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Cis-acting elements responsible for muscle-specific expression of the myosin heavy chain β gene

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ABSTRACT

The 5′ flanking region of the rabbit myosin heavy chain (HC) β gene extending 295 bp upstream from the cap site provides muscle-specific transcriptional activity. In this study, we have identified and functionally characterized cis-acting elements that regulate the muscle-specific expression within this region. By using linker-scanner (LS) mutants between −295 bp and a putative TATA box, we found five distinct positive cis-acting sequences necessary for transcription: element A, the sequences between −276 and −263, which contains a putative M-CAT motif in an inverted orientation; B, the sequences between −207 and −180; C, the sequences between −136 and −127; D, the sequences between −91 and −80; and E, a TATA consensus sequence at −28. The fragment containing both A and B elements dramatically enhanced the expression of the chloramphenicol acetyltransferase (CAT) gene driven by a heterologous promoter in differentiated muscle cells, whereas fragments containing either A or B elements alone had little or no effect in either muscle or nonmuscle cells. Therefore, these two elements appear to act cooperatively in determining a high level of muscle- and stage-specific expression. Unlike the typical enhancer element, this region functions in an orientation-dependent manner. In contrast, the fragment containing C and D elements activates the heterologous promoter in both muscle and nonmuscle cells in an orientation-independent manner.

INTRODUCTION

The myosin HC isoforms are encoded by a multigene family of distinct, but closely related members, the expression of each being controlled in a tissue- and developmental stage-specific manner (1). In mammals, one of the members, the myosin HC β gene, is expressed early during development in skeletal muscles and becomes restricted to slow twitch fibers in adults. The myosin HC β is also a major myosin HC isoform in cardiac muscle. The differential expression of the HC β gene, like that of most of the other muscle-specific genes, is regulated primarily by transcriptional mechanisms (2), indicating that the interaction of the cis-acting regulatory elements with the trans-acting nuclear factors plays an important role in determining the specific pattern of transcriptional regulation.

Multiple positive and negative cis-acting regulatory sequences that confer muscle-specific expression on marker genes have been identified in gene transfer experiments (3,4). Some shared sequence motifs have been noted among regulatory regions from many muscle-specific genes, such as the CArG/CBAR motif in the sarcomeric actin genes (5,6), the MEF-1 or MEF-2 binding sites in the muscle creatine kinase gene (7,8), and the M-CAT motif in the cardiac troponin T gene (9,10). To date, however, no universal cis-acting regulatory elements that are responsible for the expression of muscle-specific genes have yet been identified.

We have previously demonstrated that the region extending 295 bp upstream from the cap site of the rabbit myosin HC β gene is sufficient for both tissue- and stage-specific expression in primary cultures of chicken skeletal muscle cells, and that at least two distinct positive cis-acting elements are involved in the transcription of this gene (11). In this study, we further characterized the cis-acting sequences required for the transcription of the rabbit myosin HC β gene in skeletal muscle cells and identified 3 additional positive cis-acting elements involved in the transcription of this gene. Mutagenesis of any one of these sequences severely disrupted promoter activity, indicating that the myosin HC β gene is regulated by combinatorial interactions between multiple elements. We also showed that two elements are necessary for conferring muscle- and stage-specific activation on heterologous promoter.

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MATERIALS AND METHODS

Cell cultures

Primary skeletal muscle cultures were prepared from the breast muscles of 12-day-old embryonic chickens as previously described (12). The muscles were dissected and minced, and the cells were dissociated with 0.125% trypsin and 0.0125% collagenase. To minimize the number of fibroblasts, we preplated the cells twice for 30 min each on nongelatinized culture dishes (Falcon brand, Becton Dickinson Labware). The cells were placed on gelatin-coated culture plates at a density of 1×10^6 cells/ 60 mm culture dish and grown in basal medium Eagle (GIBCO Laboratories) containing 10% horse serum and 2% chicken embryo extract in 5% CO_2. For some experiments, the cultures were maintained continuously in medium containing 10 μg 5-bromodeoxyuridine (BUdR) for inhibition of muscle differentiation.

Transfections and enzyme assays

DNA transfection was performed by the calcium phosphate precipitation method as described by Wigler *et al.* (13). Primary skeletal myoblasts were transfected with 5 μg of test plasmid and 1 μg of plasmid pCH110 per 60 mm dish 24 h after plating. The pCH110, which contains the *E.coli* β-galactosidase gene driven by the SV40 early promoter, was used as an internal standard for normalization. Four hours after the addition of DNA, the medium was replaced, and the cells were cultured for an additional 72−96 hrs. The transfection experiments were repeated at least three times with independent plasmid preparations.

Cell extracts were prepared by freeze-thawing of the transfected cells three times in 150 μl of 0.25 M Tris-HCl, pH 7.8, followed by centrifugation for 10 min at 4°C. The activity of chloramphenicol acetyltransferase (CAT) in the cell extracts was assayed as described by Gorman *et al.* (14), with acetyl-coenzyme A (Sigma Chemical Co.) and [14C]chloramphenicol (Amersham Corp.). CAT assays were quantitated by excision of spots and counted in a liquid scintillation counter. The activity of β-galactosidase in the cell extracts was assayed according to the method of Miller (15), with o-nitrophenyl-β-D-galactopyranoside used as the substrate (Boehringer Mannheim Biochemicals). Typically, 20−30 μl of cell extracts from primary cultures were used for both CAT and β-galactosidase assays during a 30 min incubation.

