CHARACTERIZATION OF RAT CELLS TRANSFORMED BY HYBRID ADENOVIRUS TYPE 5/12 E1A GENES

By

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TUMORIGENICITY OF ADENOVIRUS TRANSFORMED CELLS

ABSTRACT

Previously, a series of hybrid adenovirus (Ad) type 5/12 E1A genes were constructed and used in combination with Ad12 E1B to transform primary Hooded Lister rat kidney cells. Research presented here describes the use of the resulting transformed cells lines to identify and characterize functional differences between Ad5 and Ad12 E1A proteins in an attempt to better understand the ability of Ad12 E1-transformed cells, unlike their Ad5 counterparts, to induce tumors in syngeneic immunocompetent rats.

At least two regions within the first exon of Ad12 E1A were identified which mediate tumorigenicity. Expression of either of these regions in the hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed cells was associated with a decrease in cell surface major histcompatibility complex (MHC) class I expression, a finding which suggests that the tumorigenic capacity of Ad12 E1-transformants could be due to their ability to evade lysis by class I-restricted CD8+ cytotoxic T lymphocytes (CTLs). However, neither class I down-regulation nor sensitivity to allogeneic CTLs or syngeneic natural killer (NK) cells strictly correlated with the tumorigenic capacities of the hybrids, indicating that additional factors contribute to the differences in the oncogenic potential of rodent cells transformed by the E1 regions of Ad5 and Ad12. Further study demonstrated that at least two regions of Ad5 E1A encode CTL epitopes that confer susceptibility to syngeneic Ad5 E1-specific CTLs *in vitro* while Ad12 E1A expression did not confer susceptibility to syngeneic Ad12 E1-specific CTLs.

The MHC class I enhancer is the target for Ad12 E1A-mediated down-regulation of class I transcription. In Ad12 E1-transformed rodent cells, the class I enhancer is primarily

down-regulated through decreased binding of NF-κB (activator) and increased binding of COUP-TF (repressor). Using the hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed rat cells, the same regions of Ad12 E1A implicated in tumorigenesis and down-regulation of class I expression were also found to mediate the differential binding activities of NF-κB and COUP-TF to the class I enhancer in Ad12 E1-transformants. Moreover, it was shown that reduced NF-κB binding activity was not due to decreased expression of its subunits, NF-κB1-p50 (or its precursor, NF-κB1-p105) or RelA-p65 nor to a defect in processing of NF-κB1-p105 to NF-κB1-p50. The latter discovery contradicts a previously published report in which it was suggested that decreased binding of NF-κB to the class I enhancer in Ad12 E1-transformed cells was due to impaired processing of NF-κB1-p105 to NF-κB1-p50.

Finally, during studies designed to determine whether the different tumorigenic properties of Ad5 E1- and Ad12 E1-transformed cells were a result of alterations in the composition of their E1A-cellular protein complexes, it was discovered that expression of the Ad12 E1A regions implicated in mediating tumorigenesis, down-regulation of cell surface MHC class I expression, and differential binding of NF-κB and COUP-TF to the class I enhancer also correlated with the presence of a differential phosphorylated form of p300 in Ad12 E1- compared to Ad5 E1-transformed rat cells. This finding suggests that p300 function may be altered in Ad12 E1- versus Ad5 E1-transformed rodent cells and raises the possibility that p300 may influence tumorigenicity through the regulation of MHC class I expression.

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Just to dig it all and not to wonder why,
Is just fine.
Being satisfied,
Is not to read between the lines.

- Van Morrison, 1968.

INTRODUCTION

1) Overview of Adenovirus Research

Human adenoviruses (Ads) were first isolated in 1953 from cultures of human adenoid tissue (Rowe et al., 1953). Shortly after it was recognized that Ads were responsible for the spontaneous degeneration of these cultures, they were also identified as the etiological agent of a non-influenza like case of acute upper respiratory disease (Hilleman and Werner, 1954). Since these studies, nearly 50 distinct human Ad serotypes have been identified and infection by these serotypes has been associated with a variety of human illnesses affecting the upper respiratory tract, eye, intestine, stomach, and bladder (reviewed by Horwitz, 1990).

The first demonstration that a human virus could cause malignancy in animals came when Ad serotype 12 (Ad12) was found to be tumorigenic in hamsters (Trentin et al., 1962; Huebner et al., 1962). This discovery prompted detailed studies of Ad tumorigenicity to determine whether Ads were responsible for human malignancies (reviewed in Green and Mackey, 1977). Although these studies failed to support the notion that Ads act as carcinogens in humans, other studies revealed that the tumorigenic capacity of Ads was serotype dependent despite the fact that all serotypes were capable of transforming primary rodent cells in culture (Freeman et al., 1967; McAllister et al., 1969). The ability of Ads to transform cultured cells has served and continues to serve as an excellent *in vitro* system to study and understand the processes of transformation and tumorigenesis. Among the major findings derived from these studies were: the discovery that early region 1 (E1) and particularly the E1A gene of the Ad genome is responsible for mediating transforming and tumor inducing activities; the ability of Ads to transform cells is

associated with the ability of E1A to associate with a number of cellular proteins involved in regulating gene expression and cellular proliferation (discussed in section 4).

Due to the relative ease with which the Ad genome can be manipulated by recombinant DNA techniques, Ads have also been used as recombinant vaccines and general purpose mammalian expression vectors. Very recently, Ads have attracted considerable attention as vectors for the delivery of genes in gene therapy protocols due primarily to the ability of these relatively stable vectors to grow to high titres and to transduce a variety of cell types in culture and *in vivo*. For a review on the construction and characterization of Ad vectors, see Berkner, 1992; Hitt et al., 1994, 1995; Graham and Prevec, 1995.

Finally, an overview of Ad research would not be complete without mention of other unique contributions made to the fields of molecular biology and biochemistry: the discovery of mRNA splicing; the first cell free eukaryotic DNA replication system, demonstration that virally encoded proteins control expression of cell surface major histocompatibility complex (MHC) molecules; development of DNA mediated gene transfer techniques; and the establishment of techniques for mapping mRNAs or translational open reading frames (for further reading, see Ginsberg, 1984; Horowitz, 1990).

2) General Properties of Adenoviruses

a) Classification

All Ads are members of the Adenoviridae family which is divided into two authentic genera, Mastadenoviruses (includes human and other mammalian viruses of simian, bovine, equine, porcine, ovine, canine, and opossum origins) and Aviadenoviruses (avian viruses) (Norrby et al., 1976). To date, more than 100 Ad serotypes have been reported of which approximately half are of human origin (see Horwitz, 1990 for review). Human Ads have been further partitioned into sub-groups (A-F) on the basis of chemical, immunological, and biological properties such as genome base composition and sequence

similarity, ability to hemagglutinate the red blood cells of rats or rhesus monkeys, and tumorigenic capacity in newborn hamsters (Table 1). Of the human serotypes, Ad2, Ad5, and Ad12 have been most extensively characterized.

b) Structure

A combination of studies involving electron microscopy, X-ray crystallography, and biochemical techniques have allowed the molecular composition and structural features of the Ad virion to be determined (summarized by Nermut, 1984; van Oostrum and Burnett, 1985; Horwitz, 1990; Stewart et al., 1993). These investigations revealed that the virion is icosohedral and consists entirely of protein and DNA (Fig. 1).

The capsid is composed of 252 capsomers which form 20 identical and equilateral triangular faces forming 12 edges and 12 vertices. Of the 252 capsomers, 240 are organized as hexons and the remaining 12 capsomers are organized as pentons at each of the vertices of the icosohedral capsid. Hexons are composed of 3 viral protein (VP) II (hexon) monomers which associate in planar sheets as groups of nine (GON) hexons. A penton, which occupies each of the 12 vertices, is comprised of a pentamer of VP III (penton base) proteins and 3 VP IV (fibre) proteins. The fibre molecules, which project from the vertices of the capsid, are thought to be the ligand for the cellular receptor mediating entry of Ads into cells. Taken together, the external shell of the capsid is essentially comprised of the GON hexons, unaggregated hexon trimers (peripentonal hexons), and penton base and fibre molecules. To maintain the structural integrity of the capsid, 12 VP IX molecules exert a stabilizing effect on the capsid by associating with each of the GON hexon trimers. VP IIIa molecules penetrate the facet edges of the capsid and likely function in virion assembly. VP V, VI, and VII bind DNA and may function in viral DNA packaging.

The linear double stranded DNA genome is packaged into virions in association with VP V and VII which are predicted to organize the genome into nucleosomes of repeat-

Table 1: Classification schemes for human Ad serotypes‡.

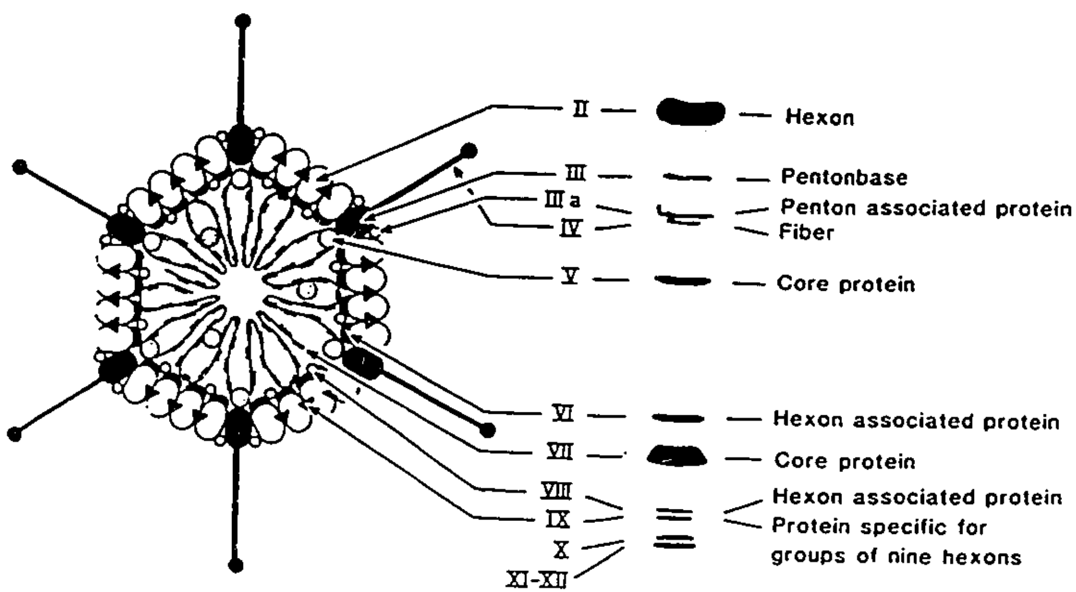
Sub Group	Hemagglutination Groups *	Serotypes	Tumors In Animals	Transformation In Culture	Percentage of G+C In DNA
A	5	12, 18, 31	High	+	48-49
B	1	3, 7, 11 14, 16, 21 34, 35	Moderate	+	50-52
C	3	1, 2, 5 6	Low/None	+	57-59
D	2	8, 9, 19 37, 10, 13 15, 17, 19 20, 22-30 32, 33, 36 37, 38, 39 42	Low/None	+	57-61
E	3	4	Low/None	+	57-59
F	3	40, 41	Unknown		

‡ Modified from Horwitz, 1990.
* The extent of agglutination decreases as the number increases.

Figure 1: **Structure of an Ad virion.**

(Taken From Horwitz, 1990)

A cross-section of the icosahedral virion is shown. Structural proteins are denoted as roman numerals. To the right, an idealized SDS-polyacrylamide gel pattern illustrates the approximate sizes and concentrations of these structural proteins in infected cells. For example, VP II, is the largest (106 kDa) and most abundant structural protein.


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
groups of nine hexons
X
XI-XII

ed subunits of DNA and protein in a chromatin-like structure. Each viral nucleosome may consist of 150 nucleotides of DNA wrapped around 3 dimers of VP VII. The individual nucleosomes are linked by a variably sized DNA segment complexed to a single VP V molecule. VP VI, which also binds DNA, has recently been shown to connect the peripentonal hexons (Stewart et al., 1993).

c) Genome Organization

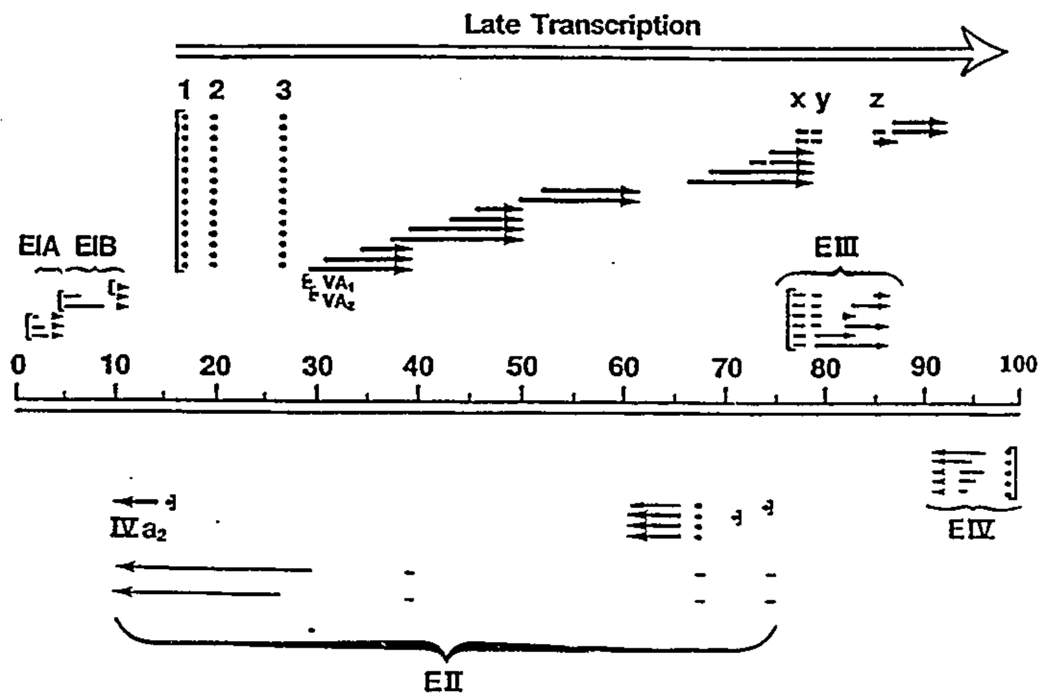
The Ad genome (Fig. 2) is a linear double stranded DNA molecule that varies in size with serotype (e. g. Ad5 = approximately 36 000 base pairs). At each end of the genome are inverted terminal repeats (ITRs) which vary in length between 50 and 170 base pairs and which serve as origins for DNA replication (Kelly, 1984). At the 5' ends of each strand a virally encoded terminal protein (TP; Rekosh et al., 1977) is covalently linked by a serine hydroxyl group to a dCMP molecule that serves to initiate viral DNA replication by a semi-conservative mechanism (Kelly and Lechner, 1977). Both genome strands encode several transcription units and are designated left (l) and right (r) according to the direction of transcription. Transcription units are situated within the early (E1, E2, E3, and E4) and late (L1-L5) regions of the genome. E1 (encodes the E1A and E1B transcription units), E3, and L1-L5 are located on the r-strand while E2 and E4 map to the l-strand. The following paragraphs briefly overview features of the E2, E3, E4, and late region transcript and translation products. A detailed description of E1 transcripts and translation products will be presented in section 3.

Transcription of E2 generates a primary mRNA that is spliced into E2A and E2B mRNAs (for review, see Sussenbach, 1984). E2A encodes a 72 kd single-strand-specific DNA binding protein which is essential for productive infection (Kruijer et al., 1981). E2B mRNAs encode the 80 kd precursor of the terminal DNA binding protein and a 140 kd DNA polymerase both of which are required for viral DNA replication and, like the 72 kd E2A protein, are necessary for the production of progeny virus (Stillman et al., 1981).

Figure 2: Organization of the Ad genome.

(Taken From Hitt et al, 1994)

The linear double stranded Ad5 genome (~36 000 base pairs) is shown as a double line. While the graduated numbers shown in bold, as multiples of ten ,above the genome represent map units (m. u.; 1 m. u.= 360 base pairs), arrows symbolize mRNAs and their direction of transcription. Messenger RNAs from early and late regions are indicated as light and bold lines repectively (Note: some E2 mRNAs are synthesized at late times). Late transcription (shown by the open arrow) originates from the major late promoter and terminates near the right end of the genome. Although this transcript is processed into five families of late mRNAs spliced to a common tripartite leader (represented by the numbers 1, 2, and 3), some mRNAs contain additional leaders.



Late Transcription
1 2 3
x y z
EIA EIB
VA1
VA2
EIII
0 10 20 30 40 50 60 70 80 90 100
IVa2
EIV
EII

E3 gives rise to one transcript which produces 9 overlapping mRNAs (Chow et al., 1979). Five E3 translation products of 19, 14.7, 14.5, 10.4, and 6.7 kd have been identified (reviewed in Wold and Gooding, 1991). Although the cassette of E3 genes is not required for productive infection in culture (Jones and Shenk, 1979), its gene products are involved in virus-host cell interactions and in mechanisms for the protection of infected cells from host immune surveillance. For instance, the Ad5 E3 19 kd protein, an integral membrane protein of the endoplasmic reticulum, inhibits the transport of major histocompatibility complexes to the cell surface, thereby preventing lysis of infected cells by cytotoxic T lymphocytes (reviewed by Andersson et al., 1985). The 14.7, 14.5, and 10.4 kd plasma membrane proteins play roles in virus-host cell interactions by preventing tumor necrosis factor- but not E1A-mediated apoptosis as well as down-regulating expression of the epidermal growth factor receptor (for review, see Wold, 1993). Additionally, the 14.5 kd and 10.4 kd proteins appear to play a role in translational control of E1A since they interfere with the translation of E1A-specific mRNA (Zhang et al., 1994).

E4, located at the extreme right end of the genome, yields a primary transcript that is processed to give rise to at least 5 mRNAs (Chow et al., 1979). Proteins of 34, 19.5, and 14 kds are translated from these mRNAs (Downey et al., 1983; Cutt et al., 1987). Polypeptides generated from this region are essential for a productive infection as they are needed for efficient viral replication and late gene expression as well as cessation of host macromolecular synthesis. To facilitate viral replication, the Ad5 E4 19.5 kd protein in direct association with a heterodimer of the transcription factors E2F-1 and DP-1 transactivates expression from the E2 promoter (Hardy and Shenk, 1989; Obert et al., 1994; Helin and Harlow, 1994). The Ad5 E4 34 kd protein is not only involved in the process of halting the synthesis of host macromolecules (Cutt et al., 1987), but interacts with the E1B 55 kd protein to allow accumulation of late viral mRNAs (Cutt et al., 1987; Bridge et al., 1991). No function has been documented for the 14 kd protein.

During the late phase of viral replication, mRNAs for all late gene products are spliced from a primary RNA molecule transcribed from the major late promoter (Ziff and Evans, 1978). Each late primary RNA transcript is processed into one of five different mRNAs (L1-L5) containing common tripartite leader sequences and 3' ends (Sussenbach, 1984). Translation products from the L1-L5 mRNAs function as capsid components as well as proteins required for the assembly of virions and packaging of the viral genome.

d) Replicative and Transforming Cycles

Human Ads undergo a series of steps during infection of permissive cells which culminate in lysis of the host cell and release of progeny virus (reviewed by Horwitz, 1990 and references therein). The first step involves attachment of the virion to the cell membrane by virtue of its fibre protein. Once attached, the virion has recently been proposed to enter the cell by internalization and/or penetration of the cytoplasmic membrane, a process which is initiated by the interaction of the penton base protein with the alpha v beta 3 and alpha v beta 5 integrins (Wickham et al., 1993, 1994; Huang et al., 1995). Following entry, virions are shuttled to the nucleus in a manner which causes loss of the capsid. In the nucleus, most of the remaining viral genome-associated proteins are shed in preparation for viral gene expression and DNA replication. Expression of viral genes is temporally regulated as a result of transcription first from early and subsequently late transcription units. Early region 1 (E1), which is transcribed immediately after infection, consists of two transcription units encoding the E1A and E1B genes. E1A is expressed approximately 1 hour after infection and is required for expression of the other early genes (E1B, E2, E3, and E4) at 1.5 to 2 hours post infection. The early genes produce proteins, prior to the onset of viral DNA replication, which play roles in viral gene expression, DNA replication, evasion from cellular immunosurveillance components, and host-protein synthesis cessation. Following the onset of viral DNA replication, at approximately 7 hours post infection, late gene transcription commences and reaches a peak

level at about 18 hours post infection. For efficient and maximum synthesis of viral structural proteins during the late phase, cellular DNA and protein synthesis is dramatically reduced. At approximately 24 hours post infection the virion is assembled and the viral genome packaged. The replication cycle of the virus concludes as the cell is lysed releasing 1000-10 000 virions, depending on the serotype which originally infected the cell.

Infection of primary rodent cells, which are not totally permissive for Ad replication, does not result in efficient production of progeny virus. However, by mechanisms that are not yet entirely understood, infection of these cells can lead to morphological transformation. When primary rodent cells were transfected with a variety of DNA fragments from the Ad genome, it was discovered that E1 (E1A and E1B genes) was solely required for transformation (Graham et al., 1974a,b; van der Eb et al., 1979). Interestingly, transfection of permissive human kidney cells with sheared Ad5 or Ad12 genomic DNA or E1 regions also resulted in transformation by E1 (Graham et al., 1977; Byrd et al., 1982). The activities of E1A and E1B gene products and their roles in transformation will be discussed in section 4. Although E1A and E1B play central roles in transformation, It should be noted that other genes also influence transformation in viral-mediated assays. For example, viruses carrying mutant viral DNA polymerases demonstrated impaired transforming activity (Miller and Williams, 1987).

3) Early Region 1 (E1) Transcripts and Translation Products

In this section, features of human Ad E1A and E1B transcription and translation products will be briefly summarized. Data presented has been obtained almost entirely from studies with the highly characterized E1A and E1B genes of the closely related Ad5 and Ad2 viruses. Because of their relevance to research presented in this thesis, features of Ad12 E1A and E1B transcripts and translation products will also be included.

a) E1A

E1A is a transcription unit within E1. The Ad5 E1A region produces two major mRNAs (12S and 13S) which share a common 3' exon and differ only in the size of the intron removed (Berk and Sharp, 1978; Chow et al., 1979; Kitchingman and Westphal, 1980; Perricaudet et al., 1979) and three minor mRNAs - 11S (Ulfendahl et al., 1987), 10S (Stephens and Harlow, 1987), and 9S (Chow et al., 1979) which also share the common 3' exon but differ in the 5' exon sequences they encode (see Fig. 3A and 3B). The translation products encoded by the 13S, 12S, 11S, 10S, and 9S mRNAs are predicted to contain 289, 243, 217, 171, and 55 amino acids respectively (Fig. 3C shows the structure of the major 289 and 243 amino acid Ad5 E1A proteins). *In vitro* translation of mRNA recovered following hybridization to the E1A region, yields many highly related polypeptides ranging in size from 35-55 kilodaltons (Lewis et al., 1976; Halbert et al., 1979; Esche et al., 1980; Halbert and Raskas, 1982). Four major E1A proteins, ranging in size from 45-52 kilodaltons, and a number of minor E1A proteins have been immunoprecipitated from Ad-infected cells (Yee et al., 1983, 1985a; Harlow et al., 1985).

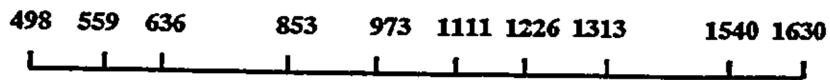
The E1A region of Ad12 (Fig. 3B) encodes two mRNAs which are very similar in structure to the 12S and 13S mRNAs of Ad2 and Ad5 (Perricaudet et al., 1980; Sawada and Fujinaga, 1980; Saito et al., 1981) but the presence of Ad12 mRNAs corresponding to the 11S, 10S, and 9S mRNAs of Ad2 or Ad5 has not been documented. Translation products encoded by the Ad12 E1A 13S and 12S mRNAs are predicted to contain 266 and 235 amino acids respectively (Perricaudet et al., 1980). As with Ad5 E1A proteins, Ad12 E1A proteins have been identified by *in vitro* translation of E1A specific mRNAs, selected by hybridization to E1A region DNA, and by immunoprecipitation from infected cells. (Segawa et al., 1980; Lucher et al., 1984; Scott et al., 1984). These studies revealed two polypeptides which range in size from 35-37 and 45-47 kilodaltons.

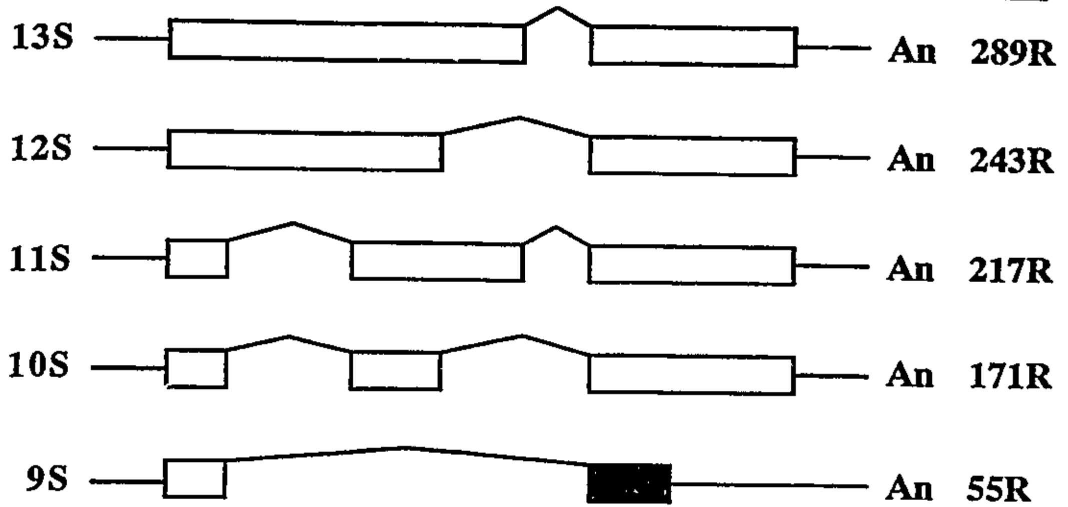
E1A gene products possess an unusually high proline content (16%). Since prolines are known to disrupt secondary structure in proteins, E1A proteins are believed to

Figure 3: Structure of the Ad5 E1A and Ad12 E1A mRNAs and of the major Ad5 E1A gene products.

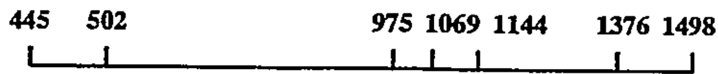
In A and B, Ad5 and Ad12 mRNAs are respectively represented by horizontal lines (open reading frames are denoted by open boxes, and introns by carets). The horizontal lines above the mRNAs show the nucleotide positions of the exon-intron boundries. By virtue of their sedimentation coefficients, mRNAs are designated 13S,12S, 11S, 10S, and 9S. The solid 9S exon represents a different open reading frame. Polyadenylation sequences are represented by "An". C is a schematic of the major Ad5 E1A proteins.

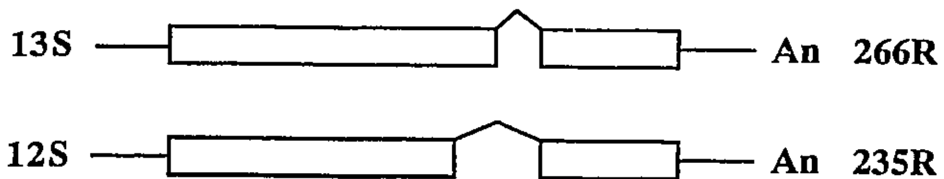
A)



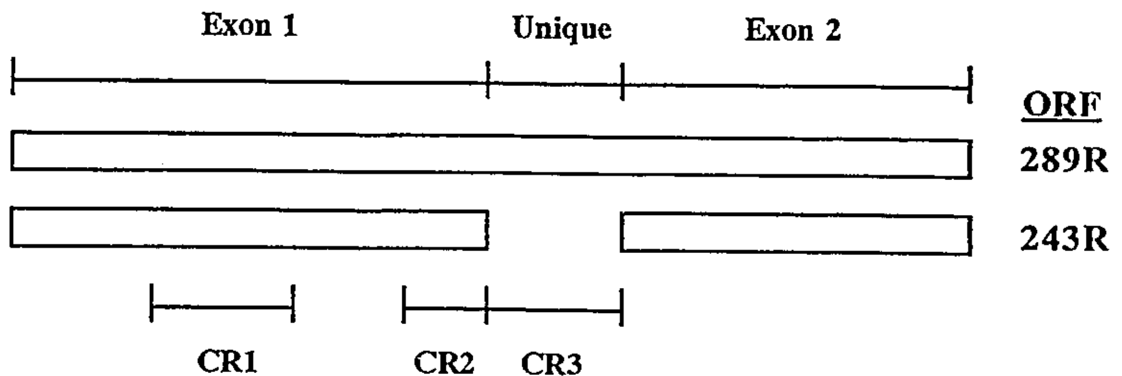


B)





C)



lack such structure. These proteins are acidic with isoelectric points ranging between pH 4 and 5 (Grand, 1987), and the acidic residues are clustered within three regions that are highly conserved among various Ad serotypes (Flint and Shenk, 1989) designated conserved region 1 (CR1), CR2, and CR3 (van Ormondt et al., 1986). While CR1 and CR2 are located in the amino terminal region common to the 13S and 12S mRNA translation products, CR3 is unique to the 13S product (see Fig 3C). The major E1A proteins are degraded rapidly with a half-life of 20-80 minutes in infected cells and approximately 2 hours in transformed cells (Rowe et al., 1983; Spindler and Berk, 1984). They are also extensively phosphorylated at a number of serine residues and consequently migrate at a retarded rate during SDS-PAGE (Tremblay et al., 1988). The significance of E1A phosphorylation is unclear, since mutation of different phosphorylation sites appears to have no dramatic effect on biological activity of the protein (Tremblay et al., 1989).

b) E1B

The primary transcripts of the Ad5 and Ad2 E1B regions are alternatively spliced into 2.2 and 1 kb mRNA products (Berk and Sharp, 1978; Chow et al., 1979; Kitchingman and Westphal, 1980; Perricaudet et al., 1980). Two minor mRNAs are also among the E1B transcripts (Virtanen and Pettersson, 1985). These transcripts contain 3 exons, the first two of which are derived from the 1 kb mRNA and the third, situated between these exons, from the 2.2 kb mRNA. Two long, partially overlapping open reading frames are contained within the 2.2 kb mRNA (Bos et al., 1981) from which proteins of 175 and 495 residues are translated. Translation products of 155, 92, and 82 amino acids which are structurally related to the 495 residue protein are also observed (Virtanen and Pettersson, 1985). As with the E1A proteins, confirmation of these E1B proteins came when their mRNAs were *in vitro* translated (Halbert et al., 1979; Esche et al., 1980; Lupker et al., 1981) and immunoprecipitated (Lassam et al., 1979; Ross et al., 1980), resulting in detection of large and small proteins of 50-65 and 15-21 kilodaltons

respectively. Using anti-peptide antisera, Green et al., 1983 and Yee et al., 1983 determined that the large and small E1B gene products were translated into 55 and 19 kilodalton proteins from the 495 and 175 amino acid open reading frames. The E1B 55 kilodalton protein was shown to be phosphorylated and by immunofluorescence and cell fractionation studies to be present in both the nucleus and cytoplasm (Sarnow et al., 1982; Yee et al., 1983). McGlade et al., 1987; 1989 demonstrated that the majority of 19 kilodalton (19 K) E1B proteins were acylated and a minor fraction phosphorylated. 19 K was also found in the nucleus and cytoplasm, and was found to be membrane associated and localized to the nuclear envelope (Persson et al., 1982).

Transcription of the E1B region of Ad12 produces 3 mRNAs, one 2.2 kb in size and similar to the large E1B mRNA of Ad5 and Ad2, and two of approximately 1 kb in size (Virtanen et al., 1982). As with the larger Ad5 E1B transcript, the corresponding Ad12 E1B transcript contains two long, partially overlapping open reading frames (ORFs) encoding 163 and 482 amino acid gene products (Bos et al., 1981). Two smaller mRNAs contain the 163 residue ORF and a 110 amino acid ORF. In addition to a large (50-59 kd) and smaller protein of 19 kd (Jochemsen et al., 1980), proteins of 17 kd, 15 kd, and 14 kd have been translated from the Ad12 E1B region (Mak and Mak, 1986). While the larger protein, referred to as the 55 kd protein, and the 19 kd product are encoded by the 482 and 163 aa ORFs, the 17, 15 and 14 kd products remain to be characterized. Like Ad5 E1B 55 and 19 kd proteins, the corresponding Ad12 E1B proteins are also phosphorylated and found in the nucleus and cytoplasm (Grand and Gallimore, 1984). Although the 19 kd Ad12 E1B protein is also membrane associated and acylated, the fatty acid moeity is linked differently (McGlade et al., 1987).

4) Functions of E1A Gene Products

a) Regulation of Gene Expression and Cellular Proliferation Through Interaction With Cellular Proteins

The major E1A proteins participate in a wide variety of viral and cellular processes. During lytic infection, they facilitate viral replication by activating transcription of other early viral genes (50-100 fold enhancement), in *trans* (Berk et al., 1979; Jones and Shenk, 1979) and they induce cellular proliferation (Braithwaite et al., 1983; Spindler et al., 1985). Since the natural host cells for Ad infection are growth arrested epithelial cells, the ability of E1A proteins to induce their proliferation creates a favorable setting for viral replication. E1A's capacity to promote cellular proliferation is believed to be linked to its potential to influence cellular gene expression. To this end, E1A proteins have not only been shown to transactivate RNA polymerase II (Gaynor et al., 1984; Svensson and Akusjarvi, 1984) and III (Hoeffler and Roeder, 1985; Gaynor et al., 1984) transcribed cellular genes (endogenous and exogenous) as well as other viral genes (Table 2), but to repress a variety of transcriptional enhancers (Table 3) (reviewed by, Branton et al., 1985; Bayley and Mymryk, 1994). During evolution, E1A proteins have also acquired the ability to immortalize a variety of primary mammalian cells in culture (Houweling et al., 1980), and, in cooperation with E1B or another oncogene such as activated ras, to fully and oncogenically transform them (Graham et al., 1974a; Ruley, 1983). Since the ability to induce tumors can not offer an obvious selective advantage to cells, it is likely a side effect of E1A's ability to promote cellular proliferation and therefore enhance viral yield. While the combination of functions which allows E1A to participate in Ad replicative and transforming cycles is uncertain, it is clear that the major function of E1A proteins is to drive cellular proliferation by influencing gene expression.

Despite the nuclear localization of the major E1A proteins, there is little evidence to suggest that they influence gene regulation via a direct DNA interaction (Ferguson et al., 1985; Chatterjee et al., 1988). In contrast, extensive evidence supports the notion that E1A

Table 2: Cellular genes transcribed by RNA polymerase II and induced by Ad5 E1A‡.

Endogenous Genes	Exogenous Genes *
c-fos	c-fos
c-jun	c-jun
nm23 for nucleoside diphosphate kinase	c-myc
Genes For:	Genes For:
β-tubulin	β-globin
creatine kinase	hsp 70
cyclin A	PCNA
heat shock proteins (hsp) 70 and 90	preproinsulin
p34cdc2 kinase	
proliferating cell nuclear antigen (PCNA)	
thymidylate synthase	
topoisomerase I	

‡ Modified from Bayley and Mymryk, 1994.
* These genes were introduced by transfection. References are shown in a similar table presented by Bayley and Mymryk, 1994.

Table 3: Viral and cellular genes repressed by Ad5 E1A‡.

DNA Tumor Virus Genes*
SV40 enhancer and early genes Polyoma enhancer plus early and late genes Adenovirus E1A enhancer and E2A late promoter
Cellular Genes*
c-myc, JE, neu <u>Genes For:</u> collagen type 1 cyclin D cytochrome P-450c fibronectin CD44 immunogobulin chains insulin interleukin 6 phosphoenolpyruvate carboxykinase stromolysin collagenases urokinase <u>Differentiation-specific genes for:</u> Muscle: α-actin, myosin heavy chain, creatine kinase, troponins 1 and T Nerve: NGF receptors, $p140^{trk}$, $p75^{NGFR}$ <u>Genes stimulated by interferon</u>

‡ Modified from Bayley and Mymryk, 1994.
* References for the genes listed are shown in a similar table presented by Bayley and Mymryk, 1994.

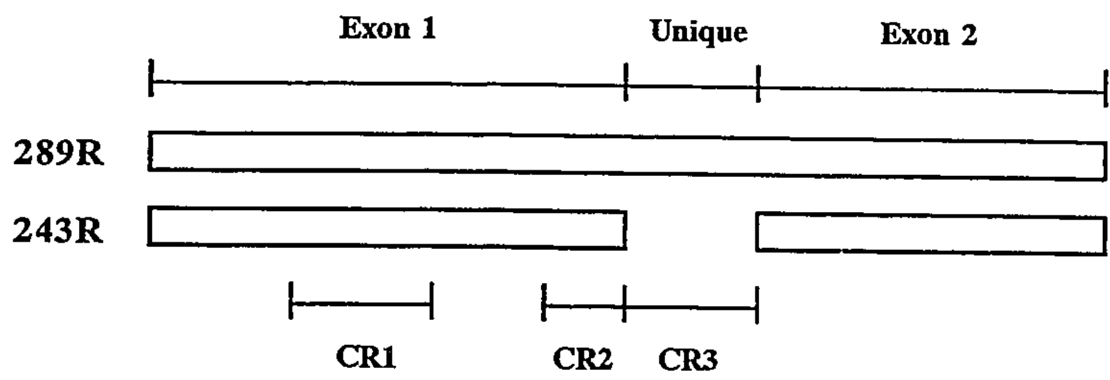
proteins mediate processes involved in regulating gene expression by forming complexes with key cellular factors. Cellular proteins of 400, 300, 130, 107, 105, 60, and 33 kds (designated p400 etc.) have consistently been found to associate with E1A proteins (Yee and Branton, 1985b; Harlow et al., 1986; Egan et al., 1987, 1988, 1989; Whyte et al., 1988; Giordano, 1989). Figure 4 shows a map of the major Ad5 E1A proteins and regions necessary for interaction with the cellular proteins mentioned above. Although identification of the E1A-associated cellular proteins was based on studies which analyzed co-immunoprecipitates from lysates of radioactively labelled cells and from *in vitro* studies examining cell lysate protein binding to bacterially produced E1A proteins, interactions with other cellular proteins which also play roles in regulating gene expression and cell growth (cyclins, cyclin-dependent kinases, and the TATA-binding protein (TBP)) were subsequently identified using more sensitive methods (reviewed by Barbeau et al., 1994; Bayley and Mymryk, 1994).

