

## mUBC9, a Novel Adenovirus E1A-interacting Protein That Complements a Yeast Cell Cycle Defect\*

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Adenovirus E1A encodes two nuclear phosphoproteins that can transform primary rodent fibroblasts in culture. Transformation by E1A is mediated at least in part through binding to several cellular proteins, including the three members of the retinoblastoma family of growth inhibitory proteins. We report here the cloning of a novel murine cDNA whose encoded protein interacts with both adenovirus type 5 and type 12 E1A proteins. The novel E1A-interacting protein shares significant sequence homology with ubiquitin-conjugating enzymes, a family of related proteins that is involved in the proteasome-mediated proteolysis of short-lived proteins. Highest homology was seen with a *Saccharomyces cerevisiae* protein named UBC9. Importantly, the murine E1A-interacting protein complements a cell cycle defect of a *S. cerevisiae* mutant which harbors a temperature-sensitive mutation in *UBC9*. We therefore named this novel E1A-interacting protein mUBC9. We mapped the region of E1A that is required for mUBC9 binding and found that the transformation-relevant conserved region 2 of E1A is required for interaction.

Early region 1A (E1A) of human adenovirus type 5 (Ad5) encodes two differentially spliced mRNAs that specify proteins of 243 and 289 amino acids, respectively (243R and 289R E1A). In infected cells, the E1A proteins are the first viral proteins that are expressed and serve two important functions. First, E1A proteins contribute to the regulation of both viral and cellular gene expression during the viral infection cycle (1, 2). Furthermore, E1A proteins stimulate quiescent cells to enter a cell division cycle following adenovirus infection, allowing the virus to use host-encoded enzymes for replication of its DNA during S phase. *In vitro*, E1A proteins can immortalize primary rodent cells and cooperate with an activated *ras* oncogene in oncogenic transformation (3, 4).

The activities of E1A proteins are mediated primarily through three functional domains that are highly conserved between the E1A proteins of different adenovirus serotypes (5). The 243R and the 289R E1A proteins share conserved regions 1 and 2 (CR1 and CR2),<sup>1</sup> while CR3 is uniquely present in the

289R E1A protein. CR1 and CR2 are largely responsible for the cell cycle stimulatory activity and transforming activity of E1A proteins (5, 6). CR3 encodes a strong transcriptional activation domain (7–10). CR1 and CR2 are required for the suppression of enhancer activity (11–13), for the induction of DNA synthesis (5, 14, 15), and for the induction of apoptosis (16). Both domains are thought to contribute to deregulation of the cell cycle by binding to a set of cellular proteins (17, 18). Subsequent functional inactivation of these proteins with growth inhibitory activity results in the transformed phenotype of the cells. Over the past decade, several of these CR1- and CR2-interacting cellular E1A-binding proteins have been identified. They include the retinoblastoma gene product pRb (19), the pRb-like proteins p107 and p130 (19–22), and the transcriptional coactivator proteins p300 and CBP (23–26). A cellular 48-kDa phosphoprotein named CtBP has also been identified that negatively modulates E1A transformation through binding to a carboxyl-terminal epitope of E1A proteins (27, 28). More recently, the p27 inhibitor of cyclin-dependent kinases has been found to interact with E1A, although it has not been reported where the p27 binding epitope is located (29).

Several cellular proteins have also been found to interact with CR3, the conserved transactivation domain of E1A. These proteins include the TATA-binding protein TBP (30, 31), the transcription factor ATF-2 (32–34), a number of TBP-associated factors (35, 36), and BS69, a protein implicated in repression of E1A-mediated transactivation (37).

We have used a yeast two-hybrid screen to clone additional cellular proteins involved in cell cycle control and that bind to CR2 of adenovirus E1A proteins. We report here the structure and characterization of mUBC9, a novel E1A-interacting protein.

### EXPERIMENTAL PROCEDURES

**Yeast Two-hybrid Screen**—Yeast strain Y190, containing the “bait” plasmid pPC97-E1A, encoding the Gal4 DNA binding domain (DBD) fused to an adenovirus 5 E1A 12 S cDNA fragment encoding amino acids 76–243 of E1A, was transformed with a day 14.5 CD1 mouse embryo library using the lithium acetate method.  $6 \times 10^5$  transformants were selected for growth on plates lacking histidine and supplemented with 25 mM 3-aminotriazole. His<sup>+</sup> colonies were subsequently analyzed for  $\beta$ -galactosidase activity as described previously (38). cDNAs library plasmids derived from double positive yeast colonies were tested for bait specificity by retransformation with different Gal4 DBD fusion plasmids: pPC97-p107, pPC97-*bmi*, and pPC97 without an insert.

**Plasmids**—pPC97-E1A was generated by cloning an adenovirus 5 E1A 12 S cDNA fragment encoding amino acids 76–243 of E1A in-frame with the Gal4 DNA binding domain (amino acids 1–147) of pPC97. Deletion mutants of E1A were constructed by subcloning fragments of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X99739.

