BIOLOGICAL ACTIVITIES OF ADENOVIRUS 5 E1A

CONTROL OF GENE EXPRESSION AND CELL CYCLE REGULATION

BY

ADENOVIRUS 5 E1A PROTEINS

By

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ABSTRACT

The primary goal of this study was to gain a better understanding of the roles of the adenovirus E1A gene in oncogenic transformation. The E1A gene encodes two related multifunctional proteins of 243R and 289R that influence a wide variety of cellular regulatory processes, including control of gene expression and regulation of DNA synthesis and mitosis. Our approach to the study of E1A was to use a series of E1A mutants - constructed by me and other researchers in Dr. Bayley's lab - to determine which of E1A's biological activities were likely to be responsible for oncogenic transformation. The mutants were also used to map the E1A binding sites for a number of cellular proteins to determine if these proteins were likely to play a role in the underlying molecular mechanisms of E1A action.

The ability of E1A to regulate the cell cycle was found to be closely correlated with the ability of the E1A proteins to interact with two cellular polypeptides, p300 and pRb (the product of the retinoblastoma susceptibility gene). Binding to either pRb or p300, by the 243R product, appeared to be sufficient for E1A to induce cellular DNA synthesis. However, association with both p300 and pRb was

required for efficient induction of cell division by 243R. In the course of these studies I found that two other E1A-associated proteins, p107 and cyclin A, form a complex normally present in cells. Studies with E1A-mutant viruses suggested that this complex may be involved in regulation of DNA replication. Transcriptional activation of gene expression by the region unique to the 289R protein was not required for induction of DNA synthesis or stimulation of mitosis. However, repression of enhancers by 243R may play an important role in cell cycle regulation.

The E1A regions required for the cell cycle effects of E1A corresponded exactly with regions of the E1A proteins which are necessary for oncogenic transformation. Therefore my results implied that the role of E1A in transformation is to interact with cellular proteins that control cell growth.

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THIS THESIS

IS DEDICATED TO MY PARENTS

ALLAN AND KATHERINE HOWE

INTRODUCTION

Human adenoviruses were first isolated by Rowe et al., (1953) as the causative agents responsible for the spontaneous degeneration of cultures of human adenoid tissue and almost simultaneously as the etiological agent of a non-influenza like case of acute upper respiratory disease (Hilleman and Werner, 1954). Since their initial discovery 41 distinct antigenic types of human adenoviruses have been identified (Horwitz, 1990). Human illnesses caused by adenoviruses are usually a result of upper respiratory tract infections, but some serotypes have been shown to be associated with ocular conjunctivitis, urinary infection and gastroenteritis (Straus, 1984).

Adenoviruses are of some clinical importance, but perhaps the greatest impetus for their detailed study came early in the 1960's when Trentin and co-workers reported that human Ad 12 was able to induce tumours when injected into hamsters (Trentin et al., 1962). Shortly after, studies revealed that although not all adenoviruses induce tumours, all could transform rodent cells in culture (Freeman et al., 1967; McAllister et al., 1969). Adenoviruses do not appear to act as carcinogens in humans

(Green and Mackey, 1977), but nevertheless their ability to transform cultured cells provides an excellent *in vitro* model system for the study of tumorigenesis.

Although the transformation of primary cells in culture is not identical to tumour induction *in vivo*, cells transformed by adenoviruses have many characteristics in common with cultured tumour cells (Tooze, 1973; Graham, 1984). These include: 1) the ability to replicate indefinitely, 2) the capability to grow to higher saturation densities than cultured primary cells, 3) the ability to grow in semi-solid media, and 4) the acquisition of specific morphological characteristics. The similarities between adenovirus transformed cells and tumour cells in culture suggests that the processes by which each evolve must involve changes in the same fundamental cell growth control mechanisms. It follows then that a detailed understanding of how adenoviruses transform primary cells in culture will help to enhance our understanding of tumour induction *in vivo*.

Adenovirus sequences required for transformation were discovered using two distinct experimental approaches (Graham, 1984). One approach used specific restriction fragments of viral DNA to probe Ad2 transformed cell lines to determine which Ad DNA sequences were common to each cell

line (Sharp et al., 1974; Gallimore et al., 1974). The other used calcium-phosphate mediated DNA-transfection to determine the smallest sections of viral DNA that would bring about transformation of primary rodent cells (Graham et al., 1974). Both studies came to the same conclusion that the left most 10-14% of the viral genome was required. This region contains three distinct gene regions which are transcribed from the right strand. Two of these, E1A and E1B, are required for transformation.

The primary goal of the studies described in this thesis was to gain a better understanding of the roles of the E1A gene in transformation. However, an understanding of products of the other viral gene regions and their roles, as well as the roles of E1A, in the normal lytic cycle is important for two reasons. Firstly, for some assays the virus has been used primarily to deliver E1A to cells and it is important to recognize that effects of other viral gene products could potentially complicate studies of E1A. Secondly, the functions of the E1A products in normal lytic infection may give us some clues to how E1A brings about transformation in rodent cells.

General Characteristics of Adenoviruses

Adenoviruses have been isolated from many mammals, birds and even frogs and fish (Ishibashi and Yasue., 1984).

All adenoviruses are members of the taxonomic family Adenoviridae which is divided into two authentic genera, the Mastadenoviruses and Aviaadenoviruses (avian adenoviruses), and one minor group.

The human adenoviruses serotypes have been further divided into sub-groups according to biological, chemical, immunological or structural properties (see Horwitz, 1990 for a review). The most widely used classification system (Table 1) partitions the 41 adenoviruses into 6 sub-groups, A-F, by a number of criteria: DNA base composition and homology, oncogenic potential in newborn hamsters, and the ability to haemagglutinate the red blood cells of rats or rhesus monkeys. The best characterized human adenoviruses are Ad2 and Ad5, closely related members of nononcogenic sub-group C, which share about 98% DNA homology. These serotypes grow quickly and to high titres in culture (Sharp, 1984) and are therefore easier to study than many other serotypes which do not grow as efficiently. Ad5 has been used exclusively for these studies and the information in this introduction pertains to this serotype and in some cases Ad2.

a) Morphology

Viral particles are composed of one double stranded DNA molecule and between 11-15 proteins encoded by the virus

Table 1. Classification of the various human adenoviruses serotypes (from Horwitz., 1990).

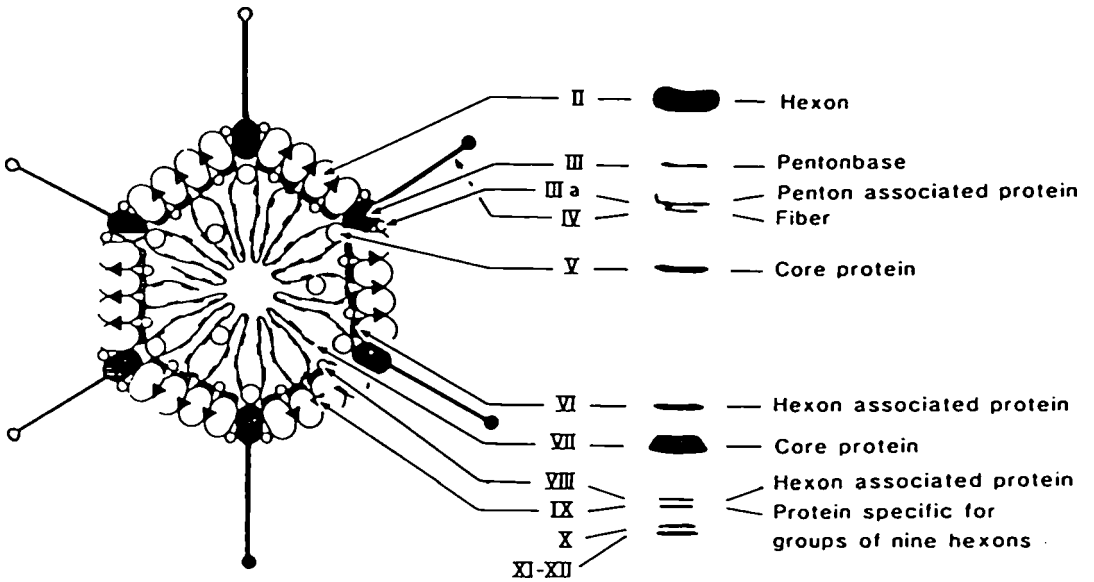
Subgroup	Hemagglutination groups	Serotypes	Oncogenic potential		Percentage of G+C in DNA
			Tumors in animals	Transformation in tissue culture	
A	IV (little or no agglutination)	12,18,31	High	+	48–49
B	I (complete agglutination of monkey erythrocytes)	3,7,11,14,16,21,34, 35	Moderate	+	50–52
C	III (partial agglutination of rat erythrocytes)	1,2,5,6	Low or none	+	57–59
D	II (complete agglutination of rat erythrocytes)	8,9,19,37,10,13,15, 17,19,20,22–30, 32,33,36,37,38, 39,42	Low or none	+	57–61
E	III	4	Low or none	+	57–59
F	III	40,41	Unknown		

(Figure 1). Electron microscopy studies revealed that the virus is icosahedral in shape and composed of a protein capsid shell and a core region containing the viral DNA and at least two proteins (see Nermut., 1984, for a review).

The icosahedral capsid shell is constructed from 252 structural units called capsomers that form 20 identical equilateral triangular faces with 12 edges and 12 vertices (Valentine and Pereira, 1965). 240 of the capsomers are called hexons because they have 6 neighbours while the 12 capsomers located at the vertices of the capsid have 5 neighbours and are called pentons. The hexons are each composed of three molecules of viral protein (VP) II, which is usually referred to as hexon (Grutter and Franklin, 1974). Pentons are assembled from 3 molecules of VP III (penton) and three molecules of VP IV (fibre). The fibre molecules form thin antenna-like projections that protrude from vertices of the capsid (Philipson et al., 1968). Four other virally encoded proteins VP IIIa, VP VI, VP VIII and VP IX associate with the capsid and are probably required for assembly and integrity of the capsid, and possibly for viral DNA packaging (Nermut, 1984).

The core region contains the viral DNA chromosome packaged in a complex with two arginine rich virus encoded proteins VP VII and VP V (Mirza and Weber, 1977). A model of the packaged viral chromosome predicts that it is organized into repeated subunits of DNA and

Figure 1. Model of an adenovirus: capsid and core. The relative sizes of the viral structural proteins (VP), and their approximate concentrations in infected cells are displayed in an idealized SDS-polyacrylamide gel pattern to the right. The largest VP, hexon, is the most abundant and has a molecular mass of about 106 kDa. The smallest, VP's XI-XII, have molecular masses between 4-5 kda (from Horwitz, 1990).


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

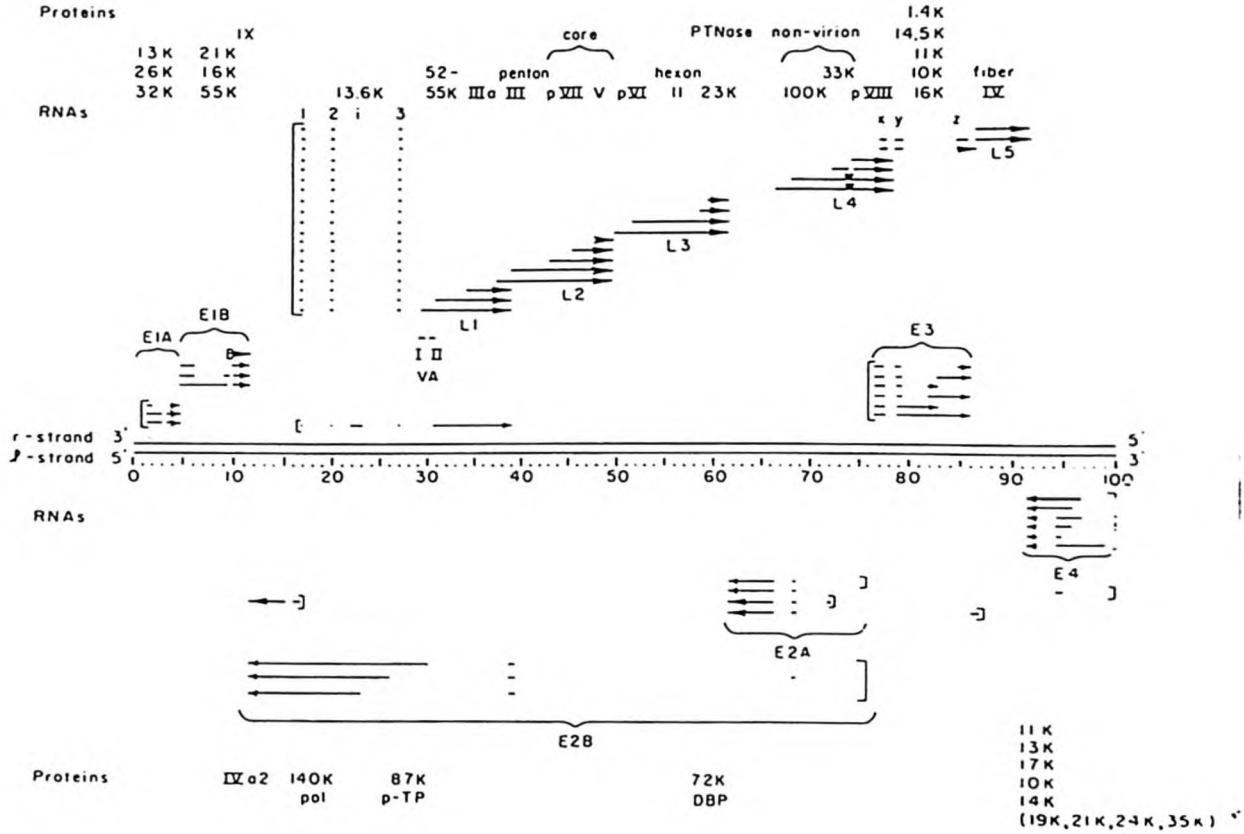
protein (e.g. nucleosomes) in a chromatin-like structure (Corden et al., 1976; Mirza and Weber, 1982). This model predicts that each viral nucleosome consists of 150 nucleotides of DNA wrapped around three dimers of VP VII and that individual nucleosomes are linked by a variable length of DNA which is associated with one molecule of VP V (see Horwitz, 1990, for a review).

b) Viral Genome

The adenovirus chromosome is a linear double stranded DNA molecule about 36 000 base pairs (bp) in length that is covalently linked at each 5′ end with one molecule of the virally encoded terminal protein (Rekosh et al., 1977). Each strand of the chromosome is identified according to the direction of transcription of the genes it encodes. The rightward strand (r-strand) contains about 69% of the genetic information encoded by the chromosome and the leftward strand (l-strand) encodes 31% (Figure 2). To facilitate mapping the genome was divided into 100 map units from left to right with each map unit (m.u.) representing about 365 bp of DNA (Sussenbach., 1984). Inverted terminal repeats (ITR) of length varying between 50 - 170 nucleotides have been identified at each end of the viral chromosome in all adenoviruses studied (Kelly, 1984).

The Ad5 transcription units have been divided into

Figure 2. Genomic map of the adenovirus type 2 mRNAs. Early and late mRNAs are represented by thin and thick lines, respectively. The early E2A MRNAS that continue to be synthesized at late times are also represented by thick lines. Arrowheads indicate direction of transcription. The primary leader sequences of the late mRNAs that are transcribed form the major late promoter are labelled 1, 2 and 3. Proteins encoded by each of the mRNAs are indicated above for genes that are transcribed rightward and below for genes that are transcribed leftward (from Horwitz, 1990).


Proteins
IX
13K 21K
26K 16K
32K 55K
13.6K
52-55K IIIa III
core
penton
pVII V pVI
hexon II 23K
PTNase
non-virion
33K
100K pVIII
1.4K
14.5K
11K
10K 16K
fiber IX
RNAs
1 2 i 3
x y
z
L5
L4
L3
L2
L1
E1B
E1A
I II
VA
E3
r-strand 3'
l-strand 5'
5'
3'
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A
E2B
Proteins
IVa2 140K 87K
pol p-TP
72K
DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

three categories, early, intermediate and late, depending on time of expression during the replicative cycle. Three early transcription units, E1A (m.u. 1.3-4.6), E1B (m.u. 4.6-11.2), and E3 (m.u. 76.6 - 86.0), are located on the r-strand while three other early regions, E2A (m.u. 75.1-61.5), E2B (m.u. 30.2-11.2) and E4 (m.u. 99.2-91.3), are located on the l-strand. Two transcription units, the IVa_2 gene (m.u. 30.2-11.2) and the gene encoding for protein IX (10-11.8), are classified as intermediate. Five late gene region families are located between map units 31.0 and 91.3 on the r-strand.

c) The Replicative Cycle in Human Cells

The viral replicative cycle in human cells can be divided into early and late phases which are punctuated by the onset of viral DNA replication. The early phase begins when viral particles attach to cells through interactions between the virion fibre protein and a specific cell membrane receptor (Philipson et al., 1968). The virion moves into the cell either by endocytosis or direct penetration of the cytoplasmic membrane (Lonberg-Holm and Philipson, 1969) and is transported to the nucleus by a process where most of the capsid is shed (Philipson, 1968). In the nucleus virion core proteins are removed yielding viral chromosomes that are almost entirely devoid of virion

proteins.

Expression of the viral genome is highly temporally coordinated and begins with the E1A region about 1 hour after infection (Nevins et al., 1979). The other early genes E1B, E2, E3, E4 are first expressed soon after E1A at 1.5-2.0 hours post infection. A number of the protein products encoded by the early genes are required for viral DNA replication, while others prepare the DNA synthesis machinery of the infected cell for efficient viral DNA replication. During infection of humans some virally encoded early proteins function to protect infected cells from host immune surveillance (Wold and Gooding, 1991).

The late phase of infection commences with the onset of DNA replication at about 7 hours after infection. Viral DNA replication requires the terminal protein for initiation and proceeds by a semi-conservative mechanism (Kelly and Lechner, 1977). With the onset of replication efficient transcription of the late gene families from the major late promoter begins and attains a maximal level at about 18 hours after infection (Nevins et al., 1979). During the late phase viral proteins block cellular DNA and protein synthesis presumably so that maximum viral macromolecular synthesis can occur (Horwitz, 1990). Intermediate gene expression, which actually begins during the early phase, reaches a maximum between 8 - 12 hours post infection (Sharp, 1984).

Assembly of the virion and packaging of the viral genome begins at about 24 hours after infection (Ginsberg, 1984). Infected cells are killed because of attrition and lyse yielding approximately 10 000 virions per cell.

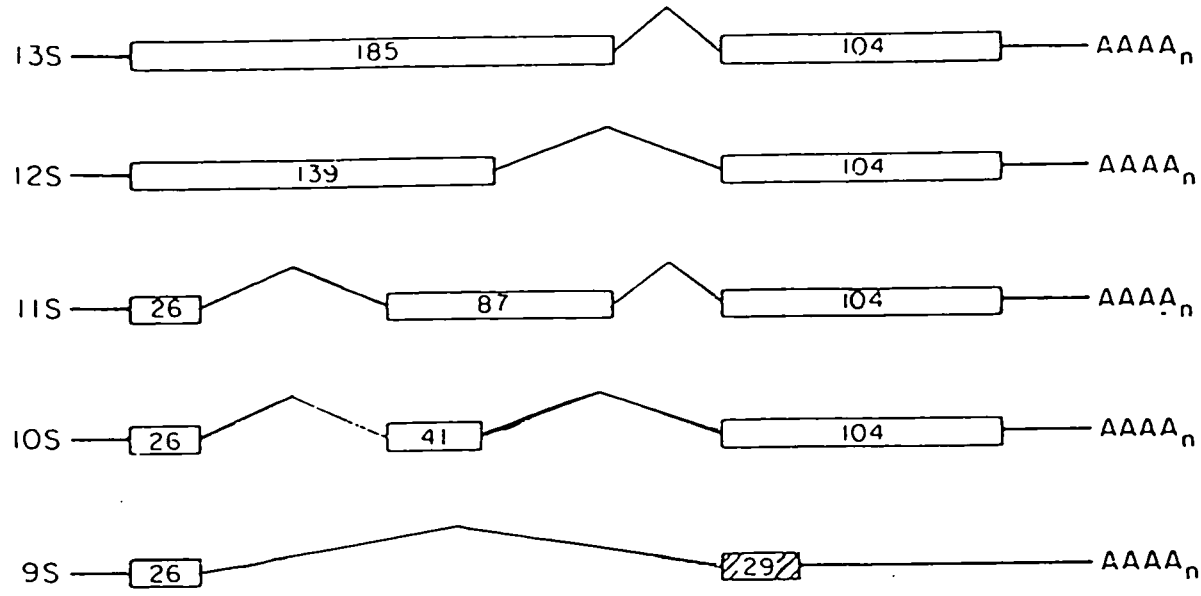
Viral Gene Products and Their Functions in Lytic Infection

a) E1A

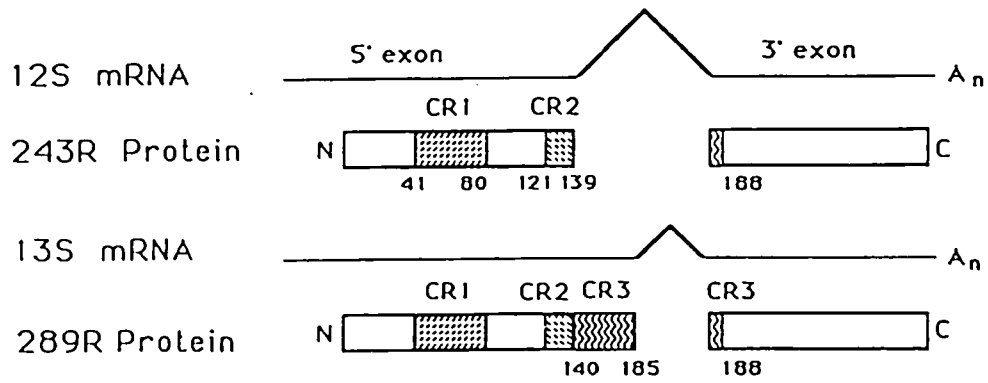
Early region 1A encodes a primary transcript from a promoter located at 1.3 map units. This transcript is differentially processed to yield 5 mRNA's which are referred to by their sedimentation coefficients of 13S, 12S, 11S, 10S or 9S (Figure 3a, Chow et al., 1979; Ulfendahl et al., 1987; Stephans and Harlow, 1987). The 13S and 12S messages are produced at early and late time after infection while the 11S and 10S mRNAs are synthesized only late in infection at very low levels (Ulfendahl et al., 1987). The 9S message, like the 10S and 11S messages, is not synthesized efficiently at early times, but is the most abundant E1A message at late times (Chow et al., 1979). Proteins of 289 residues (R), 243R, 217R and 171R corresponding to the 13S, 12S, 11S and 10s E1A mRNA's, respectively, have been detected in infected cells (Figure 3, Yee et al., 1983, Stephans and Harlow, 1987, Ulfendahl, 1987). The 9S mRNA is predicted to encode a 55R protein,

Figure 3. E1A mRNAs and predicted protein products. (A) Exons are indicated by open boxes and introns by carets. The number of amino acid residues encoded by each exon is given (adapted from Stephans et al., 1987). (B) Position of conserved region 1 (CR1), CR2 and CR3 in the 243R and 289R E1A proteins. N and C indicate the N-terminus and the C-terminus of the proteins (adapted from Shenk and Flint., 1991).

A


13S
185
104
AAAA$_n$
12S
139
104
AAAA$_n$
11S
26
87
104
AAAA$_n$
10S
26
41
104
AAAA$_n$
9S
26
29
AAAA$_n$

B


12S mRNA
5' exon
3' exon
A$_n$
CR1
CR2
243R Protein
N
C
41
80
121
139
188
13S mRNA
A$_n$
CR1
CR2
CR3
CR3
289R Protein
N
C
140
185
188

but this polypeptide has never been isolated during infection.

The 243R and 289R proteins are the most important and best characterized of the E1A proteins. These proteins share amino and carboxyl regions and differ only by the presence of a stretch of 46 unique amino acids in the larger protein (Figure 3b). This unique stretch of amino acids, together with the first three amino acid residues of the carboxyl portion of the protein (residues 185-188 in 289R), have been found to contain residues that are highly conserved among various adenovirus serotypes (Kimelman et al., 1985; van Ormondt et al., 1986). This region is referred to as conserved region 3 (CR3). Two other regions of conserved amino acids, CR1 and CR2, are located in the amino terminal region that is common to both proteins. Conserved region 1 is located between amino acids 41-80 and conserved region 2 which includes residues 121-139 is juxtaposed to CR3 in the 289R protein, and borders the carboxyl terminal portion of the protein in 243R.

The predicted molecular masses for the 289R and 243R proteins are 32 kDa and 27 kDa respectively, but when separated on one dimensional SDS polyacrylamide gels they migrate anonomously as a group of polypeptides with molecular masses of about 52 kDa, 50 kDa, 48.5 kDa, 45 kDa (Yee et al., 1983, Rowe et al., 1983). The 52 kDa and 48.5 kDa species have been shown to originate from the 13S

message and the 50 kDa and 45 kDa species from the 12S message. The aberrant migration of the E1A proteins on SDS gels probably results from incomplete denaturation under the electrophoretic conditions used (Grand, 1987). Multiple species of the 289R and 243R proteins exist as a result of differential phosphorylation on serine residues (Richter et al., 1988, Dumont et al., 1989).

The E1A proteins are acidic and have isoelectric points between pH 4 and 5 (Grand, 1987). Almost all of the acidic residues are grouped in clusters in CR1, CR2 and CR3 (Flint and Shenk, 1989). The E1A polypeptides also have an unusually high proline content of 16%. Proline is a particularity rigid amino acid that is thought to disrupt secondary protein structure. Since proline is distributed evenly through the E1A proteins it is speculated that the E1A proteins lack substantial secondary structure. Another distinguishing feature of the E1A proteins is their ability to physically associate with a number of cellular proteins (Yee and Branton, 1985; Harlow et al., 1986). Among these are proteins with approximate molecular masses of 300 kDa, 130 kDa, 107 kDA, 105 kDa, 80 kDa 68 kDa, 65 kDa, 60 kDa, 50 kDa, 40 kDa and 28 kDa.

The primary role of the E1A products in lytic infection is to initiate the coordinated expression of the viral genome by activating the expression of the other early gene regions. The ability of the E1A proteins to activate

early viral gene expression was discovered as a result of studies on adenovirus mutants that lacked a functional E1A region. These viruses were found to produce reduced levels of the other early viral mRNA's during infection of permissive cells, as a result of inefficient transcription from the early promoters (Berk and Sharp, 1978; Jones and Shenk, 1979; Nevins, 1981). Co-transfection studies showed that E1A products could stimulate transcription in *trans* from the E1A, E1B, E2, E3, E4 promoters joined to reporter genes (Imperiale et al., 1983; Weeks and Jones, 1983). Stimulation of viral promoters is independent of chromosomal organization because the adenovirus early gene promoters can be stimulated by E1A whether they are introduced as part of the viral genome, in plasmids or integrated in the mammalian cell genome (Curtois and Berk, 1984). The larger 289R E1A protein has been shown to be largely responsible for transactivation of the early viral genes (Montell et al., 1984; Winberg and Shenk, 1984). However the 243R is able to stimulate gene expression in some experimental situations (Leff et al., 1984; Winberg and Shenk, 1984).

Another role of the E1A proteins, and particularly the 243R protein, may be to stimulate maximal viral DNA replication in differentiated cells. This E1A function was discovered during studies of a mutant virus pm 975 which encodes the 289R protein, but not the 243R protein (Montell et al., 1984). Pm 975 was found to grow less efficiently

than wild type virus in quiescent human fibroblasts as a result of a reduced ability to replicate viral DNA (Montell et al., 1984; Spindler et al., 1985). Reduced viral DNA synthesis was not because of inefficient expression of the other early genes as they were present at levels equivalent to those found in wild type infected cells. Spindler et al. (1985) hypothesized that an E1A function distinct from transactivation was required for maximal viral replication. It is probable that the ability of the 243R product to stimulate cellular DNA synthesis in quiescent cells (Berk, 1986, also see below) is required to prepare the cells DNA synthesis machinery for viral DNA replication.

b) E1B

The promoter in the E1B region is located at 4.5 m.u. and is active at early and late times during infection. The primary transcript produced from this promoter is processed through a complicated series of splices into mRNAS's of 22S, 14.5S, 14S, and 13S each of which contains two open reading frames (Berk and Sharp, 1978; Chow et al., 1979; Virtanen and Petterson, 1985). One of these open reading frames is common to each message and encodes a 176R protein. Depending on the mRNA, the other open reading frames give rise to proteins of 84R, 93R, 155R and 496R (McLorie et al., 1991, and references within).

The most prominent and best characterized of the E1B proteins are 176R and 496R. The 176R polypeptide migrates on SDS gels at 19 kDa, is acylated (McClade et al., 1988) and associates in cells with cytoplasmic and nuclear membranes, with intermediate filaments in the cytoplasm, and with the nuclear lamina (Persson et al., 1982; White et al., 1984; White et al., 1989). Adenoviruses with lesions in the 176R coding sequence frequently display phenotypes characterized by enhanced cytopathic effect, large plaque size and degradation of viral and cellular DNA's (Chinnadurai, 1983; White et al., 1984; Takemori et al., 1984). Mutant 176R viruses grow as well as wild type virus in HeLa cells (Barker and Berk, 1987) and actually grow to higher titres than wild type in human W138 cells. White et al. (1991) have suggested that the 176R protein carries out three functions during infection: (1) to maintain the integrity of DNA in the nucleus; (2) to modify the morphology of the infected cell; and (3) to negatively regulate viral replication.

The 496R E1B protein has an apparent molecular mass of 55 kDa and has been shown by immunofluorescence to be localized in the nucleus and in a perinuclear spot (White and Cipriani, 1989). Viral mutants that do not produce the 496R protein grow much less efficiently than wild type virus on Hela cells (Pilder et al., 1986; Barker and Berk, 1987). The 496R protein functions to facilitate the efficient

molecular transport of late viral messages to the cytoplasm and is also required to inhibit the accumulation of host mRNAs at late times in infection (Babiss et al., 1985; Pilder et al., 1986; Leppard and Shenk, 1989). These functions appear to be related and may involve the ability of 496R to form stable complexes with the Ad E4 34 kDa protein (Halbert et al., 1985). The 496 R protein also associates with the 53 kDa product of the cellular growth suppressor gene p53, but the functional significance of this interaction is not known (Sarnow et al., 1982; Kao et al., 1990).

c) E2

The early region E2 promoter is located at map unit 75.1 on the left strand and it initiates the transcription of a primary transcript that is spliced into the E2A and E2B sub-classes of mRNAs (see Sussenbach, 1984 for a review). E2A mRNAs contain two short leaders, one encoded near the promoter and the other at 68.8 m.u. (Baker et al., 1979). These are ligated to the main body of the mRNA which is encoded between map positions 66.5 and 61.5. At late times after infection transcription of the E2A regions starts at a different promoter which is located at position 72 m.u.. The E2A mRNA encodes the 72 kDa single-strand-specific DNA binding protein which is absolutely required for productive

infection (Kruijer et al., 1981).

Two E2B mRNAs are produced and each contains leader sequences at 68 and 39 m.u. connected to a main body of mRNA encoded between 30 and 11 m.u. (Stillman et al., 1981). The E2B messages give rise to the 80 kDa precursor of the terminal DNA binding protein and the 140 kDa adenovirus DNA polymerase (Stillman et al., 1981). Both E2B proteins are required for productive infection.

d) E3

The E3 region is located on the right strand between 76.6 and 86.6 m.u. and gives rise to nine mRNAs. All nine overlapping E3 mRNAs are produced from a common primary transcript and contain a common leader sequence, encoded between map positions 76.6 to 77.6, joined to differentially spliced coding regions that begin from 78.6 m.u. (Chow et al., 1979). To date six E3 proteins of 6.7 kDa, 10.4 kDa, 14.5 kDa. 14.7 kDa and 19 kDa have been identified (see Wold and Gooding 1991 for a review).

Polypeptides encoded by the E3 region are not required for productive infection in cultured cells and in fact the various viruses used in the studies presented here do not encode the majority of the E3 proteins because of a deletion mutation (Jones and Shenk, 1979). Nevertheless this region has been maintained in all natural adenoviruses

probably because the E3 proteins appear to protect infected cells from host-immunosurveillance (reviewed in Wold and Gooding, 1991). The 19 kDa protein inhibits the transport of major histocompatibility complexes to the cell surface and thus prevents cytolysis of infected cells by cytotoxic T-lymphocytes (Andersson et al., 1985; Burget and Kvist, 1985) The 14.7 kDa, 10.4 kDa and 14.5 kDa prevent the lysis of infected cells induced by tumour necrosis factor (Gooding et al., 1988).

e) E4

This region is located at the extreme right end of the viral genome between map positions 91.2 and 99.2, and contains 7 open reading frames (Berk and Sharp, 1978; Chow et al., 1979). The E4 promoter is located at position 99.2 and directs transcription of a primary transcript which is processed to yield at least 5 mRNAs. Three polypeptides encoded in this region have been identified, 14 kDa, 19.5 kDa, and 34 kDa (Sarnow et al., 1982; Downey et al., 1983; Cutt et al., 1987).

Mutant analyses have shown that the E4 region is required for productive infection and that the E4 polypeptides are required for efficient viral replication, for late gene expression and for shut off of host macromolecular synthesis (Cutt et al., 1987). The 19.5 kDa

protein is required for efficient viral replication probably because it is able to transactivate expression of the E2 early promoter by a mechanism which involves its association with the cellular transcription factor E2F (Hardy and Shenk, 1989, Raychaudhuri et al., 1990). As described earlier the 34 kDa E4 protein associates with the E1B 55 kDa protein and this complex is thought to be required for preferential accumulation of late viral mRNAs (Sarnow et al., 1982a; Cutt et al., 1987). The 14 kDa protein is known to associate with the nuclear matrix, but no function has been ascribed to it (Sarnow et al., 1982b).

f) Late Regions

Messenger RNA's for all late gene products are spliced from a primary RNA which is transcribed from the major late promoter at position 16.5 on the r-strand (Ziff et al., 1978). The major late promoter is actually active to a limited extent in the early phase of infection, but transcription does not proceed past map position 39 (Shaw and Ziff, 1981; Akusjarvi and Perrson, 1981). During the late phase of the viral life cycle the major late promoter is fully activated and transcription continues to map position 99. Each late primary RNA transcripts is processed into one of five different mRNA's (L1-L5). These mRNA's all contain a common tripartite leader sequence 203 nucleotides

in length and a common 3' end (Sussenbach, 1984). Late mRNA's encode capsid components and proteins required for assembly of virions and packaging of the viral chromosome.

Transformation by AD5

Infection of primary rodent cells does not lead to the efficient production of progeny virus, but can result in the morphological transformation of infected cells. Transformation of primary rodent cells requires functions encoded by both E1A and E1B genes (van der Eb et. al., 1979; McKinnon et al., 1982). Human cells can also be transformed by E1A and E1B, but only in the absence of the remainder of the viral chromosome (Graham et al., 1977). As a number of others have suggested transformation appears to result from expression of the E1A and E1B genes in the absence of the cell killing that is normally associated with adenovirus infection of permissive cells (Shenk and Flint., 1991).

Roles of E1A in Transfornmation

On its own E1A can establish or immortalize primary cells so that they grow indefinitely in culture, but these cells do not display some of the growth and morphological characteristics of cells more fully transformed by E1A and E1B (Houwelling et al., 1980;

Gallimore et al., 1984). Although both E1A and E1B are required for complete morphological transformation, E1A is considered to be required for its initiation because expression of E1B without E1A results in no detectable alterations in the phenotype of rodent cells (van den Elson et al., 1983).

A number of other viral and cellular oncogenes in addition to E1B can cooperate with E1A to bring about the full morphological transformation of primary cells. Among these are activated Ha-ras and the gene for the polyoma middle T-antigen (Ruley et al., 1983). These oncogenes, like E1B, cannot transform primary cells on their own, but can bring about the transformation of established cell lines. Likewise a wide variety of viral and cellular immortalizing nuclear oncogenes, including the SV40 and polyoma large T-antigens, human papilloma virus 16 E7, c-myc, and fos, can act in concert with ras, in place of E1A, to bring about the transformation of primary cells (see Ruley et al., 1990 for a review). Taken together these observations suggest that E1A is a member of a large family of oncogenes that encode nuclear proteins that can function to extend the proliferative capacity of primary rodent cells. Once nuclear oncogenes, like E1A, have established primary cells, these cells can be more completely transformed by the products of many different oncogenes that encode non-nuclear proteins.

Studies on the individual roles of the E1A proteins in transformation have been carried out using mutants and cDNA clones that express either of the E1A proteins individually (Haley et al., 1984; Montell et al., 1984; Winberg and Shenk, 1984; Zerler et al., 1986; Stephens and Harlow, 1987; Ulfendahl et al., 1987). Taken together the results of these studies suggested that: 1) the 243R and 289R proteins are each individually able to immortalize primary cells and to cooperate with ras for transformation, 2) expression of both proteins is required for full morphological transformation by wild-type E1A, 3) the 243R protein is particularly important for anchorage independent growth, and 4) the proteins encoded by the 9S, 10S and 11S mRNA's play no role in transformation. These observations suggest that sequences that are common to both the 243R and 289R E1A proteins are required for oncogenic transformation.

At the time these studies were initiated the underlying molecular mechanisms responsible for transformation by E1A were not known. A number of other biological activities of the E1A gene, which could possibly be required for transformation, are discussed after the next section on E1B.

Roles of E1B in Transformation

Comparison of cells transformed by E1A and E1B with

cells transformed by E1A alone suggests that the E1B products are responsible for conferring on cells a number of phenotypes that are associated with tumour cells (see White and Cipriani, 1990). These characteristics include: 1) altered cell morphology; 2) growth to high cell density free from contact inhibition; 3) acceleration of cell growth rates; and 4) anchorage independent growth (Houweling et al., 1980; Shiroki et al., 1981; Subrumanian et al., 1984; Bernards et al., 1986).

