness of pPIIβ−93 by about 50% (Fig. 6*B*).

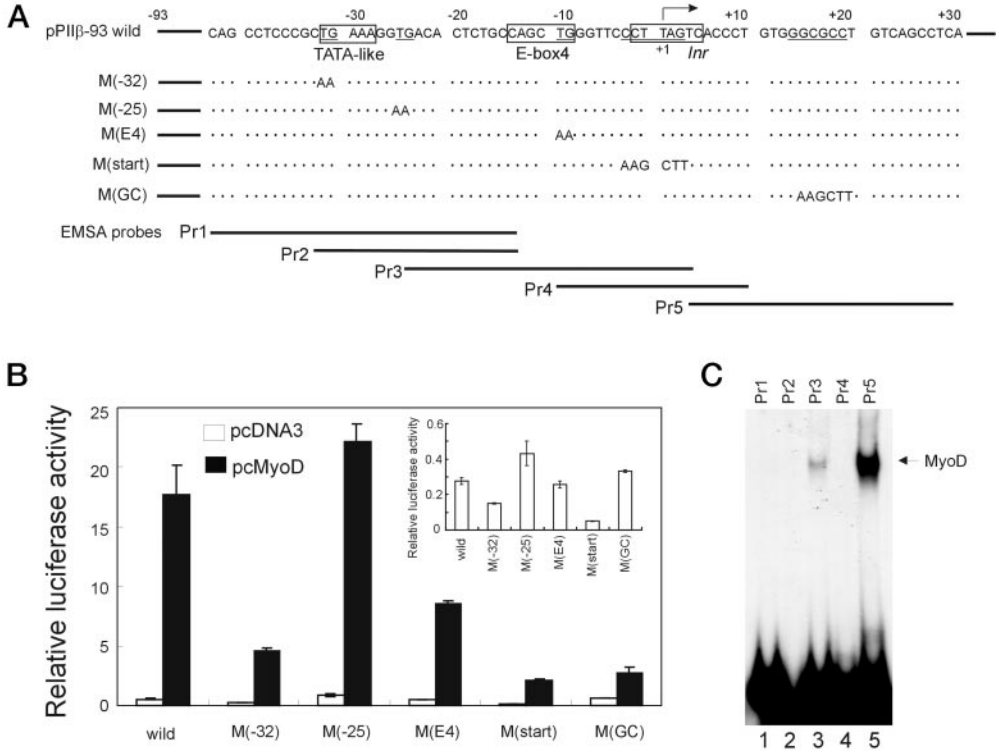

FIG. 6. **Region B has several putative *cis*-elements for the basal transcription and the MyoD responsiveness.** *A,* DNA sequences from −43 to +30 is shown to describe the five mutations introduced in region B and the five probes used in *B* and *C*. *B,* the MyoD-stimulated activities of the five mutant reporters were measured by transient transfection with co-transfection of pcMyoD into NIH3T3 cells. The basal activity of each tested mutant is shown in the *inset*. Reporter (0.4 μg) and pCMV β-galactosidase (0.2 μg) were transfected into NIH3T3 cells with pcMyoD or pcDNA3 (0.4 μg each). The reporter activities are shown as the relative luciferase activity normalized by the β-galactosidase activity. The data represents the mean ± S.D. of five independent experiments performed in triplicate. *C,* probes (Pr1 to Pr5) presented at the *bottom* of *A* were subjected to an EMSA to test the binding of MyoD (150 ng).

