Molecular cloning and expression of four actin isoforms during *Artemia* development

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**ABSTRACT**

Four cDNA clones coding for different *Artemia* actin isoforms have been isolated. Three of the clones contain the complete coding sequences while the fourth one lacks 145 bases, coding for the 49 amino terminal amino acids of the protein. The amino acid sequences predicted for the four actin isoforms identified are highly homologous to insect actins as well as to vertebrate cytoplasmic actins. The four identified cDNA clones code for mRNAs of 5.2, 1.8, 1.6 and 1.8 kb, respectively, whose expression is regulated during development. Three of the actin mRNAs are present in cryptobiotic embryos while the other is not. The steady-state levels of all four mRNAs increase during development to reach maximal levels by 10–15 hours of development and decrease thereafter. The total number of actin genes encoded in the *Artemia* genome has been estimated as 8 to 10 by Southern analysis of total DNA.

**INTRODUCTION**

Actin is involved in several basic functions of the organism such as cell division, cell motility or muscle contraction (1–3). Most organisms have genes coding for several actin isoforms specialized to fulfill some of these functions. In mammals, for example, two different isoforms are found in non muscular cells (cytoplasmic actins, named β and γ) and four isoforms in muscular cells (named α), two specific for striated muscles (skeletal and cardiac) and two specific for smooth muscles (vascular and non vascular isoforms). Although all actins isoforms are over 90% identical to each other, in vertebrates, there is a clear distinction in the amino acid sequences of muscular (α) and non muscular (β and γ) actins, specially at their amino terminus (4).

Several actin isoforms have also been identified in invertebrates. All invertebrate actins are more similar to cytoplasmic than to muscular vertebrate actins so that it is not possible to recognize invertebrate muscular actins from their amino acid sequences(5). Nevertheless, invertebrate actin isoforms expression is also tissue-specific and the study of the pattern of expression of the different isoforms during embryonic development and in adult organisms has allowed the characterization of several muscular and cytoplasmic actins in *Drosophila* (6,7), sea urchin (8) and *Dictyostelium discoideum* (9).

The family of actin genes is a very interesting system to study the regulation of gene expression since highly homologous genes of this family are expressed in different tissues and at different stages of development (10–13). Promotor sequences have been characterized from muscular actins that direct muscle specific expression when fused to reporter genes (14–16). Actually, some regions from these promoters, like the CArG box, are also present in other muscle specific genes (17). Promotor regions from cytoplasmic sea urchin actins also direct tissue specific expression of reporter genes at the proper time of development (18).

These characteristics of the actin gene family have prompted us to approach its study in the crustacean *Artemia*. This organism has been used as a model system to study embryonic development and cryptobiosis because of its property of getting into cryptobiosis at the gastrula stage of development under adverse environmental conditions (19). Cryptobiosis can be reversed by incubation of the cryptobiotic embryos (cysts) at the proper conditions of salt, light and temperature so that the activation of the cyst and its posterior embryonic development can be studied in the laboratory. *Artemia* cysts are morphologically uniform and during the first hours of development there is an active process of differentiation and morphogenesis. By 15–20 hours of incubation at 30°C swimming nauplii hatch from the cyst presenting several well differentiated organs such as the antennae, eye or salt gland. Further development of the nauplii involves the formation of the intestinal track, among other thoracic and abdominal structures, so that by about 50 hours of development the nauplii are able to feed themselves. This can be a very useful model system to study the regulation of gene expression since there is no metabolic activity in the cyst and transcription is resumed after activation of the cyst and modulated according to the pattern of embryonic development. As a first step in this project, we present in this report the isolation of cDNA clones coding for four *Artemia* actin isoforms, their nucleotide sequence and the variations of the steady-state levels of the mRNAs encoded by these genes during postgastrular embryonic and early larval development.