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<sup>1</sup> The abbreviations used are: CR, conserved region; DBD, DNA binding domain; PBS, phosphate-buffered saline; TPBS, PBS supplemented

with Tween 20 and nonfat milk; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; HA, hemagglutinin.

12 S E1A in-frame with the Gal4 DBD in pPC97. pPC97-Ad12-E1A was generated by PCR on 12 S E1A from adenovirus type 12 and subsequent subcloning of the partial cDNA encoding residues 80–154. pPC97-E1A (928/961) encodes amino acids 76–140 of 12 S E1A downstream of and in-frame with the Gal4 DBD and was generated by site-directed mutagenesis on wild type 12 S E1A (amino acids 76–140) after cloning this part into pAlter, following the directions given by the manufacturer (Promega), using the oligonucleotide: 5'-CCTCTTCATCCTTGTCGTCACTGGGTGAAAGCCAGCCTCGTGGCCGGTAAGATCG-3'. PCR was used to synthesize a full-length mUBC9 cDNA from pPC86-E1A-BP5, starting at the proper ATG codon and generating a consensus start site. This cDNA was subsequently cloned into pRc/CMV (Invitrogen) with and without an amino-terminal HA tag, recognized by the 12CA5 antibody (39) to generate two mammalian expression vectors: pCMV-mUBC9 and pCMV-HA-mUBC9. pT7-UBC9-#6 for *in vitro* transcription of yeast UBC9 was a kind gift of W. Seufert. Full-length mUBC9 cDNA was also cloned downstream of the glutathione *S*-transferase gene in pGEX-2T for expression in *Escherichia coli*. pGEX-13S-E1A was kindly provided by A. Fattaey. For E1A half-life experiments, full-length 12 S E1A cDNA was cloned into pRc/CMV (pCMV-12S-E1A) and the L122I mutant was generated by site-directed mutagenesis on a full-length 12 S E1A in pAlter, using the following oligonucleotide: 5'-CGTGGCAGGTAATATCGATCACC-3', with subsequent cloning into pRc/CMV (pCMV-L122I-E1A).

**Northern Blot Analysis**—For mUBC9 expression analysis, total cytoplasmic RNA was prepared from a panel of mouse tissues. 20  $\mu$ g of total cellular RNA was electrophoresed through a 1% formaldehyde-agarose gel, transferred to nitrocellulose, and probed with a  $^{32}$ P-labeled mUBC9 cDNA.

**Cell Culture, Transfections, and Labeling**—Human osteosarcoma (U2-OS) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed overnight using the calcium phosphate method. Total amounts of transfected DNA was 20  $\mu$ g/10-cm dish. For pulse-chase experiments, cells were starved for 1 h in methionine/cysteine-free medium and subsequently incubated with 100  $\mu$ Ci of [ $^{35}$ S]methionine/cysteine per 10-cm dish for 1 h. After this, cells were washed twice in PBS, and further maintained in normal Dulbecco's modified Eagle's medium, 10% fetal calf serum. At the indicated time points, cells were lysed by incubation in RIPA for 20 min on ice. Equal amounts of radioactive lysates were incubated on ice with 5  $\mu$ l of nonimmune serum for preclearing. Subsequently, lysates were incubated with protein A-Sepharose beads (Pharmacia Biotech Inc.), precomplexed with 50  $\mu$ l of M73 (anti-E1A) hybridoma supernatant, for 2 h at 4  $^{\circ}$ C while rocking. Then, beads were washed four times in RIPA, heated in SDS-containing sample buffer, and loaded on a 12% SDS-polyacrylamide gel. For detection of endogenous UBC9 and the size of mUBC9 encoded by the novel cDNA, U2-OS cells were transfected with the indicated plasmids overnight using the calcium phosphate method. Cells were fed the following morning, lysed 36 h post-transfection by sonication in SDS-containing sample buffer, and subjected to gel electrophoresis and immunoblotting using the mUBC9 rabbit polyclonal antiserum.

**Antibodies**—To generate antibodies against mUBC9, the production of GST-mUBC9 protein was induced in *E. coli* and purified using glutathione-Sepharose beads (Pharmacia). For immunization and three rounds of subsequent boosts, a rabbit was injected with beads carrying at least 100  $\mu$ g of purified GST-mUBC9 fusion protein to obtain polyclonal serum.

**Immunoblotting**—10-cm dishes of transfected U2-OS cells were lysed by sonication in 1 ml of SDS-containing sample buffer. 100  $\mu$ l of each sample was separated on a 15% SDS-polyacrylamide gel, and transfer was performed overnight to nitrocellulose. The filter was blocked in TPBS, 5% milk (PBS supplemented with 0.1% Tween 20 and 5% nonfat milk; Protifar, Nutricia) for 2 h at room temperature, incubated with anti-mUBC9 polyclonal rabbit serum in a dilution of 1:10,000 in TPBS, 2% milk for 2 h at room temperature, and incubated with secondary antibody (GARPO) in TPBS, 2% milk. The filter was washed three times in TPBS, and visualization was performed by enhanced chemiluminescence (Amersham).

