Protein synthesis rates in *Drosophila* associate with levels of the *hsr-omega* nuclear transcript

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Abstract Transcripts of the *Drosophila hsr-omega* gene are known to interact with RNA processing factors and ribosomes and are postulated to aid in co-ordinating nuclear and cytoplasmic activities particularly in stressed cells. However, the significance of these interactions for physiological processes and in turn for whole-organism fitness remains an open question. Because *hsr-omega*'s cellular expression characteristics suggest it may influence protein synthesis, and because both genotypic and expression variation of *hsr-omega* have been associated with thermotolerance, we characterised 30 lines for variation in the rates of protein synthesis, measured in ovarian tissues, both before and after a mild heat shock, and for basal levels of the two main *hsr-omega* transcripts, *omega-n* and *omega-c*. As expected, the mild heat shock reduced protein synthesis rates. Large variation occurred among lines in levels of *omega-n* which was negatively associated with rates of basal protein synthesis—a result that supports the model for the cellular function of *omega-n*. Furthermore, *omega-n* levels were associated with *hsr-omega* genotype of the line parents. Little variation occurred among lines for *omega-c* levels and no associations were detected with protein synthesis or genotype. Since protein synthesis is a fundamental process for growth and development, we characterised the lines for several life-history traits; however, no associations with protein synthesis, *omega-n* or *omega-c* levels were detected. Our results are consistent with the idea that natural variation in *hsr-omega* expression influence rates of protein synthesis in this species.

Keywords *hsr-omega* · Protein synthesis · *Drosophila melanogaster*

Introduction

The ecological, physiological and molecular-genetic bases of heat tolerance in *Drosophila* have been the subject of thorough investigation (see reviews by Morimoto et al. 1997, Parsell and Lindquist 1993 and Feder and Hofmann 1999). Central to understanding the processes that protect organisms from heat stress is the cellular heat shock response, and two defining features of this response involve the changes to protein synthesis that follow heat shock—heat shock proteins (hsps) are synthesised and general protein synthesis is shutdown (Lindquist 1980; Storti et al. 1980). A convincing association between the control of protein synthesis and high levels of heat tolerance in laboratory-selected lines was first demonstrated in the early 1980s by Alahiotis and Stephanou (1982) and Stephanou et al. (1983). In these studies the kinetics of protein synthesis that was assessed in ovarian tissues following a heat shock was associated with changes in the timing and extent of hsp production, with timing and extent of housekeeping protein shutdown, and with heat stress survival differences between the lines. While a few other studies have asked if protein synthesis relates to thermal tolerance (Dingley and Maynard-Smith 1968; Burton et al. 1988; Misener et al. 2001), and considerable research has shown how hsps and their genes are up-regulated following thermal stress (Lis and Wu 1994; Yost et al. 1990), little effort has been made to investigate the shutdown of general protein synthesis and how this impinges on hsp production and thermotolerance.

Protein synthesis is a fundamental biological process that may affect many aspects of an individual's fitness and how

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this is controlled, particularly under heat stress conditions, is likely to be important to an individual's fitness. For example, mutations in a number of genes that directly or indirectly affect protein synthesis capacity have effects on body size, development rate, fertility and longevity (Marygold et al. 2007; Teleman et al. 2005; Syntichaki and Tavernarakis 2006). However, whether or not naturally occurring, heritable and ecological significant variation in protein synthesis capacity exists and is related to fitness or life-history traits is an open question.

While the molecular and expression patterns of one of the classic heat shock genes of *Drosophila*, *hsr-omega*, have been well described (Pardue et al. 1990), the significance of this gene to the cell and for the whole organism are not well understood. A growing body of data suggest that it may influence both protein synthesis (summarised in Lakhota 2003) and heat tolerance (McKechnie et al. 1998). *Hsr-omega* produces no protein product but encodes two main RNA transcripts that are expressed constitutively in almost all cell types and at nearly all life stages (Fig. 1). These transcripts are rapidly up-regulated in response to heat treatment, during recovery from cold exposure and by exposure to various other environmental stressors (Bendena et al. 1989; Tapadia and Lakhota 1997; Collinge et al. 2008).

