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PCR amplification and cloning of metallothionein complementary DNAs in temperate and Antarctic sea urchin characterized by a large difference in egg metallothionein content

R. Scudiero[a], C. Capasso[b], V. Carginale[b], M. Riggio[b], A. Capasso[b], M. Ciaramella[b], S. Filosa[a] and E. Parisi[b,*]

[a]*Department of Evolutionary and Comparative Biology, University of Naples (Italy)*
[b]*CNR Institute of Protein Biochemistry and Enzymology, Via Marconi 10, I-80125 Naples (Italy),*
Fax +39 81 2396525, e-mail: parisi@iigbna.iigb.na.cnr.it
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Abstract. Metallothionein levels were determined in the eggs of two sea urchin species, the Mediterranean *Sphaerechinus granularis* and the Antarctic *Sterechinus neumayeri*. While appreciable levels of metallothionein were found in *S. granularis* eggs, a negligible amount was detected in *S. neumayeri*. Two metallothionein isoforms were purified from *S. granularis*, and metallothionein cDNAs were obtained by means of reverse transcriptase-polymerase chain reaction (RT-PCR). Two distinct cDNA species were cloned and sequenced. The translated amino acid sequences of these two forms consisted of 67 residues and differed in two amino acid substitutions. Despite the lack of metallothionein in *S. neumayeri* eggs, a metallothionein cDNA was obtained by RT-PCR amplification and a single amino acid sequence coding for a 63 residues MT was deduced. A comparative analysis of the primary structure of *S. granularis* and *S. neumayeri* metallothioneins with those of the other sea urchin metallothioneins has been performed. Sea urchin metallothioneins appear to be less similar to each other than metallothioneins of closely related vertebrates.
Key words. Metallothionein; cDNA; mRNA; primary structure; sea urchin; Antarctica.

Metallothioneins (MTs), low molecular weight cysteine-rich metal-binding proteins, are present in many phylogenetically diverse organisms [1]. These proteins have generally been considered to play a regulatory role in the homeostasis of zinc and copper [2–5], and to be involved in detoxification of non-essential highly toxic metals such as cadmium and mercury [6–9].

Owing to the ability of many invertebrates to accumulate toxic heavy metals, considerable interest has arisen in using marine and freshwater species for monitoring the pollution level in aquatic ecosystems [10–13]. In many aspects, such as cysteine content and lack of aromatic residues, invertebrate MTs resemble the vertebrate counterparts. An exception is provided by sea urchin MTs, which lack any homology to mammalian MTs [14]. Sea urchin MTs display the same type of metal-thiolate clusters present in mammalian MTs, which, however, are at different locations within the protein chain [15, 16].

In a previous work, we presented evidence for high variability in MT content in the eggs of different sea urchins [17]. In the present report, we describe the results of a comparative study on MTs in two sea urchin species, the Mediterranean echinoid *Sphaerechinus granularis* and the Antarctic *Sterechinus neumayeri*. Such a comparison is of interest as these two species, albeit related phylogenetically, live in entirely different natural habitats, and display distinctive developmental patterns.

* Corresponding author.
The cDNA sequences which we present in this report have been submitted to EMBL, and have the following accession numbers: *S. neumayeri* MT, Y08621; *S. granularis* MT-a, Z66530; *S. granularis* MT-b, Z67877.

Materials and methods

Collection of the eggs and preparation of acetone powder. Adult specimens of sea urchin *S. granularis* (Echinoida, Temnopleurina, Toxopneustidae) were obtained from the Zoological Station of Naples (Italy). Samples of *S. neumayeri* (Echinoida, Echinina, Echinidae) were collected at the McMurdo station (USA) in Antarctica. *S. granularis* eggs were obtained by gently shaking the dissected gonads in Millipore-filtered sea water. *S. neumayeri* eggs were obtained by injection of mature specimens with 0.5 M KCl. Eggs were washed three times with sea water and packed by centrifugation.

Packed eggs were homogenised in 5 volumes of acetone prechilled at −20 °C. The homogenate was filtered on a Buchner funnel using Whatman 3MM filter paper. The resulting residue was extracted five times in the same way, dried and stored at 4 °C.