**MATERIALS AND METHODS**

**Artemia culture and nucleic acid isolation**

Cryptobiotic *Artemia* embryos (cysts) were purchased from San Francisco Bay Brand (Neward, CA) in February, 1987. Embryos were dechorionized and cultured as described previously (20). RNA was isolated from cysts or nauplii cultured at 30°C for
the period of time indicated in each experiment by the method of Adams et al (21).

Total DNA was purified from 20-hour-old nauplii by the method of Cruces et al (22).

Screening of cDNA libraries
A cDNA library made up from adults mRNA in the lambda vector λgt11 (23) was screened by the method of Benton and Davis (24). A 1.8 kb long HindIII-HindIII fragment containing the actin coding sequences from the *Drosophila* actin clone DmA2 (25) was labelled according to Feinberg and Vogelstein (26) and used as probe in this screening. Filters were hybridized under standard conditions (6×SSC, 0.5% SDS, 2×Denhardt’s solution, 100 μg/ml calf thymus DNA at 65°C for 15 hours; SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7; Denhardt’s solution is 0.2 mg/ml Ficoll, type 400, 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml bovine serum albumin, fraction V) and washed in 2×SSC, 0.1% SDS at 65°C.

One of the cDNA clones isolated in the first screening (pArAct211) was used as probe in two more screenings of a cDNA library made up from 20-hours-old-nauplii mRNA in the plasmid vector pUC18 (27). These screenings were made according to the method of Hanahan and Meselson (28). Labelling of the probe and hybridization and washing of the filters were made as previously described for the first screening of the cDNA libraries.

Northern and Southern blotting analyses
Ten micrograms of total RNA from *Artemia* cysts or nauplii at different stages of development were analyzed in 1.5% agarose-2.2% formaldehyde gels and transferred to nylon membranes according to Thomas (29). RNA blots were hybridized to oligonucleotide probes labelled at their 5’ end by the enzyme polynucleotide kinase (30). Washing of the filters was made in 6×SSC, 0.1% SDS, 0.05% sodium pyrophosphate at 37°C.

For the analyses of genomic DNA, total DNA was isolated from 20-hours-old nauplii and ten micrograms of the DNA digested with each indicated enzyme. DNA fragments were analyzed on 0.7% agarose gels and transferred to nylon membranes according to the method of Reed and Mann (31). Filters were hybridized to pArAct211 insert. Labelling of the insert and hybridization of the filters were made under the same conditions described for the screening of the cDNA libraries. Washing of the filters was at 65°C in 2×SSC, 0.1% SDS or 0.1×SSC, 0.1% SDS, as indicated for each experiment.

DNA sequencing
The nucleotide sequence of the DNA was determined by the termination inhibitor method of Sanger et al. (32) modified by Chen and Seeburg (33) for sequencing double-stranded DNA. The plasmid pUC18 (34) was used as vector for all the constructions needed for sequencing of the cDNA clones. Either internal restriction sites, determined by restriction mapping of the clones, or progressive deletions of the clones made up by the use of the Exonuclease III-Nuclease S1 method (35) were used to obtain the complete nucleotide sequence of both strands of each cDNA clone.

RESULTS

Isolation of cDNA clones
The *Drosophila* actin gene DmA2 has been used as a probe to isolate *Artemia* actin cDNA clones under low stringency hybridization conditions. Initially, 100,000 clones of an adult cDNA library made in the lambda vector λgt11 were screened. Four of the positive clones were chosen for characterization. The inserts from these four clones were cloned in the EcoRI site of the plasmid vector pUC18. Restriction map and partial nucleotide sequencing of these clones showed that three of them were overlapping so that the longest of these three (pArAct211) and the other clone (pArAct205), whose restriction maps are shown in Figure 1, were chosen for further characterization.