Plasmid constructions

Linker-scanner (LS) mutations were constructed as described previously (11). The 727 bp HindIII promoter fragment was subcloned into the linker-cassette vector pBRN/B (16) in both orientations. Both 5′ and 3′ deletions were made with the exonuclease Bal31, followed by digestion with NruI and ligation of the subclone so that the BglII site was placed at the end of the deleted sequences. Deletion end points were determined by sequencing (17), and matching 5′ and 3′ deletions were combined for creation of mutants in which the sequences between the two deletion end points were replaced by the BglII sequence CGAG-ATCTCG. The recombined myosin HC promoter sequences were cloned into $pA_{10}CAT_{3M}$ for transfection. The sequence alterations of all LS mutants were confirmed by DNA sequencing (18).

A plasmid pTKCAT contains the 5′ flanking region (−109 to +56) of the herpes simplex virus thymidine kinase (TK) gene (19), which is linked to the reporter CAT gene in $pA_{10}CAT_{3M}$ (20). Plasmids pMHC-TKCAT contain various fragments of the myosin HC β gene promoter which were isolated from various 3′ deletion mutants with the BglII linker at the 3′ end as described above (See Fig. 3a). The fragments were blunt-ended with the Klenow fragment of DNA polymerase I or T4 DNA polymerase (Pharmacia LKB Biotechnology, Inc.) and ligated into the blunt-ended XbaI site 122 bp upstream of the initiation site of the TK gene in pTKCAT. The orientation of each inserted fragment with respect to the TK promoter was determined by restriction mapping. Insertion mutants were generated by ligation of the 105, 207, or 317 bp Sau3A-digested fragments of pBR322 into the BglII site of the mutant LS6 (see Fig. 1a and b).

All recombinant-DNA methods were performed according to standard protocols (21). Plasmid DNA was prepared by alkaline lysis and banded by cesium chloride-ethidium bromide gradient centrifugation.

RNase protection experiments

Total RNA was isolated from transfected primary cultures as described by Chirgwin *et al.* (22). Labeled antisense RNA probe was generated from a plasmid, pBS-TKCAT, containing 152 bp of the CAT gene and 165 bp of the 5′ flanking sequence (−109 to +56) of the TK gene. The pBS-TKCAT was constructed by insertion of the BamHI-PvuII fragment from the plasmid pTKCAT between the BamHI and SmaI sites of Bluescribe M13+ (Stratagene). This plasmid was linearized with BamHI, and a 347-nucleotide antisense ^{32}P-labeled probe was transcribed with T7 RNA polymerase, as described by the manufacturer (Stratagene), in the presence of [α-^{32}P]UTP (see Fig. 4a). The probe (30,000 cpm) was hybridized with 20 μg of total RNA in 30 μl of a solution containing 80% formamide, 40 mM PIPES (1,4-piperazine-diethanesulfonic acid), pH 6.7, 0.4 M NaCl, 1 mM EDTA at 45°C for 16 h. The hybridization mixture was diluted in 300 μl of a solution containing 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.3 M NaCl and digested with 150 units of RNase T1 (Bethesda Research Laboratories) at 37°C for 1 h. The samples were then treated with 40 μg/ml Proteinase K (Pharmacia LKB Biotechnology Inc.) at 37°C for 15 min and extracted with phenol/chloroform mixture. The RNA hybrids were precipitated with ethanol, and protected fragments were analyzed on 5% denaturing polyacrylamide gels and visualized by autoradiography and a Lightning Plus intensifying screen (Du Pont Co.).

RESULTS

Analysis of LS mutations

Our previous study using 5′ deletion mutagenesis demonstrated that the region extending 295 bp upstream from the cap site of the myosin HC β gene is necessary for muscle- and stage-specific expression (11). To localize more precisely the cis-acting elements that are responsible for muscle-specific expression, we constructed LS mutations spanning −295 bp and a putative TATA box (Fig. 1a and b). Although some of the mutants contain deletions or insertions as well, we still refer to them as LS mutants. The mutants were transiently transfected into the primary chicken skeletal muscle cells, and they were analyzed for their effect on the reporter CAT gene expression (Fig. 1a and c). Based on the CAT activities, we have identified five elements (A to E) which dramatically affect promoter activity (Fig. 2).

The mutations in element A between nucleotides −276 and −263 (LS3: −277/−266, and LS4: −277/−262) reduced the

activity to 10−20% of the wild-type activity in primary myotubes (Fig. 1a and reference 11). This element included the sequence 5′ CATTCCA 3′ at positions −264 to −270 on the anti-sense strand. Interestingly, this sequence was similar to the M-CAT motif 5′ CATTCCT 3,' which is essential for muscle-specific expression in chicken cardiac troponin T promoter (9,10). Although not the entire A element was conserved between the rabbit and human genes, the CATTCCA sequence was well conserved (Fig. 2).