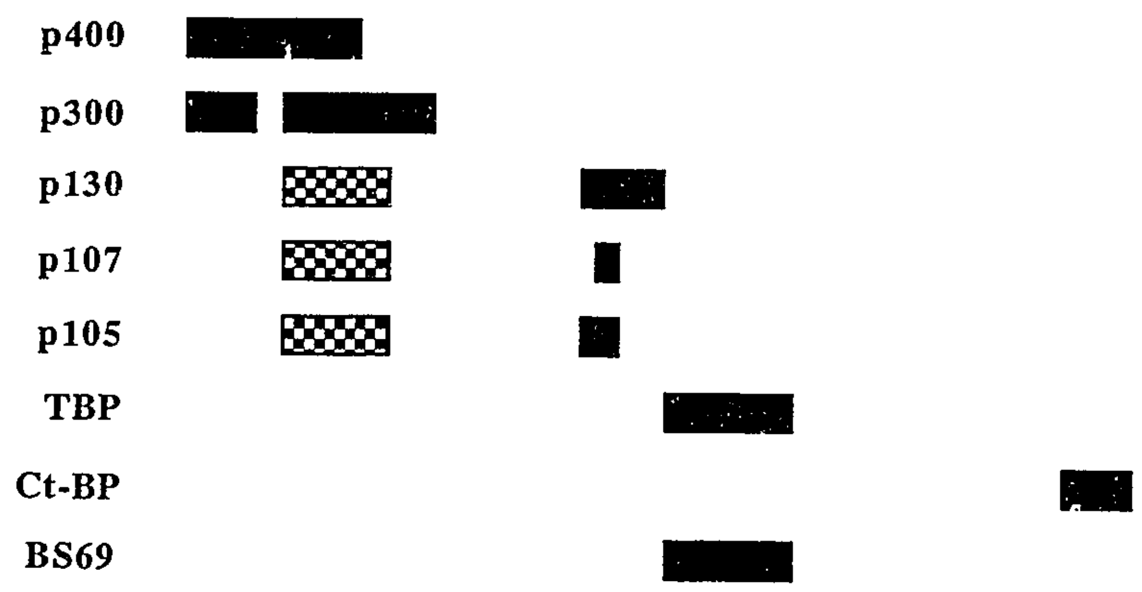
Rb Protein Family

p105 (the product of the retinoblastoma gene, Rb-1 (Lee et al., 1987)), p107 (Ewen et al., 1991), and p130 (Li et al., 1993) are a group of related proteins belonging to the Rb protein family. Members of this family are thought to function as tumor suppressors since the chromosomal regions containing the p105 and p130 genes are frequently deleted in retinoblastomas and variety of human tumors (Weinberg, 1991). Interestingly, these tumor suppressors appear to regulate the cell cycle and are themselves regulated by phosphorylation in all phases of the cell cycle. The phosphorylation state of p105, for example, is un- or hypo-phosphorylated in G_0/G_1, hyperphosphorylated through S/G_2, and hypo-phosphorylated during mitosis (Buchkovich, 1989). This suggests that the hypo-phosphorylated forms of p105 and of the other Rb family members negatively regulate the cell cycle. When complexed to E1A, however, the normal activities of the Rb family members and the proteins they interact with, are presumably altered, a change which

Figure 4: Major Ad5 E1A proteins and regions necessary for cellular protein binding.

Translation of the 13S and 12S Ad5 E1A mRNAs give rise to the 289 residue (289 R) and 243 R protein products respectively. The boundries of conserved protein sequence regions 1 (CR1), CR2, and CR3 are marked by horizontal and vertical lines which also outline the boundries of protein regions encoded by exon 1, 2, and the unique segment (found only in 289 R protein). Regions of the major Ad5 E1A protein products necessary for cellular protein binding are shown by closed and checkered boxes which respectively represent regions of primary and secondary importance. Cellular proteins other than Ct-BP and BS69 are designated on the basis of size (e. g. p400).


Exon 1
Unique
Exon 2
289R
243R
CR1
CR2
CR3


p400
p300
p130
p107
p105
TBP
Ct-BP
BS69

which positively influences gene expression and cell growth through multiple pathways (pathways reviewed by Bayley and Mymryk, 1994; Moran, 1994). One such pathway involves the ability of E1A-p105/107/130 complexes to affect the availability of the E2F/DRTF1 family of transcription factors. E2F/DRTF1 factors bind only the hypo-phosphorylated G_0/G_1 forms of Rb family members (Bandara and LaThangue, 1991). By virtue of its interaction with these members, E1A can liberate E2F/DRTF1 factors. Once free, E2F factors, for example, can activate transcription from the Ad E2 promoter (Bagchi et al, 1990) and from a variety of cellular promoters which drive expression of S-phase specific genes (e.g. c-myc (Hiebert et al., 1989)). Alternatively, E1A-Rb family member complexes can positively or negatively influence gene expression and cell growth by affecting the synthesis and phosphorylation states of a variety of transcription factors including the E2F/DRTF1 family and c-Jun, a component of the AP-1 family. This finding is linked to the ability of particular E1A-p105/p107/p130 complexes to associate with different cyclins (A-E) and cyclin-dependent kinases (cdks) which play key regulatory roles at different stages of the cell cycle (for review, see Bayley and Mymryk, 1994; Moran, 1994).

p300

Another pathway by which E1A can influence gene expression and cellular proliferation is through its interaction with p300. Several features of its gene sequence, derived from a recently cloned cDNA, and studies analyzing its protein product suggest that p300 is involved in regulating transcription: 1) the p300 protein which is actively phosphorylated in all phases of the cell cycle and present in the nucleus of a variety of mammalian cell types (Yaciuk and Moran, 1991) also possesses intrinsic DNA-binding activity with specific affinity for enhancer motifs, including MHC class I H2TF1 enhancer motif (Rikitake and Moran, 1992); 2) p300 is a component of TATA-binding protein (TBP) complexes (Abraham et al., 1993); 3) a so-called bromodomain protein sequence motif in

p300 mediates protein-protein interactions and is present in other transcriptional activators such as the TBP-associated factor, $TAF_{II}250$/CCG1 (Eckner et al., 1994); 4) p300 is a functional homologue of CREB's transcriptional co-activator CBP (Lundblad et al., 1995), and like CBP, p300 belongs to a family of transcriptional adaptor proteins which can stimulate transcription (Arias et al., 1994; Arany et al., 1994, 1995); 5) experiments where p300 cDNAs were over-expressed not only revealed that p300 could partially overcome repression of the SV40 transcriptional enhancer by E1A, but suggested that p300 participates in activating this enhancer (Eckner et al., 1994).

The latter point suggests that binding to p300 offers another means by which E1A proteins can modulate transcription. This is supported by two main bodies of evidence. First, E1A-p300 complexes, like E1A-Rb family complexes, have been associated with the repression of a variety of viral and tissue specific promoters (Jelsma et al., 1989; Stein et al., 1990; Wang et al., 1993; Arany et al., 1995). Second, the ability of E1A to interact with p300 and affect its phosphorylation state has been shown to mediate increased transcription of c-Jun during the differentiation of F9 cells (Kitabayashi et al., 1995), a finding which also suggests that the phosphorylation state of p300 may influence whether certain cells will differentiate or remain undifferentiated.

TATA-Binding Protein

Other regions of E1A besides the first exon are able to bind cellular proteins and influence transcription. Conserved region 3 (CR3), for example, endows E1A with a trans-activation potential which far exceeds that of the first exon alone; it allows the larger major E1A protein to transactivate most of the Ad early genes and many cellular genes (reviewed by Berk, 1986; Shenk and Flint, 1991). To serve as a powerful transactivator, two domains within CR3 promiscuously bind cellular factors involved in transcriptional regulation. By virtue of a zinc finger protein-binding motif in its amino-terminal domain, CR3 can bind TBP (Geisberg et al., 1994) and activate transcription by initiating the

formation of a multiprotein transcription complex with RNA polymerase II. E1A can also target several promoters through interactions between a carboxy-terminal domain of CR3 and the DNA-binding domains of a variety of cellular transcription factors including ATF-2, c-Jun and Sp1 (Liu and Green, 1994).

To date, only two cellular proteins, Ct-BP and BS69 have been found to interact with the second exon of E1A. While Ct-BP, a nuclear phosphoprotein, appears to induce immortalization and transformation (Boyd et al., 1993), the BS69 protein has been shown to inhibit E1A transactivation (Hateboer et al., 1995).

b) Inhibition of Cellular Differentiation

Since E1A proteins promote cellular proliferation, it is not surprising that they inhibit differentiation of neuronal (Maruyama et al., 1987) and muscle (Webster et al., 1988) cells. The smaller major E1A protein (lacks CR3) is thought to mediate suppression of cellular differentiation by down-regulating the expression of differentiation-specific genes such as those of the DNA-binding MyoD family of transcription factors (MyoD, myogenin, Myf-5, and Myf-6) that regulate muscle differentiation. Although evidence exists supporting a mechanism whereby E1A inhibits cellular differentiation via association with p105 or p300 (Heasley et al., 1991; Caruso et al., 1993; Kitabayashi et al., 1995), the specific details are far from understood.

c) Induction of Apoptosis

Although it is well established that the principal function of the major E1A proteins is to drive cellular proliferation during lytic infection or transformation, these proteins can also trigger apoptosis (cell death) in certain host cell types (White and Stillman, 1987). Apoptosis begins with the appearance of enhanced cytopathic effect (cell shrinkage, membrane blebbing, detachment from culture dish) and concludes with the degradation of viral and cellular DNA as well as condensation of chromatin within the nucleus (Whyllie,

1980). E1A-mediated induction of apoptosis is most apparent in the absence of E1B 19 kd (19K) or 55 kd (55K) proteins, suggesting that 19K and 55K serve to inhibit apoptosis (see section 5). The regions of E1A required for induction of cellular proliferation are also needed for apoptosis induction, suggesting that p300 and the Rb protein family may participate in apoptosis induction by E1A (White et al., 1991). In addition, several lines of evidence suggest that E1A triggers apoptosis through the activities of the cellular tumor supressor protein, p53 (reviewed by White, 1994). Taken together, it is likely that E1A induces apoptosis through p300, Rb family members, and/or p53 by activating and/or repressing expression of apoptosis inducing and suppressing genes respectively. Alternatively, it has been proposed that apoptosis may simply arise due to an imbalance in the cell cycle created by the opposing growth signals of E1A and p53 (Debbas and White, 1993). This imbalance may explain the observation that apoptosis induced by Ad5 E1A in rat cells is concomitant with a block in cellular proliferation (Mymryk et al., 1994).

5) Functions of E1B Gene Products

a) Regulation of mRNA Processing and Transport

The Ad5 E1B gene encodes two major products (55K and 19K) that play significant roles in infected and transformed cells. By forming stable complexes with the E4 34 kd protein (Halbert et al., 1985) in productively infected human cells, 55K facilitates viral replication by mediating efficient transport of late viral messages to the cytoplasm and inhibiting accumulation of host mRNAs (Pilder et al., 1986).

b) Inhibition of Apoptosis

E1B 55K also binds to and inhibits the function of the p53 tumor supressor gene product (Sarnow et al., 1982), an event which is thought to inhibit the ability of p53 to mediate apoptosis induced by E1A. Thus, 55K has been proposed to function as an inhibitor of apoptosis. E1B 19K proteins are also thought to inhibit apoptosis since

permissive cells infected by Ads carrying defective E1B 19K proteins undergo apoptosis (Pidler et al., 1984; White et al., 1984) as do cells transformed by E1A in the absence of E1B 19K or 55K (Rao et al., 1992). Moreover, 19K proteins may maintain DNA integrity and therefore inhibit apoptosis due to their association with cytoplasmic and nuclear membranes, intermediate filaments in the cytoplasm, and the nuclear lamina (Persson et al., 1982; White et al., 1984; White and Cipriani, 1989). It is likely that 19K acts to inhibit p53-dependent and probably p53-independent apoptosis pathways in a manner resembling that of the potent apoptosis inhibitor, the human Bcl-2 protooncogene (for review see, White, 1994).

As with the major E1A proteins, the endpoint of E1B 55K and 19K protein activity is to drive cellular proliferation and facilitate viral replication. That the major E1B proteins play a significant role in driving cellular proliferation is evident by their cooperation with E1A proteins to fully and oncogenically transform a variety of primary cells in culture.

6) Adenovirus Mediated Tumorigenesis

a) E1A - A Major Determinant

On the basis of their ability to induce tumors in hamsters, the human Ad serotypes have been classified into three groups: highly tumorigenic (e.g. types 12, 18, and 31), weakly tumorigenic (e.g. types 3 and 7), and non-tumorigenic (e.g. types 2 and 5) (Trentin et al., 1962; Huebner et al., 1962). While the tumorigenic capacity of Ad-infected cells requires co-expression of E1A and E1B, the serotypic origin of E1A is the major determinant for tumorigenicity (Bernards et al., 1983; Jochemsen et al., 1984). This conclusion was based on experiments which examined the tumorigenicity of rat cells transformed by different combinations of Ad5 and Ad12 E1A and E1B proteins in syngeneic immunocompetent and nude hosts (Bernards et al., 1983) (for results, see Table 4). These experiments also demonstrated the ability of Ad12 E1B proteins to enhance the tumorigenic capacity of Ad12 E1-transformed rodent cells.

Table 4: Tumorigenicity of rat cells transformed by different combinations of Ad5 and Ad12 E1A and E1B genes‡.

E1A	E1B	Syngeneic Rats	Nude Mice
5	5	0/51	15/31
12	12	18/18	23/23
5	12	0/26	18/18
12	5	6/60	2/19
5+12	12	0/18	12/12

‡ Modified from Bernards et al., 1983.
Transformed cell lines were established following transfection of primary baby rat kidney cells with different combinations of individual plasmids carrying Ad5 and Ad12 E1A and E1B genes. These lines were subcutaneously injected into litters of newborn syngeneic rats and nude mice. Results are represented as the number of tumorigenic animals / number of animals injected.

b) **MHC Class I Expression**

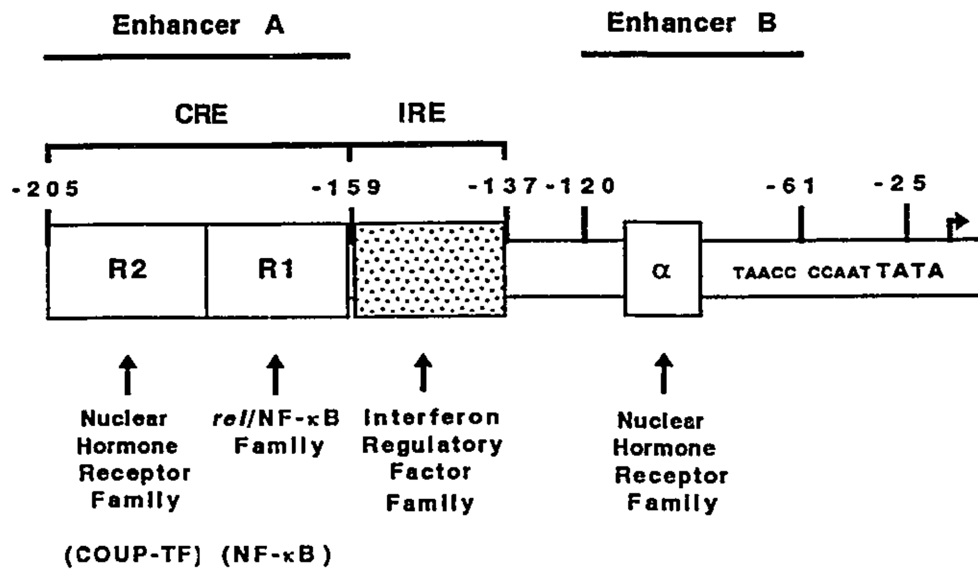
Many studies directed at identifying and understanding functional differences between Ad5 and Ad12 E1A have suggested that E1A influences tumorigenicity of Ad5 (non-tumorigenic) and Ad12 (tumorigenic) transformed rodent cells by modulating the host immune response of the immunocompetent rodent. For instance, while both the Ad5 and Ad12 E1A products can determine viral specificity of tumor specific transplantation immunity (Sawada et al., 1986, 1994), Ad12 E1A, unlike Ad5 E1A participates in down-regulating expression of MHC class I molecules in rodent (Schrier et al., 1983; Vaessen et al., 1987; Ackrill and Blair, 1988; Friedman and Ricciardi, 1988) and human cells (Vasavada et al., 1986).

At the transcriptional level (Ackrill and Blair, 1988; Friedman and Ricciardi, 1988), Ad12 E1A mediates MHC class I down-regulation through the H-2K^{b} class I enhancer (Fig. 5) which binds many transcription factors: NF-κB (Israel et al., 1987), KBF1 (Yano et al., 1987), H2TF1 (Baldwin and Sharp, 1988), MBP-1 (Baldwin et al, 1990), H2RIIBP (Hamada et al., 1989), and COUP-TF (Kralli et al., 1992; Liu et al., 1994) as well as other uncharacterized factors (Ackrill and Blair, 1989; Proffitt et al., 1994). In contrast to Ad5 E1-transformed cells, a drastic reduction of NF-κB binding to the class I enhancer has been reported in Ad12 E1-transformants (Nielsch et al., 1991; Meijer et al., 1992), a discovery which may explain their reduced class I levels and increased tumorigenic capacities.

NF-κB, which binds to the positive regulatory R1 element in the class I enhancer (Baldwin and Sharp, 1988) and activates transcription (Plaksin et al., 1993; Segars et al., 1993), is ubiquitously expressed and composed of 50 (p50) and 65 (p65) kd subunits. The genes encoding p50 (NF-κB1), its precursor, p105, and p65 (RelA) are members of the intensively studied NF-κB/Rel family of DNA binding transcription factors. Other family members and the genes encoding them (in parentheses) include NF-κB2 (p52 and its precursor, p100), Rel (c-Rel), and RelB (review by Nolan and Baltimore, 1992).

Figure 5: H2-K^{b} MHC class I enhancer.

This schematic represents the upstream regulatory nucleotide sequences of the murine H2-K^{b} class I enhancer. Elements (α, IRE, and CRE (R1 and R2)) are represented by the largest boxes. Cellular protein families which bind the enhancer elements are listed beneath the arrows, NF-κB and COUP-TF (shown in parentheses) are examples of cellular factors binding the R1 and R2 elements respectively.



Enhancer A
Enhancer B
CRE
IRE
-205
-159
-137
-120
-61
-25
R2
R1
α
TAACC CCAAT TATA
Nuclear Hormone Receptor Family
rel/NF-κB Family
Interferon Regulatory Factor Family
Nuclear Hormone Receptor Family
(COUP-TF)
(NF-κB)

c) Cellular Immune Response

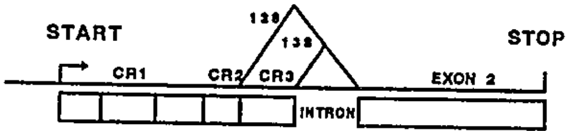
Because Ad12 E1A mediates MHC class I repression, it has been generally accepted that Ad12 E1-transformed cells induce tumors in syngeneic immunocompetent rodents by evading the surveillance of host MHC class I-restricted $CD8^+$ CTLs. Several reports, however, have failed to find a strict correlation between reduced class I expression and tumorigenicity (Mellow et al., 1984; Haddada et al., 1986; Shemesh *et al.*, 1991; Soddu and Lewis, 1992; Shemesh and Ehrlich, 1993). Consequently, it has been proposed that non-specific effectors of the immune system, such as NK cells, may play an important role in tumorigenesis since rodent cells transformed by Ad5 or Ad12 E1A have respectively demonstrated susceptibility and resistance to lysis by NKs (Raska and Gallimore, 1982; Sawada et al., 1985; Cook et al., 1986, 1987; Routes, 1993). In addition, differences in the abilities of Ad5 and Ad12 E1A molecules to serve as immunogenic determinants on transformed cells may also contribute to the oncogenicity of Ad5 and Ad12 E1 transformants. This hypothesis is reasonable since CTL stimulating epitopes encoded by Ad5 E1A gene products, have been found on cell surfaces in context with class I antigens (Bellgrau et al., 1988; Kast et al., 1989; Urbanelli et al., 1989; Rawle et al., 1991; Routes et al., 1991).

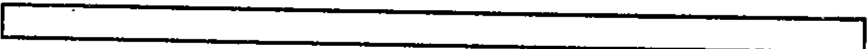
7) Research Objectives

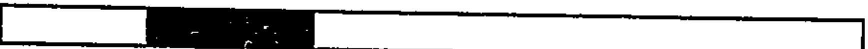
In this laboratory, a series of hybrid Ad5/12 E1A genes (Fig. 6) were previously constructed and used to create hybrid Ad5/12 E1A-containing Ads and to transform primary Hooded Lister baby rat kidney (BRK) cells in combination with an Ad12 E1B gene (Jelinek and Graham, 1992). The major conclusions derived from this study were: 1) recombinant Ads expressing all these hybrid E1A proteins were capable of efficiently supporting viral replication, with the exception of one construct (p1036) which contained a hybrid CR3 transactivation domain; 2) plasmids containing Ad12 sequences encoding the amino terminus of E1A were approximately 20-fold less efficient at transforming BRK

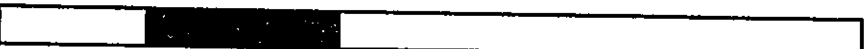
Figure 6: Schematic of Ad5 E1A (pXC1), Ad12 E1A (pHAB6), and hybrid Ad5/12 E1A plasmids used to transform Hooded Lister rat kidney cells.

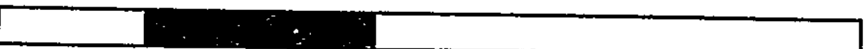
Ad5 and Ad12 E1A gene sequences are shown as open and closed boxes respectively and are aligned with the structure of the Ad5 E1A protein (shown above genes). The three conserved regions (CR1, CR2, and CR3) in the first exon of E1A are also denoted. Precise locations of crossovers are expressed in nucleotides below the crossover junctions, where Ad5 and Ad12 nucleotides are shown on the outer and inner sides of the "/" respectively. All hybrid E1A constructs, with the exception of p917-975 and p917-1227, contain a first crossover junction in which the leftmost 353 base pairs of Ad5, encompassing the viral inverted terminal repetition and enhancer/packaging regions, are followed by Ad12 E1A sequences from nucleotide 290, encompassing the Ad12 E1A promoter, transcriptional start, and amino terminus, to a second junction point where crossover again occurs into Ad5 E1A sequences to 16% of Ad5 genome length. In addition to the first 353 nucleotides of Ad5 common to the hybrid E1A plasmids, the p917-975 and p917-1227 constructs contain Ad5 gene sequences to nucleotide 917 (815; Ad12) followed by Ad12 E1A gene sequences to nucleotides 932 (975; Ad5) and 1142 (1227; Ad5) respectively. It should be noted that the p975 and p917-975 hybrids lack the 12s splice donor sites of Ad5 and Ad12 E1A, therefore these hybrids encode only the 13S E1A product. Plasmids used to transform primary BRKs and representative transformed cell lines are listed.











Plasmid	Representative Transformed Cell Line
pXC1	DP5-2
p690	690B12C1
p753	753B12C4
p827	827B12C1
p975	975B12C4
p1036	1036B12C1
p1227	1227B12C2
p917-975	917-975B12C10
p917-1227	917-1227B12C6
pHAB6	12-1

cells than their Ad5 E1-encoding counterparts, and 3) at least two regions within the first exon of Ad12 E1A (excluding CR3) mediate the difference in transforming efficiency.

By assaying their potential to induce tumors in syngeneic immunocompetent rats, hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed rat cells offer a unique opportunity to identify regions of the major Ad12 E1A proteins which mediate tumorigenesis. The ability of Ad12 E1A, unlike Ad5 E1A, to down-regulate MHC class I expression has generally been accepted as a principal feature determining the different tumorigenic capacities of Ad12 E1- and Ad5 E1-transformed rat cells. However, the regions of E1A responsible for class I down-regulation have not been determined. Thus, measurement of cell surface MHC class I levels on the hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed rat cells would not only allow the identification of Ad12 E1A regions involved in down-regulating class I expression, but may reveal a correlation between these levels and oncogenic potential. Moreover, by studying class I expression in these transformed cells, questions regarding the molecular basis of Ad12 E1A-mediated class I down-regulation may be answered. For example, why is NF-κB binding to the positive regulatory R1 element in the class I enhancer drastically reduced in Ad12 E1 versus Ad5 E1 transformed cells ?

The notion that the tumorigenic capacity of cells transformed by the E1 regions of Ad5 and Ad12 is solely dependent on down-regulation of class I expression is a topic of great debate since the relationship between class I levels and tumorigenicity is poorly defined. To better define these relationships and to determine whether other factors contribute to the different tumorigenic capacities of Ad5 E1- and Ad12 E1-transformed rodent cells, I decided to measure the susceptibility of the hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed rat cells to syngeneic NKs and allogeneic and syngeneic CTLs in order to identify the regions of E1A responsible.

Differences in the tumorigenicity of Ad5 and Ad12 E1-transformants could be explained by differences in the ability of their E1A proteins to associate with cellular proteins. This hypothesis, however, has not been extensively explored. Therefore, the

final objective of my research was to examine the ability of the hybrid Ad5/12 E1A proteins to associate with the cellular proteins that normally interact with Ad5 E1A.

MATERIALS AND METHODS

1. Materials

a) Bacterial Strains

E. coli DH5-α strain (Hanahan, 1983; genotype: *sup*E44, Δ*lac*U169 (φ80*lacZ*ΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*1, *rel*A1) was obtained from Bethesda Research Laboratories and used for routine plasmid cloning procedures.

Due to their ability to form unstable secondary and tertiary structures, many segments of DNA are deleted in conventional *E.coli* strains. A cruciform, caused by inverted repeats such as those found at the termini of the Ad12 genome, is an example of an unstable DNA structure which is very difficult to clone. Consequently, *E. coli* SURE cells (Stratagene) (Greener, 1990; genotype: *e*14-(McrA-), Δ(*mcr*CB-*hsd*SMR-*mrr*)171, *end*A1, *sup*E44, *thi*-1, *gyr*A96, *rel*A1, *lac*, *rec*B, *rec*J, *sbc*C, *umu*C::Tn5 (Kan^r), *uvr*C[F', *pro*AB, *lacI*^{q}ZΔM15, Tn10 (Tet^r) Amy Cam^r]) which were engineered to carry mutations inactivating the pathways catalyzing the elimination of inverted repeats, were utilized to clone Ad12 genomic plasmids and their inverted repeats.

b) Plasmids

The pUC19 (Yanisch-Perron et al., 1985) and pNEB193 (New England Biolaboratories) plasmids were frequently used as vectors for routine cloning procedures.

pJM17 (McGrory et al., 1988), a non-infectious Ad5 genomic plasmid, was used to rescue the p917-975 and p917-1227 hybrid Ad5/12 E1A (plus Ad12 E1B) encoding plasmids into virus.

pXC1 (McKinnon et al, 1982), contains Ad5 sequences from base pair 22 to 5790 (includes E1A and E1B) and was used to transform primary BRK cells.

pHAB6 (Mak et al., 1986), contains the left most 5573 base pairs of the Ad12 Huie strain genome (includes E1A and E1B) inserted into the BamHI site of pBR322. This plasmid was used as a source of Ad12 E1 sequences for general cloning.

To generate Ad12 genomic plasmids capable of replicating in bacteria, the bacterial origin of replication and ampicillin resistance gene present in the pUC19 derived plasmid, pABS.3 (gift of A. Bett), was inserted into pHAB6 Ad12 E1 sequences.

The kanamycin resistance gene contained in the pUC19 derived plasmid, pABS.4 (gift of A. Bett), was used for ampicillin/kanamycin double selection purposes during the construction of Ad12 genomic plasmids.

c) Enzymes

All DNA and protein modifying enzymes were purchased from Boehringher Mannheim, Bethesda Research Laboratories, New England Biolaboratories, or Pharmacia and used according to manufacturers' specifications.

d) Mammalian Cell Lines

A series of Hooded Lister rat kidney cell lines (Fig. 5) transformed by and constitutively expressing Ad5 E1 genes (DP5-2 cells (Pereira et al., 1994)), Ad12 E1 genes (12-1 (Jelinek and Graham, 1992)), and hybrid Ad5/12 E1A plus Ad12 E1B genes (Jelinek and Graham, 1992) were characterized during this research.

702-C2, HABaC1 and HABaC2 (Mak et al., 1979), rat lines transformed by and constitutively expressing Ad12 E1 genes were utilized as comparison controls for cell surface MHC class I expression.

293, a transformed human embryonic kidney cell line which constitutively expresses human Ad5 E1 genes (Graham et al., 1977) was used to propagate and titre wild

type and recombinant Ad5 and Ad12 viruses as well as for the generation of recombinant Ad5 and Ad12 viruses following transfection.

HER3, a human embryonic retinal line which constitutively expresses the Ad12 E1A and E1B genes (Byrd et al., 1982) were employed during transfection procedures aimed at generating recombinant Ad12 viruses.

A2T2C4, an Ad2 Hooded Lister rat embro cell line used as a positive control in syngeneic NK cytolytic assays due to its high susceptibility to lysis by both rat and hamster NK cells (Gallimore et al., 1977).

The following human cell lines served to grow and titre Ad5 and Ad12 wild type and recombinant viruses: KB, a human oral epidermoid carcinoma line; A549, a human lung carcinoma line; and HeLa, a human cervical epithelioid carcinoma line.

All cell lines were grown in monolayers on 100 or 150 mm plastic tissue culture dishes (Nunclon) and unless otherwise indicated were maintained in α-minimum essential medium (α-MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), 100 units/ml penicillin G (Gibco), 100 μg/ml streptomycin sulfate (Gibco) and 2.5 μg/ml Fungizone (Squibb Canada).

e) Viruses

Huie, the wild type human Ad12 strain (Kibrick et al., 1957) was used as a source of viral DNA to create recombinant Ad12 viruses.

f) Animals

Hooded Lister rats (MHC haplotype C) used for transformation, tumorigenicity, allogeneic/syngeneic CTL assays and natural killer cell cytolytic assays were bred by brother-sister mating and housed in the Central Animal Facility, McMaster University. Fischer 344 rats (MHC haplotype L) used for allogeneic CTL assays were purchased from Charles Rivers Canada (Quebec, Canada).

g) Antibodies

M73 (gift, Dr. S. T. Bayley), an anti-Ad2/5 E1A monoclonal antibody and α-p300 (gift from Dr. E. Moran, Temple University), an anti-p300 polyclonal antiserum were used in immunoprecipitation and co-immunoprecipitation studies.

CL007A (Cedarlane Laboratories), an anti-rat RT1.A monoclonal antibody and CL6002F (Cedarlane Laboratories), a rabbit anti-mouse immunoglobulin antisera conjugated to fluorescein isothiocyanate (FITC) which reacts with all mouse Immunoglobulin classes/subclasses respectively served as primary and secondary antibodies in FACS analyses designed to measure cell surface MHC class I antigen levels.

sc-114 (Santa Cruz Biotechnology), an affinity-purified rabbit polyclonal antibody raised against a human NF-κB1-p50 peptide (CTPEIKPKEEVQRKR) which maps within a domain encompassing part of the basic NLS sequence and the N-terminal adjacent 11 amino acids (Henkel et al., 1992) was used in Western blot analyses.

NR1157 (gift, Dr. N. Rice, NCI) is also a rabbit polyclonal antibody raised to a human NF-κB1-p50 peptide (DLETSEPKPFLYYPEIKDKC) that detects NF-κB1-p50 and its precursor NF-κB1-p105 by Western blot analysis.

sc-109 (Santa Cruz Biotechnology), an affinity-purified rabbit polyclonal antibody raised against a human RelA-p65 peptide (ELFPLIFPAEPAQASGP) was employed in Western blotting protocols.

sc-2004 (Santa Cruz Biotechnology), an affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugated polyclonal antiserum served as a secondary antibody in Western blot analyses.

CL020AP (Cedarlane Laboratories), a purified anti-rat CD3 monoclonal antibody was used to inhibit and confirm the authenticity of lysis by syngeneic DP5-2 specific CTLs.

2. Methods

a) Preparation of Competent Bacterial Cells

The bacterial strains, DH5-α and SURE were made competent for transformation by electroporation or by treatment with $CaCl_2$ (Sambrook et al., 1989). One litre cultures were grown to an absorbace (600 nm) of 0.5 to 0.7 and harvested by centrifugation for 10 minutes at 5000 rpm. To harvest, the cells were chilled on ice for 15 minutes and centrifuged (4000 rpm) for 15 minutes. For electroporation competence, the cell pellets were resuspended twice in sterile ice cold 10% glycerol (0.5 litres) and centrifuged as above. Next, the cells were resuspended in approximately 20 ml 10% glycerol and centrifuged as above. Finally, cell pellets were resuspended in about 2-3 ml 10% glycerol to a final concentration of 3×10^{10} cells/ml, aliquotted, frozen in a dry ice/ethanol bath, and stored at -70°C. For $CaCl_2$ competence, pelleted cells were resuspended twice in sterile ice cold 0.1 M $CaCl_2$ (100 ml) and centrifuged at 4000 rpm for 15 minutes. Cell pellets were resuspended in 2 ml ice cold 0.1 M $CaCl_2$, dispensed into aliquots, slowly frozen in dry ice/ethanol bath, and placed at -70°C.

b) Transformation of Bacteria with Plasmid DNA

When transforming $CaCl_2$ competent bacterial cells, the cells were incubated with 10-50 μl of ligation reactions or Ad12 genomic plasmids. Following 30 minutes, cells were heat shocked at 42°C for 2 minutes. Transformation of electrocompetent cells was accomplished using an *E. coli* Pulser Electroporator and 0.2 cm electoporation cuvettes (BioRad). 50 μl aliquots of electrocompetent cells in addition to 2-100 ηg of plasmid DNA were placed in a cuvette and electroporated at 2.25 kvolts and resuspended in 1 ml LB. Next, the transformation solution was placed on a shaking incubator at 37°C. After 1 hour, 10^{-2}, 10^{-1} and 10^{0} dilutions were plated on LB agar plates containing the appropriate

antibiotics and placed at 37°C overnight to allow colony formation derived from transformed bacteria.

c) Small Scale Plasmid DNA Preparation

With some modifications, small scale preparations of plasmid DNA were made following the alkaline lysis method outlined by Sambrook et al., 1989. Two ml aliquots of Luria Broth (LB: 0.5% yeast extract (Difco), 1% bacto tryptone (Difco), 0.5% NaCl (BDH), 0.1% glucose (BDH)) supplemented with either 0.8 mg/ml ampicillin or 0.5 mg/ml kanamycin sulphate were inoculated with transformed bacteria and incubated on a shaker (225 rpm) at 37°C for 12-24 hours. Cultures were dispensed in 1.5 ml eppendorf tubes and pelleted by microcentrifugation. Following aspiration of the supernatant, cells were lysed on ice for 5 minutes with 100μl of solution I (100 μg/ml RNase A, 50 mM Tris/HCl, 10 mM EDTA, pH 8.0) and a futher 5 minutes with 200 μl of solution II (200 mM NaOH, 1% SDS). To precipitate chromosomal DNA and proteins, 150 μl of solution III (3M potassium acetate, pH 5.5) was added to the lysates and allowed to stand on ice for an additional 20 minutes. Once precipitates of chromosomal DNA and cellular debris were pelleted for 10 minutes by micro-centrifugation, plasmid DNA, contained in the supernatants, was precipitated with 2 volumes of 96% ethanol, pelleted by a 5 minute microcentrifugation, rinsed with 70% ethanol, dried, and resuspended in 50-100 μl 0.1X SSC (0.088% NaCl, 0.044% sodium citrate).

d) Large Scale Plasmid DNA Preparation and Purification

The method followed for large scale plasmid DNA preparation was that of Birnboim and Doly, 1979 (with slight modifications). Log phase cultures (500 ml) were grown at 37°C overnight in super broth (SB: 2% LB premix (1% select pancreatic digest of casein, 0.5% select yeast extract, 0.5% NaCl (Becton Dickinson)), 2.2% bacto tryptone, 1.5% yeast extract, and 0.1% glucose) containing either ampicillin or kanamycin sulphate

in concentrations described above. These cultures were harvested at 4°C by centrifugation (4240 rpm) for 10 minutes in a cryofugc and the supernatants discarded. Cells were lysed on ice for 5 minutes with 40 ml solution I (containing 200 mg/ml lysozyme (BMC)) and a further 5 minutes with 80 ml solution II. Next, 40 ml solution III was added for 20 minutes to precipitate chromosomal DNA and proteins. Precipitates were pelleted by centrifugation (as described above) and the supernatants were filtered through cheese cloth into a fresh centifuge bottle containing 100 ml 2-proponal. Following a 30 minute incubation period, low molecular weight nucleic acids were pelleted by centrifugation (5000 rpm) for 10 minutes in a cryofuge and the supernatants were discarded. Once dry, the pellet was resuspended in 7 ml 0.1X SSC and incubated at 4°C with 8.6 grams cesium chloride (CsCl; ICN). After 30 minutes, excess CsCl was pelleted at 4000 rpm for 20 minutes at 4°C and the supernatant was transferred to a 13 ml 3" x 5/8" heat-sealable ultra centrifuge tube. Addition of 5.8 ml light parraffin oil to the tube was followed by 200 μl ethidium bromide (10 mg/ml). Finally, the tubes were heat-sealed and plasmid DNA was purified by equilibrium centrifugation overnight at 55 000 rpm in a VTi 65.1 rotor (Beckman). Plasmid bands were collected in syringes fitted with 16 gauge needles and transferred into 15 ml conical tubes. Ethidium bromide was extracted several times with an equal volume of CsCl-saturated 2-propanol until no traces could be seen. The aqueous phase was diluted with 3 volumes of 0.1X SSC and 8 volumes of 96% ethanol. Purified plasmid DNA was collected by centifugation (4000 rpm) at room temperature for 5 minutes, rinsed with 70% ethanol, and resuspended in 0.5-2.0 ml 0.1X SSC.

e) Separation and Purification of DNA Fragments

DNA fragments generated from restriction enzyme digests were separated by electrophoresis through 0.5-2% agarose (Gibco) gels containing 2.5 μg ethidium bromide (Sigma) per 100 ml. Following electrophoresis and visualization of DNA fragment bands using a UV illuminator, desired bands were excised from gels using a scalpel and placed in

a microcentrifuge tube. Purification of DNA from agarose was accomplished using a protocol and reagents supplied in the Magic PCR preps kit (Promega). Once pure, DNA was resuspended in ddH_2O and used in subsequent cloning procedures.

f) Plasmid DNA Sequencing

Prior to sequencing, 2 μg of plasmid DNA was placed in a 1.5 ml microcentrifuge tube, lyophilized, pelleted by vacuum centrifugation, and resuspended in 20 μl ddH_2O. To denature plasmid DNA, 20 μl 0.4 M NaOH was added and allowed to stand at room temperature. After 5 minutes, the mixture was neutralized by adding 4 μl 5 M ammonium acetate and plasmid DNA was precipitated with 100 μl 96% ethanol. Following a 15 minute micocentrifugation at 4°C, the supernatant was carefully discarded and the DNA pellet rinsed with 70% ethanol. Once dry, the denatured DNA pellet was stored at -20°C until needed.