Determination of MT content. One hundred mg of acetone powder prepared from 2.3×10^7 eggs was homogenised in 2 ml of 0.25 M sucrose. Samples were centrifuged for 30 min at 15,000 g. MT content in 0.5 ml supernatant was determined by silver saturation assay [18].

Gel-permeation chromatography of Ag-MT. For the analysis of Ag-MT, 100 mg of acetone powder was processed exactly as described in the section for the

determination of MT content. One ml of supernatant was fractionated on a Sephadex G-75 column (0.9×30 cm) equilibrated with Tris-HCl 20 mM, pH 8.6. The column was eluted with the same buffer at a flow rate of 1 ml/min. Fractions of 0.6 ml were analyzed for silver content.

Purification and sequencing of MT from *S. granularis*. MT was purified from 3.5×10^9 *Sphaerechinus granularis* eggs following a previously described procedure [19]. S-pyridylethylated MT samples were subjected to automated Edman degradation on a pulsed phase sequencer Applied Biosystems, model 120A, as described [19].

Isolation of RNA. Packed eggs obtained from mature females were immediately frozen in liquid nitrogen, and then ground into a fine powder in a mortar. RNA was extracted from 100 mg of this powder using an RNA extraction kit (Tri-Reagent, Molecular Research Center). The final RNA pellet was dissolved in H_2O and the total RNA concentration was determined by measuring absorbance at 260 nm.

Amplification of MT-specific cDNA. First strand cDNA was produced from 0.25–5 µg of total RNA using reverse transcriptase and the oligo(dT)-adaptor primer CGGAGATCTCCAATGTGATGGGAATTC$(T)_{17}$ as a primer. Reverse transcription was performed for 2 h at 42 °C, followed by heat inactivation of the enzyme for 5 min at 65 °C. PCR was performed with the single-stranded cDNA using as primers the 29-mer CCAGGACCAGACGTCAAGTG(C/T)GTITG(C/T)TG (where I = inosine) derived from the N-terminal amino acid sequence of *S. granularis* MT and designed on the basis of best codon usage for sea urchin [20], and the adaptor-primer described above. Denaturation, annealing and chain extension were performed using a program consisting of 30 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C, concluding with a period of 15 min at 72 °C.

Rapid amplification of 5′-cDNA ends (5′-RACE). A RACE protocol for PCR amplification was carried out with the 5′-Marathon cDNA amplification kit of Clontech Laboratories. Single-stranded cDNA from *S. neumayeri* was obtained as described above. The second strand synthesis was performed according to the Marathon kit protocol. The resulting double-stranded cDNA was ligated with the Marathon Adaptor primer overnight at 16 °C by T4 DNA ligase. PCR amplification was performed using the AP1 primer and the *S. neumayeri* MT-specific primer (5′-GCAACATTCTCCAGTTGTACAGCA-3′); the Marathon Adaptor primer and the AP1 primer were provided by the kit manufacturer. The conditions for PCR reaction were essentially identical to those described in the Marathon kit protocol.

Cloning and sequencing of PCR-amplified cDNA. The PCR fragments were purified using the QIAQUICK gel extraction kit (Qiagen). After purification, the PCR-amplified cDNA and the pGEM-T vector (Promega) were ligated with T4 DNA ligase. *Escherichia coli* (strain TG2) cells were transformed with the ligation mixture. Each plasmid DNA was denatured and the cloned cDNA fragment sequenced on both strands by the dideoxy method [21] using the T7 sequencing kit (Pharmacia).

Table 1. Quantification of MT content in sea urchin eggs.

	pg MT/egg
S. granularis	2.09 ± 0.04
S. neumayeri	<0.01

MT content was determined by silver saturation assay. Quantification of MT was performed on the basis of standard curve obtained with rabbit MT. Assays were run in triplicate, and results are measured ±S.E.M.

