Two very closely related human E2 ubiquitin conjugating enzymes, UbcH5B and UbcH5C, have been identified. These enzymes are products of distinct genes and are 88–89% identical in amino acid sequence to the recently described human E2, UbcH5 (now designated UbcH5A). UbcH5A-C are homologous to a family of five ubiquitin conjugating enzymes from Arabidopsis thaliana, AtUBC8–12. They are also closely related to Saccharomyces cerevisiae ScUBC4 and ScUBC5, which are involved in the stress response, and play a central role in the targeting of short-lived regulatory proteins for degradation. mRNAs encoding UbcH5A-C were co-expressed in all cell lines and tissues evaluated, with UbcH5C transcripts generally expressed at the highest levels. Analysis of Southern blots suggests that there are at least three classes of enzymes in the conjugation of ubiquitin to target proteins in the presence of the human ubiquitin protein ligase E6-AP. These results establish the existence of a highly conserved, and widely expressed, family of human ubiquitin conjugating enzymes.

The modification of proteins with ubiquitin constitutes an important cellular mechanism for targeting proteins for degradation by the 26 S proteasome (reviewed in Ref. 1). Proteins known to be degraded in this fashion include abnormal polypeptides and a number of short-lived regulatory proteins including plant phytochrome A (2), c-Myc (3), c-Jun (4), cyclins (5), p53 (6), and components of the NF-kB complex (7). In Saccharomyces cerevisiae, this modification has been extensively studied and found to lead to the degradation of abnormal and test proteins, with the susceptibility of some proteins to ubiquitin-mediated degradation dependent on the nature of their amino termini (reviewed in Ref. 8). Several transmembrane receptors are also ubiquitinated specifically in response to receptor engagement (9–12), but in these cases, the relationship between ubiquitination and degradation is less clear. Ubiquitinated proteins are found at increased levels in neuro-pathological states including Alzheimer's disease (13).

At least three classes of enzymes are involved in the conjugation of ubiquitin to proteins. Ubiquitin activating enzyme (E1), is responsible for the ATP-dependent charging of ubiquitin through the formation of a high energy thiol ester bond between the carboxyl terminus of ubiquitin and a cysteine within E1. The thiol ester-linked ubiquitin is transferred from E1 to a cysteine residue in an E2, or ubiquitin conjugating enzyme. E2 enzymes either by themselves or in combination with E3 enzymes (ubiquitin protein ligases) then transfer ubiquitin monomers or mult ubiquitin chains to target proteins, where stable isopeptide linkages are formed (reviewed in Refs. 1 and 14–17).

E1 enzymes have been characterized in several species including yeast (18), wheat (19), and man (20). Thus far, two gene products encoding E3 enzymes have been cloned, one is from S. cerevisiae (21), and the other is a human gene product termed E6-AP, named because it associates with the human papilloma virus E6 oncoprotein (22). This E3 has been shown to catalyze the E6-dependent ubiquitination of p53 (6). E3 activities have also been characterized in rabbit reticulocytes (23) and in wheat (24).

A multitude of E2s exist. In S. cerevisiae there are at least 10 E2 genes (16), whereas in Arabidopsis thaliana over 30 are likely present (17). Functions for these enzymes include roles in DNA repair, cell cycle progression, organelle biogenesis, secretion, and stress response (reviewed in Ref. 16). There is at least one example where two ubiquitin conjugating enzymes function in concert to transfer ubiquitin to a specific target protein (25). Two closely related S. cerevisiae E2s, ScUBC4 and ScUBC5, play important roles in the turnover of normal and abnormal proteins (16, 26). The levels of ScUBC4/5 are increased in response to stress, and their loss has severe effects on cellular functions; the concomitant loss of a third E2, ScUBC1, is lethal. A single homolog of ScUBC4/5 has been characterized in Caenorhabditis elegans (27) and in Drosophila melanogaster (28). In A. thaliana, a set of five closely related gene products with over 88% similarity to ScUBC4/5 and over 94% similarity to each other have been identified, with genomic evidence for at least one additional family member (29).