Because the destruction of the sole E-box in pPIIβ−93 did not abolish its entire MyoD responsiveness, we presumed there might exist other important sites for MyoD action in this short region, and prepared several mutant reporters to identify those motifs. We substituted the first and second TG sequences with AA, and designated the resulting mutants as M(−32) and M(−25) according to their locations. Mutation of the −32/−31 TG drastically decreased the MyoD responsiveness and the basal transcription by 74 and 50%, respectively, whereas mutation of the −25/−24 TG showed no significant change on the basal promoter activity and the stimulation by MyoD (Fig. 6, *A* and *B*). The TGAAA sequence from −32 to −28 appears to play a role of TATA box in the PIIβ promoter because it is located in the typical TATA box position and its mutation significantly affects the basal transcription. Next, the transcription start site (CCTT^{+1}AG) and a palindromic hexanucleotide (GGCGCC) located at +14 to +19 were substituted with AAGCTT. Those mutants, designated as M(start) and M(GC), showed remarkably decreased MyoD responsiveness by 89 and 85%, respectively. M(start) also showed significantly decreased basal transcription by over 80% while M(GC) did not show decrease in basal transcription, suggesting that the transcription start region might serve as an Inr element. To discern the *cis*-elements for MyoD from those for the general transcription complex, we examined where MyoD can bind in this region by EMSA using the probes shown in Fig. 6*A*. Probe Pr3 containing E-box 4 and probe Pr5 containing the GGCGCC palindrome could form a complex with recombinant MyoD, whereas the other probes could not (Fig. 6*C*).

Probe Pr5 (+4/+29) Is Preferentially Bound by MyoD/MyoD Homodimer—The transient transfection assay of the M(GC) mutant and the EMSA with Pr5 (Fig. 6) indicate the presence of a MyoD-responsive element, that is not a canonical E-box, in the region around +4 to +29. To clarify the precise core sequence for MyoD-binding, competitive EMSA was performed using mutant Pr5 oligonucleotides harboring substitutions (Fig. 7*A*). Competitors containing mutations from +17 to +24 could not compete effectively against Pr5, suggesting that the sequence of GCCTGTCA (+17 to +24) is important for MyoD binding.

From the previous EMSA (Fig. 6*C*), MyoD seems to bind to this element more avidly than to E-box 4. We compared this novel *cis*-element with the E-box *in vitro* by EMSA. As shown at Fig. 7B, E47 had strong affinity to Pr3 (*lanes 1* and *2*) and very poor affinity to Pr5 (*lane 7* and *8*), while MyoD showed much higher affinity to Pr5 than to Pr3 (*lanes 3, 4, 9*, and *10*). Heterodimerization of E47 and MyoD did not affect the high affinity of E47 to Pr3 (*lanes 5* and *6*), while it did significantly decrease complex formation to Pr5 in comparison with MyoD homodimer (*lane 11* and *12*). These results suggested that the novel *cis*-element in Pr5 prefers the MyoD homodimer to E47 homodimer or MyoD/E47 heterodimer. Moreover, these results corresponded to the *in vivo* results of transient transfection assays using artificial promoter-reporter constructs, which contained the E-box clustering region of PIIβ (−569 to −204) (pE-box-tk-luc) or six copies of +8 to +27 sequence (p6×(+8/+27)-tk-luc) upstream of tk minimal promoter (Fig. 7*C*). The tk minimal promoter itself was not affected by overexpression of E12 or MyoD. E12 alone did not stimulate pE-box-tk-luc or p6×(+8/+27)-tk-luc, while MyoD-overexpression enhanced the transcription of both reporter constructs. Coexpression of E12 with MyoD synergistically activated the transcription in pE-box-tk-luc compared with the transcription driven by MyoD alone. However, coexpression of E12 significantly decreased the transcription of p6×(+8/+27))-tk-luc stimulated by MyoD. Taken together, this novel *cis*-element is more apt to respond to

