Two Distinct Factor-binding DNA Elements in Cardiac Myosin Light Chain 2 Gene Are Essential for Repression of Its Expression in Skeletal Muscle

ISOLATION OF A cDNA CLONE FOR REPRESSOR PROTEIN NISHED*

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The expression of the cardiac myosin light chain 2 (MLC2) gene is repressed in skeletal muscle as a result of the negative regulation of its transcription. Two regulatory elements, the cardiac specific sequence (CSS) located upstream (~360 base pairs) and a downstream negative modulatory sequence (NMS), which function in concert with each other, are required for repression of the MLC2 promoter activity in skeletal muscle. Individually, CSS and NMS have no effect. Transient transfection analysis with recombinant plasmids indicated that CSS- and NMS-mediated repression of transcription is position- and orientation-dependent and is transferable to heterologous promoters. A minimal conserved motif, GAAG/CTTC, present in both CSS and NMS, is responsible for repression as the mutation in the core CTTC sequence alone was sufficient to abrogate its repressor activity. The DNA binding assay by gel mobility shift analysis revealed that one of the two complexes, CSSBP2, is significantly enriched in embryonic skeletal muscle relative to cardiac muscle. In extracts from adult skeletal muscle, where the cardiac MLC2 expression is suppressed, both complexes, CSSBP1 and CSSBP2, were present, whereas the cardiac muscle extracts contained CSSBP1 alone, suggesting that the protein(s) in the CSSBP2 complex accounts for the negative regulation of cardiac MLC2 in skeletal muscle. A partial cDNA clone (Nished) specific for the candidate repressor factor was isolated by expression screening of the skeletal muscle cDNA library by multimerized CSS-DNA as probe. The recombinant Nished protein binds to the CSS-DNA, but not to ΔCSS-DNA where the core CTTC sequence was mutated. The amino acid sequence of Nished showed a significant structural similarity to the sequence of transcription factor "runt," a known repressor of gap and pair-rule gene expression in Drosophila.

The acquisition of the differentiated phenotype of eukaryotic cells is a consequence of activation of tissue-specific genes and repression of other genes, both of which are precisely controlled during development of multicellular organisms (1). Since only a small population of genes is expressed at any given time in the differentiated cell, it is becoming increasingly clear that the mechanisms by which genes are repressed are as important as those that activate them (2–4). Repression of transcription is commonly achieved via binding of the negative regulators to cis-elements where the degree of repression is controlled by the location and/or orientation (5–9), structure (10), or copy number (11) of the regulatory elements. Other mechanisms of transcriptional repression involve occupation of the activator binding site (12–14), squelching of factors via protein–protein interaction (15), and formation of a dimeric complex that binds DNA but lacks the activation domain (16–18).

The coordinate expression of muscle-specific genes during myogenesis in differentiated myocytes suggests the existence of a tightly controlled regulatory program involving a cascade of expression of specific positive and negative transcription factors (19). We have shown previously that the tissue-specific expression of the chicken cardiac myosin light chain 2 (MLC2) gene is regulated by both positively and negatively acting cis-elements and their cognate DNA-binding factors (20–24). The regulation is due to the activators CArG box (20, 25) and the myocyte enhancer factor 2 binding sites (26), and a negative regulatory region, cardiac specific sequence (CSS), responsible for repression of cardiac MLC2 transcription in skeletal muscle (21). Removal of CSS alone restores cardiac MLC2 expression in skeletal muscle without impairing its function in cardiac muscle cells (21). An upstream negative regulatory domain distinct from CSS also exists in the rat MLC2 gene promoter, mutation of which led to ectopic expression of the gene in transgenic animals (27).

