Muscle-specific (CArG) and serum-responsive (SRE) promoter elements are functionally interchangeable in *Xenopus* embryos and mouse fibroblasts

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Summary

The *Xenopus* cardiac actin gene contains four copies of a promoter element, the CArG box, which is conserved amongst striated muscle actin genes and is essential for tissue-specific expression in the developing *Xenopus* embryo. Our aim is to identify embryo and muscle proteins that interact with the CArG box as a step towards understanding the molecular basis of this developmentally regulated gene expression. The CArG box shares some sequence similarity with the Serum Response Element (SRE), which mediates the transcriptional activation by serum of genes such as *c-fos* and cytoskeletal actin. We show here that the most proximal cardiac actin CArG box is recognized by the same binding activity as the cytoskeletal actin SRE in nuclear extracts from both *Xenopus* embryos and mammalian muscle cells. This activity is indistinguishable from the previously characterized HeLa cell SRE-binding activity, Serum Response Factor (SRF). Importantly, we extend these *in vitro* studies to demonstrate that the CArG box and SRE are functionally interchangeable, both in *Xenopus* embryos and mouse fibroblasts. This implies that the CArG box and SRE can bind the same protein *in vivo*, as well as *in vitro*. Our results identify an SRF-like protein as a CArG box-binding factor and we discuss the implication that a common mechanism may be utilized in both muscle-specific gene expression and serum-responsive transcription.

Key words: actin, *Xenopus* embryo, transcription factors, serum-responsive genes, muscle specificity, mesoderm induction.

Introduction

In the amphibian, *Xenopus laevis*, embryonic expression of the actin multigene family commences shortly after gastrulation. The cytoskeletal actin genes are expressed in all cells of the embryo whilst those encoding striated muscle actin isoforms (cardiac and skeletal actins) are expressed only in those cells that will form embryonic muscle (Mohun et al. 1984). These cells arise in the mesoderm, which forms as a consequence of an induction between vegetal and animal cells of the *Xenopus* embryo (Sudarwati & Nieuwkoop, 1971; Gordon et al. 1985). We are interested in determining the mechanism that controls these patterns of actin gene activity and, as a first step, have sought to analyse both the promoter elements required in *cis* for this regulated gene expression and also the protein factors that bind to these DNA sequences. This will provide information on the control of muscle-specific gene expression in general and on the control of cardiac actin gene expression in the embryo in particular. As the cardiac actin gene is only expressed in a localized area of the developing mesoderm, this may in turn shed light on the problem of how regionalization of the embryonic mesoderm is achieved.

When cloned actin genes are introduced by microinjection into the fertilized *Xenopus* egg, the DNA is partitioned by cell division and becomes distributed throughout all regions of the embryo. Copies of the cloned gene are activated at the appropriate time during development and transcription from the injected muscle-specific actin genes is restricted to embryonic muscle cells (Mohun et al. 1986; Wilson et al. 1986). We have used this procedure to define a sequence within the cardiac actin gene promoter that is essential for muscle-specific expression in the early embryo (Mohun et al. 1989). Thus, transcription is abolished by a mutation that disrupts the most proximal of four copies of a ten nucleotide motif, the CArG box. Moreover, a single CArG box, in the presence of upstream sequences, is sufficient to confer tissue-specific expression. This motif is conserved amongst vertebrate
striated muscle actin genes (Minty & Kedes, 1986; Mohun et al. 1986) and resembles the central region of a previously identified transcription factor binding site, the Serum Response Element (SRE) (Treisman, 1986; Gilman et al. 1986; Mohun et al. 1987; Greenberg et al. 1987). However, a similarity of part of a binding site in promoter sequences from different genes does not necessarily indicate that these sites bind the same proteins, as recently demonstrated for the ‘CCAAT’ box (Dorn et al. 1987; Oikarinen et al. 1987; Chodosh et al. 1988). Amongst vertebrates, SREs are found upstream of the c-fos gene and within the promoters of cytoskeletal actin genes. The SRE forms the binding site for the Serum Response Factor (SRF) and mediates the transcriptional activation of these genes by serum growth factors.

In this paper, we have used embryo and muscle cell nuclear extracts and DNA probes from the cardiac actin gene promoter to analyse a CArG box-binding activity, the identification of which is an essential step towards understanding the developmental regulation of the cardiac actin gene. Our results show that the cardiac actin CArG box motif binds the same factor in vitro as the SRE of serum-responsive genes such as c-fos and cytoskeletal actin and that the protein bound has the characteristics of the SRF purified from cultured HeLa cells. To make the important link between in vitro binding data and functional activity, our approach has been to show that the CArG box and SRE are functionally interchangeable in vivo, both in the context of the developing Xenopus embryo and in cultured mouse fibroblasts. These studies raise the possibility that the SRE/CArG box motif binds a protein that can mediate not only the transcriptional regulation of serum-responsive genes in cultured fibroblasts, but can also restore the tissue-specific expression of the cardiac actin gene during muscle cell differentiation in the Xenopus embryo.

