Identification of Two Nuclear Factor-Binding Domains on the Chicken Cardiac Actin Promoter: Implications for Regulation of the Gene

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Received 3 January 1989/Accepted 28 April 1989

The *cis*-acting regions that appear to be involved in negative regulation of the chicken α -cardiac actin promoter both in vivo and in vitro have been identified. A nuclear factor(s) binding to the proximal region mapped over the TATA element between nucleotides –50 and –25. In the distal region, binding spanned nucleotides –136 to –112, a region that included a second CArG box (CArG2) 5' to the more familiar CCAAT box (CArG1) consensus sequence. Nuclear factors binding to these different domains were found in both muscle and nonmuscle preparations but were detectable at considerably lower levels in tissues expressing the α -cardiac actin gene. In contrast, concentrations of the β -actin CCAAT-box binding activity were similar in all extracts tested. The role of these factor-binding domains on the activity of the cardiac actin promoter in vivo and in vitro and the prevalence of the binding factors in nonmuscle extracts are consistent with the idea that these binding domains and their associated factors are involved in the tissue-restricted expression of cardiac actin through both positive and negative regulatory mechanisms. In the absence of negative regulatory factors, these same binding domains act synergistically, via other factors, to activate the cardiac actin promoter during myogenesis.

Actin is a major cytoskeletal component of all eucaryotic cells. It is the subunit of microfilaments in nonmuscle cells and the major component of the thin filament in striated muscle cells. On the basis of amino sequence analysis, at least six different isoforms of actin have been identified in the higher vertebrates (35). The various isoforms are tissue specific and differentially regulated during development (2, 4, 8, 15, 30, 36). During myogenesis, β -actin is the major isoform expressed in dividing myoblasts and is subsequently replaced, first by α -cardiac and then by α -skeletal actin, as myogenesis proceeds to form adult skeletal muscle (15, 25, 30).

The molecular basis for this isoform switch during development remains obscure at present but is thought to involve the specific interaction of regulatory proteins with various *cis* sequence elements that control the expression of a particular actin isoform (10). All muscle-specific actin genes contain a highly conserved CCAAT-box element with the consensus sequence CCAATAAGG. It was initially proposed that this element might be important for expression of the muscle-specific actin genes (29). Indeed, factors that bind specifically to the CCAAT element of the α -skeletal promoter have been identified, and the binding activity is correlated with promoter function (37, 38). Multiple sequence elements of similar structure with the consensus CC(A+T)₆GG have been reported in various α -actin promoters. The presence of these elements, termed CArG boxes (24) or CBAR elements (6), was found to be correlated with promoter function. However, the contribution of these diverse elements to promoter function during differentiation was never completely resolved, and their precise roles in gene regulation and expression remain open.

In this study, we addressed questions concerning the developmentally regulated expression of the chicken α -

cardiac actin gene, using 5'-directed promoter deletions and chimeric promoter constructs with specific regions of the α -cardiac and β -actin promoters. We identified two different factor-binding domains on the cardiac actin promoter and determined that factors in crude nuclear extracts binding to these regions are more abundant in tissues that do not express the α -cardiac actin gene. The putative role of these factors and their respective binding domains in promoter function are discussed.

MATERIALS AND METHODS

Cell cultures and transfection. Culture conditions for chicken primary myoblasts and skin fibroblasts were essentially as described elsewhere (31). Briefly, tissues were removed from 12-day chicken embryos, trypsinized, and plated on collagen-coated 25-cm² flasks in Dulbecco modified Eagle medium containing 2% chicken embryo extract and 10% horse serum, supplemented with glutamine and gentamicin. At 12 to 18 h after plating, the cells were transfected by the calcium phosphate-DNA coprecipitation method as described by Gorman (13). Fused cultures were treated with 100 μ g of cytosine arabinoside per ml at 45 h, just at the start of fusion. Fusion was routinely greater than 75%. Cultures were cotransfected with the chloramphenicol acetyltransferase (CAT) and luciferase genes. The CAT gene served as the reporter activated by the various promoter constructs. The luciferase gene, driven by an enhancerless simian virus 40 promoter, was introduced as an internal standard for normalization (9). Each flask was exposed to a total of 20 μ g of plasmid, and the ratio of luciferase to CAT plasmid in the transfection mixture was 1:10. All plasmids to be compared were transfected into primary cultures derived from the same plating to ensure that the developmental stage and cell composition were identical for all constructs. This procedure was carried out five times (Table 1). Cells were harvested 96 h after plating.

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TABLE 1. CAT activities of the promoter constructs described in Fig. 1, 4, and 5^a

Construct	% Activity (SD)		M/F ratio ^b
	Myotubes	Fibroblasts	
WT	100 (5.7)	7.6 (0.9)	13.2 (3.1)
DEL1	101 (5.6)	7.7 (1.0)	13.1 (2.8)
DEL2	95 (4.6)	6.9 (0.7)	13.8 (2.3)
DEL3	51 (4.2)	13.6 (0.8)	3.8 (0.5)
DEL4	6.5 (0.7)	4.8 (0.9)	1.4 (0.4)
DEL1beta	52 (4.7)	6.8 (0.4)	7.7 (1.2)
DEL2beta	51 (5.0)	7.5 (0.4)	6.8 (1.1)
DEL3beta	23 (3.0)	16.9 (0.9)	1.4 (0.2)
DEL1-[<i>NarI</i> - <i>SacII</i>]	45 (3.0)	6.2 (0.8)	7.3 (1.6)
DEL2-[<i>NarI</i> - <i>SacII</i>]	31 (2.8)	6.9 (0.4)	4.5 (0.7)
DEL1b-[<i>NarI</i> - <i>SacII</i>]	9.5 (1.5)	1.2 (0.3)	7.5 (4.1)

^a Five independent transfection events were assayed. All conversions to the monoacetylated forms of chloramphenicol were normalized to the WT construct, which was arbitrarily assigned 100% activity. The absolute conversion by the WT construct was approximately 50%, which lies within the linear range of the assay. Values are means of five determinations.

^b Activity in fused myotubes divided by activity in fibroblasts.

