Cloning and Characterization of a *Saccharomyces cerevisiae* Gene Encoding a New Member of the Ubiquitin-conjugating Protein Family*

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Ubiquitin-conjugating enzymes (E2s), which participate in the post-translational conjugation of ubiquitin to proteins, are encoded by a multigene family in the yeast *Saccharomyces cerevisiae*. E2s function in a variety of cellular activities including intracellular protein degradation, DNA repair, sporulation, and cell cycle traverse. Here, we report the cloning and characterization of a new member of the yeast UBC gene family, *UBC8*. *UBC8* encodes a 206-amino-acid protein containing a highly acidic carboxyl terminus. The primary structure of the protein is similar to that of all other known 23-kDa E2s, with the highest homology being to the E2 (23 kDa) from wheat germ. Haploid strains in which the *UBC8* gene is disrupted are viable, and the disruption does not produce any obvious phenotype. The *UBC8* protein, produced in *Escherichia coli*, forms thiol ester adducts with ubiquitin and, apparently, diubiquitin, but does not transfer ubiquitin to histones.

Ubiquitin, a highly conserved 76-amino acid polypeptide found in all eukaryotes, functions in intracellular protein degradation and a variety of other cellular processes (1-3). The post-translational conjugation of ubiquitin to proteins requires the initial ATP-dependent activation of ubiquitin. This reaction is catalyzed by ubiquitin-activating enzyme (E1) (4-7) and results in the formation of a ubiquitin-E1 thiol ester. Transfer of ubiquitin to one of a number of ubiquitin-conjugating proteins (E2s) then occurs by transestification to give a ubiquitin-E2 thiol ester. Multiple E2s have been identified in a variety of cell types (8-11), and these fall into at least two classes. One class is capable of directly transferring ubiquitin from E2 thiol ester linkage to an appropriate protein acceptor. Transfer of ubiquitin from a second class of E2s is catalyzed by ubiquitin-protein ligase (E3). The resulting multiubiquitin-protein conjugates (12, 13) are then degraded with the release of free ubiquitin. The selectivity of proteolysis is mediated by E3(s) via distinct sites with high affinity for specific classes of amino-terminal amino acids, and probably other domains, of potential substrates for degradation (14-18).

In the yeast *Saccharomyces cerevisiae*, seven genes (designated *UBC1*-7) encoding E2s have been described (11). The genes encode related proteins, including a region of substantial amino acid identity around a conserved cysteine residue essential for ubiquitin thiol ester formation (11). In addition, a subset of *UBC* genes encode proteins with a carboxy-terminal extension. Two of these, *UBC2* and *UBC3*, are identical to the previously identified genes *RAD6* and *CDC34*, respectively (19, 20). The *UBC2* (RAD6) gene product functions in DNA repair, induced mutagenesis, and sporulation (21-24), whereas the *UBC3* (CDC34) gene product is required for the G1 to S transition of the cell cycle (20, 25). The E2s encoded by these two genes are capable of ubiquitinating bovine histones H2A and H2B in vitro. However, the in vivo substrate(s) for these E2s remain unknown; and in fact, recent evidence suggests that yeast histone H2A is not normally ubiquitinaturated (26). Three other yeast *UBC* genes, *UBC1*, *UBC4*, and *UBC5*, encode E2s of the type that require E3 for transfer of ubiquitin to substrates. Gene disruption experiments have demonstrated that these E2s are important for the turnover of normally short-lived and abnormal proteins (11, 27, 28).

We now report the cloning and analysis of an additional *UBC* gene from *S. cerevisiae*, designated *UBC8*. The *UBC8* gene encodes a 23-kDa protein containing 206 amino acids. The deduced amino acid sequence of the *UBC8* gene product is most similar to that of the E2 (23 kDa) from wheat germ (10) and includes a highly acidic carboxy-terminal extension also found in the *UBC2* (RAD6) and *UBC3* (CDC34) gene products. The *UBC8* protein forms an E1-dependent ubiquitin thiol ester, but does not transfer ubiquitin to bovine histones in vitro. Disruption of the *UBC8* gene does not produce any readily detectable phenotype.

