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An essential ubiquitin-conjugating enzyme with tissue and developmental specificity in the nematode *Caenorhabditis elegans*

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The *ubc-2* gene in *Caenorhabditis elegans* encodes a ubiquitin-conjugating enzyme (E2) homologous to yeast UBC4 and UBC5. UBC4 and UBC5 are individually dispensable class I E2 enzymes involved in the degradation of short-lived and abnormal proteins. Transgenic analysis using *ubc-2–lacZ* fusions and *in situ* immunofluorescence indicate that *ubc-2* is abundantly expressed in most tissues of embryos and early larvae, but becomes specific to the nervous system in L4 larvae and adults. This suggests that the functions of this type of E2 are developmentally regulated in *C.elegans*. This hypothesis is supported by antisense analysis, which shows that blocking the expression of *ubc-2* has a more severe effect in early developmental stages than in later stages. Through complementation of previously identified essential genes in the vicinity of *ubc-2*, we demonstrate that *ubc-2* corresponds to *let-70*, a gene essential for *C.elegans* larval development. One *let-70(ubc-2)* allele contains a His75→Tyr substitution, while another has an altered splice donor site.

Keywords: *C.elegans*/E2/*let-70(ubc-2)*/ubiquitin-conjugating enzyme

Introduction

The small, highly conserved protein ubiquitin serves as a tag to mark intracellular proteins for degradation by the proteasome, and probably for other functions (Rechsteiner, 1988; Hershko and Ciechanover, 1992; Jentsch, 1992a). Ubiquitin functions through its covalent attachment to target proteins via isopeptide bonds, a process which involves activation of its C-terminal by a ubiquitin-activating enzyme (E1), and transfer to ε amino groups of lysyl residues on the target, catalyzed by ubiquitin-conjugating enzymes (UBCs or E2s). By regulating the half-lives of key proteins in eukaryotic cells, the ubiquitin conjugation system has been shown to play roles in a wide variety of cellular processes such as DNA repair (Jentsch, 1992b), cell cycle control (Goebl *et al.*, 1988; Seufert *et al.*, 1994), yeast mating type control (Hochstrasser *et al.*, 1991; Chen *et al.*, 1993), yeast sporulation (Jentsch *et al.*, 1987), peroxisome biogenesis (Wiebel and Kunau, 1992), antigen processing (Driscoll and Finley, 1992; Michalek *et al.*, 1993), cadmium resistance (Jungmann *et al.*, 1993) and the stress response (Seufert and Jentsch, 1990). Multiple ubiquitin-conjugating enzymes (E2s) have been isolated from all eukaryotes studied to date (Bartling *et al.*, 1993; Girod and Vierstra, 1993; Mahaffey *et al.*, 1993). The system has been most thoroughly studied in yeast, where 10 E2s have been cloned and show surprisingly diverse functions (for reviews, see Jentsch, 1992a; Ciechanover, 1994). *ubc-2* was the first ubiquitin-conjugating enzyme gene described in the nematode *Caenorhabditis elegans* (Zhen *et al.*, 1993). Its amino acid sequence shares a high degree of similarity with that of the yeast E2 enzymes UBC4 and UBC5. When expressed in a yeast *ubc4/5* null mutant background, *ubc-2* complements the growth deficiency as well as the temperature-sensitive phenotype (Zhen *et al.*, 1993). This suggests that *ubc-2* also shares similar functions with its yeast homolog, namely the degradation of short-lived and abnormal proteins (Seufert and Jentsch, 1990). An important mating type control protein in yeast, MATα2, has been identified as one of the substrates subject to UBC4-mediated degradation (Hochstrasser *et al.*, 1991; Chen *et al.*, 1993). The mammalian tumor suppressor protein p53 has also been shown to be degraded specifically through a UBC4-related ubiquitin pathway (Scheffner *et al.*, 1993; Seufert *et al.*, 1994; Shkedy *et al.*, 1994). In *Xenopus*, cyclin B ubiquitination and subsequent degradation is mediated by an E2 homologous to yeast UBC4 (Mahaffey *et al.*, 1993; King *et al.*, 1995). Despite the above progress, very little is known about the functions, substrate specificities or tissue distributions of ubiquitin-conjugating enzymes in multicellular animals. Here, we examine the temporal and spatial expression patterns of *C.elegans ubc-2* using transgenic and immunochemical approaches. The effects of *ubc-2* antisense RNA expression on *C.elegans* development are also described, and it is shown that *ubc-2* corresponds to *let-70*, a gene essential for larval development in *C.elegans*.

Results

Construction of ubc-2–lacZ fusions and the expression of the transgene in the C.elegans life cycle

All *ubc-2–lacZ* translational fusion constructs are illustrated in Figure 1. Details of the construction of these fusions are given in Materials and methods. Each construct was injected as a circular plasmid together with a selection marker pRF4 (*su1006*) into *C.elegans* gonads, and extrachromosomal transgenic lines were established. The expression of β-galactosidase in all lines was assayed by histochemical staining with X-gal. Among these constructs, pZMI.1 and pZMII.1 gave similar expression patterns and the strongest, most tissue-general staining. Two integrated lines were therefore generated from a line

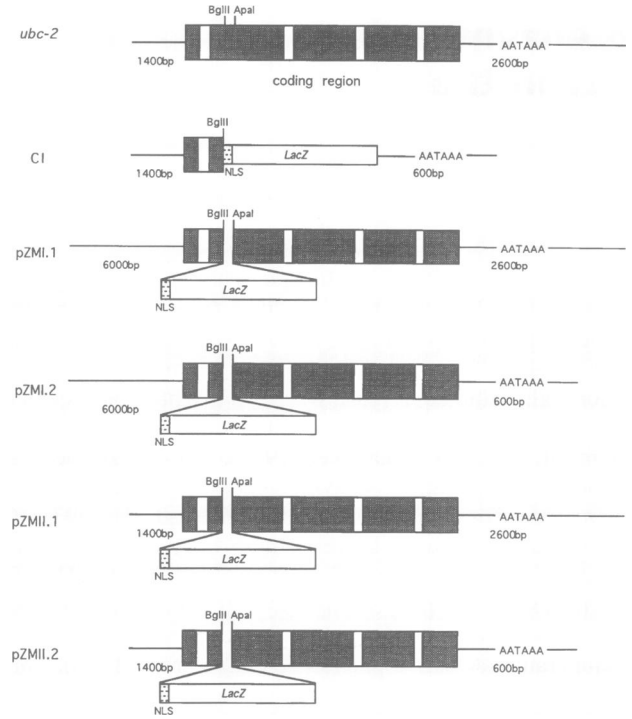

Fig. 1. Construction of *ubc-2–lacZ* transgenes. With the exception of CI, all constructs consist of the complete *ubc-2* coding region with varying 5′- and 3′-non-coding sequences, and the *E.coli lacZ* gene inserted in-frame into the second exon of *ubc-2*. CI includes 5′- and 3′-non-coding sequences of *ubc-2*, but only the first 12 residues of the coding region, as well as the first intron. Shaded rectangles represent *ubc-2* exons, while clear rectangles indicate introns. NLS is the SV40 nuclear localization signal.