Plasmids. The plasmids containing the luciferase and Rous sarcoma virus *neo* genes have been described in detail elsewhere (9, 13). The α -actin promoter region, designated wild type (WT), was isolated from the gene (11) as a fragment from positions -1091 to +15, delineated by the restriction sites *Bgl*II and *Bst*EII. The *Bst*EII end was modified with *Sall* linkers, and the entire fragment was cloned into p8CAT, a derivative of plasmid pEMBL (7). The α -actin promoter deletions were obtained by cutting this subclone with the restriction enzymes *Sma*I and *Sall* (DEL1) and with *Bal*I and *Sall* (DEL2). The fragments were recloned into p8CAT. DEL3 was obtained from DEL1 by cutting with the restriction enzyme *Nar*I. The *Nar*I site was modified with *Bam*HI linkers, and the *Bam*HI-*Sall* fragment was recloned into p8CAT. DEL4 was obtained by cutting DEL1 with the restriction enzymes *Sma*I and *Sac*II, the upstream sequence was removed by electrophoresis, the *Sac*II site was flush ended and religated with the *Sma*I site. The β TATA-region replacements were obtained by cutting DEL1, DEL2, and DEL3 with the restriction enzymes *Sac*II and *Sall*. The *Sac*II-*Sall* fragment was removed and replaced with an oligonucleotide with matching sticky ends (see Fig. 4). The CCAAT region was removed by cutting DEL1, DEL2, and DEL1beta with *Nar*I and *Sac*II. The ends were flush ended and religated. The construct containing the myosin light-chain 1 TATA box in front of CAT was obtained from a myosin light-chain 1f promoter construct (positions -66 to +1 [3]).

Expression assays. CAT assays and luciferase assays were generally performed as described elsewhere (3, 9). For the CAT and luciferase assays, 0.1 to 1 and 5%, respectively, of the cell extract from a 25-cm² plate were used. Extracts (prepared at 96 h after plating) used to compare the CAT activities of different promoter constructs were all obtained from transfected cultures derived from the same plating. This eliminated variations in promoter activities due to differences in cell composition or developmental stage. All extracts were adjusted to identical luciferase activities in a given plating before the CAT assays. Procedures were performed in a manner that minimized variations resulting from handling and processing.

Total RNA was isolated from cultured cells as described by Maniatis et al. (21). S1 nuclease probes (21) were obtained from pRSVneo (13) and the α WT promoter in p8CAT

(Fig. 1) (11). pRSVneo was 5' end labeled at the *Bgl*II site and recut with *Eco*RI to yield a fragment of 411 nucleotides (nt) and a protected fragment of 322 nt. The α -cardiac actin promoter in p8CAT was 5' end labeled at the *Eco*RI site in the CAT gene (13) and recut with *Sma*I to generate a fragment of 580 nt, of which 260 and 280 nt were protected when the gene was transcribed from the β - and α -actin promoters, respectively.

In vitro transcription was performed as described by Cao et al. (5), with mouse L-cell nuclear extracts prepared as described by Heberlein and Tjian (16). Briefly, assays were performed in 20 to 30 μ l with 200 ng to 1 μ g of supercoiled template. Transcripts were analyzed by primer extension assay, using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc.) and a CAT oligonucleotide primer from positions 4921 to 4944 in pSV2-CAT (13).

Band shift assays. Kinase-reacted (21) oligonucleotides or restriction fragments (10,000 to 40,000 cpm per reaction) were incubated for 10 min at room temperature with 10 μ g of protein from nuclear extracts in binding buffer [75 mM NaCl, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 15 mM Tris (pH 7.5), 0.5 mM dithiothreitol, 5% glycerol] supplemented with 1 μ g of poly(dI-dC) and 10 μ g of tRNA in a total volume of 20 to 30 μ l. The incubation mixture was electrophoresed in 1% agarose gels with 0.5 $\times$ TBE at 30-mA constant current for 2.5 h. Gels were dried and exposed for 4 to 12 h at -70°C.

Mapping of binding sites. All mapping procedures shown were carried out with nuclear extracts derived from embryonic chicken brain. DNase footprinting (12) was carried out on the kinase-reacted *Nar*I-*Sall* fragment and the 50-mer oligonucleotide containing the region immediately upstream of the *Nar*I site. These fragments were incubated with nuclear extracts from embryonic brain as described above, and 10 U of DNase I was added for 2 min. The reaction was stopped by addition of EDTA to a final concentration of 20 mM. After electrophoresis in 1% agarose-0.5 $\times$ TBE, the content of the gel was transferred to DEAE paper. The bound and free fragments were eluted in 1.5 M NaCl overnight, extracted with phenol-chloroform, and precipitated with ethanol. The DNase I-digested bound and free fragments were electrophoresed in an 8% sequencing gel. Footprinting by chemical cleavage with a 1,10-phenanthroline-copper complex was performed in 1% agarose gels after band shift electrophoresis as described by Kuwabara and Sigman (19). After the reaction was completed, the content of the gel was transferred to DEAE paper and processed as described above. Methylation interference was assayed by partially methylating the fragment with dimethyl sulfate before bandshift electrophoresis (23). After isolation of bound and free fragments as described above for DNase footprinting, the methylated fragments were cleaved with pipidine and separated in an 8% sequencing gel.

Oligonucleotides and DNA fragments. The following double-stranded oligonucleotides were used either as competitors or as labeled fragments in band shift assays. Oligonucleotide I (oligo I) consisted of 50 base pairs from the chicken β -actin gene, positions -106 to -56, that included the CCAAT box (18; W. W. Quitschke, Z.-Y. Lin, L. DePonti-Zilli, and B. M. Paterson, J. Biol. Chem., in press): CGGCGGAGCCAATCAGAGCGGCGGCTCCGAAAGTTTCCTTTTATGGCC. Oligo II consisted of 55 base pairs from the chicken β -actin gene, positions -55 to +1, that included the TATA box (18): GGGAGGCGGCGGCGGCG

supernatant was dialyzed against binding buffer for 3 h and then recentrifuged for 5 min at $100,000 \times g$ to remove precipitated material. The clear supernatant was stored in equal portions at -70°C . All operations were carried out at 0 to 4°C . Nuclear extracts from L cells were prepared by the method of Heberlein and Tjian (16).

RESULTS

Deletion analysis of the chicken α -cardiac actin promoter. The chicken α -cardiac actin promoter (Fig. 1A) contains a TATA element at position -33 5' to the transcriptional start site at position $+1$. The sequence element CCAAT, at position -86 , is considered to be the classical CCAAT-box element found in several of the muscle-specific actin promoters in vertebrates (29). Another common sequence motif is the so-called CArG box (24) or CBAR element (6). This element is represented by the general consensus sequence $\text{CC(A+T)}_6\text{GG}$. Using this general consensus, the conventional CCAAT element at position -86 of the α -cardiac actin promoter is a CArG or CBAR element. Similar elements are located not only at position -86 (CArG1) but also at positions -124 (CArG2), -157 (CArG3), and -215 (CArG4) (Fig. 1A).

To determine the contribution of these various sequence motifs to the regulated activity of the chicken cardiac actin promoter, a series of 5'-directed deletions was constructed, cloned into the CAT vector p8CAT (3), and analyzed by transient transfection in primary cultures of embryonic chicken breast muscle and skin fibroblasts. The skin fibroblasts were used as a nonmuscle cell background that does not substantially activate muscle-specific promoters. The low level of background expression observed in these cultures may have been largely due to contaminating myoblasts. Indeed, a low percentage (approximately 1%) of myosin-positive cells was detected with the skeletal myosin-specific monoclonal antibody MF20 in the skin fibroblast cultures (data not shown).

