

***Drosophila UbcD1* encodes a highly conserved ubiquitin-conjugating enzyme involved in selective protein degradation**

Mathias Treier¹, Wolfgang Seufert and Stefan Jentsch²

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstrasse 37–39, D-7400 Tübingen, Germany

¹Present address: European Molecular Biology Laboratory Meyerhofstrasse 1, D-6900 Heidelberg, Germany

²Corresponding author

Communicated by G. Gerisch

Ubiquitin-dependent selective protein degradation serves to eliminate abnormal proteins and provides controlled short half-lives to certain cellular proteins, including proteins of regulatory function such as phytochrome, yeast MAT α 2 repressor, p53 and cyclin. Moreover, ubiquitin-dependent proteolysis is thought to play an essential role during development and in programmed cell death. We have cloned a gene from *Drosophila melanogaster*, *UbcD1*, coding for a protein with striking sequence similarity to the yeast ubiquitin-conjugating enzymes UBC4 and UBC5. These closely related yeast enzymes are known to be central components of a major proteolytic pathway of *Saccharomyces cerevisiae*. By doing a precise open reading frame replacement in the yeast genome we could show that the *Drosophila UbcD1* enzyme can functionally substitute for yeast UBC4. *UbcD1* driven by the *UBC4* promoter rescues growth defects and temperature sensitivity of yeast *ubc4 ubc5* double mutant cells. Moreover, expression of *UbcD1* restores proteolysis proficiency in the *ubc4 ubc5* double mutant, indicating that the *Drosophila* enzyme also mediates protein degradation. This structural and functional conservation suggests that the *UbcD1*–*UBC4*–*UBC5* class of enzymes defines a major proteolytic pathway in probably all eukaryotes.

Key words: *Drosophila*/evolutionary conservation/protein degradation/ubiquitin-conjugating enzyme/yeast

Introduction

Selective protein degradation mediated by the ubiquitin system plays a vital role in probably all eukaryotic cells. Proteins degraded by this system are first earmarked by the covalent attachment of ubiquitin and are finally degraded by specific proteases. Turnover rates for individual proteins range from a few minutes to a couple of hours and can vary considerably depending on the cell type, nutritional and other influences, and the position within the cell cycle. Functionally linked to the cellular stress response, one essential role of this degradative system is the elimination of abnormal proteins caused, for example, by environmental stresses such as heat (Finley *et al.*, 1987; Seufert and Jentsch, 1990). Furthermore, ubiquitin-dependent protein breakdown serves a regulatory function by controlling the levels of proteins, including crucial cellular regulators.

Recent examples for regulatory proteins which are degraded by the ubiquitin system are the far-red light-absorbing form of phytochrome of higher plants (Shanklin *et al.*, 1987), the tumor suppressor p53 (Scheffner *et al.*, 1990), the yeast transcriptional repressor MAT α 2 (Hochstrasser *et al.*, 1991) and cyclin, a key factor for cell cycle control (Glotzer *et al.*, 1991).

Regulation and selectivity of protein turnover is largely mediated by the activities and substrate specificities of the enzymatic components of the ubiquitin–protein ligase system (for a review see Jentsch *et al.*, 1991). Ubiquitin–protein conjugate formation is catalysed by a ubiquitin-activating enzyme, E1, and a ubiquitin-conjugating enzyme, E2, and requires in some cases the activities of additional factors known as E3 proteins. Previously, we have shown that ubiquitin–protein conjugation is essential for cell viability (McGrath *et al.*, 1991). Furthermore, our genetic analysis has revealed that yeast cells have an ample set of distinct ubiquitin-conjugating enzymes that are involved in surprisingly different cellular functions (reviewed by Jentsch *et al.*, 1990), including DNA repair, induced mutagenesis and sporulation (Jentsch *et al.*, 1987), retrotransposition (Picologlou *et al.*, 1990), cell cycle control (Goebel *et al.*, 1988), resumption of growth after quiescence (Seufert *et al.*, 1990), and the stress response (Seufert and Jentsch, 1990).

Recently, we have identified the closely related ubiquitin-conjugating enzymes UBC4 and UBC5 of *Saccharomyces cerevisiae* as central components of a major pathway for selective protein degradation (Seufert and Jentsch, 1990). In *ubc4 ubc5* double mutants turnover of short-lived and abnormal proteins is strongly impaired. These mutants grow poorly at the normal growth temperature and they are not viable under stress conditions (Seufert and Jentsch, 1990). Together with UBC1, UBC4 and UBC5 constitute a subfamily of three yeast ubiquitin-conjugating enzymes essential for cell viability (Seufert *et al.*, 1990). Single genes are dispensable; however, the *ubc1 ubc4 ubc5* triple mutant is not viable.

