Expression patterns of heat shock genes during population dynamics of the rotifer *Brachionus plicatilis*

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KEY WORDS: *Brachionus plicatilis*, GRP94, HSP70, lifespan, rotifer, ubiquitin-conjugating enzyme.

INTRODUCTION

Due to their short lifespan and fertility rotifers have been used as a model organism in ecology and gerontology.¹⁾ Yoshinaga *et al.*²⁾ demonstrated that the population dynamics of the rotifer *Brachionus plicatilis* shows a typical sigmoid curve. Under starved conditions in the stationary phase, individuals reduce their lifetime fecundity and prolong lifespan and reproductive period to maintain a stable population. This suggests that rotifers can control their lifespan to adapt themselves to various environmental conditions. Although such a trade-off between lifespan and reproduction is found in various species, the underlying molecular mechanisms are still unclear. To cope with such ambiguities, we cloned the rotifer HSP70, GRP94 and ubiquitin-conjugating enzyme (E2), which have been suggested to prolong lifespan of fruit fly³⁾ and yeast,⁴⁾ and further examined for phase-specific expression patterns.

MATERIALS AND METHODS

We used a clonal *Brachionus plicatilis* established from a single amictic female of the Ishikawa strain.⁵⁾ The rotifers were cultured in Brujewicz artificial seawater (BAS) at 25 °C, under total darkness except feeding time. The feeding algae (*Nannochloropsis oculata*) was suspended in the medium at 7x10⁶ cells/mL. Rotifers were collected through filtration on days 10 (exponential phase) and 15 (stationary phase) after inoculation of 10 rotifers bearing 3-4 eggs into 400mL BAS following which total RNA were extracted using ISOGEN solution (Nippon Gene). Poly (A)⁺ RNA was fractionated using Oligotex-dT30 super (Takara).

First strand cDNA was synthesized as follows. An aliquot of 1 µg total RNA

prepared each from the exponential and stationary phases was dissolved in 12 µL water and heated at 70 °C for 10 min, then quickly chilled on ice before synthesis. The *NotI* oligo dT primer was used at 50 µg/mL to initiate first strand cDNA synthesis with 0.5U Superscript II reverse transcriptase (Gibco BRL) in a buffer containing 50mM Tris-HCl (pH 8.3), 75mM KCl, 3mM MgCl₂, 500mM dNTP, and 10mM DTT. The reaction was carried out at 42 °C for 1 h in total 20 µL and then the enzyme was heat-inactivated at 70 °C for 15 min. Ten µg of RNase H was then added to remove RNA templates.

Degenerate primers of HSP70F1 and HSP70R1 for HSP70, rGRP94 and rGRPR1 for GRP94, and rE2F1 and rE2F2 for ubiquitin-conjugating enzyme were designed with reference to DNA nucleotide sequences from various species (GenBank accession number XM_004189, U09861, M76613, AB062281, AJ001312 for HSP70; AF012437, M10051 for GRP94; and AF020344, AF114261, AF032885 for ubiquitin-conjugating enzyme). PCR consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for HSP70 and GRP94 or 42 °C for ubiquitin-conjugating enzyme for 30 s, and polymerization at 72 °C for 1 min, with a final polymerization step at 72 °C for 5 min. The composition of the reaction mixture was according to manufacturer's protocol using 1U Ex *Taq* DNA polymerase (Takara). The amplified products were subcloned into the pGEM-T Easy Vector (Promega) and sequenced from both 5' and 3' strands using primers complementary to vector arms with Dye DeoxyTM terminator cycle sequencing kit using a DNA sequencer model 373S (Applied Biosystems). Sequence homology was analyzed using BLAST search.

For Northern blot analysis, 10 µg of total RNA, prepared each from rotifers in exponential and stationary phases were

resolved in electrophoresis on a 0.9% agarose gel containing 0.44M formamide and blotted onto a Hybond N⁺ nylon membrane (Amersham). The membrane was baked at 120 C for 20 min, then prehybridized in a solution containing 0.5M Na₂HPO₄ (pH 7.2), 1mM EDTA, and 7% sodium dodecyl sulfate (SDS) at 65 C for 5 min. Hybridization was performed at 65 C for 16 h in the same solution containing cDNA probes described below. The membrane was washed with 1 x SSC (0.15 M NaCl, 15mM sodium citrate, pH 7.0) containing 0.1% SDS for 20 min at room temperature and subsequently with the same buffer at 65 C for 20 min, then exposed to X-ray films at -80 C for 24 h. The hybridized membrane was scanned with a Fujix BAS 1000 computerized densitometer scanner and quantified using a recommended scanning program. cDNA fragments amplified by RT-PCR for rotifer HSP70, GRP94 and ubiquitin-conjugating enzyme were used as probes. Twenty ng/ μ L of the fragments were labeled with [α^{32} -P] dCTP using Takara random primer DNA labeling kit Ver. 2 (Takara).

RESULTS AND DISCUSSION

cDNA fragments encoding rotifer HSP70, GRP94 and ubiquitin-conjugating enzyme were successfully cloned by PCR amplifications using primers described before (GenBank accession number AB076052, AB076053 and AB076054, respectively). Three lots of total RNA were extracted each from rotifers in the exponential and stationary phases and subjected to Northern blot analysis for HSP70, GRP94 and ubiquitin-conjugating enzyme. The mRNA accumulated levels of HSP70 and GRP94 in the exponential phase were 2.5 and 1.6 times higher, respectively, than those in the stationary phase, whereas the levels of ubiquitin-conjugating enzyme did not differ between the two phases.

Expression of stress proteins induced by several origins has been claimed to be associated with lifespan extension beside their participation in refolding or selective degradation of denatured proteins. In transgenic fruit fly *Drosophila melanogaster* with increased copy numbers of the *hsp70* gene, nonlethal heat-treatments induced HSP70 protein to levels greater than 30% of wild-type control and prolonged life expectancy.³⁾ In this study, the accumulated mRNA levels of HSP70 and GRP94 in the exponential phase

were higher than those in the stationary phase, indicating that expression of these stress proteins are not directly related to the extension of lifespan in the stationary phase. An alternate explanation may be derived from the fact that populations in the exponential phase consisted of mainly immature and young adult females, whereas those in the stationary phase consisted of old females.¹⁾ Since induction of HSPs shows age-associated decline in certain species such as rats,⁶⁾ our data may reflect the increased ratio of old rotifer females in the stationary phase.

Our results about the expression change of HSP70 and GRP94 in concert with the population dynamics suggest that these stress proteins are useful molar markers for monitoring the population phases of the rotifer. However, it appears that the expression of HSPs is not directly related to the extension of lifespan in the rotifer, as mentioned before.³⁻⁴⁾ Further studies are necessary to disclose the mechanisms involved in the regulation of rotifer lifespan.

ACKNOWLEDGMENTS

This study was partly supported by a Grant-in-Aid for Creative Scientific Research No. 12NP0201 from the Ministry of Education, Culture, Sports, Science and Technology of Japan. SK and TY were supported by Research Fellowships of the Japan Society for the Promotion of the Science for Young Scientists.

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