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Tissue-Specific Transcription of the Cardiac Myosin Light-Chain 2 Gene Is Regulated by an Upstream Repressor Element

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Physiological expression of the cardiac muscle myosin light-chain 2 (MLC-2) gene in chickens is restricted to cardiac muscle tissue only, at least during the late embryonic to adult stages of development. The mechanism by which cardiac MLC-2 gene expression is repressed in differentiated noncardiac muscle tissues is unknown. Using sequential 5′-deletion mutants of the cardiac MLC-2 promoter introduced into primary skeletal muscle cells in culture, we have demonstrated that a 89-bp region, designated the cardiac-specific sequence (CSS), is essential for repression of cardiac MLC-2 expression in skeletal muscle. Removal of the CSS sequence alone allows transcription in skeletal muscle cells without affecting the transcriptional activity of the promoter in cardiac muscle cells. DNase I footprinting and gel shift assays indicate that protein binding to sequences in the CSS domain occurs readily in nuclear extracts obtained from skeletal muscle but not in extracts isolated under identical conditions from cardiac muscle. Thus, it appears that a negative regulatory mechanism accounts for the lack of expression of the cardiac MLC-2 gene in skeletal muscle and that the CSS element and its binding proteins are important functional components of the regulatory apparatus which ensures the developmental program for cardiac tissue-specific gene expression.

The mechanism of regulation of tissue- and developmental stage-specific expression of eukaryotic genes involves specific interactions of *cis*-acting DNA segments with regulatory protein factors (see references 42, 45, and 53 for reviews). Among the *cis*-acting elements, enhancers, conserved promoter elements such as TATA, CAAT, and SP-1 binding sites, and several nonconserved regulatory elements (10, 38, 42, 45, 68) have been characterized. Protein factors that interact with these and other target DNAs with sequence specificity have been identified (3, 10, 11, 22, 33, 34, 41, 47, 50, 52, 61, 62, 68). Genes containing positive and negative regulatory elements are known (5, 18, 28, 51, 54, 55, 65, 72), and it is understood that a concerted activity of both positive and negative *cis*-acting elements and their interaction with factors ensure cell specificity and the appropriate level of expression during development (2, 4, 9, 12, 69, 70).

The genes for muscle contractile proteins are differentially expressed in different muscle and nonmuscle cell types and provide a useful system for investigation of regulatory mechanisms (see references 21 and 64 for reviews). Several *cis*-acting elements have been implicated in regulation of muscle tissue-specific transcription (4, 8, 17, 28, 29, 32, 35, 40, 63, 66, 67). Among these elements, an evolutionarily conserved element, $CC(A/T)_6GG$ (CArG box or C/BAR), has been assigned a role in up regulation of muscle tissue-specific gene transcription (4, 46), and an enhancer element in the creatine kinase gene, which recognizes a myocyte-specific enhancer-binding factor (MEF-2) in skeletal muscle, has been described (25). The cardiac troponin T promoter contains two copies of a conserved motif, M-CAT, which are involved in muscle tissue specificity (43). However, not all muscle genes have homologies to MEF and M-CAT sequences, and no single element(s) or factor(s) has been identified that would suggest the existence of a common regulatory mechanism for muscle tissue-specific transcription. The discovery of several skeletal muscle-specific determination and differentiation factors, including myogenin (20), MyoDI (14), and Myf-5 (7), has provided a key step toward an understanding of myogenic lineage, and the data suggest that these factors may be involved in mechanisms by which expression of diverse genes is regulated. The lack of expression of these factors in cardiac muscle, however, appears to preclude their requirement for cardiac muscle development (20, 59).

The physiological expression of cardiac myosin light-chain 2 (MLC-2) is restricted to cardiac cells, at least in the late embryonic to adult stages (1, 57). However, it is believed that the cardiac MLC isoforms appear in both the cardiac and skeletal tissues during early fetal cell development (71), suggesting that the cardiac MLC-2 gene is capable of expression in early development but is repressed in late embryonic and adult noncardiac muscle tissues. To define the mechanism which controls the cardiac tissue- and developmental stage-specific expression of the cardiac MLC-2 gene, or its lack of expression in skeletal muscle, we isolated and characterized the chicken (75) and rat (30) cardiac MLC-2 genes. The promoters of the two genes share identical upstream nucleotide elements in the proximal promoter region of ≈70 bp, which is sufficient for the optimal level of transcription in muscle cells (8, 76). However, the proximal promoter alone does not confer cardiac muscle tissue specificity (see below). In this study, we have identified an upstream domain, designated the cardiac-specific sequence (CSS), located between −282 to −371 bp in the chicken MLC-2 gene, which is required for repression of MLC-2 promoter activity in skeletal muscle cells in transient expression assays. Deletion of the CSS domain alone allows transcription of the cardiac MLC-2 promoter in skeletal muscle without affecting the activity in cardiac muscle. By using DNase I footprinting and gel shift assays, we detected CSS-specific nuclear protein factors (CSFs) in skeletal mus-

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cle cells but not at the same level in cardiac extracts isolated under identical conditions, suggesting that the interaction of CSFs with the CSS element is involved in negative transcriptional regulational of the cardiac MLC-2 gene in skeletal muscle. Thus, on the basis of the repressor activity of the CSS element and the presence of CSFs in skeletal muscle, we believe that the CSS and CSFs serve as important regulatory components of a complex mechanism which ensures the developmental program for cardiac tissue-specific expression of the MLC-2 gene.

