MOLECULAR AND CELLULAR BIOLOGY, Nov. 1995, p. 5991–6002 Vol. 15, No. 11
0270-7306/95/$04.00+0

Transcription of the Human β Enolase Gene (*ENO-3*) Is Regulated by an Intronic Muscle-Specific Enhancer That Binds Myocyte-Specific Enhancer Factor 2 Proteins and Ubiquitous G-Rich-Box Binding Factors

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Received 15 March 1995/Returned for modification 12 May 1995/Accepted 3 August 1995

To provide evidence for the *cis*-regulatory DNA sequences and *trans*-acting factors involved in the complex pattern of tissue- and stage-specific expression of the β enolase gene, constructs containing fragments of the gene fused to the chloramphenicol acetyltransferase gene were used in transient-transfection assays of C2C12 myogenic cells. Deletion analysis revealed the presence of four major regions: two negative regions in the 5′-flanking sequence, a basal promoter region which directs expression at low levels in proliferating and differentiated muscle cells, and a positive region within the first intron that confers cell-type-specific and differentiation-induced expression. This positive regulatory element is located in the 3′-proximal portion of the first intron (nucleotides +504 to +637) and acts as an enhancer irrespective of orientation and position from the homologous β enolase promoter or the heterologous thymidine kinase promoter, conferring in both cases muscle-specific expression to the linked reporter gene. Deletion of a putative myocyte-specific enhancer factor 1 (MEF-1) binding site, containing a canonical E-box motif, had no effects on muscle-specific transcription, indicating that this site is not required for the activity of the enhancer. Gel mobility shift assays, competition analysis, DNase I footprinting, and mutagenesis studies indicated that this element interacts through an A/T-rich box with a MEF-2 protein(s) and through a G-rich box with a novel ubiquitous factor(s). Mutation of either the G-rich box or the A/T-rich box resulted in a significantly reduced activity of the enhancer in transient-transfection assays. These data indicate that MEF-2 and G-rich-box binding factors are each necessary for tissue-specific expression of the β enolase gene in skeletal muscle cells.

During terminal differentiation of myogenic cells, the transition from precursor proliferating myoblasts to multinucleated contractile muscle fibers is accompanied by changes in the pattern of protein synthesis. Most prominent among these changes are the synthesis of myofibrillar and sarcoplasmic proteins and the accumulation of distinctive metabolic enzymes that are unique to muscle tissue (1, 9). It is now clear that many enzymes present in muscle fibers are isoforms different from those expressed in myoblasts and nonmuscle cells, and in many instances they are encoded by a muscle-specific member of a multigene family. The expression of these muscle-specific genes might be required for the maintenance of proper levels of enzymes and might contribute to the determination of the muscle phenotype.

In the last few years, we have been studying the human gene family encoding the glycolytic enzyme enolase (EC 4.2.1.11) as a model system for investigating the molecular mechanisms involved in developmentally regulated as well as tissue-specific expression. Three structurally related enolase isoforms, referred to as α, β, and γ enolase, are present in mammals (61). Each isoform is encoded by a distinct gene whose expression is regulated in a tissue-specific and development-specific manner (17, 19, 20, 45). The gene encoding the muscle-specific β isoform of enolase displays an interesting pattern of expression in the course of cardiac and skeletal muscle development, when the β isoform progressively replaces the nearly ubiquitous α isoform (18). Early in mouse embryogenesis, β enolase transcripts are first detectable, by in situ hybridization, in the cardiac tube and in the myotome (27), and expression remains extremely low in skeletal primary fibers, while a striking increase occurs in the second generation of fibers at the fetal stage of development (2, 27). A further increase is observed after birth, and, in the adult muscle, higher levels of expression of both β enolase proteins and transcripts are detected in fast-twitch fibers than in slow-twitch fibers (27). Expression of β enolase increases with terminal differentiation as established by studies of myogenic cell lines and primary myoblasts induced to differentiate in vitro, but expression already occurs in proliferating myoblasts (30, 49), in contrast to what is observed for the majority of the muscle-specific genes. Furthermore, it has been reported that, in mutated myogenic cells which do not differentiate into myotubes and do not express muscle-specific gene products as well as the members of the basic helix-loop-helix (bHLH) family of myogenic regulators (MyoD, myogenin, MRF-4, and Myf-5) (7, 15), β enolase transcripts are present at the same level as that detected in wild-type cells (49). These data suggest that other muscle-specific regulators may play a role in the transcriptional activation of the β enolase gene. Recently, a study of the regulation of the expression of the β enolase gene in proliferating myoblasts indicated a functional role for the Ets domain transcription factors (55).

Taken together, these data suggest that regulation of β enolase gene expression could take place at multiple levels and

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involve molecular mechanisms different from those proposed for other muscle genes. As a first step in understanding these mechanisms, we have determined the complete primary structure of the human β enolase gene (20). Here, we describe the identification, by using transient-transfection experiments with β enolase-chloramphenicol acetyltransferase (CAT) chimeric constructs in C2C12 myogenic cells, of a muscle-specific transcriptional enhancer located in the first intron of the gene between nucleotides (nt) +504 and +637 relative to the transcription start site. A putative myocyte-specific enhancer factor 1 (MEF-1) binding site (8) present within this element is not required for the activity of the enhancer.

Gel mobility shift assays, competition analysis, DNase I footprinting, and mutagenesis studies indicated that this first-intron enhancer contains a functional binding site for members of the MEF-2 family of transcription factors (59) and a G-rich binding site (AGTGGGGGAGGGGGCTGCG) that we have designated β enolase element 1 (BEE-1), which binds specifically nuclear protein factors present in myogenic and nonmyogenic cells. The data reported indicate that these factors are all required for tissue-specific expression of the β enolase gene in skeletal muscle cells. Furthermore, evidence that supports the hypothesis that BEE-1-like and MEF-2 binding activities may also control the expression of other muscle genes is presented.

MATERIALS AND METHODS

Cell cultures and media. The mouse myogenic cell line C2C12 (5) was a gift from S. Alemà (Istituto di Biologia Cellulare, Consiglio Nazionale delle Ricerche, Rome, Italy). Proliferating myoblasts were cultured in a growth factor-rich medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories) supplemented with 20% fetal calf serum (FCS) (Gibco Laboratories). Differentiation into myotubes was induced by exposure of subconfluent cultures to a medium containing 5% horse serum (Gibco Laboratories) and 5 μg of insulin (Sigma) per ml for 48 to 72 h. Hep-G2 human hepatoma cells and PAF human fibroblasts, from laboratory stocks, were maintained in DMEM with 10% FCS; GM1500 lymphoblastoid cells were maintained in RPMI 1640 supplemented with 10% FCS. All cells were cultured at 37°C in a 5% CO_2 atmosphere.

Construction of β enolase-CAT expression vectors. For construction of the β enolase-CAT expression vectors, appropriate restriction sites were utilized to insert fragments of the human β enolase gene into the polylinker of the promoterless plasmid pBLCAT-3 (36), 5′ to the bacterial CAT gene. All fragments were inserted in a 5′-to-3′ orientation with respect to the CAT gene. pB5-CAT, pB8-CAT, pB12-CAT, pB6-CAT, pB9-CAT, and pB10-CAT constructs were generated by using genomic fragments spanning from the *Bam*HI (nt −3300), *Xba*I (nt −1867), *Xho*I (nt −1367), *Bgl*II (nt −867), *Bst*EII (nt −345), and *Hin*cII (nt −172) sites, respectively, to the *Pst*I site at nt +706 relative to the transcription initiation site of the gene (see Fig. 2). A 200-bp *Hin*cII-*Sac*I fragment (nt −172 to +30) containing the β enolase promoter was inserted into pBLCAT-3 to produce plasmid pB3-CAT. When necessary, protruding 5′ termini were filled with deoxynucleoside triphosphates by using the Klenow fragment of DNA polymerase I (Boehringer), and protruding 3′-termini were blunt ended with T4 DNA polymerase (Boehringer) according to standard procedures (52). Construct pB3SV-CAT, containing the simian virus 40 (SV40) enhancer, was obtained by inserting a 233-bp *Nco*I-*Pvu*II genomic fragment containing 72- and 21-bp repeats (28) into the unique *Sma*I site of pB3-CAT downstream of the CAT transcription unit (see Fig. 2). Genomic fragments from the first intron were cloned, in both orientations, by blunt-end ligation into the *Sma*I site of plasmid pB3-CAT or pBLCAT-2 (36), containing the β enolase and herpes simplex virus thymidine kinase (TK) promoter, respectively (see Fig. 3 and 4). Construct pB3-5′HH1 was generated by inserting the 134-bp *Hin*fI-*Hin*fI (HH1) fragment with enhancer activity into the *Hin*dIII site of the pB3-CAT plasmid, upstream of the β enolase promoter. Similarly, recombinant plasmids pB3-5′PCR1, pB3-5′AA1m, and pB3-5′BEE1m were obtained by blunt-end ligation into the *Hin*dIII site of the pB3-CAT plasmid of DNA fragments generated by PCR (see Fig. 7). For pB3-5′PCR1, the following pair of oligonucleotide primers, B and M (see Fig. 5), were used: forward, 5′-AGCTGTTCTGAGTGGGGGAGGGGGCTGCGCCTGC-3′; reverse, 5′-GTGCTGGAGCTAAAAATACCCTACA-3′ (complementary to the putative MEF-2 binding site). For pB3-AA1m, oligomer B and oligomer Mmut (5′-GTGCTGGAGCTAAACATACCCTACA), containing a single nucleotide substitution with respect to oligomer M, were used as the forward and reverse primers, respectively. The mutation introduced in the MEF-2 consensus sequence corresponds to the one present in the MEF-2 mut6 previously described (22, 59). To generate pB3-5′BEE1m, oligomer Bmut (5′-AGCTGTTCTGAGTGGGACTCTAGGCTGCGCCTGC-3′), containing six nucleotide substitutions with respect to oligomer B, and oligomer M were used for amplification. PCRs were performed using 100 pmol of each primer and as template 1 ng of the Bluescript (Stratagene) plasmid pVA-11, containing the 134-bp HH1 fragment (see Fig. 3). PCR conditions were as previously described (16). The nucleotide sequences of all constructs obtained by PCR and the mutations introduced were verified by sequence analysis using modified T7 DNA polymerase (Sequenase; U.S. Biochemicals).