More recently a human gene product that is 78–79% identical to ScUBC4/5 in amino acid sequence has been identified. This enzyme, UbcH5 (now designated UbcH5A), stimulates the conjugation of ubiquitin to the tumor suppressor p53 in the presence of E6-AP and E6 (30). At least one member of the A. thaliana family of ScUBC4/5-related ubiquitin conjugating enzymes, AtUBC8, also can serve in this role, while an unrelated A. thaliana E2 does not (30). In this study we report the characterization of two additional human members of this class of closely related and highly conserved ubiquitin conjugating enzymes.
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FIG. 2. Amino acid comparison of closely related ubiquitin conjugating enzymes. Deduced amino acid sequence of UbcH5B and UbcH5C and comparison to UbcH5(A), and closely related E2s from D. melanogaster (DmUbcD1), C. elegans (CeUBC-2), S. cerevisiae (ScUBC4/5), and A. thaliiana (AtUBC9/10). Amino acids that are identical among all members of the family are shaded. The percent identity of UbcH5A-C to the related E2s is shown below the alignment.

Fig. 2. Amino acid alignment of UbcH5B and UbcH5C. Sites of translation initiation and stop codons are underlined.

MATERIALS AND METHODS

Generation of UBC Clones—First strand cDNA was synthesized from 0.5 μg of RNA purified from human PBLs using a cDNA Cycle kit (Invitrogen, San Diego, CA) with random primers and Moloney leukemia virus reverse transcriptase (LTI, Gaithersburg, MD). PCR amplification of 10% of the reaction mixture was carried out for 35 cycles using previously described degenerate primers based on conserved sequences in other E2s (27). These oligonucleotides contained built-in 5' BamHI and 3' Sall restriction sites. PCR was carried out for 35 cycles with the following parameters: 94°C for 30 s, 55°C for 90 s, and 72°C for 90 s, with a final 10-min extension at 72°C. The product was purified using QiAquick Spin PCR Purification Kit (Qiagen, Chatsworth CA), re-amplified as above, and ligated into pGEM7z (Promega, Madison, WI). A human PBL cDNA library made from cells stimulated for 4 h with phytohemagglutinin and phorbol myristate acetate and cloned into a Zap (Stratagene, La Jolla, CA) was a gift from Drs. K. Kelly and U. Siebenlist, National Institutes of Health. Phage DNA was prepared from 240,000 plaques by platelysis (31). Plasmid DNA from a purified from human PBLs using a cDNA Cycle kit (Invitrogen, San Diego, CA) with random primers and Moloney leukemia virus reverse transcriptase (LTI, Gaithersburg, MD). PCR amplification was carried out on 1 μl of the cDNA product and on multiple dilutions thereof, using 50 ng of each of two primers in a final volume of 50 μl. PCR conditions were as follows: 94°C for 1 min; 55°C for 2 min; 72°C for 3 min for 25 cycles. After resolution on 1.5% agarose gels and transfer to nylon-backed nitrocellulose, hybridization was carried out using standard conditions (31) at 40°C. Oligonucleotides used for amplification were: for UbcH5A (30) cgccatccctgacccatggc and tgatacagtcagagct; for UbcH5B (30) cacggcatcacaccatggctctg and ggttatccaataatttgttaatta; for UbcH5C (30) tgaggagccagacgacaagca and caggttattctgtggt; for UbcH5A-C to the related E2s is shown below the alignment.

Genomic DNA from human placenta (Oncor, Gaithersburg, MD) and from Balb/c mice was exhaustively digested prior to separation on 0.8% agarose gels. Blots were transferred to nylon-backed nitrocellulose (Schleicher and Schuell) and hybridized as above with PCR-generated probes (Bios, New Haven CT) prior to a final wash at 0.2 SSC at 30°C prior to autoradiography and quantitation by PhosphorImager (Molecular Dynamics, Sunnyvale, CA), using ImageQuant software. Oligonucleotides used for amplification were: for UbcH5A (30) gggatcgttgatattggtggtcagtctgtagtgaggg; for UbcH5B cagcagcagcagcagctgggtagttgtggttagcttgttctctcagctg and for UbcH5C tggagcagcagcagcag and caggttatgttatgttagttagctgttacgtg.

