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Identification and Characterization of a *Drosophila* Homologue of the Yeast *UBC9* and *hus5* Genes[1]

Shunji Ohsako[2] and Yoshiki Takamatsu

Department of Cell Biology, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526

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The yeast *UBC9* and *hus5* gene products have been identified as putative E2 members of the ubiquitin-conjugating enzyme (UBC) family and have been shown to play an essential role in cell cycle progression. We have identified a *Drosophila* Ubc9/Hus5 homologue (termed dUBC9) in an attempt to identify proteins that interact with the amino-terminal transcriptional repression domain of the Groucho corepressor by use of the yeast two-hybrid system. The predicted dUBC9 protein consists of 159 amino acids and shows 85, 68, and 54% amino acid sequence identities with human UBC9 homologue, *Schizosaccharomyces pombe* Hus5, and *Saccharomyces cerevisiae* Ubc9 proteins, respectively. Expression of dUBC9 cDNA complements a temperature-sensitive *ubc9-1* mutation of *S. cerevisiae* to fully restore normal growth, indicating that the dUBC9 protein can act as a substitute for the yeast Ubc9 protein. The dUBC9 transcripts were about 1.2 kb and were detected at all stages of *Drosophila* development and in ovaries and Schneider cells. However, an increased level was observed in early embryos and ovaries. The dUBC9 gene is present as a single copy in the genome and localized in segment 21C-D on the left arm of the second chromosome.

Key words: *Drosophila*, Groucho, UBC9, ubiquitin-like proteins, yeast two-hybrid.

Modification of proteins by ubiquitin, a highly conserved protein of 76 amino acid residues, is a crucial step in the targeting of proteins for degradation through proteasomes in eukaryotes. The ubiquitin-dependent proteolytic system has pivotal roles in the regulation of a variety of fundamental cellular processes such as cell cycle, transcriptional regulation, DNA repair, and signal transduction (*1-4*). Ubiquitin conjugation involves the formation of an isopeptide bond between the carboxyl-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue of an acceptor protein. In the initial step, ubiquitin-activating enzyme (E1) hydrolyzes ATP and forms a thioester bond between a cystine residue of its active site and the carboxyl terminus of ubiquitin. Ubiquitin is then transferred from the E1 to a cystine residue in a ubiquitin-conjugating enzyme (E2). Finally, ubiquitin is covalently attached to a substrate protein by the E2 enzyme, either alone or together with a ubiquitin-protein ligase (E3). Polyubiquitinated proteins are bound by the 26S proteasome and progressively degraded. However, protein ubiquitination functions not only as a signal for degradation but also to alter the properties of the linked protein in some instances (*3*).

In *Saccharomyces cerevisiae*, 13 different genes for putative E2 enzymes (Ubc) have been identified that encode proteins bearing a highly conserved UBC domain with about 160 amino acid residues (*1*). Two of these *UBC* genes, *UBC3* and *UBC9*, have been shown to be individually essential for cell viability. Loss of function mutation of the *UBC9* gene prevents cell cycle progression at the G2 or early M phase, causing the accumulation of large budded cells with a single nucleus, a short spindle, and replicated DNA and the stabilization of both Clb5, an S-phase cyclin, and Clb2, an M-phase cyclin (*5*). The *Schizosaccharomyces pombe hus5* gene product highly homologous to *UBC9* is also required for normal mitosis (*6*). Recently, mammalian homologues of Ubc9/Hus5 have been isolated repeatedly by using the yeast two-hybrid system to identify proteins that interact with a wide variety of proteins (*7-20*). More recent studies have shown that Ubc9 and its mammalian homologue conjugate the ubiquitin-like proteins Smt3 and SUMO-1, but not ubiquitin (*21-26*). SUMO-1 was identified as a covalent modification of the Ran GTPase-activating protein, RanGAP1, and SUMO-1 modification has been proposed to function to target RanGAP1 to the nuclear pore complexes (*27, 28*). Thus, SUMO-1/Smt3 modification appears to have novel functions distinct from those of ubiquitination.

