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A Single Transcription Factor Binds to Two Divergent Sequence Elements with a Common Function in Cardiac Myosin Light Chain-2 Promoter

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The cardiac myosin light chain-2 (MLC-2) gene promoter contains several positive and negative *cis*-acting sequences that are involved in the regulation of its expression. We describe here the properties of two activator sequences, elements A and P, and their DNA-binding factors (ABFs). Element A (CCAAAAGTGG), located at −61, has homology with the evolutionarily conserved sequence CC(A/T)$_6$GG, present in the genes of many contractile proteins. Element P (TAACCTTGAAAGC), located 114 bp upstream of element A, is conserved in both chicken and rat cardiac MLC-2 gene promoters. Deletion mutagenesis demonstrated that these two elements are involved in the positive regulation of MLC-2 gene transcription. At least two sequence-specific element A-binding proteins, ABF-1 and ABF-2, were identified by gel shift analysis of the fractionated cardiac nuclear proteins. ABF-1 binds to element A with strict dependence on the internal element A sequence AAAAGT. In contrast, ABF-2 exhibits a relaxed sequence requirement, as it recognizes the consensus CArG and CCAAT box sequences as well. ABF-2 also recognizes the distal element P despite the fact that the sequences of elements A and P are divergent. DNase I footprinting, methylation interference, and gel shift analyses demonstrated unequivocally that the element A-DNA affinity-purified protein ABF-2 binds to element P with sequence specificity. Since both elements A and P play a positive regulatory role in MLC-2 gene transcription and bind to a single protein (ABF-2), it would appear that ABF-2 is a key transcription factor with the ability to recognize divergent sequence elements involved in a common regulatory pathway during myogenesis.

Eukaryotic genes are regulated primarily at the level of transcription initiation, which involves specific interactions between regulatory nuclear proteins and target gene sequence elements (see references 4, 26, and 29 for review). DNA-protein interactions in eukaryotic promoters are complex, as transcription may need to be regulated positively as well as negatively in both a temporal and spatial manner in response to developmental and environmental signals. It is now known that in many cases, multiple nuclear proteins recognize a single DNA element and thereby introduce a considerable degree of functional diversity to the promoter (1, 12, 34, 40). It is also known that nuclear proteins which exhibit relaxed stringency in DNA binding are involved in the regulation of transcription (6, 9, 37). The functional significance of degenerate-sequence recognition in transcriptional regulation is not yet clear.

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 15, 39, and 43 for review). Recent studies on the genes of muscle contractile protein have led to the identification of potential *cis*-acting regulatory sequences. Among these sequence elements, the motif CC(A/T)$_6$GG (CArG box or C/BAR) is found at least once in the 5′-flanking regions of contractile protein genes in many species (30, 31). This motif, which is recognized by serum response factor, has been proposed to be involved in muscle-specific regulation of transcription (19, 32). Nuclear factors are known to interact with this element with sequence specificity (18, 45). An enhancer element in the creatine kinase gene, which recognizes a myocyte-specific enhancer-binding factor (MEF-2) (18); a motif, M-CAT, in the troponin-T gene, involved in muscle tissue specificity (27); and a repressor element, CSS, involved in repression of the cardiac myosin light chain-2 (MLC-2) gene in skeletal muscle (42) have been described. The discovery of several skeletal-muscle-specific determination and differentiation factors, including myogenin (13), MyoD1 (8), and myf-5 protein (2), has provided a key step toward our understanding of the development of myogenic lineage and its control. The lack of expression of these factors in cardiac tissues, however, appears to preclude the requirement for these factors in cardiac-muscle-specific development (13, 41).

We have isolated and characterized the MLC-2 genes from chicken (48) and rat (20) cardiac muscles. The promoters of both genes have identical sequences in the proximal region, including an element, CCAAAAGTGG, designated element A, resembling the evolutionarily conserved CArG box, and an upstream element, element P, with sequence TAACCT TGAAAGC. Recent studies on sequential 5′ deletions of the MLC-2 promoter (3) and those in our laboratory (42, 49) have demonstrated that the chicken cardiac MLC-2 promoter contains several functional *cis* elements, including those mentioned above, which either activate or repress MLC-2 promoter activity. Among these elements, the elements A and P sequences have pronounced effects as activators of the MLC-2 promoter in transient expression of the reporter genes in cardiac muscle cells.

In an attempt to gain an understanding of how activators elicit their effect, we have isolated and analyzed the element A-specific DNA-binding proteins. We show here that there are at least two different proteins, activator sequence DNA-

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binding factors (ABFs), which bind to element A with different sequence requirements. In view of the sequence similarity of element A to the CArG and CCAAT box sequences, we tested the binding of ABFs to these sequences and found that ABF-2 has a relaxed sequence requirement, as it recognizes the CArG and CCAAT sequences in addition to element A, whereas ABF-1 binds to element A with strict dependence on the internal AAAGT sequence and does not recognize the CArG and CCAAT box sequences. Footprinting, methylation interference, and competition analyses demonstrated that ABF-2 also binds with high affinity to element P and thereby displays a considerable sequence degeneracy. The ability of ABF-2 to recognize two motifs of divergent sequences which mediate a common function suggests that the factor ABF-2 plays a key role in the regulation of cardiac muscle gene transcription.

MATERIALS AND METHODS

Cell culture, transfection, and CAT assay. Chicken embryonic hearts (13-day) were isolated aseptically, washed with phosphate-buffered saline (PBS), and treated four times with 0.1% pancreatin in PBS at 37°C for 15 min. The dissociated cells from the last three treatments were combined, washed, and resuspended in growth medium (F-10 with 10% fetal bovine serum, 10% horse serum, 100 U of penicillin per ml, and 100 U of streptomycin per ml). Fibroblast cells were eliminated by two differential attachments, 60 min each. Cell cultures were plated at a density of 10^6 cells per 100-mm plate and refed with growth medium the following day. Two days after being plated, the cells were transfected by the calcium phosphate precipitation method (17) with 10 μg of DNA per plate. Cells were refed with fresh medium 4 h prior to the addition of DNA. At 3 h after the addition of calcium phosphate-precipitated DNA, the medium was replaced with fresh medium, and cells were harvested after 48 h to prepare cell extracts.

