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Author(s)	Nakatsuji, Yuji
Citation	大阪大学, 1993, 博士論文
Version Type	VoR
URL	https://doi.org/10.11501/3065817
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Note	

MOLECULAR AND CELLULAR BIOLOGY, Oct. 1992, p. 4384–4390
0270-7306/92/104384-07$02.00/0

Vol. 12, No. 10

A Single MEF-2 Site Is a Major Positive Regulatory Element Required for Transcription of the Muscle-Specific Subunit of the Human Phosphoglycerate Mutase Gene in Skeletal and Cardiac Muscle Cells

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Received 4 June 1992/Accepted 16 July 1992

In order to analyze the transcriptional regulation of the muscle-specific subunit of the human phosphoglycerate mutase (PGAM-M) gene, chimeric genes composed of the upstream region of the PGAM-M gene and the bacterial chloramphenicol acetyltransferase (CAT) gene were constructed and transfected into C2C12 skeletal myocytes, primary cultured cardiac muscle cells, and C3H10T1/2 fibroblasts. The expression of chimeric reporter genes was restricted in skeletal and cardiac muscle cells. In C2C12 myotubes and primary cultured cardiac muscle cells, the segment between nucleotides −165 and +41 relative to the transcription initiation site was sufficient to confer maximal CAT activity. This region contains two E boxes and one MEF-2 motif. Deletion and substitution mutation analysis showed that a single MEF-2 motif but not the E boxes had a substantial effect on skeletal and cardiac muscle-specific enhancer activity and that the cardiac muscle-specific negative regulatory region was located between nucleotides −505 and −165. When the PGAM-M gene constructs were cotransfected with MyoD into C3H10T1/2, the profile of CAT activity was similar to that observed in C2C12 myotubes. Gel mobility shift analysis revealed that when the nuclear extracts from skeletal and cardiac muscle cells were used, the PGAM-M MEF-2 site generated the specific band that was inhibited by unlabeled PGAM-M MEF-2 and muscle creatine kinase MEF-2 oligomers but not by a mutant PGAM-M MEF-2 oligomer. These observations define the PGAM-M enhancer as the only cardiac- and skeletal-muscle-specific enhancer characterized thus far that is mainly activated through MEF-2.

Phosphoglycerate mutase (PGAM) (EC 5.4.2.1) is a glycolytic enzyme that catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate using 2,3-bisphosphoglycerate as a cofactor (14, 15). There are two isoforms of mammalian PGAM: a muscle-specific form (PGAM-M) and a non-muscle-specific, or brain, form (PGAM-B) (32). Three types of PGAM dimers may be found in mammalian tissues: the homodimer MM form, which is found mainly in mature muscle; the BB form, which is found mainly in liver, kidney, and brain tissues; and the heterodimer MB form, which is found mainly in the heart. In addition, the isozyme pattern of PGAM is developmentally regulated during the development of human skeletal muscle. Early in development, fetal muscle contains almost exclusively PGAM-B; PGAM-M is first seen at approximately 80 to 100 days of gestation, and it predominates thereafter (28, 32). However, the molecular mechanisms for alterations of the isozyme patterns of PGAM are not yet known. We have cloned human PGAM-M (43) and PGAM-B (39) cDNAs and described human PGAM-M genomes (47), and we have used the PGAM-M gene as a model system to study differential gene regulation.

