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Molecular Characterization of the First Heat Shock Protein 70 from a Reef Coral

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The branching coral *Stylophora pistillata*, one of the most abundant hermatypic corals along the coasts of the Red Sea, has been used for many years as a model species for coral biological studies. Here we characterize the first coral heat shock protein 70 gene (SP-HSP70), cloned from *S. pistillata*, to be used as a tool for studying coral stress response. The cloning was carried out by a combination of PCR methods using heterologous, degenerate HSP70-based primers, followed by plaque-lift screening of a genomic library. The sequenced clone (5212 bp), contains a complete 1953 bp, intronless open reading frame, and 5′ and 3′ flanking regions of 1,935 and 1,324 bp, respectively. TATA, CAAT, and ATF boxes as well as 11 putative heat shock elements were identified in the SP-HSP70 5′ flanking region. A polyadenylation site was identified in the 3′ flanking region. SP-HSP70 protein sequence resembles the cytosolic/nuclear HSP70 cluster. RT-PCR studies confirmed SP-HSP70 mRNA expression in corals grown within their normal physiological conditions. Furthermore, SP-HSP70 has been shown to belong to the coral genome and not to its symbiotic algae one, as revealed by SP-HSP70 PCR amplification, using purified algal and coral DNA templates.

Key Words: **HSP70; corals; Gulf of Eilat; *Stylophora pistillata*.**

Members of the phylum Cnidaria which contain $>10^4$ contemporary species are primarily marine organisms that inhabit a wide variety of habitats, from the tropics to the arctic and from shallow waters down to the ocean's depths. Scleractinian (reef building) corals, a major cnidarian group, are key members of coral reef communities, in which they form massive structures that thrive in environmentally unpredictable waters. A central environmental issues in the context of global climate change is the susceptibility of scleractinian corals to temperature changes, UV radiation and other natural and anthropogenic stress inducers. Such perturbations are probably the causes for the recent worldwide documentation of coral bleaching (the loss of symbiotic algae) and mortality (1–3). Cellular stress responses protect organisms from damage following exposure to stressors. Therefore, understanding these responses in corals and evaluating their protective limits, would assist the explanation of the stress-related biological phenomena.

Stress responses entail rapid synthesis of a highly-conserved proteins, the heat-shock proteins 70 (HSP70s), which are found in diverse organisms from bacteria to mammals (4–6), found in every species in which they have been sought including aquatic organisms (7, 8). HSP70s are induced by stressful factors such as extremes of temperatures, cellular energy depletion, UV radiation, presence of heavy metals and amino acid analogs and others. Their gene expression is also cell cycle dependent, serum responsive, and is modulated by several oncogenes. Other HSP70s, designated heat shock cognates are constitutively expressed (reviewed in 8–11). A major mechanism of HSP70 stress-related transcription induction, operates through binding of regulatory proteins, the trimeric heat shock factors (HSFs) to HSP70 5′ flanking heat shock elements (HSEs), located upstream of the TATA box (9, 12). Heat-, metal-, or amino acid analog transcription induction of HSP70s is all mediated via the HSF-HSE route (10). HSP70s stress-related response is understandable in view of their cellular function as protein chaperones, involved in proper folding or unfolding of nascent polypeptides, proteins translocated through membranes or denatured proteins. HSP70s contain a peptide binding domain which is attached to the chaperoned protein, preventing malfolding and is