Expression of the chloramphenicol acetyltransferase (CAT) gene was assayed as described previously (42). The CAT signal was measured by direct counting of radioactivity from the thin-layer chromatograms, and the CAT assay results were normalized to the pSV2CAT expression level, used as a positive control. Equal amounts of protein extracts were used for all measurements, and DNA from multiple plasmid preparations was used for most experiments. Occasional problems in the reproducibility of the CAT assay due to variations in DNA uptake were overcome by the use of a marker plasmid, pSV2ALΔ5 (10), harboring the luciferase gene, as a cotransfectant. The level of luciferase was measured by the method of Rodriguez et al. (38).

Site-directed mutagenesis and 5′ sequential deletions. Construction of element A substitution mutants was done by the method of Morinaga et al. (33). The parent plasmid pBC12LC5.2f (48) was digested separately with *Hin*dIII and *Pvu*II. The large fragment of the *Hin*dIII-digested plasmid was annealed with the *Pvu*II-linearized plasmid in the presence of a chemically synthesized oligonucleotide. Following ligation, the mixture was used to transform competent cells of *Escherichia coli* MC1061, and the transformants were screened with the ^{32}P-labeled oligonucleotide used in mutant construction. Retransformation, screening, and hybridization were done as described before (33), and the DNA sequence of the recombinant, pBC12LC5.2A′, was confirmed by chemical sequencing (28). A 284-bp *Hin*dIII-*Hin*dIII fragment containing the mutated element A sequence (TTGGGCCCGG) obtained from this plasmid was substituted for the original 284-bp *Hin*dIII-*Hin*dIII DNA in pLC106CAT. The resultant recombinant was designated pLC106A′CAT. 5′ sequential deletion mutants were constructed by digesting *Xho*I-linearized pLC106CAT with Bal 31 exonuclease and cutting with *Sma*I, followed by polymerase end-filling and self-ligation of the large fragment. The ligation mixture was then transformed into *E. coli* JM109, and the ends of the deletions were determined by DNA sequencing.

Preparation of nuclear extracts. Preparation of nuclear extracts was done by a modification of the method of Dignam et al. (11). Heart tissues were minced and washed with PBS two times, resuspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5)–10 mM KCl–1.5 mM $MgCl_2$–0.5 mM dithiothreitol (DTT), and lysed with 10 strokes in a glass homogenizer. The cell lysate was centrifuged at 3,000 × *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 above. The nuclear lysate was centrifuged at 12,000 × *g* for 30 min, and the supernatant was dialyzed against nuclear extract storage buffer (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 20 min. The extract usually contains 2 to 4 mg of protein per ml and remains stable in liquid N_2 for several months.

Gel mobility shift assay. Double-stranded DNA fragments obtained by renaturation of chemically synthesized single-stranded DNA were labeled at the 5′ ends by polynucleotide kinase and [^{32}P]ATP and used for the binding assay. DNA fragments (4,000 cpm), 2 μg of poly(dI-dC), and 6 μg of crude nuclear extract protein were incubated in 10 mM DTT–12.5% glycerol at room temperature for 30 min and separated on an 8% polyacrylamide gel with circulating buffer, as described previously (42).

Fractionation of nuclear extract proteins. Nuclear extract containing 2 to 4 mg of protein was dialyzed against 20 mM HEPES (pH 7.5)–50 mM KCl–0.2 mM EDTA–0.1 mM PMSF–0.1 mM DTT–2.5% glycerol and loaded onto a heparin-Sepharose CL-6B column (10 by 1 cm) equilibrated with the same buffer at 4°C. The column was initially eluted with 25 ml of the same 0.05 M KCl buffer, and then the KCl concentration was increased to 0.1 M. The proteins were step eluted at 0.1 M increments until the final concentration reached 1.0 M KCl. Twenty fractions, 5 ml each, were collected. The final elutions were at 1.5 and 2 M KCl. Proteins in all fractions were desalted, concentrated by centrifugation with Centricon-10 microconcentrators with a cutoff of 10 kDa (Amicon), and balanced with nuclear extract storage buffer by repeating this procedure at least three times.

Protection analysis with MPE-Fe. Protection analyses were also carried out with methidium propyl EDTA-iron(II) (MPE-Fe) solution as described by Van Dyke and Sawadogo (44). A 74-bp *Hin*dIII-*Pst*I fragment of the chicken MLC-2 promoter region containing element P and a synthetic oligonucleotide containing three element A sequences in tandem (nucleotide AAA) were end-labeled and incubated with crude nuclear extract (10 to 25 μg of total protein) or with DNA affinity-purified protein (0.5 to 10 ng of total protein) in a 60-μl total volume containing 20 mM HEPES (pH 7.9), 2% (vol/vol) glycerol, 0.1 M KCl, 0.5 mM PMSF, 0.5 mM DTT, and 1 μg of poly(dI-dC). Following incubation at room temperature for 30 min, the DNA mixture was treated with 6 μl of MPE-Fe/DTT solution for 5 min. MPE-Fe/DTT solu-

tion is composed of 1 mM MPE, 1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and 40 mM DTT and is made immediately prior to use by combining the MPE stock (stored at −70°C in H_2O at 1.2 mM) with freshly prepared stock solutions of the other two reagents. After MPE treatment, the DNA samples were loaded onto a 6% polyacrylamide–0.37× TBE gel with circulating buffer at 4°C. Following electrophoresis, protein-DNA complexes and unbound DNA fragments were eluted as described before (24). The DNA was then analyzed on an 8% acrylamide sequencing gel. DNase I footprinting with a 160-bp *Hin*dIII-*Hin*fI (−144 to +16) fragment containing elements S, A, B, and C was carried out as described before (27).

Methylation interference. The ^{32}P-end-labeled 74-bp *Hin*dIII-*Pst*I probe DNA was partially methylated with dimethyl sulfate before complex formation. The binding reaction was carried out as described above. The complexed and free DNA was localized on an 8% polyacrylamide gel, eluted and treated with piperidine, and analyzed on a sequencing gel.