Recently, muscle-specific gene regulation has been studied extensively, and several myogenic factors have been identified. The first members of this group are helix-loop-helix proteins of the MyoD family, including MyoD (7), myogenin (9, 49), Myf 5 (4), and MRF4/Myf6/herculin (3, 27, 37). Although the MyoD family regulates most of the muscle-specific genes, some other myogenic factors, such as the muscle-specific chloramphenicol acetyltransferase (M-CAT) motif) 5′-CATTCCT-3′)-binding factor (26) and the myocyte-specific enhancer-binding factor MEF-2 (6, 13), also participate in their regulation. MEF-2 interacts with the muscle-specific enhancers of muscle creatine kinase (CKM) and myosin light chain 1/3 (13), and several potential MEF-2 sites in other muscle-specific regulatory regions have been identified (6). However, it is not certain whether those potential MEF-2 sites have any effect on muscle-specific enhancer activity. Although multimers of the MEF-2 site can activate muscle-specific transcription, a single MEF-2 site is a relatively weak enhancer in skeletal muscle (13). In addition, there is a controversy regarding the muscle specificity of the factors that bind to the MEF-2 site (6, 13, 19, 20, 36). Although several muscle-specific genes including PGAM-M are expressed in cardiac muscle cells as well as in skeletal muscle cells, most studies on the transcriptional regulation thus far have been carried out with skeletal muscle cells but not with cardiac muscle cells. Thus, the study of the transcriptional regulation in cardiac muscle cells has lagged behind that of the transcriptional regulation in skeletal muscle cells. It is therefore of interest to investigate whether either divergent or overlapping regulatory programs specify the expression of the PGAM-M gene in cardiac and skeletal muscles. To address this issue, we characterized the regu-

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latory element of the PGAM-M gene using primary cultured cardiac muscle cells and C2C12 myotubes.

MATERIALS AND METHODS

Plasmid construction. PGAM-M-CAT fusion genes were constructed by inserting the upstream 5′-flanking and untranslated region (−3.3 kbp to +41 bp) of the human PGAM-M gene (47) into a site in the CAT gene (12) located immediately upstream of the vector pSV00CAT (1) in the correct orientation. For various external deletions, the upstream 5′-flanking region of the PGAM-M gene was digested with appropriate restriction endonucleases or *Bal* 31 exonuclease. When *Bal* 31 exonuclease was used for these deletions, the 5′ endpoint of the PGAM-M enhancer region of each construct was identified by sequencing. Site-directed mutagenesis was performed by the oligonucleotide-mediated method (40) and confirmed by sequencing.

Cell culture. Mouse skeletal muscle cells (line C2C12) were grown in high-glucose (4.5-g/liter) Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, and COS medium (Cosmo Bio) was used as a differentiation medium. Fibroblast C3H10T1/2 cells were cultured in the low-glucose (1-g/liter) DMEM supplemented with 10% fetal bovine serum. Primary culture of cardiac muscle cells was prepared from the hearts of Wistar rat embryos (day 18) according to the method of Simpson and Savion (44) with minor modifications. The hearts were cut into small cubes and dissociated by phosphate-buffered saline (PBS) containing 0.05% trypsin and 0.01% collagenase at 37°C for 15 min. The dissociation procedure was repeated twice before the cells were cultured in the low-glucose DMEM with 10% fetal bovine serum.

DNA transfection and CAT assay. Expression vectors were introduced into all cells by the cationic liposome (Lipofectin; Bethesda Research Laboratories) (10, 11)-mediated method. Ten to 20 μg of DNA and 30 to 50 mg of Lipofectin were dissolved in 1.5 ml of Opti-MEM (GIBCO), mixed, and used for one transfection (60-mm-diameter dish). Tissue culture plates were washed twice with 3 ml of Opti-MEM, and then 3 ml of the DNA-liposome mixture was added. After incubation at 37°C for 12 to 16 h, 3 ml of DMEM with 20% fetal bovine serum was added. The cells were harvested by scraping 60 to 72 h after transfection. Ten micrograms of PGAM-M-CAT, 10 μg of pSV-β-galactosidase (Promega), and 50 mg of Lipofectin were used for C2C12 transfection. Either 10 μg of PGAM-M-CAT, 10 μg of pEMSV-MyoD1, and 50 mg of Lipofectin or 10 μg of PGAM-M-CAT and 30 mg of Lipofectin were used for C3H10T1/2 transfection. Ten micrograms of PGAM-M-CAT and 30 mg of Lipofectin were used for cardiac muscle cells. CAT assays were performed as described by Gorman et al. (12), and CAT activity was quantitated by scintillation spectrometry. The plasmid pSV-β-galactosidase was used as an internal control for transfection into C2C12, and the total protein, measured by the modified Bradford assay (Bio-Rad), was used as an internal control for transfection into C3H10T1/2 and cardiac myocytes.