The individual roles of the 176R and 496R E1B proteins have been studied by cotransfecting BRK cells with E1A plasmids and with plasmids encoding either of the E1B proteins individually (White and Cipriani, 1990; McLorie et al., 1991). Separately both E1B proteins caused focus formation in cooperation with E1A, but at a reduced frequency compared to wild type E1B. The results suggest that the individual E1B proteins can cooperate with E1A for transformation using independent, but additive pathways (White and Cipriani, 1990, Mclorie et al., 1991). Individually both proteins induced morphological alterations and accelerated growth rates, and allowed growth to high densities (White and Cipriani, 1990). The 176R protein however was found to be responsible for promotion of anchorage independent growth, possibly through its ability to associate with and disrupt intermediate filaments in the cytoplasm and in the nuclear lamina (White and Cipriani.,

1989; White and Cipriani, 1990). The ability of the 176R polypeptide to protect cells from the cytotoxicity of the E1A proteins probably also plays a major role in transformation (White et al., 1991).

Other Biological Activities of the E1A Proteins

In addition to the ability of the E1A products to transactivate the other Ad early promoters, and to cooperate with other non-transforming genes for transformation, a number of other biological activities have been identified for the E1A products. These include: a) transactivation of some viral and cellular genes, b) transcriptional repression of a number of other viral and cellular genes, and c) induction of DNA synthesis and mitosis in non-permissive rodent cells.

a) Transactivation of Non-Adenovirus Promoters

A host of other viral promoters are transactivated by E1A, including those of the human cytomegalovirus IE gene (Gorman et al., 1989), the bovine papilloma virus type 1 LCR (Bernard et al., 1990) and the long terminal repeats of the human immunodeficiency virus (Rice and Mathews, 1988). In addition transcription from a variety of non-related cellular promoters is also stimulated by E1A in transient

expression assays. Among these are the promoters of the genes encoding globin, preproinsulin, β-tubulin, HSP70, c-myc, c-fos and the H-2K^b class major histocompatibility complex (see Shenk and Flint, 1991 for references). Kingston and Taylor found that 23 different artificial promoters, that were constructed by combining different combinations of upstream transcription factor binding sites and or nonsense sequences with the hsp 70 TATA box, were transactivated to increase expression 10 times. Transactivation of cellular genes by E1A is generally restricted to transfected promoter sequences, but there are exceptions as the endogenous hsp 70 and β-tubulin promoters are transactivated by E1A during infection (Kao and Nevins, 1983; Stein and Ziff, 1984; see Berk, 1986, for a review). Of special interest was the finding that adenovirus VA genes, which are transcribed by RNA polymerase III, could be transactivated by E1A (Berger and Folk, 1985; Gaynor et al, 1985; Hoeffler and Roeder, 1985).

The wide variety of promoters, both natural and artificial, which are responsive to E1A suggests that E1A can enhance the general transcription machinery (Taylor and Kingston, 1990). Shenk and Flint (1991) hypothesized that if E1A were to modify a basal transcription then TFIID would be the most likely candidate because binding of this factor to the TATA box is the rate-limiting step in transcription. The finding that E1A can physically interact with TFIID

(Horikoshi et al., 1991; Lee et al., 1991) provides strong biochemical evidence for a model in which transactivation is as a result of stimulation of the general transcription machinery.

b) Transcriptional Repression

E1A proteins can repress the activity of viral enhancers such as the SV40 and polyoma virus early promoter/enhancer regions (Borelli et al., 1984; Velcich and Ziff, 1985) and the HIV long terminal repeat (Ventura et al., 1990). In addition a number of tissue type-specific cellular enhancers can also be repressed by E1A, including the control elements of the genes encoding the immunoglobulin heavy chain (Hen et al., 1986), insulin (Stein and Ziff et al., 1987), cytochrome P-450c (Sogawa et al., 1989) and the muscle protein troponin (Enkemann et al., 1990). Interestingly, suppression of the immunoglobulin heavy chain enhancer by E1A is cell type dependant as the endogenous enhancer is repressed in a lymphocyte cell line (Hen et al., 1985), but activated in a fibroblast line containing stably integrated copies of a recombinant reporter gene linked to the heavy chain gene enhancer (Borelli et al., 1986).

The mechanism responsible for repression by the E1A proteins is not known although it may involve the activation

or suppression of proteins that interact with enhancer elements. Borelli et. al. (1984) found that repression of the SV40 enhancer by E1A could be relieved by adding extra copies of the 72 base pair enhancer element. They hypothesized that the co-transfected 72 base pair elements competed for a repressor protein that was somehow activated by E1A, and when the repressor protein was titrated out the enhancer was free to function normally. Tissue specific repression of the immunoglobulin heavy chain enhancer could also result from interactions between E1A and enhancer binding proteins. In lymphoid cells the E1A proteins may interact with and nullify the activity of a protein required for activation of heavy chain enhancer (Hen et al., 1985). On the other hand in the fibroblast line where immunoglobulin genes are normally dormant, E1A proteins may suppress a repressor of the heavy chain enhancer (Borelli et al., 1986).

In the initial papers in which enhancer repression by E1A was described, both the 289R and 243R proteins were reported to repress at roughly equal efficiencies in transient expression assays (Borreli et al., 1984; Velcich and Ziff, 1985). More recent reports however have suggested that the 243R E1A protein is a more effective repressor than the 289R proteins in both transient expression assays (Schneider et al., 1987; Kuppuswamy and Chinandurai, 1987) and in assays where E1A was expressed by viral infection

(Lillie and Green, 1986). The reasons for these discrepancies are not clear, but the use of different enhancers, cell lines and assays may partially explain them. One consensus from these studies does emerge however and it is that the unique region of the 289R protein which contains the primary transactivation domain is not required for repression.

c) Induction of Cellular DNA Synthesis

Growth arrested primary and secondary rodent cells are stimulated by infection with human adenovirus to re-enter the cell cycle and to replicate their DNA (Shimajo and Yasmastata, 1968; Strohl, 1969; Younghusband et al., 1979). E1A was shown to be the only adenovirus gene required for induction of DNA synthesis by analyzing a series of mutants lacking each of the other viral gene regions (Bellet et al., 1985). Another study in which plasmid DNA containing only the E1A region was microinjected into serum-starved mouse 3T3 cells confirmed that the E1A gene on its own was able to induce cellular DNA synthesis (Stabel et al., 1985).

Analysis of the biochemical events during E1A stimulated progression from G_0/G_1 to S-phase has shown that a number of G_1 events that normally precede S-phase are not required for the induction of DNA synthesis by the E1A proteins. For example selective inhibition of rRNA

synthesis or polyamine synthesis prevents serum-induced cell cycle progression, but it does not inhibit adenovirus-induced cellular DNA synthesis (Cheetham et al., 1982). In fact Pochron et al. (1980) demonstrated that quiescent baby hamster cells were induced to enter S-phase in the absence of any RNA synthesis. If the G_1 events that lead to S-phase are considered to constitute a sequential pathway, then E1A must act to induce S-phase at a point downstream of the effects of serum growth factors or by a completely different mechanism.

Studies on induction of cellular DNA synthesis by E1A have led to rather ambiguous conclusions concerning the roles of 243R and 289R. Infection of growth arrested secondary rat embryo fibroblasts with a virus producing only the 13S E1A message induced much higher levels of cellular DNA synthesis than virus producing only the 12S E1A mRNA (Bellet et al., 1985). On the other hand in cloned rat embryo fibroblasts (Spindler et al., 1985), and in baby rat kidney cells (Quinlan and Grodzicker, 1987; Zerler et al., 1987), infection with virus producing only the 12S message induced DNA synthesis more efficiently than infection with virus producing only the 13S mRNA. However, microinjection of E1A genes encoding either the 12S or the 13S message into growth arrested 3T3 cells stimulated cellular DNA synthesis efficiently (Stabel et al., 1985). The ability of the different E1A genes to induce DNA synthesis to different

degrees appears to depend on cell type and the mode of delivery of E1A. In general the majority of work on induction of DNA synthesis by E1A has been carried out, when the cell type permitted, using virus encoding only the 12S E1A message. This avoids the potential complications arising from cytotoxicity as a result of expression of the 13S message which encodes a fully competent transactivating 289R E1A protein (Quinlan and Grodzicker, 1987).

d) Stimulation of Mitosis and Cell Proliferation

E1A can induce mitosis and cell proliferation in BRK cells (Quinlan and Grodzicker, 1987; Zerler et al, 1987), but not rat embryo fibroblasts (REF) (Murray et al., 1982). A high proportion of BRK cells infected with virus producing only the 243R E1A protein undergo mitosis within the first 24 hours after infection and these cells can continue to proliferative for extended periods of time in culture (Quinlan and Grodzicker, 1987; Zerler et al., 1987). REF cells infected with wild type virus do not undergo mitosis at a faster rate than mock infected cells and the virally induced cell cycle in these cells is abnormal (Murray et al., 1982). Numerous chromosome aberations are visible at mitosis in REF cells and a significant proportion of cells are induced to overreplicate their DNA so that cells with a greater than G_2 content of DNA accumulate (Murray et al.,

1982, Bellet et al., 1989). Chromosomal abberations and overreplication are not a result of viral replication because both occur after infection with a virus temperature sensitive for viral replication at the non-permissive temperature (Brathwaite et al., 1981; Murray et al., 1982). These contradictory effects observed after infection of BRK cells and REF cells are most probably due to the different cell types used. One possibility is that BRK cells produce a factor which augments E1A function (Bellet at al., 1989). In fact Ad infected BRK cells produce an epithelial-like growth factor, which is not produced in mock infected BRK cell cultures or in cells infected with virus dl312 which does not produce E1A proteins (Quinlan et al., 1987).

Rationale and Goals for These Studies

At the time these studies were initiated it was not known how the relatively small E1A proteins could bring about transformation and such a wide range of other biological effects in cells. However we hoped that a detailed understanding of the molecular mechanisms responsible for E1A function would contribute greatly to the understanding of a number of fundamental cellular processes including regulation of gene expression, control of the cell cycle and the breakdown of cell cycle regulation that can result in oncogenic transformation and tumorigenesis.

Attempts had been made to define domains essential for particular functions. CR1 appeared to be required for transformation and repression (Schneider et al., 1987; Lillie et al., 1987). Residues in CR2 were required for transformation (Lillie et al., 1986; Moran et al., 1986; Kuppaswamy and Chinnadurai, 1987) and some groups had reported that this region was also required for repression (Lillie et al., 1986; Schneider et al., 1987). The major domain for transactivation was known to be located in CR3 (Moran et al., 1986; Lillie et al., 1986; Schneider et al., 1987) although a number of studies had suggested that the 243R product was able to activate transcription in certain cases (Leff et al., 1984; Winberg and Shenk, 1984; Simon et al., 1987; Zerler et al., 1987).

Results with point mutants and deletion mutants had led to the tentative identification of three critical domains which are roughly equivalent to the three conserved regions discussed above (Moran and Mathews, 1987). The work presented in this thesis is part of a large project initiated by Dr. S.T. Bayley to gain a better understanding of the functional domains and functions of the E1A gene products. The experimental approach was to use a series of small deletion mutants spanning the entire coding region of the E1A gene in a number of biological assays to survey E1A domains. This study was carried out at the same time as a number of similar studies by other groups, but our approach

was different as we used small deletion mutations which we hoped would have a minimal effect on the overall structure of the E1A proteins. In addition we used a series of mutations that spanned the entire E1A coding region so that we could carry out a more thorough and systematic study of E1A domains than had been previously attempted. My particular contribution to this study was to use the E1A mutants to help to define domains for transactivation and repression and to study the ability of the 243R protein to stimulate cellular DNA synthesis and cell proliferation.

Data collected from these assays could enable us to begin to understand the molecular mechanisms responsible for biological activities of E1A and to determine which E1A functions were likely to share the same underlying mechanisms. In particular one potential result would be the identification of E1A functions that are likely to be directly responsible for oncogenic transformation.

MATERIALS AND METHODS

Enzymes

All modifying enzymes were purchased from Boehringher Mannheim, BRL, or Pharmacia and used according to the suppliers' specifications.

Antisera

The antisera used were: M73, an anti-E1A monoclonal antibody that recognizes an epitope at the carboxyl terminus of the E1A proteins was purified from hybridoma cell cultures (Dr. Ed Harlow, Massachusetts General Hospital, Boston); 9C10, an anti-E1B 55K monoclonal antibody (Oncogene Science); anti-A and anti-B, which are polyclonal rabbit antisera directed against the human cyclin A and cyclin B proteins, respectively (Dr. J. Pines, Salk Institute, San Diego, CA); and anti-p34, a polyclonal rabbit antiserum directed against a carboxyl terminal peptide of the human cdc2 p34 protein (Dr. J. Th'ng, Davis CA).

Plasmids

JM17, a gift from Dr. F. L. Graham (McMaster University), is a non-infectious 40 kb plasmid containing the entire Ad5 dl309 genome. It was derived from the infectious plasmid pFG140 by insertion of the 4.4 kb plasmid pBRX into the unique XbaI site at position 1339 of the *dl*309 genome (McGrory et al., 1988).

pLE2, constructed by Dr. T. N. Jelsma (Jelsma et al., 1988), contains the Ad5 E1A gene inserted into the tetracycline gene of pBR322 and is the parental plasmid used for all of the work described here. This plasmid is essentially identical to pCD2 (McKinnon, 1984) except that the ClaI site at position 24 in the pBR322 sequence was inactivated (Jelsma et al., 1988). PLE2 contains Ad5 sequences 22 to 1774, starting at the BamHI site in pBR322, which is joined to a short stretch of Ad5 sequence from 5644 to 5788 that ends at the SalI site in pBR322.

pLE2dl520 was constructed by J. Mymryk (Jelsma et al., 1989) by replacing the XmaI to XbaI fragment of E1A in pLE2 with the cognate fragment of Ad5 DNA from dl 520 (Haley et al., 1984).

M13mp19E1A was constructed by C. Evelegh in Dr. Bayley's lab. It was constructed by inserting the Bam HI - Xba I fragment of E1A from pLE2 into the M13mp19 multiple cloning site.

pSV2CAT (Gorman et al., 1982) was obtained from Dr. F. L. Graham and contains all of the bacterial chloramphenicol acetyl transferase gene including 3' flanking control sequences. Expression of CAT mRNA is dependant on the SV40 early promoter/enhancer region.

pKCAT23 came from Dr. N. Jones (Imperial Cancer Research Laboratories, London). This plasmid is a derivative of pSV2CAT in which the SV40 promoter/enhancer region has been replaced with the Ad5 early region 3 promoter.

Bacterial Strains

The *E. coli* strain MAX efficiency DH5α (BRL) was used in routine gene cloning procedures for transformation.

E. coli CJ236 (Bio Rad) contains mutations in the dUTPase (dut^-) and uracil N-glcosylase (ung^-) genes. This strain was maintained on LB agar plates supplemented with chloramphenicol (30 μg/ml).

E. coli MV1190 (Bio Rad) was maintained on glucose minimal medium plates. Well isolated colonies were used to inoculate liquid cultures which were freshly prepared for each application.

Viruses

Human adenovirus type 5 mutants dl 309 and dl 312 (Jones and Shenk, 1979) were obtained originally from Dr. F.L. Graham. Dl 309 contains a number of deletions, but grows in culture as efficiently as wt Ad5. Dl 312 contains a deletion of all of the E1A region. Ad5 dl 520 (Haley et al., 1984), obtained from Dr. P.E. Branton (McGill University, Montreal), is a derivative of dl309 that contains a small deletion of base pairs 1107-1117. This deletion removes the 5′ splice site for the 13S E1A mRNA. All of the other E1A mutant viruses used are listed in Table I.

Cell Lines

Unless otherwise stated all cell lines were maintained on 100mm or 150mm plastic tissue culture dishes (Nunclon) in Dulbecco's modified minimal essential growth medium (Gibco) supplemented with 10 % fetal bovine serum or 10 % newborn calf serum (Gibco or CanSerum), 100 units/ml

penicillin (Gibco) and 100 μg/ml streptomycin (Gibco).

Hela cells were obtained from Dr. F. L. Graham. They were originally derived from a human cervical carcinoma (Gey et al., 1952).

293 cells were a gift from Dr. F. L. Graham. They are a human embryonic kidney cell line which constitutively express the human adenovirus type 5 E1A and E1B genes (Graham et al., 1977).

Baby Rat Kidney Cells

Primary BRK cells were prepared using the method of van der Eb and Graham (1980). Twelve 3 - 7 day old Wistar rats (Charles River) were killed by cervical dislocation. Their kidneys were dissected, minced, added to 50 ml of a 0.5% trypsin (Gibco)/PBS solution and incubated with stirring at 37°C for 20 minutes. The resulting supernatant was transferred to a bottle containing 10 ml of 4°C fetal bovine serum, to inhibit the trypsin, and the bottle was held on ice. 50 ml of trypsin/PBS solution was added to the undigested kidney fragments and incubated with stirring for a further 20 minutes. The entire contents of this digestion were pooled with the supernatant from the first

digestion and centrifuged for 15 minutes at 3000 rev/minute in a Beckman TJ6 centrifuge. The cell pellet was resuspended in 100 ml of growth medium, incubated at 37°C for 15 minutes, and then filtered through cheese cloth. The suspension of cells was diluted with growth medium to a final volume between 400 and 600 ml, depending on the size of the kidneys, and 5 or 10 ml of this suspension was plated on 60 mm or 100 mm tissue culture plates (Nunclon) respectively. After 24 hours incubation the cells were rinsed with PBS and fed with fresh growth medium.

Small Scale Isolation of Plasmid DNA

Small scale preparations of plasmid DNA were made using the alkaline lysis method described by Sambrook et al., (1989). Two ml aliquots of LB broth and appropriate antibiotics were inoculated with transformed bacteria and incubated overnight at 37°C. Cells were harvested by centrifugation in a microfuge and then rinsed by resuspension in 0.5 ml of STE. After recentrifugation, the cells were lysed in 100 μl of solution I at 4°C and 200 μl of solution II. The lysates were then mixed with 150 μl of solution III and incubated on ice for 5 minutes in order to precipitate chromosomal DNA. The precipitates were removed by centrifugation for 5 minutes in a microfuge at 4°C. Low molecular weight nucleic acids were precipitated from the supernatants by adding 2 volumes of 95% ethanol, followed by incubation at room temperature for 5 minutes and centrifugation for another 5 minutes at 4°C. The pellet of nucleic acids was rinsed with 70% ethanol and again with 95% ethanol, then dried and resuspended in 50 μl of TE containing 20 μg/μl of RNAase A (Boehringer Mannheim).

Large-Scale Isolation of Plasmid DNA

For large scale isolation of plasmid DNA the method of Birnboim and Doly (1979) was used as modified by Sambrook et al., 1989. 500 ml bacterial cultures were grown in a 50:50 mixture of LB and terrific broth (TB), supplemented with antibiotics (in most cases ampicillin at 20 μg/μl) to an absorbance at 600 nm of 0.6. Chloramphenicol was added to a final concentration of 170 μg/μl, and the cultures were incubated overnight at 37°C.

To harvest the cells, these cultures were centrifuged at 4000 rpm for 15 minutes in a Cryofuge 6000 (4°C). The bacterial cell pellets were then rinsed in 100 ml of STE (4°C) and collected again by centrifugation. To lyse the cells, the rinsed cell pellets were resupended in 10 ml of solution I, 1 ml of lysozyme solution (10 mg/ml in 10 mM Tris-HCl pH 8.0) and 20 ml of solution II. After incubation at room temperature for 10 minutes, 15 ml of solution III was added to precipitate chromosomal DNA and protein. The centrifuge bottles were shaken vigorously and held on ice for 10 minutes. The lysates were cleared by centrifugation (as described above) and the supernatants were removed to fresh centrifuge tubes by filtering through two layers of cheese cloth. A 0.6 volume of isopropanol was then added and mixed with the supernatants and the tubes were stored at room temperature for 10 minutes. Low

molecular weight nucleic acids were pelleted by centrifugation at 7000 rpm in a Sorval SS34 rotor for 15 minutes, rinsed once with 70 % ethanol, dried and resuspended in 8 ml of TE (pH 8.0).

Purification of Plasmid DNA

Plasmid DNA was purified by equilibrium centrifugation using CsCl-ethidium bromide gradients. 1 g/ml of CsCl was added to the 8 ml nucleic acid solution prepared above and the resulting solution was loaded into sealable ultra centrifuge tubes (Seton). Ethidium bromide was added to a final concentration of 750 μg/ml and the space between the CsCl/DNA solution and the top of the tube was filled with paraffin oil. The tubes were heat sealed and centrifuged overnight at 55 000 rpm in a VTi 65.1 rotor (Beckman). Plasmid bands were collected into syringes fitted with 21 gauge needles and then transferred to 15 ml conical tubes. Ethidium bromide was extracted by mixing the plasmid DNA with an equal volume of CsCl saturated propanol, followed by centrifugation in a Beckman TJ6 to separate the aqueous and organic phases and then removing the upper (organic) phase. This procedure was repeated until no trace of ethidium bromide could be detected. The aqueous phase was diluted with 3 volumes of water and was then ethanol precipitated. Plasmid DNA was collected by centrifugation

at 8000 rpm at 4°C for 15 minutes in a swinging bucket rotor. The plasmid pellet was washed twice with 70% ethanol and once with 95% ethanol and resuspended in 1 ml of TE (pH 8.0)

Preparation of Competent Bacterial Cells

The bacterial strain DH5α and MV1190 were made competent for transformation by treatment with $CaCl_2$. 50 ml cultures of cells were grown to an absorbance (600 nm) of between 0.5 and 0.8 and were then harvested by centrifugation for 10 minutes at 5000 rpm in an SS34 rotor. The growth medium was completely removed and the pellets of bacterial cells were resuspended in 20 ml of cold 50 mM $CaCl_2$. After incubation on ice for 30 minutes the bacterial cells were collected by centrifugation as above. The supernatant was removed and the cell pellets were then resuspended in 4 ml of 50 mM $CaCl_2$ solutions. Competent cells were used for transformation within 30 hours after preparation.

Mutagenesis

The in-frame deletion mutations dl1101, dl1143, dl1144, and dl119 were constructed using the oligonucleotide site-directed technique of Zoller and Smith, (1984) with

modifications by Kunkel (1985). All reagents, bacterial strains, and M13 vectors used for mutagenesis were provided in the Muta-Gene in vitro mutagenesis kit (Bio Rad).

a) Oligonucleotides

23 nucleotide long oligonucleotides were designed to hybridize to 11 or 12 nucleotides of the E1A template on either side of the regions which were to be removed. The oligonucleotides were synthesized in the Central Facility of the Institute for Molecular Biology (McMaster University).

b) Phosphorylation of Oligonucleotides

Oligonucleotides were phosphorylated at the 5′ end with T4 polynucleotide kinase in the following reaction:

Oligonucleotide (50 pmol/μl)	4 μl
10X Kinase Buffer	3 μl
T4 Kinase (5 U/μl)	1 μl
dd H_2O	to 30 μl

The reaction mixtures were incubated at 37° C for 45 minutes, stopped by heating at 65° C for 10 minutes, and then diluted to 6 pmol/μl with TE.

c) Preparation of Template M13mp19E1A DNA

To propagate M13mp19E1A bacteriophage, 30 ml of 2X YT was inoculated with 1 ml of an overnight culture of CJ36 and incubated at 37°C, for about 2 hours, until the cultures had an optical density at 600 nm (A_{600nm}) of 0.3. The cells were then infected with M13mp19E1A bacteriophage at a multiplicity of 0.2 phage/cell by adding the phage directly to the CJ36 culture to produce phage containing uracil. After incubation at 37° C for 4 hours the cultures were centrifuged at 12 000 revs/min for 15 minutes in an SS34 rotor. The supernatants were transferred to fresh tubes, recentrifuged, and incubated with 150 μl of RNase A (Boehringer Mannheim) for 15 minutes at room temperature. Phage particles were precipitated with 3.5 M ammonium acetate (0.25 volumes) and 20% polyethylene glycol (PEG 8000, BDH). After incubation on ice for 30 minutes, precipitated phage particles were isolated by centrifugation for 15 minutes as above, and resuspended in 200 μl of high salt buffer. Insoluble material in the resuspended phage solution was removed by centrifugation for 2 minute in a microcentrifuge. To purify M13mp19E1A DNA, the entire phage solution was phenol/chloroform extracted with back extractions to improve yields. DNA was precipitated by adding 0.1 volumes of 7.8 M ammonium acetate and 2.5 volumes

of 95% ethanol. The samples were incubated at -20° C for 30 minutes and centrifuged in a microfuge for 15 minutes at 4°C. The ssDNA was rinsed once with 95% ethanol and resuspended in 20 μl of TE.

d) Annealing of Primer to Template

The following reaction was prepared using M13mp19E1A ssDNA and a number of different oligonucleotides:

DNA template	200 ng
Oligonucleotide	3 pmol
10 X Annealing Buffer	1 μl
dd H_2O to final volume	10 μl

In addition a control reaction that contained all of the above ingredients except the priming oligonucleotide was prepared. The reactions were heated to 70°C, allowed to cool slowly to 30° C over a 40 minute period and held in an ice water bath.

e) Complementary Strand Synthesis

For complementary strand synthesis, 1 μl of 10X synthesis buffer, 2-5 units of T4 DNA Ligase and 1 unit of T4 DNA polymerase were added to both the annealing reaction

and the control reaction. The reactions were kept in an ice-water bath for 5 minutes and then incubated at 25°C for 5 minutes and 37°C for 90 minutes. The reaction was stopped by adding 90 µl of TE and incubation at -20°C. To analyse the efficiency of the complementary synthesis reactions, 10 µl aliquots were separated on 1% agarose gels to determine the conversion rate of single stranded template to covalently closed circular replicative form (RF) plasmids. If sufficient amounts of RF DNA were present in the synthesis reactions as compared to the control reactions, the products of both were used to transform competent MV1190 cells.

f) Transformation of MV1190 Cells With Synthesis Reactions

For transformation 6 µl aliquots of the synthesis or control synthesis reactions were mixed with 300 µl samples of competent MV1190 cells and incubated on ice. After 40 minutes the cells were heat-shocked by incubating at 42° C for 3 minutes and then held on ice. To propagate the bacteriophage, 100 µl aliquots of the transformed cells were mixed with 300 µl of an overnight culture of MV1190 and 2.5 ml of 44°C top LB agar. This solution was poured onto LB agar plates and after the agar had solidified the plates were incubated overnight at 37° C. The next day 6 well separated plaques were picked with a pasteur pipette and

dispersed in 3 ml of TE by vortexing. Phage in these plaques were screened to isolate potential mutants by restriction enzyme analysis of replicative form (RF) DNA and by DNA sequencing of single stranded bacteriophage DNA.

g) Preparation of RF DNA and Restriction Enzyme Analysis

To prepare RF DNA, 6 μl of M13mp19E1A phage/TE suspensions were inoculated into 2 ml of LB containing 10 μl of an overnight culture of MV1190 cells. After incubation overnight at 37°C, RF DNA was prepared the next day using the mini-plasmid DNA procedure described above. RF DNA was analyzed with appropriate restriction enzymes and DNA fragments were analyzed on 1 % agarose gels.

Preparation of Single Stranded DNA for Sequencing

M13mp19E1A phage(s) which potentially contained the desired E1A mutations were propagated so that single stranded DNA could be prepared for sequencing. For propagation loops of the phage solution were streaked across LB agar plates and then covered with 2.5 ml of molten (44 °C) LB top agar containing 0.3 ml of MV1190 overnight culture. After the molten agar had solidified the plates were incubated for 6 hours at 37°C when phage plaques and top agar were scooped from about 1/4 of the surface area of

the plates and inoculated into 20 ml of LB medium. These cultures were incubated overnight at 37° C and phage DNA was prepared the next day. The procedure used to isolate single stranded phage DNA was exactly the same as for isolation of single stranded uracil containing template DNA as described above.

DNA Sequencing

The Sanger dideoxynucleotide chain termination method was used for all DNA sequencing. All reagents and protocols used were provided in the T7 DNA sequencing kit (Pharmacia).

The sequencing primers used were either the universal M13 primer (Pharmacia) or various other oligonucleotides complememtary of E1A sequences that Dr. Bayley's lab has had made by The Institute for Molecular Biology McMaster University. To anneal the primers to the single stranded phage DNA template the following were added to a 0.5 ml microcentrifuge tube:

Template DNA (2 μg/μl)	2 μl
Oligonucleotide primer (0.5 pmol)	3 μl
Annealing buffer (supplied)	2 μl
dd H_2O to final volume	14 μl

The annealing reactions were incubated at 60° C for 10 minutes followed by a 10 minute incubation at room temperature when 3 μl of the supplied labelling mix (dGTP, dTTP, dCTP), 1 μl of [^{35}S]dATP (NEN, 500 Ci/mol) and 3 units of T7 polymerase were added. The labelling reactions were first incubated at room temperature for 5 minutes to extend the primers about 20-30 nucleotides and ensure efficient incorporation of the radiolabelled dATP. For further extension 4.5 μl aliquots of the labelling reactions were added to four 0.5 ml prewarmed tubes containing high concentration dNTP's and a single dideoxynucleotide (ddATP, ddCTP, ddGTP or ddTTP). Following incubation at 37° C for 5 minutes, the extension reactions were stopped by adding 5 μl of the provided stop/gel loading solution. To separate double stranded DNA for sequencing the stopped reactions were incubated at 85° C for 2 minutes.

PAGE Analysis of the Sequencing Reactions

The extended radiolabelled DNA fragments were analyzed by electrophoresis on 6% polyacrylamide gels (19:1 acrylamide:bis-acrylamide) containing 7M urea, using a LKB macrophor electrophoresis unit and TBE gel running buffer. After electrophoresis the gels were rinsed for 30 minutes in a 10% acetic acid solution, dried at 60°C overnight, and then exposed to X-Omat K film (Kodak).

Transfer of E1A Mutations From M13 to pLE2 and pLE2dl520

E1A mutants were transferred from the M13mp19E1A background in which they were constructed into pLE2 or pLE2/dl520 by replacing an E1A fragment in the wild type pLE2 plasmids with the cognate fragment from M13mp19E1A.

Mutated M13mp19E1A and pLE2 or pLE2/dl520 were cleaved first with Sma I and then Sst II. Both restriction enzyme digests generate two fragments, a large one containing mostly vector sequences, and a small fragment which is common to both vectors and spans E1A nucleotide positions 356 - 1009. The DNA fragments were separated on 0.6% agarose/TAE gels. Agarose slices corresponding to the small M13mp1pE1A fragment and the large pLE2 or pLE2/520 vector fragments were cut from the gel and chopped into small pieces with a scalpel. DNA fragments were purified using the materials and instructions contained in a Geneclean kit (Bio 101).

For ligation the small E1A fragment and the pLE2 vector fragments were mixed at an approximate molar ratio of 1 : 1, in ligation buffer, 10 mm ATP and water to a final volume of 20 μl. T4 DNA ligase (Pharmacia) was then added and the reactions were incubated overnight at room temperature. The ligation reaction was stopped by addition of 80 μl of TE and used to transform competent DH5α *E. coli* cells.

Transformation of Bacteria

For transformation 10 - 50 μl of the ligation reactions were mixed with 100 μl of competent DH5α cells and incubated on ice for 30 minutes. The cells were then heat shocked for 2 minutes at 42° C, diluted with 1 ml of LB and incubated at 37° C for 30 minutes. 100 μl and 300 μl aliquots of the cells were plated on LB agar plates, containing 15 μg/ml ampicillin, and incubated overnight at 37° C. For screening, a number of colonies were used for small scale isolation of plasmid DNA. The plasmids were then digested with appropriate restriction enzymes to isolate recombinant pLE2 plasmids containing the mutations of interest.

Transfer of E1A Mutations into Ad 5

Mutated E1A genes were transferred from either pLE2 or pLE2d1520 backgrounds into d1309 using the technique of JM17 of Graham (McGrory et al., 1988). For transfection of 293 cells 10 μg of JM17, 5 μg E1A containing plasmid and 5 μg of sonicated salmon sperm DNA were added to 500 μl of a 1X HeBS and mixed. 25 μl of 2.5 M $CaCl_2$ was added to this solution, mixed and incubated at room temperature until a fine precipitate formed, usually after 15-30 minutes. This suspension was dripped into the growth medium of confluent

60 mm dishes of 293 cells. After a 5 hour incubation at 37°C, the growth medium was replaced with 10 ml of molten plaquing medium at 44°C and allowed to solidify at room temperature. The cells were maintained at 37° C for about 7 to 10 days until viral plaques were visible. Plaques were picked with sterile pasteur pipettes, resuspended in 0.5 ml of PBS^{++}, checked by Sma I restriction digest of viral DNA, and plaque purified twice.

Infection of Cell Lines

Monolayer cultures of cells were infected at the multiplicities indicated for each technique or experiment. Viral particles were diluted from concentrated stocks in 200 μl of growth medium (without serum) for infections of 60 mm dishes, 500 μl of medium for 100 mm dishes, or in 1ml for infections of 150 mm dishes, and carefully dripped over the cells after removal of the growth medium. Following absorption for 1 hour at 37°C warm fresh, or spent, medium was added to the plates.

Large Scale Preparation of Virus

All human adenovirus type 5 derivatives were propagated on 293 cells. For large scale preparation of virus five 150 mm dishes of confluent 293 cells were

infected at a multiplicity of 2 plaque forming units (p.f.u.) per cell and harvested 48 hours after infection into 50 ml plastic tubes (Falcon). The cells were removed by centrifugation for 15 minutes at 3000 rpm in a Beckman TJ6 centrifuge. Cell pellets were resuspended in 5 ml of Tris Saline and lysed by sonication at maximum power with a microtip attachment for 5 seconds. After sonication deoxycholic acid was added to a final concentration of 0.5% and the lysates were incubated at 37°C for 30 minutes when 50 μg/μl RNase (Boehringer), 50 μg/μl DNase (Sigma) and 0.2 M $MgCl_2$ were added. The lysates were incubated at 37°C for an additional 60 minutes and then sonicated for 15 seconds.

Viral particles were purified by centrifugation on CsCl gradients. The cell lysates were adjusted to a CsCl concentration 1.34 g/cc, loaded into ultra-centrifuge tubes (Seton), and centrifuged in a Ti 65 fixed angle rotor (Beckman) for 16 hours at 33 000 rpm. Viral bands were removed by needle and syringe, diluted with one-third volumes of glycerol and 5 times concentrated Tris/saline, aliquotted, and stored at -70°C.

Titration of Virus

Confluent 293 cells growing on 60 mm dishes were infected with 200 μl of 10^{-7}, or 10^{-8}, dilutions of large

scale viral stocks. The cells were then covered with 10 ml of a 1:1 solution (44°C) of plaquing medium and 1.0% agarose which was allowed to solidify at room temperature for 15 minutes. After incubation for one week at 37°C plaques were scored for two or three consecutive days.

Immunopreciptiation

a) Metabolic Cell Labelling and Preparation of Cell Lysates

Monolayer cultures of cells, growing in 10 cm dishes, were metabolically labelled with [^{35}S]methionine. The procedure was the same for all cell types, whether infected or uninfected, and involved first removing the growth medium and rinsing the cells with PBS to remove residual medium. For labelling 4 ml of D-MEM medium without methionine (Gibco, cat. no. 320-1970AJ) and 200 μCi translation grade [^{35}S]methionine (Dupont) were added. The cells were then incubated at 37°C for between 3 and 4 hours. To prepare cell lysates the labelling medium was removed and the cells were rinsed once with 3 ml of PBS. An additional 1 ml of PBS was pipetted onto the plates and the cells were scraped into 1.5 ml test tubes. For lysis cells were collected by centrifugation in a microfuge and resuspended in 400 μl of lysis/wash buffer X by vortexing for 20 seconds. The lysates were incubated on ice for 20 minutes

and then centrifuged in a microfuge for 15 minutes at 4°C to remove nuclei and cell debris. The supernatants were then carefully recovered to fresh tubes and precleared to remove proteins which associate non-specifically with protein A Sepharose beads. To preclear a 100 μl slurry of protein A Sepharose beads (Pharmacia, 300 mg/ml in Buffer X) was added to the cell lysates and this suspension was incubated for 15 minutes on a rotating wheel at 4°C. The protein A beads were removed by centrifuging the tubes in a microfuge for 5 minutes and then carefully transferring the supernatants to fresh tubes.

b) Antibody Reaction

Appropriate antibodies were added to the precleared supernatants and incubated at 4°C on a rotating wheel for between 30 minutes and 1 hour depending on the avidity of the antibody. Antigen-antibody complexes were collected on protein A beads by adding 100 μl of protein A Sepharose slurry (300 mg/ml) and mixing on a rotating wheel for 30 minutes at 4°C. Protein A beads were recovered by centrifugation and removal of the supernatant by aspiration with a bent 23-gauge needle. The beads were then washed four times with 700 μl of buffer X. The wash procedure involved: 1) vortexing the buffer X/protein A suspension for 20 seconds, 2) centrifugation of the tubes for 10 seconds in

a microfuge and, 3) aspiration of the wash buffer. Two further rounds of washing were carried out using wash buffer X without BSA added. After the final wash the wash buffer was completely removed by inserting the needle directly into the pellet of beads. 50 μl of SDS gel loading buffer was added to the beads and the suspension was boiled for 5 minutes to break up the antigen/antibody/protein A complexes by denaturation.

c) Discontinuous SDS Polyacrylamide Gel Electrophoresis (PAGE)

Denatured immunoprecipitated proteins were resolved by SDS PAGE electrophresis (Laemmli, 1970) on vertical slab gels using 7.5% - 10.5% polyacrylamide separating gels 18 cm in length and 4% stacking gels. The gels were run overnight at 75 volts in electrophoresis running buffer until the bromophenol blue tracking dye front had run out of the bottom of the gel. The gels where then treated with diphenyloxazole (PPO), as desribed in Harlow and Lane (1989), to enhance the radioactive signal emitted from the [^{35}S] methionine labelled proteins. After PPO treatment the gels were rinsed under a steady stream of water for 45 minutes, dried and exposed to Kodak XAR-5 film.

Analysis of E1A Associated Proteins From BRK Cells

Preparation of infected BRK cells and immunoprecipation of E1A proteins was carried out essentially as described above except for the following modifications: 1) cell lysates were prepared from two 60 mm dishes, 2) the lysates were precleared with 200 μl of protein A sepharose slurry instead of 100 μl, and 3) the vortex times for washing the protein A sepharose-antibody complexes were 10 seconds instead of 20 - 30 seconds. An increase in the amount of protein A Sepharaose removed the high concentration of background bands from BRK lysates. A decrease in the wash period probably decreased loss of E1A and associated proteins.

Peptide Mapping by Partial Proteolytic Cleavage

The procedure used was that of Clevland (1977), as described in Harlow and Lane (1988). [^{35}S]methionine labelled proteins for analysis were resolved on SDS-PAGE gels as described above, dried without PPO fluorography, and exposed to XAR-5 film overnight. Bands containing the proteins for proteolytic digestion were excised from the dried gels using the developed film as a template and pushed into the wells of a 15% polyacrylamide gel. The dried gel pieces were covered with swell buffer and incubated for 10

minutes at room temperature when 20 μl of peptide mapping sample buffer and 10 μl of protease sample buffer, containing various dilutions of Endoproteinase Glu-C (Boehringer Mannheim), were added.

