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SELENOPROTEINS, SELENOPROTEIN mRNA EXPRESSION, FERTILITY, AND MORTALITY*

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Selenocysteine is a rare amino acid in protein that is encoded by UGA with the requirement of a downstream mRNA stem-loop structure, the selenocysteine insertion sequence element. To detect selenoproteins in *Drosophila*, the entire genome was analyzed with a novel program that searches for selenocysteine insertion sequence elements, followed by selenoprotein gene signature analyses. This computational screen and subsequent metabolic labeling with ^{75}Se and characterization of selenoprotein mRNA expression resulted in identification of three selenoproteins: selenophosphate synthetase 2 and novel G-rich and BthD selenoproteins that had no homology to known proteins. To assess a biological role for these proteins, a simple chemically defined medium that supports growth of adult *Drosophila* and requires selenium supplementation for optimal survival was devised. Flies survived on this medium supplemented with 10^{-8} to 10^{-6} M selenium or on the commonly used yeast-based complete medium at about twice the rate as those on a medium without selenium or with $>10^{-6}$ M selenium. This effect correlated with changes in selenoprotein mRNA expression. The number of eggs laid by *Drosophila* was reduced approximately in half in the chemically defined medium compared with the same medium supplemented with selenium. The data provide evidence that dietary selenium deficiency shortens, while supplementation of the diet with selenium normalizes the *Drosophila* life span by a process that may involve the newly identified selenoproteins.

Selenium is recognized as an important dietary micronutrient in mammals. It has anticarcinogenic and antiviral properties, a role in reproductive function, a role in preventing heart disease, and a role in development (reviewed in Ref. 1). This element has been also implicated in enhancing immune function and in slowing the progression of AIDS in human immunodeficiency virus-positive patients (1). At optimal and suboptimal levels in the diet, selenium occurs in animals primarily as selenocysteine (Sec)[1] in Sec-containing proteins. A deficiency in dietary selenium results in decreased levels of selenoproteins, thus compromising biological processes that are maintained by these proteins.

Sec is the 21st naturally occurring amino acid in protein (reviewed in Refs. 2–4). It has its own tRNA, which is first aminoacylated with serine. Serine serves as the backbone for Sec synthesis on its tRNA, and the tRNA is therefore designated tRNA$^{[Ser]Sec}$. Sec also has its own code word, UGA, which serves a dual function of dictating Sec or the cessation of protein synthesis. The feature that distinguishes UGA as a Sec codon is the presence of a stem-loop structure downstream of UGA called a Sec insertion sequence (SECIS) element (2). In eukaryotes, the SECIS element is located in the 3′-untranslated region of selenoprotein mRNAs (2), and a specific factor, the SECIS-binding protein (SBP2), recognizes the SECIS element for insertion of Sec into protein (5). Furthermore, a second factor, the specific Sec elongation protein (EFsec), recognizes Sec-tRNA$^{[Ser]Sec}$, and the resulting complex between EFsec-Sec-tRNA-SBP2 governs the donation of Sec to the nascent selenopeptide in response to UGA (6, 7). In eubacteria, a single factor, SELB, recognizes both the SECIS element, which is located immediately downstream of UGA, and Sec-tRNA$^{[Ser]Sec}$ for Sec insertion (3), while in archaea, the factors required for Sec insertion resemble those found in eukaryotes (8).

Seventeen selenoproteins are currently known in mammals (9). The function of most of these selenoproteins is not known, and thus many of the beneficial effects of selenium remain elusive. However, the number of selenoproteins appears to be fewer in lower eukaryotes, and their identification will help elucidate functions of specific eukaryotic selenoproteins.

Among lower eukaryotes, *Drosophila* emerges as a very useful organism to characterize roles of selenium in biology and medicine. Fruit flies have been used as a model organism for studying a variety of maladies in humans, including cancer (10), neurological disorders (11), and the aging process (12). *Drosophila* has also provided a powerful tool in the investigation of genetics (13) and development (14).

Several studies support the idea that *Drosophila* may also serve as a model system for studying many parameters of selenium metabolism. For example, two groups have detected a gene encoding a homolog of selenophosphate synthetase (15, 16). The gene has a role in imaginal disc morphogenesis, brain

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF396454 (G-rich) and AF396455 (BthD).

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[1] The abbreviations used are: Sec, selenocysteine; SECIS, Sec insertion sequence; SPS2, selenophosphate synthetase; bp, base pair(s); ORF, open reading frame; PCR, polymerase chain reaction; RT, reverse transcription; EST, expressed sequence tag; MSGS, mammalian selenoprotein gene structure.

development, and cell proliferation (16, 17), although studies thus far have failed to detect selenophosphate synthetase activity (15). It is of interest to note that two forms of selenophosphate synthetase, designated SPS1 and SPS2, have been described in mammals (18–20). SPS2 is a selenoprotein (20). Recently, the occurrence of a SPS2 homolog was described in *Drosophila* (21), marking the first identification of a selenoprotein in this organism. Thus, *Drosophila* also has two selenophosphate synthetase forms, a homolog of SPS1 (18–20) and a homolog of SPS2 (21). In addition, Sec tRNA$^{[Ser]Sec}$ has been isolated and sequenced from *Drosophila*, and the tRNA$^{[Ser]Sec}$ population has been analyzed (22).

Computer algorithms were recently developed that are capable of identifying selenoprotein genes by searching for SECIS elements in nucleotide sequence data bases (9, 23). In this study, we employed a more highly sophisticated computer approach to scan the recently sequenced *Drosophila* genome for the presence of selenoprotein genes. Only three genes were found. One of these encoded a homolog of SPS2 (20), which was recently reported by Hirosawa-Takamori *et al.* (21), and two genes encoded novel selenoproteins. To analyze the role of selenium in the expression of these selenoproteins and in fly mortality, a chemically defined diet lacking selenium was devised. The addition of 10^{-8} to 10^{-6} M selenium to the medium resulted in a longer life span than that on the same medium without selenium, indicating a role of dietary selenium in normalizing the life span of *Drosophila*.

