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Cloning and Developmental Expression of a Nuclear Ubiquitin-Conjugating Enzyme (DmUbc9) That Interacts with Small Heat Shock Proteins in *Drosophila melanogaster*

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In a two hybrid screen designed to identify proteins that interact with small heat shock proteins (sHsps), a *Drosophila melanogaster* homologue of yeast and human *ubc9* (Dm*ubc9*) was found to interact with *Drosophila* Hsp23. Further, two-hybrid system analysis reveals DmUbc9 interaction with *Drosophila* and mammalian Hsp27. *In situ* hybridization localizes Dm*ubc9* as a doublet at locus 21D on chromosome 2L, and genomic cloning of the gene reveals a single open reading frame without introns. The predicted Dm*ubc9* protein sequence shares a very high level of homology with mouse (85.4%) and human (≥82.9%) Ubc9. Genetic complementation analysis show that Dm*ubc9* functionally rescues a temperature-sensitive *S. cerevisiae ubc9ts* mutant. Co-immunoprecipitation with antibody raised against DmUbc9 confirms the interaction with *Drosophila* Hsp23 and Hsp26 and preferentially with Hsp27. The DmUbc9 protein, which localizes primarily to the nucleus in *Drosophila* S2 cells, is found at high levels in embryos but is also present at lower levels throughout development. The significance of the sHsp-Ubc9 interaction is discussed.

Key Words: **ubiquitin conjugating enzyme; Ubc9; *Drosophila melanogaster;* heat shock protein; Hsp; two-hybrid system.**

Small heat shock proteins (sHsps) have been shown to protect cells from the adverse effects of high temperature exposure (thermotolerance) or of various toxic compounds (reviewed in 1). Such resistance has been shown to be conferred by overexpression of various sHsps from mammals (2-4) and from insects (4,5). In addition to their induction following stress, these proteins are also expressed in a tissue-, cell-, and developmental stage-specific manner in the absence of stress (see 6,7). The functions of sHsps during development and under normal growth conditions remain to be determined.

The exact mechanisms by which sHsps confer cellular resistance remain unclear. Human Hsp27 has been suggested to act by protection of the actin cytoskeleton (3). Expression of mammalian and *Drosophila* Hsp27 has alternatively been reported to protect against TNFα or other forms of oxidative stress by reducing the reactive oxygen species formed within the cell (8). For the mammalian protein at least this appears to result from its ability to increase the intracellular content of glutathione (8). Other reports suggest that sHsps act as molecular chaperones like many of the other Hsps (9).

In *Drosophila melanogaster* there are four related sHsps of apparent molecular weights of 22, 23, 26, and 27 kDa (10). These four proteins share homologies but they show distinct patterns of expression during embryogenesis and differentiation and are localized in different cell compartments (6,7,11). In an attempt to identify functions for these sHsps we initiated a search for their possible intracellular protein partners using the yeast two-hybrid system (12,13). Here we report the cloning of a *D. melanogaster* homologue of ubiquitin conjugating-enzyme 9 (Ubc9) which is shown to interact with certain sHsps of *D. melanogaster* as well as mammalian Hsp27. Our data provide an alternative pathway to explain the cellular protective properties of sHsps as well as providing clues to their function during normal development.

MATERIALS AND METHODS

Two-hybrid system analysis. The HybriZAP Two-Hybrid cDNA Gigapak Cloning Kit (Stratagene) was used for the generation of the

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cDNA library and two-hybrid screening, done as per the manufacturer's protocol. A HybriZAP *Drosophila* embryo cDNA library was produced from mRNA kindly provided by Dr. Paul Lasko (McGill University). Bait plasmid vectors were designed with sHsp-coding sequences inserted in-frame to the 3′-end of the Gal4 binding domain on vector pBDGal4, with the expression of the fusion protein under the control of the ADH1 promoter. *Drosophila hsp23* was cleaved with *Ssp*I and subcloned into *Sal*I digested, blunted and dephosphorylated pBDGal4 to generate pBDGal4-Hsp23. The pBDGal4-Hsp23 construct contained all the coding sequence of Hsp23 with the exception of the first three N-terminal amino acids (MAN). *Drosophila hsp27* was cleaved with *SmaI* and *PstI* and subcloned into similarly digested pBDGal4 to generate pBDGal4-Hsp27. The pBDGal4-Hsp27 construct contained all the coding sequence of Hsp27 with the exception of the first nine N-terminal amino acids (MSIIPLLHL). Expression of the pBDGal4-Hsp23 and pBDGal4-Hsp27 fusion proteins was verified in transformed yeast by immunoblotting with the monoclonal antibody 10C9 (14) which recognizes both sHsps (data not shown). The compatible bait plasmid pGBT9-Hsp27 expressing the mammalian (Chinese hamster ovary) Hsp27 was a gift of Dr. Jacques Landry (Université Laval).

