The Journal of Experimental Biology 207, 1607-1613
Published by The Company of Biologists 2004
doi:10.1242/jeb.00935

Stress gene (*hsp70*) sequences and quantitative expression in *Milnesium tardigradum* (Tardigrada) during active and cryptobiotic stages

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Accepted 12 February 2004

Summary

The eutardigrade *Milnesium tardigradum* can undergo cryptobiosis, i.e. entry into a reversible ametabolic stage induced by dehydration, cooling and, probably, osmotic and anoxic stress. For the first time in tardigrades, we described partial sequences of three heat-shock protein (*hsp70* family) genes and examined gene expression on the way from an active to a cryptobiotic and back to an active stage again. Results showed different patterns of gene expression in the *hsp70* isoforms. All three isoforms seem to be true heat-shock proteins since transcription could be clearly enhanced by temperature elevation. Isoform 1 and, at a lower level, isoform 3 do not seem to have a specific function for cryptobiosis. By contrast, transcription of isoform 2 is significantly induced in the transitional stage between the active and the cryptobiotic stage, resulting in a comparatively high mRNA copy number also during cryptobiosis. This pattern of induction implies that isoform 2 is the most relevant *hsp70* gene for *M. tardigradum* individuals entering the cryptobiotic stage.

Key words: anhydrobiosis, cryptobiosis, Eutardigrada, heat-shock protein, stress protein, *hsp70*.

Introduction

The eutardigrade *Milnesium tardigradum* Doyère 1840 is a well-known cosmopolitan species and a typical inhabitant of moist environments that facilitate the animal's gaseous exchange and avoid desiccation. However, such habitats frequently undergo seasonal changes that impact animal life. *M. tardigradum* is able to survive these periods of adverse conditions due to its ability to enter into a cryptobiotic state. In adverse environments, all terrestrial and freshwater tardigrades arrest their metabolic activity and get dehydrated to form the 'Tönnchenform' or 'tun' state (Baumann, 1922). In this state, they are capable of surviving for very long periods. Although it has been assumed that tardigrades have a very long life span, little information is available concerning their longevity. Reports that a tardigrade has revitalised from the cryptobiotic stage after a period of 120 years are not scientifically proven (Jönsson and Bertolani, 2001). Suzuki (2003) found the longest life span of *M. tardigradum* to be 58 days after hatching. Nevertheless, it is possible to estimate the life span by assuming repetitive cycles of activity and cryptobiosis. In the cryptobiotic stage, tardigrades show extraordinary tolerance to physical extremes including high-energy radiation, immersion in organic solvents (Ramløv and Westh, 2001), brief exposure to high temperatures (Doyère, 1842; Ramløv and Westh, 2001) and prolonged exposure to indefinitely low temperatures (Rahm, 1921; Westh and Hvidt, 1990; Ramløv and Westh, 1992; Westh and Kristensen, 1992; Sømme and Meier, 1995). When environmental conditions are adequate, tuns rehydrate and the animals resume metabolic activity.

Cryptobiosis in tardigrades and other invertebrates is characterized by several major events that still remain largely unidentified. On the one hand, research has focused on cryptobiotic cells that accumulate large amounts of either one or both of the disaccharides trehalose or sucrose (Clegg and Jackson, 1992; Crowe et al., 1998; Viner and Clegg, 2001; Crowe, 2002; Oliver et al., 2002; Watanabe et al., 2003). In this context, the 'water-replacement hypothesis' has been developed to explain how cellular components may be protected during extreme drying. Essentially, the hypothesis says that polyhydroxyl compounds, such as trehalose, replace the shell of water around macromolecules, circumventing any damaging effects during drying. However, the tardigrade *Adorybiotus coronifer* showed rather low trehalose accumulation (1.6% of the dry mass) compared with several anhydrobiotic species from other taxa, such as the nematode *Aphelenchus avenae*, with a trehalose level of 12–13%, or cysts of the brine shrimp *Artemia franciscana*, which contain 15–18% (Liang et al., 1997). Nevertheless, Westh and Ramløv (1991) estimated the ability of *A. coronifer* to accumulate the mentioned concentration of trehalose approximately 10 times

