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**Regulation of the p53 Tumor Suppressor
by Early Products of Adenovirus Serotype 5**

by

Emmanuelle Querido

B. Sc.

Departement of Biochemistry

McGill University

Montreal, Quebec, Canada

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Faculty of Graduate Studies and Research
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for the degree of
Doctor of Philosophy**



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Abstract

DNA tumor virus oncoproteins have evolved to regulate the p53 tumor suppressor. They must overcome p53-dependent cell cycle arrest and apoptosis, which would interfere with viral replication. Upon cellular stress, signalling to p53 takes the form of a large increase in the stability of the protein, by inhibition of the MDM2 protein which normally targets p53 for rapid degradation. Expression of the adenovirus type 5 early region 1 A (E1A) polypeptides can stimulate a quiescent cell to reenter the cell cycle and induces the accumulation of p53 protein and p53-dependent cell death under certain conditions. We mapped the regions of E1A necessary to induce p53 stabilization. Binding of either the p300 or pRb family of proteins can signal to p53, and these are the same activities required for E1A to push the cell to enter S phase. To replicate, the virus must combat this accumulation of p53, and we discovered that two other early viral proteins, E1B55K and E4orf6, can target p53 for ubiquitin-mediated degradation by the 26S proteasome. We found that when cells are infected with adenovirus, no significant accumulation of p53 occurs, and this is due to a large reduction in the half-life of the protein. E4orf6 and E1B55K each bind p53 and also bind each other, and we generated a series of E4orf6 mutations to study the regions necessary for this interaction. We identified a region at the amino terminus of E4orf6 that is the minimal domain permitting interaction with E1B55K. We also determined that binding E1B55K along with functions requiring most of the E4orf6 carboxy terminus have to be intact for E4orf6 to mediate p53 degradation. We tested the activity of the viral proteins in MDM2-deleted cells, and found that E4orf6/E1B55K don't require MDM2 to induce p53 turnover. A series of cellular proteins that interact with E4orf6 were discovered in our lab, and we identified this complex as Cullin 5, Elongin B, Elongin C, Rbx1, E4orf6 and E1B55K. This complex is very similar to the one formed by the cellular tumor suppressor VHL with Cullin 2, Rbx1, and Elongins B and C. Like other Cullin-containing E3 ubiquitin ligases, our E4orf6 complex interacted with an E2 ubiquitin conjugating enzyme (E2cdc34)

and was able to increase poly-ubiquitination of p53 in an *in vitro* reaction. We confirmed that Cullin 5 was located at the plasma membrane and the cytoplasm when transiently expressed in cells, and we showed that co-expression of E4orf6 leads to the nuclear localization of Cullin 5. Finally, with our E4orf6 mutants we observed a correlation between the ability to interact with Cullin 5 and Elongin B/C, and the ability to mediate p53 turnover. Human adenoviruses make use of a cellular degradation machinery designed to keep tight control of cell cycle regulatory proteins for their own ends, and this discovery may lead us to a novel mechanism by which cells regulate p53 levels.

Résumé

Les virus oncogéniques ont du évoluer pour produire des protéines capable d'affecter l'anti-oncogène cellulaire p53. Les virus doivent combattre l'arrêt de croissance et la mort cellulaire programmée induits par p53, qui nuiraient à leur replication. Quand la cellule perçoit un stress, un signal est transmis à p53 qui produit une grande augmentation de sa stabilité, en interférant avec la protéine MDM2 qui maintient normalement les niveaux de p53 bas. La protéine E1A de l'adénovirus humain agit en stimulant une cellule non-proliférante à se diviser et induit une accumulation de la protéine p53 ainsi que l'apoptose de la cellule dans certaines conditions. Nous avons étudié les régions de la protéine E1A nécessaires pour mener à la stabilisation de p53. La formation de complexes avec p300 ou pRb et les protéines de leurs familles peut induire p53, et ce sont ces mêmes régions qui sont nécessaires pour que E1A mène la cellule à entrer en phase S du cycle cellulaire. Pour se répliquer le virus doit contrer cette accumulation de p53, et nous avons découvert que deux autres protéines virales, E1B55K et E4orf6, peuvent dégrader p53. Quand les cellules sont infectées par un adénovirus, aucune accumulation de p53 ne se produit, dû à une grande réduction de la demi-vie de la protéine. Les protéines E4orf6 et E1B55K interagissent avec p53 ainsi que l'une avec l'autre, et nous avons crée une série de mutations dans la séquence de E4orf6 pour étudier les régions nécessaires pour cette interaction. Nous avons identifié un région amino terminale de E4orf6 essentielle pour que la protéine se lie à E1B55K. Nous avons aussi trouvé que cette région ainsi que d'autres fonctions qui requièrent presque toute la partie carboxy terminale de E4orf6 doivent être intactes pour que la protéine puissent participer à la dégradation de p53. E4orf6 et E1B55K agissent par un mécanisme indépendant de la protéine MDM2, puisque les protéines virales peuvent fonctionner dans des cellules n'exprimant pas MDM2. Notre laboratoire a découvert plusieurs protéines cellulaires qui interagissent avec E4orf6. Nous avons identifié les composantes de ce complexe: Cullin 5, Elongin B, Elongin C, Rbx1, E4orf6 et E1B55K. Ce complexe

ressemble a celui composé par l'anti-oncogène cellulaire VHL avec Cullin2, Rbx1, et Elongin B et C. Comme les autres ubiquitine ligases E3 qui contiennent une Cullin, le complexe formé par E4orf6 se lie à une enzyme de conjugaison de l'ubiquitine (E2cdc34) et est capable d'allonger les chaînes d'ubiquitine sur p53 dans une réaction *in vitro*. Nous avons confirmé le résultat que Cullin 5 est situé à la membrane cellulaire et dans le cytoplasme lorsque la protéine est introduite dans la cellule, et nous avons démontré que E4orf6 pouvait amener Cullin 5 dans le noyaux. Finalement, avec l'aide des mutants de E4orf6, nous avons observé une corrélation entre la capacité de se lier à Cullin 5 et Elongin B/C et la capacité de dégrader p53. Les adénovirus humains ont usurpé une machinerie de dégradation cellulaire normalement dédiée au contrôle des régulateurs du cycle cellulaire, et cette découverte nous mènera peut-être à celle d'un nouveau mécanisme cellulaire pour dégrader p53.

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List of Abbreviations

¹²⁵ I	iodine-125
243R	243 residue E1A protein
289R	289 residue E1A protein
³⁵ S	sodium-35
aa	amino acid (standard 3 letter abbreviations also used)
Ab	antibody
ADP	adenovirus death protein
Ad	adenovirus
AdCB	<u>Ad</u> E4orf6/E1B55K/ <u>Cul</u> 5/Elongin <u>B</u> C
APC	anaphase promoting complex
ARF	alternative reading frame
AT	ataxia telangiectasia
ATM	ataxia telangiectasia mutated protein
ATR	ATM-Rad3-related protein
<i>bcl-2</i>	B cell lymphoma gene
bHLH	basic helix-loop-helix
BMK	baby mouse kidney cells
C-terminus	carboxy terminus
CAR	coxsackie and adenovirus receptor
CAT	chloramphenicol acetyl transferase
CBP	CREB-binding protein
CDK	cyclin dependent kinase
CF	Cystic fibrosis
CKII	casein kinase II
CMV	cytomegalovirus

CPE	cytopathic effect
CR1/CR2/CR3	conserved region 1/2/3
CREB	cAMP responsive element binding protein
CtBP	C-terminal binding protein
Cul 1/2/3/4/5	Cullin 1/2/3/4/5
dl	deletion
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
ds	double-stranded
DTT	dithiothreitol
E1/2/3/4	early region 1/2/3/4
E1B19K	E1B-19 kDa protein
E1B55K	E1B-55 kDa protein
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
HAT	histone acetyl transferase
HBx	Hepatitis B virus X protein
HDAC	histone deacetylase
<i>hect</i>	homologous to E6-AP C-terminus
HH55K	His-HMK-tagged E1B55K
HIV	human immunodeficiency virus
hnRNP	heterogeneous nuclear ribonucleoprotein
hpi	hours post infection
HPV	human papillomavirus
HSV	herpes simplex virus
HTLV	Human T-cell lymphotropic/leukemia virus

kb	kilobases
kDa	kilodaltons
L1/2/3 etc...	late region 1/2/3 etc...
LXCXE	Leu-X-Cys-X-Glu
μCi	microCurrie
μg	microgram
μl	microLitre
mCi	milliCurrie
<i>mdm2</i>	mouse double minute-2 gene
MDM2	protein encoded by <i>mdm2</i>
MEF	mouse embryo fibroblasts
mg	milligrams
MLP	major late promoter
mM	milliMolar
mRNA	messenger RNA
nM	nanoMolar
NES	nuclear export signal
NLS	nuclear localization signal
NrLS	nucleolar localization signal
NRS	nuclear retention signal
N-terminus	amino terminus
orf	open reading frame
p53RE	p53 response element
PAF400	PCAF-associated factor of 400 kDa
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PFU	plaque forming unit

PKC	protein kinase C
PKR	protein kinase R / RNA-dependent protein kinase
POD	PML oncogenic domain
PML	promyelocytic leukemia protein
PP2A	protein phosphatase 2A
Rb	retinoblastoma
RBP1	pRb-binding protein 1
RID	receptor internalization and degradation complex
RNA	ribonucleic acid
S	sedimentation coefficient
S phase	synthesis phase
SCF	<u>SKP</u> / <u>Cullin</u> / <u>E</u> -box protein
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
snRNP	small nuclear ribonucleoprotein particle
SV40	simian virus 40
SV40 LT	SV 40 large T antigen
TAF	TBP-associated factor
TBP	TATA box-binding protein
TF	transcription factor
TNF	tumor necrosis factor
TP	terminal protein
TRRAP	TRansactivation/tRansformation-domain Associated Protein
Tris	tris (hydroxymethyl) aminomethane
TS	thymidilate synthase
TSA	trichostatin A
UTR	untranslated region
UV	ultra violet

VCB

VHL/Cul 2/Elongin BC

VEGF

vascular endothelial growth factor

VHL

von Hippel-Lindau protein

*wt**wild-type*

Preface

In compliance with Section 2 of the Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and Research of McGill University, Chapters II and III represent the text of published papers. Chapter IV is the text of a paper submitted for publication to the Journal of Virology. Chapter V is based on the text of a paper submitted for publication to Genes & Development.

All the work presented in this thesis is that of the author with the following exceptions: Jose G. Teodoro performed the EMSA assays shown in figure 2.3C, and he also generated figures 2.5 and 2.6; Richard Marcellus designed on his computer and generously provided figure 3.4; Albert Lai and Richard Marcellus generated figure 3.6; Richard Marcellus also made the AdE4orf6 virus and Rachel Charbonneau made the AdHH55K virus; Sarah Thirlwell made the E4orf6 mutants dl 13-23, dl 35-43, dl 49-64, dl1-108 and R240E/R241E, and determined if they were able to interact with E1B55K; Huan Chu-Pham-Dang helped me make and sequence several of the other E4orf6 mutants; Megan Morrison affinity purified the p84, p19 and p14 cellular proteins to be sequenced by ion mass trap spectrometry and mapped their binding to the E4orf6 mutants summarized in table 4.2, she also generated panels A and B of figure 5.1 ; and finally Qin Yan, Takumi Kamura, and Joan Weliky Conaway generated figures 5.4 and 5.5.

Original contributions to knowledge

- E1A binding to either p300 or pRb family proteins can induce p53 stabilization.
- E1B55K and E4orf6 together are necessary and sufficient to degrade p53 in adenovirus-infected cells.
- Amino acids 39 to 107 of E4orf6 encompass the minimal region necessary for interaction with E1B55K, while a larger region defined between amino acids 44 to 274 is necessary for E4orf6 to induce p53 degradation when expressed with E1B55K.
- E4orf6/E1B55K operate via a different mechanism to degrade p53 than either MDM2 or the human papilloma virus E6 proteins.
- E4orf6/E1B55K bind a Cullin 5/Elongin BC/Rbx1 complex that functions as a novel E3 ubiquitin ligase for p53.
- E4orf6 binds Cullin 5 and none of the other Cullins (1 to 4).
- E4orf6 can direct the nuclear localization of Cullin 5.

CHAPTER I

General Introduction

The journal Science once elected p53 "molecule of the year" (Culotta and Koshland 1993). Mutations in *p53* are the most frequent somatic genetic alteration in human cancer, and since 1993 work on this gene has only increased in intensity. Recent breakthroughs notwithstanding, there are still major gaps in our knowledge of how p53 effects all its activities. The work in this thesis is about the action of several oncoproteins of human adenovirus type 5 on the p53 tumor suppressor. It illustrates the strategies this virus has evolved to control p53, "the guardian of the genome", and will hopefully lead to a better understanding of p53 itself.

1.1 The p53 tumor suppressor

A cellular protein of 53 kilodaltons (kDa) was first observed in a complex with the large T antigen of simian virus 40 (SV40) (Lane and Crawford 1979, Linzer and Levine 1979). This p53 protein also accumulated in the nucleus of neoplastic rodent cells (Benchimol *et al.* 1982). The *p53* cDNA was first cloned in 1983 from tumor cells and p53 was shown to have oncogenic activity (Oren and Levine 1983, Eliyahu *et al.* 1984, Matlashewski *et al.* 1984). In 1989 researchers discovered that they were studying p53 mutants instead of the *wild-type* (*wt*) protein. It became evident in fact that the *p53* gene was frequently mutated in human cancers (Nigro *et al.* 1989, Takahashi *et al.* 1989). Whereas many p53 mutants acted as dominant oncogenes, the *wt* gene encodes a tumor suppressor. Recent studies have focused on the role of p53 in transcription, growth arrest, DNA repair, and programmed cell death (reviewed in Ko and Prives 1996, Levine 1997, Sherr 1998, Lohrum and Vousden 1999, Prives and Hall 1999).

1.1.1 The *p53* gene

The murine *p53* cDNA was first cloned from a mouse cell line transformed by simian virus 40 (SV40) (Oren and Levine 1983) and the gene was mapped to chromosome 11 (Czosnek *et al.* 1984, Rotter *et al.* 1984). Human *p53* cDNAs were cloned by several groups from transformed cell lines (Zakut-Houri *et al.* 1983, Matlashewski *et al.* 1984, Harlow *et al.* 1985b). The gene for human *p53* was mapped to the short arm of chromosome 17, band 17p13 (Isobe *et al.* 1986, Miller *et al.* 1986). Loss of heterozygosity of the short arm of chromosome 17 has since been frequently reported in cancer (Baker *et al.* 1989, Takahashi *et al.* 1989). The Li-Fraumeni syndrome, in which affected individuals develop cancers at a young age, is associated with *p53* germline mutations (Malkin *et al.* 1990).

The human *p53* gene is split into 11 exons that are spread over 20 kilobases (Kb) of DNA (Lamb and Crawford 1986). The first and second exons are separated by a long 10 Kb intron. The *p53* promoter is located upstream of the non-coding first exon (Reisman *et al.* 1988, Reisman and Rotter 1989). A group of six major transcription initiation sites lies at position - 114, numbered with respect to the 3' end of the first exon, which together account for 95% of the human *p53* transcripts. More minor sites exist further upstream, the most distant being at position - 175 (Tuck and Crawford 1989). A region required for full basal *p53* promoter activity which is highly conserved within the rat and murine *p53* genes has been mapped from positions - 88 to + 20 relative to the major transcription initiation sites (Bienz-Tadmor *et al.* 1985, Tuck and Crawford 1989). This promoter has no TATA box, which may account for the heterogeneous start sites. The basal *p53* promoter contains potential binding motifs for several important transcription factors such as the DNA sequences CACGTG or CATGTG, which are recognized by the basic-helix-loop-helix and leucine zipper (bHLH-Zip) family of DNA-binding proteins (Ronen *et al.* 1991). The c-Myc/Max heterodimer binds to a CATGTG motif which maps from -34 to -29 upstream of the major transcription start site of the human *p53* gene and results in up to 12-fold enhanced expression from the human *p53* promoter (Roy *et al.* 1994). Another bHLH-containing

factor, USF, has been shown to bind the CACGTG motif in the murine *p53* promoter (Reisman and Rotter 1993). The region spanning positions -70 to -46 contains a putative κ B binding site and is required for *p53* promoter activation induced by genotoxic stress as well as basal promoter activity (Sun *et al.* 1995). The human *p53* gene has a PAX binding site within its first exon which is also required for basal promoter activity (Stuart *et al.* 1995), but binding of some of the PAX developmental regulators represses *p53* transcription. Transcriptional regulation of the *p53* gene has been proposed to play a role in the elevated expression of mutant *p53* in tumor cells (reviewed in Reisman and Loging 1998).

The *p53* gene is expressed constitutively at relatively low levels in all organs of the body, as reflected by its mRNA abundance, which is approximately the same in all normal tissues (Rogel *et al.* 1985). The murine *p53* mRNA can be predicted to form a stable stem-loop structure that involves the 5'-untranslated region (UTR) and some of the coding sequence (Mosner *et al.* 1995). Murine p53 protein can bind this 5'-UTR structure and inhibit the translation of its own mRNA. This negative feedback regulation could be quickly released and large amounts of p53 then translated to act as required. The human *p53* mRNA 5'-UTR does not form such a stem-loop structure, but its 3'-UTR has been demonstrated to inhibit translation (Fu *et al.* 1996). Relief of 3'-UTR repression of translation may have a role in inducing accumulation of human p53 protein following γ -irradiation of human cells (Fu and Benchimol 1997). The thymidylate synthase (TS) protein and *p53* mRNA have been found associated in a ribonucleoprotein complex in cultured human cancer cells (Chu *et al.* 1999). This interaction between TS and the protein-coding region of *p53* mRNA could repress *p53* mRNA translation, providing an additional mechanism for regulation of p53 protein expression.

Alternative splice products of *p53* mRNA have been described, mainly for murine *p53* (Wu *et al.* 1995), but their prevalence and function remain unclear.

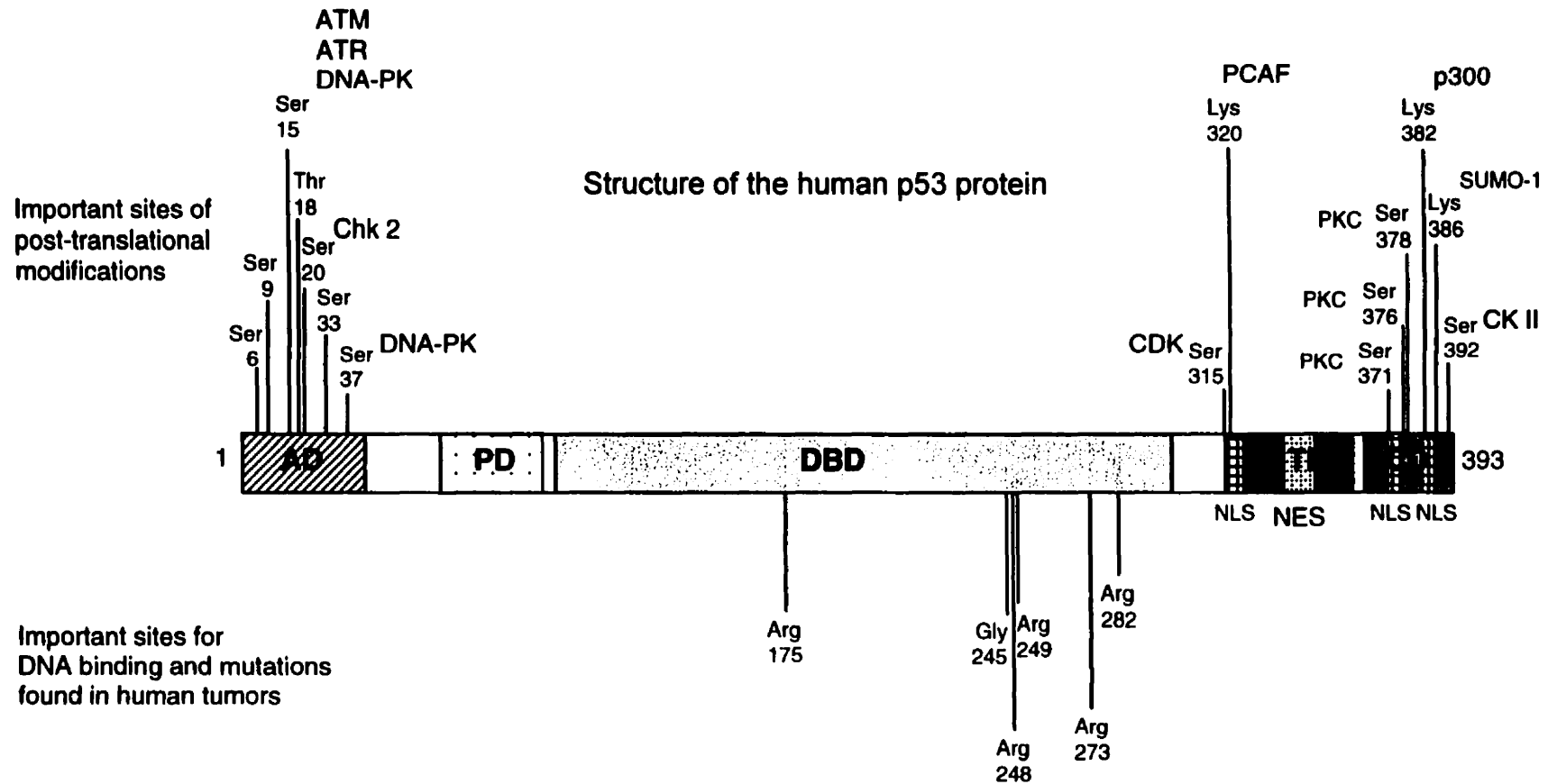


Figure 1.1 Structure of the human p53 protein.

Domains of p53 are indicated as follows: activation domain (AD), residues 1-43; growth suppression PXXP region (PD), residues 63-97; DNA-binding domain (DBD), residues 100-300; tetramerization domain (TD), residues 323-356; and carboxy terminal regulatory region (CD), residues 363-393. The positions of NLS (nuclear localization signal) and NES (nuclear export signal) sequences are represented by shaded boxes. Stress-induced modifications are indicated above the figure (the lysines are acetylated or sumolated, the serines and threonines are of course phosphorylated). Frequently mutated residues in human tumors are noted below the figure (higher frequency of mutations at a site are represented by longer bars).

1.1.2 Structure of the p53 protein

As illustrated in Figure 1.1, p53 can be separated into three physical and functional domains: (1) residues 1 to 43 form the amino terminal transcriptional activation domain ; (2) residues 100 to 300 delineate the core DNA-binding domain ; and (3) residues 320 to 393 are the carboxy terminal domain that regulates the DNA binding and tetramerization of p53.

1.1.2.1 Amino terminus: the transcriptional activation domain

The *wt* p53 protein is a transcription factor and, depending on the promoter, p53 can enhance the rate of transcription of a gene adjacent to the classical p53 response element (p53RE) (Fields and Jang 1990, O'Rourke *et al.* 1990, Raycroft *et al.* 1990) , or repress the transcription of some genes whose p53 binding sites are less well defined (Ginsberg *et al.* 1991, Santhanam *et al.* 1991, Subler *et al.* 1992). The primary structure of the p53 activation domain (residues 1-83) is similar to that of other acidic activation domains. The minimal region required for transcriptional activation has been mapped to the amino (N) terminal residues 1-43 of human p53 (Unger *et al.* 1992). This N-terminal domain is rich in acidic amino acids but they are not critical for transcriptional activation. Two hydrophobic residues at positions leucine-22 and tryptophan-23 are critical for transcriptional activation however, and a double point mutation is required for total abrogation of activity (Lin *et al.* 1994). The region (residues 40-83) adjacent to the primary activation domain of p53 is another activation subdomain. Both subdomains (1-40 and 40-83) are active in both yeast and mammalian cells (Candau *et al.* 1997). Possible mechanisms of transcriptional activation and repression by p53 will be expanded on in sections 1.1.3 and 1.1.4.

The N-terminus of p53 can be phosphorylated on serine residues 6, 9, 15, 20, 33 and 37, and on threonine residue 18. The role of N-terminal phosphorylation for the regulation of p53 activity will be discussed in section 1.1.6 on functions of p53. The region of human p53 located between residues 63 and 97 is also important because it contains five PXXP Src-homology-3 (SH3) binding motifs. This region has been implicated in growth

suppression (Walker and Levine 1996) and in the ability of p53 to induce apoptosis in response to E1A (Sakamuro *et al.* 1997). The human p53 protein has a primary structure polymorphism at amino acid residue 72, which can be either an arginine, a proline or a cysteine (Matlashewski *et al.* 1987). This single codon difference results in electrophoretically distinct forms. One of the most interesting differences found so far is that individuals with both arginine (Arg) -72 p53 alleles seem to be more prone to human papilloma virus (HPV)-associated tumors (Storey *et al.* 1998, Thomas *et al.* 1999b).

1.1.2.2 p53 core: the DNA binding region

The central core of p53 contains the sequence-specific DNA binding domain. The p53 response element (p53RE) consists of the consensus sequence 5'- R R R C A/T T/A G Y Y Y -3' repeated twice (el Deiry *et al.* 1992, Vogelstein and Kinzler 1992) as found in the promoters of genes like *p21/WAF1* and *cyclinG1* (el Deiry *et al.* 1993, Zauberman *et al.* 1995b). The structure of the p53 core-DNA cocrystal has been solved by X-ray diffraction (Cho *et al.* 1994). The structure does not contain any standard DNA-binding motifs. The cocrystal consists of two core molecules packed head to tail bound to an oligomer consisting of one p53RE half-site and a weaker site one turn of the DNA away. A third core molecule was detected, and was involved in crystal-packing contacts. DNA binding is mediated through contacts in the major groove by the helix and loop of a loop-sheet-helix motif, as well as by a contact in the minor groove of Arg-248 which is positioned by another loop. As illustrated in Fig. 1.1, most missense p53 mutations found in tumors occur within the DNA binding region, with hotspots for mutations at residues Arg-175, glycine (Gly) -245, Arg-248, Arg-249, Arg-273 and Arg-282 (Hollstein *et al.* 1994, Walker *et al.* 1999). Arg-248 and Arg-273 directly contact DNA while the others are necessary for the integrity of the DNA-protein interface (Cho *et al.* 1994). The unusual nature of the DNA-binding surface makes it so vulnerable to single amino acid (aa) substitutions. Since these p53 core mutants

no longer bind DNA but retain the ability to oligomerize with wild-type p53, they function as dominant oncogenes.

1.1.2.3 Carboxy terminus: the regulatory domain

The structure of a peptide (residues 319-360) that contains the minimal oligomerization domain of human p53 was solved by nuclear magnetic resonance spectroscopy (Clore *et al.* 1994). The peptide contains a turn, a β strand, a second turn, and an α helix. Each subunit interacts with another such that the helices and β strand are antiparallel. Two such dimers interact so that the resulting tetramer is formed of a dimer of dimers.

Figure 1.1 shows that the carboxy (C) terminus of p53 contains three nuclear localization signals (NLS), spanning residues 316-325, 369-375 and 379-384 (Shaulsky *et al.* 1990). It was recently demonstrated that p53 also contains a leucine-rich nuclear export signal (NES) located between the first and second NLS (residues 340-351) (Stommel *et al.* 1999). The NES lies within and shares key residues with the tetramerization domain, and point mutations that affect tetramerization also inactivate the NES. As will be discussed in section 1.1.5, p53 may engage in nuclear-cytoplasmic transport as part of the normal regulation of its protein levels and activity.

The extreme C-terminus of p53 (residues 363-393) is a region that may allosterically inhibit the ability of p53 to bind specifically to DNA (Halazonetis and Kandil 1993, Hupp and Lane 1994, Waterman *et al.* 1995). p53 is activated to bind to the p53RE when this carboxy-terminal region is either deleted (Hupp *et al.* 1992); bound by antibody pAb421 (Hupp *et al.* 1992); bound by peptides (Hupp *et al.* 1995) or ssDNA (Jayaraman and Prives 1995); phosphorylated by casein kinase II (CKII) (Hupp *et al.* 1992) or protein kinase C (PKC) (Takenaka *et al.* 1995); glycosylated (Shaw *et al.* 1996); acetylated (Gu and Roeder 1997); or SUMOylated (Gostissa *et al.* 1999, Rodriguez *et al.* 1999).

1.1.3 Mechanisms of transcriptional activation by p53

Eukaryotic DNA is tightly packed in repressive chromatin structures that hamper access by the transcriptional machinery. Each nucleosome in the chromatin string contains about 200 base pairs of DNA associated with a histone octamer that consists of two copies each of H2A, H2B, H3 and H4. These are known as the core histones. The hyperacetylation of the N-terminal tails of core histones has been linked to the destabilization and restructuring of nucleosomes, and the transcriptional activation of certain genes (reviewed in Wolffe and Pruss 1996, Struhl 1998).

The N-terminus of p53 is very acidic and contains two mapped transcriptional activation domains. Both subdomains (1-40 and 40-83) are active alone, in both yeast and mammalian cells (Candau *et al.* 1997). Full function of both subdomains in yeast requires the adaptors ADA2, ADA3 and GCN5 (Candau *et al.* 1997). GCN5 is a yeast histone acetyltransferase (HAT) (Brownell *et al.* 1996) and full activity of both p53 subdomains requires intact HAT activity in yeast.

The p300 protein and the related CREB-binding protein (CBP), two transcriptional coactivators of p53, have HAT activity (Bannister and Kouzarides 1996, Ogryzko *et al.* 1996, Avantaggiati *et al.* 1997, Gu *et al.* 1997, Lill *et al.* 1997). p300/CBP bind the p53 transactivation domain directly, they support p53 transactivation of the *bax*, *p21WAF1*, and *mdm2* promoters, and participate in p53-dependent growth arrest and apoptosis (Avantaggiati *et al.* 1997, Gu *et al.* 1997, Lill *et al.* 1997, Scolnick *et al.* 1997, Thomas and White 1998). p300/CBP are transcriptional adaptor molecules, and have a bromodomain, a KIX domain, and three cysteine-histidine rich regions which provide binding sites for a variety of factors (reviewed in Giordano and Avantaggiati 1999). p300 and CBP are histone acetyltransferases themselves but they also bind specifically to the PCAF and SRC-1 HAT proteins (Yang *et al.* 1996, Yao *et al.* 1996). These enzymatic activities contribute to the p300/CBP coactivation of some promoters but it is not known if the HAT activity of p300/CBP is required for their enhancement of p53 transactivation. p300/CBP can acetylate

p53 *in vitro* on lysine-382 (Figure 1.1), which activates p53 sequence-specific DNA binding (Gu and Roeder 1997).

The p300/CBP-associated factor (PCAF) and the related PCAF-B protein (human GCN5-L) have high homology to the yeast GCN5 protein (reviewed in Schiltz and Nakatani 2000). PCAF exists in a complex of more than 20 proteins, of sizes ranging from 400 kDa to 10 kDa. p300 is not stoichiometrically present in the complex, suggesting that PCAF interacts with p300 only on promoters. The PCAF-associated factor of 400 kDa (PAF400) is a novel member of the ATM superfamily that is nearly identical to TRRAP, a protein that binds to c-Myc and E2F1 (McMahon *et al.* 1998, Vassilev *et al.* 1998, McMahon *et al.* 2000). PAF400 and TRRAP will be discussed further in the E1A section. PCAF is able to acetylate p53 *in vitro* on Lys-320 (Sakaguchi *et al.* 1998). p53 could play a role in bringing the PCAF complex to promoters, which would efficiently acetylate nucleosomal histones, and p53 could then recruit the basal transcription machinery, also through its N-terminal activation domain. p53 binds to TBP (the TATA-binding protein) (Seto *et al.* 1992), TFIIF (Xiao *et al.* 1994) and TAFII31 (Lu and Levine 1995).

1.1.4 Mechanisms of transcriptional repression by p53

Alteration of the chromatin structure may also underlie the ability of p53 to repress transcription. The human p53 protein has recently been shown to interact with histone deacetylases (HDACs), and this association is mediated by the corepressor mSin3a (Murphy *et al.* 1999). Two domains of p53 had previously been implicated in transcriptional repression (Sang *et al.* 1994, Subler *et al.* 1994, Horikoshi *et al.* 1995) and they overlap with the regions necessary for the p53-mSin3a interaction (residues 40-160 and 320-360) (Murphy *et al.* 1999). It has been known for some time that *wt* p53 can repress gene transcription in a non-sequence-specific manner, particularly in reporter assays using overexpressed p53 and target promoters. Several groups have now identified genes whose endogenous expression is decreased following *wt* p53 induction (Murphy *et al.* 1996, Ahn *et*

al. 1999). Murphy *et al.* propose that a novel p53 binding site, as well as p53RE-half-sites, may be involved in gene repression (Murphy *et al.* 1999).

1.1.5 Regulation of p53 protein levels by the MDM2 and p19ARF proteins

p53 is a short-lived protein with a half-life of 5-20 minutes in most cell types studied. The MDM2 protein targets p53 for ubiquitin-mediated degradation by the proteasome (Haupt *et al.* 1997, Honda *et al.* 1997, Kubbutat *et al.* 1997, Midgley and Lane 1997). The *mdm2* gene was originally identified by virtue of its amplification in murine double minute chromosomes in various tumor cells (Cahilly-Snyder *et al.* 1987, Fakharzadeh *et al.* 1991). It is now known that the *mdm2* gene is amplified in a significant fraction of the most common human sarcomas. Its oncogenic effect is explained, at least in part, by its ability to overcome tumor suppression by p53. The human *mdm2* gene is regulated by two promoters, P1 and P2 (Zauberman *et al.* 1995a). *mdm2*-P1 regulates the level of transcripts initiating in exon 1 of the human *mdm2* gene while the *mdm2*-P2 promoter possesses two p53REs and controls transcript initiation from exon 2. By blocking the activity of p53, MDM2 shuts off its own expression through an autoregulatory feedback loop. The time interval between p53 activation and the consequent induction of *mdm2* may define the window within which p53 exerts its effects. Hydrophobic residues at codons Leu-22 and Trp-23 are required for the binding of MDM2 to p53, along with additional hydrophobic residues at codons leucine (Leu) -14 and phenylalanine (Phe) -19 (Lin *et al.* 1994). Phosphorylation of p53 N-terminal sites can disrupt MDM2 binding, as will be discussed in section 1.1.6.1. Tight binding of MDM2 to the transactivation domain of p53 blocks its ability to activate transcription (Momand *et al.* 1992) in addition to targeting p53 protein for degradation. MDM2 is an E3 ubiquitin ligase for p53 (Honda *et al.* 1997).

Figure 1.2 A illustrates the *INK4a/ARF* locus and its two products. The *INK4a/ARF* locus is frequently deleted or mutated in cancers. It codes for two tumor

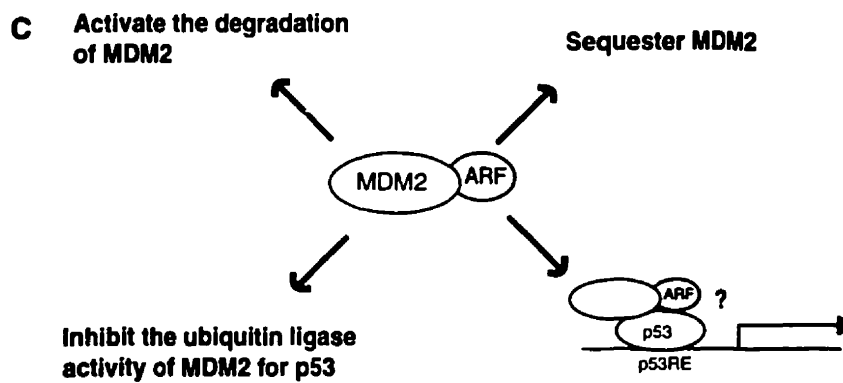
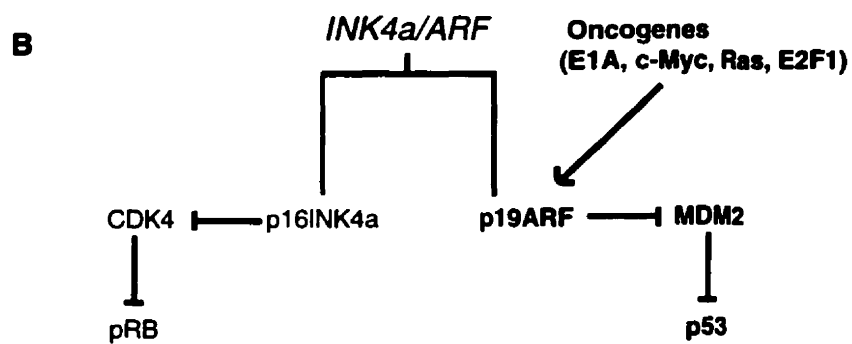
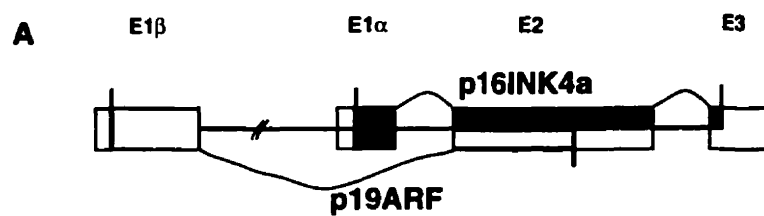


Figure 1.2 The *INK4a/ARF* locus and p53

(A) Structure of the human *INK4a/ARF* gene. (B) The *INK4a/ARF* locus regulates both the pRb and the p53 tumor suppressor proteins. Oncogenes that have recently been shown to activate p53 in a p19ARF-dependent pathway are indicated. (C) Recent theories on the mechanism of p19ARF activation of p53.

suppressor proteins of unrelated sequence: p16INK4a and p19ARF (reviewed in Sherr 1998). The α transcript encodes p16INK4a, while the β transcript initiates at an upstream exon and splices into exon 2 in an alternative reading frame (ARF) (Quelle *et al.* 1995). The p16INK4a protein is an inhibitor of cyclin D-dependent kinases (Serrano *et al.* 1993). The p19ARF protein has recently been shown to be required for E1A, Myc, E2F1, and activated Ras or MEK signaling to induce p53 accumulation (Bates *et al.* 1998, de Stanchina *et al.* 1998, Lin *et al.* 1998, Palmero *et al.* 1998, Zindy *et al.* 1998). p19ARF inhibits the ubiquitin ligase activity of MDM2 for p53 (Honda and Yasuda 1999), thus stabilizing p53. p19ARF is not essential for the p53 response to γ - or UV-induced DNA damage, indicating that it functions in another pathway. Mice with deletions in the *INK4a/ARF* locus (Serrano *et al.* 1996, Kamijo *et al.* 1997) develop tumors. Their mouse embryo fibroblasts (MEFs) do not undergo replicative senescence and are transformed by oncogenic Ras alone. Figure 1.2 C illustrates that several models exist explaining the potentiation of p53 activity by p19ARF. We will come back to this point in section 6.1.2 of the general discussion. One interesting model is that p19ARF binds MDM2 and sequesters it in the nucleolus (Weber *et al.* 1999). Both ARF and MDM2 have nucleolar localization signals (NrLS), and ARF binding to MDM2 may induce a conformational change that facilitates nucleolar import of the complex (Weber *et al.* 2000). MDM2 had previously been shown to have a nuclear export signal (NES) (Roth *et al.* 1998a) in addition to the more classical nuclear localization signal (NLS).

Stommel *et al.* have recently suggested that p53 may engage in nuclear-cytoplasmic shuttling as part of the normal regulation of its activity (Stommel *et al.* 1999). Exportin 1 (Crm1) binds to the p53 NES, the leucine at position 348 being critical for binding. Inactive, non-DNA-binding, monomeric p53 would be competent for export. Stommel *et al.* propose that when cells are cycling normally and not stressed, very low levels of p53 are present, mainly in a monomer or dimer. p53 shuttles from the nucleus to the cytoplasm during the cell cycle and has a short half-life. Upon activation of p53, levels of p53 increase in the nucleus: p53 forms a tetramer and becomes transcriptionally active. Active, DNA-binding, tetrameric

p53 is incapable of nuclear export due to the masking of the NES deep within the interface of the four monomers, resulting in its inaccessibility to the export machinery. Eventually the *mdm2* gene is turned on by p53, MDM2 protein binds to the tetramer and with its own NES transports the tetrameric p53 to the cytoplasm for degradation.

MDMX, a protein with high homology to MDM2, was cloned as a novel p53-binding protein (Shvarts *et al.* 1996, Shvarts *et al.* 1997). Like MDM2, MDMX inhibits the transcriptional activation domain of p53. MDMX is capable of hetero-oligomerization with MDM2 through their carboxy terminal RING finger domains (Tanimura *et al.* 1999). This hetero-oligomerization appears to be more stable than the homo-oligomerization of each protein. Interaction of MDMX with MDM2 inhibits the ubiquitin-proteasome-mediated degradation of MDM2, indicating that MDMX may function as a regulator of MDM2.

1.1.6 Some functions of p53

p53 activation can have three outcomes: growth arrest followed by repair of the damage and reentry into the cell cycle, irreversible growth arrest, or programmed cell death. Mice without any *p53* gene are viable: the animals develop normally, are fertile, and immunologically competent (Donehower *et al.* 1992). This underlines that p53's role is not in developmentally programmed apoptosis but rather to effect the emergency shutdown of the cell when things go wrong.

1.1.6.1 Response to DNA damage and other stress

p53 has a critical role to play in the cellular response to stress. DNA strand breaks induce the accumulation of p53 protein and activation of p53-dependent transcription (Levine 1997). The current model for the activation of p53 by DNA damage is that serine (Ser) -15, Ser-20, and Ser-33 of the N-terminus are phosphorylated first; this increases the ability of p53 to recruit p300/CBP and reduces the binding of MDM2; p300/CBP and PCAF then

acetylate the C-terminus of p53 and p53 is activated for specific DNA binding (see Fig. 1.1, and (Shieh *et al.* 1997, Shieh *et al.* 1999)). Several stimuli have been used experimentally to induce DNA damage: (ionizing) γ -radiation, UV irradiation, several chemotherapeutic agents, and microinjection of a restriction endonuclease that produces blunt-ended double-strand cuts. Cells undergo cell cycle arrest in G1 following γ -irradiation. Several kinases that have been implicated in the cellular response to DNA insult are potential effectors for the N-terminal phosphorylation of p53 in this response to γ -irradiation.

The double strand break-activated DNA-dependent protein kinase (DNA-PK) is not indispensable for the p53-dependent response to DNA damage (Abraham *et al.* 1999, Burma *et al.* 1999, Jimenez *et al.* 1999). However, DNA-PK has been shown to phosphorylate p53 *in vitro* on serine-15 and serine-37 (Lees-Miller *et al.* 1992) and there is compelling evidence that DNA-PK acts upstream of p53 in response to DNA damage (reviewed in Prives and Hall 1999). Irradiated DNA-PK catalytic subunit -/- primary mouse embryonic fibroblasts undergo p53-dependent cell cycle arrest like wild-type MEFs, still display accumulation of p53 protein, reactivity to anti-p53-phosphoSer-15 antibodies, and activation of sequence specific DNA binding in response to ionizing radiation, suggesting that other kinases are involved in phosphorylation of Ser-15 *in vivo*.

The ATM protein, encoded by the gene mutated in the genetic disorder ataxia telangiectasia (AT), is a member of the phosphatidylinositol 3 (PI-3) family of serine/threonine kinases (reviewed in Prives 1998, Levrero *et al.* 2000). The ataxia telangiectasia mutated protein (ATM) is required for the phosphorylation of p53 at Ser-15 in response to ionizing radiation (Banin *et al.* 1998, Canman *et al.* 1998). Double-strand breaks caused by the microinjection of a restriction endonuclease (blunt ends) also induces Ser-15 phosphorylation of p53, which does not occur in AT fibroblasts (Nakagawa *et al.* 1999). The ATM-related protein (ATR) is also involved in the *in vivo* phosphorylation of Ser-15 in response to irradiation (Tibbetts *et al.* 1999). Overexpression of a catalytically inactive ATR inhibits Ser-15 phosphorylation.

The Chk2 protein kinase has recently been shown to phosphorylate p53 *in vivo* on Ser-20 in response to DNA damage (Chehab *et al.* 2000, Hirao *et al.* 2000). Like Ser-15, phosphorylation at Ser-20 disrupts MDM2 binding and stabilizes p53. Chk2-deficient embryonic stem cells failed to maintain p53 γ irradiation-induced G2/M arrest. The p53 protein is also phosphorylated by S and G2/M cyclin-dependent kinases (CDK) at serine 315 (Marx 1994). UV irradiation but not ionizing radiation induces p53 phosphorylation at Ser-392. It is not known if phosphorylation of the C-terminus at the casein kinase II (CKII) site (Ser-392) or the protein kinase C (PKC) sites (Ser-371, 376, and 378), regulates DNA binding of p53 (reviewed in Prives and Hall 1999).

The central role of p53 in the cellular response to DNA damage is well established (reviewed in Ko and Prives 1996). Ionizing radiation creates double-stranded DNA breaks. p53 is stabilized by the mechanisms mentioned above and induces *p21/waf1*, which inhibits CDK-cyclin activity and thereby causes cell cycle arrest, giving the cell time to engage the DNA repair program. p53 induces *Gadd45*, which can directly associate with mononucleosomes that have been altered by irradiation and facilitate DNA accessibility to other proteins (Carrier *et al.* 1994, Carrier *et al.* 1999). *Gadd45* is also found in complexes with another p53-induced protein, the proliferating cell nuclear antigen (PCNA) (Jackson *et al.* 1994, Smith *et al.* 1994). PCNA, a normal component of Cdk complexes, is a protein involved in normal DNA replication, and is also needed for the resynthesis of DNA after damaged portions are removed by the cell's nucleotide excision repair system. p53 can also bind to ERCC3, XPB and XPD in the TFIIH transcription repair complex and modulate nucleotide excision repair (Wang *et al.* 1995b). ERCC3 is one of several excision repair molecules that can recognize and remove damaged segments of DNA (reviewed in Marx 1994). ERCC3 might also be involved in p53's gene control activities, as it allows TFIIH to unwind DNA locally in an ATP-dependent reaction.

Hypoxia, heat shock and starvation are also well-studied stress signals known to induce p53 reviewed in (Prives and Hall 1999).

1.1.6.2 Effectors of p53-induced apoptosis

Apoptosis is widespread in multicellular organisms, and underlies such processes as organogenesis, tissue homeostasis and the removal of autoreactive clones in the immune system (see Adams and Cory 1998, Evans and Littlewood 1998, Raff 1998 for recent reviews). Apoptosis can also be induced by toxic insults and, in particular, by agents that damage DNA. Programmed cell death is frequently seen in hypoxic regions of tumors and may play a role in the kinetics of tumoral growth. Elegant work on the nematode *Caenorhabditis elegans* has identified three genes responsible for controlling the apoptotic pathway called *ced4*, *ced3*, and *ced9*. Ced-4 and Ced-3 are inducers of cell death during nematode development, whereas Ced-9 blocks apoptosis. Bcl-2 is a mammalian homolog of Ced-9. The *bcl-2* gene was known to be activated in many follicular lymphomas. A family of proteins called caspases are the mammalian homologs of Ced-3, while Apaf-1 is the Ced-4 homolog. Caspases are the central players in programmed cell death; they catalyse many steps in the death pathway by cleavage of proteins at specific sites containing aspartic acid.

Caspase-9 and Apaf-1 have recently been shown to be essential downstream mediators of p53-dependent apoptosis in response to some oncogenes (Soengras *et al.* 1999). Cytochrome c release from the mitochondria is essential for some apoptotic programs and is required for the activation of pro-caspase-9 (reviewed in Green and Reed 1998). The p53-induced gene *bax* can facilitate cytochrome c release (Gross *et al.* 1998). An intense search is now ongoing for p53-induced genes involved in apoptosis (Polyak *et al.* 1997, Wyllie 1997). A newly identified p53-induced gene, Noxa, mediates apoptosis by interacting with anti-apoptotic Bcl-2 family members at the mitochondria resulting in the activation of caspase-9 (Oda *et al.* 2000). A role for p53 in direct signalling at the mitochondria has also recently been proposed (Marchenko *et al.* 2000).

The transcriptional repression function of p53 has also been implicated in the pathway to apoptosis (Caelles *et al.* 1994). The inhibition of p53-mediated repression with

the drug trichostatin A (TSA, an inhibitor of HDACs) markedly inhibits apoptosis induction by p53 (Murphy *et al.* 1999). Certain tumor-derived mutants of p53 that are almost *wild-type* in transcriptional activation but are not able to mediate repression are also defective in inducing apoptosis (Ryan and Vousden 1998). The following genes are repressed following p53 induction: *Map4* (Murphy *et al.* 1996), *stathmin* (Ahn *et al.* 1999), *cdc2* (Yun *et al.* 1999), *DNA topoisomerase II α* (Wang *et al.* 1997), *DP-1* (Gopalkrishnan *et al.* 1998), *weel* (Leach *et al.* 1998), and *presenilin-1* (Roperch *et al.* 1998).

1.1.7 p53 family members

Until 1997, researchers working on p53 thought it was unique. Recently two genes have been identified that encode proteins which display remarkable similarity to p53. They are called *p73* and *p63* and each gene produces several splice variants. The p53, p63 and p73 proteins share high amino acid sequence identity in the core DNA-binding domain, and may thus regulate a common set of genes in response to different extracellular signals (reviewed in Levrero *et al.* 2000).

1.1.7.1 p73

Two main forms of p73 are transcribed from a first promoter, p73 α encoding 636 amino acids (aa) and p73 β which has 499aa. Exon 13 of the *p73* gene is spliced out of the p73 β form. A third protein, p73 γ , has a deletion of the last 5 exons. The second promoter produces two products: dN α of 576aa and dN β of 449aa, which lack the N-terminal activation domain and are potential negative regulators. Additional shorter forms of p73, termed p73 γ , p73 δ and p73 ϵ , can be observed with the p73 monoclonal antibodies (reviewed in Levrero *et al.* 2000).

p73 α displays remarkable homology to the larger squid p53 that has 564 aa. The core domain of human p73 has a 63% amino acid sequence identity with the core of human

p53, and all the amino acids that contact DNA and are mutational hot spots are conserved between the two proteins. p73 is localised in the nucleus and displays a dotted pattern. p73 can transactivate p53 target genes, and suppress the growth of neuroblastoma cells which lack endogenous p73 expression. Ectopically expressed p73 recapitulates functions attributed to p53 such as cell cycle arrest, differentiation and apoptosis.

The p73 α protein shows little ability to oligomerize whereas p73 β does form oligomers. Neither p73 α or p73 β are able to interact with wt p53; however, they are able to form hetero-oligomers with some p53 core domain point mutants such as p53 143. This could explain the gain of function in transformation of such p53 mutants; however, the story is not clear cut since not all p53 point mutants that display gain of function are able to interact with p73. Co-expression of p53 273mut can inhibit the transcriptional activation of p21Waf1 by wt p73.

The loss of p73 in mice leads to fundamental neurological, immunological, behavioral and reproductive defects (Yang *et al.* 2000). The pups show growth retardation (they are small) and a 60% lethality at 3 weeks. They have hydrocephaly and dysgenesis of the hippocampus, as well as severe neurological defects. The mice display an inflammatory syndrome and hearing and sex drive defects. There is a striking correlation between sites of p73 expression and the functional impairments manifest in the p73 knockout mouse. Importantly, p73 $^{-/-}$ mice fail to develop spontaneous tumors as those seen in p53-deficient mice (Donehower *et al.* 1992). Like p53 and MDM2, p73 also contains a nuclear export signal (NES).

A role for p73 mutations in tumor suppression was suggested by its localization to chromosome 1p36 (Kaghad *et al.* 1997), a region subject to frequent loss of heterozygosity in a wide range of human cancers. Subtelomeric deletions, spanning but not focusing on the p73 locus, occur in neuroblastoma (about 30%), breast cancer, melanoma and some other tumors. An imprinted neuroblastoma suppressor was mapped to 1p36, and p73 was a candidate. However, there is currently a lack of evidence to support the candidacy of the p73

gene as the long sought-after neuroblastoma tumor suppressor. *p73* might however have a role in some types of neuroblast differentiation.

Recently, two groups found that the c-Abl tyrosine kinase may be involved in signaling to *p73* (Gong *et al.* 1999, Yuan *et al.* 1999). They propose that, in response to DNA damage, the ATM protein interacts with and phosphorylates c-Abl, activating c-Abl's tyrosine kinase activity, which then phosphorylates *p73* on a tyrosine (Tyr) residue. *p53* is also downstream of ATM in the response to DNA damage, but unlike *p73*, its activation is not impaired in c-Abl-deficient cells. *p53* is not phosphorylated on tyrosine (reviewed in White and Prives 1999).

