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Regulation of promoter occupancy during activation of cryptobiotic embryos from the crustacean *Artemia franciscana*

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Summary

***Artemia franciscana* embryos can suspend their development and metabolism at the gastrula stage to enter a state of cryptobiosis, forming cysts. Embryonic development and metabolism can be resumed under favorable environmental conditions to give rise to free-swimming larvae or nauplii. The mechanisms that mediate these processes are not completely known. Here, we report our studies of the mechanisms that regulate transcriptional activation upon exiting cryptobiosis. Regulatory regions of several *A. franciscana* gene promoters were identified. Functional analyses in mammalian cells allowed the identification of transcriptional activator regions in the *Actin302* promoter and in promoter 2 of the sarco/endoplasmic reticulum Ca^{2+}-ATPase-encoding gene. These regions were shown to specifically bind protein factors from nuclear extracts of *A. franciscana* nauplii by means of electrophoretic mobility shift assays. Several protein-binding regions were also detected by DNase I protection analysis in the promoters of the genes encoding the α1 subunit of Na^+/K^+-ATPase, actin 302 and sarco/endoplasmic reticulum Ca^{2+}-ATPase. Specific DNA-binding proteins in nauplius nuclear extracts were detected for all the promoter regions analyzed. These proteins were either not present in cyst nuclear extracts or were present in much smaller concentrations. Three of the five regions analyzed also bound proteins present in cyst nuclear extracts. These data indicate that transcriptional activation upon exiting cryptobiosis in *A. franciscana* involves the expression/activation of DNA-binding transcription factors that are not present in cyst nuclei**

Key words: actin, *Artemia franciscana*, Ca^{2+}-ATPase, cryptobiosis, development, gene expression, Na^+/K^+-ATPase, promoter, transcription.

Introduction

Cryptobiosis is a reversible biological process that allows some organisms to arrest their metabolic activity under adverse environmental conditions, favoring their survival (Keilin, 1959). Activity is resumed when favorable environmental conditions return. The process is complex and requires coordinated regulation of the arrest and subsequent activation of many cellular activities such as energetic metabolism, protein synthesis, DNA replication and transcription (Clegg, 2001). Despite the fact that cryptobiosis is a vital process for many important biological systems, such as plant seeds, the molecular bases of cryptobiosis are largely unknown.

The branchiopodan crustacean *Artemia franciscana* is a model system that has frequently been used to study this phenomenon (Browne et al., 1991). Embryos of these animals can enter cryptobiosis at the gastrula stage and remain viable for years. The process involves complete dehydration of the embryos (anhydrobiosis), which are surrounded by a hard shell and are known as cysts (Drinkwater and Clegg, 1991). Reversion of cryptobiosis requires hydration of the embryos but also additional activation steps. For example, hydrated embryos can continue under cryptobiosis for several years under anoxia (anoxybiosis; Clegg, 1997). Subsequently, development continues through the formation of a swimming nauplius, which hatches from the cyst shell in approximately 24 h.

Artemia cysts present undetectable metabolic activity under anhydrobiosis. Even in anoxybiosis, only a metabolic rate 50 000 times slower than the aerobic rate could be detected (Clegg, 1997). Several studies have demonstrated that intermediary metabolism and protein synthesis are reinitiated within minutes of cyst activation (Tate and Marshall, 1991). In these early stages of reactivation, protein synthesis is dependent on the existence of a large pool of stored mRNA (Amaldi et al., 1977). Reinitiation of protein synthesis is also possible because of the existence of large pools of stored ribosomes and accessory molecules, such as initiation and elongation factors (Sierra et al., 1974). *In vitro* experiments have shown that cyst extracts are not competent for protein synthesis, despite the presence of most of the necessary components, as mentioned above, and several activation

mechanisms have been proposed (Wahba and Woodley, 1984; Moreno et al., 1991). One of the signals involved seems to be a rapid increase in intracellular pH, which could be determinant in the processes that mediate cyst activation (Busa and Crowe, 1983).

Transcription activation during cyst re-activation has been studied in less detail than has protein synthesis. Studies of steady-state mRNA levels have shown that some RNAs accumulate as soon as 2 h after cyst activation but most of the mRNAs studied only start to accumulate after 4 h of development (Díaz-Guerra et al., 1989; Escalante et al., 1994). These results indicate a relatively late onset of gene transcription after cyst activation compared with the rapid resumption of intermediate metabolism and protein synthesis. The mechanisms involved in transcriptional activation are largely unknown. One possibility is that transcription is repressed in the cyst and activation is the consequence of releasing repression. Alternatively, factors required for transcription could be limiting in the cyst, and their expression/activation may be induced after exit from cyst cryptobiosis. Studies of two transcription factors from *Artemia* corroborate the latter hypothesis. The expression of the TATA-binding protein (TBP) general transcription factor doubled between the cyst and developing embryo (nauplius) stages (Sastre, 1999). DNA-binding activity of the transcription factor SRF (serum response factor) increased very significantly between cyst and nauplius stages (Casero and Sastre, 2000). Moreover, van Breukelen et al. (2000) have reported activation of transcription through the increase in intracellular pH that occurs after cyst activation.

In this article, we confirm and extend previous results by analyzing the mechanisms involved in the activation of three *A. franciscana* genes whose expression is induced during development: those coding for the Na^+/K^+-ATPase α1 subunit (García-Sáez et al., 1997), the actin 302 isoform (Ortega et al., 1996) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA; Escalante and Sastre, 1994, 1995). The intron/exon structure, promoter regions and transcription initiation sites of these genes have previously been characterized. Transcription regulatory regions of these promoters were identified by means of analysis of *in vitro* protein–DNA interactions and by functional analysis in cultured mammalian cells. The presence of protein factors that bind to the determined promoter regulatory regions in cyst and nauplius extracts was analyzed by electrophoretic mobility shift assays. The results obtained indicate that protein factors binding to these regulatory regions are expressed after cyst activation. The existence of repressor transcription factors specifically expressed in cyst nuclei also cannot be excluded for some of the promoter regions.