The insert of the clone pArAct211 was used as probe for two more screening of a second *Artemia* cDNA library, made from 20-hour-old nauplii mRNA and inserted in the BamHI site of the plasmid vector pUC18 (27). A total of 20,000 colonies of this library were screened and 14 positive clones chosen for characterization. Restriction mapping and terminal sequencing of these clones showed that 8 of these clones were overlapping with the previously isolated pArAct211 clone and one more with the pArAct205 clone. Besides these clones, two new types of cDNA clones were identified. Two cDNA clones were isolated for each of these new types and the longest ones, pArAct302 and pArAct403 selected for characterization. The restriction map of the cDNA clones that were selected is shown in Figure 1.

The nucleotide sequence of the four selected cDNA clones has been established and is shown in Figure 2. Each of the four cDNA clones contained an open reading frame whose encoded amino acid sequences were over 90% similar to the *Drosophila* actin used as probe. The nucleotide sequence of the clone pArAct205 is shown in Figure 2 together with the amino acid sequence predicted from this clone. The nucleotide sequence of the other three cDNA clones are also shown in Figure 2, underneath the pArAct205 sequence. Three of the cDNA clones (pArAct205, pArAct211 and pArAct403) contain their complete coding regions as well as 5’ and 3’ untranslated sequences while the clone pArAct302 is missing 145 nucleotides at its 5’ end that would code for the 49 N-terminal amino acids of the protein. Figure 2 shows that the four cDNA clones are very homologous in their coding sequences while the homology is rather low in their untranslated sequences, as has been shown to be the case for clones coding for different actin isoforms from other organisms.

The amino acid sequences encoded by the four cDNA clones are compared in Figure 3. The degree of identity between the coding sequences of the four clones at the nucleotide and amino acid levels is rather high, with the majority of the differences occurring near the amino terminus.
Figure 2: Nucleotide sequence of the *Artemia* actin cDNA clones. The nucleotide sequence of the clones was determined as indicated in the section Materials and Methods. The complete nucleotide sequence of the clone pARAct205 is shown in the lane 205 in capital letters. The nucleotide sequence of the other cDNA clones (lanes 211, 302 and 403) is shown underneath that of the clone pARAct205. A dot indicates the same nucleotide as that determined for pARAct205 for that position. The amino acid sequence predicted from pARAct205 nucleotide sequence is shown over the nucleotide sequence of this clone (lane Prot). Numbers at the right indicate the position of the last nucleotide of each lane (plain numbers) or the last amino acid of the lane (bold numbers).

Acid levels is summarized in Table I. The identity between the predicted amino acid sequences is over 94% and the identity between the nucleotide sequences over 78%, values currently found in actin isofoms. Besides, most of the amino acid differences are conservative (Serine for Threonine, Aspartic for Glutamic, Isoleucine for Leucine or Valine) as shown in Figure 3. The predicted N-terminal sequences are similar to other invertebrate N-terminal sequences in that they code for Methionine-Cysteine and three acidic amino acids in the first five positions, a Valine at position 11 and a Cysteine at position 18 (36). These data confirm previous results obtained by peptide analyses of total *Artemia* actin (5).

The amino acid sequence predicted from the four cDNA clones has been compared to that of actin isoforms from other organisms contained in the EMBL protein bank using the fast program (37) and the degree of homology found is shown in Table II. *Artemia* actin isoforms show a similar degree of homology to insect actsins and to vertebrate cytoplasmic actsins. The degree of homology with vertebrate muscular actsins is lower, in agreement with the results obtained for other invertebrate actins.
Figure 3. Comparison of the amino acid sequences coded for *Artemia* cDNA clones. The amino acid sequence predicted from the nucleotide sequence of the clone pArAct205 is shown in the upper lane (labelled as 205) using the one letter amino acid designation system. The predicted sequences coded by the clones pArAct211 (lane 211), pArAct302 (lane 302) and pArAct 403 (lane 403) are shown underneath. Dots indicate the presence of an amino acid identical to the one coded by pArAct205 at that position.

Table I. Comparison of the nucleotide sequences of the coding regions of *Artemia* actin cDNA clones and their predicted amino acid sequences.

