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PhD Thesis

ADAPTATIVE EVOLUTION OF THE HEAT SHOCK RESPONSE IN ANTARCTIC CILIATES

PhD Candidate
Valerio Passini

Tutor
Antonietta La Terza

to my patient parents

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General Index

Premise

Research interest in the cell response to stress was raised in the early '60 by the Italian geneticist Ferruccio Ritossa, who detected a new puffing pattern in the polytene chromosomes of heat-shocked *Drosophila buschii*. Later observations correlated these changes in gene activity with the synthesis of a new set of proteins: the heat-shock proteins.

The induction of heat-shock proteins, a powerful tool to counteract the negative effects of stresses, has readily become important to ecologists and evolutionary biologists as a model of environmentally controlled gene expression and a source of phenotypic plasticity.

Current research is addressed to investigate the processes underlying the plasticity of the heat-shock response from an integrate point of view, comprehensive of the molecular, evolutionary and ecological aspects.

To this regard, useful models for this analysis are represented by two Antarctic species of ciliates, *Euplotes focardii* and *E. nobilii*, which showed up marked differences in their capacity to mount an effective heat-shock response following thermal challenges. Ongoing research activities in the laboratory in which I have started my PhD work, are mainly deputed to unveil the reasons of this eccentricity in the stress-induction capacity between the two species and more precisely regard: (1) the characterization

of their major heat-shock genes, and (2) the study of how the interplay between the *cis*-regulatory regions of these genes and the respective *trans*-acting regulators “tunes” the final stress gene expression in response to different environmental cues.

Introduction

Antarctica

Environment and its Characteristics

Its geographical position and its peculiar geologic, climatic and morphological characteristics together with political and strategic aspects make of Antarctica a fascinating and unique continent.

The climatic role of this land is object of many researches because it's known its influence in defining the world's climate through a series of processes that impact the atmosphere and the oceans (Juckes et al., 1994). It is important to underline that in Antarctica the strong inclination of sunlight together with the high albedo coefficient (given by the ratio between the reflected and the incident light and that it is more than 80%) cause very low temperatures during summer, the maximum ranging from +5/+20°C on the coasts to -30°C in the continent, while, during winter, the sun is always under the horizon and temperatures range from -20°C on the coasts to -90°C in the continent (Petit et al., 1999).

The difference between the polar and equatorial temperatures causes the so called pressure gradient force which, in combination with the Coriolis' Force, determines the general movement of air masses on which depends the distribution of the heat all over the world. Moreover, the transition from the

cold season to the warm season causes the melting of the ice along the coasts. The cold waters originated from melted ice are dense and sink beneath the relatively warmer waters of the coasts. This process feeds the deep streams and the exchange among ocean waters and it is responsible for the formation of the Antarctic Convergence (also called Polar Front Fig.1) (Orsi et al., 1995; Juckes et al., 1994). This is a kind of barrier, that physically separates the southern oceanic waters of Antarctica to those of other oceans even if they are contiguous. The Antarctic Convergence has temperatures ranging between 2,8 and 5,5°C in average and it surrounds the South Pole as a ring wide from 30 to 50 km that sometimes reaches the 46° parallel, in which temperature, salinity, nutrient concentration and oxygen are peculiar. The presence of the Antarctic Convergence renders very difficult for many species to move among the oceans and leads to isolation of plankton and fishes populations which are clearly different from those present in near marine waters. The oceanic portion enclosed by the Polar Front establishes a marine system that is protected from the worst of the extremes, and the sea coastal water reaches a stable minimum of -1.8°C. On the contrary, the environment represented by the sea coastline is less stable during the year in regard of temperature: there are cold winds and the sunlight can warm up the surface up to 20°C. Going from the coast to the inner side of the continent the conditions worsen. The land is always covered by ice and is

subjected to temperatures even colder than those of coasts (-30 /-70°C depending on seasons), to strong winds which reach speeds over 300km/h, to intense UV irradiation that nowadays is even increased thanks to the ozone hole and the scarcity of precipitations (may sound strange but that is) that almost inhibits life (http://www.antarctica.ac.uk/met/jds/weather/weather.htm). The isolation of the Antarctic continent began when the supercontinent Gondwana broke-up (167 million years ago) and then it continued until the establishment of the Antarctic Convergence and its associated oceanographic regime in the Early Cenozoic. This isolation have meant that the evolution of both the marine and terrestrial biotas has taken place almost without any exchange with species living in the surrounding environments (Eastman, 1993; Shaw et al., 2004; Clarke and Johnston, 1996).

On the basis of these consideration the Antarctic environment represents an exciting place for studying cold adaptation, evolution in Antarctic species and the threat posed by global climate change to species.

Even if its macrofauna, like penguins, blue whales and seals, is very popular and well known thanks to mass media, Antarctic ecosystem is dominated by microorganisms, that is a wide range of small and microscopic lifeforms including protozoa, fungi, bacteria and microalgae, which interact to form dynamic and

sometimes highly structured communities. The great part of these lifeforms concentrates along the waters and the coasts of Antarctica which have a more stable and favourable conditions, while in the continental part, the environment is too hostile to life[1]. They are an important node of Antarctic food web, as it has been discovered recently, and thanks to them, the sea below the ice hosts a rich and colourful variety of species that can be compared to that of coral reefs, and which really contrasts with the continental surface that is almost deserted. Antarctic microorganisms have evolved practically in complete geographic and genetic isolation, adopting specific morphological and physiological strategies to withstand these harsh environmental conditions. Furthermore cold adaptation includes a complex range of structural and functional adaptations at the level of all cellular constituents, such as membranes, proteins, metabolic activity and mechanisms to avoid the destructive effect of intracellular ice formation .

1 Little is known about the lakes under the icecap, like the Lake Vostok, which have been sealed 500.000 years ago and might host a large unknown number of lifeforms.

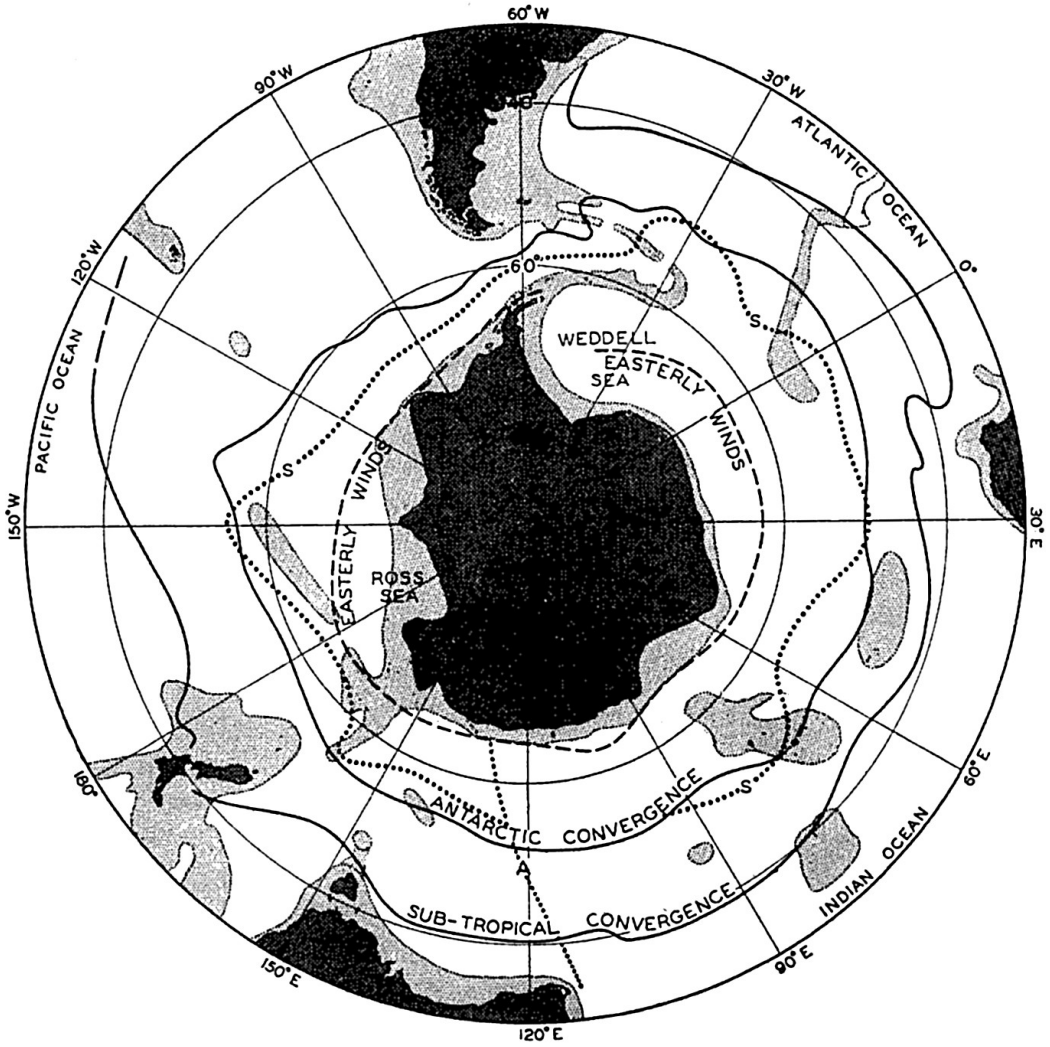

Fig.1: Location of Antarctic Convergence (from: "The Oceans: Their Physics, Chemistry and General Biology" H. U. Sverdrup et al., 1942.

Adaptive Strategies of Cold-Adapted Organisms

The first issue posed by Antarctic environment is intracellular ice formation. Sodium chloride is responsible for about 85% of the freezing point depression, and the remaining 15% is due to other small solutes. Synthesis of antifreeze glycoproteins and peptides can further depress the freezing point of body fluids or cellular water. These proteins lower the freezing point by a non-colligative mechanism without altering the melting point significantly, and are therefore also referred to as hysteresis proteins (Raymond et al., 1989; Jia et al., 1996). Other strategies to adapt to the Antarctic environment, include modification in the membrane structure, in the plasticity of the enzymes and in gene regulation.

Membrane lipids

The membrane is both the interface and the barrier between the internal and external environment of the cell. Cold-adapted bacteria respond and adapt to low temperature by modulating the fluidity of their membrane (Chintalapati et al., 2004). This is mainly achieved by altering the fatty acid composition. The most important strategy is to increase the proportion of unsaturated fatty acids, which help to maintain a semi-fluid state of the membrane at low temperatures (Aguilar et al., 1998; Suzuki et al., 2001).

Enzymes

According to the currently accepted hypothesis, cold-active enzymes must increase the flexibility of some or all parts of the protein to compensate for the lower thermal energy provided by the low temperature habitat (Somero, 2004). Flexibility induces a decrease in the activation energy and thus provides high catalytic efficiency at low temperature; in return, this increased flexibility is responsible for the generally low stability of the protein structure of cold-active enzymes, due to an inverse relationship between stability and activity (D'Amico et al., 2002; Marx et al., 2004).

All the available data regarding cold-adapted enzymes indicate that a high specific activity is almost always associated with a low thermostability. The thermostability derived from the pronounced rigidity of the molecular edifice is thought to impair the interaction between substrate and enzyme, leading to a weak specific activity. By contrast, flexibility or plasticity of the molecular structure would enable greater complementarity at a low energy cost, thus explaining the high specific activity of cold-adapted enzymes.

Psychrophily and Psychrotrophy

Even among extremophilic organisms there are differences regarding the preference to cold environments like Antarctica. In relation with temperatures and growth rates, most researches distinguish between psychrophilic (cold-loving) or psychrotrophic

(cold-tolerant) microorganisms. According to the most widely accepted definition, psychrophilic are unable to grow above 20°C and grow fastest at 15°C or below. They persist in permanently cold habitats, such as in polar regions, at high altitudes, or in the deep sea. Environments with periodic, diurnal, or seasonal temperature fluctuation (e.g. area in continental climates with high summer and low winter temperatures) are favourable to psychrotrophics, which can grow at temperatures close to the freezing point of water, but have fastest growth rates above 20°C. Psychrophiles live at the lowest temperatures allowed for the development of living organisms. According to this definition, we can classify the two *Euplotes* objectives of our researches. *E. nobilii* is psychrotrophic, because it tolerates 0-4°C, but it grows better at 12-18°C, while *E. focardii* is psychrophilic because on the opposite its growth rate is faster at 4-5°C and sharply decreases at 8-10°C.

The Heat Shock Response and the Heat Shock Proteins

Research interest in the cellular response to stress was raised in the early 60 by the Italian geneticist Ferruccio. M. Ritossa, who observed a new puffing pattern in the polytene chromosomes of the salivary glands of accidentally heat-shocked larvae of the fruit fly *Drosophila buschii* (Ritossa, 1962).

Since then, it has been established that the Heat Shock Response is a cellular response comprising of transient but

complex reprogramming of gene expression that takes place when cells or organisms are subjected to a wide variety of stresses able to denature proteins. Thus, it can be properly considered a general stress response. The puffs in the polytene chromosomes in *Drosophila* observed by F. Ritossa, are DNA regions in intense transcriptional activity and have been later correlated with the massive induction of a set of proteins, named thereafter: Heat Shock Proteins or HSPs. In details, the Heat Shock Response consists in depression of ongoing chromosomal transcription, induction of transcription of the heat shock genes, inhibition of RNA processing, inhibition of translation of general mRNAs and a preferential translation of the heat shock proteins mRNAs (Lakhotia, 1998).

As pointed before, the HSR is mainly induced in response to a large variety of stresses able to denature proteins. From a molecular point of view, during the exposure to a stress, the denatured proteins expose regions composed by hydrophobic aminoacid residues; these exposed regions can bind to those of other denatured proteins and so lead to formation of aggregates that at worst are cytotoxic and at best reduce the pool of functional proteins in the cell (Feder, 1999). The HSPs act as chaperones recognizing the exposed hydrophobic chains in non-native proteins and then the HSPs bind to them allowing the bound proteins to acquire or to return to their native conformation, otherwise to be targeted for degradation and

removal from the cell (Feder, 1999).

Besides their role as stress inducible proteins, now it is also recognized that these proteins are expressed at basal levels in unstressed cell to allow folding, assembly, intracellular localization, secretion, regulation, and degradation of other proteins (Feder and Hofmann, 1999)[2]. Owing to this important role in general stress response and in basic cellular activities these genes have been maintained, at high levels of sequence conservation, in the genomes of every eukaryotic organism examined to date (Buckley et al., 2004).

The Heat Shock Protein 70 family: importance and role

The HSPs are broadly classified, on the basis of their apparent molecular weights, amino acid sequences and functions into distinct families, HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, small HSPs (sHSP) and HSP10 (Arya et al., 2007; Feder and Hofmann, 1999). Gething (1997) recognizes 7 additional families and 12 genes/proteins for which families have not yet been described. In eukaryotes, many families comprise multiple members that differ in inducibility, intracellular localization, and function (Feder and Hofmann, 1999).

In this work I have focused on HSP70 because large bodies of evidences suggest that this HSPs can be considered as a key regulator of the Heat Shock Response as well as, with few

2 Maybe interesting to report that in higher organisms, the heat shock response can also induce a death signal that leads to apoptosis or rapid necrosis (Lila Pirkkala et al., 2001).

exceptions, the main heat-inducible molecular chaperon in every eukaryotic organism examined so far (Lerman et al., 2003; Hofmann et al., 2000). Besides the inducible isoforms, the 70kDa heat shock proteins family owns constitutively expressed members which are generally known as Heat Shock Cognate 70 proteins (HSC70) (Mayer and Bukau, 2005). In absence of stress conditions, these isoforms are called to assist a wide range of folding processes, including the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of organellar and secretory proteins, and control of the regulatory activity and through one of its members, it has also housekeeping function in the cell, in fact its constitutive member called HSC70 is regularly expressed in the cell (Mayer and Bukau, 2005).

