Analysis of the Structure and Function of Adenovirus Type 5 Early Region 4 Protein

by

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B.Sc.

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Abstract

The E4orf4 protein of adenovirus type 5 has been shown to induce p53-independent apoptosis. The only known function of E4orf4 is to bind to the Bα subunit of protein phosphatase 2A (PP2A). A series of experiments were performed with both wild-type and mutant forms of E4orf4 to examine Bα subunit binding and cell killing following introduction into p53-deficient cells. Deletion mutant analysis indicated residues 12-18 and 103-114 were not required for cell killing. Point mutant analysis indicated that residues required for Bα binding and cell killing are dispersed throughout the E4orf4 sequence. Analysis of these results showed that binding to PP2A via its Bα subunit is essential for induction of p53-independent apoptosis by E4orf4. Binding assays showed that E4orf4 cannot bind B' subunits of PP2A. In human cells, E4orf4 induces an apoptotic pathway that involves caspase-3 independent cleavage of poly(ADP-ribosyl) polymerase. E4orf4's activity may be controlled through post-translational modification since E4orf4 was found to be phosphorylated on serine residues.
Résumé

La protéine E4orf4 d'adénovirus de type 5 induit l'apoptose d'une façon indépendante de p53. La seule fonction connue de E4orf4 est de se lier à la sous-unité Bα de la protéine phosphatase 2a (PP2A). Une série d'expériences furent réalisées avec des formes normales et mutantes de E4orf4 pour examiner sa capacité de se lier à Bα et de tuer les cellules. Une analyse par délétions a indiqué que les résidus 12-18 et 103-114 n'étaient pas requis pour l'induction d'apoptose. Une analyse par mutations ponctuelles a indiqué que les résidus requis pour la liaison à Bα et l'induction d'apoptose étaient dispersés à travers toute la séquence de E4orf4. L'analyse de ces résultats montre que la liaison à PP2A via la sous-unite Bα est essentielle pour l'induction d'apoptose indépendante de p53. Des essais de liaison ont démontrés que E4orf4 ne peut se lier aux sous-unités B' de PP2A. Dans les cellules humaines, E4orf4 induit l'apoptose par un mécanisme qui implique la coupure de la polymerase poly(ADP-ribosyle) (PARP) d'une façon indépendante de caspase-3. L'activité de E4orf4 est sans-doute contrôlée par une modification post-traductionnelle puisqu'il a été découvert que E4orf4 est phosphorylé sur les résidus sérine.
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# Table of Contents

Abstract.................................................................................................................................................. i
Résumé.................................................................................................................................................. ii
Acknowledgements................................................................................................................................... iii
Table of Contents..................................................................................................................................... iv
List of Figures and Tables....................................................................................................................... v
List of Abbreviations............................................................................................................................. vii

Chapter 1: Introduction........................................................................................................................... 1
  1.1 The History of Adenoviruses........................................................................................................... 1
  1.2 Classification.................................................................................................................................. 3
  1.3 Structure of the Virion..................................................................................................................... 3
  1.4 Structure of the Adenovirus Genome.............................................................................................. 6
  1.5 The Adenovirus Infectious Cycle.................................................................................................... 8
    1.5.1 Adsorption, Uptake and Uncoating.......................................................................................... 9
    1.5.2 Genome Delivery...................................................................................................................... 10
    1.5.3 Early Gene Expression............................................................................................................ 11
      1.5.3.1 Early Region 1A................................................................................................................ 11
      1.5.3.2 Early Region 1B................................................................................................................ 22
      1.5.3.3 Early Region 2.................................................................................................................. 26
      1.5.3.4 Early Region 3.................................................................................................................. 27
      1.5.3.5 Early Region 4.................................................................................................................. 30
    1.5.4 Viral DNA Replication.............................................................................................................. 38
    1.5.5 Late Gene Expression............................................................................................................... 39
    1.5.6 Host Cell Shut off.................................................................................................................... 39
    1.5.7 Virion Assembly and Release................................................................................................. 41
1.6 Protein Phosphatase 2A................................................................. 43
1.7 Apoptosis...................................................................................... 46
1.8 Thesis Proposal............................................................................ 52

Chapter 2: Materials and Methods.................................................. 54
2.1 Cell lines...................................................................................... 54
2.2 Plasmids...................................................................................... 54
2.3 In vitro protein expression............................................................ 54
2.4 In vivo protein expression............................................................. 55
2.5 Luciferase Assay.......................................................................... 57
2.6 Binding Assay............................................................................... 57
2.7 Viruses.......................................................................................... 57
2.8 Cell Viability Assay....................................................................... 58
2.9 Phosphoaminoacid analysis.......................................................... 58

Chapter 3: Results............................................................................. 60
3.1 E4orf4 deletion mutants............................................................... 60
3.2 Expression of the E4orf4 deletion mutants................................. 60
3.3 Analysis of cell killing by the deletion mutants............................ 63
3.4 The first generation of E4orf4 point mutants................................. 65
3.5 Analysis of cell killing by the first generation
of E4orf4 point mutants...................................................................... 65
3.6 The complete set of E4orf4 point mutants.................................... 68
3.7 Expression of the complete set of E4orf4 point mutants.............. 70
3.8 Analysis of E4orf4/PP2A Bα-binding........................................ 70
3.9 Analysis of cell killing by inducible adenovirus vectors
expressing wild-type and mutant E4orf4........................................ 76
3.10 Analysis of protein expression in cells infected by
adenoviral vectors and mutant adenovirus.................................... 76
3.11 Analysis of caspase-3 cleavage and PARP cleavage
in response to E4orf4-induced apoptosis...................................... 77
3.12 Analysis of the specificity of E4orf4 binding to the Ba subunit of PP2A ................................................................. 82
3.13 Analysis of phosphorylation status of E4orf4 ...................... 89

Chapter 4: Discussion and Future Experiments .......................... 87
References ................................................................................................................................. 93
List of Figures

Figure 1.1 Structural Components of the Adenovirus Genome.... 4
Figure 1.2 Adenovirus Genome Structure........................................ 7
Figure 1.3 Products of Early Region 4........................................... 31
Figure 3.1 Ad5 E4orf4 deletion mutants........................................ 61
Figure 3.2 Expression of the HA-tagged E4orf4 wild-type and deletion mutant proteins........................................ 62
Figure 3.3 Analysis of cell killing by measurement of luciferase activity.................................................. 64
Figure 3.4 First generation of E4orf4 point mutants................. 66
Figure 3.5 Analysis of cell killing by measurement of luciferase activity.................................................. 67
Figure 3.6 The complete set of E4orf4 point mutants............. 69
Figure 3.7 Expression of the complete set of E4orf4 point mutants.................................................. 71
Figure 3.8 Analysis of binding of Bα to the panel of E4orf4 point mutants.................................................. 72
Figure 3.9 Viability of H1299 cells infected with inducible adenoviral vectors expressing wild-type and mutant E4orf4.................................................. 75
Figure 3.10 Analysis of protein expression by adenoviral vectors and mutant adenovirus.................................................. 78
Figure 3.11 Analysis of caspase-3 cleavage in response to infection by inducible adenoviral vectors expressing wild-type and mutant E4orf4.................................................. 80
Figure 3.12 Analysis of PARP cleavage in response to inducible adenoviral vectors expressing wild-type and mutant E4orf4.................................................. 81
Figure 3.13 Analysis of binding of E4orf4 to PP2A-B’ subunit family members.................................................. 83
Figure 3.14 Phosphoamino acid analysis of HA-E4orf4............ 86

List of Tables

1.1 Human Adenovirus Classification.................................................. 2
List of Abbreviations

243R 243 residue E1A protein from the 12S mRNA
289R 289 residue E1A protein from the 13S mRNA
AA arachidonic acid
Ad2 Adenovirus type 2
Ad5 Adenovirus type 5
Ad9 Adenovirus type 9
Ad12 Adenovirus type 12
Ad40 Adenovirus type 40
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
cAMP cyclic adenosine monophosphate
CAR coxsackievirus and adenovirus receptor
CARD caspase recruitment domain
Caspase cysteinyI aspartate-specific proteinase
cB P CREB binding protein
cdk cyclin-dependent kinase
cDNA complementary DNA
ced 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
DNA deoxyribonucleic acid
ds double-stranded
E1A early region 1A
E1B early region 1B
E2 early region 2
E3 early region 3
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>E4</td>
<td>early region 4</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>G0</td>
<td>gap 0</td>
</tr>
<tr>
<td>G1</td>
<td>gap 1</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>hnRNP</td>
<td>heterogeneous ribonucleoprotein</td>
</tr>
<tr>
<td>ICE</td>
<td>interleukin-1β-converting enzyme</td>
</tr>
<tr>
<td>ITR</td>
<td>inverted terminal repeat</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kd</td>
<td>kilodalton</td>
</tr>
<tr>
<td>M phase</td>
<td>mitosis phase</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>mdm-2</td>
<td>murine double minute-2</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLP</td>
<td>major late promoter</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino terminal</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>orf</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95, discs-large, ZO-1</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PH</td>
<td>plexrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>poly(A)</td>
<td>polyadenylation</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PT</td>
<td>permeability transition</td>
</tr>
<tr>
<td>pTP</td>
<td>preterminal protein</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma susceptibility gene product</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine, glycine, aspartic acid</td>
</tr>
<tr>
<td>RID</td>
<td>receptor internalization and degradation complex (E3-14.5K / E3-10.4K)</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>S phase</td>
<td>DNA synthesis phase</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>TAF</td>
<td>TBP-associated factor</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA binding protein</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TP</td>
<td>terminal protein</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
</tbody>
</table>

ix
wt  wild-type
1.1 The History of Adenoviruses

Adenoviruses were fortuitously discovered in 1953 as Rowe and his colleagues searched for the etiological agent of the common cold (Rowe et al., 1953). This group identified and isolated human adenovirus as a cytopathogenic agent which induced the spontaneous degeneration of primary human adenoid and tracheal cell cultures. Adenoviruses did not prove to be the main causative agent of the common cold (Maller et al., 1966) but various adenovirus serotypes were shown to be responsible for a wide range of diseases including acute febrile respiratory disease (group B), epidemic keratoconjunctivitis (group D), infantile gastroenteritis (group F), and pneumonia (group E) (reviewed in Horwitz, 1996). Adenoviruses are ubiquitous in humans and are fairly innocuous as most individuals acquire neutralizing antibodies against at least one serotype within their first year of life (Huebner et al., 1954).

A major interest in adenoviruses was sparked by the discovery that human adenovirus type (Ad) 12 induced malignant tumor formation in hamsters (Trentin et al., 1962). This was the first demonstration that a human pathogenic agent was capable of producing oncogenesis in animals. Fortunately, the capacity of adenoviruses to induce tumors is confined to rodents. No epidemiological evidence has linked adenoviruses to human cancer development and exhaustive studies of human cancer cells have failed to find any adenovirus deoxyribonucleic acid (DNA) sequences (Green et al., 1980). Adenoviruses are a safe model system to study oncogenesis and serve as an invaluable tools for the study of all biological processes. Studies of adenovirus-
# Table 1-1 Human Adenovirus Classification

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

*a. Modified from Shenk, 1996*
infected cells have advanced our understanding of viral and cellular gene expression, DNA replication, cell cycle control, and cellular growth regulation. In addition, adenoviruses are becoming important tools in human gene therapy as they are amenable and efficient vectors (reviewed in Shenk, 1996).

1.2 Classification

The virus family, Adenoviridae, comprises over 100 members and is divided into the genera Mastadenovirus and Aviadenovirus, based on their isolation from mammals or birds, respectively (Norrby et al., 1976).

Human adenoviruses of the Mastadenovirus genus are classified into serotypes according to their resistance to neutralizing antibodies against other serotypes. Currently, 47 human serotypes have been isolated and classified into six groups based primarily on their ability to agglutinate red blood cells (Rosen, 1960) as well as their guanine and cytosine nucleotide (GC) content (Pina and Green, 1965), oncogenicity (Green, 1970), electrophoretic mobility of virion proteins (Waddell, 1979) and genetic variability (Waddell et al., 1980). Each group also has distinct tropisms leading to its tissue specific clinical syndromes. Table 1-1 shows the overall classification of human adenoviruses. The work presented in this study involved Ad5 of group C.

1.3 Structure of the Virion

Adenoviruses are non-enveloped and icosahedral in shape and span 70-100 nm in diameter (Horne et al., 1959). An icosahedron
Figure 1.1 Structural Components of the Adenovirus Genome

This schematic diagram shows the composition and location of proteins within the virion capsid. The relative mobility of the 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

- Hexon
- Core protein
- Hexon associated protein
- Protein specific for groups of nine hexons
- Penton base
- Penton associated protein
- Fiber
consists of 20 triangular surfaces and 12 vertices and is the most efficient geometric organization to create a closed shell. An adenovirus particle is composed of the capsid, an outer protein shell created from seven structural proteins termed II, III, IIIa, IV, VI, VIII, IX, surrounding an inner DNA-containing core with four proteins (reviewed in Stewart et al., 1995) (see Figure 1-1).

The capsid is composed of 252 subunits or capsomeres, of which 240 are hexons which make up the sides of the icosahedron and 12 are pentons which create the vertices (Ginsberg et al., 1966). The hexon capsomere is formed by a polypeptide II trimer which is stabilized by polypeptides VI, VIII, and IX (Everitt et al., 1973). The penton capsomere is composed of a pentameric structure of polypeptide III which acts as a base for the fiber protein which is composed of a trimer of polypeptide IV and forms a prominent spike at each vertex of the icosahedron (van Oostrum and Burnett, 1985). Polypeptide IIIa is a key element of the viral structure which links adjacent facets of the icosahedron and functions to bridge the capsid and the virion core (Everitt et al., 1975). The virus-encoded protease is also incorporated within the mature capsid and functions to specifically process these structural proteins (reviewed in Weber, 1995).

The virion core contains a single copy of the viral genome in association with polypeptides V, VII, the terminal protein, and μ protein. Polypeptide VII is a histone-like protein rich in arginine residues which complexes with the viral genome and organizes it into nucleosome-like super coiled domains (Chatterjee et al., 1986). Polypeptide V and μ protein are basic and non-covalently bound to the viral DNA (Anderson et al., 1989). Polypeptide V contacts both the penton base and the viral genome and is essential.
for genome entry into the nucleus (Everitt et al., Matthews and Russell, 1998). The function of μ protein is still unknown. The final protein in contact with the viral genome is the terminal protein. The terminal protein is covalently attached to the 5' ends of the genome and plays a critical role in viral DNA replication (Challberg et al., 1980).

1.4 Structure of the Adenovirus Genome

The adenovirus genome is a double-stranded DNA molecule which assumes a linear conformation upon entry into the nucleus of an infected cell (Green and Pina, 1963). The DNA molecules of all members of the adenovirus family have the same approximate size and transcriptional organization and in the case of Ad5, the genome is 36 kilobases (kb) in length (reviewed in Pettersson and Roberts, 1986). At each end of the viral genome, there is a region of inverted terminal redundancy (ITR) which serves for viral packaging as it contains a cis-acting packaging sequence (Hearing et al., 1987). In addition, the ITR is essential for viral DNA replication as it contains the adenoviral origin of replication and can form a panhandle structure (Wolfson and Dressler, 1972).

The adenovirus genome is divided by convention into 100 map units from left to right of the genome (see Figure 1.2). The strands of the genome have been defined by the direction of transcription, “r” for rightward and “l” for leftward transcribed strands. Adenovirus genes are classified according to their temporal expression during the course of a productive infection. Early and late transcriptional units are so named based on their expression before and after DNA replication, respectively. There are five early
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)
transcriptional units (E1A, E1B, E2, E3, and E4), two delayed early units (IX and IVa2), and one major late unit that produces five families of late mRNAs (L1-L5). All of these genes are transcribed by RNA polymerase II and each gene undergoes alternative splicing to produce multiple gene products. In addition, the genome contains one or two, depending on the serotype, specialized virus-associated (VA) RNA molecules which are transcribed by RNA polymerase III.