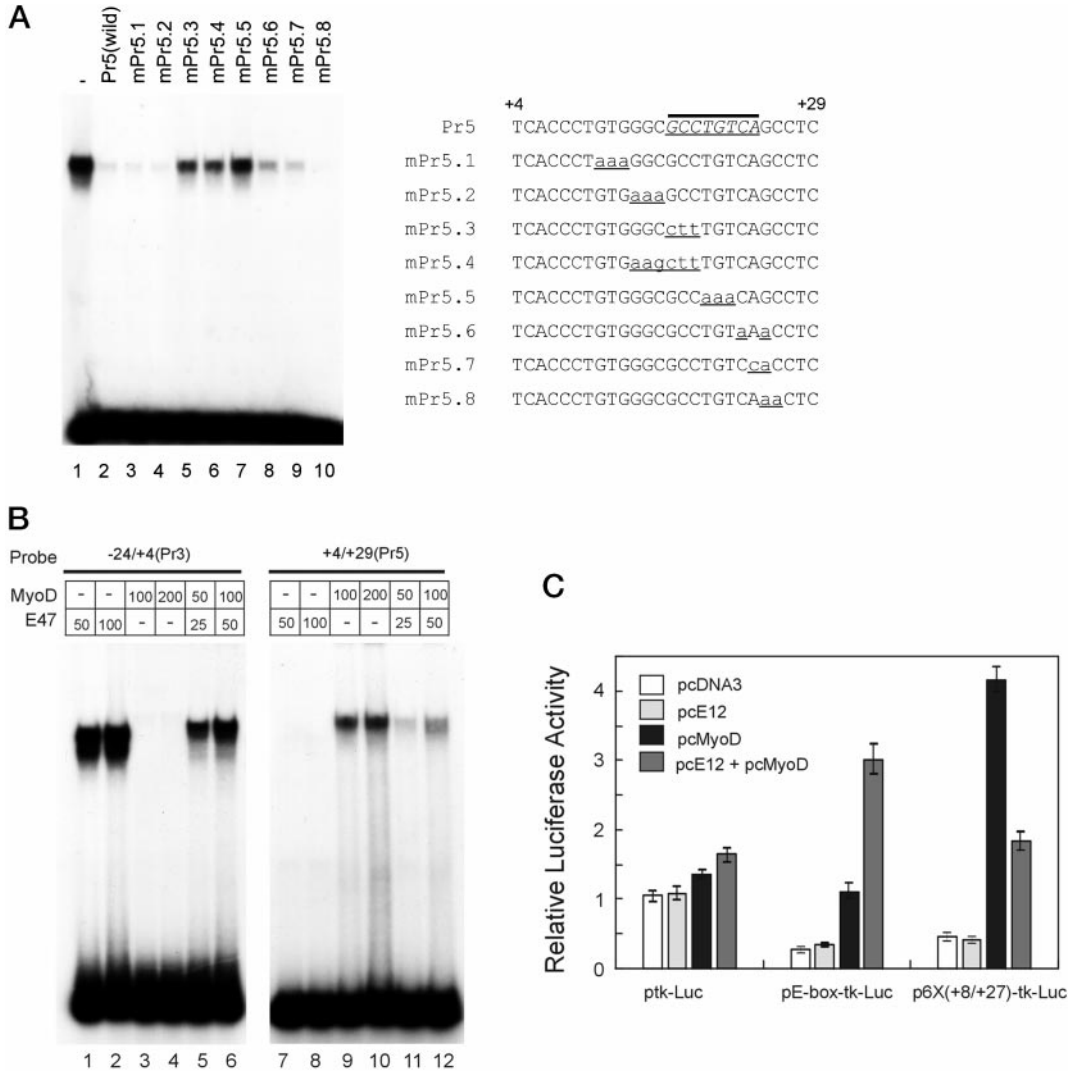

FIG. 7. **Pr5 contains a novel *cis*-element for MyoD binding whose characteristics are different from those of the E-box.** *A,* the novel *cis*-element has GCCTGTCA as its core motif. The Pr5 band shifted by MyoD (100 ng) was competed by mutant cold oligonucleotides shown in the *right panel*. Eight mutant oligonucleotides (*lanes 3–8*) and one wild type (*lane 2*) oligonucleotide were added to the binding reaction in the 200-fold molar excess. *B,* comparison of E-box 4 and the novel *cis*-element *in vitro*. ^{32}P-Labeled Pr3 (*lanes 1–6*) and Pr5 (*lanes 7–12*) were incubated with 50 or 100 ng of E47 (*lanes 1, 2* and *7, 8*), 100 or 200 ng of MyoD (*lanes 3, 4* and *9, 10*), and 50/25 or 100/50 ng of the MyoD/E47 mixture (*lanes 5, 6* and *11, 12*). *C,* comparison of the E-box 4 and the novel *cis*-element *in vivo.* The E-box clustered region (−569 to −204) or 6 copies of the +8 to +27 sequence were inserted upstream of the minimal tk promoter of ptk-luc construct as described under "Materials and Methods." Each reporter (0.4 μg) with pCMV β-galactosidase (0.2 μg) was co-transfected with pcDNA3 (0.4 μg, *white bar*), pcE12/pcDNA3 (0.1/0.3 μg, *light gray bar*), pcMyoD/pcDNA3 (0.1/0.3 μg, *black bar*), or pcE12/pcMyoD/pcDNA3 (0.1/0.1/0.2 μg, *dark gray bar*) into NIH3T3 cells. The control reporter (ptk-luc) was transfected in the same way to check the effect of MyoD or E12 on the minimal tk promoter. The reporter activities are shown as the relative luciferase activity normalized by the β-galactosidase activity. The data represents the mean ± S.D. of five independent experiments performed in triplicate.

the MyoD homodimer rather than to the E-protein/MyoD heterodimers and therefore is distinct from E-boxes.

