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Adaptive evolution of the heat-shock response in the Antarctic psychrophilic ciliate, *Euplotes focardii*: hints from a comparative determination of the *hsp70* gene structure

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Abstract: The Antarctic psychrophilic ciliate *Euplotes focardii* manifests a dramatic reduction in the activation of its *hsp70* gene in response to a heat-shock, while oxidative and chemical stresses activate the transcription of this gene to appreciable extents. To investigate the genetic causes of this eccentric behaviour of *E. focardii* in the hsp70 gene transcription activation, we carried out a comparative structural analysis of this gene between *E. focardii* and another Antarctic *Euplotes*, *E. nobilii*, which manifests a psychrotrophic behaviour and an inducible thermal response. No substantial difference was detected in the organization of the hsp70 5' promoter region, both species bearing canonical regulatory *cis*-acting elements deputed to bind transcriptional *trans*-activating factors. Adenine-rich elements favouring mRNA degradation were instead detected in the hsp70 3' regulatory region of *E. nobilii*, but not in that of *E. focardii*. These observations lend further support to the hypothesis that the causes of the *Euplotes focardii* unresponsiveness to thermal stress resides in some structural, or functional modifications of transcriptional *trans*-activating factors.

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Key words: ciliates, *cis*- and *trans*-regulatory elements, cold-adaptation, extremophiles, stress-inducible genes

Introduction

Antarctic waters host a rich variety of micro-eukaryotes, of which ciliated Protozoa represent a major component in terms of biomass and species number (Petz *et al.* 1995, Wilbert & Song 2005, Petz 2005). Particularly common are species of the most cosmopolitan and ubiquitous ciliate, *Euplotes* (Borror & Hill 1995). Easy to collect, in the laboratory they expand into massive cultures that reproduce true to type indefinitely under controlled conditions, and thus represent optimal organisms to investigate, at reduced levels of complexity, relevant aspects of the genetic basis of adaptation of the eukaryotic life to Antarctica. Of a set of *Euplotes* species isolated from the coast of Terra Nova Bay in the Ross Sea (Valbonesi & Luporini 1990, 1993, unpublished results), *E. focardii* showed a closer adaptation to cold than did the other species. This presumably depended on a remote colonization of Antarctica that comparative rDNA sequence analysis with sub-Antarctic *Euplotes* species would probably date before the definitive ecological separation of Antarctica from Gondwana (F. Dini & G. Di Giuseppe, personal communication 2006). Indeed, *E. focardii* thrives optimally at a temperature of 4–5°C typical of psychrophiles (Valbonesi & Luporini 1993), utilizes unique genetic solutions to ensure microtubule stability (Pucciarelli *et al.* 1997, Pucciarelli & Miceli 2002) and, of more interest in this context, is no longer able to appreciably enhance the transcription of its *hsp70* gene, i.e. the gene encoding the heat shock protein 70, in response to a thermal stress (La Terza *et al.* 2000, 2001). Nevertheless, this gene is represented by thousands of sub-chromosomic copies in the somatic (expressed) genome of the macronucleus and its transcription is undertaken at appreciable levels in cells subjected to an oxidative stress, or exposed to noxious chemicals such as tributyltin and sodium arsenite (La Terza *et al.* 2004).

To look into the molecular basis of this eccentricity in the stress-induction of *E. focardii hsp70* gene expression, we carried out a comparative analysis of the full-length structures between this gene and its homolog cloned from another Antarctic *Euplotes*, *E. nobilii*. This behaves like a psychrotrophic rather than a psychrophilic organism, and readily enhances its *hsp70* gene transcription in response to heat shock, as well as oxidative and chemical stresses (our unpublished observations). The 5' promoter region showed canonical (*cis*-acting) binding sites of transcriptional (*trans*-acting) activators in the *hsp70* gene of both species. Instead, only in the *hsp70* 3' region of *E. nobilii* did we detect adenine-rich elements, known to be responsible for enhanced mRNA decay rates in a variety of genes regulating cell response to environmental stimuli (Chen & Shyu 1995, Wilusz & Wilusz 2004). The absence in *E. focardii* of these elements thus weakens the hypothesis of a causal association between the *E. focardii* unresponsiveness to heat shock and an *hsp70* mRNA instability, while it reinforces the hypothesis that the cause(s) reside at the transcriptional level, probably in a mutated structure and/or activity of *trans*-acting transcriptional activators.

