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Distinct Enhancers Regulate Skeletal and Cardiac Muscle-Specific Expression Programs of the Cardiac α-Actin Gene in *Xenopus* Embryos

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During vertebrate embryonic development, cardiac and skeletal muscle originates from distinct precursor populations. Despite the profound structural and functional differences in the striated muscle tissue they eventually form, such progenitors share many features such as components of contractile apparatus. In vertebrate embryos, the α-cardiac actin gene encodes a major component of the myofibril in both skeletal and cardiac muscle. Here, we show that expression of *Xenopus* cardiac α-actin in the myotomes and developing heart tube of the tadpole requires distinct enhancers within its proximal promoter. Using transgenic embryos, we find that mutations in the promoter-proximal CArG box and 5 bp downstream of it specifically eliminate expression of a GFP transgene within the developing heart, while high levels of expression in somitic muscle are maintained. This sequence is insufficient on its own to limit expression solely to the myocardium, such restriction requiring multiple elements within the proximal promoter. Two additional enhancers are active in skeletal muscle of the embryo, either one of which has to interact with the proximal CArG box for correct expression to be established. Transgenic reporters containing multimerised copies of CArG box 1 faithfully detect most sites of SRF expression in the developing embryo as do equivalent reporters containing the SRF binding site from the c-*fos* promoter. Significantly, while these motifs possess a different A/T core within the $CC(A/T)_6GG$ consensus and show no similarity in flanking sequence, each can interact with a myotome-specific distal enhancer of cardiac α-actin promoter, to confer appropriate cardiac α-actin-specific regulation of transgene expression. Together, these results suggest that the role of CArG box 1 in the cardiac α-actin gene promoter is to act solely as a high-affinity SRF binding site.

Key Words: **cardiac α-actin; muscle; SRF; *Xenopus*.**

INTRODUCTION

In all vertebrates, skeletal muscle is derived from paraxial mesoderm, while cardiac muscle progenitors form from a distinct region of anterolateral mesoderm. The relative timing of skeletal and cardiac differentiation varies between different vertebrate classes, reflecting different strategies for embryonic development. For mammalian embryos, an early dependence on the maternal supply of nutrients and the requirements of gas exchange both necessitate rapid development of a functional cardiovascular system. In contrast, embryos of most fish and amphibia require skeletal muscle early during embryogenesis in order to swim, while the cardiovascular system develops relatively slowly, being required only later in larval stages of development.

During skeletal muscle differentiation, critical early roles are played by myogenic factors belonging to the basic–loop–helix family, including MyoD and Myf5. These factors are remarkable since not only are they required for skeletal muscle development in the embryo, but their expression is also sufficient to convert nonmyogenic cell type into myotubes in culture. Myogenic factors execute their roles, at least in part, by transactivating skeletal muscle-specific differentiation genes, and by down-regulating genes in-

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volved in cell cycle and growth control (Puri and Sartorelli, 2000). In contrast, no cardiogenic factors of equivalent potency have, so far, been described. Instead, numerous studies on regulation of cardiac-specific genes suggest that their transcription is regulated in a more complex manner, mediated by several families of transcription factors, none of which is strictly restricted to myocardial muscle tissue. The homeobox gene Nkx2.5, MADS family proteins MEF2 and Serum Response Factor (SRF), along with the zinc-finger factors GATA 4–6 have all been implicated in regulation of myocardial-specific genes (Fishman and Chien, 1997; Mohun and Sparrow, 1997). These transcription factors appear to act in concert in a variety of combinations to transactivate their target genes. Although poorly understood, the basis of their interaction is most likely the formation of heterodimers, or even higher-order complexes (Belaguli *et al.*, 2000; Biesiada *et al.*, 1999; Chen and Schwartz, 1996; Morin *et al.*, 2000, 2001).

In addition to having a role in regulating expression of cardiac differentiation genes, each of these transcription factors is involved in other developmental pathways or differentiated cell types. For example, Nkx2.5 is involved in development of the pharynx (Tanaka *et al.*, 2001), GATA-4 in gut formation (Zaret, 1999), and MEF2 in skeletal muscle differentiation (Black and Olson, 1998; Puri and Sartorelli, 2000). SRF in particular, has been implicated in the regulation of diverse transcriptional pathways. As well as participating in skeletal and cardiac muscle differentiation, it also plays a role in the regulation of genes that are inducible by serum growth factors and other extracellular stimuli (Treisman, 1995). In each role, SRF acts by binding to a DNA recognition site of consensus sequence $CC(A/T)_6GG$ (termed CArG box), present in the regulatory regions of its targets (Treisman, 1995).

How is specificity of SRF function achieved when the protein can regulate such a large number of dissimilar target genes in different cellular and developmental contexts? Studies to date have suggested that SRF activity is likely to be regulated on several levels. Of critical importance is the capacity of SRF to interact directly with numerous accessory proteins. For example, ETS-family proteins such as Elk-1 simultaneously bind SRF and a sequence adjacent to the serum response element (SRE) in the promoters of several cellular immediate-early genes, including the c-*fos* proto-oncogene (Treisman, 1994). This ternary complex mediates the response of c-*fos* to a range of stimuli (Treisman, 1994). Additionally, experiments with cultured cells indicate that SRF can interact with tissue-restricted cofactors such as MyoD (Groisman *et al.*, 1996) and GATA-4 (Belaguli *et al.*, 2000; Morin *et al.*, 2001), and such interactions may play a role in restricting activity of SRF during muscle differentiation *in vivo*. Posttranslational modifications of SRF protein are also known to regulate its activity and can be triggered by such diverse stimuli as growth factors and cytoskeletal actin polymerisation (Sotiropoulos *et al.*, 1999; Treisman, 1995). Finally, multiple splicing pathways for the SRF gene transcript may produce functionally distinct products, including at least one that appears to possess dominant-negative activity (Belaguli *et al.*, 1999; Kemp and Metcalfe, 2000).

Among striated muscle-specific genes identified as targets of SRF, one of the best studied is the cardiac α-actin gene. This encodes a major structural component of the striated muscle myofibril in vertebrate embryos. Four conserved CArG boxes are present in the promoters of the human, mouse, chick, and amphibian genes along with binding sites for the MyoD family of myogenic factors. Previous studies have identified the most proximal CArG box as essential for activity of the human cardiac α-actin promoter in cultured cardiomyocytes, and a combination of this CArG box and adjacent binding sites for SP1 and MyoD are necessary for activation of the cardiac α-actin promoter in differentiating myogenic cell lines (Biesiada *et al.*, 1999; Sartorelli *et al.*, 1990). In *Xenopus*, microinjected copies of the gene require the proximal CArG box and a distal myogenic factor binding site for expression in embryo explants that have been induced to form skeletal muscle (Mohun *et al.*, 1989b). In transgenic mouse embryos, a proximal enhancer of the mouse gene, including the four CArG boxes, is sufficient for correct but weak expression of a transgene, while more distal sequences are required for appropriate expression in the adult (Biben *et al.*, 1996).

Since the cardiac α-actin gene is activated during formation of both cardiac and skeletal muscle in vertebrates, its study may also shed light on the mechanisms that distinguish terminal differentiation in these two muscle types. In previous studies, we have examined expression of a reporter driven by the cardiac α-actin gene promoter using a direct DNA injection assay (Mohun *et al.*, 1989b). However, injected episomal DNA is unstable in embryos, and while we were able to examine expression of the reporter in embryonic skeletal muscle, we were unable to extended the assay to monitor expression in the developing heart. Transgenesis (Kroll and Amaya, 1996) overcomes such limitations and we have now used this approach to delineate sequences necessary for transgene expression in striated muscle of the embryo. Our results indicate that distinct but overlapping sets of regulatory sequences are responsible for expression of the cardiac α-actin gene in myotomal and cardiac muscle.

MATERIAL AND METHODS

Recombinant DNA

A series of 8-bp linker scan (LS) mutations have been described previously (Mohun *et al.*, 1989b). An internal deletion, LS10/13, was constructed by replacing the *Kpn*I-*Eco*RI fragment from LS13 with the equivalent fragment from LS10. All cardiac α-actin promoter variants were cloned in front of a reporter comprising the mGFP5 coding region and the SV40 polyadenylation sequence (Sparrow *et al.*, 2000a).