EXPERIMENTAL PROCEDURES

Materials—[^{75}Se]Selenite (1000 Ci/mmol) was purchased from the University of Missouri Research Reactor; the RNAeasy purification kit was from Qiagen (Valencia, CA); Monloney murine leukemia virus reverse transcriptase was from Ambion (Austin, TX); RNase-free DNase was from Promega (Madison, WI); *Pwo* DNA polymerase and Complete™ inhibitor mixture were from Roche Molecular Biochemicals; UltraPure low melting point agarose was from Life Technologies, Inc.; and Instant Drosophila Medium (127.95 g/liter corn meal, 22.38 g/liter brewers yeast, 73.89 g/liter glucose, 36.88 g/liter sucrose, 182.69 g/liter soybean fiber, and 1.21 g/liter *p*-hydroxybenzoic acid methyl ester), Grace's insect medium (G-8142), sodium selenite, and *p*-hydroxybenzoic acid methyl ester were from Sigma. The Canton S. strain of *Drosophila* was obtained from Dr. Florence Davidson and used throughout this study. Total poly(A) RNA from adult, larvae, and embryo *Drosophila* (Canton S. strain) was purchased from CLONTECH.

Diets—Chemically defined medium contained Grace's insect medium (15 ml/vial) and *p*-hydroxybenzoic acid methyl ester (100 μl/ml of a 200 mg/ml solution). Low melting point agarose (0.45%) was added as the support matrix. Either no selenium or selenium at a final concentration of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M was also included. Newborn flies (70–75 flies/vial, and 8–10 vials for every experimental condition) were collected from complete medium (instant *Drosophila* medium) and placed on the chemically defined medium with or without selenium supplementation or on complete medium. Flies were changed to fresh chemically defined medium every 3 days.

tRNA Analysis—Total tRNA was isolated from adult *Drosophila*, 0.005 A_{260} units were loaded into each lane of a 15% TBE-urea polyacrylamide gel, the tRNA was electroblotted from developed gels onto nylon membranes, and filters were hybridized with an 188-bp *Dra*III–*Nde*I fragment encoding the *Drosophila* tRNA$^{[Ser]Sec}$ gene (22). Filters were stripped and reprobed with *Drosophila* serine tRNA^{Ser} probe (5′-CGCAGCCGGTAGGATTCGAAC-3′) (22). Amounts of probe attached to filters in response to tRNA were determined using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Genomic Search—The entire *Drosophila melanogaster* genomic sequence determined by Celera (24) was analyzed for the presence of SECIS elements using an advanced version of SECISearch (9).[2] Candidate sequences that resembled eukaryotic SECIS elements (*i.e.* satisfied primary sequence and secondary structure consensus) were subjected to the free energy criterion, which allowed filtering predicted unstable structures. The sequence, structure, and energy parameters of the program that were used in the searches allowed identification of ~90% (53 out of 59) selenoproteins from our collection of known eukaryotic selenoproteins and SECIS elements. This collection of natural SECIS elements from nine eukaryotes (human, mouse, rat, bovine, zebrafish, macaque, Ovis, *Caenorhabditis elegans*, and *Dictyostelium*) was used to tune the SECISearch program. The annotated version of the *Drosophila melanogaster* genome was then used to determine the location of each candidate SECIS element. The sequences that fell to the 5′-untranslated region, introns, or coding regions of previously predicted proteins on either DNA strand were discarded. Finally, ORFs closest to the predicted candidate SECIS elements were manually analyzed with MSGS criteria (9). The principal component of these criteria was BLAST-based searches for homologs in *Drosophila* or other eukaryotes that contained Cys codons (TGC or TGT) in place of Sec-encoding TGA codons in *Drosophila* selenoproteins. Two possibilities were considered for each candidate sequence. 1) If the ORF was terminated with TGA, we tested whether this codon could encode a Sec residue. Such analysis resulted in identification of a 12-kDa selenoprotein (G-rich), in which Sec was the C-terminal penultimate residue. 2) All candidate ORFs were also analyzed for the possible extension of their N-terminal sequences that may include in-frame TGA codons. These analyses identified SPS2 and a 28-kDa selenoprotein (BthD).

mRNA Expression and Northern Hybridization—Total RNA was extracted from adult flies, purified with an RNAeasy kit, and treated with RNase-free DNase, and the reverse transcription (RT) reaction was carried out with Moloney murine leukemia virus reverse transcriptase and an oligo(dT)$_{18}$ primer. A portion of the RT product (typically 1 μl) was used for PCR amplification with *Pwo* polymerase using specific primers for each gene. Forward primers contained an *Eco*RI digestion site, and reverse primers contained a *Bam*HI site for cloning PCR products in pUC19. Primers for each cDNA were: SPS2, forward (5′-CGGAATTCCGTAGAAATTCGCGCACAATG-3′) and reverse (5′-CGGGATCCTCGTTAATCATCAAAATTATC-3′); 28 kDa, forward (5′-CGGAATTCCGATTCTGATTACTTTAGGATCATGC-3′) and reverse (5′-CGGGATCCCGATATATTTTGTAATAACAATGCTC-3′); 12 kDa, forward (5′-CGGAATTCCGCCTGAGGATAGCGTGGCGCCATGG-3′) and reverse (5′-CGGGATCCCGGAAGCAGCGACCCAGGAATCAGTG-3′); SPS1, forward (5′-CGGAATTCGCAAACCTATAAAGCAAGATG-3′) and reverse (5′-CGGGATCCCTGAATGTAGCAGCCGTTACG-3′); and SBP2, forward (5′-CGGAATTCCACTAAGAATCCACAATAAATG-3′) and reverse (5′-CGGGATCCTTACTCACTTTCGCCGGGGC-3′).

Quantification of mRNA expression was accomplished by RT-PCR using a competitive strategy. The exogenous standard (competitor) was constructed by creating a small deletion in the cloned cDNA. Cloned SPS2 cDNA was digested with *Eco*RV and *Bsg*I and religated in the same vector, BthD cDNA was digested with *Bst*XI and *Bst*EII, G-rich cDNA was digested with *Eco*RV and *Xho*I, SPS1 cDNA was digested with *Nco*I, and SBP2 cDNA was digested with *Eco*RV and *Bsg*I. All cloned cDNAs were religated in the same vector. Using 1 μl of the RT product as template, different amounts of competitors were added to the PCR mix to calculate the relative amount of each gene after amplification.

PCR products were separated in agarose gels, stained with ethidium bromide, and scanned using a Molecular Imager FX System (Bio-Rad). Signals were quantified by volumetric integration of raw data using the Quantity One Software following the instructions of the manufacturer. The amount of each PCR product was also compared with the amount of RT-amplified cDNA for the constitutive gene *rp49* (forward primer, 5′-ATGACCATCCGCCCAGCATAC-3′; reverse primer, 5′-CTTGGCGCGCTCGACAATCTC-3′) in the same sample under the same experimental conditions.