Materials and methods

Cells

Cultures of the wild-type strains TN-1 of *E. focardii* and AC-6 of *E. nobilii* were used. They were grown in a cold room, at 4°C, on the green alga *Dunaliella tertiolecta*.

Chemicals

Routine reagents were from Sigma-Aldrich (Milan, Italy); DNA-modifying and restriction enzymes, Proteinase K, Rnase A, protease inhibitors, [α32 P] dATP and [γ32P] ATP from Amersham Pharmacia Biotech (Cologno Monzese, Milan). Oligonucleotides were synthesized by Labtek Eurobio (Milan). Sources of other materials are indicated below, where appropriate.

DNA purification

Total DNA was purified from cells lysed by overnight incubation at 55°C in one volume of NDS (0.5 M EDTA, 1% SDS, 10 mM Tris-HCl, pH 9.5) containing 200 μg ml^{-1} of proteinase K. The lysate was extracted twice with a 1:1 (v/v) mixture of phenol and chloroform, and once with chloroform, and then incubated for 30 min with Rnase A at a concentration of 50 μg ml^{-1} at 37°C. Purified DNA was extensively dialysed against 1 mM EDTA and 10 mM Tris-HCl, pH 8, on Type-VS membranes of 0.025 μm pore size (Millipore, Bedford, MA), before being used.

Polymerase chain reaction for rapid amplification of telomeric ends (RATE-PCR)

This RATE-PCR strategy is specific to clone the *Euplotes* sub-chromosomic macronuclear genes, characterized as small molecules (in the range from 500 to 20 000 bp) and the presence of conserved tandem repeats C_4A_4 at their telomeric extremities (Hoffman *et al*. 1995). The *hsp70* gene sequence at the 5' end was obtained by subjecting macronuclear DNA samples of *E. focardii* and *E. nobilii* to a first set of 30–35 cycles of amplification. This amplification used, as reverse primer, the oligonucleotide 5'-GCATCIATATCIAAIGTIACITCIAT-3' (where I stands for Inosine used in relation with degenerated triplets) specific for the HSP70 motif Q_{481}IEVTFDID$_{489}$ and, as forward primer, the telomeric oligonucleotide 5'-$(C_4A_4)_4$-3'. The sequence at the 3'end was then obtained by a second set of 30–35 amplification cycles using, as forward primer, the oligonucleotide 5'-AAIGATCAAGGIAAIAGAACICC-3' specific for the HSP70 motif N_{31}DQGNRTTP$_{39}$ and again, as reverse primer, the telomeric oligonucleotide 5'-$(C_4A_4)_4$-3'. The two full-length sequences were eventually reconstructed by overlapping the sequences of the cloned PCR products, and their uniqueness was confirmed by direct sequence analysis of PCR products obtained using two additional oligonucleotides spanning the sequence segment immediately adjacent to the 5' and 3' telomeric repeats of the cloned molecules.

Gene cloning, DNA labeling, screening, and sequencing

Amplified products were cloned into the pCR 2.1-TOPO vector of the TOPO TA cloning Kit (Invitrogen) following the procedures suggested by the supplier. Colony blotting and double strand DNA labeling by the random priming method were performed according to Sambrook & Russell (2001). Hybridization signals were detected by means of a personal Molecular Imager FX (Bio-Rad). Sequence reactions were carried out with the ABI Prism sequence analyzer, Model 373A, by using the Big Dye Terminator Methodology (PE Applied Biosystems). Sequence alignments and structure modeling were performed using Clustal W (Thompson *et al*. 1994) and the Swiss-PdBViewer (Guex & Peitsch 1997).

GeneBank database

The complete sequences of the *E. focardii* and *E. nobilii hsp70* genes are available at the National Centre for Biotechnology Information (NCBI) with the accession numbers AY295877 and DQ866998, respectively. The HSP70 sequences used to generate the HSP70 consensus sequence and their accession numbers at the NCBI are from: *Euplotes crassus* AJ344550, *Euplotes eurystomus* L15292 and L15291, *Stylonychia lemnae* AF227962, *Oxytricha nova* U37280, *Saccharomyces cerevisiae* AAC04952, *Rattus norvegicus* AAA17441, *Sus scrofa* CAA48295, *Bos taurus* AAA73914, *Escherichia coli* DnaK BAA01595.