1.5 The Adenovirus Infectious Cycle

The natural targets of adenovirus 5 are non-replicating differentiated human cells of the upper respiratory tract (reviewed in Horwitz, 1996). These cells are known as permissive because both early and late genes of the virus are expressed and the full infectious cycle is implemented. Human adenoviruses can also infect rodent cells, presumably due to the presence of a CAR receptor homolog on these cells (Bergelsson et al., 1998). Most rat or hamster cells do not produce progeny virions and are therefore known as non-permissive. The adenovirus infectious cycle proceeds through adsorption, entry, and early viral gene expression but is aborted prior to viral replication in non-permissive cells. Abortive infection can cause transformation and can induce tumor infection. For example, as stated previously, Ad12 can induce tumor formation in newborn hamsters (Trentin et al., 1962). Studies of abortive infection indicate that although only class A adenovirus can induce tumors in rodents, all serotypes are able to transform primary rodent cells in culture (Freeman et al., 1967). Abortive infection causes transformation due to retention of a fraction of the viral genome in adenovirus-transformed cells.
Introduction of viral DNA via calcium-phosphate transfection to rodent cells in culture demonstrated that the minimal transforming genes of adenovirus are located within the E1 transcription unit (Graham et al., 1974). The role of E1A and E1B genes in transformation relates to their role, in the full replicative cycle of adenovirus, to induce cell cycle progression and block p53-dependent apoptosis, respectively. Adenovirus infection of permissive cells occurs via a step-wise process involving virus adsorption to the host cell, cell entry, uncoating, genome delivery to the nucleus, early gene expression, DNA replication, late gene expression, virion packaging, and finally, virion release from the host. The total replicative cycle takes about 24 hours in HeLa cells and approximately $10^4$ virions are produced per infected cell.

1.5.1. Adsorption, Uptake, And Uncoating

Adenovirus adsorption and uptake into cells occurs via separate but cooperative events that result from the interaction of the penton fiber protein with a receptor for attachment and the penton base protein with a receptor for internalization. The carboxy (C-) terminal knob of the fiber protein confers the specificity of cellular recognition by binding to a receptor termed CAR, so named as it was cloned as the Coxsackie virus and adenovirus receptor (Bergelsson et al., 1997). CAR is a cell surface protein of the immunoglobulin gene superfamily and its cellular function has yet to be elucidated. Human CAR mediates binding of Ad Subgroups A, C, D, E, and F (Roelvink et al., 1998). Murine CAR is 91% identical to its human homologue and thus explains the ability
of human adenovirus to infect mouse cells (Bergelsson et al., 1998). The specific tropism of Ad5 for epithelial cells of the upper respiratory tract (Horwitz, 1990) may be mediated by an auxiliary interaction of the Ad5 fiber knob with the α2 domain of the cellular MHC (major histocompatibility complex) class I receptor (Hong et al., 1997). MHC class I receptors are cell surface proteins responsible for antigen presentation and cytotoxic thymocyte cell recognition (reviewed in Bjorkman and Parham, 1990) and are an ideal target for Ad5 attachment.

Internalization of the virion occurs within 30 minutes post-infection and is initiated by the interaction of polypeptide III of the penton base with the integrin family of cell surface receptors (Wickham et al., 1993). This interaction is mediated by binding of an arginine, glycine, aspartic acid (RGD) sequence present in polypeptide III to αvβ3 and αvβ5 vitronectin-binding integrins. This mimics the normal cellular recognition of integrins by RGD sequence-containing extracellular adhesion molecules (Wickham et al., 1993).

Internalization of adenovirus occurs via receptor-mediated endocytosis (Greber et al., 1993) and release of the virion into the cytosol occurs prior to lysosome formation (Mellman, 1992). A drop in pH which occurs in the endocytic pathway aids the disassembly of the virion capsid as penton capsomers are lost (Greber et al., 1993). In the cytosol, the virion continues to disassemble as hexon constituents disassociate and are proteolytically digested.

1.5.2 Genome Delivery

The viral DNA core is transported across the cytoplasm via
microtubules (Luftig and Weihig, 1975) and enters the nucleus through the nuclear pores (Bodnar et al., 1989); these processes have been postulated to occur via the interaction of core protein V, which is associated with viral DNA, and cellular protein, p32. p32 function is not fully defined but it is associated with mitochondria in the cytosol and is present in the nucleus in association with splicing factor ASF/SF2 (Krainer et al., 1991). It is thought that p32 is a component of a shuttle which imports proteins to the nucleus and the adenovirus hijacks this process to deliver its genome to the nucleus. Upon arrival in the nucleus, the viral genome associates with the nuclear matrix via the terminal protein (Bodnar et al., 1989; Shaack et al., 1990). This association correlates with the initiation of viral gene expression (Shaack et al., 1990).

1.5.3 Early Gene Expression

In the period of 0-6 hours post-infection h.p.i., (Nevins et al., 1979) early gene expression occurs and products of the E1A, E1B, E2, E3, and E4 transcriptional units function to convert the host cell into a factory for viral DNA replication.

1.5.3.1 Early Region 1A

Upon adenoviral infection, E1A genes are the first to be expressed and are major regulators of the early events of infection. 

E1A Structure

The E1A transcription unit yields five mRNAs 13S, 12S, 11S, 10S, and 9S, named according to their Svedberg sedimentation coefficient. Each mRNA arises from a large primary E1 transcript due to alternate splicing. During the early stage of infection, 13S and 12S, the two major E1A mRNAs, are produced and give rise to
proteins of 289R and 243R, respectively (Pericaudet et al., 1980a, b). At later times, 11S, 10S and 9S E1A species appear but no clear role has been assigned to these products (Stephens and Harlow, 1987).

The mRNAs, 13S and 12S, produce identical proteins, except for a 46aa internal segment which is present only in the 13S product. These proteins are composed of three regions, conserved regions one, two, and three (CR1, CR2, CR3) that are conserved in all Ad serotypes and are important for protein-protein interactions (van Ormondt et al., 1980) (Figure 1.5). The 13S product contains all three of these regions whereas the 12S product lacks CR3, which corresponds to the 46aa segment absent in the 12S product. The extreme amino (N-) terminus and two non-conserved elements, termed auxiliary region 1 (AR1) and auxiliary region 2 (AR2), towards the C-terminus of E1A are also important regions.

E1A protein products are highly acidic and believed to be linear in structure due to a high proline content and an amenability to analysis by deletion mutagenesis (Barbeau et al., 1994). 13S and 12S protein products have a half-life of 20-30 minutes (Branton and Rowe, 1985), are located in the nucleus (Douglas and Quinlan, 1996) and are extensively serine phosphorylated (Yee et al., 1983; Yee and Branton, 1985).

E1A Function

An important role of the major E1 products is to activate transcription of early viral genes and to stimulate DNA synthesis. Both of these functions derive from the ability of E1A products to interact with an array of cellular proteins. Conserved regions 1, 2, and 3 mediate complex formation with host proteins and subsequent modification, inhibition, or utilization of cellular
protein function by E1A.

**E1A Transactivational Activity**

E1A's role as a transcriptional activator was discovered during studies using adenoviral mutants containing mutations in the E1A coding region. Most E1A mutant viruses were defective in early mRNA production (Berk et al., 1979; Shenk et al., 1979) and further studies showed that E1A proteins increase the rate of transcription of early viral genes remarkably (Nevins, 1981b). E1A is not a classical transcription factor as it does not possess any sequence-specific DNA-binding activity (Hearing and Shenk, 1985). E1A functions by interacting with a variety of cellular DNA-binding transcription factors and retargeting or remodeling these factors at viral promoters (Chatterjee et al., 1988).

Functional analysis has shown that CR3 in the 13S E1A product is an autonomous transactivating domain and is responsible for much of E1A's transactivational activity (Lillie et al., 1987). CR3 can be divided into two subdomains: a transactivation domain at the N-terminus and a promoter targeting region at the C-terminal (Lillie and Green, 1989). The transactivation domain contains a zinc-finger motif (Culp et al., 1998) that is involved in binding the TATA box binding protein (TBP). TBP is the DNA-binding component of the TFIID basal transcription complex and is essential for the formation of the transcription initiation complex at the TATA box present in most promoters (reviewed in Ptashne and Gann, 1997). E1A-289R potently activates transcription of viral and some cellular TATA-containing promoters via the ability of CR3 to contact TBP (Wu et al., 1987).

The C-terminal promoter targeting region of CR3 helps E1A enhance transcription of early viral genes via its direct interaction...
with promoter-bound transcription factors such as ATF-2, YY1 and AP-1. ATF-2 is a member of the cAMP-responsive element binding protein (CREB) family. CR3 binds to the bZ1P DNA-binding motif of ATF-2 (Abdel-Hafiz et al, 1993) and mediates transactivation by E1A through promoters containing ATF sites (Liu and Green, 1990). The E1A, E2, E3, and E4 promoters contain ATF binding sites and, therefore, E1A can activate each of the different promoters by interacting with a single upstream factor (Lee et al., 1987). The model of CR3-mediated transactivation is that CR3 acts as a bridge between TBP and ATF-2 or other transcription factors, stabilizing and stimulating the formation of the initiation complex at the promoter (Lee et al., 1991). Transactivation of the E1A and E3 transcription units appears to rely exclusively on such CR3-mediated interactions at ATF sites within their promoters. E1B is expressed efficiently by read-through from the E1A promoter and is activated by CR3 through the E1B TATA motif (Nevins, 1989). Expression from the E2 promoter depends in part on CR3 but mainly on separate E1A-dependent mechanisms of activation.

The E2 promoter contains ATF sites as well as E2F binding sites. E2F is a cellular transcription factor which was initially characterized as an activator of the E2 promoter (Kovesdi et al., 1986). E2F transcription factors exist as a family of heterodimers consisting of one of six E2F proteins bound to one of three DP proteins and function to stimulate transcription of genes required for cell cycle progression (Bandara et al., 1991). In non-dividing cells, E2F's transactivational activity is inhibited by interaction with the retinoblastoma susceptibility gene product (Rb) (Bandara et al., 1991). However in infected cells, the interaction of Rb with E1A stimulates the release of E2F, allowing it to interact with the
E2 promoter. E1A possesses a third mechanism for efficient E2 expression which relies upon E1A-mediated transactivation of the E4 region. The E4 orf6/7 protein binds to E2F and stabilizes E2F-containing complexes bound at the E2F sites in the E2 promoter, and thus enhances E2 expression (Raychaudhuri et al., 1990).

Expression of the E4 transcriptional unit is stimulated in a CR3-dependent manner but also requires the auxiliary regions 1 and 2 (AR1, AR2). This is due to the presence of binding sites for E4F in addition to ATF-2 sites (Liu and Green, 1990). E4F is a cellular transcription factor and, although its overexpression prevents entry into S phase of the cell cycle (Fernandes et al., 1998), E4F stimulation requires the binding of E1A via AR1 and AR2 (Bondesson et al., 1992). AR1 consists of six tandem glutamic acid-proline repeats while AR2 is acidic and contains a high serine and threonine content. The exact mechanism of stimulation is unknown but the net negative change of AR1 is essential (Fernandes et al., 1998). E1A also modulates E4F activity via an uncharacterized E1A- stimulated phosphorylation event on E4F that stimulates its DNA-binding activity (Raychaudhuri et al., 1989).

E1A also controls early viral gene expression by modulating the activity of transcription factor, AP-1. AP-1 is a family of heterodimeric transcription factors composed of various combinations of Fos, Jun, and ATF family members (reviewed in Karin et al., 1997). E1A stimulates the binding of AP-1 complexes composed of cJun/ATF heterodimers to ATF sites in the viral early promoters (Hardy and Shenk, 1988). The mechanism of AP-1 stimulation is not fully characterized, but in cooperation with cAMP, E1A increases transactivation of c fos and junB genes via their TATA motifs. Therefore, E1A upregulates levels of AP-1.
(Muller et al., 1992; Kleinberger and Shenk, 1993) and leads to increased transactivation of viral genes (Muller et al., 1989).

In addition to the regulation of RNA polymerase II-mediated expression, E1A also regulates RNA polymerase III-mediated expression of the VA RNAs. E1A stimulates the activity of transcription factor IIIC, which plays a role in transcription by RNA pol III (Hoefler et al., 1985).

Stimulation of Host DNA Synthesis

The natural targets of Ad5 are terminally differentiated epithelial cells. E1A is a powerful mitogen which stimulates infected cells to enter S-phase and synthesize the cellular DNA synthetic machinery required for viral DNA replication. The modulation of the cell cycle is carried out via the amino terminus, CR1, and CR2 regions of E1A. When expressed in isolation, both the 12s and 13s E1A proteins can induce DNA synthesis in quiescent human and rodent cells (Howe et al., 1990).

The mechanism by which E1A induces cell cycle progression was first revealed when E1A proteins were found to coimmunoprecipitate with cellular proteins that play important roles in regulating the cell cycle (Yee and Branton, 1985; Howe et al., 1990; Barbeau et al., 1993; 1994). The major E1A-associated proteins have molecular weights of 33, 60, 105, 107, 130, 300, and 400 kD.

The 105 kD protein was the first to be identified as the retinoblastoma susceptibility gene product (Rb) (Whyte et al., 1988). pRb is a tumor suppressor protein which causes cells to arrest in mid-to-late G1 (Goodrich et al., 1991) by binding to the transactivation domain of E2F (Bagchi et al., 1991) and suppressing
E2F-mediated transactivation (Flemington et al., 1993). pRb/E2F complexes are able to bind to E2F-responsive promoters to prevent transcription (Weintraub et al., 1992). Active repression is maintained via Rb's interaction with histone deacetylase enzymes (HDACs) (Brehm et al., 1998; Lai et al., 1999). HDACs function by deacetylating histones to promote chromatin condensation and possibly by deacetylating and inhibiting basal and activating transcription factors (Imhof et al., 1997). The activity of Rb is regulated through phosphorylation and its levels of phosphorylation fluctuate during the cell cycle (DeCaprio et al., 1989). Rb is hypophosphorylated during G0 and G1, hyperphosphorylated during S and G2, and dephosphorylated during M. Hyperphosphorylation of Rb abrogates its growth arrest function (Hinds et al., 1992) by preventing the association of Rb with E2F (Chellappan et al., 1991).

The other E1A-associated proteins, p107 and p130 are both related to Rb and the three proteins represent the Rb family of tumor suppressors (Hu et al., 1991; Ewen et al., 1991; Li et al., 1993). Like Rb, p107 induces growth arrest in G1 by binding and inhibiting E2F (Zamamian and La Thange, 1993). p130 is involved in negative regulation of E2F in G0 and early G1 (Cobrinik et al., 1993).

The Rb members contain regions of sequence similarity called A and B, separated by a variable length spacer region. This represents a protein-binding module termed 'the pocket' which binds proteins containing the sequence leucine-X-cysteine-X-glutamic acid, where X is any amino acid (LXCXE) (DeCaprio et al., 1988; Dyson et al., 1990). The pocket is responsible for association of Rb family members with E2F transcription factors and other proteins involved in cell cycle regulation.
E1A induces cell cycle progression via interactions of CR1 and CR2 with Rb family members. CR2 is the primary binding site and contains an LXCXE motif (Egan et al., 1988; Whyte et al., 1989) and CR1 is important in displacing E2F from Rb family members (Fattaey et al., 1993). E1A frees E2F to activate genes involved in cell cycle progression and DNA synthesis such as cdc-2, DNA polymerase α, dihydrofolate reductase, ribonucleotide reductase, thymidine kinase, c-fos, c-myb, c-myc, insulin-like growth factor 11, N-myc, neu, and transforming growth factor β1 (reviewed in Nevins, 1992). As stated previously, free E2F also activates the viral E2 promoter.

The small p60 and p30 proteins associated with E1A are also involved in E2F regulation. p60 is cyclin A (Pines and Hunter, 1990) and p30 is cyclin-dependent kinase 2 (Tsai et al., 1991). Cyclins A-H are a family of proteins whose levels vary throughout the cell cycle (reviewed in Hunter and Pines, 1994). They function in complex with the family of cyclin dependent kinases (cdks) to phosphorylate substrates such as Rb and thus control cell cycle progression. Different combinations of cyclins and cdks are present at various times during the cell cycle depending on the cyclin expression pattern. Cyclin A and cdk-2 bind E1A indirectly through their direct interaction with the spacer region of p107 or p130 which is not occluded by E1A binding (Giordano et al., 1997; Howe and Bayley, 1992). As a result, E1A is associated with cdk kinase activity and may contribute to the dramatic elevation in phosphorylation of p107, p130 and Rb upon E1A binding (Barbeau et al., 1994). The exact role of cdk2 and cyclin A in complex with E1A is unknown.