Two-hybrid screening (12,13) was performed in yeast strain YRG2. Yeast containing bait plasmids were transformed with prey vectors of the embryo cDNA library. *HIS3* and *lacZ* reporter gene activation, indicating protein-protein interaction, was determined first as growth on histidine-deficient SD media followed by the determination of β-galactosidase activity using a filter-lift assay as described by the manufacturer. Target plasmids from transformants were rescued and amplified from positive yeast clones in competent *E. coli* DH5α or XL1 bacteria. Sequencing of both strands of cDNA inserts in pADGal4 vectors was performed using RT-pAD (5′-TCGATGATGAAGATACCCCACC-3′) and T7 synthetic primers with the ABI prism dye terminator cycle sequencing ready reaction kit and a 373A DNA sequencer (Applied Biosystems).

In situ hybridization. *In situ* hybridization was carried out by the *In situ* hybridization service of the University of Alberta (Edmonton, Canada). A random-primed, digoxygenin-labeled probe was made from pADGal4-DmUbc9 and used to hybridize to salivary gland polytene chromosomes. Hybridized material was detected using the Boehringer Mannheim Colour Detection System.

Southern blotting. Genomic DNA was extracted from S2 culture cells using standard methods. A [α-^{32}P]-dCTP labeled (Multiprime DNA labeling system, Amersham) *Eco*RI-*Xho*I fragment of pADGal4-DmUbc9 was used to probe restriction enzyme digested genomic DNA. Probing was done in hybridization buffer containing 6X SSC, 50% formamide, and 0.1% (w/v) skim milk for 16 h at 45°C, and the membrane was then serially washed with 0.2X SSC + 0.2% SDS (3 times 5 min at 50°C), 0.1X SSC + 0.2% SDS (twice for 15 min at 50°C), and 2X SSC (3 times 5 min at room temperature). Autoradiography was performed at −80°C.

DmUbc9 genomic clone isolation. DNA fragments containing sequences complementary to DmUbc9 cDNA were obtained by repeated screening a *Drosophila* (Canton S) genomic library in Lambda II FIX (Stratagene; a gift of Dr. Ulrich Nauber, Göttingen, Germany) with a [α-^{32}P]-dCTP labeled (Multiprime DNA labeling system, Amersham) *Eco*RI-*Xho*I fragment of pADGal4-DmUbc9. Vectors excised from purified positive phagemids were amplified and sequenced using T3 and T7 primers.

Yeast complementation analysis. An *Eco*RI-*Xba*I fragment of pADGal4-DmUbc9 containing the entire DmUbc9 ORF was subcloned downstream of the ADC1 promoter in the yeast expression vector pTY316 (15) (kindly provided by Dr. Peter M. Howley) to yield pTY316-DmUbc9. The temperature sensitive *S. cerevisiae* strain YWO103 (*MATa, bar1::HIS3, ubc9Δ1::TRP1, LEU2::ubc9-1*) and the control strain YWO85 (wild type *ubc9; MATa, bar1::HIS3*) (both gifts of Dr. Stefan Jentsch) were used for complementation analysis. YWO103 was transformed with pTY316-DmUbc9 and transformants were selected on uracil-deficient SD plates at 23°C. YPD plates were divided into quarters and streaked with control and test strains for complementation. Identical plates were incubated at 37°C and 23°C for 2 to 3 days.

Polyhistidine-tagged Ubc9 and the generation of antisera. An *Eco*RI-*Xho*I fragment of pADGal4-DmUbc9 containing the entire Ubc9 coding sequence was directionally inserted into a similarly digested pET-30a(+) expression vector (Novagen), creating an in-frame 63 amino acid N-terminal tag containing a poly-His sequence. *E. coli* XL1-Blue competent cells were transformed with the ligated construct, and the identity of plasmid DNA isolated from the transformants by FlexiPrep (Pharmacia) was confirmed by restriction enzyme digests. *E. coli* DE3 competent cells were subsequently transformed with the purified plasmid DNA for expression of the fusion protein. Induction of expression with 1 mM IPTG and purification of the His-tagged protein with a Ni-resin column were carried out following the manufacturer's protocol (pET System Manual, Novagen).

Two anti-Ubc9 sera (#75 and #76) were obtained following immunization of two female rabbits with purified His-tagged Ubc9 (200μg) emulsified with Freund's complete adjuvant. Three weeks later, animals were given a booster injection with the same amount of protein in Freund's incomplete adjuvant. Antisera were obtained 1 week after the booster injection. Subsequently rabbits were boosted every 4 weeks with an intramuscular injection of 200 μg of purified His-tagged Ubc9, and antisera were obtained 7-10 days following injection. Serum #75 was used for all experiments.