faster than *A. avenae*. Furthermore, several stress proteins (Clegg et al., 1999; Liang and MacRae, 1999; Clegg et al., 2000; Ramløv and Westh, 2001; Viner and Clegg, 2001; Willsie and Clegg, 2001) and 'late-embryogenesis-abundant' (LEA) proteins that have been found in the nematode *A. avenae* (Browne et al., 2002) and in plant seeds exhibiting desiccation tolerance during maturation (Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Chandler and Bartels, 1999) seem to be further keys in understanding the cryptobiotic mechanisms.

Drying of cells generally leads to massive damage to cellular membranes and proteins, which eventually results in cell death and, consequently, the death of the entire organism. Upon drying, intracellular proteins and membranes may compensate the loss of hydrogen bonds to water by hydrogen bonds to other molecules and can further compensate by protein–protein interactions (Carpenter and Crowe, 1989; Prestrelski et al., 1993a,b; Dong et al., 1995). These protein–protein interactions, however, can lead to irreversible conformational changes and, in enzymes, to a loss of enzymatic activity (Carpenter et al., 1987).

Heat-shock proteins and their molecular partners are known to play diverse roles, even in unstressed cells, in successful folding, assembly, intracellular localization, secretion, regulation and degradation of other proteins (Gething and Sambrook, 1992; Gething, 1997). Alamillo et al. (1995) studied the resurrection plant *Craterostigma plantagineum*, a desert species that expresses heat-shock proteins in vegetative tissues during water stress; this expression is thought to contribute to desiccation tolerance. Similarly, rice seedlings express two proteins of the Hsp90 family upon exposure to water stress and elevated salinity (Pareek et al., 1997). Several case studies in encysted brine shrimp (*A. franciscana*) embryos showed that they undergo development arrest, in which they may survive for years without environmental water or oxygen. These cryptobiotic embryos accumulate enormous concentrations of a small heat-shock protein p26, belonging to the α-crystallin family (de Jong et al., 1998) and being restricted to this stage of the life history (Jackson and Clegg, 1996; Liang and MacRae, 1999). Clegg et al. (1994) also showed that p26 underwent extensive stress-induced translocation to nuclei and other sites. Information on the role of a major stress protein in cryptobiosis, Hsp70, however, is still scarce.

In the present study, a complementary focus is on tardigrades undergoing stress in nature and on the roles of stress genes of the *hsp70* family in the stress physiology of whole organisms in different life history stages. Using real-time RT-PCR, the levels of expression of *hsp70* isoforms in the active and cryptobiotic animals and in intermediate stages have been studied in the tardigrade species *M. tardigradum*.

Materials and methods

Experimental design

Thirty individuals of an *M. tardigradum* stock, cultured in the laboratory on agar plates, were used to study *hsp70* gene expression on the way from an active to a cryptobiotic and again to an active stage. Five animals were sampled in the active stage (I) (Fig. 1A), 20 animals were allowed to undergo cryptobiosis by dehydration for 6 h on an agar plate, while five animals were sampled in the transitional stage (II). For drying the specimens, a method according to Lapinski and Tunnacliffe (2003) was used. The tardigrades were pipetted into a Petri dish with agar and left to dry to completion at room temperature (21°C) and approximately 34% relative humidity. Stage II was defined as the stage at which the legs were drawn in but the body still showed distinct movements. After 14 days of cryptobiosis, five animals were sampled in the cryptobiotic stage (III) (Fig. 1B). The remaining 15 animals were rehydrated. Five of them were sampled in the following transitional stage (IV), which was defined as the stage at which the legs protruded and the body showed movements again. Stage V was the active stage in which the tardigrades moved around on the agar plate after 90 min of stage IV. Individual duration of rehydration time from the cryptobiotic to the active stage was measured in five specimens. To study the inducibility of the stress proteins, five animals were kept at 37°C for 90 min (stage VI).