Preparation of nuclear extracts and gel mobility shift assays. Nuclear extracts of C2C12 cells and C3H10T1/2 cells were prepared by the method of Dignam et al. (8) with minor modifications according to the work of Buskin and Hauschka (5). The additional protease inhibitors leupeptin (1 μg/ml) and pepstatin (1 μg/ml) were added to solutions A, C, and D; aprotinin (1 μg/ml) was added to solution D. For the preparation of nuclear extracts from embryonal rat hearts, 140 hearts from day 18 rat embryos were removed, trisected, and washed three times with PBS. The subsequent procedure was essentially the same as the method of Mar and Ordahl (26).

For gel mobility shift assays, a combination of 8 μg of nuclear extract and 1 μg of poly(dI-dC) was preincubated with 4 μl of 5× binding buffer (final concentrations, 15 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 30 mM KCl, 15% glycerol, 0.5 mM EDTA, and 0.5 mM dithiothreitol) in a volume of 18 μl at room temperature for 15 min. Then, 2 μl of ^{32}P-labeled probe (10 fmol of PGAM-M MEF-2 oligomer per μl [see Fig. 6D] filled by DNA polymerase with [α-^{32}P]dCTP) was added to the assay mixture, and the mixture was incubated at room temperature for an additional 20 min. The reaction mixture was separated by electrophoresis on a 5% polyacrylamide gel in TGE (50 mM Tris [pH 8.5], 380 mM glycine, 2 mM EDTA) buffer at 15 V/cm at room temperature for 1.5 to 2 h. For competition studies, a 100-fold molar excess of the unlabeled competitor DNA fragment was added during preincubation of the reaction mixture.

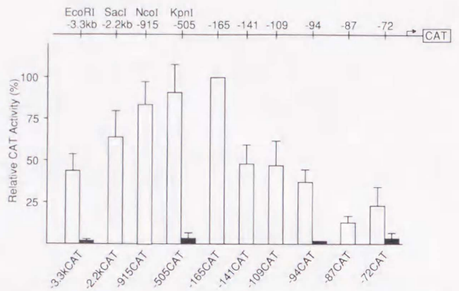

FIG. 1. Expression of CAT activities in C2C12 myoblasts and myotubes from constructs carrying deletions of the upstream region of PGAM-M. The names assigned to the chimeric plasmids are shown under the corresponding bars. All deletion constructs were generated by cleaving with restriction enzymes shown at the top or by deletion with *Bal* 31. Relative CAT activities are given as percentages of the activity of −165CAT. Open and filled bars, transfections into myotubes and myoblasts, respectively. Values are means ± standard deviations for 4 to 13 experiments with myotubes and 3 experiments with myoblasts. Cotransfection with pSV-β-galactosidase was used as a control for transfection efficiency.

RESULTS

Transfection of PGAM-M-CAT genes into skeletal muscle cells. (i) Deletion mutagenesis. As the first step toward identifying potential regulatory elements responsible for governing the expression of the human PGAM-M gene during myogenesis, a series of 5′ deletions from −3.3 kbp to −72 bp of the PGAM-M enhancer region were constructed and linked to the CAT gene (Fig. 1). As shown in Fig. 1, significant expression of CAT activity was not observed in myoblasts. The expression of CAT activities with all constructs other than the four CAT constructs shown in Fig. 1 was also very low in myoblasts (data not shown), though experiments using those constructs were performed fewer than three times. In contrast, the chimeric genes expressed a high level of activity when transfected into myotubes. The −165CAT construct expressed the highest activity. The

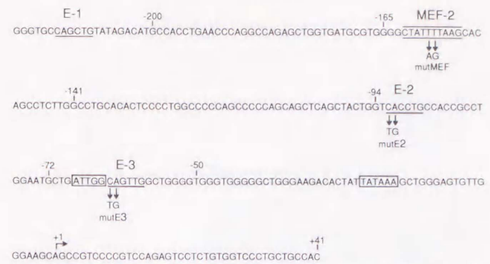

FIG. 2. Nucleotide sequence of the upstream region of the human PGAM-M gene showing nucleotide positions −221 through +41 relative to the transcription initiation site. Three E boxes (E-1, -2, and -3) are underlined, and the MEF-2 motif is shown between two horizontal lines. The inverted CCAAT box and the TATA box are shown in rectangles. Substitution mutations of E-2 (mutE2), E-3 (mutE3), and MEF-2 (mutMEF) are indicated by arrows.