Gel electrophoresis and immunoblotting. Proteins were separated on one-dimensional SDS-PAGE gels using the method of Thomas and Kornberg (16) with modifications as previously described (14). Proteins were electrophoretically transferred to nitrocellulose (Gelman) and immunoblotted with primary antibodies for 1 hour. A horseradish peroxidase-conjugated goat anti-rabbit secondary (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:10000 was used and the reaction detected using a chemiluminescence substrate (POD, Boehringer Mannheim).

Immunocytochemistry. S2 cells fixed in methanol (20 minutes, −20°C) were washed with phosphate-buffered saline, and incubated with anti-Ubc9 antibody #75 (dilution 1:500) followed by an FITC-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc.). As controls, cells were incubated either in the absence of primary antibody or with serum pre-absorbed with the His-tagged Ubc9 antigen. Slides were examined using a DAS Leitz Microscope equipped with epifluorescence optics.

S2 cell culture, heat shock, and ^{35}S-labeling. *Drosophila* S2 cells were cultured at 23°C in Schield's:D22 (1:1) medium supplemented with 10% heat inactivated fetal bovine serum. For heat shock, cells were submerged in a 35°C water bath for 1 hour, and allowed to recover at 23°C for prescribed periods to allow the synthesis of heat shock proteins. For ^{35}S-labeling, cells were heat shocked in methionine-deficient D22 and 50μCi of L-^{35}S-Met (Dupont NEN) was added immediately following heat shock. Control S2 cells were labeled with 50μCi of L-^{35}S-Met for 3 hours at 23°C in methionine deficient D22 medium.

Immunoprecipitation. For immunoprecipitation, confluent S2 cells ($\sim 20\times10^6$) were lysed by 1 ml of lysis buffer (150 mM NaCl, 1 % NP-40, 50 mM Tris-HCl, pH 8.0)(17). The lysate was centrifuged for 10 min at 10,000 $\times$ *g* at 4°C and the supernatant (450 to 600 μL) incubated for 1 hour with polyclonal antiserum (typically 2 μL of anti-Ubc9 #75). Protein A-Sepharose beads (Sigma) were then added to the mixture and incubated for 1 hour. Beads were collected by centrifugation, and washed 3 times with lysis buffer. Proteins were recovered from the beads by heating at 95°C for 10 minutes in SDS sample buffer.

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D. melanogaster     MSGIAITRLG EERKAWRKDH PFGFVARPAK NPDGTLNLMI WECAIPGKKS    50
H. sapiens          MSGIALSRLA QERKAWRKDH PFGFVAVPTK NPDGTMNLMN WECAIPGKKG    50
S. cerevisiae       MSSLCLQRLQ EERKKWRKDH PFGFYAKPVK KADGSMDLQK WEAGIPGKEG    50
Consensus           MSGIAL.RL. EERKAWRKDH PFGFVA.P.K NPDGTMNLM. WECAIPGKKG    50

D. melanogaster     TPWEGGLYKL RMIFKDDYPT SPPKCKFEPP LFHPNVYPSG TVCLSLLDEE   100
H. sapiens          TPWEGGLFKL RMLFKDDYPS SPPKCKFEPP LFHPNVYPSG TVCLSILEED   100
S. cerevisiae       TNWAGGVYPI TVEYPNEYPS KPPKVKFPAG FYHPNVYPSG TICLSILNED   100
Consensus           TPWEGGLYKL RM.FKDDYPS SPPKCKFEPP LFHPNVYPSG TVCLSIL.ED   100

D. melanogaster     KDWRPAITIK QILLGIQDLL NEPNIKDPAQ AEAYTIYCQN RLEYEKRVRA   150
H. sapiens          KDWRPAITIK QILLGIQELL NEPNIQDPAQ AEAYTIYCQN RVEYEKRVRA   150
S. cerevisiae       QDWRPAITLK QIVLGVQDLL DSPNPNSPAQ EPAWRSFSRN KAEYDKKVLL   150
Consensus           KDWRPAITIK QILLGIQDLL NEPNI.DPAQ AEAYTIYCQN R.EYEKRVRA   150

D. melanogaster     QARAMAATE                                               159
H. sapiens          QAKKFAPSX                                               159
S. cerevisiae       QAKQY--SK                                               157
Consensus           QAK..A.S.                                               159
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FIG. 1. Alignment of the deduced DmUbc9 protein sequence (Genbank Acc. AF030444) with the gene product of human (Genbank Acc. X96427) and yeast (Genbank Acc. X82538) Ubc9. Note the conserved region around cysteine 93, the active site.