Heterologous Promoter Fusions

A minimal promoter fragment comprising 48 bp of the cytoskeletal actin promoter (Mohun and Garrett, 1987) was fused to the mGFP5/SV40 cassette; an equivalent construct comprising the thymidine kinase minimal promoter has been described previously (Sparrow *et al.*, 2000a).

The following oligonucleotides were used to construct synthetic promoters comprising sequences from the cardiac α-actin promoter: CArG box 1 L; TACCAAATAAGGGTCACCTGCCTTTCC: CArG box 1 R; TACCAAATAAGGGCAGGCTATGATTCC: CArG Box 1 + DS; TACCAAATAAGGGCAGGCTGCCTTTCC: mut Box 1-5; CTACCAAATTTGGGCAGGCTGCCTTTCC: mut Box 1-6; CTACCTTTTAAGGGCAGGCTGCCTTTCC: E-CArG box 1; GCACCTGTCTACTCCATTTGCCAAATAAGGGCAGGC; c-*fos* SRE oligo sequence: GGATGTCCATATTAGGACATCT (Taylor *et al.*, 1989). Complementary oligonucleotides were annealed and cloned in vectors (as indicated) via *Kpn*I or Asp718 cohesive ends. All constructs were sequenced to establish the number and orientation of oligonucleotide copies cloned.

A promoter fragment spanning nucleotides $-275/-92$ of the cardiac α-actin promoter was amplified by PCR. pF711 comprises the entire human c-*fos* gene and promoter; pF100 possesses a truncated promoter in which sequences upstream of -100 (including the SRE) have been removed (Treisman, 1985). Cardiac α-actin promoter variants were cloned as *Eco*RI/*Bam*HI fragments in front of pF100.

Xenopus Embryo Manipulations

Blastula animal pole explants were dissected from *Xenopus* embryos and treated with activin as described previously (Sive, 2000; Smith *et al.*, 1988). DNA injections were performed at the two-cell stage by using 100 pg of DNA (Mohun *et al.*, 1989a).

Transgenic embryos were generated according to method of Amaya and Kroll (Kroll and Amaya, 1996), with modifications as described (Sparrow *et al.*, 2000b). To confirm successful generation of transgenic embryos when using very weak or inactive cardiac α-actin promoter variants, we included a γ-crystallin/GFP reporter (Offield *et al.*, 2000) with the test construct. We found that using an equimolar ratio of the full-length cardiac α-actin/GFP and γ-crystallin/GFP reporters, approximately 75% of transgenic embryos coexpressed both transgenes.

Analysis of Transgene Expression

Embryos expressing GFP were analysed either by direct observation of fluorescence or by whole-mount *in situ* hybridisation to detect GFP mRNA (Sparrow *et al.*, 2000a). GFP activity in transgenic embryos was documented by using a CoolSnap digital camera (Roper Scientific) attached to a Leica MZFLIII dissecting microscope with fluorescence illumination. Images were processed in Adobe Photoshop 5.0 and weak expression resolved from background autofluorescence by using the "auto levels" function. This procedure transforms the green colour of GFP fluorescence to blue, which is then readily distinguished from auto-fluorescence of yolk, which acquires an orange–yellow colour. Whole-mount *in situ* hybridisation was performed as previously described (Harland, 1991), with probes specific for GFP (Sparrow *et al.*, 2000a) and XSRF (Mohun *et al.*, 1991). A second XSRF probe encompassing the 3′ half of the cDNA gave identical results to those presented here.

Electrophoretic Mobility Shift Analysis

Double-stranded oligonucleotides with 5′GTAC overhangs (20 ng) were labelled with [α-^{32}P]-dCTP and Klenow DNA polymerase as described (Sambrook, 1989). Human SRF (Norman *et al.*, 1988) was translated *in vitro* by using a coupled transcription-translation system (Promega) according to manufacturer's instructions. Binding reactions and competitions were as described (Norman *et al.*, 1988).

Oligonucleotides used for probes and competition assays are described above, except CArG mut (see Fig. 6B): CTAGGAAATAAGGGCAGGCTGCCTTTC.

Cell Transfections and RNase Protection Assays

Serum stimulation assays using transfected NIH3T3 cells were performed and analysed by RNase protection assays exactly as described previously (Taylor *et al.*, 1989), using the expression of a transfected α-globin construct as an internal reference (Treisman, 1985).

RESULTS

580 bp of Cardiac α-actin Promoter Is Sufficient to Recapitulate the Endogenous Expression Pattern during Embryonic Development

In previous studies, 580 bp of the cardiac α-actin promoter were sufficient to confer high levels of reporter gene expression from episomal, plasmid DNA in skeletal muscle derived from *Xenopus* embryonic tissue (Mohun *et al.*, 1989b). We therefore tested the activity of the same promoter fragment fused to a green fluorescent protein (GFP) reporter in transgenic frog embryos. GFP expression was first seen in the somites during neurula stages (Figs. 1A and 1C), and subsequently detected in cardiac, facial, body wall and tail muscles (Figs. 1B, 1D–1G). In later embryos, transgene expression was also detected in developing limb buds (data not shown).

We next tested the ability of activin to induce expression of the GFP transgene in animal pole explants from F_1 transgenic embryos. By the time sibling control embryos had reached neurula stages, GFP expression was readily detected in the explants treated with activin, confirming that skeletal muscle differentiation was induced (Figs. 1H and 1I). Similarly, the cardiac α-actin GFP transgene provided a sensitive marker for cardiac muscle differentiation. Prolonged culture of anteroventral tissue explants encompassing the heart field of early tailbud embryos (Sater and Jacobson, 1990) resulted in the formation of primitive heart tubes. These were readily visible by GFP fluorescence (Fig. 1K) long before their detection was possible by morphology alone. Larger explants subsequently developed facial and body wall muscles, patterned in a manner remarkably similar to that detected in the whole embryo (Figs. 1J and 1K). These results demonstrate that 580 bp of proximal promoter sequence direct spatiotemporal expression during embryogenesis in a manner indistinguishable from the endogenous gene.