CAT activity was significantly reduced by the deletion of nucleotides through position −141. These results indicated that the PGAM-M-CAT gene expressed in a differentiation-specific manner and the region between positions −165 and −141 significantly influenced the PGAM-M gene expression during myogenesis in vitro.

(ii) Substitution mutagenesis. To define the region of the human PGAM-M enhancer in more detail, we tested mutants of the suspected sequences contributing to the differentiation-specific expression. Figure 2 shows the nucleotide sequence of the human PGAM-M gene between positions −221 and +41 relative to the transcription initiation site. This region contains one MEF-2 motif and three E-box motifs (CANNTG; designated E-1, -2, and -3 in Fig. 2), though among these three E boxes only E-2 (CACCTG) is a preferred binding site for the MyoD-E2A heterodimer (2, 29). Since the CAT activities of −505CAT and −165CAT were almost the same, E-1, which is located between positions −505 and −165, did not contribute to the enhancer activity of PGAM-M. To examine the significance of the MEF-2 motif and E-2 as well as E-3, we made substitution mutations of each element, as shown in Fig. 2, on the −165 construct, which yielded the highest CAT activity. When the resulting mutants, −165mutMEFCAT, −165mutE2CAT, and −165mutE3CAT, were transfected into C2C12 myotubes (Fig. 3), only the MEF-2 mutant revealed an approximately 65% reduction in activity, while the two other mutants yielded almost the same activity as their wild type, −165CAT. These results indicate that E boxes have little or no effect on the enhancer activity in the transcriptional regulation of the human PGAM-M gene in skeletal muscle cell C2C12 myotubes but the MEF-2 site plays a substantial role in it.

Transfection into nonmuscle cells. A cotransfection experiment to test whether forced expression of MyoD in nonmuscle cells influences the PGAM-M gene enhancer was performed. As shown in Fig. 4, C3H10T1/2 fibroblasts did not support expression of PGAM-M-CAT. In contrast, when cotransfected with MyoD, the chimeric genes expressed high activities. The profile shown in Fig. 4 was similar to that observed with C2C12 myotubes, shown in Fig. 1. Interestingly, −165mutMEFCAT showed reduced CAT activity, though the mutations of E-2 and E-3 in the same −165 construct did not. These results indicated that the PGAM-M

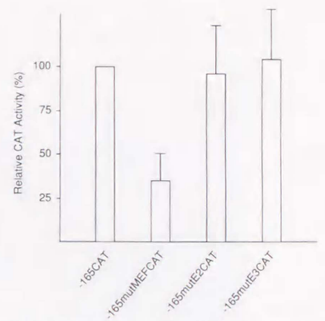

FIG. 3. Effects of mutagenesis of E-2, E-3, and the MEF-2 motif of −165CAT on CAT activities in C2C12 myotubes. Relative CAT activities are given as percentages of the activity of −165CAT. Values are means ± standard deviations for 6 to 10 experiments; pSV-β-galactosidase was used as a control for transfection efficiency.

enhancer in such cells was probably activated through the transactivation of MEF-2 by MyoD (6, 24).

Transfection into cardiac muscle cells. Since PGAM-M is also expressed in cardiac muscle (33), we examined the transcriptional regulatory mechanisms in cardiac myocytes. Several PGAM-M-CAT plasmids that have variously deleted PGAM-M enhancer regions were transfected into primary cultured cardiac myocytes. As shown in Fig. 5, −165CAT expressed the highest CAT activity again. When the enhancer region was deleted up to position −141, reduction of CAT activity was more prominent than that in C2C12 myotubes (Fig. 1 and 5). Mutation of the MEF-2 site resulted in a marked loss of the activity, as shown in Fig. 5. In addition, the CAT activities of −505CAT, −915CAT, and −2.2kCAT were remarkably low compared with that of −165CAT. These results demonstrated that a single MEF-2 site was the major positive regulatory element and played a crucial role as a cardiac muscle enhancer and that the cardiac-muscle-specific negative regulatory element(s) is located between nucleotides −505 and −165.