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Characterization of UBC Expression and Genomic Analysis—Cell lines were grown at 37°C in 5% CO2. HeLa (32) was grown in Dulbecco's modified Eagle's-based media (Biofluids, Rockville, MD), while Jurkat (33) was grown in media containing RPMI 1640 (Biofluids) (34). Outdated human PBL were obtained from the NIH Blood Bank. Tissue RNA samples were provided by F. Collins, M. Erdos, and R. Pozzatti (NIH). For analysis of E2 expression, first strand cDNA was synthesized using previously described degenerate primers based on conserved sequences in other E2s (27). These oligonucleotides contained built-in restriction sites to allow for cloning into pGEM7z.

1 The abbreviations used are: PBL, peripheral blood lymphocyte; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

Fig. 1. Sequence alignment of UbcH5B and UbcH5C. Sites of translation initiation and stop codons are underlined.

Expression of Recombinant Human Ubiquitin Conjugating Enzymes—UbcH5A and UbcH5B in pGEM7z+ were digested with Ncol and BamH1 and ligated into pET-15b (Novagen, Madison, WI). UbcH5C was amplified by PCR with a 3'- oligonucleotide corresponding to bases 457 to 479 and a 5'- oligonucleotide which created an Ncol site at the 5' end without modifying the coding sequence. The resultant product was digested with Ncol and BamH1 and ligated into pET-15b. Purified

% Identity with: UbcH5B UbcH5C UbcH5A
UbcH5B 100 97 89
UbcH5C 97 100 88
UbcH5A 89 86 100
DmUbcD1 95 94 89
Calu-2 93 92 88
SuUBC 79 79 79
SuUBC5 79 79 79
AtUBC8 79 79 76
AtUBC9 79 79 77
plasmids were transfected into BL21 Escherichia coli (Novagen). Induction of recombinant protein with isopropylthio-
-mercaptoethanol prior to resolution on 12% SDS-PAGE. Gels were transferred to Immobilon-P (Millipore, Bedford, MA), incubated with

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Fig. 3. Expression of mRNA for UbcH5A-C in human cell lines. A, schematic representation of oligonucleotides used for selective amplification of UbcH5A-C. Oligo pairs are designated A, B, or C, based on their predicted ability to amplify UbcH5A-C. The position of the 60-base probe used for hybridization is indicated by the hatched area (see “Materials and Methods” for specific sequences). B, specificity of amplification. Plasmid DNA encoding UbcH5A-C was linearized and 0.2 pg subject to PCR amplification for 25 cycles with each of the three oligo pairs. Amplified fragments were resolved on 1.5% agarose gels followed by transfer to nitrocellulose membrane and hybridization with an endlabeled 60-base probe that was equally mismatched against the three members of the family (see “Materials and Methods” for details regarding hybridization and wash conditions). C, total RNA from colon DNA was reverse transcribed and various dilutions of first strand cDNA subject to 25 cycles of PCR amplification with each set of oligonucleotide pairs as described under “Materials and Methods.” This was followed by hybridization with the oligonucleotide probe described above. D, quantification of the relative expression of UbcH5A-C. PCR products generated as described for C were quantified by PhosphorImager. After subtraction of background the values obtained for UbcH5B and UbcH5C for each tissue or cell type were normalized to UbcH5A (represented by the thick line). The data shown are representative of at least two independent PCR reactions carried out at a dilution of 1:16 of first strand cDNA. In all cases multiple dilutions of first strand cDNA were amplified to assure that the results used for this analysis were within a linear range, , UbcH5B; , UbcH5C. Affinity-purified polyclonal antibody to ubiquitin was incubated with 

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expression with 

125I-protein A (ICN, Costa Mesa, CA) and autoradiography (35). For thiol ester assays using radiolabeled ubiquitin, reaction mixtures containing the various E2s (0.05 to 10 pg), 1 pg of purified recombinant wheat E1, 0.9 pg of 125I-ubiquitin (2 pg), and 1 unit of inorganic pyrophosphatase in 20 ml of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM MnCl2, 1 mM ATP, 0.1 mM dithioerythritol were incubated for 20 s (or as indicated) at 0°C. Reactions were terminated by incubation in 25 mM Tris-HCl (pH 6.8), 5% glycerol, 4% lithium dodecyl sulfate, 4 mM urea for 15 min at 30°C. Samples were subjected to SDS-PAGE and the gels were stained with Coomassie Brilliant Blue, dried between cellophane, and visualized by autoradiography. Quantification of E2-ubiquitin thiol ester adducts was accomplished by counting gel slices in a Packard Multi-Prias 1 y-counter, following localization by autoradiography.

