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Regulation of Apoptosis by Adenovirus

Type 5

by

Richard Charles Marcellus

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

McGill University

Montreal, Quebec, Canada

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Abstract

Adenoviruses have proven to be particularly helpful in unraveling the intricacies of cell growth regulation and apoptosis, greatly increasing our understanding of oncogenesis. At the onset of this work, adenoviruses were thought to induce apoptosis via a p53-dependent cell death pathway exclusively, and we set out to develop an assay system to study the ability of viral proteins to inhibit p53-induced apoptosis. We started using the p53-null human Saos-2 osteogenic carcinoma cells when we found that p53 expression led to rapid cell death. The morphology of these cells by electron microscopy confirmed that death was by apoptosis and this death was significantly reduced with Bcl-2 overexpression. We then demonstrated that the E1B-55K protein of human adenovirus type 5 (Ad5), which was known to bind and repress p53 transcriptional activity, blocks cell death following p53 expression. These results directly show that E1B-55K is able to inhibit p53-induced apoptosis. This ability is important for adenovirus replication because alone, the 289-residue (289R) and 243R E1A products of Ad5 induce p53-dependent apoptosis which would inhibit progeny production.

During the course of this work, we also found that the 289R E1A protein is able to induce p53-independent apoptosis, and the remainder of this thesis work is focused on that question. Preliminary results suggested that p53-independent cell death required expression of additional Ad5 early gene products. We found that E1B-19kDa and Bcl-2 inhibited this p53-independent killing. Neither early regions E2 or E3 were necessary for cell death, but analysis of a series of E1A mutants indicated that mutations disrupting transactivation of E4 gene expression abolished killing. Furthermore, Saos-2 cells infected with an E1B⁺/E4⁻ viral mutant remained viable for considerably longer times than those infected with *wt* Ad5. In addition, an E1⁻/E4⁻ adenovirus vector was unable to induce cell

killing in E1A-expressing cell lines, indicating that an E4 product is essential for E1A-induced p53-independent apoptosis.

To identify which E4 products are involved, studies were conducted in p53-deficient human Saos-2 cells infected with various Ad5 E4 mutants. An E4orf6-deficient mutant was defective in cell killing whereas another which expressed only E4orf6 and E4orf4 killed like *wt* virus, suggesting that E4orf6 may be responsible for cytotoxicity; however, a mutant expressing only E4orf4 induced high levels of cell death, indicating that this E4 product may also be cytotoxic. To define the E4 cell death-inducing functions more precisely, cDNAs encoding individual E4 products were introduced into cells by DNA transfection together with a cDNA encoding firefly luciferase. Enzymatic activity was high in all cases except with E4orf4 where levels were less than 20% of controls. In addition, drug selection of cells following transfection with a puromycin-containing retroviral vector encoding individual E4 products yielded a high number of cell colonies except when E4orf4 was expressed. These data demonstrated that E4orf4 is the only E4 product capable of independent cell killing. Death was due to apoptosis, as evidenced by the morphology of DAPI stained cell nuclei. This identification of a p53-independent apoptosis pathway has offered considerable insight into how the virus may ultimately be killing the host cell. Additionally, it has directed us to another cellular apoptosis pathway which will contribute to our understanding of the life and death balance in the cell.

Résumé

Les adénovirus se sont avérés particulièrement utiles dans l'étude des mécanismes impliqués dans la régulation de la croissance cellulaire et de l'apoptose, ce qui a grandement contribué à notre compréhension de l'oncogenèse. Alors que nous débutions ces travaux, il était généralement accepté que les adénovirus induisent l'apoptose exclusivement par une voie dépendante de p53, ce qui nous avait amenés à développer un modèle pour étudier l'aptitude de protéines virales à inhiber l'apoptose dépendante de p53. Nous avons d'abord utilisé comme modèle la lignée cellulaire humaine Saos-2 qui n'exprime pas la protéine p53. Nous avons observé que la réintroduction de p53 dans ces cellules mène à une mort cellulaire précoce. L'aspect morphologique de ces cellules, tel que révélé par microscopie électronique, a confirmé que la mort cellulaire était due à l'apoptose et qu'elle est significativement réduite lorsque la protéine Bcl-2 est surexprimée. Nous avons ensuite démontré que la protéine E1B-55K codée par l'adénovirus de type 5 (Ad5), une protéine qui se lie à p53 et l'inactive, bloque la mort cellulaire observée suite à la réintroduction de p53 dans les cellules Saos-2. Cette dernière propriété est importante pour la réplication virale puisqu'en l'absence d'E1B-55K les protéines E1A-289R (289 résidus d'acides aminés) et E1A-243R (243 résidus d'acides aminés) induisent l'apoptose dépendante de p53, compromettant la production de virus dans la cellule hôte.

Au cours de ce travail, nous avons aussi observé que la protéine E1A-289R est capable d'induire l'apoptose indépendante de p53. Des résultats préliminaires suggéraient alors que la mort cellulaire indépendante de p53 requière l'expression d'autres protéines codées par Ad5. Nous avons observé que E1B-19K et son homologue cellulaire Bcl-2 inhibent la mort cellulaire indépendante de p53. Les régions E2 et E3 de l'adénovirus humain de type 5 ne sont ni l'une ni l'autre requise mais l'analyse d'une série de mutants d'E1A a indiqué qu'il y a une corrélation entre l'induction de la mort cellulaire et la transactivation de la région E4

par E1A. De plus, les cellules Saos-2 infectées avec un adénovirus mutant E1B⁺/E4⁻ sont demeurées viables durant des périodes de temps beaucoup plus longues que celles infectées avec l'adénovirus de type sauvage. Un adénovirus mutant E1⁻/E4⁻ n'a pas induit la mort cellulaire dans des lignées cellulaires exprimant E1A, indiquant qu'une protéine codée par la région E4 est essentielle dans l'apoptose indépendante de p53 induite par E1A.

Afin d'identifier quelle protéine codée par E4 est impliquée dans la mort cellulaire induite par l'adénovirus, nous avons mené des études dans des cellules humaines Saos-2 infectées avec différents adénovirus mutants dans la région E4. Un adénovirus qui n'exprime pas la protéine E4orf6 s'est avéré inapte à induire la mort cellulaire alors qu'un autre adénovirus qui n'exprime qu'E4orf6 et E4orf4 l'induit aussi bien que l'adénovirus de type sauvage. Ces résultats suggéraient que la protéine E4orf6 puisse être la cause de la cytotoxicité mais, d'autre part, un adénovirus mutant qui n'exprime qu'E4orf4 induit la mort cellulaire, indiquant que ce produit pourrait aussi être cytotoxique. Afin d'identifier de façon précise quelle protéine codée par E4 est responsable de la cytotoxicité, les ADNc qui codent pour les protéines de la région E4 ont été introduits séparément dans des cellules par transfection, en combinaison avec un ADNc qui code pour l'enzyme luciférase. L'activité de la luciférase était élevée dans tous les cas à l'exception des cellules transfectées avec l'ADNc codant pour E4orf4 où les niveaux mesurés étaient moins de 20% par rapport au contrôle. De plus, E4orf4 est la seule parmi les protéines codées par la région E4 à être capable d'inhiber la formation de colonies cellulaires. Ces résultats démontrent qu'E4orf4 est la seule protéine codée par la région E4 capable d'induire la mort cellulaire. Celle-ci était due à l'apoptose ainsi que le montre la morphologie des noyaux révélée par une sonde fluorescente (DAPI) qui se lie spécifiquement à l'ADN. L'identification d'une voie apoptotique indépendante de p53 nous a permis de mieux comprendre comment les adénovirus tuent leur cellule hôte après la réplication et contribuera à une meilleure compréhension de la balance entre la vie et la mort cellulaire.

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I would like to start by thanking Phil for his supervision and support over the years. The freedom he gave us, allowed me to pursue the opportunities that I found. Yet he was always there to provide focus, and thoughtful insights when needed. Your enthusiasm also provided the fuel when fires were running low.

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I must credit the other students and staff in the lab. You created an enjoyable environment in which to work, learn and grow.

I would like to thank my parents for their support throughout my academic studies. They taught me a solid respect for intellectual pursuits, while leaving me free to chase my own interests.

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List of Abbreviations

243R	243 residue E1A protein from the 12S mRNA
289R	289 residue E1A protein from the 13S mRNA
aa	amino acid
Ad2	Adenovirus type 2
Ad5	Adenovirus type 5
Ad9	Adenovirus type 9
Ad12	Adenovirus type 12
AIF	apoptosis-inducing factor
APAF	apoptosis protease activating factor
AR1	auxiliary region 1
AR2	auxiliary region 2
ATM	ataxia-telangiectasia gene
BH	Bcl-2 homology
bp	base pair
CAD	caspase-activated DNase
cAMP	cyclic adenosine monophosphate
CAR	coxsackievirus and adenovirus receptor
CARD	caspase recruitment domain
Caspase	cysteinyI aspartate-specific proteinase
CBP	CREB binding protein
cdk	cyclin-dependent kinase
cDNA	complementary DNA
<i>ced</i>	cell death gene
CR1	conserved region 1
CR2	conserved region 2
CR3	conserved region 3
cPLA2	phospholipase A2
CREB	cyclic AMP responsive element binding protein
C-terminal	carboxy terminal
CTL	cytotoxic T lymphocyte
DBP	DNA-binding protein
DD	death domain
DED	death effector domain
DISC	death inducing signalling complex
DNA	deoxyribonucleic acid
ds	double-stranded
E1A	early region 1A
E1B	early region 1B
E2	early region 2
E3	early region 3
E4	early region 4
EGF-R	epidermal growth factor receptor
FADD	Fas-associated death domain

FAP	Fas-associated phosphatase
FLIP	FLICE inhibitory protein
HDAC	histone deacetylase
HPV	human papillomavirus
ICAD	inhibitor of CAD
ICE	interleukin-1 β -converting enzyme
ITR	inverted terminal repeat
JNK	Jun kinase
kb	kilobase
kd	kilodalton
M phase	mitosis phase
MAP	mitogen activated protein kinase
MHC	major histocompatibility complex
MLP	major late promoter
moi	multiplicity of infection
mRNA	messenger RNA
N-terminal	amino terminal
nm	nanometer
orf	open reading frame
PDZ	PSD-95, discs-large, ZO-1
pfu	plaque forming unit
PH	plextrin homology
PI3K	phosphatidylinositide-3-kinase
PKR	protein kinase R
poly(A)	polyadenylation
PP2A	protein phosphatase 2A
PT	permeability transition
pTP	preterminal protein
RAIDD	RIP-associated ICH-1 homologous protein with death domain
Rb	retinoblastoma susceptibility gene product
RGD	arginine, glycine, aspartic acid
RID	receptor internalization and degradation complex (E3-14.5K / E3-10.4K)
RIP	receptor-interacting protein
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S phase	DNA synthesis phase
ss	single stranded
TAF	TBP-associated factor
TBP	TATA binding protein
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TP	terminal protein
TRADD	TNFR-associated death domain
UTR	untranslated region
wt	wild-type
WT-1	Wilms tumour-1

Preface

In accordance with the McGill Guidelines for Thesis Preparation, I am including the following excerpt to inform the external examiner of Faculty regulations:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidates's interest to make perfectly clear the responsibilities of all authors of the co-authored papers.

Included in this thesis are the texts from three published manuscripts. These papers have been formatted in a manner consistent with the rest of the thesis, and for convenience the references from all the chapters have been placed at the end of the thesis.

Chapter 2: Richard C. Marcellus, Jose G. Teodoro, Rachel Charbonneau, Gordon C. Shore, and Philip E. Branton. (1996). Expression of p53 in Saos-2 osteosarcoma cells induces apoptosis which can be inhibited by Bcl-2 or the adenovirus E1B-55 kDa protein. *Cell Growth & Differentiation* 7:1643-1650.

This paper was intellectually a collaborative effort between myself and Joe Teodoro. Joe did the bandshift for Figure 5. Kathy Teng did the electron microscopy for Figure 3. Rachel Charbonneau made the two E1B-55 kDa adenovectors used in Figure 6.

Chapter 3: Richard C. Marcellus, Jose G. Teodoro, Tim Wu, Douglas E. Brough, Gary Ketner, Gordon C. Shore, and Philip E. Branton. (1996). Adenovirus type 5 early region 4 is responsible for E1A-induced p53-independent apoptosis. *Journal of Virology* 70:6207-6215.

All of the figures in this paper were done by myself. Jose Teodoro had made the first observation of p53-independent death (Teodoro *et al.*, 1995) and provided helpful advice. Tim Wu helped with the subcloning of the E1A mutants that I used to make the viruses utilized in Figures 4 and 5. Doug Brough provided the E4 minus adenovector used in Figure 8 and Gary Ketner supplied the E4 minus adenovirus used in figure 7.

Chapter 4: Richard C. Marcellus, Josée N. Lavoie, Dominique Boivin, Gordon C. Shore, Gary Ketner, and Philip E. Branton. (1998). The early region 4 orf4 protein of human adenovirus type 5 induces p53-independent cell death by apoptosis. *Journal of Virology* 72:7144-7153.

For this paper, the adenovirus E4 viral mutants used in figure 2 were provided by Gary Ketner. The DAPI and immunofluorescence work of Figure 6 was done by Josée Lavoie, and the immunoblotting in Figure 7 was done with the assistance of Dominique Boivin.

Original Contributions to Knowledge

1. Demonstrated that a p53 containing viral vector kills the p53^{-/-} human osteosarcoma line Saos-2.
2. Set-up an assay system where the induction of apoptosis by p53 can be directly studied with a minimum of peripheral effects.
3. Directly demonstrated that E1B-55K blocks apoptosis by p53.
4. Determined that p300 binding was important for E4 promoter activation.
5. Identified E4 as the viral early region responsible for inducing p53-independent apoptosis.
6. Identified E4orf6 as being essential for viral killing, but not itself apoptotic.
7. Identified E4orf4 as having p53-independent apoptosis inducing activity.

CHAPTER 1

General Introduction

1.1 Introduction

Investigating the processes of life is a pursuit that is fundamental to the field of medicine. However, the complexity of life favours a systematic and reductionist approach to get at the core principles and mechanisms from which we can construct a conceptual framework. Upon this foundation, we can then layer additional details towards a more complete understanding of life's machinery, leading ultimately to the point where we can manipulate these proceedings to our own ends. This type of approach can be greatly facilitated by the use of probes that specifically target distinct cellular functions. Assisting us in this regard is the field of virology.

Viruses have evolved over the millennia to perform the simplest of life's functions. They are obligate intracellular parasites, and have developed the ability to exert exquisitely precise control over the cells that they infect. Additionally, size constraints have encouraged maximum efficiency at carrying out their essential functions. Aside from our interest in their pathogenic properties, they provide us with the powerful ability to modulate many functions of the individual cell as well as more complex systems in multicellular organisms. A number of virus families have been useful in the medical sciences, and among them adenoviruses have proven to be particularly helpful in unravelling the intricacies of cell growth regulation, which has greatly increased our understanding of oncogenesis.

1.2 Adenovirus History and Etiology

Adenoviruses were first isolated in 1953 (Rowe *et al.*, 1953) during the establishment of tissue culture lines derived from tonsils and adenoidal tissues removed from children. The same etiological agent was soon identified as the cause of a respiratory epidemic in army recruits (Hilleman & Werner, 1954; Huebner *et al.*, 1954). Adenoviruses were subsequently shown to be responsible for a number of respiratory syndromes, along with conjunctivitis, and infantile gastroenteritis, although adenoviruses were found not to be the cause of the common cold (reviewed in Horwitz, 1996). A seminal discovery was made in 1962 (Trentin *et al.*, 1962) when human adenovirus type 12 was discovered to induce malignant tumours in hamsters. This was the first instance in which a human virus was linked to cancer, and it established adenovirus as a valuable probe in the study of oncogenesis. Although adenoviruses have not been found to be involved in the genesis of human cancer (Green *et al.*, 1980; Mackey *et al.*, 1976) they have been firmly established as an important model system and biological tool in the fields of mRNA splicing, gene expression, cell cycle

control, DNA replication, and apoptosis, along with their growing usefulness as a vector for gene therapy (reviewed in Shenk, 1996).

1.3 Classification

Numerous adenovirus serotypes have been identified in humans, and family members have been found in a variety of animal species. In 1956, the current adenovirus name and nomenclature were adopted, acknowledging the original source of the virus (adenoids) (Enders *et al.*, 1956). The adenoviruses compose the Adenoviridae family which is subdivided into two genera: the Aviadenovirus genus which only infects birds; and the Mastadenovirus genus which includes human, simian, bovine, equine, porcine, ovine, canine, and opossum viruses (Norrby *et al.*, 1976). At this point there are around 100 family members, with 47 human serotypes, delineated by their resistance to neutralizing antibodies raised against other serotypes. The human serotypes are classified into six subgroups based on their hæmagglutinin properties (Rosen, 1960; Hierholzer, 1973; Hierholzer *et al.*, 1988) (see Table 1-1). Most adenovirus research has concentrated on the non-tumourigenic serotypes 2 and 5 (which are nearly identical) and the highly oncogenic serotype 12. The work presented herein has been done with adenovirus type 5 (Ad5).

Table 1-1 Human Adenovirus Classification^a

Subgroup	Hæmagglutination group	Serotypes	Tumours in Animals	Transformation in Tissue Culture
A	little or no agglutination	12, 18, 31	high	yes
B	complete agglutination of monkey erythrocytes	3, 7, 11, 14, 16, 21, 34, 35	moderate	yes
C	partial agglutination of rat erythrocytes	1, 2, 5, 6	low or none	yes
D	complete agglutination of rat erythrocytes	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-47	low or none (mammary tumours with Ad9)	yes
E		4	low or none	yes
F		40, 41	unknown	

a. Modified from Shenk, 1996

1.4 Virion Structure

Adenoviruses are non-enveloped icosahedral particles that are 70-100 nm in diameter (Horne *et al.*, 1959). An icosahedron is the most efficient geometric shape in which subunits can be used to form a closed shell, and is made up of twenty equally-sized triangular facets with twelve vertices where the corners meet. The virion is composed of a protein shell composed of seven structural proteins, with an inner DNA-containing core with four known proteins (reviewed in Stewart *et al.*, 1993)(see Figure 1-1 on page 5).

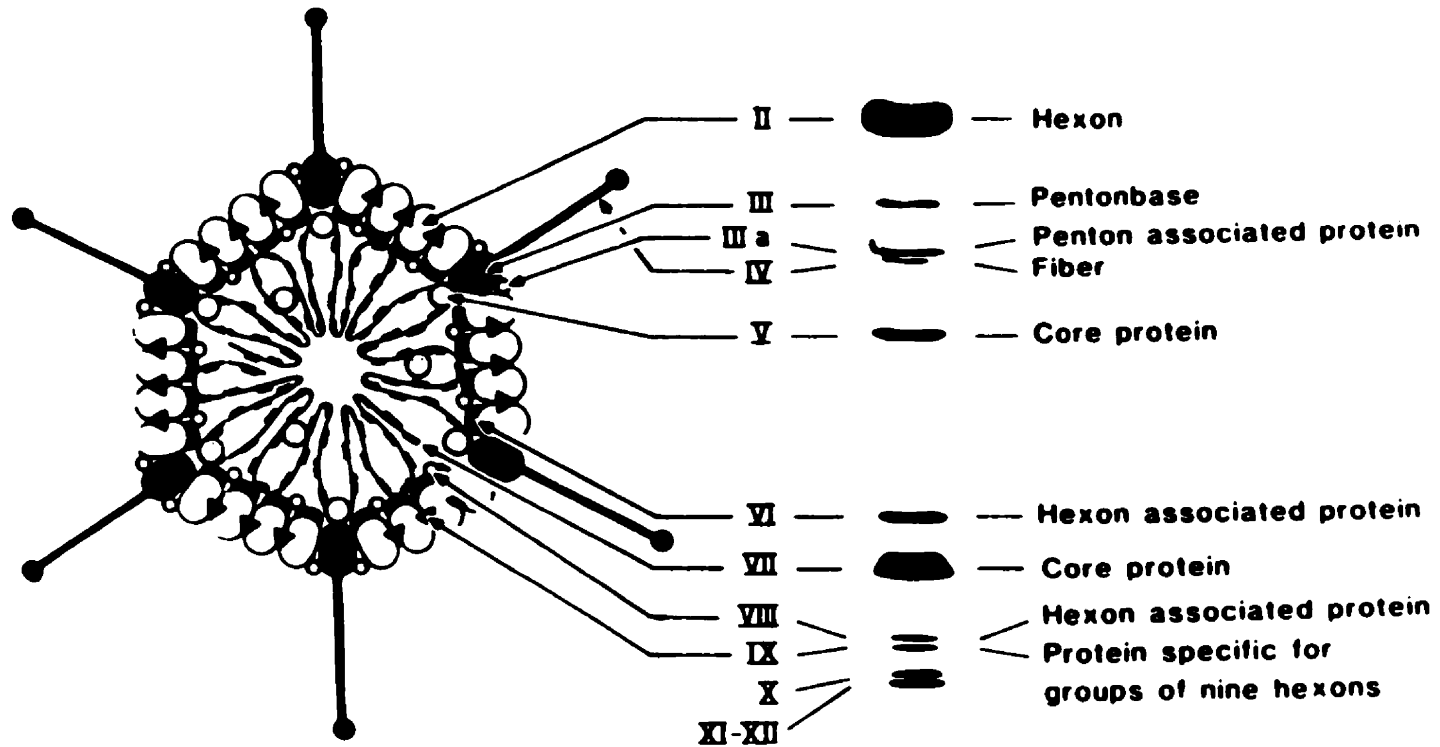
The protein shell is constructed from 252 subunits (or capsomers), 240 termed hexons (six neighbours) and 12 pentons (five neighbours) (Ginsberg *et al.*, 1966). The hexons make up the facets of the icosahedral while the pentons are located at each vertex (Philipson, 1984; Stewart *et al.*, 1991). A hexon is composed of an interwoven trimer of polypeptide II (967 aa) (Horwitz *et al.*, 1970) which is stabilized by three other polypeptides: VI (217 aa), VIII (134 aa) and IX (139 aa) (Everitt *et al.*, 1973). Polypeptide IIIa (571 aa) appears to link adjacent facets of the capsid (Everitt *et al.*, 1973; 1975) and along with polypeptide VI probably bridges the capsid to the core (Stewart *et al.*, 1991; 1993). The penton is made up of five copies of polypeptide III (571 aa) (van Oostrum & Burnett, 1985) and is bound to the fiber protein which projects outward from each penton (Norrby 1966; 1969a; 1969b). The fiber itself is composed of three copies of polypeptide IV (582 aa) (Ruigrok *et al.*, 1990; van Oostrum & Burnett, 1985) and is predicted to form a triple helical structure (Stouten *et al.*, 1992) anchored into the penton base at one end and forming a terminal bulb at the other (Devaux *et al.*, 1987; Weber *et al.*, 1989).

The virion core contains a single copy of the viral genome which is organized into supercoiled domains by the histone-like polypeptide VII (176 aa) (Chatterjee *et al.*, 1986; Mirza & Weber, 1982; Vayda & Flint, 1987). Polypeptides V (368 aa) and mu (19 aa), like VII, are basic and are non-covalently bound to the DNA (Russel *et al.*, 1968; Hosakawa & Sung, 1976; Anderson *et al.*, 1989). Mu has no known function (Hosakawa & Sung, 1976), while V appears to act as a bridge between the core and the capsid (Everitt *et al.*, 1975). The final protein in the core is the terminal protein (671 aa) which is covalently attached to each of the 5' ends of the viral DNA (Rekosh *et al.*, 1977) and acts as a primer during DNA replication (Challberg *et al.*, 1980; 1982; Enomoto *et al.*, 1981; Tamanoi & Stillman, 1982).

Figure 1-1 Structural Components of the Adenovirus Virion

This schematic diagram shows the composition and location of proteins within the virion capsid. The relative mobility of these proteins as they migrate on SDS-PAGE is also indicated. Each protein is designated by a roman numeral (Maizel *et al.*, 1968) (figure adapted from Everitt *et al.*, 1975).

Polypeptide SDS-gel Structural Unit



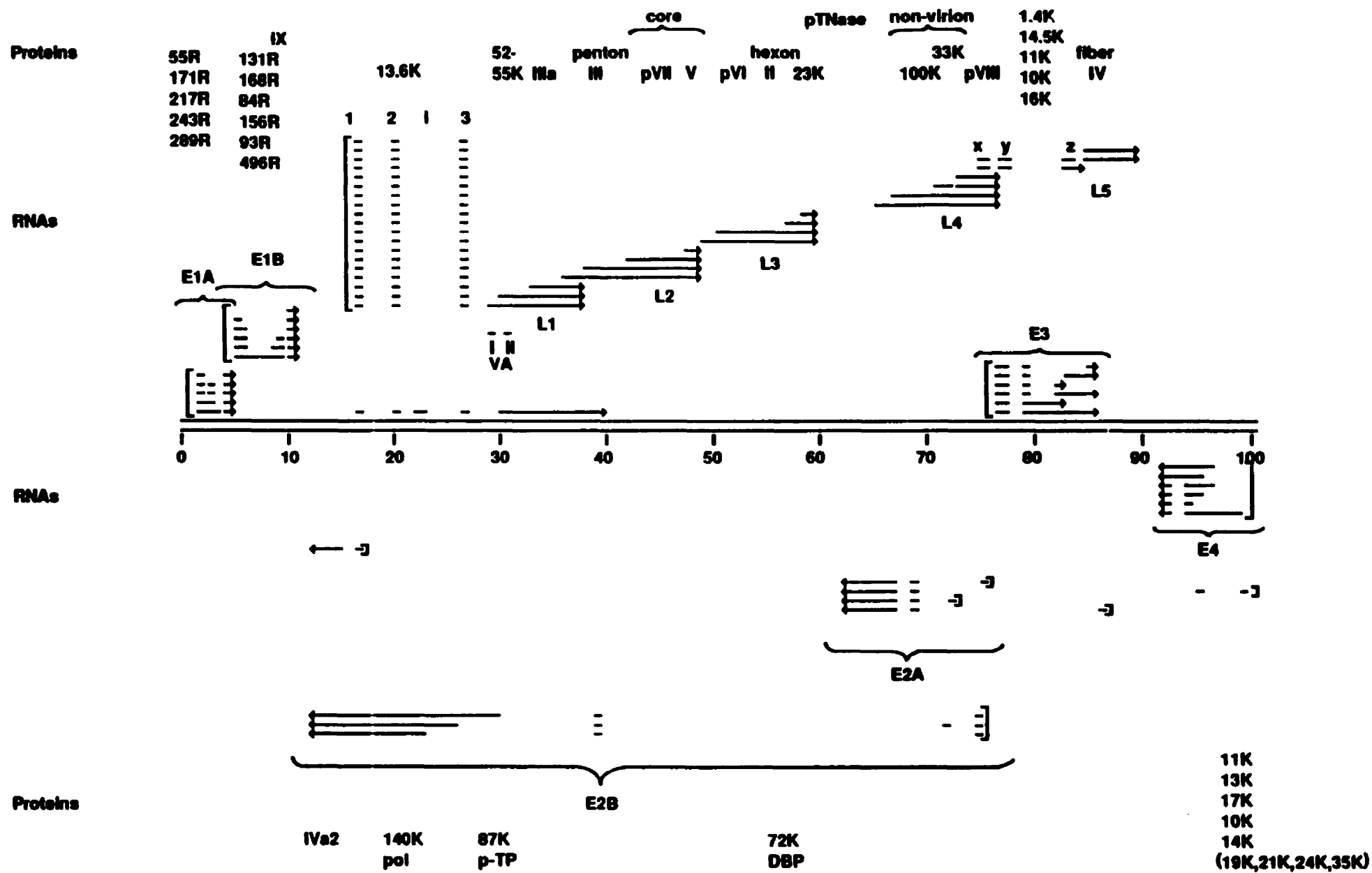
1.5 Genome Layout

The adenovirus chromosome is a linear, dsDNA molecule with the same approximate size and transcriptional organization throughout the Adenoviridae family (reviewed in Pettersson & Roberts, 1986) (see Figure 1-2 on page 7). In the case of Ad5 and Ad2, the genome is 36 kb in length, and both have been completely sequenced and share 95% sequence identity (Roberts *et al.*, 1984; Chroboczek *et al.*, 1992). The adenovirus genome has inverted terminal repeat (ITR) sequences from 100 to 140 bp in size, which are required for viral DNA replication, since they contain the two origins of replication, and during replication they circularize the genome into an important panhandle structure from single strands of viral DNA (section 1.6.9 on page 40)(Garon *et al.*, 1972; Wolfson & Dressler, 1972; Steenberg *et al.*, 1977). The viral genome is conceptually divided into early and late transcriptional units which are defined as being expressed before and after DNA replication, respectively. There are five early transcription units (E1A, E1B, E2, E3 and E4), two delayed early (IX and IVa2) and one late unit (major late) which is subdivided into five groups of late mRNAs (L1-L5). All of these genes are transcribed by RNA polymerase II, and each gene gives rise to multiple mRNAs that are alternatively spliced. In some cases the protein products from single transcription units are related in sequence, but in others they are completely different. Additionally, the genome carries one or two copies of the VA gene (varies by serotype) which is transcribed by RNA polymerase III. The analysis of adenovirus mRNA structure turned out to be of great general value, since it resulted in the discovery of splicing (Berget *et al.*, 1977; Chow *et al.*, 1977).

By convention, the viral genome is divided into 100 map units, with E1A placed at the left end of the genome. Genes are transcribed in both directions using the two DNA strands. The viral DNA contains a packaging signal several hundred base pairs from the leftmost end of the genome (Hammariskjold & Winberg, 1980; Grable & Hearing, 1992). Many adenovirus mutants have been made to study viral functions and, as will be discussed later, these mutants have also provided significant insights into many functions of mammalian cells. Complementing cell lines have been constructed to propagate viruses with mutations in the E1 (293 cells) (Graham *et al.*, 1977), E2 (Klessig *et al.*, 1984) and E4 regions (W162 cells) (Weinberg & Ketner, 1983). Recently a number of double complementing cell lines have been constructed for the production of therapeutic adenovirus vectors (section 1.8.1 on page 48) with both E1 and E4 deletions (Krougliak & Graham, 1995; Brough *et al.*, 1996; He *et al.*, 1998). The adenovirus genome recombines

Figure 1-2 Adenovirus Genomic Structure

A map of the adenovirus genome illustrating the transcription and translation products. Early (E) and late (L) transcripts are defined according to whether they are transcribed before or after the onset of viral DNA replication, respectively (figure adapted from Shenk, 1996).



readily in co-infected cells, which has promoted the evolution and diversity of viral serotypes.

1.6 Infectious Cycle

The adenovirus replicative cycle has been primarily studied using Ad5 and Ad2, though other serotypes when tested have appeared similar. This research has also mostly been done using HeLa and KB cells, which are transformed human cell lines, and the infectious cycle in these rapidly growing cells is less than half the length of the cycle in normal diploid human cells. One further item is that infections are routinely carried out at a high multiplicity of infection (moi) using from 10 to 100 plaque forming units (pfu) per cell to ensure that the cells are synchronously infected.

1.6.1 Overview

As mentioned previously, the replicative cycle is divided into early and late phases which are separated by the start of viral DNA replication. The early events include adsorption of the virus to the cell, penetration, expression of early viral genes, induction of cellular DNA synthesis, blockage of apoptosis, and antagonization of host immune responses. In HeLa cells this phase lasts about 6 hours, then viral DNA synthesis is first detected. At this point the late phase begins with the expression of the late viral genes, leading to the assembly of the virus particles and viral spread. The division of the cycle into early and late phases is actually a bit blurred, since the early genes are still expressed after the onset of viral DNA replication. The total replicative cycle takes about 24 hours in HeLa cells and approximately 10^4 virions are produced per infected cell.

1.6.2 Adsorption, Cell Entry and Uncoating

Adenovirus attaches to cells via the fiber protein (Philipson *et al.*, 1968; Londberg-Holm & Philipson, 1969) making specific contacts with the cellular receptor using the distal knob of the fiber protein (Devaux *et al.*, 1987; Henry *et al.* 1994; Louis *et al.*, 1994; Stevenson *et al.*, 1995). Virus attachment and infection can be blocked with soluble fiber protein and some fiber-specific antibodies (Defer *et al.*, 1990; Pettersson *et al.*, 1968; Philipson *et al.*, 1968; Wohlfart *et al.*, 1985). Ad2 and coxsackievirus B3 have long been known to compete for the same attachment site on HeLa cells (Lonberg-Holm *et al.*, 1976) and not surprisingly, a

common receptor for the two viruses was recently cloned and termed CAR, for coxsackievirus and adenovirus receptor (Bergelson *et al.*, 1997). CAR is a 46 kd cell surface protein from the immunoglobulin gene superfamily, with no known cellular function. CAR shows considerable conservation with its murine homologue (91% identical) which likely explains why Ad2 infects mouse cells efficiently. Ad2 and Ad5 are known to have a common cellular receptor (Philipson *et al.*, 1968; Defer *et al.*, 1990) but the amino acid sequence of the knob region of the fibre protein varies widely among adenoviruses, so it is likely that other serotypes have different cellular receptors.

The observation that Ad2 binds to some cells yet does not efficiently enter them (Silver & Anderson, 1988) led to the identification of a second virus-cell interaction between the penton base and some members of the integrin family of cell-surface receptors (Wickham *et al.*, 1993). This interaction involves binding of an arginine, glycine, aspartic acid sequence (RGD) present in the penton to the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ vitronectin-binding integrins. Interestingly, this RGD motif is the same sequence that a number of extracellular adhesion molecules use to interact with integrins. This secondary viral association with the cell surface is also important for efficient virus internalization (Wickham *et al.*, 1993; Bai *et al.*, 1993) and leads to the migration of Ad2-receptor complexes to clathrin-coated pits from where they are internalized by receptor-mediated endocytosis (Chardonnet & Dales, 1970a; 1970b; FitzGerald *et al.*, 1983; Varga *et al.*, 1991; Wang *et al.*, 1998). The internalization mechanism is an unknown, but highly efficient process, with approximately 80% of adsorbed virus internalized within endosomes in 10 minutes (Greber *et al.*, 1993). The virus then escapes to the cytosol with a half time of around 5 minutes, prior to the formation of a lysosome (Melman, 1992) in a process that is dependent upon the acidic pH of the endosome and the penton base (Pasten *et al.*, 1986; Seth *et al.*, 1984; Svensson, 1985). Adenovirus can even facilitate the uptake of naked DNA into the cell, through its internalization and endosome disrupting entry mechanism (Yoshimura *et al.*, 1993). Virus particles are transported across the cytoplasm to the nucleus by a process that appears to involve hexon-microtubule interactions (Dales & Chardonnet, 1973; Luftig & Weihing, 1975) and by 40 minutes after cell entry, virions can be seen at nuclear pores. The virions are disassembled in a sequential manner throughout the internalization process (Greber *et al.*, 1993) and after about 2 hours, almost half of the internalized virions have released their DNA. The viral genome appears to be in a chromosomal type of structure, though viral proteins likely function in place of cellular histones (Tate & Philipson, 1979; Vayda & Flint, 1987). Once in the nucleus, the viral DNA associates with the nuclear matrix via the terminal protein (Bodnar *et al.*, 1989; Fredman *et al.*, 1993; Schaack *et al.*, 1990). This

interaction correlates with the activation of transcription, suggesting a functional link (Schaack *et al.*, 1990).

1.6.3 Early Gene Expression

1.6.3.1 Overview

There are four main tasks that the virus has to complete during the early stage of growth, before viral DNA replication can start. Firstly, the virus needs to push the cell into the cell division cycle to make the various components of the DNA replication apparatus available. The E1A gene is primarily involved in carrying out this goal (section 1.6.4 on page 19). Secondly, the virus needs to block the apoptotic response of the cell to this inappropriate induction of DNA synthesis. This is accomplished by the products of the E1B region and a protein from the E4 region (section 1.6.5 on page 26). Thirdly, the virus needs to control the host immune response directed against the infected cell. This function is taken care of by the E3 region of the virus (section 1.6.7 on page 31). Fourthly, the virus needs to produce the viral gene products that are directly required for viral replication. These are the E2 proteins (section 1.6.6 on page 30).

1.6.3.2 E1A activation

Once the viral genome is in the nucleus, and associated with the nuclear matrix, viral gene expression commences. The first gene expressed is E1A, which can be seen as early as one hour post-infection (Nevins *et al.*, 1979). The E1A transcriptional unit is driven by a constitutively active promoter that contains two enhancer elements (Hearing & Shenk, 1983; 1986). E1A increases the transcription from all of the early viral promoters by 50-100 fold (Berk *et al.*, 1979; Jones & Shenk, 1979b) and is the major regulator of the early stage of viral growth.

1.6.3.3 E1A structure

Like most adenovirus genes, E1A produces multiple protein products due to alternative splicing of a primary mRNA transcript (see Figure 1-3 on page 12). During the early stage of infection, the two major E1A mRNAs are produced. These are named the 13S and 12S mRNAs (as measured by their Svedberg sedimentation coefficients) and give protein

products of 289 (289R) and 243 aa (243R), respectively (Perricaudet *et al.*, 1979; 1980; 1980b). At later times, there are an additional three mRNAs of 11S, 10S and 9S, but no clear function has been defined for their products (Stephens & Harlow, 1987; Ulfendahl *et al.*, 1987).

The two major E1A mRNAs produce identical proteins, except that the larger 13S derived product has an additional internal stretch of 46 aa. These proteins have been extensively studied, and their functions have been well documented. The E1A proteins are located in the nucleus due to the presence of a strong nuclear targeting sequence at the extreme C-terminus (Douglas & Quinlan, 1996) and they have short half lives of around 20-30 minutes (Spindler & Berk, 1984; Branton & Rowe, 1985). They are very acidic proteins, with a large number of glutamic and aspartic acid residues; they are also rich in proline residues (close to 20%) spread throughout the sequence. The combination of hydrophilicity and high proline content suggests a linear structure for the E1A proteins, which likely explains why they have been so amenable to analysis by deletion mutagenesis (Egan *et al.*, 1988; Howe *et al.*, 1990; Barbeau *et al.*, 1994).

Alignments of the amino acid sequences from E1A proteins from several adenovirus serotypes revealed three regions of high conservation—termed conserved region 1 (CR1) (spanning aa 40-80), CR2 (spanning aa 121-138) and CR3 (from aa 139-188) separated by areas of lower homology (see Figure 1-4 on page 13)(van Ormondt *et al.*, 1980; Kimelman *et al.*, 1985; Moran & Mathews, 1987; Shenk & Flint, 1991). CR1 and CR2 are involved in the stimulation of cellular DNA synthesis (section 1.6.4 on page 19) and also in the stimulation of transcription from the E2 promoter (section 1.6.3.4 on page 14). The CR3 region includes the unique 46 aa region found in the 289R protein, plus a few downstream residues, and encodes a potent transcriptional activator (section 1.6.3.4 on page 14)(Berk *et al.*, 1979; Jones & Shenk, 1979b).

E1A products are extensively serine phosphorylated (Yee *et al.*, 1983; Yee & Branton, 1985; Harlow *et al.*, 1985; Tsukamoto *et al.* 1986); however, most functional analysis has failed to ascribe any significant consequence to these modifications (Tsukamoto *et al.*, 1986; Tremblay *et al.*, 1988; 1989; Dumont *et al.*, 1989; 1992; 1993; Whalen *et al.*, 1996). One study on two sites in CR3 indicated that phosphorylation at these residues was important for the transactivation of the E4 promoter by E1A, but a mechanism has yet to emerge (Whalen *et al.*, 1997).

Figure 1-3 E1 Transcription Map

An illustration of the transcription and translation products produced by the E1 region. Both E1A and E1B give rise to at least 5 differentially spliced mRNAs which are indicated according to their sedimentation coefficients.

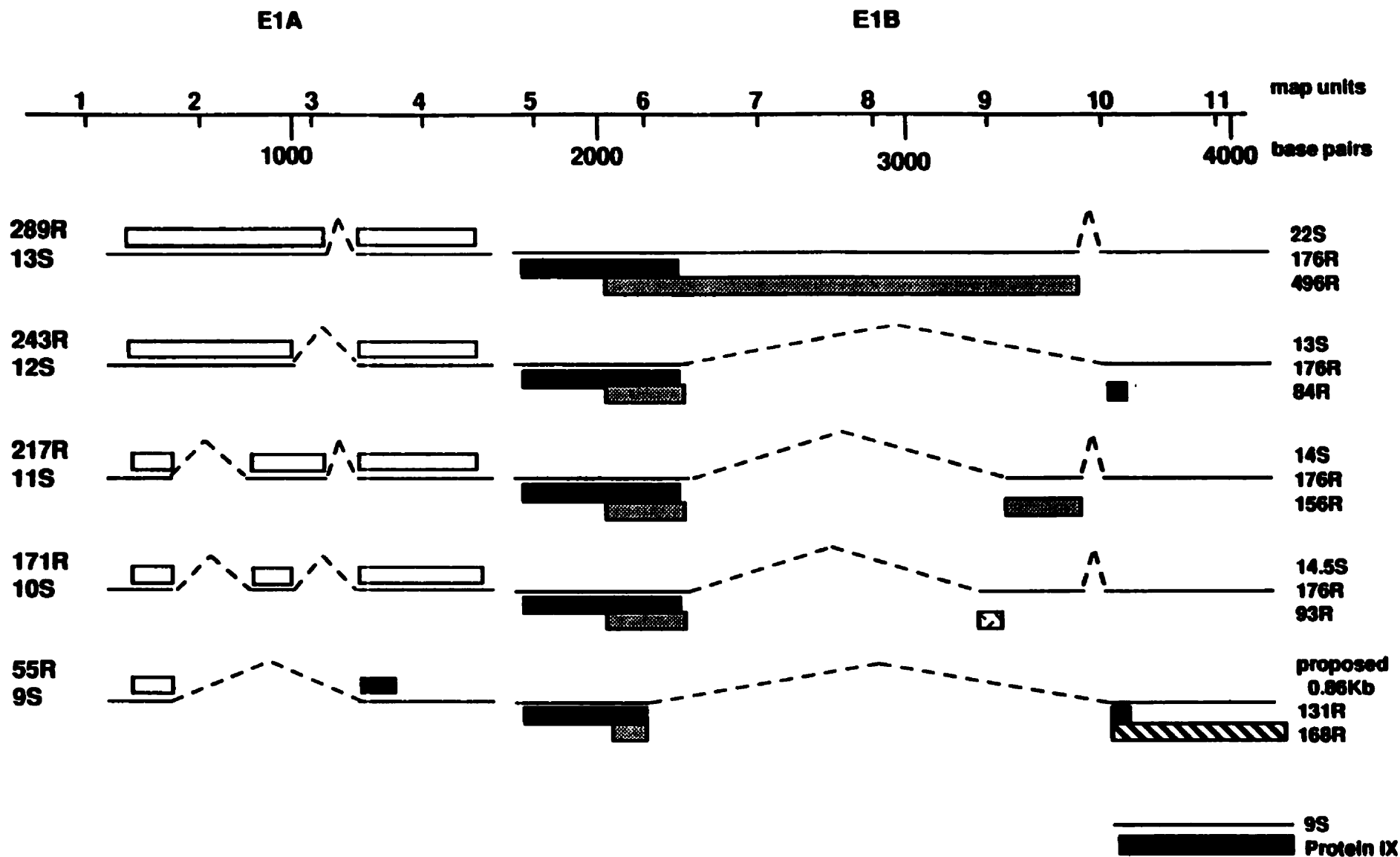
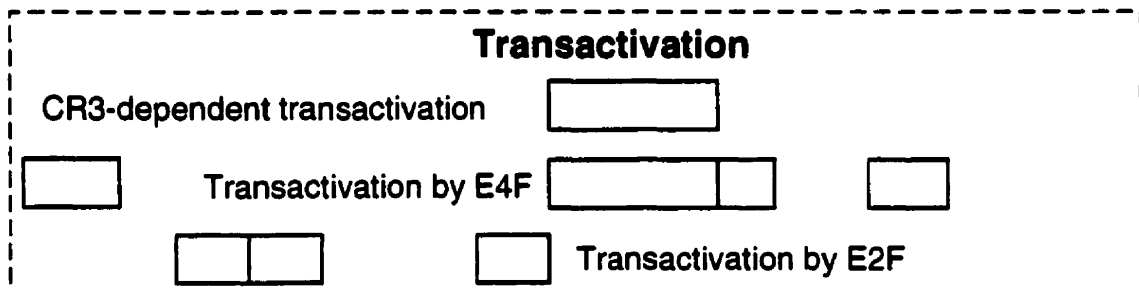
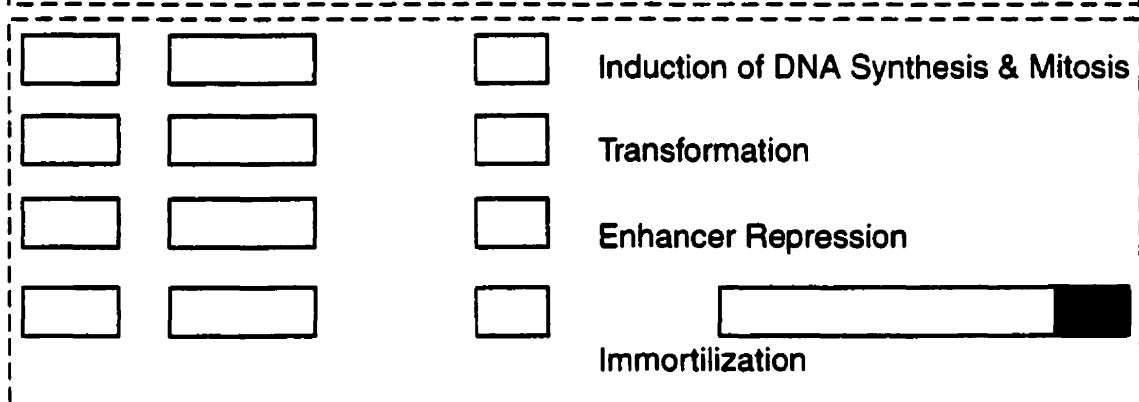
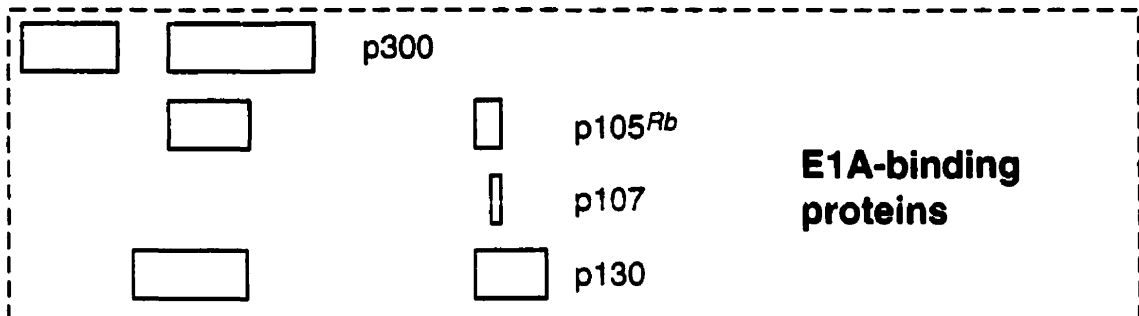
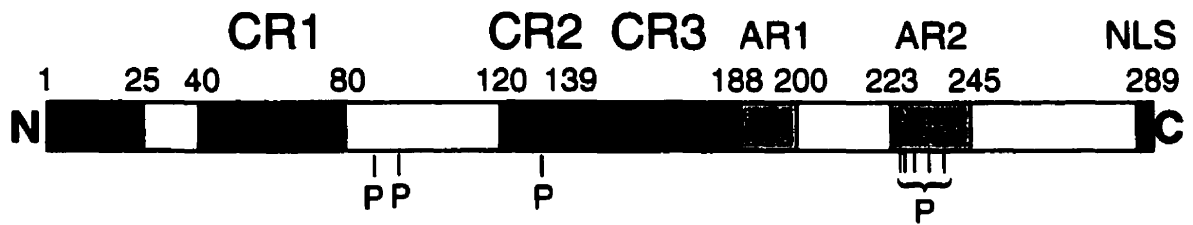


Figure 1-4 E1A protein structure and binding proteins

A schematic diagram of the 289R E1A product. The conserved regions (CR1-CR3) are indicated with black boxes, and auxillary regions (AR1 & AR2) are marked with grey boxes. The identified phosphorylation sites have been indicated. The major E1A-binding proteins are given below, corresponding to their binding sites, followed by the functional mapping.



