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Identification of a human ubiquitin-conjugating enzyme that mediates the E6-AP-dependent ubiquitination of p53

(papillomavirus/E6-associated protein/tumor-suppressor protein)

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ABSTRACT **The E6 protein of the oncogenic human papillomavirus types 16 and 18 facilitates the rapid degradation of the tumor-suppressor protein p53 via the ubiquitin-dependent proteolytic pathway. The E6 protein binds to a cellular protein of 100 kDa termed E6-AP. The complex of E6 and E6-AP specifically interacts with p53 and induces the ubiquitination of p53 in a reaction which requires the ubiquitin-activating enzyme (E1) and a cellular fraction thought to contain a mammalian ubiquitin-conjugating enzyme (E2). This mammalian E2 activity could be replaced with bacterially expressed UBC8 from *Arabidopsis thaliana*, which belongs to a subfamily of E2s including yeast UBC4 and UBC5 which are highly conserved at the amino acid level. In this paper we describe the cloning of a human cDNA encoding a human E2 that we have designated UbcH5 and that is related to *Arabidopsis* UBC8 and the other members of this subfamily. We demonstrate that UbcH5 can function in the E6/E6-AP-induced ubiquitination of p53.**

The ubiquitin-dependent proteolytic system is a major pathway in eukaryotes involved in the selective degradation of abnormal and short-lived proteins (1, 2). The hallmark of this pathway is the covalent attachment of ubiquitin to a target protein prior to degradation. Ubiquitin is a 76-aa polypeptide which is highly conserved among eukaryotic organisms. Ubiquitin-conjugate formation requires the combined action of three classes of proteins. These are the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and in some cases additional proteins (E3) referred to as ubiquitin–protein ligases, believed to play a role in substrate recognition. Ubiquitin is first activated by E1 through the ATP-dependent formation of a thioester between the C-terminal glycine of ubiquitin and the active-site cysteine residue of E1. The activated ubiquitin is then transferred to a cysteine residue of an E2, preserving the high-energy thioester bond. Finally the E2 itself, or in concert with an E3 protein, catalyzes the formation of an isopeptide bond between the C-terminal glycine of ubiquitin and an ε-amino group of a lysine residue on a target protein. The role of E3s in the transfer of ubiquitin from an E2 to a target protein is not clear. However, it seems likely that E3s bind specifically to proteins which are otherwise not recognized by E2s (2). Ubiquitin can be linked to itself primarily through a lysine residue at position 48, resulting in multiubiquitinated proteins (3). Multiubiquitinated proteins are finally recognized and degraded by a multisubunit protease complex.

The E2s are a family of proteins characterized by a highly conserved catalytic site. In the yeast *Saccharomyces cerevisiae* at least 10 different E2s have been identified that are involved in various cellular processes such as DNA repair, cell cycle control, and stress response (4). At least some members of the E2 family appear to be evolutionally conserved. Diverse organisms such as *Arabidopsis thaliana*, *Drosophila melanogaster*, and *Caenorhabditis elegans* encode E2s which are highly similar to UBC4 and UBC5 of *S. cerevisiae* at the protein sequence level (5–8). In addition, it has been shown that in yeast these related E2s of other species can functionally substitute for UBC4 and UBC5, which play a major role in the selective turnover of short-lived and abnormal proteins (6, 7).

Although genetic analyses in yeast have revealed that ubiquitin conjugation is essential to cell viability (9), only a few cellular targets of the ubiquitin system have been identified, including cyclin B and the yeast transcription factor MATα2 (10, 11). Another prominent example is the tumor-suppressor protein p53. The cancer-associated human papillomavirus (HPV) types 16 and 18 utilize the ubiquitin system to target the degradation of p53. *In vitro* studies have shown that the HPV E6 oncoprotein binds to a cellular protein of 100 kDa, termed E6-AP, for E6-associated protein (12, 13). This complex can then bind to p53 and facilitate the rapid degradation of p53 via the ubiquitin system (13, 14). This *in vitro* property of E6 is reflected in the reduced half-life as well as reduced levels of p53 in HPV-immortalized cells (15–17). In addition, there is some evidence that the ubiquitin system may be involved in the regulation of p53 in normal cells (18, 19).

