Cloning of a novel ubiquitin-conjugating enzyme (E2) gene from the ciliate *Paramecium tetraurelia*

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Abstract We isolated a 1.7 kb gene (UbcP1) for a ubiquitin-conjugating enzyme from a *P. tetraurelia* cDNA library and sequenced it. Its deduced polypeptide sequence consists of 425 amino acid residues (48 kDa). The UbcP1 protein contains novel N- and C-terminal extensions in addition to a UBC domain, and within the UBC domain it shares low identity with sequences of other known E2s. A constructed phylogenetic tree suggests that the UbcP1 protein may represent a member of a distinct subfamily of E2s. Southern blot analysis showed that the N-terminal extension of the UbcP1 is conserved in *P. multi-micronucleatum*.

Key words: Ubiquitin-conjugating enzyme (E2); cDNA cloning; Nucleotide sequence; Phylogenetic tree; *Paramecium tetraurelia*

1. Introduction

The covalent attachment of ubiquitin to proteins is involved in numerous processes in eukaryotic cells, such as selective protein degradation [1], DNA repair [2], cell cycle control [3], and possibly the regulation of chromatin structure [4].

Ubiquitin-conjugating enzymes (E2s) participate in the conjugation of ubiquitin to proteins [5,6]. So far the molecular genetics of the E2 gene family have been studied most extensively in the budding yeast *S. cerevisiae*. To date, 10 E2 genes with diverse functions have been isolated [5]. For instance, the UBC4 and UBC5 genes are involved in stress-related functions and in the turnover of regulatory proteins [7,8], and UBC3 (CDC34) is involved in the transition from the G_1 to the S phase of the cell cycle [3]. UBC2 (RAD6) functions in DNA repair, sporulation, and induced mutagenesis [2]. All of these E2s contain a conserved 'core' sequence composed of approximately 150 residues called the UBC domain. The cysteine residue, which is involved in the formation of a thiol ester bond with the C-terminus of ubiquitin, is also found in this region [9–11].

Paramecia are unicellular ciliated protozoa, but they do have something in common with higher organisms. For instance, they have evolved separate somatic and germ-line functions using two distinct nuclei, macro- and micronuclei, respectively [12]. In this respect, they can be used as a model system for multicellular organisms. Paramecia are quite suitable for cell cycle research, because meiosis can easily be synchronized. To clarify the roles of ubiquitin conjugation in cell cycle control, we have begun to clone and characterize the E2 gene from *P. tetraurelia*. The vast amount of genetic information available on this organism makes it an excellent model organism. Here we report the cloning, the sequence, and the phylogeny of UbcP1, a novel E2 gene found in *P. tetraurelia*.

2. Materials and methods

2.1. Strains

P. tetraurelia stock 51s and *P. multimicronucleatum* stock CH were used for DNA preparation.

2.2. Isolation of genomic DNA

Paramecium genomic DNA was isolated according to Tsukii's method (Dr. Y. Tsukii, Laboratory of Biology, Hosei University, Tokyo, Japan; personal communication). Cells (400 ml culture) were collected by low-speed (about $400 \times g$) centrifugation and washed in distilled water. Packed cells (about 50 μl) were suspended in 1 ml of a lysing solution (1% acetic acid, 1% Nonidet P-40, 0.25 M sucrose) by pipetting. The sample was centrifuged at about $190 \times g$ for 5 min to collect macronuclei. The precipitate was suspended in 0.5 ml of another lysing solution (1% sodium dodecyl sulfate (SDS), 0.1 M NaCl, 0.1 M Tris (pH 9.0)), and treated with 0.5 ml of phenol:chloroform (1:1). The DNA in aqueous layer was precipitated with an equal volume of 2-propanol, then rinsed with 70% ethanol, and dried. The sample was then suspended in 50 μl of TE (10 mM Tris (pH 7.5), 1 mM EDTA).