Results and discussion

Basic features of the coding regions of the E. focardii and E. nobilii of hsp70 genes

The full-length sequences of the *E. focardii* and *E. nobilii hsp70* genes, of 2506 and 2279 bp respectively, were in each case reconstructed by overlapping the sequences (one containing the gene 5' end and, the other, the 3' end) of two amplification products of macronuclear DNA preparations utilized in a RATE-PCR strategy based on two independent PCR reactions (Seegmiller *et al.* 1996). In these reactions, the same oligonucleotide specific for the telomeric ends of the sub-chromosomic macronuclear genes of *Euplotes* was alternatively used, as forward and reverse primer, in combination with either one of two oligonucleotides specific for the two universally conserved HSP70 sequence stretches N_{31}DQGNRTTP$_{39}$, and Q_{481}IEVTFDID$_{489}$ (numerations according to the *Drosophila melanogaster* HSP70 sequence). The determined sequences did not contain introns and bear open reading frames: in *E. focardii*,

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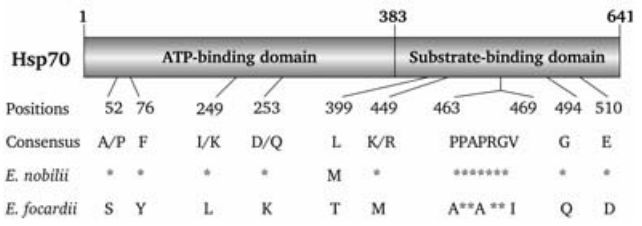

Fig. 1. Amino acid substitutions that occur in *E. focardii* at the level of its HSP70 ATP- and substrate-binding domains, and are unique with respect to *E. nobilii* and other organisms. Numbers of the amino acid positions are reported essentially according to Sriram *et al.* (1997) and Morshauser *et al*. (1999). Asterisks indicate identities. The consensus sequence was produced from an alignment of HSP70 sequences available at the NCBI GeneBank database.

of 1983 bp specific for a protein of 660 amino acids; in *E. nobilii*, of 1980 bp specific for a protein of 659 amino acids. The HSP70 protein of *E. focardii* has a calculated molecular mass of 71.8 KDa and a theoretical pI of 4.8; in *E. nobilii*, it has a calculated molecular mass of 71.6 KDa and a theoretical pI of 4.9.

The degree of HSP70 sequence identity between *E. focardii* and *E. nobilii* is 84.5% and, as shown in Fig. 1, it is only the *E. focardii* HSP70 sequence that bears potentially significant amino acid substitutions at the level of its two major functional domains, i.e. the ATP-binding and substrate-binding domains. Four substitutions in particular, i.e. M for K/R449, A for P463 and P466, and I for V469 (numeration according to the rat HSP70 sequence), appear of more immediate interest as they lie, as shown in Fig. 2, in the two loops that delimit the substrate-binding pocket. These loops are in fact directly involved in the conformational changes induced by ATP hydrolysis and needed for the HSP70 chaperonic activity (Sriram *et al.* 1997, Morshauser *et al.* 1999). Less clear is the functional relevance of the other substitutions, mostly falling in the ATP-binding domain, that reveals a strict conservation of all its functional key residues for ATP hydrolysis and ATP/ADP conversion (Zhang & Zuiderweg 2004).

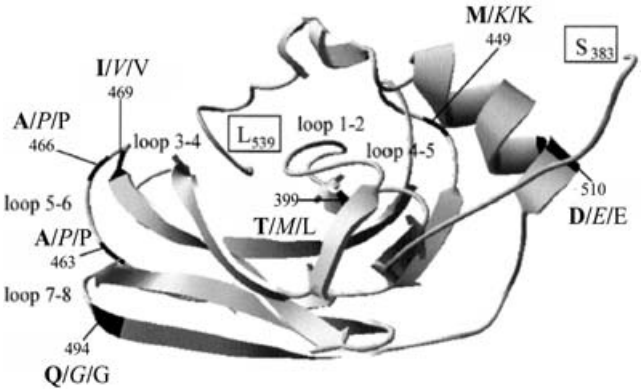

Fig. 2. Molecular architecture of the HSP70 substrate-binding domain showing the positions of the amino acid substitutions that distinguish this domain in *E. focardii* with respect to *E. nobilii* and other organisms. Amino acid positions and loops are numbered according to the rat HSP70 structure represented by Morshauser *et al.* (1999). Of each triplet of amino acids, the first one (bold) is present in the HSP70 sequence of *E. focardii*, the second one (italics) in *E. nobilii*, and the third one (normal style) in other organisms. The two amino acid residues delimiting the substrate binding domain are enclosed in boxes.