In this report, we have delineated the regulatory domains within CSS essential for repression of the cardiac MLC2 promoter in skeletal muscle. There are three distinct protein binding sites, CSS-A, CSS-B, and CSS-C, each of which contains a common sequence motif, GAAG/CTTC. Mutation in the CTTC motif in CSS-B alone was sufficient to abrogate totally both DNA-protein interaction and the inhibitory function of CSS. CSS-mediated repression, however, requires the presence of another downstream sequence element, the negative modulatory sequence (NMS), which also contains the conserved GAAG motif and serves as the binding site for nuclear proteins. Neither of the two motifs alone can repress transcription. The

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1 The abbreviations used are: MLC2, myosin light chain 2; CSS, cardiac specific sequence; NMS, negative modulatory sequence; MCK, muscle creatine kinase; bp, base pair(s); CAT, chloramphenicol acetyltransferase; ANG, angiotensinogen; LUC, luciferase; CMV, cytosme-lovirus; GMSA, gel mobility shift assay; MOPS, 4-morpholinopropane-sulfonic acid; IRE, intron-responsive; IPTG, isopropyl-1-thio-β-D-galactopyranoside; β-Gal, β-galactosidase.
CSS/NMS-binding protein complex. CSSBP2, is present at a significantly higher level in nuclear extracts from skeletal muscle relative to cardiac muscle, suggesting that CSSBP2 binding to CSS/NMS accounts for repression of the MLC2 promoter function in skeletal muscle. In an attempt to identify the protein(s) involved in the repression mechanism, we have isolated a partial CDNA clone (Nished; Sanskrit for “negative”) by expression screening of the cDNA library derived from chicken skeletal muscle mRNA by multimierized CSS-DNA as a probe. The predicted amino acid sequence of Nished shows a significant similarity to two previously described repressors, runt from Drosophila (28) and SP3 from human (29).

**MATERIALS AND METHODS**

**Construction of Plasmids**—To construct CSS-containing muscle creatine kinase (MCK) promoter/reporter recombinant, the proximal MLC2 promoter (−160 to +158) in pMLC410CAT was replaced by the 246-bp HindIII fragment containing the MCK basal promoter from plasmid E4 (30). MCK enhancer contained within a 300-bp BamHI restriction fragment was then introduced into the unique BamHI site to generate MCKCSS. Likewise, the proximal MLC2 promoter (−160 to +158) of pMLC410CAT was replaced by the 2,500-bp MCK promoter/enhancer fragment (26) to construct MCKCSS. The resultant restriction fragment from pMLC371CAT that contains the CSS domain was cloned upstream to the pANG700 promoter (31) and designated as pANG700CSS. To construct pANGCSS, the HindIII-PstI fragment from pMLC371CAT was cloned into the plasmid pBasic CAT in the HindIII and PstI sites in the polylinker. The 264-bp Ncol-XhoI fragment of ANG proximal promoter was then cloned downstream to CSS in the polylinker at SalI-XhoI to produce pANGCSS. The PstI fragment (−130 bp to +40 bp) from the MLC2 promoter was cloned into the PstI site in pBasic CAT to construct PST. A 160-bp HindIII-PstI fragment containing the CSS domain was spliced in the corresponding sites in the polylinker of pBasic CAT; the resultant plasmid was then linearized with PstI and ligated to the PstI fragment (−160 to +158) containing the MLC2 promoter to create plasmid CSSPST. A synthetic oligonucleotide harboring the CSS sequence (GAAATTCGTTATTCTGATACGGGTGTGGAACACCT3′) was cloned into the HindIII site of polylinker of pBluescript-SK±. Plasmid with the CSS domain in forward and reversed orientations was constructed into host cells BL(21)DE3. Induction was done according to manufacturer’s (Novagen) instruction.

**Northern Blot Hybridization**—Poly(A)± was made from cardiac and skeletal muscle of an adult chicken following the FastTrack mRNA isolation kit (Invitrogen). Briefly, 1 g of tissue was homogenized in 15 ml of lysis buffer containing RNase protein degrader and incubated at 450 °C for 60 min. The lystate was centrifuged at 4,000 × g for 5 min at room temperature. The supernatant was recovered and 950 µl of 5 µ M NaOAc and ethanol was added and incubated with gentle rocking for 60 min at room temperature. Then, the samples were centrifuged at 3,000 × g for 5 min, and the supernatant was removed carefully from the oligo(dT) bead. The beds were washed three times with 20 ml of binding buffer; the supernatant was removed after a spin of 3,000 × g for 5 min, followed by a wash in low salt buffer (three times). The poly(A)± was eluted in 200 µl of 3 M NaOAc. 10 µg of poly(A)± from cardiac and skeletal muscle was loaded in 1.3% agarose/formaldehyde in 1× MOPS, as described previously (32, 33).