The interaction of E1A with p300 and related proteins, p400...
and CBP, allows E1A to promote growth by a mechanism independent of E2F activation. The interaction requires CR1 and a less conserved region at the extreme N-terminus of E1A (Egan et al., 1988; Barbeau et al., 1993; 1994). p300 has been cloned (Eckner et al., 1994) but its cellular function has not been clearly defined. The protein contains a bromodomain (a domain typical of transcriptional regulators but of unknown function), a histone acetyltransferase (HAT) domain, and putative zinc fingers formed by three cysteine/histidine rich regions. E1A binds p300 via the most C-terminal cysteine/histidine rich region (Eckner et al., 1994). p400 has not been characterized but is likely related to p300 as V8 protease mapping revealed a similar pattern (Barbeau et al., 1994). p300 is closely related to CREB binding protein (CBP) (Chrivia et al., 1993) and the two species are members of a family of transcriptional coactivators (Arany et al., 1994).

p300 and CBP act at the transcriptional level to stimulate the process of differentiation and maintain cells in G0 in response to a variety of signals, such as the second messenger cAMP, steroid hormones, and those involved in AP-1 signaling. For example, when cAMP is released in the cell, protein kinase A is activated and phosphorylates the cAMP-responsive element (CRE) binding protein (CREB) (Gonzalez and Montminy, 1989). PKA-phosphorylated CREB associates with CBP and p300 and this complex can activate transcription of CRE-containing promoters (Chrivia et al., 1993) and results in cell cycle arrest and stimulation of the differentiation phenotype (Moran, 1993). E1A binds both p300 and CBP and prevents the interaction with CREB and subsequent transcriptional activation (Arany et al., 1995).

E1A proteins repress transcription of cellular enhancers from
a variety of tissue-specific genes involved in terminal differentiation. In the process of blocking muscle specific promoters, E1A proteins block myogenic differentiation (Webster et al., 1988). Growing evidence suggests that E1A proteins mediate these effects through binding with p300. The regions of E1A required for enhancer repression are the same as those required for interaction of p300 (Wang et al., 1993).

The mechanism of transcriptional activation by p300 is believed to rely on p300's intrinsic histone acetyltransferase activity and the ability of p300 and CBP to bind to p300/CBP associated factor (P/CAF) which is a histone acetyltransferase enzyme (Yang et al., 1996). Acetylation of histones is believed to disrupt chromatin structure and facilitate DNA binding of transcription factors, leading to increased gene expression (reviewed in Wolfe, 1997). E1A abrogates p300 and CBP function by inhibiting HAT enzymatic activity of both p300 and P/CAF (Hamamori et al., 1999) and by preventing binding of P/CAF which binds the same site on p300 as E1A (Yang et al., 1996).

In summary, E1A proteins induce DNA synthesis in quiescent, differentiated epithelial cells by interacting with two major classes of proteins, the Rb and p300/CBP families.

Consequences of E1A Activity

In normal, uninfected cells, cell cycle progression is highly regulated and control mechanisms exist to suppress tumor growth in response to hyperproliferation signals. The cellular tumor suppressor protein, p53, plays a key role in protecting multicellular organisms from individual aberrant cells which threaten the entire organism due to their neoplastic potential or
presence of infectious organisms (reviewed in Levine, 1997). p53 is a transcription factor containing a potent acidic transactivation domain that induces gene expression following binding to a sequence-specific p53-responsive element (Ko and Prives, 1996). p53 also has the ability to repress certain promoters but the mechanism has yet to be identified.

In normal cells, p53 levels and function are regulated by the protooncogene, murine double minute 2 (MDM2). MDM2 binds p53, prevents p53-mediated transactivation and targets p53 for ubiquitin-dependent proteolysis (Haupt et al., 1997). In response to DNA damage, cellular stress, or aberrant growth control, p53 is upregulated and activated to protect the cell in two ways. First, p53 induces G1 arrests by activating genes such as that encoding an inhibitor of cdks, which prevents phosphorylation and inactivation of Rb family members (el diery et al., 1993). Second, p53 induces cell death by apoptosis in part by activating apoptosis-promoting protein Bax (Miyashita et al., 1993). In cells expressing E1A, the ability of E1A to induce unscheduled DNA synthesis triggers the activation of p53 leading to the induction of apoptosis (Brathwaite et al., 1990; Grand et al., 1993; 1994). E1A stabilizes and upregulates p53 by increasing p53's half life from 20 minutes in uninfected cells to over 2 hours in E1A-expressing cells (Querido et al., 1997b). Recent studies have shed light on E1A's mechanism of p53 activation. In response to binding either Rb or p300/CBP family members E1A proteins induce the tumor suppressor p19-ARF (de Stanchima et al., 1988). p19-ARF is a protein encoded by the INK4a locus in an alternate reading frame than that of p16-INK4a, a cdk-4 inhibitor. p19-ARF is a potent regulator of the cell cycle and a critical component of the p53 pathway (reviewed in Sharpless and

E1A's capacity to override cell controls also sensitzes host cells to apoptosis by tumor necrosis factor (TNF) (Shister et al., 1996). TNF is a cytokine which functions in innate immunity by multiple mechanisms including induction of apoptosis of virus-infected cells. TNF-cytotoxicity is dependent upon the cell cycle as TNF must bind its receptor between G1/S or G2/M for death to occur (Coffman et al., 1989). It is likely that E1A inadvertently renders an infected cell susceptible to TNF. At later times in infection, E1A is responsible for induction of another apoptotic pathway. Following E1A-mediated transactivation of the E4 region, expression of E4orf4 induces p53-independent apoptosis (Marcellus et al., 1996b; 1998; Lavoie et al., 1998; Shtrichman and Kleinberger, 1998).

In adenovirus-infected cells, the induction of cell death by E1A at early stages in the infection cycle would severely limit progeny production. Therefore, adenoviruses have evolved multiple mechanisms to counter this cellular apoptotic response. E1A can partially inhibit p53 transactivation by binding and sequestering p300, a cofactor for p53 transactivation (Gu et al., 1997). Other mechanisms to block apoptosis early in infection rely on E1A-dependent transactivation of early viral genes and will be discussed in further detail below.

1.5.3.2 Early Region 1B

E1B Structure
The E1B transcription unit produces multiple mRNA's from alternative splicing of a primary transcript (see fig. 1-2). Early in infection the major Ad5 E1B transcript is a 22S mRNA that encodes a 19KDa polypeptide named E1B-19K and it also encodes an unrelated 55KDa species named E1B-55K using an internal initiation site in a different reading frame (Bos et al., 1981). Other E1B transcripts of varying size, 13S, 14S, and 14-5S, also encode 19K and a number of 55K and 19K related proteins of 84R, 156R, and 93R (Takayesu et al., 1994; Logan et al., 1984). E1B-55K and E1B-19K are well characterized. They are required for maximal viral growth and serve to overcome the toxic effects of E1A expression (White and Cipriani, 1990; McLorie et al., 1991; Rao et al., 1992). As discussed, transcription of E1B proceeds relatively independently of E1A transactivation and significant levels of E2B-55K and E1B-19K are present when E1A proteins are present.

**E1B-55K**

E1B-55K is a phosphoprotein located predominantly in the perinuclear region (Rowe et al., 1983; Yee et al., 1983; Sarnow et al., 1982b). E1B-55K is highly stable with a half-life greater than 4 hours (Branton and Rowe, 1985). The E1B-55K protein performs several critical functions in viral replication. Early in infection, E1B-55K inhibits E1A-induced p53-mediated apoptosis via association with p53 (Marcellus et al., 1996a; Teodoro and Branton, 1997b). E1B-55K binds to the acidic transactivation domain of p53 (Lin et al., 1994) and inhibits p53-mediated transcription (Yew and Berk, 1992). The interaction of E1B-55K and p53 is necessary but not sufficient for repressing transcriptional activity of p53. Studies in which E1B-55K has been fused to the DNA-binding
domain of the Gal4 transcription factor indicated that E1B-55K functions as a potent transcriptional repressor (Yew et al., 1994). The repression activity of E1B-55K has been mapped to a region near the carboxy terminus (Assefi, unpublished observations). The mechanism of repression is unknown. In addition to repression, binding of E1B-55K to p53 increases the affinity of p53 for DNA by approximately tenfold (Martin and Berk, 1998). This helps guarantee that p53-regulated gene expression is reduced below basal levels (Martin and Berk, 1998).

E1B-55K regulates p53 by a second mechanism seemingly independent of its repression function. E1B-55K functions in complex with E4orf6 to cause a dramatic decrease in p53 protein levels (Moore et al., 1996; Nevels et al., 1997; Querido et al., 1997a). The mechanism by which the E1B-55K/E4orf6 complex promotes p53 degradation will be discussed in the section on E4orf6 function.

E1B-55K has two additional functions which are critical to the progression of the adenovirus infectious cycle. Later in infection, E1B-55K functions in shut-off of host cell synthesis and the selective stabilization, transport, and translation of viral mRNAs. These functions will be discussed in later sections.

E1B-19K

E1B-19K is modified by fatty acylation (McGlade et al., 1987; Takayesu, unpublished observations) and phosphorylation (McGlade et al., 1989). E1B-19K is localized to nuclear and cytoplasmic membranes, and intermediate filaments and nuclear lamins (McGlade et al., 1987; 1989; Rowe et al., 1983; White and Cipriani, 1989).
E1B-19K is essential for successful adenoviral infection as infection of cells by Ad mutants defective in 19K causes the phenotype termed cyt/deg characterized by poor virus yield, degradation of DNA, and extensive cytopathic effects (Ezoe et al., 1981). More recent studies have shown that the cyt/deg phenotype is the result of induction of apoptosis (Rao et al., 1992) and therefore, E1B-19K functions to inhibit apoptosis induced in response to adenoviral infection.

The mechanism by which 19K prevents apoptosis was revealed by the identification of two bcl-2 homology (BH) domains in the 19K sequence. BH domains mediate dimerization amongst members of a family of cellular apoptosis modulators, the Bcl-2 family (reviewed in Chao and Korsemeyer, 1998). The Bcl-2 family contains both inducers and inhibitors of apoptosis and the balance of homodimers and heterodimers of inducers and inhibitors determines cell fate. Like Bcl-2, E1B-19K is an anti-apoptotic member of the Bcl-2 family. Bcl-2 can replace E1B-19K in inhibiting E1A-induced apoptosis (Rao et al., 1992). E1B-19K can bind and inhibit inducers of apoptosis such as Bax, Bak, Bik (Farrow et al., 1995; Han et al., 1996a, b) and thus prevent the induction of apoptosis in a manner similar to Bcl-2. E1B-19K can block both E1A-induced p53-dependent apoptosis and p53-independent apoptosis induced by the host immune response to infection. E1B-19K blocks cell death activated by TNFα and Fas which are both involved in cellular antiviral defense (White et al., 1992; Perez and White, 1998). E1B-19K and Bcl-2 can also block the p53-independent apoptotic pathway mediated through E1A transactivation of the E4 region (Subramanian et al., 1995; Teodoro et al., 1995).
In summary, the two major proteins of the E1B transcription unit, function early in infection to maintain host cell viability and thus, allow the adenovirus infectious cycle to proceed. E1B-55K and e1B-19K block the cell apoptotic response to E1A expression and help provide a cellular environment conducive to viral replication.

1.5.3.3 Early Region 2

The E2 transcription unit produces two mRNA families, E2A and E2B, which are initiated at similar sites but utilize different polyadenylation signals and undergo alternative splicing (see fig. 1-2) (Berk and Sharp, 1978; Horwitz, 1990). The E2A family encodes the E2 DNA binding proteins (E2-DBP) and the E2B family encodes the pre-terminal protein (E2-pTP) and the DNA polymerase (E2-DNA pol). These three proteins are necessary for synthesis of viral DNA.

The pre-terminal protein is synthesized as an 80 kD and is involved in the initiation of DNA replication (Challberg et al., 1980). E2-pTP is able to interact with the 140 Kd E2-DNA polymerase and directs the formation of the replication initiation complex at the origin of viral DNA replication (Challberg et al., 1980). E2-DNA polymerase catalyses the covalent linkage of E2-pTP to the 5' end of the viral genome where E2-pTP functions as a primer for DNA synthesis and to protect the integrity of the viral termini over multiple rounds of infection (Rekosh et al., 1977). Late in infection, E2-pTP is cleaved by the L4-protease to generate the 55KDa terminal protein (TP) which remains attached to the viral DNA core in mature virion (Challberg and Kelly, 1981).

E2-DNA polymerase replicates adenoviral DNA with high
fidelity and efficiency. E2-DNA polymerase possesses 5' to 3' DNA polymerase activity and 3' to 5' exonuclease activity which serves a proofreading function during DNA polymerization (Field et al., 1984).

E2-DNA binding protein is a 59 Kd phosphopeptide which migrates as a 72 Kd species during SDS-PAGE. E2-DBP has non-specific DNA binding activity (van der Vliet and Levine, 1973) and functions to coat and stabilize ssDNA-intermediates during viral replication (Field et al., 1984). E2-DBP enhances E2-DNA polymerase processivity and is essential in the elongation step of replication (van der Vliet et al., 1975).

1.5.3.4 Early Region 3

The E3 region produces 9 mRNA species from a primary transcript and seven protein products have been identified of which, five proteins have been characterized (Fig. 1-2). The E3 cassette of genes is dispensable for adenovirus replication in tissue culture (Cladaras et Wold, 1985), but it is indispensable for productive adenovirus infection in humans (reviewed in Ploegh, 1998). E3 protein products modulate the host immune response to adenoviral infection (reviewed in Wold and Gooding, 1991).

The E3-19KDa protein (gp19K) is a transmembrane glycoprotein that is localized to the endoplasmic reticulum (ER) (Wold et al., 1985). The function of gp19K is to protect infected cells against cytotoxic T-lymphocyte (CTL)-mediated lysis (Andersson et al., 1987). CTLs recognize and lyse cells that present viral antigenic peptides in complex with a cellular MHC class I antigen on the cell surface and in this manner, CTLs function to severely limit viral propagation. gp19K acts within the
ER to bind to MHC class I antigens, via their antigen-binding domain (Herminston et al., 1993), and hence prevent their transport to the cell surface.

Lymphocytes and activated macrophages also threaten the survival of adenovirus-infected cells through secretion of tumor necrosis factor-α, an apoptosis-inducing cytokine (Duerksen-Hughes et al., 1991; TNF reviewed in Wallach et al., 1997). TNFα binds its receptor on the host cell surface and induces a calcium-dependent translocation of phospholipase A2 (cPLA₂) to the plasma membrane where it catalyses the release of arachidonic acid (AA). AA release stimulates the host inflammatory response and host cell death by apoptosis. Adenoviruses have evolved several mechanisms to prevent this progeny-limiting host response. These include the aforementioned E1B-19K and three E3 proteins. The E3-dependent mechanisms are essential in preventing this antiviral response as TNFα efficiently lyses cells infected with adenovirus mutants which lack the E3 region (Goody et al., 1988).

The E3-14.7 kD protein is located in the cytoplasm and nucleus (Tollefson and Wold, 1988). By an unknown mechanism, E3-14.7 kD inhibits cPLA₂ activity and prevents the release of AA, and thus blocks TNF-induced cytolysis (Krajcisi et al., 1996).

The E3-14.5 kD and E3-10.4 kD proteins also function to prevent TNFα activity. E3-14.5K and E3-10.4K are integral membrane proteins that associate as heterodimers in the plasma membrane (Tollefson et al., 1991). The E3-14.5K/E3-10.4K heterodimer is known as the receptor internalization and degradation (RID) complex. The RID complex blocks TNFα signaling by inhibiting the translocation of cPLA₂ from the cytoplasm to the
plasma membrane (Krajicsi et al., 1996; Dimitrov et al., 1997). In addition, the RID complex can inhibit apoptosis induced in response to the Fas ligand, a TNFα family member. RID stimulates the endosome-mediated internalization and the lysosomal degradation of the cell surface Fas receptor (Tollefson et al., 1998). The RID complex also downregulates surface expression of epidermal growth factor receptor (EGFR) via internalization and degradation (Tollefson et al., 1992). The purpose of EGFR degradation during adenoviral infection is not known.