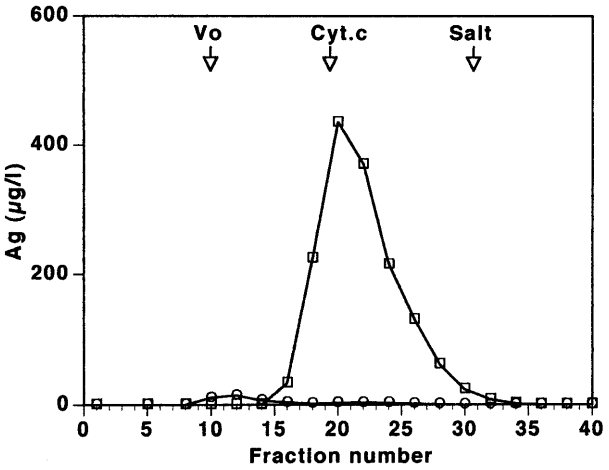


Figure 1. Gel-permeation chromatography of Ag-MT. Extracts from *S. granularis* (□–□) and *S. neumayeri* (○–○) eggs were treated with Ag^+ and chromatographed on a Sephadex G-75 column as described under Methods. The eluates were monitored for silver content.

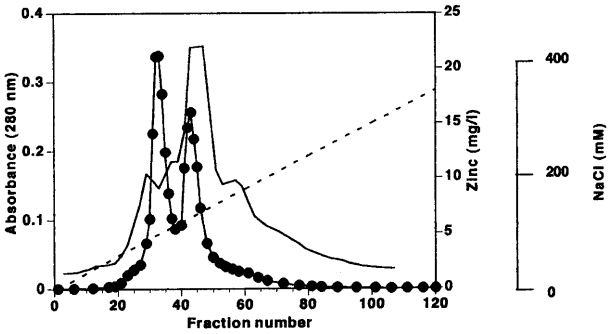


Figure 2. Anion-exchange chromatography of *S. granularis* metallothionein. The low molecular weight zinc-containing peak eluted from a Sephadex G-75 column was applied to a DEAE-cellulose column equilibrated with 20 mM Tris-HCl pH 8.6 containing 2 mM DTT. The column (1.6×25 cm) was eluted with a linear gradient of NaCl (0–400 mM) in equilibration buffer. Fractions of 3 ml were collected at a flow rate of 3 ml/min. The eluate was monitored for zinc content. Absorbance (——); zinc (●–●), electric conductivity (– – –).