UV-photoactivated protein-DNA cross-linking. Binding of radiolabeled probe DNA (synthetic oligonucleotide AAA or synthetic oligonucleotide P), 50,000 cpm each, with affinity-purified protein (10 to 20 ng of total protein) was carried out in a 30-μl reaction volume at room temperature for 30 min. For cross-linking, glass reaction vials were sealed with Saran Wrap and irradiated at 5 cm for 5 to 30 min at 240 nM in the UV Stratalinker 2400 (Stratagene), giving an equivalent of 4,000 μW/cm^2. The resulting complexes were separated from the unbound DNA on a 5% polyacrylamide gel and identified by exposure of the gel to X-ray film. The gel slices containing the protein-DNA complex were layered onto a 12% polyacrylamide–sodium dodecyl sulfate (SDS) protein stacking gel and electrophoresed along with protein markers. The gel was dried and exposed to X-ray film at −70°C with intensifying screens. Irradiation with a medium-wavelength UV light box (Photodyne) gave similar results.

Purification of element A-binding protein. Chicken cardiac nuclear extracts were prepared from 10- to 12-day-old embryonic hearts as described by Dignam et al. (11) and dialyzed against buffer (25 mM HEPES-KOH [pH 7.9], 12.5 mM $MgCl_2$, 20% glycerol, 0.1% [vol/vol] Nonidet P-40, 10 mM $ZnSO_4$, 1 mM DTT, 0.5 mM PMSF containing 0.1 M KCl). Elution buffers contained 1 μg each of aprotinin and leupeptin per ml. All procedures were performed at 4°C. Crude nuclear extract (≈250 mg [30 ml]) was applied to a wheat germ agglutinin (WGA) resin (Vector Laboratories) contained in a preequilibrated Bio-Rad Econo-column in the above-mentioned buffer at a flow rate of 15 ml/h. The resin was washed four times with 10 ml of buffer, and the *N*-acetylglucosamine-bearing proteins were recovered by elution with 10 ml of buffer containing 0.1 M KCl and 0.3 M *N*-acetylglucosamine (Sigma). This eluate (10 ml; total protein, ~1.1 mg) was mixed with poly(dI-dC) (25 μg; Boehringer Mannheim) for 15 to 30 min and then subjected to sequence-specific DNA affinity chromatography.

For DNA affinity column chromatography, oligonucleotide A was phosphorylated, annealed and concatenated (23), and then coupled to activated CNBr-derivatized Sepharose CL-4B (Pharmacia). Element A DNA affinity resin (2 ml) was equilibrated with the buffer alone containing 0.1 M KCl and mixed with 10 ml of eluate from the WGA column. The mixture was allowed to stand for 30 to 45 min and was then washed four times with 5 ml of the above-mentioned buffer containing 0.1 M KCl. The element A-binding protein factor(s) was eluted with 1.0 M KCl in buffer (1.5 ml), dialyzed, and stored in liquid N_2 in small aliquots.

RESULTS

Cardiac MLC-2 promoter activity is stimulated by elements A and P. Figure 1 shows the partial sequence of the chicken cardiac MLC-2 promoter (48) and its comparison with the rat cardiac MLC-2 promoter (20). The proximal region of the promoter contains sequence elements, indicated as P, S, A, B, and C, which are conserved in both promoters, suggesting that these sequences are functionally important. The identification of S, A, B, and C sequences as putative regulatory elements was also based on DNase I protection analysis with nuclear proteins from chicken cardiac muscle tissue (see Fig. 8A) (49). The element A sequence has a striking similarity to the evolutionarily conserved $CC(A/T)_6GG$ motif, which is implicated in transcriptional regulation of muscle genes (30).

The activator role of elements A and P in the chicken cardiac MLC-2 promoter was initially evident in a transient-expression assay with sequentially 5′-deleted promoter DNA fused to the CAT gene following transfection into chicken embryonic primary cardiac muscle cells in culture (49). The mutational analysis shown in Fig. 2 confirmed that elements A and P are involved in positive regulation of MLC-2 gene transcription. The level of CAT expression in chicken cardiac primary myoblasts driven by the sequence contained in pLCΔ58, which lacks element A, was lower (49%) than that driven by pLCΔ74, which contains element A (Fig. 2, top). A comparable effect was observed when the element A sequence alone was deleted from plasmid pLC106CAT and substituted with a nonspecific sequence (TTGGGCCCGG) by site-directed mutagenesis (see Materials and Methods) (Fig. 2, bottom). The resultant plasmid, pLCA′106CAT, exhibited 54% of the activity of the parent plasmid pLC106CAT, which contains ≈1.3 kb of the upstream sequence. A significant decrease in CAT expression was also observed when a 47-bp sequence was deleted from plasmid pLCΔ205CAT. Plasmid pLCΔ158CAT was only 40% as active as pLCΔ205CAT. Subsequent experiments (see below) showed that a 13-bp segment (element P) located in the 47-bp region was protected against chemical digestion. The level in pLCΔ74CAT was higher than that in pLCΔ158CAT, suggesting the presence of a negative element(s) between −158 and −74. Several proximal and distal positive and negative elements have previously been observed in the chicken cardiac MLC-2 promoter after sequential deletion analysis (3, 49). Several precautions were taken to minimize variations in CAT assays (see Materials and Methods). To exclude the possibility of artifacts caused by contaminating fibroblasts, transfection was also done in a myogenic cell line, H9C2, derived from embryonic rat cardiac muscle cells (22), which was shown to support human cardiac α-actin gene regulation in a transient-expression assay (19). Almost identical levels of reduced activity in pLCΔ58CAT relative to pLCΔ74CAT were obtained in H9C2 cells (data not shown).