Zeng *et al.* found that MDM2 binding disrupts the interaction of *p73* with the transcriptional coactivator p300/CBP (Zeng *et al.* 1999), and MDM2 does not target *p73* for ubiquitin-proteasome mediated degradation as it does *p53*. Another report (Ongkeko *et al.* 1999) confirms that *p73* α and *p73* β bind MDM2 and they also find that *p73* α and *p73* β bind the MDM2-related protein MDMX. However, in contrast to *p53*, the half-life of *p73* was found to be increased by binding to MDM2 or MDMX, and the growth suppression functions and induction of *p21/waf1* by *p73* were enhanced in the presence of MDM2.

1.1.7.2 *p63*

The *p63* gene was recently cloned and is also known as KET or *p51* (Osada *et al.* 1998, Yang *et al.* 1998). High levels of *p63* mRNA have been detected in circumvallate taste papilloma keratinocytes, hence the name KET for keratinocyte. RT-PCR and Northern blot analysis have confirmed the expression of *p63* mRNA in keratinocytes growing in primary culture. The gene maps to chromosome 3 q27-29, and produces four possible splice variants. Like *p53*, *p73* and MDM2, *p63* also contains a NES.

Comparison of the deduced amino acid sequence to the *p63* protein revealed strong similarity in all evolutionary conserved regions of *p53*. The identity reaches 75% in the sequence-specific DNA binding domain. *p63* also shares a 58% overall protein sequence

identity with p73 α . p63-deficient mice have a defect in the apical ectodermal ridge essential to limb development and they have truncated limbs (Mills *et al.* 1999, Yang *et al.* 1999). The p63 $-/-$ mice also have no hair follicles, no mammary glands, no teeth, no lachrymal and no salivary glands. Recently, p63 mutations have been identified in children affected by ectodermal dysplasia and facial clefts (reviewed in (Levrero *et al.* 2000)).

Both p73 and p63 are independent members of a new family of p53-related proteins. The emerging picture is that the p53/p63/p73 family of transcription factors may have overlapping as well as distinct biological functions in tumor suppression and development.

1.1.8 Viruses and p53

Many viruses can only replicate by inducing infected cells to enter S phase. This leads to the induction of p53, and thus viruses have evolved ways to counter the p53 response; most often they encode proteins that bind to p53 and block its induction of growth arrest and apoptosis. The following is a short enumeration of viral proteins known to target p53.

Simian virus 40 large T antigen (SV40 LT) binds to the DNA-binding domain of p53 and prevents transcriptional activation by p53 (Mietz *et al.* 1992). SV40 LT also relocalizes p53 from the nucleus to dense cytoplasmic bodies, and inhibits site-specific DNA binding of p53 (Bargonetti *et al.* 1992). Human papillomavirus E6 protein binds to the carboxy terminus of p53 (Werness *et al.* 1990) and HPV type 16 and 18 E6 proteins target p53 for ubiquitin-mediated degradation (Scheffner *et al.* 1990b). Hepatitis B virus X protein (HBx) binds to the carboxy terminus of p53 and inhibits p53 DNA-binding and transactivation (Wang *et al.* 1995a). HBx binding to p53 also sequesters it in the cytoplasm (Elmore *et al.* 1997). The hepatitis C virus core protein binds to the p53 promoter and represses transcription of p53 mRNA (Ray *et al.* 1997). Human cytomegalovirus (HCMV) IE2 protein causes an accumulation of p53 by transactivating the p53 promoter (Muganda *et al.* 1994,

Muganda *et al.* 1998), but IE2 is also a repressor of transcription and inhibits p53-mediated gene expression (Tsai *et al.* 1996). Human T-cell lymphotropic/leukemia virus type 1 (HTLV-1) Tax protein causes the up-regulation of p53 protein, but also blocks p53 transcriptional activation by binding the co-activator of p53, p300 (Mulloy *et al.* 1998). Finally, adenovirus E1A protein causes the stabilization of p53 (Lowe and Ruley 1993) but inhibits p53 transactivation by binding to p300 (Somasundaram and El-Deiry 1997). Adenovirus E1B55K and E4orf6 proteins each bind to p53 and also block its ability to activate transcription (Yew and Berk 1992, Dobner *et al.* 1996), and together target p53 for degradation (Querido *et al.* 1997a).

Viruses seem to target p53 but not family members p73 and p63 (Marin *et al.* 1998, Wienzek *et al.* 2000).

Table 1.1 Classification of human adenoviruses

Adapted from Shenk 1996

Subgroup	Hemagglutination group	Serotypes	Oncogenic potential for tumors in animals	DNA homology within group
A	IV	12, 18, 31	High	48 - 69 %
B	I	3, 7, 11, 14, 16, 21, 34, 35	Moderate	89 - 94 %
C	III	1, 2, 5, 6	Low or none	99 - 100 %
D	II	8, 9, 10, 13, 15, 17, 19, 20, 22 - 30, 32, 33, 36 - 39, 42 - 47	Low or none (mammary tumors)	94 - 99 %
E	III	4	Low or none	(100 %)
F	III	40, 41	Not determined	Not determined

1.2 Adenovirus etiology and classification

Adenoviruses were first isolated and characterized by two groups of researchers searching for the causative agent of the common cold (Rowe *et al.* 1953, Hillerman and Werner 1954). They are endemic in human populations. In western Europe and the United States, between one third and one half of children have acquired antibodies to one or more subgroup C adenovirus (see Table 1.1) by the time they are one year old (Fox *et al.* 1969). Few adults lack immunological experience with these ubiquitous adenoviruses. An individual can shed infectious particles intermittently for months after an initial infection. They were initially discovered because of a spontaneous expression of latent virus in human adenoid tissue (Rowe *et al.* 1953). Adenoviruses, as we now know, are not the cause of the common cold. Indeed, only 5-10% of the respiratory illnesses of children are associated with adenovirus infections (Brandt *et al.* 1969). Immune clearance of infecting virus presumably occurs by cell-mediated mechanisms requiring successful interaction among macrophages, lymphocytes, and NK cells. Most cases of acute respiratory adenovirus infections have been documented in individuals lacking adequate cellular immunity. This includes newborns, children with T-cell disorders, and immuno-depressed patients (Horwitz 1996).

There are two families of these viruses: mastadenoviruses, which infect mammals, and aviadenoviruses, which infect birds. Adenoviruses have been isolated from mice, cats, dogs, tree shrews, pigs, sheep, monkeys and cows, and these belong to the mastadenovirus family along with all the human adenovirus serotypes. Human adenoviruses are species specific for permissivity to replication, as are most avian and mammalian adenoviruses. So far, 49 serotypes of human adenovirus have been isolated. A new serotype is defined by resistance to neutralizing antibodies against other serotypes. The most widely accepted classification of human adenoviruses is based on the ability of specific immunotypes to partially or completely hemagglutinate rat or rhesus erythrocytes (Rosen 1960), and they have thus been classified into six groups lettered A to F (Table 1.1).

Adenoviruses are associated with the entire spectrum of respiratory illness, but they usually cause relatively mild illnesses such as feverish colds and sore throats. Different adenoviruses possess unique tissue tropism. The lower-numbered virus types are predominantly recovered from ocular and respiratory tissues. The higher-numbered types (Ad25-41) are almost always found in stools, and their major and primary site of infection may be the gastrointestinal tract. Adenoviruses have also been associated with acute hemorrhagic cystitis and myocarditis in children (Martin *et al.* 1994).

A recent report suggests that adenovirus type 36 may be linked to obesity (Dhurandhar *et al.* 1992, Dhurandhar *et al.* 1997). Laboratory animals injected with Ad36 were found to develop obesity in significant numbers; paradoxically Ad36 infection was associated with lower levels of cholesterol and triglycerides along with obesity. Dhurandhar *et al.* tested 154 obese and 45 lean human volunteers for the presence of antibodies to Ad36. About 15 % of the obese subjects had antibodies to Ad36 while the lean volunteers showed none. The antibody-positive obese subjects had significantly lower cholesterol and triglycerides than the antibody-negative obese subjects, a pattern similar to that seen in the animals injected with Ad36.

Adenovirus type 12 was the first human virus shown to have oncogenic properties when Trentin *et al.* (1962) reported the induction of tumors following injection of the virus in newborn hamsters. Their results were soon confirmed for other serotypes and extended to other rodents. A new field of study opened, its goals to understand the mechanisms by which adenoviruses cause tumors and to determine whether they play any role in human malignancy. As summarized in table 1.1, group A adenoviruses, to which Ad12 belongs, are strongly oncogenic. Ad12 induces tumors in rodents one to two months after injection. Group B adenoviruses are weakly oncogenic. Groups C (which contains adenovirus serotypes 2 and 5), D, E and F are not tumorigenic in rodents. Both Ad12 and Ad5 DNA can transform cells *in vitro*, but when cells transformed by Ad12 are injected into nude mice they form tumors while Ad5-transformed cells do not grow into tumors in nude mice.

The oncogenic potential of these serotypes has been mapped to the leftmost 11% of the genome. Expression of the E1 region (see Figure 1.5), encoding the E1A and E1B proteins, is all that is required for transformation of cells that are non-permissive for replication (reviewed in Branton *et al.* 1985). Such transformed cells typically are capable of indefinite cell growth *in vitro*, they are not contact-inhibited at high densities, and are capable of growth in soft agar.

Many studies have since looked for adenovirus DNA in human tumors: they have failed to find any such sequences (Mackey *et al.* 1976, Green *et al.* 1980). Adenoviruses are very poor at transforming human cells, and the initial explanation was that rodent cells are not permissive for human adenovirus replication and can thus survive the infection and go on to form transformed clones. However, cloned E1A and E1B genes are also much more efficient at transforming rodent cells. When Graham *et al.* (1977) transformed human embryonic kidney cells with Ad5 DNA and obtained the commonly used 293 cell line, it was indeed a rare and lucky event.

All of the work described in this thesis was done with adenovirus serotype 5 or in some special cases with the nearly identical serotype 2 (Roberts *et al.* 1984, Chroboczek *et al.* 1992).

1.3 The Adenovirus virion

Adenovirus is a non-enveloped DNA virus. The virion has an icosahedral shape (see Figure 1.3 from Shenk 1996) and consists of the capsid, an icosahedral protein shell, and the core, an internal body that comprises the virus DNA and at least two major proteins. The icosahedron is 20-sided with triangular-shaped facets and 12 vertices where the corners meet. The diameter of the capsid is 70 to 90 nm. It is made up of 252 capsomeres; 240

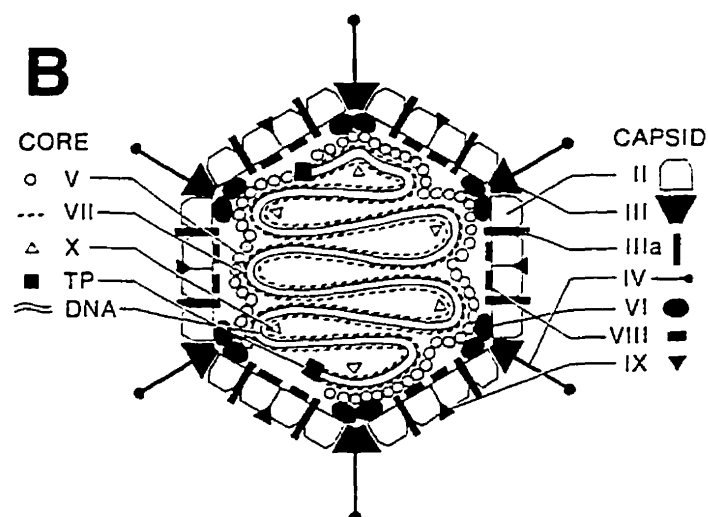
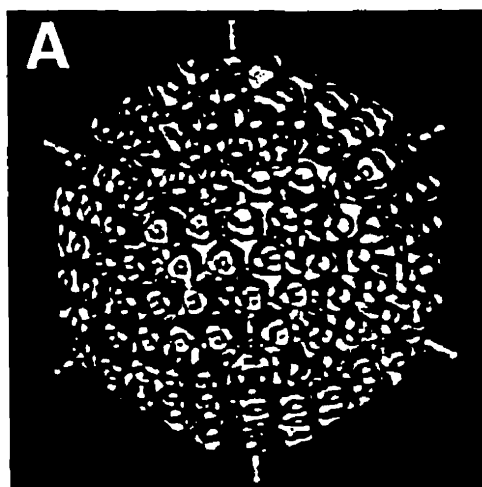


Figure 1.3 Models of the adenovirus virion.

(A) Three-dimensional image reconstruction of the intact adenovirus particle viewed along an icosahedral three-fold axis. (B) Schematic representation of the adenovirus particle based on current understanding of its polypeptide components and DNA. No real section of the icosahedral virion would contain all components. Virion constituents are designated by their polypeptide numbers with the exception of the terminal protein (TP). Adapted from Shenk, 1996.

hexons and 12 pentons. Hexons are surrounded by 6 other subunits and make up the facets of the icosahedron, while the pentons are surrounded by 5 subunits and are located at the vertices (reviewed in Shenk 1996).

Adenoviruses contain 11.6-13.5% of double-stranded DNA, the rest being protein. No lipid is present, and only about 1% is glycosylated (fiber). When the adenovirus particle is stripped of the icosahedral shell, what remains is the core, and much less is known about its structure. The DNA molecule is linear, but within the virion it may exist in a circular form mediated by the terminal proteins. The viral core is composed of 5 major proteins: polypeptides V, VII, X, μ and the terminal binding protein (reviewed in Shenk 1996). Polypeptide VII is the major component of the core with about 1000 copies per virion. It is an 18 kDa protein extensively associated with viral DNA. Studies have found that the packaging of viral DNA within the core is similar to nucleosome structures in chromatin and that polypeptide VII may play the role of a histone (Chatterjee *et al.* 1986).

1.4 The Adenovirus Genome

Adenovirus type 5 has a double stranded linear DNA genome of 36 kilobases (Chroboczek *et al.* 1992). Each extremity consists of a 103 base pair (bp) inverted terminal repetition. The 5' end of the DNA is a dCMP residue. The 55 kDa terminal proteins (TP) are covalently attached at each 5' end of the genome (Lichy *et al.* 1981).

The distribution of AT and GC base pairs along the adenovirus DNA molecule is asymmetrical. By convention, the AT-rich half of an adenovirus DNA molecule is oriented to the right and the strand transcribed to the right is called the r-strand, while the leftward-transcribed strand is designated the l-strand. The DNA can be divided into 100 map units (m.u.) from left to right on the viral genome (see Figure 1.4).

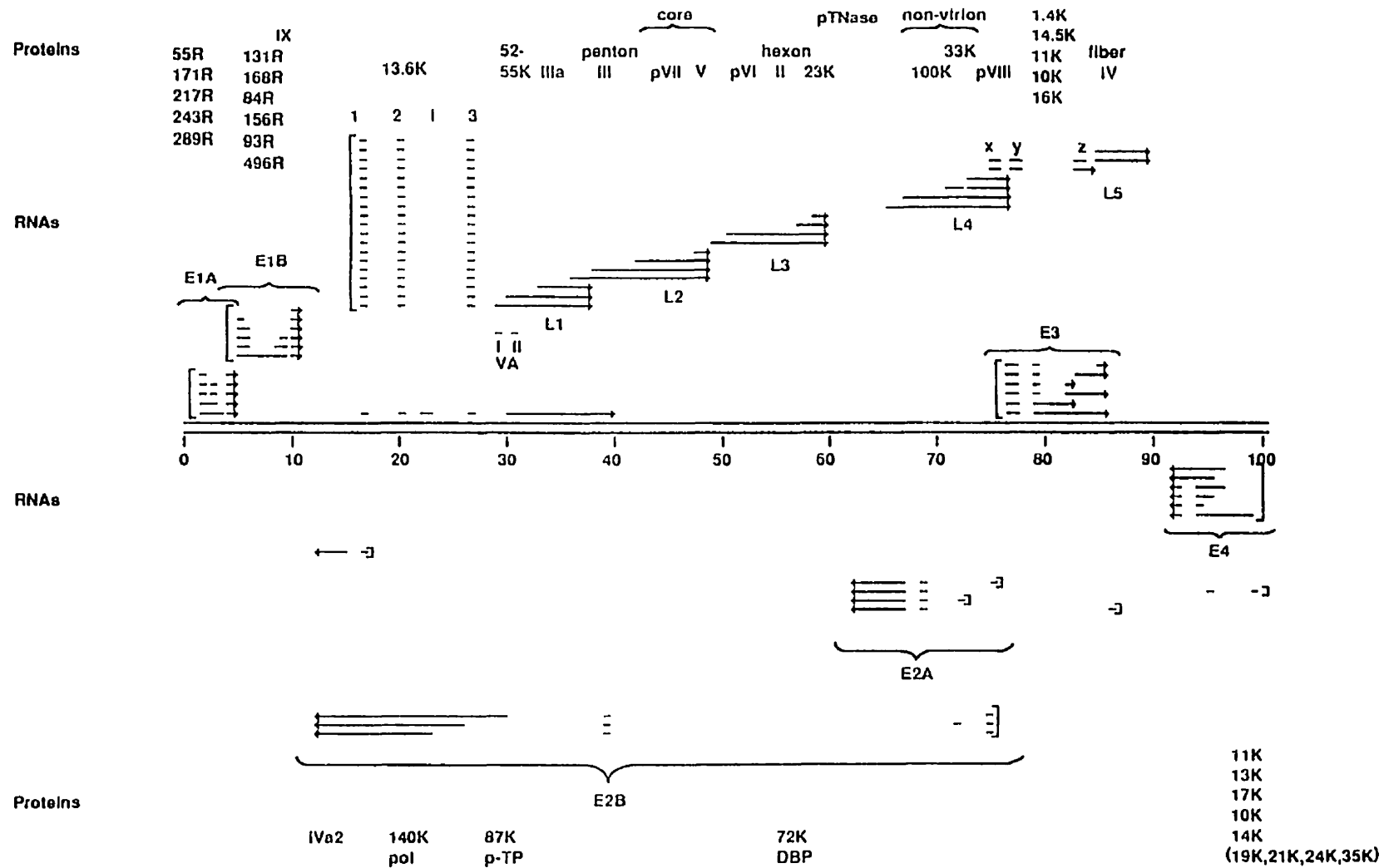


Figure 1.4 Transcription and translation map of adenovirus type 5.

The genome is divided into 100 units in this schematic representation. Transcription units are designated E for early if they are transcribed before the onset of viral replication, and the late transcription units are designated L. The promoters are indicated by the brackets at the 5' end of each set of mRNAs. Splicing is represented by a gap and the arrows indicate polyadenylation tails. Most late mRNAs originate at 16.3 map units and contain the tripartite leader whose components are labeled 1, 2, and 3. Some late mRNAs also contain a fourth leader segment i. Polypeptides encoded by the different messages are displayed above and below the transcription units. Adapted from Shenk, 1996.

The adenovirus genes are traditionally subdivided into early genes, which are expressed before the onset of viral DNA replication, and late genes, which are transcribed after replications has started. All primary transcription products of adenovirus DNA are processed in the nucleus before entering the cytoplasm. They are capped with m⁷GpppX at the 5' end, and they are polyadenylated at the 3' end. With one exception (pIX mRNA), all primary transcription products are processed into families of related mRNAs that share common 5' and 3' ends, but differ by alternative splicing (reviewed in Shenk 1996).

1.5 The infective cycle of human adenovirus

1.5.1 Adsorption and entry into the cell

Adenovirus attachment to cells is mediated by the fibers projecting from the capsid (Defer *et al.* 1990). Globular knob domains at the tips of the fibers directly contact the cellular receptor (Henry *et al.* 1994, Stevenson *et al.* 1995). The receptor shared by the coxsackie virus and adenovirus (CAR) was cloned recently (Bergelson *et al.* 1997). CAR is a 365 aa transmembrane protein. Its extracellular portion consists of two immunoglobulin-like domains. The murine CAR homolog displays 91% aa sequence identity to human CAR, most likely explaining the ability of human adenovirus to infect rodent cells. All the adenovirus serotypes from subgroups A, C, D, E, and F use CAR as a cellular fiber receptor (Roelvink *et al.* 1998). The structure of the adenovirus fiber knob domain in complex with domain I of CAR has recently been solved, and confirms that they form a high affinity complex (Bewley *et al.* 1999). Once fiber-mediated virus attachment to the receptor has occurred, internalization involves a secondary interaction between the penton base protein and integrins on the cell surface (Wickham *et al.* 1993). The virus is then rapidly internalized by receptor-mediated endocytosis and contained within endosomes from which is it released into the cytosol in a process which is dependent on the low pH of the endosome and the viral penton base protein (Seth *et al.* 1984, Svensson 1985). Uncoating happens in the cytoplasm

and requires about two hours. Pentons are removed first, then hexons, and the virion core is transported to the nuclear membrane where further uncoating occurs (Greber *et al.* 1993), following which the viral DNA is propelled into the nucleus through nuclear pores. The process of adsorption, penetration, and nuclear uptake is completed within 60-90 minutes.

1.5.2 E1A proteins

Early in infection, during the 6 to 8 hours before viral DNA replication begins, adenovirus mRNAs that represent 25 to 30 percent of the genome are made. They are derived from scattered regions of the genome called early regions 1-4 (see Fig. 1.4). As early as one hour post-infection (hpi), the first viral mRNAs transcribed from the early region 1 A (E1A) gene can be observed (Nevins *et al.* 1979). E1A proteins then enhance transcription from the early transcription units by 50-100 fold (Berk 1986).

The E1A transcription unit produces a single transcript that is spliced into five different mRNAs, which are referred to according to their sedimentation coefficients of 13S, 12S, 11S, 10S and 9S (as illustrated in Figure 1.5). All five mRNAs are translated from the same initiation codon and possess identical N-terminal regions (Green *et al.* 1979, Perricaudet *et al.* 1979). The 13S and 12S messages encode 289 and 243 residue proteins respectively. The two major E1A products of 289- and 243-residues(R) are identical apart from a unique 46 aa internal region in the 289R E1A. The 11S and 10S E1A are made during the late phase of infection. Both products have a 72 residue deletion near the N-terminus, but the 10S product also has the same 46 amino acid internal deletion as in the 243R product (Stephens and Harlow 1987, Ulfendahl *et al.* 1987). The 9S message differs from all the others as it is spliced into a different reading frame after amino acid 27, producing a 55R protein (Virtanen and Pettersson 1983). The products of the five messages of Ad5 E1A are acidic nuclear phosphoproteins. The 289R E1A protein migrates as a 52 kDa and a 48.5 kDa species by SDS-PAGE, while the 243R protein migrates as 50 kDa and 45

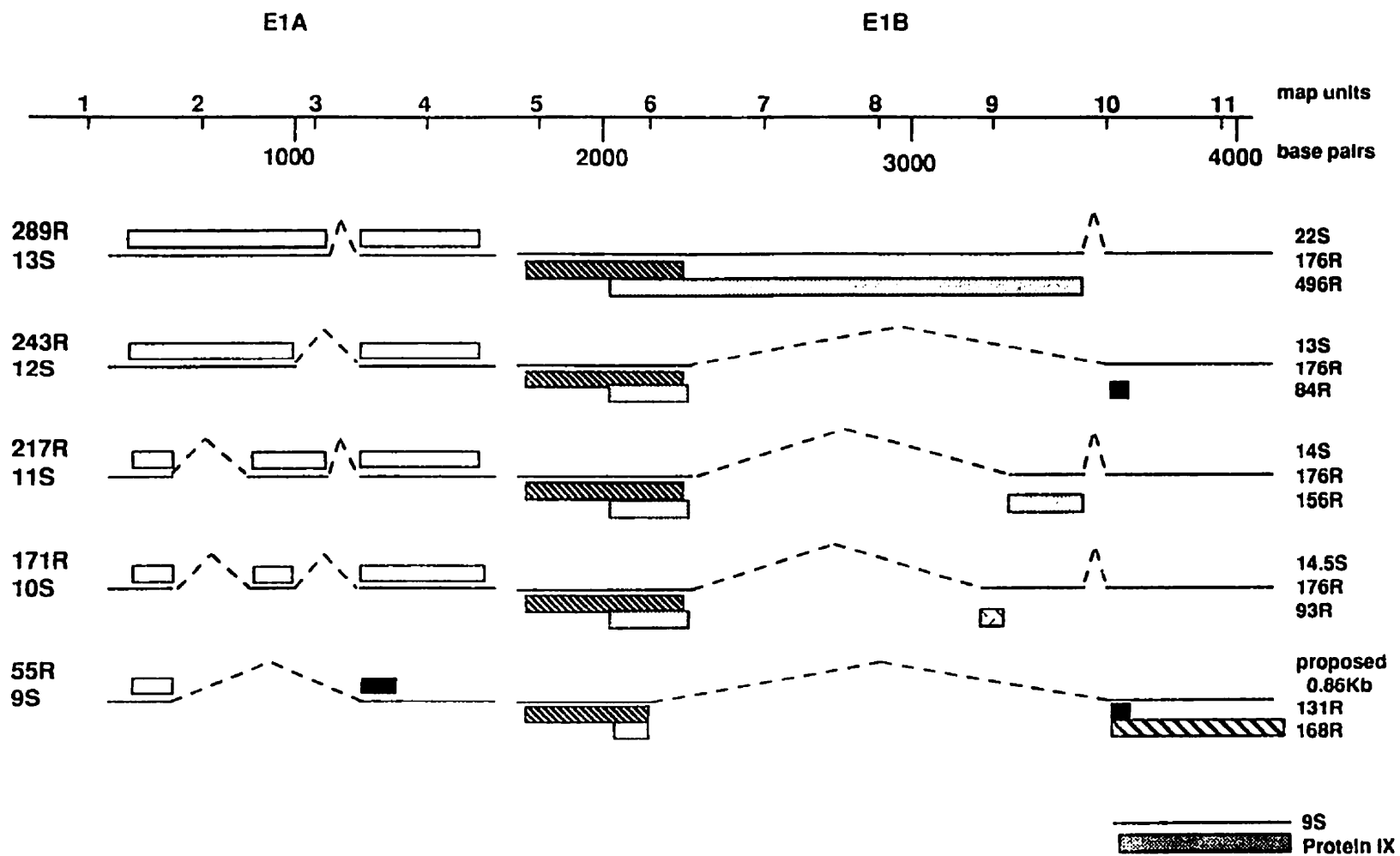


Figure 1.5 Transcription map of the E1 region

Early region 1 covers the leftmost 11% of the adenovirus type 5 genome (11 map units). The proteins encoded by the alternatively spliced mRNAs are identified both by their sedimentation coefficient (S) and the number of amino acid residues (R) they contain. E1A proteins are listed at the left side of the figure, and E1B proteins are listed on the right. Protein IX is expressed in the late phase of infection.

kDa proteins. The multiple species observed for the 289R and 243R products result from differential phosphorylation at Ser- 89 and neighboring serine sites (Dumont *et al.* 1989, Smith *et al.* 1989, Dumont *et al.* 1993). E1A proteins are primarily localized in the nucleus, though they can also be found in the cytoplasm (Feldman and Nevins 1983, Yee *et al.* 1983). The nuclear localization signal is a pentapeptide sequence located at the extreme carboxy terminus (Lyons *et al.* 1987). E1A undergoes rapid turnover with a half-life of 40 minutes (Branton and Rowe 1985). The E1A proteins are phosphorylated almost exclusively on serine residues (Tremblay *et al.* 1989). Ser-219 appears to be the major phosphorylation site (Tsukamoto *et al.* 1986, Tremblay *et al.* 1988). Phosphorylation also occurs at Ser-89 and -96, as well as serines 132 (Whalen *et al.* 1993a), 227, 228, 231, and 237 (Whalen *et al.* 1993b).

When the amino acid sequences of E1A proteins from several adenovirus serotypes were compared, three regions that were highly conserved were identified and designated conserved regions 1, 2 and 3 (CR1, CR2 and CR3). These regions are illustrated in Figure 2.1. The unique 46 aa sequence present in 289R E1A corresponds almost exactly to CR3, and this region is spliced out of the 243R E1A molecule. The CR3 region has been identified as a potent transcriptional activation domain that functions during infection to enhance the transcription of early viral promoters (Berk 1986). CR1 spans aa 40-80 and CR2 was defined as aa 121-138. Both the 289R and the 243R E1A can form complexes with two classes of cellular proteins: the tumor suppressor protein pRb and its related proteins p107 and p130; and the transcriptional co-activators p300/CBP/p400. p300/CBP binding requires the N-terminus (aa 1-25) as well as residues within CR1 (aa 36-76). p400 binding is mediated by the N-terminus (aa 1-48). pRb binding requires aa 121-127 within CR2 containing the Leu-X-Cys-X-Glu motif (LXCXE), the minimal p107 binding domain is aa 124-127, and p130 binding requires aa 121-139. pRb/p107/p130 binding is also affected by mutations in aa 36-60 within CR1 (Barbeau *et al.* 1992, Barbeau *et al.* 1994).

1.5.2.1 Mechanisms of E1A transcriptional activation

E1A proteins appear to activate transcription through multiple mechanisms, including modification of cellular transcription factors, binding to transcription factors, and causing an increase in the levels of transcription factors. The E1A CR3 region contains two kinds of domains: a promoter binding region that has been mapped to the carboxy terminal end of CR3 between residues 175 and 189, and an activation domain in the amino terminal portion of CR3 between residues 139 and 153 (Lillie and Green 1989). The central portion of CR3 (aa 154-174) contains a zinc binding domain with four conserved cysteine residues. Zinc fingers are found in many transcription factors, and they promote DNA binding or increase the stability of the transcription complex on DNA. Since E1A has no or very low affinity for DNA, it seems likely that the carboxy terminal portion of CR3 serves to recognize DNA-bound proteins.

E1A proteins can function within transcription complexes by direct interaction with a variety of transcription factors. The amino terminal activation domain of E1A has been shown to interact directly with TFIID, the transcription factor that binds to the TATA box and consists of TBP (TATA-binding protein) and a number of TAFs (TBP-associated factors) (Horikoshi *et al.* 1991, Lee *et al.* 1991). E1A can also bind to AP-1 (Lee *et al.* 1991, Maguire *et al.* 1991), which is composed of polypeptides from the *fos* and *jun* gene families, and E1A can also increase the levels of AP-1 (reviewed in Shenk and Flint 1991). The cAMP responsive element serves as a binding site for the AP-1 and the ATF/CREB families of transcription factors, and members of both families have been implicated in mediating the transcriptional activation by E1A and cAMP. E1A has also been shown to physically interact with ATF (Maguire *et al.* 1991) and Oct-4 (Scholler *et al.* 1991), reviewed in Liu and Green (1994).

By binding to the pRb family of proteins, E1A disrupts pRb-E2F complexes, enabling E2F to activate transcription (Bagchi *et al.* 1990, Nevins 1992). pRb can repress transcription by at least two mechanisms: it binds to and blocks the transactivation domain of

E2F, and it binds to the promoter as a complex with E2F which can actively repress transcription (reviewed in Harbour and Dean 2000). pRb represses by histone deacetylase (HDAC)-dependent and independent mechanisms, and recruits a binding protein (RBP1), which can also mediate repression of E2F-dependent promoters (Lai *et al.* 1999).

1.5.2.2 Transactivation of the early viral promoters

E1A first transactivates its own promoter (Berk 1986), then activates early viral genes in the following sequence: E2, E3, E4, and then E1B, as determined by detailed kinetic studies of the appearance and accumulation of early viral mRNAs (Glenn and Ricciardi 1988). The E1A promoter contains a pair of E2F sites, in addition to multiple ATF sites, and a TATA box (Osborne *et al.* 1982, Hearing and Shenk 1983, Osborne and Berk 1983, Hardy and Shenk 1988, Mudryj *et al.* 1990). Activation of the E1A promoter involves the synergistic action of E1A and cAMP.

The E1B promoter is exclusively transactivated by the 289R E1A product (Kraus *et al.* 1992). The TATAA sequence of the E1B promoter has been shown to mediate control by E1A (Nevins 1989). The E1B promoter also contains an Sp1 site; however, the importance of this element in conferring E1A responsiveness has not been shown.

The E2 promoter is activated by both 243R and 289R E1A. The E2 promoter can be regulated by E2F (Kovesdi *et al.* 1986), and it can also be activated by E1A via the ATF-2 and ATF α factors. Two E2F sites are located in the E2 promoter. As we will cover later, the E4orf6/7 19 kDa protein forms complexes with E2F and also activates E2 transcription.

The E3 promoter is exclusively transactivated by the 289R E1A product (Kraus *et al.* 1992), through a TATA box (Nevins 1989). The E3 promoter also has an AP-1 site and an ATF site as upstream promoter elements, and all appear to confer E1A inducibility.

The E4 promoter has five ATF sites and transactivation is mediated by E1A-289R via the ATF-2 and E4F factors (Gilardi and Perricaudet 1986, Lee and Green 1987). E4F recognizes a subset of ATF elements and binds to only two of the five ATF sites, and does

not bind to ATF sites in the E2 and E3 promoters. E1A transactivation of the E4 promoter via E4F requires the CR3 plus auxiliary regions 1 (AR1) and 2 (AR2). AR1 and AR2 could present negatively charged surfaces to mediate protein-protein interactions. cAMP also acts in synergy with E1A to activate transcription of the adenovirus early genes E4 and E1A (Engel *et al.* 1988).

1.5.2.3 Mechanisms of transcriptional repression by E1A

We introduced the p300/CBP and PCAF acetyl transferases in section 1.1.3 on mechanisms of transcriptional activation by p53. The PCAF complex has intrinsic histone acetyltransferase (HAT) activity and is also capable of binding to the transcriptional co-activators p300 and CBP, which are themselves HATs (reviewed in Schiltz and Nakatani 2000). Now we will discuss how E1A interacts with these factors.

E1A and PCAF bind to the same region of p300/CBP, thus E1A displaces the PCAF complex from p300/CBP (Reid *et al.* 1998, Chakravarti *et al.* 1999, Hamamori *et al.* 1999). E1A binds to either p300/CBP or PCAF through its N-terminus (residues 1 to 76). E1A can inhibit the HAT activity of p300/CBP and PCAF, and blocks acetylation of p53. The C-terminus of E1A is the HAT inhibitory domain. By disrupting the complexes responsible for acetylating histone tails, E1A favors nucleosome packing that inhibits access of the transcriptional machinery at those promoters. Both 243R and 289R E1A proteins can repress enhancer-driven transcription. The 243R product appears to be the most effective of the two (Lillie *et al.* 1986, Dery *et al.* 1987) and can repress transcription of other early viral genes as well as cellular promoters. Enhancer repression was mapped to the N-terminal p300 binding domain of E1A (Stein *et al.* 1990).

The largest subunit of the PCAF complex is PAF400/TRRAP (reviewed in Schiltz and Nakatani 2000). The 400 kDa E1A-binding protein (p400) has not yet been conclusively identified, and it is tempting to speculate that E1A binds to PAF400/TRRAP. It was recently shown that TRRAP recruits HAT activity to the c-Myc transcription factor

(McMahon *et al.* 2000) , thus E1A might repress Myc-dependent promoters by binding TRRAP.

A C-terminal binding protein (CtBP) of E1A (Boyd *et al.* 1993) is a transcriptional repressor that can interact with HDAC1 (Sundqvist *et al.* 1998) and with a class of vertebrate Polycomb proteins (Sewalt *et al.* 1999). The association with CtBP is important for E1A-mediated repression of transformation, tumorigenesis and metastasis.

BS69, a protein that interacts directly with the transactivation domain (CR3) of E1A-289R (Hateboer *et al.* 1995), represses transcription, at least in part, through the interaction with a co-repressor, N-CoR (Masselink and Bernards 2000). E1A represses transcription from p300/CBP-regulated genes such as PCNA (Kannabiran *et al.* 1999). The insulin enhancer, the neu protooncogene, differentiation-specific genes, fibronectin, genes stimulated by interferon, and members of the matrix metalloprotease family, are some of the cellular genes repressed by E1A (reviewed in Bayley and Mymryk 1994). Some of these activities contribute to the tumor suppression properties of E1A (reviewed in Mymryk 1996).

1.5.2.4 Some functions of E1A: induction of S phase and transformation

Adenovirus infects differentiated non-dividing cells in the host, and makes use of the cellular DNA synthetic machinery to replicate its genome. E1A-243R and E1A-289R can thus both induce DNA synthesis in quiescent primary cells (Spindler *et al.* 1985, Kaczmarek *et al.* 1986), even in low serum (Quinlan and Grodzicker 1987). Binding of E1A to either the pRb or the p300 family proteins is sufficient to induce DNA synthesis (Whyte *et al.* 1988, Whyte *et al.* 1989, Howe *et al.* 1990, Shepherd *et al.* 1993). For E1A to induce mitosis, cell proliferation, or immortalization of primary cells, however, association with both p300 and pRb family proteins is required (Howe and Bayley 1992, Quinlan and Douglas 1992).

E1A can transform primary baby rat kidney cells (BRK) or primary mouse embryo fibroblasts (MEF) in cooperation with another oncogene: the E1B gene or an activated Ras

allele (reviewed in Bayley and Mymryk 1994). E1A alone is able to immortalize cells, but without inducing the morphological changes associated with full transformation (reviewed in Branton *et al.* 1985). E1A induces p53-dependent apoptosis when it is expressed in cells that cannot proliferate and this is dependent on binding pRb and p300 (Mymryk *et al.* 1994). E1B55K or E1B19K can block this p53-dependent apoptosis and cooperate with E1A to transform BRK cells (Debbas and White 1993). Activated H-ras can also rescue cells from E1A-induced apoptosis and cooperate with E1A in transformation (Lin *et al.* 1995).

1.5.3 E1B proteins

As can be seen in Figure 1.5, two major messages are produced from the E1B region (4.6 to 11.2 map units): a 22S mRNA which is abundant in early infection, and a 13S message which accumulates at later times during infection (Glenn and Ricciardi 1988). In addition, two minor species of mRNA of 14S and 14.5S are produced late in infection (Anderson *et al.* 1984, Virtanen and Pettersson 1985, Lewis and Anderson 1987). These four differentially spliced mRNAs all give rise to a 176-residue protein (E1B19K). These four messages can also be translated from a second internal initiation codon in a different reading frame to yield a family of related proteins (Virtanen and Pettersson 1985). The 22S message gives rise to a 496R protein (E1B55K), the 13S message yields a 156R protein (25 kDa doublet), the 14S message produces a protein of 93 amino acids (about 20 kDa), and the 14.5S message gives rise to an 84-residue protein (about 20 kDa). All four of these proteins share 79 common residues at the amino terminus. In addition, the 156R and 496R protein also share 78 carboxy terminal residues. The 93R and 84R proteins have unique carboxy terminal sequences.

1.5.3.1 E1B19K

The 19 kDa E1B protein is modified both by fatty acid acylation and phosphorylation. E1B19K is associated with nuclear and cytoplasmic membranes, and intermediate filaments of the cytoplasm (vimentin) and the nuclear lamina (lamins). Infection of cells with adenovirus mutants lacking E1B19K expression results in the appearance of large plaques, characteristic of cytotoxic effects and the rapid degradation of both viral and cellular DNA (Subramanian *et al.* 1984, White *et al.* 1984, McGlade *et al.* 1987, McGlade *et al.* 1989).

E1B19K has domains which are homologous to the Bcl-2 family proteins (reviewed in Adams and Cory 1998), and it is now clear that the E1B19K protein functions to protect cells against programmed cell death induced by E1A (Rao *et al.* 1992, Teodoro *et al.* 1995). E1B19K can also inhibit apoptosis induced by tumor necrosis factor α (Gooding *et al.* 1991), FAS antigen (Hashimoto *et al.* 1991) and nerve growth factor withdrawal (Martinou *et al.* 1995).

E1A-induced apoptosis involves the activation of Apaf1, procaspase-9 (Soengras *et al.* 1999), and procaspase-8 (Nguyen *et al.* 1998). Bap31 is an integral protein of the endoplasmic reticulum that forms a complex with Bcl-2/Bcl-XL and procaspase-8 (and perhaps other procaspases). E1A induces the cleavage of Bap31 to produce a pro-apoptotic p20 fragment (Ng *et al.* 1997); thus Bap31 may serve a bridging function between pro- and anti-apoptotic proteins.

Bcl-2 can replace E1B19K in blocking E1A-induced apoptosis (Chiou *et al.* 1994), and E1B19K and Bcl-2 can interact with some of the same cellular proteins (Boyd *et al.* 1994). E1B19K can interact with Bax and block its anti-apoptotic function (Han *et al.* 1996a), but it is not affected by the Bcl-2 inhibitor Bad (Chen *et al.* 1996). E1B19K can however interact with the Bcl-2 antagonists Bak (Farrow *et al.* 1995) and Nbk/Bik (Han *et al.* 1996b), and block their proapoptotic functions. E1B19K can block the processing of procaspase-3 and poly(ADP-ribose)polymerase induced by E1A (Boulakia *et al.* 1995). It

was recently shown that E1B19K could bind and inhibit Apaf-1 (CED-4), the activator of caspases (Han *et al.* 1998).

E1B19K and Bcl-2 are both able to relieve transcriptional repression by p53 (Shen and Shenk 1994, Sabbatini *et al.* 1995), providing another mechanism by which they might block p53-dependent apoptosis.

1.5.3.2 E1B55K

The Ad5 E1B 55 kDa protein binds to the p53 tumor suppressor (Sarnow *et al.* 1982a, Kao *et al.* 1990). E1B55K was shown to inhibit p53 transcriptional activity by binding to the N-terminal activation domain of p53 (Yew *et al.* 1990, Yew and Berk 1992). E1B55K binding to p53 is mediated by contacts at residues 22, 23 and 27 in the N-terminus of p53 (Lin *et al.* 1994) and overlaps with the MDM2 binding site. E1B55K is a transcriptional repressor (Yew *et al.* 1994, Teodoro and Branton 1997), and the inhibition of transactivation by p53 is required for E1B55K to have transformation activity with E1A (Yew and Berk 1992). E1B55K blocks apoptosis induced by p53 (Marcellus *et al.* 1996a, Teodoro and Branton 1997). *In vitro* reconstitution experiments have shown that E1B55K is a specific repressor of transcription from a promoter with a bound p53 molecule (Martin and Berk 1998, Martin and Berk 1999).

Most deletion mutants of E1B55K are unstable, so analysis of the functional domains was done primarily by insertion mutagenesis (Yew *et al.* 1990). The domains of E1B55K involved in p53 binding have been mapped to the central portion of the protein (aa 170-190 and aa 240-340) (Kao *et al.* 1990). The C-terminus of E1B55K is phosphorylated at Ser-490, Ser-491 and Thr-495, possibly by casein kinase II (Teodoro *et al.* 1994, Teodoro and Branton 1997). Double or triple point mutations at these sites do not affect E1B55K complex formation with p53, but E1B55K is no longer able to effectively repress p53

transactivation activity. Perhaps phosphorylation regulates the association of E1B55K with histone deacetylases.

The E1B55K protein forms a complex in virally infected cells with the E4orf6-34 kDa protein, and the regions necessary for the interaction with E4orf6 include residues 130-150 and 240-340 (Sarnow *et al.* 1984, Rubenwolf *et al.* 1997). When expressed alone, E1B55K is predominantly localized in the cytoplasm, but coexpression of E4orf6 directs the nuclear localization of E1B55K (Goodrum *et al.* 1996). This nuclear colocalization of E4orf6/E1B55K seems to require a primate-specific factor, since it does not occur in most rodent cells but is observed in monkey and human cell lines (Goodrum *et al.* 1996). In E1A/E1B-transformed cells, Ad5 E1B55K sequesters p53 into dense cytoplasmic bodies while Ad12 E1B55K does not (Zantema *et al.* 1985a, van der Eb 1993). Ad12 E1B55K is nuclear on its own and form complexes with p53 that are difficult to immunoprecipitate (van den Heuvel *et al.* 1990). Ad12 E1B55K is more oncogenic than Ad5 E1B55K in transformation assays (van den Heuvel *et al.* 1990) and this may be due to its ability to induce genomic instability (Li *et al.* 1998). It is possible that association with p53 enables Ad5 E1B55K to enter the nucleus and travel with p53 to the transcription sites where it acts as a specific repressor (Martin and Berk 1998, Martin and Berk 1999).

The E1B55K/E4orf6 complex regulates the stability and transport of viral mRNA and inhibits the transport of cellular mRNA during adenovirus replication (Babiss and Ginsberg 1984, Halbert *et al.* 1985, Pilder *et al.* 1986, Leppard and Shenk 1989). E4orf6 has a nuclear export signal (NES) and can mediate nuclear-cytoplasmic shuttling of the E1B55K/E4orf6 complex which may be required for viral replication (Dobbelstein *et al.* 1997, Weigel and Dobbelstein 2000). Kratzer *et al.* recently showed that E1B55K also has a NES at residues 83 to 93 and may also have a nuclear localization signal in the C-terminal residues 364 to 496 (Kratzer *et al.* 2000). The E1B55K protein can shuttle efficiently in the absence of E4orf6 and p53. This would then suggest that when E1B55K is expressed

alone, its NES directs complete nuclear export of the protein, but when E1B55K is in a complex with E4orf6, it may be retained in the nucleus.

The E4orf3-11 kDa protein also forms complexes with E1B55K in infected cells (Leppard and Everett 1999). E4orf3 localizes E1B55K to the nuclear matrix and the interaction with E4orf6 and E4orf3 seems to be mutually exclusive (Konig *et al.* 1999, Leppard and Everett 1999). E4orf3 complex formation with E1B55K is also sufficient to relocalize E1B55K to the nucleus. The E1B55K/E4orf3 complex prevents E1B55K from repressing p53-dependent transactivation and p53 becomes properly nuclear (Konig *et al.* 1999). E4orf3 is expressed a little earlier than E4orf6 during infection (Konig *et al.* 1999). As will be discussed in chapters III, IV, V, and VI of this thesis, E1B55K/E4orf6 complexes target p53 for degradation by the ubiquitin-proteasome pathway. E1B55K/E4orf3 complexes do not.

E1B55K has intrinsic RNA-binding activity (Horridge and Leppard 1998) and can disrupt mRNA export in yeast (Liang *et al.* 1995). Recently a novel cellular protein has been identified that binds specifically to E1B55K *in vitro* and *in vivo* (Gabler *et al.* 1998). This E1B55K-associated protein (E1B-AP5) is a novel member of the hnRNP protein family that is highly related to hnRNP-U/SAF-A (see Fig. 1.6). The E1B55K/E1B-AP5 protein interaction is mediated by two segments of the 55K protein which partly overlap regions responsible for p53 binding. The E4orf6 protein may interfere with the E1B55K/E1B-AP5 interaction, because substantially more E1B55K can bind to E1B-AP5 in the absence of E4orf6. E1B-AP5 is able to partially block E1A/E1B-mediated transformation, and stable overexpression of E1B-AP5 overcomes the host cell shutoff effect in Ad5 infected cells (Gabler *et al.* 1998). Host cell shutoff will be discussed in more detail in section 1.5.8.

An exciting new cancer therapy has recently been developed by ONYX Pharmaceuticals, that uses an E1B55K deficient adenovirus (dl1520). They were able to show that such a virus will preferentially replicate in p53 *-/-* tumor cells and will cause significant reduction and regression of tumors in animal models (Bischoff *et al.* 1996). This

virus has shown some promise in clinical trials in the treatment of solid tumors of the head and neck . However, Goodrum *et al.* have shown that the selective replication of the E1B55K-deleted virus in tumor cells was not dependent on p53 status, but rather reflected a cell cycle restriction for replication. Indeed, dl1520 (ONYX-015) is able to replicate efficiently only when cells are infected in G1 or S phase (Goodrum and Ornelles 1997, Goodrum and Ornelles 1998). E4orf6 and E4orf3 may also play a role in cell cycle-independent growth (Goodrum and Ornelles 1999). Thus, ONYX-015 works (it replicates in and kills tumor cells and not surrounding tissues), because tumor cells are rapidly cycling and not because of their p53 status.

1.5.4 E2 proteins

The E2 region produces two differentially spliced transcripts from two regions of the I strand of the genome; E2A (67.9 to 61.5 map units) and E2B (29 to 14.2 map units). They share the E2 promoter with the same leader sequence, but they use different polyadenylation signals. The adenovirus E4orf6/7 gene, in addition to the E1A gene, is important for trans-activation of E2 transcription and for E2F activation (Reichel *et al.* 1989). The E2A and E2B transcripts code for proteins directly involved in DNA replication (section 1.5.7). E2A codes for the 72 kDa DNA binding protein that is required for elongation during viral DNA replication, possibly through its ability to unwind DNA. The E2B transcript codes for an 80 kDa protein that is cleaved to yield the 55kDa terminal protein (TP). The E2B transcript also produces the 140 kDa DNA-dependent DNA polymerase of adenovirus.

1.5.5 E3 proteins

The E3 transcription unit, transcribed from the r strand of the genome (76.6 to 86.2 map units), is a cassette of genes that has evolved to counteract the host immune response to adenovirus infection (reviewed in Wold and Gooding 1991, Wold *et al.* 1994).

Two of the E3-encoded polypeptides, the receptor internalization and degradation (RID) α (E3-10.4K) and RID β (E3-14.5K) proteins, are integral membrane proteins that function together as a heterodimer (Stewart *et al.* 1995). The RID complex functions to: (1) downregulate the epidermal growth factor receptor (EGFR) from the cell-surface, (2) inhibit tumor necrosis factor α (TNF α)-induced apoptosis, (3) inhibit TNF-induced translocation of cytosolic phospholipase A2 (cPLA2) from cytosol to membranes, and (4) downregulate cell surface Fas and inhibit Fas agonist-induced apoptosis. RID targets selected cell surface receptors for degradation in lysosomes inside the cell. It has recently been shown that RID also mediates the internalization of cell-surface Fas and its destruction inside lysosomes (Tollefson *et al.* 1998).

The E3-14.7K protein also has anti-tumor necrosis (TNF) factor activity (Tollefson and Wold 1988, Ginsberg *et al.* 1989). E3-14.7K is located in the nucleus and the cytoplasm, and it prevents TNF- α cytolysis, probably by preventing synthesis of leukotrienes and prostaglandins by cPLA₂ (reviewed in (Wold *et al.* 1994)).

The E3-gp19K glycoprotein is the most abundant E3 protein, and it inhibits lysis by cytotoxic T cells. E3-19K interacts with the class I histocompatibility (MHC) antigens in the rough endoplasmic reticulum, thereby preventing transport of MHC molecules to the cell surface and, consequently, MHC-restricted T cell recognition (Wold and Gooding 1991). The E3-6.7K protein is a type III integral membrane protein (Wilson-Rawls *et al.* 1990), that can direct E3-gp19K into the membrane of the endoplasmic reticulum (Wilson-Rawls *et al.* 1994).

The E3-11.6K protein is also known as the adenovirus death protein (ADP) (Wold *et al.* 1984, Ginsberg *et al.* 1989). ADP/E3-11.6K is synthesized from the E3 promoter at low

levels early in infection but, at very late stages of infection, it is made in large amounts from the major late promoter (MLP). ADP is located at the nuclear membrane and it may function to destabilise the nuclear membrane and allow release of the progeny virus. Adenovirus deleted for the ADP gene displayed a small plaque phenotype, and accumulated in swollen nuclei (Tollefson *et al.* 1992, Tollefson *et al.* 1996).

The other E3 protein is a 12.5 kDa protein (Hawkins and Wold 1992).

1.5.6 E4 proteins

The E4 transcription unit lies between map units 91.3 and 99.3 of the Ad5 genome and is transcribed from the I strand (see Fig 3.4). At least nine messages are produced, which share initiation and polyadenylation sites. Alternative splicing produces 7 open reading frames (orf), with the corresponding proteins identified as orf1, orf2, orf3, orf3/4, orf4, orf6 and orf6/7 (Rigolet and Galibert 1984). Figure 3.4 contains a schematic representation of the E4 open reading frames as well as the E4 deletion mutants used in chapter III. The products from the E4orf2 and E4orf3/4 open reading frames have not been characterized. E4orf1, E4orf3, E4orf6 and E4orf6/7 are oncoproteins, while E4orf4 encodes a tumor suppressor.

1.5.6.1 E4orf1

E4orf1 is a 14 kDa cytoplasmic protein that has weak sequence similarity with dUTP pyrophosphatase enzymes (Weiss *et al.* 1997b). Ad9 E4orf1 promotes focus formation of the rat embryo fibroblast cell line CREF (Weiss *et al.* 1996) while the Ad5 and Ad12 E4orf1 do not (Javier 1994). Adenovirus type 9 induces exclusively estrogen-dependent mammary tumors when injected into rats, and the essential oncogene responsible is E4orf1 (Javier *et al.* 1991, Javier *et al.* 1992). Mutational analysis of Ad9 E4orf1 has revealed three regions necessary for cellular transformation of CREF cells (Weiss *et al.* 1997a), and several

cellular phosphoproteins of 220, 180, 160 and 155 kDa associate with a C-terminal region required for transformation (Weiss and Javier 1997).