MRFs Bind to the Novel cis-Element in Vivo—To isolate the trans-acting factors acting on region B, we screened a human skeletal muscle cDNA library using the yeast one-hybrid system. Three bait constructs were generated by insertion of three copies of the nucleotide sequence corresponding to the regions of −42 to −23, −10 to +10, and +8 to +27 upstream of the *HIS3* gene. We isolated the clones expressing the GAL4 activation domain fused to DNA-binding proteins, which bind to the bait sequences, and thus activate the *HIS3* gene expression allowing selection in histidine-depleted media. We could not obtain any positive clones as for the −42 to −23 or −10 to +10 regions, while several positive clones were isolated for the +8 to +27 region. Sequencing of those isolated clones revealed that one of them contained the full-length Myf4 cDNA and the other seven clones contained various lengths of Myf6 cDNA. These data suggest that MRFs bind to the +8 to +27 region *in vivo*, where the E-box consensus sequence does not exist. To confirm their binding ability to this DNA sequence *in vitro* by EMSA, we prepared recombinant Myf4, Myf6, and MyoD expressed in bacteria (Fig. 8B). MyoD, Myf4, and Myf6 could bind to this region *in vitro*. Those bindings were effectively competed by a 200-fold molar excess of the unlabeled wild type Pr5 (Fig. 8*A, lanes 3, 6,* and *9*), while the addition of excess cold mPr5.4, an oligonucleotide containing GGCGCC → AAGCTT mutation, did not decrease complex formation (Fig. 8*A*, *lanes 4, 7*, and *10*).

We tested whether MyoD and Myfs induce transcriptional activation on the promoters containing this novel *cis*-element. pPIIβ−93 and p6×(+8/+27)-tk-luc were greatly activated by MyoD or Myf6, while tk promoter itself was not activated by MyoD or Myf6 (Fig. 8*C*). All together these *in vivo* and *in vitro* data, we conclude that the GCCTGTCA motif truly is a novel *cis*-element for MRF transcription factors.