Recently all the factors necessary and sufficient for the E6/E6-AP-induced ubiquitination of p53 *in vitro* have been identified (20). In addition to ubiquitin and ATP, there was a requirement for the ubiquitin-activating enzyme E1 and a specific E2 activity present in mammalian cell extracts. Since E6 and E6-AP were the only other proteins required for ubiquitination of p53, it was concluded that the complex of E6 and E6-AP represents the E3 activity in this particular system. In addition, it was shown that E6-AP could facilitate the ubiquitination of cellular proteins in the absence of E6, suggesting that the normal function of E6-AP is that of an E3 protein. The E2 activity required for the E6-dependent ubiquitination of p53 had chromatographic properties which distinguished it from all other previously described mammalian E2s; however, it could be functionally replaced by *A. thaliana* UBC8. Here we report the isolation of a human cDNA encoding an E2 which is active in the E6/E6-AP mediated ubiquitination of p53.‡ It is highly similar to the subfamily of E2s comprising *A. thaliana* UBC8 and *S. cerevisiae* UBC4, suggesting that it may play a major role in selective protein degradation in human cells.

MATERIALS AND METHODS

cDNA Cloning. Cytoplasmic RNA was prepared from primary human foreskin keratinocytes, from the HPV-

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Abbreviations: GST, glutathione *S*-transferase; HPV, human papillomavirus; ORF, open reading frame; RACE, rapid amplification of cDNA ends.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X78140).

negative cervical carcinoma cell line C-33A, and from the HPV-positive cervical carcinoma cell line HeLa by standard procedures (21). The source and maintenance of the cell lines have been described (15). Two degenerate primers similar to the primers described by Zhen *et al.* (7), which correspond to regions conserved among yeast UBC4, *D. melanogaster* UbcD1, *C. elegans* ubc-2, and *A. thaliana* UBC8 (aa 147–141 and 45–51; for numbering see Fig. 2), were used for reverse transcription followed by PCR amplification with the RNA PCR kit from Cetus. PCR products were cloned into pGEM-1 (Promega). The sequence of several clones derived from independent PCRs was determined with modified T7 DNA polymerase (Sequenase; United States Biochemical).

Phage λ clones comprising the 3′ end of the open reading frame (ORF; Fig. 1) were isolated from a random-primed cDNA library from normal human keratinocytes (Clontech). ^{32}P-labeled probes extending from nt 132 to nt 441 (see Fig. 1) were prepared by random-primed labeling (Bethesda Research Laboratories). Positive λ clones were amplified and the inserts were subcloned into pGEM-1 for sequencing.

The 5′ end of the ORF was cloned using a 5′ RACE (rapid amplification of cDNA ends) kit (Bethesda Research Laboratories). Reactions were performed according to the suggestions of the manufacturer. For reverse transcription a primer extending from nt 378 to nt 358 (Fig. 1) was used followed by PCR amplification. An aliquot of the PCR mixture was taken and a second round of PCR amplification was performed with a primer extending from nt 356 to nt 339. In both PCRs the opposing primer was supplied by the 5′-RACE kit. PCR products were cloned into pGEM-1 and sequenced.

The complete ORF designated UbcH5 was cloned in a single fragment by reverse transcription followed by PCR amplification. The primer for reverse transcription spanned nt 444–421 of the ORF with a *Bam*HI site at the 5′ end for cloning. The opposing primer used for PCR amplification covered nt 1–21 with an *Nde* I site at the 5′ end. The PCR products were cloned into pET-3a (Novagen) and sequenced.

Protein Expression. E1, *A. thaliana* UBC8, *A. thaliana* UBC1, and UbcH5 were expressed in *Escherichia coli* BL21 using the pET expression system (5, 22, 23). Glutathione *S*-transferase (GST) fusion proteins were expressed in *E. coli* DH5α. Construction, purification, and radioactive labeling of GST–E6–E7, GST–p53, and GST–ubiquitin have been described (20). Preparation of E6-AP from Sf9 insert cells infected with a recombinant baculovirus expressing E6-AP has been described (24).