The mutations in element B between nucleotides −207 and −180 (LS9: −208/−195, LS10: −198/−184, and LS11: −188/−179) reduced the activity to 2−9% in primary myotubes (Fig. 1a). Within this element, the sequence 5′ ACCCCATGCC 3′ at positions −202 to −193 was similar to the binding site of a transcriptional activator, AP-2 (23). However, in the human gene this sequence showed less homology with the consensus sequence of the AP-2 recognition site than did that in the rabbit gene (Fig. 2).

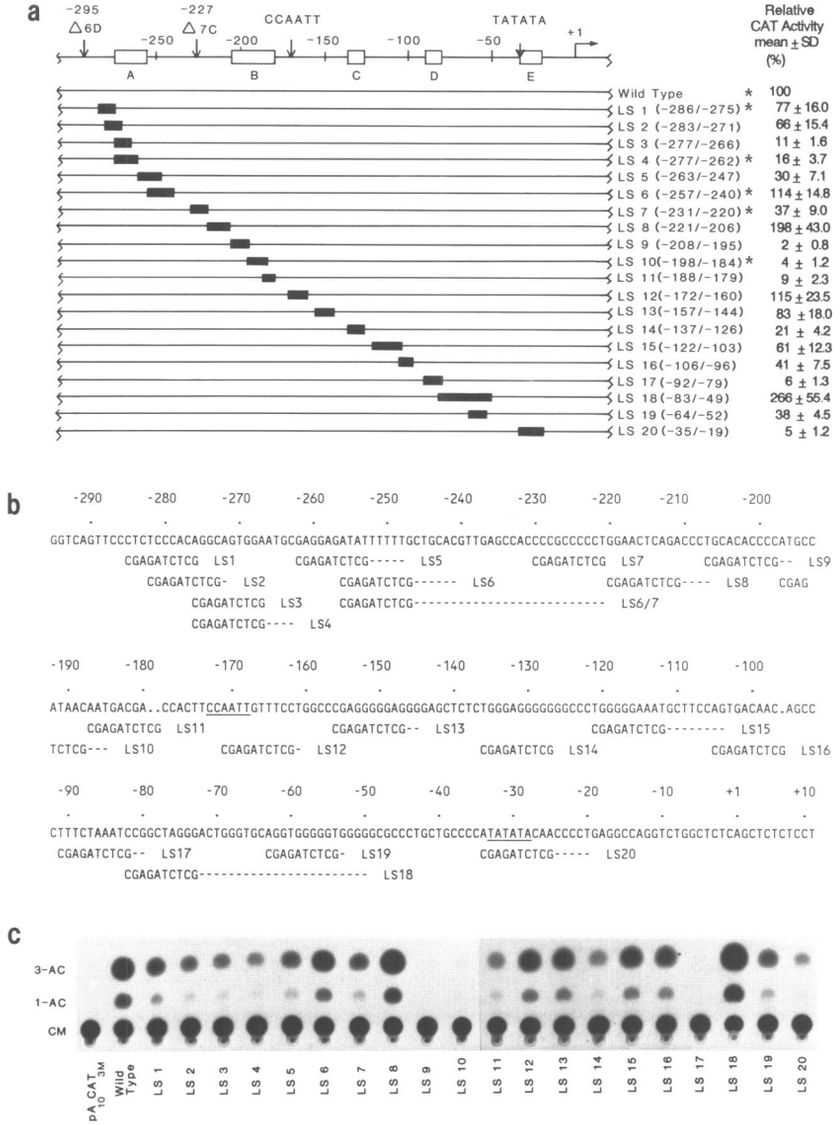

Fig. 1. Functional assay of linker-scanner mutants of myosin HC β promoter regions. (a) Positions of Bg1II linker mutations spanning the region between −295 bp and the TATA box. The black boxes represent the regions replaced by the Bg1II linker sequence CGAGATCTCG. The numbers in parentheses indicate the positions of the 5′ and 3′ nucleotides that are immediately adjacent to the substituted sequences. The relative CAT activity of each mutant transfected into primary skeletal myoblasts is listed. As internal standard the primary skeletal myoblasts were co-transfected with each LS mutant and with pCH110 containing the β-galactosidase gene and were harvested 72 h after transfection at the myotube stage. The CAT activity was normalized by dividing the% conversion ([1-AC + 3-AC]×100 / [CM + 1-AC + 3-AC] by the β-galactosidase activity expressed as A420 units. All CAT activities are given relative to the normalized values obtained for the wild-type plasmid, which is set at 100%. Values given are the averages of 4−6 transfections and plasmid preparations per gene construct ± standard deviation. (Data marked by an asterisk has been published previously (11). (b) Sequence of the myosin HC β gene promoter region between nucleotides −295 and +11, and positions of Bg1II linker mutations. (c) Transient CAT expression of LS mutants. Primary skeletal myoblasts were co-transfected with each LS mutant and with pCH110 and were harvested 72 h after transfection. CM, [^{14}C]chloramphenicol; 1-AC and 3-AC, acetylated forms of [^{14}C]chloramphenicol. Data concerning the analysis of LS4,6,7 and 10 are from our previously published study (11).