1.6.3.4 E1A transactivational activity

The discovery that E1A was required to get transcriptional activation of the early viral genes emerged with the examination of some E1A mutants which were substantially defective in early mRNA production (Berk *et al.*, 1979; Jones & Shenk, 1979b; Shenk *et al.*, 1979). Subsequent work demonstrated that the E1A proteins greatly increased the rate of transcription of early viral genes (Nevins, 1981b). Initial studies attempting to find the E1A-responsive promoter element were unsuccessful (Hearing & Shenk 1985; Kingston *et al.*, 1984; Murthy *et al.*, 1985) until it was realized that the E1A proteins do not possess sequence-specific DNA binding activity, but rather function by interacting with a number of different cellular transcription factors (Ferguson *et al.*, 1985; Chatterjee *et al.*, 1988).

Much of the E1A transactivational activity relies on CR3, positioning the 289R protein as the major early viral transcriptional regulator. CR3 is an autonomous transactivating domain, since even a synthetic peptide corresponding to this region will stimulate transcription when microinjected into cells (Lillie *et al.*, 1987). CR3 can be subdivided into two main sections: a transactivation domain at the N-terminal end (residues 141-178), and a promoter targeting region at the C-terminal end (residues 183-188) (Glenn & Ricciardi, 1987; Lillie & Green, 1989; Webster & Ricciardi, 1991). A major feature of the transactivation domain is a zinc finger with four conserved cysteine residues (Culp *et al.*, 1988; Martin *et al.*, 1990; Webster & Ricciardi, 1991) which has been shown to directly contact the TATA binding protein (TBP) (Horikoshi *et al.*, 1991; Lee *et al.*, 1991). Metal binding domains, including zinc fingers, are often found in transcription factors and are thought to be involved in DNA binding or protein-protein interactions (Culp *et al.*, 1988, Mitchell & Tjian, 1989). The ability of CR3 to contact TBP allows E1A 289R to activate transcription through the TATA motif (Green *et al.*, 1983; Simon *et al.*, 1988; Wu *et al.*, 1987) though some TATA-containing promoters are not responsive to E1A (Pei & Berk, 1989; Simon *et al.*, 1988). TBP is the DNA binding subunit of the basal transcription factor TFIID, which is central in forming the transcriptional initiation complex at the TATA box (Pugh & Tjian, 1992; reviewed in Ptashne & Gann, 1997). The 243R E1A protein can also activate transcription through the TATA motif by binding and inactivating Dr1 (Kraus *et al.*, 1994), an inhibitor of TBP (Inostroza *et al.*, 1992), but this effect is much weaker than that observed with the 289R protein (Kraus *et al.*, 1992).

As mentioned previously, E1A does not directly bind to DNA; instead the promoter targeting region of CR3 interacts with a number of cellular, DNA-binding transcription factors. One of the best studied examples of this involves the transcription factor ATF-2, a

member of the cAMP-responsive element binding protein (CREB) family (reviewed in Montminy, 1997). E1A 289R can transactivate promoters containing ATF sites (Liu & Green, 1990) through its ability to bind to the bZIP DNA-binding domain of ATF-2 (Lillie & Green, 1989; Liu & Green, 1990; 1994; Abdel-Hafiz *et al.*, 1993). The model of CR3-mediated transactivation is that by binding to both an upstream transcription factor and TBP, CR3 is able to bridge the two, stabilize the complex, and stimulate transcriptional initiation (Lee *et al.*, 1991). With the exception of the E1B promoter, all of the other early viral promoters contain ATF binding sites, allowing E1A to activate these different promoters by interacting with this single upstream transcription factor (Lee & Green, 1987; Lee *et al.*, 1987).

In addition to CR3-mediated transcriptional activation, the 243R product is also able to stimulate some cellular transactivators. A prime example is the E2F transcription factor, which was initially characterized as the activator of the adenovirus E2 promoter (Kovesdi *et al.*, 1986). E2F is normally found complexed with the retinoblastoma susceptibility gene product (Rb) (section 1.6.4.2 on page 20) (Bagchi *et al.*, 1991; Bandara *et al.*, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991) which inhibits the transactivational ability of E2F (Chellappan *et al.*, 1992; Dalton, 1992; Hamel *et al.*, 1992; Hiebert *et al.*, 1992; Flemington *et al.*, 1993; Helin *et al.*, 1993). Both E1A products can interact with Rb (Whyte *et al.*, 1988; Egan *et al.*, 1989) using the CR1 and CR2 domains (Egan *et al.*, 1988; Whyte *et al.*, 1989; Dyson *et al.*, 1992) and this releases E2F from the inhibitory complex with Rb (Bagchi *et al.*, 1990; 1991; Bandara *et al.*, 1991; Bandara & La Thangue, 1991; Chellappan *et al.*, 1991). Once released, E2F is available to activate the viral E2 promoter, and is also involved in activating cellular promoters required for cell cycle progression (section 1.6.4 on page 19) (Bagchi *et al.*, 1990; Bandara *et al.*, 1991; Chellappan *et al.*, 1991).

Both E1A products have also been found to both activate and repress the AP-1 transcription factor, a family of heterodimeric transcription factors composed of various combinations of Fos, Jun and ATF family members (reviewed in Karin *et al.*, 1997). This E1A function is dependent upon binding to the cellular transcriptional co-activator p300 (section 1.6.4.3 on page 24) which involves CR-1, and the relatively unconserved region at the N-terminus of E1A (Engel *et al.*, 1991; Gedrich *et al.*, 1992; Offringa *et al.*, 1990). E1A represses the activity of AP-1 at its binding sites in the collagenase and stromelysin genes (Offringa *et al.*, 1990) by inhibiting the DNA binding activity of the c-fos / c-jun AP-1 heterodimer (Hagmeier *et al.*, 1993). In contrast, E1A stimulates the binding of AP-1 to ATF sites in the viral early genes (Engel *et al.*, 1988; Hardy & Shenk, 1988). Most likely the ATF-binding, AP-1 complexes are composed of c-jun / ATF complexes (Benbrook & Jones,

1990). The mechanism by which E1A acts to modulate AP-1 activity is not fully understood, and is probably indirect. E1A cooperates with cAMP to upregulate the levels of AP-1, via an increase in the transactivation of the *c-fos* and *junB* genes via their TATA motifs (Müller *et al.*, 1992; Kleinberger & Shenk, 1993) and this leads to increased transactivation of viral genes (Müller *et al.*, 1989). In this manner, E1A is able to stimulate viral promoters via ATF sites by two mechanisms: indirectly by activating AP-1, as well as directly through the CR3-mediated activation of ATF-2.

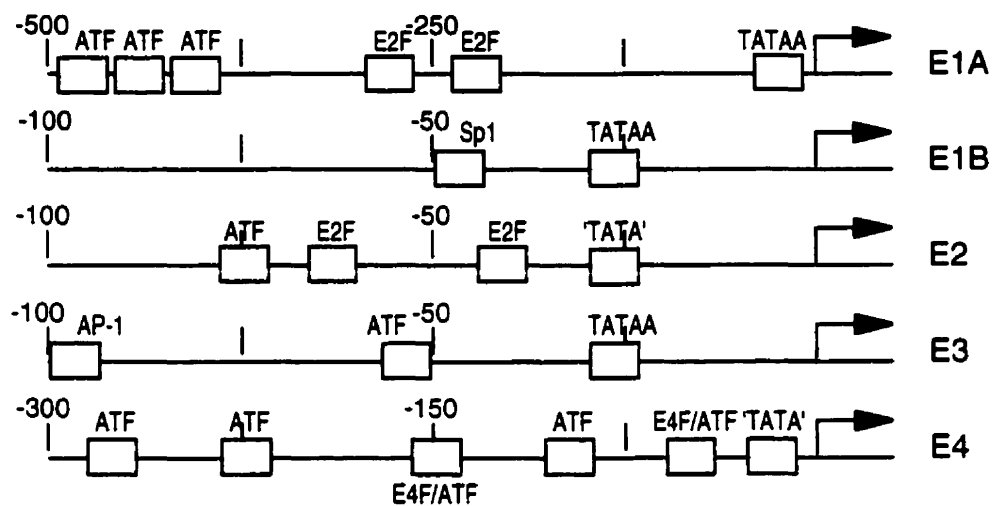
E1A also interacts with the YY1 transcription factor (Shi *et al.*, 1991; reviewed in Shi *et al.*, 1997) via the p300 binding region (Lee *et al.*, 1995). This association is stabilized, in the case of the 289R product, by CR3 (Lewis *et al.*, 1995). E1A activates promoters containing the YY1 consensus site by relieving the transcriptional repression activity of YY1 (Shi *et al.*, 1991).

The E1A promoter contains a pair of E2F sites, multiple ATF sites, a YY1 site, and a TATA box (see Figure 1-5 on page 17) (Hardy & Shenk, 1988; Hearing & Shenk, 1983; Mudryj *et al.*, 1990; Osborne *et al.*, 1982; Osborne & Berk, 1983). E1A is the first viral promoter to get activated during an infection, and has a high basal activity in most cell types. Both E1A products transactivate their own promoter in a positive feedback loop early in the growth cycle; however, later in infection, E1A down-regulates its promoter by repressing an enhancer that lies upstream of the E1A gene (Borelli *et al.*, 1984; Velcich & Ziff, 1985). This enhancer repression function of E1A maps to the binding of p300 (section 1.6.4.3 on page 24) (Rochette-Egly *et al.*, 1990; Stein *et al.*, 1990; Wang *et al.*, 1993). The E1B promoter is transcribed partly by read-through from the E1A promoter and it is activated by the 289R E1A product, through the E1B TATA motif (Nevins, 1989). The E1B promoter also contains an Sp-1 site, though its role in conferring E1A responsiveness has not been determined (Wu *et al.*, 1987; Nevins, 1989).

The E2 promoter contains both ATF-2 and E2F binding sites. The 289R E1A product can transactivate this promoter directly via the ATF-2 sites (Chatton *et al.*, 1993) and both E1A products can stimulate transcription via the two E2F sites (Mudryj *et al.*, 1990). As will be discussed later, the E4orf6/7 product is also involved in increasing the production of E2 transcripts (section 1.6.8.7 on page 40). The E4orf6/7 protein dimerizes and specifically activates E2F transactivation at the E2 promoter by binding to two E2F-1 / DP-1 heterodimers and fixing their spacing so they exactly match the layout on the viral E2 promoter (Hardy & Shenk, 1989; Huang & Hearing, 1989b; Marton *et al.*, 1990; Raychaudhuri *et al.*, 1990; Neill *et al.*, 1990; 1991). This assistance by the E4orf6/7 protein

Figure 1-5 Adenovirus Early Region Promoter Schematics

An illustration of the layout of the different promoter elements and their relative positions with the adenovirus early promoters (adapted from Nevins, 1989).



appears unnecessary for cycling cells in tissue culture; however, in a natural infection of non-dividing differentiated cells, it may play a more important role (Hemström *et al.*, 1991).

The E3 promoter contains AP-1 and ATF-2 sites along with a TATA box, and all seem to be involved in transactivation by the 289R E1A product (Nevins, 1989). Most of the previously mentioned work characterizing the CR3 region of E1A was done using the E3 promoter, since it is primarily activated through this mechanism.

The E4 promoter contains five ATF-2 sites, which are activated in an E1A CR3-dependent manner as discussed previously, as well as two binding sites for a previously unknown transcription factor called E4F (Rooney *et al.*, 1990; Liu & Green, 1990; Jones & Lee, 1991). The E4F DNA binding site contains an ATF-2 consensus, but encompasses additional residues that are not required for ATF-2 binding (Rooney *et al.*, 1990). In the E4 promoter, two of the ATF-2 sites are also E4F sites, but the ATF-2 sites in the other viral promoters do not allow E4F binding (Raychaudhuri *et al.*, 1987; Rooney *et al.*, 1990). E1A activates E4F differently from the mechanism used to transactivate through ATF-2 sites. While the CR3 region of E1A is sufficient for ATF-2 activation, E4F stimulation also requires two auxiliary regions (AR) termed AR1 and AR2 (Bondesson *et al.*, 1992). AR1 (aa 189-200) consists of six pairs of alternating proline and glutamic acid residues, while AR2 (aa 223-245) has a high serine and threonine content (33%) and contains a number of acidic residues (see Figure 1-4 on page 13). The recent characterization of AR1 has indicated that it may actually be important for the full activity of CR3 in all contexts, but the function of AR1 and AR2 is unknown, and independently they do not have any transactivation activity, nor do they appear to interact with upstream transcription factors (Bondesson *et al.*, 1992; Ström *et al.*, 1998).

The activation of E4F by E1A also involves an uncharacterized phosphorylation event on E4F that increases its DNA-binding activity (Raychaudhuri *et al.*, 1989). E4F is a 50 kD transcription factor that is proteolytically generated from a larger 783 aa product (p120E4F) (Fernandes & Rooney, 1997). Interestingly, while the E1A stimulated phosphorylation of p50E4F increases its DNA binding activity, the p120E4F product is inhibited by this phosphorylation, and normally in its hypophosphorylated form, represses the E4 promoter (Fernandes & Rooney, 1997). The kinases involved and the mechanism by which E1A influences E4F activity are yet to be determined. Analogous to the situation with E2F, adenovirus may have pointed the way to another critical cell cycle regulating transcription factor with p120E4F, as it has recently been found to promote growth arrest

by stabilizing the cdk inhibitor p21^{waf-1} (Fernandes *et al.*, 1998). In addition to the stimulatory effects of E1A, the E4 promoter is also down-regulated through a negative feedback loop. As will be described in detail later, the E4orf4 protein, once transcribed from the E4 region, acts to antagonize E4 expression (section 1.6.8.5 on page 37). The deletion of this gene from the virus led to an increased detachment of infected cells from the plate, indicating that tight control of the E4 promoter is essential for proper viral growth (Muller *et al.*, 1992) (see Chapter 4 on page 112).

Once the early mRNAs are transcribed they are translated on polysomes, together with cellular messages. Unlike the late viral mRNAs, the early transcripts are not favoured over cellular mRNAs (section 1.6.11 on page 43).

In addition to the stimulation of RNA polymerase II-mediated transcription, E1A is also able to activate the expression of the VA RNAs (section 1.6.11.2 on page 43) (Hoeffler *et al.*, 1988; Hoeffler *et al.* 1985; Yoshimura *et al.*, 1986). These specialized RNA molecules are transcribed by RNA polymerase III, and E1A seems to stimulate the activity of a transcription factor called IIIc which is involved in their expression.

1.6.4 Stimulation of Host DNA Synthesis by E1A

Adenoviruses normally infect differentiated epithelial cells of the upper respiratory tract, and in order to carry out their replicative functions, they need to override the differentiation phenotype, and push the cells into the cell division cycle. This allows the virus to make use of the cellular DNA replicative machinery, permitting the virus to carry the minimum functions necessary to ensure that the viral genome is preferentially replicated. The modulation of the cell cycle is primarily a function of the E1A proteins and in isolation, both the 12S and 13S E1A products can induce DNA synthesis in quiescent human and rodent cells (Spindler *et al.*, 1985; Kaczmarek *et al.*, 1986; Quinlan & Grodzicker, 1987; Nakajima *et al.*, 1987; Zerler *et al.*, 1987; Moran & Zerler, 1988; Smith & Ziff, 1988; Howe *et al.*, 1990).

1.6.4.1 E1A binding proteins

The mechanism by which E1A induces cell cycle progression was first revealed when E1A proteins were observed to coimmunoprecipitate with a number of cellular proteins (Yee & Branton, 1985; Harlow *et al.*, 1986; Egan *et al.*, 1988; 1989; Giordano *et al.*, 1989; 1991; Howe

et al., 1990; 1992; Barbeau *et al.*, 1993; 1994). The major E1A-associated proteins have molecular weights of 400, 300, 130, 107, 105, 60 and 33 kd. This finding raised the possibility that the mitogenic activity of E1A involved interactions with cellular growth regulators, and now that these proteins have been identified, this model appears to be correct.

1.6.4.2 The Retinoblastoma Family, E2F, Cyclins, and Cdk's

The 105 kd protein was the first to be identified, and it is the retinoblastoma susceptibility gene product (Rb) (Whyte *et al.*, 1988; Egan *et al.*, 1989). The SV40 and polyoma large T antigens as well as the human papillomavirus E7 protein were also found to bind to Rb, implicating it as an important target for the DNA tumour viruses (Decaprio *et al.*, 1988; Dyson *et al.*, 1989; Ludlow *et al.*, 1989). The pattern of retinoblastoma tumour formation among children with hereditary predispositions, compared to the sporadic occurrences in adults, led to the "two hit hypothesis" in which Knudson proposed that the development of tumours followed from the disruption of both of the Rb alleles (Knudson, 1971). The cloning of the responsible gene confirmed his theory, when mutations were found in each Rb allele in retinoblastoma tumours, one of which was a germline mutation in hereditary cases (Dryja *et al.*, 1986; Friend *et al.*, 1986; Lee W.-H. *et al.*, 1987; Fung *et al.*, 1987). Rb was the first tumour suppressor gene to be cloned and has been found to be involved in other cancers as well, including osteosarcomas, and small cell lung carcinomas (Sellers & Kaelin, 1997). Reintroduction of Rb into retinoblastoma tumour lines can lead to a loss in tumorigenicity, attesting to its role as tumour suppressor (Huang *et al.*, 1988; Brookstein *et al.*, 1990; Takahashi *et al.*, 1991).

As mentioned previously, the E1A association with Rb releases free E2F, which then activates the viral E2 promoter (section 1.6.3.4 on page 14); however, there is an additional benefit for the virus. E2F activates many genes involved in cell cycle progression and DNA synthesis, including *cdc2*, *c-fos*, *c-myc*, *c-myb*, insulin-like growth factor II, *N-myc*, the *neu* protooncogene, dihydrofolate reductase, DNA polymerase α , ribonucleotide reductase, thymidine kinase, thymidylate synthetase, and transforming growth factor β 1 (reviewed in Nevins, 1992; Bernards, 1997). The release of free E2F is a simple yet powerful means for E1A to stimulate cell growth, and this is likely why other unrelated DNA tumour viruses also target this control point.

Rb is a nuclear protein that is phosphorylated on both serine and threonine residues (Lee W.-H. *et al.* 1987) and is a substrate for the cyclin dependent kinase (cdk) family of protein kinases with which it also associates (Lees *et al.*, 1991; Lin *et al.*, 1991; Hu *et al.*, 1992; Kitagawa *et al.*, 1992; Williams *et al.*, 1992). Rb phosphorylation is regulated during the cell cycle (Buchovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989; Ludlow *et al.*, 1990). It is hypophosphorylated during G₀ and G₁, hyperphosphorylated during S and G₂, and dephosphorylated during M. The phosphatases involved include protein phosphatase type 1 and type 2A (Alberts *et al.*, 1993; Ludlow *et al.*, 1993). Phosphorylation of Rb negatively regulates its function by preventing the association with E2F (Chellappan *et al.*, 1991; Helin *et al.*, 1992; Schwarz *et al.*, 1993), abrogating its growth arrest function (Hinds *et al.*, 1992). The hypophosphorylated Rb binds directly to the E2F transactivation domain (Bagchi *et al.*, 1990; Bagchi *et al.*, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991) and suppresses E2F-mediated transcriptional activity (Flemington *et al.*, 1993; Helin *et al.*, 1993). Rb has been shown to block the cell cycle in late G₁ (Goodrich *et al.*, 1991; Hinds *et al.*, 1992) and this activity correlates with its ability to bind to E2F and inhibit its transactivational activity (Hiebert *et al.*, 1992; Qian *et al.*, 1992; Qin *et al.*, 1992).

Rb is not the only tumour suppressor involved in E2F regulation. Two of the other E1A-associated proteins are also involved—p107 and p130—and they both show homology to Rb (Hu *et al.*, 1990; 1991; Kaelin *et al.*, 1990; Ewen *et al.*, 1991; Hannon *et al.*, 1993; Li *et al.*, 1993). Analysis of the amino acid sequences of these three Rb family members revealed two highly conserved domains, termed the A and B boxes (or the “pocket”) which are separated by a variable length spacer. E1A and E2F both bind the pocket, making the interaction mutually exclusive, such that E1A binding releases E2F (Ewen *et al.*, 1991; Hu *et al.*, 1990; 1991; Kaelin *et al.*, 1990). Not surprisingly, the pocket is also the target of many Rb mutations in human cancers (Hu *et al.*, 1990; Huang S. *et al.*, 1990). Like Rb, p107 has also been demonstrated to induce growth arrest in G₁ by binding and inhibiting E2F (Schwarz *et al.*, 1993; Zamanian & La Thangé, 1993; Zhu *et al.*, 1993), and p130 is also involved in the negative regulation of E2F (Cobrinik *et al.*, 1993); however these two Rb family members have not been implicated in cancer to this point.

As mentioned earlier E1A has a number of conserved domains, termed CR1, CR2, and CR3 (section 1.6.3.3 on page 10). CR3 contacts components of the transcriptional apparatus, while CR1 and CR2 are involved in the interactions with the Rb protein family. CR2 contains the primary Rb family binding site from 120-127 aa, with a core conserved sequence Asp¹²¹-Leu-X-Cys-X-Glu (the LXCXE motif, where X is any amino acid) (Egan *et al.*, 1988; Whyte *et al.*, 1989) which is also found in the oncoproteins from the other DNA

tumour viruses, and in the cellular proteins that bind to the pocket (DeCaprio *et al.*, 1988; Figge *et al.*, 1988; Phelps *et al.*, 1988; Dyson *et al.*, 1989; 1989b; 1990). The N-terminal section of CR1, from aa 36-60, is also involved in stabilizing the Rb interaction (Egan *et al.*, 1988; Whyte *et al.*, 1989; Stein *et al.*, 1990; Svensson *et al.*, 1991) and is involved in displacing E2F from Rb after CR2 has made the primary high-affinity interaction (Fattaey *et al.*, 1993).

E2F is actually a family of six currently identified members: E2F-1 to -6 (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Helin *et al.*, 1992; Ivey-Hoyle *et al.*, 1993; Kaelin *et al.*, 1992; Lees *et al.*, 1993; Shan *et al.*, 1992). These transcription factors are active as heterodimers, with an E2F protein complexed with a member of the DP transcription factor family (DP-1, DP-2, and DP-3) (Bandara *et al.*, 1993; Helin *et al.*, 1993b; Huber *et al.*, 1993). The E2F-1, E2F-2 and E2F-3 heterodimers complex with Rb, E2F-4 interacts with Rb, p107 and p130, and E2F-5 only binds to p130 (Lees *et al.*, 1991; Helin *et al.*, 1992; Kaelin *et al.*, 1992; Ivey-Hoyle *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Buck *et al.*, 1995; Hijmans *et al.*, 1995; Vairo *et al.*, 1995; Moberg *et al.*, 1996). In this fashion, the different Rb family members regulate E2F activity throughout the cell cycle, with different complexes predominating at each stage (Weinberg, 1995). Little is known about the function of E2F-6.

Interestingly, E2F remains associated with DNA when bound to the Rb family members, and this may serve a repression function to keep E2F responsive promoters at very low levels (Weintraub *et al.*, 1992; Sellers *et al.*, 1995). Rb has recently been shown to bind to histone deacetylase-1 (HDAC-1) through a pocket / LXCXE motif interaction (Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Acetylation of core histones is believed to promote chromatin unraveling, leading to greater accessibility for the transcriptional apparatus, and increasing gene expression (reviewed in Wolfe., 1997). The HDACs are thought to reverse this process to promote chromosomal condensation, and additionally they deacetylate and thereby inhibit basal and activating transcription factors (Imhof *et al.*, 1997; Gu & Roeder, 1997). Since HDAC1 binds to Rb via an LXCXE-like motif, E1A would be expected to displace it from Rb, and in the case of HPV E7 and SV40 largeT, this was found to be true (Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). p107 and p130 likely function in a similar manner, and some recent work has demonstrated that p130 and Rb bind another LXCXE motif-containing protein—RBP-1—which itself is a transcriptional repressor (Lai *et al.*, 1999).

Gene knockouts have been performed on the different Rb family members to examine their roles in development. The Rb^{-/-} embryos died between days 14 and 15 with extensive developmental defects in nervous tissue and in the haematopoietic system (Jacks *et al.*, 1992;

Lee *et al.*, 1992). The p107^{-/-} (Lee *et al.*, 1996) and p130^{-/-} (Cobrinik *et al.*, 1996) mice were phenotypically normal; however a double knockout of p107 and p130 exhibited severe skeletal limb defects arising from chondrocyte growth abnormalities (Cobrinik *et al.*, 1996). From this, there appears to be substantial functional overlap among the Rb family in the mouse, though individual members probably play slightly different roles in the development of various cell types, and in the normal turnover of adult tissues.

Two of the smaller E1A-associated proteins—p60 and p33—are also involved in the E2F regulatory pathway. They are cyclin A (Pines & Hunter, 1990; Ewen *et al.*, 1992; Faha *et al.*, 1992) and cdk2 respectively (Giordano *et al.*, 1989; Hermann *et al.*, 1991; Kleinberger & Shenk, 1991; Tsai *et al.*, 1991). E1A associates with these proteins indirectly; cdk2 binds to cyclin A and cyclin A in turn interacts with p107 and p130 (Giordano *et al.*, 1989; 1991; Howe & Bayley, 1992). E1A does not displace cyclin A from p107 or p130 because they bind to the spacer region between the A and B boxes (Devoto *et al.*, 1992; Ewen *et al.*, 1992; Faha *et al.*, 1992). The Rb spacer is smaller and does not associate with any known cyclins.

The cyclins make up a family (cyclin A through H) of proteins that undergo dramatic shifts in protein levels throughout the cell cycle (reviewed in Hunter & Pines, 1994) and function by binding and regulating the group of cyclin dependent kinases (cdks). The cyclin / cdk complexes specifically phosphorylate substrates involved in cell cycle progression, and different combinations of cyclins and cdks predominate at each stage of the cell cycle, corresponding to the cyclin expression pattern. The Rb family members are all regulated by cyclin / cdks to control E2F release. Both p107 and p130 are found in complexes with cycA or cycE, and cdk2 (Lees *et al.*, 1992; Devoto *et al.*, 1992; Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993; Faha *et al.*, 1993; Li *et al.*, 1993).

As mentioned, Rb does not bind cyclins through its spacer region, but rather binds to a different group of cyclins—the D-type cyclins—via an LXCXE motif which is present in the N-terminus of the D cyclins (Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Kato *et al.*, 1993). The D cyclins associate with cdk4 and cdk6 and push the cell through the G1 checkpoint (Jiang *et al.*, 1992; Quelle *et al.*, 1993; Baldin *et al.*, 1993; Bates *et al.*, 1994; Meyerson & Harlow, 1994), which in part likely involves the phosphorylation and inactivation of Rb (Kato *et al.*, 1993; Fagan *et al.*, 1994; Matsushime *et al.*, 1994; Lukas *et al.*, 1994; 1995). The D cyclins have short half-lives, but are highly induced during G₁ in the presence of growth factors (Matsushime *et al.*, 1991; Ajchenbaum *et al.*, 1993; Winston & Pledger, 1993; Won *et al.*, 1992) and likely function as a link between growth factors and the cell cycle—via Rb (Sherr, 1993). By disrupting the function of the Rb family members, E1A can prime the cell cycle in one

step, bypassing the need for growth factors and activated cyclin / cdk complexes. The release of free, active E2F is the key.

1.6.4.3 The p300 Family

E1A does not rely exclusively on E2F activation to stimulate DNA synthesis. The E1A interaction with the p300 co-immunoprecipitating protein is also sufficient to induce cell cycle progression from G₁ to S (Zerler *et al.*, 1987; Lillie *et al.*, 1987; Howe *et al.*, 1990); however, progression to M phase requires both p300 binding and Rb family interactions (Zerler *et al.*, 1987; Howe *et al.*, 1990; Howe & Bayley, 1992). The ability of E1A to promote growth through two separate pathways may simply lead to increased effectiveness, or perhaps in different cell types one pathway may be more efficient. In any case, to progress through the G2/M checkpoint requires E1A to stimulate both pathways, and this may be important in a natural infection, where virus replication is much slower, and the virus may need multiple S phases to complete its replication.

For p300 binding, E1A requires CR1, spanning 36-76 aa, and the less conserved region at the N-terminus, 1-25 aa (Egan *et al.*, 1988; Whyte *et al.*, 1989; Stein *et al.*, 1990; Svensson *et al.*, 1991; Wang *et al.*, 1993). The p400 E1A-binding protein also binds to the same region, but the exact residue requirements vary slightly (Barbeau *et al.*, 1993; 1994). The p300 and p400 proteins are likely related, as V8 protease mapping revealed a similar pattern; however, p400 has not been otherwise characterized (Barbeau *et al.*, 1994). p300 has been cloned and encodes a 2414 residue protein with homology to transcriptional coactivators (Eckner *et al.*, 1994), particularly a central bromo domain, which has no known function, but is found in several transcriptional modulators, such as TAF_{II}250, a TBP-associated factor (Sekiguchi *et al.*, 1991; Hisatake *et al.*, 1993; Ruppert *et al.*, 1993). p300 also contains three cysteine / histidine rich regions, the most C-terminal of which contains the E1A binding site (Eckner *et al.*, 1994).

p300 is closely related to the CREB binding protein (CBP) (Chrivia *et al.*, 1993), and these two species are believed to form a family of transcriptional regulatory proteins (Arany *et al.*, 1994). E1A associates with both proteins, and they both interact with CREB (Arany *et al.*, 1995; Lundbland *et al.*, 1995). When the second messenger cAMP is released in the cell, protein kinase A is activated and phosphorylates CREB (Gonzalez & Montminy, 1989), which then becomes competent to interact with p300 / CBP and activate transcription of CRE containing promoters (Chrivia *et al.*, 1993; Kwok *et al.*, 1994;

Lundbland *et al.*, 1995). When E1A interacts with p300 / CBP the association with CREB is prevented, and transcriptional activation is blocked (Arany *et al.*, 1995; Lundbland *et al.*, 1995). p300 / CBP are also involved as transcriptional coactivators with the nuclear receptor superfamily (reviewed in Beato *et al.*, 1995; Kastner *et al.*, 1995; Mangelsdorf & Evans, 1995; Mangelsdorf *et al.*, 1995) and they have been shown to interact directly with a number of receptors, including those for retinoic acid, estrogen and retinoid X (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996). They also interact with the AP-1 components c-Jun and c-Fos, and CBP / p300 appear to be essential coactivators, since the E1A association with them downregulates AP-1 driven promoters (Arias *et al.*, 1994; Banister & Kouzarides, 1995).

The E1A proteins are known to repress a number of viral transcriptional enhancers (Borrelli *et al.*, 1984; Velcich & Ziff, 1985) and this maps to the p300 binding region (Rochette-Egly *et al.*, 1990; Stein *et al.*, 1990; Wang *et al.* 1993). Additionally, E1A has been shown to repress a variety of differentiation specific enhancers, including promoters for the immunoglobulin heavy chain (Hen *et al.*, 1985), insulin (Stein & Ziff, 1987) and some muscle specific genes, blocking myogenic differentiation (Webster *et al.*, 1988). p300 / CBP are thus thought to function by promoting differentiation and maintaining differentiated cells in G₀. E1A likely forces cells out of their quiescent state by inactivating p300 / CBP, but how this process overcomes the G₁ restriction point and promotes DNA synthesis is not currently known.

One mechanism by which transcriptional coactivators function was revealed when p300 and CBP were shown to bind to p300/CBP associated factor (P/CAF) a histone acetylase (Yang *et al.* 1996). As mentioned earlier, acetylation of the core histones and transcription factors is believed to promote gene expression by opening up otherwise inaccessible chromatin and stimulating transcription. E1A and P/CAF compete for the same site on p300; however, p300 also possesses intrinsic histone acetylase activity, and the E1A-p300 complex can exist with other factors bound to CR2 and presumably CR3 (Barbeau *et al.*, 1994), suggesting that E1A may bring p300 to E1A-activated promoters to enhance transcription. This would provide an additional benefit to the virus, beyond the inhibition of differentiation-specific gene expression. Recent work has also shown that E1A binds and targets P/CAF to promoters as well, through a p300-independent interaction that also involves CR1 (Reid *et al.*, 1998).

In summary, E1A pushes cells into the cell cycle by inactivating two major classes of proteins—the Rb and p300/CBP families. The mechanism by which Rb family binding leads to cell cycle progression is quite well characterized, and primarily involves the

activation of E2F, a critical cell cycle promoting transcription factor. The E1A association with p300 is understood from the perspective of blocking the expression of tissue-specific genes and shifting the cell from G_0 to G_1 , but how this leads to a stimulation of DNA synthesis is not known. In addition to promoting growth in a viral infection, E1A can also transform cells in conjunction with the E1B products, and this activity maps to the ability of E1A to stimulate the cell cycle (section 1.7 on page 46).

1.6.5 Blockage of Apoptosis

1.6.5.1 E1A and apoptosis

As discussed, E1A alone can induce DNA synthesis and cellular proliferation in quiescent cells; however, this unscheduled growth promotion triggers the stabilization and upregulation of the cellular tumour suppressor p53, leading to the induction of apoptosis (Braithwaite *et al.*, 1990; Debbas & White, 1993; Lowe & Ruley, 1993; Grand *et al.*, 1993; 1994; de Stanchina *et al.*, 1998). This activity maps to the ability of the E1A protein to bind either Rb family members or p300/CBP, and may be an unavoidable consequence of the viral growth promotion process (Querido *et al.*, 1997b; Chiou & White, 1997). One major role of p53 is to protect the integrity of multicellular organisms by preventing the survival of individual aberrant cells that can arise through DNA damage, and in this capacity, it is considered the guardian of the genome (reviewed in Levine, 1997) (section 1.9.10 on page 64). In addition, p53 also detects the inappropriate stimulation of proliferation and / or the presence of viral replication. In brief, p53 has the ability to induce growth arrest in G_1 to allow time for DNA mutations to be repaired, but if this is not sufficient to fix the problem, p53 will then induce apoptosis.

The cellular apoptotic response (section 1.9 on page 51), if uncountered, would prevent or limit progeny production, so the virus has developed a number of strategies to block this host defense. One of the primary functions of the E1B region is to block apoptosis during viral replication; however, at the end of an adenoviral infection, there is the suggestion that the virus uses apoptosis as a release mechanism (section 1.6.12 on page 45). Thus the inhibition and induction of apoptosis by the virus must be carefully balanced. Like adenoviruses, many viruses have evolved mechanisms to both block apoptosis at early stages of replication and induce apoptosis late in the lytic cycle, by targeting similar control points (reviewed in Teodoro & Branton, 1997a; Granville *et al.*, 1998b).

E1A is partially responsible for inhibiting apoptosis by inducing the expression of the E1B gene products (section 1.6.5.2 on page 27) and E4orf6 (section 1.6.8.6 on page 38) which are all involved in abrogating the cellular apoptotic response. E1A itself can also partially inhibit the transactivational capacity of p53 (one of its primary mechanisms of action) by binding and sequestering p300, an essential cofactor for p53 transactivation (Avantaggiati *et al.*, 1997; Gu *et al.*, 1997b; Lill *et al.*, 1997), but the E1B products and E4orf6 are the major viral defense against apoptosis (Rao *et al.*, 1992; Dobner *et al.*, 1996).

1.6.5.2 E1B Products

Transcription of the E1B region produces multiple mRNAs from alternative splicing of a primary transcript (see Figure 1-3 on page 12). Two major messages are produced: a 2.2 kb message (22S) early in infection, and a 1.05 kb transcript (13S) at later times (Perricaudet *et al.*, 1980; Bos *et al.*, 1981; Glenn & Ricciardi, 1988). Late in infection two minor species of 1.26 kb and 1.31 kb are also made (Anderson *et al.*, 1984; Lewis & Anderson, 1987; Virtanen & Pettersson, 1983). All four messages code for a 19 kd, 176 residue protein (E1B-19K) which runs as a doublet by SDS-PAGE (Rowe *et al.*, 1983; McGlade *et al.*, 1987; 1989), and, from a second internal initiation site in a different reading frame, they produce a series of related proteins. This group of products includes a 496 aa, 55 kd product (E1B-55K) from the 2.2 kb mRNA, a 25 kd, 156 residue protein (156R) from the 1.05 kb mRNA, and 93 aa and 84 aa proteins from the 1.26 kb and 1.31 kb mRNAs, respectively. All four products share 79 aa at the N-terminus, and E1B-55K and 156R share 78 aa at their C-termini. The smaller two proteins have unique C-termini (Bos *et al.*, 1981; Green *et al.*, 1982; Matsuo *et al.*, 1982; Lewis & Anderson, 1987). The E1B-55K and E1B-19K products have been well characterized. They are required for maximal viral growth, and will cooperate independently with E1A to transform cells by blocking apoptosis (Bernards *et al.*, 1983; Barker & Berk, 1987; White & Cipriani, 1990; McLorie *et al.*, 1991; Rao *et al.*, 1992). The functions of the other three E1B products are not known.

1.6.5.3 E1B-55K

As will be discussed, E1B-55K is required for a number of distinct roles in virus replication: (1) splicing and transport of late viral messages (section 1.6.10 on page 41); (2) host cell shut-off (the disruption of cellular mRNA transport and translation) (section 1.6.11 on page 43); and (3) the inhibition of E1A-induced apoptosis, as will be detailed in this

section. The first two E1B-55K functions are carried out in a complex with E4orf6 (section 1.6.8.6 on page 38) while the third can be performed independently of E4orf6, though some activities require the complex.

E1B-55K has been shown to directly bind p53 (Sarnow *et al.*, 1982a; Kao *et al.*, 1990) and mutants that are defective in this function are completely incapable of cooperating with E1A to transform cells (Yew & Berk, 1992). As will be discussed in more detail later (section 1.9.10 on page 64) p53 is a sequence-specific DNA-binding transcription factor, and the interaction of E1B-55K with p53 blocks transactivation activity (Yew & Berk, 1992). E1B-55K binds to the N-terminal acidic region of p53 which is involved in transcriptional transactivation (Kao *et al.*, 1990; Lin *et al.*, 1994) and carries with it a potent transcriptional repression activity (Yew *et al.*, 1994). The repression function of E1B-55K is regulated through the phosphorylation of three sites at the extreme C-terminus of the protein, such that the hypophosphorylated form of E1B-55K is competent to bind p53, but does not inhibit p53-mediated transcription nor apoptosis (Teodoro *et al.*, 1994; Teodoro & Branton 1997b). How phosphorylation regulates the repression function is not known, but recent work has indicated that the repression domain on E1B-55K is in the C-terminus, close to the phosphorylation sites (Assefi *et al.*, 1998). *In vitro* studies have shown that E1B-55K likely exists as a dimer (Martin & Berk, 1998). Additionally, binding to p53 by the E1B-55K dimer was shown to increase the affinity of p53 for DNA by approximately 10-fold, which may help to ensure that p53-regulated promoters are kept below basal levels (Martin & Berk, 1998). E1B-55K repression activity was demonstrated by *in vitro* reconstitution not to involve histones, suggesting direct or indirect interactions with the RNA pol II transcriptional machinery (Martin & Berk, 1998). As expected, the E1B-55K association with p53 blocks p53-mediated apoptosis (Marcellus *et al.*, 1996a; Teodoro & Branton, 1997b)(see Chapter 2 on page 72).

E1B-55K has a second mechanism for downregulating p53 activity involving complex formation with E4orf6. This complex causes a dramatic decrease in p53 protein levels (Moore *et al.*, 1996; Nevels *et al.*, 1997; Querido *et al.*, 1997a) reminiscent of the human papilloma virus-mediated degradation of p53 via the E6 protein (Scheffner *et al.*, 1990). The mechanism by which E1B-55K / E4orf6 promotes this degradation is currently unknown, and does not appear to involve an E6-style ubiquitination of p53 (Scheffner *et al.*, 1993). In addition, E4orf6 has been suggested by one group to independently bind and inhibit transactivation by p53 (Dobner *et al.*, 1996) (section 1.6.8.6 on page 38).

In summary, the apoptosis-preventing activity of E1B-55K seems to arise from its ability to repress p53-activated transcription (by both direct binding and destabilization), presumably blocking the production of proteins involved in growth arrest and apoptosis. Attesting to the importance of suppressing apoptosis, E1B contains a second gene that is even more potent at blocking death—E1B-19K.

1.6.5.4 E1B-19K

Early studies had indicated that some adenovirus mutants exhibited reduced virus yield, extensive cytopathic effect, and degradation of viral and cellular DNA, termed *cyt/deg* (Ezoe *et al.*, 1981). The mutation was mapped to the E1B-19K gene, and the phenotype was eventually shown to result from the induction of apoptosis (Pilder *et al.*, 1984; Takemori *et al.*, 1984; Subramanian *et al.*, 1984a ; 1984b; White *et al.*, 1984; 1991; 1992; Rao *et al.*, 1992). The E1B-19K protein is modified by both fatty acylation and phosphorylation, and is associated with nuclear and cytoplasmic membranes, intermediate filaments of the cytoplasm (vimentin), and nuclear lamins (Grand *et al.*, 1985; McGlade *et al.*, 1987; 1989; Persson *et al.*, 1982; Rowe *et al.*, 1983; White & Cipriani, 1989). E1B-19K is partially homologous to a family of cellular apoptosis modulators, called the Bcl-2 family, and Bcl-2 (the prototype member) can substitute for E1B-19K in blocking E1A-induced apoptosis (Rao *et al.*, 1992). The Bcl-2 family contains both inducers and inhibitors of apoptosis, and individual members function to control a crucial check-point in the life / death balance of the cell (section 1.9.4 on page 53). E1B-19K functions like a pro-life member of the Bcl-2 family, and both Bcl-2 and E1B-19K interact with a similar set of proteins, suggesting some functional overlap (Boyd *et al.*, 1994). E1B-19K has also been shown to bind and inhibit some of the pro-apoptotic Bcl-2 family members: Bak, Bax and Bik (Farrow *et al.*, 1995; Han *et al.*, 1996; 1996b).

Unlike E1B-55K, E1B-19K can block both p53-dependent and -independent apoptosis pathways. E1B-19K is known to block death mediated by tumour necrosis factor α (TNF α , an antitumour / antiviral cytokine secreted by activated macrophages and lymphocytes) which functions through a p53-independent mechanism (White *et al.*, 1992) (section 1.9.6 on page 58). E1B-19K can also block cell death induced by Fas, a member to the TNF receptor family, by interfering with FADD function (section 1.9.6 on page 58) (Perez & White, 1998). This property of E1B-19K would be useful during a viral infection to control the host immune response. In addition, E1A activates a p53-independent apoptosis pathway which is blocked by E1B-19K and Bcl-2 (Subramanian *et al.*, 1995; Teodoro *et al.*,

1995). This cell death pathway is induced by E4orf4 and may play a role late in infection to facilitate virus release (section 1.6.12 on page 45)(Marcellus *et al.*, 1996; 1998; Lavoie *et al.*, 1998; Shtrichman & Kleinberger, 1998) (see also Chapters 3, 4 & 5).

E1B-55K and E1B-19K function to inhibit the apoptotic response by the infected cell to allow the virus time to complete its replicative cycle. The primary activity of E1B-55K is to inactivate the cellular tumour suppressor p53, while E1B-19K functions further downstream in the apoptosis pathway to block both p53-dependent and -independent cell death. Once the virus has taken care of this critical problem early in the infection, it can proceed with its replicative functions.

1.6.6 E2 region functions

The E2 region of adenovirus encodes a group of three proteins that are directly involved in replicating the viral DNA (section 1.6.9 on page 40). The E2 transcriptional unit is expressed from two regions on the left strand of the genome—E2a from 67.9 to 61.5 map units, and E2b from 29 to 14.2 map units (see Figure 1-2 on page 7). As mentioned previously, E1A activates the E2 promoter, then alternate splicing and the differential use of poly(A) signals results in the production of several mRNAs (Berk & Sharp, 1977). The E2 DNA binding protein (E2-DBP) is produced from the E2a region, while both the pre-terminal protein (E2-pTP) and the DNA polymerase (E2-DNA polymerase) are encoded in E2b.

1.6.6.1 E2-pTP

The pre-terminal protein (pTP) is synthesized as an 80 kd polypeptide that is involved in the initiation of DNA replication (Challberg *et al.*, 1980; Stillman *et al.*, 1981). The pTP is found as a heteromeric complex with the E2-DNA polymerase in solution (Enomoto *et al.*, 1981; Lichy *et al.*, 1982; Stillman *et al.*, 1982; Temperley & Hay, 1992). The pTP / DNA polymerase unit binds to the origin of replication to initiate viral DNA synthesis. During this process the E2-pTP becomes covalently attached to the 5' end of the viral genome where it functions both to prime DNA synthesis by the E2 DNA polymerase, and to protect the integrity of the viral termini (Rekosh *et al.*, 1977). During the assembly of the virions, the pTP is processed by proteolysis to generate a 55 kd form known as the terminal protein (TP) which remains covalently attached to the genome (Challberg & Kelly, 1981) and which stays associated with the cleaved peptide (Schaack *et al.*, 1990)

1.6.6.2 E2-DNA polymerase

The E2-DNA polymerase is a 140 kd protein with several unique properties compared to other DNA polymerases (Enomoto *et al.*, 1981; Field *et al.*, 1984). It contains both a 5' to 3' DNA polymerase activity and a 3' to 5' exonuclease function which likely serves in proof-reading (Field *et al.*, 1984). As mentioned above, the DNA polymerase exists in a complex with the E2-pTP. This complex possesses sequence-specific DNA binding activity with both duplex (Temperley & Hay, 1992) and single-stranded templates (Chen *et al.*, 1990; Kenny & Hurwitz, 1988) and recognizes the origin of replication in the viral ITRs.

1.6.6.3 E2-DBP

The E2-DBP is a 59 kd protein which runs on SDS-PAGE gels with an apparent weight of 72 kd. It associates tightly with single-stranded DNA (ssDNA), in a cooperative and sequence independent manner (van der Vliet & Levine, 1973). During replication the E2-DBP coats the single-stranded intermediates produced, and stabilizes them, enhancing the E2-DNA polymerase processivity (Field *et al.*, 1984; Lindenbaum *et al.*, 1986). A temperature sensitive mutation in this gene has been used to demonstrate that it is essential for chain elongation (van der Vliet *et al.*, 1975).

1.6.7 E3 region functions

The E3 region of the virus encodes a cassette of genes that are mostly involved in the modulation of the host immune response (reviewed in Wold & Gooding, 1991; Gooding, 1992). These genes are completely dispensable in tissue culture (Cladaras & Wold, 1985); however, they are critical in a productive infection to hold off the host anti-viral defenses and enable the virus to complete its life cycle (reviewed in Ploegh, 1998). The immune avoidance functions of the E3 region are undoubtedly also involved in the long term persistence of adenoviruses in lymphocytes (Ginsberg *et al.*, 1987).

1.6.7.1 E3-19 kd

The E3-19 kd protein is a transmembrane glycoprotein that is localized to the endoplasmic reticulum and is expressed at high levels early in infection (Wold *et al.*, 1985). The function of E3-19 kd is to reduce the sensitivity of infected cells to cytotoxic T-lymphocyte (CTL)-

mediated lysis (Andersson *et al.*, 1987; Burget & Kvist, 1987) by binding to the peptide-binding domain of major histocompatibility (MHC) class I antigens (Kvist *et al.*, 1978; Burget & Kvist, 1987; Hermiston *et al.*, 1993) and retaining them in the endoplasmic reticulum (Pääbo *et al.*, 1987). This activity should protect infected cells from premature lysis, and in support of this hypothesis, viral mutants lacking this gene induce increased inflammation in a rat model system (Ginsberg & Prince, 1989). In other work, however, the inflammatory response in mice is not sufficiently modified by E3-19 kd during an acute infection, but it may play a role in blocking immune recognition during a persistent infection (Sparer *et al.*, 1996). Interestingly, human cytomegalovirus also encodes a protein which blocks cell surface accumulation of MHC class I antigens (Browne *et al.*, 1990).

1.6.7.2 E3-14.7 kd

Infection of cells by adenovirus induces susceptibility to TNF- α (Duerksen-Hughes *et al.*, 1991), an apoptosis-inducing cytokine that is secreted by activated macrophages and lymphocytes (reviewed in Wallach *et al.*, 1997). Due to the importance of blocking this progeny-limiting host response, the virus has three ways of dealing with it: the E3-14.7 kd protein, the E3-14.5 / E3-10.4 kd complex and E1B-19K (section 1.6.5.2 on page 27). The E3 proteins play an important role in preventing this anti-viral immune response, since cells infected with E3 deletion viruses are lysed by TNF- α (Gooding *et al.*, 1988). The E3-14.7 kd protein is abundantly expressed early in infection, and is found in both the cytoplasm and nucleus (Tollefson & Wold, 1988). It was demonstrated to block cell lysis by TNF- α under a number of stresses (Gooding *et al.*, 1988; 1990), but its mechanism of action does not involve complete inhibition of TNF- α signalling. By an unknown method, it appears to specifically block the TNF-induced cytolysis pathway by inhibiting the release of arachidonic acid by phospholipase A2 (cPLA2), which is activated indirectly by the TNF- α receptor (Horton *et al.*, 1991; Zilli *et al.*, 1992; Krajcsi *et al.*, 1996).

