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Molecular characterization and expression analysis of *hsp60* gene homologue of sheep blowfly, *Lucilia cuprina*

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ABSTRACT

The 60 kDa heat shock protein (Hsp60) or chaperonin is one among the highly conserved families of heat shock proteins, known to be involved in variety of cellular activities, including protein folding, thermal protection, etc. In this study we sequence characterized *hsp60* gene homologue of *Lucilia cuprina*, isolated and cloned from the genomic library as well as by genomic PCR, followed by RACE- PCR. The *L. cuprina hsp60* gene/protein expression pattern was analyzed in various tissues, either at normal temperature (25 ± 1 °C) or after exposure to heat stress (42 °C). The analysis of nucleotide sequence of *Lchsp60* gene revealed absence of intron and the nuclear localizing signal (NLS). The deduced amino acid sequence showed presence of unique conserved sequences, such as those for mitochondrial localization, ATP binding, etc. Unlike *Drosophila*, *Lucilia* showed presence of only one isoform, i.e., *hsp60A*. Phylogenetic analysis of *hsp60* gene homologues from different species revealed *Lchsp60* to have $>88.36\%$ homology with *D. melanogaster*, 76.86% with *L. sericata*, 58.31% with mice, 57.99% with rat, and 57.72% with human. Expression analysis using Real Time PCR and fluorescence imaging showed significant enhancement in the expression level of *Lchsp60* upon heat stress in a tissue specific manner, indicating its likely role in thermo-tolerance as well as in normal cellular activities.

1. Introduction

Heat shock proteins are highly conserved and ubiquitously present from prokaryotes to eukaryotes (Heikkila, 1993a,1993b). Heat shock has global effects on various cellular activities under stressed and unstressed conditions. Among the major classes of Hsps, Hsp60 chaperonin (s), also named as GroEL/Cpn60/Hsp60, has an important but passive role in protein folding (Radford, 2006). Chaperonins are a conserved class of octameric double-ring complexes with a molecular mass of 800 kDa, shaping a central cavity for binding of native and misfolded proteins (Hartl and Hayer-Hartl 2002). There are two major groups of chaperonins: (1) Group I chaperonins, also known as Hsp60, are generally found in eubacteria and in organelles of endosymbiotic origin, explicitly the mitochondria and chloroplasts. They cooperate with cofactors of the GroES or Hsp10 family of proteins (co-chaperone of GroEL or Hsp60, respectively); 2) Group II chaperonins are GroES independent, and abundantly present in the eukaryotic cytosol (Nitsch et al., 1998). In protein folding, the process of substrate binding to GroEL/Hsp60 and dissociation of chaperonin from central cavity is ATP dependent (Ditzel et al., 1998). The non-native substrate proteins are captured through hydrophobic interactions of inner cavity and are then entrapped in the central ring cavity, where they get folded and prevented from further aggregation (Ellis and Hartl, 1996; Fenton and Horwich, 1997; Bhutani and Udgaonkar, 2002). The significant structural and functional similarities across various species from prokaryotes to eukaryotes and the striking variations in *hsp60* gene structure and its isoforms suggest that this gene has evolved with functional diversity (Kozlova et al., 1997; Pochan and Mach, 1996; Poss et al., 2000; Timakov and Zhang, 2001; Venner and Gupta 1990; Werner et al., 1997). The above information indicates *hsp60* to be one of the most enigmatic molecular chaperones of great significance.

The blowfly, *Lucilia cuprina* (Diptera), is a sheep pest, known to be uniquely adapt in nature to a wide range of environmental stress conditions, including high temperature, heavy metals, pesticides, host immune response, etc. (Tiwari et al., 1995; Joshi and Tiwari, 2000; Sharma et al., 2006), and thus, can be used as a

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suitable biological model to study the mechanism of stress response. The fly exhibits unique ability to withstand considerably increased level of thermal stress, accompanied with increased expression of different Hsps, including Hsp60, Hsp70, Hsp90 and small Hsps. Our earlier studies have demonstrated various unique properties of stress response and high temperature tolerance in this fly (Tiwari et al., 1995; Joshi and Tiwari, 2000; Sharma et al., 2006). We showed that the expression of Hsp60 protein in *L. cuprina* varies with tissue type and developmental stage (larva and adult) (Reddy et al., 2006; Sharma et al., 2006). The present study is focused on the molecular characterization of *hsp*60 gene homologue of *L. cuprina* in order to understand its evolutionary relation with other species and analyze its likely role and functional significance during development, differentiation and tolerance (adaptability) towards heat stress.

2. Material and methods

2.1. Fly culture and maintenance

The *L. cuprina* fly stock was reared in the insectary in a humidified chamber (50–60% relative humidity) at 26 ± 2 °C temperature. The adults were fed on raw goat meat and sugar, whereas, larvae were fed only on goat meat till pupation.