The inducible HSP70, in cooperation with small HSPs, has been shown to enhance the survival of mammalian cells exposed to numerous types of stimuli that induce stress and apoptosis (Pirkkala et al., 2001)[3]. Other researches have found an important role of this chaperone in its inducible form in different processes like long term memory and immune response[4].

3 The antiapoptotic HSP27 and HSP70 are abundantly expressed in many malignant human tumors (Pirkkala et al., 2001).

4 The pattern of heat shock proteins in mammalian brain, either synthesized in a developmentally regulated manner or in response to stress, is non-random. This has been related to specific functions of different parts of the brain, including role of HSPs, particularly the HSP70, in short- and long-term memory and making different parts of the brain more or less susceptible to stress-induced injuries . [...] A number of HSPs such as HSP70, HSP90 and Gp96/Grp94 have been shown to chaperone a broad array of peptides, derived from different cellular proteins, from the cytosol to the Major Histocompatibility Complex I molecules which in turn display the peptides on the cell surface. The HSPs therefore play a key role in

HSP70 Gene regulation

The activation of heat shock gene transcription, including that of *hsp70*, is mediated mainly by heat shock transcription factors (HSFs), which binds heath shock elements (HSEs) in the promoter region and transactivates the heat shock genes (Baler et al., 1993; Morimoto, 1993; Sarge et al., 1993; Hung et al., 1998). HSEs consist of an array of inverted/alternate repeats of the pentameric sequence NGAAN. This sequence has been found in many promoters of stress inducible genes and now is generally considered a sort of “marker” of inducible stress genes. The arrangement and number of these NGAAN units can vary (Abravaya et al., 1992) and affect *hsp70* gene expression (Lis and Wu, 1994). It is generally assumed that at least three repetitions of this basic sequence are required to have a working HSE (Morimoto, 1993). Among the many possible functional arrangements studied so far, there are the so-called gapped HSEs, containing a variable number of bases inserted between the pentameric units. For example, the *S. cerevisiae* MDJ1 promoter represents a functional gapped HSE, as it carries an insertion of 11bp between the first and the second repetition (nTTCn-(11bp)-nGAAn-(5bp)-nGAAn). This promoter also contains two functional contiguous nGAAn direct repeats. Recently, several examples of functional HSEs with direct repeats of nTTCn or nGAAn interrupted by 5bp insertions were characterized in the promoter regions of several *S. cerevisiae*

antigen presentation (Lakhotia, 1998).

genes. (Tachibana et al., 2002).

Activation of the heat-shock transcription factor via trimerization, phosphorylation and nuclear localization is a key step in heat-shock protein expression. HSF is negatively regulated through interaction with HSP70 family members and some other co-chaperones (Cotto and Morimoto, 1999; Lerman and Feder, 2001; Zatsepina et al., 2000). When activated, HSF can bind heat-shock response elements (HSEs) in the *hsp70* promoter to induce transcription. In very general model (Fig. 2), HSP70 forms complexes with HSF in the citosol[5], during non-stressing conditions. Following stress, denaturated proteins compete for the binding to the Hsp70 normally present in steady state level in the cell freeing the HSFs. The monomers of HSFs trimerize and migrate into the nucleus. In the trimeric state, HSF binds to the HSE, forming a complex that has the potential to activate the transcription of *hsp* genes (Zuo et al., 1994; Zou et al., 1998; Lee et al., 2000; Pirkkala et al., 2000; Rieger, 2005). As shown in Fig.2, a negative feedback provides control over *hsp70* expression. When the amount of HSP70s exceeds that of denatured proteins in the citosol, Hsp70 proteins are free to move in the nucleus and bind the transcriptional active HSFs positioned on HSE element. This could happen either when the stress is finished or when the expression is too intense. This

5 Other Authors report that HSF's are bound to other chaperones too (like HSP90) or to chaperones in general: “metazoans sequester the majority of HSF as chaperone-bound monomers within the cytoplasm; upon heat-shock, HSF monomers undergo an intramolecular rearrangement that allows HSF subunits to trimerize”(Lee et al., 2000).

binding destabilizes the association between HSF and HSE, halting or greatly reducing the transcription of stress genes. This model is considered valid for higher eukaryotes from *Drosophila* where this mechanisms was firstly described, to humans[6].

However, exceptions to this model have been described in unicellular eukaryotes like *Saccharomyces cerevisiae*, fungi (Xavier et al., 1999), *Tetrahymena piriformis (*do Carmo Avides et al., 1990) and *Tetrahymena thermophila* too (Barchetta et al., submitted manuscript). In these organisms, HSF is constitutively bound to HSE and its transcriptional activity is induced by heat shock (Abravaya et al., 1990; Zou et al., 1995) through a mechanism of phosphorylation (or hyper phosphorylation) by specific kinases[7].

Besides canonical HSE, other stress regulatory elements have been identified in the promoter region of *hsp* genes. In yeast, the promoter regions of several stress genes yeast bears Stress Responsive Elements (StRE) denoted by the sequence motifs CCCCT and AGGGG (Kobayashi and McEntee, 1993). These motifs work well even in a single copy, however when two or more StRE are present, the induction effect increases in a way that is not simply proportional to the number of StRE, but it is greater (Kobayashi and McEntee, 1993; Estruch, 2000). Genes containing StRE elements in their promoters are inducible by

6 This result suggests that factors are not bound to the HSE region prior to heat shock in HeLa cells (Abravaya et al., 1991).

7 Richard I. Morimoto calls these kinases: stress-dependent kinase (Rieger et al., 2005).

various different environmental and metabolic stresses and the transcriptional activation mediated through this element results in the acquisition of a tolerant state towards other stress conditions (Estruch, 2000)[8]. These sequences are known to be targets of *trans*-acting transcriptional activators characterized in a variety of organisms (Boy-Marcotte, 1998). In yeast, the MSN2/4P factors, with a characteristic zing-finger domain, have been identified for being able to bind the StRE elements (Schmitt and McEntee, 1996; Estruch, 2000). In yeast, StRE motifs represent an alternative way to HSE, to produce a stress-induced response, and thus to activate Hsp70 expression too (Feder and Hofmann, 1999; Estruch, 2000). Interestingly in the protozoan ciliate *Tetrahymena thermophila*, has been recently demonstrated that HSE elements do not represent the minimal regulatory elements for heat shock induction. In this ciliate the heat shock activation of one of its *hsp70* isoforms, *hsp70*-1, is achieved by means of a combination of two regulatory elements: the conventional HSE and GATA elements (Barchetta et al., submitted manuscript).

8 It is the so called cross-protection.

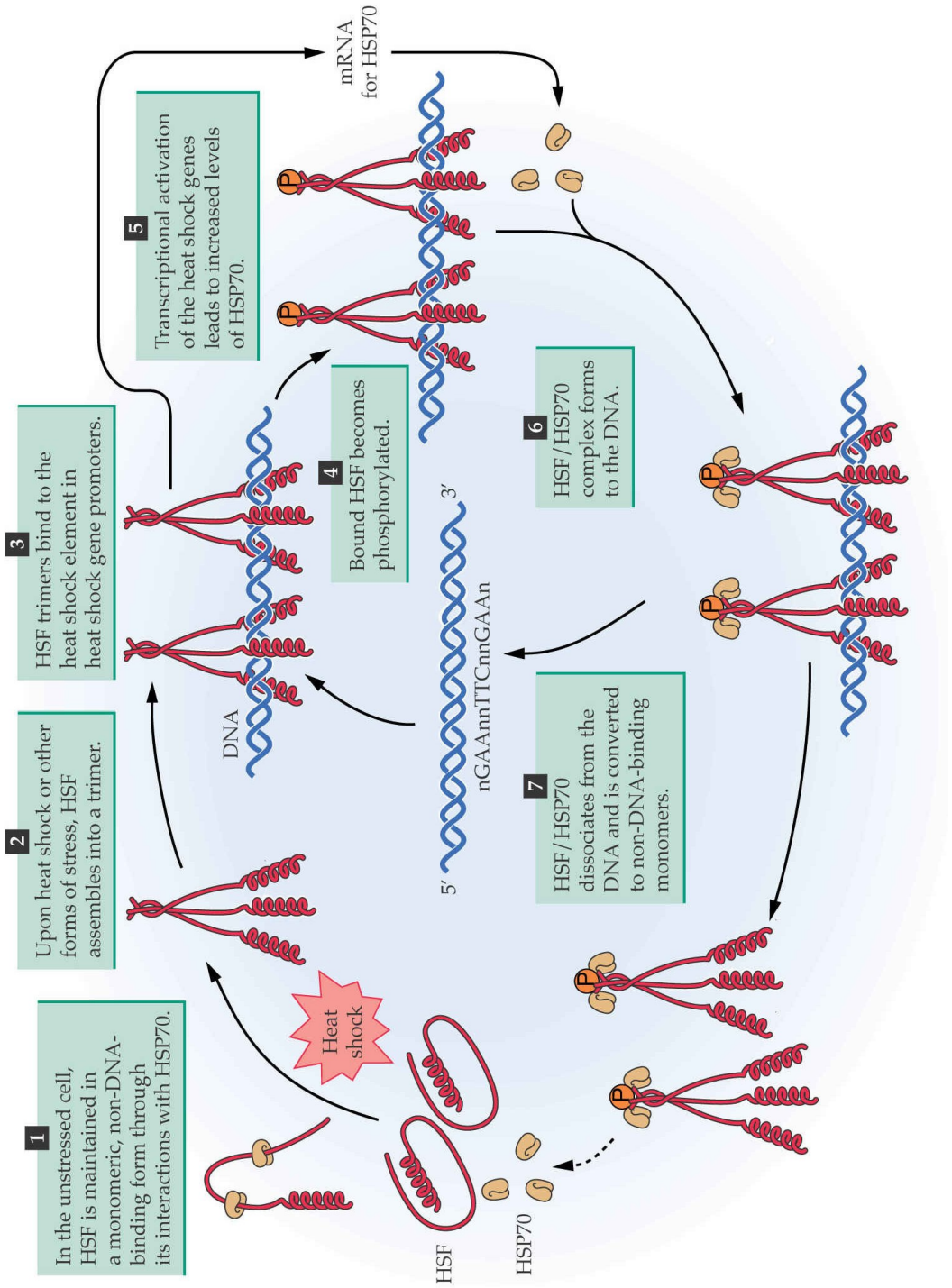

Fig. 2: Mechanism of heat shock gene transcriptional induction in eukaryotic cells

HSP70 protein structure

Actually in most publications are reported two principal domains: a N-terminal ATPase domain of 45kDa and a C-terminal substrate binding domain of ca. 25kDa, which is further subdivided into a β-sandwich subdomain of 15kDa and a C-terminal α-helical subdomain (Mayer and Bukau, 2005). The way these two domains are involved in the correct folding of non native proteins is not completely clear and has been studied only in few organisms, like *E. coli* which owns a HSP70 like protein, called DnaK, and in some eukariotes like yeast and *Drosophila*. The ATPase domain binds ATP and has a very low activity. In this bound state the HSP70 is able to interact with a non native protein or a growing A.A. chain, emerging from ribosomes, which exposes hydrophobic aminoacids. The binding domain recognizes these exposed residues, at least in chains 7-8 AA long, and binds to them. At this point the ATPase activity raises, but not enough to hydrolyze the ATP. The presence of so called co-chaperones of the JDPs family is required to increase by 1000-fold the ATPase activity in HSP70. Thus the ATP is hydrolyzed to ADP, there is a conformational change in the HSP70 that tightly binds the protein and prevents its aggregation to other nascent or denaturated proteins (Laufen et al., 1999; Liberek et al., 1991; Gamer et al., 1996). Finally, the release of ADP is possible. In some organisms it's known that some nucleotide exchange factors like BAG-1 and HSPBP1/FES1P are needed and help the

HSP70 to release ADP and to begin a new cycle (Dragovic et al., 2006; Kabani et al., 2002).

Recently a new domain has been reported and confirmed in the HSP70 structure: the Histone deacetylases (or HDAC) interacting domain. HDAC is an enzyme whose activity removes acetyl groups from specific lysine residues in the histones, tightening the DNA to the histones and regulating the state of the chromatin. So it inhibits the access to DNA by RNA polymerase complexes and, as a consequence, the transcription. The HDAC interacting domain in HSP70 seems responsible for formation of complexes with many members of HDAC family. This must be further investigated, but HSP70 seems also responsible for enhancing the activity of the HDAC itself *in vitro*, suggesting that HSP70 may play a role in the reconfiguration of the whole transcriptome during the early stage of a stress (Johnson, 2002 , Lee et al., 2006)[9].

mRNA stability and distinctive features

HSP70 expression enhances thermotolerance, but it is known that too high level of HSP70 in the cytoplasm can affect growth or decrease individual fitness (Feder et al., 1992; Krebs and Feder, 1997). Therefore organisms must limit expression at periods when damage may result (Krebs and Bettencourt, 1999). That is why steady state level of HSP70 are quickly reached after

9 The role of this HDAC interacting domain is not clear and some data seem in contrast with previous one (e.g.: in a very extensive a complete review by M.E. Feder and G.E. Hofmann about heat shock response it's said: HSP70 [it is necessary] for recovery from translational and transcriptional inhibition following heat shock.

heat shock, and mRNA is fine regulated during and after transcription. In many organisms have been observed that in the initial phase of an heat shock there is an increasing transcriptional activity, then this activation reaches a peak and slowly decreases, but the level of HSP70 proteins does not follow the decreasing level of mRNA. It's known that there is a mechanism of degradation of unstable messengers that operates through the 3' UTR of *hsp70* mRNA that contains a class III AU-rich element called also ARE (Malter, 2001)[10]. During heat shock, this mechanism is inactivated (probably by the heat shock itself) and the messengers cumulates as a consequence of not being degraded. After this initial stabilization of the messenger, HSP70 transcription is induced. When thermal stress ends, the induction mechanism involving the promoter region stops and the degradation mechanism of *hsp70* messengers is activated again. Following these events the translation of new HSP70 reaches basal level. In a recent study, a number of indirect and *in vitro* evidences[11] also suggests that HSP70 is involved in the degradation of its own mRNA (Balakrishnan and De Maio, 2006), and this adds a new negative feedback mechanism of regulation. It's interesting to report what Ullmann et al., (2004) have discovered about HSP70 expression in a ciliate: *Moneuplotes*

10 The presence of an ARE can affect the stability of an mRNA by decapping, deadenylation and 3'-->5' decay. [...] These effects are mediated through *trans*-acting factors that associate with the ARE (Wilusz and Wilusz, 2004).

11 HSP70 coimmunoprecipitates with hsp70 mRNA in lysates from stressed cells and *in vitro* ATP competes for binding with mRNA to HSP70. HSP70 is also able to bind AU rich regions in mRNA and *hsp70* mRNA may contain a similar region in the 3'UTR.

crassus. The heat shock response could be dissected into two phases. An initial protein-dependent stabilization of the mRNA is followed by an increase of the steady state transcript level that is dependent on continued transcription. They conclude that the regulation of the heat shock response is a two-step process that occurs at the transcript level (Ullmann et al., 2004).