1.6.7.3 E3-14.5 kd / E3-10.4 kd

In addition to E3-14.7, the E3-14.5 kd and E3-10.4 proteins can also prevent apoptosis by TNF- α (Gooding *et al.*, 1991). The E3-14.5 kd and E3-10.4 kd proteins exist in a tight complex (termed RID for receptor internalization and degradation) and both are integral membrane proteins found in the plasma membrane, with their C-termini in the cytoplasm (Tollefson *et al.*, 1990a; 1990b; Gooding *et al.*, 1991; Krajcsi *et al.*, 1992a; 1992b; Stewart *et al.*,

1995). Like E3-14.7 kd, RID is able to block the TNF- α cytolytic signalling pathway by interfering with the release of arachidonic acid by cPLA2; however, its mechanism of action is different, involving the inhibition of the translocation of cPLA2 from the cytoplasm to the plasma membrane, where its substrates lie (Krajcsi *et al.*, 1996; Dimitrov *et al.*, 1997).

The RID complex has a second function: the down-regulation and internalization of the epidermal growth factor receptor (EGF-R) (Carlin *et al.*, 1989; Tollefson *et al.*, 1991; Hoffman *et al.*, 1992; Stewart *et al.*, 1995). The E3-10.4 kd protein has a short stretch of amino acids that are homologous to a region on the cytoplasmic side of the transmembrane region of the EGF-R, and some sort of heterooligomer may be involved in the internalization process. RID has also been recently found to force the internalization and lysosomal degradation of Fas (Shisler *et al.*, 1997; Tollefson *et al.*, 1998), a pro-apoptotic cell surface receptor in the TNF receptor superfamily (section 1.9.6 on page 58) (Nagata, 1997). This would serve to protect infected cells from CTL-mediated killing via the Fas pathway (reviewed in Clark *et al.*, 1995) and indeed RID-expressing cells are resistant to mouse CTLs. The advantages of protecting infected cells from Fas are clear, and this may also play a role in adenovirus persistence in lymphocytes, but the purpose of degrading the EGF-R is still not obvious. Other viruses including the Epstein-Barr virus and Shope fibroma virus also target the TNF pathways for immune avoidance (Hsu *et al.*, 1990; Smith *et al.*, 1991).

1.6.7.4 E3-11.6 kd

The E3-11.6 kd protein is unusual among early genes because it is expressed at very low levels during the early phase of replication and then amplified 400-fold at the late stage, when it is transcribed from the major late promoter, rather than the E3 promoter (Tollefson *et al.*, 1992). The E3-11.6 kd protein is a type III membrane protein, with a luminal N-terminus and a cytoplasmic C-terminus (Wold *et al.*, 1984; Scaria *et al.*, 1992). It is found in golgi, endoplasmic reticulum and most predominantly in the nuclear membrane (Scaria *et al.*, 1992). It also undergoes extensive post-translational modification with complex glycosylation (Scaria *et al.*, 1992) and palmitoylation (Hausmann *et al.*, 1998). The role of the E3-11.6 kd protein is different from the other E3 proteins, which are all involved in immune modulation. The E3-11.6 kd function was revealed when deletion mutants were found to be defective in cell killing and virus release (Tollefson *et al.*, 1996a). Cells infected with E3-11.6 kd mutants exhibit an intact nucleus, swollen with virus, at times when wild-type (wt) virus has disrupted the nucleus and killed the cell (Tollefson *et*

al., 1996b). The mechanism by which this protein contributes to cell killing and progeny spread is unknown, but it has been seen to partially co-localize with E1B-19K (Bill Wold, personal communication) and may function to inactivate the apoptosis blocking activity of E1B-19K to promote cell killing. Additionally, E3-11.6 kd may function in a more direct manner to facilitate nuclear membrane break-down, and consistent with this, the E3-11.6kd protein is toxic to both mammalian cells and bacteria (Ann Tollefson, personal communication).

1.6.7.5 E3-12.5 kd and E3-6.7 kd

The E3-12.5 kd and 6.7 kd proteins are poorly characterized. The E3-6.7 kd is a type III membrane glycoprotein located in the endoplasmic reticulum, with a luminal N-terminus and a cytoplasmic C-terminus (Wilson-Rawls *et al.*, 1993; 1994) and while relatively conserved among serotypes, its functional importance is unknown (Wilson-Rawls *et al.*, 1990). The E3-12.5 kd protein is expressed at low levels during an infection, at both early and late stages of growth (Hawkins & Wold, 1992). It has a long half-life, and is highly conserved among serotypes, but its function is also unknown.

1.6.8 E4 region functions

Unlike the other early regions of adenovirus, the E4 gene contains open reading frames (orfs) with very different functions, and no obvious common theme. There is a total of at least seven expressed proteins. Early work demonstrated that the E4 region was required for viral growth, providing functions that regulate DNA replication, facilitate accumulation of late viral messages, and promote host cell shutoff (Halbert *et al.*, 1985; Weinberg & Ketner, 1986). More recent work has shown that the E4 region is also involved in transformation (Javier *et al.*, 1992; 1994; Dobner *et al.*, 1996; Moore *et al.*, 1996; Nevels *et al.*, 1997), and transcriptional regulation (Müller *et al.*, 1992; Bondesson *et al.*, 1996; Whalen *et al.*, 1997).

1.6.8.1 E4orf1

The Ad5 E4orf1 mRNA codes for a protein product of 14 kd, and is regulated like a late viral message, requiring viral DNA replication and the functions of E4orf6 and E1B-55K, in order to accumulate to high levels late in the infectious cycle (Dix & Leppard, 1993). The

role of E4orf1 in viral growth is unknown, and it is dispensable in tissue culture (Halbert *et al.*, 1985; Bridge & Ketner, 1989). The first insights into the function of this protein have emerged from work done with adenovirus type 9 (Ad9), a D subgroup adenovirus. Ad9 is unique among adenoviruses in that it exclusively induces estrogen-dependent mammary tumours in rats (Ankerst *et al.*, 1974; Ankerst & Jonsson, 1989; Javier *et al.*, 1991). This oncogenic activity was mapped to the E4 region of the virus (Javier *et al.*, 1992) then narrowed down to E4orf1 (Javier, 1994; Weiss *et al.*, 1996). E4orf1 from Ad5 and Ad12 was not found to be tumourigenic in these studies; however, this appears to be due to poor protein expression levels in rat cells, since E4orf1 proteins from Ad5, Ad12 and Ad9 are equally transforming in human cells (Weiss *et al.* 1997a). E4orf1 is a cytoplasmic protein that has moderate sequence homology with a variety of dUTP pyrophosphatases, though it does not have any of the expected enzymatic activity, suggesting that it may have just evolved from this structural framework (Weiss *et al.* 1997a). Three essential regions of Ad9 E4orf1 have been mapped (Weiss *et al.*, 1997b) and a number of unidentified cellular binding proteins have been observed (Weiss & Javier, 1997). Ad5 and Ad12 E4orf1 proteins were also found to interact with some of these proteins, suggesting that they may all share a common mechanism of action (Weiss & Javier, 1997). One of the Ad9 E4orf1 binding proteins was identified as hDig/SAP97 (DLG), a PDZ domain containing protein, which is the mammalian homologue of the *Drosophila* discs large tumour suppressor gene (Lee *et al.*, 1997). This interaction was found to be via a PDZ consensus binding motif that is present in the C-terminus of E4orf1 (Lee *et al.*, 1997). Interestingly, the human T cell leukemia virus type 1 Tax protein and human papillomavirus type 18 E6 proteins also possess PDZ domain binding motifs and interact with DLG *in vitro* (Lee *et al.*, 1997; Rousset *et al.*, 1998). This indicates that a diverse group of viruses have targeted DLG, but the functional importance of this interaction in viral growth is still unknown.

1.6.8.2 E4orf2

E4orf2 codes for a 14.6 kd protein that is currently uncharacterized. From Blast search analysis we know that it is 65% identical to the RNA polymerase β subunit over half its length (Marcellus, unpublished) but what function this plays in viral growth is unknown. Deletion of this region has no effect on the viral life cycle in tissue culture (Halbert *et al.*, 1985; Bridge & Ketner, 1989). An antiserum raised against E4orf2, was used to demonstrate that it is expressed during the early phase of the lytic cycle, and revealed that it is a soluble cytoplasmic component (Dix & Leppard, 1995).

1.6.8.3 E4orf3

E4orf3 is an 11 kd nuclear protein (Sarnow *et al.*, 1982c) that is primarily involved in late gene expression. Early results demonstrated that either E4orf3 or the E4orf6 / E1B-55K complex (section 1.6.8.6 on page 38) was essential in the viral replication cycle, and that these two factors appeared to function in redundant pathways involved in the regulation of late protein synthesis (Bridge & Ketner 1989; Huang & Haring, 1989a; Ketner *et al.*, 1989; Bridge & Ketner, 1990). Their function was subsequently found to involve the stimulation and regulation of late mRNA accumulation (Öhman *et al.*, 1993)(section 1.6.10 on page 41). All late viral messages have a common 201 base pair 5' untranslated leader sequence called the tripartite leader, which is composed of three exons. This leader has two forms, due to the presence or absence of the i-leader exon which is spliced between the second and third exons. In addition to the general stimulation of late mRNA accumulation, E4orf3 favours i-leader inclusion while E4orf6 facilitates i-leader omission (Nordqvist *et al.*, 1994). The functional importance of this difference is not known.

While E4orf3 was not believed to play any direct role in viral DNA replication (Bridge & Ketner, 1989), deletion of both E4orf3 and E4orf6 results in irregularities in viral DNA synthesis, and leads to the formation of viral genome concatamers (Weiden & Ginsberg, 1994). This suggests that these E4 products may play some accessory role in maintaining genomic integrity during the multiple rounds of viral DNA replication.

As mentioned, E4orf3 is nuclear (Sarnow *et al.*, 1982c) and has been found to reorganize PML, a nuclear matrix associated protein from spherical nuclear bodies, termed PODs to fibrous structures (Carvalho *et al.*, 1995; reviewed in Lamond & Earnshaw, 1998). E4orf3 co-localizes with PML in these fibers, and E1A, through the CR2 domain, is also found co-localized with PML (Carvalho *et al.*, 1995). In the process of this reorganization, E4orf3 disrupts the PODs, releasing a number of other POD proteins which relocate to the viral inclusion bodies where viral replication and late transcription occur (Doucas *et al.*, 1996). Interestingly, other DNA tumour viruses also target PODs, so this E4orf3 function may be of general significance (Carvalho *et al.*, 1995). E4orf3 has also been shown to associate with E1B-55K, and to relocalize it to the PODs (König *et al.*, 1998). This interaction disrupts the E1B-55K / p53 complex, relieving p53 inhibition, but it is only apparent when E4orf6 is not present (König *et al.*, 1998). When expressed, E4orf6 preferentially interacts with E1B-55K, and p53 activity is inhibited, but under certain conditions, E4orf3 may cause a temporary liberation of p53 to fulfil some role in viral DNA replication, since p53 is found in the viral replication centers when E4orf6 is absent (König *et al.*, 1998).

1.6.8.4 E4orf3/4

E4orf3/4 is completely uncharacterized. Disruption of this coding sequence from the virus does not result in any obvious aberration in replication in tissue culture (Halbert *et al.*, 1985; Bridge & Ketner, 1989). It is a small protein of 7.1 kd composed of 33 aa from the amino-terminus of E4orf3 and 27 aa from the carboxy-terminus of E4orf4.

1.6.8.5 E4orf4

E4orf4 produces a 114 aa protein of 14 kd. The activity of E4orf4 was first revealed following from some work on the synergistic activation of the AP-1 transcription factor (c-Fos and JunB, reviewed in Karin *et al.*, 1997) by E1A and cAMP (Engel *et al.*, 1988; 1991; Müller *et al.*, 1989). The combination of adenovirus infection together with cAMP addition was observed to decrease the phosphorylation levels of both E1A and c-Fos, and this dephosphorylating activity was mapped to E4orf4 (Müller *et al.*, 1992). This hypophosphorylation resulted in a decrease in AP-1 transcriptional activity, which was not observed in E4orf4 minus viral mutants (Müller *et al.*, 1992). The kinetics of this activity implied that E4orf4 was acting to inhibit a cellular kinase which was involved in the phosphorylation of E1A and c-Fos, and E4orf4 was additionally shown to suppress the protein levels of JunB (through a transcriptional mechanism) and c-Fos (at both transcriptional and translational control points). One further finding from this work was that E4orf4 minus viral mutants were seemingly more cytotoxic than *wt* virus, suggesting that E4orf4 serves a protective role (see Chapter 4 on page 112). As mentioned previously, AP-1 is involved in the activation of a number of early viral genes including E1A and E4 (Engel *et al.*, 1988), indicating that E4orf4 is involved in downregulating viral promoters. This negative feedback loop apparently limits the production of one or more toxic early viral proteins.

An insight into the mechanism of action of E4orf4 came with the discovery that it stably associates with the B α subunit of the cellular protein phosphatase 2A (PP2A; reviewed in Mumby & Walter, 1993) leaving the holoenzyme—composed of A, B α and C subunits—intact (Kleinberger & Shenk, 1993). This work led to a model in which E4orf4 was activating PP2A which was then inactivating a kinase required to stimulate AP-1 activity, and in support of this concept, PP2A overexpression alone was seen to inhibit AP-1 activity in these studies. PP2A has been reported to antagonize the MAP kinase pathway

which may be involved in activating AP-1 (Anderson *et al.*, 1990; Gomez & Cohen, 1991; Jaiswal *et al.*, 1993; Braconi Quintaje *et al.*, 1996).

As has been mentioned previously (section 1.6.3.4 on page 14) the major transcription factor involved in activating the E4 promoter is not AP-1, but is rather E4F (Raychaudhuri *et al.*, 1987; Rooney *et al.*, 1990) and this transcription factor is also activated by E1A through a phosphorylation event (Raychaudhuri *et al.*, 1989). Additionally, the E4 promoter is responsive to the ATF family of transcription factors (Hai *et al.*, 1988) which can also be activated by phosphorylation via an indirect effect of E1A (Liu & Green, 1990; Martin *et al.*, 1990). Experiments looking at the inhibition of the E1A and E4 promoters by E4orf4 has shown strong repression, which can be relieved by the addition of okadaic acid (Bondesson *et al.*, 1996), a well characterized inhibitor of PP2A (Cohen *et al.*, 1990). This suggests that other transcription factors—likely including E4F—may also be targets of the E4orf4 / PP2A complex, but this idea has not been examined directly. Furthermore, there is evidence that the E4orf4 / PP2A-induced dephosphorylation of E1A (Müller *et al.*, 1992) may be involved in decreasing the ability of E1A to activate the E4 promoter (Whalen *et al.*, 1997). Most likely, a number of these inhibitory dephosphorylation events are triggered by the E4orf4 / PP2A complex to suppress E4 expression.

In addition to its effect on viral transcription, E4orf4 is also involved in regulating splicing of mRNAs late in infection. Through dephosphorylation, the E4orf4 / PP2A complex inactivates SR proteins (Kanopka *et al.*, 1998) which are an essential group of splicing factors that are involved in spliceosome assembly and recognition of splice sites (Zahler *et al.*, 1992). At early stages of viral growth, the SR proteins bind to an intronic repressor element and prevent usage of a secondary splice site in the L1 unit (Kanopka *et al.*, 1996). This alteration in SR protein activity by E4orf4 appears to be involved in the temporal shift in the L1 viral pre-mRNA splicing patterns, and has offered a valuable insight into the regulation of splicing by phosphorylation in mammalian cells.

Recent work has linked E4orf4 to the induction of p53-independent apoptosis (Marcellus *et al.*, 1998; Lavoie *et al.*, 1998; Shtrichman & Kleinberger, 1998) and this observation will be extended in Chapters 4 (page 112) and 5 (page 135).

1.6.8.6 E4orf6

E4orf6 is a 34 kd, 294 amino acid nuclear protein (Cutt *et al.*, 1987) that is found predominantly in a complex with the E1B-55K protein (Sarnow *et al.*, 1984). As mentioned

in the section on E4orf3 (section 1.6.8.3 on page 36) both E4orf3 and E4orf6 / E1B-55K are involved in parallel pathways that stimulate expression from the late viral transcription unit (Bridge & Ketner 1989; Huang & Hearing, 1989a; Ketner *et al.*, 1989; Bridge & Ketner, 1990) as well as regulate splicing patterns from the major late promoter (Öhman *et al.*, 1993; Nordqvist *et al.*, 1994). Unlike E4orf3, however, the E4orf6 / E1B-55K complex is additionally required for the efficient transport of viral mRNAs from the nucleus to the cytoplasm in the late phase of viral replication (Babiss *et al.*, 1985; Halbert *et al.*, 1985; Pilder *et al.*, 1986; Weinberg *et al.*, 1986; Leppard & Shenk, 1989; Bridge & Ketner, 1990). The complex of E1B-55K and E4orf6 also plays an important role in host cell shut-off, which in part involves a block in transport of cellular mRNAs to the cytoplasm (section 1.6.11 on page 43). In human cells, the presence of E4orf6 localizes E1B-55K to distinct nuclear viral inclusion bodies, where viral replication and transcription are thought to occur (Ornelles & Shenk, 1991; Goodrum *et al.*, 1996). This complex has been shown to shuttle back and forth between these inclusion bodies and the cytoplasm, and may act in this fashion to transport the viral mRNAs out of the nucleus (Dobbelstein *et al.*, 1997). E4orf6 contains both nuclear import and export signals, and utilizes the same nuclear export pathway as the retroviral proteins rev and rex (Dobbelstein *et al.*, 1997). Additionally, E4orf6 contains a nuclear retention signal, made up of an amphipathic alpha-helix, but its activity appears to be inhibited by the interaction with E1B-55K (Dobbelstein *et al.*, 1997). Mutational analysis has demonstrated that the N-terminal 55 amino acids of E4orf6 are sufficient to bind to the central region of E1B-55K directly (Rubenwolf *et al.*, 1997).

As previously discussed, E1B-55K is involved in negatively regulating the activity of p53 (section 1.6.5.3 on page 27). E4orf6 has now also been shown to interact with p53 independently and suppress its transactivational activity (Dobner *et al.*, 1996). E4orf6 binds to the C-terminal regulatory domain of p53 (Dobner *et al.*, 1996;) while E1B-55K binds to the N-terminal activation domain (Kao *et al.*, 1990; Yew & Berk, 1992; Lin *et al.*, 1994), raising the possibility that the three proteins exist in a complex. In isolation, E4orf6 can function to block p53-induced apoptosis, and will cooperate with E1A to transform cells (Moore *et al.*, 1996; Nevels *et al.*, 1997). Additionally, the E1B-55K / E4orf6 complex reduces the half-life of p53, substantially decreasing its protein level in the cell (Moore *et al.*, 1996; Nevels *et al.*, 1997; Querido *et al.*, 1997a). This function requires that both E1B-55K and E4orf6 retain the ability to bind to each other as well as to p53 (Roth *et al.*, 1998; Rubenwolf *et al.*, 1998). E4orf6 has been seen to coimmunoprecipitate with a number of other viral and cellular proteins in addition to E1B-55K and p53, but they still have not been identified (Boivin *et al.*, 1999).

1.6.8.7 E4orf6/7

E4orf6/7 produces a protein product of 19.5 kd that is created by splicing the coding region for the N-terminal 58 aa of E4orf6 onto the E4orf7 reading frame (Cutt *et al.*, 1987). During an adenovirus infection, the E1A-induced activation of the E2 promoter via E2F is greatly assisted by E4orf6/7 (section 1.6.3.4 on page 14) (Hardy & Shenk, 1989; Huang & Hearing, 1989b; Marton *et al.*, 1990; Raychaudhuri *et al.*, 1990; Neill *et al.*, 1990; 1991). Two E4orf6/7 proteins bind directly to two E2F-1 / DP-1 heterodimers on the adenovirus E2a promoter and induce cooperative and stable binding to the DNA (Obert *et al.*, 1994). The E4orf6/7 interaction requires correctly spaced and inverted E2F sites, found in the E2a promoter, but which differ in spacing from the known cellular promoters (Hardy & Shenk, 1989). The E4orf6/7 induced activation of the E2 promoter is not needed in rapidly growing lines such as HeLa cells, but this function is presumably much more important in the differentiated, growth-arrested cells that the virus would normally infect (Hemström *et al.*, 1991).

1.6.9 Replication of Viral DNA

Once E1A has stimulated the cell to enter S phase and the early gene products have accumulated, the virus is ready for DNA replication (reviewed in Challberg & Kelly, 1989; Stillman, 1989). Replication is first seen approximately six hours after HeLa cells have been infected and continues until cell death. The adenovirus genome was the first eukaryotic DNA to be replicated *in vitro* (Challberg & Kelly, 1982) and these cell-free studies have been instrumental in constructing the existing 2-stage model for adenovirus replication (Lechner & Kelly, 1977).

In the first stage of viral DNA replication, synthesis is initiated at either end of the linear viral genome, and proceeds in a semi-conservative manner to the other end of the chromosome. This process produces a DNA duplex with a daughter and parental strand, and a displaced parental single-stranded DNA molecule. In the second stage, the single-stranded DNA circularizes by annealing its homologous ITRs (section 1.5 on page 6) to produce a panhandle structure with a terminus that looks exactly like that found on either end of a duplex genome. The same replication apparatus then initiates on this template to produce a linear DNA duplex.

The viral origins of replication are located in the terminal 51 bp of each ITR and have been divided into three domains. The terminal 18 bp comprise Domain A which is the

minimal origin of replication (Challberg & Rawlins, 1984; Lally *et al.*, 1984; Tamanoi & Stillman, 1983; van Bergen *et al.*, 1983). The cellular protein ORP-A binds in the first 12 bp (Rosenfeld *et al.*, 1987), and a complex of the E2-pTP and the E2-DNA polymerase binds to a highly conserved stretch from 9 to 18 bp (Chen *et al.*, 1990; Mul & Van der Vliet, 1992; Temperley & Hay, 1992). Domains B and C are located from 19 to 39 bp and from 40 to 51 bp respectively, and while not absolutely essential for replication, they enhance the efficiency of initiation. Domain B contains a binding site for a cellular transcription factor—nuclear factor I (NFI; Jones *et al.*, 1987). The E2-DBP recruits NFI (Cleat & Hay, 1989; Stuiver & van der Vliet, 1990) and NFI then stabilizes the E2-pTP / E2-DNA polymerase complex at the origin (Bosher *et al.*, 1990; Chen *et al.*, 1990; Mul *et al.*, 1990; Mul & van der Vliet, 1992). A second cellular transcription factor known as nuclear factor III (NFIII) or Oct 1 (O'Neill *et al.*, 1988; Pruijn *et al.*, 1989) binds to Domain C. The mechanism of action of NFIII is not known, though it induces DNA bending which may be involved in initiation (Verrijzer *et al.*, 1991).

At the start of replication, the E2-pTP / E2-DNA polymerase complex binds to the DNA duplex in Domain A (Temperley & Hay, 1992). The complex is then thought to open up the duplex and then associate with ssDNA (Chen *et al.*, 1990; Kenny & Hurwitz, 1988). At this point the E2-pTP protein becomes covalently linked with the first residue at the 5' end of the DNA, via an ester bond between the β -OH of a serine and the α -phosphoryl group of dCMP (Challberg *et al.*, 1980; Lichy *et al.*, 1981). The 3'-OH group of the E2-pTP/dCMP unit is then used to prime DNA synthesis of the daughter strand by the polymerase. The E2-pTP also acts to maintain the integrity of the genomic ends during the many rounds of replication (Rekosh *et al.*, 1977). For chain elongation the virus requires the E2-DNA polymerase, the E2-DBP, and a cellular DNA topoisomerase, nuclear factor II (NFII; Nagata *et al.*, 1983). The E2-DBP coats and stabilizes the ssDNA produced during replication to greatly increase the processivity of the polymerase (Field *et al.*, 1984; Lindenbaum *et al.*, 1986). NFII is probably involved in resolving structural problems with the viral chromosome.

1.6.10 Late Gene Expression

Late gene expression is activated by the onset of viral DNA replication. The late viral coding region is expressed as a single primary transcript of about 29 kb in length (Evans *et al.*, 1977; Nevins & Darnell, 1978). The combination of splicing and the use of different polyadenylation (poly(A)) sites results in the production of at least 18 distinct mRNAs (see

Figure 1-2 on page 7). These mRNAs have been divided into 5 families, termed L1 to L5, depending on which poly(A) site was utilized (Chow *et al.*, 1977b; Nevins & Darnell, 1978; Ziff & Fraser, 1978). The major late promoter (MLP) controls the expression of this region. Early in infection the MLP has very low activity, but it increases dramatically during the late phase of growth (Shaw & Ziff, 1980).

There are at least two major occurrences that lead to the activation of the MLP. Firstly, there is a *cis*-acting change in the viral chromosome that enhances late gene expression (Thomas & Mathews, 1980). The mechanism behind this chromosomal change is not understood; however, after replication the viral DNA associates with a distinct group of proteins compared to unreplicated DNA (Chatterjee *et al.*, 1986; Dery *et al.*, 1985), suggesting that the chromatin is structurally different after replication. The MLP may become more accessible to transcription factors after the viral DNA has been opened up and disassociated from the viral core proteins during replication. One transcription factor named USF or MLTF (Carthew *et al.*, 1985; Miyamoto *et al.*, 1985; Sawadago & Roeder, 1985) is found associated with the MLP after the onset of DNA replication (Toth *et al.*, 1992), though its presence does not appear to be absolutely essential (Reach *et al.*, 1990; 1991).

The second component leading to MLP stimulation is the delayed early gene IVa2. The IVa2 protein product binds downstream of the MLP start site in the region +85 to +120 where it cooperates with the upstream USF/MLTF element to activate transcription (Leong *et al.*, 1990; Mansour *et al.*, 1986; Mondesert & Keding, 1991; Mondesert *et al.*, 1992; Tribouley *et al.*, 1994). The IVa2 gene is a delayed early gene because its promoter is initially repressed by an unknown factor and this repression is thought to be relieved by a viral early protein (Chen *et al.*, 1994). The combination of the delayed production of the IVa2 product and the requirement for cellular transcription factors that only bind the replicated viral genome likely provide much of the temporal regulation of the MLP. One further check against early expression from the MLP is premature termination of transcription by RNA polymerase II (Nevins & Wilson, 1981). Only late in infection will the polymerase transcribe through all of the late coding region (Fraser *et al.*, 1979). Even at late times, however, there are many premature terminations at different sites in the late region (Evans *et al.*, 1979, Fraser *et al.*, 1979b, Seiberg *et al.*, 1987). The mechanism behind these premature terminations is unknown, but it likely involves the viral chromatin structure.

1.6.11 Regulation of Host Protein Synthesis

With the onset of viral DNA replication, and the shift to late gene expression, the virus initiates a block in host protein synthesis (Beltz & Flint, 1979). This process, often termed host cell shut-off, likely functions to maximize the rate at which the virus can make the structural proteins required for virus assembly. The underlying mechanisms fall into a number of categories: (1) the selective transport of viral mRNAs, coupled with a block in cellular mRNA transport; (2) preferential translation of viral messages through the inhibition of host mRNA translation; (3) specific activation of viral protein synthesis.

1.6.11.1 Selective Transport of Viral mRNAs

The virus uses two proteins—E1B-55K and E4orf6—to carry out the double task of efficiently shuttling late viral mRNAs from the nucleus to the cytoplasm while blocking the transport of cellular mRNAs (Beltz & Flint, 1979; Babiss & Ginsberg, 1984; Babiss *et al.*, 1985; Halbert *et al.*, 1985; Pilder *et al.*, 1986; Weinberg & Ketner, 1986; Bridge & Ketner, 1990; Dobblestein *et al.* 1997). These two nuclear proteins (Cutt *et al.*, 1987; Sarnow *et al.*, 1982b) function in a complex (Sarnow *et al.* 1984) which appears to be located in viral transcription centers (Ornelles & Shenk 1991). The synthesis and processing of cellular mRNAs continues in the nucleus, but their transfer to the cytoplasm is blocked immediately prior to transport (Leppard & Shenk, 1989). This block is not based on the identity of the mRNAs (Gaynor *et al.* 1985; Hearing & Shenk 1985) but rather seems to depend on where they were transcribed. The current model is that the E1B-55K / E4orf6 complex sequesters a limiting mRNA transport factor for use in shuttling the viral mRNAs, leaving the cellular mRNAs trapped in the nucleus (Ornelles & Shenk 1991). Recent work has identified an RNA binding protein (a novel member of the hnRNP family) that interacts with E1B-55K (Schütt *et al.*, 1998) and this may be the unknown mRNA transport factor. E1B-55K and E4orf6 also have intrinsic, but non-specific, mRNA binding ability, which may be involved in this process (Leppard & Horridge, 1998).

1.6.11.2 Preferential Translation of Viral mRNAs

Once the viral late mRNAs have been selectively transported to the cytoplasm they are also preferentially translated, while host mRNAs are excluded (Babich *et al.*, 1983; Yoder *et al.*, 1983; reviewed in Zhang & Schneider, 1993). The block in cellular mRNA translation is not

dependent on the inhibition of transport as even cellular messages that make it to the cytoplasm are not translated (Moore *et al.* 1987). Furthermore, this inhibition does not involve mRNA degradation (Thimmappaya *et al.*, 1982). For assistance, the virus makes use of two cellular regulatory mechanisms to inhibit cellular mRNA translation. The first is a cellular anti-viral defense strategy. During adenovirus replication, double-stranded RNA accumulates and this activates the cellular protein kinase R (PKR) (O'Malley *et al.*, 1986; Maran & Mathews, 1988). Once stimulated, PKR phosphorylates and inactivates the translation initiation factor eIF-2 α , which results in a translation block (de Haro *et al.*, 1996). As expected, cells lacking PKR do not exhibit this phenotype in response to adenovirus infection (Huang & Schneider, 1990; O'Malley *et al.*, 1989). To allow viral mRNA translation, Ad5 codes for two copies of a small RNA called VA RNA, which associate with viral messages (Mathews, 1980) and block PKR activation (O'Malley *et al.*, 1986). The VA RNAs are believed to stimulate viral protein production selectively because they are localized to viral translational centers.

Another cellular response to viral infection results in the dephosphorylation and inactivation of eIF-4F during the late phase of viral growth (Huang & Schneider, 1991). This translation initiation factor binds to the mRNA cap and uses its helicase activity to remove secondary structures from the 5' untranslated region (UTR) of the mRNA allowing the 40S ribosome to scan for the initiator AUG codon (reviewed in Jackson & Wickens, 1997). Viral late messages are unaffected by its inactivation, however, because they all contain a 201 nucleotide 5' noncoding sequence termed the tripartite leader (Berget *et al.*, 1977). This region is important for translation at late times during infection (Logan & Shenk 1984; Berkner & Sharp 1985) and because, unlike the 5' UTRs of cellular mRNAs, it is completely free of secondary structure (Dolph *et al.*, 1990), the 40S ribosome is thought to be able to scan for the AUG without the eIF-4F helicase activity (Huang & Schneider, 1991).

1.6.11.3 Stimulation of Viral Translation

The final method the virus utilizes for selective translation of viral mRNAs involves a 100 kd protein encoded from the L4 family of late mRNAs (Hayes *et al.*, 1990). The L4-100 kd protein binds mRNA nonspecifically (Adam & Dreyfuss, 1987; Riley & Flint, 1993) and mutant analysis indicates that its role is to promote efficient translation of viral products rather than to inhibit host mRNA translation (Hayes *et al.*, 1990).

1.6.12 Virion Assembly and Release

The combination of viral DNA replication and the large-scale production of structural proteins leads to virion assembly (reviewed in Philipson, 1984). The L4-100 kd protein is instrumental in this process (Liebowitz & Horwitz, 1975; Oosterom-Dragon & Ginsberg, 1981) as it forms a multimeric complex which binds hexon monomers after their production in the cytoplasm (Cepko & Sharp, 1982) and acts as a scaffold to facilitate the formation of trimeric hexon capsomers (Horwitz *et al.*, 1969). Penton base monomers also assemble into their final pentameric form in the cytoplasm then associate with the independently assembled trimeric fiber (Horwitz *et al.*, 1969; Velicer & Ginsberg, 1970). After assembly of the penton and hexon capsomers, they concentrate in the nucleus and form empty capsids (Sundquist *et al.*, 1973). The viral chromosome is inserted into the capsid through a DNA-capsid recognition event which relies on a packaging signal that is centered 260 bp from the left end of the genome (Grable & Hearing, 1992; Hammarskjöld & Winberg, 1980; Hearing *et al.*, 1987; Tibbetts, 1977). Capsid construction is dependent upon the presence of this genomic packaging signal, suggesting that the DNA interaction is involved in assembly (Hasson *et al.*, 1992; Hearing & Shenk, 1983). The L1-52/55 kd phosphoprotein assists in the encapsidation process by acting as a scaffold protein (Hasson *et al.*, 1992; Hasson *et al.*, 1989) while the L3-coded cysteine protease functions at a late stage of virion assembly to cleave a number of viral proteins, stabilizing the structure and making the virus infectious (Mangel *et al.* 1993; Tihanyi *et al.*, 1993; Webster *et al.*, 1993).

Viral release is not currently very well characterized. Adenovirus is not a typical lytic virus, but it rather seems to cause a breakdown of the cellular cytoskeleton, leading to a form of cell death that resembles apoptosis. Early in infection, virus entry (Belin & Boulanger, 1987) and E1B-19K expression (White & Cipriani, 1989; 1990) induce the cleavage of vimentin, which causes this intermediate filament system to collapse into the perinuclear region (Defer *et al.*, 1990; Zhai *et al.*, 1988). In the late stages of infection the L3 viral protease cleaves the cellular cytokeratin K18, disrupting its polymerizing activity, and further destroying the intermediate filament network (Chen *et al.*, 1993). Other targets of the L3 protease are not yet known. As was mentioned earlier, the E3-11.6 kd protein is required for efficient nuclear membrane disruption, though its mechanism is undetermined (see 1.6.7.4 on page 33) and as will be discussed in Chapter 4, evidence is starting to accumulate, suggesting that the virus may continue this cell breakdown by inducing apoptosis in the host cell. This would have the distinct advantage of allowing

spread in a manner that would cause a minimal inflammatory response, attracting little attention from the immune system.

1.7 *Adenovirus and Transformation*

1.7.1 Transformation

The oncogenic properties of adenoviruses (and the other DNA tumour viruses) arise from the necessity to induce DNA synthesis in the host cell, to make the cellular replication machinery available for the virus (reviewed in Nevins & Vogt, 1996). A normal viral infection results in the death of the host cell, so the only time this viral property is revealed is in infections of non-permissive rodent cells, or in artificial situations where selected viral genes are introduced into cells. Adenoviruses have never been found to be linked to human tumours (Mackey *et al.*, 1976); however, all serotypes tested can induce morphological transformation of rodent cells in culture (McBride & Weinart, 1964; Freeman *et al.*, 1967; Castro, 1968; Gilden *et al.*, 1968; McAllister & Macpherson, 1968; McAllister *et al.*, 1969a; 1969b) and viruses from the highly oncogenic class A such as Ad12 can induce tumours when injected into newborn hamsters (Trentin *et al.*, 1962). The difference between the highly and weakly oncogenic viruses is likely just a matter of immune system evasion and does not seem to reflect a difference in growth promoting potential (Cook & Lewis, 1979).

The identification of the adenoviral genes retained in transformed cells revealed that the left end of the genome was always present (Doeffler 1968; Gallimore *et al.*, 1974; Sharp *et al.*, 1974). The development of a technique to introduce DNA into cells in a CaPO_4 precipitate led to the verification that the transforming function of the virus resides in this region (Graham & van der Eb 1973a; 1973b; Graham *et al.*, 1974). Adenoviral DNA does not integrate into the cellular genome with any specificity, leading to poor transformation efficiency, with a frequency of less than 10^{-5} . The transforming region of the virus encompasses about 10% of the genome and was found to contain two distinct transcription units—E1A and E1B (Berk & Sharp, 1977).

As has been discussed previously, E1A has two main functions in viral replication: the transactivation of the early viral genes, and the stimulation of the cell cycle. The transforming function of adenovirus only requires the growth inducing properties of E1A, namely the modulation of the Rb (section 1.6.4.2 on page 20) and p300 families (section 1.6.4.3 on page 24) via the N-terminus, CR1 and CR2 (Moran *et al.*, 1986; Lillie *et al.*, 1986;

1987; Kuppuswamy & Chinnadurai, 1987; Schneider *et al.*, 1987; Subramanian *et al.*, 1988; Whyte *et al.*, 1988b; Jelsma *et al.*, 1989; Howe *et al.*, 1990; Svensson *et al.*, 1991). E1A mutants that deregulate only one of the two pathways are quite defective for transformation, and this effect likely echoes previous viral analyses which demonstrated that these mutants could induce entry into S phase, but could not push the cell past the G2 checkpoint (Egan *et al.*, 1988; 1989; Whyte *et al.*, 1988b; 1989; Jelsma *et al.*, 1989; Howe & Bayley, 1992).

Alone E1A can immortalize some rodent cell types, but it cannot fully transform cells (Dijkema *et al.*, 1979; Houweling *et al.*, 1980). E1A must be expressed together with the E1B region or an activated *ras* gene to fully transform primary rodent cells (Ruley, 1983; Franza *et al.*, 1986). This failure to transform is a consequence of the extreme toxicity of E1A which is the result of apoptosis (Rao *et al.*, 1992; Debbas & White, 1993; Lowe & Ruley, 1993) (section 1.6.5.1 on page 26). Both of the E1B proteins can cooperate with E1A to produce transformed cells, but the process is more efficient with both (Bernards *et al.*, 1983; Barker & Berk, 1987; McLorie *et al.*, 1991; White & Cipriani, 1990). As discussed, the E1B proteins block the cellular apoptotic response that would otherwise be induced by E1A (section 1.6.5.3 on page 27) (section 1.6.5.4 on page 29), allowing the cells to become transformed.

Interestingly, adenovirus is very poor at transforming human cells when compared to rodent cells. This is not simply a matter of permissivity of virus replication, since even cloned E1A and E1B genes show this effect. The commonly used 293 human kidney epithelial line expresses the viral E1 region, and functions well in complementing for E1A and E1B deficiencies for growing viruses, but it does not exhibit much of a transformed phenotype (Graham *et al.*, 1977). This line has also proven to be very hard to duplicate. The difference between rodent and human cells that gives rise to this variation in transforming efficiency is not known. While rodent and human cells contain analogous proteins that interact with E1A and E1B, human cells must contain extra safeguards that prevent long-term growth promotion. The characterization of these differences should provide us with knowledge of an additional layer of growth controls beyond our current understanding.

1.8 Therapeutic Adenovirus Use

Clinically adenoviruses are primarily thought of as the etiological agents behind a number of respiratory syndromes, and some forms of conjunctivitis and gastroenteritis. Recently, however, the virus has also been gaining attention due to its direct potential in disease treatment. Adenoviruses have the ability to infect many human cell types, and this coupled

with our ability to easily modify their genomic structure, has resulted in a very effective viral vector for vaccines and gene therapy work. Secondly, encouraging results have arisen from the use of a viral mutant that replicates selectively in cancer cells. Thirdly, because viral proteins are highly evolved to modulate specific processes in the host cell, exploiting these proteins directly, or mimetics based on them, is adding another angle to drug development.

1.8.1 Vaccines and Gene Therapy

Adenovirus vectors are very useful tools for the introduction of heterologous genes into mammalian cells. They have been used in this fashion for many years in basic research, and are more recently also being used clinically (Mulligan, 1993). Adenoviruses have a number of advantages over other potential viral vectors: (1) virus replication is not dependent on the initial growth state of the infected cell; (2) the virus efficiently infects many human cell types; (3) vectors can be designed to be replication competent or defective as desired; (4) adenoviruses can be grown to high titres; (5) there is a large cloning capacity for heterologous inserts; and (6) millions of people have been safely immunized with adenoviruses.

For vaccines, replication competent vectors are normally used to enhance the amount of immunogen to which the patient is exposed. The E3 region of the virus (section 1.6.7 on page 31) is dispensable in tissue culture, and can be deleted to provide room for the heterologous sequences. Numerous genes have been inserted into adenoviral vectors for vaccination including: hepatitis B surface and core antigens (Morin *et al.*, 1987), herpes simplex gB glycoprotein (McDermott *et al.*, 1989), rabies virus gP glycoprotein (Prevec *et al.*, 1989), respiratory syncytial virus F protein (Collins *et al.*, 1990), and HIV-1 gag, env and p24 genes (Dewar *et al.*, 1989)(reviewed in Grunhaus & Horwitz, 1992). These genes are normally expressed to very high levels in adenovirus vectors due to the presence of strong upstream promoters, such as the MLP (section 1.6.10 on page 41). For booster immunizations, different viral serotypes can be used with identical inserts, to avoid a strong host immune response directed against the vectors (Hse *et al.*, 1991).

For gene therapy use, replication defective viral vectors are used for gene delivery (reviewed in Kovesdi *et al.*, 1997). Vectors with the E1 region deleted are unable to express viral genes effectively, and consequently only the transgene is expressed. To increase the cloning capacity, the E1 deletion can be combined with deletions in other viral regions, as

long as the appropriate complementing cell lines are used to provide the essential viral functions for vector production. One of the first clinical uses for these gene therapy vectors was the introduction of the normal allele of the gene that is defective in cystic fibrosis (CFTR) to correct the pulmonary defect in these patients (Rosenfeld *et al.*, 1992; Zabner *et al.*, 1993). Adenovectors have also been used to reintroduce the tumour suppressor p53 into p53 null cancer cells (reviewed in Breau & Clayman, 1996).

There are still some issues which need to be addressed with adenovectors. The most important issue is the short duration of transgene expression. The adenovirus genome does not integrate into the host chromosome, and is eventually cleared by the cell, sometimes rapidly. Some cell types, such as muscle, have been seen to provide long-term expression, but much work is still to be done in this area. Problems with cloning capacity are currently being resolved. The first generation vectors with only E1 deletions (pJM17; McGrory *et al.* 1988) had a maximum insert size of around 5 kb; however, the second generation vectors with E1/E3 (pBHG11; Bett *et al.*, 1994) or E1/E4 (Krougliak & Graham, 1995; Brough *et al.*, 1996; Gao *et al.*, 1996; Dedieu *et al.*, 1997; He *et al.*, 1998) double deletions can handle greater than 11 kb of foreign sequences, which is sufficient for most applications, and the latest “gutless” constructs can hold 35 kb, almost the full adenovirus genome in size (Haecher *et al.*, 1996; Lieber *et al.*, 1996; Chen *et al.*, 1997). A final issue is the activation of the host immune response, which can further reduce the persistence of the vector. This is especially problematic in E3 deleted vectors, because this region is involved in down-regulating the host immune response (section 1.6.7 on page 31)(Ali *et al.*, 1994). To get around this problem, the E3 region is being left in some vectors, but others are taking the opposite approach and using the gutless vectors which lack all viral genes, offering the promise of a weak immune response. In practice, however, there are conflicting reports regarding the efficacy of the gutless vectors (Kaplan *et al.*, 1997).

1.8.2 Cancer Treatment using Replicating Adenovirus

While attention was focused on the use of adenoviruses for gene therapy, a completely novel, viral-based therapy has emerged in the field of oncology (Bischoff *et al.*, 1996). Unlike a gene therapy approach to cancer, where defective viruses are used to deliver a heterologous anti-tumour gene to a cell, this unconventional approach taken by ONYX Pharmaceuticals Inc. uses a replication competent adenovirus which selectively grows in tumour cells. The conceptual basis behind this novel therapy involves the cellular tumour suppressor p53, and the necessity during viral replication to inactivate its apoptosis-

inducing function. The ONYX virus (ONYX-015, originally *dl* 1520) is a mutant which has an E1B-55K deletion (Barker & Berk, 1987). This virus is replication defective in most normal cells, because p53 is able to block the viral replication cycle, possibly through cellular growth arrest, even though E1B-19K inhibits p53-induced apoptosis. The selectivity of ONYX-015 derives from the fact that over 50% of all human tumours have a defect in p53 function (Brennan *et al.*, 1995; Chang *et al.*, 1993; 1995; Bergh *et al.*, 1995). In many p53 defective cells, ONYX-015 can grow relatively normally because the growth arresting function of p53 is not present to inhibit viral replication (Bischoff *et al.*, 1996; Heise *et al.*, 1997). In this situation, ONYX-015 replication should kill the infected p53 null tumour cells, allowing spread to surrounding tumour cells, until the tumour is cleared. The infection of normal cells should be unproductive. The utility of this p53-independent strategy is great because much conventional cancer chemotherapy relies on intact p53 functionality (Lowe *et al.*, 1993a; 1994; Fujiwara *et al.*, 1994) which is often not present in tumour cells. Additionally ONYX-015 is benign to normal cells, while chemotherapy can be quite toxic.

ONYX-015 has done well in Phase I and II trials for head and neck tumours, and Phase III trials will be starting soon. Additional trials are underway for liver, pancreatic, and ovarian cancers. While this approach has broken new conceptual ground, problems do exist with the current ONYX-015 virus. E1B-55K has multiple roles in viral replication and a complete deletion of this gene is not ideal. A selective mutation in E1B-55K which disrupted the ability of E1B-55K to inhibit p53 activity, while retaining its other activities, would be preferable, and would permit much more efficient growth of the virus in tumour cells. In addition, the E4orf6 protein also inhibits p53 activity (see 1.6.8.6 on page 38) potentially allowing growth in some normal cell types. Deficiencies in the ONYX-015 virus do not detract from this overall approach though. ONYX is working on improving their therapeutic adenovirus, and additionally, ONYX and others are trying this general strategy with different virus families.

1.8.3 Exploiting Individual Viral Proteins

Viral proteins have evolved to efficiently modulate many aspects of cell growth, apoptosis, and immune function. Taking advantage of these functions provides a rational approach to drug development, in which viral products, or mimetics based on them, can be used in disease treatment. Currently, the immunosuppressive activity of the E3 region is being retained in many adenovectors designed for gene therapy, and future research will

undoubtedly look at mimicking their activities for more general use. Another area that is drawing a lot of attention is the control of apoptosis by viral gene products. The E1B-55K and E1B-19 products could both find uses in diseases characterized by excessive apoptotic cell death, and the E3-11.6 kd and E4orf4 products might be employable to kill cancer cells that are resistant to other means of treatment.

1.9 Apoptosis

1.9.1 Introduction and Historical Observations

Apoptosis (or programmed cell death) is of fundamental importance to multicellular organisms. It is a mechanism for removing unwanted cells in an orderly directed fashion, that avoids both inflammation and damage to surrounding cells. Apoptosis plays an essential role in development and in the maintenance of the adult organism. Tissue modelling in embryogenesis, organ development, and cell number homeostasis, all involve apoptosis in otherwise healthy tissues, while the elimination of nonfunctional, abnormal, misplaced or virally-infected cells is equally important, when there are disturbances. Apoptosis must be tightly controlled as too much can lead to degenerative diseases, and deficiencies can lead to hyperplasia and cancer.

Apoptosis was likely first observed in 1885 as chromatin condensation and a gradual degradation and disappearance of nuclei in epithelial cells in regressing ovarian follicles (Flemming, 1885), and the basic concept of programmed cell death in embryology was first detailed as a specific, temporally-regulated occurrence during the development of an organism (Glucksmann, 1951). However, the distinction between apoptosis and necrosis was not made until 1965, when Kerr noted that ischemic damage to the liver resulted in the conversion of hepatocytes into small round cytoplasmic masses with traces of chromatin, that did not exhibit a necrotic histology (Kerr, 1965). Kerr went on to observe the formation of vesicles containing intact organelles and condensed chromatin (Kerr, 1969). The term apoptosis, from the Greek word depicting the process of leaf or petal shedding, was coined to describe this novel form of cell death (Kerr, 1971; Kerr *et al.*, 1972).

1.9.2 Apoptosis verses Necrosis

Apoptosis and necrosis differ in most respects (reviewed in Wyllie, 1987; Schwartzman & Cidlowski, 1993; Majno & Joris, 1995; Uchiyama, 1995; Bosman *et al.*, 1996; Cory & Adams, 1998; Granville *et al.*, 1998). At the tissue level, apoptosis is often observed in scattered isolated cells with a complete lack of immunological attention. At the cellular level, apoptosis is an active, ATP-consuming process (Eguchi *et al.*, 1997; Leist *et al.*, 1997) which is characterized by a morphological progression starting with the loss of cell-cell contacts, collapse of the cytoskeleton, cytoplasmic shrinkage, membrane blebbing, condensation of DNA, nuclear fragmentation, and break-up of the cell into membrane-bound vesicles (apoptotic bodies) containing intact organelles and nuclear fragments (Kerr *et al.*, 1972; Russell *et al.*, 1972). During apoptosis, DNA is destroyed by the activation of a specific DNase—CAD—which produces a characteristic DNA ladder (due to intranucleosomal cleavage) when the DNA is separated on an agarose gel (Enari *et al.*, 1998; Sakahira *et al.*, 1998; Wyllie, 1998). Finally, the apoptotic bodies are phagocytosed by surrounding cells and macrophages (Duvall *et al.*, 1985; Savill *et al.*, 1990).