Several lines of evidence suggest that not only controlled gene expression but also post-translational control of protein levels by selective breakdown is important for cell differentiation and developmental processes. Highly selective protein degradation is thought to be required for the spatial and temporal distribution of key regulators of development. A prominent example is the transcription factor encoded by the *Drosophila* gene *bicoid*. A prerequisite for its graded distribution within the embryo is the short half-life of the *bicoid* protein (Driever and Nüsslein-Volhard, 1988). Moreover, the development of distinct cell types and tissues often requires drastic alterations in protein compositions which is mediated by both protein synthesis and degradation. Ubiquitin-dependent proteolysis seems also essential for developmentally programmed cell death (Schwartz *et al.*, 1990).

To study the role of ubiquitin-mediated proteolysis in the

```

XbaI
TCTAGAGGCGCAACG -181

CAGCTTTGTGTTTCGTTTTTCGTTCAATTCTAAGCAGGAGGATTATATATTGAATAAAAC -121

CCAATTCTGTGCGAAGAACAAGAAATACGCGAGACACCAACACCAGCACACAACCAAC -61

CAACACACACAACCAACCAACACACACACACACACACCAACCAACCAATTTCAATCAGAAA -1

ATGGCGTTAAAAAGAATCAATAAGGAACGCAAGATCTGGGCAGAGATCCACCTGCACAA 60
M A L K R I N K E L Q D L G R D P P A Q

TGTTCCAGCGGTCCAGTTGGAGATGATTTATTTCACTGGCAAGCTACAATAATGGGCCCG 120
C S A G P V G D D L F H W Q A T I M G P

CCGGACAGCCCTTATCAAGGAGGTGATTTCTTCTTAACATACATTTTCCAACAGACTAT 180
P D S P Y Q G G V F F L T I H F P T D Y

CCCTTTAAACCACCAAGTGGCTTTTACAACGCGCATATACCATCCAACATCAACAGC 240
P F K P P K V A F T T R I Y H P N I N S

AATGGATCGATTGTCTCGATATATTAAGATCTCAGTGGTCGCCAGCATTAACTATTTCA 300
N G S I C L D I L R S Q W S P A L T I S

AAAGTTTTATTATCAATTTGCTCTCTACTCTGTGATCCCAATCCAGACGATCCTCTGT 360
K V L L S I C S L L C D P N P D D P L V

CCAGAGATTGCCAGAATATATAAACCGATCGGGAAAAATACAATGAGCTGGCAGGAGAG 420
P E I A R I Y K T D R E K Y N E L A R E

TGGACTAGAAAGTATGCTATGTGATGCGTTCCAGATTGCAGCAGTCCAACAGAATCAGCA 480
W T R K Y A M

AATCAACATCAACAATAGCAGAAGTAAAGCAGATGCAACAACAACAGTAAAGCAACA 540

PstI
AAGATAACATCAACAGTTTACAATAGTCAGATTCAGAAGAGGAGGAGCTGCAG 593

```

Fig. 1. Nucleotide sequence of a *Xba*I–*Pst*I DNA fragment of the cDNA clone (see Figure 3a, right) containing the *UbcD1* gene and the deduced amino acid sequence of the UbcD1 protein. Nucleotide numbers starting at the first nucleotide of the *UbcD1* coding region are given on the right.

control of developmental processes, we have chosen *Drosophila melanogaster* as a model system. In this paper we describe the gene cloning and functional characterization of a *Drosophila* homologue of the yeast UBC4–UBC5 enzyme pair. *Drosophila* UbcD1 is remarkably similar in structure to the yeast counterpart, showing 80% amino acid sequence identity. By replacing the open reading frame of *UBC4* in the yeast genome by *Drosophila UbcD1* we show that UbcD1 is functionally similar to the yeast UBC4 and UBC5 enzymes, and that the *Drosophila* enzyme also mediates protein degradation.

Results

Cloning of *Drosophila UbcD1*

Ubiquitin-conjugating enzymes are related in sequence, showing at least 30% amino acid sequence identity. Predominantly sequences flanking the essential cysteine of these enzymes are conserved (Jentsch *et al.*, 1990). Primers were designed for a polymerase chain reaction (PCR) which were specific for the UBC1, UBC4, UBC5 enzyme subfamily (see Materials and methods). Using *Drosophila* genomic DNA as a template we amplified a DNA fragment of a size equivalent to the corresponding regions of the yeast genes. Subsequent DNA sequencing suggested that this cloned PCR fragment corresponded to a part of one *Drosophila* gene encoding a ubiquitin-conjugating enzyme.