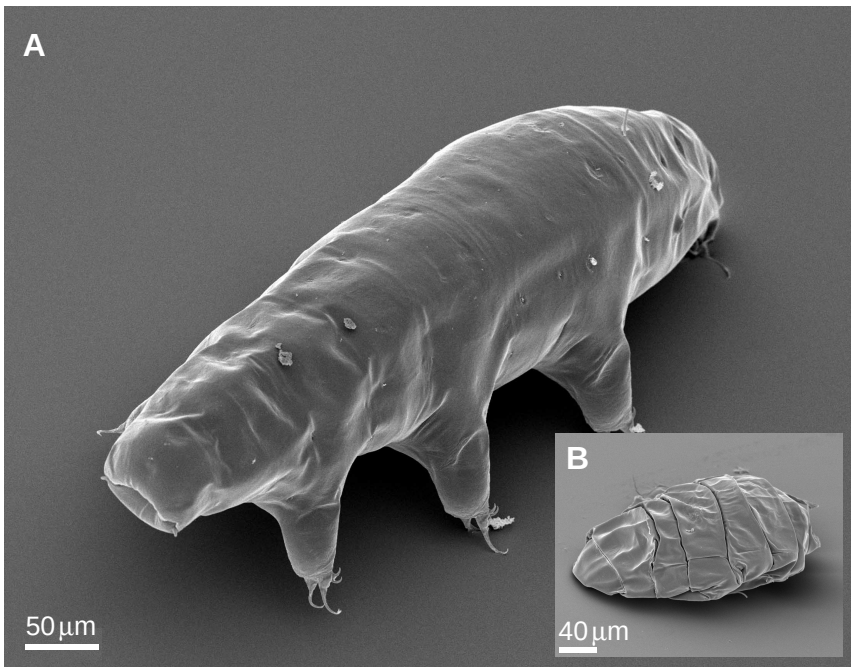

Fig. 1. SEM images of the tardigrade *Milnesium tardigradum*. (A) Active hydrated individual, stage I. (B) Dehydrated, cryptobiotic individual ('tun'), stage III.

Thermogravimetric analysis of the dried

tardigrades to measure the residual moisture content was not performed, although other authors observed a maximum residual moisture content in the range of 6–10% in different anhydrobiotic organisms when the above-mentioned drying method was used (Potts, 1994; Lapinski and Tunnacliffe, 2003).

RNA/DNA extraction

RNA was extracted from individual tardigrades (*N*=5) in the different stages (I–VI) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Specimens were incubated in 200 µl TRIzol reagent for 5 min at room temperature to achieve complete dissociation of nucleoprotein complexes. 40 µl of chloroform was added, the tubes were shaken vigorously by hand for 15 s and incubated for a further 5 min at room temperature. After centrifugation (15 min, 12 000 ***g***, 4°C), the aqueous phase containing RNA was separated from the other phases, which were stored for DNA preparation (see below). The colorless upper aqueous phase was transferred into fresh vials to precipitate the RNA by addition of 100 µl isopropyl alcohol. The samples were incubated for 10 min and centrifuged (20 min, 12 000 ***g***, 4°C). The RNA precipitates were then washed twice with 75% ethanol (in DEPC-treated water), air-dried and resolved in DEPC-treated water for the DNA digestion with RNase-free DNase I (Promega, Madison, WI, USA).

DNA in the interphase and phenol phase of the initial homogenate was pooled and isolated by precipitation with ethanol and centrifugation (10 min, 2000 ***g***, 4°C). The pellets were washed twice with 0.1 mol l^{-1} sodium citrate in 10% ethanol and centrifuged (5 min, 2000 ***g***, 4°C). Following these two washes, the pellet was suspended in 75% ethanol, centrifuged (5 min, 2000 ***g***, 4°C), air-dried and resolved in DEPC-treated water for RNA digestion with RNase H (Invitrogen).