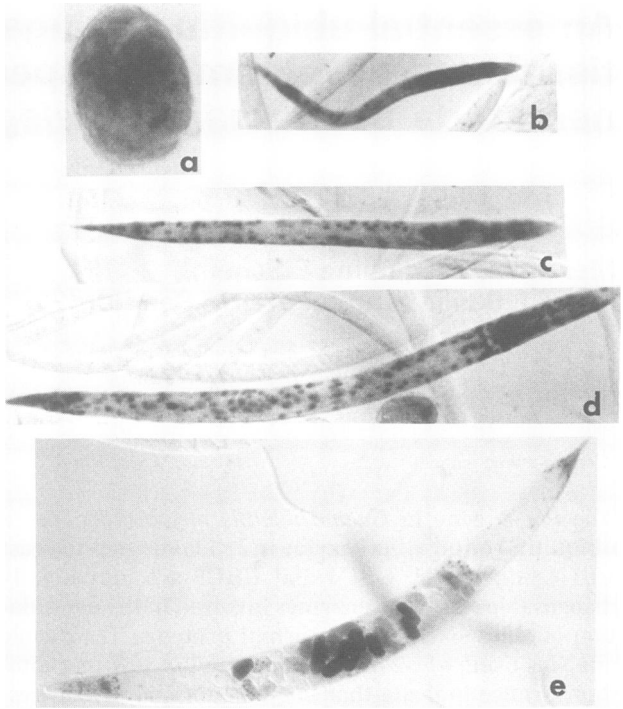

Fig. 2. *lacZ* expression of the pZMI.1 and pZMI.2 transgene in different stages of *C.elegans*. (**a**) Comma stage embryo. Note X-gal staining throughout the embryo. Magnification 500×. (**b**), (**c**) and (**d**) Staining in L1, L2 and L3 larvae respectively. Blue precipitate is seen in body muscle, hypodermal, neural and some intestinal nuclei. Magnification 500×. (**e**) Adult animal showing specific expression in the nervous system and in a few hypodermal cells in the head and tail, and strong expression in embryos. Magnification 100×.

carrying pZMI.1 and pRF4 mixed extrachromosomal arrays.

Analysis of the transgenic lines carrying pZMI.1 or pZMII.1 extrachromosomal arrays and the pZMI.1 integrated lines showed that β-galactosidase was expressed in embryos from gastrulation onward, and in the larval, dauer larval and adult stages (Figure 2). X-gal staining was very intense, suggesting that *ubc-2* expression is driven by a strong promoter. Staining of embryos and L1 larvae was visible within 2 h, and was saturated in 5 h. Staining in L4 larvae and young adults was first visible after 3 h, whilst older adults began to show staining after 4–5 h.

The expression of ubc-2–lacZ transgenes is tissue-general in early larvae but is restricted mainly to the nervous system in adults

The expression of UBC-2–β-galactosidase fusion protein from pZMI.1 and pZMII.1 was observed consistently in embryos, larvae and adults. β-Galactosidase was expressed in most cells in the embryos (Figure 2a). In L1, L2, L3 and dauer larvae, most somatic tissues including neurons, pharynx, hypodermis and body muscle were intensely stained. In a small percentage of animals, intestinal cells were also stained (Figure 2b–d). At the onset of the L4 stage, staining was more restricted to neurons, pharynx and hypodermis. β-Galactosidase staining was seen only in the nervous system in adults.

ubc-2 is expressed in the nervous system at all post-embryonic stages

An interesting feature of the expression patterns from pZMI.1 and II.1 is that, despite differing tissue specificities in larval and adult stages, *ubc-2–lacZ* expression is seen constitutively in the nervous system at all post-embryonic stages. From L1 onward, intense β-galactosidase staining was observed in the ventral nerve cord, including P-cells and neurons, pharyngeal ganglia and retrovesicular ganglia (Figures 2 and 3).

To determine if the expression patterns of the above transgenes resembled those of endogenous *ubc-2*, the localization of UBC-2 protein was examined by *in situ* immunofluorescence staining. Rabbit polyclonal antibodies were raised against a UBC-2 fusion protein expressed in *Escherichia coli*. The anti-UBC-2 antiserum was affinity purified on a UBC-2 column, and the specificity of the affinity-purified antibodies was verified by Western blot analysis. In *C.elegans* extracts the antiserum reacted with a single polypeptide of the size expected for UBC-2. Other *C.elegans* UBCs, such as UBC-1 (Leggett *et al.*, 1995) and several identified by the genome sequencing project (Sulston *et al.*, 1992), are distinctly larger than UBC-2. Furthermore, the anti-UBC-2 antiserum recognizes the yeast homolog UBC4, but not the related yeast UBC5 (data not shown). Thus, although the existence of another *C.elegans* UBC very similar in size and sequence to UBC-2 cannot be ruled out entirely, these data suggest that the antiserum is specific for UBC-2. The affinity-purified antiserum was used for *in situ* immunofluorescence staining of *C.elegans*. Figure 4 clearly shows that this antibody specifically reacts with the nervous sytem, including the ventral nerve cord and pharyngeal ganglia. Staining was also seen in the retrovesicular ganglia in

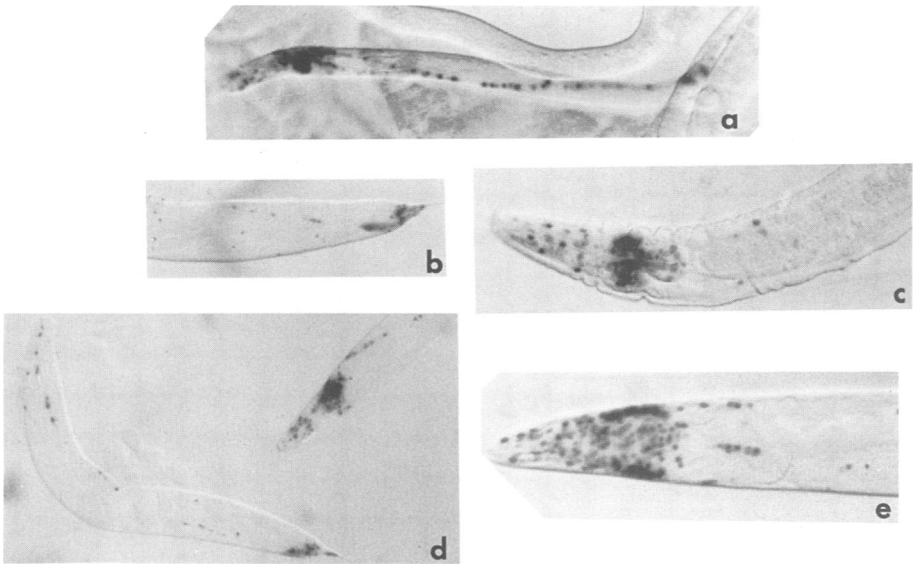

Fig. 3. *lacZ* expression of the pZMI.1 and pZMI.2 transgenes in the nervous system. Magnification 100× (a, b and d), 200× (c and e). (**a**) L4 larva carrying the pZMI.1 or pZMI.2 transgene. Staining is seen in ventral nerve cord, pharyngeal ganglia and retrovesicular ganglia. A few hypodermal cells in the head and tail are also stained. (**b–e**) X-gal staining in adults carrying pZMI.1 or pZMI.2. The same staining pattern as in (a) can be observed.

adults (not shown). Interestingly, staining was seen not only in the ventral nerve cord, but also in the nerve bundles connecting the ventral and dorsal cords.