The results for the various constructs and deletions are presented as the percentage of chloramphenicol conversion compared with the level of conversion in the original 1,091-base pair promoter fragment designated WT (Fig. 1B and Table 1). Quantitative results for promoter activities (Fig. 1, 3, and 5) were determined directly from the CAT assays illustrated in those figures. The reproducibility of these results was tested by comparing the relative CAT activities from five independent transfection events. The averages and standard deviations of these determinations are presented in Table 1. All CAT activities were normalized to the activity of a cotransfected plasmid containing the luciferase gene activated by an enhancerless simian virus 40 promoter (9). The ratio of activity in myotubes versus fibroblasts, the M/F ratio, was used to establish the degree of tissue-restricted expression conferred by the various constructs.

The deletions were generated by using convenient restriction sites within the original promoter construct; the restriction enzymes *Sma*I, *Bal*I, *Nar*I, and *Sac*II yielded promoter fragments with lengths of 315, 200, 100, and 58 base pairs, respectively. Removal of the entire sequence 5' to DEL2 (-215) had little effect on the level or tissue-restricted activity of the cardiac actin promoter (Fig. 1). Therefore, the promoter fragment in DEL2 was sufficient to maintain both tissue-restricted and differentiation-dependent regulation. However, removal of the region from -200 to -100 , including all of the CArG boxes except the primary CCAAT element, resulted in a decrease in promoter activity to 50%

of the level of the complete promoter and a decline in the M/F ratio to 4 (DEL3; Fig. 1). This decline in the M/F ratio not only was a result of a decrease in the level of differentiation-dependent expression but also reflected an increase in fibroblast expression (Fig. 1 and Table 1). Removal of the primary CCAAT box or CArG1 in DEL4 caused a decrease in promoter activity to roughly 7% of the original value, a level close to background activity in fibroblasts (Fig. 1B and Table 1). Consequently, *cis*-acting sequence elements controlling tissue-restricted and differentiation-dependent expression of the cardiac actin promoter are located predominantly between -200 and -58 . This region is operationally subdivided by the *Nar*I site at -100 , which separates the three upstream CArG elements from the original CCAAT box and demonstrates that both regions contribute to normal promoter activity in a synergistic fashion.

Specific binding of nuclear factors over the TATA region. To identify nuclear *trans*-acting factors that might interact with the cardiac actin promoter, the 100-base-pair fragment 3' to the *Nar*I restriction site in DEL3 was first analyzed for factor binding by band shift assay because this deletion maintained a moderate M/F ratio of 4 (Fig. 1B and Table 1). Since factors that interact with the promoter may be positive or negative, tissues that expressed as well as tissues that did not express cardiac actin were used as sources for nuclear extracts. To normalize for variations in the quality of the nuclear extracts, all extracts were adjusted for similar binding activity to the CCAAT element in the β -actin promoter. The β -actin promoter is active in all tissues examined to date and is activated by a factor(s) binding to the CCAAT element (Quitschke et al., in press). This binding activity was used as an internal control to normalize extracts for possible variations resulting from procedural differences in preparation. On the basis of the amount of labeled β -promoter fragment shifted per microgram of protein in the nuclear extracts, the concentration of β -actin CCAAT binding activity was similar in nuclear extracts from 14-day embryonic chicken heart, brain, and breast muscle (Fig. 2B). In contrast, the -100 fragment of the cardiac promoter showed the most prominent band shift with brain extracts (Fig. 2A, lane 1) in comparison with extracts from either heart or breast muscle (Fig. 2A, lanes 2 and 3); therefore, brain nuclear extracts were used for further studies except where noted. The slight increase in binding observed with breast muscle extracts compared with heart extracts likely reflects the different levels of fibroblasts and myoblasts in the breast tissue. The two distinct bands observed with the cardiac actin promoter fragment (Fig. 2A, b1 and b2) represent factors binding to a common domain (see below). Similar analysis with the promoter fragment of DEL4 (-58) gave the identical shift pattern (Fig. 2C); thus, the recognition sequence for factor binding was located within this shorter sequence element, which contained no CArG boxes.

Binding specificity was determined by band shift competition. The end-labeled fragment derived from DEL4 was incubated with nuclear extracts from embryonic chicken brain and competed with a variety of double-stranded oligonucleotides and restriction fragments. Binding competition was observed only with the promoter deletions from DEL3 and DEL4 that contained the homologous sequence region (Fig. 2D). Fragments that were tested and found unable to compete included the β -actin promoter, positions -106 to $+1$, with the CCAAT and TATA elements, the *Sma*I-*Nar*I fragment of the cardiac actin promoter, positions -315 to -100 , containing the three upstream CArG elements, and the *Nar*I-*Sac*II fragment of the cardiac actin promoter,

FIG. 2. Band shift and competition assays on the -100 cardiac actin promoter fragment with muscle and nonmuscle nuclear extracts. (A) The labeled -100 fragment from DEL3 was incubated with nuclear extracts from 15-day embryonic chicken brain (lane 1), heart (lane 2), and breast muscle (lane 3). (B) Same as above except that oligo I (see Materials and Methods), containing the chicken β -actin CCAAT domain (-106 to -56), was used as the labeled probe. (C) Band shift competition assay using the DEL4 fragment (-56 to $+15$) as the labeled probe. Competitor DNA was added at 0-, 25-, 50-, and 100-fold molar excess. Fragments unable to compete at 100-fold molar excess included oligos I through IV (see Materials and Methods). Oligo III is shown here. (D) Specific competition with the DEL3 (-100 to $+15$) and DEL4 (-56 to $+15$) fragments. Competition with DEL4 is shown.

positions, -100 to -58 , containing the primary CCAAT or CARG1 element (Fig. 2C). Therefore, binding was specific for the region 3' to the *Sac*II site at position -58 and could not be competed for by any of the heterologous CARG or TATA elements used.

To delineate the binding site, again with nuclear extracts from embryonic chicken brain, the promoter fragment of DEL3 was analyzed by DNase I and methylation interference footprinting techniques. DNase I footprinting indicated a protected region on the coding strand of approximately 24 base pairs extending roughly 6 base pairs from the 3' end of the *Sac*II restriction site to 3 base pairs beyond the TATA element (Fig. 3A). The protected region on the noncoding strand was somewhat shorter on the 5' end, and it partially overlapped the TATA box. Similarly, methylation interference demonstrated that particular G residues 5' to the TATA element in both strands were involved in factor binding (Fig. 3B). Similar results were obtained with extracts from breast muscle (data not shown). The two distinct shifted bands (Fig. 2, b1 and b2) gave identical patterns with both assays (data not shown). The similarities in the shift patterns and the footprints for the various extracts, although different in intensity, suggested a quantitative difference in the amount of binding activity present in each nuclear extract. The binding domain in DEL4 included regions in and adjacent to the TATA box, and this binding was specific for the cardiac actin promoter, as determined by competition assay.