One of the *hsr-omega* transcripts, the cytoplasmic *omega-c* resembles a typical messenger RNA, having a small ORF, being spliced and transported to the cytoplasm where it associates with ribosomes (Fini et al. 1989). According to Pardue et al. (1990), the polysomal-associated turnover of *omega-c* may serve to monitor or regulate some aspect of protein synthesis in the cell. The second, nuclear-restricted transcript, *omega-n*, may also influence protein synthesis by controlling release into the cytoplasm of messenger RNAs—the essential templates for building proteins. *Omega-n* shares the same transcriptional start site as *omega-c*; however, it is much longer and remains unspliced (Fig. 1). What makes *omega-n* unusual is that it

contains a large 3' region consisting of a variable number of ~280-bp tandem repeats resulting in transcripts between ~8 kb and ~20 kb in length depending on the strain (Hogan et al. 1994). Under heat stress, the nuclear distribution of *omega-n* rapidly shifts and associates with several heterogeneous ribonucleoproteins (hnRNPs), many of which are involved with processing and transport of pre-mRNAs and mRNAs (Lakhota et al. 1999; Krecic and Swanson 1999). In the current model (Fig. 2), the binding of a subset of these hnRNPs to *omega-n* is likely to be dependent upon their high affinity for a short, but highly conserved motif AUAGGUAGG that occurs twice within each of the numerous *omega-n* repeats (Zu et al. 1998). In this model the tandem repeats of *omega-n* serve to quantitatively bind specific hnRNPs, while those remaining unbound are available for nuclear processing of pre-mRNAs with ensuing transport of mature mRNAs to the cytoplasm for protein synthesis. Under heat stress, when *omega-n* is elevated, we would expect a reduction in delivery of mature mRNAs to the cytoplasm and less protein synthesis.

Geographical patterns of genotype variation in *hsr-omega* suggest a variable adaptive role for this gene along a climatic gradient. First, an 8-bp indel polymorphism (the L/S variation, Fig. 1) occurs in the first exon, with the S (short) allele being strongly and positively associated with latitude along the Australian eastern coastline (Anderson et al. 2003). Second, the multi-allele tandem-repeat variation at the 3' end of the gene (Fig. 1) also clines with latitude along the same transect, largely independent of the L/S variation, with average repeat number being consistently and negatively associated with latitude (Collinge et al. 2008). In addition, both variation in *hsr-omega* genotype and *hsr-omega* transcript levels have been related to variation in heat and cold tolerance (McColl et al. 1996; McKechnie et al. 1998; Rako et al. 2007; Collinge et al. 2008). Also, both heat and cold tolerance show latitudinal clines along the Australian transect, clines that are opposite in direction (Hoffmann et al. 2002). These data suggest that *hsr-omega* variation contributes to adaptation to different thermal environments; however, there is a lack of evidence for a cellular mechanism by which *hsr-omega* influences thermotolerance or associated fitness traits.

To address this issue, we characterise a set of *Drosophila melanogaster* lines for variation in rates of protein synthesis and ask if protein synthesis levels relate to variation in the *hsr-omega* gene. We use 30 single-pair mating lines derived from a single mass-bred population that was recently collected at a central latitude location along the Australian east-coast. At such a location genotype and phenotype variation is likely to be maximised, and the use of single-pair lines both limits variation within lines and maximises variation among lines. Associations between protein synthesis capacity, measured in ovarian tissue (both before and

Fig. 1 Structure of the *hsr-omega* gene with the positions of the *hsr-omega*-L/S and tandem repeat polymorphisms indicated (modified from Pardue et al. 1990). Positions of *omega-n* and *omega-c* transcript-specific primers used for qRT-PCR are indicated by arrows

Fig. 2 Model of *omega-n* function in the nucleus. When *omega-n* level is high more sequestration of hnRNPs occurs, removing them from normal nuclear activities that include splicing of intron-containing primary transcripts and nuclear export of mature mRNAs (Lakhotia et al. 1999). Consequential reduction in rates of mRNA maturation and export result in less total translation of hnRNP-processed messages and decreased protein synthesis. This may free-up ribosomes for imminent arrival of HSP mRNAs and hsp synthesis

after a mild heat shock) and variation in both *hsr-omega* transcript levels and genotype are explored. We also investigated links between protein synthesis rates and four basic fitness/life-history traits. Our data suggest that for *D. melanogaster* variation in protein synthesis capability may be a process influenced by natural heritable variation in the *hsr-omega* gene.