**GST Pull-down Assay**—mUBC9, HA-mUBC9, and yeast UBC9 proteins were made by *in vitro* transcription and translation of the cDNAs using rabbit reticulocyte lysates (Promega) and [ $^{35}$ S]methionine. Equal amounts of proteins were incubated with 1.0  $\mu$ g of either purified GST-13S-E1A protein or GST alone, for 4 h in 0.5 ml of ELB (18) on ice. 50  $\mu$ l of glutathione-Sepharose beads (Pharmacia, 30% slurry) was added and rocked for 1 h at 4  $^{\circ}$ C. Beads were washed four times in ELB, heated in SDS sample buffer, and loaded on a 15% SDS-polyacrylamide gel.

**Yeast Complementation Assay**—A BamHI/XbaI fragment of the E1A-BP5 cDNA was fused to the GAL1 promoter in vector YCplac33 (40). The resulting plasmid was used for transformation of wild type (YWO1) and *ubc9-1* mutant (YWO102) strains (41).

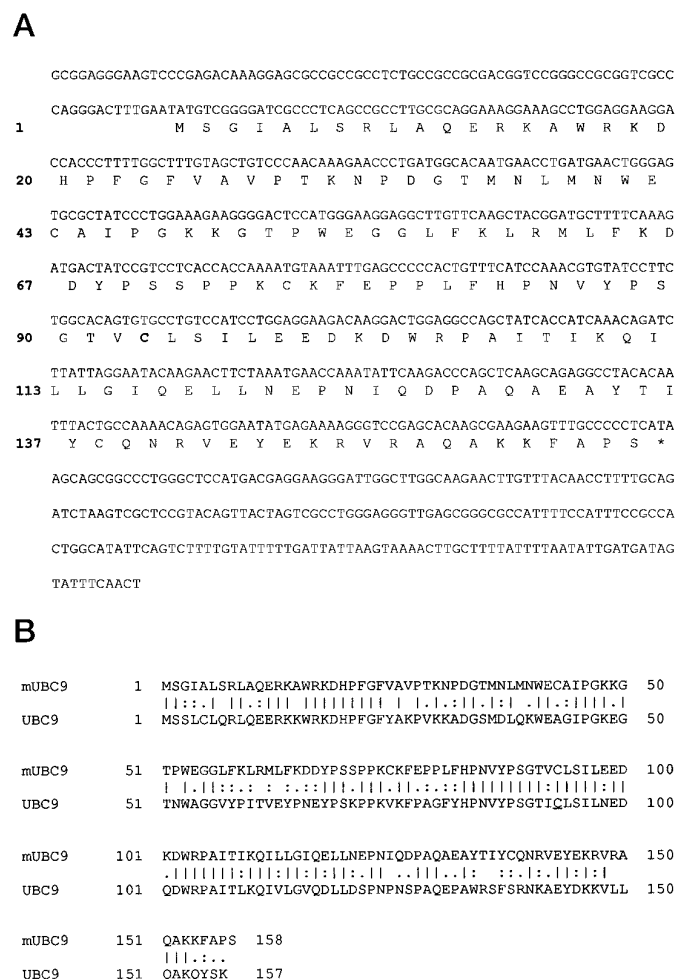
**Screening E1A Mutants, Defective in mUBC9 Binding**—PCR was performed on pPC97-E1A with a 5' primer, identical to a sequence present in the Gal4 DBD and a 3' primer of which the sequence is located downstream of the multiple cloning site. PCR was performed under normal conditions using recombinant *Taq* polymerase (Life Technologies, Inc.) as the mutating agent, following the instructions of the manufacturer. In the first screen the entire PCR pool, containing the randomly point mutated cDNAs, was directly transformed with competent Y190 yeasts in combination with a gapped pPC97. The Y190 strain contained already the pPC86-E1A-BP5 plasmid, carrying the *TRP1* gene, which enables this strain to grow on plates without tryptophan. After homologous recombination and thus recirculation of pPC97, yeast clones were selected for growth on plates lacking leucine and tryptophan. 257 colonies were subsequently analyzed for  $\beta$ -galactosidase activity as described (38). Six clones did not turn blue, suggesting loss of interaction with mUBC9. White colonies were lysed; the lysates were separated on a 12% SDS-polyacrylamide gel, and protein size was checked by immunoblotting, using an antibody directed against the carboxyl terminus of E1A (M73). Plasmid DNA was extracted from the yeast clone that still produced full-length but mutated E1A, and the insert was sequenced completely using the Pharmacia T7 sequencing kit.