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                1
cgccatccctgacccATGGCGCTGAAGAGGATTCAGAAAGAATTGAGTGAT   36
                M  A  L  K  R  I  Q  K  E  L  S  D

CTACAGCGCGATCCACCTGCTCACTGTTCAGCTGGACCTGTGGGAGATGAC   87
 L  Q  R  D  P  P  A  H  C  S  A  G  P  V  G  D  D

TTGTTCCACTGGCAAGCCACTATTATGGGGCCTCCTGATAGCGCATATCAA  138
 L  F  H  W  Q  A  T  I  M  G  P  P  D  S  A  Y  Q

GGTGGAGTCTTCTTTCTCACTGTACATTTTCCGACAGATTATCCTTTTAAA  189
 G  G  V  F  F  L  T  V  H  F  P  T  D  Y  P  F  K

CCACCAAAGATTGCTTTCACAACAAAAATTTACCATCCAAACATAAACAGT  240
 P  P  K  I  A  F  T  T  K  I  Y  H  P  N  I  N  S

AATGGAAGTATTTGTCTCGATATTCTGAGGTCACAATGGTCACCAGCTCTG  291
 N  G  S  I  C  L  D  I  L  R  S  Q  W  S  P  A  L

ACTGTATCAAAAGTTTTATTGTCCATATGTTCTCTACTTTGTGATCCTAAT  342
 T  V  S  K  V  L  L  S  I  C  S  L  L  C  D  P  N

CCAGATGACCCCTTAGTACCAGATATTGCACAAATCTATAAATCAGACAAA  393
 P  D  D  P  L  V  P  D  I  A  Q  I  Y  K  S  D  K

GAAAAATACAACAGACATGCAAGAGAATGGACTCAGAAATATGCAATGtaa  444
 E  K  Y  N  R  H  A  R  E  W  T  Q  K  Y  A  M  *
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FIG. 1. Complete nucleotide and amino acid sequence of the cloned ORF. The coding sequence is represented in capital letters. The stop codon located 5′ to the start codon is represented in bold type. Amino acids are given in the single-letter code.

Thioester and Ubiquitination Assays. Formation of thioester adducts of the various E2s with ubiquitin was determined as described by Haas *et al.* (25). Reaction mixtures contained 5–10 ng of E1, 1 μg of ^{32}P-labeled GST–ubiquitin, and 20–40 ng of the respective E2 in 20 mM Tris·HCl, pH 7.6/50 mM NaCl/4 mM ATP/10 mM $MgCl_2$/0.1 mM dithiothreitol. After 2 min at 25°C, reactions were terminated either by boiling the mixtures in SDS sample buffer (50 mM Tris·HCl, pH 6.8/100 mM dithiothreitol/2% SDS/10% glycerol) or by incubating the mixtures for 15 min at 30°C in SDS sample buffer containing 4 M urea instead of dithiothreitol. Reaction mixtures were electrophoresed in SDS/10% polyacrylamide gels at 4°C and radioactively labeled bands were visualized by autoradiography.

Ubiquitination assays using GST–E6-E7 or GST–p53 as substrate were performed as described (20).