**RESULTS**

**CSS Element Represses Transcription of Heterologous Promoters**—Previous studies in our laboratory (21) have identified a negative regulatory region, CSS, located between −371 and −282 bp in the chicken cardiac MLC2 gene promoter, which is required for repression of cardiac MLC2 gene transcription in skeletal muscle cells. To test the potential role of CSS in repressing the transcription of heterologous promoters such as skeletal MCK (30) and the non-muscle rat ANG (31) promoters, we used plasmid pMCKCSS and pANGCSS containing CSS in the respective promoters in a transient transfection assay in skeletal muscle cells in culture. pMCKCSS expression was repressed effectively (80%) compared with that of parent plasmid pMCK without CSS (Fig. 1A). When CSS was placed 2.5 kilobases upstream to the MCK promoter (see “Materials and Methods”), the activity of the resultant plasmid, MCK2.5CSS, was the same as the parent plasmid pMCK2.5, suggesting that repression of transcription by CSS is position-dependent (Fig. 1A). Similar results were obtained with the CSS-containing ANG promoter, which is expressed optimally in liver and at a lower level in skeletal muscle. As shown in Fig. 1B, pANGCSS activity was repressed significantly (52%) relative to the level of the ANG promoter lacking CSS (pANG). The expression of plasmid containing CSS placed 700 bp upstream in the ANG promoter (pANG700CSS) was not repressed. When CSS was fused to the with a Wheaton dounce homogenizer. Nuclei were collected, and protein extracts were prepared as described previously (23, 33).

**Gel Electrophoretic Mobility Shift Assay (GMSA)**—Double-stranded oligonucleotide was end labeled with [γ-32P]ATP and 0.5 ng of labeled oligonucleotide, incubated with 2 µg of poly(I·dC), 1–12 µg of protein in 20 µl Hepes, pH 7.5, 3% mouse embryonic and MglCl1, 1 mM dithiothreitol, 2 mM EDTA, and 50 mM KCI at 4 °C for 30 min. Competitor DNA was added in a 100-fold excess following incubation for 30 min on ice, and the reaction mixtures were analyzed by electrophoresis as described previously (23).

**DNase-I Footprinting Assay**—A footprinting assay was performed using embryonic and adult skeletal muscle essentially as described earlier (24). A 160-bp EcoRI-XhoI fragment containing the CSS domain was incubated with nuclear extract (10–60 µg of protein) from embryonic and adult cardiac and skeletal muscle in a 50-µl reaction buffer containing 20 µl Hepes, pH 7.5, 5 µg MglCl1, 0.1 mM EDTA, 50 mM KCI, 0.5 mM dithiothreitol, and 10% glycerol. Following incubation at room temperature freshly diluted DNase-I (1 µg/ml) was added and then allowed to incubate for 60 s. DNA was extracted with phenol/chloroform and analyzed on an 8% sequencing gel. For footprinting of the NMS domain, a PstI-HindIII fragment spanning the NMS region was labeled by end filling.