The E3-11.6 kD protein has an unusual expression pattern during adenoviral infection. Early in infection, E3-11.6K is expressed in minute levels from the E3 promoter and then late in infection, E3-11.6K expression is initiated at the major late promoter and levels increase 400-fold (Tollefson et al., 1992). This change in E3-11.6K expression levels parallels its change in localization from its initial site in the ER and Golgi apparatus to its predominant location in the nuclear membrane later in infection (Scaria et al., 1992). E3-11.6K is a type III membrane protein that is post-translationally modified by glycosylation (Scaria et al., 1992) and palmitoylation (Hausmann et al., 1998).

Unlike the other E3 proteins, E3-11.6K does not function to counteract host immune response to adenoviral infection. Infection of cells by adenoviral mutants which lack E3-11.6K are defective in viral progeny release and host cell lysis (Tollefson et al., 1996a) and exhibit an intact nucleus, swollen with virus, at times post-infection when wild-type virus has disrupted the nucleus and killed the cell (Tollefson et al., 1996b). These results suggest that E3-11.6K plays a role in the final stages of the adenovirus infection cycle and since those experiments, E3-11.6K has been termed the
adenovirus death protein (ADP). The mechanism by which ADP contributes to cell killing and progeny release is unknown. Late in infection, ADP partially colocalizes with E1B-19K (Bill Wold, personal communication) and may function to inactivate E1B-19K and promote cell death by apoptosis.

1.5.3.5 Early Region 4

The E4 transcription unit is located at the extreme right end of the adenovirus genome (Fig. 1.2). Studies conducted using a complementary DNA library indicate that 12 unique mRNAs are transcribed and encode at least seven polypeptides as determined by the identification of open reading frames (orf) and differential splice sites (Virtanen et al., 1984). The E4 products possess a wide and varied range of activities that are crucial to successful adenoviral replication (Bridge et al., 1993).

E4orf1

The Ad5 E4 orf1 mRNA encodes a protein of 14 kD. The function of E4orf1 in the lytic cycle is unknown but studies of E4orf1 from different adenovirus serotypes indicate that it is a novel transforming protein. The oncogenic activity of E4orf1 was first revealed when Ad9 E4orf1 was shown to be responsible for the induction of mammary carcinomas in rats in response to Ad9 infection (Javier, 1994; Weiss et al., 1996). Although there is some variation in the sequence of E4orf1 among different serotypes, E4orf1 from Ad5, Ad12, and Ad9 are equally capable of transforming human cells (Weiss et al., 1997a). The transforming potential of Ad9 E4orf1 has been mapped to three regions within the protein, including a region present in the C-terminus which is a PDZ consensus binding motif (Weiss et al., 1997b). PDZ domains are
Figure 1.3 Products of Early Region 4

Boxes and lines indicate protein products and spliced products, respectively. Adapted from Querido et al., (1996b)
motifs found in a variety of signaling proteins. The motif derives its name from the three proteins originally found to contain these sequences which are as follows: post-synaptic density protein, PSD-95, the Drosophila septate junction protein, discs-large suppressor, Dlg, and the epithelial tight junction protein, ZO-1 (reviewed in Ranganathan and Ross, 1997). These regions are responsible for binding a series of unidentified cellular proteins and the mammalian homologue of the Drosophila disc large tumour suppressor (Lee et al., 1987). Ad5 E4orf1 binds a subset of these same proteins, suggesting that E4orf1 of Ad5 and Ad9 share a common mechanism of action in transformation (Weiss and Javier, 1997). The functional importance of these interactions with respect to viral growth remains to be determined.

E4orf2

The E4orf2 mRNA encodes a 14.6 kD protein that is localized to the cytoplasm in adenovirus-infected cells (Dix and Leppard, 1995). The function of this protein is unknown and it is not required for viral growth in tissue culture (Bridge and Ketner, 1989).

E4orf3

The E4orf3 mRNA encodes an 11 kD protein which is localized to the nucleus (Sarnow et al., 1982). E4orf3 stimulates and regulates late gene expression. Its functions in adenovirus infection are redundant with those of E4orf6 which can complement the growth of E4orf3-defective mutants (Bridge and Ketner, 1989). In the nucleus of adenovirus-infected cells, E4orf3 localizes to and reorganizes the structure of promyelocytic leukemia oncogenic domains (PODs) which are macromolecular protein complexes active in cellular gene expression, cell cycle control, and
regulation of DNA damage (reviewed in Sternsdorf et al., 1997). E4orf3 disrupts the POD structure and mediates the relocation of proteins from PODs to viral inclusion bodies where viral replication and late gene transcription occur (Doucas et al., 1996). E4orf3 may relocate cellular proteins involved in gene expression and thus, contribute to late gene transcription. E4orf6 in complex with E1B-55K has also been suggested to relocate a cellular protein to sites of viral replication (Ornelles and Shenk, 1991). This may explain the ability of E4orf6 to compensate for E4orf3's absence. Studies of E4orf3 activity in cells transformed by the E1 region show that E4orf3 can also bind to E1B-55K and relocalize to PODs (Konig et al., 1998). This interaction of E1B-55K with E4orf3 disrupts its association with p53 and relieves p53 inhibition by E1B-55K (Konig et al., 1999). When expressed, E4orf6 preferentially interacts with E1B-55K and p53 activity is inhibited (Koenig et al., 1998). In adenovirus-infected cells, E4orf3 may cause a temporary relief of p53 inhibition so that p53 can fulfill a role in infection that has yet to be determined.

**E4orf6**

The E4orf6 mRNA encodes a 34 kD protein that contributes to viral gene expression late in infection and acts in concert with E1B-55K to overcome E1A's toxic effects early in infection. E4orf6 is localized in the nucleus and is found predominantly in complex with E1B-55K (Sarnow et al., 1984). E4orf6 also coprecipitates with other viral and cellular proteins in infected cells (Boivin et al., 1999). The E4orf6 protein sequence has three signaling domains (Dobbelstein et al., 1997): a classical nuclear localization signal, a rev-like signal sequence that mediates nuclear export, and an arginine-faced amphipathic alpha helix (Orlando and Ornelles, 1997).
that functions as a nuclear retention signal. These domains are essential for the function of E4orf6 in selective viral mRNA transport and host cell shut-off. In complex with E1B-55K, E4orf6 shuttles between the cytoplasm are viral replication centers and this requires all three domains.

E4orf6 possesses two mechanisms to inhibit E1A-induced p53 activation. E4orf6 binds directly to the C-terminus of p53 and displaces TAF131 from the N-terminal activation domain of p53 (Dobner et al., 1996). In this manner, E4orf6 can suppress p53 transactivation and block p53-mediated apoptosis in some systems (Dobner et al., 1996; Nevels et al., 1997).

In addition, the E1B-55K-E4orf6 complex prevents p53 accumulation in response to E1A expression (Nevels et al., 1997; Querido et al., 1997a). The reason for this effect is that E4orf6 and E1B-55K, possibly bound to p53 in a trimeric complex, stimulate p53 turnover (Querido et al., 1997a; Dobner et al., 1996). This effect may be achieved through the targeting of p53 to the ubiquitin system of intracellular protein degradation, as some of the E4orf6 cellular binding proteins (Boivin et al., 1999) have recently been identified as members of an E3 ubiquitin ligase family (Morrison, unpublished observations).

E4orf6/7

E4orf6/7 is a 19.5 kD protein expressed from a spliced mRNA that encodes the N-terminus of E4orf6 linked to the unique E4orf7 sequence. E4orf6/7 contributes to viral DNA synthesis by ensuring production of high levels of E2 products. Two E4orf6/7 molecules bind directly to two E2F-DP heterodimers and induce cooperative and stable binding to the two E2F sites located in the E2 promoter.
E4orf3/4

E4orf3/4 is expressed from a spliced mRNA and encodes a small protein of 7.1 kD composed of the N-terminus of E4orf3 and the C-terminus of E4orf4. The function of this protein is not known and it is not required for viral growth in tissue culture (Bridge and Kettner, 1989).

E4orf4

The E4orf4 mRNA encodes a 114 residues protein of 14 kD. The activity of E4orf4 was first revealed through studies showing a synergistic effect on transcription factor AP-1 (reviewed in Karin et al., 1997) by E1A and cAMP (Engel et al., 1988; 1991; Muller et al., 1989). During the course of adenovirus infection in the presence of cAMP, a decrease in the phosphorylation levels of E1A and c-Fos, an AP-1 family member, was observed (Muller et al., 1989). Hyperphosphorylation of c-fos resulted in a concomitant decrease in AP-1 transcriptional activity (Muller et al., 1989; Kleinberger and Shenk, 1993). This dephosphorylation activity was mapped to E4orf4 and it was not observed in E4orf4 defective viral mutants (Muller et al., 1992). The kinetics of this effect implied that E4orf4 was acting to inhibit a cellular kinase involved in the phosphorylation of E1a and c-Fos. E4orf4 was additionally shown to suppress JunB and c-Fos (another AP-1) protein levels at both transcriptional and translational levels (Bondesson et al., 1994).

A major insight into the mechanism of action of E4orf4 came with discovery that E4orf4 associates with cellular protein phosphatase 2A (PP2A; reviewed in Mumby and Walter, 1993) by interaction with its Bα subunit (Kleinberger and Shenk, 1993). The effects of E4orf4 on AP-1 are believed to be caused by PP2A-
dependent dephosphorylation and inactivation of the mitogen-activated kinase (MAPK), which can act as a PP2A substrate and which is believed to play a role in regulation of AP-1 (Braconi Quintaje et al., 1996). AP-1 is involved in the activation of a number of early viral genes, indicating that E4orf4 is involved in downregulating viral promoters. The effect of E4orf4 on PP2A and its ability to regulate MAPK has direct implication on regulation of E4 gene expression. As discussed previously, E4 expression is dependent upon the E1A-mediated phosphorylation and activation of E4F (Raychaudhuri et al., 1989; Rooney et al., 1990) and of ATF-2 (Liu and Green, 1990; Martin et al., 1990). E4orf4 inhibits E4 expression but treatment with okadaic acid, a potent inhibitor of PP2A (Cohen et al., 1990), results in upregulation suggesting that the PP2A/E4orf4 complex reverses the phosphorylation events required for E4 expression. While direct effects on E4F phosphorylation play a role (Bondesson et al., 1996), the ability of E1A to transactivate the E4 promoter may also be affected. Two MAPK dependent sites in E1A have been identified that are required for E1A-mediated activation of E4 expression (Whalen et al., 1997).

In addition to its ability to regulate viral transcription, E4orf4 is also involved in regulation of alternative splicing. E4orf4 modulates the activity of SR proteins which are splicing factors involved in spliceosome assembly and recognition of splice sites (Zahler et al., 1992). Studies of the L1 pre-mRNA indicated that early in adenovirus infection, hyperphosphorylated SR proteins bound and prevented the production of the IIIA mRNA and then later in infection, the E4orf4/PP2A complex can dephosphorylate and inactivate SR proteins which allows the usage of the secondary
splice site and the preferential splicing of the IIIA mRNA (Kanopka et al., 1998).

Another activity of E4orf4 was revealed during studies undertaken to define the apoptosis pathways induced in response to adenovirus infection. It was observed that E1B-defective viruses expressing the E1A-289R induce apoptosis in p53-null cells (Teodoro et al., 1995). No such response was observed with virus expressing E1A 243R. This effect was shown to rely on the ability of E1A-289R to transactivate the E4 region, indicating that one or more E4 proteins is cytotoxic (Marcellus et al., 1996). Furthermore, in the presence of the E1B region, cell killing at the final stages of the infectious cycle is prevented or greatly delayed in the absence of E4 products (Marcellus et al., 1996). Studies using mutants defective in expression of various E4orfs implicated both E4orf6 and E4orf4 in this process (Marcellus et al., 1998). However, only E4orf4 induces apoptosis when expressed in the absence of other adenovirus products (Marcellus et al., 1998, Lavoie et al., 1998; Schitrichman and Kleinberger., 1998). This function may play a role in the ultimate death of infected cells and spread of progeny virions by inducing p53-independent apoptosis. Cells infected with mutants defective in E4orf4 remain intact and survive longer than wild-type infected cells (Marcellus et al., 1998) presumably because E4orf4 is not present to induce apoptotic death. Infected cells also show an elevated cytopathic effect which may be due to the high expression of a toxic protein whose levels are usually down regulated by E4orf4. E4orf4's role at the end of infection may explain its ability to autoregulate its expression so as to prevent cell death at early times in infection.

E4orf4 induces a p53-independent cell death process that is
characterized by classic apoptotic features including DNA degradation, chromatin condensation, and loss of mitochondrial potential (Lavoie et al., 1998; Shtrichman and Kleinberger, 1998). Studies conducted in hamster cells indicate that this process can be inhibited by Bcl-2 or Bcl-X\textsubscript{L}, but not by zVAD-fmk, a potent pan-inhibitor of caspases (Lavoie et al., 1998). These results suggest E4orf4 triggers apoptosis in a manner which is independent of caspase activation and may act at the terminal mitochondrial-permeability transition phase of apoptosis (Lavoie et al., 1998). The interaction of E4orf4 with PP2A may be important to the mechanism of E4orf4-induced apoptosis as mutant E4orf4 protein which lost the ability to induce apoptosis also lost the ability to bind PP2A (Shtrichman and Kleinberger, 1998).

1.5.4 Viral DNA Replication

Viral DNA replication begins approximately 6 hours after infection and reaches its maximal level by 18 h.p.i (Pina et al., 1969). Replication is initiated at the core origin located within the two terminal repeats of the adenovirus genome (Tolun et al., 1979). Initiation is achieved at the origin through the interaction of E2 pre-terminal protein in complex with E2-DNA polymerase. E2-DNA polymerase catalyses the formation of an ester linkage between the beta hydroxyl group of a serine residue in pTP and the alpha phosphoryl group of the deoxycytidylate residue at the extreme 5' ends of the viral genome (Rekosh et al., 1977). Then, E2-DNA polymerase initiates DNA synthesis using E2-pTP as a primer (Rekosh et al., 1977). Efficient initiation also requires two cellular proteins, nuclear factor I and III (NF\textsubscript{I}, NF\textsubscript{III}) (Chen et al., 1987).
1990; Bosher et al., 1990). NF-I binds to E2-pTP and serves to stabilize the preinitiation complex (Chen et al., 1990). NF-III has DNA-bending activity which is believed to aid DNA strand separation at the origin (Verrijzer et al., 1991). Viral DNA replication proceeds via leading strand synthesis and displacement of the non-template strand. Elongation is dependent upon the presence of E2-DNA binding protein, which stabilizes the non-template leading strand (Van der Vliet et al., 1995) and the presence of cellular protein NF-2, which possesses topoisomerasers activity to relieve torsional strain induced in the DNA structure during replication (Nagata et al., 1983).

1.5.5 Late Gene Expression

Once viral DNA replication has commenced, a dramatic shift occurs from the expression of early genes to the expression of late genes. The late viral coding region is expressed as a single primary transcript of about 29 kb in length driven by the major late promoter (see Fig 1.1.4) (Nevins and Darnell, 1978). Through differential splicing and use of alternative polyadenylation signals, this large transcript is processed into five families of late transcripts, L1-L5 (Nevins and Darnell, 1978). Two E4 proteins stimulate and regulate late mRNA accumulation. E4orf3 and E4orf6 are involved in viral RNA processing as they control splice site usage in alternatively spliced transcripts derived from the major late promoter (Nordqvist et al, 1994). The transcripts are translated into the structural proteins of the adenovirus virion and expression reaches a peak at 18 hours post-infection.

1.5.6 Host Cell Shut off
With the onset of DNA replication and the shift to late gene expression, the virus initiates a block in host protein synthesis (Beltz and Flint, 1979). This process, known as host cell shut-off, prevents translation of host cellular proteins and is achieved by two mechanisms. First, the selective transport of viral mRNAs occurs in which the transport of cellular mRNAs from the nucleus to the cytoplasm is blocked while viral mRNAs are efficiently shuttled to the cytoplasm (Beltz and Flint, 1985; Bridge and Ketner, 1990; Dobbelstein et al, 1997). This phenomenon is achieved by the activities of the E1B-55K and E4orf6 which function in a complex (Sarnow et al., 1984) that shuttles between the cytoplasm and viral replication centers (Ornelles and Shenk, 1991). The current model is that the E1B-55K/E4orf 6 complex sequesters an unknown cellular factor required for mRNA export and directs it to sites of viral transcription, thereby inhibiting host mRNA export and promoting viral mRNA export (Ornelles and Shenk, 1991). A novel nuclear RNA-binding protein of the heterogeneous ribonucleoprotein (hnRNP) family, called E1B-AP5, was isolated as a E1B-55K associated protein and has been implicated as the unknown cellular factor (Gabler et al., 1988).