Normal regulation of the cardiac actin promoter requires the cardiac actin TATA region. Results from the footprinting experiments and band shift competition assays indicated that a nuclear factor(s) bound specifically in the vicinity of the cardiac actin TATA box. To determine whether there was a requirement for this particular TATA domain for function in vivo, a series of chimeric promoters was made by replac-

ing the *Sac*II-*Sal*I fragment in DEL1, -2 , and -3 , positions -58 to $+15$, with an oligonucleotide representing the corresponding region from the chicken β -actin gene. This oligonucleotide and the corresponding new constructions are shown in Fig. 4A. The CAT activities obtained with these new constructions, designated DEL1beta, DEL2beta, and DEL3beta, were normalized to the activity of the original (WT) promoter (Fig. 1 and 4; Table 1). A direct comparison of DEL1 and DEL1beta showed a roughly 50% drop in the normal differentiation-dependent increase in CAT activity and a 30% decrease in the M/F ratio, from 12.0 to 8.4 (Fig. 4B). Unlike DEL3, however, the CAT activity of DEL3beta declined from 50% to 25% of the control value, and the M/F ratio dropped from 4.0 to 1.5, with 17% activity in fibroblasts compared with 25% in myotubes. An M/F ratio of 1.5 is similar to that observed for the β -actin promoter alone, which does not display any tissue-specific regulation (3; Quitschke et al., in press). Therefore, replacement of the cardiac actin TATA region with the TATA domain from the β -actin gene altered the regulatory pattern, especially in DEL3beta, and indicated that, in addition to the CARG1 element defined by DEL4 (Fig. 1), the cardiac TATA region plays a role in regulation. Thus, sequence elements 5' to the *Nar*I site at position -100 , the CARG1 element at position -86 , and the TATA domain at -33 are all involved in regulated expression from the cardiac actin promoter.

The proximal CARG1 element can be functionally replaced by upstream CARG elements. Since deletion of the CARG1 sequence in DEL4 (Fig. 1) resulted in the loss of promoter activity, it was of interest to determine whether the upstream CARG elements at positions -124 , -157 , and -205 were capable of complementing the deletion. To test this, DEL1 and DEL2 were cut with the restriction enzymes *Nar*I and *Sac*II and religated after the appropriate repair. This effectively removed the CARG1 element in a 42-base-pair fragment and brought the CARG2 sequence into approximately the same position (within 2 nt) previously occupied by CARG1 (Fig. 5A). In addition, the upstream CARG3 and CARG4 elements were now shifted further 3'. The TATA replacement, DEL1beta, was treated in a similar fashion. Although the overall promoter activity of the internal deletion constructs was lower than that of the control (WT), a reasonable level of differentiation-dependent activity was evident, with M/F ratios of 4.3 to 7.6 (Fig. 5C and Table 1). The CARG2 substitution in the TATA replacement, DEL1beta, containing only the promoter sequences from the *Sma*I site at -315 to the *Nar*I site at -100 , was capable of regulating a low level of differentiation-dependent activity (9%), as shown by an M/F ratio of 7.6 (Fig. 5C). Taken together with the results shown in Fig. 1 and 4, the data indicate that the CARG2 element, possibly in coordination with the upstream CARG3 element, can functionally replace the CARG1 domain to a certain extent and that the sequences in the cardiac actin promoter from the *Bal*I site at -200 to the *Nar*I site at -100 are crucial for the normal pattern of tissue-restricted and differentiation-dependent promoter activity. However, the level of expression is further dependent on the homologous CCAAT (CARG1) and TATA regions.

The transcriptional start for the promoter deletions and substitutions is determined by the particular TATA element. Since the constructs described above represent major rearrangements of the promoter region, it was considered essential to test whether the transcriptional start sites were correctly maintained in vivo. For this purpose, S1 nuclease protection assays were performed on examples of each type of promoter construct (Fig. 6). These included DEL1, representing the original cardiac actin promoter (lane 1), the

FIG. 3. DNase footprinting and methylation interference analyses for factor binding with embryonic brain nuclear extracts on the -100 fragment from DEL3. (A) DNase footprint on the coding (C) and noncoding (N) strands of DEL3. After exposure to DNase, the bound (b) and free (f) fragments were isolated by band shift and processed individually. Brackets indicate the approximate protected areas. (B) Methylation interference. The methylated G residues that interfered with binding are indicated by arrows. The coding strand contains five G residues in a row (bracketed) that do not separate because of compression. (C) Summary of the results.

β -actin promoter (lane 2), DEL1beta and the β -actin TATA substitution in the cardiac deletion (lane 3), and the internal deletion of CARG1, called DEL1-[NarI-SacII] (lane 4). As expected, the transcriptional starts were correct and were correlated with the corresponding TATA domain in each construct.

Binding of nuclear factors over the distal CARG2 region. The deletion and exchange studies on the cardiac actin promoter defined at least two regions involved in regulation of the gene, one in the vicinity of the TATA box and the other within the 100-base-pair *BalI-NarI* restriction fragment (-200 to -100). In the former case, the argument that the TATA region was important for proper regulation was strengthened by the exchange experiments and the demonstration of specific factors binding to this domain. Therefore,

similar binding studies were carried out with the *BalI-NarI* fragment (-200 to -100).

Just as in the case of the TATA-region binding factor, only brain nuclear extracts were capable of generating a substantial band shift with the *BalI-NarI* fragment (Fig. 7A, lane 3), whereas factors binding to the β -actin CCAAT element were equally prominent in all extracts tested (Fig. 7B). To narrow down the binding domain even further, a double-stranded oligonucleotide containing the 50 base pairs immediately 5' to the *NarI* site (oligo IV) was synthesized (see Materials and Methods). Oligo IV maintained all binding properties of the entire fragment and was used for subsequent analyses (Fig. 7C, lane 0X). The specificity of binding was determined by competition with a variety of oligonucleotides and restriction fragments (see Materials and Methods for oligonucleo-

FIG. 4. TATA-region substitutions in the cardiac actin promoter. (A) Sequence in which all elements in the cardiac actin promoter 3' of the *Sac*II site (–58) have been replaced with the β -actin TATA region, using oligo II (see Materials and Methods). The lower portion indicates the upstream deletions described in Fig. 1. (B) CAT activities of the substituted deletions described above (see Table 1).

tide sequences): oligo III, the 3' untranslated region of the β -actin gene; oligo I, the β -actin CCAAT region; oligo II, the β -actin TATA region; oligo IV, the cardiac actin CArG2 region; the *Nar*I-*Sac*II fragment (–100 to –58) with the CArG2 element; and the *Sac*II-*Sal*I fragment of DEL4 (–58 to +15). Competition was observed only with the homologous oligonucleotide itself, oligo IV (Fig. 7C and D). These competition studies indicated that factors binding to the CArG2 region were distinct from the factors binding either to the cardiac TATA domain or to the β -actin CCAAT element. Furthermore, the inability of the CArG1 element to compete for factor binding to the CArG2 domain demonstrated that even though the two elements appeared functionally similar by substitution analysis (Fig. 5), CArG1 did not bind the same factor by this assay.