Transfection and CAT assays. Cells were transfected by the calcium phosphate method (23) according to standard procedures. For C2C12 cells, approximately 24 h before transfection, 1.5×10^5 to 2×10^5 cells were plated on 5.8-cm-diameter dishes in DMEM with 20% FCS (growth medium). Calcium phosphate precipitates containing 10 μg of recombinant CAT plasmid and 2 μg of the β-galactosidase expression plasmid pON1 (54), used as an internal control, were incubated with the cells for 16 h, and then cultures were transferred to growth medium. Undifferentiated cultures (myoblasts) were generally harvested 24 h later, and at the same time a parallel set of cultures were exposed to DMEM with 5% horse serum and 5 μg of insulin per ml (differentiation medium) for at least 48 h. All transfections were performed with multiple sets of cultures with at least two different DNA preparations for each plasmid. Cell lysates were prepared by three cycles of freeze-thawing in 0.25 M Tris-HCl, pH 7.5. Lysates were assayed for β-galactosidase activity (54), normalized, and then assayed for CAT activity by the method of Gorman et al. (21) with 0.1 μCi of [^{14}C]chloramphenicol (Amersham). Acetylated and unacetylated forms of chloramphenicol were separated by thin-layer chromatography and visualized by autoradiography. The extent of conversion of substrate to products was quantitated by excising labeled spots from thin-layer plates and subjecting them to liquid scintillation counting.

Preparation of nuclear extracts and in vitro transcription and translation. Nuclear extracts were prepared as described by Dignam et al. (13) and modified by Gosset et al. (22). Protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride [PMSF] and 1.5 μg each of leupeptin, aprotinin, and pepstatin A per ml) were added to all buffers. Nuclear extracts were dialyzed against 500 volumes of 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9)–40 mM KCl–1 mM EDTA–0.5 mM dithiothreitol–0.5 mM PMSF–20% glycerol for 6 h at 4°C. In vitro transcription was carried out with 2 μg of linearized MEF-2/pGEM plasmid (59) (a generous gift of B. Nadal-Ginard, Harvard Medical School, Boston, Mass.) by using an mRNA capping kit (Stratagene). In vitro translation was performed with a commercially available kit according to the instructions of the manufacturer (Promega). Nuclear extracts and translation mixtures were kept frozen in small aliquots until used. Protein concentration was determined by the Bradford method with a Bio-Rad kit.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed by incubating end-labeled probes (0.1 to 0.5 ng, about 10,000 to 50,000 cpm) with either 2 μg of poly(dI-dC) (Pharmacia) and 5 μg of nuclear protein extract, unless otherwise stated, or 0.5 μg of poly(dI-dC) and 7 μl of translation mixture, containing in vitro-translated proteins, in a total volume of 20 μl. The binding reaction mixtures contained 15 mM HEPES (pH 7.9)–40 mM KCl–1 mM EDTA–0.5 mM dithiothreitol–5 mM $MgCl_2$–10% glycerol; in some experiments, $MgCl_2$ was replaced by 5 mM spermidine (Sigma). The binding reaction mixtures were incubated for 10 min at 4°C before the probe was added, and the mixtures were further incubated for 20 min on ice, before being loaded onto a 5% polyacrylamide gel (20:1 acrylamide-bisacrylamide). Electrophoresis was carried out at 8 V/cm for 3 to 3.5 h at 4°C in 25 mM TBE (25 mM Tris [pH 8.3], 20 mM boric acid, 0.5 mM EDTA). After electrophoresis, gels were vacuum dried and exposed to Kodak X-AR film. For antibody interference, EMSAs were performed under the conditions described above except for the addition to the reaction mixture of 1 μl of anti-RSRFC4 serum (a generous gift of R. Treisman, Imperial Cancer Research Fund, London, United Kingdom) or preimmune rabbit serum. For competition in EMSAs, 25- to 800-fold molar excesses, with respect to the labeled probe, of unlabeled specific and nonspecific competitor DNAs were included in the binding reaction mixtures. The following double-stranded oligonucleotides were synthesized and used in the EMSAs: β-ENO/MEF-2 (5′-TGTAGGGTATTTTTAGCTCCAGCAC-3′), MCK/MEF-2 (5′-AAGCTCGCTCTAAAAATAACCCTGCCCTGGT-3′), MCK/MEF-1 (5′-CCCCAACACCTGCTGCCTGAGCCTC-3′), β-ENO/G-rich or CTCCC box (5′-AGCTGTTCTGAGTGGGGGAGGGGGCTGCGCCTGC-3′), β-ENO/mutG-rich (5′-AGCTGTTCTGAGTGGGACTCTAGGCTGCGCCTGC-3′), MCK/CACCC-box (5′-TCACCCCCACCCCGGTGCA-3′), cTnC/CACCC-box (5′-TAACACTGCCCCACCCCCTGCAG-3′), myoglobin/CACCC-box (5′-CGCACAACCACCCCACCCCCTGTGG-3′), β-globin/CACCC-box (5′-TAGAGCCACACCCTGGTAA-3′), H4-histone/CTCCC-box (5′-GATTTCCCCTCCCCCACCGG-3′), and Sp1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′).

DNase I footprint analysis. DNase I protection assays were performed using the 134-bp HH1 fragment as a probe (see Fig. 5). End-labeled HH1 fragment was obtained by cleavage of the Bluescript recombinant plasmid pVA-11 with *Eco*RI and *Xba*I restriction enzymes in order to label the coding and noncoding strand, respectively. After treatment with alkaline phosphate, the 5′ ends were labeled with [γ-^{32}P]ATP, and after the digestion with the appropriate enzyme, each labeled fragment was purified by polyacrylamide gel electrophoresis and electroelution. Binding reactions were carried out as described above with the exception that 75 to 100 μg of nuclear extract was used. DNA-protein complexes were treated with 0.2 to 0.8 μg of DNase I (Boehringer) for 1.5 min at room temperature and then analyzed by electrophoresis through an 8% polyacryl-

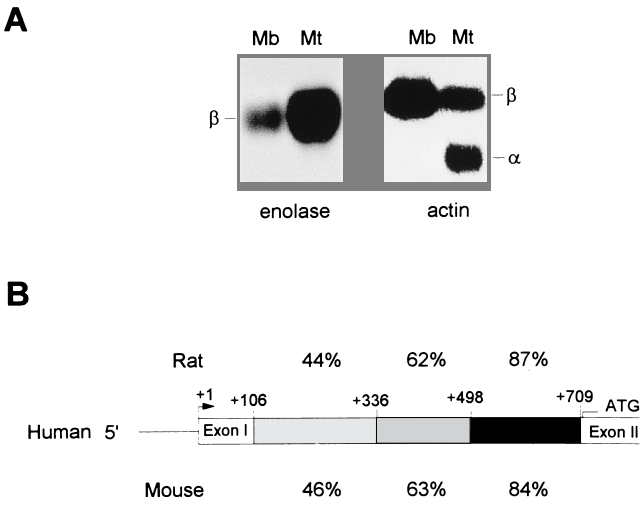


FIG. 1. (A) Expression of β enolase mRNA in mouse C2C12 myogenic cells. Total RNA was isolated from C2C12 myoblasts (Mb) and differentiated myotubes (Mt) as described by Chomczynski and Sacchi (10). A 10-μg aliquot of RNA was electrophoresed, transferred to a nylon membrane, and hybridized with ^{32}P-labeled mouse β enolase and chicken actin probes as previously described (2). The same filter was washed and rehybridized. The positions of β enolase (β) and actin (α and β) mRNAs are indicated on the left and right sides, respectively. (B) Schematic representation of nucleotide sequence similarity in the first intron of human, mouse, and rat β enolase genes. A diagram of the pertinent region of the human β enolase gene is shown. The first noncoding exon (Exon I) and the second exon (Exon II), with the initiation codon ATG, are indicated. Nucleotide positions are numbered relative to the transcriptional start site (arrow). First-intron regions of the human gene that show different percentages of similarity with the homologous regions of the rat (top) and mouse (bottom) gene are indicated (shaded boxes). Sequence alignment and maximum homology were obtained by using the HBIO DNAsis software package (Pharmacia).

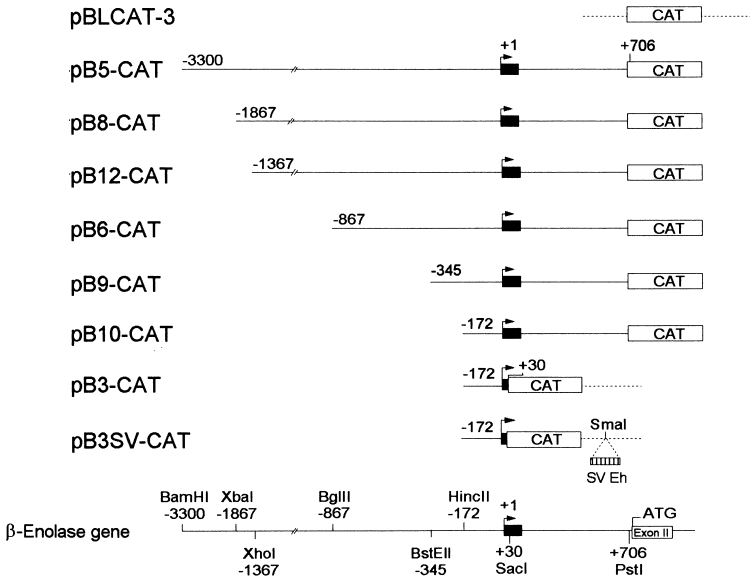
amide–7 M urea sequencing gel. Maxam-Gilbert A+G reactions were performed with the end-labeled probes, and the reaction products were electrophoresed on the same gel to determine the sequences that were protected from DNase I digestion.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers for the human, rat, and mouse β enolase genes in the EMBL data bank are X56832, X57774, and X61600, respectively.