## RESULTS

**Cloning of a Novel Adenovirus E1A-interacting Protein**—To identify cDNAs encoding polypeptides that interact with adenovirus E1A, we first tested which domains of the 243R adenovirus 5 E1A protein activate transcription in yeast. The largest fragment of the 243R E1A protein that failed to activate transcription in yeast specified amino acids 76–243 (data not shown). Since this peptide harbors the transformation-relevant CR2 of E1A, a yeast two-hybrid screen was performed with this fragment of E1A as a bait. Yeast strain Y190, which contains two Gal4-inducible reporter genes, *HIS3* and *LacZ*, was co-transformed with the E1A bait plasmid and a day 14.5 CD1 mouse embryo cDNA library. Of  $6 \times 10^5$  yeast transformants, 66 colonies appeared on plates lacking histidine, 19 of which stained positive when tested for expression of  $\beta$ -galactosidase. To test whether E1A was required for interaction with the products of the 19 identified cDNAs, all these clones were retransformed in yeast strain Y190 together with plasmids encoding other Gal4 DBD fusions. All hybrid proteins were found to interact only with Gal4-E1A. Subsequent analyses indicated that 12 of the 19 clones contained an approximately 1-kilobase insert and were derived from the same gene.

We determined the complete nucleotide sequence of the insert of E1A-BP5, one of the 12 identical E1A-interacting clones. Analysis of the cDNA sequence (Fig. 1A) shows a single open reading frame of 158 amino acids. Comparison of the deduced amino acid sequence with GenBank revealed that the protein encoded by the newly identified cDNA had significant homology to a class of related proteins, named ubiquitin-conjugating enzymes (42). These proteins play a role in the proteasome-mediated targeted proteolysis of short-lived cellular proteins. In particular, the E1A-interacting protein showed high homology to *Saccharomyces cerevisiae* UBC9 and a protein from *Schizosaccharomyces pombe*, *hus5* (identities: 56 and 66%, respectively; Refs. 41 and 43). Fig. 1B shows the predicted amino acid sequence of E1A-BP5 and its comparison to the amino acid sequence of *S. cerevisiae* UBC9. Although E1A-BP5 is more closely related to *hus5* than to UBC9, we named this novel E1A-interacting protein mUBC9 (see below).

To address whether mUBC9 also interacts with E1A in another context than in yeast, we performed an *in vitro* binding assay with a bacterially expressed GST-E1A fusion protein and different radioactively labeled UBC9 proteins generated by sequential *in vitro* transcription and translation. Fig. 2A (lanes

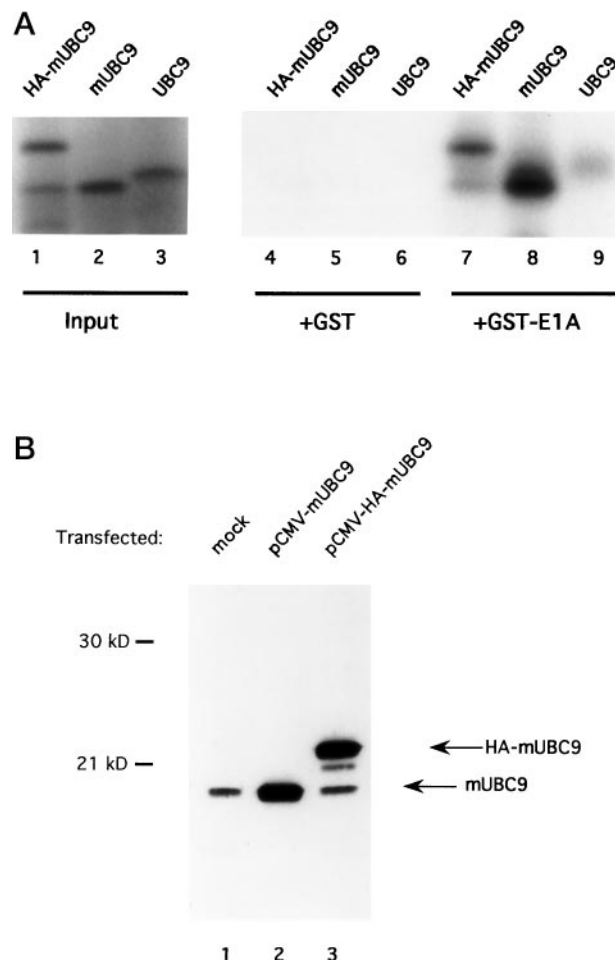


**FIG. 1. Predicted amino acid sequence of mUBC9 and its comparison to yeast UBC9 from *S. cerevisiae*.** A, nucleotide and deduced amino acid sequence of mUBC9. No in-frame stop codons were identified upstream of the ATG start site. The cysteine residue most likely acting as the ubiquitin acceptor site is depicted in **bold** at position 93. B, comparison between mUBC9 (upper strand) and UBC9 from *S. cerevisiae* (lower strand). The identity between these two polypeptides is 56%. The cysteine at position 93 in both proteins is underlined.

1–3) shows 10% of the input of the *in vitro* translated proteins. After incubation, full-length and HA-tagged mUBC9 (lanes 1 and 2) interacted with GST-E1A (lanes 7 and 8), but not with GST alone (lanes 4 and 5). Moreover, the highly homologous UBC9 protein (lane 3) from *S. cerevisiae* also interacted specifically with GST-E1A (lane 9), but not with GST alone (lane 6).