Materials and methods

Embryos

Xenopus embryos were cultured by standard methods (Gurdon, 1977; Mohun et al. 1986) and staged as described by Nieuwkoop & Faber (1956). Embryos were microinjected in each blastomere at the 2-cell stage with linearized plasmid DNA. We have previously shown that transcription driven by the cardiac actin gene promoter is restricted to muscle-containing tissue of injected embryos irrespective of how the muscle differentiation was obtained. Thus, transcripts of the injected gene are detected in both the somites of neurula embryos and in the muscle-containing tissue obtained by experimental manipulations of the embryo (Gurdon et al. 1985; Mohun et al. 1986). One such manipulation is dissection of injected embryos at the blastula stage into animal, equatorial and vegetal portions, each of which is then cultured in isolation. Equatorial explants differentiate to form elongated structures containing mesodermal derivatives such as muscle tissue, whilst the animal and vegetal explants form ectodermal derivatives and atypical endoderm, respectively (Sudarwati & Nieuwkoop, 1971; Nakamura, 1978). This procedure was used in the experiments described here to obtain embryo fragments containing differentiated muscle tissue. These were tested for the expression of the cardiac actin/β-globin fusion gene and compared with explants of the remaining portions of the injected embryo using an RNAse protection assay described elsewhere (Mohun et al. 1989). In agreement with our earlier results (Mohun et al. 1986), expression of the fusion genes was only detected in those embryo fragments that contained embryonic muscle tissue.

Cell culture

NIH 3T3 fibroblasts were cultured, transfected and analysed as described previously (Treisman, 1985). 20 μg of total RNA from each sample were assayed for transcripts from the transfected human c-fos and α-globin genes by RNAse protection.

Nuclear extracts

The nuclear extract from Xenopus neurula embryos (stage 18) was made essentially as described by Gorski et al. (1986) for rat tissues and is described elsewhere (Mohun et al. 1989). The FAF2 cell line is a fast-fusing subclone selected from the mouse myoblast line G8-1 (Sugiyama, 1977), and was kindly provided by Dr D. Bennett. Nuclear protein extracts were made from FAF2 myoblasts, FAF2 myotubes, C2 myocytes and HeLa cells essentially as described by Dignam et al. (1983).

Plasmids

A series of 5' deletions of a Xenopus cardiac actin–globin gene created between the promoter of the cardiac gene and the body of the human β-globin gene was obtained (Mohun et al. 1988). One such clone (end-point –116) was cut with SacI (polylinker and natural site at –51), digested with T4 Polym-erase to give a 61 bp fragment containing CArG box1. This was cloned into a EcoRI-cut pUC18 plasmid filled-in with Klenow fragment, which recreated the EcoRI site. Digestion with EcoRI released the CArG box1-containing fragment for use as a competitor DNA.

pXYDAD contains a synthetic copy of the Xenopus c-fos gene created between the promoter of the cardiac gene and the SRE of the human c-fos gene (T. Mohun, unpublished results) cloned into the EcoRI site of pUC18. The oligonucleotides used were 5'-AATTGTTTTCCTTATAAGGTTATCT-3' and 5'-AATTAGATAACCTTATAAGGAAAACA-3'. A number of other SRE-like sequences cloned in a similar manner into pUC12 have been described previously (Mohun et al. 1987; Treisman, 1987); these are pACT (Xenopus cytoskeletal actin SRE), pACT.R (inverted repeat of the right half of the cytoskeletal actin SRE), pACT.L (inverted repeat of the left half of the cytiskeletal actin SRE), pACT.R* (a variant of pACT.L with G-C transversions at positions –4 and +4 relative to the centre of dyad symmetry) and pDYAD (human c-fos SRE). Digestion of all these plasmids with BamHI and BsmNI released the SRE-containing fragment for use as a competitor DNA.

For transfection of NIH3T3 cells, the Xenopus cardiac actin promoter constructs were made as follows: 5' deletions (end-points –123 and –166) of the cardiac actin gene promoter were digested with SacI to yield 79 and 122 bp fragments containing CArG box1 and CArG boxes 1 and 2, respectively. These were inserted into the SacI and BamHI sites of pUC12. The same two 5' deletions of the cardiac actin promoter were digested with MspI for use as competitor DNAs in the experiments shown in Fig. 4A. Derivatives of the plasmid pF261 containing cloned SRE sequences ACT, ACT.L and ACT.R adjacent to a truncated human c-fos gene have been described previously (Treisman, 1987).

Plasmids for microinjection into fertilized Xenopus eggs
were made using a Xenopus cardiac actin/human β-globin fusion gene (LS #29, Mohun et al. 1989) containing an 8 nucleotide linker scan mutation (KpnI linker -79 to -86) that disrupts the promoter CARG box 1. A copy of the Xenopus c-fos SRE was cloned into the position of CARG box 1 using the XDYAD oligonucleotides described above. A 30 bp fragment obtained from filling in the EcoRI-cohesive ends of the annealed 26-mer was ligated into the blunt site created by T4 Polynucleotase treatment of the KpnI site in plasmid LS #29. The orientation of the SRE sequence in the recombinant promoter was determined by DNA sequencing.

Preparation of probes
Probes for use in the DNA mobility shift assays were prepared from the plasmids described above by end-labelling with [α-32P]dATP using Klenow fragment after cutting with restriction enzymes as follows: CARG box 1 with EcoRI to release a 71 bp fragment; pACT, pACT.L and pDYAD with BamHI and BstNI to release a 145 bp fragment. Probes for the DMS interference assays were as follows.