MATERIALS AND METHODS

Cell culture. Heart and skeletal (thigh) muscle tissues excised from 13-day-old chicken embryos were used to prepare primary cell cultures. Heart tissue isolated aseptically was washed with phosphate-buffered saline (PBS), and cells were dissociated by successive digestion with 0.1% pancreatin. Dissociated cells from the first treatment were discarded, and those from the subsequent three treatments were combined, washed, and resuspended in growth medium (F-10; 10% fetal bovine serum [FBS], 10% horse serum, 100 U of penicillin per ml, 100 U of streptomycin per ml). Skeletal myoblasts were prepared by mechanically dissociating the tissue from the thigh muscle of the same embryo, and cells were cultured as described by Grichnik et al. (27), with minor modifications. The medium for skeletal myoblast contains 10% horse serum, 2% chicken serum, 100 U of penicillin per ml, and 100 U of streptomycin per ml. Both cell cultures were plated at a density of 1.5×10^6 cells per 100-mm plate. The cell line H9C2 (39) was grown in Dulbecco modified Eagle medium (DMEM)–10% FBS–1% penicillin-streptomycin, and the mouse skeletal muscle cell line C2C12 was cultured in DMEM–10% FBS–1% penicillin-streptomycin and plated at a density of 10^6 cells per 100-mm plate.

Transfection of cells and CAT assay. Cells were transfected by the calcium phosphate precipitation method (24). One day before transfection, cells were plated as described above and refed with fresh medium 3 h prior to the addition of DNA. Calcium phosphate-DNA precipitate was added, and 3 h later the medium was replaced with fresh medium; 20 μg of plasmid DNA per plate was used in each case. Cells were harvested after 48 h to prepare cell extracts. Chloramphenicol acetyltransferase (CAT) expression was assayed as described by Gorman et al. (24). The protein concentration of each cell extract was measured, and equal amounts of protein were used for all assays. To ensure reproducibility, experiments were repeated three to five times, using at least three different DNA preparations. When variations were encountered, results were expressed after normalization of DNA uptake, using a cotransfectant plasmid containing the luciferase-coding sequence (15).

Construction of deletion mutants. The construction of plasmid pLC106CAT, previously designated pLC5.2CAT, containing ≈1.3 kb of 5′-flanking sequence of the chicken cardiac MLC-2 gene fused to the CAT-coding sequence, was described earlier (75). pLC106CAT was linearized with *Sac*I and treated with Bal 31 for different lengths of time, and the deleted DNAs were then digested with *Bgl*I. The *Bgl*I-*Hin*dIII fragment containing the ampicillin resistance gene and the origin of replication obtained from pSV0CAT was ligated to the Bal 31-digested fragments and used to transform *Escherichia coli* HB101. The transformants were analyzed by restriction digestion with *Nde*I and *Eco*RI, and the deletion sites were confirmed by sequence analysis.

To construct the internal deletion mutant pLC431ΔCSS CAT, pLC431CAT was digested with *Xho*I and blunt ended by using the Klenow fragment of DNA polymerase I. The linearized pLC431CAT was digested with *Nde*I, and the resulting fragments were separated on an agarose gel. The small *Nde*I-*Xho*I fragment was then digested with *Rsa*I, and the purified *Nde*I-*Rsa*I fragment was ligated to the large *Nde*I-*Xho*I DNA vector described above. The resulting CSS-free plasmid is designated pLC431ΔCSSCAT.

Nucleotide sequence analysis of recombinant DNA molecules. Nucleotide sequence analysis of the recombinant DNA molecules was performed according to Maxam and Gilbert (44) or by the dideoxynucleotide procedure of Sanger et al. (58) by using Sequenase (U.S. Biochemical Corp.) as instructed by the supplier.

Preparation of nuclear extracts. Nuclear extracts were prepared by a modification of the method of Dignam et al. (16). All steps were performed at 4°C. Confluent cells were washed with PBS twice, resuspended in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–10 mM KCl–1.5 mM $MgCl_2$–0.5 mm dithiothreitol (DTT), and lysed with 10 strokes in a Teflon homogenizer. The cell lysate was centrifuged at 12,000 × *g* for 20 min, and the nuclear pellet was suspended in 20 mM HEPES (pH 7.5)–420 mM NaCl–1.5 mM $MgCl_2$–0.2 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride (PMSF)–0.5 mM DTT–25% glycerol and lysed as described above. The nuclear lysate was centrifuged at 12,000 × *g* for 30 min; the supernatant was dialyzed against 20 mM HEPES (pH 7.5)–100 mM KCl–0.2 mM EDTA–0.5 mM PMSF–0.5 mM DTT–20% glycerol and centrifuged at 12,000 × *g* for 5 min. The extract usually contained 4 μg of protein per μl and remained stable in liquid nitrogen for several months.

DNase I footprinting assay. DNase I footprinting was performed essentially as described by Jones et al. (36). A ^{32}P-end-labeled DNA fragment of 161 bp obtained from deletion mutant pLC371CAT was incubated with crude nuclear extracts (100 μg of protein each) of cardiac and skeletal myocytes in 50 μl of reaction buffer containing 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM KCl, 0.5 mM DTT, and 10% glycerol. Following incubation at room temperature for 45 min, DNase I digestion was performed with freshly diluted DNase I (1 U/μl; Bethesda Research Laboratories) for 60 s. The reaction was terminated by addition of 100 μl of buffer containing 200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, and 20 μg of carrier tRNA per ml. The mixture was subjected to phenol-chloroform extraction and analyzed on an 8% sequencing gel.