Other researches have found that in some organisms (Human and *Drosophila melanogaster*)[12] the translation of the HSP70 messenger is possible without the 5'-cap because of the presence of the so called internal ribosome entry site (or IRES) in 5' UTR. This is an alternative mechanism of positioning the 40S ribosomal subunit nearby or directly onto the first AUG codon in the coding sequence. The characteristic elements in a generic IRES were studied first in picornaviruses until their discoveries in eukaryotic genes (Hellen and Sarnow, 2001; Baird et al., 2006). At the beginning it was believed that IRES should have specific sequence like a polypyrimidine region 25 nucleotides upstream the AUG. Now that data have increased in number of genes and organisms represented, the investigators focus on the secondary/tertiary structure instead of the sequence: the general rule seems that to operate as an IRES, a 5'UTR should form a structural scaffold in which precisely positioned RNA tertiary structures contact the 40S ribosomal subunit through a number of specific intermolecular interactions (Vagner et al.,

12 A similar research performed in *Zea mays* L., has found IRES in the HSP101 mRNA (Dinkova et al., 2005).

2001). This mechanism is useful when, for instance, cap-dependent translation is impaired during various forms of stress, including heat shock. The presence of IRES in the non coding region in *hsp70* mRNA (in human and fruit fly, to date) seems to confirm again the key role of this gene during heat shock and to explain why translation of mRNAs encoding heat shock proteins (HSPs) is favoured during heat shock, while general cap-dependent translation appears severely inhibited (Rubtsova et al., 2003; Hernandez et al., 2004).

Ecological Role of Heat Shock Response

The heat shock response is greatly responsible for the acclimatization of an organism to the thermal changes in the environment. In fact, it is the way a cell can survive momentary adverse conditions and thus it is a homeostatic response, not meant for long time changes[13]. Usually, as stated by Prasanta K. Ray et al., (1999) “the urge of an organism is to adjust itself to the prevalent conditions to come back to a normal or steady state, conducive enough for perpetuation of life processes”.

The thermal threshold for heat shock protein expression is correlated with the temperatures that organisms normally

13 There is an interplay between adaptation and acclimatization. If evolution affects the sequences of the genes encoding the other proteins such that these proteins resist stress-induced deformation or denaturation at species-appropriate temperatures (that leads to the so called adaptation), then one outcome could be countergradient variation in threshold temperature [in heat shock response](thus affecting acclimatization) (Feder).
Acclimatization is a physiological change that allows organisms to cope with a changed condition. Adaptation is Evolution. Adaptation involves the evolutionary selection of gene alleles.

encounter, and the magnitude of HSP expression is correlated with the variability of the thermal environment (Feder; Zatsepina et al., 2001). The heat shock response greatly contributes to the capacity of an organism to survive a specific environment and thus to withstand thermal variations.

As mentioned before, the heat shock response depends on the “normal” conditions to which an organism is exposed. The temperatures on earth range from -100°C to 100°C and more, and even if organisms can escape adverse conditions exploiting microhabitats, the microhabitats themselves are possible source of stress. Even equable environments can contain HSP-inducing microhabitats, and even mild stresses can induce HSPs when multiple stresses act in combination (Feder and Hofmann, 1999). It is widely accepted and the actual data support a correlation existing among HSP expression, stress tolerance, and gradients of environmental stress (Feder and Hofmann, 1999). From a general point of view, organisms that grow, reproduce and have their optimal life conditions in a cold environment, have a lower activation threshold than those which prefer high temperatures. There are many example of this behavior. A northern species of mussel (*Mytilus trossulus*) that lives in cold waters has a lower threshold for HSP70 expression than its congener, *M. galloprovincialis*, a warm-water species with a more southern distribution (Hofmann and Somero, 1996). Similar differences in the threshold temperature for *hsp70* gene expression, have been

described between the ant *Cataglyphis bicolor* inhabiting the desert of Sahara and *Formica polyctena* living in temperate climate. In *Cataglyphis bicolor* the threshold temperature is higher in respect to that of *Formica polyctena* (37°C instead of 33°C). In *Cataglyphis has been* discovered that even the range to which HSP70 is induced is different from that of the temperate climate species, and HSP70 is still produced in response to temperature until 45°C while in other species it stops at 39°C. This seems due to the fact that the desert ant encounters a quick shift on temperature when it passes from the underground anthill (<30°C) to the desert surface(>50°C). Moreover, in *Cataglyphis* the basal level of HSP70 proteins it is higher than in other ants, so that *Cataglyphis* can be ready to encounter high temperature (Gehring and Wehner, 1995). A similar pattern (although not as extreme) is evident for desert and non-desert *Drosophila* (Huey and Bennett, 1990).

In extremely hot environment, like black smokers, there are Archaea which are capable of heat shock response, but only when the temperature raises over 88°C, as in *Sulfolobus*, and even more than 100°C as in a species called ES4, a heterotrophic sulfur reducer isolated from a deep-sea hydrothermal vent (Feder and Hofmann, 1999).

A special case: Heat-Shock Response in Antarctic organisms

Antarctica is characterized by at least three mains habitats: the

marine environment, the intertidal environment and the terrestrial environment; everyone with its peculiarities.

The marine waters are a cold but a stable environment, in which temperature and many other parameters are constant. The changes in temperature never exceed 3.5°C and they almost range between -1.8 and 0°C for the great part of the year. Along with the deep sea, these are amongst the most thermally stable environments on Earth. Salinity is remarkably constant, even in nearshore waters because there is not any major river flowing into the Southern Ocean. On the other hand, the intertidal zone is less stable during the year in regard of temperature and salinity: there are cold winds and the sunlight can warm up the surface up to 20°C, while during winter the temperature reaches -20°C. Salinity may vary a lot in dependence of flows belonging alternatively from melted ice (freshwater) or from marine water. This harsh conditions worsen going from the coasts to the continent, that constitutes the great part of the terrestrial environment. In this part of Antarctica, temperature ranges from -30° to -90°C (Peck et al., 2005). These extreme but different environments have influenced the evolution of the heat shock response in many species. The first example is a midge, *Belgica antarctica*, that according to its life cycle stages occupies two different terrestrial habitats. In the larval stage, this midge is unable to move freely and it lives under rocks where the temperature is almost stable (from -4°C to 4°C), but other

parameters such as pH and osmotic pressure are less stable and there are frequent freezing/thawing periods. In these changing conditions, the larva has a high basal level of HSP70 expression and there is not any further increase in the expression upon heat shock. When it turns in an adult midge, it becomes able to escape from these environmental changes while it can encounter rocks warmed up to 20°C by direct sunlight. In this stage of its life, there is no more the need for a continuous *hsp70* expression. In fact HSP70 reaches a low basal level and it also returns inducible (Rinehart et al., 2006).

An interesting example of convergent evolution of the Heat Shock Response at sub-zero temperatures is offered by two organisms inhabiting the cold coastal sea water of Antarctica, the fish *Trematomus bernacchii (*Hofmann et al., 2000; Buckley et al., 2004) and the ciliate *Euplotes focardii* (La Terza et al., 2001). Both species have lost the capacity to induce the expression of their *hsp70* genes in response to thermal variation[14]. This shared behavior can be due to the large energetic cost associated with the HSP70 expression that could be saved in very stable environment. In fact, current estimates regarding both *Trematomus bernacchii* and *E. focardii,* suggest that they have evolved in an extremely cold and stable thermal environment for approximately 14–25 million years (Eastman,

14 In *Trematomus bernacchii*, the Heat Shock Response is not detectable in response to increase of temperature and/or to exposure to chemicals while HSP70 it's constantly expressed at a basal level (Hofmann et al., 2000; Buckley et al., 2004).

1993; Clarke and Johnston, 1996; Di Giuseppe et al., unpublished results).

Heat Shock Response and Evolution

As shown in the several examples illustrated in the previous chapters, although the heat-shock response might be considered among one of the most ancient and highly-conserved features of living things, it greatly varies within and among populations, species, and higher taxa. Feder and Hofmann (Feder, 1999; Feder and Hofmann, 1999) have characterized the major axes of variation. Typically the variation is "countergradient". In terms of microenvironmental temperature, for example, the thermal threshold for heat-shock protein (HSP) expression is correlated with the temperatures that organisms normally encounter, and the magnitude of HSP expression is correlated with the variability of the thermal environment.

The genetic encoding of this variation might reside at several locations in the genome:

1. *Variation in the hsp coding sequence*: Non-synonymous change in the coding sequence of *hsp* genes could affect the structure and function of Hsps, and thereby the cell's requirement for HSPs.
2. *Variation in hsp gene copy number*: Gene duplication is a major feature of molecular evolution in general and of many *hsp* genes. In opposition, duplicated *hsp* genes can become pseudogenes.

3. *Variation in cis-regulatory elements*: The nucleotide sequence of the proximal promoter and untranslated regions can affect the magnitude of *hsp* gene expression. Variation in any such sequence could result in evolutionary change in HSP levels.
4. *Variation in trans-regulatory factors*: The various transcription factors and the kinase-based signaling pathways whose activity is needed and influence the heat-shock response could themselves evolve in their *trans*-activation domains or in their own regulatory domains, both *cis* and *trans*.
5. *Variation in co-chaperons and co-factors*: Many heat-shock proteins do not function as monomers or as homo-oligomers, but as components of a chaperon machinery comprising many proteins and any of them could evolve in an independent way.
6. *Variation in chaperon targets*: The number and sequence of the sites at which HSPs recognize and interact with their client proteins could themselves be targets of evolutionary change.

Genomic Organization in ciliates

The organisms that are objects of our research on HSP70 are hypotrichs and belongs to the Ciliates phylum, part of the Protists reign. The whole phylum is characterized by an

interesting example of genomic organization.

They have all two different nuclei called macronucleus and micronucleus named after their difference in size. They share the same origin because they both derive from the same zygotic nucleus but they are different in function and structure.

The macronucleus, also called somatic nucleus, is polyploid and it is active in transcription. From its activity depends the cell life and it is not able to divide mitotically, but only through a process called amitosis.

The micronucleus, or germ nucleus, is diploid and it is almost inactive during cell life, it becomes operative only during sexual processes. The micronucleus divides itself mitotically and then it performs a meiotic division during sexual process producing four gametic pronuclei among which one is exchanged with another conjugating cell, one is retained to form the zygotic nucleus while the others are degraded. Next, the zygotic nucleus splits usually two times producing four nuclei, then some of them will be degraded while one will become the macronucleus and one will become the micronucleus depending on the position they occupy in the cell. The macronucleus development starts from the formation of polytenic chromosomes, after it is followed by process called fragmentation that breaks the chromosomes, then specific DNA sequences are eliminated or transposed, followed by a selective amplification of them and the final addition of

telomeric ends. The result is a completely different macronuclear genome from that is in the micronucleus by quantity and quality of its DNA.

At the beginning of the studies in the macronuclear organization, the ciliates were divided in two groups in the conviction that there were two different types of macronuclear reorganization. Now its known that the differences only regard the extent of the reorganization, not is quality.

The various *Euplotes* we are going to present in the next chapters are ciliates belonging to the hypotrichs group one of the most evolved group in the ciliates phylum and for this reason they have the finest reorganization of the macronucleus in comparison with the micronucleus. In fact, the macronuclear genome is constituted by many minichromosomes that are called “genesize” bearing only one coding sequence that are 2000bp long on the average. The coding sequence is flanked by two non coding regions in 5' and 3', called “leader” and “trailer”, and closed by two telomeric ends composed by several repetition of the C_4A_4 motif.

Our Models: *Euplotes focardii* and *Euplotes nobilii*

Antarctic waters host an unexpectedly rich variety of eukaryotic microbes (Vincent, 1988). A common component is represented by species of ciliates, in particular of *Euplotes* (Petz et al., 1995), which is one of the most successful bottom-dwelling hypotrich

ciliates distributed world-wide in both freshwater and marine habitats (Curds, 1975; Borror and Hill, 1995). Two of these *Euplotes* species, *E. focardii* (Fig.3), and *E. nobilii* (Fig. 4), which have been described as new species endemic in Antarctica, all derive from benthonic communities inhabiting freezing (–1.8 ° C), interstitial waters along the coasts of Terra Nova Bay (Ross Sea) (Valbonesi and Luporini, 1990a,b). In assessing the environmental parameters favouring higher rates of survival and multiplication of these *Euplotes* species in captivity, clearcut variations emerge in their responses to the warming of their environmental (cold room) temperature. While *E. nobilii* manifests a behaviour which is typical of psychrotroph micro-organisms, as they start multiplying faster at 12–18 ° C rather than at 4°C, a marked psychrophile behaviour distinguishes *E. focardii* (Valbonesi and Luporini, 1990a, 1993; unpublished). It was later demonstrated (La Terza et al., 2001) that these species deeply diverges in their capacities to respond to thermal stress with an activation of the transcription of their *hsp70* genes, a universally conserved HSPs whose function is to assist cells in defence against environmental stresses (Frydman and Hartl, 1994). Following a thermal stress, only *E. nobilii* is able to strongly activate the transcription of its *hsp70* genes, while *E. focardii* does not show any appreciable induction of its *hsp70* genes, thus suggesting that the *hsp70* genes of this Antarctic ciliate have greatly reduced a function which is apparently

useless in the thermally immutable environment where it lives. This and other major features such as the structural modifications of tubulin genes to ensure microtubule polymerization in the cold (Pucciarelli et al., 2002; Pucciarelli and Miceli, 2002) strongly support the idea that *E. focardii* is an ancient colonizer of Antarctica and among the several species of *Euplote*s collected from different site in Terra Nova Bay, one of the most cold-adapted. This hypothesis is further sustained by data from SSrRNA analysis that date the separation of this species from an ancestral Gondwanan species much earlier than the biogeographical isolation of Antarctica caused by the circum-Antarctic Current about 25 million year ago (Di Giuseppe et al., unpublished results). Differently, the SSrRNA data and the typical psychrotroph behaviour suggest that *E. nobilii* can be regarded as a secondary colonizer of Antarctica. This species has retained the capacity to switch on the transcription of its *hsp70* genes when it is transferred from an environmental temperature of 4°C to 20°C. Thus, it may be regarded as one of the many organisms that face thermal stress with new synthesis of heat shock proteins.

All these peculiarities, makes ciliates a fascinating biological material to investigate at reduced level of complexity, relevant aspects of the genetic bases of adaptation of the eukaryotic life to Antarctica and of more interest for my PhD work, to analyse

potential mechanisms of transcriptional adaptation to chronically cold environments.