Top strand Xenopus cardiac actin
A series of 3' deletions of the cardiac actin promoter was obtained by Bal31 digestion and cloned between the EcoRI and KpnI sites of pUC18 (Mohn et al. 1989). One such clone (end-point -63) was cut with BamHI, end-labelled with Klenow fragment and [α-32P]dATP, recut with HphI (natural site in promoter) to release a fragment that contained -63 to -147 of the cardiac promoter, which includes CARG boxes 1 and 2.

Bottom strand cardiac actin
A 5' deletion to -165 of the cardiac actin promoter–globin fusion (see above) was cut with EcoRI, end-labelled as described above and recut with HindIII (natural site in promoter). The fragment contained -165 to -45 of the promoter.

Top strand Xenopus cytoskeletal actin SRE
A 5' deletion of a cytoskeletal actin promoter–CAT fusion (pA114CAT; Mohun et al. 1987) was cut with BamHI, end-labelled as described above and recut with EcoRI. The fragment comprised -114 to +21 of the cytoskeletal actin gene containing the SRE.

Bottom strand cytoskeletal actin SRE
As for the coding strand, except that the DNA was cut with EcoRI first, end-labelled and then recut with BamHI.

DNA mobility shift assay
The standard binding reactions (15 μl) contained 9 μl nuclear protein (added last) in Gorski's dialysis buffer, 4 fmol of end-labelled probe and 0.75 μg nonspecific DNA. The mix was brought to a final concentration of 45 mM-KCl, 15 mM-Hepes (pH 7.6), 5 mM-spermidine, 1 mM-MgCl2, 1 mM-DTT, 0.5 mM-PMFS, 0.1 mM-EDTA, 12% glycerol. The nonspecific DNA was 0.53 μg sheared salmon sperm DNA and 0.22 μg MspI/Sau3A-digested pUC18 DNA. Sheared salmon sperm DNA gave cleaner reactions than either digested pUC18 DNA or poly dIdC. Competitor plasmid DNAs were restriction enzyme digested to release the cloned DNA fragment, their concentration estimated by ethidium bromide staining and substituted for the appropriate amount of the pUC18 DNA in the competition binding assays. After incubation on ice for 30 min, the binding reactions were analysed on acrylamide gels (Treisman, 1986). The assay was quantified by scanning densitometry.

The assay with antibody was as described above, except the nonspecific DNA was 0.5 μg of MspI cut pUC plasmid plus 0.25 μg sheared salmon sperm DNA, 1 mM-EDTA replaced the 1 mM-MgCl2 and the incubation was for 15 min on ice. For incubations with antibody, 2 μl of 1:5 dilution of a polyclonal antibody raised in mice against purified HeLa cell SRF (Norman et al. 1988) was included after 5 min incubation. The DNA mobility shift assay with purified protein including 500 μg ml⁻¹ BSA and 0.1% NP-40 and otherwise was as described (Treisman, 1987), except that the nonspecific DNA was 0.5 μg MspI-cut pUC plasmid for assays with the cardiac actin probe.

DMS interference assay
End-labelled DNA probe was partially methylated by DMS (2 min, room temperature) as described (Maxam & Gilbert, 1980). The binding reaction was scaled up and contained about 50 fmol probe. Free and complexed DNA were separated on acrylamide gels, located by autoradiography (4 h, -80 °C), the DNA eluted and then processed further as described (Treisman, 1986) before analysis on 6% sequencing gels. A quantitative measure of interference was obtained by scanning densitometry.

Results

The CARG box 1 and the SRE bind an SRF-like protein in embryo extracts
The first step in this investigation was to use a range of in vitro procedures to analyse a CARG box-binding activity present in Xenopus embryos. Nuclear protein extracts were prepared from neurula stage Xenopus embryos by a procedure not previously used for embryonic material (see Materials and methods). At the neurula stage in early development, embryonic muscle tissue has begun to differentiate from the dorsal mesoderm along the axis of the embryo and both the cardiac and skeletal actin genes are active in these early muscle cells (Mohun et al. 1984). Extracts of nuclei from whole embryos contain a binding activity which will form a specific complex with a DNA probe containing the most proximal Xenopus cardiac actin promoter CARG box, hereafter referred to as box 1 (Fig. 1, lanes 1–4). The yield of labelled complex was reduced by excess, unlabelled DNA containing the Xenopus cytoskeletal actin SRE, pACT, (lanes 5–7). In the reciprocal experiment, the yield of specific complex formed between the SRE probe and the extract was reduced by excess, unlabelled cloned CARG box 1 (lanes 9–11). These results suggest that CARG box 1 and the SRE are recognized by the same or related binding activities in the embryo extract.