**EXPERIMENTAL PROCEDURES**

Materials

All chemicals were reagent-grade. Restriction endonucleases and other enzymes were purchased from commercial sources and used according to the supplier's recommendations. Bovine histone H2B was from Boehringer Mannheim. Bovine ubiquitin (Sigma) was radioiodinated using the chloramine-T method as described (29). L-[^35]S]Methionine was from Amersham Corp.

Methods

Plasmids, Strains, and Growth Conditions—The YEp24-based yeast genomic library (80), obtained from David Botstein via Toshio Fukasawa, was used for cloning of the *UBC8* gene. The *S. cerevisiae* diploid strain NOY397 (MATα/a ade2-1/2-1 ura-3-1 his3-11/13 trpl-1/1-f1 leu2-3,112/2-3,112 con1-100/-100) was used for one-step gene disruption experiments (31). Strain S173-6B (MATα leu2-3,112 his3-11 ura3-52 trpl-289) was the source of E1 and E2s. Yeast...
strains were cultivated on YEPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic dextrose medium prepared as described (32) containing supplements as needed to satisfy auxotrophic requirements.

The plasmid used for expression of the UBC8 protein in Escherichia coli contains the UBC8 coding sequence just downstream from the T7 promoter and ribosome-binding site in vector pT7-7 (33). pT7-7 was digested with NdeI and BamHI, and a synthetic double-stranded DNA fragment containing the sequence from the coding region of the unique SpeI site of UBC8 (see Fig. 1) was cloned in to give pT7-Q. After digestion of pT7-Q with SpeI and HindIII, the 1.5-kb SpeI-HindIII fragment of UBC8 was inserted to give pNOY3119. pNOY3119 was introduced into the E. coli λ lysogen BL21(DE3) (34), which contains a single copy of the T7 RNA polymerase gene under the control of the inducible lacUV5 promoter, to obtain strain NO3916. A control strain in which pT7-7 lacking any insert was introduced into BL21(DE3) was also constructed.

Synthesis of UBC8 Protein in E. coli— Cultures of NO3916 and the control strain were grown to early log phase at 37 °C in LB medium supplemented with 50 μg/ml ampicillin and induced by the addition of isopropyl-1-thio-β-d-galactopyranoside to a concentration of 1 mM. After 30 min, rifampicin (100 μg/ml) was added, and incubation was continued for 3 h. Cells were harvested by centrifugation; resuspended to 204th of the original culture volume in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride; and lysed by freezing and thawing. SDS-PAGE of the lysates was performed according to Laemmli (35).

Preparation of 35S-labeled proteins, 5-ml cultures of NO3916 and control cells were grown to early log phase at 37 °C in M9 medium supplemented with 50 μg/ml ampicillin and a 50 μg/ml concentration of all amino acids except methionine and cysteine. After isopropyl-1-thio-β-d-galactopyranoside induction as described above, rifampicin (100 μg/ml) was added; and 30 min later, [35S]methionine (70 μCi/ml of culture) was added. Incubation was continued for 1.5 h, and the cells were harvested by centrifugation. Cells were washed once in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and then suspended in 25 μl of lysate buffer plus lysozyme (300 μg/ml) and lysed by freezing and thawing. SDS-PAGE of the lysates followed by Klenow allowed to sporulate for tetrad analysis. Chromosomal disruptions of the diploid strain NOY397. Ura+ transformants were selected and identified by SDS-PAGE and separately concentrated using Centriprep 10 concentrators, and stored in small aliquots at -70 °C.