Multiple nuclear proteins recognize element A sequence. Nuclear extract isolated from chicken embryonic heart tissue was fractionated on a heparin-Sepharose column with stepwise KCl elution as described above. An aliquot from each fraction was tested by the mobility shift assay for binding to a chemically synthesized double-stranded oligonucleotide probe containing three copies of the element A sequence (Fig. 1). Two distinct patterns of binding complexes were detected (Fig. 3), fraction 1 (ABF-1, eluting at 50 mM KCl) and fraction 6 (ABF-2, eluting at 500 mM KCl). The sequence specificity of these proteins was examined by competition with oligonucleotides containing an element A

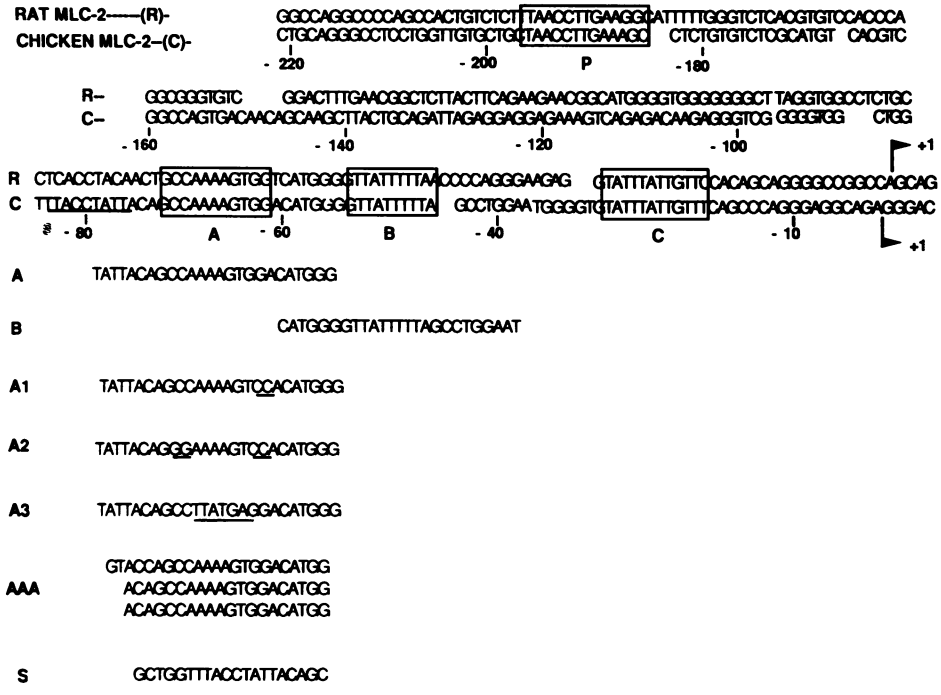


FIG. 1. Partial DNA sequence of chicken and rat cardiac MLC-2 promoter. Transcription initiation site is indicated as +1. The consensus elements P, A, B, and C (see text) common to both genes are boxed. The 63-bp oligonucleotide probe designated AAA contains three copies of element A and a linker, GTAC, at the 5′ end. The sequences of oligonucleotides A, B, A_1, A_2, A_3, and S are indicated.

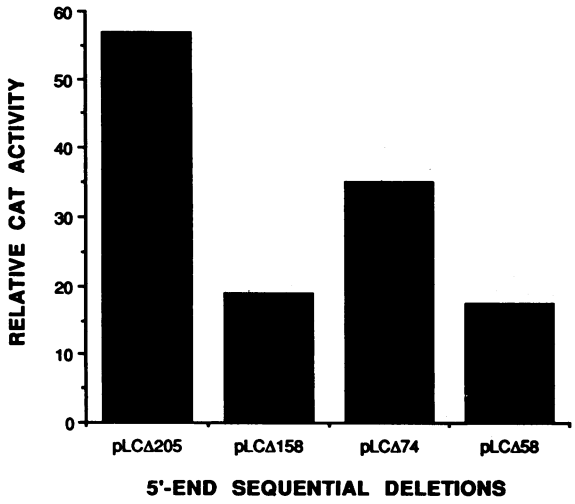
or element B sequence (Fig. 1). Each of these two complexes (indicated by arrowheads) was inhibited by competition with element A-containing DNA but not by element B DNA. A DNA probe containing a single copy of the element A sequence was also tested, and no qualitative difference was found in complex formation (data not shown).

The possibility that the protein may recognize a new sequence generated in the A triplet probe was discounted, as competitor DNA containing only a single copy of the element A sequence competed efficiently even at a 20-fold excess (see below). Also, to rule out the possibility that element S-specific factors are present in the complexes, since the element A competitor oligonucleotide overlaps with element S (Fig. 1), competition was done with element S. No inhibition was detected at a 100-fold excess of the S probe (data not shown). It appears, therefore, that there are at least two protein factors present in the embryonic cardiac nuclear extracts which recognize element A with sequence specificity.

Element A-binding proteins have distinct sequence requirements. To dissect the sequence requirements of ABF-1 and ABF-2, competition was performed with synthetic oligonucleotides containing specific mutations in element A sequence (Fig. 1). Since it was reported that for protein binding and in vivo function of the CArG box, the 5′- and 3′-flanking dinucleotides, CC at the 5′ end and GG at the 3′ end, are required, whereas the requirement for the middle A+T-rich sequence is less stringent (18), mutant sequences were prepared to test whether element A displays a structural requirement similar to that of the CArG box. As shown in Fig. 1 and 3, the mutant oligonucleotides A_1, in which GG was converted to CC, and A_2, in which the GG and the CC dinucleotides were reversed, competed effectively for binding with both factors. Mutant A_3, with four A→T and T→A substitutions in the internal region of the CArG box, on the other hand, did not compete for binding to ABF-1 but competed effectively in binding to ABF-2 (compare the signals with and without competitor). Therefore, it appears that ABF-1 is dependent on the sequence AAAAGT in element A, whereas ABF-2 has a relaxed sequence requirement. In this respect, element A appears to be different from the CArG box as defined in the cardiac α-actin gene (19), at least in its recognition by factors ABF-1 and ABF-2.

Element A is a distinct promoter element. The striking similarity in sequence between element A and the CArG box and to some extent with the CCAAT box (see Discussion) prompted us to compare the binding of ABFs to these two sequence elements. Oligonucleotides containing the CArG box sequence of the human cardiac α-actin promoter and the CCAAT box sequence of the murine sarcoma virus long terminal repeat promoter were chemically synthesized and tested in a competition assay at a 20- and 100-fold excess as above (Fig. 4). ABF-1 was strongly competed with by the single-copy element A competitor at a 20-fold excess, and binding was eliminated at a 100-fold excess but was not affected by either the CCAAT box or the CArG box competitors even at a 100-fold excess. On the other hand, ABF-2 binding was inhibited by all three probes. This is consistent with the observation above that ABF-1 has a high sequence stringency in binding to element A, with dependence on the internal A/T sequence, whereas ABF-2 appears to be a distinct protein with the ability to recognize divergent DNA sequences.