In order to visualize the interaction between the CARG box probe and the embryo binding activity, we examined the DNA–protein complex using DMS interference assays (Fig. 2A). Methylated of either G residue in the GG pair on the top strand of the CARG box 1 sequence blocks formation of the complex; a smaller effect is detected at the GG pair on the bottom strand. Methylated also has an inhibitory effect at G(-80) on the top strand, G(-79) on the bottom strand, and at the A residues in the centre of the binding site. These results suggest that the embryo protein(s) interacts with the minor as well as the major groove of the DNA (Siebenlist & Gilbert, 1980). This pattern of DMS interference is very similar to that
Fig. 1. CArG box1 and the SRE bind a similar protein in embryo extracts. End-labelled cardiac actin CArG box1 or cytoskeletal actin SRE (pACT) probe (4 fmol) was incubated with 3-8 µg or 2-2 µg Xenopus embryo nuclear protein, respectively, and 3-fold (lanes 2, 5, 9, 12), 10-fold (lanes 3, 6, 10, 13) and 30-fold (lanes 4, 7, 11, 14) molar excess of cloned CArG box1 or pACT competitor DNA. Protein–DNA complex formation was determined by the DNA mobility shift assay (Materials and methods). Lanes 1 and 8 show complex formation in the absence of specific competitor DNA. Competition by unlabelled CArG box1 DNA (lanes 2-4) and pACT DNA (lanes 12-14) shows that the complexes on the CArG box1 and SRE probes, respectively, are specific.

obtained with the embryo extract and the cytoskeletal actin SRE (Fig. 2A). This is emphasized by comparing the reverse complement of the CArG box1 sequence and the SRE (Fig. 2B). The pattern of DMS interference with the cytoskeletal actin SRE and the Xenopus extract is also strikingly similar to that previously obtained for SREs and HeLa cell SRF (Treisman, 1986; Mohun et al. 1987).

To test the sequence specificity of the embryo binding activity on each probe, we carried out a series of binding competition experiments, using as competitors a variety of SRE-like sequences of different binding affinity listed in Table 1. Complexes were formed with either the CArG box1 or cytoskeletal actin SRE (pACT) probes in the presence of increasing amounts of competitor sequences and the yield of complex evaluated by gel electrophoresis. All these sites compete with the same relative efficiency for complex formation with either CArG box1 or cytoskeletal actin SRE probes (compare Fig. 3A and 3B). These results indicate that these two sites bind a factor in the embryo extract with a very similar DNA-binding-sequence specificity. The relative affinities of the sites shown in Table 1 for the embryo binding factor are ACT.L>ACT>DYAD=XYDAD>box1. The same order for the affinity of these sites has been found with

Fig. 2. DMS interference analysis of binding to CArG box1 and the cytoskeletal actin SRE in embryo extracts. (A) Binding reactions were carried out with partially methylated end-labelled CArG box1 and cytoskeletal actin SRE probes and Xenopus embryo nuclear extract: free and bound DNA were resolved as described in Materials and methods. The DNA from the free (F) and the bound (B) was electrophoresed next to a marker lane (M) of an A+G sequencing reaction (Maxam & Gilbert, 1980) of the same probe. Sites where methylation interferes with protein binding are under-represented in the bound lane relative to the free. (B) The CArG box1 sequence motif is shown underlined with numbering in base-pairs upstream from the transcriptional start site. The reverse complement of the CArG box1 sequence is shown immediately above the sequence of the cytoskeletal actin SRE, with points of binding interference by methylation indicated by triangles (G residues: ▲ >10-fold difference between free and bound, ▲ 2- to 10-fold; A residues: ▲ >10-fold, ▲ >2- to 10-fold). Quantification was by scanning densitometry of autoradiographs.
The Xenopus CArG box1 (box1) and the Xenopus cytoskeletal actin (pACT), human c-fos (pDYAD) and Xenopus c-fos (pXDYAD) SREs are all listed in their natural orientation. Also listed is a synthetic symmetric SRE (pACT.L), which has a greatly increased affinity for HeLa cell SRF, and a mutated derivative of this that cannot bind SRF (pACT.L*). The conserved CC and GG pairs in each motif are underlined. References: (1) Mohun et al. 1986; (2) Mohun et al. 1987; (3) Treisman, 1985; (4) T. Mohun, unpublished results; (5) Treisman, 1987.

The similarity of the complexes formed in the embryo extract with the CArG box1 and SRE probes is illustrated further by the different binding affinities of the synthetic SRE variants, pACT.L and pACT.L*. The two GG pairs, which were identified as points of close contact with the binding protein by the DMS interference assays (Fig. 2) and are symmetrically placed with respect to the centre of dyad symmetry in pACT.L, are each disrupted by a single base change (GG to CG) in pACT.L*. This results in a sequence that is incapable of binding SRF (Treisman, 1987). Fig. 3 shows that pACT.L* fails to compete for complex formation with either CArG box1 or pACT probes, whereas pACT.L is the most effective competitor tested. This indicates that the Xenopus embryo binding activity, in the same way as HeLa cell SRF, requires the symmetrically located GG pairs within its recognition sequence.

Taken together, the competition DNA mobility shift and DMS interference experiments suggest that the GG pairs of the binding sites lie in close apposition to the bound protein and are essential for complex formation. These in vitro studies also suggest that the CArG box and SRE sequences bind the same factor in the Xenopus embryo extract. Moreover, this binding activity has a sequence specificity indistinguishable from that of SRF, suggesting that it may correspond to the Xenopus SRF.