RESULTS AND DISCUSSION

Identification and characterization of DmUbc9. In studies designed to identify the functions of *Drosophila* small heat shock proteins (sHsps) the yeast two-hybrid system was used to rescue cDNA clones from cell and tissue libraries that encoded proteins that interact with the sHsps. Recovered from an embryonic cDNA library with Hsp23 as bait was a *Drosophila* homologue of a ubiquitin-conjugating enzyme. The primary sequence of this clone, termed Dm*ubc9*, encoded an open reading frame (ORF) of 159 amino acids with a predicted molecular mass of 18 kDa (Figure 1). The amino acid sequence was found to be highly homologous to Ubc9 from other organisms, with 85.4%, 85.4%, 82.9%, 54.9%, and 34.3% homology to that of mouse (Genbank acc. U76416), human (X96427), human (U31882), *S. cerevisiae* (X82538), and *A. thaliana* (Z14990), respectively. The cysteine residue required for Ubc9-ubiquitin thioester formation, at position 93 (18), is also conserved in this *Drosophila* homologue. *In situ* hybridization localized the Dm*ubc9* gene to two distinct bands in region 21D of chromosome 2L (Figure 2). Southern blotting analysis with S2 cell DNA confirmed the likely presence of two related genes; digestion with *Pst*I showed two distinct bands even though no such restriction site is located within the gene (Figure 2). Whether both genes code for Ubc9 or are instead closely related proteins, and if they are both expressed, is not known. One Dm*ubc9* gene was recovered from a genomic library and sequenced (Figure 3). Comparison with the recovered cDNA clone showed the DmUbc9 gene to be uninterrupted by introns, unlike its yeast counterpart which contains one intron (18).

Dmubc9 expression functionally complements the yeast gene. To establish a functional role for Dm*ubc9 in vivo*, we used yeast complementation analysis.

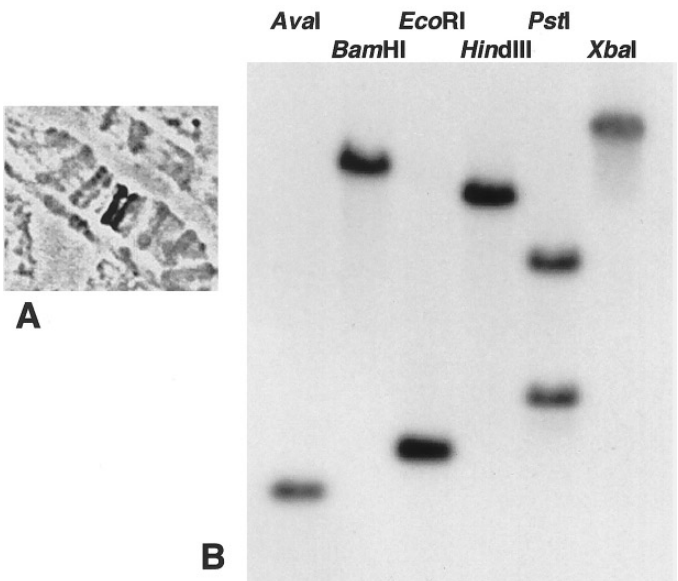

FIG. 2. Chromosomal location of Dm*ubc9*. (A) Detail of *in situ* hybridization of DmUbc9 DNA to *D. melanogaster* salivary gland polytene chromosomes showing a doublet at locus 21D on the left arm of the second chromosome. (B) Southern blots with genomic DNA of S2 cells probed with an [α-^{32}P]-dCTP labeled *Eco*RI-*Xho*I fragment of pADGal4-DmUbc9.