RESULTS

Cloning of a Human E2 Highly Similar to Yeast UBC4. *C. elegans* ubc-2 has been cloned by using degenerate oligonucleotide primers corresponding to highly conserved regions of yeast UBC4 and UBC5, *Drosophila* UbcD1, and *Arabidopsis* UBC8 (7). Since the mammalian E2 involved in E6/E6-AP-induced ubiquitination of p53 could be replaced with *Arabidopsis* UBC8 (20), it seemed likely that it represents a member of this subfamily of E2s. Therefore two similar degenerate primers corresponding to aa 45–51 and 141–147 (for numbering, see Fig. 2) were used to amplify cDNA synthesized from cytoplasmic RNA by reverse transcription. The RNA was isolated from normal primary human keratinocytes, from the HPV-negative cervical carcinoma cell line C-33A, or from HeLa, an HPV-positive cervical carcinoma cell line. In all three cases a 325-bp PCR product was isolated, cloned, and sequenced. The DNA sequence between the oligonucleotide primers was identical in all clones and, as expected, encoded an amino acid sequence highly similar to aa 51–141 of yeast UBC4. To obtain the sequence of the complete ORF, the 325-bp PCR fragment was used to probe a random-primed cDNA library made from primary human keratinocytes. However, the clones isolated contained only the C-terminal sequence of the putative E2 including the stop codon. The reason for the inability to isolate clones expanding in the 5′ direction is unknown. To gain information about the 5′ end of the ORF another PCR approach, 5′ RACE, was used (26). Cytoplasmic RNA was transcribed by reverse transcription using an oligonucleotide primer corresponding to aa 120–126 of the ORF (according to numbering in Fig. 2). The resulting cDNA was tailed with poly(dC) and terminal nucleotidyltransferase. The tailed cDNA was used as a template for PCR amplification using the primer above and an opposing primer containing an oligo(dG) stretch (Bethesda Research Laboratories). The PCR products were used as templates in a second round of PCR using the primer with the oligo(dG) stretch and a primer corresponding to aa 114–119 of the ORF (Fig. 1). This approach allowed for the specific accumulation of PCR products containing portions of the 5′ region of the E2 ORF. The PCR products were cloned and sequenced. The cDNA sequence shown in Fig. 1 contains only those sequences that were present in multiple clones from independent PCRs. It contains an ORF that encodes 147 aa with a total predicted molecular mass of 16.6 kDa. The initiating methionine codon appears to be present within this ORF (at nucleotide position 1) since there is a stop codon located immediately upstream and in frame with the putative start codon. The E2 was

```
hsUbcH5     MALKRIQKELSDLQRDPPAHCSAGPVGDDLFHWQATIMGPPDSAYQGGVF  50
dmUbcD1     M-----N---Q--G-----Q----------------------P------
scUBC4     MSSS---A------E----TS----------Y----S----A--P-A----
ceUBC2      M---------Q--G-E---Q----------------------E-P------
atUBC8      M-S---L---K---K---TS------AE-M----------AE-P-S----
atUBC1   MSTP-R--LMRDFKR--Q----GI-GA-QDNNIML-N-V-F--D-TPWD--T-
hsHHR6A  MSTP-RR-LMRDFKR--E----GV-GA-SENNIMV-N-V-F--EGTPFGD-T-

                                                    *
hsUbcH5   FLTVHFPTDYPFKPPKIAFTTKIYHPNINSNGSICLDILRSQWSPALTVS  100
dmUbcD1   ---I------------V----R--------------------------I-
scUBC4    --SI-------------S-----------A--N------KD-------L-
ceUBC2    ---I------------V----R----------------------------I-
atUBC8    LV-I---P---------V--R--VF---------------KE-------I-
atUBC1    K-SLQ-SE---N---TVR-VSRMF----YAD--------QN----IYD-A
hsHHR6A   K--IE-TEE--N---TVR-VS-MF---VYAD--------QNR---TYD--


hsUbcH5   KVLLSICSLLCDPNPDDPLVPDIAQIYKSDKEKYNRHAREWTQKYAM*     147
dmUbcD1   ----------------------E--R---T-R----EL-----R----*
scUBC4    ---------T-A---------E--H---T-RP--EAT-----K---V*
ceUBC2    ----------------------E--R---T-R-R--QL----------*
atUBC8    ---------T-----------E--HM--T-RA--EAT--N-------G*
atUBC1    AI-T--Q--------NS-ANSEA-RMYSES-RE---RV-DVVEQSWTAD*
hsHHR6A   SI-T--Q---DE--SPANSQAAQLYQENKREYEKRVS-IVEQSWRDC*
```

FIG. 2. Comparison of amino acid sequence of human (*Homo sapiens*, hs) UbcH5 with homologous E2s of other organisms: *D. melanogaster* UbcD1 (6), *S. cerevisiae* UBC4 (8), *C. elegans* ubc-2 (7), and *A. thaliana* UBC8 (5). Also shown are the amino acid sequences of functionally less similar E2s: *A. thaliana* UBC 1 (5) and *Homo sapiens* HHR6A (28). Differences in amino acid sequence are indicated, with identities denoted by dashed lines. The active-site cysteine residue is marked with a star, as are the C-terminal ends of the sequences.

termed UbcH5 because, to our knowledge, it is the fifth E2 isolated from human cells (27–30).

Sequence comparison demonstrates that UbcH5 is highly similar to the members of the E2 subfamily mentioned above, with the highest similarity to the E2s isolated from *D. melanogaster* and *C. elegans* (89% identity, 95% similarity) (Fig. 2). In contrast, the sequence of UbcH5 shows 35–55% similarity to the family of known human E2s, which is typical for the functionally less related E2s.