Two procedures were utilized for plasmid DNA sequencing during these studies:

1) Chain-termination DNA sequencing using Sequenase

In a total volume of 10 μl, 4 nanograms of primer DNA in a volume of 1 μl was annealed to the lyophilized template DNA (described above) in the presence of Sequenase buffer (200 mM Tris HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) at 65°C for 2 minutes followed by slow cooling to < 35°C. During cooling, 2.5 μl of each termination mixture (80 μM dGTP, 80 μM dATP, 80 μM dTTP, 80 μM dCTP plus 50 mM NaCl and 8 μM dd GTP or ddATP or ddTTP or ddCTP) was added to separate tubes. Next, the labelling mixture (7.5 μM dGTP, 7.5 μM dTTP, 7.5 μM dCTP) was diluted 1:15 with ddH_20 and Sequenase (a modified bacteriophage T7 DNA polymerase, United States Biochemical) was diluted 1:8 with enzyme dilution buffer (10 mM Tris HCl pH 7.5, 5 mM DTT, and 0.5 mg/ml BSA). The tubes containing the 4 separate termination mixtures were pre-warmed in a 37°C heating block. To the 10 μl annealed primer/template DNA mixture, 1 μl 0.1 M DTT, 2 μl 1:15 diluted labelling mixture, 1 μl [^{32}P] dATP, and 2 μl diluted Sequenase was

added, mixed, and incubated at room temperature for 5 minutes. To terminate the reactions, 3.5 μl of labelling reaction was added to each of the 4 termination mixture tubes and incubated for 5 minutes at 37°C. Finally, reactions were stopped upon addition of 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% Xylene Cyanol FF), denatured at 100°C for 5 minutes, placed on ice for 2 minutes and typically electrophoresed on a 7% polyacrylamide gel (for 100 ml: 6.65 grams acrylamide, 0.35 grams BIS, 42 grams urea).

2) Automated DNA Sequencing

Plasmid DNA samples (200 ng/μl) and primer samples (0.2 picomoles/μl) were provided to the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University where sequencing reactions were carried out using a cycle sequencing procedure (annealing, elongation, and denaturation temperatures were 50°C, 60°C, and 96°C respectively).

g) Mammalian Cell Passaging

To passage mammalian cells, medium was removed from confluent or near confluent monolayer cultures and the cells were rinsed with 1-5 ml PBS (137 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and .027 mM KCl). Once PBS was removed from the cells, they were detached from culture dishes using 0.5-2 ml trypsin (Gibco), appropriately diluted with fresh growth media, and seeded onto new dishes. 293 cells were passaged as just described with the exception that citrate saline (134 mM KCl and 15 mM sodium citrate) was used to rinse and detach cells.

h) Ad Infection, Titration, and Purification

Infection of Mammalian Cells in Monolayer or Suspension

Infection of 80-90% confluent monolayer cell cultures was conducted at various multiplicities. Viral particles were diluted from concentrated stocks or plaque isolates in

0.2 ml PBS^{2+} (PBS plus 0.1% $CaCl_2$ and 0.1% $MgCl_2$) for infection of 60 mm dishes, 0.5 ml for 100 mm dishes, and 1.0 ml for 150 mm dishes. Following removal of medium from cells, diluted virus particles were dispersed dropwise on the monolayer and allowed to absorb at 37°C for 30-60 minutes after which fresh media was added and the cells returned to 37°C. The cells were subsequently monitored for cytopathic effect (CPE). Once cells were rounded and CPE was complete, cells were scraped from dishes into the surrounding medium using a rubber policeman. After a five minute 2000 rpm centrifugation, medium was removed from the pelleted infected cells and a concentrated viral stock prepared by resuspending the infected cell pellet in 2 ml PBS^{2+} + 10% glycerol/150 mm dish. The medium removed from the infected cell pellet also contained infectious virus albeit at a lower titre.

To obtain more concentrated stocks of Ad12 for subsequent isolation of viral DNA, KB cells were infected in 2-4 litre suspension cultures. In Joklik's modified MEM + 10% horse serum, KB spinner cultures were grown to a concentration of 2-4 x 10^5 cells/ml. After pelleting cells at 2000 rpm for 10 minutes, they were resuspended in a fresh medium (1/10 volume) and infected at 37°C with virus at a multiplicity of 10-20 plaque forming units (PFU) per cell. After 1 hour, the infected cells were brought to the original culture volume using a 1:1 mixture of fresh medium and conditioned medium, returned to a 37°C incubator, and monitored daily for the presence of inclusion bodies in 80-90% of cells which is indicative of peak viral production (3-4 days). A 5 mL aliquot from the infected spinner culture was removed, pelleted, and resuspended in 0.5 ml 1% sodium citrate at room temperature. After 10 minutes, cells were fixed with 0.5 ml Carnoy (3:1 methanol:glacial acetic acid mixture) at room temperature for 10 minutes. Next, 1 ml 1% sodium citrate was added to cells, centrifugation as above, and resuspended in a few drops of 1% sodium citrate. Finally, a drop of fixed cells were placed on a slide, allowed to dry for 1 hour, overlayed with Orcein stain (2% orcein in 50% acetic acid (filtered through #1 filter paper)) and a cover slipe, and examined under a light microscope for densely stained

nuclear structures. When ready, cells were harvested by centrifugation and resuspended in 0.1M Tris-HCl, pH 8.0 (10-20 ml for 1-4 litres) for preparation of virus to be used for CsCl banding and DNA extraction (when necessary, cells were stored at -70°C until needed).

Plaque Assays

One day prior to use, appropriate cells (293 or HER-3) were seeded in 60 mm culture dishes to be approximately 80-90% confluent at the time virus was added. Virus dilutions of concentrated stocks or medium were prepared in PBS^{2+} and added to cells (0.2 ml/dish) after removal of medium from the monolayer. Once virus particles had adsorbed to cells as above, 10 ml 44°C overlay (1:1 mixture of 1% agarose and 2X MEM or F-11 plus 2-5% horse serum or FBS) was added to cells and allowed to solidify at room temperature. The cells were incubated at 37°C until plaques appeared (4-14 days). Titres, expressed as the number of plaque forming units per ml of viral preparation, were calculated using the following calculation: ((number of plaques / mls of viral dilution added to dish) x dilution factor).

Purification of Ad Virions

To disrupt cells but not virions, Ad12 stocks prepared from infected KB suspension cultures were treated with 1/10 volume 5% sodium deoxycholate, mixed well, and incubated at room temperature. After 30 minutes, 0.01 volume 2M MgCl2, 0.005 volume DNAse I, and 0.005 volume RNase A solutions were added, mixed thoroughly, and allowed to stand for 30-45 minutes at 37°C. Once cellular DNA and RNA were digested, 1.8 ml saturated CsCl (in 0.01M Tris-HCl, pH 8.0, 0.001M EDTA) was added to each 3.1 ml portion of virus suspension at room temperature. This solution was placed into Beckman 50Ti heat seal tubes and spun in a Beckman 50Ti angle rotor at 35000 rpm for 16-20 hours at 4°C. Viral bands were collected, pooled, and centrifuged in a Beckman

SW50.1 rotor as above. As before, viral bands were collected in a very small volume and dialyzed in boiled dialysis tubing at 4°C against two 1 hour changes of 100 volumes 0.01M Tris-HCl, pH 8.0 to remove all traces of CsCl.

i) Ad DNA Purification

Following dialysis of CsCl banded Ad12 virions, viral DNA was extracted and purified. To a petri dish containing a volume of digestion buffer (1 mg/ml pronase in 0.01M Tris-HCl, pH 7.5, 0.01M EDTA, 1% SDS) an equal volume of dialyzed virus was added and incubated at 37°C for 4 hours. The mixture was extracted once with 0.01M Tris-HCl, pH 8.0, 0.01M EDTA saturated phenol and the DNA precipitated by adding 1/10 volume 30% sodium acetate and 2 volumes 96% ethanol. Once pelleted, the DNA was extensively rinsed with 96% ethanol and the dried pellet was resuspended in 0.1X SSC.

j) Primary Baby Rat Kidney (BRK) Cell Preparation

BRK cells were prepared for two main purposes: 1) To be transformed by pXC1, an Ad5 E1-encoding plasmid; and 2) To be infected with Ad12Ampr (a wild type Ad12 Huie strain virus encoding a bacterial origin and ampicillin resistance gene in E1) in an attempt to create an Ad12 genomic plasmid. BRK cells were prepared essentially as described by van der Eb and Graham, 1980 with some modifications. A litter of 5-7 day old Hooded Lister rats were sacrificed by cervical dislocation. Once the kidneys were dissected, minced, and added to 30 ml of a 2X trypsin (Gibco)/PBS solution, the solution was incubated at room temperature for 20 minutes. The resulting supernatant was transferred to 20 ml FBS at 4°C to inhibit trypsin. A further 30 ml of 2X trypsin/PBS was added to the remaining undigested kidney fragments and incubated at room temperature for 20 minutes. This supernantant was pooled with the supernatant from the first trypsinization and centrifuged for 5 minutes at 2000 rpm. The cell pellet was resuspended in 50 ml α-MEM plus 10% FBS and filtered through cheese cloth. Using α-MEM plus

10% FBS, the cell suspension was diluted appropriately and plated on 60, 100, or 150 mm tissue culture plates (Nunclon). After 24 hours cells were rinsed and fed with fresh α-MEM plus 10% FBS in preparation for infection or transformation 1 to 2 days later.

k) Transformation of BRK Cells

Primary BRK cells were transformed by pXC1 (Ad5 E1-encoding plasmid) using the calcium phosphate technique (Graham and van der Eb, 1973 a,b) modified by Wigler et al., 1978. Into a 50 ml conical tube, 1.5 ml of 2.5M $CaCl_2$ and a volume of salmon sperm carrier DNA (10 μg/60 mm dish) were diluted with 0.1 M TE pH 8.0 to a volume which is equivalent to the number of plasmids multiplied by the number of concentrations used. One ml aliquots of this solution were dispensed into 15 ml conical tubes where appropriate concentrations of plasmid DNA were added to 1 ml 2X Hepes buffer pH 7.1 (To 1 litre, add 10 grams Hepes, 16 grams NaCl, 0.74 grams KCl, 0.25 grams Na_2HPO_4-$2H_2O$, 2 grams glucose). Next, using a sterile pasteur pipet, oxygen was bubbled slowly and constantly through the $CaCl_2$/salmon sperm carrier DNA/plasmid DNA/Hepes solution. After 15-60 minutes, 0.5 ml of the $CaCl_2$-treated plasmid DNA solution was added to the appropriate number of 60 mm BRK dishes and incubated at 37°C in complete α-MEM plus 10% FBS. In certain instances, 250 μl of freshly prepared BRK cells (2.5×10^8) were transformed by electroporation in 0.4 cm electrode gap cuvettes (Bio Rad), at 220 V and 960 μF (Chu et al., 1987).

Whether BRKs were transformed using the calcium phosphate or electroporation procedures, selection for Ad E1-transformed rat cells followed incubation with complete Joklik's medium (Gibco) supplemented with 5% horse serum (Gibco). After 1-3 weeks, transformed foci were either subcloned to establish lines or fixed in 75% methanol/25% acetic acid and stained with Giemsa stain.

l) Tumorigenicity Assays

Cell lines transformed by the hybrid Ad5/12 E1A constructs plus Ad12 E1B were expanded through 10-15 passages in culture for subcutaneous injection into newborn syngeneic Hooded Lister rats. Each line was injected into single litters of newborn Hooded Lister rats at 10^7 cells per rat and monitored for tumor formation at the site of injection for up to 6 months.

m) Detection of Protein Expression in Mammalian Cells

Immunoprecipitation Analysis

Prior to ^{35}S methionine labeling, 10^6 cells/sample were seeded onto 100 mm monolayer tissue culture plates. At 50-60% confluence, cells were metabolically labelled at 37°C for 4 hours in 199 medium lacking methionine and cysteine and supplemented with 300 μCi ^{35}S-translabel (ICN) per sample. The radioactive cells were washed with PBS, centrifuged, resuspended in "lysis buffer X + BSA" (50 mM Tris (pH 8.8), 250 mM NaCl, 1% NP-40, 2 mM EDTA, 10 μg/ml aprotinin, 0.5 mM sodium metavanadate, and 2 mg/ml bovine serum albumin), and placed on ice for 20 minutes. Following a 15 minute/15000 rpm centrifugation at 4°C, 3% protein-A sepharose (resuspended in "lysis buffer X + BSA") was added to the lysate, mixed at 4°C for 15 minutes and pelleted. Depending on the experiment, the pre-cleared lysate was incubated with anti-Ad2/5 E1A M73 monoclonal antibody (Harlow et al., 1985) or anti-p300 polyclonal serum at 4°C for 1 hour, followed by incubation with 3% protein-A sepharose for a further 30 minutes. Next, the protein-A sepharose was pelleted and washed three times with "lysis buffer X + BSA" and finally with "lysis buffer X - BSA". Samples were boiled for 5 minutes in Laemmli loading buffer and loaded on either 7 or 8% SDS-polyacrylamide gels.

Western Blot Analysis

50 μg of thymic whole cell extracts from p105 +/+, +/-, and -/- cells were electrophoresed on a 10% SDS-polyacrylamide gel and electro-blotted onto an Immobilon-P transfer membrane (Millipore Corporation, Bedford, MA, USA). The blot was blocked for 1 hour in 5% nonfat dry milk/PBS and probed for 1 hour with the primary NF-κB1-p50 specific antibodies, NR1157 (1:1000 dilution) (lanes 1-3) (N. Rice, NCI, Frederick, MD, USA) and sc-114 (1:400 dilution) (lanes 4-6) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following two 7 minute washes in PBS/0.05% Tween-20, the blot was incubated for 1 hour with a 1:2000 dilution of a horseradish peroxidase conjugated goat anti-rabbit polyclonal secondary antibody (Santa Cruz Biotechnology). After three 5 minute PBS/0.05% Tween-20 washes and one PBS wash, the blot was developed using Enhanced Chemiluminescence Western blotting reagents (Amersham International plc., Buckinghamshire, England) and exposed to film for 1 minute.

n) **Characterization of Protein Phosphorylation States**

Protein Phosphatase Treatment

Proteins were immunoprecipitated as outlined above, using α-p300 polyclonal antiserum. p300-containing immunocomplexes were washed with lambda protein phosphatase buffer (50 mM Tris-HCl, 5 mM DTT, pH 7.8), divided in two equal portions, pelleted, and resuspended in 50 μL 2 mM $MnCl_2$/100 μg/ml BSA. One portion was incubated at 30°C for 30 minutes with 1000 units lambda protein phosphatase (New England Biolabs) which releases phosphate groups from serine, threonine, and tyrosine residues. Incubations were halted and denatured by addition of 50 μl of 2X Laemmli loading buffer and heated at 100°C for 5 minutes. p300 proteins were analyzed on a 20 cm long 7% SDS-polyacrylamide gel after electrophoresis for 36 hours at 9 mAmps.

Two Dimensional Tryptic Phosphopeptide Mapping

DP5-2 and 12-1 cells (5 x 10^6) were metabolically labelled with 10 mCi orthophosphate ^{32}P/cell line. After 4 hours, cell extracts were prepared in RIPA buffer, immunoprecipitated with α-p300 antibody, and electrophoresed through a 7% polyacrylamide gel as described earlier. Once briefly rinsed in ddH_2O, the gel was blotted onto Whatman paper, dried, and visualized by a autoradiography. Using a scalpel, ^{32}P-labelled p300 proteins were excised from the gel, deposited into a microfuge tube and homogenized with a pestel in the presence of 0.4 ml 50 mM ammonium bicarbonate, pH 7.6. Next, p300 proteins were vortexed and incubated at 98°C in the presence of a further 0.8 ml 50 mM ammonium bicarbonate (pH 7.6), 12 µl 2-mercaptoethanol, and SDS to 0.1%. After 3 minutes, samples were incubated at 37°C on a rotating platform for 2 hours. Once p300 proteins were denatured and gel pieces were removed by extensive centrifugation, RNA was digested and proteins precipitated using 10 µg RNAse A and TCA respectively. Following a 2 hour incubation at 4°C, denatured proteins were pelleted for 10 minutes, dried, vortexed with 0.4 ml 1:1 ethanol:ether, and pelleted again. The protein pellet was vortexed in the presence of 200µl performic acid (10:1 formic acid:30% H_2O_2) and allowed to stand at 4°C. After 1.5 hours, samples were diluted with 0.4 ml ddH_2O, frozen on dry ice, and lyophilized in a vacuum centifuge for 3-4 hours. Protein pellets were neutralized following addition of 20 µl 50 mM ammonium bicarbonate, pH 7.6. Next, proteins were fragmented into peptides by a 37°C incubation in the presence of 10 mg TPCK-treated trypsin (Worthington Biochemical). After 12-18 hours, 5 mg TPCK-treated trypsin was again added, allowed to act for 4 hours, and repeated. Samples were diluted with 0.4 ml ddH_2O and lyophilized in a vacuum centrifuge. By vortexing, lyophilized peptides were solubilized in 400 µl pH 1.9 Buffer (25 ml 88% formic acid, 78 ml acetic acid per litre) at 60°C. Once 5 minutes had expired, samples were centrifuged for 10 minutes and pellets were resuspended in 4 µl pH 1.9 buffer at 37°C with vortexing. Using a capillary tube, this peptide mixture was spotted on a lower corner of a 20 cm x 20

cm cellulose thin layer chromatograpy plate (VWR Scientific). Next, Whatman paper was saturated with pH 1.9 buffer and overlayed on the cellulose plate. The plate was placed in a HTLE-7000 electrophoresis apparatus (CBS Scientific) and electrophoresed at 1500 volts in pH 1.9 buffer. Once 25 minutes had elapsed, the plate was liberated from the electrophoresis apparatus, dried, and placed in a vertical liquid chromatography tank containing phosphochromo buffer (375 ml n-butanol, 250 ml pyridine, 75 ml acetic acid per litre) in order that ^{32}P-labelled p300 peptides could be resolved in the second dimention. After 6 hours, plates were dried and exposed to film.

o) Detection of Cell Surface MHC Class I Expression

Cell surface expression of MHC class I proteins was determined by flow cytofluorimetry using a FACScan (Becton Dickinson Canada Incorporated, Ontario, Canada) and antibodies purchased from Cedarlane Laboratories Limited (Ontario, Canada). Single cell suspensions were prepared from monolayer cultures and resuspended in PBS supplemented with BSA (10 mg/ml) and sub-divided into three aliquots (unstained, stained by secondary antibody only, stained by both primary and secondary antibodies) containing 10^6 cells/aliquot. Where appropriate, single-cell suspensions were incubated with primary antibodies for 30 minutes on ice using either the mouse anti-rat RT1.A monoclonal antibody, CL007A, which recognizes a monomorphic determinant of rat class I MHC antigen, or the mouse anti-human antibody, W6/32, (gift from I. York) which is directed against human HLA (A, B, or C). Subsequent or independent staining was conducted using FITC conjugated rabbit anti-mouse Ig antisera (reacts with all mouse Ig classes and subclasses).

p) Electrophoresis Mobility Shift Assays (EMSAs)

Two oligonucleotides were synthesized corresponding to base pair -159 to -180 of the $H2K^b$ MHC class I R1 enhancer element: 5'-GCCCAGGGCTGGGGATTC-3' and 5'-

TGGGGAATCCCCAGCCCTGGG-3'. A 1:1 mixture of these oligonucleotides were annealed following a 5 minute 65°C incubation, and slow cooling to room temperature. When annealed, the double stranded oligonucleotides were radioactively labelled by filling in recessed 3' ends with [α-^{32}P] dCTP and [α-^{32}P] dATP using the Klenow fragment of DNA polymerase I. Using NAP-5 Sephadex G-25 columns (Pharmacia) labelled oligonucleotides were purified from unlabelled oligonucleotides. Whole cell extracts were prepared from 10^6-10^7 monolayer cells which were rinsed, trypsinized, pelleted, resuspended in 1 ml EMSA lysis buffer (20 mM Hepes, pH 7.9, 600 mM KCl, 0.2 mM EDTA, 1 mM DTT, 100 mM aprotinin, and 10 mM sodium vanadate), and placed in a dry ice/ethanol bath for one minute. To liberate cellular contents, samples were thawed and frozen twice and stored at -20°C until needed.

EMSAs were performed by incubating 10 μg of whole cell extract proteins with 1 μg poly (dI-dC) and 5 fmol radioactively labelled oligonucleotides. Following a 30 minute incubation at room temperature, samples containing putative DNA-protein complexes were loaded onto a 4% non-denaturing polyacrylamide gel and electrophoresed for 2 hours at 10V/cm. To determine whether p300 existed in protein-R1 complexes, the α-p300 (anti-p300) antibody was also added to incubations.

q) Cytotoxic T Lymphocyte (CTL) and Natural Killer (NK) Cell Cytolytic Assays

Generation of Allogeneic and Syngeneic CTLs and Syngeneic NKs

Allogeneic effector CTLs were generated following co-culture of single cell suspensions of splenic lymphocytes isolated and prepared from 8 week old Hooded Lister rats (stimulators; MHC haplotype C) and Fischer 344 rats (responders; MHC haplotype L) at a responder:stimulator ratio of 1:1. Three days prior to co-culture, stimulator Hooded Lister rat splenic lymphocytes were γ-irradiated at 5000 rads.

To generate syngeneic Ad5 E1- and Ad12 E1-specific CTLs, 8 week old Hooded Lister rats were respectively immunized intraperitoneally with 10^7 DP5-2 (Ad5 E1-transformed Hooded Lister rat line) and 10^7 12-1 (Ad12 E1-transformed Hooded Lister rat line) cells which were resuspended in 1 ml of PBS. Three weeks later, splenic lymphocytes were isolated, made into a single cell suspension, and restimulated by co-culture with γ-irradiated (3000 rads) DP5-2 or 12-1 cells at a responder:stimulator ratio of 50:1. Co-cultured cells were suspended in complete RPMI media (supplemented with 10% FCS, 2mM L-glutamine, 100 units/ml of penicillin G, 100 μg/ml of streptomycin sulfate, and 5 x 10^{-5} M 2-mercaptoethanol) and incubated in upright 25 cm^2 tissue culture flasks for 3 days in a humidified environment containing 5% CO_2. Gamma irradiation was conducted using a Gammacell 1000 containing a ^{137}Cs source (Atomic Energy of Canada Limited, Ontario, Canada).

Syngeneic NK cells were isolated from Ficoll gradients of lymphocytes and isolated from the spleens of 8 week old Hooded Lister rats. Effectors were resuspended to an appropriate concentration with complete RPMI.

Chromium Release Cytolytic Assays

5 x 10^4 target cells/ml were seeded in 96-well microtitre plates (Falcon 3072) and subsequently labelled with ^{51}Cr-sodium chromate (2μCi/well) for 1 hour at 37°C. Once labelled, target cells were washed three times with complete RPMI (supplemented as described above) to remove excess label. Meanwhile, effector cells were pelleted at 2000 rpm for 5 minutes, resuspended in complete RPMI media, and counted in the presence of trypan blue to determine the number of viable lymphocytes. These effector cells were then incubated with ^{51}Cr-labelled targets (in a total volume of 200 μl/well) at various E:T ratios for 6 hours at 37°C under co-culturing conditions described above. For allogeneic CTL assays, E:T ratios were 136:1, 68:1, and 34:1, while for syngeneic CTL assays, ratios of 100:1, 50:1, and 25:1 were utilized. E:T ratios of 140:1, 70:1 and 35:1 were chosen for

NK assays. Next, 100 μl samples of the supernatants were removed from each well, placed in borosilicate tubes, and counted in a LKB Wallac 1282 universal gamma counter. Percentage specific lysis was calculated using the following formula: (T-S/M-S) X 100, where T = cpm released by targets in the presence of CTLs or NKs, S = cpm released spontaneously by targets in the absence of CTLs or NKs, and M = maximum cpm released from targets following complete lysis using Triton X-100.

RESULTS

Major findings derived from my research were described in three published journal articles, one manuscript in press, and one submitted manuscript listed below in the order to be discussed:

1) Jelinek, T., **Pereira, D. S.**, and Graham, F. L. 1994. Tumorigenicity of adenovirus-transformed rodent cells is influenced by at least two regions of adenovirus type 12 early region 1A. **J. Virol.** *68*, 888-896.

2) **Pereira, D. S.**, Rosenthal, K. L., and Graham, F. L. 1995. Identification of two regions of adenovirus E1A which affect MHC class I expression and susceptibility to cytotoxic T lymphocytes. **Virology** *211*, 268-277.

3) Kushner, D. B., **Pereira, D. S.**, Liu, X., Graham, F. L., and Ricciardi, R. P. The first exon of Ad12 E1A excluding the transactivation domain mediates differential binding of COUP-TF and NF-κB to the MHC class I enhancer in transformed cells. **Oncogene.** In Press.

4) **Pereira, D. S.**, Kushner, D. L., Ricciardi, R. P., and Graham, F. L. Testing NF-κB1-p50 antibody specificity using knockout mice. **Submitted.**

5) **Pereira, D. S.**, Jelinek, T., and Graham, F. L. 1994. The adenovirus E1A-associated p300 protein is differentially phosphorylated in Ad12 E1A- compared to Ad5 E1A-transformed rat cells. **Int. J. Oncol.** *5*, 1197-1205.

Preceeding each article, a brief description of background, purpose, and relevance is presented. An outline of specific contributions and a summary of major findings follows each article. Where appropriate, additional unpublished data which are related to a specific publication, also follow that publication.

1) Jelinek, T., Pereira, D. S., and Graham, F. L. 1994. Tumorigenicity of adenovirus-transformed rodent cells is influenced by at least two regions of adenovirus type 12 early region 1A. J. Virol. *68*, 888-896.

Preface:

While all known human Ad serotypes are able to transform primary rodent cells *in vitro*, their ability to induce tumors in syngeneic immunocompetent rodents is serotype-specific. The importance of the E1 region of the Ad genome, and particularly E1A, in tumorigenesis is exemplified by the discovery that primary rodent cells transformed by the E1 region of Ad12 are tumorigenic in contrast to their Ad5 counterparts. Since the specific regions of Ad12 E1A mediating tumorigenicity had not been identified, the tumorigenic capacity was determined for a series of previously described Hooded Lister rat kidney cell lines transformed by hybrid Ad5/12 E1A genes (plus Ad12 E1B) (Jelinek and Graham, 1992) and two additional lines transformed by second generation Ad5/12 E1A genes (encoded by the plasmids, p917-975 and p917-1227).

Identification of the Ad12 E1A regions mediating tumorigenesis was the starting point for future efforts directed at understanding the relative importance of E1A-modulated phenotypic properties which govern the differential oncogeneic potentials of Ad5 E1- and Ad12 E1- transformed rodent cells.

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Tumorigenicity of Adenovirus-Transformed Rodent Cells Is Influenced by At Least Two Regions of Adenovirus Type 12 Early Region 1A

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Chimeric adenovirus type 5 (Ad5)/Ad12 early region 1A (E1A) genes were used to transform primary baby rat kidney cells in cooperation with Ad12 E1B, and the resulting cell lines were assayed for tumorigenicity in syngeneic rats. It was found that lines were nontumorigenic when transformed by hybrid E1A genes consisting of the amino-terminal 80 amino acids from Ad12 including conserved region 1 (CR1), with the remaining portion from Ad5. In contrast, cell lines transformed by hybrids containing Ad12 E1A sequences from the amino terminus to the leftmost border of CR3 or beyond were tumorigenic. To extend these results, sequences spanning CR2 and CR3 of Ad5 E1A were replaced with the homologous regions of Ad12 E1A and additional transformed cell lines were established. These lines were weakly-to-moderately tumorigenic, suggesting that Ad12 E1A sequences between CR2 and CR3 may be involved in tumorigenicity but are not the sole factors influencing it. Interestingly, examination of an E1A sequence alignment indicated that the region between CR2 and CR3 of Ad12 E1A is also conserved in the corresponding sequence of simian adenovirus type 7, which, like Ad12, is highly oncogenic. This region is characterized by the presence of a stretch of several alanine residues and is similar to a motif present in a number of proteins with transcriptional repression activity. The possibility that this region may influence tumorigenicity by means of a transcriptional regulatory mechanism is discussed.

While all known human adenovirus (Ad) serotypes are able to transform primary baby rat kidney (BRK) cells in vitro, cells transformed by Ad type 2 (Ad2) or Ad5 are not tumorigenic in syngeneic rats unless the rats are immunosuppressed (13). In contrast, Ad12-transformed cells are tumorigenic in immunocompetent syngeneic rats (18), suggesting that cells transformed by oncogenic Ad serotypes are able to evade one or more host immune responses which efficiently reject cells transformed by nononcogenic Ad serotypes. While the nature of the host immune function(s) responsible for the elimination of Ad5-transformed cells is not clear, experiments involving transformation of cells with chimeric constructs such as Ad5 E1A/Ad12 E1B or vice versa demonstrated that the identity of the E1A gene is the major factor which determines whether the transformed cells are tumorigenic in immunocompetent animals (4, 37). This suggests that the immunological properties which discriminate between Ad-transformed cells with various degrees of tumorigenicity may be controlled by E1A.

One immunological difference between cells transformed or infected by nononcogenic and oncogenic Ad serotypes is their differential sensitivity to lysis by natural killer (NK) cells and activated macrophages. Cells expressing the Ad2 or Ad5 E1A protein are highly susceptible to lysis, whereas those expressing the Ad12 E1A protein are not (5–7, 25, 33, 36). Moreover, the introduction of Ad5 E1A into a tumorigenic hamster sarcoma cell line results in the loss of tumorigenicity concomitant with increased sensitivity to lysis (43), suggesting that the E1A protein of nononcogenic Ad serotypes actively induces nonspecific, NK cell-mediated cytolysis of expresser cells, while E1A of oncogenic serotypes is inactive in this respect.

Cells transformed by the E1 regions of Ad5 and Ad12 also differ in their expressions of class I major histocompatibility complex (MHC) molecules, which are essential for antigen-specific cytolysis mediated by cytotoxic T lymphocytes (CTL). While class I MHC transcription is transiently elevated following infection of mouse cells with either serotype (35), stable expression of the Ad12 E1A protein in transformed cell lines leads to transcriptional down regulation of class I MHC genes (1, 8, 9, 12, 30, 39, 40, 42). The down regulation is promoter specific (29) and correlates with reduced levels of CTL killing of Ad12-transformed cells in vitro, suggesting that tumorigenicity of these cells occurs as a consequence of active CTL evasion (4). Because a reduction in class I MHC expression is not seen in cells transformed by Ad2 or Ad5, those cells would be susceptible to cytolysis by CTLs reactive against tumor antigens. Both the Ad5 and Ad12 E1A proteins are potential antigens and can give rise to tumor-specific transplantation immunity, which is mediated by CTLs (3, 24, 34, 38). However, quantitation of class I MHC expression levels in Ad2- and Ad5-transformed cell lines which had acquired various degrees of tumorigenicity following serial passage in animals showed no correlation between levels of class I MHC expression and degree of tumorigenicity (15, 30). Furthermore, transfection of genomic *H-2D*^{d} or *H-2L*^{d} fragments into tumorigenic Ad12-transformed BALB/c mouse cells not only increased the levels of cell surface class I proteins in these cells but also increased their tumorigenicity 16-fold beyond that of the parental cells in syngeneic adult mice (41). These results suggest that a property of Ad12-transformed cell lines other than, or in addition to, their decreased class I MHC expression levels is involved in tumorigenicity.

As a further step in understanding the relative importance of E1A-modulated phenotypic properties in the tumorigenicity of Ad-transformed cells, we undertook to map the regions of the

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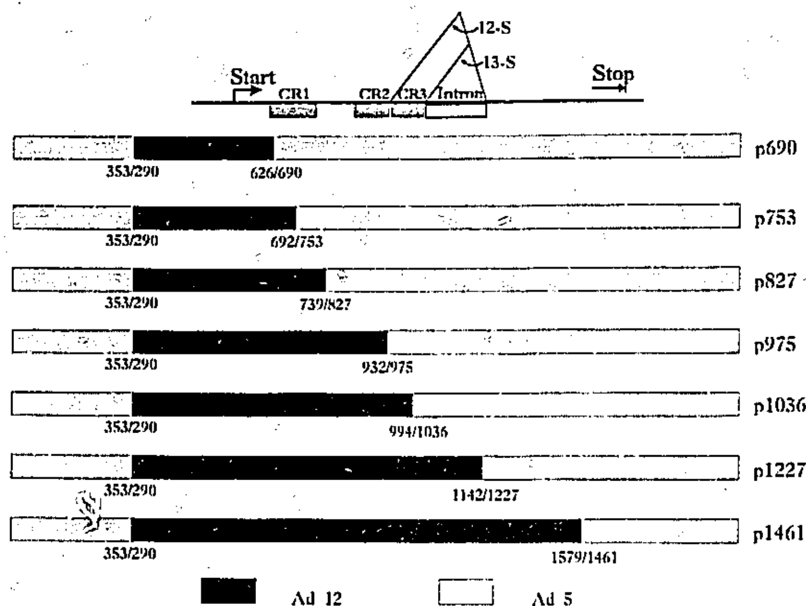


FIG. 1. Hybrid E1 plasmids used. The constructs shown here have been described previously (22) and depict only the E1A region. The precise locations of crossovers in nucleotides are indicated beneath each construct, at the junctions of Ad5 and Ad12 sequences. All constructs contain the leftmost 353 bp of Ad5 joined to Ad12 sequences from nucleotide 290 and contain the Ad12 E1A promoter, transcriptional start site, and the amino terminus of the Ad12 E1A protein. At the indicated site in the 3′ direction, a crossover event has taken place and Ad5 sequences are present from that point to 16% of the genome length. When used to transform cells for tumorigenicity assays, these plasmids were digested at Ad5 nucleotides 1770, 3641, and 5644 with *Sac*I and religated, resulting in the elimination of sequences between the *Sac*I sites and corresponding to most of the E1B transcription unit.

Ad5 and Ad12 E1A proteins which influence tumorigenicity. To accomplish this, a series of hybrid Ad5/Ad12 E1A regions were constructed and characterized for their transforming and tumorigenic properties. The construction of several of these hybrid E1A plasmids, as well as their transforming properties have been described previously (22). Studies with cells transformed by these hybrids, presented here, have identified a region of the Ad12 E1A protein which, when introduced into Ad5 E1A, confers a modest degree of tumorigenicity to transformed cells. A similar region is present in the unrelated and highly oncogenic simian adenovirus type 7 E1A sequence but is absent in the known E1A sequences of other human Ads.

MATERIALS AND METHODS

Cells. Primary BRK cells were prepared, and cell lines were established as previously described (22). BRK lines transformed by the p917-975 and p917-1227 hybrid Ad5/Ad12 E1A constructs (see Fig. 4A) were newly established for this study.

Tumorigenicity assays. Approximately 0.1 ml of a cell suspension containing 10^8 cells per ml (10^9 cells per ml for some experiments) was injected subcutaneously near the right shoulder of newborn Hooded Lister rats, which were monitored for tumor formation at the site of injection for up to 6 months. Tumor-bearing rats were bled by cardiac puncture, and the serum was retained for Western blots (immunoblots) and other uses.