Thus, both the transgenic analysis and *in situ* immunofluorescence data indicate that *ubc-2* is expressed specifically in the nervous system in *C.elegans* adults.

Both the coding and 3′-non-coding sequences of ubc-2 are important for transgene expression

The β-galactosidase expression levels of the five constructs in *C.elegans* were compared. CI is an in-frame translational fusion of the *lacZ* coding region with the second exon of *ubc-2*, and contains 1.4 kb of the *ubc-2* 5′-non-coding sequence, extending to codon 12 (Asp) of the coding region. A polyadenylation signal is contained within a 0.6 kb 3′-non-coding region. In constructs pZMI.1, pZMI.2, pZMII.1 and pZMII.2, the *lacZ* coding region was inserted in-frame into the second exon of the *ubc-2* genomic clone. These constructs all contain the complete *ubc-2* coding sequence, including five exons and four introns, but differ in the extent of their 5′- and 3′-non-coding sequences, as described in Materials and methods. CI failed to produce β-galactosidase, pZMI.2 and pZMII.2 were weakly expressed only in nerve ganglia around the pharynx (data not shown), while pZMI.1 and pZMII.1 showed the strongest and most tissue-general staining pattern (Figure 2). This suggests that both the coding region and distal 3′-non-coding sequences of *ubc-2* are required for the proper expression of the transgene.

Expression of ubc-2 antisense RNA results in significant larval lethality and arrested development

To determine if *ubc-2* is required for *C.elegans* development, we attempted to interfere with its expression using antisense RNA expression. Two antisense constructs and two sense constructs were made (Figure 5). ANTI-I contains an inversion of the entire *ubc-2* cDNA under control of the *hsp16-2* promoter, which drives expression

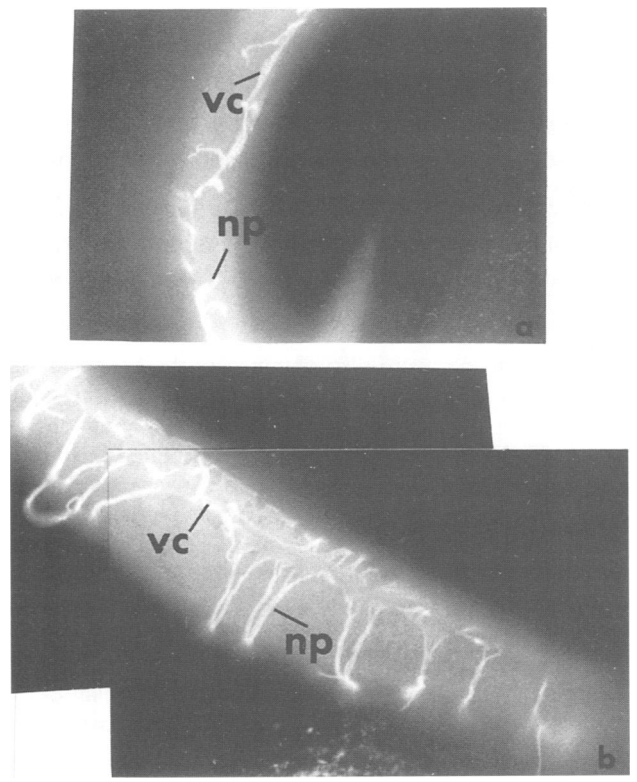

Fig. 4. *In situ* immunofluorescence staining of adults with polyclonal antibodies against UBC-2 protein. Staining is seen in the ventral nerve cord (**a**) and also in neuronal processes connecting the ventral and dorsal cords (**b**). Magnification 600×.

in all somatic tissues at temperatures higher than 29°C (Stringham *et al.*, 1992). Construct *hsp–ubc-2* is similar to ANTI-I except that the *ubc-2* cDNA was placed in the sense orientation. ANTI-II was constructed directly from the *ubc-2* genomic clone, and contains the complete *ubc-2* genomic sequence with a large part (nucleotides 658–1514, numbering from Zhen *et al.*, 1993) of the coding region inverted.

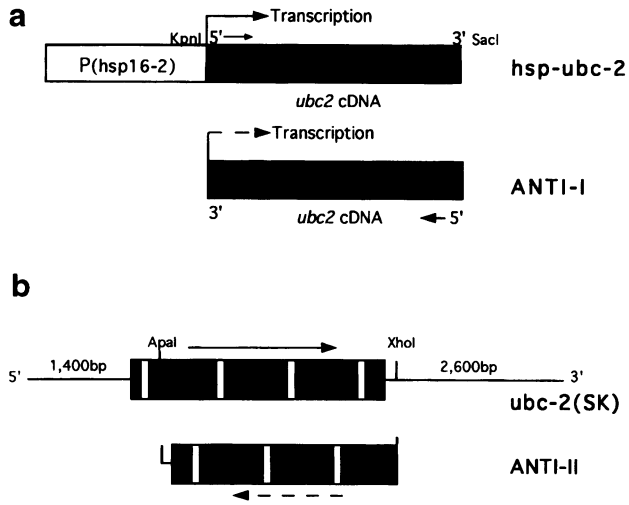

Fig. 5. Construction of *hsp–ubc-2*, *ubc-2*(SK), ANTI-I and ANTI-II. (**a**) A 1.0 kb *Kpn*I–*Sac*I fragment containing the complete *ubc-2* cDNA sequence was inserted into pPD16.49 in two orientations. The *hsp16-2* promoter in this construct drives the expression of antisense RNA under heat-shock conditions. (**b**) ANTI-II was derived directly from a *ubc-2*(SK) genomic clone. The *Apa*I–*Xho*I fragment was blunted and inverted. Shaded regions represent exons, while clear regions indicate introns.

Each of the four constructs was injected into a *rol-6* null strain along with the pRF4 marker. No transformants were obtained using ANTI-II, at concentrations of 40–200 μg/ml, and injected animals gave rise to dead larvae and unhatched embryos. Injecting the original *ubc-2* genomic construct at the same concentrations resulted in normal numbers of transformants (20–30 rollers per successful injection).

Transformants were also obtained at the expected frequency with *hsp–ubc-2* and ANTI-I, and several stable extrachromosomal lines were established. From one of the ANTI-I extrachromosomal lines, integrated lines were derived. The expression of sense and antisense *ubc-2* was induced by heat-shocking worms carrying either construct at 30°C for 2 h. Upon heat-shock, *ubc-2* transcript levels increased dramatically in worms carrying the *hsp–ubc-2* (sense) construct, confirming that the heat shock promoter functioned as intended (Figure 6a). Western blot analysis showed that, after heat-shock, UBC-2 protein levels decreased by at least 50% in worms carrying the antisense construct (Figure 6b). Antisense RNA expression was then induced at various developmental stages, and the development of the worms was monitored. The results are summarized in Table I. When *ubc-2* expression was inhibited in the embryonic or early larval stages (L1 and L2), 65% of the animals died within 1 day, and the remaining 35% developed into adults within 4 days. In contrast, worms of the parental strain subjected to the same treatment all developed into adults within 2 days. When antisense RNA expression was induced in later larval stages (L3 and L4), most of the animals survived and developed into adults, although their developmental time was lengthened by 2 days relative to identically treated control worms. Expression of antisense RNA in adults produced no apparent effects.