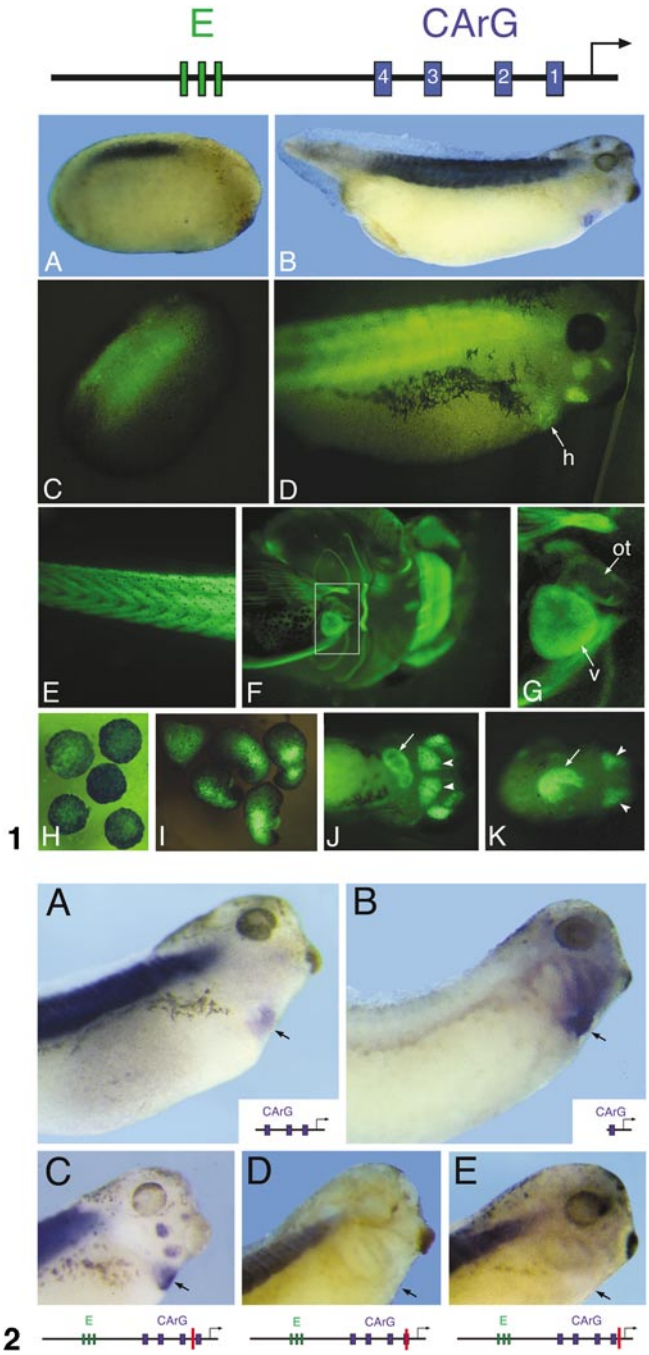

FIG. 1. A portion of cardiac α-actin promoter that is −580 bp is sufficient for correct expression in *Xenopus* embryos, tadpoles, and explants. (Top) Schematic presentation of −580 cardiac α-actin promoter. Transgene expression can be detected in the somites of the late neurula embryo and in myotomes, facial muscle and heart (h) of the larval tadpole, either by whole-mount *in situ* for GFP RNA (A, B) or by GFP fluorescence (C, D). (E) GFP activity in the tail of 7-day-old tadpole. (F) Ventral view of a 7-day-old tadpole, showing strong GFP activity in the heart (boxed), body wall, and jaw muscles. Anterior is to the right. (G) Close-up of the heart region boxed in (G). ot, outflow tract; v, ventricle. (H, I) Animal cap explants from F_1 transgenic embryos cultured in the absence (H) or presence (I) of 8 U/ml activin until sibling embryos reached stage 24. GFP activity is only seen in animal caps treated with activin, demonstrating that the transgene is activated by the mesoderm-inducing signal. GFP expression can be used to monitor heart and head muscles in explants from F_1 transgenic embryos. (J) Stage 34 control embryo, ventral view, anterior to the right. Strong expression is detected in the heart (arrow) and head muscles (arrow heads). (K) A heart field explant excised at stage 22, oriented in the same way as an embryo in (J).

CArG Box1 and Immediate Proximal Sequences Are Necessary for Expression in the Heart

We next tested the effect of individual 8-bp linker scan mutations on expression of the cardiac actin-GFP transgene in skeletal and cardiac muscle of transgenic embryos. Surprisingly, our results proved strikingly different from those obtained by direct DNA injection. In those studies, mutation of CArG box 1 (linker scan mutation LS29) abolished any expression of the reporter in embryonic skeletal muscle (Mohun *et al.*, 1989b). By contrast, when tested by transgenesis, the same mutation had little or no apparent effect on GFP expression in the myotomes, rather it selectively abolished later expression in differentiating heart muscle (Fig. 2D). Mutation of flanking sequences immediately upstream of CArG box1 (LS25) had no comparable effect but, unexpectedly, an equivalent mutation affecting proximal flanking nucleotides (LS13) also resulted in specific reduction or loss of heart-specific expression (Figs. 2C and 2E).

The *Xenopus* gene contains two other CArG motifs upstream of CArG box 1, each of which can bind SRF (Taylor *et al.*, 1989) and functionally replace CArG box 1 when moved to the equivalent position in the promoter. Not surprisingly, removal of all CArG boxes by deletion of sequences from −216 to −80 (internal deletion LS10/13) also abolished expression of the transgene in cardiac muscle (Fig. 3A). Insertion of two copies of a short oligonucleotide comprising only CArG box 1 and the immediate downstream sequence into the internal deletion was sufficient to restore strong levels of transgene expression in the tadpole heart (Fig. 3B). Furthermore, the temporal pattern of transgene expression was comparable with that of the full-length promoter (data not shown).

FIG. 2. E-boxes are dispensable for normal expression of cardiac α-actin in embryos, while CArG box 1 and sequence immediately downstream of it are necessary for expression in the heart. (A) Deletion of sequences upstream of −216 results in expression indistinguishable from that obtained with the entire 580-bp promoter. (B) 5′Δ-103 is expressed in the heart but not in the myotomes. It also gives inappropriate expression in the head. (C) Linker scan mutation LS25 shows no effect on expression. (D) In contrast, LS29, which destroys CArG box 1, results in loss of heart expression. (E) A sequence downstream of CArG box 1 is essential for expression in the heart, as revealed by linker scan mutation LS13.

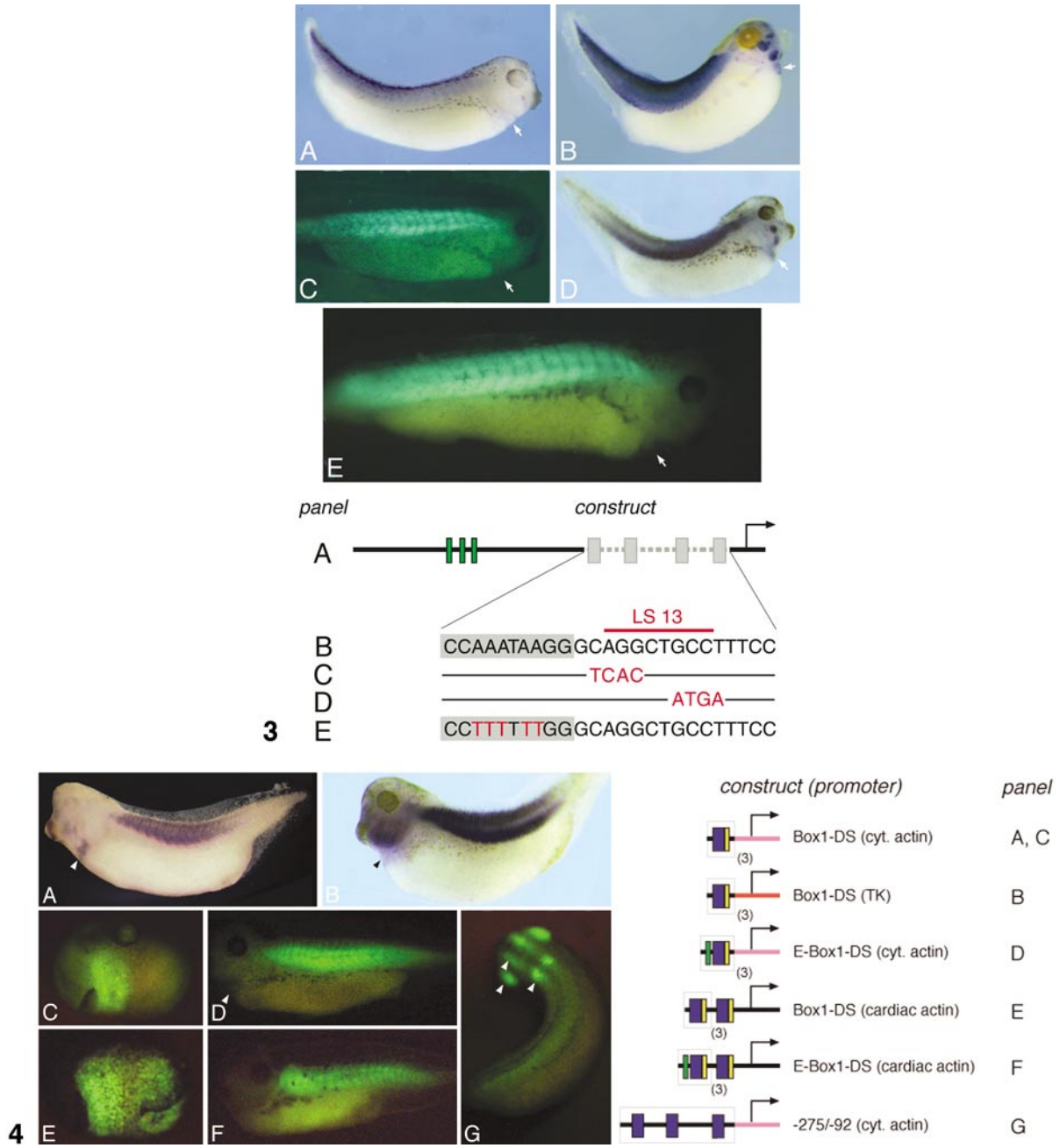

FIG. 3. (A) Internal deletion LS10/13 removes CArG boxes 1-4, resulting in weak expression in the myotomes and loss of expression in the heart. (B) An oligonucleotide comprising CArG box 1 and the adjacent 10 bp downstream are sufficient to restore normal expression to LS10/13. (C), L-LS10/13, in which 5 bp most proximal to CArG box 1 are changed, is not expressed in the heart but is expressed normally in the myotomes. (D) Mutation of the more distal 5 bp has no effect on transgene activity in the heart. (E) Mutant T-LS10/13 shows expression in the myotomes, but not in the heart (arrow). (Transgenic constructs shown in B–E all contain two copies of the oligonucleotides; see Table 1.)