The second mechanism of host cell shutoff is the preferential translation of viral mRNAs which is achieved in concert with the host cell's normal antiviral defense strategies (reviewed with Zhang and Schneider, 1993). In response to viral infection, the interferon-inducible cellular protein kinase R (PKR) is stimulated (O'Malley et al., 1989) and phosphorylates, thereby inactivating, the translation initiation factor eIF-2α (de Haro et al., 1996). This causes a global block in protein synthesis which is overcome specifically at viral translation centers by the localization of
adenovirus VA RNA. In Ad5, VA RNA I and II associate with viral mRNAs (Matthews, 1980) and block PKR activation (O'Malley et al., 1989) allowing the preferential translation of viral mRNAs.

In addition, in the late phase of adenoviral infection, the mRNA cap binding translation initiation factor, eIF-4F, is dephosphorylated and inactivated and this blocks translation of most cellular mRNAs (Huang and Schneider, 1991). eIF-4F’s helicase activity is necessary to remove secondary structures in the 5’ untranslated region (UTR) of mRNA and allow translation to initiate (reviewed in Jackson and Wickens, 1997). Unlike most cellular mRNAs, the 5’UTR of late adenoviral messages, known as the tripartite leader, is completely free of secondary structure (Dolph et al., 1990) and translation occurs in the absence of eIF-4F activity (Huang and Schneider, 1991). Finally, a viral protein aids in the preferential translation of adenoviral transcripts. A L4-100, 100 Kd protein encoded by L4 (Hayes et al., 1990) binds mRNA (Riley and Flint, 1993) and by an unknown mechanism, stimulates efficient translation of viral products (Hayes et al., 1990).

1.5.7 Virion Assembly and Release

Once viral DNA replication and translation of late structural proteins has occurred, virion assembly begins (reviewed in Philipson, 1984). Hexon formation is mediated by the L4-100 kD protein which acts as a scaffold to aid polypeptide II trimerization (Liebowitz and Horwitz, 1975). The penton is formed in a stepwise fashion in which the polypeptide III pentamerizes to create the base then associates with the independently-assembled fiber, the
polypeptide IV trimer (Velicer and Ginsberg, 1970). Hexon and penton capsomeres assemble in the nucleus to form empty capsids (Sundquist et al., 1973). The mature virion is complete following insertion of the viral genome into the capsids. The encapsidation process and virion maturation is dependent upon the cis-acting packaging signal located in the ITR (Grable and Hearing, 1992), and two late viral proteins, L1-52/55 kD and L3-coded viral protease. L1-52/55 kD is required as a scaffold protein (Hasson et al., 1992). The L3 protease is essential as it proteolytically digests viral proteins to provide stability and infectivity to the mature virion (Webster et al., 1993).

The adenovirus infectious cycle does not terminate in the same fashion as traditional lytic viruses. Virion release is not fully characterized but does not occur due to bursting of the host-cell membrane under the pressure of mature virion. Adenoviral infection causes cytoskeletal breakdown and active membrane disruption leading to a form of cell death which resembles apoptosis (Defer et al., 1990). Recent studies support the hypothesis that adenoviruses kill the host cell by the process of apoptosis and that viral progeny spreads to neighboring cells via phagocytosis of virus-filled apoptotic bodies. E4orf4 has been implicated in this process as it is capable of inducing apoptosis and E4orf4-null mutants survive longer times than those infected by wild-type virus (Marcellus et al., 1996; 1998; Lavoie et al., 1998, Shtrichman et Kleinberger, 1998). Mutants defective in E3-11.6 kD protein also exhibit a similar phenotype and thus this protein may cooperate with E4 orf4 in cell killing. Death by apoptosis would be beneficial to the virus as it would minimize the host immune inflammatory response and help guarantee further
rounds of infection and is a mechanism used by a number of viruses (reviewed in Teodoro and Branton, 1997).

1.6 **Protein Phosphatase 2A**

Protein phosphatase 2A is an abundant serine/threonine phosphatase present in all eukaryotes. Its holoenzyme is a trimer of catalytic C subunit and A and B regulatory subunits (Cohen et al., 1998). The A subunit is a 65 kD rod-like protein comprised of 15 non-identical repeats (Kremmer et al., 1997). The catalytic C subunit is a 37 KDa protein that binds to repeats 11-15, whereas B subunits bind to repeats 1-10 (Ruediger et al., 1994). A and C are ubiquitously expressed and each exists in two isoforms (DePaoli-Roach et al., 1994). Thus far, 18 B subunit variants have been cloned and exist in three classes. The B class comprises three members of about 55 kD, Bα, Bβ, and By (Khew-Goodall et al., 1991; Pallas et al., 1992; Healy et al., 1991; Zolnierowicz et al., 1994). The B’ contains at least 13 isoforms, varying in size from 54-68 kD (McCright and Virshup, 1995; Csortos et al., 1996; Tehrani et al., 1996; Zolnierowicz et al., 1996). The B’’ contains two isoforms of 72 kD and 130 kD formed by alternative splicing (Hendrix et al., 1993). B subunits are unrelated to each other by primary sequence but can bind overlapping regions of A to bind the core AC dimer. B subunits are phosphorylated and are located throughout the cell (McCright et al., 1996). Some, like Bα, are expressed in many tissues while others have more limited expression. B subunits are believed to function not only in defining the substrate specificity and activity of the PP2A holoenzyme (DePaoli-Roach et al., 1994;
Cegielska et al., 1994; Mayer-Jaekel., 1994; Sontag et al., 1993) but also in intracellular targeting, tissue specificity. B subunits can also act as binding partners for interacting proteins and second messengers. The regulatory subunits of PP2A produce different holoenzymes that dephosphorylate distinct substrates in distinct cellular compartments. This complexity may explain PP2A's ability to regulate such diverse functions as cellular metabolism, DNA replication, transcription, RNA splicing, translation, cell-cycle progression, morphogenesis, development, transformation, and apoptosis (reviewed in Millward et al., 1999). Complementation data from studies in yeast have confirmed that the members of the B and B' class perform non-redundant functions (Zhao et al., 1997). In yeast, the Bα homolog, CDC55 is essential for proper regulation of cell cycle progression, the spindle checkpoint, cytokinesis, and bud morphogenesis (Zhao et al., 1997). In contrast, the B'α homolog, RTS-1 plays a role in the regulation of transcription in response to anaerobic conditions (Zhao et al., 1997). The search is currently underway to determine the holoenzyme(s) responsible for each of PP2A's diverse functions in mammalian cells. Recent advances have been made in the role of the Bα subunit of PP2A. Studies in mammalian fibroblasts have shown that the Bα regulatory subunit specifically targets the intermediate filament protein, vimentin, for dephosphorylation by PP2A (Turowski et al., 1999). Thus, the holoenzyme of PP2A containing Bα helps mediate rearrangement of the cytoskeleton and the nuclear envelope since reversible phosphorylation plays a key role in the assembly of intermediate filament networks of the cytoskeleton and nuclear envelope (reviewed in Inagaki et al., 1996). Specific depletion of Bα from cells by antisense Bα RNA is accompanied by disassembly
of vimentin intermediate filament networks (Turowski et al., 1999). Bα may also play a role in the growth inhibitory pathway activated by transforming growth factor β (TGFβ; reviewed in Derynck and Feng, 1997). Bα contains five WD-40 repeat motifs (reviewed in Neer et al., 1994) that mediate the interaction of Bα to the cytoplasmic domain of type I TGFβ in a receptor-dependent manner (Griswold-Prenner et al., 1998).

The B′α and B′β subunits of PP2A have been shown to mediate complex formation between PP2A and cyclin G (Okamoto et al., 1996). The cyclin G gene is a transcriptional target of p53 (Okamoto and Beach, 1994; Zauberman et al., 1995) and interaction of cyclin G and PP2A appears to require p53 induction (Okamoto et al., 1996). These findings support the existence of cross-talk between p53-mediated pathways and the PP2A holoenzyme containing B′ family members.

The importance of PP2A in cellular function is highlighted by the fact that it is a target during infection by SV40, polyoma, and adenovirus DNA tumour viruses (reviewed in Millward et al., 1999). The small T antigens of SV40 and polyoma and the middle T antigen of polyoma virus bind PP2A via the A subunit and cause the release of the B subunit and the inhibition of AC core enzyme activity. These effects contribute to the transforming activity of SV40 and polyomavirus through modulation of the MAPK pathway of signal transduction (reviewed in Mumby, 1995). As mentioned, adenovirus E4orf4 protein binds PP2A and increases its activity to a variety of substrates.

**PP2A and Apoptosis**

Recent studies have suggested that PP2A plays a direct role in control of the apoptotic program. Treatment of cell with
survival agonist interleukin-3 induces phosphorylation at a conserved serine residue which is required for Bcl-2’s ability to suppress apoptosis (Ito et al., 1997). It was observed that a direct interaction between Bcl-2 and PP2A occurs in response to Bcl-2 phosphorylation and that the AC dimer can dephosphorylate Bcl-2 in vitro (Deng et al., 1998). Thus, PP2A is believed to act in concert with a Bcl-2 kinase to mediate rapid regulation of Bcl-2 function by reversible phosphorylation.

Studies undertaken to understand the mechanism by which caspases elicit their effect in apoptosis led to the discovery that PP2A activity is regulated by caspase-3 during apoptosis (Santoro et al., 1998). The yeast two-hybrid system was used to detect the interaction of caspase-3 enzyme and putative substrates and the A subunit was isolated. Studies of Fas-induced apoptotic pathways indicate that caspase-3 is activated and then cleaves the regulatory A subunit of PP2A, resulting in increased activity of the C subunit (Santoro et al., 1998). The role of activated PP2A in apoptosis is unknown but provides a link between the caspases and signal transduction pathways.

1.7 Apoptosis

Apoptosis is a genetically programmed process of cell death which is of fundamental importance to multicellular organisms. The process of apoptosis is important in a wide variety of biological systems, including embryonic development, the immune system, and normal cell turnover. Inappropriate stimulation of apoptosis is associated with such diseases as Alzheimer’s and Huntington’s and inappropriate inhibition of apoptosis is associated
with cancer and autoimmune disorders.

Apoptosis is characterized by a series of morphological changes including loss of cell-cell contacts, disruption of the cytoskeleton, cytoplasmic shrinkage, membrane blebbing, condensation of DNA, nuclear fragmentation, and in the final stages, fragmentation of the cell into membrane-bound vesicles that are phagocytosed by neighboring cells (Kerr, et al., 1972).

The core program of apoptosis was discovered during studies of the nematode worm, *C. elegans* (reviewed in Hengartner and Horvitz, 1994). During development, 131 cells are eliminated by apoptosis. By observing the fate of these cells in *C. elegans* mutants, genes involved at several stages in apoptosis were identified. Two genes, *ced-3* and *ced-4* (*ced* for cell death abnormal) are required for cell death and a third gene *ced-9* is required to suppress cell death (Ellis and Horvitz, 1986). Ced-3 was identified as an effector of cell death, Ced-4 as an activator of Ced-3, and Ced-9 as a suppressor of apoptosis by inhibiting Ced-4 activity. Mammalian homologs have been identified for all three proteins indicating that the apoptotic machinery has been conserved throughout evolution. Ced-3 was found to be very similar to a mammalian protease interleukin-1B-converting enzyme (ICE/caspase-1) (Yuan et al., 1993). This was the first indication that apoptosis depends on proteolysis. Ced-9 was found to be related to the protooncogene Bcl-2, providing a link between suppression of apoptosis and oncogenesis (Hengartner et al., 1994b). The Ced-4 homolog was isolated and cloned following studies of caspase-1 activation and was named apoptosis protease activating factor-1 (*Apaf-1*) (Zou et al., 1997).
Caspases

Since the recognition of the similarity between caspase-1 and Ced-3, 10 additional members of the caspase family have been identified (Wang et al., 1998). The 11 caspase family members can be classified into two sub-groups: the Ced-3 group (caspases-2, -3, -6, -7, -8, -9, -10) which are involved in apoptosis (reviewed in Thornberry and Lazebnik, 1998) and the ICE group (caspase-1, -4, -5, -11) which are involved in the inflammatory response. Caspases are so-named because they have cysteine in their active site and cleave their target proteins after specific aspartic residues (Alnemri et al., 1996). Caspases are synthesized as large, inactive precursors, or procaspases, which are activated by cleavage at aspartic acids, usually by another caspase. Cleavage occurs at two sites causing the release of an N-terminal pro-domain and two additional fragments which heterotetramerize with two fragments from another precursor molecule to create one active molecule.

The Ced-3 subgroup of caspases can be further subdivided into initiator caspases (8, 9,) and effector caspases (3, 6, 7) depending on the length of their pro-domain. The role of caspase-2 and -10 is unknown. Initiator caspases have long pro-domains which contain a death effector domain (DED) or a caspase recruitment domain (CARD). The DED and CARD motifs promote binding of the inactive caspase to proteins which also contain these motifs and which are located in caspase activation complexes. Activated initiator caspases then activate via a proteolytic cascade the effector caspases which have short pro-domains. Effector caspases trigger the morphological changes associated with apoptosis through cleavage of specific proteins involved protection
of cells from apoptosis (such as Bcl-2), maintenance of cell membrane integrity, DNA repair, and cell cycle regulation (reviewed in Thornberry and Lazebnik, 1998). For example, effector caspase-3 and -7 have been shown to cleave poly (ADP-ribose) polymerase in response to apoptosis induction (Lazebnik et al., 1994; Kaufmann et al., 1993).

**Caspase Activation Complexes**

Two caspase activation complexes have been identified in mammalian cells (reviewed in Raff, 1998). The first involves the cell surface transmembrane TNF receptor (TNFR) superfamily of "death receptors" (reviewed in Ashkenazi and Dixit, 1998). The two most studied receptors are TNFR1, which is ubiquitously expressed, and Fas, which is predominantly expressed on activated T cells. Ligand binding to the extracellular domains promotes receptor trimerization. The cytoplasmic domains of these receptors contain death domains (DD) that dimerize with other DD-containing adapter proteins, such as FADD. FADD which binds directly to Fas, also contains DEDs which interact with DEDs located in the pro-domain of procaspase-8. This oligomerization results in the autoproteolytic activation of caspase-8 and subsequent activation of downstream effector caspases.

The second caspase activation complex identified consists of Apaf-1, procaspase-9, and cytochrome c (reviewed in Green and Reed, 1998). Cytochrome c normally resides within the mitochondria but in response to apoptotic stimuli, cytochrome c is released into the cytoplasm. The mechanism of release is unknown but it may correlate with the loss of transmembrane potential across the inner mitochondrial membrane and the opening of a large conductance channel known as the mitochondrial permeability-
transition (PT) pore. Both these events occur when cells are committed to undergo apoptosis. Cytoplasmic cytochrome c binds to Apaf-1, the mammalian Ced-4 homolog. Apaf-1 contains a CARD motif through which it also interacts with pro-caspase-9. In the presence of ATP, the formation of this complex, known as the apoptosome, leads to caspase-9 activation and subsequent activation of downstream effector caspases and death by apoptosis.

**The Bcl-2 Family**

Apoptosis induction is regulated by the Bcl-2 family of proteins which has 15 known members (reviewed in Adams and Cory, 1998). The role of Bcl-2 in apoptosis was discovered when its overexpression in human B-cell lymphoma was shown to prevent cell death (reviewed in Yang and Kosemeyer, 1996). Bcl-2 family members act to integrate and respond to a diverse array of negative and positive survival signals. All members posses one of at least four conserved motifs known as Bcl-2 homologue domains (BH1 to BH4) and some members have a C-terminal transmembrane domain. Family members can be divided into two groups: suppressors of apoptosis, which include Bcl-2, Bcl-X\textsubscript{L}, Bcl-w, Mcl-1, A1 and Bfl-1, and promoters of apoptosis, which include Bax, Bak, Bok, Bid, Bik, Bcl-X\textsubscript{S}, Hrk, Bim, and Bad. Pro- and anti-apoptotic members can heterodimerize and interfere with each other's function, suggesting that the ratio of suppressors to promoters determines cell fate (Oltavi et al., 1993). The mechanism(s) by which Bcl-2 members act in vivo to modulate apoptosis induction are unknown. Biochemical evidence suggests that pro-survival members bind to Apaf-1 and prevent formation of the apoptosome and thus block caspase-9 activation. Pro-apoptotic members may act to prevent
the interaction of Apaf-1 and death inhibitors. Structural studies of Bcl-X<sub>L</sub> indicated a similarity with pore-forming bacterial toxins. This suggests that Bcl-2 family members may function through pore formation and pro-apoptotic members may induce the release of cytochrome c from mitochondria.