As for Ubiquitin-E2 Thiol Ester and Ubiquitin-Histone Conjugate Formation—Ubiquitin-E2 thiol ester formation was assayed essentially as described (19, 20, 24). Reaction mixtures (20 μl) contained 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM ATP, 0.2 mM dithiothreitol, 300 μg of pure E1, and either an extract of 35S-labeled NO3916 cells (6 × 105 cpm) and 1 μg of unlabeled ubiquitin or an extract of unlabeled NO9316 cells and 35I-labeled ubiquitin (6 × 105 cpm). Reaction mixtures were incubated at 30 °C for 15 min, and the reaction was stopped by the addition of SDS sample buffer (35) lacking reducing agent. SDS-PAGE analysis was conducted in the absence of reducing agent at 4 °C. In some experiments, parallel reaction mixtures were stopped by the addition of complete SDS sample buffer (containing 2-mercaptoethanol), and samples were boiled for 5 min followed by SDS-PAGE at room temperature in the presence of reducing agent.

Ubiquitin-histone conjugate formation was assayed as described above using extracts of unlabeled NO3916 cells and [35I]-ubiquitin, except that 1 μg of bovine histone H2B was also included.

RESULTS

Cloning of UBC8 Gene—During the course of studies of mutants of S. cerevisiae defective in RNA synthesis, we screened a yeast genomic library in YEp24 (30) for complementation of a temperature-sensitive mutant and obtained several independent candidate clones. Upon sequence analysis, one of these clones was found to have an open reading frame that showed a high degree of similarity to known UBC genes. Although further experiments showed that this open reading frame was not responsible for the apparent complementation of the mutation in question, we carried out a series of experiments to be described below and found that it is a previously unidentified member of the S. cerevisiae UBC gene family (11), and we propose the name UBC8 for this gene.

Nucleotide and Amino Acid Sequence—A partial restriction map of a portion of the cloned DNA fragment carrying the UBC8 gene is shown in Fig. 1. The sequence of the Sall-BgII fragment was determined completely on both strands. A portion of this sequence is shown in Fig. 2. An ATG codon at nucleotide +1 begins an open reading frame of 618 nucleotides, which is followed by a TAA termination codon.

The open reading frame has the potential to encode a protein of 206 amino acids with a calculated molecular mass of 23,217 Da. A computer search of protein sequence data banks identified the wheat germ E2 (23 kDa) and the UBC2 (RAD6) protein as having strong overall similarity to the UBC8 protein.

The thinner lined portion of the map represents vector pT7-7. The open arrow designates the open reading frame of the gene. The sites at which a portion of the UBC8 gene was deleted and replaced by the URA3 gene are indicated. Bg, BgII; E1, EcoRI; E5, EcoRV; HI, HindIII; Hp, HpaI; S, Sall; Sp, SphI.

![Fig. 1. Partial restriction map of DNA segment carrying UBC8 gene. The thinner lined portion of the map represents vector sequence. The open arrow designates the open reading frame of the gene. The sites at which a portion of the UBC8 gene was deleted and replaced by the URA3 gene are indicated. Bg, BgII; E1, EcoRI; E5, EcoRV; HI, HindIII; Hp, HpaI; S, Sall; Sp, SphI.](image-url)
New Ubiquitin-conjugating Protein

Amino acid sequence identity ranging from 20 to 35% is seen when the UBC8 protein core sequence is compared to that of other UBC proteins from yeast and higher eukaryotes. A comparison of the codon frequency usage of UBC8 with the average frequency of codon usage in 454 S. cerevisiae genes (43) shows a relatively high frequency of rare codon usage in UBC8, suggesting that the gene is expressed at low levels. Northern blot analysis revealed a single transcript of ~0.9 kb when total yeast RNA was probed with the 1.3-kb EcoRI DNA fragment (Fig. 1) that contains the complete UBC8 coding region (data not shown).

The single cysteine residue at amino acid 73 is found in all E2s for which sequence information is available, and the sequence around this residue is the most highly conserved region in E2 proteins. Mutation of the analogous cysteine residue in UBC2 (RAD6) to alanine or valine abolishes ubiquitin thiol ester formation (24). Cys73 of the UBC8 gene product presumably also serves as the site of ubiquitin thiol ester formation. Experiments described below provide more direct evidence that the UBC8 gene encodes a ubiquitin-conjugating enzyme.