The Ad9 E1A and E1B proteins are closely related to those of Ad5 and Ad12. It was recently shown that an Ad9 E1 region plasmid could promote focus-formation in rat embryo fibroblasts, like Ad5 and Ad12 E1 plasmids (Thomas *et al.* 1999a). Deletions in either the Ad9 E1A or E1B gene impaired the transformation activity of the plasmid. However, when similar E1 deletions were introduced into the Ad9 virus, Ad9 retained the ability to induce mammary tumors in rats. This elegant experiment showed that adenovirus serotype 9 is unique in that the E4 region rather than the E1 region encodes the major oncogenes in the rat (Thomas *et al.* 1999a).

1.5.6.2 E4orf3 and E4orf6

The E4orf3 11 kDa protein and the E4orf6 34 kDa protein are both required for efficient expression from the major late transcription unit (Nordqvist *et al.* 1990, Ohman *et al.* 1993, Nordqvist *et al.* 1994). E4orf3 and E4orf6 stimulate constitutive splicing from the MLP. E4orf3 facilitates i-leader exon inclusion (see Fig. 1.4), while E4orf6 favors i-leader exon skipping (Nordqvist *et al.* 1994). In cells infected by E4 mutants lacking both E4orf3 and E4orf6 a severe defect in late viral protein synthesis is observed (Bridge and Ketner 1989).

The deletion of the E4 region results in the production of adenovirus DNA concatemers during replication (Weiden and Ginsberg 1994). It was recently shown that E4orf3 and E4orf6 are both responsible for suppressing concatemer formation. E4orf3 and E4orf6 can interact with DNA-PK and function to inhibit DNA-PK-dependent double strand break repair (Boyer *et al.* 1999).

E4orf3 is able to cooperate with E1A, or with E1A/E1B, in the transformation of primary baby rat kidney (BRK) cells, by inducing morphological alterations and enhanced growth rates which increase the tumorigenicity of the BRK cells in nude mice (Nevels *et al.*

1998). E4orf3 expression induces the reorganisation of PML oncogenic domains (PODs) in the nucleus. E1B55K and E4orf3 have been shown to associate with the POD in infected cells, which in turn undergoes a dramatic morphological change (Doucas *et al.* 1996). E4orf3 protein colocalizes with the putative tumor suppressor protein PML in novel structures resembling tracks in transformed BRK cells (Nevels *et al.* 1998), and also in transient transfection assays (Doucas *et al.* 1996).

Wild-type PML (promyelocytic leukemia protein), and at least four other proteins, are normally localized within discrete nuclear structures known as PODs. The POD is a macromolecular multiprotein complex that is present in all cultured cell lines examined and appears to be widely expressed in the body. PODs can vary in number from 5 to 30 per nucleus depending on cell type. They can also vary substantially in size after interferon treatment or heat shock (reviewed in Melnick and Licht 1999). Adenovirus DNA replication and late RNA transcription occurs in viral inclusion bodies containing E4orf3, E4orf6, E1B55K and the displaced PML and Sp100 POD proteins. E4orf3 alone will induce POD reorganization and reciprocally, viruses carrying mutations in the E4 domain fail to do so. Cell lines engineered to overexpress PML protect PODs from viral-induced reorganization and block or severely delay adenovirus replication (Doucas *et al.* 1996)

The E4orf6/E1B55K complex (Sarnow *et al.* 1984) is required for host cell shutoff, stability, and transport of viral mRNA (Babiss and Ginsberg 1984, Halbert *et al.* 1985). A complete description of host cell shutoff and viral mRNA transport can be found in section 1.5.8.

In the presence of E4orf3, p53 is dissociated from E1B55K and distributed over the nucleoplasm. Under these conditions, p53-dependent transcriptional activity is no longer repressed by E1B55K (Konig *et al.* 1999). It has been proposed that E4orf3 temporarily liberates p53 from its association with E1B55K, possibly to facilitate a supportive role of p53 during virus DNA replication, and that the subsequent expression of E4orf6 induces the degradation of p53. As will be shown and discussed in great detail in chapters III, IV, V,

and VI of this thesis, E1B55K/E4orf6 complexes target p53 for degradation by the ubiquitin-proteasome pathway (Querido *et al.* 1997a, Steegenga *et al.* 1998, Nevels *et al.* 1999, Nevels *et al.* 2000, Querido *et al.* 2000b, Querido *et al.* 2000c).

E4orf6 has oncogenic activity and can promote transformation together with E1A/E1B proteins (Moore *et al.* 1996, Nevels *et al.* 1997, Nevels *et al.* 1998, Nevels *et al.* 1999, Nevels *et al.* 2000). In addition, E4orf3 can synergise with E4orf6 to enhance E1A plus E1B-induced focus formation in BRK cells (Nevels *et al.* 1998). These "super-transformed" E1 cells grow faster, form bigger and more colonies in focus assays, and have increased capacity to form tumors in nude mice.

In 1996, E4orf6 was shown to be able to bind to the p53 tumor suppressor directly, in the absence of E1B55K (Dobner *et al.* 1996). E4orf6 interacts with the C-terminal residues 318-360 of p53, right in the tetramerization domain, and this interaction displaces TAFII31 from p53. E4orf6 was shown to repress transcriptional activation by p53 (Dobner *et al.* 1996, Nevels *et al.* 1997, Querido *et al.* in preparation); however, some other groups have found that E4orf6 cannot repress p53 transactivation but may repress transactivation by the p53 family member p73 (Higashino *et al.* 1998, Roth *et al.* 1998b, Steegenga *et al.* 1999, Wienzek *et al.* 2000). The reason for these conflicting results is not clear. In some cases different cell lines were consistently used to perform the reporter assays, and there may be some cell line-specific effects.

Figure 4.1 includes a schematic representation of the E4orf6 protein. The N-terminus of E4orf6 was shown to mediate the interaction with E1B55K (Rubenwolf *et al.* 1997, Querido *et al.* 2000b), but an amphipathic arginine-faced alpha helical region at the C-terminus, termed nuclear retention signal (NRS), mediates the colocalization of E1B55K with E4orf6 in the nucleus (Orlando and Ornelles 1999). The E4orf6 nuclear export signal (NES) is located at residues 83-94 (Dobbelstein *et al.* 1997) and is required for late protein expression and viral replication (Weigel and Dobbelstein 2000). E4orf6 is a very stable protein with a half-life of over 24 hours in infected cells (Boivin *et al.* 1999), and high levels

are maintained even at late stages of infection. E4orf6 was recently shown to be a zinc metalloprotein (Boyer and Ketner 2000). Conserved histidine and cysteine residues are important for the interaction with E1B55K, for late protein synthesis, and for the ability to cooperate with E1B55K in p53 degradation (Boyer and Ketner 2000). Nevels *et al.* also recently showed that p53 destabilization mapped to multiple region within E4orf6 and was correlated to the ability to support increased growth in E1A/E1B/E4orf6-transformed BRK cells (Nevels *et al.* 2000). The ability of E4orf6 to promote focus formation and morphological transformation, however, required only a C-terminal segment of E4orf6, implying that p53 degradation was not important for these activities in BRK cells (Nevels *et al.* 2000). The domains of E4orf6 mediating the interaction with p53 have not yet been identified.

1.5.6.5 E4orf6/7

E4orf6/7 is a 17 kDA protein that shares the first 58 N-terminal residues with E4orf6 (see Fig. 3.4). E4orf6/7 can bind to E2F family members 1-5 through a conserved domain termed the marked box (Jost *et al.* 1996). The C-terminal 70 residues of E4orf6/7 mediate the interaction with E2F (Cress and Nevins 1994). E4orf6/7 interacts with a heterodimer of the E2F-1 and DP-1 transcription factors (Helin and Harlow 1994) and this stable complex activates E2F-1-dependent transcription of the viral E2 (Obert *et al.* 1994) or the cellular E2F1 promoters (Schaley *et al.* 2000). Phosphorylation of E2F-1 on serine residues 332 and 337 prevents its interaction with pRb but is a prerequisite for interaction with E4orf6/7 (Fagan *et al.* 1994). Phosphorylation at those sites may be mediated by the p34cdc2 kinase. The interaction of pRb with the E2F-1/DP-1 heterodimer would prevent E4orf6/7 binding (O'Connor and Hearing 1994); thus E1A also contributes to E2 transactivation by disrupting E2F-1/pRb complexes.

Similar to E2F-1 overexpression, E4orf6/7 expression in cells was shown to be able to promote both transformation and p53-dependent apoptosis, depending on experimental design (Yamano *et al.* 1999).

E4orf6/7 is much less stable than E4orf6 in infected cells (Boivin *et al.* 1999). It was recently shown that, when E4orf6 is expressed from a cDNA instead of the E4 gene, the splice donor which normally creates E4orf6/7 will splice aberrantly into the C-terminus of E4orf6, creating a novel 8 kDa product containing the N-terminal 58 aa shared by E4orf6/7 and E4orf6 (Querido *et al.* 2000a, and see figure 7 in the Appendix). This product is not observed in wt E4-adenovirus infected cells.

1.5.6.3 E4orf4

The E4orf4 mRNA encodes a 14 kDa nuclear protein. E4orf4 has several roles during infection and current knowledge indicates that all its functions may be mediated through interaction with the cellular protein phosphatase 2A (PP2A). PP2A consists of a catalytic C subunit and a regulatory A subunit, which form the core enzyme, to which one of several B subunits can bind. The B subunits fall into four families called B, B', B'' and the recently cloned B''', which are unrelated by protein sequence. The B subunits seem to confer specificity to the PP2A phosphatase. E4orf4 binds to the B α subunit of PP2A (Kleinberger and Shenk 1993). PP2A may be the principal regulator of protein kinase cascades in signal transduction pathways (reviewed in Schontal 1998, Millward *et al.* 1999). PP2A is on the Src pathway with downstream effectors SHC, PI3K and PLC γ , and can inactivate the MAP kinase (MAPK) and its regulator MAPKK (Anderson *et al.* 1990, Gomez and Cohen 1991). Cyclin G1 has been shown to interact with the B' α and β subunits of PP2A in a p53-dependent manner (Okamoto *et al.* 1996), and this may be implicated in the role of PP2A in mitosis and cell cycle progression. PP2A may have also a role in the initiation of DNA replication, and PP2A targets include phospholipase A and Histone H1 (reviewed in Millward *et al.* 1999).

During infection, E4orf4 regulates the phosphorylation of E1A and c-Fos proteins (Muller *et al.* 1992). One of the main functions of the E4orf4-driven dephosphorylation of E1A is to inhibit E1A's ability to transactivate the E4 promoter (Bondesson *et al.* 1996). Thus E4orf4 regulates its own expression. Upon cAMP treatment of adenovirus-infected cells, E1A and c-Fos accumulate in hypophosphorylated forms, and this is dependent on the presence of E4orf4. Phosphorylation at Ser-185 and Ser-188 in the CR3 transactivating domain of E1A is decreased in response to cAMP (Whalen *et al.* 1997). These are believed to be MAPK sites (Whalen *et al.* 1997), and thus E4orf4 may direct PP2A to inactivate MAPK and thus inhibit phosphorylation at Ser-185 and Ser-188. E4orf4 also blocks the increase in AP-1 DNA binding activity due to E1A-enhanced accumulation of JunB and c-Fos (which then associate to form the AP-1 transcription factor). The dephosphorylation of a target molecule is probably responsible for the down regulation of AP-1 by E4orf4. For all these reasons, mutant viruses with an E4orf4 deletion exhibit increased AP-1 activity and are much more toxic to the cell (Muller *et al.* 1992). E4orf4 also inhibits E1A activation of the E2 promoter. The inhibitory effect of E4orf4 was relieved by treatment with a PP2A phosphatase inhibitor, and apparently results from dephosphorylation of transcription factors and reduced stability of E2F/DNA complexes (Mannervik *et al.* 1999). E4orf4 did not affect E4orf6/7 transactivation of the E2 promoter.

E4orf4 also activates the dephosphorylation of SR proteins by PP2A, thus inactivating them (Kanopka *et al.* 1998). SR proteins are a family of essential splicing factors required for early recognition of splice sites during spliceosome assembly. The E4orf4-PP2A complex might thus play a role in regulating adenovirus RNA splicing.

It was recently discovered that E4orf4 induces p53-independent apoptosis characterized by chromatin condensation, DNA degradation and cell death (Marcellus *et al.* 1996b, Marcellus *et al.* 1998). E4orf4-induced apoptosis may operate by a novel mechanism that is not blocked by caspase inhibitors (Lavoie *et al.* 1998). E4orf4 induces apoptosis only in primary cells that have been transformed with oncogenes such as E1A and Ras

(Shtrichman and Kleinberger 1998, Shtrichman *et al.* 1999). The induction of apoptosis by E4orf4 seems to correlate with PP2A binding, though the function of this is not yet clearly understood. E4orf4 protein doesn't alter the localization of the bulk of the phosphatase (Kleinberger and Shenk 1993), but it may relocalize specific subpopulations of PP2A. E4orf4 may thus act to target PP2A to new substrates to induce apoptosis, and the search for these substrates may provide targets for cancer therapy.

Other DNA tumor viruses encode proteins that bind PP2A. Polyoma virus small t and middle T antigens and SV40 small t also form stable complexes with PP2A (Pallas *et al.* 1990).

1.5.7 Replication of Viral DNA

Replication of Ad5 DNA begins about 5 hours after infection and it continues until the cell dies. The inverted terminal repeats of the viral chromosome function as replication origins. Synthesis is first initiated at either terminus of the linear DNA and proceeds in a continuous manner to the other end of the genome, thus no Okasaki fragments are generated. The displaced single strand circularizes through annealing of its self-complementary termini, and the resulting "panhandle" has the same structure as the termini of the duplex viral genome. This structure allows it to be recognized by the same initiation machinery that replicates the double-stranded linear genome in the first step (reviewed in Shenk 1996).

The E2-coded terminal protein is synthesized as an 80 kDa polypeptide (the preterminal protein or pTP) that is covalently attached to the 5' ends of the viral chromosome and is active in the initiation of DNA replication (Challberg *et al.* 1980, Stillman *et al.* 1981). During assembly of the virion, it is processed by proteolysis to generate a 55 kDa fragment (the terminal protein or TP) that is covalently attached to the genome. The E2 viral polymerase is a 140 kDa protein with distinct biochemical properties. It has both 5' to 3' polymerase activity and 3' to 5' exonuclease activity that serves a proofreading function

(Field *et al.* 1984). The preterminal protein and the polymerase form a complex that can bind in a sequence specific fashion to the replication origin (Temperley and Hay 1992). The preterminal protein serves as a primer for DNA replication, beginning with the formation of an ester bond between the β -OH of a serine residue in the pTP and the α -phosphoryl group of dCMP, the first residue at the 5' end of the DNA chain (Lichy *et al.* 1981). The 3'-OH group of the preterminal protein-dCMP complex then serves to prime synthesis of the nascent strand by the DNA polymerase. The E2-coded 72 kDa DNA-binding protein binds tightly and cooperatively in a sequence-independent fashion to single-stranded DNA. The viral polymerase is highly processive only in the presence of the DNA-binding protein which coats single-stranded replication intermediates, and this is probably the basis for its requirement in chain elongation (Lindenbaum *et al.* 1986).

The adenovirus E4 gene also encodes one or more products required for efficient DNA replication but they probably have an indirect role (Bridge *et al.* 1993).

1.5.8 Shutoff of host protein synthesis

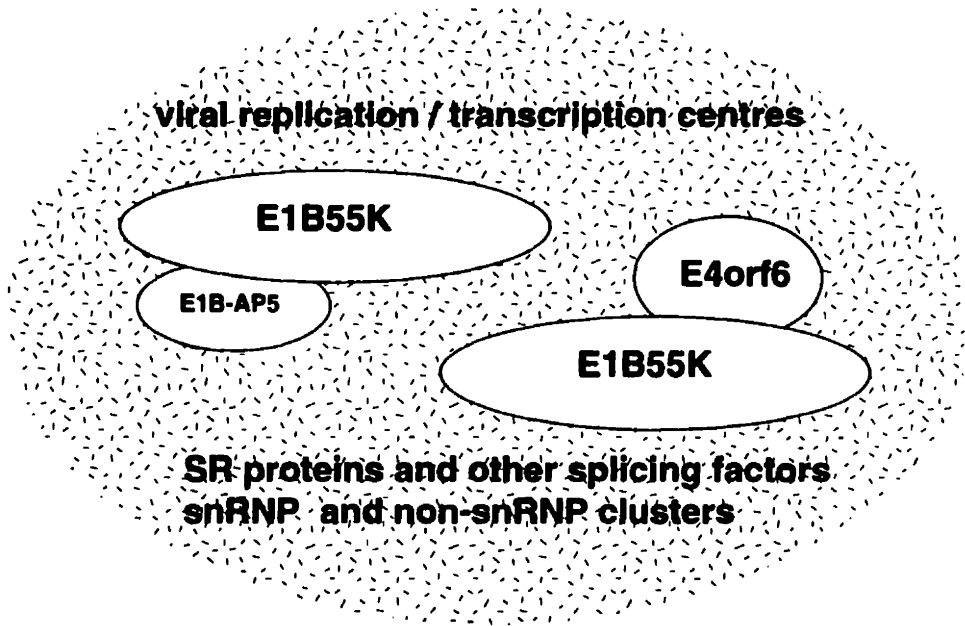
Host cell shutoff begins only after adenovirus has entered the late phase of infection. As illustrated in Fig 1.6, shutoff happens at 2 levels: in the nucleus, transcription of cellular genes is not substantially altered, but newly synthesized mRNAs fail to be transported to the cytoplasm. In the cytoplasm, steady state levels of preexisting mRNAs are little changed, but they are not actively translated (Beltz and Flint 1979). Synthesis and processing of cellular transcripts continue, but the mRNAs fail to accumulate in the cytoplasm, suggesting that their transport is blocked (Leppard and Shenk 1989).

The adenovirus proteins E1B55K (Babiss and Ginsberg 1984, Pilder *et al.* 1986) and E4orf6 (Halbert *et al.* 1985, Weinberg and Ketner 1986) mediate the block to cellular

HOST CELL SHUTOFF BY ADENOVIRUS

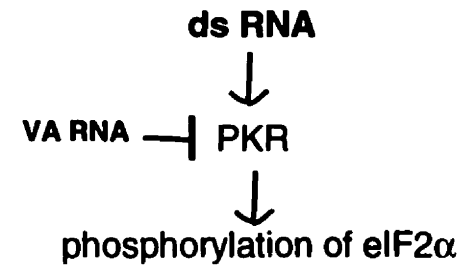
nucleus

cytoplasm



shutoff requires
translation of
Ad late mRNA

eIF4E is dephosphorylated



phosphorylation of 4E-BP1 and 2

Figure 1.6 Host cell shutoff by adenovirus

The shutoff of host cell translation caused by adenovirus occurs at two levels: new cellular mRNAs are not transported to the cytoplasm, and cellular mRNAs already there are not actively translated. The E1B55K/E4orf6 complex complex is depicted in the nucleus at the sites of viral replication and transcription. SR proteins and other splicing factors are sequestered into these nuclear domains in infected cells. The newly discovered E1B55K associated protein E1B-AP5, a novel hnRNP protein, is also shown. A late viral protein or at least translation of late RNA, is required for host cell shutoff in addition to E1B55K/E4orf6. Adenovirus mRNA translation occurs in regions rich in the VA RNA I adenovirus product, which inactivates PKR in those regions. The eIF-4E binding proteins 1 and 2 (4E-BP1 and 2) are negative regulators of translation. Their phosphorylation inactivates them, and is an early event attributed to E1A, probably to allow maximal translation of viral products.

mRNA accumulation. The E1B55K/E4orf6 complex is involved in the splicing of early viral mRNA and in the transport and stability of mature viral mRNA with unused splice donor/acceptor sites (Bridge and Ketner 1989, Ohman *et al.* 1993, Nordqvist *et al.* 1994). Cellular mRNAs are blocked immediately prior to movement from the nuclear compartment to the cytoplasm (Leppard and Shenk 1989). The discrimination between host and viral mRNAs is not based on the identity of individual mRNAs. Cellular genes expressed from recombinant viral chromosomes accumulate in the cytoplasm late after infection as if they were viral mRNAs. Immunoelectron microscopy revealed that the E1B55K/E4orf6 complex is localized within and surrounding intranuclear inclusions believed to represent viral transcription and replication centers (Ornelles and Shenk 1991). These observations suggest a model in which the E1B55K/E4orf6 complex relocates a cellular factor required for the transport of mRNAs from their site of synthesis to the nuclear pore. This proposal can also explain the inhibition of host mRNA transport since a transport factor would be moved from the many sites of host transcription and processing to the viral centers. A recently cloned E1B55K associated protein (E1B-AP5) may be such a factor (Gabler *et al.* 1998). E1B-AP5 encodes a novel nuclear RNA-binding protein of the hnRNP family that is highly related to hnRNP-U/SAF-A. Stable overexpression of E1B-AP5 in cells protects them from adenovirus host cell shutoff. E1B55K also exhibits endogenous RNA binding activity (Horridge and Leppard 1998), and when expressed in yeast it disrupts mRNA export (Liang *et al.* 1995).

In addition to their facilitated transport from the nucleus, viral mRNAs are preferentially translated when they reach the cytoplasm late after infection (reviewed in (Zhang and Schneider 1993)) to the exclusion of host mRNAs (Babich *et al.* 1983). Host mRNAs are not degraded, and the translational block is not dependent on the inhibition of host cell mRNA accumulation in the cytoplasm, since some host mRNAs such as β -tubulin continue to accumulate in the cytoplasm, yet they are not translated (Moore *et al.* 1987). The critical event for shutoff of translation by adenovirus is the dephosphorylation of the cap

binding subunit eIF-4E (Huang and Schneider 1991) which occurs at 16 hours post infection when shutoff starts. Adenovirus works by inhibiting the phosphorylation of eIF-4E rather than enhancing its dephosphorylation (Zhang *et al.* 1994), and the L4-100K nonstructural protein (Hayes *et al.* 1990) was recently shown to be responsible for inhibiting eIF-4E phosphorylation. L4-100K binds to eIF-4G and displaces the eIF-4E kinase Mnk1 thus preventing it from phosphorylating 4E (Cuesta *et al.* 2000). The 100 kDa polypeptide is a phosphoprotein present in large quantities late after infection, its mRNA being one of the most abundant viral messages in the infected cell at that time. Mutant virus expressing defective L4-100K protein fails to efficiently translate its late mRNAs (Hayes *et al.* 1990).

The major late promoter tripartite leader sequence confers cap-independent translation to late viral mRNAs (Dolph *et al.* 1988). Ad late mRNAs are capped and initiate ribosome binding through a cap-dependent process. However, the adenovirus tripartite leader sequence directs a novel form of translation initiation described as ribosome jumping or shunting (Yueh and Schneider 1996). The hypophosphorylation of eIF-4E thus probably contributes to this selective translation of viral messages. The eIF-4E inhibitory proteins 4E-BP1 and 4E-BP2 are also inactivated following adenovirus infection (Gingras and Sonenberg 1997), but this is an early event probably mediated by E1A in order to enhance general translation.

The cellular protein kinase R (PKR) is activated by double-stranded RNA that accumulates within adenovirus-infected cells (O'Malley *et al.* 1986, Maran and Mathews 1988). PKR can phosphorylate eIF-2 α , inactivating the initiation factor and blocking translation. Translation of host cell mRNAs is not inhibited after infection of cells deficient in PKR activity (O'Malley *et al.* 1989), suggesting that PKR, and presumably inactivation of eIF-2 α , are key components of the block to host cell translation. The adenovirus-coded VA RNAs inhibit the activation of PKR. These small RNAs have been shown to copurify with viral mRNAs (Mathews 1980) and, as a result, they might protect viral but not cellular protein synthesis, providing a functional compartmentalization. VA RNA I and VA RNA II

are each about 160 nucleotides in length, they are G-C rich, they adopt stable secondary structures that is important for their function, and they are transcribed by RNA polymerase III. VA RNA synthesis begins during the early phase of the infectious cycle and dramatically accelerates during the late phase. Analysis of a mutant Ad5 virus in which the VA RNA I gene was deleted revealed that the virus grew poorly (Thimmappaya *et al.* 1982).

1.5.9 Expression of late proteins and assembly of viral particles

After the onset of viral DNA replication, transcription from the Major Late Promoter (MLP) is unattenuated and fully activated, and early gene expression is repressed. The mechanism underlying the DNA replication-coupled early-to-late switch is not fully understood (Fessler and Young 1998). A 28 kb mRNA is transcribed from the MLP, and it is spliced 20 different ways. The structural proteins are numbered from II to IX in order of highest to lowest molecular weight. Polypeptides V and VII, are associated with the core. Protein IIIa is a minor component of the virion. Polypeptides VI, VIII, and IX are also minor capsid components. The replication of viral DNA and the preferential translation of viral mRNA into large quantities of structural polypeptides signal the beginning of virus assembly. Trimeric hexon capsomeres are rapidly assembled from monomers after their synthesis in the cytoplasm. Penton capsomeres, consisting of a pentameric penton base and trimeric fiber, assemble somewhat more slowly in the cytoplasm (Horwitz *et al.* 1969). Assembly of the hexon requires the participation of L4-100K proteins that act as a scaffold to facilitate assembly of trimers (Oosterom-Dragon and Ginsberg 1981). After their production, hexon and penton capsomeres accumulate in the nucleus where assembly of the virion occurs. Assembly is inefficient; only 20% of the hexon trimers are incorporated into virions and only 10% of viral DNA enters virus particles. The excess pool of capsid components and viral genomes are thus enormous because around 100,000 infectious virions are produced per HeLa cell.

The first step in viral morphogenesis is the association of viral proteins (some in precursor form) with scaffolding proteins, to form the empty capsid. The viral DNA and core proteins are then inserted into the capsid. Adenovirus type 5 DNA packaging is initiated from the left end of the viral genome and depends on the presence of a cis-acting packaging domain located between nucleotides 194 and 380. Two cellular binding activities have been described which interact in a specific manner with Ad5 packaging elements; COUP-TF and the P-complex (Schmid and Hearing 1998). Physical association between the components bound to inverted terminal repeats (ITR) and packaging sequences may result in the formation of a nucleoprotein complex within the viral left end, marking the respective molecule as a bona fide packaging substrate. Subsequent capsid recognition may involve recognition of this complex; and the viral genome is inserted into the preformed capsid.

The L1-coded 52/55 kDa protein is present in many copies in early assembly intermediates, which gradually lose the 52/55 kDa proteins as they mature into virions (Hasson *et al.* 1989, Hasson *et al.* 1992). The 52/55 kDa protein is essential for association between the viral genome and the empty capsid or in the encapsidation process itself (Gustin and Imperiale 1998). The final step of maturation involves proteolytic cleavage of precursor polypeptides. The L3-coded 23 kDa proteinase cleaves at least four virion constituents, generating the mature VI, VII, VIII, and terminal proteins. The 80K precursor terminal protein is cleaved into the 55K species found in infective virions. These cleavage events stabilize the structure of the particle and render it infectious (reviewed in Shenk 1996).

The Ad5 gene IX is an intermediate gene encoding a small polypeptide (pIX) of 140 residues that participates to the assembly of the viral capsid and is located at the outer surface of mature virions (see Fig. 1.5). pIX, in addition to its structural contributions, exhibits transcriptional activation properties (Ghosh *et al.* 1987, Furcinitti *et al.* 1989). pIX stimulates the activity of the major late promoter (MLP) (Lutz *et al.* 1997).

Adenovirus induces the disruption of the intermediate filament system that helps maintain the mechanical integrity of cells, making the infected cell more susceptible to lysis

and release of progeny virus. Vimentin is cleaved very rapidly after infection, in response to E1B19K (White and Cipriani 1989, White and Cipriani 1990) and also by a reaction initiated by the absorption process that does not require viral gene expression (Belin and Boulanger 1987). Late in the infectious cycle, the viral proteinase cleaves the cellular cytokeratin K18 (Chen *et al.* 1993). Adenoviruses may also facilitate the transmission of infection from cell to cell by inducing apoptosis with the E4orf4 product (Marcellus *et al.* 1998). The virions contained in the fragmented nucleus and apoptotic bodies could then be absorbed into the surrounding cells without inflammation. Additionally, expression of the E3-11.6K protein or adenovirus death protein, in the late phase of infection, seems to mediate efficient cell lysis and release of the virus (Tollefson *et al.* 1996).

1.6 Adenovirus vectors: gene therapy and research

Human adenoviruses have attracted considerable attention because of their potential utility for gene transfer and gene therapy. The capacity of this virus to infect cells and deliver a foreign gene even in the absence of viral and cellular replication makes it attractive to deliver a transgene to quiescent cells. Bett *et al.* (1993) tested the packaging capacity of human adenovirus type 5 vectors. Serial passages in tissue culture revealed that vectors with inserts resulting in viral DNA with a net genome size of close to or less than 105% of the *wt* genome grew well and were relatively stable, but if the insert was bigger the vectors grew poorly and were subject to rearrangements. These findings suggest that there is a relatively tight constraint on the amount of DNA which can be packaged into virions. To overcome this limitation, several groups have developed vectors that permit the insertion of foreign genes into the E3 region or the E4 region. The E3 region genes are dispensable for growth in tissue culture, so the deletion of this region in vectors was attempted first (Bett *et al.* 1994). However, the E3 gene cassette could be useful to fight host immune responses to Ad vectors, believed to be one of the major causes of attenuation of expression of foreign genes

in infected tissues. If the essential functions of the E4 region for viral growth could be complemented in a cell line, vectors carrying deletions of the E4 region could be made. Cell lines based on 293 cells were made, and it was found that overexpression of the E4orf6 protein alone was sufficient to permit high titer growth of E1/E4 deleted adenoviruses (Brough *et al.* 1996). However deletion of the entire E4 region may not be desirable. A recent study has found that both the E3 and the E4 region may protect adenovirus vector-infected cells from lysis by cytotoxic T lymphocytes (Kaplan *et al.* 1999) and that some E4 proteins may be required to maintain long term expression from the transgene promoter (Armentano *et al.* 1997, Armentano *et al.* 1999).

Duchenne muscular dystrophy (DMD) and Cystic fibrosis (CF) are two of the pathologies considered for treatment with adenovirus vectors. The protein dystrophin is absent in the muscles of patients with DMD. Recombinant adenovirus vectors hold promise as a means for delivering a functional dystrophin gene to muscle (reviewed in Hartigan-O'Connor and Chamberlain 2000). Human protocols have demonstrated that it is feasible to transfer the normal human CF transmembrane conductance regulator cDNA to the respiratory epithelium and that this will lead to production of the normal CF protein and in some cases to a physiological response. Other examples of disorders that might one day be treated by gene therapy with Ad vectors are: hemophilia, Parkinson's disease, rheumatoid arthritis, and some cancers (reviewed in Brenner 1999). In addition to the airways, adenovirus vectors have been used to mediate *in vivo* gene delivery to cells of the liver, blood vessels, brain, skeletal muscle, and heart (reviewed in Trapnell and Gorziglia 1994, Brenner 1999, Curiel 1999). Adenoviruses expressing the p53 protein are being widely considered for cancer therapy since they are surprisingly effective at killing even cancer cells expressing p53 mutants (reviewed in Roth 1998, Lang *et al.* 1999, McCormick 1999).

Key impediments to effective gene therapy with Ad vectors are the host immune response and low vector efficiency. Neutralizing antibodies are often generated that prevent re-administration of the virus. Several groups have considered the applicability of adenovirus

as a vaccine vector. Recombinant adenovirus vectors expressing glycoproteins of herpes simplex virus (HSV) induce humoral and cellular immunity to HSV in mice (Endresz *et al.* 1995).

Adenovirus vectors are also very useful in the laboratory to achieve high efficiency synchronized introduction of one or more transgenes into cultured cells, and study their activity (Bacchetti and Graham 1993, Schwarz *et al.* 1995). Some of the work in chapters III, IV, and V of this thesis was done with the aid of adenovirus vectors.

1.7 Thesis proposal

Human adenoviruses infect non-dividing epithelial cells, and in order to replicate they have to force infected cells to enter S-phase. It had been known for some time that this process is a function of the transforming E1A oncogene. Expression of E1A also induces the stabilization of the cellular tumor suppressor p53 and could lead to apoptosis and premature death of the infected cell. The N-terminus of E1A products binds two very important classes of cellular proteins, the p300/CBP/p400 and pRb/p107/p130 families of proteins. Adenoviruses expressing E1A deletion mutants with well characterized defects in p300 and pRb binding were available. In Chapter II of this thesis, we used these adenovirus E1A mutations to identify what regions of E1A are involved in inducing the accumulation of p53. This work led to the conclusion that binding of pRb or p300 by E1A proteins caused both the induction of DNA synthesis and the accumulation of p53.

All the experiments pursued in the rest of this thesis, Chapters III, IV and V, derive from an observation made while studying the accumulation of p53 protein induced by E1A. We observed that *wild-type* adenovirus expressing both E1A as well as the other early viral genes did not induce the accumulation of p53.

In Chapter III, I used adenovirus mutants to map the products responsible for the attenuation of E1A-induced p53 stabilization. These studies indicated that the E1B55K and E4orf6 proteins were responsible for the block in p53 accumulation. E4orf6 is known to form complexes with E1B55K and to function both in the transport and stabilization of viral mRNA and in shutoff of host cell protein synthesis.

In chapter IV, I pursued further the mechanism of p53 degradation by E1B55K/E4orf6. I showed that both E4orf6 and E1B55K together were needed to greatly reduce the levels of p53 in cell extracts. I also determined which mutant forms of p53 were susceptible to such degradation, including the newly discovered p53-related proteins, p73 α and p73 β . I also generated an extensive series of mutations in E4orf6, and established a preliminary mapping of three functions of E4orf6 that mediate p53 degradation. To probe into the mechanism of degradation, I used an inhibitor of the proteasome as well as another drug that blocks nucleo-cytoplasmic shuttling. I also investigated the differences between adenovirus-induced p53 degradation, and that induced by two other proteins, the cellular MDM2 and the human papilloma virus E6 proteins. These studies led to the possibility that p53 is degraded by E1B55K/E4orf6 complexes not in the cytoplasm, but in the nucleus.

Our group had recently detected several cellular E4orf6-interacting proteins. In Chapter V, we identified, using mass spectrometry, four such polypeptides that form a novel E3 ubiquitin ligase complex. The identity of these cellular proteins was confirmed by performing binding assays *in vivo* and with baculovirus-expressed proteins in insect cells. I also verified that E4orf6 colocalizes with them in the nucleus, and that mutants of E4orf6 that no longer bind these proteins are not functional for p53 degradation. Finally, we verified that this novel E3 ubiquitin ligase complex could ubiquitinate p53 in an *in vitro* reconstitution assay. These studies indicated that a novel mechanism of regulation exists that utilizes a unique cellular ubiquitination system.

CHAPTER II

Accumulation of p53 induced by the adenovirus E1A protein requires regions involved in the stimulation of DNA synthesis

2.1 Abstract

It has been known for some time that expression of the 243-residue (243R) human adenovirus type 5 early region 1A (E1A) protein causes an increase in the level of the cellular tumor suppressor p53 and induction of p53-dependent apoptosis. Deletion of a portion of conserved region 1 (CR1) had been shown to prevent apoptosis, suggesting that binding of p300 and/or the pRb retinoblastoma tumor suppressor and related proteins might be implicated. To examine the mechanism of the E1A-induced accumulation of p53, cells were infected with viruses expressing E1A-243R containing various deletions that have well characterized effects on p300 and pRb binding. It was found that in human HeLa cells and rodent cells, complex formation with p300 but not pRb was required for the rise in p53 levels. However, in other human cell lines, including MRC-5 cells, E1A proteins which were able to form complexes with either p300 or pRb induced a significant increase in p53 levels. This same pattern was also apparent in p53-null mouse cells coinfecting by Ad5 mutants and an adenovirus vector expressing either wild-type or mutant human p53 under a cytomegalovirus promoter, indicating that the difference in importance of pRb binding may relate to differences between rodent and human p53 expression. The increase in p53 levels correlated well with the induction of apoptosis and, as shown previously, with the stimulation of DNA synthesis. Thus, it is possible that accumulation of p53 is induced by the induction of unscheduled DNA synthesis by E1A proteins and that increased levels of p53 then activate cell death pathways.

2.2 Introduction

The products of early region 1A (E1A) of human adenoviruses play several critical roles in viral replication. The largest E1A product, 289 residues (289R), which is encoded by the 13S mRNA, is important in the activation of transcription of all early viral genes, largely through the action of the 46-residue conserved region 3 (CR3) domain (Flint and Shenk 1989). Adenoviruses normally replicate in terminally differentiated epithelial cells and thus have evolved mechanisms to induce DNA synthesis in order to permit replication of the viral DNA genome. Work by our group (Howe *et al.* 1990) and others (Zerler *et al.* 1987, Bellett *et al.* 1989) showed that E1A proteins induce DNA synthesis by two probably independent mechanisms involving interactions with two classes of cellular proteins. Complex formation with the pRb tumor suppressor and related proteins p107 and p130 was shown to stimulate DNA synthesis in serum-starved baby rat kidney (BRK) cells (Howe *et al.* 1990). Such interactions result in the release of transcription factor E2F heterodimers which regulate the expression of genes involved in DNA synthesis (Nevins 1992). However, binding of E1A proteins to the transcriptional modulator p300 (Eckner *et al.* 1994) and/or related proteins p400 (Barbeau *et al.* 1994) and the CREB-binding protein (Arany *et al.* 1995) also results in the activation of the S phase in quiescent cells (Howe *et al.* 1990). Figure 2.1 shows that complex formation with the pRb family of proteins involves a primary binding site within conserved region 2 (CR2) and a secondary but functionally important site in conserved region 1 (CR1) (Egan *et al.* 1988, Whyte *et al.* 1989, Corbeil and Branton 1994). Binding of p300 requires both CR1 and the extreme amino terminus of E1A proteins (Egan *et al.* 1988, Whyte *et al.* 1989, Barbeau *et al.* 1994). The 243-residue (243R) product of the 12S E1A mRNA is identical to 289R except that it lacks CR3, and thus both 289R and 243R are capable of inducing unscheduled DNA synthesis. It is likely that the



<i>dl</i> 1101 4-25	<i>dl</i> 1143 38-60	<i>dl</i> 1107 111-123	<i>dl</i> 520 140-185
		<i>dl</i> 1108 124-127	

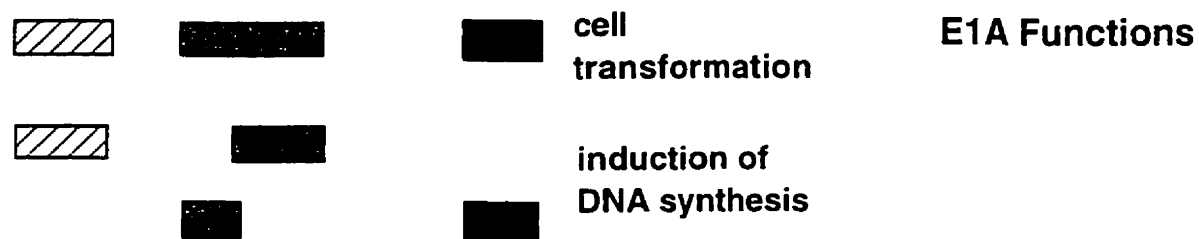
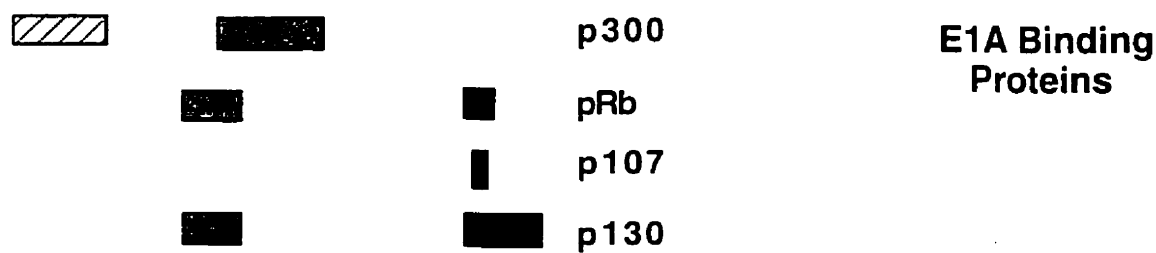


Figure 2.1 Ad5 E1A functions and mutants

The Ad5 E1A protein containing CR1, CR2, and CR3 is illustrated, along with the sequences deleted in a series of E1A mutants. The sequences required for the binding of p300- and pRB-related proteins are indicated, as are those shown to play a role in cell transformation and induction of DNA synthesis.

stimulation of DNA synthesis via these two mechanisms constitutes the basis for the transforming activity of these two E1A products.

E1A alone transforms cells very poorly because E1A proteins induce apoptosis and cell death. Stable E1A-dependent transformation occurs most efficiently in the presence of products of early region 1B (E1B) which encodes two major polypeptides of 19 and 55 kDa. These proteins play important roles in both productive infection and cell transformation, most probably by preventing rapid cell death due to E1A-induced apoptosis. The 19-kDa product is functionally homologous to the cellular Bcl-2 protein, which blocks apoptosis, at least in part, through complex formation with the Bax protein regulator of terminal death pathways (Han *et al.* 1996a). The 55 kDa E1B protein binds to and inactivates the p53 tumor suppressor (Sarnow *et al.* 1982a, Kao *et al.* 1990, Yew and Berk 1992) which has been linked to the regulation of growth arrest and apoptosis (Ko and Prives 1996). Expression of either E1B product allows E1A-dependent cell transformation to occur, although expression of both proteins results in much higher transforming efficiencies.

It is now known that E1A induces both p53-dependent and p53-independent apoptosis. The latter is produced only by the 289R E1A product and requires expression of an early region 4 (E4) protein (Teodoro *et al.* 1995, Marcellus *et al.* 1996b). The 243R E1A polypeptide is unable to induce apoptosis in 53-null cells (Teodoro *et al.* 1995). However, in the presence of wild-type p53 protein, either expressed endogenously or introduced ectopically into p53-null cells, E1A-243R induces the full range of apoptotic events including cell death, degradation of DNA to nucleosome-sized fragments, and all of the classic morphological hallmarks (Teodoro *et al.* 1995). Expression of E1A-243R leads to a large increase in the levels of p53, and it is likely that this increase causes apoptosis (Braithwaite *et al.* 1990, Debbas and White 1993, Grand *et al.* 1993, Lowe and Ruley 1993, Chiou *et al.* 1994, Grand *et al.* 1994, Sanchez-Prieto *et al.* 1995, Grand *et al.* 1996). Previous studies have shown that induction of apoptosis by E1A-243R is prevented by deletions within CR1

and the amino terminus (White *et al.* 1991, Mymryk *et al.* 1994), thus implicating complex formation with the p300 or pRb families of proteins or both.

In the present study, we have taken a genetic approach to investigate further the mechanism by which E1A-243R induces the accumulation of p53. The data indicated that complex formation with the pRb family and/or p300 appears to be responsible and that such accumulation induces apoptosis.

2.3 Materials and methods

2.3.1 Cells and viruses

Normal MRC-5 diploid human embryonic lung fibroblasts (ATCC CCL 171), IMR-90 (ATCC CCL 186), human HeLa cells, baby rat kidney (BRK) cells, mouse 10T1/2 cells (Reznikoff *et al.* 1973), and mouse 10(1) cells which fail to express p53 (Harvey and Levine 1991) were cultured on 60 mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in α -modified minimal essential medium (α -MEM) supplemented with 10% fetal calf serum (FCS). Human 293 cells derived from human embryonic kidney cells and expressing Ad5 E1A and E1B proteins (Graham *et al.* 1977) were also used in some experiments and for preparation and titer determination of all virus stocks. Cells were infected with wild-type (*wt*) or mutant virus at a multiplicity of 35 PFU per cell, as described previously (Rowe *et al.* 1984b). Almost all viruses used in the present studies were derived from *dl520* which produces the E1A 12S mRNA but not the 13S mRNA and thus produces E1A 243R but not 289R (Haley *et al.* 1984). Construction of the series of Ad5 deletion mutants containing in-frame deletions within the E1A coding sequence of exon 1 of *dl520* has been described previously (Egan *et al.* 1988, Jelsma *et al.* 1988, Jelsma *et al.* 1989, Howe *et al.* 1990). In addition, most of the E1A mutants used have been introduced into a background of mutant 12S/E1B⁻ (originally *dl520T*) which produces only E1A-243R and no E1B products (Shepherd *et al.* 1993). As illustrated in Fig. 2.1, these include 01/12S/E1B⁻ (amino acids 4

to 25 deleted), 43/12S/E1B⁻ (38 to 60 deleted), 07/12S/E1B⁻ (111 to 123 deleted), 08/12S/E1B⁻ (124 to 127 deleted), and 01/08/12S/E1B⁻ (4 to 25 and 124 to 127 deleted). In one experiment (see Fig. 2.7), two viruses which express both 12S and 13S E1A mRNAs were used. These included *wt* Ad5 (Harrison *et al.* 1977) and mutant E1B⁻, which expresses neither the 19 kDa nor the 55 kDa E1B product (Teodoro *et al.* 1995). The Ad5 vectors used in this study included Adp53wt, in which the E1 (E1A and E1B) region of Ad5 was replaced by the *wt* human p53 gene under the cytomegalovirus (CMV) promoter; Adp53mut, which is identical except that the p53 sequence contains dominant negative mutations affecting the codons for Pro-72 and Cys-135; and AdLacZ, which is a similar construct but expresses *Escherichia coli lacZ* (Bacchetti and Graham 1993).

2.3.2 Western blotting analysis.

Whole-cell extracts were prepared from mock- or adenovirus-infected cells at 24h postinfection. Cells were lysed in buffer [50mM HEPES, pH 7.9, containing 400mM KCl, 0.1% NP40 (vol/vol), 4mM NaF, 4mM NaVO₄, 0.2mM EDTA, 0.2 mM EGTA, 1 mg/ml each of aprotinin, leupeptin and pepstatin, 0.5 mM PMSF, and 1mM DTT]. Total protein was measured by Bio-Rad protein assay as specified by the manufacturer, and 50µg of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% polyacrylamide gel. The material was transferred to nitrocellulose membranes for 1h in a semidry transfer apparatus, and the membranes were probed with primary antibodies which recognize p53 or E1A proteins. Anti-p53 mouse monoclonal antibodies Ab1801 (Ab-2, Oncogene Science), Ab240 (Ab-3) and Ab421 (Ab-1) were concentrated by precipitation with ammonium sulfate from supernatants obtained from hybridoma cell cultures grown in α -MEM. Ab1801 was used for immunoblotting of human p53 and a mixture of Ab240 and Ab421 was used for detection of rodent p53. Mouse monoclonal antibody M73 against E1A proteins (Harlow *et al.* 1985a) was collected as an ascites fluid. Binding patterns were determined by the addition of a secondary antibody,

either horseradish peroxidase-conjugated goat anti-mouse Immunoglobulin G (Jackson Immuno Research Laboratories), which was detected by enhanced chemiluminescence (chemiluminescence reagent; Dupont NEN), or ^{125}I -labeled anti-mouse antibody from sheep (Amersham), which was detected by a Fuji BAS-III imaging plate. The amount of activity was quantified with a Fujix Bas 2000 Phosphor-Imager.

2.3.3 EMSA

Electrophoretic mobility shift assay (EMSA) for p53 was performed with whole-cell extracts from mock-infected and adenovirus-infected cells at 24 h postinfection. The oligonucleotide used for these experiments containing the consensus binding site for p53, 5'-tcgagAGGCATGTCTAGACATGCCTc-3', has been described previously (el Deiry *et al.* 1992). The double-stranded oligonucleotides were labeled with $[\gamma\text{-}^{32}\text{P}]\text{dCTP}$ (Amersham Corp.) and Klenow polymerase (Pharmacia). EMSAs were performed in 20 μL volumes containing 10 μg of whole-cell extract in 20mM HEPES-KOH (pH 7.9), containing 40mM KCl_2 , 0.1mM EGTA, 0.4mM DTT, 4 μg of bovine serum albumin, 2.5% (vol/vol) Ficoll, 3 μg sonicated salmon sperm DNA (Pharmacia), 100 μg oligo dT and labeled probe containing 20,000 to 50,000 counts per min. In order to activate p53 DNA binding activity, 100ng of the anti-p53 monoclonal antibody pAb421 (Oncogene Science) was used, as described previously (Hupp *et al.* 1992). The EMSA reaction mixtures were incubated for 30 min at room temperature and then analyzed on a 4% polyacrylamide gel (30:1, acrylamide-to-bisacrylamide) in 0.25 x TBE (Tris-borate-EDTA) and subjected to electrophoresis at 200V for 2 h. The gels were dried and analyzed by autoradiography with Kodak-X-Omatic film.

2.3.4 Cell viability assays

Cells were infected with wt or mutant Ad5 in 24-well plates containing HeLa cells at about 80% confluence. At various times following infection, adherent and non-adherent cells

were pooled and viability was assessed by Trypan Blue exclusion. At least 300 cells were counted at each time point.

2.3.5 Electron Microscopy

Mock and 12S/E1B- infected cells were prefixed *in situ* with 2% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2 to 7.4) at 48h postinfection and then post-fixed with 1% (wt/vol) osmium tetroxide in cacodylate buffer. The cells were stained with uranyl acetate and photographs of representative fields were taken with a Philips 410 electron microscope.