In an attempt to identify the proteins involved in the transcriptional repression by the Groucho corepressor protein, we performed a yeast two-hybrid screen of a *Drosophila* embryonic library using the Groucho amino-terminal transcription domain as bait and identified the *Drosophila* homologue of the yeast Ubc9 and Hus5. Here

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[2] To whom correspondence should be addressed. Phone: +81-42-325-3881, Fax: +81-42-321-8678, E-mail: sohsako@tmin.ac.jp

Abbreviations: DIG, digoxigenin; GST, gultathione S-transferase; HA, hemagglutinin; TLE, transducin-related enhancer of split; UBC, ubiquitin-conjugating enzyme.

we report the identification and characterization of the *Drosophila* homologue of the yeast *UBC9* and *hus5* genes.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen—To identify the proteins involved in the transcriptional repression by the Groucho-related corepressors, we employed the yeast two-hybrid system which was developed and provided by Dr. S. Hollenberg (Vollum Institute, Portland, Oregon) (*29*). The amino-terminal transcription repression domain (amino acids 1-264) of the Groucho protein was inserted into pBTM116 vector, which fuses with the LexA DNA-binding domain, resulting in pLexA-Groucho 1-264. A random-primed cDNA library was constructed in the *Not*I site of pVP16 vector, which fuses with the viral transcriptional activation domain VP16, using cDNAs generated from poly(A)$^+$ RNA from 3-8 h *Drosophila melanogaster* (Oregon R) embryos, as described (*30*) with some modifications. Yeast strain L40 was transformed with pLexA-Groucho 1-264 and the prey library plasmids and analyzed for interaction of the proteins as described (*29*). The nucleotide sequences were determined by the dideoxyribonucleotide chain-termination method using an Autoread sequencing kit (Pharmacia) and analyzed with an A.L.F. DNA sequencer (Pharmacia).

Rapid Amplification of cDNA Ends (RACE)—To isolate longer cDNAs, the 5′ and 3′ RACE analyses were carried out using a Marathon cDNA Amplification kit (Clontech) and an Expand High Fidelity PCR system (Boehringer, Mannheim) following the manufacturer's instructions. The primers used were 5′-GCAGGTCCTGGATGCCCAGCAGGAT-3′ (complementary to nucleotides 334-358) for the 5′ RACE and 5′-ACCCTCAACCTGATGATCTGGGAGT-3′ (nucleotides 103-127) for the 3′ RACE. The RACE products were purified, inserted into the pGEM-T vector (Promega), and sequenced.

Genomic PCR Analysis—Drosophila melanogaster (Canton S) genomic DNA was amplified by PCR using the Expand High Fidelity PCR system with primers 5′-GTAAAAATTGCGAAAAGCGCGAGTT-3′ (nucleotides −192 to −170) and 5′-TTTTATTGAAATTACATAGGTTTGTA-3′ (complementary to nucleotides 814 to 839). The PCR products were purified, inserted into the pGEM-T vector and sequenced.

Yeast Complementation Analysis—The dUBC9 cDNA was amplified by PCR using the Expand High Fidelity PCR system with primers 5′-CGGGATCCACCATGTCCGGCATTGCT-3′ (*Bam*HI site and nucleotides −3 to 12) and 5′-GCTCAGATTTTATTGAAATTACATAGGTT-3′ (*Xba*I site and complementary to nucleotides 818 to 839). The PCR product was digested with *Bam*HI and *Xba*I, and the resultant DNA fragment was fused to the ADH1 promoter in pYCplac33 vector. The ADH1 promoter was shuttled from the pAS2-1 vector (Clontech) into the *Eco*RI and *Bam*HI sites in pYCplac33. The resulting plasmid or control plasmid was used for transformation of wild type (YW01) and *ubc9-1* mutant (YWO102) strains (*5*). The transformants were streaked out on plates containing selective minimal glucose medium and incubated at 30 or 37°C for 3 days.