Materials and methods

DNA constructs

A 389-nucleotide (nt) fragment from the *Actin302* gene promoter was amplified by PCR using the oligonucleotides 5′-GGTTGTGTGGTCCTCATGCAG-3′ and 5′-CAAAGAAGTAACACAAGAAGC-3′ as primers. The PCR product was cloned in the luciferase reporter vector pXP2 (Nordeen, 1988). Deletions from the 5′ end of the *Actin302* promoter were performed with the *Exo*III/Mung Bean kit (Stratagene, La Jolla, USA) in the pBlueScript vector. The nucleotide sequence of the deleted fragments was determined, and some fragments were selected and cloned into the pXP2 vector. The 1.4 kb *Hin*dIII/*Bam*HI fragment of promoter 2 of the SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase)-encoding gene was cloned into the pXP2 vector using these same restriction sites. Fragments of promoter 2 of the SERCA-encoding gene, deleted from its 5′ end to the *Bst*EII, *Bal*I and *Cla*I restriction sites, were generated by double digestion with each of these enzymes and *Sal*I, which cuts the pXP2 vector upstream of the promoter. The fragments generated were blunt-ended at both ends by treatment with the Klenow fragment of *Escherichia coli* DNA polymerase I and re-ligated. Fragments of the distal region of SERCA promoter 2 were generated by PCR. A common oligonucleotide was used as 5′ primer for fragments F1–F5 (5′-GCCCTAGACGGTTGGCCG-3′), with the following oligonucleotides as 3′ end primers: F1, 5′-GAAAG-GTCGAGATGGCTAAGGC-3′; F2, 5′-CGAAAACTGTGG-TTCAACCCTG-3′; F3, 5′-ACTGTAGGATGACGAAAAC-TG-3′; F4, 5′-GACCGTATTTTAAACTGTAGGC-3′; F5, 5′-GGTTACCCGGCTGACTGACCG-3′. Fragments F6–F8 were generated with a common 3′ end primer (5′-GGTTACCCG-GCTGACTGACCG-3′) and the following 5′ end primers: F6, 5′-GCCTTAGCCATCTCGACCTTTC-3′; F7, 5′-CAGTTT-TCGTCAGCCTACAG-3′; F8, 5′-GCCTACAGTTTAAAAT-ACGGTC-3′. The fragments generated by PCR were cloned into the pT109 vector (Nordeen, 1988). All fragments were sequenced using the Taq dye deoxyterminator cycle sequencing kit (Applied Biosystems, Foster City, USA) and a 377 sequencer (Applied Biosystems) to ascertain the absence of mutations.

Cell culture and transfection

Monkey kidney Bsc40 cells were cultured in Dulbecco's modified Eagle's medium (Dulbecco and Freeman, 1959) supplemented with 10% newborn bovine serum and 2 mmol l^{-1} glutamine. Cells were transfected with 5 μg of the luciferase reporter vectors and 1 μg of the β-galactosidase expression vector pCMVβ (Clontech Laboratory Inc., Palo Alto, CA, USA) by the calcium phosphate precipitation method (Chen and Okayama, 1987). Cells were harvested 48 h after transfection, lyzed and the luciferase and β-galactosidase activities determined (Murguía et al., 1995). Luciferase activity was determined with a commercial kit (Promega, Madison, USA) according to the manufacturer's instructions. The luciferase/β-galactosidase ratio was determined for each sample to correct for transfection efficiency. Each experiment was repeated at least three times with duplicate samples and means ± S.D. are represented in the figures.