Species-specific PCR primers

To obtain specific *hsp70* gene family members, PCR was carried out using degenerated oligonucleotide primers (Table 1a), specified in Köhler et al. (1998), for a highly conserved region of the *hsp70* gene. As an internal standard, *beta-actin* was chosen as the housekeeping gene. Conserved *beta-actin* primers (Table 1b) were designed by hand, based on the National Center for Biotechnology Information (NCBI) GenBank (*beta-actin* accession no. BC014861). After using DNA of *M. tardigradum* as a template, the PCR products were cloned with the TOPO TA Cloning® Kit for Sequencing (Invitrogen), and the inserts checked by digestion with *Eco*RI. Clones of interest were sequenced twice and identified by BLAST® (Basic Local Alignment Search Tool) in the NCBI GenBank. Partial sequences of three *hsp70* family genes and a *beta-actin* family gene were described for the first time in tardigrades. Species-specific oligonucleotide primers were designed with the Primer 3 software (Rozen and Skaletsky, 2000) based on these partial gene sequences, which have been submitted to the NCBI GenBank (*hsp70* isoform 1, accession no. AJ579531; *hsp70* isoform 2, accession no. AJ579532; *hsp70* isoform 3, accession no. AJ579533) and for *beta-actin* (accession no. AJ579530). The used primers, purchased from MWG Biotech AG (Ebersberg, Germany), are summarized in Table 1c–f.

Real-time RT-PCR

Reverse transcription (RT) of first-strand cDNA was performed with the total RNA of each specimen. The RNA was incubated with 10 mmol l^{-1} dNTP mix and 50 ng oligo$(dT)_{12–18}$ primer at 65°C for 5 min. After cooling on ice, the reaction mixture [0.1 mol l^{-1} dithiothreitol, 5× 1st strand buffer and 40 units of RNaseOUT™ (Invitrogen)] was added, mixed gently and incubated at 42°C for 2 min. 50 units of *SuperScript*™ II (Invitrogen) were added and incubated at 42°C for 50 min and inactivated by heating to 70°C for 15 min. The cDNA was precipitated in 75% ethanol and washed twice with 75% ethanol, air-dried and resolved in DEPC-treated water.

Table 1. *Primer characteristics used to amplify various sequences of* beta-actin *and* hsp70 *genes*

Primer	Sequence
(a) Conserved *hsp70*	for 5′-AT(C/T) AA(C/T) GA(G/A) CCI AC(G/T) GCI GCI GC(T/A) ATT GCI TAT GG-3′ rev 5′-GA(T/C) GAA (A/G)G C(T/A/C)G TTG C(A/G)T A(T/C)G G(A/T/C)G C(A/T)G C(T/A/C)G TAC AAG C-3′
(b) Conserved *beta-actin*	for 5′-CAT TGC CGA CAG GAT CCA GA-3′ rev 5′-GAC TCG TCG TAC TCC TGC TTG-3′
(c) *hsp70* isoform 1	for 5′-CTT ATC AGT GAA ATT AAG CGA GAG C-3′ rev 5′-ACA AGG ATA ACT TCA TCA ACC TTT G-3′
(d) *hsp70* isoform 2	for 5′-GAG GTG GTA CTT TTG ATG TTT CTG T-3′ rev 5′-GTC AAT TCC TTC GTA GAG AGA GTC A-3′
(e) *hsp70* isoform 3	for 5′-TGG TGT TGA TAT TTC TAA AGA CCG T-3′ rev 5′-ACA AGG ATA ACT TCA TCA ACC TTT G-3′
(f) *beta-actin*	for 5′-ATG AAG ATC AAG ATC ATC GCT CC-3′ rev 5′-GAG ATC CAC ATC TGC TGG AAG-3′

for, forward; rev, reverse.