RESULTS

Characterization of *cis*-acting regulatory elements that control β enolase gene expression. The isolation of genomic clones containing the human β enolase gene, nucleotide sequence, exon-intron boundaries, and localization of the transcription start site have been previously described (20). To identify the *cis*-acting sequences that regulate transcription of the human β enolase gene, a series of transient-transfection assays was performed. The expression of β enolase-CAT chimeric constructs was tested in the established cell line C2C12, derived from satellite skeletal muscle cells of adult mice (5). The pattern of β enolase mRNA expression in C2C12 cells is shown in Fig. 1A. When C2C12 myoblasts are maintained at subconfluent density in growth medium, they proliferate and express low levels of β enolase mRNA, while no skeletal α-actin mRNA is detectable. β enolase mRNA increases after exposure of confluent myoblasts to differentiation medium and reaches steady-state levels within 48 to 72 h after induction, when α actin is already expressed (Fig. 1A). As in C2.7 cells (30) and primary cultures of mouse fetal myoblasts (2), following differentiation β enolase mRNA is induced at least 10-fold, indicating that C2C12 cells represent a suitable system with which to study the tissue-specific and differentiation-controlled expression of the gene. CAT constructs including 5′ upstream sequences of the β enolase gene from nt −3300, relative to the transcription start site, and progressive 5′ deletions up to nt −345, all ending at nt +30 (see Fig. 2), were relatively inactive in both proliferating and differentiated C2C12 cells (data not shown). These results indicate that these sequences are not sufficient to promote expression and that additional transcriptional regulatory element(s) located upstream of position −3300 or intragenic sequences might be required for the expression of the human β enolase gene in C2C12 cells. A comparison of the human β enolase gene nucleotide sequence (20) with the sequences of the homologous mouse (EMBL Databank accession no. X61600) and rat (51) genes revealed a remarkable percentage of sequence similarity in the first intron of the three genes. There is a homology gradient from the 5′ to the 3′ end of the intron that reaches almost 90% similarity in the 3′-proximal portion (Fig. 1B). This observation suggested that conserved sequences required for transcriptional activation of β enolase genes might be present in the first intron. To investigate the role of these DNA sequences in controlling expression of the gene, another series of β enolase-CAT chimeric constructs, all ending at nt +706 and including the first intron of the gene, were generated and tested. The constructs containing 5′ upstream sequences between nt −3300 and nt −1367 showed a low level of activity; only a two- to fourfold increase in transcription of the CAT reporter gene, compared with that of the promoterless pBLCAT-3 plasmid, was detected in both C2C12 myoblasts and myotubes (Fig. 2, pB5-CAT, pB8-CAT, and pB12-CAT). Further deletions from nt −1367 to nt −867 and to nt −345 resulted in a seven- to eightfold increase of CAT activity in myotubes but not in myoblasts (Fig. 2, pB6-CAT and pB9-CAT). Deletion of upstream sequences up to nt −172 resulted in a further threefold increase of CAT activity in both myoblasts and myotubes (Fig. 2, pB10-CAT). None of the constructs tested was active in human hepatoma Hep-G2 cells (Fig. 2). Low levels of CAT activity were detected in myoblasts and myotubes when a construct containing sequence upstream from nt −172 and lacking the first-intron sequence was used in transfection assays (Fig. 2, pB3-CAT), indicating that sequence between nt −172 and +30 may represent the basal promoter region of the gene. To investigate whether the basal promoter region itself is subject to muscle-specific regulation, sequences from the promiscuous SV40 enhancer were cloned into the pB3-CAT construct, downstream of the CAT transcription unit. This new construct was found to be expressed at the same level in C2C12 myoblasts and myotubes as well as in Hep-G2 cells (Fig. 2, pB3SV-CAT).

Taken together, the results of the CAT assays indicated that several distinct regulatory regions are present in the human β enolase gene: an upstream region, located between nt −1367 and −867, that seems to have inhibitory effects on transcription only in myotubes (Fig. 2, compare pB12-CAT and pB6-CAT); a second inhibitory element, contained in the region between nt −345 and −172, that affects transcription in both myoblasts and myotubes (Fig. 2, compare pB9-CAT and pB10-CAT); a basal promoter region located between nt −172 and +30 that is relatively inactive on its own but is able to promote constitutive transcription when associated with a promiscuous enhancer (Fig. 2, compare pB3-CAT and pB3SV-CAT); and, finally, a positive intronic regulatory element between nt +30 and +706 that is critical for high-level expression. Removal of the positive regulatory element resulted in a >20-fold decrease in expression of CAT in differentiated myotubes and a 6-fold decrease in expression in undifferentiated myoblasts (Fig. 2, compare pB10-CAT and pB3-CAT). Given the prob-



	Relative CAT Activity		
	C2C12		Hep-G2
	Mb	Mt	
pBLCAT-3	1	1	1
pB5-CAT	2.9 ± 0.3	2.5 ± 1.0	0.9 ± 0.2
pB8-CAT	4.8 ± 0.5	3.8 ± 0.9	1.2 ± 0.4
pB12-CAT	3.5 ± 0.6	2.2 ± 0.8	-
pB6-CAT	4.5 ± 0.9	15.0 ± 1.2	0.8 ± 0.3
pB9-CAT	4.1 ± 1.0	16.4 ± 1.4	-
pB10-CAT	12.0 ± 0.6	44.0 ± 6.1	-
pB3-CAT	2.0 ± 0.7	2.0 ± 0.7	0.8 ± 0.3
pB3SV-CAT	10.2 ± 1.5	9.3 ± 2.1	11.4 ± 2.3

FIG. 2. Identification of transcriptional regulatory elements in the human β enolase gene by transient CAT assays. Schematic representations of the promoterless plasmid pBLCAT-3 and the 5′-flanking region of the β enolase gene are shown at the top and bottom, respectively. Restriction sites relevant for the preparation of the deletion constructs are shown, and the nucleotide positions relative to the transcription initiation site (+1) are indicated. The nucleotide positions of the *Bam*HI, *Xba*I, and *Xho*I sites were deduced by restriction analysis. The first untranslated exon is indicated (black box). Names assigned to the β enolase-CAT fusion genes are indicated at the left of the corresponding constructs. Plasmid DNAs were transfected in hepatoma Hep-G2 cells and proliferating C2C12 myoblasts; 24 h after transfection, one set of myogenic cultures was transferred to differentiation medium for 48 h. Transient levels of CAT activity in myoblasts (Mb), myotubes (Mt), and Hep-G2 cells were determined after adjustments for β-galactosidase activity as described in Materials and Methods. To facilitate comparison, CAT activities are expressed in arbitrary units relative to the basal level obtained with the promoterless vector pBLCAT-3 (assigned a value of 1, which corresponds to 0.8 to 1% conversion of [^{14}C]chloramphenicol). The data are averages ± standard deviations of three to six independent experiments. The SV40 enhancer region (SV Eh) was inserted into the unique *Sma*I site of the pB3-CAT construct, downstream of the CAT transcription unit, to generate plasmid pB3SV-CAT (see Materials and Methods for details).

able importance of this intronic region in driving muscle-specific transcription in C2C12 muscle cells, we first concentrated our efforts on the fine analysis of this positive regulatory element.

Identification of an intronic enhancer required for muscle-specific expression of the β enolase gene. The experiments described above defined the β enolase intronic region between nt +30 and +706 as essential for high-level expression in muscle cells but not in hepatocytes. In order to define the boundaries of this enhancer-like sequence within the first intron, a series of constructs was made by insertion of overlapping fragments of this region in the *Sma*I site downstream of the CAT gene in plasmid pB3-CAT, containing the homologous promoter (nt −172 to +30), and was tested by transfection of C2C12 cells. As shown in Fig. 3, almost no variation in CAT expression in comparison with that of the pB3-CAT basic construct was observed in myoblasts transfected with this series of recombinant plasmids, while a severalfold increase in expression was detected in myotubes. Construct pB3-HH1, containing the 134-bp HH1 fragment between nt +504 and +637, retained almost 100% of the CAT activity of construct pB3-Sp1, containing the entire intron (Fig. 3). A basal level of CAT activity was obtained with plasmid pB3-SB1, containing the 5′ half of the intron, and no relevant additive effects were observed with constructs containing sequences flanking the 134-bp HH1 fragment (Fig. 3, compare pB3-BP1, pB3-DP1, and pB3-HH1). Similar results were obtained with constructs containing the DNA fragments inserted in the opposite orientation (data not shown). Further characterization of the sequences promoting muscle-specific expression was obtained by inserting the relevant fragments of the first intron, in both orientations, downstream of the CAT gene driven by the TK gene promoter. Following transfection of C2C12 cells, CAT activity in proliferating myoblasts and differentiated myotubes was determined. The results of these experiments are expressed relative to the CAT activity detected in the cells transfected with the parental, insertless pBLCAT-2 vector (Fig. 4). The CAT activity of the constructs with overlapping intronic fragments containing the 134-bp HH1 sequence was higher in myotubes, irrespective of the orientation of the sequence. As expected, the enhancement effect of this β enolase element on the TK promoter was limited to differentiated C2C12 cells and was not observed in proliferating myoblasts. The β enolase enhancer linked to its homologous promoter was almost twice as active as when linked to the TK promoter. This different behavior in differentiated C2C12 cells may be related to the TK promoter function at this stage of muscle differentiation or may be indicative of positive muscle-specific interactions between homologous enhancer and promoter. However, these results demonstrate that the region from nt +504 to +637 in the first intron of the β enolase gene contains the sequence information required to confer the tissue-specific and differentiation-induced expression of the β enolase gene itself, whether linked to its homologous promoter or a heterologous promoter, irrespective of orientation and position. It is noteworthy that the construct pB3-HH1, containing the enhancer fragment downstream of the CAT reporter, restored not more than 25% of the activity of the construct containing the first