To detect endogenous mUBC9 protein and to ask whether the protein encoded by the mUBC9 cDNA is full-length, we generated a rabbit polyclonal mUBC9 antiserum. We then used this serum to detect UBC9 in whole cell extracts from mock transfected cells and in extracts from cells transfected with an mUBC9 expression vector. Fig. 2B shows that the mUBC9 antiserum detects a protein of approximately 20 kDa in mock-transfected cells (lane 1), the abundance of which is increased substantially in the mUBC9-transfected cells (lane 2). Furthermore, an HA-tagged version of mUBC9 is also recognized by the rabbit serum, which migrates somewhat slower due to the presence of the HA tag (lane 3). A preimmune serum from the same rabbit did not detect any protein of the indicated sizes (data not shown). We conclude that endogenous UBC9 is of similar molecular weight as the cDNA-encoded mUBC9, indicating that the cDNA specifies the full-length mUBC9 protein.

**mUBC9 Complements a *S. cerevisiae* *ubc9* ts Mutation**—To test whether mUBC9 can functionally replace *S. cerevisiae*



**FIG. 2. *In vitro* association between E1A and full-length mUBC9 in a GST pull-down assay.** A, full-length mUBC9, HA-mUBC9, and UBC9 from *S. cerevisiae* were *in vitro* transcribed and translated using [<sup>35</sup>S]methionine/cysteine. Equal amounts of translated products (lanes 1–3, 10% of the input) were mixed with either a purified fusion between 13 S E1A and the GST protein (lanes 7–9) or GST alone (lanes 4–6), incubated and pulled down with glutathione-Sepharose beads. B, the novel cDNA encodes a full-length UBC9 protein. Two expression vectors (pCMV-mUBC9 and pCMV-HA-mUBC9) were transfected in the human osteosarcoma cell line U2-OS. Whole cell lysates were separated on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. The blot was then incubated with a rabbit polyclonal antiserum directed against GST-mUBC9. Positions of endogenous UBC9 (lane 1), transfected mUBC9 (lane 2), and HA-tagged mUBC9 are indicated by arrows.

UBC9, we used a yeast strain (*ubc9*–1) with a temperature-sensitive mutation in *UBC9* (41). Wild type yeast and *ubc9*–1 mutants (YWO1 and YWO102, respectively) were transformed with either vector plasmids alone or vector expressing murine UBC9 and were streaked out on galactose-containing agar plates and incubated at either 23 °C or 37 °C for 3 days. Fig. 3 shows that the temperature-sensitive growth of the yeast *ubc9*–1 mutant is suppressed by expression of the murine UBC9 homologue. Surprisingly, mUBC9 did not complement a mutation in the *S. pombe* *hus5* gene<sup>2</sup> This was unexpected since mUBC9 is more homologous to *hus5* than to UBC9. These data indicate that mUBC9 can functionally replace the *S. cerevisiae* UBC9, but not *S. pombe* *hus5*. We therefore named this novel E1A-interacting protein mUBC9.

**mUBC9 Is Broadly Expressed**—To analyze the pattern of expression of mUBC9 mRNA, the murine *UBC9* cDNA was

<sup>2</sup> A. M. Carr, personal communication.



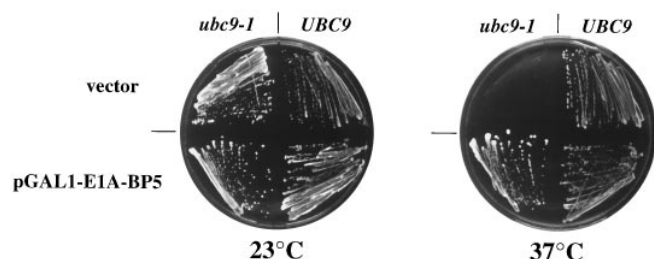


FIG. 3. **Murine E1A-BP5 complements yeast *ubc9-1*.** Yeast wild type (UBC9; right side of the plates) and *ubc9-1* mutants (left side) transformed with either vector plasmids (top) or vector expressing murine E1A-BP5 (pGAL1-E1A-BP5; bottom) were streaked out on galactose-containing agar plates and incubated at either 23 °C or 37 °C for 3 days. The temperature-sensitive growth of the yeast *ubc9-1* mutant is suppressed by expression of the UBC9 homologue E1A-BP5 from mice.

used to probe a Northern blot containing total cytoplasmic RNA from several rat tissues. The *mUBC9* probe detected two transcripts of 1.0 and 3.2 kilobases in all tissues tested (Fig. 4). We have recently cloned several additional *mUBC9* cDNAs from a mouse embryo cDNA library and found that the larger transcript only differs in the 3'-noncoding region from the 1.0-kilobase mRNA (data not shown). Immunostaining of cells transiently transfected with a plasmid encoding HA-tagged *mUBC9* revealed that transfected HA-*mUBC9* was present in the cytosol as well as in the nucleus (data not shown).