Materials and methods

Line derivation, *hsr-omega*-L/S genotyping and maintenance

D. melanogaster were collected in March 2005 from Coff's Harbour (30° 18' S; 153° 08' E), and maintained as 25 isofemale lines for 5 months prior to pooling into a single mass bred laboratory population. This was cultured in duplicate bottles for eight generations prior to establishing 150 single-pair mating lines using virgin females. The parental flies from these lines were genotyped for *hsr-omega*-L/S variation as previously described (Anderson et al. 2003) and a set of 30 lines with wide variation in *hsr-omega*-L frequency were selected. This resulted in zero, one, three and four copies of the *omega*-L allele occurring in the parents of three, 15, six and six lines, respectively. All cultures were maintained at 25°C under a 12:12 h light–dark regime in 250 ml bottles containing a treacle-semolina medium. Adults used for measuring protein synthesis and RNA extraction were raised at constant uncrowded densities for at least one generation prior to testing. Protein synthesis estimates were performed within four generations of line

derivation and transcript level analysis and life-history trait measurements were conducted four and eight generations later, respectively.

Protein synthesis

Protein synthesis rates were assessed by measuring the amount of ^{35}S -methionine incorporation into dissected *Drosophila* ovaries in 1 h following a method modified from Stephanou et al. (1983) and Peterson et al. (1979). All lines were raised in multiple cohorts staggered by 2 days to minimise effects of female age which was between 2 and 8 days. In each dissection session (18 sessions at the rate of two per day, all within a 14-day period) five lines were sampled at random (until three replicates of each line were obtained). Ten assays occurred per session, unstressed females being assayed immediately before the heat-stressed females of the same line. For the heat stress treatment, five adult females were placed in a 1.7-ml centrifuge tube and held in a heating block at 37°C for 15 min immediately prior to dissection. Ovaries from four of these females were pooled per sample. Immediately following dissections, samples were incubated with 6 μl Grace's Insect Medium (Invitrogen) and 6 μCi of ^{35}S -methionine (Amersham Biosciences) for 1 h at 25°C. Protein synthesis was halted by the addition of 300 μl of ice-cold phosphate-buffered saline (PBS), agitated, held on ice for 1 min, centrifuged and supernatant discarded. While holding on ice, pellets were thoroughly washed a further three times to remove unincorporated ^{35}S -methionine (each time mixing by vortex, centrifuging and careful removal of all possible supernatant without disturbing the pellet). Ovaries were

dried in a Speedvac (Savant Speedvac SC100) set at medium drying temperature for 20 min. Proteins were extracted from dry ovaries by incubation at 95°C for 6 min in 40 µl of sample buffer (0.0625 M Tris-HCl pH 6.8, 1% sodium dodecyl sulphate, 1% β-mercaptoethanol (added on day of use), 10% glycerol) before being placed briefly on ice and centrifuged. The supernatant was transferred to a new tube and 5 µl removed to 5 ml liquid scintillate (Ecolume, MP Biomedicals) for scintillation counting (LKB Rackbeta, Wallac). Counts per minute (CPM) of radioactive emissions were corrected for session effects by multiplying each by grand mean/session mean. Incorporation of label into protein and its successful recovery were confirmed using autoradiography by the detection of labelled protein bands from the supernatant run on electrophoretic gels. In addition, control assay in which ovaries were pre-incubated with 3 mM cycloheximide (a protein synthesis inhibitor; Sigma-Aldrich Co, St Louis, C-7698; Bendena et al. 1989; Marcos et al. 1982) at 25°C for 20 min prior to and during labelled methionine incorporation confirmed that >75% of recovered label was due to protein synthesis.

Quantification of *hsr-omega* transcripts

Levels of *omega-n* and *omega-c* were determined by real-time RT-PCR as previously described (Collinge et al. 2008). The positions of *omega-n* and *omega-c* transcript-specific primers are indicated in Fig. 1. Two RNA extracts were made from each line, each consisting of 30 6-day-old adult females. For transcript level analysis, cycle-threshold (Ct) differences between *cycK* and *omega-n* and *cycK* and *omega-c* for each RNA extract were converted to fold differences. Lines and duplicate assays were included at random in real-time thermocycle runs. Between run effects were corrected by adjusting according to the run mean differences from the grand mean. Line differences and associations with *hsr-omega-L/S* variation were assessed using one-way ANOVA, using a weighted linear term to account for differences in sample size among the *hsr-omega-L* frequency categories (SPSS 14.0) for the latter.

