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# Molecular Cloning of Chick UCH-6 Which Shares High Similarity with Human UCH-L3: Its Unusual Substrate Specificity and Tissue Distribution

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**A full-length cDNA encoding ubiquitin C-terminal hydrolase-6 (UCH-6) was isolated from the chick skeletal muscle cDNA library. The sequence of two peptides generated from purified UCH-6 matched perfectly with the predicted amino acid sequence. Nucleotide sequence analysis of the cDNA containing an open reading frame of 690 base pairs revealed that the protease consists of 230 residues with a calculated molecular mass of 26,315 Da. UCH-6 belonged to members of the UCH family containing highly conserved Cys, His, and Asp domains and showed 86% amino acid identity to human UCH-L3. Interestingly, most tissues examined contained significant amounts of UCH-6 mRNA, while human UCH-L3 is expressed only in the brain, lungs, and red cells. Moreover, UCH-6, unlike other UCH family enzymes including UCH-L3, could release free ubiquitin from ubiquitin-β-galactosidase fusion proteins both *in vivo* and *in vitro*. The ubiquitous expression pattern and unusual substrate specificity of UCH-6 suggest that the enzyme may represent a distinct subfamily of UCH-L3.** 

Covalent modification of proteins by the 76-residue ubiquitin (Ub) polypeptide is involved in many aspects of protein metabolism and cellular functions, including elimination of abnormal proteins, cell cycle progression, signal transduction, transcription, and antigen presentation (1, 2). The known substrates of this ubiquitination pathway include protein kinase (Mos), cyclins, transcription factors (Matα2, GCN4, c-Jun, p53, and NF-κB), inhibitors of cyclin-dependent protein kinases (Sic1, Far1, and Rum1), and subunits of trimeric G proteins (3). Ligation of Ub to other proteins is catalyzed by a family of Ub-conjugating enzymes, called E2s (4), through an isopeptide linkage between the C-terminal Gly residue of Ub and the ε-amino group of Lys residue(s) of the proteins. Ubs by themselves or in conjugation to proteins may also be ligated to additional Ub molecules to form branched poly-Ub by the linkage between the ε-amino group of Lys-48 of one Ub and the C-terminus of the other. Proteins ligated to multiple units of Ub are recognized by the 19S regulatory complex of the 26S proteasome. Proteins bound to the 19S complex are then probably unfolded and translocated into the central cavity of the 20S proteasome where they are degraded to small peptides in an ATP-dependent fashion (1–3, 5).

Ubs are encoded by two distinct classes of genes, neither of which encodes a monomeric form of Ub (6). One is a poly-Ub gene which encodes a linear polymer of Ubs that are linked through peptide bonds between the C-terminal Gly and N-terminal Met of contiguous Ub molecules. The other encodes a fusion protein in which a single Ub is linked to a ribosomal protein consisting of 52 or 76–80 amino acids (7). Thus, generation of free Ub from the linear poly-Ub and Ub-fusion proteins, and recycling of Ub from the branched poly-Ub ligated to proteins should be essential for Ub-requiring processes, including intracellular protein breakdown of ubiquitinated proteins by the 26S proteasome.

Deubiquitinating enzymes can be subdivided into two groups: Ub C-terminal hydrolases (UCHs) and Ub specific proteases (UBPs) (8). As for UBP family, many UBPs have been implicated in numerous cellular processes, such as eye development in fly (9), transcriptional silencing (10), cytokine response (11), and growth regulation (12). As far as UCH family is concerned, a neuron-specific UCH in Aplysia has been reported to be essential for long-term facilitation (13). Recently, Leroy and co-workers have identified in a German family with Parkinson's disease an $Ile^{93}$/Met

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mutation in the UCH-L1 gene, implicating the involvement of Ub pathway in Parkinson's disease (14). Johnston *et al.* (15) determined the crystal structure of UCH-L3 and showed a central antiparallel $\beta$-sheet flanked on both sides by $\alpha$-helices. The structure resembles the well-known papain-like cysteine proteases with the greatest similarity to cathepsin B.

