Characterization of protein complexes interacting with adenovirus type 5 E1A proteins and identification of novel E1A phosphorylated forms within these complexes.

Characterization of protein complexes interacting with adenovirus type 5 E1A proteins and identification of novel E1A phosphorylated forms within these complexes.

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TITLE: Characterization of protein complexes interacting with adenovirus type 5 E1A proteins and identification of novel E1A phosphorylated forms within these complexes.

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Abstract

The transforming potential of adenovirus E1A oncogene products derives largely from the formation of complexes with cellular proteins, including the p105^{Rb} tumour suppressor and a related p107 species, p130 and p300 proteins, and cyclin A (p60^{cycA}). Extensive quantitative analyses using E1A deletion mutants identified unique binding patterns for each of these polypeptides within the amino terminus and conserved regions 1 and 2 (CR1 and CR2) of E1A proteins. A novel protein, termed p400, was found by peptide mapping to be related to p300, and, like p300, to require the E1A amino terminus and a portion of CR1 for binding. p130 was shown to be related to p107, and like p107, to associate with p60^{cycA}. p107, p130 and p105^{Rb} all interacted primarily with CR2, however, sequences within CR1 and the amino terminus were capable of weak interactions and appeared to function cooperatively with CR2 to bind these proteins. Protein kinase activity exists in E1A complexes and probably derives in part from p33^{cdk2}-p60^{cycA} heterodimers associated with p107 and p130. In vitro phosphorylation of complexes purified by immunoprecipitation resulted in labeling of several proteins. p60^{cycA} was phosphorylated to about the same extent in cyclin A complexes prepared from either Ad5- or mock-infected KB cells, however, that of p130 and p107 was dramatically higher in p60^{cycA} complexes from infected cells. p300 was also phosphorylated in complexes containing E1A proteins. Thus one role of E1A proteins in signal transduction and regulation of the cell cycle may be to control the biological activity of p107, p130 and p300 by enhancing their phosphorylation through complex formation.

Both major proteins encoded by the E1A region of human adenoviruses are phosphorylated at serine residues located at amino acids 89, 96, 132 and 219 of the 289-residue E1A product and additional serine sites exist between residues 227 and 237. Mutants containing

alanine residues in place of these serines formed complexes with a series of cellular polypeptides in an fashion analagous to *wt* Ad5. Phosphorylation at Ser-89 was known to cause a significant "shift" in mobility of E1A proteins in SDS polyacrylamide gels. A significant proportion of E1A proteins were phosphorylated at these additional sites near the carboxy terminus which induced a further "supershift" in gel mobility. However, whereas E1A proteins associated with the p105^{Rb} tumour suppressor in *wt*-infected KB cells were found in both the normal and "supershifted" forms, the latter species were present preferentially in complexes containing p60^{cycA}. These data suggested that phosphorylation near the carboxy terminus may play a role in complex formation or may be either enhanced or stablized through associations with protein complexes containing p60^{cycA}.

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List of Abbreviations

243R	243 residue E1A protein
289R	289 residue E1A protein
aa	amino acid
Ad	Adenovirus
ATF	Activating transcription factor
bHLH motif	basic helix-loop-helix motif
bZIP motif	basic leucine zipper motif
cAMP	cyclic adenosine mono-phosphate
cdc2	cell division cycle-2 protein kinase
cdks	cell cycle dependent kinases
cDNA	complementary DNA (to sense strand)
CR1/CR2/CR3	conserved region 1/2/3
CREB	cAMP responsive element binding protein
C-terminus	carboxy terminus
CTLs	cytotoxic T lymphocytes
cycA	cyclin A
cycE	cyclin E
DNA	deoxyribonucleic acid
E1A	Early region 1A of Adenovirus
E1B	Early region 1B of Adenovirus
gp	glycoprotein
HPV E6/E7	Human papillomavirus early region 6 &7
hsp70	heat shock protein 70
HSV-1 VP 16	Herpes simplex virus type 1 viral poduct 16
IL-6	interleukin 6
kb	kilobases
kDa	kilodaltons
LTAg	Large T antigen
M phase	Mitosis phase
mdm2	murine double minute 2
MDR1	multi-drug resistance gene
MHC I	Major histocompatibility complex class I
MES	2-[N-morpholino]ethanesulfonic acid
μM	micromolar
mM	millimolar
mRNA	messenger RNA
N-terminal	amino terminal
NF-κB	Nuclear factor-κB
NK cells	Natural killer cells
PCNA	Proliferation cell nuclear antigen
p.i.	post-infection
PMSF	phenyl methyl sulfonyl fluoride
PP2A	Phosphatase 2A
$pp60^{c\text{-}src}$	phophoprotein 60 c-src kinase
Rb/RB-1	Retinoblatoma gene
RCE	Retinoblastoma control element
RNA	ribonucleic acid
S phase	Synthesis phase

SDS	sodium dodecyl sulfate
SV40	Simian virus type 40
TAFs	TBP-associated factors
TBP	TATA box binding protein
TFIID/TFIIB	Transcription factor IID/IIB
TGF-β1/β2	Transforming growth factor β1/β2
TNF	Tumour necrosis factor
TPA	12-O-tetradecanoyl phorbol 13-acetate
TRE	TPA-responsive element
TSTA	Tumour transplantation antigen
wt	wild type

CHAPTER 1: Introduction

1.1. Adenovirus structure

1.1.1 Etiology and Adenovirus Classification

Adenoviruses (Ad) were the first human viruses shown to be oncogenic as they were found to be able to induce tumours in rodents (Trentin *et al.*, 1962). They do not appear to be tumourigenic in humans, however, they give rise to common cold-like symptoms, various respiratory illnesses, as well as keratoconjunctivitis, cystitis (urinary bladder inflammation) and gastroenteritis (Gary *et al.*, 1979; Hilleman and Werner, 1954; Jawetz *et al.*, 1955; Retter *et al.*, 1979; Rowe *et al.*, 1953; reviewed in Horwitz, 1990b). There is little evidence to suggest that they are of importance in human cancer since there is only a single report which documented adenovirus sequences in neurogenic tumours (Ibelgaufts *et al.*, 1982). All other studies have failed to detect adenoviral sequences in association with human tumours, suggesting that adenoviruses are not major human cancer-causing agents (Horwitz, 1990b).

Approximately 42 different human serotypes have been identified. These have been classified into six groups according to a number of properties: tumourigenic potential, DNA homology and G + C content, DNA restriction patterns, molecular characteristics of virion proteins, and haemagglutination ability (Horwitz, 1990b). Groups A (which contains Ad 12) and B are highly and weakly tumourigenic, respectively, whereas groups C (which contains Ad serotypes 2 and 5), D, E, and F are non-tumourigenic in rodents (Table 1). A number of other serotypes have been isolated from various species including cats, dogs, mice, tree shrews, pigs, sheep, monkeys, cows (Green, 1985; Horwitz, 1990b). These mammalian viruses all belong to the Mastadenovirus genera of the Adenoviridae family.

Table 1. Classification of human adenoviruses.

Sub-genus	Species	DNA			Apparent molecular weight of the major internal polypeptides			Hemagglutination pattern[3]	Oncogenicity in newborn hamsters
		Homology (%)[1]	G+C %	# of SmaI fragments[2]	V	VI	VII		
A	12,18 31	43-69 (8-20)	48	4-5	51-51.5K 46.5-48.5K[4]	25.5-26K	18K	IV	High (tumours in most animals in 4 months
B[5]	3,7,11,14, 16,21,34, 35	89-94 (9-20)	51	8-10	53.5-54.5K	24K	18K	I	Weak (tumours in few animals in 4-8 months
C	1,2,5,6	99-100 (10-16)	58	10-12	48.5K	24K	18.5K	III	nil
D[5]	8,9,10,13, 15,17,19, 20,22,23, 24,25,26, 27,28,29, 30,32,33, 36,37,38,39	94-99 (4-17)	58	14-16	50-50.5K[6]	23.2K	18.2K	II	nil
E	4	(4-23)	58	16-19	48K	24.5K	18K	III	nil
F	40	n.d.	n.d.	9	46K	25.5K	17.2K	IV	nil
G	41	n.d.	n.d.	11-12	48.5K	25.5K	17.7K	IV	nil

not classified: Ad 42

1 Per cent homology within the group and in brackets: homology with members of other groups

2 DNA fragments were analysized on 0.8-12% agarose gels. DNA fragment smaller than 400bp not resolved

3 I, complete agglutination of monkey erythrocytes ; II complete agglutination of monkey erythrocytes; III, partial agglutination of rat erythrocytes (fewer receptors); IV agglutination of rat erythrocytes only after addition of heterotypic antisera.

4 Polypeptide V of Ad 31 was a single band of 48K

5 Only DNA restriction and polypeptide analysis have been performed on Ad 32 - Ad 39

6 Polypeptides V and VI of Ad8 showed apparent molecular weights of 45K and 22K respectively. Polypeptide V of Ad30 showed an apparent molecular weight of 48.5K.

* Modification of Wadell *et al.*, 1980

Serotypes isolated from avian species have been classified into a different genus, Aviadenovirus, since these do not possess group-specific determinants shared among the Mastadenovirus family (Horwitz, 1990a).

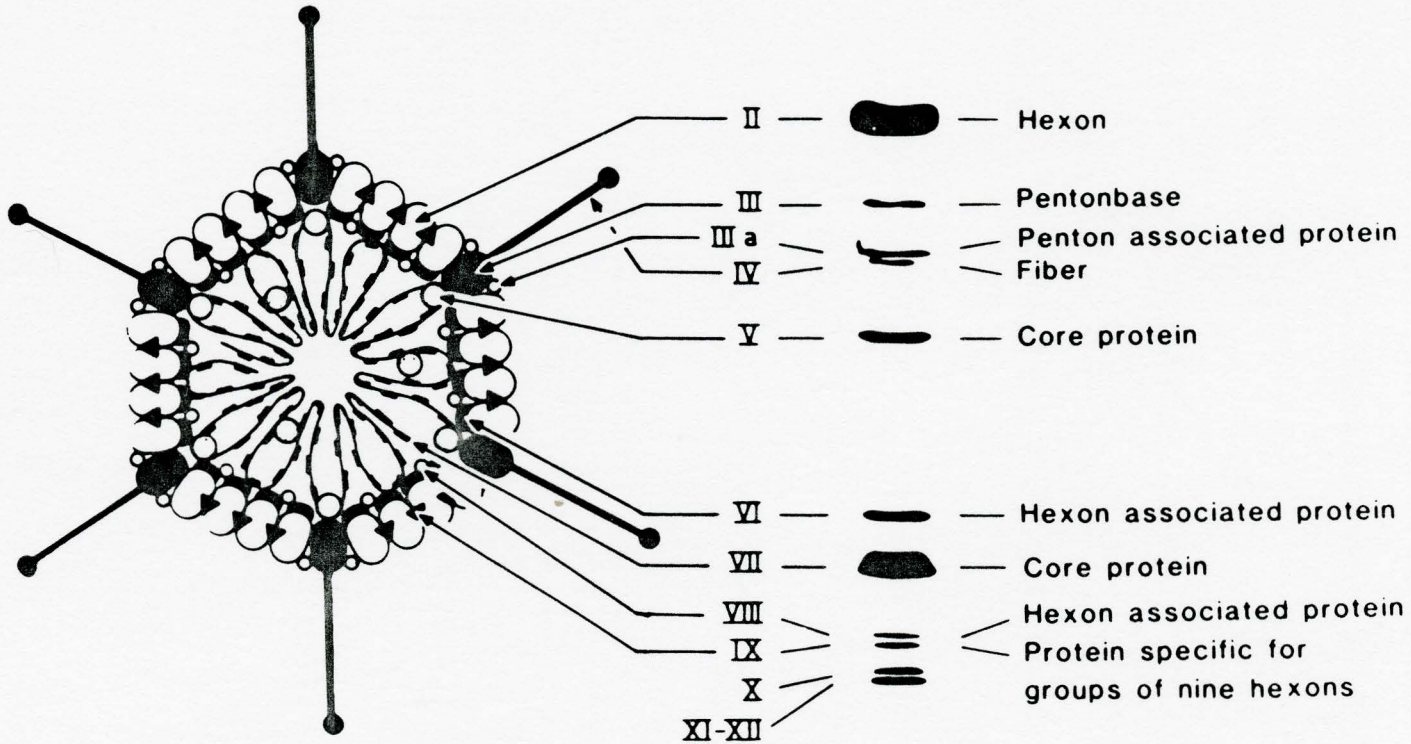
1.1.2 Virion Structure

Adenoviruses are non-enveloped, icosahedral viruses containing a linear, double-stranded DNA genome of 36 kb in length (Green and Pina, 1963; Horne *et al.*, 1959; van der Eb and van Kesteren, 1966; reviewed in Horwitz, 1990a). The structural proteins forming the capsid (20 triangular surfaces and 12 vertices) which encloses the viral core containing the viral genome, consists of hexons (protein II), pentons (protein III) and fibers (protein IV) (Ginsberg *et al.*, 1966; Norby, 1966), as well as a number of associated proteins which are minor capsid components (proteins IIIa, VI, VIII, and IX) (Everitt *et al.*, 1973; 1975; Maizel *et al.*, 1968). The virion core is composed of at least three proteins (proteins V, VII, X), which play a role in compacting adenoviral DNA into nucleosome-like subunits, although these are not covalently attached to DNA (Cupo *et al.*, 1987; Hosakawa and Sung, 1976; Mirza and Weber, 1982; Russell *et al.*, 1968). Figure 1 illustrates the proteins which form the virion core and capsid, as well as the relative gel mobilities of these proteins (from Horwitz, 1990a). A 55kDa terminal protein is covalently linked to the 5' ends of the viral genome (Rekosh *et al.*, 1977; Robinson *et al.*, 1973). The ends also have inverted terminal repeats of approximately 100 nucleotides which upon denaturation can form panhandle structures that are thought to promote replication of the virus (Garon *et al.*, 1972; Wolfson and Dressler, 1972).

1.1.3 Productive Infection and Genome Structure: An Overview

Human adenoviruses normally infect non-replicating differentiated epithelial cells of the upper respiratory tract (Horwitz, 1990b). Upon infection these viruses undergo

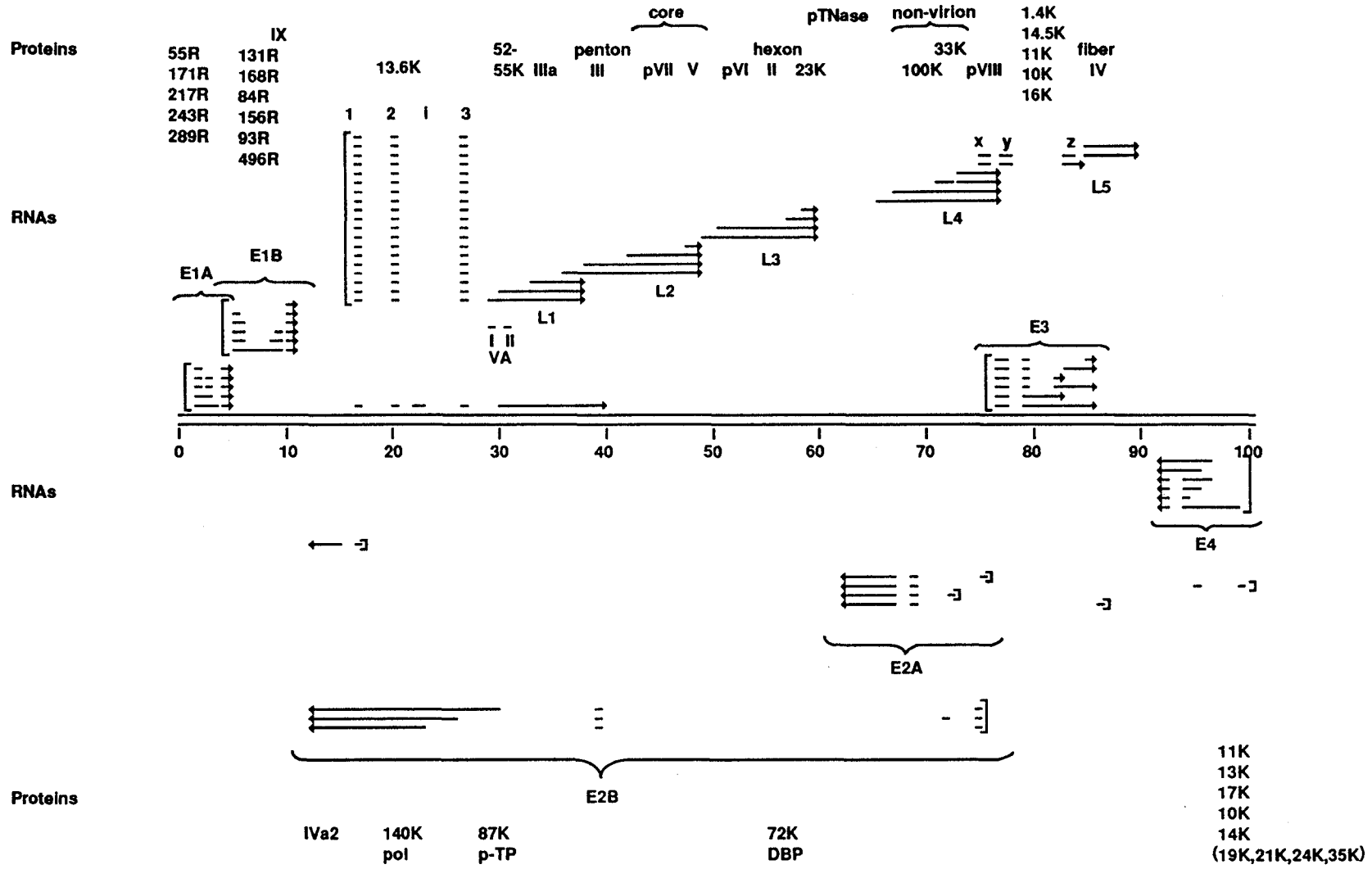
Figure 1. Structural components of the adenovirus virion particle. The schematic diagram shows the composition and location of proteins within the virion capsid. The relative mobilities of these various proteins as they migrate in SDS-PAGE gels is also indicated. Each protein is designated by a roman numeral as described by Maizel *et al.*, 1968. Adapted from Everitt *et al.*, 1975.


Polypeptide SDS-gel Structural Unit
II
Hexon
III
Pentonbase
III a
Penton associated protein
IV
Fiber
V
Core protein
VI
Hexon associated protein
VII
Core protein
VIII
Hexon associated protein
IX
Protein specific for
X
groups of nine hexons
XI-XII

a lytic cycle, producing progeny virions by lysing the host cells (Horwitz, 1990a). Human adenoviruses can also infect rat or hamster cells, however these cells are not permissive for viral replication. As a result, a few of these cells will become transformed through integration of the viral genome and expression of early viral genes.

Infection of permissive host cells such as KB or HeLa cells occurs as a result of adsorption of viral fiber proteins to receptors on the cell surface (Hennache and Boulanger, 1977; Lonberg-Holm and Philipson, 1969; Meager *et al.*, 1976; Svensson., 1981), penetration by endocytosis, and release of the virion core within the cytoplasm (Chardonnet and Dales, 1970a; Defer *et al.*, 1990; Pastan *et al.*, 1987; Patterson and Russell, 1983; Svensson, 1985; Varga *et al.*, 1991). The virion core is transported to the nuclear membrane where further uncoating occurs, following which the viral DNA is propelled into the nucleus through nuclear pores (Chardonnet and Dales, 1970a; 1972). The process of adsorption, penetration, and nuclear uptake is completed within 60-90 minutes following infection at 37°C (Chardonnet and Dales, 1970a,b). A number of viral regions are transcribed at different times following infection. The early (E) genes are transcribed before the onset of viral DNA replication, whereas late (L) genes are transcribed once DNA replication begins (see Figure 2; Sharp *et al.*, 1975). By convention, nucleotides are numbered starting from the left end of the genome and the r and l strands refer to the direction of transcription towards the right or the left. Early messages are produced from both the r and l strands, whereas the late messages are produced mainly from a single region on the r strand (Chow *et al.*, 1977; Sharp *et al.*, 1975). Each of the regions contains its own promoter and gives rise to a number of diffentially spliced mRNAs encoding various protein products (Berk and Sharp, 1977; Horwitz, 1990a). The E1A region is the first to be transcribed, encoding proteins which appear two hours after infection, and are required for the activation of the other early viral genes which are transcribed in the following temporal sequence: E2, E3, E4, and E1B (Berk *et al.*, 1979; Glenn and Ricciardi, 1988; Jones and

Figure 2. Map of the adenovirus genome showing 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 replication, respectively. Adaptation from Horowitz, 1990a.


Proteins
IX
55R
131R
171R
168R
217R
84R
243R
156R
289R
93R
496R
13.6K
core
pTNase
non-virion
1.4K
14.5K
11K
10K
16K
52-
55K IIIa
penton
III
pVII V
hexon
pVI II 23K
33K
100K pVIII
fiber
IV
RNAs
1 2 I 3
x y
z
L5
L4
L3
L2
L1
E1B
E1A
I II
VA
E3
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A
E2B
Proteins
IVa2
140K
pol
87K
p-TP
72K
DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

Shenk, 1979a; Rowe *et al.*, 1984). Expression of these early proteins is required for viral DNA replication which begins at about 8 hrs post-infection (p.i.) (Chow *et al.*, 1980; Horwitz, 1990a). The late structural proteins forming the virion capsid and core are synthesized at maximal rates at about 20 hrs p.i. (Horwitz, 1990a). Completion of the viral lytic cycle results in cell lysis 32-36 hours following infection, releasing 4,000-10,000 progeny virus particles per infected cell (Green and Daesch, 1961; Horwitz, 1990a).

1.1.4 Organization of the Early Region 2, 3, and 4, and Late Region

E2 Region

The E2 region produces a number of differentially spliced transcripts from two portions of the l strand of the genome. E2a (67.9-61.5 map units) and E2b (29 to 14.2 map units) are transcribed from the same promoter with the same leader sequence, but utilize a different polyadenylation signal (Berk and Sharp, 1978; Horwitz, 1990a). The E2a transcripts code for the 72kDa DNA-binding protein that is required for elongation during viral DNA replication, possibly through its ability to unwind DNA (Lewis *et al.*, 1976; Lichy *et al.*, 1983; Stuiver and van der Vliet, 1990). The 72kDa protein also appears to play a role in down-regulation of early gene expression (Carter and Blanton, 1978; Handa *et al.*, 1983; Lazaridis *et al.*, 1988; Nevins and Winkler, 1980), regulation of late gene expression (Brough *et al.*, 1985; Klessig and Grodzicker, 1979), and assembly of virion particles (Nicolas *et al.*, 1983). The E2B transcipts give rise to the 105kDa viral DNA polymerase and the 80kDa precursor to the 55kDa terminal protein, which together with the 72kDa DNA-binding protein are responsible for viral DNA replication (Alestrom, *et al.*, 1982; Smart and Stillman, 1982; Stillman *et al.*, 1981; 1982).

E3 Region

The E3 region is transcribed from the r strand of the genome (76.6-86.2 map units) and produces at least nine messages with similar 5' untranslated regions but different open reading frames, through the use of alternative splice sites, and one of two polyadenylation sites (Carlin *et al.*, 1989; Ginsberg *et al.*, 1989). This region is not required for viral growth in tissue culture cells, but presumably plays a role in pathogenesis during infection of normal host cells. At least seven proteins have been identified: a 19kDa glycoprotein (Chow *et al.*, 1980; Persson *et al.*, 1980), a 14.7kDa protein (Tollefson and Wold, 1988; Ginsberg *et al.*, 1989), a 10.4kDa membrane protein (Carlin *et al.*, 1989; Ginsberg *et al.*, 1989; Tollefson *et al.*, 1990a), a 14.5kDa polypeptide (Tollefson *et al.*, 1990b), an 11.6kDa product (Ginsberg *et al.*, 1989; Wold *et al.*, 1984), a 12.5kDa protein (Hawkins and Wold., 1992), and a 6.7kDa membrane protein (Wilson-Rawls *et al.*, 1990). However, the functions for only some of these proteins have been established. The E3 gp19kDa species protects Ad-infected cells against cytotoxic T cells by binding and retaining the major histocompatibility complex class I antigens within the endoplasmic reticulum, thus preventing their expression on the cell surface (Burgert and Kvist, 1987; Ginsberg *et al.*, 1989; reviewed in Wold and Gooding, 1991). The E3 14.7kDa, 10.4kDa, and 14.5kDa proteins protect mouse cells from lysis mediated by tumour necrosis factor (TNF), a cytokine secreted from activated macrophages and lymphoid cells (Gooding *et al.*, 1988; 1990; 1991a; reviewed in Wold and Gooding, 1991). The epidermal growth factor receptor appears to be downregulated by both the E3 10.4kDa and 14.5kDa proteins (Carlin *et al.*, 1989; Tollefson *et al.*, 1991).

E4 Region

The E4 region is transcribed from the l strand of the genome (99.3-91.3 map units) and produces at least nine messages which share initiation and polyadenylation sites, but differ through alternative splicing (Chow *et al.*, 1980). Although this region appears to be

important for viral growth, viral DNA replication, and accumulation of late messages and proteins, specific protein products have not yet been linked to all of these various functions (Bridge and Ketner, 1989; Halbert *et al.*, 1985; Hemstrom *et al.*, 1988). Five proteins have been identified thus far: a 14kDa protein (Downey *et al.*, 1983); proteins with molecular masses of 19kDa and 21kDa (Harter and Lewis, 1978); and 17kDa and 34kDa proteins (Cutt *et al.*, 1987). The 34kDa protein forms a complex with E1B-55kDa protein, which is involved in host cell shut off and late viral protein synthesis (Cutt *et al.*, 1987; Sarnow *et al.*, 1984). The E4-19kDa protein binds to and activates the E2F transcription factor which is required for maximal expression of the viral E2 region (refer to E2F transactivation section; Hardy *et al.*, 1989; Huang and Hearing, 1989; Marton *et al.*, 1990; Neill *et al.*, 1990; Neill and Nevins, 1991; Raychaudhuri *et al.*, 1990; Reichel *et al.*, 1989)

Late Region

Most of the late structural proteins are encoded by the r strand of the genome, where a single nuclear transcript, extending from 16.4 to 99 map units, is produced late in infection from the major late promoter (Berget and Sharp, 1979; Shaw and Ziff, 1980). Through differential splicing of nuclear transcripts and termination at one of five polyadenylation sites, twenty or more transcripts are generated, sharing a common tripartite leader sequence (Chow *et al.*, 1977a; 1980; Klessig, 1977; Lewis *et al.*, 1977). These have been grouped into "families" of transcripts (L1-L5) depending on the polyadenylation signal utilized (Chow *et al.*, 1980). A number of messages from the L1 region have been detected early in infection, although at very reduced levels, indicating that transcription from the major late promoter in not restricted to the late phase of the infectious cycle (Lewis and Mathews, 1980; Shaw and Ziff, 1980).

Two additional structural proteins, IX and IVa2, are not transcribed from the major late promoter. Protein IX, is translated from a transcript between map units 9.4 and 11.1 on the

r strand of the genome (Pettersson and Mathews, 1977), whereas protein IVa2 is produced from a message between map units 18.1 and 11.3, on the l strand of the genome (Persson *et al.*, 1979).

Two small virus associated RNA species, transcribed by host RNA polymerase III, are encoded within the late region at around 30 map units on the r strand of the genome and are thought to function in splicing of viral messages (Weinmann *et al.*, 1976; Horwitz, 1990a).

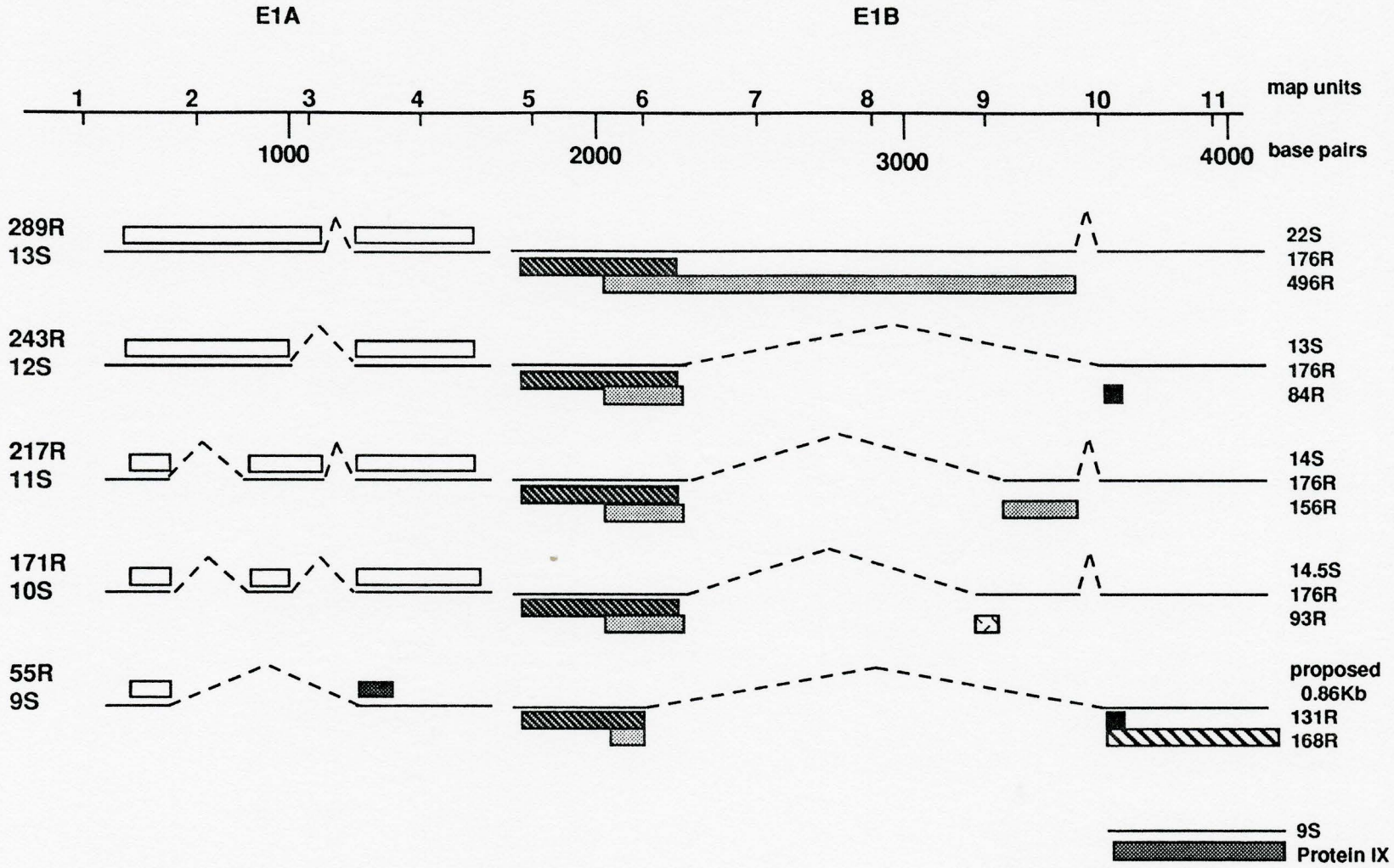
1.2. Early Region 1 (E1A/E1B)

The expression of the E1 region, encompassing the leftmost 11% of the genome and encoding the E1A and E1B proteins, is all that is required for transformation of non-permissive cells (reviewed in Branton *et al.*, 1985; Horwitz, 1990a). Such transformed cells typically are capable of indefinite cell growth *in vitro*, they are not contact-inhibited at high densities, and are capable of growth in soft agar, and at low levels of serum or calcium (0.1mM) (Freeman *et al.*, 1967; McAllister *et al.*, 1969). The mechanism by which E1A and E1B proteins transform cells has recently begun to be elucidated and appears to involve the association of these viral products with a number of cellular proteins (see below).

1.2.1 E1B mRNA Species and Protein Products

Two major messages are produced from the E1B region (4.6-11.2 map units): a 2.2kb message (22S) which is most abundant early in infection, and a 1.05kb message (13S) which accumulates at later times during infection (see Figure 3, Bos *et al.*, 1981, Glenn and Ricciardi, 1988; Horwitz, 1990a, Perricaudet *et al.*, 1980). In addition, two minor species of 1.26kb (14S) and 1.31kb (14.5S) are produced late in infection (Anderson *et al.*, 1984; Lewis and Anderson, 1987; Virtanen and Pettersson, 1983b). These four messages all give rise to a 176-residue protein,

Figure 3. Map of the adenovirus early region 1 showing transcription and translation products. Both E1A and E1B give rise to at least 5 differentially spliced mRNAs which are indicated according to their sedimentation coefficient.