Fitness/life-history traits

For measuring development time adults from each line were allowed to lay eggs for 12 h (overnight) on culture media containing three times the standard agar concentration in near darkness. The following day, ten replicate groups of 20 eggs each were picked and transferred to 42-ml culture vials containing fresh media for all lines. Vials were placed at 25°C and checked for emerging adults initially at eight hourly intervals and more frequently during the period of peak emergence. Development time for each line was calculated as

the mean number of hours from egg to adult for all replicate vials.

To assess female fecundity, ten pairs of 3-day-old adults from each line (reared under constant density) were mated in separate vials. Females laid eggs on standard media set onto a small plastic spoon with live yeast that was placed in the empty vial with the flies. Spoons were replaced every 24 h for four consecutive days and frozen upon removal until egg numbers could be counted. Fecundity was taken as the mean number of eggs laid per female over the 4-day period for each line.

For body size estimates, wing centroid sizes were measured. Bottle cultures were kept at constant density for two consecutive generations prior to wing mounting. Approximately ten pairs of wings from 6-day-old male and female adults from each line were removed and mounted onto microscope slides and held in place using double-sided tape and a coverslip. Wing images were captured using a Nikon DS digital camera (Nikon Australia Pty. Ltd.) attached to a Wild M3 dissection microscope (Wild Heerbrugg, Heerbrugg, Switzerland). Digital landmarks were then placed on every wing at eight vein intersections following Hoffmann and Shirriffs (2002) using TpsDig v1.2 (Rohlf 2001). Centroid size was determined using CoordGen6D morphometrics software (IMP) and mean centroid sizes calculated for each sex for each of the 30 lines.

To measure longevity, newly emerged adults reared under constant density were sorted into ten vials, each containing ten males and ten females for each line. Every 2 or 3 days flies were placed onto fresh media and deaths scored until no survivors remained (90 days). We used the survival analysis component of JMP4.0 (SAS Institute, Cary, NC, USA) to test for line differences in survival curves and calculate the mean lifespan for each line.

Results and discussion

In the association study, we used a recently established large out-bred population and derived, without inbreeding, a set of single-pair-mating family lines. The genetic variation of the natural population is therefore spread at random across lines and the effects of inbreeding and artificial linkage associations are minimised (Weeks et al. 2002). Basal rates of protein synthesis showed a 2.1-fold level of variation across lines (Fig. 3) and a marginally non-significant effect of line was evident ($F_{29, 120}=1.53$, $p=0.058$). A significant change in protein synthesis occurred following a mild heat shock ($F_{1,120}=4.77$, $p=0.031$)—basal rates of protein synthesis averaged 208.8×10^3 cpm/extract declining to 189.7×10^3 cpm/extract after the heat shock treatment, consistent

Fig. 3 Basal and heat-shocked rates of protein synthesis ranked in order of increasing basal CPM. Error bars represent +1 standard error. A positive association is suggested between basal (*black*) and heat-shocked (*white*) rates of protein synthesis ($r=0.349$, $p=0.059$)

with the expected cellular heat stress response whereby general protein synthesis is shut down (Lindquist 1980). No interaction between line and heat treatment ($F_{29,120}=0.75$, $p=0.810$) was indicated. This measurement does not separate general protein synthesis shutdown from hsp induction, but given the short time and mild intensity of the heat shock and the well-characterised dynamics of the cellular heat stress response (Yost et al. 1990), we interpret the net decrease to reflect an efficient shutdown of general protein synthesis. The data suggested that basal protein synthesis rates were associated with heat-shocked rates ($r=0.349$, $p=$

0.059) as might be expected if some common factors influenced both basal and heat-shocked rates.