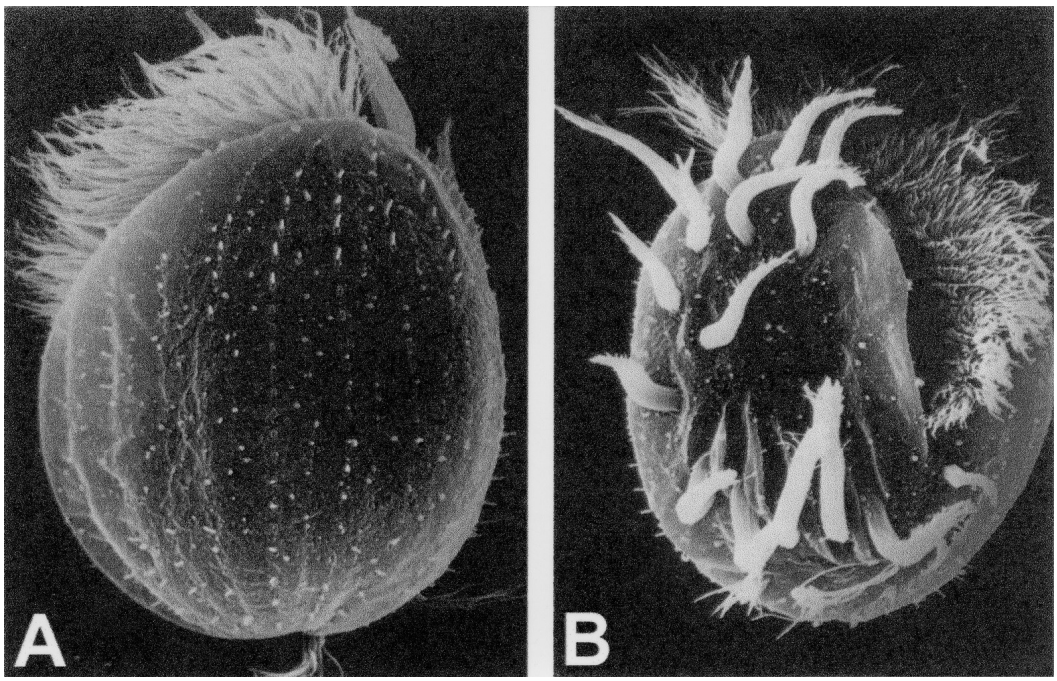

*Fig. 3: Pictures of giant individuals of E. focardii, taken with a Scanning Electron Microscope. In **A** it is shown the dorsal surface (x770); in **B** it is shown the ventral surface (x750).*

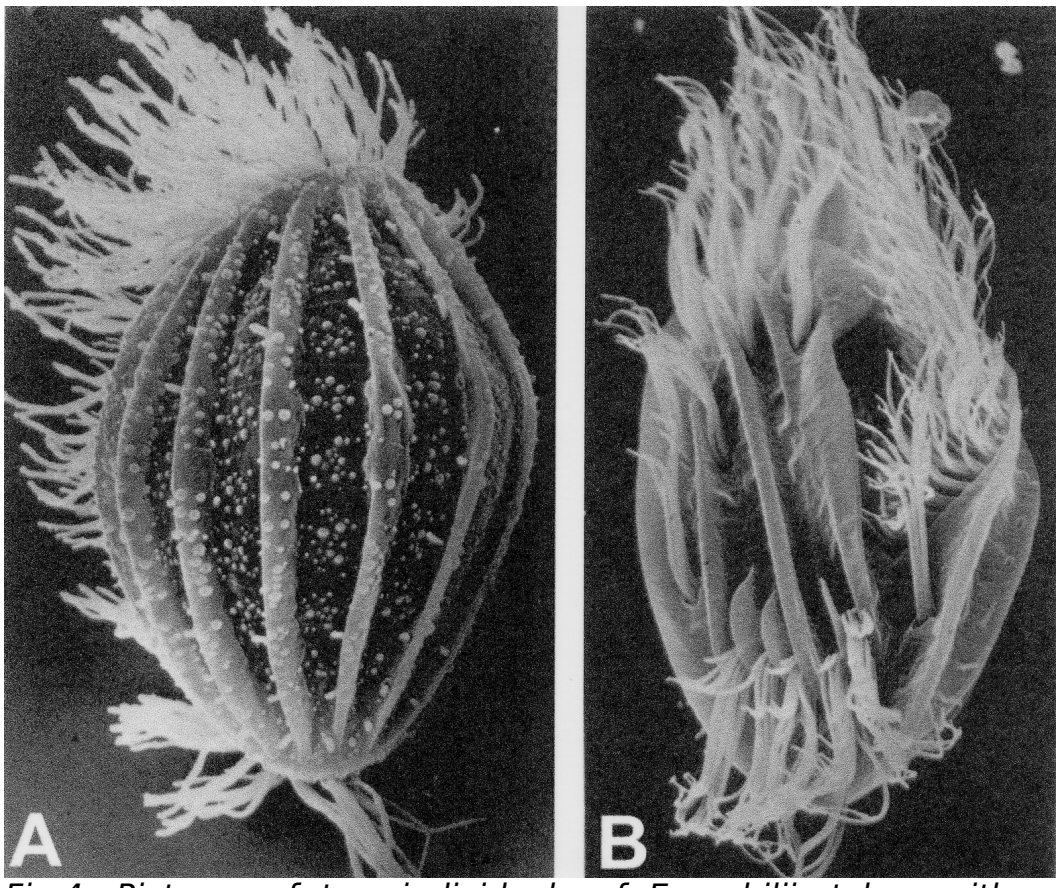

*Fig.4: Pictures of two individuals of E. nobilii, taken with a Scanning Electron Microscope. In **A** it is shown the dorsal surface (x2250); in **B** it is shown the ventral surface (x2250).*

Objectives

During my PhD, the general objective of my work was to contribute to a better knowledge of the molecular basis of Heat Shock Response in eukaryotic microorganisms. In particular, I was interested in analyzing the plasticity of *hsp70* gene expression in marine species of ciliates inhabiting the cold and thermally stable coastal sea water of Antarctica. Useful organism models for analysis of adaptation of gene expression were represented by two Antarctic species of *Euplotes*, *E. focardii* and *E. nobilii*, which showed up marked differences in their capacity to activate *hsp70* gene expression in response to thermal insults. The specific aims of my PhD projects were:

1. To perform a comparative analysis of *hsp70* gene structures (at level of coding and non-coding, regulatory regions) between the two Antarctic species of *Euplotes*, *E. focardii* and *E. nobilii,* with the ultimate goal to identify the causes of the unresponsiveness to thermal stress in *E. focardi*. (Chapter 1)
2. To perform a comparative analysis of *hsp70* gene structures (at level of coding and non coding, regulatory regions) and a preliminary examination of the DNA-binding activity of Heat Shock Factors by means of Electrophoretic Mobility Shift Assay approaches in *E. nobili* and *E. raikovi* a species inhabiting temperate waters and thus, adapted to a fluctuating thermal environment. The possibility to

compare the transcriptional machinery of closely related species adapted to different thermal regimes, might provide valuable clues for unraveling the molecular mechanisms of transcription in the cold. (Chapter 2)

References

Abravaya, K.; Myers, S. & Morimoto, R. (1992), The human heat shock protein HSP70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes & Development* **6**, 1153-1164.

Abravaya, K.; Phillips, B. & Morimoto, R. (1991), Heat shock-induced interactions of heat shock transcription factor and the human hsp70 promoter examined by in vivo footprinting. *Molecular and Cellular Biology* **11**(1), 586-592.

Aguilar, P.; Cronan, J. & de Mendoza, D. (1998), A Bacillus subtilis Gene Induced by Cold Shock Encodes a Membrane Phospholipid Desaturase. *Journal of Bacteriology* **180**(8), 2194-2200.

Arya, R.; Mallik, M. & Lakhotia, S. (2007), Heat shock genes - integrating cell survival and death. *Journal of Biosciences* **32**(3), 595-610.

Baird, S.; Turcotte, M.; Korneluk, R. & Holcik, M. (2006), Searching for IRES. *RNA* **12**(10), 1755.

Balakrishnan, K. & De Maio, A. (2006), Heat shock protein 70 binds its own messenger ribonucleic acid as part of a gene expression self-limiting mechanism. *Cell Stress & Chaperones* **11**(1), 44-50.

Baler, R.; Dahl, G. & Voellmy, R. (1993), Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. *Molecular and Cellular Biology* **13**(4), 2486-2496.

Barchetta, S.; La Terza, A.; Ballarini, P.; Pucciarelli, S. & Miceli, C. (2007 in review), A combination of two regulatory elements in the Tetrahymena thermophilaHSP70-1 gene controls heat shock activation. *Eukaryot Cell.*.

Boy-Marcotte, E.; Perrot, M.; Bussereau, F.; Boucherie, H. & Jacquet, M. (1998), Msn2p and Msn4p Control a Large Number of Genes Induced at the Diauxic Transition Which Are Repressed by Cyclic AMP in Saccharomyces cerevisiae. *Journal of Bacteriology* **180**(5), 1044-1052.

Buckley, B.; Place, S. & Hofmann, G. (2004), Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, Trematomus bernacchii. *Journal of Experimental Biology* **207**(21), 3649-3656.

Chintalapati, S.; Kiran, M. & Shivaji, S. (2004), Role of membrane lipid fatty acids in cold adaptation. *Cellular and Molecular Biology (Noisy-le-grand)* **50**(5), 631-42.

Clarke, A. & Johnston, I. (1996), Evolution and adaptive radiation of Antarctic fishes. *Trends in Ecology and Evolution* **11**(5), 212-218.

Cotto, J. & Morimoto, R. (1999), Stress-induced activation of the heat-shock response: cell and molecular biology of heat-shock factors. *Biochem Soc Symp* **64**, 105-18.

Dinkova, T.; Zepeda, H.; Martinez-Salas, E.; Martinez, L.; Nieto-Sotelo, J. & Jimenez, E. (2005), Cap-independent translation of maize Hsp 101. *The Plant Journal* **41**(5), 722-731.

Do Carmo Avides, M.; Sunkel, C.; Moradas-Ferreira, P. & Rogrigues-Pousada, C. (1990), Properties and partial characterization of the heat-shock factor from Tetrahymena pyriformis. *European Journal of Biochemistry* **194**, 331-336.

Dragovic, Z.; Broadley, S.; Shomura, Y.; Bracher, A. & Hartl, F. (2006), Molecular chaperones of the Hsp110 family act as nucleotide exchange factors of Hsp70. *The EMBO Journal* **25**, 2519-2528.

D'Amico, S. (2002), Molecular basis of cold adaptation. *Philosophical Transactions: Biological Sciences* **357**(1423), 917-925.

Eastman, J.T. Antarctic Fish Biology. Evolution in a Unique Environment. (1993).

Eastman, J. & Clarke, A. (1998), A comparison of adaptive radiation of Antarctic fish with those of non-Antarctic fish. *Fishes of Antarctica a Biological Overview.*, 3-28.

Estruch, F. (2000), Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiology Reviews* **24**(4), 469-86.

Feder, J.; Rossi, J.; Solomon, J.; Solomon, N. & Lindquist, S. (1992), The consequences of expressing hsp70 in Drosophila cells at normal temperatures. *Genes & Development* **6**(8), 1402-1413.

Feder, M. (1999), Organismal, Ecological, and Evolutionary Aspects of Heat-Shock Proteins and the Stress Response: Established Conclusions and Unresolved Issues. *Integrative and Comparative Biology* **39**(6), 857.

Feder, M. & Hofmann, G. (1999), Heat-Shock Proteins, Molecular Chaperones, and The Stress Response: Evolutionary and Ecological Physiology. *Annual Review of Physiology* **61**(1), 243-282.

Frydman, J. & Hartl, F.U. (1994), Molecular chaperone functions of hsp70 and hsp60 in protein folding. *The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Preuss, Cold Spring, New York, NY* 251-283.

Gamer, J.; Multhaup, G.; Tomoyasu, T.; McCarty, J.; Ruediger, S.; Schoenfeld, H.; Schirra, C.; Bujard, H. & Bukau, B. (1996), A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates activity of the *Escherichia coli* heat shock transcription factor sigma super 32. *EMBO Journal* **15**(3), 607-617.

Gehring, W. & Wehner, R. (1995), Heat Shock Protein Synthesis and Thermotolerance in Cataglyphis, an Ant from the Sahara Desert. *Proceedings of the National Academy of Sciences* **92**(7), 2994-2998.

Gething, M. (1997), *Guidebook to molecular chaperones and protein-folding catalysts*, Oxford University Press New York.

Hellen, C. & Sarnow, P. (2001), Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes & Development* **15**(13), 1593-1612.

Hernandez, G.; Vazquez-Pianzola, P.; Sierra, J. & Rivera-Pomar, R. (2004), Internal ribosome entry site drives cap-independent translation of reaper and heat shock protein 70 mRNAs in Drosophila embryos. *RNA* **10**(11), 1783-1797.

Hofmann, G. & Somero, G. (1996), Interspecific variation in thermal denaturation of proteins in the congeneric musselsMytilus trossulus andM. galloprovincialis: evidence from the heat-shock response and protein ubiquitination. *Marine Biology* **126**(1), 65-75.

Hofmann, G.E., Buckley, B. A., Airaksinen, S., Keen, J. E. & Somero, G. N. (2000) The Antarctic fish Trematomus bernacchii lacks heat-inducible heat shock protein synthesis. *J. Exp. Biol* **203**, 2331-2339.

Huey, R. & Bennett, A. (1990), Physiological adjustments to fluctuating thermal environments: an ecological and evolutionary perspective. *Stress Proteins in Biology and Medicine*, 37-59.

Hung, J.; Cheng, T.; Dah-Tsyr Chang, M.; Chen, K.; Huang, H. & Lai, Y. (1998), Involvement of heat shock elements and basal transcription elements in the differential induction of the 70-kDa heat shock protein and its cognate by cadmium chloride in 9 L rat brain tumor cells. *Journal of Cellular Biochemistry* **71**(1), 21-35.

Jia, Z.; DeLuca, C.; Chao, H. & Davies, P. (1996), Structural basis for the binding of a globular antifreeze protein to ice. *Nature* **384**(6606), 285-288.

Johnson, C.; White, D.; Lavender, J.; O'Neill, L. & Turner, B. (2002), Human Class I Histone Deacetylase Complexes Show Enhanced Catalytic Activity in the Presence of ATP and Co-immunoprecipitate with the ATP-dependent Chaperone Protein Hsp70. *Journal of Biological Chemistry* **277**(11), 9590-9597.

Juckes, M.; James, I. & BLACKBURN, M. (1994), The influence of Antarctica on the momentum budget of the southern extratropics. *Quarterly Journal of the Royal Meteorological Society* **120**(518), 1017-1044.

Kabani, M.; Beckerich, J. & Brodsky, J. (2002), Nucleotide Exchange Factor for the Yeast Hsp70 Molecular Chaperone Ssa1p. *Molecular and Cellular Biology* **22**(13), 4677-4689.

Kobayashi, N. & McEntee, K. (1993), Identification of *cis* and *trans* components of a novel heat shock stress regulatory pathway in Saccharomyces cerevisiae. *Molecular and Cellular Biology* **13**(1), 248-256.

Krebs, R. & Bettencourt, B. (1999), Evolution of Thermotolerance and Variation in the Heat Shock Protein, Hsp70. *Integrative and Comparative Biology* **39**(6), 910.

Krebs, R. & Feder, M. (1997), Deleterious Consequences of Hsp70 Overexpression in Drosophila melanogaster Larvae. *Cell Stress & Chaperones* **2**(1), 60-71.

La Terza, A.; Miceli, C. & Luporini, P. (2004), The gene for the heat-shock protein 70 of *Euplotes focardii*, an Antarctic psychrophilic ciliate. *Antarctic Science* **16**(01), 23-28.

La Terza, A.; Passini, V.; Barchetta, S. & Luporini, P. (2007), Adaptive evolution of the heat-shock response in the Antarctic psychrophilic ciliate, *Euplotes focardii*: hints from a comparative determination of the hsp70 gene structure. *Antarctic Science* **19**(02), 239-244.

Lakhotia, S. (1998), Stress biology: A challenging area in

integrated biology. *Biology International*, 18-30.

Lau, D.; Saeed-Kothe, A.; Parker, S. & William Detrich III, H. (2001), Adaptive Evolution of Gene Expression in Antarctic Fishes: Divergent Transcription of the 5'-to-5' Linked Adult alpha- and beta-Globin Genes of the Antarctic Teleost Notothenia coriiceps is Controlled by Dual Promoters and Intergenic Enhancers. *American Zoologist* **41**(1), 113-132.

Laufen, T.; Mayer, M.; Beisel, C.; Klostermeier, D.; Mogk, A.; Reinstein, J. & Bukau, B. (1999), Mechanism of regulation of Hsp70 chaperones by DnaJ cochaperones. *Proceedings of the National Academy of Sciences* **96**(10), 5452-5457.

Lee, H.; Sengupta, N.; Villagra, A.; Rezai-Zadeh, N. & Seto, E. (2006), Histone Deacetylase 8 Safeguards the Human Ever-Shorter Telomeres 1B (hEST1B) Protein from Ubiquitin-Mediated Degradation. *Molecular and Cellular Biology* **26**(14), 5259-5269.