The binding domain was determined by DNase footprinting, copper-phenanthroline chemical cleavage, and methylation interference techniques. All operations were carried out with oligo IV (see Materials and Methods) and nuclear extracts from embryonic chicken brain. Binding covered approximately 24 base pairs on the coding strand and 16 base pairs on the noncoding strand and included the CArG2 element (Fig. 8A and B). Weak methylation interference also indicated that three G residues on the coding strand and one G residue on the noncoding strand were within the binding site (Fig. 8C). A summary of the binding region is shown in Fig. 8D.

Removal of the CArG2 and exchange of the TATA-region binding domains in the cardiac actin promoter increases *in vitro* transcription activity in nuclear extracts from nonmuscle cells. The deletion and substitution results combined with the factor-binding data demonstrated that at least two distinct regions on the cardiac actin promoter were involved in modulating the tissue-restricted and differentiation-depen-

dent activity of the cardiac actin promoter. The distal region included the CArG2 element around position –124 in the region from –200 to –100, which is important for tissue-restricted expression (Fig. 1B, DEL2 versus DEL3; Fig. 5C, DEL1beta). The proximal region contained the TATA domain between –58 and –33 and played a role in both the level of expression and the tissue restriction of promoter activity (Fig. 1B, DEL3; Fig. 4B, DEL3beta). Surprisingly, the binding studies indicated that the factors interacting with these two regions were more prevalent in tissues that did not express the cardiac actin gene, suggesting a negative regulatory function for these factors. If the binding factors detected are indeed involved in negative regulation of the cardiac actin gene, the *in vitro* transcriptional activity should increase as each binding site is removed or replaced, since negative regulatory factors can no longer repress transcription. To test this possibility directly, the promoter constructs DEL2, DEL3, DEL2beta, and DEL3beta were analyzed by *in vitro* transcription with nuclear extracts prepared from L cells, a nonmuscle cell line. Previous studies with L cells transfected with a variety of muscle genes (33) have shown that the genes are properly expressed at very low levels. In addition, factors binding to the CArG2 and TATA domains of the cardiac actin promoter are qualitatively similar in both L-cell and chicken brain nuclear extracts, as determined by band shift and footprint assays (data not shown). A short description of these constructs (Fig. 1 and 4) is given again for clarification: DEL2 contains all of the binding and regulatory domains for maximal promoter activity (from –200), DEL3 is missing the CArG2 domain, DEL2beta contains the CArG2 element with the β -actin TATA region, and DEL3beta contains the β -actin TATA region but is missing the CArG2 element. Transcription reactions were carried out with an additional CAT construct containing a

FIG. 5. Internal deletion of the CarG1 element in the cardiac actin promoter. (A) Removal of the 42-base-pair (bp) segment between the *NarI*-*SacII* sites (–100 to –58) from DEL1 and DEL2 (see Fig. 1); (B) deletions from DEL1beta (see Fig. 4); (C) CAT activities for the constructs described above (see Table 1).

myosin light-chain 1f promoter deletion (3) as an internal transcription standard. The transcriptional activities of the various constructs, after adjustment to the internal standard, were compared with the activity of DEL2, which was given an arbitrary value of 1 (Fig. 9). Transcriptional activity was lowest with DEL2, which contained both the CarG2- and

TATA-binding domains. Removal of the cardiac actin TATA domain and substitution of the β -actin TATA region resulted in an approximately 20-fold increase in the transcriptional activity with DEL2beta. Elimination of just the CarG2-binding site and substitution with DEL3 increased transcriptional activity roughly fivefold. However, removal of the CarG2 element and substitution of the β -actin TATA region with DEL3beta increased transcriptional activity nearly 50-fold over DEL2 values. Thus, the results obtained in the transcription studies are compatible with the idea that the regulatory regions defined in the transfection studies could also interact with negative regulatory factors and lend credence to a putative negative regulatory role for the nuclear factors binding to the CarG2 and TATA regions of the cardiac actin promoter reported here.

DISCUSSION

5' Deletion analyses of the chicken α -cardiac actin promoter fragment truncated from nt –1091 to –58 (relative to the transcriptional start site at +1) and joined to the CAT structural gene have defined a minimal promoter region starting at –200 that gives essentially the same tissue-restricted and developmentally regulated activity found with the longest fragment (Fig. 1) in primary cultures of embryonic chicken breast muscle. Similar results were obtained when the promoter constructs were first linearized at the 5' promoter-plasmid junction before transfection (data not

FIG. 6. S1 nuclease protection analysis of promoter deletions and substitutions. Lanes: 1, DEL1; 2, β -actin promoter (–339 to +1) in p8CAT; 3, DEL1beta; 4, internal deletion DEL1-[*NarI*-*SacII*]; 5, untransfected control; 6, molecular weight markers. All constructs were cotransfected with pRSVneo as an internal control. The protected fragments generated in constructs containing the cardiac actin and β -actin TATA regions are 280 and 260, respectively, base pairs long.

FIG. 7. Band shift and competition assays on the region of the cardiac actin promoter from -200 to -100 with muscle and non-muscle nuclear extracts. (A) The *Ball-Nar1* restriction fragment (-200 to -100) was used as a labeled probe and was incubated with nuclear extracts from 15-day embryonic chicken breast muscle (lane 1), heart (lane 2), and brain (lane 3). (B) The chicken β -actin CCAAT region, oligo I (see Materials and Methods), was used as a labeled probe (see Fig. 2). Lane designations are as for Fig. 2. (C) Band shift competition assay using oligo IV (-150 to -100) from the cardiac actin promoter as the labeled probe. Competitor DNA was added at 0-, 25-, 50-, and 100-fold molar excess. Only the *Ball-Nar1* fragment (-200 to -100) or oligo IV was able to compete for binding. Oligo IV is shown. (D) Inability of oligo I to compete at 100-fold molar excess. Also unable to compete under this condition were oligo II, oligo III, and the *Nar1-SacII* fragment (-100 to -56).

shown). Thus, varying the proximity of the plasmid sequences to the promoter had little consequence.

