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## Cloning and sequencing of an $\alpha$ -tubulin cDNA from *Artemia franciscana*: evidence for translational regulation of $\alpha$ -tubulin synthesis<sup>1</sup>

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### Abstract

The brine shrimp, *Artemia franciscana*, exhibits a limited number of tubulin isotypes which change little during early postgastrula growth. In order to better understand the synthesis of  $\alpha$ -tubulins during *Artemia* development, a cDNA termed  $\alpha$ AT1 was cloned and sequenced. Alignment analyses revealed that the polypeptide encoded by  $\alpha$ AT1 is similar to  $\alpha$ -tubulins from other species. Hybridization of  $\alpha$ AT1 to restriction-digested DNA on Southern blots produced a simple banding pattern, indicating that *Artemia* have a small number of  $\alpha$ -tubulin genes. Probing of Northern blots demonstrated an abundant supply of  $\alpha$ -tubulin mRNA in dormant cysts, emerging nauplii and instar I larvae. However, it was not until instar I larvae were produced that the amount of polysomal  $\alpha$ -tubulin mRNA increased, suggesting that synthesis of the tubulin corresponding to  $\alpha$ AT1 is translationally controlled. This work provides one of the few examples where tubulin synthesis is thought to be translationally regulated. Moreover, when considered in the light of previous analyses, the findings imply that cell differentiation in postgastrula *Artemia* and the diversification of microtubule function certain to accompany this process occur with little or no change in  $\alpha$ -tubulin composition. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:**  $\alpha$ -Tubulin gene; Tubulin isoform; Translational regulation; Brine shrimp

Microtubules are dynamic polymers that influence the shape and polarity of eukaryotic cells, mediate movement of chromosomes and spatially organize

cytoplasmic membrane-bound organelles [1]. The microtubule wall displays a single structural protein termed tubulin, a heterodimer formed of  $\alpha$ - and  $\beta$ -tubulin [2,3]. These two polypeptides are typically present within eukaryotic cells as batteries of closely related isotypes/isoforms, arising from differential gene transcription and posttranslational modifications [4–7]. The tubulin superfamily also includes  $\gamma$ -tubulin, a protein important in nucleation of microtubules at microtubule-organizing centers [8,9], and a prokaryote homologue, FtsZ, [10]. There is evidence that isotubulin composition modulates microtubule formation and stability [11–14]. This is interesting because polymerization into microtubules, a

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process best described by the dynamic instability model [15], is the defining characteristic of tubulin. Moreover, developmental regulation of gene expression may yield tubulin isoforms required to assemble microtubules of unusual morphology (a particular protofilament number, for example) or which are arranged into discrete intracellular arrays [5,7,14,16,17]. Purified *Artemia* tubulin exhibits unusual assembly properties, and as revealed by Coomassie blue staining of two-dimensional gels, it consists of three  $\alpha$ - and two  $\beta$ -tubulins [18,19]. It is not until encysted embryos develop into free-swimming nauplius larvae, in the virtual absence of cell division (see [20] for a description of *Artemia* development), that isotubulin composition changes. Specifically,  $\alpha$ -tubulin is detyrosinated, probably by a posttranslational mechanism [21–23]. These observations demonstrate that a complex metazoan animal, composed of many different cell types, has a limited need for detyrosinated tubulin. Moreover, differentiation of cells during formation of nauplius larvae from encysted gastrulae occurs essentially in the absence of tubulin changes. The purpose of this work was to further describe tubulin genes and their expression during *Artemia* development, as well as to address broader issues of isotubulin synthesis and function.

Dormant cysts of the brine shrimp *Artemia franciscana*, obtained from Sanders Brine Shrimp Co. (Ogden UT), were hydrated in distilled H<sub>2</sub>O at 4°C for 6 h, collected by suction and washed several times with cold H<sub>2</sub>O in a Buchner funnel. Hydrated cysts were either homogenized immediately or incubated in hatch medium [24] containing 2.6 mM Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> at 27°C on a rotary shaker at 250 rpm.