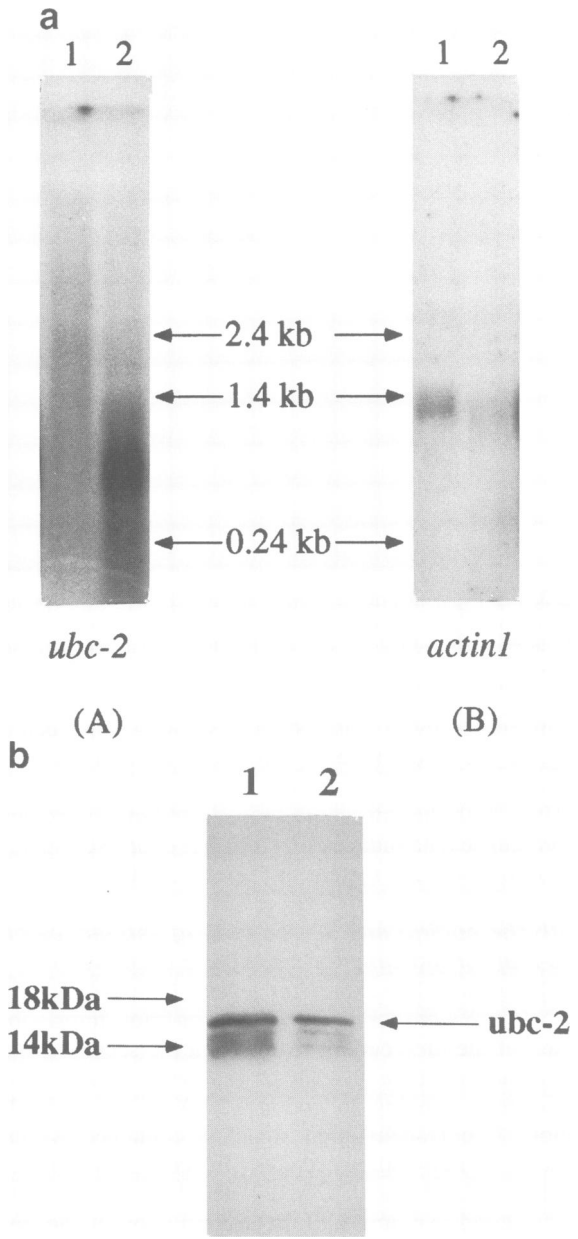

Fig. 6. (**a**) Northern blot analysis of strains carrying *hsp–ubc-2* before and after heat-shock treatment. Total cellular RNA was prepared from 200 worms carrying *hsp–ubc-2* both before and after heat shock treatment, separated on a 1% formaldehyde–agarose gel, and subjected to Northern blot analysis. Lane 1 (in both panels), RNA prepared from worms before heat-shock; lane 2 (both panels), RNA prepared after heat-shock for 2 h at 30°C. A M13 ssDNA probe specific to *ubc-2* sense transcripts was hybridized with the blot (A). The same blot was stripped and probed with an *actin1*-specific probe to check the RNA loading (B). (**b**) Western blot analysis of strains carrying ANTI-I before and after heat-shock treatment. Protein lysate was prepared from worms carrying the ANTI-I construct before and after heat-shock treatment (2 h, 30°C) and separated by electrophoresis on a 12.5% SDS–polyacrylamide gel. ECL Western blotting was carried out using UBC-2 antibodies. Lane 1, extract from worms before heat-shock; lane 2, extract from worms after heat-shock. The total amount of protein loaded in both lanes was similar as determined by A_{280} reading and Coomassie blue staining (not shown).

ubc-2 corresponds to let-70, an essential C.elegans gene

ubc-2 was mapped previously to the right arm of chromosome IV. (Shown in ACEDB; Richard Durbin and Jean

Table I. The effect of *ubc-2* antisense RNA expression on the development of *C.elegans*

Stage of heat shock	*rol-6* null	*rol-6* null carrying integrated ANTI-I
embryo, L1 and L2 larva	100% survival and development into adults within 2 days	85% growth arrest and death after heat-shock, 15% survival and development into adults within 4 days
L3 and L4 larva	100% survival and development into adults within 1 day	95% survival and development into adults in 3 days
adult	no detectable effect	no detectable effect

rol-6 null and integrated ANTI-I/*rol-6* null strains were subjected to a 2 h 30°C heat-shock at various life stages. The proportion of animals surviving and developing to adults was scored in each case.

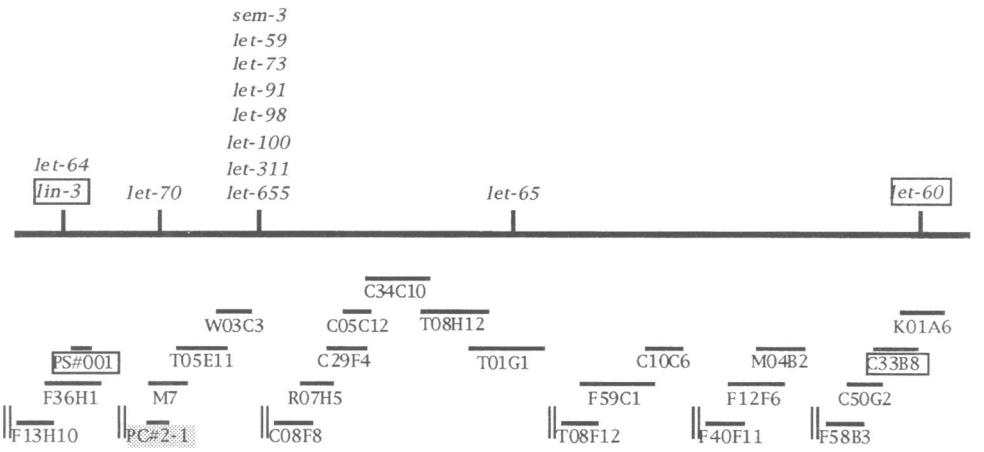

Fig. 7. A portion of the genetic and physical maps of chromosome IV, in the vicinity of *ubc-2*. Genes with recessive lethal alleles which map in the 0.4 map unit *lin-3–let-60* interval are shown (Rogalski *et al.*, 1982; Rogalski and Baillie, 1985; Clark *et al.*, 1988; Clark and Baillie, 1992). The physical map (Coulson *et al.*, 1988) is shown below the genetic map, and is represented by selected cosmid and phage clones. (Adapted from ACEDB; Richard Durbin and Jean Thierry-Mieg, 1991. A *C.elegans* database. Documentation, code and data available from anonymous FTP servers at lirmm.lirmm.fr and ncbi.nlm.nih.gov.) Double vertical lines indicate gaps between cosmid contigs. Clone PC#2-1, indicated with a shaded box, contains the *ubc-2* gene (Zhen *et al.*, 1993). The nearest flanking clones to PC#2-1 which had been correlated previously to the genetic map are indicated with open boxes.