Total poly(A) RNA from adult *Drosophila* was electrophoresed on formaldehyde-agarose gels, and the RNA was transferred onto nylon membranes and hybridized with a 550-bp *Eco*RI-*Bam*HI fragment encoding a portion of the G-rich gene, a 900-bp *Eco*RI-*Xho*I fragment encoding a portion of the BthD gene, or a 450-bp *Bam*HI-*Eco*RV fragment encoding a portion of the SPS2 gene. Filters were stripped and reprobed with a 550-bp *Eco*RI-*Hin*dIII fragment encoding a portion of the ribosomal protein gene (*rp49*) gene as a control, since RP49 is constitutively expressed at all stages of development.

^{75}Se Labeling—Flies maintained on complete and chemically defined medium with and without selenium supplementation were transferred to defined medium without selenium and labeled with 100 μCi of ^{75}Se for 24 h. Protein extracts were prepared from 30–35 flies using radioimmune precipitation buffer (0.3 M NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 0.1 M Tris-HCl, pH 7.4); 40 μg of total protein from each sample were loaded onto a 10% polyacrylamide gel; and the gels were run, dried, and exposed to PhosphorImager screens for 48 h.

[2] G. V. Kryukov, A. V. Lobanov, and V. N. Gladyshev, unpublished data.

TABLE I
In silico analysis of the Drosophila genome for the presence of selenoprotein genes

Pattern criteria applied[a]	Energy criteria applied[b]	Location criteria applied[c]	MSGS criteria applied[d]
4908	179	14	3

[a] Number of candidate sequences after the initial analysis of the entire *Drosophila* genome for primary sequences and secondary structures.
[b] Number of candidate sequences after application of the free energy criterion.
[c] Number of candidate SECIS elements that satisfied location criteria (not present in 5′-untranslated region, introns, or coding regions of known *Drosophila* proteins).
[d] Number of selenoproteins that satisfied MSGS criteria (9).

RESULTS

Analysis of the Drosophila Genome—The entire *Drosophila* genome was analyzed with an advanced version of SECISearch, a computer program that identifies SECIS elements in large nucleotide sequence data bases, including completely sequenced eukaryotic genomes (9). This program entails searching genomic DNA for the primary SECIS element consensus sequence, followed by analyses of their secondary structure and then an evaluation of the free energy of identified candidate SECIS structures (9). Parameters of the program were set such that the absolute majority of known eukaryotic selenoprotein genes could be recognized. These automatic analyses of the *Drosophila* genome were followed by manual analyses of selected sequences using MSGS criteria (9)[3] and BLAST searches to identify possible EST or non-redundant sequences as well as ORFs within these sequences. In particular, we searched for homologs that contained Cys residues in place of putative Sec residues in *Drosophila* ORFs. Three ORFs encoding proteins of 12, 28, and 43 kDa were detected that contained in frame TGA codons predicted to encode Sec (Table I). Genes for these proteins (Fig. 1*A*) have three or four exons separated by small introns with each exon containing a coding sequence. SECIS elements in the three selenoprotein genes (Fig. 1*B*) contained all features characteristic of eukaryotic SECIS elements, including the quartet of non-Watson-Crick-interacting nucleotides UGAN . . . NGAN preceded by an unpaired A as well as an unpaired AA motif in a bulge separated from the Quartet by an 11–12 nucleotide stem (Fig. 1*B*).

The 43-kDa selenoprotein was homologous to SPS2 (see also Ref. 21), a protein that was previously discovered in mammals (20). Like mammalian SPS2, this *Drosophila* protein has a conserved Sec residue in the N-terminal portion of the protein. Two other proteins have no homology to known proteins. The 28-kDa protein, designated BthD, has a C*XX*U motif (*i.e.* Cys and Sec are separated by two residues) in the N-terminal portion, similar to that found in the mammalian selenoproteins SelT and SelW. Interestingly, the 12-kDa protein, designated G-rich, contained a C-terminal penultimate Sec residue. This was similar to mammalian thioredoxin reductases, which also contain the C-terminal penultimate Sec.

Homology searches revealed additional Cys-containing *Drosophila* homologs of 12- and 28-kDa selenoproteins (Fig. 2), consistent with MSGS criteria (9) for selenoprotein genes. Furthermore, Cys-containing and Sec-containing homologs of 12- and 28-kDa selenoproteins were found in other eukaryotes including higher plants (Cys) and vertebrates (Sec) (data not shown). Expression of the three selenoprotein mRNAs was initially assessed by the presence of multiple *Drosophila* ESTs and subsequently by Northern blot and RT-PCR analyses (see below).

[3] G. V. Kryukov and V. N. Gladyshev, unpublished data.