Gel shift assay. Double-stranded DNA fragments obtained by digestion of plasmid DNA with restriction enzymes were radiolabeled at the 5′ end by polynucleotide kinase and [γ-^{32}P]ATP and used for the binding assay. DNA fragments (2,000 cpm), 2 μg of poly(dI-dC), and 6 μg of nuclear extract protein were incubated in 10 mM HEPES (pH 7.5)–50 mM KCl–5 mM $MgCl_2$–0.5 mM EDTA–1 mM DTT–12.5% glycerol at room temperature for 30 min and separated on an 8% polyacrylamide gel with circulating Tris-borate buffer.

RESULTS

Tissue-specific expression of cardiac MLC-2 promoter/CAT recombinants. Consistent with the physiological expression of cardiac MLC-2, we have observed that the cardiac MLC-2 gene promoter fused to the CAT-coding sequence is expressed in cardiac but not skeletal muscle cells in a transient

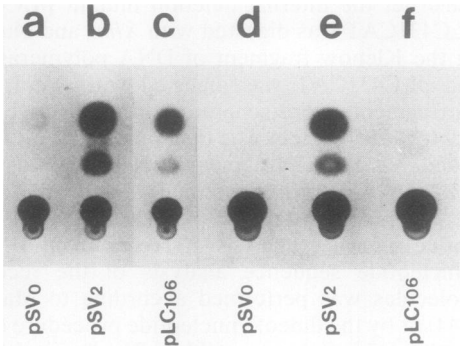

FIG. 1. Differential expression of plasmid pLC106CAT in chicken primary muscle cells. Chicken primary cardiac (lanes a to c) and skeletal (lanes d to f) myoblasts in culture were transfected with pLC106CAT containing the ≈1.3-kb 5′-flanking region of the chicken MLC-2 gene fused to the CAT-coding sequence. Cell extracts were processed for CAT assays as described in Materials and Methods. pSV0CAT and pSV2CAT were used as negative and positive controls, respectively.

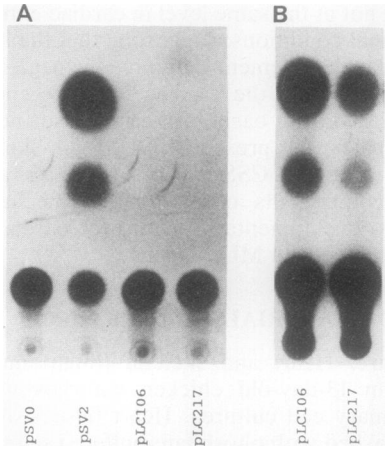

FIG. 2. MLC-2/CAT expression in cell lines C2C12 and H9C2. Plasmids pLC106CAT and pLC217CAT, containing ≈1.3 kb and 217 bp, respectively, of the 5′-flanking region of the MLC-2 promoter, were used to transfect mouse skeletal C2C12 (A) and rat cardiac H9C2 (B) myoblasts. Cell extracts obtained from differentiated myocytes 48 h after transfection were processed for CAT assays (see Materials and Methods). pSV2CAT and pSV0CAT served as positive and negative controls, respectively.

expression assay. Figure 1 shows CAT expression with extracts from 13-day-old primary cultures of chicken cardiac muscle cells transfected with pLC106CAT DNA. Plasmid pLC106CAT has a ≈1.3-kb DNA segment containing the promoter and 5′-flanking region of the cardiac MLC-2 gene fused to the CAT reporter sequence (75). No CAT activity was observed in skeletal muscle cells prepared from the same embryos in repeated transfections under identical conditions. This was true when the transfected cells were assayed for CAT expression either as proliferating myoblasts or as differentiated and multinucleated myotubes. Chicken fibroblast cells in culture, NIH 3T3 and HeLa cells, and the mouse and rat skeletal muscle cell lines L6 and C2C12 (see below) were also inactive for CAT expression driven by the MLC-2 promoter (data not shown). Several precautions were taken to minimize variations in CAT expression (see Materials and Methods). Equal amounts of protein extracts were used in duplicate from different plasmid preparations. Normalization of data for DNA uptake and quantitation of assays by scanning of underexposed films, direct measurements of radioactivity, or both indicated that variations were less than 12% in experiments done under identical conditions. Fibroblasts were routinely removed by differential attachment. To further exclude the possibility of artifacts caused by contaminating fibroblasts, transfection was also done in mouse skeletal muscle (C2C12) and rat cardiac muscle (H9C2) cell lines. Although derived from rat cardiac muscle, H9C2 is in several respects similar to skeletal muscle (39). Nevertheless, it was shown to support human cardiac α-actin gene expression and its regulation in transient transfection assays (31). The construct pLC106CAT did not express in the skeletal muscle cell line C2C12, whereas H9C2 cells supported MLC-2/CAT expression (Fig. 2). The lack of expression of cardiac MLC-2/CAT constructs in skeletal muscle cell lines is in agreement with earlier observations (1).