In contrast, necrosis normally occurs in a contiguous group of cells, usually initiated by some sort of acute physical damage, and is accompanied by an inflammatory response. Necrosis is a passive process in which cells swell prominently due to the loss of selective membrane permeability, leading to membrane damage of organelles. This also results in swelling of the endoplasmic reticulum and mitochondria, polysome dissociation, and the eventual lysis of the plasma membrane, with the release of lysosomal hydrolases into the extracellular milieu. (Hawkins *et al.*, 1972; Kerr *et al.*, 1972; Farber & El-Mofty, 1975). The lysosomal disruption accelerates cell destruction, and causes non-specific protein and nucleic acid degradation, producing a DNA smear on gels (Kerr, 1971; Sun *et al.*, 1994). The nucleus swells, but remains relatively intact throughout much of the necrotic death process. Cellular debris is eventually cleared by the immune system.

1.9.3 Evolutionary conservation and *C. elegans*

The core underlying components of the apoptotic machinery were first determined genetically using the microscopic nematode worm, *C. elegans* (reviewed in Hengartner & Horvitz, 1994a). During development, 131 cells from a total of 1090 are functionally redundant and are eliminated by apoptosis. By observing the fate of these cells in *C. elegans* mutants, a dozen cell death (*ced*) genes were identified. Three of these genes are of

particular interest: *ced-3* and *ced-4* are required for cell death, while *ced-9* antagonizes the activity of the other two proteins (Ellis & Horvitz, 1986). *Ced-9* was found to be related to the mammalian Bcl-2 protein, a known inhibitor of cell death (section 1.9.4 on page 53), demonstrating that apoptosis is highly conserved from the nematode to humans (Vaux *et al.*, 1992; Hengartner *et al.* 1994b). *Ced-3* was characterized as a cysteine protease with aspartic acid specificity (caspase) and was found to be homologous to a mammalian protease—interleukin-1 β -converting enzyme (ICE/caspase-1)—the first link between the caspases and apoptosis (section 1.9.5 on page 57) (Yuan *et al.*, 1993). *Ced-4* was the last of the three nematode genes to be identified in a mammalian system. From work in *C. elegans*, it was known to physically associate with both *Ced-9* and *Ced-3*, linking the upstream protector to the downstream effector, and was required (together with ATP) for the activation of *Ced-3* function (Ellis & Horvitz, 1986; Shaham & Horvitz, 1996; Chinnaiyan *et al.*, 1997a; Seshagiri & Miller, 1997; Irmeler *et al.*, 1997; James *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997). Evidence for a mammalian *Ced-4* came with the finding that *in vivo* a Bcl-2 related protein (Bcl-X_L) associated with caspase-1, whereas *in vitro* they had no mutual affinity (Chinnaiyan *et al.*, 1997b). This was shortly followed by the biochemical isolation and cloning of a mammalian *Ced-4* homologue—apoptosis protease activating factor-1 (Apaf-1) (Liu *et al.*, 1996; Zou *et al.*, 1997). Apaf-1 exhibits areas of high homology with *Ced-4* and appears to function in a similar manner (section 1.9.7 on page 59). The *C. elegans* work has demonstrated that the primary apoptotic mechanism has high evolutionary conservation, though in mammalian cells, there are many additional layers of complexity.

1.9.4 The Bcl-2 Family

Bcl-2 was the first mammalian protein linked to apoptotic regulation (reviewed in Yang & Korsmeyer., 1996; White, 1996; Kroemer, 1997; Reed, 1997a) and was originally identified as an overexpressed protein in B-cell lymphomas, due to a t(14;18) chromosomal translocation (Pegoraro *et al.*, 1984; Bakhshi *et al.*, 1985; Tsujimoto *et al.*, 1985; Tsujimoto & Croce, 1986; Cleary *et al.*, 1986). Bcl-2 has been shown to block apoptosis induced by a diverse range of stimuli, and can cooperate with known oncogenes such as c-myc or adenovirus E1A to transform cells (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990; Rao *et al.*, 1992). Bcl-2 also plays an important role in blocking apoptosis during development, as Bcl-2 knockout mice exhibited severe immunodeficiency, due to a lack of B and T cells, and additionally had kidney defects (Nakayama *et al.*, 1993; 1994; Veis *et al.*, 1993; Kamada *et al.*, 1995).

Bcl-2 was found to belong to a family of proteins that function as integrators of a diverse array of positive and negative survival signals. Bcl-2, Bcl-X_L (Boise *et al.*, 1993), Bcl-w (Gibson *et al.*, 1996), A1 (Lin *et al.*, 1993), Bfl-1 (Choi *et al.*, 1995; D'Sa-Eipper *et al.*, 1996), and Mcl-1 (Reynolds *et al.*, 1994) are apoptosis inhibitors, while Bax (Oltvai *et al.*, 1993), Bak (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995), Bok/Mtd (Hsu *et al.*, 1997), Bik/NBK (Boyd *et al.*, 1995; Han *et al.*, 1996), Bid (Wang K. *et al.*, 1996), Bcl-X_s (Boise *et al.*, 1993), Hrk (Inohara *et al.*, 1997), Bad (Yang *et al.*, 1995), and Bim (O'Connor *et al.*, 1998) are death promoters (section Figure 1-6 on page 55). There are also a number of viral apoptosis inhibiting proteins that are related to Bcl-2: adenovirus E1B-19K (Rao *et al.*, 1992), Epstein Barr virus BHRF1 (Henderson *et al.*, 1993), African swine fever virus 5HL (Neilan *et al.*, 1993), herpes virus saimiri Orf16 (Smith, 1995), and human herpes virus 8 Bcl-2 (Russo *et al.*, 1996). The two opposing Bcl-2 sub-families form heterodimers, and appear to titrate out one another's functions, acting as a rheostat to determine the cell's fate (Oltavi *et al.*, 1993). Homodimers are formed by many family members as well, and this may be their functionally active form.

1.9.4.1 Bcl-2 Structure

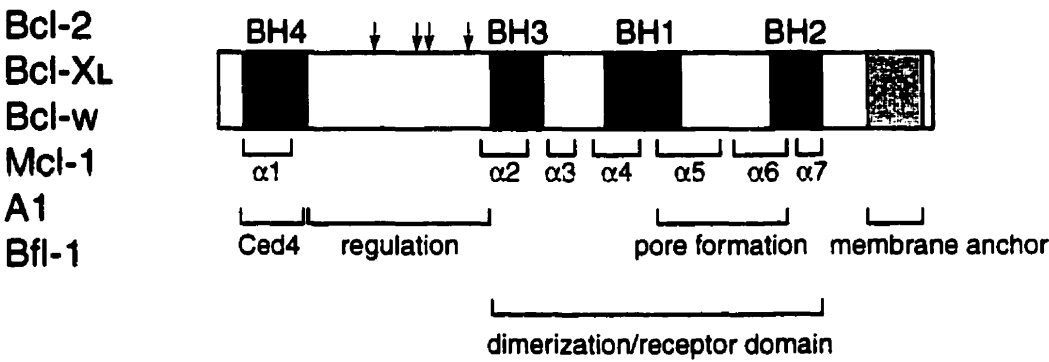
Among the Bcl-2 family, there are four Bcl-2 homology domains (BH1-BH4) with one or more domains in each member. All four regions are required for Bcl-2 to inhibit apoptosis. The BH1, BH2 and BH3 domains are involved in homo- and heterodimerization (Yin *et al.*, 1994) while BH4 is not (Huang *et al.*, 1998). Three death agonists—Bax, Bak and Bok—closely resemble Bcl-2, just lacking the BH4 domain, which only appears to be present in the anti-apoptotic family members. The other apoptosis promoters—Bad, Bid, Hrk, Bim, and Bik—just have a BH3 domain, and it is central to their function. The Bcl-2 family members are largely membrane-associated (with the exception of Bad and Bid). Bcl-2 localizes to the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear envelope (Baffy *et al.*, 1993; Lam *et al.*, 1994; Ryan *et al.*, 1994). The C-terminus of the membrane-associated members is highly hydrophobic, likely functioning as a membrane anchor, and has been demonstrated to be important in the case of Bcl-2 (Oltavi *et al.*, 1993; Nguyen *et al.*, 1994). Bax, however, appears to be cytosolic prior to an apoptotic stimulus, and only a fraction of Bcl-X_L is membrane-bound (Hsu *et al.*, 1997b; Hsu & Youle, 1997).

Structural studies with Bcl-X_L have indicated that the BH1, BH2 and BH3 regions create a hydrophobic cleft on the surface of the protein (Muchmore *et al.*, 1996) to which a

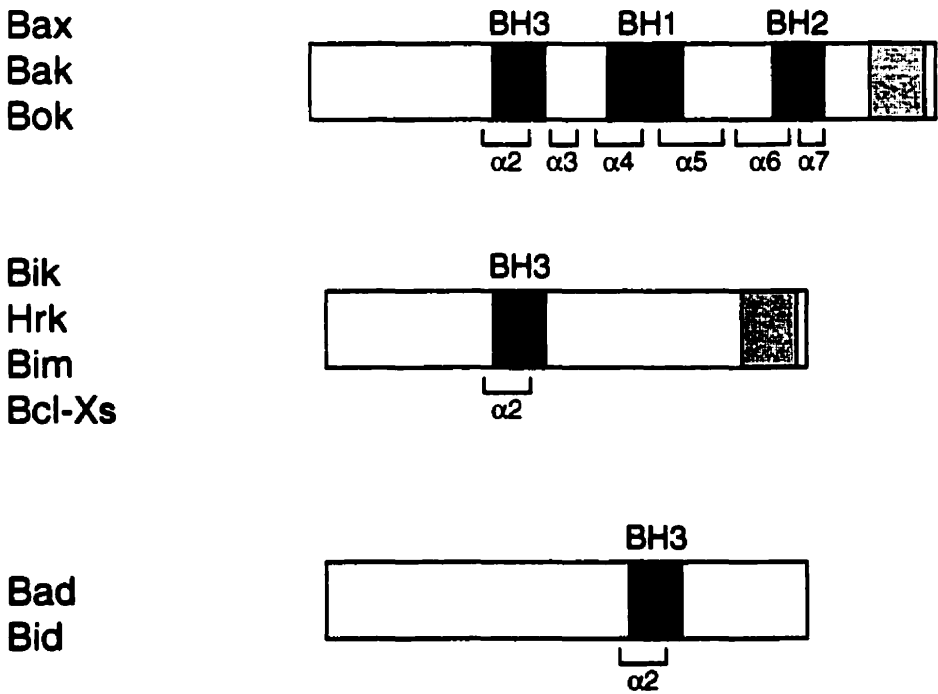
Figure 1-6 The Bcl-2 Family of Apoptosis Regulators

A generalized schematic representation of the members of the Bcl-2 family. The Bcl-2 homology domains (BH1 to BH4) are indicated with solid dark boxes, and the membrane anchor is represented by a lighter box. The helical structures in Bcl-2 are labelled $\alpha 1$ - $\alpha 7$. The phosphorylation sites are indicated by arrows. Functional regions are as indicated. Adapted from Cory & Adams (1998).

Pro-survival



Pro-apoptosis



BH3 amphipathic helix can bind (Sattler *et al.*, 1997). This has suggested a model whereby the BH3-only death promoters, bind via their BH3 domain to the survival promoting members and neutralize their activities (Kelekar *et al.*, 1997), but the Bax-like death promoters with three BH domains almost certainly have additional functions. There is some specificity in heterodimerization among the family members, but how this modulates the life / death balance within the cell is not yet known.

1.9.4.2 Regulation by Phosphorylation

Phosphorylation plays a role in regulating at least some members of the Bcl-2 family. The best characterized example involves the BH3-only Bad death promoter (reviewed in Gajewski & Thompson, 1996; Franke & Cantley, 1997). In its hypophosphorylated state, Bad can bind to Bcl-2 or Bcl-X_L via its BH3 domain and inactivate their survival activity (Yang *et al.*, 1995). However, in response to IL-3 Bad becomes phosphorylated on serines 112 and 136, and is sequestered into the cytosol where it interacts with 14-3-3, a protein which recognizes specific phosphoserine sites (Zha *et al.*, 1996). The mechanism leading to Bad phosphorylation is partially understood and involves the binding of survival factors to cell surface receptors, which activates the phosphoinositide 3-kinase (PI3K) (del Peso *et al.*, 1997). The resulting phosphoinositide second messengers recruit the kinases Akt and PDK1 through their plextrin homology (PH) domains (reviewed in Hemmings, 1997) then PDK1 activates Akt via phosphorylation (Alessi *et al.*, 1997a; 1997b; Stokoe *et al.*, 1997). Akt subsequently translocates and phosphorylates Bad, inactivating it (del Peso *et al.*, 1997; Datta *et al.*, 1997b). However, this is not the whole story; Akt only phosphorylates Bad on serine 136, which while inactivating alone leaves room for a second survival pathway to inhibit Bad via serine 112 (del Peso *et al.*, 1997). Raf-1 has also been reported to phosphorylate Bad, but the physiological relevance of this is still unknown (Wang H.-G. *et al.*, 1996). Whether other death promoters are inhibited by the growth and survival signal transduction pathways is currently unnoted, but seems plausible.

Bcl-2 has been described as being both positively and negatively regulated by phosphorylation (Halder *et al.*, 1996; Blagosklonny *et al.*, 1997; Ito *et al.*, 1997). Microtubule damage by chemotherapeutic agents was found to inhibit Bcl-2 function through phosphorylation (Halder *et al.*, 1996; Blagosklonny *et al.*, 1997). Additionally, activation of the Jun kinase (JNK) which is linked in some systems to apoptosis induction, promotes the phosphorylation of Bcl-2 on four inhibitory sites in the non-conserved flexible loop between the BH4 and BH3 domains (see Figure 1-6 on page 55) (Maundrell *et al.*, 1997).

1.9.5 The Caspase Family

Beginning with the identification of *ced-3* as an important mediator of death in *C. elegans* (Yuan *et al.*, 1993), the caspases have become established as key factors in apoptotic signalling and execution (reviewed in Salvesen & Dixit, 1997; Cryns & Yuan, 1998). The human caspase family now includes eleven members (caspase-1 to -11) (Alnemri *et al.*, 1996; Wang *et al.*, 1998) and can be broken into two sub-families phylogenetically: the ICE group (caspases-1, -4, -5, -11) which are primarily involved in the inflammatory response; and the *ced-3* group (encompassing the remaining caspases) which are involved in apoptosis. Caspases exist in the cytoplasm as inactive zymogens, which require proteolytic processing (often by another caspase) to become activated. This processing releases a N-terminal pro-domain (which can be large or small depending on the caspase type) and generates two additional fragments, generally in the range of 20 kd (p20) and 10 kd (p10), which heterodimerize to form the active enzyme. The caspases with large pro-domains appear to be regulatory caspases, while those with small pro-domains are regarded as effector caspases. The current generalized model of activation is that the regulatory caspases receive the apoptotic signals via interactions with their pro-domains, become proteolytically processed (probably autocatalytically in many cases) then activate, via a proteolytic cascade, the downstream effector caspases, which amplify the signal through positive feedback, and start the execution stage of apoptosis.

The different caspases can be divided into three groups based on their tetrapeptide substrate specificity: Group I is the ICE family (-1, -4, -5) and recognizes a WEHD peptide sequence; Group II is composed of effector caspases (-2, -3, -7, and *ced-3*) and prefers DEXD; while Group III contains the regulatory caspases (-6, -8, -9, and granzyme B) and cleaves at (I/L/V)EXD, which corresponds to the cleavage sites in the effector procaspases (Thornberry *et al.* 1997). The caspases show considerable overlap in specificity, which may indicate some redundancy in function, and work with knockout mice has supported this view, as well as illustrated the role of some caspases in inflammation (Kuida *et al.*, 1995; 1996; Li *et al.*, 1995; Zheng *et al.*, 1995). Specific chemical inhibition of caspase activity can in many cases block apoptosis (Hay *et al.* 1994; Beidler *et al.* 1995; Enari *et al.*, 1995; Los *et al.*, 1995; Tewari & Dixit 1995; White *et al.*, 1996; Sabbatini *et al.*, 1997) and a number of viruses encode caspase antagonists (Clem & Miller, 1994; Komiyama *et al.*, 1994; Bump *et al.*, 1995). However, some apoptotic pathways appear to be caspase-independent or involve yet to be described caspases (Xiang *et al.*, 1996; McCarthy *et al.*, 1997; Lavoie *et al.*, 1998).

1.9.6 Death Receptor Signalling

The best characterized pathways leading to caspase activation and apoptosis involve Fas and TNF receptor signalling (reviewed in Nagata, 1997). Fas and TNFR1 are related cell surface receptors that homotrimerize when presented with ligand (Fas ligand and TNF- α respectively) (Banner *et al.*, 1993) and initiate an apoptotic signal. Protein synthesis is not required for apoptosis, indicating that all of the apoptotic machinery is ready to be activated with this system (Yonehara *et al.*, 1989; Itoh *et al.*, 1991). These pathways are important in immune system regulation, and immune disorders have been associated with Fas pathway defects (Watanabe-Fukunaga *et al.*, 1992; Takahashi *et al.*, 1994; Bellgrau *et al.*, 1995; Fisher *et al.*, 1995; Giordano *et al.*, 1997).

Apoptotic signalling by Fas and TNFR requires a death domain (DD) in the cytoplasmic portion of the receptor. When the ligand binds to the receptor, the DD recruits an adaptor protein—FADD/MORT-1 (Fas-associated death domain)—through DD dimerization (Boldin *et al.* 1995; Chinnaiyan *et al.* 1995). FADD in turn contains a second dimerization motif known as the death effector domain (DED) and retrieves caspase-8 (also called FLICE) through DED dimerization with the DED in the pro-domain of caspase-8. This provides the link between the receptor and an initiator/regulatory caspase, producing a complex termed DISC, for death-inducing signalling complex (Boldin *et al.*, 1996; Muzio *et al.*, 1996). A Fas-associated phosphatase (FAP-1) also binds to the activated receptor and may play a role in DISC assembly (Yanagisawa *et al.*, 1997). In addition to FADD, there is another adapter protein that functions in a slightly different manner named RIP (receptor-interacting protein) which binds to the TNFR and weakly to Fas (Hsu *et al.*, 1996b). RIP associates with another adapter protein RAIDD (RIP-associated, ICH-1-homologous protein with DD) via a DD interaction, and RAIDD brings along caspase-2 (ICH-1) through DED dimerization, linking TNFR and Fas to caspase-2 activation (Ahmad *et al.*, 1997; Duan & Dixit, 1997).

TNFR interactions differ slightly from Fas, with the addition of another adapter protein called TRADD (TNFR-associated DD) (Hsu *et al.*, 1995). TRADD lacks a DED domain, and functions by binding to the TNFR and recruiting FADD through a DD association, providing a second way for the receptor to associate with FADD (Hsu *et al.*, 1996a). With both receptors, formation of the DISC results in the activation of caspase-8, which ultimately induces apoptosis, through the stimulation of downstream effector caspases.

TNFR-induced apoptosis is suppressed under certain circumstances by the activation of the transcription factor NF- κ B, which presumably transcribes genes involved in survival (Beg & Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang C. *et al.*, 1996). In addition, the Fas and TNFR DISC complex can be inhibited by proteins known as FLIPs (FLICE-inhibitory proteins). These proteins are composed of two DDs which interact with FADD and prevent the recruitment of caspase-8 (FLICE). Both cellular and viral FLIPs have been identified (Goltsev *et al.*, 1997; Hu *et al.*, 1997a; 1997b; Irmeler *et al.*, 1997; Shu *et al.*, 1997; Srinivasula *et al.*, 1997; Thome *et al.*, 1997). The viral FLIPs are from several γ -herpesviruses and molluscipoxvirus, and presumably enhance viral persistence by down-regulating this component of the host immune response. Adenoviruses also target these signalling pathways, by downregulating Fas protein levels with RID (Tollefson *et al.*, 1998) (section 1.6.7.3 on page 32) and by interfering with FADD function with E1B-19K (section 1.6.5.4 on page 29) (Perez & White, 1998). Many viruses have methods of inhibiting this critical host defense using a wide array of mechanisms (Teodoro & Branton, 1997a; Granville *et al.*, 1998b).

1.9.7 Mitochondria and the Apoptosome

The mitochondria are the power plant of the cell, using the electron transport chain to produce the ATP that drives most cellular processes (reviewed in Attardi *et al.*, 1988). However, they also play a major role in the commitment to apoptosis (reviewed in Kroemer *et al.*, 1997; 1998). Mitochondria must maintain a transmembrane potential across their inner membrane ($\Delta\Psi_m$) for proper function, but in cells undergoing apoptosis, the $\Delta\Psi_m$ is lost prior to many of the morphological hallmarks of apoptosis, but only once a cell is fully committed to die (Vayssière *et al.*, 1994; Zamzami *et al.*, 1995a; 1995b; Petit *et al.*, 1995; Castedo *et al.*, 1996; Marchetti *et al.*, 1996a; 1996b; Susin *et al.*, 1996; 1997). The loss of $\Delta\Psi_m$ has been linked to a mitochondrial permeability transition (PT) likely involving the opening of a large pore (Zamzami *et al.*, 1995b; Castedo *et al.*, 1996). Inhibitors of $\Delta\Psi_m$ loss or PT formation block apoptosis, and direct chemical disruption of $\Delta\Psi_m$ induces apoptosis, suggesting that the PT has some causal link to downstream apoptotic events, and is not just a consequence (Castedo *et al.*, 1996; Marchetti *et al.*, 1996a; 1996b; Zamzami *et al.*, 1996; Macho *et al.*, 1997; Susin *et al.*, 1997). The exact composition of the PT pore complex has not been determined, but it appears to be located at contact points between the inner and outer mitochondrial membranes, and consists of several proteins in each membrane. The mitochondrial swelling that accompanies PT pore opening leads to rupture of the smaller outer membrane, and release of proteins from the inner membrane

space, which are believed to induce further apoptotic progression, as well as destroy the cell's capacity to make ATP (Zamzami *et al.*, 1996).

The most prominent apoptotic protein released from mitochondria is cytochrome c, a protein normally involved in oxidative phosphorylation (reviewed in Reed, 1997b). Following exposure to apoptotic stimuli, cytochrome c is released resulting in caspase activation, which can be blocked by Bcl-2 or Bcl-X_L (Kharbanda *et al.*, 1997; Kim *et al.*, 1997; Kluck *et al.*, 1997a; Yang *et al.*, 1997). The mechanism behind both the release and the Bcl-2 block is unknown, but the cytochrome c efflux appears to precede PT, indicating that a more specific release pathway exists (Kluck *et al.*, 1997a; 1997b; Yang *et al.*, 1997). Active caspases can then induce PT (Susin *et al.*, 1997) suggesting that this may be the sequence of events. Structural studies of Bcl-X_L indicated a similarity with pore-forming bacterial toxins (Muchmore *et al.*, 1996), suggesting that the Bcl-2 family members, which have the requisite $\alpha 5$ and $\alpha 6$ helices (see Figure 1-6 on page 55) may function in part through pore formation. Bcl-2 and Bcl-X_L have been shown to have cation transport specificities *in vitro*, though assay conditions were far from physiological (Minn *et al.*, 1997; Schendel *et al.*, 1997). Conversely, Bax has been seen to exhibit anion specificity *in vitro* at physiological pH (Schlesinger *et al.*, 1997). Bcl-2 has been reported to block Bax channel formation, suggesting that Bcl-2 may function by blocking Bax pores rather than forming its own (Antonsson *et al.*, 1997). Whether Bax can form pores large enough to allow cytochrome c efflux is not known, but it is a possibility. Alternately Bax may regulate another cytochrome c pore.

The mechanism by which cytochrome c acts was determined using an *in vitro* system in which dATP addition would activate caspase-3, an effector caspase. Using biochemical purification, three factors were found to be required for this activation: apoptotic protease activating factor (Apaf) -1, -2, and -3 (Zou *et al.*, 1997). Apaf-1 was identified as the first mammalian Ced-4 homologue (Zou *et al.*, 1997). Apaf-2 was found to be cytochrome c (Liu *et al.*, 1996) and Apaf-3 was identified as procaspase-9 (Li *et al.*, 1997), a regulatory caspase (Duan *et al.*, 1996; Srinivasula *et al.*, 1996). The 130 kd Apaf-1 consists of an N-terminal caspase recruitment domain (CARD) which binds to the DED motif in the pro-domain of regulatory caspases (including caspase-9) (Hofmann *et al.*, 1997; Zou *et al.*, 1997) followed by a Ced-4 homologous region containing among other motifs the nucleotide binding domain, then 12 putative WD40 repeats, which are thought to mediate protein-protein interactions (Neer *et al.*, 1994; Wall *et al.*, 1995; Sondek *et al.*, 1997). Caspase-9 is bound and activated by Apaf-1 in a cytochrome c- and dATP-dependent fashion, followed by caspase-9-mediated cleavage and activation of caspase-3 (Li *et al.*, 1997). This caspase-

activation complex has been named the apoptosome, and is strongly reminiscent of the Ced-4/Ced-3 complex in *C. elegans*. Bcl-X_L has been shown to bind and inhibit Ced-4 through a BH4 interaction (Huang *et al.*, 1998) and recent data has shown that Bcl-X_L can also bind and inhibit Apaf-1 (Hu *et al.*, 1998; Pan *et al.*, 1998), analogous to the situation with Ced-9 in the nematode. Additionally, Bcl-X_L has been reported to bind directly to cytochrome c in the cytosol restricting its availability to Apaf-1, and as mentioned, Bcl-2 and Bcl-X_L can block cytochrome c release from the mitochondria (Kharbanda *et al.*, 1997; Kim *et al.*, 1997; Kluck *et al.*, 1997; Yang *et al.*, 1997). This combination provides tight control over apoptosome formation, which is possibly relieved during apoptosis by a shift of the Bax / Bcl-2 setpoint, as Bax, Bak and Bik can attenuate the association of Bcl-2 with Ced-4 (Chinnaiyan *et al.*, 1997b).

The importance of the apoptosome was recently demonstrated with the characterization of caspase-9 knockout mice (Hakem *et al.*, 1998; Kuida *et al.*, 1998). This deletion resulted in embryonic lethality, with a greatly enlarged brain due to a lack of apoptosis. Embryonic stem cells and fibroblasts exhibited resistance to DNA damage-induced apoptosis and dexamethasone treatment. This apoptotic resistance was accompanied by the retention of $\Delta\Psi_m$, although cytochrome c was released, supporting the existing model. The work also strongly supported the role of caspase-9 in effector caspase activation.

In addition to cytochrome c, mitochondria release a second apoptosis-inducing factor (AIF) when undergoing PT (Susin *et al.*, 1996; 1997). AIF has not been identified, but initial characterization indicates that it is an unstable, 50 kd protein, which can induce nuclear hallmarks of apoptosis through a caspase-dependent pathway (Susin *et al.*, 1996).

Through these functions, the mitochondria form an important control point in the induction of apoptosis, though exactly how upstream signals are linked is not currently known. There are probably caspase sensitive proteins on the outer mitochondrial membrane that can initiate this stage of apoptosis when cleaved, or antagonists that lose function upon digestion. Once initiated, an amplifying loop is likely formed with the activated caspases further inducing mitochondrial changes.

1.9.8 The Endoplasmic Reticulum Control Point

While much work has concentrated on mitochondrial apoptosis control, Bcl-2 also regulates events at the endoplasmic reticulum. A search for Bcl-2 binding proteins during

adenovirus E1A-induced apoptosis pulled out a membrane-spanning endoplasmic reticulum protein, called Bap31 (Ng *et al.*, 1997). Bap31 was shown to indirectly interact with procaspase-8 (a regulatory caspase) but not procaspase-3 (an effector caspase) and also to directly bind Bcl-2 or Bcl-X_L (Ng *et al.*, 1997). During apoptosis Bap31 is proteolytically processed to a p20 form, which when ectopically expressed is an inducer of cell death (Ng *et al.*, 1997). Bcl-2 appears to normally prevent the cleavage of Bap31, but Bax/Bcl-2 heterodimerization sequesters Bcl-2, blocking its protective action and promoting p20 generation and apoptosis (Ng *et al.*, 1997). *C. elegans* Ced-4 was shown to substitute for the unidentified adapter between Bap31 and procaspase-8, and also to enhance Bcl-X_L binding, suggesting that a Ced-4-like protein is a normal component of the complex (Ng & Shore, 1998). The Bap31 complex like the apoptosome is likely an important caspase control point in the cell, and may be additionally involved in regulating Ca⁺⁺ release from the endoplasmic reticulum, which could then potentially influence events at the mitochondria (Hajnoczky *et al.*, 1995; Jouaville *et al.*, 1995; Kroemer *et al.*, 1997). A second endoplasmic reticulum localized integral membrane protein has been recently identified—BI-1—and acts as an inhibitor of Bax (Xu & Reed, 1998). BI-1 does not bind to Bax directly, however, but rather associates with Bcl-2 or Bcl-X_L, and may also play a role in Ca⁺⁺ regulation (Xu & Reed, 1998).

1.9.9 The Downstream Execution Stage

The execution stage of cell death is beginning to be unravelled. Many caspase substrates have been identified, and their link to apoptosis and cell disassembly is emerging. Caspases do not randomly chew up cellular components, but rather specifically dismantle cells by cleaving defined targets. Many of the targets identified so far are involved in cell signalling and survival, homeostasis and DNA repair (reviewed in Cryns & Yuan, 1998).

One logical category of caspase substrates is the death antagonists, such as Bcl-2 and Bcl-X_L, which are both cleaved during some types of apoptosis (Cheng *et al.* 1997; Clem *et al.*, 1998; Grandgirard *et al.*, 1998). This cleavage removes the N-terminal BH4 domain from the two proteins, producing a C-terminal Bax-like fragment which promotes rather than antagonizes cell death. These proteolytically generated killers can be blocked by caspase inhibitors, suggesting that they function in a death-amplifying loop rather than by contributing to the downstream execution stage. As mentioned, Bap31 is also a substrate for caspases, and is likewise turned into a killer by proteolysis (Ng *et al.*, 1997)

A second category of substrates consists of a number of apoptosis-inducing kinases. In this case, caspase cleavage is directed at removing an N-terminal regulatory domain, which generates a constitutively active kinase. PAK2/hPAK65, PKC δ and θ , and MEKK-1 all fall into this group (Emoto *et al.* 1995; Ghayur *et al.* 1996; Cardone *et al.* 1997; Datta *et al.*, 1997a; Lee *et al.*, 1997; Rudel & Bokoch, 1997). Overexpression of the C-terminal domain of PAK2/hPAK65 (a kinase involved in p21 Rac signalling to the actin cytoskeleton) has been shown to induce cellular detachment, nuclear and cytoplasmic condensation, and the hallmark phosphatidyl serine flip from the inner face of the plasma membrane to the outer surface of the cell. These effects have been shown to be downstream of the caspases, and a protease resistant kinase mutant has a dominant negative effect on the induction of these apoptotic features (Lee *et al.*, 1997). The PKC isoforms also induce apoptosis when the C-terminal fragments are overexpressed, which is not the case with the intact kinases (Ghayur *et al.* 1996; Datta *et al.*, 1997a). Similarly, MEKK-1 which is at the top of the JNK kinase pathway is cleaved and activated by caspases when cell matrix attachments are lost (Cardone *et al.*, 1997). Overexpression of the caspase cleavage form of the enzyme is apoptotic, while a caspase resistant mutant inhibits cell death and caspase-7 activation, indicating that MEKK-1 may be in an important death amplifying loop (Cardone *et al.*, 1997). The critical substrates of these kinases are not currently known, but this demonstrates that some morphological effects of apoptosis do not involve direct caspase activity.

As expected, the breakdown in cell structure does involve the proteolytic cleavage of a number of structural proteins. Gelsolin, an actin filament organizing factor (Kwiatkowski *et al.*, 1986) is cleaved to produce a N-terminal fragment that disrupts the actin filament network, and can induce apoptosis without further caspase involvement (Kothakota *et al.*, 1997). Gas2 cleavage also leads to actin filament disturbances, accompanied by cell rounding and cytoplasmic condensation (Brancolini *et al.*, 1995). The lamins, which play an important structural role in the nucleus, are also caspase targets (Lazebnik *et al.*, 1995; Orth *et al.*, 1996; Rao *et al.*, 1996; Takahashi *et al.*, 1996) and caspase-resistant lamins can delay many of the nuclear manifestations of apoptosis (Rao *et al.*, 1996). Many other structural proteins are cleaved by caspases, but the functional importance of these events has not yet been documented.

The mechanism behind the characteristic degradation of DNA into nucleosome sized fragments has now also been linked to caspase activation. The caspase activated deoxyribonuclease (CAD/CPAN/DFF40) exists in a cytoplasmic complex with an inhibitor (ICAD/DFF45). Active caspase-3 was found to cleave ICAD, allowing CAD to translocate

to the nucleus to degrade the genomic DNA (Liu *et al.*, 1997; Enari *et al.*, 1998; Halenbeck *et al.*, 1998; Sakahira *et al.*, 1998).

The characterization of the terminal stage of apoptosis is reinforcing the importance of the effector caspases; however in some settings, caspase inhibitors do not block cell death, indicating the presence of uncharacterized, possibly caspase-independent apoptosis pathways (Xiang *et al.*, 1996; McCarthy *et al.*, 1997; Lavoie *et al.*, 1998). There has been an enormous amount of progress in the field of apoptosis signalling over the last couple of years. The parallel studies of growth promotion and apoptosis are coming together, but one major player in apoptosis has yet to be firmly incorporated into the models—p53.

1.9.10 p53

1.9.10.1 Introduction and Early Work

p53 is one of the cell's primary guardians against DNA damage, loss of growth control, and viral infection (reviewed in Levine, 1997; Ko & Prives, 1996). Not surprisingly, the p53 tumour suppressor is probably the most commonly mutated gene in cancer, as disruptions are found in over 50% of human cancers (Hollstein *et al.*, 1994). p53 is most often observed to suffer point mutations, followed by a reduction to homozygosity, but the null phenotype also predisposes to cancer, as has been confirmed in the mouse knockout and with humans defective in the p53 gene (Malkin *et al.*, 1990; Donehower *et al.*, 1992). p53 plays such an important role in the defense against cancer, that all cancer cells may have p53 pathway defects at some level. p53 is also commonly inactivated by viral proteins during the infectious process to prevent p53-mediated cell cycle inhibition and apoptosis induction (section 1.6.5.3 on page 27)(reviewed in Teodoro & Branton, 1997; Granville *et al.*, 1998b).

p53 was originally misclassified as an oncogene since it had immortalizing activity in primary cells and transforming ability in cooperation with the activated *ras* oncogene (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984). However, the original p53 cDNA was subsequently found to be a mutant and the *wt* p53 did not share this activity (Hinds *et al.*, 1989). Furthermore, p53 was found to repress transformation mediated by other oncogenes (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989), growth suppress transformed lines upon overexpression (Baker *et al.*, 1990; Diller *et al.*, 1990; Martinez *et al.*, 1991; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990), and inhibit tumourigenicity of cells in nude mice (Chen *et al.*, 1990). p53 was shown to block the cell cycle at the G₁/S boundary (Baker *et al.*, 1990;

Diller *et al.*, 1990) and to act as a cell cycle checkpoint, guarding against various forms of genotoxic stress. DNA damage will cause an increase in p53 protein levels, which leads to growth arrest (Maltzman & Czyzyk, 1984; Kastan *et al.*, 1991; 1992; Kuerbitz *et al.*, 1992; Fritsche *et al.*, 1993).

1.9.10.2 p53 Structure

p53 is a 393 aa DNA-binding transcription factor (Funk *et al.*, 1992; Kern *et al.*, 1992) which is composed of a number of functional domains. The N-terminus is an acidic transcriptional transactivating region (1-42 aa) (Fields & Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990; Unger *et al.*, 1992). The central core of this domain from residue 13 to 23 is highly conserved, and a number of the essential amino acids have been shown to contact the TATA-associated factors TAF_{II}70 and TAF_{II}31, both subunits of TFIID (Lu & Levine, 1995; Thut *et al.*, 1995).

The DNA binding domain occupies the middle of the protein from 102 to 292 aa. It is composed of two β -sheets that support two α -helical loops stabilized by a zinc atom, and a loop-sheet-helix motif (Cho *et al.*, 1994). The two loops and the loop-sheet-helix motif are the conserved core of the DNA-binding domain, and compose the DNA-binding surface of p53. The majority of p53 mutations are in these DNA-binding loops demonstrating the importance of DNA-binding for p53 function (el-Deiry *et al.*, 1992; Farmer *et al.*, 1992; Kern *et al.*, 1991; 1992; Scharer & Iggo, 1992). p53 specifically binds to a 5 bp motif present as two sets of inverted repeats (Kern *et al.*, 1991; el-Deiry *et al.*, 1992).

p53 normally exists as a tetramer in the cell, and requires residues 324-355 for this interaction (Shaulian *et al.*, 1992; Iwabuchi *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993b; 1994). p53 binds DNA in this tetrameric form, explaining the requirement for 4 consensus sequences in tandem (Hainaut *et al.*, 1994; Hupp & Lane, 1994; Wang *et al.*, 1995b). The oligomerization domain is the minimum region required for transformation, suggesting that p53 mutants function in a dominant negative fashion by forming inactive tetramers with *wt* p53 (Shaulian *et al.*, 1992; Wang *et al.*, 1994). The DNA binding domain is connected to the tetramerization domain by a flexible linker of 37 residues (Jeffrey *et al.*, 1995).

In the cell, p53 requires a structural change to make it competent to bind DNA, and the C-terminal region appears to function in a regulatory manner to control this. It is a basic region of 26 aa which, through some allosteric or steric mechanism, suppresses sequence

specific DNA-binding (Hupp *et al.* 1992). Many modifications to this region including antibody binding by pAb421, deletion, or phosphorylation by casein kinase II, G₂ cyclin / CDKs or protein kinase C will activate p53 for DNA-binding (Bischoff *et al.*, 1990; Sturzbecher *et al.*, 1990; Hupp *et al.* 1992; Hupp & Lane, 1994; Wang & Prives, 1995). Additionally, ssDNA fragments (as you might find during DNA damage or replication errors) will interact with this domain and activate specific DNA-binding by p53 (Jayaraman & Prives, 1995). The C-terminus likely plays a role in regulating p53 activity, but so far the *in vivo* signalling network that modifies it is poorly characterized. p53 is also regulated by N-terminal phosphorylation and this will be discussed in the next section (reviewed in Meek, 1998).

1.9.10.3 Upstream Activators

p53 has a short half life of about 20 minutes and normally this keeps the p53 protein levels low. Additionally, p53 appears to primarily exist in a latent, non DNA-binding form. However once a signal reaches p53, the half-life lengthens increasing the protein levels, and p53 becomes active for DNA-binding and transcriptional activation. p53 responds to a number of different cellular stresses, the best characterized being DNA damage from UV or γ -irradiation. Cellular proteins that recognize and repair damaged DNA are thought to communicate with p53. The ataxia-telangiectasia gene (ATM) is one example of DNA damage-recognizing protein that is thought to activate p53 through phosphorylation (Kastan *et al.*, 1992). Since p53 itself can also bind to ssDNA ends, p53 and the DNA repair enzymes may become co-localized which would simplify the cross-talk (Lee *et al.*, 1995b). Recent work has shown that p53 can undergo phosphorylation at serine 15 in response to DNA damage, catalyzed by the DNA-dependent protein kinase (DNA-PK) (Ikeda *et al.*, 1998). This modification appears to induce a conformational change in p53 that reduces its interaction with the p53-inhibitor mdm-2 (section 1.9.10.6 on page 70). In addition the CAK kinase heterotrimer, which is part of TFIIH a multiprotein complex involved in RNA pol II transcription and nucleotide excision repair, can phosphorylate p53 at serine 33 *in vivo*, though whether this occurs in response to DNA damage is not known (Shieh *et al.*, 1997). p53 functions as an integrator of all genotoxic stresses to the cell, presumably to prevent mutations that will alter cellular function, and one reason many viruses may need to inactivate p53 is to avoid stimulation caused by unusual replication intermediates.

A second p53 activating stress is a drop in ribonucleotide triphosphate levels (Linke *et al.*, 1996). During DNA replication when nucleotide supply is critical, some cellular factor

appears to monitor the levels and signal p53 if shortages are detected. The signalling mechanism and proteins involved in this process are still unknown, however.

A third p53 triggering cellular stress is hypoxia (Graeber *et al.*, 1996). When cells become cancerous and form tumours they reach a size where simple diffusion is not adequate to supply oxygen, and they become hypoxic. This stops further growth until the tumour cells develop the ability to promote angiogenesis; however, in the interval p53 gets activated and will act to suppress tumour growth. p53 has also been reported to transcriptionally activate the thrombospondin gene, which is an antiangiogenic factor which would then further limit oxygen supply (Dameron *et al.*, 1994).

The final method of p53 activation is like the others poorly understood. As mentioned previously, oncogene stimulated cell growth can often trigger apoptosis (section 1.6.5.1 on page 26). Viral growth promoters such as adenovirus E1A or SV40 large T, and cellular oncogenes like Myc activate p53 as a consequence of their cell cycle stimulation. Somehow p53 appears to detect this unscheduled DNA synthesis. This is a major problem for many viruses, and likely for a lot of cancers as well, which is another reason why both have developed means of neutralizing p53.

Once activated, p53 appears to initiate two primary pathways: growth arrest and apoptosis. In situations with minor DNA damage or nucleotide shortages, p53 appears to induce growth arrest to allow the cell time to rectify the problem before continuing with the cell cycle. In cases with more severe DNA damage, viral infection, or oncogene expression, p53 activates the cellular apoptotic program to clear the aberrant cell. As p53 requires DNA binding activity for many functions, transcriptional regulation has been a major research focus.

1.9.10.4 Growth Arrest

The cell cycle block by p53 has been linked to the transcriptional upregulation of two cellular proteins: p21^{waf1/cip1} and GADD45 (El-Diery *et al.*, 1993; Kastan *et al.*, 1992). p21^{waf1/cip1} is a cyclin/CDK antagonist that binds to a number of G1 cyclin complexes, and inhibits their kinase activity (section 1.6.4.2 on page 20) (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993). This effect is likely behind the ability of p53 to induce G1 arrest in response to genotoxic stresses. p21^{waf1/cip1} has also been shown to interact with PCNA, and block its role as a DNA polymerase processivity factor without inhibiting its function in DNA repair (Li *et al.*, 1994; Waga *et al.*, 1994; reviewed in Prosperi, 1997). Mice deleted for

p21^{waf1/cip1} show a partial reduction in p53-mediated growth arrest, indicating that there are additional pathways (Deng *et al.*, 1995). GADD45 may function in this capacity, as it has been linked to G₁ growth arrest and DNA polymerase inhibition, again through interactions with PCNA (Smith *et al.*, 1994). G₁ cell cycle arrest likely works predominantly through Rb family regulation, as E1A and SV40 large T can block p53-mediated G₁ arrest, via their inactivation of the Rb family (Hansen *et al.*, 1995).

p53 has also been linked to a second checkpoint at the G₂/M transition (Cross *et al.*, 1995). p53 has been shown to associate with centrosomes (Brown *et al.*, 1994) and is also known to be involved in regulating their number during division (Fukasawa *et al.*, 1996). Whether this ploidy monitoring function is linked to the G₂/M checkpoint is not known, but it does contribute to genomic stability (Donehower *et al.*, 1995). Involvement in a third cell cycle checkpoint has come from the demonstration that Gas1, a G₀ arrest factor, requires p53 for its function, though p53 DNA-binding activity is not necessary (Del Sal *et al.*, 1995). This suggests that p53 has activities that do not involve direct transcriptional regulation.

1.9.10.5 Apoptosis Induction

The introduction of p53 into p53^{-/-} murine leukemia cells provided the first demonstration that p53 could induce apoptosis (Yonish-Rouach *et al.*, 1991). Further work with thymocytes from p53^{-/-} mice showed that p53 was required for γ -irradiation-induced apoptosis (Clarke *et al.*, 1993; Lowe *et al.*, 1993b). Notably these mice were still sensitive to apoptosis induction by glucocorticoids, establishing the existence of both p53-dependent and -independent cell death pathways. As mentioned, oncogene-stimulated cell growth triggers p53 activation. Adenovirus E1A causes an increase in p53 protein levels which leads to apoptosis and likely explains why E1A growth promotion is not sufficient to transform cells (section 1.6.5.1 on page 26) (Rao *et al.*, 1992; Debbas & White, 1993; Lowe & Ruley, 1993). Many other viral oncoproteins induce p53-dependent apoptosis and in compensation viruses encode proteins to block p53 action, like the E1B products of adenovirus (section 1.6.5.2 on page 27) (reviewed in Teodoro & Branton, 1997; Granville *et al.*, 1998). In addition to the viral genes, some cellular oncoproteins have been shown to induce apoptosis dependent upon p53 including c-Myc (Hermeking & Eick, 1994; Wagner *et al.*, 1994) and E2F-1 (Wu & Levine, 1994).

The mechanism of p53-induced apoptosis appears to be multi-faceted. p53 can transcriptionally activate Bax, a pro-apoptotic Bcl-2 family member (section 1.9.4 on page 53) and this could certainly contribute to cell death (Miyashita *et al.*, 1994; Miyashita & Reed, 1995). However, the induction of Bax is not essential, since cells from Bax^{-/-} mice are still responsive to p53-induced death (Knudson *et al.*, 1995) and in some systems, the transcriptional activity of p53 appears to be dispensible (Caelles *et al.*, 1994; Wagner *et al.*, 1994; Haupt *et al.*, 1995). In contrast the same basic stimuli do require p53 transcriptional activity when tested in different cell types or under slightly different conditions, causing some controversy (Sabbatini *et al.*, 1995; Haupt *et al.*, 1995; Attardi *et al.*, 1996). p53 also stimulates expression of IGF-BP3, a protein that blocks the IGF mitogenic signalling cascade, and this may serve to enhance apoptosis by reducing the proliferative response of cells (Buckbinder *et al.*, 1995). p53 has been reported to increase Fas protein levels, but a direct transcriptional effect has not yet been demonstrated (Owen-Schaub *et al.*, 1995).

p53 has been demonstrated to transcriptionally repress promoters in the absence of the p53 consensus binding site (Ginsberg *et al.*, 1991; Santhanam *et al.*, 1991; Shiio *et al.*, 1992; Subler *et al.*, 1992; Jackson *et al.*, 1993). This requires the presence of a TATA box, and may be related to the ability of p53 to contact the basal transcriptional machinery and perhaps inhibit its function or sequester limiting factors (Mack *et al.*, 1993). Whether this is of biological significance is unknown, but one could conceive of model in which p53 inhibition of survival genes would contribute to cell death.

There have not been many insights into how p53 could regulate apoptosis in a non-transcriptional manner. p53 has not been observed to interact with the apoptotic signalling proteins from the Bcl-2 or caspase families, yet these pathways do get activated by p53. One link between p53 and signal transduction pathways emerged with the demonstration of binding to c-Abl, a nuclear tyrosine kinase (Yuan *et al.*, 1996). p53 has 5 copies of a SH3-binding consensus sequence which could facilitate the c-Abl interaction (residues 61-94), and deletion of this region significantly inhibits p53-induced apoptosis or growth arrest, without altering the transactivation activity of p53 (Walker & Levine, 1996). c-Abl can induce growth arrest dependent upon p53 suggesting that they are in a common pathway, but how c-Abl activates p53 is not understood (Goga *et al.*, 1995).