Purified SRF binds to the cardiac actin CArG box1 in vitro

We used two approaches to test whether the CArG box is capable of binding to purified HeLa cell SRF protein. Fig. 4A shows the results of binding competition assays using purified SRF together with various SRE-like sequences. Both the cytoskeletal actin SRE (pACT) and its high affinity variant, pACT.L, reduce the yield of the labelled complex formed between SRF and a human c-fos SRE probe. Similarly, fragments of the cardiac actin gene promoter containing either the most proximal (box1) or the two most proximal CArG box sequences (box1–2) also compete for SRF. The cardiac

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### Table 1. SRE/CArG sequences

<table>
<thead>
<tr>
<th>Binding site</th>
<th>Plasmid</th>
<th>Sequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac actin CArG Box1</td>
<td>BOX1</td>
<td>AAGCTACCAAATAAGGGCAGGC</td>
<td>1</td>
</tr>
<tr>
<td>Cytoskeletal actin SRE</td>
<td>pACT</td>
<td>AGATGCCCATATTTGGCGATCT</td>
<td>2</td>
</tr>
<tr>
<td>c-fos* SRE</td>
<td>pDYAD</td>
<td>GGAAGCCTATATTTAGGACATCT</td>
<td>3</td>
</tr>
<tr>
<td>c-fos SRE</td>
<td>pXDYAD</td>
<td>TGGTTTCCCTTAAAGGTATCT</td>
<td>4</td>
</tr>
<tr>
<td>Synthetic 'SRE'</td>
<td>pACT.L</td>
<td>AGATGCCCATATTTGGGCTC</td>
<td>5</td>
</tr>
<tr>
<td>Synthetic 'SRE'</td>
<td>pACT.L*</td>
<td>AGATGCCCATATTTGGGCTC</td>
<td>5</td>
</tr>
</tbody>
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Fig. 3. CArG box1 and the SRE bind an SRF-like protein in embryo extracts. (A) Protein–DNA complex formation with an end-labelled CArG box1 probe was determined as described (Fig. 1 legend), but with 3-, 10- and 30-fold molar excess of the following competitor DNAs: CArG box1, pACT, pDYAD, pXDYAD, pACT.L and pACT.L*. 0-3- and 1-fold competitor was also used for pACT.L. The resulting autoradiograph was quantified by scanning densitometry and the results plotted with the mean value from incubations with no specific competitor taken as the 100% reference. The results presented are the means from 2 (pACT.L and pACT.L*) or 3 (CArG box1, pACT, pDYAD, pXDYAD) separate determinations. (B) As A but using end-labelled cytoskeletal actin SRE (pACT) probe. All the DNAs compete more efficiently against the CArG box1 probe than the SRE probe because CArG box1 has an approximately three-fold lower binding affinity as estimated by the concentration of competitor DNA required for 50% inhibition of binding.
To visualize CArG box1–SRF complexes directly, we carried out the experiment shown in Fig. 4B. Increasing amounts of the complex are formed with CArG box1 probe as the amount of purified SRF in the binding reaction is increased. Again, unlabelled CArG box1 DNA competes effectively against its labelled counterpart, as does the synthetic SRE, pACT.L.

**The CArG box1 and SREs bind an SRF-like protein in muscle cell extracts**

Detailed analysis of both the human (Miwa & Kedes, 1987) and *Xenopus* cardiac actin gene promoters (Mohun et al. 1989) has demonstrated that the most proximal CArG box sequence is indispensable for expression of the gene in differentiating muscle cells. We therefore attempted to identify CArG box-binding activities present in muscle cells by examining the complexes formed by the CArG box1 and SRE sequences with muscle cell nuclear extracts.

Since embryonic muscle tissue constitutes only about 10% of the total number of cells of the *Xenopus* neurula stage embryo, we prepared nuclear extracts from cultured myogenic cell lines for binding assays. The murine cell line, C2, has previously been used to map the human cardiac actin gene promoter by a transient transfection procedure (Minty & Kedes, 1986; Miwa & Kedes, 1987). However, we have found that differentiation of the C2 myoblast line is rapid, spontaneous at high cell densities and frequently incomplete, making it difficult to obtain pure cultures of either myoblasts or myotubes. In contrast, myoblast cultures of the murine cell line, FAF2, differentiate in a slower, but more complete, manner enabling the preparation of homogeneous myoblast and myotube cell populations.

It has been reported that a CArG box sequence and the human c-fos SRE can compete for a binding activity in C2 cells (Phan-Dinh-Tuy et al. 1988; Gustafson et al. 1988). In our experiments, we used a more detailed competition assay in FAF2 myotube nuclear extracts to determine the binding sequence specificity of a muscle cell CArG box-binding activity by assessing its relative affinity for the CArG box motif and various SRE sequences. Cardiac actin CArG box1 and the cytoskeletal actin SRE (pACT) were again used as probes and the results are shown in Fig. 5. The relative effectiveness of each competitor with either probe was the same, suggesting that the CArG box1 and the SRE bind the same factor in this extract. Similar results have been obtained with extracts from FAF2 myoblast and C2 cell cultures (data not shown). Alternatively, the amount of CArG box-binding activity in FAF2 myoblasts was also the same as that in FAF2 myotubes. The relative binding affinities of pACT, pDYAD and CArG box1 determined with the CArG box1 probe in the FAF2 myoblast extract are 1:1:3:2:8. Almost identical values were obtained with the *Xenopus* embryo extract, indicating that the binding activity in the muscle cell and embryo extracts has a similar sequence specificity. Moreover, binding is again dependent on the pairs of G residues common to both sequences since the SRE
CArG and SRE promoter elements are interchangeable