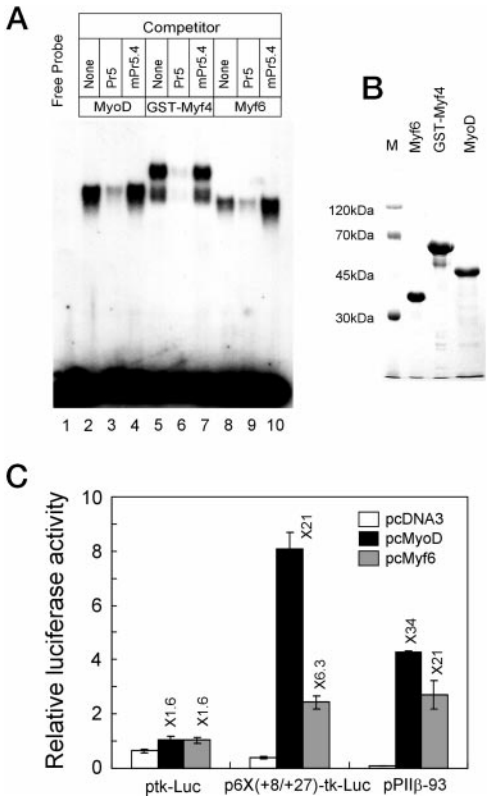

FIG. 8. **The novel *cis*-element containing GCCTGTCA sequence shows MRFs responsiveness.** *A,* EMSA of Pr5 with 100 ng of MyoD (*lanes 2–4*), GST-Myf4 (*lanes 5–7*), or Myf6 (*lanes 8–10*). Both wild type (*lanes 3, 6,* and *9*) and mutant (mPr5.4) unlabeled double-stranded oligonucleotides (*lanes 4, 7,* and *10*) were used as competitors. *B,* SDS-PAGE showing the purified recombinant proteins used in the EMSA. *Lanes 1–4,* the molecular weight size markers (Genepia, Korea), MyoD, GST-Myf4, and Myf6, respectively. *C,* the reporter constructs (0.4 μg each), such as ptk-luc, p6×(+8/+27)-tk-luc, and pPIIβ−93, were transfected into NIH3T3 cells with pcMyoD, pcMyf6, or pcDNA3 (0.4 μg each), and pCMV β-galactosidase (0.2 μg) was co-transfected. Fold increase of the MyoD- or Myf6-mediated induction compared with the basal activity of each reporter is shown *above* the *bars*. The reporter activities are shown as the relative luciferase activity normalized by the β-galactosidase activity. The data represents the means ± S.D. of five independent experiments performed in triplicate.

DISCUSSION

In this study, we have introduced the muscle-type promoter of ACCβ and characterized the *cis*-elements and *trans*-acting factors related. Moreover, we are suggesting the presence of the liver-type ACCβ promoter. The PIIβ promoter in the present study was cloned according to the information of 5′-UTR sequence of ACCβ cDNA originating the from skeletal muscle. However, because ACCβ is expressed not only in skeletal muscle but also in liver, another promoter different from the PIIβ seems to induce the ACCβ expression in liver. The following evidence supports this suggestion. First, the PIIβ promoter was highly active in myoblast but not in the hepatoma cell line. Second, the sequences of exon 1b transcribed by the PIIβ exists only in the ACCβ mRNA of skeletal muscle and not in that of hepatoma cell lines such as Alexander and HepG2, while the sequences for the C terminus region of ACCβ could be detected in both mRNAs from skeletal muscle and hepatoma cell lines. Conclusively, ACCβ expression might employ another promoter in liver, and the PIIβ is its muscle-specific promoter.

The *trans*-acting factors involved in the PIIβ promoter are identified as MRF family members, such as MyoD, myogenine/Myf4, and MRF4/Myf6. The myogenic regulatory factor-mediated transcription in this promoter depends on four E-boxes and one novel *cis*-element immediately downstream from the transcription start site, and at least two *cis*-elements which are not fully characterized but appear to be important for basal transcription. Among the four E-boxes, three of them were located in a distal region (−498 to −403), and one resides in a very proximal region (−14 to −9). The distal E-box clustered region (region A) is responsible for up to 50% of the MyoD responsiveness of this promoter, and the proximal region (region B), harboring the fourth E-box and a novel downstream *cis*-element, is responsible for the other 50% of MyoD responsiveness. E-box 1 of the clustered three E-boxes is thought to be most critical for the MyoD responsiveness of region A, although E-boxes 2 and 3 are also required for full activity. This distribution of triple E-boxes and the finding that, while all are necessary, one of the three is more crucial, has been reported by Wentworth *et al.* (26) in the myosin light chain enhancer. In addition, the E-box 1 (caTCtg) has never been reported as an MRF-binding site as far we know, while E-box 3, the less critical MyoD responsive site (caGCtg) has been repeatedly reported as a binding motif for MyoD/E-protein heterodimers in several muscle gene promoters (29, 30). E-box 2 (caGAtg) is reported to exist in the promoter of mouse acetylcholine receptor α subunit (35), but its weak binding affinity for MyoD/E47 do not allow ignoring the possibility that other transcription factors bind to this region and help the action of neighboring MyoD.