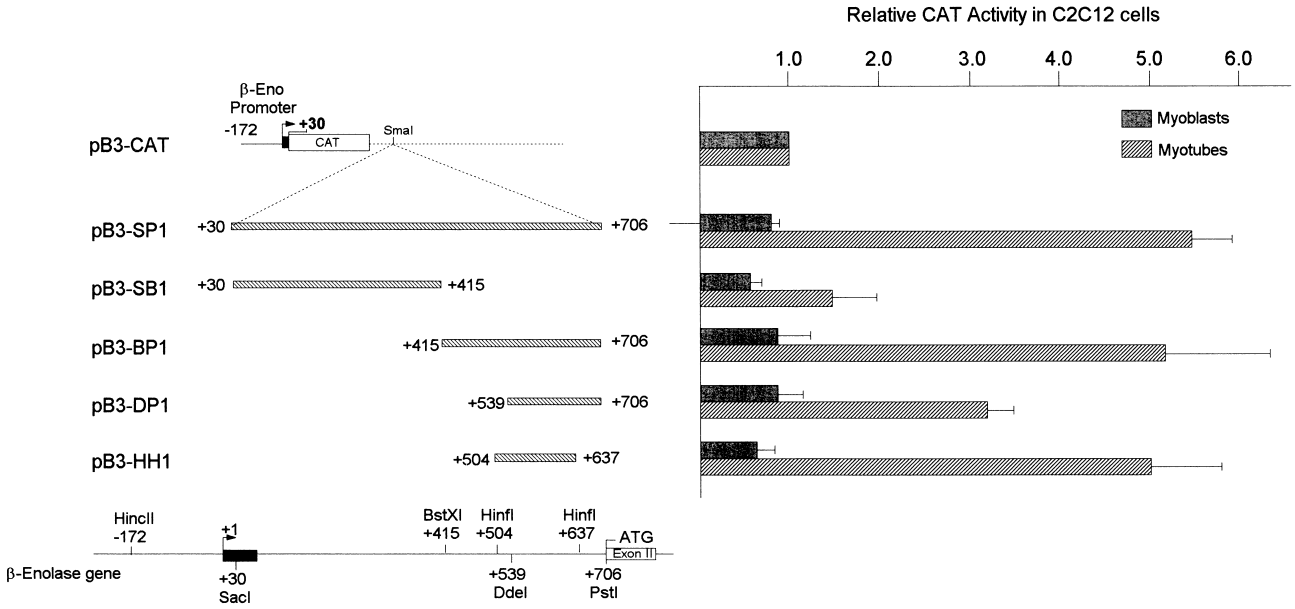


FIG. 3. Identification and localization of a transcriptional enhancer in the first intron of the human β enolase gene. Schematic representations of plasmid pB3-CAT, containing the human β enolase promoter linked to the CAT gene, and the 5′ end of the gene are shown at the top and bottom, respectively. Restriction sites relevant for the construction of the CAT plasmids are shown, and nucleotide positions relative to the transcription initiation site (+1) are indicated. The first untranslated exon is indicated (black box). Overlapping restriction enzyme fragments from the first intron were subcloned into the *Sma*I site downstream of the CAT gene in plasmid pB3-CAT. Names assigned to the β enolase-CAT fusion genes are indicated at the left of the corresponding constructs. Relative CAT activities, corrected for differences in transfection efficiencies, were determinated for extracts from C2C12 myoblasts and myotubes. Values are expressed in arbitrary units relative to the CAT activity produced by the control plasmid pB3-CAT, which was assigned a value of 1. The data are averages of at least three independent experiments, and the error bars represent standard deviations.

intron in its wild-type location (Fig. 2 and 3, compare pB10-CAT and pB3-HH1). To address the question whether the distance between enhancer and promoter could have been responsible for the lower activity, the 134-bp HH1 fragment was inserted into pB3-CAT just upstream of the β enolase basal promoter. The resulting recombinant, pB3-5′HH1, gave a CAT activity comparable to that obtained with pB10-CAT (see Fig. 7), and all subsequent constructs were generated by following this strategy.

Protein binding sites in the human β enolase enhancer. Sequence analysis of the enhancer element defined above revealed the presence of at least two potential binding sites for previously described transcription factors (Fig. 5A). A sequence motif centered at nt +611 corresponds to the MEF-1 site first identified by Buskin and Hauschka (8) in the enhancer of the mouse muscle creatine kinase (MCK) gene. This sequence is a nearly perfect fit (13 of 14 nt) to the MEF-1 consensus sequence. The core sequence of the MEF-1 motif, CACCTG, is a typical E-box recognized by transcription factors of the bHLH family of transcriptional regulators that include many muscle-determining factors such as MyoD, myogenin, MRF-4, and Myf-5 (7, 15). In addition, located between nt +593 and +602, there is an A/T-rich region whose sequence matches the consensus sequence for the MEF-2 transcription factor, whose activity has been found to be important for the transcriptional activation of many muscle-specific genes (22, 59). To identify the *trans*-acting proteins that bind the β enolase enhancer, EMSAs were performed using the 134-bp end-labeled HH1 fragment as a probe. Two specific protein-DNA complexes, indicated as C1 and C2 in Fig. 5B, were retarded in the presence of nuclear extract from both C2C12 myoblasts and myotubes (Fig. 5B, lanes 1 and 2). The specificity of the binding was demonstrated by competition in the EMSA (Fig. 5B, lanes 3 and 6). Lower autoradiographic exposures of these EMSAs revealed that complex C1 is composed of two distinct closely migrating complexes (not shown). Several nonspecific DNA complexes that could not be eliminated by competition with a large excess of unlabeled probe or with nonspecific competitor, such as calf thymus DNA or poly(dA-dT) (data not shown), were reproducibly seen. We observed that C1 and C2 complexes require $MgCl_2$ or spermidine in the binding reaction mixture to be formed on the HH1 DNA fragment. Addition of spermidine to the binding reaction mixture resulted in a reduction of the nonspecific DNA complexes, with no effect on the formation of the C1 and C2 complexes. Specific complexes due to direct binding of MEF-2 proteins or the bHLH family of transcription factors were not detected under the conditions used, as demonstrated by competition in EMSAs with a 100-fold excess of unlabeled oligonucleotides containing the canonical MEF-2 motif or the MEF-1 binding site (Fig. 5B, lanes 4 and 5) derived from the mouse MCK enhancer sequence. These results suggested that nuclear proteins other than MEF-2 and bHLH factors were directly responsible for the formation of specific complexes C1 and C2 on the HH1 fragment. To identify the sequences involved in the formation of these complexes, DNase I footprint analyses were carried out with the radiolabeled HH1 fragment and nuclear extracts from proliferating (myoblast) and differentiated (myotube) C2C12 cells (Fig. 6A). The only DNase I-protected region which was clearly visible as a footprint with both extracts corresponds to a 26-bp G-rich sequence located between nt +537 and +562 in the enhancer (Fig. 5A). To assess the number and specificities of the nuclear proteins that bind to this G-rich region of the β enolase enhancer, a 34-bp double-stranded synthetic oligonucleotide containing the region identified by DNase I footprinting was used as a competitor in an EMSA performed with the entire HH1 fragment as a probe. As shown in Fig. 6B, the 34-bp oligonucleotide containing the