Purification of element A-binding factor. In order to understand the molecular mechanisms underlying the element A-mediated activation of the MLC-2 promoter, we purified the element A-binding protein by using WGA and DNA affinity column chromatography. The affinity column was prepared by coupling oligonucleotide A to cyanogen bromide-activated Sepharose CL-4B as described in Materials



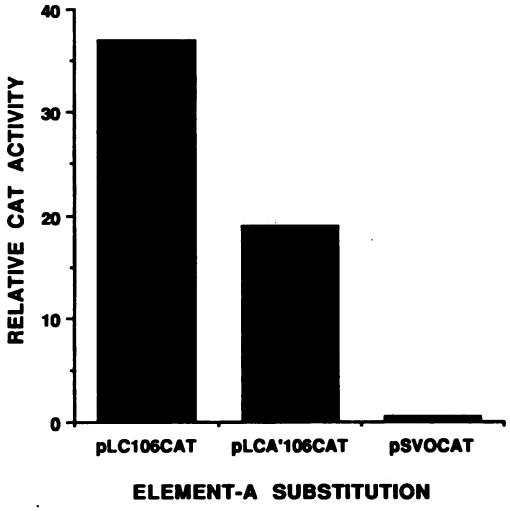


FIG. 2. Expression of the CAT gene driven by mutant MLC-2 promoters. The MLC-2 promoter mutants were generated by 5'-end and site-directed deletions as described in the text and fused to the CAT gene in pSV0CAT. (Top) The endpoints of deletions are indicated as 205, 158, 74, and 58, indicating sequential 5' deletions up to −205, −158, −74, and −58, respectively. (Bottom) The parent plasmid pLC106CAT contains ≈1.2 kb of upstream sequence. pLCA'106CAT is identical to pLC106CAT except that the element A sequence was deleted and substituted as described in the text. CAT activity was normalized to that with pSV2CAT, used separately as a positive control. pSV0CAT was used as a negative control.

and Methods. The protein(s) was eluted with KCl and analyzed by the gel mobility shift assay. The purified protein was examined by SDS-polyacrylamide gel electrophoresis (PAGE), which showed a single band of ≈50 kDa (Fig. 5, lanes 1 and 2). The faint bands of larger molecular size are presumably protein aggregates. The sequence specificity of the purified element A-binding factor was analyzed by gel mobility shift assay with various oligonucleotides as competitors. As shown in Fig. 6, a single DNA-protein complex was formed with the purified protein which was competed

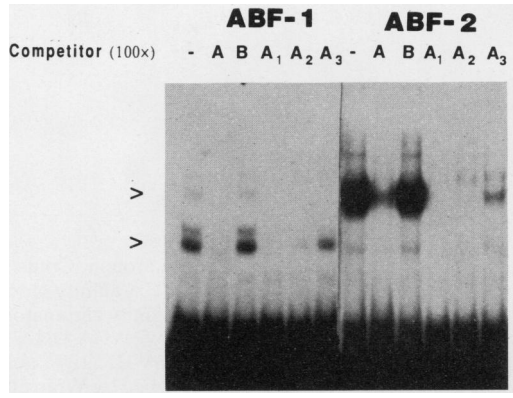


FIG. 3. Mobility shift assay with fractionated cardiac nuclear extracts. The synthetic oligonucleotide AAA (Fig. 1) was used to assay the promoter-binding activities of the fractionated cardiac nuclear extract in fractions 1 (ABF-1) and 6 (ABF-2) after an initial screening by gel retardation of all fractions. Competition was done with unlabeled oligonucleotides A and B DNA and with mutants A_1, A_2, and A_3 (Fig. 1). All competitors were present in a 100-fold excess in molar ratio. Specific binding complexes are marked by arrowheads. For competition, compare lanes with and without competitors.

out by an excess of oligonucleotide A, but not with oligonucleotides S, B, and C.

In order to ascertain which of the two previously identified proteins, ABF-1 or ABF-2, was obtained by purification, the binding properties of the purified factor were examined by competition with the CArG and CAAT box sequences and with oligonucleotides A_1, A_2, and A_3 as before (Fig. 1). The results shown in Fig. 7A indicate that, like ABF-2, the affinity-purified protein complex was competed out by mutants A_1, A_2, and A_3 as well as by a 20-fold excess of the CAAT and CArG box oligonucleotides but not by element B, demonstrating that the purified protein is the same as ABF-2. The purified protein-DNA complex was also identical in mobility to the complex obtained with ABF-2 in fraction 6 above (Fig. 3).

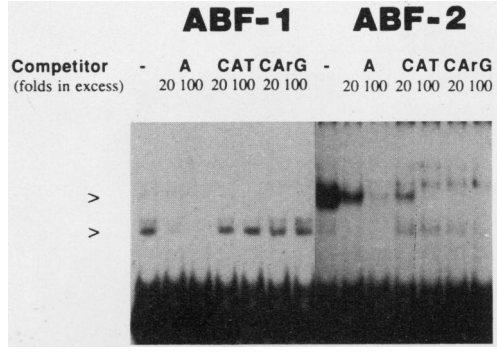


FIG. 4. Competition by oligonucleotides containing CCAAT box and CArG box sequences in gel shift assay. The binding-competition experiment was carried out as described in Fig. 3 with ABF-1 and ABF-2. The competitors were the single element A sequence, the CCAAT box oligonucleotide TTATTTGAACTAACCAATCAGT from the murine sarcoma virus long terminal repeat promoter (30), and the CArG box oligonucleotide GAAGGGGACCAAATAAG GCAAGGTG from the human cardiac α-actin promoter (31), present in a 20- or 100-fold excess, as indicated. Specific binding complexes are marked by arrowheads.

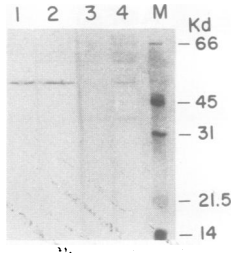


FIG. 5. Purification of element A-binding protein. Crude nuclear extract of cardiac tissue was subjected to WGA affinity chromatography, followed by sequence-specific DNA affinity chromatography. Duplicate samples of fractions obtained by WGA-DNA affinity chromatography were analyzed by SDS-PAGE (12% polyacrylamide). The gels were silver stained as described by Wray et al. (46) and developed in 10% citric acid and 38% formaldehyde. The gel was fixed in 45% methanol and 10% acetic acid. Lanes 1 and 2 contain 10 and 20 μl (10 and 20 ng), respectively, of the eluate from the DNA affinity column. Lanes 3 and 4 contain 10 and 20 μl (~2.5 and 5 μg of total protein), respectively, of the eluted material from WGA chromatography. Lane M, protein standards (sizes in kilodaltons).