The real-time PCR reaction mixture contained the following items in a final volume of 20 µl: 50 ng cDNA, 1 unit Taq DNA Polymerase D1806 (Sigma-Aldrich, Inc., St Louis, MO, USA) with 10× reaction buffer supplemented to a final concentration of 3.9 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTP, 2 µmol l^{-1} of each oligonucleotide primer and 2 µl of SYBR Green® I 1:1000 (Molecular Probes, Inc., Eugene, OR, USA). The PCR amplification profiles were as follows:

hsp70 isoform 1: initial denaturation for 8 min at 95°C, followed by 45 cycles of 30 s at 94°C, 30 s at 63°C and 60 s at 72°C;

hsp70 isoform 2: 30 s at 94°C, 30 s at 64°C, 30 s at 72°C;

hsp70 isoform 3: 30 s at 94°C, 30 s at 50°C, 60 s at 72°C, and final extension of 8 min at 72°C;

beta-actin: 30 s at 94°C, 30 s at 64°C, 60 s at 72°C, and final extension of 8 min at 72°C.

Negative control reactions containing water in place of cDNA were included in each batch of PCR reactions to ensure that contamination was not a problem. For the positive control and standard curve, a standard of the particular sequences was amplified in three different dilutions (10^2, 10^3 and 10^4 sequence copies).

Product analysis

In the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), analysis of the real-time fluorescence signal of SYBR Green® I (Molecular Probes, Inc.) bound to double-stranded DNA was performed using the iCycler iQ™ Real-Time PCR Detection System software (Bio-Rad Laboratories). A threshold position was user-defined for the samples, using the exponential growth phase and baseline cycles of the fluorescent amplification plots. The quantity of RNA was expressed in relation to the internal reference of *beta-actin* and compensated for variation in the quantity and quality of the cDNA samples. Standard curves were generated by plotting the log of the cDNA copy number against respective threshold cycles (C_T) and covering the orders of magnitude in variation of cDNA template concentrations. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis on a 2% gel in Tris-borate-EDTA buffer and stained with CYBR Gold® (Molecular Probes, Inc.).

Statistics

The statistical significance of differences in the *hsp70* transcript levels between the samples was tested using Mann–Whitney–Wilcoxon's *U*-test. Significance levels were $P>0.05$ (not significant), $0.01<P<0.05$ (weakly significant, *), $0.001<P<0.01$ (significant, **), and $P<0.001$ (highly significant, ***).

Results

The objective of the study was the quantification of the expression of isoforms of the *hsp70* gene family during cryptobiosis in *M. tardigradum*. In order to quantify the *hsp70* transcripts, the *hsp70* genes and the housekeeping gene *beta-actin* had to be partially sequenced and a sensitive and reliable real-time RT-PCR quantification method had to be developed. The resulting sequences can be obtained from the NCBI GenBank (accession nos. AJ579530–AJ579533).

The expression of *hsp70* isoform 1 (Fig. 2A) decreased in a highly significant manner from stage I to stage II, stage III and stage IV. The copy number of this sequence was very low at the transitional stage (II), cryptobiotic stage (III) and consecutive transitional stage (IV) compared with at the active stage. Between the cryptobiotic stage (III) and the transitional stage (IV) there was no significant difference. By contrast, the copy number of isoform 1 in the active stage (V) 90 min after the transitional stage (IV) was increased more than twofold compared with stage I, with a significant difference to stage IV and a weakly significant difference to the active stage (I). In contrast to *hsp70* isoforms 2 and 3, the expression of isoform 1 in *M. tardigradum*, compared with the *beta-actin* housekeeping gene copy numbers, was very high (Table 2).