**Screening of Expression Library from Skeletal Muscle with CSS-50**—Chicken skeletal muscle cDNA expression library cloned in the α zap expression vector was screened with a DNA sequence with nucleotides altered to introduce specific mutations and through sieves of 90 µm and 45 µm sequentially. Preplating was done three times, for 1 h each, to facilitate differential removal of fibroblasts. Cells were plated at a density of 2 × 106 cells/10-cm dish. Cultures were lysed in lysis buffer (20 mM Hepes, pH 7.6, 20% glycerol, 1 mM NaCl, 1.5 mM MglCl1, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (10 µg/ml)
FIG. 1. CSS represses heterologous promoter activity. Panel A, recombinants MCK, MCK2.5, MCKCSS, and MCK2.5CSS (see “Materials and Methods”) were transiently transfected into primary skeletal muscle cell cultures. CAT assays were performed after 48 h of transfection, and the CAT activity was normalized using CMVβ-Gal expression as an internal control. Panel B, plasmids pANG, pANGCSS, pANG700, and pANG700CSS (see “Materials and Methods”) were transfected in primary skeletal muscle culture as in panel A along with CMVβ-Gal plasmid as an internal control. Panel C, plasmids pTKCAT and pTKCSSCAT were transfected in skeletal muscle culture as in panel A. Each histogram represented by error bars is a mean of four separate experiments with standard deviations. A schematic diagram of each plasmid and the position of the CSS domain in each construct are shown (bottom). kb, kilobases.
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Identification of Protein Binding Domains in CSS—To identify the nucleotide sequence within CSS involved in protein-DNA interaction, a DNase-I footprinting assay was performed using a 160-bp fragment that contains the CSS domain (−410 to −250) and nuclear extracts from adult and embryonic (13-day-old) chicken skeletal (soleus) muscle (Fig. 2). Three protected regions (CSS-A: 5′-CGATTGGAAGGAC-3′; CSS-B: 5′-GATACTTC-3′; and CSS-C: 5′-CTGAAGCAAAAG-3′), which together span between −360 and −310, and at least one hypersensitive site were detected. A common nucleotide sequence motif, GAAG, is present in regions A and C and the complementary sequence, CTTC, in region B. We have denoted this motif, GAAG, is present in regions A and C and the complementary sequence, GTTC in region B. The first and second lanes of each nuclear extract contained 3 and 6 µg of protein, respectively. The specificity of the complex formation was evaluated with a 160-bp fragment that contains the CSS domain (−371 to −227) of MLC2 promoter was end labeled and incubated with nuclear extracts from embryonic (Emb.) and adult (Ad.) skeletal muscle and subjected to partial digestion with DNase-I (1 µg/ml) as described under “Materials and Methods.” Lanes C and G represent the Maxam and Gilbert sequencing reaction; lane (−) is free DNA cleaved by DNase-I. 10 and 60 µg of total nuclear extracts were incubated with the CSS probe. Sequences of the protected regions are indicated on the right.

To define further the role of CSS-50, GMSA were done with recombinant DNA fragment containing an activator element in the first intron of the MLC2 gene, which also contains the GAAG/CTTC motif. When nuclear extracts from the adult (4 weeks old) skeletal and cardiac muscle tissues were compared as above, only CSSBP1 binding activity was present in the cardiac muscle, whereas the skeletal muscle tissue contained both CSSBP1 and CSSBP2 complexes (Fig. 3A). Since cardiac MLC2 is down-regulated in skeletal tissue, one may speculate that the relative abundance of CSSBP2 accounts for negative regulation of MLC2 gene in skeletal muscle. A barely visible complex was observed when CTTC in CSS-B was mutated to GGTC (CSS-B′) (Fig. 3B), suggesting that the binding activity of CSS-50 is primarily due to the CSS-B sequence.

The CTTC/GAAG Motif Is Essential for Repression—To investigate whether the sequence contained in CSS-50 alone is sufficient for repression or whether it acts in concert with other elements, we used the minimal basal MLC2 promoter containing the −121 to +158 fragment containing the three cis-regulatory elements, CArG box, myocyte enhancer factor 2 site, and TATA box, which are required for its optimal expression in cardiac muscle cells (21, 23). As shown in Fig. 4, the presence of the CSS-50 alone produced an effective (70%) inhibition of MLC2 transcription, as measured by the luciferase assay. Recombinant plasmid pMLC2CSS3'LUC, with CSS-50 in reverse orientation, was totally ineffective, suggesting that CSS is not a conventional silencer, as it is both position- and orientation-dependent. Since it was reported that the regulatory sequences of myocyte enhancer factor 2 (44), Sp1 (45) and E box (25) have an additive effect on transcription when present in multiple copies, we made recombinants with four copies of CSS, arranged in tandem, placed upstream in plasmid pMLC4CSSLUC. However, the presence of multiple copies of CSS in plasmid pMLC4CSSSLLUC did not cause additional repression of transcription compared with repression observed with the single CSS copy in pMLC2CSSLUC. When a substitution mutation (CTTC→GGTC) was introduced in window B in plasmid pMLC2CSSSLLUC, the repression due to CSS was disrupted, and the expression level of the mutant plasmid reached 80% of the activity of the parent plasmid pMLC2LUC. Additional mu-