Thierry-Mieg, 1991. A *C.elegans* database. Available from anonymous FTP servers at lirmm.lirmm.fr and ncbi.nlm.nih.gov.) The *C.elegans* genetic map indicates >10 unidentified essential genes in the vicinity of *ubc-2* (Figure 7). These genes were identified either as *unc-22*-linked lethal mutations or as lethal mutations generated by ethylmethane sulfonate (EMS) mutagenesis over the nT1 balancer (Moerman and Baillie, 1979; Rogalski and Baillie, 1985; Clark *et al.*, 1988; Clark and Baillie, 1992). Since the antisense RNA analysis suggested that *ubc-2* might be important for early development of *C.elegans*, complementation tests were carried out to investigate the possibility that one of these genes corresponds to *ubc-2*. Two transgenic lines carrying *ubc-2* and pRF4 mixed extrachromosomal arrays were established by microinjection, and the arrays were crossed into *C.elegans* strains carrying balanced recessive lethal alleles of each candidate gene (Figure 8). The appearance of viable rolling adult progeny homozygous for the marked mutant chromosome would indicate the rescue of the lethal mutation by *ubc-2*. Of the essential genes tested, only *let-70* was rescued, suggesting that *let-70* corresponds to *ubc-2*.

Both alleles of let-70 contain single mutations in the ubc-2 locus

To confirm that *let-70* mutants are altered in the *ubc-2* gene, the *ubc-2* coding region of two alleles, *s1132* and *s689*, was PCR amplified from single worms homozygous for the *let-70* mutations. DNA sequencing revealed that *let-70(s689)* contains a G→A transition in the splice donor site of the last intron, while *s1132* contains a C→T change resulting in substitution of histidine 75 for a tyrosine (Figure 9). Interestingly, His75 is conserved in all ubiquitin-conjugating enzymes sequenced to date, with the exception of yeast UBC6. The splice site mutation in *s689* could result in a mutant UBC-2 protein either lacking the last exon or containing an altered and extended C-terminal sequence, depending upon whether or not potential cryptic splice sites (GTGA) are utilized. More than 10 worms from each *let-70* strain were individually tested by PCR amplification and sequencing analysis and the same mutations were found in the *ubc-2* locus in each animal.

let-70(ubc-2) alleles show growth arrest in their larval stages

The majority of homozygous *let-70(s689)* hermaphrodites arrest at mid-larval stages, although a few individuals reach adulthood. These adult hermaphrodites appear to carry a few oocytes, but are infertile. The stage of arrest of the *let-70(s1132)* allele is earlier (L1 to L2 larval stage), but its assessment requires further examination since it arrests later (L3 and L4) when placed over a deficiency. This suggests that either *s1132* has a weak dominant

negative effect, or that a second site mutation exists on the same chromosome which contributes to the earlier developmental arrest in this strain.

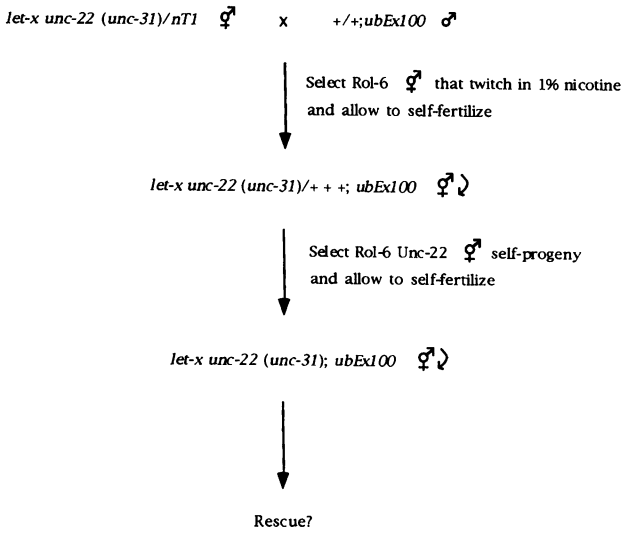

Fig. 8. *C.elegans* complementation test. The extrachromosomal array *ubEx100*, which contains the wild-type *ubc-2* gene (a 5 kb *Eco*RI fragment with 1.3 kb of upstream and 2.6 kb of downstream sequence) and marker plasmid pRF4, was first crossed with N2 males. The resulting roller males were mated with hermaphrodites heterozygous for lethal mutations (twitch in 1% nicotine), and the F1 roller hermaphrodites that twitched in nicotine were picked and allowed to self-fertilize. The progeny of these hermaphrodites were screened for the presence of phenotypically Unc-22 Rol-6 or Unc-22 Unc-31 Rol-6 individuals, which were also presumed to be homozygous for the lethal mutation. If these individuals developed past the normal blocking stage associated with the lethal mutation, then the mutation was considered to be rescued by the presence of the transgenic array.

Discussion

Ubiquitin-conjugating enzymes (E2s) are key components of the ubiquitin ATP-dependent proteolysis system. Members of a large family of E2 genes have been cloned from yeast, and different E2s prove to be involved in very different cellular functions. The surprising diversity of E2 enzymes raises interesting questions regarding their functions in multicellular organisms. For example, how many different E2s are required in a multicellular animal? How are their expression and function regulated, and do they exhibit tissue or developmental specificity?

The results presented here demonstrate that *ubc-2*, a gene encoding an E2 in *C.elegans*, is expressed in all life stages, beginning early in embryonic development and continuing to the adult stage. The spatial pattern of gene expression, however, undergoes alteration during development: while *ubc-2* expression is quite tissue-general in larvae, it is largely restricted to the nervous system in adults. Given that UBC-2 is both structurally and functionally homologous to yeast UBC4 and UBC5, it is possible that this enzyme is involved in the degradation of abnormal and short-lived proteins, including key regulatory proteins such as transcription factors and cell cycle regulators essential for embryonic and early development in *C.elegans*. In later stages, restricted distribution of the gene product suggests either that these functions are no longer required in some tissues or that they are taken over by other E2s. In support of this hypothesis, a human ubiquitin-conjugating enzyme which mediates the degradation of the tumor suppressor protein p53 is closely related to yeast UBC4/5 and *C.elegans* UBC-2 (Scheffner *et al.*, 1993; Scheffner *et al.*, 1994; Seufert *et al.*, 1994; Shkedy *et al.*, 1994). In *Xenopus*, the ubiquitination and subsequent