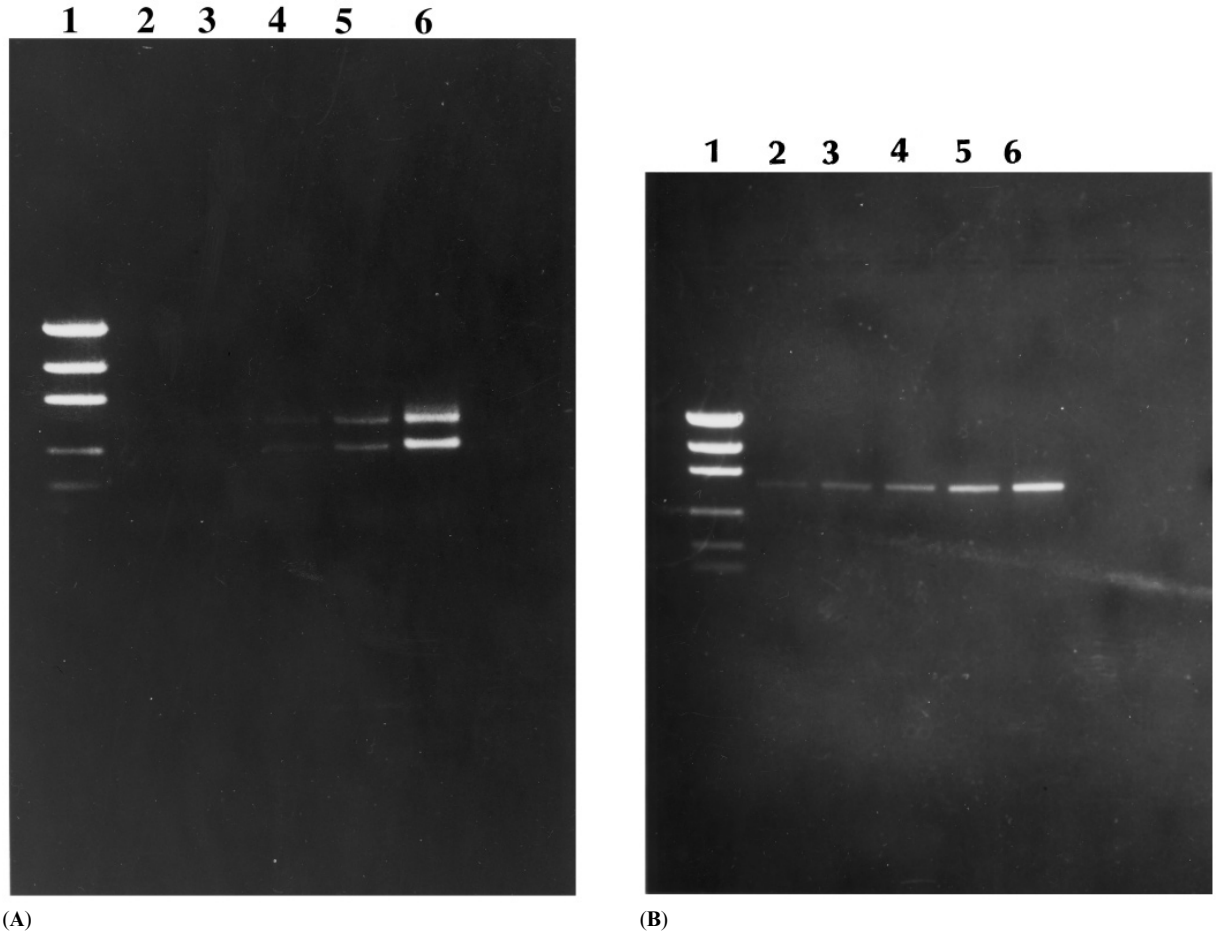
Other methods. The net charge of MT sequences deduced from cDNAs was estimated on the basis of the sum of positive and negative charges of non-cysteine residues plus the six negative charges of the cysteine-zinc complex [15].

Metal content was determined by a Perkin-Elmer 5100 atomic absorption spectrophotometer, equipped with a Zeeman graphite furnace. Amino acid analyses were performed on an Carlo Erba automatic analyzer model 3A30. Cysteine was determined as cysteic acid after performic acid oxidation [22]. Following oxidation, samples were hydrolyzed under nitrogen in 6 N HCl for 1 h at 150 °C. Recovery was determined by means of norleucine added as internal standard to all samples. Tricine-SDS-PAGE was performed according to Schägger and von Jagow [23]. The gel (6 × 8 cm; 16.5% T, 3% C) was run for 3 h at 90 V and stained with Coomassie Brilliant Blue R-250.

Results

MT content in *S. granularis and S. neumayeri* eggs. The MT levels in unfertilized eggs were determined by silver saturation assay. The results in table 1 show that in *S. granularis* the amount of MT is more than two hundred-fold the amount detected in *S. neumayeri*. These results were validated by gel-filtration chromatographic analyses carried out on silver-treated egg extracts. The elution profile of *S. granularis* extract shows a peak of silver with an M_r value of about 12,000, typical of MT. Such a peak was not found in the eluates of the *S. neumayeri* extract (fig. 1).

Isolation of MT from *S. granularis* eggs. A low molecular weight Zn-containing protein was isolated from an *S. granularis* extract fractionated on a Sephadex G-75 column. This Zn-containing protein was further resolved into two components by anion-exchange



(A) (B)