1.9.10.6 p53 regulation

p53 activity is kept in check by a negative feedback loop. Once activated p53 transactivates the gene for mdm-2 (Barak *et al.*, 1993; Wu *et al.*, 1993) which then binds to the p53 transactivation domain (like adenovirus E1B-55K) and down-regulates p53 transcriptional activity (Momand *et al.*, 1992; Oliner *et al.*, 1992; 1993; Lin *et al.*, 1994) and protein levels (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). The importance of this regulation was demonstrated by the embryonic lethality of the mdm-2^{-/-} mouse, which can be rescued when put in a p53^{-/-} background (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). Not surprisingly, mdm-2 is often found to be amplified in certain tumour types and was first isolated from a transformed murine cell line (Oliner *et al.*, 1992). p53 regulation has an additional layer of complexity. p19Arf, a product of the *INK4a* locus, is able to potently suppress transformation by binding mdm2 and promoting its degradation (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998). This has the effect of p53 stabilization and accumulation and p19Arf activation is likely one of the ways in which p53 is activated. The adenovirus E1A-induced accumulation of p53 has been shown to require the presence of p19Arf, but the link remains uncharacterized (de Stanchina *et al.*, 1998). p19Arf deletion does predispose mice to tumours and may play a role in human cancers as well (Serrano *et al.*, 1996; Kamijo *et al.*, 1997). As mentioned, a second way of limiting the effectiveness of mdm-2 is the phosphorylation of p53 at serine 15. This modification seems to alter p53 structure such that mdm-2 binding affinity drops, and p53-driven transactivation increases (Shieh *et al.*, 1997).

p53 has also been shown to interact with WT-1, the product of the Wilms tumour suppressor locus, when both are overexpressed (Maheswaran *et al.*, 1995). This interaction stabilizes p53, and produces increased transcriptional activity by p53, but it inhibits p53-mediated apoptosis. The physiological relevance of this interaction has not been determined, however.

1.10 Summary

The study of the adenovirus life cycle has offered us many insights into the processes governing growth control, the cell cycle, apoptosis and general transcriptional mechanisms. This information will undoubtedly lead to better clinical approaches for the treatment of cancer, but the workings of the cell are complex, and we still have much to discover. The virus has not lost its relevance though; we are still learning more with this useful biological probe. Adenoviruses are also entering clinical trials for vaccine

development, gene therapy, replicating anti-cancer therapies, and in the future hopefully the exploitation of individual viral products will be possible.

1.11 Thesis Proposal

The goal at the beginning of this project was to better understand the phenomenon of cellular apoptosis and how adenovirus modulates it in order to replicate effectively. The introduction provides the framework of viral replication, presenting the challenges faced by the virus. Against this has been set our understanding of apoptosis.

At the onset of these studies, adenovirus was thought to induce only p53-dependent apoptosis. In Chapter 2 is presented the development of an assay system in which viral and cellular apoptosis inhibitors were tested against p53-induced apoptosis. These studies revolved around the use of a p53-expressing adenovirus vector that we found capable of killing Saos-2 cells, a p53 null osteosarcoma line. This work also reinforces the importance of p53 downregulation in the genesis of cancer.

During the course of this research, the discovery of an adenovirus-induced p53-independent apoptosis pathway was made. In the third and forth chapters we set out to map the viral proteins responsible for this killing. We used a number of viral mutants in this endeavor, and ended up identifying the E4orf4 protein. This work has revealed a novel p53-independent apoptosis pathway, and has provided us with a possible explanation of how the virus escapes from infected cells at the end of its replicative cycle.

In the discussion I will attempt to incorporate these findings into the requirements for a productive viral infection, and additionally integrate it into our knowledge of mammalian cell biology.

CHAPTER 2

Expression of p53 in Saos-2 Osteosarcoma
Cells Induces Apoptosis Which Can Be
Inhibited by Bcl-2 or the Adenovirus E1B-55
Kilodalton Protein

2.1 Abstract

Studies were carried out to determine the effects of introducing p53 using an adenovirus gene transfer vector into p53 null human Saos-2 osteogenic carcinoma cells. Expression of p53 led to cell death within 30 to 40 hours. The morphology of these cells as determined by electron microscopy indicated that death was by apoptosis. Such death was significantly reduced in Saos-2 variants which express high levels of the Bcl-2 suppressor of apoptosis. We also found that the E1B-55kDa protein of human adenovirus type 5, which was known to bind and inactivate p53, blocks Saos-2 cell death following expression of p53. These results thus directly demonstrate that this viral protein is able to inhibit p53-induced apoptosis.

2.2 Introduction

The p53 tumor suppressor has been implicated in a variety of physiologic responses to DNA damaging agents and aberrant growth signals (reviewed in Levine *et al.*, 1994). p53 was first identified through its co-immunoprecipitation with the SV40 large T antigen (Linzer & Levine, 1979). Somewhat later it was also shown to interact with the adenovirus early region 1B (E1B) 55kDa product (Sarnow *et al.*, 1982), and such complex formation was found to be necessary for cooperation with adenovirus early region 1A (E1A) in cell transformation (Yew *et al.*, 1990). In addition, the E6 protein of papillomavirus has been found to activate ubiquitin-mediated degradation of the p53 protein (Sheffner *et al.*, 1993). The observation that p53 is a common target of several viral oncogenes resulting in its inactivation supports the belief that p53 plays a major role in cell transformation by DNA tumor viruses. Furthermore, the finding that many forms of human cancers carry a high frequency of p53 mutations indicates a prominent role for p53 in human neoplasia (reviewed in Chang *et al.*, 1993). Mice lacking expression of p53 through gene targeting have also been shown to possess a clear predisposition to tumors at a very early age (Donehower *et al.*, 1992).

p53 induces several cellular responses, including the suppression of transformation, cell cycle and growth arrest, and the induction of apoptosis (Finlay *et al.*, 1989; Diller *et al.*, 1990; Ginsberg *et al.*, 1991; Kastan *et al.*, 1992; Yonish-Rouach *et al.*, 1991). p53 is a transcription factor with both sequence-specific and nonspecific DNA binding activity which can result in either activation or repression of gene expression (Ginsberg *et al.*, 1991;

Farmer *et al.*, 1992; Subler *et al.*, 1992; Zambetti *et al.*, 1992). The amino terminus of p53 contains an acidic activation domain which, when fused to the GAL4 DNA-binding region, is capable of transcriptional activation both in mammalian cells and yeast (Fields & Jang, 1990). However, under certain conditions, p53 can also repress basal transcription (Santhanam *et al.*, 1991; Chin *et al.*, 1992; Kley *et al.*, 1992). p53 plays an essential role in induction of apoptosis in response to ionizing radiation or expression of dominant oncogenes such as E1A or *c-myc* (Debbas & White, 1993; Lowe *et al.*, 1993b). p53 has been reported to activate expression of the *Bax* gene which is implicated in induction of apoptosis, and this effect could at least in part explain the role of p53 (Miyashita & Reed, 1995). However, p53 is also linked to growth arrest and appears to activate expression of genes important in growth regulation, such as that encoding p21^{cip1/waf1} which inhibits cyclin-dependent protein kinases and promotes cell cycle arrest in G1 (el-Deiry *et al.*, 1993). Complex formation with the adenovirus E1B-55kDa protein inhibits p53 transactivation activity and represses basal transcription from p53-specific promoters (Yew *et al.*, 1990; Kao *et al.*, 1990; Yew & Berk, 1992; Yew *et al.*, 1994; Teodoro *et al.*, 1995; 1994) and thus the role of this viral protein in cell transformation may be to block p53-dependent growth arrest and apoptosis.

The human Saos-2 cell line was derived from an osteogenic sarcoma from a patient undergoing extensive treatment with chemotherapeutic agents including methotrexate, adriamycin, vincristine, cytoxan, and aramycin-C. Such compounds have been shown to induce apoptosis via p53-dependent mechanisms (Lowe *et al.*, 1993a). Saos-2 cells do not express p53, a property that could explain their survival during aggressive chemotherapy (Masuda *et al.*, 1987). Previous studies have addressed the consequences of reintroducing p53 into human tumor cell lines which have lost p53 expression (Chen *et al.*, 1990; Wang *et al.*, 1995). One limited study introduced p53 using an adenovirus vector, while other studies have generally been limited to expressing p53 by transfection followed by selection for drug resistant clones. The inherent difficulty in analyzing selected clones expressing genes which could potentially induce apoptosis or growth arrest is that in the process of selection, new defects in the pathways regulating these processes may be introduced. In the present studies Saos-2 cells were reconstituted with mutant or wild-type p53 using an adenovirus vector. In this manner virtually all cells in the culture were effectively reconstituted without the need for clonal selection, thus providing a more suitable system for the analysis of p53 function. Our results indicated that expression of p53 led to

induction of rapid cell death due to apoptosis which was prevented by expression of the Bcl-2 suppressor of cell death. Furthermore, we show for the first time that the adenovirus E1B-55kDa product blocks p53-dependent apoptosis.

2.3 Methods and Materials

Cells and viruses. Human Saos-2 cells (ATCC HTB 85), and Saos-2 derived cell lines were cultured on 60 mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in Dulbecco's modified eagle media (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were normally infected with adenovirus vectors expressing either *wt* (Adp53wt) or mutant (Adp53mut) p53, at a multiplicity of infection of 35 plaque-forming units per cell, as described previously (Rowe *et al.*, 1983). Both viruses express p53 proteins under the cytomegalovirus promoter in a vector that lacks both E1A and E1B. The construction of the p53-expressing adenovirus vectors used in these studies has been described in detail previously (Bacchetti and Graham, 1993). The vector expressing adenovirus type 5 E1B-55kDa was made with an amino-terminal 6xHis and heart muscle kinase site (HMK) tag and driven by the CMV promoter in an adenovirus vector similar to that used to express p53(Ad55k/His). The A₂₆₂ mutant was created by subcloning the KpnI-BglII restriction fragment from the previously described Ad2 A₂₆₂ construct (Kao *et al.*, 1990) into the corresponding sites at positions 2048 and 3328 bp in Ad5. This Ad5 A₂₆₂ mutant was similarly tagged with amino-terminal 6xHis and HMK sites and inserted into the CMV-driven adenovirus vector (Ad55k/A₂₆₂/His).

Transfection and selection of Bcl-2 expressing clones. Bcl-2 expressing cell lines were derived by transfecting Saos-2 cells at a confluency of 20% using the calcium phosphate co-precipitation method (Graham & van der Eb, 1972) with a construct which expresses a HA-tagged Bcl-2 protein and the neomycin resistance gene under the RSV promoter (Nguyen *et al.*, 1994). At 48 hours following DNA transfection, cells were selected with G418 at 1 mg/mL. Resistant clones were isolated and maintained in G418 at 400 µg/mL. The level of Bcl-2 expression was determined by Western blotting analysis using the 6C8 hamster antihuman Bcl-2 monoclonal antibody (Hockenbery *et al.*, 1990).

Western blotting for p53. The levels of p53 expression following infection with the recombinant adenoviruses were measured by Western blotting using the pAb1801 antihuman p53 monoclonal antibody (Oncogene Science) (Banks *et al.*, 1986). Whole cells

extracts were prepared from mock- or adenovirus vector-infected cells at 24 h post-infection, and were separated on a 10% (30:1, acrylamide:bis) SDS polyacrylamide gel prior to transfer to nitrocellulose sheets.

Cell viability assay. Cells were infected with Adp53wt or Adp53mut in 24 well plates at 80% confluence. At various points post-infection both adherent and non-adherent cells were pooled and assessed for viability by the Trypan Blue exclusion assay. At least 300 cells were counted for each time point.

Electron Microscopy. Mock- and adenovirus vector-infected cells were prefixed *in situ* at 40 h post-infection with 2% (v/v) glutaraldehyde and then post-fixed using 0.1M sodium cacodylate (pH 7.4) containing 1% (w/v) osmium tetroxide and 1% potassium ferrocyanide. Cells were stained with uranyl acetate and lead citrate and photographs were taken of representative fields using a Phillips 300 electron microscope.

Electromobility Shift Assay (EMSA). EMSA for p53 was performed using whole cell extracts from mock-infected cells and Adp53wt- or Adp53mut-infected cells at 24 h post-infection. 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 using [γ^{32} P]dCTP (New England Nuclear) and Klenow polymerase (Pharmacia). Electrophoretic mobility shift assays (EMSA) were performed in 20 μ L volumes containing 10 μ g of whole cell extract in 50mM HEPES-KOH (pH 7.9), containing 400mM KCl, 0.2mM EGTA, 0.2mM EDTA, 1mM DTT, 4 μ g BSA, 2.5% (v/v) Ficoll, 3 μ g sonicated salmon sperm DNA (Pharmacia), 100 μ g oligo dT and labeled probe containing 20,000-50,000 counts per min. In order to activate p53 DNA binding activity, 100 ng of the anti-p53 monoclonal antibody pAb421 (Oncogene Science) were used, as described previously (Hupp *et al.*, 1992). The EMSA reaction mixtures were incubated for 30 minutes at room temperature and then analyzed on a 4% polyacrylamide gel (30:1, acrylamide:bis) in 0.25 x TBE and subjected to electrophoresis at 200V for 2 h. Gels were dried and analyzed by autoradiography using Kodak X-OMAT AR film.

2.4 Results

Expression of p53 in human osteosarcoma cell lines induces rapid cell death. Human Saos-2 osteogenic sarcoma cells are immortal and multiply rapidly in cell culture. One possible reason for the failure of these cells to undergo normal growth arrest and apoptosis is the absence of functional p53. However, additional mutations in cellular oncogenes, tumor suppressor genes or cell death suppressor genes may also have accumulated which contribute to the altered proliferation of these cells and render them insensitive to the effects of p53. To determine if they are still capable of responding to p53-dependent regulation, Saos-2 cells were infected with adenovirus vectors expressing high levels of p53 under the cytomegalovirus (CMV) promoter and cell viability was measured by Trypan Blue exclusion assays. Fig. 2-1 shows that following infection by the vector Adp53wt which expresses the wild-type form of human p53, Saos-2 cells began to die rapidly such that by 60 h less than 10% of the culture was still viable. No significant death was observed in mock-infected cells or in cells infected with Adp53mut vector expressing a nonfunctional mutant form of p53. The specific p53 mutant used in these studies was a Cys to Ser conversion at position 135 which is a "hot-spot" mutation frequently observed in many human tumors (Chiou *et al.*, 1994; Zhuang *et al.*, 1995). To ensure that the failure of mutant p53 to induce cell death was not due to differences in expression, p53 levels present in Saos-2 cells were examined by Western blotting using p53-specific antibodies. Fig 2-2 shows the results obtained with cell extracts prepared 24 h following infection. No p53 was detected in mock-infected Saos-2 cells (lane 1), but high levels were found in Adp53wt-infected cells (lane 2) and Adp53mut-infected cells (lane 3). Thus introduction of wild-type p53 into Saos-2 cells appears to induce a p53-dependent cell death pathway.

Ultrastructural analysis of cells undergoing p53-dependent death. To distinguish if the p53-induced death of Saos-2 cells was due to apoptosis or necrosis, the morphology of Saos-2 cells infected with adenovirus vectors expressing mutant or wild-type p53 was examined by electron microscopy. The morphological changes of cells undergoing apoptosis have been studied by many groups and are clearly different from the effects of necrosis (Kerr *et al.*, 1972; Searle *et al.*, 1975). Fig. 2-3C shows that Saos-2 cells expressing wild-type p53 exhibit extensive cytoplasmic blebbing and, in this case, contain at least one darkly staining region of highly condensed chromatin. Both of these characteristics are classic hallmarks of apoptosis and were not apparent in mock-infected cells (Fig. 2-3A) or those infected adenovirus vector expressing mutant p53 (Fig. 2-3B). Thus the p53-induced cell death in Saos-2 cells appeared to be due to apoptosis.

Figure 2-1 Viability of Saos-2 cells following expression of p53.

Viability of Saos-2 cells following expression of p53. p53-deficient human Saos-2 cells were mock-infected or infected with adenovirus vectors Adp53wt or Adp53mut which express wild-type or mutant forms of human p53. Cells were tested for viability by a Trypan Blue exclusion assay at various times following infection. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in the Figure.

Saos-2 cells

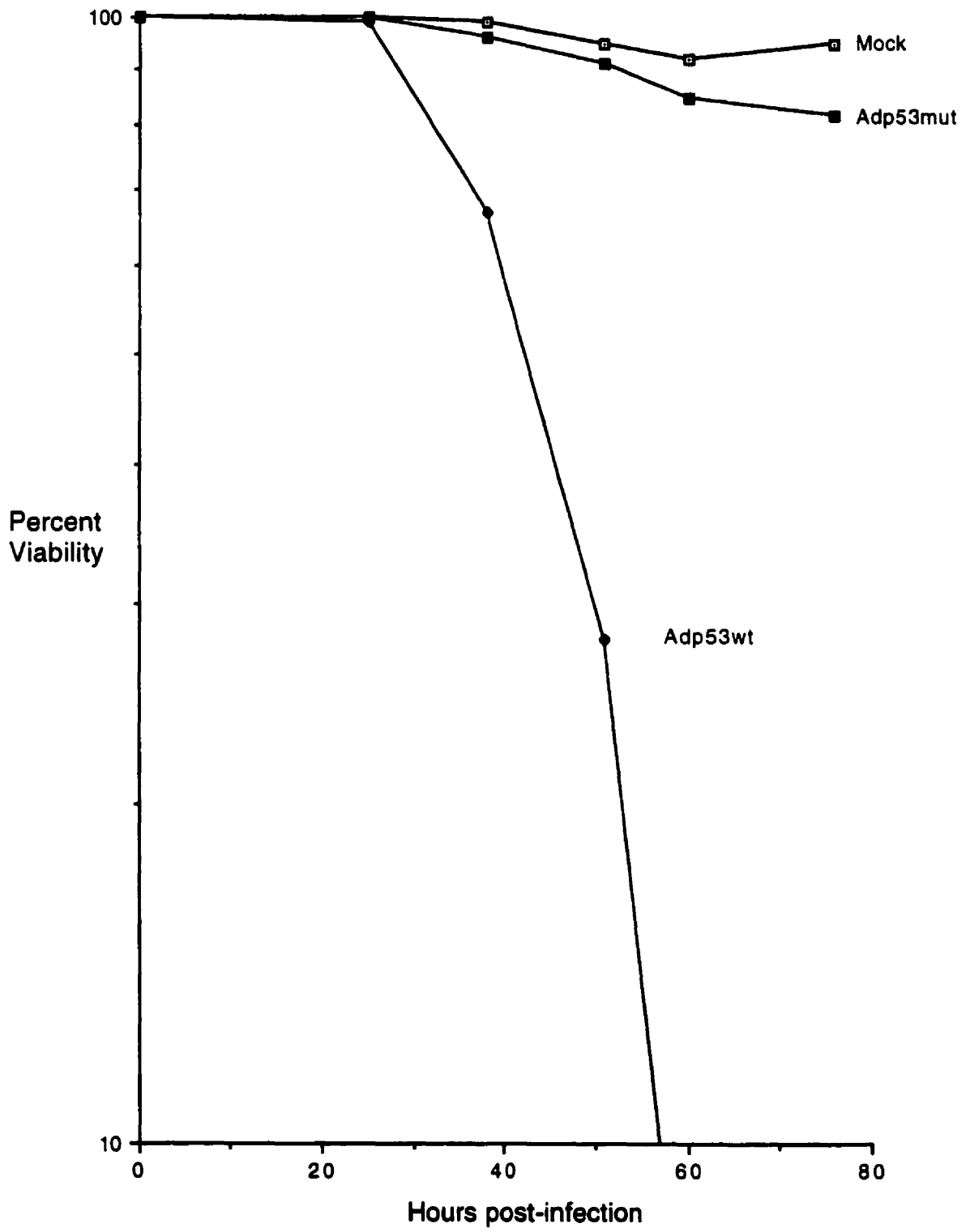


Figure 2-2 Analysis of p53 levels by Western blotting.

Equal amounts of Saos-2 cell extracts from the parental line used in Fig 2-1 and Bcl-2/neo (3g4) and control neo (2a1) expressing derivatives were separated by SDS-PAGE, transferred to nitrocellulose and analyzed by Western blotting using anti-p53 antibody. The lanes are as indicated in the figure.

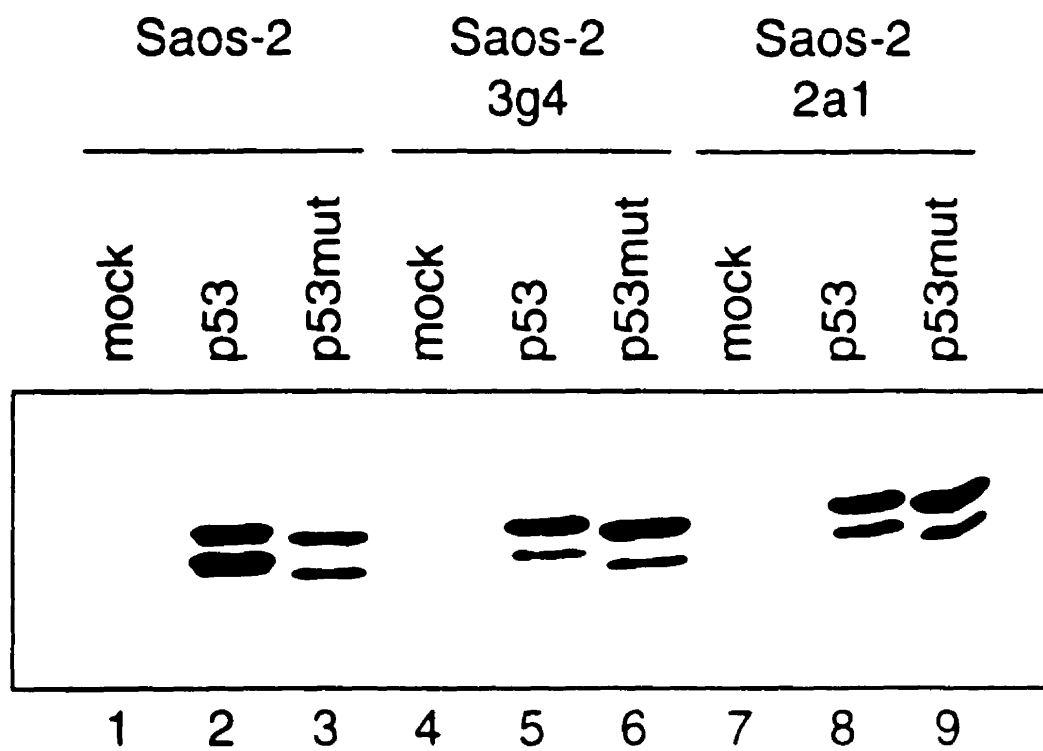


Figure 2-3 Analysis of cell morphology by electron microscopy.

Saos-2 cells from cultures described in Fig. 2-1 were harvested at 50 hours post-infection and analyzed by electron microscopy, as described in the Materials and Methods. Mock-infected (A); Adp53mut (B); Adp53wt (C). All photos show cells at an identical magnification. The bar in panel A represents 1 micron.

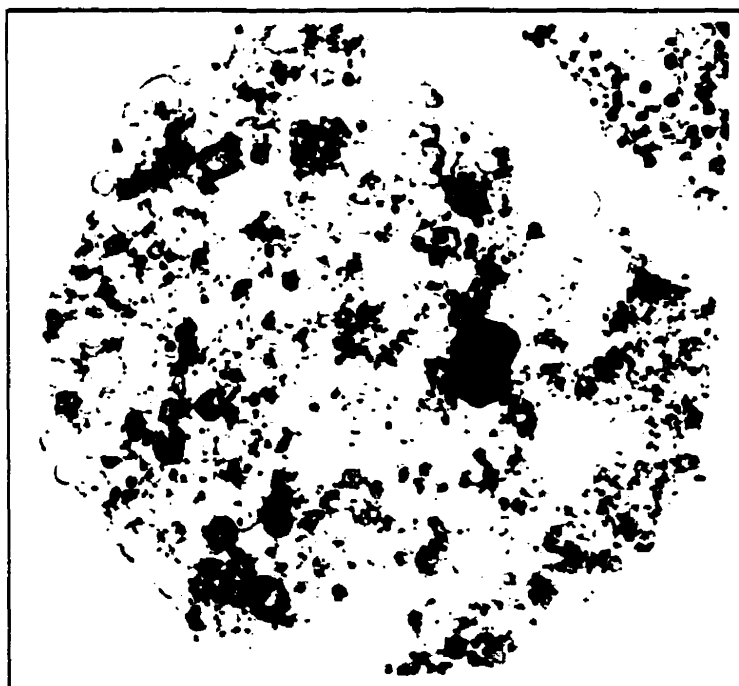
A



B



C



The Bcl-2 suppressor blocks p53-mediated death of Saos-2 cells. To establish further the mechanism of cell death induced by p53 expression in Saos-2 cells, Saos-2 cell lines were created which constitutively express Bcl-2 which is known to be an effective suppressor of apoptosis. Cells were transfected with plasmid DNA expressing the neomycin resistance gene and a hemagglutinin (HA)-tagged version of human Bcl-2 (see Materials and Methods). A series of cell lines expressing Bcl-2 at varying levels was isolated as were several control cell lines transfected with plasmid DNA containing *neo* alone (data not shown). Two representative cell lines were Saos2-3g4 which expresses high levels of Bcl-2 and a control *neo* cell line Saos2-2a1. These cells were infected with adenovirus vectors Adp53wt or Adp53mut, or they were mock-infected. Fig. 2-4 shows that whereas Saos2-2a1 control cells were readily killed by Adp53wt (Fig. 2-4A), Saos2-3g4 cells were largely protected, and even after 80 hours post-infection the majority of the cells were still viable (Fig. 2-4B). Again, neither cell line was affected by infection with Adp53mut or by mock infection. Fig 2-2 shows that the levels of p53 expression in the Bcl-2 expressing Saos2-3g4 and control Saos2-2a1 cells were similar (compare lanes 5 and 8 for Adp53wt, and lanes 6 and 9 for Adp53mut), and thus the observed protection was not due to reduced p53 levels. Similar results were obtained with other Bcl-2 expressing cell lines, and protection was generally in proportion to the level of Bcl-2 expression (data not shown). Bcl-2 is known to prevent apoptosis, but not necrosis, and has been shown to prevent apoptosis induced by p53 in previous studies in other cell systems (Chiou *et al.*, 1994). In Saos-2 cells, Bcl-2 protection of p53-induced apoptosis has only been shown previously in transient transfection experiments with a small population of transfected cells (Zhuang *et al.*, 1995). These data confirmed that the killing effect observed upon reconstitution with p53 is due to p53-induced apoptosis.

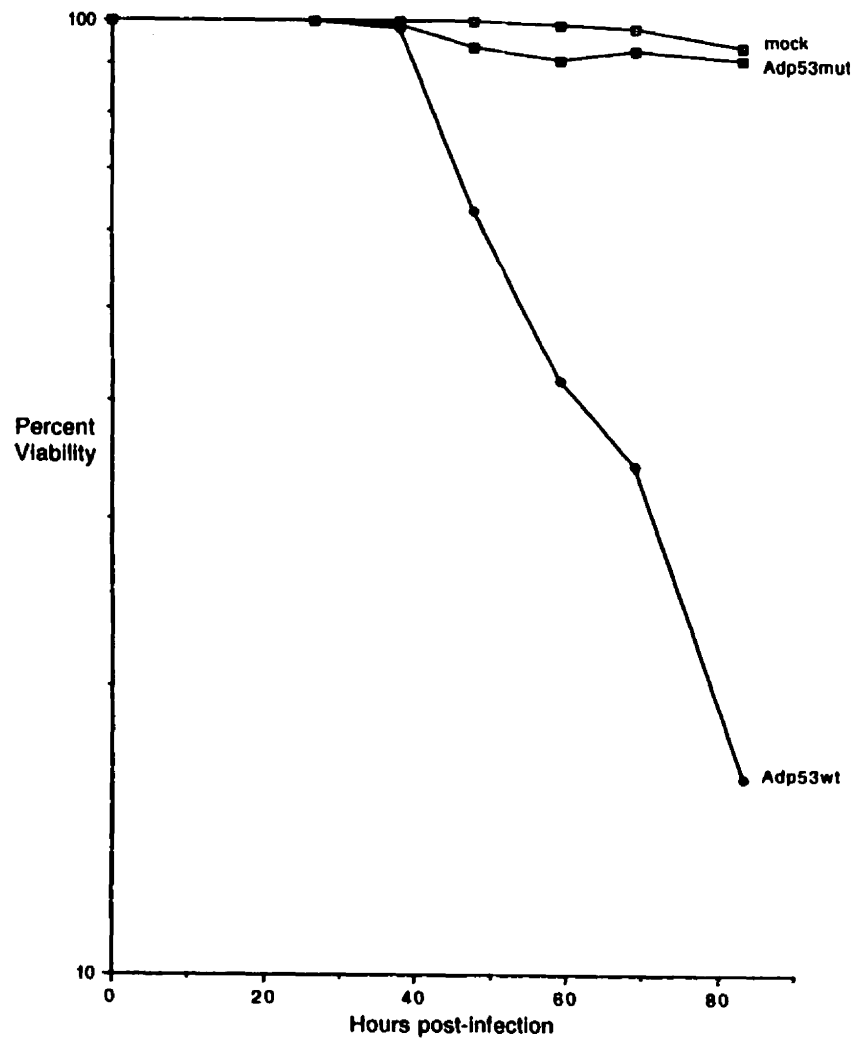
Ability of p53 to bind DNA correlates with induction of apoptosis. p53 binds to DNA in a sequence specific manner and many of its biological activities have been attributed to control of cellular genes involved in apoptosis such as that encoding Bax (Miyashita & Reed, 1995), and growth arrest such as p21^{cip1/waf1} (el-Deiry *et al.*, 1993). To study the biochemical properties of p53 expressed by the adenovirus vector in control and Bcl-2 expressing Saos-2 cells, the ability of p53 to bind to DNA in a sequence specific manner was determined. Whole cell extracts from cells which had either been reconstituted with wild-type or mutant p53 were subjected to electrophoretic mobility shift assays (EMSA) using a p53-specific radiolabeled oligonucleotide (see Materials and Methods). Such assays required addition of pAB421 antibody, and thus do not measure directly the DNA binding activity of p53 *in vivo*, but rather the ability of p53 molecules to be activated in the *in vitro*

Figure 2-4 Viability of normal and Bcl-2 expressing Saos-2 cells following expression of p53.

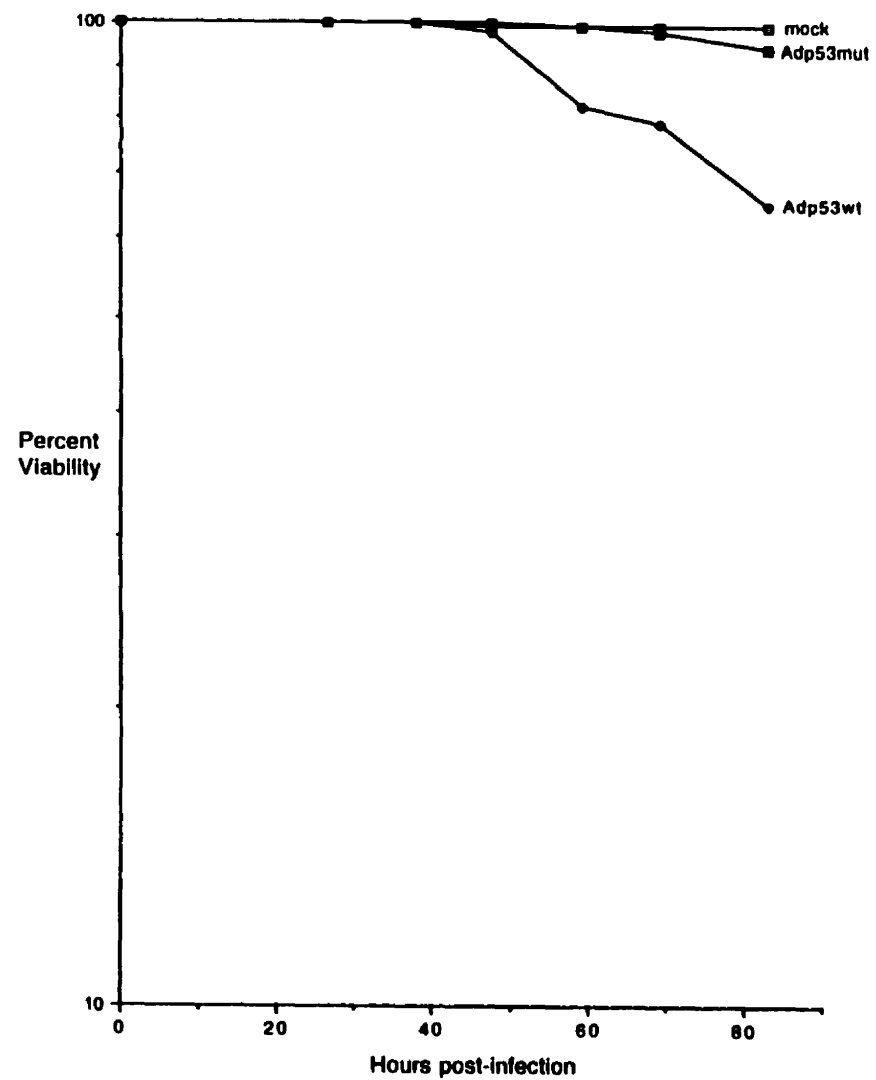
Control Saos-2/neo(2a1) cells (panel A) or Saos-2/Bcl-2(3g4) which express human Bcl-2 constitutively (panel B) were mock-infected or infected with adenovirus vectors Adp53wt or Adp53mut, and as in Fig. 2-1 cells were tested for viability by a Trypan Blue exclusion assay at various times following infection. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in the Figure.

A

Saos-2 / Neo (2A1)

**B**

Saos-2 / Bcl-2 (3g4)



assay. Fig. 2-5 shows that with extracts from cells expressing wild-type p53, a p53-specific DNA binding complex was detected in both Bcl-2 expressing Saos2-3g4 and control Saos2-2a1 cells (lanes 6 and 3, respectively). Fig. 2-5 also shows that mutant p53 possessed very little potential for DNA-binding (lanes 4 and 7). Thus p53 reconstituted into Saos-2 cells retains the potential for specific DNA-binding activity which was evident at similar levels even in Bcl-2 expressing cells which were protected from p53-induced apoptosis. Furthermore, mutant p53 which was shown to be defective for induction of apoptosis was also defective in DNA-binding activity, suggesting that such may be required for induction of apoptosis in this system.

Expression of the adenovirus E1B-55kDa protein blocks p53-dependent cell death in Saos-2 cells. Expression of the adenovirus early region 1A (E1A) oncogene induces both p53-dependent (Debbas & White, 1993; Lowe *et al.*, 1993b) and p53-independent (Teodoro *et al.*, 1995; Subramanian *et al.*, 1995; Marcellus *et al.*, 1996b) apoptosis in both rodent and human cells, including Saos-2 cultures (Teodoro *et al.*, 1995; Marcellus *et al.*, 1996b). Adenovirus E1B 19kDa and 55kDa proteins in both productive infection and cell transformation are believed to block E1A-induced apoptosis (White *et al.*, 1992; Rao *et al.*, 1992; Yew & Berk, 1992). The E1B-19kDa polypeptide is a functional analog of Bcl-2 and thus presumably functions largely downstream of p53 in the cell death pathway (White *et al.*, 1992; Rao *et al.*, 1992; Boyd *et al.*, 1994; Nguyen *et al.*, 1994). However, the E1B-55kDa protein specifically targets p53 by binding and inactivating p53 transactivation activity (Sarnow *et al.*, 1982; Yew *et al.*, 1990; Koa *et al.*, 1990; Yew & Berk, 1992; Yew *et al.*, 1994; Teodoro *et al.*, 1994). Although formal proof has been lacking, the E1B-55kDa protein was believed to inhibit p53-dependent apoptosis. We therefore examined this question directly by determining if p53-induced cell death in Saos-2 cells is affected by expression of the E1B-55kDa protein. Studies were conducted using Saos-2 cells co-infected by adenovirus vectors expressing p53 and either the wild-type E1B-55kDa polypeptide or a mutant, A₂₆₂, which is defective for binding p53 (Kao *et al.*, 1990). Fig. 2-6 shows that expression of wild-type E1B-55kDa protein suppressed cell death in cells expressing wild-type p53. Expression of the E1B mutant A₂₆₂ had no effect as cells died at the same rate as those which received wild-type p53 alone. In both cases the level of p53 expression was not affected by coexpression of the E1B-55kDa protein (data not shown). These results indicated that the E1B-55kDa protein was able to block p53-dependent apoptosis and suggested that direct p53 binding is essential for E1B-55kDa protection.

Figure 2-5 Electromobility-shift assays of Saos-2 cells expressing p53.

Extracts were prepared from Saos-2/neo(2A1) and Saos-2/Bcl-2(3g4) cells which had been mock-infected or infected with adenovirus vectors Adp53wt or Adp53mut as in Fig. 2-4, and were mixed with a ^{32}P -labeled p53-specific oligonucleotide probe and analyzed by polyacrylamide gel electrophoresis, as described in the Materials and Methods. Lane 1- no extract; lanes 2 to 4 - Saos-2/neo(2A1) cells; lanes 5 to 7 - Saos-2/Bcl-2(3g4) cells. Individual lanes are as indicated in the Figure. The positions of the 53-specific complex, a nonspecific complex and free probe have been presented at the right.

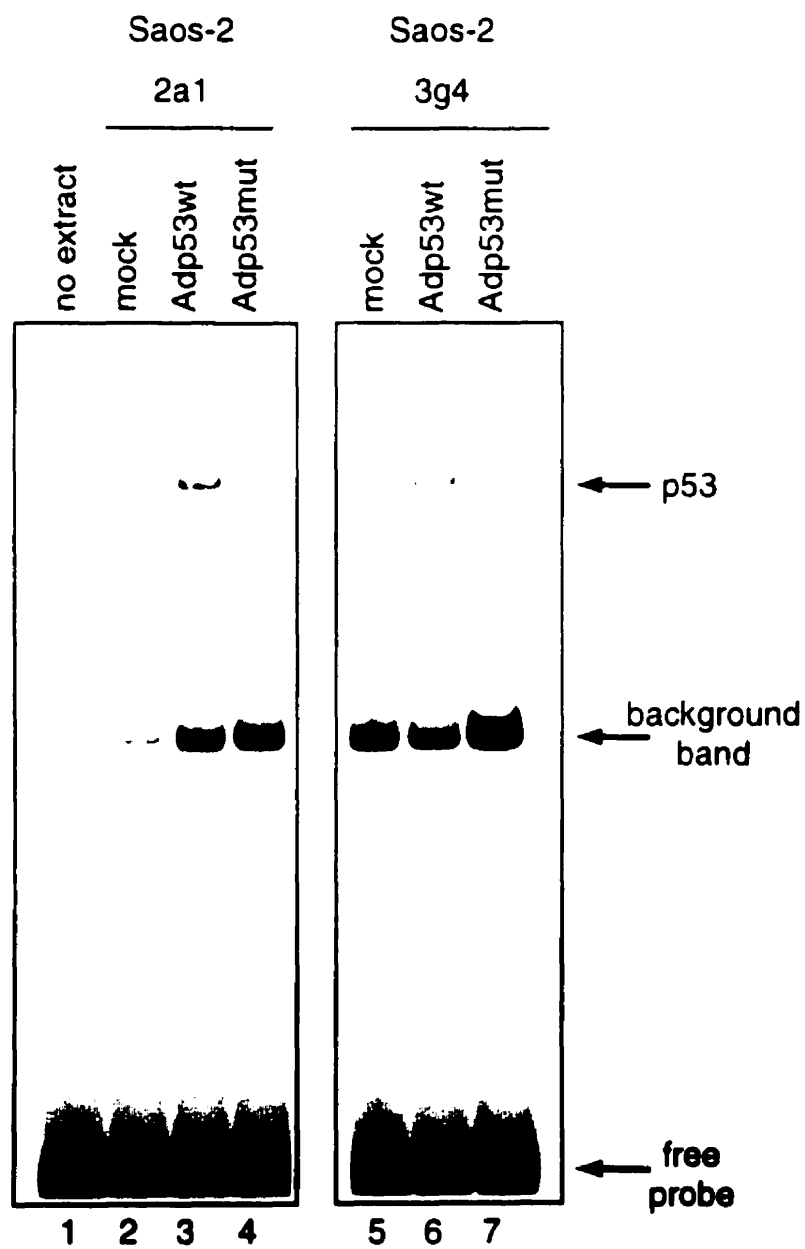
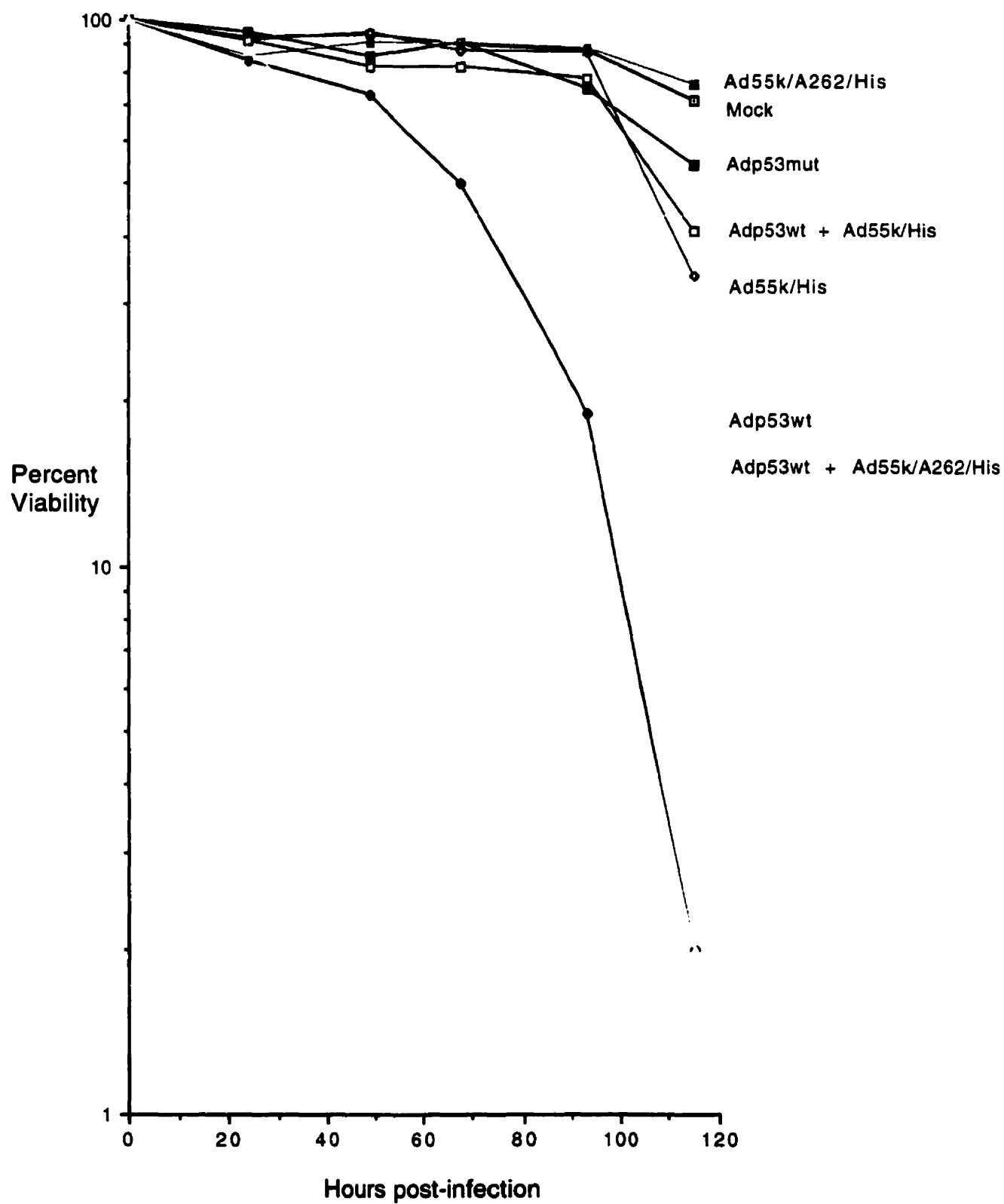


Figure 2-6 Effect of E1B-55kDa protein on viability of Saos-2 cells.

An experiment similar to that described in Fig. 2-1 was conducted except that some Saos-2 cells infected with Adp53wt or Adp53mut were coinfecting with adenovirus vectors expressing either wild-type or A₂₆₂ a mutant form of the adenovirus type 5 E1B-55kDa protein. Cells were tested for viability by a Trypan Blue exclusion assay at various times following infection. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in the Figure.

Saos-2 Cells



2.5 Discussion

The p53 protein induces apoptosis in response to various stimuli, including ionizing radiation and the expression of dominant oncogenes such as adenovirus E1A. In the present studies we demonstrated that reconstitution of p53 in human osteosarcoma Saos-2 cells using an adenovirus gene transfer vector results in the spontaneous induction of apoptosis without the need of any further stimulus. These data extend our understanding of the cell and molecular biology of the Saos-2 cell line which has become of increasing interest to several groups (Buckbinder *et al.*, 1994; Radinsky *et al.*, 1994; Yamoto *et al.*, 1995; Maheswaran *et al.*, 1995; Zhuang *et al.*, 1995; Wang *et al.*, 1995). Expression of a mutant form of p53 found frequently in human cancers had no effect on Saos-2 cells whereas with cultures reconstituted with wild-type p53 nearly all cells were dead by 80 hours. The observation that Saos-2 cells require no stimulus other than p53 suggests that the mechanism within these cells which triggers the apoptotic response is already primed and requires only p53 to execute. Saos-2 cells have also been shown to lack the expression of the *Rb* retinoblastoma tumor suppressor gene (Masuda *et al.*, 1987). Loss of pRB protein has been shown to cause apoptosis in a transgenic mouse system which is dependent upon p53 (Morgenbesser *et al.*, 1994). pRB functions at least in part to regulate the E2F family of transcription factors (reviewed in Nevins, 1991), and thus the *Rb*-negative state of Saos-2 cells would lead to large amounts of free E2F and aberrant triggering of DNA synthesis. Such inappropriate signals could activate apoptosis and may have lead to the original selection of p53-defective cells in the original tumor from which Saos-2 cells were derived.

Several previous studies attempted to determine the consequences of reconstituting p53 negative cells with p53 by conventional techniques, including infection by a retrovirus expressing p53 and a selectable marker to yield drug-resistant cell clones (Chen *et al.*, 1990). Cell death was not apparent in these studies; however, the limitation in such an approach is that if the introduction of p53 results in apoptosis, any cell clones derived either must contain defects in the p53 apoptosis pathway or must overexpress suppressors of apoptosis. The clear advantage of reconstituting cells using adenovirus vectors is that it allows the study of an unselected population of cells.

In the present studies we also showed that the Bcl-2 proto-oncogene protein is able to block p53-induced apoptosis in Saos-2 cells. Bcl-2 has been demonstrated to prevent apoptosis induced by a wide variety of stimuli including p53 (Chiou *et al.*, 1994; Zhuang *et al.*, 1995). The ability of Bcl-2 to block apoptosis induced by such a wide variety of stimuli

supports the belief that it functions at a downstream stage in the apoptotic pathway (Oltvai & Korsmeyer, 1994). This also confirms that the death of Saos-2 cells in response to p53 does in fact occur via apoptosis and not necrosis, as also indicated by the ultrastructure of the dying cells we observed by electron microscopy. Of even greater novelty were results obtained with the adenovirus E1B-55kDa protein. The E1B-19kDa product is functionally homologous to Bcl-2 and is able to block apoptosis induced by a variety of agents (White *et al.*, 1992; Rao *et al.*, 1992; Boyd *et al.*, 1994; Nguyen *et al.*, 1994). The E1B-55kDa protein is known to bind to the activation domain of p53 and block expression of p53-responsive genes (Yew *et al.*, 1990; Yew & Berk, 1992; Yew *et al.*, 1994; Teodoro *et al.*, 1994). The mechanism of inhibition is believed to derive from transcriptional repression activity resident in the 55kDa polypeptide which inhibits the p53 transactivation function (Yew & Berk, 1992; Teodoro *et al.* 1997b). The finding that expression of the wild-type E1B-55kDa protein prevented p53-induced apoptosis in Saos-2 cells, whereas the nonbinding mutant form encoded by A₂₆₂ did not, directly confirmed for the first time that the E1B-55kDa protein is able to suppress p53-dependent apoptosis, and that such inhibition correlates with p53 binding. Previous studies indicated that cells infected with adenovirus mutants defective in expression of the E1B-19kDa protein succumb rapidly to death by apoptosis whether or not the E1B-55kDa protein is expressed (White *et al.*, 1992; Nguyen *et al.*, 1994; Ezoe *et al.*, 1981; Pilder *et al.*, 1984; Subramanian *et al.*, 1984; White *et al.*, 1991). However, recent studies by our group and others indicated that adenovirus E1A proteins induce both p53-dependent and p53-independent apoptosis (Teodoro *et al.*, 1995; Subramanian *et al.*, 1995). The E1B-19kDa protein would be expected to suppress either pathway, but the E1B-55kDa polypeptide would only be effective against the former. The present results confirm this hypothesis, suggesting that the major role for this protein both in lytic infection and cell transformation is to reduce E1A-induced p53-dependent apoptosis.