At least 10 different UCHs in chick skeletal muscle have been identified using $^{125}$I-labeled Ub-$\alpha$NH-MHISPPEPESEEEEEHYC (referred to as Ub-PESTc) as a substrate (16, 17). Since the Tyr residue next to the C-terminal Cys can be exclusively radio-iodinated, we could assay the UCH activity by a simple measurement of the radioactivity of the peptide portion that is released into acid-soluble products. Of these, UCH-1 (18), UCH-6 (17), and UCH-8 (19) have been purified and their biochemical properties characterized. Moreover, cDNAs for UBP41, UBP46, UBP52, and UBP66 have recently been isolated from chick skeletal muscle cDNA library using *E. coli*-based *in vivo* screening method (20, 21). Here we report the cDNA cloning, tissue distribution, and unusual substrate specificity of UCH-6 in chick skeletal muscle.

## MATERIALS AND METHODS

*Peptide sequencing of the purified UCH-6.* UCH-6 was purified to apparent homogeneity from chick muscle extracts according to Woo *et al.* (17). Lys-C digestion, peptide separation by HPLC, and peptide sequencing with a sequencer (Applied Biosystems 473A Instrument) were carried out according to standard methods.

*cDNA cloning.* Using obtained two peptide sequences (NACGTIG, GQTEAP), degenerated primers (5′-AATGC(A/G/C/T)TG(C/T)GG(A/G/C/T)AC(A/G/C/T)ATIGG-3′ and 5′-GG(A/G/C/T)GC(C/T)TC(A/G/C/T)-GT(C/T)TGICC-3′, respectively) were designed. PCR was performed using Taq DNA polymerase (Promega) in a GeneAmp 2400 Thermocycler. Reaction mixtures (50 $\mu$l) contained 4 mM $MgCl_2$, 200 $\mu$M each dNTP, 50 mM Tris–HCl (pH 9.0), 50 mM NaCl, and 2.5 units *Taq* DNA polymerase. Approximately 1 $\mu$g of phage DNA from chick muscle cDNA library (Stratagene) was used as a template. The PCR conditions were 94°C/5 min, followed by 30 reaction cycles of 94°C/30 s, 52°C/30 s, and 72°C/1 min. After completion of the cycle, the samples were held at 72°C for 5 min and then cooled to 4°C. About 200 bp-PCR product was ligated into pGEM-T vector (Promega) and named it as pGEM-T/uch-6. The 5′ anchored-PCR method using the 5′-RACE PCR kit (BRL) was employed to clone the ultimate 5′ end. Three primers (5′-AAGCTTCATAAGTTTCTAG-3′, 5′-AAGAATCTTCTAGGAACTTTTTCAG-3′, and 5′-TTTCTCTCTGTTGTTGGCAATAGC-3′) were used, and reactions were performed according to the manufacturer's instructions. About 300 bp-5′-RACE PCR product was obtained and ligated into *Apa*I/*Mun*I site of pGEM-T/uch-6. The 3′ anchored-PCR method to clone the ultimate 3′ end was also employed using two primers (5′-CGAGCCAACAACAGAGAGAAAATG-3′ and 5′-CGAGGCAAAATATCTAGAAACTTATG-3′), and it allowed us to amplify a band of 500 bp. This 500-bp 3′-RACE PCR product was ligated into *Xba*I/*Spe*I site of pGEM-T/uch-6. Full length of UCH-6 cDNA was ligated into *Sph*I/*Sac*I site of pQE31 vector (QIAGEN) for expression, and named it as pQE31/uch-6. The DNA fragments of the cDNAs were sequenced using an automated DNA sequencer (Applied Biosystems 373A Instrument). All sequences were determined on both DNA strands.

*Northern blot analysis.* Myoblasts obtained from breast muscle of 12-day-old chick embryos were cultured as described previously (22). The cells were harvested at 24, 48, 60, 72, and 96 h. Various tissues were prepared from an adult chicken. Skeletal muscle tissues from 12-, 15-, 18-, 21-day-old chick embryos and adult chicken were prepared. From these, total RNAs were obtained, and aliquots (20 $\mu$g each) of them were electrophoresed and transferred to Hybond $N^+$ membranes (Amersham). Hybridization was performed using $^{32}$P-labeled UCH-6 cDNA probe followed by washes under standard conditions and detection by autoradiography.