E1A
E1B
1
2
3
4
5
6
7
8
9
10
11
map units
1000
2000
3000
4000
base pairs
289R
13S
22S
176R
496R
243R
12S
13S
176R
84R
217R
11S
14S
176R
156R
171R
10S
14.5S
176R
93R
55R
9S
proposed
0.86Kb
131R
168R
9S
Protein IX

which migrates as a 19kDa doublet on SDS-polyacrylamide gels (McGlade *et al.*, 1987; McGlade *et al.*, 1989; Rowe *et al.*, 1983a). These four messages can also be translated from a second internal initiation codon positioned in a different reading frame to yield a family of related proteins. With Ad 5, the 2.2 kb and 1.05 kb messages give rise to 496-residue (55kDa) and 156-residue (25kDa doublet) proteins, respectively, whereas the 1.26 kb and 1.31 kb minor messages produce proteins of 93 (about 20kDa) and 84 (20kDa) amino acids, respectively. All four of these proteins share 79 common residues at the amino terminus. In addition, the 156R and 496R protein also share 78 carboxy terminal residues. The 93R and 84R proteins have unique carboxy terminal sequences as they arise from messages that are spliced into different reading frames (Bos *et al.*, 1981; Green *et al.*, 1982; Lewis and Anderson, 1987; Matsuo *et al.*, 1982)

E1B 55kDA and 19kDa products

Expression of both the 55kDa and 19kDa proteins is required for maximal productive infection, and mutants affecting either protein can be host range (Harrison *et al.*, 1977). The 19kDa protein, which is modified both by fatty acid acylation and phosphorylation, is associated with nuclear and cytoplasmic membranes, and intermediate filaments of the cytoplasm (vimentin) and the nuclear lamina (lamins) (Grand *et al.*, 1985; McGlade *et al.*, 1987; 1989; Persson *et al.*, 1982; Rowe *et al.*, 1983a; White and Cipriani, 1989). Infection of cells with viral mutants carrying defects in the 19kDa coding region leads to the appearance of a large plaque phenotype characteristic of cytocidal effects, and to rapid degradation of both viral and cellular DNA (*cyt/deg* phenotype; Barker and Berk, 1987; Chinnadurai, 1983; Ezoe *et al.*, 1981; Mak and Mak, 1983; McLorie *et al.*, 1991; Pilder *et al.*, 1984; Subramanian *et al.*, 1984a; 1984b; Takemori *et al.*, 1984; White *et al.*, 1984). It now seems clear that the 19kDa protein functions to protect cells against programmed cell death induced by E1A products (see below; Rao *et al.*, 1992; White *et al.*, 1991; 1992). 55kDa is a phosphoprotein which is primarily associated with the perinuclear region of

infected cells (Grand and Gillmore, 1984; Rowe *et al.*, 1983a; Sarnow *et al.*, 1982b; Schughart *et al.*, 1985; Yee *et al.*, 1983). Through association with a 25kDa protein from the E4 region, the E1B-55kDa protein promotes shutoff of host cell protein synthesis, controls late viral protein synthesis and accumulation of late viral mRNAs, and possibly ensures efficient transport of viral mRNAs to the cytoplasm (Babiss and Ginsgerg, 1984; Babiss *et al.*, 1985; Bernards *et al.*, 1986; Bridge and Ketner, 1989; Cutt *et al.*, 1987; Halbert *et al.*, 1985; Leppard and Shenk, 1989; Pilder *et al.*, 1984; Samulski and Shenk, 1988; Sandler and Ketner, 1989; Yew *et al.*, 1990). E1B-55kDa mediated-transformation, in cooperation with E1A, is thought to be achieved largely through its association with and inactivation of the p53 tumour suppressor (Kao *et al.*, 1990; Sarnow *et al.*, 1982a).

Function of the p53 Tumour Suppressor

p53 is believed to function as a tumour suppressor since it is frequently inactivated in a large number of cancers (Bartek *et al.*, 1991; Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Hollstein *et al.*, 1991; Lane and Benchimol, 1990; Levine *et al.*, 1991; Mowat *et al.*, 1985). Recent experiments have suggested that p53 may function as a transcriptional regulator. It contains an acidic N-terminal region, which, when fused to a GAL4 DNA-binding domain, can activate transcription from reporter genes (Fields and Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990; Unger *et al.*, 1992). In addition, a number of specific DNA sequences are recognized by wild-type but not mutant p53 (Bargonetti *et al.*, 1991; 1992; El-Deiry *et al.*, 1992; Funk *et al.*, 1992; Kern *et al.*, 1991). In one study, p53 was shown to activate transcription of creatine phosphokinase by specifically binding a DNA element (Zambetti *et al.*, 1992). p53 can also, directly or indirectly, down-regulate transcription of various promoters including *Rb*, interleukin-6 (IL-6), the multi-drug resistance gene (MDR1), PCNA (proliferation cell nuclear antigen), and transcription factors c-*fos* and c-*jun* (Chin *et al.*, 1992; Ginsgerg *et al.*, 1991; Mercer *et al.*, 1991; Santhanam *et al.*, 1991; Shiio *et al.*, 1992). p53 itself is subject to regulation by the cellular

protein *mdm2*, which was initially isolated as a gene amplified in murine double minute chromosomes in various tumour cells (Cahilly-Snyder *et al.*, 1987; Fakharzadeh *et al.*, 1991). mdm2 binds to the transcriptional activation domain of p53 and blocks its activity (Momand *et al.*, 1992; Oliner *et al.*, 1993). E1B-55kDa was also shown to inhibit p53 transcriptional activity by binding to the N-terminal activation domain, and therefore E1B-55kDa may simply mimic inhibition by *mdm2* (Yew and Berk., 1992).

1.2.2 E1A mRNA Species and Protein Products

A single nuclear transcript is produced from the E1A region (1.3-4.5 map units) and is spliced into five different mRNAs designated 13S, 12S, 11S, 10S and 9S, according to their sedimentation coefficients (Figure 3). The four largest messages share the same reading frame, but differ in size according to the number and position of internal splices (Horwitz, 1990a; Perricaudet *et al.*, 1979; Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987). All five mRNAs are translated from the same initiation codon and possess identical N-terminal regions (Green *et al.*, 1979b; Horwitz, 1990a; Perricaudet *et al.*, 1979; Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987; Virtanen and Pettersson, 1983a). The protein products synthesized from the 13S and 12S messages, referred to as the 289R and 243R products, respectively, are the major proteins produced early in infection (Chow *et al.*, 1980; Glenn and Ricciardi, 1988). The 289R product contains an additional 46 amino acids which is not found in the 243R product (Green *et al.*, 1979b, Horwitz, 1990a). The products of the 11S and 10S mRNAs are synthesized at low levels, predominantly during the late phase of infection. Both products have a 72 residue deletion near the N-terminus, but the 10S product also has the same 46 amino acid internal deletion as in the 243R product (Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987). The 9S message differs from all the others as it is spliced into a different reading frame after amino acid 27, producing a 55R protein (Virtanen and Pettersson, 1983a).

The products of the five messages of Ad5 E1A are acidic nuclear phophoproteins and migrate as a collection of discrete bands on SDS-polyacrylamide gels, including 52kDa and 48.5kDa species from the 13S message, 50kDa and 45kDa proteins from the 12S message (Green *et al.*, 1979a; Rowe *et al.*, 1983b; Yee *et al.*, 1983), a 35kDa polypeptide from the 11S message, a 30kDa product from the 10S message (Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987), and a 6.1kDa species from the 9S message (Virtanen and Pettersson, 1983a). The multiple species observed for the 289R and 243R products occur as a result of differential phosphorylation (Richter *et al.*, 1988; Tremblay *et al.*, 1988; 1989; Tsukamoto *et al.*, 1986; Yee *et al*, 1983). Phosphorylation at serine residue 89 and neighboring serine sites was found to induce a molecular mass shift of about 5kDa (i.e. 48.5 to 52kDa and 45 to 50kDa) for both 289R and 243R products (Dumont *et al.*, 1989; 1993; Smith *et al.*, 1989).

E1a proteins are primarily localized in the nucleus, although they can also be found in the the cytoplasm (Branton *et al.*, 1984; Chatterjee and Flint, 1986; Feldman and Nevins, 1983; Rowe *et al.*, 1983a; Schmitt *et al.*, 1987; Yee *et al.*, 1983). The predominant nuclear localization signal is a pentapeptide sequence located at the extreme carboxy terminus (Lyons *et al.*, 1987), although additional sequences spanning the E1A protein may also be important (Quinlan *et al.*, 1988; Quinlan and Douglas, 1992). E1a proteins undergo rapid turnover with a half-life of 40 minutes (Branton and Rowe, 1985; Spindler and Berk, 1984).

1.2.3 Structure of the E1A Protein

Conserved regions

The 289R and 243R E1A proteins are produced from 2 exons with the 289R protein containing an additional unique 46 amino acid sequence. A number of regions are

conserved among various serotypes of adenovirus, and are referred to as conserved region 1 (CR1) spanning aa 40-80, conserved region 2 (CR2) spanning aa 121-138, and conserved region 3 (CR3) spanning the 289R unique sequence from residues 139-188 (Figure 4; Kimelman *et al.*, 1985; van Ormondt *et al.*, 1980). Numerous functions have been mapped to these regions, including induction of cellular DNA synthesis and mitosis, cellular transformation in cooperation with E1B or *Ha-ras*, transactivation of both viral and cellular genes, repression of enhancer activity, and most of these functions correlate with the formation of complexes with a variety of cellular proteins (see below).

Phosphorylation sites

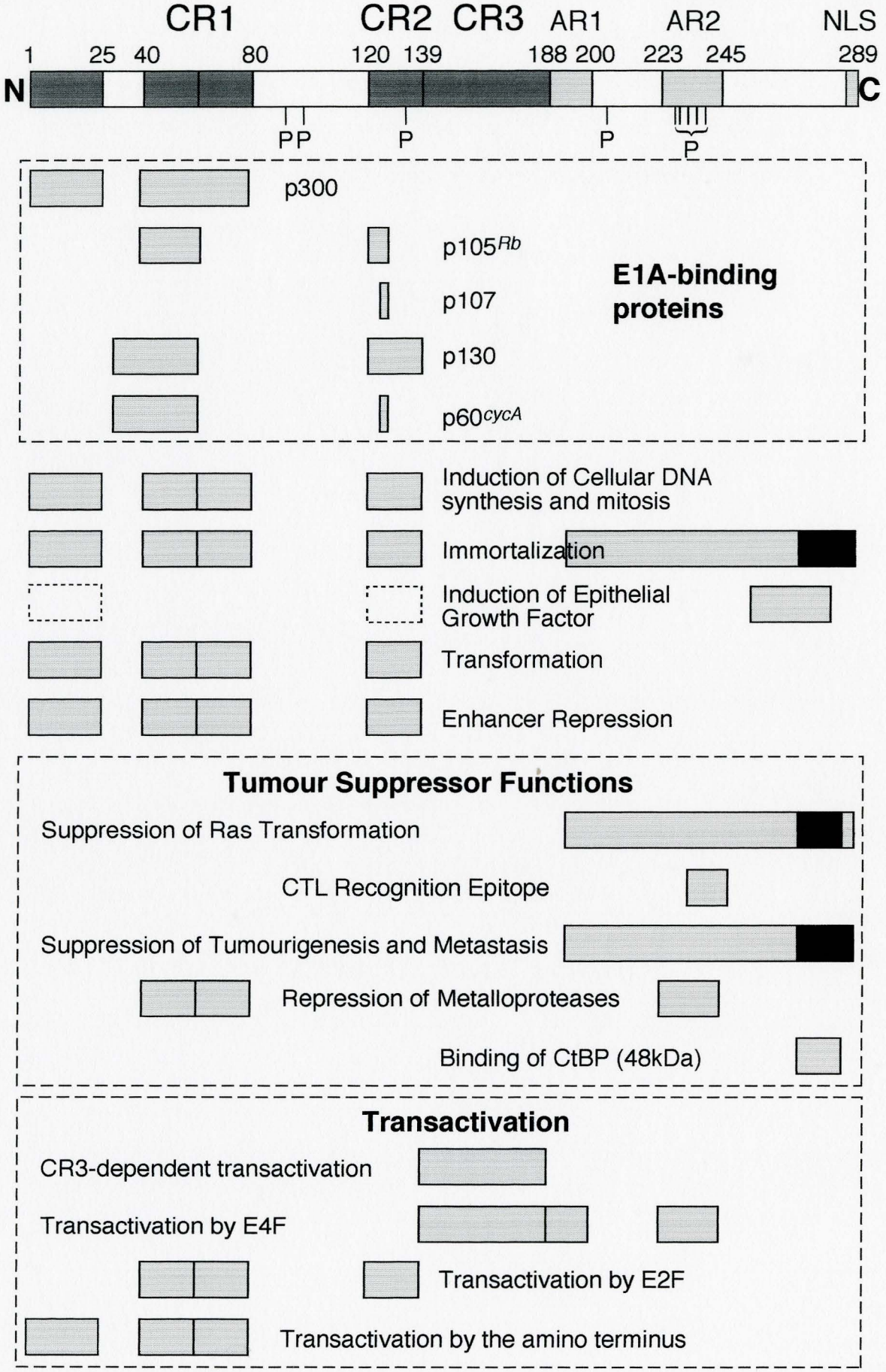
E1A proteins are phophorylated almost exclusively on serine residues (Tremblay *et al.*, 1988; Tsukamoto *et al.*, 1986). A number of sites have been mapped, of which serine 219 appears to be the major phosphorylation site (see Figure 4) (Tsukamoto *et al.*, 1986; Tremblay *et al.*, 1988). Phosphorylation also occurs at serine 89 and 96, and more specifically, phophorylation at serine 89 appears to regulate phosphorylation at 96 and perhaps at other unidentified neighboring sites, and to be responsible for a major shift in gel migration of E1A products (Dumont *et al.*, 1989; 1993; Smith *et al.*, 1989). Additional sites have been mapped to serine 132 (S.G. Whalen, D. Barbeau, H.B. Corbeil, R.C. Marcellus, and P.E. Branton, in preparation), as well as a number of sites within the carboxy terminus, serines 227, 228, 231, 234, and 237 (Tremblay *et al.*, 1988; S.G. Whalen, D. Barbeau, R.C. Marcellus, J.A. Howe, S.T. Bayley and P.E. Branton, submitted).

1.3. E1A function

1.3.1 Association with Cellular Proteins

E1A products associate with several cellular proteins, including phosphoproteins of 300, 107, and 105kDa (p300, p107, p105), and minor species which include p60,

Figure 4. Functions of the E1A products. The Ad5 289R E1A protein has been illustrated and phosphorylation sites as well as the positions of CR1, CR2, CR3, AR1 and AR2 have been indicated with amino acid numbers shown above. The region required for the numerous functions carried out by E1A are indicated.


CR1
CR2 CR3
AR1
AR2
NLS
1
25
40
80
120
139
188
200
223
245
289
N
C
P
P
P
P
P
p300
p105Rb
p107
p130
p60cycA
E1A-binding proteins
Induction of Cellular DNA synthesis and mitosis
Immortalization
Induction of Epithelial Growth Factor
Transformation
Enhancer Repression
Tumour Suppressor Functions
Suppression of Ras Transformation
CTL Recognition Epitope
Suppression of Tumourigenesis and Metastasis
Repression of Metalloproteases
Binding of CtBP (48kDa)
Transactivation
CR3-dependent transactivation
Transactivation by E4F
Transactivation by E2F
Transactivation by the amino terminus

p130 (Egan *et al.*, 1988; 1989; Giordano *et al.*, 1989; 1991a; 1991b; Harlow *et al.*, 1986; Howe *et al.*, 1990; Stein *et al.*, 1990; Svensson *et al.*, 1991; Whyte *et al.*, 1988a; 1989; Yee and Branton, 1985). The p105 protein was identified as the product of the *RB-1* retinoblastoma gene (Whyte *et al.*, 1988b). Mutations in this gene have been associated with a number of cancers, including retinoblastoma, osteosarcoma, small cell lung carcinoma, as well as others (Knudson, 1971; Weinberg, 1990). Hypophosphorylated $p105^{Rb}$ is a growth suppressor which negatively regulates entry into the S phase of the cell cycle (Buchkovich *et al.*, 1989; DeCaprio *et al.*, 1989; Goodrich *et al.*, 1991; Kim *et al.*, 1991; 1992a; 1992b; Ludlow *et al.*, 1989; 1990; Wagner and Green, 1991). If it is mutated, sequestered by E1A, or phosphorylated in response to mitogens, the constraints on growth control are released (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; 1992; Ludlow *et al.*, 1990; Mihara *et al.*, 1989; Templeton *et al.*, 1991; Templeton, 1992). Binding of $p105^{Rb}$ to E1A products requires aa 120-127 within CR2 containing the core sequence Asp^{121}-Leu-X-Cys-X-Glu (Egan *et al.*, 1988; Whyte *et al.*, 1989; Dyson *et al.*, 1989a; 1989b; 1990; Stein *et al.*, 1990; Svensson *et al.*, 1991). However, binding is also affected by mutations spanning aa 36-60 within CR1 (Egan *et al.*, 1988; Stein *et al.*, 1990; Svensson *et al.*, 1991; Whyte *et al.*, 1989) and it has been suggested that the residues Glu^{39}-X-X-X-Leu-X-Glu/Asp-Leu-X-(X)-Leu may play a major role (Dyson *et al.*, 1990; 1992a; 1992b).

Two other cellular proteins, p107 and p130 have recently been cloned and shown to be similar to $p105^{Rb}$ in two regions, termed the A and B box regions (or "pocket binding" domain), which are involved in interacting with E1A proteins (Ewen *et al.*, 1991; Hu *et al.*, 1990; 1991; Kaelin *et al.*, 1990; Whyte, personal communication). $p105^{Rb}$, p107 and p130 are commonly referred to as the $p105^{Rb}$ family of proteins. The A and B boxes of $p105^{Rb}$ are also the regions that are frequently mutated in numerous cancer cells (Hu *et al.*, 1990; Huang *et al.*, 1990). p107 relies largely on sequences from residues 124-127 on the E1A

molecule for binding, whereas p130 requires residues spanning amino acid 30-60 as well as the entire CR2 region (aa 121-138; refer to Figure 4) (Egan *et al.*, 1988; 1989; Giordano *et al.*, 1991b; Stein *et al.*, 1990; Svensson *et al.*, 1991; Whyte *et al.*, 1989).

The p60 protein has been identified as cyclin A and binds to sequences 124-127 and to a lesser extent residues 30-60 (Giordano *et al.*, 1989; 1991b; Howe and Bayley, 1992; Pines and Hunter, 1990). A number of different cyclin molecules exist and are thought to regulate specific points of the cell cycle, as they appear during distinct periods and are then rapidly degraded (reviewed in Hunter and Pines, 1991; Pines, 1991). Cyclins are normally associated with *cdc2*-like (cell division cycle-2) protein kinases and are thought to be regulatory subunits of these kinases (Pines and Hunter, 1989; reviewed in Hunter and Pines, 1991; Pines, 1991). There also appears to be a family of cell cycle dependent kinases (cdks including *cdc2*) which are active at different phases of the cell cycle and elicit their effects by phosphorylating specific substrates (Meyerson *et al.*, 1992). $p60^{cycA}$ can associate with two cdks, $p34^{cdc2}$ and $p33^{cdk2}$, however, in E1A complexes, only the latter type is found (Giordano *et al.*, 1991a; Herrmann *et al.*, 1991; Hunter and Pines, 1991; Kleinberger and Shenk, 1991; Pagano *et al.*, 1992b; Pines and Hunter, 1990; Tsai *et al.*, 1991). It has been reported recently that $p60^{cycA}$ does not bind to E1A directly but rather interacts indirectly through the p107 protein. The binding site on p107 for $p60^{cycA}$ has been localized to the spacer region between the A and B boxes (Ewen *et al.*, 1992; Faha *et al.*, 1992; Howe and Bayley, 1992).

A p300 E1A-binding protein has also been characterized and found to bind to E1A within aa 1-25 and 36-76 (Egan *et al.*, 1988; Stein *et al.*, 1990; Svensson *et al.*, 1991; Whyte *et al.*, 1989). The identity of p300 has not been determined, however it has been shown to exhibit sequence-specific DNA binding activity (to NF-κB sites) and is differentially

phosphorylated throughout the cell cycle (Rikitake and Moran, 1992; Yaciuk and Moran, 1991).

Numerous E1A functions (which will be described below) map to the same regions involved in the binding of these cellular proteins, and thus complex formation is believed to underlie much of the biological activity of E1A proteins.

1.3.2 Induction of Cellular DNA Synthesis and Mitosis

Adenoviruses usually infect non-dividing cells, and thus need to induce the cellular DNA synthetic machinery in order to replicate. Both 243R and 289R products can induce DNA synthesis in quiescent, non-transformed human cells (Kaczmarek *et al.*, 1986; Spindler *et al.*, 1985) and primary epithelial rat cells (Moran and Zerler, 1988; Nakajima *et al.*, 1987; Quinlan and Grodzicker, 1987; Smith and Ziff, 1988; Zerler *et al.*, 1987). Studies with the 243R E1A protein have shown that induction of cellular DNA synthesis and cell cycle progression in non-replicating cells can occur both in the presence and absence of serum, indicating that E1A bypasses the need for growth factors in serum. Experiments conducted by Quinlan's group suggest that 243R may potentiate this effect by inducing a cellular growth factor (see "immortalization" section; Bellett *et al.*, 1985; Quinlan and Grodzicker, 1987; Quinlan *et al.*, 1988; Quinlan and Douglas, 1992; Spindler *et al.*, 1985; Stabel *et al.*, 1985).

An extensive series of studies demonstrated that induction of cellular DNA synthesis and mitosis have different requirements (Howe *et al.*, 1990; Howe and Bayley; 1992). Induction of DNA synthesis is completely unaffected by individual mutations in the N-terminal, CR1, or CR2 domains, however, if two regions are mutated simultaneously, such as the N-terminus and CR2 and/or CR1 and CR2, which would affect the binding of both p300 and the $p105^{Rb}$ protein family, cells do not enter S phase (Figure 4; Howe *et al.*,

1990; Lillie *et al.*, 1987; Moran and Zerler, 1988; Quinlan and Douglas, 1992; Quinlan *et al.*, 1988; Smith and Ziff, 1988; Subramanian *et al.*, 1988; Zerler *et al.*, 1987). In several cases when the deletion was fairly extensive in the CR1 region (at least 25 residues), such that it affected binding of both $p105^{Rb}$ and p300, induction of DNA synthesis was not observed (Howe *et al.*, 1990; Lillie *et al.*, 1987; Quinlan and Douglas, 1992; Quinlan *et al.*, 1988). These results suggested that E1A association with either p300 or the $p105^{Rb}$ protein family is sufficient for induction of DNA synthesis (Howe *et al.*, 1990). One discrepancy is that Quinlan's group also found that a region between aa 119-127 affected DNA synthesis (Quinlan and Douglas, 1992; Quinlan *et al.*, 1988). This larger deletion may encompass the binding site for a protein yet to be discovered and which is important for induction of DNA synthesis. For induction of mitosis or cell proliferation, however, if any of these regions are mutated, cells do not progress through the M phase of the cell cycle, indicating that association with both p300 and $p105^{Rb}$ protein family is indispensible (Bellett *et al.*, 1985; 1989; Howe and Bayley, 1992; Moran and Zerler, 1988; Quinlan and Douglas, 1992; Zerler *et al.*, 1987). These results suggested that the controls for completion of the cell cycle are more compex than the controls for the induction of cellular DNA synthesis.

1.3.3 Immortalization, Induction of Epithelial Cell Growth Factor and Transformation

E1A can immortalize and partially transform primary rodent cells, providing indefinite growth in culture (Dijkema *et al.*, 1979; Houweling *et al.*, 1980). However, cooperation with E1B or an activatd T24 *ras* gene (c-HAR-*ras*-1) is required for full transformation (Franza *et al.*, 1986; Ruley, 1983; reviewed in Branton *et al.*,1985 and Graham, 1984). In the case of E1B, either E1B-19kDa or E1B-55kDa alone can cooperate with E1A to transform primary rodent cells, but in the presence of both E1B products, an increase in transforming efficiency was observed, suggesting that these two E1B proteins function in

independent but additive pathways (Edbauer *et al.*, 1988; McLorie *et al.*, 1991; Telling and Williams, 1993; White and Cipriani, 1990; Yew *et al.*, 1990; Zhang *et al.*, 1992). Unlike *ras*, E1B alone is unable to transform established cell lines (Solnick and Anderson, 1982; Ruley, 1983; van del Elsen *et al.*, 1982; 1983; reviewed in Ruley, 1990). Since expression of E1B is regulated by E1A (see below), most transformation assays have been conducted with *ras*, presumably to measure more directly the sequences in E1A involved in transformation (Bautista *et al.*, 1991). Most of these studies were conducted with the 243R protein since it appears to be more efficient than the 289R protein in immortalization and cooperation with other oncogenes in primary rat epithelial cells (Bautista *et al.*, 1991; Cone *et al.*, 1988; Kuppuswamy and Chinnadurai, 1988).

Cellular immortalization requires the same domains of E1A products as induction of DNA synthesis and mitosis, but in addition requires carboxy terminal residues spanning aa 193-289 (Boyd *et al.*, 1993; Quinlan and Douglas, 1992; Quinlan *et al.*, 1988; Subramanian *et al.*, 1989). A critical region has been located between aa 271-289 (Boyd *et al.*, 1993). The proliferative response induced in the absence of the N- and/or C-terminus is only transient and does not give rise to sustained growth characteristic of immortalized cells (Quinlan and Douglas, 1992). Sequences spanning aa 254-282 are required for the production of an epithelial cell growth factor which may play a role in immortalization (Quinlan and Grodzicker, 1986; 1987; Quinlan and Douglas, 1992; Quinlan *et al.*, 1988; Subramanian *et al.*, 1988). Additionally, N-terminal and CR2 sequences are involved in growth factor induction, however, it is not clear whether these sequences affect growth factor induction directly, or whether prior expression of an N-terminal activity is necessary to elicit growth factor induction (Quinlan *et al.*, 1988; Quinlan and Douglas, 1992; Subramanian *et al.*, 1988).

Expression of three critical E1A exon 1 regions, the N-terminus, CR1 and CR2, is both necessary and sufficient for transformation of primary epithelial cells in cooperation with Ha-*ras* (Jelsma *et al.*, 1989; Kuppuswamy and Chinnadurai, 1987; Lillie *et al.*, 1986; 1987; Moran *et al.*, 1986; Schneider *et al.*, 1987; Stein *et al.*, 1990; Subramanian *et al.*, 1988; Svensson *et al.*, 1991; Whyte *et al.*, 1988a). Transformation correlates with complex formation with cellular proteins, and E1A mutants that fail to bind either p300 or $p105^{Rb}$ or p107 are transformation defective (Egan *et al.*, 1988; 1989; Jelsma *et al.*, 1989; Whyte *et al.*, 1988a; 1989). Exon 2, although not necessary for transformation, plays a modulatory role (Boyd *et al.*, 1993; Douglas *et al.*, 1991; Subramanian *et al.*, 1989), since a region between aa 193-289 including a critical aa 271-284 region, suppresses *ras*-mediated transformation (Boyd *et al.*, 1993; Douglas *et al.*, 1991). Similar negative regulatory effects by exon 2 were also observed with the 289R protein (Subramanian *et al.*, 1989). Enhanced cooperation of E1A exon 2 deletion mutants with E1B has not been detected, and appears to be specific to the *ras* pathway (Subramanian *et al.*, 1989).

1.3.4 Suppression of Tumourigenesis and Metastasis

Loss of cell growth control as mediated by the region of E1A proteins involved in immortalization and transformation does not necessarily lead to the creation of metastatic cells (reviewed in Liotta *et al.*, 1991). Additional events are required to permit metastasis, including escape from immunological detection and invasion of secondary target organs . Cells transformed by Ad12 induce tumours in immunocompetent syngeneic rats whereas Ad2 or Ad5 (group C) are non-tumourigenic (Bernards *et al.*, 1983). In athymic animals, however, both Ad5- and Ad12-transformed cells are tumourigenic, suggesting that a component of T cells is required to mediate the difference in oncogenicity between Ad5 and Ad12 in immunocompetent hosts. In fact, cells transformed by Ad12 express reduced levels of MHC I (major histocompatibility complex class I) antigens (Eager *et al.*, 1985; Schrier *et al.*, 1983), resulting in decreased recognition and lysis by CTLs (cytotoxic T

lymphocytes) and therefore increased tumour formation in immunocompetent animals (Bernards *et al.*, 1983; Vasavada *et al.*, 1986). Other reports link the difference in oncogenicity between the group A and C adenoviruses to differences in resistance to natural killer (NK) cells, with Ad12 transformed cells being more resistant to NK lysis (Cook *et al.*, 1987; Kenyon and Raska, 1986; Sawada *et al.*, 1985). Both suppression of MHC I and NK lysis by Ad12 has been shown to require the E1A region (Bernards *et al.*, 1983; Cook *et al.*, 1987; Kenyon and Raska, 1986; Sawada *et al.*, 1985; Schrier *et al.*, 1983).

In Ad5, the C-terminal domain of E1A is important for induction of cytotoxic T lymphocytes (CTLs), since the recognition epitope, at least in the case of one mouse haplotype, has been mapped to residues 232-247 (Kast *et al.*, 1989). In addition, E1A exon 2 has been shown to be involved in tumour transplantation (TSTA) immunity, although the precise region has not been delineated (Kast *et al.*, 1989; Urbanelli *et al.*, 1989).

Deletion of sequential groups of E1A C-terminal residues between aa 193-289 of Ad5 E1A, resulted in not only an increased transformation efficiency in cooperation with *ras* (as described earlier), but also increased tumourigenesis and metastasis as evidenced by tumour formation in syngeneic rats and athymic mice at the injection site, and tumour formation in lungs of athymic mice (indicative of metastasis), respectively (Boyd *et al.*, 1993; Subramanian *et al.*, 1989). The critical regions for suppression of tumourigenesis and metastasis map to residues 271-289 (Figure 4; Boyd *et al.*, 1993). The importance of the expression of matrix metalloproteases, which mediate the dissolution of basement membranes surrounding organs, has been shown for the invasive capacity of tumour cells (reviewed in Liotta *et al.*, 1991). However, it has been shown that during adenovirus infection, a number of matrix metalloproteases (stomelysin , collagenase type I and IV) are repressed and the regions of E1A responsible have been mapped to CR1 and C-terminal

residues 223-245 (see repression section below; Engel *et al.*, 1992; Frisch *et al.*, 1990; Linder *et al.*, 1992; Offringa *et al.*, 1990; van Dam *et al.*, 1989). Using a reconstituted basal membrane *in vitro* invasiveness assay, with tumour cells transfected with E1A, repression of stromelysin and the 92kDa type IV collagenase and suppression of *in vitro* invasiveness comapped to residues 223-245 (Linder *et al.*, 1992). When parental non-transfected tumour cells were tested in the *in vitro* invasiveness assay with antibodies to collagenase type IV, these lost their invasive capacity, further indicating a relationship between repression of secreted proteases and repression of metastasis (Frisch *et al.*, 1990).

A 48kDa cellular phosphoprotein, CtBP, which binds to the C-terminal region involved in suppressing tumourigenesis and metastasis (aa 271-284; Boyd *et al.*, 1993) may be more directly involved in suppression. In addition, E1A can activate the expression of the cellular gene, *NM23*, which is a suppressor of metastatic progression (Steeg *et al.*, 1988), but it has not been determined whether C-terminal sequences are required for this function or if NM23 plays a role in suppression of tumourigenesis and metastasis.

1.3.5 Transactivation

Adenovirus E1A can mediate the transcriptional activation of early viral genes and certain cellular genes (Berk *et al.*, 1979; Jones and Shenk, 1979a; Nevins, 1981; 1982). The CR3 region present in the 289R protein has classically been thought to be responsible for transactivation of early viral genes (Lillie *et al.*, 1986; Jelsma *et al.*, 1988; Moran *et al.*, 1986; Schneider *et al.*, 1987; reviewed in Flint and Shenk, 1990). The ability of a synthetic peptide comprising this region to independently transactivate an early viral promoter demonstrated that the CR3 domain was sufficient (Lillie *et al.*, 1987). However, it has become apparent in the last few years that the 243R protein can also mediate transcriptional activation, indicating that the entire E1A molecule may contain multiple activation functions (Kaddurah-Daouk *et al.*, 1990; Simon *et al.*, 1987; Zerler *et al.*, 1987). E1A is thought to

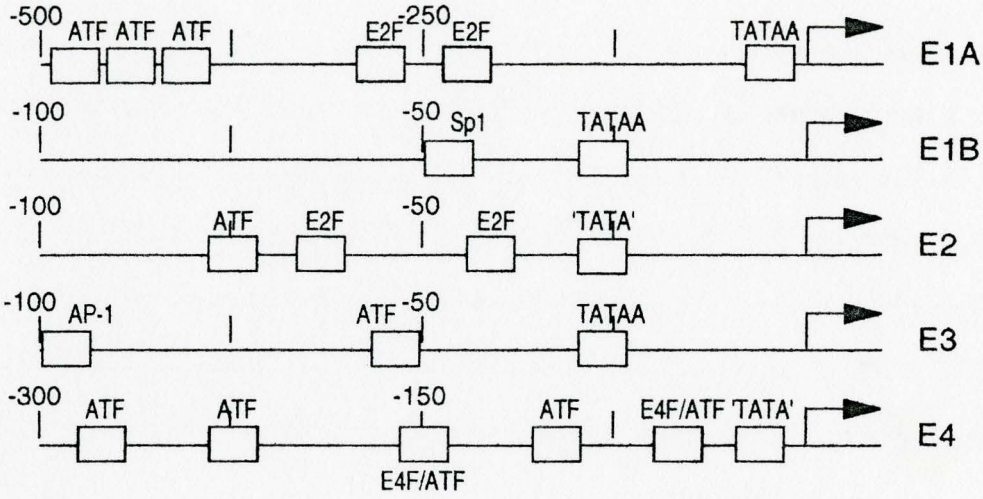
mediate transcriptional activation through interaction with, or modification of, cellular transcription factors because it cannot bind to DNA in a sequence-specific manner (Chatterjee *et al.*, 1988; Ferguson *et al.*, 1985; Ko *et al.*, 1986). In addition, there is no single DNA responsive element that is common to all the adenovirus-inducible promoters (Pei and Berk, 1989; reviewed in Nevins, 1989).