Using this fragment as a probe we identified and subcloned from a plasmid-borne *Drosophila* oligo(dT) primed library the complete cDNA, which had a size of ~1.5 kb in agreement with the size of the corresponding mRNA (data not shown). We named this gene *UbcD1* (ubiquitin-conjugating enzyme from *Drosophila melanogaster*; the numbering reflects the order of gene identification and does not follow the numbering of yeast *UBC* genes).

UbcD1 is highly similar in sequence to yeast UBC4 and UBC5 enzymes

Sequencing of the *UbcD1* cDNA indicated an open reading frame of 441 bp with the coding capacity for a protein of 16.7 kDa (Figure 1). The initiator methionine was assigned to the first ATG codon preceded by stop codons in all three reading frames. The amino acid sequence of UbcD1 is related to the sequences of all known yeast E2 enzymes (not shown). Unlike the enzymes UBC1 (Seufert *et al.*, 1990), UBC2/RAD6 (Jentsch *et al.*, 1987) and UBC3/CDC34 (Goebel *et al.*, 1988) that possess unrelated carboxy-terminal extensions, UbcD1 is structurally a class I ubiquitin-conjugating enzyme (Jentsch *et al.*, 1990), consisting almost entirely of the conserved ubiquitin-conjugating enzyme domain. UbcD1 is nearly identical in size and remarkably similar in sequence to yeast UBC4 and UBC5 enzymes (Seufert and Jentsch, 1990), sharing 80% and 81% amino acids, respectively (Figure 2). Taking similar amino acids

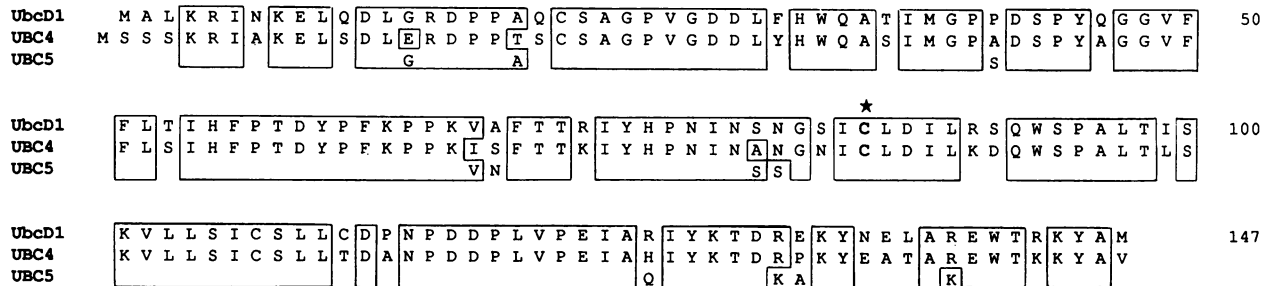


Fig. 2. Amino acid sequence similarity of UbcD1 to yeast UBC4 and UBC5 proteins. Amino acids of UbcD1 identical to UBC4 or UBC5 proteins are boxed. Only those residues of UBC5 which differ from UBC4 are shown. The tentative active-site cysteine of UbcD1 required for thiolester formation with ubiquitin is marked by an asterisk. The numbers on the right give the numbers of amino acids of the UbcD1 protein.

into consideration, the sequence similarity reaches almost 90%, pointing to an unusually strong conservation bias during evolution.

Open reading frame replacement in the yeast genome of UBC4 by Drosophila UbcD1

The striking structural similarity between *Drosophila* UbcD1 and yeast UBC4 and UBC5 led us to suggest that UbcD1 is also functionally equivalent to these yeast enzymes. To test this hypothesis we tested if the *Drosophila* gene when expressed in yeast would complement the phenotypic deficiencies of *ubc4 ubc5* double mutants. Such complementation studies aimed to identify similar *in vivo* functions are usually done using extra-chromosomal plasmid vectors as templates and heterologous promoters for gene expression. However, in such experiments high level expression of more distantly related enzymes might also lead to a complementation if the enzymes are only functionally overlapping. In fact, *UBC1* when expressed from a high-copy number plasmid improves growth and restores temperature resistance of the *ubc4 ubc5* double mutant (Seufert *et al.*, 1990). To avoid any high-copy number effects we decided to integrate the *Drosophila* gene into the yeast genome. To ensure comparable gene expression, the *UBC4* open reading frame was precisely replaced in the yeast genome by the open reading frame of the *Drosophila* UbcD1 cDNA. Using site-directed mutagenesis, a plasmid was constructed which carries the upstream promoter region of *UBC4* fused to the *UbcD1* open reading frame followed by non-coding *UBC4* downstream sequences (Figure 3). Linear DNA bearing such a chimeric gene was used to transform a haploid yeast *ubc4 ubc5* double mutant (strain YWO45) which is unable to grow at 37°C. In this recipient the *UBC4* gene is replaced by a *TRP1* marker. If UbcD1 can functionally replace the yeast UBC4 enzyme recombinants should be phenotypically similar to a *ubc5* single mutant which grows at 37°C. Thus, homologous recombinants at the *UBC4* locus were selected by virtue of their ability to grow at 37°C, without a need for an additional selectable yeast marker. This selection scheme resulted in several independent temperature resistant recombinants. Integration of the *Drosophila* sequences at the *UBC4* locus was indicated by the observed tryptophan auxotrophy and was confirmed by Southern hybridization (not shown).

