JOURNAL OF VIROLOGY, Jan. 2001, p. 699–709 Vol. 75, No. 2
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.2.699–709.2001

Identification of Three Functions of the Adenovirus E4orf6 Protein That Mediate p53 Degradation by the E4orf6-E1B55K Complex

EMMANUELLE QUERIDO,[1]† MEGAN R. MORISON,[1] HUAN CHU-PHAM-DANG,[1] SARAH W.-L. THIRLWELL,[1] DOMINIQUE BOIVIN,[1]‡ AND PHILIP E. BRANTON[1,2]*

Departments of Biochemistry[1] and Oncology,[2] McGill University, Montreal, Quebec, Canada H3G 1Y6

Received 18 May 2000/Accepted 10 October 2000

Complexes containing adenovirus E4orf6 and E1B55K proteins play critical roles in productive infection. Both proteins interact directly with the cellular tumor suppressor p53, and in combination they promote its rapid degradation. To examine the mechanism of this process, degradation of exogenously expressed p53 was analyzed in p53-null human cells infected with adenovirus vectors encoding E4orf6 and/or E1B55K. Coexpression of E4orf6 and E1B55K greatly reduced both the level and the half-life of wild-type p53. No effect was observed with the p53-related p73 proteins, which did not appear to interact with E4orf6 or E1B55K. Mutant forms of p53 were not degraded if they could not efficiently bind E1B55K, suggesting that direct interaction between p53 and E1B55K may be required. Degradation of p53 was independent of both MDM2 and p19ARF, regulators of p53 stability in mammalian cells, but required an extended region of E4orf6 from residues 44 to 274, which appeared to possess three separate biological functions. First, residues 39 to 107 were necessary to interact with E1B55K. Second, an overlapping region from about residues 44 to 218 corresponded to the ability of E4orf6 to form complexes with cellular proteins of 19 and 14 kDa. Third, the nuclear retention signal/amphipathic arginine-rich α-helical region from residues 239 to 253 was required. Interestingly, neither the E4orf6 nuclear localization signal nor the nuclear export signal was essential. These results suggested that if nuclear-cytoplasmic shuttling is involved in this process, it must involve another export signal. Degradation was significantly blocked by the 26S proteasome inhibitor MG132, but unlike the HPV E6 protein, E4orf6 and E1B55K were unable to induce p53 degradation in vitro in reticulocyte lysates. Thus, this study implies that the E4orf6-E1B55K complex may direct p53 for degradation by a novel mechanism.

The cellular p53 tumor suppressor protein could represent a major obstacle to the replication of all small DNA tumor viruses. Activation of p53 following infection would result in inhibition of viral and cellular DNA synthesis and premature cell death through apoptosis (64, 81). Regulation of the stability of p53 protein is known to be crucial in the control of p53 function in uninfected cells. In the early stages of human adenovirus infection, the products of early region 1A (E1A) form complexes with two classes of cellular proteins, the retinoblastoma tumor suppressor product, pRb, and related proteins p107 and p130, as well as the histone acetyltransferase p300 and related proteins p400 and the cyclic AMP-responsive element-binding protein (CREB)-binding protein (CBP) (5, 18, 33). Binding of E1A to either class of proteins stimulates cellular DNA synthesis and activates S phase in quiescent cells. Expression of E1A has been shown by several groups to trigger the accumulation of p53 protein and p53-dependent apoptosis (8, 12, 13, 23–25, 44, 67). This stabilization of p53 is dependent on the same E1A regions involved in complex formation with p300-CBP-p400 and pRb (11, 15, 61, 66). It is likely that p53 stabilization induced by E1A binding of pRb results from the activation of E2F transcription factors and the subsequent up regulation of expression of *ink4a*/*arf* (14, 15). Stabilization therefore ensues through inhibition by p19ARF of MDM2-mediated p53 degradation (32). Nevertheless, E1A can still induce p19ARF protein levels in $pRb^{-/-}$ cells, suggesting that additional mechanisms exist (15). Binding of p300 by E1A proteins reduces the ability of p53 to transactivate *mdm2* (82) and diminishes MDM2-mediated p53 degradation, which may require p300 as a cofactor (26).

The accumulation of p53 induced by E1A is not normally evident in wild-type-adenovirus-infected cells, and several studies have shown that expression of both the 34-kDa early-region 4 orf6 protein (E4orf6) and the early-region 1B 55-kDa protein (E1B55K) is responsible (50, 52, 60, 75). E4orf6 and E1B55K have been known for some time to interact (10, 69) and to be involved in such late functions as selective transport and translation of viral mRNAs and shutoff of host cell protein synthesis (1, 2, 9, 28, 55, 68). Both E1B55K (70) and E4orf6 (17) also bind independently to p53. The interaction of E1B55K with the amino-terminal acidic activation domain of p53 results in inhibition of p53 transactivation activity through the introduction of an E1B55K repression function (47, 79, 80, 87). This interaction also has recently been suggested to inhibit the acetylation of a carboxy-terminal lysine of p53 by the histone acetyltransferase P/CAF (D. Liao, personal communication). The p53-mediated repression activity originating from

* Corresponding author. Mailing address: Departments of Biochemistry and Oncology, McGill University, McIntyre Medical Sciences Building, 3655 Promenade Sir William Osler, Montreal, Quebec H3G 1Y6, Canada. Phone: (514) 398-8350. Fax: (514) 398-7384. E-mail: branton@med.mcgill.ca.

† Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11274.

‡ Present address: GeminX Biotechnology Inc., Montreal, Quebec, Canada H2W 2M9.

associated histone deacetylase complexes may also be inhibited by E1B55K (51). Such events block p53-induced growth arrest and apoptosis (13, 45, 79, 81) and thus enhance transformation by E1A and other oncogenes (88). E4orf6 binding toward the carboxy terminus of p53 also enhances E1A/E1B-mediated transformation (50, 52, 54). The mechanism of this effect is still not clear. Two studies have shown that E4orf6 binding blocks p53-mediated gene expression (17; E. Querido, S. Gurd, and P. E. Branton, unpublished data), although two other studies found little effect of E4orf6 on several p53-regulated promoters, at least in certain cell types (63, 76). It has been suggested that inhibition of p53 transcriptional activity might result from the disruption of interactions with $TAF_{II}31$ (17). A recent study by our group found that E4orf6 did not effectively block p53-mediated apoptosis, even though it blocked p53 transactivation of several promoters (Querido et al., unpublished).

E4orf6 is a nuclear protein and contains both a putative nuclear localization signal (NLS) and an amphipathic arginine-rich α-helical nuclear retention signal (NRS) that are believed to be responsible for the targeting of E4orf6 and E4orf6-E1B55K complexes to the nucleus (21, 57). In addition, E4orf6 contains a nuclear export signal (NES) believed to play a role in nuclear-cytoplasmic shuttling of the E1B55K-E4orf6 complex (16) and to be essential for the ability of E4orf6 to support viral replication (83); however, the results from a more recent study have questioned the ability of this E4orf6 domain to function in nuclear export, at least in some cells, and have demonstrated that E1B55K itself contains an NES and can shuttle independently of E4orf6 (39). It remains unclear which functional domains of the E1B55K and E4orf6 proteins participate in p53 degradation. E4orf6 was recently identified as a zinc-binding protein (7), and several cysteine and histidine residues are critical for E4orf6 to interact with E1B55K and to participate in p53 degradation. Several studies have found that E1B55K and E4orf6 selectively target p53 but not the related p73 or p63 proteins (30, 63, 85). Recently our group detected a series of proteins that interact with E4orf6, including cellular products of 84, 19, 16, and 14 kDa (6). Some insights into the mechanism of p53 degradation may therefore be derived through the identification of novel E1B55K- or E4orf6-binding proteins.

In this study we have used a mainly genetic approach to investigate some of the parameters of E4orf6-E1B55K-mediated p53 degradation. Interactions of E1B55K with both p53 and E4orf6 appeared to be required, but degradation was independent of both MDM2, which controls p53 degradation in uninfected cells by a ubiquitin-dependent pathway (29, 31, 40), and p19ARF, which promotes p53 stability (32, 58, 89). E4orf6-E1B55K-mediated p53 degradation was inhibited by the drug MG132, an inhibitor of the 26S proteasome. Our results suggest the E4orf6-E1B55K complexes may induce p53 turnover by a unique mechanism.

MATERIALS AND METHODS

Cells. Human $p53^{-/-}$ H1299 cells (48) and primary mouse embryo fibroblasts (MEF) were cultured on 60-mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in Dulbecco's modified Eagle's medium (GIBCO BRL) supplemented with 10% fetal bovine serum (CanSera). Primary MEFs derived from wild-type, $p53^{-/-}$ and $p53^{-/-}$ $MDM2^{-/-}$ (34, 49), and $p19ARF^{-/-}$ (37) mice were used in some experiments. All viral stocks were subjected to titer determination on 293 cells (22).

Vectors. Various $E1^-$ $E3B^-$ adenovirus vectors were used for this study. In the control AdLacZ vector, the E1 region (E1A and E1B) was replaced by the *Escherichia coli lacZ* gene under the control of the cytomegalovirus (CMV) promoter, as was the case with the Adp53wt and Adp53S135 vectors, which, instead of LacZ, express wild-type p53 or a p53 Cys-135-to-serine mutant, respectively (3). All these vectors were provided by Frank Graham (3). The Adp53KEEK (mutated at residues 341, 344, 348, and 355) and Adp53S135KEEK adenovirus vectors were produced by cloning each cDNA (74) into a shuttle vector under the control of the CMV promoter, and viruses were generated in 293 cells by homologous recombination of the shuttle vector DNA with the adenovirus $E1^-$ $E3B^-$ vector DNA backbone (3). The AdE4orf6 and AdHH55K adenovirus vectors were produced in a similar fashion and express E4orf6 or histidine-HMK-tagged E1B55K, respectively, under the control of the CMV promoter (60). The AdE2F1 vector expressing human E2F-1 was provided by Joe Nevins and has been described elsewhere (38). In cases of coinfection with multiple adenovirus vectors, such vectors were first premixed in serum-free medium and then used to infect H1299 cells at 35 PFU of each virus. MEFs were infected with 100 PFU of each virus. Total virus input was kept constant in all experiments by addition of the appropriate amount of AdLacZ vector. For infection, cell culture medium was removed and 0.5 ml of medium containing the appropriate vectors was added followed by gentle rocking every 15 min. Medium containing 10% fetal calf serum was added after 1 h, and the cultures were incubated until harvesting. Constructs containing the cDNA for hemagglutinin antigen (HA)-tagged p73α and p73β were obtained from Bill Kaelin (35). The pRcCMV p53 22-23 plasmid was provided by Arnold Levine (43). The pcDNA3p53wt plasmid was made by cloning the cDNA encoding human p53 into pcDNA3. The pCA14HH55K plasmid, expressing His-tagged E1B55K from the CMV promoter, is the same plasmid used to make the AdHH55K vector (60).