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  1 ATG GCT CTC AAA AGA ATC CAG AAG gtaaggtttcaacacttaacaacaataa 52
  1 M   A   L   K   R   I   Q   K                               8
 53 aattattatgtacgtttcag GAA CTC CAA GAT CTC GGC CGT GAT CCA CCC 102
  9                      E   L   Q   D   L   G   R   D   P   P  18
103 GCA CAA TGC TCC GCT GGA CCA GTT GGT GAT GAT TTG TTC CAT TGG  147
 19 A   Q   C   S   A   G   P   V   G   D   D   L   F   H   W   33
148 CAA GCT ACG ATT ATG GGC CCA CCA GAG TCT CCC TAT CAG GGA GGT  192
 34 Q   A   T   I   M   G   P   P   E   S   P   Y   Q   G   G   48
193 GTC TTC TTC CTC ACT ATC CAC TTC CCA ACA GAC TAT CCA TTC AAA  237
 49 V   F   F   L   T   I   H   F   P   T   D   Y   P   F   K   63
238 CCA CCA AAG gtattgatcgaaattggaaaaaaataaatttaatttgttttcttcag 293
 64 P   P   K                                                   66
                              s1132  T (Y)
                                     ↕
294 GTT GCC TTC ACC ACT CGA ATT TAT CAT CCG AAC ATC AAT TCA AAC  338
 67 V   A   F   T   T   R   I   Y   H   P   N   I   N   S   N   81
339 GGA AGC ATC TGC CTT GAC ATT CTC CGT TCG CAG TGG TCG CCG GCT  383
 82 G   S   I   C   L   D   I   L   R   S   Q   W   S   P   A   96
384 CTG ACC ATT TCG AAA G gttgatattatcattattgttcgcgttctaacttttaa 437
 97 L   T   I   S   K   V                                       102
438 ttttcag TT CTG CTT TCG ATC TGC TCG CTG CTC TGT GAT CCA AAT   482
103           L   L   S   I   C   S   L   L   C   D   P   N     114
483 CCG GAT GAT CCA CTT GTG CCA GAG ATT GCA CGC ATC TAC AAG ACG  527
115 P   D   D   P   L   V   P   E   I   A   R   I   Y   K   T  129
          s689  a
                ↕
528 GAT CGT GAA AG gtgaatttagattttcttttaactactaaaaataatcgattttca 583
130 D   R   E   R                                               133
584 g G TAC AAT CAA TTG GCT AGA GAA TGG ACG CAA AAG TAC GCT ATG  627
134     Y   N   Q   L   A   R   E   W   T   Q   K   Y   A   M  147
628 TGA ggaggctaacaccattcatataagaacgcagccaaaccaatcaataaaccatgtgt 686
148 *                                                           148
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Fig. 9. Sequences of *let-70(ubc-2)* wild-type and mutant alleles. Exon sequences are shown in upper case, intron and 3′ non-coding sequences in lower case. Protein sequence is indicated in upper case below the nucleotide sequence, in single letter code. The nucleotide and amino acid changes for *let70(s1132)* and *let-70(s689)* are indicated.

degradation of cyclin B is also mediated by an E2 enzyme that is closely related to yeast UBC4/5 (Mahaffey *et al.*, 1993; King *et al.*, 1995).

The prominent expression of UBC-2 in the nervous system suggests a role for this protein in neuronal function. The involvement of the ubiquitin-conjugating system in neural tissue has been noted in several other systems. In humans, the cerebral soluble ubiquitin content in patients with Alzheimer's disease is significantly higher than that of normal individuals (Tadder *et al.*, 1993). In cultured rat DRG neurons, stress results in increased formation of ubiquitin conjugates (Morandi *et al.*, 1989) and, in the moth *Manduca sexta*, the level of ubiquitin increases markedly in neurons undergoing programed cell death (Fahrbach and Schwartz, 1994). More recently, *bendless*, a *Drosophila* mutation affecting the connectivity between the giant fiber and the tergotrochanter motor neuron, was shown to encode an E2 homolog (Muralidhar and Thomas, 1993; Oh *et al.*, 1994). Although the function of UBC-2 in *C.elegans* is as yet unknown, given the known functions of E2s in other systems, we suggest that UBC-2 may act by targeting for degradation specific proteins which are required for neuronal development and/or activity. In this regard, recent data on $G_o\alpha$, the α subunit of the heterotrimeric GTP binding protein G_o, show that this protein has an almost identical expression pattern to that of UBC-2 in *C.elegans* adults, and that alteration of $G_o\alpha$ activity affects nematode behavior controlled by a specific type of neuronal transmitter (Mendel *et al.*, 1995). Since the yeast Gα subunit was shown to be degraded (at least partially) by a RAD6-mediated N-end rule pathway (Madura and Varshavsky, 1994), it would be interesting to determine whether $G_o\alpha$ is one of the substrates degraded via UBC-2-mediated ubiquitination.

Maximum expression of *ubc-2* requires sequences present in three regions: 1.4 kb of upstream DNA, the coding region and 3 kb of downstream DNA. When only the 1.4 kb upstream sequence and the polyadenylation signal were present, no expression was seen. The addition of the coding region, including four introns and five exons, only partially restored expression: not only was the level of β-galactosidase low, but prominent staining was restricted to nerve ganglia around the pharynx, even in larvae. Only when additional downstream 3′-non-coding sequence was included in the translational fusion was maximum expression seen. This suggests that important regulatory elements, perhaps enhancers or sequences conferring message stability, exist in the 3′-non-coding region.

The finding that *ubc-2* corresponds to an essential gene was unexpected. Since yeast *UBC4* and *UBC5* encode proteins with redundant functions and knocking out either gene alone reveals no apparent cellular defect, it seemed likely that this type of E2 would also be redundant in multicellular organisms and that mutations in single genes would therefore have little or no effect on viability. However, both the antisense experiments and the genetic analysis presented here show that *ubc-2* is essential in *C.elegans*. This suggests that the regulation and function of this type of E2 differs considerably between yeast and a multicellular animal such as *C.elegans*. Furthermore, in yeast, *UBC4/5* are required for survival under stress conditions, while the expression of *C.elegans ubc-2* has tissue specificity and is not induced by heat-shock or cadmium treatment (M.Zhen, unpublished results). Thus, it seems likely that, in multicellular organisms, related E2 proteins may have become specialized for different life stages, different tissues or different environmental conditions.

The identification of *let-70(ubc-2)* as an essential gene will allow the powerful tools of *C.elegans* genetics and molecular biology to be brought to bear in elucidating the functions of this important class of ubiquitin-conjugating enzymes in multicellular animals.

Materials and methods

Maintenance of C.elegans strains

The strain used for DNA transformation was *rol-6* (*n1270e187*), which carries a putative null allele of *rol-6*, a collagen gene (Kramer *et al.*, 1990). All strains were cultured either on nematode growth medium (NG) plates or in liquid medium at 20°C as previously described by Brenner (1974).