Characterization of the E2. The UbcH5 cDNA was inserted into the pET-3a vector and expressed in *E. coli*. After induction of logarithmic-phase cultures, a 16-kDa protein was produced as predicted from the calculated molecular mass of UbcH5 (Fig. 3*A*). Like all E2s characterized to date, UbcH5 contains a conserved cysteine residue (position 85, Fig. 2) that most likely represents the active site for thioester formation with ubiquitin. To demonstrate that UbcH5 is indeed an E2, its ability to form a stable adduct with radioactively labeled ubiquitin in an E1- and ATP-dependent reaction was tested (25). A GST–ubiquitin fusion protein has been shown before to efficiently substitute for free ubiquitin (20). As shown in Fig. 3*B*, a linkage between GST–ubiquitin and UbcH5 could be observed. The linkage formed was labile to reducing agents such as dithiothreitol or 2-mercaptoetha-

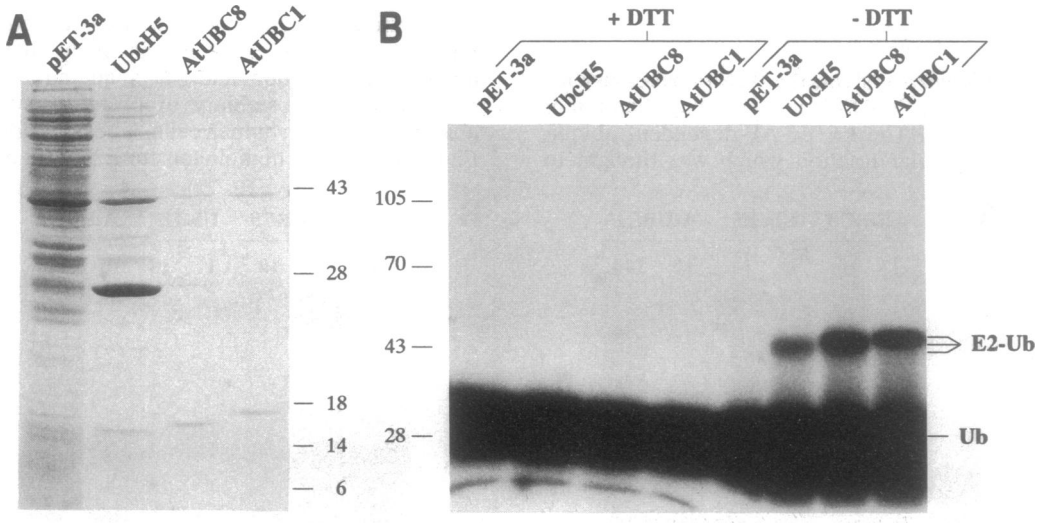

FIG. 3. Thioester adduct formation between ubiquitin and bacterially expressed UbcH5. (*A*) The ORF encoding UbcH5 was cloned in the pET-3a expression system. After induction, crude lysates from cells harboring the expression vector only (pET-3a) or recombinant expression vectors encoding UbcH5 or *A. thaliana* (At) UBC8 or UBC1 were subjected to SDS/polyacrylamide gel electrophoresis and proteins were stained with Coomassie blue. Running positions of molecular size (kDa) markers are indicated. UbcH5 migrates at ≈16 kDa. The identity of the band migrating at ≈28 kDa is unknown, but the band is not related to UbcH5. (*B*) Thioester reactions contained ^{32}P-labeled ubiquitin expressed as a GST fusion protein in *E. coli* (see *Materials and Methods*), bacterially expressed E1, ATP, and crude extracts from bacteria expressing the various E2s as indicated. Similar amounts of the respective E2s were used as determined by staining with Coomassie blue (see *A*). After 2 min at 25°C reactions were stopped, and the products were subjected to SDS/polyacrylamide gel electrophoresis in the absence or presence of dithiothreitol (DTT), followed by autoradiography. Positions of free ^{32}P-labeled GST–ubiquitin (Ub) and of the respective E2 thioester adducts are indicated.

nol (data not shown), consistent with its identity as a thioester bond. This establishes that UbcH5 has the properties of an E2.