Fig. 2. DNase-I protection assay. An EcoRI-XhoI DNA fragment from plasmid pLC371 CAT (−371 to −227) of MLC2 promoter was end labeled and incubated with nuclear extracts from embryonic (Emb.) and adult (Ad.) skeletal muscle and subjected to partial digestion with DNase-I (1 µg/ml) as described under “Materials and Methods.” Lanes C and G represent the Maxam and Gilbert sequencing reaction; lane (−) is free DNA cleaved by DNase-I. 10 and 60 µg of total nuclear extracts were incubated with the CSS probe. Sequences of the protected regions are indicated on the right.

Fig. 3. GMSA with CSS-DNA. Panel A, the GMSA shows the DNA-protein complex formed with nuclear extracts from 13-day-old embryonic and adult chicken heart and skeletal muscles. The resulting complexes were resolved on 8% nondenaturing polyacrylamide gel. The first and second lanes of each nuclear extract contained 3 and 6 µg of protein, respectively. The specificity of the complex formation was evaluated with 50-fold molar excess of the unlabeled oligonucleotide containing CSS (C) or IRE (I); both contain the GAAG/CTTC motifs. Panel B, GMSA performed as in panel A with CSS and CSSΔB where core B-element was deleted, as probes.
tations in the reverse complement GAAG sequence in window C exhibit no further loss of repression (data not shown). Since mutation in CSS-B window alone caused a loss of protein binding in CSS-50, it would appear that the conserved sequence CTTC in CSS-B is the primary target for binding the repressor protein(s), which also accounts for its loss of function upon mutation.

**CSS-mediated Function Is Dependent upon a Downstream NMS**—The plasmid pMLCLUC, used in the experiments above, contains the downstream sequence up to +158. An examination of the downstream sequence revealed that the conserved GAAG/CTTC motif is also present once in the 5'-untranslated region at +160 in the MLC2 gene. To test the potential involvement of this motif in repression, we constructed the plasmids PST and CSSPST, containing the basal promoter alone extending to +42, without and with CSS, respectively (see “Materials and Methods”) (Fig. 5). Plasmid CSSPST, which lacks the downstream sequence containing the GAAG motif but has the upstream CSS element, was surprisingly as active as PST, which lacks both the upstream and downstream elements. This would mean that CSS alone was unable to repress MLC2 transcription in skeletal muscle cells and suggests the requirement of the downstream GAAG sequence, which was absent in both constructs. To test this possibility, we inserted the GAAG contained in a short (17-mer) oligonucleotide (5'-tctagacctagaagacttctaga-3') downstream of the MLC2 promoter in CSSPST (see “Materials and Methods”). The results showed that CSSPSTNMS was active in repression of the promoter activity almost to the same extent as the native sequence NMS containing the motif in plasmid pMLC371. The downstream motif alone (PSTNMS) was inactive as repressor. These findings thus confirm the requirement of the downstream GAAG sequence which was supported further when insertion of another cis-regulatory sequence, the IRE region, present in the first intron of the MLC2 gene containing GAAGCTTC motif, also led to repression of the promoter.