Deletion studies with the human cardiac actin promoter transfected into mouse C2C12 cells (24) resulted in a two- to threefold decrease in promoter activity as deletions progressed to position -177 . However, neither the human nor the chicken cardiac actin promoter showed tissue-regulated expression in mouse C2C12 myoblasts or myotubes (24, 33). Differences in promoter behavior due to cell background have also been observed with deletion studies on the human skeletal actin gene in mouse C2C12 and rat L8 myogenic lines (28) and in studies with the chicken cardiac actin gene in different mouse myogenic cell lines (20). Thus, direct comparisons with the results reported here for primary cultures of embryonic chicken breast muscle, where both promoter activity and regulation can be studied, are not possible.

The proximal promoter regions of both the chicken and human cardiac actin genes have roughly a 70% sequence homology from nt -200 and -177 , respectively, and further 5' deletions beyond these boundaries result in a significant decline in promoter activity (Fig. 1; 24). In the chicken cardiac actin promoter, removal of sequences from -200 to -100 reduced CAT expression by 50% and lowered tissue-restricted expression threefold (Fig. 1, DEL3). This deletion also resulted in the removal of three of the four CARG elements (24, 32) in the -200 promoter fragment, leaving just the CARG1 domain or traditional CCAAT box. Although the presence of CARG1 was sufficient for low-level regulated activity with the homologous TATA region, deletion of this last CARG box reduced activity almost 20-fold (Fig. 1, DEL4). In vivo competition assays with various fragments

of the human cardiac actin promoter have also implicated the two CARG regions at -140 and -100 , corresponding to the CARG1 and CARG2 elements reported here, as binding sites for positive-acting factors (26).

The proximal promoter region of the human cardiac actin gene was reported to bind nuclear factors present in C2C12 cells that were absent in Ltk⁻ cells (26). More recent work by Phan-Dinh-Tuy et al. (32) has identified a single factor-binding domain, corresponding to the CARG1 element or CCAAT box at -100 , with nuclear extracts from C2C12 myoblasts and L cells. Mutations within the CARG1 element eliminated factor binding and in vivo activity of the promoter. No factor binding to the CARG2 element could be demonstrated. Furthermore, deletion of the CARG2 element at -140 still gave 50 to 75% of the proximal promoter activity. This result is similar to our observations with DEL3 (Fig. 1), which contained only the CARG1 element. Although the sequence of CARG1 is the same for the human and the chicken cardiac actin genes, CCAATAAGG (24), we were not able to detect substantial binding of nuclear factors to this element with nuclear extracts from C2C12 cells, L cells, or embryonic chicken brain, heart, or breast muscle extracts. Lack of binding may have been due to subtle differences in the binding assay or variations in nuclear extract preparation. Alternatively, differences in the compositions of the sequences flanking the various CARG regions may modulate factor affinity for a particular CARG element. Indeed, methylation of a single G residue immediately flanking CARG1 of the chicken (37) and *Xenopus* (27) skeletal actin promoters inhibits protein binding. This effect is even more pronounced in the case of the *Xenopus* cytoskeletal actin serum response element, in which two nucleotide substitutions in the two bases flanking the CARG sequence within this element causes a 30-fold decrease in affinity for the binding factor (34).

Our efforts to detect nuclear factors binding to the proximal regulatory region of the chicken cardiac actin gene began with DEL3, the shortest promoter fragment that gave a moderate degree of regulation (Fig. 1). Nuclear extracts from both muscle and nonmuscle sources were able to form specific protein-DNA complexes (Fig. 2); however, the complex was more readily formed with nuclear extracts from tissues not expressing the cardiac actin gene. This result could reflect the absence of the factor or a factor modification such that binding no longer occurred. By comparison, binding activity to the β -actin CCAAT domain was very similar in all extracts tested, suggesting that procedural variations were not responsible for the cardiac promoter-specific differences seen with the various extracts. The -100 promoter fragment still contained a single CARG1 element, so it was surprising to find that the factor-binding domain mapped over and adjacent to the TATA region, not the CARG1 region (Fig. 3) as reported for the human cardiac actin gene (32). The specificity of this binding further supported the idea that binding to this proximal region was unique for the cardiac actin promoter, since various CARG, CCAAT, and TATA regions were not able to compete for factor binding.

Replacing the cardiac actin TATA domain with the same region from the β -actin gene while maintaining only the CARG1 element resulted in a further 50% drop in promoter activity and a complete loss of tissue-restricted expression (compare DEL3beta in Fig. 4 with DEL3 in Fig. 1). Thus, the TATA regions are not equivalent, as supported by results of the competition studies, and the data indicate that the cardiac TATA domain is involved specifically in pro-

FIG. 8. DNase I footprinting, chemical cleavage, and methylation interference analyses of the CARG2 binding domain with nuclear extracts from embryonic chicken brain. (A) DNase footprint of the coding (C) and noncoding (N) strands of oligo IV (–150 to –100). Free (f) and bound (b) fragments were separated by band shift after treatment with DNase I as described in Materials and Methods. Brackets indicate the approximate binding domains. (B) Chemical footprint with a 1,10-phenanthroline-copper complex. (C) Methylation interference assay. Arrows indicate the methylated G residues that showed some degree of binding interference. (D) Summary of the results.

motor regulation. In fact, all deletions containing the β -actin TATA region displayed activity roughly 50% lower than that of the unsubstituted deletions.

Even though nuclear proteins binding to the CARG1 domain of the chicken cardiac actin promoter could not be demonstrated, deletion studies suggested that the sequences including the CARG1 region are important for activity and regulated expression (Fig. 1, DEL3 versus DEL4). In addition, the promoter sequence from –315 to –100 in the chicken cardiac actin gene, which contains three of the four CARG elements (CARG4 to CARG2), excluding CARG1 (Fig. 1), can appropriately regulate tissue-restricted promoter activity when joined simply to the α - or β -actin TATA region (Fig. 5). The upstream region containing these CARG domains is therefore particularly important for tissue-restricted, regulated promoter activity.

Nuclear proteins binding to this distal regulatory region were detected in band shift assays using the *Ball-NarI* restriction fragment from –200 to –100. Similar to the results with nuclear proteins binding to the TATA region, nuclear extracts from tissues expressing the cardiac actin gene showed little detectable binding to this fragment, whereas extracts from tissues not expressing the cardiac

gene produced a specific protein-DNA complex (Fig. 7A, lane 3). Specific competition for binding to this distal regulatory region was achieved only with the homologous fragment (Fig. 7C and D). Further definition of the binding domain by DNase I footprint analysis, chemical cleavage, and methylation interference indicated that the CARG2 element was within the binding site (Fig. 8) and that binding involved the two G residues within the CARG2 sequence.