The mammalian E2 involved in E6/E6-AP-induced ubiquitination was believed to represent an E2 not previously described in mammals, because of its chromatographic behavior (20). Unlike mammalian E2s described in the literature, this E2 did not bind to anion-exchange resins, but rather to cation-exchange resins. Consistent with such chromatographic characteristics UbcH5 has a predicted isoelectric point (pI) of 7.4. Furthermore, bacterially expressed UbcH5 shows a chromatographic behavior similar to that of the E2 activity present in cell extracts (data not shown). To demonstrate that UbcH5 is functional in E6/E6-AP-induced ubiquitination, its ability to mediate ubiquitination of an E6–E7 fusion protein or of p53 was assayed (Fig. 4). Bacterially expressed, radioactively labeled GST–E6–E7 fusion protein or GST–p53 (see *Materials and Methods*) were incubated with E1, E6-AP, ubiquitin, and ATP in the presence or in the absence of UbcH5 or *A. thaliana* UBC8. In the assays utilizing GST–p53 as the substrate, E6 activity was provided by bacterially expressed GST–E6 protein. After 3 hr the whole reaction mixtures were separated by polyacrylamide gel electrophoresis and radioactively labeled proteins were visualized by autoradiography. As judged by the appearance of high molecular weight bands (marked with an asterisk), UbcH5 could mediate the ubiquitination of the E6–E7 fusion protein as well as of p53 with a similar efficiency as *A. thaliana* UBC8. As a negative control, bacterially expressed *A. thaliana* UBC1 was used, which was previously shown to be inactive in these particular ubiquitination assays (20). Even at a 100-fold greater concentration of UBC1 compared with *A. thaliana* UBC8 or UbcH5, little or no ubiquitination of the E6–E7 fusion protein or p53 was observed, even though UBC1 was at least as active as the other E2s in the thioester assay (see Fig. 3*B*). The ability of UbcH5 to ubiquitinate p53 was still dependent upon the presence of E6, E6-AP, and E1 (data not shown). This shows the high specificity of the E6/E6-AP-dependent ubiquitination for UbcH5 and the related E2s.

DISCUSSION

With the use of an *in vitro* system, the factors necessary for the HPV-16 E6-induced, E6-AP-dependent ubiquitination of p53 were identified (20). The E6/E6-AP-dependent ubiquitination required a cellular fraction which was thought to contain a mammalian E2 activity that could be replaced by *A. thaliana* UBC8. Now, by a combination of RNA PCR methods and cDNA library screening, we have cloned a human E2 designated UbcH5 that is functional in E6/E6-AP-dependent ubiquitination. The protein sequence of UbcH5 shows high similarity to *A. thaliana* UBC8 and its behavior on ion-exchange chromatography is similar to that of the E2 activity present in mammalian cell extracts. Therefore it seems likely that UbcH5 represents the mammalian E2 activity involved in E6/E6-AP-induced ubiquitination. However, since it has been shown that *A. thaliana* encodes several E2s which are closely related to each other (5), it cannot be excluded that there are other mammalian E2s highly related to UbcH5 that may function in this particular system as well.

Two recent papers published while this manuscript was under review describe a mammalian E2 activity present in the flowthrough from an anion-exchange column (31, 32). This activity may be similar to the activity we previously described that was involved in the E6/E6-AP-dependent ubiquitination of p53 (20). The E2 activity described by Ciechanover and colleagues (31, 32) is involved in the ubiquitination of non-"N-end-rule" substrates and appears to be involved in the ubiquitination of p53. The HPV E6-dependent ubiquitination of p53 does not involve the N-end-rule pathway (33). The specific E2 purified by Blumenfeld *et al.* (31) referred to as E2-F1, however, is different from UbcH5 described in this manuscript, as shown by a comparison of the sequence of UbcH5 and the partial amino acid sequence of the proteolytic fragments of E2-F1. This may mean that there are more than one species of E2 in the flow through from the anion exchange column with similar activities. However, it should be noted that because E2-F1 has not been cloned, it has not been established that the peptide sequences are encoded by the E2-F1 gene.

The protein sequence of UbcH5 shows high similarity to *A. thaliana* UBC8 as well as to E2s from other organisms, such as *S. cerevisiae* UBC4 and UBC5, *D. melanogaster* UbcD1, and *C. elegans* ubc-2. As mentioned earlier, yeast UBC4 and UBC5 are necessary for cell viability and play a general role in the degradation of short-lived and abnormal proteins (8, 9). Similarly most of the ubiquitination activity detected in wheat germ extracts is dependent on E2s related to *A. thaliana* UBC8 (34). Therefore it seems likely that UbcH5 has a homologous function and plays an important role in the regulation of the stability of proteins recognized by the ubiquitin system in human cells. UbcH5 is not the first human E2 isolated that is homologous to E2s from other organisms.