The mutation in element C (LS14: −137/−126) also reduced the activity to 21% in primary myotubes (Fig. 1a). The sequence in element C was similar to the binding site of SP-1 transcription factor, 5′ GGGGAGGGGG 3′, which is reported for the human cardiac α-actin promoter region (24) and is slightly different from a typical SP-1 binding site 5′ GGGCGG 3′ (25) (Fig. 2). Another G-rich sequence was also found slightly upstream of element C; however, its mutation (LS13: −157/−144) had little effect on the transcriptional activity of the promoter (Fig. 1a). In addition, the typical consensus sequence of SP-1 binding site 5′ GGGCGG 3′ was located at positions −224 to −229 on the anti-sense strand (Fig. 2) and its mutation (LS7: −231/−220) reduced the activity to about 40% (Fig. 1a). All of these G-rich regions were well conserved between the rabbit and human genes (Fig. 2).

The mutation in element D (LS17: −92/−79) inactivated the promoter in primary myotubes (Fig. 1a). This element contained the sequence 5′ CTAAAT 3′ at positions −88 to −83. This sequence may be a variant of the CCAAT box, because the CCAAT box has been known to be located at −50 to −100 in many eukaryotic genes. This element showed 100% homology between the rabbit and human genes (Fig. 2). In contrast, the sequence 5′ CCAATT 3′ at positions −173 to −168, although showing homology with the concensus sequence of the CCAAT box (Fig. 2), did not lose its promoter function following alteration of this sequence (Fig. 1a, LS12: −172/−160). In addition, a potential E box consensus sequence 5′ CANNTG 3′ at positions −172 to −167 (Fig. 2) which is the recognition site of the helix-loop-helix muscle regulatory factors, such as MyoD1 and myogenin (26), was not required for transcriptional activity of the myosin HC β gene (Fig. 1a, LS12). Another potential E box consensus sequence was located at positions −64 to −59 (Fig. 2) and its mutation (LS:19: −641/−52) reduced the activity to about 40% (Fig. 1a). The former potential E box (−172/−167) was found in both rabbit and human genes, and the latter potential E box (−641/−59) was found in the rabbit gene, but not in the human gene.

The mutation in element E (LS20: −35/−19) reduced the activity to 5% (Fig. 1a). This element contained the sequence 5′ TATATA 3′ at positions −33 to −28, which is similar to the TATA box sequence. The TATA box has been known as the recognition site of the general transcription factor TFIID that is shared by most genes transcribed by RNA polymerase II and that appears to play a key role in eukaryotic transcriptional initiation (27,28). This TATA box sequence was well conserved between the rabbit and human genes (Fig. 2).

In undifferentiated myoblasts (BUdR+) as well as nonmuscle HeLa cells, the wild type and all mutants showed the background level of CAT activity (data not shown).

Effects of the myosin HC regulatory elements on the heterologous promoter

For characterization of these cis-acting elements, various fragments from the myosin HC β gene promoter region were placed upstream of the heterologous TK promoter in the reporter

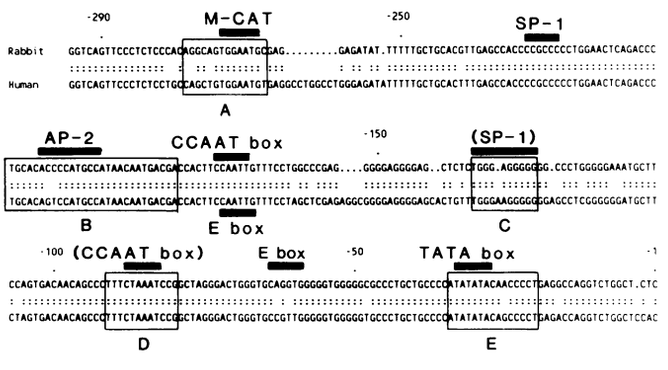

Fig. 2. Comparison of nucleotide sequences in the rabbit and human myosin HC β gene promoter regions. The rabbit myosin HC β gene sequence is aligned with the human myosin HC β gene sequence reported by Yamauchi-Takihara *et al.* (41). The rabbit and human HC β genes showed high homology, with an 85.4% match in the sequence between −295 and −1 (rabbit numbering system). Gaps marked by dots are introduced to maximize homology. Colons indicate nucleotides that are identical. A to E elements identified by mutation analysis of the rabbit myosin HC β gene are in boxes. Putative regulatory sequences such as TATA, CCAAT, and E boxes, M-CAT motif, and SP-1 and AP-2 binding sites are indicated.