The basal levels of *omega-n* and *omega-c* were determined for all 30 lines (Fig. 4) and a strong line effect was detected for *omega-n* ($F_{29, 57}=4.92$, $p<0.001$), but not for *omega-c* ($F_{29, 57}=0.554$, $p=0.94$). *Omega-n* abundance varied more than fivefold across lines whereas *omega-c* levels varied only twofold between the highest and lowest expressing lines. The mean *omega-n* and *omega-c* transcript levels were not associated ($r=0.025$, $p=0.897$). Since these transcripts share a common transcription start site and

Fig. 4 Levels of the *hsr-omega* transcripts in unstressed flies across 30 lines, with lines ranked in order of increasing *omega-n* abundance. Both measures were internally normalised against levels of *cyclin K*. Error bars represent +1 standard error

common regulatory elements, we might have expected their levels to be related. However, earlier studies have emphasised the independent nature of their expression and turnover under various culture and treatment regimes (Bendena et al. 1989; Hogan et al. 1994; Lakhota and Sharma 1995) so this result is perhaps not so surprising.

Whilst no association was detected between *omega-n* and rates of heat-shocked protein synthesis ($r=0.058$, $p=0.759$), we found high *omega-n* levels to be associated with low rates of basal protein synthesis ($r=-0.454$, $p=0.012$, Fig. 5). Also, an association occurred between *omega-n* level and the level of decrease in protein synthesis after the mild heat shock ($r=-0.48$, $p=0.007$). However, this was accounted for by the association of *omega-n* with basal levels of protein synthesis since the association level was reduced and not significant after we adjusted the protein synthesis decrease to account for regression to the mean of the heat-shocked measures of protein synthesis ($r=-0.26$, $p=0.16$; Kelly and Price 2005). The association was also reduced after partial correlation controlling for basal levels of protein synthesis ($r=-0.26$, $p=0.17$). It remains a possibility that lines with lower *omega-n* levels show a larger decrease in protein synthesis following heat shock but our data only provide weak evidence for this. *Omega-c* transcript levels were not associated with basal protein synthesis rates ($r=0.132$, $p=0.488$), heat-shocked rates ($r=0.216$, $p=0.252$), nor with adjusted decrease in rates following heat shock ($r=0.18$, $p=0.37$).

The negative direction of the *omega-n* association with basal rate of protein synthesis is consistent with the model presented in Fig. 2—high *omega-n* decreasing availability of processed mRNA resulting in reduced protein synthesis. Appealing about this model is that most of the heat shock genes, such as *hsp70*, *hsp68* and the *small hsps*, do not contain introns and their nascent mRNAs may therefore

require less processing, perhaps by-passing the ‘processing blockade’ caused by elevated *omega-n*, and allowing for their uninterrupted translation. Our data support this model and provide insight into a possible role of *hsr-omega* in the control of the cellular heat stress response—thus, the *omega-n* transcript may help control the shut down of general protein synthesis that occurs following heat shock, by applying a ‘brake’ to the processing of the *pre-mRNAs* of non-stress-related genes. This may also facilitate ribosomes being more readily available for hsp synthesis.

As *hsr-omega-L/S* genotype was determined for each of the parental flies that generated each line, we compared the average *hsr-omega* transcript levels among lines grouped into different *omega-L* frequency categories. While *omega-c* levels displayed no pattern with respect to *omega-L/S* genotype (Fig. 6b), *omega-n* levels decreased significantly with increasing parental *omega-L* frequency category ($F_{1, 26}=5.89$, $p=0.022$; Fig. 6a). It is possible that sequence variation in linkage disequilibrium with the 8-bp *omega-L* insert, either elsewhere in the genome, near the *hsr-omega* intron or the insert itself, can affect levels of the transcript. Whatever the basis for this association, the result suggests that expression levels of *omega-n* may be less in cooler regions, since the *omega-L* allele shows a strong positive association with latitude (Anderson et al. 2003)—an idea that needs to be tested. It is also worth noting that the likely major functional component of the *omega-n* transcript, the tandem repeat unit, also decreases in number at cooler latitudes (Collinge et al. 2008). Both of these observations are consistent with the idea that the number of *hsr-omega* repeats per cell are fewer in flies adapted to cool, high-latitude conditions along the Australian coastal transect. At the very least our data provide evidence that natural variation in basal levels of the *omega-n* transcript depends on common allelic variation in the *hsr-omega* gene.