2.4 Results

2.4.1 Expression of E1A-243R induces accumulation of p53

Previous studies had already indicated that expression of adenovirus E1A protein results in the production of increased levels of the p53 cellular tumor suppressor protein (Braithwaite *et al.* 1990, Debbas and White 1993, Grand *et al.* 1993, Lowe and Ruley 1993, Chiou *et al.* 1994, Grand *et al.* 1994, Sanchez-Prieto *et al.* 1995, Grand *et al.* 1996). To analyze this phenomenon further, HeLa cells, which are derived from a human cervical cancer, were studied. These cells express human papilloma virus (HPV) proteins E6 and E7 and were known to contain very low levels of p53, at least in part because of the effects of HPV-E6, which enhances its degradation via ubiquitin pathways (Scheffner *et al.* 1990a). HeLa cells were mock infected or infected with mutants *dl520*, which expresses the E1A-243R protein but not 289R, or 12S/E1B⁻, which also expresses only 243R and in addition fails to produce E1B polypeptides. Because of the absence of E1A-289R, such cells fail to express significant levels of products of early regions 3 and 4 (E3 and E4). Cell extracts were collected, separated by SDS-PAGE, and following transfer to nitrocellulose, analyzed for p53 content by Western blotting with a p53-specific monoclonal antibody. Figure 2.2

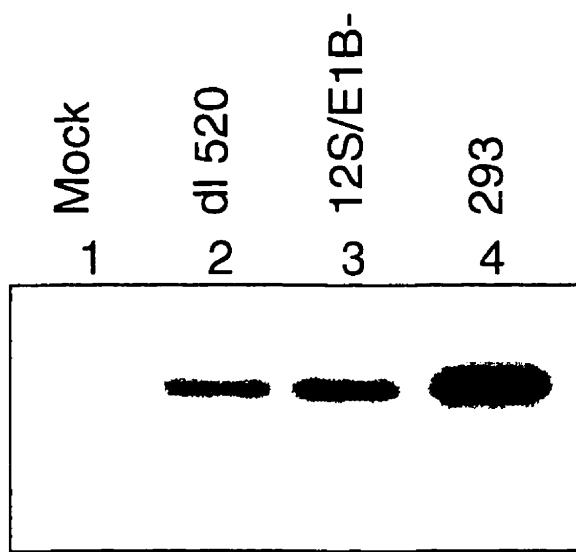


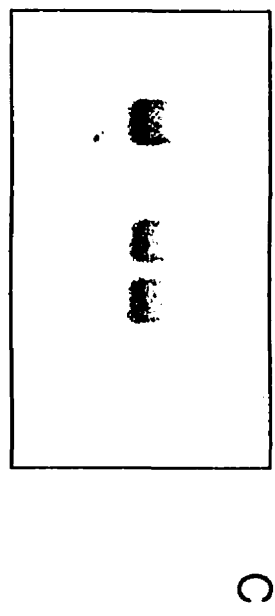
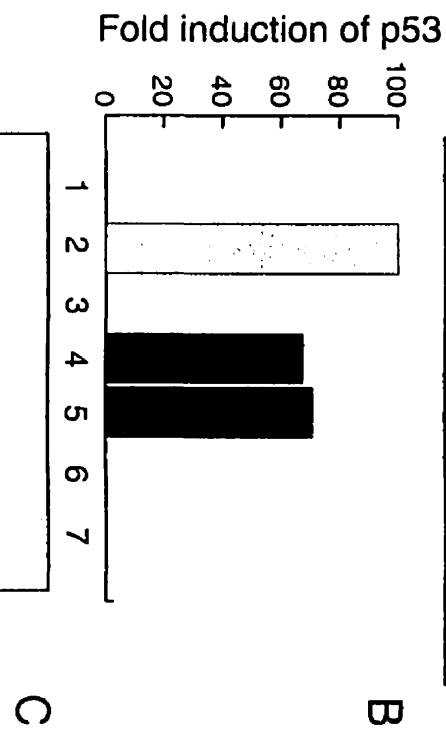
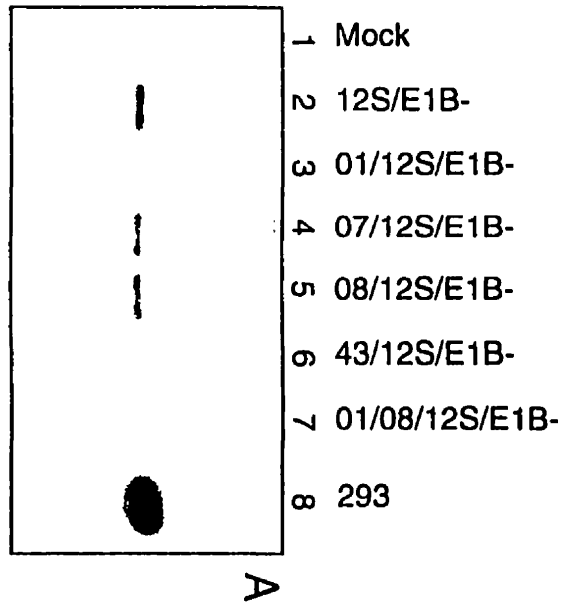
Figure 2.2 Analysis of E1A-induced accumulation of p53 in HeLa cells by Western blotting

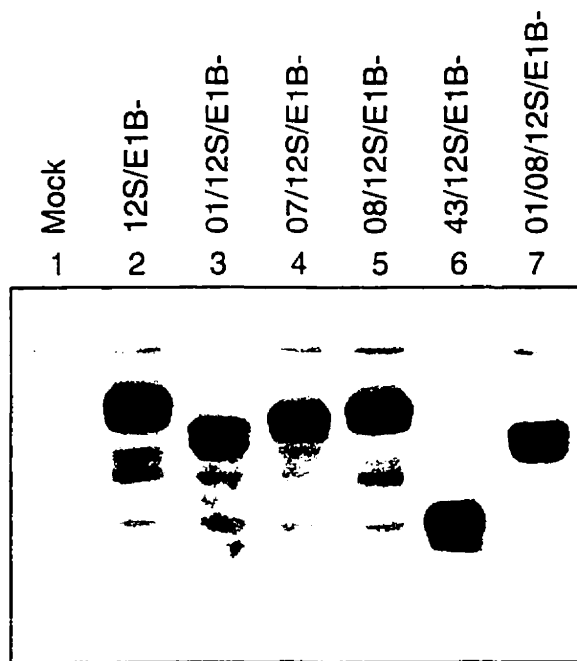
HeLa cells were infected with *dl520* or 12S/E1B⁻ or mock-infected, and cell extracts were prepared at 24 hpi and separated by SDS-PAGE. Following transfer to nitrocellulose, p53 was detected with anti-p53 Ab1801 and ¹²⁵I-labeled anti-mouse antibody. An extract from Ad5-transformed 293 cells was included as a control.

shows that infection with *dl520* (lane 2) results in a large increase in p53 relative to mock-infected cells (lane 1), approaching the levels observed in Ad5-transformed 293 cells (lane 4). Quantitative studies indicated that the increase in p53 in HeLa cells is a minimum of 8- to 10-fold (data not shown). A similar increase was also observed with mutant 12S/E1B⁻ (lane 3), suggesting that expression of neither the E1B 19 kDa protein nor the 55 kDa polypeptide, known to interact with p53 (Sarnow *et al.* 1982a, Yew and Berk 1992), is important for the rise in p53 levels.

2.4.2 Identification of the domains of E1A products involved in the accumulation of p53

To determine the molecular basis for the E1A-induced accumulation of p53, HeLa cells were infected with a series of mutants which express E1A-243R proteins containing various deletions (Fig. 2.1) and which fail to express E1B products. Previous detailed studies on these mutants had established the binding patterns with p300 and the related p400 proteins and with pRb and the related p107 and p130 polypeptides (Barbeau *et al.* 1994, Mymryk *et al.* 1994). Mutant 01/12S/E1B⁻ lacks an amino terminal region required for p300/p400 binding but interacts with pRb and related proteins at fairly normal levels. Mutants 07/12S/E1B⁻ and 08/12S/E1B⁻ contain deletions in the primary pRb binding site in CR2 and thus form complexes with these proteins very poorly; however, they interact with p300 and p400 normally. Mutant 01/08/12S/E1B⁻ lacks binding sites for both p300 and pRb and thus binds to neither. Mutant 43/12S/E1B⁻ contains a deletion within CR1 and thus is at least partially defective for binding p300, pRb and related proteins (Barbeau *et al.* 1994). Figure 2.3D shows the results of a Western blotting analysis with E1A-specific M73 antibody and indicates that the synthesis of E1A proteins by all of these mutants occurred at quite comparable levels. Cell extracts were prepared from HeLa cells infected by these viruses and they were analyzed for p53 content by Western blotting as in Fig. 2.2. Figure 2.3A shows again that infection with 12S/E1B⁻ (lane 2) induced a large increase in p53





D

Figure 2.3. Identification of the E1A regions required for the accumulation of p53 in HeLa cells

Extracts from HeLa cells infected with Ad5 12S/E1B⁻ or a series of deletion mutants present in a 12S/E1B⁻ background were prepared as in the experiment in Fig. 2.2. (A) Some of the extracts were analyzed by Western blotting using anti-p53 Ab1801 and ¹²⁵I-labeled anti-mouse antibody as in Fig. 2.2. (B) Such analyses were quantified with a PhosphorImager. (C) Some aliquots were combined with Ab421 anti-p53 antibody, mixed with a ³²P-labeled p53-specific oligonucleotide, and then subjected to EMSA. (D) Other aliquots were analyzed by Western blotting with M73 anti-E1A monoclonal antibody and ECL.

relative to mock-infected cells (lane 1). Infection with mutant 01/12S/E1B⁻ (lane 3) resulted in no increase in p53 levels. After infection with mutants 07/12S/E1B⁻ and 08/12S/E1B⁻ (lanes 4 and 5, respectively), increased levels of p53 were noted. Quantitative analysis of these samples with a PhosphorImager indicated that these mutants increased the p53 levels to about 70% of those obtained with *wt* 12S/E1B⁻ (see Fig. 2.3B). Infection with mutants 01/08/12S/E1B⁻ (Fig. 2.3A, lane 7) and 43/12S/E1B⁻ (lane 6), which produce E1A proteins that fail to bind or bind poorly to both p300 and pRb, did not alter the p53 levels. Thus these results suggested that in HeLa cells, complex formation with p300 but not pRb correlated with E1A-induced accumulation of p53.

To analyze this effect further, similar studies were conducted in other cell types which were known to express *wt* p53 and to lack endogenous HPV sequences. Fig. 2.4A shows that a very similar pattern of p53 accumulation was observed following infection of BRK cells with the same series of E1A mutants. The levels of p53 in mock-infected BRK cells (lane 1) were higher than those found previously in HeLa cells (Figs. 2.2 and 2.3); nevertheless, following infection with *wt* 12S/E1B⁻ (Fig. 2.4A, lane 2), an increase of about eightfold was observed. Similar increases were again found with mutants 07/12S/E1B⁻ (lane 4) and 08/2S/E1B⁻ (lane 5), suggesting that complex formation with pRb and related proteins was not critical for the effect. Levels similar to those in mock-infected cells were observed with 01/12S/E1B⁻ (lane 3) and 01/08/12S/E1B⁻ (lane 6), and only partially increased levels were seen with 43/12S/E1B⁻ (lane 8), indicating that interactions with p300 were important. A virtually identical pattern was also observed in mouse 10T1/2 cells (Fig. 2.4B). As was the case in HeLa cells (Fig. 2.3D), the levels of E1A expression by all mutants in these and other cells discussed below were similar (data not shown). Thus results obtained with these rodent cells indicated that the E1A-induced increase in p53 levels appeared to require complex formation with p300 and related proteins but not with the pRb family. Similar results were obtained recently with E1A proteins expressed in PAM212 murine keratinocyte cells (Sanchez-Prieto *et al.* 1995).

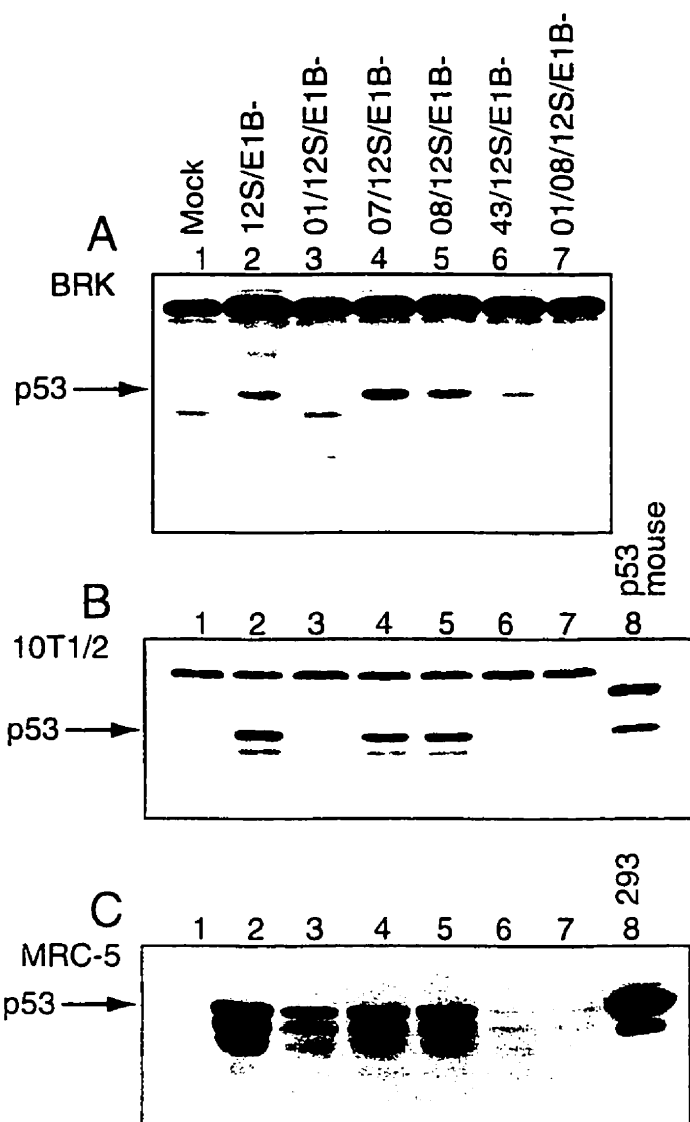


Figure 2.4. Identification of the E1A regions required for the accumulation of p53 in rodent cells and in human MRC-5 cells

An experiment similar to that described the legend to Fig. 2.3 was performed with BRK cells, mouse 10T1/2 cells, and human MRC-5 cells. (A and B) p53 present in cell extracts was detected by Western blotting analysis with a mixture of Ab240 and Ab421; (C) Ab1801 was used to detect p53. Binding patterns were determined by ECL. (B) *In vitro* -transcribed translated p53 from mouse cDNA was included in lane 8 as a control. (C) An extract from Ad5-transformed 293 cells was included in lane 8 as a control.

Analysis of additional human cell lines which are known to express wt p53 gave a somewhat different response than human HeLa cells (Fig. 2.3) and rodent cells (Figs. 2.4A and B). Figure 2.4C shows the results of an experiment with human MRC-5 cells in which the p53 detected by Western blotting analysis appeared as a triplet, presumably because of degradation, as is sometimes observed in these and other cells. Infection of MRC-5 cells with wt 12S/E1B⁻ (Fig. 2.4C, lane 2) caused about a 10-fold increase in the amount of p53 relative to mock-infected cells (lane 1). Significant increases in p53 levels were also observed with mutants 01/12S/E1B⁻ (lane 3), 07/12S/E1B⁻ (lane 4), and 08/12S/E1B⁻ (lane 5) relative to mock-infected cells. However, mutants 43/12S/E1B⁻ (lane 6) and 01/08/12S/E1B⁻ (lane 7), which fail to interact or interact poorly with both the p300 and pRb protein families, showed only a modest increase in p53 levels. An identical pattern was also observed in human IMR-90 cells (data not shown). These results suggested that in normal human cells, accumulation of p53 correlated with binding to either p300 or pRb. It appeared possible that the results obtained with human HeLa cells were complicated by the fact that these cells express HPV-E7 (Inagaki *et al.* 1988), which is known to interact with pRb (Dyson *et al.* 1989) but has not been demonstrated to bind to p300. We also considered the possibility that in HeLa cells E1A affects the expression of the HPV-E6 protein, thus altering the rate of p53 turnover; however, this cannot explain our similar results with several rodent and human cell lines which totally lack HPV sequences.

2.4.3 Effects of E1A-243R on ectopic expression of human p53 in p53-null mouse cells

To clarify further the ability of E1A-243R to induce the accumulation of p53, studies were carried out with mouse 10(1) cells which lack endogenous p53 (Harvey and Levine 1991). Such cells were shown to be resistant to the induction of apoptosis by E1A-243R unless p53 was expressed ectopically with an adenovirus gene transfer vector (Teodoro *et al.* 1995). Experiments were conducted in which 10(1) cells were coinfecting by the series of

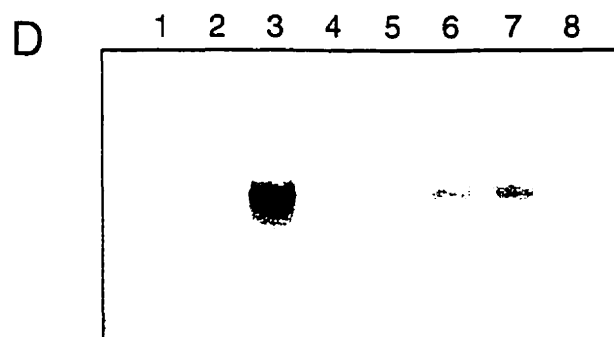
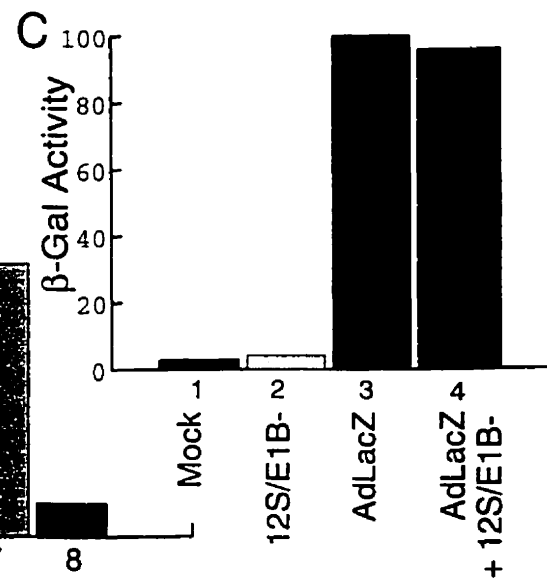
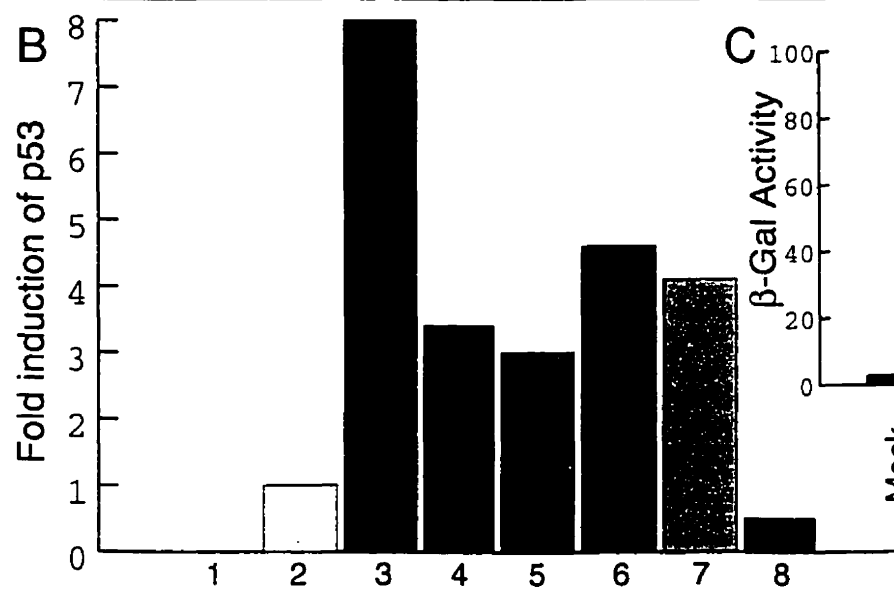
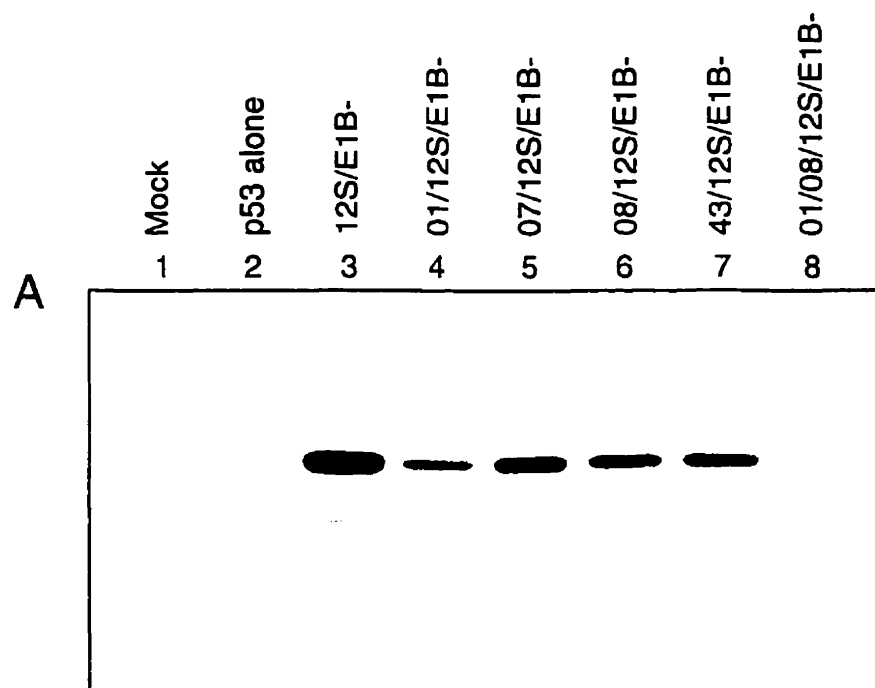


Figure 2.5. Analysis of E1A-induced p53 accumulation in mouse 10(1) cells expressing human p53

Mouse 10(1) cells, which are p53 null, were coinfectd by *wt* 12S/E1B⁻ or various E1A mutants and the adenovirus vector Adp53wt, which expresses human p53 by using a CMV promoter. Cell extracts were prepared and analyzed for p53 content by Western blotting analysis and for p53-specific DNA binding activity, as described in the legend to Fig. 2.3. As a control for expression from the CMV promoter, some cells were infected with the AdLacZ vector, which expresses bacterial LacZ instead of p53. In this case, cell extracts were analyzed for β -galactosidase activity. (A) Western blotting with anti-p53 Ab1801 and ¹²⁵I-labeled anti-mouse antibody. (B) Quantitation of results in panel A by PhosphorImager analysis. (C) Analysis of β -galactosidase expression in extracts from cells infected by the AdLacZ vector. (D) Analysis of p53 DNA-binding activity by EMSA. Lanes in panel A, B, and D are as indicated in panel A.

12S/E1B⁻ mutants and Adp53wt, which expresses *wt* p53 under the control of the CMV promoter (Bacchetti and Graham 1993). As in previous experiments, cell extracts were assessed for p53 content by Western blotting analysis with anti-p53 monoclonal antibody. Fig. 2.5A shows that 10(1) cells express no p53 (lane 1); however, following infection with Adp53wt (lane 2), appreciable levels were apparent. Coinfection with 12S/E1B⁻ (lane 3) led to a significant rise in p53, which, when quantified by PhosphorImager analysis (Fig. 2.5B), was about eightfold. These results indicated that E1A-243R was able to induce the accumulation of human p53 which was expressed ectopically from the CMV promoter in these mouse cells. This increase was not due to enhanced transcription of p53 mRNA induced by the E1A protein, because no difference in expression of a CMV-driven *lacZ* gene in the AdLacZ vector was observed following coinfection with 12S/E1B⁻ (Fig. 2.5C). Thus, accumulation must relate either to the stabilization of 53 or to some other function related to synthesis of the p53 protein. Induction of p53 accumulation was partially defective with mutant 01/12S/E1B⁻ (Fig. 2.5A, lane 4), 07/12S/E1B⁻ (lane 5), and 08/12S/E1B⁻ (lane 6). Quantitative analyses indicated that only about a three- to fourfold increase was evident with all of these mutants (Fig. 2.5B). In the case of mutant 43/12S/E1B⁻ (Fig. 2.5A, lane 7), which binds both p300 and pRb at reduced levels, a similar effect was observed. However, with 01/08/12S/E1B⁻ (lane 8), which is completely defective for p300 and pRb binding, no increase whatsoever was evident. These results suggested again that induction of accumulation of human p53 occurred via complex formation with either p300- or pRb-related proteins, even if it occurred in mouse cells. Thus, the difference in the importance of interactions with the pRb family of tumor suppressors in these experiments (and those with human MRC-5 cells described in Fig. 2.4C), compared with those obtained by using rodent cells (Fig. 2.4A and B), related to the human origin of the cDNA, its transcript, or its protein product, and not to the cell type in which it is expressed.

An additional experiment was conducted in 10(1) cells in which both *wt* p53, expressed by Adp53wt, and a dominant negative mutant form of p53, expressed by

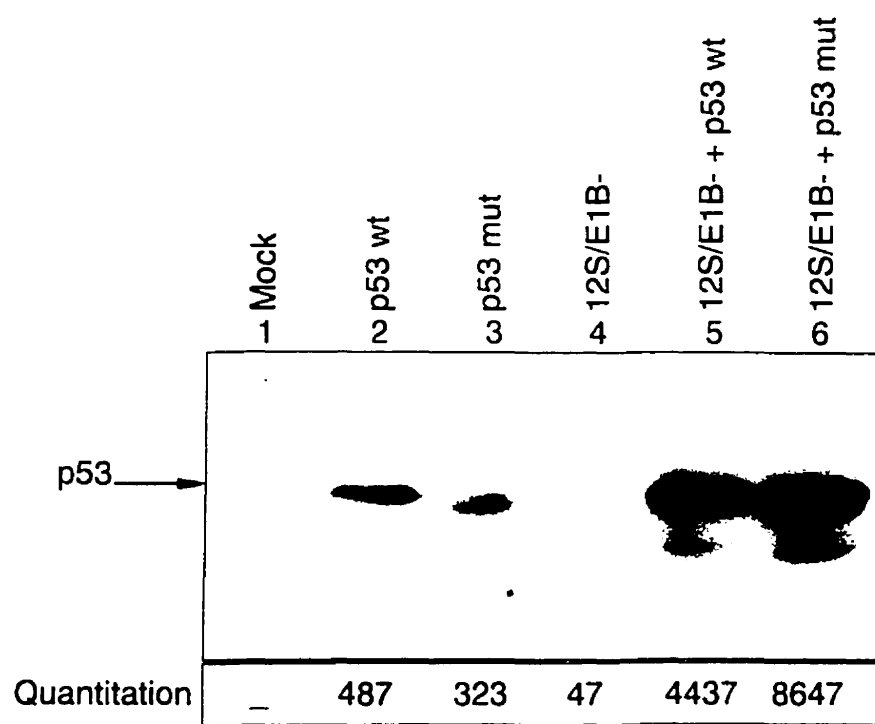


Figure 2.6. Analysis of E1A-induced p53 accumulation in mouse 10(1) cells expressing human wild-type and mutant p53

An experiment similar to that described in the legend to Fig. 2.5 was performed, except that 10(1) cells were infected or coinfectd with Ad5 12S/E1B⁻ and either Adp53wt or Adp53mut. Cell extracts were analyzed for p53 content by Western blotting using anti-p53 Ab1801 and ¹²⁵I-labeled anti-mouse antibody as in the experiment in Fig. 2.2. The amount of p53 present was quantified by PhosphorImager analysis (shown below the blot).

Adp53mut, were tested in coinfection experiments with 12S/E1B⁻. Figure 2.6 shows that the levels of both *wt* and mutant p53 (compare lanes 5 and 2 and lanes 6 and 3, respectively) increased after the expression of E1A-243R. Thus, the rise in p53 levels did not require a functional p53 protein.

2.4.4 E1A induces accumulation of functional p53 protein

To determine if the increased quantities of p53 molecules induced by E1A protein are capable of interacting with target DNA sequences, EMSAs were carried out with a p53-specific oligonucleotide and extracts from 10(1) cells which had been coinfecting with the Adp53wt vector and the series of 12S/E1B⁻ E1A mutants. In these assays, as has in many other similar studies, addition of the anti-p53 antibody pAb421 was required to obtain efficient DNA-binding activity. Figure 2.5D shows that no p53-DNA complexes were apparent in mock-infected 10(1) cells (lane 1). Low levels were evident in cells infected with Adp53wt alone (lane 2), and these were greatly increased upon coinfection with 12S/E1B⁻ (lane 3). Detection of such p53-oligonucleotide complexes with various E1A mutants followed the same pattern as total p53 levels shown in Fig. 2.5A and B. A similar study was also carried out with endogenous p53, which was induced in HeLa cells by expression of *wt* and mutant E1A-143R. Figure 2.3C shows that little p53-DNA complex was observed following infection by 01/12S/E1B⁻ (lane 3), 01/08/12S/E1B⁻ (lane 7) or 43/12S/E1B⁻ (lane 6) which produce E1A proteins which bind poorly or not at all to p300. Conversely, high levels of p53-DNA complexes were detected with *wt* (lane 2) and with mutants 07/12S/E1B⁻ (lane 4) and 08/12S/E1B⁻ (lane 5), which yield E1A products that do not interact efficiently with pRb and related proteins. Thus, in both cases, the increased quantities of p53 molecules induced by *wt* E1A-243R in mouse 10(1) cells expressing human p53 or in human HeLa cells expressing endogenous p53 could be induced to bind DNA in a normal fashion.

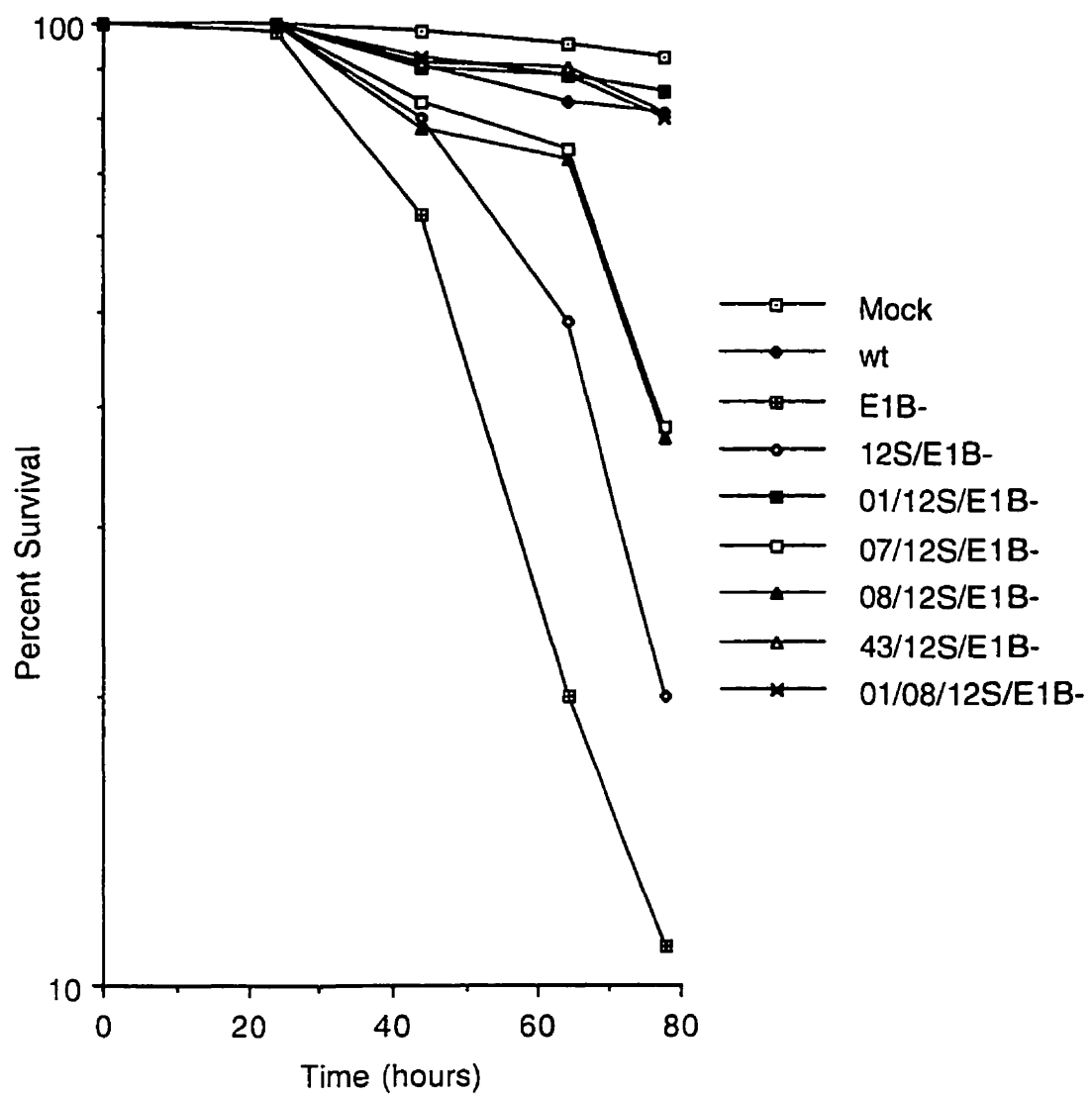


Figure 2.7. Cell viability assay

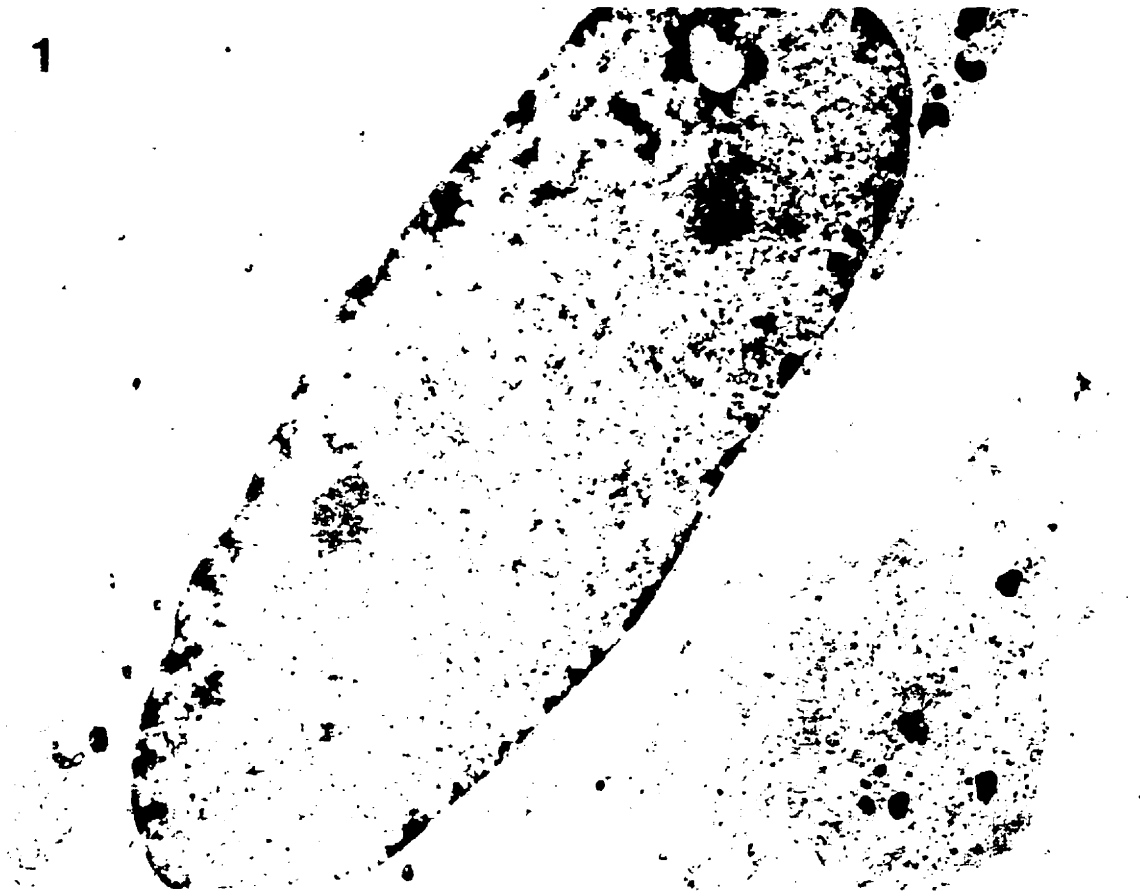
HeLa cells were mock-infected or infected by *wt* Ad5, E1B⁻, 12S/E1B⁻, or a series of mutants prepared in a 12S/E1B⁻ background. The cells were tested for viability by a Trypan Blue exclusion assay at various times following infection. The data are presented as the log percent viable cells.

2.4.5 Induction of cell death by E1A deletion mutants

To correlate the accumulation of p53 with E1A-induced p53-dependent cell death, cell-killing assays were carried out with HeLa cells by using viruses which lack E1B expression and thus induce p53-dependent apoptosis. Figure 2.7 shows that in HeLa cells, very rapid cell death was produced by infection with mutant E1B⁻, which expresses both major E1A products but no E1B proteins and induces both p53-dependent and p53-independent apoptosis (Teodoro *et al.* 1995). Mutant 12S/E1B⁻, which yields only E1A-243R and no E1B polypeptides and is able to induce only p53-dependent apoptosis, produced cell death somewhat less rapidly but nevertheless, impressively. Significant levels of killing were also observed with mutants 07/12S/E1B⁻ and 08/12S/E1B⁻, which cause the accumulation of p53. Little or no cell death was seen in mock-infected cells and in cells infected by *wt* Ad5, 01/12S/E1B⁻, 01/08/12S/E1B⁻, or 43/12S/E1B⁻, all of which fail to induce accumulation of p53. These data indicated that induction of cell killing correlated with the accumulation of p53.

To verify that induction of the p53 levels reported above in human MRC-5 cells also results in apoptosis, the morphologies of mock-infected MRC-5 cells and those infected with 12S/E1B⁻ virus were examined by electron microscopy. Figure 2.8 shows that the infected cells (Fig. 2.8B) displayed highly condensed chromatin and vacuolization of the cytoplasm as is typical of apoptotic cells but absent in normal MRC-5 cells (Fig. 2.8A).

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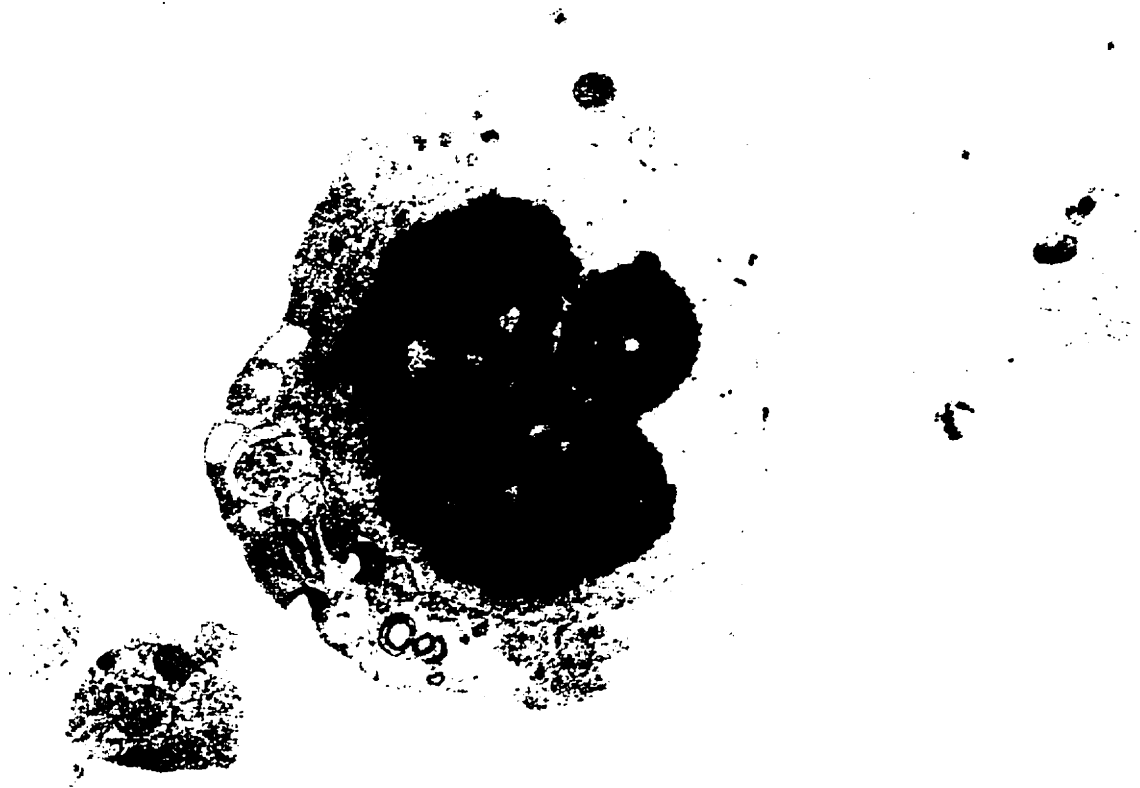


Figure 2.8. Analysis of cell morphology by electron microscopy

Mock-infected (A) or 12S/E1B⁻-infected (B) MRC-5 cells were harvested at 24 h.p.i and analyzed by electron microscopy. Magnification, x3,432..

2.5 Discussion

It has been known for some time that the expression of adenovirus E1A protein results in an increase in the intracellular concentration of p53 protein and the induction of p53-dependent apoptosis (Braithwaite *et al.* 1990, Debbas and White 1993, Grand *et al.* 1993, Lowe and Ruley 1993, Chiou *et al.* 1994, Grand *et al.* 1994, Sanchez-Prieto *et al.* 1995, Grand *et al.* 1996). It is likely that these effects relate to interactions with one or more cellular polypeptides, since the major function of E1A products seems to be to mediate biological responses through the formation of complexes with a variety of cellular proteins. Induction of apoptosis has been shown to be suppressed by deletion of a portion of the CR1 region of E1A protein (White *et al.* 1991, Mymryk *et al.* 1994) and thus may depend on complex formation with p300 and related proteins, the pRb family of tumor suppressors, or both. In the present studies, we have used a genetic approach to examine the accumulation of p53 induced by the 243R E1A protein and to relate this information to the induction of p53-dependent cell death and apoptosis. Our studies indicated clearly that in rodent cells, accumulation of p53 corresponded to the formation of E1A-p300 complexes and interactions with the pRb family were not required. Similar results were obtained recently with murine PAM212 cells, in which the increase in p53 levels obtained by expressing E1A protein was much more dependent on interactions with p300 than with the pRb family (Sanchez-Prieto *et al.* 1995). We also found a similar pattern with human HeLa cells to that found by another group in an accompanying paper (Chiou and White 1997). However, we were concerned that the expression of HPV E6 and E7 proteins in HeLa cells may have affected this response. E7 proteins, which are expressed at least at low levels in HeLa cells, bind to pRb-related proteins (Dyson *et al.* 1989) but have not been demonstrated to interact with p300. Thus, it is possible that pRb complexes are present constitutively in these cells. This concern may have been justified as analysis of other human cells indicated that interactions with either p300 or pRb induced the accumulation of p53, although higher levels were obtained when E1A molecules were able to interact with both. Thus, a difference exists in the role of the

pRb family in mouse and human cells. Studies of mouse cells expressing human p53 encoded by an adenovirus gene transfer vector indicated that the difference was not caused by the cell type but, rather, derived from the origin of the p53 gene and its transcript. Thus, regardless of the species of the infected cell, accumulation of human p53 was at least partially induced by complex formation with pRb-related proteins whereas that of mouse p53 was not. At present, we do not know the basis for this difference. The pRb family plays a role in the regulation of the cell cycle and entry into the S phase in both human and rodent cells (Goodrich *et al.* 1991, Qin *et al.* 1992). We (Corbeil *et al.* 1995, Corbeil and Branton 1996) and others (Buchkovich *et al.* 1989, Chen *et al.* 1989, DeCaprio *et al.* 1989, Mihara *et al.* 1989) have noted that the levels of p107 and p130 and of E2F complexes containing these proteins vary during the cell cycle. However, whereas pRb-E2F complexes were detected at high levels in human and monkey cells throughout the cell cycle, such complexes were only detected at significant levels in growth arrested or terminally differentiated mouse cells (Corbeil *et al.* 1995, Corbeil and Branton 1996). It is possible that this difference may underlie a functional difference in the role of pRb in the two species. Further experiments are needed to understand the precise basis for this effect.

The mechanism by which p53 accumulates is unknown. It has been suggested that the E1A protein may alter the rate of transcription of the p53 gene (Braithwaite *et al.* 1990). Although we have not studied this question directly, our results with p53-null mouse 10(1) cells infected with adenovirus vectors indicated that E1A-243R has little effect on the CMV promoter which controls p53 expression. Thus, at least in this case, the accumulation of p53 must be due to some other mechanism. Some increase in the half-life of p53 has been reported (Lowe and Ruley 1993, Grand *et al.* 1996), and it is possible that this effect could account for the large (8- to 10-fold) increase in the levels of p53 reported here. In a recent report, it has been suggested that translation of mouse p53 mRNA can be regulated via a sequence in the 5' untranslated region of the p53 mRNA (Mosner *et al.* 1995). Furthermore, these results suggested that the p53 protein itself may be involved in this translational

regulation. Even more recently, it was suggested that the 3' untranslated region may play a role in controlling human p53 mRNA translation (Fu *et al.* 1996). Our data would generally be consistent with such control, except for the fact that mutant p53, which is functionally inactive, also accumulated in response to E1A. Further studies are necessary to clarify this phenomenon. In addition, it should be noted that all the experiments carried out in the present study were performed with Ad5 strains that express the E1A-243R protein and not the 289R product. Thus, under these conditions of infection, little expression of the E3 and E4 genes takes place, since 243R lacks the CR3 transactivation domain. In recent experiments with wt Ad5 in our laboratory, we have found that the expression of E4orf6 and the 55 kDa E1B protein limits the accumulation of p53 induced by E1A protein (Querido *et al.* 1997a).

The p53 molecules accumulated by expression of E1A appear to be functional, at least in terms of their ability to be induced to interact with p53-specific binding sequences. We also found that induction of cell death correlates with the accumulation of p53. In mouse cells, cell killing was greatly reduced by mutations which blocked the E1A-induced increase in p53 levels. Previous studies indicated that E1A-243R is able to induce only p53-dependent apoptosis (Teodoro *et al.* 1995). Thus, it is likely that apoptosis results from the accumulation of active p53 molecules. It was of great interest that the pattern of induction of accumulation of p53 by E1A proteins resembles that observed for the stimulation of DNA synthesis. Previous studies indicated that E1A proteins able to bind either p300 or pRb were able to induce DNA synthesis in serum-starved cells (Howe *et al.* 1990). Thus, induction of unscheduled DNA synthesis through complex formation with either p300- or pRb-related proteins may represent the actual trigger for the accumulation of p53, and the resulting high levels of active p53 therefore induce apoptosis. Further experiments are underway to elucidate this mechanism further.

2.6 Acknowledgments

We thank Stan Bayley for mutant 12S/E1B⁻ and other E1A mutants; Andy Bett and Frank Graham for the gift of vectors AdLacZ, Adp53wt and Adp53mut; Ed Harlow for M73 hybridoma cells; Arnold Levine for 10(1) cells; and Jim Pipas for 10T1/2 cells. We also thank Rachelle Charbonneau and Dennis Takayesu for some technical assistance.

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As we have just seen, E1A can induce the accumulation of p53 protein in the cell, and this seems to involve two regions in the amino terminus of E1A which are involved in the binding of p300 and pRb family proteins. However, during infection with wild-type adenovirus, we observed no significant rise in p53 protein levels. This prompted us to find out which adenovirus proteins might be involved in the suppression of p53 accumulation during infection.

CHAPTER III

Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells

3.1 Abstract

The adenovirus type 5 243R E1A protein induces p53-dependent apoptosis in the absence of the 19 and 55 kDa E1B polypeptides. This effect appears to result from an accumulation of p53 protein and is unrelated to expression of E1B products. We now report that in the presence of E1B55K, the 289R E1A protein does not induce such p53 accumulation and, in fact, is able to block that induced by E1A-243R. This inhibition also requires the 289R-dependent transactivation of E4orf6 expression. E4orf6 is known to form complexes with E1B55K and to function both in the transport and stabilization of viral mRNA and in shutoff of host cell protein synthesis. We demonstrated that the block in p53 accumulation is not due to the generalized shutoff of host cell metabolism. Rather, it appears

to result from a mechanism targeted specifically to p53, most likely involving a decrease in the stability of p53 protein. The 55 kDa E1B protein is known to interact with both E4orf6 and p53, and as demonstrated recently by others, we showed that E4orf6 also binds directly to p53. Thus, multiple interactions between all three proteins may regulate p53 stability, resulting in the maintenance of low levels of p53 following virus infection.

3.2 Introduction

The most common targets of human adenoviruses are terminally differentiated epithelial cells of the bronchial tract. Adenoviruses possess relatively small genomes, and thus in order to produce progeny virus, they must first activate host cell DNA synthetic machinery which is then utilized to replicate viral DNA. This induction of DNA synthesis by the products of early region 1A (E1A) appears to underlie the oncogenic potential of human adenoviruses in rodent cells in which lytic infection fails to occur, and cells which exhibit unregulated proliferative capacities sometimes survive. Although E1A represents the major viral oncogene, products of early region 1B (E1B) are usually required to yield stable transformants (reviewed in (Bayley and Mymryk 1994)). Both E1A and E1B encode multiple products due to alternative splicing. The two major adenovirus type 5 (Ad5) E1A proteins of 289 and 243 residues (289R and 243R, respectively) are identical apart from an internal region, termed CR3, which transactivates expression of early viral transcription units 3 and 4 (E3 and E4) and, to some extent, early region 2 (E2) and some cellular genes (Shenk and Flint 1991). The 289R and 243R E1A proteins can both induce DNA synthesis and transform rodent cells through the formation of complexes with two classes of cellular proteins, the retinoblastoma (RB) family of growth suppressors and the transcriptional modulator p300 and related proteins (reviewed in (Bayley and Mymryk 1994)). However, expression of these E1A proteins is toxic, and cells die rapidly from apoptosis unless E1B products are also expressed. The E1B 19 kDa protein is able to block apoptosis induced by a

wide variety of agents and seems to be functionally analogous to the cellular Bcl-2 protein (Rao *et al.* 1992, Boyd *et al.* 1994, Nguyen *et al.* 1994). The 55 kDa E1B protein (E1B55K) binds to and inhibits p53 (Sarnow *et al.* 1982a, Kao *et al.* 1990, Yew and Berk 1992, Yew *et al.* 1994), a cellular transcriptional regulator which induces growth arrest and/or apoptosis following the induction of unscheduled DNA synthesis or DNA damage (reviewed in (Ko and Prives 1996)). We have shown recently that E1B55K is able to block p53-dependent apoptosis induced by E1A (Teodoro and Branton 1997) or by expression of p53 in p53-null Saos-2 human osteosarcoma cells (Marcellus *et al.* 1996a). Thus, one role of E1B products in both productive infection and cell transformation appears to be to prevent untimely E1A-induced cell death, thus permitting production of high yields of progeny or survival of transformants. In productively infected human cells, E1B55K also interacts with the E4orf6 protein to form complexes which enhance accumulation and transport of late viral mRNAs and induce shutoff of host cell protein synthesis (Babiss and Ginsberg 1984, Sarnow *et al.* 1984, Babiss *et al.* 1985, Halbert *et al.* 1985, Pilder *et al.* 1986, Weinberg and Ketner 1986, Cutt *et al.* 1987, Leppard and Shenk 1989, Sandler and Ketner 1989, Bridge and Ketner 1990, Ornelles and Shenk 1991).

It is now clear that E1A induces apoptosis by two mechanisms, one requiring p53 (Debbas and White 1993, Lowe *et al.* 1993) and the other via a p53-independent pathway involving one or more viral E4 proteins (Subramanian *et al.* 1995, Teodoro *et al.* 1995, Marcellus *et al.* 1996b). It has been known for some time that expression of E1A induces p53 protein accumulation (Braithwaite *et al.* 1990, Grand *et al.* 1993, Lowe and Ruley 1993, Grand *et al.* 1994, Grand *et al.* 1996). Recently, we have found that such increases in p53 levels are induced by complex formation with p300 or, in the case of human cells, with pRb family members (Querido *et al.* 1997b). The molecular basis of this p53 accumulation remains uncertain. One possibility is that E1A products somehow increase the stability of p53, which has a half-life of about 20 minutes or so in most normal cells. In stable E1A-transformed cell lines, p53 half-lives of over 2 h have been reported, especially in cells that

also express E1B55K (Grand *et al.* 1993, Lowe and Ruley 1993, Grand *et al.* 1994, Grand *et al.* 1996). p53 is usually cytoplasmic in cells containing E1B55K, whereas it is largely nuclear in the absence of this E1B product (Zantema *et al.* 1985b, van den Heuvel *et al.* 1990). In some cell lines, two populations of p53 appear to exist, one with a normal short half-life and another containing more long-lived species (Grand *et al.* 1993, Grand *et al.* 1996). In addition, the 55 kDa E1B protein, or the equivalent 54 kDa product in serotype 12 (Ad12) has been shown to stabilize p53 in the absence of E1A (van den Heuvel *et al.* 1990, Grand *et al.* 1993). However, transformants in which little change in p53 half-life is apparent also exist (Grand *et al.* 1993, Grand *et al.* 1995). A second possibility is that E1A products increase expression of p53 mRNA. Braithwaite *et al.* have reported such an induction of p53 transcription following adenovirus infection (Braithwaite *et al.* 1990). More recently, it has also been suggested that p53 expression may be regulated at the translational level (Mosner *et al.* 1995, Fu *et al.* 1996).

In the present study, we report that although the E1A 243R protein induces the accumulation p53, such is not the case with E1A-289R. We show that this suppression of p53 accumulation requires the E4orf6 protein, which we confirm is able to bind directly to p53 *in vitro*. Furthermore, the E1B 55 kDa species is also required, suggesting that multiple interactions involving p53, E1B55K, and E4orf6 might play a role in preventing the E1A-induced accumulation of p53.

3.3 Materials and methods

3.3.1 Cells and viruses

Normal MRC-5 (ATCC CCL 171) and IMR-90 (ATCC CCL 186) diploid human embryonic lung fibroblasts were cultured on 60 mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in α -modified minimal essential medium (α -MEM) supplemented with 10% fetal calf serum. The MRC-5 cell line was shown to contain wild-type p53 and is not

immortalized (Crook *et al.* 1991). The cells used for the present studies were from passages 6 to 11. Human 293 cells were derived from human embryonic kidney cells and express Ad5 E1A and E1B proteins (Graham *et al.* 1977). Human HeLa and KB cells, as well as mouse 10T1/2 cells (Reznikoff *et al.* 1973), were also cultured as described above. Cells were infected with wild-type (*wt*) or mutant virus at a multiplicity of 35 PFU per cell, as was described previously (Rowe *et al.* 1984a). For double infections, the viruses were premixed before infection at a multiplicity of 35 PFU per cell for each virus. The *wt* Ad5 used in most of the present studies was described by Harrison *et al.* (Harrison *et al.* 1977), although in some cases, including for the preparation of all E1A and E1B mutants, *dl309* (Jones and Shenk 1979) was used as *wt*. Some experiments required mutant *pm975* (Montell *et al.* 1982) which expresses E1A-289R but not 243R, or mutant *dl520* (Haley *et al.* 1984) which produces E1A-243R but not 289R. The mutant 12S/E1B⁻ (originally *dl520T*) produces only the E1A-243R protein and no E1B products (Shepherd *et al.* 1993). The mutant E1B⁻ expresses both major E1A products but no E1B species (Teodoro *et al.* 1995). Mutant E1B/55K⁻ (originally *pm2019/2250*) fails to express the E1B55K protein and mutant E1B/19K⁻ (originally *pm1716/2072*) does not express the E1B19K polypeptide (McLorie *et al.* 1991). Additional E1B55K protein mutants included *pm490/1A* and *pm490/1/5A* which contain Alanine residues in place of the Ser-490, Ser-491 and Thr-495 phosphorylation sites, thus reducing transforming and transcriptional repression activities and the ability to inhibit p53 activity, but not affecting complex formation with p53 (Teodoro *et al.* 1994, Teodoro and Branton 1997), and mutants A262 and R309 which contain insertions next to residues Ala-262 and Arg-309 which block interactions with p53 (Kao *et al.* 1990, Yew *et al.* 1990). E1A and E1B mutant viruses were propagated on 293 cells. Figure 3.4 illustrates a series of Ad5 E4 deletion mutant viruses used for mapping studies, including *dl1010*, *dl1011*, *dl1013*, *dl1014*, *dl1015* and *dl1019*, as described previously (Bridge and Ketner 1990, Bridge *et al.* 1993). These viruses, which express all E1A and E1B products, were propagated on W162 monkey cells, as described previously (Weinberg and Ketner 1983).