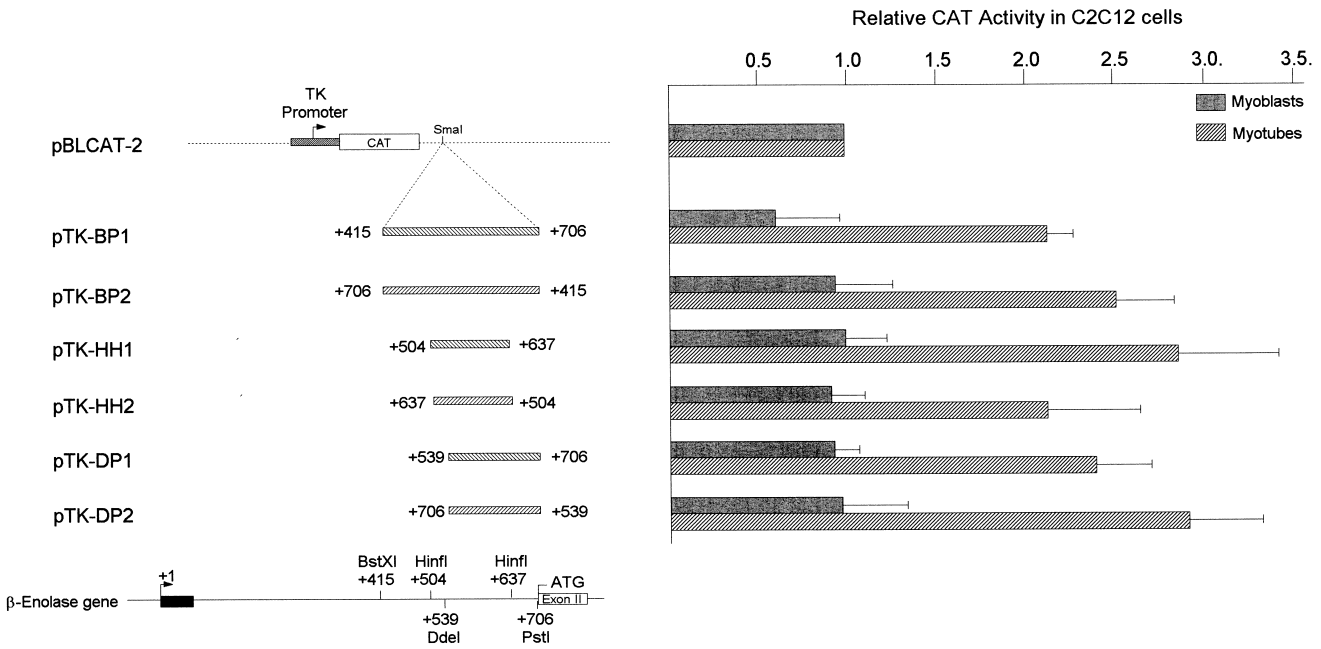


FIG. 4. Analysis of the β enolase first-intron enhancer. Schematic representations of plasmid pBLCAT-2, containing the TK promoter linked to the CAT gene, and the 5′ end of the human β enolase gene are shown at the top and bottom, respectively. Restriction sites relevant for the construction of the CAT plasmids are shown, and nucleotide positions relative to the transcription initiation site (+1) are indicated. Overlapping restriction enzyme fragments from the first intron were subcloned into the *Sma*I site downstream of the CAT gene in plasmid pBLCAT-2. Names assigned to the β enolase-CAT fusion genes are indicated at the left of the corresponding constructs. Transient levels of CAT activity, corrected for differences in transfection efficiencies, were determined for C2C12 myoblasts and myotubes and expressed in arbitrary units relative to the CAT activity produced by the control plasmid pBLCAT-2, which was assigned a value of 1. The data are averages of at least three independent experiments, and the error bars represent standard deviations.

G-rich box was able to compete for the formation of both specific DNA-nuclear protein complexes C1 and C2 (Fig. 6B, lanes 2 to 4). An oligonucleotide with a mutated G-rich box did not affect the formation of the complexes (Fig. 6B, lanes 5 to 7). Taken together, these data define a novel nuclear protein binding site, which we have designated BEE-1, which interacts with at least two different proteins expressed in both C2C12 myoblasts and myotubes and in nonmyogenic cells, as demonstrated by EMSAs with nuclear extracts from different cell types (Fig. 6C). Competition analysis using the 34-bp oligonucleotide containing the G-rich box confirmed the specificity of the binding observed with nuclear extracts from nonmyogenic cells (data not shown).

Functional analysis of the human β enolase muscle-specific enhancer. The identification of a G-rich binding site or BEE-1 in the β enolase enhancer able to interact specifically with at least two apparently ubiquitous nuclear proteins was in conflict with the results obtained by transient-transfection experiments, which showed high levels of CAT expression with constructs containing this region only in differentiated muscle cells. Therefore, it was of interest to determine whether MEF-2 and MEF-1 sites present in the HH1 fragment with enhancer activity played a regulatory role in β enolase gene expression in C2C12 cells. To address this question, the effects of deletions or mutations of BEE-1, MEF-2, and MEF-1 binding sites on CAT expression were investigated. Deleted and mutated constructs were assayed by transfection in proliferating C2C12 cells, and CAT activity was measured after differentiation into myotubes. Deletion of the region containing the MEF-1 site had no effects on the transcriptional activity of the pB3-5′PCR1 construct in comparison with that of the pB3-5′HH1 construct (Fig. 7), indicating that this site is not required for the transcriptional activity of the enhancer.

Constructs containing mutations within the MEF-2 or the BEE-1 binding site gave levels of CAT activity just above the level obtained with the pB3-CAT enhancerless construct (Fig. 7, pB3-5′AA1m and pB3-5′BEE1m), indicating that both BEE-1 and MEF-2 sites contribute to the activity of the β enolase enhancer and suggesting a functional cooperation between the transcription factors involved.

MEF-2 proteins bind the A/T-rich site present in the β enolase enhancer. The results of the functional analysis demonstrate the involvement of the MEF-2 site in the transcriptional activation of the β enolase gene in muscle cells. These findings are in contrast with the results of the EMSA analysis, in which no specific binding related to MEF-2 was detected (Fig. 5B). To clarify this point, we decided to further investigate whether the MEF-2 site present in the β enolase enhancer was able to bind the factor in vitro. A 25-bp radiolabeled synthetic oligonucleotide containing the putative MEF-2 binding site was used as probe in an EMSA. As shown in Fig. 8A, an abundant DNA-protein complex occurred with nuclear extracts from C2C12 myotubes, but it was barely detectable in myoblasts. The complex was specific, since oligonucleotides containing the functional β enolase MEF-2 (self) or the MCK MEF-2 competitively blocked, at comparable molar excesses, the formation of the complex, while an unrelated oligonucleotide could not (Fig. 8A). Supershift experiments with anti-RSRFC4 antibodies, which recognize the COOH terminus of MEF-2A/RSRFC4 gene products (50, 59), were performed to confirm the identities of the proteins complexed with the β enolase MEF-2 site. As shown in Fig. 8B, the formation of the complex obtained with myotubes nuclear extract was specifically prevented by addition of anti-RSRFC4 antibodies, while the addition of a preimmune serum did not affect the formation of the complex; conversely, the binding was inhibited by

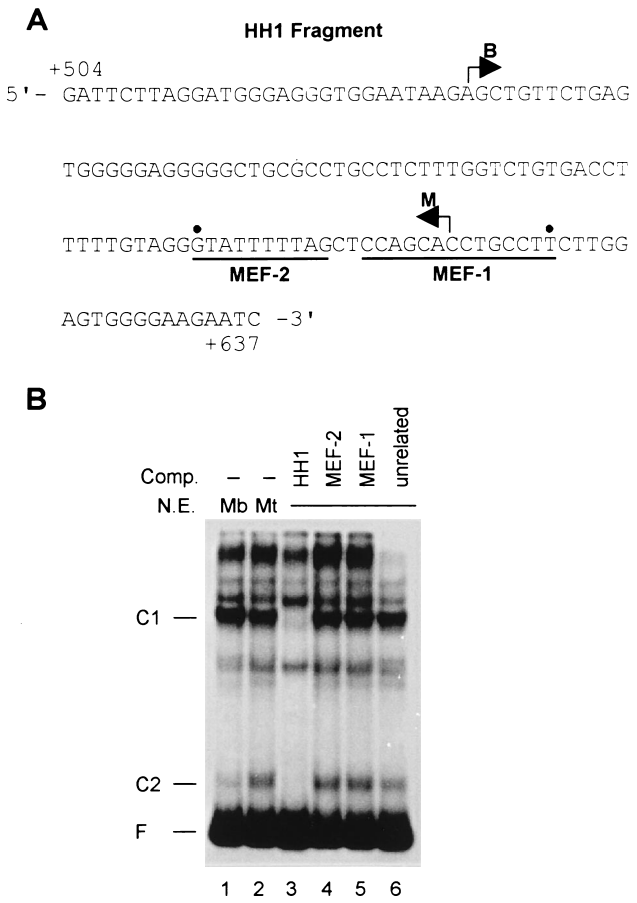


FIG. 5. Identification of DNA binding activities in the β enolase enhancer. (A) Nucleotide sequence of the intronic enhancer region of the β enolase gene. The 134-bp HH1 fragment spans nt +504 to +637 relative to the transcription start site of the gene. MEF-2 and MEF-1 binding sites (underlined), nucleotides differing from the reported consensus sequences (22, 59) (dots), and the first β enolase gene nucleotide included within each primer (B and M) used to generate subregions by PCR (see Materials and Methods) are indicated. (B) Specific DNA-protein interactions in the human β-enolase intronic enhancer. EMSAs were performed with the ^{32}P-labeled HH1 fragment and nuclear extract (N.E.) from C2C12 myoblasts (Mb; lane 1) or myotubes (Mt; lanes 2 to 6). For competition in the EMSA, 100-fold molar excesses of unlabeled HH1 fragment (lane 3), MCK/MEF-2 oligonucleotide (lane 4), MCK/MEF-1 oligonucleotide (lane 5), and an unrelated 200-bp DNA fragment (lane 6) were added to the binding reaction mixtures. C1 and C2, positions of the shifted probe due to specific complexes; F, position of the free probe; Comp., competitor.

addition of an excess of unlabeled oligonucleotide (Fig. 8B, lane 4). These results indicate that the β enolase MEF-2 binding site contained in the muscle-specific enhancer interacts with MEF-2A gene product(s) or an immunological related factor. In addition, in vitro-translated MEF-2 proteins (59) were able to bind the 134-bp HH1 fragment, and the formation of the complex was inhibited to the same extent by oligonucleotides containing the β enolase MEF-2 or the MCK/MEF-2 binding site (Fig. 8C, lanes 3 and 4 and lanes 5 and 6, respectively). The identity of the protein in the complex was further confirmed by addition of anti-RSRFC4 or preimmune serum (Fig. 8C, lanes 9 and 10, respectively). Therefore, the inability to identify by EMSAs HH1-MEF-2-specific complexes in crude cell extracts may be due to the lesser abundance of MEF-2 protein(s) compared with the G-rich binding protein(s) and/or to the presence of nonspecific DNA-binding complexes that comigrate with the MEF-2-specific complex in our assays or, in addition, to the fact that MEF-2 proteins do not favorably interact in vitro with a such large fragment.