The cardiac MLC-2 promoter contains a transcriptional repressor element. To localize the *cis* element potentially involved in repression of MLC-2 gene activity in skeletal cells, a series of unidirectional 5′-deletion mutants was constructed (Fig. 3) and tested for CAT expression in both cardiac and skeletal primary cells in culture under identical conditions (Fig. 4). We started our deletions from ≈500 bp upstream of the mRNA start site, assuming that tissue-specific regulatory elements are located within this domain in the MLC-2 gene as found in the α-cardiac actin gene (26, 46). Tissue-specific expression of the promoter is maintained up to −410 bp relative to the mRNA start site, since plasmid pLC410CAT was active in cardiac cells but not in skeletal cells. On the other hand, plasmid pLC217CAT promoted almost the same level of CAT activity in both cardiac and skeletal primary muscle cells in culture. However, pLC217 CAT was consistently less active (≈45%) than pLC410CAT and pLC162CAT in both types of muscle cells, suggesting the existence of a positive regulatory element(s) within this domain. The presence of multiple positive and negative regulatory elements within 300 bp relative to the mRNA site in MLC-2 has previously been noted (8, 76). After normalization of the data (see Materials and Methods), the level of pLC162CAT expression was higher in cardiac muscle than in skeletal cells (cardiac/skeletal = 1.27 ± 0.09).

Additional unidirectional deletion mutants were tested for promoter activity (Fig. 5). Plasmids pLC410CAT, pLC381 CAT, and pLC371CAT, containing 410, 381, and 371 bp, respectively, of the 5′-flanking region, failed to express in skeletal muscle, whereas pLC282CAT and pLC264CAT, containing 282 and 264 bp, respectively, of the 5′-flanking region, promoted CAT expression effectively. The data in Fig. 5 represent averages of three separate experiments using two separate plasmid preparations. CAT activity is expressed as percent conversion of [^{14}C]chloramphenicol in each assay normalized to the level of pSV2CAT (24), used as a positive control. These results thus delimited a region, designated CSS, located between −371 and −282, which appears to be essential for repression of MLC-2 gene promoter activity in skeletal muscle cells. The sequences of the CSS domain and of other putative regulatory elements identified recently in our laboratory (41a) are shown in Fig. 6. The transcription start site in the MLC-2 gene (Fig. 6), established previously in this laboratory following transfection of COS cells, is 14 bp upstream of that proposed by Arnold et al. (1). Although variations in transcription starts

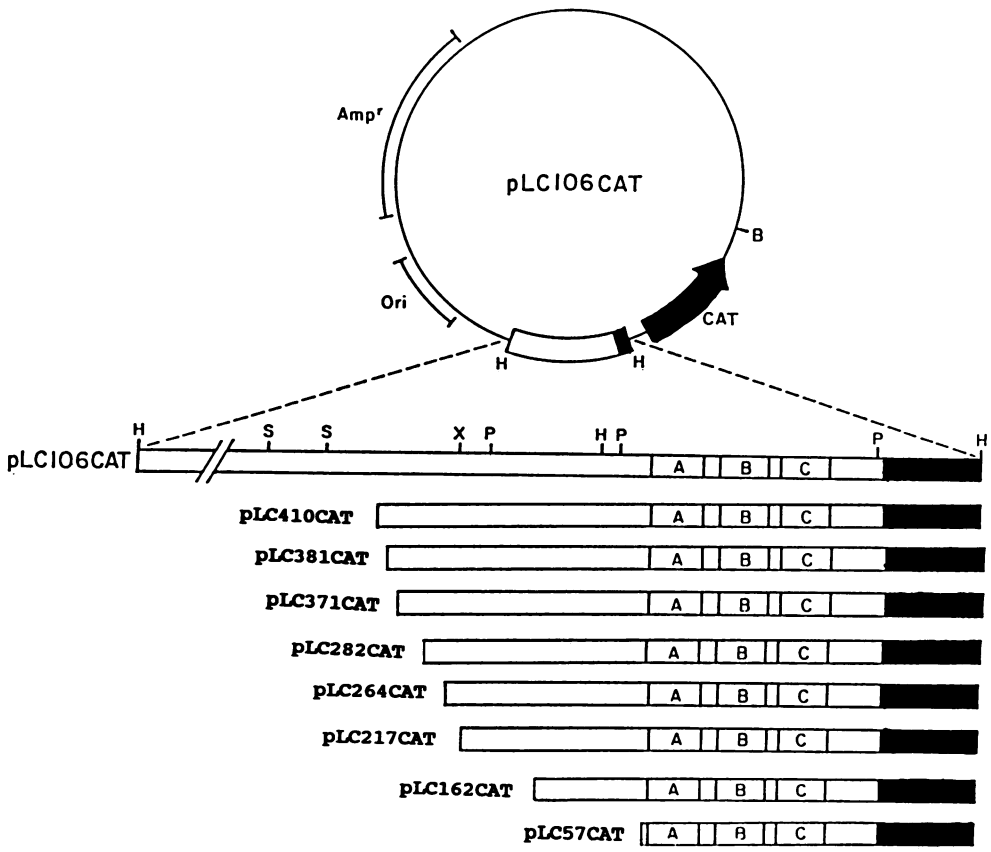

FIG. 3. Construction of various 5'-deletion mutants. The *Sac*I-linearized DNA of the parent plasmid pLC106CAT was digested with Bal 31 for different time periods and digested with *Bgl*I. The digestion products were ligated to a *Bgl*I-*Hin*dIII fragment of pSV0CAT and subsequently transformed into *E. coli* HB101. The cloned deletion mutants were analyzed by restriction mapping, and the deletion sites indicated were identified by nucleotide sequence determination. Restriction sites: S, *Sac*I; X, *Xho*I; P, *Pst*I; H, *Hin*dIII. A, B, and C are the functional domains in the proximal MLC-2 gene promoter (41a).

are known to occur in different host cells, the potential of two promoters in the MLC-2 gene would need to be established. We arbitrarily use the +1 site identified in our laboratory for the purpose of this study.