E2 Activity of UBC8 Gene Product—The homology between the deduced amino acid sequence of the UBC8 gene product and known ubiquitin-conjugating enzymes from various sources strongly suggested that it, too, was a ubiquitin-conjugating enzyme. These enzymes are characterized by their ability to form ubiquitin thiol ester bonds in the presence of added E1 and ATP. We have used extracts from E. coli expressing the UBC8 gene to test for ubiquitin conjugating activity. NO3916 cells were grown and induced as described under “Experimental Procedures.” The appearance with time of a 23-kDa protein whose presence is dependent upon the presence of UBC8 coding sequence is shown in Fig. 4A. The size of this protein is in excellent agreement with that predicted from the deduced amino acid sequence of UBC8. When rifampicin-treated cultures are labeled with [35S]methionine, >90% of the radioactivity is found in the expected UBC8 gene product (Fig. 4B), so that an extract of labeled cells could be used directly to assay thiol ester bond formation between the UBC8 protein and ubiquitin.

Figs. 5 and 6 show the results of experiments in which the predicted UBC8 protein. The homology to the wheat germ E2 (23 kDa), and UBC2 (RAD6) protein. Numbers to the right refer to the final amino acid residue in each line relative to the start of each respective primary sequence. Amino acids that are identical or chemically similar (D = E, R = K, S = T, F = Y, I = L, W = M) to the corresponding residue in the “E2 core” (11) sequence (residues 1-145) of the UBC8 protein are boxed. The unique cysteine residue is underlined.

predicted UBC8 protein. The homology to the wheat germ E2 (23 kDa) is particularly striking, with 64% amino acid identity (76% with conservative substitutions) in the conserved domain (11) common to all E2 proteins (amino acids 1-145 in UBC8), as shown in Fig. 3. In addition, both proteins have carboxyl-terminal extensions consisting of ~50% acidic amino acids. The UBC8 carboxyl-terminal extension is also rich in hydroxyamino acids (12 of 67 residues are serine or threo-

Fig. 2. Nucleotide and predicted amino acid sequence of UBC8. The nucleotide sequence of the non-transcribed strand is shown. The coding sequence starts at nucleotide 1 and ends at nucleotide 619. The unique cysteine residue is marked by an arrow.

Fig. 3. Comparison of deduced amino acid sequences of UBC8 protein, wheat germ E2 (23 kDa), and UBC2 (RAD6) protein. Numbers to the right refer to the final amino acid residue in each line relative to the start of each respective primary sequence. Amino acids that are identical or chemically similar (D = E, R = K, S = T, F = Y, I = L, W = M) to the corresponding residue in the "E2 core" (11) sequence (residues 1-145) of the UBC8 protein are boxed. The unique cysteine residue is underlined.
New Ubiquitin-conjugating Protein

**Fig. 5.** UBC8-ubiquitin thiol ester formation. Extracts of \[^{35}S\]methionine-labeled induced NOY3916 cells were incubated with purified yeast E1, ubiquitin, and ATP, as described under “Experimental Procedures” and then subjected to SDS-PAGE on 12% acrylamide gels in the absence (A and B) or presence (C) of 2-mercaptoethanol and subjected to autoradiography. A, lane 1, extract of NOY3916 cells only; lane 2, complete reaction mixture; lane 3, same as lane 2 but with E1 omitted; lane 4, same as lane 2 but with ubiquitin omitted. B, lane 1, extract of NOY3916 cells only; lane 2, complete reaction mixture; lane 3, same as lane 2 but with ATP omitted. The experiments in A and B were performed at different times. C, lanes 1 and 2, same as in A but samples were boiled in SDS sample buffer containing 2-mercaptoethanol and subjected to SDS-PAGE in presence of 2-mercaptoethanol. Arrows in A and B indicate the positions of UBC8-ubiquitin thiol ester adducts.

