Characterization of a functional serum response element in the Actin403 gene promoter from the crustacean Artemia franciscana

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The serum response factor (SRF) activates expression of several genes in response to growth factors present in serum. SRF also regulates the expression of tissue-specific genes, including those in vertebrate muscles. An SRF-binding site (CArG box) present in the Artemia franciscana Actin403 promoter was shown to be necessary for transcriptional activity in cultured cells from Drosophila melanogaster and mammals. This DNA region bound mammalian and Drosophila SRFs in vitro and mediated transcriptional activation of the Actin403 promoter in response to serum, phorbol esters and lysophosphatidic acid in transfected cultured mammalian cells. Mutations in the CArG box greatly reduced promoter activity and stimulation by extracellular compounds.

Keywords: Artemia franciscana; CArG box; serum response element; serum response factor (SRF).

The crustacean Artemia franciscana develops through a biological cycle characterized by the possible existence of cryptobiotic embryos, also named cysts [1]. These resistance forms are produced when embryos at the gastrula stage, interrupt their metabolism and development, become surrounded by a hard shell, and are released from the maternal ovisac to the external medium. These embryos remain viable for years. Placed under favorable conditions, the cysts reinitiate development in a complex process that requires an activation step followed by hydration and resumption of metabolism and embryonic development [2]. Several functions are activated during this process, including gene transcription. Studies of steady-state expression levels of several mRNAs revealed transcription activity 2–4 h after cysts had resumed development [3,4]. The mechanisms that regulate transcription activation are largely unknown, although some possibilities have been proposed, such as activation of the transcriptional machinery through an increase in intracellular pH [5] and induction and/or activation of transcription factors [6,7].

The Actin403 gene, coding for one of four actin isoforms identified in A. franciscana, is transcribed a few hours after cyst development begins, with a sixfold increase in mRNA levels between 4–16 h of cyst growth [4]. Actin403 mRNA is expressed in ectodermal tissues of the embryo including the epidermis, foregut and hindgut [7]. Genomic clones containing the complete coding region and the promoter region of the Actin403 gene have been isolated [8]. Analysis of the nucleotide sequence of the promoter indicated the presence of a putative CArG box, a region with a nucleotide sequence very similar to the consensus binding site of the vertebrate serum response factor (SRF). The possible relevance of this element in regulation of Actin403 expression was suggested by the observation that an A. franciscana SRF homolog exhibited a similar pattern of mRNA expression as Actin403 in the embryos [7].

The transcription factor, SRF, plays important roles in several processes in vertebrates, including cell proliferation [9], muscle cell differentiation [10], and mesoderm induction [11]. In cultured cells, SRF activity is dependent on cell activation by growth factors. These signals are transduced to SRF through phosphorylation of cofactors of the Ets family of transcription factors, named ternary complex factors (TCFs), by mitogen-activated protein kinases [9,12]. Binding of phosphorylated TCF to SRF on the promoter triggers gene transcription. Alternatively, SRF can be activated by extracellular signals through weakly defined TCF-independent pathways [12].

The available data make Actin403 one of the more interesting genes to study from the perspective of transcriptional regulation during A. franciscana cyst development. The analysis of this gene is continued here by characterization of promoter region functional elements. The absence of cultured A. franciscana cell lines and the inability to incorporate exogenous DNA into the organism necessitated the use of cultured mammalian and Drosophila cells in these studies. The data obtained demonstrate the functionality of the CArG box in this promoter and its conserved regulation in mammalian cells.

MATERIALS AND METHODS

DNA constructs

Actin403 promoter-luciferase reporter vectors. The largest Actin403 promoter fragment (~1362 to ~38) was generated by PCR from the original genomic clone [8] and inserted upstream of the luciferase gene in the reporter vector pXP2 [13]. Progressive deletions from the 5' end of
the fragment were achieved by the exonuclease III, nuclease S1 method [14]. The Δ6 deletion of the Actin403 promoter (−176 to −38) was used as a substrate to generate a double mutation in the CArG box [15]. The oligonucleotide 5′-ACATGACCATATAAGGTATTGCA-GCT-3′ and the universal sequencing primers were used as primers in the PCRs required to generate this mutation. The nucleotide sequences of all fragments generated by PCR were determined to confirm that no polymerase errors were introduced. The FAM dye deoxynucleotide polymerase sequencing kit and an Applied Biosystems 373A sequencer were used to determine nucleotide sequences.