## RESULTS AND DISCUSSION

*Cloning of a cDNA encoding UCH-6.* Previously, we have identified at least 10 different UCH activities from the extracts of chick skeletal muscle, and have tentatively named them as UCH-1 to UCH-10 (17). Of these, UCH-1 (18), UCH-6 (17), and UCH-8 (19) have been purified and their biochemical properties have been characterized. In the present studies, we have cloned a cDNA that encodes UCH-6 in chick skeletal muscle. Purified UCH-6 was digested with Lys-C enzyme, and the resulting peptides were separated by HPLC using a $C_{18}$ column. These peptides were then subjected to automated Edman degradation for determining their amino acid sequences. Degenerated primers, synthesized on the basis of two peptide sequences (NACGTIG and GQTEAP) were used to amplify chick muscle cDNAs by PCR. About 200 bp-PCR product was obtained. Several independent clones having a 200-bp cDNA insert were also sequenced on both ends, and two peptide sequences used were verified as well. They did not exhibit any sequence difference indicating no PCR error. The 5′ and 3′ end of the cDNA were obtained through the 5′ and 3′ anchored PCR technique. The position of the start (ATG) codon was inferred to yield the 690-base pair open reading frame, which suggest that the cDNA clone encodes a protein of 230 amino acids (calculated $M_r$ 26,315 Da) with a p*I* of 4.91 (Fig. 1). The BLAST search revealed the overall amino acid sequence of UCH-6 is highly homologous to human UCH-L3 with an identity of 86%. The amino acid identity of UCH-6 to human UCH-L1 is 52%, and to yeast Yuh1 is 32%. Three residues, Cys-95, His-169, and Asp-184, which are necessary for catalytic activity (15), are conserved well in the chick UCH-6 amino acid sequence. ELDGR, a conserved sequence in UCH family that is believed to participate in substrate binding, is also present (Fig. 2).

*Ubiquitous expression of UCH-6 mRNA.* Wilkinson *et al.* (23) have reported that there is considerable tissue specificity in the distribution of UCH isozyme L1, L2, and L3. UCH-L1 was found in brain and testis, highly innervated tissues. Trace amounts of UCH-L1 were detected in heart. Most tissues examined contained significant amount of UCH-L2, and hence UCH-L2 has been regarded as a constitutive or 'housekeeping' enzyme. UCH-L3 was found only in the red cells, lungs, and brain. To determine the expression pattern of UCH-6 mRNA in various tissues, we per-

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                                                    CGCTGTCAGCATCGTGCTGCCCGCCGCC(-28)
ATGGAGCCGCATCGCTGGCTGCCGCTGGAGGCCAACCCCGACGTCACCAATCAGTTTCTC 60
 M  E  P  H  R  W  L  P  L  E  A  N  P  D  V  T  N  Q  F  L  20
AAACAGTTAGGTATACATCCTGACTGGCAATTTGTAGATGTCTATGGTATGGAACCAGAA 120
 K  Q  L  G  I  H  P  D  W  Q  F  V  D  V  Y  G  M  E  P  E  40
CTGCTTAGCATGGTGCCAAGACCTGTATGTGCAGTGCTTCTTCTCTTTCCAATAACAGAA 180
 L  L  S  M  V  P  R  P  V  C  A  V  L  L  L  F  P  I  T  E  60
AAGTATGAGACCTTCAGAACAGAAGAAGAAGAGAGGATAAAAGCTAAGGGACAAGATGTC 240
 K  Y  E  T  F  R  T  E  E  E  E  R  I  K  A  K  G  Q  D  V  80
AAATCATCAGTGTATTTCATGAAGCAAACTATCAACAATGCTTGTGGGACAATTGGGCTT 300
 K  S  S  V  Y  F  M  K  Q  T  I  N  N  A  C  G  T  I  G  L  100
ATTCATGCTATTGCCAACAACAGAGAGAAAATGAACTTTGAAACCAATTCATCACTGAAA 360
 I  H  A  I  A  N  N  R  E  K  M  N  F  E  T  N  S  S  L  K  120
AAGTTCCTAGAAGATTCTTTATCTATGACTCCTGAAGAGAGGGCAAAATATCTAGAAACT 420
 K  F  L  E  D  S  L  S  M  T  P  E  E  R  A  K  Y  L  E  T  140
TATGAAGCAATTCGTGTCACTCATGAATCCAGTGCCCATGAAGGTCAGACTGAGGCACCA 480
 Y  E  A  I  R  V  T  H  E  S  S  A  H  E  G  Q  T  E  A  P  160
AGTATAGATGAAAAAGTAGATCTTCACTTTATTGCATTAGTTAACGTAGGTGGTCATCTC 540
 S  I  D  E  K  V  D  L  H  F  I  A  L  V  N  V  G  G  H  L  180
TATGAATTGGATGGACGCAAGCCATTTCCAATAAACCACGGGGAAACCAGCGATGATTCT 600
 Y  E  L  D  G  R  K  P  F  P  I  N  H  G  E  T  S  D  D  S  200
TTTTTAGAGGATGCAATAGAGGTTTGCAAGAAATTCATGGAACGTGACCCAGAAGAATTA 660
 F  L  E  D  A  I  E  V  C  K  K  F  M  E  R  D  P  E  E  L  220
AGATTTAATGCAATCGCACTGTCTGCAGCTTAAAAACTTTTCCACTTGGGCTAACTAACC 720
 R  F  N  A  I  A  L  S  A  A  *                              230
TATTGCAATGTATTGTAACGAGATAACTGTTGCCATATGTTAATAGTACAAGTTTTGATA 780
CTTCCCTAACATGTTGATTAATTGTAGCACATGTGGGAATAAACTTTGCATTTGTCCTTG 840
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  891
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**FIG. 1.** Nucleotide sequence of a cDNA for UCH-6 and deduced amino acid sequence of the UCH-6 protein. The nucleotides are numbered on the right, beginning at the A of the presumed start codon. The amino acid residues are also numbered on the right. Two peptide sequences obtained by Edman degradation were underlined. The asterisk indicates the TAA stop codon (GenBank Accession No. AF167995).