2.3. cDNA cloning and sequencing

Three degenerate oligonucleotides, 5′-A(A/G)TTT(A/G)(G/T)C(A/T)AT(G/T)(G/T)C(A/T)GATTT-3′ (primer A), 5′-AT(A/T)TC(A/T)AT(A/T)GATAGATA-3′ (primer B), and 5′-TGGATTAT(G/A)AA(T/C)(A/T)GC(T/A)G-3′ (primer C) were designed based on the conserved regions of E2s. Polymerase chain reaction (PCR) was carried out in 100 μl containing 1 μg genomic DNA, 200 pmol of each primer, 20 nmol of each dNTP and 2.5 U of AmpliTaq DNA polymerase (Takara) in PCR buffer provided by the manufacturer. The amplified 489 bp DNA fragment was purified and cloned into the PCR-II plasmid vector (Invitrogen).

P. tetraurelia total RNA was isolated, using an RNA extraction kit (Pharmacia), from cultures at the logarithmic and stationary phases of growth. Poly(A)$^+$ RNA was isolated from total RNA using a mRNA purification kit (Pharmacia). Complementary DNA was synthesized with oligo(dT) primer by reverse transcription, and adapter-ligated using a Timesaver cDNA synthesis kit (Pharmacia). A λ-gt10 library was constructed using a λ-DNA in vitro packaging module and a cDNA rapid cloning module λ-gt10 (Amersham) following manufacturer's protocols.

For the initial screening, the recombinant phages were plated on *E. coli* NM514 (Amersham) at 10000 plaque forming units/150 mm Petri dish, and plaques were transferred to nylon membranes (Hybond N$^+$, Amersham). The 489 bp PCR fragment was radiolabelled using a Nick Translation Kit (Takara), and then used to screen the cDNA library, and two positive clones were identified. The cDNA inserts were subcloned into the M13mp18 phage vector or the PUC19 plasmid vector.

The nucleotide sequences were determined using an Applied Biosystems 373A automated DNA sequencer. Nucleotide or amino acid sequence searches, and protein motif searches were performed in the GenBank, SWISS-PROT, and PROSITE data bases using the FAS-

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The nucleotide sequence presented here has been submitted to the EMBL/GenBank/DDBJ database under accession number D50499.

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TA and MOTIF network services at the Human Genome Center, Institute of Medical Science, The University of Tokyo. The protein sequence alignment was obtained using the pileup program of the Wisconsin Sequence Analysis Package made by the Genetics Computer Group (GCG). A phylogenetic tree was constructed using the ODEN programs of the DNA Research Center, National Institute of Genetics, Japan by the maximum parsimony method [13].

2.4. Southern analysis

Various genomic DNAs were digested completely with various restriction enzymes and electrophoresed in 0.7% agarose gels. The DNA fragments were then transferred by vacuum blotting to nylon membrane filters (Hybond-N^+). Two kinds of DNA fragments were labeled and used to probe the filter using the ECL direct nucleic acid labeling and detection systems (Amersham). Stringency of hybridization and washing were controlled by altering the salt concentrations in the hybridization buffer and the urea and SSC (150 mM NaCl, 15 mM sodium citrate) concentration in the primary washing buffer according to the manufacturer's instructions (Amersham).

2.5. Hybridization probes

Using the UbcP1 cDNA subclone as a template, two kinds of DNA fragments were amplified by PCR and used as probes. A 484 bp PCR fragment, which covers almost the same region as the 489 bp fragment, was generated using primer D (5′-TATGAAAAAAGAAGAAATTTTAAAG-3′) and primer E (5′-CAATTCTGGCTCATGC-3′). A 594 bp PCR fragment, which corresponds to a part of the N-extension of UbcP1, was generated using primer F (5′-CTATCTAGAATTGGAGCTGTC-3′) and primer G (5′-TTAGACATCCATTGATACAAC-3′).

3. Results and discussion

We screened for E2 genes in *P. tetraurelia*. Primer pairs covering the conserved part of the UBC domain were designed and used for PCR with *P. tetraurelia* genomic DNA as the template. A distinct fragment, roughly equivalent in size to the corresponding region within yeast E2 genes, was amplified. DNA sequence analysis indicated that the amplified fragment shared sequence homology with the E2 gene family. Using the cloned PCR fragment as a probe, we screened 170000 plaques of a *P. tetraurelia* cDNA library, and two positive clones were obtained. We sequenced the longer, 1.7 kb cDNA, clone. We named the gene UbcP1 (ubiquitin-conjugating enzyme from *Paramecium*; the numbering reflects the order of identification).