UbcD1 is functionally similar to yeast UBC4 and UBC5 enzymes

Yeast cells deficient in the enzyme pair UBC4 and UBC5, grow at a markedly reduced rate at normal temperatures and

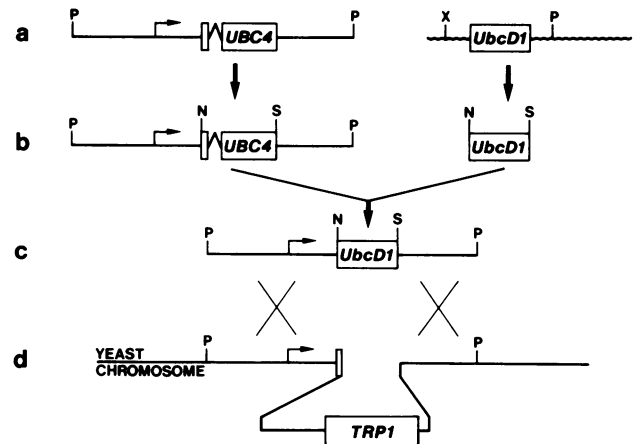


Fig. 3. Precise gene replacement of the *UBC4* open reading frame by the *UbcD1* open reading frame. (a) A genomic *PstI*(P)–*PstI* fragment carrying the intron-containing *UBC4* open reading frame driven by the *UBC4* promoter (arrow) and the *Drosophila* cDNA (wavy line) bearing the *XbaI*(X)–*PstI* *UbcD1*-containing fragment shown in Figure 1 were used for site-directed mutagenesis. (b) *NcoI* (N) and *SpeI* (S) sites were introduced directly in front and after the open reading frames of *UBC4* and *UbcD1* (see Materials and methods for details) and used (c) to construct a chimeric *UbcD1* gene flanked by *UBC4* upstream and downstream sequences. (d) This construct was used to transform a yeast *ubc4 ubc5* double mutant where the *UBC4* gene is replaced by the *TRP1* marker. By homologous recombination (large crosses) via identical flanking sequences a yeast recombinant was generated with the *Drosophila* *UbcD1* gene integrated into the yeast genome, precisely at the position of the original *UBC4* gene.

are unable to grow at elevated temperatures (Seufert and Jentsch, 1990; Seufert *et al.*, 1990). In contrast, *ubc4* or *ubc5* single mutants show only moderate growth defects and are able to grow even at heat-shock temperatures. In particular, *ubc5* mutants are almost devoid of any functional defect as long as a functional copy of *UBC4* is present (Seufert and Jentsch, 1990). As shown in Table I and Figure 4, the recombinant *ubc4 ubc5* double mutant, in which the expression of the *Drosophila* UbcD1 gene is driven by the *UBC4* promoter, were phenotypically similar to *ubc5* mutants. They grew at rates near to wild type cells and were viable at elevated temperatures. This result suggests that the *Drosophila* enzyme functions in yeast cells during normal growth and during the stress response similar to the UBC4 enzyme.

UBC4 and UBC5 enzymes are known to mediate *in vivo* the formation of high mol. wt ubiquitin–protein conjugates (Seufert and Jentsch, 1990). Such conjugates are most likely multi-ubiquitinated proteins which are thought to be necessary intermediates of ubiquitin-dependent protein

Table I. Phenotypes of *ubc* mutants and *UbcD1*-expressing *ubc* mutants

	Doubling time (h) ^a	Resistance to canavanine (%) ^b
wild type	1.5	82
<i>ubc4</i>	2.0	18
<i>ubc5</i>	1.5	79
<i>ubc4 ubc5</i>	6.2	<0.02
<i>ubc4::UbcD1 ubc5</i>	1.8	67

^aCells were grown in YPD liquid medium at 30°C. OD₆₀₀ was followed for determination of doubling times.