formed Northern hybridization analysis. Interestingly, the UCH-6 mRNA was detected in all adult tissues tested (Fig. 3A), despite our finding that chick UCH-6 shares high sequence similarity with UCH-L3. Rather, its ubiquitous expression pattern suggests that UCH-6 may belong to the family of UCH-L2. So far, only four peptide sequences of UCH-L2 from bovine liver have been reported in the GenBank database. However, the amino acid sequence of UCH-6 shares much lower similarity with the partial sequence of UCH-L2 than that of UCH-L3. One other possible explanation is that UCH-6 represents a distinct subfamily of UCH-L3 that shows different expression patterns.

It has been reported that the amount of UCH-L3 mRNA is the highest in the 11-day-old mouse embryos, and markedly decreased in the 15- and 17-day-old embryos. Therefore, it has been suggested that expression of UCH-L3 is down-regulated during development (24). To determine whether the expression of UCH-6 is also developmentally regulated, Northern blot analysis was performed using muscle tissues obtained from 12-, 15-, 18-, and 21-day chick embryos (Fig. 3B) as well as using cultured myoblasts in myogenic differentiation (Fig. 3C). However, little or no change in the level of UCH-6 mRNA was observed during the development of embryonic muscle or differentiation of myoblasts. Thus, UCH-6, unlike UCH-L3, appears to be a constitutive or “housekeeping” enzyme.

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Chick UCH-6    MEPHRW--LPLEANPDVTNQFLKQLGIHPDWQFVDVYGM-EPELLSMVPRPVCAVLLLFP
Human UCH-L3   MEGQRW--LPLEANPEVTNQFLKQLGLHPNWQFVDVYGM-DPELLSMVPRPVCAVLLLFP
Human UCH-L1   MQLK-----PMEINPEMLNKVLSRLGVAGQWRFVDVLGL-EEESLGSVPAPACALLLLFP
Yeast Yuh1     MSGENRAVVPIESNPEVFTNFAHKLGLKNEWAYFDIYSLTEPELLAFLPRPVKAIVLLFP

Chick UCH-6    ITEKYETFRTEEEERIKAKGQDVKSSVYFMKQTINNPCGTIGLIHAIANNREKMNFETNS
Human UCH-L3   ITEKYEVFRTEEEEKIKSQGQDVTSSVYFMKQTISNACGTIGLIHAIANNKDKMHFESGS
Human UCH-L1   LTAQHENFRKKQIEELK--GQEVSPKVYFMKQTIGNSCGTIGLIHAVANNQDKLGFEDGS
Yeast Yuh1     INED----RKSSTSQQITSSYDV---IWF-KQSVKNACGLYAILHSLSNNQSLL--EPGS