The DNA sequence and the deduced amino acid sequence are shown in Fig. 1. The 1.7 kb cDNA contains an open reading frame which encode a protein of 425 amino acids. The calculated molecular mass of the deduced amino acid sequence of UbcP1 is 48 kDa. We assigned the translation start codon, ATG, to position 349 since there is no in-frame ATG codon between the 5′ terminal and this one. Sixteen of the 20 glutamines of this gene were encoded by TAA or TAG. It has been shown that TAA and TAG encode glutamine instead of causing termination in the genes of several ciliates [12]. The predicted UbcP1 protein contains the conserved active site region consensus pattern (FYWLS)-H-(PC)-(NH)-(LIV)-X3,4-G-X-(LIV)-C-(LIV)-X-(LIV), which is found in almost every known E2 protein (PROSITE data base, accession number PS00183). The conserved cysteine residue at position 325, within the consensus region, is the active site amino acid that is essential for the transfer of ubiquitin. UbcP1p (the product of the UbcP1 gene) is, however, less related to the E2s isolated previously. A comparison of UbcP1p with E2s from a number of species is shown in Fig. 2.

So far, E2 enzymes have been classified into different classes

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GTAAAACAAAAATTAATTATTGATGATCCTTAAAAACAGATAAATGACTTAAAAAAATAA   60
ATATTTAAGAACTTTTTAGAGAATGGAATTAATATTGATGAGAATTAAGTTTTGCTTTAA  120
GATGAAGAAGGATTTATTTTAAATGAAAGTGAAACAATTGGAAATTTACTGAAAACAAAT  180
GATATAGTAAAGGTAGTGTAATAGGAATAAAAATAAAATATAGTAAATCAGTAATAATAA  240
CAACAAATAGTAGCTTAAGAAGAATAATAAGAATAATTATAGTAAGATCTCAATCAAAAT  300
AATGATCCTGTAGAAGCTATAGTTGTGTTATTTGATATTTCAGGATCAATGGGCGGAATG  360
                                          M  G  G  M      4

TATTTTAAAGAAGAGGAACTATCTAGAATTGGAGCTGTCAATGCGTTTTTCTCTGCCTTT  420
Y  F  K  E  E  E  L  S  R  I  G  A  V  N  A  F  F  S  A  F     24

GCCGATAAAACATTGGCTTTTGAATTTAATCACATAGTAAAACTTGTATGGTTTGAATCC  480
A  D  K  T  L  A  F  E  F  N  H  I  V  K  L  V  W  F  E  S     44

TTTATAACAGATAAATGTGACTTCACAAACGATTTTAATAATTTCATAAAATTAGTCGAT  540
F  I  T  D  K  C  D  F  T  N  D  F  N  N  F  I  K  L  V  D     64

GATGCATCCCCAAGAGGTGGAACTAAATGTTATGATGCAATAGCCTATGCAATTGAATAG  600
D  A  S  P  R  G  G  T  K  C  Y  D  A  I  A  Y  A  I  E  Q     84

TTGAAAGAAATCAAGAAAAAATATCCAAATATTATCTTAAGAATAATTGCATTAACTGAT  660
L  K  E  I  K  K  K  Y  P  N  I  I  L  R  I  I  A  L  T  D    104

GGAGACGATAATTAATCAAAAGAAAATCCTTAAAGTTTAGTTAATAGAATATTTGAAAAT  720
G  D  D  N  Q  S  K  E  N  P  Q  S  L  V  N  R  I  F  E  N    124

TAAATTATAATAGATTCCTTTGTTGTAAATAATGATTGTGTTGGTTTAAAAACTTTGACT  780
Q  I  I  I  D  S  F  V  V  N  N  D  C  V  G  L  K  T  L  T    144

CATGCAACAAATGGTAGATGCTATTGTCCTTAAACTTTGGCTGAAGGCATGTCTTTATTT  840
H  A  T  N  G  R  C  Y  C  P  Q  T  L  A  E  G  M  S  L  F    164

GAAATAGAAAGCATATTAAGTATAAGCCATAGAGAACAAAAAGAGTATCCAAAAGAATTG  900
E  I  E  S  I  L  S  I  S  H  R  E  Q  K  E  Y  P  K  E  L    184

CAAGATTTAAATTCATTGAAAGATAAACCTTTTGATACTGATGGAATGAAAGTTGTATCA  960
Q  D  L  N  S  L  K  D  K  P  F  D  T  D  G  M  K  V  V  S    204