The degenerate primers AT16 (5'-AT(T/C/A)-GG(T/C/A)AA(T/C)GC(T/C/G/A)TG(T/C)TGGG-3') and AT412 (5'-(A/G)AA(T/C)TC(T/A/G)CC-(T/C)TC(T/C)TCCAT(T/G/A)CC-3') were employed to amplify an  $\alpha$ -tubulin DNA fragment by PCR using Taq polymerase (Life Technologies) and cDNA from a commercially prepared library that represented several developmental stages of *Artemia* as template. The amplification protocol, utilizing buffer supplied by Life Technologies supplemented with 1.25 mM MgCl<sub>2</sub>, consisted of 36–40 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1.5 min. A DNA fragment of about 1.3 kb was obtained, which when cloned, sequenced and analyzed by the proce-

dures described below, proved to contain  $\alpha$ -tubulin sequence (data not shown), and it was termed  $\alpha$ L1. The  $\alpha$ L1-containing plasmid, <sup>32</sup>P-labelled with the <sup>32</sup>P-Quick Prime kit (Pharmacia) following the manufacturer's instructions, was utilized to screen a cDNA library prepared from *Artemia* that had undergone 20 h of postencystment development. The change in cDNA libraries was made because analysis of clones indicated the possibility of contamination within the initial library. The second library, constructed in the *Bam*HI site of pUC18, was a gift from Dr. Leandro Sastre (Instituto de Investigaciones Biomédicas del C.S.I.C., Madrid, Spain). Before use, this library was transferred into *Escherichia coli* strain NM522 and amplified once on LB plates supplemented with 60–100  $\mu$ g/ml of ampicillin to achieve a titer of  $1 \times 10^9$  cells/ml. For screening, colonies were grown on 2  $\times$  YT agar plates containing ampicillin, lifted onto dry Hybond N (Amersham) filters, soaked in 2  $\times$  SSC containing 5% (w/v) SDS for 2 min and exposed at full setting (750 W) in a microwave oven. The filters were incubated for 1 h at 65°C in hybridization medium (5  $\times$  SSC, 5  $\times$  Denhardt's solution, 2% (w/v) SDS), the <sup>32</sup>P-labelled probe was added and incubation continued at 65°C for 12–16 h. The filters were washed twice in 2  $\times$  SSC with 0.1% (w/v) SDS at room temperature, then twice in 1  $\times$  SSC containing 0.1% (w/v) SDS at 65°C, before overnight exposure to Kodak X-OMAT AR film at –70°C. Plasmid DNA, prepared from several colonies that hybridized to the <sup>32</sup>P-labelled probe, was sequenced, either manually with the <sup>32</sup>P-Sequencing kit (Pharmacia) or with an ABI373A stretch liner automated sequencer. A recombinant plasmid (AN10), containing the *Artemia*  $\alpha$ -tubulin cDNA termed  $\alpha$ AT1, was cloned and used in subsequent analysis as described. The compositions of SSC and Denhardt's solution are described in Maniatis et al. [25].

Sequences were sent to data bases at the National Center of Biological Information (NCBI) for the determination of significant matches with known sequences using the Basic Local Alignment Search Tool (BLAST) [26] including blastn and blastx for DNA and blastp for protein. For multiple alignments, protein sequences of other  $\alpha$ -tubulins were retrieved from NCBI data bases using the Web browser based Entrez program and analyzed with 'Clustal' version V and W [27,28].

Genomic DNA and cytoplasmic and polysomal poly(A)<sup>+</sup> mRNA were made from *Artemia* [22,29]. Fractionated, restriction enzyme digested genomic DNA was transferred from TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) buffered, 0.7% agarose gels to Hybond N membrane (Amersham) following the manufacturer's instructions. Filters were then incubated for at least 1 h at 60°C in hybridization buffer, then  $\alpha$ AT1 recovered from AN10 by digestion with *Bam*HI and <sup>32</sup>P-labelled with the T<sup>7</sup>QuickPrime kit (Pharmacia), was added.  $\alpha$ AT1 is an *Artemia*  $\alpha$ -tubulin cDNA obtained as outlined previously and AN10 is the recombinant plasmid containing  $\alpha$ AT1. Both  $\alpha$ AT1 and AN10 are described in more detail elsewhere in the paper. Hybridization was at 60°C for 12 h. Filters were washed twice with 2×SSC containing 0.1% (w/v) SDS at room temperature and once with 1×SSC containing 0.1% (w/v) SDS at 65°C, followed by exposure to Kodak X-OMAT AR film at –70°C.