The gels were run at 150 volts until the dye front was about 1 cm from the separating gel and the power pack was turned off. After a 30 minute incubation period the power pack was turned back on and the gels were run until the dye fronts had just run out. The gels were PPO enhanced and dried as described above.

Immunoblotting

Proteins were transferred from 8-10% polyacrylamide gels to nitrocellulose transfer membranes using a semi-dry electrophoretic transfer apparatus (Tyler Research Instruments) by the method described in Harlow and Lane (1988). Transfer was carried out for between 30 and 90 minutes, depending on the molecular weight of the protein of interest, at constant voltage (30 V).

Immunodetection was carried out using the ECL enhanced chemilumimescence technique (Amersham). Nitrocellulose filters were prehybridized in TBST wash buffer supplemented with 5% dried milk powder (Carnation) for between 1 and 16 hours, and then washed with multiple changes of TBST. Primary antibodies were hybridized to the

filters for 1 hour at room temperature in TBST with 5% dried milk powder. Detection was by horse-radish peroxidase conjugated anti-mouse, or anti-rabbit secondary antibodies (Amersham), which were hybridized to the filters in TBS-T with milk for 1 hour at room temperature. Following washing as above the filters were immersed in a 1:1 solution of detection reagents 1 and 2 (Amersham) for 1 minute. Excess detection solutions were then drained, and the filters were wrapped in Saran Wrap and exposed to XAR-5 or XOMAT-K1 (Kodak) film for between 15 seconds and one hour.

Transfection for Transactivation Assay

The calcium phosphate technique was used to co-transfect 10 cm dishes of rapidly growing HeLa cells with CAT reporter plasmids and pLE2 containing either wild type or mutated forms of E1A. For each transfection the following solutions were added to 15 ml screw top Falcon tubes.

2x Hepes (pH 7.1)	0.5 ml
dd H_2O	0.5 ml
sonicated salmon sperm DNA (5 mg/ml)	10.0 μl

Plasmid DNA was then added (for transactivation assays 5 μg of E1A plasmid and 10 μg of pKCAT23; for repression assays 2

or 3 μg of E1A plasmid and 5 μg of pSV2CAT), followed by 50 μl of a 2.5 M $CaCl_2$ solution. The resulting solutions were incubated at room temperature until a fine precipitate had formed (20-30 minutes) and then added to the cell medium.

After a 4 -8 hour incubation period at 37° C the cells were glycerol shocked to help improve the efficiency of transfection. For this procedure the growth medium was aspirated from the dishes and 2 ml of 20 % glycerol in 1 X Hepes (pH 7.1) was added. After exactly 90 seconds the glycerol solution was removed by aspiration and 30 seconds later 4 ml of 1 X Hepes was added to rinse the cells. The Hepes solution was swirled over the plates and then replaced with 10 ml of fresh medium.

Chloramphenicol Acetyl Transferase (CAT) Assay

CAT assays were performed using the methods of Gorman and Howard, (1982) with modificatons by Crabb and Dixon (1987). At 48 hours after transfection the growth medium was removed and the cells were rinsed with 5 ml of PBS which was replaced with 750 μl of CAT solution 1. After a 5 minute incubation at room temperature the cells were scraped from the plates and transferred to microcentrifuge tubes. The plates were then rinsed with an additional 750 μl of CAT solution 1. Cells were collected by centrifugation for 30 seconds in a microfuge and resuspended

in 165 μl of TED buffer. Cell lysates were prepared by sonication on ice using 5 short bursts at maximum power with a microtip attachment. The lysates were heated at 60°C for 10 minutes and then centrifuged for 5 minutes at 4°C in a microfuge.

For the CAT reactions a substrate stock solution was prepared as follows:

Acetyl COA (1 μg in 57 μl ddH_2O)	26 μl
[^{14}C]Chloramphenicol (50 mCi/mmole, Amersham)	26 μl
TED Buffer	273μl

50 μl of the substrate stock solution was added to either half or all of the cell lysate and this solution was incubated for between 15 to 60 minutes at 37° C. The reactions were stopped by addition of 1 ml of ethyl acetate. Chloramphenicol derivatives were extracted by vortexing the lysate/ethyl acetate mixture for 30 seconds, removing the upper organic phase to a fresh test tube and then speed-vac evaporation of the ethyl acetate. The resulting residue was resuspended in 20 μl of ethyl acetate. Radiolabelled chloramphenicol and acetylated derivatives were separated by chromatography by spotting the entire sample onto a silica gel thin layer chromatography plate (Analtech Silica gel G) with a micropipette tip. The samples were run in a solvent

solution of 95 : 5 chloroform : methanol until the solvent front was just below the top of the plate. The plates were dried and then exposed to X-OMAT film (Kodak) overnight. ^{14}C chloramphenicol and acetylated derivatives were scraped from the plates using the autoradiograph as a template, dissolved in 10 ml of Ready Safe scintillation fluid (Beckman) and counted in a scintillation counter.

Measurement of Induction DNA Synthesis

Induction of DNA synthesis was measured by incorporation of [^{3}H]thymidine into newly replicated DNA (Zerler et al., 1987). For the assay triplicate 60 mm dishes of 3 day old quiescent BRK cells were infected at an moi of 10 pfu/cell with mutant Ad5 virus. The BRK growth medium was replaced with DMEM containing 5% fetal bovine serum after infection, and at 0, 24 and 48 hours after infection 10 μl of [methyl-^{3}H]thymidine (5 Ci/mmol, Amersham) was added. Following 24 hour incubation periods, at 24, 48 and 72 hours after infection, the cells were rinsed and 1 ml of PBS was added. The cells were then scraped from the plates with a rubber policeman, transferred into 1.5 ml microfuge tubes and collected by centrifugation for 30 seconds in a microfuge. The pellet of cells was resuspended in 700 μl of 0.3 M NaOH by pipetting up and down. This solution was held on ice for 30 minutes and then

700 µl of 20 % trichloroacetic acid was added. After thorough mixing this solution was incubated for 20 minutes on ice. The TCA precipitates were collected under suction on Whatman glass fiber filters, washed 3 times with 5 ml of 10% TCA at 4°C and then 3 times with 5 ml of 95% ethanol at 4°C. The filters were dried under suction, transferred to 3.5 ml scintillation vials and overlayed with 3 ml of Ready Safe scintillation fluid (Beckman). Samples were stored in the dark overnight and counted the following day.

Estimation of Viral DNA replication in BRK Cells

To estimate the efficiency of viral replication in BRK cells the total amount of viral DNA synthesized for 3 days after infection with dl 520 or dl 309 was compared with the total amount of newly synthesized DNA over this same time period. For the assay, duplicate sets of infected BRK cell monolayers were labelled with [^{3}H]thymidine for three 24 hour labelling periods as described above. Total incorporation of [^{3}H]thymidine was determined for one set of infected cells (as described above). The other set of cells were used to determine the extent of viral DNA replication using a modified Hirt extraction procedure (Hirt, 1967).

To isolate viral DNA the cells were rinsed with PBS and lysed by adding 2 ml of pronase buffer, containing 1 mg/ml of pronase, to the culture dishes. Following

incubation for 2 hours at 37°C, 250µl of a 3 M solution of NaAC was added to the dishes. The lysates were then transferred into microcentrifuge tubes and held overnight at 4° C. High molecular weight DNA and other debris were pelleted by centrifugation at 4° C for 15 minutes in a microfuge. The supernatants extracted twice with a 1:1 mixture of phenol and chloroform and once with chloroform. Low molecular weight DNA was ethanol precipitated and separated on 0.6% agarose gels containing 10 µg/ml ethidium bromide. Agarose bands containing the marker Ad5 dl309 DNA were visualized with UV light, cut from the gels, chopped into small pieces and added to 3.5 ml scintillation vials together with 3 ml of Ready Safe scintillation fluid. The vials were stored in the dark overnight and counted the next day. The counts per minute (cpm) from the Hirt experiments gave relative proportions of viral DNA for dl 309 and dl 520 infections. These were then used to estimate the cpm of TCA precipitations as viral DNA for dl 520.

Measurement of Mitotic Index

The method of Zerler et al (1987) was used to measure the mitotic index of BRK cells infected with E1A mutants virus. Duplicate sets of BRK cells, propagated on microscope cover slips, were infected 3 days after plating with Ad5 dl520 (wild type or mutant) at a multiplicity of 10

plaque forming units per cell. After absorption fresh DMEM growth medium containing 5 % fetal bovine serum was added to the cells. One set of cells was incubated in the presence of 1 μM nocodazole (Sigma) from 0 to 24 hours after infection while the other set was treated with nocodazole from 24-48 hours after infection.

After 24 hours in the presence of nocodazole the cells were rinsed twice with 5 ml of PBS at 4°C and then fixed by adding 3 ml of Carnoy's fixative (3 : 1 ethanol : acetic acid, 4° C) and incubating the cells at -20° C for 20 minutes. After incubation the Carnoy's fixative was removed from the dishes and the cells were air dried. The fixed cells were stained with propidium iodide (5 μg/ml) and a solution containing 38 mM Na citrate and 100 μg/ml RNase A (Boerhinger Mannheim) for 5 minutes. Stained cells were rinsed twice for 5 minutes with water and mounted on microscope slides in a solution containing 50 % glycerol and 50 % PBS. The mitotic index was determined by counting the number of cells in mitosis, with the aid of a fluorescence microscope (Leitz), for random batches of at least 300 cells.

Fluorescence Activated Cell Sorting

Triplicate sets of confluent BRK cells were infected two or three days after plating with Ad5 dl520 (mutant or

wild type) at a multiplicity of 5 or 10 pfu per cell. At 24, 48 and 72 hours after infection the cells were rinsed with PBS and 1 ml of a 0.05% solution of trypsin was added. After a 5 minute incubation the cells were agitated from the surface of the culture dishes, resuspended in 600 μl of PBS$^+$ and carefully passed through 25% gauge needles to break up cell clumps.

To fix the cells 1.8 ml of 95% ethanol was added dropwise with mixing and the cells were incubated overnight at 4° C (Morris and Mathews, 1987). Fixed cells were collected by centrifugation at 2000 rpm for 5 minutes in a Beckman TJ6 centrifuge and washed twice by resuspending the cells in 2 ml of PBS$^+$, recentrifugation as above and removal of the PBS$^+$ by aspiration. After the second wash the cells were resuspended in 0.9 ml of PBS$^+$, 0.1 ml of a solution of propidium iodide solution (50 μg/ml) in 38 mM sodium citrate and 10 μl of RNase A (10 mg/ml) was added. The cells were incubated for 30 minutes at 37° C and quantitative fluorescence analysis was carried out by B. Bagnard on batches of 20 000 cells using a Coulter Epics 5.

SOLUTIONS

The following is an alphabetically ordered list of the solutions used in these studies.

Annealing Buffer (10X)

20 mM Tris-HCl (pH 7.4)

2 mM $MgCl_2$

50 mM NaCl

Buffer A

100 mM Tris-HCl (pH 7.5)

100 mM $MgCl_2$

50 mM dithiothreitol (DTT)

Buffer X

50 mM Tris (pH 8.2)

250 mM NaCl

1 % NP40

2 mM EDTA

1 μg/ml Aprotinin

CAT Solution I

40 mM Tris-HCl (pH 7.8)

1.0 mM EDTA

150 mM NaCl

DNA Gel Loading Buffer

0.25% Bromophenol blue (BDH)

0.25% Xylene Cyanol (Kodak)

30% Glycerol (in H_2O)

Glucose Minimal Medium

10.5 g/L KH_2PO_4

4.5 g/L K_2HPO_4

1.0 g/L NH_4Cl

0.5 g/L NaCl

After sterilization by autoclaving, the following filter sterilized solutions were added to this solution:

1.0 ml/L 1 M $MgSO_4.H_2O$

0.5 ml/L 2% Thiamine HCl

10.0 ml/L 20% Glucose

Glucose Minimal Agar Plates

15 g/L Bacto-Agar in glucose minimal medium, Sterilized by autoclaving

Hepes buffer (2X)

170 mM NaCl

10 mM KCl

1.8 mM Na_2HPO_4

42 mM Hepes (Boehringer Mannheim)

11 mM Glucose

pH to 7.05 with NaOH

High Salt Buffer

300 mM NaCl

100 mM Tris-HCl (pH 8.0)

1.0 mM EDTA

Hirt Buffer

10 mM EDTA

10 mM Tris

0.6% SDS

50 μg/μl Pronase (Sigma)

Kinase Buffer (10X)

1.0 M Tris-HCl pH 8.0

100 mM $MgCl_2$

100 mM DTT

Luria Broth

10 g/L Tryptone

5 g/L Yeast Extract

5 g/L NaCl

1 g/L Glucose

Sterilized by autoclaving

Luria Agar

15 g/L Bacto-Agar (Difco) in Luria broth,

Sterilized by autoclaving

Luria Top Agar

7 g/L Bacto-Agar (Difco) in Luria Broth,

Sterilized by autoclaving

PAGE Run Buffer (5X)

15 g/L Tris Base

72 g/L Glycine

3 g/L SDS

PAGE Separating Gel Solution (8.5%)

H_2O	31.4 ml
1.5 M Tris-HCl (pH 8.8)	15.0 ml
10% SDS	0.6 ml
40% Acrylamide/Bis (37.5 : 1, BioRad)	12.6 ml
10% Ammonium persulfate(BRL)	300 µl
TEMED (BioRad)	30 µl

PAGE Stacking Gel Solution (4%)

ddH_2O	12.8 ml
0.5 M Tris-HCl (pH 6.8)	5.0 ml
10% SDS	200 µl
40% Acrylamide/Bis (37.5 : 1, BioRad)	2.0 ml
10% Ammonium persulfate	100 µl
TEMED	20 µl

Phosphate Buffered Saline (PBS)

140 mM NaCl

2.5 M KCl

8 mM Na_2HPO_4

1.5 mM KH_2PO_4

Sterilized by autoclaving

PBS^{++}

To sterile PBS were added:

10 ml 1% $CaCl^2$ (filter sterilized)

10 ml 1% $MgCl_2$ (filter sterilized)

Plaquing Medium

2 X Minimal Essential Medium (Gibco, cat. #410-1100EB), filter sterilized, supplemented with:

10% Horse Serum (Gibco)

200 units/ml Penicillin (Gibco)

200 μg/ml Streptomycin (Gibco)

0.2% sterilized Yeast Extract (Difco)

4 mM L-Glutamine (Gibco)

Potassium Salt Solution

0.17 M KH_2PO_4

0.72 M K_2HPO_4

Sterilized by autoclaving

Pronase Buffer

0.01 M Tris-HCl (pH 7.6)

0.01 M EDTA

0.5 % SDS

Protease Sample Buffer I

20% Glycerol

0.1% SDS

1 mm EDTA

2.5 mM DTT

0.125 mM Tris-HCl (pH 6.8)

Protease Sample Buffer II

10% Glycerol

0.1% SDS

1 mM EDTA

2.5 mM DTT

0.125 mM Tris-Cl (pH 6.8)

0.001 mg Phenol Red (Gibco)

SDS Gel Loading Buffer

63 mM Tris-HCl (pH 6.8)

50 mM 2-β mercaptoethanol

10% Glycerol

2% SDS

0.2% bromophenol blue

SDS Gel Run Buffer

0.025 M Tris Base

0.192 M Glycine

0.1 % SDS

Salt/Tris/EDTA (STE)

0.1 M NaCl

10 mM Tris-HCl (pH 8.0)

1.0 mM EDTA (pH 8.0)

Sequencing Gel Loading Buffer

99.4% deionized formamide

0.3% xylene cyanol

0.3% bromophenol blue

Solution I

50 mM glucose

25 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0)

Solution II

0.2 N NaOH

1.0% SDS

Solution III

600 ml/L 5 M potassium acetate

114 ml/L glacial acetic acid

Swell Buffer

0.1 % SDS

1 mM EDTA

2.5 mM dithiothreitol

1.25 M Tris-HCl (pH 6.8)

Synthesis Buffer (10X)

100 mM Tris-HCl pH 7.4

50 mM $MgCl_2$

20 mM DTT

10 mM ATP

5 mM each of: dATP, dCTP, dGTP, dTTP

TAE Gel Run Buffer (20 X)

96.8 g/L Tris Base

13.6 g/L NaAc

7.4 g/L EDTA

26.0 ml/L Glacial Acetic Acid

TBE Gel Run Buffer (20 X)

242.2 g/L Tris Base

102.7 g/L Boric Acid

7.4 g/L EDTA

TBST Western Blot Wash Buffer

20 mM Tris-HCl (pH 7.6)

137mM NaCl

0.1% Tween 20

TED Buffer

250 mM Tris-HCl (pH 7.8)

5 mM EDTA

Terrific Broth

24 g/L yeast extract

12 g/L tryptone

0.4% v/v glycerol

Sterilized by autoclaving

Tris/EDTA (TE)

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

Tris Saline

140 mM NaCl

25 mM Tris-HCl (pH 7.6)

5 mM KCl

5 mM Glucose

0.7 mM Na_2PO_4

YT (2X) Medium

16 g/L Tryptone

10 g/L Yeast extract

5 g/L NaCl

Sterilized by autoclaving

RESULTS

Most of the work described in this thesis has been published in five primary research articles. These publications are presented here, preceded in each case by a brief description of the experimental approach and followed by a list of my specific contributions and a synopsis of the main findings.

1) Use of Deletion and Point Mutants Spanning the Coding Region of the Adenovirus 5 E1A Gene to Define a Domain That Is Essential for Transcriptional Activation (Jelsma et al., 1988, Virology 163, 494-502)

This report describes the construction of a series of E1A mutants and their initial use to identify regions of the E1A proteins required for transactivation. The panel of mutants created included sixteen in-frame deletion and 4 mis-sense point mutations that together cover essentially the entire E1A coding region. Each of these mutants was used in plasmid form to test the ability of the mutated E1A genes to transactivate an Ad5 E3 promoted CAT gene.

VIROLOGY 163, 494–502 (1988)

Use of Deletion and Point Mutants Spanning the Coding Region of the Adenovirus 5 E1A Gene to Define a Domain That Is Essential for Transcriptional Activation

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To help in identifying functional domains within Ad5 E1A proteins, we have constructed a series of mutants that create deletions throughout these products. We have also produced several mis-sense point mutations in the unique 13 S mRNA region. These mutated E1A regions have been tested in plasmid form for their ability to activate transcription of an E3-promoted CAT gene. From the results, a major domain for transactivation has been identified. This begins between residues 138 and 147, ends between residues 188 and 204, and encompasses the unique 13 S region. This domain is sensitive to mis-sense mutations. Transactivation was unaffected by small deletions in the N-terminal half of E1A proteins between residues 4 and 138, but was destroyed when this whole region was deleted. The C-terminal 71 residues may affect transactivation, but the results with the mutant in which this region was deleted were unstable. The results obtained with these mutants are discussed in relation to the transactivation obtained by J. W. Lillie *et al.* [(1987). *Cell* 50, 1091–1100] with a synthetic peptide similar to the domain described here.

INTRODUCTION

The E1A region of adenovirus 5 (Ad5), like that of the closely related Ad2, is able to influence a variety of viral and cellular functions. It can activate in *trans* the transcription of other early viral genes (Berk *et al.*, 1979; Jones and Shenk, 1979), as well as cellular genes, such as those for heat shock protein (Kao and Nevins, 1983; Wu *et al.*, 1986) and β-tubulin (Stein and Ziff, 1984). It can repress the effect on transcription of viral and cellular enhancers (Borrelli *et al.*, 1984; Hen *et al.*, 1985; Stein and Ziff, 1987; Velcich and Ziff, 1985; Velcich *et al.*, 1986). E1A, together with region E1B, is necessary for the oncogenic transformation of rodent cells by adenovirus, but on its own, E1A can bring about partial transformation (Houweling *et al.*, 1980; Ruley, 1983). In primary baby rat kidney cells, E1A is able to stimulate DNA synthesis, to induce mitosis, and to activate the expression of cyclin, or proliferating cell nuclear antigen (PCNA) (Zerler *et al.*, 1987). Recently E1A has been shown to induce differentiation of F9 teratocarcinoma cells (Montano and Lane, 1987), possibly because an E1A protein product resembles a factor in these cells controlling gene expression (Reichel *et al.*, 1987; La Thangue and Rigby, 1987).

These effects of E1A result from the action of polypeptide products generated from two mRNAs, 13 and 12 S, made early in infection. These mRNAs are read in the same reading frame and differ only in the size of intron excised from the original transcript. As a result, their predicted polypeptide products, of 289 and 243 residues (289R, 243R), respectively, differ only by an extra internal sequence of 46 amino acids present in the 13 S mRNA product.

As a beginning to understanding the molecular mechanisms by which E1A products exert their effects, attempts have been made to define domains in the proteins that are essential for particular functions. Results with E1A mutants (Krippl *et al.*, 1985; Lillie *et al.*, 1986, 1987; Moran *et al.*, 1986b; Schneider *et al.*, 1987; Zerler *et al.*, 1987), coupled with the identification of regions of the products that are highly conserved between adenovirus serotypes (Kimelman *et al.*, 1985), have led to the tentative identification of three domains. Two of these, conserved regions 1 and 2 (CR1, CR2), which are essential for transcriptional repression, for transformation and for the induction of DNA synthesis, are in the first exon common to 12 and 13 S mRNA products, while the third, CR3, for transactivation, encompasses the region unique to the 13 S mRNA product.

To provide a means of systematically surveying E1A proteins for domains, we have made a series of in-frame deletion and mis-sense point mutations, which together cover the whole of the coding region of the E1A gene. Here we describe the construction of these mutants and their use in a transient expression assay

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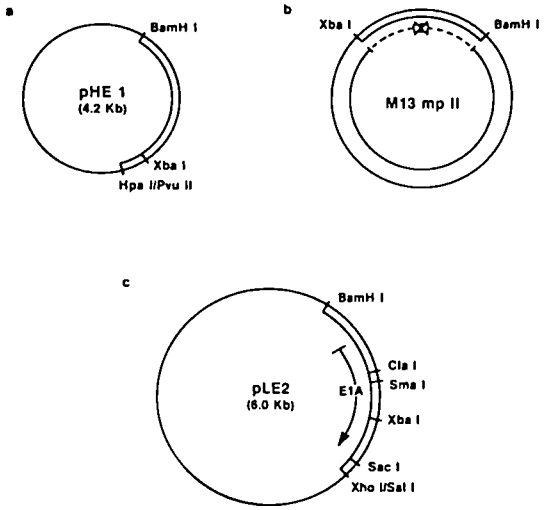

FIG. (a) Pl[illegible]id pHE1, a recombinant of pBR322 in which the *Hpa*I E[illegible]agme[illegible] Ad5, with a *Bam*HI linker at nucleotide 22, has been i[illegible]erted [illegible]een the *Bam*HI and *Pvu*II sites in pBR322. (b) Oligon[illegible]leotic[illegible] tagenesis of the Ad5 *Bam*HI–*Xba*I fragment from pHE1 i[illegible]erted i[illegible]13 mp11, using as primers a synthetic oligonucleotid[illegible] bearin[illegible] mutation and a complementary strand from *wt* Rf M13 mp1[illegible] with *Bam*HI and *Xba*I and denatured. (c) Plasmid pLE2 a recombinant of pBR322 containing the whole of E1A in the form of a modified *Xho*I C fragment of Ad5 inserted between the *Bam*HI and *Sal*I sites. In this plasmid, the *Xho*I C fragment lacks the sequence between the *Sac*I sites at nucleotides 1770 and 5644; in addition, the *Cla*I site at nucleotide 24 in the pBR322 sequence has been removed. In these diagrams, double lines represent Ad5 sequences, single lines represent pBR322 or M13 mp11 sequences.

to investigate in detail the contribution of different regions of the E1A proteins to transactivation.

MATERIALS AND METHODS

Enzymes were obtained from Bethesda Research Laboratories, Boehringer-Mannheim, and Pharmacia, and were used as recommended by the suppliers. Oligonucleotides were synthesized in the Central Facility of the McMaster Institute for Molecular Biology and Biotechnology.

Plasmids were cloned and grown in *Escherichia coli* strain LE392. Strain GM 119 (dam$^-$) was obtained from A. E. Smith. *E. coli* strain JM103 and phage M13 mp10 and mp11 were obtained from Pharmacia. Plasmids pHE1 and pCD2 (gifts from F. L. Graham) were both derived from plasmid pXC1, a derivative of pBR322 with the *Xho*I C fragment of Ad5 (0–16%) inserted between the *Bam*HI and *Sal*I sites (McKinnon *et al.*, 1982). pHE1 was produced by cutting pXC1 in the pBR322 sequence with *Pvu*II and in the Ad5 sequence with *Hpa*I and ligating the blunt ends (Fig. 1a). pCD2 was constructed from pXC1 by removing the Ad5 sequence between the *Sac*I sites at positions 1770 and 5644. Plasmid pLE2 (Fig. 1c) is the same as pCD2 except that the *Cla*I site at position 23 in the pBR322 sequence has been removed by cleaving with *Cla*I, treating with the Klenow fragment of DNA polymerase, and ligating the blunt ends. (The *Cla*I site at position 920 in the Ad5 sequence is cleaved by *Cla*I only when the plasmid is grown in dam$^-$ bacteria.) Plasmid pKCAT23 (Weeks and Jones, 1983) was a gift from M. B. Mathews.

Oligonucleotide-directed mutagenesis

Mutants *dl* 1101–1107, 1109, 1112–1116, 1119, *pm* 1131, and *sub* 1117 were constructed by oligonucleotide mutagenesis using the two-primer method of Zoller and Smith (1984). The template for mutagenesis was M13 mp11, into which had been inserted the Ad5 sequence from plasmid pHE1 between the *Bam*HI and *Xba*I sites (Figs. 1a, 1b). The two primers were the synthetic mutagenic oligonucleotide phosphorylated at the 5′ end, and a complementary strand of RF DNA from *wt* M13 mp11, digested with *Bam*HI and *Xba*I and denatured (Fig. 1b).

To create the deletion mutants, oligonucleotides 23 nucleotides long, consisting of sequences of 11 or 12 nucleotides of the sense strand of Ad5 DNA on either side of the deletion, were synthesized. For the point mutant *pm* 1131, an oligonucleotide 20 nucleotides long was made spanning C1331, but altering it to G to create the termination codon TGA. Construction of *sub* 1117 (see below) began as an attempt to remove the *Cla*I site by making a silent point mutation of T922 to C, using a procedure similar to that for *pm* 1131.

Plaques were screened by filter hybridization using the end-labeled mutagenic oligonucleotide as a probe. With deletion mutants, positive plaques were further checked for the reduction in size of an appropriate restriction fragment. Finally, phage DNA prepared from selected plaques was sequenced by the dideoxynucleotide method, using suitable oligonucleotides as primers, ^{35}S-ATP from NEN, and other materials and methods supplied by Pharmacia and NEN. For every mutant, the whole portion of the mutagenized Ad5 sequence destined to be rescued into plasmid pLE2 was sequenced. With *pm* 1131, the point mutation was confirmed by sequencing the complementary strand in the vicinity of the mutation after rescue into M13 mp10.

With *sub* 1117, sequencing revealed that in addition to changing T922 to C, mutagenesis had radically altered the sequence between nucleotides 1141 and 1289. As a result of homologies between the synthetic oligonucleotide and nucleotides in the vicinities of

1141 and 1289, nucleotides 1142–1288 had been deleted to be replaced by 11 nucleotides from the middle of the oligonucleotide. A consequence of this was to remove the 3′ splice site at nucleotide 1228.

Deletion loop mutagenesis and construction of *dl* 1110 and *pm* 1120–1122

Point mutations were made in the unique 13 S region around the *Sma*I site at 1007–1012 (Fig. 2) by deletion loop mutagenesis (Kalderon *et al.*, 1982). Deletions were created at the *Sma*I site of the Ad5 sequence in pHE1 (Fig. 1a) by digesting pHE1 with *Sma*I, Exo III, and SI, and religating. The sizes of the deletions were determined by sequencing in M13. One of the deletions, *dl* 1110, was an in-frame deletion of 63 bp, and was used as part of the survey of the coding region. This deletion removed the 5′ splice site for the 12 S mRNA, so that *dl* 1110 should produce no 12 S mRNA or its product. Another plasmid that lacked 43 nucleotides from 996 to 1038 (*dl* 1111) was used for mutagenesis. It was cut with *Xba*I and used to form heteroduplexes with *wt* pHE1 linearized with *Eco*RI. These were then mutagenized with sodium bisulfite. The mutations produced were identified by sequencing both DNA strands throughout the region between the *Cla*I and *Xba*I sites (Fig. 2) that was later rescued into plasmid pLE2. Sequencing was carried out in M13 by the dideoxynucleotide method.

Rescue of mutations into plasmid pLE2

To enable assays to be carried out on the E1A mutants in plasmid form, restriction fragments of the Ad5 sequence containing the mutations were transferred from the vector in which they were created, M13 or pHE1, into plasmid pLE2, which contains the whole of region E1A (Fig. 1c). For all the mutations except *pm* 1131, the recipient was *wt* pLE2; after the transfer, the presence of a deletion in pLE2 was confirmed by the decrease in size of an appropriate restriction fragment. For *dl* 1107 and 1110, additional confirmation came from the absence of a *Cla*I or a *Sma*I site, respectively. The presence of *pm* 1120–1122 in pLE2 was confirmed by the absence of a *Sma*I site. *pm* 1131 was rescued into pLE2 *dl* 1115, the transfer being monitored by the increase in the *Sma*I–*Xba*I fragment to *wt* size with removal of the deletion.

DNA transfection and CAT assay in HeLa cells

Monolayer cultures of HeLa cells were grown in 100-mm dishes to 75–90% confluency, and were then

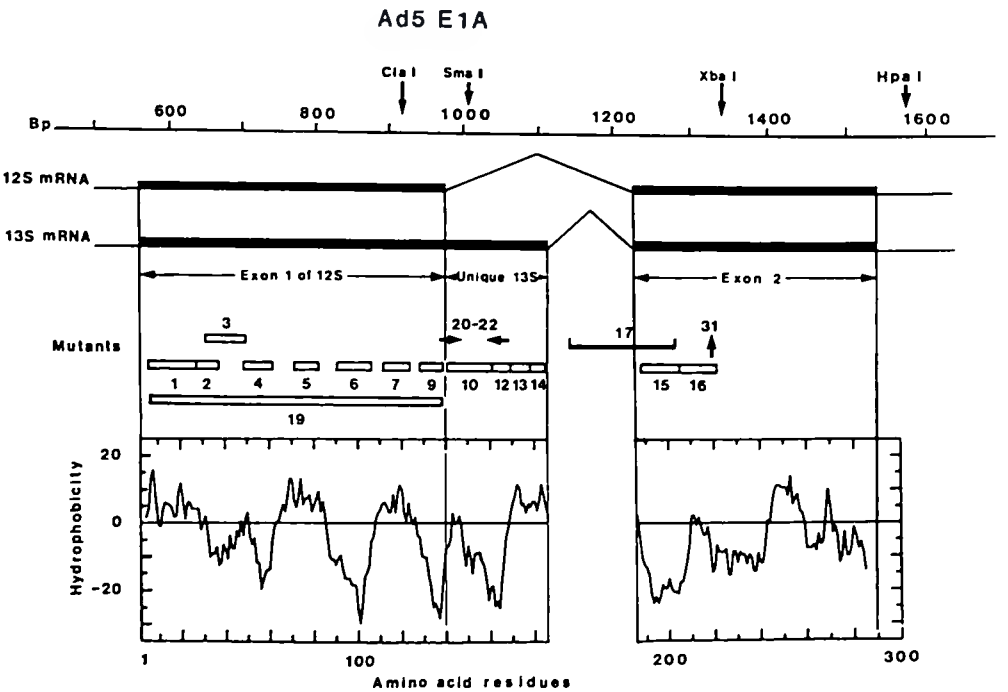

FIG. 2. Map of region E1A of Ad5 showing the locations of the deletion mutants (boxes), point mutants (arrows), and the substitution mutant (bar) listed in Table 1 and Fig. 3, and discussed in the text. For clarity, only the last one or two digits of the mutant numbers have been given. The lower part of the diagram is a hydropathy plot of the E1A 13 S mRNA (289 residue) product, constructed after the method of Kyte and Doolittle (1982) using a span of nine amino acid residues. Hydrophobic regions are positive, hydrophilic regions negative.

transfected by a modified calcium phosphate–DNA coprecipitation technique (Graham and van der Eb, 1973; Weeks and Jones, 1983). To 500 μl/dish of buffered saline (NaCl 16 g/liter; KCl 0.74 g/liter; Na_2HPO_4 0.2 g/liter; glucose 2 g/liter; HEPES 10 g/liter, pH 7.1) were added 500 μl water, 5 μg pKCAT23, 10 μg pLE2 (*wt* or mutant), and 20 μg sonicated salmon sperm DNA, followed by 50 μl 2.5 *M* $CaCl_2$. A precipitate was allowed to form for 15–30 min, and the mixture was then added dropwise to the medium in each dish. After 6 hr, the medium was removed from each dish, and 2 ml 20% glycerol was added for 1–2 min and then was replaced by fresh medium. Forty to forty-eight hours later, the cells were harvested and sonicated.

CAT assays were performed as outlined by Weeks and Jones (1983) with the following modifications. The assay mixture contained the entire cell extract from one dish, together with 0.1 μCi [^{14}C]chloramphenicol (50 mCi/mmol; NEN) and 1 m*M* acetyl coenzyme A. After autoradiography of chromatograms on thin-layer plates (Analtech), the spots were scraped and the radioactivity was quantified by scintillation counting. Results were not corrected for the protein contents of different cell extracts within an experiment, as measurements showed that they did not differ significantly.

Between separate assays, we found significant variation in the CAT activity given by the controls, pKCAT23 alone and with *wt* pLE2, probably due to differences in the state of the cells. Because of this, it was impossible to control properly the kinetics of the acetylation reaction. In the following section, results are quoted only from assays in which the stimulation by *wt* pLE2 was at least eight times that of pKCAT23 alone, and in which not more than 50–70% of the [^{14}C]chloramphenicol was acetylated.

RESULTS

The E1A mutants to be described are listed in Table 1 and their map positions are shown in Fig. 1.

Using mainly oligonucleotide mutagenesis (Zoller and Smith, 1984), we have constructed a series of in-frame deletion mutants, *dl* 1101–1107, 1109, 1110, 1112–1116, that span the E1A coding region up to codon 221 of the 13 S, 289R product. Each of these mutants removes between 7 and 22 amino acid residues. So far as possible, and particularly in 12 S exon 1, codons 1–139, the regions deleted were chosen to remove either a hydrophobic amino acid sequence, representing possibly an interior part of the protein, or a hydrophilic sequence, representing a more exposed section of chain (see Table 1 and the hydropathy plot, Fig. 2). To delete the C-terminal region, residues 219–289 of the 13 S product, a point mutant, *pm* 1131, was produced to alter codon 219 from serine to termination. Mutant *sub* 1117 lacks the normal 3′ splice site, so that it should produce a read-through product that terminates at an in-frame TGA at nucleotides 1118–1120, and that consists of the first 185 residues of the 13 S product, ending with a mis-sense lysine.

In addition to the above, a series of mis-sense point mutants affecting individual residues around the *Sma*I site in the unique 13 S region were created by deletion loop mutagenesis (Kalderon *et al.*, 1982). Three of these are described here: *pm* 1120 with a single conservative amino acid change of Val_{147} to Ile, *pm* 1121 with six altered residues, and *pm* 1122 with Pro_{150} changed to Phe (Fig. 3).

All the mutations were rescued into pLE2, a recombinant pBR322 plasmid containing the whole of the Ad5 region E1A (Fig. 1c).

CAT assay with pLE2 and pKCAT23

The ability of mutated E1A genes to cause the efficient transcription of genes under the control of early Ad5 gene promoters was tested using pKCAT23, a pBR322 derivative containing the structural gene for chloramphenicol acetyltransferase (CAT), linked to the E3 promoter of Ad5 (Weeks and Jones, 1983), a promoter that responds well to E1A transactivation. For the assay, HeLa cells were transfected with pKCAT23

TABLE 1

MUTANTS PRODUCING DELETIONS IN THE E1A 13 S mRNA PROTEIN PRODUCT

Mutant	bp deleted	aa deleted	Type of sequence removed
dl 1101	569–634	4–25 (22)	Hydrophobic
dl 1102	635–664	26–35 (10)	—
dl 1103	647–706	30–49 (20)	Hydrophilic
dl 1104	701–739	48–60 (13)	Hydrophilic
dl 1105	767–802	70–81 (12)	Hydrophobic
dl 1106	827–874	90–105 (16)	Hydrophilic
dl 1107	890–928	111–123 (13)	Hydrophobic
dl 1109	941–973	128–138 (11)	Hydrophilic
dl 1119	569–973	4–138 (135)	—
dl 1110	976–1038	140–160 (21)	—
dl 1112	1040–1063	161–168 (8)	Hydrophilic
dl 1113	1064–1090	169–177 (9)	Hydrophobic
dl 1114	1091–1111	178–184 (7)	Hydrophobic
dl 1115	1229–1287	188–204 (17)	Hydrophilic
dl 1116	1288–1338	205–221 (17)	Hydrophobic
pm 1131	C → G at 1331	219–289 (71)	—
sub 1117	1142–1288	185–289 (107)	—

Note. Mutants are listed in order across the 289 residue protein from the N- to the C-terminal.

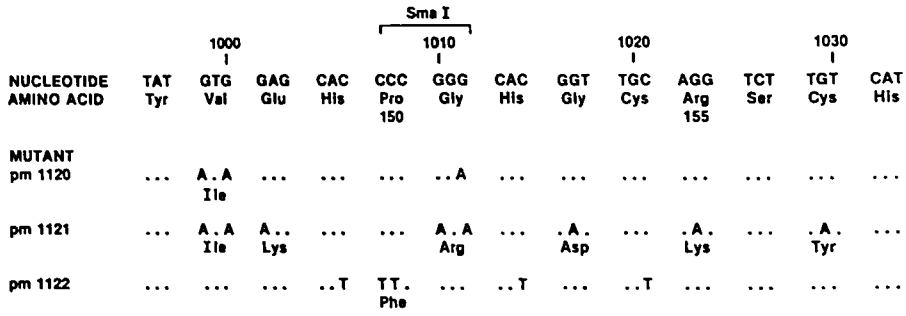

FIG. 3. Map of the mutated nucleotides and amino acid residues in *pm* 1120, 1121, and 1122, obtained by deletion-loop mutagenesis. In each case, the mutations resulted in the loss of the *Sma*I site.

together with a mutant or *wt* pLE2, and extracts were tested for CAT activity using [^{14}C]chloramphenicol, as described under Materials and Methods. Each mutant was tested at least three times with different plasmid preparations. Results are given in Table 2, and representative results are shown in Fig. 4.