The role of the CSS domain was also documented by an internal deletion of 112 bp, encompassing the CSS segment, from plasmid pLC431CAT. The resultant plasmid, pLC431 ΔCSSCAT (see Materials and Methods), was tested for CAT expression as described above. The internal deletion of the sequence from −339 to −227 alone in plasmid pLC431Δ CSSCAT removed the repressor effect, resulting in expression of the MLC-2 promoter in skeletal muscle cells (Fig. 7). Removal of the CSS sequence had no deleterious effect on CAT expression in cardiac cells. Two separate transfections were done using separate plasmid preparations, and identical results were obtained. To exclude the possible effects of the sequence upstream to −431, we also removed the CSS-containing fragment from the parent plasmid pLC106CAT and obtained identical results (data not shown).

The CSS domain contains DNA-binding sites for skeletal muscle nuclear factors. DNase I footprinting was performed on the CSS-containing DNA segment of the MLC-2 promoter to demonstrate interaction with putative regulatory protein factors in nuclear extracts isolated from skeletal and cardiac myocytes. We used a 161-bp DNA fragment obtained by *Xho*I-*Eco*RI digestion of the parent plasmid containing the entire CSS sequence. The nuclear extracts were prepared under identical conditions from well-differentiated skeletal and cardiac myocytes. The results (Fig. 8) indicate that extracts from skeletal muscle cells protect the DNA sequence spanning a region from −271 to −309 with intermittent hypersensitive sites. Two areas of protection (II and III), located between −271 and −278 (5'-AGATAACC-3') and between −289 and −295 (5'-GGGCACA-3'), are visible, and a third region (I) with a weak protection was observed at −299 to −309 (5'-GCACCGTGACG-3'). No other protection area was found. The lack of defined protection by several cardiac extracts prepared under identical conditions suggests that the CSFs are either absent or present in amounts below the threshold level required for DNA binding in cardiac muscle cells (see Discussion).

Nuclear extracts isolated from cardiac and skeletal muscle cells were also tested by mobility shift assays for binding to the 161-bp DNA containing the CSS element as described above. Equal amounts of nuclear proteins prepared under identical conditions were used for all assays. At least three and possibly four different complexes were obtained with extracts of skeletal muscle cells (Fig. 9). The cardiac muscle extracts were relatively inactive. To ascertain whether the absence of defined complexes in the cardiac extracts reflects the absence of CSFs and not poor activity of the extract itself, we tested another DNA probe containing the element A sequence, which binds to multiple proteins present in both cardiac and skeletal cells (41a). The element A-specific

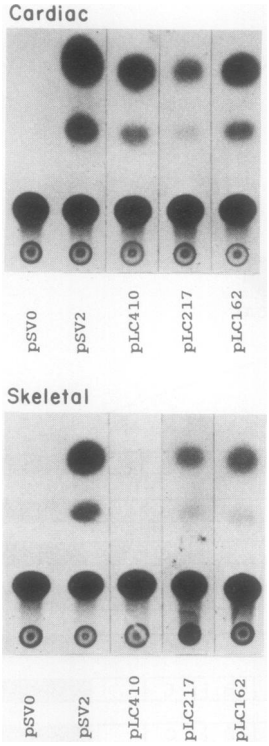

FIG. 4. Analysis of CAT activities of 5′-deletion mutants in chicken cardiac and skeletal myoblasts. Various 5′-deletion mutants derived from pLC106CAT by Bal 31 digestion as described in the legend to Fig. 3 and Materials and Methods were used to transfect primary cultures of chicken cardiac and skeletal cells prepared from 13-day-old chicken embryos. CAT activity was analyzed 48 h posttransfection.

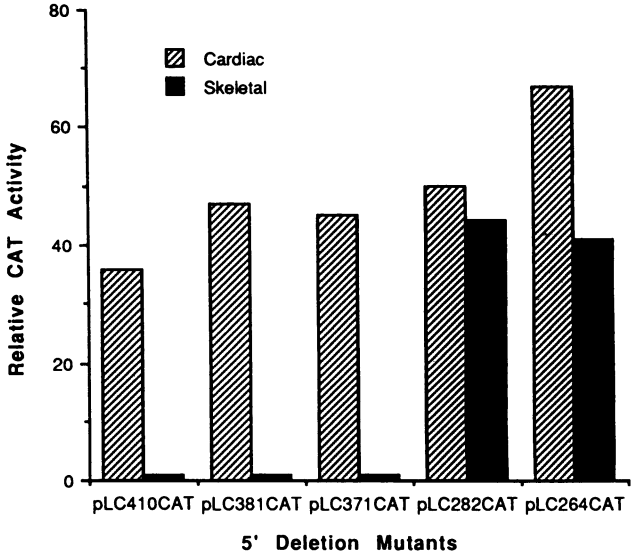

FIG. 5. Relative CAT activities of 5′-deletion mutants. Relative CAT activity represents the conversion of [^{14}C]chloramphenicol into its acetyl derivatives. Spots corresponding to chloramphenicol and its acetylated derivatives were cut out from the thin-layer chromatography plates, and radioactivity was measured. The relative CAT activity of each deletion mutant (20 μg per plate) is expressed as percentage of the average activity obtained by transfection of cardiac and skeletal cells by pSV2CAT (20 μg per plate). Deletion mutants were obtained by unidirectional 5′ deletion of pLC106CAT by Bal 31 endonuclease as shown in Fig. 3.

complexes were produced in approximately equal intensity in both extracts (data not shown). Oligonucleotides of 70 and 30 bases, containing the sequences in protection areas I plus II and III, respectively (Fig. 8), were synthesized and used as competitors in the binding assay. Protein complexes I and III were inhibited effectively by the 70-mer but not by the 37-mer, indicating that complexes I and III are produced by protein binding to sequences in protection areas I and II (Fig. 8).