E4orf6 mutants. Figure 1 illustrates the E4orf6 mutants used in the present studies. The cDNA for adenovirus type 5 E4orf6 was cloned into pcDNA3 and expressed in vivo from the CMV promoter. The T7 promoter and an SP6 promoter were located at the amino terminus and at the carboxy terminus, respectively, of E4orf6. The internal deletion mutants were prepared by a four-oligonucleotide PCR method, as follows. A PCR amplification was performed with a T7-specific oligonucleotide and with another complementary to the region bordering the amino-terminal limit of the deletion, while another PCR amplification was done with an oligonucleotide to the carboxy-terminal border of the deletion and an SP6-specific oligonucleotide. The two PCR fragments were ligated, and a second PCR amplification was done with the T7 and SP6 oligonucleotides, so that only correctly ligated products were amplified. E4orf6 mutants were then cloned into pcDNA3. The G198A, R240E/R241E, and E255A/E256A point mutants were made by three-oligonucleotide PCR amplification using an oligonucleotide containing the desired point mutation(s) as well as those specific for T7 and SP6. The *dl*1–13, *dl*1–38, and *dl*1–55 mutants were made by a two-oligonucleotide PCR amplification using amino-terminal oligonucleotides engineered to start at the desired amino acid, and the SP6 oligonucleotide. The *dl*1–108 mutant was made by restriction enzyme digestion with *Eco*RV, which cleaves at a unique site, thus placing the methionine at position 109 to position 1. The *dl*108–294, *dl*143–394, *dl*219–294, and *dl*275–294 deletions were obtained by first engineering three stop codons in the three reading frames at the C terminus of E4orf6, and then digesting with the unique cutters *Eco*RV, *Pvu*II, *Ssp*I, and *Bss*HII, respectively, as well as the restriction site right before the stop codons; this was followed by blunt end ligation. All E4orf6 mutants were verified by sequencing. A list of the oligonucleotides used will be provided upon request. Point mutants with mutations affecting the NES (L90A/I92A) and the NRS (R248E), as well as an NLS-NRS double mutant (L90A/I92A/R248E), were generously provided by Matthias Dobbelstein (16).

Antisera. Anti-p53 mouse monoclonal antibodies Ab1801 and Ab421 were purified by precipitation using ammonium sulfate from supernatants obtained from hybridoma cell cultures grown in Dulbecco's modified Eagle's medium supplemented with 10% low-immunoglobulin G (IgG) fetal calf serum (Bethesda Research Laboratories). A goat polyclonal antibody to p53 (Santa Cruz) was also used for Western blotting of p53 in the binding assays. Anti-E1B55K mouse monoclonal antibody 2A6 (71) was collected as an ascites fluid generated in mice by using appropriate hybridoma cells. Production of E4orf6-N (Ab1808) and E4orf6-C (Ab1807) rabbit polyclonal antisera has been described previously (6). The 1D5 anti-E4orf6 mouse monoclonal antibody was raised against a peptide corresponding to the 14 carboxy-terminal amino acids of E4orf6 by using standard protocols. Anti-HA.11 mouse monoclonal antibody (BabCo) was used to detect HA-tagged p73 proteins. Anti-E2F1 and anti-cMyc monoclonal anti-

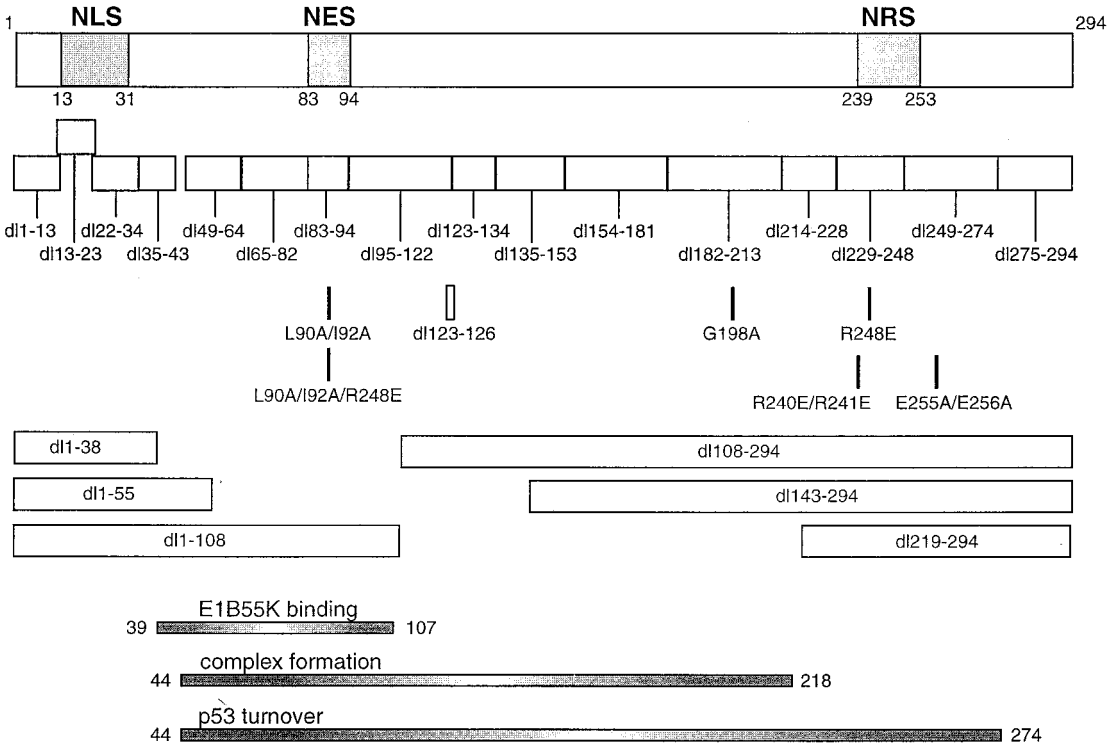

FIG. 1. Model of the Ad5 E4orf6 protein. The positions of the putative NLS, NES and NRS are indicated by shadowed boxes. All the deletion and point mutants used in the present study are indicated by boxes or lines. The minimal regions necessary for interaction with E1B55K, for complex formation with the cellular p84, p19, and p14 proteins, and for p53 degradation are also indicated.

bodies were purchased from Santa Cruz. Anti-MDM2 2A10 mouse monoclonal antibody has been described previously (56).

Western blotting analysis. Whole-cell extracts were prepared on ice in Western blot lysis buffer (50 mM HEPES [pH 7.9] containing 400 mM KCl, 0.1% [vol/vol] NP-40, 4 mM NaF, 4 mM $NaVO_4$, 0.2 mM EDTA, 0.2 mM EGTA, 2 mg each of aprotinin, leupeptin, and Pefabloc SC per ml, and 1 mM dithiothreitol). Total protein was measured by Bradford protein assay as specified by the manufacturer (Bio-Rad), and 40 μg of total cell protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Nuclear and cytoplasmic extracts were prepared as described previously (84). Proteins were electroblotted onto nitrocellulose membranes for 1 h by using a semidry transfer apparatus. After blocking overnight, the membranes were incubated with the appropriate dilution of primary antibody for 2 h at room temperature, followed by a 45-min incubation with secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Jackson ImmunoResearch Laboratories), or with anti-goat IgG (Santa Cruz). Immunoreactive bands were revealed using an enhanced chemiluminescence (ECL Plus) kit (Renaissance; DuPont-NEN).

Binding assays. In many cases, H1299 cells growing on 60-mm-diameter dishes were infected with adenovirus vectors coding for p53, E1B55K, or E4orf6, as described above. In some cases, cells were subjected to lipofection by the Lipofectamine Plus (GIBCO) method with 2 μg of pRcCMV p53 22-23, pcDNA3 HAp73α, or pcDNA3 HAp73β and the adenovirus vectors AdE4orf6, AdHH55Kwt, or AdlacZ were added with the plasmid DNA during lipofection. Finally, for all the binding assays with E4orf6 mutants, cells were subjected to lipofection by the standard Lipofectamine Plus method with 2 μg of pcDNA3 E4orf6 (or mutants) and 2 μg of pCA14HH55Kwt plasmid. Cell extracts were prepared 24 h later in buffer X (50 mM Tris-HCl [pH 8.0] containing 250 mM NaCl, 1% (vol/vol) NP-40, 1 mM EDTA, 1 mM EGTA, and 2 μg each of aprotinin, leupeptin and pepstatin per ml). Extracts were precleared and immunoprecipitated with the indicated antibody for 2 h at 4°C, and a minimum of five washes in buffer X were performed. Since E1B55K and E4orf6 proteins tended to form high-molecular-weight aggregates when boiled in SDS sample buffer, samples were instead incubated for 15 min at 55°C before being loaded on SDS–8 or 14% polyacrylamide gels. For E4orf6 binding to p53 or HA-p73, cell lysates were immunoprecipitated with anti-E4orf6 antibody (Ab1807) and immunoblotted with anti-p53 (Ab1801 and Ab421) antibody or anti-HA antibody. For E1B55K binding to E4orf6, lysates were immunoprecipitated with 2A6 antibodies and precipitates were immunoblotted using either E4orf6-N or E4orf6-C sera. Western blotting was performed as above.