ATGGATGTCTAAAAAATTGCAGTTATGAAAAAAGAAGAAATTTTAAAGAAAATATCACAA 1020
M  D  V  Q  K  I  A  V  M  K  K  E  E  I  L  K  K  I  S  Q    224

ATCGAAAGCGCCCCTTAAAATTCTAGCTCAACCTCTTAGAGTGTAAATAACACAGCAAGA 1080
I  E  S  A  P  Q  N  S  S  S  T  S  Q  S  V  N  N  T  A  R    244

ATTTTAAAAGAACTCCAAGATGTTACAGCATAGGGTGATAAATTGAATTTTAAATGTTAT 1140
I  L  K  E  L  Q  D  V  T  A  Q  G  D  K  L  N  F  K  C  Y    264

CCCACTGCTGATGATATCAAAACATGGAAGATCTTATTATATGGTCCTAAAGGCACAGTT 1200
P  T  A  D  D  I  K  T  W  K  I  L  L  Y  G  P  K  G  T  V    284

TATGAAGGTGGATTATACATTTAAGTTATGTTTTCACTTAAAATTATCCTTTCAGACCT 1260
Y  E  G  G  L  Y  I  L  S  Y  V  F  T  Q  N  Y  P  F  R  P    304

CCAAAAGTGTAGTTCATCACTAAATTATATCATCCTAATGTCAGTAGAGGCGGATCACTT 1320
P  K  V  Q  F  I  T  K  L  Y  H  P  N  V  S  R  G  G  S  L    324

TGCTTGGATGTTTTAAATACTTCATGGAGTCCTTTACTCACAACTACAAAAGTACTTGAT 1380
C  L  D  V  L  N  T  S  W  S  P  L  L  T  T  T  K  V  L  D    344

GCAGTTTCAGTGATGCTTTAAAATCCAAATGCTGATGATGCTTTGGATTGCAATATAGCA 1440
A  V  S  V  M  L  Q  N  P  N  A  D  D  A  L  D  C  N  I  A    364

GCAATTTATAAGCATGAGCCAGAATTGTTTAAATAAAATGCCTTAAAAGAGAAATTAGAG 1500
A  I  Y  K  H  E  P  E  L  F  K  Q  N  A  L  K  E  K  L  E    384

GCTGCTTCTCCATCAGAGGACAATCTATTAGCTGATATCTTAGGAGCTGTTGATATTAAT 1560
A  A  S  P  S  E  D  N  L  L  A  D  I  L  G  A  V  D  I  N    404

TCGTAAGAGTATCTAGATACAAAGAAAGAATTATAAACCTGGTTGGAATATTAAAAATCA 1620
S  Q  E  Y  L  D  T  K  K  E  L  Q  T  W  L  E  Y  Q  K  S    424

AATTGAAGATATATATATTTGTCATTAGAAATAATTAAATTTAAAAAAAAAAAA        1676
N  *                                                           425
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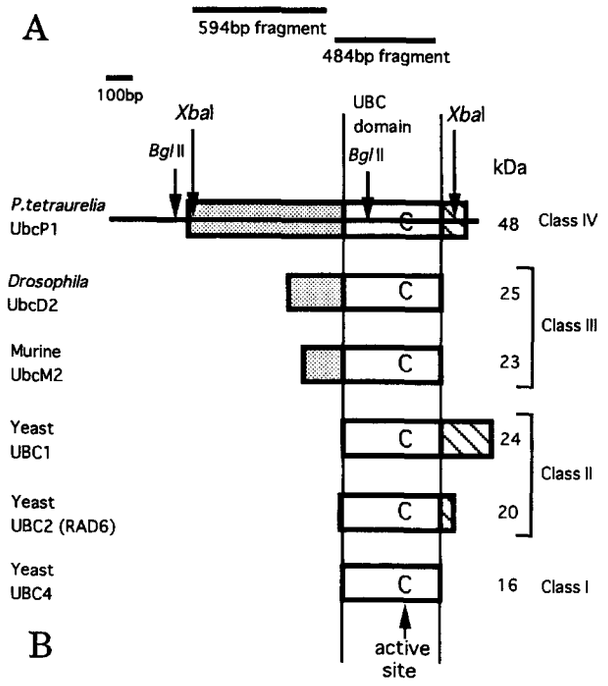
Fig. 1. The nucleotide and deduced amino acid sequences of the UbcP1 gene of *P. tetraurelia*. The asterisk represents a stop codon. The underlined amino acid region corresponds to the UBC domain. The consensus pattern of the active site region has been doubly underlined. The cysteine residue (the putative active site) is highlighted.