The AdLacZ adenovirus vector in which the E1A and E1B regions have been replaced by the *Escherichia coli* gene *lacZ* under the control of the cytomegalovirus (CMV) promoter (Bacchetti and Graham 1993) was provided by Frank Graham. The AdHH55K vector, expressing the Ad5 E1B55K protein under the CMV promoter in an adenovirus vector that lacks both E1A and E1B, was described previously (Marcellus *et al.* 1996a). The AdE4orf6 vector expressing adenovirus type 2 (Ad2) E4orf6 under the control of the CMV promoter was made by introducing an appropriate cDNA (Ohman *et al.* 1993) into the same adenovirus vector.

3.3.2 Western blotting analysis

Cell extracts were prepared on ice in lysis buffer (50 mM HEPES, pH 7.9, containing 400 mM KCl, 0.1% [vol/vol] Nonidet P-40, 4 mM NaF, 4 mM NaVO₄, 0.2 mM EDTA, 0.2 mM EGTA, 1 µg [each] of aprotinin, leupeptin and pepstatin per ml, 0.5 mM PMSF, and 1 mM DTT). Total protein was measured by a Bio-Rad protein assay according to the manufacturer's specification, and 50 µg of total cell protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with either 10% or 12% polyacrylamide gels. The material was transferred to nitrocellulose membranes for 1 h in a semidry transfer apparatus, and the membranes were probed with primary antibodies which recognize p53, E1A proteins, E1B55K, or certain E4 proteins. Anti-p53 mouse monoclonal antibody Ab1801 (Ab-2; Oncogene Science) was purified by precipitation with ammonium sulfate from supernatants obtained from hybridoma cell cultures grown in Dubelcco's minimal essential medium (D-MEM) supplemented with 10% low IgG fetal calf serum (BRL). Anti-p53 mouse monoclonal antibody Ab421 was obtained from Oncogene Science or, as in the cases of mouse monoclonal antibodies M73 against E1A proteins (Harlow *et al.* 1985a) and 2A6 against E1B55K (Sarnow *et al.* 1982b), was collected as ascites fluid generated in mice, with appropriate hybridoma cells. The M45 mouse monoclonal antibody against the amino-terminal portion of the Ad2 E4orf6 and E4orf6/7 proteins (Obert *et al.*

1994) was generously provided by Pat Hearing. Binding patterns were determined by addition of a secondary antibody, horseradish peroxidase conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories), and the signal was detected by an enhanced chemiluminescence (ECL) reagent (Dupont NEN).

3.3.3 Measurement of p53 half-life

293 cells were grown on 60 mm-diameter plates and mock infected or infected with AdLacZ virus, and at 7.5 hours postinfection (hpi), the cells were labeled for 15 minutes with 100 μ Ci of [^{35} S]methionine (1175 Ci/mmol; Dupont NEN) per plate and then incubated further for various times with α -MEM supplemented with 15 mg of L-methionine (ICN Biochemicals) per ml. The cells were then lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl, pH 7.2, containing 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 500 mM Sodium vanadate, 50 mM NaF, 1 mM β -glycerol phosphate, and 100 KIU of aprotinin] and immunoprecipitated with a mixture of Ab1801 and Ab421. The labeled p53 was detected on a Fuji BAS-III imaging plate, and the amount of radioactivity was quantified with a Fujix Bas 2000 PhosphorImager.

3.3.4 Host cell shutoff and p53 translation

Cells were grown on 60 mm-diameter plates and infected with *wt* or mutant Ad5. At the indicated times following infection, cells were labeled for 1 h with 100 μ Ci of [^{35} S]methionine (1,175 Ci/mmol; Dupont NEN) per plate and harvested. To analyze host cell shutoff, cell lysates were prepared in the same lysis buffer used for Western blotting, and 50 μ g of total cell protein, as determined by Bio-Rad assays, were analyzed by SDS-PAGE (10% polyacrylamide). To analyze synthesis of p53, cell lysates were prepared in RIPA buffer and immunoprecipitated with a mixture of Ab1801 and Ab421. In all cases, protein patterns were visualized by autoradiography with Kodak X-OMAT AR film. In

addition, the labeled p53 was detected as described above on a Fuji BAS-III imaging plate, and the radioactivity was quantified with a Fujix Bas 2000 PhosphorImager.

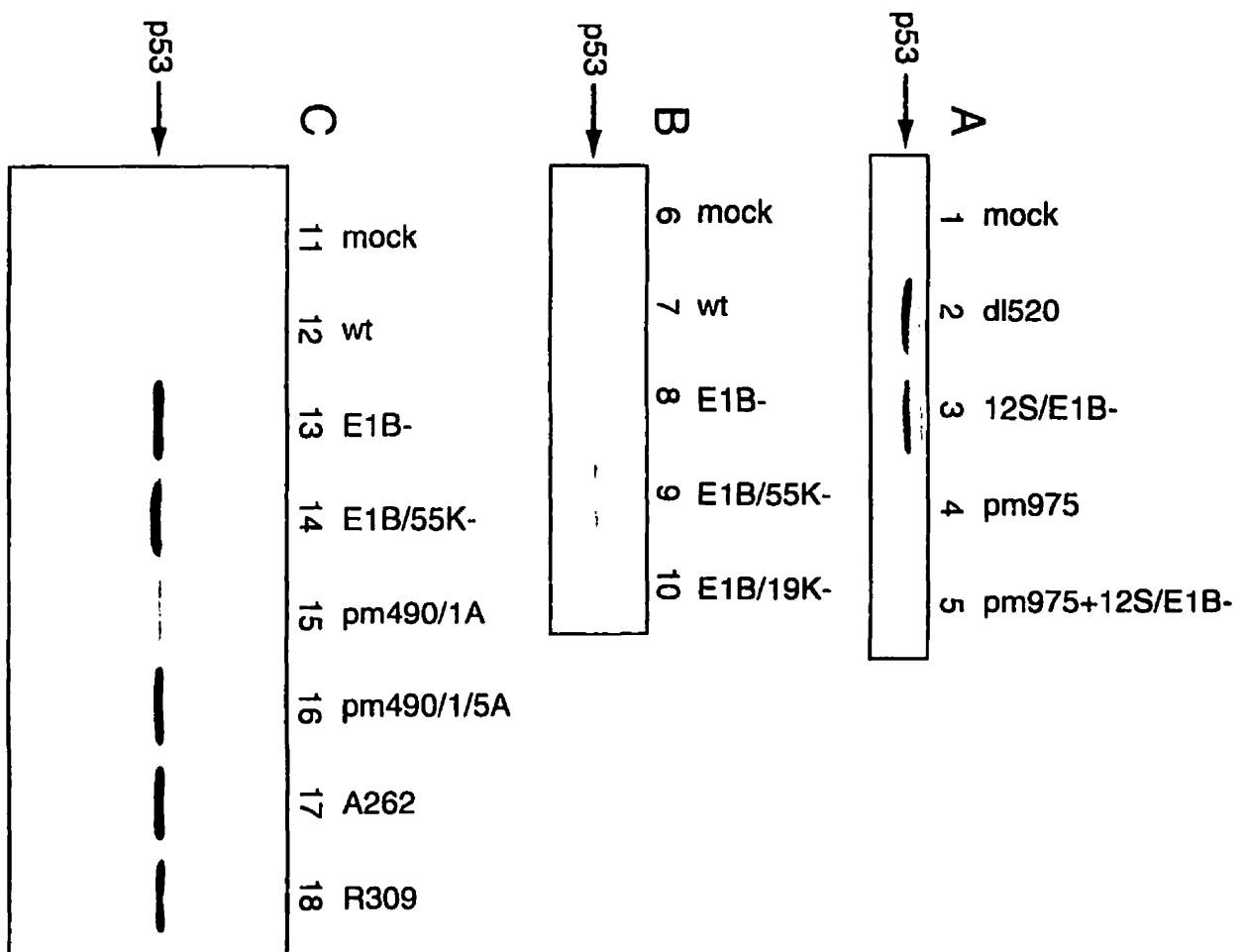
3.3.5 Immunoprecipitation and E4orf6 protein binding assays

For *in vitro* E4orf6 binding assays, cell extracts were prepared in 500 μ l of RIPA buffer from 293 cells grown on 100 mm-diameter plates and harvested at 7 and 16 hpi with *wt* Ad5. E4orf6 and E4orf6/7 proteins were immunoprecipitated with M45 antibody in the presence of a mixture of protein A and protein G-Sepharose (Pharmacia). Following extensive washing with RIPA buffer, the precipitates were incubated in buffer G (20 mM Tris-HCl, pH 6.8, containing 137 mM NaCl, 0.1% [vol/vol] Nonidet P-40, 1 mM EDTA, 2 mg/ml BSA, 1 μ g [each] of aprotinin, leupeptin, and pepstatin per ml, and 0.5 mM PMSF) containing only nonionic detergent, in the presence of [^{35} S]methionine-labeled proteins synthesized by an *in vitro* transcription-translation system. DNA from expression plasmids P53pSP65 containing the murine p53 cDNA (Kao *et al.* 1990) and pGEM-55K containing the Ad5 E1B55K open reading frame under the T7 promoter (Teodoro *et al.* 1994) was linearized, and transcription was performed with 2 μ g of DNA and SP6 or T7 RNA polymerase (Pharmacia). Translation of the resulting mRNAs was performed in a 50 μ l translation reaction volume containing 35 μ l of pretreated rabbit reticulocyte lysate (Promega) in the presence of 40 μ Ci of translation grade [^{35} S]methionine (1,175 Ci/mmol; Dupont NEN). The precipitates were incubated for 2 h in the presence of [^{35} S]methionine-labeled p53 and E1B55K proteins before extensive washing in buffer G. Bands were visualized by autoradiography after separation by SDS-PAGE on a 10% polyacrylamide gel.

3.4 Results

3.4.1 Expression of the 243R but not the 289R E1A protein induces an accumulation of p53

It has previously been reported that expression of human adenovirus E1A protein results in the accumulation of p53 (Braithwaite *et al.* 1990, Debbas and White 1993, Grand *et al.* 1993, Lowe and Ruley 1993, Chiou *et al.* 1994, Grand *et al.* 1994, Sanchez-Prieto *et al.* 1995, Grand *et al.* 1996). Figure 3.1A shows the results of an experiment in which extracts of human MRC-5 cells infected with various Ad5 viruses or mock-infected were analyzed following SDS-PAGE by Western blotting with an anti-p53 antibody. After infection with mutant *dl520* which produces the E1A-243R protein but not E1A-289R, the level of p53 was seen to increase by about 8- to 10-fold relative to that in mock-infected cells (compare lanes 2 and 1, respectively). A similar increase was observed with mutant *12S/E1B⁻*, which produces only E1A-243R and no E1B products (lane 3). Such was not the case in cells infected with *pm975*, which produces the E1A-289R protein but not E1A-243R. In these cells little increase in p53 was observed (lane 4) and levels were similar to those in mock-infected cells (lane 1). Analysis of the levels of E1A proteins synthesized by these (and all other) mutants in Fig. 3.1D, by Western blotting with anti-E1A M73 monoclonal antibody, indicated that *pm975* actually yielded slightly smaller amounts than did *dl520* (Fig. 3.1D, compare lanes 4 and 2). Thus, the failure to accumulate p53 could possibly have been due to the presence of smaller amounts of E1A protein; however, such did not appear to be the explanation, as in cells doubly infected with *pm975* and *dl520*, the accumulation of p53 induced by E1A-243R of *dl520* was completely blocked by expression of even lower levels of 289R by *pm975* (Fig. 3.1A, lane 5). Figure 3.1B shows that infection of MRC-5 cells by *wt* Ad5, which expresses both E1A-289R and 243R, also does not result in any increase in p53 compared with the case for mock-infected cells (Fig 3.1B, compare lanes 7 and 6, respectively). However, infection with mutant E1B⁻ (lane 8), which produces both major E1A products but no E1B proteins, resulted in the accumulation of p53, suggesting that one



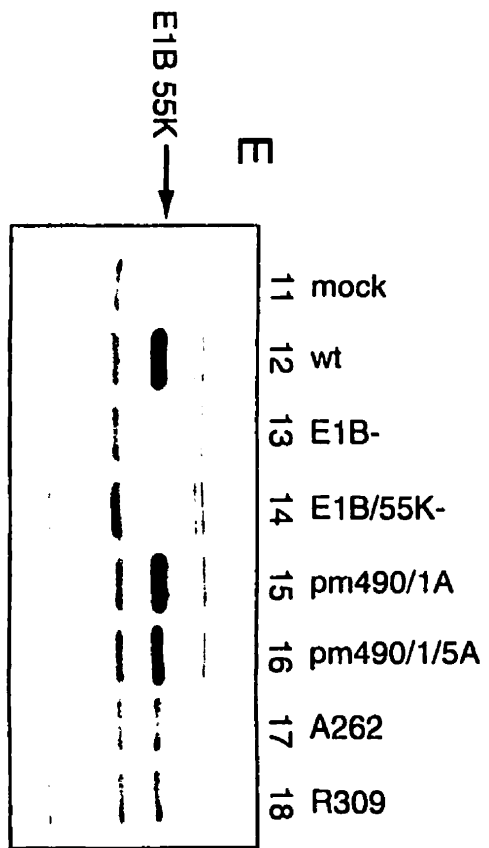
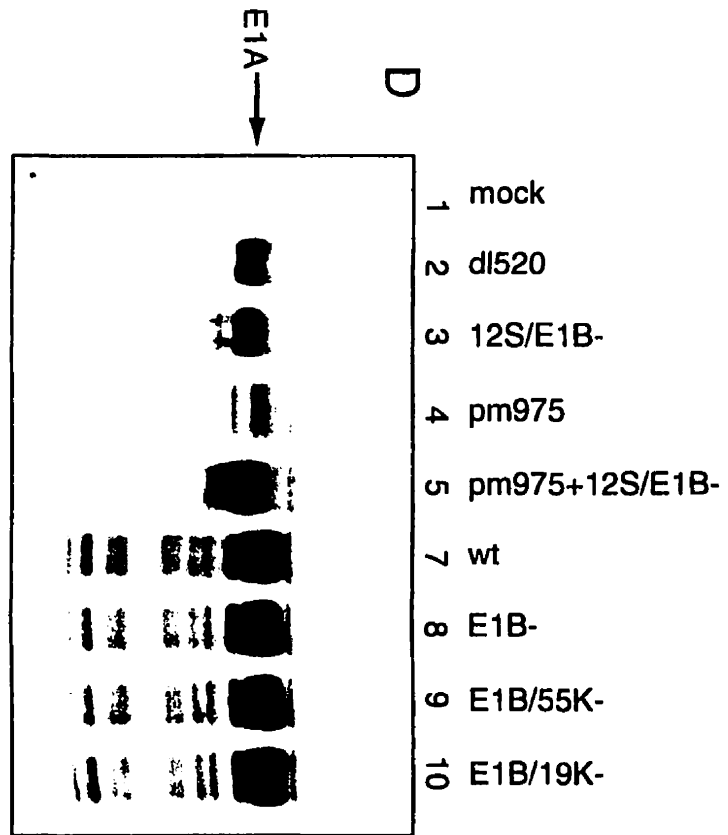


Figure 3.1. Effects of E1A and E1B products on the accumulation of p53

MRC-5 cells were infected with *wt* or mutant Ad5, and cell extracts were prepared at 24 hpi and separated by SDS-PAGE. (A through C) Analysis of p53 levels. p53 levels were determined by Western blotting with Ab1801 monoclonal antibody. (D) Analysis of E1A protein levels. The amounts of E1A proteins present were determined by immunoblotting with M73 monoclonal antibody. (E) Analysis of E1B-55K protein levels. Levels of the 55 kDa protein were determined by immunoblotting with 2A6 monoclonal antibody. Binding patterns were determined by ECL.

or more E1B proteins were required for the inhibition of p53 accumulation. To determine which E1B product was responsible for this effect, cells were infected with mutants that expressed both major E1A products, but only one of the major E1B proteins. Expression of the E1B19K protein but not the E1B55K product, with mutant E1B/55K⁻, resulted in the accumulation of p53 (lane 9), whereas in the presence of the 55 kDa protein but not the 19 kDa polypeptide with mutant E1B/19K⁻, this accumulation did not occur (lane 10). Identical results were also obtained with the IMR-90 human cell line (data not shown). Thus, these results indicated that expression of E1A-289R prevented the accumulation of p53 induced by the E1A-243R protein alone and that this inhibition required the expression of E1B55K.

3.4.2 Inhibition of E1A-induced accumulation of p53 requires a functional E1B55K protein

The 55 kDa E1B product possesses intrinsic repression activity which is believed to block the action of the p53 activation domain following complex formation with p53 (Yew *et al.* 1994, Teodoro and Branton 1997). To examine the role of this E1B protein in the inhibition of E1A-induced accumulation of p53, the levels of p53 protein were examined in extracts from E1B mutant-infected cells by Western blotting. Figure 3.1C shows again that in MRC-5 cells infected with *wt* Ad5 (lane 12), the levels of p53 were similar to those in mock-infected cells (lane 11), whereas with mutants E1B⁻ and E1B/55K⁻, which express no E1B55K (lanes 13 and 14, respectively), increased levels of p53 were evident. Studies were also conducted with mutants *pm490/1A* and *pm490/1/5A*, which contain Ala residues in place of the Ser-490, Ser-491, and Thr-495 phosphorylation sites of E1B55K. These alterations have been found to diminish cell transformation dependent on the 55 kDa protein, reduce its ability to inhibit p53 activation activity, and eliminate its transcriptional repression activity without affecting the efficiency of binding to p53 or the induction of host cell shutoff (Teodoro *et al.* 1994, Teodoro and Branton 1997). The precise role of phosphorylation in the regulation of these functions has still not been established. Figure 3.1C (lanes 15 and 16)

shows that, in both cases, significant increases in p53 levels were observed. The increase was greater with *pm490/1/5A*, which encodes an E1B55K that is also more defective than that produced by *pm490/1A*, both in endogenous repression activity and in cooperation with E1A in cell transformation (Teodoro and Branton 1997). Thus, complex formation with p53 is not sufficient for inhibition of accumulation of p53, which appears to require a functional E1B55K repression domain. To analyze the role of the 55 kDa protein further, two additional mutants A262 and R309, which contain insertions in the p53 binding region, thus blocking complex formation with p53 but not affecting transcriptional repression, were examined (Kao *et al.* 1990, Yew *et al.* 1990). Figure 3.1C (lanes 17 and 18) shows that, with both of these mutants, accumulation of p53 occurred at high levels, suggesting that binding to p53 may be required. Levels of expression of 55 kDa proteins by all the E1B55K mutants were measured by immunoblotting with 2A6 monoclonal antibody. Figure 3.1E shows that mutants yielded quantities approaching those of the wt (lane 12), although as shown previously (Yew *et al.* 1990), the levels of both A262 and R309 (lanes 17 and 18) were somewhat lower, due possibly to reduced protein stability. Thus, inhibition of E1A-induced accumulation of p53 requires an E1B55K protein that possesses intrinsic repression activity and, most likely, that can bind to p53.

3.4.3 Inhibition of E1A-induced accumulation of p53 requires an additional viral protein and a shortening of the half-life of p53

The data just presented indicated that expression of the E1A-289R protein and the E1B55K product resulted in the inhibition of E1A-induced accumulation of p53. Of some interest in this context are 293 cells which express Ad5 E1A 289R and 243R proteins and E1B products, yet accumulate very high endogenous levels of p53 protein (Grand *et al.* 1995). It was possible that this accumulation in the presence of E1A-289R (and that found in other E1A/E1B transformed cells) could occur either because 293 cells lack a cellular protein required for the inhibition of E1A-induced p53 accumulation or because such inhibition

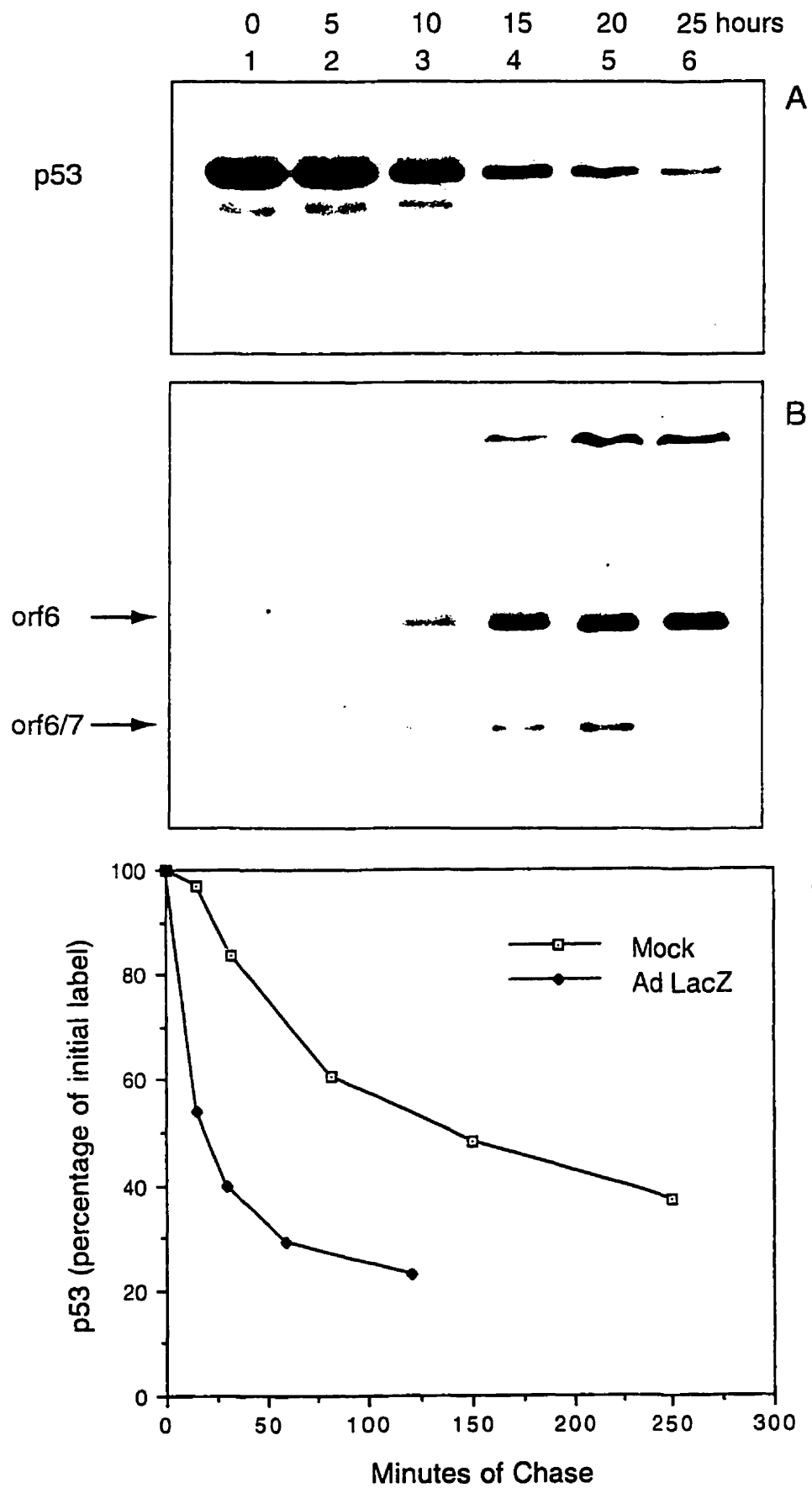
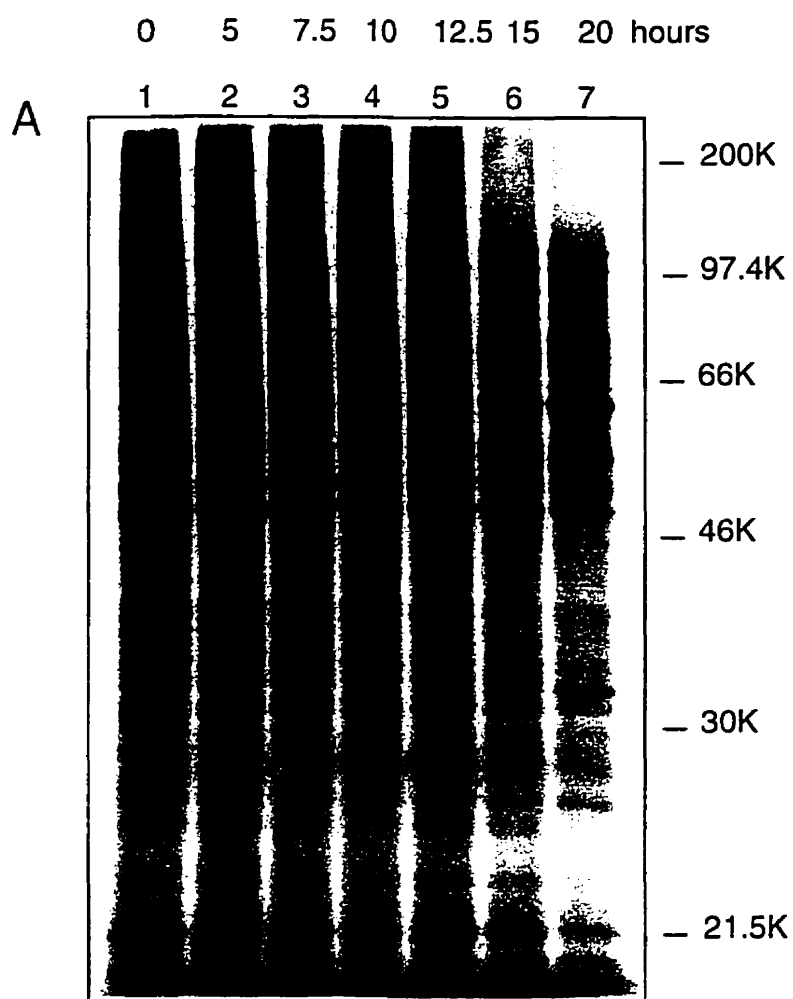


Figure 3.2. Analysis of p53 and E4orf6 levels in 293 cells infected by AdLacZ vector

Human 293 cells were infected with AdLacZ. In some cases, cells were harvested at various time after infection and whole-cell extracts were separated by SDS-PAGE. Following transfer to nitrocellulose, the levels of p53 and E4orf6 were determined by Western blotting. In other cases cells were labeled at 7.5 hpi for 15 min with [³⁵S]methionine, and the half-life of p53 proteins was measured in pulse-chase experiments. (A) Analysis of p53 levels. Amounts of p53 were determined by Western blotting with anti-p53 antibody Ab1801 and ECL. The position of migration of p53 has been shown at the left. (B) Analysis of E4orf6 levels. Amounts of E4orf6 were determined by Western blotting with M45 antibody and ECL. The positions of migration of E4orf6 and E4orf6/7 are shown at the left. (C) Determination of p53 half-life. Mock- and AdLacZ-infected 293 cells were analyzed in a pulse-chase experiment commencing at 7.5 hpi. Labeled p53 was immunoprecipitated with a mixture of Ab1801 and Ab421, and the amount of labeled p53 was measured with a PhosphorImager and was plotted as a percentage of the value at time zero. The data presented are from one representative experiment of four independent analyses.

requires an additional viral protein encoded by a region other than E1A and E1B. To address this question, 293 cells were infected with the adenovirus vector AdLacZ which contains the β -galactosidase gene in place of E1A and E1B, and the levels of p53 were determined by Western blotting of extracts prepared at various times following infection. Figure 3.2A shows that high levels of p53 were evident in uninfected 293 cells; however, following infection, the amount began to diminish by as early as 10 hpi (lane 3) and continued to decrease up to 25 hpi (lane 6). Thus, a reduction in the accumulation of p53 occurred in 293 cells after introduction of the viral genome, suggesting that diminished levels of p53 resulted from the expression of one or more viral proteins in addition to E1A and E1B products.

It was possible that infection of 293 cells by AdLacZ provided some factor that rendered p53 proteins less stable, thus reducing p53 levels. Figure 3.2C shows the results of a representative pulse-chase experiment in which the half-life of p53 was determined in both AdLacZ-infected and mock-infected 293 cells. p53 half-life in mock-infected 293 cells was found to be about 2.5 h, while in AdLacZ-infected cells the half-life, as measured at 7.5 hpi, was about 20 minutes. Thus, expression of additional viral genes is responsible for a decrease in the stability of the p53 protein. This decreased stability could therefore account for some or all of the reduction in p53 levels. It was also possible that some of the decrease in p53 levels following infection with AdLacZ could be linked to generalized host cell shutoff, a process requiring both the E1B55K protein and E4orf6. To examine the relationship between host cell shutoff and the observed decreases in p53 levels, whole-cell extracts from 293 cells which had been labeled for 1 h with [^{35} S]methionine at various times after infection with AdLacZ were analyzed by SDS-PAGE. Figure 3.3A shows that shutoff of host protein synthesis began between 12.5 and 15 hpi, as was evident by the decrease in labeling of most cellular proteins (see lanes 5 to 7). Thus, this effect did not correlate with decreases in p53 levels which, as shown previously in Fig. 3.2A, began several hours earlier, between 5 and 10 hpi. In addition to examining the whole-cell protein pattern (Fig. 3.3A), extracts from these [^{35} S]-labeled AdLacZ-infected 293 cells were immunoprecipitated



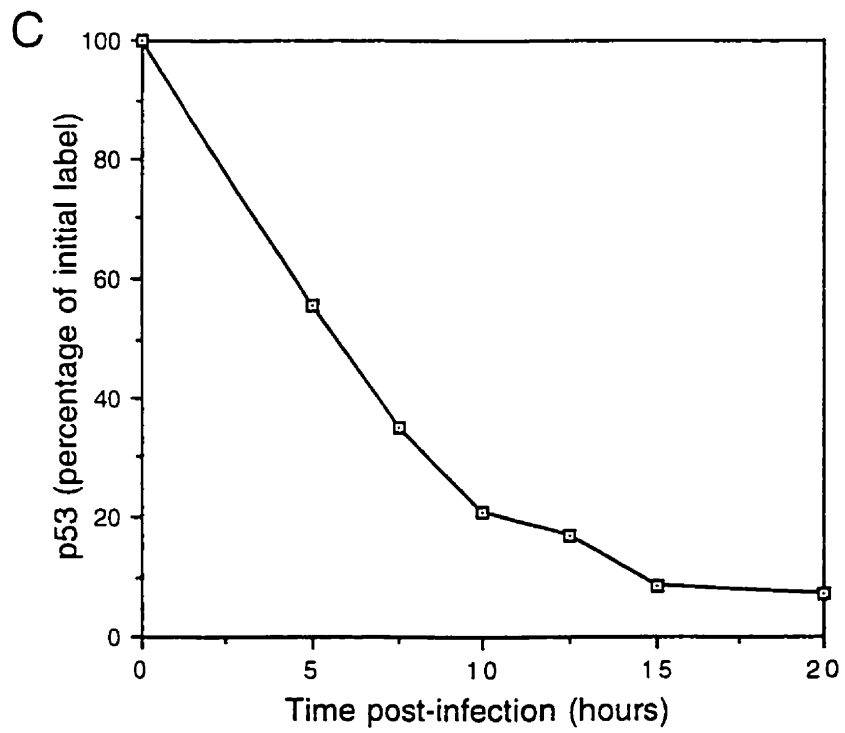
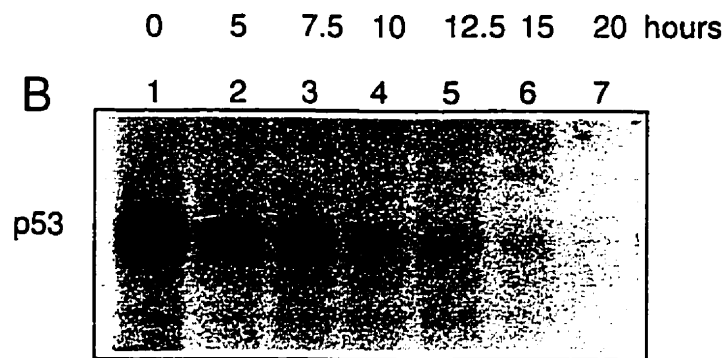


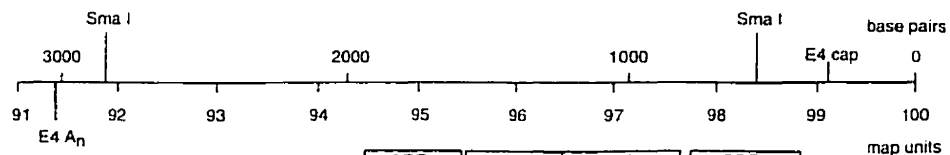
Figure 3.3. Time course of host cell shutoff and p53 synthesis

Human 293 cells were infected with AdLacZ, and at various times postinfection they were labeled for 1 h with [^{35}S]methionine and cell extracts were prepared. (A) Analysis of whole-cell protein synthesis. From each sample, 50 mg of total cell protein was analyzed by SDS-PAGE followed by autoradiography. The positions of ^{14}C -labeled molecular mass markers are shown on the right. (B) Analysis of p53 synthesis. Equal aliquots of the cell extracts shown in panel A were immunoprecipitated using a mixture of Ab1801 and Ab421 anti-p53 antibodies, and the precipitates were examined by SDS-PAGE. The position of migration of p53 is shown at the left. (C) Quantitative analysis of p53 synthesis. The amount of labeled p53 was measured by PhosphorImager and was plotted as a percentage of the value at time zero (uninfected cells).

with a mixture of Ab1801 and Ab421 anti-p53 antibodies, and the precipitates were analyzed by SDS-PAGE. Figures 3.3B and C show that synthesis and accumulation of p53 during the various 1 h intervals were reduced to approximately 50% of initial levels by about 5 to 6 hpi and continued to decrease up to about 15 to 20 hpi. Thus, following infection of 293 cells with AdLacZ, a specific decrease in the production of p53 appeared to take place, commencing several hours before the generalized shutoff of host cell protein synthesis in these productively infected cells. It remains possible, however, that host cell shutoff affects synthesis of p53 more acutely than that of other proteins. This possibility will be readdressed in separate experiments described below (see Fig. 3.9). Thus, while a decrease in the rate of translation of p53 could account for some of the drop in p53 production, it is quite possible that the 7- to 8-fold reduction in p53 half-life reported above in Fig. 3.2C could account for all or most of this effect.

3.4.4 The E4orf6 protein is required to inhibit the E1A-induced accumulation of p53

Although it was possible that any of a number of proteins encoded by the E2, E3 and E4 regions, or even late viral products, could function in the prevention of accumulation of p53, the E4orf6 protein was a prime candidate, because it was already known to interact with the E1B55K protein and to play a cooperative role in host cell shutoff (Weinberg and Ketner 1986, Bridge and Ketner 1989, Sandler and Ketner 1989, Bridge and Ketner 1990, Sandler and Ketner 1991, Bridge *et al.* 1993). In addition, recent studies had argued that E4orf6-dependent host cell shutoff may be involved in the regulation of p53 levels (Grand *et al.* 1996). To analyze this possibility, we used a series of Ad5 deletion mutants which are defective in expression of various combinations of the seven E4 products. Figure 3.4 shows that *d11011* and *d11019* fail to produce any E4 products, whereas *d11013* produces E4orf4 and E4orf6, *d11014* yields E4orf4 alone, *d11015* synthesizes only E4orf3 and E4orf4, and



		Expressed Proteins
dl 1011	————— —————	none
dl 1013	■————— —————	Orf 4, 6
dl 1014	————— —————	Orf 4
dl 1015	————— —————	Orf 3, 4
dl 1019	————— ————— x ————— —————	none
dl 1010	————— —————	Orf 1, 2, 3, 4, 6/7

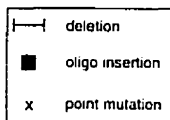


Figure 3.4. E4 open reading frames and deletion mutants

The E4 region occupies about 3,000 bp at the right end of the adenovirus genome and contains open reading frames (Orfs) for at least seven proteins. The Ad5 E4 deletion mutant viruses used in the present studies, including *dl1010*, *dl1011*, *dl1013*, *dl1014*, *dl1015*, and *dl1019*, have been described previously.

dl1010 expresses all E4 products except E4orf6 (Bridge and Ketner 1990, Bridge *et al.* 1993). Studies similar to those whose results are shown in Figs. 3.1 and 3.2 were conducted in human MRC-5 and 293 cells which were infected by *wt* Ad5 or various E4 deletion mutants. Cell extracts were analyzed for the amount of p53 and E4orf6 proteins by Western blotting with anti-p53 monoclonal antibodies or a mouse monoclonal antibody M45 which recognizes the amino termini of the E4orf6 and E4orf6/7 proteins (Obert *et al.* 1994). The levels of E1A products were determined by Western blotting with M73 anti-E1A monoclonal antibody and found to be comparable in all cases (data not shown). Figure 3.5A shows that no accumulation of p53 occurred following expression of *wt* Ad5 (lane 2) and that the amounts of p53 were low and similar to those in mock-infected cells (lane 1). The levels of p53 were similarly low in cells infected with mutant *dl1013* (Fig. 3.5A, lane 4), which produces only E4orf4 and E4orf6. However, with all other E4 mutants, the accumulation of p53 to high levels was evident. This effect was also observed with *dl1010* (Fig. 3.5A, lane 5), which produces all viral proteins with the exception of E4orf6.

These results indicated that E4orf6 is required for the inhibition of p53 accumulation. This conclusion was confirmed in experiments involving 293 cells infected by these E4 mutant viruses. Figure 3.5B shows that the high levels of p53 observed in mock-infected 293 cells (lane 1) were greatly reduced by infection with *wt* Ad5 (lane 2) and mutant *dl1013* (lane 4) but not with the other mutants (lane 3 and 5 to 8). Figure 3.5C shows the pattern of expression of the E4orf6 and E4orf6/7 proteins, which share common amino-terminal sequences recognized by the M45 monoclonal antibody employed in this analysis. Reduction of p53 levels occurred only following infection with viruses that express E4orf6, including *wt* Ad5 (Fig. 3.5C, lane 2) and *dl1013* (Fig. 3.5C, lane 4). Further analysis of the time course of infection of 293 cells by AdLacZ shown previously in Fig. 3.2 also indicated that the reduction in p53 levels closely paralleled the appearance of the E4orf6 protein (compare to Fig. 3.2B and A). Thus, E4orf6 appeared to be required, along with E1B55K, for the reduction in p53 levels.

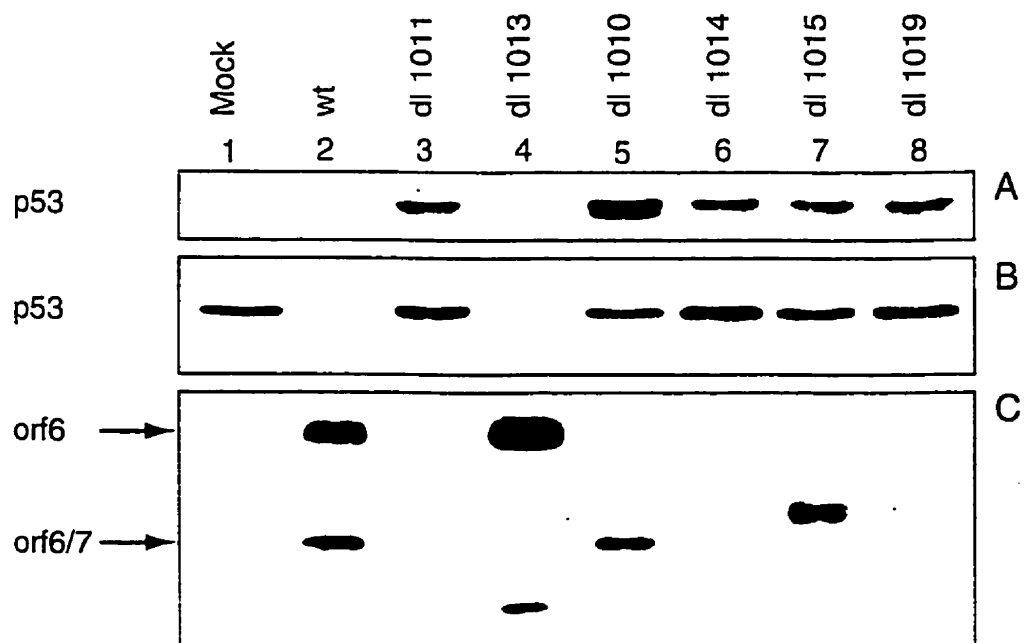


Figure 3.5. Analysis of p53 accumulation and E4orf6 expression in *wt* and E4 mutant-infected cells

Human MRC-5 or 293 cells were infected with *wt* Ad5 or a series of Ad5 E4 mutants described in the legend to Fig. 3.4. At 24 hpi, cell extracts were prepared, proteins were separated by SDS-PAGE and transferred to nitrocellulose. (A) p53 in MRC-5 cells; (B) p53 in 293 cells; (C) E4orf6 (and E4orf6/7) in 293 cells. Amounts of p53 were determined by Western blotting with Ab1801 and ECL. The membrane used for panel B was stripped and reprobed with anti-E4orf6 monoclonal antibody M45 and ECL. The positions of migration of p53, E4orf6, and E4orf6/7 are shown at the left.

Figure 3.5C also contains additional information. The small truncated form of the E4orf6/7 protein encoded by mutant *dl1013* and containing amino-terminal sequences (see Fig. 3.4 for reference) was evident in lane 4 of Fig. 3.5C. Of more interest was the truncated form of E4orf6 produced by mutant *dl1010* (Fig. 3.5C, lane 5). Figure 3.4 illustrates that the out-of-frame deletion present in *dl1010* is predicted to yield an E4orf6 protein which contains only the first 96 amino-terminal residues of the 294-residue product of molecular mass of about 34 kDa (Bridge and Ketner 1989). This truncated product appeared to be synthesized and to be relatively stable (see Fig. 3.5C, lane 4), but it did not appear to retain the p53 inhibitory function of the full-length E4orf6.

3.4.5 E4orf6, p53 and E1B55K each form complexes individually

It is known that the E1B55K protein interacts directly and independently with both p53 (Sarnow *et al.* 1982a) and the E4orf6 product (Sarnow *et al.* 1984). To investigate the molecular basis of the inhibition of E1A-induced accumulation of p53 further, interactions between E4orf6 and p53 or E1B55K were investigated with both *in vitro* binding assays and coimmunoprecipitation from cell extracts. Extracts from mock-infected 293 cells or those infected with *wt* Ad5 and harvested at either 7 or 16 hpi were combined with M45 monoclonal antibody, which recognizes the amino terminus of the E4orf6 (and E4orf6/7) protein, and subjected to immunoprecipitation in the presence of ionic detergent, as described in Materials and Methods. Following extensive washing, precipitates were resuspended in buffer lacking ionic detergent and thus capable of maintaining protein-protein interactions, and the mixtures were incubated with [³⁵S]methionine-labeled p53 which had been transcribed and translated *in vitro* from a p53-specific cDNA. E4orf6 proteins were repurified by immunoprecipitation under mild conditions and analyzed by SDS-PAGE. Figure 3.6 shows that high levels of p53 as well as several truncated forms of this species were synthesized (lane 1). Addition of labeled p53 to precipitates from mock-infected cells was without effect, as no labeled species were apparent (lane 2). However, in the case of

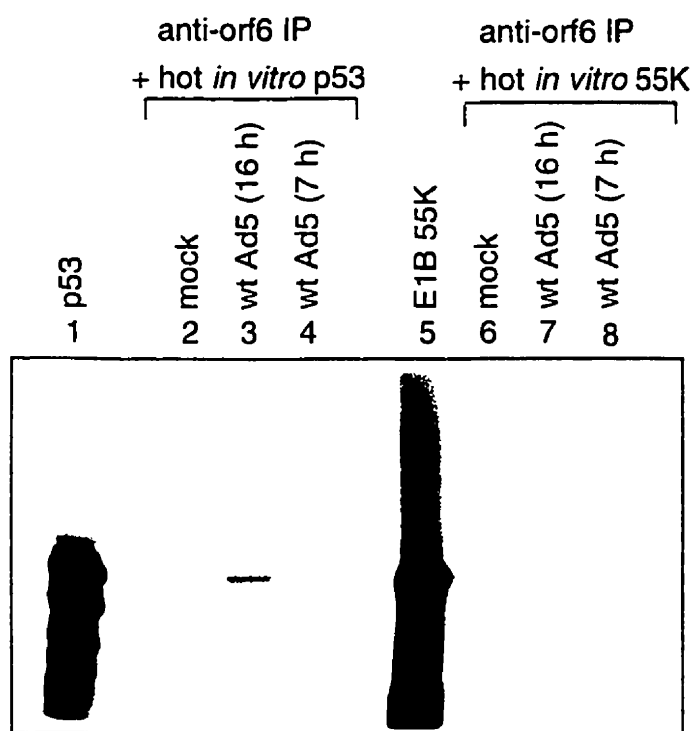


Figure 3.6. *In vitro* binding of E4orf6 to p53 and E1B-55K

Mock- or Ad5-infected 293 cells were harvested at 7 and 16 hpi, and E4orf6 protein was purified by immunoprecipitation (IP) with M45 antibody. Following extensive washing in buffer containing ionic detergent, the precipitates were incubated with *in vitro* -synthesized [³⁵S]methionine-labeled p53 or E1B-55K protein, and the amount of binding was determined by SDS-PAGE followed by autoradiography.

precipitates from infected cells, material which comigrated with full-length p53 was present (lanes 3 and 4). More p53 was present in the sample prepared with material isolated from cells at 16 hpi (lane 3) than at 7 hpi (lane 4), in proportion to the relative amounts of E4orf6 present at these times (data not shown). Parallel experiments were also performed with *in vitro* transcribed and translated E1B55K. Figure 3.6 shows that this protein also interacted with E4orf6 precipitates from infected cells (lanes 7 and 8) but not mock-infected cells (lane 6) in a similar fashion. (Note: the low levels present in the sample from mock-infected cells in Fig. 3.6, lane 6, were due to spillover from lane 5 and were not present in other analyses.) While the present work was being completed, a report was published which also established the existence of complex formation between p53 and E4orf6 and suggested that such interactions could lead to the inhibition of p53 transactivation activity (Dobner *et al.* 1996).

Studies were also carried out to determine the role of complex formation between E1B55K and E4orf6. The 55 kDa polypeptide was immunoprecipitated under mild conditions from [³⁵S]methionine-labeled *wt* Ad5-infected human KB cells with 2A6 monoclonal antibody, which recognizes the amino-terminus of this E1B species as well as the related E1B-84R polypeptide. Figure 3.7 shows that in addition to the E1B55K protein and the 84R product, a species migrating at about 34 kDa was detected (lane 2). This protein appears to be E4orf6, as in other studies (data not shown) it was recognized by immunoblotting with E4orf6-specific M45 serum and to be absent in extracts from cells infected with mutant *d11010*, which fails to synthesize E4orf6. Neither the E1B species nor E4orf6 was detected in mock-infected cells (lane 1) or cells infected with mutant E1B⁻, which produces no E1B products (lane 3). Figure 3.7, lanes 4 and 5, shows results of a similar experiment with the E1B mutants *pm490/1A* and *pm490/1/5A* which were shown in Fig. 3.1C to be defective in the 289R-dependent block in accumulation of p53. Clearly, both mutant 55 kDa proteins were able to form complexes with E4orf6 at normal levels, in agreement with the finding that they induce host cell shutoff normally (Teodoro *et al.* 1994).

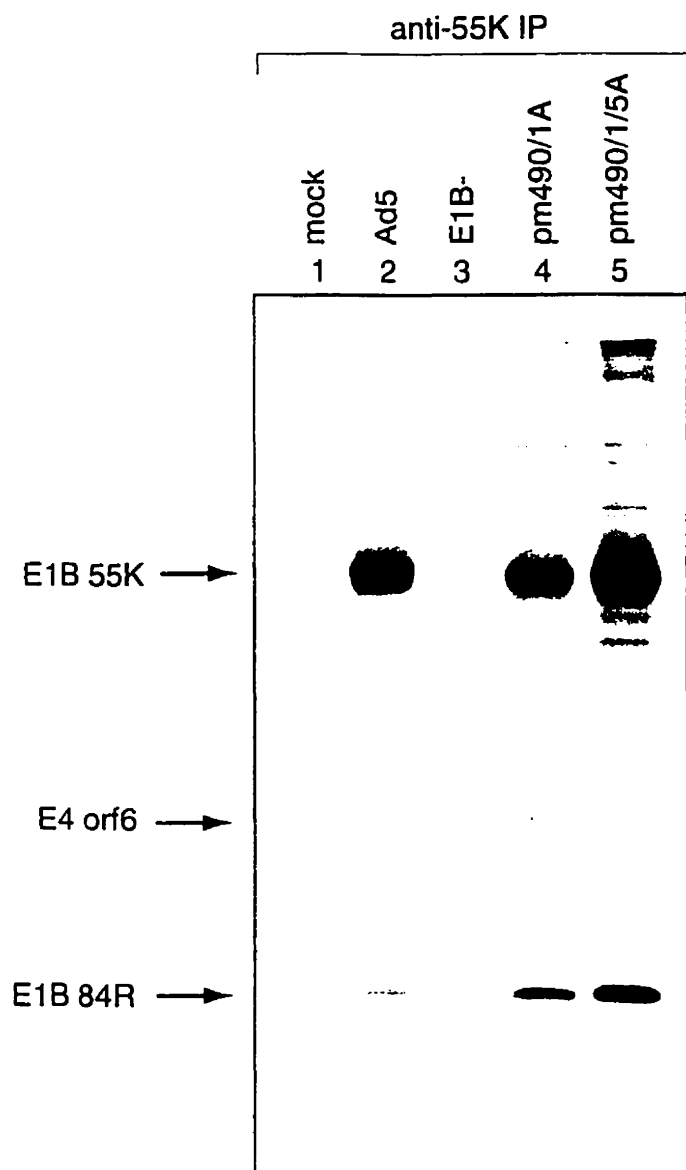


Figure 3.7. Complex formation between E4orf6 and mutant and *wt* E1B-55K

Mock-infected KB cells or those infected by *wt* Ad5 or E1B mutants were labeled at 16 hpi with [³⁵S]methionine for 1 h. Cell extracts were prepared under mild conditions, and immunoprecipitation (IP) was performed with 2A6 antibody, which recognizes the 55 kDa E1B protein and related products. Immunoprecipitated proteins were visualized by SDS-PAGE followed by autoradiography. The positions of migration of the 55 kDa protein, 84R, and E4orf6 proteins are shown at the left.

These results also indicated that complex formation between E4orf6 and E1B55K is not sufficient to induce a reduction in p53 levels and that inhibition of p53 transactivation activity may be necessary.

3.4.6 Host cell shutoff is not responsible for the block in accumulation of p53 by E4 orf6 and E1B55K

Although the experiments described above suggested that the block in accumulation of p53 did not appear to result from generalized shutoff of host cell protein synthesis induced by E1B55K and E4orf6, we conducted further experiments to examine this possibility, as others (Grand *et al.* 1996) had concluded that this process plays a role in the regulation of p53 levels. To establish the roles of these viral proteins more clearly, an adenovirus vector which expresses the Ad2 E4orf6 protein under the control of the CMV promoter was constructed. It was, therefore, possible to express E4orf6 in the absence of other viral proteins. HeLa cells were mock-infected or infected with one or more Ad5 mutants and/or the AdE4orf6 vector, and extracts were analyzed for p53 and E4orf6 protein levels by Western blotting, as in Fig. 3.1 and 3.2. Figure 3.8 shows that infection by the AdE4orf6 vector alone resulted in high levels of expression of E4orf6 (Fig. 3.8B, lane 2), but no effect on p53 levels (Fig. 3.8A, lane 2). Infection with mutant *dl520* (Fig. 3.8A, lane 3), which expresses E1A-243R, and mutant 12S/E1B⁻ (Fig. 3.8A, lane 4), which also expresses 243R but no E1B products, resulted in a large increase in p53 protein levels, as reported previously (Chiou *et al.* 1994, Querido *et al.* 1997b) and as shown above in Fig. 3.1A. In these cases, only very low levels of E4orf6 were detected (see Fig. 3.8B, lanes 3 and 4, respectively), as E1A-243R alone does not induce high levels of expression of E3 and E4 products or synthesis of late viral proteins. Coinfection by *dl520* and the AdE4orf6 vector completely suppressed this accumulation of p53 (Fig. 3.8A, lane 5). However, such was not the case following coinfection with 12S/E1B⁻ and AdE4orf6 (Fig. 3.8A, lane 6), as high levels of p53, which were at best only slightly reduced relative to 12S/E1B⁻ alone (Fig.