In vivo p53 degradation assay. H1299 cells growing on 60-mm-diameter dishes were subjected to lipofection by the Lipofectin (GIBCO/BRL) method with a total of 9 μg of plasmid DNA per plate, made up of 1 μg of pcDNA3 p53wt plasmid DNA, 4 μg of pCA14 HH55Kwt, and 4 μg of pcDNA3 E4orf6. Empty pcDNA3 vector DNA was used to keep the quantity of DNA constant in all plates when either E1B55K or E4orf6 was omitted. After 24 h, the cells were harvested and quantitative Western blotting was performed, as described above. For all the p53 turnover assays, the expression level of E4orf6 and E1B55K species was always confirmed by stripping the membranes of p53 antisera and then reblotting with antibodies against these polypeptides.

In vitro p53 degradation assay. Human papillomavirus type 18 (HPV18) E6, p53, E1B55K, and E4orf6 proteins were transcribed and translated in vitro in reticulocyte lysates (TnT; Promega) with either T7 or SP6 polymerase, depending on the particular plasmid (details of plasmids used are available upon request). E1B55K, E4orf6, p53, and luciferase proteins were generated in the presence of [^{35}S]methionine, while HPV18 E6 was labeled with [^{35}S]cysteine (translation grade; DuPont-NEN). The proteins were then separated by SDS-PAGE and visualized by autoradiography to verify the efficiency of the reaction (data not shown). Unlabeled HPV18 E6, E1B55K, E4orf6, and luciferase proteins were generated in a parallel reaction in the presence of nonradioactive methionine-cysteine. The p53 degradation assay was performed as described by Scheffner (73) in 40-μl volumes containing 2 μl of radioactively labeled p53, 8 μl of each unlabeled protein, and 10 μl of unprogrammed reticulocyte lysate in 25 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 3 mM dithiothreitol. The reaction mixtures were incubated for 3 h at 25°C, the reactions were stopped by boiling in SDS sample buffer, the products were separated by SDS-PAGE, and p53 was visualized by autoradiography.

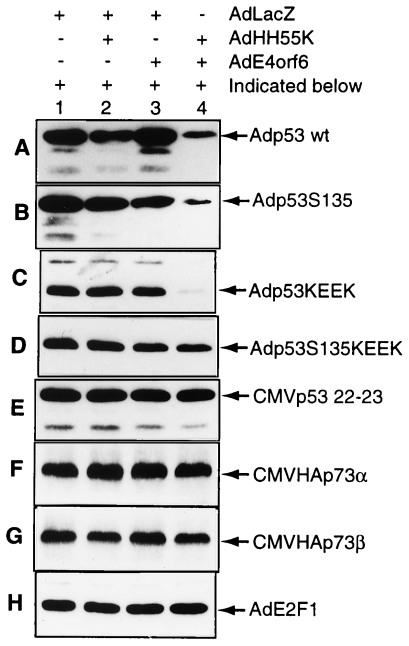

FIG. 2. E4orf6-E1B55K-mediated degradation of wild-type and mutant p53 and of p73. (A) Degradation of wild-type p53. p53-null H1299 cells were infected with an adenovirus vector expressing wild-type p53 (lanes 1 to 4) along with adenovectors coding for LacZ (as a control), E4orf6, or E1B55K. Whole-cell extracts were prepared at 24 h postinfection and subjected to Western blotting using anti-p53 Ab1801 antibodies. (B to E) Degradation of mutant p53. Similar assays were performed using various p53 mutants expressed from adenovirus vectors (Ad) or following colipofection with appropriate cDNAs (CMV). (F and G) Degradation of p73. Degradation of p73 proteins was assessed in similar assays following colipofection of p73-specific cDNAs. (H) Lack of degradation of control protein E2F1.

RESULTS

Analysis of E4orf6-E1B55K-induced degradation of mutant and wild-type p53 and related p73 proteins. Previous studies showed that E4orf6 in combination with E1B55K protein induces the degradation of p53 (50, 52, 60, 75). To study this process further, the degradation of both wild-type and mutant p53 proteins as well as of the p53-related proteins p73α and p73β (35, 36) was assessed. p53-null human H1299 cells infected with adenovirus vectors expressing E4orf6 and/or E1B55K were either coinfected with vectors expressing various forms of p53 or subjected to lipofection with plasmid DNA expressing p53 or p73 species, and the amounts of each product were determined by quantitative Western blotting of whole-cell extracts. Figure 2 shows that E2F-1, expressed as a control from AdE2F1 vector and detected by anti-E2F-1 serum, was unaffected by E4orf6 and E1B55K (Fig. 2H); however, both wild-type p53 (Fig. 2A, Adp53wt), and mutant forms with defects in the DNA-binding domain (Fig. 2B, Adp53S135) and the tetramerization domain (Fig. 2C, Adp53KEEK) were readily degraded. As shown previously (51, 53, 63, 78), such degradation required the expression of both viral proteins. Using the adenovirus vectors described for Fig. 2A, we verified that the half-life of wild-type p53 was reduced from 4 h to 20 min when both E1B55K and E4orf6 were expressed (data not shown). The half-life of p53 expressed from the Adp53wt vector has been described previously as being unusually long (3), presumably because the levels of p53 expressed from the CMV promoter exceeded the endogenous degradative capacities of the cell.

Interestingly, p53 molecules that contained mutations affecting both the DNA-binding and tetramerization domains (Fig. 2D, Adp53S135KEEK) or the p53 22-23 mutation (Fig. 2E, CMVp53 22-23) were resistant to degradation by E1B55K-E4orf6. The reason for this effect appeared to be related to the ability of p53 to interact with E1B55K. Table 1 summarizes results of quantitative p53-binding assays (primary data not shown), in which the ability of p53 molecules to interact with E4orf6 or E1B55K was assessed by immunoprecipitation with 2A6 anti-E1B55K serum or rabbit polyclonal E4orf6-C sera followed by Western blotting with the appropriate antibody. Both wild-type p53 and the p53S135 mutant proteins interacted with both E4orf6 and E1B55K; however, the p53KEEK mutant was found to interact only with E1B55K and the p53S135KEEK mutant form interacted with neither. The p53 mutations at codons 22 and 23 are known to disrupt both MDM2 and E1B55K binding (43). These results suggested that the interaction of p53 with E1B55K alone is sufficient for p53 degradation, since the p53 KEEK mutant, which failed to bind to E4orf6, was still able to be degraded by the E1B55K-E4orf6 complex. The present analysis was extended to the p53-related p73α and p73β proteins. Two studies have shown that these molecules do not interact with E1B55K (46, 63), although another report indicated that E4orf6 does bind p73 (30). Table 1 shows that in cells expressing HA-tagged p73 products, neither E4orf6 nor E1B55K interacted with p73 proteins. As might be expected, Fig. 2F and G indicate that no p73 degradation took place in response to E4orf6 and E1B55K.

Generation of E4orf6 deletion mutants. To analyze the regions of E4orf6 required for degradation of p53, a series of deletion mutants was generated by PCR-directed mutagenesis. E4orf6 is a 294-residue 34-kDa nuclear protein predicted to contain a large central hydrophobic region with more hydrophilic carboxy and amino termini. Figure 1 shows that two signal sequences have been identified that can mediate import into the nucleus: an NLS near the amino terminus (residues 13

TABLE 1. Binding of wild-type and mutant p53 proteins and p73 to E4orf6 and E1B55K

Binding Protein	Degree of binding to[a]:	
	E4orf6	E1B55K
p53 (wild type)	+++	+++
p53S135	+	+++
p53KEEK	−	+
p53S135/KEEK	−	−
p73 α	−	−
p73 β	−	−

[a] H1299 cells were infected with adenovirus vectors encoding the indicated proteins, except for HAp73 α and β, for which lipofection and infection was performed, as described in Materials and Methods. For each binding assay, a negative control was conducted by infecting the cells with AdLacZ instead of AdE4orf6 or AdHH55K. Binding of p53 proteins was assessed by immunoprecipitation with anti-E4orf6 (Ab1807) or anti-E1B55K (2A6) antibodies, and immunoblots were performed using goat polyclonal p53 antibody (Santa Cruz). The ability of HA-tagged p73 proteins to bind E1B55K or E4orf6 was assessed in a similar fashion, except that blots were probed using HA.11 antibody (BabCo).

TABLE 2. Mapping of E4orf6 regions necessary for interaction with E1B55K, complex formation with cellular proteins, and p53 degradation

E4orf6 region	Degree of:		
	p53 degradation[a]	Complex formation[b]	Binding to E1B55K[c]
E4orf6 (wild type)	+++	+++	+++
*dl*1–38	+++	+++	+++
*dl*1–55	−	±	−
*dl*1–108	−	−	−
*dl*108–294	−	−	+++
*dl*143–294	−	−	+++
*dl*219–294	−	+++	+++
*dl*275–294	++	+++	+++
*dl*1–13	+++	ND[d]	+++
*dl*13–23	+++	ND	+++
*dl*22–34	+++	ND	+++
*dl*34–43	+++	+++	+++
*dl*49–64	−	−	+++
*dl*65–82	−	−	+++
*dl*83–94	−	−	+++
*dl*95–122	−	−	+++
*dl*123–126	−	ND	+++
*dl*123–134	−	ND	+++
*dl*135–153	−	ND	+++
*dl*154–181	−	ND	+++
*dl*182–213	−	ND	+++
*dl*214–229	−	ND	+++
*dl*229–248	−	ND	+++
*dl*249–274	−	ND	+++
L90A/I92A	++	ND	+++
G198A	+++	+++	+++
R240E/R241E	−	+++	+++
R248E	++	ND	+++
L90A/I92A/R248E	++	ND	+++
E255A/E256A	+++	+++	+++
E4orf6/7 (wild type)	−	−	−