structurally [5,6]. Class I E2s are small proteins of about 16–18 kDa and have a conserved UBC domain. Class II E2s have C-terminal extensions of various lengths. Several extensions have a high content of charged residues [2,3,14–16]. Recently a subfamily of E2s with distinct N-terminal extensions has been identified (class III) [17,18]. These enzymes possess highly charged N-terminal extensions which contain clusters of serine/threonine residues, which are thought to be phosphorylation sites. A murine UbcM1 protein was reported to have both N- and C-terminal extensions (class IV) [5,6]. The UbcM1 protein has, in addition to a small C-terminal extension, a long N-terminal extension with several putative transmembrane regions.

UbcP1p possesses a C-terminal extension (37 amino acids) and a long N-terminal extension (238 amino acids) in addition to the UBC domain, so it belongs to class IV. The sequence of UbcM1 has not been revealed yet, so it is unclear whether

UbcP1 shares sequence homology with UbcM1 or not. Computer aided sequence comparisons of the UBC domain of UbcP1p with the sequences of other known E2s in the data bases revealed that homology is not very high, but it shares the highest degree of homology with yeast UBC4/5 (an enzyme pair expressed from duplicated genes [7,19]) and their respective homologues in higher eukaryotes, including the *Drosophila* UbcD1 [20], *Caenorhabditis elegans* ubc-2 [21], human UbcH5 [22], and plant *Arabidopsis* AtUBC8 [23] gene products, which all belong to class I. These homologues share about 80% homology with the yeast UBC4 protein sequence. The UBC domain of UbcP1p only shares about 40–44% identity with this evolutionarily conserved subfamily. Moreover UbcP1p has N- and C-terminal extensions. These suggest that UbcP1 may not be functionally related to UBC4/5. As shown in the phylogenetic tree in Fig. 3, UbcP1p is less related to previously isolated E2s than these proteins are to each other. Thus UbcP1p may represent a member of a distinct subfamily of E2s.



B

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Yeast UBC4          MSSSKRIAKELSDLE-RDPPTSCSAGPVGDDLYHWQASI
Arabidopsis AtUBC8   MASKRILKELKDLQ-KDPPTSCSAGPVAEDMFHWQATI
C.elegans ubc-2      MALKRIQKELQDLG-RDPPAQCSAGPVGDDLFHWQATI
Murine UbcM2        .STSAKRIQKELAEIT-LDPPPNCSAGPKGDNIYEWRSTI
Human UbcH2         .SPGKRRMDTDVVKLIESKHEVTILGG-----LNEFVVKF
Yeast UBC2          .TPARRRLMRDFKRMK-EDAPPGVSASPLPDNVMVWNAMI
Drosophila bendless .SSLPRRIIKETQRLM-QEPVPGINAIPDENNARYFHVIV
P.tetraurelia UbcP1 .VNNTARILKELQDVTAQGDKLNFKCYPTADDIKTWKILL
                                                        *
MGPADSPYAGGVFFLSIHFPTDYPFKPPKISFTTKIYHPNIN-ANGNICLDILKDQ
MGPAESPYSGGVFLVTIHFPPDYPFKPPKVAFRTKVFHPNIN-SNGSICLDILKEQ
MGPPESPYQGGVFFLTIHFPTDYPFKPPKVAFTTRIYHPNIN-SNGSICLDILRSQ
LGPPGSVYEGGVFFLDITFSSDYPFKPPKVTFRTRIYHCNIN-SQGVICLDILKDN
YGPQGTPYEGGVWKVRVDLPDKYPFKSPSIGFMNKIFHPNIDEASGTVCLDVINQT
IGPADTPYEDGTFRLLLEFDEEYPNKPPHVKFLSEMFHPNVY-ANGEICLDILQNR
TGPNDSPFEGGVFKLELFLPEDYPMSAPKVRFITKIYHPNID-RLGRICLDVLKDK
YGPKGTVYEGGLYILSYVFTQNYPFRPPKVQFITKLYHPNVS-RGGSLCLDVLNTS