Except for *pm* 1120 and *pm* 1131, the mutants gave clear-cut results. All the mutants in 12 S exon 1 as well as *dl* 1116 in exon 2 induced levels of CAT activity similar to those obtained with *wt* pLE2. On the other hand, all the mutants in the unique 13 S region except *pm* 1120, together with *dl* 1115 and *sub* 1117 in exon 2 gave CAT activities similar to those with pKCAT23 alone, and usually less than 5% of the level with *wt* pLE2. These low levels of activity were not due to a failure of these mutant E1A regions to produce proteins, as infections of cells with mutants *dl* 1110 and *dl* 1112–1114 rescued into virus gave similar amounts of E1A proteins to those with *wt* virus (Egan *et al.*, in preparation). These results suggest that the 13 S unique region of the 289R protein plus some or all of the region deleted in *dl* 1115 define a functional domain for transcriptional activation.

TABLE 2

RESULTS WITH MUTANTS IN THE CHLORAMPHENICOL ACETYLTRANSFERASE ASSAY

Mutant	Activity with mutant / Activity with *wt* pLE2	Mean
dl 1101	1.3; 1.3; 0.7; 1.0	1.1
dl 1102	0.6; 0.6; 0.8; 1.2	0.8
dl 1103	0.9; 1.2; 1.2	1.1
dl 1104	1.2; 1.0; 1.7	1.3
dl 1105	0.9; 0.9; 1.3	1.0
dl 1106	0.7; 1.1; 2.2; 0.9	1.2
dl 1107	1.9; 1.1; 1.1	1.3
dl 1109	0.8; 1.4; 4.7; 0.7	1.9
dl 1110	0.02; 0.01; 0.01	0.01
pm 1120	0.05; 0.08; 0.2; 0.06; 0.4; 0.6; 0.4	0.24
pm 1121	0.02; 0.03; 0.14; 0.03	0.06
pm 1122	0.01; 0.01; 0.02; 0.06	0.03
dl 1112	0.01; 0.01; 0.03; 0.01	0.02
dl 1113	0.01; 0.03; 0.01; 0.05; 0.03	0.03
dl 1114	0.02; 0.02; 0.03; 0.02	0.02
sub 1117	0.06; 0.04; 0.01; 0.04	0.04
dl 1115	0.05; 0.02; 0.04; 0.03	0.04
dl 1116	1.0; 0.8; 0.9; 0.9; 1.0	0.9
pm 1131	1.1; 0.2; 0.2; 0.2	—
dl 1119	0.08; 0.01; 0.06; 0.1	0.06

Note. Mutants are listed in the same order as in Table 1 except for *dl* 1119. The values shown were calculated from the results of different experiments, quantified by scintillation counting as described under Material and Methods.

Of the exceptions, *pm* 1120, with a single conservative Val_{147} to Ile change, gave about 25% of the activity obtained with *wt* E1A. (This estimate of relative activity is very inaccurate because, as discussed under Materials and Methods, the kinetics of the reaction could not be controlled.) This partial reduction may mean that, although Val_{147} is important to the functioning of the domain, a conservative change prevents total loss of activity. Alternatively, the relative importance of particular residues may gradually increase from nonessential for Glu_{138}, whose removal in *dl* 1109 causes no loss of activity, to essential for Pro_{150}, which when changed to Phe in *pm* 1122 causes total loss of activity.

Together, these results define the limits of a functional transactivation domain. Its N-terminal must lie between the end of the deletion in *dl* 1109 at residue 138 and the mutation at residue 147 in *pm* 1120. The C-terminal lies within the deletion in *dl* 1115, i.e., between residues 188 and 204.

The point mutant, *pm* 1131, which deletes the 70 C-terminal residues of the protein, gave results that varied in different experiments from *wt* levels to about 20% of *wt*. The cause of this variation is not clear, but it suggests that the C-terminal end of exon 2 can affect transactivation. This point is discussed more fully below.

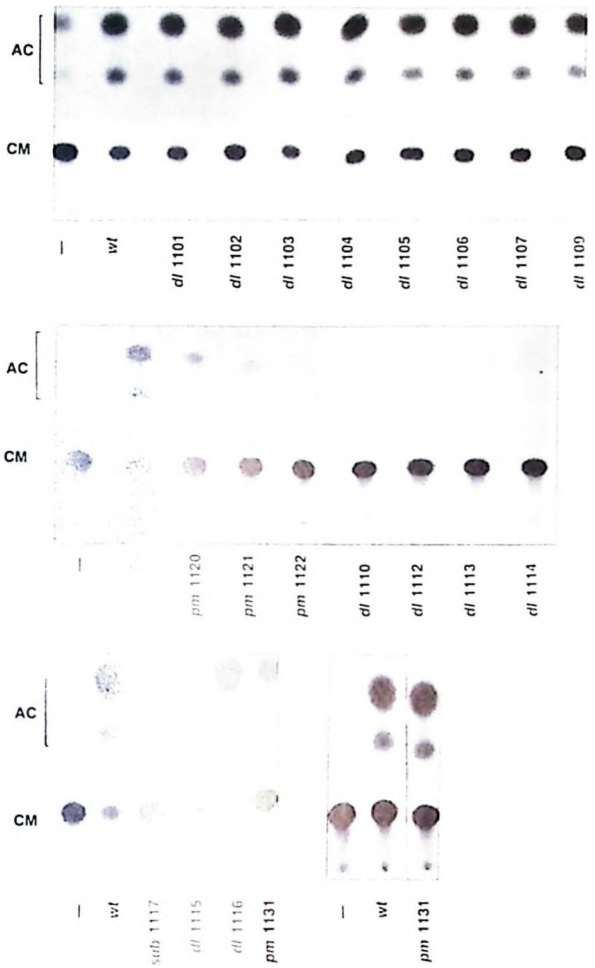

FIG. 4. Transactivation of the E3-promoted chloramphenicol acetyltransferase gene by E1A mutants. The assays contained pKCAT23 with the addition of pLE2, *wt* or mutant, as indicated. Autoradiographs from one set of experiments on the mutants in sequence across the 289R protein from the N- to the C-terminal are shown. A duplicate experiment on *pm* 1131 is also shown to illustrate the variability with this mutant, as described in the text. CM, chloramphenicol; AC, acetylated chloramphenicol.

The results with deletions in the 12 S exon 1 suggested that the region up to residue 138 was not involved in transactivation by the 289R protein. To test this further, we constructed *dl* 1119 to delete residues 4 to 138, and tested this in plasmid pLE2 for activation of CAT. The results, given in Table 2, show that it had essentially no ability to transactivate.

DISCUSSION

Several studies with mutated E1A genes (Glenn and Ricciardi, 1985; Lillie *et al.*, 1986; Moran *et al.*, 1986b; Schneider *et al.*, 1987) have shown that the domain for transactivation of early viral genes encompasses the unique 13 S region and continues into exon 2. The most recent results of Lillie *et al.* (1987), published while the present paper was in preparation, show that a synthetic peptide of 49 amino acids, corresponding to residues 140–188 in the unique region of the 289 residue protein, has transactivation activity when injected into cells.

The results presented here further confirm that part of the 289R protein encompassing the unique 13 S region constitutes the principal domain for transactivation. This domain extends from between residues 138 and 147 to somewhere between residues 188 and 204. In our work, the functioning of this domain was not affected by limited deletions in the part of the protein N-terminal to this domain or in the part immediately to the C-terminal side that is deleted in *dl* 1116. However, when essentially the whole of the 12 S exon 1 was removed in *dl* 1119, transactivation was lost. The reason for the loss of activity may be that the radically altered protein product of *dl* 1119 was rapidly degraded by the cell: we are rescuing this mutant into virus to investigate this possibility. An alternative explanation may be that 12 S exon 1 provides a scaffolding for holding the domain in its correct form, and this scaffolding cannot be removed entirely without loss of activity.

Our results with *dl* 1119 differ from those of Lillie *et al.* (1987) who obtained transactivation with a 49 amino acid peptide. To produce a product more closely resembling their peptide, we combined *dl* 1119 with *pm* 1131 so as to remove much of exon 2; the product of this double mutant should contain only residues 1–3 and 139–218 of the 289R protein. It also failed to transactivate (not shown). The explanation for the transactivation observed with the 49 amino acid peptide may be that the domain on its own has very low activity that becomes detectable only when comparatively large amounts of it are injected into a cell. Alternatively, if truncated proteins are degraded, perhaps enough of the injected peptide escapes degradation to produce a detectable effect.

The role of the C-terminal end of exon 2 in transactivation remains uncertain. As Table 2 shows, *pm* 1131, which deletes the 71 C-terminal residues, gave variable results in the transactivation reaction, whereas other authors have reported essentially *wt* levels with similar deletion mutants (e.g., Schneider *et al.*, 1987). The signal for rapid nuclear localization appears to be located in the five C-terminal residues of E1A proteins (Lyons *et al.*, 1987), and it is possible that in some of our experiments, the truncated protein was transported poorly into the nucleus to activate the CAT gene, or was degraded before it entered the nucleus. The possibility must also be considered that the *wt*

levels we observed were fortuitous and that the C-terminal end of exon 2 affects transactivation directly. We are examing this point further.

The present results provide additional evidence that the integrity of the principal transactivation domain is essential for activity. This requirement was first demonstrated with the mis-sense mutants *hr* 3, 4, and 5 (Glenn and Ricciardi, 1985), which do not transactivate and which map in the C-terminal half of the domain at residues 173, 176, and 185, respectively. Here we find that activity is lost in the mis-sense mutant *pm* 1122 when Phe replaces Pro_{150}, a conserved residue at the N-terminal end of the domain. Even a single conservative change of Val_{147} to Ile in *pm* 1120 resulted in loss of activity, even though Val_{147} is not one of the residues conserved between adenovirus serotypes, being replaced by Pro in human Ad7 and Ad12, and simian adenovirus 7 (SA7) (Kimelman *et al.*, 1985). However, despite the sensitivity of the domain to amino acid changes and to deletions, it is interesting that Kappuswamy and Chinnadurai (1987) found that the insertion of four amino acids between residues 150 and 151 had no effect on transactivation.

The domain defined here corresponds closely to conserved region 3, a sequence of residues that is highly conserved between Ad5, Ad7, Ad12, and SA7 (Kimelan *et al.*, 1985; Moran and Mathews, 1987; Schneider *et al.*, 1987). However, there are some interesting differences between the predicted E1A proteins of these viruses. It is only in Ad5 that the N-terminal end of the conserved sequence corresponds to the beginning of the unique 13 S region. In Ad7 and Ad12, some 13 residues of the conserved region lie before the 12 S 5′ splice site and so are common to both 12 and 13 S products. In SA7, on the other hand, the unique 13 S region is enlarged by about 29 nonconserved residues, which precede the conserved region. From the present results on Ad5, it seems likely that in these other viruses, the domain for transactivation corresponds to the conserved region alone, but it would be interesting to know how close this correspondence is.

Although its significance is not apparent, one noticeable feature of the transactivation domain in Ad5 is that it is flanked by highly acidic sequences. At the N-terminal end, all but one residue from 132 to 140 is acidic, while at the C-terminal end, the sequence Glu–Pro repeats six times from residues 189 to 200.

The region discussed here must constitute a dominant domain for the transactivation of early viral genes, and would clearly account for the considerable activity of the 13 S product compared to the very low activity of the 12 S product reported by many groups (Haley *et al.*, 1984; Montell *et al.*, 1984; Moran *et al.*, 1986a; Svensson and Akusjarvi, 1984; Zerler *et al.*, 1986). This domain also would account for the results obtained with the products of two other E1A mRNAs of 11 and 10 S, that have recently been reported (Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987). These 11 and 10 S mRNAs resemble the 13 and 12 S mRNAs, respectively, except for the removal of a second intron during their maturation. As a result, the products of these mRNAs are effectively deletion mutants of those from 12 and 13 S mRNAs, lacking residues 27 to 98. The 11 S mRNA product, like that of the 13 S mRNA, contains the transactivation domain described here and shows activity, whereas the 10 S product lacks the domain and shows no activity (Ulfendahl *et al.*, 1987). However, it is interesting that the activity of the 11 S mRNA product is reported to be lower than that of the 289R product. This reduction is not due to a failure of the 11 S protein product to accumulate (Ulfendahl *et al.*, 1987). A possible explanation for the reduction is that if, as suggested above, the N-terminal 138 residues of the 289R protein act as a scaffold holding the transactivation domain in its functional form, then in the 11 S product the loss of 72 residues from this portion of the protein has reduced its effectiveness in this respect.

Despite the evident importance of the domain encompassing the unique region of the 289R protein, other studies have shown that the 12 S, 243R product transactivates early adenoviral genes as efficiently as the 13 S product (Leff *et al.*, 1984; Richter *et al.*, 1985; Winberg and Shenk, 1984). In the present work, no evidence was obtained that any sequences present in the 12 S product, except for the C-terminal end of the domain, were at all necessary for transactivation of the Ad5 E3 promoter. This would suggest that if there is a 12 S domain, its activity is less than about 5% that of the 13 S domain. However, there is another possible explanation for our failure to detect another domain in the 12 S sequence. Glenn and Ricciardi (1987) have shown that the Ad5 EIA mutant *hr* 5, in which Ser_{185} of the 13 S product is changed to AsN, blocks transactivation by *wt* E1A, possibly by the mutant protein competing with the *wt* protein for a cellular transcription factor. If the 12 S product interacts with the same factor, our nonfunctional mutants may block this interaction, preventing us from detecting 12 S activity. We have looked for interference from *dl* 1112–1115 in transactivation by *wt* pLE2, but we have failed to detect the clearly defined effect reported for *hr* 5 (not shown).

Recently, Zerler *et al.* (1987) have shown that expression of PCNA is activated by the 12 S product and independently of the 13 S unique region. It seems likely, therefore, that the 12 and 13 S products contain

different transactivation domains for different sets of targets genes. The array of mutants described here is being used to study this possibility, as well as to investigate domains for other E1A functions.

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My Contributions to Jelsma et al. (1988)

1) Construction of the deletion mutations dl 1101 and dl 1119 using oligonucleotide mutagenesis and transfer of the mutated sequences from the M13mp19E1A vector in which they were constructed to the wild type E1A plasmid pLE2. Construction of the double mutant dl 1119/pm 1131 in plasmid pLE2 by combining the single mutants dl 1119 and pm 1131 using restriction enzyme digestion and ligation.

2) Dideoxynucleotide DNA sequencing to check for extraneous mutations in the E1A regions transferred from M13mp19E1A to pLE2 for almost all of the mutants used in this study. In two cases, irrelevant mutations were found. These probably resulted from non-specific hybridization of the mutagenic oligonucleotide. The erroneously mutated regions were removed and replaced with wild type sequences.

3) Assayed mutants dl 1101, dl 1102, dl 1103, dl 1119 and dl 1119/pm 1131 for their ability to stimulate transcription of the Ad5 E3 promoter using transient expression CAT assays.

In Jelsma et al. (1988) the major domain of the 289R protein required for transactivation was found to be contained between residues 139 - 204 (see Figure 5, pg. 130). This region contains all of the residues unique to the 289R protein (CR3), and the first 10 residues immediately following CR3 which are common to both 234R and 289R. Small deletion mutations in this region (residues 139 - 204) rendered the E1A proteins defective for transactivation, while small deletions in the N-terminal and C-terminal regions of the E1A proteins were found to have no effect. However, mutants constructed to encode for E1A proteins lacking either all of the N-terminal region or most of the C-terminal of the E1A protein were defective for transactivation.

This study demonstrated that our series of E1A deletion mutants could be used to systematically survey domains in the E1A proteins. Having mapped the E1A region required for transactivation we next used the mutants to study a number of other E1A functions.

2) **Sequences in E1A Proteins of Human Adenovirus 5 Required for Cell Transformation, Repression of a Transcriptional Enhancer and Induction of Proliferation Cell Nuclear Antigen (Jelsma et al., 1989, Virology 171, 120-130)**

This paper describes a series of mapping studies in which our panel of E1A mutants was used to define regions of the E1A proteins required for: 1) transformation of primary BRK cells in cooperation with activated ras; 2) trans-repression of the SV40 early promoter/enhancer in Hela cells; and 3) induction of proliferating cell nuclear antigen (PCNA) in BRK cells.

VIROLOGY 171, 120–130 (1989)

Sequences in E1A Proteins of Human Adenovirus 5 Required for Cell Transformation, Repression of a Transcriptional Enhancer, and Induction of Proliferating Cell Nuclear Antigen

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A range of deletion and other mutants in the coding region of the E1A gene of Ad5 has been assayed for transformation of baby rat kidney (BRK) cells in cooperation with *ras*, repression of the SV40 enhancer, and induction of proliferating cell nuclear antigen (PCNA). Transformation efficiency was drastically reduced by deletion of residues 4–25, 36–60, or 111–138 in exon 1 of the 289 residue (289R) and 243R E1A proteins. Deletion of other residues in exon 1 had little effect. With mutants in the region unique to the 289R protein, and in exon 2, the only effect on transformation seemed to be an increased tendency of mutant transformants, compared to *wt*, to migrate to form secondary foci. Repression assays, performed with E1A plasmids producing only the 243R protein, showed that deletion of residues 4–25 or 36–60 inhibited repression completely. Deletion of residues 128–138 reduced repression, but deletions elsewhere in exon 1 had little effect. Deletion of residues 188–204 in exon 2 reduced repression slightly, and deletion of all of exon 2 reduced it to about one-half. It is concluded that for transformation, there are two functional domains in E1A proteins, both in exon 1, both involved in binding different cellular proteins, and both probably concerned with different transforming functions. One of these domains, involving residues 4–25 and 36–60, also functions in repression, but the role of the second in repression is much less critical. All of the deletion mutants in exon 1 induced PCNA synthesis in BRK cells. This result, together with previously published work, suggests that the active site for PCNA induction either involves residues 61–69 or 82–85 in exon 1, which have not been deleted, or it does not depend on any single limited region of the E1A proteins.

INTRODUCTION

Among the transforming proteins of mammalian DNA tumor viruses, the 289 residue (289R) and 243R proteins from the E1A region of human adenovirus 5 (Ad5), and of the closely related Ad2, are of particular interest for several reasons: together or individually, they are almost certainly responsible for most of the range of effects that the E1A region can produce in infected cells; they are relatively small; and they appear to consist structurally of a series of relatively independent functional domains. This paper describes experiments designed to provide more information on the relationship between structure and function in these proteins.

The 289R and 243R E1A products are acidic, nuclear phosphoproteins encoded by mRNAs of 13 and 12 S, respectively, and are identical to one another except for an internal sequence of 46 amino acids unique to the 289R. They are the major products of the E1A region and are able to bring about the immortalization or establishment of cells, the transformation of cells in cooperation with the E1B region of adenovirus or with an activated oncogene such as *ras*, the activation of transcription in *trans* of viral and cellular genes, and the repression of viral and cellular transcriptional enhancers. In quiescent cells, they induce mitosis, and the synthesis of DNA, proliferating cell nuclear antigen [PCNA or cyclin, the auxiliary protein of DNA polymerase δ (Bravo *et al.*, 1987; Prelich *et al.*, 1987)] (Zerler *et al.*, 1987), and an epithelial cell growth factor (Quinlan *et al.*, 1987). (For reviews and earlier references, see Branton *et al.*, 1985; Berk, 1986.)

The molecular mechanisms underlying the activities of these proteins are largely unknown, although some clues have been obtained. One example is in the stimulation of gene transcription, where the effect of E1A is apparently not to increase synthesis of transcription factors (Green *et al.*, 1988), but to activate preexisting factors. Thus in transcription by RNA polymerase III, transcription factor IIIC appears to be activated by phosphorylation (Hoeffler *et al.*, 1988). Another example relates to the ability of E1A proteins to bind to a number of cellular proteins (Yee and Branton, 1985;

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Harlow *et al.*, 1986; Egan *et al.*, 1988), one of which, with molecular mass 105kDa, has now been identified as the retinoblastoma protein p105-RB (Whyte *et al.*, 1988a; Egan *et al.*, 1989). The binding to this protein, which we have shown to occur between residues 111 and 127 of E1A (Egan *et al.*, 1988), may be directly involved in the oncogenic transformation of cells (Whyte *et al.*, 1988a; Egan *et al.*, 1988, 1989).

An approach used by a number of different groups to understand more about the structure and function of E1A proteins has been to examine E1A mutants created by site-directed mutagenesis. These studies have made it possible to localize segments of the proteins that are essential for different functions (Moran and Mathews 1987). In general, these segments coincide with three protein regions that are conserved between several human and simian serotypes (Kimelman *et al.*, 1985), namely conserved regions 1, 2, and 3. Thus conserved region 2 (CR2) and particularly CR1, both in exon 1 and common to both 289R and 243R proteins, are required for cell transformation (Zerler *et al.*, 1986; Moran *et al.*, 1986; Kuppuswamy and Chinnadurai, 1987; Whyte *et al.*, 1988b) and for enhancer repression (Schneider *et al.*, 1987; Lillie *et al.*, 1987; Kuppuswamy and Chinnadurai, 1987). On the other hand, CR3, which encompasses the region unique to 289R, is involved in transcriptional activation (Moran *et al.*, 1986; Lillie *et al.*, 1987; Schneider *et al.*, 1987; Jelsma *et al.*, 1988).

In order to be able to survey the coding region of E1A systematically, we have created a series of in-frame deletion mutants by oligonucleotide mutagenesis that spans the entire coding region. Here we report results with these and other E1A mutants that have identified three regions, all in exon 1, that are essential for transformation. In enhancer repression, two of these same regions are also critical, but the third is less important. Surprisingly, although E1A protein is required for the induction of PCNA, we have been unable to find any region of the protein which by itself is essential for this induction.

MATERIALS AND METHODS

Plasmids, viruses, and cells

The construction of E1A mutants *dl* 1101–1110, *dl* 1112–1116, *sub* 1117, *pm* 1120–1122, and *pm* 1131 and their rescue into plasmid pLE2, containing all of E1A, has been described previously (Jelsma *et al.*, 1988; Egan *et al.*, 1988). Ad5 *dl* 520 (Haley *et al.*, 1984) was obtained from P. E. Branton, McMaster University. pLE2 *dl* 520 was constructed by replacing the *wt* E1A *Xma*I to *Xba*I fragment of pLE2 with the same fragment from *dl* 520. Mutants *dl* 1101–1109 in pLE2 were cloned into pLE2 *dl* 520 by cleaving both plasmids with *Bam*HI and *Xma*I, recovering the appropriate fragments from agarose gels, and ligating. [In pLE2, the *Bam*HI site is joined to the beginning of the Ad5 sequence at nucleotide 22 (Jelsma *et al.*, 1988).] Mutated E1A regions were rescued into Ad5 *dl* 309 by the method of Stow (1981) or McGrory *et al.* (1988). pSV2CAT (Gorman *et al.*, 1982), Ad5 *dl* 312 (Jones and Shenk, 1979), and Ad2/Ad5 *dl* 1504 (Osborne *et al.*, 1982) were obtained from F. L. Graham, McMaster University, and an EJ-*ras* plasmid (Shih and Weinberg, 1982) from S. Mak, McMaster University.

HeLa cells were maintained in minimal essential medium (MEM) supplemented with 10% newborn calf serum. Primary baby rat kidney (BRK) cells were prepared from 2- to 6-day-old Wistar rats according to van der Eb and Graham (1980) with minor modifications and were plated and maintained as described (Ruley, 1983).

Transformation assays

BRK cells were transfected (Ruley, 1983; Graham and Bacchetti, 1983) on the second day after plating, when the dishes were 60–70% confluent. Cells were stained by treatment with Carnoy's fixative and Giemsa stain.

Lines of transformed cells were tested for growth on soft agar according to Macpherson and Montagnier (1964).

CAT assay for enhancer repression

The CAT assay for enhancer repression was carried out on extracts of transfected HeLa cells in the same manner as that for transactivation (Jelsma *et al.*, 1988) with the following changes. In transfection, the DNA of interest was 5 μg of pSV2CAT and 2 or 3 μg of either pLE2 or pUC18 per 100-mm dish of cells. To increase the sensitivity of the assay itself, harvested cells were resuspended in 150 μl/dish 0.25 *M* Tris, pH 7.8, 5 m*M* EDTA (TED buffer), and after sonication, extracts were incubated at 60° for 10 min (Crabb and Dixon, 1987). Reaction mixtures contained 10 to 20 μl of extract, 0.2 μCi [^{14}C]chloramphenicol (50 mCi/mmol; NEN), 1.6 m*M* acetyl-coenzyme A, and TED buffer to a final volume of 50 μl. After thin-layer chromatography and autoradiography, radioactive spots were scraped and quantified by liquid scintillation counting as before (Jelsma *et al.*, 1988).

Analysis of PCNA induction

Confluent monolayers of BRK cells, 2 days after plating, were infected with virus at an m.o.i. of 10 PFU/cell and labeled from 12 to 14 hr postinfection with 100 μCi

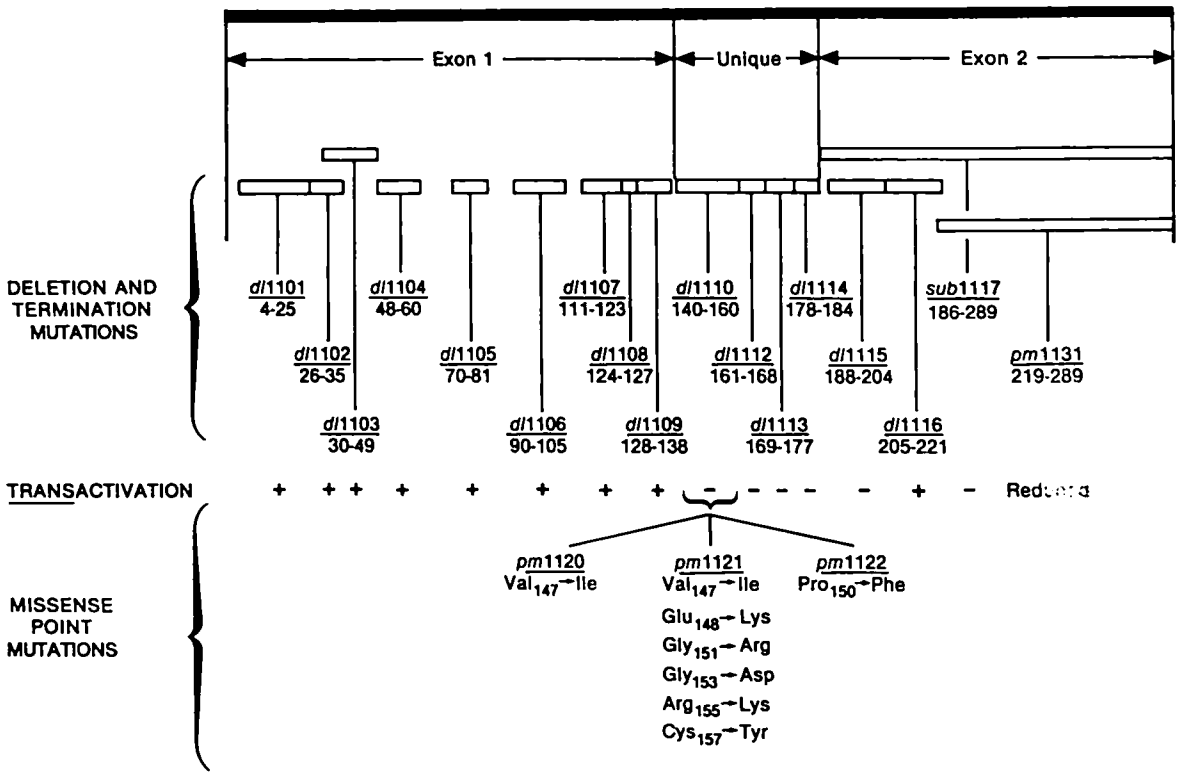

FIG. 1. Map of a series of mutations spanning the coding region of Ad5 E1A. Regions deleted in terms of the 289R protein are indicated by open boxes. The ability of mutants to transactivate transcription (Jelsma *et al.*, 1988) is also shown.

[^{35}S]methionine (ICN Biomedicals) per 60-mm dish in methionine-free medium. Cells were harvested, and extracts were prepared and immunoprecipitated as described (Mathews *et al.*, 1984). Extracts in 40 μl/dish buffer A (10 m*M* Tris–HCl, pH 7.5, 0.14 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.5% Nonidet-P40) were precleared by adding 10 μl 10% protein A–Sepharose CL-4B (Pharmacia) in buffer C (50 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl, 5 m*M* EDTA, 0.5% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mg/ml bovine serum albumin) and then diluting 1:1 with buffer C. Cleared lysates were immunoprecipitated with 5 μl of a human antiserum directed against PCNA (ImmunoConcepts, Sacramento, CA), mouse monoclonal antibody H219 to the Ad5 72KD DNA binding protein (Rowe *et al.*, 1984), or mouse monoclonal antibody M73 to Ad2 E1A proteins (Oncogene Science, Inc., Manhasset, NY), together with 30 μl 10% protein A–Sepharose in buffer C. The immunoprecipitates were analyzed by electrophoresis on 12.5% SDS–polyacrylamide gels and fluorographed.

RESULTS

Most of the work described below was carried out with the E1A mutations shown in Fig. 1. These mutations, described previously (Jelsma *et al.*, 1988; Egan *et al.*, 1988), fall into two categories. *Pm* 1120–1122 contain mis-sense mutations affecting 1, 6, and 1 residues respectively in the unique region of the 289R protein between residues 147 and 157 (Fig. 1). All the other mutations cause deletions in the E1A protein products, as a result either of deletions in the genome (*dl* 1101–1110 and *dl* 1112–1116), or of premature termination during translation (*sub* 1117 and *pm* 1131). All the mutated E1A regions should produce 289R and 243R proteins, except *dl* 1110, which lacks the 5′ splice site for 12 S mRNA and therefore probably produces no 243R protein, and *sub* 1117, which lacks the 3′ splice site and therefore probably produces a single readthrough product consisting of the first 185 residues of the 289R protein followed by a mis-sense lysine (Jelsma *et al.*, 1988).

For experiments on repression of the SV40 transcriptional enhancer, we constructed a series of double mutants, in which *dl* 1101–1109 were each combined with *dl* 520. Mutant *dl* 520 lacks nucleotides 1107–1117, which span the 13 S 5′ splice site, and therefore produces no 13 S mRNA or its 289R protein product (Haley *et al.*, 1984). This series of double mutants, designated *dl* 1101/520, *dl* 1102/520 and so on, therefore produces only the 243R protein mutated in exon 1.

TABLE 1

TRANSFORMATION OF BRK CELLS BY MUTANTS IN THE UNIQUE REGION AND IN EXON 2 of E1A

Number of dishes with foci†
Total number of foci
Average number of foci/dish

	Normal culture			With agar overlay		
	Mutant	*wt* control	Number of experiments	Mutant	*wt* control	Number of experiments
pm 1122	51/52 833 16.0	47/57 301 5.3	5	8/8 31 3.9	6/7 33 4.7	2
dl 1110	38/54 440 + 7* 8.3	30/55 105 1.9	5	7/8 38 4.8	6/7 33 4.7	2
dl 1112	39/47 565 12.0	29/52 129 2.5	6	19/19 154 8.1	17/18 83 4.6	4
dl 1115	51/53 732 + 1* 13.8	43/53 165 + 2* 3.2	5	8/8 45 5.6	6/7 33 4.7	2
dl 1116	43/45 224 + 2* 5.0	39/45 146 + 2* 3.3	4	3/4 6 1.5	3/4 7 1.8	1
pm 1131	54/54 523 + 6* 9.8	49/55 323 + 1* 5.9	5	4/4 11 2.8	3/4 7 1.8	1
dl 520	37/37 752 20.3	29/36 142 3.9	6	31/32 448 14.0	28/32 193 6.0	6
ras alone	8/145 21 0.14	— — —	18	— — —	— — —	—

* Number of dishes in which the foci were too numerous to count. Most of these were probably secondary foci (see text) and for the calculation of average number of foci/dish were treated as one focus/dish.

† Results are totals for the number of experiments indicated.

In the assays for transformation and for enhancer repression, the mutant E1A regions, including *dl* 520, were tested in plasmid pLE2, a pBR322 derivative containing the whole of the Ad5 E1A region (Jelsma *et al.*, 1988). For the study on the induction of PCNA synthesis, mutated E1A regions were rescued into the phenotypically *wt* Ad5 virus *dl* 309.

Transformation of BRK cells by E1A mutants in cooperation with EJ-*ras*

E1A mutants were assayed for their ability to function in cell transformation by transfecting primary baby rat kidney cells with pLE2 and a plasmid containing the EJ-*ras* oncogene. In early experiments, 0.12, 0.25, and 0.50 μg per 60-mm dish of both the E1A and *ras* plasmids were used in each assay, but different concentrations did not give significantly different numbers of foci. The results of these assays therefore have been pooled, and in later experiments only 0.25 μg of each plasmid DNA per dish was used.

Cultures were monitored for foci throughout each experiment. In many experiments, particularly with mutants in the unique region and in exon 2, which are discussed first, transformed cells tended to lift off and seed secondary foci elsewhere in the dish very rapidly. To minimize this, wherever possible dishes were stained as soon as the number of foci rose above 10. All remaining dishes were stained after about 4 weeks. Because of the complication with secondary foci, results are given for the fraction of dishes with foci, the total number of foci, and the average number of foci/dish. In a few instances, foci became too numerous-

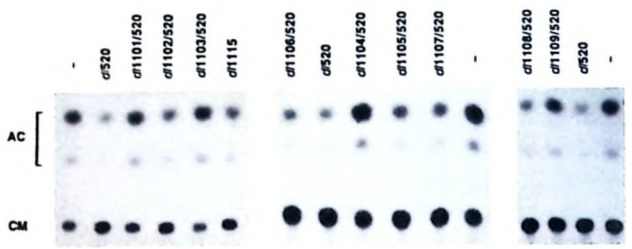

FIG. 3. Repression by E1A mutants of expression of the chloramphenicol acetyltransferase gene driven by the SV40 enhancer–promoter. The assays contained pSV2CAT alone or with the mutant pLE2 indicated. Autoradiographs of three separate experiments are shown. CM, chloramphenicol; AC, acetylated chloramphenicol.

protein as well as a 289R protein that is defective in transactivation (Jelsma *et al.*, 1988), CAT activity was reduced significantly. From measurements of the percentage of chloramphenicol acetylated, activity with pLE2 *dl* 520 was 24.5 ± 7.6% (in 17 experiments), and with pLE2 *dl* 1112 was 24.1 ± 6.0% (in 7 experiments) of the activity with carrier. Thus in our hands repression of CAT expression was only seen in the absence of transactivation (cf. Lillie *et al.*, 1986), but the extent of repression was the same with the 243R protein alone as with 243R together with a transactivation-defective 289R protein. For the repression experiments, we assayed the exon 1 mutants after they had been rescued into pLE2 *dl* 520.

Figure 3 illustrates the results from repression assays on mutants *dl* 1101/520–*dl* 1109/520. The repressions meaured for these mutants in a series of experiments are shown in Table 3 as percentages of the repression obtained with *dl* 520. The averages of these results are plotted in Fig. 2B for comparison. Some of the mutants, such as *dl* 1109/520, gave a wide spread in the results, and almost all showed some reduction in repression compared to *dl* 520. Nevertheless, it is clear that the ability of the 243R protein to repress the SV 40 early promoter–enhancer was critically affected by deletions in two separate regions of exon 1, namely residues 4–25 in *dl* 1101, and residues 30–60 in *dl* 1103 and *dl* 1104. Repression was affected less critically but still significantly by a deletion in a third area, namely residues 128–138 in *dl* 1109. Sequences separating these critical regions in the 243 residue protein appeared to be less necessary, as *dl* 1102, which deletes residues 26–35, and *dl* 1105–*dl* 1108, which together delete almost all of the residues between 70 and 127, had smaller effects on enhancer repression.

Results obtained with two exon 2 mutants defective in transactivation, *dl* 1115 and *sub* 1117, are given in the lower part of Table 3, and an assay for *dl* 1115 is shown in Fig. 3. Both of these mutants showed less repression than *dl* 520 or *dl* 1112, but the reduction in repression was greater with *sub* 1117, which removes the whole of exon 2, than with *dl* 1115, which deletes 17 residues at the N-terminal end of exon 2.

Induction of PCNA by E1A exon 1 mutants

The induction of PCNA synthesis in primary BRK cells by Ad5 infection has been studied by Zerler *et al.* (1987). These authors found, first, that it could be brought about by a virus that expressed only 12 S E1A mRNA, indicating that the activation was not due to the transactivation domain that spans the region unique to the 289R product of the 13 S mRNA. Second, they found that it occurred on infection with viral deletion mutants that together deleted all of the E1A proteins except for the N-terminal 85 residues, suggesting indirectly that this may have been the region responsible for induction.

We have examined more directly the role of the N-terminal 85 residues on the stimulation of PCNA synthesis, by using mutants with deletions in this region. BRK cells were infected with mutant virus for 12 hr and were then labeled with [^{35}S]methionine for 2 hr. Extracts were prepared and portions were immunoprecipitated with antiserum against PCNA, against E1A proteins, or against the E2A 72-kDa DNA-binding protein. The immunoprecipitates were then analyzed on SDS–polyacrylamide gels.

TABLE 3

TRANSCRIPTIONAL REPRESSION BY EXON 1 MUTANTS IN *dl* 520 SERIES (243R PROTEIN ALONE)

Mutant	Repression as % of *dl* 520*	Mean
dl 1101/520	−16, −15, 3, −5, −17, 2	−8 (±9)
1102/520	52, 74, 76, 66, 37, 58, 67, 52, 89, 70	64 (±15)
1103/520	−35, 10, 3, −21	−11 (±20)
1104/520	−13, −5, −1, 2, 1, −3	−3 (±5)
1105/520	76, 85, 61, 75, 76, 86, 78	77 (±8)
1106/520	64, 84, 87, 103, 96, 89, 94	88 (±12)
1107/520	86, 82, 98, 85, 81, 55, 69, 70, 88, 88	80 (±12)
1108/520	89, 95, 106	97 (±9)
1109/520	23, −15, −2, 30, 13, 38, 53, 12, 57	23 (±24)
Exon 2 mutants defective in transactivation		
dl 1115	63, 92,† 105†	87 (±22)
sub 1117	58, 43,† 32†	44 (±13)

* (% chloramphenicol acetylated with pSV2CAT − % with pSV2-CAT and mutant pLE2)/(% with pSV2CAT − % with pSV2CAT and pLE2 *dl* 520) × 100. Negative values represent increased acetylation relative to pSV2CAT alone. The results of independent experiments are shown. In each case, at least two, and usually three, different plasmid preparations were tested.