Electrophoretic mobility shift assays

Nuclear extracts from *Artemia franciscana* cysts and

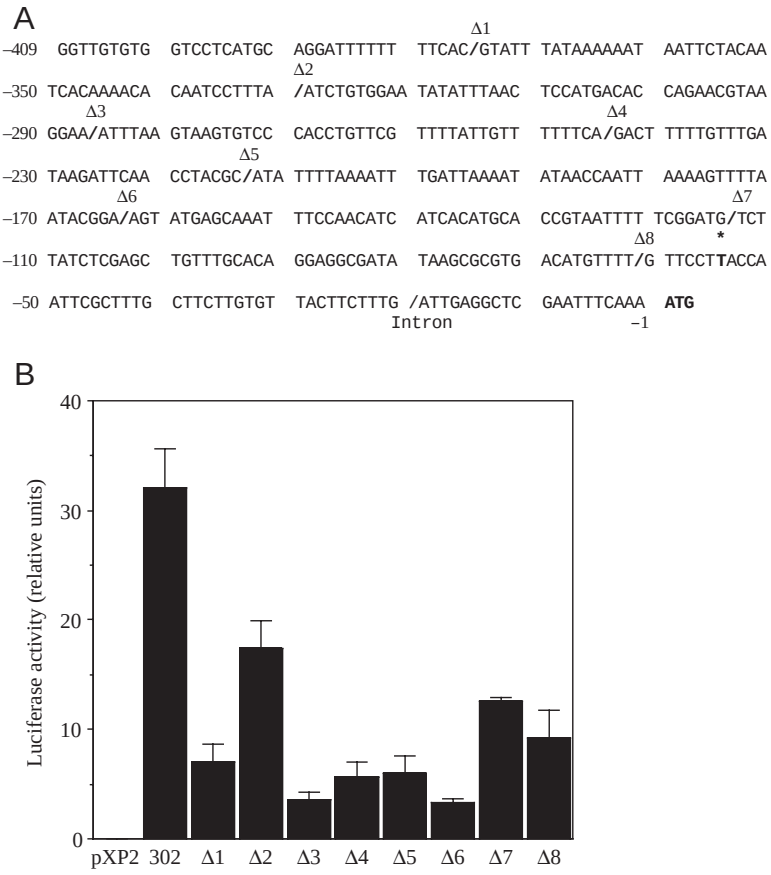

Fig. 1. Functional analysis of the *Actin302* gene promoter in mammalian cells. (A) Several fragments (Δ1 to Δ8) of the *Actin302* promoter region were generated and cloned into the pXP2 luciferase reporter vector. The fragments differed in their 5′ ends, which are indicated by a solidus on the nucleotide sequence of the promoter with the corresponding name shown above. The 3′ end of all the fragments was coincident with the end of exon 1, also indicated by a solidus. Nucleotides are numbered from the ATG initiation codon in exon 2 (the intron is excluded for the numeration). (B) The different fragments indicated in A were cloned into the pXP2 reporter vector and subsequently transfected into Bsc40 cells. Cells were collected 48 h after transfection and luciferase activity was determined. Abbreviations: pXP2, the activity of the empty reporter vector; 302, the activity of the complete promoter shown in A; Δ1 to Δ8, the activity of fragments Δ1 to Δ8.

nauplii, obtained after 20 h of cyst culture, were prepared as previously described (Sastre, 1999). 10–20 μg of each extract (as indicated in each experiment) were incubated with 1–3 ng of ^{32}P-labeled double-stranded oligonucleotide at 4°C for 20 min in 20 mmol l^{-1} Hepes, pH 7.0, 70 mmol l^{-1} NaCl, 2 mmol l^{-1} dithiothreitol (DTT), 0.005% NP40, 50 μg ml^{-1} bovine serum albumin (BSA), 2% Ficoll and 200 μg ml^{-1} Poly(dI-dC) and analyzed in 6% polyacrylamide gels. Fifty times excess of unlabeled oligonucleotides were added to the indicated incubation mixtures 10 min before the labeled oligonucleotide.

DNase I protection assays

DNase I protection assays were performed according to Ausubel et al. (1994). Double-stranded DNA probes were generated by PCR reactions, where one of the oligonucleotide primers was labeled using [γ-^{32}P]ATP and polynucleotide kinase. Aliquots consisting of 100 000 c.p.m. of the probe were incubated with 7.5 μg or 15 μg of nuclear extracts from *A. franciscana* cysts, nauplii or both, as indicated in each sample. After 60 min at room temperature, the samples were incubated with 0.02 mg ml^{-1} of DNase I for 1 min, phenol/chloroform extracted, ethanol precipitated and analyzed in 6% polyacrylamide–7 mol l^{-1}urea gels. Sequencing reactions of the same DNA fragment, using the labeled oligonucleotide as primer, were carried out with the AmpliCycle sequencing kit (Perkin Elmer, Norwalk, USA) and run in parallel to the DNase I protection assays to identify the nucleotide sequence of protected regions.

Results

Functional analyses of A. franciscana promoters in mammalian cells

Several *A. franciscana* genes whose expression is induced during the initial hours after cyst activation, including those coding for the α1 subunit of Na^+/K^+-ATPase, actin 211, actin 302 and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), have previously been isolated and characterized (Escalante et al., 1994; Escalante and Sastre, 1994, 1995; Ortega et al., 1996; García-Sáez et al., 1997). In order to determine the main regulatory regions present in the promoters of these genes, pXP2 reporter vectors containing the luciferase gene under control of the corresponding *A. franciscana* promoters were transfected into cultured cells. Progressive deletions from the 5′ end of these promoters were also generated, cloned into the pXP2 reporter vector and transfected into mammalian cells. Promoters of the genes encoding the Na^+/K^+-ATPase α1 subunit and actin 211 and the muscle-specific SERCA promoter region 1 showed low activity in mammalian cells, and no significant differences were found among different fragments, which impaired the identification of possible regulatory regions (data not shown).

By contrast, analysis of the *Actin302* promoter showed that a fragment containing the more proximal 354 nt, upstream of the transcription start site, had transcriptional activity that was similar to that of the longest genomic fragment available (1063 nt). The 354-nt fragment was further deleted from its 5′ end to generate deletions Δ1 to Δ8, whose 5′ ends are indicated in Fig. 1A. Deleted fragments were cloned into the pXP2 reporter vector and the constructs were transfected into Bsc40 cells. Luciferase activity obtained for each construct is shown in Fig. 1B. A significant difference in activity was observed between the 354-nt fragment and the longest deletion fragment (Δ1), indicative of the existence of an activator region between

nucleotides –375 and –409 of the *Actin302* promoter. The rest of the deletions analyzed gave more similar promoter activities, which were significantly higher than that of the empty reporter vector. These results are indicative of the presence of a proximal basal promoter in the *Actin302* gene.

In order to identify the possible relevance of this finding in *Artemia*, rather than in transfected mammalian cells, we studied the existence of *Artemia* proteins from cryptobiotic cysts and developing nauplii, which could specifically bind to the putative regulatory regions *in vitro*, by means of electrophoretic mobility shift assays (EMSAs). The results of these assays for the –409/–388 *Actin302* promoter region are shown in Fig. 2. Similar prominent complexes could be observed after incubation with cyst and nauplius nuclear extracts (lanes 2, 5). These complexes are specific for this DNA region, since their formation was inhibited by a 50-fold excess of the same (S) oligonucleotide (lanes 3, 6) but not with a non-related (N) oligonucleotide (lanes 4, 7). In addition to these complexes, three other slower-migrating, specific complexes were observed when the probe was incubated with nauplius nuclear extracts but not with cyst extracts (labeled with arrows in Fig. 2). The formation of these nauplius-specific complexes was significantly reduced when cyst nuclear extracts were added together with the nauplius extracts (lanes 8, 9). These results indicate that the activator region identified in mammalian cells is also a protein-binding region in *Artemia* and that some of the corresponding binding proteins are present in nauplius but not in cyst nuclear extracts.