Northern Blot Analysis—Total RNA was prepared from each developmental stage and from the ovaries and Schneider 2 cells as described (*30*). Twenty micrograms of total RNA was electrophoresed through a 1.5% formaldehyde-agarose gel, transferred to nylon membrane, and probed with a ^{32}P-labeled dUBC9 cDNA as described (*30*).

In Situ Hybridization of Whole-Mount Embryo—In situ hybridization to whole mount embryos was performed using digoxigenin (DIG)-labeled anti-sense and sense dUBC9 RNA probes as described (*31*). RNA probes were generated by T3 RNA polymerase and a DIG RNA-labeling system (Boehringer, Mannheim) from linearized pGEM-dUBC9 DNA templates.

In Situ Chromosome Mapping of the dUBC9 Gene—In situ hybridization on squashes of polytene chromosome was performed using the DIG-labeled dUBC9 DNA probe as described (*30*). The DIG-labeled DNA probe was generated by PCR using the Expand High Fidelity PCR system, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.13 mM dTTP, and 0.07 mM DIG-11-dUTP (Boehringer, Mannheim) with M13 universal and reverse primers from pGEM-dUBC9.

RESULTS AND DISCUSSION

In an attempt to identify proteins involved in the transcriptional repression by the Groucho corepressor protein, we used the Groucho amino-terminal transcriptional repression domain (amino acids 1-264) (*32*) as bait to screen a *Drosophila* embryonic cDNA prey library. From approximately 2×10^6 transformants, 8 candidate clones were isolated. Two of these contained identical cDNA sequences (nucleotides 3-538 in Fig. 1a) homologous to the yeast *UBC9* and *hus5* genes as described below. To obtain longer cDNAs, we performed 5′ and 3′ RACE using primers described in "MATERIALS AND METHODS." Five of each RACE clone had the same sequences but different lengths. The nucleotide sequence of two representative overlapping cDNA clones was determined and the amino acid sequence was deduced as shown in Fig. 1a. The DNA sequence surrounding the first ATG was in agreement with the consensus sequence of translation initiation except T to G at position 4 (*33*). No other ATGs and two in-frame-stop codons were identified upstream of the apparent initiator ATG in the cDNA. Two optimal polyadenylation signals (AATAAA) were observed in the 3′-noncoding region, of which one is followed by 20 nucleotides before the poly(A) start. The predicted open reading frame from position 1 to 477 of the cDNA encoded a protein of 159 amino acids with a calculated molecular weight of ~18 kDa. The predicted protein shared 85, 68, and 54% amino acid sequence identities with human UBC9 homologue, *S. pombe* Hus5, and *S. cerevisiae* Ubc9 proteins (ScUbc9), respectively (Fig. 1b). Thereafter we termed it dUBC9, because this sequence similarity and a complementation analysis described below suggested that it was a *Drosophila* homologue of the yeast Ubc9. To further confirm the DNA sequence, *Drosophila* genomic DNA was amplified by PCR using primers described in "MATERIALS AND METHODS," and the resultant products were purified and cloned into pGEM-T vector. Their nucleotide sequences determined from two independent clones were identical both with each other and with that of the cDNA, except for the presence of an intron in the 5′ non-coding region and three nucleotide substitutions in 3′ non-coding regions. The nucleotide sequence of the intron of the dUBC9 gene and substituted nucleotide

a.

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                                                GTAAAAATTGCGAAAAGCGCGAGTTCCCCCC -164
GCGATTGCAAATAATTAATTTGCTTGGAACCACTGGAGACGCAAGGACGAACAGAGAGgt -103
aggtgctctcgcacttagcgcgccaaaaggcgacaaagcccctcgcaggccacttaaaca
cacacacattcgcagACAGACGCGCGTGCAAAAAAGGCCAAGTGGGACTGCTAGCGGA  -61
AATCCAGGACTCGAGGACACGTTCGGGCGGCAGTAGAGAAGCGAGCAAGCAGCGACGACC   -1