Western blots. Transformed cells were lysed in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.1–0.1% Nonidet P-40–250 mM NaCl, and 50 μg of protein was electrophoresed on sodium dodecyl sulfate–10% polyacrylamide gels which were then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) by electroblotting. Membranes were incubated in a solution of 5% powdered skim milk in phosphate-buffered saline (PBS) for 2 h at room temperature, followed by a further 2 h in the same solution containing antitumor serum or M73 monoclonal antibody specific for Ad5 E1A (Oncogene Science) (17). Membranes were then washed three times in PBS, and proteins were detected by enhanced chemiluminescence (Amersham).

PCR primers required for construction of additional hybrid Ad5/Ad12 E1A genes. AB1508 (5′-GAAGATATCGATTTAT TGTGCTACGAGATG-3′) and AB416 (5′-CGCACACACC AATTGCG-3′) are synthetic oligonucleotides used in PCRs with either p975 or p1227 as a template to create p917-975 or p917-1227, respectively. AB1508 anneals to Ad12 sequences from nucleotides 808 to 837 and introduces a *Cla*I site suitable for splicing of PCR products to the *Cla*I site at nucleotide 917 in Ad5 E1A. AB416 anneals to Ad5 sequences from nucleotide 1579 to 1563.

RESULTS

Establishment of cell lines containing hybrid Ad5/Ad12 E1A genes and Ad12 E1B. The primary purpose of establishing transformed cell lines was to assay them for tumorigenicity in syngeneic rats. The hybrid E1 plasmids constructed previously (22) contain various amounts of Ad12 E1A from the beginning of the E1A transcription unit to the specific crossover site and Ad5 sequences through the remainder of E1A plus E1B (Fig. 1). Previous work has indicated that while the tumorigenicity of Ad-transformed cells is influenced primarily by the identity of the E1A transcription unit in immunocompetent animals, the E1B transcription unit is also important. Specifically, Ad12 E1B mediates a higher degree of tumorigenicity in a shorter time period than Ad5 E1B (4, 37). To study the role of E1A in tumorigenicity, it is therefore helpful to transform cells with combinations of a hybrid E1A gene and the Ad12 E1B

transcription unit, a strategy which permits the identification of tumorigenic cell lines within a shorter time. To eliminate Ad5 E1B coding sequences, all hybrid E1A plasmids were digested at positions 1770, 3641, and 5644 with *Sac*I and religated to remove all but the first 120 nucleotides of the E1B transcription unit. Primary BRK cells were then electroporated with the hybrid E1A plasmids plus pHAB13, which encodes the Ad12 E1B transcription unit (28a). Four to six weeks after transfection, individual colonies of transformed cells were isolated and expanded.

E1 protein expression in transformed cell lines. Protein samples from each transformed cell line were subjected to electrophoresis and Western blotting to verify that the cells expressed E1 proteins encoded by the hybrid E1A and pHAB13 plasmids. In one analysis, the membranes were probed with sera from tumor-bearing rats injected with cell line 1227B12C1. This cell line contains a chimeric E1A protein with exon 1 of Ad12 E1A, exon 2 of Ad5 E1A, and Ad12 E1B. Antitumor sera collected from rats injected with this cell line were found to have antibody with high avidity for Ad5 E1A proteins, hybrid E1A proteins, and Ad12 E1B proteins and little or no avidity for Ad12 E1A or Ad5 E1B proteins (unpublished data). This serum was thus convenient for the analysis of E1 protein expression in transformed cells. Results from analyses of several lines are shown in Fig. 2A. Lane 1 contained protein from line 5-1, a cell line transformed by Ad5 E1, and as expected, the antibody reacted only with E1A proteins of these cells. Lanes 12 and 13, containing protein from Ad12-transformed cells showed reactivity with the Ad12 E1B 55,000-molecular-weight protein (55K protein) and a faint band (lane 12) which may correspond to an Ad12 E1A product. Lanes 2 through 11 contained protein from cells transformed by various hybrid E1A genes plus Ad12 E1B. Although the intensity of the signals varied from one line to another, all cell lines, with the possible exception of 1227B12C2, appeared to be expressing the Ad12 E1B 55K protein as well as one or more forms of E1A protein. To confirm E1A expression, additional analyses were carried out in which blots were probed with the M73 monoclonal antibody specific for the C terminus of Ad5 E1A proteins. As shown in Fig. 2B, E1A expression was readily detected in cells transformed by the Ad5 E1 region (lanes 2 to 4), in cells transformed by hybrid E1A plasmids p827, p975, p1036, and p1227 (lanes 5 to 14), and in cells transformed by p690 and p753 (data not shown). The E1A protein was not detected in the lane corresponding to 1461B12C1 because p1461 encodes Ad12 E1A, which is not recognized by the M73 monoclonal antibody. It is noteworthy that both mobility and heterogeneity of E1A products varied considerably among the different hybrid constructs. Similar effects on gel migration patterns were seen previously in studies with a series of insertion mutants of Ad5 E1A, where it was found that the mobility of E1A proteins shifted unpredictably depending on the site of insertion (2).

Tumorigenicity assays of transformed cell lines. Selected cell lines were expanded through 10 to 15 passages in culture for injection into syngeneic rats. Tumorigenicity assays were generally carried out with newborn rats, but in some experiments certain cell lines were also assayed in weanling rats. The results from assays with newborn rats and one experiment with weanling rats are summarized in Table 1. Cell lines transformed by p827 E1A plus Ad12 E1B were not tumorigenic even at an input dose of 10^8 cells per rat. In two animals, small tumors appeared at the site of injection after 4 months but in both animals these subsequently regressed. These rats were sacrificed at the age of 8 months and dissected to examine tissues at the site of injection, and no tumors were visible.

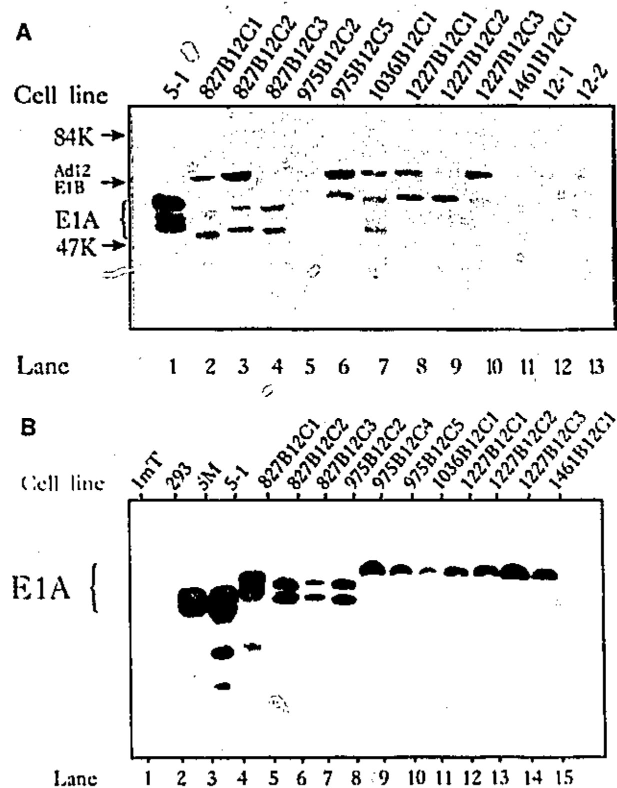


FIG. 2. Western blot analysis of E1 proteins from transformed cell lines. (A) Membrane filters were probed with tumor antiserum from rats bearing tumors induced by the 1227B12C1 cell line containing exon 1 of Ad12 E1A, exon 2 of Ad5 E1A, and Ad12 E1B. (B) Filters were probed with the M73 monoclonal antibody against the carboxy terminus of Ad5 E1A. The negative control used was cell line 1mT (Rat-1 cells transformed by polyomavirus middle T antigen [21]).

These results indicate that p827 either lacks Ad12 sequences necessary for tumorigenicity or contains Ad5 sequences which preclude tumorigenicity. Some clonal variation was seen with cells transformed by p975 E1A plus Ad12 E1B. Cell line 975B12C2 did not produce any tumors within 6 months, while cell line 975B12C4 was highly tumorigenic, giving rise to tumors within 2 months. Lastly, cell line 975B12C5 was weakly tumorigenic, giving rise to one tumor in each of two separate experiments. Several additional rats developed what appeared to be small tumors which subsequently regressed. One of the tumor-bearing rats injected with 975B12C5 was dissected, revealing a disc-shaped tumor at the site of injection, approximately 15 mm in diameter and 3 mm thick. Three different cell lines transformed by p1227 were tumorigenic, showing clonal variation only in the time of tumor appearance (1227B12C2 induced tumors slowly, but five of five rats eventually developed tumors). The hybrid E1A gene containing a crossover at Ad5 nucleotide 1036 has previously been shown to be defective for E1A-mediated transactivation, and p1036 did not give rise to any transformed colonies in previous transformation assays using plasmids with chimeric E1A and Ad5 E1B sequences (22), but when cotransfected with Ad12 E1B, the E1A derivative of p1036 did give rise to a single transformed colony from which it was possible to establish a cell line (1036B12C1) expressing E1 proteins (Fig. 2). This cell line was tumorigenic (Table 1), suggesting that the transactivation function of E1A is not required for tumorigenicity.

TABLE 1. Tumorigenicity of cell lines transformed by hybrid E1A plasmids plus Ad12 E1B

Cell line[a]	No. of tumors/total no. of rats after month:					
	1	2	3	4	5	6
690B12C1	0/9	0/9	0/9	0/9	0/9	0/9
753B12C1	0/9	0/9	0/9	0/9	0/9	0/9
827B12C1	0/7	0/7	0/7	1/7	0/7	0/7
827B12C2	0/10	0/10	0/10	0/10	0/10	0/10
827B12C2[b]	0/10	0/10	0/10	1/10	0/10	0/10
975B12C2	0/11	0/11	0/11	0/11	0/11	0/11
975B12C4	0/15	15/15	14/15	14/15	14/15	14/15
975B12C5	0/11	0/11	3/11	1/11	1/11	1/11
975B12C5	0/8	1/8	3/8	1/8	1/8	1/8
1036B12C1	0/12	3/12	5/12	5/12	5/12	5/12
1227B12C1	0/9	7/9	7/9	7/9	7/9	7/9
1227B12C2	0/5	1/5	1/5	3/5	5/5	5/5
1227B12C3	0/9	6/9	6/9	6/9	6/9	6/9
12-1	0/8	7/8	7/8	7/8	7/8	7/8
1227B12C1[c]	0/7	3/7	4/7	4/7		
12-1[c]	0/10	0/10	6/10	3/10		
12-2[c]	2/10	1/10	2/10	3/10		

[a] Each row represents the injection of a cell line into a single litter of newborn rats at 10^7 cells per rat, except as noted below.
[b] This litter was injected with 10^8 cells per rat.
[c] Weanling rats were injected with 10^7 cells per rat.

The results presented in Table 1 suggest that hybrid E1A proteins containing Ad12 sequences from the amino terminus up to the region between conserved region 1 (CR1) and CR2 (e.g., hybrids p690, p753, and p827) were unable to give rise to tumorigenic cells, while those containing additional Ad12 sequences at least to the leftmost border of CR3 (e.g., hybrids 975, 1036, and 1227) were tumorigenic (the DNA sequences of relevant hybrids are shown in Fig. 3).

Construction of additional hybrids. The results presented above indicated that a region of the Ad12 E1A protein surrounding CR2 or between CR2 and CR3 is important for tumorigenicity. A question arising from this observation was whether this region was the sole factor determining the oncogenicity of different E1A proteins. It is noteworthy that the region of the Ad12 E1A protein located between CR2 and CR3 has no counterpart in Ad5 E1A (Fig. 3). To assess the role of this region in tumorigenicity, CR2 of Ad5 E1A was replaced with the corresponding region of Ad12 E1A containing CR2 and the region between CR2 and CR3. To create such plasmids, we used PCR to amplify specific fragments with engineered restriction sites. Since the existing hybrid E1A plasmids p975 and p1227 contained crossovers near the region of interest, these crossover sites were used to define the

```
Residue                                                              Serotype

  1  MRHIICHGGVITEEMAASLLDQLIEEVLAD--N-LPPPSHFEPPTLHELYDLD  Ad  5
  1  MRTEMTPLVLSYQEA-----DDILEHLVDNFFNEVPSDDDLYVPSLYELYDLD  Ad 12
                                                   p690

                CR1
 51  V-TAPEDPNEEAVSQIFPDSVMLAVQEGIDLLTFPPAPGSPEPPHLSRQPEQP  Ad  5
 49  VESAGEDNNEQAVNEFFPESLILAASEGLFL---------PEPPVLS-----P  Ad 12
                p753                            p827

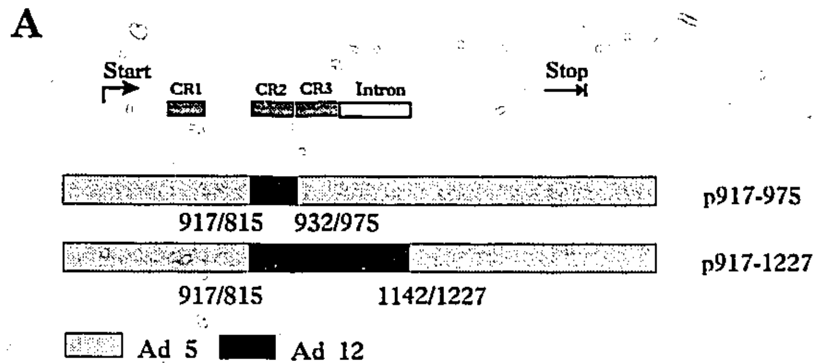
                        ⇓      CR2
103  EQRALGPVSMPNLVPEVIDLTCHEAGFPPSDDEDEEG----------------  Ad  5
 88  VCEPIGGECMPQLHPEDMDLLCYEMGFPCSDSEDEQDENGMAHVSASAAAAAA  Ad 12

                                 CR3
140  ----EEFVLDYVEHPGHGCRSCHYHRRNTGDPDIMCSLCYMRTCGMFVYSPVS  Ad  5
141  DREREEFQLDHPELPGHNCKSCEHHRNSTGNTDLMCSLCYLRAYNMFIYSPVS  Ad 12
         p975                p1036                  p1227

189  EPEPEPEPEPEPARPTRRPKMAPAILRRPTSPVSRECNSSTDSCDSGPSNTPP  Ad  5
194  DPEPEP-------------------------------NSTLDGDERPSPPKLG  Ad 12

242  EIHPVVPLCPIKPVAVRVGGRRQAVECIEDLLNEPG--Q--PLDLSCKRPRP-  Ad  5
220  SAVPEGVIKPVPQR-VT-GRRRCAVESILDLIQEEEREQTVPVDLSVKRPRCN  Ad 12
```

FIG. 3. Alignment of Ad5 and Ad12 E1A protein sequences. The amino acid sequences of Ad12 and Ad5 E1A were aligned to reflect regions of maximum conservation while introducing a minimum number of discontinuities. Residues at crossover sites common to both serotypes and each particular hybrid are indicated in boldface type, and the identity of the hybrid is shown beneath the alignment at each crossover site. The arrow corresponds to the location of the unique *Cla*I site in Ad5 E1A DNA.



B

Tumorigenicity of cell lines transformed by p917-975 and p917-1227.

Cell Line[a]	Time (Months) 1	2	3	4	5	6
917-975B12C6	0/10	0/10	2/10	3/10	0/10	0/10
917-975B12C10	0/11	0/11	0/11	2/11	2/11	2/11
917-1227B12C6	0/6	1/6	0/6	0/6	0/6	0/6
917-1227B12C6	0/16	0/16	4/16	7/16	6/16	8/16
917-1227B12C10	0/9	1/9	1/9	0/9	0/9	0/9

[a]Newborn rats were injected with 10^7 cells per rat.

FIG. 4. Tumorigenicity of cells transformed by the p917-975 and p917-1227 hybrids. (A) Structure of p917-975 and p917-1227 hybrid Ad5/Ad12 E1A genes. Hybrid E1A plasmids p975 and p1227 were linearized and used as templates in PCRs with the synthetic oligonucleotides AB1508 and AB416 (see Materials and Methods). Amplified products were treated with the Klenow fragment of DNA polymerase I to create blunt ends and cloned into the *Hinc*II site of pUC19. pXC38 containing the leftmost 16% of the Ad5 genome (2) was purified from *dam dcm* mutant *E. coli* GM48. Following digestion with *Cla*I and *Xba*I, the large fragment of pXC38 was ligated with the appropriate cloned, PCR-generated, hybrid E1A *Cla*I-*Xba*I fragment to generate plasmids p917-975 and p917-1227 containing Ad12 sequences (solid segments) from the start of CR2 (defined by the *Cla*I site in Ad5 E1A) to the crossover point of the original hybrid; the remaining sequences were derived from pXC38 (shaded segments). (B) Results of tumorigenicity assays with cells transformed by p917-975 and p917-1227. Tumorigenicity is given as the number of tumors found for the total number of rats in each group.

rightmost borders of Ad12 sequences in second-generation hybrids. Although the results shown in Table 1 suggested that p975 contained the minimum amount of Ad12 E1A necessary for tumorigenicity, two of three cell lines transformed by this plasmid were not highly oncogenic. For this reason, p1227 was also used for these experiments, since it contains all of CR3 of Ad12 E1A, and all cell lines transformed by it were highly tumorigenic.

The Ad5 E1A gene contains a unique *Cla*I restriction site, which is methylated in most strains of bacteria. The location of the restriction site is at nucleotide 917 and overlaps amino acid residues 120 and 121 (Ile and Asp) at the left border of CR2 (arrow in Fig. 3). Ad12 has Met and Asp residues at the analogous positions (residues 105 and 106) and lacks a *Cla*I site. To generate an Ad12 E1A PCR fragment with a *Cla*I site at this position, an oligonucleotide (AB1508; see Materials and Methods) identical to the coding strand of Ad12 but containing a *Cla*I site near its 5′ end was synthesized. This oligonucleotide and AB416 (complementary to the Ad5 E1A coding strand [see Materials and Methods]) were used in PCRs with either p975 or p1227 as a template to amplify fragments of DNA containing the crossovers in p975 and p1227. The resulting fragments were cloned into the *Cla*I-*Xba*I region of pXC38 which contains the left 16% of Ad5 DNA (2) (pXC38 was grown in *Escherichia coli* GM48 to avoid methylation of the Ad5 E1A *Cla*I site at position 917 and then cleaved with *Cla*I and *Xba*I). The resulting hybrid E1A regions are shown in Fig. 4A. The first construct, p917-975, contains Ad5 E1A residues 1 to 121 followed by Ad12 CR2 and the region between CR2 and CR3 (Ad12 residues 107 to 147), in place of Ad5 CR2, and ends with Ad5 E1A residues 141 to 289. The second construct, p917-1227, contains Ad5 E1A residues 1 to 121, Ad12 E1A sequences corresponding to CR2 and CR3 (residues 107 to 193), and Ad5 E1A from residues 184 to 289.

Transforming properties of new hybrids and establishment of transformed lines. Ad12 E1 transforms primary rodent cells about 1/10 as efficiently as Ad5 E1, and we have previously shown that two regions of E1A mediate this differential transforming efficiency (22). One of these appeared to be located in the N-terminal region of E1A, while the other was identified as the region corresponding to CR2 plus flanking sequences. Transforming activities of p917-975 and p917-1227 were determined to assess the role of CR2 in the differential transforming efficiencies of Ad5 and Ad12. Primary BRK cells

were electroporated with each hybrid plasmid, and transformed colonies were counted after 2 weeks. Transformation efficiencies of p917-1227 and pXC1 (Ad5 E1) were not significantly different, and p917-975 was approximately two- to threefold less efficient (data not shown), a characteristic which is shared by the hybrid p975, than p1227 and pHAB6 (22). Since the 12S splice donor site is not present in p975 or p917-975, the lower transforming efficiencies of these plasmids could be due to the lack of a 12S mRNA product, which is known to be important for transformation (16, 19, 20, 22, 23, 26, 28, 31, 32). These results and our previous observations (22) indicated that differences in transforming efficiencies between the E1A proteins of Ad5 and Ad12 are due to at least two regions of Ad12 E1A which result in decreased transformation efficiency. The first region, present in all hybrid plasmids with the exception of p917-975 and p917-1227, consists of Ad12 E1A amino-terminal sequences up to nucleotide 626 (including Ad12 CR1). The transforming efficiencies of p690, p753, and p827 containing this region are approximately threefold lower than that of pXC1 (22). The second region, present in plasmids p917-975, p917-1227, p975, p1036, p1227, and p1461 but absent in p690, p753, and p827, encompasses Ad12 nucleotides between 815 and 932 and includes Ad12 CR2 (reference 22 and this paper). When both these E1A regions are Ad12 derived as in hybrids containing Ad12 sequences from the N terminus up to CR3, the efficiency of transformation is similar to that of plasmids containing all of Ad12 E1A, i.e., approximately 5% that of pXC1. While the latency time (i.e., the time required for the detectable appearance of foci) for transformation by Ad12 and previously characterized hybrid E1 plasmids p690 through p1461 was 14 days (22), both p917-975 and p917-1227 had latency times of 6 days, indistinguishable from that of Ad 5. This indicates that the latency time is determined by a region of Ad12 E1A located to the left of CR2 and likely to the left of Ad12 nucleotide 626, since the latency time of p690 is 14 days. This is in agreement with our previous results (22).

To establish transformed cell lines for tumorigenicity assays, the Ad5 E1B regions of both new hybrid E1A plasmids were deleted and BRK cells were electroporated with the resulting hybrid E1A plasmids plus pHAB13. Cell lines were established and assayed for protein expression by Western blot analysis. The results (Fig. 5A) indicated that all three cell lines expressed E1A proteins reacting with M73 antibody. To detect E1B proteins, this blot was reprobed with 12-1 antitumor serum directed against the Ad12 E1 region (Fig. 5B). Cell lines transformed by the Ad12 E1 region (Fig. 5B, lanes 4 to 6) expressed Ad12 E1A proteins and the E1B 55K protein. Similarly, cell lines transformed by hybrid E1A plasmids plus Ad12 E1B (Fig. 5B, lanes 1 to 3) expressed both E1A and the E1B 55K protein.

Tumorigenicity of new cell lines. Newborn rats were injected with 10^7 cells for each line transformed by p917-975 and p917-1227 plus pHAB13. The results, shown in Fig. 4B, indicate that cells transformed by p917-975 were, at best, weakly tumorigenic, since the 917-975B12C10 line was tumorigenic in only 2 of 11 rats after 4 months and any tumors which arose following injection with 917-975B12C6 regressed by 5 months postinjection. Cells transformed by p917-1227 were tumorigenic at a significant efficiency with the 917-1227B12C6 line. These results suggest that while the region of Ad12 E1A introduced into Ad5 E1A in these plasmids was able to confer some tumorigenicity, it was not sufficient for a highly tumorigenic phenotype. Thus, one or more additional regions of the E1A protein may be involved in the tumorigenicity of transformed cells.

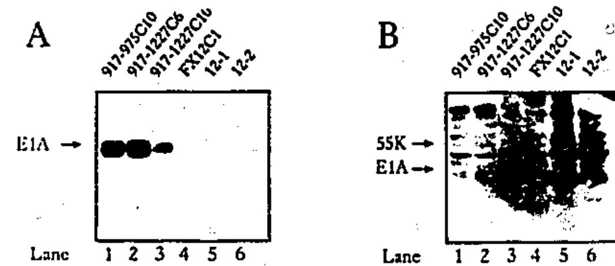


FIG. 5. Western blot analysis of E1 proteins from cell lines transformed by p917-975 and p917-1227 in cooperation with Ad12 E1B. (A) Filters were probed first with the M73 monoclonal antibody to detect E1A proteins. (B) The same filters were washed and reprobed with antitumor serum directed against the E1 region of Ad12 to detect expression of the Ad12 E1B 55K protein.

DISCUSSION

Rat cells transformed by E1 of Ad2 or Ad5 or by Ad5 E1A plus Ad12 E1B are nontumorigenic in syngeneic immunocompetent rats (4, 13). In an attempt to map the regions of Ad5 and Ad12 E1A responsible for differences in tumorigenicity, we have transformed rat cells with chimeric Ad5/Ad12 E1A genes plus Ad12 E1B. There was some clonal variation in the tumorigenicity of different cells transformed by the same plasmid (e.g., 975B12C2, C4, and C5 ranged from nontumorigenic to highly tumorigenic), and one cell line, 917-1227B12C6, failed to induce tumors in one assay but was moderately tumorigenic in a second experiment. Despite this variation, for which we have no explanation, the overall pattern which emerged from this study was fairly clear. Cells transformed by hybrid E1A genes encoding amino-terminal Ad12 residues up to amino acid 49, 66, or 83 were nontumorigenic (no tumors at 6 months in a total of 56 injected animals), whereas cells transformed by hybrids encoding Ad12 residues to the right of CR2 or beyond were moderately tumorigenic (43 tumors in 87 animals). Included in the region of Ad12 E1A which is present in the oncogenic p975 but absent from the nononcogenic p827 are a portion of the stretch between CR1 and CR2, CR2 itself, and the region bordered by CR2 and CR3. Ad5 has no counterpart to the sequence between CR2 and CR3 of Ad12, and in fact, the two conserved regions abut each other in Ad5 (Fig. 3). Examination of the E1A sequences for a number of Ad serotypes indicated that this region, characterized by the presence of a stretch of alanine residues, is absent from other human Ads for which the E1A sequence is known. However, the amino acids between CR2 and CR3 of Ad12 bear a close resemblance to the analogous region in the E1A sequence of the highly oncogenic simian adenovirus type 7 (Fig. 6), suggesting that the region between CR2 and CR3, which is unique to these two unrelated oncogenic Ads, may play a role in their oncogenicity. To investigate this possibility, Ad12 E1A sequences encompassing CR2 and the CR2-CR3 region were introduced into Ad5 E1A sequences. One cell line transformed by each of the resulting plasmids, p917-975 and p917-1227, was weakly or moderately oncogenic, and one was nononcogenic. Our data suggest, therefore, that the Ad12 E1A region bordered by CR2 and CR3 may be involved in tumorigenicity but is likely not the sole factor influencing it, since cells transformed by hybrids consisting of Ad12 E1A sequences from the amino terminus up to and beyond CR3 were more strongly tumorigenic. Moreover, our data suggest that when acting in concert with Ad12 E1A sequences bordered by CR2

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          CR2                                 CR3
Ad 4      DED--EQIAQNAASHG------------VQAVS--ESF
Ad 5      DED--EEG---------------------------EEF
Ad 7      DED--GETEQSIHTAV---------NEGVKAAS--DVF
Ad 12     SED-------EQDENGMAHVSASAAAAAADRER--EEF
Ad 40     PET------DEATEAEEE----AAMPTYVNENE--NEL
Ad 41     SEA------DEAEERAEEEETAVSNYVNIAEGA--SQL
SA7       SEE--GEHSQVETERKMAEAAAAGAAAAARREQ--DDF