*Regulation of Bcl-2 and caspase function*

Many 'survival factors' are growth factors that regulate cell proliferation through phosphorylation involving tyrosine kinase receptors and S/T kinase cascades. Bcl-2 and Bcl-X<sub>L</sub> are positively regulated by Ser phosphorylation (Chen and Faller, 1996). Bcl-2 binds the S/T kinase Raf-1 which regulates the Ras/MAPK pathway and targets it to mitochondria (Wang et al., 1996). It is still unknown if Raf-1 acts on Bcl-2. Phosphorylation of Bad at S112 and S136 blocks binding to Bcl-2 and promotes association with 14-3-3 in the cytoplasm (Wang et al., 1996; Zha et al., 1996). PI3' kinase is linked with phosphorylation of Bad. PI3' kinase is activated by binding to phosphotyrosylated receptors and the resulting phosphoinositides recruit the kinases Akt and PDK 1 via PH domains (reviewed in Hemmings, 1997). PDK1 activates Akt by phosphorylation (Alessi et al., 1997). Akt subsequently translocates and inactivates Bad by phosphorylating some sites (del Peso et al., 1997). Thus phosphorylation of Bcl-2 and Bad promotes cell survival. Dephosphorylation of these proteins would promote apoptosis.

It was recently hypothesized that alternative splicing plays a role in apoptosis (Jiang and Wu, 1999). Alternative splicing of genes that encode some members of the Bcl-2 family and the caspase family results in the generation of functionally distinct
products. For example, the \textit{bcl-x} gene is alternatively spliced to produce Bcl-x\textsubscript{L} and Bcl-x\textsubscript{S}. Bcl-x\textsubscript{L} is similar to Bcl-2 in its ability to inhibit cell death whereas Bcl-x\textsubscript{S} antagonizes the anti-apoptotic function of Bcl-x\textsubscript{L} and Bcl-2. This hypothesis is supported by the fact that targeted disruption of alternative splice factor/splicing factor 2 results in cell death (Wang et al., 1996). ASF/SF2 is a SR protein which plays an important role in constitutive and alternative splicing of pre-mRNAs and may be important in the generation of splice products which encode suppressors of apoptosis.

\textbf{1.8 Thesis Proposal}

The goal of this project was to better understand the structure and function of Ad5 E4orf4 protein. As mentioned, E4orf4 is capable of inducing p53-independent apoptosis (Marcellus et al., 1998; Lavoie et al., 1998; Shtrichman and Kleinberger, 1998). The mechanism by which E4orf4 induces apoptosis is not known but in rodent cells, E4orf4-induced apoptosis is not accompanied by caspase activation and is not blocked by a broad spectrum caspase inhibitor (Lavoie et al., 1998). The only biochemical function associated with E4orf4 is its ability to bind the B\textalpha subunit of protein phosphatase (PP) 2A and activate the PP2A holoenzyme (Kleinberger and Shenk, 1993). This activity accounts for the role of E4orf4 in regulation of E4 expression and late viral mRNA splicing through PP2A-dependent dephosphorylation of factors required for these processes (Bondesson et al., 1992; 1996; Whalen et al., 1997; Kanopka et al., 1998). At the onset of this study it was unknown if the interaction of E4orf4 with PP2A was important
to the mechanism of E4orf4-induced apoptosis. My project involved a structural and genetic analysis of E4orf4 to identify the domains which are responsible for its role in apoptosis and those responsible for its interaction with the Bα subunit of PP2A and to determine if these regions overlap. Studies were also undertaken to determine the ability of E4orf4 to bind other B subunits of PP2A.
2 Materials and Methods

2.1 Cell lines

Ad5-transformed human embryonic kidney 293 cells (Graham et al., 1977), human p53-null C33A cells (ATCC HTB-31), and human p53-null H1299 cells (ATCC CRL-5803) were cultured in α-minimal essential medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin and 0.292 mg/ml L-glutamine, using Falcon tissue culture dishes. Ad5 E1A-expressing, p53-null 1A.A3 mouse embryo fibroblasts were grown in the same medium under selective conditions in the presence of 100 mg/ml hygromycin (Lowe et al., 1994).

2.2 Plasmids

pcDNA3 plasmids expressing HA-tagged wild-type E4orf4, HA-tagged E4orf4 deletion mutants, HA-tagged E4orf4 point mutants, FLAG-tagged wild-type E4orf4, HA-tagged Bα, and FLAG-tagged Bα were created by Dennis Paquette (GeminX Biotechnologies Inc, Montreal) (Marcellus et al., 1999). pCEP plasmids expressing HA-tagged B'α, HA-tagged B'β, HA-tagged B'γ1, HA-tagged B'δ, and HA-tagged B'ε were obtained from David Virshup (University of Utah, Salt Lake City) (McCright et al., 1996). The plasmid used in the luciferase assay expresses the firefly luciferase enzymes under the control of the RSV promoter and has been described previously (Goping et al., 1995).

2.3 In vitro expression

Fifty nanograms of plasmid expressing wild-type or deletion mutants of E4orf4 was transcribed with T7 RNA polymerase and translated in the presence of stabilized L-[^35]S)methionine
(DuPont-NEN) with Promega's TNT T7 Coupled Reticulocyte Lysate System (10 μl) at 30°C for 2 hrs. The volume of the sample was increased to 1 ml in RIPA buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% deoxycholate, 1% Triton X-100), clarified and immunoprecipitated using 2 μl of anti-HA antibody (HA.11, BAbCO) together with protein A sepharose CL-4B beads (Pharmacia Biotech). The immunoprecipitates were washed six times with RIPA buffer, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using gels composed of 15% polyacrylamide. Gels were treated with 2,5-diphenyloxazole in dimethyl sulfoxide, dried and exposed to Kodak X-Omat film at -80°C.

2.4 In vivo protein expression

293 cells were transfected using the calcium phosphate transfection method (Graham and van der Erb, 1973) with 10 μg of plasmid DNA and 6 μg of sonicated salmon sperm carrier DNA. Starting at 46 hours post-transfection, cells were labeled for 2 hrs with 200 μCi of [35S]methionine-[35S]cysteine EasyTag Express protein-labeling mix (specific activity, greater than 1000 Ci/mmol [DuPont-NEN]) per main methionine- and cysteine-free medium. Cell extracts were prepared in RIPA buffer, clarified, and immunoprecipitated using 2 μl of anti-HA antibody (HA.11, BAbCO) together with protein A sepharose CL-4B beads (Pharmacia Biotech). The immunoprecipitates were washed six times with RIPA buffer, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using gels composed of 12% polyacrylamide. Gels were treated with 2,5-diphenyloxazole in dimethyl sulfoxide, dried and exposed to Kodak X-Omat film at -
C33A cells were lipofected using LipofectAMINE PLUS (Gibco-BRL) with 1.25 μg of plasmid DNA and at 24 hours post-lipofection, cell extracts were prepared in a volume of 50 μl of Buffer K (0.1% IGEPAL, 0.4 M KCl, 50 mM HEPES pH 7.9, 10% glycerol, 0.2 M EDTA, 1mM DTT, 0.5 mM PMSF). Protein levels were quantified using the Bio-Rad protein assay reagent, and 10 μg of total protein per lane was resolved by SDS-PAGE using a 15% polyacrylamide gel. Separated proteins were transferred to nitrocellulose and probed with anti-HA antibody (HA.11, BabCO) at 1/2500 dilution. Visualization was completed using a goat anti-mouse IgG antibody linked to horseradish peroxidase (Jackson ImmunoResearch) at 1/20 000 dilution followed by ECL detection (NEN Life Science Products). Cell extracts were also prepared from H1299 cells following viral infection and treated in the same manner as the C33A cell extracts except that some were resolved in different percentages of polyacrylamide gels and different antibodies were used as indicated, anti-E1A antibody (M73, Harlow et al., 1985) following 12% SDS-PAGE, anti-p53 antibody (pAb1801, Oncogene Science, Inc.) following 10% SDS-PAGE and anti-procaspase-3 antibody (E-8, Santa Cruz Biotechnologies, Inc.) following 12% SDS-PAGE. Cell extracts were also prepared following viral infection according to the protocol for utilization of the anti-PARP antibody (C2-10, Bio-Mol Research Laboratories, Inc.) in which cell extracts are prepared in 50mM TrisHCl [pH 6.8], 6M urea, 6% β-mercaptoethanol, 3% SDS, sonicated on ice then subjected to 8% SDS-PAGE, transferred to nitrocellulose, and probed with C2-10 then detected in the same manner as the C33A extracts.
2.5 Luciferase assay

The luciferase killing assay was carried out using 1A.A3 cells plated the day before at a density of 1.5 x 10^5 cells per well in six-well plates. The DNA mixture was introduced into the cells by calcium phosphate transfection and consisted of 0.5 ug of RSV luciferase plasmid DNA, 3 ug of pcDNA3 plasmid DNA with 2.5 ug sonicated salmon sperm DNA as a carrier. Cells were harvested 48 hours post-transfection and luciferase activity was determined following freeze/thaw disruption of the cells, as described previously (Lavoie et al., 1996).

2.6 Binding Assays

C33A or H1299 cells were co-lipofected using LipofectAMINE PLUS (Gibco BRL) with 1.25 ug of each differentially tagged protein of interest. At 24 hours post-lipofection one ml of cell extract was prepared in 50 mM Tris HCl [pH 7.5] containing 0.5% IGEPA, 0.1% Triton X-100, 250 mM NaCl and 5 mM EDTA, clarified and immunoprecipitated using 2 ul of anti-HA antibody (HA.11, BabCo) together with protein A Sepharose CL-4B beads (Pharmacia Biotech). The immunoprecipitate was washed six times with extraction buffer, boiled in SDS-PAGE sample buffer and separated by 8% or 15% SDS-PAGE. Proteins were transferred to nitrocellulose then probed with anti-FLAG antibody (M2, Sigma-Aldrich) at a dilution of 1/1000. Proteins were visualized using goat anti-mouse IgG linked to horseradish peroxidase (Jackson ImmunoResearch) at a dilution of 1/20 000 followed by ECL treatment (NEN Life Science Products).

2.7 Viruses

Ad rTTA and Ad ind HA-tagged wild-type or mutant E4orf4
viruses were obtained from GeminX Biothechnologies, Inc. (Marcellus et al., 1999). Ad p53 expressing wild-type human p53 (Bachetti and Graham, 1993) and pm2/3 (McLorie et al., 1991) were also used. All viruses were titred on 293 cells.

2.8 Cell Viability Assay

H1299 cells were co-infected at 50 total pfu/ cell with Ad rTTA and Adind orf4 and point mutants or Ad p53 or pm2/3 and doxycycline (Sigma) was added to induce E4orf4 expression. At various times following infection, adherent and non-adherent cells were harvested by trypsinization and viability was assessed by Trypan Blue exclusion. At least 100 cells were counted at each time point.

2.9 Phosphoaminoacid analysis

H1299 cells were lipofected using LipofectAMINE PLUS (Gibco BRL) with plasmid DNA and starting at 20 hours post-lipofection, cells were labeled for four hours with 0.33 mCi of [32P]orthophosphate (specific activity, 8,500 to 9,120 Ci/mmol; DuPont-NEN) per ml in phosphate-free medium. Cell extracts were prepared in 1ml RIPA Buffer, clarified and immunoprecipitated using 2 ul of anti-HA antibody (HA.11, BAabCO) together with protein A sepharose CL-4B beads (Pharmacia Biotech). The immunoprecipitates were washed six times with RIPA buffer, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using gels composed of 12% polyacrylamide. Labeled extracts were transferred to PVDF and following exposure to Kodak X-Omat AR film at -80°C. The band corresponding to HA-tagged E4orf4 was excised and a band at the equivalent height in pcDNA3 transfected control sample. The samples were then hydrolyzed and analyzed by
two-dimensional thin-layer chromatography at pH 1.9 in the first dimension then pH 3.5 in the second dimension as described previously (Hunter and Sefton, 1991). Phosphamino acid standards (Sigma) were visualized following by staining with ninhydrin.
3 Results

3.1 E4orf4 deletion mutants

It was shown previously that Ad5 E4orf4 induces p53-independent apoptosis (Marcellus et al., 1998; Lavoie et al., 1998; Shtrichman and Kleinberger, 1998). To determine the regions of E4orf4 involved in cell killing, a series of HA-tagged deletion mutants were made (Figure 3.1). The carboxy terminus and certain internal regions of E4orf4 were chosen for deletion based on the likelihood of these regions of E4orf4 to be on the surface of the protein and therefore accessible for interaction with targets necessary for apoptosis induction. The HA epitope was linked to the N-terminus of wild-type and mutant E4orf4 proteins to facilitate experimentation since no high affinity anti-E4orf4 antibody exists. The HA epitope does not affect E4orf4 activity (Lavoie et al., 1998).

3.2 Expression of the E4orf4 deletion mutants

The expression of the E4orf4 deletion mutants was verified by two methods (Figure 3.2). Plasmid DNA encoding wild-type and mutant HA-tagged E4orf4 proteins was in vitro transcribed and translated in the presence of [35S]-methionine in rabbit reticulocyte lysates and lysates were immunoprecipitated with an anti-HA antibody and resolved by 15% SDS-PAGE (Figure 3.2, panel A). In addition, plasmid DNA encoding wild-type and mutant HA-tagged E4orf4 proteins was introduced by calcium phosphate precipitation into 293 human embryonic kidney cells that express the Ad5 E1A and E1B products. Cells were labeled with a mix of [35S]-methionine and [35S]-cysteine and extracts were
Figure 3.1 Ad 5 E4orf4 deletion mutants

At the top is shown the accessible surface area of Ad 5 E4orf4 protein which was prepared using Gene Inspector Software, Textco, Inc., USA. Below is shown the coding sequence of wild-type E4orf4 and of various C-terminal and internal deletions constructed as described in Materials and Methods. The residues from E4orf4 present in each C-terminal mutant have been indicated. The residue numbers from E4orf4 removed in each internal mutant are indicated by the closed rectangle.
C-Terminal deletion mutants

- E4 orf4 dl 103-114
- E4 orf4 dl 81-114
- E4 orf4 dl 55-114

Internal deletion mutants

- E4 orf4 dl 12-18
- E4 orf4 dl 34-48
- E4 orf4 dl 50-66
- E4 orf4 dl 68-78
- E4 orf4 dl 87-99
Figure 3.2 Expression of HA-tagged E4orf4 wild-type and deletion mutant proteins

The plasmids pcDNA3 and pcDNA3HA expressing wild-type E4orf4 and the indicated mutants were transcribed and translated in vitro in the presence of $[^{35}\text{S}]$-methionine, immunoprecipitated with anti-HA antibody, and separated by 15% SDS-PAGE (panel A). 293 cells were transfected with the same plasmids and 48 hours post-transfection, cells were labeled with a mix of $[^{35}\text{S}]$-cysteine and $[^{35}\text{S}]$-methionine for 2 hours and cell extracts were immunoprecipitated with anti-HA antibody and separated by 12% SDS-PAGE.
immunoprecipitated with anti-HA antibody and resolved by 12% SDS-PAGE (Figure 3.2, panel B). The E4orf4 deletion mutants were expressed at levels comparable to wild-type following in vitro transcription and translation, with the exception of mutants, dl19-114 and dl34-48, which were not detected (Fig 3.2, panel A) Following in vivo expression, levels of the deletion mutants varied greatly as compared to wild-type (figure 3.2, panel B) dl34-48 and dl68-78 were expressed at reasonable levels, but dl12-18, dl50-66, dl87-99, and dl103-114 were expressed at low levels and dl19-114, dl55-114, and dl81-114 were not expressed at all. dl34-48 was observed in vivo but not in vitro; there was difficulty with sample during in vitro synthesis. dl19-114, was not detected in either assay and therefore, this mutant was not employed in further studies of E4orf4 function.