**Fig. 6.** UBC8-ubiquitin thiol ester adducts of same size are formed from \[^{35}S\]labeled UBC8 protein and from \[^{125}I\]labeled ubiquitin. Reaction mixtures containing extracts of \[^{35}S\]methionine-labeled induced NOY3916 cells, unlabeled ubiquitin, purified yeast E1, and ATP (lane 1) or extracts of unlabeled induced NOY3916 cells, \[^{125}I\]ubiquitin, purified yeast E1, and ATP (lane 2) were resolved on 12% acrylamide gels in the absence of 2-mercaptoethanol and subjected to autoradiography. Positions of the UBC8-ubiquitin thiol ester adducts are indicated by arrows.

UBC8 gene product, produced in E. coli, was assayed for ubiquitin conjugating activity. Experiments were performed with \[^{35}S\]labeled UBC8 gene product and unlabeled ubiquitin (Fig. 5 and Fig. 6, lane 1) as well as with unlabeled UBC8 gene product and \[^{125}I\]labeled ubiquitin (Fig. 6, lane 2). In both cases, two radioactive bands with apparent molecular masses of 32 and 37 kDa (indicated by arrows in Figs. 5 and 6), both of which are labile to boiling in the presence of mercaptoethanol (compare Fig. 5C, lane 2, to Fig. 5A, lane 2), were formed. These thiol ester adducts were not found in the absence of added purified E1 or ATP or with control E. coli extracts harboring vector alone (Fig. 5A). The mobilities of the two radioactive bands correspond reasonably well to the predicted sizes of thiol ester complexes formed between the UBC8 gene product and one and two molecules of ubiquitin, respectively.

Several species of E2s are capable of transferring ubiquitin from ubiquitin-E2 thiol ester linkage to appropriate acceptors in an E3-independent reaction. Those E2s with polyacidic carboxyl termini, wheat germ E2 (23 kDa), UBC2 (RAD6), and UBC3 (CDC34) are all capable of transferring ubiquitin to histones in vitro. When histones were added as substrates to reaction mixtures capable of forming UBC8-ubiquitin thiol ester conjugates, no ubiquitin-histone conjugates were detected (Fig. 7). When the reaction mixtures were supplemented with a partially purified preparation of total yeast E2s, such conjugates were readily detected (Fig. 7, A and B, lane 5). In addition, in separate experiments in which an extract of E. coli expressing the UBC3 (CDC34) protein was substituted for the extract expressing the UBC8 protein, ubiquitin-histone conjugate formation was also demonstrable (data not shown), confirming results obtained by previous workers (20).

Disruption of UBC8 Gene—Disruption of the UBC8 gene in the diploid strain NOY397 was carried out with the URA3 gene as described under “Experimental Procedures.” Stable Ura+ transformants were isolated and sporulated. Seven tetrad were dissected, and all gave four fully viable spores. The chromosomal disruption was confirmed by Southern blot analysis by EcoRI digestion of genomic DNAs and probing with the 1.3-kb EcoRI fragment carrying UBC8 (Fig. 8). The results demonstrate that disruption of the UBC8 gene is not lethal to the cell. We have tested a variety of phenotypes including generation time on various carbon sources, sensitivity to amino acid analogs, DNA-damaging agents, and heat shock and cellular density in stationary phase and find no significant difference between haploid strains with the disrupted UBC8 gene and the wild-type strain.

**DISCUSSION**

In this paper, we report the isolation and characterization of a new member of the S. cerevisiae UBC gene family, UBC8. The deduced amino acid sequence of the UBC8 gene product is similar to that of the sequences of other ubiquitin-conjugating proteins from S. cerevisiae (11, 19, 20, 27, 28), Schizo-
saccharomyces pombe (44), and higher eukaryotes (10, 45).