Figure 5. CArG box1 and the SRE bind an SRF-like protein in muscle cell extracts. (A) Protein-DNA complex formation was assayed between end-labelled CArG box1 probe (4 fmol) and nuclear protein (5-5 µg) from FAF2 myotubes in the presence of 3-, 10- and 30-fold molar excess of a series of competitor DNAs (CArG box1, pACT, pDYAD, pACT.L, pACT.L*). 0-3- and 1-fold competitor was also used for pACT.L. The results were processed as described in the legend for Fig. 3A and are the means from at least 3 separate determinations. The relative affinity of each binding site may be estimated by the concentration of competitor DNA required for 50% inhibition of binding. (B) As A but using end-labelled pACT probe (4 fmol) and 3-1 µg nuclear protein from FAF2 myotubes. The results presented are the means from at least 3 separate determinations.

variant pACT.L is the most effective competitor of complex formation whilst its mutated counterpart, pACT.L*, is completely inactive (Fig. 5). Thus, cultured muscle cells, in common with HeLa cells and Xenopus embryos, contain a binding activity that recognizes both the SRE and CArG box motif in vitro, and has a DNA sequence specificity very similar to that of SRF.

These results suggest that the SRE- and CArG box1-binding activity that we detect in muscle cells may be SRF. We therefore tested whether complexes formed between SRE or CArG box1 probes and muscle cell extracts would react with a polyclonal antibody raised against purified HeLa SRF. This antibody reacts with SRF from a variety of mammalian sources, including murine cell lines (Norman et al. 1988). Fig. 6A shows the complex formed by the SRE probe, pACT.L, with extracts from muscle cell (C2, FAF2) and HeLa cell nuclei. In each case, addition of antibody to the binding reaction results in a further decrease in complex mobility. Preimmune serum has no effect (data not shown), indicating that retardation of complex results from binding of the antibody. Fig. 6B shows a similar series of binding reactions in which the probe is the CArG box1 sequence. The antibody also recognizes the complex formed between the CArG box1 probe and both muscle and nonmuscle cell extracts, demonstrating the presence of SRF, or a very closely related protein, in each.

Xenopus c-fos SRE can substitute for the cardiac actin CArG box1 in vivo

The experiments described above suggest that the CArG box1 and the SRE bind the same SRF-like protein in vitro. The next step was to determine whether the CArG box and the SRE can also bind the same protein in vivo, that is whether they are functionally interchangeable. The first question asked was whether the Xenopus c-fos SRE could functionally substitute for CArG box1 in restoring muscle-specific expression in the developing Xenopus embryo.
We have shown elsewhere that a chimeric gene containing the cardiac actin promoter and transcription start site attached to the protein-coding portion of the human \(\beta\)-globin gene is correctly expressed in the muscle tissue of transgenic Xenopus embryos (Mohun et al. 1989). Transcription is completely abolished by an 8-nucleotide linker scan mutation (LS#29) that disrupts the most proximal CArG box sequence, located at position −80 within the cardiac actin gene promoter (see Fig. 7A), despite the presence of three other more distally located versions of the motif (Mohun et al. 1986). We therefore inserted a synthetic oligonucleotide comprising the Xenopus c-fos SRE (see Materials and methods) into the site of the disrupted CArG box of LS#29. LS#29.XDYAD(+) contains the c-fos SRE in its normal orientation; in LS#29.XDYAD(−) the orientation is reversed (Fig. 7B). A third variant LS#29.XDYAD(*) was also obtained in which a single nucleotide change converts a G in the SRE core of LS#29.XDYAD(+) into an A residue.

The transcriptional activity of each variant was tested by microinjection into Xenopus embryos. Both LS#29.XDYAD(+) and LS#29.XDYAD(−) are transcribed in embryo explants that contain embryonic muscle tissue (Fig. 7A). Neither variant is expressed in cultured embryo fragments that fail to differentiate into muscle tissue (data not shown), nor is the reference cardiac actin/globin gene (see legend Fig. 7). Together these results show that the c-fos SRE, in either orientation, can indeed replace the proximal CArG box required for the muscle-specific expression of the cardiac actin gene promoter in vivo. Moreover, the point mutation present in LS#29.XDYAD(*) is sufficient to inhibit nearly all transcriptional activity of the chimeric gene. The GG pairs of the SRE are therefore essential for its CArG box-like activity in embryos, just as they are required for complex formation in the embryonic extract.

The cardiac actin CArG box can function as an SRE in fibroblasts

Since the SRE can replace the CArG box in vivo and the CArG box sequence binds the purified SRF protein in vitro, we tested whether the CArG box motif can function as an SRE, that is, restore transcriptional activation to a truncated human c-fos gene in response to serum. DNA fragments carrying either CArG box1 or CArG boxes 1+2 were inserted into a plasmid, pF261X, containing a truncated human c-fos gene (c-fosX) that lacks an SRE and is not activated following serum stimulation. Each plasmid was transfected into mouse NIH3T3 cells and their expression monitored before and after serum stimulation. For comparison, cells were transfected with pF261 alone or three derivatives containing SRE sequences inserted at the same site as the cardiac actin promoter fragments. Plasmid pF261ACT contains the Xenopus cytokeratin actin SRE, and pF261L and pF261R contain SRE variants with high and low affinity, respectively, for SRF (ACT.L and ACT.R; Treisman, 1987). Cells transfected with plasmids carrying either the cytokeratin