3.3 Analysis of cell killing by the E4orf4 deletion mutants

It was shown previously that E4orf4’s cytotoxic effect could be measured indirectly using the luciferase assay. High luciferase activity is consistent with a low degree of cell killing; whereas if extensive death has occurred, luciferase activity would be reduced (Marcellus et al., 1998). Therefore, the cytotoxicity of each HA-tagged E4orf4 deletion mutant was assessed in this manner. Plasmid DNA encoding wild-type or mutant HA-tagged E4orf4 was used to cotransfect p53-null 1A.A3 cells expressing Ad5 E1A products, along with plasmid DNA encoding firefly luciferase and, after 48 hours, cell extracts were assayed for luciferase activity. Figure 3.3 shows the result from an experiment with triplicate samples. In cells co-transfected with plasmid expressing HA-tagged E4orf4, luciferase activity was reduced by 86%, as
Figure 3.3  Analysis of cell killing by measurement of luciferase activity

IA.A3 cells were cotransfected with pRSV expressing firefly luciferase and pcDNA3 plasmid individual HA-tagged E4orf4 proteins. After 48 hours the cells were harvested and the extracts were analyzed for luciferase activity as described in Materials and Methods. The data represent an experiment involving triplicate samples. Luciferase activity obtained with pcDNA3 empty vector control was arbitrarily set at 100%. The plasmid expressing HA-tagged E4orf4 is indicated as pc304.
compared to that obtained with pcDNA3 alone. This result is consistent with results obtained by Marcellus et al. (1998) using an untagged E4orf4 construct and confirms that the HA epitope does not interfere with E4orf4's cytotoxic effect. Cells cotransfected with plasmid expressing the mutant lacking 12-18 or the mutant lacking residue 103-114 exhibited luciferase activity almost as low as with wild-type. In the case of the other deletion mutants, dl 81-114 and dl 55-114, which lacked greater C-terminal fragments, and mutants dl 34-48, dl 50-66, dl 68-78, and dl 87-99, which lacked residues in the center of E4orf4, coexpression in cells caused only partial decreases in luciferase activity.

3.4 The first generation of E4orf4 point mutants

Following the analysis of cell killing by the E4orf4 deletion mutants, a new mutational approach was taken to identify the residues of E4orf4 involved in cell killing. Alignments of the E4orf4 sequence of adenovirus serotypes 5, 9, 12, and 40 were performed and residues conserved amongst the serotypes were identified (Figure 3.4, upper panel). The conserved residues presumably have a role in E4orf4 activity and were targeted for mutagenesis. A series of HA-tagged single point mutants as well as some groupings of point mutants were made, within the 114 residue sequence of E4orf4. The conserved residues were substituted for the amino acid alanine (Figure 3.4, lower panel).

3.5 Analysis of cell killing by the first generation of point mutants

The first generation of point mutants was subjected to the luciferase death assay to identify which residues were required for the cytotoxic effects of E4orf4. Experiments were performed as
Figure 3.4  First generation of E4orf4 point mutants

At the top is shown the alignment of the E4orf4 protein from the adenovirus serotypes 5, 9, 12, 40 and the consensus sequence of these four individual sequences. At the bottom is shown the E4orf4 point mutants. The residues indicated by the lines directed upward are the locations of mutations of multiple point mutants. The residues indicated by lines directed downward are the sites for a series of single point mutations. The single letter amino acid codes have been used to show the original residue and all mutants have been constructed with alanine substitutions.
### E4 Point Mutants

#### Consensus Sequences

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<td>Ad5 E4orf4</td>
<td>MLPLPALPP PCDQNECV WLGVAYSAV DVIHKAHEG VYIPEAEGR</td>
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<tr>
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<td>Ad12 E4orf4</td>
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<tr>
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<td>Ad40 E4orf4</td>
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<td>LALRLEWMY AFITTERQCK QKGRGATSG RTWFCFFKYE DARSUVYDA</td>
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<tr>
<td></td>
<td>Ad40 E4orf4</td>
<td>LSGLQWENLY ALKTERARQR NRFFANICWQ RVRLKQKYE AVRELLYIDA</td>
</tr>
</tbody>
</table>

#### E4 Point Mutants Diagram
Figure 3.5 Analysis of cell killing by measurement of luciferase activity

IA.A3 cells were cotransfected with pcDNA3 plasmids expressing firefly luciferase and individual HA-tagged E4orf4 wild-type or mutant proteins. After 48 hours the cells were harvested and the extracts were analyzed for luciferase activity as described in Materials and Methods. The data represent the average of four separate experiments each involving duplicate samples. Luciferase activity obtained with the pcDNA3 empty vector control was arbitrarily set at 100%.
described in section 3.3. Figure 3.5 shows the combined results from four separate experiments. Coexpression of individual HA-tagged E4orf4 point mutants with luciferase in 1A.A3 cells had varying effects on luciferase activity. The alanine substitution of many residues had no obvious effect on E4orf4 cytotoxicity and decreased relative luciferase activity to levels similar to that of wild-type. The mutants ptD31A/V32A/R34A, ptP45A, ptE46A/R48A, ptT64A/E65A/R66A, ptR69A/R70A, ptC78A, ptR81A/F84A, ptC85A, and ptD90A all decreased relative luciferase activity by 70-85%, indicating that these residues are not essential for E4orf4 to induce cell death. Other mutants, ptP7A/P9A/P10A, ptL43A, ptE56A/W57A, ptR73A/R74A/R75A, and ptR93A/R94A increased relative luciferase activity above 40% and are considered partially defective for cytotoxic effects. Most importantly, certain residues were identified that are essential for E4orf4 activity. The double mutation, replacing leucines at residues 51 and 54 for alanines (pt L51A/L54A), had a drastic effect on E4orf4's ability to kill and caused luciferase activity to return to levels of empty vector. The single mutant at residue 51 (pt L51A) or residue (ptL54A) had less drastic effects, indicating that both leucines are required for killing. The double mutation of lysine at residue 88 and tyrosine at residue 89 to alanines (pt K88A/Y89A) was also defective for killing. In this case, the single mutation of tyrosine at residue 89 (pt Y89A) had a more pronounced individual effect than the lysine at residue 88 (ptK88A). It is also interesting to note that the mutation of tyrosine at residue 59 to alanine enhanced the ability of E4orf4 to kill.

3.6 The complete set of E4orf4 point mutants

To further characterize the structure and function of E4orf4,
The complete set of Ad5 E4orf4 point mutants is shown. The residues indicated by the lines directed upward are the locations of mutations of multiple point mutants. The residues indicated by the lines directed downward are the sites for a series of single point mutations. The single letter amino acid codes have been used to show the original residue and all mutants have been constructed with alanine substitutions.
E4 Point Mutants
another generation of alanine point mutants were created. Several conserved residues which had not been previously altered were targeted and triple or double point mutants of the first generation of point mutants, which had interesting phenotypes, were isolated into single mutants. The complete set of point mutants are indicated in Figure 3.6.

3.7 Expression of the complete set of E4orf4 point mutants

To verify that the HA-tagged E4orf4 point mutants were stably expressed, the plasmid DNA encoding each mutant was introduced by lipofection into the human, p53 mutant cervical carcinoma line C33A. Cell extracts were prepared 24 hours after lipofection and following 15% SDS-PAGE and transfer to nitrocellulose, the expression was analyzed by immunoblotting using anti-HA antibody. Figure 3.7 shows that all of these mutants yielded stable products, with the exception of a triple proline mutant (pt P7A/P9A/P10A) and a double mutant (pt K88A/Y89) which showed consistently decreased expression levels. The decreased expression of these two mutants may explain their inability to decrease relative luciferase activity as shown in Figure 3.5.

3.8 Analysis of E4orf4/PP2A Bα subunit binding

It was shown previously that E4orf4 interacts with the Bα subunit of PP2A (Kleinberger and Shenk, 1993) and that this interaction leads to decreased phosphorylation of adenovirus E1A products (Kleinberger and Shenk, 1993; Whalen et al., 1997), transcription factor E4F (Bondesson et al., 1996), and SR proteins present in spliceosomes (Kanopka et al., 1998). These results suggested that association of E4orf4 with PP2A either activates or
Figure 3.7 Expression of the complete set of E4orf4 point mutants.

The expression of the HA-tagged E4orf4 mutants was analyzed. C33A cells were lipofected with the indicated HA E4orf4 mutant cloned into pcDNA3. Cells were harvested 24 hours post-lipofection and cell extracts were separated by 15% SDS-PAGE, transferred to nitrocellulose and immunoblotted using an HA-antibody.
Figure 3.8  Analysis of binding to Bα to the panel of E4orf4 point mutants

C33A cells were co-lipofected with pcDNA3 plasmids expressing FLAG-tagged Bα protein and the indicated HA-tagged E4orf4 mutant proteins. Cell extracts were prepared 24 hours post-lipofection and HA-antibody immunoprecipitations were performed and separated by 8% SDS-PAGE, transferred to nitrocellulose, then immunoblotted against the FLAG epitope. In parallel, duplicate plates were harvested and extracted proteins were similarly separated and immunoblotted to examine overall FLAG-Bα expression.
redirects the activity of this phosphatase. To determine the residues required for Bα binding and to help determine if such interactions were also required for E4orf4-induced apoptosis, the complete set of E4orf4 point mutants were used to define the residues of E4orf4 required for PP2A binding. C33A cells were co-lipofected with plasmids expressing FLAG-tagged Bα and HA-tagged wild-type and mutant E4orf4 and extracts were immunoprecipitated with anti-HA antibodies (HA IP). Precipitates were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted for the presence of FLAG-Bα using anti-FLAG antibodies. To ensure that comparable amounts of these proteins were present in lipofected cells, the levels of E4orf4 and Bα in whole cell extracts (WCE) were also examined by western blotting using anti-HA and anti-FLAG antibodies, respectively. Figure 3.8 shows that Bα expression was similar in all cells, and that of E4orf4 paralleled results presented in Figure 3.7. Figure 3.8 also shows that with most of the mutants, the levels of Bα subunit present in E4orf4 complexes were similar to wild-type. However, a number of mutants were either partially or completely defective in Bα binding.


When this binding data was correlated with the luciferase
data in figure 3.5, the first generation of point mutants could be divided into four classes in terms of cytotoxicity. The first class comprised mutants that failed to bind Bα and failed to induce cell death. These mutants include pt L51A/L54A and pt Y89A. The second class comprised mutants that showed decreased Bα binding and decreased cytotoxicity relative to wild-type E4orf4. These mutants include pt L51A, pt E56A/W57A, pt R73A/R74A/R75A, and pt R93A/R94A. The third class comprised mutants that exhibited relatively normal Bα binding yet were significantly impaired in cell killing. These mutants include pt I43A and pt K88A. The fourth class comprised a mutant that failed to bind Bα yet is capable of cell killing, pt R81A/F84A.

3.9 Analysis of cell killing by inducible adenovirus vectors expressing wild-type and mutant E4orf4

In order to further characterize if the interaction of E4orf4 with PP2A was required for E4-induced apoptosis, the cytotoxic effect of inducible adenovirus vectors expressing certain E4orf4 mutants was determined. Vectors expressing E4orf4 mutants pt L51A/L54A and pt Y89A, which failed to bind Bα (Figure 3.8) and failed to kill (Figure 3.5), were analyzed. Vectors expressing E4orf4 mutants pt L3A and pt F84A, which failed to bind Bα (Figure 3.8) but whose cytotoxicity was not previously tested, were also analyzed. A vector expressing mutant pt K88A, which exhibited wild-type Bα binding activity yet was impaired in cell killing as determined by the luciferase assay (Figure 3.5 and 3.8), was analyzed.

p53-null human lung carcinoma cell H1299 were coinfectected with AdrTTA, an adenoviral vector expressing the reverse tet-transactivator driven by the CMV promoter, and Adind wild-type or
Figure 3.9 Viability of H1299 cells infected with inducible adenoviral vectors expressing wild-type and mutant E4orf4

H1299 cells were untreated (mock) or infected with AdrTTA vector alone or in combination with the indicated Adind vector expressing HA-tagged E4orf4 protein or with pm2/3, an EIB-19K minus adenovirus, or with Ad p53. The cells were treated with 5mM DOX throughout the course of the experiment. Cells were trypsinized at the indicated time points and cell viability was monitored by Trypan Blue exclusion.
H1299 Trypan Blue Survival Assay

<table>
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<th>Time Post-Infection (hrs)</th>
<th>Percent Survival (%)</th>
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<td>0</td>
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- **mock**
- **Ad rTTA**
- **Adind orf4**
- **Adind ptL3A**
- **Adind ptL5154A**
- **Adind ptF84A**
- **Adind ptK88A**
- **Adind ptY89A**
- **pm 2/3**
- **Ad p53**
mutant E4orf4, an adenoviral vector expressing HA-tagged E4orf4 driven by the tetracycline inducible operon. Protein expression was induced at the start of infection by addition of doxycycline, a tetracycline analog. Cells were harvested by trypsinization and cell viability was measured by exclusion of Trypan Blue stain at various hours post-infection (h.p.i.). Two positive controls were included in this experiment. H1299 cells were infected with pm2/3, an adenovirus E1B-l9K-null mutant which can induce p53-independent apoptosis (Boulakia et al., 1995). H1299 cells were also infected with Adp53, an adenoviral vector which induces apoptosis in H1299 cells (Nguyen, et al., 1997). The results are depicted in Figure 3.9. As expected, pm2/3 and Ad p53 rapidly kill H1299 cells and few cells survive past 50 h.p.i. Ad ind orf4 expressing HA-tagged E4orf4 kills H1299 cells more slowly and more moderately than pm2/3 and Adp53 and at 124 h.p.i., 53% of cells have died. Ad ind pt L51L54, Ad ind pt L3A, Ad ind pt F84A, and Ad ind pt Y89A were slightly more toxic than AdrTTA but defective in cell killing as compared to Ad ind orf4. These results indicate residues L3, L51, L54, F84, and Y89 are essential for E4orf4's cytotoxic effect in addition to Bα binding.

Ad ind pt K88A was more cytotoxic than the other vectors expressing mutant orf4 and at 124 h.p.i. 26% of cells are no longer viable. This is consistent with the intermediate cytotoxicity of E4orf4 pt K88A as measured by the luciferase assay (Figure 3.5). Though K88 is not involved in binding to Bα, it is required to induce cell death.

3.10 Analysis of protein expression in cells infected by adenoviral vectors and mutant virus

Whole cell extracts were prepared from a portion of the cell
cultures which were harvested to analyze cell viability (Figure 3.9). Extracts were resolved by SDS-PAGE and following transfer to nitrocellulose, samples were immunoblotted using anti-HA antibodies, anti-E1A antibodies, or anti-p53 antibodies. Expression of wild-type and mutant E4orf4 from inducible adenoviral vectors is first observed at 24 h.p.i. and increases over the course of the experiment (Figure 3.10, panel A). Mutants are expressed at levels similar to wild-type E4orf4 by 124 h.p.i. The expression of adenoviral proteins by pm2/3 in H1299 cells was confirmed by the presence of E1A proteins at 24 h.p.i. (Figure 3.10, panel B). Multiple forms of E1A are detected due to the presence of at least five sites of phosphorylation (Whalen et al., 1997). By 72 h.p.i., E1A is no longer detected as all cells have died (Figure 3.9). p53 expression is detected at 24 h.p.i., then decreases over the course of the experiment as cells begin to die (Figure 3.10, panel C; Figure 3.9).

3.11 Analysis of caspase-3 cleavage and PARP cleavage in response to E4orf4-induced apoptosis

Previous studies conducted in Chinese hamster ovary cells showed that E4orf4-induced apoptosis is not accompanied by caspase-3 activation nor cleavage of poly (ADP-ribosyl) polymerase (PARP), a substrate for active caspase-3 (Lavoie et al., 1998). In addition, E4orf4-induced apoptosis is not blocked by zVAD-fmk, a broad spectrum caspase inhibitor, suggesting that E4orf4 induces a novel caspase-independent apoptotic pathway (Lavoie et al., 1998).