Although the CARG1, CARG2, and TATA regions are involved in the differentiation-dependent regulation of the chicken cardiac actin promoter, presumably via both positive and negative *trans* activators, a subset of the nuclear proteins binding to these regions is clearly not restricted to muscle tissue. Factors binding to CARG1 of the human cardiac actin promoter were also present in cells not expressing the gene (32). Gustafson et al. (14) recently described factor binding to the CARG1 element of the human cardiac actin promoter with extracts prepared from muscle and nonmuscle cells, even though this domain is apparently involved in muscle-specific transcriptional regulation. Methylation interference studies indicated that the identical nucleotides interact with the factor(s) from both cell types, similar to the CARG2 binding reported here for brain and

FIG. 9. In vitro transcription of the cardiac actin promoter: role of the CArG2- and TATA-region nuclear factor-binding domains. Supercoiled DNA was transcribed in L-cell extracts and assayed by primer extension with a CAT-specific primer. (A) In vitro transcription of DEL2 (lane 1), DEL3 (lane 2), DEL2beta (lane 3), and DEL3beta (lane 4) plasmids. A myosin light-chain 1f promoter deletion construct, cloned in p8CAT, was cotranscribed as an internal control. (B) Transcriptional activity quantitated by densitometric scanning and normalized to the activity of the myosin light-chain 1f construct. Transcriptional activities are expressed relative to that of DEL2, which was assigned a value of 1.

breast muscle nuclear extracts. Competition studies confirmed that the same factor(s) bound the serum response element of the *c-fos* gene; therefore, the factor(s) was neither gene nor tissue specific. The factors we describe binding to the chicken cardiac actin promoter are apparently different in that they are more abundant in tissues not expressing the cardiac gene and the binding is not competed for by any of the heterologous CArG, TATA, or additional sequence motifs tested.

In vitro transcription of promoter constructs lacking various combinations of these CArG2 and TATA binding regions demonstrated increased transcriptional activity as the binding regions were removed (Fig. 9), resulting in a 50-fold increase in transcription for the construct lacking both regions. This observation is consistent with the idea that these binding factors serve as negative regulators in cells that do not express α -cardiac actin. Repression of the chicken α -smooth muscle actin gene in skeletal muscle cultures appears to involve a DNA segment containing a CArG element (6) that may interact with similar negative factors.

A search for the CArG2- and TATA-region binding-domain sequences in the cardiac actin genes from other species revealed that CArG2 is highly conserved in human, mouse (24), and *Xenopus* (27) species in reverse orientation (Fig. 10A), with the exception of a single nucleotide replacement in the mouse and *Xenopus* sequences. The CArG2 sequence is also reminiscent of a consensus sequence, CATTCCT, that has been found to be important in troponin I expression (22). The GCCGGGGGC sequence motif immediately 5' to the chicken cardiac TATA box, which displays various degrees of methylation interference, is also found in reverse orientation in the human and mouse promoters but not in the *Xenopus* promoter (Fig. 10B). However, this sequence is located further upstream of the TATA box in the human and mouse promoters than in the chicken promoter.

The overlap of the binding site with the TATA box in the chicken promoter may therefore not be functionally relevant. The precise role of these sequence elements in other promoters must be studied further.

In summary, the results presented here define two distinct regulatory domains in the chicken α -cardiac actin promoter. The proximal domain includes the CArG1 or CCAAT box and the TATA region. The distal domain is more complex and is characterized by the presence of three additional CArG-box elements (CArG2 to -4). Deletion and substitution analyses with the chicken cardiac actin promoter demonstrate that these two domains interact to control differentiation-dependent expression. The nuclear factors binding to these two domains over CArG2 and in the vicinity of the TATA element may possibly act as negative regulators, since they are more abundant in cells not expressing the cardiac actin gene and removal of the binding domains from the cardiac actin promoter relaxes regulation in vivo (high fibroblast expression in DEL3 and DEL3beta) and increases transcriptional activity in nuclear extracts from nonmuscle cells in vitro.

To date, there has been no characterization of a nuclear protein that specifically activates the transcription of a particular muscle gene. Although positive-acting factors likely play a role in muscle gene activation during myogenesis (17), the removal of specific repressors as a first step in this activation cannot be ruled out, and our results support this interpretation. An example of this type of regulation is provided by the histone H2B-1 gene in sea urchins. This gene is under strict developmental control and is expressed only during spermatogenesis. However, the promoter is activated by a CCAAT-binding protein that is common to other tissues. The control of expression is mediated by a sequence-specific negative regulatory factor that prevents the interaction of the CCAAT-binding protein with the promoter (1). This type of mechanism may also be used in

FIG. 10. Interspecies sequence comparison of the core binding domains for the CArG2 and TATA elements in the chicken cardiac actin promoter. (A) The CArG2 sequence element, which is highly conserved between species, is underlined. The chicken (C) sequence element is conserved on the opposite strand in the reverse orientation in the human (H), mouse (M), and *Xenopus* (X) actin promoters. (B) The core binding domain for the chicken (C) TATA-box region is underlined. The same sequence element is found further 5', in the reverse orientation, in the human (H) and mouse (M) promoters. This element is not present in this region in the *Xenopus* promoter.

gene regulation during myogenesis, and the factors described here may be such negative regulatory proteins.