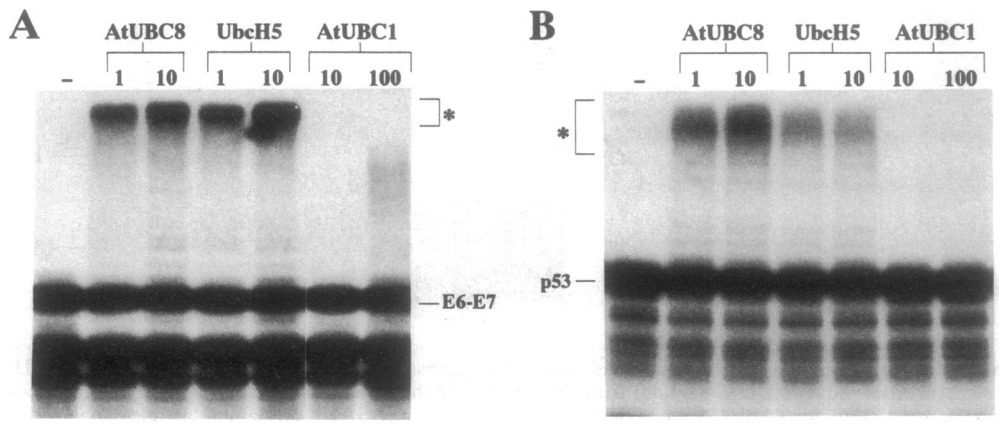

FIG. 4. Effectiveness of UbcH5 in E6/E6-AP-dependent ubiquitination. (*A*) Ubiquitination of an E6–E7 fusion protein. (*B*) Ubiquitination of p53. Radioactively labeled p53 and E6–E7 fusion protein expressed GST fusion proteins in *E. coli* were used as substrates (20). Ubiquitin-conjugation mixtures also contained bacterially expressed E1, ubiquitin, E6-AP, ATP, and, in the case of the reactions with p53, GST–HPV-16 E6 protein. Reactions were started by addition of crude extracts of bacteria expressing the various E2s as indicated. Relative amounts of the respective E2s are given as determined by staining with Coomassie blue. Positions of GST–E6–E7, GST–p53, and the respective ubiquitinated forms are indicated.

Genes referred to as HHRA and HHRB encode E2s homologous to the *S. cerevisiae RAD6* gene product, which is involved in DNA repair (27, 28, 35). Furthermore, a human homolog to *S. cerevisiae* UBC3, which is encoded by the yeast gene *CDC34*, has been cloned by complementation experiments (30, 36).

In some cases E2s can facilitate ubiquitination of target proteins only in the presence of E3s. In contrast to the E2s there is not much known about E3s. The first E3 to be described was UBR1 of *S. cerevisiae*, which is involved in the ubiquitination of proteins recognized according to the N-end rule (37) (for a review of the N-end rule, see ref. 38). UBR1 has been shown to specifically interact with both substrate proteins and a specific E2, the product of the *rad6* gene, UBC2 (39). A current model therefore is that E3 proteins may be involved in substrate recognition for proteins which are otherwise not recognized by E2s. The complex of E6 and E6-AP represents the E3 function in the ubiquitination of p53. In addition, E6-AP has been shown to induce ubiquitination of cellular proteins in the absence of E6, suggesting that E6-AP itself has the properties of an E3 (20). Based on the model of UBR1 and UBC2, we can postulate that E6-AP might physically interact with UbcH5. In preliminary coprecipitation experiments, however, an interaction of E6-AP with UbcH5 could not be detected in either the absence or the presence of E6. This may indicate that there is only a weak interaction between UbcH5 and E6-AP which cannot be detected under the relative stringent conditions of a coprecipitation experiment. Alternatively, E6-AP and UbcH5 may function or interact differently than UBR1 and UBC2. Regardless of the mechanism, the high conservation among the E2s from different organisms at both the sequence level and the functional level indicates that they recognize common structural features or sequence motifs either on target proteins or on proteins with E3 function.

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