Promoter region 2 of the SERCA-encoding gene, which directs expression in non-muscle tissues, was similarly analyzed. The analysis of a 1376-nt fragment and of several deletions originated from its 5′ end is shown in Fig. 3A. The deletion of 166 nt from the 5′ end of this fragment reduced promoter activity by 85%. These data suggest the presence of an activator region between nucleotides –1311 and –1477 of this promoter. A second activator region was detected between nucleotides –837 and –1120. Deletion of both regions almost completely abolished promoter activity. The –1311/–1477 region was analyzed in more detail through its subdivision into smaller fragments. To this end, the pT109 reporter vector, which contains the luciferase reporter gene under control of the minimal thymidine kinase promoter, was employed. A diagram of the fragments analyzed is shown in Fig. 3B, together with the luciferase activities obtained in Bsc40 cells. These data suggest the existence of both activator and repressor elements in this promoter region. Deletion of the more 3′ region (fragments F1–F4) increased promoter activity, suggesting the presence of a promoter region that represses transcription in mammalian cells. There is also evidence of the presence of additional activator sequences, which are present in fragments F2–F4 but only partially in fragments F1 and F6. The activity of this element is, at least partially, orientation independent, since fragment F5, which contains the complete analyzed region inverted with respect to the reporter gene, showed significant activity. This characteristic is typical of eukaryotic enhancer elements.

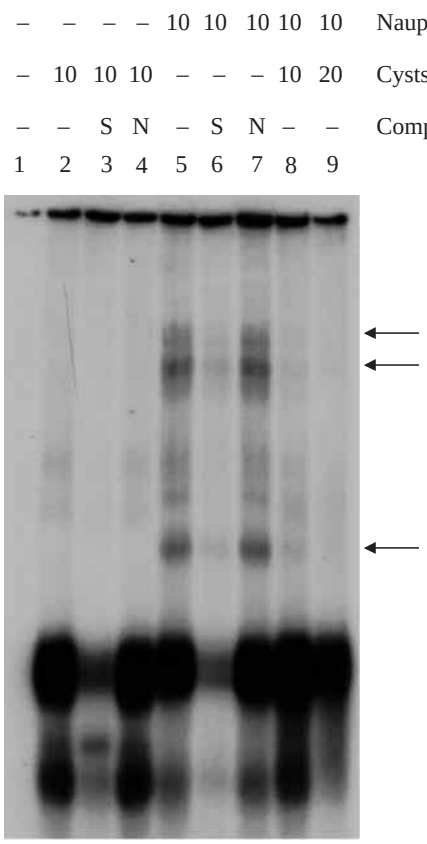

Fig. 2. Analysis of proteins binding to the activator region of the *Actin302* promoter. An oligonucleotide corresponding to nucleotides –409 to –388 of the *Actin302* promoter was used as a probe in an electrophoretic mobility shift assay. Either cyst nuclear extracts (10 μg; lanes 2–4), nauplius nuclear extracts (10 μg; lanes 5–7) or both (lanes 8,9) were incubated with the probe before electrophoretic analysis. Fifty times excess unlabeled oligonucleotide was added as a competitor. Lanes marked 'S' correspond to samples in which the same oligonucleotide was used as competitor, while lanes marked 'N' contain samples in which a non-related oligonucleotide, corresponding to nucleotides –1397 to –1349 of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) promoter 2, was used as competitor. Arrows indicate the migration of nauplius-specific retardation complexes.

An oligonucleotide probe was designed from the identified activator region (nucleotides –1397 to –1349) and used in EMSAs to check for the presence of proteins in *Artemia* nuclear extracts, which would bind to this DNA region. A specific retardation complex was observed after incubation of the probe with nauplius nuclear extracts (Fig. 4; lanes 2–5). The formation of this complex was reduced by a 50-fold excess of the same unlabeled oligonucleotide but not by two non-related oligonucleotides. This specific complex was also observed, but in much smaller amounts, when the probe was incubated with the same amount of cyst nuclear extract, (Fig. 4; lanes 6–9). An additional, slower-migrating retardation complex was observed with cyst extracts but not with nauplius nuclear extracts.