Constructs and DNA transformation in worms

CI is an in-frame translational fusion of the *lacZ* coding region with the second exon of *ubc-2*. A 1.4 kb *Hin*dIII–*Bgl*II DNA fragment from a *ubc-2* genomic clone was inserted into the *Hin*dIII–*Bam*HI region of the pPD16.43 vector (Fire *et al.*, 1990) as the promoter fragment. A polyadenylation signal was provided by a 0.6 kb *Xho*I fragment downstream of the stop codon of *ubc-2*. To make constructs pZMI.1, pZMI.2, pZMII.1 and pZMII.2, the *lacZ* coding region, a 3.3 kb *Bam*HI–*Apa*I fragment from pPD16.43, was inserted in-frame into the second exon (*Bgl*II–*Apa*I sites) of the *ubc-2* genomic clone. All four constructs contain a complete *ubc-2* coding sequence, including five exons and four introns. In pZMI.1, a 6 kb *Bgl*II fragment preceding the initiation methionine codon and 2.6 kb of sequence downstream of the TAG stop codon were included in the construct to provide regulatory elements for expression. pZMI.2 included the same 5′-non-coding sequence but less 3′-non-coding sequence (a 0.6 kb *Xho*I fragment). pZMII.1 contained the 1.4 kb *Hin*dIII–*Bgl*II upstream sequence and the 2.6 kb 3′-non-coding sequence. pZMII.2 is identical to pZMII.1, except that the 0.6 kb *Xho*I fragment was ligated to the 3′ end of the fusion. A nuclear localization signal (NLS) from SV40 was included in all constructs at the beginning of the *lacZ* coding sequence. Two sense constructs *hsp–ubc-2* and *ubc-2*(SK) and two antisense RNA constructs ANTI-I and ANTI-II were made as follows: For *hsp–ubc-2*, a 1.0 kb *Kpn*I–*Sac*I fragment containing the *ubc2* cDNA was inserted into pPD49.78 (Fire *et al.*, 1990). ANTI-I contains the same insert in pPD49.78 but in the opposite orientation. *ubc-2*(SK) is a *ubc-2* genomic clone in pBluescriptSK vector; ANTI-II was made directly from this clone by digestion with *Apa*I and *Xho*I. This resulted in two DNA fragments, one containing the *ubc-2* coding sequence and the other containing the vector and *ubc-2* upstream and downstream sequences. Both fragments were blunt-ended and religated together. Restriction digestions and sequencing were used to identify clones containing the inverted *ubc-2* coding region sequence. All constructs were transformed into a *C.elegans rol-6* null strain with the marker plasmid pRF4 as described by Mello *et al.* (1991). The pRF4 plasmid carries a dominant *rol-6* allele, *su1006*, encoding a mutant collagen that causes the worm to roll clockwise as it moves forward.

Generation of stable, integrated transgenic strains

Transgenic strains carrying mixed extrachromosomal arrays of the *rol-6* plasmid and pZMI.1 were mutagenized with 3800 rads of γ-rays emanating from a ^{60}Co source. Individual F2 animals were screened for those producing only rolling progeny. Homozygous rolling F3s were then outcrossed to the N2 (Bristol) strain for seven generations to eliminate other potential mutations in the genome. Two integrated lines, namely pZMI.1In1 and pZMI.1In2, were obtained.

In situ localization of β-galactosidase expression in transgenic C.elegans

Transgenic nematodes carrying *ubc-2–lacZ* fusions were fixed and stained with X-gal to detect β-galactosidase expression as described by Fire (1992). The stained cells were identified on the basis of their shapes,

sizes and positions using Nomarski microscopy (Sulston, 1976; Sulston and Horvitz, 1977).

Production of antibodies and Western blot analysis

A 450 bp *Bam*HI–*Hin*dIII fragment containing the *ubc-2* open reading frame was inserted into pRSETC (Invitrogen) to create the plasmid pRSETC-ubc-2. This plasmid expresses a 21 kDa fusion protein in the *E.coli* strain BL21 (λ*DE3 pLysS*) by induction with 1–2 mM isopropyl β-D-thiogalactopyranoside (IPTG). This fusion protein consists of a 4 kDa amino-terminal sequence containing six histidyl residues and the 16.7 kDa UBC-2 protein. The protein was purified on a Ni-NTA resin column according to the protocol of the supplier (Qiagen). New Zealand White rabbits were immunized with 0.4 mg of 0.1% SDS-denatured fusion protein in an emulsion of Freund's complete adjuvant. The rabbits were boosted with antigen at 2 week intervals using Freund's incomplete adjuvant. Antiserum was affinity purified using UBC-2 fusion protein affixed to an Affi-gel 10 column (Bio-Rad). To prepare protein samples for Western blot analysis, worms were washed in phosphate-buffered saline (PBS) and resuspended in 1× sample buffer [50 mM Tris pH 6.8, 100 mM dithiothreitol (DTT), 2% SDS and 10% glycerol]. After boiling for 5 min, the supernatant was collected, and the protein concentration was determined by mini-Bradford assay (Bio-Rad). Proteins separated on SDS–polyacrylamide gels were electroblotted to polyvinylidene difluoride membranes (Immobilon-P, Millipore), and incubated with affinity-purified anti-ubc-2 antiserum. Development of the immunoblots was carried out using the enhanced chemiluminescence system (ECL) of Amersham.

In situ immunofluorescent staining

This procedure was based on that of McIntire *et al.* (1992). Worms were harvested from NG plates and fixed with freshly prepared 4% paraformaldehyde in 100 mM potassium phosphate buffer (pH 7.4) for 4–6 h. The animals were then incubated in 1% Triton X-100, 100 mM Tris (pH 7.0), 1% β-mercaptoethanol overnight to reduce disulfide bonds in the cuticle. After several washes with PBS, the worms were permeabilized by incubation in 1000 U/ml collagenase IV (Sigma) at 37°C for 3 h. The permeabilized worms were incubated with horse serum for 6 h at room temperature in AbA buffer followed by overnight incubation with antibodies against UBC-2 protein. Both serum and antibody were used at 1:500 dilution. After three washes in AbB buffer (PBS, 0.1% Triton X-100, 0.1% bovine serum albumin, 0.05% NaN_3), worms were incubated with a 1:100 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma) for 6 h. After three washes in AbB buffer, worms were mounted on slides in 70% glycerol, 100 mM potassium phosphate (pH 7.4), 5% *n*-propylgallate to minimize photobleaching, and 1 mg/ml 4,6-diamidino-2-phenylindole (DAPI). For immunofluorescence, a Zeiss filter set consisting of a BP450–490 excitation filter, a 510 nm dichroic and a BP515–565 emission filter was used.

C.elegans complementation crosses

Rol-6 males of the genotype +/+; *ubEx100* were mated to hermaphrodites of the genotype *let-x unc-22/nT1(IV)*; *+/nT1(V)* or *let-x unc-22 unc-31/nT1(IV)*; *+/nT1(V)* (Moerman and Baillie, 1979; Clark *et al.*, 1988). In the F1 generation, L3–L4 stage Rol-6 hermaphrodites that twitched in 1% nicotine were selected to separate plates (*let-x unc-22/ + +*; *ubEx100* or *let-x unc-22 unc-31/+ + +*; *ubEx100*) and allowed to self-fertilize. The progeny of these hermaphrodites were screened for the presence of phenotypically Unc-22 Rol-6 or Unc-22 Unc-31 Rol-6 individuals, which were also presumed to be homozygous for the lethal mutation. If these individuals developed past the normal blocking stage associated with the lethal mutation, then the mutation was considered to be rescued by the presence of the transgenic array.

PCR and sequencing of the ubc-2 gene from single worms

The region of *let-70(ubc-2)* corresponding to nucleotides 496–1133 (Zhen *et al.*, 1993) was amplified from each mutant using PCR with the primer pair oZM3 (5′ GA*GGATCC*ATGGCTCTCAAAAGAATCCA-GAAG 3′) and oZM4 (5′ AGTA*CTAGT*TTAGCCTCCTCACATAGC-GTA 3′). *Bam*HI and *Spe*I sites included in the primers to facilitate cloning are shown in italics. Single worms homozygous for the *let-70* mutation were recognized by the twitching phenotype in the early larval stages, and lysed in 1× Pfu buffer (Stratagene) supplemented with 2.5 mg/ml protease K. PCRs were then performed in 25 μl volumes containing 25 pmol of each oligonucleotide primer, 50 mM of each deoxynucleoside triphosphate and 1 U of *Pfu* DNA polymerase (Stratagene) in PCR buffer as described by Plasterk and Groenen (1992). PCR products were subcloned into pBluescript SK vectors (Stratagene), and the inserts were sequenced using a Sequenase kit (U.S. Biochemical) and primers T3, T7, MEI20-2 (5′ GGTTGCCTTCACCACTCGAAT 3′) and MEI20-4 (5′ GGTCAGAGCCGGCGACCACTG 3′).