The UB8C protein sequence is most similar to the sequence of the wheat germ E2 (23 kDa) protein, which, like the S. cerevisiae UBC2 (RAD6) and UBC3 (CDC34) proteins, also has an acidic carboxyl-terminal extension. The acidic carboxyl-terminal domains of UBC2 (RAD6) and UBC3 (CDC34) have been implicated in the transfer of ubiquitin from E2 thiol ester linkage to protein substrates (20, 41). In vitro, both of these gene products and the wheat germ E2 (23 kDa) protein can catalyze the E3-independent transfer of ubiquitin to histones. Several E2 proteins from reticulocytes, the primary structures of which are unknown, also form ubiquitin-histone conjugates (8, 46, 47).

To confirm that the UB8C product functions as a ubiquitin-conjugating protein, we tested the ability of the cloned gene product to form thiol ester conjugates with ubiquitin and also to transfer ubiquitin to histones in vitro. In the presence of purified E1 and ATP, two UB8C-ubiquitin conjugates, both of which are labile to boiling in the presence of mercaptoethanol prior to electrophoresis, are formed. The apparent molecular masses of these labile conjugates are 32 and 37 kDa, suggesting that they consist of UB8C protein conjugated to one and two molecules of ubiquitin, respectively. Since both conjugates have the properties of thiol esters and UBC8 protein contains a single cysteine residue, they most likely represent UB8C-ubiquitin and UB8C-diubiquitin thiolester adducts. Chen and Pickart (48) have demonstrated that calf thymus E2ub8 catalyzes the formation of an isopeptide bond between the carboxyl-terminal glycine of one ubiquitin and the e-amino group of Lys46 of another. The diubiquitin so formed is capable of being activated by E1 and transferred to form the diubiquitin thiol ester adduct. Although we do not have direct experimental evidence, it is probable that a similar mechanism accounts for UB8C-diubiquitin thiol ester adduct formation.

It has been suggested that diubiquitin and larger mult ubiquitin chains generated in this way may be transferred to specific proteins in an E3-independent or E3-dependent manner to generate proteolytic substrates (48, 49). It should be noted, however, that we cannot exclude the possibility that the putative diubiquitin is formed via isopeptide linkage between the carboxyl terminus of one ubiquitin and lysine residues other than Lys46 of a second ubiquitin or even via peptide linkage between the carboxyl terminus of one ubiquitin and the amino terminus of another. Nevertheless, the results suggest that UB8C protein can apparently function in transferring one ubiquitin molecule to another in the absence of E3. Thus, UB8C appears to belong to the class of ubiquitin-conjugating proteins capable of E3-independent transfer of ubiquitin, as suspected from its structural similarities to UBC2 and UBC3.

In contrast to the other E2s with polycydic carboxyl termini, the UB8C protein does not form ubiquitin-histone conjugates. Conjugation of ubiquitin to histone was demonstrable when a partially purified preparation of total yeast E2s was assayed. Sung et al. (41) have suggested that the UBC2 (RAD6) gene product is responsible for most of the histone ubiquitinat ing activity in E2 fractions prepared from wild-type S. cerevisiae. The natural substrate(s) for ubiquitin transfer from UB8C thiol ester adducts is presently unknown.

The UB8C gene appears to be dispensable for normal viability. Cells in which the gene is disrupted grow at the same rate as wild-type cells under a variety of conditions. The most plausible explanation for the observed lack of phenotype is functional redundancy, although it remains possible that UB8C gene function becomes essential under conditions that we have not yet identified. Several examples of redundancy already exist in ubiquitin-dependent processes. Thus, there is evidence for multiple E1 genes in wheat (7), for alternative paths for the degradation of short-lived proteins in yeast (18, 50), and for overlapping function of the subsets of E2s (encoded by the UBC1, UBC4, and UBC5 genes) required for the degradation of short-lived and abnormal proteins in yeast (11, 27, 28). Cells harboring a disruption in the UB8C gene may prove useful in genetic approaches to identify mutations in genes that become essential only in a ubc8 background or whose products interact with the UB8C product (51, 92).

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New Ubiquitin-conjugating Protein