Kr(53-85)       AMGLQQAAAASAFGMLSPTQLLAANRQAAAFMA
en(63-77)       AMAFD-AAAAAAAAAA-----------------
en(229-237)     ---QQQAAAAAA---------------------
eve(146-177)    AASILQAAANSV-GMPYPPYAPAAAAAAAAAAA
```

FIG. 6. Alignment of the sequences of several Ad serotypes in the region between CR2 and CR3. The sequences were obtained from D. S. Bautista (2), and only the region between CR2 and CR3 is shown, illustrating the unusual repeat of alanine residues common to Ad12 and simian adenovirus type 7 (SA7). Shown below for comparison are alanine-rich portions of the primary sequences of the *Drosophila* transcriptional repressors: Kruppel (*Kr*), engrailed (*en*), and even-skipped (*eve*) (27). Residues 53 to 85 of *Kr* constitute a portion of the amino-terminal repression domain aligned to two segments of *en* and one segment of *eve*.

and CR3. Ad12 E1A sequences to the left of CR2 are involved in strongly and positively influencing tumorigenicity or, alternatively, Ad5 E1A sequences to the left of CR2 negatively influence tumorigenicity. The latter possibility may explain the lower degree of tumorigenicity of cell lines transformed by p917-975 and p917-1227.

The finding that the Ad12 E1A region bordered by CR2 and CR3 is an oncogenic determinant of Ad12 is also suggested by recent studies of J. Williams and G. C. Telling (41a). They have constructed Ad12-based chimeric viruses in which this region was replaced with the corresponding type 5 sequence and found that the viruses or cells transformed by them were greatly reduced in oncogenic capacity (41a).

We have suggested previously that the mechanism by which E1A transforms may not be simply by sequestration of cellular proteins (22). Rather, the complexes of E1A with p300, p130, p107, p105, and p60 may carry out specific functional roles which could differ depending on the serotypic origin of E1A in the complexes. In this case, it is likely that complexes containing the Ad5 E1A protein may be functionally different from those consisting of the Ad12 E1A protein. Interestingly, the amino terminal residues of Ad5 E1A have been shown to be required for transforming functions and repression of viral enhancers. Since Ad5 and Ad12 E1A proteins differ greatly at the amino terminus, with the exception of residues 2 and 20 (19 in Ad12) which among others have been shown to be critical for p300 binding (44), it is possible that sequence differences between the Ad5 and Ad12 E1A amino-terminal residues may alter the functions associated with E1A-300K complex in some way that alters tumorigenicity of the resulting transformed cells.

All in all, the differential tumorigenic potentials of cells transformed by Ad5 and Ad12 may be attributable to several regions, with unique functions, nested within the E1 region. At least one function influencing tumorigenicity is specified by the E1B region, since cell lines expressing Ad12 E1B are more tumorigenic than those expressing Ad5 E1B (4, 37). While the nature of E1A functions that influence tumorigenicity is not clear at present, it is possible that individual functions of both the Ad5 and Ad12 E1A proteins influence the tumorigenic phenotype. As an example, it is very likely that Ad5 E1A has an antitumorigenic function related to the immune response of the host animal, since cells expressing Ad5 E1A are tumorigenic in nude mice but nontumorigenic in syngeneic immunocompetent animals (4). Furthermore, transformed lines transfected with Ad5 E1A exhibit decreased tumorigenicity (43).

The mechanism by which the region of Ad12 E1A between CR2 and CR3 may influence tumorigenicity is not clear, but this region has features which suggest that it may be involved in transcriptional regulation. First, it is adjacent to the transactivation domain of the Ad12 E1A protein, raising the possibility that it may interact with or influence the function of this domain. Secondly, several proteins with transcriptional repressor activity in which the repression function maps to a region containing a stretch of alanine residues similar to that seen between CR2 and CR3 of Ad12 and simian adenovirus type 7 E1A have been characterized (27) (Fig. 6). A previously characterized transcriptional repression activity of Ad12 which may influence tumorigenicity is class I MHC repression. Further study may determine whether this function localizes to the region between CR2 and CR3.

In addition to the establishment of cell lines transformed by p917-975 E1A and p917-1227 E1A with Ad12 E1B, these plasmids were assayed for transforming efficiency in combination with Ad5 E1B. Both plasmids transformed BRK cells at an efficiency significantly greater than that of Ad12, with p917-1227 approximating Ad5 E1A even though both encode CR2 of Ad12 E1A. This suggests that CR2, which is critical for the transforming activity of both E1A proteins, is not involved in the differential transforming efficiency of Ad5 and Ad12 E1A. This is in agreement with the observation that the cellular proteins whose interactions with CR2 of Ad5 E1A are critical for transformation (10, 11, 14, 17, 45, 46) interact equally well with CR2 of Ad5 and Ad12 E1A (22). The observation that a region or regions of E1A besides those corresponding to known cellular protein binding sites may be involved in the transforming efficiency of the protein supports the idea mentioned previously that transformation of primary cells by E1A involves properties in addition to the sequestration of cellular proteins.

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Contributions to Jelinek et al., 1994:

(1) Assisted in establishing BRK cell lines transformed by p917-975 and p917-1227.

(2) Assessed the tumorigenic capacity of the p917-975- and p917-1227-transformants following subcutaneous injection into newborn syngeneic rats.

(3) Assisted in rescuing hybrid Ad5/12 E1A genes encoded by p917-975 and p917-1227 into wild type Ad5 virus. To determine whether these hybrid E1A proteins could support viral replication, the ability of these viruses to form plaques in 293 cells (constitutively express Ad5 E1A and complements growth of E1A deletion or mutation viruses) and HeLa cells (do not complement growth of E1A deletion or mutation viruses) was compared by T. Jelinek.

Conclusions:

Cells transformed by the hybrid Ad5/12 E1A- (plus Ad12 E1B) genes: p690, p753, and p827 were non-tumorigenic when expressing up to 80 amino acids of the Ad12 E1A amino terminus (includes CR1) whereas cells transformed by the amino-terminal 144 residues of Ad12 E1A (includes CR1 and CR2) or beyond were tumorigenic (e. g. p975-, p1036-, and p1227-transformants).

To extend these results, Ad12 E1A sequences spanning a stretch of 20 amino acids (124-144; EQDENGMAHVSASAAAAAADRER) between CR2 and CR3, which is absent from the analogous Ad5 E1A CR2-CR3 region were substituted for homologous sequences of Ad5 E1A and used to establish the p917-975- and p917-1227- transformed cell lines. These lines were approximately two-fold less tumorigenic than Ad12 E1-transformed cells, suggesting that Ad12 E1A sequences in addition to the CR2-CR3 residues also contribute to tumorigenicity. The view that the Ad12 CR2-CR3 residues served as an oncogeneic determinant were corroborated by a complementary approach using Ad12-based chimeric

viruses and transformed cell lines in which the Ad12 E1A CR2-CR3 region was replaced by corresponding Ad5 E1A sequences (Telling and Williams, 1994).

It should be noted that the different tumorigenic capacities observed by the hybrid Ad5/12 E1A (plus Ad12 E1B) transformants was not due to differences in E1A expression as measured by Western blot analysis.

2) Pereira, D. S., Rosenthal, K. L., and Graham, F. L. 1995. Identification of two regions of adenovirus E1A which affect MHC class I expression and susceptibility to cytotoxic T lymphocytes. Virology *211*, 268-277.

Preface:

Since the discovery that cells transformed by the E1 regions of Ad5 and Ad12 differ in oncogenic potential, many subsequent studies attempted to identify and understand functional differences between their E1A proteins. Conclusions drawn from these investigations suggested that the Ad5 and Ad12 E1A proteins, present in transformed cells, differed in their potential to modulate the host immune response to these cells. Specifically, Ad12 E1A, unlike Ad5 E1A, was found to down-regulate expression of MHC class I mRNA and cell surface molecules, a property which would endow Ad12 E1-transformants with the ability to evade host class I-restricted CD8+ CTLs thereby favoring tumorigenesis.

Although the ability of Ad12 E1A to down-regulate MHC class I expression of transformed cells has generally been accepted as the principal feature determining tumorigenicity, the notion that the tumorigenic capacity of these cells depends solely on class I down-regulation is a topic of great debate since the relationship between class I levels, susceptibility to CTLs and tumorigenicity is poorly defined. To better define these relationships and obtain a further understanding of the basis for the different tumorigenic capacities of Ad5 E1- and Ad12 E1-transformed rodent cells, the series of hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed Hooded Lister rat cell lines, which differ in tumorigenic capacity, were used to identify E1A regions which affect MHC class I expression and susceptibility to allogeneic and syngeneic CTLs.

VIROLOGY 211, 268–277 (1995)

Identification of Adenovirus E1A Regions Which Affect MHC Class I Expression and Susceptibility to Cytotoxic T Lymphocytes

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To map and characterize functional differences between the E1A oncoproteins of Ad5 and Ad12, we previously constructed a series of hybrid Ad5/12 E1A genes and used them in combination with Ad12 E1B to transform Hooded Lister rat cells. At least two regions within the first exon of Ad12 E1A which influenced tumorigenicity were identified. In this report, again using the hybrid Ad5/12 E1A (plus Ad12 E1B) transformants, we further examined the role of these regions in tumorigenicity by analyzing their effect on cell surface major histocompatibility complex class I expression and sensitivity to class I-restricted CD8$^+$ cytotoxic T lymphocytes (CTLs). Results of these studies suggest that expression of either of the Ad12 E1A regions implicated in tumorigenicity could down-regulate cell surface class I levels. However, neither class I down-regulation nor sensitivity to allogeneic CTLs was shown to strictly correlate with the tumorigenic capacities of the transformed rat cells. Another factor influencing the tumorigenicity of Ad5 E1 and Ad12 E1 transformants may be the ability of their E1A products to encode CTL epitopes. To this end, we provide evidence suggesting that CTL epitopes may be encoded by Ad5 E1A but not by Ad12 E1A, since expression of certain portions of the Ad5 E1A protein conferred susceptibility to syngeneic Ad5 E1-specific CTLs *in vitro*, while Ad12 E1A expression did not confer susceptibility to syngeneic Ad12 E1-specific CTLs.
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INTRODUCTION

Human adenoviruses (Ads) are nonenveloped viruses which contain dsDNA genomes and which replicate in the nucleus of infected cells. Expression of viral genes is temporally regulated as a result of transcription first from early and subsequently from late transcription units. Early region 1 (E1), which is transcribed immediately following infection, consists of two transcription units encoding the E1A and E1B oncogenes. Cooperatively, E1A- and E1B-encoded oncoproteins are responsible for the ability of Ads to transform primary rodent cells in culture. On the basis of *in vivo* tumorigenicity, the human Ad serotypes have been classified into three groups: highly tumorigenic (e.g., types 12, 18, and 31), weakly tumorigenic (e.g., types 3 and 7), and nontumorigenic (e.g., types 2 and 5) (Huebner, 1967). Although tumorigenicity of Ad-infected and Ad-transformed cells requires coexpression of E1A and E1B, the serotypic origin of E1A is the major determinant for tumorigenicity (Bernards *et al.*, 1983; Jochemsen *et al.*, 1984). In addition to mediating the processes of transformation and tumorigenicity, the nonstructural E1A oncoproteins also play key roles in viral replication, regulation of viral and cellular gene expression, and interactions with host immune components (for reviews see Graham, 1984; Williams, 1986; Barbeau *et al.*, 1994; Bayley and Mymryk, 1994).

Earlier studies directed at identifying and understanding functional differences between Ad5 and Ad12 E1A oncoproteins have suggested that E1A influenced tumorigenicity of Ad5 (nontumorigenic)- and Ad12 (tumorigenic)-transformed rodent cells by modulating the host immune response of the immunocompetent rodent. For instance, while both the Ad5 and Ad12 E1A products can determine viral specificity of tumor-specific transplantation immunity (Sawada *et al.*, 1986, 1994), Ad12 E1A, unlike Ad5 E1A, participates in down-regulating expression of major histocompatibility complex (MHC) class I mRNA and cell surface molecules in rodent (Schrier *et al.*, 1983; Vaessen *et al.*, 1987; Ackrill and Blair, 1988; Friedman and Ricciardi, 1988) and human cells (Vasavada *et al.*, 1986). The E3 region of the group B–E Ads (types 2–5, 9, 11, 19, and 34) encode a 19-kDa glycoprotein which is also capable of down-regulating cell surface class I expression by retaining class I antigens in the endoplasmic reticulum (for review see Rinaldo, 1994). Other viruses in the DNA (e.g., herpesviridae, papovaviridae, and poxviridae) and RNA (e.g., retroviridae, coronaviridae, rhabdoviridae, and paramyxoviridae) virus families also down-regulate MHC class I expression (for review see Rinaldo, 1994). It is thought that down-regulation of class I expression is a mechanism many persistent and tumorigenic viruses, such as Ad, have evolved as a strategy to evade immunosurveillance by class I-restricted CD8$^+$ cytotoxic T lymphocytes (CTLs) (Anderson *et al.*, 1985). In the context of tumorigenicity, Ad5 E1-transformed cells are thought to fail to form tumors in immunocompetent rodents because they are more antigenic than Ad12 E1 transformants. This notion is partially

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supported by the fact that cells transformed by non-oncogenic Ads are tumorigenic in immunoincompetent rodents (Gallimore *et al.*, 1977; Bernards *et al.*, 1983). Several reports which have failed to find a strict correlation between reduced class I expression and tumorigenicity (Mellow *et al.*, 1984; Haddada *et al.*, 1986; Soddu and Lewis, 1992) suggest that nonspecific effector cells of the immune system, such as natural killer (NK) cells, may also play an important role in affecting tumorigenicity. Rodent cells transformed by Ad5 or Ad12 E1A have, respectively, demonstrated susceptibility or resistance to lysis by NK cells (Raska and Gallimore, 1982; Sawada *et al.*, 1985; Cook *et al.*, 1986, 1987; Routes, 1993). Since the association between MHC class I expression and tumorigenicity is not strictly defined, yet another factor determining tumorigenicity may be the ability of Ad E1A molecules to serve as immunogenic determinants on transformed cells. This hypothesis is reasonable since CTL-stimulating epitopes, encoded by Ad5 E1A gene products, have been found on cell surfaces in context with class I antigens (Bernards *et al.*, 1983; Bellgrau *et al.*, 1988; Kast *et al.*, 1989; Urbanelli *et al.*, 1989; Rawle *et al.*, 1991; Routes *et al.*, 1991).

Previously, using primary baby rat kidney cell lines cotransformed with hybrid Ad5/12 E1A genes plus Ad12 E1B (Jelinek and Graham, 1992), we identified at least two regions of Ad12 E1A which influenced tumorigenicity (Jelinek *et al.*, 1994). Our findings were corroborated by a complementary approach using Ad12-based chimeric viruses in which one of these Ad12 E1A regions was replaced by corresponding Ad5 E1A sequences (Telling and Williams, 1994). In this report, the functional importance of these regions in modulating cell surface MHC class I expression and sensitivity to CTLs was determined to better understand the tumorigenic capacity of Ad5 E1- and Ad12 E1-transformed cells.

MATERIALS AND METHODS

Animals

Hooded Lister rats (MHC haplotype C) used for allogeneic and syngeneic CTL assays were bred by brother–sister mating and housed in the Central Animal Facility, McMaster University. Fischer 344 rats (MHC haplotype L) used for allogeneic CTL assays were purchased from Charles Rivers Canada (Quebec, Canada).

Cell culture

Hooded Lister rat cells transformed by Ad5 E1, Ad12 E1, and hybrid Ad5/12 E1A (plus Ad12 E1B) genes (Jelinek and Graham, 1992), as well as the human 2H (Ad12 E1-transformed embryonic kidney) (S. Mak, unpublished) and the rat 702-C2, HABaC1, and HABaC2 (Ad12 E1 transformants) (Mak *et al.*, 1979) cell lines were grown in α-MEM supplemented with 10% FCS. The human cell lines 293 (Ad5 E1-transformed embryonic kidney) (Graham *et al.*, 1977) and HER3 (Ad12 E1-transformed embryonic retinal) (Byrd *et al.*, 1982) were maintained as monolayer cultures in F11 medium supplemented with 10% newborn calf serum and DMEM supplemented with 7% FCS. All media were also supplemented with 2 m*M* L-glutamine, 100 μg/ml of penicillin G, and 100 μg/ml of streptomycin sulfate.

Tumorigenicity assays

Cell lines transformed by hybrid Ad5/12 E1A constructs plus Ad12 E1B were expanded through 10–15 passages in culture for subcutaneous injection into newborn syngeneic Hooded Lister rats. Each line was injected into single litters of newborn rats at 10^7 cells per rat and monitored for tumor formation at the site of injection for up to 6 months (Jelinek *et al.*, 1994). Data in Fig. 1B are a compilation of multiple assays for each line, thus accounting for the different numbers of rats injected per cell line.

Detection of cell surface MHC class I proteins

Cell surface expression of MHC class I proteins was determined by flow cytofluorimetry using a FACScan (Becton–Dickinson Canada, Inc., Ontario, Canada) and antibodies purchased from Cedarlane Laboratories, Ltd. (Ontario, Canada). Single-cell suspensions were prepared from monolayer cultures and resuspended in PBS supplemented with BSA (10 mg/ml) and subdivided into three aliquots (unstained, stained by secondary antibody only, stained by both primary and secondary antibodies) containing 10^6 cells/aliquot. Where appropriate, single-cell suspensions were incubated with primary antibodies for 30 min on ice using either the mouse anti-rat RT1.A monoclonal antibody CL007A, which recognizes a monomorphic determinant of rat class I MHC antigen, or the mouse anti-human antibody W6/32 (gift from I. York), which is directed against human HLA (A, B, or C). Subsequent or independent staining was conducted using FITC-conjugated rabbit anti-mouse Ig antiserum (reacts with all mouse Ig classes and subclasses).

Generation of cytotoxic T lymphocytes

Allogeneic effector CTLs were generated following coculture of single-cell suspensions of splenic lymphocytes isolated and prepared from 8-week-old Hooded Lister rats (stimulators; MHC haplotype C) and Fischer 344 rats (responders; MHC haplotype L) at a responder:stimulator ratio of 1:1. Three days prior to coculture, stimulator Hooded Lister rat splenic lymphocytes were γ-irradiated at 5000 rads. To generate syngeneic Ad5 E1- and Ad12 E1-specific CTLs, 8-week-old Hooded Lister rats were immunized ip with 10^7 DP5-2 (Ad5 E1-transformed Hooded Lister rat line) or 10^7 12-1 (Ad12 E1-transformed Hooded Lister rat line) cells which were resuspended in 1 ml of PBS. Three weeks later, splenic lymphocytes were isolated, made into a single-cell suspension, and

restimulated by coculture with γ-irradiated (3000 rads) DP5-2 or 12-1 cells at a responder:stimulator ratio of 50:1. Cocultured cells were suspended in complete RPMI media (supplemented with 10% FCS, 2m*M* L-glutamine, 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulfate, and 5×10^{-5} *M* 2-mercaptoethanol) and incubated in upright 25-cm^2 tissue culture flasks for 3 days in a humidified environment containing 5% CO_2. Gamma irradiation was conducted using a Gammacell 1000 containing a ^{137}Cs source (Atomic Energy of Canada, Ltd., Ontario, Canada).

Chromium release cytolytic assays

Target cells (5×10^4 cells/ml) were seeded into 96-well microtiter plates (Falcon 3072) and subsequently labeled with [^{51}Cr]sodium chromate (2 μCi/well) for 1 hr at 37°. Once labeled, target cells were washed three times with complete RPMI (supplemented as described above) to remove excess label. Meanwhile, effector cells were pelleted at 2000 rpm for 5 min, resuspended in complete RPMI media, and counted in the presence of trypan blue to determine the number of viable lymphocytes. These effector cells were then incubated with ^{51}Cr-labeled targets (in a total volume of 200 μl/well) at various effector:target (E:T) ratios for 6 hr at 37° under coculturing conditions described above. For allogeneic CTL assays, E:T ratios were 136:1, 68:1, and 34:1, while for syngeneic CTL assays, ratios of 100:1, 50:1, and 25:1 were utilized. Next, 100-μl samples of the supernatants were removed from each well, placed in borosilicate tubes, and counted in a LKB Wallac 1282 universal gamma counter. The percentage of specific lysis was calculated using the following formula: $(T - S/M - S) \times 100$, where T = cpm released by targets in the presence of CTLs, S = cpm released spontaneously by targets in the absence of CTLs, and M = maximum cpm released from targets following complete lysis using Triton X-100.

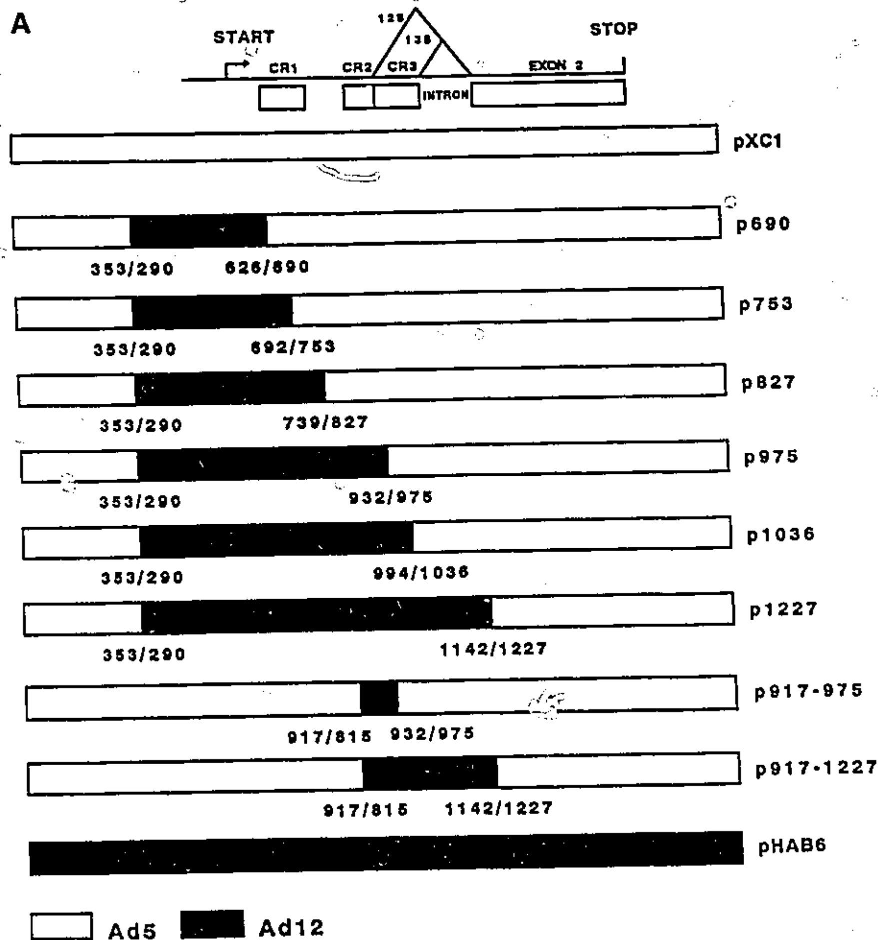
RESULTS

A series of Hooded Lister rat cells transformed by hybrid Ad5/12 E1A plus Ad12 E1B genes

The hybrid Ad5/12 E1A genes, shown in Fig. 1A, were constructed previously and used in combination with the Ad12 E1B gene to transform primary Hooded Lister rat kidney cells (Jelinek and Graham, 1992). For a complete description of these hybrid E1A genes, see the legend to Fig. 1. Previous analysis of hybrid Ad5/12 E1A and Ad12 E1B protein expression by Western blots revealed that comparable levels of hybrid Ad5/12 E1A and Ad12 E1B proteins were expressed by all the transformed rat cells (Jelinek and Graham, 1992; Jelinek *et al.*, 1994). Furthermore, all recombinant viruses expressing these hybrid E1A proteins were capable of replicating efficiently in HeLa cells with the exception of one virus (T1036) which contained a hybrid E1A gene in which the crossover from Ad12 to Ad5 coding sequences was in the transactivation domain located within the third conserved region (CR3) of E1A (Jelinek and Graham, 1992; Jelinek *et al.*, 1994). Recently, the hybrid Ad5/12 E1A (plus Ad12 E1B) transformants were assayed for their ability to form tumors in newborn syngeneic rats (Jelinek *et al.*, 1994). The tumor induction data, shown in Fig. 1B, are a compilation derived from multiple 6-month assays in which 10^7 cells were injected subcutaneously into newborn syngeneic rats (see Materials and Methods). As expected, the Ad5 E1-transformed line (DP5-2; transformed by pXC1) and the Ad12 E1 transformant (12-1; transformed by pHAB6) were respectively nontumorigenic (0 of 9 rats) and tumorigenic (7 of 8 rats). Cells transformed by the p690, p753, and p827 hybrid Ad5/12 E1A genes, shown in Fig. 1A, which respectively encode 5′-terminal Ad12 E1A sequences up to nucleotides 626 [amino acid (aa) 42], 692 (aa 64), and 739 (aa 80) were nontumorigenic, whereas the presence of Ad12 E1A sequences up to nucleotides 932 (aa 144), 994 (165), and 1142 (aa 193) conferred tumorigenicity to the p975, p1036, and p1227 transformants, respectively. Therefore, Ad12 E1A sequences necessary for tumor induction appeared to lie between nucleotides 739 and 932 (aa 80–144). To determine whether this region alone was sufficient for tumor induction when present in an Ad5 E1A background, we constructed the p917-975 and p917-1227 hybrid Ad5/12 E1A-encoding constructs which contained Ad12 E1A nucleotides 815–932 (aa 105–144) and 815–1142 (aa 105–193), respectively, embedded in Ad5 E1A coding sequences as illustrated in Fig. 1A. The p917–975 and p917–1227 transformants was less tumorigenic than the p975, p1036, p1227, and pHAB6 transformants, suggesting that expression of Ad12 E1A sequences encoded by p917–975 (aa 105–144) were necessary but not sufficient to confer the same degree of tumor incidence observed in rats transformed by the Ad12 E1-encoding construct pHAB6 (7 of 8 rats). Therefore, in addition to Ad12 E1A residues 105–144, amino acids to the left of residue 105 (nucleotide 815) may also be required for tumorigenicity (Jelinek *et al.*, 1994) (see Fig. 2A).

Cell surface MHC class I expression

Since it has been proposed that the difference in tumorigenic capacity of Ad5 E1 and Ad12 E1 transformants is due to the ability of Ad12 E1A, but not Ad5 E1A, to down-regulate class I expression below levels necessary for recognition by CTLs, we used the hybrid Ad5/12 E1A transformants to map E1A regions which affect cell surface class I expression and susceptibility to allogeneic CTLs. Compared to levels expressed on cells transformed by Ad5 E1A (DP5-2 or 5-M), all the hybrid Ad5/12 E1A-transformed lines exhibited decreased cell surface class I levels (see Fig. 3A). The decrease appeared to occur in two steps as Ad5 E1A sequences were progressively replaced with Ad12 E1A sequences from the amino



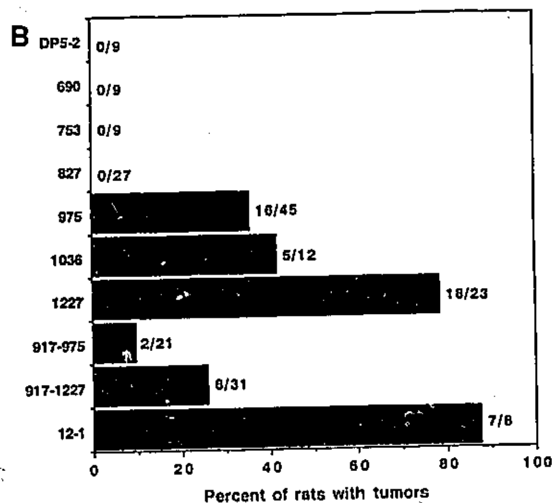


FIG. 1. (A) Cartoon of Ad5 E1A (pXC1), Ad12 E1A (pHAB6), and hybrid Ad5/12 E1A plasmids used to transform Hooded Lister rat kidney cells. These hybrid E1A constructs, shown with Ad5 and Ad12 E1A gene sequences in white and black, respectively, are aligned with the structure of Ad5 E1A (top). The three conserved regions (CR1, CR2, and CR3) in the first exon of E1A are also shown. Precise locations of crossovers are expressed in nucleotides below the crossover junctions, where Ad5 and Ad12 nucleotides are shown on the outer and inner sides of the slashes, respectively. All hybrid E1A constructs, with the exception of p917-975 and p917-1227, contain a first crossover junction in which the leftmost 353 bp of Ad5, encompassing the viral inverted terminal repetition and enhancer/packaging regions, are followed by Ad12 E1A sequences from nucleotide 290, encompassing the Ad12 E1A promoter, transcriptional start, and amino terminus, to a second junction point where crossover again occurs into Ad5 E1A sequences to 16% of Ad5 genome length. In addition to the first 353 nucleotides of Ad5 common to the hybrid E1A plasmids, the p917-975 and p917-1227 constructs (described previously) contain Ad5 gene sequences to nucleotide 917 (815; Ad12) followed by Ad12 E1A gene sequences to nucleotides 932 (975; Ad5) and 1142 (1227; Ad5), respectively. It should be noted that the p975 and p917-975 hybrids lack the 12s splice donor sites of Ad5 and Ad12 E1A, therefore these hybrids encode only the 13S E1A product. (B) Tumorigenicity of Hooded Lister rat cells transformed by hybrid Ad5/12 E1A plus Ad12 E1B genes. Data are a compilation derived from multiple assays in which 10^7 cells per clone per newborn rat was injected subcutaneously into newborn syngeneic rats and monitored for tumor formation at the site of injection for up to 6 months (Savada *et al.*, 1985). The number of tumor bearing rats/total number of injected rats are shown.

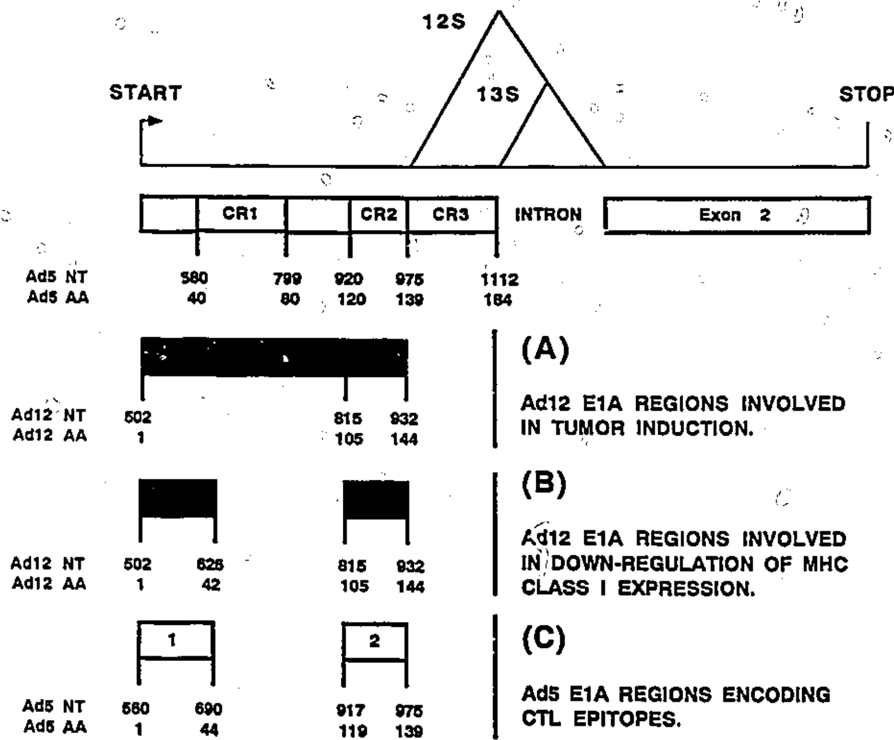


FIG. 2. Schematic representing functional regions of the adenovirus E1A first exon. Ad5 and Ad12 E1A regions are depicted in white and black, respectively. The open reading frame of Ad5 E1A and locations of conserved regions (CRs) are shown atop. (A) Ad12 E1A regions involved in tumor induction and (B) down-regulation of MHC class I expression. (C) Ad5 E1A regions (1 and 2) potentially encoding CTL epitopes.

terminus rightward. The first decrease, seen with the nontumorigenic lines transformed by the p690, p753, and p827 constructs, resulted in class I levels an average of 5-fold lower than those of the DP5-2 or 5-M lines. Tumorigenic p975, p1036, and p1227 transformants demonstrated a second average decrease represented by an approximately 2- to 3-fold reduction compared to the nontumorigenic hybrids and 10-fold compared to the non-

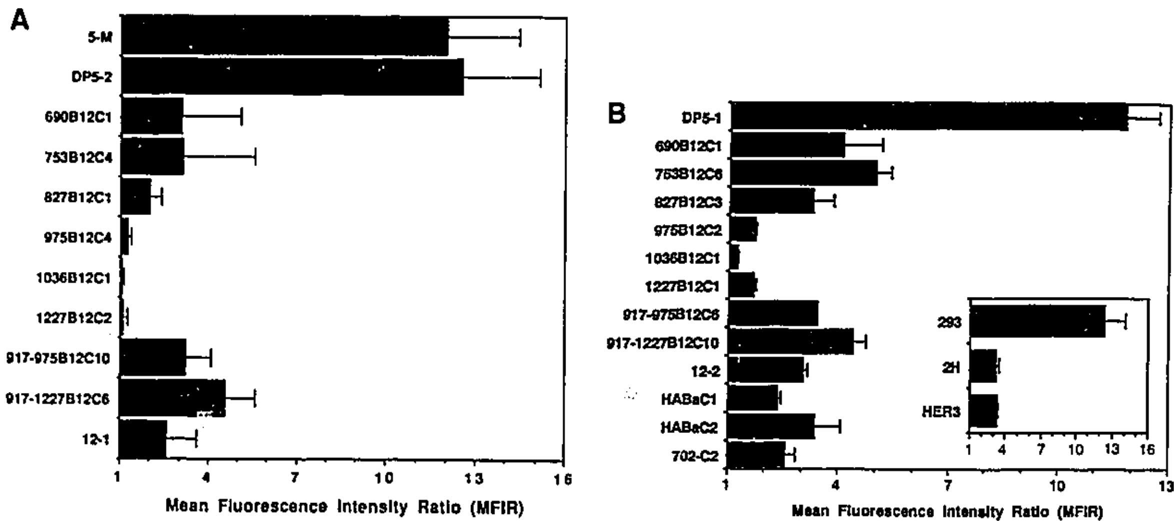


FIG. 3. Cell surface expression of MHC class I proteins on Hooded Lister rat cells transformed by Ad5 E1, Ad12 E1, and the hybrid Ad5/12 E1A plus Ad12 E1B genes. Cell surface expression of MHC class I molecules was determined by flow cytofluorimetry using a primary monoclonal antibody specific for the rat MHC class I proteins (RT1.A) and a secondary antibody conjugated to FITC. For a detailed explanation of sample preparation and analysis, see Materials and Methods. The mean fluorescence intensity ratio was determined according to the following calculation: (mean fluorescence intensity of cell line in the presence of primary and secondary antibody)/(mean fluorescence intensity of cell line in presence of secondary antibody only). (A and B) Cell surface MHC class I expression of the transformed Hooded Lister rat cells; however, different clones of the respective cell lines are shown in A and in B. Results from FACS analyses used to measure cell surface class I expression in the human cell lines transformed by Ad5 E1 (293) or Ad12 E1 (2H and HER3) are shown in the inset in B.

tumorigenic DP5-2 and 5-M lines. Class I levels on the tumorigenic hybrid p917-975 and p917-1227 transformants, which contain Ad5 E1A-derived amino terminal sequences, were an average of 3- to 4-fold lower than those of the nontumorigenic DP5-2 or 5-M lines and were comparable to levels expressed on the nontumorigenic hybrids transformed by the p690, p753, and p827 constructs. While 12-1 cells (transformed by the Ad12 E1-encoding plasmid pHAB6) expressed class I levels comparable to those on the nontumorigenic p690, p753, and p827 transformants and tumorigenic p917-975 and p917-1227 transformants, they exhibited approximately 2-fold greater cell surface class I levels than those of the tumorigenic p975, p1036, and p1227 transformants. To ensure that differences observed in MHC class I expression among the transformants were not the result of clonal variation, we measured cell surface levels of additional cell lines by FACS analysis (Fig. 3B) and found the levels to be in agreement, within experimental error, with those of the clones transformed by the same E1A constructs shown in Fig. 3A. Also included in the experiment were additional Ad12 E1-transformed rat cells, HABaC1, HABaC2, and 702-C2, to verify that class I down-regulation occurs in other Ad12 E1 transformants. Cell surface class I levels were also measured on human Ad5 E1 (293) and Ad12 E1 (2H and HER3) transformed cells (see inset of Fig. 3B). Class I levels on 2H and HER3 lines were nearly 4-fold lower than those of the 293 line and paralleled the differences seen between the rat Ad5 E1 and Ad12 E1 transformants shown in Fig. 3A.