Assay for Ubiquitin Conjugate Formation Using E6-AP—Expression and preparation of E6-AP was generally as described (36). Recombinant baculovirus expressing a 95-kDa truncated E6-AP was used to infect Trichoplusia Ni insect cells plated at a multiplicity of infection = 10. A “mock” infection to which no baculovirus was added was carried out as a control. Infected cells were grown in TC-100 (Life Technologies) + 10% fetal bovine serum. At 36 h postinfection, expressed protein was harvested by sonication in 1 ml of 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% Nonidet P-40, 1 mM dithioerythritol, 10% glycerol per 8 x 106 cells.

Reactions mixtures containing the various E2s (0.05 to 10 pg of bacterial lysate), 0.1 pg of purified recombinant wheat E1, 0.5 units of phosphocreatine kinase, and 0.9 pg of 125I-ubiquitin were incubated with 0.1 pg of either E6-AP extract, extract from mock infected cells, or...
RESULTS

Prior to the initiation of this study, no mammalian homologs of the yeast ScUBC4/5 and the A. thaliana AtUBC8–12 E2 enzymes had been identified. To determine if there are related members of this E2 class in man, oligonucleotides based on conserved regions in ScUBC5 and the related D. melanogaster DmUbdC1 were synthesized. Similar oligonucleotides had previously been used for the identification of a C. elegans E2, CeUbc-2, by PCR (27). The use of these oligonucleotides in the PCR with first strand cDNA from PBL as template resulted in a 325-base pair fragment. Cloning and sequencing of this fragment revealed a product with an open reading frame that was most homologous to the region encoding amino acids 52 to 139 of DmUbdC1 and CeUbc-2. Using DNA prepared from a human PBL library as template, PCR was carried out in which sense and antisense oligonucleotides synthesized based on the sequence of the 325-base pair fragment were paired with oligonucleotides corresponding to the T3 or T7 regions flanking the λ Zap poly linker. Overlapping fragments extending 5’ and 3’ were identified, sequenced, and found to contain parts of the original product. Oligonucleotides based on regions predicted to be the 5’- and 3’-untranslated regions were then synthesized and used to clone the entire open reading frame by PCR (Fig. 1). A search of the GenBank data base found that the deduced amino acid sequence of this gene product was 89% identical to a recently described human E2, UbcH5 (30) (now designated UbcH5A), and was thus designated UbcH5B.

Oligonucleotides generated based on the original 325-base pair PCR fragment also yielded overlapping fragments that were distinct from UbcH5B. This second sequence was truncated at its 5’ end prior to the predicted site of translation initiation, thus it did not encode a complete E2 enzyme. However, it appeared to overlap a partial sequence found in GenBank that had been designated EST06924. EST06924 had previously been identified by random sequencing of a human infant brain cDNA library (accession number: T09032) (37), and had been noted to have homology to the 5’ ends of cDNAs encoding ubiquitin conjugating enzymes. A 5’-oligonucleotide based on EST06924 was synthesized and used in combination with an antisense oligonucleotide based on the 3’ region of our sequence in the PCR, with DNA from the PBL library as template. This resulted in the generation of a full-length clone encoding an E2 related to UbcH5A and UbcH5B that we denote UbcH5C. The correct sequence of this clone was independently confirmed by PCR amplification and cloning from a cDNA library made from YT-1, a human natural killer cell line.