Relation between β enolase BEE-1 binding site and homologous C(A/T)CCC sites. The sequence of the BEE-1 binding site is similar to the consensus sequence for the ubiquitous transcription factor Sp1 (26) and resembles a CACCC box present in the transcriptional regulatory regions of many muscle and nonmuscle genes. To assess the relationships between nuclear proteins that bind the human β enolase first-intron enhancer and other factors recognizing consensus sequences similar to the BEE-1 element, a 34-bp radiolabeled synthetic oligonucleotide containing the G-rich box, indicated as CT CCC according to the sequence of the noncoding strand, was used in an EMSA with nuclear extract from C2C12 myotubes. The β enolase CTCCC box oligonucleotide yielded one major protein complex and two minor, faster-migrating complexes (Fig. 9B, lane 1). Competition analyses were performed using unlabeled oligonucleotides (Fig. 9A) corresponding to the CA CCC boxes of muscle genes such as those encoding MCK (22, 25), cardiac troponin C (48), and myoglobin (3), as well as those of nonmuscle genes such as human β-globin (38) and human histone H4 (12) genes. The experiments demonstrated differences in the specificity of the binding. The MCK and β-globin CACCC boxes did not compete for the formation of the three complexes (Fig. 9B, lanes 4 and 5 and lanes 12 and 13, respectively); cardiac troponin C and myoglobin CACCC boxes partially competed for formation of all three complexes only at high molar excesses (Fig. 9B, lanes 6 and 7 and lanes 14 and 15, respectively), and the histone H4 CTCCC box competed as successfully as the β enolase box (Fig. 9, lanes 17 and 18 and lanes 2 and 3, respectively). Of note, an unlabeled oligonucleotide containing an Sp1 binding site did not compete for formation of the complexes (Fig. 9B, lanes 8 and 9), suggesting that Sp1 factor is not involved in the binding. This is of particular importance since the sequence motif GGGAGGG, present in the BEE-1 site (Fig. 5A), has been shown to be an Sp1 binding site (32). These results were further confirmed by an EMSA in which a 200-fold excess of an unlabeled DNA fragment from the SV40 early promoter containing six functional Sp1 binding sites (26) was not able to compete for the formation of C1 or C2 complexes (Fig. 5) obtained with the HH1 fragment (data not shown). It has been reported that Sp1 factor (26), Sp1-related proteins (29), and the H4TF1 factor (11), which binds the histone H4 CTCCC box, require zinc for their specific interaction with DNA. We observed that the formation of C1 and C2 complexes is dependent on the addition of bivalent ions but not preferentially of zinc in binding assays performed in the presence of 1,10-phenantroline or EDTA as a chelating agent (data not shown). All together, these results indicate that the β enolase G-rich-box-binding protein(s) is not related to Sp1, Sp1-like factor(s), or H4TF1 factor and might be only partially related to other previously identified C(A/T)CCC box binding activities.

DISCUSSION

The major finding of this study is that the expression of the human β enolase gene in skeletal muscle is regulated by a tissue-specific transcriptional enhancer located in the first intron of the gene between nt +504 and +637 relative to the transcription start site. The nucleotide sequence of this 134-bp enhancer region shows almost 90% similarity with the homologous regions of rat and mouse β enolase genes, suggesting that its functional role might have been conserved through evolution. Although this intronic region has an essential role in the muscle-specific expression of the human β enolase gene,

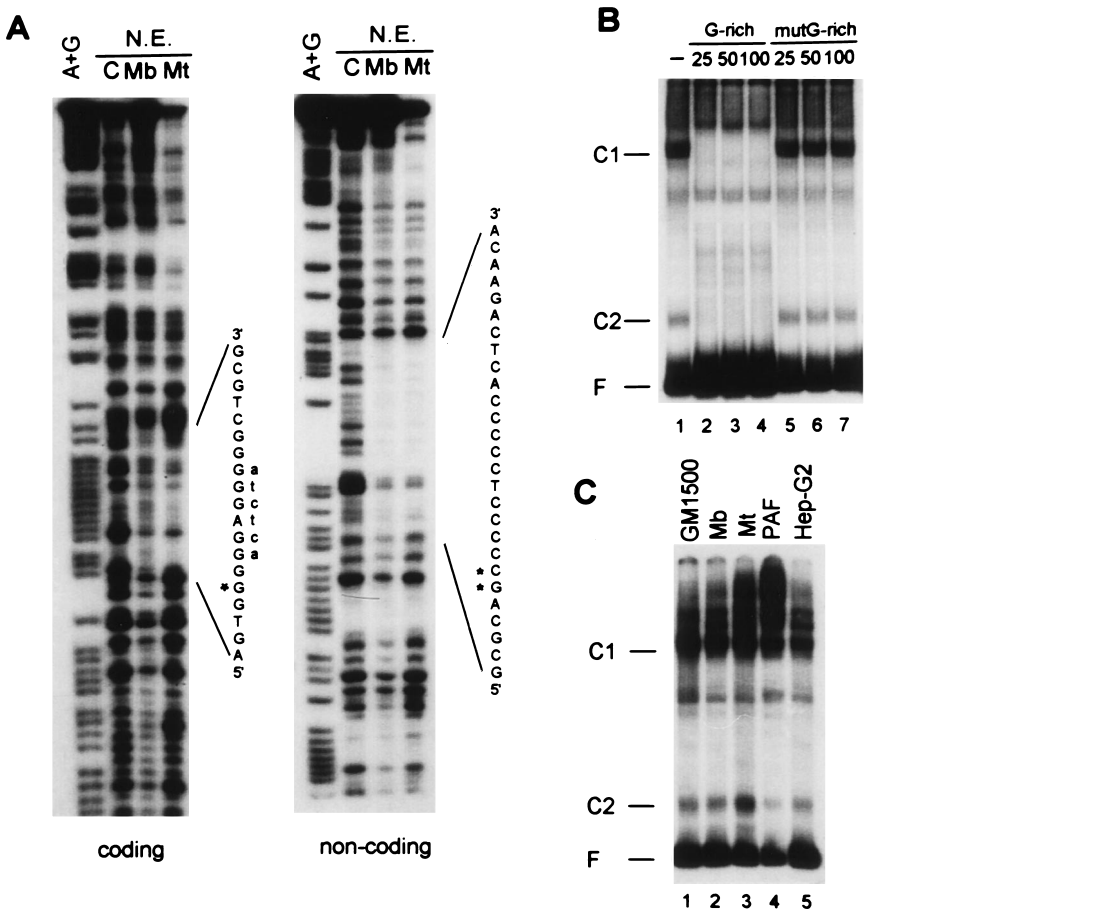


FIG. 6. DNase I footprint and EMSA analyses of the human β enolase first-intron enhancer. (A) DNase I footprint analyses. The ^{32}P-labeled 134-bp HH1 fragment was incubated in the absence (C) or presence of C2C12 myoblast (Mb) or myotube (Mt) nuclear extract (N.E.) before partial digestion with 0.8 μg of DNase I for 1.5 min at room temperature and analyzed by 8% polyacrylamide–7 M urea gel electrophoresis (only the relevant region of the 134-bp sequence is shown). Both coding and noncoding strands are shown. Standard Maxam-Gilbert purine (A+G) sequencing reaction mixtures of the same fragments were run in parallel. Protected sequences are shown on the right, and DNase I-hypersensitive sites (asterisks) and nucleotide substitutions used to produce a mutated binding site (mutG-rich in panel B) (lowercase letters) are indicated. (B) Competition analysis by EMSA demonstrates the specificity of the binding. The HH1 probe was incubated with nuclear extracts from C2C12 myotubes (3 μg) in the presence of no competitor (lane 1) or 25-, 50-, and 100-fold molar excesses of a 34-bp oligonucleotide containing the wild-type G-rich box identified by DNase I footprinting (G-rich) or a mutated binding site (mutG-rich) and analyzed by EMSA. (C) Cell type specificity of nuclear protein binding. The HH1 probe was incubated with nuclear extracts from C2C12 myoblasts and myotubes, GM1500 lymphoblastoid cells, PAF fibroblasts, and Hep-G2 hepatoma cells. C1 and C2 binding activities are indicated. F, free probe.

other control elements that reside elsewhere in the gene may have important regulatory functions. From the present study, two putative negative regulatory elements in the upstream region of the gene have been identified, one located between nt −1367 and −867 that inhibits transcription only in differentiated myotubes and the other between nt −345 and −172 that affects transcription in both myoblasts and myotubes. In addition, a basal promoter region has been identified between nt −173 and +30.

The β enolase enhancer contains a sequence almost identical to that of the MEF-1 binding site first identified by Buskin and Hauschka (8) in the enhancer of the mouse MCK gene. The core sequence of the MEF-1 motif, CACCTG, is a typical E-box recognized by transcription factors of the bHLH family of transcriptional regulators, which include many muscle-determining factors such as MyoD, myogenin, MRF-4, and Myf-5 (7, 15). Results obtained by transient-transfection assays with C2C12 myotubes and constructs containing the β enolase enhancer lacking the MEF-1 site clearly indicated that the binding site for the myogenic bHLH proteins (E-box motif) is not needed for the activity of the β enolase enhancer.