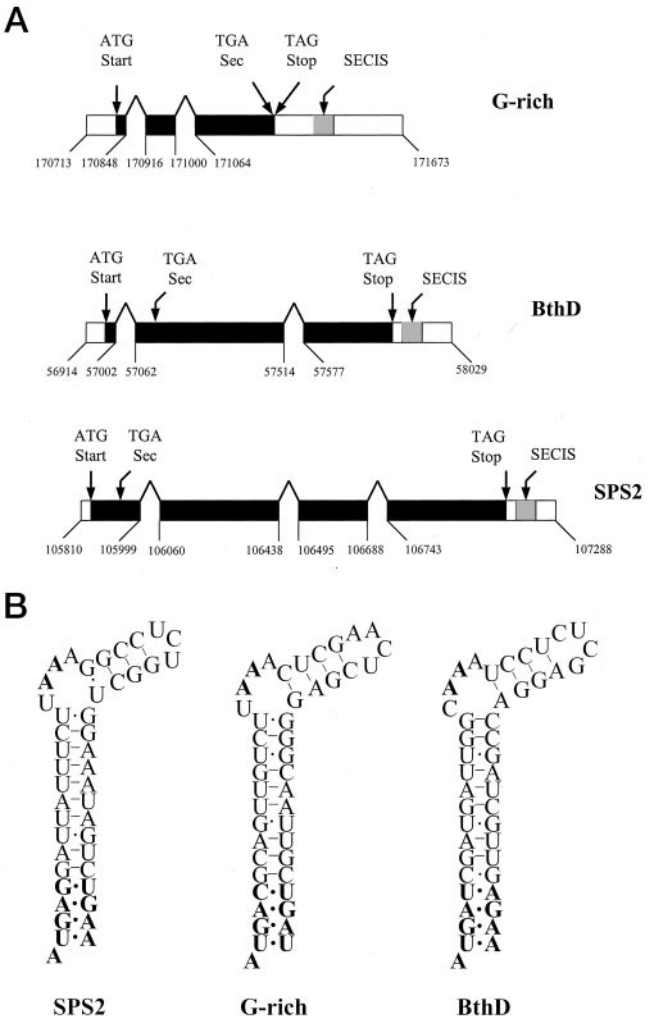

FIG. 1. **Organization of the *Drosophila* selenoprotein genes and their SECIS element structures.** *A*, selenoprotein genes are depicted as *boxes*, which represent exons, and *lines* connecting *boxes*, which represent introns. *Black boxes* represent coding regions, and *open boxes* represent untranslated regions within exons. The locations of initiator ATG codons, Sec-encoding TGA codons, and terminator signals in the three selenoprotein genes are indicated. *Gray boxes* indicate the locations of SECIS elements (also indicated by *arrows*) within the 3′-untranslated region. *Numbers* correspond to nucleotides within *Drosophila* genomic scaffolds containing selenoprotein genes (G-rich, accession number AE003487; BthD, accession number AE003493; and SPS2, accession number AE003628). *B*, SECIS elements in the *Drosophila* selenoprotein genes are shown. The quartet of non-Watson-Crick interacting nucleotides, an unpaired A preceding the quartet, and an unpaired AA motif in the bulge are shown in *boldface type*. These SECIS elements are further discussed under "Results."

The *Drosophila* genome was also screened by homology search analyses for the presence of fruit fly selenoproteins homologous to known eukaryotic selenoproteins. However, no additional selenoproteins were detected by this approach.

Chemically Defined Medium—Dietary selenium is known to regulate expression levels of selenium-containing proteins. To assess the possible significance of the newly discovered selenoproteins and, in particular, their role in the life span of *Drosophila*, we devised a chemically defined diet that supported the growth of adult flies. In developing the diet, the following considerations were taken into account. 1) We reasoned that the yeast-based medium may not be sufficient to obtain low selenium levels in the diet, especially if the requirement for selenium is low in *Drosophila*. This is because selenium can enter sulfur pathways and is present as a contaminant in a number of compounds used in the medium. It should be noted

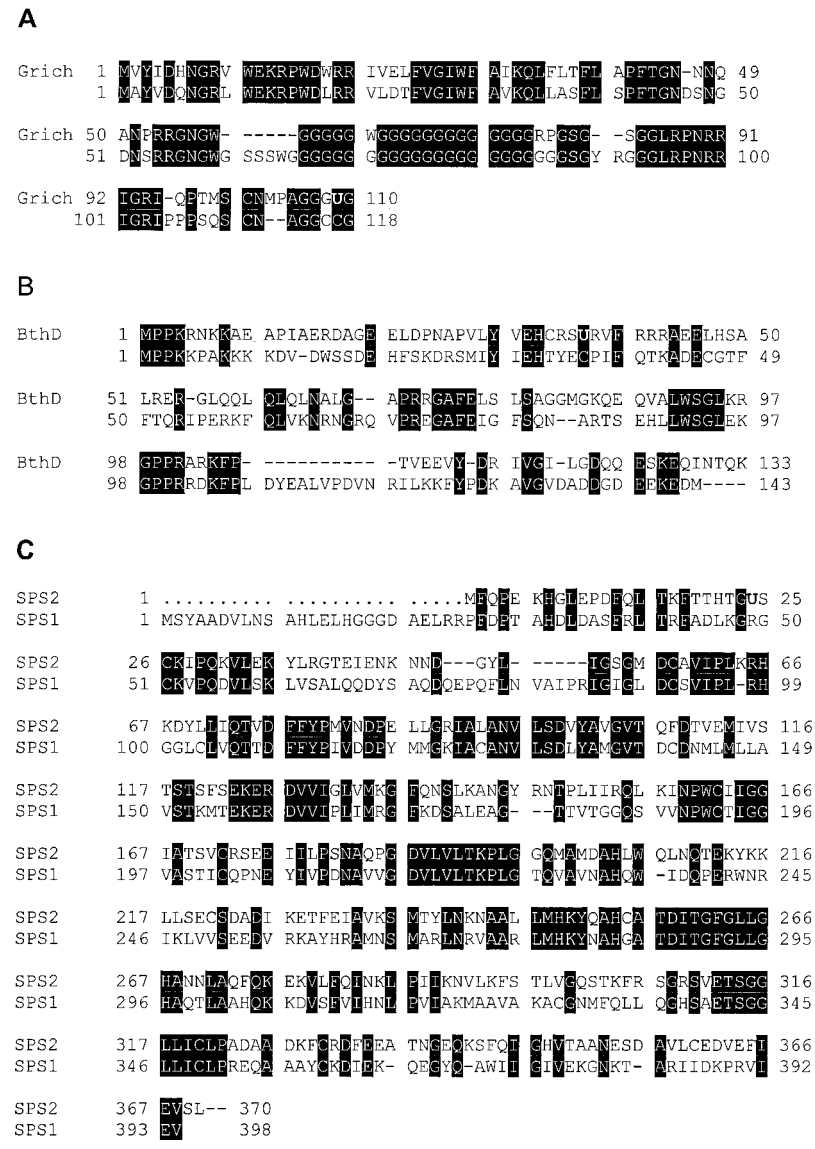

FIG. 2. **Alignments between *Drosophila* selenoproteins and their *Drosophila* homologs.** *A*, alignment between G-rich and its Cys homolog (accession number AAF48112). *B*, alignment between BthD and its Cys homolog (accession number AAF58585). *C*, alignment between SPS2 and *Drosophila* SPS1 (accession number AAF58266). Identical residues in each alignment are *highlighted*. *U*, which has been used previously to designate Sec (1–9), indicates Sec. *Numbers* correspond to amino acids in proteins. Only the N-terminal Sec-containing domain of BthD is shown in the alignment shown in *B*.