The cDNAs encoding UbcH5B and UbcH5C are 87% identical on a nucleotide level within the predicted coding region. In contrast, their 3’-untranslated regions are only 23% conserved. Both UbcH5B and UbcH5C are 78% identical to UbcH5A within their coding regions. Alignment of the deduced amino acid sequences of UbcH5B and UbcH5C shows only four amino acid differences, the only non-conservative change being amino acid 11 where there is an asparagine in UbcH5B and a serine in UbcH5C. When compared to the amino acid sequence of UbcH5A, UbcH5B is 89%, and UbcH5C is 88% identical (Fig. 2). These three human E2s are 77-80% identical to ScUBC4/5 (26) and to members of the AtUBC8–12 family of enzymes had been identified. To determine if there are related family members in man, oligonucleotides based on the published UbcH5A sequence in the PCR, with DNA from the PBL library as template. This resulted in the generation of a full-length clone encoding an E2 related to UbcH5A and UbcH5B that we denote UbcH5C. The correct sequence of this clone was independently confirmed by PCR amplification and cloning from a cDNA library made from YT-1, a human natural killer cell line.

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sequence were generated and used to isolate cDNAs encoding UbcH5A from both the PBL and YT libraries. The PBL isolate was sequenced and found to be identical to the published sequence (30).

To determine the tissue distribution and relative expression of transcripts encoding UbcH5A-C, first strand cDNA was synthesized from RNA followed by PCR amplification with pairs of oligonucleotides specific to individual members of the family (Fig. 3A). The resultant products were hybridized with a 32P-labeled 60-base oligonucleotide probe that was equally mismatched against UbcH5A-C (Fig. 3A), and therefore should hybridize equally well with PCR products encoding UbcH5A-C. To establish the specificity of the oligonucleotide pairs and the ability of the probe to hybridize with the PCR products, amplification of linearized plasmid DNA encoding each of the three E2s was carried out. As shown (Fig. 3B), the oligonucleotide pairs behaved as expected, with products detected only in samples having the appropriate combination of oligonucleotide and template. To assess the expression of UbcH5A-C in samples reverse-transcribed from human RNA, conditions were optimized to achieve a degree of amplification that was within a linear range. A typical result using 25 cycles of PCR and three different dilutions of template is shown (Fig. 3C). Results obtained using 1:16 dilutions of first strand cDNA are presented in Fig. 3D. For each source of RNA, the values obtained for UbcH5B and UbcH5C were normalized to UbcH5A. The results of this analysis demonstrate that messages encoding UbcH5A-C are co-expressed in a number of different tissue and cell lines (Fig. 3D). While UbcH5C was consistently found at higher levels than UbcH5A, the ratio of these two family members varied from 9:1 (PBL) down to 2:1 (prostate and HeLa). In most cases UbcH5B was found at levels between UbcH5A and UbcH5C. However, there was significant variation in the expression of UbcH5B relative to UbcH5A and UbcH5C. For example, in PBL, the level of UbcH5B was almost equal to UbcH5C, while in other samples, such as Jurkat and prostate, UbcH5B more closely approximates UbcH5A.

To determine if there are other closely related members of the UbcH5A-C gene family, Southern blots were carried out on genomic DNA from human placenta and from Balb/c mice. DNA was digested with either HindIII or EcoRI and the resultant blots hybridized first with a radiolabeled probe correspond-

**Fig. 3. Ubiquitin conjugation of UbcH5A-C.** To carry out conjugation assays, volumes of lysate from E. coli expressing the various E2s being assayed were determined that resulted in equivalent thiol ester adduct formation in the presence of E1. Reaction volumes were equalized using lysate from E. coli not expressing an E2. Panel A demonstrates equivalent thiol ester adduct formation between 125I-ubiquitin and the indicated E2s. Adducts of 125I-ubiquitin with E2s and with E1 are indicated. Panel B illustrates the conjugation of cellular proteins with 125I-ubiquitin in an E6-AP and E2-dependent fashion using equivalent thiol ester adduct activity for the four different E2s. After incubation as indicated for 2 h at 30°C, samples were heated to 95°C in sample buffer containing β-mercaptoethanol, and resolved by SDS-PAGE. E. coli transformed with empty pET-15b vector is shown as a control. Assays were carried out with lysates from Trich Ni cells that express E6-AP. Lysates from cells not expressing E6-AP are shown as controls (Mock). As an additional control, samples to which no E. coli lysate was added are shown (No E2 lanes). The species seen in the AtUBC8 lanes at ~25 and 32 kDa were found reproducibly, and are of unknown significance. In Panel C, conjugate formation was carried out for the indicated times. The area of the gel corresponding to molecular masses greater than 50 kDa was excised and quantified by γ-counting. ○, UbcH5B; ●, UbcH5C; □, UbcH5A; ■, AtUBC8.
Fig. 7. Evolutionary relationship of ubiquitin conjugating enzymes. This illustrates the likely evolutionary relationship between human E2s, designated by an asterisk (*), and E2s related to UbcH5A-C from other species (only two of the five A. thaliana members of this family are shown). Rn refers to Rattus norvegicus. This analysis was generated using GeneWorks (Intelligenetics, Mountain-view, CA), using a progressive alignment method.