ATGTCCGGCATTGCTATTACACGATTGGGGGAGGAGCGCAAGGCCTGGCGCAAGGATCAC   60
MetSerGlyIleAlaIleThrArgLeuGlyGluGluArgLysAlaTrpArgLysAspHis   20

CCATTCGGGTTCGTCGCACGACCCGCCAAGAACCCTGACGGCACCCTCAACCTGATGATC  120
ProPheGlyPheValAlaArgProAlaLysAsnProAspGlyThrLeuAsnLeuMetIle   40

TGGGAGTGCGCCATTCCCGGCAAGAAGTCCACCCCCTGGGAGGGCGGGCTCTACAAGCTG  180
TrpGluCysAlaIleProGlyLysLysSerThrProTrpGluGlyGlyLeuTyrLysLeu   60

CGCATGATCTTCAAGGACGACTACCCCACCTCGCCGCCCAAGTGCAAGTTCGAACCGCCG  240
ArgMetIlePheLysAspAspTyrProThrSerProProLysCysLysPheGluProPro   80

CTGTTCCACCCGAACGTCTATCCCTCGGGCACCGTTTGCCTGTCGCTGCTGGACGAGGAG  300
LeuPheHisProAsnValTyrProSerGlyThrValCysLeuSerLeuLeuAspGluGlu  100

AAGGACTGGCGCCCCGCCATCACCATCAAGCAAATCCTGCTGGGCATCCAGGACCTGCTC  360
LysAspTrpArgProAlaIleThrIleLysGlnIleLeuLeuGlyIleGlnAspLeuLeu  120

AACGAGCCGAACATCAAGGACCCGGCCCAGGCGGAGGCCTACACCATCTACTGCCAGAAC  420
AsnGluProAsnIleLysAspProAlaGlnAlaGluAlaTyrThrIleTyrCysGlnAsn  140

CGACTGGAGTACGAGAAGCGCGTGCGTGCCCAGGCCCGCGCCATGGCGGCCACCGAGTAG  480
ArgLeuGluTyrGluLysArgValArgAlaGlnAlaArgAlaMetAlaAlaThrGlu***  159

TCGGGCTCCTGCCAACCACCTTTACCACATAGACATTAAATACACACATCTAGCCGGTAA  540
GCGTTTGTTTGGAATGCTCTCCACGTCTACTTACTTTTAAAGAATCGACTAATTTTGGAT  600
AGCCCCCACGGCCCGAAGCATCTAATTTTTTAATGTTCTGCACTTGCTTAATTACCAGCA  660
TGTACCATCTGAATAAATACAAATAAGTCCTGTAATTTTTCGATGAGGCCCAAAGTGGCC  720
ACGTGGCACCCAGAATTCGACGCTCTTTTCAGCTTCCATTCTGGGTGCAGGTGCCGATTA  780
TTCTACGGGCTATTTTGTATCGCGAGTACTACGTACAAACCTATGTAATTTCAATAAAAA  840
          c           ga
TTTATTAGTTGTAAAAACAAAAAAAAAAAAAAAAAAAAAAAAAAAA                887
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b.

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dUBC9      1 MSGIAITRLGEERKAWRKDHPFGFVARPAKNPDGTLNLMIWECAIPGKKS
hUBC9      1 .....LS..AQ..............V.T......M...N.........G
SPHus5     1 ..SLCK...Q....Q..R......Y.K.C.SS..G.D..N.KVG....PK
SCUBC9     1 ..SLCLQ..Q....K.........Y.K.V.KA..SMD.QK..AG....EG

dUBC9     51 TPWEGGLYKLRMIFKDDYPTSPPKCKFEPPLFHPNVYPSGTVCLSLLDEE
hUBC9     51 .......F....L......S.......................I.E.D
SPHus5    51 .S........T.A.PEE...R....R.T................I.N..
SCUBC9    51 .N.A..V.PITVEPNE..SK...V..PAGFY..........I...I.N.D