[a] p53 degradation assays were performed as described for Fig. 3D and E.
[b] For binding to p19, and p14, H1299 cells were subjected to lipofection with cDNAs encoding wild-type or mutant E4orf6, and following labeling with [^{35}S]methionine-[^{35}S]cysteine, E4orf6 was immunoprecipitated with appropriate amino- or carboxy-terminal-specific antisera and the presence of E4orf6 and its binding proteins was determined by SDS-PAGE and autoradiography, as described in the legend to Fig. 4. The ability of the p19 and p14 cellular proteins (6) to associate with E4orf6 polypeptides is summarized.
[c] E4orf6-E1B55K binding assays were performed as described for Fig. 3A to C.
[d] ND, not determined.

to 31) and an NRS toward the carboxy terminus (residues 239 to 253). Each is believed to play a role in directing the nuclear localization of E1B55K (21, 57). E4orf6 also contains a putative Rev-like NES sequence that may mediate nuclear-cytoplasmic shuttling, at least under some circumstances (16). Point mutants with mutations affecting the NES (L90A/I92A) and the NRS (R240E/R241E and R248E), as well as an NLS-NRS double mutant (L90A/I92A/R248E), have been described by others (16, 57). Mutants with the mutations G198A in the central region and E255A/E256A in a sequence just carboxy terminal to the NRS were produced, as well as a series of large and small deletion mutants (a complete list is given in Fig. 1). The early-region 4 E4orf6/7 protein, which shares 58 amino-terminal residues with E4orf6 but reportedly none of its functions, was also included in our assays. All the E4orf6 mutant proteins listed in Table 2 and Fig. 1 could be detected by Western blotting using either the E4orf6-N or E4orf6-C polyclonal serum, and they were stable and expressed at similar levels (59). E4orf6 mutants *dl*108–294 and R240E/R241E (containing an intact NLS) and E4orf6 *dl*1–108 (containing an intact NRS) were properly localized to the nucleus when transfected into H1299 cells, as shown by immunofluorescence (data not shown).

Analysis of E1B55K binding and p53 turnover using E4orf6 mutants. To determine what regions of E4orf6 are functionally important, E4orf6 mutant products were analyzed for their ability to bind to E1B55K and to participate in the degradation of p53. It should be noted that we have made repeated and unfortunately unsuccessful attempts to use these mutants in detailed mapping studies of the regions in E4orf6 involved in binding to p53 in the absence of E1B55K. One reason for this failure in studies involving immunoprecipitation with anti-p53 serum and Western blotting with E4orf6 sera was that the level of binding to p53 alone is considerably lower than that with E1B55K. Therefore it became difficult to separate many of the E4orf6 mutant proteins from various minor contaminating nonspecific species during the prolonged exposure times required. In addition, the amino and carboxy terminus-specific E4orf6 rabbit polyclonal sera do not immunoprecipitate with the same efficiency. Thus, performance of the study using the reverse approach with anti-p53 serum for Western blotting was also difficult. We have also been unsuccessful in purifying significant quantities of E4orf6 synthesized in bacteria to permit in vitro binding assays. Further studies are being pursued to rectify these problems; however, we are not able to address this important aspect of the regulation of p53 degradation in this report.

The ability of E4orf6 mutant proteins to bind to E1B55K was examined in coimmunoprecipitation studies following lipofection of p53-null H1299 cells with appropriate cDNAs. The capacity of E4orf6 products to degrade p53 in cooperation with E1B55K was determined by measuring p53 levels by Western blotting of whole-cell extracts of H1299 cells that had been subjected to lipofection with cDNAs encoding human p53, E1B55K, and E4orf6 (wild type or mutants). Figure 3A to C show the primary results for some of the E4orf6 mutants, and results for all mutants are summarized in Table 2. Figure 3B shows the expression levels for the series of larger deletion mutants, as determined by immunoblotting of whole-cell extracts with the appropriate amino- or carboxy-terminal polyclonal sera. Figure 3A shows the presence of similar total levels of E1B55K in all whole-cell extracts. Figure 3C shows that all mutant E4orf6 proteins interacted with E1B55K, with the exception of the *dl*1–55 and *dl*1–108 mutants. Since the *dl*1–38 and *dl*108–294 products associated with E1B55K, it appeared that the binding site for this protein on E4orf6 is located between residues 39 and 107 or at least that this region is required to establish the appropriate conformation for binding. Table 2 shows that all of the smaller deletion mutants with deletions toward the amino terminus still bound E1B55K, suggesting that it may interact with several points in this region of E4orf6. None of the point mutations affected E1B55K binding, and E4orf6/7 (residues 1 to 58) did not interact.

Analysis of the ability of E4orf6 mutants to support p53 degradation in cooperation with E1B55K is shown in Fig. 3D and E. Figure 3D (lanes 2 to 5) again indicated that both E4orf6 and E1B55K were essential. Figure 3D (lanes 6 to 12) shows results obtained with the larger E4orf6 deletions. Mu-

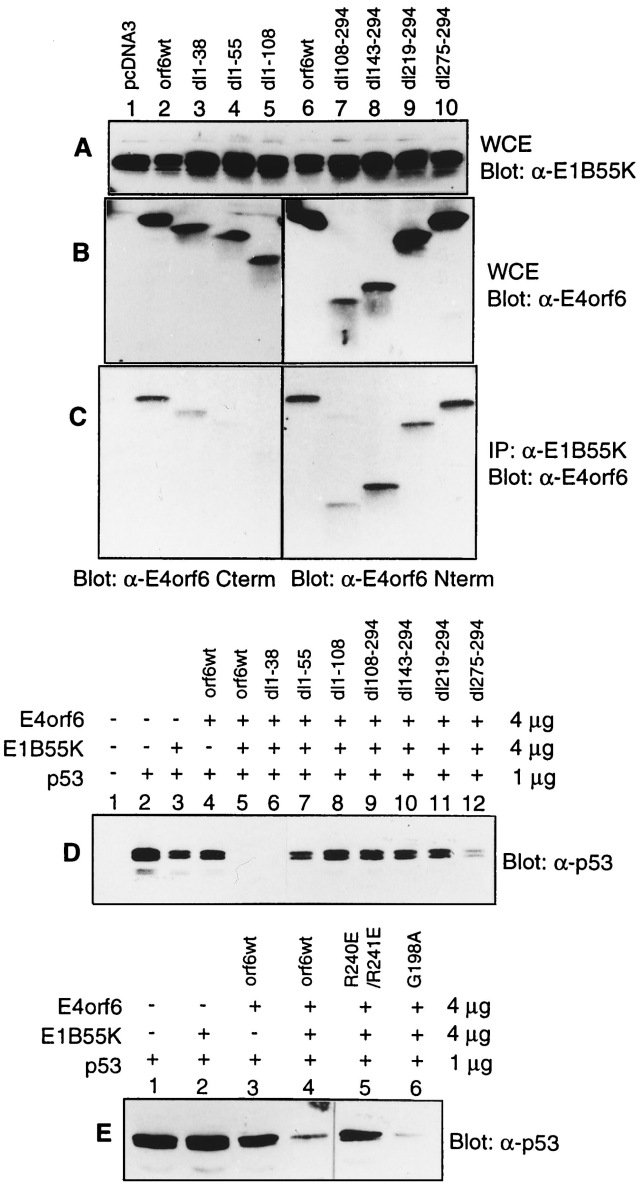

FIG. 3. Mapping of E4orf6 regions required for binding of E1B55K and p53 degradation. H1299 cells were subjected to colipofection with cDNAs encoding E1B55K and wild-type or mutant E4orf6, and cell extracts were prepared at 24 h postinfection. (A to C) E4orf6-E1B55K binding. Whole-cell extracts (WCE) were separated by SDS-PAGE and immunoblotted for E1B55K using 2A6 antibodies (A) or E4orf6-C (lanes 1 to 5) or E4orf6-N (lanes 6 to 10) antibodies (B). Extracts were also immunoprecipitated (IP) using 2A6 antibodies and immunoblotted using E4orf6-C (lanes 1 to 5) or E4orf6-N (lanes 6 to 10) antibodies (C). (D) p53 degradation by E4orf6 deletion mutants. H1299 cells were subjected to colipofection with cDNAS expressing E1B55K, mutant or wild-type E4orf6, and wild-type p53, as listed above the figure. The levels of p53 were determined by quantitative Western blotting using a mixture of anti-p53 Ab1801 and Ab421 antibodies. (E) p53 degradation by E4orf6 point mutants. An experiment similar to that in panel D was performed using wild-type E4orf6 and E4orf6 point mutants.

tant *dl*1–38 (lane 6) exhibited the same efficacy as did wild-type E4orf6 for p53 degradation, and mutant *dl*275–294 (lane 12) exhibited a very high degree of p53 turnover. Mutant *dl*1–55 was almost totally defective, as were all the other E4orf6 mutant products. Table 2 shows that several of the smaller amino-terminal deletions, including mutations affecting residues between 1 and 43, were able to cause efficient p53 degradation. All other deletion mutants were totally defective for p53 degradation. These results suggested that an extended region from residues 44 to 274 is required for E4orf6 to function in p53 degradation. Further analysis of p53 turnover by using deletion and point mutants revealed the role of E4orf6 signal sequences. Deletion of the NLS (residues 13 to 31; see mutant *dl*1–38, Fig. 3D, lane 6) had no effect on turnover. Although deletion of the region containing the NES (residues 83 to 94) in mutants *dl*1–108 (Fig. 3D, lane 8) and *dl*83–94 (Table 2) eliminated p53 turnover, the point mutant L90A/I92A, which blocks E4orf6-mediated nuclear export (16), supported high levels (see Table 2). Therefore, neither the E4orf6 NLS nor its NES is essential. The R240E/R241E mutant, which is predicted to disrupt the arginine-faced amphipathic α-helix and thus affect nuclear targeting (57), was greatly deficient in p53 degradation (Fig. 3E, lane 5). Interestingly, another mutant with a mutation in the NRS, R248E, which is believed to have little or no effect on NRS function (D. A. Ornelles, personal communication), supported p53 turnover well (Table 2). Thus, p53 turnover appears to require a functional NRS.