FIG. 4. CArG Box 1 + DS is sufficient for expression in myotomes and head and heart region. (A) A chimeric construct comprising oligonucleotide CArG Box 1 + DS adjacent to the minimal cytoskeletal actin promoter gave expression in the myotomes, heart (arrowed), and branchial arches of the stage 33 tadpole. (B) Similar expression was obtained with this oligonucleotide in front of the thymidine kinase minimal promoter. (C) However, at earlier stages, transgene expression directed by CArG Box 1 + DS is premature and too broad, as seen in the presumptive heart field of stage 22 embryo. (D, F) Using either the cardiac actin or cytoskeletal actin basal promoter, an E-box placed adjacent to the CArG box 1 + DS restricts activity of transgene to the myotomes. No expression is seen in the heart (arrowed). (E) The CArG Box 1 + DS oligonucleotide also directs premature and broad expression in the stage 22 embryo when placed in front of the 5′Δ-103truncation of the cardiac α-actin promoter. (G) Nucleotides spanning −275 to −91 of the cardiac α-actin promoter can act as a weak myotome-specific enhancer. Dorsolateral view of a stage. 25 embryo showing feint, but detectable fluorescence in the myotomes. (NB, Strong fluorescence in the forebrain, hindbrain, and eyes (arrows) results from the presence of a γ crystallin/GFP cotransgene). (A–E) Anterior to left. (G) Anterior at top.

In an effort to delineate more precisely the nucleotides downstream of CArG box 1 responsible for restoring expression in the heart, we next tested CArG box 1 oligonucleotides that included variant flanking sequence. Since the entire DS sequence except a central C is conserved between the two amphibian species *Xenopus laevis* and *Xenopus borealis*, we compared mutation of four nucleotides on either side of this central residue. Two copies of each oligonucleotide were cloned into the LS10/13 vector. Mutation of the nucleotides most distal to CArG box 1 (variant R-LS 10/13) had no apparent effect on the expression pattern in either myotomes or heart (Fig. 3D). Conversely, alteration of four nucleotides most proximal to CArG box 1 (variant L-LS 10/13) completely abolished transgene expression within the heart (Fig. 3C). Together, these results demonstrate that the most proximal CArG box, along with five nucleotides downstream, are critical for expression of the cardiac α-actin promoter in cardiac muscle of the embryo.

E Boxes Are Dispensable for Expression of the Cardiac α-Actin Gene in Vivo

We next investigated sequences necessary for transgene expression in embryonic skeletal muscle. Several previous studies of the cardiac actin gene have emphasised importance of E boxes for cell-specific promoter activity, including assays using differentiation of cultured myogenic cell lines and skeletal muscle induction in *Xenopus* embryo explants (Mohun *et al.*, 1989b; Sartorelli *et al.*, 1990). Once again, however, our results with transgenic embryos proved quite different. Truncation of the promoter region from 580 bp to the most proximal 216 bp removes all E box motifs along with CArG box 4, yet transgenic embryos showed GFP expression that was qualitatively indistinguishable from the full-length promoter (Fig. 2A). Therefore, within the embryo, sequences distal from −216 are not essential for correct embryonic expression of the cardiac α-actin gene.

Further truncation to remove all sequences upstream of CArG box 1 (−103) had a profound impact on promoter activity, severely reducing or abolishing expression in myotomal muscle (Fig. 2B, and data not shown). This transgene gave variable levels of GFP expression within the heart along with ectopic expression throughout the head region. Sequences lying between −580 and −103, encompassing the E boxes and CArG boxes 2–4, are therefore essential for proper regulation of the cardiac α-actin gene in both myotomal and cardiac muscle.

Sequences Encompassing CArG Boxes 1–4 Are Required for Strong and Uniform Expression in Skeletal Muscle

Since removal of sequences upstream of CArG box 4 had no apparent effect on expression within the myotomes, while truncation to CArG box 1 severely impaired or abolished expression in this tissue, we reasoned that the intervening sequences, while apparently unimportant for expression in the heart (Figs. 3B–3D), must be important for myotomal expression. Consistent with this, internal deletion of sequences encompassing CArG boxes 1–4 (LS10/13) significantly altered transgene expression in the tadpole myotomes. Instead of uniform, strong expression, staining for GFP transcripts and GFP fluorescence itself were weak, appearing stronger in the posterior portion of the embryo gradient of signal (Fig. 3A, and data not shown). This resembles the graded expression of the myogenic factors during normal development (Hopwood *et al.*, 1991, 1992).

Interestingly, replacement of the entire promoter region containing CArG boxes 1–4 by two copies of the CArG box 1-containing oligonucleotide not only restored transgene expression within the heart, but also reestablished strong, uniform expression throughout the myotomes (compare Figs. 3A and 3B). This result could not be attributed to the presence of the CArG box 1 sequence alone since a truncated promoter comprising only CArG box 1 and more proximal sequences (5′Δ-103; Fig. 2B) showed little or no expression in the myotomes. This suggests that normal expression in striated muscle was restored by the combination of CArG box 1 and the distal, E-box-containing region of the promoter.

Taken together, our results suggest that expression of the full-length cardiac α-actin promoter in skeletal muscle depends on several sequences dispersed throughout the promoter, none of which is apparently indispensable. In the absence of more proximal sequences, the most distal region, containing E box motifs, gives weak and graded expression along the A-P axis. The presence of proximal sequences encompassing the CArG boxes is sufficient to establish a uniform pattern of expression, irrespective of whether distal sequences are present. Finally, the same result can be obtained in the absence of such sequences, as long as both the E-box-containing region and the most proximal CArG box 1 sequence are present.

CArG Box1 + DS Is Sufficient for Expression in the Myotomes, Head, and Heart Region

Having identified sequences within cardiac α-actin promoter that are necessary for expression in the heart and myotomes, we next asked whether they were also sufficient to confer appropriate expression upon a heterologous promoter. A fragment encompassing all identified cardiac α-actin regulatory sequences (−580/−73) gave a pattern of transgene expression indistinguishable from the endogenous gene when placed upstream of a minimal cytoskeletal actin promoter (Table 1, and data not shown). We therefore tested the activity of individual cardiac α-actin promoter elements within the same context.

Three copies of the CArG box1 + DS sequence gave a pattern of expression within the myotomes that was identical to that obtained with the full-length promoter (Fig. 4A). Expression in the heart region, however, was prema-

TABLE 1
Summary of Transgenesis Experiments

Transgene promoter	Insert	Insert copy no.	Embryos analysed	Figure
Cardiac 5′Δ-216	—	NA	16/107	2A
Cardiac LS29	—	NA	34/109	2D
Cardiac LS13	—	NA	42/81	2E
Cardiac LS25	—	NA	11/43	2C
Cardiac LS 10/13	—	NA	16/40	3A
Cardiac LS 10/13	(CArG box1 + DS)	2	64/177	3B
Cardiac LS 10/13	(CArG box1 R)	2	54/109	3D
Cardiac LS 10/13	(CArG box1 L)	2	15/30	3C
Cardiac LS 10/13	(CArG box1 T)	2	12/35	3E
Cardiac LS 10/13	(SRE)	1	7/15	7A
Cardiac LS 10/13	(SRE)	2	12/40	7B,C
Minimal cyt. actin	Cardiac prom −580/−73	1	10/25	n/s
Minimal cyt. actin	Cardiac prom. −275/−91	1	10/37	4G
Minimal cyt. actin	(CArG box1 + DS)	3	66/261	4A,C
Minimal cyt. actin	(CArG box1 L)	3	0/20*	n/s
Minimal cyt. actin	E + CArG box1 + DS	3	28/124	4D
Minimal cyt. actin	(CArGbox1 T)	3	2/12*	n/s
Minimal cyt. actin	(E box)	3	3/15	n/s
Minimal cyt. actin	(CArG box1 m5)	3	0/15*	n/s
Minimal cyt. actin	(CArG box1 m6)	5	2/13*	n/s
Minimal cyt. actin	(SRE)	3	4/14	7D,E
Cardiac 5′Δ-103	—	NA	5/30	2B
Cardiac 5′Δ-103	(CArG box1 + DS)	3	7/8	4E
Cardiac 5′Δ-103	(E + CArG box1 + DS)	3	22/47	4F