WSPALTLSKVLLS-ICSLLTDANPDDPLVPEIAHIYKTDRPKYEATA-REWTKKYAV
WSPALTISKVLLS-ICSLLTDPNPDDPLVPEIAHMYKTDRAKYEATA-RNWTQKYAM.
WSPALTISKVLLS-ICSLLCDPNPDDPLVPEIARIYKTDRERYNQLA-REWTQKYAM
WSPALTISKVLLS-ICSLLTDCNPADPLVGSIATQYLTNRAEHDRIA-RQWTKRYAT
WTALYDLTNIFESFLPQLLAYPNPIDPLNGDAAAMYLHRPEEYKQKI-KEYIQKYAT.
WTPTYDVASILTS-IQSLFNDPNPASPANVEAATLFKDHKSQYVKRV-KETVEKSWE.
WSPALQIRTILLS-IQALLSAPNPDDPLANDVAELWKVNEAEAIRNA-REWTQKYAV.
WSPLLTTTKVLDA-VSVMLQNPNADDALDCNIAAIYKHEPELFKQNALKEKLEAASP.
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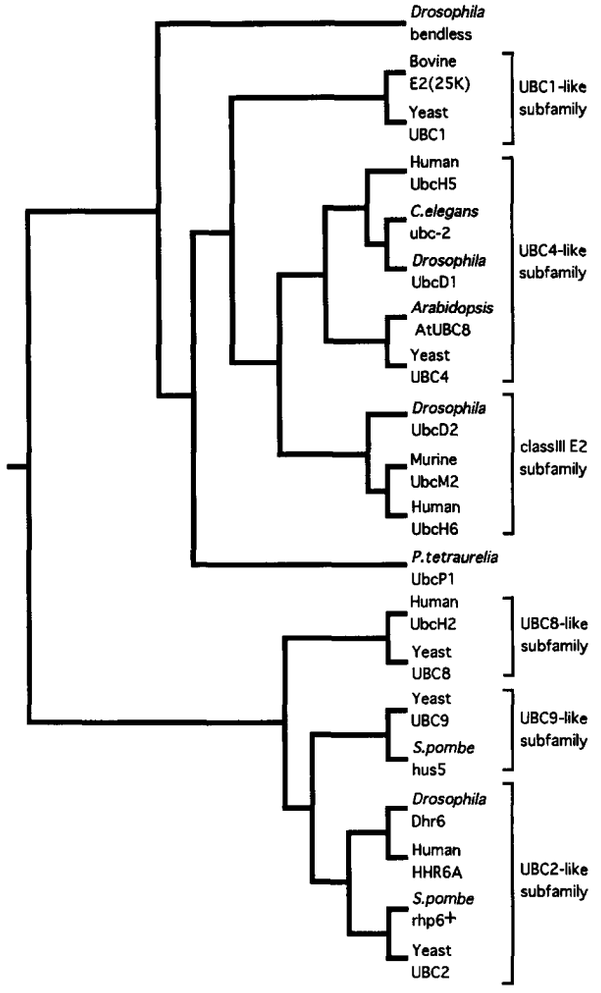

Fig. 3. Phylogenetic tree of the E2 family. Only the UBC domains were compared.

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Fig. 2. Comparison of UbcP1p sequence with sequences of other E2 proteins. A: Schematic diagram of the structures of E2 proteins. The protein sequences of the six different E2s are shown as long boxes. The size of the proteins is given in kDa. The central regions (white boxes) are the conserved domains of the E2 proteins (UBC domains). The position of the cysteine residues (the active sites) are indicated by a C. The N-terminal extensions of class III E2s and UbcP1p are indicated by gray boxes. The C-terminal extensions of class II E2s and UbcP1p are indicated by the striped boxes. The nucleotide sequence of UbcP1 is shown as a line drawn through the box representing the protein sequence. The positions of the major restriction enzymes sites are indicated by arrows. Two DNA regions, which were used as hybridization probes, are also indicated as lines on the upper part of the diagram. B: Sequence alignment of E2s. Only the UBC domains were compared. The bold-face residues are the same in all eight proteins. The cysteine residue in the UBC domain (the active site) is indicated with an asterisk. Broken lines denote gaps in the protein sequence alignment. Dots indicate a continuation of the protein sequence (not shown). Sequence information was taken from the sources indicated: UbcD2 and UbcM2 [17], UBC1 [29], UBC2 [2], UBC4 [7], AtUBC8 [23], ubc-2 [21], UbcH2 [30], and bendless [31].