As mentioned earlier, E1A activates early viral genes in the following sequence; E2, E3, E4, and then E1B (Glenn and Ricciardi, 1988), and can also modulate activity from its own promoter (reviewed in Berk, 1986). The E3 and E4 promoters are exclusively transactivated by the 289R product of E1A (Kraus *et al.*, 1992), implicating the CR3 region of the 289R in the transactivation mechanism, whereas the E2 promoter appears to be activated by both 243R and 289R E1A products (see E2F transactivation section). Most of the early viral adenovirus promoters, including E1A, E2, E3 and E4, contain ATF/CRE sites (Activating transcription factor, also called cAMP-responsive element; Figure 5a) (Hardy and Shenk, 1988; Nevins, 1989). All of these also have a TATA box, however, the importance of the TATA box in transactivation appears to differ according to the promoter (Nevins, 1989). The *TATAA* sequence has been shown to mediate E1A control of the E1B, E3 and *hsp70* promoters (Nevins, 1989). E1B also contains an Sp1 site, however, the importance of this element in conferring E1A responsiveness has not been determined (Nevins, 1989; Wu *et al.*, 1987). The E4 promoter appears to be activated by two different mechanims involving ATF and E4F transcription factors (see below under Transactivation by E4F). The E3 region has an AP-1 site in addition to an ATF site and a TATA box as upstream promoter elements, and all appear to confer E1A inducibility (reviewed in Nevins, 1989). The E2 promoter has E2F sites in addition to an ATF site, both of which are important for transactivation (Nevins, 1989). The E1A promoter contains a pair of E2F sites, in addition to multiple ATF sites, and a TATA box (Hardy and Shenk, 1988; Hearing and Shenk, 1983; Mudryj *et al.*, 1990; Osborne and Berk, 1983; Osborne *et al.*, 1982).

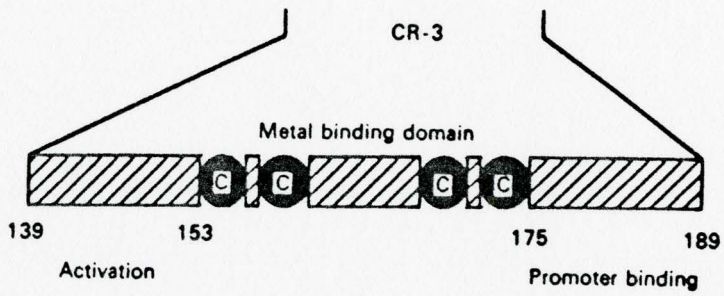
Figure 5. Transactivation by adenovirus E1A.

Figure 5a shows the different promoter elements and their relative positions within the various adenovirus early promoters (adaptation of Nevins, 1989). Figure 5b is an illustration of CR3, depicting the activation, metal binding and promoter binding domains with the amino acids shown below. The four cysteine residues within the metal binding domain have been indicated (taken from Boulanger and Blair, 1991). Figure 5c describes a model for transcriptional activation by adenovirus E1A. E1A is thought to function by interacting simultaneously with ATF-2 and TFIID providing a link between upstream regulatory elements and the basal transcription machinery (taken from Lee *et al.*, 1991).

A.



B.

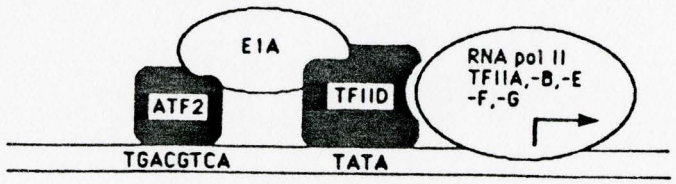


Potential Zn-finger in box 3

154 174

Cys-Xaa$_2$-Cys-Xaa$_{13}$-Cys-Xaa$_2$-Cys

C.



CR3 dependent transactivation

Two functional domains have been defined for cellular transcriptional activators: a DNA-binding region which directs activators to target promoters; and an acidic activating region which enables the bound activator to stimulate transcription by contacting elements of the transcriptional machinery (Ptashne, 1988; Mitchell and Tjian, 1989). For E1A, both domains have been mapped within CR3 (Lillie and Green, 1989).The promoter binding region has been mapped towards the C-terminal end of CR3 between residues 175 and 189 (refer to Figure 5b). Since E1A has no or very low affinity for DNA (Chatterjee *et al.*, 1988; Ferguson *et al.*, 1985; Ko *et al.*, 1986), it seems likely that this region recognizes a DNA-bound protein. A potential target protein has been identified as ATF-2 (also known as CREB2, CRE-BP1; Hai *et al.*, 1989; Maekawa *et al.*, 1989), a member of the ATF/CREB family of transcription factors which binds to the ATF/CREB homology region. The region of E1A proteins involved in this interaction appears to map within aa 183-188 at the end of CR3 (Liu and Green, 1990; Maguire *et al.*, 1991; Webster and Ricciardi, 1991). The ATF/CREB family of proteins is quite extensive and 10 different cDNAs have been described encoding proteins containing a relatively basic region followed by a leucine zipper dimerization domain (bZIP class of transcription factors). An additional member of the ATF/CREB family, ATFa, can also bind to E1A and specifically activate transcription from E2 and E4 promoters (Chatton *et al.*, 1993; Gaire *et al.*, 1990). Although the precise regions of contact on E1A products have not been delineated, CR3 appears to be required, in addition to other regions (Chatton *et al.*, 1993). Other members of the ATF family, CREB and ATF1, are not responsive to E1A proteins and are therefore not likely to be invoved in the E1A-mediated activation of viral early genes (Flint and Jones, 1991).

The N-terminal portion of CR3 (aa 139 and 153) harbors the activation domain (Lillie and Green, 1989). Recent studies have shown the direct interaction of E1A molecules with TFIID, the transcription factor complex that binds to the TATA box and consists of TBP

(TATA-binding protein) and a number of TAFs (TBP-associated factors). The area of contact on E1A proteins has been localized to the activation domain within CR3 (Dynlacht *et al.*, 1991; Horikoshi *et al.*, 1989; 1991; Lee *et al.*, 1991). The central portion of CR3 (aa 154-174) contains a zinc binding domain with four conserved cysteine residues (C154, C157, C171, and C174; Culp *et al.*, 1988) which are required for CR3-mediated transactivation and may be involved in binding TFIID (Martin *et al.*, 1990; Webster and Ricciardi, 1991). Metal binding domains (zinc fingers) are motifs found in many transcription factors, and are believed to promote DNA binding or increase the stability of a transcription complex on DNA (Culp *et al.*, 1988; Mitchell and Tjian, 1989). Extensive mutational analyses have indicated that mutations affecting critical residues within any of the three CR3 domains, result in a severely reduced capacity for transactivation (Culp *et al.*, 1988; Glenn and Ricciardi, 1987; Green *et al.*, 1988; Jelsma *et al.*, 1988; Webster and Ricciardi, 1991).

A number of activators, including GCN4, GAL4, VP16, have been described that all require acidic residues within the activating domain (reviewed in Mitchell and Tjian, 1989). Following an acidic region at the carboxy terminal end of the CR2 region of E1A, the activation domain of CR3 contains four acidic residues (E140, E141, D145 and E148) however, with the exception of D145, mutation of these amino acids does not appear to affect transactivation (Martin *et al.*, 1990; Webster and Ricciardi, 1991). Thus E1A proteins may not function exactly like typical acidic transcriptional activators such as HSV-1 VP16 (Triezenberg *et al.*, 1988), GAL4 (Ma and Ptashne, 1987), and GCN4 (Hope and Struhl, 1986). Other activators have been described which do not contain acidic activating domains, including Sp1 (a transcription factor that recognizes a 'GC' box). E1A may function in a fashion analogous to these non-acidic transactivators (Ptashne and Gann, 1990). A common mechanism for transactivation is emerging which suggests that activators function by contacting TFIID as well as other components of the transcriptional

machinery (Berger *et al.*, 1990; Stringer *et al.*, 1990). For example, VP16 has been shown to bind TFIID (Stringer *et al.*, 1990; Ingles *et al.*, 1991) as well as TFIIB (Lin and Green, 1991), both of which are important for VP16-mediated transactivation. Although TFIID binds the activating region of E1A, this interaction is not sufficient since a mutation affecting residue 145 (D -> A) remains capable of binding to TFIID but is transactivation defective (Lee *et al.*, 1991; Martin *et al.*, 1990). These findings suggested that additional processes are required.

Figure 5c shows a model which describes the current view on E1A-mediated transactivation (adaptation of Lee *et al.*, 1991). E1A via CR3 is envisioned to function at the promoter by bridging ATF-2 and TFIID proteins. Thus E1A proteins would function as adaptors linking TFIID and specific DNA-binding transcription factors (reviewed by Lewin, 1990). Additional components which have not yet been isolated may also be required. This mode of activation most likely applies for the regulation of the E1A, E2, E3 and E4 viral promoters, all of which contain ATF sites (Hardy and Shenk, 1988; Nevins, 1989). In addition, 243R may transactivate through a similar mechanism, since physical interaction between E1A and TFIID, and E1A and ATF-2 (and ATFa) has also been detected with the 243R product, although at much lower affinity than with the larger 289R product (Chatton *et al.*, 1993; Lee *et al.*, 1991; Maguire *et al.*, 1991). Thus E1A proteins may contact these transcription factors either directly or indirectly through sites outside of the CR3 region.

Activation of E4 and E1A promoters, which contain 4-5 CRE/ATF-like sequences, has been described involving the synergistic action of both E1A and cAMP (Engel *et al.*, 1988; Hardy and Shenk, 1988). The CRE/ATF binding sites can respond to transcriptional activation by both cAMP and E1A, however, members of the CREB/ATF protein family show differential responses to these two activators. CREB1 (also known as CREB;

Gonzales *et al.*, 1989) is strongly activated by cAMP, but weakly by E1A (Flint and Jones, 1991) and does not bind to E1A (Chatton *et al.*, 1993). ATF-2 , in contrast, is not activated by cAMP, but is strongly activated by E1A (Flint and Jones, 1991). ATF1 (Hai *et al.*, 1989) is weakly activated by cAMP and not activated by E1A (Flint and Jones, 1991). Experiments were performed using a mouse lymphoma cell line (S49) which is deficient for adenylate cyclase but responds to exogenously added cAMP (Horibata and Harris, 1970). It was found that cAMP in the presence of E1A greatly induces the mRNA levels of c-Fos and JunB, which are components of the AP-1 transcription factor, leading to a tremendous increase in AP-1 activity (Müller *et al.*, 1989). As with the ATF protein family, Jun and Fos proteins also contain a bZIP motif, with leucine repeats mediating dimerization and a basic region allowing DNA binding (Gentz *et al.*, 1989; Landschulz *et al.*, 1989; Turner and Tjian, 1989). Although the ATF protein family is immunologically related to the AP-1 family of transcription factors (Hai *et al.*, 1988), they do not share significant similarity outside of the basic leucine zipper region (Hai *et al.*, 1989). The CRE element which binds ATF/CREB proteins differs by only one nucleotide from the TRE [TPA (12-O-tetradecanoyl phorbol 13-acetate)-responsive element] recognized by AP-1. It has been shown that AP-1 can bind to CRE sites, although at lower efficiency (Hai and Curran, 1991), and can transcriptionally activate CRE-containing promoters (Hoeffler *et al.*, 1989; Sassone-Corsi *et al.*, 1990), which may include E4 and E1A (Engel *et al.*, 1988; Müller *et al.*, 1989). The synergistic response observed with cAMP and E1A may originate from the activation of CREB and ATF-2 by cAMP and E1A, respectively, in addition to AP-1 (Flint and Jones, 1991). AP-1 could function in a mechanism analagous to ATF-2 (as described in the model above) as components of AP-1 transcription factor have been shown to bind E1A (Maguire *et al.*, 1991).

Transactivation by E4F

The E4 promoter of adenovirus appears to be transactivated by alternate mechanisms involving two different transcription factors, ATF-2 and E4F (Jones and Lee, 1991; Liu and Green, 1990; Rooney *et al.*, 1990). The E4F transcription factor consists of a single polypeptide of 50K (Raychaudhuri *et al.*, 1989) which is distinct from the ATF family as it does not have the bZIP motif (Rooney and Nevins, unpublished results) and binds only to a subset of ATF elements. E4F does not bind to ATF sites present in E2 and E3 promoters, but does to two of the five ATF sites within the E4 promoter (Raychaudhuri *et al.*, 1987; Rooney *et al.*, 1990). The E4F DNA sequence (5'-TGACGTAAC-3') overlaps the core sequence recognized by ATF (5'-TGACGT-3') plus an additional three nucleotides . In contrast to ATF, which requires the E1A CR3 region only, E1A transactivation via E4F is also dependent upon either auxiliary regions 1 (AR1) or 2 (AR2) within exon 2 (Bondesson *et al.*, 1992). The acidic AR1 (aa 189-200) consists of alternating glutamic and proline residues whereas AR2 (aa 223-245) contains four acidic residues plus several potential serine and threonine phosphorylation sites. It has been postulated that AR1 and AR2 could represent negatively charged surfaces that mediate protein-protein interactions involved in transcriptional activation, as found in other systems (reviewed in Mitchell and Tjian, 1989 and Ptashne and Gann, 1990). The precise mechanism by which E1A activates E4F is not known, however, increased E4F phosphorylation and a concommitant increase in DNA binding activity occurs following adenovirus infection (Raychaudhuri *et al.*, 1987; 1989). Results generated from several labs are somewhat contradictory as to the relative importance of ATF-2 and E4F in E4 transactivation (Jones and Lee, 1991; Rooney *et al.*, 1990). Bondesson *et al.* (1992), showed that although both pathways are active in HeLa cells, E4 transactivation seemed to be mediated primarily by E4F. The predominance of one transcription pathway over another may be dependent on the cell type or the state of growth of cells.

Transactivation by E2F

The E2 promoter of adenovirus can be regulated by the E2F factor (Kovesdi *et al.*, 1986), however, it can also be activated by E1A via the ATFa factor (Chatton *et al.*, 1993). It has been shown that an E2-CAT promoter construct can be equally activated by 243R or 289R products alone. With the 243R product, mutation of CR1 blocks E2F-mediated transactivation whereas with the 289R product transactivation occurs but at half the wild-type efficiency (Kraus *et al.*, 1992). These results imply that the 289R E1A product can activate E2 transcription in a number of ways.

Two E2F sites (TTTCGCG) are located both in the E1A and E2 promoters (Mudryj *et al.*, 1990). Transcription factor E2F complexes with a 19kDa product of the E4 gene (E4 ORF 6/7) and binds to E2 promoter sequences in a cooperative fashion to generate a stable complex which stimulates E2 transcription (Hardy *et al.*, 1989; Hardy and Shenk, 1989; Huang and Hearing, 1989; Marton *et al.*, 1990; Neill *et al.*, 1990; Neill and Nevins, 1991; Raychaudhuri *et al.*, 1990; Reichel *et al.*, 1989). E2F has been shown, in a number of cell types, to be present in complexes with other cellular proteins which prevented the association with E4-19kDa (Bagchi *et al*, 1990). The 243R product of E1A can disrupt these complexes, releasing free E2F which can then interact with E4-19kDa (Bagchi *et al.*, 1990). p105^{Rb} is one of the cellular proteins found to complex with E2F (Bagchi *et al.*, 1991; Bandara and LaThangue, 1991; Bandara *et al.*, 1991; Cao *et al.*, 1992; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991; Pagano *et al.*, 1992a; Shirodkar *et al.*, 1992). p107-p60^{cycA}-p33^{cdk2} complexes have also been found in association with E2F (Bandara *et al.*, 1991; Cao *et al.*, 1992; Devoto *et al.*, 1992; Mudryj *et al.*, 1991). Dissociation of these complexes and transactivation of E2F-dependent promoters by E1A proteins are dependent both on CR1 and CR2 (Bagchi *et al.*, 1990; Raychaudhuri *et al.*, 1991) which are the same regions required for direct physical interaction with p105^{Rb} and p107-p60^{cycA}-p33^{cdk2} (Egan *et al.*, 1988; 1989; Whyte *et al.*, 1988b; 1989, Giordano *et al.*, 1991b; Howe *et al.*,

1990; Stein *et al.*, 1990; Svensson *et al.*, 1991). Phosphorylation of the E2F factor, which occurs in the presence of E1A, has also been found to correlate with increased activation of the viral E2 promoter (Bagchi *et al.*, 1989; Reichel *et al.*, 1988).

In addition to transactivating the E2 promoter, cellular targets for activation by E2F are becoming known and include c-*myc*, c-*myb*, *cdc2*, N-*myc* and a number of DNA synthetic enzymes (reviewed in Nevins, 1992). All of these gene products are involved in control of entry into S phase , and will be discussed in greater detail below (see "Transcriptional activation as the mechanism of E1A-mediated transformation").

Transactivation mediated by the amino terminus

It has been shown that the 243R product can activate transcription via E2F. There are, however, other cellular promoters that are activated by the 243R product which do not contain E2F sites, including *hsp70* (heat shock protein 70), PCNA and c-*jun* (Draetta *et al.*, 1988; Fahnestock and Lewis, 1989; Jelsma *et al.*, 1989; Kaddurah-Daouk *et al.*, 1990; Simon *et al.*, 1987; van Dam *et al*, 1990; Zerler *et al.*, 1987). Activation of *hsp70* requires the *TATAA* sequence and both the N-terminus and CR 1 of E1A proteins (Kraus *et al.*, 1992) which are regions required for binding of p300. Thus p300 may play a role in transactivation, especially since it has been found to associate directly or indirectly with TBP (Abraham *et al.*, 1993). An additional element within the *hsp70* promoter, CAAT, is involved in the binding of CBF (CAAT binding factor) and has also been shown to be important for E1A responsiveness (Lum *et al.*, 1992). *In vitro* binding studies have shown that both the 243R and 289R products can interact with CBF, although 289R binds much more efficiently (Lum *et al.*, 1992). E1A proteins are thought to function as co-activators linking CBF and TFIID in a mechanism analagous to that described for ATF-containing promoters (refer to Figure 5c). In support of this model, 243R binding to TFIID has also been detected , although with a lower affinity than with 289R (Lee *et al.*, 1991).

PCNA, a replication factor for DNA polymerase δ, also referred to as cyclin, is important for proliferating cells. PCNA expression is activated by 243R and by 289R plus E1B-19kDa (Jelsma *et al.*, 1989; Morris and Mathews, 1989; 1991; Zerler *et al.*, 1987). An ATF site in the PCNA promoter has been shown to be E1A responsive (Morris and Mathews, 1991). Although PCNA is a TATA-less promoter, the mechanism of activation by E1A may still be through cooperation of ATF-2 with TFIID as TBP is known to be important for transcription of TATA-less polymerase II promoters (Pugh and Tjian, 1991). Additional elements appear to be required (Labrie *et al.*, 1993) and mutations in the N-terminus, CR1 or CR2 of E1A partially affect PCNA transactivation but do not abolish the response (Jelsma *et al.*, 1989; Kannabarin *et al.*, 1993).

Individual components of the AP-1 transcription factor, c-Fos and c-Jun, can be activated by the both 243R and 289R E1A products (deGroot *et al.*, 1991; Müller *et al.*, 1989; Engel *et al.*, 1991; Kitabayashi *et al.*, 1991). Generally, c-*jun* appears to be activated by E1A in all cell types tested (Kitabayashi *et al.*, 1991; deGroot *et al.*, 1991; van Dam *et al.*, 1990), whereas c-*fos* is activated only in certain cell types in the presence of mitogen (deGroot *et al.*, 1991; Müller *et al.*, 1989). The N-terminus and CR1 of E1A are required for the activation of c-*fos* transcription in mouse S49 cells in response to cAMP and 243R (Gedrich *et al.*, 1992). The mechanism of activation may be analogous to that described in Figure 5c as the c-*fos* promoter contains an ATF site as well as a *TATAA* sequence (Frisch *et al.*, 1987; Simon *et al.*, 1990). CR1 is also required for induction of the c-*jun* promoter, whereas deletion of CR2 or CR3 does not affect c-*jun* activation (van Dam *et al*, 1990). It is thought that E1A acts by modifying existing AP-1 complexes which mediate the initial transcriptional activation of c-*jun* for example, through the TRE element, followed by a positive feedback mechanism (Kitabayashi *et al.*, 1991; Müller *et al.*, 1989).

In summary, E1A appears to activate transcription through a number of mechanisms which include post-translational modification, direct association, and increased synthesis of transcription factors. Modification can be achieved through phosphorylation of transcription factors such as E2F and E4F (Bagchi *et al.*, 1989; Raychaudhuri *et al.*, 1989) perhaps through E1A-associated kinases (Giordano *et al.*, 1991a; Kleinberger and Shenk, 1991; Herrmann *et al.*, 1991; Tsai *et al.*, 1991). Modification could also be achieved through alterations in components of transcription complexes, such as with $p105^{Rb}$ and E2F, which are disrupted by E1A to generate transcriptionally active E2F (Bagchi *et al.*, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991; Raychaudhuri *et al.*, 1991). Direct physical interaction of E1A with a number of transcription factors has been described and include TFIID (Horikoshi *et al.*, 1991; Lee *et al.*, 1991), AP-1 (Maguire *et al.*, 1991), ATF-2 (Maguire *et al.*, 1991; Liu and Green, 1990), and CBF (Lum *et al.*, 1992), suggesting that E1A proteins can function within a transcription complex. In addition, EIA products can cause an increase in the level of transcription factors as in the case of AP-1 (deGroot *et al.*, 1991; Müller *et al.*, 1989; Engel *et al.*, 1991; Kitabayashi *et al.*, 1991).

1.3.6 Enhancer Repression

Both 243R and 289R E1A protein products can repress enhancer-driven transcription, including that of E1A itself (Borrelli *et al.*, 1984; Smith *et al.*, 1986). The 243R product appears to be the most effective (Dery *et al.*, 1987; Lillie *et al.*, 1986) and can repress transcription of other viral early genes as well as of cellular promoters (Borrelli *et al.*, 1984; Dery *et al.*, 1987; Guilfoyle *et al.*, 1985; Hen *et al.*, 1985; Jelsma *et al.*, 1989; Lillie *et al.*, 1986; Stein and Ziff, 1987; Webster *et al.*, 1988; Velcich and Ziff, 1985). More specifically, the N-teminus, CR1, and to some extent CR2 appear to be required for repressing the SV40 (simian virus 40) enhancer (Jelsma *et al.*, 1989; Lillie *et al.*, 1987; Rochette-Egly *et al.*, 1990; Svensson *et al.*, 1991), whereas the N-terminus and CR1 appear to mediate repression of the insulin enhancer (Stein *et al.*, 1990). For the *neu*

protooncogene and fibronectin, CR2 is absolutely required for repression. The importance of the N-terminus and CR1 has not been determined for *neu* , whereas the CR1 region but not the N-terminus may be required for repression of fibronectin (Nakajima *et al.*, 1992; Yu *et al.*, 1990). These results have been summarized in Figure 4. Of the three conserved regions tested, CR1 alone was found to be involved in transcriptional repression of some of the members of the matrix metalloprotease family (which comprises collagenase type 1 [interstitial collagenase], 72kDa and 92kDa type IV collagenase and stromelysin), of which stromelysin and collagenase type I were found to be affected (Offringa *et al.*, 1990; van Dam *et al.*, 1989). While other proteases (72kDa type IV collagenase) have also been shown to be repressed by E1A, the precise E1A regions involved have not been delineated (Frisch *et al.*, 1990). In the case of stromelysin and the 92kDa type IV collagenase, carboxy terminal sequences (aa 223-245) also mediate repression (Linder *et al.*, 1992). Deletion of these sequences correlated with increased invasiveness as described above (Linder *et al.*, 1992).

The mechanism involved in E1A-mediated repression is not understood. A common DNA element mediating CR2-specific E1A repression of fibronectin and *neu* has been identified, although the factor binding to this sequence has not been determined (Nakajima *et al.*, 1992; Yu *et al.*, 1990). In addition, there are other sequences in the fibronectin gene which are more important and appear to be the primary E1A-responsive elements. However, in the case of the matrix metalloprotease family, the mechanism is thought to involve AP-1. These metalloproteases appear to be important for the metastatic process as degradation of the extracellular matrix is required for invasion of secondary target sites (Liotta *et al.*, 1991). Most of the matrix metalloprotease genes are repressed by E1A at a TRE element except the 72kDa type IV collagenase promoter (Frisch *et al.*, 1990; Offringa *et al.*, 1990; Sato and Seiki, 1993; van Dam *et al.*, 1993; reviewed in McDonnell and Matrisian, 1990). This element is normally responsive to TPA which generally leads to increased

transcription of components of AP-1 and increased activity of the AP-1 transcription factor. However, in the presence of E1A, transcriptional repression via TRE occurs through inhibition of the transacting function of AP-1 without affecting the synthesis of AP-1 components or AP-1 DNA binding activity (reviewed in Chinnadurai, 1992). The cellular p300 protein may be involved in the regulation of AP-1 as repression of some genes involves CR1, which is an essential binding site for this protein (Offringa *et al.*, 1990; van Dam *et al.*, 1989). A parallel study has also shown that CR1 is required for activation of c-*jun* (van Dam *et al.*, 1990; see above in Transactivation by the amino terminus), indicating that E1A can mediate either a positive or a negative response, depending on the promoter sequence. In addition, because C-terminal sequences (aa 223-245) are also involved in repression of some metalloprotease genes, there are most likely other mechanisms of transcriptional repression (Engel *et al.*, 1992; Linder *et al.*, 1992).

1.4. Mechanism of Oncogenesis by DNA Tumour Viruses

Adenovirus appears to transform cells through associations with at least three classes of cellular proteins, including the p105^{Rb} family, p300 and p53. As described earlier, E1A binding to both p105^{Rb} and p300 is required for transformation. E1B-55kDa protein associates with and inactivates p53 (Sarnow *et al.*, 1982a), and this interaction may account for most of the transforming activity of E1B-55kDa (Kao *et al.*, 1990). Although 19kDa can substitute for E1B-55kDa, the mechanism through which it acts has not been clearly defined (Bernards *et al.*, 1986; McLorie *et al.*, 1991; White and Cipriani, 1990), but may involve the inhibition of E1A-induced apoptosis (Rao *et al.*, 1992; White *et al.*, 1991; 1992).

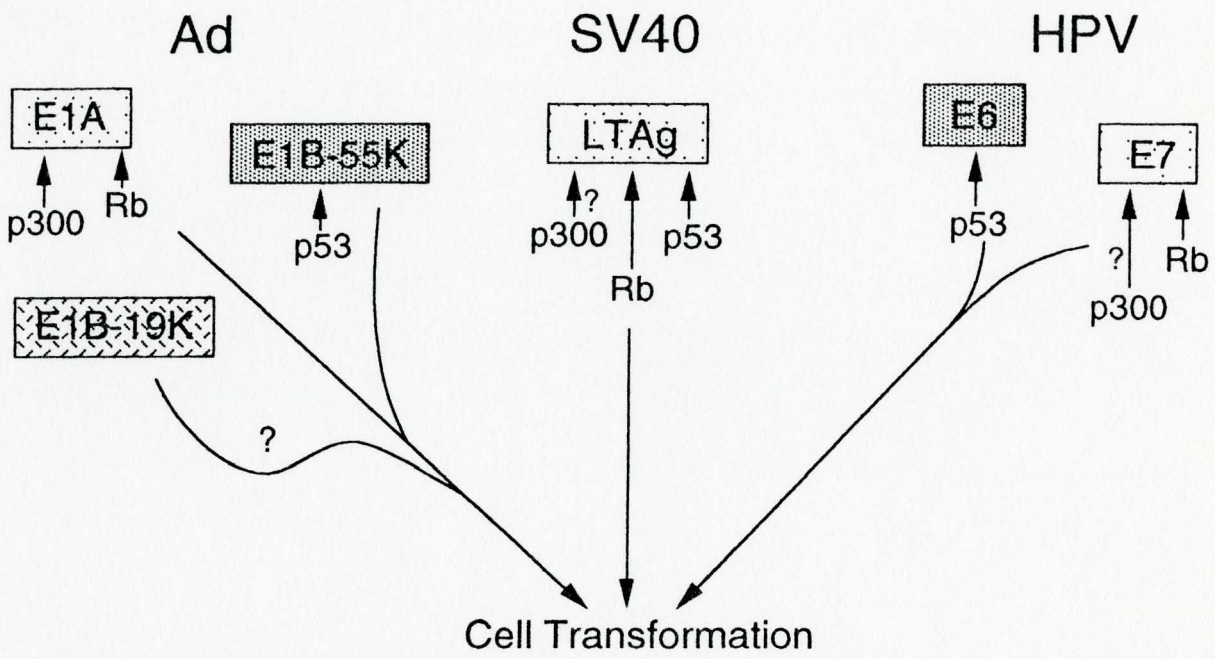
1.4.1 Transformaton by DNA Tumour Viruses

Oncogene products of DNA tumour viruses of the papovavirus family including SV40, polyomavirus, and papillomavirus also appear to interact with a similar array of cellular proteins (Figure 6a). p105^{Rb} and p107 bind to large T antigen (LTAg) of SV40 and E7 of HPV (human papillomavirus) through a region required for transformation which shares homology with E1A CR2 (Banks *et al.*, 1990; Barbosa *et al.*, 1990; Davies *et al.*, 1993; DeCaprio *et al.*, 1988; 1989; Dyson *et al.*, 1989a; 1989b; 1990; 1992a; Ewen *et al.*, 1989; Harada and Yanagi, 1991; Imai *et al.*, 1991; Jones *et al.*, 1990; Ludlow *et al.*, 1989; Münger *et al.*, 1989; Phelps *et al.*, 1991; Srinivasan *et al.*, 1989; Watanabe *et al.*, 1990). The core sequence in CR2 that mediates these interactions, Asp^{121}-Leu-X-Cys-X-Glu, is also found in the products of the other DNA tumour viruses (Egan *et al.*, 1988; Whyte *et al.*, 1989; Münger *et al.*, 1989; Dyson *et al.*, 1989a; 1989b; 1990; Larose *et al.*, 1991). SV40 LTAg and HPV E7 also contain sequences similar to a portion of CR1, but, although these residues are important for transformation by both viruses, they have not thus far been implicated in the binding of p105^{Rb}/p107 (Banks *et al.*, 1990; Dyson *et al.*, 1992a; 1992b; Edmonds and Vousden, 1989; Ewen *et al.*, 1989; Imai *et al.*, 1991; Marsilio *et al.*, 1991; Montano *et al.*, 1990; Münger *et al.*, 1989; Phelps *et al.*, 1992; Srinivasan *et al.*, 1989; Thompson *et al.*, 1990; Watanabe *et al.*, 1990). p130 has also been shown to associate with E7 of HPV (Dyson *et al.*, 1992a). One study also suggested that HPV-E7 can bind to p60^{cycA}-p33^{cdk2} directly rather than through p107 (Tommasino *et al.*, 1993). p300 has not yet been reported to be present in complexes with either HPV-E7 or LTAg of SV40, however, in the case of SV40 an activity analogous to p300 appears to be present (Yaciuk *et al.*, 1991). For polyomavirus, only p105^{Rb} has so far been shown to bind to LTAg (Dyson *et al.*, 1990; Larose *et al.*, 1991). Transformation by polyomavirus, additionally requires an association with protein phosphatase 2A (PP2A; reviewed by Mumbry and Walters, 1991) and the induction of pp60$^{c\text{-}src}$ protein kinase activity via an interaction with

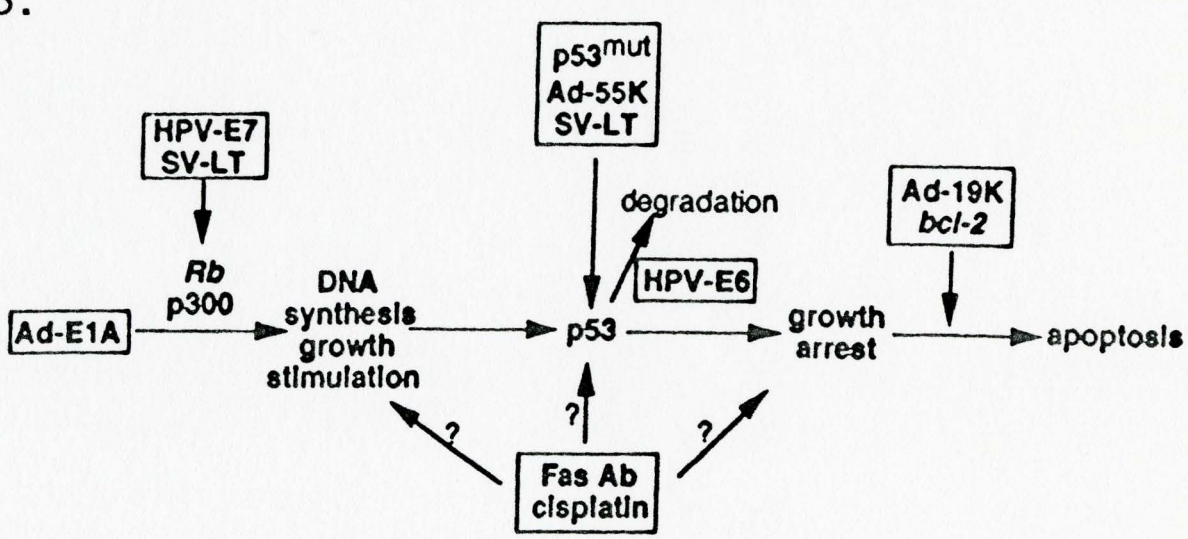
Figure 6. Oncogenesis mediated by DNA-tumour viruses.