^bResistance to canavanine was determined with cells grown in SD medium containing required nutrients. Appropriate aliquots were spread on supplemented SD plates with or without canavanine at 1.5 µg/ml. Resistance is given as the fraction of colonies formed in the presence of the amino acid analogue.

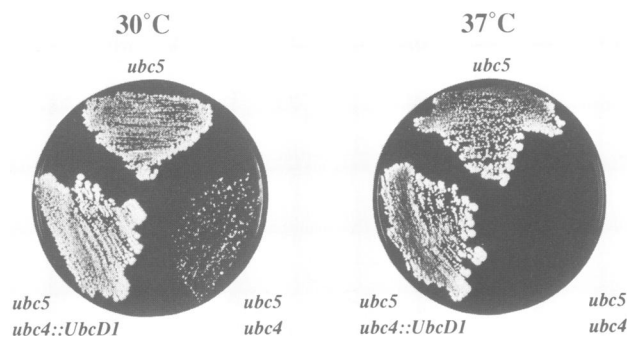


Fig. 4. Growth on plates of yeast *ubc5*, *ubc5 ubc4* double mutants and *UbcD1*-expressing double mutants (*ubc5 ubc4::UbcD1*) at normal growth temperature (30°C) and heat-shock temperature (37°C).

degradation (Chau *et al.*, 1989). Most high mol. wt conjugates which are detectable in wild type and *ubc4* and *ubc5* single mutant cells are absent in *ubc4 ubc5* double mutants and free ubiquitin is accumulating instead (Seufert and Jentsch, 1990). This defect was cured by the expression of the *Drosophila* enzyme mediates the multi-ubiquitination of proteolytic substrates, suggesting that the enzyme functions in a mechanistically similar fashion to the yeast UBC4 and UBC5 enzymes.

UBC4 and UBC5 enzymes mediate the degradation of naturally short-lived and abnormal proteins (Seufert and Jentsch, 1990). *ubc4 ubc5* double mutants and *ubc4* single mutants, not however, *ubc5* single mutants, are hypersensitive to canavanine (Seufert and Jentsch, 1990). This arginine analogue gets incorporated *in vivo* into proteins and results in abnormal proteins. The *ubc4 ubc5* double mutant expressing *Drosophila UbcD1* is canavanine resistant like wild type (or *ubc5*) cells, indicating that UbcD1 mediates the degradation of abnormal proteins in yeast.

To determine the turnover of canavanyl proteins in yeast wild type, mutant and rescued mutant cells, proteins of canavanine treated cells were pulse-labelled and the release of radioactivity from these cells during chase was measured. Degradation of abnormal proteins was severely affected in *ubc4 ubc5* double mutants and significantly reduced in *ubc5* single mutants (Seufert and Jentsch, 1990). *UbcD1* expression in the *ubc4 ubc5* double mutant rescued the proficiency to degrade canavanyl proteins almost to the level

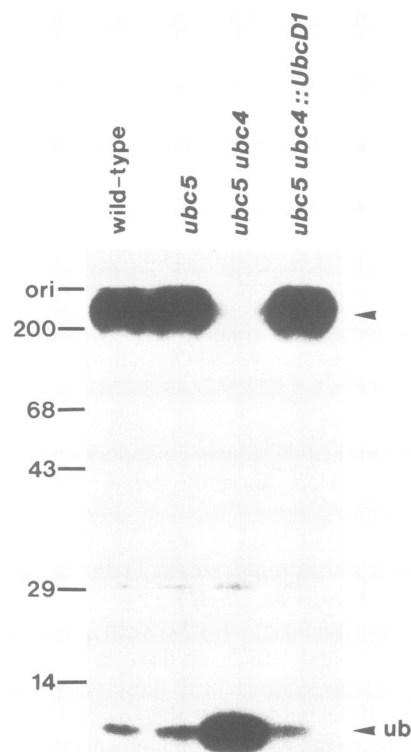


Fig. 5. Western blot analysis of ubiquitin and ubiquitin protein conjugates in wild type, *ubc5*, *ubc4 ubc5* double mutant and *UbcD1*-expressing *ubc4 ubc5* double mutant (*ubc4::UbcD1 ubc5*) cells. Proteins were prepared from exponentially growing cells. Identical amounts of protein (10 µg/lane) were separated on an 18% SDS-polyacrylamide gel, and after transfer reacted with an anti-ubiquitin serum. The blot was developed with [¹²⁵I]protein A followed by autoradiography. Free ubiquitin (ub) and high mol. wt ubiquitin-protein conjugates are indicated by arrows. Other bands may represent stable ubiquitin-protein conjugates (Seufert and Jentsch, 1990). Mol. wts of marker proteins are given in kDa and the origin of the gel (ori) is indicated.

of the *ubc5* single mutant (Figure 6). Together, these data strongly imply that the *Drosophila* ubiquitin-conjugating enzyme UbcD1 is structurally, functionally and mechanistically similar to the yeast enzymes UBC4 and UBC5 and that UbcD1 also mediates protein degradation.