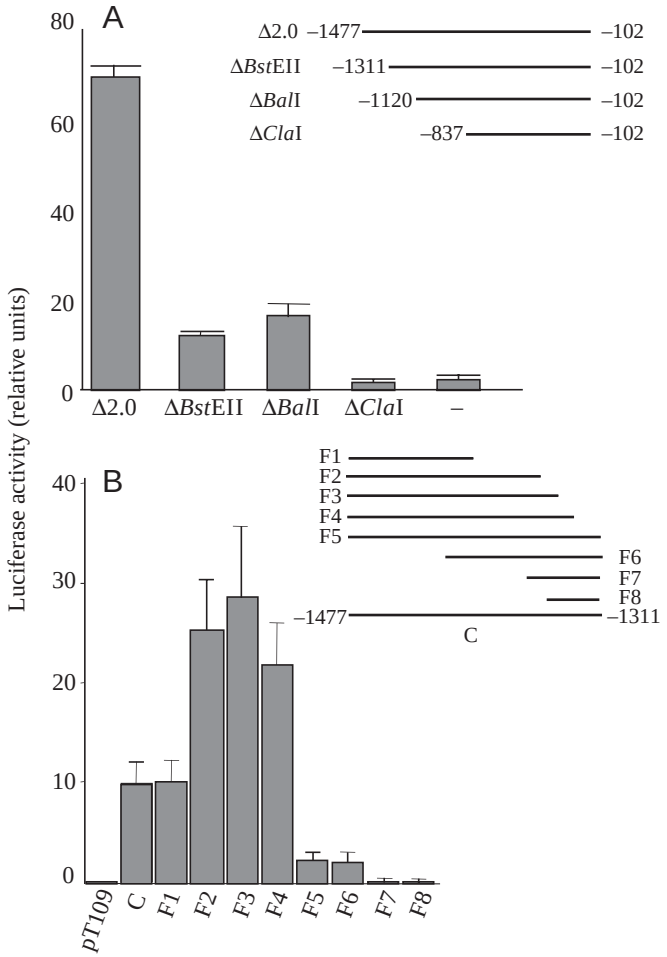

Fig. 3. Functional analysis of promoter 2 of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-encoding gene in mammalian cells. (A) A 1376 nt fragment of SERCA promoter 2, from nucleotides –1477 to the end of exon 1 (nucleotide –102), named Δ2.0, and smaller fragments generated from the internal restriction sites *Bst*EII, *Bal*I and *Cla*I to nucleotide –102, were cloned into the pXP2 luciferase reporter vector. DNA from these plasmids was transfected into Bsc40 cells, and luciferase activity was determined 48 h after transfection. Columns Δ2.0, Δ*Bst*EII, Δ*Bal*I and Δ*Cla*I indicate the activity obtained after transfection with these vectors, while column (–) indicates the result of transfecting the reporter vector without the promoter region. (B) The region –1477 to –1311 of SERCA promoter 2 was isolated and divided into smaller fragments through PCR reactions. The complete fragment, either in its natural orientation (C) or inverted (F5) with respect to the transcription initiation site, or the smaller fragments (F1–F4 and F6–F8) were cloned into the pT109 reporter vector, which contains the minimal thymidine kinase promoter. DNA from the different plasmids was transfected into Bsc40 cells, and the associated luciferase activity was determined 48 h later. The relative activities obtained after transfection of the reporter vector without promoter (pT109) or containing the complete fragment (C, F5) or smaller fragments (F1–F4, F6–F8) are indicated.

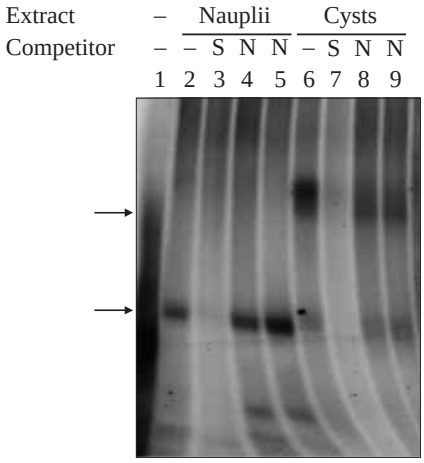

Fig. 4. Analysis of protein binding to the activator region of promoter 2 of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-encoding gene. An oligonucleotide corresponding to nucleotides –1397 to –1349 of SERCA promoter 2 was used as probe in an electrophoretic mobility shift assay. The probe was incubated with either 10 μg of nauplius or cyst nuclear extracts. A fifty times excess of the same (S) unlabeled oligonucleotide (lanes 3, 7) or two different non-related (N) oligonucleotides (lanes 4, 8 and 5, 9, respectively) were used as competitors. The first non-related oligonucleotide corresponds to nucleotides –1347 to –1316 of the same SERCA promoter 2 (lanes 4, 8) and the second to the consensus binding site for the transcription factor Oct-1 (lanes 5, 9). Arrows indicate the migration of specific retardation complexes.

Analysis of protein-binding regions in A. franciscana promoters

Analysis of *A. franciscana* promoters in cultured mammalian cells is limited by the fact that only those regions recognized by mammalian transcription factors are detectable in these assays. To circumvent this limitation, we employed DNA footprinting in which protein-binding regions of the promoters were identified by DNase protection after their incubation with cyst or nauplius nuclear extracts. These experiments also allowed a comparison of DNA-binding proteins present in nuclear extracts from cysts or nauplii.

An end-labeled fragment of the proximal promoter region of the Na^+/K^+-ATPase α1-subunit-encoding gene promoter was incubated with different amounts of cyst or nauplius nuclear extracts, or with mixtures of both of them, and submitted to partial digestion with DNase I (Fig. 5A). Some nucleotide bonds were protected from DNase digestion after incubation with nauplius extracts but not with cyst nuclear extracts. Addition of cyst nuclear extracts together with nauplius extracts did not alter the protections observed with nauplius extracts alone. Some of the additional bands observed after incubation with nauplius nuclear extracts could be due to the activity of an *A. franciscana* DNase whose expression is induced during development (Domingo et al., 1986). These results indicate the presence of nauplius-specific DNA-binding proteins that are not detected in cyst nuclear extracts. To confirm these results, oligonucleotide probes were designed from one of the protected regions (nucleotides –207 to –188, indicated by an open box in Fig. 5A) and used in EMSAs. The results (Fig. 5B) indicate the formation of specific