Formation of complexes with cellular proteins by E4orf6 mutant products. We recently reported that E4orf6 forms complexes with a series of cellular proteins (6). We speculate that at least some of these proteins play a role in E4orf6-induced p53 degradation, and studies are under way to identify certain of these species. Of some interest are the cellular 84-, 19-, 16-, and 14-kDa E4orf6-binding proteins. The 84-kDa species is difficult to resolve unless special gel conditions are employed, and the levels of the 16-kDa protein vary considerably from experiment to experiment (6); however, the 19- and 14-kDa species, which have been observed in parallel with the 84- and 16-kDa proteins, reproducibly coimmunoprecipitate with E4orf6. To determine the relationship between complex formation with these proteins and E4orf6-dependent p53 turnover, H1299 cells were subjected to lipofection with cDNAs encoding wild-type or mutant E4orf6 and labeled with [^{35}S]methionine-[^{35}S]cysteine, and E4orf6 was immunoprecipitated using antisera directed against the carboxy or amino terminus of E4orf6. Figure 4 shows the pattern of E4orf6 and coimmunoprecipitating proteins following SDS-PAGE and autoradiography for several of the E4orf6 mutants. Results for additional mutants are summarized in Table 2. Using both E4orf6-C (Fig. 4, lane 2) and E4orf6-N (lane 9) sera, both the 19- and 14-kDa cellular species were present in association with wild-type E4orf6, but they were not present in precipitates from cells subjected to lipofection with empty plasmid vector DNA (lanes 1 and 8). The E4orf6 product encoded by mutant *dl*1–38 (lane 3) also associated with these 19- and 14-kDa proteins. Mutant E4orf6 from *dl*1–55 showed little or reduced levels of binding (lane 4), but mutants produced by *dl*219–294 (lane 12) and *dl*275–294 (lane 13) associated with the 19- and 14-kDa species consistently. Such was not the case with *dl*1–108 (lane 5), *dl*49–64 (lane 7), *dl*108–294 (lane 10), and *dl*143–294 (lane 11). Among the mutants tested (see the summary in Table 2), the patterns of p53 degradation and complex formation corresponded exactly, with the exception of *dl*219–294 and R240E/R241E, which appeared to interact with these cellular species

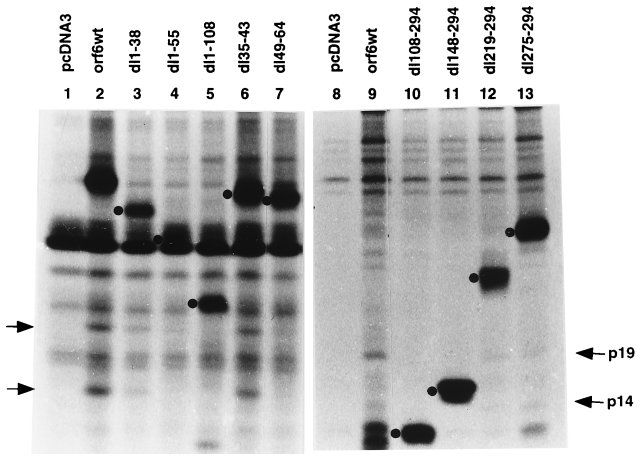

FIG. 4. Mapping of the region of E4orf6 involved in complex formation with cellular proteins. H1299 cells growing on 100-mm-diameter plates were subjected to lipofection with 10 μg of pcDNA3 plasmid DNA encoding wild-type or mutant E4orf6. Following labeling from 18 to 22 h postinfection with 200 μCi of [^{35}S]methionine-[^{35}S]cysteine EasyTag Express protein-labeling mixture (>1,000 Ci/mmol) per plate in methionine-cysteine-free medium, whole-cell extracts were prepared in buffer X (see Materials and Methods), immunoprecipitated using 1808-E4orf6-N (lanes 8 to 13) or 1807-E4orf6-C (lanes 1 to 7) rabbit polyclonal antisera, and analyzed by SDS-PAGE and autoradiography, as described previously (6). The identities of the E4orf6 proteins in each lane are indicated, as are the positions of migration (●) of wild-type or mutant E4orf6 products and the 19- and 14-kDa E4orf6-binding proteins (6).

but were defective in the NRS and failed to induce p53 turnover. These results suggested that complex formation may be necessary but is insufficient for p53 degradation. The identities of these cellular proteins will be the subject of a future communication.

In vitro p53 degradation assays using reticulocyte lysates. It is well known that the HPV E6 protein induces the degradation of p53 through interactions with a cellular ubiquitin ligase, E6AP (72). For HPV E6 this process can be reconstituted in vitro by incubation of E6 and p53 proteins with reticulocyte lysates (73). To determine if E4orf6 and E1B55K function through a similar mechanism, comparable in vitro studies were conducted with reticulocyte lysates. Figure 5 shows that the HPV18 E6 protein stimulates the degradation of p53 in either the absence (lane 2) or presence (lane 3) of E1B55K; however, whether alone (lane 4) or in the presence of E1B55K (lane 5), E4orf6 was not functional in this assay. These results suggest that E4orf6-induced p53 degradation occurs via a mechanism different from that of HPV E6 and may require factors that are not present or not reconstitutable in reticulocyte lysates.

Role of cellular p19ARF and MDM2 proteins in E4orf6-mediated p53 turnover. It has recently been shown that the alternative-reading-frame protein p19ARF from the *ink4a*/*arf* locus plays a role in the stabilization of p53 by interacting with MDM2 to inhibit the ubiquitin-mediated degradation of p53 by MDM2 (32, 37, 58, 78). Adenovirus E1A proteins signal through p19ARF to activate and stabilize p53 (15). To determine if E4orf6/E1B55K-mediated p53 turnover requires either p19ARF or MDM2, studies were carried out in MEF cells derived from knockout mice deficient in the expression of p53, p53 and MDM2 (34, 49), and p19ARF (37). The MEFs were coinfected with Adp53wt and combinations of AdE4orf6 and AdHH55Kwt vectors, and the levels of p53 and MDM2 in whole-cell extracts were determined by Western blotting using a mixture of anti-p53 and anti-MDM2 monoclonal antibodies. The same blots were then reanalyzed for E1B55K and E4orf6 proteins using appropriate antibodies. Figure 6A shows that in p53$^{-/-}$ MEFs, degradation of exogenously expressed p53 was efficient only in the presence of both E1B55K and E4orf6 (lane 5). As in Fig. 2, some reduction was observed with AdHH55Kwt alone (Fig. 6A, lane 4), due presumably to the presence of low levels of E4orf6 from the vector backbone. The slightly reduced level of p53 observed with AdE4orf6 alone (lane 3) was not reproducible and may have resulted from experimental variation, which can occur in studies involving infection with three or four different viral vectors. Previous studies failed to show any effect on p53 stability using E4orf6 alone (75, 76); however, neither these results nor those presented in Fig. 6 can exclude the possibility that E4orf6 alone has some small effect on p53 degradation. Because *mdm2* expression is regulated by p53 (4), MDM2 protein was detected in proportion to the levels of p53. Analysis of extracts from both p53$^{-/-}$ MDM2$^{-/-}$ (Fig. 6B) and p19ARF$^{-/-}$ cells (Fig. 6C) indicated patterns of p53 degradation very similar to those shown in Fig. 6A. In both cases, the levels of p53 were greatly reduced in the presence of E4orf6 and E1B55K, indicating that neither MDM2 nor ARF was required for p53 degradation. Similar high levels of p53 degradation were also detected in U2OS human osteosarcoma cells that lack (78) p19ARF (data not shown). It is interesting that although p19ARF-null cells were fully able to support E4orf6-E1B55K-dependent degradation, somewhat higher levels of p53 appeared to be present than in p19ARF-positive cells following infection by Adp53wt and AdHH55Kwt. These small differences probably resulted from experimental variation (discussed above), since they were not evident in all experiments.

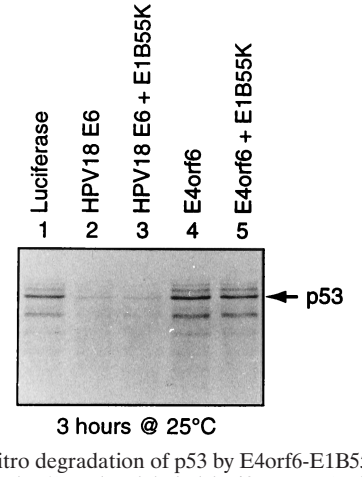

FIG. 5. In vitro degradation of p53 by E4orf6-E1B55K and HPV18 E6. Radiolabeled p53 and unlabeled luciferase, E1B55K, E4orf6, or HPV18 E6 protein were synthesized by in vitro transcription-translation. Labeled p53 was incubated for 3 h at 25°C and then analyzed by SDS-PAGE and autoradiography, as described in Materials and Methods. The position of migration of p53 is indicated on the right.