To test whether NMS is recognized by DNA-binding proteins, we performed the DNase-I footprinting assay with the PstI-HindIII fragment, which includes the NMS region spanning from +40 to +158. As shown in Fig. 6A, there was a protected window corresponding to CTTCATG in the noncoding strand. Although a partial protection also occurred in regions flanking the CTTCATG sequence, the repression activity in plasmid CSSPSTNMS containing only the protected GAAG sequence (see Fig. 5) was sufficient to restore repression of the MLC2 promoter activity. The sequence flanking the GAAG motif has no resemblance to the native sequence flanking GAAG in the MLC2 gene. Additional analysis of the NMS-DNA-binding protein interaction was done by GMSAs with an oligonucleotide containing the NMS domain (140 to 170) and nuclear extracts from the embryonic and adult heart and skeletal muscles. As seen in Fig. 6B, the NMS-binding proteins are present in both cardiac and skeletal embryonic tissues. As with the CSS-binding proteins, the NMSBP1 binding activity was abundantly present in the skeletal extracts but to a lesser extent in cardiac extracts. The specificity of the complex formation was established by self-competition, with CSS and another GAAG-containing regulatory domain, IRE, all of which competed out the complex formation.

2 M. Dhar, E. M. Mascareno, and M. A. Q. Siddiqui, unpublished observations.
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**Fig. 6. Protein binding to the NMS domain.** Panel A, a Pst-HindIII fragment (+36 to +158) from MLC2 promoter was end labeled and incubated with nuclear extract isolated from adult chicken skeletal muscle for footprinting. Lanes G, A, T, and C indicate the Maxam and Gilbert sequencing reaction; lane (−) is free DNA cleaved by DNase-I. 10, 30, and 60 µg of total nuclear extract were used separately for protection. Panel B, a GMSA showing the binding profile between cardiac (C) and skeletal (Sk) from 13-day-old embryo or adult chicken. Liver (L) and brain (Br) nuclear extracts from adult chicken were also tested. The specificity of complex formation was evaluated using 50-fold molar excess of unlabeled oligonucleotides containing NMS (N), CSS (C), and IRE (I), as competitors. The DNA-protein complexes NMSBP1 and NMSBP2 are indicated by arrows.

**Isolation of a cDNA Clone (Nished) for CSS-binding Protein.**—As a prelude to identifying the CSS- and NMS-binding proteins and delineating their role in repression of transcription, we isolated a cDNA clone by screening an expression cDNA library made from the adult chicken skeletal muscle mRNA (Stratagene) with a high affinity DNA containing the multimerized CSS-50 element. Of several candidate clones, one with the largest insert was identified as a candidate for CSS-specific protein coding cDNA clone, referred to from here on as Nished, which contains an insert of 1,217 bp in length. The nucleotide and the predicted amino acid sequences are shown in Fig. 7. Plasmid pET29Nished, containing the Nished cDNA in-frame with the ATG from the pBS vector spans 205 amino acids.

**Fig. 7. Nucleotide sequence and predicted amino acid sequence of Nished cDNA.** The sequence for the Nished cDNA is shown. The cDNA contains 1,217 nucleotides. The predicted amino acid sequence in-frame with the ATG from the β-galactosidase fusion peptide of the pBS vector spans 205 amino acids.

**Fig. 8. GMSA with recombinant protein Nished.** The GMSA showed the partially purified recombinant protein Nished to CSS-50. The specificity of DNA binding was demonstrated by lack of binding to ACSS where the core sequence in CSS-B was mutated (see "Results"). The arrow indicates the position of the IPTG-induced DNA-protein complex. (+), induced; (−), uninduced.

**DISCUSSION**

The physiological expression of the chicken cardiac MLC2 is restricted to cardiac muscle (44–46). In this report, we identified the functional sequence elements within the upstream negative regulatory domain (CSS) essential for repression of the cardiac MLC2 gene expression in skeletal muscle (21). In addition, a downstream sequence domain, NMS, was identified which functions in concert with CSS. The conserved CTTC motif in CSS-B alone is essential for CSS-mediated repression of the cardiac MLC2 promoter, as the substitution mutation in this motif caused the loss of DNA-protein interaction and led to a significant reduction in CSS-mediated repression of tran-
scription. This motif is also present in the negative regulatory element (HF3) of the rat cardiac MLC2 promoter, mutation of which led to ectopic expression of the reporter luciferase activity in transgenic animals (27). A comparison of the nucleotide sequence of CSS and NMS with other genes indicated that GAAG/CTTC motif common to both elements is present in other known negative regulatory domains of several genes, such as retinoblastoma, human hypoxanthine phosphoribosyltransferase, yeast RME1, mouse albumin, rat MLC2, human neurotropic papovavirus JC virus late promoter (27, 28, 37, 41). Thus, it appears that the negative regulation of transcription, at least in the genes tested here, is mediated by a conserved sequence element (GAAG/CTTC). The prevalence of this motif in disparate promoters suggests that a common protein, presumably a family of proteins, is responsible for down-regulation of the respective genes. In this context, CSSBP appears to be the member of an active repressor family of proteins that have intrinsic repression activity by inhibition of transcription initiation. Repression is presumably achieved with specific modulation in the level of CSSBPs, most likely of CSSBP2, which exhibits increased binding activity in the adult skeletal muscle where the expression of cardiac MLC2 is optimally repressed.