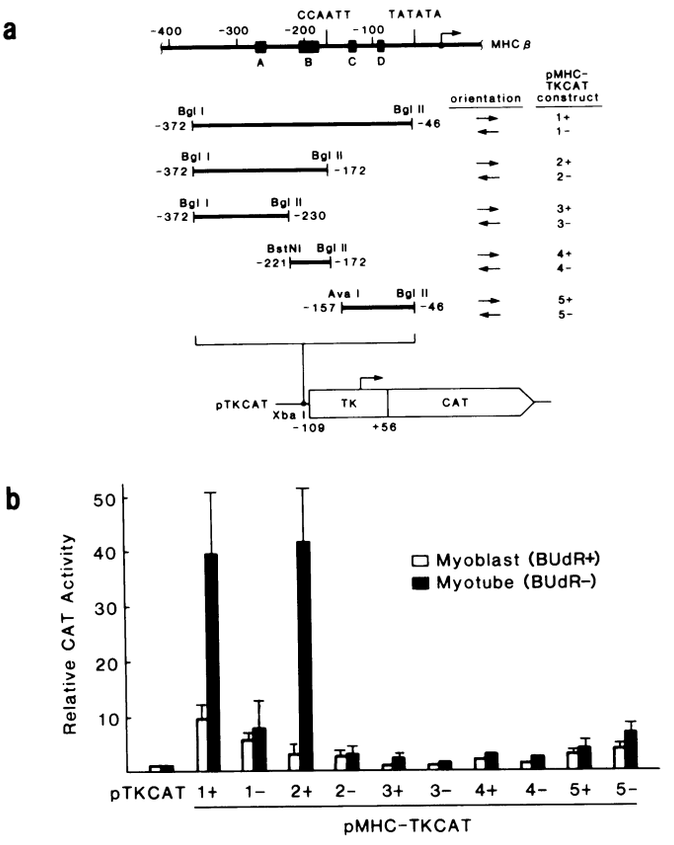

Fig. 3. Effect of the myosin HC β regulatory elements on the heterologous thymidine kinase promoter. (a) Construction of myosin HC-TKCAT chimeric plasmids (pMHC-TKCAT). The various fragments from the regulatory region of the myosin HC β gene were cloned upstream of the thymidine kinase promoter in the pTKCAT vector in both positive (+) and negative (−) orientations relative to their normal positions in the myosin HC β gene. The restriction enzymes which we used to generate fragments and the positions of the 5′ and 3′ end of the fragments in the gene are indicated. The solid boxes mark the regulatory elements defined by analysis of LS mutants. (b) Relative CAT activities of pMHC-TKCAT plasmids in primary skeletal myoblasts cultured in the absence (−) and presence (+) of BUdR were co-transfected with each test plasmid and with pCH110 and were harvested 72 h after transfection. All normalized CAT activities (see Fig. 1) are scaled to the normalized values obtained for pTKCAT (set at 1.0 for each cell type). Expression values represent the averages of 4−6 transfections and plasmid preparations per gene construct ± standard deviations. Standard deviations that are less than 0.5 are not shown on the graph).

CAT gene vector in both orientations (Fig. 3a). These constructs were tested for transient expression in primary skeletal muscle cells at two different stages, myoblasts and myotubes (Fig. 3b). The myoblasts were obtained by treatment of the cells with the thymidine analogue BUdR, which inhibits muscle differentiation.

The pMHC-TKCAT 2+ and 2− contain a fragment between nucleotides −372 and −172, in which both A and B elements are located. In a positive orientation (pMHC-TKCAT 2+), relatively little effect was observed on the CAT expression driven by TK promoter in myoblasts. Following differentiation, however, the expression was enhanced, *i.e.*, the level of activation in myotubes was about 13-fold higher than that in myoblasts. In addition, a fragment −372/−46 in a positive orientation (pMHC-TKCAT 1+), in which all cis-acting elements identified by LS mutation analysis except for the TATA box were present, contributed to the stage-preferential expression from the TK promoter, *i.e.*, the level of activation in myotubes was 4-fold higher than that in myoblasts. However, stage-specific activation was not seen when the fragments were in a negative orientation (pMHC-TKCAT 1− and 2−). Furthermore, neither the fragment −372/−230, containing the A element alone (pMHC-TKCAT 3+ and 3−), nor the fragment −221/−172, containing the B element alone (pMHC-TKCAT 4+ and 4−), had any effect on the CAT expression driven by the TK promoter in both myoblasts and myotubes. Therefore, two cis-acting elements, A and B, are essential for conferring stage-specific expression on the basal heterologous promoter, and they function together in an orientation- or position-dependent manner.

The pMHC-TKCAT 5+ and 5− contain the fragment between nucleotides −157 and −46, in which the C and D elements reside. The fragment −157/−46 in both orientations increased the CAT expression driven by the TK promoter about 4- to 7-fold in primary skeletal muscle cells (Fig. 3b) as well as HeLa cells (data not shown).