In the β enolase enhancer there is also present an A/T-rich box whose sequence matches the consensus of the MEF-2 binding site found in many muscle-specific promoters and enhancers (22). Factors responsible for MEF-2 activity in vivo have recently been cloned and were found to be the previously characterized non-muscle-related SRF (RSRF) proteins, all containing a MADS box domain (50, 59). Four different genes transcribed in a wide range of cell types and giving rise to several differentially spliced isoforms of MEF-2 have recently been characterized (6, 39, 40, 59). Products of the MEF-2A/RSRFC4 gene are specific to differentiated skeletal and cardiac muscle cells. Although multimers of the MEF-2 site can activate transcription in muscle cells when linked to the MCK promoter or the TK basal promoter, a single MEF-2 site represents a relatively weak enhancer (22). Transactivation by MEF-2 appears to require cooperation with one or more other factors. Several ubiquitous factors have been reported to support the function of MEF-2 in the muscle-specific enhancers of several genes, for instance, MEF-3 for the rat aldolase A gene (24), MEF-3 and a CACCC-box binding activity for the mouse cardiac troponin C gene (48), myocyte nuclear factor (MNF)

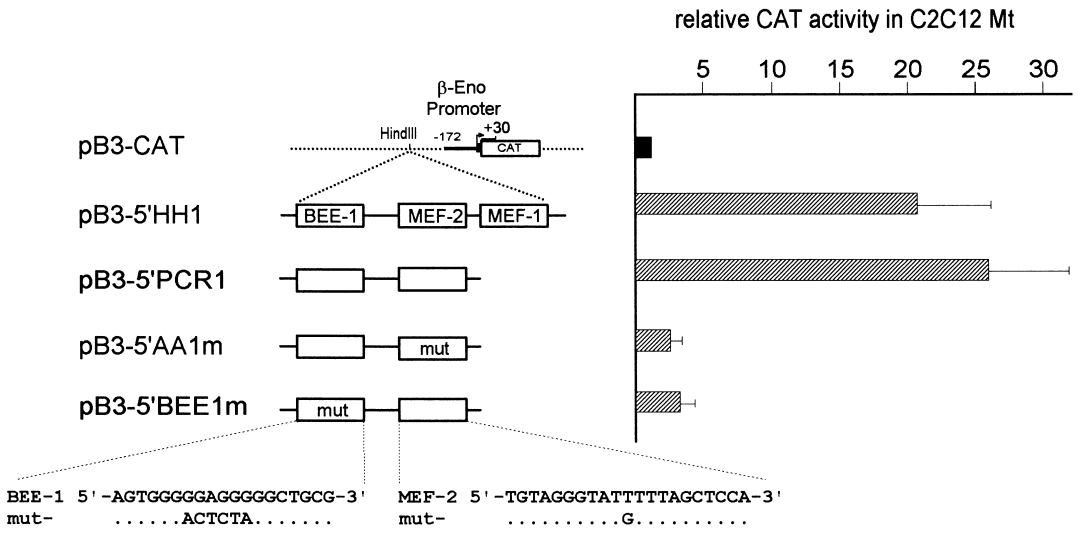


FIG. 7. Functional analysis of the nuclear protein binding sites of the β enolase enhancer. Effects of deletions and mutations of the BEE-1, MEF-2, and MEF-1 protein binding sites on the activity of the β enolase enhancer were determined by transient-transfection assays. Deleted and mutated fragments were generated by PCR as described in Materials and Methods. Nucleotide sequences of the wild-type BEE-1 nuclear protein binding site determined by DNase I footprint analysis and of the MEF-2 binding site are shown at the bottom. Nucleotide substitutions used to produce mutated binding sites (mut) are shown below the sequence. All fragments were cloned into the *Hin*dIII site upstream of the CAT gene in plasmid pB3-CAT, containing the β enolase basal promoter, and the resulting plasmids were transfected into C2C12 myotubes (Mt). Relative CAT activities, corrected for differences in transfection efficiencies, are shown at the right and are in reference to the activity of the pB3-CAT construct, which was assigned a value of 1. The data are averages of at least three independent experiments, and error bars represent standard deviations.

for the human myoglobin gene (3, 4), HF-1a for the rat cardiac myosin light-chain 2 gene (44), DRF-1 for the human skeletal α-actin gene (42), and NFe for the rat embryonic myosin heavy-chain gene (58). It has been reported that deletion of the MEF-2 binding site from regulatory regions of the mouse muscle MCK gene (22), the rat cardiac myosin light-chain 2 gene (44), the human muscle-specific phosphoglycerate mutase gene (43), and the aldolase A gene (24) results in a dramatic reduction in transcription of the associated reporter genes in differentiated muscle cells, indicating that MEF-2 plays a pivotal role in the transcriptional regulation of these genes.

The A/T-rich MEF-2 motif present in the β enolase en-

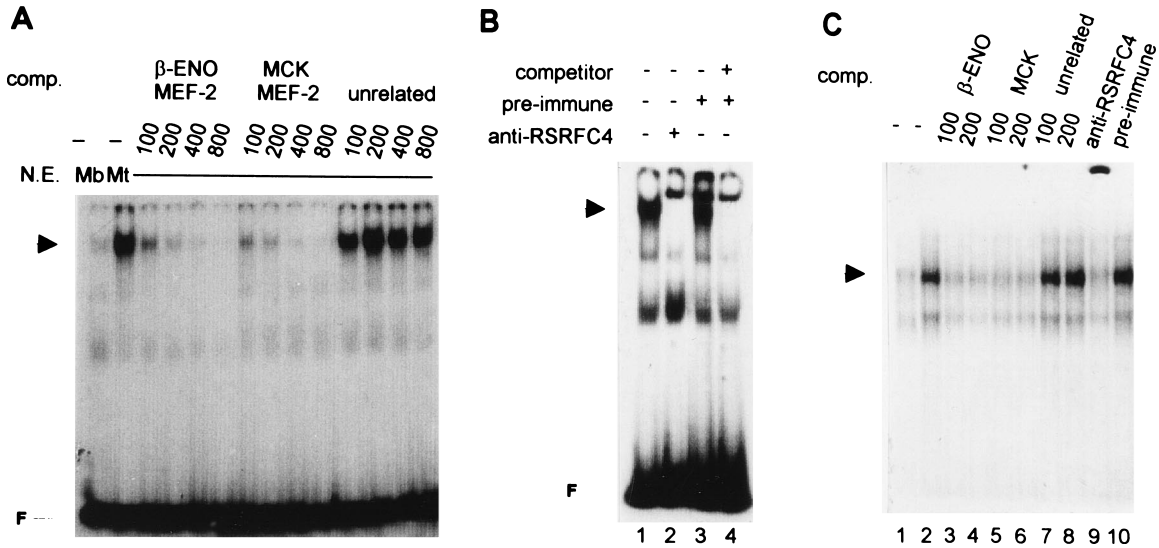


FIG. 8. EMSA analyses of the MEF-2 binding site in the β enolase enhancer. (A) Competition in EMSA demonstrates specificity of the binding. A 25-bp labeled oligonucleotide containing the MEF-2 binding site of the β enolase enhancer was incubated with nuclear extracts (N.E.) from C2C12 myoblasts (Mb) and myotubes (Mt). For competition in the EMSA, a 100-, 200-, 400-, or 800-fold molar excess of unlabeled oligonucleotide containing the MEF-2 binding site from the β enolase enhancer (β-ENO) or from the MCK enhancer (MCK) or an unrelated oligonucleotide was added to the binding reaction mixture. comp., competitor. (B) Effect of MEF-2 antibodies on the β enolase–MEF-2 sequence and protein complex. Nuclear proteins from C2C12 myotubes were incubated with anti-RSRFC4 serum (lane 2) or preimmune serum (lanes 3 and 4). The competitor used is the β enolase MEF-2 sequence (lane 4). A more slowly migrating nonspecific complex is due to the addition of the serum (lanes 2 to 4). (C) The in vitro-translated MEF-2A gene product binds the β enolase enhancer. The labeled 134-bp HH1 fragment was used in EMSAs with reticulocyte lysates programmed with no RNA (lane 1) or in vitro-translated MEF-2A (lanes 2 to 10). For competition in the EMSA, a 100- or 200-fold molar excess of unlabeled oligonucleotide containing the MEF-2 binding site from the β enolase enhancer (β-ENO) or from the MCK enhancer (MCK) or an unrelated oligonucleotide was added to the binding reaction mixture. To test the effect of MEF-2 antibodies on the HH1 fragment and protein complex, the in vitro-translated MEF-2A proteins were incubated with anti-RSRFC4 serum (lane 9) or preimmune serum (lane 10). Arrows, MEF-2 specific complex; F, free probe.

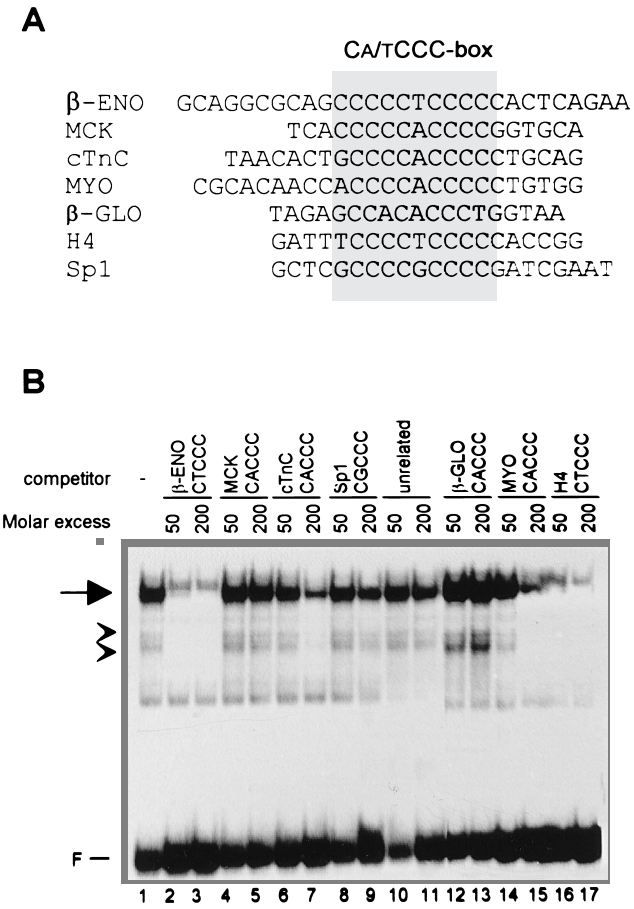


FIG. 9. Relation between β enolase G-rich box and homologous C(A/T)CCC boxes. (A) Sequences of the six oligonucleotides, each containing a C(A/T)CCC box and derived from transcriptional regulatory regions of muscle and nonmuscle genes, that were used as competitors in EMSAs. Sequences for MCK, cardiac troponin C (cTnC), and myoglobin (MYO) muscle genes and for β-globin (β-GLO) and histone H4 (H4) nonmuscle genes are shown. The homologous sequence in the β enolase enhancer (β-ENO) is shown as it appears in the noncoding strand. The consensus sequence for Sp1 transcription factor is also shown. The shaded box indicates a common core sequence. (B) EMSA analysis of the C(A/T)CCC-binding proteins. A labeled synthetic oligonucleotide corresponding to the β enolase CTCCC box was incubated with nuclear extract from C2C12 myotubes, and a 50- or 200-fold excess of the indicated unlabeled competitor oligonucleotide was included in the binding reaction mixture. Specific major and minor complexes (big and small arrows, respectively) and the position of the free probe (F) are indicated.

