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# Hsp56 mRNA in *Paracentrotus lividus* embryos binds to a mitochondrial protein

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**Abstract**

We previously demonstrated that *Paracentrotus lividus* Hsp56 mitochondrial chaperonin is constitutively expressed during development, that it has a specific territorial distribution, both in normal and heat-shocked embryos, and that its amount increases after heat shock [Roccheri MC, Patti M, Agnello M, Gianguzza F, Carra E, Rinaldi AM. Localization of mitochondrial Hsp56 chaperonin during sea urchin development. Biochem Biophys Res Commun 2001;287:1093–98] and cadmium treatment [Roccheri MC, Agnello M, Boneventura R, Matranga V. Cadmium induces the expression of specific stress proteins in sea urchin embryos. Biochem Biophys Res Commun 2004;321:80–7]. In this study, we looked at Hsp56 mRNA during normal development and under stress conditions. The messenger is almost constantly expressed at all stages of development and its amount is steadily increased in stressed embryos. Moreover, we found, using T1 RNase protection assay, that the most proximal region of the 3′-UTR of the Hsp56 mRNA binds a 40 kDa protein: this factor is more abundant in the mitochondrial extract and, more specifically, in the outer membrane of the organelle.


*Keywords:* Cadmium; Chaperonin; Embryo development; Heat shock; Mitochondria

## 1. Introduction

Heat-shock proteins (Hsps) have an essential role in most organisms, participating in many basic processes, such as protein folding (Ellis and Hartl, 1999). Hsps expression is activated by different stress conditions and Hsps activity probably counteracts apoptosis pathways (Beere, 2004). Mitochondrial Hsp60 chaperonin was studied in different organisms and shown to be involved in the folding of proteins imported into the mitochondrial matrix, assembly of protein complexes when required, and sorting of proteins to different locations in mitochondria, favoring the transport of imported proteins across membranes (Soltys and Gupta, 1999). Marine invertebrates are directly exposed to ever-changing environmental conditions and the induction of Hsps may constitute an important defense mechanism.

As shown by sequence similarity at protein as well as at mRNA level, *Paracentrotus lividus* mitochondrial Hsp56 is the homologue of vertebrate Hsp60 chaperonin and, given the evolutionary conservation of the sequence, it is believed to perform similar functions in sea urchin embryos.

In a previous study, we showed that: (i) Hsp56 is constitutively expressed at all stages of sea urchin development, (ii) in heat-shocked embryos, the chaperonin concentration increases, as shown by Western blot analysis and *in situ* (whole mount) immunoreaction, and (iii) its presence becomes evident also in the ectodermal layer (Roccheri et al., 2001). As previously reported, sublethal cadmium concentrations also have a clear effect on Hsp56 expression (Roccheri et al., 2004). In this paper, we analyzed the Hsp56 mRNA content, both during normal sea urchin development and in embryos that underwent heat-shock or cadmium treatment. Moreover, we identified an RNA-binding protein, more abundant in the outer mitochondrial membrane, that interacts with the Hsp56 mRNA 3′-UTR.

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## 2. Materials and methods

### 2.1. Embryo cultures

Embryo cultures were carried out as described by Roccheri et al. (2001).

### 2.2. Protein extracts

Cell fractionation was carried out as described elsewhere (Cannino et al., 2004). Preparation of mitochondrial sub-fractions was carried out essentially as described by Schnaitman and Greenawalt (1968). Protein concentration was calculated by the method of Bradford (1976).

### 2.3. Western analyses

Western blotting was performed as described by Cannino et al. (2004). Protein markers were from Fermentas, polyclonal anti-rabbit Mn-SOD antibodies were from Stressgen and polyclonal anti-rabbit GAPDH antibodies were from Santa Cruz Biotechnologies. Goat anti-rabbit secondary antibodies were from Stressgen.

### 2.4. Northern analyses

Total RNA was prepared from *P. lividus* eggs or embryos at different stages of development, by the method of Chomczynski and Sacchi (1987). Northern analyses were performed according to Castiglia et al. (1994). DNA fragments used to make probes for hybridizations were radiolabeled using the "random primed DNA labeling kit" purchased from Roche, following the manufacturer's instructions. Autoradiographs were scanned and planimetries of scans were used to calculate the relative abundance of mRNAs using the ImageJ program.