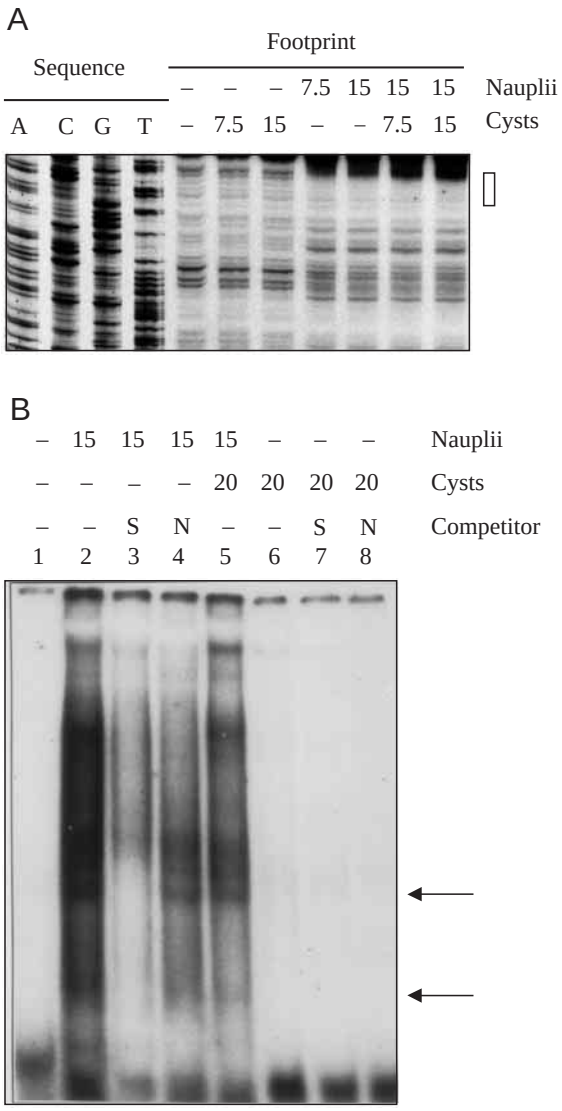

Fig. 5. *In vitro* analysis of protein-binding regions of the promoter of the Na^+/K^+-ATPase α1 subunit-encoding gene. (A) The indicated amounts of cyst or nauplius nuclear extracts were incubated with an asymmetrically labeled probe from the proximal region of the Na^+/K^+-ATPase α1-subunit-encoding gene promoter. After incubation, the samples were subjected to partial DNase I digestion and analyzed in 6% polyacrylamide–7 mol l^{-1} urea gels. Nucleotide sequencing reactions of the same fragment were used to locate the digestion products. The open box on the right indicates the protected region that was analyzed in B. (B) An oligonucleotide probe of the region indicated in A, corresponding to nucleotides –207 to –188, was incubated with nauplius (lanes 2–4; 15 μg), cyst (lanes 6–8; 20 μg) or both (lane 5) nuclear extracts and analyzed by polyacrylamide gel electrophoresis (PAGE). The same unlabeled oligonucleotide was used as a specific competitor (S; lanes 3, 7), while an oligonucleotide from nucleotides –240 to –221 of the *Actin302* promoter was used as a non-related competitor (N; lanes 4, 8). Arrows indicate the migration of nauplius-specific retardation complexes.

DNA–protein complexes when the probe is incubated with nauplius nuclear extracts but not when incubated with cyst nuclear extracts. The addition of cyst nuclear extracts together with nauplius extracts did not affect the formation of DNA–protein complexes (Fig. 5B; lane 5).

The proximal region of the *Actin302* promoter was also analyzed by footprinting. Protection from DNase I digestion was observed after incubation with nauplius nuclear extracts but not with cyst nuclear extracts (Fig. 6A). Simultaneous incubation with both extracts did not alter the protection observed with nauplius extracts. An oligonucleotide probe that includes the protected region shown in Fig. 6A (open box) was used for EMSAs. A specific protein–DNA complex was detected after incubation with nauplius nuclear extracts, indicated by an arrow in Fig. 6B. No specific complexes were observed after incubation of the probe with cyst nuclear extracts. Incubation with cyst and nauplius nuclear extracts slightly decreased the intensity of the protein–DNA complex obtained with nauplius extracts.

Finally, similar footprinting experiments were carried out with a fragment of the proximal region of promoter 2 of the SERCA-encoding gene. A representative region of the footprint is shown in Fig. 7A. DNase I protection was once again observed after incubation of the DNA with nauplius nuclear extracts but not with cyst nuclear extracts. Simultaneous incubation with both extracts produced the same protection as with nauplius extracts alone. An oligonucleotide probe was designed for the protected region shown in Fig. 7A (indicated by the open box) and used for EMSAs. A specific protein–DNA complex was observed after incubation with cyst extracts (Fig. 7B). A similarly migrating complex was also observed with nauplius nuclear extracts. In addition, a faster-migrating complex was specifically observed after incubation with nauplius extracts.

Discussion

The mechanisms that mediate the reduction of biological activity at the onset of cryptobiosis and its reactivation upon exit from this state are not completely understood. We have focused on the mechanisms that mediate repression of transcription in the cryptobiotic *A. franciscana* cyst and its activation after biological activity and embryonic development are resumed. Two extreme models can be considered. According to the first one, transcription could be actively repressed as the embryo enters cryptobiosis. Several mechanisms could be involved, including changes in chromatin structure and accumulation of transcription repressor molecules. Alternatively, a decrease in the levels of activator transcription factors may occur, and their paucity would lead to the absence of transcription. Activation of transcription, once cryptobiosis has ceased, would require removal of the repressor molecules, according to the first model, or induction of activator molecules, if the second model holds true. Nevertheless, it is likely that both models are valid to different degrees, depending on the gene under study.