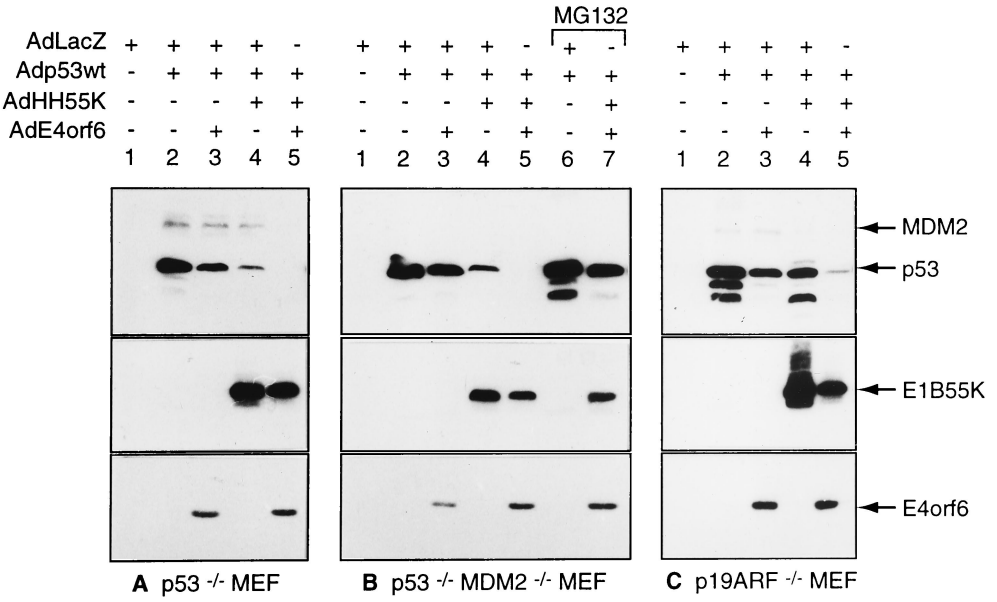

FIG. 6. Role of MDM2, p19ARF, and the 26S proteasome in E1B55K-E4orf6-induced p53 degradation. p53 degradation was assessed in cells coinfected with combinations of adenovirus vectors expressing LacZ (control), wild-type p53, E1B55K, or E4orf6. Such cells included p53-null MEFs (A), p53- and MDM2-null MEFs (B), and p19ARF-null MEFs (C). Panel B (lanes 6 and 7) also includes extracts from cells treated at 18 h postinfection with medium containing 50 mM MG132, an inhibitor of the 26S proteasome. Cell extracts were prepared at 24 h postinfection, separated by SDS-PAGE and analyzed by Western blotting using antibodies against MDM2 (antibody 2A10) and p53 (a mixture of antibodies Ab1801 and Ab421) (top panels), E1B55K (2A6) (middle panels), and E4orf6 (1807-4 E4orf6-C) (bottom panels). The combinations of vectors used are listed at the top.

Role of the 26S proteasome in E4orf6-E1B55K-mediated p53 degradation. To determine if the 26S proteasome is involved in E4orf6-E1B55K-induced p53 degradation, studies were conducted using a proteasome inhibitor, MG132. The drug MG132 (*N*-carbobenzoxy-leucyl-leucyl-leucinal) is a cell-permeable transition-state inhibitor of chymotrypsin-like and postacidic activities of the proteasome (MG132 also inhibits cathepsins and calpains). It was successfully used by Roth et al. to inhibit the degradation of p53 by MDM2 in vivo (62). Analyses of whole-cell extracts from $p53^{-/-}$ $MDM2^{-/-}$ MEFs (Fig. 6B) showed that addition of MG132 greatly inhibited E4orf6-E1B55K-induced p53 turnover (compare lanes 6 and 7 with untreated lanes 2 and 5). Thus, as with the degradation of many proteins, the 26S proteasome appeared to be important for E4orf6-E1B55K-mediated p53 turnover.

DISCUSSION

The present studies have addressed the ability of E4orf6 to function in E4orf6-E1B55K complexes to degrade p53 and the p53-related p73 products. Many previous studies have shown that this complex functions in the regulation of p53 activity and stability, but the data on interactions with p73 are conflicting. We found no evidence that E1B55K or E4orf6 interacts with either form of p73, a finding in agreement with two previous reports (46, 63) but contrary to another for E4orf6 (30). In any case, using exogenously expressed p73α and p73β, E4orf6-E1B55K complexes were clearly unable to induce p73 degradation.

Our other studies made use of p53 and E4orf6 mutants to reveal further details of the mechanism of E4orf6-E1B55K-induced p53 degradation. Mutations in p53 affecting DNA-binding activity or the tetramerization motif had no effect on degradation; however, forms of p53 that failed to associate with E1B55K were resistant. Would p53 and E1B55K proteins that are incapable of interacting directly, but that retain the ability to complex with E4orf6, still be active in degradation? We are still uncertain about the answer to this question since it has been difficult to map the p53-binding site(s) on E4orf6 and thus to generate the appropriate E4orf6 mutants. In addition, all existing E1B55K mutants that fail to bind p53 are defective for E4orf6 binding (65, 86). The p53 22-23 point mutations that disrupt the E1B55K-binding site should not affect E4orf6 binding, and as we and others (63) have shown, p53 22-23 is resistant to degradation. These results suggest that binding of p53 to E1B55K, but not to E4orf6, is absolutely required for p53 degradation. This point was also well illustrated by Roth et al. (63) in an ingenious experiment in which a small region in the amino terminus of p73 was replaced with the region of p53 responsible for E1B55K binding. They found that this p73mut20–24 form of p73 could be degraded by E1B55K-E4orf6. Since E4orf6 does not bind p73 (see above), it must mean that E1B55K binding was sufficient for p73mut20–24 to be degraded.

The present studies also indicated that E4orf6-E1B55K-mediated degradation of p53 took place at high levels in the presence or absence of two cellular regulators of p53 stability, p19ARF and MDM2. These results suggested that the mechanism of p53 degradation may differ from cellular pathways regulated by MDM2. One complication of these studies was that they were performed with knockout MEFs. One previous study indicated that although E4orf6 and E1B55K colocalize

efficiently in human cells, convincing colocalization was not apparent in mouse cells (21). Colocalization of E4orf6 and E1B55K in other rodent cells has been observed at high levels using transformed baby rat kidney cells (52–54). We have not investigated this issue directly; however, the fact that E4orf6 and E1B55K cooperate to induce p53 turnover in mouse cells suggests that sufficient functional interactions must take place.

The production of a large series of E4orf6 mutants permitted the analysis of the role of E4orf6 in p53 turnover. All E4orf6 mutants studied yielded products that were stable in mammalian cells (see above) (Querido et al., unpublished); however, fine mapping in the central, generally hydrophobic region of E4orf6 was not highly informative since all such mutants lost the ability to cooperate in p53 degradation. These results may suggest a functional requirement for extended regions of E4orf6 or may reflect a high degree of conformational effects elicited by even small deletions. Detailed analysis of this region will require either high-resolution structural information or the production of specific point mutants. Conversely, deletions at the hydrophilic amino and carboxy termini were reasonably informative.

A recent study (7) found that E4orf6, like HPV E6, is a zinc-binding protein. Several critical histidine and cysteine residues were identified, and mutations affecting those sites interfered with E1B55K binding and p53 degradation, although none of the mutations seemed to disrupt p53 binding. It is interesting that Boyer and Ketner (7) found that the amount of E4orf6 that coimmunoprecipitated with p53 was smaller than the amount with E1B55K and that none of the E4orf6 mutations disrupted p53 binding. We have made similar observations (data not shown), and thus further kinds of analyses must be used to identify the regions of E4orf6 that mediate interaction with p53.

Mapping studies confirmed a previous report that binding of E1B55K requires a region toward the amino terminus of E4orf6 (65). This region includes residues 44 to 107, and the lack of binding to the E4orf6/7 protein, which contains E4orf6 residues 1 to 58, indicated that residues 44 to 58 were clearly not sufficient. Fine mapping of this binding region using small E4orf6 deletion mutants suggested that many contact points may exist, since all were capable of E1B55K binding. Figure 1 shows that this region is downstream of the NLS but spans the putative NES sequence; however, the latter is not required for binding since mutant *dl*83–94 was wild type both for binding and for p53 degradation in cooperation with E1B55K. It should be noted that although we found that *dl*108–294 and all other carboxy-terminal E4orf6 mutants were able to interact with E1B55K, another group reported on the importance of the NRS for E4orf6 to direct E1B55K into the nucleus (21). We believe that the region identified in the present study, residues 39 to 107, corresponds to the minimal domain necessary for interaction with E1B55K and that it remains possible that the E1B55K-E4orf6 complexes we observed may be created in cell extracts following lysis. Therefore, the data from these separate studies may not be contradictory. The strong interaction between E1B55K and E4orf6 was not disrupted by any of the internal deletion mutations, perhaps because there are many points of contact between the two proteins or because the interaction may be reinforced in vivo by E1B55K- or E4orf6-binding proteins.

An extended region of E4orf6 from residues 44 to 274 was required to support p53 degradation. As just discussed, one function of this region appeared to be to interact with E1B55K; however, residues 108 to 294 were dispensable for E1B55K binding. We believe that this region provides at least two additional functions required for p53 degradation. We have recently reported the coimmunoprecipitation of a series of cellular and possibly viral proteins by using E4orf6-specific antibodies (6). In the present studies using E4orf6 deletion mutants, complex formation with 19- and 14-kDa cellular proteins was mapped to an extended region from about residues 44 to 218. It will be important to identify these species since they may provide insights into the mechanism of E4orf6-dependent p53 degradation. The second critical function appeared to be the amphipathic arginine-rich α-helical NRS located between residues 239 and 253 (16, 21, 54). Disruption of this region, by either deletion or point mutations, blocked p53 degradation without affecting either E1B55K binding or complex formation with the series of cellular proteins. This region may simply be involved in nuclear localization of E4orf6; however, NRS mutants that contain the strong amino-terminal NLS were nonetheless defective for p53 degradation. It seems more likely that this region may target E4orf6-E1B55K-p53 complexes to specific locations in the nucleus required for the degradation process. Overall, our results are in general agreement with those published by Nevels et al. (53, 54), who found that in rodent cells transformed with E1A-E1B and E4orf6, the amino-terminal region, a conserved cysteine-rich motif (CCR), the NRS, and a carboxy-terminal region of E4orf6 were all required for p53 levels to be kept low. We found that deletion of residues 123 to 126, the core of this newly described CCR motif (7, 53), was sufficient to disrupt p53 degradation.

It remains unclear if E4orf6-E1B55K-mediated degradation takes place in the nucleus or the cytoplasm. Removal of the putative E4orf6 NES had no effect on p53 turnover; however, nuclear export could still be possible via E1B55K, since this protein has been reported to contain a functional NES (39). Although further studies are necessary, preliminary results using the drug leptomycin B, which is a potent inhibitor of the CRM1 nuclear export receptor (41, 42), indicated that leptomycin B had little effect on E4orf6-E1B55K-mediated p53 degradation (E. Querido, unpublished results), suggesting that nuclear export may not be required for adenovirus-induced p53 degradation. Some further support that p53 degradation may occur in the nucleus was derived from the failure of reticulocyte lysates to facilitate E4orf6-dependent p53 degradation in vitro. Such lysates are derived from cells that lack nuclei and thus possibly critical nuclear factors. Additional experiments are under way to investigate the mechanism further.