Taken together, these results suggest that two regions of Ad12 E1A are involved in down-regulation of cell surface class I expression: aa 1–42 and aa 105–144 (see Fig. 2B). Interestingly, these two regions lie within the regions of Ad12 E1A involved in tumor induction (see Fig. 2A). It should be noted, however, that reduced cell surface class I expression did not appear to strictly correlate with tumorigenicity since the tumorigenic Ad12 E1 transformant 12-1 expressed class I levels comparable to those of the nontumorigenic p690, p753, and p827 transformants.

Allogeneic cytotoxic T lymphocyte activity

In the context of tumorigenicity, it was important to determine whether the reduced (but nonzero) class I levels expressed on some of these lines were sufficient for recognition and lysis by allogeneic CTLs (Fig. 4). With the exception of the tumorigenic p975, p1036, and p1227 transformants, all the hybrid Ad5/12 E1A (plus Ad12 E1B) transformants were significantly lysed by allogeneic Fischer 344 splenic CTLs, suggesting that these lines expressed sufficient levels of cell surface class I molecules for CTL recognition/lysis. Although the susceptibility of the 917-975, 917-1227, and 12-1 transformants to allo-CTLs did not correlate with their tumorigenic capacities, their sensitivity to these CTLs was expected since class

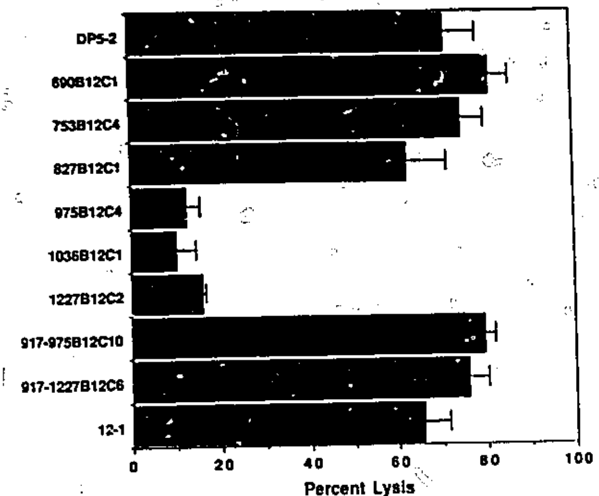


FIG. 4. Susceptibility of Hooded Lister rat cells transformed by hybrid Ad5/12 E1A plus Ad12 E1B genes to allogeneic Fischer 344 rat CTLs. ^{51}Cr-labeled transformed Hooded Lister rat cell lines (MHC haplotype C) served as targets for Fischer 344 rat (MHC haplotype I) splenic CTLs which were sensitized to γ-irradiated (5000 rads) stimulator Hooded Lister rat splenic lymphocytes 3 days prior. For experimental details see Materials and Methods. Collected data were averaged from two separate 6-hr allogeneic CTL chromium release assays conducted in triplicate using E:T ratios of 136:1, 68:1, and 34:1. Only data collected for the 37:1 E:T ratio are shown.

I levels on these lines were comparable to levels expressed on the nontumorigenic p690, p753, and p827 transformants.

Syngeneic cytotoxic T lymphocyte activity

The notion that the ability of Ad12 E1 transformants to induce tumors is dependent on down-regulation of class I expression to levels below those necessary for CTL recognition is inconsistent with the observation that the 12-1 (Ad12 E1 transformant) line expressed sufficient cell surface class I levels for recognition by allo-CTLs. Thus, another factor influencing tumorigenicity of Ad E1-transformed cells may be the ability of their E1A products to serve as CTL epitopes. To assess this possibility, we determined the susceptibilities of the hybrid Ad5/12 E1A transformants to syngeneic Ad5 E1- and Ad12 E1-specific CTLs (Figs. 5A and 5B) which were generated by immunizing syngeneic Hooded Lister rats with DP5-2 (Ad5 E1 transformant) and 12-1 (Ad12 E1 transformant) cells, respectively. As expected, the DP5-2 line showed >80% lysis by DP5-2-specific CTLs while lysis of primary syngeneic Hooded Lister baby rat kidney cells was trivial (<5%) (see Fig. 5A). Lysis of DP5-2 cells was indeed due to CTLs, since anti-rat CD3 monoclonal antibodies which inhibit T-cell effector function *in vitro* decreased lysis of DP5-2 cells from >80% to approximately 10% (data not shown). The nontumorigenic lines transformed by p690, p753, and p827 exhibited approximately 35% lysis by DP5-2-specific CTLs which was similar to the level of sensitivity observed for the p917-975 (35% lysis) and

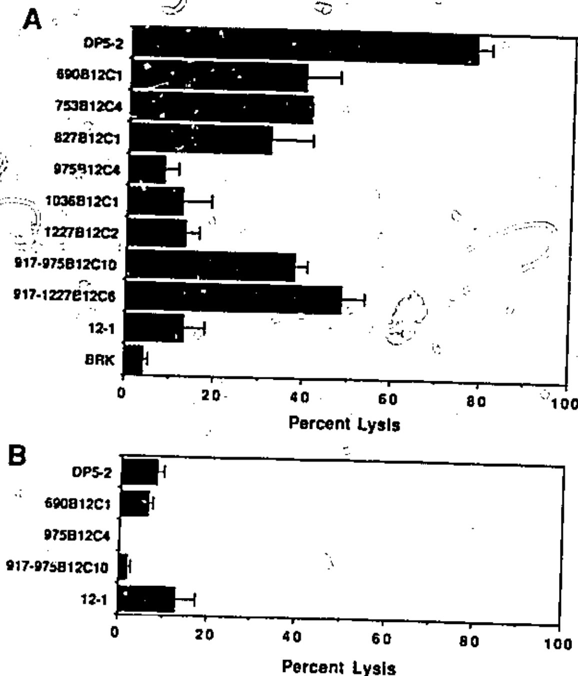


FIG. 5. Susceptibility of hybrid Ad5/12 E1A plus Ad12 E1B-transformed Hooded Lister rat cells to (A) syngeneic Ad5 E1-specific CTLs and (B) syngeneic Ad12 E1-specific CTLs. Hooded Lister rats (from which the hybrid Ad5/12 E1A-transformed cell lines were derived) were immunized with the Ad5 E1A-expressing DP5-2 line (A) or the 12-1 line which expresses Ad12 E1A (B). Three weeks later, splenic CTLs were isolated and resensitized for 3 days in a coculture with γ-irradiated (3000 rads) DP5-2 or 12-1 cells to ensure proliferation of syngeneic CTLs directed against Ad5 and Ad12 E1A epitope(s). Detailed explanation of procedures is outlined under Materials and Methods. These effectors were subsequently incubated with ^{51}Cr-labeled Hooded Lister rat cell lines transformed by the hybrid Ad5/12 E1A genes shown in Fig. 1A. Collected data were averaged from three 6-hr syngeneic CTL chromium release assays conducted in triplicate using E:T ratios of 100:1, 50:1, and 25:1. Only data collected for the 50:1 E:T ratio are shown.

p917-1227 (45% lysis) transformants. Despite expressing sufficient levels of class I antigens for recognition and lysis by allo-CTLs, the tumorigenic Ad12 E1 transformant 12-1 was not significantly lysed (<10%) by DP5-2-specific CTLs. The tumorigenic p975, p1036, and p1227 transformants were also resistant to DP5-2-specific CTL lysis, as expected from the observation that their cell surface class I levels were not sufficient for recognition/lysis by allo-CTLs (see Fig. 4).

The above findings suggest that the Ad5 E1A residues 1–44, region 1, and 119–139, region 2 (see Fig. 2C) may encode CTL epitopes which confer susceptibility to Ad5 E1-specific CTLs. Following immunization of syngeneic rats with Ad12 E1-transformed cells (12-1 cells) and subsequent restimulation with γ-irradiated 12-1 cells, proliferation of Ad12 E1-specific lymphocytes was not evident when compared to the marked proliferation observed for Ad5 E1-specific lymphocytes. Moreover, when these potential Ad12 E1-specific secondary lymphocytes were tested for their ability to lyse DP5-2 and 690 (nontumorigenic) and 975, 917-975, and 12-1 (tumorigenic) cells, <15% lysis was observed (Fig. 5B). Interestingly, 12-1 cells, which were used for immunization and generation of Ad12 E1-specific CTLs, exhibited the same degree of susceptibility to these CTLs (<15%) as they did to syngeneic Ad5 E1-specific CTLs (see Figs. 5A and 5B). Thus, it is unlikely that Ad12 E1A encodes CTL epitopes presented in conjunction with class I antigens.

DISCUSSION

The ability of Ad12 E1A to down-regulate MHC class I expression of transformed cells has generally been accepted as a principal feature determining tumorigenicity. That the tumorigenic capacity of these cells depends solely on class I down-regulation, however, is a topic of great debate since the relationship between class I levels, susceptibility to CTLs, and tumorigenicity is poorly defined. To better define this relationship and to obtain a further understanding of the basis for the different tumorigenic capacities of Ad5 E1- and Ad12 E1-transformed rodent cells, we used a series of previously described hybrid Ad5/12 E1A (plus Ad12 E1B)-transformed Hooded Lister rat cell lines, which differ in tumorigenic capacity, to map E1A regions influencing MHC class I expression and susceptibility to allogeneic and syngeneic CTLs.

MHC class I expression was measured on the cell surface of hybrid Ad5/12 E1A (plus Ad12 E1B)-transformed rat cells and human Ad5 E1- and Ad12 E1-transformed cells. Compared to Ad5 E1 transformants, Ad12 E1-transformed cells exhibited significantly reduced cell surface class I levels, a finding which is in agreement with previous reports (for review see Gallimore *et al.*, 1977). All the hybrid Ad5/12 E1A (plus Ad12 E1B) transformants also showed significantly reduced class I levels, suggesting that expression of Ad12 E1A residues 1–42 and/or 105–144, which lie within the Ad12 E1A sequences previously shown to be involved in tumor induction (Fig. 2A), induce down-regulation of cell surface class I expression (Fig. 2B). Within the region encoding Ad12 E1A amino acids 105–144, there exists a stretch of 20 amino acids (124–144; EQDENGMAHVSASAAAAA-ADRER) between CR2 and CR3 which is absent from corresponding Ad5 E1A sequences, but bears >55% homology to the corresponding region of the highly oncogenic simian Ad7 E1A protein. Intriguingly, a feature of this region is the presence of several consecutive alanine residues which appear to be characteristic of some transcriptional repressors (Jelinek *et al.*, 1994; Telling and Williams, 1994). Whether this region of Ad12 E1A and/or residues 1–42 play functional roles in transcriptional repression of class I expression or function in another manner which influences tumorigenicity remains to be determined.

Down-regulation of class I expression by Ad12 E1-transformed cells is thought to aid in evasion of CTL

immunosurveillance and thereby contribute to tumorigenicity. However, a strict correlation between reduced cell surface class I expression and tumorigenicity was not observed among the hybrid Ad5/12 E1A (plus Ad12 E1B) transformants used in this study. With the exception of the tumorigenic p975, p1036, and p1227 transformants, all other rat cells transformed by the hybrid Ad5/12 E1A plus Ad12 E1B genes, regardless of tumorigenic capacity, were found to express sufficient levels of class I antigens for recognition and lysis by allogeneic CTLs. Our data are therefore in agreement with previous studies that failed to find a strict correlation between class I expression, susceptibility to allogeneic CTLs, and tumorigenicity among Ad5 E1 and Ad12 E1 transformants (Shemesh *et al.*, 1991; Shemesh and Ehrlich, 1993). In fact, results from one study suggest that transfection of class I genes into already tumorigenic Ad12-transformed BALB/c mouse cells enhanced rather than abrogated tumorigenicity (Soddu and Lewis, 1992). It should be noted that differences in class I levels among various transformants were not the result of clonal variation following transformation by the hybrid Ad5/12 E1A and Ad12 E1B proteins since two or more independently derived cell lines transformed by the same E1 sequences always displayed similar class I levels. Moreover, differences in class I levels between clones could not be attributed to differing levels of E1A expression since comparable E1A levels were detected by Western blot analysis in all transformed cell lines (Jelinek and Graham, 1992; Jelinek *et al.*, 1994).

Taken together, the relationship between class I down-regulation, susceptibility to allo-CTLs, and tumorigenicity suggests that factors in addition to reduced class I expression contribute to the tumorigenic capacities of Ad E1-transformed cells. For example, cells transformed by Ad2 or Ad5 E1A are known to be susceptible to NK cells (Raska and Gallimore, 1982; Sawada *et al.*, 1985; Cook *et al.*, 1986, 1987; Routes, 1993). When the sensitivity of the hybrid Ad5/12 E1A (plus Ad12 E1B) transformants to syngeneic NK lysis was measured (data not shown), however, susceptibility did not correlate with tumorigenicity and was observed only by transformants expressing levels of cell surface class I molecules which were insufficient for recognition by allogeneic CTLs, a finding which is in agreement with reports suggesting that cell surface class I expression interferes with NK cell recognition/lysis (Storkus *et al.*, 1989; Ljunggren and Karre, 1990; Kaufman *et al.*, 1992; Pena and Solona, 1992).

Another factor which may contribute to the tumorigenicity of Ad E1-transformed cells is whether their E1A products can serve as CTL epitopes. The susceptibilities of cells transformed by hybrid Ad5/12 E1A (plus Ad12 E1B) genes to syngeneic Ad5 E1-specific CTLs suggests that Ad5 E1A potentially encodes CTL epitopes in two regions: the amino-terminal 44 amino acids (region 1) and residues 119–139 (region 2) (see Fig. 2C). These are in excellent agreement with previous results which identified CTL epitopes between nucleotides 625–810 (aa 21–83) and 916–974 (aa 119–138) (Routes *et al.*, 1991). Independent expression of either region 1 (present in the tumorigenic p917–975 and p917–1227 transformants) or region 2 (present in the nontumorigenic p690, p753, and p827 transformants) resulted in approximately 40% lysis by these CTLs, suggesting that the epitopes encoded by region 1 or 2 are equivalent in their ability to elicit CTL lysis *in vitro*. The fact that these two groups of transformants, containing either region 1 or 2, demonstrate similar sensitivities to lysis by syngeneic Ad5 E1-specific CTLs *in vitro* yet differ in ability to induce tumors *in vivo* may suggest that the Ad5 E1A epitope encoded by region 2 (present in nontumorigenic transformants) is dominant to the region 1 epitope in eliciting an *in vivo* CTL response. That a hierarchy exists among peptides encoded by the same protein for induction of a response by T-cells is not without precedent (for review see Sercarz *et al.*, 1993). When used to immunize syngeneic rats, Ad12 E1-transformed cells (12-1 cells) were unable to generate syngeneic Ad12 E1-specific CTLs despite expressing sufficient cell surface class I levels for recognition by allo-CTLs. It should be noted that Ad12 E1-specific syngeneic CTLs have been generated following *in vitro* stimulation of spleen cells isolated from tumor-bearing Hooded Lister rats injected with Ad12 E1-transformed cells (Raska *et al.*, 1980). However, the percentage specific lysis of target cells, at an effector:target ratio of 400, ranged from approximately 8–30% and was associated with standard errors as great as 14.9%. This range of lysis is comparable to the lysis we observed for the 12-1 line (12%) at a far lower effector:target ratio of 50. Thus, it seems unlikely that Ad12 E1A encodes CTL epitopes in conjunction with class I antigens. We cannot exclude the possibility that the inability of these cells to generate CTLs may be due to down-regulation of peptide transporter genes, a recent finding in Ad12-transformed cells (Rotem-Yehudar *et al.*, 1994).

In conclusion, we show that transformation of rat cells by Ad E1A modifies two functionally related properties of the cells each of which influences host immunosurveillance mechanisms. First, two Ad12 E1A regions seem to participate in down-regulating cell surface class I expression. Second, our results from studies in the Hooded Lister rat strain (MHC haplotype C) indicate that Ad5 E1A contains two CTL epitopes encoded in noncontiguous regions, whereas Ad12 E1A was unable to elicit a CTL response. Although the ability of Ad12 E1A, but not Ad5 E1A, to down-regulate MHC class I expression in transformed cells has generally been accepted as the major factor determining tumorigenicity, results presented in this report clearly suggest that class I down-regulation does not, in every case, solely account for the tumorigenicity of resulting cells. Factors in addition to class I repression, such as the ability of Ad5 E1A, unlike Ad12 E1A, to encode CTL epitopes, may be responsible for the different tumorigenic capacities of Ad5 E1- and Ad12

E1-transformed cells (depending on the species and strains of transformed cells and host rodents).

ACKNOWLEDGMENTS

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Contributions to Pereira et al., 1995:

(1) Using the hybrid Ad5/12 E1A- (plus Ad12 E1B) transformants, cell surface MHC class I levels were measured by FACS analysis with the assistance of Barb Bagnarol (Clinical Immunology Section, McMaster University Medical Center).

(2) Determined susceptibility of the hybrid E1A transformants to allogeneic and syngeneic CTLs in order to map E1A regions encoding CTL epitopes.

Conclusions:

Results of this study suggest that transformation of rat cells by Ad E1A modifies two functionally related properties of cells each of which influence host immuno-surveillance mechanisms. First, two Ad12 E1A regions (amino acids 1-42 and 105-144) seem to participate in down-regulating cell surface class I expression. Second, results from studies in the Hooded Lister rat strain (MHC haplotype C) indicate that Ad5 E1A contains two CTL epitopes (within amino acids 1-44 and 119-139) whereas Ad12 E1A was unable to elicit a CTL response. Although the ability of Ad12 E1A, but not Ad5 E1A, to down-regulate MHC class I expression in transformed cells has generally been accepted as the major factor determining tumorigenicity, results presented in this report clearly suggest that class I down-regulation does not, in every case, solely account for the tumorigenicity of resulting cells. Factors in addition to class I repression, such as the ability of Ad5 E1A, unlike Ad12 E1A, to encode CTL epitopes, also contribute to the different tumorigenic capacities of Ad5 E1- and Ad12 E1-transformed cells (depending on the species and strains of transformed cells and host rodents).

The identification of the Ad12 E1A regions mediating tumorigenicity and class I down-regulation will undoubtedly facilitate future studies aimed at understanding the molecular basis of MHC class I down-regulation.

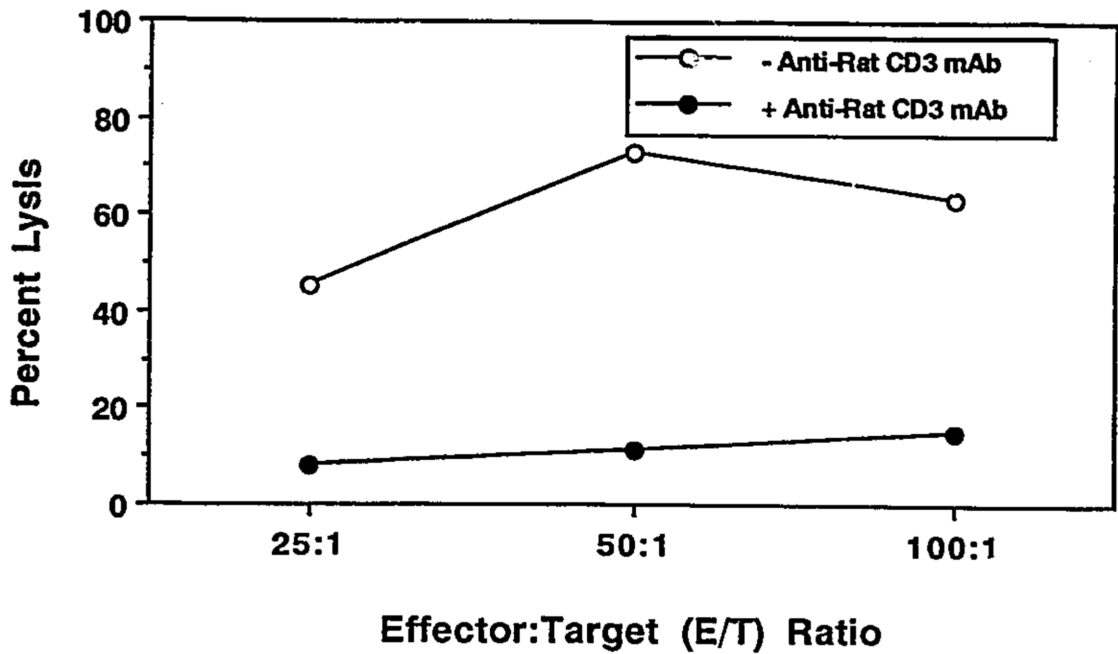
Unpublished Data Related To Pereira et al., 1995

Since the Ad5 E1-transformed rat line, DP5-2, demonstrated nearly 80% susceptibility to DP5-2-specific CTLs in syngeneic CTL chromium release assays, the possibility that lysis observed was due to other components of cellular immunity needed to be determined. To confirm that lysis observed in the aforementioned assay was indeed due to CTLs, an anti-CD3 antibody (CL020AP) was used to inhibit lysis of DP5-2 cells by putative DP5-2-specific CTLs (Fig. 7). In the presence of CL020AP, lysis was decreased six-fold at an E/T ratio of 50:1, thereby confirming the authenticity of CTL lysis.

Following several reports which failed to find a strict correlation between reduced class I expression and the tumorigenic capacity of Ad12 E1-transformed cells it has been suggested that other cellular immune factors which are not specific for MHC class I antigens may also contribute to tumorigenesis. For example, the difference in tumorigenic potential between rodent cells transformed by the E1 regions of Ad5 and Ad12 has been attributed to the susceptibility of Ad5 E1- but not Ad12 E1-transformants to NK cells. To test this observation in our system, I determined whether the sensitivity of the hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed rat lines to NK cells correlated with their ability to induce tumors in syngeneic immunocompetent rats (Fig. 8). In the event that tumorigenicity and NK susceptibility correlates, the hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed rat lines could be utilized to identify regions of E1A modulating sensitivity to NKs. As is demonstrated in Fig. 8A, the susceptibility of the Ad5 E1- (DP5-2) and Ad12 E1- (12-1) transformed cells and of the hybrid lines, 827B12C1, 1227B12C2 and 917-1227B12C6 did not correlate with tumorigenicity. For example, DP5-2 cells exhibited an approximately three-fold lower sensitivity to NK cells than 12-1 cells. In comparison to 12-1 cells, DP5-2 cells exhibited a 4 to 5 fold increase in cell surface MHC class I levels (Fig. 8B), a finding which suggests that cell surface class I expression interferes with NK lysis. This trend is very apparent when cell surface MHC class I levels and susceptibilities to NKs are compared among the lines as shown in Fig. 8.

Figure 7: Susceptibility of DP5-2 cells (Ad5 E1-transformed) to syngeneic DP5-2-specific CTLs in the presence and absence of anti-rat CD3 monoclonal antibodies. Syngeneic Hooded Lister rats were immunized with DP5-2 cells (Ad5 E1-transformed rat kidney cells) to generate CTLs sensitized to epitopes encoded by E1A. Splenic lymphocytes were isolated three weeks later, restimulated with γ-irradiated DP5-2 cells for 4 days, and tested for their ability to lyse ^{51}Cr-labelled DP5-2 cells for 6 hours at effector:target ratios of 25:1, 50:1, and 100:1 (A, open circles). To ensure that the lysis observed was indeed due to DP5-2-specific CTLs and not to other cellular immune components, anti-rat CD3 monoclonal antibodies were added to effector:DP5-2 mixtures in an attempt to inhibit DP5-2-specific CTL lysis (A, closed circles). **B**) Bar graph illustrating the susceptibility of DP5-2 cells to DP5-2-specific CTLs at a 50:1 ratio in the presence (closed bar) and absence (open bars) of anti-rat CD3 monoclonal antibodies.

A)



B)

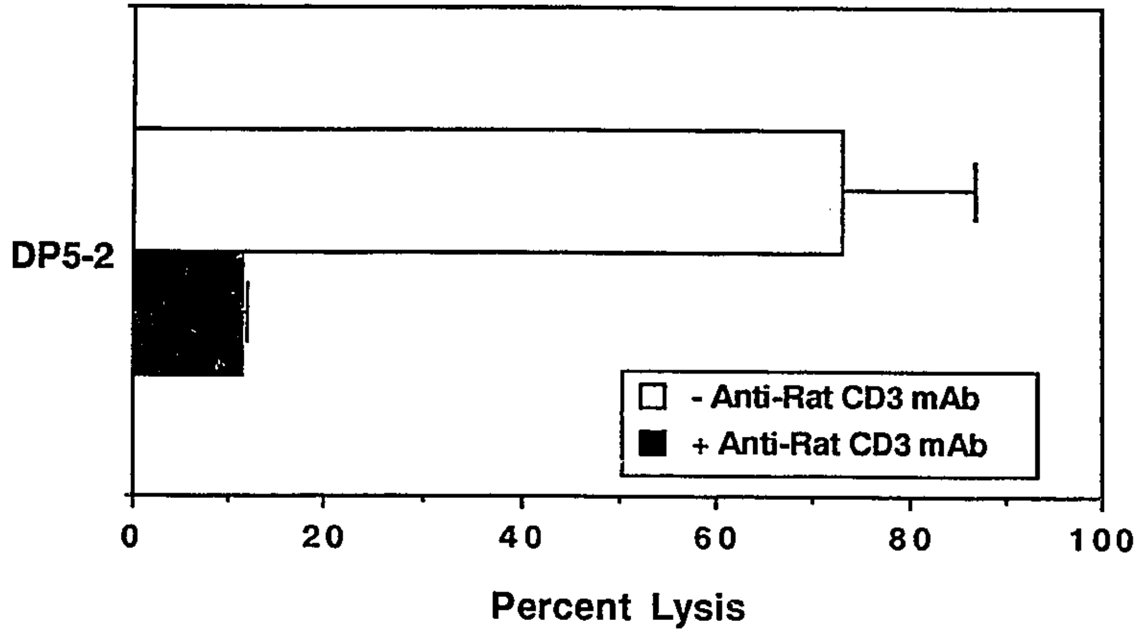
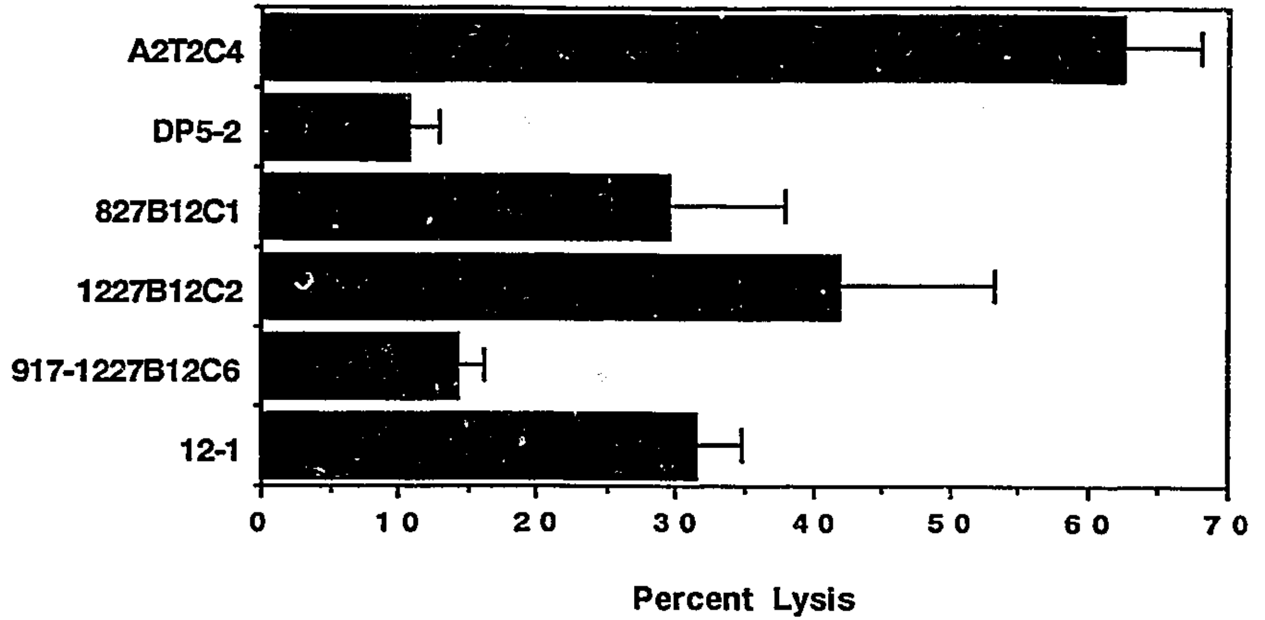


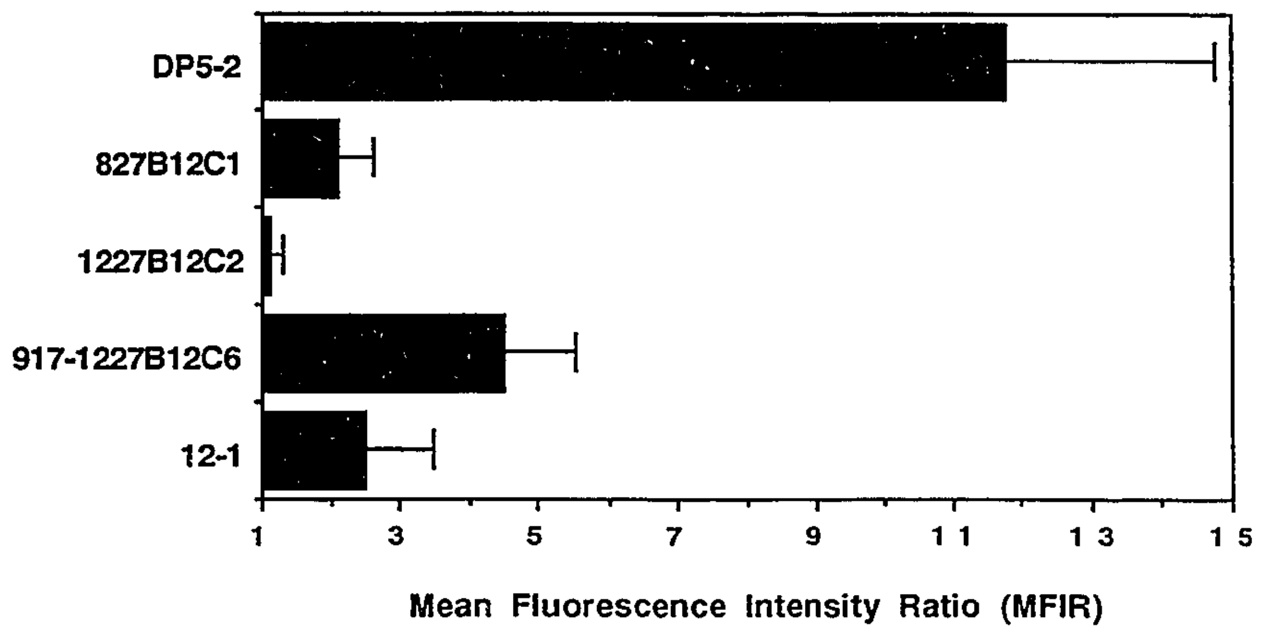
Figure 8: Susceptibility of Ad5 E1-, Ad12 E1-, and hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed rat cells to syngeneic NK cells.

A) Hooded Lister rat splenic NK cells were isolated from Ficoll gradients and incubated with ^{51}Cr-labelled Ad5 E1- (DP5-2), Ad12 E1- (12-1), and p827-, p1227-, and p917-1227- transformed Hooded Lister rat cells and A2T2C4 (Ad2 transformed Hooded Lister rat cell line which is highly susceptible to rodent NKs). The percentage lysis shown was derived from 4 hour assays at a 140:1 effector:target ratio. **B)** Cell surface MHC class I levels of the lines tested in panel A are shown for comparison purposes.

A)



B)



3) **Kushner, D. B., Pereira, D. S., Liu, X., Graham, F. L., and Ricciardi, R. P.**
The first exon of Ad12 E1A excluding the transactivation domain mediates differential binding of COUP-TF and NF-κB to the MHC class I enhancer in transformed cells.
Oncogene. In Press.

Preface:

At the beginning of this research, it was postulated that reduced levels of MHC class I antigens in Ad12 E1-transformed cells was due in large part to differences in the binding activity of NF-κB (activator) and COUP-TF (repressor) to the class I gene enhancer. Specifically, the binding activity of NF-κB in contrast to the binding activity of COUP-TF was very low. In collaboration with Dr. R. P. Ricciardi (University of Pennsylvania) the hybrid Ad5/12 E1A (plus Ad12 E1B) transformed rat cells were used to map regions of Ad12 E1A which mediate the differential NF-κB and COUP-TF binding activities observed in Ad12 E1-transformed cells. Since NF-κB is a transcription factor composed of 50 (p50) and 65 (p65) kilodalton subunits, the second objective of this study was to determine whether the decreased NF-κB1 binding activity observed in Ad12 E1-transformed cells was due to altered expression of its subunits, p50 (and its precursor p105) and p65.

The first exon of Ad12 E1A excluding the transactivation domain mediates differential binding of COUP-TF and NF-κB to the MHC class I enhancer in transformed cells

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RUNNING TITLE: Ad12 E1A region mediates COUP-TF and NF-κB binding

Abstract

The major histocompatibility complex class I enhancer is the target for adenovirus-12 E1A-mediated down-regulation of class I transcription. In Ad12 transformed rodent cells, the class I enhancer is down-regulated through increased binding of the repressor COUP-TF to the R2 element and decreased binding of the activator NF-κB (p50/p65) to the R1 element. The reduced surface levels of class I antigens contribute to the tumorigenic potential of Ad12 transformed cells by favoring their immunoescape from cytotoxic T-lymphocytes. Previous studies using transformed cells containing hybrid Ad5/Ad12 E1A (plus Ad12 E1B) genes have indicated that sequences within the first exon of the 266R Ad12 E1A gene are required for tumorigenesis. In this study we demonstrate that these same sequences, which do not include the Ad12 CR3 transactivation domain, are also required for the increase of COUP-TF binding to the R2 element and the decrease of NF-κB binding to the R1 element of the class I enhancer. We further show that diminished NF-κB binding is not due to a lack of NF-κB1-p50 in the nuclei of Ad12 transformed rat cells.

Introduction

Adenovirus (Ad) early region 1 (E1A and E1B) gene products are capable of transforming non-permissive rodent cells (Gallimore *et al.*, 1974; Graham *et al.*, 1974). Although all of the approximately fifty known serotypes can transform cells *in vitro*, only a subset (e.g. Ad12, Ad18, and Ad31) can efficiently induce tumors in immunocompetent animals following inoculation of transformed cells or virus. The highly tumorigenic phenotype of Ad12 was found to correlate with diminished cell surface levels of the major histocompatibility complex (MHC) class I antigens on Ad12 transformed cells (Bernards *et al.*, 1983; Schrier *et al.*, 1983; Eager *et al.*, 1985). Notably, non-tumorigenic Ad5 transformed cells do not exhibit a reduction in cell surface class I antigens. The reduced amounts of cell surface class I molecules on Ad12 transformed rodent cells are believed to contribute to their tumorigenic potential by enabling them to evade recognition and lysis by host cytotoxic T- lymphocytes (Bernards *et al.*, 1983; Tanaka *et al.*, 1985; Yewdell *et al.*, 1988).

The block in class I antigen expression on Ad12 transformed cells occurs at the level of transcription (Ackrill & Blair, 1988; Friedman & Ricciardi, 1988) and is mediated by products of the E1A gene of Ad12 (Vasavada *et al.*, 1986). Molecular genetic analysis has shown that the target of Ad12 E1A mediated down-regulation of class I transcription is the class I enhancer (Ge *et al.*, 1992). Recent studies have revealed that the down-regulation of class I transcription in Ad12 transformed cells correlates with strong binding of the transcriptional repressor COUP-TF to the R2 element of the enhancer, as compared to weak binding observed in non-tumorigenic Ad5 transformed cells (Ge *et al.*, 1992; Kralli *et al.*, 1992; Liu *et al.*, 1994). Also,

down-regulation of the class I enhancer correlates with reduced binding of the transcriptional activator NF-κB to the R1 element in Ad12 transformed cells as compared to strong binding in Ad5 transformed cells (Ackrill & Blair, 1989; Nielsch *et al.,* 1991; Meijer *et al.,* 1992; Liu *et al.,* submitted). These findings suggest that the MHC class I enhancer is globally down-regulated in Ad12 transformed cells.

The differential binding activities in tumorigenic Ad12 versus non-tumorigenic Ad5 transformed rodent cells of COUP-TF and NF-κB have prompted us to determine the portions of the Ad12 E1A coding region which are required for these phenotypes. Previous studies with transformed rat cells containing hybrid Ad5/Ad12 E1A (plus Ad12 E1B) genes have demonstrated that the tumorigenic potential of Ad12 E1A is mediated by sequences within the first exon (Jelinek *et al.,* 1994; Telling & Williams, 1994). It has been further suggested that the sequences responsible for the tumorigenic phenotype might be encoded by two separate regions of Ad12 E1A; the first containing CR2 and the 20 amino acid (aa) spacer region between CR2 and CR3 which is unique to Ad12, and the second extending from the N-terminus to the leftmost border of CR2 (Jelinek *et al.,* 1994). These regions are also thought to contribute to a decrease in class I expression (Pereira *et al.,* 1994, 1995). In addition, these regions of Ad12 E1A are believed to lack CTL epitopes encoded by the corresponding regions of Ad5 E1A (Pereira *et al.,* 1995). Here, we provide evidence that the regions of Ad12 E1A implicated in class I down-regulation and tumorigenesis are also required for strong binding activity of COUP-TF to the R2 element and diminished binding activity of NF-κB to the R1 element of the class I enhancer in Ad12 E1 transformed rat cells. Our results further indicate that the dramatic decrease

in NF-κB binding activity is not due to a lack of NF-κB1-p50 (p50) in the nuclei of these cells.

Results

The first exon of Ad12 E1A contains sequences required for increased binding of COUP-TF to the R2 element of the MHC class I enhancer

The COUP transcription factor is an orphan member of the nuclear hormone receptor superfamily (reviewed in Qiu *et al.,* 1994). In tissue culture, COUP-TF has been shown to be a repressor of transactivation by the retinoic acid, thyroid hormone, and vitamin D_3 receptors (Cooney *et al.,* 1993). Recently, COUP-TF was shown to bind to the R2 element of the MHC class I enhancer (Figure 1). Of particular significance, increased binding of COUP-TF to the R2 element in Ad12 versus Ad5 transformed mouse cells suggested that COUP-TF may also play a role in repressing transcription of the MHC class I antigens in Ad12 transformed mouse cells (Liu *et al.,* 1994).

To identify what regions of Ad12 E1A are responsible for the strong binding of COUP-TF to the R2 element, we made use of chimeric Ad5/Ad12 E1A (plus Ad12 E1B) transformed rat cell lines (Figure 2; Jelinek & Graham, 1992; Jelinek *et al.,* 1994; Pereira *et al.,* 1994). Band-shift experiments were performed using a labeled R2 oligonucleotide and nuclear extracts from parental Ad5, Ad12, and the hybrid Ad5/Ad12 E1A transformed rat cells. As expected from previous studies with transformed mouse cells (Liu *et al.,* 1994), the parental Ad12 E1 transformed 12-1 cell line exhibited much stronger COUP-TF binding activity than the parental Ad5 E1 transformed DP5-2 cell line (Figure 3, compare lanes 5 and 1). Since Ad12 E1A contains a 20 aa unique spacer region between CR2 and CR3 which is absent in Ad5 E1A (Perricaudet *et al.,* 1980), and which has been implicated in class I down-regulation (Pereira *et al.,* 1994, 1995), we suspected that this region might play a role in increased COUP-TF binding to the R2 element. However, compared

to parental Ad5 E1 transformed cells, no increase of COUP-TF binding activity was observed in the cell line 917-975B12C10 (Figure 2), which was transformed by a hybrid Ad5/Ad12 E1A containing the 20 aa unique spacer region as well as Ad12 CR2 (Figure 3, compare lanes 1 and 2). Likewise, the cell line 690B12C1, transformed by a hybrid containing the first 42 aa of Ad12 E1A (Figure 2), exhibited no increase in COUP-TF binding activity (Figure 3, lane 3). By contrast, strong COUP-TF binding activity was observed in the 975B12C4 cell line, which was transformed by a hybrid Ad5/Ad12 E1A that includes both of the regions of Ad12 E1A tested independently in the aforementioned two hybrid lines (Figure 3, lane 4). These results indicate that aa residues 1-144 of the first exon of Ad12 E1A contain sequences which are required for increased of COUP-TF binding to the R2 element of the MHC class I enhancer. Importantly, the CR3 activation domain of Ad12 E1A does not appear to be specifically required.