![Figure 7](image_url)

**Fig. 7.** An SRE can replace the cardiac actin promoter CArG box1 in vivo. (A) Fertilized Xenopus eggs were injected with plasmids containing the Xenopus cardiac actin gene promoter fused to the human \(\beta\)-globin gene. Embryos were cultured, dissected and RNA extracted as described (see Materials and methods). An RNase protection assay to monitor transcription from the injected fusion genes is shown. 5 μg of tRNA were assayed as a control (tRNA). In the remaining lanes, RNA from the equivalent of a single embryo fragment was used. Each embryo also received a reference plasmid comprising the normal cardiac actin gene promoter attached to a truncated human \(\beta\)-globin gene (Mohun et al. 1989). This was co-injected in a 1:4 ratio with a variety of test fusion genes: a control plasmid containing the unmodified cardiac actin gene promoter (control); LS#29 consisted of the same fusion gene with an 8 nucleotide linker scan mutation disrupting CArG box1 (LS#29); the remaining test plasmids contained a single copy of the Xenopus c-fos SRE inserted into the linker site of LS#29 in either its normal (XDYAD+) or inverted (XDYAD−) orientation with respect to the direction of transcription. The effect of a point mutation in the SRE of plasmid LS#29.XDYAD(+) was also tested (XDYAD*). Protected fragments resulting from correctly initiated transcripts of both the test and reference genes are indicated. The level of chimeric actin/globin mRNA detected is comparable with that transcribed from the wild-type cardiac actin promoter. The two larger bands result from the presence of residual test and reference DNA in the embryo RNA samples. Approximate size markers were HinfI fragments of pBR322, labelled using Klenow fragment. (B) The sequence of the cardiac actin promoter CArG box1 (underlined) is shown with the resulting changes (in bold type) introduced by the linker scan mutation (LS#29) and the insertion of the c-fos SRE.
actin or ACT.L SREs accumulate large amounts of c-fos transcripts in response to serum stimulation. In contrast, cells transfected with the plasmid carrying the low affinity SRE produce relatively small amounts of c-fos RNA (Fig. 8, compare lanes 3-4 with lanes 5-8). Both plasmids carrying cardiac actin CArG boxes generate large amounts of c-fos RNA upon serum stimulation (Fig. 8, lanes 9-12).

CArG box1 is a weaker binding site than ACT.L or the cytoskeletal actin SRE and yet is as effective in this transcription assay. We have no simple explanation for these results, but note that a disparity has also been found between the binding affinity and transcriptional activity of the lymphoid-specific ‘octamer’ element of the immunoglobulin gene promoter (Wirth et al. 1987). In summary, the CArG box-containing fragments of the cardiac actin promoter are at least as effective as the actin SREs in restoring serum responsiveness to the c-fos gene, demonstrating that the CArG box motif can substitute for the SRE in vivo.

Discussion

The CArG box is a key element in the tissue-specific expression of the cardiac actin gene (Miwa & Kedes, 1987; Mohun et al. 1989). In the Xenopus embryo, mutation of the most proximal of four copies of the CArG box motif abolishes transcription and a single CArG box, in the position of box1 and in the presence of upstream sequences, is sufficient to confer muscle-specific expression (Mohun et al. 1989). This paper identifies a CArG box-binding factor in extracts from both Xenopus embryos and mammalian muscle cells that is very similar to HeLa cell SRF as shown by a detailed in vitro analysis of its binding sequence specificity. Moreover, the muscle cell factor shares an antigenic determinant with SRF and purified HeLa cell SRF will bind to CArG box1 in vitro. Together these studies indicate that the embryo and muscle cell SRF-like, CArG box-binding factor identified here may be a trans-regulator of muscle-specific cardiac actin gene expression.

The CArG box sequence resembles the central region of the Serum Response Element (SRE), which is a necessary and sufficient upstream promoter element for the transcriptional activation of c-fos and cytoskeletal actin genes by serum growth factors (Treisman, 1985, 1986; Gilman et al. 1986; Mohun et al. 1987; Greenberg et al. 1987). Thus, the CArG box and the SRE are critical sequences in the regulated expression of muscle-specific and serum-responsive genes, respectively. As well as showing that the CArG box and SRE can bind the same factor in vitro, our results imply that they can also bind the same factor in vivo as we have demonstrated that these sequences are functionally interchangeable in both Xenopus embryos and mouse fibroblasts. Thus, in the context of the developing Xenopus embryo, the SRE can interact with the factor that restores muscle-specific transcription and, in the context of the fibroblast, the CArG box can interact with the factor that confers serum responsiveness.

It has been proposed that the transcriptional activity of the CArG box is orientation-dependent on the basis of experiments reversing the orientation of long, CArG box-containing fragments of the human cardiac actin promoter (Miwa & Kedes, 1987). The observed orientation-dependence could be due to sequences other than the CArG box itself. To address this question we have tested the ability of an SRE or CArG box1 oligonucleotide, in either orientation, to functionally replace CArG box1 (this paper) or the promoter region containing all four CArG boxes (Mohun et al. 1989). In both cases these direct tests show the SRE/CArG motif to be orientation-independent.