Figure 6a. Transformation of non-permissive cells is thought to result from interactions with at least two tumor suppressors, p105^{Rb} and p53 In the case of adenovirus, a third class, p300 is also required. Figure 6b shows the current model summarizing how interactions between viral oncogenic products and cellular proteins lead to transformation.

A.


Ad
SV40
HPV
E1A
p300
Rb
E1B-55K
p53
E1B-19K
?
LTAg
?
p300
p53
Rb
E6
p53
E7
?
p300
Rb
Cell Transformation

B.


HPV-E7
SV-LT
Rb
p300
Ad-E1A
DNA
synthesis
growth
stimulation
p53mut
Ad-55K
SV-LT
degradation
HPV-E6
p53
growth
arrest
Ad-19K
bcl-2
apoptosis
?
?
?
Fas Ab
cisplatin

polyomavirus middle T antigen (reviewed by Cheng *et al.*, 1989). For most DNA tumour viruses, oncogenesis is thought to result from interactions with at least two classes of tumour suppressors, the p105^{Rb} family and p53. p53 has been shown to associate with SV40 LTAg (Lane and Crawford, 1979; Linzer and Levine, 1979) and the product of the E6 oncogene of HPV (Werness *et al.*, 1990). In the case of HPV-E6, binding to p53 was shown to be mediated indirectly via a 100K protein, which promotes degradation of p53 through the ubiquitin pathway (Huibregtse *et al.*, 1991; 1993; Scheffner *et al.*, 1990).

1.4.2 Model of Transformation

A current model that summarizes how interactions between viral oncogenic products and cellular proteins lead to transformation is described in Figure 6b. In order to replicate in growth arrested cells, DNA tumour viruses have to induce entry into the S phase of the cell cycle. This appears to be achieved by interactions with one or both classes of growth suppressors, p105^{Rb} and p300. As a result of this aberrant induction of the cell cycle, which may lead to DNA damage, p53 protein levels are induced (Kastan *et al.*, 1991). Increased levels of p53 have been reported in response to DNA damage induced by chemotherapeutic drugs, ultraviolet light and γ-irridiation treatments and ultimately lead to arrest of the cell cycle in G1 (Fritsche *et al.*, 1993; Kastan *et al.*, 1991; reviewed in Lane, 1992). If the DNA cannot be sufficiently repaired, the process of programmed cell death or apoptosis is activated, leading to induction of a number of enzymes including an endonuclease which cleaves the DNA into specific fragment lengths (Bursch *et al.*, 1990; Lane, 1992; Owens *et al.*, 1991). The involvement of p53 in induction of apoptosis was recently demonstrated in experiments which showed that wild-type p53 overexpression in tumour cells resulted in cell death (Ryan *et al.*, 1993; Shaw *et al.*, 1992; Yonish-Ruach *et al.*, 1991). Elevated p53 levels may also be due to the direct action of E1A as transcriptional activation of p53 mediated by the 289R product has been described

(Braithwaite *et al.*, 1990). E1B-55kDa, SV40 LTAg, and HPV-E6 all interact with p53, and by a variety of mechanisms inactivate it and thus prevent p53-mediated cell death.

Adenoviruses have evolved an additional strategy to counteract the apoptotic pathway and thus ensure uninterrupted viral replication. Earlier experiments described protection by E1B-19kDa of *cyt/deg* phenotype which is induced by E1A and resembles apoptosis (Barker and Berk, 1987; Chinnadurai, 1983; Ezoe *et al.*, 1981; Mak and Mak, 1983; McLorie *et al.*, 1991; Pilder *et al.*, 1984; Subramanian *et al.*, 1984a; 1984b; Takemori *et al.*, 1984; White *et al.*, 1984; 1991). E1B-19kDa has recently been shown to block apoptosis induced by the E1A CR1 region and by tumour necrosis factor α (TNF-α) (White *et al.*, 1991; 1992). TNF-α is a cytokine secreted from activated macrophages and lymphoid cells in response to signals from virus-infected cells and is induced by the CR1 region of E1A during adenovirus infection (Duerksen-Hughes *et al.*, 1991; Gooding *et al.*, 1991b; White *et al.*, 1992). It is possible that induction of apoptosis observed with E1A may be mediated through the activation of this cytokine. A number of E3 viral products can also protect against TNF-mediated lysis (Gooding *et al.*, 1988; 1990; 1991a). The product of the cellular gene *bcl-2* efficiently blocks apoptosis and has been shown recently to substitute for E1B-19kDa in transformation assays (Hockenbery *et al.*, 1990; Nunez *et al.*, 1991; Rao *et al.*, 1992; reviewed in Williams, 1991). The precise mechanism by which E1B-19kDa and Bcl-2 block apoptosis is not known.

c-Myc has also been shown to induce apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992, Shi *et al.*, 1992). c-*myc* is one of the immediate-early class of genes that are activated in G1 and required for entry into S phase (Eilers *et al.*, 1991; Evans *et al.*, 1992; Marcu *et al.*, 1992; Resnitzky and Kimchi, 1991). Recent evidence has suggested that c-Myc is a transcription factor which contains an activation domain and a leucine zipper similar to the Fos/Jun/CREB transcription factor families, and a bHLH (basic helix-loop-helix) motif for

dimerization and DNA binding (Blackwood and Eisenman., 1991; Kato *et al.*, 1990; reviewed in Marcu *et al.*, 1992; Spencer and Goudine, 1991). Comparisons between Ad-E1A and c-Myc have shown some sequence similarities (Ralston and Bishop, 1983). Like E1A, c-Myc is able to interact with the retinoblastoma gene product *in vitro* (Rustgi *et al.*, 1991). E1A proteins bind to $p105^{Rb}$ and release the E2F transcription factor, hence promoting transcription of c-*myc* which contains E2F sites within its promoter (reviewed in Nevins, 1992). Thus, one of the ways in which Ad-E1A, SV40 LTAg and HPV-E7 induce cellular DNA synthesis may be through the activation of c-*myc*. Apoptosis induced by c-Myc, is also inhibited by Bcl-2 (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; Wagner *et al.*, 1993). It still remains to be proven, however, whether c-Myc-induced cell death involves elevated levels of p53. A recent report suggested that such may be the case as c-Myc was shown to activate the p53 promoter which contained a c-Myc binding site (Blackwood and Eisenman, 1991; Halazonetis and Kandil, 1991; Reisman *et al.*, 1993). A number of tumours display increased levels of c-*myc* expression as well as mutated p53 genes, implying that these functions or lack of functions are important in oncogenesis (Wagner *et al.*, 1993).

A variety of factors, have been demonstrated to induce apoptosis, including TNF-α, anti-Fas antibodies and cisplatin (Figure 6b; Fritsche *et al.*, 1991; Itoh *et al.*, 1991; Laster *et al.*, 1988; Marx, 1993). Cisplatin, an anti-cancer drug which causes DNA damage, has been shown to induce accumulation of p53 protein (Fritsche *et al.*, 1991; 1993). Anti-Fas antibodies, which are directed against a cell surface receptor called APO-1 or Fas, a member of the TNF-receptor family, have been shown to cause regression of tumours (Marx, 1993). Cytolysis induced by this antibody can be inhibited by E1B-19kDa, but it is not known whether this antibody induces an increase in p53 levels (Hashimoto *et al.*, 1991).

1.5. Transcriptional Activation as the Mechanism of E1A-Mediated Transformation

1.5.1 $p105^{Rb}$, a Cell Cycle Regulator

$p105^{Rb}$ plays a negative regulatory role in controlling cell growth and has been proposed to negatively regulate transcription of c-*myc*, and c-*fos* through the the RCE element (retinoblastoma control element) (Pietenpol *et al.*, 1990; 1991; Robbins *et al.*, 1990). Both of these cellular genes are immediate-early response genes, and are activated prior to DNA replication in response to mitogens (reviewed in Wagner and Green, 1991). c-Myc and c-Fos are also both transcription factors, as described previously, and do not require new protein synthesis for activation in response to growth factors. Recent studies have shown that $p105^{Rb}$ can also positively regulate expression of c-*myc*, c-*fos*, transforming growth factor β1 and β2 (TGF-β1 and TGF-β2), and Kim *et al.* report that positive or negative regulation by $p105^{Rb}$ appears to be cell type-specific (Kim *et al.*, 1991; 1992a; 1992b). The transcription factor Sp1 can specifically activate the c-*fos* promoter in the presence of $p105^{Rb}$ through the RCE element (Kim et al., 1992a). $p105^{Rb}$ can also mediate positive regulation on the TGF-β2 promoter via the transcription factor ATF-2 (Kim *et al.*, 1992b). It is possible that $p105^{Rb}$ acts as a coactivator bridging upstream transcription factors to the transcriptional regulatory machiney, however, it is not known whether $p105^{Rb}$ binds Sp1 or ATF-2 directly. Negative regulation by $p105^{Rb}$ may be mediated through other factors such as E2F since it has been shown that $p105^{Rb}$ binds to and inactivates E2F (Bagchi *et al.*, 1991; Bandara and La Thangue, 1991; Bandara *et al.*, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991). However, the E2F sites in c-*myc* and other promoters are not located within the RCE element, indicating that other factors may be involved in negative regulation (Hiebert *et al.*, 1989; Kim *et al.*, 1992a; Pientenpol *et al.*, 1991).

1.5.2 Association of p105^{Rb} with Transcription Factor E2F

A major target of p105^{Rb} is the E2F factor. The hypophosphorylated form of p105^{Rb} associates with E2F during the G_0/G_1 of the cell cycle thereby inactivating it (Bagchi *et al.*, 1991; Bandara and La Thangue, 1991; Bandara *et al.*, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991). It is the underphosphorylated form of p105^{Rb} that is thought to be active in mediating cell cycle regulation and it is predominantly this form of p105^{Rb} that binds to Ad-E1A, SV40 LTAg and HPV-E7 (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; 1992; Egan *et al.*, 1989; Imai *et al.*, 1991; Ludlow *et al.*, 1989; 1990; Mihara *et al.*, 1989; Moses *et al.*, 1990; Templeton *et al.*, 1991; Templeton, 1992). p105^{Rb} is differentially phosphorylated during the cell cycle. It is hypophosphorylated during G_0 and early G1, hyperphosphorylated at the G1/S boundary and during the S and G2/M phases, and is dephosphorylated during late M phase (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; 1992; Ludlow *et al.*, 1990; Mihara *et al.*, 1989). During the the cell cycle, different residues on p105^{Rb} are phosphorylated, indicating that p105^{Rb} is phosphorylated in at least three steps (DeCaprio *et al.*, 1992). p105^{Rb} has been shown to associate with a *cdc2*-like kinase which phosphorylates p105^{Rb} at a number of physiologically relevant sites (Hu *et al.*, 1992; Kitagawa *et al.*, 1992; Lees *et al.*, 1991). Quiescent cells do not transcribe *cdc2* which is only activated as cells enter the S phase, indicating that the initial phosphorylation of p105^{Rb} in mid G1 is probably not mediated by p34^{cdc2}, but perhaps by some other *cdc2*-like kinase (Dalton, 1992; Welch and Wang, 1992). Indeed, recent studies have shown that p105^{Rb} may be bound and phosphorylated by cyclin D-p34^{cdk4} which is active in G1 (Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Hall *et al.*, 1993; Kato *et al.*, 1993; Matsushime *et al.*, 1992).

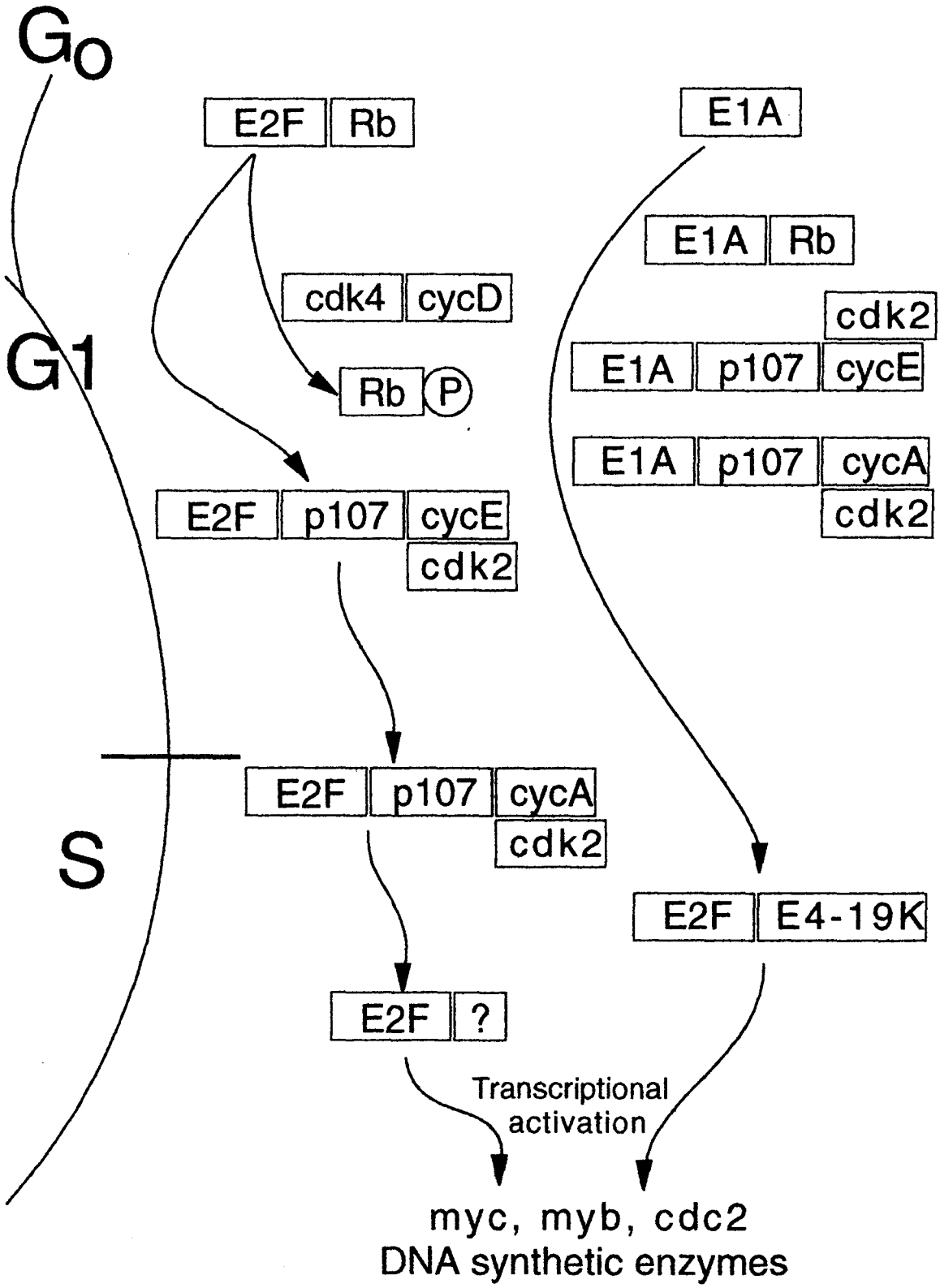
When cells are activated by growth factors, p105^{Rb} is phosphorylated and presumably releases free E2F such that it can activate transcription of cellular genes required for entry

into S-phase (Figure 7). A number of cellular genes have been reported to be transactivated by E2F including c-*myc*, c-*myb*, *cdc2* as well as the following DNA synthetic enzymes; dihydrofolate reductase (DHFR), thymidine kinase, DNA polymerase α (Dalton, 1992; Hiebert *et al.*, 1989; 1991; Mudryj *et al.*, 1990; Thalmeier *et al.*, 1989; reviewed in Nevins, 1992). It has been shown that p105^{Rb} converts the E2F site in the promoter of these genes from a positive to a negative regulatory element (Weintraub *et al.*, 1992). p105^{Rb}-E2F complexes require the association of an accessory factor to bind to the E2F site and mediate repression (Girling *et al.*, 1993; Hiebert *et al.*, 1992; Ray *et al.*, 1992; Zamanian and La Thangue, 1992). Transcriptional repression by hypophosphorylated p105^{Rb}-E2F complexes have been reported for promoters including viral E2, *cdc2*, c-*myc*, and *Rb* itself (Dalton, 1992; Hamel *et al.*, 1992).

Upon adenovirus infection, Ad-E1A binds to p105^{Rb} and presumably releases E2F from negative regulatory complexes (Bagchi *et al.*, 1990; Bandara *et al.*, 1991; Hiebert *et al.*, 1992; Raychaudhuri *et al.*, 1991; Zamanian and La Thangue, 1992). Release of E2F can be mediated by either 289R or 243R products, and the required regions on E1A have been mapped to CR1 and CR2 (Bagchi *et al.*, 1990; Raychaudhuri *et al.*, 1991), which are the same regions involved in p105^{Rb} binding and transformation (Egan *et al.*, 1988; 1989; Stein *et al.*, 1990; Svensson *et al.*, 1991; Whyte *et al.*, 1989). SV40 LTAg and HPV-E7 protein are functionally similar to 243R as CR2-like sequences in these proteins can also disrupt complexes between E2F and p105^{Rb} (Chellappan *et al.*, 1992; Chittenden *et al.*, 1991; Huang *et al.*, 1993; Morris *et al.*, 1993; Phelps *et al.*, 1991). The E2F binding sites on p105^{Rb} overlap with the regions required for binding Ad-E1A, SV40 LTAg and HPV-E7 and include the "binding pocket" (A and B boxes) as well as additional sequences extending towards the carboxy terminus (Bandara *et al.*, 1991; Chittenden *et al.*, 1991; Hiebert *et al.*, 1992; Qian *et al.*, 1992;.Qin *et al.*, 1992). These DNA tumour viruses have

Figure7. Protein complexes and the cell cycle. Interactions between proteins involved in regulation of entry into S phase have been depicted. As displayed on the right part of the diagram, E1A displaces these complexes and allows E2F to activate transcription of cellular genes required for entry into S phase.

The E2F Pathway



therefore evolved a common mechanism to stimulate entry into S phase to allow efficient viral replication.

There appear to exist different E2F-complexes throughout the cell cycle as both p107-p60^{cycA}- p33^{cdk2} and p107-p51^{cycE}- p33^{cdk2} have been found in association with E2F during the S and the G1 phase, respectively (Bandara *et al.*, 1991; Cao *et al.*, 1992; Devoto *et al.*, 1992; Dulic *et al.*, 1992; Lees *et al.*, 1992; Mudryj *et al.*, 1991; Pagano *et al.*, 1992a; Shirodkar *et al.*, 1992). As mentioned previously, p107 associates with p60^{cycA}-p33^{cdk2} kinase (Ewen *et al.*, 1992; Faha *et al.*,1992; Howe and Bayley, 1992), however, recent studies have also shown that p107 can bind to the p51^{cycE}-p33^{cdk2} kinase (Lees *et al.*, 1992). It is not known whether p107 acts as a suppressor or an inducer of the cell cycle, however its association with protein kinases could suggest that it facilitates phosphorylation of E2F (Nevins, 1992).

1.6. Thesis Proposal

The purpose of the present studies was to determine more precisely the roles in complex formation of various domains of adenovirus E1A proteins and of individual phosphorylated forms of these viral products.

Quantitative analyses of the binding patterns of cellular proteins to E1A products were performed using E1A exon 1 deletion mutants. Binding of most proteins was affected at least partially by mutations within the amino terminal 25 residues, amino acids 36-69 within CR1, and residues 121-138 in CR2. However, the specific binding characteristics of each protein varied considerably and showed that complex formation for all proteins required at least two domains. Complementation experiments showed that these critical binding domains could not be provided in *trans*. An additional E1A-binding protein, termed p400, was shown to be similar but not identical to p300 both in V8 peptide pattern and E1A binding properties, suggesting that p400 may either be a related but distinct relative, or a modified form of p300. p130 was shown to be related to p107, and like p107, to associate with $p60^{cycA}$.

Protein kinase activity exists in E1A complexes and probably derives in part from $p33^{cdk2}$-$p60^{cycA}$ heterodimers associated with p107 and p130. *In vitro* phosphorylation of complexes purified by immunoprecipitation resulted in labeling of several proteins. $p60^{cycA}$ was phosphorylated to about the same extent in cyclin A complexes prepared from either Ad5- or mock-infected KB cells, however, that of p130 and p107 was dramatically higher in $p60^{cycA}$ complexes from infected cells. p300 was also phosphorylated in complexes containing E1A proteins. Thus the role of complex formation by E1A proteins may be to increase phosphorylation of p107, p130 and p300, possibly by enhancing interactions with $p60^{cycA}$-bound kinases or through the introduction of other kinases into the complex.

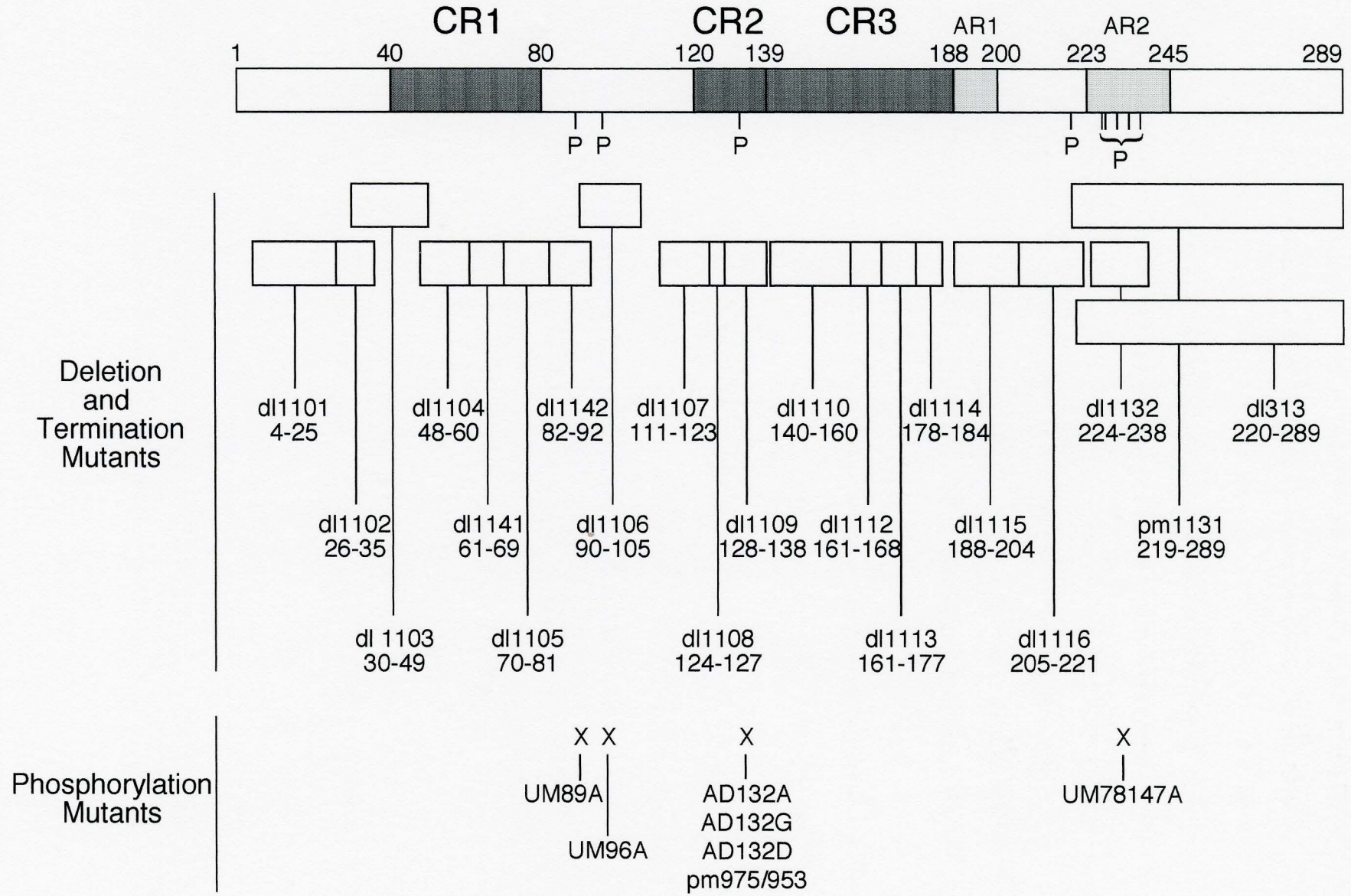
E1A proteins are multiply phosphorylated and one site, serine 89, has been linked to a major shift in gel mobility and shown to enhance E1A-mediated transformation (Dumont *et al.*, 1989; 1993; Richter *et al.*, 1988; Smith *et al.*, 1989). Studies carried out with mutants affecting phosphorylation at serine 89 and other sites indicated that none had any major effect on the overall binding of p300, p400, p130, p107, $p105^{Rb}$, p107 or $p60^{cycA}$. These studies indicated that E1A phosphorylation does not appear to regulate complex formation. An additional supershift in gel migration of E1A products was found to be due to the phosphorylation of carboxy terminal sites, serine residues 227,228, 231, 234, 237 with the phosphorylation of at least two residues being required to induce the supershift. Analysis of E1A species contained in $p60^{cycA}$ complexes revealed that there was a preponderance of the supershifted E1A species present in cyclin A complexes.

CHAPTER 2: Materials and Methods

2.1 Cells and viruses

Human KB cells were maintained in α-modified minimal essential medium containing with 10% fetal calf serum. Cells were either mock-infected or infected with mutant or *wt* Ad5 at 35 plaque-forming units (pfu) per cell, as described previously (Rowe *et al.*, 1983b). For complementation studies involving two infecting viruses, a total of 35 pfu/cell with equal amounts of each virus was used. Viruses were propagated and titred on 293 cells which express Ad5 E1A and E1B products (Graham *et al.*, 1977). *dl* 520 (Haley *et al.*, 1984), which produces 12S but not 13S E1A mRNA, and thus only the 243R protein, was used as *wt* in some studies. Construction of the series of Ad5 deletion mutants containing in-frame deletions within the E1A coding sequence of exon 1 of *dl* 520 (*dl*1101/520, *dl*1102/520, etc.) has been described previously (Jelsma *et al.*, 1988; 1989; Egan *et al.*, 1988; Howe *et al.*, 1990; summary in Figure 8). Mutants combining two of these deletions (eg. *dl*1103/520 & *dl*1108/520) have been named using the two last digits of each mutant (eg. *dl* 03/08/520). *pm*1131/520 gives rise to a 71 amino acid truncated 243R product due to the substitution of Ser-219 to a termination codon (Jelsma *et al.*, 1988; 1989). Some of these deletion mutants have also been introduced into a *wt* strain *dl*309 (Jelsma *et al.*, 1988;1989) which produces both 13S and 12S mRNAs (Jones & Shenk, 1979b). Additional mutants carrying deletions in CR3 and the region encoded by the second E1A exon and introduced into *dl*309 (see Figure 1A) have also been described previously (Jelsma *et al.*, 1988; 1989). Deletion mutant *dl*313 (Colby and Shenk, 1981) which lacks the carboxy terminal 70 residues of 289R and 243R (following Ser-219) was used in some experiments, as was *dl*1132 which contains a deletion affecting residues 224-238 (Mymryk & Bayley, in press). *pm* 975 contains a point mutation at the splice donor site of 12S mRNA so that only the 13S mRNA and thus 289R is produced (Montell *et al.*, 1982).

Figure 8. Ad5 E1A and E1A deletion and phosphorylation mutants. The Ad5 289R E1A protein (243R is identical except that CR3 is absent) has been illustrated and phosphorylation sites as well as the positions of CR1, CR2, CR3, AR1 and AR2 have been indicated with amino acid numbers shown above. Below are indicated the relative positions of deletion mutants spanning the entire E1A molecule. Mutants *dl* 1101-*dl* 1109 including *pm*1131 are present in the *dl* 520 background; all other mutants are present in the *dl* 309 background. Mutants affecting phosphorylation sites 89, 96, 132, 227, 228, 231, 234 and 237 are shown in the lower part of the figure. The UM series of viruses are present in the 243R background (*dl* 520), whereas *pm*975/953 and the AD series of mutants are present in the 289R (*pm*975), and the 289R and 243R (*dl* 309) backgrounds, respectively.


CR1
CR2
CR3
AR1
AR2
1
40
80
120
139
188
200
223
245
289
P
P
P
P
P
Deletion and Termination Mutants
dl1101 4-25
dl1102 26-35
dl 1103 30-49
dl1104 48-60
dl1141 61-69
dl1105 70-81
dl1142 82-92
dl1106 90-105
dl1107 111-123
dl1109 128-138
dl1108 124-127
dl1110 140-160
dl1112 161-168
dl1113 161-177
dl1114 178-184
dl1115 188-204
dl1116 205-221
dl1132 224-238
pm1131 219-289
dl313 220-289
Phosphorylation Mutants
X
X
UM89A
UM96A
X
AD132A
AD132G
AD132D
pm975/953
X
UM78147A

Amino acid substitution mutants affecting phosphorylation at serine residues 89, 96, 132, 227, 228, 231, 234, and 237 were used in some experiments. Mutants are named according to whether they were constructed in the *dl* 520 (UM series) or *dl* 309 (AD series) background. The single letter at the end of the mutant name refers to the amino acid type, that is the serine residue was either converted to an alanine (A), an aspartic acid (D), or a glycine (G) residue. The mutants included: UM89A, UM96A, UM89A96A, UM89D96A, UM90A96A (Dumont *et al.*, 1989; 1993; Tremblay *et al.*, 1989), AD132A, AD132G, AD132D (S.G. Whalen, D. Barbeau, H.B. Corbeil, R.C. Marcellus, and P.E. Branton, in preparation) as well as a combined mutant with the 5 carboxy serines 227, 228, 231, 234, 237 converted to alanines termed UM78147A [This and other mutants with alterations at multiple carboxy terminal phosphorylation sites have been named using the final digit of the affected residues.](S.G. Whalen, D. Barbeau, R.C. Marcellus, J.A. Howe, S.T. Bayley, and P.E. Branton, submitted). Individual mutants have also been generated in which serine codons for each of these residues were reintroduced to yield UM8147A (which contains Ser-227 intact), UM7147A (Ser-228), UM7847A (Ser-231), UM7817A (Ser-234) and UM7814A (Ser-237), respectively. UM-1 is essentially *wt-dl* 520 except that a restriction site was incorporated at the DNA level in order to help in screening virus mutants (Dumont *et al.*, 1993; 1989; Tremblay *et al.*, 1989). *pm*975/953 is a derivative of *pm* 975, and therefore produces 289R only but with an additional mutation at Ser-132 which has been converted to glycine (Lillie *et al.*, 1986).

2.2 Immunoprecipitation of E1A polypeptides and associated cellular proteins

KB cells growing on 100-mm diameter plates were either mock-infected or infected with mutant or *wt* Ad5 and labelled from14 to 17 h post-infection with 150 μCi of [^{35}S]methionine (New England Nuclear Corp., 800 Ci mmol- 1) in 2 ml of medium lacking methionine. Cells were harvested and lysed in 0.5 ml of lysis buffer [50 mM Tris-

HCl, pH 8.5, containing 250 mM NaCl, 1 mM EDTA, 1% (vol/vol) nonidet P-40, 100 KIU aprotinin] and clarified by centrifugation. Cell extracts were pre-cleared by incubation with 80 μl of Protein A-Sepharose beads (50% vol/vol; Phamacia) for 1 h. For immunoprecipitations, samples were incubated for 1 to 2 hours with a similar amount of protein A-Sepharose beads in the presence of 2 mg/ml BSA and one of the following monoclonal antibodies: 1.5 μl of E1A-specific M73 (Harlow *et al.*, 1985); 6 μl of E1B-55K-specific 9C10 (Oncogene Science); 12μl of p105^{Rb} -specific C36 (Oncogene Science); and 42μl of p60^{cycA}- specific C160 (Giordano *et al.*, 1989) in the presence of 1μl rabbit anti-mouse IgG (ICN; 3mg/ml). Under these conditions these antisera immunoprecipitated essentially all of the relevant proteins present in the cell extract. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using gels containing 7.5% polyacrylamide.

2.3 Quantitative analysis of E1A-binding proteins

To quantify binding of cellular proteins, equal amounts of immunoprecipitates from extracts prepared using equal numbers of infected KB cells were analyzed by SDS-PAGE. Dried gels were fluorographed and then submitted to autoradiography using pre-flashed Kodak XAR-5 film (Laskey & Mills, 1975). The intensity of individual bands was quantified using a Millipore Corporation Bio-Image Densitometer System. In all cases data were obtained and averaged from 8 separate experiments in which a number of autoradiographs representing different exposures of each gel were analyzed to ensure that the densities from the various bands in the gel fell in the linear range of the characteristic curve of the film. Band intensities (using the appropriate exposure) produced by p400, p300, p130, p107, p105^{Rb} and p60^{cycA} were determined. These were then corrected for the amount of cell extract using the band density of a non-specific species migrating at about 75kDa. Results represented the averages (± standard error) of a minimum of three experiments for each mutant.