These data, taken together, suggest that a negative transcriptional mechanism operates in skeletal muscle cells which represses transcription of the cardiac MLC-2 gene by way of binding of repressor proteins to a specific sequence element in protection areas I and II in the CSS domain. Further experiments, in progress, on functional characterization of the sequences with purified binding proteins will clarify the precise role(s) of the individual factors in control of cardiac tissue-specific transcription.

DISCUSSION

The tissue- and developmental stage-specific expression of muscle proteins is accompanied by a complex array of gene switches during myogenic differentiation (see reference 19), which suggests that both activation and repression of gene expression are involved in control mechanisms operating in developing muscles. There are multiple examples in which accumulation of contractile muscle gene products is directly attributable to activation of gene transcription. However, little is known about the process by which muscle gene products are repressed. Cardiac MLC-2 gene expression provides an excellent opportunity for investigation of these mechanisms, since the physiological expression of MLC-2 is restricted to cardiac muscle tissue in the late embryonic to adult stages, although it appears that during early development the cardiac MLC isoforms are present in noncardiac myogenic cells as well. The goal of the experiments presented here was to define the regulatory element(s) responsible for lack of expression of the cardiac MLC-2 gene in skeletal muscle.

Through the use of 5′-deletion mutants of the cardiac MLC-2 gene, we have established that the repression of MLC-2/CAT expression occurs in skeletal muscle and is mediated by an element contained in a 89-bp upstream sequence (CSS) of the gene promoter. All 5′ deletions containing the promoter sequence up to −371 bp retained cardiac tissue specificity, whereas plasmid pLC282CAT and other downstream deletion mutants expressed in both cardiac and skeletal cells. Therefore, a negative regulatory element (CSS) located between −282 and −371 is involved in the repression of CAT expression in skeletal cells. Negative regulation plays a role in a variety of eukaryotic genes (see reference 60 for a review), the molecular basis of which is not clear. Some negative elements are involved in regulation of tissue-specific gene expression (5, 13, 23, 37, 49, 55), while others have no apparent role in tissue specificity (56, 60). Several of these elements exert their influence in response to a particular stimulus. The findings reported here strongly suggest that in the negative regulation of cardiac MLC-2 gene expression in skeletal myocytes, the CSS element and the skeletal muscle-specific CSS-binding proteins play an important role.

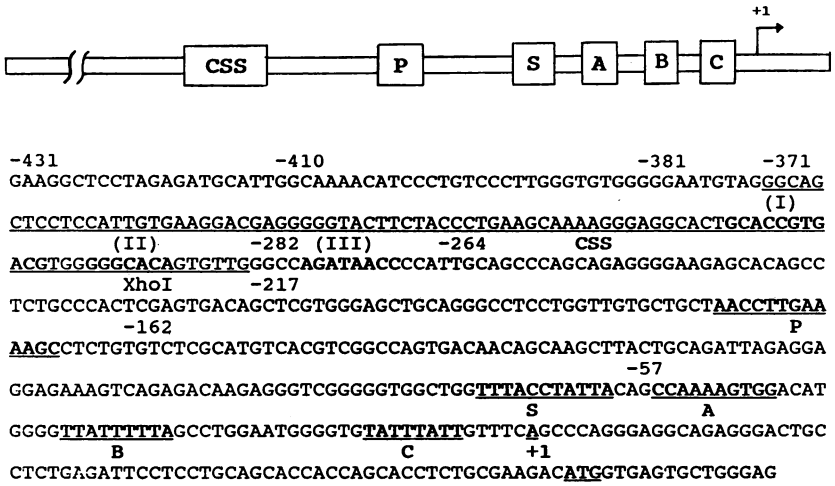

FIG. 6. Partial sequence of the chicken cardiac MLC-2 gene promoter. The nucleotide sequence of 431 bases of the 5'-flanking region of the chicken cardiac MLC-2 gene is shown. The transcription initiation site is marked +1 (see text). The *cis*-acting regulatory elements A, B, C, S, P (41a), and CSS are underlined. The positions of various 5'-deletion mutants derived from pLC106CAT by Bal 31 digestion are also indicated. Nucleotide sequences within the CSS element that are protected by nuclear factors in DNase I footprinting assays are indicated as I, II, and III.