Northern blots were prepared by transferring approximately equal amounts of poly(A)<sup>+</sup> mRNA, as determined by absorption of samples at 260 nm, from dormant cysts, emerging nauplii and instar I larvae to Hybond N [29,30]. The membranes were incubated in RNA hybridization buffer (5×SSC, 5×Denhardt's solution, 2% (w/v) SDS and 50% (v/v) deionized formamide) for 1 h, <sup>32</sup>P-labelled probes (described below) were added and hybridization was at 42°C for 12 h. The blots were washed twice with 2×SSC containing 0.1% (w/v) SDS at room temperature for 15 min, twice with 1×SSC containing 0.1% (w/v) SDS at 50°C for 15 min and twice with 0.1×SSC containing 0.1% (w/v) SDS at 50°C for 10 min, before exposure to Kodak X-OMAT AR film at –70°C. Northern blots were probed with pAT1200, a DNA fragment amplified by PCR using AN10 as template and the degenerate primers AT16 and AT412. pAT1200 encompassed nucleotides 106–1314 of  $\alpha$ AT1, equivalent to most of the  $\alpha$ -tubulin sequence in AN10. Northern blots were also probed with a PCR generated DNA fragment termed pATNC which included 141 bp of the 3' noncoding region from  $\alpha$ AT1. Again, AN10 was the template, but the primers were:  $\alpha$ NCS (sense) (5'-TAGTGCCTGTGCTTATATC-3') and  $\alpha$ NCA (antisense) (5'-CTTGTTATGTATTTACATT-3').

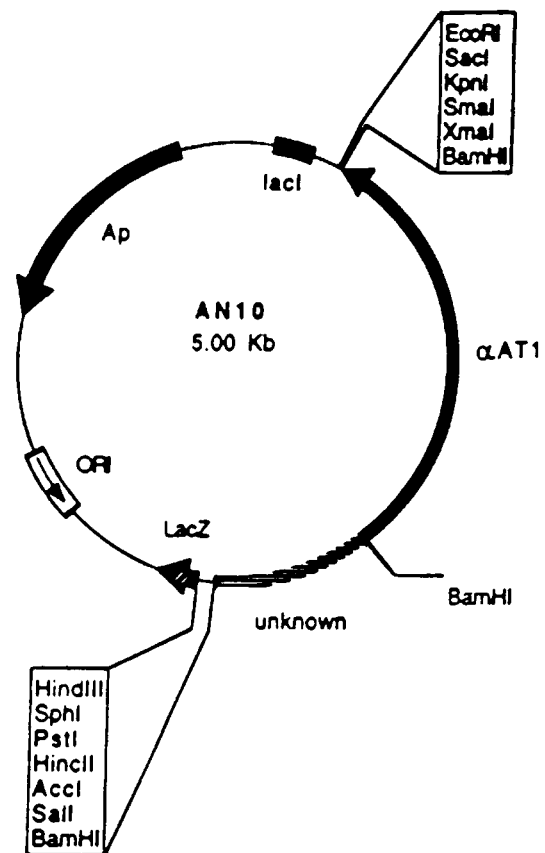


Fig. 1. Structure of plasmid AN10. The *Artemia*  $\alpha$ -tubulin cDNA,  $\alpha$ AT1, represented by the solid black arrow on the right side of the figure, was attached at its 5' end through a *Bam*HI site to a cDNA fragment of 600 bp, labelled 'unknown'. The arrow indicates the 5'-3' direction of the insert. The cDNA fragment was inserted into the *Bam*HI site of the pUC18 polylinker region, producing a plasmid of approximately 5.0 kb.