dUBC9    101 KDWRPAITIKQILLGIQDLLNEPNIKDPAQAEAYTIYCQNRLEYEKRVRA
hUBC9    101 ................E.......Q..............V........
SPHus5   101 EG.K..............DD...AS...T....MFKKDKV........
SCUBC9   101 Q.......L...V..V....DS..PNS...EP.WRSFSR.KA..D.K.LL

dUBC9    151 QARAMAATE 159
hUBC9    151 ..KKF.PS  158
SPHus5   151 ...EN.P   157
SCUBC9   151 ..KQYSK   157
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Fig. 1. **Nucleotide and deduced amino acid sequences of cDNA and genomic DNA encoding *Drosophila* dUBC9 and amino acid comparison between dUBC9 and human UBC9 homologue, ScUbc9 and SpHus5 proteins.** (a) Nucleotide and deduced amino acid sequences of cDNA and genomic DNA encoding *Drosophila* dUBC9. Nucleotide residues are numbered in the 5′ to 3′ direction starting with the initiation signal ATG as numbers 1 to 3. The nucleotide sequence of the intron and substituted nucleotide sequences of the dUBC9 gene are shown as lower-case letters. The exon/intron boundary consensus sequences are underlined. A branch site with the *Drosophila* consensus sequences, t-t-a-a-c, is dot-underlined. The poly(A) signals (AATAAA) in the 3′ noncoding region are underlined twice. (b) Amino acid comparison between dUBC9 and human UBC9 homologue, ScUbc9 and SpHus5. Single letter amino acid notation is used. Identical amino acids are denoted by dots. The dUBC9 protein shared 85, 68, and 54% amino acid sequence identities with human UBC9 homologue, *Schizosaccharomyces pombe* Hus5, and *Saccharomyces cerevisiae* Ubc9 proteins, respectively.

sequences are shown as lower-case letters in Fig. 1a. These nucleotide substitutions could be due to differences between strains (Oregon R *versus* Canton S). The intron of the dUBC9 gene consisted of 79 nucleotides and contained the intron/exon boundary consensus sequences and a branch site with the *Drosophila* consensus sequences, C/T-T-A/G-A-C/T (*34*).

The structural similarity between dUBC9 and ScUBC9

suggested that dUBC9 might function in place of ScUBC9 *in vivo*. To test this possibility, we used a yeast strain YWO102 (*ubc9-1*) with a temperature-sensitive mutation in *ScUBC9* gene. *ubc9-1* mutant cells halt cell progression at the G2 or early M phase when grown at the nonpermissive temperature (*5*). These cells were transformed with either yeast expression vector alone or vector expressing dUBC9, streaked out on plates containing selective minimal glucose medium and incubated at 30 or 37°C for 3 days. As shown in Fig. 2, expression of dUBC9 rescued the temperature-sensitive growth of the yeast *ubc9-1* mutant. This result strongly supported the idea that dUBC9 is a *Drosophila* homologue of ScUBC9 and suggested that dUBC9 is also required for cell cycle progression in *Drosophila*, its function possibly being mediated through its SUMO-1/Smt3-related protein conjugation activity.

To gain an insight into the function of dUBC9 in *Drosophila*, we analyzed the expression of dUBC9 mRNA by Northern blot analysis using total RNA isolated from *Drosophila* at different developmental stages, the ovaries, and the Schneider line 2 cells (Fig. 3). The transcripts of dUBC9 were approximately 1.2 kb and expressed widely throughout development and in the ovaries and Schneider cells, but an increased level was detected in early embryos and ovaries.

We next determined the distribution of dUBC9 mRNA during *Drosophila* embryogenesis by *in situ* hybridization with anti-sense digoxigenin-labeled RNA probes (Fig. 4). dUBC9 mRNA was uniformly detected at high levels in all embryos before cell formation and the onset of zygotic transcription (stages 1 to 5) (Fig. 4, a and b), indicating that dUBC9 mRNA is maternally derived. During germ band extension (Fig. 4c), dUBC9 transcripts continued to be present throughout the embryo. The weakly stained central region of the embryo contained mainly yolk. After germ band shortening, strong expression of dUBC9 transcripts was observed in areas of the primitive brain (supraesophageal ganglion), while the transcripts were still present throughout the embryo (Fig. 4d). The weakly stained dorsal middle region of the embryo contained mainly yolk sac. In later stages, expression gradually decreased throughout the embryo, but a relatively increased level of expression persisted in areas of the brain (data not shown). Sense digoxigenin-labeled RNA probes showed no specific staining (data not shown).