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-612  TACATTGCATTTCTTAAAACTAATGCAGTAATTTGTGTAAACAGTAATGCGAATAAATTT
-552  TAATTCTAACGTTTAATTAAACTAGTTTTGTATTGAGTAGAGGGTTTTTAGGAATGTAAC
-492  GGCTTAAGAGAAAAACCTAATACATTTTAAGTTGAGCTTACCAAAATCCAAAGAATTTTT
-432  AAATTTAAAACATAATACAAAAGTGCTTTGAAAAAGTCAGTATTTTAATACCACTGTTTA
-372  TCCACGGCCACACTAAATATCGATAACCGCATAGTGAGACCCTTTGTGTAGAAAAACTTA
-312  TACCTTTAGCGAGCACAAGCGTTCATTCCTGAAAAATTGCGGTAAAAATTGCGAAAAGCG
-252  CGAGTTCCCCCCGCGATTGCAAATAATTAATTTGCTTGGAACCACTGGAGACGCAAGGAC
-192  GAACAGAGAGGTAGGTGCTCTTGCACTTAGCGCGCCAAAAGGCGACAAAGCCCCTCGTAG
-132  GCCACTTACCCACACACACACATTCGCAGACAGACGCGCGTGCAAAAAAGGCCAAGTGGG
-72   ACTGCTAGCGGAAATCCAGGACTCGAGGACACGTTCGGGCGGCAGTAGAGAAGCGAGCAA
-12   GCAGCGACGACCATGTCCGGCATTGCTATTACACGATTGGGGGAGGAGCGCAAGGCCTGG
                M  S  G  I  A  I  T  R  L  G  E  E  R  K  A  W
43   CGCAAGGATCACCCATTCGGGTTCGTCGCACGACCCGCCAAGAACCCTGACGGCACCCTC
     R  K  D  H  P  F  G  F  V  A  R  P  A  K  N  P  D  G  T  L
103  AACCTGATGATCTGGGAGTGCGCCATTCCCGGCAAGAAGTCCACCCCCTGGGAGGGCGGG
     N  L  M  I  W  E  C  A  I  P  G  K  K  S  T  P  W  E  G  G
163  CTCTACAAGCTGCGCATGATCTTCAAGGACGACTACCCCACCTCGCCGCCCAAGTGCAAG
     L  Y  K  L  R  M  I  F  K  D  D  Y  P  T  S  P  P  K  C  K
223  TTCGAACCGCCGCTGTTCCACCCGAACGTCTATCCCTCGGGCACCGTTTGCCTGTCGCTG
     F  E  P  P  L  F  H  P  N  V  Y  P  S  G  T  V  C  L  S  L
283  CTGGACGAGGAGAAGGACTGGCGCCCCGCCATCACCATCAAGCAAATCCTGCTGGGCATC
     L  D  E  E  K  D  W  R  P  A  I  T  I  K  Q  I  L  L  G  I
343  CAGGACCTGCTCAACGAGCCGAACATCAAGGACCCGGCCCAGGCGGAGGCCTACACCATC
     Q  D  L  L  N  E  P  N  I  K  D  P  A  Q  A  E  A  Y  T  I
403  TACTGCCAGAACCGACTGGAGTACGAGAAGCGCGTGCGTGCCCAGGCCCGCGCCATGGCG
     Y  C  Q  N  R  L  E  Y  E  K  R  V  R  A  Q  A  R  A  M  A
463  GCCACCGAGTAGTCGGGCTCCTGCCAACCACCTTTACCACATAGACATTAAATACACACA
     A  T  E  *
523  TCTAGCCGGTAAGCGTTTGTTTGGAATGCTCTCCACGTCTACTTACTTTTAAAGAATCGA
583  CTAATTTTGGATAGCCCCCACGGCCCGAAGCATCTAATTTTTTAATGTTCTGCACTTGCT
643  TAATTACCAGCATGTACCATCTGAATAAATACAAATAAGTCCTGTAATTTTTCGATGAGG
703  CCCAAAGTGGCCACGTGGCACCCAG
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FIG. 3. Primary structure of a Dm*ubc9* gene (Genbank Acc. AF030443) and the predicted amino acid sequence of DmUbc9. Comparison with the cDNA sequence shows the gene to code a single ORF, uninterrupted by introns. Nucleotide numbers (on the left) start at the first nucleotide of the coding region. The active site cysteine at position 93 is shown in bold. Double underline, start codon. Underline, polyadenylation signal. The polyA tail of the cDNA begins at nucleotide 688 of this sequence.

A *ubc9* temperature sensitive (ts) mutant strain (YWO103) of *S. cerevisiae* (18), which grows normally at 25°C but fails to do so at high (>35°C) temperatures, was used for these studies. Introduction of the pTY316-DmUbc9 vector into the mutant strain restored growth at 37°C (Figure 4A), showing that the recovered clone rescues the function of mutant yeast gene. Growth at 37°C was not restored in yeast transformed with pTY316 alone (data not shown). The expression of DmUbc9 in yeast transformed with pTY316-DmUbc9 was confirmed by immunoblotting (Figure 4B).