When probed with <sup>32</sup>P-labelled  $\alpha$ L1, the *Artemia* cDNA library obtained from L. Sastre yielded many cross-reactive colonies. Forty isolates were passed through a minimum of two additional screens and if all colonies on the final test plate gave a hybridization signal the size of the cDNA insert in the recombinant plasmid was determined. Twenty-four of the colonies, with plasmids containing inserts larger than 600 bp, were selected, and about 250 bp at both ends of each cDNA insert were determined. Comparison to sequences in the NCBI data base revealed that 22 of the isolates possessed  $\alpha$ -tubulin cDNAs with variant 5' ends due to size differences in the cloned fragments, but the same 3' end, including a poly(A) tail (data not shown). One clone, termed



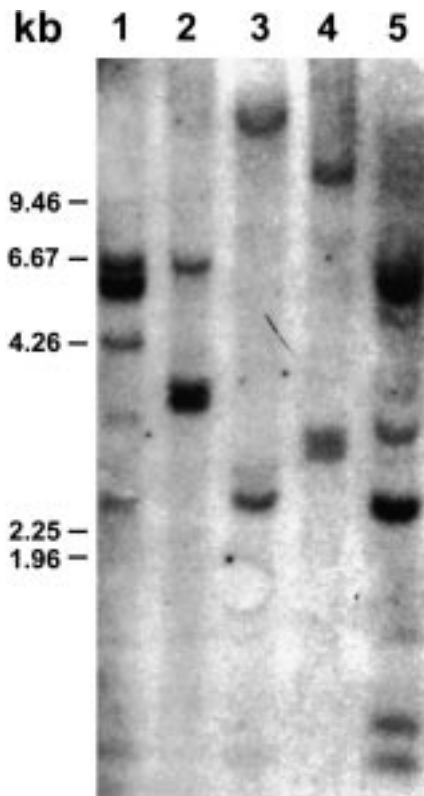


Fig. 4. Estimation of  $\alpha$ -tubulin gene number in *Artemia*.  $\alpha$ AT1 was recovered from AN10,  $^{32}$ P-labelled and hybridized to approximately 30  $\mu$ g of restriction digested *Artemia* DNA which had been electrophoresed in 1% agarose and blotted to nylon membranes. The restriction enzymes were: 1, *BstEII*; 2, *KpnI*; 3, *BglII*; 4, *SalI*; 5, *EcoRI*. Size markers in kb are on the left side of the figure.

tional acetylation. There was a potential GTP-binding site composed of residues 142–148 and 182–185.

Alignment analyses (Fig. 3) demonstrated that amino acid identities between *Artemia*  $\alpha$ -tubulin and the  $\alpha$ -tubulins from human (K00558, g340021), frog (Z31591, g468607), nematode (U65429, g1854669), fruit fly (M14643, g158731), and lobster (U41810, g1272240) were 94%, 87%, 91%, 93% and 96%, respectively. Nucleotide identities, presented in the same order as for amino acids, were 78%, 73%, 75%, 78%, and 81%. The most divergent region was the extreme carboxy-terminus beginning at residue 437 of  $\alpha$ AT1.

To examine  $\alpha$ -tubulin gene number, Southern blots of restriction enzyme digested *Artemia* DNA were hybridized to  $^{32}$ P-labelled  $\alpha$ AT1 (Fig. 4). Digestion by *BglII*, with one restriction site in  $\alpha$ At1, yielded two bands, whereas digestion with *KpnI*,

which also has one restriction site in  $\alpha$ AT1, gave three fragments, the lower two forming a tight doublet. Incubation with *SalI*, which has two sites in  $\alpha$ AT1, produced two bands, of which the lower is possibly a doublet. A 135 bp fragment generated by *SalI* digestion was not visible on the blots and must have run off the gel. Digestion with *EcoRI*, for which there are two sites in  $\alpha$ AT1 separated by 489 bp, provided five bands on Southern blots and *BstEII* also gave five bands, but there is only one restriction site in  $\alpha$ AT1 for this enzyme.