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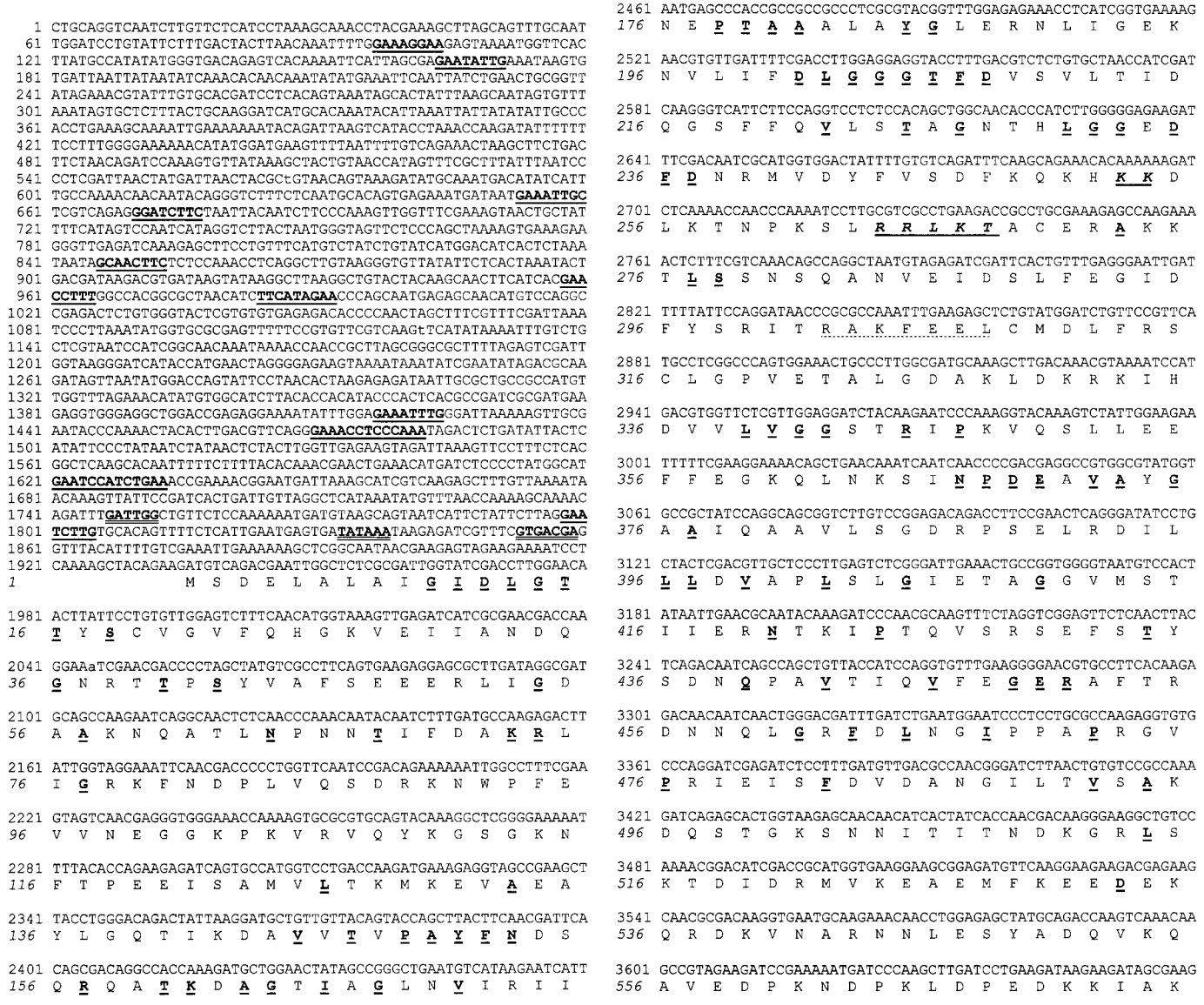

FIG. 1. The sequence of SP-HSP70 and its flanking regions. Promoter transcriptional elements are double underlined. Putative HSEs at the 5′ UTR region are underlined. Consensus amino acids according to Boorstein *et al.* (1994) are underlined, putative NLS is italicized and underlined and the nonorganellar eukaryotic consensus (Rensing and Maier, 1994) is dotted underlined. The polyadenylation site at the 3′ UTR is double underlined.

released through ATPase activity carried out by their ATPase domain (13–16).

Eukaryotic HSP70 sequences are divided into four clusters, corresponding to their intracellular localization, cytosolic/nuclear HSP70s, endoplasmic reticulum (ER) residents, termed also glucose-regulated proteins 78 (GRP78), and mitochondrial group. Plants HSP70 fourth group is located in the chloroplast (5).