To help clarify this issue, we examined protein binding to different gene promoters in cryptobiotic embryos and in embryos that have resumed development. We analyzed the

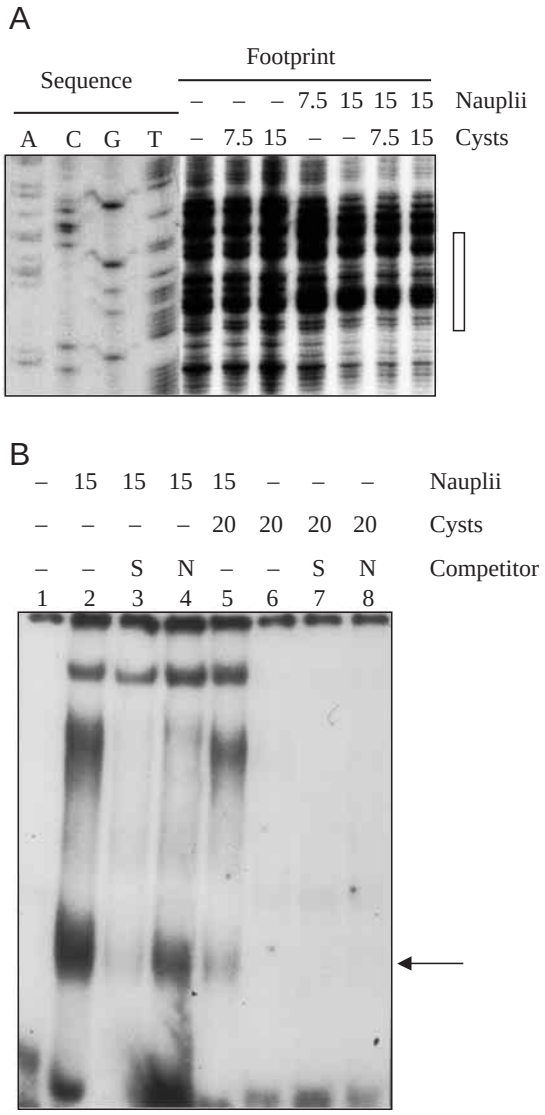

Fig. 6. *In vitro* analysis of protein-binding regions of the *Actin302* proximal promoter region. (A) Nauplius or cyst nuclear extracts, or both, were incubated with an asymmetrically labeled probe from the proximal region of the *Actin302* gene promoter. After incubation, samples were partially digested with DNase I and analyzed in 6% polyacrylamide–7 mol l^{-1} urea gels. Sequencing reactions of the same promoter region were analyzed in parallel to determine the nature of the digestion products. The open box to the right indicates the migration corresponding to the probe used in B. (B) The region corresponding to the footprint shown in A (nucleotides –240 to –221) was used to synthesize an oligonucleotide probe, which was incubated with nauplius (15 μg; lanes 2–4), cyst (20 μg; lanes 6–8) or both nuclear extracts (lane 5). Complexes were analyzed by polyacrylamide gel electrophoresis (PAGE). 50 times excess of the same unlabeled oligonucleotide was used as a specific competitor (S; lanes 3, 7). An oligonucleotide corresponding to nucleotides –207 to –188 of the Na^+/K^+-ATPase α1-subunit-encoding gene promoter was used as a non-specific competitor (N; lanes 4, 8). The arrow indicates the specific retardation complex obtained after incubation with nauplius nuclear extracts.

promoters of three genes whose expression is known to be induced a few hours after cyst activation. We identified putative regulatory regions of these promoters and subsequently analyzed the presence of proteins from cryptobiotic (cysts) or developing (nauplii) embryos that would bind to these regions.

Regulatory regions were initially identified by means of a functional analysis of the promoters in cultured mammalian cell lines. It is likely that some promoter regions that play an important regulatory role in *Artemia* are not recognized in mammalian cells, since the functionality of *Artemia* promoters in this heterologous system depends on the conservation of DNA–transcription factor interactions. Nevertheless, *Artemia* cell lines are not currently available. One example of the utility of this assay came from the study of the *Actin403* gene promoter. Functional analysis in mammalian cells allowed the identification of an activator element homologous to mammalian serum response element (Casero and Sastre, 2001). Subsequent studies led to the identification of cDNA clones coding for an *A. franciscana* protein homologous to the serum response factor, the transcription factor that binds to the serum response element (Casero and Sastre, 2000).

Our second approach consisted of identifying protein-binding regions in the promoters through footprinting analysis. This is a homologous system in which *Artemia* proteins are assayed on *Artemia* DNA, but the system does not provide information about the functionality of the interactions. Putative protein-binding regions identified by either of these two criteria were confirmed by EMSAs. This confirmation is particularly relevant when the regions were previously identified by functional assays in mammalian cells, since it shows that these regions also have the capacity to specifically bind proteins from *Artemia* extracts, suggesting the conservation of this interaction. Because of these limitations, the regions identified by these techniques may not be the most physiologically relevant in terms of gene regulation. Nevertheless, both techniques can detect regulatory regions that are useful for a comparative study of transcriptional regulation during cyst activation.

The presence of DNA-binding proteins in cyst and nauplius nuclear extracts was compared by EMSAs for those regions identified by functional assays. EMSAs and footprinting assays were used for the remaining regions. In all the cases analyzed, specific protein–DNA complexes were detected using nauplius nuclear extracts. These complexes were not detected using cyst nuclear extracts or were present in much smaller amounts. These results indicate that the expression of these DNA-binding proteins is induced after activation of cryptobiotic cysts. Footprinting analyses also detected other promoter regions that were protected when incubated with nauplius extracts and not when incubated with cyst extracts, although these data were not confirmed by EMSA (not shown). Analysis of the expression of the basal transcription factor TBP (Sastre, 1999) and of the DNA-binding activity of the sequence-specific transcription factor SRF (Casero and Sastre, 2000) also showed a significant increase between the cyst and nauplius