Figure 3. Electrophoresis of RT-PCR products. RT-PCR was carried out as described under Methods. The products were analyzed on a 1.2% agarose gel stained with ethidium bromide. (*A*) Lane 1: DNA ladder (2,000; 1,000; 800; 400; 200 bp); lane 2–6: RT-PCR products obtained with 10, 15, 25, 50, 250 µg/ml RNA from *S. granularis* eggs. (*B*) Lane 1: DNA ladder (2,000; 1,000; 800; 400; 200; 100 bp); lane 2–6: RT-PCR products obtained with 10, 15, 25, 50, 250 µg/ml RNA from *S. neumayeri* eggs.

```
          1                                                  50
pSg-400   CCAGGACCAG ACGTCAAGTG CGTGTGCTGC CAAGATGGCA AAGAGTGTCC
pSg-600   CCAGGACCAG ACGTCAAGTG CGTGTGCTGC CAAGATGGCG AAGAGTGTCC
pSn-600   ......CCTG ATGTCAAGTG TGTCTGTTGC AAAGAGGGTA AGGAGTGTGC
          51                                                 100
pSg-400   TTGCAAGGGT GGGGAATGCT GCATTACAGG ATCATGCTGT AAGGAAGGAG
pSg-600   TTGCAAGGGT GGGGAATGCT GCATTACAGG AACATGCTGT AAGGAAGGAG
pSn-600   CTGCAAGGGA AAAGAGTGCT GTACAACTGG AGAATGTTGC AAG......G
          101                                                150
pSg-400   ATGGACTATG CTGTGGAAAA TGTTCAAATG CTGCATGTAA GTGTGCCGAT
pSg-600   ATGGACTATG CTGTGGAAAA TGTTCAAATG CTGCATGTAA GTGTGCCGAT
pSn-600   ACGGAACCTG CTGTGGAAAA TGCACAAATG CTGCATGCAA GTGTGCTGAT
          151                                                200
pSg-400   GGCTGCAAAT GTGGAAGTGG ATGTTCGTGT ACTTTGGGAA ACTGTACATG
pSg-600   GGCTGCAAAT GTGGAAGTGG ATGTTCGTGT ACTTTGGGAA ACTGCACATG
pSn-600   GGCTGCAAAT GTGGAAGCGG GTGCTCGTGC ACTGAGGGAA ACTGTGCATG
          201                                                250
pSg-400   CTAGA..TCA GCAAG..... .......... .......... ..........
pSg-600   CTAGC..TGC ACAGTCTGG. ACTATCAAGG CCTTCTTCGA TAGAGTCAGA
pSn-600   CTAGACTTTA GCAAGGCCCT TATCCACTGG GATGAAAGAT TGTATATACA
          251                                                300
pSg-400   .......... .......... .......... .......... ..........
pSg-600   CCTAAAGTCA T......... .......... .CGATCACTT ATGTAATTGA
pSn-600   GCTGAAGAAG TCGGTCAACA ATGCTGCCAA CGTGCTCCAA GCAGACATGA
          301                                                350
pSg-400   .......... .......... .......... .......... ..........
pSg-600   TCGACGAC.T CACCTGCACA TATAGATATG ACTTTATGTA TTATTTGTAA
pSn-600   TGTTATACAT TATCAATATG ACGTCAAATA TTTGTTTGTA ATATAAATTG
          351                                                400
pSg-400   .......... .......... .......... .......... ..........
pSg-600   CATAATGTAT TATTTGTTTA TATAAAAAAG TTAAACTTAT TAAAAAAAAA
pSn-600   TAAAAAACAG ATTAAATTAA TCGTCATTCT ATTTTTTAGA AAAGTAAAAC
          401                                                450
pSg-400   .....GCCCT TTCTTCCGGA TAGAAAGATT GAACATAC.. .AACCAAAGA
pSg-600   ACCATGAAAT TGAGGCATTT CAAAGGTACT TAAAATGCAG AAACACTTGA
pSn-600   AATAAGAGCT TTAATAGTAT AATACTTAAA TTGCAAGGGT CTATGCCTAT
          451                                                500
pSg-400   AGTCAGTCAT CCATCGATCT CTTATGCAAA TTTGCTCC.. ..GAGCAGAC
pSg-600   AGTAAGGCTG GCCCGTACCT CTTATTTCAT TTGGAATTAT TCTTTCGGTG
pSn-600   TTTAATTCTG TCTGATTTGT AACTGCCCTT TTGAGACTTT CTTGATTTTT
          501                                                550
pSg-400   TTCACAAGCA CATTATAAAT ATGACTTCAT ATATTATTTG TAACATAATG
pSg-600   TTTTCTTTGC CTACCTTGAT ATCAAGCCAT ATGTGAGCAT TGACAGACTC
pSn-600   TTTCTGTTTC TAATTTCTAT CAAGTTGTCA TTATCTGGAT TGACAAAGCT
          551          579
pSg-400   TATATTTGTT TTATAT.... .........
pSg-600   ATAAAACAAG TATGATGCTA AAAAAATTT
pSn-600   CATGAATTAA ACCTAAATAA TCCT.....
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Figure 4. Nucleotide sequences of MT cDNAs from *S. granularis* and *S. neumayeri*. Nucleotide sequences were determined by double-strand sequencing. The sequences were aligned by means of the program PileUp (GCG-Wisconsin). The coding sequence is underlined.