To determine the cytological location of dUBC9, the longest cDNA was digoxigenin-labeled and used as a probe for *in situ* hybridization to the polytene chromosomes of larval salivary glands. The probe hybridized to a single site, which was identified as segment 21 C-D on the left arm of the second chromosome (Fig. 5). Southern blot analysis of *Drosophila* genomic DNA digested with *Eco*RI, *Bam*HI, and *Bgl*II also revealed one major band in each with a length of about 1.3, 14, and 6 kbp, respectively (data not shown). Taken together with the genomic PCR analysis, these results indicate that the dUBC9 gene is present in a single copy in the genome.

In this report, we described the identification and characterization of a *Drosophila* homologue of yeast *UBC9* and *hus5* genes. In yeast two-hybrid assays, two VP16-dUBC9 fusion clones appeared to interact specifically with pLexA-Groucho, pLexA-TLE1 (TLE1: a human Groucho homologue), and pLexA-TLE1 1-400 as well as pLexA-Groucho 1-264 but not pLexA-lamin (data not shown). In addition, both dUBC9 and Groucho transcripts are maternally derived and are expressed widely in *Drosophila* (*35, 36*), suggesting that they interact with each other *in vivo*. However, we could not detect *in vitro* interaction between GST-dUBC9 and *in vitro* translated Groucho or Groucho 1-264, or HA-tagged Groucho 1-264 fusion proteins in pull-down assays (data not shown). Therefore, it is at present unclear whether the Groucho-related corepressors are able to interact with the UBC9 family proteins and to be conjugated by SUMO-1/Smt3-related proteins *in vitro* and *in vivo*. Recently, mammalian homologues of Ubc9/Hus5 have been isolated repeatedly by using the yeast two-hybrid system to identify proteins that interact with a wide

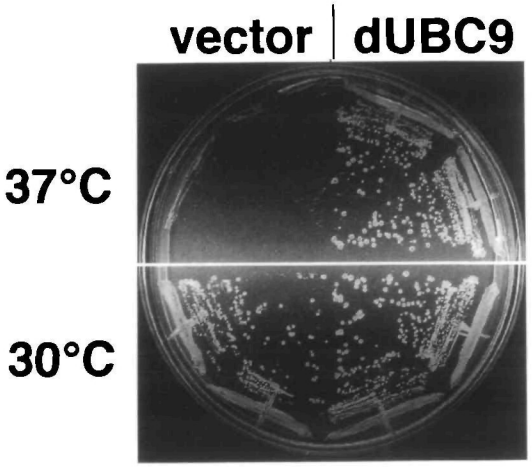

Fig. 2. **Functional complementation of the yeast *ubc9-1* mutant by expression of the dUBC9 cDNA.** Yeast *ubc9-1* mutant cells were transformed with either control vector (left) or vector containing dUBC9 cDNA (right), and incubated at either 30°C (bottom) or 37°C (top) for 3 days. The temperature-sensitive growth of the yeast *ubc9-1* mutant is suppressed by expression of the dUBC9 cDNA.