2.4 V8 protease analysis

Partial Cleveland peptide mapping (Cleveland *et al.*, 1977) was performed with *Staphylococcus aureus* V8 Protease (Boehringer Mannheim) in essentially the same manner as described in Harlow and Lane, 1988 with some modifications. Cells were infected with wild type virus in a similar way as described above except that 0.5 -1.0 mCi of [^{35}S] methionine was used per plate. In one experiment E1A proteins were labeled *in vivo* using 2.5 mCi of [^{32}P]orthophosphate (New England Nuclear, 3000 Ci/mmol) in 2 ml of phosphate-free medium, and cell extracts prepared in the usual manner. Immunoprecipitations, performed as described above were run on a gel, an autoradiogram of the unfluored dried gel was obtained and superimposed on the gel so that the bands of interest could be excised. The gel pieces were rehydrated in electrode buffer (25 mM Trizma, 192 mM Glycine, 0.1% SDS), the paper backing was removed, after which the gel pieces were loaded into the wells of a 15% polyacrylamide gel. Various concentrations of V8 protease (3μg, 10μg and 20μg) in 50 µl of Laemmli sample buffer (50 mM Tris pH 6.8, 1% SDS, 10% glycerol, 1% ß-mercaptoethanol, 0.01% bromophenol blue) were added to individual lanes. A constant current of 30 mA was applied to the gel until the dye front migrated three quarters of the way in the stacking gel, which was approximately 1 cm away from the separating portion of the gel. The gel was stopped for one hour to allow partial digestion, after which the current was reestablished and the dye front was run to the bottom of the gel. The gel was fluored with PPO/DMSO and appropriate exposures of autoradiograms were obtained.

2.5 *In vitro* phosphorylation

Immunoprecipitations were carried out on unlabeled cells in essentially the same manner as described above, with the following modifications: after precipitates were extensively washed, 150μl of lysis buffer containing 10 mM $MnCl_2$ and 10 μCi [γ^{32}P]ATP (New England Nuclear, 3000 Ci/mmol) was added to the beads and the mixture was incubated

for 1 hr at 20°C. Samples were then washed twice with lysis buffer, boiled in sample buffer, and the proteins separated on 7.5% SDS-PAGE gels.

2.6 Potato acid phosphatase treatment

Potato acid phosphatase treatment of E1A complexes was essentiallly carried out as described in Yaciuk and Moran, 1991 with some minor modifications. Proteins were coimmunoprecipitated from unlabeled extracts using E1A-specific M73 as described above, following which they were washed with 100mM MES buffer (2-[N-morpholino] ethanesulfonic acid, pH 6.0) containing 1mM PMSF. The samples were separated into two tubes, one of which was treated with 1.25mg of potato acid phosphatase (Boehringer Mannheim; specific activity-2units/mg) resuspended in 60 µl of MES containing 1mM PMSF, and the other half of the sample received buffer only. The samples were incubated at 37°C for 15 min, following which reactions were washed twice with MES to get rid of the phosphatase before resuspending in sample buffer and separating on 10% polyacrylamide gels.

2.7 E1A Western analysis

Immunoprecipitations prepared from unlabelled cells were separated on 10% polyacrylamide mini-gels and then tranferred to nitrocellulose for 1 hr at 350 mA using a Tyler semi-dry transfer apparatus. Membranes were rinsed in Tris buffered saline (TBS) [25 mM Tris-HCl, pH 7.2, containing137 mM NaCl, 2.7 µM KCl, and 0.5% (v/v) Tween 20] and then treated for 2 hrs in TBS containing 5% filtered calf serum. Membranes were washed and incubated for 12 hrs with E1A-specific M73 antibody at a dilution of 1/1000 in TBS supplemented with 1.0% filtered calf serum and 1.0% Bovine serum albumin (TBS-S). The membranes were washed again and incubated for 1hr with a 1/10000 dilution of peroxidase conjugated goat anti-mouse IgG (0.8mg/ml, Jackson Immunoresearch

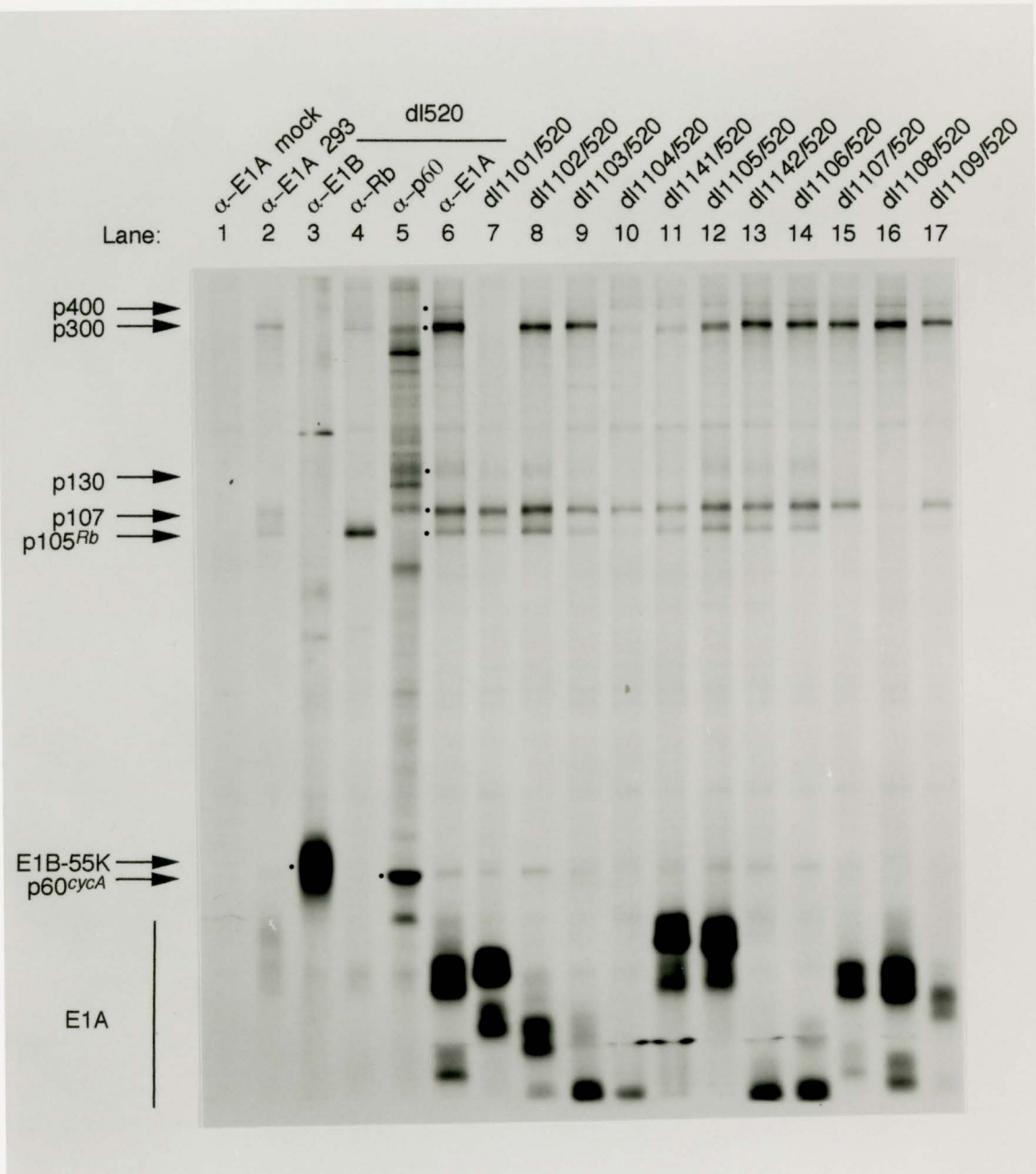
Laboratories, Inc.) in TBS-S, following which the membranes were washed extensively and E1A visualized using the enhanced chemoluminescence (ECL) system (Amersham) according to the manufacturer's specifications.

CHAPTER 3: E1A Regions involved in complex formation with cellular proteins

3.1 Interactions between Ad5 E1A products and cellular proteins

Earlier studies had implicated regions within CR2, CR1 and the amino terminus of E1A proteins in the formation of complexes with a series of cellular proteins including p105^{Rb}, p107, p300, p130, and p60^{cycA} (Yee & Branton, 1985; Harlow *et al.*, 1986; Egan *et al.*, 1987; 1988; 1989; Whyte *et al.*, 1988a;1989; Howe *et al.*, 1990; Giordano *et al.*, 1991b; Howe & Bayley, 1992; Stein *et al.*, 1990; Svensson *et al.*, 1991). However, most of these studies were basically qualitative in nature and/or employed a limited array of mutant viruses that expressed both the 243R and 289R E1A products. A large quantitative study was undertaken to determine the precise importance of various regions of Ad5 E1A proteins in complex formation. Human KB cells were infected with *wt* virus *dl*520 that synthesizes only 12S E1A mRNA (and thus 243R but not 289R) or with various E1A deletion mutants present in a *dl* 520 background (see Figure 8). After labeling with [^{35}S]methionine, cell extracts were immunoprecipitated under mild conditions using M73 mouse monoclonal antibody which recognizes the carboxy terminus of E1A proteins, and the precipitates were analyzed by SDS-PAGE. Figure 9 shows profiles obtained from a representative experiment. As can be seen, precipitates prepared from *wt* -infected cells using M73 serum (lane 6) contained, in addition to E1A products, the major p300, p107, and p105^{Rb} E1A-binding species, as well as less prominent p130 and p60 polypeptides. A similar array of proteins was also observed using Ad5-transformed 293 cells (lane 2). None of these species were precipitated from mock-infected cells by M73 antibody (lane 1), or from infected cells using a monoclonal antibody 9C10 that recognizes the 55kDa

Figure 9. Analysis of E1A complex formation with E1A exon 1 deletion mutants by SDS-PAGE. Human KB cells were infected with *wt* - *dl* 520 Ad5 or various E1A deletion mutants and after labeling with [^{35}S]methionine, cell extracts were immunoprecipitated with various antisera. Ad5-transformed 293 cells or mock-infected were labeled and processed in a similar fashion. Cells and mutants have been indicated above each lane, as have the antisera which include: α-E1A (M73 serum), α-55K (9C10 serum specific for E1B-55kDa protein), α-Rb (p105^{Rb} -specific C36 serum), and α-p60 (p60^{cycA}-specific C160 serum). The positions of E1A-binding proteins, E1A products, and the E1B 55kDa protein have been indicated at the left.

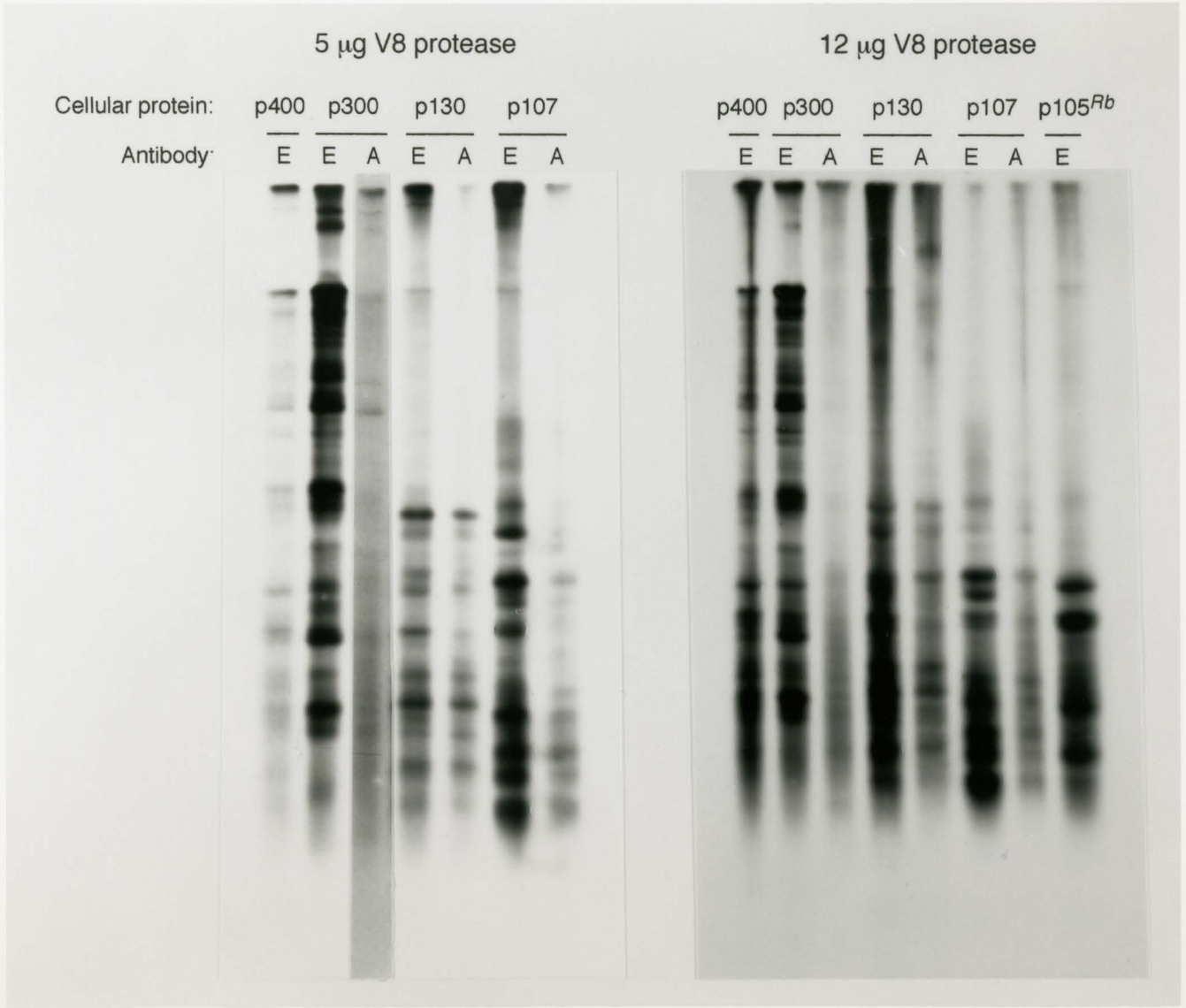

α-E1A mock
α-E1A 293
α-E1B
α-Rb
α-p60
α-E1A
dl520
dl1101/520
dl1102/520
dl1103/520
dl1104/520
dl1141/520
dl1105/520
dl1142/520
dl1106/520
dl1107/520
dl1108/520
dl1109/520
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
p400
p300
p130
p107
p105Rb
E1B-55K
p60cycA
E1A

E1B protein (lane 3). Immunoprecipitation of extracts from infected cells with monoclonal antibodies C36 and C160 identified p105^{Rb} (lane 4) and confirmed that the 60kDa protein was p60^{cycA} (lane 5).

Prior to discussing quantitative aspects of complex formation, a number of other features evident in Figure 9 should be noted. First, a protein of high molecular mass, termed here as p400, was observed in M73 precipitates from cells infected with *wt* (lane 6) and at least some of the mutants (lanes 10-17), but not in those from mock-infected cells (lane 1). In some analyses it appeared as two closely-migrating species. To study this species further, p400 and p300 labeled with [^{35}S]methionine were isolated from a gel similar to that shown in Figure 9 and the patterns of peptides on SDS-PAGE were compared following digestion with *Staphylococcus aureus* V-8 protease. Figure 10 shows that treatment of p400 at both 5 and 12μg of V-8 protease yielded peptide patterns that were very similar but not identical to those obtained with p300. As discussed below (Figure 12), the E1A-binding profiles obtained with p400 and p300 were also similar These results indicated that p400 could represent the product of an alternatively spliced mRNA of the gene encoding p300, the product of a novel but related gene, or a post-translationally modified form of p300.

A second feature evident in Figure 9 was that with infected cells, C36 anti-p105^{Rb} serum precipitated not only p105^{Rb}, but also some p300 protein (lane 4). Such was not the case with uninfected cells (data not shown). Thus p300 and p105^{Rb} probably do not interact directly, but rather may bind to E1A molecules simultaneously In addition, previous studies had indicated that a significant amount of p105^{Rb} in infected KB cells was present in E1A protein complexes (Egan *et al.*, 1989), and this observation can be confirmed by comparing the total amount of p105^{Rb} precipitated by p105^{Rb}-specific serum (lane 4) with that complexed with E1A proteins recognized by E1A-specific serum (lane 6). While E1A products clearly were able to bind much of the p105^{Rb}, the reciprocal effect was quite

Figure 10. Partial *S. aureus* V-8 peptide mapping. Immunoprecipitations were carried out as described in Figure. 9 and individual bands were excised from gels and submitted to partial V-8 protease digestion in 15% polyacrylamide gels using 5 or 12μg of enzyme, as described in Materials and Methods. The identities of cellular E1A-binding proteins have been given at the top, and the antibodies involved given just below, including E1A-specific M73 serum (E) and $p60^{cycA}$-specific C160 serum (A).


5 μg V8 protease
12 μg V8 protease
Cellular protein:
p400
p300
p130
p107
p400
p300
p130
p107
p105Rb
Antibody:
E
E
A
E
A
E
A
E
E
A
E
A
E
A
E

different. Only a very small amount of E1A protein, less than 1%, was present in precipitates prepared using p105^{Rb}-specific serum (lane 4).

A third feature was that precipitates prepared using C160 serum contained not only p60^{cycA}, but also high amounts of proteins that co-migrated with p300, p130 and p107, as well as small quantities of E1A products (lane 5). V-8 peptide analysis confirmed that these proteins were identical to p300, p130 and p107 precipitated by M73 antibodies (see Figure 10). The patterns obtained from p130 and p107 were very similar, though not identical, and indicated that these proteins are highly related. These and other experiments, including those described below in Figure 15, indicated that, like p107 (Ewen *et al.*, 1991), p130 also appears to interact directly with p60^{cycA}. Studies shown in Figure 15, as well as other experiments (not shown) indicated that, as was the case with anti-p105^{Rb} C36 serum, p300 was probably present in C160 precipitates because it interacts with E1A molecules that are linked to p60^{cycA} via interactions with p107 or p130.

3.2 Quantitative analysis of E1A complex formation using E1A deletion mutants

The goal of the present quantitative studies was to provide comprehensive data on the importance of regions within E1A proteins for complex formation. As noted above, the approach taken was to analyze binding through detection of cellular proteins present in immunoprecipitates obtained using E1A-specific M73 serum and extracts from cells infected with *wt* virus or a series of mutants lacking various E1A sequences. The amounts of radioactivity in appropriate bands of gels such as that shown in Figure 9 were determined as described in Materials and Methods and used to measure binding. We considered normalizing binding to levels of E1A proteins, but this method was not used because E1A products appeared to be present in large excess. Instead, binding was

determined by assessing the total amount of each of the cellular proteins present in the precipitates obtained from equal amounts of cell extract, and relating this value to that obtained with *wt-dl* 520. Figure 11 shows that this method was appropriate. By changing the multiplicity of infection it was possible to alter the levels of E1A proteins synthesized in infected cells and thus to compare the effects of increasing E1A concentrations on complex formation. With the amounts of M73 antibodies used, 95 to 100% of the E1A products were precipitated, even at higher levels of virus input. The data indicated that within the range of E1A protein concentration obtained in experiments using *wt* and essentially all of the mutants, binding of p300, p107 and $p105^{Rb}$ was maximal. As indicated in Figure 11, the only exception was *dl*1109/520 which produced levels at or slightly less than that required for maximal binding.

Previous studies had indicated that deletions in CR3 and in the carboxy terminus of E1A products had little effect on interactions with the series of cellular proteins under investigation (Egan *et al.*, 1988; Whyte *et al.*, 1989), as confirmed by data presented later in Figure 15 and Table 2 in related studies. Thus binding studies were confined to mutants with deletions in the amino terminal region encoded by the first exon of the 243R product. Although this study was done using *dl* 520 as *wt* , similar results were obtained in a more limited series using mutants that produce both 13S and 12S E1A mRNAs (data not shown). Binding was measured in 8 separate quantitative assays which included a minimum of 3 for any individual mutant. Figure 12 summarizes the results both numerically and graphically. As summarized at the bottom of Figure 12 and as described previously (Egan *et al.*, 1988; 1989; Jelsma *et al.*, 1989), the ability of mutants to transform primary baby rat kidney cells correlated well with binding of both the p300 and $p105^{Rb}$ classes of proteins.

Figure 11. Complex formation and E1A protein concentration. KB cells were infected with dl 520 at different multiplicities of infection, and after labeling with [^{35}S]methionine from 14-16 h post-infection, extracts were immunoprecipitated with M73 monoclonal antibody and analyzed by SDS-PAGE, as described in Figure 9. The amounts of radioactivity present in p300, p107, p105^{Rb} and E1A species were determined from autoradiographs, as described in Materials and Methods. The % maximal binding for each cellular protein has been plotted against the concentration of E1A proteins (in arbitrary densitometric units). (X- - -X) p300; (^____^) p107; (•_ _ _ •) p105^{Rb}.

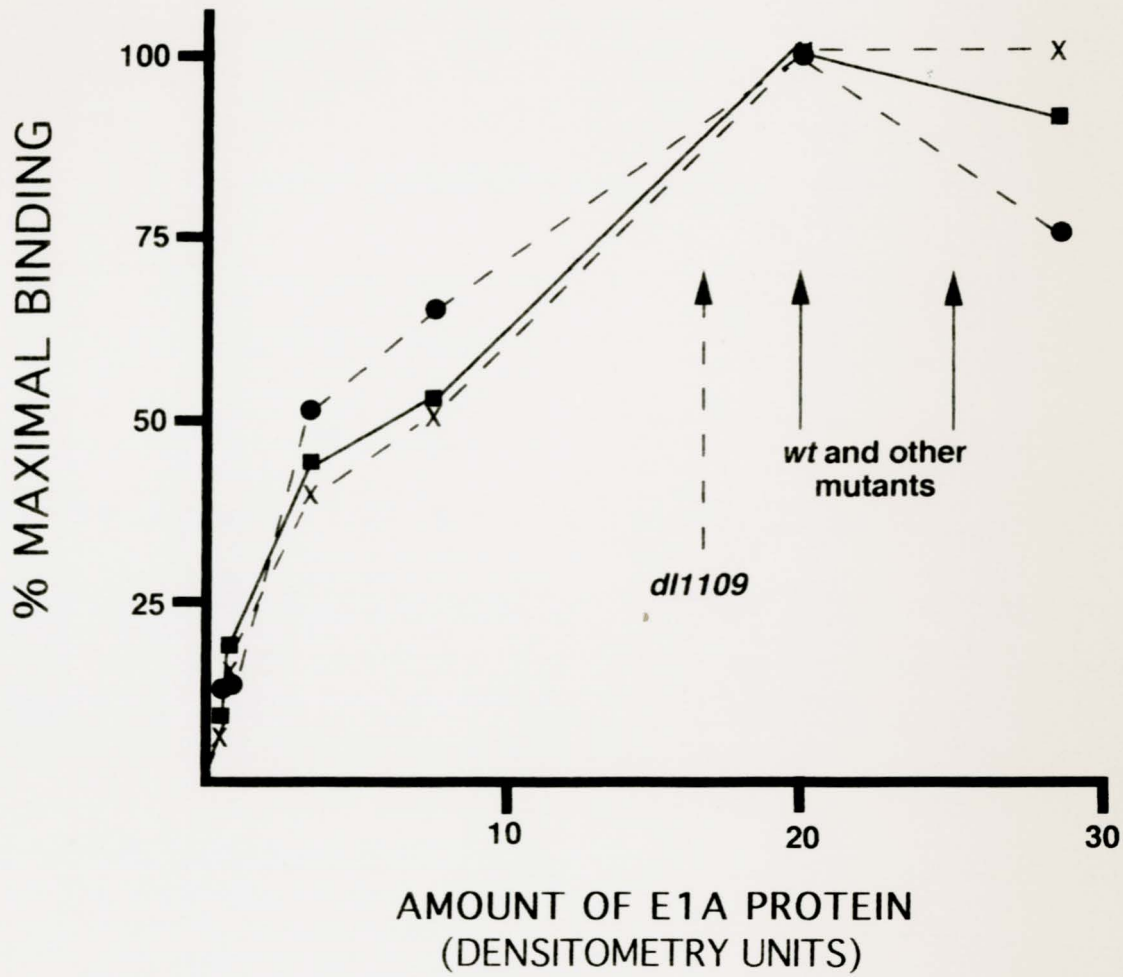
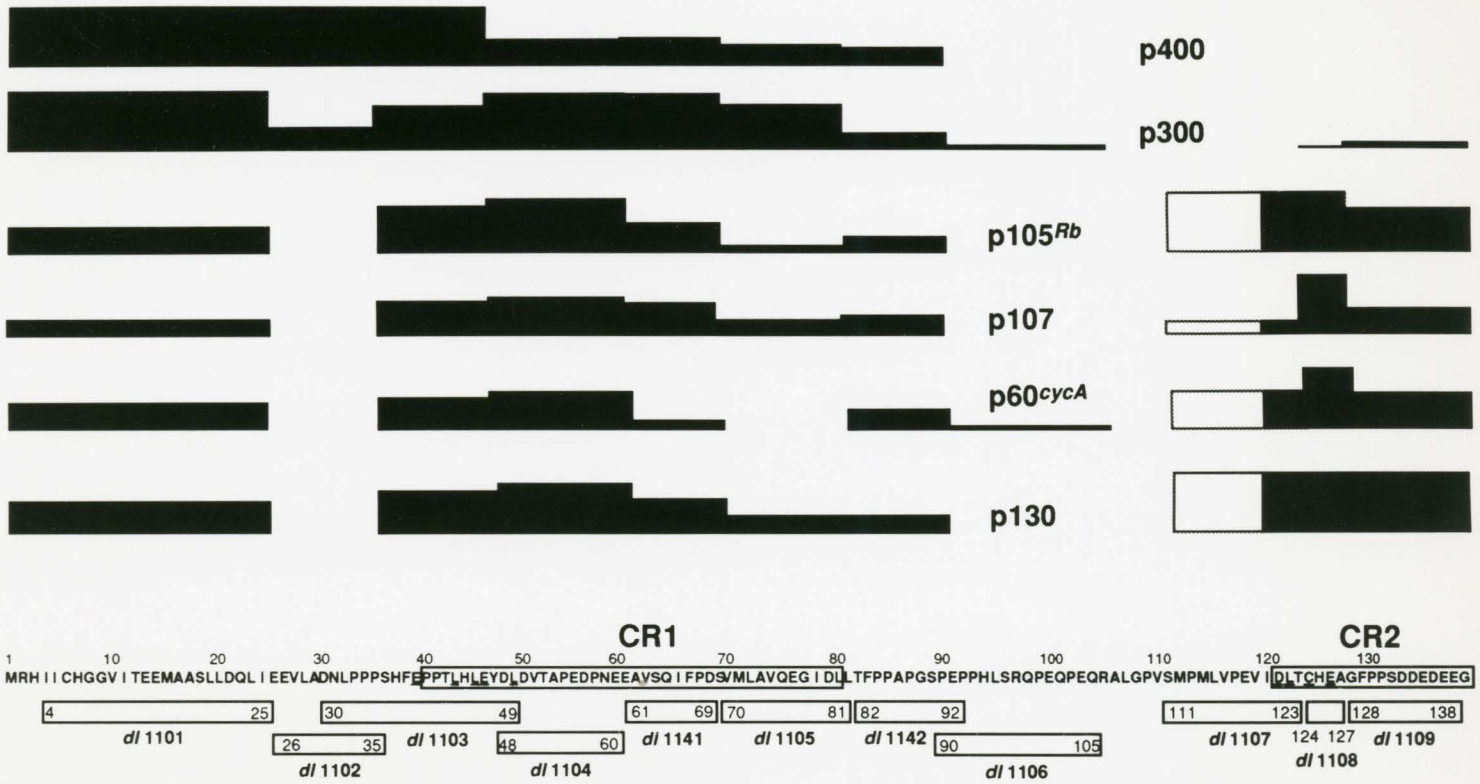

% MAXIMAL BINDING
100
75
50
25
10
20
30
wt and other mutants
dl1109
AMOUNT OF E1A PROTEIN
(DENSITOMETRY UNITS)

Figure 12. Quantitative analysis of binding between cellular proteins and E1A polypeptides. Binding of cellular proteins to E1A products was assessed in 8 separate studies involving *wt-dl*520 and various E1A deletion mutants, as described in Figure 2. The amounts of each protein were quantified from autoradiographs, as described in Materials and Methods and in the text, and then expressed as a percentage of levels obtained with *wt-dl* 520. Some data from a more limited study (Barbeau *et al.*, 1993) have been incorporated into the figure. The schematic diagram at the top is a summary of binding data in which the importance of regions of the E1A molecule in interactions with $p105^{Rb}$, p107, $p60^{cycA}$, p130, p300 and p400 has been indicated by the height of the boxes representing the % inhibition of binding relative to E1A amino terminal sequences and the locations of the various E1A deletion mutants (first and last residues) which are indicated just below. The open boxes with *dl*1107/520 indicate that certain residues within this mutant are not required for binding of the $p105^{Rb}$-family of proteins, as described by Whyte *et al.*, 1989 and Giordano *et al.*, 1991b. The average values for % binding relative to *wt* derived from 3 to 8 separate experiments (± standard error) have been provided in a table towards the bottom of the figure. At the bottom have been listed the abilities of these mutants to transform baby rat kidney cells in combination with activated p21Ha-ras in DNA-mediated assays published in previous studies (Jelsma *et al.*, 1989) with ++ being the efficiency obtained with *wt* E1A . nd, not determined.



% Binding	dl1101	dl1102	dl1103	dl1104	dl1141	dl1105	dl1142	dl1106	dl1107	dl1108	dl1109
p400	0(±0)	0(±0)	0(±0)	53(±12)	50(±28)	62(±35)	69(±19)	102(±31)	114(±83)	120(±68)	155(±79)
p300	0(±0)	58(±11)	27(±10)	3(±5)	6(±5)	21(±15)	65(±27)	89(±18)	106(±34)	89(±27)	65(±23)
p105Rb	56(±11)	135(±9)	20(±9)	10(±10)	50(±22)	83(±6)	74(±32)	100(±22)	0(±0)	0(±0)	23(±15)
p107	74(±12)	128(±5)	41(±8)	36(±9)	43(±11)	71(±7)	66(±22)	103(±11)	73(±14)	4(±4)	55(±12)
p60cycA	57(±11)	120(±20)	43(±19)	38(±20)	86(±33)	115(±37)	65(±37)	91(±34)	36(±24)	0(±0)	40(±35)
p130	45(±6)	98(±8)	29(±10)	14(±13)	40(±9)	69(±12)	69(±35)	99(±31)	0(±0)	0(±0)	0(±0)
Transformation		++			nd	++	nd	++			

The mutations that affected binding of p300 included those at the amino terminus (*dl* 1101/520) and within CR1 from residues 36 to 81 (*dl* 1103/520, *dl* 1104/520, *dl* 1141/520 and *dl* 1105/520). Binding of p300 absolutely required a sequence within the first 25 residues at the amino terminus, and was greatly dependent on CR1, especially residues 48-69. Elimination of residues 36-47 and 70-81 had moderate or small effects, respectively. The region between CR1 and CR2, and CR2 itself were of little or no importance. [The slight decrease seen with *dl*1109/520 may have resulted from the reduced levels of E1A proteins synthesized by this mutant.] The binding pattern for p400 was somewhat similar except that regions within the entire 48 amino terminal residues were essential, and CR1 was less important. These results indicated that although these proteins were highly related, they interacted with E1A molecules in distinctive fashions.

Binding of p105^{Rb} absolutely required a region containing the first 7 residues of CR2 which comprise the conserved Asp^{121}-Leu-X-Cys-X-Glu binding motif, and to some extent the remainder of CR2, although the role of the carboxy terminal portion of CR2 (aa 128-138) could have been overestimated because *dl*1109/520 synthesizes lower levels of E1A proteins. In addition, mutants lacking the first 20 amino acids of CR1 (residues 36-60) were considerably impaired in complex formation. Previous studies had also implicated sequences within CR1 in binding p105^{Rb} but the precise region had not been well defined. Certain conserved residues located between amino acids 39 and 49 and found in other p105^{Rb}-binding proteins had been proposed to play a role. The results presented here clearly indicated that the entire region between residues 36 to 60 was important including the relatively acidic region between amino acids 50 and 60. Mutant *dl* 1104, in which this latter region had been deleted, had the lowest binding capacity. Residues 61-69 were of some importance, but the remaining portion of CR1 did not appear to be critical. In addition, it was noted that deletion of the amino terminus had some effect on p105^{Rb} binding. This region had never been found to be of importance in previous more limited

studies. As discussed below, these sequences may play a direct role in p105^{Rb} binding, or they may function in a cooperative fashion with the CR2 and CR1 binding sites.

Binding of p130 displayed a pattern virtually identical to that of p105^{Rb} except that the entire CR2 region, including residues 128-138, was essential.