Cell lines and transfections

Monkey kidney Bsc40 cells and mouse fibroblast NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn bovine serum and 2 mM glutamine. Mouse teratocarcinoma F9 cells were maintained in a 1 : 1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s medium, supplemented with 10% fetal bovine serum and 2 mM glutamine. Drosophila Schneider cells were cultured in M3 medium (Gibco-BRL) with 10% fetal bovine serum, supplemented with penicillin and streptomycin, at 25 °C. Cells were transfected by the calcium phosphate precipitation method in 60-mm dishes [16,17]; 5 μg luciferase reporter vectors and 1 μg of the β-galactosidase expression vector pCMVβ (Clontech Laboratory Inc., Palo Alto, CA, USA) were transfected per plate. In Schneider cells, 10 μg pSVβgal was used instead of pCMVβ. Cells were harvested 48–72 h after transfection, and β-galactosidase activities determined [18]. Luciferase activity was determined with a commercial kit (Promega) according to the manufacturer’s instructions. Transfection efficiencies were corrected by obtaining the luciferase/β-galactosidase ratio for each sample. Each experiment was repeated at least three times, with duplicate samples. Means ± SD are presented in the figures. Transfected cells in stimulation experiments were changed to Dulbecco’s modified Eagle’s medium supplemented with 0.5% fetal bovine serum 15–16 h after transfection and kept in this medium for 24 h. After this period of serum depletion, cells were stimulated by the addition of serum (10% fetal bovine serum for F9 and NIH3T3 cells, 20% for Bsc40 cells), phorbol 12,13-dibutyrate (PDBu; 350 nM for F9 and NIH3T3 cells, 700 nM for Bsc40 cells), or lysophosphatidic acid (LPA; 20 μM for F9 and NIH3T3 cells, 40 μM for Bsc40 cells). Cells were harvested after 6–8 h of stimulation. Control dishes were maintained in 0.5% fetal bovine serum until harvesting. Luciferase activities were not corrected for β-galactosidase activity in the stimulation experiments. Statistical analyses were performed with the PRISM 2.0 program, using the one-way ANOVA and Turkey tests. The statistical significance of activities observed after each treatment, compared with control cells maintained in low serum, are indicated in Figs 2 and 4.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts of NIH3T3 and Schneider cells were prepared [19], and 10 μg incubated with 1–3 ng 32P-labeled double-stranded oligonucleotide serum response element (SRE; 5′-ACATGACCATATAAGGTATTGCA-GCT-3′) [20]. The competitor oligonucleotides SRE, SRE* (5′-ACATGACCATATAAGGTATTGCA-TTCGAGCT-3′) or Act (5′-CCACCATCATACATGGCACC-3′) were added to incubation mixtures 10 min before the labeled oligonucleotide. As indicated for each experiment, 1 μL antibody including anti-(mouse SRF) (G20; Santa Cruz Biotechnology), anti-(mouse Myc) (C8; Santa Cruz Biotechnology) or anti-(Drosophila SRF) was added to the binding reaction mixture 10 min before the labeled oligonucleotide. The binding reaction mixtures were incubated at 4 °C for 20 min after addition of the labeled oligonucleotide and analyzed on a 6% polyacrylamide gel [20].

<table>
<thead>
<tr>
<th>Construct</th>
<th>Promoters</th>
<th>NIH 3T3</th>
<th>Bsc40</th>
<th>Schneider</th>
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<tr>
<td>Δ0</td>
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<td>100 ± 20</td>
<td>100 ± 34</td>
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<td>Δ1</td>
<td>130 ± 24</td>
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<td>Δ3</td>
<td>180 ± 72</td>
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<td>Δ4</td>
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<td>96 ± 22</td>
<td>416 ± 121</td>
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<td>Δ5</td>
<td>141 ± 42</td>
<td>105 ± 24</td>
<td>150 ± 56</td>
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<td>Δ6</td>
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<tr>
<td>Δ7</td>
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<td>1.4 ± 0.3</td>
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<tr>
<td>Δ8</td>
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<td>1.3 ± 0.3</td>
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<tr>
<td>Δ9</td>
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Table 1. Transcriptional activity of A. franciscana Actin403 promoter in cultured mammalian and D. melanogaster cells. The mean ± SD luciferase activity, normalized to the activity obtained with the empty pXP2 vector, is presented for each cell line in relative units.