chromatography (fig. 2). Each of these two peaks was further purified by HPLC on a C_{18} reverse-phase column and characterized by SDS-PAGE and amino acid composition. SDS-PAGE showed the presence of a single band of about 6 kDa in both fractions. The results of the amino acid analysis showed a high cysteine content (about 30%) and absence of histidine, arginine, methionine and aromatic residues.

N-terminal sequence of S-pyridylethylated MTs. The sequence of the first 24 residues from the N-terminal of the two MT isoforms, termed MTa and MTb on the basis of the order of elution from the DEAE-cellulose column, was obtained by automatic sequence analysis. The amino-terminal sequence of MTa is PGPDVKCVCCQDGKECPCKGGECC; that of MTb is PGPDVKCVCCQDGEECPCKGGECC. They differ in a single amino acid substitution at position 14 (K → E).

PCR amplification of MT-specific cDNA. Total RNA extracted from the eggs of each species was reverse-transcribed as described in Methods. The resulting ss-cDNA was amplified by PCR and the product was electrophoresed on agarose gel. The pictures of the ethidium bromide-stained gels show the presence of two bands of 400 and 600 bp for *S. granularis* (fig. 3A), and a single 600 bp band for *S. neumayeri* (fig. 3B). These cDNAs, hereafter denoted pSg-400, pSg-600 and pSn-600, were cloned into a pGEM-T sequencing vector.

cDNA sequences. Four *E. coli* colonies containing pSg-400, five containing pSg-600 and five containing pSn-600, were grown for plasmid sequencing. All the clones derived from the same cDNA gave identical sequences. The sequence of the 5′-terminus of the *S. neumayeri* cDNA was determined by 5′-RACE as described in Methods. The full nucleotide sequences of pSg-400, pSg-600 and pSn-600 are reported in figure 4; they show large differences at the level of 3′-untranslated regions (3′-UTRs) preceding the polyA stretch. The two cDNAs from *S. granularis* encode proteins made of 67 amino acid residues, 20 of them being cysteines; the two coding sequences differ only in two amino acid substitutions at positions 14 and 28. By comparing these two sequences with the amino terminal sequences of proteins, pSg-400 should correspond to MTa and pSg-600 to MTb. The protein sequence deduced by pSg-400, identified as MTa, has an estimated net charge of −6, whilst the sequence deduced from pSg-600, identified as MTb, has a net charge of −8. Such a difference in charge may account for the behaviour of the proteins on DEAE-cellulose described in figure 2. The *S. neumayeri* cDNA encodes for an MT made of 63 amino acid residues. In figure 5 the amino acid sequences of *S. granularis* and *S. neumayeri* MTs are reported, deduced from the nucleotide sequences reported in figure 4 together with other available sea urchin MT sequences, i.e. the two MT isoforms from *P. lividus* [19], the two *S. purpuratus* MT isoforms [24], and the MT from *Litechinus pictus* [25].

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S. purpuratus MT1    --PDVKCVCCTEGNECACFGQDCCVTGKCCKDG--TCCGICTNAACK-CANGCKCGSGCSCTEGNCAC
S. neumayeri MT      --PDVKCVCCKEGKECACKGKECCTTGECCKDG--TCCGKCTNAACK-CADGCKCGSGCSCTEGNCAC
S. purpuratus MT2    --PDVKCVCCKEGNECACTGQDCCTIGKCCKDG--TCCGKCSNAACKTCANGCTCGSGCSCTEGNCPC
P. lividus MT1       --PDTKCVCCQDGKQCPCAGQECCITGKCCKDGASVCCGTCSNAACK-CTDGCKCEGGCQCTEGNCTC
P. lividus MT2       --PDTKCVCCQDGKQCPCAGQECCITGKCCKDGASVCCGTCSNAACK-CTDGCKCEGGCVCTEGNCTC
S. granularis MTa    PGPDVKCVCCQDGKECPCKGGECCITGSCCKEGDGLCCGKCSNAACK-CADGCKCGSGCSCTLGNCTC
S. granularis MTb    PGPDVKCVCCQDGEECPCKGGECCITGTCCKEGDGLCCGKCSNAACK-CADGCKCGSGCSCTLGNCTC
L. pictus MT         PGPDVKCFCCQDGKQCACGGGECCITGKCCQEGDGTCCGKCSNAACK-CADGCKCEGACACTMGNCTC