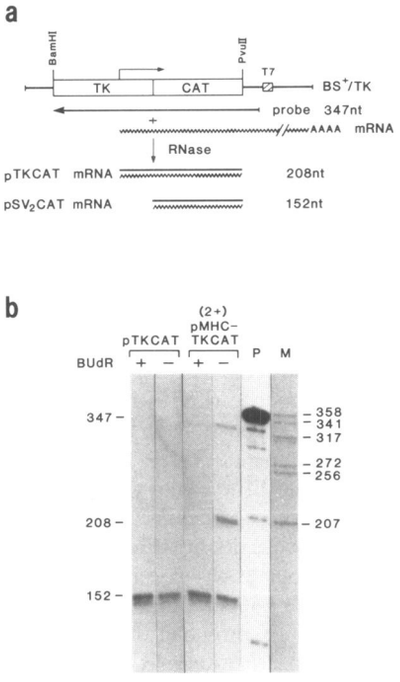

Fig. 4. RNase protection of mRNAs prepared from transfected cells. (a) 32P-labeled RNA probe was generated by transcription *in vitro* from the T7 promoter of the linearized pBS-TKCAT (BS+/TK) template. The predicted sizes of fragments protected from RNase digestion are indicated. nt, nucleotides. (b) Autoradiogram of an RNase protection analysis. Primary skeletal myoblasts cultured in the absence (−) and presence (+) of BUdR were co-transfected with 5 μg of either pTKCAT or pMHC-TKCAT 2+ along with 1.0 μg of pSV2CAT as an internal control. Total RNA was extracted after 96 h of culture. Hybridization and digestion with RNase T1 were performed as described in Materials and Methods. Fragments protected from RNase digestion were resolved on a denaturing 5% polyacrylamide gel. Molecular size markers (M) and a ^{32}P-labeled Sau3A digest of pBR322. P, probe.

Transcription from MHC-TKCAT plasmid

RNA transcripts initiated at cryptic start sites can cause artifactual changes in the levels of CAT mRNA and its protein, if their initiation site is in frame with the CAT gene. To determine the transcriptional start site and the levels of CAT mRNA transcribed from plasmid pMHC-TKCAT 2+ in a muscle- and stage-specific fashion, we performed RNase protection experiments. As shown in Fig. 4a, RNA correctly initiated at the TK cap site should protect 208 nucleotides of the probe from digestion by RNase T1. This probe also detects the expression of the co-transfected pSV2CAT by yielding a 152-nucleotide fragment consisting of CAT gene sequences.

As shown in Fig. 4b, RNA from differentiated muscle cells transfected with pMHC-TKCAT 2+ protected a 208-nucleotide fragment, verifying that transcription was initiated from the correct TK cap site. In agreement with the enzymatic analysis, the pMHC-TKCAT 2+ transcript was absent or present at a very low level in myoblasts (BUdR+), but was expressed after differentiation. In contrast, the level of parental plasmid pTKCAT transcripts remained very low, regardless of the stage of muscle cells. In addition, the expression of the co-transfected pSV2CAT identified by a 152-nucleotide fragment was comparable before and after differentiation. Therefore, two myosin HC regulatory elements together enhance the TK promoter activity in a stage-specific manner at the transcriptional level.

Effect of distance between two elements

The A and B elements of the myosin HC β promoter are separated by 55 bp. To determine whether this spacing is important for promoter activity, we changed the distance between the two elements by either deleting or inserting nucleotides (Fig. 5). The

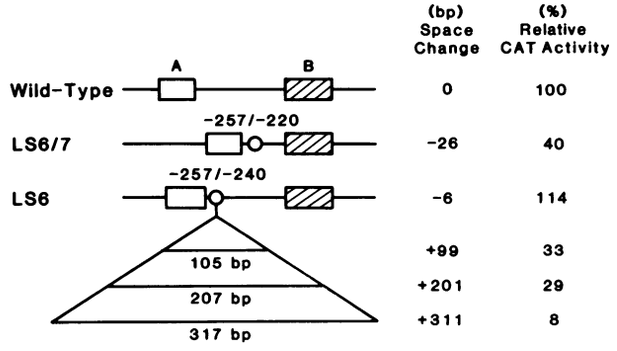

Fig. 5. Effect of changing distance between two elements on transcriptional activity. Primary skeletal myoblasts were co-transfected with each test plasmid and with pCH110 and were harvested after 96 h of culture. All normalized CAT activities are given relative to that of the wild-type plasmid set at 100% (see Fig. 1).

mutant LS6 (−257/−240), which contains a deletion of 6 bp between two elements, had no effect on CAT activity. The mutant LS6/7 (−257/−220), which contains a deletion of 26 bp, reduced the activity to 40% of the wild-type activity. This decline was due to deletion of the sequences between −231 and −220, including the putative SP-1 binding site, rather than to a change in the distance between the two elements. This can be seen from the fact, that in mutant LS7 (−230/−221), the promoter activity is reduced to 37% by substitution of the putative SP-1 binding sequence without any change in their spacing. The insertion of 99 bp or more resulted in a progressive decrease of promoter activity, and the insertion of 311 bp reduced the activity to 8% of the wild-type activity. These results indicate that an appropriate distance between two elements is necessary for a high level of promoter activity.

DISCUSSION

By using linker-scanner mutagenesis, we identified at least five cis-acting elements of the myosin HC β promoter necessary for transcriptional activity. It has been reported that multiple cis-acting elements, both positive as well as negative are involved in tissue-restricted transcription of other muscle genes (3,4,29). Thus, the tissue- and developmental stage-specific gene regulation seems to be a complex process, probably involving the concerted interaction of multiple cis-acting elements with trans-acting factors in various combinations.