Discussion

A major pathway for selective protein degradation of eukaryotic cells is mediated by the ubiquitin system (for a review, see Hershko, 1991). The involvement of a post-translational protein modification system is thought to provide the proteolytic pathway with fidelity and control features to secure the remarkable selectivity and temporal control of protein breakdown. Members of the large family of ubiquitin-conjugating enzymes (UBC enzymes) are primarily responsible for the substrate selectivity and the functional diversity of this system (Jentsch *et al.*, 1990). Previously, we have shown that the closely related enzyme pair UBC4 and UBC5 of *S.cerevisiae* mediate the degradation of a large fraction of naturally short-lived and abnormal proteins and that these enzymes become essential under stress conditions (Seufert and Jentsch, 1990).

This work characterizes *UbcD1*, a *Drosophila* gene,

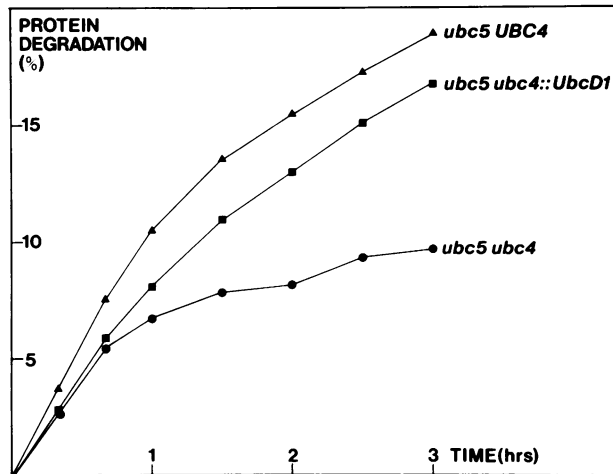


Fig. 6. Protein turnover in *ubc5* (triangles), *ubc5 ubc4* double mutants (dots) and *UbcD1*-expressing *ubc5 ubc4* mutants (squares). Cells were pretreated for 90 min with canavanine at 20 μ g/ml and labelled for 5 min to determine canavanine-protein turnover. Protein degradation is given as the fraction of total incorporated radioactivity released from cells during the chase period.

coding for a ubiquitin-conjugating enzyme which is structurally and functionally similar to the yeast UBC4 and UBC5 enzymes. The primary structure of this enzyme class is remarkably conserved, showing 80% identical amino acid residues between the *Drosophila* and yeast enzymes. When expressed in yeast, *Drosophila UbcD1* can substitute the function of the yeast *UBC4* gene. *UbcD1* was integrated into the yeast genome by replacing the *UBC4* open reading frame, such that *UbcD1* expression was under the control of *UBC4* regulatory sequences. In these recombinants growth, temperature resistance, canavanine resistance, and proteolysis proficiency was restored close to the levels of *UBC4*-expressing cells. The high degree of conservation of the *UbcD1-UBC4-UBC5* protein class suggests a strong evolutionary bias on the structural and functional integrity of this enzyme. This differs from UBC2/RAD6-like proteins which mediate more specific functions, including DNA repair, induced mutagenesis and sporulation. These enzymes are less conserved and can replace each other only partially, even when expressed from high-copy number plasmids (Reynolds *et al.*, 1990; Koken *et al.*, 1991).

UbcD1, *UBC4* and *UBC5* probably depend on E3-proteins for substrate recognition (class I enzymes; Jentsch *et al.*, 1990). Since these enzymes mediate a large portion of cellular protein degradation (Seufert and Jentsch, 1990) this enzyme class (in contrast to RAD6-like enzymes) might have to interact with a large set of different E3-proteins with distinct substrate specificities. Such multiple protein-protein interactions might impose structural constraints on these enzymes leading to their remarkable conservation.

During development of an organism cells undergo changes in the content and concentration of cellular proteins which are not only controlled by gene expression but also by the regulated breakdown of proteins. Cell cycle control (Glotzer *et al.*, 1991), cell differentiation (Hochstrasser *et al.*, 1991) and possibly cell proliferation (Scheffner *et al.*, 1990) are mediated in part through ubiquitin-dependent proteolysis. Studies on the role of protein degradation in the early development of *Drosophila* are attractive because morphogens that determine the body plan of an organism,

such as the protein bicoid, have tightly controlled half-lives (Driever and Nüsslein-Volhard, 1988). The use of specific probes and antibodies to monitor expression of the *UbcD1* protein may help to define the role of ubiquitin-dependent protein degradation during development.