Note. Each GFP transgene promoter construct used in the study is listed along with any inserted DNA sequence and its copy number within the promoter. Oligonucleotide insertions (detailed in Materials and Methods) are shown in parentheses. For each construct, the total number of embryos showing GFP expression is shown along with the total of developing embryos. In some experiments (*), inclusion of a γ-crystallin/GFP cotransgene allowed selection of transgenic embryos, and in such cases, this total is shown. n/s, not shown; NA, not applicable.

ture, commencing during early tailbud stages (stage 22–24 rather than stage 26); it was also too broad, encompassing an anteroventral region far larger than the tailbud heart field (Fig. 4C). By the early tadpole stage (stage 32–34), expression of this transgene was evident in the myotomes, the heart, and branchial arches (Fig. 4A).

The expression pattern seen with CArG box 1 + DS sequence could reflect the limited regulatory capacity of this sequence or it could result from an unexpected interaction of this regulatory element with the minimal cytoskeletal actin promoter. To differentiate between these two possibilities, we also tested CArG box 1 + DS in front of a minimal thymidine kinase promoter. Similar results were obtained with this transgene (Fig. 4B), indicating that the CArG box 1 and the sequence downstream of it act as an enhancer active in both the myotomes and a broad anterior domain of the embryo including the heart. These data also demonstrate that other sequences within the promoter are necessary to restrict anterior expression in the embryo to cardiac and facial (skeletal) muscle.

Interestingly, when we used the same assay to test mutated versions of the CArG box 1 + DS sequence, we found that alteration of the most proximal sequences variant L, in which sequences most proximal to CArG box 1 were altered (see Fig. 3) was inactive in the myotomes (Table 1). This mutation therefore blocks activity of the CArG box 1 + DS sequence as an independent myotome-specific enhancer, although the variant can still support transgene expression in the myotomes in the presence of the distal E-box-containing enhancer (Fig. 3, L-LS10/13).

Promoter Region −275/−92, Including CArG Boxes 2–4 Is a Myotome-Specific Enhancer

Our deletion data described above indicate that the promoter region encompassing CArG boxes 2–4 (nucleotides −275/−92) is important for expression of the cardiac α-actin promoter in the myotomes. For example, the E-box-containing distal portion of the promoter can only direct weak myotomal expression in a posterior-to-anterior gradient (Fig. 3A), yet specific mutation of CArG box 1 within the full promoter (LS29; Fig. 2D) leaves the normal, uniform expression in the myotomes unaffected. We therefore tested the activity of promoter region −275/−92 when fused

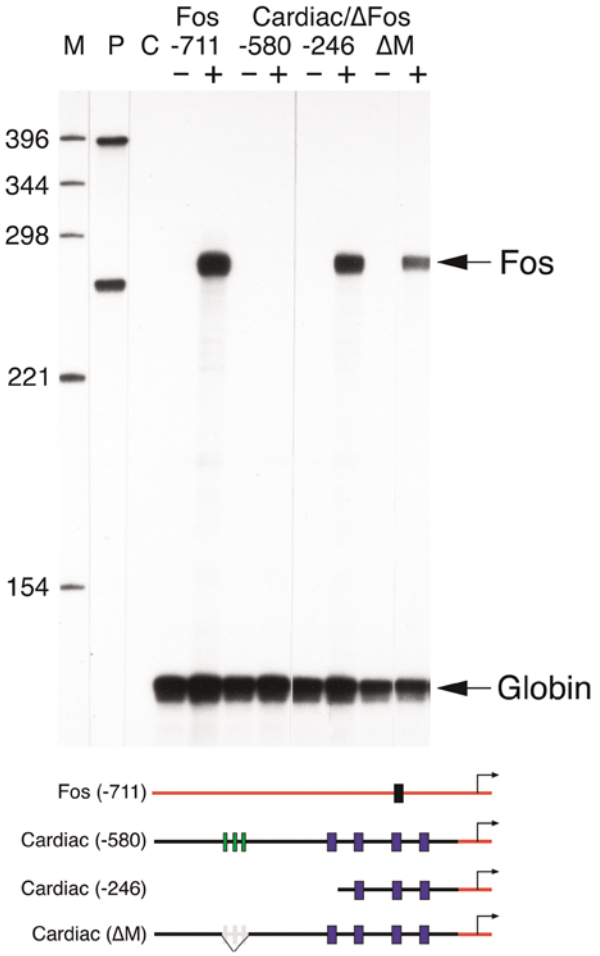

FIG. 5. E-boxes mediate silencing of cardiac α-actin promoter in serum-stimulated fibroblasts. NIH3T3 fibroblasts were transfected with test plasmids comprising the human c-*fos* gene driven by the full-length c-*fos*^{h} promoter (Fos-711) or a chimeric promoter containing cardiac actin promoter sequences fused upstream of the c-*fos*^{h} 5′Δ-100. Three cardiac actin promoter regions were tested; the full-length promoter (− 580), a 5′ truncation that removes the E-box motifs (−246), and an internal deletion of the 580-bp promoter that removes 31 bp encompassing the three E-box motifs (ΔM). Cells were serum-starved (−) and then stimulated with serum for 40 min (+). Total RNA was analysed by RNase protections. M-DNA markers; P-undigested riboprobes; C-probes hybridised to tRNA. Diagrams showing essential features of test plasmids are shown below the gel. Protected *Fos* and globin transcripts are indicated.

upstream of the minimal cytoskeletal actin promoter. The resulting transgene showed weak but uniform expression in the myotomes (Fig. 4G), indicating that the −275/−92 region, encompassing CArG boxes 2–4, can act independently, as a myotome-specific enhancer. The combination of strong and uniform expression seen in the myotomes of LS29 transgenic embryos presumably results from the combined activities of this enhancer and sequences within the distal, E-box-containing portion of the promoter.

Regulatory Interactions between Distal and Proximal Enhancers

Two distinct effects of the distal, E-box-containing region of the promoter can be distinguished in our results. In isolation, this region acts as a weak myotomal-specific enhancer (see above), but a second activity is detected using constructs that combine this region with the CArG box 1 + DS sequence. Such transgenes show correct spatiotemporal restriction of transgene expression in the heart, rather than the more widespread expression throughout the head region displayed by constructs containing the CArG box1-DS sequence alone (Fig. 3B). The distal enhancer thus appears to restrict expression driven by the proximal CArG box 1 enhancer. We therefore tested whether either of these results could be replicated by using an E-box oligonucleotide rather than the entire distal region of the cardiac α-actin promoter.

Three copies of the principal functional E-box motif (Mohun *et al.*, 1989b; Taylor *et al.*, 1991) placed in front of the cytoskeletal actin promoter gave rise to an expression pattern similar to that attributable to the entire distal promoter region (Table 1, and data not shown). In contrast, a transgene comprising the E-box motif directly adjacent to the CArG box 1 + DS sequence on either the minimal cytoskeletal or cardiac actin promoters gave strong, uniform myotomal expression (Figs. 4D and 4E). This is similar to that obtained with CArG box 1 + DS sequence alone, suggesting either that the effects of the two myotome enhancers is additive or that the activity of the CArG box 1 + DS sequence predominates. However, such a synthetic promoter did not recapitulate the entire expression programme of the cardiac actin promoter. No detectable expression was obtained with this promoter either in the heart, or in ectopic sites within the head (Figs. 4D and 4F). One explanation for this may be inappropriate spacing between the E-box and CArG box 1 motifs; alternatively, sequences present in the distal enhancer (−580/−216), but absent in the E-CArG box 1 + DS oligo, may also be required for the interactions necessary for expression in cardiac muscle. We cannot yet distinguish between these alternatives.