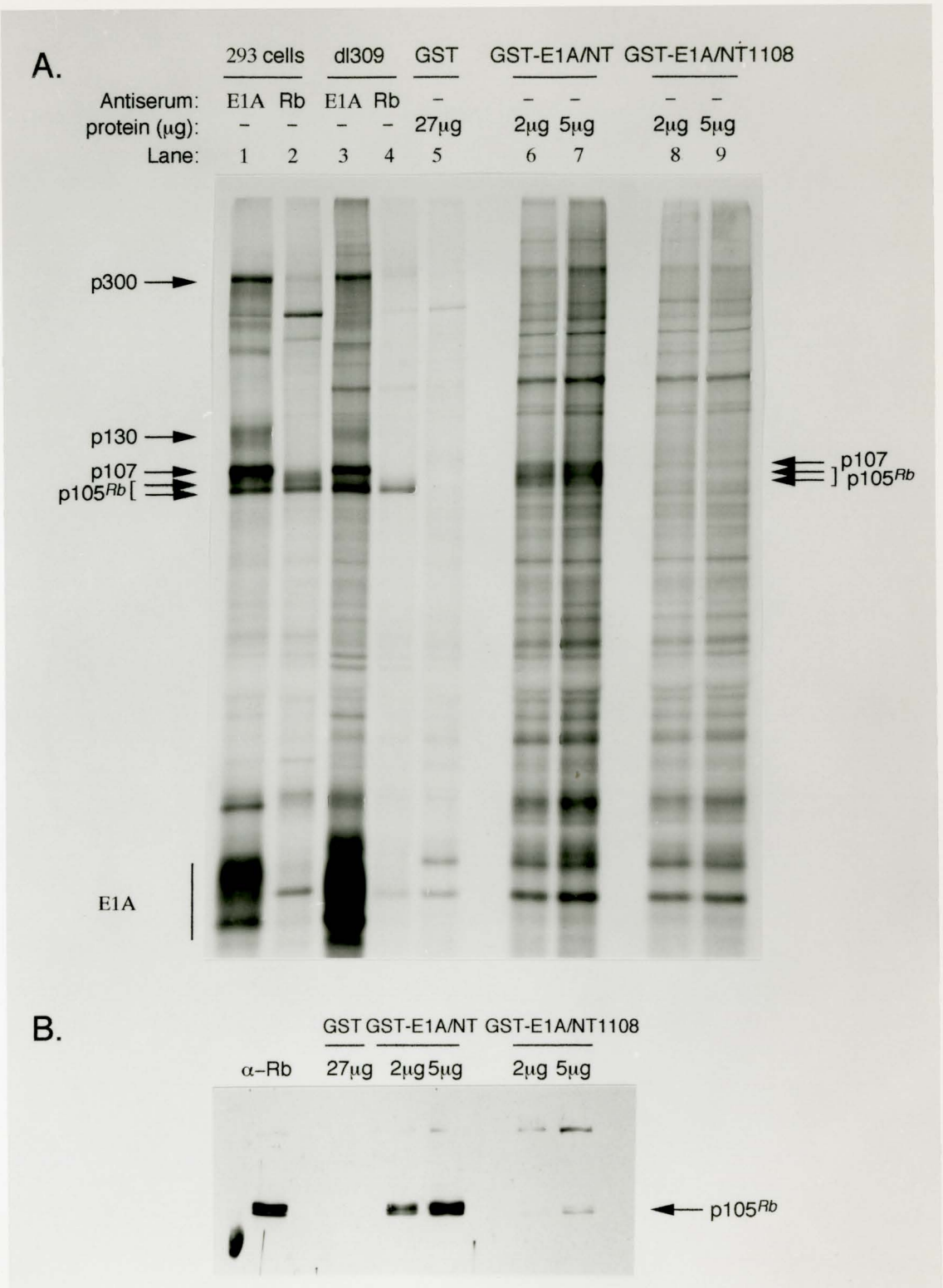
p107 binding was known to be affected by mutations in CR2 that overlapped the binding domain of p105^{Rb} (Egan *et al.*, 1988; Whyte *et al.*, 1989; Svensson *et al.*, 1991). In addition, some effect of mutations within CR1 had been observed by some groups (Egan *et al.*, 1988; Svensson *et al.*, 1991). Figure 12 shows that binding of p107 generally paralleled that of p105^{Rb} and p130 except that there was much less dependence on CR1 and the amino terminus, and the region within CR2 essential for complex formation was much more limited in that deletion of Asp^{121}-Leu122 (*dl*1107) within the p105^{Rb} binding core clearly had little effect on binding. In addition, removal of the acidic downstream region in *dl* 1109/520 at best only partially reduced binding. p107 was also unique in that no mutant, including a large series of recently constructed point mutants (H. B. Corbeil & P.E. Branton, unpublished), failed completely to interact with this protein. Previous studies suggested that p105^{Rb} and p107 are similar in sequence within the 'binding pocket' that interacts with E1A proteins and SV40 T antigen (Ewen *et al.*, 1991). Present results suggested that p130 also resembles p107, presumably within 'binding pocket' sequences. Nevertheless, each of these proteins displayed distinct patterns of binding.

The pattern of binding observed with p60^{cycA} clearly resembled that of p107 and p130, as was expected because p60^{cycA} probably interacts with E1A proteins indirectly through associations with these two proteins (Ewen *et al.*, 1992; Faha *et al*, 1992; Howe & Bayley, 1992).

3.3 Role of E1A protein domains in complex formation

Although little is known about their individual functions, complex formation with all of the E1A-binding proteins clearly requires at least two domains within E1A polypeptides. To study the roles of these domains further, the amino terminal 159-residue portion of E1A 289R was expressed as a fusion protein with glutathione S-transferase (GST) by Rachel Charbonneau in our lab. In addition to this product, termed GST-E1A/NT, a mutant lacking residues 124-127 within CR2, termed GST-E1A/NT1108, was also expressed. These proteins were mixed with glutathione-Sepharose beads and incubated with extracts from [^{35}S]methionine-labeled KB cells to assess complex formation *in vitro*. Figure 13a (kindly provided by Rachel Charbonneau) shows a comparison of *in vitro* binding patterns with those obtained by immunoprecipitation of KB cells infected with Ad5 *wt -dl* 309 or Ad5-transformed 293 cells using either E1A- or p105^{Rb}-specific sera. A species confirmed as p300 by V-8 protease digestion (data not shown, Rachel Charbonneau, unpublished results) was detected using both GST-E1A/NT and GST-E1A/NT1108 (lanes 6-9) but not with GST alone (lane 5), indicating again that binding was unaffected by the absence of CR2. Binding of p130 or p60^{cycA} was not studied in these experiments because the levels of p130 were too low and several species were present in the region of the gel containing p60^{cycA}. Proteins migrating in the positions of p107 and p105^{Rb} were observed in material binding to GST-E1A/NT (lanes 6 and 7). A similar pattern was seen with GST-E1A/NT1108 (lanes 8 and 9) except that the amounts of most of these species were considerably reduced. One appeared to bind non-specifically as it was detected with GST alone (lane 5). The protein that co-migrated with p107 was identified as p107 by V-8 peptide analysis (data not shown, Rachel Charbonneau, unpublished results). Three species which bound to GST-E1A/NT but not to GST alone were recognized in Western

Figure 13. Analysis of affinity purified E1A-binding proteins by SDS-PAGE. Figure 13a: 35S-labeled proteins. GST-E1A fusion proteins GST-E1A/NT and GST-E1A/NT1108 (2 or 5 μg), or GST alone (27 μg) were used to affinity purify proteins from extracts of [^{35}S]methionine-labeled KB cells, as described in Materials and Methods. Bound material was analyzed by SDS-PAGE along with immunoprecipitates from labeled 293 or dl 309-infected cells prepared using anti-E1A (M73) or anti-p105^{Rb} (C36) sera. The identity of cells, antibodies and fusion proteins have been given in the figure. The positions of migration of p300, p130, p107, p105^{Rb}, and E1A products are shown at the right and/or the left. Figure 13b: Western blotting of affinity purified p105^{Rb}. Studies similar to those described above were carried out using extracts from unlabeled KB cells. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes and analyzed by Western blotting using Rb-3 antibody, as described in Materials and Methods.


A.
293 cells
dl309
GST
GST-E1A/NT
GST-E1A/NT1108
Antiserum:
E1A
Rb
E1A
Rb
–
–
–
–
–
protein (μg):
–
–
–
–
27μg
2μg
5μg
2μg
5μg
Lane:
1
2
3
4
5
6
7
8
9
p300
p130
p107
p105Rb
E1A
p107
p105Rb
B.
GST
GST-E1A/NT
GST-E1A/NT1108
α–Rb
27μg
2μg
5μg
2μg
5μg
p105Rb

blotting analysis using p105^{Rb}-specific serum (Figure 13b), and thus presumably represented differentially phosphorylated forms of p105^{Rb}. p105^{Rb} was also found to bind to GST-E1A/NT1108, although quantitative analysis of the Western blots shown in Figure 13b indicated that the level was only 1.5% of that found with GST-E1A/NT, and not all of the p105^{Rb} species were present. These results indicated that binding of p105^{Rb} (and p107) to E1A proteins can occur in the absence of the core binding site in CR2, but only at very low levels. The failure to detect binding of p105^{Rb} with mutant *dl*1108/520 in the studies described in Figure12 may have been due to differences in complex formation occurring *in vivo* and *in vitro* , or in the sensitivity of detection in the two experiments. Thus it is likely that weak interactions can occur between p105^{Rb} and p107 and regions outside of CR2, presumably within the sequences in CR1 or the amino terminus identified in Figure 12.

Another approach to study the roles of different domains of E1A proteins was to carry out complementation experiments in which different portions of E1A products involved in complex formation were provided on separate E1A molecules. Figure 14 shows results obtained in cells doubly infected with mutants containing deletions in the amino terminus, CR1, or CR2. None of the combinations tested resulted in restoration of *wt* levels of binding of any of the E1A-associated proteins. For example, provision of the amino terminus and the entire CR1 region by co-infection with *dl*1101/520 and *dl*1103/520 or *dl*1104/520 resulted in no increase in binding of p300 over levels seen with individual mutants. Similarly, binding of p105^{Rb} was unchanged using a combination of CR2 (*dl*1107/520 or *dl*1108/520) and CR1 (*dl*1103/520 or *dl*1104/520) mutants. These results suggested that interactions between two or more regions within E1A proteins and these cellular polypeptides may result in complex formation through cooperative interactions.

Figure 14. Analysis by SDS-PAGE of E1A complex formation in cells infected by combinations of E1A deletion mutants. Cells were infected with E1A deletion mutants, either singly or in combination, and immunoprecipitates were analyzed by SDS-PAGE, as described in Figure 9. Mutants (lanes 3-12) or mutant combinations (lanes 14-26) for each lane have been given at the top, and the positions of migration of relevant proteins at the left.

CHAPTER 4: Phosphorylation within E1A protein complexes

E1A complexes contain protein kinase activity which originates at least in part from cyclin -associated $p33^{cdk2}$ (Tsai *et al.*, 1991, Giordano *et al.*, 1991a; Herrmann *et al.*, 1991, Kleinberger & Shenk, 1991). Experiments were carried out in which complexes purified using either E1A- or $p60^{cycA}$-specific sera (M73 and C160) were incubated *in vitro* with [γ^{32}P]ATP Figure 15 shows results from a study representative of 6 separate experiments. With M73 precipitates from *wt-dl* 520-infected cells (lane 3), phosphorylation of p130 and p107 occurred at very high levels, and labeling of a 300kDa species was also observed. These proteins were confirmed as being p300, p130 and p107 by V-8 peptide mapping which indicated the presence of phosphopeptides that were similar to a subset of those present in p300, p130 and p107 labeled *in vivo* with [^{32}P]orthophosphate (Figure 16). Interestingly, upon examination of phosphopeptides generated from *in vivo* labeled p300, p130 and p107, these all appeared to be related, whereas no such similarities were observed with *in vitro* phosphorylated p300, p130 and p107 The relatedness of p130 to p107 was therefore not as apparent with peptide patterns obtained from *in vitro* phosphorylated p130 and p107 (Figure 17) as compared to those obtained from [^{35}S] labeled proteins (Figure 10) and *in vivo* phosphorylated proteins (Figure 17). Given the limited value of V8 protease analysis, additional experiments would be required to address this point. In Figure 15, labeling of $p60^{cycA}$ and E1A products was also observed at substantial levels (lane 3). Phosphorylation of a 200kDa protein was present at similar levels in M73 precipitates from cells infected by mutants affecting regions across the entire E1A molecule and thus will not be discussed further Little or no phosphorylation took place using material precipitated from uninfected KB cells by M73 serum (lane 1).

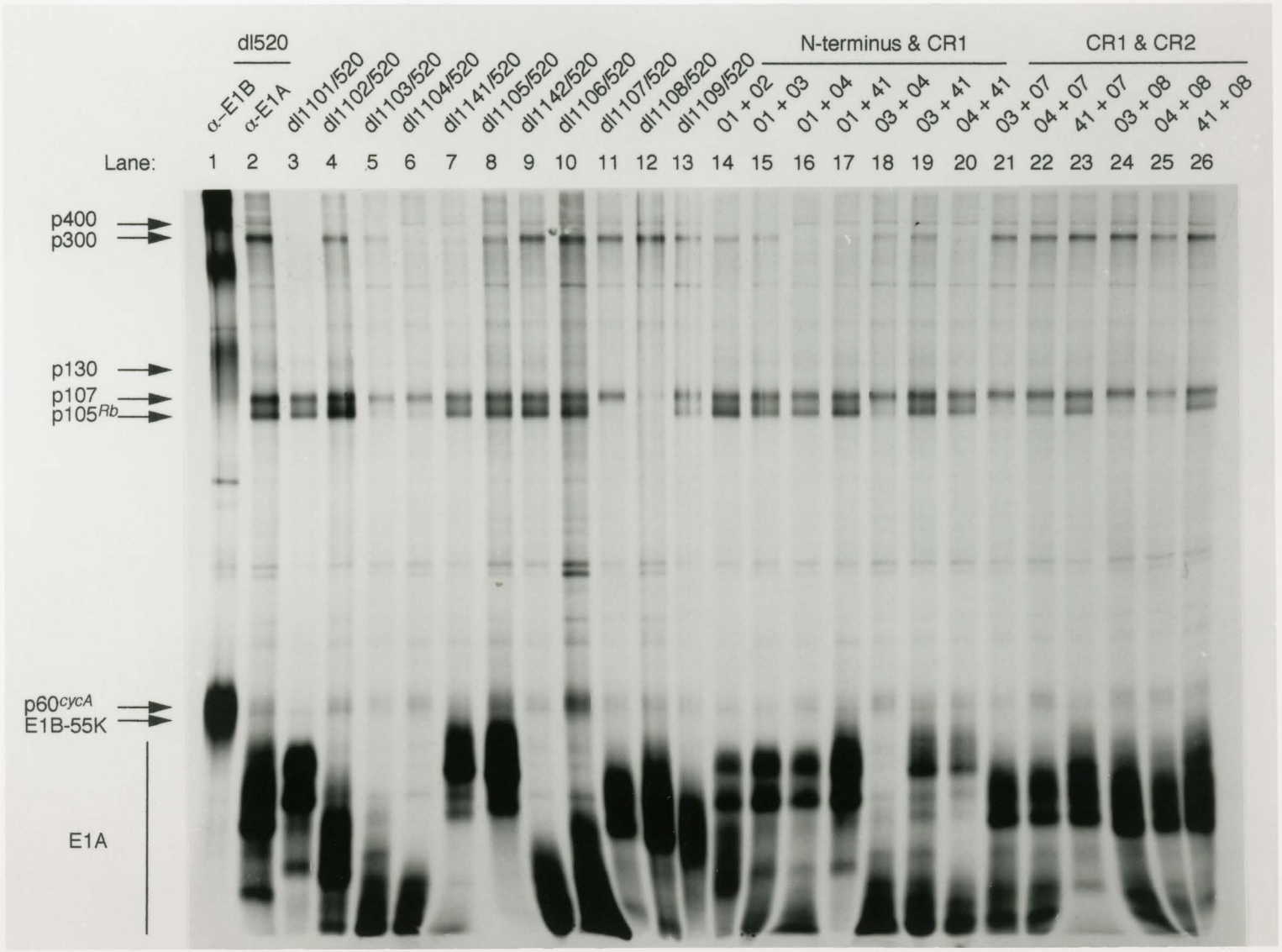

dl520
N-terminus & CR1
CR1 & CR2
α–E1B
α–E1A
dl1101/520
dl1102/520
dl1103/520
dl1104/520
dl1141/520
dl1105/520
dl1142/520
dl1106/520
dl1107/520
dl1108/520
dl1109/520
01 + 02
01 + 03
01 + 04
01 + 41
03 + 04
03 + 41
04 + 41
03 + 07
04 + 07
41 + 07
03 + 08
04 + 08
41 + 08
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
p400
p300
p130
p107
p105Rb
p60cycA
E1B-55K
E1A

Figure 15. Analysis of E1A complexes by *in vitro* phosphorylation. Protein complexes were purified by immunoprecipitation using M73 anti-E1A (E) or C160 anti-$p60^{cycA}$ (A) antibodies, precipitates were incubated with [γ^{32}P]ATP, and the mixtures were analyzed by SDS-PAGE, as described in Materials and Methods. Names of mutants for each lane have been given at the top and the positions of migration of [^{35}S]methionine-labeled p300, p130, p107, $p105^{Rb}$, and E1A products (lanes not shown) at the left. The open arrow shown for $p105^{Rb}$ indicates the position where $p105^{Rb}$ would be detected if it were phosphorylated by E1A-associated kinases.

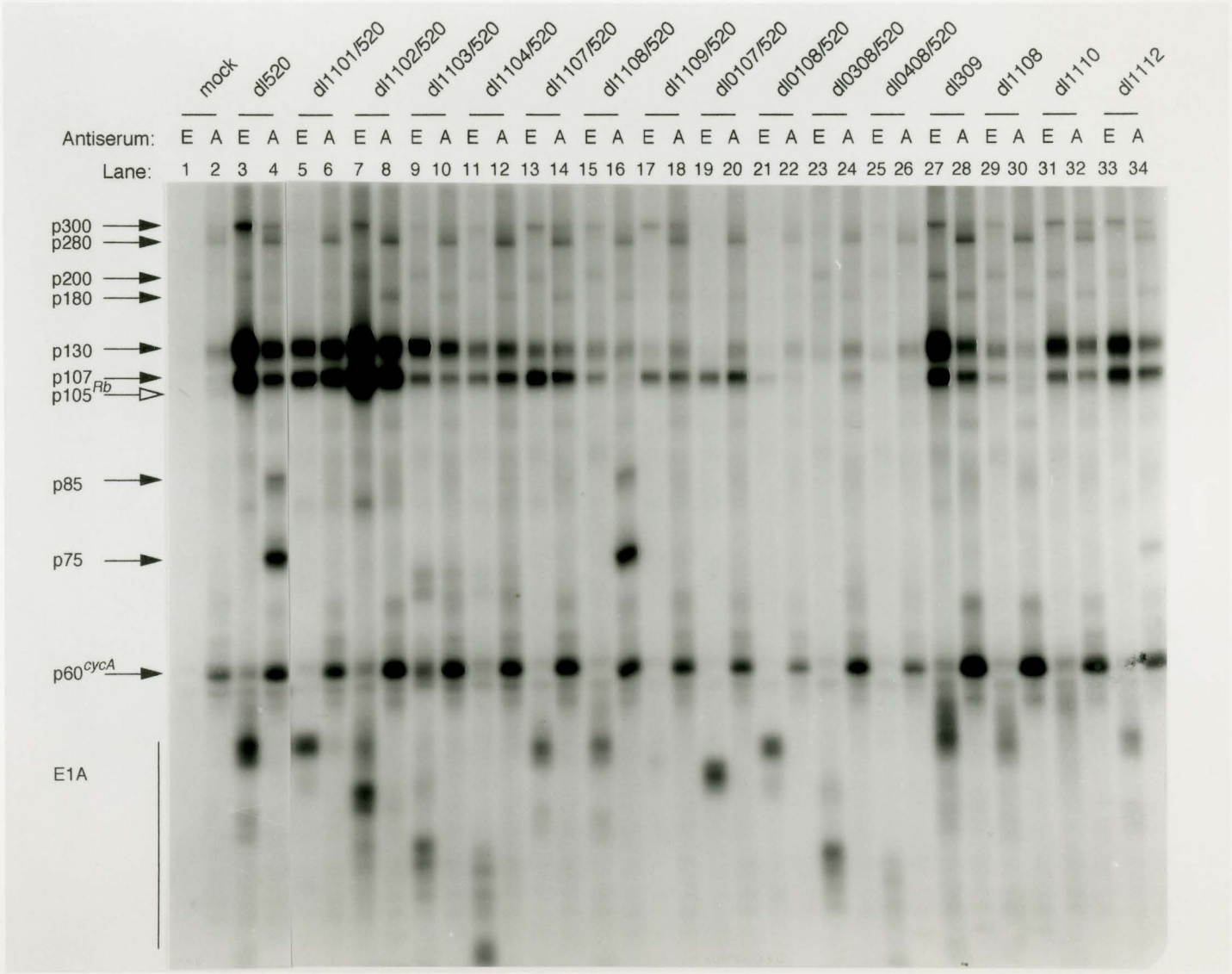

mock
dl520
dl1101/520
dl1102/520
dl1103/520
dl1104/520
dl1107/520
dl1108/520
dl1109/520
dl0107/520
dl0108/520
dl0308/520
dl0408/520
dl309
dl1108
dl1110
dl1112
Antiserum: E A E A E A E A E A E A E A E A E A E A E A E A E A E A E A E A E A E A E A E A
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
p300
p280
p200
p180
p130
p107
p105Rb
p85
p75
p60cycA
E1A

Figure 16. V8 peptide analysis of *in vivo* and *in vitro* [^{32}P] labeled E1A protein complexes. Cells infected with *dl* 520 were either unlabeled or labeled *in vivo* with [^{32}P]orthophosphate and cell extracts were immunoprecipitated with E1A-specific M73 serum. *In vitro* kinase assays as described in Materials and Methods were performed on unlabeled extracts for one hour at 37^{0}C. V8 peptide patterns from *in vivo* and *in vitro* labeled p300, p130, p107 cellular proteins were compared. Samples were treated with 3 and 12μg of V8 protease.

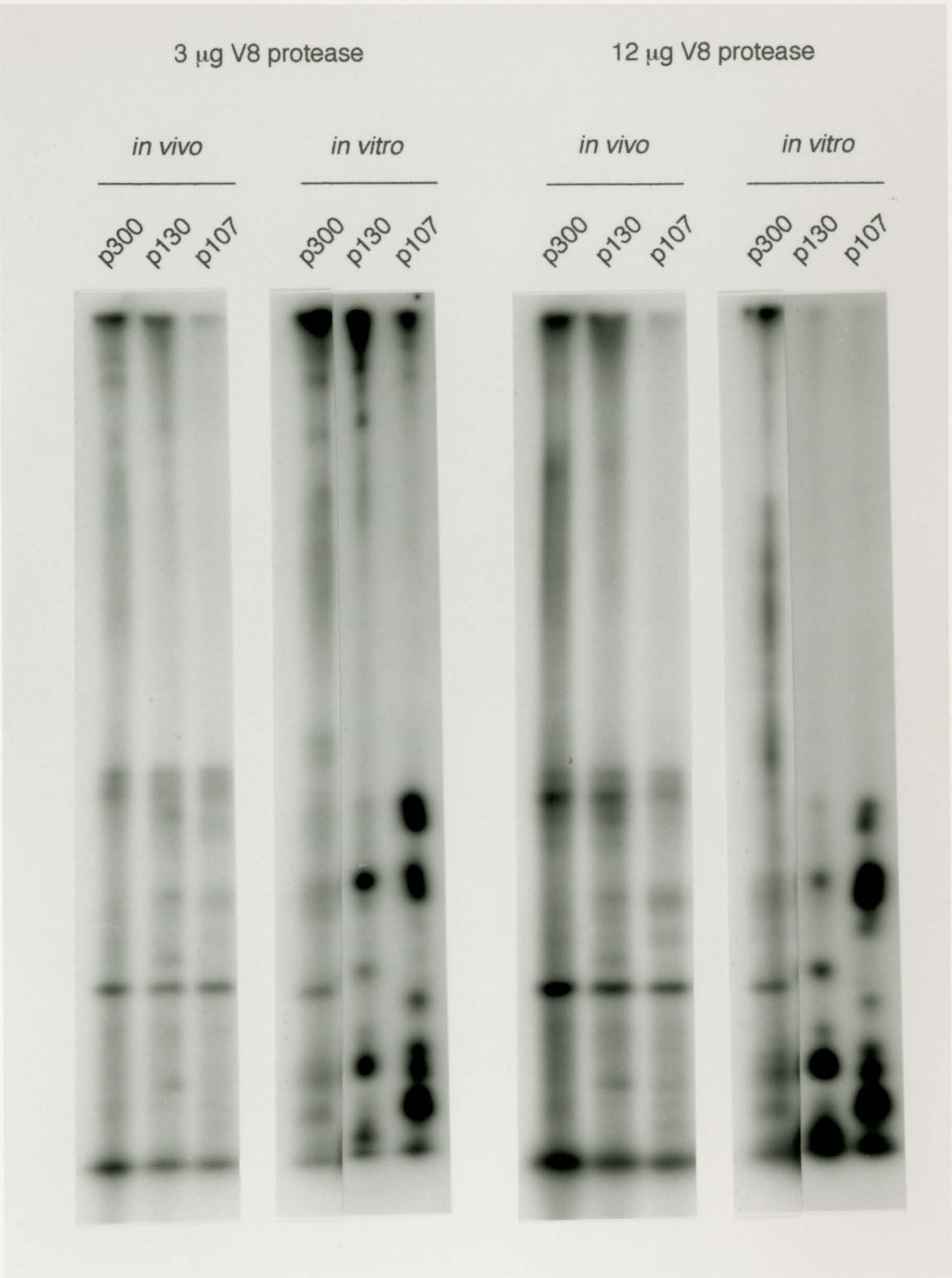
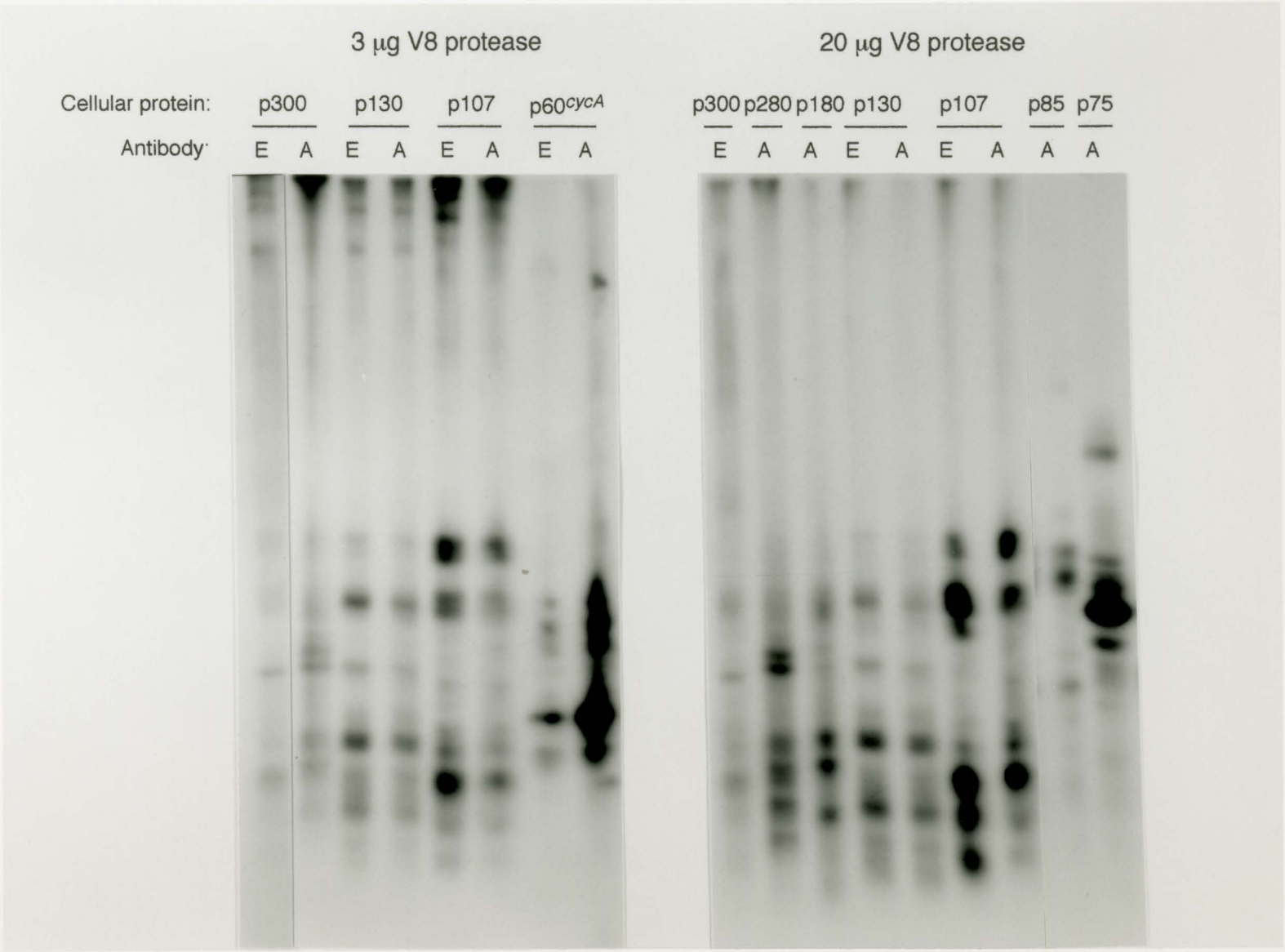

3 μg V8 protease
12 μg V8 protease
in vivo
in vitro
in vivo
in vitro
p300
p130
p107
p300
p130
p107
p300
p130
p107
p300
p130
p107

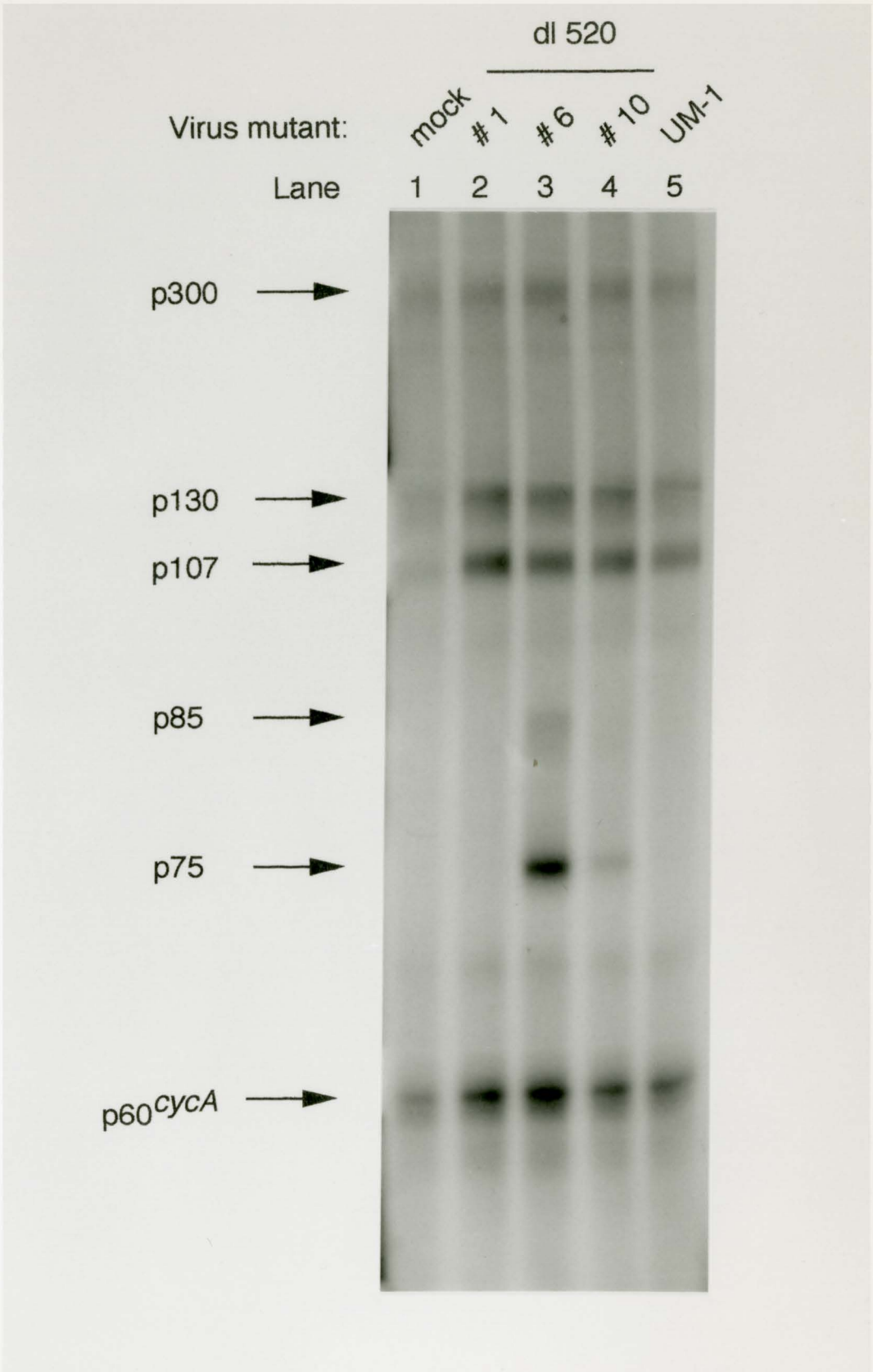
Figure 17. V8 Peptide analysis of *in vitro* [^{32}P] labeled cellular proteins demonstrating relatedness between p60^{cycA} and E1A immunoprecipitated complexes. E1A-specific M73 and p60^{cycA}-specific immunoprecipitates were prepared as descibed in Figure 16 and analyzed using 5 and 12μg of V8 protease. The identities of cellular E1A-binding proteins have been given at the top, and the antibodies involved given just below, including E1A-specific M73 serum (E) and p60^{cycA}-specific C160 serum (A). A number of additional proteins detected with p60^{cycA}, p280 p180, p85 and p75, were also analyzed.