Gel mobility shift analysis of PGAM-M MEF-2. Since the MEF-2 motif of PGAM-M is crucial for the muscle-specific expression of the PGAM-M gene according to the results of CAT analysis, gel mobility shift analysis was performed to ascertain whether MEF-2 was in fact bound to the PGAM-M MEF-2 site in a muscle-specific manner. As shown in Fig. 6A, nuclear extracts from C2C12 myotubes interacted with the PGAM-M MEF-2 site. The unlabeled PGAM-M MEF-2 oligomer competed effectively for the binding of MEF-2, while the PGAM-M mutMEF-2 oligomer failed to compete. Furthermore, the oligomer corresponding to the mouse CKM MEF-2 site also competed effectively. In contrast, the PGAM-M MEF-2 site did not effectively interact with MEF-2 when nuclear extracts from C2C12 myoblasts were used. This result is compatible with the previous studies (6) showing that MEF-2 was up-regulated during the conversion of C2 myoblasts to myotubes. When nuclear extracts from cardiac muscle cells were used (Fig. 6C), the PGAM-M MEF-2 oligomer formed the same shift of the band as that of C2C12 myotubes and was inhibited by unlabeled PGAM-M MEF-2 and CKM MEF-2 oligomers. Thus, the PGAM-M MEF-2 site interacted with the protein, probably MEF-2,

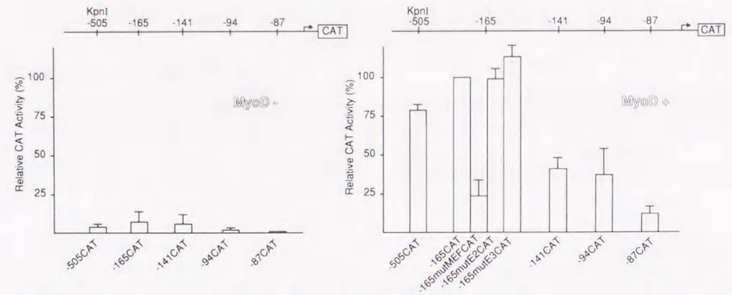

FIG. 4. Effects of MyoD cotransfection on CAT activities in C3H10T1/2. Values are means ± standard deviations for at least three experiments; the total protein was used as a control for transfection efficiency.

existing in both C2C12 myotubes and cardiac myocytes. As shown in Fig. 6B, the oligonucleotide PGAM-M MEF-2 interacted with the nuclear extract from MyoD-transfected C3H10T1/2 cells. Unlabeled PGAM-M MEF-2 and CKM MEF-2 also competed for the binding of MEF-2. In contrast, no apparent interaction was seen in the case of parent C3H10T1/2 cells. Thus, MEF-2 induced by MyoD activated the PGAM-CAT fusion gene in MyoD-transfected C3H10T1/2 cells (Fig. 4).

DISCUSSION

In this report, we have demonstrated that the glycolytic enzyme PGAM-M is transcribed in a tissue-specific and developmental-stage-specific manner. This specificity is mainly mediated by a single MEF-2 site. E boxes have little or no effect on specificity in this gene, though recent studies have shown that most muscle-specific genes have multiple E boxes and are regulated mainly by the MyoD protein family (23, 25, 35, 41, 48), with some exceptions, such as cardiac troponin T (26) and skeletal myosin heavy-chain (45) genes.