that even when selenium-deficient Torula yeast was used in studies on rats and mice, many selenoproteins, especially those expressed in the endocrine organs, were apparently expressed in sufficient levels to maintain essential processes. Thus, a simple diet for *Drosophila* was required that would eliminate as many selenium-containing, contaminant compounds as possible. 2) In addition to designing a medium that is chemically defined (to be able to regulate the amount of selenium intake by *Drosophila*), it was important to obtain a different support matrix for the medium, since the agar routinely used for this purpose contained selenium. We therefore used a highly purified agarose as matrix (see "Experimental Procedures"). 3) We considered and/or tested a number of supplement compounds and growth conditions that could be sufficient to maintain the flies for a period long enough to assess their life span characteristics. For example, we tested several concentrations of a regular agarose (0.4–2%) as a matrix for the media. However, the only kind of agarose that could be used without affecting normal life in flies was low melting point agarose. We also tested the survival of flies using a selenium-deficient Torula yeast extract. This extract failed to support life of adult flies even after supplementation with Grace's vitamin solution and a lipid mixture. As a result of numerous attempts to find a diet that was dependent on selenium, we found that Grace's medium supplemented with *p*-hydroxybenzoic acid methyl ester provided a simple, chemically defined medium that supported growth of adult flies and manifested a growth response to selenium. Flies could be maintained on this medium without any other supplementation for more than 5 weeks.

Drosophila Mortality—The life span of flies maintained on the chemically defined medium with varying levels of selenium was compared with that of flies maintained on the yeast-based complete medium routinely used for growing *Drosophila* (Fig. 3). Selenium levels varied from none to 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M. Flies maintained on defined medium without selenium or with 10^{-5} M selenium died at more than twice the rate of flies maintained on complete medium or on defined medium with 10^{-8}, 10^{-7}, or 10^{-6} M selenium. The rate of survival in the medium with the latter levels of selenium was similar to those in the complete medium. More than 90% of the flies maintained on chemically defined medium in the presence of 10^{-4} M selenium died at the end of the 35-day period (data not shown). These data suggested that selenium in the 10^{-8} to 10^{-6} M range was beneficial to the life span of flies under these growth conditions, whereas higher levels of selenium (10^{-5} and 10^{-4} M) were toxic. It also appears that the requirement for selenium in *Drosophila* was less than 10 nM.

Drosophila Fertility—To assess the relationship between selenium and fertility in *Drosophila*, we determined the number and the hatching of laid eggs in flies maintained on defined and

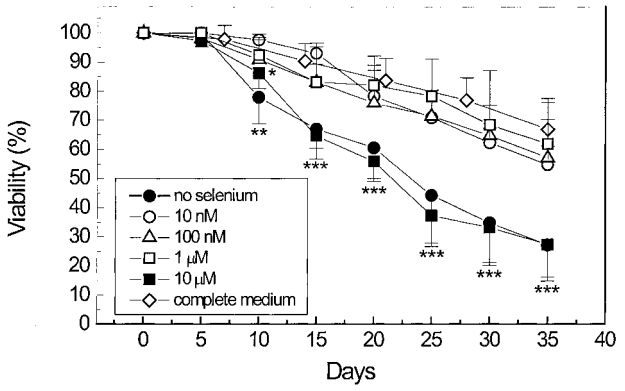

FIG. 3. **Survival rates of adult *Drosophila* in response to selenium.** Newborn flies were transferred from complete medium to defined medium supplemented with or without selenium or to complete medium. A total of 600–700 flies (70–75 flies/vial and a total of 8–10 vials containing each medium condition) were used to assess survival of flies in each medium, and the number of surviving flies was counted at the time intervals shown. *Error bars* correspond to S.D. for the 8–10 vials in every experimental condition. Statistical analyses were done using an analysis of variance test. p values of significance were represented as follows: *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

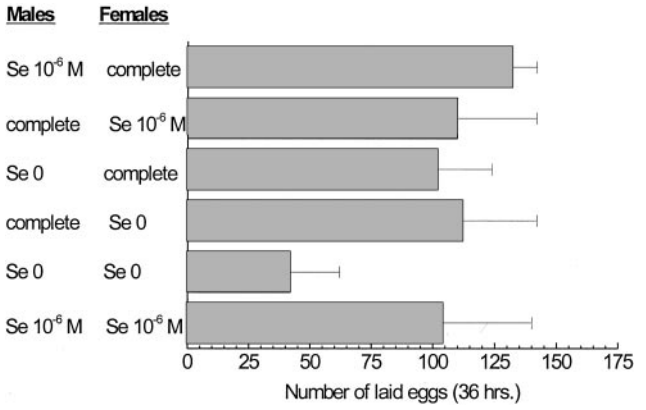

FIG. 4. **Effect of selenium on fertility.** Fresh newborn flies were collected, and males and females were maintained separately on chemically defined media with 10^{-6} M selenium or without selenium during the first postnatal 14 days. Subsequently, 40 males and 40 females from each group were combined in different combinations and kept on selenium-free medium for 36 h. The total number of eggs laid in each combination was counted. Analysis of variance was performed in order to describe significant differences between groups. (Se0/Se0, $p < 0.05$, significant difference between chemically defined medium without selenium for both males and females and any combination).

complete media. Both sexes were kept separately for 14 days on either complete or chemically defined medium with 10^{-6} M or without selenium. Subsequently, populations of flies were mixed in different combinations, and the number of laid eggs was counted in each case (Fig. 4). The number of laid eggs was similar if either males or females were maintained on complete diet or diet supplemented with selenium, but this number dropped to less than 50% when both sexes were maintained on chemically defined medium without selenium. The viability of eggs (hatching) was higher than 90% under all conditions examined. The data suggest a role of dietary selenium in egg production and/or fertilization.

Sec tRNA$^{[Ser]Sec}$ Levels—The Sec tRNA$^{[Ser]Sec}$ population in *Drosophila* migrates as two bands on gel electrophoresis (18). The primary sequences of both bands are identical, suggesting that *Drosophila* tRNA$^{[Ser]Sec}$ exists in two stable conformations. Furthermore, the levels of these two isoforms did not change throughout development (22). We investigated whether the amounts of the two tRNA$^{[Ser]Sec}$ isoforms from flies maintained on complete medium or on defined medium with and without different levels of selenium varied. As an internal control, we used serine tRNA^{Ser}. The total amount of tRNA$^{[Ser]Sec}$ for each sample relative to the amount of tRNA^{Ser} was determined. The amounts of two tRNA$^{[Ser]Sec}$ isoforms did not change in response to selenium status after 7, 21, or 35 days of growth on the various media (data not shown). It should also be noted that a sufficient number of flies that were grown on defined media containing 10^{-5} or 10^{-4} M selenium did not survive for 35 days to perform tRNA analysis.