Our results show that a key sequence in muscle-specific transcription can also mediate growth factor-dependent transcription in fibroblasts. In the Xenopus embryo, the muscle-specific cardiac actin gene is activated in presumptive muscle tissue following gastrulation, while the growth factor-responsive, type 5 cytoskeletal actin gene is transcribed in all cell types (Mohun et al. 1984). Expression of the c-fos gene is detectable only in later stages of development, after the completion of organogenesis (T. Mohun, unpublished results). Thus, the same SRE/CArG promoter element appears to be involved in diverse patterns of developmentally regulated gene expression. Similarly, in cultured mammalian cells, c-fos and cytoskeletal actin
gene transcription is inducible by growth factors in many cell types (Elder et al. 1984; Greenberg & Ziff, 1984; Krujver et al. 1984; Muller et al. 1984; Greenberg et al. 1985; Curran & Morgan, 1985), whilst the cardiac actin gene remains uninducible in fibroblasts (Elder et al. 1984).

Another example of the involvement of the same or a very similar regulatory sequence in different modes of gene expression is the "octamer" motif found in the promoters of histone and immunoglobulin genes. In this case, at least two proteins recognize the regulatory sequence. One factor is ubiquitous and is implicated in the transcriptional control of the histone H2B gene, which is expressed in all cell types (Staudt et al. 1986; Fletcher et al. 1987), whilst a second factor is specific to lymphoid cells and may mediate the tissue-specific expression of immunoglobulin genes (Staudt et al. 1986; Wirth et al. 1987; Scheidereit et al. 1987). These two proteins appear to be identical in their binding sequence specificities, but differ immunologically (Fletcher et al. 1987). Several other examples of regulatory sequences being recognized by more than one protein are known, although in many of these the precise sequence specificity of each binding protein differs in contrast to the octamer binding proteins and our findings with the SRE/CArG motif. These include the group of elements known as the CCAAT box (Dorn et al. 1987; Oikarinen et al. 1987; Chodosh et al. 1988), the AP-1 recognition site (Lee et al. 1987; Rauscher et al. 1988a, b; Ryder et al. 1988) and the NF-xB/H2TF1 recognition site (Baldwin & Sharp, 1988). In the case of the SRE/CArG motif, we can envisage two models to explain the interchangeability of these two promoter elements.

In the first model, a number of different proteins, each with the same or a similar sequence specificity, recognize the SRE/CArG motifs. For example, muscle-specific transcription could result from the binding of a muscle cell-specific transcription factor to the CArG box, whilst serum-inducible transcription could result from binding of an inducible factor. Additional levels of specificity might be introduced by other control mechanisms which would ensure that cardiac actin genes are inactive in nonmuscle cell types. Since the SRE/CArG boxes are imperfect palindromes and SRF binds as a dimer (Norman et al. 1988), a large number of different binding factors could be produced as heterodimers from a limited number of proteins. Our in vitro data are apparently inconsistent with this first model. In extracts from Xenopus embryos, muscle cell lines and HeLa cells (Mohn et al. 1987; R. Treisman, unpublished results), we detect a single SRE/CArG-box-binding activity with the binding specificity of SRF. Moreover, in the murine muscle cell extracts, the complex formed with a CArG box probe is recognized by antibodies raised against purified HeLa cell SRF. Nevertheless, we cannot exclude the possibility that there exists a second SRE/CArG-binding activity which is either indistinguishable from SRF or remains undetected in our binding assays. Indeed our results are consistent with the hypothesis of two or more SRE/CArG-binding factors which share a DNA-binding domain, but have a different domain for effector function (see Santoro et al. 1988).

A second model compatible with our data is that a single SRE/CArG box-binding activity, SRF, is involved in both serum-responsive and muscle-specific transcription. This is consistent with our observation of apparently identical SRE/CArG box-binding proteins in extracts from different cell types. One interpretation of this model is that a common signalling pathway involving SRF is shared by both types of gene regulation. Muscle excitation results in phosphoinositide hydrolysis (Vergara et al. 1985), which is one signalling event implicated in SRE-mediated transcriptional activation in fibroblasts (Fisch et al. 1987; Gilman, 1988). Perhaps CArG boxes might function to link muscle-specific RNA synthesis to the rate of muscle excitation in this way. It is intriguing to speculate that a similar second messenger system might also operate to activate transcription of the cardiac actin gene via SRF in the developing frog embryo.

Alternatively, SRF activity could be regulated differently according to cell type. In this case, another protein might interact with SRF to form an active transcription complex in muscle cells. In nonmuscle cells, an active complex would only arise following growth factor stimulation. In both interpretations of this second model, other mechanisms could restrict activity of particular SRE-containing genes to the appropriate cell type (see DePONT-ZILLI et al. 1988). Both are consistent with our observations that there is no substantial change in SRE/CArG box-binding activity during early Xenopus development (Mohun et al. 1989), muscle cell differentiation (this paper) or serum stimulation of HeLa cells (Treisman, 1986). The injection of genes into developing Xenopus embryos is a powerful procedure for studying the basis of tissue-specific transcription. This approach coupled with the purification of the CArG box-binding protein(s) should enable the models outlined above for differential expression of muscle-specific and serum-responsive genes to be tested. In turn this will help uncover the molecular basis for the regulated activation of the cardiac actin gene in the developing mesoderm of the Xenopus embryo.

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