ABF-1 was not present in proteins eluted from the WGA column. ABF-1 activity was present in the WGA unbound fraction, which was recovered and assayed for CArG and CAAT competition on a gel shift assay. CArG- and CCAAT-containing DNAs failed to compete (data not shown), indicating that this protein was identical to ABF-1 of fraction 1 above (Fig. 3). That the WGA-bound and DNA affinity-purified protein was element A specific was confirmed by the DNase I protection assay. When a 160-bp *Hin*dIII-*Hin*fI fragment encompassing elements S, A, B, and C was subjected to DNase I protection in the presence of crude nuclear extract, well-defined protection of all four sites (S, A, B, and C) was obtained, as expected (Fig. 8A). With the affinity-purified protein, the protection was restricted primarily to element A only. A synthetic oligonucleotide containing three element A sequences was also examined for protection following treatment with the DNA intercalator MPE (see Materials and Methods). In the presence of purified protein, protection of the element A sequence was evident (Fig. 8B). These results, taken together, clearly demonstrate that the

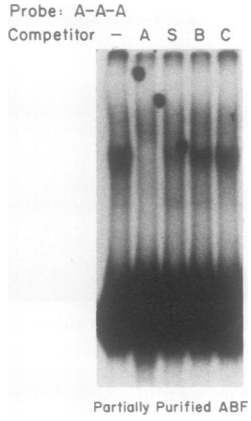


FIG. 6. DNA-binding properties of purified ABF. The DNA binding of purified factor was analyzed by a protein-DNA mobility shift assay as discussed in the legend to Fig. 3. The competitors were double-stranded oligonucleotides corresponding to elements A, S, B, and C (Fig. 1).

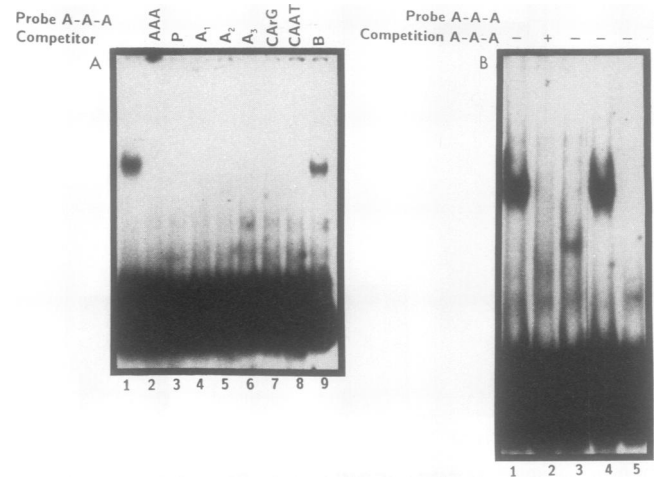


FIG. 7. (A) Competition of DNA affinity-purified protein by oligonucleotides containing mutants A_1, A_2, and A_3 and the CAAT box and CArG box. The DNA-binding competition experiment was carried out as described in Fig. 3. Lanes 2 and 3, competition with the AAA sequence and a 74-bp *Hin*dIII-*Pst*I fragment containing element P, respectively; lanes 4 through 9, competition with mutant oligonucleotides A_1, A_2, A_3, CArG box-, CAAT box-, and B element-containing DNAs, respectively. All competitors were present in 50-fold excess except for AAA and P, of which only a 20-fold excess of cold DNA was used. (B) Comparison of ABF-1 and ABF-2 with affinity-purified protein. Gel shift mobility analysis of the affinity-purified ABF with synthetic oligonucleotide AAA was done as above. Lane 1, without competition; lane 2, competition with oligonucleotide AAA; lanes 3 and 4, comparison of ABF-1 and ABF-2 complexes with the same probe; lane 5, AAA probe alone (with no nuclear extract).

affinity-purified protein is indeed an element A-binding protein identical to ABF-2.

ABF-2 binds to element P. Because of the functional similarity between elements A and P, we speculated that a common protein(s) may interact with the two sequence elements to mediate their activator role. We therefore included element P (TAACCTTGAAAGC)-containing DNA in the competition assay (Fig. 7A) and found that the purified factor ABF-2/DNA complex was competed out effectively by element P. In order to confirm whether ABF-2 binds to element P, we performed a heterologous competition experiment (Fig. 9), in which the DNA-protein complex formed with the element P-containing fragment was competed with increasing concentrations of oligonucleotide A DNA. In a reciprocal experiment, the DNA-protein complex formed with oligonucleotide A was competed with increasing concentrations of element P DNA. A 100-fold molar excess of oligonucleotide A was required to inhibit formation of the element P DNA-protein complex, whereas a 5-fold molar excess of element P DNA was sufficient to inhibit formation of the element A DNA-protein complex, indicating that the binding affinity for the element P sequence is at least 20-fold higher than that for the element A sequence.

DNA protection and methylation interference assays confirm that ABF-2 binds to element P motif. The MPE protection analysis methylation interference assays further confirmed that ABF-2 binds to element P and delineated the boundary of the DNA-protein interaction. We performed a DNA protection assay with the DNA intercalator MPE and the labeled 74-bp *Hin*dIII-*Pst*I fragment encompassing the element P sequence. The results shown in Fig. 10 (left panel)