The studies described here suggest that adenovirus vectors can serve as valuable vehicles for the delivery and study of potentially cytotoxic genes which would be difficult to express constitutively in tissue culture systems by more classical techniques of transfection and clonal selection. Such genes are expressed at high levels in virtually all cells in the culture, and their effects can be evaluated quite rapidly. Studies to elucidate E1A-induced cell death and the mechanism of suppression by E1B proteins are continuing in our group using this approach.

2.6 Acknowledgements

We wish to thank Frank Graham for the gift of vectors Adp53wt and Adp53mut. We are also indebted to Kathy Teng for her impeccable electron microscopy. This work was supported through grants from the Medical Research Council of Canada and the National Cancer Institute of Canada. R.C.M. held a Student Research Award from the Glaxo/Burroughs-Wellcome Corp. and J.G.T. a Research Studentship from the NCIC.

CHAPTER 3

Adenovirus 5 Early Region 4 Is Responsible
For E1A-Induced p53-Independent Apoptosis

3.1 Abstract

In the absence of E1B, the 289- and 243-residue (289R and 243R) E1A products of human adenovirus type 5 (Ad5) induce p53-dependent apoptosis. However, our group has shown recently that the 289R E1A protein is also able to induce apoptosis by a p53-independent mechanism (Teodoro *et al.*, 1995). Preliminary results suggested that p53-independent cell death required expression of one or more additional adenovirus early gene products. Here we show that both the E1B-19kDa protein and cellular Bcl-2 inhibit or significantly delay p53-independent apoptosis. Neither early regions E2 or E3 appeared to be necessary for such cell death. Analysis of a series of E1A mutants indicated that mutations in the transactivation domain and other regions of E1A correlated with E1A-mediated transactivation of E4 gene expression. Furthermore, p53-deficient human Saos-2 cells infected with a mutant which expresses E1B but none of the E4 gene products remained viable for considerably longer times than those infected with wt Ad5. In addition, an adenovirus vector lacking both E1 and E4 was unable to induce DNA degradation and cell killing in E1A-expressing cell lines. These data showed that an E4 product is essential for E1A-induced p53-independent apoptosis.

3.2 Introduction

Replication of human adenoviruses in terminally differentiated epithelial cells requires an efficient mechanism to induce cellular DNA synthesis to permit replication of viral DNA and production of progeny virus. Products of early region 1A (E1A) induce cell DNA synthesis and are largely responsible for cell transformation by adenoviruses (see reviews Bacchetti & Graham, 1993; Bayley & Mymryk, 1994; Branton *et al.*, 1985; and references therein). E1A produces two major mRNAs of 13S and 12S which encode proteins of 289 and 243 residues (289R and 243R, respectively) that are identical except for a central 46-amino acid sequence, termed conserved region 3 or CR3 (Kimelman *et al.*, 1985). Two additional regions present in the common sequence encoded by exon 1 of both E1A mRNAs are also conserved in all human serotypes and have been termed CR1 and CR2 (see Fig. 1A). E1A products induce DNA synthesis through complex formation between CR2 and CR1 and the retinoblastoma tumor suppressor pRB and related p107 and p130 proteins, or between the amino terminus and CR1 and the transcriptional modulator p300 and possibly related proteins (Barbeau *et al.* 1994; 1993; Dyson & Harlow, 1992b; Eckner *et al.*, 1994; Egan *et al.*, 1989; Egan *et al.*, 1988; Harlow *et al.*, 1986; Moran & Mathews, 1987;

Whyte *et al.*, 1988a; 1988b; 1989; Yee & Branton, 1985). E1A-289R also activates expression of the early viral transcription units E2, E3 and E4, and certain cellular genes at least in part through interactions with transcription factors and basal transcription machinery requiring CR3 (see reviews Akusjärvi, 1993; Bayley & Mymryk, 1994; Braithwaite *et al.*, 1991; Flint & Shenk, 1989; Nevins, 1991; Shenk & Flint, 1991). In addition to CR3, transactivation of the E4 promoter has also been shown to rely to some degree on two regions encoded by the second exon of 13S mRNA, termed auxiliary regions 1 and 2, or AR1 and AR2 (Bondesson *et al.*, 1992). Production of stably transformed cells requires early region 1B (E1B) which encodes polypeptides of 19 and 55kDa that are individually capable of cooperating with E1A via separate but additive pathways (Barker & Berk, 1987; Bernards *et al.*, 1986; McLorie *et al.*, 1991; White & Cipriani, 1989; Yew *et al.*, 1990; Zhang *et al.*, 1992).

Considerable evidence indicates that a major function of E1B proteins in lytic infection and cell transformation is to suppress cytotoxic effects and apoptosis induced by expression of E1A. Without E1B, the toxicity of E1A products results in the death of E1A-transformed cells and a reduction in the yield of progeny due to the early demise of productively infected cells. E1A proteins can cause apoptosis by a process mediated by the tumor suppressor p53 (Debbas & White, 1993; Lowe & Ruley, 1993), which controls growth arrest and programmed cell death pathways (Diller *et al.*, 1990; Finlay *et al.*, 1989; Ginsberg *et al.*, 1991; Youish-Rouach *et al.*, 1991). Expression of E1A products results in the elevation of p53 levels (Braithwaite *et al.*, 1990; Lowe & Ruley, 1993; Querido *et al.*, 1997). The 55kDa E1B protein binds to p53 (Sarnow *et al.*, 1982; Yew & Berk, 1992) and blocks both p53-mediated activation of gene expression (Teodoro *et al.*, 1994; Yew *et al.*, 1994) and apoptosis (Marcellus *et al.*, 1996a; Teodoro *et al.*, 1997b). The 19kDa E1B protein appears to suppress apoptosis by a mechanism that is functionally analogous to that of the cellular proto-oncogene product Bcl-2 (Boyd *et al.*, 1994; Nguyen *et al.*, 1994; Rao *et al.*, 1992; White *et al.*, 1992). Cells infected with adenovirus mutants which fail to express the 19kDa protein display enhanced cytotoxicity and extensive degradation of both cellular and viral DNA into nucleosome sized fragments (Ezoe *et al.*, 1981; Nguyen *et al.*, 1994; Pilder *et al.*, 1984; Subramanian *et al.*, 1984; White *et al.*, 1991; 1992).

Recently both our group (Teodoro *et al.*, 1995) and Subramanian *et al.* (1995) showed that in the absence of E1B, E1A products also induce p53-independent apoptosis. Our results indicated that such apoptotic cell death was only induced by the 289R E1A protein. Furthermore, when p53-null mouse cells constitutively expressing E1A products were

infected by an adenovirus vector lacking the entire E1A and E1B coding regions but containing early regions E2, E3 and E4, rapid cell death was observed (Teodoro *et al.*, 1995). Such cell death exhibited all of the hallmarks of apoptosis, including degradation of DNA to nucleosome-sized fragments, extensive chromosomal condensation, and formation of cytoplasmic vacuoles (Teodoro *et al.*, 1995). These results indicated that the role of E1A-289R may be to transactivate expression of an additional early transcript whose product actually induces p53-independent apoptosis. In the present studies we report that one or more E4 gene products appear to be responsible for such cell killing.

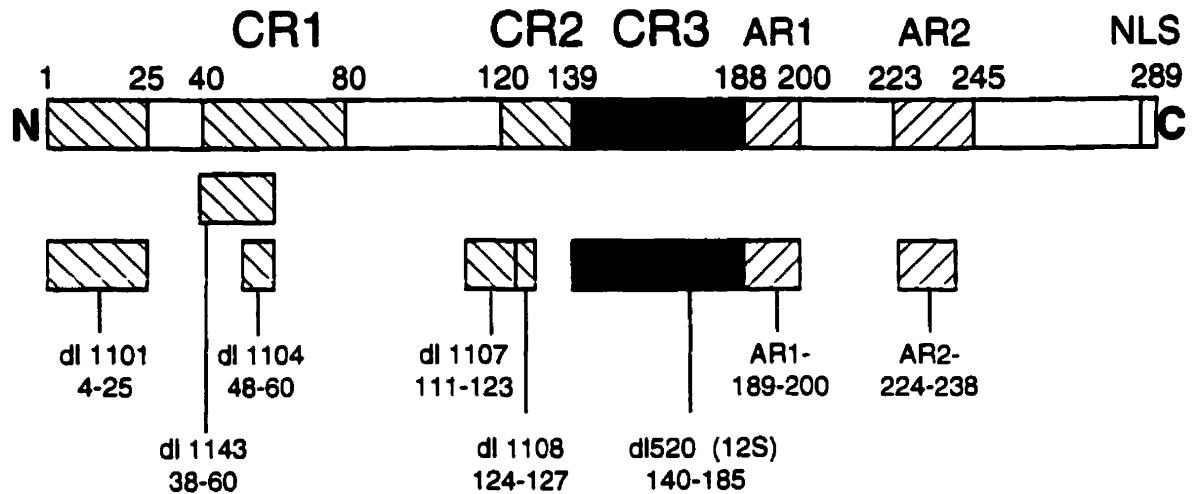
3.3 Materials and Methods

Cells and viruses. Human Saos-2 cells (ATCC HTB 85) and 10(1) mouse embryo fibroblast-derived cells (Harvey & Levine, 1991) which are both deficient for p53 expression were cultured on 60mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in Dulbecco's modified MEM (D-MEM) supplemented with 10% fetal calf serum (FCS) as were both NIH-3T3 and CHO cells. The cell line Saos-2/Bcl-2(3g4) which stably expresses Bcl-2 was derived for this study from Saos-2 cells by selection with G418, as previously described (Chen *et al.*, 1995), as was the control line Saos-2/Neo(2a2). 1A.A3, 1A.A6 and 1A.A12 mouse embryo fibroblast cell lines expressing Ad5 E1A proteins, and Hy.A3 hygromycin-selected control lines, have been described previously (Lowe *et al.*, 1994), and were cultured in D-MEM containing 10% FCS and 100 µg/mL of hygromycin. Normally cells were infected with mutant or wild-type (*wt*) Ad5 at a multiplicity of 100 pfu per cell, as described previously (Teodoro *et al.*, 1994). The virus used as *wt* has been described by Harrison *et al.* (1977), although in many cases the *wt* strain was *dl*309 (Jones & Shenk, 1979) which also was used in the preparation of E1A and E1B mutants which were propagated in 293 cells (Graham *et al.*, 1977). Ad5 E1A mutants are illustrated in Fig. 1A (see Figure 3-1 on page 93) and include deletion mutants *dl*1101 (residues 4-25 deleted), *dl*1143 (38-60), *dl*1107 (111-123), *dl*1108 (124-127), *dl*1143/08 (38-60 plus 124-127) and *dl*1132 (224-238) which have all been described previously (Jelsma *et al.*, 1988). A new E1A mutant was constructed as described previously (Dumont *et al.*, 1989), termed AR1⁻/E1B⁻, which lacks the entire AR1 region (residues 189-200) and also fails to express E1B products. Mutant AR2⁻/E1B⁻ was generated by introducing *dl*1132 (Mymryk & Bayley, 1993), which lacks residues 224-238, into a background that fails to express E1B proteins. Mutant AR1⁻/AR2⁻/E1B⁻ represents a combination of the latter two mutants. Additional E1A mutants containing single amino acid substitutions at various sites within CR3 were

Figure 3-1 Ad5 E1A mutants

A. Proteins encoded by some of the mutants used in the present studies have been presented, including the residues removed in deletion mutants. CR1, CR2, CR3, AR1 and AR2 (see text) have also been indicated. **B.** Other adenovirus mutants. A list providing the names and defects of E1B and other mutants has been presented.

A



B

Virus Mutant	Description
wt Ad5	wt E1A (12S & 13S mRNAs), wt E1B. In some cases <i>dl</i> 309 which has a partial deletion of E3 was used
12S / E1B-	12S E1A mRNA only, no E1B expression
13S / E1B-	13S E1A mRNA only, no E1B expression
E1B / 55K-	wt E1A (12S & 13S mRNAs), no E1B 55K expression, wt E1B 19K
E1B / 19K-	wt E1A (12S & 13S mRNAs), no E1B 19K expression, wt E1B 55K
<i>dl</i> 1101 / E1B-	12S/13S E1A mRNAs, E1A mutation as in fig. 1A, no E1B expression
<i>dl</i> 1104 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
<i>dl</i> 1107 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
<i>dl</i> 1108 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
<i>dl</i> 1143 / 08 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR1- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR2- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR1- / AR2- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AD147VL / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
AD171CS / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
AD185SG / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
<i>dl</i> 1019	wt E1A, E1B, E2 and E3, no E4 expression
AdLacZ	no E1A or E1B expression, wt E2, E3 and E4
Ad5 <i>dl</i> 70-8	no E1A, E1B or E3 expression, wt E2 and E4
AdRSVβ-gal.11	no E1A, E1B or E4 expression, wt E2 and E3

produced by subcloning appropriate restriction enzyme fragments from mutant E1A cDNA plasmids (Webster & Ricciardi, 1991) into genomic viral DNA, followed by rescue into virus by the method of McGrory *et al.* (1988) to form mutants AD147VL (Val-147 converted to Leu), AD177CS, and AD185SG. All other mutants have been summarized in Fig. 1B (see Figure 3-1 on page 93). Two were produced previously by our group (McLorie *et al.*, 1991) and fail to express E1B proteins of 19kDa (originally termed *pm1716/2072* but now called E1B/19K⁻) and 55kDa (originally *pm2019/2250*, now E1B/55K⁻). Mutant 12S/E1B⁻ (originally *dl520E1B⁻*) produces only the E1A-243R protein encoded by the 12S mRNA and no E1B products (Shepherd *et al.*, 1993). Mutant E1B⁻ which expresses both major E1A products but neither the 19kDa or 55kDa E1B species was described previously (Teodoro *et al.*, 1995), and a similar mutant that expresses only 289R in the absence of E1B, termed 13S/E1B⁻, was prepared for the present studies. A series of E1A mutants (*dl1101/E1B⁻*, *dl1107/E1B⁻*, AD147VL/E1B⁻, etc.) which express no E1B products was also produced by introducing E1A mutations into mutant E1B⁻ which expresses both 289R and 243R E1A products but no E1B (Teodoro *et al.*, 1995). The presence of mutations in all mutants was confirmed by DNA sequencing, restriction enzyme digestion, or Southern blotting, as described previously (Dumont *et al.*, 1989). Ad5 vectors used in this study included AdLacZ in which the E1 (E1A + E1B) region was replaced with the *E. coli* gene *lacZ* under the CMV promoter (Bacchetti & Graham, 1993), and Ad5dl70-8 which was generated by cotransfection of plasmids pAB7 and pBHG10 (Bett *et al.*, 1994) and which lacks both E1 and the entire E3 region (Bett & Graham, unpublished). These vectors and other E1A and E1B mutants were grown on human 293 cells (Graham *et al.*, 1977). Adenovirus vector AdRSVβgal.11 which lacks the entire E1 and E4 regions was prepared and propagated as described elsewhere (Brough *et al.*, 1996). In addition, some experiments were carried out with human adenovirus type 2 (Ad2) mutant *dl1019* which contains deletions that eliminate expression of all E4 products and which was propagated on W162 monkey cells, as described previously (Bridge & Ketner, 1989).

DNA fragmentation. Low molecular weight DNA was isolated from mock- or Ad5-infected cells using a modified Hirt extraction procedure (Hirt, 1967). For such experiments, 60mm-diameter plates of cells were harvested at 40 h post-infection and lysed in pronase lysis buffer comprised of 10mM Tris-HCl (pH 8) containing 5mM EDTA, 100mM NaCl, and 1 mg/mL (w/v) pronase to which SDS was added to 0.5% (w/v). Cell lysates were incubated at 37° C for 2 h and NaCl was added to a final concentration of 1M. Samples were then incubated overnight at 4° C and centrifuged at 15,000 x g for 30 min.

Extracted nucleic acids were treated with RNAase A and analyzed on 1% agarose gels stained with ethidium bromide. In all cases DNA extracted from equal numbers of cells was analyzed.

Cell viability assays. Cells were infected with *wt* or mutant Ad5 in 24-well plates containing 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.

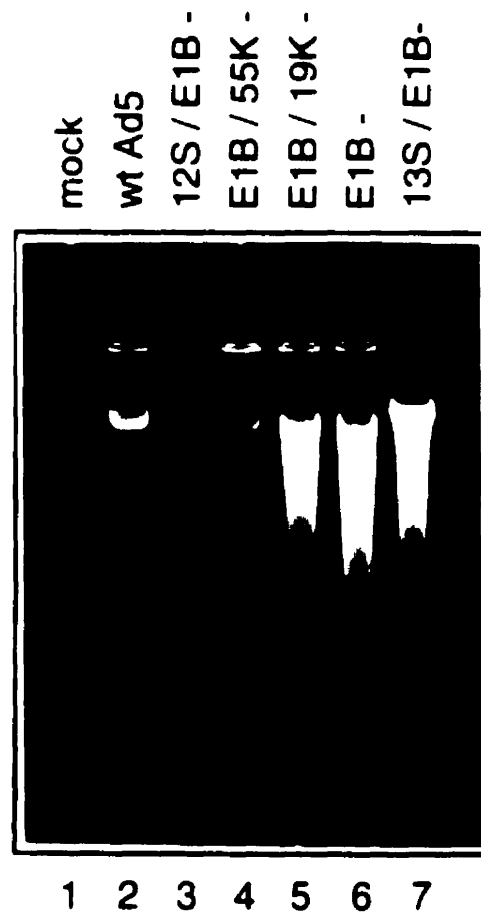
Measurement of E1A-mediated transactivation of the adenovirus E4 promoter. Transactivation assays were performed using NIH 3T3 or CHO cells plated at a density of 2×10^5 cells on 60 mm-diameter dishes. The E4 reporter plasmid was E4-CAT containing the E4 promoter upstream of the chloramphenicol acetyltransferase (CAT) gene (Weeks and Jones, 1983). Transient co-transfections were performed by the calcium phosphate precipitation method (Graham & van der Eb, 1973) using 2.5 μ g of reporter plasmid DNA and 2.5 μ g of DNA from plasmids expressing *wt* or mutant E1A products. The plasmid pSV2CAT (Gorman *et al.*, 1982) was used as a positive control. In addition, 3 μ g of RSV β -Gal plasmid (Pöpperl & Featherstone, 1992) was included to allow normalization of transfection efficiency by measuring β -galactosidase activity. Cells were glycerol shocked after 12 h and then harvested 36 h later. CAT assays were performed using cell extracts containing equal amounts of β -galactosidase activity, essentially as described previously (Gorman *et al.*, 1982). The amount of activity was quantified from TLC plates using a Fujix Bas 2000 Phosphorimager.

3.4 Results

E1A-induced p53-independent apoptosis is inhibited by both the E1B-19kDa protein and cellular Bcl-2. Previous studies indicated that whereas both major Ad5 E1A products could induce apoptosis in cells expressing p53, only the 289R E1A protein could do so in cells lacking p53 (Teodoro *et al.*, 1995; Subramanian *et al.*, 1995). Fig. 2 shows the pattern of DNA fragmentation in p53⁻ mouse 10(1) cells infected by various Ad5 mutants. Extracts from mock-infected cells (lane 1) and those infected with *wt* Ad5 (lane 2) which expresses E1B products displayed reduced levels of extracted DNA of low molecular mass and little or no degraded DNA, as did those from cells infected with mutant E1B/55K⁻ (lane 4) which produces the E1B-19kDa protein but not the E1B-55kDa product. With

Figure 3-2 Induction of DNA fragmentation by Ad5 mutants in the absence of p53

10(1) cells which fail to express p53 were infected with various Ad5 mutants, or they were mock-infected, and at 40 h p.i., low molecular weight DNA was analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in the Figure.



cells infected with mutant E1B⁻ which synthesizes both the 289R and 243R E1A proteins but which produces no E1B products (lane 6), large amounts of DNA were extracted and high levels of nucleosome-sized DNA fragments were evident. Similar results were also obtained with cells infected with E1B/19K⁻ (lane 5) which produces the E1B-55kDa species but not the 19kDa protein. Induction of DNA degradation in these p53⁻ cells did not occur following infection with 12S/E1B⁻ (lane 3) which produces only E1A-243R and no E1B, but it did occur with 13S/E1B⁻ (lane 7) which yields only E1A-289R in the absence of E1B products. Thus as shown previously, E1A-289R but not 243R induces p53-independent apoptosis in the absence of E1B proteins. In addition the results indicated that the E1B 19kDa polypeptide but not the 55kDa E1B product is able to protect against apoptosis induced by E1A in the absence of p53.

To examine the specificity of inhibition of apoptosis further, studies were conducted to determine if the cellular Bcl-2 protein is also able to prevent p53-independent apoptosis as several previous studies had shown that Bcl-2 and the E1B-19kDa protein may be functionally similar (Boyd *et al.*, 1994; Nguyen *et al.*, 1994; Rao *et al.*, 1992; White *et al.*, 1992). Human Saos-2 cells which are defective for synthesis of p53 were transfected with cDNAs encoding the human Bcl-2 protein and the neomycin resistance marker and several cell lines were selected using G418 (see Materials & Methods). One such Bcl-2 expressing clone, termed Saos-2/Bcl-2(3g4), and a control Saos-2 clone, Saos-2/neo(2a2) selected only for resistance to G418, were infected with wt Ad5, mutants 12S/E1B⁻ or E1B/19K⁻, or were mock-infected, and cell viability assays were conducted at various times after infection. Fig. 3A shows that Saos-2/neo(2a2) control cells were killed by the E1B/19K⁻ virus that expresses E1A-289R, but those infected with wt or 12S/E1B⁻ remained almost as viable as mock-infected cells during the test period. Fig. 3B shows that with Saos-2/Bcl-2(3g4) cells which stably express high levels of Bcl-2 (data not shown), little cell death was induced by the E1B/19K⁻ virus. Similar results were obtained with three other control and Bcl-2 producing Saos-2 cell lines (data not shown). Thus like the E1B-19kDa protein, Bcl-2 also blocks E1A-induced p53-independent apoptosis.

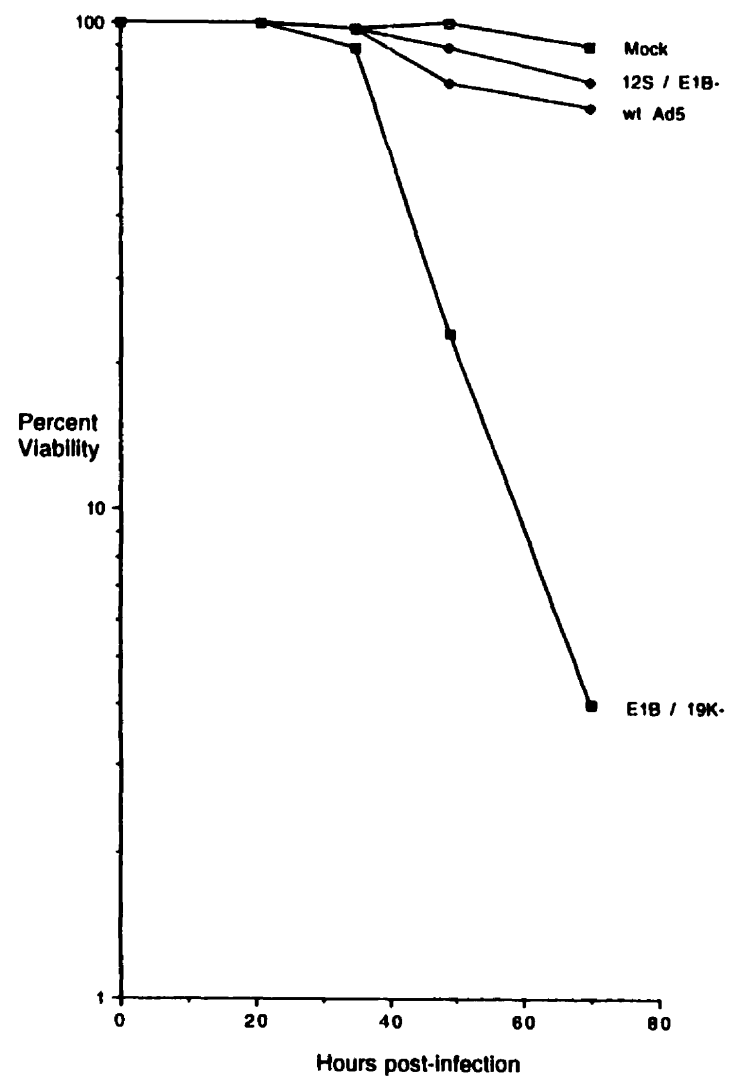
Role of E1A domains in p53-independent apoptosis. To investigate the regions of E1A products involved in causing p53-independent cell death, p53⁻ mouse 10(1) cells were infected with Ad5 mutants which fail to express E1B and which harbor a variety of defects at various regions of the E1A molecule. Extracts were harvested and analyzed on gels to determine the extent of degradation of low molecular weight DNA. The levels of E1A expression were analyzed in parallel by Western blotting using E1A-specific M73

Figure 3-3 Viability of infected normal and Bcl-2 expressing SAOS-2 cells

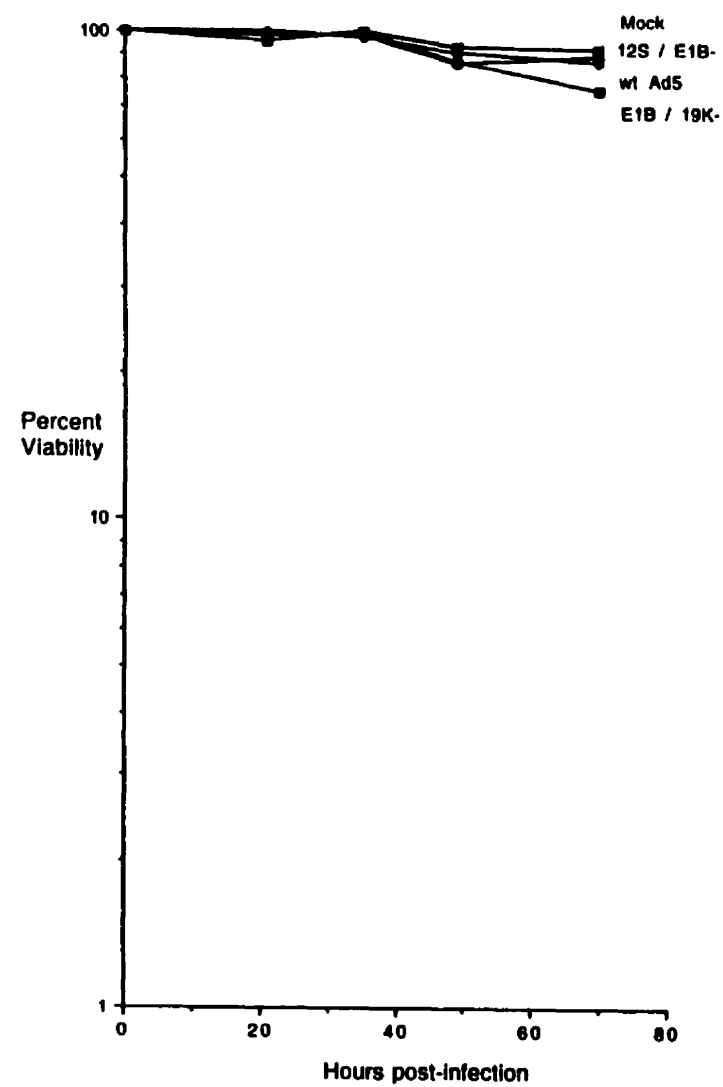
p53-deficient human Saos-2/neo(2a2) cells (panel A) or Saos-2/Bcl-2(3g4) which express human Bcl-2 constitutively (panel B) were mock-infected or infected with *wt*, E1B/19K⁻ or 12S/E1B⁻ and were tested for viability by a Trypan Blue exclusion assay at various times following infection, as described in Materials and Methods. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in the Figure.

A

Saos-2 / Neo (2a2) cells

**B**

Saos-2 / Bcl-2 (3g4) cells



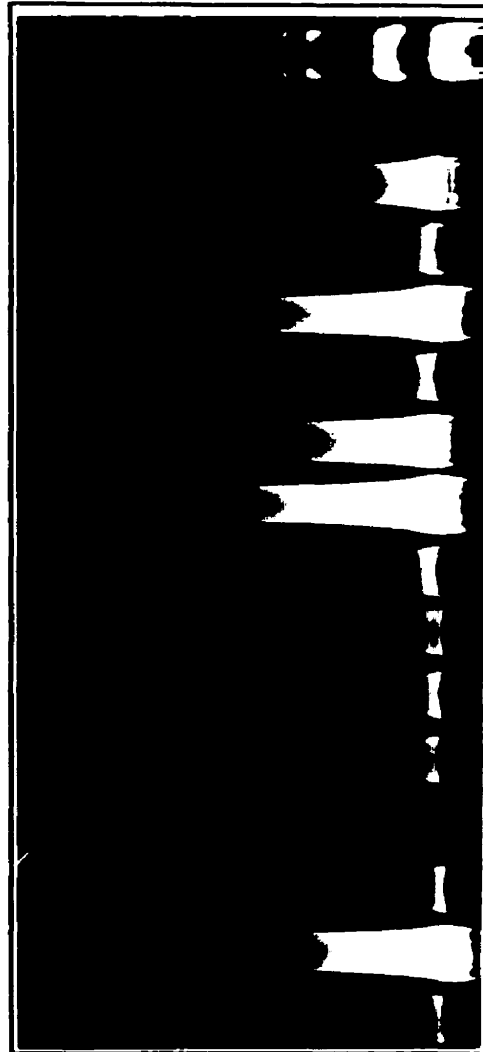
monoclonal antibody and were found to be comparable in all cases (data not shown). Fig. 4 shows that again mutant E1B/19K⁻ (lane 3) induced DNA degradation characterized by the appearance of high levels of extracted DNA and nucleosome-sized DNA fragments whereas such did not occur with *wt* Ad5 (lane 2) or mock-infected cells (lane 1). Mutants which affected the E1A transactivation function associated with CR3 all failed to induce DNA degradation. These included 12S/E1B⁻ (lane 8), and point mutants AD147VL/E1B⁻, AD171CS/E1B⁻ and AD185SG/E1B⁻ (lanes 9 to 11, respectively) which carry single residue substitutions at critical residues in CR3 that eliminate E1A transactivation activity (Webster and Ricciardi, 1991). In addition, deletion of AR1 or both AR1 and AR2 (AR1⁻/E1B⁻ and AR1⁻/AR2⁻/E1B⁻ in lanes 12 and 14) also eliminated DNA degradation whereas removal of AR2 alone (AR2⁻/E1B⁻ in lane 13) had little effect. Interestingly, mutants in CR2 which eliminate complex formation with pRB and related proteins (*dl*1107/E1B⁻ and *dl*1108/E1B⁻ in lanes 5 and 6) had no effect on the induction of DNA degradation, whereas those that eliminated binding of p300 by removal of the N-terminus (*dl*1101/E1B⁻ in lane 4) or a portion of CR1 as well as the pRB binding site (*dl*1143/08/E1B⁻ in lane 7) no longer caused this effect. These results suggested that E1A-induced p53-independent apoptosis required the CR3 transactivation domain, AR1, and the regions necessary for binding of p300 but not pRB-related proteins. Fig. 5 shows that similar results were obtained with these mutants in cell killing assays. Cell death was induced by the E1B/19K⁻ virus which expresses both E1A products and by *dl*1107/E1B⁻. Mutant AR2⁻/E1B⁻ which lacks AR2 also killed, but was consistently less toxic than the former viruses. All other mutants affecting CR3, AR1 and the p300 binding sites failed to kill significantly during the test period.

Activation of E4 expression and apoptosis. The requirement for AR1 suggested that E4 products might somehow be involved in the induction of p53-independent apoptosis as this region is not important in the activation of other early viral transcription units (Bondesson *et al.*, 1992). Studies were therefore carried out to examine the pattern of E1A transactivation of the E4 promoter in which plasmid DNA encoding various mutant forms of E1A-289R was co-transfected into NIH-3T3 or CHO cells along with DNA from E4-CAT, a construct that encodes CAT under the control of the Ad5 E4 promoter (Weeks & Jones., 1983). Table 3-1 shows that in addition to CR3, activation of the E4 promoter required AR1 and to some extent AR2. In addition, regions at the N-terminus and in CR1

Figure 3-4 Induction of DNA fragmentation by E1A mutants

An experiment in p53-deficient 10(1) cells similar to that described in Fig. 2 was performed using a series of Ad5 E1A mutants defective in expression of E1B products. The contents of individual lanes are as indicated in the Figure.

1
2
3
4
5
6
7
8
9
10
11
12
13
14



Markers

Mock

wt Ad5

E1B / 19k-

dl 1101 / E1B-

dl 1107 / E1B-

dl 1108 / E1B-

dl 1143 / 08 / E1B-

12S / E1B-

147VL / E1B-

171CS / E1B-

185SG / E1B-

AR1- / E1B-

AR2- / E1B-

AR1- / AR2- / E1B-

Figure 3-5 Analysis of viability of SAOS-2 cells infected with E1A mutants

An experiment similar to that described in Fig. 3 was carried out in SAOS-2 cells infected with various E1A mutants defective in expression of E1B products. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in the Figure.

[illegible]

involved in binding of p300 also were of some importance. These results closely paralleled the pattern of E1A-induced p53-independent apoptosis and suggested that E4 products might be involved.

Table 3-1 E4 Transactivation by E1A mutants

Virus	Residues deleted	Regions affected	E4 CAT activity (% wt $\pm$ SD) ^a
wt	None	None	100
<i>dl</i> 1101	4-25	N terminus	30 $\pm$ 11
<i>dl</i> 1104	48-60	CR1	40 $\pm$ 5
<i>dl</i> 1107	111-123	CR2	85 $\pm$ 5
<i>dl</i> 1108	124-127	CR2	81 $\pm$ 14
<i>dl</i> 520	140-185	CR3	10 $\pm$ 7
AR1 ⁻	189-200	AR1	25 $\pm$ 7
AR2 ^b	224-238	AR2	64 $\pm$ 16

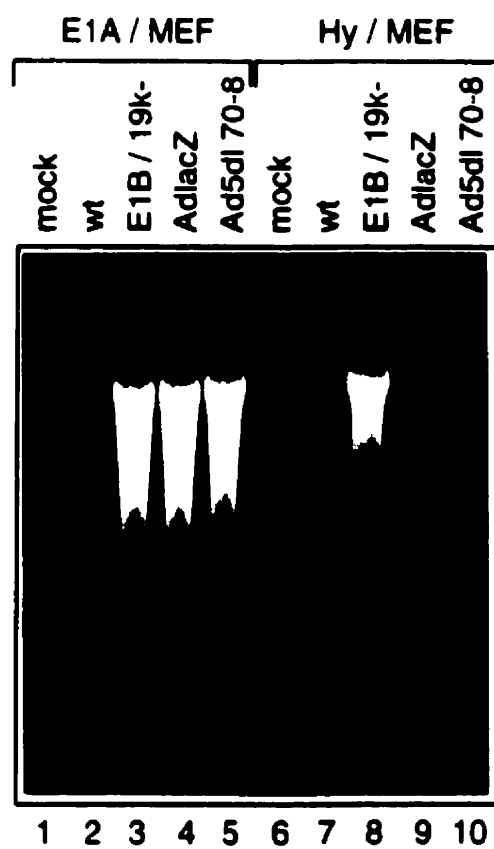
a. CHO or NIH 3T3 cells were transfected with plasmid DNA encoding various E1A mutants and CAT under the control of the Ad5 E4 promoter. Cell extracts were assayed for CAT activity as described in Materials and Methods. Three independent assays were done for each mutant.

b. *pm* 1132

E2 and E3 products are not required for apoptosis. E2 products were probably not responsible for the induction of p53-independent apoptosis for two reasons. First, in addition to CR3, complex formation involving CR2 and the pRB family of proteins activates E2 expression (Kovesdi *et al.*, 1987), and CR2 was shown to be of little importance in cell killing. Second, reasonably high levels of expression of E2 proteins are known to be induced by the E1A-243R protein (Tremblay *et al.*, 1985) which is completely unable to induce p53-independent apoptosis. Thus experiments were carried out to determine if any E3 products were involved. The 1A.A3 mouse embryo fibroblast cell line lacking p53 but expressing Ad5 E1A proteins, and Hy.A3 hygromycin-selected p53⁻ control cells (Lowe *et al.*, 1994), were infected with *wt* Ad5, the E1B/19K⁻ virus, adenovirus vector AdlacZ which contains *lacZ* in place of E1A and E1B, or with vector Ad5dl70-8 which lacks both the entire E1 and E3 regions. Cell extracts were assayed for the presence of degraded DNA as before. Fig. 6 shows that high levels of DNA degradation were induced in 1A.A3 cells with the E1B/19K⁻ mutant (lane 3) as well as both adenovirus

Figure 3-6 Induction of DNA degradation in the absence of E3

Cell lines expressing 289R and 243R E1A proteins constitutively, or the Hy.A3 non-expressing control cell line, were mock-infected or infected with *wt* Ad5 or adenovirus vectors AdLacZ or d5dl70-8. After 40 h, DNA was extracted and analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in the Figure.



vectors AdlacZ (lane 4) and Ad5dl70-8 (lane 5). Similar results were also obtained with two other comparable p53-null, E1A-expressing cell lines, 1A.A6 and 1A.A12 (data not shown). Fig. 6 also shows that in the control cells lacking constitutive E1A expression, only the E1B/19K⁻ virus (lane 8) induced DNA degradation. These results indicated that E3 products were not required for induction of p53-independent apoptosis by E1A under these conditions.

E4 proteins are essential for p53-independent apoptosis. To determine directly if E4 products are involved in the induction of cell death, as suggested by experiments described above, two approaches were taken. In the first, human p53⁻ Saos-2 cells were infected with *wt* Ad5 or Ad2, or with Ad2 mutant *dl1019* which produces no E4 proteins (Bridge & Ketner, 1989), or they were mock-infected. Although such viruses express E1B proteins and thus are protected from E1A-induced apoptosis, it was thought that if E4 products were essential for p53-independent cell death, some difference in long term cell survival might be observed, and thus at various times up to 10 days, infected cultures were tested for cell viability. Fig. 7 shows that cells infected by *wt* Ad5 virus (or Ad2, data not shown) began to die at about 100 h p.i., and by 240 h p.i. almost all of the cells were dead. Such was not the case with *dl1019*-infected cells which remained almost as viable as mock-infected cells even 10 days after infection. These results indicated that an E4 product was required for cell killing in the absence of p53. This idea was confirmed in experiments involving infection of E1A-expressing p53⁻ 1A.A3 cells with the adenovirus vector AdRSVβgal.11 in which both the E1 and E4 regions had been completely deleted (Brough *et al.*, 1996). Fig. 8 shows that in control Hy.A3 p53⁻ cells which do not express E1A, only the E1B/19K⁻ Ad5 mutant caused DNA degradation (lane 3), and neither *wt*, 12S/E1B⁻ (lane 4) nor the AdRSVβgal.11 vector (lane 5) had any significant effect. With 1A.A3 cells, both the E1B/19K⁻ (lane 8) and 12S/E1B⁻ (lane 9) viruses induced DNA degradation. Importantly, with the AdRSVβgal.11 vector (lane 10), even though somewhat higher levels of DNA were present, little or no nucleosome-sized DNA fragments were apparent. Similar results were obtained with the other two sister cell lines, 1A.A6 and 1A.A12 discussed above (data not shown). The ability of this virus to induce apoptosis in 1A.A3 cells was analyzed further in cell killing experiments. Fig. 9A shows that in the Hy.A3 control cells, only the E1B/19K⁻ virus induced cell death, whereas in 1A.A3 cells both the E1B/19K⁻ and 12S/E1B⁻ viruses did so. However in both cases the AdRSVβgal.11-infected cells remained as fully viable as mock-infected cultures. In addition, these cultures were seen by light microscopy to be virtually indistinguishable from mock-

Figure 3-7 Role of E4 products in p53-independent cell killing

SAOS-2 cells were mock-infected or infected with *wt* Ad5 or Ad2, or *dl*1019 which expresses no E4 products. At various times up to 10 days cell viability was assessed by Trypan Blue exclusion. Data have been expressed as % cell viability and symbols are as indicated in the Figure.

Saos-2 cells

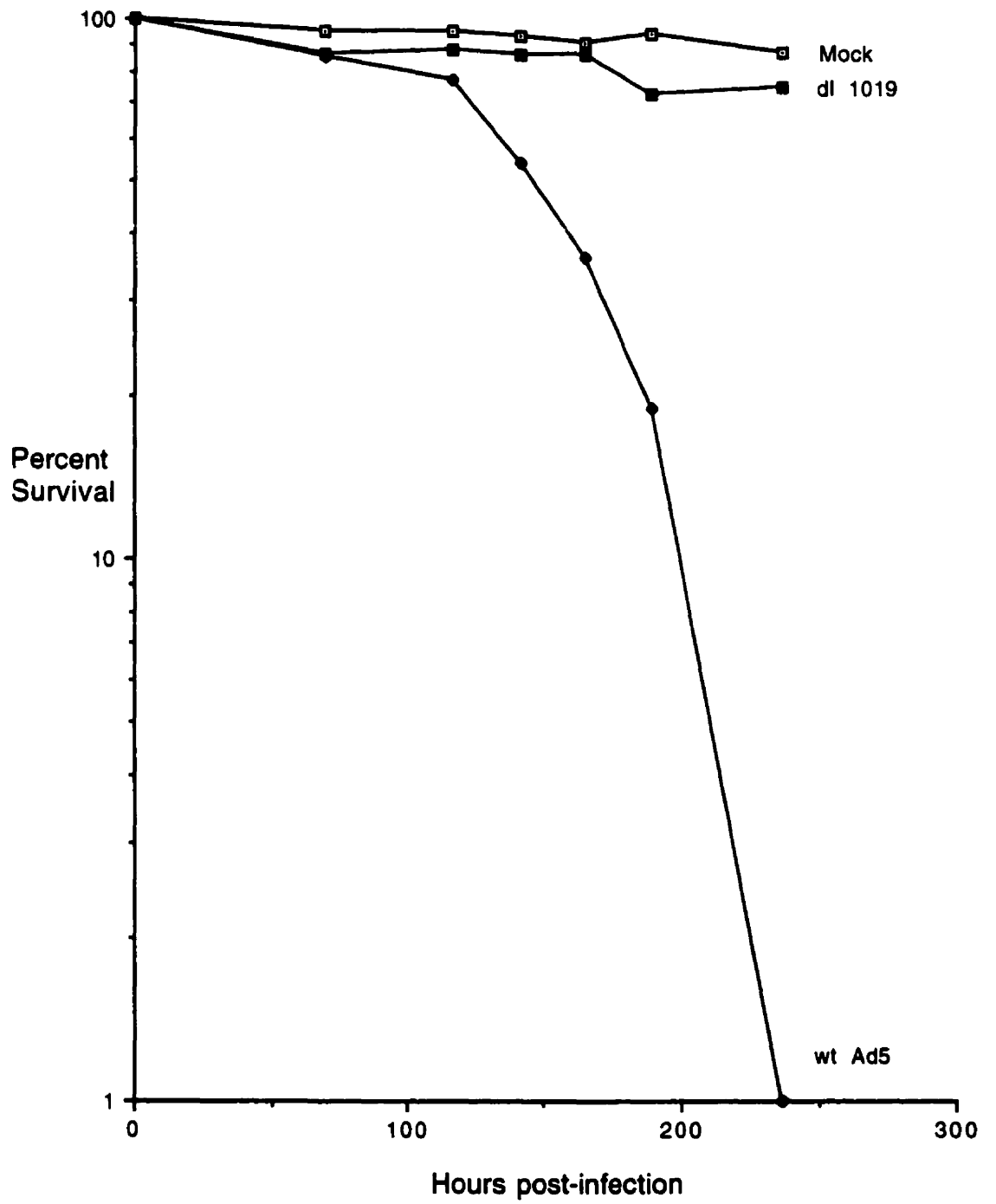


Figure 3-8 Analysis of DNA degradation in the absence of E4

Cell lines expressing 289R and 243R E1A proteins constitutively, or the Hy.A3 non-expresser control cell line, were mock-infected or infected with *wt* Ad5, E1B/19K⁻, 12S/E1B⁻, or the adenovirus vector AdRSV β gal.11 which lacks both E1 and E4. After 40 h, DNA was extracted and analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in the Figure.

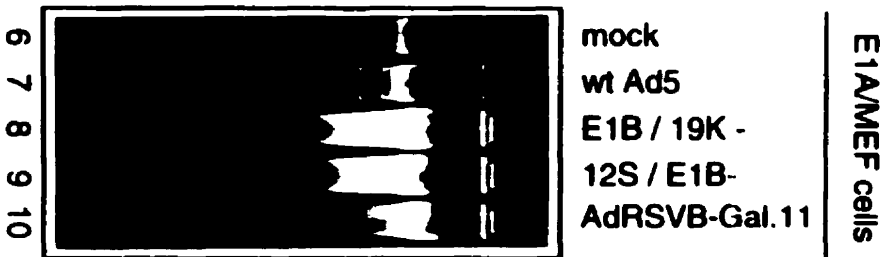
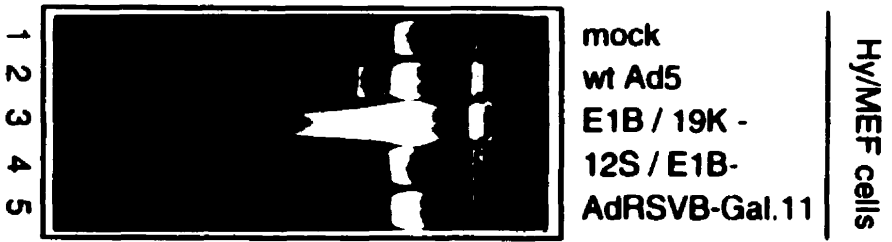
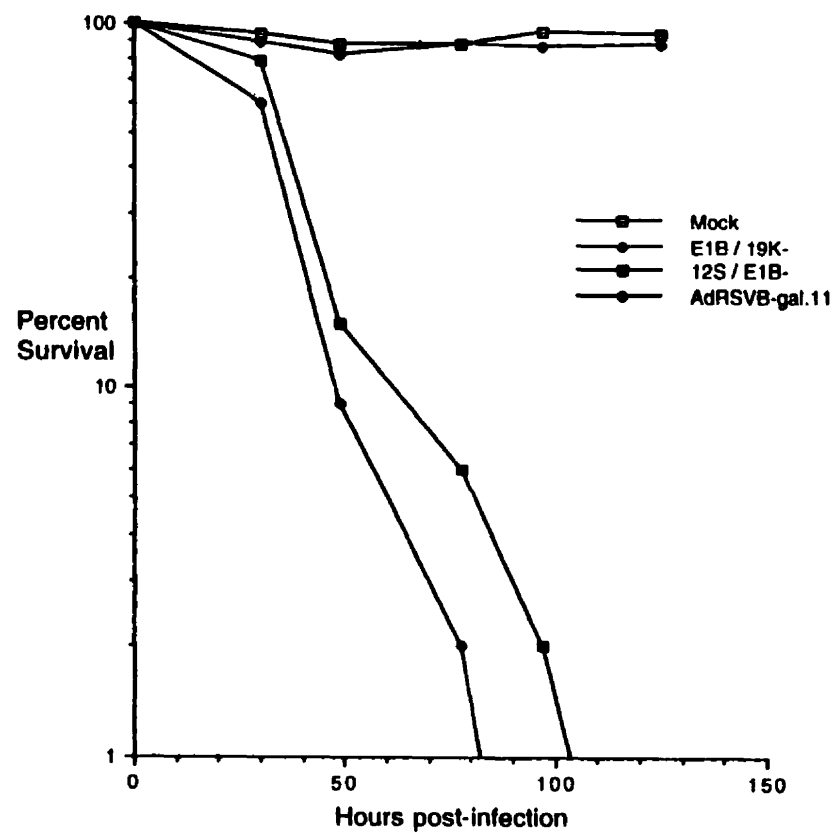


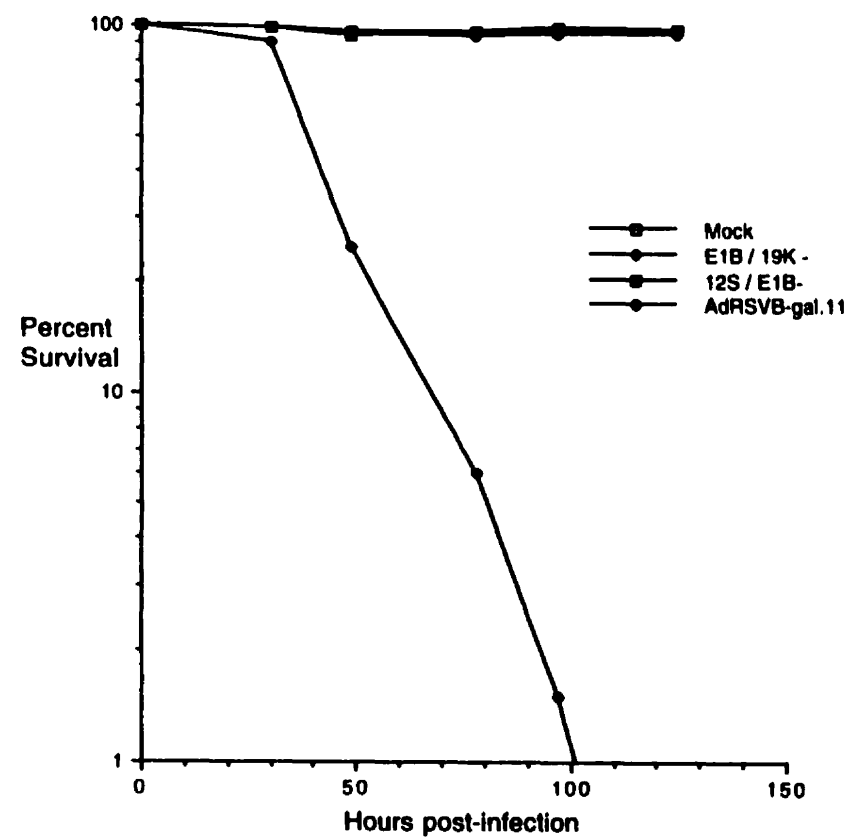
Figure 3-9 Analysis of p53-independent cell killing in the absence of E4 products

Cell lines expressing 289R and 243R E1A proteins constitutively (panel A), or the Hy.A3 non-expresser control cell line (panel B) were mock-infected or infected with *wt* Ad5, E1B/19K⁻, 12S/E1B⁻, or the adenovirus vector AdRSV β gal.11. At various times after cell viability was assessed by Trypan Blue exclusion. Data have been expressed as % cell viability and symbols are as indicated in the Figure.