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Fig. 1. Analyses of functional regions in the A. franciscana Actin403 promoter. (A) Nucleotide sequence of the A. franciscana Actin403 gene promoter with indication of the 5′ end of the deletions analyzed (Δ1–Δ8), shown as gaps in the sequence. The nucleotide sequence is numbered from the protein translation initiation codon as in Ortega et al. [8]. The putative CArG box is underlined. (B) Transcriptional activity of the Actin403 promoter deletions. The longest fragment available from the Actin403 promoter (Δ0) and the deletions generated from it (Δ1–Δ8) were cloned in the pXP2 luciferase expression vector. The 5′ end of each deletion was numbered as shown in (A); the 3′ end (nucleotide −38) was common to all constructs. NIH3T3, Bsc40 and Schneider cells were cotransfected with each of the reporter vectors, and a plasmid expressing β-galactosidase was used to check for transfection efficiency. Mean ± SD luciferase/β-galactosidase activities obtained, relative to those of the longest promoter fragment (Δ0 = 100), are indicated.
RESULTS

Characterization of a CArG box in the A. franciscana Actin403 promoter

Functionality of the A. franciscana Actin403 gene promoter was analyzed by transient expression of a plasmid in which a luciferase reporter gene was placed under the control of a 1323-nt fragment that included the first exon of the gene and the immediate upstream region. Several mammalian cell lines and Drosophila Schneider cells were tested for reporter gene expression (Table 1). A significant induction of luciferase activity, as compared with the vector without promoter region, was observed in all cell lines tested, indicating functionality of the A. franciscana promoter in Drosophila and mammalian cells. Successive deletions of the Actin403 promoter were generated from its 5′ end and tested for transcriptional activity to identify functional regions. Deletion of a region between nucleotides −2176 and −2122 almost completely abolished promoter activity in the three cell lines tested (Fig. 1B). This deletion eliminates the first two nucleotides of a putative CArG box, suggesting that this is an important functional region of the promoter. To test this proposal, the CArG box was mutagenized

Fig. 2. Characterization of a putative SRE in the A. franciscana Actin403 promoter. (A) The putative Actin403 CArG box was mutated in the Δ6 deletion vector (5′-CctaaTtaagg-3′ to 5′-AcaataCtaagg-3′). The luciferase reporter vector (−), the longest Actin403 promoter construct (Δ0), the wild-type Δ6 deletion (Δ6), the mutated Δ6 deletion (Δ6mut) and the deletion Δ7 which interrupts the CArG box were cotransfected with the β-galactosidase expression vector in NIH3T3 (left), Bsc40 (middle) or Schneider (right) cells. The relative luciferase activities obtained, corrected by β-galactosidase expression, are shown. The value 100 was arbitrarily assigned to the Δ0 deletion. Bars represent standard deviations. (B) NIH (left) and Bsc40 (right) cells were transfected with the luciferase reporter vector (−), the longest Actin403 promoter (Δ0), the Δ6 or Δ7 deletion reporter constructs. Transfected cells were stimulated with fetal bovine serum (columns + serum) or left in 0.5% fetal bovine serum (columns − serum). The relative luciferase activities obtained are represented. The value 100 was assigned to the activity of the longest promoter construct (Δ0) in cells maintained in 0.5% fetal bovine serum. Bars represent standard deviations. ***P < 0.001 compared without serum.