3 μg V8 protease
20 μg V8 protease
Cellular protein:
p300
p130
p107
p60cycA
p300
p280
p180
p130
p107
p85
p75
Antibody:
E
A
E
A
E
A
E
A
E
A
A
E
A
E
A
A
A

With $p60^{cycA}$ complexes from uninfected cells (lane 2), $p60^{cycA}$ was labeled as were, at low levels, species confirmed to be p130 and p107 by V-8 peptide mapping. Figure 17 indicates that [γ^{32}P]ATP *in vitro* labeled p130, p107 and $p60^{cycA}$ generated similar digestion patterns whether isolated from M73 or $p60^{cycA}$ precipitates. In addition, several minor labeled species of about 280, 180, 106, 90, 68, and 65kDa were detected (Figure 15) at similar low levels in all $p60^{cycA}$ precipitates. These polypeptides could represent $p60^{cycA}$ -binding proteins, but could also be present non-specifically. V-8 protease digestion of p280, p180 yielded peptides with some similarities to those generated from p300 but not to p130 or p107 (Figure 17). The identity of these proteins is not known and thus will not be discussed further.

p130 and p107 were phosphorylated to much higher levels in $p60^{cycA}$ precipitates from *dl* 520-infected cells (Figure 15; lane 4) than in those from uninfected cells (lane 2). Labeling of $p60^{cycA}$ was somewhat higher in this particular experiment, but over the course of several experiments, neither the level of $p60^{cycA}$ protein detected by C160 serum nor *in vitro* labeling of $p60^{cycA}$ differed substantially in infected and uninfected cells. In addition, a species confirmed to be p300 by V-8 peptide mapping (Figure 17) was found to be phosphorylated only in $p60^{cycA}$ precipitates from Ad5-infected cells. Labeling of E1A products also occurred in these preparations, but at very low levels evident only upon much longer exposures of the autoradiographs. These results suggested that the presence of E1A proteins in complexes containing $p60^{cycA}$ led to the phosphorylation of p300 and a substantial increase in that of p130 and p107. Phosphorylation of species of about 85 and 75kDa was also observed in $p60^{cycA}$ precipitates prepared from *dl* 520- and *dl* 1108/520-infected cells in this experiment (lanes 4 and 16, respectively). We attempted to characterize these proteins. Phosphopeptide patterns generated from these two proteins bore no similarities to any of the E1A-binding proteins or to each other (Figure 17). It was

Figure 18. Analysis of the 85 and 75kDa species present in $p60^{cycA}$ complexes. *In vitro* kinase assays were preformed on $p60^{cycA}$ immunoprecipitates using various stocks of *dl* 520. Stock #6 was the one used in experiments as shown in Figure 15. UM-1 is essentially like *dl* 520 but contains a silent mutation to facilitate detection and recovery of viruses. The position of migration of $p60^{cycA}$-associated proteins, including p85 and p75, is indicated on the left.


dl 520
Virus mutant:
mock
#1
#6
#10
UM-1
Lane
1
2
3
4
5
p300
p130
p107
p85
p75
p60cycA

observed, however, that the 85 and 75kDa species were only present using certain stocks of these two viruses and not others. As illustrated in Figure 18, only two out of four stocks of *dl*520 showed the presence of these bands. We have no explanation for their appearance, and they will not be discussed further.

It was interesting to note that $p105^{Rb}$ was not phosphorylated in any *in vitro* study. It has been suggested that $p105^{Rb}$ is associated with *cdc2* -like kinase activity (Kitagawa *et al.*, 1992; Hu *et al.*, 1992; Qian *et al.*, 1992), but the conditions employed in the present experiments may not have been appropriate to detect this activity (see Discussion).

Results obtained using a large series of E1A deletion mutants (Figures. 8 and 15; Table 2) indicated that phosphorylation of p107 generally corresponded to the levels of p107 shown to be present in the complexes (Figure 12). Mutants with deletions in CR3 or the carboxy terminus of E1A yielded *wt* levels of p107 phosphorylation (Table 2). In the case of *pm*1131/520, negative results were obtained with E1A immunoprecipitates, since the M73 epitope maps within the amino acids deleted in this mutant (Table 2). E1A complexes isolated from cells infected by CR1 or CR2 mutants which showed reduced binding of p107 (e.g. *dl*1103/520, *dl*1104/520, and *dl*1109/520, lanes 9, 11, and 17, respectively), displayed reduced ^{32}P labeling, whereas with mutants containing deletions in both CR1 and CR2 and which were shown to bind very small quantities of p107 (e.g. *dl*1101/08/520, *dl*1103/08/520, and *dl*1104/08/520; lanes 21, 23 and 25, respectively), labeling was extremely low or almost undetectable. In the case of $p60^{cycA}$ complexes, with CR1/CR2 mutants which bind virtually no p107 (lanes 22, 24 and 26) labeling of p107 was at low levels and very similar to those observed with uninfected cells (lane 2). Phosphorylation in complexes purified using $p60^{cycA}$-specific C160 serum was always less than that obtained using anti-E1A M73 antibodies. Under the conditions employed, virtually all $p60^{cycA}$ and E1A products were immunoprecipitated by these two sera. Thus

Table 2. Phosphorylation in E1A and $p60^{cycA}$ complexes.

* *In vitro* phosphorylation was carried out as described in Figure 15 using E1A and $p60^{cycA}$ complexes isolated from KB cells infected by mutant or wt Ad5, or from mock-infected or 293 cells. The amino acid regions lacking in these deletion mutants have been indicated in the Table and have been illustrated in Figure 1A. The data represent a summary of 8 separate experiments and have been presented qualitatively with [++++] representing the highest level of phosphorylation, [±] representing an extremely low level, and [-] representing the absence of label.

Table 2: Phosphorylation in E1A and cyclin A complexes

Mutant	Serum	p300	p130	p107	p105Rb	p60cycA	E1A	Deletion
mock	E1A	-	-	-	-	-	-	-
	p60	-	+	+	-	+++	-	
wt(dl520)	E1A	+++	++++	++++	-	+	+++	none
	p60	+	+++	+++	-	+++	+	
dl1101/520	E1A	-	+++	+++	-	+	+++	4-25 *
	p60	-	+++	+++	-	+++	+	
dl1102/520	E1A	++	++++	++++	-	+	+++	26-35
	p60	+	+++	+++	-	+++	+	
dl1103/520	E1A	+/-	+++	++	-	+	++	30-49
	p60	-	+++	++	-	+++	+/-	
dl1104/520	E1A	+/-	++	++	-	+	++	48-60
	p60	-	++	++	-	+++	+/-	
dl1141/520	E1A	nd	nd	nd	nd	nd	nd	61-69
	p60	-	+++	+++	-	+++	+/-	
dl1105/520	E1A	nd	nd	nd	nd	nd	nd	70-81
	p60	-	+++	+++	-	+++	+/-	
dl1142/520	E1A	nd	nd	nd	nd	nd	nd	82-92
	p60	+	+++	+++	-	+++	+/-	
dl1106/520	E1A	nd	nd	nd	nd	nd	nd	90-105
	p60	+	+++	+++	-	+++	+/-	
dl1107/520	E1A	++	++	+++	-	+/-	++	111-123
	p60	+	++	+++	-	+++	+/-	
dl1108/520	E1A	+	+	++	-	+/-	++	124-127
	p60	-	+	+/-	-	+++	+/-	
dl1109/520	E1A	+	+/-	++	-	+/-	+	128-138
	p60	+	+	++	-	+++	+/-	
dl0107/520	E1A	-	-	++	-	-	++	4-26 + 111-123
	p60	-	+	++	-	+++	+/-	
dl0108/520	E1A	-	+/-	+	-	-	++	4-26 + 124-127
	p60	-	+/-	+/-	-	+++	+/-	
dl0308/520	E1A	+/-	+/-	-	-	+/-	++	30-49 + 124-127
	p60	-	+/-	+	-	+++	+/-	
dl0408/520	E1A	-	+/-	-	-	+/-	+	48-60 + 124-127
	p60	-	+	+/-	-	+++	+/-	
pm1131/520	E1A	-	-	-	-	-	-	219-289
	p60	++	+++	+++	-	+++	+/-	
wt(dl309)	E1A	++	++++	++++	-	+	++	none
	p60	+	++	++	-	+++	+/-	
dl313	E1A	-	-	-	-	-	-	220-289
	p60	+	++	++	-	+++	+/-	
dl1108	E1A	+	+	+	-	+/-	++	124-127
	p60	-	+	+/-	-	+++	+/-	
pm975	E1A	+	++	++	-	+	++	289R only
	p60	+/-	+	+	-	+++	+/-	
dl1110	E1A	+	+++	++	-	+	++	140-160
	p60	+	++	++	-	+++	+/-	
dl1112	E1A	++	+++	+++	-	+	++	161-168
	p60	+	++	++	-	+++	+/-	
dl1113	E1A	++	++++	++++	-	+	+++	169-177
	p60	+	++	++	-	+++	+/-	
dl1114	E1A	++	++++	++++	-	+	+++	178-184
	p60	+	++	++	-	+++	+/-	
dl1115	E1A	++	++++	++++	-	+	++	188-204
	p60	+	++	++	-	+++	+/-	
dl1116	E1A	++	++++	++++	-	+	++	205-221
	p60	+	++	++	-	+++	+/-	
dl1132	E1A	++	++++	++++	-	+	++	224-238
	p60	+	++	++	-	+++	+/-	
293 cells	E1A	++	++++	++++	-	+	+	none
	p60	+	+++	+++	-	+++	-	

p107 must be linked to other molecules capable of enhancing its phosphorylation (see Discussion).

Phosphorylation of p130 closely paralleled the patterns described for p107 and correlated well with the levels of p130 associated with E1A products. p130 was clearly a better substrate than p107, considering that the former was apparently present in E1A precipitates in quite low amounts. Thus p130 may possess more phosphorylation sites or it may be more accessible to kinases.

$p60^{cycA}$ was phosphorylated in complexes purified using C160 serum, and this level was not significantly affected by infection with *wt* virus or with mutants defective in binding to p107 and p130. These results suggested that E1A proteins have little effect on $p60^{cycA}$ phosphorylation which presumably occurs largely via associated $p33^{cdk2}$. Labeling of $p60^{cycA}$ in E1A complexes was lower than with precipitates prepared using C160 serum and was greatly reduced with mutants showing impaired abilities to complex with p107 and p130. These results suggested that $p60^{cycA}$ interacts with E1A proteins exclusively through indirect associations with p107, p130, or perhaps other proteins with similar binding patterns.

Phosphorylation of p300 appeared to result at least in part through the binding of p300 to E1A molecules that were simultaneously linked to either p107 or p130. This idea was evident because phosphorylation of p300 did not occur in $p60^{cycA}$ complexes from uninfected cells (lane 2) or cells infected with mutants that failed to bind high amounts of p107 or p130 (lanes 16 and 30). As expected, E1A complexes from cells infected with mutants that failed to bind significant amounts of p300 (*dl*1101/520, *dl*1103/520 and *dl*1104/520; lanes 5, 9 and 11, respectively) showed little or no phosphorylation of p300. However, with mutants that showed defects in binding of p107 and p130 but which

associated with p300 normally (e.g. lanes 13, 15 and 17), reduced phosphorylation of p300 was observed. Nevertheless, some phosphorylation of p300 still took place, suggesting that p300 or E1A molecules are associated with kinases other than those linked to p107, p130, or p105^{Rb}.

E1A proteins were labeled to high levels in M73 precipitates, but were poorly phosphorylated in p60^{cycA} complexes. This difference was not surprising because E1A products are present in great excess. Nevertheless, they were clearly phosphorylated less efficiently than were p107 and p130. In addition, E1A proteins were labeled quite well even in E1A complexes prepared from cells infected with mutants that bind p107, p130 and p300 very poorly (e.g. lanes 21, 23 & 25). Thus in addition to p107- and p130-linked p33^{cdk2}, additional kinases may be present in E1A complexes. E1A molecules have previously been shown to be phosphorylated at serine residues 89, 96, 132, 219, as well as a number of serines located between residues 227-237 within AR2 of the C-terminus (Dumont *et al.*, 1989; 1993; Dumont & Branton, 1992; Richter *et al.*, 1988; Smith *et al.*, 1989; Tremblay *et al.*, 1988; 1989; S.G. Whalen, D. Barbeau, R.C. Marcellus, J.A. Howe, S.T. Bayley and P.E. Branton, submitted; S.G. Whalen, D. Barbeau, H.B. Corbeil, R.C. Marcellus, and P.E. Branton, in preparation). Previous studies by our group identified phosphorylation sites at Ser89 and Ser219 that exist within *cdc2* substrate consensus sequences that could be phosphorylated *in vitro* by purified p34^{cdc2} (Tremblay *et al.*, 1988; 1989; Dumont & Branton, 1992; Dumont *et al.*, 1993). In *in vitro* kinase studies similar to those mentioned above, analysis of tryptic peptides from E1A molecules labeled *in vitro* with [γ^{32}P]ATP in M73 complexes indicated that Ser-89 and Ser-219 were the only sites phosphorylated, possibly via p107- and p130-linked p33^{cdk2} (D. Barbeau, R. Charbonneau, S.G. Whalen, S.T. Bayley, & P.E. Branton, in press). Thus, although E1A proteins are known to be phosphorylated by other classes of kinases, these experiments indicated that *cdc2*-like enzymes may be the only class present in E1A complexes.

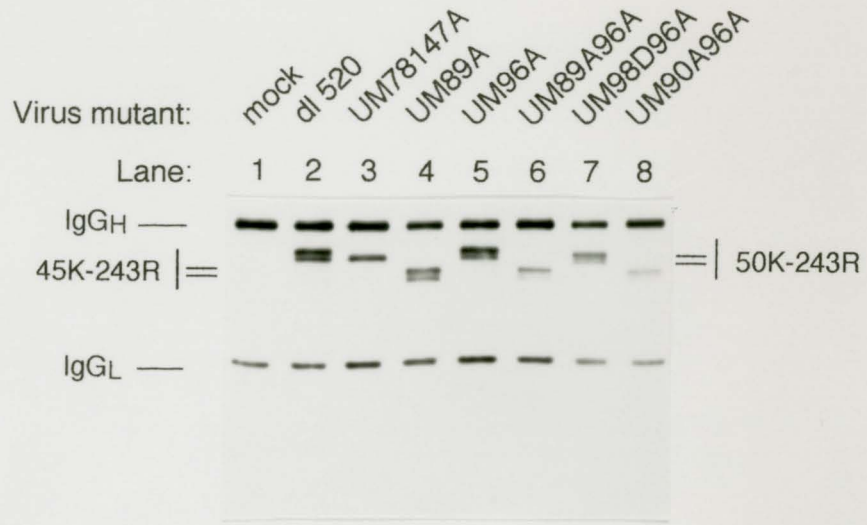
CHAPTER 5: Phosphorylation of E1A proteins and complex formation

5.1 Serine residues in AR2 play a role in a "supershift" in gel mobility of Ad5 E1A proteins.

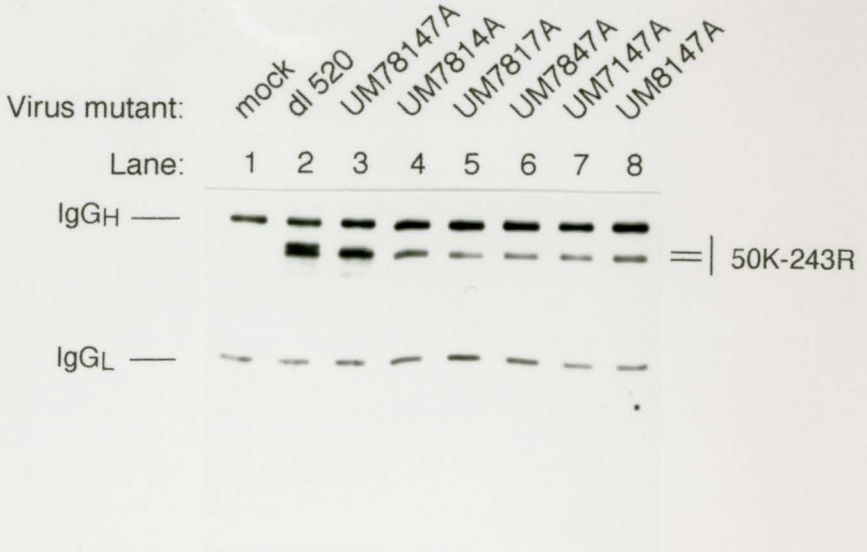
Phosphorylation at Ser-89 causes a "shift" in the migration of E1A proteins in SDS polyacrylamide gels by an amount corresponding to about 5kDa (Dumont and Branton, 1992; Dumont *et al.*, 1989; 1993; Richter *et al.*, 1988; Smith *et al.*, 1989; Tremblay *et al.*, 1989; Yee *et al.*, 1983). In the case of the 289R protein, a species of an apparent molecular mass of 48.5kDa was shown to be converted to one of about 52kDa, whereas with 243R, a 45kDa species was altered to one of 50kDa (Branton and Rowe, 1985; Dumont *et al.*, 1989). This large "shift" in migration probably results from addition of two or more phosphate groups as phosphorylation at Ser-89 appears to regulate that at Ser-96 and perhaps other nearby unidentified sites (Dumont *et al.*, 1993). In recent studies using slightly different conditions of SDS-PAGE, we observed that each of these major E1A species migrates in two forms. To determine if differences in phosphorylation were responsible for this "supershift" in mobility, E1A proteins were examined in human KB cells infected with *wt-dl* 520, or with two series of mutants in a *dl* 520 background. E1A proteins were immunoprecipitated from unlabeled infected cells using E1A-specific M73 mouse monoclonal antibody, separated by SDS-PAGE, and then identified by Western blotting using M73 antibody. Figure 19a shows that, in addition to the immunoglobulin heavy and light chains which were detected in all preparations, including those from mock-infected cells, two closely-migrating E1A species were present in a position corresponding to that of the 50kDa product. Prolonged treatment also revealed two minor species in the position of the 45kDa E1A species (not shown). Figure 19a also shows that several

Figure. 19. Analysis of E1A "supershifted" proteins by SDS-PAGE. Extracts from KB cells infected with *wt-dl* 520 or various mutants in a *dl* 520 background, or mock-infected, were immunoprecipitated with M73 monoclonal antibody and following separation by SDS-PAGE, E1A proteins were transferred to nitrocellulose membranes and identified by Western blotting with M73 using the ECL technique. Lanes are as indicated in the Figure. The positions of the 50kDa- and 45kDa-243R proteins and immunoglobulin heavy (IgG_H) and light (IgG_L) chains have been indicated at the right and left. (a) shows E1A mutants spanning various sites of the molecule. (b) was conducted with a series of mutants with defects affecting potential phosphorylaton sites in AR2.

A


Virus mutant:
mock
dl 520
UM78147A
UM89A
UM96A
UM89A96A
UM98D96A
UM90A96A
Lane:
1 2 3 4 5 6 7 8
IgGH
45K-243R
50K-243R
IgGL

B


Virus mutant:
mock
dl 520
UM78147A
UM7814A
UM7817A
UM7847A
UM7147A
UM8147A
Lane:
1 2 3 4 5 6 7 8
IgGH
50K-243R
IgGL

mutants with defects affecting phosphorylation at Ser-89, including UM89A (Ser-89 to Ala), UM89A96A (Ser-89 and -96 to Ala), and UM90A96A (Pro-90 and Ser-96 to Ala), yielded no "shifted" 50kDa E1A products, but rather those that migrated in the position of the 45kDa form, as shown previously (Dumont *et al.*, 1989; 1993). However, these species again were present as two closely-migrating forms. Elimination of the Ser-96 phosphorylation site (UM96A) had little effect on gel mobitity Introduction of an acidic residue at amino acid 89 was shown previously to induce only a partial "shift" in gel mobility (Dumont *et al.*, 1993), and Figure 19a shows a similar result with UM89D96A (Ser-89 to Asp, Ser-96 to Ala), except that two species were present which migrated in a position intermediate between the 50 and 45kDa forms. These results confirmed that phosphorylation involving Ser-89 was responsible for the major "shift" in gel mobility and showed that it played no part in the generation of the "supershifted" form of E1A proteins. To determine if the potential sites in AR2 at residues 227, 228, 231, 234 and 237 were involved, a mutant, termed UM78147A was constructed in which all of these serines were converted to alanine residues. Figure 19a shows that only the faster-migrating 50kDa E1A species was apparent. These results suggested that phosphorylation at one or more of these residues was responsible for the "supershift" in gel mobility To examine this phenomenon further, the pattern of E1A proteins was studied using a series of mutants into which codons for serine residues 227, 228, 231, 234 and 237 had been reintroduced individually into UM78147A. Figure 19b shows that all of these mutants yielded a single major E1A species present in the position of the faster-migrating 50kDa form observed with *wt-dl*520. As with the previous study, prolonged ECL treatment revealed a single minor 45kDa species (not shown). These results indicated that the "supershift" in gel migration of E1A proteins was not due to phosphorylation at a single site within the carboxy terminal AR2 region, but that two or more serine residues appeared to be involved.

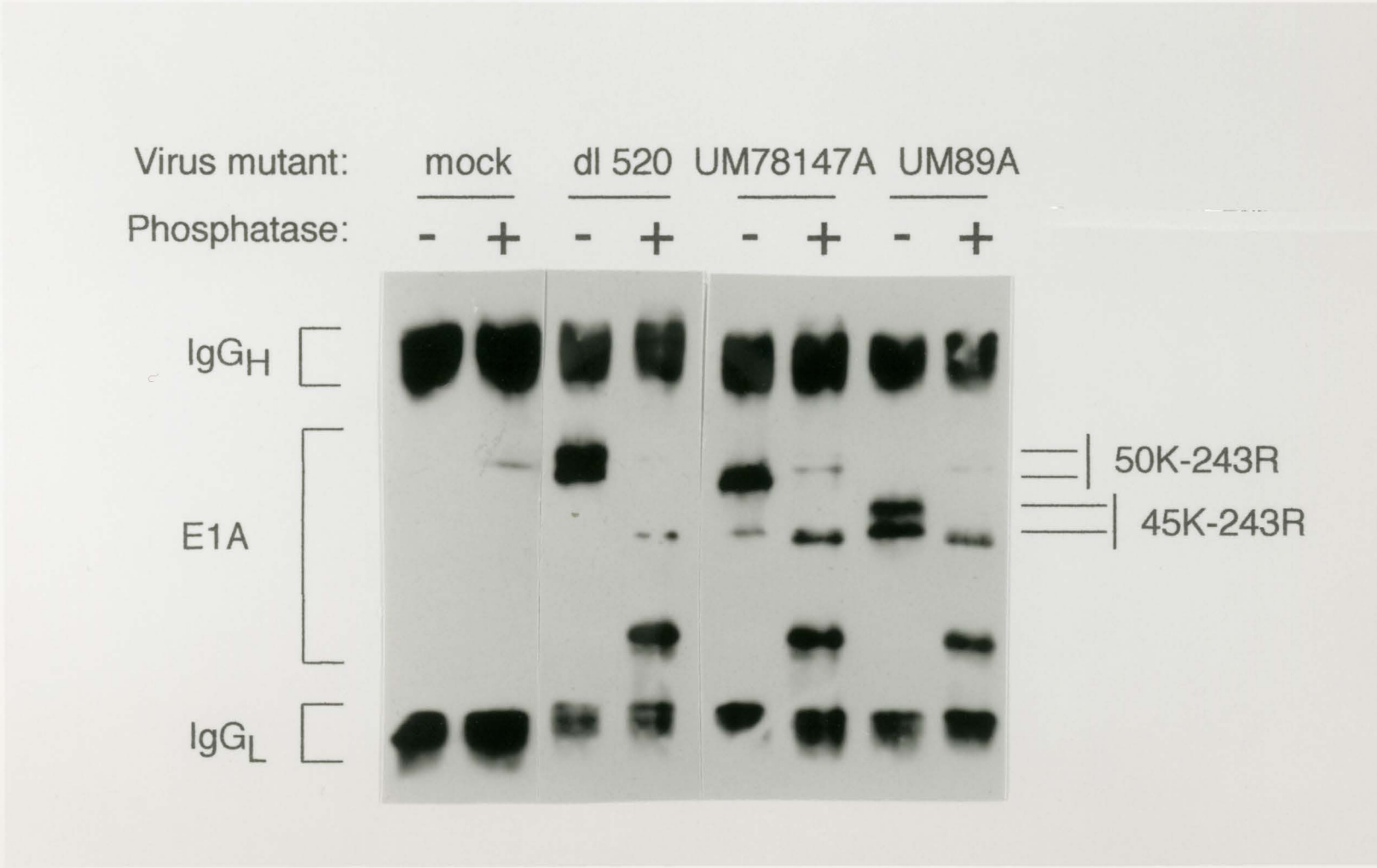
5.2 Phosphorylation is responsible for the "supershift" in gel mobility of E1A proteins.

To determine if phosphorylation was responsible for the "supershift" in gel migration, E1A proteins were immunoprecipitated from cells infected with *wt-dl* 520, UM78147A, or UM89A and then the precipitates were either subjected directly to SDS-PAGE and examined by Western blotting using M73 serum, or first treated with 1.25mg of potato acid phosphatase prior to analysis. Figure 20 shows that the patterns obtained with the untreated samples were identical to those described above, with *wt* and UM89A giving rise to faster-migrating and "supershifted" forms of the 50 and 45kDa E1A proteins, respectively, and UM78147A yielding only the faster-migrating form. Phosphatase treatment of UM78147A E1A protein eliminated the 50kDa species and yielded a single 45kDa form resulting from the dephosphoryation of Ser-89, as shown previously (Tremblay *et al.*, 1989). In addition, a single new and even faster-migrating species was also observed which was consistent with the dephosphorylation of other sites in the E1A protein molecule. With *wt* , both 50kDa forms were eliminated to yield the single faster-migrating 45kDa form as well as the new species. With UM89A, the "supershifted" form of the 45kDa species was eliminated and only the faster-migrating 45kDa protein as well as the new species remained. These results clearly suggested that the "supershift" in gel mobility is the result of phosphorylation.

5.3 "Supershifted" E1A proteins predominate in cyclin A complexes.

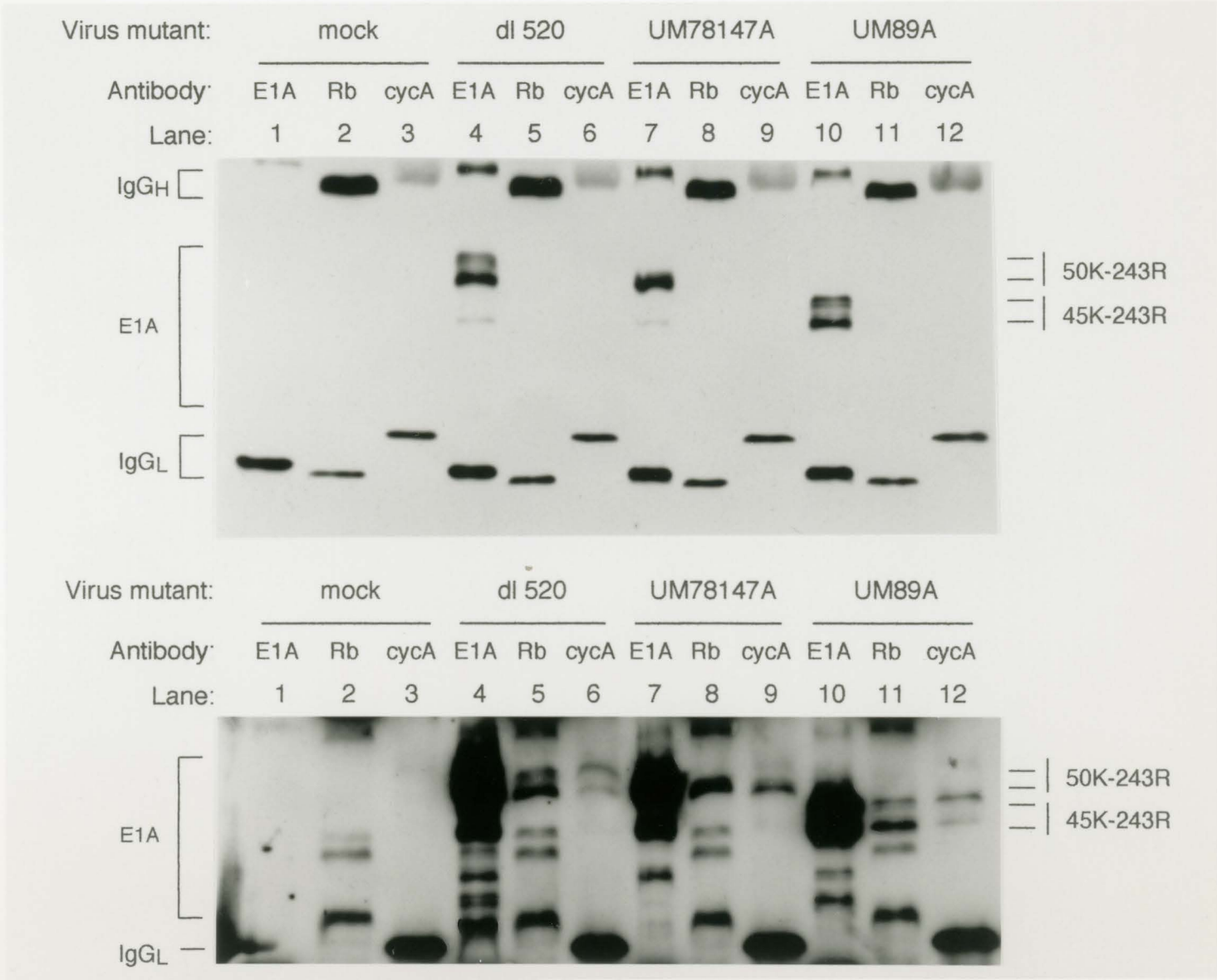
E1A products appear to be present at a considerable molar excess to the cellular proteins with which they interact. For example, the majority of $p105^{Rb}$ in infected cells exists within E1A complexes, but only about 1% of E1A products is associated with this cellular

Figure. 20. Phosphatase treatment of E1A proteins. Extracts from KB cells infected with *dl* 520, UM78147A, UM89A, or mock-infected cells, were immuno-precipitated using M73 serum, and, following incubation for 15 min at 37°C in the presence (+) or absence (-) of 1.25mg of potato acid phosphatase, E1A proteins were analyzed by SDS-PAGE and Western blotting using M73 serum. Lanes are as indicated and the positions of immunoglobulin heavy (IgG_H) and light (IgG_L) chains and of migration of the faster-migrating and "supershifted" forms of the 50 and 45kDa proteins have been shown.


Virus mutant:
mock
dl 520
UM78147A
UM89A
Phosphatase:
- + - + - + - +
IgG$_H$
E1A
IgG$_L$
50K-243R
45K-243R

protein (Egan *et al.*, 1989; Barbeau *et al.*, 1993). To determine if certain E1A species are favored in complex formation, extracts from KB cells infected with *wt-dl* 520 or mutants UM89A or UM78147A were immunoprecipitated using E1A-specific M73 serum or monoclonal antibodies which recognize $p105^{Rb}$ or $p60^{cycA}$, and proteins were separated by SDS-PAGE. Following transfer to nitrocellulose membranes, E1A polypeptides present in these precipitates were identified by Western blotting using M73. Figure 21 shows the results of one such experiment, the bottom panel representing a longer exposure of the gel shown at the top. Total 243R E1A protein precipitated by M73 from *wt-dl* 520-infected cells migrated largely in the position of the 50kDa polypeptide, the faster-migrating species present in excess of the "supershifted" form (lane 4). With UM89A these two species migrated in the position of the 45kDa form, again with the faster-migrating form in excess (lane 10). With UM78147A, as before, the "supershifted" species was absent and only the faster-migrating 50kDa form was detected (lane 7). None of the preparations from mock-infected cells contained E1A products and only proteins which reacted non-specifically were apparent (lanes 1-3). The patterns of E1A products present in complexes precipitated by $p105^{Rb}$-specific serum (lanes 5, 8 and 11) were exactly as those just described for total E1A protein with the faster-migrating species predominating. With complexes recognized by $p60^{cycA}$-specific serum the case was different. In precipitates from cells infected with *wt-dl* 520 or UM89A, such complexes contained predominantly the "supershifted" form of the 50kDa and 45kDa E1A species (Figure 21, lanes 6 and 12). In some cases, the "supershifted" form was actually resolved into two bands (UM89A). The results shown in Figure 21 represent the most conservative data obtained in several experiments in which, in some cases, only the "supershifted" form was detected in $p60^{cycA}$ complexes. Thus it appeared that the "supershifted" species either forms complexes more efficiently with $p60^{cycA}$ or is more stable in association with $p60^{cycA}$. Reasonable levels of the faster-migrating 50kDa E1A protein were detected with UM78147A (lane 9), suggesting that "supershifted" E1A products did not appear to be essential for complex formation. It was

Figure. 21 Analysis of E1A proteins within p105^{Rb} and p60^{cycA} protein complexes. Extracts from KB cells infected with *dl* 520, UM78147, UM89A, or mock-infected cells, were immunoprecipitated under mild conditions with one of the following sera: M73 which recognizes E1A proteins; C36 which recognizes p105^{Rb}; or C160 which recognizes p60^{cycA} . Following SDS-PAGE and transfer to nitrocellulose, E1A proteins were identified by Western blotting with M73 serum. Lanes are as indicated at the top. The positions of the immunoglobulin heavy (IgG_H) and light (IgG_L) chains are shown at the left and those of the two 50 and 45kDa E1A proteins at the right. The botton panel represents a longer exposure of the central portion of the gel shown at the top.