Lee, S.; Pelham, H.; Carlson, T.; Christian, N.; Lea, K.; Kedzie, J.; Reilly, J. & Bonner, J. (2000), The Yeast Heat Shock Transcription Factor Changes Conformation in Response to Superoxide and Temperature. *Molecular Biology of the Cell* **11**(5), 1753-1764.

Lerman, D. (2001), Laboratory selection at different temperatures modifies heat-shock transcription factor (HSF) activation in Drosophila melanogaster. *Journal of Experimental Biology* **204**(2), 315-323.

Lerman, D.; Michalak, P.; Helin, A.; Bettencourt, B. & Feder, M. (2003), Modification of Heat-Shock Gene Expression in Drosophila melanogaster Populations via Transposable Elements. *Molecular Biology and Evolution* **20**(1), 135-144.

Liberek, K.; Marszalek, J.; Ang, D.; Georgopoulos, C. & Zylicz, M. (1991), *Escherichia coli* DnaJ and GrpE Heat Shock Proteins Jointly Stimulate ATPase Activity of DnaK. *Proceedings of the National Academy of Sciences* **88**(7), 2874-2878.

Lis, J. T. & Wu, C. (1994), Transcriptional regulation of heat shock genes. *Transcription: Mechanisms and Regulation*, 459-475.

Malter, J. (2001), Regulation of mRNA stability in the nervous system and beyond. *Journal of Neuroscience Research* **66**(3), 311-6.

Marx, J.; Blaise, V.; Collins, T.; D'Amico, S.; Delille, D.; Gratia, E.; Hoyoux, A.; Huston, A.; Sonan, G.; Feller, G. & others (2004), A perspective on cold enzymes: current knowledge and frequently asked questions. *Cellular and Molecular Biology (Noisy-le-grand)*

50(5), 643-55.

Mayer, M. & Bukau, B. (2005), Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences (CMLS)* **62**(6), 670-684.

Morimoto, R. (1993), Cells in stress: transcriptional activation of heat shock genes. *Science* **259**(5100), 1409-10.

Orsi, A.; Whitworth, T. & Nowlin, W. (1995), On the meridional extent and fronts of the Antarctic Circumpolar Current. *Deep Sea Research Part I: Oceanographic Research Papers* **42**(5), 641-673.

Peck, L.S., Convey, P. & Barnes, D.K.A. Environmental constraints on life histories in Antarctic ecosystems: tempos, timings and predictability". *Biological Reviews* **81**, 75-109 (2005).

Petit, J., Jouzel, J., Raynaud, D., Barkov, N., Barnola, J., Basile, I.; Bender, M.; Chappellaz, J.; Davis, M.; Delaygue, G. & others (1999), Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature* **399**, 429-436.

Pirkkala, L.; Nykanen, P. & Sistonen, L. (2001), Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *The FASEB Journal* **15**(7), 1118-1131.

Pucciarelli, S., Di Giuseppe, G., Dini, F., Luporini, P., Miceli, C. (2003), *Euplotes focardii*, an Antarctic psychrophilic ciliate of presumed Gondwanan origin. *Journal of Eukaryotic Microbiology* **50**, 38-38.

Ray, P. (1999), Stress genes and species survival. *Molecular and Cellular Biochemistry* **196**(1), 117-123.

Raymond, J.; Wilson, P. & DeVries, A. (1989), Inhibition of Growth of Nonbasal Planes in Ice by Fish Antifreezes. *Proceedings of the National Academy of Sciences* **86**(3), 881-885.

Rieger, T.; Morimoto, R. & Hatzimanikatis, V. (2005), Mathematical Modeling of the Eukaryotic Heat-Shock Response: Dynamics of the hsp70 Promoter. *Biophysical Journal* **88**(3), 1646-1658.

Rinehart, J.; Hayward, S.; Elnitsky, M.; Sandro, L.; Lee Jr, R. & Denlinger, D. (2006), Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proceedings of the National Academy of Sciences* **103**(38), 14223-14227.

Ritossa, F. A (1962) A new puffing system induced by temperature shock and DNP in Drosophila. *Experientia* **18**, 571-

573.

Rubtsova, M.; Sizova, D.; Dmitriev, S.; Ivanov, D.; Prassolov, V. & Shatsky, I. (2003), Distinctive Properties of the 5'-Untranslated Region of Human Hsp70 mRNA. *Journal of Biological Chemistry* **278**(25), 22350-22356.

Sarge, K.; Murphy, S. & Morimoto, R. (2003), Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Molecular and Cellular Biology* **1993**, 1392-1407.

Schmitt, A. & McEntee, K. (1996), Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences* **93**(12), 5777-5782.

Shaw, P.; Arkhipkin, A. & Al-Khairulla, H. (2004), Genetic structuring of Patagonian toothfish populations in the Southwest Atlantic Ocean: the effect of the Antarctic Polar Front and deep-water troughs as barriers to genetic exchange. *Molecular Ecology* **13**(11), 3293-3303.

Somero, G. (2004), Adaptation of enzymes to temperature: searching for basic strategies. *Comp Biochem Physiol B Biochem Mol Biol* **139**(3), 321-33.

Suzuki, I.; Kanesaki, Y.; Mikami, K.; Kanehisa, M. & Murata, N. (2001), Cold-regulated genes under control of the cold sensor Hik 33 in Synechocystis. *Molecular Microbiology* **40**(1), 235-244.

Tachibana, T., Astumi, S., Shiod,R., Uenom, M., Uritani, M., Ushimaru, T. (2002) A Novel Non-conventional Heat Shock Element Regulates Expression of MDJ1 Encoding a DnaJ Homolog in Saccharomyces cerevisiae. *Journal of Biological Chemistry* **277**, 22140-22146.

Vagner, S. & others (2001), Irresistible IRES: Attracting the translation machinery to internal ribosome entry sites. *EMBO Reports* **2**(10), 893-898.

Valbonesi, A. & Luporini, P. (1993), Biology of *Euplotes focardii*, an Antarctic ciliate.” *Polar Biology* **13**, 489-493.

Wilusz, C. & Wilusz, J. (2004), Bringing the role of mRNA decay in the control of gene expression into focus. *Trends in Genetics* **20**(10), 491-497.

Xavier, I.; Khachatourians, G. & Ovsenek, N. (1999), Constitutive

and Heat-Inducible Heat Shock Element Binding Activities of Heat Shock Factor in a Group of Filamentous Fungi. *Cell Stress & Chaperones* **4**(4), 211-222.

Zatsepina, O. (2000), Thermotolerant desert lizards characteristically differ in terms of heat-shock system regulation. *Journal of Experimental Biology* **203**(6), 1017-1025.

Zatsepina, O.; Velikodvorskaia, V.; Molodtsov, V.; Garbuz, D.; Lerman, D.; Bettencourt, B.; Feder, M. & Evgenev, M. (2001), A Drosophila melanogaster Strain From Sub-Equatorial Africa Has Exceptional Thermotolerance But Decreased Hsp70 Expression. *Journal of Experimental Biology* **204**(11), 1869-1881.

Zou, J.; Guo, Y.; Guettouche, T.; Smith, D. & Voellmy, R. (1998), Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* **94**, 471-480.

Zuo, J.; Baler, R.; Dahl, G. & Voellmy, R. (1994), Activation of the DNA-binding ability of human heat shock transcription factor 1 may involve the transition from an intramolecular to an intermolecular triple-stranded coiled-coil structure. *Molecular and Cellular Biology* **14**(11), 7557—7568.

Zuo, J.; Rungger, D. & Voellmy, R. (1995), Multiple layers of regulation of human heat shock transcription factor 1. *Molecular and Cellular Biology* **15**(8), 4319-4330.

Chapter 1

Adaptive evolution of the heat-shock response in the Antarctic psychrophilic ciliate, Euplotes focardii: hints from a comparative determination of the HSP70 gene structure

Antonietta La Terza, Valerio Passini, Sabrina Barchetta and

Pierangelo Luporini

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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 obtain initial information on the genetic causes of this eccentric behavior 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 behavior 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 favoring mRNA degradation were instead detected in the *hsp70* 3' regulatory region of *E*. nobilii, but not in that of *E. focardii*. Overall these observations were reputed to lend further support to the hypothesis that the causes of the *Euplotes focardii* unresponsiveness to thermal stress reside in some structural, or functional modifications of transcriptional *trans*-activating factors.

Key words: Ciliates, extremophiles, cold-adaptation, *cis*- and

trans-regulatory elements, stress-inducible genes, HSP70.

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 and Song, 2005; Petz, 2005). Particularly common are species of the most cosmopolitan and ubiquitous ciliate, *Euplotes* (Borror and 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 Ross Sea (Valbonesi and 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 date far antecedent to the definitive ecological separation of Antarctica from Gondwana (F. Dini and G. Di Giuseppe, personal communication). Indeed, *E. focardii* thrives optimally at a temperature of 4-5°C as any psychrophilic organism (Valbonesi and Luporini, 1993), utilizes unique genetic solutions to ensure microtubule stability (Pucciarelli et al., 1997; Pucciarelli and 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 elicited 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 seek 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*, that 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 and Shyu, 1995; Wilusz and 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.

Material 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 [γ32 P] 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 (0.5M EDTA, 1% SDS, 10mM Tris-HCl, pH 9.5) containing 200µg/ml 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 50µg/ml 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 by small dimension (in the range from 500 to 20,000bp) 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 involving, 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 involving, 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 labelling, 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 and 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 and 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 2279bp 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*, of 1983bp specific for a protein of 660 amino acids; in *E. nobilii*, of 1980bp 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. 5, 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 P446, and I for V469 (numeration according to the rat HSP70 sequence), appear of more immediate interest as they lie, as shown in Fig. 6, 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 and Zuiderweg, 2004).

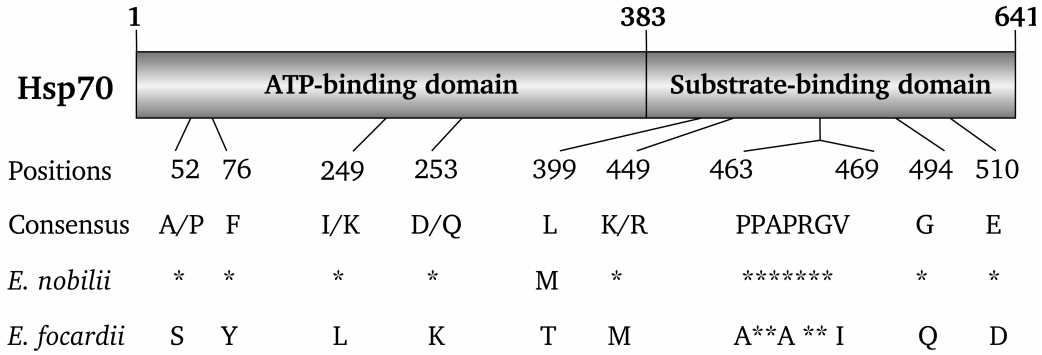

Fig. 5: Amino acid substitutions that occur in E. focardii at the level of its HSP70 ATP- and substrate-binding domains, and are unique with respect to E. nobilii and other organisms. Numbers of the amino acid positions are reported essentially according to Sriram et al., (1997) and Morshauser et al., (1999). Asterisks indicate identities. The consensus sequence was produced from an alignment of HSP70 sequences available at the NCBI GeneBank database.

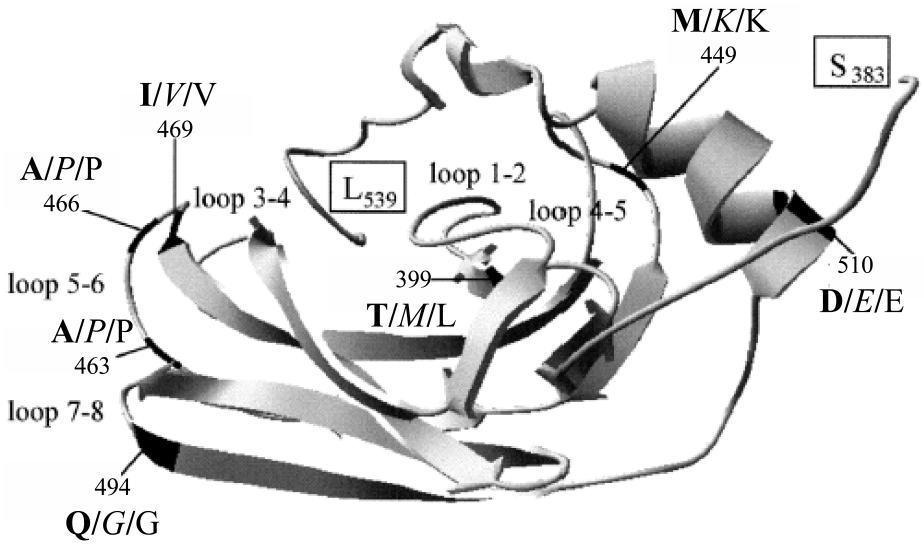

Fig. 6: Molecular architecture of the HSP70 substrate-binding domain showing the positions of the amino acid substitutions that distinguish this domain in E. focardii with respect to E. nobilii and other organisms. Amino acid positions and loops are numbered according to the rat HSP70 structure represented 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 $5'C_4A_43'/3'G_4T_45'$ telomeric repeats typical of the subchromosomic macronuclear genes of *Euplotes* (Hofmann et al., 1995; Jahn and Klobutcher, 2002).

As shown in Fig. 8, 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 harbors 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 (Kobayashi and McEntee, 1993; Fernandes et al., 1994; Ruis and 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 and 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 63, 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., 2006). One of these ARE elements is present in the *hsp70* 3’ region of *E. nobilii* between positions 2176 and 2180; none in *E. focardii*.

Euplotes focardii

CCCCAAAACCCCAAAACCCCAAAACCCCGGAATTCTAAATCAACATTGAA 50

TATTTCAACATAAAAATATACTATTAATTCCACTCTTTATTTCTAAAAAC 100

GAGATGAAAATCCATTAAAAAGTTCCATTTCATTTTGAGAATATTCGAGA 150

ATCTTCCTTTATCTCTAAAATTAATTATAAAATTTGCCAAATACGTCGTT 200

TTCCCTCTTTAATCATCTCCTTGAGCCTTCTATGATGTCTGAACTTGCTG 250

AAAGAGGATTAAAAGGATGGATTTTGGTTCTATTTCCGCTAAAATGATAT 300

TTTTCAAAAATCCAGATTTTTCTCCTAAATTCTAGAATTTTTCCAGGATA 350

GGTATTTAAAAATCCGAAAATAGTCTTCATTAATTAAAAATTAAAAACAC 400

TAACAAA**ATG**(410)

Euplotes nobilii

CCCCAAAACCCCAAAACCCCAAAACCCCGGTAATAGGTGAATTGTTGAAT 50

TTTCACTGTAAAAATTAATCGAAGTTTCCCTAAAGTTCGATTTAAATTAG 100

AATTATCTAGATTTGGGGGATTAAATACTATTTAAGTAAAATTCTGGGAGA 150

CTAATTAATTAATAAATTTTAATAAATAAT**ATG**(183)

Fig. 7: 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.

Euplotes focardii

TAATGATATAACTTAAGTTGTAATTCTTTAATATCTTTAACAAAATCCAT 2437

TTGTTCTTATAAACCTAGTTTTTAGTTTGATTTTACATAATGGGGTTTTG 2487

GGGTTTTGGGGTTTTGGGG(2506)

Euplotes nobilii

TAAACATATAAATAACCCATTTATTGAGTTTTAATAAGCAAATTGCACAA 2207

AAAGCAGTGTCTACCATTTATGTTCAACCATACTAGATATATGAGGGGTT 2257

TTGGGGTTTTGGGGTTTTGGGG(2279)

Fig. 8: Nucleotide sequences of the 3'regulatory regions of the E. HSP70 genes. Telomeric G_4T_4 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.