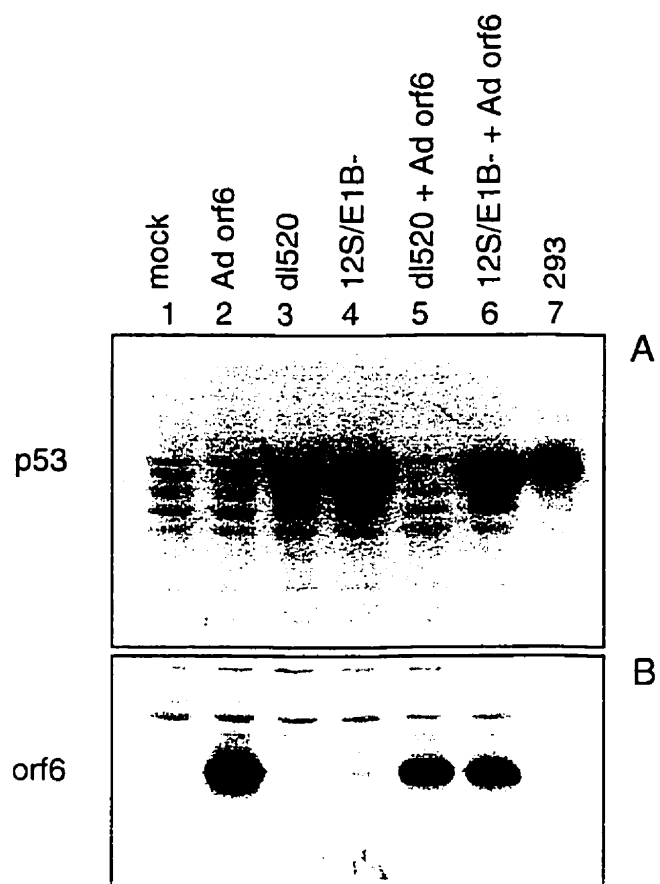


Figure 3.8. Accumulation of p53 in HeLa cells infected with Ad5 mutants and AdE4orf6

Human HeLa cells were infected with various Ad5 mutants or the AdE4orf6 vector, and cell extracts were prepared at 24 hpi and separated by SDS-PAGE. Following transfer to nitrocellulose, p53 (A) and E4orf6 (B) were detected by Western blotting with Ab1801 and M45 antibodies, respectively, and ECL. The positions of migration of p53 and E4orf6 have been shown at the left.

3.8A, lane 4), were present. These results confirmed again that both E4orf6 and E1B55K are necessary to block the E1A-induced accumulation of p53 efficiently. Similar results were also obtained in mouse 10T1/2 cells and human MRC-5 cells (data not shown).

Using the AdE4orf6 vector, it was also possible to examine more precisely the role of generalized host cell shutoff in the control of p53 levels. HeLa cells were infected by various combinations of Ad5 and adenovirus vectors, and after being labeled with [³⁵S]methionine for 1 h at 36 hpi, whole-cell extracts were analyzed by SDS-PAGE followed by autoradiography. Figure 3.9 shows that, in *wt* Ad5-infected cells (lane 1), a significant decrease in synthesis of cellular proteins was evident but that high levels of late viral polypeptides were being produced. This pattern was typical of the late host cell shutoff observed commonly in virus-infected cells. In the case of mutant E1B⁻, which produces both major E1A products but no E1B proteins, late viral protein expression was evident, but synthesis of cellular proteins was not shut down significantly (lane 2). This effect was reversed when the E1B55K protein was supplied by coinfection with adenovirus vector AdHH55K, which expresses a histidine-tagged version of this viral protein (lane 3). Of considerable interest was the analysis of cells infected by *dl*520 and the AdE4orf6 vector. Synthesis of late viral proteins was poor in all cases (lanes 4 to 9). In addition, no host cell shutoff was apparent relative to mock (lane 4) in cells infected by AdE4orf6 alone (lane 5), *dl*520 (lane 6), or 12S/E1B⁻ (lane 7) or in those coinfecting by *dl*520 or 12S/E1B⁻ and AdE4orf6 (lanes 8 and 9, respectively). These results indicated that whereas it was possible to block accumulation of p53 induced by *dl*520 by supplying E4orf6 (Fig. 3.8A, lane 5), induction of generalized host cell shutoff appeared to require at least some additional viral protein, possibly one encoded by E3, E4, or a late product. Thus, the decrease in E1A-induced accumulation of p53 by E1B55K and E4orf6 is not due to host cell shutoff regulated by these proteins.

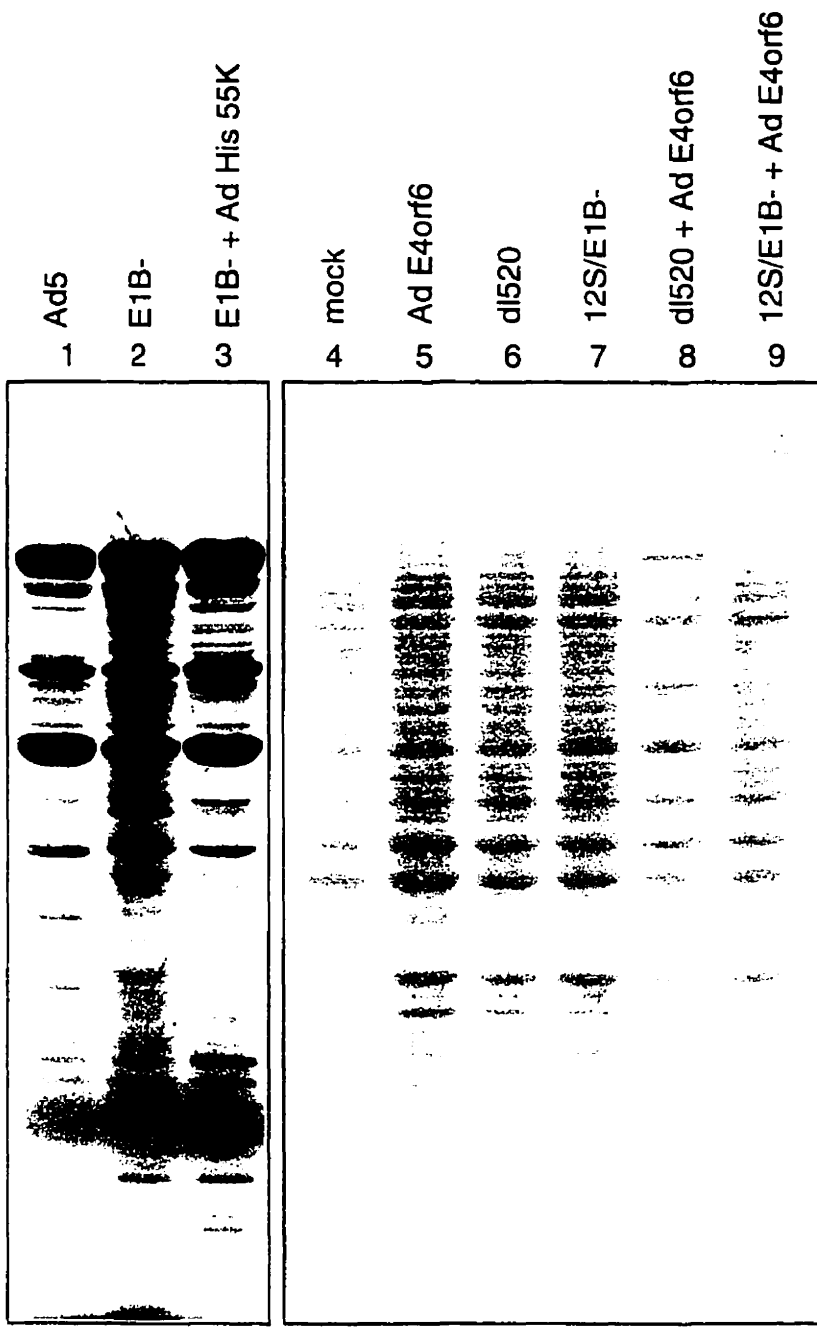


Figure 3.9. Host cell shutoff in HeLa cells infected with Ad5 mutants and vectors

Human HeLa cells were infected with Ad5 mutants or the adenovirus vectors AdE4orf6 or AdHis55K. At 36 hpi, the cells were labeled with [^{35} S]methionine for 1 h, cell extracts were separated by SDS-PAGE, and proteins were visualized by autoradiography.

3.5 Discussion

Expression of E1A proteins is known to induce the accumulation of p53, and other studies by our group have linked this increase to complex formation between E1A molecules and the p300 transcriptional modulator (and possibly related proteins p400 and the CREB binding protein) or the pRb tumor suppressor and related polypeptides (Querido *et al.* 1997b). Previous reports indicated that complex formation with either class of protein induces DNA synthesis in serum-starved primary rodent cells (Howe *et al.* 1990, Howe and Bayley 1992, Shepherd *et al.* 1993), and thus it is possible that in Ad5-infected cells, the rise in p53 levels results from stimulation of unscheduled DNA synthesis. The mechanism of accumulation of p53 has not been established. As discussed above, increased protein stability has been observed in the case of E1A-transformed human and rodent cells (Grand *et al.* 1993, Lowe and Ruley 1993, Grand *et al.* 1994, Grand *et al.* 1996), however, such is not always the case (Grand *et al.* 1993, Grand *et al.* 1995). Increases in p53 levels induced by simian virus 40 large T antigen have been linked to increased stability of p53 as a result of complex formation with this viral protein (Linzer and Levine 1979). However, neither the 289R or 243R E1A product is believed to bind directly to p53, and expression of E1B55K, which does interact with p53, is not required for p53 accumulation. It has been suggested that p53 expression may also be regulated at the translational level (Mosner *et al.* 1995, Fu *et al.* 1996) and that the p53 protein may play some autoregulatory role (Mosner *et al.* 1995). Further studies will be required to establish the molecular basis for E1A-induced accumulation of p53.

Of some interest was the observation that whereas infection with adenovirus encoding only the E1A-243R protein induced about an 8- to 10-fold rise in p53 levels, expression of the E1A-289R protein blocked this effect. Thus, infection with *wt* Ad5 or coinfection with viruses expressing each E1A protein individually resulted in no overt change in p53 quantities relative to the endogenous levels of uninfected cells. This inhibitory process required an active E1B55K, as in its absence, high levels of p53 were detected

regardless of the E1A species expressed. Mutational analysis indicated that complex formation with p53 may be required, and in addition, the transcriptional repression activity resident in the 55 kDa species must be functional. These results could suggest that suppression of p53 transactivation activity is necessary. Of further interest was the fact that expression of E1A-289R and E1B55K was not sufficient to prevent E1A induction of p53 accumulation. Human 293 cells contain high endogenous levels of p53, presumably due to the effects of E1A proteins, and such cells express both the E1A-289R and E1B55K proteins (Graham *et al.* 1977, Grand *et al.* 1995). We showed that the reduction of p53 accumulation requires expression of the E4orf6 product. We were also able to show that expression of the E4orf6 and E1B55K proteins is sufficient to block the accumulation of p53 induced by E1A and that additional E3, E4 or late proteins do not appear to be required. In addition, expression of E4orf6 in the absence of E1B55K blocked p53 accumulation only very poorly, if at all.

The mechanism by which this inhibition occurs remains to be established. As discussed above, it is possible that a decrease in the rate of translation could play a role in blocking the accumulation of p53. The present experiments were not designed to examine this question in detail. However, interactions between E4orf6 and E1B55K have been linked to shutoff of host cell protein synthesis and stabilization and transport of viral mRNA (Babiss and Ginsberg 1984, Sarnow *et al.* 1984, Babiss *et al.* 1985, Halbert *et al.* 1985, Pilder *et al.* 1986, Weinberg and Ketner 1986, Cutt *et al.* 1987, Leppard and Shenk 1989, Sandler and Ketner 1989, Ornelles and Shenk 1991). It was possible that such inhibition could play a role in the drop in p53 levels, as suggested in an earlier study (Grand *et al.* 1996). However, three observations argued strongly that generalized shutoff of host protein synthesis induced by complexes of E4orf6 and E1B55K plays little or no role in the block in p53 accumulation. The first was that mutants *pm490/1A* and *pm490/1/5A* which encode 55 kDa proteins lacking two or all three of the carboxy-terminal phosphorylation sites, fail to prevent the rise in p53 levels even though these mutant E1B products induce host cell shutoff

effectively (Teodoro *et al.* 1994). Second, generalized host cell shutoff in infected 293 cells started over 7 h after the beginning of the decline in p53 levels. And third, expression of E4orf6 via an adenovirus vector in cells infected with *dl520*, which expresses only very low quantities of E3, E4, and late proteins, induced the block in p53 accumulation but not host cell shutoff. Thus, we believe that the effects on p53 are not the result of global inhibition of host cell protein synthesis, but, rather, are caused by some other process that is targeted uniquely to p53.

The major cause of the block in E1A-induced accumulation of p53 is likely related to a change in the stability of p53 molecules. Results obtained in AdLacZ-infected 293 cells indicated that the extended half life of about 2.5 h in uninfected cells diminished to about 20 min following virus infection and expression of E4orf6. Thus, the reduction in the rate of p53 production during 1 h intervals could possibly be explained entirely by this 7- to 8-fold decrease in p53 stability. The presence of E4orf6 and E1B55K therefore may function to block increased p53 stability induced by E1A protein.

These effects probably involve the formation of multiprotein complexes. The E1B55K protein has been known for some time to bind to both p53 and E4orf6 (Sarnow *et al.* 1982a, Sarnow *et al.* 1984). We show here that E4orf6 is also capable of binding to p53, an observation reported recently by others who also demonstrated that such complex formation led to the inactivation of p53 transactivation activity (Dobner *et al.* 1996). Thus, formation of trimeric complexes containing p53, E4orf6 and E1B55K could be responsible for preventing accumulation of p53 induced by E1A, although at present there is no direct evidence that such complexes are formed. Just how such complex formation could affect p53 stability remains to be established, although the present studies indicated that a functional E1B55K repression domain (and thus perhaps inhibition of the p53 transactivation activity) seems to be necessary.

As the present manuscript was being submitted Moore *et al.* (Moore *et al.* 1996) reported that E4orf6 is able to cooperate with E1A in cell transformation, presumably

through the inactivation of p53, as reported earlier (Dobner *et al.* 1996). This study also reported that expression of E4orf6 in stable transformants induces both a decrease in p53 stability and a reduction in p53 levels. These data are in complete accord with the present results. However, our results differed in one significant way. Whereas in the stable transformants E4orf6 was able to produce these effects alone, in the present studies with virus-infected cells, the E1B55K protein was clearly also required. Further studies will be necessary to determine the reason for these differences and the molecular basis for these profound effects on p53.

3.6 Acknowledgments

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Early in adenovirus infection, E1A binds the p300 and pRb family proteins, and can induce non-proliferating cells to enter S phase. One consequence of this is the accumulation of p53 protein, a cellular response to oncogenic stress. As we have just demonstrated in chapter III, two other adenoviral early proteins, E1B55K and E4orf6, collaborate to block this accumulation of p53. They do this by reducing the stability of the p53 protein. In this following chapter we will continue to study the mechanism by which the E4orf6/E1B55K complex induces p53 turnover.

CHAPTER IV

Identification of three functions of the adenovirus E4orf6 protein that mediate p53 degradation by the E4orf6/E1B55K complex

4.1 Abstract

Complexes containing adenovirus E4orf6 and E1B55K proteins play critical roles in productive infection. Both interact directly with the cellular tumor suppressor p53 and in combination 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. Co-expression 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 involving 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 between residues 44 and 218 corresponded with the ability of E4orf6 to form complexes with cellular proteins of 84, 19, 16 and 14kDa. The third contained the nuclear retention signal (NRS)/amphipathic arginine-rich α -helical region from residues 239 to 253. Interestingly, neither the E4orf6 nuclear localization (NLS) nor the nuclear export (NES) signals were essential. These results, and those from inhibitor experiments utilizing leptomycin B, suggested that p53 degradation may not require nuclear-cytoplasmic shuttling. 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* using reticulocyte lysates. These results suggested that the E4orf6/E1B55K complex may direct p53 for degradation in the nucleus by a novel mechanism.

4.2 Introduction

The cellular p53 tumor suppressor protein represents 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 (Teodoro *et al.* 1995, Roulston *et al.* 1999). In uninfected cells, regulation of the stability of p53 protein is known to be crucial in the control of p53 function. 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 (HAT) p300 and related proteins p400 and the CREB-binding protein (Howe *et al.* 1990, Barbeau *et al.* 1994, Eckner *et al.* 1994). 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 (Braithwaite *et al.* 1990, Debbas and White 1993, Grand *et al.* 1993, Lowe and Ruley 1993, Chiou *et al.* 1994, Grand *et al.* 1994, Sanchez-Prieto *et al.* 1995, Grand *et al.* 1996). This stabilization of p53 is dependent on the same E1A regions involved in complex formation with p300/CBP/p400 and pRb (Chiou and White 1997, Querido *et al.* 1997b, Samuelson and Lowe 1997, de Stanchina *et al.* 1998). 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* (DeGregori *et al.* 1997, de Stanchina *et al.* 1998). Stabilization therefore ensues through inhibition by p19ARF of MDM2-mediated p53 degradation (Honda and Yasuda 1999). E1A can still induce p19ARF protein levels in pRb^{-/-} cells, suggesting that additional mechanisms exist (de Stanchina *et al.* 1998). Binding of p300 by E1A proteins reduces the ability of p53 to transactivate *mdm2* (Thomas and White 1998), and diminishes MDM2-mediated p53 degradation, which may require p300 as a co-factor (Grossman *et al.* 1998).

The accumulation of p53 induced by E1A is not normally evident in wild-type adenovirus infected cells, and several genetic studies have shown that expression of both the 34kDa early region 4 orf6 protein (E4orf6) and the early region 1B 55kDa protein (E1B55K) are necessary (Moore *et al.* 1996, Nevels *et al.* 1997, Querido *et al.* 1997a, Steegenga *et al.* 1998). E4orf6 and E1B55K have been known for some time to interact (Sarnow *et al.* 1984, Bridge and Ketner 1990), and to be involved in such late functions as selective transport and translation of viral mRNAs and shutoff of host cell protein synthesis (Babiss and Ginsberg 1984, Babiss *et al.* 1985, Halbert *et al.* 1985, Bridge and Ketner 1989, Sandler and Ketner 1989, Ohman *et al.* 1993). Both E1B55K (Sarnow *et al.* 1982a) and E4orf6 (Dobner *et al.* 1996) 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 (Teodoro *et al.* 1994, Yew *et al.* 1994, Teodoro and Branton 1997, Martin and Berk 1998). This interaction also has recently been suggested to inhibit the

acetylation of a carboxy terminal lysine of p53 by PCAF (Daiqing Liao, personal communication). The p53-mediated repression activity originating from associated histone deacetylase (HDAC) complexes (Murphy *et al.* 1999) may also be inhibited by E1B55K. Such events block p53-induced growth arrest and apoptosis (Debbas and White 1993, Teodoro *et al.* 1995, Marcellus *et al.* 1996a, Teodoro and Branton 1997) and thus enhance transformation by E1A and other oncogenes (Zhang *et al.* 1992). E4orf6 binding towards the carboxy terminus of p53 also enhances E1A/E1B-mediated transformation (Moore *et al.* 1996, Nevels *et al.* 1997, Nevels *et al.* 1999). The mechanism of this effect is still not clear. Two studies have shown that E4orf6 binding blocks p53-mediated gene expression (Dobner *et al.* 1996, Querido *et al.* in preparation), although two other reports found little effect of E4orf6 on several p53-regulated promoters, at least in certain cell types (Roth *et al.* 1998b, Steegenga *et al.* 1999). It has been suggested that inhibition of p53 transcriptional activity might result from the disruption of interactions with TAF_{II}31 (Dobner *et al.* 1996). A recent study from our group found that E4orf6 did not effectively block p53-mediated apoptosis, even though it blocked p53 transactivation of several promoters (Querido *et al.* in preparation).

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 (Goodrum *et al.* 1996, Orlando and Ornelles 1999). In addition, E4orf6 has a nuclear export signal (NES) that was shown to play a role in nuclear-cytoplasmic shuttling of the E1B55K/E4orf6 complex (Dobbelstein *et al.* 1997) and to be essential for the ability of E4orf6 to support viral replication (Weigel and Dobbelstein 2000). Another group found that E1B55K itself contains an NES and may shuttle independently of E4orf6 (Kratzer *et al.* 2000). 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 (Boyer and Ketner 2000), 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, and not

the related p73 or p63 proteins (Higashino *et al.* 1998, Roth *et al.* 1998b, Wienzek *et al.* 2000). Recently our group detected a series of proteins that interact with E4orf6, including cellular products of 84, 19, 16 and 14kDa (Boivin *et al.* 1999). Some insights into the mechanism of p53 degradation may be derived through the identification of novel E1B55K- or E4orf6-binding proteins.

In this report we have used a mainly genetic approach to investigate some of the parameters of E4orf6/E1B55K-mediated p53 degradation. Interactions between E1B55K and 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 (Haupt *et al.* 1997, Honda *et al.* 1997, Kubbutat *et al.* 1997), and p19ARF, which promotes p53 stability (Pomerantz *et al.* 1998, Zhang *et al.* 1998, Honda and Yasuda 1999). E4orf6/E1B55K-mediated p53 degradation also did not appear to require nuclear export, but was inhibited by the MG132 drug, an inhibitor of the 26S proteasome. These results suggest the E4orf6/E1B55K complexes may induce p53 turnover by a unique mechanism.

4.3 Materials and methods

4.3.1 Cells

Human p53^{-/-} H1299 cells (Mitsudomi *et al.* 1992) and primary mouse embryo fibroblasts (MEF) were cultured on 60mm diameter dishes (Corning Glass Works, Corning, N.Y.) in Dulbecco's modified Eagle medium (D-MEM, GIBCO BRL) supplemented with 10% fetal bovine serum (CanSera). Primary MEFs derived from wild-type, p53^{-/-} and p53^{-/-}MDM2^{-/-} (Montes de Oca Luna *et al.* 1995, Jones *et al.* 1996), and p19ARF^{-/-} (Kamijo *et al.* 1997) were used in some experiments. All viral stocks were titrated on 293 cells (Graham *et al.* 1977).

4.3.2 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* gene *lacZ* under the control of the cytomegalovirus (CMV) promoter, as were the cases with Adp53wt and Adp53S135 vectors which express wild-type p53 or a p53 Cys-135 to serine mutant, respectively (Bacchetti and Graham 1993). These vectors were provided by Frank Graham (Bacchetti and Graham 1993). The Adp53KEEK (mutated at residues 341, 344, 348 and 355) and Adp53S135KEEK adenovirus vectors were produced by cloning each cDNA (Slingerland *et al.* 1993) into a shuttle vector under the control of the CMV promoter, and viruses were generated in 293 cells by homologous recombination of the shuttle vectors with the adenovirus E1⁻/E3B⁻ vector backbone (Bacchetti and Graham 1993). 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 (Querido *et al.* 1997a). The AdE2F1 vector expressing human E2F-1 was provided by Joe Nevins and has been described elsewhere (Kowalik *et al.* 1995). In cases of co-infection with multiple adenovirus vectors, such vectors were first pre-mixed in serum-free media 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 minutes. 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 p73 α and p73 β were obtained from Bill Kaelin (Jost *et al.* 1997). The pRcCMV p53 22-23 plasmid was provided by Arnold Levine (Lin *et al.* 1994). 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 (Querido *et al.* 1997a).

4.3.3 E4orf6 mutants

Figure 4.1 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. A T7 promoter was located at the amino terminus and an SP6 promoter at the carboxy terminus of E4orf6. The internal deletion mutants were prepared by a four-oligonucleotide PCR method as follows: a PCR amplification was performed with a T7 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 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 dl1-13, dl 1-38, and dl1-55 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 *EcoRV*, which cleaves at a unique site, thus placing the methionine at position 109 to position 1. The dl108-294, 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, followed by digestion with unique cutters *EcoRV*, *PvuII*, *SSpI*, and *BSSHII*, respectively, as well as the restriction site right before the stop codons, blunting and then ligating. All the E4orf6 mutants were verified by sequencing. A list of the oligonucleotides used will be provided upon request. Point mutants 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 (Dobbelstein *et al.* 1997).

Ad5 E4orf6

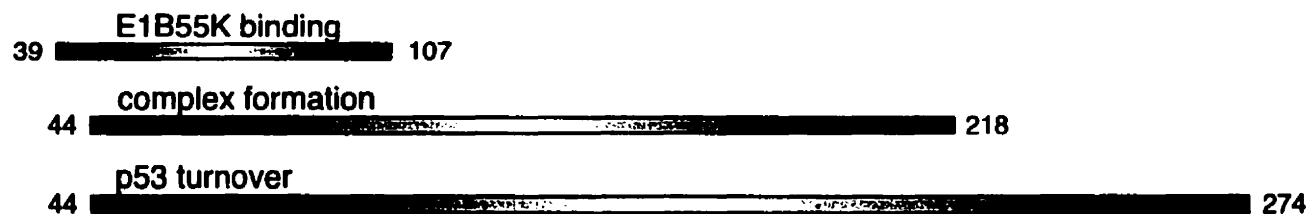
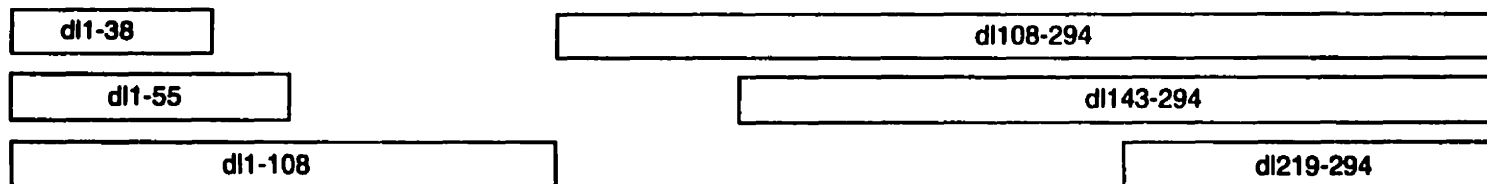
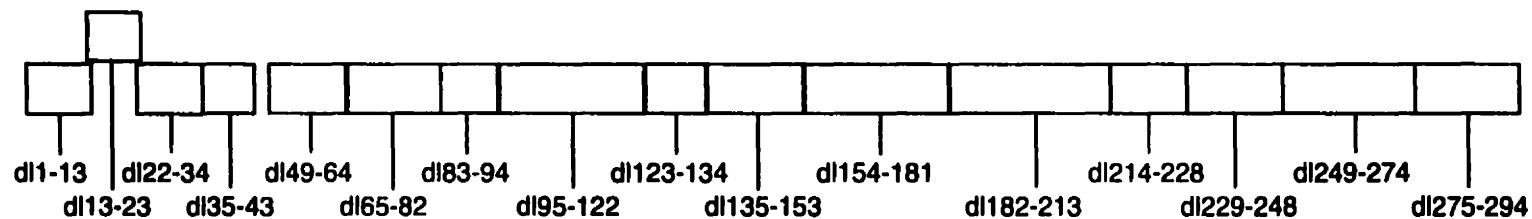
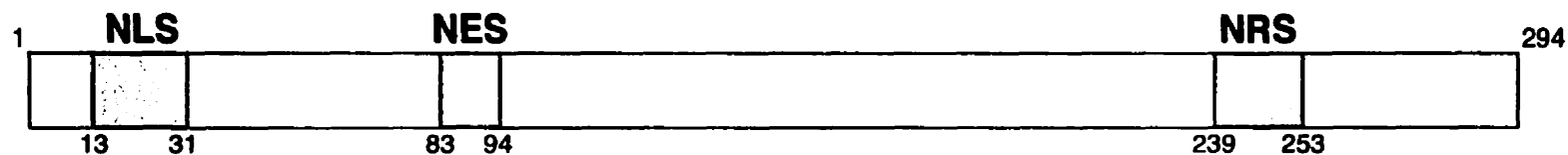


Figure 4.1 Model of adenovirus type 5 E4orf6 protein.

The positions of the putative nuclear localization signal (NLS), the nuclear export signal (NES) and the nuclear retention signal (NRS) are indicated by shadowed boxes. All the deletion and point mutants used in the present studies have been indicated by boxes or lines. The minimal regions necessary for interaction with E1B55K, for complex formation with the cellular p84, p19 and p14 proteins, as well as for p53 degradation are also indicated.

4.3.4 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 D-MEM supplemented with 10% low IgG fetal calf serum (BRL). 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 (Sarnow *et al.* 1982b) was collected as an ascites fluid generated in mice using appropriate hybridoma cells. Production of E4orf6-N (1808) and E4orf6-C (1807) rabbit polyclonal antisera have been described previously (Boivin *et al.* 1999). The 1D5 anti-E4orf6 mouse monoclonal antibody was raised against a peptide corresponding to the 14 carboxy terminal amino acids of E4orf6. Anti-HA.11 mouse monoclonal antibody (BabCo) was used to detect HA-tagged p73 proteins. Anti-E2F1 and anti-cMyc monoclonal antibodies were purchased from Santa Cruz. Anti-MDM2 2A10 mouse monoclonal antibody has been described (Olson *et al.* 1993).

4.3.5 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% NP40 (v/v), 4 mM NaF, 4 mM NaVO₄, 0.2 mM EDTA, 0.2 mM EGTA, 2 mg/ml each aprotinin, leupeptin and pepstatin, and 1 mM DTT]. Total protein was measured by Bradford protein assay as specified by the manufacturer (Bio-Rad), and 40 µg of total cell protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Nuclear and cytoplasmic extracts were prepared as described previously (Whalen *et al.* 1997). Proteins were electroblotted onto nitrocellulose membranes for 1 h using a semi-dry transfer apparatus. After blocking overnight, the membranes were incubated with the appropriate dilution of primary antibody for 2 hours at room temperature, followed by a 45 minute incubation with secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG, or anti-rabbit IgG (Jackson Immuno Research Laboratories), or with anti-goat IgG (Santa

Cruz). Immunoreactive bands were revealed using an enhanced chemiluminescence (ECL Plus) kit (Renaissance, DuPont-NEN).

4.3.6 Binding assays

In many cases H1299 cells growing on 60mm-diameter dishes were infected with adenovirus vectors coding for p53, E1B55K or E4orf6, as described above. In some cases, cells were lipofected according to the Lipofectamine Plus (GIBCO) protocol 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 plasmids during lipofection. Finally, for all the binding assays with E4orf6 mutants, cells were lipofected according to the standard Lipofectamine Plus (GIBCO) protocol with 2 µg of pcDNA3 E4orf6 (or mutants) and 2 µg of pCA14HH55Kwt plasmid. Cell extracts were prepared 24 h later in buffer X [50mM Tris-HCl, pH 8.0, containing 250mM NaCl, 1% NP40 (v/v), 1 mM EDTA, 1mM EGTA, 2µg/ml each of aprotinin, leupeptin and pepstatin]. Extracts were pre-cleared, immunoprecipitated with the indicated antibody for two hours at 4 °C, and a minimum of five washes in buffer X were performed. As E1B55K and E4orf6 proteins tended to form high molecular weight aggregates when boiled in SDS sample buffer, samples were incubated for 15 minutes at 55 °C before loading on 8% or 14% SDS-polyacrylamide gels. For E4orf6 binding with p53 or HA-p73, cell lysates were immunoprecipitated with anti-E4orf6 antibody (1807) and immunoblotted with anti-p53 (1801 and 421) antibody or anti-HA antibody. For E1B55K binding with 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.

4.3.7 *In vivo* p53 degradation assay

H1299 cells growing on 60mm-diameter dishes were lipofected according to the Lipofectin (GIBCO/BRL) protocol with a total of 9 µg of plasmid DNA per plate, involving 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 were omitted. After 24 hours the cells were harvested and quantitative western blotting was performed, as described above. For all the p53 turnover assays in chapters IV and V, the expression level of E4orf6 and E1B55K species was always confirmed by stripping the membranes of p53 antisera and reblotting.

4.3.8 *In vitro* p53 degradation assay

Human papilloma virus (HPV) 18 E6 protein, p53, E1B55K, and E4orf6 proteins were *in vitro* transcribed/translated 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 [³⁵S]methionine while HPV18 E6 was labeled with [³⁵S]cysteine (translation grade; DupontNEN). 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 non-radioactive methionine/cysteine. The p53 degradation assay was performed as described by Howley and colleagues (Scheffner *et al.* 1990b) in 40 µl volumes containing 2 µl of radioactively labeled p53, 8 µl of each unlabeled protein, 10 µl of unprogrammed reticulocyte lysate, in 25 mM Tris-HCl, pH7.5, containing 100 mM NaCl, and 3 mM DTT. The reactions were incubated for 3 h at 25 °C, then stopped by boiling in SDS sample buffer, separated by SDS-PAGE, and p53 was visualized by autoradiography.

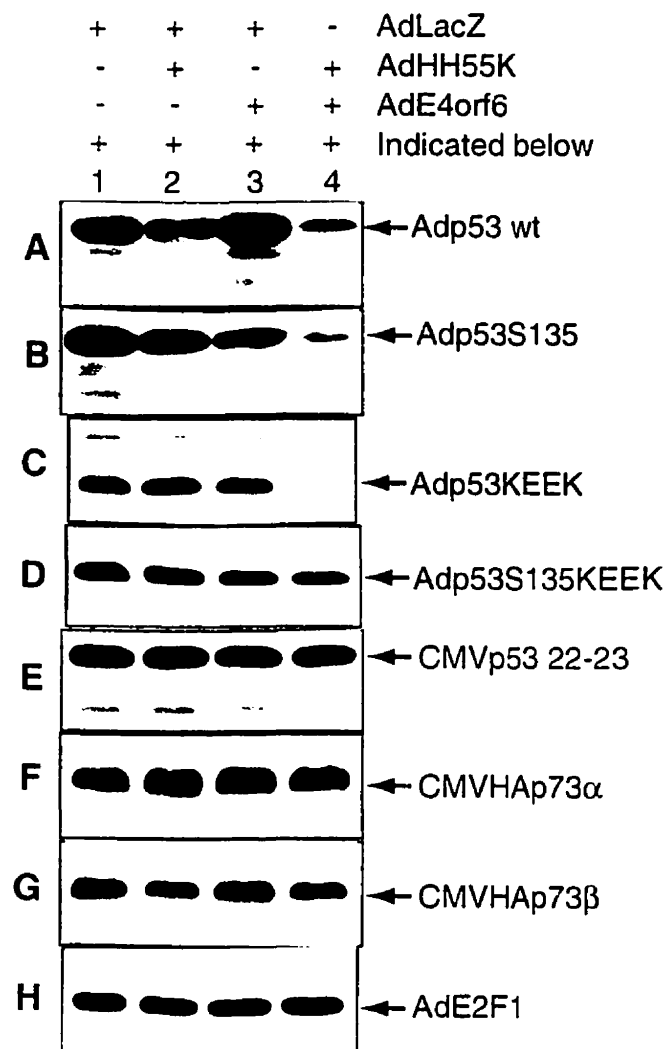


Figure 4.2 E4orf6/E1B55K-mediated degradation of wild-type and mutant p53 and of p73.

(A) Degradation of wild-type p53. p53-null H1299 cells were infected with an adenovirus vector expressing *wt* p53 (lanes 1-4) along with adenovectors coding for LacZ (as a control), E4orf6 or E1B 55K. Whole cell extracts were prepared at 24 hpi 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 co-lipofection with appropriate cDNAs (CMV). (F-G) Degradation of p73. Degradation of p53-related p73 proteins was assessed in similar assays following co-lipofection of p73-specific cDNAs. (H) Lack of degradation of control protein E2F1.

4.4 Results

4.4.1 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 (Moore *et al.* 1996, Nevels *et al.* 1997, Querido *et al.* 1997a, Steegenga *et al.* 1998). To study this further, degradation of both wild-type and mutant p53 proteins as well as the p53-related proteins p73 α and p73 β (Jost *et al.* 1997, Kaghad *et al.* 1997) was assessed. p53-null human H1299 cells infected with adenovirus vectors expressing E4orf6 and/or E1B55K were either co-infected with vectors expressing various forms of p53 or lipofected with plasmid DNA expressing p53 or p73 species, and the amounts of each product were determined by quantitative western blotting of whole cell extracts. Fig. 4.2 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. 4.2H); however, both wild-type p53 (Fig. 4.2A - Adp53wt), and mutant forms with defects in the DNA binding domain (Fig. 4.2B - Adp53S135) and the tetramerization domain (Fig. 4.2C - Adp53KEEK) were readily degraded. As shown previously (Moore *et al.* 1996, Nevels *et al.* 1997, Querido *et al.* 1997a, Steegenga *et al.* 1998), such degradation required expression of both viral proteins. Using the adenovirus vectors as described for Fig. 4.2A, we verified that the half-life of wt p53 was reduced from 4 hours to 20 min when both E1B55K and E4orf6 were expressed (data not shown). The half-life of p53 expressed from the Adp53 wt vector has been described as being unusually long (Bacchetti and Graham 1993), 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 the tetramerization domains (Fig. 4.2D - Adp53S135KEEK) or the p53 22-23 mutation (Fig. 4.2E - 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 4.1 summarizes results of quantitative p53 binding assays (primary data not shown) in which the

Table 4.1 Binding of wild-type and mutant p53 proteins to E4orf6 and E1B55K

Binding Protein	Degree of Binding To*	
	E4orf6	E1B55K
p53 (wild-type)	+++	+++
p53S135	+	+++
p53KEEK	-	+
p53S135/KEEK	-	-
p73 α	-	-
p73 β	-	-

* H1299 cells were infected with adenovirus vectors encoding the indicated proteins, except in the cases of HAp73 α and β , for which lipofection/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 though immunoprecipitated with anti-E4orf6 (1807) or anti-E1B55K (2A6) antibodies, and immunoblots were performed using goat polyclonal p53 antibody (Santa Cruz). The ability of the 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).

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 (Lin *et al.* 1994). These results suggested that the interaction of p53 with E1B55K alone is sufficient for p53 degradation, as 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 p73 α and p73 β proteins. Two studies have shown that these molecules do not interact with E1B55K (Marin *et al.* 1998, Roth *et al.* 1998b), although another report indicated that E4orf6 does bind p73 (Higashino *et al.* 1998). Table 1 shows that in cells expressing hemagglutinin antigen (HA)-tagged p73 products, neither E4orf6 nor E1B55K interacted with p73 proteins. As might be expected, Fig. 4.2F and 4.2G indicate that no p73 degradation took place in response to E4orf6 and E1B55K.

4.4.2 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 34kDa nuclear protein predicted to contain a large central hydrophobic region with more hydrophilic carboxy and amino termini. Fig. 4.1 shows that two signal sequences have been identified that can mediate import into the nucleus: an NLS near the amino terminus (residues 13-31); and a nuclear retention signal (NRS) towards the carboxy terminus (residues 239-253). Each is believed to play a role in directing the nuclear localization of E1B55K (Goodrum *et al.* 1996, Orlando and Ornelles 1999). E4orf6 also contains a Rev-like NES sequence that can mediate nuclear-cytoplasmic shuttling of the E4orf6/E1B55K complex (Dobbelstein *et al.* 1997). Point mutants 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 (Dobbelstein *et al.* 1997, Orlando and Ornelles 1999). Mutants 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 (see Fig. 4.1 for a complete list). 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 4.2 and Fig. 4.1 could be detected with either the E4orf6-N or E4orf6-C polyclonal serum. They were stable and expressed at similar levels, as can be seen in Figure 7 appended at the end of the thesis. The E4orf6 splicefix (SF) and the 8K product are described in (Querido *et al.* 2000a). E4orf6 mutants dl108-294 and R240E/R241E (containing an intact NLS) and E4orf6 dl1-108 (containing an intact NRS) were properly localized to the nucleus when transfected into H1299 cells, as shown by immunofluorescence (data not shown).

4.4.3 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. The binding of E4orf6 to E1B55K was examined in co-immunoprecipitation studies following lipofection of p53 minus H1299 cells with appropriate cDNAs. The capacity for 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 lipofected with cDNAs encoding human p53, E1B55K and E4orf6 (wt or mutants). Figs. 4.3A-C show the primary results for some of the E4orf6 mutants, and results for all mutants have been summarized in Table 4.2. Fig. 4.3B 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. Fig. 4.3A shows similar total levels of E1B55K in all whole cell extracts. Fig. 4.3C shows that all mutant E4orf6 proteins interacted with E1B55K, with the exception of the dl1-55 and dl1-108 mutants. As the dl1-38 and dl108-294 products associated

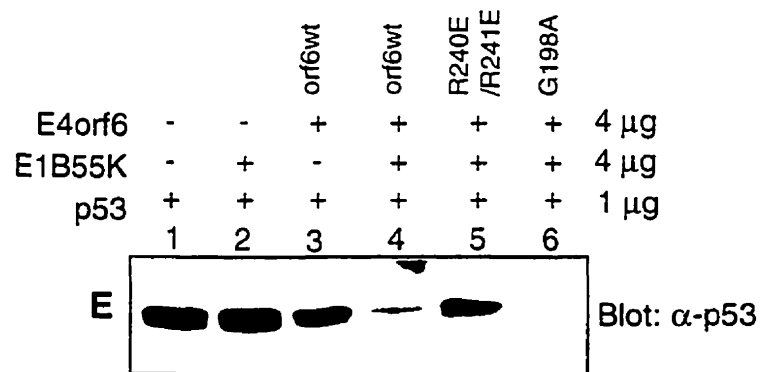
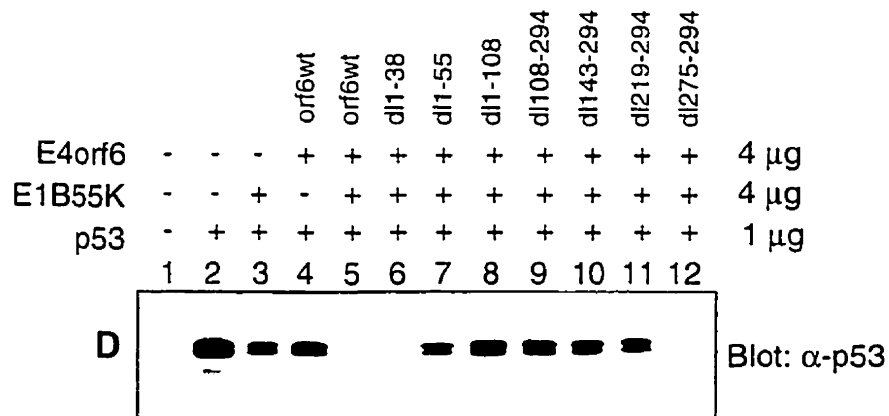
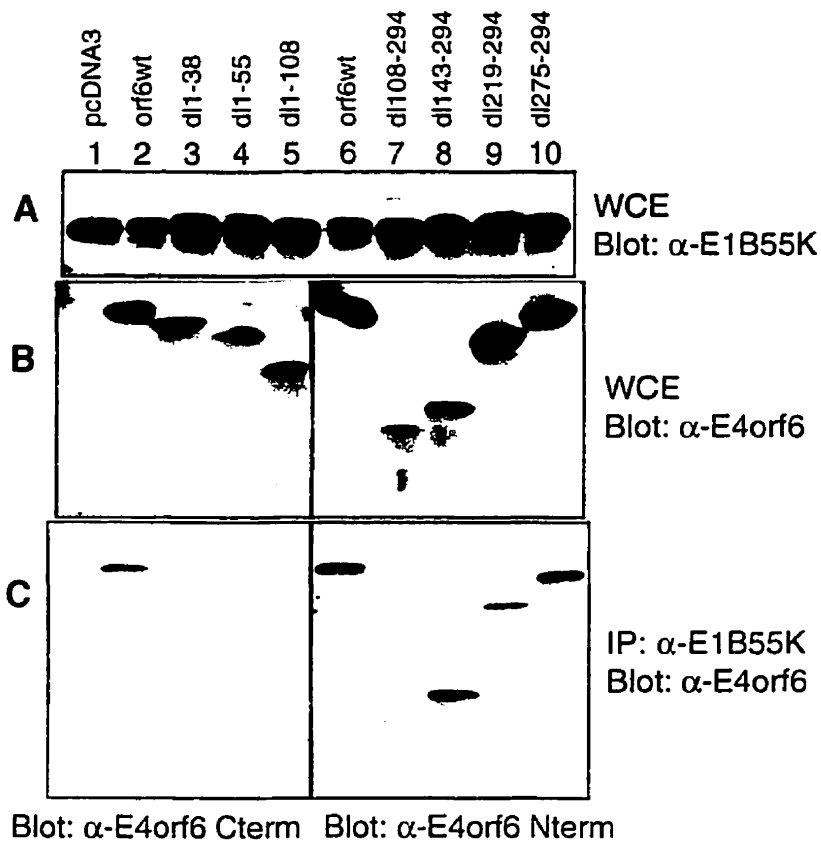


Figure 4.3 Mapping of E4orf6 regions required for binding of E1B55K and p53 degradation.

H1299 cells were co-lipofected with cDNAs encoding E1B55K and wild-type or mutant E4orf6 and cell extracts were prepared at 24 hpi. (A-C) E4orf6-E1B55K binding. Whole cell extracts were separated by SDS-PAGE and immunoblotted for E1B55K using 2A6 antibodies (A) or E4orf6-C (lanes 1-5) or E4orf6-N (lanes 6-10) antibodies (B). Extracts were also immunoprecipitated using 2A6 antibodies and immunoblotted using E4orf6-C (lanes 1-5) or E4orf6-N (lanes 6-10) antibodies (C). (D) p53 degradation by E4orf6 deletion mutants.

H1299 cells were co-lipofected 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 of Fig. 4.3 D was performed using wild-type E4orf6 and E4orf6 point mutants.

with E1B55K, it appeared that the binding site for this protein on E4orf6 is located between residues 39-107, or at least that this region is required to establish the appropriate conformation for binding. Table 4.2 shows that all of the smaller deletion mutants towards 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 1-58) did not interact.

Analysis of the ability of E4orf6 mutants to support p53 degradation in cooperation with E1B55K is shown in Figs. 4.3D-E. Fig. 4.3D (lanes 2-5) again indicated that both E4orf6 and E1B55K were essential. Fig. 4.3D (lanes 6-12) shows results obtained with the larger E4orf6 deletions. Mutant dl1-38 (lane 6) exhibited the same efficacy as wild-type E4orf6 for p53 degradation, and mutant dl275-294 (lane 12) exhibited a very high degree of p53 turnover. Mutant dl1-55 was almost totally defective, as were all the other E4orf6 mutant products. Table 4.2 shows that several of the smaller amino terminal deletions were able to cause efficient p53 degradation, including mutants affecting residues between 1 and 43. 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 deletion and point mutants revealed the role of E4orf6 signal sequences. Deletion of the NLS (residues 13-31; see mutant dl1-38, Fig. 4.3D, lane 6) had no effect on turnover. Although deletion of the region containing the NES (residues 83-94) in mutants dl1-108 (Fig. 4.3D, lane 8) and dl83-94 (Table 4.2) eliminated p53 turnover, the point mutant L90A/I92A, which has been shown to block nuclear export (Dobbelstein *et al.* 1997), supported high levels (Fig. 4.3E, lane 6). Thus neither the NLS nor NES is essential. A mutant R240E/R241E, which is predicted to disrupt the arginine-faced amphipathic alpha helix and thus affect nuclear targeting (Orlando and Ornelles 1999), was greatly deficient in p53 degradation (see Fig. 4.3E, lane 7). Interestingly, another mutant in the NRS, R248E, which is believed to have little or no effect on NRS function (Ornelles Personal communication), supported p53 turnover well (Table 4.2). Thus p53 turnover appears to require a functional NRS.

Table 4.2 Mapping of E4orf6 regions necessary for the interaction with E1B55K, complex formation with cellular proteins, and p53 degradation

E4orf6	Degree of		
	p53 degradation*	Complex formation [#]	Binding to E1B55K [†]
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	+++	n.d.	+++
dl 13-23	+++	n.d.	+++
dl 22-34	+++	n.d.	+++
dl 34-43	+++	+++	+++
dl 49-64	-	-	+++
dl 65-82	-	-	+++
dl 83-94	-	-	+++
dl 95-122	-	-	+++
dl 123-126	-	n.d.	+++
dl 123-134	-	n.d.	+++
dl 135-153	-	n.d.	+++
dl 154-181	-	n.d.	+++
dl 182-213	-	n.d.	+++
dl 214-229	-	n.d.	+++
dl 229-248	-	n.d.	+++
dl 249-274	-	n.d.	+++
L90A/I92A	++	n.d.	+++
G198A	+++	+++	+++
R240E/R241E	-	+++	+++
R248E	++	n.d.	+++
L90A/I92A/R248E	++	n.d.	+++
E255A/E256A	+++	+++	+++
E4orf6/7 (wild-type)	-	-	-

Table 4.2 Mapping of E4orf6 regions necessary for the interaction with E1B55K, complex formation with cellular proteins, and p53 degradation

* p53 degradation assays were performed as described for Fig. 4.3 D-E.

Binding to p84, p19, and p14: H1299 cells growing in 100mm-diameter plates were lipofected with 10µg of pcDNA3 plasmid DNA encoding wild-type or mutant E4orf6.

Following labeling from 18 to 22 h p.i. with 200µCi per plate of

[³⁵S]methionine/[³⁵S]cysteine EasyTag Express protein labeling mixture (>1000 Ci/mmol;

DuPont NEN) in methionine/cysteine-free medium, whole cell extracts were prepared in buffer

X (see Materials and Methods), immunoprecipitated using 1D5 anti-E4orf6 antibody and

analyzed by SDS-PAGE and autoradiography. The ability of the p84, p19 and p14 cellular proteins to associate with E4orf6 polypeptides has been summarized in the Table.

† E4orf6-E1B55K binding assays were performed as described for Fig. 4.3 A-C.

n.d. not determined

4.4.4 Formation of complexes with cellular proteins by E4orf6 mutant products

E4orf6 has recently been reported to form complexes with a series of cellular proteins (Boivin *et al.* 1999). We speculate that at least some of these proteins play a role in E4orf6-induced p53 degradation. The ability of mutant E4orf6 molecules to interact with a subset of these proteins, including those of 84, 19, and 14kDa, was analyzed in co-immunoprecipitation experiments with anti-E4orf6 sera, and the results have been summarized in Table 4.2 (see also Figure 5.1B for the primary data for some of the E4orf6 mutants). Among the mutants tested, the patterns of p53 degradation and complex formation corresponded exactly, with the exception of dl219-294 and R240E/R241E, which appeared to interact with these cellular species 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. Further studies on the identities of these cellular proteins will be the subject of a future communication.

4.4.5 *In vitro* p53 degradation assays using reticulocyte lysates

It is well known that the human papillomavirus (HPV) E6 protein induces the degradation of p53 through interactions with a cellular ubiquitin ligase, E6AP (Scheffner *et al.* 1993). For HPV E6 this process can be reconstituted *in vitro* by incubation of E6 and p53 proteins with reticulocyte lysates (Scheffner *et al.* 1990b). To determine if E4orf6 and E1B55K function through a similar mechanism, comparable *in vitro* studies were conducted in reticulocyte lysates. Fig. 4.4 shows that HPV18 E6 protein stimulates the degradation of p53 either in the absence (lane 2) or presence of E1B55K (lane 3); however, whether alone (lane 4) or in the presence of E1B55K (lanes 5 and 6), E4orf6 was not functional. 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.

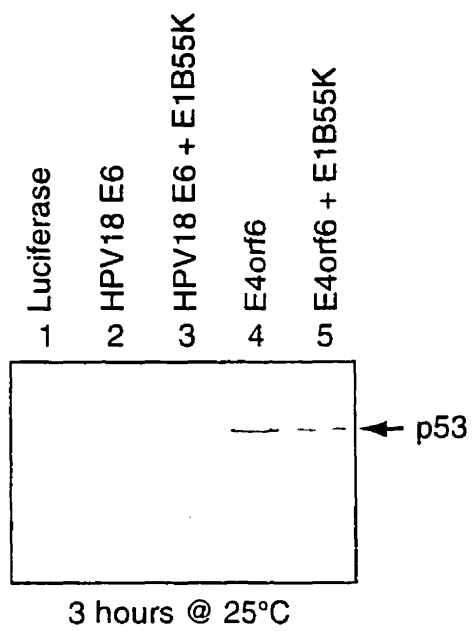


Figure 4.4 *In vitro* degradation of p53 by E4orf6/E1B55K and HPV18 E6.