mRNA Expression—Total RNA was isolated from flies maintained on the various diets for 21 days. Expression levels of the three selenoprotein mRNAs (SPS2, G-rich, and BthD) as well as mRNAs for proteins involved in selenocysteine biosynthesis (SPS1 and SBP2) were examined by competitive RT-PCR in flies grown on media supplemented with 0–10^{-4} M selenium (Fig. 5). The amounts of each mRNA expressed in complete medium relative to SPS1, which was assigned a value of 1.0, were as follows: SPS2, 3.0; SBP2, 2.0; G-rich, 14.0; and BthD, 6.0. Thus, the selenoprotein mRNAs were expressed in higher levels than mRNAs of the two nonselenium containing proteins with G-rich and BthD mRNAs being expressed at highest levels. The levels of SPS1 mRNA manifested only a slight variation in relation to selenium status. SBP2 showed slightly

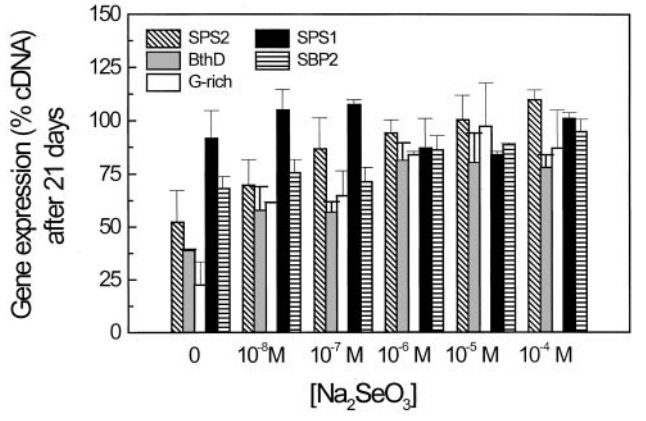

FIG. 5. **Expression of SPS2, the 12- and 28-kDa selenoprotein genes, and SPS1 and SBP2 in response to selenium.** Total RNA was isolated from flies maintained on complete and chemically defined medium at the selenium concentrations shown for 21 days, and the amounts of each cDNA were determined by RT-PCR as given under "Experimental Procedures." Values obtained for each cDNA on complete medium were assumed to represent optimal or 100% expression, and the amounts of each cDNA relative to those found in complete medium are shown.

greater changes but not nearly as much as those found with the three selenoprotein mRNAs. The amounts of SPS2 and BthD mRNAs increased 2-fold in the presence of selenium, and that of G-rich mRNA increased 4-fold. Comparison of mRNA levels in the chemically defined medium with those in the complete medium revealed that selenium deficiency resulted in the decrease in selenoprotein mRNA levels, whereas supplementation of the diet with selenium normalized selenoprotein mRNA expression.

Selenoprotein mRNAs were analyzed by Northern blotting from adults, larva, and embryos (Fig. 6) to assess the expression of these mRNAs during development and to better assess their relative sizes. The constitutively expressed *rp49* gene was used as a control. G-rich protein and SPS2 mRNAs were expressed throughout development, although SPS2 appeared to be more weakly expressed at the embryonic and larval stages. BthD was expressed in embryos and adults, but very weakly in larvae. The relative sizes of each mRNA are given in the legend of Fig. 6, and they agree with the predicted sizes of each mRNA

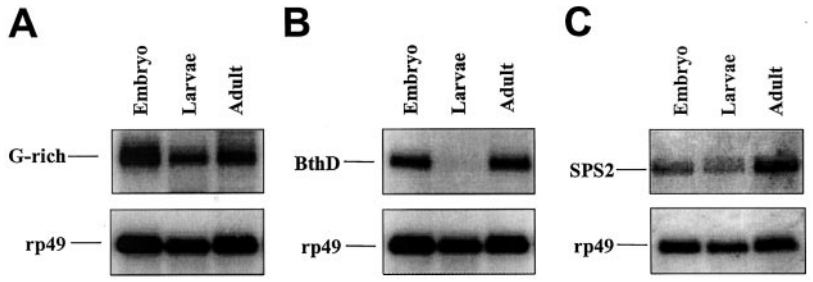

FIG. 6. **Northern blot analysis of selenoprotein mRNAs during development.** 1.0 μg of total poly(A) RNA from embryos, larva, and adults was electrophoresed on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized with the probe (see "Experimental Procedures") specific for G-rich mRNA (A), BthD mRNA (B), and SPS2 mRNA (C), and the amount of probe attached to the corresponding mRNA was determined using a PhosphorImager. RNA markers were also run on gels, and mRNA sizes were estimated to be 0.8 kilobases for G-rich mRNA, 1.1 kilobases for BthD mRNA, and 1.3 kilobases for SPS2 mRNA. These sizes are in agreement with those estimated from sequencing DNA clones, aligning EST sequences found in the *Drosophila* data base, and analyzing selenoprotein gene structures, which were 876 bases for G-rich, 994 bases for BthD, and 1309 bases for SPS2 excluding poly(A) tails.

determined by sequencing cDNA clones, aligning all EST sequences found in the *Drosophila* EST data base and analyzing selenoprotein gene structures (see legend to Fig. 6).

Metabolic Labeling of Flies with ^{75}Se—Flies were maintained on the chemically defined medium with and without selenium or on complete medium for 7 days and were then labeled with ^{75}Se for 24 h. The resulting protein extracts were analyzed by SDS-polyacrylamide gel electrophoresis and PhosphorImager analysis (Fig. 7). In the absence of added selenium (Fig. 7*B*, *lane 1*), three bands were clearly discernible. The strongest labeled band corresponded in size to the selenoprotein that we identified in this study as the 12-kDa (G-rich) protein. It was present as a double band, as is clearly shown in *lane 5*, that contained labeled extract from flies maintained on complete medium. The reason why the 12-kDa selenoprotein migrated as a double band is not clear. The slower migrating portion of this band is far more enriched in flies maintained on complete medium than on defined medium either with or without supplemented selenium. In addition, a 43-kDa band and an ~80-kDa band were visible in the samples. The 43-kDa band corresponds to SPS2, one of the three selenoproteins we identified in this study. The 80-kDa protein was not detected in the *Drosophila* genome by SECISearch, and the possibility exists that it might be of bacterial origin (see "Discussion").