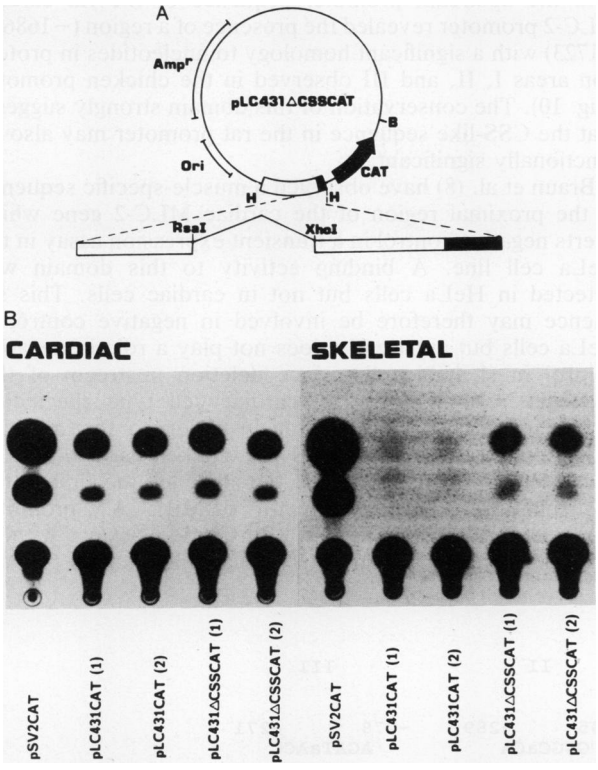

FIG. 7. Loss of tissue specificity by internal deletion of the CSS domain. Construction of the internal deletion mutant pLC431ΔCSS-CAT (A) is described in Materials and Methods. (B) CAT assay. pLC431ΔCSSCAT and pLC431CAT (with the CSS element) were used to transfect primary cultures of chicken cardiac and skeletal cells. Transfection conditions and the CAT assay, done in duplicate (1 and 2), are described in Materials and Methods. pSV2CAT was used as a positive control.

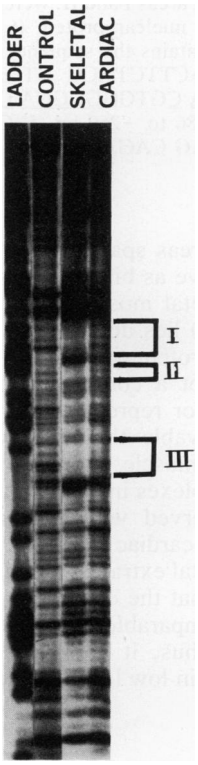

FIG. 8. DNase I footprinting of CSS DNA. A 161-bp DNA fragment containing the CSS element obtained from pLC371CAT was end labeled with [^{32}P]dATP and polynucleotide kinase. The footprinting assay was performed with freshly diluted DNase I (see Materials and Methods). The reaction mixture was analyzed by 8% polyacrylamide gel electrophoresis. Lanes: ladder, G-specific reaction product obtained in a Maxam-Gilbert (44) sequencing reaction; control, DNase I digestion product of the fragment without addition of nuclear proteins; skeletal, DNase protection of CSS DNA by the skeletal nuclear extract; cardiac, DNase protection of CSS DNA by the cardiac nuclear extract. The same amount of nuclear extract was added in cardiac and skeletal lanes. The three nucleotide regions protected by skeletal nuclear extracts are shown as I, II, and III.

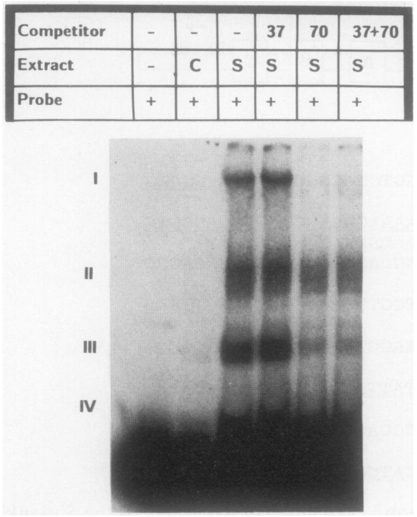

FIG. 9. Gel shift assay. A 161-bp DNA fragment produced by *Xho*I-*Eco*RI digestion of pLC371CAT containing the CSS element was used. A ^{32}P-labeled fragment was allowed to react with nuclear extracts from cardiac (C) and skeletal (S) cells separately and then fractionated on an 8% polyacrylamide gel. Competitor DNAs, a 37-mer containing the sequence in protection area III (see Fig. 8) and a 70-mer containing areas I and II, were used in 100-fold excess, and equal amounts of nuclear protein (6 μg) were added in all assays. The 70-mer contains the sequence from −350 to −281 bp (5′-GACGAGGGGGTACTTCTACC CTGAAGCAAA AGGGAG GCAC TGCACCGTGA CGTGGGGGCA CAGTGTTGGG-3′), and the 37-mer is from −286 to −250 bp (5′-GTTGGGCCAGATAAC CCCAT TGCAGCCCAG CAGAGGG-3′).

Three sequence areas spanning −309 to −271 bp within the CSS domain serve as binding sites for potential regulatory factors of skeletal muscle origin. One of the binding sites (−278 to −271) lies downstream of the CSS domain, suggesting that the protein which binds to the sequence from −278 to −271 is not a component of the transcriptional apparatus required for repression of promoter activity, although it is conceivable that it plays an auxiliary role. Binding of skeletal muscle nuclear proteins to CSS DNA produces three complexes in gel shift assays. Poor complex formation was observed with the cardiac extracts. The possibility that the cardiac extracts used here were less active than the skeletal extracts was discounted on the basis of the observation that the complexes formed with another DNA probe were comparable in intensity in both cardiac and skeletal extracts. Thus, it appears that the CSS-binding proteins are present in low levels in cardiac extracts, which may facilitate the optimum expression of MLC-2 gene in cardiac muscle cells.