Radiolabeled p53 and unlabeled E1B55K, E4orf6 and HPV18 E6 protein were synthesized by *in vitro* transcription/translation, and various combinations were 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 has been indicated at the right.

4.4.6 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 (Pomerantz *et al.* 1998, Zhang *et al.* 1998) by interacting with MDM2 to inhibit the ubiquitin-mediated degradation of p53 by MDM2 (Honda and Yasuda 1999). Adenovirus E1A proteins signal through p19ARF to activate and stabilize p53 (de Stanchina *et al.* 1998). 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 expression of p53, p53 and MDM2 (Montes de Oca Luna *et al.* 1995, Jones *et al.* 1996), and p19ARF (Kamijo *et al.* 1997). The MEFs were co-infected 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 re-analyzed for E1B55K and E4orf6 proteins using appropriate antibodies. Fig. 4.5A 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. 4.2, some reduction was observed with AdHH55Kwt alone, due presumably to the presence of low levels of E4orf6 from the vector backbone. Because *mdm2* expression is regulated by p53 (Barak *et al.* 1993), MDM2 protein was detected in proportion to the levels of p53. Analysis of extracts from p53^{-/-}/MDM2^{-/-} (Fig. 4.5B) and p19ARF^{-/-} cells (Fig. 4.5C) indicated patterns of p53 degradation very similar to those shown in Fig. 4.5A. In both cases the levels of p53 were greatly reduced in the presence of E4orf6 and E1B55K, indicating that neither MDM2 nor ARF were required for p53 degradation. Similar high levels of p53 degradation were also detected in U2OS human osteosarcoma cells that lack (Stott *et al.* 1998) p19ARF (data not shown).

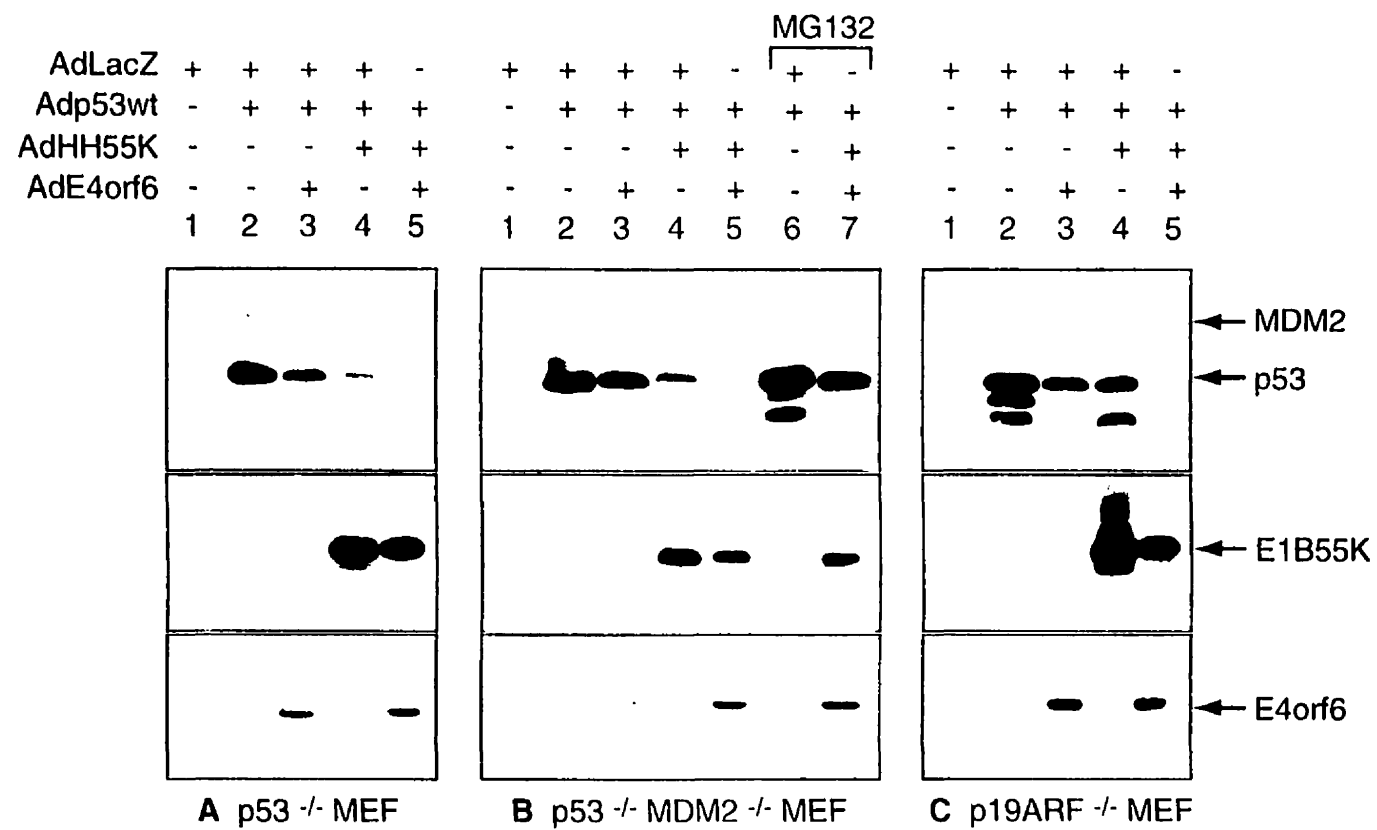


Figure 4.5 Role of MDM2, p19ARF and the 26S proteasome in E1B55K/E4orf6-induced p53 degradation.

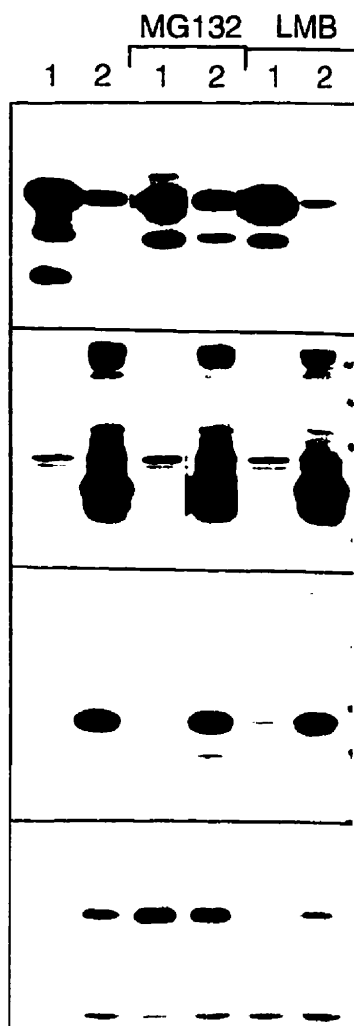
p53 degradation was assessed in cells co-infected with combinations of adenovirus vectors expressing LacZ (control), wild-type p53, E1B55K or E4orf6. Such cells included p53-null MEFs (panel A), p53-/MDM2-null MEFs (panel B) or p19ARF-null MEFs (panel C). Panel B (lanes 6 and 7) also includes extracts from cells treated at 18 hpi with medium containing 50 μ M MG132, an inhibitor of the 26S proteasome. Cell extracts were prepared at 24 hpi, separated by SDS-PAGE and analyzed by western blotting using antibodies against MDM2 (antibody 2A10) and p53 (a mixture of antibodies 1801 and 421) (top panels), E1B55K (2A6) (middle panels), and E4orf6 (1807-4 E4orf6-C) (bottom panels). The combinations of vectors used have been listed at the top of the figure.

4.4.7 Role of the 26S proteasome and nuclear export in E4orf6/E1B55K-mediated p53 degradation

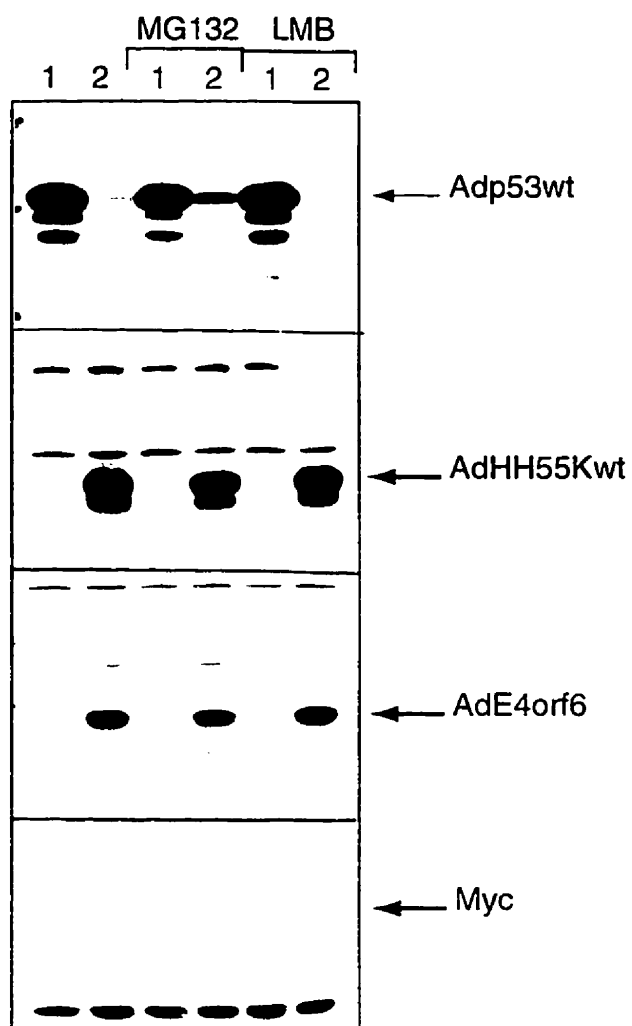
To inquire further into the mechanism of E4orf6/E1B55K-induced p53 degradation, we used drugs that inhibit the 26S proteasome and nuclear export. The MG132 drug (*N*-carbobenzoxy-leucyl-leucyl-leucinal) is a cell permeable transition state inhibitor of chymotrypsin-like and post-acidic activities of 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* (Roth *et al.* 1998a). Analyses of whole cell extracts from p53^{-/-}/MDM2^{-/-} MEFs (Fig. 4.5B) showed that addition of MG132 greatly inhibited E4orf6/E1B55K-induced p53 turnover (compare lanes 6 and 7 with untreated lanes 2 and 5). Thus the 26S proteasome appeared to be important for this process. Fig. 4.6 shows the results of similar studies conducted in human H1299 cells. Cells were co-infected with Adp53wt and either control vector AdLacZ or a combination of AdHH55Kwt and AdE4orf6 in the presence or absence of MG132. Following collection, cells were separated into nuclear and cytoplasmic fractions and then analyzed for p53, E4orf6 and E1B55K by western blotting as in Fig. 4.5. The levels of endogenous c-Myc protein were measured using anti-c-Myc serum as this nuclear protein is known to be degraded via the 26S proteasome (Flinn *et al.* 1998, Gross-Mesilaty *et al.* 1998). Fig. 4.6 shows that c-Myc is detected in nuclear (Fig. 4.6A) but not cytoplasmic (Fig. 4.6B) fractions, indicating the integrity of the fractionation protocol. Fig. 4.6A shows that treatment with MG132 increased the levels of endogenous c-Myc, confirming that MG132 was effective in inhibiting the 26S proteasome (Gross-Mesilaty *et al.* 1998). It was noted that the levels of c-Myc in untreated cells were somewhat higher in the presence of E4orf6 and E1B55K. The reasons for this effect are unknown, though we can speculate that the highly expressed viral proteins could monopolize cellular degradative machinery. Analysis of p53 levels in untreated cells indicated that significant quantities were present in both the nucleus (Fig. 4.6A) and the cytoplasm (Fig. 4.6B). This result could be expected as p53 itself contains a nuclear export signal and shuttles between nuclear and cytoplasmic compartments (Stommel *et al.* 1999). It

H1299

A Nuclear Extracts



B Cytoplasmic Extracts



1 - Adp53wt + AdLacZ

2 - Adp53wt + AdHH55Kwt + AdE4orf6

Figure 4.6 Role of nuclear export and the 26S proteasome in E1B55K/E4orf6-mediated p53 degradation.

H1299 cells were infected with Adp53wt and AdLacZ (lanes 1) or Adp53wt, AdHH55Kwt and AdE4orf6 (lanes 2) and nuclear and cytoplasmic extracts were prepared at 24 hpi, as described in Materials and Methods. As indicated in the figure, in some cases cells were treated from 18 hpi to 24 hpi with medium containing 50 μ M MG132 inhibitor, or 20nM leptomycin B (LMB), an inhibitor of nuclear export. Extracts were separated by SDS-PAGE and analyzed by western blotting using antibodies against p53 (top panel), E1B55K (second panel), E4orf6 (third panel), or c-Myc (bottom panel). The positions of migration of these proteins has been indicated at the right.

should be noted that even though equal protein was loaded in all lanes, the intensity of the signal detected does not represent the relative abundance of proteins in each compartment. Expression of E4orf6 and E1B55K caused a significant reduction of p53 in both cell fractions. Treatment with MG132 blocked this reduction both in the nucleus, where levels increased only slightly, and in the cytoplasm where the recovery of p53 levels was somewhat greater. One explanation for this observation is that p53 was constantly being transported into the nucleus for degradation and thus more depletion was seen in the cytoplasmic compartment. Also MG132 may not inhibit the nuclear proteasomes as efficiently, and thus the relief of E4orf6/E1B55K-directed p53 degradation was not complete (see also Fig. 4.5).

Like p53, E1B55K, and E4orf6, MDM2 also contains a functional NES (Roth *et al.* 1998a). Unbound p53 may also be constantly shuttling. To direct p53 degradation, MDM2 may need to export p53 into the cytoplasm, as suggested by the fact that inhibition of nuclear export results in the accumulation of active p53 in the nucleus (Lain *et al.* 1999a, Lain *et al.* 1999b). As shown in Table 4.2, the E4orf6/E1B55K-induced turnover of p53 was unaffected by mutations of the E4orf6 NES. To confirm that E4orf6-dependent nuclear export of complexes containing E1B55K and p53 is not required, use was made of leptomycin B (LMB) cytotoxin, a potent inhibitor of the CRM1 nuclear export receptor (Lain *et al.* 1999a, Lain *et al.* 1999b). Fig. 4.6 shows that LMB had no effect on p53 degradation, either in nuclear or cytoplasmic fractions. Thus E4orf6-mediated nuclear export did not appear to be essential for p53 degradation.

4.5 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. We found no evidence that E1B55K or E4orf6 interact with either form of p73, a finding in agreement with two previous reports (Marin *et al.* 1998, Roth *et al.* 1998b), but contrary to another for E4orf6 (Higashino *et*

al. 1998). In any case, using exogenously expressed p73 α and p73 β , E4orf6/E1B55K complexes were clearly found to be unable to induce p73 degradation.

The present 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 (Fig. 4.2 B and C). The forms of p53 that were resistant to such degradation were those that failed to associate with E1B55K. Would p53 and E1B55K proteins that are incapable of interacting directly, but which retain the ability to complex with E4orf6, still be active in degradation? We are still uncertain about the answer to this question as it has been difficult to map the p53 binding site(s) on E4orf6, and thus far all existing E1B55K mutants that fail to bind p53 are also defective for E4orf6 binding (Yew *et al.* 1990, Rubenwolf *et al.* 1997). The p53 22-23 point mutations (Fig. 4.2E) in the E1B55K binding site should not affect E4orf6 binding, and as we and others (Roth *et al.* 1998b) 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 (Table 4.1). This point was also well illustrated by Roth *et al.* (Roth *et al.* 1998b) 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 then found that this p73mut20-24 could be degraded by E1B55K/E4orf6. As E4orf6 does not bind p73 (see above), it must mean that E1B55K binding was sufficient for p73mut20-24 to be degraded.

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 (Querido *et al.* 2000a); however, fine mapping in the central, generally hydrophobic region of E4orf6, was not highly informative as all such mutants lost the ability to cooperate in p53 degradation. These results may suggest a functional requirement for extended regions of E4orf6, or 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 many specific point mutants. Conversely, deletions at the hydrophilic amino and carboxy termini are reasonably informative.

Mapping studies confirmed a previous report that binding of E1B55K required a region towards the amino terminus of E4orf6 (Rubenwolf *et al.* 1997). 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 probably not sufficient. Fine mapping of this binding region using small E4orf6 deletion mutants suggested that many contact points may exist as all were capable of E1B55K binding. Fig. 4.1 shows that this region is downstream of the NLS, but spans the NES signal sequence; however, the latter is not required for binding as mutant dl83-94 was wild-type both for binding and for p53 degradation in cooperation with E1B55K. It should be noted that although we found that dl108-294 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 (Goodrum *et al.* 1996). 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 is possible that the E1B55K/E4orf6 complexes we observed may be created in cell extracts following lysis of the cell. Thus 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 mutants (Table 4.2), perhaps because there are many points of contact between the two proteins, and 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 found to be 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 co-immunoprecipitation of a series of cellular and possibly viral proteins using E4orf6-specific antibodies (Boivin *et al.* 1999). In the present studies using E4orf6 deletion mutants, complex formation with 84, 19, and 14kDa cellular

proteins was mapped to an extended region from residue 44 to 218. It will be important to identify these species as 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 to 253 (Goodrum *et al.* 1996, Dobbelstein *et al.* 1997, Orlando and Ornelles 1999). Disruption of this region, either by 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. Studies with the drug leptomycin B suggested that nuclear export was not required for p53 degradation. In addition, removal of either the NES or the NLS had no effect on p53 degradation, suggesting, in contrast to a previous model (Weigel and Dobbelstein 2000), that E4orf6-dependent nuclear-cytoplasmic shuttling is not required, and that p53 degradation may take place in the nucleus. Support for this possibility 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 underway to investigate the mechanism further. Our results are in agreement with those published by Nevels *et al.* (Nevels *et al.* 1999, Nevels *et al.* 2000) who found that in rodent cells transformed with E1A/E1B and E4orf6, the amino terminal region, the NES, 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 showed that deletion of residues 123-126, the core of this newly described CCR motif (Boyer and Ketner 2000, Nevels *et al.* 2000), was sufficient to disrupt p53 degradation (Table 4.2). Boyer and Ketner recently reported that E4orf6, like HPV E6 (Grossman and Laimins 1989), is a zinc binding protein (Boyer and Ketner 2000). Several critical histidine (H) and cysteine (C) residues of E4orf6 were identified and mutations at those residues interfered with E1B55K binding and p53 degradation.

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 (Gabler *et al.* 1998) and E4orf6 (Boivin *et al.* 1999); and genetic analyses using appropriate viral mutants.

4.6 Acknowledgments

Dennis Takayesu prepared the mouse monoclonal antibody against the carboxy terminus of E4orf6. We thank Allan Bradley and Guillermina 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 the AdE2F1 virus, Matthias Dobbstein for the E4orf6 mutants L90A/I92A, R248E and L90A/I92A/R248E, Bill Kaelin for the p73 α and p73 β 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. a Studentship from McGill University.

As the adenovirus E4orf6 protein was being characterized in our laboratory, Dominique Boivin and I discovered several E4orf6 binding proteins that were cellular in origin. These proteins had apparent molecular weights of 84, 19, 16 and 14 kDa, and could be observed when immunoprecipitations were performed either with E4orf6 antibodies (Boivin *et al.* 1999) or with an E1B55K antibody when E4orf6 was also present (E. Querido, data not shown). To further characterize them, Megan Morrison isolated three of these cellular proteins from silver-stained SDS-PAGE gels and sent them to Brian Cox at Borealis Biosciences Inc. (Toronto, Canada), to be identified by ion trap mass spectrometry. This final data chapter contains work that was done both by me in our laboratory, and in our collaborator Joan Weliky Conaway's laboratory, to confirm the identification of these cellular proteins and study their function.

CHAPTER V

Human Adenovirus E4orf6 and E1B55K Proteins are Subunits of a Cullin-containing E3 Ubiquitin Ligase That Targets p53.

5.1 Abstract

Although MDM2 plays a major role in the regulation of the stability of the p53 tumor suppressor protein, MDM2-independent pathways also appear to exist in cells, and little is known about these processes. The adenovirus E1B55K and E4orf6 proteins collaborate to promote viral replication by targeting p53 for degradation, preventing early death of infected cells. To shed light on the mechanism of adenovirus-induced p53 degradation, and perhaps novel cellular pathways, we immunoaffinity purified a multi-protein E4orf6-containing complex and identified its components. This complex contains E4orf6, E1B55K, p53, and a novel E4orf6-associated

Cullin-containing E3 ubiquitin ligase that is: (1) composed of Cullin family member Cul5, Elongins B and C, Cdc34 E2 ubiquitin-conjugating enzyme, and the RING-H2 finger protein Rbx1; (2) remarkably similar to the recently described von Hippel-Lindau (VHL) tumor suppressor and SCF (Skp1-Cul1/Cdc53-F-box) E3 ubiquitin ligase complexes and; (3) capable of stimulating ubiquitination of p53 *in vitro* in the presence of E1/E2 ubiquitin activating and conjugating enzymes. Our results identify a novel role for the Cullin-based ubiquitination machinery in regulation of p53.

5.2 Introduction

Regulation of the stability of the p53 tumor suppressor protein is crucial in the control of p53 function. Several studies have shown that the rapid increase in p53 protein levels upon cellular stress is due to a sharp rise in protein half-life. Several groups have used protease inhibitors and cells lacking components of the proteasome to demonstrate that ubiquitin-dependent proteolytic degradation is the most important mechanism of p53 turnover (reviewed in (Lohrum and Vousden 1999)). MDM2, which functions as an E3 ubiquitin ligase for p53 (Honda *et al.* 1997) is the principal regulator of p53 stability. The Jun-N(amino)-terminal kinase (JNK), however, has been shown to mediate MDM2-independent p53 degradation (Fuchs *et al.* 1998). JNK is likely an adapter that targets c-Jun, ATF2 or p53 to an E3 ubiquitin ligase complex (reviewed in (Lohrum and Vousden 1999)), the components of which have yet to be determined.

DNA tumor virus oncoproteins target activities involved in the normal growth control of cells. The adenovirus early region 1A (E1A) protein binds to the retinoblastoma tumor suppressor and the p300/CBP/p400 proteins to stimulate DNA synthesis, and but these interactiona also induce the activation, stabilization, and accumulation of p53 (Chiou and White 1997, Querido *et al.* 1997b, Samuelson and Lowe 1997). Nonetheless, p53 accumulation is not usually apparent in wild-type adenovirus-infected cells, even though these cells express high

levels of E1A products. We and others have shown that the adenovirus type 5 (Ad5) E4orf6 and E1B55K proteins function together to induce efficient p53 degradation (Moore *et al.* 1996, Nevels *et al.* 1997, Querido *et al.* 1997a, Steegenga *et al.* 1998). E4orf6 is a nuclear protein containing both nuclear localization and nuclear retention signals, which are believed to be responsible for targeting E4orf6 and E4orf6/E1B55K complexes to the nucleus (Goodrum *et al.* 1996, Orlando and Ornelles 1999). In addition, like HIV Rev and MDM2, E4orf6 contains a putative nuclear export signal that is believed to play a role in nuclear-cytoplasmic shuttling (Dobbelstein *et al.* 1997, Roth *et al.* 1998a). Nuclear export of p53 via MDM2 appears to be required for its degradation by the 26S proteasome, and blocking this nuclear export of p53 by the drug leptomycin B (Lain *et al.* 1999a) or the p53-binding protein p19ARF (reviewed in (Lohrum and Vousden 1999)) results in p53 stabilization. In contrast, E4orf6/E1B55K-mediated p53 degradation does not require MDM2 and is not affected by leptomycin B (see Figures 4.5 and 4.6), suggesting that adenovirus-mediated degradation of p53 may occur in the nucleus by a novel mechanism (Querido *et al.* 2000b).

In the present studies we have characterized a series of E4orf6-binding proteins that appear to be essential for adenovirus-induced p53 degradation. Their identity as members of a unique Cullin-containing E3 ubiquitin ligase complex identifies a novel role for the Cullin-based ubiquitination machinery in regulation of p53.

5.3 Materials and methods

5.3.1 Detection of E4orf6 binding proteins in [³⁵S] labeled extracts

Human small cell carcinoma H1299 cells (Mitsudomi *et al.* 1992) growing on 100mm-diameter plates were infected at a multiplicity of infection of 30 plaque-forming units per cell with E1/E3B-deleted adenovirus vectors containing inserts in the E1 region under the cytomegalovirus (CMV) promoter and expressing either *E. coli* LacZ, AdLacZ (Bacchetti and Graham 1993) , or the human Ad5 E4orf6 protein, AdE4orf6 (Boivin *et al.* 1999). Following

labeling from 18 to 22 hours post-infection with 200 μ Ci per plate of [^{35} S]methionine/[^{35} S]cysteine EasyTag Express protein labeling mix (>1000 Ci/mmol; DuPont NEN) in methionine/cysteine-free medium, whole cell extracts were prepared in buffer X [50mM Tris-HCl, pH 8.0, containing 250mM NaCl, 1% NP40 (v/v), 1 mM EDTA, 1mM EGTA, 2 μ g/ml each of aprotinin, leupeptin and pepstatin], immunoprecipitated using 1D5 mouse monoclonal antibody raised against the carboxy terminus of E4orf6, and analyzed by SDS-PAGE and autoradiography, as described previously (Boivin *et al.* 1999). Prestained standard size markers (Bio-Rad) were used and their positions are indicated in Fig. 1A. The potential identities shown for the p84, p19 and p14 species were derived from mass spectroscopy analysis of tryptic peptides derived from gel purified material (Borealis Biosciences Inc., Toronto, Canada). For the analysis of complex formation using E4orf6 deletion mutants, a study similar to that of Fig. 5.1A was carried out in H1299 cells except that cells in 100mm-diameter plates were lipofected according to the GIBCO/BRL Lipofection protocol with 10 μ g of pcDNA3 plasmid DNA encoding wild-type or mutant E4orf6. In-frame deletion mutants were generated using PCR-based protocols, as described in chapter IV (see Fig. 4.1).

5.3.2 Binding assays for endogenous or overexpressed proteins

In some cases H1299 cells growing in 100mm-diameter dishes were infected with the indicated adenovirus vectors, while in other cases H1299 cells were seeded in 60mm plates and lipofected according to the GIBCO/BRL Lipofection protocol with 2 μ g total of plasmid DNA. After 24 hours, whole cell extracts were prepared in buffer X, immunoprecipitations were done with the indicated antibodies, and they were separated by SDS-PAGE. Following transfer to nitrocellulose, western blotting was performed and species were visualized by enhanced chemiluminescence (NEN LifeScience). The adenovirus vectors AdHH55K, expressing His-tagged Ad5 E1B55K (Querido *et al.* 1997a) and Adp53wt, expressing human wild-type p53 (Bacchetti and Graham 1993), were used in addition to AdLacZ and AdE4orf6. The following plasmids were also used: MycRbx1 encodes myc-tagged murine Rbx1 (Kamura *et al.* 1999);

HA-E2cdc34 encodes HA-tagged human E2cdc34 (Lisztwan *et al.* 1998); FLAG-VHL encodes human FLAG-tagged VHL (Kamura *et al.* 1999). Anti-Myc 9E10 antibody (Santa Cruz), anti-HA.11 antibody (BabCo), as well as anti-FLAG M2 antibody (SIGMA) were used to detect the tagged epitopes. Anti-p53 pAb421 and pAb1801 hybridoma supernatants concentrated by ammonium sulfate precipitation were used to immunoblot p53. E1B55K was detected with 2A6 monoclonal antibody (Sarnow *et al.* 1982b). E4orf6-specific 1807 rabbit polyclonal antibody was described previously (Boivin *et al.* 1999). Anti-Elongin A, anti-Elongin B, and anti-Elongin C goat polyclonal antibodies were purchased from Santa Cruz Biotechnology, and Ig32 anti-VHL mouse monoclonal antibody was from Pharmingen. The rabbit polyclonal antibody generated against the carboxy terminus of rabbit VACM-1 (Burnatowska-Hledin *et al.* 1995) was obtained from M. Burnatowska-Hledin. A rabbit polyclonal antibody prepared for us by Genemed Synthesis Inc. (CA, USA) against a synthetic peptide (EHKIRRDESDINTFIYMA) corresponding to the carboxy terminus of human Cul5 was also used in this study.

5.3.3 Binding assay for *in vitro*-translated Cullins

Human Cul1, Cul2, Cul3 and Cul5, and mouse Cul4A (Michel and Xiong 1998) were synthesized using a standard *in vitro* transcription/translation kit (TnT, Promega) in the presence of 40μCi of translation grade [³⁵S]methionine (Dupont-NEN). Aliquots of these labeled proteins were also combined with immunoprecipitates prepared from AdLacZ (middle panel) or AdE4orf6 (bottom panel) using 1D5 anti-E4orf6 antibody. Following the final wash the precipitates were divided into five equal portions, resuspended in 100μl of buffer X and each combined with a 10μl aliquot of each Cullin *in vitro* translation preparation. The mixture was incubated at room temperature for 1 hour and after five washes with buffer X the precipitates were separated by SDS-PAGE using a 10% polyacrylamide gel. The presence of labeled Cullins was assessed by autoradiography.

5.3.4 Intracellular co-localization of E4orf6 and Cul5

H1299 cells were seeded in 4-chamber culture slides (Falcon) and lipofected with 0.4 μ g of plasmid according to the Gibco/BRL protocol. After 24 hours cells were fixed and prepared for immunohistochemistry. E4orf6 was detected using rabbit polyclonal 1807 antibody and Alexa 594 (red) anti-rabbit IgG antibody (Molecular Probes). HA-tagged human Cul2 and Cul5/VACM-1 (T. Kamura) were detected using mouse monoclonal anti-HA.11 antibody (BabCo) and Alexa 488 (green) anti-mouse antibody (Molecular Probes). The fluorophores were visualized using a laser confocal Zeiss microscope.

5.3.5 Reconstitution of E4orf6/E1B55K/Cul5/Rbx1/Elongin B and C complexes in Sf21 cells

Plates containing 1×10^6 Sf21 cells were infected with baculoviruses encoding various proteins. Cells were lysed in 1 ml 40 mM Hepes-NaOH (pH 7.9), containing 150 mM NaCl, 1 mM DTT, 0.5% (v/v) Triton X-100, 10% (v/v) glycerol, 5 μ g/ml leupeptin, 5 μ g/ml antipain, 5 μ g/ml pepstatin A, and 5 μ g/ml aprotinin. Approximately 100 μ g of Sf21 cell lysate was immunoprecipitated with either 2 μ g of anti-HA (12CA5, Boehringer-Mannheim) or 2 μ g of anti-E4orf6 (1D5) antibody and 10 μ l of protein A Sepharose. Crude lysates and washed immune complexes were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

5.3.6 In vitro ubiquitination of p53 by E4orf6 complexes

Lysates from cells infected with baculoviruses encoding E1B55K, Elongin B, Elongin C, HA-Cul5, and myc-Rbx1, with or without baculovirus encoding E4orf6, containing ~100 μ g of Sf21 were immunoprecipitated with 2 μ g of 1D5 antibody and 10 μ l protein A Sepharose beads. To the precipitates were added 0, 20, or 65 ng of murine histidine-tagged p53 (Wang *et al.* 1993) that had been expressed in insect cells and purified by nickel chromatography, as previously described (Kamura *et al.* 1999), and the mixtures

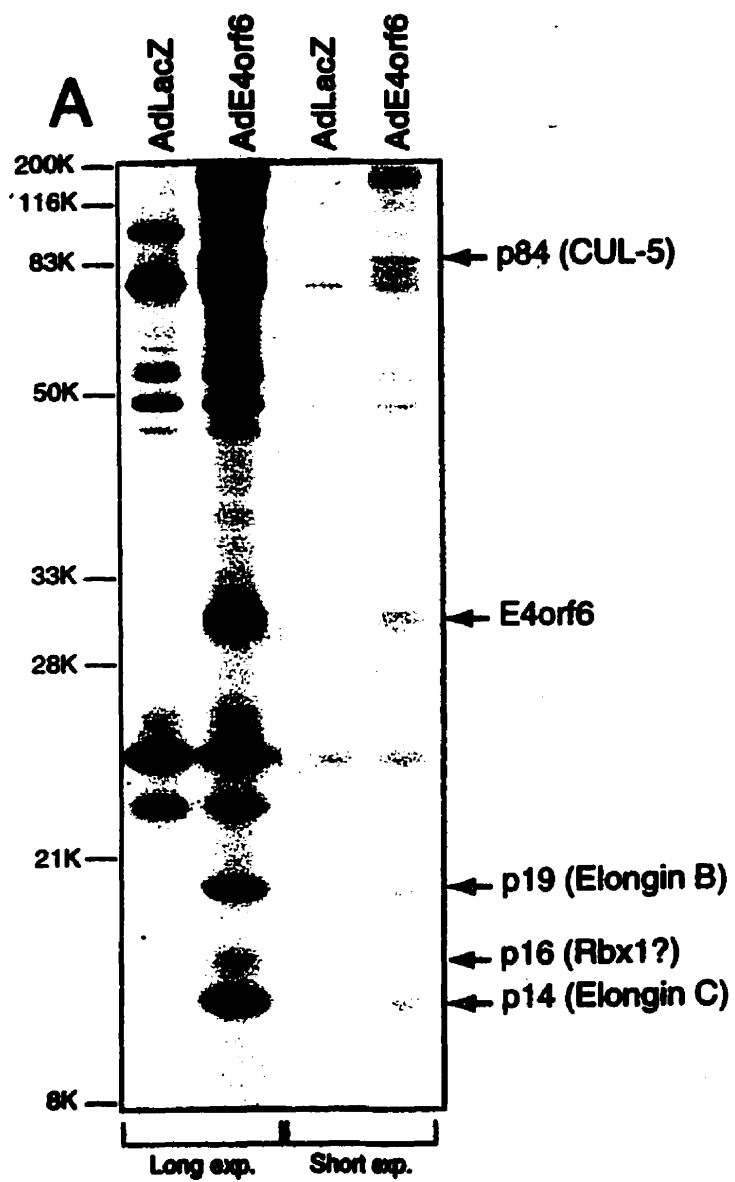
were incubated with ~50 ng yUba1, ~100 ng hUbc5a, and ~500 ng GST-UbK48R in 10 μ l of 40mM Hepes-NaOH (pH7.9), containing 60 mM potassium acetate, 0.1% (v/v) Triton X-100, 2 mM DTT, 6 mM MgCl₂, 0.5 mM EDTA (pH 7.9), 10% (v/v) glycerol, and 1.5 mM ATP. Reaction mixtures were incubated for 30 minutes at 25^o C, separated by SDS-PAGE, and immunoblotted with Ab421 anti-p53 antibodies.

5.4 Results

5.4.1 Identification of novel E4orf6/E1B55K binding proteins

To investigate the roles of the E1B55K and E4orf6 proteins in regulating p53 degradation in adenovirus-infected cells, E4orf6-containing complexes were immunoprecipitated from whole cell extracts of [³⁵S]methionine/ [³⁵S]cysteine-labeled p53-null human H1299 cells infected with either an adenovirus vector expressing Ad5 E4orf6 (AdE4orf6) or a control vector (AdLacZ). Using this procedure, we observed that stoichiometric amounts of ³⁵S-labeled cellular proteins of ~ 84 kDa, 19 kDa, and 14 kDa, as well as a substoichiometric amount of an ~16 kDa protein were reproducibly and specifically immunoprecipitated with E4orf6 (Fig.5.1, panel A and also previous work (Boivin *et al.* 1999)). Ion trap mass spectrometry of the E4orf6-associated proteins revealed that the ~84 kDa protein was the Cullin family member Cul5 (Byrd *et al.* 1997) (also known as the vasopressin-activated calcium mobilization receptor or VACM-1) and that the ~19 kDa and 14 kDa proteins were Elongins B and C, respectively.

Cullin family members have recently been shown to be components of the architecturally similar VHL tumor suppressor and SCF (Skp1-Cullin-F box protein) complexes, which are E3 ubiquitin ligases that target a variety of transcriptional, cell cycle, and other regulatory proteins for ubiquitination and subsequent degradation by the proteasome (reviewed in (Tyers and Willems 1999). The Cullin family member Cul2 is linked to the VHL protein by the Elongin BC complex, which functions as an adapter that



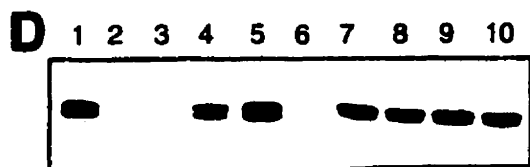
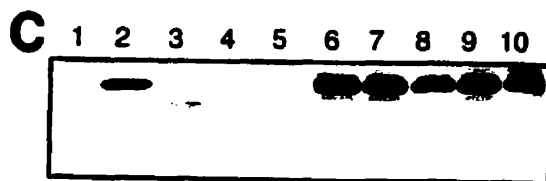
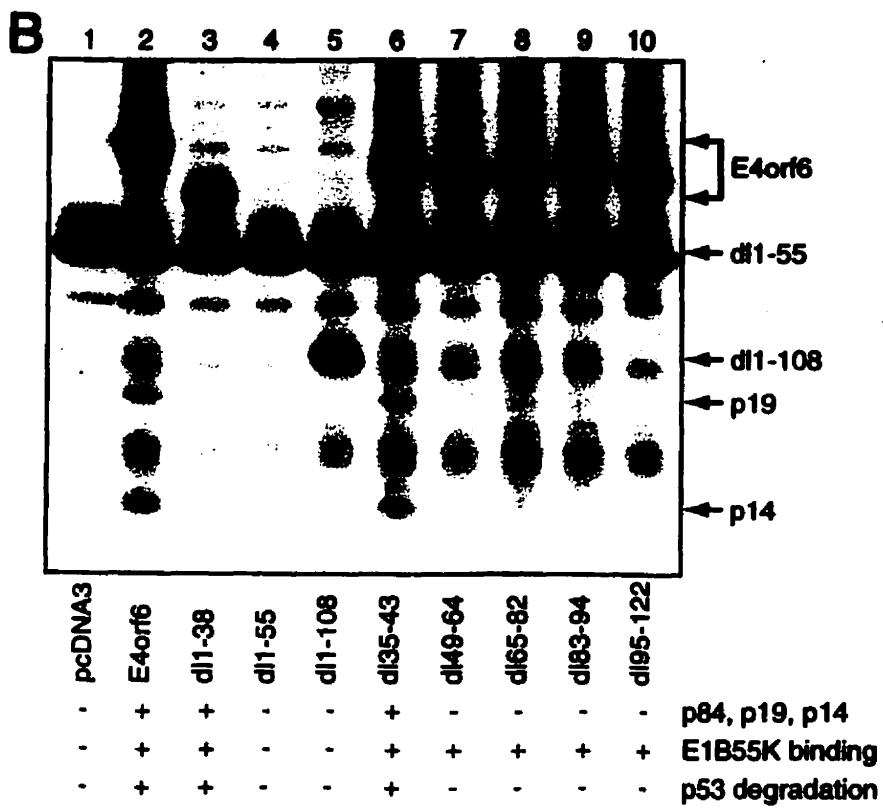


Figure 5.1 Analysis of E4orf6 complex formation.

(A) Detection of E4orf6 complexes. Human small cell carcinoma H1299 cells were infected with adenovirus vectors AdLacZ or AdE4orf6. Following incubation with [³⁵S]methionine/[³⁵S]cysteine protein labeling mix, whole cell extracts were prepared, immunoprecipitated with anti-E4orf6 (1D5) antibody, and analyzed by SDS-PAGE and autoradiography. Two exposure times have been included. At the left are shown the positions of migration of prestained standard size markers. At the right, in addition to E4orf6, are indicated the positions of co-immunoprecipitating proteins. The potential identities shown for these species were derived from mass spectroscopy analysis of tryptic peptides. (B) Analysis of complex formation using E4orf6 deletion mutants. A study similar to that of Fig. 1A was carried out in H1299 cells except that cells were transfected with plasmid DNA encoding wild-type or mutant E4orf6. The ability of the p84, p19 and p14 cellular proteins to associate with E4orf6 polypeptides, have been summarized below as + and -. In addition, the results of binding experiments with E1B55K (shown in Fig. 1C) and p53 turnover experiments with E4orf6 and E1B55K (Fig. 1D) have also been indicated by + and -. (C) Complex formation between E4orf6 mutants and E1B55K. H1299 cells were transfected with plasmids expressing E1B55K (pCA14 HH55K) and wild-type or mutant E4orf6 proteins (pcDNA3E4orf6). Cell lysates were immunoprecipitated with anti-E1B55K (2A6) antibody and E4orf6 was detected with rabbit polyclonal (1807) antibody. (D) E4orf6/E1B55K-induced turnover of p53. H1299 cells were transfected with the following plasmids: 1μg of pcDNA3 p53wt plasmid DNA (expressing human p53), 4μg of pCA14 HH55K, and 4μg of pcDNA3E4orf6 (expressing wild-type or mutant E4orf6). Equal protein samples from whole cell extracts were separated by SDS-PAGE and immunoblotted with a mixture of anti-p53 (1801 and 421) antibodies.

binds to both Cul2 and a conserved ~12 amino acid 'BC-box' motif present in VHL (Kamura *et al.* 1998). In SCF complexes, the Cullin family member Cul1/Cdc53 is linked to a variety of F-box proteins by the Elongin C-like protein Skp1, which binds to both Cul1/Cdc53 and a conserved ~40 amino acid 'F-box motif' present in F-box proteins. As components of Cul1/Cdc53-containing SCF complexes, F box proteins bind to and recruit proteins targeted for ubiquitination to the complex.

5.4.2 The E4orf6 protein binds Cul5 and Elongins B and C

To confirm the interaction of Elongins B and C with E4orf6, H1299 cells were infected with either the control adenovirus vector AdLacZ (Fig. 5.2A, lanes 1) or with vectors encoding E4orf6 (AdE4orf6, lanes 2) or a combination of E4orf6, His-tagged E1B55K, and p53 (lanes 3). Western blotting with the anti-E4orf6 antibody 1807, and with antibodies against Elongin B or Elongin C was then carried out on whole cell extracts (left) or on proteins immunoprecipitated with anti-E4orf6 1D5 antibodies (right). As expected, E4orf6 was detected in only in whole cell extracts from E4orf6-expressing cells (top panel). Elongin B (middle panel) and Elongin C (bottom panel) were specifically precipitated with E4orf6 whether or not E1B55K and p53 were present.

Elongins B and C have been shown previously to bind to Elongin A and to the VHL protein, raising the possibility that E4orf6 might be linked to one or both of these proteins through its interaction with the Elongin BC complex. As shown in Fig. 5.2D, however, neither Elongin A nor the VHL protein could be coimmunoprecipitated with E4orf6. In confirmation that VHL is not required for E4orf6/E1B55K-mediated p53 degradation, human VHL-positive and -null tumor cell lines were tested and both were found to support p53 turnover by the viral proteins at similar levels (data not shown).

To verify the interaction of E4orf6 and Cul5, extracts were prepared from control cells infected with AdLacZ and from cells infected with AdE4orf6, separated into nuclear and

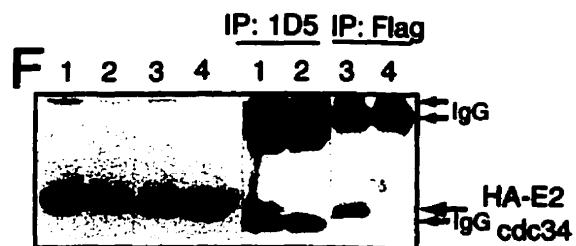
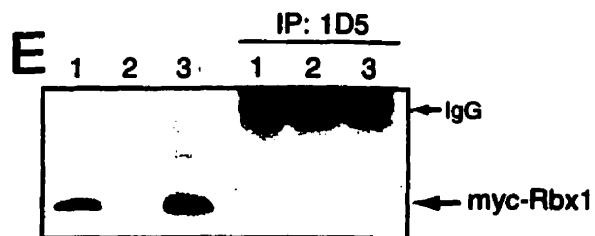
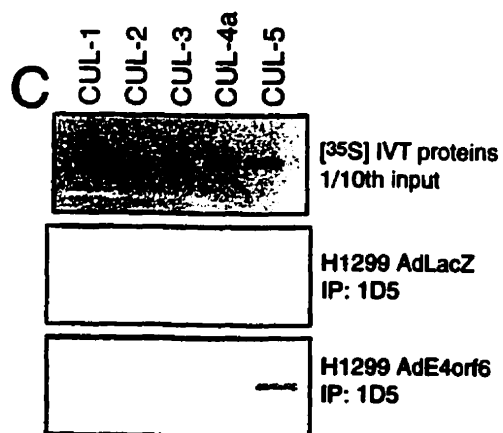
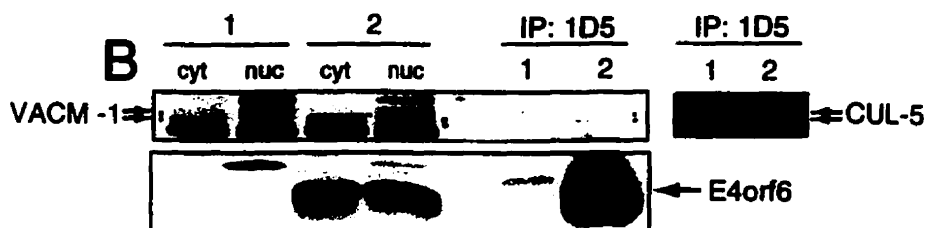
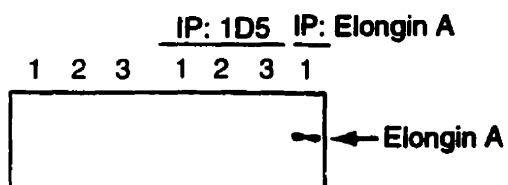
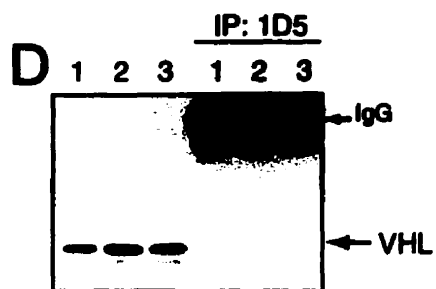


Figure 5.2 Identification of E4orf6 binding proteins.

(A) H1299 cells were infected with AdLacZ (lane 1), AdE4orf6 (lane 2), or AdE4orf6 plus AdHH55K plus Adp53wt (lane 3). Whole cell extracts (left three lanes) and 1D5 immunoprecipitates (right three lanes) were separated by SDS-PAGE, transferred to nitrocellulose, and then subjected to western blotting using: (top panel) 1807 rabbit polyclonal anti-E4orf6 antibodies; (middle panel) anti-Elongin B goat antibody; (bottom panel) anti-Elongin C goat polyclonal antibody. (B) H1299 cells were infected with AdLacZ (lane 1) or AdE4orf6 (lane 2), and after harvesting, some cells were fractionated into cytoplasmic and nuclear extracts while the remainder were prepared as whole cell extracts and immunoprecipitated with 1D5 anti-E4orf6 antibody. VACM-1/Cul5 (upper panels) was detected by immunoblotting using either a rabbit polyclonal antibody to rabbit VACM-1 (left six lanes), or a rabbit polyclonal antibody against human Cul5 (right two lanes). E4orf6 (lower panel) was detected using 1807 rabbit polyclonal antibody. (C) Human Cul1, Cul2, Cul3 and Cul5, and mouse Cul4A were synthesized (top panel) using a standard *in vitro* transcription/translation kit in the presence of [³⁵S]methionine. Aliquots of these labeled proteins were also combined with immunoprecipitates prepared from AdLacZ (middle panel) or AdE4orf6 (bottom panel) infected H1299 cells using 1D5 anti-E4orf6 antibody, and after washes the precipitates were separated by SDS-PAGE. The presence of labeled Cullins was assessed by autoradiography. (D) H1299 cells were infected by AdLacZ (lane 1), AdE4orf6 (lane 2) or AdLacZ plus AdHH55K plus Adp53wt (lane 3), and whole cell extracts (left three lanes) or 1D5 immunoprecipitates (right lanes) were separated by SDS-PAGE. The presence of VHL (upper panel) was assessed by immunoblotting using anti-VHL (Ig32) antibody. Elongin A (bottom panel) was detected with a goat polyclonal antibody. The right lane in the bottom panel shows an immunoprecipitate prepared from AdLacZ-infected cells using anti-Elongin A antibody followed by immunoblotting with the same antibody. (E) H1299 cells were transfected with (lane 1) pcDNA3 + Myc-Rbx1; (lane 2) pcDNA3 + pcDNA3E4orf6; and (lane 3) Myc-Rbx1 + pcDNA3E4orf6. Whole cell extracts (left lanes) or 1D5 immunoprecipitates (right lanes) were separated by SDS-PAGE and immunoblotted with anti-Myc (9E10) antibody. (F) H1299 cells were transfected with (lane 1) pcDNA3E4orf6 + HA-E2cdc34; (lane 2) pcDNA3 + HA-E2cdc34; and (lane 3) FLAG-VHL + HA-E2cdc34; and (lane 4) pcDNA3 + HA-E2cdc34. Cells were harvested and analyzed as above, except that anti-HA (HA.11) antibody was used for immunoblotting.

cytoplasmic fractions, and immunoprecipitated with 1D5 antibodies against E4orf6 (Fig. 5.2B). Following western blot analysis, an antibody raised against rabbit Cul5 (VACM-1, (Burnatowska-Hledin *et al.* 1995)) detected two closely-migrating Cul5 species in cytoplasmic fractions from AdLacZ- (lane 1) and AdE4orf6-infected cells (lane 2); however, in 1D5 immunoprecipitates and in nuclear fractions, Cul5 was only detected when E4orf6 was present (compare lanes 1 and 2). To confirm that this species represented Cul5, an anti-peptide polyclonal antiserum was raised against a synthetic peptide corresponding to the carboxy terminus of human Cul5. Fig. 5.2B shows that similar results were obtained with this antiserum (see lanes 1 and 2 at right). To determine whether E4orf6 can associate with other members of the Cullin family, *in vitro* translated human Cul1, Cul2, Cul3 and Cul5 or mouse Cul4a were tested for the ability to bind 1D5 immunoprecipitates from AdLacZ or AdE4orf6-infected H1299 cells. As shown in Fig. 5.2C, all Cullins were synthesized at similar levels (Fig. 5.2C, top); however, only Cul5 was specifically precipitated with E4orf6.

5.4.3 The E4orf6/Cul5/ElonginBC complex interacts with Rbx1 and E2cdc34

The ~16 kDa Rbx1 protein is a component of both the SCF and VHL tumor suppressor complexes (Kamura *et al.* 1999). As shown in Fig. 5.2E, myc-tagged Rbx1 can be specifically immunoprecipitated with E4orf6 from cells transfected with cDNAs expressing these two proteins. Thus, Rbx1 associates with E4orf6 and probably represents the 16kDa species detected at low levels in Fig. 5.1A.

To confirm the interaction of E4orf6 and E1B55K with Cul5, Elongins B and C, and Rbx1, Sf21 insect cells were infected with various combinations of baculoviruses encoding E4orf6, E1B55K, Elongins B and C, HA-tagged Cul5 (HA-Cul5), and c-Myc-tagged Rbx1 (cmyp-Rbx1), and complexes were immunoprecipitated from cell lysates with 1D5 antibodies against E4orf6 or with antibodies against the HA-epitope and subjected to immunoblotting. As shown in Fig. 5.4A, complexes containing E4orf6, E1B55K, Cul5,

Elongins B and C, and Rbx1 could be isolated from insect cells. Consistent with the results obtained in mammalian cells, the interaction of E4orf6 with Cul5, Elongins B and C, and Rbx1 does not require E1B55K. In addition, Cul5 and Elongins B and C were able to interact with E4orf6 in lysates from cells not overexpressing other complex components. Taken together, our data indicate that E4orf6 can assemble with Elongins B and C, Cul5, and Rbx1 in a complex that is distinct from the Elongin BC-containing complexes formed with VHL and Elongin A.

The Cul5-containing E4orf6 complex shares remarkable architectural similarity with the Cul1/Cdc53-containing SCF and the Cul2-containing VHL ubiquitin ligase complexes. The human Cdc34 E2 ubiquitin-conjugating enzyme functions with both the SCF and VHL complexes, so the possibility that it might associate with E4orf6 complexes as well was tested. H1299 cells were transfected with cDNAs expressing E4orf6 and human HA-E2cdc34 (lanes 1), HA-E2cdc34 alone (lanes 2 and 4), or FLAG-tagged VHL and HA-E2cdc34 (lanes 3), and whole cell extracts were prepared and subjected to immunoprecipitation with anti-E4orf6 or anti-FLAG antibodies. As shown in Fig. 5.2F, Cdc34 was specifically immunoprecipitated with both E4orf6 (lane 1) and with FLAG-VHL (lane 3).