† Repression as percentage of pLE2 *dl* 1112.

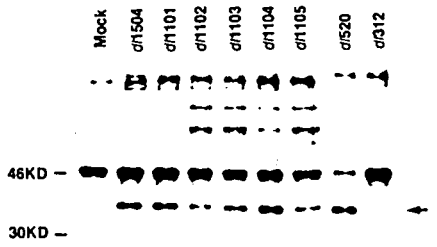

FIG. 4. Induction of PCNA synthesis by E1A mutants of Ad5. Extracts of BRK cells, mock-infected or infected with the mutant viruses indicated and labeled with [^{35}S]methionine, were immunoprecipitated with anti-PCNA antiserum and the immunoprecipitates were analyzed on SDS–polyacrylamide gels and fluorographed. The positions of molecular mass markers are shown. The PCNA band is indicated by an arrow.

Figure 4 shows typical results with immunoprecipitates obtained using anti-PCNA serum for deletion mutants spanning the entire N-terminal 85 residue region, namely Ad5 *dl* 1101-1105 and Ad2/Ad5 *dl* 1504 (Osborne *et al.*, 1982), which lacks residues 1–15 (Downey *et al.*, 1984), compared to that obtained with the 12 S virus Ad5 *dl* 520. All of these precipitates gave a band of 35–37 kDa, typical of PCNA (Zerler *et al.*, 1987; Bravo *et al.*, 1987; Prelich *et al.*, 1987). This band could be detected only faintly in extracts of mock-infected cells and of cells infected with Ad5 *dl* 312, which lacks most of E1A (Jones and Shenk, 1979) (Fig. 4). In other experiments (not shown), this band was found after infection with Ad5 *dl* 309, which produces both 13 and 12 S E1A mRNAs; this band comigrated with a band precipitated from infected cell extracts by a sample of PCNA antiserum from M. B. Mathews (Cold Spring Harbor Laboratory); and this band was not detected in similar extracts precipitated with unrelated antisera against E1A proteins and the 72-kDa E2A protein. Occasionally in these experiments, a few of the infections gave a less intense 35 to 37-kDa band than did the rest, as is the case with *dl* 1102 and *dl* 1105 in Fig. 4. Over all the experiments, however, we found no consistent evidence to suggest that any of these exon 1 mutants was appreciably less efficient at inducing this PCNA band than *dl* 520 or *dl* 309. Rather, it was clear that infection with these mutants always gave a PCNA band that was significantly stronger than the background level seen in mock or *dl* 312 infections, indicating that the ability of E1A proteins to activate PCNA synthesis had not been abolished by the deletions studied. Results similar to these were also obtained (but are not shown) for mutants that delete all of the rest of the 243R and 289R proteins, namely the other exon 1 mutants, Ad5 *dl* 1106–1109, and exon 2 mutants Ad5 *dl* 1115–1116 and Ad5 *dl* 313, which lacks sequences for the C-terminal 70 residues of E1A proteins as well as most of E1B (Graham, 1984).

These results, together with earlier work (Zerler *et al.*, 1987), confirm that E1A is required to induce PCNA synthesis in BRK cells, but they also show that none of the deletions so far studied abolishes induction completely, i.e., none of the deleted portions of the E1A protein products appears to be essential by itself for this induction.

Production of E1A proteins

Cells transfected with the different pLE2 mutants used in this work were not checked for the production of E1A proteins, but it is unlikely that any of the results obtained could have been due to the failure of some mutants to produce these proteins, for the following reasons. First, in KB cells infected with mutant viruses containing each of the deletions in exon 1, either alone or in combination with *dl* 520, or containing the deletions in the unique region, E1A proteins have been detected in amounts comparable to those infected with *wt* virus (Egan *et al.*, 1988; C. Egan, unpublished observations). Second, every mutant studied gave a positive result, indicating protein function, in at least one, and usually two, of four assays, the three described here and that for transactivation in Jelsma *et al.* (1988).

DISCUSSION

Site-directed mutagenesis is a valuable technique for studying adenovirus E1A proteins, as it enables us to identify domains that are important for different phenotypic effects, and from this to distinguish which of these effects are likely to share the same underlying molecular mechanisms. Using a series of in-frame deletion mutants spanning the whole of the coding region of E1A, we have identified, more systematically and precisely than has been possible before, regions in E1A proteins which when deleted adversely affect the ability of these proteins to transform BRK cells in cooperation with *ras*, and to repress the SV40 transcriptional enhancer. By contrast, we found no region of these proteins which by itself was necessary for the stimulation of PCNA synthesis in BRK cells.

In exon 1, there were three regions in which deletions markedly reduced transformation, namely at the N-terminal end between residues 4 and 25, between residues 36 and 60 in CR1, and at the C-terminal end from residues 111 to 138 in CR2 (Figs. 2A and 2C). In enhancer repression assays of the *dl* 520 series of double mutants, which express the 243R protein only, deletions of residues 4–25 and 36–60 effectively abolished repression, whereas in CR2 only deletion of residues 128–138 had any significant effect, reducing but

not abolishing repression; mutants *dl* 1107 and *dl* 1108 repressed the enhancer efficiently, even though they transformed cells poorly (Fig. 2). By contrast, deletions of residues separating these critical regions, that is, 26–35, 70–81 at the C-terminal end of CR1, and 90–105, had comparatively little effect on either transformation or repression.

Previous work has also shown that in transformation, the N-terminal end of E1A (Subramanian *et al.*, 1988; Whyte *et al.*, 1988b; Velcich and Ziff, 1988), CR1 (Lillie *et al.*, 1987; Schneider *et al.*, 1987; Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987; Velcich and Ziff, 1988), and CR2 (Moran *et al.*, 1986; Lillie *et al.*, 1986; Kuppuswamy and Chinnadurai, 1987; Schneider *et al.*, 1987) were important, and that in enhancer repression, the N-terminal end (Velcich and Ziff, 1988) and particulary CR1 (Schneider *et al.*, 1987; Lillie *et al.*, 1987; Velcich and Ziff, 1988) were involved, but that mutations in CR2 had limited effects (Schneider *et al.*, 1987; Kuppuswamy and Chinnadurai, 1987). However, our results extend these observations by demonstrating clearly that for both transformation and repression, the N-terminal half of exon 1 contains two critical regions, the N-terminal end and CR1, and that these regions are distinctly separate. The existence of these two separate, functionally important regions is further reflected in the ability of mutants in this area to bind a 300-kDa cellular protein. E1A products from *dl* 1101, 1103, and 1104 bind this protein poorly, whereas with *dl* 1102, the binding is the same as that with *wt* E1A (Egan *et al.*, 1988) (Fig. 2C).

Of the three important regions identified in exon 1, that in CR2 is probably functionally independent of the other two. The *structural* independence of CR2 has been shown by complementation studies (Moran and Zerler, 1988), but these do not prove that CR2 is involved in a separate function from the others. At present, the strongest evidence for this is the differences in the properties of the regions. While mutations in all three regions affect transformation, those in CR2 affect enhancer repression much less than mutations in the other two (Fig. 2B). Furthermore, CR2 appears to contain the principal binding sites for cytoplasmic proteins of 107 and 105 kDa, but not for one of 300 kDa, whereas the reverse is the case for the two regions in the N-terminal half of exon 1 (Egan *et al.*, 1988; Fig. 2C).

This same evidence indicates that the N-terminal region and CR1 share properties and so are probably involved with the same functional domain. This would mean that residues 26–35 form a joining loop that is not essential to function. There are two possible ways in which the N-terminal region and CR1 are organized: one is that they form complementary parts of one domain; an alternative is that the N-terminal region forms a structural foundation for the correct folding of a functional domain in CR1. At present we cannot choose between these possibilities.

Mutations in the unique region and in exon 2 had no effect on transformation, except for an increase in the seeding of secondary foci compared to that found with *wt* E1A, probably due to altered adhesion of transformants to the plastic substrate. It is not clear whether this phenomenon could have affected the results of other authors using similar mutants. The lack of sensitivity of transformation to mutations in the unique region and exon 2 confirms the earlier finding that a truncated product consisting of the first 140 residues of the 289R protein is able to transform BRK cells in cooperation with *ras* (Zerler *et al.*, 1986; Whyte *et al.*, 1988b). In addition, this lack of sensitivity demonstrates that, functionally, the regions in exon 1 that are critical for transformation must be totally independent of the rest of the polypeptide chain beyond exon 1. This is shown most clearly by *dl* 1110, which deletes residues 140–160 in the 289R protein and presumably makes no 243R product as the 5′ splice site for the 12 S mRNA has been removed. With this mutant, producing only a mutated 289R product, removal of the section of the polypeptide chain immediately adjacent to the critical CR2 at its C-terminal had no effect on transformation (Table 1). The functional independence of the unique region from exon 1 is also apparent from results on transcriptional activation: the region required, corresponding closely to CR3 (Lillie *et al.*, 1987; Jelsma *et al.*, 1988), is not detectably affected by limited deletions in exon 1 (Jelsma *et al.*, 1988).

It is interesting to compare the results depicted in Fig. 2 with a comparison of the hydropathy profiles of E1A 289R protein and the product of the avian retrovirus MC29 *myc* oncogene (Branton *et al.*, 1985), a nuclear protein that, like E1A, is able to transform in cooperation with *ras* (Ruley, 1983; see also Berk, 1986). These profiles show regions of close similarity, between residues 7 and 85 in E1A and 112 and 190 in v-*myc*, and between residues 115 and 166 in E1A and 213 and 264 in v-*myc*, which may reflect similarities in function. Within exon 1 of E1A, these regions correspond closely to the sequences required for transformation and enhancer repression. However, all that is known of the functions of the similar v-*myc* sequences is that residues 112–143 are required for transformation, but the rest are not (Stone *et al.*, 1987).

In two exon 2 mutants, *dl* 1115 and *sub* 1117, which were defective in *trans* activation and deleted part or all of exon 2 respectively, repression of the SV40 enhancer was reduced but not abolished. Because removal of the whole of exon 2 does not eliminate repres-

sion entirely, it seems likely that exon 2 is not directly involved in repression, but has only a limited effect on the folding of active sites in exon 1. Alternatively, a deletion or a premature termination in exon 2 could cause the C-terminal portion of the protein to block an active site in exon 1. The results with *dl* 1115 and *sub* 1117 agree with those of Schneider *et al.* (1987), using a mutant very similar to *sub* 1117, but not with Velcich and Ziff (1988), who found that two mutants similar to *dl* 1115 and *sub* 1117 were unable to repress transcriptional enhancers. The reason for the discrepancy is not apparent, as although the repression assay of Velcich and Ziff (1988) differed from ours, it was similar to that of Schneider *et al.* (1987).

Results with mutations in the C-terminal end of exon 1, which repress the SV40 enhancer but do not transform, and with mutations in exon 2, which transform but have reduced repression, confirm observations of others (Subramanian *et al.*, 1988; Velcich and Ziff, 1988) that transformation and enhancer repression are separable functions. In the present work, no mutants were found that transformed and yet completely lacked the ability to repress the SV40 enhancer. On this basis, it is possible that enhancer repression is necessary, but alone is not sufficient, for transformation.

The conclusion that probably two separate domains in exon 1 are required for transformation is interesting in light of the recent finding of Whyte *et al.* (1988a) that the 105-kDa cellular protein, which binds to residues 111–127 of E1A proteins (Egan *et al.*, 1988), is the retinoblastoma protein p105-RB (see also Egan *et al.*, 1989). Clearly this binding, which involves one of these two domains, could be one of the activities of E1A necessary for transformation, but the necessity for a second, functionally separate domain suggests that at least one other E1A function is also required. The discovery of the identity of the 300-kDa cellular protein that binds to this second domain is eagerly awaited.

So far, no functional domain has been identified for the induction of PCNA synthesis. Induction was not appreciably reduced by any of the E1A deletions studied here or previously (Zerler *et al.*, 1987) that collectively delete the whole of the 243R and 289R proteins, with two minor exceptions. These exceptions are residues 61–69, between *dl* 1104 and *dl* 1105, and residues 82–85, between *dl* 1105 and the beginning of the regions deleted by the mutants of Zerler *et al.* (1987). It is possible that one of these two regions is the active site for induction and we are investigating this. An alternative is that no single region of the E1A proteins alone is responsible for activating PCNA synthesis. Rather, activation may depend on some repetitive feature of protein structure and is able to tolerate the deletion of one of the repeats without noticeable effect. For example, by analogy with a recent model for the initiation of transcription (Sigler, 1988), the repetitive feature may possibly be regions of negative charge, of which three of the most extensive in the 289R protein (excluding phosphorylation) are residues 45–60, 133–141, and 189–199. (In the 243R protein, these are residues 45–60, 133–138, and 143–153.) To detect a loss of PCNA stimulation would then require the deletion of at least two of these regions. We are at present investigating this possibility also. Whatever the outcome of future studies, however, results to date suggest that the mechanism by which E1A proteins induce PCNA is entirely different from those used in processes for which functional domains have been identified.

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My Contributions to Jelsma et al. (1989)

1) Modified a transient CAT assay to measure trans-repression of the SV40 early promoter/enhancer region by the E1A mutants.

2) Assayed the entire set of exon 1 mutants (dl 1101 - 1109), in a dl 520 (243R E1A only) background, to map E1A regions required for trans-repression. Because of the number of experiments required, Dr. Tony Jelsma completed a portion of these assays.

In Jelsma et al. (1989), E1A sequences required for transformation and repression were mapped to the amino terminal half of the E1A proteins (Figure 5). Transformation efficiency was greatly reduced by deletion mutations in three regions between residues 4 - 25, 36 - 60 and 111 - 128. Enhancer repression appeared to be a function primarily of the 243R protein and repression activity was eliminated by deletion mutation in two regions between residues 4 - 25 and 36 - 60. Deletion of residues 128 - 138 in dl 1109/520 also affected the efficiency of repression, but the results were variable; in some assays dl 1109/520 repressed at near wild type levels and in others it was as defective as the completely defective mutants. All of the small exon 1 mutants in viral form induced PCNA

synthesis.

Repression by Transactivation Defective Double Mutants

In a series of experiments that were not included in Jelsma et al. (1989) but which are relevant here, I tested the ability of the exon mutants to affect repression by a transactivation defective mutant. For these experiments the exon 1 mutants were transferred to dl1112, which deletes residues 161 - 168 in CR3. The resulting double mutants as well as expressing mutated 243R products also express transactivation defective 289R products that contain the same exon 1 mutation as the 243R protein. In general the dl 1112 series of exon 1 mutants were less effective than the dl520 series for repression. However, the results with these mutants confirmed that residues at the amino terminus and in CR1 were required for repression (Table 2). On the other hand mutant dl 1109/1112, unlike dl 1109/520, repressed consistently at wild type levels.

This finding could be explained if dl 1109 affected the structure of the 243R and 289R proteins differently. It is possible that dl 1109 destabilizes the structural integrity of the protein in the 243R product, but not in the 289R product because exon 1 is followed by the unique region and not directly by exon 2 as in the 243R protein. Subsequent characterization of the E1A protein produced by

Table 2. Enhancer repression by exon 1 mutants in the dl 1112 series. The ability of each mutant to repress the SV40 enhancer was calculated as in Jelsma et al., (1989) for at least three separate experiments.

Deletion in dl 1112 series	Repression as a Percentage of dl 1112	Mean
dl 1101	39, 34, 5, 45	31 ±18
dl 1102	88, 94, 95, 90	92 ± 3
dl 1103	43, 25, 32, 37	34 ± 8
dl 1104	39, 25, 24, 13	25 ±11
dl 1105	88, 52, 91	77 ±22
dl 1106	88, 77, 97	87 ±10
dl 1107	102, 100, 99	100 ± 1
dl 1109	72, 120, 89	94 ±24

the dl1109/520 virus has revealed that it is detected much less efficiently than any of the other mutated 243R proteins (Howe and Bayley, 1992). Together these results suggest that residues 128 - 138, which are deleted in dl 1109, do not constitute a functional domain for repression, but instead contribute to the overall stability of the 243R product. Due to the nature of this finding, I will not further discuss the use of the dl 1109/520 mutant in other mapping studies.

With the completion of the work described in Jelsma et al. (1988, 1989), domains in the E1A proteins for transactivation, enhancer repression and transformation had been mapped. By comparing the results of these studies we concluded that transactivation by the 289R protein was not required for transformation and that enhancer repression could be required, but was not sufficient for transformation. Although these correlations are interesting, they did not contribute to a better understanding of the underlying molecular mechanisms responsible for E1A function. At the time these studies were in progress however, it was known that the E1A proteins could physically interact with a number of cellular polypeptides including ones of 105 kDa, 107 kDa and 300 kDa. These proteins are referred to as p105, p107 and p300, respectively. To determine if the binding of these proteins to the E1A polypeptides could be potentially important for

E1A function, our series of E1A mutants was used to map cellular protein-binding sites on the E1A polypeptides.

3) Mapping of Cellular Protein-Binding Sites on the Products of Early-Region 1A of Human Adenovirus (Egan et al., 1988, M.C.B. 8, 3955-3959)

The experimental approach for mapping cellular protein binding sites on the E1A polypeptides involved infecting human KB cells with the dl 309 series of E1A mutant viruses, immunoprecipitating E1A proteins with an E1A-specific monoclonal antibody and analysing the immunoprecipitates by SDS PAGE.

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Mapping of Cellular Protein-Binding Sites on the Products of Early-Region 1A of Human Adenovirus Type 5

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The binding sites for the 300-, 107-, and 105-kilodalton cellular proteins which associate with human adenovirus type 5 E1A products were studied with E1A deletion mutants. All appeared to bind to the amino-terminal half of E1A products in regions necessary for oncogenic transformation. These results suggest that these cellular species may be important for the biological activity of E1A products.

Early-region 1A (E1A) of human adenovirus type 5 (Ad5) encodes two major proteins of 289 and 243 residues (proteins 289R and 243R), which are identical except for the presence of an additional 46 internal amino acids in the larger species (27). Structurally, 289R can be divided into three regions: residues 1 to 139, which are common to both 289R and 243R (exon 1); residues 140 to 185, which are unique to 289R (unique); and residues 186 to 289 (exon 2), which are common to both products (Fig. 1). E1A proteins activate transcription of viral (1, 16, 17, 24) and cellular (18, 25, 31) genes, and this function has been mapped to the unique region (15, 21–23, 30). E1A products inhibit some transcriptional enhancers (2, 13, 14, 33), and this activity maps to the exon 1 region (21, 22, 30). E1A products also accomplish a number of other functions, including the induction of cell division and immortalization, participation with early-region 1B (E1B) in oncogenic transformation (see reference 3 and references therein), and stimulation of expression of an epithelial growth factor (28). These activities appear to require at least two separate regions encoded by exon 1 (21, 22, 28, 30, 34).

Little is known about the molecular mechanisms which underlie these various E1A functions. As one approach, we (6, 36) and others (11) have identified several cellular proteins that associate specifically with E1A polypeptides. The three major species observed by our group and that of Harlow are phosphoproteins of 300, 107, and 105 kilodaltons (kDa) (6, 11, 36). The identity, function, and biological importance of these cellular E1A-associated proteins are unknown. In the present report, we have investigated the binding sites of these species on E1A proteins by using a variety of E1A deletion mutants. It is difficult to define binding sites accurately without a detailed knowledge of the three-dimensional structure of E1A proteins. Nevertheless, if particular regions of the E1A protein molecule are involved in binding, either directly or indirectly, deletion of these sequences should reduce the association of one or more of the cellular species. We report that all of these species appear to bind in exon 1.

Role of the E1A amino and carboxy termini. To examine the importance of the amino and carboxy termini of E1A products in the binding of cellular proteins, human KB cells (ca. 2×10^7 in 150-mm-diameter dishes) were infected either with wild-type (wt) Ad5 or with mutant *dl*1504 or *dl*313, which lacks sequences encoding the first 14 residues at the amino terminus (5, 23) or the last 70 amino acids at the carboxy terminus (4), respectively. Infected KB and mock-infected cultures were labeled with [^{35}S]methionine (Amersham Corp.; specific activity, 1,300 Ci/mmol) from 7 to 11 h postinfection, and cell extracts prepared in either the presence (buffer B, 200 mM Tris [pH 7.0]–137 mM NaCl–1.0 mM $CaCl_2$–0.4 mM $MgCl_2$–10% [vol/vol] glycerol–1% [vol/vol] Nonidet P-40–100,000 IU of aprotinin per ml) or absence (buffer A, 50 mM Tris [pH 7.2]–150 mM NaCl–1% [vol/vol] Triton X-100–100,000 IU of aprotinin per ml) of divalent cations were immunoprecipitated with anti-peptide serum E1A-C1 or E1A-N1, which is specific for the carboxy or amino terminus, respectively, of both 289R and 243R (35, 37). In some cases, the peptides to which the sera were generated were also added. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by using discontinuous 12% polyacrylamide gels, and migration patterns were determined by autoradiography, as described previously (29, 36). Figure 2 (lane B) shows that with extracts from wt Ad5-infected cells, E1A-C1 serum precipitated the 300-, 107-, and 105-kDa cellular proteins, in addition to E1A products. As shown previously (36), the 300-kDa species was absent when extracts were prepared in the presence of divalent cations (lane E). The three cellular proteins were not precipitated from mock-infected cells (lanes A, G, and J), and addition of E1A-C peptide blocked their precipitation (lanes C, I, and L). With E1A-C1 serum and extracts from cells infected with mutant *dl*1504, the 107- and 105-kDa species were detected, but the 300-kDa protein was not (lanes H and K). With extracts from wt- or *dl*313-infected cells precipitated with E1A-N1 serum (lanes N and Q, respectively), the 107- and 105-kDa species were present, but the 300-kDa protein was not. These results were compatible with previous data that indicated that E1A protein complexes containing the 300-kDa species were not precipitated by serum prepared against the amino terminus (36). In total, these data suggested that the amino terminus of E1A products may be involved in binding the 300-kDa protein, but that neither this region nor the last 70 residues at the carboxy terminus play a significant role for the 107- and 105-kDa species.

Studies with other deletion mutants and *hr*5. Cells infected with wt Ad5, with *hr*5, or with one of a series of deletion

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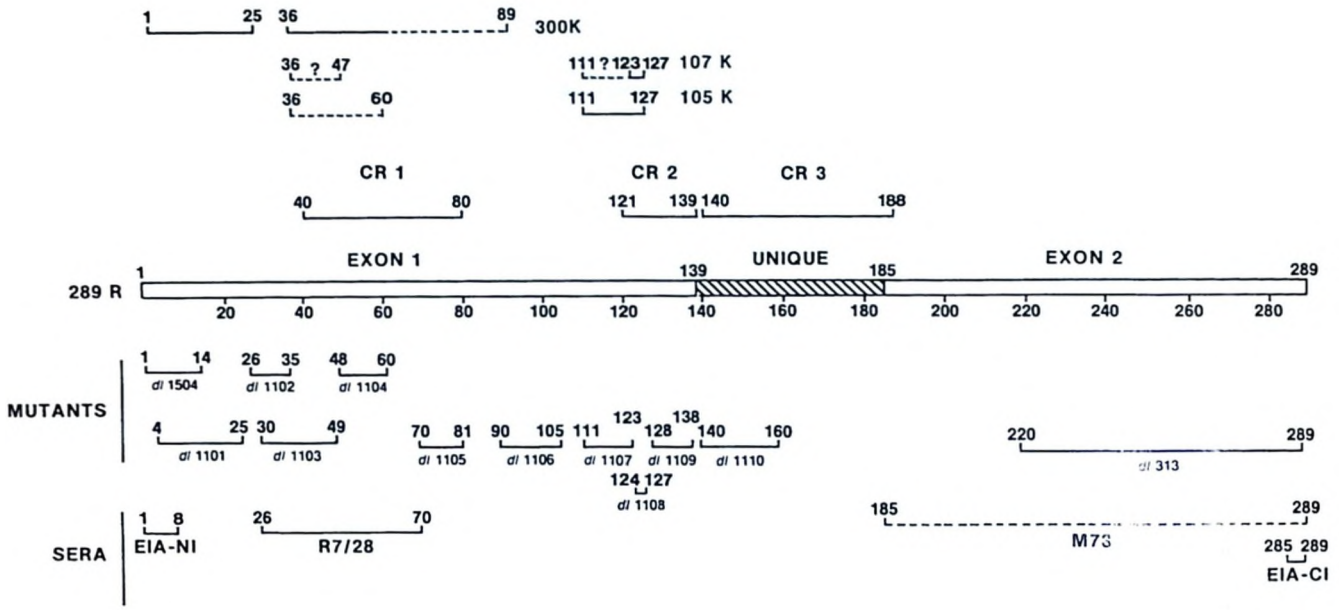

FIG. 1. Ad5 E1A 289R protein, mutants, antisera, conserved regions, and binding sites. The 289R protein is shown with its three structural regions: that encoded by exon 1 of the 0.9-kilobase mRNA (residues 1 to 138), the region unique to the 289R product of the 1.1-kilobase mRNA (residues 139 to 185 of the 289R protein), and the region encoded by exon 2 of both E1A mRNAs (residues 186 to 289 of the 289R protein). Shown below are the regions believed to be recognized by various E1A-specific sera and the locations of deletions in the various Ad5 mutants. Shown above are the positions of conserved regions 1, 2, and 3 (CR1, CR2, and CR3, respectively) and the locations of the regions which play a role in the binding of E1A-associated proteins (300-, 107-, and 105-kDa proteins [300 K, 107 K, and 105 K, respectively), as interpreted from data obtained in the present report. ——, Sequences essential for binding; – – –, sequences having some effect on binding.

mutants were labeled with [^{35}S]methionine, and extracts were precipitated with E1A-specific monoclonal antibody M73 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Mutant *hr5* (8, 12) contains a point mutation at nucleotide 1229, which alters Gly-139 in 243R to Asp and alters Ser-185 in 289R to Asn (7). The other mutants contain in-frame deletions and yield E1A products lacking various regions (15; see Fig. 1, Table 1, and the legend to Fig. 3 for complete details). All of these mutants produced reasonable amounts of E1A proteins, although migration rates were anomalous and increased or even decreased in a fashion unrelated to the size of the deletion (Fig. 3). An analysis (not shown) indicated that removal of acidic, and to a lesser extent, proline residues was the principal cause of increased gel mobility of mutant E1A proteins. These results suggested that high contents of these two types of amino acids cause E1A proteins to migrate much slower than would be predicted from their molecular masses.

Figure 3 shows that the 300-, 107-, and 105-kDa species coprecipitated with E1A products from cells infected with wt Ad5 and *hr5* but were reduced or absent with some of the deletion mutants. To quantify the amount of binding, appropriate exposures of the gel shown in Fig. 3 were scanned by a microdensitometer. The amounts of 300-, 107-, and 105-kDa proteins were normalized to the total amount of E1A products present, and then results were expressed relative to the values obtained with wt Ad5 (Table 1). The 300-kDa species was undetectable with *dl*1101 and *dl*1104, greatly reduced with *dl*1103, and partially reduced with *dl*1105. These results, together with that obtained with *dl*1504, showed that binding was affected by deletions within two regions of exon 1, namely, between residues 1 and 25 and between amino acids 36 and at least 60 (Fig. 1). The involvement of the first 14 to 25 residues at the amino terminus supported observations made previously (36) and in the present study (Fig. 2), which indicated that E1A-N1 anti-peptide serum failed to recognize E1A protein complexes containing the 300-kDa species. This failure may have resulted from the binding of the 300-kDa protein to this region, although it could also have been due to a conformational change induced by binding at another site. The 105-kDa species was undetectable with *dl*1107 and somewhat reduced with *dl*1103 and *dl*1104, indicating that the region between residues 111 and 123 (and perhaps the region between residues 36 and 60) plays a role in binding this protein. The increased binding with *dl*1102 was not seen in other experiments in which levels similar to wt Ad5 were observed (data not shown). With the 107-kDa species, binding was somewhat reduced with mutants *dl*1101, *dl*1103, and *dl*1107. It is unlikely that residues 1 to 25 are involved, as reduced binding was not seen consistently in other experiments with mutant *dl*1101 (data not shown) and was never seen with *dl*1504 (Fig. 2), which lacks residues 1 to 14. Less binding was seen reproducibly in several experiments with *dl*1103 and *dl*1107, thus suggesting that residues 36 to 47 and 111 to 123 may be of some importance. The region between residues 36 and 60 was also implicated in the binding of the 107- and 105-kDa proteins by results obtained with the E1A-specific rat monoclonal antibody R28. This antibody (and a similar monoclonal antibody, R7) was known to interact with an epitope mapping between residues 23 and 120 (32), and by using the present set of E1A deletion mutants, we have now shown that this epitope requires all of the region between amino acids 26 to 60 (data not shown). This serum immunoprecipitated E1A complexes containing the 300-kDa protein but failed to recognize those bound to the 107- and 105-kDa species (data not shown).

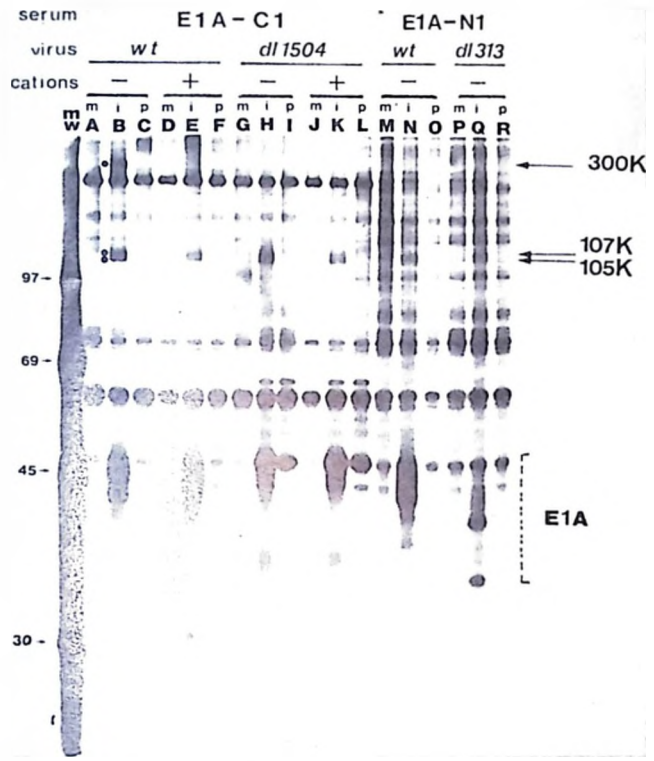

FIG. 2. E1A-associated proteins in cells infected with the mutants *dl*1504 and *dl*313. Human KB cells infected with wt Ad5 or with mutant *dl*1504 or *dl*313 were labeled with [^{35}S]methionine, and cell extracts prepared either in the presence or absence of divalent cations were immunoprecipitated by using either E1A-C1 or E1A-N1 anti-peptide serum, in some cases in the presence of the appropriate synthetic peptides. Lanes: mw, ^{14}C-labeled molecular weight markers (Du Pont NEN Research Products); m, mock-infected cells; i, Ad5-infected cells; p, precipitations carried out in the presence of 10 μg of appropriate peptide. Extracts were prepared in the presence (+) or absence (−) of divalent cations. To the right of the gel are shown the positions of the 300-, 107-, and 105-kDa proteins (300K, 107K, and 105K, respectively); to the left are shown the molecular masses of the ^{14}C-labeled markers.

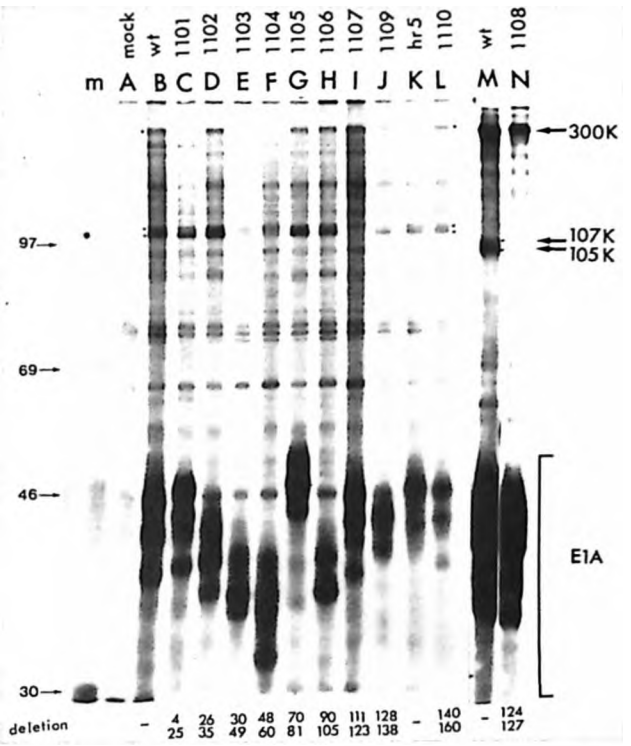

FIG. 3. E1A-associated proteins in cells infected with various deletion mutants and *hr5*. Cells were infected with wt or mutant Ad5 or they were mock-infected. The Ad5 deletion mutants *dl*1101 to *dl*1107 (1101 to 1107), *dl*1109 (1109), *dl*1110 (1110) (15), and *dl*1108 (1108) all possess in-frame deletions (see Fig. 1 and Table 1 for details of the deleted sequences), and their production has been described previously (15). Rescue of mutations into virus was by standard methods (Jelsma et al., in preparation). These mutants were all propagated in cell line 293 (9). Cells were labeled with [^{35}S]methionine, and extracts prepared in buffer A were immunoprecipitated by using the E1A-specific mouse monoclonal antibody M73, which reacts with an epitope in the exon 2 region (10). Lanes: m, molecular weight markers; A, mock-infected cells; B, wt Ad5-infected cells; C through L, mutant-infected cells (as indicated); M and N, wt Ad5 (M) and *dl*1108 (N) from a separate experiment. The residues missing in the deletion mutants are indicated at the bottom of the figure. Positions of the 300-, 107-, and 105-kDa proteins (300K, 107K, and 105K, respectively) are shown to the right of the gel; to the left are shown the molecular masses of the ^{14}C-labeled markers.

Because none of the previous mutants completely eliminated binding of the 107-kDa species, we constructed mutant *dl*1108, in which residues 124 to 127 between those removed in *dl*1107 and *dl*1109 were deleted. With this mutant, binding of the 300-kDa species was similar to that seen with wt Ad5 (Fig. 3, lane M), but both the 107- and 105-kDa proteins were absent (Fig. 3, lane N; Table 1). These data showed that the region necessary for binding of the 105-kDa protein extends to residue 127 and that amino acids 124 to 127 are required for the 107-kDa species.

It is unlikely that the unique region of E1A products plays a significant role in binding of the 300-, 107-, and 105-kDa proteins. Previous results have shown that the 243R protein is as efficient in binding these species as is 289R (6, 36), and binding of all three proteins to E1A products harboring deletions in the unique region, such as *dl*1110 (Fig. 3, lane L) and others (data not shown), was as with wt E1A. None of exon 2 appeared to be necessary for binding of the 300-kDa species, as preliminary studies with mutant E1A proteins produced in and purified from *Escherichia coli* containing the plasmid 410X, which encodes a protein lacking residues 150 to 289 (19), indicated that binding of the 300-kDa species was as efficient as with full-length E1A protein (C. Egan, B. Ferguson, M. Rosenberg, and P. E. Branton, unpublished results). Although the role in binding of the 107- and 105-kDa species of residues 186 to 220 in exon 2 has not been examined directly, results with *dl*313 suggested that residues 221 to 289 are of little importance (Fig. 2).

While conclusions from experiments such as these must be regarded with caution, the data suggest that exon 1 is the only portion of the E1A protein molecule involved in the binding of these cellular polypeptides, and furthermore, that regions in exon 1 in which deletions eliminate binding completely could represent the actual binding sites. For the 300-kDa species, two such regions exist which are located within residues 1 to 25 and 36 to 49. It is not clear if these two regions are combined by tertiary structure to form a single binding site or if one of these regions is necessary only for the formation of an appropriate protein structure at the other site. For each of the 107- and 105-kDa proteins, only a single

TABLE 1. Correlation between binding of E1A-associated proteins and transformation by Ad5 E1A mutants

Virus	Residues deleted	Binding (% wt) of 300-kDa protein[a]	Binding (% wt) of 107-kDa protein[a]	Binding (% wt) of 105-kDa protein[a]	Transformation with *ras*[b]
wt	0	100	100	100	+
*dl*1101	4–25	0	60	120	−
*dl*1102	26–35	160	150	380	+
*dl*1103	30–49	10	40	50	−
*dl*1104	48–60	0	90	50	−
*dl*1105	70–81	50	90	120	+
*dl*1106	90–105	130	120	170	+
*dl*1107	111–123	140	50	0	−
*dl*1108	124–127	100[c]	0[c]	0[c]	−
*dl*1109	128–138	130	80	130	−
*dl*1110	140–160	160	120	100	+
hr5[d]		110	120	100	+
*dl*1504	1–14	−	+	+	+[e]
*dl*313	220–289	+	+	+	+

[a] To quantify binding, the polyacrylamide gels shown in Fig. 3 were autoradiographed with preflashed film (20). From exposures in which all film densities fell on the linear part of the characteristic curve of the film, the amounts of the 300-, 107-, and 105-kDa cellular proteins and all of the E1A products were measured by microdensitometry with a Hoefer Scientific GS 300 apparatus. Values were normalized to the amount of total E1A protein and expressed as a percentage of the values obtained with wt Ad5. In cases in which deletions eliminated a methionine residue, an appropriate correction was made. Binding with the mutants *dl*504 and *dl*313 was not quantified in this study; the presence (+) or absence (−) of binding is indicated.

[b] Transformation assays were carried out with BRK cells with plasmid pLE2 containing mutated E1A regions and an activated *ras* gene, as described previously (21, 30). The specific transformation data will be published separately (Jelsma et al., in preparation). +, Transformation; −, no transformation.

[c] Values were obtained in a separate experiment.