Cnidarian HSP70 genes were cloned and characterized only from two *Hydra* species (17, 18). In both cases they were heat-induced, although expressing different mRNA half-life patterns and consequent difference in the level of HSP70 protein expression. However, HSP proteins were detected in other cnidarians following heat shock, by PAGE-SDS, and Western blot using heterologous HSP antibodies. The examined species include a scyphozoan jellyfish (19), sea anemones (20, 21), hermatypic corals (21–24), and symbiotic algae (zooxanthellae) residing within a variety of cnidarians (25).

Not much is known of the cellular-biochemical bases of the cnidarians stress-related biological responses (26), and studying the stress-related function of coral HSP70 may contribute to our understanding of the coral environmental physiology (7, 8).

Our target species is the common Red Sea hermatypic coral *Stylophora pistillata* (a branching species),

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3661 AACAAGGCCCAAGAAGTGATTGCTTGGTTGGAACGCAATCAAGAAGCTGAAAAAGAGGAG
576  N  K  A  Q  E  V  I  A  W  L  E  R  N  Q  E  A  E  K  E  E

3721 TATGAAGAAAAGCAAAGAGATCTACAGCAAGTGTGTTCACCAATCATGTCCAAGGTAAAA
596  Y  E  E  K  Q  R  D  L  Q  Q  V  C  S  P  I  M  S  K  V  K

3781 CAACAGGAGACCCCGGAaCAaGGGCGGGGAAACAGCTGTGGCAATGGACCTTCGAGTGCA
616  Q  Q  E  T  P  E  Q  G  R  G  N  S  C  G  N  G  P  S  S  A

3841 ATGCCTGCCCCTAACAAATATAATTCTAAAATCGAAGAAATTGATTGACTGAACGCTCCT
636  M  P  A  P  N  K  Y  N  S  K  I  E  E  I  D  *  -        650

3901 AAGGAGTGTTATTATTTGGCATAGTCTAGTTGGCATAGACTTTAAAAATATTTTAAATTA
3961 GTTTTTGATAGTTAGTTTTAGATCAAATCACGAACTATAGTGGGAGATTGGATAACAAAC
4021 ATTATGAATAACTAAGGTTTGGAAAATTTCTTTTTGTAGTACAAGAAACGATTTTGTTTT
4081 TAATTTAGGTTTCTCGAATCTAGTTTCGCCTATCGGGCATGTTATTCGTGTCACGCATAG
4141 CGACTCGTTGCATATGTTTTCAGTTTCCATTAAAGGTAGTTTGACACCAAACTTCTTTCG
4201 TGATCTGTGAGATATATTGAAAGAGGTGCCAAGAAGGTTAGTAGTGGGAAAGATCTTCGA
4261 TGATGTAAAATAAAACCAATCAACACATGAATATATGAGAATACATCAAAAAAACATATT
4321 CATTTCGAGCGTTACCAAAACTTTGAAGAAAACTATTTTACTTTCCACTATCCTCAAATT
4381 GATTGGATGAATCGACCTTCATTTTCGCTGCGTATATGACGCACTTTCCTTTTAACAAGT
4441 AGAGCGTGAGTATAAGCGTGACTGGGCGTGATTCACTGATGTAAGCGCACATAGCCATAT
4501 GGGCTGTGCGATCTACTGCTTCTGCTTCTGAAAGAAATCCTCACTTGTGTTGCGTTTGTA
4561 AGTACAGTCCCGAGTTGTAAGATCAAATGTTTATAATGGAACCTGGACTGGACCTAATTT
4621 ACTTTCCAACATAGAGGGATAGGGTAAACCTAAAAATAATCTGAGGCGCTGATTGACCTT
4681 CTGAGCGTCAGCTTTCGCTTGTAGTGTCACGGTGACTTACGATGTAAACAACCCTCCAAA
4741 GTTCGGACTTTGGTTTCACCCACGTGTACGAACTTGTAGTTGCTATTTCAGATTTCCGTT
4801 CCCATAGCTGGTTTCTTCCATGTCTTTAAAATTCGAATATCGAATAGAACATCTGTTCTT
4861 ACCCACTTACTGTACGTGGTCGGACCACGAGTCTCATATATTGGTAAAAAAAAAACACCC
4921 GAAAGTTCGGACCCTATATATGCGGCTTTAACACTCCTTAAAAAGAAATTCGAATATCGA
4981 AAAGAAGATCATAAGGGTCAAAATGTCGCTTCAGAAGAACACTAGCGACTTCAAGGCTAA
5041 GAATGTGGCAGAACTGTTGAAATGTCTTGTCGTTGTTATTTCCTAGTTCAAAAATAGAAG
5101 GAAGCCTTCCGAAATATTTACTATCCTTTCAGAATAGTAAACATGAGTTTAAGGGAGTTT
5161 GATGCTAGATTGTACCCGTAATTTATTTTTGGTACACCATGGACTGCTGCAG  - 5212
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FIG. 1—*Continued*