2.2. Construction of Fosmid Library

The genomic DNA from adult flies was isolated using phenol: chloroform method (Ashburner, 1989). The quantity and quality of DNA was analyzed by UV–visible spectrophotometer. The genomic DNA was fragmented by mechanical shearing. The 25–40 kb long DNA fragments were separated by Pulse field Gel electrophoresis (BioRad CHEF DR-II, Electrophoresis cell Power supply-BioRad Pulse wave 760, USA). The electrophoresis was carried out using following parameters: 1% LMT agarose in 1 × TBE buffer (45 mM Tris-Borate, 1 mM EDTA), the current applied being forward 9 V/cm and reverse 6 V/cm, with switch time 0.1 s (initial) and 21.8 s (final), start ratio 1.0, ramping linear, run time 16 h at 14 °C and voltage set at 180 V. The fragmented DNA was made blunt ended at the 5' end. The gel extracted fragments were ligated into Fosmid Vector pCC2FOS™, following manufacturer's instructions (Copy-Control™ HTP Fosmid Library production kit, Epicentre Technologies, Madison, WI, USA) (Rowe-Magnus, 2009). The ligated DNA was packaged using MaxPlax™ Lambda packaging extract. Packaged fosmid clones were either used for immediate transformation or stored at 4 °C over chloroform in 1.0 ml phage dilution buffer (10 mM Tris–HCl pH 8.3, 100 mM NaCl, 10 mM $MgCl_2$). The clones were mixed with EPI300-T1® cells and incubated at 37 °C for overnight. The separated individual colonies were picked up and transferred to individual well of 96-well microtitre plates containing Luria Bertani medium, supplemented with 20% glycerol and 12.5 μg/ml chloramphenicol using sterile tooth pick. The plates were stored at −80 °C for further use.

2.3. Size estimation of Fosmid clones

The size of cloned fragmented DNA was determined by using control fosmid DNA (~40 kb) run in adjacent lanes of obtained positive clones from genomic library using Fosmid DNA purification kit (Fosmid Max™ DNA purification kit, USA). The isolated fosmid DNA was restriction digested using *Not I* enzyme and the digested products were electrophoresed in 1.0% TAE-agarose gel. The size of transferred DNA was estimated under UV trans-illuminator, which were in the range of 25–40 kb in each clone.

2.4. Screening of Lucilia genomic library for hsp60

The *Lchsp60* positive colonies were screened by colony hybridization using α-^{32}P-dATP labelled *hsp60* (60-1F-3R, 1668 bp) probe obtained from genomic PCR using primers designed from *Drosophila hsp60* cDNA (GenBank accession no. X99341). The phage colonies were transferred onto the nylon membrane (Hybond-N, Amersham) and incubated with labelled probe for hybridization followed by autoradiography. The genomic library was confirmed by screening of few constitutive genes specific to *Lucilia*, such as acidic ribosomal phosphoprotein PO (RPLO) and Glutathione S transferase (GST), (Bagnall and Kotze, 2010) labelled with α-^{32}P-dATP. The obtained positive RPLO and GST specific phage colonies were subjected to PCR amplification and the products were sequenced commercially and aligned with other species.

2.5. Gene amplification and sub-cloning of Lchsp60 related sequences and hsp60 isoforms

The full length *Lchsp60* gene sequences were amplified by PCR (60-1F-3R, 1668 bp) using forward 5' GTC GCA ATG TGA TCA TTG AG 3' and reverse 5' AGT CGC TTA CAT CAT GCC AC 3' primers, followed by RACE-PCR. The primers were designed from *hsp60* cDNA sequence of *D. melanogaster*. The amplified fragments were sub-cloned in pTZ57R/T vector with a T overhang end and sequenced commercially.

Drosophila hsp60 isoforms, *hsp60A, B, C*, and *D* were used to search out or amplify isoforms related colonies or sequences from *Lucilia* genomic library/gDNA. Further, confirmations of colonies were done by using Southern hybridization, followed by autoradiography. Simultaneously, RT-PCR (reverse transcriptase PCR) were used to identify and isolate positive colonies for their isoforms by using *Dmhsp60* isoforms specific primers. The sequences of the primers/DNA probes were designed from mRNA sequences of *D. melanogaster hsp60* isoforms A, B, C and D (GenBank accession nos. NM_078560.3; NM_167266.1; NM_135104; NM_135812 respectively). The primer sequences used were: (*hsp60A* F-5'-TTGCTCGCTCCTCCATTA-3' and R-5'-CGCTTACATCATGCCACC-3'; *hsp60B F-5'*-GATTACCTCTAGTCGCTGTT-3' and R-5'-GTCAATGTCACGCACCTCTA-3'; *hsp60C* F-5'-GTTCCGTTACACCAACACAT-3' and R-5'-GGCAACTCCGTAACTACAG-3'; *hsp60D* F5' TTAGCAAGATCCTT GGTGAC 3' and R-5'-GGATGCTGAGCAGATTGG-3').

2.6. PCR amplification of Lchsp60

Three sets of primers spanning the entire coding region of *hsp60A* were designed from *Drosophila hsp60* cDNA (60-IF 5' GTCGCAATGTGATCATTGAG 3'; 60-1R 5' GAGGAGTTGATGAAGTACGG 3'; 60-2F 5' GATCTCGTCAAGCTGGAGGA 3'; 60-2R 5' AATCGCTAA CCTTGACGTCC 3' and 60-3R 5' AGTCGCTTACATCATGCCAC 3'). The primers designed from *Dmhsp60* sequences were also used for library screening (see above). The PCR amplification was performed in 25 μl reaction using 100 ng genomic DNA as template. The cycling conditions were, denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min and extension at 72 °C for 2 min, total 30 cycles (EP500 Thermocycler, Eppendrof, Germany).

2.7. RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) PCR for 5' and 3' UTRs

RNA was isolated from different tissues of larva or adult, using Trizol reagent (Invitrogen, USA) and cDNA was prepared. To isolate 5' and 3' UTRs, 5' and 3' RACE PCR was performed using RLM-RACE kit (RNA Ligase Mediated Random Amplification of cDNA Ends,