To date, there is no evidence for a factor(s) which mediates the muscle cell-type-specific transcription of muscle genes. The CArG-box-binding factor, which is involved in up regulation of muscle-specific genes, does not appear to be muscle tissue specific (73). The chicken MLC-2 gene contains a sequence element (element A) with a striking similarity to the CArG box. However, element A is not required for cardiac muscle expression, although its removal reduces MLC-2 gene transcription significantly in cardiac muscle (41a). The myocyte-specific enhancer-binding factor MEF (48) of the creatine kinase gene recognizes other conserved elements in multiple genes. The cardiac troponin T gene, which is transcribed constitutively throughout cardiac development but only transiently in skeletal muscle, contains two copies of a conserved sequence motif (5′-CATTCCT-3′) which are required for cardiac troponin T expression in skeletal muscle (43). The binding factors are, however, present in both muscle and nonmuscle tissues. Thus, to date no single factor which dictates muscle tissue specificity has been reported, and clearly the mechanism by which the gene discriminates the cardiac cell environment from that of skeletal muscle is unknown. Our results have demonstrated that cardiac MLC-2 tissue specificity is rendered via a negative regulation mechanism in skeletal muscle in which the CSS element and the binding factor(s) CSF play a pivotal role. A comparison of the sequences in the three DNase I protection areas in the chicken cardiac MLC-2 promoter with the upstream promoter sequence of the rat cardiac MLC-2 promoter revealed the presence of a region (−1686 to −1723) with a significant homology to nucleotides in protection areas I, II, and III observed in the chicken promoter (Fig. 10). The conservation of this domain strongly suggests that the CSS-like sequence in the rat promoter may also be functionally significant.

Braun et al. (8) have observed a muscle-specific sequence in the proximal region of the cardiac MLC-2 gene which exerts negative control in a transient expression assay in the HeLa cell line. A binding activity to this domain was detected in HeLa cells but not in cardiac cells. This sequence may therefore be involved in negative control in HeLa cells but apparently does not play a role in negative control in skeletal cells, since deletion upstream of this sequence results in loss of cardiac cell type specificity. Arnold et al. (1) have previously observed that plasmid LCpUC.CAT, which contains the chicken cardiac MLC-2 promoter, as in pLC106CAT, but includes the first intron sequence inserted into the vector, pEMBLCAT, promotes CAT expression in transient transfection assays without tissue specificity, i.e., in both cardiac and skeletal primary

	I	II	III
	−309 −299	−295 −289	−278 −271
Chicken	gCACcGtGACg	GGGCaCA	AGATaACC
	−1723 −1712	−1700 −1693	−1693 −1705
Rat	cCACaGgGACa	GGGCcCA	AGATcACC

FIG. 10. Sequence comparison showing that the rat cardiac MLC-2 promoter contains CSS-like sequences. Comparison of the rat and chicken cardiac MLC-2 promoters reveals sequences homologous to protection areas I, II, and III (see Fig. 8) in the CSS domain at positions −1686 to −1723. The conserved nucleotides are in block letters. The element I-like sequence is present in the rat promoter at positions −1723 to −1712. The element II- and III-like sequences are present at positions −1693 to −1700 and −1712 to −1705, respectively.

muscle cells, in culture. In the same study, the authors noted that the in vivo expression of cardiac MLC-2 was nonetheless restricted to cardiac cells. We reexamined the activity of LCpUC.CAT (kindly provided by H. H. Arnold) along with that of pLC106CAT in our laboratory and confirmed that while pLC106CAT is inactive, LCpUC.CAT is expressed readily in skeletal muscle (data not shown). Since the difference between the two constructs lies in the presence of additional ≈0.8-kb intron sequence in LCpUC.CAT, we assume that another regulatory element resides in the intron region which apparently compensates for the negative regulatory effect of the CSS domain in skeletal muscle. Preliminary experiment in our laboratory suggested that the intron contains a potent activator element which has the ability to override the negative effect of the CSS sequence in skeletal muscle. This observation explains why Arnold et al. (1) failed to demonstrate muscle cell type specificity with the construct that they used, which includes the first intron. There are several examples in which muscle-specific genes with a large first-intron sequence contain functionally significant sequence domains within the intron which are required for tissue- and differentiation-specific expression (40, 67). One could envisage that expression and repression of the MLC-2 gene are related to the availability of repressor- and activator-binding proteins. The lack of the optimum level of CSFs in cardiac muscle extracts, as demonstrated in these studies, may account for expression of the gene in cardiac cells by extinguishing the repression mechanism. However, the optimal gene activity is achieved by way of participation of the activator element in transcription. A balance between the repressor- and activator-binding proteins may be necessary for programmed expression of the MLC-2 gene during development. Conceptually, cell-type-specific expression and developmental changes of MLC-2 promoter activity may be achieved via a preferential or combinatorial use of the positive and negative factors. Identification of the CSS and the putative activator in the intron provides a means to study the developmental changes in binding proteins, and we anticipate that such variations can be experimentally demonstrated. Ultimately, however, the overall developmental program involves not only multiple regulatory regions but a multiplicity of regulatory protein-protein and protein-DNA interactions as well. A variety of positive and negative elements with disparate functions have been described in several muscle genes (6, 12, 40, 48, 67, 74). However, myogenic cell-specific motifs critical for one gene (43) are not necessarily required for the transcription activity of others (12). Therefore, the presence or absence of binding proteins specific for a presumptive regulatory domain does not necessarily guarantee a single unifying mechanism for muscle tissue-specific gene transcriptions. Ongoing studies in our laboratory are aimed at identifying the specific regulatory protein factors and exploring their roles in MLC-2 gene regulation.

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