Different mechanisms are used for the negative regulation of eukaryotic genes (for review, see Ref. 36). One of them involves interference with the binding of activators that recognize sequences that overlap the binding sequences of negative factors. The fact that CSS and NMS do not overlap with any known activator site in the MLC2 gene, nor do they serve as binding sites for other known regulatory factors, suggests that the mechanism of negative regulation based on binding of factors to the same sequence, but with the opposite effect (47–50), or via sterical hindrance due to overlapping sites (51), can be excluded. For repressors, as for activators, interaction with the components of the basal transcription machinery is common, although not essential (52). Some factors can repress transcription of certain promoters but not of others, suggesting the involvement of specific intermediary factors or cofactors (16). It is not uncommon for transcription factors to have different effects on transcription based on different locations within the same promoter or in different promoters. Some repressors become active only when they are overexpressed (17, 36, 54–56). CSS appears to function when positioned proximal to the promoter but requires, at the same time, the downstream GAAG motif. Thus the tissue-specific expression of the MLC2 gene is not the function of CSS alone; it depends on cooperative interaction with NMS.

Cooperation among multiple regulatory domains is, in general, the basis of negative regulation in several genes (36, 52, 55, 57–59), although bipartite promoter elements have also been identified in negative regulation (38, 60, 61). Interaction between distinct domains, presumably by virtue of the folding of DNA, leads to repression of transcription, such as of the insulin-like growth factor I receptor under negative regulation by Wilms' tumor-1 gene (62). The effect of Wilms' tumor-1 depends on the number of Wilms' tumor-1 binding sites located both upstream and downstream of the insulin-like growth factor I receptor transcription initiation site (63). Repressors that pose interference with the function and assembly of initiation complex without binding DNA have also been described (47–50). However, in our case, at least two protein-binding DNA motifs, located upstream and downstream of the transcription initiation site, are needed for repression, a situation analogous to the requirement for multiple sites for silencing of insulin-like growth factor I receptor or vimentin gene expression (63, 64). Interaction of the putative repressor protein(s) with multiple sites (CSS and NMS) located on both sides of the transcription initiation site might be a requirement for the physical and structural rigidity needed for the negative control of transcription initiation.

To understand further the CSS-mediated repression, we obtained a partial 1.2-kilobase cDNA encoding the CSS binding factor, Nished. Although the evidence on its involvement in repression of transcription is not yet available, the primary sequence analysis of the Nished cDNA revealed a similarity to other known repressors, such as runt of Drosophila melanogaster (28, 65), and SP3 (29). Runt is required for suppression of certain abdominal genes to limit the domains of engrailed expression in parasegmental pattern in Drosophila (16, 53). It has a conserved domain RHD, with an ATP binding box located within, which is essential for DNA binding and heterodimerization. Thus, Nished, based on its sequence similarity to these functional domains, appears to belong to a family of transcriptional inhibitors. Activation of Nished binding activity in skeletal muscle may be an important prerequisite for suppression of cardiac MLC2 gene expression in skeletal muscle. It is not yet known whether Nished is one of the two CSSBPs observed in skeletal nuclear extracts. The generation of Nished-specific antibodies, presently in progress in our laboratory, may help in elucidation of its role in repression mechanism(s) underlying the tissue-specific expression of cardiac MLC2.

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