<table>
<thead>
<tr>
<th>% Protein homology</th>
<th>205</th>
<th>211</th>
<th>302</th>
<th>403</th>
</tr>
</thead>
<tbody>
<tr>
<td>205</td>
<td>---</td>
<td>96.4</td>
<td>94.5</td>
<td>97.1</td>
</tr>
<tr>
<td>211</td>
<td>85.5</td>
<td>---</td>
<td>94.8</td>
<td>97.1</td>
</tr>
<tr>
<td>302</td>
<td>79.2</td>
<td>78.7</td>
<td>---</td>
<td>96.6</td>
</tr>
<tr>
<td>403</td>
<td>80.1</td>
<td>80.3</td>
<td>78.9</td>
<td>---</td>
</tr>
</tbody>
</table>

% Nucleotide identity

Table II. Comparison of the amino acid sequences of *Artemia* actin isoforms with those of other organisms.

<table>
<thead>
<tr>
<th>Actin origin</th>
<th>pArAct205</th>
<th>pArAct211</th>
<th>pArAct302</th>
<th>pArAct403</th>
<th>Number of sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insects</td>
<td>95.8</td>
<td>96.1</td>
<td>95.3</td>
<td>96.5</td>
<td>3</td>
</tr>
<tr>
<td>Vertebrate α</td>
<td>92.4</td>
<td>92.6</td>
<td>92.3</td>
<td>93.7</td>
<td>13</td>
</tr>
<tr>
<td>Vertebrate β</td>
<td>95.7</td>
<td>96.1</td>
<td>96.0</td>
<td>97.8</td>
<td>7</td>
</tr>
<tr>
<td>Vertebrate γ</td>
<td>95.3</td>
<td>95.6</td>
<td>96.0</td>
<td>97.4</td>
<td>2</td>
</tr>
<tr>
<td>D. discoideum</td>
<td>92.1</td>
<td>92.6</td>
<td>93.4</td>
<td>94.4</td>
<td>3</td>
</tr>
<tr>
<td>Yeast</td>
<td>88.1</td>
<td>88.1</td>
<td>88.4</td>
<td>89.7</td>
<td>2</td>
</tr>
<tr>
<td>Plants</td>
<td>83.1</td>
<td>83.7</td>
<td>85.3</td>
<td>85</td>
<td>4</td>
</tr>
</tbody>
</table>

Expression of actin isoforms during *Artemia* development

In order to analyze the steady-state levels of the mRNAs coded by the actin cDNA clones that we have isolated, oligonucleotide probes were designed that specifically hybridize to each of the clones. The use of oligonucleotide probes was necessary given the high homology of the coding regions of the cDNAs and the small length of the untranslated regions of some of the clones (for example, clone pArAct302). The oligonucleotide probes were complementary to 3' untranslated sequences of the clones and their names and sequences are as follows:

- Act-1: CACACCAAGTAACCAGTCA, complementary to nucleotides 1394 to 1413 of the clone pArAct205.
- Act-2: GATCTAGATAGGGAGCCCT, complementary to nucleotides 1285 to 1304 of the clone pArAct211.
- Act-3: CGGGCAGTGATTTAGAATG, complementary to nucleotides 1005 to 1024 of the clone pArAct302.
- Act-4: GCCCGTTAACTACCTTTCAAC, complementary to nucleotides 1198 to 1218 of the clone pArAct403.

The specificity of each oligonucleotide was tested by hybridization of every oligonucleotide probe to the insert of each of the four cDNA clones (data not shown).

Total RNA was obtained from *Artemia* cysts or nauplii at different stages of development and analyzed for the presence of the mRNA encoded by each actin isoform. The results of these experiments are shown in Figure 4. The amount of probe hybridized to each RNA lane was quantified by densitometry of the autoradiograms and divided by the amount of ribosomal RNA present in that lane, quantified by ethidium bromide staining of the gels. The amount of ribosomal RNA was used to correct possible differences in the amount of RNA applied to each lane and because it has been shown that the amount of ribosomal RNA present per cell does not change during the period of development that we have studied in *Artemia* (38). The normalized quantity of the mRNAs expressed at different stages of development are shown in Figure 5. Hatching of 50% of the *Artemia* nauplii took place by 15 hours of incubation at 30°C in these experiments.