**Mapping of the UBC9 Binding Site on E1A**—To determine which domain of 243R E1A is responsible for binding to *mUBC9*, we generated a set of expression vectors in which different parts of the 243R E1A protein are fused to the Gal4 DBD. These proteins were then coexpressed in yeast strain Y190 with a vector that directs the synthesis of *mUBC9* fused to the Gal4 transactivation domain. The results of these experiments are summarized in Table I. Interaction (as evidenced by the blue staining of the yeast colonies) was seen with 243R E1A residues 76–140, but not 120–140 or 76–120. These data indicate that CR2 (residues 121–127) is likely to be involved in *mUBC9* binding, but is not sufficient for interaction. In support of a role for CR2 in the binding of UBC9, we found that residues 80–154 of adenovirus type 12 E1A also mediate binding to *mUBC9*, indicating that *mUBC9* binding is a conserved feature of different adenovirus serotypes. However, a double point mutant in CR2 of Ad5 E1A that abolishes binding to pRb (928/961) still binds *mUBC9*, indicating that the pRb binding site and the *mUBC9* binding site are not identical (Table I).

To further study the amino acid residues of E1A that are involved in *mUBC9* interaction, we performed a yeast PCR-based mutagenesis screen. This screen is schematically represented in Fig. 5. This procedure takes advantage of the high frequency of recombination between homologous DNA fragments in yeast (44). In short, a part of the DNA encoding Gal4-E1A (amino acids 76–243) fusion protein was amplified by PCR under conditions in which random mutations are induced at low frequency. This mutagenized PCR product was then introduced in yeast, together with a gapped expression vector that lacks an E1A insert. After homologous recombination of sequences up- and downstream of the E1A insert, a functional Gal4-E1A expression vector is created *in vivo*. Using this approach, we created a “library” of mutated Gal4-E1A expression vectors that were tested for interaction with *mUBC9* using the standard two-hybrid assay. In the initial screen 257 yeast colonies were assayed, of which six failed to interact with *mUBC9*. From these six, five yeast colonies turned out to express a truncated Gal4-E1A fusion protein, resulting from the introduction of an early stop codon by the mutagenesis procedure. One white colony expressing full-

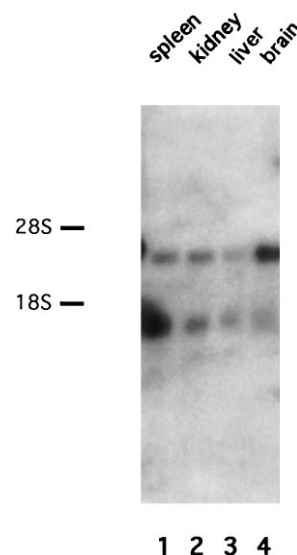


FIG. 4. **Expression of *mUBC9*.** Northern blot containing total RNA derived from the following rat tissues: spleen (lane 1), kidney (lane 2), liver (lane 3), and brain (lane 4). Equal amounts of RNA were loaded. The positions of the 18 and 28 S rRNAs are depicted on the left.

TABLE I

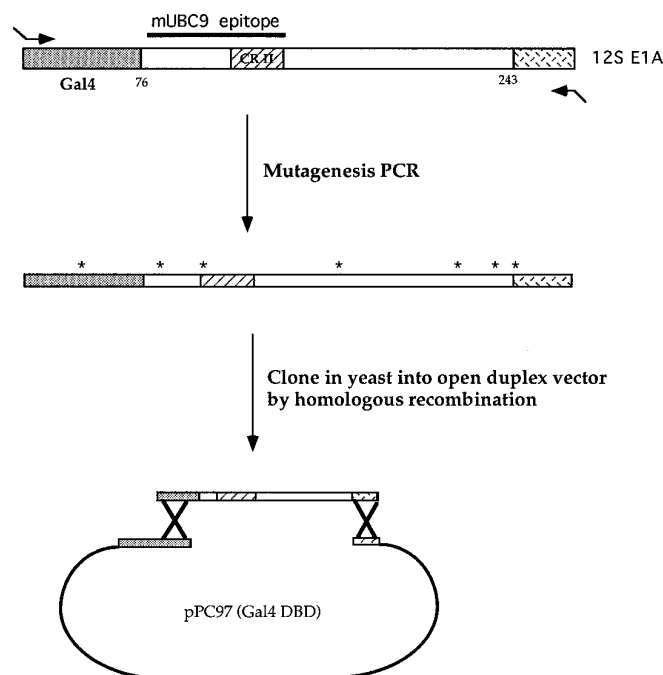
Mapping of the *mUBC9* binding epitope of adenovirus E1A in a yeast two-hybrid assay

A set of yeast expression vectors encoding Gal4-DBD-12S E1A deletion mutants was generated (left column). These vectors were introduced in the yeast strain Y190 with and without pPC86-*mUBC9* (center), and  $\beta$ -galactosidase activity was determined. Only two E1A fusion proteins, comprising residues 1–76 and the full-length 12 S E1A (1–243), transactivated in yeast (blue, right column). The largest E1A part that did not transactivate (white) contained amino acids 76–243 of 12 S E1A. This deletion mutant of E1A was used in the initial two-hybrid screen. All non-transactivating fusions were checked for the interaction with a fusion of *mUBC9* and the transactivation domain of Gal4 (Gal4-TA). The smallest part of E1A that still interacts with *mUBC9* contains residues 76–140. The 928/961 mutant contains residues 76–140 of Ad5 E1A, except for two mutations: C124G and E135K, together resulting in an E1A mutant that is unable to interact with the family of pocket proteins (pRb, p107, and p130). Residues 80–154 of Ad12 E1A are the equivalent amino acids of 76–140 in Ad5 E1A, both including CR2.