DmUbc9 expression during development. Western blot analysis of S2 cell extracts showed that polyclonal antibody #75 recognized a single major band with the predicted size of Ubc9, approximately 18 kDa. Cross-reactivity with Ubc9 from other species, expected given the high homology of the protein across species, was verified by using MCF7 cell (human) and YRG-2 (yeast) extracts. In both species a major band at approximately the same molecular weight was observed, indicating that the antibody cross-reacts with Ubc9 from different species (data not shown). Pre-absorption of the anti-

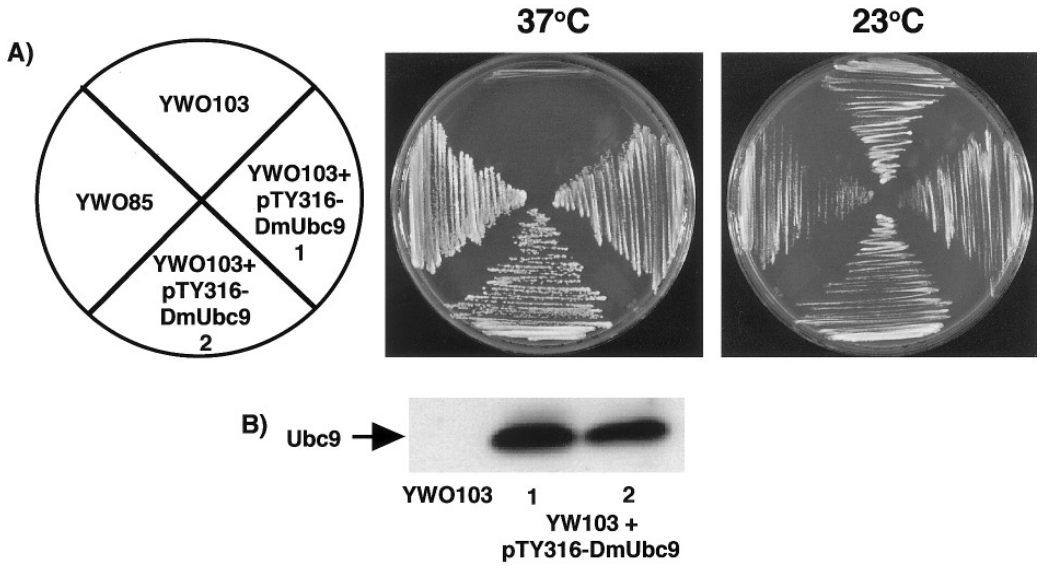

FIG. 4. Functional complementation of Sc*ubc9* by Dm*ubc9*. Wild type (YWO85), *ubc9-1ts* mutant yeast (YWO103), and transformants of YWO103 with the indicated plasmids were incubated at 23°C or 37°C on YPD plates. (A) Growth of yeast at different temperatures. (B) Immunoblot showing DmUbc9 expression in YWO103 transformed with pTY316-DmUbc9. Anti-DmUbc9 #75 was used at a dilution of 1:2000.

DmUbc9 antibody with homogenates from IPTG-induced His-tagged DmUbc9-producing *E. coli* resulted in the loss of the 18 kDa band.

Immunoblotting with the antibody was used to determine *Drosophila* Ubc9 expression during development. The protein was found to be present in high levels in embryos and in adult female flies (Figure 5). Overexposure of the chemiluminescence signal showed the protein to be present during all other developmental stages, but at a much lower level than in embryos. Although we cannot provide a specific role for Ubc9 in *Drosophila*, a particular function in the embryo seems likely given the levels of Ubc9 throughout the embryonic stages. Since the protein is found in both early embryos and adult females, a maternal origin for the expression of the protein seems likely, at least in the early stages of embryo development.

DmUbc9 localization in cultured cells. Immunocytochemical analysis showed *Drosophila* Ubc9 to be a mainly nuclear protein in S2 cells (Figure 6). Little or no staining was observed in the cytoplasm or nucleoli of these cells. An identical staining pattern was obtained with S2 cells fixed with paraformaldehyde and permeabilized with Triton X-100 (data not shown). The nuclear localization of Ubc9 in S2 cells is identical to that observed for a Ubc9-β-galactosidase fusion protein in yeast (18). Heat shock and recovery from heat shock had little or no effect on DmUbc9 levels in S2 cells (Figure 7), showing that DmUbc9 does not behave as a heat shock protein.

DmUbc9 interaction with small heat shock proteins. First recovered as a protein interacting with DmHsp23 using the yeast two-hybrid system, DmUbc9 was also shown to interact with DmHsp27 and mammalian Hsp27, as well as with itself (Table 1). The tumor suppressor p53, normally used as a negative control in the system, was also found to interact with DmUbc9. Reporter genes were not activated in the presence of