Virus mutant:
mock
dl 520
UM78147A
UM89A
Antibody:
E1A
Rb
cycA
Lane:
1
2
3
4
5
6
7
8
9
10
11
12
IgGH
E1A
IgGL
50K-243R
45K-243R

more likely therefore that complex formation with $p60^{cycA}$ stabilizes "supershifted" E1A proteins.

5.4 Effect of E1A phosphorylation on complex formation

E1A products appear to induce cell transformation largely through interactions with a series of cellular proteins, including p300, $p105^{Rb}$, p130, and p107, and with $p60^{cycA}$ which may associate indirectly via interactions with p107 and p130 (Yee & Branton, 1985; Harlow *et al.*, 1986; Egan *et al.*, 1987; 1988; 1989; Whyte *et al.*, 1988a; 1988b; 1989; Barbeau *et al.*, 1992; Ewen *et al.*, 1991; Giordano *et al.*, 1989; 1991b; Pines & Hunter, 1990; Stein *et al.*, 1990; Svensson *et al.*, 1991). To determine if complex formation is affected by phosphorylation at N-or C-terminal sites, extracts from KB cells infected with *wt* or various phosphorylation mutants were immunoprecitated using E1A-specific M73 serum under appropriate conditions, and the resulting protein complexes were analyzed by SDS-PAGE. The phosphorylation mutants shown in Figure 22 are present in different E1A backgrounds. The UM series of mutants as described earlier are contained within the 243R background only (*dl* 520), whereas *pm*975/953 and the AD series of mutants are present in the 289R (*pm* 975), and 289R and 243R (*dl* 309) backgrounds, respectively. At first glance, E1A proteins produced by all mutants appeared to form complexes with these cellular polypeptides in a fashion similar to that of *wt* . A quantitative analysis of complex formation with these E1A mutants was performed as described earlier (Figure 12) and was expressed as a percent of levels obtained with the appropriate *wt* virus. The results are summarized in Table 3 along with transformation data . Mutants affecting phosphorylation at Ser-89 have been found to induce cell transformation with two- to three-fold reduced efficiencies (Dumont *et al*, 1989; 1993) but appear to form complexes with several cellular proteins at basically wild type efficiencies, with perhaps the exception of p130 and $p60^{cycA}$ which were mildly affected with mutants other than UM89A. Similar binding efficiencies

Figure 22. Analysis of complex formation with E1A phosphorylation mutants by SDS-PAGE. Extracts from KB cells infected with *wt* or mutants with defects at various potential phosphorylation sites in exon 1 and 2, and labeled with [^{35}S]methionine were immunoprecipitated under mild conditions with E1A-specific M73 serum and precipitates analyzed by SDS-PAGE followed by autoradiography. The UM series of viruses are present in the 243R background (*dl* 520), whereas *pm*975/953 and the AD series of mutants are present in the 289R (*pm*975), and the 289R and 243R (*dl* 309) backgrounds, respectively. The positions of E1A proteins and E1A-binding proteins have been shown at the left.

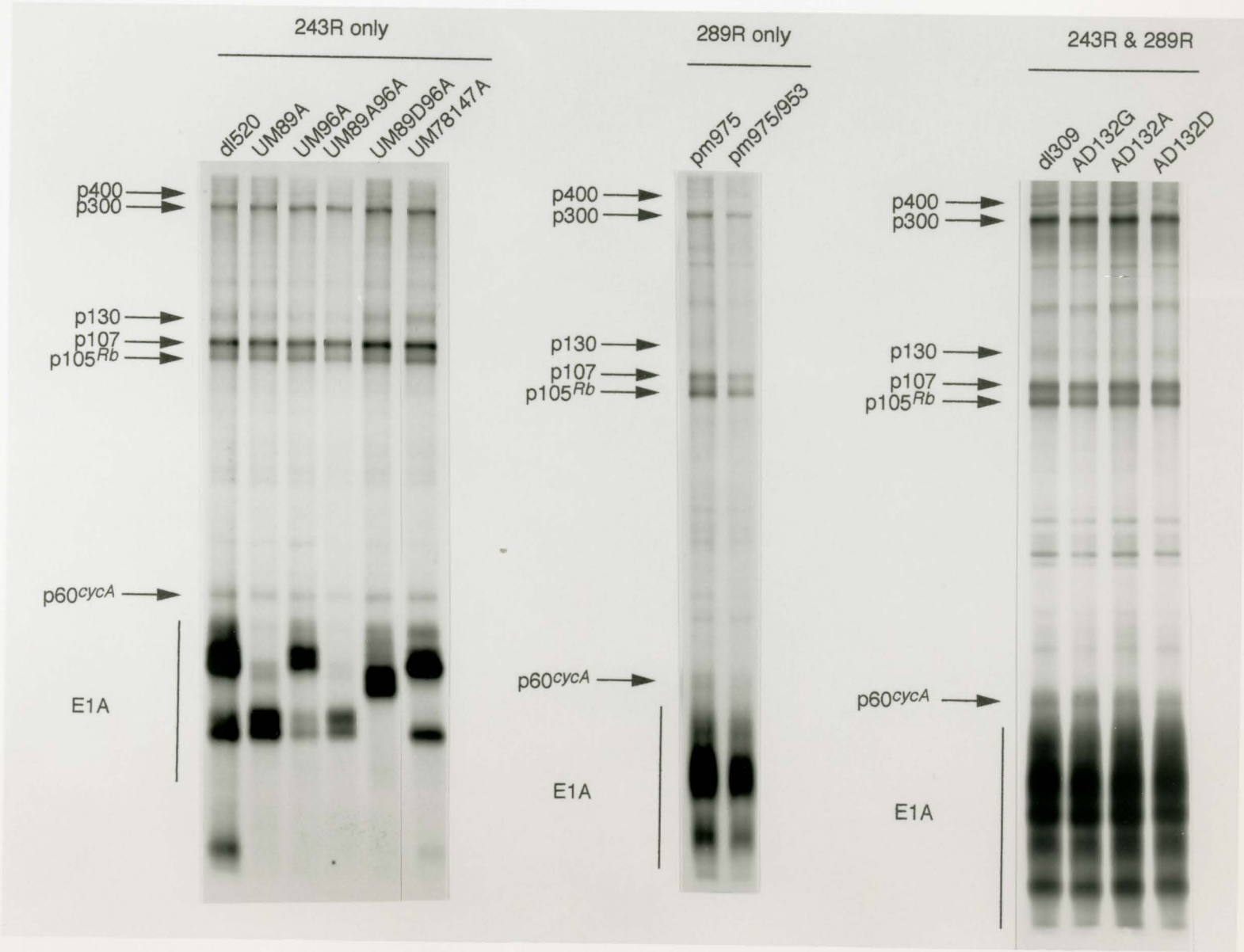

243R only
dl520
UM89A
UM96A
UM89A96A
UM89D96A
UM78147A
p400
p300
p130
p107
p105Rb
p60cycA
E1A
289R only
pm975
pm975/953
p400
p300
p130
p107
p105Rb
p60cycA
E1A
243R & 289R
dl309
AD132G
AD132A
AD132D
p400
p300
p130
p107
p105Rb
p60cycA
E1A

Table 3: Quantitative analysis of complex formation with E1A phosphorylation mutants. Immunoprecipitations were carried out as described in Figure 22 and analyzed in the same manner as Figure 12. The UM series of viruses are present in the 243R background (*dl* 520), whereas *pm*975/953 and the AD series of mutants are present in the 289R (*pm*975), and the 289R and 243R (*dl* 309) backgrounds, respectively. The average values for % binding relative to the appropriate *wt* derived from 3 to 8 separate experiments (± standard error) have been provided. At the bottom have been listed the abilities of these mutants to transform baby rat kidney cells in combination with activated p21Ha-ras in DNA-mediated assays obtained from previous results and results to be published by our laboratory (Dumont *et al.*, 1993; S.G. Whalen, D. Barbeau, R.C. Marcellus, J.A. Howe, S.T. Bayley & P.E. Branton, submitted; S.G. Whalen, D. Barbeau, H.B. Corbeil, R.C. Marcellus, & P.E. Branton, in preparation).

Table 3: Quantitative analysis of complex formation with E1A phosphorylation mutants

% Binding	UM89A	UM96A	UM89A96A	UM89D96A	UM90A96A	pm975/953	AD132G	AD132A	AD132D	UM78147A
p400	82(±32)	106(±32)	102(±49)	111(±30)	144(±60)	80(±21)	92(±24)	98(±65)	88(±40)	142(±99)
p300	101(±46)	87(±50)	105(±73)	105(±60)	123(±79)	84(±16)	91(±25)	92(±38)	93(±17)	114(±96)
p105-Rb	91(±47)	84(±45)	92(±63)	92(±45)	88(±49)	76(±24)	78(±19)	68(±20)	99(±33)	99(±70)
p107	94(±30)	84(±27)	101(±49)	101(±41)	113(±55)	76(±22)	93(±27)	90(±18)	93(±28)	100(±61)
p60-cyclinA	76(±28)	63(±20)	58(±32)	66(±29)	82(±57)	49(±15)	66(±23)	72(±31)	75(±13)	99(±62)
p130	90(±43)	69(±26)	64(±33)	62(±20)	76(±29)	52(±13)	70(±19)	66(±23)	90(±18)	82(±40)
Tranformation % of wt	26.9	78.3	39.6	100.5	43.4	??	223	130	162	107

were also observed with *dl* 1142/520 which deletes aa 82-92 (Figure 12), except that binding of all the cellular proteins appeared to be slightly affected. A mutant with defects at carboxy terminal sites (UM78147A) induced transformation at wild type efficiencies (S.G. Whalen, D. Barbeau, R.C. Marcellus, J.A. Howe, S.T. Bayley and P.E. Branton, submitted) and formed complexes with cellular polypeptides in a fashion quantitatively similar to that of *wt-dl*520. Interestingly, mutants affecting phosphorylation at Ser-132, transformed at slightly higher efficiencies than *wt* , whereas complex formation with p105^{Rb} and p130 was slightly affected (20-30%). This site is located within the downstream acidic region in CR2 that is required for the binding of both p130 and p105^{Rb}. On the whole, these results suggested that phosphorylation does not affect complex formation to a significant degree.

CHAPTER 6: Discussion

These studies represent the first extensive quantitative analysis of the interactions between E1A products and cellular proteins. To varying degrees, all complex formation was found to involve three regions of E1A proteins: the amino terminus and portions of CR1 and CR2. These sequences are known to be functionally important in cell transformation. Although similarities in binding were evident, the patterns were not identical. $p105^{Rb}$ and p107, which are related in the E1A-binding domains (Ewen *et al.*, 1991), exhibited clear differences in the requirement for the Asp^{121}-Leu^{122} residues in the conserved binding core in CR2, and for regions within CR1. Preliminary studies have identified several cellular proteins that interact with the E1A-binding domains of $p105^{Rb}$ (Kaelin *et al.*, 1991; 1992; Helin *et al.*, 1992; Huang *et al.*, 1991; Shan *et al.*, 1992). Thus p107 and $p105^{Rb}$ could interact with different classes of these E1A-like cellular proteins. The binding characteristics of p130 resembled those of $p105^{Rb}$, which required CR2 and the amino terminal portion of CR1, except that the downstream acidic region in CR2 appeared to be essential for the former. Thus, like p107, p130 may be related to $p105^{Rb}$. Mutant *dl* 1109/520 which lacks the downstream acidic region is transformation defective (Egan *et al.*, 1988; 1989; Jelsma *et al.*, 1989), an effect that could result either from decreased binding of $p105^{Rb}$ or from the complete inhibition of complex formation with p130. More specific mutants would be needed to resolve this question.

As found previously, the interactions of E1A proteins with p300 differed somewhat from those with the other cellular species. Quantitative binding analyses showed that p300 and a novel p400 species required sequences within the amino terminal 25 residues and most of CR1, but had little or no requirement for CR2. p400 was demonstrated to be related to p300, possibly the product of a novel transcript or a post-translationally modified form of

p300, with slightly different binding characteristics. The identity of p300 is not known, but like p105^{Rb}, it may limit entry into S-phase (Howe *et al.*, 1990). A previous study had suggested that p105^{Rb} and p300 interact with different regions within CR1, the former requiring sequences within the amino terminal portion of CR1 whereas that latter depends upon sequences further downstream (Wang *et al.*, 1993). The present data indicated that the sequences in CR1 required by these proteins were clearly overlapping. Earlier studies had suggested that residues Glu^{39}-X-X-X-Leu-X-Glu-Leu-X-(X)-Leu are involved in interactions with p105^{Rb} (Dyson *et al.*, 1990; 1992a; 1992b), however, results obtained with mutant *dl*1104/520 indicated that the acidic region between residues 50-60 is also important.

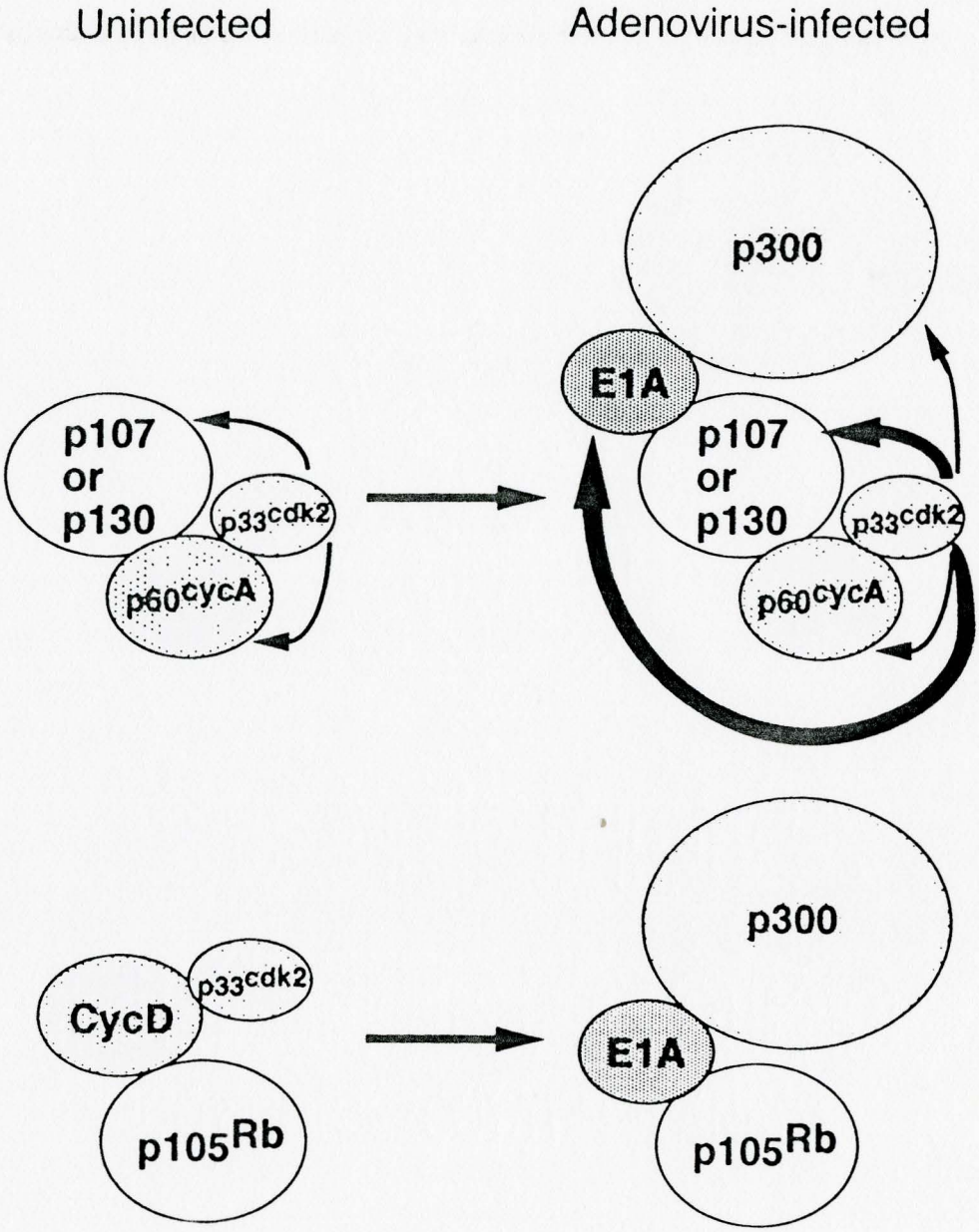
The role of the amino terminus in complexing with p300 and p400 is unclear. This region is not highly conserved in adenoviruses and it seems more likely to play a conformational role in regulating access to a binding site in CR1. Binding of p105^{Rb}, p107 and p130 was also affected by removal of a region at the amino terminus of E1A proteins, an effect not noted in previous more limited studies. Such sequences could contain minor binding sites, as was suggested by results obtained with mutants *dl*1103/08/520 and *dl*1104/08/520 which, although encoding E1A proteins lacking portions of both CR1 and CR2, nevertheless still interacted with small amounts of p130 and p107. Quantitative analysis of *in vitro* binding using GST-E1A fusion proteins indicated that in the absence of a portion of CR2, binding of *p105^{Rb}* (and p107) occurred at only a very low level, about 1.5% that found in the presence of CR2. Similar results were obtained with p105^{Rb} , p130 and p107 using synthetic peptides (Dyson *et al.*, 1992a; 1992b). However, removal of critical regions within CR1 reduced binding of p105^{Rb} *in vivo* by 90%, that of p130 by 86%, and that of p107 by 64%. Removal of amino terminal residues also reduced binding of these proteins by 44%, 55%, and 26%, respectively. These results clearly indicated that binding must occur through cooperative interactions involving all three regions. Such cooperativity

could relate to the three dimensional structure of E1A proteins. However, because regions essential for binding of p300 were also implicated in interactions with p105^{Rb}, p130 and p107, another possibility existed. As illustrated in Figure 23, p300 appears to bind to E1A proteins that are also linked to these proteins because: 1) antiserum against p105^{Rb} also coprecipitated p300; 2) p60^{cycA} serum coprecipitated not only p107 and p130, but also p300; and 3) *in vitro* phosphorylation of p300 in p60^{cycA} complexes was affected by deletions in CR2. Binding of p300 was unaffected by removal of CR2 which greatly reduced binding of p105^{Rb}, p130, and p107, thus indicating that p300 can interact with E1A proteins that cannot bind these other proteins. However, such may not be the case with p105^{Rb}, p130 and p107, which could form stable complexes more efficiently with E1A molecules already linked to p300. Thus one of the roles of p300 may be to enhance interactions between E1A proteins and these cellular polypeptides.

Present studies indicated that p130 is highly related to p107, and, like p107, it seems to interact with p60^{cycA}. While this thesis was being prepared, a report appeared which indicated that both p107 and p130 are associated with p60^{cycA} and also cyclin E which also forms heterodimers with p33^{cdk2} (Faha *et al.*, 1993). During this same period, other reports appeared confirming an earlier study (Matsushime *et al.*, 1992) showing that p105^{Rb} and p107 interact with members of the cyclin D family and *cdc2* -like kinases (Ewen *et al* ., 1993; Dowdy *et al* ., 1993; Hall *et al.*, 1993; Kato *et al.*, 1993). It has also been shown recently that complex formation may require interaction with cyclin-*cdc2* kinase heterodimers (Peeper *et al.*, 1993). Thus E1A proteins may form many different kinds of complexes.

Incubation of complexes purified by E1A- or p60^{cycA} -specific antisera *in vitro* with [γ^{32}P]ATP led to the phosphorylation of several proteins. One notable absentee was p105^{Rb} which had been shown to be phosphorylated *in vitro* by an associated

Figure 23: Model of complex formation and phosphorylation. Complexes containing p105^{Rb}, p107, and p130 in adenovirus-infected and uninfected cells have been depicted. Thickness of the arrows indicate the levels of phosphorylation proposed to occur within such complexes (according to Figure 15 and Table 2).


Uninfected
Adenovirus-infected
p107
or
p130
p33cdk2
p60cycA
p300
E1A
p107
or
p130
p33cdk2
p60cycA
CycD
p33cdk2
p105Rb
p300
E1A
p105Rb

cdc2 -related kinase (Kitagawa *et al.*, 1992; Hu *et al.*, 1992; Qian *et al.*, 1992). Previous studies had suggested that p105^{Rb} can associate with p60^{cycA} (Hu *et al.*, 1992; Qian *et al.*, 1992), however, we failed to detect p105^{Rb} in precipitates prepared using anti-p60^{cycA} serum C160 (Figure 2, lane 5), and p105^{Rb} was never phosphorylated *in vitro* in such precipitates (Figure 8). It is possible that this serum fails to recognize or disrupts p105^{Rb}-p60^{cycA} complexes. p105^{Rb} was also not phosphorylated in complexes purified using E1A-specific M73 serum. This failure may have occurred because the binding sites on p105^{Rb} for cyclin D and E1A proteins are similar, and thus interaction with E1A products may release cyclin D and its associated kinase from the complex (Dowdy *et al.*, 1993).

p130 and p107 were phosphorylated in p60^{cycA} complexes, presumably by associated p33^{cdk2} activity. As illustrated in Figure 23, such phosphorylation was greatly enhanced in Ad5-infected cells, suggesting that the association of E1A molecules with p60^{cycA} complexes either stimulated phosphorylation of p107 and p130, either by enhancing interactions with p60^{cycA}-bound kinases or through the induction of other kinases into the complex, or blocked their dephosphorylation by phosphoprotein phosphatases. In this regard, p105^{Rb} has recently been shown to associate with the catalytic subunit of protein phosphatase type 1 (Durfee *et al.*, 1993). It is not known if similar interactions occur with p107 and p130. It is also possible that E1A proteins enhance interactions of p60^{cycA} with p107 and p130. Phosphorylation of p60^{cycA} was at best only slightly affected by the presence of E1A proteins. The biological activity of p105^{Rb} is controlled in a cell cycle fashion by phosphorylation which inhibits complex formation with transcription factor E2F (Ludlow *et al.*, 1989; 1990; DeCaprio *et al.*, 1992). p105^{Rb}-related proteins p107 and p130 may also be regulated in a similar fashion, and thus one function of E1A proteins may be to inactivate these polypeptides by increasing their levels of phosphorylation. Another function of E1A proteins may be to induce p300 phosphorylation. p300 did not appear to bind directly to p60^{cycA} as it was not detected in p60^{cycA} complexes from uninfected

cells, however, it could be introduced into such complexes by E1A molecules that are also linked to p107 or p130 . Phosphorylation of p300 appeared to be catalyzed at least in part by cyclin-linked kinases because it was diminished with E1A mutants defective in complex formation with p107, p130 and $p105^{Rb}$. However, substantial levels of p300 phosphorylation still occurred in complexes isolated from cells infected by these mutants, suggesting that p300 or E1A proteins could be linked to one or more other kinases. It is likely that such kinases are *cdc2/cdk* enzymes as *in vitro* phosphorylation of E1A proteins occurred only within tryptic peptides containing Ser-89 and Ser-219 which contain *cdc2* consensus sites (D. Barbeau, R. Charbonneau, S.G. Whalen, S.T. Bayley, & P.E. Branton, in press). Additional E1A peptides containing other potential phosphorylation sites are also labelled *in vivo* (Dumont *et al.*, 1989; 1993; Tremblay *et al.*, 1988; S.G. Whalen, D. Barbeau, H.B. Corbeil, R.C. Marcellus, & P.E. Branton, in preparation; S.G. Whalen, D. Barbeau, R.C. Marcellus, J.A. Howe, S.T. Bayley, & P.E. Branton, submitted), and so it is probable that other classes of kinases could be present, especially as *in vitro* phosphorylation of E1A proteins occurred at reasonable levels in mutants that failed to associate efficiently with p300, p107, p130 and $p105^{Rb}$. Several additional proteins bind to E1A products through interactions involving other regions of the E1A molecule. CR3 has been implicated in complex formation with TATA-binding protein and other transcription factors (Horikoshi *et al.*, 1991; Lee *et al.*, 1991). In addition, a 48kDa species has been reported to interact with a region near the carboxy terminus of E1A products (Boyd *et al.*, 1993). Thus additional kinases could be associated with E1A molecules through a variety of other interactions. Further information will be required to determine the mechanism of E1A-mediated control of phosphorylation.

A previous study had suggested that phosphorylation at carboxy terminal sites may contribute to the "shift" in gel mobility of E1A products (Schneider *et al.*, 1987), but results by our group (Dumont *et al.*, 1989) and others (Richter *et al.*, 1988; Smith *et al.*,

1989) indicated that phosphorylation at Ser-89 was largely responsible. Phosphorylation at multiple sites including Ser-89, Ser-96 and perhaps other residues near the amino terminus seems to be responsible for the major "shift" in gel migration (Dumont *et al*, 1993). However, we now show that phosphorylation within the carboxy terminal region gives rise to an additional "supershift" in gel mobility which was absent in the E1A products of mutant UM78147A. Reintroduction of individual serine codons into UM78147A failed to restore the "supershift", indicating that this change in gel mobility probably resulted from multiple phosphorylation events within this region. It will be difficult to determine precisely which serine residues in this region are phosphorylated, but it is possible that all serve as sites. All are present within sequences characteristic of casein kinase I and II phosphorylation sites (Pearson & Kemp, 1991). Phosphorylation at Ser-237 could activate a phosphorylation cascade at serines 234, 231 and 228 through the introduction of a negative charge at position +3 which is ideal for casein kinase II (Pearson & Kemp, 1991). Ser-227 is a natural casein kinase I or II site. In addition to these serine sites, threonines at amino acids 229 and 239 could also serve as sites. Although phosphoserine is the major phosphoamino acid detected in E1A proteins, small amounts of phosphothreonine have also been observed in some experiments (D. Dumont & S.G. Whalen, unpublished results). Of the 12 amino acids in the region between residues 227 and 239, none are basic, two are acidic, and seven are potential serine or threonine phosphorylation sites, which, if utilized, would generate a highly acidic domain.

The function of the carboxy terminus encoded by exon 2 of the 289R and 243R is unclear. It has been linked to the production of epithelial growth factor (Quinlan *et al.*, 1988; Quinlan & Douglas, 1992), and deletions spanning aa 193-289 within this region have been found to increase the efficiency of E1A-mediated transformation, with the critical region lying between aa 271-284 (Subramanian *et al.*, 1989; Douglas *et al.*, 1991). In the present studies conversion of carboxy terminal serine phosphorylation sites to alanines did

not affect the efficiency of transformation of primary rodent cells by DNA from plasmids expressing E1A and E1B, nor the association of cellular proteins with E1A products. Therefore, the AR2 serine residues appeared to play no role in cell transformation. Recent experiments have suggested a potential molecular basis for functional activity within the carboxy terminus of E1A products. This region contains two domains, termed auxilliary regions 1 and 2 (AR1 and AR2) comprising residues 189-200 and 223-245 that function in combination with CR3 to regulate transcription of the adenovirus E4 region (Bondesson *et al*, 1992). It was postulated that AR1 and AR2 could represent negatively charged surfaces that mediate protein-protein interactions involved in transcriptional activation, as found in other systems (Berger *et al*, 1990; Stringer *et al.*, 1990; Lin & Green, 1991). Although serines at positions 185 and 188 just upstream of AR1 represent potential sites of phosphorylation by casein kinase I or II, AR1 is highly negatively charged because of the presence of a series of repeated Glu-Pro sequences. The phosphorylation site at Ser-219 lies just upstream of AR2 which, in addition to four negatively charged amino acids, also contains all of the phosphorylation sites discussed in the present report. Clearly phosphorylation at these multiple sites would create an extended negatively charged surface. During preparation of this manuscript, a report appeared that showed that E1A proteins expressed in S49 cells become hypophosphorylated upon treatment with cyclic AMP in a process involving E4 products (Müller *et al.*, 1992). This effect was linked to a small decrease in mobility of E1A products and loss of tryptic phosphopeptides corresponding to the C-terminal region encompassing aa 227-237. These results suggested that phosphorylation at carboxy terminal sites might be regulated by cyclic AMP and play an important role in the control of E4 transcription. Further studies would be required to confirm this possibility.

Mutation of serine residue 132, which is contained within the binding sites of p105^{Rb} and p130, was found to increase transforming efficiency by at least 30% (S.G. Whalen, D.

Barbeau, R.C. Marcellus, J.A. Howe, S.T. Bayley, and P.E. Branton, submitted). It is possible that the acidic downstream portion of CR2 also mediates suppression of transformation analagous to the C-terminus of E1A. Complex formation of various cellular proteins with such mutant E1A products was found to be essentially unaffected, with perhaps the exception of p130 and $p105^{Rb}$ as a decrese in binding of about 20-30% was detected. The increase in transformation observed may not be related to the small differences seen in binding, but rather some other unknown function involving CR2 which leads to a decrease in the cytotoxicity of E1A proteins. Results obtained from another system, the E7 protein of papillomavirus, were quite different (Barbosa *et al.*, 1990). HPV-E7 contains a region of homolgy to CR2 located between aa 21 and 26, as well as two serine residues at positions 31 and 32 within a CK II consensus sequence. Mutation of these two serine residues within HPV-E7 resulted in lower transforming activity without affecting the binding of $p105^{Rb}$ (Barbosa *et al.*, 1990). The flanking sequences may influence the binding of $p105^{Rb}$, and it is possible that the region required for binding $p105^{Rb}$ to HPV-E7 is not as extensive. Differences in complex formation of cellular proteins with various DNA tumour viruses have previously been detected. Conserved sequences within CR1 of Ad5 E1A, were found to influence the binding of the $p105^{Rb}$ family of proteins. However, mutational analysis has not indicated a clear role of CR1 sequences contained within the SV40 LTAg and the HPV E7 protein (Ewen *et al.*, 1989; DeCaprio *et al.*, 1988; 1989; Münger *et al.*, 1989; Jones *et al.*, 1990; Marsilio *et al.*, 1991; Imai *et al.*, 1991). It is possible that this domain is only important for interactions with adenovirus E1A proteins.

In terms of transcriptional control, the observations presented here concerning the nature of E1A products present in complexes containing $p60^{cycA}$ are highly provocative. Whereas only some of the 243R-E1A proteins present in infected cells and those within complexes containing $p105^{Rb}$ exist in the "supershifted" form characteristic of carboxy terminal

phosphorylation, those found in complexes containing p60^{cycA} were preferentially in this hyperphosphorylated state. One possibility to explain this difference was that phosphorylation at carboxy terminal sites regulates or enhances complex formation with p60^{cycA}. However, the detection of E1A-p60^{cycA} complexes in cells infected by mutant UM78147A suggested that such phosphorylation may not be essential. Another possibility was that phosphorylation within this region occurs exclusively or preferentially within p60^{cycA}-E1A complexes. As mentionned earlier, E1A products appear to interact indirectly with p60^{cycA}-p33^{cdk2} through interactions with p107 (Ewen *et al.*, 1992; Faha *et al.*, 1992; 1993; Howe & Bayley, 1992) and p130 (Faha *et al.*, 1993; D. Barbeau, R. Charbonneau, S.G. Whalen, S.T. Bayley, & P.E. Branton, in press). Kinase activity with characteristics of p33^{cdk2} has been detected in complexes containing E1A products (Lassam *et al.*, 1979; Branton *et al.*, 1981; Tsai *et al.*, 1991; Giordano *et al.*, 1991a; Herrmann *et al.*, 1991; Kleinberger & Shenk, 1991), and such activity appeared to be exclusively of the *cdc2/cdk* class (D. Barbeau, R. Charbonneau, S.G. Whalen, S.T. Bayley, & P.E. Branton, in press). With the exception of Thr-239 which exists within a sequence resembling a *cdc2* site, all other carboxy terminal sites are typical of those phosphorylated by casein kinase I or II. Thus either p60^{cycA} complexes also contain a casein kinase-like enzyme, or E1A phosphorylation at AR2 sites is unrelated to complex formation. The stoichiometry of complex formation also argues against this possibility. A significant proportion of E1A products were detected in the "supershifted" form, whereas only a small amount of E1A molecules exist within complexes containing p60^{cycA}. A third possibility is that E1A products present in such complexes are resistant to dephosphorylation at carboxy terminal sites. Increases in cyclic AMP levels have been linked with hypophosphorylation at sites corresponding to the C-terminal region spanning AR2 sites (Müller *et al.*, 1992). It is unclear whether this effect is due to an inhibition of the kinases involved in such phosphorylation or to increased phosphoprotein phosphatase activity. KB cells contain fairly high constitutive levels of cyclic AMP and thus it is possible that complex formation

with $p60^{cycA}$ either alters the intracellular location of E1A products or induces a conformational change such that these AR2 sites are no longer accessible to the action of phosphatases. Further experiments would be necessary to distinguish between these possibilities.

The function of E1A-$p60^{cycA}$ complexes is not known. Interactions between E1A proteins and $p105^{Rb}$ appear to activate transcription factor E2F by releasing it from inactive complexes (Bagchi *et al.*, 1991; Bandara and La Thangue, 1991; Bandara *et al.*, 1991; Cao *et al.*, 1992; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991). While p107-$p60^{cycA}$ complexes have been found in association with E2F (Cao *et al.*, 1992; Devoto *et al.*, 1992; Mudryj *et al.*, 1991), the role of such complexes is not understood. p107-$p60^{cycA}$ complexes could also play a role in the regulation of other transcription factors. An appealing hypothesis is that such complexes, which contain E1A products in the "supershifted" form containing a highly acidic domain within AR2, could function specifically in E1A-mediated activation of E4 expression through interactions with E4F or some other transcription factor.

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