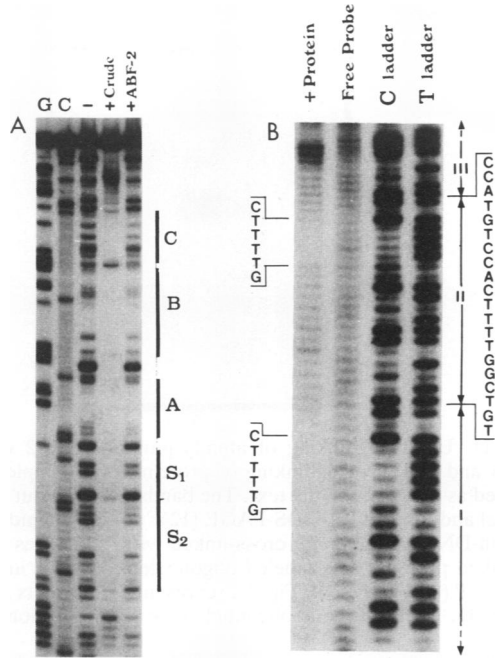


FIG. 8. (A) DNase I footprinting of the proximal MLC-2 region. A 160-bp *Hin*dIII-*Hin*f fragment (−144 to +16), encompassing elements S, A, B, and C, was 5′-end labeled and used for protection analysis. Lanes G and C represent the sequence G and C ladder of the coding strand. DNase I digestion pattern without nuclear extract is shown in the lane marked —; DNA was digested with 1 μl of a 1:200 dilution of DNase I stock solution (1 mg/ml). Lanes +Crude and +ABF-2 represent DNase I patterns of the coding strand incubated with 25 μg of crude nuclear extract and 10 ng of affinity-purified ABF-2, respectively. Bars indicate protected regions C, B, A, S_1, and S_2. (B) Chemical footprinting of element A-containing DNA. A 63-bp synthetic oligomer containing three repeats (I, II, and III) of element A sequence was end labeled and used for footprinting as described in the text. Lanes T and C are products of Maxam-Gilbert reactions (28). The lane marked +protein shows the MPE cleavage pattern of the DNA obtained from the protein-DNA complex precisely at the core sequence of element A (CTTTTG). The lane marked free probe shows the MPE cleavage pattern of DNA not bound by the protein. A single element A sequence is shown on the right side of the panel. The repeat protected sequences are bracketed on the left side of the panel.

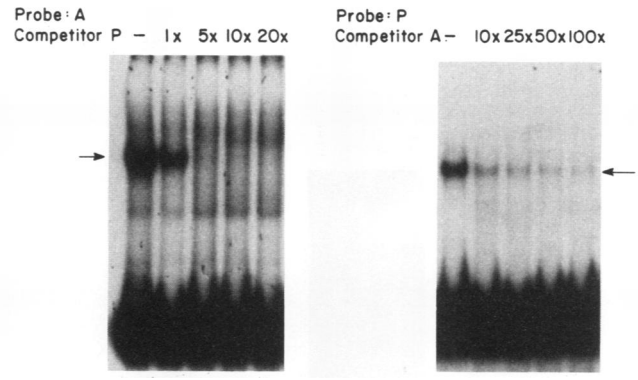


FIG. 9. Heterologous competition with element A- and P-containing DNAs. End-labeled oligonucleotide AAA and a 74-bp *Pst*I-*Hin*dIII fragment containing element P were used as DNA probes for binding of the affinity-purified protein ABF. All competitors were present in molar ratio as indicated for each lane. The first lane in each panel is without competitor. Arrows indicate the ABF/DNA complex.

indicate that the protected region extends from −196 to −184 and contains a nucleotide segment of TAACCT TGAAAGC. Twelve of the 13 nucleotides in this segment are conserved in both rat and chicken MLC-2 promoters. A methylation interference assay was also performed with the same 74-bp fragment (Fig. 10, right panel) and confirmed the involvement of two G residues at positions −185 and −189 in the binding region. Two additional G residues located 5′ to the protected region also appear to block protein binding.

Elements A and P bind to the same transcription factor. Finally, we examined the possibility that ABF-2 may not be a pure protein and that one of the two elements binds to the putative contaminant protein. To confirm that elements A and P bind to the same protein, purified ABF-2 was cross-linked to the oligonucleotide by UV irradiation, and the resulting DNA-protein complex was analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 11. The protein cross-linked to oligonucleotide AAA (Fig. 11, lane 2) and to oligonucleotide P (Fig. 11, lane 3) migrated with identical mobilities. The non-cross-linked product apparently dissociated in the denaturing gel (lane 1). These results thus demonstrate that a single protein, ABF-2, is capable of binding to two distinct elements with divergent sequences.

DISCUSSION

At least two distinct sequence elements, A and P, act as activators of cardiac MLC-2 gene transcription. The goal of the present experiments is to gain an understanding of how activators elicit their effects. The sequence of element A has striking homology with that of another positive regulatory element, the CArG box, present in the genes for many contractile proteins. This motif was originally designated the CCAAT box because of a limited sequence homology with the consensus CCAAT sequence (14, 35). Subsequently, it was designated the CArG box and implicated in muscle-specific regulation, as it activates the cardiac and skeletal α-actin promoters in muscle cell lines (31). Recently, it was shown that the consensus CArG box sequence binds to the serum response factor, which requires the flanking CC and GG dinucleotides at the 5′ and 3′ ends respectively. Binding of other less-defined protein factors to the CArG box, particularly to the distal CArG box in multiple CArG box-containing muscle genes, involves mainly the sequences immediately upstream from the CArG sequence (19).

The cardiac MLC-2 promoter contains a single CArG-like sequence (element A) which appears to be a distinct entity in itself. Element A is recognized by at least two nuclear proteins, ABF-1 and ABF-2, with different sequence requirements. ABF-1 recognition is dependent upon the internal AAAAGT sequence, while ABF-2 is promiscuous, as it binds to both the CArG and CCAAT boxes and to element P as well. The existence of multiple ABFs with distinct sequence requirements thus points to the complexity of the role of element A in gene regulation. One may speculate that the ABFs either compete with each other to mediate different regulatory functions in response to different physiological signals or bind synergistically to effect transcriptional activation. In many eukaryotic systems, multiple activators and their cognate proteins elicit synergistic effects, the mechanism of which remains unknown. Transcriptional synergy may not simply reflect cooperative binding of activators to DNA, but multiple activator elements may simultaneously