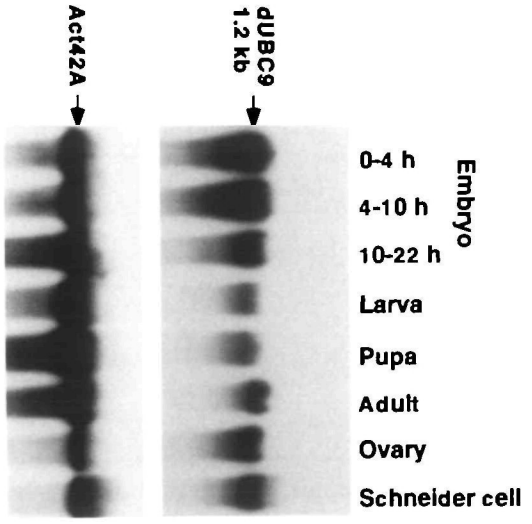

Fig. 3. **Northern blot analysis of dUBC9.** Twenty micrograms of total RNA prepared from each developmental stage and from the ovaries and Schneider 2 cells, was hybridized with a ^{32}P-labeled dUBC9 cDNA as described in "MATERIALS AND METHODS." Act42A probe was used as an internal control.

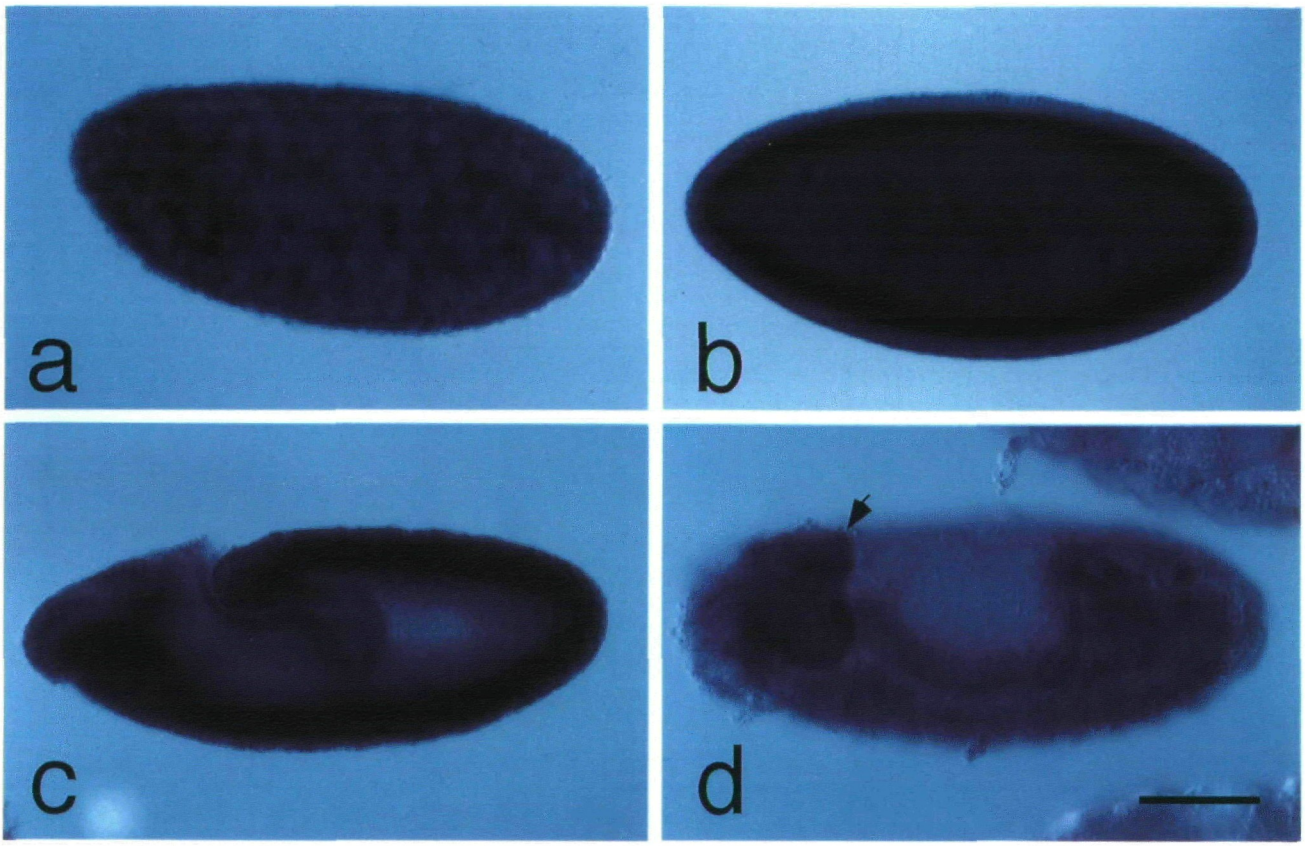

Fig. 4. **Expression patterns of the dUBC9 transcript during embryonic development.** Whole mount embryos were hybridized with a dUBC9 digoxigenin-labeled RNA probe. Staging of embryos was determined according to Campos-Ortega and Hartenstein (*37*). The embryos are oriented with the anterior to the left and the ventral down. a, stage 2 embryo contained high levels of mRNA; b, stage 5 embryo. dUBC transcripts were seen uniformly. These mRNA expression patterns before zygotic transcription (stages 1 to 5) suggest that dUBC9 transcripts are a maternal contribution. c, stage 10 embryo. dUBC9 transcripts were observed in essentially all cells during germ band extension. The weakly stained central region of the embryo contained mainly yolk. d, stage 14 embryo (Dorsoventral view). High levels of dUBC9 transcripts were found in areas of the primitive brain (supraesophageal ganglion) as indicated by an arrow, although the transcripts were still present throughout the embryo. The weakly stained dorsal middle region of the embryo contained mainly yolk. The bar represents 100 μm.

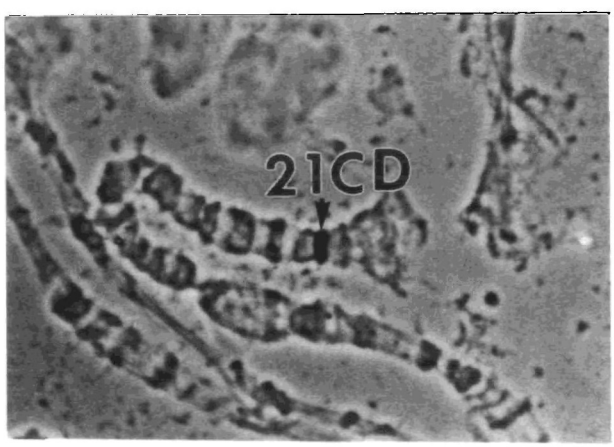