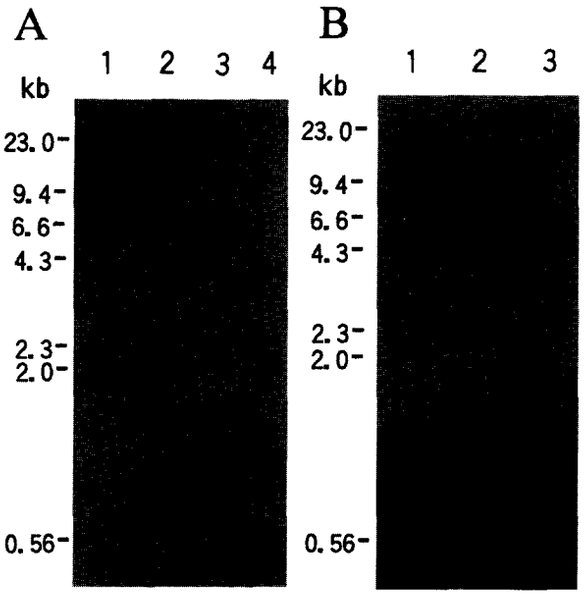

Fig. 4. Southern analysis of UbcP1. A: Genomic DNA (about 10 μg) prepared from *P. tetraurelia* was digested with *Eco*RI, *Hin*dIII, *Xba*I, and *Bgl*II (lanes 1, 2, 3 and 4, respectively). B: Genomic DNA (about 5 μg) prepared from *P. multimicronucleatum* was digested with *Eco*RI, *Hin*dIII, and *Bam*HI (lanes 1, 2 and 3, respectively). Sizes (in kb) of the markers are indicated at the left.

No significant similarity between the N-extension of UbcP1p and known sequences in the data bases were found. No conserved sequence motifs were found in the N-extension. The C-terminal extension of class II E2s is known to determine its substrate specificity (UBC2, UBC3) [24–26], or mediate intracellular localization (UBC6) [27]. The significance of the extensions of UbcP1p is not clear yet.

Using the 484 bp DNA fragment as a probe, which corresponds to most of the UBC domain of UbcP1, Southern hybridization of *P. tetraurelia* genomic DNA digested with different restriction enzymes was performed. Under high stringency conditions the hybridization results agreed with those predicted by the presence or absence of restriction sites in the UbcP1 sequence, except for *Eco*RI (Fig. 4A). UbcP1 has no *Eco*RI site, but two bands appeared. This result is not due to the existence of a short intron which contains the *Eco*RI site, because we confirmed that there were no introns within the probe region of genomic DNA by sequencing the PCR fragment derived from genomic DNA. An explanation is that a region of the UbcP1 or the neighboring region might be differentially processed when micronuclei develop into macronuclei at autogamy or conjugation; such a case of alternative DNA processing has already been reported [28]. Under low stringency conditions no changes in the hybridization patterns were observed (data not shown). This result indicates that there are no other UbcP1-like genes in the paramecium genome that hybridize with this probe.

Under low stringency conditions a genomic Southern blot of another paramecium, *P. multimicronucleatum*, was also probed with the 484 bp DNA fragment (Fig. 4B). With *Hin*dIII, two bands were detected. This also might be due to an alternative DNA processing. *P. multimicronucleatum* was also examined using a 594 bp fragment as a probe, which corresponds to a part of the N-extension region of UbcP1. In this case also the same bands appeared (data not shown). These results indicate that the N-terminal extension of UbcP1 is conserved also in *P. multimicronucleatum*. So we expect that the N-extension region of UbcP1p plays some specific role in ubiquitin-conjugating systems mediated by UbcP1p in at least the two paramecium species. To analyze the function of UbcP1 in the cell, we are now trying to delete this gene using an electroporation-mediated gene replacement technique.

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