Interactions Mediated by E-Boxes and CArG Sites also Restrict Activity in Serum-Induced Fibroblasts

Our finding of a regulatory interaction mediated by the combination of E-box and CArG box sites in embryonic muscle prompted us to ask whether the same sequences might also be responsible for regulating transcription of the cardiac α-actin promoter in other contexts. We have previously shown that the CArG box 1 sequence of the cardiac α-actin gene can replace the related SRE motif of the *c-fos* proto-oncogene promoter and mediate transcriptional induction of *c-fos* by serum response factor (Taylor *et al.*, 1989). However, transcription of striated muscle-specific genes containing CArG boxes, such as cardiac α-actin, is not induced in fibroblasts by serum growth factors and we

therefore sought to establish what might restrict SRF activity on such promoters.

Using a serum induction assay, we found that a chimeric reporter comprising a 580-bp region of the cardiac α-actin promoter fused to the *c-fos*^{h} gene remained transcriptionally inactive in transfected fibroblasts (Fig. 5). In contrast, 5′ truncations (Δ-246) or an internal deletion (ΔM) that removed the E-boxes from the cardiac α-actin promoter portion resulted in rapid induction by serum growth factors. These results demonstrate that, in two distinct contexts, (embryonic muscle differentiation and serum induction of fibroblasts), the activity of SRF at its CArG box 1/SRE binding site is modulated by factors binding to distal E-box motifs of the cardiac α-actin promoter.

Functionally Impaired CArG Box 1 Mutants Have Reduced Affinity for SRF

We have observed that mutating sequences downstream of CArG box 1 results in loss of heart expression from the full-length cardiac α-actin promoter (LS13; Fig. 2E) and gives a similar result when tested with heterologous promoters (CArG-L; Fig. 3C). One explanation could be that such mutations impair the binding of an essential, regulatory transcription factor. However, we have been unable to identify any consistent, qualitative difference in the capacity of wild-type and CArG-L variant sequences to bind proteins in embryonic extracts, despite testing a variety of binding conditions and extract preparation procedures (data not shown). We have, however, consistently observed a lower apparent affinity of the CArG-L variant for SRF present in embryo extracts (as identified by anti-SRF antibodies), suggesting that the effect of such mutations is to reduce SRF binding to CArG box 1 (data not shown). This effect is evident both in direct binding of each sequence to recombinant SRF (Fig. 6A) and from competition assays (Fig. 6B).

Reduced Affinity CArG Variants Are Not Expressed in Cardiac Muscle

Mutation of the A/T rich core within CArG box 1 to an all-T sequence had little effect on the ability of the motif to interact with the distal enhancer of LS10/13, as judged by uniform expression in the myotomes (Fig. 3E; T-LS10/13). However, this construct gave no expression within the developing tadpole heart, indicating that the core region of the CArG box 1 is important for its function as heart enhancer. In the absence of the upstream enhancer provided by the LS 10/13 promoter construct, CArG-T oligonucleotides, including the downstream DS element, were incapable of supporting any detectable transgene expression from the minimal cytoskeletal actin promoter (data not shown; Table 1). Two additional constructs that introduce less radical changes in A/T-rich core of the CArG box 1 (Fig. 6C; mutations m5 and m6) were also tested by using the heterologous promoter assay. Mutation of two A residues in the proximal half of the core (mutation m5) showed no expression in the embryo. Mutation of the distal three A residues (mutation m6) also generally showed lack of expression, although occasional embryos showed very weak expression in the myotomes and heart region. This may reflect an intrinsic difference between the two mutations, but may also be a consequence of the increased oligonucleotide copy number (5 rather than 3) in the m6/cyt/GFP transgene. When either mutant sequence was tested for SRF binding in a competition assay, each showed an affinity for the protein which was significantly reduced from that of the wild-type sequence, although somewhat greater than the CArG-L variant (Fig. 6).

These results suggest that a reduction in affinity of the CArG box 1 sequence for SRF can specifically block expression of the cardiac promoter within the developing heart. Such reductions can be achieved by mutations within the CArG box itself, (CArG-T, m5, and m6). They can also be obtained by mutations within adjacent downstream sequences (LS13, CArG-L) which are not thought to be involved in specific binding to SRF (Pellegrini *et al.*, 1995).

If this interpretation is correct, as long as no other factors required for the CArG-SRF interaction are limiting, it should be possible to activate the low affinity, inactive CArG-L variant simply by increasing concentration of SRF. Consistent with this, coinjection of the lower affinity CArG-L cyt/GFP reporter with CMV-SRF greatly increased both the frequency of GFP-expressing embryos and their levels of expression (Fig. 6D). These results suggest that the high affinity of SRF for the CArG box 1 sequence is itself a major determinant of cardiac α-actin regulation.

The c-fos SRE Can Replace CArG Box 1 in the Cardiac α-Actin Promoter

As a further test of the role of CArG box as SRF binding site, we examined whether a heterologous, high-affinity SRF binding site could interact with the distal, E-box-containing enhancer of cardiac α-actin promoter to restore normal expression on the LS10/13 internal deletion construct. Using the SRE sequence from the c-*fos* gene, which has different core and flanking sequences from CArG box 1, we compared the activity of transgenes containing either one or two copies of the SRE oligonucleotide with that obtained from two copies of the wild-type CArG box 1 sequence (Fig. 3). Constructs containing a single copy of the SRE gave uniform expression in the myotomes with no other sites of expression (Fig. 7A). Such a pattern of expression was also seen with two copies of the CArG-L variant (Fig. 3C) and with a single copy of the wild-type CArG box 1 (data not shown). In contrast, constructs containing two copies of the SRE were also expressed in other regions of the embryo. In approximately half the cases (5/12), this additional expression was observed only in the heart (Fig. 7B), indicating that the c-*fos* SRE can indeed interact with the distal enhancer from cardiac α-actin gene to direct expression to myotomes and heart. In the remaining embryos, two

copies of the SRE yielded a broader pattern of expression, encompassing heart and branchial arches (Fig. 7C).

A simple explanation of these results is that their outcome reflects the relative affinities of CArG box 1/SRE variants for SRF and the distribution of SRF within the embryo. To investigate the latter, we used whole-amount *in situ* hybridisation to determine the distribution of SRF RNA in *Xenopus* embryos. In the late neurula (stage 19), whole-mount *in situ* hybridisation indicates that SRF mRNA is present at the highest levels in the somites. A lower level is detected in the anterior region of the embryo, including the heart field (Figs. 7F and 7G) and still lower levels can be detected uniformly in the surface ectoderm. In the hatching tailbud (stage 26), expression is strongest in the myotomes and presumptive heart tube, but is readily detectable in the head (Fig. 7H). By stage 33, strong sites of expression are evident throughout the embryo, including the myotomes, heart, body wall musculature, branchial arches, and restricted areas of the brain. Lower levels of SRF mRNA remain in the pronephros and in surface ectoderm (Figs. 7I and 7J).

The distribution of active SRF protein need not, of course, match the distribution of its transcripts and we have no quantitative assay of SRF distribution. However, we might expect that transgenes driven by multimerised, high-affinity SRF binding sites such as the c-*fos* SRE, or its even higher affinity variant, ACTL (Taylor *et al.*, 1989), would act broadly as sensors for SRF localisation. The expression of such transgenes is indeed similar to that found for SRF RNA distribution (Fig. 7E, and data not shown).

In this interpretation, when CArG box 1 variants with lower affinity (such as CArG-L and CArG-T) are cloned into the CArG-deficient promoter LS10/13, the resulting transgenes show expression in myotomes, but not heart, because the tadpole myotomes have higher levels of SRF expression than the developing cardiac muscle. Higher affinity variants (such as the wild-type CArG box 1-DS sequence or the c-*fos* SRE) give correct expression in both myotomes and heart. The c-*fos* SRE appears to have a higher affinity for SRF than the CArG box 1 sequence *in vitro* (Taylor *et al.*, 1989) and the widespread expression that can also be obtained when two copies of the c-*fos* SRE are present may then simply reflect the ability of such transgenes to respond to even lower levels of SRF in a wide range of other tissues in the embryo. Consistent with this view, three copies of the c-*fos* SRE in front of the cytoskeletal actin promoter result in a pattern of expression which is broader then expression driven by three copies of the CArG box 1-DS sequence (compare Figs. 7D and 7E with Figs. 4A and 4B).