A E1A/MEF (1AA6) cells



B Hy/MEF (A3) cells



infected cells and did not exhibit the rounded morphology and loss of adhesion typical of 1A.A3 cells infected by E1B/19K⁻ and 12S/E1B⁻ viruses. These data thus confirmed that an E4 product is responsible for E1A-induced p53-independent cell death.

3.5 Discussion

Adenovirus E1A products are known to induce DNA degradation, rapid cell death and other hallmarks of apoptosis when expressed in the absence of E1B products whose major role in lytic infection and transformation is to suppress E1A toxicity. Both the 289R and 243R E1A proteins are able to induce apoptosis through p53-dependent pathways (Debbas & White, 1993; Lowe & Ruley, 1993). This function appears to require regions of E1A proteins involved in complex formation with the pRB and p300 families of proteins and may be linked with the induction of cell DNA synthesis (Querido *et al.*, 1997; White, 1996; White *et al.*, 1991) and increase in levels of p53 (Braithwaite *et al.*, 1990; Lowe & Ruley, 1993; Querido *et al.*, 1997). This form of apoptosis is effectively blocked by expression of either the 19kDa (Ezoe *et al.*, 1981; Nguyen *et al.*, 1994; Pilder *et al.*, 1984; Subramanian *et al.*, 1984; White *et al.*, 1991; 1992) or 55kDa (Teodoro *et al.*, 1997b) E1B proteins. Thus infected or transformed cells are efficiently protected against this form of E1A-induced cell death.

E1A proteins also induce apoptosis in cells lacking p53 (Subramanian *et al.*, 1995; Teodoro *et al.*, 1995). We found that this p53-independent apoptosis was elicited only by the 289R E1A protein, and preliminary evidence suggested that expression of one or more additional early viral genes regulated by E1A-289R was required (Teodoro *et al.*, 1995). The present experiments indicated that the E1B-55kDa protein is unable to block this effect, but both the E1B-19kDa product and the cellular suppressor of apoptosis Bcl-2 significantly inhibited this response.

The major goal of this work was to identify which early viral transcription units were required to induce cell death in the absence of p53. Results obtained with E1A mutants clearly indicated the CR3 is important. Furthermore, CR3-mediated transactivation activity appeared to be required, as several point mutants in CR3 which were known to eliminate transactivation of target genes were defective for induction of DNA degradation and cell killing. Of great interest were results obtained with mutants with defects outside CR3. Mutant *dl1108* which lacks the core binding site for pRB and related proteins

induced p53-independent apoptosis like *wt*. However, mutant *d11101* which binds pRB at reasonably normal levels but fails to bind the p300 transcriptional modulator (Barbeau *et al.*, 1993; 1994) was totally defective. These results may suggest that interactions between p300 and 289R are essential to institute cell death pathways. Another possibility was offered by results obtained with two additional mutants with defects in the AR1 and AR2 regions encoded by the second exon of the 13S E1A mRNA. The AR1-defective mutant was unable to induce p53-independent apoptosis, and that lacking AR2 was somewhat impaired. These results corresponded exactly to the relative abilities of these mutant E1A molecules to transactivate the E4 promoter. We also found that *d11101* was partially defective for transactivation of E4, thus suggesting both that E4 products might be involved in induction of cell death and that interactions of 289R with p300 may reflect more a requirement for transactivation of E4 transcription than a direct role in apoptosis. This question will only be answered by further experiments using a new series of mutants.

Early regions E2, E3 and E4 encode a variety of products which could play some role in cell death. E2 proteins are largely involved in viral DNA synthesis (Horwitz, 1990). Although we did not examine the specific requirement for E2 products directly, it is unlikely that any play an essential role in cell death. First, E2 transcription requires not only CR3, but also the formation of complexes with pRB which result in the activation of the E2F family of transcription factors and E2 gene expression. Our results clearly indicated that complex formation with pRB was not essential for apoptosis. Second, the adenovirus vector AdRSV β gal.11 contains a *wt* E2 region and yet was defective for induction of p53-dependent apoptosis in E1A-expressing cells. The E3 region encodes several proteins which affect virus-host interactions, however, the adenovirus vector Ad5dl70-8 was fully capable of inducing apoptosis in E1A expressing p53-deficient cells. As discussed above, the pattern of apoptosis observed with E1A mutants suggested that the early viral proteins associated with cell death are encoded by E4. Direct evidence that an E4 protein is responsible was obtained from experiments in which the pattern of death was observed in p53-null Saos-2 cells infected by *wt* virus or a mutant defective in E4 expression. Because E1B products were expressed by these viruses, cell death occurred only at late times, but the observation that E4 mutant-infected cells displayed considerably retarded death clearly implicated an E4 product in the death process. Possibly cell death did not occur because E4 products are required for virus replication (Bridge *et al.*, 1993; Sandler & Ketner, 1989); however, induction of E1A-induced p53-dependent apoptosis does not require replication as the effect has been demonstrated to occur in the absence of the E1B-55kDa protein which is essential for replication, and in rodent cells in which

replication does not occur. Final confirmation came from results with the AdRSV β gal.11 adenovirus vector which was defective for cell killing. This virus was unable to induce DNA degradation or cell killing in p53-deficient cells expressing E1A.

At present we do not know which Ad5 E4 products are involved in the induction of p53-independent apoptosis. E4 is believed to encode seven proteins as determined by identification of open reading frames and sequencing of cloned E4 cDNAs (Fryer *et al.*, 1984; Hérisse *et al.*, 1981; Virtanen *et al.*, 1984). The E4orf6 product is a 34kDa protein known to associate with the E1B-55kDa polypeptide and to be involved with host shut off and transport of viral late mRNAs to the cytoplasm (Bridge & Ketner, 1990; Challberg & Ketner, 1981; Pilder *et al.*, 1986; Sandler & Ketner, 1989; Sarnow *et al.*, 1984). Induction of apoptosis is likely not the result of host cell shut off as cell death was induced efficiently in the absence of the E1B-55kDa protein which is absolutely required for this effect (Bridge & Ketner, 1990; Pilder *et al.*, 1984; Sandler & Ketner, 1989; Sarnow *et al.*, 1984). The E4orf3 protein is an 11kDa species is associated with the nuclear matrix and involved in accumulation of late viral mRNA (Downey *et al.*, 1983; Sarnow *et al.*, 1982), and along with E4orf4 and E4orf6 appears to affect viral DNA synthesis (Bridge *et al.*, 1993). E4orf6/7 is a 17kDa protein that binds as a homodimer to transcription factor E2F to promote E2 promoter activity by ensuring correct spacing and orientation (Cutt *et al.*, 1987; Huang & Hearing, 1989; Marton *et al.*, 1990; Neill *et al.*, 1990; Obert *et al.*, 1994; Raychaudhuri *et al.*, 1990). The E4orf4 protein is a 13kDa species that binds to and activates protein phosphatase 2A (Kleinberger & Shenk, 1993) and may have a role in the regulation of DNA synthesis (Bridge & Ketner, 1993) and AP-1 activity (Müller *et al.*, 1992). Mutants defective in E4orf4 show increased cytotoxicity (Müller *et al.*, 1992). Little is known about the Ad5 E4orf1, E4orf2 or E4orf3/4 products, although one or more products in the region encoding these proteins in the Ad9 serotype cooperates with E1A in cell transformation (Javier *et al.*, 1992). Any of the E4 species could be linked to apoptosis, and future ongoing studies are designed to identify which are involved and to determine the precise mechanism of induction of p53-independent apoptosis.

3.6 Acknowledgements

We are indebted to Andy Bett and Frank Graham for the gift of adenovirus vectors AdLacZ and Ad5dl70-8, to Stan Bayley for mutant dl520(12S)/E1B⁻, dl1132 and other E1A mutants, to Arnold Levine for 10(1) cells, to Scott Lowe for cell lines Hy.A3, 1A.A3, 1A.A6

and 1A.A12, to Rob Ricciardi for E1A plasmids containing point mutations in CR3 which allowed construction of some CR3 mutants, and to Tom Shenk for *dl309*. We also thank Rachel Charbonneau and Dennis Takayesu for some technical assistance. This work was supported by grants to P.E.B. from the National Cancer Institute of Canada and the Medical Research Council of Canada. J.G.T. holds a Research Studentship from the NCIC and R.C.M. a Student Research Award from the Glaxo/Burroughs-Wellcome Corp.

CHAPTER 4

The Early Region 4 orf4 Protein of Human
Adenovirus Type 5 Induces p53-Independent
Cell Death by Apoptosis

4.1 Abstract

Previous studies by our group showed that infection of human and rodent cells by human adenovirus type 5 results in the induction of p53-independent apoptosis and cell death that are dependent upon transactivation of early region 4 (E4). To identify which E4 products are involved studies were conducted in p53-deficient human Saos-2 cells infected with various Ad5 E4 mutants. An E4orf6-deficient mutant was defective in cell killing whereas another that expressed only E4orf6 and E4orf4 killed like wild-type virus, suggesting that E4orf6 may be responsible for cytotoxicity; however, a mutant expressing only E4orf4 induced high levels of cell death, indicating that this E4 product may also be able to induce cytotoxicity. To define the E4 cell death-inducing functions more precisely, cDNAs encoding individual E4 products were introduced into cells by DNA transfection in the absence of other Ad5 proteins. In co-transfections with a cDNA encoding firefly luciferase, enzymatic activity was high in all cases except with E4orf4 where luciferase levels were less than 20% of controls. In addition, drug selection of several cell types following transfection with retroviral vector DNA encoding individual E4 products as well as puromycin resistance yielded a high number of cell colonies except when E4orf4 was expressed. These data demonstrated that E4orf4 is the only E4 product capable of independent cell killing. Cell death induced by E4orf4 was due to apoptosis, as evidenced by DAPI staining of cell nuclei in E4orf4-expressing cells. Thus although E4orf6 may play some role, these results suggested that E4orf4 may be the major E4 product responsible for induction of p53-independent apoptosis.

4.2 Introduction

Many viruses induce apoptosis as part of their natural life cycle (Teodoro & Branton, 1997a). Killing of infected cells by this process is advantageous to the virus as it reduces both the inflammatory response and exposure to the host immune system and degradative enzymes. Human adenoviruses have developed complex processes which regulate both induction and suppression of apoptosis. Expression of early region 1A (E1A) products causes a rise in p53 levels, due at least in part to the stabilization of p53, and induces p53-dependent apoptosis (Braithwaite *et al.*, 1990; Debbas & White, 1993; Grand *et al.*, 1993; 1994; 1996; Lowe & Ruley, 1993; Sanchez-Prieto *et al.*, 1995). Recently we (Querido *et al.*, 1997b) and others (Chiou & White, 1997) mapped such effects in rodent cells to the region of E1A products involved in binding the p300 family of cellular transcriptional

modulators. We have also found that in normal human cells, binding of the retinoblastoma tumor suppressor (RB) family alone can elicit this effect (Querido *et al.*, 1997b), suggesting that p53 stabilization may be an unavoidable by-product of the induction of unscheduled DNA synthesis caused by complex formation with p300 or RB family members (Howe *et al.*, 1990; Yew *et al.*, 1994). The occurrence of p53-dependent apoptosis early during infection would severely reduce virus production and thus limit the spread of virus in the infected host. E1A is oncogenic in rodent cells, but its ability to yield stable transformants is greatly limited by this p53-dependent cell death (reviewed in Bayley & Mymryk, 1994). Adenoviruses have evolved at least three individual viral products which suppress p53-dependent pathways and thus promote viral replication and cell transformation. Early region 1B (E1B) encodes two such proteins. The E1B-55kDa protein binds to p53 (Sarnow *et al.*, 1982a) and blocks both p53-mediated activation of gene expression (Teodoro & Branton, 1997b; Teodoro *et al.*, 1994; Yew *et al.*, 1990; 1992; 1994) and apoptosis (Marcellus *et al.*, 1996a; Teodoro & Branton, 1997b). The E1B-19kDa protein appears to suppress apoptosis by a mechanism functionally analogous to that of the cellular proto-oncogene product Bcl-2 (Boyd *et al.*, 1994; Nguyen *et al.*, 1994; Rao *et al.*, 1992; White *et al.*, 1992). Cells infected with adenovirus mutants which fail to express the 19kDa protein display enhanced cytotoxicity and extensive degradation of both cellular and viral DNA into nucleosome sized fragments, a characteristic of apoptosis (Ezoe *et al.*, 1981; Nguyen *et al.*, 1994; Pilder *et al.*, 1984; Subramanian *et al.*, 1984; White *et al.*, 1991; 1992). Recently the orf6 protein encoded by early region 4 (E4orf6) has also been found to bind to and inactivate p53 (Dobner *et al.*, 1996; Querido *et al.*, 1997a). Such interactions block p53-dependent transactivation activity (Dobner *et al.*, 1996; Nevels *et al.*, 1997) and induce the rapid degradation of p53 protein (Nevels *et al.*, 1997; Querido *et al.*, 1997a).

In addition to p53-dependent apoptosis, both our group (Teodoro *et al.*, 1995) and Subramanian *et al.* (1995) showed that in the absence of E1B, E1A products also cause p53-independent cell death. We found that such cell death exhibited all of the hallmarks of apoptosis, including degradation of DNA to nucleosome-sized fragments, extensive chromosomal condensation, and formation of cytoplasmic vacuoles (Teodoro *et al.*, 1995). This effect was found to rely on the ability of the large E1A protein to transactivate E4 (Marcellus *et al.*, 1996b), suggesting that one or more E4 products may be cytotoxic. Furthermore, in the presence of E1B, cell killing at the final stages of the infectious cycle was prevented or greatly delayed in the absence of E4 products (Marcellus *et al.*, 1996b), suggesting that this E4-dependent, p53-independent apoptosis may represent a major mechanism for the ultimate death of the infected cell and spread of progeny virions.

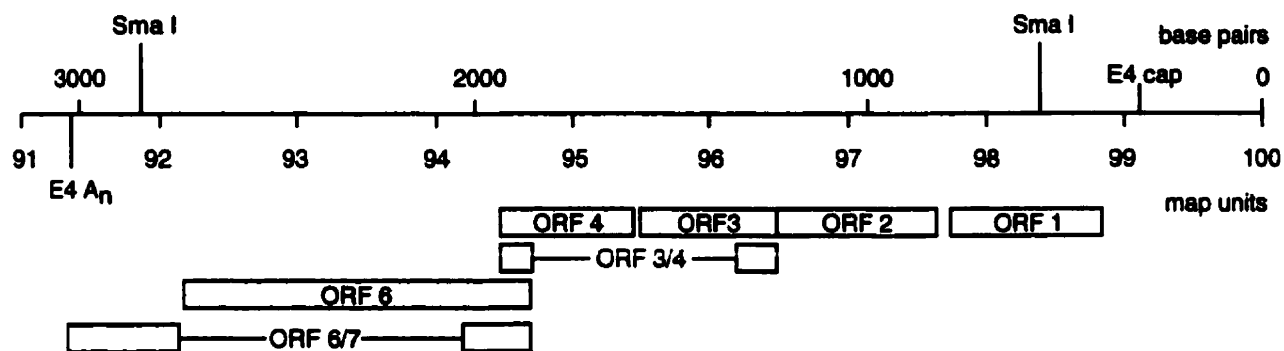
Figure. 1 shows that E4 encodes seven known proteins as determined by analysis of cloned cDNAs deriving from several open reading frames (Fryer *et al.*, 1984; Hérisse *et al.*, 1981; Virtanen *et al.*, 1984). E4orf6 is a 34kDa protein that is involved with host shut off and transport of viral late mRNAs following complex formation with the E1B-55kDa polypeptide (Bridge & Ketner 1990; Challberg & Ketner, 1981; Pilder *et al.*, 1986; Sandler & Ketner, 1989; Sarnow *et al.*, 1984). As noted above, E4orf6 also binds to and inactivates p53 (Dobner *et al.*, 1996; Querido *et al.* 1997a) and can cooperate with E1A to enhance cell transformation (Nevels *et al.*, 1997). E4orf3 is an 11kDa species that also interacts with the E1B-55kDa protein (Leppert, 1997) and is associated with the nuclear matrix (Leppert, 1997; Sarnow *et al.*, 1982b). It is involved in accumulation of late viral mRNA (Downey *et al.*, 1983; Sarnow *et al.*, 1982b), and, along with E4orf4 and E4orf6, appears to affect viral DNA synthesis (Bridge *et al.*, 1993). E4orf6/7 is a 17kDa species that binds as a homodimer to transcription factor E2F to promote E2 promoter activity by ensuring correct spacing and orientation (Cutt *et al.*, 1987; Huang & Haring 1989; Marton *et al.*, 1990; Neill *et al.*, 1990; Obert *et al.* 1994; Raychaudhuri *et al.*, 1990). E4orf4 is a 14kDa species that has been reported to bind to and activate protein phosphatase 2A (PP2A) (Kleinberger & Shenk, 1993). It may have a role in the regulation of DNA synthesis (Bridge *et al.*, 1993) and AP-1 activity (Müller *et al.*, 1992). Mutants defective for E4orf4 produce an enhanced cytopathic effect (Müller *et al.*, 1992). E4orf1 of Ad9 appears to be capable of cell transformation (Javier *et al.*, 1992; Javier, 1994; Weiss *et al.*, 1997a; 1997b; Weiss & Javier, 1997), possibly through the formation of complexes with several unidentified cellular proteins (Weiss & Javier, 1997). Little is known about the E4orf2 or E4orf3/4 products. In the present studies we report that E4orf4, even when expressed in the absence of other viral proteins, is highly cytotoxic, although E4orf6 may also have some role in adenovirus-induced cell death.

4.3 Materials and Methods

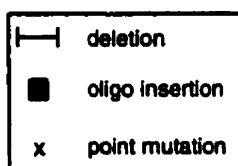
Cells and viruses. Human Saos-2 cells (ATCC HTB 85) that are deficient for p53 (and RB) expression were cultured on 60mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in alpha-modified MEM (α -MEM) supplemented with 10% fetal calf serum (FCS), as were LR73 Chinese hamster ovary (CHO) cells, human HeLa cells and Ad5 E1A-expressing 1A.A3 and 1A.A6 mouse embryo fibroblast cells that were derived from p53-null mice and grown under selective conditions in the presence of 100 μ g/mL of hygromycin (Lowe *et al.*, 1994). Normally cells were infected with mutant or wild-type Ad5 at a multiplicity of 35 pfu per cell (if titered on 293 cells) or 10 pfu per cell (if titered on

Figure 4-1 Ad5 early region 4 and E4 mutants

At the top is shown the right end of the Ad5 genome including positions in base pairs and map units. The locations of two *Sma*I restriction enzyme sites used in the preparation of E4 mutants have also been presented. Below is shown the positions of several open reading frames for E4 proteins that are encoded from right (amino terminus) to left (carboxy terminus). At the bottom is shown the structure of several E4 deletion mutants. The E4 proteins expressed by these mutants have been summarized at the right. A legend showing the symbols for deletions, insertions and point mutations is shown in the figure.



		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



W162 cells), as described previously (Teodoro *et al.*, 1994). The virus used as wild-type has been described by Harrison *et al.* (Harrison *et al.*, 1977). Ad5 E4 mutants are illustrated in figure 1 and include: *dl1019* and *dl1011* that express no E4 products; *dl1013* that expresses only E4orf4 and E4orf6; *dl1014* that expresses only E4orf4; *dl1015* that expresses only E4orf3 and E4orf4; *dl1010* that expresses all E4 products except E4orf6; and *dl359* (Halbert *et al.*, 1985) that is defective for E4orf4 alone. The profiles of these mutants in terms of expression of E4orf4, E4orf6 and E4orf6/7 were confirmed by Western blotting analysis using antisera that recognize these viral products. All of these mutants were propagated on W162 monkey cells, as described previously (Bridge & Ketner, 1989), with the exception of *dl359* which was grown on 293 cells (Graham *et al.*, 1977).

Adenovirus vectors expressing E4 products. cDNAs expressing E4orf6 (or other E4 products) were subcloned into pCA14, a CMV promoter-containing plasmid that was then used to produce adenovirus vectors expressing E4 products (AdE4orf6, AdE4orf1, etc.), as has been previously described (Bett *et al.*, 1993; Marcellus *et al.*, 1996a; Querido *et al.*, 1997a).

Cell viability assays. Cells were infected with wild-type or mutant Ad5 in 24-well plates containing 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.

Plasmids. cDNAs expressing individual E4 products were obtained from Göran Akusjärvi who showed that all yielded appropriate mRNA products (Öhman *et al.*, 1993). The E4 cDNAs were subcloned into the multicloning site of pcDNA3, a CMV-driven mammalian expression vector that also expresses the *neo* drug resistance gene (Invitrogen). From pcDNA3, the E4-specific cDNAs were subcloned into pBABEpuro (Morgenstern & Land, 1990), a retrovirus expression vector that contains the puromycin drug resistance gene. In addition, a hemagglutinin (HA) epitope tag was inserted at the 5' end of the E4orf4 coding sequence by standard PCR techniques (Chen *et al.*, 1995). The HA-tag was found to have no effect on the ability of E4orf4 to induce cell killing (Lavoie *et al.*, 1997). The RSV luciferase plasmid used in the firefly luciferase assay has been described previously (Goping *et al.*, 1995).

Luciferase assay. The luciferase killing assay was carried out using 1A.A3 or 1A.A6 cells plated the day before at a density of 1.5×10^5 cells per well in six-well plates. The DNA mixture was introduced into cells by calcium phosphate transfection (Graham & van der Eb, 1973) and consisted of 0.5 μ g of RSV luciferase plasmid DNA, 3 μ g of pcDNA3 plasmid DNA (or pcDNA3 containing an E4orf) with 2.5 μ g of sonicated salmon sperm DNA as a carrier. Cells were harvested 48 h post-transfection and luciferase activity was determined after freeze/thaw disruption of the cells, as described previously (Lavoie *et al.*, 1996).

Cell colony forming assays. Mouse 1A.A3 and 1A.A6 cells and human Saos-2 and HeLa cells were plated at a density of 10% in six-well dishes and each well was transfected with 1.5 μ g of DNA from pBABEpuro (or an E4- containing derivative) using 1.5 μ g of sonicated salmon sperm DNA as a carrier. Cells were replated 48 h post-transfection on 100 mm dishes in the presence of puromycin (1 μ g/ml). Two weeks after the onset of drug selection, cells were fixed in methanol:acetic acid (3:1 v/v), stained with Giesma (0.15 mg/ml in PBS), and colonies were counted.

Analysis of DNA content by DAPI staining. CHO cells were transfected with 12 μ g of DNA from pcDNA3 expressing HA-tagged E4orf4 together with 3 μ g of pHOOK vector DNA (Invitrogen), and 24 h after transfection the cells were magnetically sorted using Capture-Tec beads (Invitrogen) to isolate pHOOK-expressing cells. Cells were then plated on glass coverslips and 24 h later they were washed in PBS containing 1 mM $MgCl_2$ and then fixed for 20 min in 3.7% formaldehyde in PBS. Permeabilization of the cells was performed in 0.02% Triton X-100/PBS for 5 min after which mouse anti-HA HA.11 (Babco) was added followed by Texas Red-conjugated anti-mouse immunoglobulin G (Molecular Probes) to detect HA-orf4. The nuclear morphology of the cells was analyzed by DNA staining with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes).

Antisera and immunoblotting. HeLa cells growing in 60mm-diameter dishes were infected with wild-type or E4 mutant Ad5, and 16 h p.i. cells were harvested by scraping into PBS, washed, and lysed in 10mM Hepes/KOH (pH 7.4), containing 142mM KCl, 5mM $MgCl_2$, 1mM EDTA, and 0.2% NP40. Clarified cell extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 50 μ g of protein per lane. Separated proteins were transferred to nitrocellulose and filters were immunoblotted with appropriate antibodies. For E1A proteins mouse monoclonal antibody M73 (Harlow *et al.*, 1985) was used at a 1/2000 dilution. For E4orf4 a rabbit polyclonal antibody raised against

the carboxy terminal 28 residues fused to glutathione-S-transferase (GST) was prepared for this study and used at a 1/200 dilution. For E4orf6, a rabbit polyclonal serum raised against the amino terminal 46 residues fused to GST was used at a 1/500 dilution.

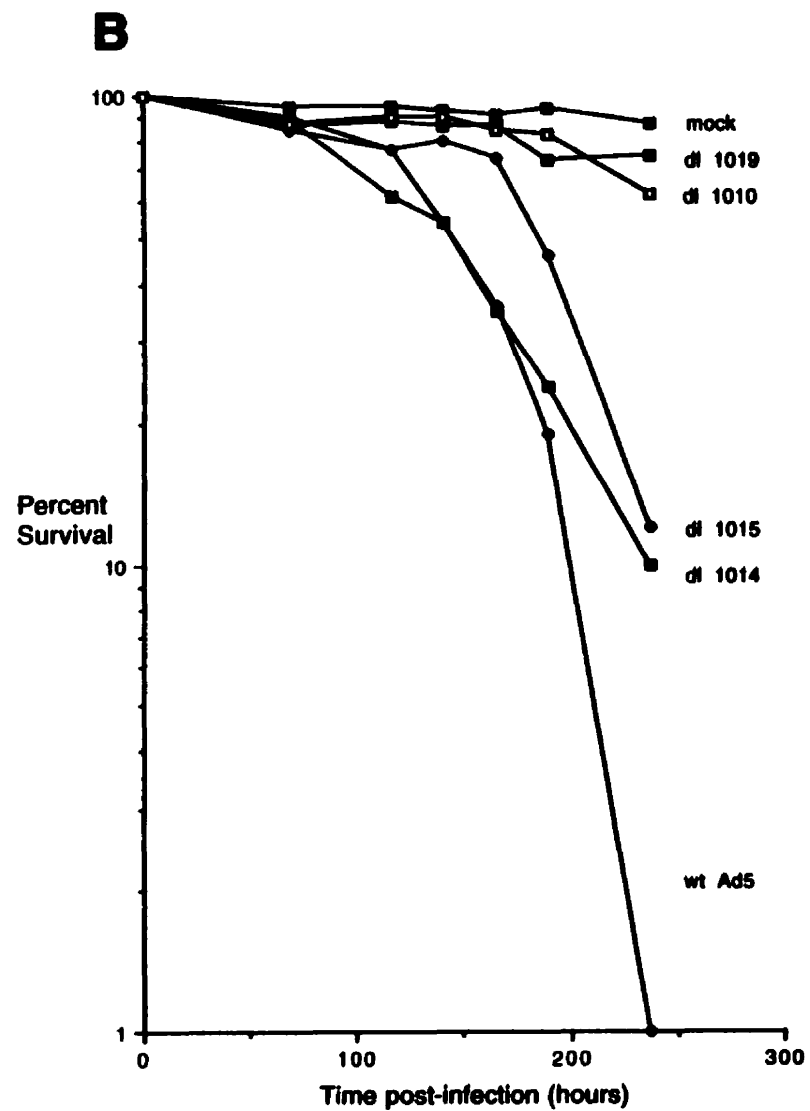
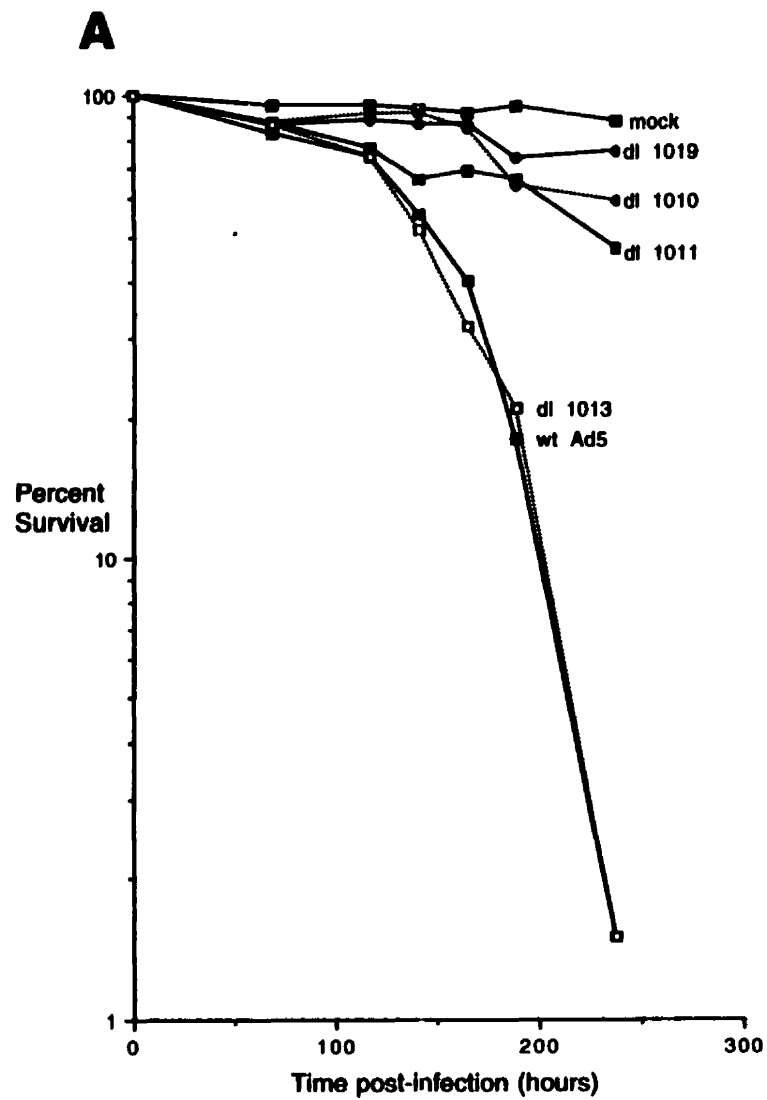
4.4 Results

Analysis of cell killing by Ad5 E4 mutants. Previous studies indicated that in the presence of E1A proteins, one or more E4 products was required for Ad5 E1A-induced p53-independent apoptosis (Marcellus *et al.*, 1996b). To identify which E4 products are involved in this p53-independent cell killing, experiments were carried out using various E4 mutants. Examining rapid cell killing which occurs in the absence of E1B was not possible because E1B/E4 double mutants were not available and were difficult to construct. Therefore human p53-deficient Saos-2 cells were infected with wild-type Ad5 or with mutants with defects in the E4 coding region, and cell viability was measured by exclusion of Trypan Blue stain. These viruses all express wild-type E1B products and thus cell death did not occur until late during infection. Figure 2A shows, as found previously (Marcellus *et al.*, 1996b), that wild-type virus began to kill cells at about 125 h p.i. and that by 240 h p.i. virtually all cells were dead, whereas with mutants *dl1019* and *dl1011*, which produce no E4 products, cells remained almost as viable as in mock-infected cultures. A similar effect was also noted with mutant *dl1010* that expresses all E4 products except E4orf6. Cell killing was similar to that of wild-type virus using mutant *dl1013* that expresses only E4orf6 and E4orf4. These results suggested that E4orf6 may be responsible for Ad5-induced cell death, but left open the possibility that E4orf4 might also play some role.

Figure 2B shows results of a similar experiment using additional E4 mutants. Again mutant *dl1019* failed to kill infected cells efficiently as did mutant *dl1010* that produces all E4 products except E4orf6; however, mutant *dl1014*, that produces only E4orf4 and none of the other E4 products, killed quite efficiently. Infection with mutant *dl1015*, which produces only E4orf4 and E4orf3, also resulted in significant cell killing, although, over several experiments (data not shown) such was always found to be somewhat delayed relative to wild-type virus or *dl1014*. These results indicated that in virus-infected cells, E4orf4 alone is able to support efficient cell death and that the presence of E4orf3 might limit this effect somewhat. Thus the data in Figs. 2A and 2B suggested that in virus-infected cells E4orf4 and E4orf6 may synergize to produce efficient killing. e4orf4 appears to harbor the major killing activity; however, this effect seems to be reduced by another E4

Figure 4-2 Viability of SAOS-2 cells infected with mutant and wild-type Ad5

p53-deficient human SAOS-2 were mock-infected or infected with wild-type Ad5 or a series of mutants which contain defects in E4 and at various times following infection they were tested for viability by a Trypan Blue exclusion assay, as described in Materials and Methods. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in the figure. Figures. 2A and 2B show two separate representative studies involving two sets of E4 mutants.



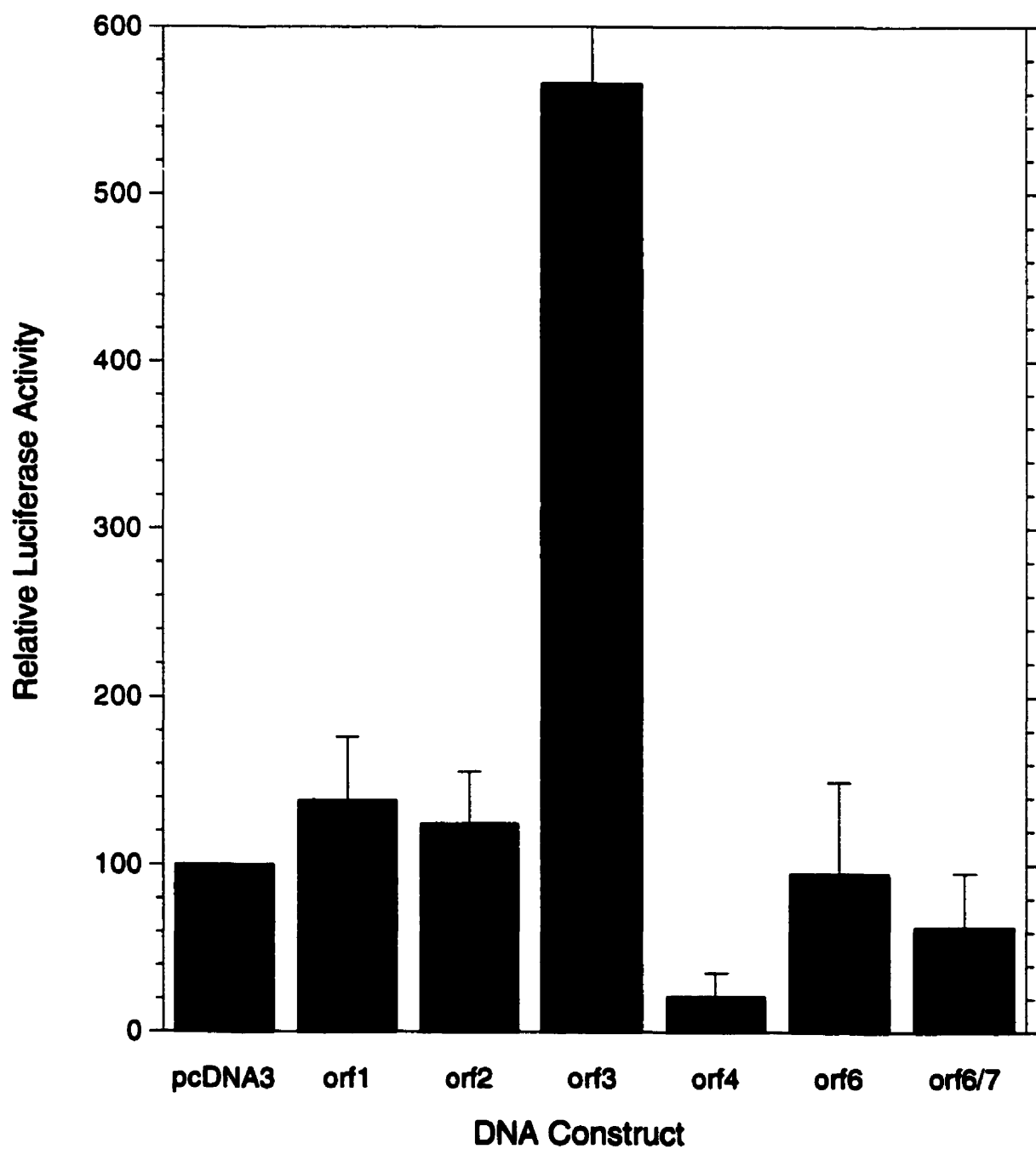
product in the absence of E4orf6. Although the toxic effect of E4orf6 was not tested directly in these experiments, studies have been described below that address this issue. In addition we were aware of a previous report (Müller *et al.*, 1992) that suggested that a mutant *dl359* which expresses all E4 products except E4orf4 produces a greater cytopathic effect than wild-type virus (see further below in figure 8).

Analysis of cell killing by individual E4 products. To examine the specific potential of E4 products to induce cell death, cDNAs expressing individual E4 products were subcloned into the plasmid pcDNA3 which expresses inserts under the CMV promoter. Previous studies (Öhman *et al.*, 1993) indicated that these cDNAs express appropriate E4 mRNAs. We have confirmed directly by Western blotting analysis that high levels of E4orf4, E4orf6 and E4orf6/7 proteins are synthesized from the corresponding constructs (data not shown). DNA from such plasmids was used to co-transfect p53-null 1A.A3 and 1A.A6 cells expressing Ad5 E1A products, along with DNA encoding firefly luciferase, and after 48 h cell extracts were assayed for luciferase activity. We assumed that high luciferase activity was consistent with a low degree of cell killing, whereas if extensive cell death occurred, luciferase activity would be reduced. Figure 3 shows the combined results from five separate experiments. Cells co-transfected with plasmids expressing E4orf1 or E4orf2 exhibited luciferase activities equal to or slightly greater than with pcDNA3 alone, suggesting that these E4 products were not highly toxic. Co-expression of E4orf3 resulted in considerably increased levels of luciferase activity. E4orf3 is known to induce effects on mRNA metabolism and splicing (Bridge & Ketner, 1989; 1990) and thus this increase may have resulted from an enhancement of expression of luciferase mRNA from the RSV promoter (see more below). Co-expression of E4orf6/7 reduced luciferase activity by a modest amount. Interestingly, co-expression of E4orf6 generally had little effect on luciferase activity, although in some experiments a modest reduction was observed. In the case of E4orf4, luciferase activity was reduced by 80 to 90%, suggesting that this E4 product might induce cell death. Co-expression of both E4orf4 and E4orf6 resulted in even slightly lower levels (data not shown). These data were consistent with the conclusion that E4orf4 is the only E4 product capable of inducing p53-independent cell death when expressed alone. Interpretation of these assays assumed that the E4 products had no direct effect on luciferase expression from the CMV promoter, but such may not be the case. As already described above, increased luciferase activity found with E4orf3 may have resulted from an increase in luciferase expression. Effects of this nature with other E4 products might also affect our ability to interpret cell killing accurately. For example, E4orf4 has been shown to reduce transcription from certain promoters (Bondesson *et al.*, 1996;

Figure 4-3 Analysis of cell killing by measurement of luciferase activity

1A.A6 cells were co-transfected with pcDNA3 plasmids expressing firefly luciferase and individual Ad5 E4 proteins. After 48 hours the cells were harvested and the extracts analyzed for luciferase activity as described in Materials and Methods. The data represent the average of five separate experiments each involving duplicate samples. Luciferase activity obtained with the pcDNA3 alone control was arbitrarily set at 100%.

Luciferase Killing Assay Results E1A 1AA6 MEF cells



Kleinberger & Shenk, 1993) although there is no evidence that the CMV promoter is affected. In addition, E4orf6, in association with the E1B-55kDa protein, plays a role in enhancing viral mRNA stability and transport (Bridge & Ketner, 1989; 1990; Dobbelstein *et al.*, 1997; Goodrum *et al.*, 1996), and thus cell killing by this protein might be balanced by a concomitant increase in luciferase expression. Clearly an assay which more directly assesses cell death was warranted.

One approach was to construct adenovirus vectors which express each of the E4 products. Such vectors were easily constructed for E4orf1, E4orf2, E4orf3 and E4orf6/7. Only a single plaque was obtained for E4orf6 during five separate attempts; however, as shown previously, such a construct, AdE4orf6, was produced (Querido *et al.* 1997a) and shown to express wild-type E4orf6 at levels two- to three-fold higher than in Ad5-infected cells (data not shown). We were unable to generate a vector expressing E4orf4 even after many attempts, as might be expected if E4orf4 is highly toxic. The adenovirus vectors were used to infect Saos-2 cells; however, none caused any cytopathic effect even after several days (data not shown). Because the data presented above indicated that E4orf6 might play a role in inducing cell death, the AdE4orf6 vector was studied in more detail. Saos-2 cells were infected by AdE4orf6 or with wild-type virus and cell killing was measured by Trypan Blue exclusion assay as in figure 2. Figure 4 shows that cell viability was maintained in AdE4orf6-infected cells to a level similar to mock-infected cells, whereas with wild-type virus, cell death occurred as before. These results confirmed that E4orf6 possesses little cytotoxic activity when expressed alone.

The next approach was to measure the effects of E4 products on cell viability during colony formation following drug selection. For this assay, cDNAs encoding E4 products were inserted into the pBABE retroviral vector, which also contains the gene for puromycin resistance, and colony formation was measured following selection by puromycin of transfected 1A.A6 mouse cells and human Saos-2 or HeLa cells. Figure 5A shows that colony formation with mouse 1A.A6 cells following puromycin selection was similar to controls with plasmids expressing all of the E4 products except for E4orf4, with which colony formation was inhibited by over 60%. Similar results were also obtained in a more limited study with human Saos-2 and HeLa cells (figures 5B and C, respectively). In these cases colony formation was inhibited by 80 to over 90% by E4orf4, although some reduction was also seen with E4orf6 and E4orf6/7 with HeLa cells. These data indicated that E4orf4 exhibits the greatest capacity to block colony formation, and that the other E4 products had either no effect or produced only a modest reduction. Reduction in colony

Figure 4-4 Analysis of cell killing using an adenovirus vector expressing E4orf6

SAOS-2 cells were infected either with wild-type Ad5 or with adenovirus vector AdE4orf6 that expresses only E4orf6. Cell killing was measured by Trypan Blue exclusion as in figure 2. Symbols are as indicated in the figure.

Survival Assay in Saos-2 cells

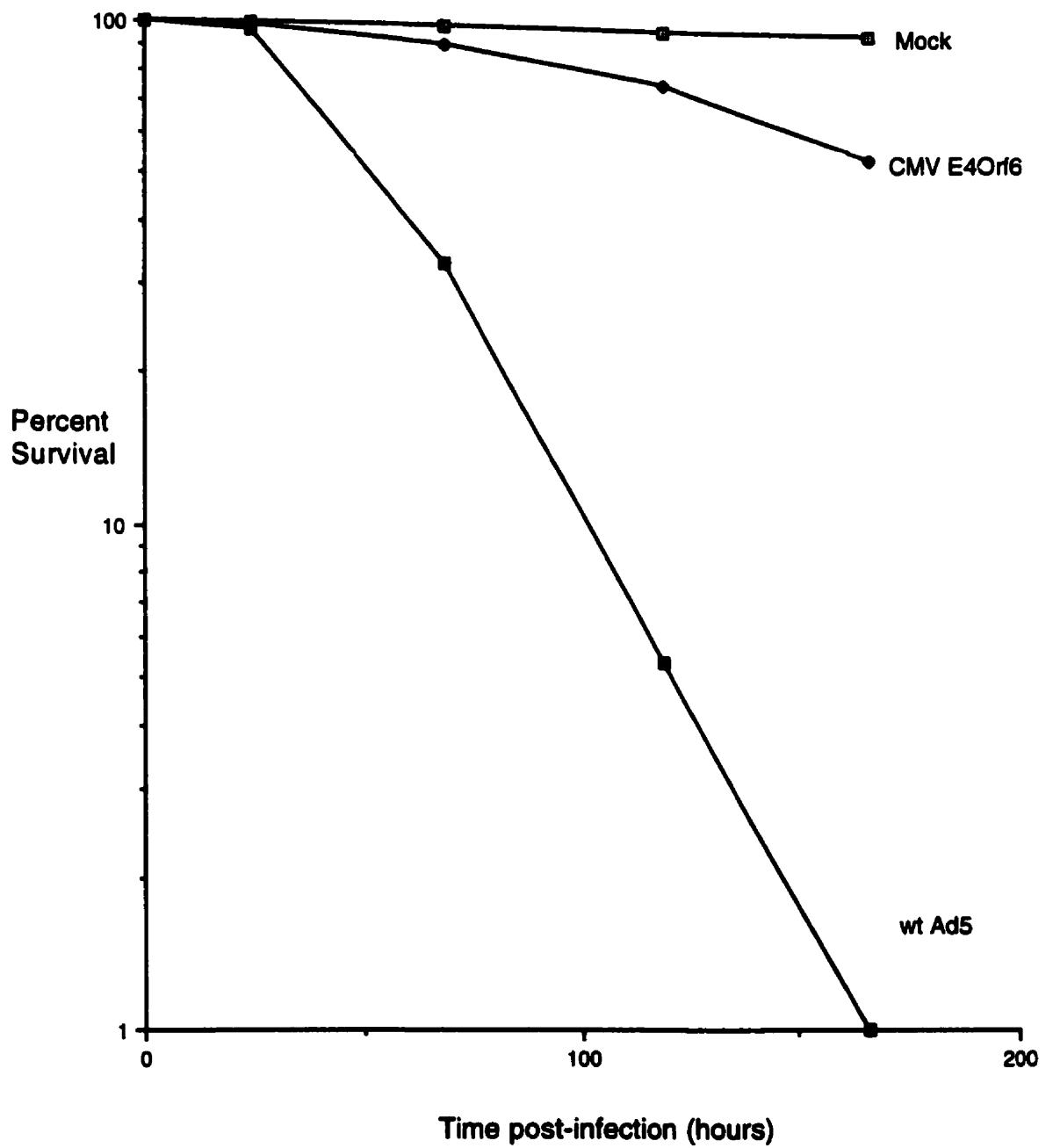
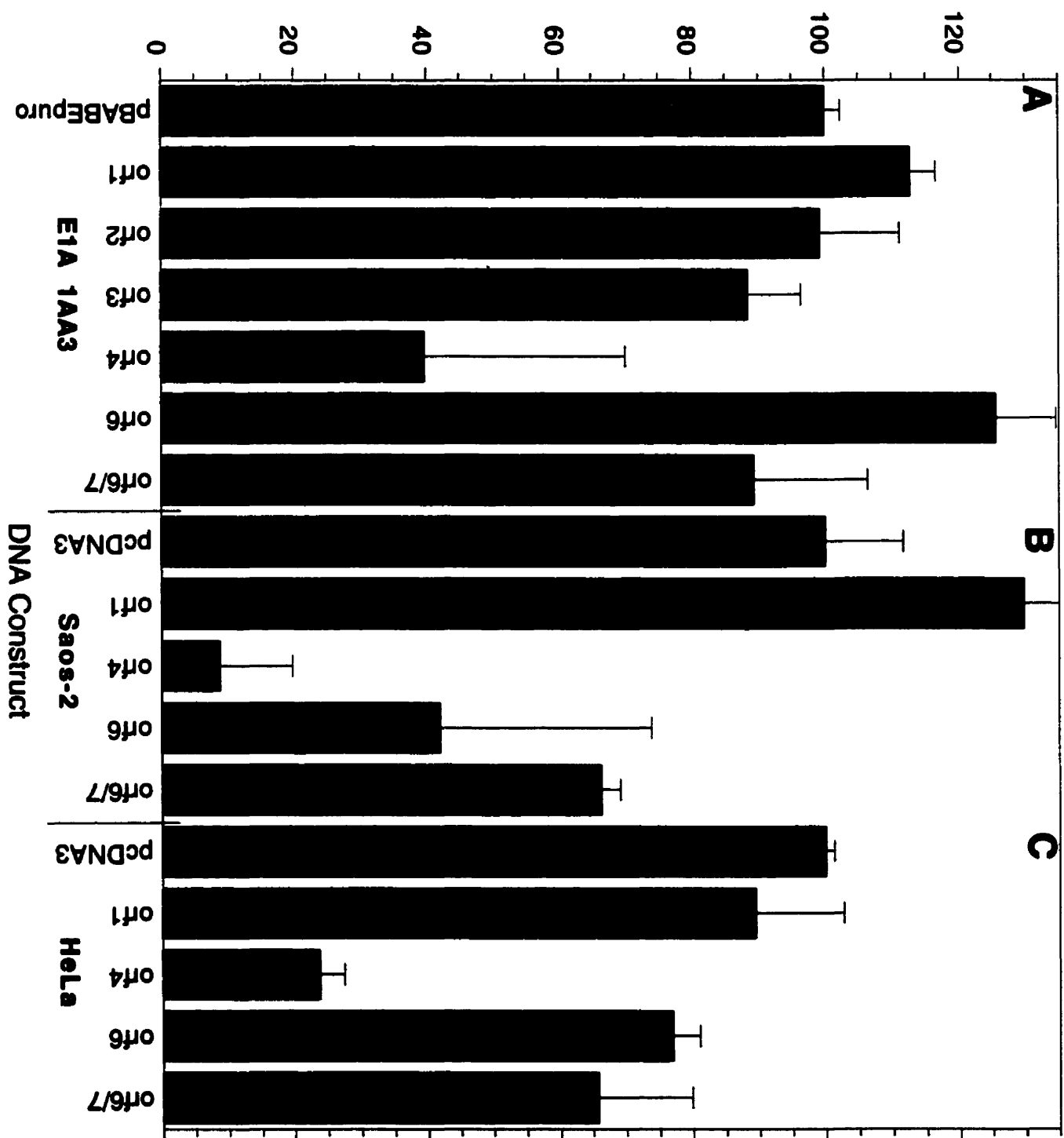


Figure 4-5 Analysis of E4 killing by colony inhibition

E1A expressing, p53 minus 1A.A6 cells (panel A) SAOS-2 (panel B) or HeLa cells (panel C) were transfected with the puro containing pBABE retroviral plasmid expressing individual E4 products. Cells were plated and then grown in the presence of puromycin for 14 days, as described in Materials and Methods, after which time the numbers of colonies were tabulated. The results show the average of four (panel A) or two (panels B and C) experiments involving two plates of each E4orf per experiment (standard error indicated by bars). Colony formation in the presence of pcDNA3, which varied between about 80 and 120 per plate, was set at 100%.