The sequences of Ad12 E1A required for increased COUP-TF binding to the R2 element also mediate decreased NF-κB binding to the R1 element

The transcription factor NF-κB belongs to the Rel/NF-κB family of proteins (reviewed in Miyamoto & Verma, 1995; Thanos & Maniatis, 1995). NF-κB is a heterodimer (Urban *et al.*, 1991) composed of RelA (p65) (Nolan *et al.*, 1991; Ruben *et al.*, 1991), and NF-κB1-p50 (p50). NF-κB1-p50 is post-translationally processed from its precursor, NF-κB1-p105 (p105) (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991) by a ubiquitin-dependent 26S proteosome (Palombella *et al.*, 1994). Since RelA contains a C-terminal transactivation domain which is not found in NF-κB1-p50, only NF-κB (p50/p65), and not the NF-κB1-p50 homodimer (p50/p50), is

transcriptionally active *in vivo* (Schmitz & Baeuerle, 1991; Perkins *et al.*, 1992; Ballard *et al.*, 1992).

NF-κB binds to the R1 element of the MHC class I enhancer (Baldwin & Sharp, 1988; Israël *et al.*, 1989), and is required for activation of class I expression (Plaksin *et al.*, 1993; Segars *et al.*, 1993). In Ad12 transformed cells, diminished NF-κB binding activity correlates with reduced class I transcription (Ackrill & Blair, 1989; Nielsch *et al.*, 1991; Meijer *et al.*, 1992; Liu *et al.*, submitted). To identify the region(s) of Ad12 E1A responsible for decreased NF-κB binding to the R1 element, we again employed nuclear extracts from the chimeric Ad5/Ad12 E1A cell lines (Figure 2) in band-shift experiments using a labeled R1 oligonucleotide as the probe. In agreement with previous studies (Ackrill & Blair, 1989; Nielsch *et al.*, 1991; Meijer *et al.*, 1992; Liu *et al.*, submitted), NF-κB (p50/p65) binding activity was greatly reduced in the parental Ad12 E1 transformed cells (12-1) compared to the parental Ad5 E1 transformed cells (DP5-2) (Figure 4, compare lanes 7 and 3). Additionally, it was noted that the binding activity of the more rapidly migrating NF-κB1-p50 homodimer was also diminished in the nuclear extract from the Ad12 E1 transformed cells. Neither the cell line 917-975B12C10 (Figure 2), which was transformed by the hybrid Ad5/Ad12 E1A containing the 20 aa unique spacer region (and CR2), nor the cell line 690B12C1 (Figure 2), transformed by a hybrid containing aa 1-42 of Ad12 E1A, exhibited reduced NF-κB binding activity (Figure 4, lanes 4 and 5). However, a dramatic decrease in NF-κB binding activity, similar to the reduction seen in Ad12 E1 transformed cells (12-1), was observed in the 975B12C4 cell line, which was transformed by the hybrid including both of the regions of Ad12 E1A tested in the 917-975B12C10 and 690B12C1 cell lines (Figure 4, lane 6). These results indicate that the first exon of Ad12 E1A contains sequences which are

required for diminished NF-κB binding to the R1 element of the MHC class I enhancer. Taken together with the COUP-TF binding results, our results suggest that the first exon of Ad12 E1A, exclusive of the Ad12 CR3 transactivation domain, mediates global down-regulation of the class I enhancer in Ad12 E1 transformed rat cells.

It is of interest to note that we repeatedly observed a more rapid migration of the NF-κB1-p50 homodimer in the nuclear extracts from the DP5-2, 917-975B12C10, and 690B12C1 rat cell lines, as compared to its migration in the 975B12C4 and 12-1 rat cell lines, and the previously characterized Ad12 and Ad5 transformed mouse cell lines (Liu *et al.*, submitted) used here as migration controls (Figure 4, compare lanes 3, 4, and 5 with lanes 6, 7, 1, and 2). To confirm that the faster migrating complex was comprised of NF-κB1-p50 subunits, supershift experiments were performed using an antibody against NF-κB1-p50. Figure 5 shows that in each case, the faster migrating complex was supershifted by the anti-NF-κB1-p50 antibody (lanes 4, 6, 8, 10, 12). As expected, NF-κB, which also contains NF-κB1-p50, was also supershifted. Neither the faster migrating NF-κB1-p50 homodimer complex nor the slower migrating NF-κB heterodimer was supershifted by a control antibody, anti-COUP-TF (lane 1; Liu *et al.*, 1994). While it is not clear why the faster complex migrates differently in the DP5-2, 917-975B12C10, and 690B12C1 cell lines, the supershift experiments confirmed that this complex indeed contains NF-κB1-p50.

The decrease in NF-κB binding is not due to a lack of NF-κB1-p50 in nuclei of Ad12 E1 transformed cells

The above band shift experiments using nuclear extracts from the 975B12C4 and 12-1 transformed cells showed significantly decreased R1

binding activities of both the NF-κB heterodimer and the NF-κB1-p50 homodimer. Since NF-κB1-p50 is a common subunit of the two complexes, it is conceivable that a reduction in the level of NF-κB1-p50 could account for the decrease in the binding activities of both dimers in 12-1 and 975B12C4 cells. To address this possibility, Western blot analyses were performed to determine the levels of NF-κB1-p50 in nuclear extracts of all the cell lines using the 1157 anti-NF-κB1-p50 antibody. As shown in Figure 6A, NF-κB1-p50 is clearly present in nuclear extracts of all the cell lines. These Western blot results were repeated using a different anti-NF-κB1-p50 antibody (1613), with similar results (data not shown). Furthermore, no major differences in the levels of RelA (p65) were observed (data not shown). These results demonstrate that the low NF-κB binding activity observed in nuclear extracts of 975B12C4 and 12-1 cells is not due to significantly reduced expression of either NF-κB1-p50 or RelA in the nuclei of these cells.

While these studies were in progress, an independent study using whole cell extracts suggested that the low NF-κB binding activity observed in Ad12 E1 transformed cells was due to a reduction in the processing of precursor NF-κB1-p105 to NF-κB1-p50 (Schouten *et al.*, 1995). Because our findings described above relied on nuclear extracts to conclude that NF-κB is not limiting in Ad12 E1 transformed cells, we therefore examined the levels of NF-κB1-p105 and NF-κB1-p50 in whole cell extracts from our transformed cells. Consistent with our findings using nuclear extracts, Western blot analysis of whole cell extracts using the 1157 anti-NF-κB1-p50 antibody not only revealed that there are similar steady-state levels of NF-κB1-p50, as well as NF-κB1-p105, in all of the rat cell lines (Figure 6B), but that processing of NF-κB1-p105 to NF-κB1-p50 in Ad12 E1 transformants is not defective as previously suggested (Schouten *et al.*, 1995) (see discussion).

Recently, a commonly used anti-NF-κB1-p50 antibody (sc-114) was shown to have an incorrect specificity for NF-κB1-p50 (Pereira *et al.*, submitted). To confirm that the 50 kD protein detected in our Western analyses of nuclear and whole cell extracts from the five Ad12 E1 transformed rat cell lines was authentic NF-κB1-p50, we decided to test the specificity of the 1157 anti-NF-κB1-p50 antibody using NF-κB1-p50/NF-κB1-p105 knockout mouse cell extracts (Sha *et al.*, 1995). The rationale behind this experiment was that any anti-NF-κB1-p50 antibody with proper specificity for NF-κB1-p50 would recognize NF-κB1-p50 in Western analysis of NF-κB1-p50/NF-κB1-p105 +/+ and +/- cell extracts, but should not detect a band representative of NF-κB1-p50 in NF-κB1-p50/NF-κB1-p105 -/- cell extracts. Figure 7 reveals that this was the case when the 1157 anti-NF-κB1-p50 antibody (lanes 4-6; Pereira *et al.*, submitted) was used. Importantly, the band observed in the wild-type (+/+) and heterozygous (+/-) cell extracts co-migrates with a 50 kD band detected in DP5-2 and 12-1 E1 transformed rat cell extracts (lanes 2 and 3), as well as 293 human cell extracts (lane 1). The 293 extracts were included as a positive control as the sequence of the peptide that was used to generate the 1157 antibody is derived from human NF-κB1-p50. These results confirm the specificity of the 1157 anti-NF-κB1-p50 antibody for NF-κB1-p50 and reinforce the conclusion that NF-κB1-p50 is present in Ad12 E1 transformed rat cells (Figure 6).

Discussion

We have demonstrated that the first exon of Ad12 E1A, excluding the CR3 activation domain, is sufficient for mediating increased binding of the repressor COUP-TF and diminished binding of the activator NF-κB to the R1 and R2 elements, respectively, of the MHC class I enhancer in Ad12 transformed rat cells. This portion of Ad12 E1A is comprised of the conserved domains CR1 and CR2, as well as non-conserved sequences, the most salient of which is the 20 aa unique spacer region located between CR2 and CR3 which is not present in Ad5 E1A (Perricaudet *et al.*, 1980). This 20 aa unique spacer region appears to be essential but not sufficient for both tumorigenesis and repression of class I transcription (Telling & Williams, 1994; Jelinek *et al.*, 1994; Pereira *et al.*, 1995). Our results demonstrate that even though the CR3 transactivating domain could play a role in altering the binding activities of COUP-TF and NF-κB, the viral serotype from which it is derived is irrelevant. Furthermore, preliminary experiments suggest that if CR3 is needed to achieve this E1A mediated effect, it is most likely a structural requirement (e.g., maintaining the structure of the 20 aa unique spacer region), since a 4 aa insertion placed in the center of the Ad12 CR3 region destroys transactivation but does not affect class I expression (Vasavada and R.P.R., unpublished observations). Interestingly, the first exon of Ad12 E1A excluding CR3 has also been shown to mediate tumorigenicity and decreased class I expression (Telling & Williams, 1994; Jelinek *et al.*, 1994; Pereira *et al.*, 1994, 1995). A more detailed analysis of this region may help discriminate whether single or multiple domains within the first exon modulate the binding activities of COUP-TF and NF-κB.

How might sequences within the first exon of Ad12 E1A mediate the down-regulation of NF-κB binding to R1 in Ad12 transformed cells? One possibility is that the nuclear translocation of NF-κB is blocked as a result of being retained in the cytoplasm by association with IκBs (Beg & Baldwin, 1993; reviewed in Miyamoto & Verma, 1995). However, this does not appear to be the case since the nuclei of Ad12 transformed rat cells contain levels of NF-κB1-p50 (p50) and RelA (p65) which are similar to those seen in Ad5 transformed rat cells, where decreased NF-κB binding activity is not observed. These results suggest a second possibility, in which a nuclear inhibitor is responsible for blocking the binding of NF-κB to R1 in Ad12 transformed rat cells. Indeed, NF-κB binding activity was restored in nuclear extracts from Ad12 transformed mouse and rat cells following treatment with the mild detergent sodium deoxycholate, providing further evidence for the presence of a nuclear inhibitor (Liu *et al.*, submitted; D.B.K. and R.P.R., unpublished observations). Interestingly, a nuclear inhibitor has also been postulated to block NF-κB binding to the HIV-LTR in THP-1 monocytic cells, negatively regulating NF-κB activity and therefore restricting HIV-1 gene expression (Raziuddin *et al.*, 1991). The existence of nuclear inhibitors of NF-κB might serve as one mechanism by which certain viruses block NF-κB function.

During the course of this study, other evidence was proposed suggesting that diminished NF-κB binding activity in Ad12 E1 transformed rat cells is due to an interference in the processing of NF-κB1-p105 to NF-κB1-p50, resulting in significantly reduced levels of NF-κB1-p50 (Schouten *et al.*, 1995). This is in contradiction with our findings which clearly show that NF-κB1-p50 and NF-κB1-p105 are present at similar levels in Ad12 E1 and Ad5 E1 transformed rat cells. The notion that processing of NF-κB1-p105 to NF-κB1-p50 is defective in Ad12 E1 transformed rat cells is not consistent with our

findings which show that steady-state levels of NF-κB1-p50 are comparable in nuclear and whole cell extracts prepared from Ad12 E1 and Ad5 E1 transformed rat cells (Figure 6). While the discrepancy between our data and the findings of Schouten *et al.* seem unexpected in light of the fact that the E1 transformed cells examined in both cases were derived from primary rat kidney cells, it may be explained on the basis of differences in the specificity of the anti-NF-κB1-p50 antibodies used. In this study we have employed the 1157 anti-NF-κB1-p50 antibody, which was raised against a human NF-κB1-p50 derived peptide. This antibody is specific for NF-κB1-p50, and not another Rel family member, because NF-κB1-p50 was not detected by Western blot analysis using cell extracts derived from NF-κB1-p105/NF-κB1-p50 knockout mice (Figure 7; Pereira *et al.*, submitted). We feel that this stringent test for antibody specificity validates our analyses using both nuclear and whole cell extracts, which indicate that the decreased NF-κB binding activity observed in Ad12 E1 transformed rat cells is not due to a significant reduciton in the expression of either NF-κB1-p105 or NF-κB1-p50.

It is unclear how the sequences within the first exon of Ad12 E1A are able to mediate both increased COUP-TF binding to R2 and decreased NF-κB binding to R1. It needs to be determined whether or not these E1A-mediated effects are regulated independently. In addition, it is of interest to consider if the DNA binding activities of COUP-TF and NF-κB are influenced by their potential to directly interact with one another. It may be significant that the glucocorticoid nuclear hormone receptor has been implicated in repressing the ability of NF-κB to activate gene expression by preventing NF-κB binding (Ray & Prefontaine, 1994; Mukaida *et al.*, 1994; Scheinman *et al.*, 1995; Caldenhoven *et al.*, 1995). It needs to be determined if COUP-TF, which is

also a member of the nuclear hormone receptor superfamily, functions similarly to diminish NF-κB binding.

Regardless of the actual mechanisms, the apparent net effect of increased COUP-TF binding to R2 and decreased NF-κB binding to R1 is to produce global down-regulation of the MHC class I enhancer (Kralli *et al.*, 1992; Liu *et al.*, submitted), effectively diminishing class I transcription. In Ad12 transformed cells, this reduced class I expression favors their escape from CTL mediated immune detection, and contributes to their ability to form tumors. Importantly, the sequences within the first exon of Ad12 E1A that mediate these events correlate with the same regions that are required for class I down-regulation and tumorigenesis (Jelinek *et al.*, 1994; Pereira *et al.*, 1994, 1995). It is most interesting to consider that the mechanism by which Ad12 E1A achieves tumorigenesis through down-regulation of the class I enhancer might also be used during infection to accomplish persistent infection in the host.

Materials and methods

Cell lines

The parental Ad5 (DP5-2), Ad12 (12-1), and hybrid Ad5/Ad12 (690B12C1, 917-975B12C10, and 975B12C4) E1A (plus Ad12 E1B) transformed Hooded Lister rat cell lines were constructed previously (Jelinek & Graham, 1992; Jelinek *et al.*, 1994; Pereira *et al.*, 1994). Ad5 (KAd-5) and Ad12 (12A1) transformed cell lines were derived from primary mouse BALB/c (H-2^d) cells (Eager *et al.*, 1985). Monolayer cultures of rat cells were grown in α-minimal essential medium (α-MEM, GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS, HyClone), 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 μg/ml fungizone. Monolayer cultures of mouse cells were grown in Eagle's Minimal Essential Medium (EMEM, BioWhittaker) supplemented with 10% FBS, 2 mM L-glutamine, and 50 μg/ml gentamycin sulfate (GIBCO-BRL).

Nuclear and whole cell extract preparation

Nuclear extracts of the above cells were prepared as described (Liu *et al.*, 1994; Liu *et al.*, submitted) with slight modifications. Briefly, cells were washed and lysed in two packed cell volumes of Triton-lysis buffer (9 mM Tris-HCl, pH 7.5, 135 mM NaCl, 0.9 mM $MgCl_2$, 0.3% Triton X-100, 0.5 mM DTT, 0.5 mM PMSF) for 4-6 min on ice. Nuclei were pelleted at 3000 r.p.m. for 5 min at 4°C, washed once in lysis buffer (9 mM Tris-HCl, pH 7.5, 135 mM NaCl, 0.9 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF), then resuspended in two packed cell volumes of Dignam Buffer C (25% glycerol, 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT [Dignam *et al.*, 1983]). Nuclear proteins were extracted by rocking at 4°C for 45 min. Insoluble material was pelleted at 14,000 r.p.m. for 15 min at 4°C, and the

supernatant dialyzed for 1 h against Shapiro's buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 0.5 mg/l leupeptin, 0.7 mg/l pepstatin A [Shapiro *et al.*, 1988]) for 60 min at 4°C. Precipitated proteins were removed by centrifugation at 12,000 r.p.m. for 15 min at 4°C and the supernatant frozen at -80°C.

Whole cell extracts were prepared by lysing a confluent monolayer of cells in RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 10 μg/ml aprotinin, 200 μM PMSF). Extracts were stored at -20°C.

Electrophoretic Mobility Shift Analysis

Band shift assays using nuclear extracts were performed as previously described (Kralli *et al.*, 1992), with slight modifications. Four micrograms of nuclear extract was incubated at 30°C for 30 min with $3x10^4$ cpm of ^{32}P-labeled R1 or R2 oligonucleotide (Kralli *et al.*, 1992) and 4 μg poly [d(I-C)] (Boehringer Mannheim) in band shift buffer (for R1, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol; for R2, 10 mM HEPES pH 7.9, 60 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 10% glycerol). For supershift experiments, an antibody against NF-κB1-p50 (1613; kindly provided by Dr. Nancy Rice, NCI) was preincubated with the nuclear extract and poly [d(I-C)] in R1-binding band shift buffer for 20 min at room temperature, followed by addition of the ^{32}P-labeled R1 probe with further incubation at 30°C for 30 min. DNA-protein complexes were fractionated on a 5% non-denaturing polyacrylamide gel in 0.5 x TBE buffer (45 mM Tris-HCl, pH 8.3, 45 mM boric acid, 0.5 mM EDTA) for R1, or 1.0 x TBE buffer (90 mM Tris-HCl, pH 8.3, 90 mM boric acid, 1 mM EDTA) for R2. Gels were dried and autoradiographed to analyze the DNA-protein complexes.

Western Blots and antisera

Ten micrograms of nuclear extract or 50 μg of whole cell extract in sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 200 mM DTT, 20% glycerol) was boiled and then electrophoresed on SDS - 10% polyacrylamide gels. Separated proteins were transferred to Immobilon-P membranes (Millipore) by electroblotting. Membranes were blocked in TBS-Tween (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) with 5% powdered milk (Carnation) for at least one hour. Membranes were incubated for 1 h with primary antibody (see below), then washed three times with TBS-Tween. Membranes were incubated for 1 h with goat anti-rabbit IgG horseradish peroxidase (Boehringer Mannheim), then washed three times with TBS-Tween and once with TBS. Proteins reactive to the primary antibody were detected using enhanced chemiluminescence (ECL; Amersham). The following are the peptide-antibodies (gift of Dr. Nancy Rice) and their dilutions used in these studies: #1157 anti-NF-κB1-p50 (p50); 1:3000 (nuclear extract); 1:1000 (whole cell extract). #1613 anti-NF-κB1-p50 (p50); 1:1000. #1226 anti-RelA (p65); 1:1000.

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Figure 1 Regulatory elements of the H-2K^b MHC class I promoter. Indicated are the transcriptional start site (arrow), the TATA box (closed circle), interferon response sequence (IRS; black rectangle), and class I enhancer. The class I enhancer is comprised of the R2, R1′, and R1 elements. R2 contains a recognition-site for COUP-TF binding. R1 contains a κB consensus sequence, to which members of the NF- κB/Rel protein family members can bind.

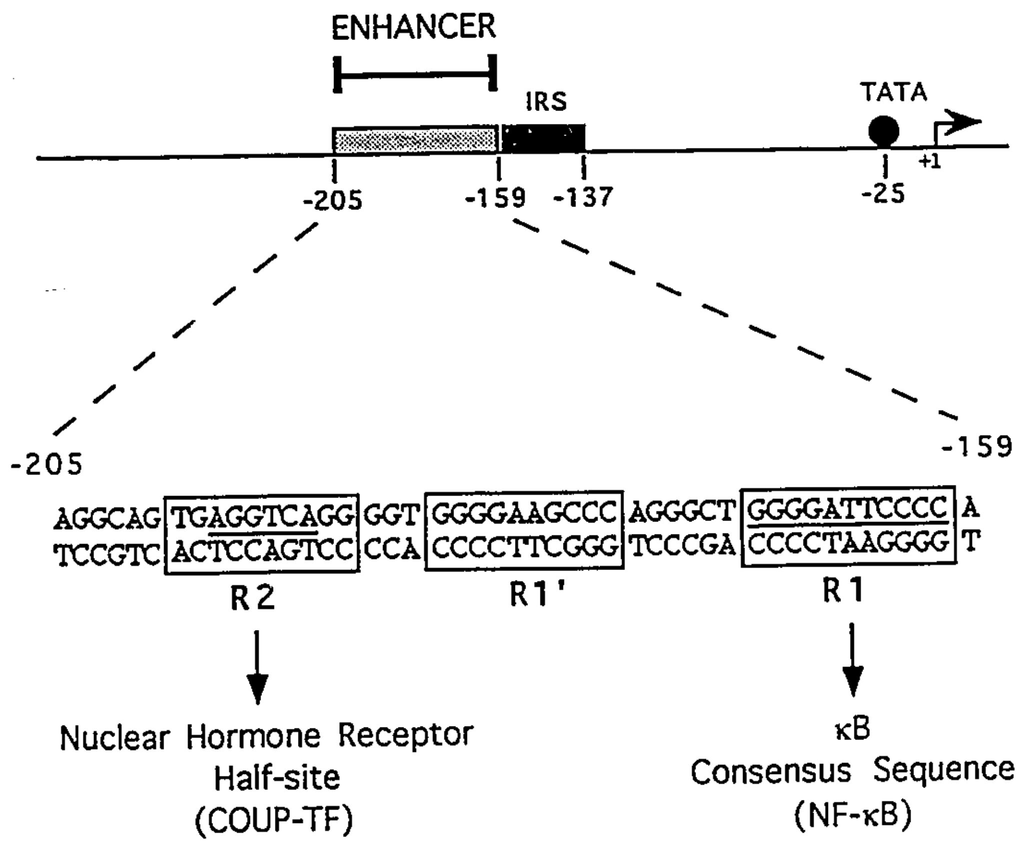
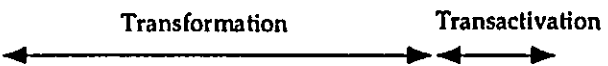
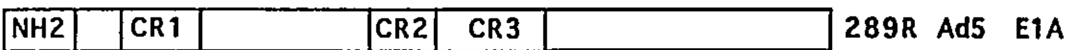
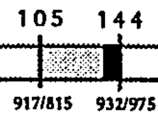
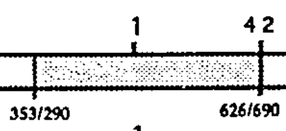

ENHANCER
IRS
TATA
+1
-205
-159
-137
-25
-205
-159
AGGCAG TGAGGTCAGG GGT GGGGAAGCCC AGGGCT GGGGATTCCCC A
TCCGTC ACTCCAGTCC CCA CCCCTTCGGG TCCCGA CCCCTAAGGGG T
R2
R1'
R1
Nuclear Hormone Receptor
Half-site
(COUP-TF)
κB
Consensus Sequence
(NF-κB)

Figure 2 Hybrid Ad5/Ad12 E1A constructs used to generate adenovirus-transformed cells. The DNA constructs used are aligned below the coding regions of the 289R Ad5 E1A and the 266R Ad12 E1A. The regions of E1A required for transformation and transactivation are indicated. CR1, CR2, and CR3 denote the three conserved regions of E1A. A 20 aa unique region in Ad12 located between CR2 and CR3 is indicated by the small black box. For each construct depicted, the Ad5 sequences are shown in white; the Ad12 sequences are shaded. The numbers below each Ad5/Ad12 hybrid construct indicate the locations of the crossovers between Ad5 and Ad12; Ad5 and Ad12 nucleotides are indicated on the outside and inside of the "/", respectively. The corresponding codons of Ad12 E1A are indicated above each construct. These E1A constructs were used in cooperation with Ad12 E1B to transform Hooded Lister rat kidney cells and create immortalized cell lines harboring hybrid E1 genes.

Transformation
Transactivation
NH2
CR1
CR2
CR3
289R Ad5 E1A
266R Ad12 E1A
Construct
DP5-2
105
144
917/815
932/975
917-975B12C10
1
42
353/290
626/690
690B12C1
975B12C4
12-1

Figure 3 The first exon of Ad12 E1A contains sequences which mediate increased COUP-TF binding to the R2 element of the MHC class I enhancer. A ^{32}P-labeled R2 oligonucleotide was incubated with 4 μg of nuclear extract from Ad5 (DP5-2; lane 1), Ad12 (12-1; lane 5), and Ad5/Ad12 hybrid (917-975B12C10, lane 2; 690B12C1, lane 3; 975B12C4, lane 4) E1 transformed rat cells. SC; R2/COUP-TF shifted complex. F; free probe.

DP5-2 917-975B12C10 690CB12C1 975B12C4 12-1

SC

F

Figure 4 The first exon of Ad12 E1A contains sequences which mediate reduced NF-κB binding to the R1 element of the MHC class I enhancer. **(A)** A ^{32}P-labeled R1 oligonucleotide was incubated with 4 μg of nuclear extract from Ad5 (DP5-2; lane 3), Ad12 (12-1; lane 7), and Ad5/Ad12 hybrid (917-975B12C10, lane 4; 690B12C1, lane 5; 975B12C4, lane 6) E1 transformed rat cells. The labeled R1 oligonucleotide was also incubated with nuclear extract from Ad12 (12A1; lane 1), and Ad5 (KAd5; lane 2) transformed mouse cells (Liu *et al.*, 1994) to serve as controls for the migration of the NF-κB1-p50 homodimer and the NF-κB heterodimer. 50/50; NF-κB1-p50 homodimer. 50/65; NF-κB heterodimer. F; free probe. The bracket denotes the range of differential migration of the shifted NF-κB1-p50 homodimer complexes in lanes 3-5. **(B)** A darker exposure of (A) to show the low levels of NF-κB binding to the R1 element in 975B12C4 and Ad12 transformed rat cells.

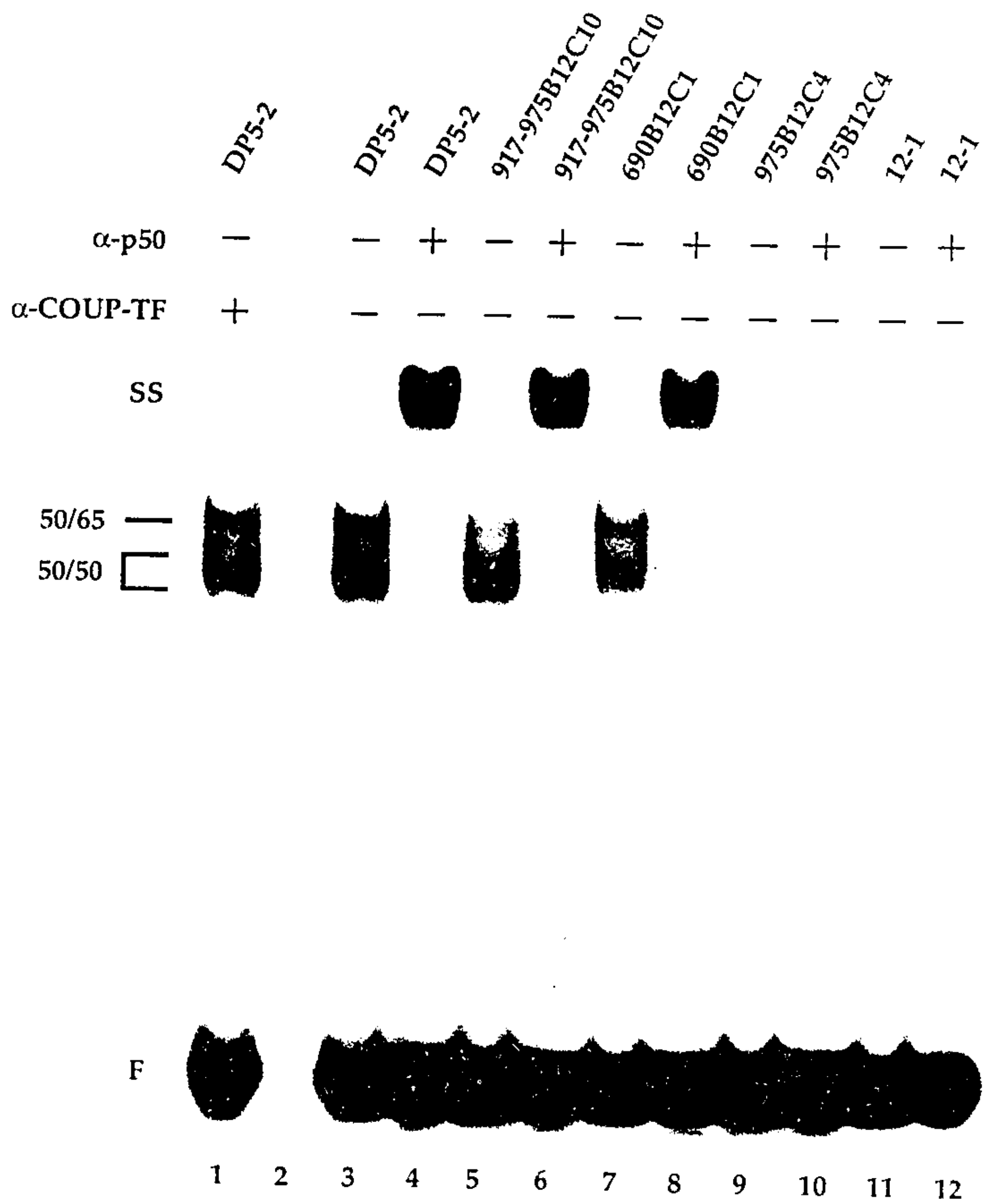
12A1
KAd5
DP5-2
917-975B12C10
690B12C1
975B12C4
12-1
50/65
50/50

F
1
2
3
4
5
6
7

12A1
KAd5
DP5-2
917-975B12C10
690B12C1
975B12C4
12-1
50/65
50/50

F
1
2
3
4
5
6
7

Figure 5 NF-κB1-p50 is a component of the R1-protein complexes in the Ad5, Ad12, and hybrid Ad5/Ad12 E1 transformed rat cells. **(A)** Four micrograms of nuclear extract from Ad5 (DP5-2), hybrid Ad5/Ad12 (917-975B12C10, 690B12C1, 975B12C4), or Ad12 (12-1) E1 transformed cells was incubated with the ^{32}P-labeled R1 oligonucleotide (lanes 3, 5, 7, 9, and 11), or pre-incubated with the 1613 anti-NF-κB1-p50 antibody (lanes 4, 6, 8, 10, and 12) or the COUP-TF antibody (Liu *et al.*, 1994) as a control (lane 1). Lane 2 was intentionally unused. To verify that the rapidly migrating complexes in lanes 3, 5, and 7 (denoted by the bracket) contain NF-κB1-p50, the 1613 anti-NF-κB1-p50 antibody was used to completely supershift both the NF-κB1-p50 homodimer band, as well as the NF-κB heterodimer band, in nuclear extracts from the E1 transformed cells. Note that the band-shifted complexes in lanes 9 and 11 are visible upon longer exposure. 50/50; NF-κB1-p50 homodimer. 50/65; NF-κB. F; free probe. SS; supershifted complexes.



DP5-2
DP5-2
DP5-2
917-975B12C10
917-975B12C10
690B12C1
690B12C1
975B12C4
975B12C4
12-1
12-1
α-p50
α-COUP-TF
SS
50/65
50/50
F
1
2
3
4
5
6
7
8
9
10
11
12

Figure 6 (A) Diminished NF-κB binding to R1 is not due to a lack of NF-κB1-p50 in the nuclei of the 975B12C4 and Ad12 E1 transformed cells. Ten micrograms of nuclear extract were electrophoresed on a SDS - 10% polyacrylamide gel. Separated proteins were transferred to membrane, probed with anti-NF-κB1-p50 antibody (1157) for 1 h, and detected using enhanced chemiluminescence. NF-κB1-p50 is detected in the nuclei of Ad5 (DP5-2), Ad5/Ad12 hybrid (917-975B12C10, 690B12C1, and 975B12C4), and, most importantly, Ad12 (12-1) E1 transformed rat cells (lanes 1 through 5, respectively). The lower amount of NF-κB1-p50 detected in the 975B12C4 cell line is due to a underloading of the sample. Weights of prestained molecular weight markers are shown at left. The arrow at right indicates the position of NF-κB1-p50. (B) Western analysis indicates both NF-κB1-p50 and NF-κB1-p105 are present in whole cell extracts from the five E1 transformed rat cell lines. Fifty micrograms of whole cell extract were separated by PAGE, transferred to membrane, and probed with the 1157 anti-NF-κB1-p50 antibody. Weights of prestained molecular weight markers are shown at left. Arrows at right indicate the positions of NF-κB1-p50 and NF-κB1-p105.

DP5-2
917-975B12C10
690B12C1
975B12C4
12-1

121.0 kDa

82.0 kDa

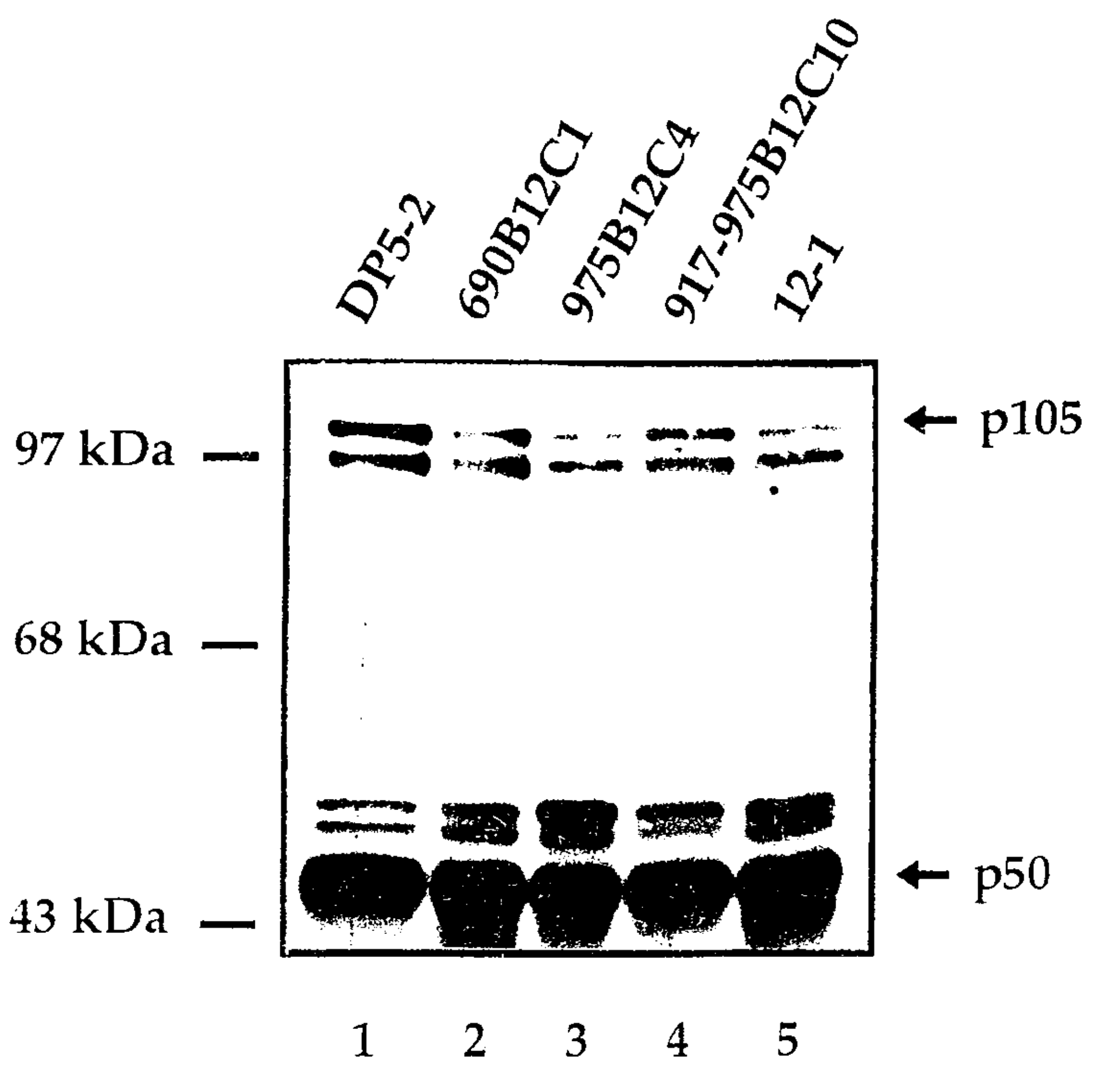
50.2 kDa

← p50

34.2 kDa

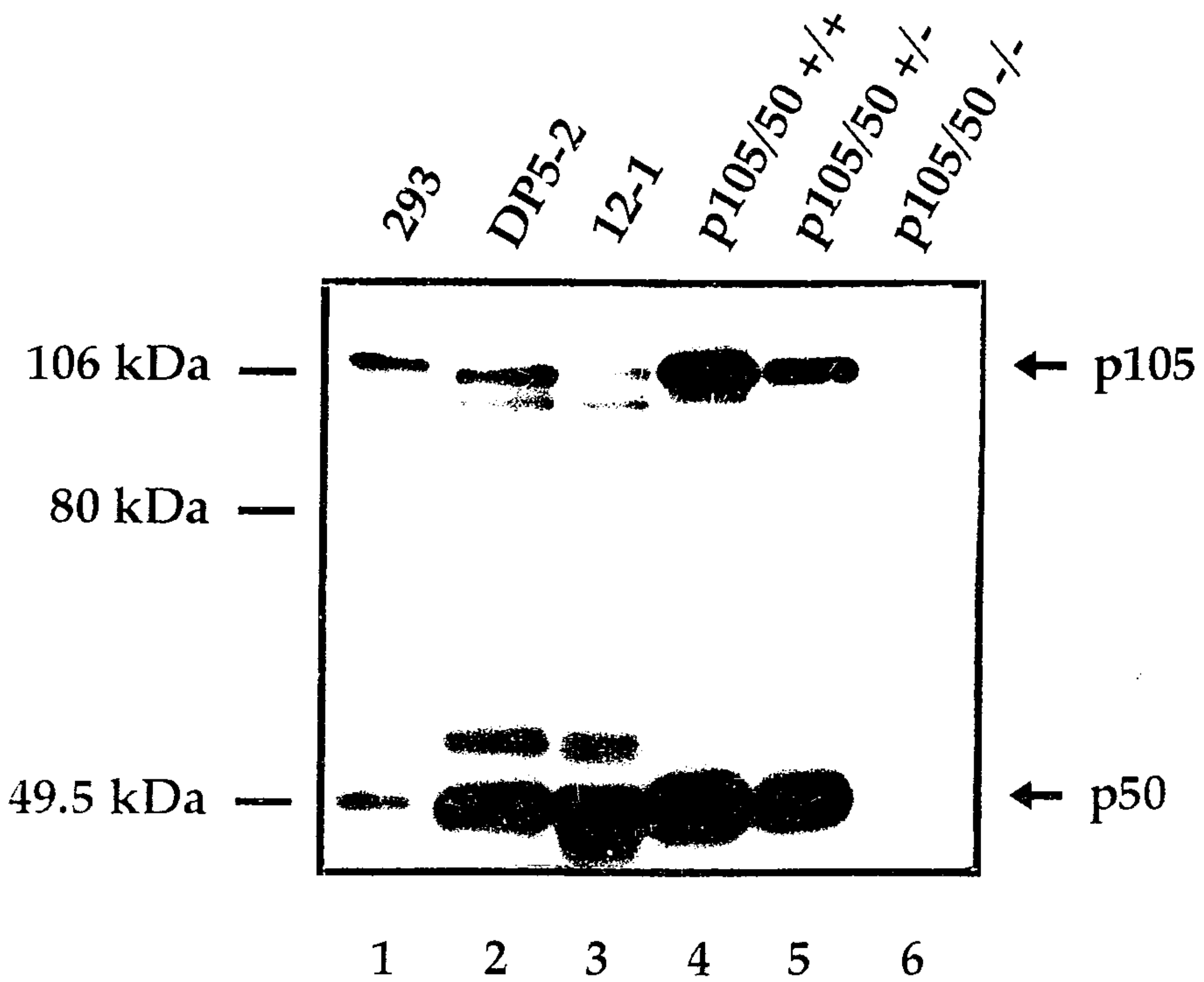
28.1 kDa

1 2 3 4 5



DP5-2
690B12C1
975B12C4
917-975B12C10
12-1
97 kDa
68 kDa
43 kDa
p105
p50
1
2
3
4
5

Figure 7 The 1157 antibody is specific for NF-κB1-p50. Fifty micrograms of whole cell extracts from human 293 (lane 1), rat DP5-2 (lane 2), rat 12-1 (lane 3), and NF-κB1-p105 +/+, +/-, and -/- (lanes 4-6) cells were electrophoresed on a SDS - 10% polyacrylamide gel. Separated proteins were transferred to membrane, probed with the 1157 antibody for 1 h, and detected by enhanced chemiluminescence. NF-κB1-p50 is detected in all the cell extracts except the NF-κB1-p105 -/- cell extracts (lane 6), indicating that the 1157 antibody used in these studies is specific for NF-κB1-p50. Weights of prestained markers are shown at left. Arrows at right indicate the positions of NF-κB1-p50 and NF-κB1-p105.


293
DP5-2
12-1
p105/50 +/+
p105/50 +/-
p105/50 -/-
106 kDa
80 kDa
49.5 kDa
p105
p50
1
2
3
4
5
6

Contributions to Kushner et al., In Press:

(1) To determine whether reduced NF-κB1 binding in Ad12 E1-transformed cells was due to a reduction in p50, p65, and p105 expression, I assessed steady state levels of p50 (and its precursor, p105) in whole cell extracts of Ad12 E1-transformants as well as of hybrid Ad5/12 E1A- (plus Ad12 E1B) transformants by Western Blot analysis.

(2) Tested the specificity of the NF-κB1-p50 antibody (NR1157) used in this study by Western blot analysis using NF-κB1-p50 +/+, +/-, and -/- mouse cell extracts.

Conclusions:

Expression of the first exon of Ad12 E1A, excluding CR3, which influences tumorigenicity and MHC class I down-regulation in transformed rat cells was found to correlate with increased binding of COUP-TF (repressor) and decreased binding of NF-κB (activator) to the MHC class I gene enhancer. Moreover, it was determined that reduced NF-κB binding activity in Ad12 E1-transformants was not due to a decrease in steady state levels of the NF-κB subunit proteins: NF-κB1-p50, RelA p65, or NF-κB1-p105, a finding which was crucial to the validity of the binding data in this study in view of the fact that an indepenedent study using an independent NF-κB1-p50-specific antibody suggested otherwise (Shouten et al., 1995).

4) Pereira, D. S., Kushner, D. L., Ricciardi, R. P., and Graham, F. L. 1995.
Testing NF-κB1-p50 antibody specificity using knockout mice. Submitted.

Preface:

In comparisons of NF-κB1-p50 expression in wild type Ad5 E1- and Ad12 E1-transformed cells for the Kushner et al. study described above, two NF-κB1-p50-specific antibodies (sc-114 and NR1157), used to detect p50 expression by Western blot analysis, produced contradictory results. To resolve which antibody recognized authentic NF-κB1-p50, we compared the specificity of sc-114 and NR1157 by Western blot analysis using whole cell extracts (WCE) derived from p105/p50 -/-, +/- and +/+ mice (Sha et al., 1995).

Testing NF-κB1-p50 Antibody Specificity Using Knockout Mice.

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Running Title: NF-κB1-p50 Antibody Specificity

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Key Words: NF-κB1, p50, p105, transcription factor, antibody specificity, knockout mice.

NF-κB is a ubiquitously expressed transcription factor composed of 50 (p50) and 65 (p65) kilodalton subunits. p50 and p65 are members of the intensively studied NF-κB/Rel family of transcription factors of which there are five members identified in vertebrates: NF-κB1 (p50 and its precursor, p105), NF-κB2 (p52 and its precursor, p100), p65 (RelA), Rel (c-Rel), and RelB (for review, see Nolan and Baltimore, 1992). In *in vitro* studies, most members of this family have been shown to dimerize, directly interact with DNA (κB motifs), and positively and negatively affect transcription of κB reporter genes. Cellular genes targeted for regulation by NF-κB are involved in acute-phase responses, inflammation, lymphocyte activation, and cell growth and differentiation. That NF-κB is a critical component for both specific and non-specific immune responses is apparent from studies using mice lacking the p50 subunit of NF-κB1. Although these mice show no developmental abnormalities, they demonstrate multifocal defects in immune responses involving B lymphocytes and non-specific responses to pathogen challenge (Shaw et al., 1995).