                       ** ** **  *  * * *  **  * **  *  *** * ***** *  ** *   * ** *** *
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Figure 5. Sequence comparison of metallothionein from different sea urchin species. Primary sequences of *S. granularis* and *S. neumayeri* MTs were deduced from the coding regions of the nucleotide sequences reported in figure 4. The sequences were aligned by means of program PileUP (GCG-Wisconsin). The asterisks indicate identity between the sequences.

Discussion

Although metallothioneins have been the subject of a large number of investigations both in vertebrate and invertebrate species, they have been less extensively studied in echinoderms [14–17, 19, 24–27].

In the present report, we provide evidence for large differences in MT content in the eggs of two sea urchin species, *S. granularis* and *S. neumayeri*. In the Mediterranean species a high MT content was detected, whilst the MT level in the Antarctic species was negligible. It is worth noting that such a marked difference in MT content is not dependent on the phylogenetic position of the species examined: in fact, *S. neumayeri* belongs to the same family of *P. lividus*, in which the MT level was found to be comparable to *S. granularis* [17]. On the basis of the present results, however, it is not possible to establish whether such a difference in MT content reflects a similar difference in MT mRNA level. Nevertheless, the fact that in both species comparable transcript amplifications could be obtained by using the same scalar amounts of RNA in the PCR reactions indicates that the expression of the MT gene in *S. neumayeri* is not impaired.

The absence of an appreciable amount of MT in the egg of the Antarctic sea urchin may be explained by the developmental pattern of this species in comparison to the species living in temperate habitats. It is known, in fact, that the development of the *S. neumayeri* egg is much slower than that of other sea urchin species: the hatching of the blastula occurs approximately 6 days after fertilization, and the pluteus stage is reached in about three weeks [28]. Conversely, in *S. granularis*, full development to the larval stage takes about three days. Clearly, to cope with such a fast development a good deal of nutrients, enzymes and mRNAs must be initially stored in the cytoplasm of the unfertilized eggs during oogenesis. In *S. granularis*, about 80% of the total zinc present in the egg is bound to MT (unpublished observations), and it is probable that MT constitutes the main zinc depot of the developing embryo. A large amount of zinc is expected to be required for the metabolic processes occurring during the cell divisions which take place after fertilization. In the *S. neumayeri* egg, characterized by a slower development, such a bulk amount of maternal MT is probably not necessary, a hypothesis which is supported by the observation that other sea urchins living in cold habitats, such as *Strongylocentrotus intermedius*, lack appreciable levels of MT [27].

A comparison of the metallothionein sequences derived from different sea urchin species shows the existence of some differences. The most remarkable is the insertion/deletion of one or two residues in the amino acid sequences. In the two *S. granularis* MTs and in the *L. pictus* MT, the first two residues are P and G, which have not been found in the MTs of the other sea urchin species. In addition, *S. granularis*, *L. pictus* and *P. lividus* show two residues at positions 35 and 36 which are lacking in *S. purpuratus* and *S. neumayeri* MTs. From this it can be deduced that, among sea urchins, MTs are more variable than in different classes of vertebrates.

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