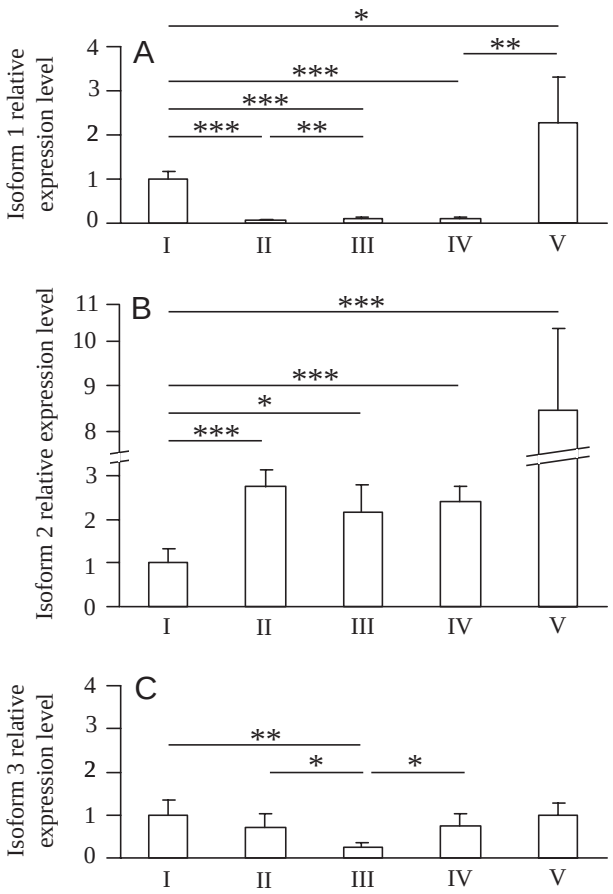

Fig. 2. *hsp70* expression at the different life cycle stages (I–V) of the tardigrade *Milnesium tardigradum*: (A) *hsp70* isoform 1, (B) *hsp70* isoform 2 and (C) *hsp70* isoform 3. The mRNA copy number was calculated based on the *beta-actin* housekeeping gene as described in the Materials and methods. Relative expression levels refer to stage I=100%=1.0. Results are presented as means ± S.D. The stages are as follows: I, active before cryptobiosis; II, transitional before cryptobiosis; III, cryptobiotic; IV, transitional after cryptobiosis; V, active after cryptobiosis.

Table 2. hsp70 *expression at the different life cycle stages of the tardigrade* Milnesium tardigradum *(I–V) and heat-shocked animals*

Stage	Isoform 1	Isoform 2	Isoform 3
I	1980±363	10±3	91±30
II	145±21	27±4	66±27
III	230±27	22±6	24±7
IV	211±36	24±4	69±25
V	4531±2011	83±20	91±27
Heat shock	11 807±2205	205±34	759±157

The mRNA copy number was calculated based on 100 *beta-actin* housekeeping gene copies as described in the Materials and methods. Results are presented as means ± S.D.

The *hsp70* expression of isoform 2 (Fig. 2B) showed the lowest level in the active stage (I) and increased to the second highest observed level in the transitional stage (II). During the cryptobiotic stage (III), the level was slightly reduced again but increased continuously in the course of stages IV and V. The maximum level was achieved in active stage V, with a highly significant elevated expression that was about eight times as high as in active stage I.

The lowest mRNA expression of *hsp70* isoform 3 (Fig. 2C) was detected in *M. tardigradum* during the cryptobiotic stage (III), followed by a significant increase of expression during stage IV. By contrast, mRNA expression was relatively high during the active stage and reached about the same level in stage V. During the transitional stages (II and IV), mRNA expression showed no significant changes compared with the active stages (I and V). Nevertheless, transcription levels showed a clear decrease from the active stage *via* dehydration to the cryptobiotic stage and an increasing trend from the cryptobiotic stage *via* rehydration to the active stage again.

Expression of all *hsp70* family member genes was significantly (isoform 3) or even highly significantly (isoform 1 and 2) elevated during a heat shock at 37°C for 90 min (Fig. 3). Isoforms 1 and 3 showed heat-inducible expression levels that were 6–8-fold higher than the levels of the non-stressed active stages, respectively. The highest relative elevation in the studied *hsp70* isoforms was found in isoform 2, which showed a >20-fold higher level after heat shock.

In summary, three different patterns of gene expression in the studied *hsp70* mRNA isoforms were observed.