Materials and methods

Gene cloning and analysis

To clone *Drosophila* genes related to the yeast *UBC1-UBC4-UBC5* gene family we used inosine (I) containing PCR primers *MT3* (5'-GGG AAT TCG GIC CII CIG A(C/T)(A/T)(G/C)IC C(G/A/T/C)T A-3') and *MT2* (5'-ATT CTA GA(G/C)(A/G)T(T/C)(T/C)TT IA(G/A) IAT (G/A)TC IA(G/A) (GA)CA-3') corresponding to *UBC4/UBC5*-specific amino acid sequences GPADSPYAGG and CLDILKD, respectively. PCR reaction was carried out using 2.5 units of *Taq* DNA Polymerase (AmpliTaq, Perkin-Elmer Cetus) per 100 μ l sample and a thermocycler (Perkin-Elmer Cetus). The reaction buffer contained 67 mM Tris-HCl pH 8.8, 2 mM $MgCl_2$, 0.2% Tween 20, 1 mM mercaptoethanol and 0.2 mg/ml gelatine. The nucleotide concentration was 200 μ M each; primer concentration was 5 μ M. To reduce yeast DNA contamination the reaction vial was irradiated at 254 nm for 10 min. Genomic *D. melanogaster* Oregon P2 DNA was restricted with *EcoRI*, *Sall* and *XbaI* (Boehringer Mannheim, Germany), pooled and 20 ng/ μ l reaction vol was used as a template for the PCR reaction. The reaction was done with 40 cycles of 1.5 min at 94°C, 3 min at 40°C, and 2 min at 72°C. Reaction products were separated on a 6% polyacrylamide gel and DNA from bands of correct sizes were eluted, restricted with *EcoRI* and *XbaI* and subcloned into M13mp18/19 vectors (Yanisch-Perron *et al.*, 1985). A ^{32}P -labelled isolated insert fragment was used to screen pNB40-based plasmid library carrying cDNA inserts from an oligo(dT) primed *Drosophila* RNA from 0–24 hour-old embryos (kindly provided by Dr Kafatos). Positive clones were purified and the *EcoRI-HindIII* *Drosophila* insert was subcloned into M13mp18/19 vectors. DNA sequencing was carried out with a Sequenase kit (US Biochemicals). Both strands were sequenced completely with sufficient overlaps.

Precise gene replacement

Using the 'gapped duplex DNA approach' of site-directed mutagenesis (Kramer *et al.*, 1984) and a mutagenesis kit (Boehringer Mannheim) a *NcoI* restriction site was created at the initiator ATG codon (CCATGG) and a *SpeI* site downstream of the TAA stop codon (TAAACACTAGT) of the *UBC4* gene (Seufert and Jentsch, 1990). Similar *NcoI* and *SpeI* restriction sites were placed precisely in front and after the open reading frame of the *UbcD1* gene by a PCR reaction (Figure 3b). The nucleotide sequence of PCR-amplified gene fusions were confirmed by DNA sequencing. After the exchange of the *NcoI-UBC4-SpeI* open reading frame fragment with the *NcoI-UbcD1-SpeI* open reading frame fragment, linear DNA carrying the *UbcD1* gene and *UBC4* upstream and downstream sequences of ~1000 bp and 700 bp, respectively, was used to transform haploid yeast *ubc4 ubc5* double mutant cells (strain YWO45). *UbcD1* recombinants were selected by growth at 37°C on YPD medium. Since *UBC4* was disrupted with a yeast *TRP1* marker (strain YWO45) *UbcD1* recombinants acquired tryptophan auxotrophy.

Yeast strains and techniques

Wild type cells and mutant cells are haploid *MATa* strains congenic to DF5 (Finley *et al.*, 1987). *ubc4* (YWO14), *ubc5* (YWO18) and *ubc4 ubc5* (YWO22) are *MATa* derivatives of YWO13, YWO17 and YWO23, respectively (Seufert *et al.*, 1990). YWO45 used as a recipient for *UbcD1* transformation is similar to YWO22, however, *UBC4* was replaced by a *TRP1* marker (Figure 3; unpublished data). YWO56 is the resulting recombinant (*ubc4::UbcD1*, *ubc5::LEU2*). Standard protocols (Rose *et al.*, 1990) were followed for growth of yeast cells, and transformation. Rich medium (YPD) and minimal medium (SD) are standard media (Rose *et al.*, 1990).