Conclusions

Rare are the cases of organisms that no longer respond to a heat-shock by promptly activating the expression of their *hsp70* genes. They appear limited to species such as *Hydra oligactis* (Bosch et al., 1988; Geller 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, and implying 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 stably cold waters of the

Antarctic coasts would have determined a selective silencing 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 instead have maintained its activity. How might evolutionary adaptation have operated in silencing the HSE-modulated mechanism? The fact that the HSE consensus sequence presents an orthodox organization would exclude that this silencing 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. Credit for this hypothesis derives also from 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 the organism viability (Smith and Yaffe, 1991; Trinklein et al., 2004; Yamamoto et al., 2005; Yamamoto and Sakurai, 2006).

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References

Abravaya, K., Phillips, B. & Morimoto, R.I. Heat shock-induced interactions of heat shock transcription factor and the human hsp70 promoter examined by in vivo footprinting. *Molecular and Cellular Biology* **11**, 586-592 (1991).

Barreau, C, Paillard, L., Osborne, H.B., Journals, O., AU-rich elements and associated factors: are there unifying principles. *Nucleic Acids Research* **33**, 7138-7150 (2006).

Borror, A.C. & Hill, B.F. The order Euplotida (Ciliophora): taxonomy, with division of *Euplotes* into several genera. *J. Eukaryot. Microbiol* **42**, 457-466 (1995).

Bosch, T.C.G. et al., Thermotolerance and Synthesis of Heat Shock Proteins: These Responses are Present in Hydra attenuata but Absent in Hydra oligactis. *Proceedings of the National Academy of Sciences* **85**, 7927-7931 (1988).

Brennecke, T., Gellner, K. & Bosch, T.C.G. The lack of a stress response in Hydra oligactis is due to reduced hsp 70 mRNA stability. *European Journal of Biochemistry* **255**, 703-709 (1998).

Buckley, B.A., Place, S.P. & Hofmann, G.E. Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, Trematomus bernacchii. *Journal of Experimental Biology* **207**, 3649-3656 (2004).

Chen, C.Y. & Shyu, A.B. AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* **20**, 465-70 (1995).

Estruch, F. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiology Reviews* **24**, 469-86 (2000).

Fernandes, M., O'Brien, T. & Lis, J.T. Structure and regulation of heat shock gene promoters. *The Biology of Heat Shock Proteins and Molecular Chaperones* 375\uc0\u150393 (1994).

Gellner, K., Praetzel, G. & Bosch, T. Cloning and expression of a heat-inducible hsp 70 gene in two species of Hydra which differ in their stress response. *European Journal of Biochemistry* **210**, 683-691 (1992).

Guex, N. & Peitsch, M.C. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling.

Graumann, P. & Marahiel, M.A. Some like it cold: response of microorganisms to cold shock. *Archives of Microbiology* **166**, 293-300 (1996).

Hoffman, D.C. et al., Macronuclear gene-sized molecules of hypotrichs. *Nucleic Acids Res* **23**, 1279-1283 (1995).

Hofmann, G.E. Heat-shock protein expression is absent in the antarctic fish Trematomus bernacchii (family Nototheniidae). *Journal of Experimental Biology* **203**, 2331-2339 (2000).

Jahn, C.L. & Klobutcher, L.A. Genome Remodeling in Ciliated Protozoa. *Annual Review of Microbiology* **56**, 489-520 (2002).

Kobayashi, N. & McEntee, K. Identification of *cis* and *trans* components of a novel heat shock stress regulatory pathway in Saccharomyces cerevisiae. *Molecular and Cellular Biology* **13**, 248-256 (1993).

La Terza, A., Papa, G., Miceli, C. & Luporini, P. Unresponsiveness of *Euplotes focardii hsp70* genes to thermal stress. *Italian Journal of Zoology*, Supplement **1**, 111-114.

La Terza, A. Papa, G., Miceli, C. & Luporini, P. Divergence between two Antarctic species of the ciliate *Euplotes*, *E. focardii* and E. nobilii, in the expression of heat-shock protein 70 genes. *Molecular Ecology* **10**, 1061-1067 (2001).

La Terza, A., Miceli, C. & Luporini, P. The gene for the heat-shock protein 70 of *Euplotes focardii*, an Antarctic psychrophilic ciliate. *Antarctic Science* **16**, 23-28 (2004).

Morshauser, R.C., Hu, W., Wang, H., Pang, Y., Flynn, GC., Zuiderweg, E.R.P. High-resolution Solution Structure of the 18kDa Substrate-binding Domain of the Mammalian Chaperone Protein Hsc70. *Journal of Molecular Biology* **289**, 1387-1403 (1999).

Petz, W. Ciliates. in Scott, F.J. & Marchant, H.J. Antarctic marine protists. Canberra: *Australian Biological Resources Study, Goanna Print, 347-448* (2005).

Petz, W., Song, W. & Wilbert, N. Taxonomy and Ecology of the Ciliate Fauna (Protozoa, Ciliophora) in the Endopagial and Pelagial of the Weddell Sea, Antarctica. (1995).

Pirkkala, L., Nykanen, P. & Sistonen, L. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *The FASEB Journal* **15**, 1118-1131 (2001).

Place, S.P., Zippay, M.L. & Hofmann, G.E. Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible hsp70 gene in Antarctic notothenioid fishes. *American Journal of Physiology- Regulatory, Integrative and Comparative Physiology* **287**, 429-436 (2004).

Pucciarelli, S. & Miceli, C. Characterization of the cold-adapted a-tubulin from the psychrophilic ciliate *Euplotes focardii*. *Extremophiles* **6**, 385-389 (2002).

Pucciarelli, S., Ballarini, P. & Miceli, C. Cold-adapted microtubules: Characterization of tubulin posttranslational modifications in the Antarctic ciliate*Euplotes focardii*. *Cell Motility and the Cytoskeleton* **38**, 329-340 (1997).

Ruis, H. & Schuller, C. Stress signaling in yeast. *Bioessays* **17**, 959-65 (1995).

Sambrook, J. & Russell, D.W. Molecular Cloning: A Laboratory Manual. (2001).

Schmitt, A.P. & McEntee, K. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences* **93**, 5777-5782 (1996).

Seegmiller, A. et al., Internal eliminated sequences interrupting the *Oxytricha* 81 locus: allelic divergence, conservation, conversions, and possible transposon origins. *Molecular Biology and Evolution* **13**, 1351-1362 (1996).

Smith, B.J. & Yaffe, M.P. Uncoupling Thermotolerance from the Induction of Heat Shock Proteins. *Proceedings of the National Academy of Sciences* **88**, 11091-11094 (1991).

Sriram, M. et al., Human Hsp70 molecular chaperone binds two calcium ions within the ATPase domain. *Structure* **5**, 403-414 (1997).

Thompson, J.D., Higgins, D.G. & Gibson, T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680 (1994).

Trinklein, N.D. et al., The Role of Heat Shock Transcription Factor 1 in the Genome-wide Regulation of the Mammalian Heat Shock Response. *Molecular Biology of the Cell* **15**, 1254-1261 (2004).

Valbonesi, A. & Luporini, P. Description of two new species of

Euplotes and *Euplotes rariseta* from Antarctica. *Polar Biology* **11**, 47-53 (1990).

Valbonesi, A. & Luporini, P. Biology of *Euplotes focardii*, an Antarctic ciliate. *Polar Biology* **13**, 489-493 (1993).

Wilbert, N. & Song, W. New contributions to the marine benthic ciliates from the Antarctic area, including description of seven new species (Protozoa, Ciliophora). *Journal of Natural History* **39**, 935-973 (2005).

Wilusz, C.J. & Wilusz, J. Bringing the role of mRNA decay in the control of gene expression into focus. *Trends in Genetics* **20**, 491-497 (2004).

Yamamoto, A. & Sakurai, H. The DNA-binding domain of yeast Hsf1 regulates both DNA-binding and transcriptional activities. *Biochem Biophys Res Commun* **346**, 1324-9 (2006).

Yamamoto, A., Mizukami, Y. & Sakurai, H. Identification of a Novel Class of Target Genes and a Novel Type of Binding Sequence of Heat Shock Transcription Factor in Saccharomyces cerevisiae. *Journal of Biological Chemistry* **280**, 11911 (2005).

Zhang, Y., Zuiderweg, E.R.P. & Horwich, A.L. The 70-kDa Heat Shock Protein Chaperone Nucleotide-Binding Domain in Solution Unveiled as a Molecular Machine That Can Reorient Its Functional Subdomains. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 10272-10277 (2004).

Chapter 2

Plasticity of the HSR in two phylogenetically related marine species of *Euplotes: E. nobilii* and *E. raikovi*

Manuscript in preparation

Introduction

Temperature, a key factor in establishing growth, reproduction, and distribution of organisms, lacks spatial and temporal constancy in the majority of the environments with very few exceptions such as the cold coastal sea water of Antarctica where the temperature rarely varies from the freezing point of seawater: -1.86°C. For this reason, living organisms employ diverse adjustments at multiple levels of biological organization (from physiological to molecular mechanisms) to deal with the fluctuating nature of the thermal environment (Precht, 1973; Cossins and Bowler, 1987; Hochachka and Somero, 2002). One of such response, the Heat shock response (HSR), consists in the rapid induction of a specific set of proteins known as Heat shock protein (HSPs) following an acute increase in temperature (Lindquist, 1980). HSPs are molecular chaperones that play important roles in protein biosynthesis and protection from thermally induced protein denaturation and aggregation across virtually every taxa (Parsell et al.,, 1993; Hartl and Hayer-Hartl, 2002). In general, HSPs expression is considered a good marker for estimating the physiological effects of thermal variation because their expression is extremely sensitive to temperature and reflects the thermal as well as the evolutionary history of organisms (Feder and Hofmann, 1999). In the last few years, the induction of HSPs has readily become important to ecologist and

evolutionary biologists as a model of environmental controlled gene expression and source of phenotipyc plasticity. Current research (see Feder and Hofmann, 1999 as review), is addressed to investigate the processes underlying the plasticity of the HSR response from an integrate point of view, comprehensive of the molecular, evolutionary and ecological aspects. To this regard, useful models to analyse these issues are represented by two species of ciliates, which are endemic in Antarctic coastal seawater, *Euplotes focardii* and *E. nobilii* (Valbonesi and Luporini, 1990, 1993; unpublished results). In a previous study (La Terza et al., 2001) we showed that *Euplotes focardii* and *E. nobilii*, which behave as psychrophile and psychrotroph microorganisms, respectively, deeply diverge in their capacities to respond to thermal stress with an activation of the transcription of their *hsp70* genes, a universally conserved HSPs, whose function is to assist cells in defence against environmental stresses (Frydman and Hartl, 1994). Following a thermal stress, only *E. nobilii* is able to strongly activate the transcription of its *hsp70* genes while, *E. focardii* does not show any appreciable induction of its *hsp70* genes, thus suggesting that the *hsp70* genes of this Antarctic ciliate have greatly reduced a function which is apparently useless in the thermally immutable environment where it lives. Thus, *E. focardii* offers one of the most extreme case of plasticity of the HSR: it has been lost altogether.

This and other major features such as the structural

modifications of tubulin genes to ensure microtubule polymerization in the cold (Pucciarelli et al., 2002; Pucciarelli and Miceli, 2002) strongly support the idea that *E. focardii* is an ancient colonizer of Antarctica and among the several species of *Euplote*s collected from different site in Terra Nova Bay, one of the most cold-adapted. This hypothesis is further sustained by data from SSrRNA analysis that date the separation of this species from an ancestral Gondwana species much earlier than the biogeographical isolation of Antarctica caused by the circum-Antarctic Current about 25 million year ago (Di Giuseppe et al., unpublished results).

Differently, the SSrRNA data and the typical psychrotroph behaviour suggest that *E. nobilii* can be regarded as a secondary colonizer of Antarctica. This species has retained the capacity to switches on the transcription of its *hsp70* genes when it is transferred from an environmental temperature of 4°C to 20°C. Thus, it may be regarded as one of the many organisms that faces thermal stress with new synthesis of heat-shock proteins. Only the threshold temperature of 20°C at which this synthesis is switched on is somewhat unusual. Indeed, it is markedly lower than the 35°C required for the induction of *hsp70* gene transcription in other ciliates of temperate waters, such as *Oxytricha nova* (Anderson et al., 1996) and *Moneuplotes crassus* (Ullmann et al., 2004).

In order to gain insights in the molecular mechanisms regulating

thermal threshold for *hsp70* genes in *E. nobilii* we carried out a comparative analysis of the full-length structures between this gene and its homolog cloned from *E. raikovi* a phylogenetically related species, inhabiting temperate waters and thus, adapted to a fluctuating thermal environment in a range of 8-32°C (during the year in the Adriatic Sea). Moreover, we identified the minimum heat shock temperature (T_{on}) required to induce *hsp70* gene transcription in *E. raikovi* and we performed an examination of the DNA-binding activity of Heat Shock Factors by means of EMSA approaches in both species. Preliminary results from this study support the idea that the modification of the T_{on} of *E. nobilii* (20°C vs 35°C of *E. raikovi*) might reside in a mutated capacity of the transcriptional (*trans*-acting) factor (HSF) to bind and activate HSE.

Materials and Methods

Cell cultures

The results described in this article were obtained from experiments carried out on cultures of strains TN1 (*E. nobilii*) and ER1 (*E. raikovi*). TN1 strain was isolated from sediment and seawater samples repeatedly collected from the coastal waters of Terra Nova Bay (temperature, –1.8°C; salinity, 35‰; pH, 8.1–8.2) (Valbonesi and Luporini, 1990a,b, unpublished). This culture was grown in a cold room at 2– 4°C, under a rhythm of 16 h of darkness and 8 h of very weak light. *E. raikovi* was instead

collected from sea water of Porto Recanati (Italy) and it was grown at room temperature (23°C), in marine water characterized by salinity 29-30% and pH 8.1-8.2. The daily light/darkness cycle was divided equally (12h/12h). For both the cultures *Dunaliella tertiolecta* green algae was used as food. The cells were subject to stressing treatment during logarithmic phase.

Stress Conditions

Cells were stressed accordingly to the following protocol. The right amount of cells per sample (read further) was gathered by mild centrifugation (about 3000rcf) in large volume tube (150ml/175ml) from cultures raised at best conditions. Then the cells were abruptly shocked in pre-heated marine water for appropriate time and temperature: for total RNA extraction *E. raikovi* was treated 30 minutes at 30°C, 35°C and 40°C, while *E. nobilii* was treated 30 minutes at 15°C, 20°C and 25°C; for nuclear extraction *E. raikovi* was shocked at 40°C, while *E. nobilii* at 25°C, both for 30 minutes. In every experiment a control was sampled considering as a control the best growing temperature. Samples were then collected by centrifugation, separated from the culture medium, and treated for nuclear protein or RNA extraction.