Membranes containing poly(A)<sup>+</sup> mRNA from dormant cysts, emerging nauplii, and instar I larvae were hybridized with  $^{32}$ P-labelled DNA fragments obtained by PCR amplification of AN10. Fragment pAT1200, which included most of the  $\alpha$ -tubulin coding region, yielded a single band of approximately 1.8 kb on autoradiograms (Fig. 5a). Band intensity for equal amounts of cytoplasmic poly(A)<sup>+</sup> mRNA from all three developmental stages was similar. For polysomal poly(A)<sup>+</sup> mRNA from dormant cysts and

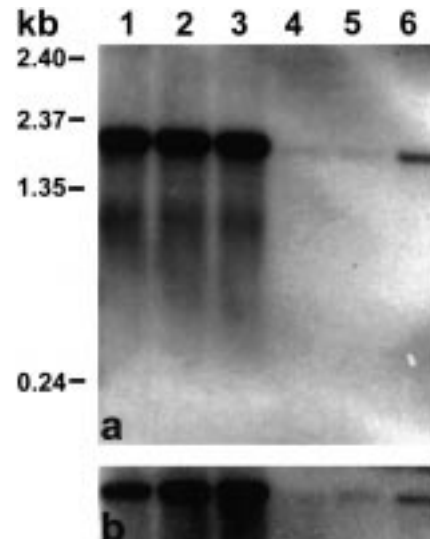


Fig. 5. Analyses of cytoplasmic and polysomal poly(A)<sup>+</sup> mRNA encoded by  $\alpha$ AT1. Equal amounts of poly(A)<sup>+</sup> mRNA from dormant cysts (lanes 1 and 4), emerging nauplii (lanes 2 and 5) and instar I larvae (lanes 3 and 6) were electrophoresed in 1.5% denaturing agarose gels, transferred to nylon membranes and hybridized with  $^{32}$ P-labelled probes. (a) Hybridization with pAT1200, a 1200 bp fragment of the  $\alpha$ AT1 coding region. (b) Hybridization with pATNC, a 141 bp probe amplified from the 3' noncoding region of  $\alpha$ AT1. 5  $\mu$ g of cytoplasmic and polysomal poly(A)<sup>+</sup> mRNA was loaded in lanes 1–3 and 4–6, respectively. Size markers in kb are on the left side of the figure.

emerging nauplii, hybridization to pAT1200 was very weak (Fig. 5a). The signal increased with mRNA from instar I larvae, although it was less than that for the cytoplasmic poly(A)<sup>+</sup> mRNA samples. Hybridization with pATNC, a 141 bp probe from the 3' noncoding region of  $\alpha$ AT1, gave the same pattern as pAT1200 (Fig. 5b). When repeated with other preparations of poly(A)<sup>+</sup> mRNA the results were the same.

$\alpha$ AT1 is the first  $\alpha$ -tubulin cDNA cloned from *Artemia*, and only the second from a crustacean, the previous coming from the lobster, *Homarus americanus* [31]. *Artemia*  $\alpha$ -tubulin is closely related to lobster  $\alpha$ -tubulin, sharing 96% and 81% identity, respectively, in amino acid and nucleotide sequences. The alignments also reveal that the *Artemia*  $\alpha$ -tubulin characterized in this study is very similar to  $\alpha$ -tubulins from other organisms. Residue 40 of the *Artemia*  $\alpha$ -tubulin is lysine, to which an acetyl group can be covalently attached posttranslationally at the  $\epsilon$ -amino moiety, thus producing acetylated tubulin [4,32,33]. The carboxy-terminal residue encoded by  $\alpha$ AT1 is tyrosine and the penultimate residue is glutamic acid, the usual situation for  $\alpha$ -tubulins [4,5]. Observation of these particular amino acid residues corroborates earlier results wherein acetylated and tyrosinated  $\alpha$ -tubulin were found in *Artemia* through immunological methods [21,23,29,34]. Additionally, in that we have not found a coding region lacking a terminal tyrosine codon, detyrosinated tubulin, which is developmentally regulated in *Artemia*, may arise posttranslationally. All 20  $\alpha$ -tubulin cDNAs sequenced were identical to  $\alpha$ AT1, other than for differences in lengths, suggesting that only one  $\alpha$ -tubulin gene encoding a carboxy-terminal tyrosine is expressed when detyrosinated tubulin first appears. However, the presence of a gene in *Artemia* that encodes detyrosinated  $\alpha$ -tubulin, although unlikely, cannot be ruled out.