one of the most abundant coral species in the Gulf of Eilat, found in shallow waters down to at least 60 m (27), and used for almost 30 years as a model coral species (28). An intimate knowledge was accumulated in regard to its life history patterns, physiology, inter-intraspecific interaction, reproductive activities, growth rates, senescence and more, which make it a preferred experimental species.

This study is aimed at revealing the molecular characterization of its HSP70, an essential tool for future cellular, biochemical and physiological studies of stress response in corals.

MATERIALS AND METHODS

General methods and sample collection. General molecular methods, agarose and agarose-formaldehyde gel electrophoresis, DNA purification from the gel and from λ phages, DNA ^{32}P random priming, Southern hybridization, plasmid manipulations, DNA phenolation, ethanol precipitation, and plaque-lift library screening were carried out according to (29) or by using commercial kits. Sequencing was carried out using the ABI-Prism Big Dye sequencing kit (Applied Biosystems). Sequence analysis and comparison to gene databases was performed by the Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin.

Collection of material was carried out in the coral reef, in front of the Marine Biological Laboratory at Eilat. Coral branches were pruned underwater, at a depth of 5–6 m, by side cutters. Fragments were washed with seawater several times to eliminate excess mucus, and placed in plastic containers. Coral planulae larvae were collected from gravid colonies *in situ* as described (30). Collected material was placed in an isolated container and transported to the National Institute of Oceanography in Haifa.

Purification of high-molecular-weight DNA and RNA. *S. pistillata* high molecular weight DNA of whole animal (coral and algal DNA) was isolated by high molecular weight DNA purification kit (G-nome DNA kit, Bio101, Vista, California) according to the manufacturer instructions. Chopped coral branch tips (0.0.5–1 cm) were incubated in the lysis solution, during the first purification step, to permit lysis of the soft coral tissue overlying the calcareous skeleton. The isolated DNA was further purified by phenolation and ethanol precipitation. High molecular weight DNA was also purified from isolated symbiotic algae cells, and from algae-free coral homogenate, as followed: batches of ~200 *S. pistillata* planulae were manually homogenized on ice, in 1 ml seawater, by glass-Teflon homogenizer, and were centrifuged for 2 min at 4°C, 12,000 g. The supernatant, which contained only the animal cell DNA, was immediately mixed with the relevant components of the G-nome DNA kit, and DNA purification proceeded according to the manufacturer instructions. The pellet which contained intact algae, and also broken algal and coral cells, was resuspended in 1 ml seawater, brought to 0.1% Triton-X-100 and incubated for 10 min at room temperature. Following incubation, the clean, intact algal cells were precipitated, the supernatant was discarded, and the cells were washed twice in seawater. Algal DNA was purified from the isolated cells using the G-nome DNA kit. DNAs integrity was verified on 1% TAE-agarose gel (Fig. 2).

Total RNA was isolated from *S. pistillata* fragments by the TriReagent isolation kit (Molecular Research Center, Inc. U.S.A.), following the manufacturer instructions. The first step was modified to permit lysis of the soft tissue overlying the calcareous skeleton, by shaking the 0.5–1 cm chopped coral branch tips in the TriReagent solution for one hour at room temperature. RNA integrity was verified on 1% agarose-formaldehyde gel.