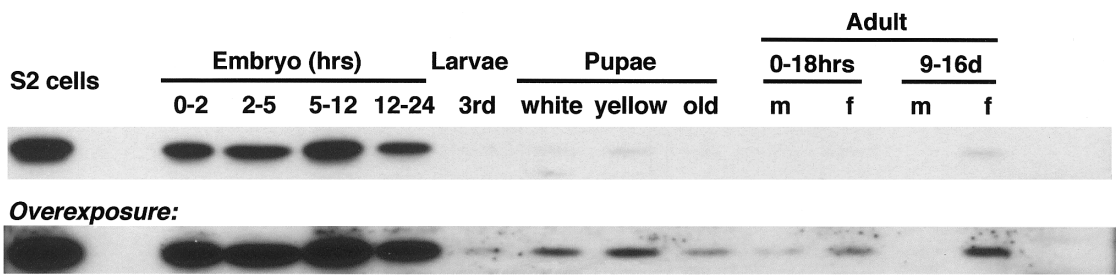

FIG. 5. Developmental expression of Ubc9 measured by immunoblotting showing the predominance of DmUbc9 protein in embryos. Equal amounts (~20 μg) were protein loaded. m, male; f, female. Anti-DmUbc9 #75 was used at a dilution of 1:2000.

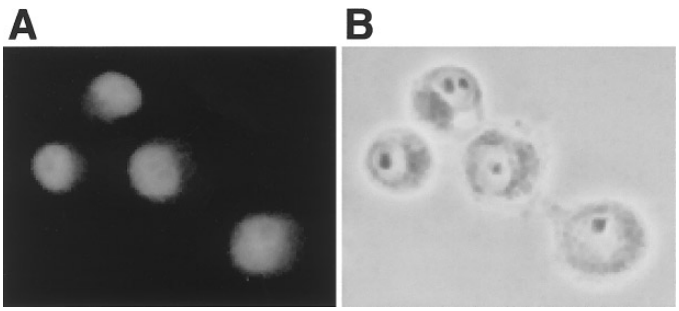

FIG. 6. Immunocytochemical analysis of DmUbc9 localization in S2 cells. (A) Immunofluorescence; (B) phase contrast microscopy. Cells were grown at 23°C and were fixed in methanol and labeled using anti-DmUbc9 #75 (at a dilution of 1:500) and FITC-labeled goat anti-rabbit secondary antibodies.

DmUbc9 plasmids (bait or prey vectors) alone, pBDLaminC plus pADGAL4-DmUbc9, or in the presence of pBDGal4-Hsp23 or pBDGal4-Hsp27 bait vectors alone.

In order to confirm the *in vivo* interaction observed with the two-hybrid system, ^{35}S-labeled heat-shocked S2 cell extracts were immunoprecipitated with anti-Ubc9 polyclonal antibody. *Drosophila* Hsp27, Hsp26, and Hsp23 were found to co-immunoprecipitate with Ubc9 (Figure 8). Experiments with pre-immune serum and with an unrelated antibody (data not shown), or in the absence of antibody (Figure 8) did not result in the co-immunoprecipitation of sHsps. From the relative ratio of the different sHsps in the precipitate it is clear that Hsp27 is preferentially recovered during co-immunoprecipitation with the anti-Ubc9 antibody. This may reflect a true difference in the binding of the different sHsps to Ubc9 or, on the other hand, this may be a reflection of the localization of the proteins, Ubc9 occupying a subcellular locale which would favour encounters with Hsp27 and its subsequent binding (19). The nature of the sHsp-Ubc9 interaction is not decipherable from the present data. It is thus possible that a larger complex involving intermediate protein partners is formed. The ability of Ubc9 to interact with mammalian Hsp27 in the two-hybrid system suggests that the interaction is not species-specific. The effects, if any, of sHsp phosphorylation (see 20,21) and their ability to interact with Ubc9 are presently unknown.

FIG. 7. Heat shock and recovery has no effect on the levels of DmUbc9 protein. Total protein extracts from *Drosophila* S2 cells were immunoblotted with anti-DmUbc9 #75 (at a dilution of 1:2000). Lanes: 1, control cells; 2 to 5, cells heat shocked at 35°C for 1 hour and recovered at 23°C for 0 (lane 2), 2 (lane 3), 8 (lane 4), and 24 (lane 5) hours.

TABLE 1

Two-Hybrid Results for DmUbc9 Clones Containing the Gal4 Binding or Activation Domain Vectors with (as Indicated) or without (None) Protein Inserts

Binding domain fusions	Activation domain fusions		
	pAD-DmUbc9	pAD-SV40	No vector
pBD-DmUbc9	+		−
pBD-DmHsp23	+		−
pBD-DmHsp27	+		−
pGBT9-Hsp27 (mammalian)	+		−
pBD-p53	+	+	−
pBD-LaminC	−		−
pBD alone	−		−
No vector	−		−

Note. A minus (−) denotes an absence of growth on Leu/Trp/His deficient SD or the absence of β-galactosidase activity, whereas a plus (+) denotes growth on Leu/Trp/His deficient medium and β-galactosidase activity measured using the filter-lift assay.