Structure of the non-coding regions

The coding regions of the *hsp70 E. focardii* and *E. nobilii* genes are flanked by short 5'-leader and 3'-trailer non-coding regions, ending with blocks of inverted 5' C_4A_4 3'/3'G_4T_4 5' telomeric repeats typical of the subchromosomic macronuclear genes of *Euplotes* (Hofmann *et al.* 1995, Jahn & Klobutcher 2002).

As shown in Fig. 3, the 5' region differs between the two species in length (379 nucleotides in *E. focardii* versus 152 in *E. nobilii*) and the sequence motif that identifies the site of the transcription initiation (GAAAA in *E. focardii* and GTAAA in *E. nobilii*). Nevertheless, in both the species it harbours the *cis*-acting elements denominated HSE (from Heat-Shock Elements) and StRE (from Stress-Response Elements), that are known to be targets of *trans*-acting transcriptional activators characterized in a variety of organisms in association with their stress-inducible genes

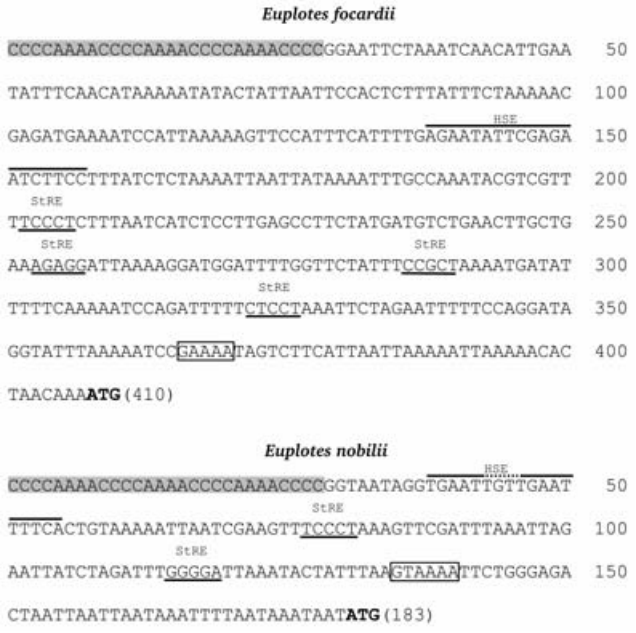

Fig. 3. Nucleotide sequences of the 5'regulatory regions of the *E. focardii* and *E. nobilii hsp70* genes. The telomeric C_4A_4 repeats are shaded; the transcription initiation ATG codons are in bold; putative sites for the transcription initiation are boxed; sequence motifs bearing agreement with HSE and StRE elements are over-lined and underlined, respectively.

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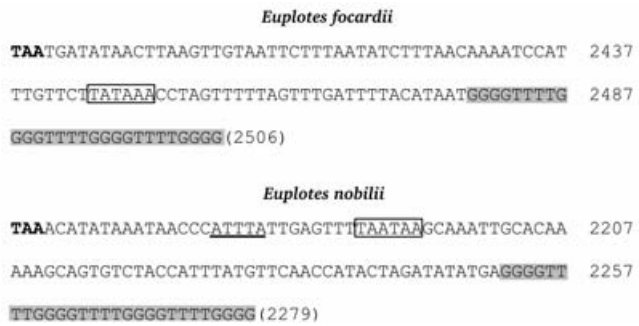

Fig. 4. Nucleotide sequences of the 3'regulatory regions of the *E. focardii* and *E. nobilii hsp70* genes. Telomeric G4T4 repeats are shaded; the stop TAA codons are in bold; putative polyadenilation motifs are boxed; the ATTTA motif, indicative of an mRNA destabilization ARE element, is underlined in the *E. nobilii* sequence.

(Kobayashi & McEntee 1993, Fernandes *et al.* 1994, Ruis & Schuller 1995). One class of these activators, designated as HSF (from Heat-Shock Factor) and first described in yeast and humans, is specific for the HSE elements (Pirkkala *et al.* 2001); a second class, represented by the Msn2p and Msn4p factors containing Zn-finger domains, is StRE-specific (Schmitt & McEntee 1996, Estruch 2000).