When selenium was added to the medium, an additional band appeared that migrated just above SPS2 (Fig. 7, *lanes 2* and *3*). This band and the one that runs as the higher of the two 12-kDa bands were more enriched in labeled extracts from flies maintained on diets containing 10^{-4} M selenium, which is a toxic level. The 28-kDa (BthD) selenoprotein that we described in this study was not detected in adult flies. It is not clear why higher levels of BthD mRNA were expressed in response to selenium in adults (see above), while expression of the corresponding selenoprotein was not detected.

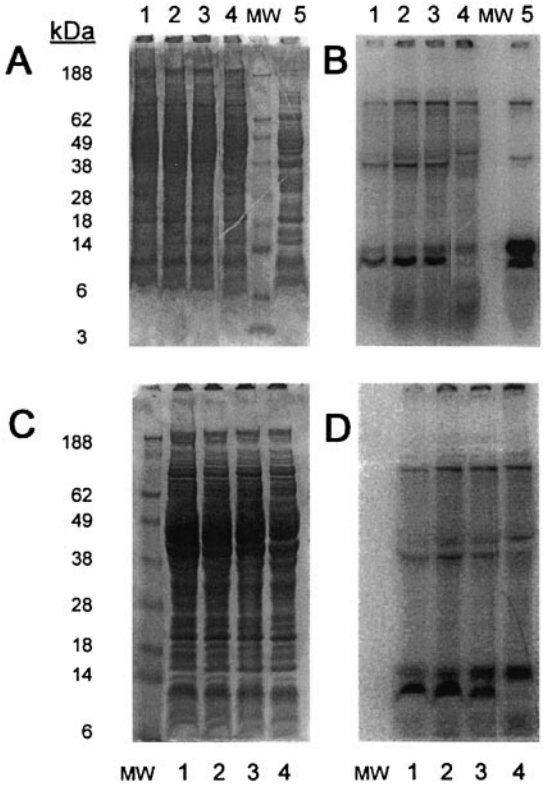

FIG. 7. **Detection of selenoproteins in adult *Drosophila*.** Flies that had been maintained on complete and chemically defined medium at the selenium concentrations shown for 21 days were transferred to medium without selenium supplementation, labeled with ^{75}Se for 24 h, protein-extracted, and electrophoresed. The gels were stained with Coomassie Blue and dried, and the amount of labeling was determined in a PhosphorImager. *A* and *C*, the Coomassie Blue-stained gels are shown from two separate labelings with ^{75}Se, and the lanes contained the following. *A*, *lane 1*, chemically defined medium without selenium supplementation; *lane 2*, chemically defined medium plus 10^{-8} M sodium selenite; *lane 3*, chemically defined medium plus 10^{-7} M sodium selenite; *lane 4*, chemically defined medium plus 10^{-4} M sodium selenite; *lane 5*, complete medium. *C*, *lane 1*, chemically defined medium without selenium supplementation; *lane 2*, chemically defined medium plus 10^{-8} M sodium selenite; *lane 3*, chemically defined medium plus 10^{-7} M sodium selenite; *lane 4*, chemically defined medium plus 10^{-4} M sodium selenite. *B* and *D*, the labeled bands are shown from two separate labelings with ^{75}Se, and the lanes correspond to those shown in *A* and *C*, respectively. *MW*, molecular mass.

DISCUSSION

This report describes the identification of selenoprotein genes in *Drosophila* and provides evidence for the role of dietary selenium in the life span of the fruit fly. It should be noted that the presence of *Drosophila* components of the Sec insertion machinery, including Sec tRNA$^{[Ser]Sec}$ (22) and SPS1 (15–17), was previously reported. In addition, the selenoprotein, SPS2, was identified recently in *Drosophila* (21). Although the completely sequenced genome of *Drosophila* is available, it does not provide correct annotation to selenoproteins. This is because selenoprotein genes contain in-frame TGA code words encoding Sec residues that are recognized as termination codons by currently available genome analysis programs. Such programs are therefore prone to incorrect annotation of sequences containing in-frame TGA codons.

To identify selenoproteins in *Drosophila*, we applied a computer program that identifies selenoproteins through recognition of SECIS elements. The initial version of this program was previously used to discover new selenoproteins in the human dbEST (9). However, analyses of completely sequenced ge-

nomes are more challenging and have not been reported. The present study constitutes the first analysis of a completely sequenced eukaryotic genome, in which new selenoprotein genes were identified by searching for mRNA structures.

Our search of the *Drosophila* genome for selenoproteins identified three such genes. Two of the selenoprotein genes that we identified, G-rich and BthD, do not have sequence homology to known proteins, and their functions are currently not known. The third protein, SPS2, was a previously identified mammalian (20) and *Drosophila* (21) selenoprotein that is involved in selenocysteine biosynthesis by activating intracellular selenium to form a selenium donor compound, selenophosphate.

Labeling of flies with ^{75}Se resulted in more bands on SDS gels than expected from the computational analysis of the genome for selenoproteins. It is possible that our computational analysis may not have identified all selenoprotein genes in *Drosophila*. However, since the program was sufficiently specific to identify the majority of selenoprotein genes from various eukaryotes, it is likely that we have identified most, if not all, of such genes in the *Drosophila* genome. This raises a question of the origin of the 80-kDa band and the occurrence of two bands in the 12- and 43-kDa ranges observed on SDS gels (see Fig. 7*B*). One possibility is that since selenium can enter sulfur pathways, then these additional bands may be proteins that are particularly rich in cysteine and/or methionine and arise by nonspecific labeling with selenium. Another possibility is that the two bands in the 12- and 43-kDa ranges may be the result of processing of these proteins. Interestingly, an 80-kDa selenoprotein has been observed in *Drosophila* extracts that was identified as bacterial formate dehydrogenase.[4] Thus, it is possible that the labeled 80-kDa band we observed may be of bacterial origin.

To elucidate the possible role of these identified selenoproteins, we developed a chemically defined medium. When this medium was supplemented with 10^{-6} to 10^{-8} M selenium, flies had the same life span as flies on a complete yeast-based medium over a 35-day period, indicating the requirement of this element for supporting the normal life in adult flies. When selenium was not included in the medium, the survival rate of flies was about one-half that of flies supplemented with optimal levels of selenium. We also found that a concentration of 10 nM selenium was sufficient to support a normal life span for the 35-day period. Concentrations over 10 μM were highly toxic.