In a previous study of *Artemia* tubulin, antibodies to tyrosinated (anti-Y) and detyrosinated (anti-E) tubulin were raised, respectively, to the peptides <sup>+</sup>H<sub>3</sub>N-GEEEGEEY-COO<sup>-</sup> and <sup>+</sup>H<sub>3</sub>N-GEEEGEE-COO<sup>-</sup> [21]. The corresponding sequence in the *Artemia*  $\alpha$ -tubulin characterized in this study is <sup>+</sup>H<sub>3</sub>N-EGEAGEEY-COO<sup>-</sup>. The four carboxy-terminal residues of the tyrosinated peptide are identical to those encoded by  $\alpha$ AT1. The remaining four residues are

less conserved, with only glutamic acid at position 6 from the carboxy-terminus conserved. If anti-Y recognizes the tubulin encoded by  $\alpha$ AT1, then antibody specificity depends on a limited number of amino acid residues and sequence degeneracy adjacent to the carboxy-terminus of  $\alpha$ -tubulin does not prevent recognition.

Hybridization of  $\alpha$ AT1 to Southern blots of restriction digested genomic DNA yielded relatively simple banding patterns, but because intron compositions are unknown, the number of  $\alpha$ -tubulin genes in *Artemia* could only be estimated. However, even though additional analyses of Southern blots and genomic cloning are required, the data indicate that *Artemia* have no more than three  $\alpha$ -tubulin genes. Possession of a relatively simple  $\alpha$ -tubulin family parallels the situation in lobster [31], the only other crustacean for which this determination has been made, and it verifies previous results for *Artemia* using a *Drosophila*  $\alpha$ -tubulin gene as probe [29]. The minor deviations in banding patterns between the two studies may arise either from sequence diversity in the probes which differently affects hybridization to  $\alpha$ -tubulin gene fragments on Southern blots, or to variations between *Artemia* populations.

$\alpha$ -Tubulin mRNA was abundant in all developmental stages of *Artemia* examined, and some of this mRNA corresponded to  $\alpha$ AT1, as shown by probing Northern blots with pATNC, a 3' noncoding sequence of 141 bp. Postdormancy development of cysts is characterized by resumption of protein synthesis [35], possibly dependent on the rapid activation of ribosomal S6 kinase [36] and/or synthesis of aminoacyl-tRNA synthetases [37]. Although the process requires formation of polysomes, presumably in part from stored mRNA, dormant cysts do exhibit polysomal  $\alpha$ AT1 at low abundance. This demonstrates that polysomes containing tubulin mRNA are present in *Artemia* embryos before they encyst, and that some of them survive the metabolic disruptions associated with encystment and diapause. Moreover, even though dormant cysts and emerging nauplii are well endowed with  $\alpha$ -tubulin mRNA, only a small fraction appears in polysomes before hatching. This exclusion from polysomes demonstrates that synthesis of  $\alpha$ -tubulin is translationally regulated as *Artemia* cysts develop and larvae emerge.

The expression of tubulin genes is regulated in other organisms, but the molecular mechanisms received little study [5]. One means of control may be differential stability of mRNAs [38], but the best characterized process is autoregulation of  $\beta$ -tubulin mRNA, requiring the tubulin amino-terminal residues, MREI [39]. The first three residues also appear in  $\alpha$ -tubulin from several species, including *Artemia*. Gonzalez-Garay and Cabral [40] describe a mechanism in which translation of  $\alpha$ -tubulin mRNA in Chinese hamster cells is modulated by free  $\alpha$ -tubulin. A related process could occur in *Artemia*, wherein there is sufficient tubulin to support early postgastrula development; mRNA is thus prevented from associating with ribosomes and its translation is inhibited.

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