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References

Bartling,D., Rehling,P. and Weiler,E.W. (1993) *Plant Mol. Biol.*, **23**, 387–396.
Brenner,S. (1974) *Genetics*, **77**, 71–94.
Chen,P., Johnson,P., Sommer,T., Jentsch,S. and Hochstrasser,M. (1993) *Cell*, **74**, 357–369.
Ciechanover,A. (1994) *Cell*, **79**, 13–21.
Clark,D.V. and Baillie,D.L. (1992) *Mol. Gen. Genet.*, **232**, 97–105.
Clark,D.V., Rogalski,T.M., Donati,L.M. and Baillie,D.L. (1988) *Genetics*, **119**, 345–353.
Coulson,A., Waterson,R., Kiff,J., Sulston,J. and Kohara,Y. (1988) *Nature*, **335**, 184–186.
Driscoll,J. and Finley,D. (1992) *Cell*, **68**, 823–825.
Fahrbach,S.E. and Schwartz,L.M. (1994) *J. Comp. Neurol.*, **343**, 464–482.
Fire,A. (1992) *Genet. Anal. Tech. Appl.*, **9**, 151–158.
Fire,A., Harrison,S.W. and Dixon,D.K. (1990) *Gene*, **93**, 189–198.
Girod,P.-A. and Vierstra,R. (1993) *J. Biol. Chem.*, **268**, 955–960.
Goebl,M.G., Yochem,J., Jentsch,S., McGrath,J.P., Varshavsky,A. and Byers,B. (1988) *Science*, **241**, 1331–1335.
Hershko,A. (1988) *J. Biol. Chem.*, **263**, 15237–15240.
Hershko,A. and Ciechanover,A. (1992) *Annu. Rev. Biochem.*, **61**, 761–807.
Hochstrasser,M., Ellison,M.J., Chau,V. and Varshavsky,A. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 4606–4610.
Jentsch,S. (1992a) *Annu. Rev. Genet.*, **26**, 177–205.
Jentsch,S. (1992b) *Trends Cell Biol.*, **2**, 98–103.
Jentsch,S., McGrath,J.P. and Varshavsky,A. (1987) *Nature*, **329**, 131–134.
Jungmann,J., Reins,H.-A., Schobert,C. and Jentsch,S. (1993) *Nature*, **361**, 369–371.
King,R.W., Peters,J.-M., Tugendreich,S., Rolfe,M., Hieter,P. and Kirschner,M.W. (1995) *Cell*, **81**, 279–288.
Kramer,J.M., French,R.P., Park,E.-C. and Johnson,J.J. (1990) *Mol. Cell. Biol.*, **10**, 2081–2089.
Leggett,D.S., Jones,D. and Candido,E.P.M. (1995) *DNA Cell Biol.*, **14**, 881–889.
Madura,K. and Varshavsky,A. (1994) *Science*, **265**, 1454–1458.
Madura,K., Prakash,S. and Prakash,L. (1990) *Nucleic Acids Res.*, **18**, 771–778.
Mahaffey,D., Yoo,Y. and Rechsteiner,M. (1993) *J. Biol. Chem.*, **268**, 21205–21211.
McIntire,S.L., Garriga,G., White,J., Jacobson,D. and Horvitz,H.R. (1992) *Neuron*, **8**, 307–322.
Mello,C.C., Kramer,J.M., Stinchcomb,D. and Ambros,V. (1991) *EMBO J.*, **10**, 3959–3970.
Mendel,J.E., Korswagen,H.C., Liu,K.S., Hajdu-Cronin,Y.M., Simon, M.I., Plasterk,R.H.A. and Sternberg,P.W. (1995) *Science*, **267**, 1652–1655.
Michalek,M.T., Grant,E.P., Gramm,C., Goldberg,A. and Rock,K.L. (1993) *Nature*, **363**, 552–554.
Moerman,D.G. and Baillie,D.L. (1979) *Genetics*, **91**, 95–104.
Morandi,A., Los,B., Osofsky,L., Autilio-Gambetti,L. and Gambetti,P. (1989) *Prog. Clin. Biol. Res.*, **317**, 819–827.
Muralidhar,M.G. and Thomas,J.B. (1993) *Neuron*, **11**, 253–266.
Oh,C.E., McMahon,R., Benzer,S. and Tanouye,M.A. (1994) *J. Neurosci.*, **14**, 3166–3179.
Plasterk,R.H.A. and Groenen,J.T.M. (1992) *EMBO J.*, **11**, 287–290.
Rechsteiner,M. (ed.) (1988) *Ubiquitin*. Plenum Press, New York.
Rogalski,T.M. and Baillie,D.L. (1985) *Mol. Gen. Genet.*, **201**, 409–414.
Rogalski,T.M., Moerman,D.G. and Baillie,D.L. (1982) *Mol. Gen. Genet.*, **201**, 725–736.

Scheffner,M., Huibregtse,J.M., Vierstra,R.D. and Howley,P.M. (1993) *Cell*, **75**, 495–505.
Scheffner,M., Huibregtse,J.M. and Howley,P.M. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 8797–8801.
Seufert,W. and Jentsch,S. (1990) *EMBO J.*, **9**, 543–550.
Seufert,W., Futcher,B. and Jentsch,S. (1994) *Nature*, **373**, 78–81.
Shkedy,D., Gonen,H., Bercovich,B. and Ciechanover,A. (1994) *FEBS Lett.*, **348**, 126–130.
Stringham,E.G., Dixon,D.K., Jones,D. and Candido,E.P.M. (1992) *Mol. Biol. Cell*, **3**, 221–233.
Sulston,J.E. (1976) *Philos. Trans. R. Soc. Lond., B. Biol. Sci.*, **275**, 287–297.
Sulston,J.E. and Horvitz,H.R. (1977) *Dev. Biol.*, **56**, 110–156.
Sulston,J.E., Du,Z., Thomas,K., Wilson,R., Hillier,L., Staden,R., Halloran,N., Green,P., Thierry-Mieg,J., Qiu,L., Dear,S., Coulson,A., Craxton,M., Durbin,R., Berks,M., Metzstein,M., Hawkins,T., Ainscough,R. and Waterston,R. (1992) *Nature*, **356**, 37–41.
Tadder,N., Liguri,G., Sorbi,S., Amaducci,L., Camici,G., Nassi,P., Cecchi,C. and Ramponi,G. (1993) *Neurosci. Lett.*, **151**, 158–161.
Wiebel,F.F. and Kunau,W.-H. (1992) *Nature*, **359**, 73–76.
Zhen,M., Heinlein,R., Jones,D., Jentsch,S. and Candido,E.P.M. (1993) *Mol. Cell. Biol.*, **13**, 1371–1377.

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