Our two-hybrid system results also indicate that DmUbc9 interacts with itself and with p53 (see Table 1). The significance of these interactions remains to be determined.

Functional significance of the sHsp-Ubc9 interaction. Small Hsps are expressed during both normal development and following cellular stress. Although they have been suggested to play some role in the acquisition of tolerance to stress, their functions in development in the absence of stress are as yet unknown. The present data provide important avenues of investigation to ascertain these functions.

In eukaryotes the ubiquitin-dependent proteolytic system plays a major role in the selective degradation of proteins. Following selective tagging via ATP-dependent ubiquitination, target proteins are recognized by a specific receptor of the 26S proteasome and subsequently degraded. Members of the ubiquitin conjugating enzyme family are responsible for the transfer of ubiquitin to specific target proteins. This proteolytic system has been shown to be important in controlling the levels of enzymes and regulatory proteins, as well as modulating a number of cellular processes including cell cycle progression, transcription, and DNA repair (reviewed in 22). Though no functional significance to the sHsp-Ubc9 interaction can be obtained directly from this work, two possibilities can be envisaged. First, sHsps may be substrates for Ubc9 activity and degraded via the proteasome following ubiquitination. At least one sHsp has been shown to be partly ubiquitinated, the αB-crystallin in Rosenthal fibers (23). On the other hand sHsps could play an active role in the modulation of Ubc9 activity. For example, modulation of Ubc9 activity could in fact explain the ability of hu-

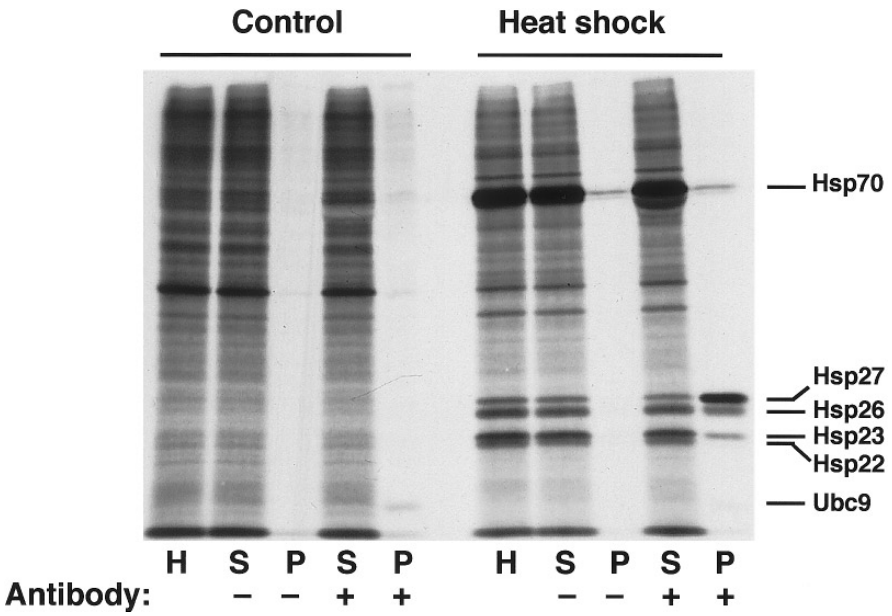

FIG. 8. *Drosophila* sHsps 23, 26, and 27 co-immunoprecipitate with DmUbc9. Homogenates of control (23°C) and heat shocked (35°C, 1 hour) S2 cells labeled with ^{35}S were immunoprecipitated with anti-Ubc9 #75. H, total homogenate; S, supernatant from the immunoprecipitate; P, precipitate (protein recovered from Sepharose-A beads).

man and *Drosophila* Hsp27 to prevent tumor necrosis factor (TNF)α and H_2O_2-induced cytotoxicity in murine L929 fibroblasts (4). Recently murine Ubc9 has been shown to be directly involved in the degradation of IκBα (24), the loss of this inhibitor leading to activation and migration to the nucleus of the transcriptional activator NF-κB. Should sHsps possess the ability to modulate Ubc9 activity, we can imagine a model where sHsps may prevent or delay the NF-κB-mediated stress-response (including apoptosis) by inhibiting ubiquitination of IκB and its subsequent degradation. Other functions for a sHsp-Ubc9 interaction could also be envisaged during development. *Drosophila* sHsps are often found associated with tissues that undergo significant growth or metamorphosis. The interaction of sHsps with Ubc9 could provide a mechanism for modulating these processes. Indeed, yeast Ubc9 has been shown to participate in the degradation of the S and M phase cyclins CLB5 and CLB2, respectively, and Ubc9 mutation prevents yeast cell-cycle progression (18).

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