Dietary selenium also had a dramatic effect on *Drosophila* fertility. If both male and female flies were maintained on a selenium deficiency diet, the number of laid eggs decreased more than twice compared with conditions in which either males or females were placed on selenium-supplemented or complete diets.

The data clearly indicate that dietary selenium has a role in normalizing the life span and fertility of *Drosophila*. Selenium was previously implicated in delaying the aging process in mammals through changes in antioxidant selenoproteins and oxidative modifications in proteins in aging cells (4). In humans, selenium deficiency was associated with early aging (25, 26). Mice and rats maintained on selenium-deficient yeast-based diets do not show any effect of selenium on aging. In these dietary studies in mammals, the diets contained trace amounts of selenium that were sufficient to maintain expression of some selenoproteins at the expense of others. The relative significance of these selenoproteins in the life span of these organisms is not known, and such studies do not seem at present to provide a means of assessing the role of selenoproteins in mortality.

Since only three selenoproteins appear to be encoded in the *Drosophila* genome, it seems likely that fruit flies will provide a simple model system to examine the role of selenoproteins in the life span of this organism. Two of the newly identified selenoproteins, G-rich and BthD, are possible candidates. The third selenoprotein, SPS2, appears to be involved in Sec biosynthesis and may provide an autoregulatory mechanism for selenoprotein expression (20). An additional mechanism, which we found to regulate selenoprotein expression, is selenium-dependent changes in the abundances of selenoprotein mRNAs. Taken together, these processes should provide dramatic responses in selenoprotein levels in response to selenium supplementation and should provide a model system for exploring the role of selenoproteins in the life span of the fruit fly.

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[4] R. A. Sunde, personal communication.

REFERENCES

1. Gladyshev, V. N., Martin-Romero, F. J., Xu, X.-M., Kumaraswamy, E., Carlson, B. A., Hatfield, D. L., and Lee, B. J. (1999) *Recent Res. Dev. Biochem.* **1,** 145–167
2. Low, S. C., and Berry, M. J. (1996) *Trends Biochem. Sci.* **21,** 203–208
3. Bock, A. (2000) *Biofactors* **11,** 77–78
4. Hatfield, D. L., Gladyshev, V. N., Park, J. M., Park, S. I., Chittum, H. S., Huh, J. R., Carlson, B. A., Kim, M., Moustafa, M. E., and Lee, B. J. (1999) *Comprehensive Natural Products Chemistry* (Kelly, J. W., ed) Vol. 4, pp. 353–380, Elsevier Science Ltd., Oxford
5. Copeland, P. R., Fletcher, J. E., Carlson, B. A., Hatfield, D. L., and Driscoll, D. M. (2000) *EMBO J.* **19,** 306–314
6. Tujebajeva, R. M., Copeland, P. R., Xu, X.-M., Carlson, B. A., Harney, J. W., Driscoll, D. M., Hatfield, D. L., and Berry, M. J. (2000) *EMBO Rep.* **1,** 158–163
7. Fagegaltier, D., Hubert, N., Yamada, K., Mizutani, T., Carbon, P., and Krol, A. (2000) *EMBO J.* **19,** 4796–4805
8. Rother, M., Wilting, R., and Bock, A. (2000) *J. Mol. Biol.* **299,** 351–358
9. Kryukov, G. V., Kryukov, V. M., and Gladyshev, V. N. (1999) *J. Biol. Chem.* **274,** 33888–33897
10. Bilder, D., Li, M., and Perrimon, N. (2000) *Science* **289,** 113–116
11. Phillips, J. P., Tainer, J. A., Getzoff, E. D., Boulianne, G. L., Kirby, K., and Hilliker, A. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8574–8578
12. Tower, J. (2000) *Mech. Ageing Dev.* **118,** 1–14
13. Vernooy, S. Y., Copeland, J., Ghaboosi, N., Griffin, E. E., Yoo, S. J., and Hay, B. A. (2000) *J. Cell Biol.* **150,** F69–F76
14. Wieschaus E., Nusslein-Volhard, C., and Kluding, H. (1984) *Dev. Biol.* **104,** 172–186
15. Persson, B. C., Bock, A., Jackle, H., and Vorbruggen, G. (1997) *J. Mol. Biol.* **274,** 174–180
16. Alsina, B., Serras, F., Baguña, J., and Corominas, M. (1998) *Mol. Gen. Genet.* **257,** 113–123
17. Alsina, B., Corominas, M., Berry, M. J., Baguña, J., and Serras, F. (1999) *J. Cell Sci.* **112,** 2875–2884
18. Low, S. C., Harney, J. W., and Berry, M. J. (1995) *J. Biol. Chem.* **270,** 21659–21664
19. Kim, I. Y., and Stadtman, T. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7710–7713
20. Guimaraes, M. J., Peterson, D., Vicari, A., Cocks, B. G., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Ferrick, D. A., Kastelein, R. A., Bazan, J. F., and Zlotnik, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 15086–15091
21. Hirosawa-Takamori, M., Jackle, H., and Vorbruggen, G. (2000) *EMBO Rep.* **1,** 441–446
22. Zhou, X., Park, S. I., Moustafa, M. E., Carlson, B. A., Crain, P. F., Diamond, A. M., Hatfield, D. L., and Lee, B. J. (1999) *J. Biol. Chem.* **274,** 18729–18734
23. Lescure, A., Gautheret, D., Carbon, P., and Krol, A. (1999) *J. Biol. Chem.* **274,** 38147–38154
24. Myers, E. W., Sutton, G. G., Delcher, A. L., Dew, I. M., Fasulo, D. P., Flanigan, M. J., Kravitz, S. A., Mobarry, C. M., Reinert, K. H., Remington, K. A., Anson, E. L., Bolanos, R. A., Chou, H. H., Jordan, C. M., Halpern, A. L., Lonardi, S., Beasley, E. M., Brandon, R. C., Chen, L., Dunn, P. J., Lai, Z., Liang, Y., Nusskern, D. R., Zhan, M., Zhang, Q., Zheng, X., Rubin, G. M., Adams, M. D., and Venter, J. C. (2000) *Science* **287,** 2196–2204
25. Li, F. S., Duan, Y. J., Yan, S. J., Guan, J. Y., Zou, L. M., Wei, F. C., Mong, L. Y., Li, L., and Li, S. Y. (1990) *Mech. Ageing Dev.* **54,** 103–120
26. Foster, H. D., and Zhang, L. (1995) *Sci. Total Environ.* **170,** 133–139