Anti-ubiquitin serum and Western analysis

For preparation of proteins yeast cells were harvested, resuspended in 100 mM Tris-HCl, pH 7.5, 10 mM Na-EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and vortexed with an equal vol of glass beads for 3 min. For Western analysis proteins were separated on 18% SDS-polyacrylamide gels and electrophoretically blotted onto PVDF membrane (Millipore). Blotted proteins were probed with anti-ubiquitin serum (Seufert and Jentsch, 1990) diluted 1:100 in 20% fetal calf serum, 1 \times PBS, washed with 1 \times PBS containing 0.2% Brij 58, and developed with [^{125}I]protein A (5 μ Ci per blot; Amersham).

Measurement of protein turnover

Cells growing exponentially in SD-medium supplemented with required nutrients were harvested and resuspended at $OD_{600} = 2.5$. Cells were pretreated with canavanine (20 $\mu\text{g/ml}$) for 90 min and then labelled for 5 min with [^{35}S]methionine (75 $\mu\text{Ci/ml}$ culture medium). Cells were washed and incubated in growth medium (chase) containing methionine (40 $\mu\text{g/ml}$) and cycloheximide (0.5 mg/ml). Samples of total culture and culture supernatant were withdrawn at given time intervals, spotted on nitrocellulose filters and radioactivity was determined by liquid scintillation counting. Protein degradation is given as the fraction of total incorporated radioactivity released from cells during the chase period. Determination of radioactivity becoming soluble in 10% TCA yielded identical results. No radioactivity soluble in 10% TCA accumulated in wild type or mutant cells during the chase period. The labelling efficiencies of wild type and mutant cells were essentially the same.

Acknowledgements

We thank Dr Manfred Frasch for his help and advice to screen the *Drosophila* plasmid library. We are grateful to Ute Ehringer for technical assistance and to Dr Günther Gerisch and Hans-Peter Hauser for comments on the manuscript. This work was partially supported by a grant to S.J. by the Deutsche Forschungsgemeinschaft (Je 134/2-2).

References

- Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K. and Varshavsky, A. (1989) *Science*, **243**, 1576–1583.
- Driever, W. and Nüsslein-Volhard, C., (1988) *Cell*, **54**, 83–93.
- Finley, D., Özkaynak, E. and Varshavsky, A. (1987) *Cell*, **48**, 1035–1046.
- Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991) *Nature*, **349**, 132–138.
- Goebel, M.G., Yochem, J., Jentsch, S., McGrath, J.P., Varshavsky, A. and Byers, B. (1988) *Science*, **241**, 1331–1335.
- Hershko, A. (1991) *Trends Biochem. Sci.*, **16**, 265–268.
- Hochstrasser, M., Ellison, M.J., Chau, V. and Varshavsky, A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4606–4610.
- Jentsch, S., McGrath, J.P. and Varshavsky, A. (1987) *Nature*, **329**, 131–134.
- Jentsch, S., Seufert, W., Sommer, T. and Reins, H.-A. (1990) *Trends Biochem. Sci.*, **15**, 195–198.
- Jentsch, S., Seufert, W. and Hauser, H.-P. (1991) *Biochim. Biophys. Acta*, **1089**, 127–139.
- Koken, M., Reynolds, P., Bootsma, D., Hoeijmakers, J., Prakash, S. and Prakash, L. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3832–3836.
- Kramer, W., Druetsa, V., Jansen, H.-W., Kramer, B., Pflugfelder, M. and Fritz, H.-J. (1984) *Nucleic Acids Res.*, **12**, 9441–9456.
- McGrath, J.P., Jentsch, S. and Varshavsky, A. (1991) *EMBO J.*, **10**, 227–236.
- Picologlou, S., Brown, N. and Liebmann, S.W. (1990) *Mol. Cell. Biol.*, **10**, 1017–1022.
- Rose, M., Winston, F. and Hieter, P. (1990) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Reynolds, P., Koken, M.H.M., Hoeijmakers, J.H.J., Prakash, S. and Prakash, L. (1990) *EMBO J.*, **9**, 1423–1430.
- Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J. and Howley, P.M. (1990) *Cell*, **63**, 1129–1136.
- Schwartz, L.M., Myer, A., Kosz, L., Engelstein, M. and Maier, C. (1990) *Neuron*, **5**, 411–419.
- Seufert, W. and Jentsch, S. (1990) *EMBO J.*, **9**, 543–550.
- Seufert, W., McGrath, J.P. and Jentsch, S. (1990) *EMBO J.*, **9**, 4535–4541.
- Shanklin, J., Jabben, M. and Vierstra, R.D. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 359–363.
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene*, **33**, 103–119.

Received on August 23, 1991; revised on October 9, 1991

Note added in proof

The *UbcD1* sequence has been deposited in the EMBL/GenBank/DBJ nucleotide sequence databases under the accession number X62575.