PCR procedures—General. PCRs were carried out on PTC-100 thermal cycler, NJ Research; or Robocycler, Stratagene. The reactions were usually performed in a volume of 25 μl PCR solution, containing adequate amount of template DNA, 5 pmol of each specific primer or 50 pmol of each degenerate primer, 0.2 mM dNTP mix, and 1.25 units of *Taq* polymerase.

Cloning of S. pistillata HSP70 partial gene. A 990 bp *S. pistillata* HSP70 (SP-HSP70) partial gene (designated clone 18) was cloned using PCR-related methodologies, in two stages:

A 683 bp fragment (designated clone 5) was cloned using a set of *Hydra* HSP70 primers (Gellner *et al.*, 1992) (forward primer: GGATCCGGNA CNACNTATTC NTGTGT; reverse primer: CTGCAGGAAA TCTTCACCGC CAAG) and 1 μg of *S. pistillata* high molecular weight DNA template. A second fragment (clone 9; 917 bp) partly overlapping clone 5, was amplified using forward primer designed according the clone 5 sequence (GTCTCTGTGCTAACCATCG) and a reverse degenerate primer, that was designed using a conserved HSP70 sequence (CCYTTRTCRT TNGTDATNG). The final probe, clone 18 (990 bp) was amplified by using designed primers in accordance with the sequences of the former two fragments (forward-GACCCCCTGG TTCAATCCGAC, reverse-CCCACCGGCA GTTTCAATCC C), and was used to screen the library.

The thermal cycler was programmed during the cloning procedures to 35 cycles of 94°C (1 min), 50–55°C (1 min), and 72°C (2 min) followed by 15 min at 72°C. The resulting products were visualized on a 1% TAE-agarose gel, isolated from the gel, cloned into pGEM-T-easy vector (Promega) and sequenced.

Construction and screening of S. pistillata genomic library. A genomic library of a 8–22 kb insert range was constructed by a commercial company (Clontech, California) in λ-DASH II vector (Stratagene) from partially MboI digested high molecular weight DNA, separated on a sucrose gradient.

The genomic library was screened for HSP70 gene by the plaque-lift method using the ^{32}P-labeled clone 18. The cloned phage was amplified in MRA(P2) *E. coli* strain (Stratagene), its DNA was purified, and digested by PstI. The resulting fragments were electrophoresed and hybridized to a labeled clone 18. The only labeled fragment

TABLE 1

SP-HSP70 Protein Sequence Comparisons, with Representative Eukaryotic HSP70s Localized in the Cytosol (Cyt.), Mitochondria (MT), Chloroplast (Chloro.), and Endoplasmic Reticulum (ER)

Species	Accession No.	Subcellular localization	Identity to SPHSP70 [%]	Similarity to SPHSP70 [%]
Rattus norvegicus	Q07439	Cyt.	69	77
Anopheles albuminus	P41827	Cyt.	69	78
Hydra magnipapillata	Q05944	Cyt.	68	77
Saccharomyces cerevisiae	P10591	Cyt.	65	75
Mus musculus	P38647	MT	48	58
Pisum sativum	P37900	MT	48	57
Pisum sativum	Q02028	Chloro.	49	57
Porphyra purpurea	P30723	Chloro.	49	60
Drosophila melanogaster	L01498	ER	59	69
Homo sapiens	P11021	ER	59	69

was cloned into pBluescript plasmid, (designated clone 27) and gradually sequenced, using vector primers and primers designed according to already sequenced parts of the insert.

Expression of SP-HSP70 transcription. HSP70 mRNA expression was examined by RT-PCR using total RNA as template. Reverse transcription (RT) was performed in two steps: a volume of 16.75 μl aqueous solution containing 100 pmol of the reverse primer CAACACGTTC TTTTCACCG (designated HSP4R) and 3 μg RNA was heated to 70°C for 5 min and chilled on ice. RT was carried out in 25 μl solution, after adding to the primer-RNA solution: 5 μl of 5× reverse transcriptase buffer (Promega), 1.25 μl of 10 mM dNTP mix, 25 units of rRNasin, and 200 units of MMLV-reverse transcriptase (Promega). The RT reaction was carried out for one hour at 42°C followed by 3 min at 94°C. Two μl of the RT solution served as PCR template. PCR amplification of the RT products was designed for 35 cycles at 94°C (1 min), 60°C (1 min), and 72°C (1 min) per cycle, preceded by 3 min at 94°C and followed by 5 min at 72°C, using the reverse primer HSP4R and the forward primer HSP4F (ATGGTAAAGT TGAGATCATC GC).