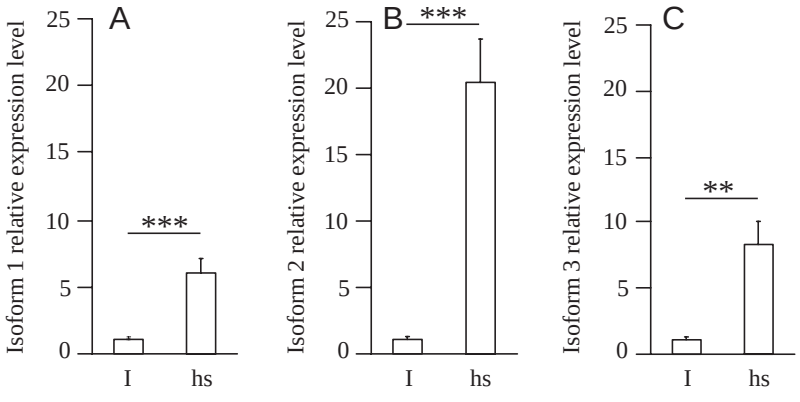

Fig. 3. *hsp70* expression of the control active stage (I) and an active stage subjected to a heat shock (hs) at 37°C for 90 min: (A) *hsp70* isoform 1, (B) *hsp70* isoform 2 and (C) *hsp70* isoform 3. The mRNA copy number was calculated based on the *beta-actin* housekeeping gene as described in the Materials and methods. Results are presented as means ± S.D.

Discussion

Tardigrades are well known for their ability to undergo a cryptobiotic stage. Mechanisms that control the entry into cryptobiosis and the later activation of the tuns are poorly understood. One of the functions that needs to be regulated during these processes is gene transcription. For the brine shrimp *A. franciscana*, there is no transcriptional activity in the cyst, but several studies have shown the induction of gene expression a few hours after their activation (Marco et al., 1991; Escalante et al., 1994). One possible explanation for the lack of transcriptional activity in the cyst of the cryptobiotic stage would be the absence of a functional transcriptional machinery (Sastre, 1999). Nevertheless, the accumulation of specific mRNA molecules associated with the cryptobiotic process has been reported in both prokaryotic (Albertson et al., 1990) and eukaryotic cells. In the ciliates *Colpoda inflata* (Benitez and Gutiérrez, 1997) and *Sterkiella histriomuscorum* (Tourancheau et al., 1999), mRNAs were found in resting cysts. Several fungal species produce dormant spores containing stored mRNA (Camonis et al., 1982) and, in *Acanthamoeba*, Byers et al. (1991) managed to isolate mRNA from resting cysts that was capable of actin translation *in vitro*. Gutiérrez et al. (2001) described transcripts encoding, among others, a heat-shock protein (Hsp70) in cryptobiotic stages of *C. inflata* and *Colpoda nova*.

To the best of our knowledge, this is the first report on different heat-shock (*hsp70*) gene transcripts stored in cryptobiotic stages in tardigrades. It is questionable whether the transcripts represent remnant mRNAs produced during transition from the active stage to the cryptobiotic stage, which will be destroyed without use or otherwise will be translated during the following rehydration, once the translation activity is restored.

It is apparent that all three isoforms seem to be true hsps since they could be clearly induced by temperature elevation. In addition, all three isoforms show a constitutive basic level, even without stress, and therefore fulfil this requirement for heat-shock cognate (hsc) genes.

Santomenna and Colberg-Poley (1990) showed the effect of heat-shock treatment upon human cytomegalovirus (HMCV) induction of *hsp70* RNA and Hsp70 protein expression. They found that there was a several-hour delay between the time of *hsp70* RNA induction and the time of

increased inducible Hsp70 protein expression. A correlation between *hsp70* RNA and Hsp70 protein in cerebral tissue of birds after heat stress has also been shown by Dionello et al. (2001). We conclude that the *hsp70* RNA of tardigrades will be translated into Hsp70 proteins to a comparable extent. Thus, the difference between hsp and hsc obviously has not been established in tardigrades, a rather basic group of 'pre-arthropods' (Garey et al., 1996). A similar situation was found in Diplopoda (Knigge, 2003), another phylogenetically 'old' group.