An increasing number of cellular proteins are being recognized as substrates for conjugation to ubiquitin (reviewed in Ref. 1). In some cases, the nature of the amino terminus determines the potential susceptibility for ubiquitin-mediated degradation, a concept known as the N-end rule (8). For the mitotic cyclins and c-j un, specific internal sequences have been identified that confer susceptibility to ubiquitination (4, 5). However, in most cases, the molecular mechanisms responsible for targeting proteins for ubiquitination are unknown. Specificity with regard to the recognition of targets would seem to lie with the E2 and E3 enzymes. In yeast and in plants, a multitude of E2s exist (16, 17). In humans, several distinct E2s have been characterized. Two very closely human related E2s, HHR6A and HHR6B, have been identified that are homologous to the product of the yeast DNA repair gene RAD6 (39–41). In addition to its function in DNA repair (42), the RAD6 gene product is also believed to be involved in the recognition of substrates via the N-end rule (43, 44). Another human E2, identified by complementation studies, is the human homolog of the S. cerevisiae CDC34, which is important in the G1 to S transition in yeast (45, 46). UbcH2, a human homologue of AtUBC4–6 (47) and ScUBC8 (48), may be involved in the ubiquitination of histones in an E3-independent manner (49). These E2s are characterized by acidic COOH termini that help in the recognition of basic histones. Studies of auto-antibodies expressed in pemphigus foliaceus has resulted in the identification of a human cDNA denoted EPF-5, which, in an alternative reading frame (EPF5-ORF2), encodes an E2 enzyme (50). The significance of this reading frame remains to be determined.

It is clear from the present study that there are at least three related human E2s that are homologous to yeast ScUBC4/5 and A. thaliana AtUBC8–12. All three of these enzymes are expressed in the same cells, and all function in an equivalent manner in a cell-free system with the human E3 enzyme E6-AP. These findings naturally raise the issue of whether these
enzymes perform redundant, partially overlapping, or distinct functions in vivo, Pertinent to a discussion of this issue is one of two related studies published while this manuscript was being prepared. In this study, Wing and J ain (51) describe two rat E2s, RnE2E and RnE2IA, from testis, that are identical in amino acid sequence to UbcH5B and UbcH5C, respectively. There is also greater than 95% nucleic acid conservation between each pair of human and rat cDNAs, which includes both the coding regions and the 3′-untranslated regions. A dendrogram, based on deduced amino acid sequence, illustrating the relationship between UbcH5A-C, related E2s from other species, and other human E2s is depicted in Fig. 7. It shows the close relationship that these ScUBC4/5-related enzymes have to each other, and also suggests that genes encoding UbcH5B/C and UbcH5A likely arose by duplication quite early in evolution. The total conservation of amino acid sequence among UbcH5B/C and their rat equivalents, the early divergence of UbcH5A-C from the closely related UbcH5A, together with the finding that the relative levels of transcripts encoding UbcH5A-C varies among tissues and cell types makes it reasonable to assume that there are likely to be in vivo differences in functions among these enzymes.

Based on genomic analysis, and the precedent in A. thaliana, there are probably other related members of this family of E2 enzymes. A second, recently published relevant study reports the cloning of a human cDNA from the a human cervical carcinoma cell line, HeLa. This cDNA encodes a protein identical to UbcH5B except for the substitution of a lysine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine for a glutamine 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