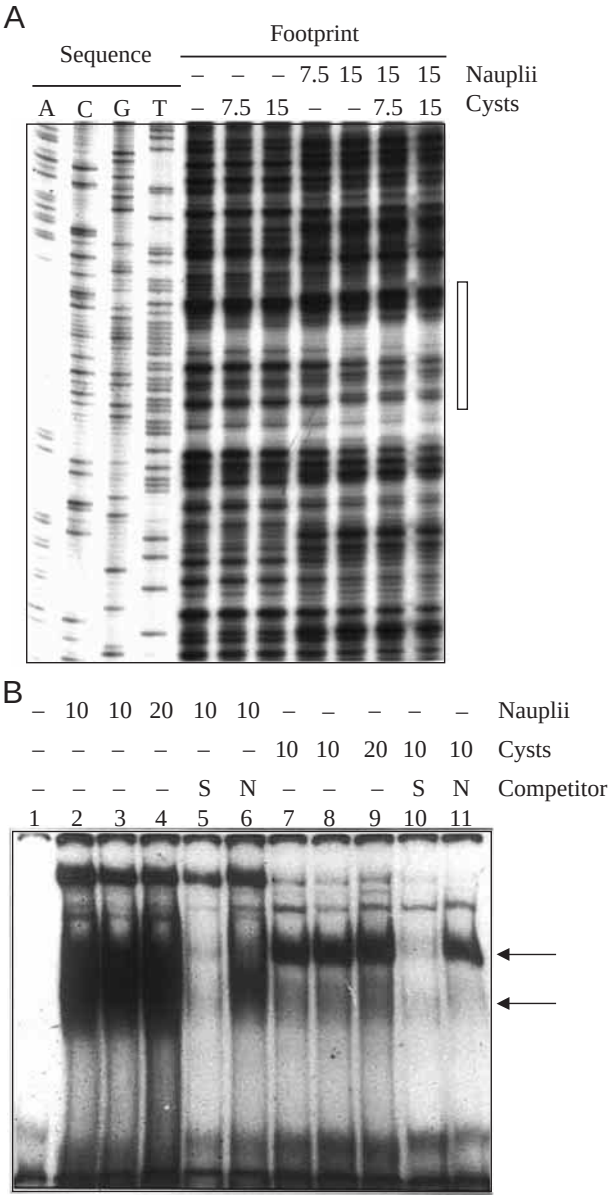

Fig. 7. *In vitro* analysis of protein-binding regions of promoter 2 of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-encoding gene. (A) The indicated amounts of cyst or nauplius nuclear extracts were incubated with an asymmetrically labeled probe derived from the proximal region of promoter 2 of the SERCA gene. Samples were subjected to limited digestion with DNase I before analysis in 6% polyacrylamide–7 mol l^{-1} urea gels. Sequencing reactions of the same promoter region were analyzed in parallel to identify DNase I digestion bands. The open box on the right indicates the protected region that was further analyzed in B. (B) An oligonucleotide probe corresponding to nucleotides –262 to –238 of promoter 2 of the SERCA gene was incubated with nauplius (lanes 2–6) or cyst (lanes 7–11) nuclear extracts and analyzed by polyacrylamide gel electrophoresis (PAGE). Fifty times excess unlabeled oligonucleotides were used as competitors. The same oligonucleotide used as probe was the specific competitor (S; lanes 5, 10), and an oligonucleotide corresponding to nucleotides –207 to –188 of the Na^+/K^+-ATPase α1 subunit gene promoter was the non-specific competitor (N; lanes 6, 11). Arrows indicate the migration of specific retardation complexes.

stages. These results are consistent with the model that proposes the induction of expression and/or activity of transcription factors after cyst activation.

EMSA experiments using the SERCA promoter 2 and *Actin302* promoter probes detected the existence of DNA-binding proteins in cyst extracts. The binding of these proteins was not detected in footprinting experiments. At present, the functional significance of these protein–DNA interactions remains unknown. These proteins might exert an inhibitory function in the cysts, as predicted by one of the two hypotheses initially considered. In any case, the existence of these putative repressive interactions was not general for all the promoter regions analyzed.

The differences observed between cyst and nauplius extracts in the formation of DNA–protein complexes could also be due to the presence of some inhibitory factors in the cysts. This possibility was studied through the addition of cyst nuclear extracts to nauplius extracts to see if there was any interference with the formation of nauplius-specific DNA–protein complexes. No interference was detected in any of the DNase I protection experiments analyzed. Similarly, EMSAs with the Na^+/K^+-ATPase α1 subunit and *Actin302* proximal promoter probes showed no variation in nauplius-specific complexes after addition of cyst extracts. Only EMSAs using the *Actin302* distal promoter probe showed inhibition of the nauplius-specific retardation complexes after addition of cyst nuclear extracts. This probe formed a very abundant retardation complex with cyst extracts (Fig. 2). Simultaneous addition of cyst and nauplius extracts could produce either inhibition of the nauplius-specific complexes or a competition between cyst and nauplius DNA-binding proteins. The higher concentration or affinity of the cyst proteins may reduce the formation of the nauplius-specific complexes.

The results obtained in this study favor a model in which transcription factors are absent, or are present in very limited amounts, in the nuclei of cryptobiotic embryos. Activation of the cysts would lead to the induction of transcription factor expression and/or activity. Increases in activity could be due to post-translational modifications of existing transcription factors. Nuclear translocation of transcription factors may also occur, in a manner similar to the rapid translocation of the crystalline homologue p26 during cyst activation (Clegg et al., 1994, 1995). Increases in expression would be dependent on protein synthesis, which is known to be induced a few minutes after cyst activation (Tate and Marshall, 1991). The synthesis of some of these transcription factors could be directed by mRNAs stored in the cyst, which would make this process independent of transcription reactivation. In agreement with this idea, mRNAs coding for the SRF transcription factor have been found stored in cryptobiotic cysts at levels similar to those found in developing nauplii (Casero and Sastre, 2000).

The existence of transcription factor induction does not rule

out the participation of other regulatory mechanisms. Some of the data obtained are compatible with the presence of repressor molecules in the cyst, as mentioned above. The existence of a repressive chromatin environment is another possibility that cannot be discarded. The DNA-binding proteins involved in these interactions may not have been detected by the footprinting and electrophoretic mobility assays utilized in this study. Despite these considerations, the data presented strongly support the idea that induction of transcription factor expression and/or activity is an important mechanism of activation of gene expression in *Artemia* embryos exiting cryptobiosis.

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