The precise mechanism of p53 turnover by Ad5 proteins remains to be established. We believe that insights will be derived from two types of approaches: identification and characterization of cellular proteins that interact with E1B55K (20) and E4orf6 (6), and genetic analyses using appropriate viral mutants.

ACKNOWLEDGMENTS

Dennis Takayesu prepared the mouse monoclonal antibody against the carboxy terminus of E4orf6. We thank Allan Bradley and Guiller-

mina Lozano for the gift of p53- and MDM2/p53-deficient mouse embryo fibroblasts and Chuck Sherr for p19ARF-defective fibroblasts as well as wild-type controls. We also thank Joe Nevins for AdE2F1; Matthias Dobbelstein for the E4orf6 mutants L90A/I92A, R248E, and L90A/I92A/R248E; Bill Kaelin for the p73α and p73β cDNAs; Sam Benchimol for cDNAs expressing mutants p53 KEEK and p53 S135KEEK; and Arnie Levine for the 2A10 monoclonal antibody and for the p53 22-23 cDNA.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada. E.Q. was supported by a Studentship from the Fonds FRSQ-FCAR-Santé du Québec, and S.W.-L.T. was supported by a Studentship from McGill University.

REFERENCES

1. **Babiss, L. E., and H. S. Ginsberg.** 1984. Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. J. Virol. **50:**202–212.
2. **Babiss, L. E., H. S. Ginsberg, and J. J. Darnell.** 1985. Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. Mol. Cell. Biol. **5:**2552–2558.
3. **Bacchetti, S., and F. L. Graham.** 1993. Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. Int. J. Oncol. **3:**781–788.
4. **Barak, Y., T. Juven, R. Haffner, and M. Oren.** 1993. mdm2 expression is induced by wild type p53 activity. EMBO J. **12:**461–468.
5. **Barbeau, D., R. Charbonneau, S. G. Whalen, S. T. Bayley, and P. E. Branton.** 1994. Functional interactions within adenovirus E1A protein complexes. Oncogene **9:**359–373.
6. **Boivin, D., M. R. Morrison, R. C. Marcellus, E. Querido, and P. E. Branton.** 1999. Analysis of synthesis, stability, phosphorylation, and interacting polypeptides of the 34-kilodalton product of open reading frame 6 of the early region 4 protein of human adenovirus type 5. J. Virol. **73:**1245–1253.
7. **Boyer, J. L., and G. Ketner.** 2000. Genetic analysis of a potential zinc-binding domain of the adenovirus E4 34K protein. J. Biol. Chem. **275:**14969–14978.
8. **Braithwaite, A., C. Nelson, A. Skulimowski, J. McGovern, D. Pigott, and J. Jenkins.** 1990. Transactivation of the p53 oncogene by E1a gene products. Virology **177:**595–605.
9. **Bridge, E., and G. Ketner.** 1989. Redundant control of adenovirus late gene expression by early region 4. J. Virol. **63:**631–638.
10. **Bridge, E., and G. Ketner.** 1990. Interaction of adenoviral E4 and E1b products in late gene expression. Virology **174:**345–353.
11. **Chiou, S.-K., and E. White.** 1997. p300 binding by E1A cosegregates with p53 induction but is dispensable for apoptosis. J. Virol. **71:**3515–3525.
12. **Chiou, S. K., C. C. Tseng, L. Rao, and E. White.** 1994. Functional complementation of the adenovirus E1B 19-kilodalton protein with Bcl-2 in the inhibition of apoptosis in infected cells. J. Virol. **68:**6553–6566.
13. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. **7:**546–554.
14. **DeGregori, J., G. Leone, A. Miron, L. Jakoi, and J. R. Nevins.** 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. Proc. Natl. Acad. Sci. USA **94:**7245–7250.
15. **de Stanchina, E., M. E. McCurrach, F. Zindy, S.-Y. Shieh, G. Ferbeyre, A. V. Samuelson, C. Prives, M. F. Roussel, C. J. Sherr, and S. W. Lowe.** 1998. E1A signaling to p53 involves the p19ARF tumor suppressor. Genes Dev. **12:**2434–2442.
16. **Dobbelstein, M., J. Roth, W. T. Kimberly, A. J. Levine, and T. Shenk.** 1997. Nuclear export of the E1B–55kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence. EMBO J. **16:**4276–4282.
17. **Dobner, T., N. Horikoshi, S. Rubenwolf, and T. Shenk.** 1996. Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. Science **272:**1470–1473.
18. **Eckner, R., M. E. Ewen, D. Newsome, M. Gerdes, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston.** 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev. **8:**869–884.
19. **Gabler, S., H. Schütt, P. Groitl, H. Wolf, T. Shenk, and T. Dobner.** 1998. E1B 55-kilodalton-associated protein: a cellular protein with RNA-binding activity implicated in nucleocytoplasmic transport of adenovirus and cellular mRNAs. J. Virol. **72:**7960–7971.
20. **Goodrum, F. D., T. Shenk, and D. A. Ornelles.** 1996. Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells. J. Virol. **70:**6323–6335.
21. **Graham, F. L., J. Smiley, W. C. Russel, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–72.
22. **Grand, R. J., M. L. Grant, and P. H. Gallimore.** 1994. Enhanced expression of p53 in human cells infected with mutant adenoviruses. Virology **203:**229–240.
23. **Grand, R. J., P. S. Lecane, S. Roberts, M. L. Grant, D. P. Lane, L. S. Young, C. W. Dawson, and P. H. Gallimore.** 1993. Overexpression of wild-type p53 and c-Myc in human fetal cells transformed with adenovirus early region 1. Virology **193:**579–591.
24. **Grand, R. J., D. Owens, S. M. Rookes, and P. H. Gallimore.** 1996. Control of p53 expression by adenovirus 12 early region 1A and early region 1B 54K proteins. Virology **218:**23–34.
25. **Grossman, S. R., M. Perez, A. L. Kung, M. Joseph, C. Mansur, Z.-X. Xiao, S. Kumar, P. M. Howley, and D. M. Livingston.** 1998. p300/MDM2 complexes participate in MDM2-mediated p53 degradation. Mol. Cell **2:**405–415.
26. **Halbert, D. N., J. R. Cutt, and T. Shenk.** 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J. Virol. **56:**250–257.
27. **Haupt, Y., R. Maya, A. Kazaz, and M. Oren.** 1997. Mdm2 promotes the rapid degradation of p53. Nature (London) **387:**296–299.
28. **Higashino, F., J. M. Pipas, and T. Shenk.** 1998. Adenovirus E4orf6 oncoprotein modulates the function of the p53-related protein, p73. Proc. Natl. Acad. Sci. USA **95:**15683–15687.
29. **Honda, R., H. Tanaka, and H. Yasuda.** 1997. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett. **420:**25–27.
30. **Honda, R., and H. Yasuda.** 1999. Association of $p19_{ARF}$ with Mdm2 inhibits ubiquitin ligase activity of Mdm2 tumor suppressor p53. EMBO J. **18:**22–27.
31. **Howe, J. A., J. S. Mymryk, C. Egan, P. E. Branton, and S. T. Bayley.** 1990. Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. Proc. Natl. Acad. Sci. USA **87:**5883–5887.
32. **Jones, S. N., A. T. Sands, A. R. Hancock, H. Vogel, L. A. Donehower, S. P. Linke, G. M. Wahl, and A. Bradley.** 1996. The tumorigenic potential and cell growth characteristics of p53-deficient cells are equivalent in the presence or absence of Mdm2. Proc. Natl. Acad. Sci. USA **93:**14106–14111.
33. **Jost, C. A., M. C. Marin, and W. G. Kaelin.** 1997. p73 is a human p53-related protein that can induce apoptosis. Nature (London) **389:**191–194.
34. **Kaghad, M., H. Bonnet, A. Yang, L. Creancier, J.-C. Biscan, A. Valent, A. Minty, P. Chalon, J.-M. Lelias, X. Dumont, P. Ferrara, F. McKeon, and D. Caput.** 1997. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell **90:**809–819.
35. **Kamijo, T., F. Zindy, M. F. Roussel, D. E. Quelle, J. R. Downing, R. A. Ashmun, G. Grosveld, and C. J. Sherr.** 1997. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell **91:**649–659.
36. **Kowalik, T. F., J. DeGregori, J. K. Schwarz, and J. R. Nevins.** 1995. E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J. Virol. **69:**2491–2500.
37. **Kratzer, F., O. Rosorius, P. Heger, N. Hirschmann, T. Dobner, J. Hauber, and R. H. Stauber.** 2000. The adenovirus type 5 E1B–55K oncoprotein is a highly active shuttle protein and shuttling is independent of E4orf6, p53 and Mdm2. Oncogene **19:**850–857.
38. **Kubbutat, M. H., S. N. Jones, and K. H. Vousden.** 1997. Regulation of p53 stability by Mdm2. Nature (London) **387:**299–303.
39. **Lain, S., C. Midgley, A. Sparks, E. B. Lane, and D. P. Lane.** 1999. An inhibitor of nuclear export activates the p53 response and induces the localization of HDM2 and p53 to U1A-positive nuclear bodies associated with the PODs. Exp. Cell Res. **248:**457–472.
40. **Lain, S., D. Xirodimas, and D. P. Lane.** 1999. Accumulating active p53 in the nucleus by inhibition of nuclear export: a novel strategy to promote the p53 tumor suppressor function. Exp. Cell Res. **253:**315–324.
41. **Lin, J., J. Chen, B. Elenbaas, and A. J. Levine.** 1994. Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes Dev. **8:**1235–1246.
42. **Lowe, S. W., and H. E. Ruley.** 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. **7:**535–545.
43. **Marcellus, R. C., J. G. Teodoro, R. Charbonneau, G. C. Shore, and P. E. Branton.** 1996. Expression of p53 in Saos-2 osteosarcoma cells induces apoptosis which can be inhibited by Bcl-2 or the adenovirus E1B-55 kilodalton protein. Cell Growth Differ. **7:**1643–1650.
44. **Marin, M. C., C. A. Jost, M. S. Irwin, J. A. DeCaprio, D. Caput, and W. G. Kaelin.** 1998. Viral oncoproteins discriminate between p53 and the p53 homolog p73. Mol. Cell. Biol. **18:**6316–6324.
45. **Martin, M. E. D., and A. J. Berk.** 1998. Adenovirus E1B 55K represses p53 activation in vitro. J. Virol. **72:**3146–3154.
46. **Mitsudomi, T., S. M. Steinberg, M. M. Nau, D. Carbone, D. D'Amico, S. Bodner, H. K. Oie, R. I. Linnoila, J. L. Mulshine, J. D. Minna, and A. L. Gazdar.** 1992. p53 gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of ras mutations and clinical features. Oncogene **7:**171–180.
47. **Montes de Oca Luna, R., D. S. Wagner, and G. Lozano.** 1995. Rescue of early embryonic lethality in *mdm2*-deficient mice by deletion of p53. Nature (London) **378:**203–208.
48. **Moore, M., N. Horikoshi, and T. Shenk.** 1996. Oncogenic potential of the adenovirus E4orf6 protein. Proc. Natl. Acad. Sci. USA **93:**11295–11301.