Between regions A and B (−349 to −39), unidentified repressor elements that might control the MyoD responsiveness seem to exist, because the deletion of this region enhanced the MyoD responsiveness. The enhancement was most prominent when the region containing an Sp1-binding site was excised out. According to our unpublished EMSA data[2] using the nuclear extracts of the H9C2 myoblasts and its differentiated myotubes, Sp1 binding to the consensus motif (−70 to −65) is severely decreased in muotubes. This result corresponds to the fact that myogenesis accompanies Sp1 decrease (36, 37). The Sp1 binding to this region might suppress the MyoD responsiveness in myoblasts and down-regulation of Sp1 during differentiation to myotube would be required for complete activation of the ACCβ gene by MyoD.

In region B, the proximal MyoD-responsive region, a well conserved E-box 4 (caGCtg) and a novel *cis*-element containing a GCCTGTCA sequence (Fig. 7) were identified as responsible for the MyoD responsiveness. This novel MyoD-binding site is very different from E-boxes where MRF binding is mediated by dimerization with E-proteins. The presence of E47 weakened the MyoD binding to this element *in vitro*, and coexpression of E12 diminished the MyoD-mediated stimulation on p6×(+8/+27)-tk-luc *in vivo* (Fig. 7). Those inhibitory effects of E-proteins seem to result from the dimerization of MyoD with E-proteins. In other words, MyoD binding to this site is not mediated by heterodimerization with E-proteins such as E47 and E12. This novel sequence, which was avidly bound by homodimeric MyoD, seems to be bound by other MRF family factors. Using the yeast one-hybrid system, we found that other myogenic transcription factors such as Myf4 and Myf6 are recruited by this novel *cis*-element. They all are bHLH proteins, responsible for muscle differentiation, bind to E-boxes, and heterodimerize with E-proteins (23, 28–32). Interaction of these Myfs with this site was successfully demonstrated *in vitro* and *in vivo* by EMSA and transient transfection assays (Fig. 8). Taken together, the novel *cis*-clement containing GCCTGTCA sequence functions as a general binding site for bHLH myogenic transcription factors without E-protein mediation.

Region B is a very proximal regulatory region and is thus, thought to contain basic elements responsible for the initiation of transcription through interactions with RNA polymerase II

and general initiator factors. The TGAAA at −32 region appears to play a role similar to a "TATA" element since it is located at the general TATA locus and the mutation at this region dramatically reduced the basal transcription. The previously reported consensus Inr sequence ($YYA^{+1}N(T/A)YY$) of metazoan (33, 34) is similar to the start region ($CTT^{+1}AGTC$) of this ACCβ promoter and disruption of this site decreased the basal transcription significantly. These findings suggest that this region might function as the Inr element of basal transcription.

A previous study reported that ACCβ expression is not observed in myoblast cells which express high levels of MRFs and is observed only after the differentiation of myoblast cells into myotubes (7). However, the promoter activity of ACCβ was very high in H9C2 myoblasts in our transient transfection assay (Fig. 3). According to our unpublished data,[2] the ACCβ promoter, stably transfected into H9C2 myoblast cells, shows very high basal activity and was not further enhanced by differentiation into myotubes (data not shown). These discrepancies between transfection assays and endogenous gene expression suggest that MRFs are required for maintnaining high level expression of ACC in muscle cells, and another switching system exist to turn on the expression of this muscle-specific gene. Further studies on the switching system might provide a general understanding for stage-specific turning on and off of muscle-specific genes. We now introduce the first report on the regulation mechanism for the muscle-type ACCβ promoter and also suggest the possible presence of the liver-type ACCβ promoter.

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