Relative Number of Drug Resistant Colonies



formation could result either from growth arrest or from cell killing. We have not studied the properties of the colonies produced with constructs containing E4orf4 in detail; however, most were much smaller than those produced by controls. Possibly these colonies are the result of low levels of E4orf4 expression.

Expression of E4orf4 induces p53-independent apoptosis. The assays thus far indicated that E4orf4 alone among the E4 products exhibited significant effects on luciferase expression and colony formation that were consistent with cell killing. Direct measurement of cell death was not possible in these types of experiments, as only a small percentage of cells in such cultures express the exogenously introduced E4orf4. We believed from previous studies (Marcellus *et al.*, 1996b; Teodoro *et al.*, 1995) that the effects caused by E4orf4 were due to p53-independent apoptosis.

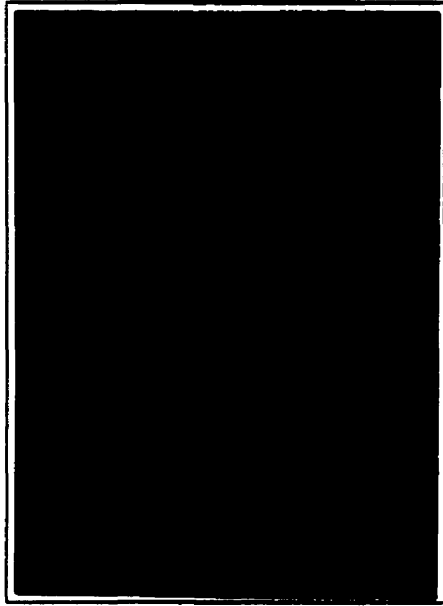
To determine if expression of E4orf4 induces apoptosis, CHO cells and human 293 cells were cotransfected with DNA from a pcDNA3 plasmid that expresses an HA-tagged version of E4orf4 together with the pHOOK vector. Cells were magnetically sorted using Capture-Tec beads to isolate cells expressing pHOOK. Selected cells were examined both by immunofluorescence using an anti-HA antibody and by DAPI staining. The DAPI technique allows identification of apoptotic cells due to the presence of irregularly shaped nuclei, in some cases containing foci of bright fluorescence. Figure 6 shows representative fields of cells treated by these two methods. The upper panels in figure 6A (CHO cells) and figure 6B (293 cells) show the pattern of immunofluorescence using anti-HA antibody. Expression of E4orf4 was largely nuclear with some diffuse cytoplasmic staining. The lower panels of these figures show the same fields visualized by DAPI staining. Clearly cells lacking detectable levels of E4orf4 exhibited DAPI-stained nuclei of normal morphology; however, those cells that expressed E4orf4 contained nuclei that were either irregular in shape or contained brightly stained regions that are characteristic of highly condensed chromatin. This correlation was consistent throughout the entire cell population. These results suggested that expression of E4orf4 induces apoptosis. This contention was confirmed in extensive separate studies using CHO cell lines expressing E4orf4 under an inducible promoter (Lavoie *et al.*, 1998). Following induction of E4orf4 expression, cells exhibited a number of changes characteristic of apoptosis, including rapid cell death, DNA degradation, and the appearance of phosphatidyl serine on the outer cell surface as shown by staining with annexin 5. Clearly E4orf4 is toxic and induces apoptotic cell death.

Figure 4-6 Immunofluorescence and DAPI staining of E4orf4 transfected cells

CHO (A) or 293 (B) cells were transfected with the plasmid pcDNA3 expressing an HA-tagged version of E4orf4 together with the pHOOK plasmid to facilitate magnetic sorting of transfected cells (see Materials and Methods). After sorting, the cells were analyzed either by immunofluorescence using an antibody directed against HA or by staining for DNA with DAPI. A single representative field for each cell line has been presented. Upper panels - immunofluorescence using anti-HA antibody. Lower panels - the same field by DAPI staining.

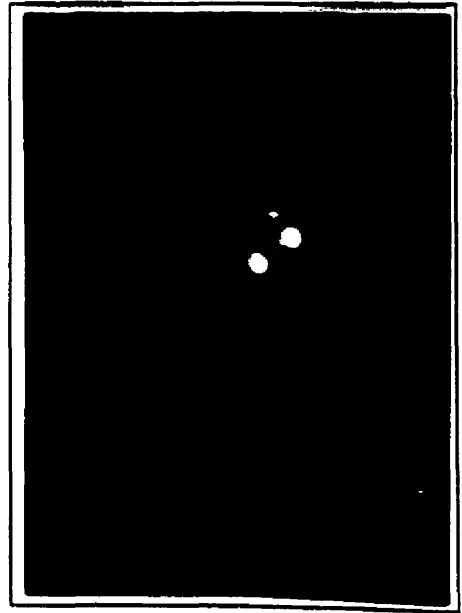
A

Immunofluorescence

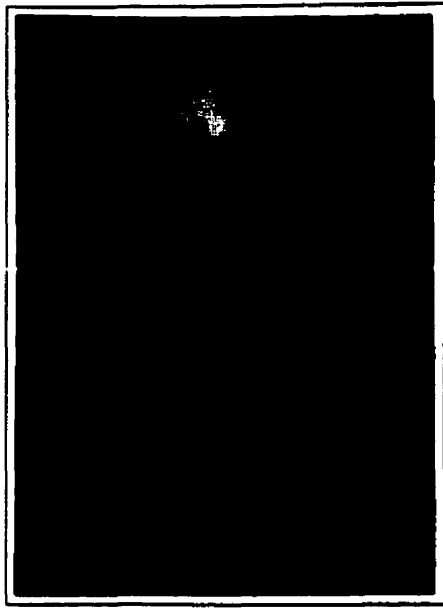


CHO

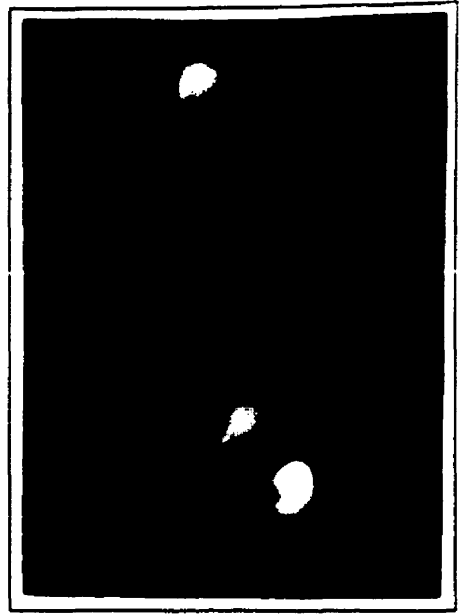
DAPI



B



293



Further analysis of the roles of E4orf4 and E4orf6 in cell killing. The results described above indicated that E4orf4 appeared to be the major E4 product involved in the induction of p53-independent apoptosis. The question remained, why did mutant *dl1010*, which encodes E4orf4 but not E4orf6, fail to kill cells in the experiments shown in figure 2. One explanation was that in the absence of E4orf6, but in the presence of the other E4 products, expression of E4orf4 could be reduced. To study this possibility, HeLa cells were infected with wild-type Ad5 or with E4 mutants *dl1010*, *dl1013*, or *dl1015*, and at 16 h p.i. cells were harvested and extracts were analyzed for the presence of E1A proteins, E4orf6 and E4orf4 by immunoblotting using appropriate antisera. Figure 7A shows that wild-type and all mutant viruses contained approximately equal amounts of E1A products. The levels of E4orf6 (figure 7B) and E4orf4 (figure 7C) with *dl1013* were somewhat elevated relative to those of wild-type. Mutants *dl1010* and *dl1015* produced no E4orf6 as expected; however, the levels of E4orf4 were similar to that seen with wild-type. Similar results were also obtained in Saos-2 cells, although the overall levels of all Ad5 proteins, including E4orf4, were lower than with HeLa cells (data not shown). The failure of cell killing by *dl1010* must be due to some mechanism other than a reduction in E4orf4 expression (see Discussion).

Another question relates to the previously observed phenotype of cells infected with mutant *dl359* that is defective for E4orf4 expression but wild-type for other E4 proteins. *dl359* has been reported to elicit a greater cytopathic effect than wild-type virus, as evidenced by the extensive destruction of cell monolayers (Müller *et al.*, 1992). How could E4orf4 represent the major cytotoxic E4 protein if, in its absence, *dl359* elicits this effect? To examine this question, Saos-2 cells were infected with either wild-type Ad5 or with *dl359* and cell viability assays were conducted as in figure 2. The kinetics of cell killing in these experiments cannot be directly compared to those in figure 2 because the viruses used for the present experiment were titrated on 293 cells, whereas in figure 2 the viruses used had to be titrated on monkey W162 cells. For this assay (as in others) both adherent and non-adherent cells were collected and examined. Microscopic examination of *dl359*-infected cultures, revealed that cells began to round up and detach much earlier than with wild-type virus, such that by about 60 h p.i., when most of the cells in wild-type-infected cultures remained attached, a large percentage of mutant-infected cells had already detached (data not shown). Nevertheless, figure 8A shows that in terms of cell viability, *dl359*-infected cells did not die more rapidly than those infected with wild-type virus, but rather they were considerably delayed in cell death. As found previously (Müller *et al.*, 1992) figure 8B shows that expression of both E4orf6 and E4orf6/7, as detected by immunoblotting with an antiserum that recognizes the amino terminus of both of these

Figure 4-7 Analysis of viral protein expression with wild-type and E4 mutant Ad5

HeLa cells were infected by wild-type Ad5 or mutants *dl1010*, *dl1013* or *dl1015* and harvested at 16 h p.i. Cell extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted. Panel A - anti-E1A M73 monoclonal antibody. Panel B - anti-E4orf6 serum. Panel C - anti-E4orf4 serum.

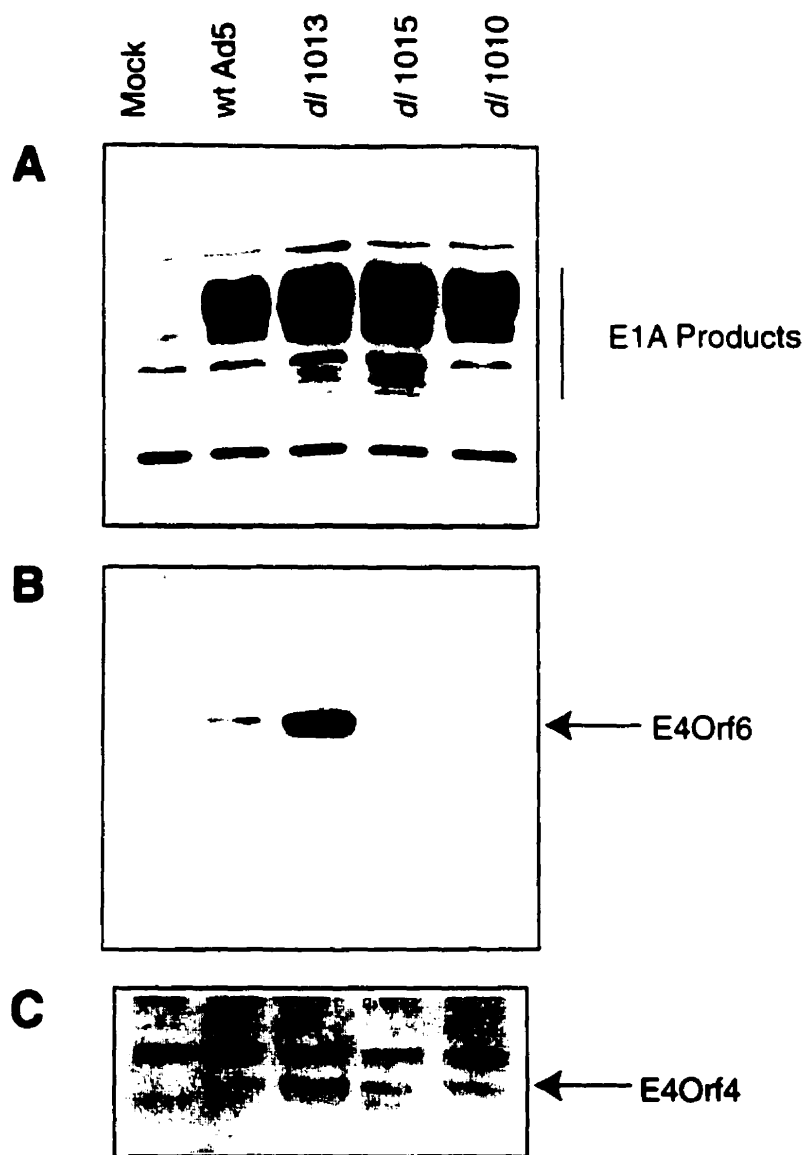
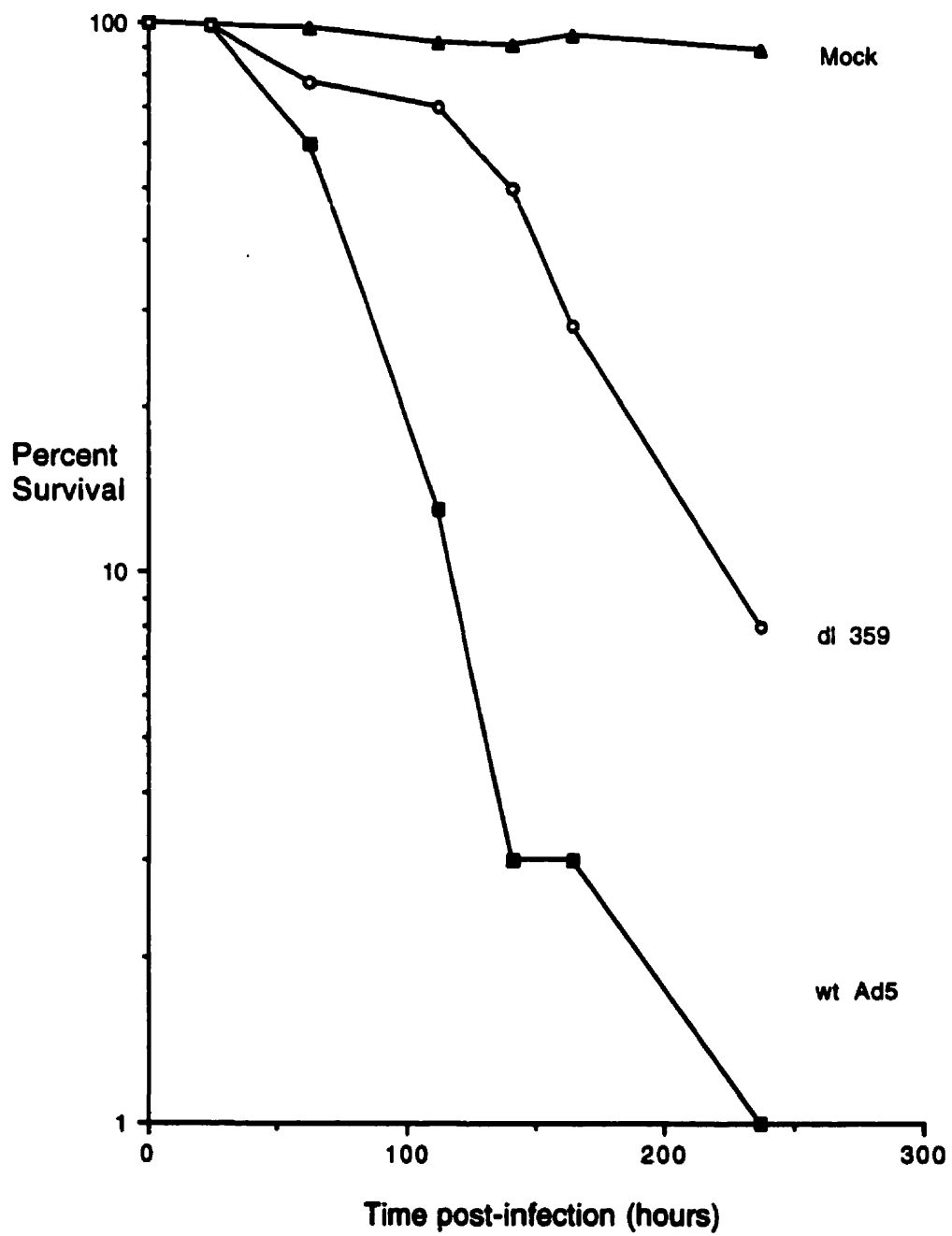
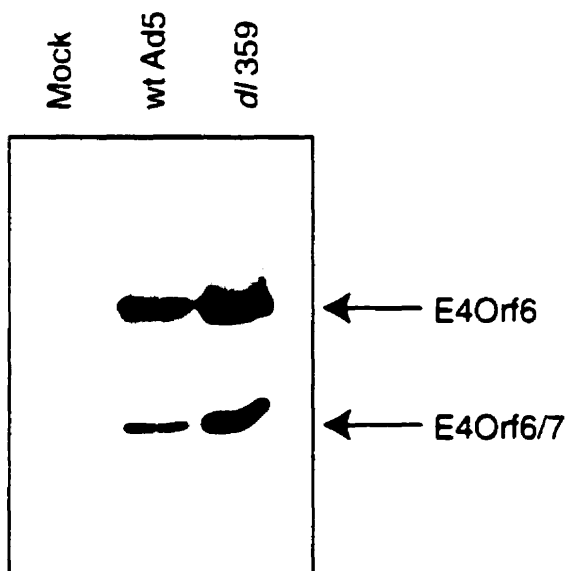


Figure 4-8 Analysis of cell killing and E4 expression by wild-type Ad5 and mutant *dl359*

SAOS-2 cells were infected with wild-type Ad 5 or mutant *dl359* that is defective for E4orf4 expression, and at various times after infection cell viability was assessed by Trypan Blue exclusion as in figure 2. Figure 8A - Cell viability by Trypan Blue staining. Data have been expressed as % cell viability and symbols are as indicated in the figure. Figure 8B - Expression of E4orf6 and E4orf6/7. Portions of the cultures described above were harvested at 16 h p.i. and analyzed by immunoblotting as in figure 7 using an antiserum that recognizes the amino terminus of E4orf6 and E4orf6/7.

Survival Assay in Saos-2 cells





proteins, was higher in *dl359*-infected cells than in those infected with wild-type Ad5. This effect is believed to be due to the role of E4orf4 in reducing expression of E4 transcripts (Bondesson *et al.* 1992; 1996; Whalen *et al.*, 1997). Thus while it appeared that *dl359* did cause the early detachment of cells from the monolayer, the cells that were released were still viable, at least by the criterion of Trypan Blue exclusion. These results were entirely consistent with a major role for E4orf4 in cell killing. The data also clearly suggested that other mechanisms for the generation of cytopathic effects must exist that involve other E4 proteins and/or additional viral products.

4.5 Discussion

The major goal of the present studies was to establish which Ad5 E4 products are responsible for the induction of p53-independent apoptosis. Analysis of cell death induced by E4 mutant viruses in the presence of E1B yielded a somewhat paradoxical result. Cell death was inhibited using a mutant that produced all E4 products except E4orf6, suggesting that this product plays a role in cytotoxicity. A mutant expressing only E4orf6 and E4orf4 killed infected cells as efficiently as wild-type virus; however, a high degree of cell killing was obtained with a virus expressing E4orf4 as the only E4 product. These results clearly indicated that E4orf4 was sufficient among E4 products for induction of cell death. We recognize that analyses involving virus-infected cells may be complicated by the effects of multiple viral products that may affect E4orf4-dependent cell death or that may participate in parallel death pathways. The results obtained with E4 mutant viruses cannot be explained by differences in the kinetics of the early phase of the infectious cycle as previous studies indicated that such mutants exhibit only slight or moderate delays relative to wild-type virus (Bridge & Ketner, 1989). In fact these earlier studies indicated that the largest effects on progression of the early phase were with *dl1014* that we found to kill almost as well as wild-type. In addition, successful entry into the late phase does not appear to be critical for killing as our previous work showed that such killing could be demonstrated in infected rodent cells that fail to replicate virus and express significant levels of late viral products (Marcellus *et al.*, 1996b; Teodoro *et al.*, 1995). We are still uncertain as to the roles of other E4 products, but the simplest explanation is that in virus-infected cells, E4orf6 may enhance killing by E4orf4, perhaps by counteracting the effects of one or more of the other E4 products that may suppress this effect. Such suppression was seen to some degree with mutant *dl1015* that expresses only E4orf4 and E4orf3 and that caused cell death at a slightly slower rate than did *dl1014* that produces only E4orf4.

The molecular basis for such suppression is unclear. One possibility was that in the absence of E4orf6 the levels of expression of E4orf4 might be reduced, limiting the toxic effects of the virus; however, this did not appear to be the case as the level of E4orf4 synthesized in mutant-infected cells was similar to that obtained with wild-type virus (figure 7). Identifying which of the E4 products suppresses E4orf4-dependent cell death, and how E4orf6 blocks this inhibition or otherwise promotes cytotoxicity, is of interest. E4orf6 is a multi-functional protein and plays a role in the regulation of p53 function and stability (Dobner *et al.*, 1996; Moore *et al.*, 1996; Nevels *et al.*, 1997; Querido *et al.*, 1997a; 1997b; Sarnow *et al.*, 1984) and cooperates with the E1B-55kDa protein in the regulation of late viral mRNA transport and host cell shut off (Dobbelstein *et al.*, 1997; Goodrum *et al.*, 1996). Generation of the appropriate E4orf6 mutants should permit the mapping of the E4orf6 effect.

E4orf4 was found to induce apoptosis when expressed alone in the absence of all other Ad5 products. The induction of apoptosis was confirmed by the appearance of abnormal DAPI-stained nuclei in E4orf4-expressing cells. In separate studies using continuous CHO cell lines expressing E4orf4 under an inducible promoter, we found that expression of E4orf4 induces cell death associated with classic morphological changes, degradation of DNA into nucleosome-sized fragments, and an exchange of phosphatidyl serine from the inside to the outside of the plasma membrane as visualized by staining with annexin 5 (Lavoie *et al.*, 1998). All of these properties are specific characteristics of apoptosis. Additionally, recent work by Tamar Kleinberger has shown that 293, H1299 and transformed NIH 3T3 cells undergo apoptosis in response to E4orf4 expression (Shtrichman *et al.*, 1998). Previous studies showed that E4-dependent cell death was due to p53-independent apoptosis (Marcellus *et al.*, 1996; Teodoro *et al.*, 1995) and it is likely that E4orf4-induced apoptosis is also unrelated to p53 status as it occurred in both CHO cells that contain very low endogenous levels of p53, and in 293 cells that express high levels of p53 due to the presence of E1A and E1B products (Lavoie *et al.*, 1998; and this report).

The role of E4orf6 in promoting cell death remains unclear. Like the other E4 products, E4orf6 exhibited little cytotoxicity when expressed in the absence of other viral products. As discussed above, perhaps its role is simply to enhance E4orf4-dependent cell death, or possibly, in the context of viral infection, it may play a more direct role in cell killing. Mutant dl359, which is defective in E4orf4, yielded an interesting phenotype. As found previously (Müller *et al.*, 1992) dl359-infected cells exhibited a greater cytopathic effect than wild-type Ad5-infected cells; however, we found that the cells that detached

rapidly from the cell monolayer were not dead by the criterion of Trypan Blue exclusion, but rather required longer times to die than those from wild-type-infected cultures. These results supported the conclusion that E4orf4 is a major inducer of cell death, but they also indicated that other mechanisms for Ad5-induced cytopathogenicity must exist. One product that has been implicated in cell death is the E3-11.6kDa product or “adenovirus death protein” (Tollefson *et al.*, 1996). Its role in this process remains unclear, but the absence of cell death with many of the E4 mutants described in Figs. 2A and 2B suggests that it may function in cooperation with E4 products. E4orf4 is known to suppress E4 transcription (Bondesson *et al.*, 1992; 1996; Kleinberger & Shenk 1993; Whalen *et al.*, 1997) and thus with dl359, expression of E4orf6 and E46/7 was found to be somewhat elevated (figure 8). Possibly these higher levels of E4orf6, in combination with one or more additional early or late viral proteins, results in a second form of Ad5-induced cytotoxicity.

The major finding in the present work remains the ability of E4orf4 to induce p53-independent apoptosis independently of other viral proteins. The only biochemical function associated with this E4 product is its ability to bind and activate the trimeric form of PP2A. This activity appears to account for the role of E4orf4 in regulating E4 expression through the dephosphorylation of transcription factors required for E4 mRNA production (Bondesson *et al.*, 1992; 1996) and/or members of the mitogen-activated protein kinase family which phosphorylate a site on E1A products required for efficient transactivation of the E4 promoter (Whalen *et al.*, 1997). Induction of cell death could relate to induction of PP2A or to some other unidentified function of E4orf4. Studies are currently underway to identify the biochemical function of E4orf4 involved in induction of apoptosis. Importantly, since this effect is independent of p53, E4orf4, or derivatives that function in a similar fashion, might be amenable to the development of reagents for the treatment of p53-null human cancers.

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CHAPTER 5

General Discussion

Viruses have proven to be extremely useful probes in our quest to unravel the intricacies of the eukaryotic cell. Adenoviruses in particular have been very helpful in our study of cellular growth regulation and apoptosis. For replication to occur, adenoviruses need to harness the cellular metabolic processes and program them for viral progeny production. To accomplish this, adenoviruses need to push the differentiated cells that they infect into the cell division cycle to facilitate viral DNA replication. This mitogenic property has provided us with great insights into the mechanisms behind the cell cycle. However, the cell responds to this unscheduled DNA synthesis with an attack of its own—apoptosis. Apoptosis is of critical importance to multicellular organisms as it provides a method for removing unwanted cells in an orderly fashion that avoids both inflammation and damage to surrounding cells. Apoptosis is also essential for the elimination of nonfunctional, abnormal, or virus-infected cells. Apoptosis is carefully controlled by the cell as too much can lead to degenerative diseases, and deficiencies can lead to hyperplasia and cancer. Our ultimate interest in cancer is what prompted our study of adenoviruses and how they modulate the cellular apoptotic program to ensure their own survival.

At the onset of this project adenoviruses were thought to induce apoptosis in a p53-dependent manner exclusively, perhaps as a consequence of the stimulation of cellular growth by E1A (section 1.6.5.1 on page 26). As discussed, the p53 tumour suppressor is an important component of the cell's apoptotic defense (section 1.9.10 on page 64) and adenovirus, like many other viruses, has needed to develop means of countering p53-induced death. The E1B-55K protein appeared to be the primary inhibitor of p53 (section 1.6.5.3 on page 27) but we had not conclusively proven that E1B-55K could block p53 induced apoptosis. We were in the process of setting up an assay system in which we wanted to introduce p53 and E1B-55K into p53 negative cells using adenovectors. Our goal was to examine how E1B-55K functioned to inhibit p53 transcriptional activity and to

investigate the mechanism by which E1B-55K and E4orf6 cooperated to down-regulate p53 protein levels.

However, to our surprise we found that the simple introduction of p53 into the osteosarcoma line Saos-2 induced rapid death, without the need of any further apoptotic stimulus. In contrast, expression of an inactive p53 mutant was benign. This observation indicated that the mechanism by which these cells had triggered the apoptotic response was still active, and the only thing saving them from death was the deletion of p53. Saos-2 cells also lack the expression of the Rb tumor suppressor gene (Masuda *et al.*, 1987) which can cause p53-dependent apoptosis in a transgenic mouse system (Morgenbesser *et al.*, 1994). As discussed, Rb is involved in the regulation of the E2F family of transcription factors (section 1.6.4.2 on page 20) and the Rb-negative state of Saos-2 cells would lead to large amounts of active E2F and aberrant triggering of DNA synthesis. Such inappropriate signals could activate apoptosis and may have led to the selection of a p53 deletion in the original tumour from which Saos-2 cells were derived. Previous work involving the reconstitution of p53 negative cells with p53 involved the production of stable lines (Chen *et al.*, 1990) and cell death was not apparent in these studies; however, the obvious result would have been that any surviving clone must have inactivated the p53 apoptosis pathway in another fashion. The clear advantage of reconstituting cells using adenovirus vectors is that it allows the study of an unselected population of cells.

Our finding was quite significant in that it demonstrated the possibility of treating p53 defective tumours with p53 replacement therapy, potentially employing adenovectors. A majority of human cancers are lacking p53 function, and since many chemotherapeutic agents function through a p53-dependent apoptosis pathway, they are not very responsive to this approach (Lowe *et al.*, 1993a). The introduction of p53 alone or in combination with

genotoxic drugs has potential with some types of otherwise refractory cancers. Recently, this approach has been taken with a number of adenovector-p53 therapies in clinical trials.

At a more basic research level we also showed that Bcl-2 is able to block p53-induced apoptosis in Saos-2 cells. Bcl-2 is known to block apoptosis by a wide variety of stimuli, indicating that it likely functions downstream of the initial triggering stage of apoptosis (Chiou *et al.*, 1994; Zhuang *et al.*, 1995; Oltvai & Korsmeyer, 1994). This also indicates that the p53-induced death that we were observing was in fact apoptosis, as further supported by results from our electron microscopic studies. Of greater novelty and more in line with our initial goals, we found that E1B-55K effectively blocked the p53-induced apoptosis. E1B-55K appears to function by binding to the p53 transactivation domain and repressing p53-driven transactivation (Yew *et al.*, 1990; 1994; Yew & Berk, 1992; Teodoro *et al.*, 1994; 1997b; Martin & Berk, 1998; Assefi *et al.*, 1998). The finding that E1B-55K prevented p53-induced apoptosis in Saos-2 cells, whereas a nonbinding mutant A₂₆₂ did not, directly confirmed for the first time that E1B-55K is able to suppress p53-dependent apoptosis, and that such inhibition correlates with p53 binding. Studies to elucidate E1A-induced activation of p53 and the mechanism of suppression by adenovirus proteins are continuing in our group using this approach. This system is also of use for the investigation of downstream p53 effects, without the issue of multiple pathway activation as one might obtain with more upstream triggering of p53.

Previous studies indicated that E1B-19K minus viral mutants would rapidly kill cells, regardless of the presence of E1B-55K (Ezoe *et al.*, 1981; Pilder *et al.*, 1984; Subramanian *et al.*, 1984; White *et al.*, 1991; 1992; Nguyen *et al.*, 1994). This caused some confusion, as the presence of E1B-55K would be expected to block p53-induced death. An explanation arrived with the finding by our group and another that adenovirus E1A can induce both p53-dependent and p53-independent cell killing (Teodoro *et al.*, 1995; Subramanian *et al.*,

1995). Both E1A products are able to induce p53-dependent apoptosis contingent upon interactions with the Rb and p300 families of proteins, which appears to be linked to the induction of DNA synthesis and concomitant increase in p53 levels (Braithwaite *et al.*, 1990; White *et al.*, 1991; 1996; Debbas & White, 1993; Lowe & Ruley, 1993; Querido *et al.*, 1997a; 1997b; Chiou & White, 1997; de Stanchina *et al.*, 1998). This form of apoptosis is effectively blocked by expression of either E1B-19K or E1B-55K (Ezoe *et al.*, 1981; Nguyen *et al.*, 1994; Pilder *et al.*, 1984; Subramanian *et al.*, 1984; White *et al.*, 1991; 1992; Marcellus *et al.*, 1996a; Teodoro *et al.*, 1997b). In contrast we found that p53-independent apoptosis was elicited only by the larger 289R E1A product, and preliminary evidence suggested that expression of one or more additional early viral genes was required (Teodoro *et al.*, 1995). As expected we found E1B-55K unable to block the effect, whereas E1B-19kDa and Bcl-2 significantly inhibited this response, indicating that it was apoptosis.

The major goal of our work was to identify which early viral product was required to induce cell death in the absence of p53. Results obtained with E1A mutants clearly indicated that the 289R transactivation function was required, and the mutational data correlated exactly with the requirements for the activation of the E4 promoter. Of peripheral interest was the finding that p300 binding to E1A appeared to be required for E4 activation, which had not been previously described. This was also necessary for the induction of p53-independent death, and complementation studies indicated that p300 binding was a requirement of E4 transactivation rather than an additional cell death stimulus. The role of p300 as a cotransactivator (section 1.6.4.3 on page 24) suggests that the E4 promoter may depend, at least in part, upon the histone acetylation activity that E1A would recruit via p300 binding. Recent work has also shown that E1A binds another histone acetyl transferase, P/CAF, using the same region of E1A as is involved in p300 binding, presenting the possibility that it is actually P/CAF that is essential for E4 promoter activation (Reid *et al.*, 1998).

Further mutational work with E3 and E4 deleted adenovectors confirmed that an intact E4 region was required for cell death and that the E3 region was dispensable. This was an important finding because the E3-11.6K protein has been reported to be essential for adenovirus induced death at the end of an infection (section 1.6.7.4 on page 33). However as we demonstrated, an E4 product is able to kill cells in the absence of E3-11.6K, when E1B-19K is not present.

To map which E4 protein was involved in the induction of p53-independent apoptosis, we utilized a number of E4 mutant viruses in a wt E1B background. We conducted these experiments in the human p53-null Saos-2 cell line to allow full viral replication to occur. The presence of E1B blocks cell killing through most of the infectious cycle, but we expected the final death of the cell to be delayed in mutants lacking the death-inducing protein. We found that E4 deleted viruses were highly defective for cell killing, as would be expected from our mapping data, but while these viruses can replicate their DNA, they are unable to complete the late stage of the viral growth cycle (Sandler & Ketner, 1989; Bridge & Ketner, 1989; Bridge *et al.*, 1993). If some late function is required to inactivate E1B-19K to allow cell killing, this would not occur. We had shown that the induction of p53-independent apoptosis did not require late viral products, replication, or host-cell shutoff. This relates to a couple of observations: (1) rodent cells are non-permissive for viral replication so late gene expression is not activated; (2) the presence of E1B-55K was not required for apoptosis, yet it is necessary for host cell shutoff. However, E1B-19K was not present in these experiments so we cannot exclude a role for a late viral product in the inactivation of E1B-19K.

Analysis of cell killing induced with more selective mutants indicated that E4orf4 and 6 were sufficient to generate a fully wt result, suggesting that full replication and the induction of cell death only required these two proteins. A mutant expressing only E4orf4

was also able to kill cells, even though it is completely defective for viral replication and late viral functions. This suggested that E4orf4 is the toxic protein. However a mutant that expressed all E4 products except E4orf6 failed to induce any cell killing, even though this virus is competent for replication and progeny production. E4orf4 expression levels appeared normal in this virus, leaving us with the possibility that in the context of an intact virus, another viral protein may inhibit E4orf4-induced killing, which is relieved at the appropriate time by E4orf6. We have not at this point followed up this observation, but the determination of this mechanism may offer further insights into the viral inhibition of apoptosis. E4orf6 is a multi-functional protein playing a role in p53 regulation, splicing and transport of late viral mRNAs, and host cell shut off (section 1.6.8.6 on page 38). Generation of the appropriate E4orf6 mutants should permit the mapping of the E4orf6 effect.

Analyses involving virus-infected human cells are complicated by the effects of multiple viral products, and the cellular dysfunction caused by host-cell shutoff, so to further investigate this finding we decided to work with isolated E4 cDNAs. Our results confirmed that E4orf4 was the only E4 product to show substantial cytotoxicity or growth suppressive effects, and the induction of apoptosis by E4orf4 was confirmed by the appearance of abnormal DAPI-stained nuclei. In separate studies expressing E4orf4 under an inducible promoter, we found that E4orf4 induces cell death associated with classic apoptotic morphological changes—degradation of DNA into nucleosome-sized fragments, and an exchange of phosphatidyl serine from the inside to the outside of the plasma membrane (Lavoie *et al.*, 1998). All of these properties are specific characteristics of apoptosis. In parallel with our work, another group has shown apoptosis induction by E4orf4 with a couple of transformed human and rodent cell lines (Shtrichman *et al.*, 1998). Our earlier work demonstrated that E4-induced cell death was due to p53-independent apoptosis and E4orf4-induced apoptosis is also unrelated to p53 status as it occurs in both

p53 positive and negative cell lines (Lavoie *et al.*, 1998; Shtrichman *et al.*, 1998; Marcellus, 1998).

Interestingly, E4orf4 had initially been the E4 product we thought least likely to be involved in killing. This relates to a previous finding that an E4orf4 minus virus elicited a greater cytopathic effect than *wt* virus (Müller *et al.*, 1992). However, when we tested this virus we found that while the CPE was high with the rapid detachment of cells from the tissue culture plate, the cells were not dead by the criterion of trypan blue exclusion, and actually required a longer time to lose membrane integrity than *wt* virus. These results are consistent with E4orf4 being the major inducer of cell death, but they do suggest that other mechanisms for Ad5-induced cytopathogenicity must exist. E4orf4 is known to suppress E4 transcription and in its absence the other E4 products are expressed at higher levels (Bondesson *et al.*, 1992; 1996; Kleinberger & Shenk 1993; Whalen *et al.*, 1997). We have not seen much evidence of toxicity with the other isolated E4 products, but perhaps together with other adenovirus proteins, there is second form of p53-independent killing.

One other viral protein that has been implicated in the induction of cell death is the E3-11.6K protein (section 1.6.7.4 on page 33) (Tollefson *et al.*, 1996). As we demonstrated, it is unnecessary for the viral induction of apoptosis, in the absence of E1B-19K; however, when E1B-19K is present it may play a role in inactivating its anti-apoptotic function. Cells infected with viruses that are deleted for E3-11.6K live for much longer than cells infected with *wt* virus, even past the point at which viral progeny have accumulated in the nucleus. E3-11.6K has been seen to co-localize with E1B-19K at the nuclear membrane, suggesting that they may interact, but this has yet to be shown.

From the recent literature, there is a strong suggestion that there is another p53-independent apoptosis pathway induced by an early adenovirus function. Work with the

E1B-19K minus adenovirus had clearly demonstrated the generation of active caspases during killing of p53^{-/-} rodent cells (Boulakia *et al.*, 1996); however, later work with E4orf4 has shown that killing is not accompanied by caspase activation, and is not blocked by a broad spectrum caspase inhibitor (Lavoie *et al.*, 1998). This indicates that there is another early viral protein that stimulates caspase activation, either alone or in combination with E4orf4. The characterization of this pathway is beginning, but nothing is currently known. The activation of a caspase-independent pathway is itself quite novel with only a few reports illustrating this occurrence; however the possibility does exist that unidentified caspases which are not inhibited by the current chemical inhibitors are involved. Ongoing work should clarify this.

The only biochemical function associated with E4orf4 is its ability to bind and activate the trimeric form of PP2A (section 1.6.8.5 on page 37). This activity appears to account for the role of E4orf4 in regulating E4 expression through the dephosphorylation of transcription factors required for E4 mRNA production (Bondesson *et al.*, 1992; 1996) and / or members of the mitogen-activated protein kinase family which phosphorylate a site on E1A products required for efficient transactivation of the E4 promoter (Whalen *et al.*, 1997). The E4orf4 / PP2A complex also regulates late viral mRNA splicing through the dephosphorylation of SR proteins (Kanopka *et al.*, 1998). Changes in phosphorylation of apoptotic regulators in response to E4orf4 has not yet been examined.

Recently, extensive mutagenesis of E4orf4 has correlated binding of E4orf4 to the B α PP2A subunit with the induction of apoptosis, but what specifically E4orf4 does to the enzyme is not yet known (Marcellus, unpublished). The mutagenesis data pointed to residues in E4orf4 that are essential for PP2A binding and for killing, but evidence for the requirement of a second site on E4orf4 was weak. This suggests that E4orf4 is not acting to recruit substrates to PP2A through directed interactions. Alterations in localization would

certainly affect substrate availability for PP2A, and E4orf4 appears to be primarily nuclear whereas PP2A is in many cases predominantly in the cytosol. Localization studies have not been carried out with PP2A together with E4orf4, but they may be interesting. Other mechanisms of action of E4orf4 include the competitive displacement of PP2A binding proteins and/or the allosteric enhancement of interactions. Both of these options are currently under investigation.

PP2A is a complex group of serine/threonine specific phosphatases that are quite abundant in the cell, and play a role in gene expression, cell division, and signal transduction (reviewed in Mumby & Walter, 1993). It is found in two primary forms with similar abundance (Kremmer *et al.*, 1996): the core enzyme, which is a dimer composed of an A regulatory subunit, and a C catalytic subunit; and a trimer, termed the holoenzyme, which has an additional B regulatory subunit. The A and C subunits exist in two isoforms, while the B subunit is from one of three unrelated families (B, B', and B'') which themselves are comprised of multiple members (Hendrix *et al.*, 1993; McCright & Virshup, 1995; Csortos *et al.*, 1996; Tanabe *et al.*, 1996; Tehrani *et al.*, 1996). The combinations of different subunits produce a heterogeneous mixture of enzymes known generically as PP2A. However, the multitude of PP2A forms do have different substrate specificities and are differentially expressed in various tissues. Much of the enzyme specificity appears to lie within the B subunit (Kamibayashi *et al.*, 1991; 1994; Sola *et al.*, 1991; Agostinis *et al.*, 1992; Ferrigno *et al.*, 1993; Cegielska *et al.*, 1994), and it may also influence subcellular localization (Sontag *et al.*, 1995; McCright *et al.*, 1996; Tehrani *et al.*, 1996). The A subunit forms a rod-like shape with 15 non-identical repeats (Chen *et al.*, 1989b; Hemmings *et al.*, 1990; Imaoka *et al.*, 1983; Walter *et al.*, 1989). The B subunit binds to repeats 1-10 on A, and the C subunit binds to repeats 11-15 (Ruediger *et al.*, 1992; 1994).

E4orf4 has only been shown to bind to the B α subunit (Kleinberger *et al.*, 1993), but a thorough examination of the others has not been done. As mentioned, the E4orf4 effect on the holoenzyme is not known. A few substrates show changes in phosphorylation, but how E4orf4 accomplishes this is speculative at this point. Whether E4orf4 actually activates PP2A in an enzymatic sense has never been demonstrated, and the changes in phosphorylation may result from increased accessibility to the enzyme due to some property of E4orf4.

The existing link between PP2A and apoptosis is confusing and seemingly contradictory, with the frequent use of broad spectrum inhibitors which have pleiotropic effects. Consistent with our E4orf4 results, PP2A has been reported to be activated during the execution stage of apoptosis induced by either growth factor withdrawal or DNA damage, leading to dephosphorylation of tau, a microtubule-stabilizing protein (Mills *et al.*, 1998). Ceramide, a signalling lipid involved in the regulation of cellular differentiation, proliferation and apoptosis (reviewed in Kolesnick & Kronke, 1998), has also been demonstrated to stimulate the heterotrimeric PP2A dependent upon the presence of the B subunit (Dobrowsky *et al.*, 1993; Kowluru & Metz, 1997; Galadari *et al.*, 1998; Leoni *et al.*, 1998). However, whether this stimulation is required for ceramide-induced apoptosis is unreported. Furthermore, in Fas-induced apoptosis, the A subunit of PP2A gets cleaved, releasing the active C subunit, which leads to a reduction in phosphorylated MAP kinase forms, and possibly contributes to the apoptotic response (Santoro *et al.*, 1998).

In contrast, however, okadaic acid and calyculin A (both inhibitors of PP1 and PP2A) were shown to work synergistically to promote TNF-induced apoptosis in a number of tumour lines, implicating PP2A as an inhibitor of the TNF signalling network (Wright *et al.*, 1993). The natural toxin tautomycin has also been shown to inhibit PP2A in the process of inducing apoptosis but the two activities may not be linked (Kawamura *et al.*, 1998). In

addition okadaic acid, calyculin A, and microcystin-LR (another PP1/PP2A inhibitor) induce apoptosis in a number of cell lines including HeLa and KB cells. (Zheng *et al.*, 1994; von Zezschwitz *et al.*, 1997; Ritz *et al.*, 1997; Solter *et al.*, 1998; Yoshida *et al.*, 1998). The problem with much of the PP2A inhibitor work is that the entire group of PP2A enzymes are affected, along with PP1 (another serine / threonine phosphatase) making the results difficult to interpret. If some PP2A forms are involved in apoptosis, and others in survival, these types of experiments will remain relatively uninformative. Likely a more directed approach, focusing on distinct PP2A forms, will be required to clarify the existing literature.

Interestingly, a number of other DNA tumour viruses also target PP2A during their replicative cycles. The small T antigens (sT) from SV40 and polyomavirus bind to the PP2A core enzyme as does the middle T antigen from polyomavirus (reviewed in Mumby, 1995). These interactions inhibit PP2A activity, which contributes to the transforming abilities of these viral proteins, possibly due to the upregulation of the MAP kinase cascade and the AP-1 transcription factor family (Sontag *et al.*, 1993; Frost *et al.*, 1994). This PP2A inhibition has also been linked to the stimulation of PKC ζ which promotes survival due to the upregulation of the NF- κ B transcription factor (Sontag *et al.*, 1997).

In contrast, adenoviruses do not target PP2A in order to transform cells, but rather apparently, to kill them. Because adenoviruses are non-lytic, non-budding viruses, they need another mechanism for cell release and progeny spread. The induction of apoptosis in the host cell would be an ideal method of achieving this, as the apoptotic bodies produced would be taken up by surrounding cells, providing a way to spread with a minimum of immune system attention. Although we have not proven that E4orf4 is involved in apoptosis at the end of a viral infection, it is an attractive model and one that we will test.

In summary, adenoviruses have taught us a lot about the stimulation of cell growth and the induction and inhibition of apoptosis. The cellular apoptotic response is a major barrier to viral replication, necessitating the evolution of viral mechanisms to block this defense. Our initial investigation demonstrating the ability of E1B-55K to block p53-induced apoptosis looked at one side of the issue, while our identification of a novel adenovirus-induced p53-independent apoptosis pathway has shown that adenoviruses may also make productive use of this cellular response. The utility of viruses as biological probes has allowed us to selectively examine many cellular processes, greatly contributing to our knowledge of cell biology. Hopefully future work will allow us to harness the properties of these viral proteins, so that we can redirect them towards the treatment of human disease. A magic bullet may be on its way.

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