DISCUSSION

Regulation of Expression in the Heart by SRF

Our promoter analysis shows that not only is the CArG box 1 and its adjacent 5 bp downstream necessary for expression of the *Xenopus* cardiac α-actin gene in the heart, when multimerised it is also sufficient for expression in both the myotomes and anterior part of the embryo, including the heart. This sequence presents the only candidate "heart-specific" enhancer within the 580-bp promoter region. Intriguingly, our data suggest that the role of this sequence may be relatively simple, acting as high-affinity SRF binding site rather than functioning as a composite site for SRF and other accessory proteins. We base this conclusion on the following three observations. Mutations of the 5 bp adjacent to the CArG box or within the A/T-rich core affect transgene expression in the heart and lower affinity of the CArG sequence for SRF (Fig. 6). A heterologous, high-affinity CArG box can interact with the distal enhancer from cardiac α-actin gene in manner similar to that of the native CArG box 1 sequence, resulting in correct expression in both the myotomes and heart (Fig. 7). Finally, the low-affinity, functionally impaired CArG box 1 variant CArG-L can be activated in embryos by providing exogenous SRF (Fig. 6).

This is a surprising finding because SRF has been implicated in regulation of many striated and smooth muscle genes, as well as in regulation of a subset of cellular immediate-early genes. Previous studies of such genes have defined CArG/SRE box-adjacent sequences that provide specificity of regulation through the accessory factors that bind them. The best example of this is provided by the c-*fos* SRE and ternary complex factors from ETS family (Treisman, 1994). We cannot, of course, exclude the possibility that nucleotides adjacent to CArG box 1 act as a binding site for a factor that modulates CArG box 1 function. However, it is striking that these sequences are poorly conserved amongst the cardiac α-actin genes of vertebrates and it is difficult to reconcile such variation with the presence of a common binding site. Furthermore, since a heterologous SRE can productively interact with the distal enhancer of cardiac α-actin gene, if the DS sequence adjacent to CArG box 1 is indeed a binding site for an additional factor, it cannot be an essential heart-specific cofactor of SRF.

Our data show that the DS sequence affects the affinity of SRF for CArG box 1, most likely by modulating the structure of the binding site. In the dystrophin promoter, a CArG-adjacent sequence essential for muscle-specific expression acts as a binding site for a factor that induces DNA bending (Galvagni *et al.*, 1997). Similarly, the DS sequence could affect the affinity of SRF for the cardiac actin promoter by modulating the structure of CArG box 1. If so, then such an interaction is either unnecessary with the heterologous c-*fos* SRE, or is supported by nucleotides included with this binding site, despite the apparent lack of sequence similarity.

Whether mutation of the DS sequence results in a change in the affinity of SRF for CArG box 1 or a change in CArG box 1 conformation, or both, such changes abolish heart-specific expression. This could be a direct consequence of reduced SRF binding or it may result from an indirect effect on the association of SRF with an accessory factor which

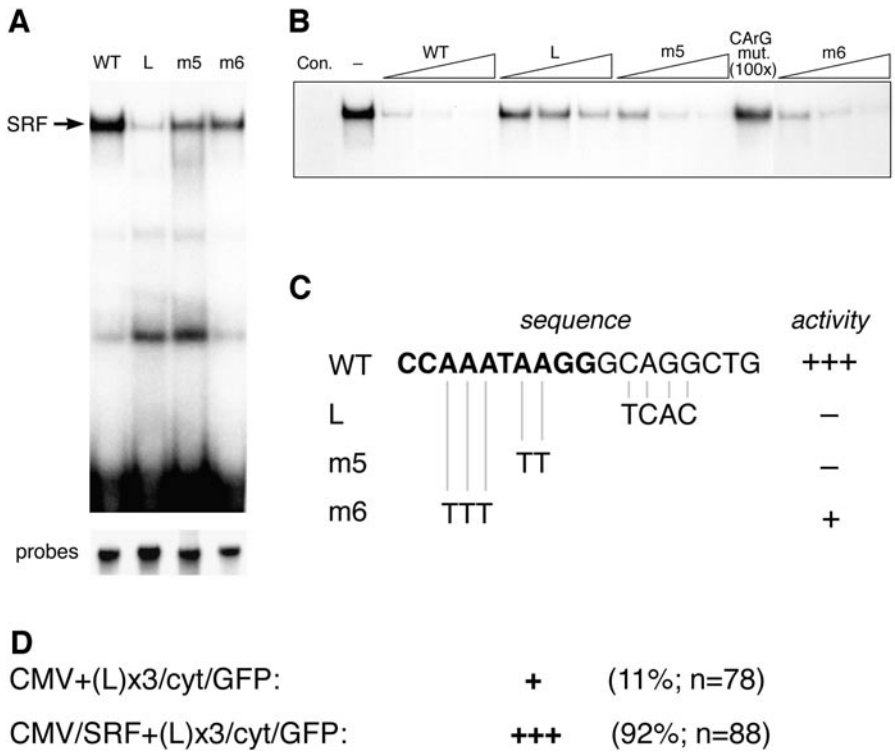

FIG. 6. CArG box 1 variants with impaired activity *in vivo* have low affinity for SRF *in vitro*. (A) Designated probes of equal specific activity and integrity (lower panel, 8% native polyacrylamide gel) were incubated with SRF obtained from coupled transcription-translation reactions. Protein–DNA complexes were resolved on 6% native acrylamide gels. (B) The relative affinities of variant CArG box 1 sequences for SRF were examined by testing the ability of a 20-, 50-, and 100-fold molar excess of unlabelled oligonucleotides to compete with the wild-type CArG box 1 probe. (C) Oligonucleotide sequences tested. The relative activity of these enhancer elements when tested by transgenesis using the minimal cytoskeletal actin promoter is indicated. (Embryos were scored at the tadpole stage.) All constructs (detailed in Fig. 4 and Table 1) had three copies of oligonucleotides, except m6, which contained 5 copies (D) Exogenous SRF can activate L-cyt/GFP in embryos. Fertilised embryos were injected with 100 pg of L/cyt/GFP and CMV or CMV-SRF DNAs at 5:1 ratio, and embryos were scored for GFP activity from stage 19 onwards.

does not itself bind DNA but is nevertheless essential for heart-specific expression. An example of such a factor is myocardin, a recently described transcriptional activator. Myocardin cooperates with SRF on muscle-specific promoters bearing multiple CArG boxes, but not on the c-*fos* promoter, which has a single CArG box (Wang *et al.*, 2001). It is noteworthy that in our experiments, correct cardiac expression was indeed only restored to transgenes when more than one CArG box motif was present in the transgene promoter (Figs. 3 and 7).

Recently, transgenic mouse studies have investigated regulatory capacity of isolated CArG boxes from smooth-muscle specific SM22 gene and c-*fos* (Chang *et al.*, 2001; Strobeck *et al.*, 2001). Chang *et al.*, (2001) found that the high-affinity c-*fos* SRE directs widespread expression within the embryo, whereas the lower affinity CArG box from the SM22 gene directs expression only in muscle cell types which are sites of high SRF expression. These results match our findings with transgenic frog embryos. In contrast, Strobeck *et al.*, (2001) found that an isolated SRE has no activity. The explanation for this discrepancy is unclear, but may in part result from the differing minimal promoters used in the transgenes.

Enhancer Interactions in the Cardiac α-Actin Promoter

Our studies have identified three distinct enhancers that are involved in regulation of cardiac α-actin in the somites and subsequently in the myotomes. The distal enhancer, containing three E-boxes, drives expression in a posterior-to-anterior gradient, resembling the expression of endogenous myogenic bHLH factors (Hopwood *et al.*, 1991, 1992). CArG box 1 + DS, discussed above, is an enhancer that gives uniform expression in the myotomes, when present in three copies. Finally, region including CArG boxes 2-4 will confer weak and uniform expression in the myotomes only. Interaction between any pair of these enhancers results in strong, uniform expression in the myotomes.