Gal4-DBD+	Gal4-TA+	Colony
E1A 1–243		Blue
E1A 1–76		Blue
E1A 76–243		White
E1A 76–243	<i>mUBC9</i>	Blue
E1A 76–140	<i>mUBC9</i>	Blue
E1A 120–140	<i>mUBC9</i>	White
E1A 76–120	<i>mUBC9</i>	White
E1A 141–243	<i>mUBC9</i>	White
E1A 928/961	<i>mUBC9</i>	Blue
E1A 80–154(Ad12)		White
E1A 80–154(Ad12)	<i>mUBC9</i>	Blue

length Gal4-E1A protein, as judged by immunoblot analysis of whole yeast protein extracts (data not shown), was then subjected to DNA sequence analysis. This mutant E1A protein turned out to have a single amino acid substitution, changing leucine 122 of E1A to an isoleucine. We named this mutant E1A-L122I. Since leucine 122 is part of the highly conserved pRb binding motif of E1A (the LXCXE motif), these data indicate that *mUBC9* interacts directly with CR2 of E1A, raising the possibility that *mUBC9* binding may contribute to trans-formation by E1A.

**E1A Protein Stability Is Not Affected by *mUBC9***—Since E1A itself is a short-lived protein (45, 46), one possibility would be that E1A itself is a target for UBC9-mediated proteolysis as was suggested by Ciechanover *et al.* (47). To investigate this, we used the L122I mutant, which fails to bind to *mUBC9*. To

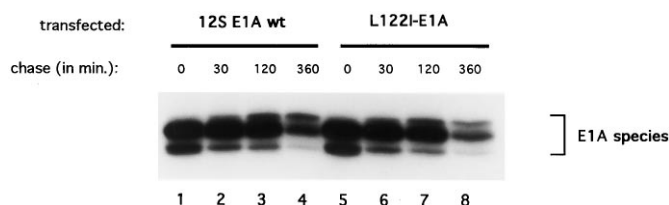


**FIG. 5. PCR-based screen for point mutants of E1A that lost the ability to interact with mUBC9 in yeast.** PCR was performed on pPC97-E1A using primers upstream and downstream of the E1A insert. To generate a pool of mutated products, it was sufficient in these studies to use recombinant *Taq* polymerase (Life Technologies, Inc.) under normal PCR conditions. Products were subsequently transformed together with a gapped pPC97 plasmid as indicated. Homologous recombination takes place in a transformed Y190 yeast strain, which already contains the pPC86-mUBC9 (pPC86-E1A-BP5) plasmid, and occurs on both sites of the point-mutated E1A cDNA. A circularized pPC97-E1A plasmid allows growth on plates without leucine. Yeast colonies are subsequently screened for  $\beta$ -galactosidase activity, where a blue colony means interaction between E1A and mUBC9, while a white colony means loss of interaction. A subsequent analysis of the white colonies is then performed to see whether the mutated Gal4-E1A fusion is full-length. If so, plasmid DNA is extracted and the insert is sequenced to locate the generated point mutation(s).

measure protein half-life, U2-OS cells were transiently transfected with expression vectors encoding full-length 12 S wild type and L122I mutant E1A proteins. After 2 days, transfected cells were pulse-labeled with [ $^{35}$ S]methionine/cysteine and chased with normal medium for various periods of time. Fig. 6 shows that both transiently expressed proteins and especially the faster migrating species have similar half-lives of approximately 2 h, which agrees well with data from previous studies (45, 46). We conclude that mUBC9 interaction does not affect the half-life of E1A protein.

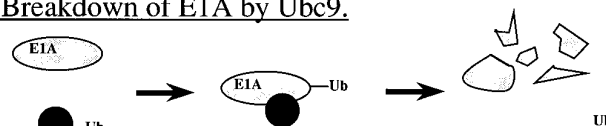
#### DISCUSSION

We have used a yeast two-hybrid screen to identify a novel murine cDNA whose encoded protein interacts with adenovirus E1A-transforming proteins. The newly identified protein is a member of a family of related proteins, named ubiquitin-conjugating enzymes (UBCs), that play a role in proteasome-mediated proteolysis of short-lived proteins (42, 48). It shares significant homology to both *S. cerevisiae* UBC9 (41) and *S. pombe* hus5 (43). However, in a functional complementation assay, the murine UBC only complements the *S. cerevisiae* *ubc9* defect, but not the *S. pombe* *hus5* mutation. For this reason, we have named the novel E1A-interacting protein mUBC9. Recently, the human homologue of UBC9 was reported by two groups. Strikingly, the amino acid sequence of human UBC9 is 100% identical to the mouse sequence reported here (49, 50).