We also showed that two elements, A and B, are essential for conferring muscle- and stage-specific activation on the homologous and heterologous promoters. Neither element alone functioned as an activator when placed upstream of the TK promoter, whereas the fragment containing both elements together enhanced TK promoter activity in a muscle- and stage-specific manner. In addition, the disruption of either element reduced the activity of the myosin HC β gene promoter. Moreover, when the intact A element was placed further upstream from the other elements by insertion of plasmid DNA between the A and B elements, the promoter activity was reduced to a low level. Thus, it is likely that cooperative interaction of these two elements is required for tissue- and stage-specific activation of the myosin HC β gene. Furthermore, because the putative SP-1 binding site is present between A and B elements, and because its mutation (LS7) had a moderate effect on the promoter activity (reduction to 40% of the wild-type), the binding of SP-1 or an SP-1-like factor is necessary for full promoter activity. It is possible that this binding modulates the effect of the A and B elements on gene expression.

It has been demonstrated that distal promoter elements and some upstream activating sequences display a critical dependence on their separation and appropriate alignment on the DNA helix for efficient transcription (30). The deletion of 6 bp (about one-half of a helix turn) between the A and B elements had no effect on the promoter activity (LS6), suggesting that such a stereospecific alignment between two elements is not necessary for transcriptional activation. However, the promoter activity was reduced by the insertion of DNA fragments of more than 99 bp between the two elements. Thus, the spacing of two elements may be flexible, but an appropriate distance is required for a high level of activation of the myosin HC β gene.

Comparison of the sequence of the A element in the myosin HC β gene with that of other characterized cis-acting elements showed that, on the anti-sense strand of this element there is a sequence homologous to that of the M-CAT motif (CATTCC-T), with one base mismatch. This motif is present in the regulatory regions of several muscle genes (31). In the chicken cardiac troponin T gene, the muscle-specific activation is dependent on two intact M-CAT motifs in the distal regulatory region, and hence on the interaction of the two motifs (10). On the other hand, in the chicken skeletal α-actin gene, which contains the single M-CAT motif, the mutation of this element had no effect on the promoter activity (4). Because the A element in the myosin HC β gene needs to associate with the B element to contribute to muscle-specific expression, the M-CAT motif may confer tissue-specific activation only in cooperation with proper elements that include the same motif. Such a situation has also been observed in the MyoD1 binding site (E box). In the muscle creatine kinase (22) and myosin alkali light chain (33) enhancers, at least two MyoD1 binding sites are essential for muscle-specific activation. Moreover, in the cardiac α-actin (34) and troponin I (35) genes, a single MyoD site cooperates with other elements, such as the CArG-box and the SP-1 binding site in the actin promoter and sites I and II in the troponin I enhancer, to provide efficient tissue-specific expression.

In the case of the B element, we have previously reported on the similarity of this sequence to the binding site of AP-2 (11). However, the gel mobility shift assay showed that the binding of protein factor to the B element was not blocked by the addition of an excess of oligonucleotides containing the high-affinity AP-2 binding site (36); thus, another factor, rather than AP-2, interacts with the B element and is involved in muscle-specific stimulation.

The mutagenesis studies described here also indicated that potential E box (−172/−167) in the myosin HC β gene was not required for transcriptional activity. It has been reported that conserved sequences which contribute to promoter function in many genes are not required for the expression of the given gene, although they are present in the regulatory region (4,10,37). Thus, the presence of any conserved elements within a regulatory region does not imply their functional roles in the particular gene. However, it is possible that these elements contribute to the regulation of gene transcription in different backgrounds or physiologic conditions, although they are not functional in cultured skeletal muscle cells. The mutation of another potential E box (−64/−59) sequence (LS19) had a moderate effect on the promoter activity (reduction to 40% of the wild-type). However, since the fragment −372/−172 which doesn't include the potential E box was sufficient to confer the muscle-specific activation on the TK promoter (Fig. 3, pMHC-TKCAT 2+), it appears that this sequence doesn't directly contribute for muscle specific activation in our assay system.

The fragment −157/−46 in either orientation enhanced the activity of heterologous TK promoter regardless of the transfected cell backgrounds. This activation may be due to the interactions of the C and D elements with general transcriptional activators, since these elements contained the sequences homologous to the SP-1 binding site and the CCAAT box, respectively. It is known that SP-1 and CCAAT box-binding proteins, such as C/EBP (38), CTF/NF1 (39) and CP1 (40), are transcriptional stimulators which interact with many genes, including the TK promoter, and its target elements seem to function bidirectionally (38). The D element especially may play an important role in general transcriptional activation, as its mutation severly blocks the promoter activity.