5.4.4 E4orf6 can direct the nuclear localization of Cul5

These results suggest that E4orf6 can assemble with Cul5, Elongins B and C, and Rbx1 into a complex that interacts with an E2 ubiquitin conjugating enzyme, thereby functioning as an E3 ligase. Because E4orf6 is found largely in the nucleus (Goodrum *et al.* 1996, Dobbelstein *et al.* 1997), and Cul5 had been previously reported as largely cytoplasmic (Burnatowska-Hledin *et al.* 1999), it was important to determine whether Cul5 and E4orf6 co-localize in cells. H1299 cells were transfected with cDNAs expressing human HA-Cul5 or HA-Cul2 and/or E4orf6, and cells were examined by laser confocal microscopy. E4orf6 was detected using a rabbit polyclonal antibody (1807) directed towards

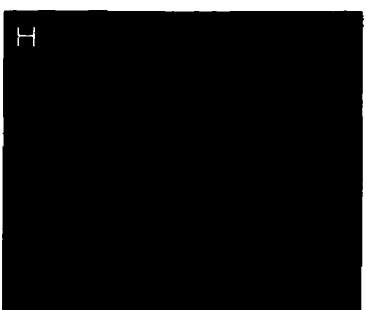
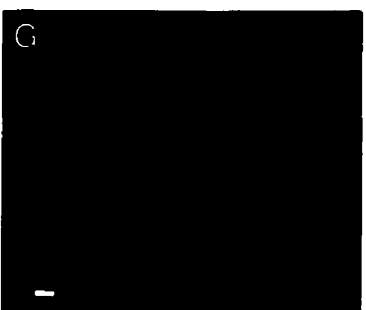
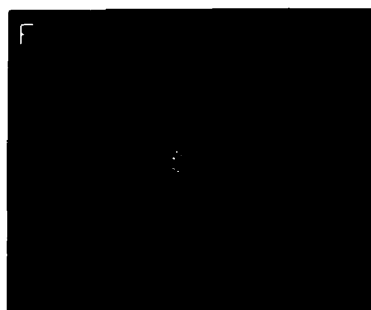
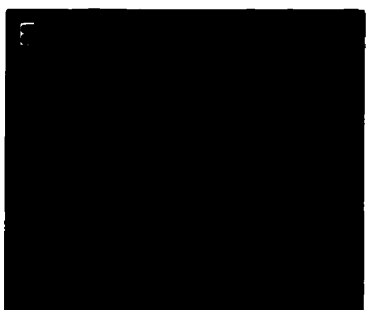
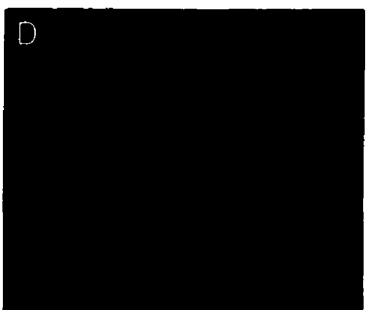
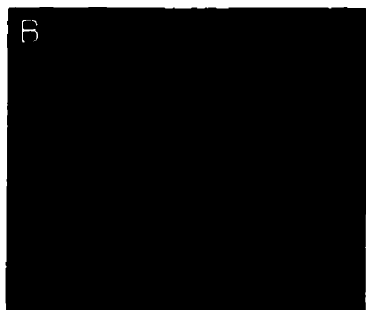
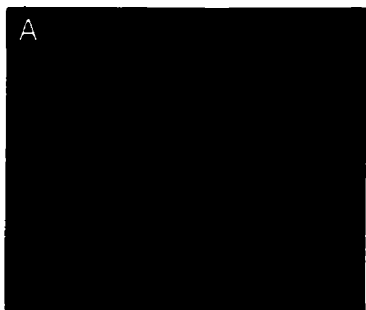


Figure 5.3 Intracellular co-localization of E4orf6 and Cul5.

H1299 cells were lipofected with various plasmid DNAs, described below. After 24 hours cells were fixed and prepared for immunohistochemistry. E4orf6 was detected using rabbit polyclonal 1807 antibody and Alexa 594 (red) anti-rabbit IgG antibody. HA-tagged human Cul2 and Cul5 were detected using mouse monoclonal anti-HA.11 antibody and Alexa 488 (green) anti-mouse antibody. The fluorophores were visualized using a laser confocal Zeiss microscope. The bar in panel G represents 2.5 μm . Panels A-C. H1299 cell expressing HA-Cul5 and E4orf6. (A) HA-Cul5 visualized in green; (B) E4orf6 visualized in red; (C) panels A and B superimposed to show co-localization in yellow. Panels D-F. H1299 cell expressing HA-Cul2 and E4orf6. (D) HA-Cul2 visualized in green; (E) E4orf6 visualized in red; (F) panels D and E superimposed. Panel G. H1299 cell expressing HA-Cul2 alone. Panel H. H1299 cell expressing HA-Cul5 alone. Panel I. H1299 cells expressing E4orf6 alone. The cells shown in this figure are representative of most positive cells in the preparation.

the E4orf6 carboxy terminus (Boivin *et al.* 1999) and visualized using Alexa 594 anti-rabbit antibody (red). The Cullins were detected using mouse monoclonal anti-HA antibodies and visualized using Alexa 488 anti-mouse antibody (green). Figs. 5.3G and H show that, when expressed independently, Cul2 (G) and Cul5 (H), were localized primarily to the cytoplasm, whereas E4orf6 (Fig. 5.3 I) was largely nuclear. Figs. 5.3A-C and 5.3D-E show cells that co-expressed E4orf6 and either Cul5 or Cul2, respectively. In the presence of E4orf6, Cul5 (Fig. 5.3A), but not Cul2 (Fig. 5.3D), was present at high levels in the nucleus. The appearance of yellow nuclear fluorescence following superimposition of Figs. 5.3A and B (Fig. 5.3C) indicated that Cul5 co-localized in the nucleus with E4orf6 (Fig. 5.3C). This was not the case with Cul2, as the nuclear fluorescence remained largely unchanged following superimposition of Figs. 5.3 D and E (Fig. 5.3F). These data are consistent with our observation that E4orf6 binds preferentially to Cul5, and they suggest that E4orf6 may transport Cul5 to the nucleus, as it does with E1B55K (Goodrum *et al.* 1996).

5.4.5 E4orf6/E1B55K binding to Cul5 and Elongins B and C correlates with their ability to target p53 for degradation

Consistent with the idea that induction of p53 degradation in cells depends not only on binding of E4orf6 to E1B55K but also on binding to Cul5, Elongin B, and Elongin C, we observed a correlation between the ability of a series of E4orf6 in-frame deletion mutants to promote degradation of p53 and to bind all four of these proteins (see Fig. 4.1, Table 4.2, and Fig. 5.1, panels B-D) (Querido *et al.* 2000b). To determine the ability of E4orf6 mutants to participate in p53 degradation, p53-null H1299 cells were co-transfected with cDNAs expressing human p53, histidine-tagged E1B55K (HH55Kwt), and wild-type or mutant E4orf6. As shown in Fig. 5.1D, p53 levels were reduced in whole cell extracts from cells expressing wild-type E4orf6 and E4orf6 mutants dl1-38 and dl35-43 (lanes 2, 3 and 6), but not from cells expressing dl1-55, dl1-108, dl49-64, dl65-83, dl83-94 or dl95-122. Similarly, endogenous Elongins B and C could be co-immunoprecipitated with wild type

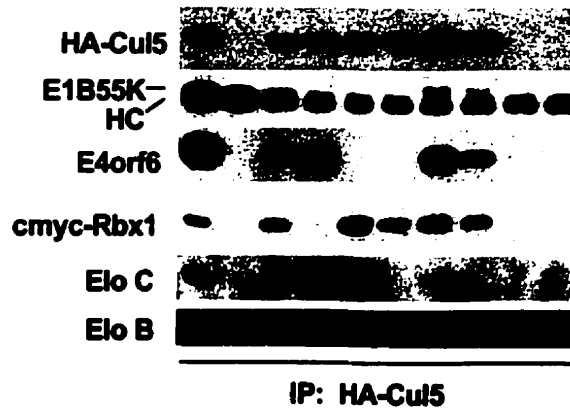
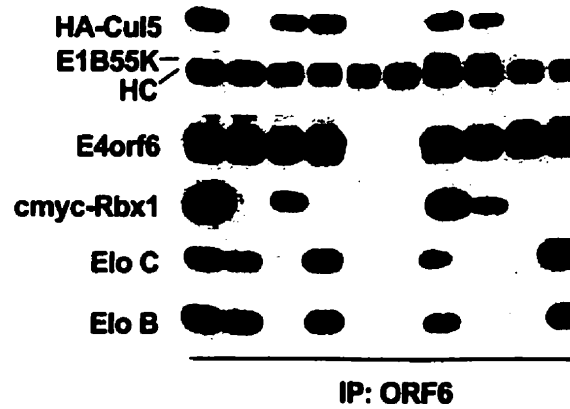
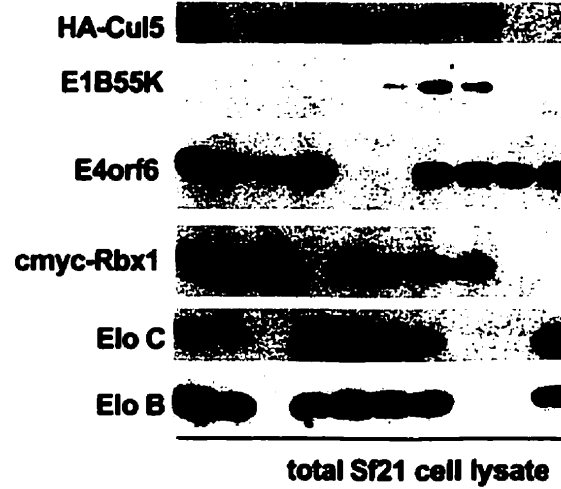
E4orf6 and mutants dl1-38 and dl35-43, but not with any of the other mutants tested, suggesting that a region required for the interaction may reside between residues 44 and 108 of E4orf6. Interaction with E1B55K is not sufficient to promote degradation of p53, since several of the mutants that fail to support p53 degradation and to bind Cul5 and Elongins B and C did successfully bind E1B55K (see Table 4.2 and Fig. 5.1C).

5.4.6 The E4orf6/E1B55K/Cul5/Elongin B/C/Rbx1 complex can ubiquitinate p53 *in vitro*

E3 ligases participate in the transfer of ubiquitin to the substrate through ubiquitin-thioester intermediates on catalytic cysteine residues (Hershko and Ciechanover 1998). We tested immunopurified E4orf6 complexes for their ability to support ubiquitination of recombinant p53 *in vitro*. Cul5, E1B55K, Rbx1, and Elongins B and C were expressed in insect cells, with or without E4orf6, and insect cell lysates were immunoprecipitated with anti-E4orf6 1D5 antibodies. Immunoprecipitated complexes were supplemented with various concentrations of recombinant p53 and with yUba1, hUbc5A, ATP, and GST-ubiquitinK48R. Ubiquitination of p53 was assessed by the appearance of slower-migrating forms of p53, as detected by western blotting using an anti-p53 monoclonal antibody. Fig. 5.4B (left three lanes) shows that in the absence of 1D5 immunoprecipitate, low levels of ubiquitinated p53 could be detected in the presence of 65ng of recombinant p53, but none was present in the absence of p53 or with 20ng of p53. Identical, background levels of p53 ubiquitination were observed in reactions containing 1D5 immunoprecipitates from cells not expressing E4orf6 (middle three lanes). In the presence of immunoprecipitates from cells expressing E4orf6 (right three lanes), significantly higher levels of ubiquitinated p53 were formed in reactions containing either 20 or 65ng of p53. Only background levels of ubiquitinated p53 were formed in reactions performed with immunoprecipitates from cells

Sf21 cells infected with:

HA-Cul5	+	-	+	+	+	+	+	+	-	-
E1B55K	-	-	-	-	-	+	+	+	-	-
E4orf6	+	+	+	+	-	-	+	+	+	+
cmymc-Rbx1	+	+	+	-	+	+	+	+	-	-
EloBC	+	+	-	+	+	+	+	-	-	+



E1, UbcH5a, ATP
GST-UbK48R

IP: E4-ORF6

E1B-p55,
EloBC, Cul5, Rbx1
- ORF6 + ORF6

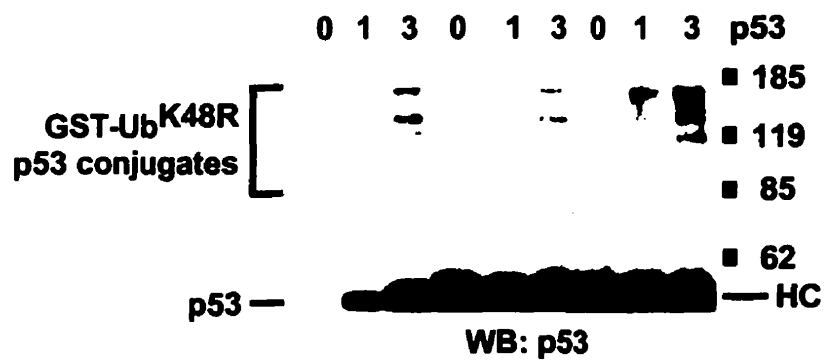


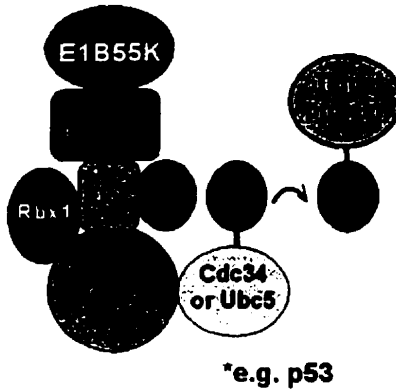
Figure 5.4 Analysis of *in vitro* p53 ubiquitination by E4orf6 complexes.

(A) Reconstitution of E4orf6/E1B55K/Cul5/Rbx1/Elongin B and C complexes in Sf21 cells. Sf21 cells were infected with baculoviruses encoding the proteins indicated in the figure. The lysates were immunoprecipitated with either anti-HA (12CA5) or anti-E4orf6 (1D5) antibody. Crude lysates (upper panel) and washed immune complexes (middle and lower panels) were separated by SDS-PAGE and immunoblotted with the indicated antibodies. (B) *In vitro* ubiquitination of p53 by E4orf6 complexes. Sf21 cells infected with the indicated mix of baculoviruses were immunoprecipitated with anti-E4orf6 (1D5) antibody. 0, 20, or 65 ng of purified p53 protein were incubated with the immunoprecipitates and with yUba1, hUbc5a, GST-UbK48R, and ATP. Reaction mixtures were incubated for 30 minutes at 25^o C, separated by SDS-PAGE, and immunoblotted with Ab421 anti-p53 antibodies.

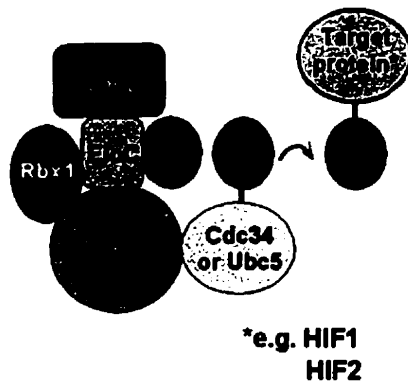
expressing E4orf6 alone, in the absence of other proteins of the E4orf6 complex (data not shown). Taken together, these results argue that the E4orf6 complex can function with a ubiquitin activating enzyme and a ubiquitin conjugating enzyme to facilitate p53 ubiquitination *in vitro*, and they are consistent with the idea the E4orf6/E1B55K complex targets p53 for ubiquitination and subsequent degradation via this Cul5/Elongin BC/Rbx1 complex in cells.

5.5 Discussion

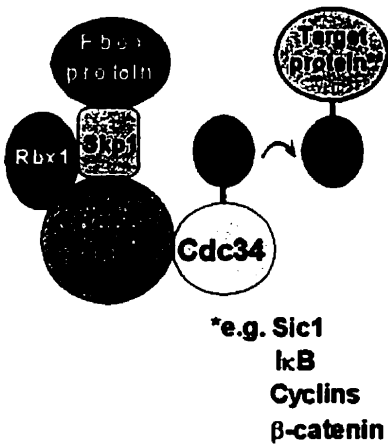
In this report, we have presented evidence that assembly of E4orf6 into a multiprotein complex containing E1B55K, Elongins B and C, Cul5, and Rbx1 is required for E4orf6/E1B55K-dependent p53 turnover in cells. In addition, we have shown that this E4orf6-containing complex functions as an E3 ubiquitin ligase that promotes ubiquitination of p53 *in vitro*. As shown in Fig. 5.5, the E4orf6 complex is strikingly similar to VHL and SCF E3 ubiquitin ligase complexes, in that each contains a substrate recognition module, which is linked to a Cullin-Rbx1 subcomplex via either Elongin BC or the Elongin C-like protein Skp1. The viral E4orf6 may have evolved functions that parallel those of cellular regulators of p53 degradation. E4orf6 is an oncogene that can cooperate most efficiently with adenovirus E1A/E1B to accelerate the growth rate of baby rat kidney cells and human 293 cells when injected into nude mice (Moore *et al.* 1996, Nevels *et al.* 1997). In these "super-transformed" cells the steady-state levels of p53 are reduced compared to cells expressing E1A/E1B alone. In light of the evidence for MDM2-independent cellular pathways of p53 ubiquitination (Fuchs *et al.* 1998), it is possible that non-infected cells contain cellular E4orf6 homologues that function with Cullin-containing ubiquitin ligases that regulate p53.



E4orf6 Complex



VHL Complex



SCF Ubiquitin Ligase

Figure 5.5 Parallels between E4orf6, VHL, and SCF ubiquitin ligase complexes.

Structurally and/or functionally related proteins are indicated in the same color. EloB, Elongin B; EloC, Elongin C. E4orf6/E1B55K, VHL, and F-box proteins function as substrate recognition subunits; Elongin B and Elongin C are structurally related to ubiquitin and Skp1, respectively; and Cul5, Cul2, and Cul1/Cdc53 are Cullin family members.

5.6 Acknowledgments

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CHAPTER VI

General Discussion

6.1 E1A signaling to p53: new insights

All small DNA tumor viruses use the cellular DNA synthetic machinery to replicate their genomes. Infected cells perceive an inappropriate mitogenic signal upon expression of early viral genes, and respond via the p53 tumor suppressor to attempt to induce cell cycle arrest and programmed cell death. DNA tumor virus early proteins have been shaped by evolution to invade and incapacitate the cell's defenses. Papillomaviruses express the E6 and E7 proteins, polyomaviruses use their T antigens, and adenoviruses have the E1A, E1B and E4 proteins. All these viruses induce the stabilization of the p53 tumor suppressor (reviewed in (Roulston *et al.* 1999)).

In Chapter II, we determined which regions of Ad5 E1A participate in inducing this accumulation of p53 protein. As illustrated in Figure 6.2, two E1A domains induce p53 stabilization and apoptosis. The first binds the p300/CBP transcriptional coactivators, whereas the second binds pRb/p107/p130 (Egan *et al.* 1988). The integrity of both domains is required for E1A's oncogenic potential (Whyte *et al.* 1988, Whyte *et al.* 1989). Since 1997, when the studies comprising Chapter II were completed, several new results have been published that built on my results in part, and that help explain how E1A induces p53 stabilization and what roles pRb and p300-binding may play. Figure 6.1 illustrates that E1A has at least five different ways of modulating p53 activity, as will be discussed in the following sections.

Inhibits HAT activity of p300 and PCAF
 Blocks p53 acetylation
 Inhibits nucleosomal histone modification by the PCAF complex

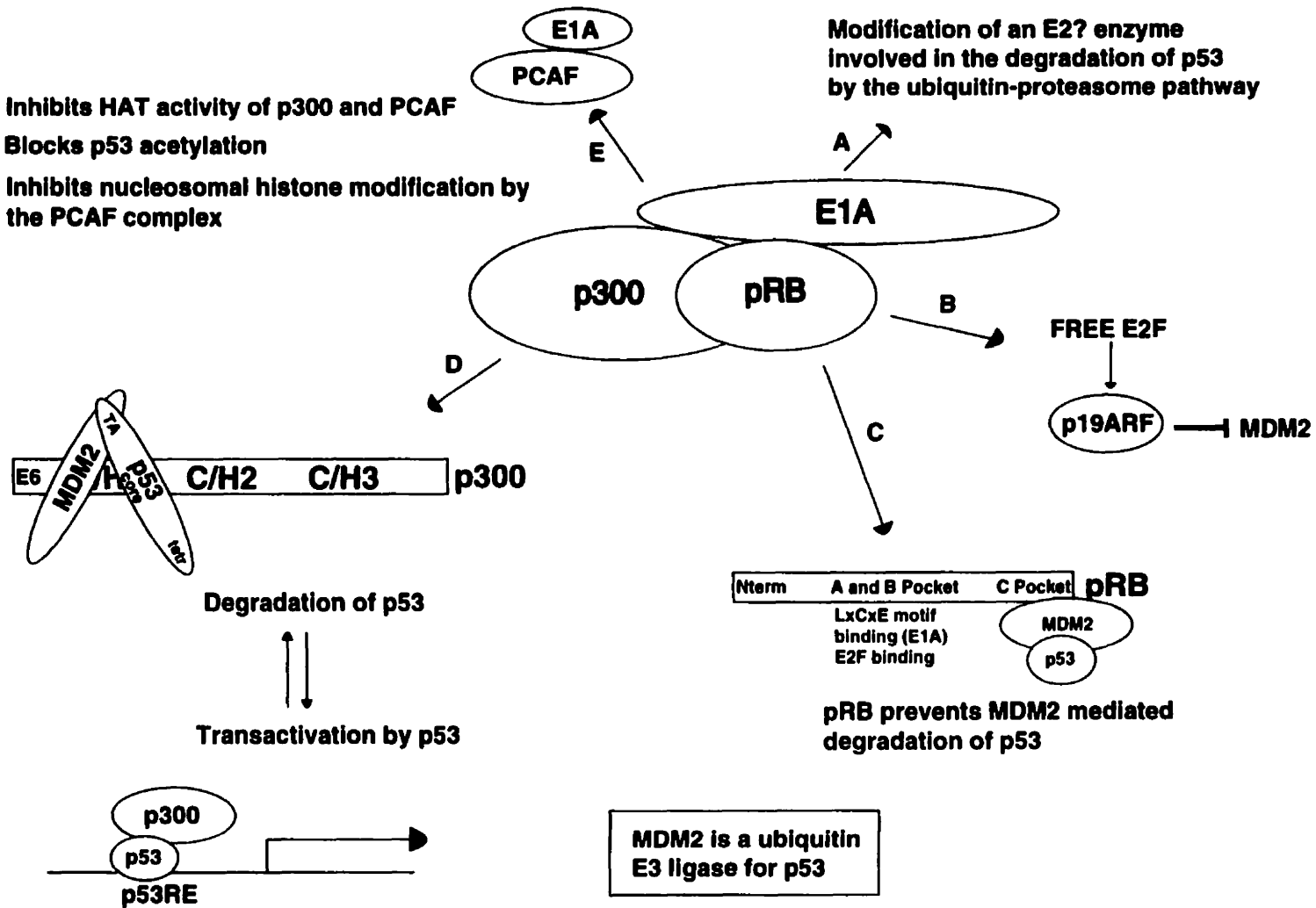


Figure 6.1 E1A has at least five different ways of modulating p53 activity.

(A) E1A directly inhibits ubiquitinating enzymes responsible for p53 degradation. (B) E1A induces the p19ARF protein. (C) E1A blocks pRB activation of p53-mediated apoptosis. (D) E1A binds p300 and prevents it from participating in p53-mediated transcriptional activation. (E) E1A inhibits the acetylation of p53 by p300 and PCAF.

6.1.1 E1A inhibits enzymes responsible for p53 degradation

The stability of the p53 protein is regulated by ubiquitin-proteasome dependent proteolysis (reviewed in (Lohrum and Vousden 1999)). A recent paper suggests that the stabilization of p53 induced by E1A occurs through modification of specific enzyme(s) in the ubiquitin-proteasome pathway (Nakajima *et al.* 1998). Nakajima *et al.* show that extracts prepared from E1A expressing cells contain an activity that directly inhibits the ubiquitination of p53 (see Fig. 6.1A). Another group also recently found that the E1A-298R and E1A-243R proteins of Ad5 and Ad12 bind to the proteasome (Grand *et al.* 1999). Ad12 E1A in particular can be co-immunoprecipitated with SUG1, a component of the 19S regulatory subunit of the proteasome. These results suggest that E1A might directly act on components of the ubiquitination machinery, presumably to prevent degradation of viral products during replication. A negative consequence of this, from the viral point of view, would be the stabilization of the p53 tumor suppressor.

6.1.2 E1A induces the p19ARF tumor suppressor.

The *INK4a/ARF* locus was described in Chapter I (see Figure 1.2). Enforced expression of p19ARF stabilizes p53 and induces p53-dependent growth arrest (Kamijo *et al.* 1997). p19ARF has recently been shown to be upregulated in response to oncogene products such as c-Myc (Zindy *et al.* 1998), E1A (de Stanchina *et al.* 1998), and activated Ras (Palmero *et al.* 1998). E1A mutants unable to bind either p300/CBP or pRb are impaired in their ability to induce p19ARF and p53 (Querido *et al.* 1997b, Samuelson and Lowe 1997, de Stanchina *et al.* 1998). How does E1A induce p19ARF? Loss of *Rb* contributes to *ARF* induction consistent with the possibility that it is an E2F-responsive gene (DeGregori *et al.* 1997). As illustrated in Fig. 6.1B, E1A releases E2F1 from pRb constraint (Bagchi *et al.* 1990, Nevins 1992) and E2F1 can selectively induce the *ARF* gene (Bates *et al.* 1998). However, E1A still induces p19ARF in *Rb*^{-/-} MEFs (de Stanchina *et al.* 1998), and p300/CBP binding by E1A must also induce p19ARF.

How does p19ARF induce p53 ? As illustrated in Fig. 1.2 C, there are several models for the mechanism of p19ARF induction of the p53 response: p19ARF binds MDM2 and MDM2 half-life is reduced (Zhang *et al.* 1998); p19ARF blocks the nuclear-cytoplasmic shuttling of MDM2 (Tao and Levine 1999, Zhang and Xiong 1999); p19ARF binds MDM2 and relocalizes it to the nucleolus (Weber *et al.* 1999); p19ARF binds both MDM2 and p53, and inhibits p53 degradation (Kamijo *et al.* 1998) ; p19ARF promotes the stabilization of p53 by inhibiting the ubiquitin ligase activity of MDM2 (Honda and Yasuda 1999); and finally, p19ARF binds p53 and participates in p53 transactivation of some promoters (Kamijo *et al.* 1998). These explanations are not necessarily contradictory, but a definitive model has not been arrived at yet, and studying how E1A induces p53 through p19ARF induction may shed light on the problem. Strong emphasis has been placed recently on the role of localization of p53/MDM2/p19ARF for their activity (Weber *et al.* 1999, Lindstrom *et al.* 2000, Weber *et al.* 2000) The nucleolus in particular may be an important site for p53 regulation. It will be interesting to pursue whether oncogenic signaling to p53 induces a change in its localization.

Another unresolved question is how p53 produces diverse biological effects, and the role of p19ARF in determining the outcome of p53 activation. In principle, p19ARF could function as a p53 activator, with the various biological outcomes being determined by how cells respond to the same set of p53 transcriptional targets. Alternatively, p19ARF could modulate p53 function, such that a different subset of p53 responsive genes are expressed in the presence or absence of ARF. Comparing the targets induced by p19ARF expression alone to the ones induced by E1A could help us understand why p19ARF induces irreversible growth arrest while E1A favors apoptosis. This approach could help identify more genes like PERP, a novel p53 target induced by E1A in cells undergoing apoptosis (Attardi *et al.* 2000).

6.1.3 E1A blocks pRb activation of p53-mediated apoptosis

pRb has been reported to interact physically with MDM2 (Xiao *et al.* 1995). Hsieh *et al.* demonstrated recently that the pRb-MDM2 interaction inhibits the degradation of p53 by MDM2 but not the inhibition of p53-dependent transcription by MDM2 (Hsieh *et al.* 1999). This is illustrated in Fig. 6.1C. The p53 in the trimeric complex with pRb and MDM2 was transcriptionally inactive but was still able to induce apoptosis (Hsieh *et al.* 1999). *Rb* deficiency leads to inappropriate S-phase entry and increased cell death associated with p53 induction (Almasan *et al.* 1995), so pRb has traditionally been associated with preventing p53-dependent apoptosis (reviewed in (Harbour and Dean 2000)). Hsieh *et al.* make the interesting argument that the pRb tumor suppressor could have a role in restoring the apoptotic function of p53. E1A binding of pRb may disrupt its regulation of p53/MDM2 activity, and this possible mechanism remains to be investigated.

6.1.4 E1A binds p300 and prevents it from participating in p53-mediated transcriptional activation and MDM2-mediated p53 degradation

Most of the MDM2 protein in normally cycling cells is bound to p300 in a stable complex (Grossman *et al.* 1998). The p300 diagram of Fig. 6.1D shows the C/H1 region which serves as a specific binding site for the p53/MDM2 complex and which, along with an N-terminal domain of p300, has significant homology to HPV E6. Specific interactions of p53 and MDM2 with p300 may be important steps in the MDM2-directed p53 turnover (Grossman *et al.* 1998). The p300-binding domain of MDM2 overlaps with the p19ARF-binding domain (Pomerantz *et al.* 1998), suggesting that p19ARF may disrupt the interaction of MDM2 with p300. This would be another explanation for the stabilization of p53 observed from p19ARF overexpression. In a recent review, Giordano and Avantaggiati propose that when p300 levels are not limiting, p53 accumulation is kept in check by MDM2 and G1 arrest is favored; but when p300 levels are limiting or it is unavailable for interaction

with p53, then p53 is stabilized and it induces *bax* and apoptosis (Giordano and Avantaggiati 1999).

The p300/CBP/PCAF transcriptional coactivators play an important role in the function of p53 as a transcription factor. PCAF acetylates p53 resulting in increased transcription of p53-regulated genes (Sakaguchi *et al.* 1998, Liu *et al.* 1999). p300 and CBP participate in p53-dependent growth arrest and apoptosis (Avantaggiati *et al.* 1997, Gu *et al.* 1997, Lill *et al.* 1997, Scolnick *et al.* 1997). p300 and CBP are histone acetyltransferases (HAT) (Ogryzko *et al.* 1996) and also bind specifically to another HAT, PCAF (Yang *et al.* 1996, Yao *et al.* 1996). As illustrated in Fig. 6.1E, E1A has been reported to inhibit the acetyl transferase activity of p300 and PCAF (Reid *et al.* 1998, Chakravarti *et al.* 1999, Hamamori *et al.* 1999). If E1A is so adept at interfering with p53's ability to activate transcription, why does the virus need to encode proteins to block p53-induced apoptosis? Perhaps p53-induced by E1A does not transactivate *p21/WAF1* but can still induce killing genes or repress survival promoters. This may also lead us to think that p53 can kill through additional mechanisms that have nothing to do with transcription.

6.1.5 E1A induced modifications of 53

Several groups have shown that p53 is phosphorylated on serine (Ser) -15 in response to radiation, but E1A induces p53 in the absence of phosphorylation on Ser-15 (de Stanchina *et al.* 1998). However, serine-20 phosphorylation also disrupts MDM2-binding and stabilizes p53 (Unger *et al.* 1999), and E1A could induce phosphorylation at that or other sites. It was recently shown that lysine-386 of p53 can be conjugated by the ubiquitin-like molecule SUMO-1/sentrin (Gostissa *et al.* 1999, Rodriguez *et al.* 1999). Sumolated p53 has activated transcriptional activity. E1A interacts with mUBC9, a component of the sumolating enzyme (Hateboer *et al.* 1996). Does E1A inhibit the sumolation of p53?

Acetylation of the p53 carboxy terminus is another signal that activates its sequence-specific DNA binding (Gu and Roeder 1997). As mentioned, E1A has been reported to

inhibit the activity of some proteins that acetylate p53 (Reid *et al.* 1998, Chakravarti *et al.* 1999, Hamamori *et al.* 1999); however, it remains to be seen if p53 is acetylated in E1A-expressing cells *in vivo*. As more modification-specific antibodies for p53 become available, assays to look for the changes induced by E1A and other signals will be easier to perform and these modifications will surely be elucidated in the coming year or two. What will then remain to work out is how these specific modifications affect p53 activity, *i.e.* do they target p53 to novel promoters and does p53 then transactivate or repress them; or do they cause p53 to associate with new binding partners? The big question always remains the same: how does p53 kill ? Despite a great deal of research throughout the world, its mechanism remains unresolved.

6.2 The E4orf6/E1B55K-mediated degradation of p53: a CULprit is found

In Chapters III and IV, I discovered that the viral E4orf6/E1B55K complex can bind p53 and target it for proteasomal degradation. In *wt* adenovirus-infected cells, E1A did not induce p53 protein levels to rise, and this was dependent on the presence of both the E1B55K and E4orf6 proteins. The degradation of p53 by the viral proteins could be prevented by an inhibitor of the 26S proteasome, but not by an inhibitor of nuclear export, and the MDM2 protein was dispensable for E4orf6/E1B55K-induced p53 turnover. In Chapter V, we identified a novel protein complex formed by E4orf6 that could reconstitute p53 ubiquitination *in vitro*. I will now discuss some unresolved aspects of the p53 degradation mediated by adenovirus.

6.2.1 A novel E3 ubiquitin ligase complex similar to the von Hippel-Lindau E3 ligase complex

Protein ubiquitination involves a cascade of ubiquitin transfer reactions and requires three activities: E1, E2, and E3 (reviewed in (Hershko and Ciechanover 1998)). The E1

ubiquitin-activating enzyme first forms a thiol-ester linkage with a ubiquitin molecule. This ubiquitin thiol-ester is then transferred to an E2 ubiquitin conjugating enzyme. An E3 ubiquitin ligase then transfers the ubiquitin to a lysine residue on the substrate. The ubiquitin can then be itself ubiquitinated in multiple rounds leading to the formation of a polyubiquitin chain, which is the signal for destruction of the substrate by the 26S proteasome. There are four main classes of E3 enzymes: the N-end rule E3 (E3 α); the *hect* (homologous to E6-AP C-terminus) domain family; the APC (anaphase promoting complex); and the phosphoprotein-ubiquitin ligase complexes (reviewed in (Hershko and Ciechanover 1998)). Two E3 ligases that target p53 were known to exist: the human papilloma virus E6 and E6-AP complex (Scheffner *et al.* 1993); and the p53-induced protein MDM2 (reviewed in (Lohrum and Vousden 1999)). Some cases of Angelman syndrome, a human hereditary disease characterized by mental retardation and seizures, are due to mutations in the *E6-AP* gene (reviewed in Hershko and Ciechanover 1998). However, E6-AP plays a role in targeting of p53 only in the presence of E6 (Beer-Romano *et al.* 1997). The MDM2 protein targets p53 for ubiquitin-mediated degradation in normal cells (Honda *et al.* 1997), but it is not yet known if MDM2 acts alone as an E3 ligase or if it needs cellular partners. The fact that deletion of *mdm2* causes early embryonic lethality in the mouse, and is rescued by deletion of *p53* (Jones *et al.* 1995, Montes de Oca Luna *et al.* 1995), suggests that MDM2 is an important player in controlling p53 levels. However it may not be the only one, as calpains (Pariat *et al.* 1997) and the JNK kinase (Fuchs *et al.* 1998), have recently been shown to regulate p53 degradation independently of MDM2.

We hoped that the study of E4orf6/E1B55K-mediated p53 degradation would lead to the identification of new E3 enzymes, in a similar way that HPV E6 lead to the discovery of E6-AP (Scheffner *et al.* 1993), and this turned out to be the case. E4orf6 binds Cullin 5 (VACM-1), Elongins B and C, and Rbx1 (Querido *et al.* 2000c). Cullins form a very conserved family of six known genes in mammals, five in *C. elegans*, and three in *S. cerevisiae* (Kipreos *et al.* 1996). Cullin family members have recently been shown to be

components of the architecturally similar von Hippel-Lindau tumor suppressor (VHL) and SCF (Skp1/Cullin/E-box protein) complexes, which are all members of the phosphoprotein-ubiquitin ligase class of complexes (Hershko and Ciechanover 1998), as they couple protein kinase signaling pathways to the control of protein abundance (Skowyra *et al.* 1997). They target a variety of transcriptional, cell cycle, and other regulatory proteins for ubiquitination and subsequent degradation by the proteasome (reviewed in (Tyers and Willems 1999)). The Cullin family member Cul2 is linked to the VHL protein by the Elongin BC complex, which functions as an adapter that binds to both Cul2 and a conserved ~12 amino acid 'BC-box' motif present in VHL (Kamura *et al.* 1998). In SCF complexes, the Cullin family member Cul1/Cdc53 is linked to a variety of F-box proteins by the Elongin C-like protein Skp1, which binds to both Cul1/Cdc53 and a conserved ~40 amino acid 'F-box motif' present in F-box proteins. F-box proteins bind to and recruit proteins targeted for ubiquitination to the complex (Skowyra *et al.* 1997). E4orf6 is thus the viral F-box protein, and it remains to be investigated whether post-translational modifications of p53 play any role in regulating its degradation by the E4orf6/E1B55K complex.

6.2.2 Role of E1B55K in the E4orf6/E1B55K/Cul5/ElonginBC complex

In Chapter IV, we tested the ability of several p53 mutants to be degraded by E1B55K/E4orf6, and the results suggested that E1B55K binding but not E4orf6 binding was absolutely required for p53 degradation (see Fig. 4.1 and Table 4.1). Roth *et al.* found that if they replaced a small region in the amino terminus of p73 with the region of p53 responsible for E1B55K binding, the p73mut20-24 form of p73 could be degraded by E1B55K/E4orf6 (Roth *et al.* 1998b). Since we determined that E4orf6 does not bind p73 (Table 4.1), E1B55K binding was thus sufficient for p73mut20-24 to be degraded. This raises the possibility that other proteins could be degraded by the E1B55K/E4orf6 complex if they were tagged with a motif that permitted E1B55K binding. Alternatively, one could fuse either E1B55K or E4orf6 with a recognition motif that would allow them to bind a novel protein, and ask whether the protein

could be degraded. There may be special considerations, such as the protein having a nuclear localization, for this effect to occur.

Interestingly, the E1B55K protein was recently described to have a functional nuclear export signal (NES) and to shuttle between the nucleus and the cytoplasm (Kratzer *et al.* 2000). Detailed immunolocalization studies will have to be conducted to determine if the E1B55K NES plays a role in shuttling the E4orf6/E1B55K complex, and what impact this has on p53 degradation.

6.2.3 Role of E4orf6 in the E4orf6/E1B55K/Cul5/ElonginBC complex

The production of a large series of E4orf6 mutants permitted the analysis of the role of E4orf6 in p53 turnover (Querido *et al.* 2000b). All E4orf6 mutants studied yielded stable products. However, the central hydrophobic region of E4orf6 was not amenable to fine mapping because all the deletions mutants from residues 44 to 274 lost the ability to cooperate in p53 degradation (see Fig. 4.3 and Table 4.2). Thus a high degree of conformational effects may be elicited by even small deletions. Detailed analysis of this region will require either high resolution structural information or the production of many specific point mutants. Conversely, deletions at the hydrophilic amino and carboxy termini are reasonably informative.

One question that remains unsolved is the binding site for p53 on E4orf6. 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 less than that with E1B55K. Thus it became difficult to separate many of the E4orf6 mutant proteins from various minor contaminating non-specific species upon the prolonged exposure times required. In addition, the amino and carboxy termini-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 were also unsuccessful in purifying significant quantities of E4orf6 in bacteria to permit *in vitro* binding assays.

Boyer and Ketner recently showed that E4orf6 is a zinc binding protein (Boyer and Ketner 2000). They identified critical histidine and cysteine residues conserved in E4orf6 of many adenovirus serotypes. Point mutations at those residues interfered with E1B55K binding and p53 degradation. It is interesting to note that Boyer and Ketner found that the amount of E4orf6 immunoprecipitated with p53 seemed to be less than that immunoprecipitated with E1B55K, and that none of the E4orf6 mutations disrupted p53 binding. This is also what we observed (data not shown). Further studies will have to address the question of which regions of E4orf6 mediate interaction with p53.

The E4orf6 protein does not have a classical BC-box binding motif (Joan Weliky Conaway, personal communication). The regions responsible for Elongin C and B binding on E4orf6 remain to be determined, as well as the regions mediating the interaction with Cullin 5. The VHL-Cullin 2 interaction is strictly dependent on the integrity of the tetrameric VHL/Cul2/Elongin BC complex (Pause *et al.* 1997, Pause *et al.* 1999), and the same may be true of the E4orf6-Cullin 5 interaction (see Fig. 5.5).

6.2.4 Speculation on E4orf6 cellular homologues

Like the E1B55K/E4orf6 complex, MDM2 shuttles between the nucleus and the cytoplasm (Roth *et al.* 1998a). As mentioned above, the MDM2 E3 ligase complex may have other components that have not yet been identified. Since the E4orf6/E1B55K seems to have evolved to mediate the same functions as MDM2 (reviewed in Roth *et al.* 1998a), it is tempting to speculate that a Cullin complex could be a component of MDM2 degradation.

JNK-induced degradation of p53 is MDM2-independent (Fuchs *et al.* 1998). Perhaps a Cullin complex is implicated in JNK-induced p53 degradation in non-stressed cell. The degradation of cyclin E by Cullin complexes is regulated by phosphorylation (reviewed in Winston *et al.* 1999). Phosphorylation is involved in the stabilization of p53 (see

introduction); however, we know that MDM2 induction lags behind p53 stabilization. It is reasonable to speculate that in untreated normally cycling cells, p53 levels could be kept low by additional mechanisms.

6.2.5 Speculation on other substrates of E4orf6/E1B55K

In Chapter IV, we showed that E4orf6 and E1B55K could not interact with p73 α and p73 β , or induce their degradation. Like other DNA tumor viruses, adenovirus thus discriminates between p53 and the related protein p73 (reviewed in Kaelin 1999). One group did find that E4orf6 was able to interact with the C-terminus of p73 and to inhibit transcriptional activation and apoptosis induced by p73 (Higashino *et al.* 1998). Another group showed that human embryonic retina cells transformed by Ad5 or Ad12 had increased levels of both p73 and p53 (Steegenga *et al.* 1999). There is some controversy in the field about the ability of E4orf6 to repress p53- and p73-transcriptional modulation. One point is undisputed, both Ad5 and Ad12 E4orf6/E1B55K proteins are involved in the degradation of p53 but not of p73 (Roth *et al.* 1998b, Querido *et al.* 2000b), or of p63 (Wienzek *et al.* 2000).

Do other substrates of the AdE4orf6/E1B55K/Cul5/ElonginBC (AdCB) ubiquitin ligase E3 complex exist ? We may already know of one: cyclin A (Grifman *et al.* 1999). Grifman *et al.* engineered a 293 cell line (E1A/E1B expressing) to express an inducible E4orf6 protein. They found that cyclin A along with p53 was selectively degraded. They did not detect a direct interaction between E4orf6 and cyclin A, so it is possible that E1B55K mediates this interaction. SCF complexes formed by Cul1 and Cul3 are involved in degrading cyclin E to regulate cell cycle progression (reviewed in Winston *et al.* 1999), so it is interesting to find that the AdCB complex could be involved in the degradation of a cyclin.

Regulation of p53 during adenovirus infection

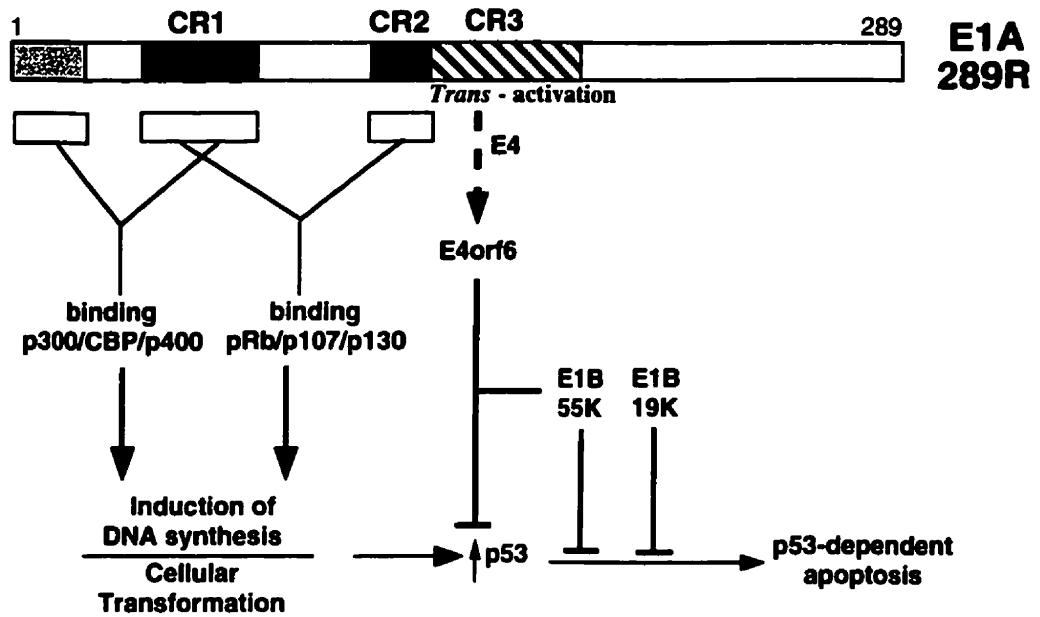


Figure 6.2 Regulation of p53 by adenovirus early proteins.

E1A induces p53 stabilization and p53-dependent apoptosis. E1B55K can bind and inhibit p53 activity. E1B55K and E4orf6 form a complex that degrades p53, also blocking p53-dependent apoptosis.

Loss of VHL is associated with hereditary and sporadic renal cell carcinomas. The VHL/Cul 2/Elongin BC ubiquitin ligase E3 complex (VCB) inhibits the accumulation of hypoxia-inducible mRNAs like the vascular endothelial growth factor (VEGF) (Lonergan *et al.* 1998) , possibly by targeting hypoxia inducible factors responsible for stabilizing such mRNAs for degradation (Levy *et al.* 1996). The E1B55K/E4orf6 proteins are involved in maintaining the stability and transport of viral mRNAs during adenovirus replication (Halbert *et al.* 1985, Pilder *et al.* 1986). E1B55K has been shown to bind a novel hnRNP protein, E1B-AP5 (Gabler *et al.* 1998), and is also reported to have endogenous RNA binding activity (Horridge and Leppard 1998). The AdCB complex, like the VCB complex, might be involved in regulating the stability of specific classes of cellular mRNAs. This point remains to be investigated.

6.2.6 Regulation of p53 by adenovirus E1A, E1B and E4orf6 proteins

The protein levels and activity of the p53 tumor suppressor increase following E1A expression and binding of the pRb and p300 family proteins. The model in Figure 6.2 shows the regulation of p53 by adenovirus early proteins. E1A induces p53 stabilization and p53-dependent apoptosis. E1B55K can bind and inhibit p53 activity. E1B19K can also inhibit p53-dependent apoptosis. E1B55K and E4orf6 form a complex (AdCB) that degrades p53, also blocking p53-dependent apoptosis.

Why does adenovirus need to degrade p53? Since the E1B19K and E1B55K proteins are both capable of blocking p53-dependent apoptosis, it seems excessive for the virus to also induce p53-degradation. One reason may be that different strategies are required for the different cell populations that adenoviruses can infect in their human host. Another may be that there are several pools of p53 in the cell, and that each must be dealt with individually and uniquely. Finally, the threshold of active p53 protein that is permissible for adenovirus replication to proceed may be so low that evolutionary pressure has created and maintained multiple mechanisms for its neutralization in adenoviruses.

Why does adenovirus encode proteins to degrade p53 (why not use the cellular degradation machinery)? The AdCB (AdE4orf6/E1B55K/Cul5/ElonginBC) ubiquitin ligase E3 complex may be more efficient than the MDM2 E3 ligase to degrade the large amounts of p53 protein induced by E1A. In fact, E1A binding of p300 and the E1A-induced disruption of p53 acetylation by p300/PCAF may severely limit the ability of p53 to transactivate the *mdm2* promoter. Also, E1A may induce post-translational modifications of p53 that would prevent MDM2 binding but not E1B55K binding.

The work presented in this thesis has yielded information that may speak to several basic cellular processes. Thus again, the study of small DNA viruses has lead to novel insights into normal cell biology, as has been the case many times over the past three decades.

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APPENDIX

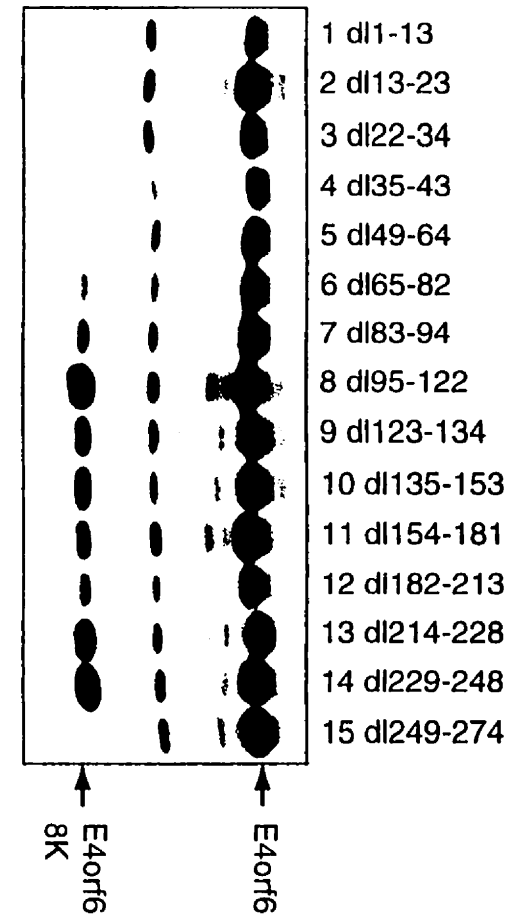
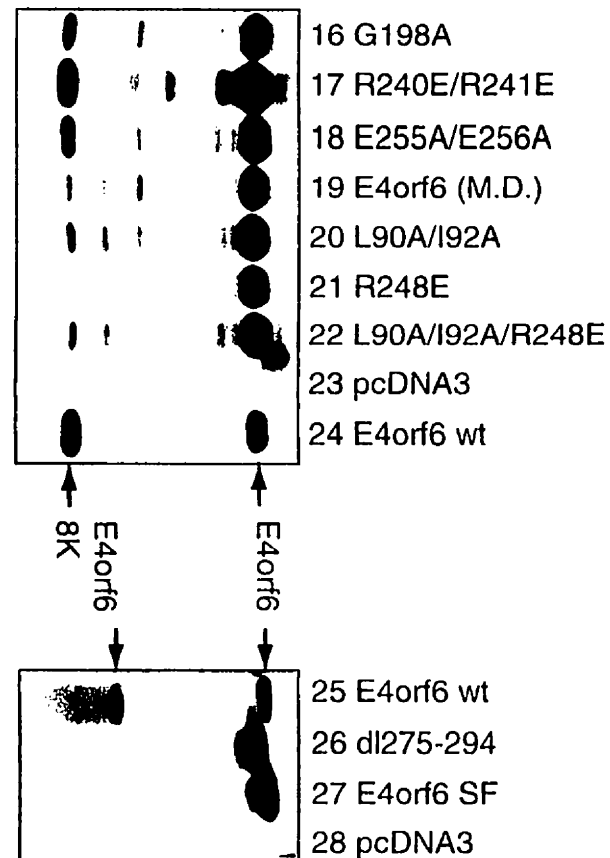


Figure 7 Analysis of the synthesis of E4orf6 products

Analysis of the synthesis of E4orf6-related proteins using cDNAs expressing wild-type and mutant E4orf6 products. H1299 cells were lipofected according to the Gibco/BRL Lipofectin protocol. Whole cell extracts were prepared and 40 µg of protein were analyzed by SDS-PAGE using gels containing either 14% or 15% polyacrylamide. After transfer to nitrocellulose, samples were immunoblotted using 1808 E4orf6 N-terminal serum as described in Boivin *et al.* 1999. The E4orf6 splicefix (SF) and the E4orf6 8K products indicated in the Figure are described in Querido *et al.* 2000a.