LITERATURE CITED

- Barberis, A., G. Superti-Furgar, and M. Busslinger. 1987. Mutually exclusive interaction of the CCAAT binding factor and of a displacement protein with overlapping sequences of a histone gene promoter. *Cell* 50:347-359.
- Bergsma, D. J., J. M. Grichnik, L. M. A. Gosset, and R. J. Schwartz. 1986. Delimitation and characterization of *cis*-acting DNA sequences required for the regulated expression and transcriptional control of the chicken skeletal α actin gene. *Mol. Cell. Biol.* 6:2462-2475.
- Billeter, R., W. Quitschke, and B. M. Paterson. 1988. Approximately 1 kilobase of sequence 5' to the two myosin light-chain 1f/3f gene cap sites is sufficient for differentiation-dependent expression. *Mol. Cell. Biol.* 8:1361-1365.
- Buckingham, M. E., and A. J. Minty. 1984. Contractile protein genes. p. 365-395. In M. Maclean, S. P. Gregory, and R. A. Flavell (ed.), *Eukaryotic genes: their structure, activity, and regulation*, vol. 21. Butterworths, London.
- Cao, Z., E. A. Barron, A. J. Carrillo, and Z. D. Sharp. 1987. Reconstitution of cell-type-specific transcription of the rat pro-lactin gene in vitro. *Mol. Cell. Biol.* 7:3402-3408.
- Carroll, S. L., D. J. Bergsma, and R. J. Schwartz. 1988. A 29-nucleotide DNA segment containing an evolutionary conserved motif is required in *cis* for cell-type-restricted repression of the chicken α smooth muscle actin gene core promoter. *Mol. Cell. Biol.* 8:241-250.
- Dente, L., M. Sollazzo, C. Baldari, G. Cesareni, and R. Cortese. 1985. The pEMBL family of single stranded vectors. p. 101-107. In D. M. Glover (ed.), *DNA cloning*, vol. 1. IRL Press, Washington, D.C.
- DePonti-Zilli, L., A. Seiler-Tuyns, and B. Paterson. 1988. A 40 base-pair sequence at the 3' end of the β actin gene regulates β actin mRNA transcription during myogenesis. *Proc. Natl. Acad. Sci. USA* 85:1389-1393.
- De Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* 7:725-737.
- Elder, K. E., C. L. French, M. Subramaniam, L. J. Schmidt, and M. J. Getz. 1988. Evidence that the functional β actin gene is single copy in most mice and is associated with 5' sequences capable of conferring serum- and cycloheximide-dependent regulation. *Mol. Cell Biol.* 8:480-485.
- Eldridge, J., Z. Zehner, and B. M. Paterson. 1985. Nucleotide sequence of the chicken α cardiac actin gene: absence of strong homologies in the promoter and 3'-untranslated regions with the skeletal α actin sequence. *Gene* 36:55-63.
- Galas, J. D., and A. Schmitz. 1978. DNase footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* 5:3157-3170.
- Gorman, C. 1985. High efficiency gene transfer into mammalian cells. p. 143-190. In D. M. Glover (ed.), *DNA cloning*, vol. 2. IRL Press, Washington, D.C.
- Gustafson, T. A., T. Miwa, L. M. Boxer, and L. Kedes. 1988. Interaction of nuclear protein with muscle-specific regulatory sequences of the human cardiac α -actin promoter. *Mol. Cell Biol.* 8:4110-4119.
- Hayward, L. J., and R. J. Schwartz. 1986. Sequential expression of chicken actin genes during myogenesis. *J. Cell Biol.* 102:1485-1493.
- Heberlein, U., and R. Tjian. 1988. Temporal pattern of alcohol dehydrogenase gene transcription reproduced by *Drosophila* stage-specific embryonic extracts. *Nature (London)* 331:410-451.
- Jaynes, J. B., J. E. Johnson, J. N. Buskin, C. L. Gartside, and S. D. Hauschka. 1988. The muscle creatine kinase gene is regulated by multiple upstream elements, including a muscle-specific enhancer. *Mol. Cell. Biol.* 8:62-70.
- Kost, T. A., N. Theodorakis, and N. Hughes. 1983. The nucleotide sequence from the chick cytoplasmic β actin gene. *Nucleic Acids Res.* 11:8287-8301.
- Kuwabara, M. D., and D. S. Sigman. 1987. Footprinting DNA-protein complexes *in situ* following gel retardation assays using 1,10-phenanthroline-copper ion: *Escherichia coli* RNA poly-

- merase-lac promoter complexes. *Biochemistry* **26**:7234–7238.
20. Lassar, R. B., B. M. Paterson, and H. Weintraub. 1986. Transfection of a DNA locus that mediates the conversion of 10T 1/2 fibroblasts myoblasts. *Cell* **47**:649–656.
 21. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 22. Mar, J. H., and C. P. Ordahl. 1988. A conserved CATTCCT motif is required for skeletal muscle-specific activity of the cardiac troponin T gene promoter. *Proc. Natl. Acad. Sci. USA* **85**:6404–6408.
 23. Maxam, A. M., and W. Gilbert. Sequencing of end-labeled DNA with base specific cleavage reactions. *Methods Enzymol.* **65**: 499–560.
 24. Minty, A., and L. Kedes. 1986. Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. *Mol. Cell. Biol.* **6**:2125–2136.
 25. Minty, A. J., S. Alonso, M. Cavavotti, and M. B. Buckingham. 1982. A fetal skeletal muscle actin mRNA in the mouse and its identity with cardiac actin mRNA. *Cell* **30**:185–192.
 26. Miwa, T., L. M. Baker, and L. Kedes. 1987. CArG boxes in the human cardiac α -actin gene are core binding sites for positive trans-acting regulatory factors. *Proc. Natl. Acad. Sci. USA* **84**:6702–6706.
 27. Mohun, T. J., N. Garrett, and J. B. Gurdon. 1986. Upstream sequences required for tissue-specific activation of the cardiac actin gene in *Xenopus laevis* embryos. *EMBO J.* **5**:3185–3193.
 28. Muscat, G. E. O., and L. Kedes. 1987. Multiple 5'-flanking regions of the human α -skeletal actin gene synergistically modulate muscle-specific expression. *Mol. Cell. Biol.* **7**:4089–4099.
 29. Ordahl, C. P., and T. A. Cooper. 1983. Strong homology in promoter and 3'-untranslated regions of chick and rat α actin genes. *Nature (London)* **303**:348–349.
 30. Paterson, B. M., and J. Eldridge. 1984. α cardiac actin is the major isoform expressed in embryonic avian skeletal muscle. *Science* **224**:1436–1438.
 31. Paterson, B. M., and R. C. Strohman. 1972. Myosin synthesis in cultures of differentiating chicken embryo skeletal muscle. *Dev. Biol.* **29**:113–138.
 32. Phan-Dinh-Tuy, F., D. Tuil, F. Schweighoffer, C. Pinset, A. Kahn, and A. Minty. 1988. The CCArGG box. A protein-binding site common to transcription-regulatory regions of the cardiac actin, c-fos and interleukin-s receptor genes. *Eur. J. Biochem.* **173**:507–515.
 33. Seiler-Tuyns, A., J. D. Eldridge, and B. M. Paterson. 1984. Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells. *Proc. Natl. Acad. Sci. USA* **81**:2980–2984.
 34. Treisman, R. 1987. Identification and purification of a polypeptide that binds to the c-fos serum response element. *EMBO J.* **6**:2711–2717.
 35. Vandekerckhove, J., and K. Weber. 1978. At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide. *J. Mol. Biol.* **126**:783–802.
 36. Vandekerckhove, J., and K. Weber. 1979. The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle, and rabbit slow skeletal muscle. A protein chemical analysis of muscle differentiation. *Differentiation* **14**: 123–133.
 37. Walsh, K., and P. Schimmel. 1988. Two nuclear factors compete for the skeletal muscle actin promoter. *J. Biol. Chem.* **262**: 9429–9432.
 38. Walsh, K., and P. Schimmel. 1988. DNA-binding site for two nuclear factors is important for expression in muscle cells. *Mol. Cell. Biol.* **8**:1800–1810.
 39. Wu, C., S. Wilson, B. Walker, I. David, T. Paisley, V. Zimarino, and H. Ueda. 1987. Purification and properties of *Drosophila* heat shock activator protein. *Science* **238**:1247–1253.