[d] *hr5* contains a point mutation that alters residue 139 in the 289R product and amino acid 185 in 243R.

[e] Data of Osborne et al. (26) by a different transformation assay involving virions.

putative binding site was apparent, between residues 124 and 127 and residues 111 to 127, respectively. Other regions of the E1A protein which, when deleted, resulted in only partial reduction in binding may play a role in the tertiary structure of the primary binding site.

The fact that the E1A-binding proteins appear to interact with exon 1 of E1A proteins is of great interest, as this region has been linked to oncogenic transformation, cellular immortalization, enhancer repression, and other activities (15, 21, 22, 28, 30, 34). For transformation in association with an activated *ras* gene, two highly conserved regions encoded by exon 1, termed CR1 and CR2 (Fig. 1), are required (21, 22, 30, 33, 34). Recently, the deletion mutants described in the present study were used in plasmid form to map the regions in exon 1 necessary for transformation in cooperation with *ras*. The results showed that in addition to CR1 and CR2, a region near the amino terminus was also essential (Jelsma, et al., manuscript in preparation). Others had previously demonstrated the importance of the amino terminus, but they did not show that this region was functionally separable from CR1 (34). There was an excellent correlation between the failure to bind one or more of the E1A-associated proteins and loss of transforming activity (Table 1). The only exceptions were *dl*1109 and *dl*1504. The former transformed poorly, even though the mutant E1A product appeared to bind the E1A-associated species at levels comparable with that of wt Ad5. The latter, which fails to bind the 300-kDa species, has been shown by others, using whole virus, to be capable of transformation (26), although it has not been tested under the present assay conditions. Thus, it is possible that at least one of the reasons why CR1, CR2, and the amino terminus are necessary for transformation may be the requirement to form functional complexes with all three E1A-associated proteins. Further clues about the roles of these proteins must await their identification and characterization.

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My Contributions to Egan et al. (1988)

1) Construction of the mutant viruses dl 1101, dl 1102, dl 1103 and dl 1104 using the method of McGrory et al. (1988). Each virus was plaque purified twice and large scale stocks were purified by centrifugation through CsCl gradients.

The E1A binding sites for each of the cellular proteins p300, p105 and p107 appeared to be located exclusively in the amino terminal half of the E1A polypeptides (Figure 5). E1A sequences required for association with p300 were found in two regions namely at the amino terminus between residues 4 - 25 and in CR1 between residues 36 - 60. The primary binding sites for p105 and p107 appeared to be contained in overlapping regions of CR2. p105 appeared to associate with residues between 111 - 127 and the primary binding site for p107 was located between residues 124 - 127. A minor decrease in binding of p105 and p107 was also observed for E1A proteins with deletions between residues 36 - 60.

These studies, together with the work described in Jelsma et al. (1989), showed that there was a strong correlation between the ability of the E1A proteins to associate with cellular polypeptides and their abilities to repress the SV40 enhancer and to transform BRK cells (see

Figure 5). Each mutant that was defective for association with p300 was impaired for transformation, as well as for repression of the SV40 enhancer, while mutants that failed to associate with p105, or p107 and p105 both, were defective for transformation, but not enhancer repression. These correlations suggested that the E1A proteins may repress enhancers by binding to p300 and bring about transformation by associating with both p300 and p105. The hypothesis that binding of cellular proteins by E1A was required for transformation was significantly strengthened shortly after publication of Egan et al. (1988) when one of the E1A associated proteins, p105, was identified as the product of the retinoblastoma growth suppressor gene (Whyte et al., 1988). Mutations in both copies of the retinoblastoma gene were known to be common characteristics of all retinoblastomas, suggesting that p105 (now referred to as pRb) normally functioned to suppress cell growth (see Weinberg, 1991 for a review). If the normal function of pRb was to regulate cell growth, then it followed that one of the roles of the E1A proteins in transformation could be to sequester and inactivate pRb to allow unregulated cell division. Since binding to pRb was probably required - but not sufficient - for transformation it was also possible that the other E1A-associated proteins played similar roles to pRb in cell growth control. In an attempt to further characterize the consequences of the interactions between

the E1A proteins and p300, p107 and pRb, we next used our series of exon 1 mutants to map sequences in the E1A proteins required for stimulation of cellular DNA synthesis in growth arrested cells.

4) **Retinoblastoma Growth Suppressor and a 300-kDa Protein Appear to Regulate Cellular DNA Synthesis (Howe et al., 1990, PNAS 87:5883-5887)**

This paper describes the use of our E1A deletion mutants to map E1A domains required for stimulation of cellular DNA synthesis in quiescent BRK cells. BRK cells were chosen for this work because they become quiescent naturally, within 2 or 3 days after plating, and also to allow us to compare the results of these studies directly with our previous studies on transformation. Since these cells are semipermissive for Ad5, and viral DNA replication would complicate studies of cellular DNA synthesis, we first transferred the exon 1 series of E1A mutants to a dl 520 background. Dl 520 produces the 12S E1A mRNA and the 243R protein, but no 13S mRNA, and in the absence of the transactivation domain of the 289R protein viral DNA replication and cell killing is limited (Quinlan and Grodzicker, 1987; Zerler et al., 1987; Howe et al., 1990).

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Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis

(adenovirus type 5/E1A mutants)

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ABSTRACT **Previous work has suggested that oncogenic transformation by the E1A gene products of adenovirus type 5 may be mediated through interactions with at least two cellular proteins, the 105-kDa product of the retinoblastoma growth suppressor gene (p105-Rb) and a 300-kDa protein (p300). By using viral mutants, we now show that the induction of cellular DNA synthesis in quiescent cells by E1A differs from transformation in that E1A products induce synthesis if they are able to bind to either p105-Rb or p300, and only mutant products that bind to neither are extremely defective. These results suggest that p105-Rb and p300 (or cellular proteins with similar E1A-binding properties) provide parallel means by which DNA synthesis can be regulated.**

The familiar explanation that cancer is due to uncontrolled cell growth has been given sharper focus recently by developments in the concept of cellular anti-oncogenes or growth suppressor genes, that is, genes whose products normally arrest cell growth. Unlimited cell growth is thought to occur if these genes are deleted or altered or if their products are inactivated. The main experimental basis for this concept has been recent findings on the retinoblastoma (*Rb*) gene, indicating that retinoblastomas arise when both alleles of this gene are lost or inactivated. This work, which essentially began with a study by Knudson (1), has been reviewed by Weinberg (2).

Further insights into the mechanisms of action of the *Rb* gene have been obtained through studies with the early region 1A (E1A) oncogene of human adenovirus type 5 (Ad5). Expression of this gene can immortalize cells, cause cells to proliferate, and, in collaboration with a second oncogene such as Ad5 E1B or activated *ras*, induce oncogenic transformation (3). Transformation seems to require specific interactions between E1A polypeptides and key cellular proteins. Several cellular proteins to which E1A proteins bind have been observed (4, 5). Two of these have molecular masses of 105 kDa and 300 kDa (p105 and p300, respectively). p105 has been identified as the product of the *Rb* gene (p105-Rb) by Whyte *et al.* (6), and this was subsequently confirmed by our group (7). It has further been found that E1A mutants whose proteins fail to bind to p105-Rb also fail to transform cells (7–10). It is widely believed that the formation of a complex with E1A products inactivates p105-Rb, thereby permitting the uninhibited growth associated with oncogenically transformed cells. However, this association is clearly not the only event required for E1A-mediated transformation as mutant E1A products that bind p105-Rb normally but fail to bind p300 are also defective for transformation (8–10). This suggests that p300 is involved in growth control as well.

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The major products of the E1A region of Ad5 are proteins of 289 and 243 residues. They are produced from 13S and 12S mRNAs, respectively, and are identical except for an extra internal sequence of 46 amino acids in the larger protein. To learn more about the way these proteins function in transformation, we have constructed and studied the properties of a series of small in-frame deletion mutants spanning the whole of the coding sequence of the E1A gene (9, 11). We have also used these mutants to map the sites on E1A proteins involved in binding to p105-Rb, p300, and another cellular protein of 107 kDa (p107) (8).

It has been known for some time that E1A induces cellular DNA synthesis in a variety of growth-arrested cells (12–14). Studies on the phosphorylation of p105-Rb during the cell cycle (15–18) led to the suggestion that this protein may help to regulate the progress of cells from G_0/G_1 phase into S phase (16, 17). In an attempt to understand more clearly the role of both p105-Rb and p300 in this regulation, we have used our series of E1A mutants to examine the relationship between the induction of cellular DNA synthesis by E1A products and their ability to bind to p105-Rb and p300. The results suggest that binding of E1A proteins to either p105-Rb or p300 (or to other cellular proteins with similar binding properties) is sufficient to induce synthesis. These data provide further suggestive evidence that p105-Rb regulates entry into S phase but that p300 is an alternative control element for DNA synthesis.

MATERIALS AND METHODS

Cells and Viruses. The 293 cell line (19) was maintained in Eagle's modified minimal essential medium (MEM) supplemented with 10% (vol/vol) newborn calf serum. KB cells were maintained in α-modified MEM supplemented with 10% (vol/vol) fetal bovine serum. Primary baby rat kidney (BRK) cells were prepared from 4- to 6-day-old Wistar rats as described (9) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum.

The construction of E1A mutations dl1101–1109 and pm1131 and their transfer into an E1A plasmid pLE2/520, which expresses only the 12S E1A mRNA, have been described (8, 9, 11). pLE2/520 contains a deletion from Ad5 dl520 that removes the 5′ splice site for the 13S E1A mRNA (9, 20). E1A mutations dl1141–1143 were constructed and transferred into pLE2/520 in a similar manner. Mutant pLE2/520 plasmids containing two deletions in exon 1 were created by combining appropriate restriction fragments from plasmids containing single exon 1 deletions. Mutated E1A regions were transferred from pLE2/520 into the phenotypically wild-type (wt) virus Ad5 dl309, by using either the

Abbreviations: Ad5, adenovirus type 5; BRK, baby rat kidney; p105-Rb, 105-kDa retinoblastoma protein; p300 and p107, cellular proteins of 300 kDa and 107 kDa, respectively; wt, wild type; pfu, plaque-forming unit(s); moi, multiplicity of infection.

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method of McGrory *et al.* (21) or the method of Stow (22), as described (9). After screening, each mutant virus was plaque-purified twice, amplified, and titrated on 293 cells.

Induction and Measurement of DNA Synthesis. The procedure for measuring DNA synthesis was that of Zerler *et al.* (14). BRK cells were plated on 60-mm dishes and infected 3 days later at a multiplicity of 10 plaque-forming units (pfu) per cell. At the time of infection or 24 or 48 hr after infection, 50 μCi of [^{3}H]thymidine (Amersham) in 5 ml of fresh medium was added. After labeling for 24 hr, the cells were harvested and resuspended in 0.3 M NaOH. DNA was precipitated with trichloroacetic acid and collected on Whatman glass-fiber filters, and radioactivity was measured in a scintillation counter.

To estimate the extent of viral DNA synthesis in BRK cells infected with 12S virus, duplicate sets of quiescent cells were infected either with the phenotypically wt Ad5 dl309 or with the 12S virus Ad5 dl520 and labeled for three 24-hr periods. Controls were provided by mock-infected cells or by cells infected with Ad5 dl312, which lacks essentially the whole E1A region. With one set of cells, incorporation of [^{3}H]-thymidine into total DNA was measured as before. From a second set of cells, viral DNA was extracted by the Hirt method (32), the samples were electrophoresed on 0.6% agarose gels, and the radioactivity in each of the viral DNA bands was measured by scintillation counting. Because of lysis, measurements on cells infected with dl309 could only be made over the first 24-hr labeling period.

Analysis of E1A and Associated Cellular Proteins. Proteins from virally infected KB cells were analyzed as described (8). KB cells were infected at a multiplicity of 70 pfu per cell and labeled with [^{35}S]methionine. To lysates of these cells was added either the E1A-specific monoclonal antibody M73 (Oncogene Science, Manhasset, NY) to precipitate E1A and associated cellular proteins or the Ad5 E1B-specific antibody 9C10 (Oncogene Science) as a control. Precipitates were analyzed by SDS/polyacrylamide gel electrophoresis, followed by fluorography.

RESULTS

Several previous studies have shown that E1A products are able to induce cellular DNA synthesis in quiescent primary BRK cells infected with adenovirus (14, 23–25). This is also the system we have chosen for examining the role of E1A protein binding in this process, as it enables us to relate results directly to our earlier work on the transformation of BRK cells by E1A mutants (9). BRK cells are semipermissive for Ad5, and cell killing was prevented by infecting with virus containing mutated E1A regions transferred into a dl520 background. dl520 expresses only the E1A 12S mRNA and its product, the smaller 243-residue protein (20), and, in the absence of the 289-residue protein, which is required to activate transcription of other early viral genes, viral replication is limited (14, 23). Throughout our work, DNA synthesis was measured by the incorporation of [^{3}H]thymidine over three 24-hr intervals, namely, 0–24, 24–48, and 48–72 hr after viral infection. Three measurements were made to discover whether the incorporation induced by each of our E1A mutants was rapid, delayed, or transient (cf., refs. 23 and 25). In no case did we detect any significant trend, and so we report below the incorporation over the total 72 hr after infection for each mutant as a percentage of that by dl520.

Estimation of Viral and Cellular DNA Synthesis in BRK Cells Infected with 12S Virus. To establish the extent to which viral DNA synthesis affected our results, quiescent BRK cells were infected with the phenotypically wt Ad5 dl309 or with dl520 and labeled with [^{3}H]thymidine. Uptake of radioactivity was then measured into total DNA and into viral DNA. Ad5 dl309 produced much more viral DNA than did dl520 (see also ref. 14). By comparing incorporation into total DNA and viral DNA for these two viruses (data not shown), it was possible to arrive at an upper limit for the contraction of the incorporation with dl520 due to viral DNA synthesis. Results from two experiments estimated this at 1% or less of total DNA synthesis and, therefore, of minor importance compared to the synthesis of cellular DNA.

Induction of DNA Synthesis by E1A Mutants Containing Single Deletions. A number of published studies with E1A mutants have attempted to define the regions of the 243-residue protein essential for the induction of DNA synthesis (14, 23, 24, 26, 27). The results suggested that the N-terminal 85 residues are required, although other evidence (25, 26) indicated that sequences at the C-terminal end of exon 1 (Fig. 1) are also necessary.

To investigate this question further, we examined a series of Ad5 mutant viruses, dl1101/520, dl1102/520, etc., in which single deletions in the coding region of E1A (Fig. 1) were combined with dl520. Initially, we tested dl1101/520 through dl1109/520, dl1141/520, dl1142/520, and pm1131/520 for their ability to stimulate incorporation of [^{3}H]thymidine into DNA in quiescent BRK cells. Except for pm1131/520, these mutants all contain a small deletion in exon 1 of E1A and include dl1101/520, dl1103/520, and dl1104/520, which should lack the principal binding sites for p300, and dl1107/520 and dl1108/520, which should lack those for p105-Rb and p107 (Fig. 1). pm1131/520 contains a termination mutation in exon 2 that deletes the C-terminal 71 residues of the E1A protein. Collectively, these mutants delete almost the entire exon 1 and a major portion of exon 2 (Fig. 1). Fig. 2 shows

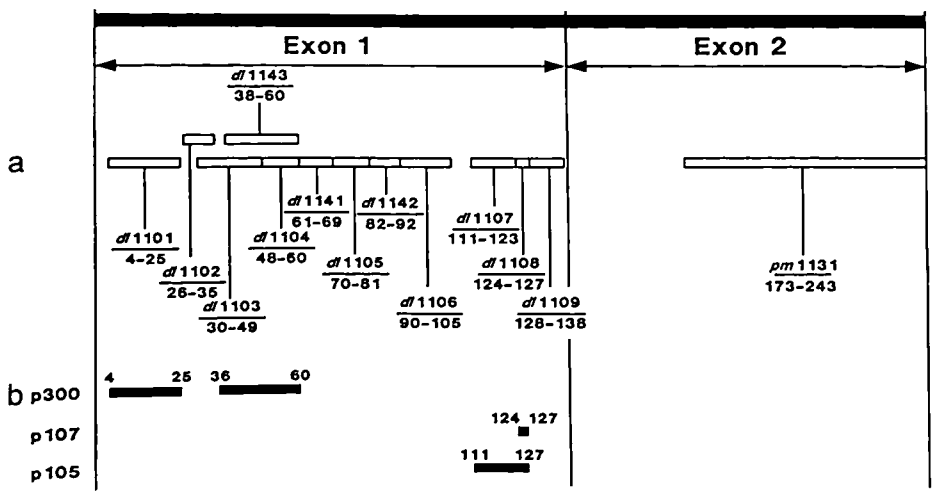

FIG. 1. Map of the Ad5 243-residue E1A protein, showing the deletions used in the present study, with the residues deleted (*a*) and the principal sites affecting binding to p105-Rb, p107, and p300 (*b*) from Egan *et al.* (8).

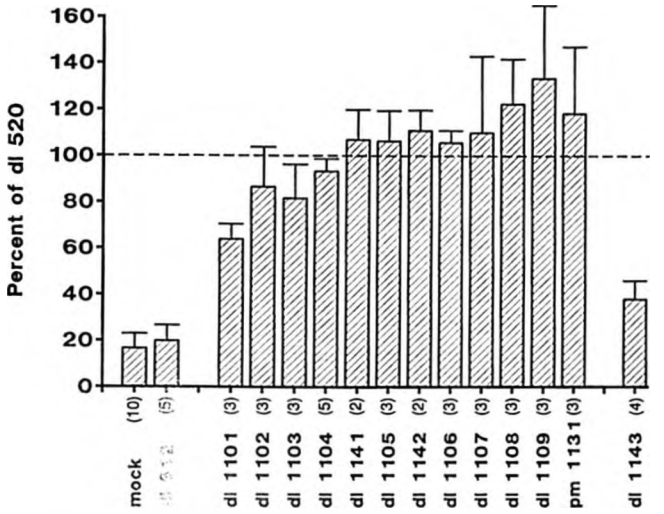

FIG. 2. Incorporation of [^{3}H]thymidine into DNA over a 72-hr period by BRK cells infected with 12S viruses containing single E1A deletions. For each mutant, the average incorporation for the number of experiments indicated in parentheses is shown as a percentage of the incorporation by the wt 12S control virus, dl520, in the same experiments. Error bars show SD.

the incorporation of [^{3}H]thymidine for each mutant over a 72-hr period after infection expressed as a percentage of that with dl520. All the mutants were able to induce DNA synthesis, although those with deletions in the N-terminal 60 residues were slightly less efficient than dl520. The most significant result, however, was that with none of these mutants was induction of DNA synthesis seriously impaired. Thus individually, none of the deletions in the 243-residue protein impeded the ability of this protein to induce DNA synthesis.

Smith and Ziff (24) found that small deletion-substitution mutants in the N-terminal region of the 243-residue protein also had no effect on induction of DNA synthesis in BRK cells. However, these authors (24) and Moran and Zerler (23) showed that, in these cells, viruses with deletions beyond residue 20 that were larger than 25 residues were defective. To confirm the adverse effect of a larger deletion on induction of DNA synthesis, we constructed dl1143/520 to delete residues 38–60 (Fig. 1). Induction with this mutant was markedly reduced, although not to the level in mock-infected cells or in cells infected with Ad5 dl312, a mutant that lacks essentially the whole E1A sequence (Fig. 2).

As part of a routine study of E1A mutants, we tested dl1143/520 for the ability of its E1A protein to bind to cellular proteins. This was done by infecting human KB cells, immunoprecipitating the E1A proteins, and analyzing the precipitates on denaturing gels (8). Fig. 3 shows that binding of p300 to the E1A protein from dl1143/520 occurred at greatly reduced levels. This was expected as we had found (8) that another mutant, dl1104, which contains a deletion in the same region, also gave reduced binding to p300 (see Fig. 1). However, dl1143/520 showed reduced binding to p105-Rb as well, but binding to another cellular protein, p107, was not noticeably affected (Fig. 3). [Whyte *et al.* (10) observed similar changes in binding with their mutant dl646N, which deleted residues 30–85.]

Induction of DNA Synthesis by E1A Mutants Containing Double Deletions in Exon 1. The results just described suggested that the reduced induction of DNA synthesis by dl1143/520 may have been related to a failure of its altered E1A protein to bind to both p105-Rb and p300. To test this hypothesis, we made a series of mutants containing two deletions in exon 1. For brevity, a double mutant combining, for example, dl1101 and dl1106 within the dl520 background was designated dl01/06/520. Among these double mutants, we combined a deletion such as dl1101, which destroys the ability of E1A proteins to bind to p300, with a deletion such as dl1107, which eliminates the p105-Rb binding site (see Fig. 1). As controls we constructed other double mutants in which the two exon 1 deletions should yield E1A products still capable of binding to p105-Rb, to p300, or to both. These mutants were tested for their ability to stimulate incorporation of [^{3}H]thymidine into DNA as before, and results obtained over a 72-hr period after infection for all the mutants are presented in Table 1. Each of these mutants was tested for its ability to bind to p105-Rb, p107, and p300 in studies such as those shown in Fig. 3. The results from the binding studies are summarized in Table 1.

It is clear that double mutants that were defective at inducing DNA synthesis—namely, dl01/07/520, dl01/08/520, and dl43/08/520—were those that failed to bind to both

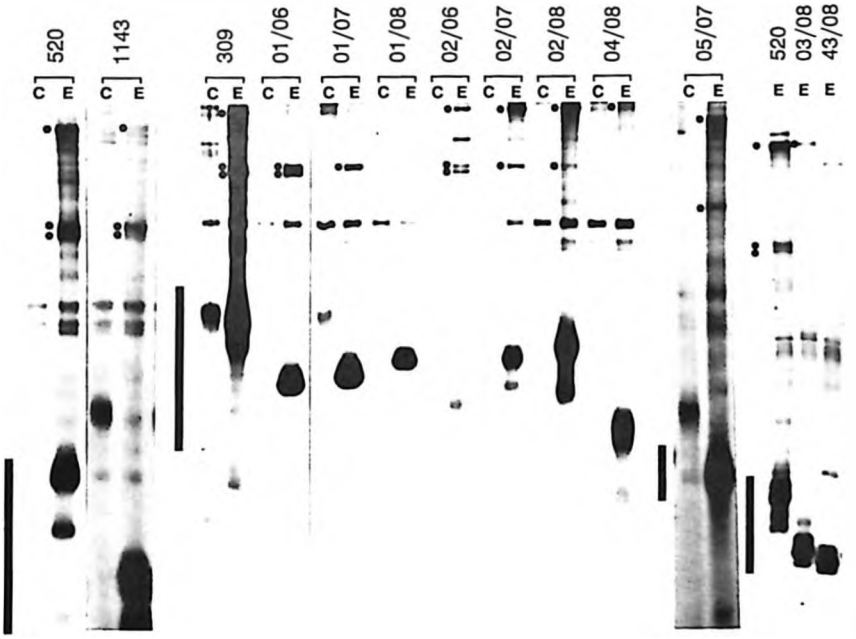

FIG. 3. Fluorographs of denaturing gels of extracts of KB cells infected with Ad5 viruses containing E1A deletions in a dl520 background, compared to infections with dl520 or with the phenotypically wt dl309. In many cases, extracts were immunoprecipitated with either an antiserum to Ad5 E1B proteins as control (lanes C) or an E1A-specific monoclonal antibody (lanes E). Bands due to p300, p107, and p105-Rb are indicated by dots, and those due to E1A proteins are indicated by bars. For some unknown reason, dl02/08/520 showed a faint band of p107. This does not normally occur when dl1108 is present: see dl01/08/520, dl04/08/520, and dl43/08/520 in neighboring lanes and dl1108 in Egan *et al.* (8).

Table 1. Ability of E1A 243-residue-related proteins from mutant Ad5 viruses to induce DNA synthesis in BRK cells compared to their ability to bind to p105-Rb, p107, and p300 in KB cells

Mutant	Binding			DNA synthesis, % of control	Experiments, no.
	p105-Rb	p107	p300		
Mock	−	−	−	15 ± 4	11
dl520	+	+	+	100	11
dl312	−	−	−	18 ± 5	4
dl01/07/520	−	+	−	20 ± 4	7
dl01/08/520	−	−	−	29 ± 6	3
dl43/08/520	−	−	−	14 ± 4	2
dl03/08/520	−	−	↓	48 ± 9	3
dl04/08/520	−	−	↓	41 ± 10	4
dl1143/520	↓	+	−	38 ± 8	4
dl01/06/520	+	+	−	69 ± 6	3
dl02/06/520	+	+	+	121 ± 15	3
dl02/07/520	−	+	+	80 ± 12	3
dl02/08/520	−	↓	+	89 ± 18	3
dl05/07/520	−	+	+	92 ± 17	3

DNA synthesis for each mutant is the incorporation of [^{3}H]-thymidine as a percentage of that for dl520 (control) (mean ± SEM). Mutants are grouped according to their ability to induce cellular DNA synthesis poorly (lines 4–6), at a reduced level (lines 7–9), or at a level comparable to that of dl520 (lines 10–14). Binding to p105-Rb, p107, and p300 is shown as similar to (+), or much reduced compared to that with dl520 (↓), or not detectable (−).

p105-Rb and p300. In contrast, the ability to bind to p107 did not correlate with induction of DNA synthesis. In no case was loss of induction due to failure to produce E1A proteins, as the defective mutants produced at least as much E1A protein as other mutants (Fig. 3). Lack of induction was also not due simply to the presence of two separate deletions in exon 1, as double mutants dl02/06/520, dl02/07/520, dl02/08/520, and dl05/07/520 stimulated DNA synthesis efficiently (Table 1) and dl01/06/520 stimulated DNA synthesis to the same level as dl1101/520 alone (Fig. 2).

From these results, it appeared, therefore, that the ability of a mutant to induce DNA synthesis correlated with its ability to bind to p105-Rb, to p300, or to both and that it was only mutants that bound to neither of these proteins that were markedly defective at inducing DNA synthesis.

Two double mutants, dl03/08/520 and dl04/08/520, induced intermediate levels of thymidine incorporation. With dl04/08/520, only weak binding to p300 was observed (Fig. 3); we discuss this mutant below. The ability of dl03/08/520 to induce DNA synthesis at the level it did was at first surprising, as results with dl1103 (ref. 8; see Fig. 1) had led us to believe that this mutant would bind poorly to p300 and not at all to p105-Rb. However, in this earlier work (8), the mutant used produced both the 289-residue and 243-residue E1A proteins, whereas the present mutant gave only a 243-residue-related product. In fact, with dl03/08/520 (Fig. 3), binding to p300 was somewhat better than that observed with dl1103 (8). These data suggest that deletion of residues 30–49 may have somewhat different effects on binding to p300 by the 289- and 243-residue proteins.

Effects of Different Multiplicities of Infection and of Mixed Infections. To investigate the effect of multiplicity of infection (moi) on the level of induction of DNA synthesis, dl520, dl01/07/520, and dl04/08/520 were assayed using up to 40 pfu per cell (Fig. 4). The plot for dl520 shows that induction was maximal at moi values above 5. dl01/07/520 gave only a slight increase in thymidine incorporation toward higher values of moi, indicating that this mutant is extremely defective at inducing DNA synthesis. However, dl04/08/520

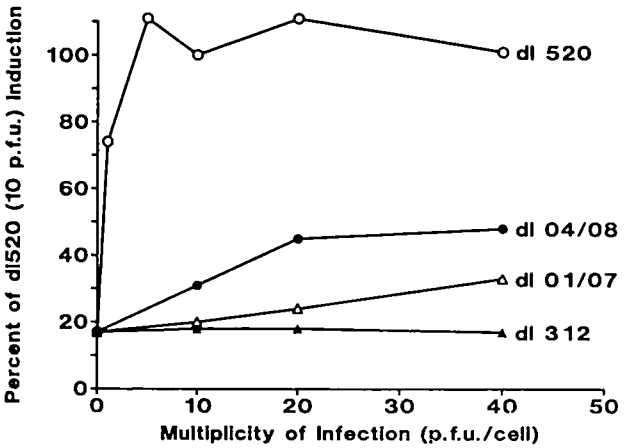

FIG. 4. Incorporation of [^{3}H]thymidine into DNA by E1A mutants in a dl520 background as a function of moi.

showed a moderate increase in induction to 20 pfu per cell. This mutant is discussed in the next section.

If the induction of cellular DNA synthesis is a consequence of the E1A protein binding to cellular proteins such as p105-Rb and p300, then, in a mixed infection the E1A product of a mutant such as dl02/08/520, which induces near wt levels of incorporation and binds p300 well, should predominate over the product of a mutant such as dl01/07/520, which is defective for induction and binds undetectable amounts of p105-Rb and p300. This was tested in an experiment in which BRK cells were infected with these two mutants. The incorporations obtained, as percentages of the level with dl520, were as follows: dl312, 25%; dl01/07/520 alone, 30%; dl02/08/520 alone, 110%; dl01/07/520 plus dl02/08/520, 120%. Thus the effect of dl02/08/520 predominated over that of dl01/07/520.

DISCUSSION

There are two significant findings in the results presented here. First, as others have found (14, 24), there exist E1A mutants such as dl1101, dl1103, dl1107, and dl1108 that in BRK cells are defective for transformation (9) but, nevertheless, induce significant levels of cellular DNA synthesis (Fig. 2). Clearly therefore, changes in the control mechanisms of the cell that permit cellular DNA synthesis are not in themselves sufficient to account for the uncontrolled continued growth associated with transformation.

The second finding is the close correlation between the induction of cellular DNA synthesis by E1A products and the ability of these products to form complexes with the cellular proteins p105-Rb and p300. Mutants that induced DNA synthesis at a level comparable to that observed with the wt 12S control virus dl520 (see Table 1) produced E1A proteins that bound readily detectable amounts of p105-Rb, p300, or both. On the other hand, mutants that were most deficient at inducing DNA synthesis—namely, dl01/07/520, dl01/08/520, and dl43/08/520—produced E1A proteins that bound undetectable or very low amounts of these two cellular proteins. The binding of E1A proteins to the cellular protein p107 did not correlate with the induction of DNA synthesis, and so this binding did not appear to be involved.

Mutants dl1143/520, dl03/08/520, and dl04/08/520 induced reduced levels of DNA synthesis. Of these, dl1143/520 and dl03/08/520 gave levels that correlated with the limited binding of their E1A proteins to both p105-Rb and p300 (Fig. 3). It is more difficult to account for the properties of dl04/08/520, which induced DNA synthesis better than expected from the very weak binding to p300 (Fig. 3). One

possibility is that the BRK equivalent to p300 binds more efficiently to this E1A product than does human p300. Alternatively, induction of DNA synthesis may be due to another, unidentified cellular protein with binding properties similar but not identical to those of p300 (see below).

A connection between the induction of DNA synthesis and binding to p105-Rb and p300 readily explains our results with double infections and with different values of moi. If binding of the cellular proteins to E1A products is responsible for inducing DNA synthesis, then, in an infection with two mutants, we would expect a mutant whose protein binds to predominate over one whose protein does not bind. This is what we found. In experiments on the induction of DNA synthesis at increasing values of moi (Fig. 4), induction by dl520 was not critically dependent on moi values above 5 pfu per cell. This suggests that if binding to p105-Rb and p300 is required for induction, the amount of E1A protein produced at these moi values is not limiting. However, if a mutant E1A protein were sufficiently weak at binding, then the amount of it produced in the cell could become limiting, and induction might then increase as the value of moi increased. This appeared to be the case with dl04/08/520, the E1A protein of which binds extremely weakly to p300.

These our results are consistent with the hypothesis that the binding of the E1A 243-residue product to p105-Rb and p300 is responsible for the induction of cellular DNA synthesis and that binding to either of these proteins is sufficient for this induction.

However, the evidence for this hypothesis is not conclusive. The present results suggest that binding of at least two cellular proteins to different sites on the 243-residue polypeptide, toward the N- and C-terminal ends of exon 1, respectively, is involved in the induction, but it is not certain that p105-Rb and p300 are these proteins. A variety of other cellular proteins besides p105-Rb, p107, and p300 can bind to the 243-residue E1A product (4, 5, 28), and any that bind to the same sites as p105-Rb and p300 could be important for induction. So far, we have not identified other cellular proteins with such binding properties.

How might E1A products affect regulatory processes in the cell? In the induction of DNA synthesis, one or both of the two cellular proteins could be inducers that are activated when the E1A product binds. Alternatively, one or both could be suppressors that are inactivated by binding the E1A product. If both are inducers, our data indicate that it is sufficient for the E1A protein to interact with either to induce synthesis. If p105-Rb is one of the proteins, it is likely to act as a suppressor. From studies on the phosphorylation of p105-Rb during the cell cycle (15–18), it has been proposed that this protein may help to block passage of cells from G_0/G_1 into S phase (16, 17) and that a viral protein like E1A may remove this block by binding to p105-Rb and inactivating it as a suppressor (16). Should one of the proteins be a suppressor and the other an inducer, induction by mutants, such as dl1101/520 and dl1107/520, requires that activation of the inducer and inactivation of the suppressor can each on their own lead to DNA synthesis. Finally, if both cellular proteins are suppressors, they must act cooperatively rather than in parallel, as these same mutants show that inactivation of either suppressor by binding to the E1A product is sufficient for DNA synthesis to occur.

Were p105-Rb and p300 to be the cellular proteins affected by E1A in the induction of cellular DNA synthesis and in cell transformation, several other questions follow. For example, it has been reported that the *Rb* gene is expressed in a wide variety of tissues (29). Is p300 equally ubiquitous? We would expect the induction of cellular DNA synthesis to be intimately associated with cell transformation. What is the relationship? Earlier work (8, 9) suggested that transformation required E1A proteins to bind to p105-Rb and p300. The evidence for this was that mutants such as dl1101, which failed to bind to p300, and dl1107, which failed to bind to p105-Rb, were defective for transformation in combination with *ras*. Thus to permit uncontrolled growth associated with transformation, the E1A protein must bind to p105-Rb and p300 whereas, to induce DNA synthesis, it must bind to either one. This would suggest that the pathways by which p105-Rb and p300 participate in the control of cell growth are more complex than those that control the onset of DNA synthesis and that in the latter case, the two proteins provide alternative means by which DNA synthesis can be induced. It is already known that sequences in exon 1 of E1A also affect mitosis and passage of cells through the cell cycle (23, 30, 31). Our E1A mutants can be used to examine the relationship of these processes to the binding of p105-Rb and p300 and to explore some of the other questions raised here.

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My Contributions to Howe et al., 1990

1) Construction of the exon 1 dl 520 series of mutant viruses by transferring plasmid sequences into dl 309 using the technique of McGrory et al. (1988).

2) Worked with Joe Mymryk to construct the dl 520 series of double E1A mutants.

3) Used a published assay to measure levels of DNA synthesis induced by the single and double E1A mutant virus series.

4) Measured dl 520 viral DNA synthesis in BRK cells.

In Howe et al. (1990) we found that all of the mutants with small deletions in exon 1, and pm 1131/520 which deletes almost all of exon 2, induced levels of cellular DNA synthesis comparable to dl 520, but dl 1143/520, a mutant with most of CR1 deleted, was defective. Dl 1143/520 was found to associate poorly with both pRb and p300 and was different from any of our small deletions which were able to bind efficiently to at least one of these cellular proteins. We therefore made a series of double exon 1 mutants by combining existing small deletions in exon 1, and tested them for their ability to induce cellular DNA

synthesis and to associate with cellular proteins. Double mutants which failed to associate with pRb and p300 were completely defective for stimulation of DNA synthesis, while double mutants which retained the ability to bind to one or both of pRb and p300 were able to induce at wild type levels. These results suggested that the binding of the 243R protein to pRb and p300 is responsible for induction of cellular DNA synthesis and that binding to either of these proteins is sufficient for this induction.

Although all of the exon 1 series of E1A mutants retained the ability to stimulate cellular DNA synthesis a number of them were previously found to be defective for transformation (Jelsma et al., 1989). Therefore stimulation of cellular DNA synthesis by E1A is not, in itself, sufficient for transformation and additional E1A functions, such as their ability to induce mitosis (Moran and Zerler, 1988) and cell proliferation (Quinlan and Grodzicker, 1987), may also be required. In our next series of experiments we further characterized the cell cycle progression in BRK cells infected with our E1A mutant viruses.

5) **Effects of Ad5 E1A Mutant Viruses on the Cell Cycle in Relation to the Binding of Cellular Proteins Including the Retinoblastoma Protein and Cyclin A (Howe and Bayley., 1992, Virology 186, 15-24)**

This report describes the use of the dl 520 series of mutant viruses to: 1) map domains in the 243R protein for stimulation of cell division in BRK cells, 2) map binding sites on the 243R E1A product for association with the cellular proteins p400, p300, p107, pRb and cyclin A.

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Effects of Ad5 E1A Mutant Viruses on the Cell Cycle in Relation to the Binding of Cellular Proteins Including the Retinoblastoma Protein and Cyclin A

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We have examined the ability of Ad5 E1A 12S viruses with deletions in E1A exon 1 to induce quiescent baby rat kidney cells to progress through the cell cycle and to undergo mitosis. Measurements of mitotic index and analyses by fluorescence activated cell sorting were correlated with the abilities of the mutant E1A proteins to bind to cellular proteins. All the mutants induced cells to leave G0/G1 and enter S phase, but two groups were defective at inducing mitosis and cells infected with them appeared to be blocked between the S and M phases. The first group of mutants, with deletions in the regions of residues 4–25 and 30–60, bound p300 poorly or not at all and gave reduced numbers of mitoses. The second group, with deletions between residues 111 and 138 in CR2, failed to bind pRb and were completely defective at inducing mitosis. In this group, mutants lacking residues between 124 and 138 bound p107 and cyclin A at much reduced levels and induced cells to overreplicate their DNA. The site in E1A required to bind cyclin A extends from residue 124 to at least 127. Cyclin A binds to a 107-kDa cellular protein, which by peptide analysis appears identical to p107.