The clone pArAct205 (probe Act-1) hybridizes to a 5.2 kb long mRNA present in cryptobiotic embryos whose steady-state level increase more than twice by 10–15 hours of development to come back to cyst levels at later stages of development. Clones pArAct211 and pArAct403 (probes Act-2 and Act-4) code for mRNAs of 1.9 and 1.8 kb, respectively and follow a similar pattern of expression during development: both are expressed at low levels in cryptobiotic embryos, their steady-state levels increase over five times to reach maximal values by 10–15 hours of development.
of development and decrease there after. In contrast, the steady-state levels of the mRNA coded by the clone pArAct302 (probe Act-3), which is 1.6 kb long, follow a more complex pattern. This mRNA is not present in cryptobiotic embryos, it is induced by ten hours of development and follows an oscillatory pattern of expression during development, with maximal expression by 15, 30 and 50 hours of development. These results were confirmed by dot-blot analyses (data non shown).

**Number of actin genes present in Artemia**

The number of genes coding for actin in the *Artemia* genome was estimated by Southern blot analyses. Total DNA from 20-hours-old nauplii was prepared and digested with the restriction enzymes BamHI, EcoRI, HindIII or PstI, as indicated in Figure 6. Ten micrograms of DNA digested with each enzyme were electrophoresed on 0.7% agarose gels. The DNAs were transferred to nylon membranes that were hybridized with the pArAct211 clone insert. Duplicate filters were washed at 65°C with either 0.1×SSC, 0.1% SDS or 2×SSC, 0.1%SDS, as indicated. Under high stringency conditions (0.1×SSC) only one or two major bands of hybridization were observed (Figure 6) while under low stringency conditions (2×SSC) 10 to 12 bands gave similar hybridization signals in each lane. These data suggest the presence of an approximated number of eight to ten genes coding for actin in *Artemia*.

**DISCUSSION**

*Artemia* cDNA clones coding for four different isoforms of actin have been characterized. Their nucleotide sequences show a high homology between their coding sequences and those of other vertebrate and invertebrate actins. The higher homology is found between *Artemia* actins, insect actins and vertebrate cytoplasmic actins. Further more, the N-terminal amino acid sequence predicted from three of the *Artemia* cDNA clones show the characteristic features of other invertebrate actins: Cysteine at

![Figure 4](image-url)  
*Figure 4.* Expression of actin isoforms during *Artemia* development. Ten micrograms of total RNA from cryptobiotic embryos (lane 0) or cysts or nauplii incubated for 5 to 50 hours at 30°C (lanes 5 to 50, respectively) were analyzed in 1.5%-agarose-2.2M-formaldehyde gels, transferred to nylon membranes and hybridized to the following oligonucleotide probes: A. Oligonucleotide Act-1, complementary to clone pArAct205. B. Oligonucleotide Act-2, complementary to the clone pArAct211. C. Oligonucleotide Act-3, complementary to the clone pArAct302. D. Oligonucleotide Act-4, complementary to the cDNA clone pArAct 403. The position in the gel of ribosomal RNAs obtained from rat (28S and 18S) and *E. coli* (23S and 16S) is indicated on lane M.