All three isoforms are endogenously regulated, following the steps active–cryptobiotic–active. Based on absolute copy numbers and on the expression pattern, isoform 1 seems to be the dominant *hsp70* isoform in active tardigrades. Expression of isoform 1 seems to cease prior to the transitional stage, and a presumably short half-life of this isoform leads to a rapid decrease in isoform 1 level. Eventually, transcription starts again at the end of stage II, right before formation of the cryptobiotic stage, but it is questionable whether the observed significant difference between stages II and III is biologically relevant. Transcription of isoform 1 starts again when tardigrades have reached the active state again. However, we regard isoform 1 as being the most important isoform in 'normal' (i.e. non-cryptobiotic) metabolism (i.e. a 'heat-inducible hsc'). The pattern of isoform 3 is similar to that of isoform 1 but at a much lower level. In contrast to the expression pattern of isoform 1, the pattern of isoform 3 is strongly induced by high temperature. Thus, isoform 3 can be regarded as a true hsp, with a rather low constitutive level and a more than eightfold increase by heat shock. As with isoform 1, isoform 3 does not seem to have a specific function for cryptobiosis, even though cessation of transcripts seems to take place later than in isoform 1: the transitional stages showed slightly and non-significantly lower levels than the active stages. Concomitantly, transcription of isoform 3 definitely starts upon 'awakening' from the cryptobiotic stage in the transitional stage (IV).

We regard isoform 2 as the classical hsp since it showed a very large constitutively transcribed copy number and a 20-fold inducibility by heat shock. Furthermore, this isoform is inducible by the stress posed to the individual when undergoing cryptobiosis. Transcription of isoform 2 is significantly induced in transitional stage II, resulting in a comparatively high mRNA copy number. The copy number remains constant throughout the cryptobiotic stage and the transitional stage of 'awakening' tardigrades, thus implying that isoform 2 is the most relevant *hsp70* gene for cryptobiosis. Based on the induction cascade of true hsps by malformed and nascent polypeptide strains, transcription of isoform 2 is elevated further when the tardigrades turn from cryptobiosis into a new active stage, and, consequently, the formation of overall new proteins starts. In the present study, stage V represents the first 90 min of the new active stage only; therefore, it is supposed that expression of isoform 2 will decrease after a while and remain constantly low, as shown for active stage I. Presumably, isoform 1 will take over some time after the return to the active stage and will cover the tasks of isoform 2, which remains at a very low level further on. Ramløv and Westh (2001) described the appearance of protein bands with a molecular mass of 71 kDa from cryptobiotic tardigrades [*Adorybiotus* (*Richtersius*) *coronifer*] and the absence of bands from active animals. However, they are not certain that the observed *de novo* protein synthesis was a heat-shock protein belonging to the Hsp70 family, but one can speculate the highly inducible protein form, deriving from *hsp70* isoform 2.

Like most nematodes, tardigrades regularly show cell constancy (Greven, 1980), and increasingly the cellular structure has to be secured by protecting mechanisms. We have shown that different expression of *hsp70* genes is involved in the cycle of dehydration, cryptobiosis and rehydration, but the role of other stress genes in this process still remains to be clarified.

Thanks are due to Eva-Maria Huber, Irina Panchuk, Roman Volkov and Markus Wunderlich for their help with the real-time quantification technique, Karl-Heinz Helmer for the SEM images and to Eva Schwörtzer for general assistance. We also wish to thank Alan Tunnacliffe and Jim Clegg for their helpful advice and critical discussion on the manuscript and Marcelo Sánchez-Villagra for proofreading. This study was financed by the Wilhelm Schuler Foundation, Tübingen, Germany.

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