Fig. 3. SRF binding to the CArG box of the A. franciscana Actin403 promoter. Nuclear extracts obtained from either NIH3T3 (left) or Schneider (right) cells were incubated with the 32P-labeled oligonucleotide SRE. A 100 times excess of the unlabeled oligonucleotides SRE, SRE* (contains changes in the CArG box identical with those of the Δ6mut promoter deletion), the unrelated oligonucleotide Act or none (−) were used as competitors. Anti-(mouse SRF) (αSRF), anti-(Drosophila SRF) (αDmSRF) or anti-(mouse Myc) (αMyc) was added to the incubation mixtures in the indicated lanes. Arrows indicate the migration of the SRF-specific complexes. The asterisk indicates the position of the more slowly migrating complexes specifically obtained after incubation with α-SRF antibodies.
in vitro. The first C of the CArG box was changed to A and the sixth T was changed to C to impair SRF binding. The promoter region with the modified CArG box was almost completely inactive in NIH and Bsc40 cells (Fig. 2A) showing that the CArG box is the main functional element of the A. franciscana Actin403 promoter in cultured mammalian cells. In Drosophila Schneider cells, mutation of the CArG box reduced promoter activity significantly although the Δmut promoter region still retained higher promoter activity than Δ7 or the empty vector.

The importance of the A. franciscana CArG box was further analyzed by studying the transcriptional response of the promoter to stimulation with serum. Bsc40 and NIH3T3 cells were transfected with the reporter vector containing the longest promoter fragment (Δ6), the more proximal 176 nt including the CArG box (Δ6), or the more proximal 122 nt (Δ7), of the Actin403 promoter. NIH3T3 cells showed strong transcriptional activation by serum (Fig. 2B). Bsc40 showed transcriptional activity in low serum which was increased by serum (Fig. 2B). The stimulations observed were similar for the longest promoter region and fragment Δ6, but fragment Δ7, with a partially deleted CArG box, showed very low transcriptional activity and no serum stimulation.

Binding of SRF to the Actin403 CArG box

The above data suggested that the Actin403 promoter contained a functional SRE, centered on the CArG box. To further confirm this result, we determined if SRF bound to this region of the promoter in vitro. EMSAs were performed using oligonucleotides containing the Actin403 CArG box and nuclear extracts from NIH3T3 or Schneider cells (Fig. 3). A more slowly migrating band was observed in the presence of nuclear extracts which could be specifically competed for with an excess of the same unlabeled oligonucleotide but not with a similar oligonucleotide containing the mutated CArG box sequence (SRE*) or an unrelated oligonucleotide (Act). Incubation with antibodies specific for mouse SRF (NIH) or Drosophila SRF (Schneider) further reduced the migration of the band. From these experiments, we concluded that mammalian and Drosophila SRF specifically recognized the CArG box contained in the Actin403 promoter.

Conserved regulation of the SRE from the Actin403 promoter

The functional conservation of the A. franciscana SRE was further analyzed by studying its capacity to mediate SRF-dependent transcriptional responses of the reporter gene under the activation of TCF-dependent and independent pathways. F9, Bsc40 and NIH3T3 cells were transfected with the reporter vector containing the most proximal 176 nt of the Actin403 promoter, including the CArG box (Δ6), which showed somewhat higher serum activation than the longest fragment analyzed (Δ0). Transfected cells were stimulated with serum (fetal bovine serum), phorbol esters and LPA. As shown in Fig. 4A, the three treatments differentially stimulated the transcriptional activity driven by the Actin403 promoter in NIH3T3 and Bsc40 cells. In F9 cells, transcriptional activation was obtained in response to serum or LPA, but not in response to PDBu (Fig. 4A), in agreement with the described inactivity of the TCF activation pathway in this cell line [21]. Bsc40 cells showed significant transcriptional activity in low serum which was increased by treatment with PDBu and LPA (Fig. 4A). Finally, NIH3T3 cells showed strong transcriptional activation by serum and lower levels of activation by LPA and PDBu, respectively (Fig. 4A). The dependence of the serum response on the CArG box was tested using the
Drosophila Schneider cells shows the important contribution of the CArG box in these cells, but the regulation is more complex than in mammalian cells. Deletion of the region −176 to −122 decreased promoter activity 6.5-fold, but mutation of the CArG box had a smaller effect, decreasing the activity about threefold. These results could be explained if the nucleotide changes introduced into the CArG box decreased Drosophila SRF binding less than mammalian SRF binding. However, EMSA experiments showed that the mutated oligonucleotide did not compete significantly for Drosophila SRF binding, suggesting that it has much lower affinity for the mutated CArG box. Alternatively, there may be other important regulatory sites in the Actin403 promoter in the −176 to −122 region. Another difference in Schneider cells is the large increase in activity observed between deletions −222 and −176, which could be explained by the presence in this region of binding sites for one or more transcription inhibitory factors. The nature of these possible repressors has not been investigated further. Similar results have been described previously for other promoters, such as the actin 5C [22,23] or the δ-aminolevulinate synthase [24] Drosophila promoters in which deletion of some regions increased promoter activity. There are no similarities in the nucleotide sequences of the Actin403 −222 to −176 promoter region and those of the other promoters mentioned above.