In the *hsp70* 5' region of *E. focardii*, the HSE elements are identified by four contiguous sequence motifs inserted between positions 138 and 158 and characterized by repeats of the pentameric consensus sequence nGAAn and the relative complement nTTCn (in which, n stands for any nucleotide); instead, the StRE elements are identified by four not contiguous motifs, i.e. T_{202}CCCT, A_{253}GAGG, C_{286}CGCT, and C_{322}TCCT, that overall bear 4/5 agreement with the consensus sequences AGGGG and CCCCT.

In *E. nobilii*, the HSE and StRE elements are practically half in number compared to *E. focardii*, most likely as a consequence of the shorter length of the HSP70 5' region. Three nGAAn pentameric units identify HSE elements that have a non-canonical, discontinuous arrangement between positions 38 and 55, the first and second units being separated from one another by a gap according to the known model nGAAn(3bp)nGAAnnTTCn (Yamamoto *et al.* 2005). Two StRE elements are, instead, identified by the motifs T_{77}CCCT and T_{116}GGGG, that bear 4/5 agreement with the consensus sequences CCCCT and AGGGG.

Unlike the 5' promoter region, as shown in Fig. 4, the *hsp70* 3' region varies between the two species with regard to at least two aspects. One is relative to the putative polyadenilation signal (represented by TATAAA and TAATAA motifs in *E. focardii* and *E. nobilii*, respectively), and the second one, more important in this context, is relative to the adenine-rich elements, designated as ARE and identified by ATTTA sequence motifs, that affect the stability of many post-transcriptionally regulated mRNA in genes encoding stress proteins, as well as cytokines and other regulatory proteins (Barreau *et al.* 2005). One of these ARE elements is present in the *hsp70* 3' region of *E. nobilii* between positions 2176 and 2180; none in *E. focardii*.

Conclusions

It is rare for organisms not to respond to a heat-shock by promptly activating the expression of their *hsp70* genes. This appears limited to species such as *Hydra oligactis* (Bosch *et al.* 1988, Gellner *et al.* 1992) and *Trematomus bernacchii* (Hofmann *et al.* 2000) that, like *E. focardii*, show a close and long adaptation to living in thermally stable waters. In *H. oligactis* the loss of an effective heat-shock response has been shown to essentially reside in an unusually rapid degradation of the *hsp70* mRNA (Brennecke *et al.* 1998), while in *T. bernacchii* the causes have been tentatively associated with alterations in the *hsp70* transcriptional mechanism (Buckley *et al.* 2004, Place *et al.* 2004, Hofmann *et al.* 2005).

In the case of *E. focardii*, the absence of ARE elements in the *hsp70* 3' region would exclude a rapid mRNA degradation, that occurs in *H. oligactis* (Brennecke *et al.* 1998). It thus appears more reasonable to accept a hypothesis based on the coexistence of regulatory *cis*-acting elements of both the HSE and StRE types in the *hsp70* 5' region. This also implies that the *hsp70* gene transcription is, at least in principle, under the control of two distinct, independent mechanisms: one, HSE-modulated, more specific for a response to a stress of thermal nature; the other, StRE-modulated, more specific for a response to a broader range of non-thermal stresses. Should this be the case, the adaptation of *E. focardii* to the stable cold waters of the Antarctic coast would have determined a selective inactivation of only the former mechanism, it being no longer useful in an organism exposed to no environmental thermal stimulus. The latter mechanism, still having a protective function against other environmental stimuli, would however have maintained its activity. How might evolutionary adaptation have inactivated the HSE-modulated mechanism? The fact that the HSE consensus sequence presents an orthodox organization would exclude the possibility that this inactivation directly depends on a defective HSE structure. Rather, it suggests that the causes are likely to reside primarily in a mutated capacity of the HSF *trans*-acting factors to bind and activate HSE. In support of this hypothesis is the knowledge that the yeast HSF, as well as the human HSF1, may lose their heat-activation functions, yet still preserve the capacity to induce the basal *hsp70* gene transcription necessary for organism viability (Smith & Yaffe 1991, Trinklein *et al.* 2004, Yamamoto *et al.* 2005, Yamamoto & Sakurai 2006).

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