INTRODUCTION

In the transformation of cells by DNA tumor viruses, the role of at least some of the viral oncogenes appears to be to synthesize products that interfere with cellular proteins controlling growth. An important line of evidence supporting this view is that the E1A proteins of human adenovirus type 5 (Ad5), large T antigens of SV40 and polyoma viruses, and the E7 protein of human papilloma virus 16, all bind to pRb, the 105-kDa protein product of the retinoblastoma growth suppressor gene (Yee and Branton, 1985; Harlow *et al.*, 1986; Whyte *et al.*, 1988b; Egan *et al.*, 1988, 1989; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989, 1990). Furthermore, mutant E1A, SV40 large T antigen, and E7 proteins that fail to bind pRb are defective for transformation (Egan *et al.*, 1988, 1989; Jelsma *et al.*, 1989; Whyte *et al.*, 1989; DeCaprio *et al.*, 1988; Banks *et al.*, 1990). It has been suggested that pRb controls the exit of cells from G0/G1 into S phase of the cell cycle, and that proteins of viral oncogenes remove such a control by binding to pRb (Whyte *et al.*, 1988b; Buchkovich *et al.*, 1989; DeCaprio *et al.*, 1989). So far, no direct connection has been established between the formation of such protein complexes and changes in cell growth, either for pRb or for another cellular protein, p300, that is bound by E1A proteins and that has also been implicated in the regulation of cell growth and in transformation (Whyte *et al.*, 1988a, 1989; Egan *et al.*, 1988; Jelsma *et al.*, 1989; Stein *et al.*, 1990). Nevertheless studies of the effects on cells of oncogenes such as E1A are likely to yield important information on the molecular mechanisms controlling cell growth.

The contribution of the Ad5 E1A oncogene in cell transformation is apparently to "immortalize" cells, that is to induce unlimited growth and division (Ruley, 1983). Aside from this, E1A has a range of other biological activities as well (Berk, 1986). These include the ability to activate transcription of some viral and cellular genes in *trans,* to repress some viral and cellular transcriptional enhancers, and to induce mitosis and cellular DNA synthesis in growth-arrested primary rodent cells. The principal products of the E1A gene are proteins of 243 and 289 residues, which differ only by an internal sequence of 46 amino acids in the larger. From the predicted sequences of E1A proteins, three regions have been found to be highly conserved between different adenovirus serotypes (van Ormondt *et al.*, 1980; Kimelman *et al.*, 1985), of which two, conserved regions (CR) 1 and 2 (Fig. 1) are pertinent here. To learn more about the molecular mechanisms underlying E1A activities, a number of laboratories, including our own, have used site-directed mutagenesis to map functional domains within the E1A proteins. The results have shown that the N-terminal end of the protein and the N-terminal end of CR1 are required for transformation in cooperation with *ras,* for enhancer repression, and for binding to p300 (Lillie *et al.*, 1987; Schneider *et al.*, 1987; Whyte *et al.*, 1988a, 1989; Egan *et al.*, 1988; Velcich and Ziff, 1988; Subramanian

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et al., 1988; Jelsma *et al.*, 1989), while CR2 is necessary for transformation and for binding to pRb and to another cellular protein, p107 (Moran *et al.*, 1986; Lillie *et al.*, 1986, 1987; Kuppuswamy and Chinnadurai, 1987; Schneider *et al.*, 1987; Egan *et al.*, 1988; Jelsma *et al.*, 1989; Whyte *et al.*, 1988a, 1989). In our laboratory, the E1A mutant proteins that failed to bind to either p300 or pRb and were defective for transformation still induced cellular DNA synthesis in quiescent baby rat kidney (BRK) cells; only mutant proteins that bound to neither of these cellular proteins were defective for this induction (Howe *et al.*, 1990). These results suggest that immortalization of cells is separable from the induction of cellular DNA synthesis, and that the controls on cell growth are more complex than those for DNA synthesis (Howe *et al.*, 1990). To investigate cell growth further, we have now extended our studies on quiescent BRK cells infected with Ad5 E1A mutants to the induction of mitosis and passage through the cell cycle, and we have related the properties of these mutants to the ability of their E1A proteins to bind to different cellular proteins.

MATERIALS AND METHODS

Cells and viruses

The preparation of BRK cells and the culture of these and of 293 cells were as described previously (Howe *et al.*, 1990). The construction of Ad5 E1A mutants *dl* 1101–*dl* 1109 and their subsequent reconstruction into Ad5 virus *dl* 520, which produces only 12S E1A mRNA, have been described (Jelsma *et al.*, 1988, 1989; Howe *et al.*, 1990). All viruses were plaque purified twice and propagated on 293 cells.

Measurement of mitotic index

The method for measuring mitotic index was essentially that of Moran and Zerler (1988). Duplicate sets of BRK cells grown on coverslips were infected at a multiplicity of 10 plaque forming units (PFU)/cell 3 days after plating, and at the time of infection or 24 hr later, nocodazole was added to a final concentration of 1 μM. After 24 hr in the presence of the drug, infected cells were rinsed with PBS at 4° and treated with Carnoy's fixative (3:1 ethanol and acetic acid) for 20 min at −20°. After air drying, the cells were stained with propidium iodide (5 μg/ml) in a solution containing 38 m*M* sodium citrate, pH 7, and 100 μg/ml RNase A. The mitotic index was then determined on batches of at least 300 cells using a fluorescence microscope.

Flow cytometry

Triplicate 60-mm dishes of confluent monolayers of BRK cells were infected at a multiplicity of 10 PFU/cell 3 days after plating. At 24, 48, and 72 hr postinfection (p.i.), cells were rinsed twice with PBS, trypsinized, and then resuspended in PBS$^+$ (0.1% glucose, 0.3 m*M* EDTA, and 1% fetal bovine serum). The cells were passed through a $25\frac{5}{8}$-gauge needle to break up clumps, and were then fixed in ethanol and stained with propidium iodide according to the procedure of Morris and Matthews (1989). Quantitative fluorescence analysis was carried out on batches of about 20,000 cells with a Coulter Epics 5.

Analysis of immunoprecipitates from cell lysates

HeLa cells in 100-mm dishes were either mock-infected or infected with virus at a multiplicity of 75 PFU/cell. They were labeled with 200 μCi tran ^{35}S-label (ICN Biomedicals, St. Laurent, Quebec, containing [^{35}S]-methionine and [^{35}S]cysteine) from 12 to 16 hr p.i., and were then lysed in 200 μl Buffer X (100 m*M* Tris, pH 8.5, 250 m*M* NaCl, 1 m*M* EDTA, 10% (vol/vol) glycerol, 1% (vol/vol) Nonidet P-40, and 2 mg/ml bovine serum albumin). After clarification, the lysates were immunoprecipitated by adding the antibody or antiserum and incubating on ice for 20 min. One hundred microliters of Protein-A Sepharose CL-4B beads (3% wt/vol) was added, the beads were washed extensively with buffer X, and the immunoprecipitates were analyzed on 8.5% SDS–polacrylamide gels followed by fluorography. Immunoprecipitations were performed with: anti-E1A monoclonal antibody M73 and anti-E1B 55-kDa antibody 9C10 (both from Oncogene Science, Manhasset, NY); polyclonal rabbit antisera directed against either human cyclin A or human cyclin B (from J. Pines, San Diego, CA); a monoclonal antibody, C160, against cyclin A (from P. Whyte, Hamilton, Ont.); and a rabbit antiserum to a peptide of p34^{cdc2} (from J. Th'ng, Davis, CA).

Partial proteolytic peptide mapping

^{35}S-labeled proteins from gels were partially digested with V8 protease, and the digests were analyzed by gel electrophoresis as described (Harlow and Lane, 1988).

RESULTS

Stimulation of mitosis by E1A

The ability of E1A to induce mitosis in growth-arrested cells cultured from rat embryos or baby rats has been described previously (Bellet *et al.*, 1985, 1989; Braithwaite *et al.*, 1983; Moran and Zerler, 1988; Zerler *et al.*, 1987). E1A mutants have been used to define the regions of E1A proteins that are required for this induction. It was reported that CR2 is necessary

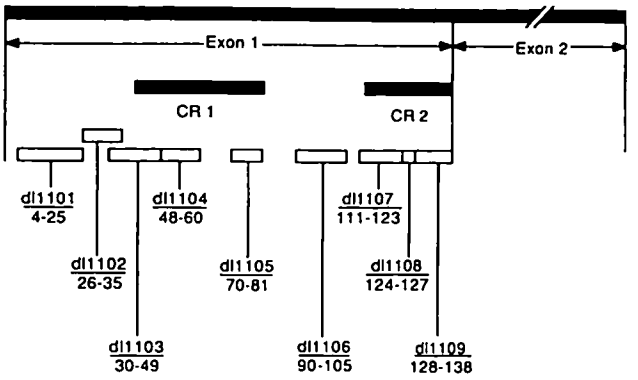

FIG. 1. Map of the Ad5 E1A 243 residue protein, showing the positions of conserved regions 1 and 2 (CR1, CR2), and the positions of the deletion mutations used in the present study, with the residues deleted.

(Zerler *et al.*, 1987), but this was not confirmed in later work (Bellet *et al.*, 1989).

To investigate further the importance of sequences in exon 1 of E1A proteins in the induction of mitosis, we studied quiescent BRK cells infected at a multiplicity of 10 PFU/cell with a series of mutant Ad5 viruses containing deletions in exon 1 of E1A. These deletions, *dl* 1101 to *dl* 1109, are shown in Fig. 1. To reduce viral replication and the cell killing that results, these exon 1 mutations were used in an Ad5 *dl* 520 background, so that only the smaller 12S E1A mRNA and its protein product were synthesized (Haley *et al.*, 1984; Howe *et al.*, 1990). These mutant viruses were designated *dl* 1101/520–*dl* 1109/520. The percentage of infected cells entering mitosis was measured by blocking cycling cells in a metaphase-like state by adding the microtubule polymerization inhibitor nocodazole for either 0–24 or 24–48 hr postinfection (p.i.). Measurements were not made later than 48 hr, because at these times dead cells confused the counting of mitoses. In five experiments, the average percentage of cells entering mitosis, summed over the whole 48-hr period, was 38 ± 12 in *dl* 520-infected cells, and 4 ± 3 in cells infected with *dl* 312, a virus lacking essentially all of E1A. Mock-infected cells gave a value similar to that for infection with *dl* 312. In these experiments, a multiplicity of infection of 10 PFU/cell was found to be optimal: at 1 PFU/cell, mutant viruses defective at inducing mitosis (see below) gave too few mitoses for accurate counts, while at 30 PFU/cell, the viruses were lethal to the cells.

To compare mutants *dl* 1101/520–*dl* 1109/520 with *dl* 312 and *dl* 520, the mitotic indices of infected cells were summed over the two 24-hr periods of treatment with nocodazole, expressed as a percentage of the index for *dl* 520, and then averaged over several experiments. These averages are shown in Fig. 2. It is clear that induction of mitosis by mutants *dl* 1101/520, *dl* 1103/520, and *dl* 1104/520 was significantly reduced, while mutants *dl* 1107/520, *dl* 1108/520, and *dl* 1109/520 were as ineffective at inducing mitosis as *dl* 312. By contrast, induction was affected relatively little in *dl* 1102/520, *dl* 1105/520, and *dl* 1106/520. Mitotic indices in individual experiments (not given) showed that *dl* 520, *dl* 1102/520, *dl* 1105/520, and *dl* 1106/520 produced most of the mitoses in the first 24 hr p.i., with 30% or less of this number in the second 24 hr p.i. For *dl* 1101/520, *dl* 1103/520, and *dl* 1104/520, all mitoses occurred in the first 24 hr p.i.

FACS analysis of cells infected with E1A mutants

In a previous study (Howe *et al.*, 1990), we found that each of the mutant viruses *dl* 1101/520–*dl* 1109/520 retained the ability to stimulate cellular DNA synthesis efficiently in quiescent BRK cells. Because not all of these viruses were able to induce BRK cells to proceed through mitosis, it was clear that other E1A functions beyond those required to stimulate DNA synthesis are needed to drive infected cells to complete the cell cycle. In an attempt to learn more about the effect of E1A on progress through the cycle, we used fluorescence activated cell sorting (FACS) to analyze BRK cells infected with viruses containing E1A exon 1 mutations.

For FACS analysis, infected BRK cells were fixed and stained with propidium iodide at 24, 48, and 72 hr p.i. Representative FACS profiles are shown in Fig. 3. Except for one feature discussed below, the profiles for cells infected with *dl* 520 or with mutants *dl* 1101/520–*dl* 1109/520 (Figs. 3B–3F) were generally similar and

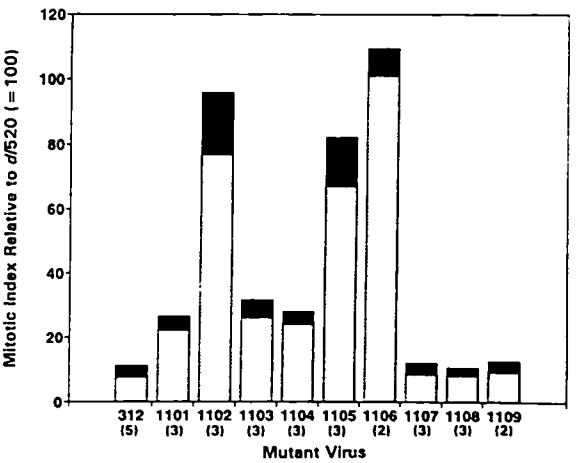

FIG. 2. Histograms of the mitotic index for BRK cells infected with different E1A mutant viruses, expressed as a percentage of that for cells infected with *dl* 520 (+SD for the numbers of experiments given in parentheses).

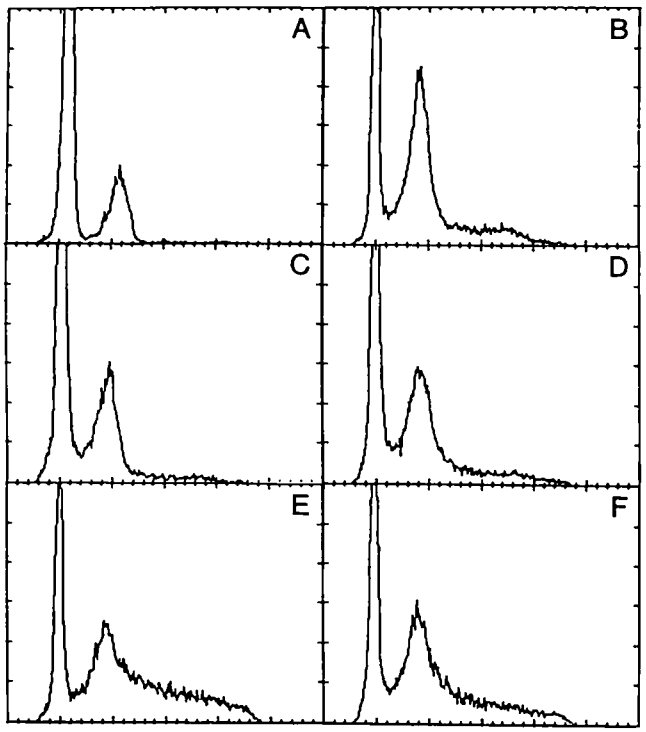

FIG. 3. FACS profiles from one experiment of BRK cells at 72 hr after infection with different E1A mutant viruses. (A) Mock-infected and infected with (B) *dl* 520; (C) *dl* 1103/520, (D) *dl* 1107/520; (E) *dl* 1108/520; (F) *dl* 1109/520.

contained a higher percentage of cells in S and G2/M than were found in mock infected cells (Fig. 3A). The percentages of cells in S and G2/M at the three 24 hr times after infection with different mutant viruses and averaged over a number of FACS analyses are shown in Fig. 4A. It is evident that all of the mutants were capable of inducing cells to leave G0/G1 and enter S phase, and this is consistent with our earlier observation that all of these mutants are able to induce cellular DNA synthesis (Howe *et al.*, 1990). A significant difference between the FACS profiles was that *dl* 1108/520 (Fig. 3E), *dl* 1109/520 (Fig. 3F), and possibly to a small extent *dl* 1107/520 (Fig. 3D), gave a higher proportion of cells with DNA contents greater than G2/M than did *dl* 520 (Fig. 3B) and the other mutants (Fig. 3C). These higher DNA contents varied in amount and were not confined to one discrete value. Histograms for the percentages of such cells for all of the mutant viruses are given in Fig. 4B.

To investigate further the overreplication of DNA caused by these mutants, FACS analyses were carried out to compare cells infected with *dl* 520, *dl* 1107/520, *dl* 1108/520, and *dl* 312 (or mock-infected). In each case, the number of cells with DNA contents >G2/M was calculated as a percentage of cells in S, G2/M, and >G2/M. Table 1 shows the results of three experiments for cells at 48 and 72 hr p.i., the times at which the effects were most marked. It is clear that the percentage of cells overreplicating their DNA was significantly higher with *dl* 1108/520 than with *wt* 12S virus, *dl* 520. With *dl* 1107/520, the percentage was greater than for *dl* 520, but the difference was barely significant, statistically.

Binding of mutant E1A proteins to cellular proteins

In previous studies on the binding of mutant E1A proteins to the cellular proteins p300, p107, and pRb, we used either single E1A mutations in a background of *dl* 309, a phenotypically *wt* virus which produces both 13S and 12S E1A mRNAs (Egan *et al.*, 1988), or double E1A mutations in a *dl* 520 background (Howe *et al.*, 1990), all of which differ from the mutants used here. To check the binding properties of the E1A proteins from the present mutants, we analyzed immunoprecipitates of these E1A proteins for the presence of p300, p107, and pRb (Fig. 5). Because of the importance of cyclins A and B in the cell cycle, we analyzed the immunoprecipitates for the presence of these proteins as well: cyclin A has been reported to bind to E1A proteins (Pines and Hunter, 1990; see also Giordano *et al.*, 1989), but there is no evidence that cyclin B does.

Significant amounts of E1A proteins were immunoprecipitated with the E1A-specific M73 antiserum from labeled cells infected with each of the mutant viruses except *dl* 1109/520 (Fig. 5). Because this mutant consistently produced much lower levels of E1A protein, it was impossible to compare it with the other mutants in the efficiency of its E1A protein to bind cellular proteins. Relative to *dl* 520 (Fig. 5, lanes 5, 13), binding of E1A to p300 was similar in *dl* 1102/520 (Fig. 5, lane 14), *dl* 1105/520 (Fig. 5, lane 15), *dl* 1106/520, *dl* 1107/520, and *dl* 1108/520 (Fig. 5, lanes 9–11), considerably reduced in *dl* 1103/520 (Fig. 5, lane 7), and negligible in *dl* 1101/520 (Fig. 5, lane 6) and *dl* 1104/520 (Fig. 5, lane 8). In the same region of the gel as p300, another protein, first observed by C. Egan and P. E. Branton (unpublished) and designated p400, was bound to proteins from *dl* 520 (Fig. 5, lanes 5, 13) and *dl* 1104/520–*dl* 1108/520 (Fig. 5, lanes 8–11, 15), but not from *dl* 1101/520–*dl* 1103/520 (Fig. 5, lanes 6, 7, 14). E1A proteins from all the mutants bound p107, except that the level was much reduced with *dl* 1108/520 (Fig. 5, lane 11). Binding of pRb with *dl* 1101/520, *dl* 1102/520, *dl* 1105/520, and *dl* 1106/520 was comparable to that with *dl* 520, but it was reduced in *dl* 1103/520 and *dl* 1104/520 (Fig. 5, lanes 7, 8), and it was negligible in *dl* 1107/520 and *dl* 1108/520 (Fig. 5,

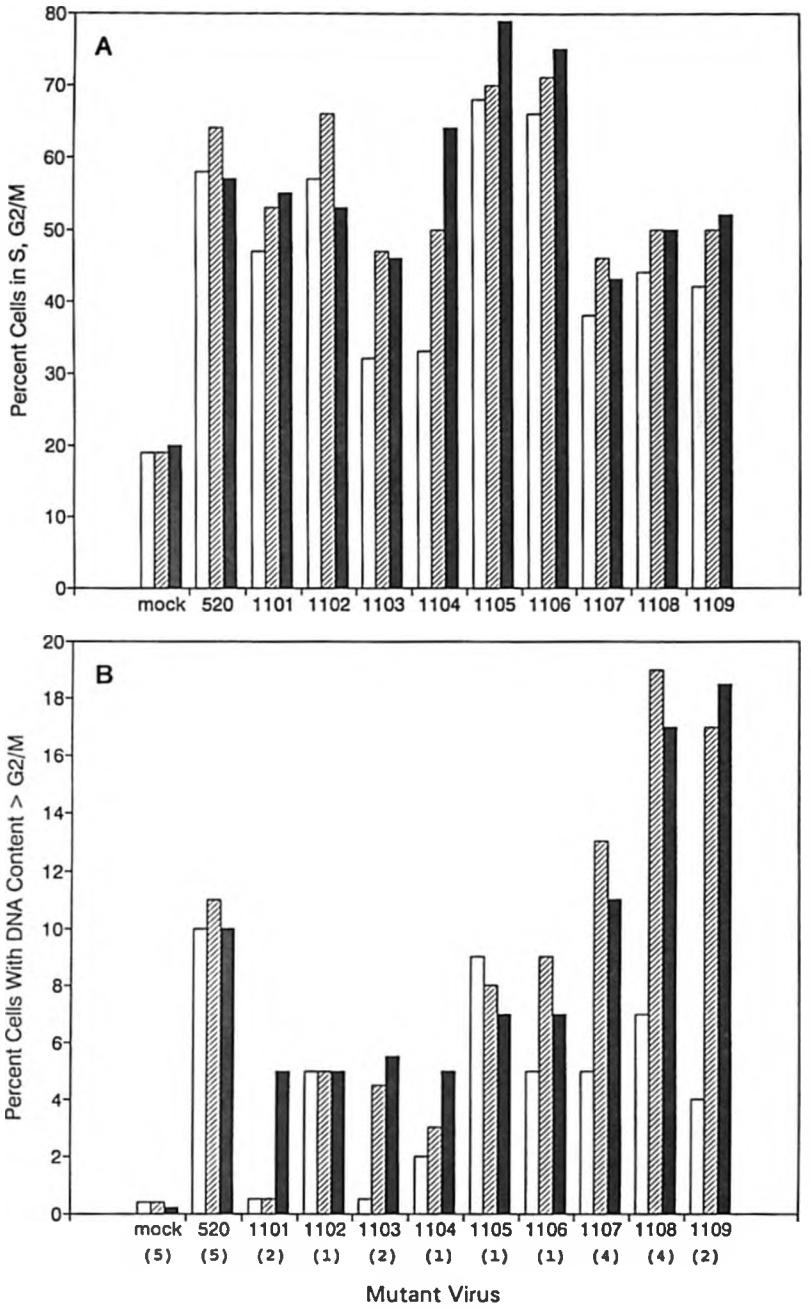

FIG. 4. Histograms of FACS analyses of cells at 24, 48, and 72 hr after infection with different E1A mutant viruses. The average percentages of cells (A) in S and G2/M, (B) with DNA contents greater than that for G2/M are shown from a range of experiments involving different groups of mutants. The numbers of experiments are given in parentheses.

lanes 10, 11). Controls with *dl* 312, which lacks E1A (Fig. 5, lane 1), and with an antibody to Ad5 E1B 55-kDa protein (lane 4), showed that the bands described in these immunoprecipitates were specific to the presence of E1A proteins.

Extracts of mock-infected cells (Fig. 5, lane 3) and cells infected with *dl* 312 (Fig. 5, lane 20) or *dl* 520 (Fig. 5, lane 21) immunoprecipitated with cyclin A antiserum showed two prominent bands, one of 60 kDa that we assumed represented native cyclin A (Pines and Hunter, 1990), and another of about 50 kDa. Relative to the 60-kDa band, the amount of 50 kDa varied between experiments. This 50-kDa protein was recognized weakly by cyclin A antiserum in Western blots,

TABLE 1

FACS ANALYSES FOR THE PERCENTAGE OF CYCLING CELLS WITH DNA CONTENTS >G2/M

Infecting virus	Percentage >G2/M[a] 48 hr p.i.	72 hr p.i.
dl 312 and mock	3 ± 3	2 ± 1
dl 520	17 ± 1	16 ± 5
dl 1107/520	22 ± 2	20 ± 4
dl 1108/520	31 ± 3	27 ± 4

[a] Calculated as number of cells in >G2/M × 100/total number of cells in S, G2/M, and >G2/M. Values shown are means ± SD for three separate experiments.

and analyses of V8 protease digests indicated that it shared some peptides with cyclin A. These results (not shown) suggest it may be related to cyclin A, but we have not investigated it further. Bands that comigrated with these two were present at significant intensities in samples from cells infected with all the mutants except *dl* 1108/520, where they were negligible (Fig. 5, lane 11). This suggests that the main region of E1A required for binding to cyclin A involves residues 124–127 deleted in *dl* 1108/520. Because of the low level of E1A protein produced by *dl* 1109/520, it was impossible to tell whether residues deleted in this mutant are required as well. In addition to 60- and 50-kDa bands, immunoprecipitates obtained with cyclin A antiserum revealed other components. Among these were bands of about 30–35 kDa (Fig. 5, lanes 20, 21; in Fig. 5, lane 3, these bands have run out of the gel), which may represent different forms of $p34^{cdc2}$ or a related protein, p33, reported to associate with cyclin A (Giordano *et al.*, 1989; Pines and Hunter, 1990). The strongest of these bands migrated slightly ahead of the strongest of several bands precipitated by anti-$p34^{cdc2}$ serum, which were associated with cyclin B (Fig. 5, lanes 18, 19).

Immunoprecipitates obtained with cyclin A antiserum from mock-infected cells (Fig. 5, lane 3) and from cells infected with *dl* 520 (Fig. 5, lane 17) showed a band comigrating with p107 observed in extracts of infected cells immunoprecipitated with M73 antiserum (see particularly Fig. 5, lanes 16, 17). A 107-kDa band

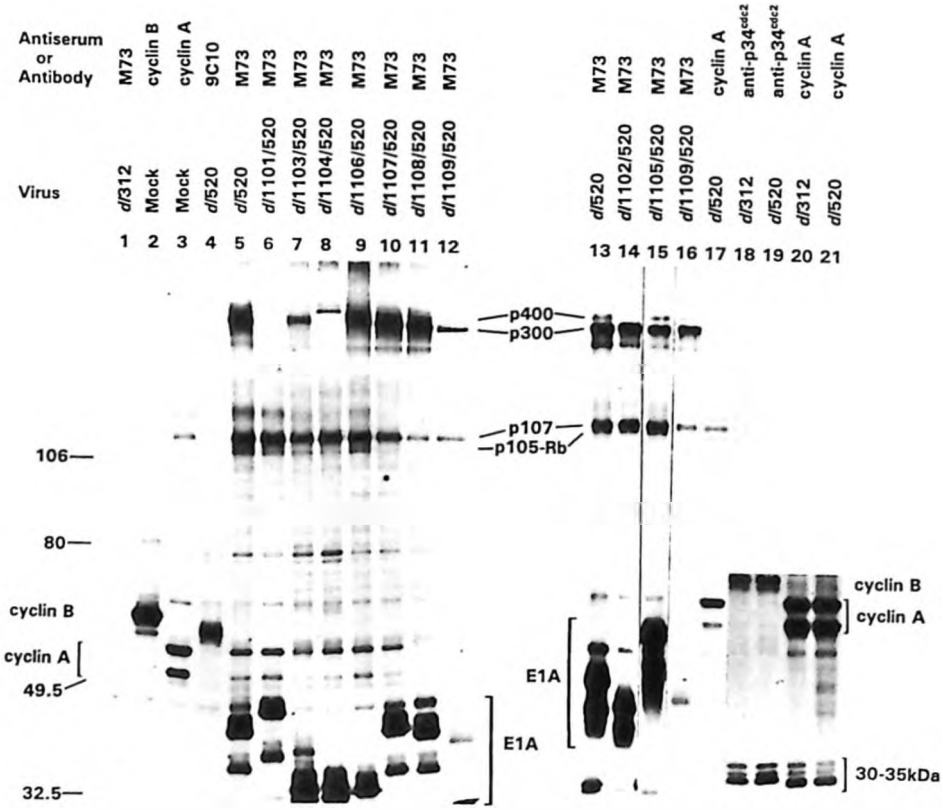

FIG. 5. Fluorographs of SDS–polyacrylamide gels of immunoprecipitates from [^{35}S]-labeled HeLa cells, mock-infected or infected with different mutant viruses. The infecting virus and the antiserum or antibody used are indicated for each lane. The positions of molecular mass markers in kDa are indicated by the numbers on the left.

FIG. 6. V8 protease digests after gel electrophoresis. Lanes 1–3, p107 digested with 0.1, 0.5, and 1.0 μg V8 protease, respectively; lanes 4–6, 107 kDa from cells infected with *dl* 520 digested with 0.1, 0.5, and 1.0 μg protease

was also obtained with C160 monoclonal anti-cyclin A serum (Giordano *et al.*, 1989) in place of the polyclonal antiserum used in Fig. 5. The polyclonal antiserum did not recognize the 107-kDa protein from either infected or uninfected cells in Western blots (not shown), and so probably had not precipitated it directly. Fig. 6 shows V8 protease digests analysed by gel electrophoresis of p107 obtained by immunoprecipitating an extract of cells infected with *dl* 520 using anti-E1A serum M73 (as in Fig. 5, lane 9) compared to similar digests of the 107-kDa protein obtained from cells infected with *dl* 520 using the polyclonal anti-cyclin A serum (as in Fig. 5, lane 17). The results suggest that the two proteins are the same. In our experience, the amount of 107 kDa in anti-cyclin A immunoprecipitates of uninfected cells (as in Fig. 5, lane 3) has been consistently less than that obtained with infected cells (we are investigating this difference further). To enable us to analyze 107 kDa from uninfected cells, we phosphorylated immunoprecipitates on protein A-Sepharose beads using the p33-p34 kinase associated with cyclin A (Fig. 5, lanes 20, 21). Comparison of tryptic and V8 digests of phosphorylated samples showed that the 107 kDa immunoprecipitated with cyclin A from uninfected cells was the same as p107 (Whyte, Howe, and Bayley, to be published). These results suggest therefore that cyclin A may bind to p107 in both infected and uninfected cells. As a consequence, it is impossible to tell from extracts of infected cells immunoprecipitated with M73 antiserum whether cyclin A bound to E1A protein directly or via p107. None of the bands in infected cell extracts immunoprecipitated with M73 corresponded to those observed in uninfected extracts immunoprecipitated with cyclin B antiserum (Fig. 5, lane 2).

The results described above for all the exon 1 mutants are summarized in Table 2.

DISCUSSION

In this work, all the E1A mutations studied were in a background of Ad5 *dl* 520 virus. This virus produces a *wt* 12 S mRNA and its protein product, but no 13 S mRNA (Haley *et al.*, 1984; Howe *et al.*, 1990). Measurements of mitotic index and DNA content on quiescent BRK cells infected with *dl* 520 itself showed that 80% of the cells reached S and G2/M (Fig. 4A), but less than 40% underwent mitosis. This suggests that under the influence of *wt* protein from 12 S E1A mRNA, the majority of cells were able to enter the cell cycle from G0/G1 and traverse S phase, but that passage through mitosis was somewhat restricted.

Measurements of mitotic index and DNA content on cells infected with viruses mutated in exon 1 of E1A (Fig. 1) showed that all the mutants caused a majority of cells to leave G0/G1 and enter S (Fig. 4A), but that compared to *dl* 520, two separate groups of these mutants were defective at inducing cells to enter mitosis (Fig. 2). The first group, comprising *dl* 1101/520 at the N-terminal end of the E1A protein, and *dl* 1103/520 and *dl* 1104/520 in the N-terminal half of CR1, were only partially defective. This suggests that cells infected with these mutants may have encountered a greater barrier to passage from G2 into M than did cells infected with *dl* 520. The second group, *dl* 1107/520–*dl* 1109/520 in CR2, were completely defective at causing cells to enter mitosis (Fig. 2), confirming earlier observations that CR2 is essential for the induction of mitosis (Zerler *et al.*, 1987). As none of the cells infected with these mutants reached mitosis in the 48-hr period of our experiments, they must have encountered a severe block between S and the G2/M border. Within this second group of mutants, *dl* 1108/520 and *dl* 1109/520 caused an appreciable number of cells to accumulate DNA contents greater than that for G2/M (Fig. 4B). As a percentage of cells entering the cell cycle, this was significantly greater in *dl* 1108/520 than in *dl* 520 (Table 1). Similar results to these for *dl* 1108/520 and *dl* 1109/520 have been reported by Moran and Zerler (1988) for a mutant virus, CXdl, with the whole of CR2 deleted.

What relation have these results to the ability of mutant E1A proteins to bind to particular cellular proteins? BRK cells were induced to enter S phase by, for exam-

TABLE 2

SUMMARY OF ABILITIES OF E1A EXON 1 MUTANTS TO INDUCE PROGRESSION THROUGH THE CELL CYCLE AND TO BIND TO CELLULAR PROTEINS

	Induction of			Binding[b] to				
	Entry into S	Entry into M[a]	Overreplication of DNA	p400	p300	pRb	p107	Cyclin A
dl 520	+	+		+	+	+	+	+
dl 1101/520	+	↓		−	−	+	+	+
dl 1102/520	+	+		−	+	+	+	+
dl 1103/520	+	↓		−	↓	+	+	+
dl 1104/520	+	↓		+	−	+	+	+
dl 1105/520	+	+		+	+	+	+	+
dl 1106/520	+	+		+	+	+	+	+
dl 1107/520	+	−	+/−	+	+	−	+	+
dl 1108/520	+	−	+	+	+	−	↓	−
dl 1109/520	+	−	+					

[a] Mitotic index: + similar to *dl* 520; ↓ markedly less than with *dl* 520; − same as mock infection or infection with *dl* 312.

[b] Binding: + significant; ↓ markedly less than in *dl* 520; − not detectable. No results are shown for *dl* 1109/520 for reasons discussed in the text.

ple, mutants *dl* 1101/520 and *dl* 1108/520, that bound to only one of the two cellular proteins, p300 and pRb. Thus, as our earlier results indicated (Howe *et al.*, 1990), neither of these proteins on their own can be responsible for preventing BRK cells from leaving G0/G1: both must be uncomplexed to E1A in the cell. Mutants *dl* 1101/520, *dl* 1103/520, and *dl* 1104/520 bound p300 at much reduced or undetectable levels (Fig. 5, lanes 6–8) and caused only a small percentage of cells to enter mitosis. This correlation suggests that p300 may affect passage of cells from S into M. Cells infected with *dl* 1107/520 and *dl* 1108/520 were blocked completely before M, and as neither of these mutants bound pRb (Fig. 5, lanes 10, 11), this protein may be responsible for the block. *dl* 1108/520 also showed much reduced binding to p107 and cyclin A, and it induced a significant increase in the number of cells accumulating excess DNA (Table 1). This may mean that p107 and/or cyclin A in some way facilitate the overreplication of DNA, although as *dl* 1107/520 caused a small, marginally significant increase as well, a role for pRb in overreplication cannot be excluded.

As *dl* 1109/520 consistently produced much less E1A protein than the other mutants (Fig. 5), it was impossible to compare this protein with those of the other mutants in the efficiencies with which it formed complexes with cellular proteins. Despite the small amount of E1A protein from *dl* 1109/520, the effect of this mutant on cells was similar to that of *dl* 1108/520, and this similarity can be explained in terms of the amounts of cellular proteins bound. The E1A proteins from both mutants bound to pRb, p107, and cyclin A at comparably low levels (Fig. 5, lanes 11, 12), and it is this weak binding in both cases that we suggest may account for the failure to induce cells to enter mitosis and for the induction of excess DNA synthesis. However, the reasons for the weak binding were different in the two mutants: *dl* 1108/520 produced normal levels of E1A protein that bound inefficiently, whereas at the same multiplicity of infection, *dl* 1109/520 produced too little E1A protein for significant binding, however efficient it may have been intrinsically in binding to these proteins. It is more difficult to explain how *dl* 1109/520 induced cells to progress from G0/G1 into S in the first place. At the multiplicity of infection used, this mutant complexed a detectable amount of p300 (Fig. 5, lane 12), and although this amount was smaller than in other mutants, it may have been sufficient to allow cells to escape from G0/G1 into S phase. None of the phenotypes observed here correlated with binding to p400.

The abilities of mutant forms of the 243 residue E1A protein to bind to p60-cyclin A (Fig. 5) showed that the principal binding site includes some or all of residues 124–127 deleted in *dl* 1108/520. We could not judge whether the site extends into the neighbouring deletion of *dl* 1109/520, because of the low level of E1A protein produced by that mutant. This site is smaller than, but consistent with, residues 121–127, one of two sites for binding to cyclin A recently reported by Giordano *et al.* (1991). Based largely on the failure of a mutant with residues 30–85 deleted to bind to cyclin A, these authors concluded that residues 30–60 are also required. In contrast, our two smaller deletions spanning this region, *dl* 1103/520 and *dl* 1104/520, bound cyclin A reasonably well. Evidently binding to cyclin A is

affected only by relatively large deletions between residues 30–60, as we recently demonstrated to be the case in the binding of E1A to pRb (Howe *et al.*, 1990).

Much more information is required on the interactions of E1A with cellular proteins before we can properly understand how E1A affects cells. Nevertheless a picture is beginning to emerge, and in this respect the work of Wang *et al.* (1991) nicely complements our present and previous results (Howe *et al.*, 1990). Wang *et al.* (1991) showed that, probably by inducing $p34^{cdc2}$ kinase activity, the 12S E1A protein can induce the phosphorylation of pRb in quiescent BRK cells without necessarily binding to it. As a consequence of this phosphorylation, the cells can enter the cell cycle. Only double E1A mutants that failed to bind both p300 and pRb were ineffective at inducing the kinase activity. These results help to explain our observations here and previously (Howe *et al.*, 1990) on the induction of entry of quiescent cells into S phase by E1A. Our present work adds to the picture by showing that both p300 and pRb act later in the cell cycle as well, and that E1A must bind to these two proteins to abrogate their effects in restricting progress through the cycle.