MEF-2 was first described by Gossett et al. (13) as a muscle-specific enhancer-binding factor which interacts with the upstream region of the CKM gene. But there has been some controversy concerning the muscle specificity of the factors that bind to the MEF-2 site. Horlick et al. (19, 20) demonstrated by the gel retardation assay that the factors binding to the MEF-2 site (designated the TA-rich segment) of the CKM enhancer region were present in a wide variety of tissues and cell types. The TA-rich recognition protein (TARP) is not muscle specific and binds to both TA-rich segments of the brain creatine kinase promoter and CKM enhancer. Pollock and Treisman (36) isolated cDNA clones encoding a family of human serum response factor (SRF)-related DNA-binding proteins (RSRF). Ubiquitously expressed RSRF proteins have binding activities specific for the CTA(A/T)$_4$TAG sequence that is homologous to TARP and the MEF-2-binding sequence. On the other hand, Cserjesi and Olson (6) demonstrated that, although ubiquitous binding factors recognized the MEF-2 site, MEF-2 extracted from C2 myotubes formed a band slightly different from others in its mobility in the gel retardation assay. There are several possible explanations of how its muscle specificity occurs. (i) MEF-2 is expressed in a muscle-specific manner and produces the muscle specificity, although ubiquitous proteins referred to as TARP or RSRF exist. (ii) The muscle specificity occurs when ubiquitous proteins such as TARP and RSRF interact with other muscle-specific factors or muscle-specific accessory proteins. (iii) Such ubiquitous proteins are modified, for example, by phosphorylation. In our CAT assay study, a single MEF-2 element regulated the muscle-specific and differentiation-specific expression of the PGAM-M gene, and in our gel mobility assay, DNA-MEF-2 complexes were formed only in C2C12 myotubes, cardiomyocytes, and MyoD-transfected fibroblasts. These results may support the notion that MEF-2 is a muscle-specific enhancer-binding factor different from other ubiquitous factors, although this conclusion is uncertain until cloning of MEF-2 is accomplished.

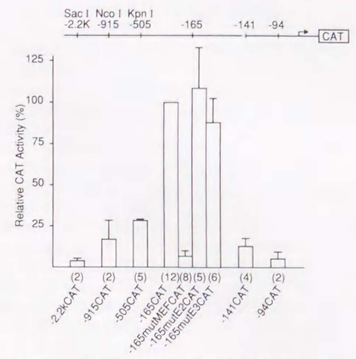

FIG. 5. CAT activities of the PGAM-M-CAT plasmids in cardiac muscle cells. Values are means ± standard deviations for 2 to 12 experiments (the number of experiments is shown in parentheses).

In the gel mobility shift assay, MyoD induced activation of MEF-2 (Fig. 6B); this result is consistent with recent reports (6, 24). The cotransfection of PGAM-M-CAT plasmids with MyoD into C3H10T1/2 fibroblasts showed that MEF-2 was a major factor governing the expression, and the profile was very similar to that obtained with C2C12 myotubes (Fig. 1, 3, and 4). Although MEF-2 has been characterized as a weak enhancer that depends on an adjacent enhancer and has not exhibited the enhancer activity in a single copy (13), a single MEF-2 site in the PGAM-M gene heavily contributed to its muscle-specific enhancer activity.