The relevance of the CArG box for activity of the Actin403 promoter in mammalian and Drosophila cells suggests that the region is important in the regulation of this gene in Artemia. cDNA clones coding for an A. franciscana SRF homolog, more than 90% identical with Drosophila and vertebrate SRFs in their DNA-binding regions (MADS boxes), have been isolated [7]. Moreover, both Actin403 and SRF mRNAs are coexpressed in the ectodermal tissues of the nauplii, in accordance with the proposed relevance of SRF in the regulation of the tissue-specific expression of Actin403 [7]. CArG-binding activity has been detected in A. franciscana nauplii that is very reduced in encysted embryos, which is in agreement with the observed induction of Actin403 mRNA expression during development [4,7]. Unfortunately, no techniques have yet been developed for transforming A. franciscana embryos, which prevents further testing of this hypothesis.

The A. franciscana Actin403 promoter is regulated as an SRE through the CArG box. The A. franciscana promoter is activated by serum, phorbol esters and LPA, which indicates its regulation by TCF-dependent and TCF-independent pathways. The stimulations observed in Bsc40 cells are much lower than those observed in NIH cells, because of the high promoter activity observed in Bsc40 cells cultured in 0.5% fetal bovine serum. This activity could be due to a higher sensitivity of Bsc40 cells to growth factors present in the serum or, perhaps, to the secretion of growth factors by these cells through an autocrine mechanism. Promoter activity and stimulation by growth factors are markedly reduced, although not completely abolished, by mutation of the CArG box in the Actin403 promoter, as shown in Fig. 4B. The low activity of the mutated promoter could be explained by stimulation through SRF-independent pathways. Alternatively, the results could be explained by residual low-affinity binding of SRF to the mutated CArG box.

Both TCF-dependent and TCF-independent pathways require the association of SRF on the promoter with other transcription factors, either the TCF family of factors (EIk1, Sap1a, Sap2) [25] or other factors such as Mbox and Nkx-2.5 [26,27]. We do not know if any of these factors bind to the Actin403 promoter region. Alternatively, some cofactors could bind SRF through protein–protein interactions, without DNA binding [28,29]. Although these interactions still have to be defined, the results suggest conservation of SRF regulatory pathways between vertebrates and invertebrates. Some of the data obtained with Drosophila Schneider cells suggest this hypothesis. Actin403 promoter activity in Schneider cells is partially dependent on the CArG box, and binding of Drosophila SRF from Schneider extracts is observed in EMSAs. Binding specificity is demonstrated by the lack of competition of the CArG mutated oligonucleotide and by the supershift produced after incubation with monoclonal antibodies to Drosophila SRF. The possible stimulation of Actin403 promoter by serum could not be studied in Schneider cells because of their high mortality in low serum.

SRF is necessary for proper development of Drosophila terminal tracheal cells and wing intervein regions [30,31]. The expression and functionality of SRF in Schneider cells suggests that this transcription factor has a role in proliferating Drosophila cells, although no defect in cell proliferation has been observed in SRF mutants. These results resemble those obtained in Srf knock-out mouse strains which display a severe defect in gastrulation [11], but embryonic stem cells obtained from these mice showed normal rates of proliferation [32]. However, SRF has been shown to have an important role in mammalian cell cycle progression [9,25]. Similarly, the data obtained in Drosophila Schneider cells suggest that SRF could also...
play a significant role in regulation of cell proliferation in invertebrates, although more experiments are needed to test this hypothesis.

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