Information is accumulating on the role of cyclin A in the cell cycle. Important functions for both cyclin A and cyclin B are to bind to and activate p34 and related histone H1 kinases (see e.g., Lewin, 1990). D'Urso *et al.* (1990) have shown that in an *in vitro* system, these kinases are necessary to activate DNA synthesis. The cyclin–kinase complex that is responsible for this activation *in vivo* has not been identified, but it is more likely to be the one associated with cyclin A as this kinase activity appears earlier in the cell cycle than does that with cyclin B (Giordano *et al.*, 1989; Pines and Hunter, 1990). It is not clear how the association of E1A with cyclin A would affect the role of cyclin A in activating DNA synthesis. Giordano *et al.* (1989) found that the level of cyclin A-kinase activity was the same in adeno-infected HeLa cells as in uninfected cells. In the present work, cells infected with either *dl* 1107/520, in which E1A associates with cyclin A, or *dl* 1108/520, in which E1A does not, both entered S phase and were both unable to progress into mitosis: the main difference between the two infections was that *dl* 1108/520 caused a higher percentage of cells to accumulate excess DNA, suggesting perhaps that in the absence of E1A, cyclin A may be able to reinitiate DNA synthesis so as to cause overreplication. It is unfortunate that we do not have a mutant that binds to pRb but not to cyclin A, as this might allow us to define the function of cyclin A and the effect of E1A binding more clearly. The results reported here suggest there may be other aspects to cyclin A function, however. Analyses of extracts of infected and uninfected HeLa cells immunoprecipitated with anti-cyclin A serum revealed the presence of a 107-kDa protein, which comigrated on SDS gels with p107 in extracts of Ad5-infected HeLa cells immunoprecipitated with M73 anti-E1A serum (Fig. 5, lanes 3, 5–10, 16, 17), and which, from peptide analyses (Fig. 6; Whyte, Howe, and Bayley, to be published), appeared identical to p107. Furthermore, the binding of E1A to p107 and to cyclin A were both markedly reduced with *dl* 1108/520. Together, these observations suggest that while cyclin A may bind directly to E1A, it could alternatively bind to E1A via p107. Clearly more work is required to establish what function the cyclin A–p107 complex serves and what interactions cyclin A makes with other proteins in infected and uninfected cells. Apart from the role of cyclin A itself, however, the association of cyclin A with p107, and the correlation between the phenotype of *dl* 1108/520 and its reduced ability to bind p107 and cyclin A discussed above, are the first hints of function to be reported for p107.

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Yee, S.-Y., and Branton, P. E. (1985). Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. *Virology* 147, 142–153.

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My Contributions to Howe and Bayley, (1992)

1) Used a published assay to measure the mitotic index of BRK cells infected with each of the exon 1 mutants.

2) Mapped sequences in the 243R protein required for association with p400, p300, p107, pRb and cyclin A.

3) Modified and used a technique to prepare cells infected with each of the exon 1 mutants for fluorescence activated cell sorting (FACS).

Analysis of the DNA contents of infected cells showed that all of the exon 1 mutant viruses were able to induce quiescent cells to move into the S-phase of the cell cycle confirming the results of Howe et al. (1990). However, analysis of the mitotic index of infected cells showed that two groups of mutants were defective for stimulation of mitosis. The first group with deletions of residues 4 - 25 and 30 - 60 gave reduced levels of mitoses and produced E1A proteins that associated poorly with p300. The second group with deletions between residues 111 - 138 were completely defective for induction of mitosis and failed to associate with pRb. Among this group a mutant lacking residues 124 - 127 induced cells to accumulate DNA content greater than the G2/M level and was defective for association with p107 and

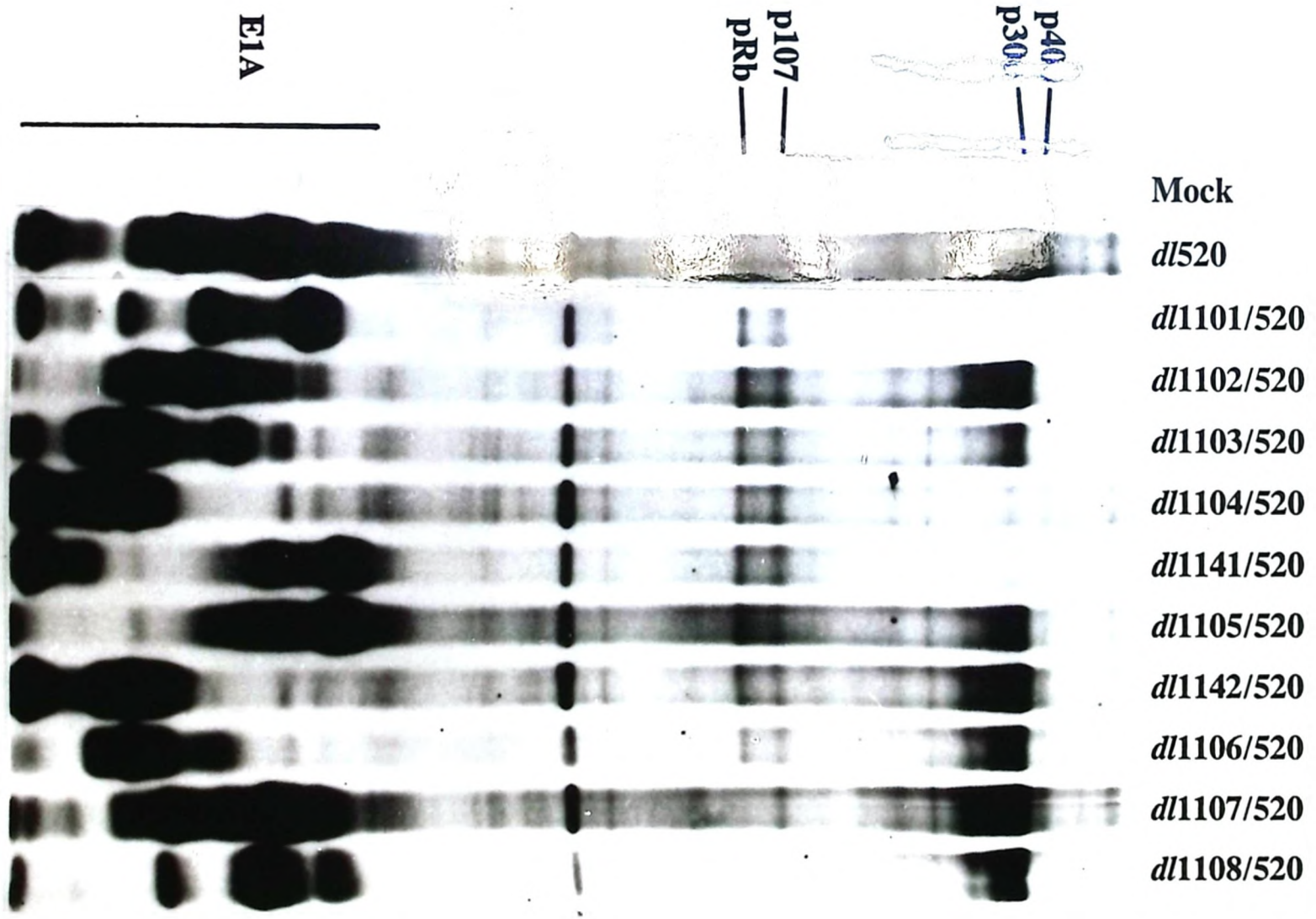
cylcin A as well as pRb. In the course of defining the E1A binding site for cyclin A we also found that cyclin A and p107 normally associate in cells.

E1A Protein-Binding Sites for Cellular Proteins in BRK Cells

Our studies of the E1A proteins have suggested that there is a strong correlation between the abilities of the E1A proteins to affect the cell cycle and to associate with cellular proteins. A major criticism of this work is that the cell cycle effects of E1A have been studied in BRK cells and the ability of the E1A proteins to associate with cellular proteins have been studied in HeLa cells. In order to draw more meaningful correlations, it was necessary to map the E1A sequences required for association with cellular proteins in BRK cells. Binding assays were not carried out in BRK cells before this time because of the technical difficulties involved in using rodent cells for E1A coimmunoprecipitations. However, a number of modifications in our immunoprecipitation technique (Methods section) allowed us to map the 243R E1A binding sites of the E1A-associated cellular proteins p300, p107 and p105 in BRK cells.

Immunoprecipitates of mutants dl 1101/520 - dl1108/520 were analyzed on SDS-PAGE gels (Figure 4) and found to contain protein bands corresponding to p400, p300,

Figure 4. Mapping of E1A protein binding sites in BRK cells using the dl 520 series of E1A mutant viruses. E1A immunopreciptates were prepared from ^{35}S-labelled BRK cells and analyzed by SDS-PAGE. A fluorogaph of the dried gel is shown. Bands corresponding to E1A polypeptides and the cellular proteins p400, p300, p107 and pRB are indicated.

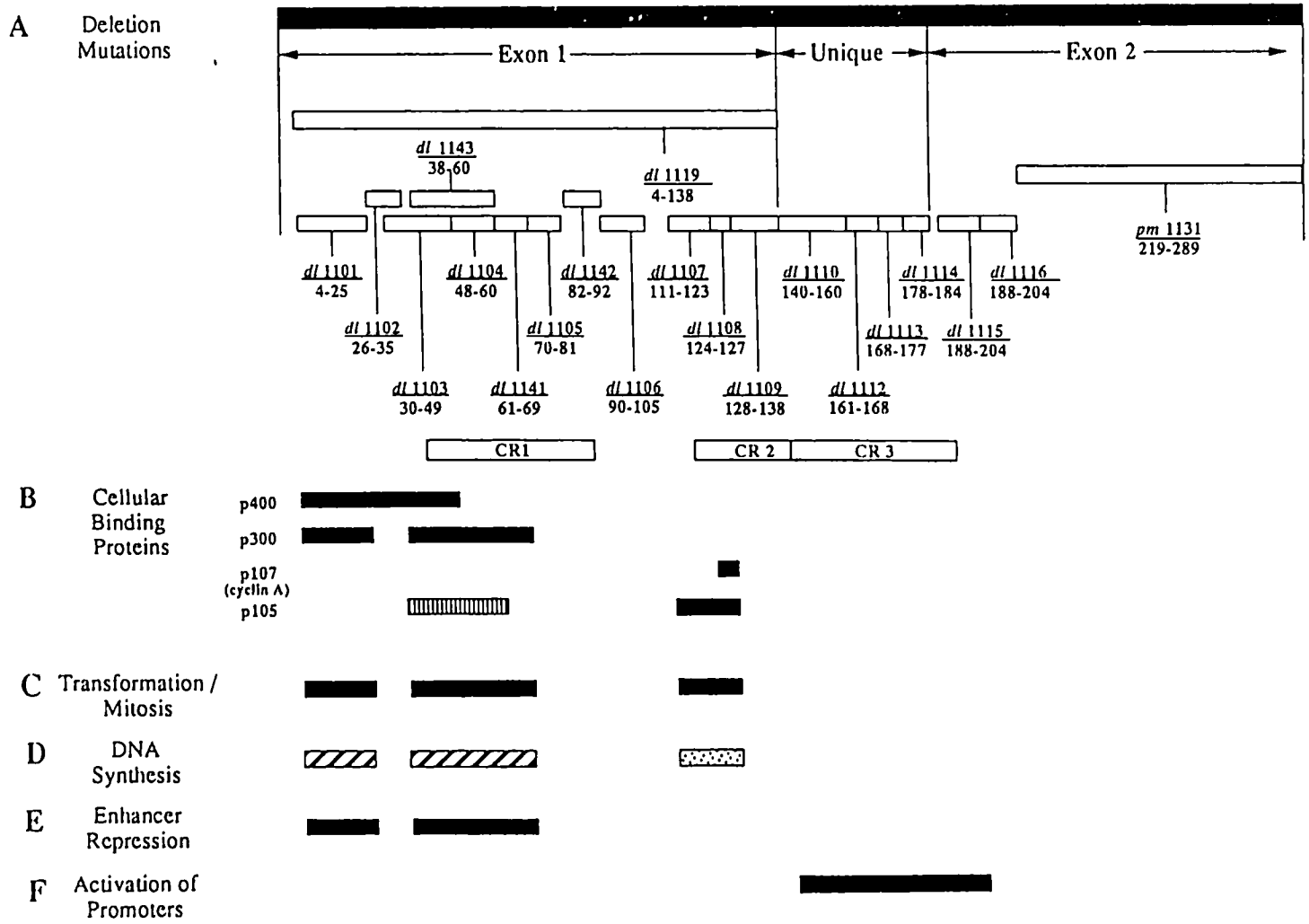

E1A
pRb
p107
p30
p40
Mock
dl520
dl1101/520
dl1102/520
dl1103/520
dl1104/520
dl1141/520
dl1105/520
dl1142/520
dl1106/520
dl1107/520
dl1108/520

p107, and p105, as well as the E1A proteins. Mutants dl 1101/520, dl 1102/520 and dl 1103/520 did not associate with p400. Mutants dl 1101/520, dl 1104/520 and dl 1141/520 did not associate with p300, and mutant dl 1103/520 bound this protein poorly. Dl1107/520 and dl 1108/520 failed to associate with p105 and mutant dl1108/520 was the only mutant that failed to associate with p107.

A comparison of the protein binding studies in BRK cells with the results in HeLa cells demonstrated that sequences required for association with p300 and p107 appeared to be identical in both cell types (Figure 5). Although pRb binding was not detected with the CR2 mutants dl 1107/520 and dl 1108/520 in both cell types, the effect of residues in CR1 on pRB binding appeared to be dependant on cell type. The E1A proteins produced by the CR1 deletion mutants dl 1103/520 and dl 1104/520 coprecipitated with low levels of pRb in Hela cells, but binding in BRK cells was not affected by these mutations. The ability of pRb to associate with the mutated 243R proteins is therefore cell-type dependent to some extent. CR1 residues play a larger role in binding human pRb than in binding rat pRb.

Our studies on E1A binding proteins in BRK cells did not require us to change or modify our models on the roles of pRb and p300 in cell cycle regulation by E1A.

Figure 5. E1A deletion mutations and functional domains. (A) The first and last amino acid residues removed by the indicated mutations are given. The approximate position of the residues removed is represented by open rectangles. (B) E1A domains required for binding to each of the indicated cellular polypeptides are represented by filled rectangles. Regions required for binding to the human and rat cellular polypeptides are the same with one exception; sequences represented by the striped rectangle are necessary for association with human pRb, but not rat pRb. (C-F) Domains required for the indicated E1A functions are represented by filled rectangles. For induction of DNA synthesis the regions represented by the striped and the stippled rectangles are each sufficient on their own.


A
Deletion Mutations
Exon 1
Unique
Exon 2
dl 1143 38-60
dl 1119 4-138
pm 1131 219-289
dl 1101 4-25
dl 1102 26-35
dl 1103 30-49
dl 1104 48-60
dl 1141 61-69
dl 1105 70-81
dl 1142 82-92
dl 1106 90-105
dl 1107 111-123
dl 1108 124-127
dl 1109 128-138
dl 1110 140-160
dl 1112 161-168
dl 1113 168-177
dl 1114 178-184
dl 1115 188-204
dl 1116 188-204
CR1
CR 2
CR 3
B
Cellular Binding Proteins
p400
p300
p107 (cyclin A)
p105
C
Transformation / Mitosis
D
DNA Synthesis
E
Enhancer Repression
F
Activation of Promoters

SUMMARY AND DISCUSSION

The results of my studies on the biological activities of the E1A proteins are summarized and discussed here in terms of functional domains in the E1A proteins; the likely underlying molecular mechanisms of these biological activities; and what role, if any, the individual functions are likely to play in transformation. This section is divided into three areas: Transcriptional Activation, Enhancer Repression and Cell Cycle Effects of E1A.

1) TRANSCRIPTIONAL ACTIVATION

At the time our studies on transcriptional activation were initiated, the 289R E1A protein was known to be mainly responsible for the transactivation of the other early adenovirus promoters and, in most cases, the 243R protein had no detectable effect (Montell et al., 1984, Moran et al., 1986, Schneider et al., 1987). These results implied that the region unique to the 289R product, which is roughly equivalent to conserved region 3, plays an important role in transactivation. This view was strengthened by the finding that point mutants in this region were trans-

activation defective (Glenn and Ricciardi, 1985). Although it was clear that the unique region was required for transactivation, the precise borders of the transactivation domain were not known and, in addition, it was unknown whether the unique region functioned on its own for transactivation or in concert with other regions. Using our series of deletion and point mutations in the E1A coding region, we set out to map more precisely the domain of the 289R protein responsible for transactivation of the Ad5 E3 promoter, and to determine if any other regions of the protein were required.

Domain for Transactivation

We found that the transactivation domain of the 289R protein is located between residues 139, in the unique region, and 204 in exon 2 (Figure 5). Mutants with deletions or point mutations in this region were defective for transactivation of the Ad5 E3 promoter. A number of small deletion mutations that collectively removed almost all of the common N-terminal regions of the 243R and 289R proteins, and a portion of the common C-terminal region did not affect transactivation. As a result of these findings I examined the effect of removing all of exon 1 with one large deletion - dl 1119. This deletion extends from amino acids 4 - 138 at the border of the unique 289R region and was

found upon testing to be completely defective for trans-activation. Recently, Joe Mymryk in Dr. Bayley's laboratory has found that this mutant produces an E1A protein, indicating that exon 1 may act as a framework which holds the transactivation domain in an active form. Small deletions within this framework do not compromise its structural integrity, while deletion of the entire framework renders transactivation domain ineffective.

The results with dl 1119 differ from those of M. Green (Lillie et al., 1987) who synthesized a 49 amino acid peptide corresponding to residues in the unique region. These researchers showed that when this peptide was micro-injected into the nuclei of cells, it was able to activate transcription from the Ad5 E2 promoter (Lillie et al., 1987). To create a product resembling this peptide, dl 1119 was combined with pm 1131. This double mutant, dl 1119/pm 1131, then was predicted to code for an 83-amino acid peptide containing all of the unique region. However this mutant, like dl 1119, was unable to activate transcription in our assay. Possible reasons for the inactivity or apparent inactivity of the 83 amino acid peptide were that the peptide was unstable and rapidly degraded, or that our assay was not sensitive enough to detect low levels of transactivation. M. Green reported that the 49R peptide was 100 times less potent than wild type E1A (personal communication). If this was also the case for our putative

83 amino acid peptide, our CAT assay would not have detected any transactivation ability it may have had. More recently dl1119/1131 as well as dl1119 have been found to activate the E2 promoter at low levels, but reproducibly, during infection of BRK cells (Joe Mymryk, personal communication). This finding suggests that the lack of activity detected in my previous study was due to the insufficient sensitivity of the CAT assay.

Mechanism of Transactivation

A wide range of studies has been carried out to determine how the CR3 region functions to activate transcription (see Shenk and Flint, 1991 for a review; Lee et al., 1991). The most compelling evidence to date suggests that E1A acts to assemble multi-component transcription factor complexes. E1A physically interacts with the general transcription factor TFIID (Horikoshi et al., 1991; Lee et al., 1991), which binds to TATA box promoter elements. There is also strong genetic evidence that it can interact with the upstream transcription factor ATF-2 (Liu and Green, 1990). Berk and colleagues have proposed that E1A functions to mediate interactions between TFIID and upstream regulators such as ATF-2 (Lee et al., 1991).

Transactivation and Transformation

The ability of the 289R E1A protein to transactivate the expression of adenoviral early genes as well as some cellular genes could play a role in transformation if the cellular genes that were transactivated included those that produced cell cycle regulatory proteins. However, studies by a number of groups have shown that the 243R protein is fully able to cooperate with activated ras or E1B to bring about the transformation of primary rodent cells (Haley et al., 1984; Zerler et al., 1987; Stephens and Harlow, 1987; Ulfendahl et al., 1987). These findings demonstrate that the transcriptional induction activity of the region unique to the 289R protein (CR3) is not required for transformation.

2) REPRESSION OF TRANSCRIPTION

The first reports describing enhancer repression by E1A suggested that both the 243R and 289R proteins were equally able to down-regulate enhancers (Borelli et al., 1984; Velcich and Ziff, 1985). Since then, other groups have shown that the 243R protein is a more effective repressor than the 289R protein (Schneider et al., 1987; Stein et al., 1990), while others still have reported that efficient repression was only detected when the 243R product

was expressed in the absence of the 289R protein (Lillie et al., 1986, Kuppuswamy and Chinnadurai, 1987; Jelsma et al., 1989; Bautista et al., 1991). The reasons for these conflicting reports are not clear, but the use of different test enhancers and different assays may be partially responsible.

During preliminary CAT repression assay experiments, we found that repression of the SV40 enhancer was only detected with transactivation defective mutants such as dl1112, or with dl520 which encodes only the 243R product (Jelsma et al., 1989). Wild type E1A failed to repress regardless of the ratio of E1A plasmid to test plasmid used, and in many experiments wild type E1A appeared to stimulate expression from the SV40 enhancer. These results could be explained if transactivation and repression were mutually antagonistic effects used by E1A to control gene expression. This view is supported by Dery et al. (1987) who showed that the early Ad promoters were repressed by the 243R in transient CAT assay experiments, while the 289R product stimulated transcription from these promoters. It should be pointed out that the 289R protein would be expected to retain the ability to repress transcription because it contains all of the sequences in the 243R protein. However, this activity may only be detected under appropriate conditions, depending on the test promoter/enhancer and the particular assay used.

Domain for Repression

Since our assay could only detect repression in the absence of transactivation by the 289R protein, we mapped E1A regions required for repression of the SV40 enhancer after the E1A mutants had been rescued into pLE2/520, so that only the 243R protein was produced. Our results indicate that an N-terminal region as well as residues within CR1 are required for effective repression (Figure 5).

A number of studies have also implicated CR2 in enhancer repression (Lillie et al., 1986; Schneider et al., 1987), but it appears to play a less significant role than CR1 (Schneider et al., 1987); others have suggested that it is not required (Rochette-Egly et al., 1990; Stein et al., 1990). In our work, two deletions which remove portions of CR2, dl 1107/520 and dl 1108/520, were found to repress at wild type levels while a third mutant in CR2, dl 1109/520, gave variable results. Two lines of evidence however suggest that the region deleted by dl 1109 (residues 128 - 138) does not play a direct role in repression. First of all, the E1A protein produced by dl 1109/520 virus was detected in much lower quantities than expected. Second, when dl 1109 was transferred to a mutant that produced a transactivation defective 289R protein, the resulting double mutant, dl 1109/1112 repressed at wild type levels (Table 2). Together these results suggest that dl 1109

destabilizes the 243R protein, but not the 289R protein, and therefore CR2 does not contain a region required for repression.

Mechanism for Repression

The E1A proteins do not possess a known intrinsic biochemical activity and they do not associate with specific DNA sequences. Therefore, the most likely mechanism by which the E1A polypeptides bring about their biological activities is through their ability to associate with and presumably modify the functions of cellular proteins (Whyte el al., 1988b, 1989; Egan et al., 1988, 1990; Howe et al., 1990; Horikoshi et al., 1991; Lee et al., 1991). Our studies on repression together with those of Dr. C. Egan, who used the exon 1 mutant series to map E1A binding sites for cellular proteins (Egan et al., 1988), suggested that there is a strong correlation between enhancer repression and the ability of the E1A proteins to associate with p300. The E1A regions required for association with p300 were found to be identical to those required for enhancer repression (Figure 5).

Although the gene which encodes p300 has not been identified, p300 is known to be a nuclear DNA binding protein (Rikitake and Moran, 1992). The consensus DNA sequence to which p300 binds is related to elements that are

recognized by the enhancer binding proteins H2TF1 and NF-KB (Rikitake and Moran, 1992). These findings indicate that p300 could be a transcription factor which associates with enhancer elements. DNA sequence motifs related to the recognition sequences for p300 have been identified in some enhancers which are repressed by E1A (Rikitake and Moran, 1992). These include the SV40 enhancer, the HIV long terminal repeat, the kappa light-chain enhancer and the E1A enhancer. These findings suggest that p300 may act normally to promote transcription from these enhancers. If this was correct, it would be tempting to speculate that the E1A protein could bring about the repression of these enhancers by modifying the ability of p300 to act as a transcription factor. The E1A proteins do not seem to affect the DNA binding activity of p300 (Rikitake and Moran, 1992), but it is possible that they prevent p300 from associating with other key transcription factors. One of these could be the TATA binding protein which is known to associate with p300 (Dr. E. Moran, personal communication).

Gene enhancers that do not contain p300 consensus binding sites can also be repressed by E1A (Rochette-Egly et al., 1990; Enkemann et al., 1990, Rikitake and Moran, 1992). These findings could be interpreted in a number of different ways. It is possible, for example, that E1A could bring about repression of various classes of enhancers by associating with cellular proteins other than p300 (Rikitake

and Moran, 1992). These proteins could associate with E1A through the same, or similar, sequences as does p300. One possibility is p400, which we have shown to associate with N-terminal sequences that overlap part of the p300 E1A binding site (Howe and Bayley, 1992). Our studies with mutant dl 1102 suggest that association with p400 by E1A is not required for repression of the SV40 enhancer. This mutant associates with p300, but not p400, and is able to repress the SV40 enhancer at near wild type levels. To implicate p400 in enhancer repression it would be necessary to identify enhancers that are not repressed by dl 1102.

Repression and Transformation

The two E1A regions required for enhancer repression, one at the N-terminus and the other at a region in CR1, are also required for transformation (Figure 5). This correlation suggests that enhancer repression may be one of the underlying mechanisms of transformation (Lillie et al., 1987; Schneider et al., 1987; Jelsma et al., 1989). We know that repression is not the only E1A function involved in transformation, since our group (and others) have described mutants with deletions in CR2 that are defective for transformation of BRK cells, but retain the ability to repress the SV40 enhancer in HeLa cells (Kuppaswamy and Chinnadurai, 1987; Jelsma et al., 1989). On

the other hand, Lillie et al. (1986) have reported that a mutant containing a point mutation of a single CR2 residue is defective for both repression of the SV40 enhancer in a human Hepatoma cell line, and for transformation of BRK cells. These seemingly contradictory results highlight the potential dependency of enhancer repression assays on cell type and they suggest that correlations drawn between E1A functions (such as enhancer repression and transformation) would be more meaningful if the assays for both were carried out in the same type of cell.

One potential role for repression in transformation could be to prevent cells from entering pathways leading to terminal differentiation. A number of studies have shown that the E1A proteins can prevent myogenic and neuronal differentiation (Webster et al., 1988; Maruyama et al., 1987) and that the ability of the E1A proteins to inhibit myoblast differentiation involves the repression of transcription of a number of muscle specific genes (Webster et al., 1988). The E1A sequences required for suppression of myogenic differentiation correlate exactly with the E1A sequences at the N-terminal end and within CR1 which are required for repression and transformation (Mymryk et al., 1992). The ability of the E1A gene to prevent cellular differentiation and hence cessation of cell growth could be advantageous for productive viral infection (Stein and Ziff, 1987; Rikitake and Moran, 1992).

3) CELL CYCLE EFFECTS OF E1A

The role(s) of the E1A proteins in oncogenic transformation most probably involve their ability to induce resting cells to move into the cell cycle and to proliferate, since an unlimited capacity to grow is a hallmark of all transformed cells. To learn more about how E1A stimulates cell proliferation we studied the ability of our mutants to induce DNA synthesis and cell cycle progression in primary quiescent baby rat kidney cells (Howe et al., 1990; Howe and Bayley, 1992). The results of our mapping studies are shown in Figure 5. Mutants with deletions at the N-terminus between residues 4 - 25, in CR1 between residues 36 - 69, and at the C-terminus of exon 1 between residues 111 - 127 were found to be defective for stimulation of mitosis. The only mutants that were defective for induction of DNA synthesis were double mutants in which an N-terminal, or CR1 mutant, was combined with an exon 1 C-terminal mutant.

Model for Induction of DNA Synthesis and Mitosis

The ability of the E1A proteins to affect cell cycle regulation appears to be tightly linked to their binding of the cellular proteins pRb and p300 in HeLa cells (Figure 5; Howe et al., 1990). We found that all of the

double mutants that were defective for induction of DNA synthesis failed to associate with both pRb and p300, and that the single mutants that were defective for stimulation of mitosis failed to associate with either pRb or p300 (Howe and Bayley, 1992). These correlations suggested that the E1A proteins could induce DNA synthesis by binding to pRb or p300, but that binding to both was necessary for stimulation of cell division.

Although pRb and p300 appear to be the most important proteins required for E1A's effect on the cell cycle, we cannot rule out the possibility that other E1A binding proteins also play important roles. One E1A associated protein, p107, is a member of the pRb family and is known to associate with cyclin A (Ewen et al., 1992; Faha et al., 1992; Howe and Bayley, 1992). Cyclin A is required to regulate S-phase. Results found with one of our mutants (discussed below) suggest that E1A might associate with p107/cyclin A for proper regulation of S-phase. In addition we have also found that another E1A binding protein, p130, which is also a member of the pRb family of polypeptides (Dr. P. Whyte, unpublished results) binds to E1A through the same residues as pRb (Mymryk et al., 1992; my unpublished results). It is possible that this protein could be responsible in whole or in part for the functions we have suggested for pRb in E1A-mediated cell cycle regulation.

Although other E1A binding proteins could be

involved in cell cycle regulation by E1A our results provide strong genetic evidence that the E1A proteins affect the cell cycle, at least in part, by associating with pRB and p300. If this is correct then we would expect that the normal roles of these proteins would be to control the cell cycle in some way. So, what are the normal roles of pRb and p300 and how does E1A affect these functions?

PRb and Cell Cycle Regulation by E1A

As mentioned in the Results section, a wide body of evidence suggests that pRb is the product of a growth suppressor gene and the E1A proteins would therefore be expected to inactivate pRb as a repressor of cell growth. Loss of pRb function by inactivating mutations in each copy of the RB gene is associated with the etiology of all human retinoblastomas. Mutated forms of the gene have also been found in a number of sarcomas, carcinomas, and leukemias (see Weinberg, 1991, for review). Reintroduction of pRb into transformed cell lines, in which both Rb genes are mutated, inhibits cell cycle progression and provides strong evidence that pRB restrains cell growth in normal cells (Bookstein et al., 1990). In addition, the transforming antigens produced by other small DNA tumour viruses (including SV40, polyoma and human papilloma) also associate with pRb (DeCaprio et al., 1988; Larose et al., 1991; Dyson

et al., 1989). Presumably by sequestering pRb, the DNA tumour viruses can mimic the RB^- state in cells to promote cell growth and, as a result, more efficient viral replication.

The role of pRb as a growth suppressor appears to be controlled by its state of phosphorylation. During the cell cycle, underphosphorylated forms of pRb are predominant from G_0 to mid G_1 when hyperphosphorylated forms accumulate and persist through the rest of the cell cycle (Buchkovich et al., 1989; DeCaprio et al., 1989, Ludlow et al., 1989; Chen et al., 1989). The underphosphorylated form is thought to actively repress cell cycle progression because it is this form which is predominantly targeted by the SV40 T antigen (Ludlow et al., 1989; Ludlow et al., 1990). The E1A proteins bind mainly to the underphosphorylated form of pRb, but can also associate with at least some of the phosphorylated forms (DeCaprio et al., 1992; my unpublished results).

How then does pRb regulate the cell cycle and how is E1A able to usurp this function? One model of pRb function suggests that it may inhibit cell cycle progression by repressing the expression of a number of genes that encode cell cycle regulatory proteins. The underphosphorylated form of pRb can associate with the transcription factor E2F, and this complex is thought to actively repress expression of genes that encode important

cell cycle regulators such as cdc2 and myc (reviewed in Cobrinik et al., 1992; Nevins, 1992). The E1A proteins may be able to induce S-phase by binding to pRb since this results in the dissociation of pRb/E2F complexes and the release of active E2F (Bagchi et al., 1990). Free E2F would be available to promote expression of genes like myc and cdc 2 which would in turn stimulate S-phase.

Our model of cell cycle progression by E1A predicts that binding of pRb may be required to stimulate progression from S-phase to mitosis. Evidence comes from our studies with mutant dl 1107/520. This mutant, which fails to associate with pRb, is able to induce cellular DNA synthesis, but is defective for stimulation of mitosis. One other line of evidence suggesting that pRb may act at points in the cell cycle other than at the G_1/S transition comes from a detailed study of the state of phosphorylation of pRb throughout the cell cycle by DeCaprio et al. (1992). These authors found that pRb is phosphorylated in three steps: once after exit from G_0 but before the G_1/S transition, once during S-phase, and once in G_2/M. These changes in phosphorylation state could represent different functional forms of pRb (DeCaprio et al., 1992). Since E1A (unlike SV40T antigen) can associate with phosphorylated forms of pRb it is possible that E1A may bind to and alter pRb's potential regulatory roles at various points in the cell cycle beyond the G_1/S transition. It is also possible that

p130 - the cellular protein that associates with E1A through the same binding sites as pRb - may play a role in the induction of mitosis.

P300 and Cell Cycle Regulation by E1A

We have found that E1A mutants which do not associate with pRb, but do associate with p300, are capable of stimulating DNA synthesis. At first glance this observation would seem difficult to explain. How could another protein activate DNA synthesis when the growth suppressor pRb was still present? One way to explain this result would be to suggest that p300 and pRb can suppress cell growth by two parallel pathways and that removal of either, by E1A, is sufficient for induction of DNA synthesis. However, a better explanation has been proposed by Dr. E. Moran and her co-workers (Wang et al., 1991). These authors have shown that the 243R protein can induce phosphorylation of pRb in cells infected with wild type 12S virus or with E1A mutant viruses which fail to associate with pRb, but do associate with p300. Perhaps when E1A binds to p300, cell cycle progression is stimulated by a pathway that would normally lead to phosphorylation of pRb. This finding suggests that p300 may be an upstream regulator of pRb phosphorylation. One observation which supports this hypothesis is that E1A mutants, which fail to associate with

pRb, take longer to induce cellular DNA synthesis than wild type dl 520 (Wang et al., 1991). Perhaps mutants that do not bind pRb directly induce DNA synthesis as they have to bring about phosphorylation of pRb before cell cycle progression can occur. Wild type or E1A mutants producing proteins that associate with pRb may bypass the need for phosphorylation by sequestering pRb directly.

Compared to pRb, relatively little is known about p300, but it is possible that this protein plays a role analogous to pRb as a suppressor of cell growth. p300 is a phosphoprotein that, like pRb, is phosphorylated in a cell-phase specific manner (Yaciuk and Moran, 1991). A phosphatase-sensitive form of p300 has been found to accumulate when cells are blocked in mitosis, suggesting that this form accumulates in phases outside of G_1 (Yaciuk and Moran, 1991). As described above, p300 has a DNA binding activity and it associates with specific enhancer elements that have been shown to be present in the promoters of a number of genes which can be repressed by E1A. The ability of E1A to repress enhancers and to suppress myogenic differentiation appears to be closely correlated with binding to p300 (Mymryk et al., 1992; Heasley et al., 1991). Therefore by binding to p300, the E1A products could prevent p300 from carrying out its likely normal role - to act as a transcription factor (Rikitake and Moran, 1992) that regulates expression of differentiation specific genes whose

products could act to suppress cell growth.

Why Does E1A Associate with p107?

Our mutant analysis has suggested that the E1A associated proteins p300 and pRb are involved in cell cycle control in the G_1 phase of the cell cycle and perhaps during later phases of the cell cycle as well. However, we have not sufficiently enhanced our knowledge of the roles of p107 in E1A-induced cell cycle progression. Although the E1A binding site of p107 is located in CR2 between residues 124 - 128, deletion of these residues in dl 1108/520 also eliminates binding to pRb. Therefore phenotypes of this mutant cannot be correlated with binding to p107 exclusively. The only phenotypic difference detected between dl 1108/520 and its neighbour dl 1107/520 (which eliminates binding to pRb but not p107) is that dl 1108/520 induces a greater number of cells to overreplicate their DNA. Therefore, when p107 and pRb are present uncomplexed in cells, mitosis is inhibited, but cells may synthesize DNA at an enhanced rate. It should be noted that dl 1107/520 can also induce overreplication of DNA to levels equivalent to dl 520 levels. Thus a role for pRb in overreplication cannot be ruled out.

The finding that p107 may be implicated in overreplication of DNA suggests that p107 may be involved in

control of S-phase. A number of other lines of evidence support this hypothesis. The gene encoding p107 has been cloned and it was found to contain sequence homology with pRb in the two pRb regions necessary for association with the E1A proteins and SV40 T antigen (Ewen et al., 1991). Our results (Howe and Bayley, 1992) along with those of Ewen et al. (1992), and Faha et al., (1992) showed that p107 can associate with cyclin A in normal cells. In addition, tetrameric complexes of p107, cyclin A , cdk2 and the transcription factor E2F have also been identified; these complexes possess kinase activity and form mainly in S-phase (Cao et al., 1992; Devoto et al., 1992; Pagano et al., 1992). Based on these findings a number of workers have suggested that these complexes activate E2F and promote the expression of a number of genes that are required for DNA synthesis (Cao et al., 1992; Branton, 1992; Cobrinik et al., 1992). A role for these complexes in the initiation of DNA synthesis has also been suggested (Dr. J. Newport, personal communication). This would suggest that the roles of pRb and p107 are opposed, with pRb acting to inhibit cell growth by sequestering transcription factors such as E2F, and p107 acting to promote DNA synthesis by activating E2F or by promoting initiation of replication (Cobrinik et al., 1992).

Regulation of Gene Expression and Cell Cycle Effects

We have shown that the E1A proteins most probably promote DNA synthesis and mitosis by associating with, and modifying, the activities of cellular proteins, such as pRb, that normally control the expression of cell cycle regulatory genes. Evidence that E1A affects the expression of genes required for DNA replication has also comes from studies on transcriptional activation by the 243R protein. One of the genes that is transactivated by 243R is the proliferating cell nuclear antigen (PCNA) gene. PCNA is an auxiliary sub-unit of the DNA polymerase δ that is essential for cellular DNA replication. Expression of the PCNA gene is activated by E1A during viral infection (Zerler et al., 1987) and also in transient expression assays (Morris and Mathews, 1990), suggesting that the induction of this gene is at least at the level of transcription (Zerler et al., 1987; Morris and Mathews, 1990).

In studies to map the E1A domains required for transactivation of the PCNA gene, J. Mymryk in Dr. Bayley's laboratory found that each of the dl 520 series of exon 1 mutants was able to induce PCNA at wild type levels. He also found that induction was only reduced in mutants in which binding to both p300 and pRb was affected. Therefore, as was the case for induction of DNA synthesis, there could be two pathways that are used by E1A to induce expression of

these genes; one that involves binding to p300 and another that involves binding to pRb. It is possible of course that these "two pathways" are actually two steps in one pathway. As described above, E1A can bring about phosphorylation and inactivation of pRb as a repressor of cell growth without binding to it directly, but by associating with p300 (Wang et al., 1991). Thus, if one of the functions of the underphosphorylated forms of pRb was to repress PCNA expression, then E1A could usurp this function either by binding to it directly or by binding to p300.

Cell Cycle Effects and Transformation

The E1A domains required for transformation, induction of DNA synthesis and stimulation of mitosis are the same. This finding leads us to believe that the ability of the 243R product to induce cell cycle progression is directly responsible for E1A's ability to cooperate with other oncogenes for transformation. E1A's role in transformation is to immortalize cells so that they can grow indefinitely in culture. The ability of E1A products to bind to cellular growth regulators is required to initiate cell cycle progression and division, but it is not clear from our studies if E1A functions beyond these are required to maintain the immortalized state.

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