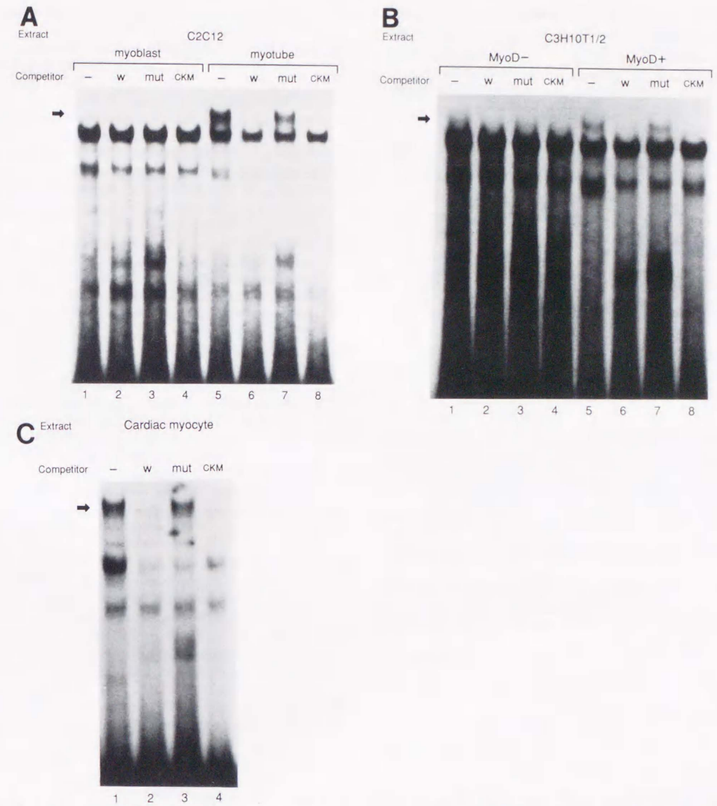

FIG. 6. Gel mobility shift analysis of MEF-2. Nuclear extracts were prepared from C2C12 myoblasts (panel A, lanes 1 through 4), C2C12 myotubes (panel A, lanes 5 through 8), C3H10T1/2 cells (panel B, lanes 1 through 4), MyoD-transfected C3H10T1/2 cells (panel B, lanes 5 through 8), and cardiac myocytes (panel C, lanes 1 through 4). Gel mobility shift assays were performed with ^{32}P-labeled probes corresponding to the PGAM-M MEF-2 site and 8 μg of nuclear extract from each cell type without competitor oligomers (−) (panel A, lanes 1 and 5; panel B, lanes 1 and 5; and panel C, lane 1) and with competitor oligomers: 100-fold molar excesses of cold PGAM-M MEF-2 (w) (panel A, lanes 2 and 6; panel B, lanes 2 and 6; and panel C, lane 2), PGAM-M mutMEF-2 (mut) (panel A, lanes 3 and 7; panel B, lanes 3 and 7; and panel C, lane 3), and mouse CKM MEF-2 (panel A, lanes 4 and 8; panel B, lanes 4 and 8; and panel C, lane 4). (D) Nucleotide sequences of the oligomers used as competitors.

PGAM-M is also found in cardiac muscle as well as in skeletal muscle (31). Thus far, a few reports on the mechanism of transcriptional regulation in cardiac muscle have suggested that the known *cis*-regulatory elements such as M-CAT, CArG, and C-rich motifs were required for cardiac muscle-specific gene expression (21, 34, 46). Other reports (16, 30, 50) suggested that the regions containing the MEF-2 motif and other known elements were required for cardiac-muscle-specific gene expression. In the cardiac troponin T gene (21), M-CAT and a cardiac element containing MEF-2 are required, and in the cardiac MLC-2 gene (30), MEF-2 and other ubiquitous factors are required for cardiac-muscle-specific gene expression. Thus, Navankasattusas et al. (30) speculated that MEF-2 must interact with other factors to confer muscle-specific expression. Our data demonstrated that a single MEF-2 site has an enhancer activity in cardiac muscle cells, though we cannot absolutely rule out the possibility that MEF-2 interacts with other factors that bind to some region between nucleotides −165 and +41. As mentioned previously, MEF-2 is induced by MyoD or myogenin in skeletal muscle. In cardiac muscle, where proteins of the MyoD family are not found (4, 9, 18, 31, 38, 42), other cardiac-muscle-specific E-box binding factors may transactivate the MEF-2 gene. Our study also suggested that the cardiac-muscle-specific negative regulatory element(s) was located someplace between nucleotides −505 and −165, although there has been no report on such elements. Identification of such elements in this region is under investigation.

The concept of gene regulatory programs operating in skeletal and cardiac muscle cells is complicated, since en-

hancers are composed of multiple positive and negative elements that interact with combinations of ubiquitous and cell-type-specific transcription factors. However, the muscle-specific gene regulatory program of PGAM-M is the simplest reported thus far. The region containing a single MEF-2 motif has a skeletal-muscle-specific enhancer activity in the rat aldolase A gene (22), and mutation of the MEF-2 site resulted in a marked loss of the enhancer activity (17). It is possible that the same concept might apply to the muscle-specific genes of other enzymes.

ACKNOWLEDGMENTS

We are grateful to N. Mimura for providing C2C12 cells and technical suggestions, to Japanese Cancer Research Resources Bank for providing C3H10T1/2 cells, to H. Weintraub for providing the MyoD expression vector, to C. Hashimoto for technical assistance, and to Y. Fujii and A. Ogata for manuscript preparation.

This work was supported by grants for specific diseases and for congenital disorders from the Ministry of Health and Welfare, Japan.

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