Chick UCH-6    SLKKFL-EDSLSMTPEERAKYLETYE-AIRVTHESSAH--EGQTEAPSIDEKVDLHFIAL
Human UCH-L3   TLKKFL-EESVSMSPEERARYLENYD-AIRVTHETSAH--EGQTEAPSIDEKVDLHFIAL
Human UCH-L1   VLKQFL-SETEKMSPEDRAKCFEKNE-AIQAAHDAVAQ--EGQC---RVDDKVNFHFILF
Yeast Yuh1     DLDNFLKSQSDTSSSKNRFDDVTTDQFVLNVIKENVQTFSTGQSEAPEATADTNLHYITY

Chick UCH-6    VNVGGHLYELDGRK---PFPINHGETSDDSFLEDAIEVCK--KFMERDPEE--LRFNAIA
Human UCH-L3   VHVDGHLYELDGRK---PFPINHGETSDETLLEDAIEVCK--KFMERDPDE--LRFNAIA
Human UCH-L1   NNVDGHLYELDGRM---PFPVNHGASSEDTLLKDAAKVCR--EFTEREQGE--VRFSAVA
Yeast Yuh1     VEENGGIFELDGRNLSGPLYLGKSDPTATDLIEQELVRVRVASYMENANEEDVLNFAMLG