Since protein synthesis is a fundamental cellular process for growth and development, we measured four fitness-related traits on the 30 lines (fecundity, development rate, body size and longevity) and looked for associations. For fecundity, the mean number of eggs laid per female over a 4-day period varied substantially among lines ($F_{28, 1081}=2.51$, $p<0.001$) with an average of 60 eggs laid per female per day. Development time also varied among lines in both males ($F_{29, 1962}=37.99$, $p<0.001$) and females ($F_{29, 2108}=60.53$, $p<0.001$). Wing centroid size as a proxy for body size varied substantially across lines in both males ($F_{28, 253}=6.72$, $p<0.001$) and females ($F_{28, 263}=9.67$, $p<0.001$). For longevity, marked and significant differences occurred in survival curves among lines (Mantel–Cox Log-rank, $\chi^2=1105.22$, $p<0.001$). Consistent with previous observations, a negative relationship was detected between fecundity and female development time ($r=-0.371$, $p=0.022$; Nunney 1996). Other associations that might have been expected

Fig. 5 Basal rates of protein synthesis ($\text{cpm} \times 10^{-3}$ per hour) in ovary tissues associate with *omega-n* transcript levels across lines ($Y=256.0-21.60X$, $r^2=0.206$, $p=0.012$)

Fig. 6 Relationship between *hsr-omega* transcript levels and *hsr-omega-L/S* genotype. Lines with the same parental *hsr-omega-L* allele contribution were averaged for estimates of *omega-n* (a) and *omega-c* (b) levels. Error bars represent +1 standard error. *Omega-n* levels were associated with *hsr-omega-L/S* genotype ($F_{1, 26}=5.89$, $p=0.022$)

among traits, such as a positive association between body size and fecundity (Zwaan et al. 1995) and a negative association between body size and development time (Zwaan et al. 1995; Nunney 1996; Partridge et al. 1999) were in the expected direction but not significant. No significant associations were detected between any of these traits and rates of basal or heat-shocked protein synthesis (Table 1) and no significant association of *omega-n* or *omega-c* levels occurred with any of the fitness/life-history traits. Each of these quantitative life-history traits is complex and influenced by multiple physiological, genetic and environmental factors. However, since ovaries are both the site of egg production and the tissue where protein synthesis was measured, the detection of an association between protein synthesis and fecundity seemed a promising idea. Only if protein synthesis was a major factor for any of these traits would we have expected to find an association since only 30 lines were involved and statistical power was not high (see Rako et al. 2007). Thus, while no associations were detected, our data by no means exclude the possibility that heritable variation in general protein synthesis, or *hsr-omega* genotype for that matter, is a factor influencing any of these traits in nature.

If *hsr-omega* directly influences protein synthesis capability, as our data and the model suggest, it may be a general stress gene and worthy of further investigation. Consistent with this view is mounting evidence that protein synthesis is important for both heat tolerance (Stephanou et al. 1983) and cold tolerance (Burton et al. 1988; Misener et al. 2001) and that variation in *hsr-omega* influences both heat tolerance (McKechnie et al. 1998; Rako et al. 2007) and cold tolerance (Collinge et al. 2008). While there is little doubt about the importance of variation in the hsp, particularly *hsp70*, in influencing thermal tolerance variation (Krebs and Feder 1997; Bettencourt et al. 1999; Sørensen et al. 2003), variation in *hsr-omega* via its effects on protein synthesis and its shutdown may also be an

Table 1 Summary of the associations examined between protein synthesis rates and life-history traits across 30 lines

Protein synthesis measure	Life history trait	<i>r</i>	<i>P</i> -value	Expect
Basal protein synthesis ^a	Development time (female)	0.005	0.489	–
	Development time (male)	0.071	0.354	–
	Longevity	0.095	0.308	–
	Body size (female)	–0.158	0.207	+
	Body size (male)	–0.100	0.303	+
	Fecundity	–0.075	0.347	+
Heat-shocked protein synthesis ^b	Development time (female)	–0.209	0.267	
	Development time (male)	–0.248	0.186	
	Longevity	0.031	0.871	
	Body size (female)	–0.244	0.203	
	Body size (male)	0.020	0.917	
	Fecundity	–0.259	0.167	

r Pearson's correlation coefficients

^a One-tailed tests

^b Two-tailed tests

important determinant of fitness in thermally stressful environments.

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