### 2.5. Preparation of in vitro transcribed RNAs for T1 RNase assay

Different regions of the *P. lividus* Hsp56 cDNA (Gianguzza et al., 2000; Genbank accession no. AJ249625) were amplified by PCR and cloned between KpnI and EcoR1 or HindIII sites of Bluescript (+) SK plasmid. The oligonucleotides used for PCR amplification were: 5′-GCGC*GGTACC*TGAGGTGC TTATGAACCCCA-3′ forward and 5′-GCGCGGTTC*GAATTC*CCCTGACTT TTCCCC-3′ reverse (fragment 1906–2413); 5′-GCGC*GGTACC*GGCACGA GCTCAGCCGTA-3′ forward and 5′-GCGCGG*GAATTC*TCTCCTGCCTCCT CGTTG-3′ reverse (fragment 54–510); 5′-GCGC*GGTACC*GTTTTGATAA GGAGCAGAAC-3′ forward and 5′-GCGCGG*AAGCTT*TGAATGAAGTGC TGATAGAT-3′ reverse (fragment 2932–3422). The cloned fragments were *in vitro* transcribed using Riboprobe Sistem-T7 (Promega). The PEP-19 unlabeled RNA was prepared by using as a template a PEP-19 cDNA cloned by Sala et al. (2007) and identical to the one reported by Sangameswaran et al. (1989; EMBL accession number: M24852).

### 2.6. T1 RNase protection assay

T1 RNase protection assay was carried out according to the method described by Zaidi and Malter (1994), as modified by Izquierdo and Cuezva (1997). Briefly, cell extracts (15 μg of total proteins) were incubated for 10 min, at 30 °C, with $1 \times 10^6$ cpm (specific activity: $1–2 \times 10^7$ cpm/pmol of RNA) of *in vitro* transcribed, radiolabeled RNA. Samples were then incubated for 30 min at 37 °C, with 100 units of T1 RNase (Roche) to degrade the whole of the RNA except the regions protected by bound proteins; the extracts were finally exposed to a spectroline UV (254 nm) lamp (Aldrich Chemical Co., Inc.), for 20 min at 4 °C, to cross-link RNA to proteins. The covalent radioactive complexes were analyzed by denaturing electrophoresis on sodiumdodecylsulfate (SDS)-polyacrylamide slab gels (PAGE), according to Laemmli (1970) and the gels were directly exposed to X-ray film for autoradiography, with intensifying screens, for 12–18 h, at −70 °C. At the end of exposure, the gels were stained with Coomassie brilliant Blue R-250 (Sigma) to confirm the loading of equal amounts of proteins per lane.

## 3. Results

First, we analyzed by Northern analysis total RNA from sea urchin *P. lividus* eggs, and embryos at different stages of development, using as a probe the $^{32}$P-labeled cDNA encoding the *P. lividus* Hsp56, previously isolated (Gianguzza et al., 2000; Genbank accession no. AJ249625).

As shown in Fig. 1, upper panel, the probe identifies a unique band (about 4000 nt) in all the samples: Hsp56 messenger appears to be almost constantly expressed during embryonic development, from fertilization up to pluteus stage. In order to be sure that identical amounts of RNA had been loaded per lane, the membranes were stripped and re-hybridized with a $^{32}$P-labeled fragment of the gene encoding the 18S *P. lividus* rRNA (a gift from Dr. R. Barbieri, Fig. 1, lower panel).

We previously showed by Western blot (Roccheri et al., 2001; Roccheri et al., 2004) that Hsp56 protein is more abundant in *P. lividus* gastrulae grown in stress conditions. To analyze the changes in Hsp56 mRNA expression under stress conditions, normal, heat-shocked and cadmium-treated embryos were grown until gastrula or pluteus stage, and total RNAs were prepared and analyzed by Northern blot.

The results of one representative experiment are shown in Fig. 2A (upper panels): the mRNA concentration sharply increases in both heat-shocked gastrulae (lane 2) and in cadmium-treated gastrulae (lane 3), when compared with control gastrulae (lane 1). An increase was also evident when plutei under stress conditions (lanes 5 and 6) were compared with control plutei (lane 4).

In order to be sure that identical amounts of RNA had been loaded per lane, the membranes were stripped and re-hybridized with a $^{32}$P-labeled fragment of the gene encoding the *P. lividus* 26S rRNA (a gift from Dr. R. Barbieri, Fig. 2A, lower panels). A graphic representation of the results of three (gastrulae) and five (plutei) independent experiments is shown

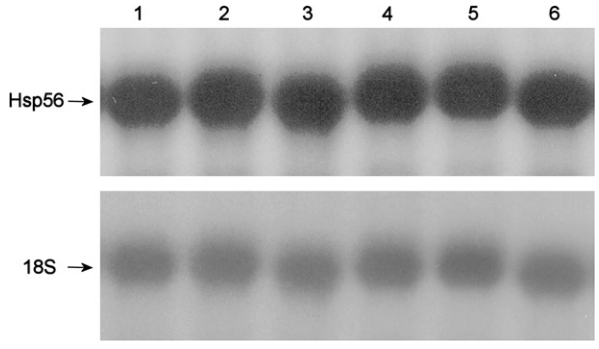


Fig. 1. Northern blot analysis of *P. lividus* Hsp56 mRNA during development. Total RNA from eggs, and embryos at different stages of development, hybridized to Hsp56 probe (upper panel), or 18S rRNA probe (lower panel): (1) unfertilized egg; (2) four blastomeres; (3) 16 blastomeres; (4) blastula; (5) gastrula; and (6) pluteus.