Genomic origin of the SP-HSP70. The purified algal and coral DNAs were served as PCR templates to identify the presence of SP-HSP70 gene, using the primer pair HSP4F/R. The PCR designed to 35 cycles at 94°C (1 min), 60°C (1 min), and 72°C (1 min) per cycle, preceded by 3 min at 94°C and followed by 5 min at 72°C.

RESULTS

Clone 27 (5212 bp) contains the entire sequence of the SP-HSP70 gene (Fig. 1), 1,935 bp in the 5′ flanking region, 1,324 bp in the 3′ flanking region and a 1,953 bp complete intronless open reading frame (ORF), including the stop codon. The characterization of the ORF amino acid sequence was accomplished by its comparison to amino acid sequences of other HSP70s, and was found to be most similar to the cytosolic/nuclear HSP70s (sampled cases in Table 1). Therefore, the SP-HSP70 amino acid sequence NSKIEEID-stop codon (amino acids 643-650, Fig. 1) which resembles the carboxy terminal signature of cytosolic/nuclear HSP70 (GP(T/K)(V/I)EEVD-stop codon; (4)), was assumed to be the stop codon site. The translation start site was identified by SP-HSP70 comparison to related cytosolic/nuclear HSP70s. Both sites defined a protein of 650 amino acids. Two additional amino acid motifs indicate the cytosolic/nuclear assignment of SP-HSP70. They are the RA · FEEL (dotted underlined; amino acids 303-309, Fig. 1), typical of non organellar eukaryotic HSP70s, and the bipartite nuclear localization signal (KK and RRLKT; amino acids 253–254 and 265–269, respectively, Fig. 1) required for nuclear targeting of cytosolic/nuclear HSP70s (5). There is a complete identity between the HSP70 conserved amino acids described by Boorstein *et al.* (4), and the present sequence (separately underlined amino acids, Fig. 1). The lack of the motif GPKH between amino acids 295-296, typical of prokaryotic HSP70s (6), render unlikely the possibility of false prokaryotic cloning.

TATA, CAAT and ATF boxes, basic elements of the transcription machinery, can be recognized in the 5′ flanking region (Fig. 1). HSEs in HSP70 promoters, are usually composed of varying numbers of the conserved 5 bp motif, 5′-GAA-3′, organized in contiguous arrays, where each unit is inverted relative to the immediately flanking units (31, 32). Using this pattern, 11 putative HSE motifs, most of them incomplete, were identified in the SP-HSP70 5′ flanking region (Fig. 1). In contrast, GRP-related regulatory domains (33) were not recognized. Comparison of the SP-HSP70 5′ flanking region to the consensus metal response element (MRE) identified in mammalian metallothionein gene (34), revealed almost complete similarity with the consensus (TGCRCNC), lacking only the 3′ C, in 6 locations between bases 256–1806 of the SP-HSP70. A putative polyadenylation site was identified in the 3′ flanking region (Fig. 1).

Constitutive expression of SP-HSP70 mRNA was shown by RT-PCR, using RNA preparations from five different *S. pistillata* colonies, maintained in their physiological conditions. The PCR results were run on 1% TAE-agarose gel, transferred to a charged nylon