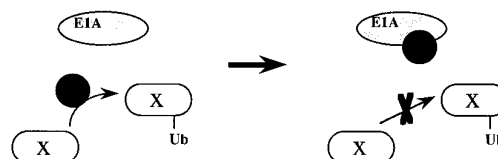


**FIG. 6. Half-lives of wild type and L122I mutant E1A proteins.** U2-OS cells, transfected with either pCMV-12S-E1A (lanes 1–4) or pCMV-L122I-E1A (lanes 5–8), were labeled with [ $^{35}$ S]methionine/cysteine for 1 h and subsequently chased in Dulbecco's modified Eagle's medium for the indicated times. E1A protein was immunoprecipitated by M73, an E1A-specific monoclonal antibody, collected with protein A-Sepharose beads, and separated on a 12% SDS-polyacrylamide gel. The different E1A species derived from the 12 S cDNAs are indicated by a bracket on the right.

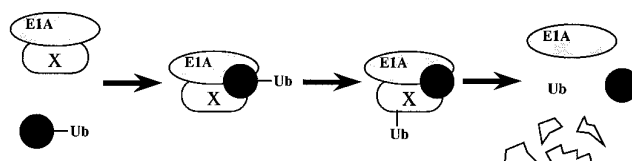
#### A. Breakdown of E1A by Ubc9.



#### B. E1A inactivates Ubc9.



#### C. E1A activates Ubc9.



**FIG. 7. Three possible models for the E1A-UBC9 interaction.** A, UBC9 (indicated as a black dot) targets E1A for breakdown through the ubiquitin-proteasome pathway, by conjugating ubiquitin molecules (Ub) to E1A. B, E1A binds and subsequently inactivates UBC9, thereby preventing the breakdown of protein X, which can no longer be ubiquitinated by UBC9. C, E1A binds a certain protein X, acting as a targeting factor, and because of the E1A-UBC9 interaction, X becomes a target for breakdown by ubiquitination through UBC9.

There are several possible explanations for the observed E1A-mUBC9 interaction, which are schematically represented in Fig. 7. First, it is possible that E1A itself is a target for UBC9-mediated ubiquitin-dependent degradation (Fig. 7A). This is unlikely, because a mutant E1A protein that fails to bind mUBC9 has the same half-life as wild type E1A protein (Fig. 6). It does, however, not exclude the possible ubiquitination of E1A through a number of essential residues in the entire amino terminus of E1A, which are involved in the rapid breakdown of E1A (51, 52). Nevertheless, we did not find evidence supporting ubiquitination of E1A using a method as was described for c-Jun (53). Second, by binding UBC9, E1A could interfere with the targeted proteolysis of certain cellular proteins, thereby stabilizing proteins with growth-stimulatory activity (Fig. 7B). Alternatively, E1A could simultaneously bind

both UBC9 and a growth-inhibitory cellular protein, thereby targeting the cellular E1A-interacting protein for ubiquitin-mediated proteolysis (Fig. 7C). In this scenario, E1A would act as a targeting factor. A possible candidate in such a model is the cyclin-dependent kinase inhibitor p27, a protein degraded through ubiquitination (54) and one which was recently shown to interact with E1A (29).

That viral infections can lead to a significant deregulation of cell cycle control was suggested in studies from the laboratory of P. Howley, in which it was shown that the tumor suppressor protein p53 is targeted for degradation by the ubiquitin pathway, through binding of the papilloma virus protein E6 to a cellular polypeptide called E6-AP (55, 56). In this case, E6-AP serves as a ubiquitin ligase targeting p53 for rapid breakdown. That binding of adenovirus E1A to UBC9 somehow contributes to deregulation of the cell cycle is suggested only indirectly by our finding that E1A uses conserved region 2 for mUBC9 interaction, a region known to be crucial for the transforming activity of E1A. Our finding that mUBC9 complements a yeast cell cycle defect is also consistent with a role for mUBC9 in cell cycle control.

Recently, a number of cell cycle regulatory proteins have been shown to be targets of ubiquitin-mediated targeted proteolysis. These proteins include the yeast cyclins CLN2, CLN3, CLB2, and CLB5 (41, 57, 58), the yeast p40<sup>sup1</sup> Cdk inhibitor (59), A and B type cyclins (60), the immediate early gene products c-Jun and c-Fos (53, 61), and the two proteins mentioned earlier, the mammalian p27 Cdk inhibitor and the tumor suppressor p53 (54, 55). We have recently found that the E2F transcription factors are also degraded by ubiquitin-mediated proteolysis. Significantly, E1A coexpression causes a significant increase in the half-life of E2F.<sup>3</sup> We are currently investigating the role of mUBC9 in E1A-mediated transformation and cell cycle progression of mammalian cells.

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