Chick UCH-6    LSAA--
Human UCH-L3   LSAA--
Human UCH-L1   LCKAA-
Yeast Yuh1     LGPNWE
```

**FIG. 2.** Sequence similarity among UCH family. Sequence similarity between chick UCH-6 (this work), human UCH-L3 (23), human UCH-L1 (23), and yeast Yuh1 (27) is shown. Identical amino acid residues are shaded, conserved Cys, His, Asp residues are bolded, and ELDGR residues for substrate binding are underlined.

*Hydrolysis of Ub-β-galactosidase by UCH-6.* The UCH family is a group of closely related thiol proteases, including the mammalian UCH-L1, L2, and L3 as well as yeast Yuh1, all of which have a relatively small size of 20–30 kDa and exhibit no apparent similarity to the UBP family (23). The UCH family enzymes prefer to cleave off small leaving groups and/or extended peptide chains from the C-terminus of Ub, whereas UBP enzymes release the C-terminal extensions regardless of their size. For example, UCH-L3 has been shown to cleave off peptide extensions of up to 20 residues from the C-terminus of Ub with high efficiency and low sequence preference, but not larger extensions, such as the β-galactosidase (gal) fusions of Ub (25). It has also been reported that UCH-L1 neither can release free Ub from Ub-β-gal fusion proteins (25).

To determine whether UCH-6 can release Ub from Ub-β-gal fusion proteins, UCH-6 and Ub-β-gal were coexpressed in the same cell. *E. coli* JM101 cells harboring pACUb-β-gal ($cm^r$) were transformed with pQE31/uch-6 ($amp^r$), and plated on LB agar containing both chloramphenicol and ampicillin to select for cotransformant. Induction with IPTG resulted in coexpression of the UCH-6 enzyme along with Ub-β-gal fusion proteins. Processing activity of UCH-6 was assessed by immunoblotting using β-gal antibody. As shown in Fig. 4A, UCH-6 was capable of generating free Ub from Ub-β-gal *in vivo.* To confirm whether Ub-β-gal can also be hydrolyzed *in vitro* by purified UCH-6, the enzyme was incubated with the extracts of *E. coli* JM101 cells transformed with pACUb-β-gal. Immunoblot analysis was performed with β-gal antibody. Figure 4B shows that UCH-6 can release Ub from Ub-β-gal *in vitro* as well. On the other hand, Yuh1, which belongs to members of UCH family, could not cleave the same substrate *in vitro.* Thus, it seems clear that chick UCH-6 is capable of cleaving the C-terminus of Ub regardless of the size of its extensions, similar to UBP enzymes but unlike to yeast Yuh1 or mammalian UCH-L1 or L3. This finding is

rather surprising, because chick UCH-6 shares high sequence similarity with UCH-L3 but not at all with any of UBP enzymes so far been reported. These results again suggest that UCH-6 may represent a distinct subfamily of UCH-L3 that has different substrate specificity.

It has recently been demonstrated that UCH-L3 is also capable of cleaving the C-terminus of NEDD8 (24). Therefore, we generated various Ub-like molecule (Ubl)-$\beta$-gal fusion proteins, such as SUMO1-$\beta$-gal, NEDD8-$\beta$-gal, Smt3-$\beta$-gal, Rub1-$\beta$-gal, and FUb-$\beta$-gal (26). UCH-6 could not hydrolyze any of these Ubl-$\beta$-gal fusion proteins (data not shown). Thus, the activity of UCH-6 appears to be specific for releasing Ub.

In conclusion, although chick UCH-6 shares high sequence similarity with human UCH-L3, we suggest that UCH-6 may represent a distinct subfamily of UCH-L3 that shows ubiquitous expression pattern and unusual substrate specificity. These differences in expression pattern and substrate specificity may be due to slight sequence differences and spatial differences wherein these isozymes are expressed. The observed tissue specificity of the UCH family enzymes may reflect a distinct set of substrates. The elucidation of physiological roles of the UCH enzymes and assessment of distribution of *in vivo* substrates will answer these questions.

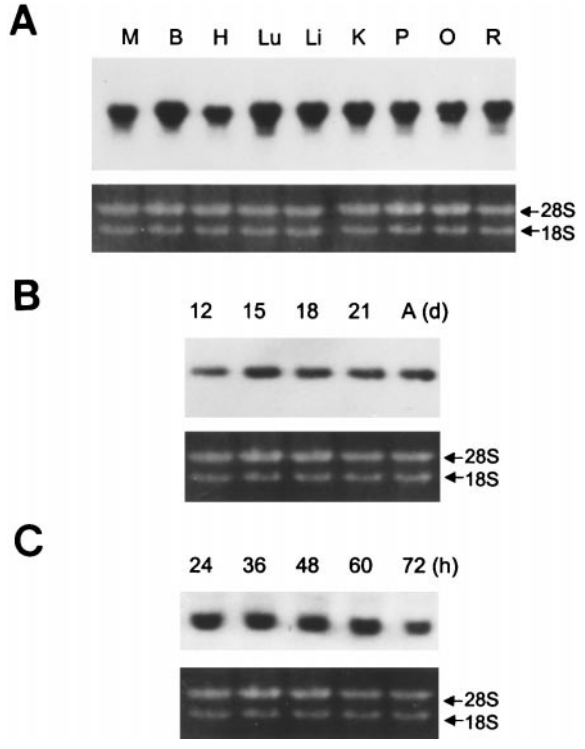


**FIG. 3.** Expression of chick UCH-6 mRNA in various adult tissues (A), muscle tissues from embryos and adult (B), and cultured myoblasts (C). Total RNA (20 $\mu$g) from the indicated sources were run on a denaturing gel, transferred to Hybond $N^+$ membranes, and hybridized with $^{32}$P-labeled cDNA of chick UCH-6. The 18S and 28S RNAs were used as a control for loading. Abbreviations are M, muscle; B, brain; H, heart; Lu, lung; Li, liver; K, kidney; P, pancreas; O, ovary; R, red cell; A, adult chicken.

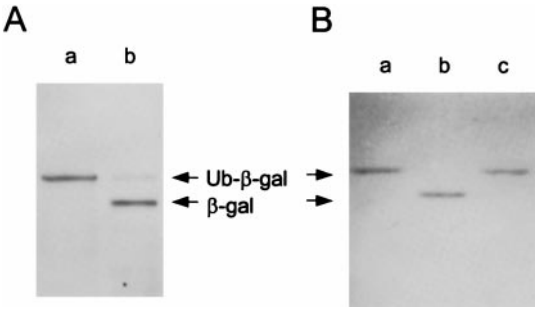


**FIG. 4.** Hydrolysis of Ub-$\beta$-gal by UCH-6 *in vivo* and *in vitro*. (A) The *E. coli* cells harboring pACUb-$\beta$-gal were cotransformed with pQE31/uch-6, and incubated for 4 h at 37°C in the absence (lane a) or presence of IPTG (lane b). The cells were harvested and subjected to SDS–PAGE followed by immunoblot analysis using $\beta$-gal antibody. (B) The extracts (50 $\mu$g) obtained from *E. coli* JM101 cells expressing Ub-$\beta$-gal were incubated for 2 h at 37°C in the absence (lane a) or presence of 0.5 $\mu$g of purified UCH-6 (lane b) or Yuh1 (lane c). After incubation, the samples were electrophoresed and subjected to immunoblot analysis as above.

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