51. **Murphy, M., J. Ahn, K. K. Walker, W. H. Hoffman, R. M. Evans, A. J. Levine, and D. L. George.** 1999. Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. Genes Dev. **13:**2490–2501.
52. **Nevels, M., S. Rubenwolf, T. Spruss, H. Wolf, and T. Dobner.** 1997. The adenovirus E4orf6 protein can promote E1A/E1B-induced focus formation by interfering with p53 tumor suppressor function. Proc. Natl. Acad. Sci. USA **94:**1206–1211.
53. **Nevels, M., S. Rubenwolf, T. Spruss, H. Wolf, and T. Dobner.** 2000. Two distinct activities contribute to the oncogenic potential of the adenovirus type 5 E4orf6 protein. J. Virol. **74:**5168–5181.
54. **Nevels, M., T. Spruss, H. Wolf, and T. Dobner.** 1999. The adenovirus E4orf6 protein contributes to malignant transformation by antagonizing E1A-induced accumulation of the tumor suppressor protein p53. Oncogene **18:**9–17.
55. **Ohman, K., K. Nordqvist, and G. Akusjarvi.** 1993. Two adenovirus proteins with redundant activities in virus growth facilitates tripartite leader mRNA accumulation. Virology **194:**50–58.
56. **Olson, D. C., V. Marechal, J. Momand, J. Chen, C. Romocki, and A. J. Levine.** 1993. Identification and characterization of multiple mdm-2 proteins and mdm-2-p53 protein complexes. Oncogene **8:**2353–2360.
57. **Orlando, J. S., and D. A. Ornelles.** 1999. An arginine-faced amphipathic alpha helix is required for adenovirus type 5 E4orf6 protein function. J. Virol. **73:**4600–4610.
58. **Pomerantz, J., N. Schreiber-Agus, N. J. Liegeois, A. Silverman, L. Alland, L. Chin, J. Potes, K. Chen, I. Orlow, H.-W. Lee, C. Cordon-Cardo, and R. A. DePinho.** 1998. The *Ink4a* tumor suppressor gene product, $p19^{Arf}$, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell **92:**713–723.
59. **Querido, E., H. Chu-Pham-Dang, and P. E. Branton.** 2000. Identification and elimination of an aberrant splice product from cDNAs encoding the human adenovirus type 5 E4orf6 protein. Virology **275:**263–266.
60. **Querido, E., R. C. Marcellus, A. Lai, R. Charbonneau, J. G. Teodoro, G. Ketner, and P. E. Branton.** 1997. Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J. Virol. **71:**3788–3798.
61. **Querido, E., J. G. Teodoro, and P. E. Branton.** 1997. Accumulation of p53 induced by the adenovirus E1A protein requires regions involved in the stimulation of DNA synthesis. J. Virol. **71:**3526–3533.
62. **Roth, J., M. Dobbelstein, D. A. Freedman, T. Shenk, and A. J. Levine.** 1998. Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. EMBO J. **17:**554–564.
63. **Roth, J., C. Konig, S. Wienzek, S. Weigel, S. Ristea, and M. Dobbelstein.** 1998. Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins. J. Virol. **72:**8510–8516.
64. **Roulston, A., R. C. Marcellus, and P. E. Branton.** 1999. Viruses and apoptosis. Annu. Rev. Microbiol. **53:**577–628.
65. **Rubenwolf, S., H. Schütt, M. Nevels, H. Wolf, and T. Dobner.** 1997. Structural analysis of the adenovirus type 5 E1B 55-kilodalton-E4orf6 protein complex. J. Virol. **71:**1115–1123.
66. **Samuelson, A. V., and S. W. Lowe.** 1997. Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the RB-related proteins. Proc. Natl. Acad. Sci. USA **94:**12094–12099.
67. **Sanchez-Prieto, R., M. Lleonart, and S. Ramón y Cajal.** 1995. Lack of correlation between p53 protein level and sensitivity of DNA-damaging agents in keratinocytes carrying adenovirus E1a mutants. Oncogene **11:**675–682.
68. **Sandler, A. B., and G. Ketner.** 1989. Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. J. Virol. **63:**624–630.
69. **Sarnow, P., P. Hearing, C. W. Anderson, D. N. Halbert, T. Shenk, and A. J. Levine.** 1984. Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. J. Virol. **49:**692–700.
70. **Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine.** 1982. Adenovirus E1b–58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell **28:**387–394.
71. **Sarnow, P., C. A. Sullivan, and A. J. Levine.** 1982. A monoclonal antibody detecting the adenovirus type 5-E1b–58Kd tumor antigen: characterization of the E1b–58Kd tumor antigen in adenovirus-infected and -transformed cells. Virology **120:**510–517.
72. **Scheffner, M., J. M. Huibregtse, R. D. Vierstra, and P. M. Howley.** 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell **75:**495–505.
73. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129–1136.
74. **Slingerland, J. M., J. R. Jenkins, and S. Benchimol.** 1993. The transforming and suppressor functions of p53 alleles: effects of mutations that disrupt phosphorylation, oligomerization and nuclear translocation. EMBO J. **12:**1029–1037.
75. **Steegenga, W. T., N. Riteco, A. G. Jochemsen, and F. J. Fallaux.** 1998. The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. Oncogene **16:**349–357.
76. **Steegenga, W. T., A. Shvarts, N. Riteco, J. L. Bos, and A. G. Jochemsen.** 1999. Distinct regulation of p53 and p73 activity by adenovirus E1A, E1B, and E4orf6 proteins. Mol. Cell. Biol. **19:**3885–3894.
77. **Stommel, J. M., N. D. Marchenko, G. S. Jimenez, U. M. Moll, T. J. Hope, and G. M. Wahl.** 1999. A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. EMBO J. **18:**1660–1672.
78. **Stott, F. J., S. Bates, M. C. James, B. B. McConnell, M. Starborg, S. Brookes, I. Palmero, K. Ryan, E. Hara, K. H. Vousden, and G. Peters.** 1998. The alternative product from the human CDKN2A locus, $p14_{ARF}$, participates in a regulatory feedback loop with p53 and MDM2. EMBO J. **17:**5001–5014.
79. **Teodoro, J. G., and P. E. Branton.** 1997. Regulation of p53-dependent apoptosis, transcriptional repression and cell transformation by phosphorylation of the 55kDa E1B protein of human adenovirus type 5. J. Virol. **71:**3620–3627.
80. **Teodoro, J. G., T. Halliday, S. G. Whalen, D. Takayesu, F. L. Graham, and P. E. Branton.** 1994. Phosphorylation at the carboxy terminus of the 55-kilodalton adenovirus type 5 E1B protein regulates transforming activity. J. Virol. **68:**776–786.
81. **Teodoro, J. G., G. C. Shore, and P. E. Branton.** 1995. Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms. Oncogene **11:**467–474.
82. **Thomas, A., and E. White.** 1998. Suppression of the p300-dependent mdm2 negative-feedback loop induces the p53 apoptotic function. Genes Dev. **12:**1975–1985.
83. **Weigel, S., and M. Dobbelstein.** 2000. The nuclear export signal within the E4orf6 protein of adenovirus type 5 supports viral replication and cytoplasmic accumulation of viral mRNA. J. Virol. **74:**764–772.
84. **Whalen, S. G., R. C. Marcellus, A. Whalen, N. G. Ahn, R. P. Ricciardi, and P. E. Branton.** 1997. Phosphorylation within the transactivation domain of adenovirus E1A protein by mitogen-activated protein kinase regulates expression of early region 4. J. Virol. **71:**3545–3553.
85. **Wienzek, S., J. Roth, and M. Dobbelstein.** 2000. E1B 55-kilodalton oncoproteins of adenovirus types 5 and 12 inactivate and relocalize p53, but not p51 or p73, and cooperate with E4orf6 proteins to destabilize p53. J. Virol. **74:**193–202.
86. **Yew, P. R., C. C. Kao, and A. J. Berk.** 1990. Dissection of functional domains in the adenovirus 2 early 1B 55K polypeptide by suppressor-linker insertional mutagenesis. Virology **179:**795–805.
87. **Yew, P. R., X. Liu, and A. J. Berk.** 1994. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. Genes Dev. **8:**190–202.
88. **Zhang, S., S. Mak, and P. E. Branton.** 1992. Overexpression of the E1B 55-kilodalton (482R) protein of human adenovirus type 12 appears to permit efficient transformation of primary baby rat kidney cells in the absence of the E1B 19-kilodalton protein. J. Virol. **66:**2302–2309.
89. **Zhang, Y., Y. Xiong, and W. G. Yarbrough.** 1998. ARF promotes MDM2 degradation and stabilizes p53: *ARF-INK4a* locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell **92:**725–734.