Fig. 5. **Mapping of the dUBC9 locus on polytene chromosomes.** The digoxigenin-labeled cDNA, the longest one, was hybridized to a squash of salivary gland chromosomes. Hybridization was visualized by staining with alkaline phosphatase. The arrow indicates the hybridized band in region 21C-D on the left arm of the second chromosome.

variety of proteins (*7-21*). However, it also remains to be determined whether these interactions are physiologically significant. Why is UBC9 isolated from yeast two-hybrid screens by such a wide variety of proteins? One possible explanation for this is that "bait" proteins are modified by Smt3 in yeast and there is an association between Ubc9 and bait proteins (*21*), because Ubc9 and its mammalian homologue have been shown to conjugate the ubiquitin-like proteins Smt3 and SUMO-1, but not ubiquitin (*21-26*). These studies also suggested that the role of yeast Ubc9 in cell cycle progression is mediated through its Smt3 conjugation activity: possibly modification of nuclear transport substrates or the nuclear transport machinery (*21-26*). Expression of dUBC9 cDNA is able to complement a cell cycle defect of a temperature-sensitive *ubc9-1* mutant of *Saccharomyces cerevisiae*, suggesting that dUBC9 also plays an essential role in cell cycle progression of *Drosophila*, possibly mediated through its SUMO-1/Smt3-related protein conjugation activity. Although the physiological role of the dUBC9 gene is undetermined in *Drosophila*, studies on dUBC9 will be useful for understanding its functions and the SUMO-1/Smt3-related protein conjugation pathway in multicellular organisms, because of the

availability of genetic and molecular tools in the *Drosophila* system.

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