![Figure 5](image-url)  
*Figure 5.* Quantification of the actin isoforms expression during *Artemia* development. The hybridization of the actin oligonucleotide probes to total RNA from different stages of *Artemia* development, shown in Figure 4, was quantified by densitometry of the autoradiograms. The amount of hybridization was corrected by the amount of ribosomal RNA present in each sample, as determined by ethidium bromide staining of the gels, and is given in arbitrary units. A. Oligonucleotide Act-1, complementary to clone pArAct205. B. Oligonucleotide Act-2, complementary to the clone pArAct211. C. Oligonucleotide Act-3, complementary to the clone pArAct302. D. Oligonucleotide Act-4, complementary to the cDNA clone pArAct 403.
the second amino acid position followed by three acidic amino acids, Valine at position 11, Methionine at position 17 and Cysteine at position 18 (36). The similarity of the N-terminal peptide of *Artemia* with those of other invertebrate and cytoplasmic vertebrate actins had been reported from paper electrophoretic analyses of the major N-terminal peptide obtained from total actin population of adult animals (5). The data presented in this report confirm these findings for three specific actin isoforms and extend the analyses to the complete sequence of the proteins. To our knowledge, these are the first complete amino acid sequences to be reported for crustacean actins. The analysis of the N-terminal peptide, together with the results presented in this article, also show that post-translational processing of actin is similar in *Artemia* to other organisms, involving the removal of the N-terminal Methionine and Cysteine and the acetylation of the N-terminal Aspartic acid residue. The existence of the enzymatic machinery necessary to achieve these processing reactions has been previously demonstrated both in muscular and non muscular cells (39).

The comparison of the nucleotide and predicted amino acid sequences of the *Artemia* actin clones shows that the most homologous clones are pArAct205 and pArAct211 (98.4 homology at the amino acid level and 85.5 identity at the nucleotide level). Despite the high level of conservation of the coding regions, there is no significant conservation of the untranslated regions of the different isoforms, not even between pArAct205 and pArAct211. These data would suggest that the different *Artemia* actin isoforms diverged long time ago, in contrast to the situation described for some sea urchin actin isoforms that seems to have diverged so recently that their untranslated and intron sequences are highly homologous and the genes are contiguous in the genome (40).

*Artemia* actin isoforms pArAct205, 211 and 302 show a similar degree of homology with insect and cytoplasmic vertebrate actins while pArAct403 is even more homologous to vertebrate β and γ isoforms than to insect isoforms. These data suggest that crustacea and insects diverged soon after the evolutionary separation of vertebrates and invertebrates. These results are in good agreement with the filogenetic trees that have been derived from the comparison to vertebrates, than the comparison of globin (41) or ribosomal RNA (42) sequences.

The number of actin genes encoded in the *Artemia* genome has been roughly estimated in 8 to 10 by Southern analyses. The number of genes estimated for *Artemia* would be intermediate between the six genes existing in *Drosophila* (7) and the 15 genes reported in sea urchin (43) and *D. discoideum* (44).

The study of the expression of the actin mRNAs during development is of particular interest since the pattern of RNA synthesis during the embryony stage of *Artemia* development is poorly understood. This fact is the consequence of two circumstances: 1. *Artemia* cysts are impermeable to radioactive precursors. 2. There are very few studies published on the expression of different genes during *Artemia* development (19). The data obtained in this report indicate an important increase in the steady-state level of the actin mRNAs between 5 and 10 hours after activation of the cyst. Similar increases in steady-state levels of Na/K and Ca ATPase mRNAs have also been found between 5 and 10 hours of development (23, 45). These results suggest an important transcriptional activity between 5 and 10 hours of development. The establishment of the existence of this period of active transcription is of great interest since comparison of this situation with the opposite, represented by the cyst, where no transcription takes place, can be very helpful to study the mechanisms that regulate the inhibition of transcription in the cyst and its resumption after cyst activation.

Further studies would be required in order to characterize the tissue-specific expression of each of the actin isoforms coded by the cDNA clones. In particular, the use of the specific oligonucleotides probes in 'in situ' hybridization experiments could be very informative. On the other hand, the isolation of actin cDNA clones makes possible the identification of their genomic clones and the characterization of the promoter sequences that regulate their expression during *Artemia* cyst activation and embryonic development. These topics are currently under study in our laboratory.

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REFERENCES