Expression in cardiac muscle of the embryo appears to

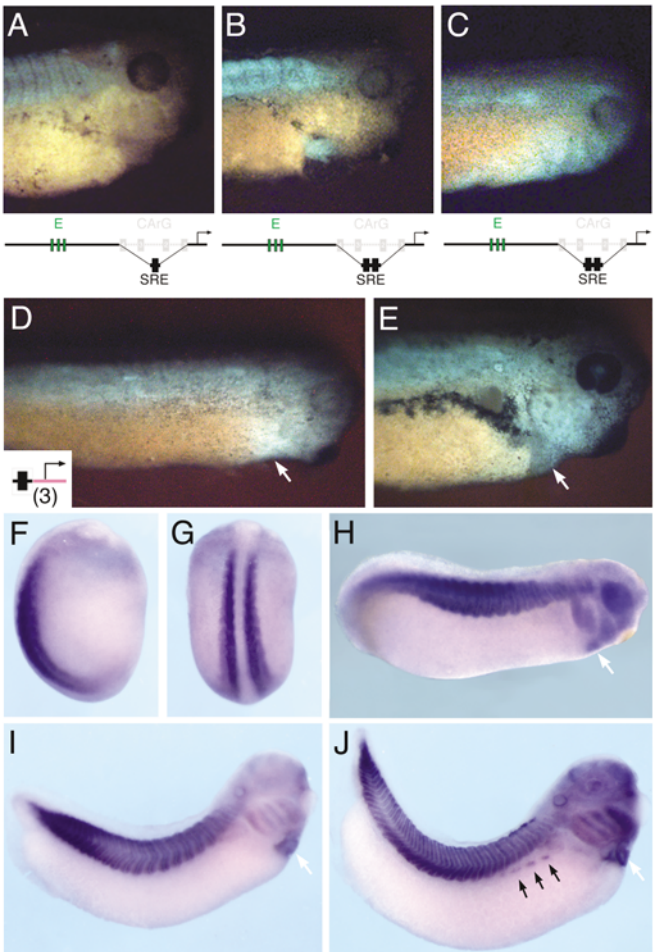

FIG. 7. c-*fos* SRE activity in embryos can be restricted to heart and myotomes by interaction with the distal enhancer from the cardiac α-actin gene. (A) A single copy of the SRE together with the −580/−275 enhancer can direct uniform expression of GFP in the myotomes, but not in the heart. Two copies of the SRE in this context also direct expression of GFP in the heart, resulting either in the correct pattern of expression for the cardiac α-actin promoter (B) or a significantly broader expression pattern (C). With three copies of c-*fos* SRE, transgenic embryos show widespread expression of GFP, encompassing the myotomes, head, heart, and pronephric region (D, E). Lateral view of stage 32 embryos, anterior to the right. (F–J) Expression pattern of endogenous SRF mRNA. (F) Lateral view of a stage 19 embryo showing strong expression in the somites, intermediate expression levels in ventroanterior region (arrow), and low uniform level of expression in the surface ectoderm. (G) Dorsal view of the same embryo. (H) Stage 26 embryo, showing expression of SRF mRNA in the myotomes, future heart (arrow) and head. (I) Stage 30 embryo showing SRF mRNA in the heart and branchial arches. (J) Stage 33 embryo, with SRF mRNA now also detectable in the body wall muscles (arrows) and distinct regions in the brain. (Anterior to the right in all panels, except F and G, where anterior is to the top).

result from further interactions between the most proximal and distal enhancers. While multimerised copies of the CArG box 1 + DS sequence will result in transgene expression in the heart, expression is not restricted to this tissue but is detected more broadly throughout the head region and commences prematurely. Addition of the E-box-containing enhancer appears to block expression of the transgene in ectopic sites within the head, yielding a pattern comparable with that of the endogenous cardiac α-actin gene (compare Figs. 4A and 4B with Fig. 3B).

Our experiments do not identify the critical sequence(s) within the distal promoter necessary for this interaction. As a myotome-specific enhancer, the E-box sequence itself is comparable with the entire distal region (Table 1). Furthermore, we have found that in an another regulatory context (the serum-stimulation of fibroblasts), interactions between factors binding at the E-box and CArG box 1 sites are indeed responsible for restricting expression from cardiac α-actin promoter. However, we were unable to replicate the interaction between distal and proximal enhancers of the promoter using oligonucleotide copies of the E-box and CArG box 1 motifs. Replacement of the entire E-box-containing enhancer with an E-box motif immediately adjacent to the CArG box 1 + DS sequence blocked all expression in the head region, whether ectopic or cardiac restricted, whilst myotomal expression was unaffected (Fig. 4). We do not understand why expression of the transgene is selectively affected in this way but it may indicate that expression in myotome and cardiac muscle is affected quite differently by the precise distance or configuration of the E-box and CArG box binding sites.

Comparison with Previous Studies of Cardiac α-Actin

In transgenic mice, the proximal portion of the cardiac α-actin promoter is sufficient for proper expression in embryos but inadequate for appropriate expression in postnatal mice (Biben *et al.*, 1996). Our results are strikingly similar. Transgenes containing sequences of the *Xenopus* promoter up to −580 are appropriately expressed in *Xenopus* embryos and tadpoles, but do not appear to be expressed in the heart muscle of adult frogs (B.L. and T.M., unpublished results). A notable difference between our studies and earlier work is our finding that E-box sequences are dispensable. For example, in cell transfection studies, the E-box was found to be critical for activity of human cardiac α-actin promoter (Sartorelli *et al.*, 1990). Similarly, our earlier studies in *Xenopus* employing a muscle induction assay also identified the distal E-box region as essential for expression in somitic muscle (Mohun *et al.*, 1989b). Our earlier studies also found that CArG box 1 was necessary for activity of cardiac α-actin reporter in induced muscle tissue (Mohun *et al.*, 1989b), while our transgenic studies indicate that it is only required for heart expression (Fig. 2). At present, we cannot adequately account for these discrepancies but note the profound differences between the *in vivo* and *in vitro* assays employed, in particular the chromosomal vs episomal status of the reporter DNA. Another

potential basis for discrepancies may be the nonquantitative nature of GFP transgenic expression.

Previous studies have suggested that multiple CArG boxes are required for activation of the chick skeletal and cardiac actin genes (Chen *et al.*, 1996; Lee *et al.*, 1991). Interaction between multiple CArG boxes within these promoters apparently leads to cooperative binding of SRF (Lee *et al.*, 1991). In the chick cardiac actin promoter, such cooperative binding might displace repressor YY1 from its' sites within CArG boxes 2 and 3 and thereby contribute to overall activity of the promoter (Chen and Schwartz, 1996). In our study, we have also found that CArG boxes 2-4 can interact with CArG box 1 to direct appropriate expression from the *Xenopus* cardiac actin promoter, but we do not yet know whether this results from cooperative binding of SRF. As in the chick promoter, potential YY1 sites are present within *Xenopus* CArG boxes 2 and 3, but whether YY1 plays a role in regulating the *Xenopus* promoter remains to be established

CArG boxes are required for regulation of all cardiac α-actin genes studied. They have been shown to interact with several adjacent sequences: SP1 and E-box in human cardiac α-actin (Biesiada *et al.*, 1999; Sartorelli *et al.*, 1990); Nkx2.5 and E-boxes in the chick gene (Chen *et al.*, 1996; Chen and Schwartz, 1996). With the *Xenopus* gene, we have no evidence indicating a role for SP1, binding either directly or through modulation of SRF binding (data not shown). Nor are any consensus Nkx2.5 binding sites present in the 580-bp portion of the *Xenopus* cardiac α-actin promoter. However, as the reported interaction of Nkx2.5 and SRF occurs in the absence of DNA binding by Nkx2.5 (Chen and Schwartz, 1996), we cannot rule out participation of Nkx2.5, or any such SRF-accessory factor. A conserved feature of cardiac α-actin gene regulation in different species appears to be the interaction of CArG box motifs with other site(s). In future, it will be of great interest to determine how the regulatory interactions we have detected occur, and to determine whether similar mechanisms regulate other striated muscle-restricted and cardiac-specific genes.

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