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REFERENCES

1. Mahdavi, V., Strehler, E.E., Periasamy, M., Wieczorek, D., Izumo, S., Grund, S., Strehler, M.-A. and Nadal-Ginard, B. (1986) p. 345–361, In C. Emerson, D. Fischman, B. Nadal-Ginard, and M.A.Q. Siddiqui (ed), Molecular Biology of Muscle Development, Alan R. Liss, Inc., N.Y.
2. Medford, R.M., Nguyen, H.T. and Nadal-Ginard, B. (1983) *J. Biol. Chem.* **258**,11063–11073.
3. Bouvagnet, P.F., Strehler, E.E., White, G.E., Strehler-Page, M.A., Nadal-Ginard, B. and Mahdavi, V. (1987) *Mol. Cell. Biol.* **7**,4377–4389.
4. Chow, K.-L. and Schwartz, R.J. (1990) *Mol. Cell. Biol.* **10**,528–538.
5. Grichnik, J.M., French, B.A. and Schwartz, R.J. (1988) *Mol. Cell. Biol.* **8**,4587–4597.
6. Minty, A. and Kedes, L. (1986) *Mol. Cell. Biol.* **6**,2125–2136.
7. Buskin, J.N. and Hauschka, S.D. (1989) *Mol. Cell. Biol.* **9**,2627–2640.
8. Gossett, L.A., Kelvin, D.J., Sternberg, E.A. and Olson, E.N. (1989) *Mol. Cell. Biol.* **9**,5022–5033.
9. Mar, J.H. and Ordahl, C.P. (1988) *Proc. Natl. Acad. Sci. USA* **85**,6404–6408.
10. Mar, J.H. and Ordahl, C.P. (1990) *Mol. Cell. Biol.* **10**,4271–4283.
11. Cribbs, L.L., Shimizu, N., Yockey, C.E., Levin, J.E., Jakovcic, S., Zak, R., and Umeda, P.K. (1989) *J. Biol. Chem.* **264**,10672–10678.
12. Clark, W.A. and Fischman, D.A. (1983) *Dev. Biol.* **97**,1–9.
13. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, R. and Chasin, L. (1979) *Proc. Natl. Acad. Sci. USA* **76**,1373–1376.
14. Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) *Mol. Cell. Biol.* **2**,1044–1051.
15. Miller, J.H. (1972) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. Haltiner, M., Kempe, T. and Tijan, R. (1985) *Nucleic Acids Res.* **13**,1015–1025.
17. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**,5463–5467.
18. Maxam, A.M. and Gilbert, W. (1980) *Methods Enzymol.* **65**,499–560.
19. McKnight, S.L. and Kingsbury, R. (1982) *Science* **217**,316–324.
20. Laimins, L.A., Gruss, P., Pozzatti, R. and Khoury, G. (1984) *J. Virol.* **49**, 183–189.
21. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. Chirgwin, J.M., Rrzybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) *Biochemistry* **18**,5294–5299.
23. Imagawa, M., Chiu, R. and Karin, M. (1987) *Cell* **51**,251–260.
24. Gustafson, T.A. and Kedes, L. (1989) *Mol. Cell. Biol.* **9**,3269–3283.
25. Kadonaga, J.T., Jones, K.A. and Tjian, R. (1986) *Trends Biochem. Sci.* **11**,20–23.
26. Olson, E.N. (1990) *Genes Dev.* **4**,1454–1461.
27. Buratowski, S., Hahn, S., Guarente, L. and Sharp, P.A. (1989) *Cell* **56**,549–561.
28. Workman, J.L. and Roeder, R.G. (1986) **51**,613–622.
29. Quitschke, W.W., DePonti-Zilli, L., Lin, Z.-Y. and Paterson, B.M. (1989) *Mol. Cell. Biol.* **9**,3218–3230.
30. Lee, D.-H. and Schleif, R.F. (1989) *Proc. Natl. Acad. Sci.USA* **86**,476–480.
31. Nikovits, W. Jr., Kuncio, G. and Ordahl, C.P. (1986) *Nucleic Acids Res.* **14**,3377–3390.
32. Weintraub, H., David, R., Lockshon, D. and Lassar, A. (1990) *Proc. Natl. Acad. Sci. USA* **87**,5623–5627.
33. Wentworth, B.M., Donoghue, M., Engert, J.C., Berglund, E.B. and Rosenthal, N. (1991) *Proc. Natl. Acad. Sci. USA* **88**,1242–1246.
34. Sartorelli, V., Webster, K.A. and Kedes, L. (1990) *Genes Dev.* **4**,1811–1822.
35. Lin, H., Yutzey, K.E. and Konieczny, S.F. (1991) *Mol. Cell. Biol.* **11**,267–280.
36. Shimizu, N., Dizon, E. and Zak, R. (1992) *Mol. Cell. Biol.* **12**,619–630.
37. Uetsuki, T., Nabeshima, Y., Fujisawa-Sehara, A. and Nabeshima, Y. (1990) *Mol. Cell. Biol.* **10**,2562–2569.
38. Johnson, P.F., Landschulz, W.H., Graves, B.J. and McKnight, S.L. (1987) *Genes Dev.* **1**,133–146.
39. Jones, K.A., Kadonaga, J.T., Rosenfeld, P.J., Kelly, T.J. and Tjian, R. (1987) *Cell* **48**,79–89.
40. Chodosh, L., Baldwin, A., Carthew, R. and Sharp, P. (1988) *Cell* **53**,11–24.
41. Yamauchi-Takihara, K., Sole, M.J., Liew, J., Ing, D. and Liew, C.C. (1989) *Proc. Natl. Acad. Sci. USA* **86**,3504–3508. 7416–7417 (Correction).