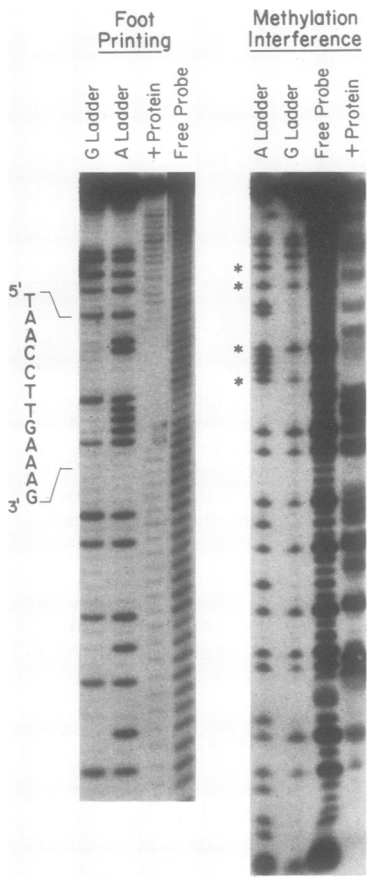


FIG. 10. Chemical footprinting of element P-containing DNA. Footprinting was done with the MLC-2 promoter (−216 to −142) with purified chicken cardiac nuclear extract. A 74-bp *Pst*I-*Hin*dIII fragment of the chicken cardiac MLC-2 gene was 5′-end labeled, which provides the noncoding strand. Lanes G and A are products of Maxam-Gilbert reactions. The lane marked +protein shows the MPE cleavage pattern of DNA obtained from the protein-DNA complex, and the lane marked free probe shows the MPE cleavage pattern of the DNA not bound by the protein. The nucleotide sequence of the protected region is shown on the left side of the panel. The methylation interference assay is shown on the right. Lanes G and A are the products of a Maxam-Gilbert reaction. Free probe and +protein are unbound DNA and bound DNA profiles, respectively; asterisks denote G residues involved in binding.

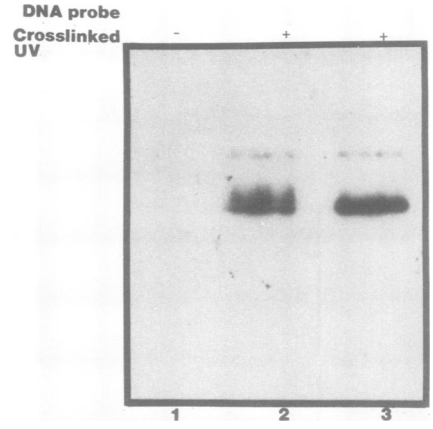


FIG. 11. UV cross-linking of affinity-purified ABF-2 with elements A and P. UV cross-linking of protein-DNA complexes was performed as described in the text. The bands were cut out from the native gel and resolved by SDS-PAGE (12% polyacrylamide). Lane 1, protein-DNA complex not cross-linked with UV; lanes 2 and 3, cross-linked proteins with labeled oligonucleotide AAA and oligonucleotide P (−211 to −150; Fig. 1), representing elements A and P, respectively. Each synthetic oligonucleotide was 63-bp long.

contact a common factor (5, 25). A variation of this model envisages that activators contact with two different proteins, which themselves interact.

During the past few years, many examples have been put forth which present the complexity of activator-target DNA interactions and their effect on transcription initiation. The binding of a GAL4 derivative to an enhancer and that of a mammalian activating transcription factor, ATF, to a proximal element can produce transcriptional synergism (25). NFκB can activate transcription synergistically with itself, even though it binds DNA cooperatively (36). The GC box sequence can recognize both activators, such as Sp-1 and the transcription factor ETF, and the repressor factor GCF (21), and competition between the thyroid hormone receptor and the estrogen receptor can inhibit induction by estrogen receptor (16).

More than one nuclear protein may independently mediate different effects on different gene promoters containing similar binding motifs. For example, binding of a lymphoid-specific factor, NF-A2, to the octamer sequence activates immunoglobulin promoters in lymphoid cells, while binding of ubiquitous factor NF-A1 to the octamer sequences in nonspecific promoters accounts for activation of nonspecific genes (34).

There are examples of proteins for which the lack of stringency in recognition of a *cis* element by regulatory factors is exhibited. C/EBP, a CCAAT/enhancer-binding protein, originally identified on the basis of its sequence-specific interaction with CCAAT pentanucleotides (40), also binds to a degenerate nonamer sequence, TGTGGAAAG, common to many animal virus enhancers (6, 7). Similarly, HAP1, a yeast regulatory protein, interacts with comparable affinity to the upstream activation site (UAS) of the *CYC1* and *CYC7* genes (37). Glucocorticoid receptor, which binds in vitro to multiple sites that display considerable sequence degeneracy (47), is another example. Such degeneracy in DNA-protein interactions perhaps provides an additional avenue for modulation of gene expression. Whether the binding of ABF-2 to elements A and P with different affinities is important in modulation of MLC-2 gene transcription in vivo is not known. One possibility is that ABF-2 contains physically distinct sites, one of which recognizes element P and the other of which recognizes element A. Alternatively, one domain of the polypeptide may recognize both sequences with subtle differences in binding affinity. Competition data indicate that an excess of P DNA could readily displace both the A and P complexes, whereas A DNA competition would require a higher concentration to destabilize the P complex.

The recognition of multiple sequence motifs by a single protein could conceptually provide a means for diversification of gene functions. Despite the divergent nature of the sequence, elements A and P display a common function, i.e., activation of transcription. Therefore, ABF-2, which binds to the two elements, must be involved in at least one function, i.e., positive regulation of the MLC-2 gene. It is conceivable that binding to one of the two sequences would result in establishment of a different level of activation than

would be achieved by binding to both. Alternatively, the formation of the more stable element P-ABF-2 complex might achieve a higher level of promoter activity than the element A-ABF-2 complex. Thus, the level of activation may depend upon a preferential or combinatorial use of these two sequence elements. Ultimately, however, overall transcriptional regulation involves a multiplicity of protein-protein and DNA-protein regulatory interactions. The transient expression of recombinant plasmids in cell culture and the DNA-binding assays may only reflect a subset of the regulatory activities governing a specific promoter domain. It is therefore likely that other regulatory factors, in cooperation with ABF-2, may be involved in activation of transcription. Alternatively, they may promote or inhibit the binding of ABF-2 to one site or another. The molecular mechanism that underlies the dual binding capacity of ABF-2 and its precise role in MLC-2 promoter function will require further functional and structural analyses of the purified protein.

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