Nuclear extracts

For every nuclear extraction about three million cells for *E. nobilii* and about four million cells for *E. raikovi* were used. The

integrity of the nuclei was controlled during every step using a fluorescence microscope and DAPI 2μg/ml for staining nuclei. Cells were first collected by centrifugation and then transferred in a 1.5ml tube, after they were resuspended in Lysis Buffer (320mM sucrose, 10mM Tris-HCl pH 8.0, 3mM CaCl2, 2mM MgOAc, 0.1mM EDTA, 0.2% v/v NP-40, 1mM DTT, and 0.5mM PMSF) and kept for 3 minutes on ice. Membranes and nuclei were then precipitated at 500rcf at 4°C for 5 minutes and the supernatant was discarded. The pellet was gently washed with Sucrose Buffer (320mM sucrose, 10mM Tris-HCl pH 8.0, 3mM CaCl2, 2mM MgOAc, 0.1mM EDTA, 1mM DTT, and 0.5mM PMSF) and centrifuged again at 500rcf at 4°C for 5 minutes. Low Salt Buffer (20mM HEPES, 1.5mM MgCl2, 20mM KCl, 0.2mM EDTA, 25% v/v glycerol, 0.5mM DTT, 0.5mM PMSF, 1X Cocktail Proteases Inhibitors) was added to resuspend gently the intact nuclei and then High Salt Buffer (20mM HEPES, 1,5mM MgCl2, 800mM Kcl, 0,2mM EDTA, 25% v/v glycerol, 0.5mM DTT, 0.5mM PMSF, 1X Cocktail Proteases Inhibitors) was added carefully and mixed slowly. The nuclei were left to shake mildly at 4°C for 45 minutes and after that period the tube was centrifuged no more than 14000rcf for 15 minutes at 4°C. Finally the supernatant was recovered and stored in liquid nitrogen. Protein concentration was estimated using the Bradford method.

DNA extraction

DNA necessary for amplification in RATE-PCR was extracted from

E. raikovi cells using urea method. The about 5 million cells were pelleted at 3000rpm for 5 minutes in a 1,5ml tube. The supernatant was wasted and cells were resuspend in 0,7ml of urea buffer (350mM NaCl, 5mM Tris-HCl ph7.4, 0,1%SDS 10mM EDTA, 7M Urea). The mixture in the tube was mixed for 5 minutes and then followed twice by extraction with 500µl of Phenol/Chloroform/Isoamilic Alcohol (1:1:1 volume ratio) to get rid of cellular debris. The aqueous phase containing DNA was collected after centrifugation at ≈16000rcf for 2 minutes. 500µl were recovered from the tube, transferred in a clean one and then mixed with 150µl of NaCl 5M. Isopropilic Alcohol was added to the solution, well mixed and then centrifuged at ≈16000rcf for 10 minutes at room temperature. Supernatant was discarded and pellet containing DNA was washed twice with Ethanol 70% and then allowed drying. The pellet was resuspended in 50µl of sterile water together with 2µl of RNAse A (3mg/ml) and incubated at 55°C for 2 hours. Incubation was followed by another extraction with Phenol/Chloroform (1:1) to remove RNAse A and digested nucleotides. The aqueous phase was recovered and then it was mixed with LiCl 0,5M and cold absolute Ethanol (1:1 volume ratio), stored at -20°C for 1hour and centrifuged at max speed for 15 minutes. The purified DNA was resuspended in sterile water, quantified and stored at -20°C for further use.

RNA extraction

RNA was extracted from a varying amount of *Euplotes* cells (ranging between 3.5 and 6 millions) using Trizol and following the protocol provided by GibcoBRL. The RNA was quantified and its purity estimated using U.V light spectrophotometry (Sambrook et al., 2003).

Northern Blot Analysis

Northern blots analysis was performed according to standard procedures (Sambrook et al., 2003), after electrophoresis on 1.2% formaldehyde agarose gels. All hybridizations were carried out on Hybond-N membranes overnight at 65°C in a solution containing 6xSSC, Denhardt solution, 0.1% SDS, and 100µg/ml denatured tRNA. Blotted membranes were washed under high stringency conditions (0.5xSSC containing 0.1% SDS) at the hybridization temperature, dried, and exposed for autoradiography. Probes from the ATPase domain of *E. raikovi hsp70* gene and from 16S ribosomal subunit gene, were labelled with 32P by the random hexamer priming method (Feinberg and Vogelstein, 1984).

RATE-PCR strategy

To amplify the whole *hsp70* gene, we exploited the gene-size structure of macronuclear DNA using degenerated primers in combination with unspecific primer matching telomeres (C_4A_4) in a technique that is called Rapid Amplification Telomeric Ends or RATE-PCR (Hoffman et al., 1995). The HSP70 gene sequence at

the 5’ end was obtained by subjecting macronuclear DNA samples of *E. raikovi* to a first set of 30–35 cycles of amplification. This used, as reverse primer, the oligonucleotide 5’-GCATCIATATCIAAIGTIACITCIAT-3’ (where I stands for Inosine) 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 obtained in a similar way 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 specific primers.

Gene cloning 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. Sequence reactions were carried out with the ABI Prism sequence analyzer, Model 373A, by using the Big Dye Terminator Methodology (PE Applied Biosystems). The sequences were performed by an external service, CRIBI, settled in Padova (PD) Italy. Sequence alignments was performed using Clustal W (Thompson et al., 1994).

Database

The domains in the translated coding sequence were found using SMART services and their databases: http://smart.embl-heidelberg.de/

PCR of promoter region

Specific primers designed to exclude putative StRE elements where used to obtain only HSE sequences from the promoter regions. Clones containing the leading telomere, the whole 5' leader sequence and part of *hsp70* gene of both *E. nobilii* and *E. raikovi* were selected as template.

To amplify *E. raikovi* HSE were used 5'-TGAGAGAATTC-CCCTAGAAGGTTCTAGACGG-3' (EcoRI restriction site at the beginning) as forward primer 5'-TGAGAGAATTC-GCATGAGCTCGTCGATTTATTGT-3' (EcoRI restriction site at the end and modification on putative StRE) as reverse primer giving a product 95bp long (76bp of amplified HSE promoter plus 19bp of restriction sites).

To amplify *E. nobilii* HSE were used 5'-TGAGAGAATTC-CCCGGTAATAGGTGAATTGT-3' (EcoRI restriction site at the beginning) as forward primer and 5'-TGAGAGAATTC-TCGAACTTTAGAGAAACTTCGA-3' (EcoRI restriction site at the end) as reverse primer, giving a products 89bp long (67bp of amplified HSE promoter plus 22bp of restriction sites). Amplification products were then digested (or accordingly double digested) with needed restriction ezyme (EcoRI or EcorRI/NotI)

using standard conditions for reaction.

Filling-in amplified HSE

Filling-in of digested fragments was the chosen method for radiolabeling the HSE promoters. DNA was mixed with buffer (provided together with Klenow's fragment by Fermentas), BSA 0.2µg/µl, dNTP (excluded ATP) 20µM, 2-3µl [α-32P]ATP, water to final volume 50µl and 5U Klenow's Fragment added at the very end to start reaction in the tube. The solution was incubated for 1,5 hours at 37°C. Finally the probe was precipitated with ammonium acetate and cold absolute ethanol, washed twice with ethanol 80% and then resuspended in sterile water for further use.

Gel retardation electrophoresis

About 30µg of nuclear extraction were incubated with 15ng of radio labelled probe (obtained with the filling-in method) in EMSA buffer (10mM Tris-HCl pH 7.5, 50mM NaCl, 2.5mM $MgCl_2$, 4% v/v Glycerol, 0,5mM DTT, Cocktail proteases inhibitors 1X, PolydI-dC) for 45 minutes at 25°C. After that, samples were transferred on ice until they had been loaded in the gel.

Gel retardation was performed in 5% polyacrylamide gel (TBE 1X, 2,5% v/v Glycerol), with TBE 0,5X as running buffer. Samples were run for 1 hour and half at constant 120V.

Screening of the sample

After run, gel was washed in destainig solution (40% methanol, 10% acetic acid) for 15 minutes waiting for bromophenol blue to

turn green. Then the gel was dried on a gel drier and exposed to a phosphor screen. Shift of the bands signals were detected by means of a personal Molecular Imager FX (Bio-Rad).

Results and Discussion

hsp70 gene activation in response to thermal stress in E. nobilii and E. raikovi

By means of Northern-blot analysis, in a preliminary phase of this study we characterized the minimum Heat shock temperature (T_{on}) required to activate *hsp70* gene expression in the sister species of *E. nobilii* inhabiting temperate waters, *E. raikovi* (La Terza et al., unpublished). Total RNA samples were obtained from cells grown at their standard temperature of 23-24°C (i.e. non heat shocked) and cells that were either exposed to a set of increased temperatures, i.e. 28, 35 and 40°C for 30 minutes, following an abrupt heat-shock protocol as described in Materials and Methods. RNA samples were also obtained by *E. nobilii* cells at their growing temperatures of 4°C and heat shocked at the temperature of 18, 20 and 25°C. All RNA preparations were fractionated by electrophoresis and blotted with homologous hsp70 probes. As shown in Fig.9**A**, *E. raikovi* cells produced an intense 2.1 Kb band, i.e., the molecular size expected for *hsp70* mRNA, starting from the temperature of 35°C. A more intense hybridization signal showed up in the RNA preparation extracted at 40°C. At this temperature, as determined by densitometric analysis of the blot, the intensity of

signal is 3/4-fold greater than that at 35°C and thus suggesting that this species is able to modulate the expression of its *hsp70* genes according to the intensity of the thermal stress stimuli. This behaviour is somewhat expected in a species living in highly fluctuating thermal environment (Tomanek and Somero, 2002). On the contrary and as shown in Fig.9**B**, *E. nobilii* is capable to activate the expression of its hsp70 genes at the unusual temperature of 20°C. Moreover, *E. nobilii* heat-shocked at 25°C increased only 1-fold the amount of its *hsp70* mRNA with respect to that at 20°C. Altogether, these observations strongly suggest that *E. nobilii* might have lost the capacity to properly modulate the expression of its *hsp70* probably, as a consequence of its evolution at sub zero temperature.

Characterization of E. raikovi hsp70 gene sequence

The full-length sequences of the *E. raikovi hsp70* macronuclear gene of 2272 base pairs (bp), was reconstructed by overlapping the sequences of the two cloned RATE-PCR products, one of 1577bp containing the 5'end and the other of 2053bp containing the 3' end. Its uniqueness was confirmed by direct sequence analysis of the product of a PCR amplification, in which an *E. raikovi* DNA preparation was used as template for primers represented by two oligonucleotides spanning the sequence segments of 25 bases immediately adjacent to the 5' and 3' telomeric repeats of the cloned *hsp70* gene sequences. The determined *hsp70* gene sequence is intronless and shows and

open reading frame of 2034bp, spanning from the initiation codon ATG at nucleotide 133 to the termination codon TAA at nucleotide 2166. The coding region is flanked by a 5'-leader and a 3'-trailer untranslated region of 104 and 78 nucleotides, respectively each one ending with a block of inverted 5'-C_4A_4-3'/3'-G_4T_4-5' telomeric repeats.

Structural and functional features

The ORF of *E. raikovi hsp70* gene encodes a protein 677 amino acids protein with a calculated molecular mass of 72.9kDa and an isoelectric point (pI) of 5. The Hsp70 protein of *E. nobilii* presents slight differences with respect to that of *E. raikovi*, since that has a calculated molecular mass of 71.6 kDa and a theoretical pI of 4.9 (La Terza et al., 2004). The degree of Hsp70 sequence identity between *E. raikovi* and *E. nobilii* is 90.4% In Fig.10 is shown a comparison of the *E. nobilii* Hsp70 sequence with that of *E. raikovi*. In this sequences, the three characteristic functional domains of all Hsp70 proteins, may be recognized: the ATPase domain, the HDAC interacting domain and the substrate binding domain. Moreover, both Hsp70 proteins present the classical signature patterns for the Hsp70 family of protein as defined in Prosite database. The first signature is centred on a conserved peptide found in the N-terminal section of these proteins ([IV]-D-L-G-T-[ST]-x-[SC], prosite ID HSP70_1 PS00297), the two others are centered on conserved regions located in the central part of the sequence ([LIVMF]- [LIVMFY]-[DN]-[LIVMFS]-G-

[GSH]-[GS]-[AST]-x(3)-[ST]- [LIVM]-[LIVMFC], prosite ID HSP70_2 PS00329 and [LIVMY]-x-[LIVMF]-x-G-G-x-[ST]-{LS}-[LIVM]-P-x-[LIVM]-x-[DEQKRSTA], prosite ID HSP70_3 PS01036). The only difference detectable in the primary structure of these proteins is localised in the C-terminal region where the Hsp70 of *E. raikovi* owns a longer stretch of glycine rich motifs.

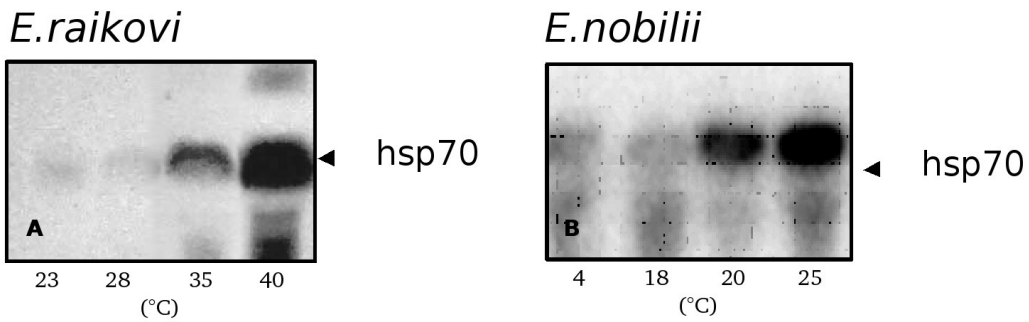

Fig. 9: Northern blot analysis of total RNA preparations from E. raikovi and E. nobilii exposed to different temperatures. Equal RNA amounts (40µg) were fractionated by electrophoresis, blotted, and hybridized with a probe represented by the hsp70 gene sequence specific for the protein catalytic domain. Probe was used at equal numbers of counts per minute in both Northern Blots.

Fig. 10: Alignment of the inferred aminoacid sequences of E. nobilii (upper) and E. raikovi (lower) HSP70. The various HSP70 signature motifs are boxed and labeled. The various domains are shaded in different kinds of grey in the following order: ATPase domain, HDAC interacting domain, Substrate Binding domain. Colon and dot marks indicate similar aminoacids. The glycine rich region is boxed and labeled.

Structural features of the hsp70 gene regulatory regions

As shown in Fig.11, the 5’ leader regions slightly differs between the two species in length (104 nucleotides in *E. raikovi* versus 152 in *E. nobilii*). Nevertheless, in both the species this region harbours two types of stress inducible motifs: HSE (from Heat-Shock Elements) and StRE (from Stress-Response Elements). HSE consist of at least three inverted repeats of the sequence motif 5’nGAAn3’ alternate with the complement sequence nTTCn (in which, n stands for any nucleotide). In general, a functional HSE can tolerate a maximum insertion of 5-7bp between each motif (Fernandes et al., 1994; Sorger, 1991). The StRE are characterised by the consensus sequences AGGGG and CCCCT (Kobayashi and McEntee, 1993; Schmitt and McEntee, 1996). Both elements are known to be targets of *trans*-acting transcriptional activators characterized in a variety of organisms in association with their stress-inducible genes (Kobayashi and McEntee, 1993; Fernandes et al., 1994; Ruis and 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 and McEntee, 1996; Estruch, 2000).

In the *hsp70* 5’ region of *E. raikovi*, we identified a canonical HSE motif lying between the nucleotides 30 and 62. This HSE element

is characterized by two contiguous repeats of the pentameric consensus sequence nGAAn and its complement nTTCn between nucleotides 30 and 39, followed by a third repeat separated from the second repeats by a gap of seven nucleotide. Moreover, a fourth units is placed six nucleotides downstream the third one. We can summarize the formula of this HSE as nGAAnnTTCn(7bp)nGAAn. A single StRE identified by the motif A_{86}GGCG that bears a 4/5 agreement to the consensus sequences AGGGG and CCCCT is positioned 24 nucleotides downstream the fourth HSE motif.