Experiments were performed to help characterize the apoptosis pathway induced by E4orf4 in human cells. Caspase-3 is an effector caspase and functions in cellular disassembly in response to activation by initiator caspases (reviewed in
Figure 3.10  Analysis of protein expression by adenoviral vectors and mutant adenovirus

Portions of the cultures described in figure 3.9 were harvested at 0 (A), 24 (B), 48 (C), 72 (D), 100 (E) and 124 (F) hours post-infection and lysed. In panel A, extracted proteins of the cultures indicated were separated by 15% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-HA antibody. In panel B, extracted proteins were separated by 12% SDS-PAGE and immunoblotted with anti-EIA antibody. In panel C, extracted proteins were separated to 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-p53 antibody. The arrows and arrowheads indicate the proteins of interest.
Thornberry and Lazebnik, 1998). Activation of caspase-3 was monitored in whole cell extracts prepared from a portion of the cell cultures harvested to analyze cell viability (Figure 3.9). Whole cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted using an antibody which recognizes pro-caspase-3 of 32kD in size but not the activated, cleaved form of caspase-3. The results of this analysis are presented in Figure 3.11. Mock-infected H1299 cells revealed a constant level of pro-caspase-3 whereas infection of cells by Ad rTTA triggered the accumulation of pro-caspase-3. In response to co-infection of H1299 with Ad rTTA and Ad ind orf4, caspase-3 was not activated, confirming that, as in rodent cells, E4orf4-induced apoptosis does not require caspase-3 activation in human cells. The expression of pro-caspase-3 was induced in all cultures coinfected with Ad rTTA and wild-type or mutant Ad ind orf4, presumably due to the presence of Ad rTTA. As previously observed, pm 2/3 and Ad p53 induce cleavage of caspase-3 (Boulakia et al., 1995; Nguyen et al., 1997).

PARP is a DNA repair enzyme that contributes to genome integrity (Burkle et al., 1992) and is cleaved and inactivated during the process of apoptosis by caspase-3 and caspase-7 (reviewed in Cohen, 1997). PARP cleavage in response E4orf4-induced apoptosis was monitored in whole cell extracts prepared from a portion of the cell cultures harvested to analyze cell viability (Figure 3.9). Whole cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-PARP antibody which recognizes the full-length 116 kD PARP protein as well as the 85 kD cleaved product. The results of this analysis are presented in figure 3.12. PARP was not cleaved in response to
Figure 3.11 Analysis of caspase-3 cleavage in response to infection by inducible-adenoviral vectors expressing wild-type and mutant E4orf4.

The proteins extracted as described in figure 3.10 were separated by 12% SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti-caspase-3 antibody. The arrow indicates the pro-caspase-3 protein of 32kD.
Figure 3.12 Analysis of PARP cleavage in response to infection by inducible-adenoviral vectors expressing wild-type and mutant E4orf4.

Portions of the cultures described in figure 3.9 were harvested at 0 (A), 24 (B), 48 (C), 72 (D), 100 (E) and 124 (F) hours post-infection and treated as per PARP Extraction Protocol, described in Materials and Methods. Cell extracts were separated by 8% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-PARP antibody. The arrows indicate the full-length p116 product and the cleaved p85 product.
mock-infection nor infection with Ad rTTA. In contrast, pm2/3 and Adp53 infection induced rapid cleavage of PARP and disappearance of the full-length 116 kD product as previously reported (Boulakia et al., 1995; Nguyen et al., 1997). Just as Adp53 killed H1299 cells more rapidly than pm2/3 (Figure 3.9), PARP cleavage was also induced more rapidly in response to Ad p53 infection. Surprisingly, Ad ind orf4 infection also induced PARP cleavage in H1299 cells. Appearance of the 85 kD cleavage is observed at 72 h.p.i. which coincides with a decrease in survival of cells expressing E4orf4. Ad ind pt K88A infection also induced PARP cleavage but to a lower extent than Ad ind orf4 infection which is consistent with its reduced cytotoxicity (Figure 3.9). PARP cleavage was not observed in cells infected by Ad ind pt L51L54A which correlates with its inability to induce cell death (Figure 3.9). Low levels of PARP cleavage were observed in cells infected by Ad ind pt L3A, Ad ind pt F84A, and Ad ind pt Y89A. This reflects the small cytotoxic effect observed in response to infection by these viruses which was slightly greater that of Ad ind pt L51L54 at 100 and 124 h.p.i. (Figure 2.9). These results indicate that in human cells, E4orf4 induces apoptosis by a pathway that does involve PARP cleavage. Taken together with the results presented in Figure 2.10, E4orf4-induced PARP cleavage occurs independently of caspase-3 activation.

3.12 Analysis of the specificity of E4orf4 binding to the Bα subunit of PP2A

Previous work suggested that E4orf4 binds exclusively to the Bα subunit of PP2A (Kleinberger and Shenk, 1993), but this work was done prior to the cloning of other subunits of the B, B’, and B” families of PP2A regulatory subunits. E4orf4 may bind other B
H1299 cells were lipofected with pcDNA3 expressing HA-tagged B-α subunit or pCEP expressing the indicated B’ subunit family member together with or without pcDNA3 expressing FLAG-tagged E4orf4, as indicated. Cells were harvested 24 hours post-lipofection and half the cell pellet was lysed, subjected to immunoprecipitation with anti-HA antibody, separated by 15% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-FLAG antibody (panel A). The other half of the cell pellet was lysed and in panel B, extracted proteins were separated by 8% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-HA antibody and in panel C, extracted proteins were separated by 15% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-FLAG antibody.
A. IP HA

- IgG_H
- IgG_L
- FLAG orf4

B. WCE

C. WCE

- FLAG orf4
subunits and thus, may interact with multiple forms of the PP2A holoenzyme in order to carry out its various functions, including induction of apoptosis. To determine if E4orf4 can also bind to members of the B' family, cDNAs encoding B'α, B'β, B'γ1, B'δ, and B'ε were obtained and *in vivo* binding experiments were conducted. H1299 cells were co-lipofected with plasmid DNA encoding FLAG-tagged E4orf4 and HA-tagged B' subunits and extracts were immunoprecipitated using anti-HA antibodies (IP HA). Precipitates were resolved by SDS-PAGE, transferred to nitrocellulose and samples were immunoblotted for the presence of FLAG-orf4 using anti-FLAG antibodies. Whole cell extracts (WCE) were also prepared from aliquots of the lipofected cells to verify that comparable amounts of these proteins were present. The extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anti-HA and anti-FLAG antibodies. Cells were also colipofected with HA-tagged Bα and FLAG-tagged orf4 which served as a positive control for binding. Figure 3.13, panel B shows that the HA-tagged B' subunits are stably expressed and are of the expected size (McCright et al., 1996). Panel C shows that FLAG-tagged E4orf4 is expressed at equivalent levels in all samples. Panel A shows that E4orf4 was present in Bα immunoprecipitates but could not be detected in immunoprecipitates of B'α, B'β, B'γ, B'δ, nor B'ε. This data indicates that E4orf4 does not interact with PP2A holoenzymes containing B' family members.

3.13 Analysis of the phosphorylation status of E4orf4

E4orf4 is known to autoregulate E4 expression and thus regulates its own activity through transcriptional control mechanisms (Bondesson et al., 1996). This raises the possibility
that E4orf4 activity may also be controlled at the post-translational level. The activities of many cellular and adenoviral proteins, including E1A, are regulated by phosphorylation (Whalen et al., 1997). The targets of phosphorylation may be Y59, T64, Y89, S95 or S106, which are residues involved in E4orf4 (Figure 3.5, 3.8, 3.9; Shtrichman and Kleinberger, 1998). To determine the phosphorylation status of E4orf4, H1299 cells were lipofected with plasmid DNA expressing HA-tagged E4orf4 and labeled with $^{32}$P orthophosphate for 4 hours commencing at 20 hours post-lipofection and extracts were immunoprecipitated with anti-HA antibodies. Precipitates were resolved by SDS-PAGE and phosphoamino acid analysis was performed. Figure 2.14 shows that HA-tagged E4orf4 is labeled with $^{32}$P (panel A) and only phosphoserine residues were detected following acid hydrolysis (panel B).
Figure 3.14 Phosphoaminoacid analysis of HA-E4orf4

H1299 cells were transfected with pcDNA3 and the pcDNA3 plasmid expressing an HA-tagged version of E4orf4, labeled with [\textsuperscript{32}P] orthophosphate, lysed, and subjected to immunopurification by anti-HA antibody, and immunoprecipitates were separated by SDS-PAGE (panel A). HAorf4 and the corresponding band of pcDNA3 were hydrolyzed and the resulting phosphoamino acids were analyzed by 2D-TLC, as described in Materials and Methods (panel B). The positions of phosphoamino acid markers were determined by the use of phosphoamino acid standards stained with ninhydrin.
4. Discussion

The present studies demonstrated that no distinct domain of E4orf4 is responsible for its ability to induce cell death. The residues in E4orf4 which are involved in the induction of cell death span the 114 residues of the protein. Leucine-3, leucine-51 in cooperation with leucine-54, phenylalanine-84 and tyrosine-89 were shown by the luciferase assay (Fig. 3.5) and/or the viability assay (Fig. 3.9) to be required for the cytotoxic effect of E4orf4 as their mutation to alanine abrogated cell death. Isoleucine-43, glutamic acid-56 in cooperation with tryptophan-57, the threesome of arginines at 73,74, and 75, and arginine-93 in cooperation with arginine-94 were shown to contribute to E4orf4 killing as their mutation to alanine caused a reduced cell killing activity (Fig. 3.5). It is interesting to note that mutation of tyrosine-59 to alanine enhanced the ability of E4orf4 to kill, indicating that this residue may be involved in negative regulation of E4orf4 activity (Fig. 3.5). Deletion mutant analysis indicated that residues 12-18 with the N-terminus, and the extreme C-terminus residues 103-114, do not play an essential role in induction of death by E4orf4. Mutants dl 12-18 and dl 103-114 were as cytotoxic as wild-type orf4 (Fig. 3.3) in spite of the observation that these mutants are expressed at lower levels than wild-type in vivo (Fig. 3.2; panel B). Other deletion mutants were uninformative due to their variable expression (Fig. 3.2) and partial defects in cytotoxicity (Fig. 3.3). These results suggest that large deletions in this small 14 kD protein disrupt its functional confirmation and decrease its stability. The threesome of prolines-7, -9, and -10 and lysine-88 in cooperation with tyrosine-89 are also involved in E4orf4 protein
stability as their substitution to alanine decreased protein levels (Fig. 3.7) and thus explains their inability to kill cells (Fig. 3.5) and to bind Bα (Fig 3.8).

E4orf4/Bα binding studies demonstrated no distinct domain of E4orf4 is responsible for the interaction with the Bα subunit of PP2A. Leucine-3, leucine-51 in cooperation with leucine-54, arginine-81 in cooperation with phenylalanine-84, phenylalanine-84 alone, the group of arginines at 69,70 and 72-75 and tyrosine-89 are required for interaction of E4orf4 to Bα (Fig. 3.8). Proline-9, cytosine-18, tryptophan-21, glutamic acid-56 in cooperation with tryptophan-57, tryptophan-57 alone, the threesome of threonine-64, glutamic acid-65 and arginine-66, the threesome of arginines 73-75, arginine-81, cytosine-85, arginine-93 in cooperation with arginine-94 and serine-106 are involved in the interaction of E4orf4 with Bα (Fig. 3.8).

These results show that Bα binding, and thus PP2A binding, is necessary for the induction of cell death in E4orf4. E4orf4 mutants which failed to bind Bα and that were assayed for cytotoxicity were highly defective for induction of cell death. Furthermore, mutants that displayed intermediate binding levels also exhibited somewhat reduced cell killing activity when assayed. The other important finding was the identification of mutants that were partially defective for cell killing, but which were wild-type for Bα binding. These mutants indicate that Bα binding, and thus PP2A binding, is necessary but not sufficient for E4orf4 death. The sole exception to these generalizations is the E4orf4 mutant, pt R81MF84A which kills cells as measured by the luciferase assay (Fig. 3.8). Further characterization of the cytotoxic effect of this mutant through colony inhibition assays, which directly measures
decreases in cell viability, showed that pt R81A/F84A cannot induce cell death (Marcellus, unpublished observations).

The discrepancy between the effects measured in the luciferase assay versus those measured in the colony inhibition assays underscores the drawbacks of this indirect cell death assay. The decrease in luciferase activity observed in response to cotransfection with pt R81A/F84A was not due to this mutant's cytotoxic effect but more likely due to transcriptional effects.

Therefore, the interaction of E4orf4 with Bα is indeed required although it is not sufficient for E4orf4 to induce cell death. This implies that killing requires some other activity in E4orf4. This effect may simply relate to the activation of PP2A. Mutant E4orf4 proteins that exhibit wild-type Bα binding but are partially defective in cell killing may be competent for binding but unable to stimulate phosphatase activity. Another possibility is that E4orf4 provides an additional function for cell killing such as altering the intracellular localization of PP2A, altering the complex formation of other Bα-interacting proteins, or linking PP2A with critical substrates.

E4orf4 does not bind members of the B' family of PP2A regulatory subunits (Fig. 3.13). This suggests that E4orf4 may mediate its activities through specific binding of the ABαC holoenzyme of PP2A and thus only induce a subset of PP2A functions. Bα has no homology with B' subunits or B” subunits but it shares 86% identity with the Bβ subunit (Mayer et al., 1991) and 81% identity with the Bγ subunit (Zolnierowicz et al., 1994). It will be of interest to determine if E4orf4 can bind these other members of the B class or members of the B” class of subunits.

The results of figure 3.11 and 3.12 demonstrated that E4orf4
induces p53-independent apoptosis via a pathway which does not induce caspase-3 activation but does involve PARP cleavage. These results differ from those obtained by Lavoie at al. during studies of E4orf4 induced death in rodent cells. In rodent cells, E4orf4 appears to induce a novel caspase-independent apoptotic pathway and PARP cleavage was not observed. This discrepancy may be due to the fact that rodent cells are non-permissive for adenoviral infection suggesting that E4orf4 and other adenoviral proteins may mediate different effects in human versus rodent cells.

The ability of E4orf4 to induce PARP cleavage in human cells independently of caspase-3 activation indicates that a different caspase must be activated in the apoptotic pathway induced by E4orf4. E4orf4 may induce the activation of a novel caspase, but of the 11 known members of the caspase family, caspase-7 is a likely candidate. Like caspase-3, caspase-7 induces the cleavage of PARP. Also, caspase-7 is constitutively expressed in adult tissues (Fernandes-Alnemri et al., 1995). Of specific interest is the fact that caspase-7 pre-mRNA generates two isoforms, Mch-3α and Mch-3β. Mch-3α encodes a peptide that can be processed into an active caspase, whereas Mch-3β is produced by alternative splicing that leads to deletion of the region encoding the caspase active site and creates an inactive caspase (Fernandes-Alnemri et al., 1995). Thus, caspase-7 activity is regulated through alternative splicing. E4orf4 is known to regulate alternative splicing of late viral mRNAs through PP2A-dependent dephosphorylation of serine-arginine-rich (SR) proteins (Kanopka et al., 1998). A potential mechanism for E4orf4-induced apoptosis may be the activation of caspase-7 through the preferential splicing of the Mch-3α isoform of the caspase-7 pre-mRNA.
Preferential splicing could be achieved by E4orf4/PP2A-dependent dephosphorylation and inactivation of SR proteins which may prevent Mch-3α accumulation in normal cells. This proposed mechanism is analogous to the preferential IIa splicing of L1 pre-mRNA controlled by E4orf4/PP2A complexes late in adenoviral infection (Kanopka et al., 1998). In this model, PP2A activity would be required but would not be sufficient for induction of cell death which is consistent with the results obtained in this study.

The ability of E4orf4 to target the E4orf4/PP2A complex to spliceosomes containing caspase-7 pre-mRNA would be required in addition to binding. Future experiments monitoring alternative splicing of caspase-7 pre-mRNA in response to E4orf4 expression could be performed to test this hypothesis. E4orf4 may also induce apoptosis through PP2A-dependent dephosphorylation and inactivation of Bcl-2 or activation of Bad.

The final result demonstrated in this study was that E4orf4 is phosphorylated in lipofected cells on serine residues exclusively. This is based on the assumption that the HA epitope does not incorporate phosphoserine but further experiments should be performed to determine the phosphorylation status of E4orf4 in adenovirus-infected cells. The Ad5 E4orf4 protein sequence contains a putative casein kinase II site at serine-14 and a putative protein kinase A site at serine-76 which maybe the targets of phosphorylation. Serine-95 and serine-106 may also be targets. Serine-95 is required for E4orf4 killing and Bα binding (Shtrichman and Kleinberger, 1998) and serine-106 is important for Bα binding (Fig. 3.8) but its removal in dl 103-114 does not affect cytotoxicity (Fig. 3.3). Mapping of the E4orf4 phosphorylation sites will help determine what role they play in
E4orf4 activity.
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