hancer binds specifically to proteins encoded by the MEF-2A gene, as demonstrated by competition in the EMSA, binding of MEF-2A in vitro-translated products, and interference experiments with anti-MEF-2/RSRFC4 antibodies. Furthermore, we identified in the β enolase enhancer a G-rich region (TGAG TGGGGGAGGGGGCT), located 30 nt upstream of the MEF-2 binding site which interacts with at least two distinct nuclear proteins that appear to be present at relatively comparable levels in muscle and nonmuscle cells. We named this G-rich box BEE-1. Functional analyses by transient transfection of CAT constructs containing the β enolase enhancer with a mutated MEF-2 or BEE-1 binding site in C2C12 myotubes demonstrated that both elements are necessary for muscle-specific expression. The MEF-2 binding site, although essential for β enolase gene expression, is not sufficient for the muscle-specific activity of the enhancer. Mutation of the BEE-1 site abolishes the enhancer activity, also in the presence of a functional MEF-2 binding site. These observations suggest that the BEE-1 site could have a lineage-restricted function, despite the fact that BEE-1 binding activities are observed in both muscle and nonmuscle cells.

Sequences homologous to the BEE-1 site found in the β enolase enhancer, named C(A/T)CCC boxes, are present in the enhancer regions of several skeletal and cardiac muscle-specific genes. In Fig. 10, a sequence comparison of the C(A/T)CCC boxes and MEF-2 binding sites present in many of these genes is shown, along with the distances between the two consensus sequences and the position of the enhancer relative to the transcriptional start site of the gene. In many of these cases, the position in the gene of the C(A/T)CCC box and the MEF-2 site, their core sequences, and their relative positions are highly conserved across species. It has been demonstrated that regulatory sequences display a critical dependence on their relative distance and appropriate alignment on the DNA helix for efficient transcription (31). It is interesting that in the enhancer region of several genes shown in the figure, the spacing between the two *cis*-acting elements is a multiple of a DNA helix turn (10.5 bp), suggesting that a stereospecific alignment between the two elements may be required for transcriptional activation. For a few of these genes, such as the rat MCK gene (25), the human myoglobin gene (3), and the mouse cardiac troponin C gene (48), it has been shown that the C(A/T)CCC box, associated (from 30 to 41 bp) with a functional MEF-2 binding site, has a functional activity. In the enhancer region of other genes, such as the human desmin gene (34), the rat cardiac myosin light-chain 2 gene (44), the rat α-myosin heavy-chain gene (37, 41), the chicken cardiac-slow skeletal alkali myosin light-chain gene (46), the human muscle-specific phosphoglycerate mutase gene (43), the mouse myogenin gene (14), and the mouse skeletal troponin C gene (47), a functional MEF-2 site is associated with a putative C(A/T)CCC binding site. These observations suggest that factors binding to G-rich or C(A/T)CCC boxes, like those binding to the BEE-1/G-rich site in the β enolase enhancer, may play an important role in conjunction with MEF-2 proteins in the transcriptional regulation of many other muscle-specific genes and form a common regulatory pathway for muscle-specific expression. Furthermore, functional G-rich or C(A/T)CCC motifs that appear to act cooperatively with regulatory elements other than MEF-2 binding sites have been identified in the transcriptional regulatory region of the quail fast skeletal troponin I gene (35), the human cardiac α-actin gene (53), and the rat β-myosin heavy-chain gene (56). The G-rich elements of the human cardiac α-actin gene and the rat β-myosin heavy-chain gene bind Sp1 factor in vitro and are required for the expression of the two genes in skeletal muscle (53, 56). We have shown that the ubiquitously expressed proteins that bind the BEE-1 site in the β enolase enhancer are not strictly related to other previously identified G-rich or C(A/T)CCC binding activities, nor to Sp1 and Sp1-like proteins, suggesting that they may represent new members of a family of transcription factors that share a common sequence binding site. At present, only the factor binding the CACCC box within the enhancer region of the myoglobin gene, MNF, has been identified by cDNA cloning and found to be a novel member of the winged-helix family of transcription factors (4).

In conclusion, the data presented here are consistent with a model in which interactions between muscle-specific members of the MEF-2 family of transcription factors and ubiquitous transcription factors, such as the BEE-1 binding activities, are required to regulate the tissue-specific and developmental expression of the β enolase gene in skeletal muscle cells. Furthermore, several muscle-specific genes, including β enolase,

Gene	CA/TCCC box	bp	MEF-2 site	Position
Hu ENO-3	CTCACCCCCTCCCCCGACG	30	TCCCATAAAAATCGAGGT	+541/+607
Mo ENO-3	TCACCCCCCTTCCCTGGTC	33	TCCCATAAAAATCGAGGT	+493/+556
Rat ENO-3	CCCCCCCCCTCCCCTGGTC	33	CCCCATAAAAATCGAGGT	+494/+563
Hu MCK	AACCCCCCCATACCCAGCG	40	CTCAATAAAAATCTCCCG	-804/-727
Mo MCK	CTCACCCCCACCCCGGTGC	36	CCCAATAAAAATCTCGCT	-1135/-1062
Rat MCK	CCCACCCCCACCCCAGTGC	32	CTCAATAAAAATCTCGTT	-1124/-1043
Hu MYO	AACCACCCCACCCCCTGTG	34	TTCGATAAAAATCCCGTG	-225/-173
Mo MYO	CCCCTCCCCACCCCCTACT	33	GTCGATAAAACCCCGTTC	-211/-150
Seal MYO	AACACCCCCACGCCCCTGT	32	TTCGATAAAACCCCGTCC	-220/-149
Hu cTnC	GGCCCACCCACCCAGCTTG	86	CCCGATAAAAATTGCTCC	+1099/+1223
Mo cTnC	CACTGCCCCACCCCCTGCA	41	CTCGATAAAAATTTCCCC	+1033/+1111
Hu Desmin	GTACCCCCCTGCCCCCACA	41	CTCTATAAATACCCGCTC	-980/-904
Ham Desmin	CTGGGCCCCAATCCCCAGA	41	CTCTATAAATACACGCTC	-920/-844
Rat cMLC-2	CCGTACCCCACCCCCGGAG	32	CCCAATAAAAATTGGGGT	-107/-37
Ch cMLC-2	TGTTCTCCCAGCCCCCACC	33	CCCAATAAAAATCGGACC	-105/-31
Rat alpha MHC	CCCATCCCCTCCACCACAC	15	ATCAATAAAAATTAGACT	-339/-289
Ch MLC1c/1s	AAAACCCCCAATCCCTTCT	37	ACCGATAAAATTTCGGTA	-105/-31
Hu PGAM-M	CTGGCCCCCAGCCCCCAGC	21	CCCGATAAAATTCGTGTC	-175/-132
Mo Myogenin	TCCCACCCCACCCCCGTTT	88	ACCGATATAAATAGAGAC	-179/-53
Mo sTnC	GGACTCGTCACCCCTTCTT	71	TCCGATAAAAATCAACCC	+563/+671
Consensus	MCCCCWCCCCY	15-88	YCRATAAAAATY	

FIG. 10. Comparison of the nucleotide sequences of the β enolase transcriptional enhancer protein binding sites with transcriptional regulatory sequence identified in previously characterized skeletal and cardiac muscle genes. The C(A/T)CCC box and MEF-2-containing sequence are aligned irrespective of their real orientation in the regulatory region of each gene, and the distances between the two elements as well as their positions relative to the transcriptional start site are indicated. Hu, human; Mo, mouse; Ham, hamster; Ch, chicken. Genes and corresponding reference numbers are as follows: human β enolase (ENO-3), 20; rat ENO-3, 51; human MCK, 57; mouse MCK, 22; rat MCK, 25; human, mouse, and seal myoglobin (MYO), 3; human and mouse cardiac troponin C (cTnC), 48; human and hamster desmin, 33; rat cardiac myosin light chain 2 (cMLC-2), 44; chicken cMLC-2, 60; rat alpha-myosin heavy chain (MHC), 37; chicken cardiac-slow skeletal alkali myosin light chain (MLC1c/1s), 46; human muscle phosphoglycerate mutase (PGAM-M), 43; mouse myogenin, 14; and mouse skeletal troponin C (sTnC), 47.

are expressed in skeletal muscle cells as well as in cardiac muscle cells. It will be of interest to investigate whether either divergent or overlapping regulatory programs specify the expression of the β enolase gene in cardiac and skeletal muscle. Further studies designed to define and identify the nuclear proteins binding the BEE-1 site and the role they play in regulating the transcription of the β enolase gene are in progress.

ACKNOWLEDGMENTS

This work was supported by grants from Telethon 416, from a CNR bilateral project to A.G., and from CNR target project "Ingegneria Genetica" to S.F.

The excellent technical assistance of Patrizia Rubino is acknowledged. We thank B. Nadal Ginard for kindly providing MEF-2/pGEM plasmid, R. Treisman for anti-RSRFC4 antibodies, and S. Ferrari for the MCK MEF-2 and MEF-1 oligonucleotides. We also thank G. Cossu, S. Ferrari, and G. Spinelli for comments and suggestions during the preparation of the manuscript.

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