On the contrary the *hsp70* 5' region of *E. nobilii*, bears a HSE motif with non-canonical, discontinuous arrangement between positions 38 and 55, and two StRe elements identified by the motifs T_{77}CCCT and T_{116}GGGG, that bear 4/5 agreement with the consensus sequences CCCCT and AGGGG. This HSE is composed by a single nGAAn repeats followed by two more repeats (nnGAAnnTTCn) after a gap of three bases. According to Yamamoto et al., (2005) this HSE can be classified as gap type. nGAAn(3bp)nGAAnnTTCn. Several authors confirmed that this variations in the architecture are fully functional (Fernandes et al., 1994a,b; Santoro et al., 1998; Tachibana et al., 2002).

Unlike the 5' promoter region, as shown in Fig.12, the *hsp70* 3' region presents a common/uniform structure in both length (78bp in *E. raikovi* and 90bp in *E. nobilii*) and type of regulatory motifs. Both regions present 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., 2006)

HSF activation

Since that *hsp70* transcription of the depends on HSE elements in response to heat shock, we assayed the functionality of the promoters and if there were factors able to bind to the HSE elements that are present in the 5' leader sequences. To exclude any other possible binding motif than HSE we have amplified only the region containing the HSE elements both in *E. nobilii* and *E. raikovi* taking care of isolating them from the putative StRE elements. For this reason, we designed primers matching the region flanking the HSE and mutating some of the bases constituting part of the putative StRE elements. Nuclear proteins, extracted from unshocked and heat-shocked cells were then tested with radiolabeled probes made as stated before. The autoradiography Fig.13 in the two *Euplotes* that both the control sample and the shocked sample have a factors able to bind the probe, which suggest that the putative HSE transcription factor is constitutively positioned on the HSE. The free probe cumulating at the gel bottom in the first lane means that the bands are not an artefact due to the probe itself, while the competitive binding with a molar excess of 25X and 50X of unlabelled probe in the last two lanes demonstrates the specificity of binding because

the strength of the signals degrades proportionally.

Euplotes nobilii

```
                                                    HSE
CCCCAAAACCCCAAAACCCCAAAACCCCGGTAATAGGTGAATTGTTGAAT  50
TTTCACTGTAAAAATTAATCGAAGTTTCCCTAAAGTTCGATTTAAATTAG 100
AATTATCTAGATTTGGGGATTAAATACTATTTAAGTAAAATTCTGGGAGA 150
CTAATTAATTAATAAATTTTAATAAATAATATG(183)
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Euplotes raikovi

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                                          HSE
CCCCAAAACCCCAAAACCCCAAAACCCCTAGAAGGTTCTAGACGGTAGAA  50
ATGGACGTTTCTAGATTTAATATTTTCACAATAAATAGGCGAGCTCATGC 100
TATAATTATTATTAGTAATTAATTAGAAAATATG(134)
```

Fig. 11: Nucleotide sequences of the 5'regulatory regions of the E. nobilii and E. raikovi HSP70 genes. The telomeric C_4A_4 repeats are shaded; the transcription initiation ATG codons are in bold; sequence motifs bearing agreement with HSE and StRE elements are over-lined and underlined, respectively.

Euplotes nobilii

TAAACATATAAATAACCCATTTATTGAGTTTTAATAAGCAAATTGCACAA 2207
*AA***A***GCAGTGTCTA*CCATTTATGTTCAACCATACTAGATATATGAGGGGTT 2257
TTGGGGTTTTGGGGTTTTGGGG(2279)

Euplotes raikovi

TAAACAAAGCTTAACCTATAATTGAGTTTAAATAAGTATTTATACTA*AA***A***G* 2213
*CAGTGTCTA*TTCAATAATTTATATTTCACTAGGGGTTTTGGGGTTTTGGG 2263
GTTTTGGGG(2272)

Fig. 12: Nucleotide sequences of the 3'regulatory regions of the E. HSP70 genes. Telomeric G_4T_4 repeats are shaded; the stop TAA codons are in bold; secondary in frame TAA codons are underlined, putative polyadenilation motifs are bold and underlined; the ATTTA motif, indicative of an mRNA destabilization ARE element, is boxed in both sequences.

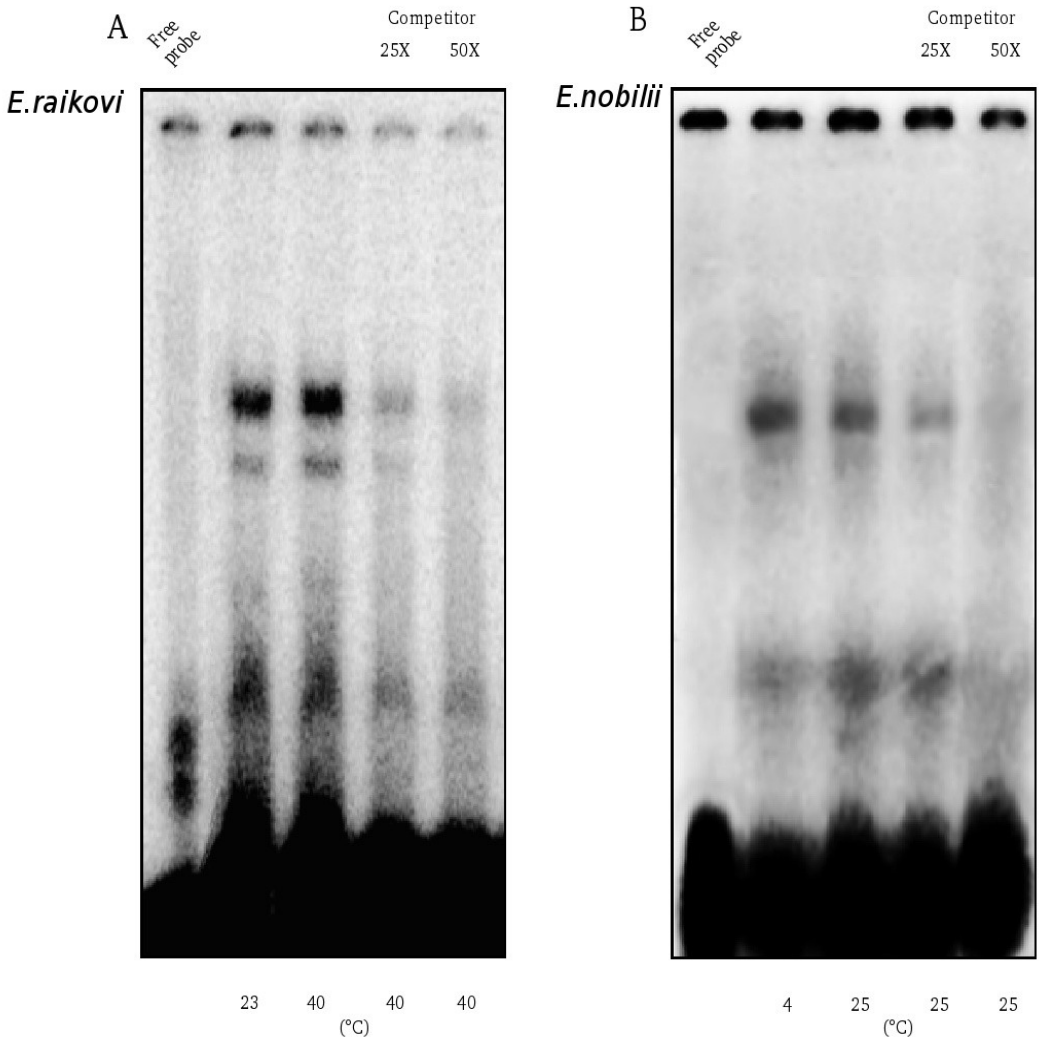

Fig. 13: Electrophoresis Mobility Shift Assay of HSE probe of hsp70 gene promoter. ***A)*** *E. raikovi* ***B)*** *E. nobilii. In both panels there is the same loading scheme. In the first lane, free probe without nuclear extract as a negative control. In the second lane probe hybridization with extract from non shocked cells. In the third lane probe hybridization with extract from shocked cells. In the fourth and fifth lanes, 25 or 50- fold molar excesses respectively of unlabelled probe as cold competitor were added to hybridization reaction with nuclear extracts from shocked cells.*

Conclusions

This study reports the complete *hsp70* gene sequence of *E. raikovi* a ciliate inhabiting temperate waters and its comparison (at level of coding and non coding, regulatory regions) with that of the sister species *E. nobilii,* inhabiting the constantly cold Antarctic waters (Eastman, 1993; Clarke and Johnston, 1996; Di Giuseppe et al., unpublished results). The latter species is able to activate the expression of its *hsp70* genes at 20°C, whereas *E. raikovi* switch on the transcription of its *hsp70* gene at 35°C, a thermal threshold shared also by other species of *Euplotes* from temperate waters (Ullmann et al., 2004). Considering that the genetic encoding of this plasticity might reside at several locations in the genome (i.e. *hsp* coding sequence, *cis*-regulatory and *trans*-regulatory elements, in co-chaperones and co-factors interactions), the simplest approach to get hints in the molecular mechanisms regulating the thermal activation of *hsp70* gene in *E. nobilii* is offered by the possibility to compare the transcriptional machinery of closely related species adapted to different thermal regimes. HSP70 sequences from the two species appeared to be very similar at level of their putative amino acid sequences and in their 3’ regulatory regions. Differences are present in 5’ promoter region: this region contains conventional HSE motifs in *E. raikovi* and non conventional gapped HSE (Fernandes et al., 1994; Sorger, 1991; Tachibana et al., 2002) in *E. nobilii*. Moreover, investigations by

means of electrophoretic mobility shift assays (EMSA), showed that putative HSF factors able to bind HSE elements are present in nuclear extracts from both heat shocked and not shocked *E. nobilii* and *E. raikovi* cells. Altogether these observations suggest that the causes of this inter-specific *hsp70* thermal plasticity, are likely to reside primarily in a mutated capacity of the HSF factors to activate HSE. This might resides in defective post-translational mechanisms of HSF, such as phosphorylation or sumoylation (Hietakangas et al., 2006; Anckar et al., 2006), or in the interactions with specific cofactors such as Heat Shock Factor Binding Proteins.

References

Anckar, J. et al., Inhibition of DNA Binding by Differential Sumoylation of Heat Shock Factors. *Molecular and Cellular Biology* **26**, 955-964 (2006).

Anderson, R.C., Lindauer, K.R. & Prescott, D.M. A gene-sized DNA molecule encoding heat-shock protein 70 in *Oxytricha nova. Gene* **168**, 103-107 (1996).

Barreau, C, Paillard, L., Osborne, H.B., Journals, O., AU-rich elements and associated factors: are there unifying principles. *Nucleic Acids Research* **33**, 7138-7150 (2006).

Clarke, A. & Johnston, I. Evolution and adaptive radiation of Antarctic fishes. *Trends in Ecology and Evolution* **11**, 212-218 (1996).

Cossins, A.R. & Bowler, K. Temperature biology of animals. (1987).

Eastman, J.T. Antarctic Fish Biology. Evolution in a Unique Environment. (1993).

Feder, M.E. & Hofmann, G.E. Heat-Shock Proteins, Molecular Chaperones, and The Stress Response: Evolutionary and Ecological Physiology. *Annual Review of Physiology* **61**, 243-282 (1999).

Fernandes, M., O'Brien, T. & Lis, J.T. Structure and regulation of heat shock gene promoters. *The Biology of Heat Shock Proteins and Molecular Chaperones* 375-393 (1994).

Fernandes, M., Xiao, H. & Lis, J.T. Fine structure analyses of the *Drosophila* and *Saccharomyces* heat shock factor-heat shock element interactions. *Nucleic Acids Res* **22**, 167-73 (1994).

Frydman, J. & Hartl, F.U. Molecular chaperone functions of hsp70 and hsp60 in protein folding. *The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Preuss, Cold Spring, New York, NY* 251-283 (1994).

Hartl, F.U. & Hayer-Hartl, M. Molecular Chaperones in the Cytosol: from Nascent Chain to Folded Protein. *Science* **295**, 1852 (2002).

Hietakangas, V. et al., PDSM, a motif for phosphorylation-dependent SUMO modification. *Proceedings of the National Academy of Sciences* **103**, 45-50 (2006).

Hochachka, P.W. & Somero, G.N. Biochemical Adaptation: Mechanism and Process in Physiological Evolution. (2002).

Hoffman, D.C. et al., Macronuclear gene-sized molecules of hypotrichs. *Nucleic Acids Res* **23**, 1279-1283 (1995).

Kobayashi, N. & McEntee, K. Identification of cis and trans components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **13**, 248-256 (1993).

La Terza, A. et al., Divergence between two Antarctic species of the ciliate *Euplotes*, *E. focardii* and *E. nobilii*, in the expression of heat-shock protein 70 genes. *Molecular Ecology* **10**, 1061-1067 (2001).

Lindquist, S. Varying patterns of protein synthesis in *Drosophila* during heat shock: implications for regulation. *Dev Biol* **77**, 463-79 (1980).

Parsell, D.A. et al., The Role of Heat-Shock Proteins in Thermotolerance and Discussion. *Philosophical Transactions: Biological Sciences* **339**, 279-286 (1993).

Pirkkala, L., Nykanen, P. & Sistonen, L. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *The FASEB Journal* **15**, 1118-1131 (2001).

Precht, H. Temperature and Life. (1973).

Pucciarelli, S. & Miceli, C. Characterization of the cold-adapted alpha-tubulin from the psychrophilic ciliate *Euplotes focardii*. *Extremophiles* **6**, 385-389 (2002).

Pucciarelli, S., Di Giuseppe, G., Dini, F., Luporini, P. & Miceli, C. (2003), *Euplotes focardii*, an Antarctic psychrophilic ciliate of presumed Gondwanan origin. *Journal of Eukaryotic Microbiology* **50**, 38-38.

Ruis, H. & Schuller, C. Stress signaling in yeast. *Bioessays* **17**, 959-65 (1995).

Sambrook, J. & Russell, D.W. Molecular Cloning: A Laboratory Manual. (2001).

Santoro, N., Johansson, N. & Thiele, D.J. Heat Shock Element Architecture Is an Important Determinant in the Temperature and Transactivation Domain Requirements for Heat Shock Transcription Factor. *Molecular and Cellular Biology* **18**, 6340-

6352 (1998).

Schmitt, A.P. & McEntee, K. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences* **93**, 5777-5782 (1996).

Sorger, P.K. Heat shock factor and the heat shock response. *Cell* **65**, 363-6 (1991).

Tachibana, T., Astumi, S., Shiod,R., Uenom, M., Uritani, M., Ushimaru, T. (2002) A Novel Non-conventional Heat Shock Element Regulates Expression of MDJ1 Encoding a DnaJ Homolog in Saccharomyces cerevisiae. *Journal of Biological Chemistry* **277**, 22140-22146.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680 (1994).

Tomanek, L. & Somero, G.N. Interspecific-and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*)*:* implications for regulation of hsp gene expression. *Journal of Experimental Biology* **205**, 677-685 (2002).

Ullmann, J., Reidt, U. & Klein, A. The Expression of the HSP70 Gene in *Moneuplotes crassus* is Controlled by a Two-Step Process at the Transcript Level. *The Journal of Eukaryotic Microbiology* **51**, 344-350

Valbonesi, A. & Luporini, P. Description of two new species of *Euplotes* and *Euplotes rariseta* from Antarctica. *Polar Biology* **11**, 47-53 (1990).

Valbonesi, A. & Luporini, P. Biology of *Euplotes focardii*, an Antarctic ciliate. *Polar Biology* **13**, 489-493 (1993).