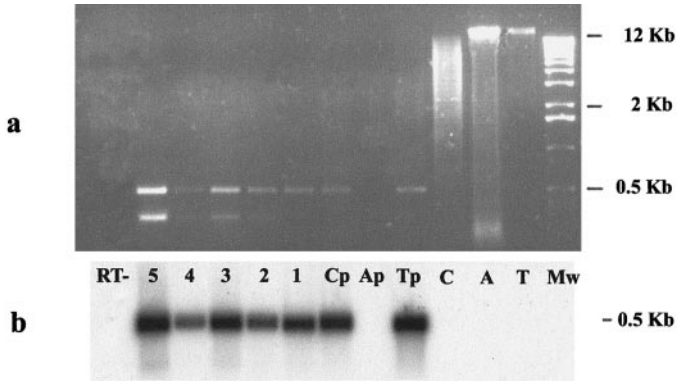

FIG. 2. 1% TEA-Agarose gel (a) and its hybridization to ^{32}P-labelled clone 5 probe (b). Mw, molecular weight marker; T, total *S. pistillata* DNA (algae + coral); A, algal DNA; C, coraligenous DNA. Tp, Ap, Cp—PCR products using the corresponding DNAs as templates; 1–5—RT-PCR products of different total RNA preparations; (RT-)—control PCR product aimed at identifying DNA contamination. HSP4F/R were used as PCR primers in all the reactions, and the PCR design was: one cycle at 94°C (3 min), 35 cycles of 94°C (1 min), 60°C (1 min), and 72°C (1 min), and final incubation at 72°C (5 min).

membrane, hybridized to a labeled clone 5 and washed under stringent conditions (0.1% SCC, 0.1% SDS, 65°C, 1 hour). The results are presented in Fig. 2, revealing expression in all colonies. An additional 300 bp PCR product was evident in some of the preparations (Fig. 2a), despite the high PCR annealing temperature. This band showed no affinity to clone 5. RT(−) controls revealed no HSP70 PCR products in most cases and faint response in others, indicating variability in the purity of the RNA preparations (only one result is shown; Fig. 2).

The genomic origin of the SP-HSP70 was detected by a PCR, using isolated coral and algal DNAs as templates, revealing coraligenous origin of the cloned gene (Fig. 2). The coral high molecular weight DNA appeared more degraded than the algal or the DNA of the whole animal, probably due to the few minutes elapsed between the planulae homogenization and the addition of the protective components of the purification kit (see Materials and Methods).

DISCUSSION AND CONCLUSIONS

The study presents the first characterized coral HSP70 gene. The described gene is a typical cytosolic/nuclear HSP70, as indicated by the cytosolic signature at its ORF 3′ terminus (4), the NLS required for nuclear translocation and an additional signature of a non organellar HSP70. Prokaryotic origin of the gene is negated by the absence of the 4 bp prokaryotic signature (5). Comparison of the SP-HSP70 to the cnidarian *Hydra* HSP70 revealed no higher similarity in comparison to other cytosolic/nuclear eukaryotic HSP70s (Table 1), which can be explained by the high degree of evolutionary conservation of this gene. TATA, CAAT, GC rich, activating transcription factor (ATF), and AP-2 motifs, which participate in modes of regulation other than heat induction were recognized in upstream regulatory domains of various HSP70s, and are required for maximal stress-induced HSP70 transcription (10). Part of these domains, TATA, CAAT and ATF motifs, were recognized also in SP-HSP70 5′ flanking region (Fig. 1). ATF may indicate cAMP involvement in the transcription regulation of this gene (35).

Binding of a trimeric HSF to a trimeric HSE is the accepted HSF-HSE binding model. The binding is cooperative, namely, HSF binding to a HSE trimer is facilitated by adjacent bound HSFs. It was shown also that HSE effect is synergistic, and higher induction is attained when more HSE arrays are present (31, 32). Most of the 11 putative HSEs identified in the 5′ flanking region of the SP-HSP70 gene do not form trimers and are slightly different from the consensus motif. In addition, the furthest ones from the TATA box are less likely to be functional HSEs. Bienz and Pelham (9) compared HSEs location from several HSP70 promoters, and found them up to 426 bp from the TATA box, and this range includes only four of the SP-HSP70 putative HSEs. It has to be emphasized that few HSE repeats can be recognized even in HSC70s which express weak stress induction response. All this evidence indicates an apparent weak stress-related SP-HSP70 induction via the HSE-HSF route, which have to be examined by direct induction experiments in future studies.

It is evident that studying the biochemical basis for biological response to stressors, would improve our ability to analyze the effects of environmental perturbations and their combinatory outcomes. The characterization of SP-HSP70, the first stress-related gene from a coral, and the demonstration of its mRNA expression by RT-PCR, may provide useful molecular tools to reach that goal.

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