Measurement of NF-κB expression/activity frequently utilizes antibodies specific for NF-κB1-p50 in techniques such as electrophoresis mobility shift/super shift assays, immunoprecipitation, cell staining, and Western blotting. This article reports on the specificity of two commonly used NF-κB1-p50 antibodies in Western blots. The first antibody, sc-114 was purchased from Santa Cruz Biotechnology and is an affinity-purified rabbit polyclonal antibody raised against a human p50 peptide (CTPEIKPKEEVQRKR) which maps within a domain encompassing part of the basic NLS sequence and the N-terminal adjacent 11 amino acids (Henkel et al., 1992). It is claimed that sc-114 detects p50 in human, mouse and rat cells in electrophoresis mobility shift/supershift assays, immunoprecipitation, cell staining, and Western blotting protocols (Santa Cruz Biotechnology Incorporated, 1995 and manufacturer's specifications sheet included with antibody). The second antibody, NR1157 (graciously supplied by Dr. Nancy Rice (NCI, Frederick, MD)) is also a rabbit polyclonal antibody raised to a human p50 peptide

(DLETSEPKPFLYYPEIKDKC) that detects p50 and p105 by Western blot analysis in human, murine, and chicken cells as well as rat cells (our unpublished data).

The motivation for comparing the specificity of sc-114 and NR1157 arose when each antibody detected different proteins of approximately 50-55 kilodaltons in Western blot analysis of transformed rat cells. To resolve which antibody recognized authentic NF-κB1-p50, we decided to compare the specificity of sc-114 and NR1157 by Western blot analysis using whole cell extracts (WCE) derived from p105/p50 -/-, +/- and +/+ mice (See Fig. 1). Briefly, duplicate sets of thymic WCEs containing 50 μg of protein from these mice were resolved using SDS-PAGE (10% gel) and transferred to an Immobilon-P transfer membrane for Western blot analysis. The membrane was cut between the duplicate sample sets to create two membranes. The first membrane was probed with NR1157 (1:1000 dilution) (Fig. 1, lanes 1-3) while the second was probed with sc-114 (1:400 dilution) (Fig. 1, lanes 4-6). The NR1157 antibody detected proteins of 105 (p105) and 50 (p50) kilodaltons in p105/p50 +/+ mouse cells (Fig. 1, lane 1). As expected, NR1157 also detected a two-fold reduction of p50 levels in p105/p50 +/- mouse cells and a complete lack of p50 expression in p105/p50 -/- cells (Fig. 1, lanes 2 and 3). Using sc-114, a 52-55 kilodalton protein was the major protein detected with equal intensity in p105/p50 +/+, +/-, and -/- mouse cells, suggesting that the sc-114 antibody, at a dilution of 1:400, does not have significant affinity for NF-κB1-p50 by Western blot analysis in murine cells (Fig. 1, lanes 4-6) nor in human and rat cells (data not shown). Although the identity of the major protein recognized by sc-114 is not yet known, it may be a p50-related protein or perhaps NF-κB2-p52. Interestingly, 8 amino acids in the human p50 peptide used to raise sc-114 are also present in the human p52 protein. It should be noted that the sc-114 antibody (at a 1:400 dilution), may have slight affinity for p50 in Western blots, but this was only apparent upon overexposure of the blot shown in Fig. 1. Finally, although the peptide used to raise sc-114 was generated to the N-terminus of human p50, which is also present

in its precursor p105, sc-114 did not appear to detect p105 when the blot shown in Fig. 1 was overexposed.

In conclusion, we have demonstrated the usefulness of cell extracts from knock-out mice to test the specificity of multiple antibodies raised to a specific protein, namely NF-κB1-p50. In addition, we wish to take this opportunity to caution users of the sc-114 antibody, not only on its lack of acceptable specificity for NF-κB1-p50, but also on its strong affinity for a 52-55 kilodalton protein which can be easily mistaken for NF-κB1-p50 by Western blot analysis.

Acknowledgments

The authors gratefully acknowledge Dr. William C. Sha of Dr. David Baltimore's laboratory for providing NF-κB1-p50 knock-out mouse cell extracts. Special thanks is extended to Dr. Nancy Rice for her helpful suggestions and generous supply of antibodies to NF-κB1-p50. Without their contributions, our findings would not have been possible. This work was supported by grants from the National Cancer Institute and Medical Research Council of Canada as well as a public health service grant CA-29797 from the National Cancer Institute to R.P.R. F.L.G is a Terry Fox Research Scientist of the National Cancer Institute of Canada and D.S.P is a recipient of a studentship from the Cancer Research Society of Canada.

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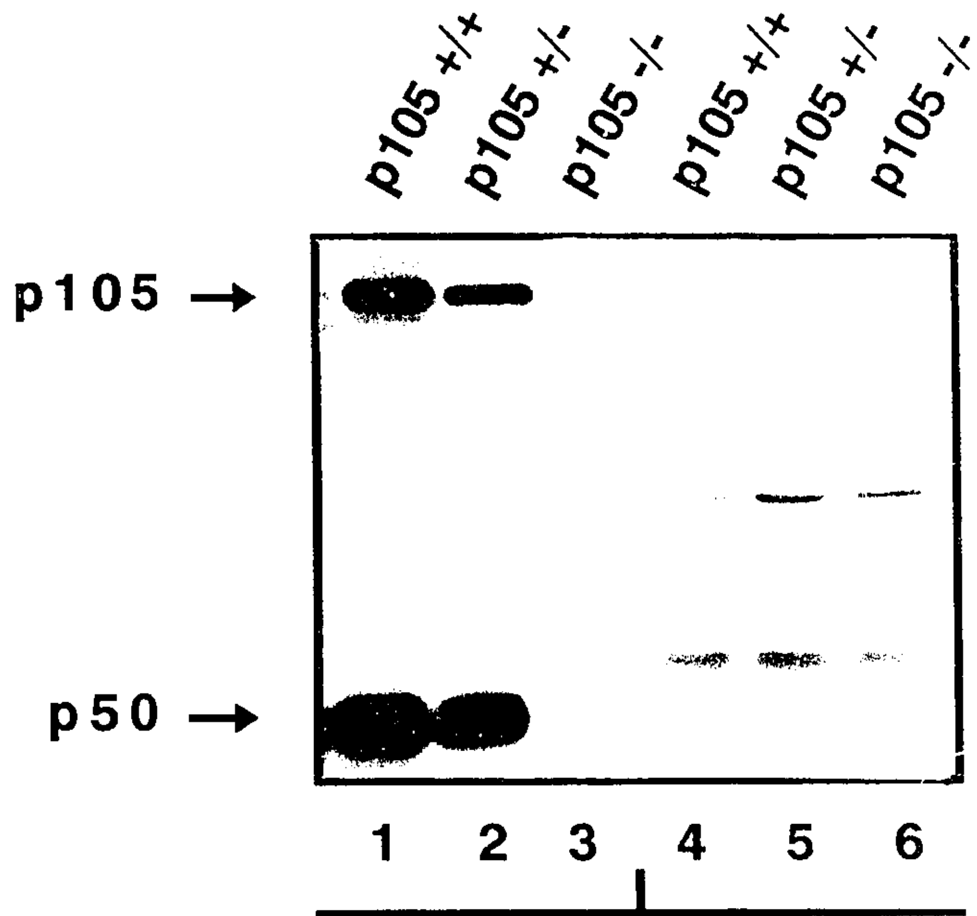
Figure 1. Western blot analysis of whole cell extracts from p105 +/+, +/-, and -/- thymic mouse cells using NF-κB1-p50 specific antibodies, NR1157 and sc-114. 50 μg of thymic whole cell extracts from p105 +/+, +/-, and -/- cells were electrophoresed on a 10% SDS-polyacrylamide gel and electro-blotted onto an Immobilon-P transfer membrane (Millipore Corporation, Bedford, MA, USA). The blot was blocked for 1 hour in 5% nonfat dry milk/PBS and probed for 1 hour with the primary NF-κB1-p50 specific antibodies, NR1157 (1:1000 dilution) (lanes 1-3) (N. Rice, NCI, Frederick, MD, USA) and sc-114 (1:400 dilution) (lanes 4-6) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following two 7 minute washes in PBS/0.05% Tween-20, the blot was incubated for 1 hour with a 1:2000 dilution of a horseradish peroxidase conjugated goat anti-rabbit polyclonal secondary antibody purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. After three 5 minute PBS/0.05% Tween-20 washes and one PBS wash, the blot was developed using Enhanced Chemiluminescence Western blotting reagents (Amersham International plc., Buckinghamshire, England) and exposed to film for 1 minute.



p105 +/+
p105 +/-
p105 -/-
p105 +/+
p105 +/-
p105 -/-
p105
p50
1
2
3
4
5
6
Antibody:
NR1157
sc-114

Contributions to Pereira et al., Submitted:

(1) Conducted Western blot analysis to test the specificity of the sc-114 and NR1157 NF-κB1-p50-specific antibodies using whole cell extracts derived from NF-κB1-p50 -/-, +/-, and +/+ mice.

Conclusions:

The major findings from this study were: 1) While sc-114 demonstrates unacceptable specificity for NF-κB1-p50 in rat whole cell extracts by Western Blot analysis NR1157 is highly specific; 2) sc-114 demonstrates a strong affinity for a 52-55 kilodalton protein which can be easily mistaken for NF-κB1-p50 in Western blot analysis.

Consequently, the major motivation for publishing this manuscript is to inform users of sc-114 about its inadequacy.

5) **Pereira, D. S., Jelinek, T., and Graham, F. L. 1994.**
The adenovirus E1A-associated p300 protein is differentially phosphorylated in Ad12 E1A- compared to Ad5 E1A-transformed rat cells.
Int. J. Oncol. 5, 1197-1205.

Preface:

Ad5 E1A associates with a number of cellular proteins such as p300 and the Rb protein family members p130, p107, and p105Rb. Whether Ad12 E1A also associates with these proteins and what the consequence of these interactions are on tumorigenicity was unknown in rat cells constitutively expressing Ad12 E1. Therefore, in keeping with the aim of characterizing Ad5 E1- and Ad12 E1-transformed rodent cells to explain differences in tumorigenicity, the hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed rat cells were examined for differences in the ability of their hybrid Ad5/12 E1A proteins to associate with cellular proteins. The notion that Ad5 E1A and Ad12 E1A may interact distinctly with cellular proteins in a manner which would correlate with the tumorigenic capacities of Ad5 E1- and Ad12 E1- transformed cells was the motivation for this study. Moreover, these studies may provide additional insight into existing theories which were put forth to explain the differential tumorigenic capacities of Ad5 E1- and Ad12 E1-transformed rodent cells.

INTERNATIONAL JOURNAL OF ONCOLOGY 5: 1197-1205, 1994

The adenovirus E1A-associated p300 protein is differentially phosphorylated in Ad12 E1A- compared to Ad5 E1A-transformed rat cells

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Contributed by F.L. Graham, September 26, 1994

Abstract. Recently, using hybrid Ad5/12 E1A-transformed rat cells, we identified at least two regions of Ad12 E1A which influence tumorigenicity. In this report, again using the hybrid Ad5/12 E1A-transformants, we show that expression of these same two Ad12 E1A regions not only correlates with down-regulation of cell surface MHC class I expression, but coincides with the presence of an altered form of the E1A-associated cellular protein, p300, detected as a more slowly migrating species in SDS-polyacrylamide gels. The decreased electrophoretic mobility of p300 from hybrid Ad5/12 E1A- and Ad12 E1A-transformants can be abolished by protein phosphatase treatment suggesting that the change in mobility results from differential phosphorylation of p300. We suggest that differential phosphorylation of p300 may be functionally significant in the context of phenotypic differences between Ad5 E1- and Ad12 E1-transformed cells.

Introduction

Human adenoviruses (Ads) are DNA tumor viruses capable of transforming rodent cells in culture. The DNA sequences responsible for transformation of rodent cells by Ad are the early region 1A (E1A) and B (E1B) oncogenes (for reviews, see refs. 15,49). Within the E1A gene product, three regions, conserved region 1 (CR1), CR2 and the amino terminus are required for transformation (for a review of E1A proteins and transformation, see refs. 6,7 and refs. therein). The amino terminus and CR2 form two independent binding sites for direct interaction with the E1A-associated cellular proteins p300, p130, p107 and p105^{Rb}. While CR2 possesses binding sites for p130, p107 and p105Rb, p300 associates with the amino terminus of E1A. Indirectly, E1A also associates with p60$^{Cyclin A}$ (through p107) and p33$^{cdk2 kinase}$ (through p60$^{Cyclin A}$). Mechanistically, transformation by E1A is thought to promote cell proliferation by total or partial sequestering of cell growth-regulating proteins in a manner which initiates DNA synthesis and influences gene expression. For example, sequestration of the p130, p107 and p105^{Rb} proteins, which are known inhibitory components of E2F transcription complexes, frees active E2F (for review see ref. 31), while E1A-mediated repression activity is dependent on the interaction of p300 with the amino terminus of E1A (23,41,47).

Although the E1 region from any Ad serotype is capable of transforming primary rodent cells, the tumorigenic capacity of these transformants in syngeneic rodents is dependent on the serotypic origin of E1A (9,19), specifically the first exon (24). For instance, Ad E1-transformed cells expressing Ad5 or Ad12 E1A are non-tumorigenic and tumorigenic respectively (9). Results from many studies directed at identifying and understanding functional differences between Ad5 and Ad12 E1A oncoproteins have suggested that E1A influences tumorigenicity by affecting the immune response of the immunocompetent rodent. For example, E1A proteins in Ad5 E1-infected and transformed cells have been observed to induce susceptibility to non MHC class I-restricted natural killer (NK) cells (12,27, 36,46), macrophages (12) and lymphocyte activated killer cells (LAKs) (27) where as Ad12 E1A proteins have been shown to confer resistance to these cells. Additionally, the Ad12 E1A 13S product, unlike the corresponding Ad5 E1A product, has been shown to down-regulate the expression of MHC class I mRNA and cell surface molecules in rodent (2,14,29,37,44) and human cells (45). Consequently, it is thought that Ad12 E1A-transformed cells become tumorigenic by evading the surveillance of MHC class I restricted CD8^{+} cytotoxic T lymphocytes (CTLs) (3,9,32,35). Other studies suggest that regulation of MHC class I expression may not be as important for susceptibility to CTLs as whether the Ad5 or Ad12 E1A proteins are directly

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immunogenic in the form of class I-restricted epitopes (8,25,33,43).

Previously, using rats cells transformed by hybrid Ad5/12 E1A plus Ad12 E1B genes, we identified at least two regions within the first exon of Ad12 E1A required for tumorigenicity (20). Our findings were corroborated by evidence from the laboratory of J. Williams (42). In keeping with the aim of characterizing Ad5 E1A- and Ad12 E1A-transformed rodent cells to explain differences in tumorigenicity, we again utilized these transformed rat cells to examine differences in cell surface MHC class I expression as well as differences in the ability of their hybrid Ad5/12 E1A proteins to associate with cellular proteins. Our findings suggest that expression of the same two Ad12 E1A regions implicated in tumorigenicity, not only correlate with decreased cell surface levels of MHC class I molecules, but coincide with the presence of a differentially phosphorylated form of p300, detected as a more slowly migrating species in SDS-polyacrylamide gels.

Materials and methods

Cell culture. Monolayer cultures of Ad5 E1A-, Ad12 E1A-, and hybrid Ad5/12 E1A-transformed Hooded Lister rat cell lines (20,22) as well as Ad12 E1-transformed rat cells, HABaC1 (28), HABaC2 (28) and 702-C2 (28) were grown in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum. The human cell lines, 293 (Ad5 E1-transformed embryonic kidney) (16) and HER-3 (Ad12 E1-transformed embryonic retinal) (11) were maintained as monolayer cultures in F11 medium supplemented with 10% newborn calf serum and Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal bovine serum. All media were also supplemented with 2 mM L-glutamine, 100 units/ml penicillin G and 100 µg/ml streptomycin sulfate.

Detection of cell surface MHC class I proteins. Cell surface expression of MHC class I proteins was determined by flow cytofluorimetry using a FACScan (Becton Dickinson Canada Incorporated, Ontario, Canada) and antibodies purchased from Cedarlane Laboratories Limited (Ontario, Canada). Single cell suspensions were prepared from monolayer cultures and resuspended in phosphate buffered saline supplemented with bovine serum albumin (10 mg/ml) and sub-divided into three aliquots (unstained, stained by secondary antibody only, stained by both primary and secondary antibodies) containing 10^6 cells/aliquot. Where appropriate, single-cell suspensions were incubated for 30 minutes on ice with anti-rat RT1. A monoclonal antibody, CL007A, which served as the primary antibody. Subsequent or independent staining with rabbit anti-mouse immunoglobulin antisera [conjugated to fluorescein isothiocyanate (FITC)], which reacts with all mouse Ig classes and subclasses, served as the secondary antibody.

Immunoprecipitations. Prior to ^{35}S methionine labeling, 10^6 cells/sample were seeded onto 100 mm monolayer tissue culture plates. At 50-60% confluence, cells were metabolically labelled at 37°C for 4 h in 199 medium lacking methionine and cysteine and supplemented with 300 µCi ^{35}S-translabel (ICN) per sample. The radioactive cells were washed with PBS, centrifuged, resuspended in 'lysis buffer X+BSA' (50 mM Tris (pH 8.8), 250 mM NaCl, 1% NP-40, 2 mM EDTA, 10 µg/ml aprotinin, 0.5 mM sodium metavanadate and 2 mg/ml bovine serum albumin) and placed on ice for 20 min. Following a 15 min/15000 rpm centrifugation at 4°C, 3% protein-A sepharose (resuspended in 'lysis buffer X+BSA') was added to the lysate, mixed at 4°C for 15 min and pelleted. Depending on the experiment, the pre-cleared lysate was incubated with anti-Ad2/5 E1A M73 monoclonal antibody (18) or anti-p300 polyclonal serum at 4°C for 1 h, followed by incubation with 3% protein-A sepharose for a further 30 min. Next, the protein-A sepharose was pelleted and washed three times with 'lysis buffer X+BSA' and finally with 'lysis buffer X-BSA'. Samples were boiled for 5 minutes in Laemmli loading buffer and loaded on either 7 or 8% SDS-polyacrylamide gels.

Protein phosphatase treatment. Proteins were immunoprecipitated as outlined above, using α-p300 polyclonal serum. p300-containing immunocomplexes were washed with λ protein phosphatase buffer (50 mM Tris-HCl, 5 mM DTT, pH 7.8), divided in two equal portions, pelleted and resuspended in 50 µl of 2 mM $MnCl_2$/100 mg/ml BSA. One portion was incubated at 30°C for 30 minutes with 1000 U of λ protein phosphatase (New England Biolabs) which releases phosphate groups from serine, threonine and tyrosine residues. Incubations were halted and denatured by addition of 50 µl of 2X Laemmli loading buffer and heated at 100°C for 5 minutes. p300 proteins were analyzed on a 20 cm long 7% SDS-polyacrylamide gel after electrophoresis for 36 h at 9 mAmps.

Phosphoamino acid analysis. [^{32}P]-orthophosphate-labelled p300 proteins from Ad5 E1A- and Ad12 E1A-transformants were isolated by SDS-PAGE following immunoprecipitation (as described above). These p300 proteins were excised from the gel, trypsinized and oxidized as described previously (10). Trypsinized lyophilisates were then hydrolysed with 5.7M HCl at 110°C for 1 h, lyophilized, resuspended and analyzed by one-dimensional thin layer electrophoresis as outlined previously (21).

Results

Tumorigenicity of hybrid Ad5/12 E1A-transformants. The hybrid Ad5/12 E1A genes, shown in Fig. 1A, were previously constructed and used in combination with the Ad12 E1B gene to transform primary baby Hooded Lister rat kidney cells (BRKs) (20,22) which were subsequently assayed for their ability to form tumors in newborn syngeneic rats (20). For a complete description of hybrid Ad5/12 E1A genes, see the legend to Fig. 1A. The ability of these BRKs transformed by the hybrid Ad5/12 E1A constructs (plus Ad12 E1B) to form tumors in newborn syngeneic rats is shown in Fig. 1B. Cells transformed by the p690, p753 and p827 hybrid Ad5/12 E1A genes [contain the 5' terminal Ad12 E1A nucleotides up to 626 (aa 49), 692 (aa 66) and 739 (aa

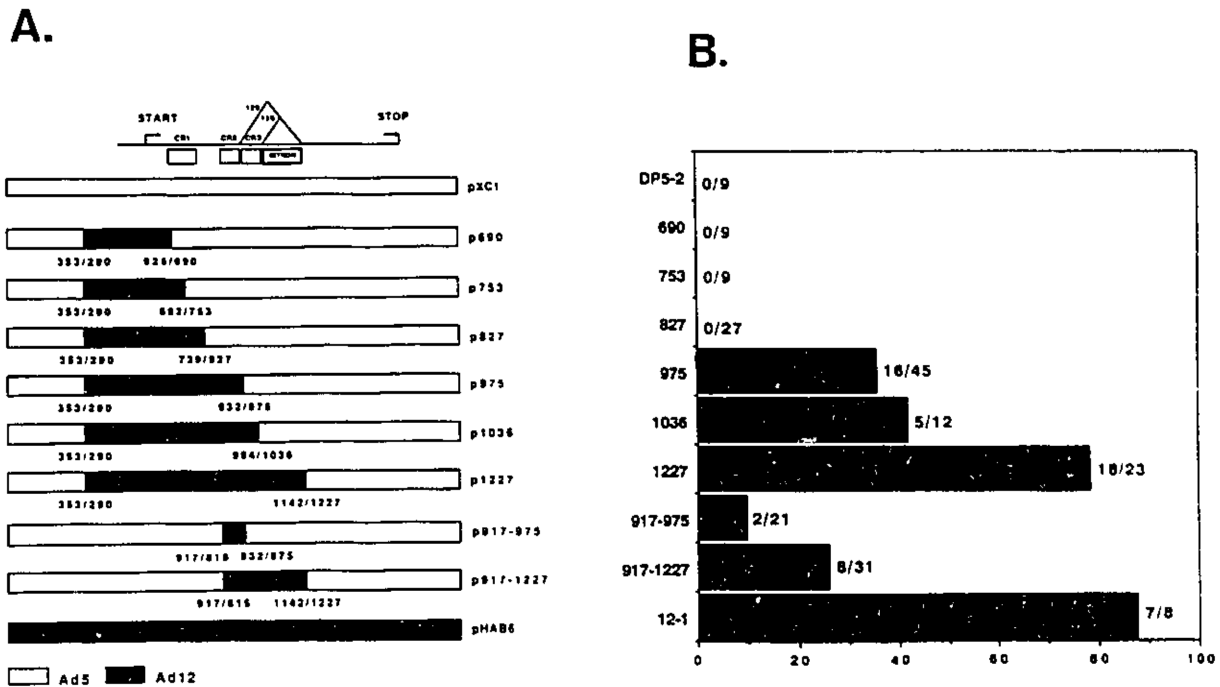

Figure 1. (A) Schematic of Ad5 (pXC1), Ad12 (pHAB6) and hybrid Ad5/12 E1A plasmids used to transform Hooded Lister rat cells in conjunction with Ad12 E1B. The previously described hybrid Ad5/12 E1A constructs (20,22), shown with Ad5 and Ad12 E1A gene sequences in white and black respectively, are aligned with the structure of Ad5 E1A. Precise locations of crossovers are expressed in nucleotides below the crossover junctions, where Ad5 and Ad12 nucleotides are shown on the outer and inner sides of the '/' respectively. All hybrid E1A constructs, with the exception of p917-975 and p917-1227, contain a first crossover junction in which the leftmost 353 base pairs of Ad5, encompassing the viral inverted terminal repeat and enhancer/packaging regions, are followed by Ad12 E1A sequences from nucleotide 290, encompassing the Ad12 E1A promoter, transcriptional start and amino terminus, to a second junction point where crossover again occurs into Ad5 E1A sequences to 16% of Ad5 genome length. In addition to the first 353 nucleotides of Ad5 common to the hybrid E1A plasmids, the p917-975 and p917-1227 constructs contain Ad5 gene sequences to nucleotide 917 (815; Ad12) followed by Ad12 E1A gene sequences to nucleotides 932 (975; Ad5) and 1142 (1227; Ad5) respectively. It should be noted that the p975 and p917-975 hybrids lack the 12s splice donor sites of Ad5 and Ad12, therefore these hybrids encode only the 13S E1A product; (B) Tumorigenicity of cells transformed by E1A genes shown in Fig. 1A. Data presented has been previously reported (20) and shows the percentage of rats with tumors following subcutaneous injection of 10^7 cells per newborn syngeneic rat. The number of tumor-bearing rats/total number of rats injected per cell line (includes different independently derived lines transformed by same plasmid) are shown right of the bars representing each cell line.

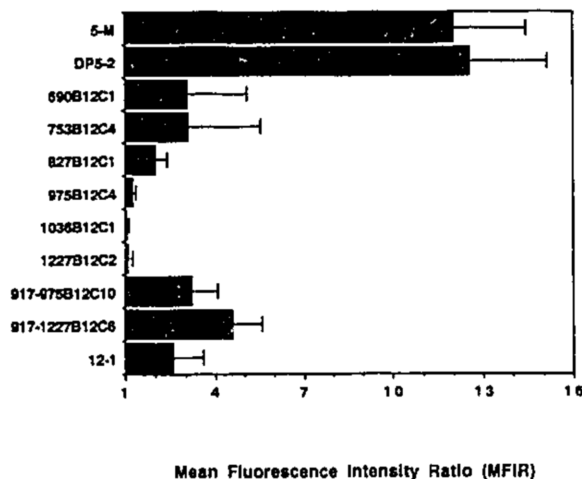

Figure 2. Determination of cell surface MHC class I expression. Cell surface expression of MHC class I molecules was determined by flow cytofluorimetry using a primary monoclonal antibody specific for the rat MHC class I proteins (RT1.A) and a secondary antibody conjugated to FITC. The mean fluorescence intensity ratio (MFIR) was determined according to the following calculation: (mean fluorescence intensity of cell line in the presence of primary and secondary antibody)/(mean fluorescence intensity of cell line in presence of secondary antibody only).

83)] were non-tumorigenic while cells transformed by p917-975 or p917-1227 [contain Ad12 E1A nucleotides 815-932 (aa 108-147) or 815-1142 (aa 108-193) respectively] exhibited weakly to moderately tumorigenic phenotypes. When most or all of Ad12 E1A exon 1 was expressed as in the p975-, p1036- and p1227-transformants, a strongly tumorigenic phenotype was observed in that the percent of rats with tumors was similar to that obtained with 12-1 cells transformed by pHAB6 which encodes the entire Ad12 E1 region.

Cell surface expression of MHC class I proteins. Reduced MHC class I levels in Ad12-transformed rodent cells appear to be due to expression of Ad12 E1A. To map the regions of Ad12 E1A responsible for down-regulation of class I expression, we measured class I levels on the surface of the hybrid Ad5/12 E1A plus Ad12 E1B transformants (Fig. 2). Compared to levels expressed on cells transformed by Ad5 E1A (DP5-2 or 5-M), all hybrid Ad5/12 E1A plus Ad12 E1B transformed lines showed decreased levels of cell surface MHC class I molecules. The decrease appeared to occur in two steps as Ad5 E1A sequences were progressively replaced with Ad12 E1A sequences from the amino terminus rightward. The first decrease, demonstrated by the non-

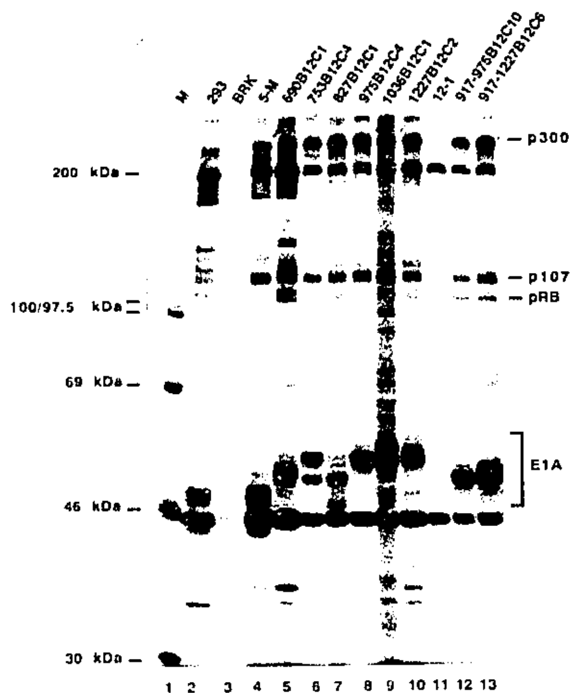

Figure 3. Coimmunoprecipitation of cellular proteins associated with E1A proteins. Cells transformed by Ad12 E1B plus the E1A genes depicted in Fig. 1, were metabolically labelled with ^{35}S-translabel, lysed, pre-cleared, and immunoprecipitated with M73 monoclonal antibody as described in the text. Samples were subjected to SDS-PAGE on an 8% polyacrylamide gel and subsequently visualized by autofluororadiography. Lanes 2 and 3 are 293 and primary BRK cell lines serving as positive and negative controls respectively. The absence of specific bands in lane 11 is due to the fact that the E1A proteins present in the 12-1 cell line [transformed by pHAB6 (Ad12 E1A encoding plasmid)] do not immunoprecipitate with the E1A-specific monoclonal antibody, M73. Molecular weight markers and identities of the E1A-associated proteins: E1A, pRB, p107 and p300 are indicated on the left and right sides of the autofluororadiogram respectively.

tumorigenic lines transformed by the plasmids, p690, p753 and p827, was an average of 5 fold lower than the DP5-2 or 5-M lines. Tumorigenic p975-, p1036- and p1227-transformants, demonstrated a further decrease represented by an approximately 2-3 fold reduction compared to the non-tumorigenic hybrids or 10 fold compared to the non-tumorigenic DP5-2 and 5-M lines. Levels of cell surface MHC class I proteins on the weakly to moderately tumorigenic hybrid p917-975- and p917-1227-transformants, which contain Ad5 E1A-derived amino terminal sequences, were an average of 3 to 4 fold lower than that of the non-tumorigenic DP5-2 or 5-M lines and were comparable to levels expressed on the non-tumorigenic hybrids transformed by the p690, p753 and p827 constructs. While 12-1 cells (transformed by the Ad12 E1A encoding plasmid, pHAB6 and the Ad12 E1B plasmid, pHAB13) expressed levels of class I antigens comparable to those on the non-tumorigenic p690-, p753- and p827-transformants and weakly to moderately tumorigenic p917-975- and p917-1227-transformants, they exhibited an approximately 2 fold greater level of class I expression than that of the tumorigenic cell lines transformed by p975, p1036 and p1227. To ensure that differences observed in MHC class I expression among the transformants were not the result of clonal variation we measured cell surface levels of additional cell lines (data not shown) and found levels to be comparable, within error, to the clones transformed by the same E1A constructs shown in Fig. 2. Taken together, the above results suggest that the same regions of Ad12 E1A important for tumor induction are also involved in down-regulation of MHC class I expression. However, reduced cell surface class I expression did not appear to strictly correlate with tumorigenicity since the strongly tumorigenic Ad12 E1-transformant, 12-1 and the weakly to moderately tumorigenic p917-975- and p917-1227-transformants expressed class I levels comparable to those of the non-tumorigenic p690-, p753- and p827-transformants.

Cellular proteins coimmunoprecipitate with hybrid Ad5/12 E1A proteins. To determine whether the hybrid Ad5/12 E1A proteins found in the transformed rat cell lines (Fig. 3; lanes 5-10 and 12-13) interact with the E1A-associated cellular proteins, these proteins were coimmunoprecipitated using the E1A-specific monoclonal antibody, M73 (18) and analysed by SDS-PAGE. Because M73 specifically recognizes the carboxy terminus of Ad2/5 E1A, it binds to all the hybrid Ad5/12 E1A proteins since they contain the second exon of Ad5 E1A, but fails to bind to Ad12 E1A proteins. Therefore, both 12-1 transformants (lane 11) and primary BRKs (lane 3) served as negative controls. The Ad5 E1-transformed 293 cell line (lane 2) served as a positive control. Regardless of whether the E1A binding domains were derived from Ad5 or Ad12 E1A protein sequences, the affinity with which the p300, p107 and pRB cellular proteins associated with the E1A proteins present in 5-M (lane 4), 1227B12C2 (lane 10) and hybrid Ad5/12 E1A transformants (lanes 5-9 and 12-13) remained relatively constant. These data are in agreement with previous results from our laboratory which demonstrated that E1A and its associated proteins, present in HeLa cells infected with hybrid Ad5/12 E1A-containing viruses, interacted with similar affinities for all the hybrids (22).

p300 from hybrid Ad5/12 E1A- and Ad12 E1A-transformed rat cells exhibits decreased electrophoretic mobility in SDS-polyacrylamide gels. Upon close examination of the p300 proteins present in the E1A-coimmunoprecipitation depicted in Fig. 3, small differences in electrophoretic mobility of p300 were apparent. In fact, as the Ad12 E1A content increased in the hybrid Ad5/12 E1A-transformants, a decrease in the electrophoretic mobility of p300 in SDS-PAGE was observed. To confirm this observation, we again coimmunoprecipitated the E1A-associated proteins with M73 (Fig. 4A) or used α-p300 polyclonal serum (Fig. 4B) to immunoprecipitate and analyze the p300 proteins on a lower percentage (7%) SDS-polyacrylamide gel subjected to a longer period of electrophoresis. This analysis showed that introduction of Ad12 E1A sequences into an Ad5 E1A background resulted in a significant decrease in p300 mobility. The 'shifted' p300 proteins present in the lines transformed by the hybrid Ad5/12 E1A genes are shown in panels A (lanes 2-4,6-8 and 10-11) and B (lanes 2-9). The consistently faster migration of Ad5 E1A-associated p300 proteins from DP5-2 cells loaded in lanes 1, 5 and 9 of panel A precludes the possibility that observed differences in p300

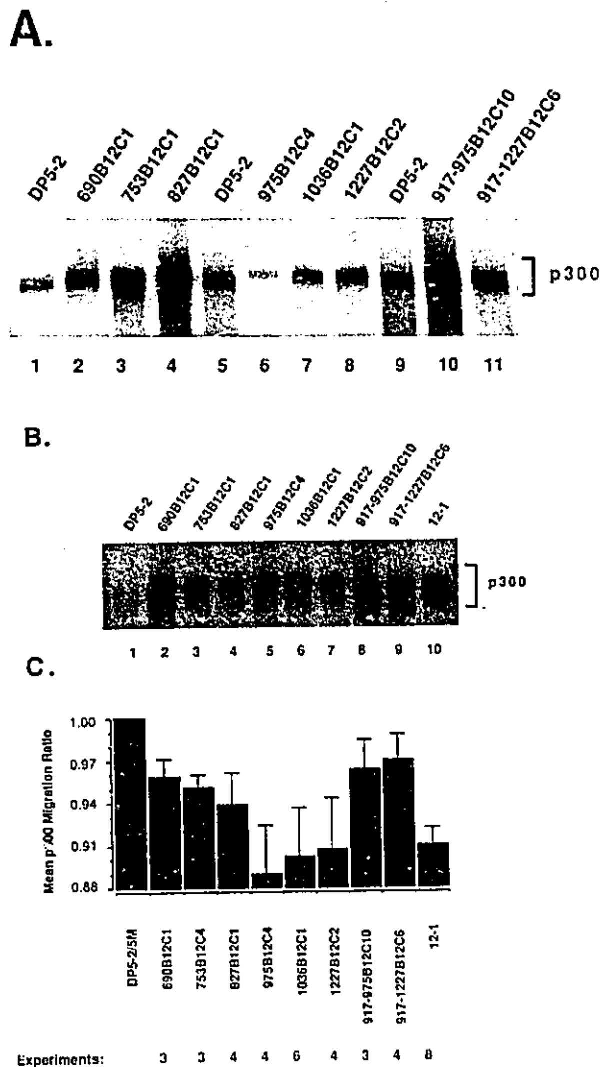

Figure 4. Electrophoretic mobility of p300 decreases as the Ad12 E1A content in hybrid Ad5/12 E1A genes increases. (A) The M73 antibody was used to coimmunoprecipitate p300 from rat cells. Lanes 1, 5 and 9 represent the p300 protein coimmunoprecipitated from the Ad5 E1A-transformed rat cell line, DP5-2 while p300 from hybrid Ad5/12 E1A-transformed rat cells are shown in lanes 2-4, 6-8 and 10-11; (B) α-p300 polyclonal antiserum was used to immunoprecipitate p300 from the DP5-2 (lane 1), 12-1 (lane 10; Ad12 E1A-transformant) and hybrid Ad5/12 E1A-transformed (lanes 2-9) rat cells. p300 proteins shown in panels A and B were analyzed on a 7% SDS-polyacrylamide gel and subsequently visualized by autofluoro-radiography; (C) The average relative mobility of p300 from BRKs transformed by wild-type and hybrid Ad5/12 E1A genes was determined by densitometric analysis. The numbers in parentheses, under the cell lines, represent the number of experiments in which p300 was immunoprecipitated from that cell line. The mean p300 migration ratio was determined according to the following calculation: [(migration distance of p300 found in cells transformed by a hybrid Ad5/12 E1A gene) divided by (migration distance of p300 found in wildtype Ad5 E1A-transformed cell lines, DP5-2 or 5M)].

mobility were due to imperfections of the gel. In panel B, lanes 1 and 10 contain p300 proteins immunoprecipitated from DP5-2 and 12-1 cells respectively. It should be noted that all detectable p300 molecules immunoprecipitated from 12-1 cells (panel B, lane 10) using α-p300 polyclonal antiserum were 'shifted' with respect to p300 immuno-precipitated from DP5-2 cells (panel B, lane 1).

The analysis presented in Fig. 4B and additional gels (not shown) were densitometrically scanned in order to accurately measure the migration distance of the respective p300 proteins in an unbiased manner. Fig. 4C illustrates the mean p300 migration ratio, calculated as the migration distance for p300 in cells transformed by various hybrid Ad5/12 E1A genes divided by the migration distance for p300 in the Ad5 E1A-transformant, DP5-2. The results suggest that retardation in p300 mobility may have occurred in two progressive steps and that maximum retardation appeared to coincide with the presence of the same two regions of Ad12 E1A which were described earlier as influencing tumorigenicity and MHC class I expression.

p300 is differentially phosphorylated in Ad12 E1A compared to Ad5 E1A-transformed rat cells. Since the mobility of proteins in SDS-PAGE is affected by post-translational modifications such as phosphorylation and p300 is known to be a phosphoprotein (50), we decided to determine whether gel retardation of p300 from Ad12 E1A transformants was due to differential phosphorylation. Fig. 5A shows an immunoprecipitation of p300 from the DP5-2 (lane 1) and 12-1 (lane 3) lines using α-p300 polyclonal antiserum. To determine whether the 12-1 and DP5-2 p300 proteins would migrate with equal mobility following phosphatase treatment, we incubated the DP5-2 (lane 2) and 12-1 (lane 4) p300 proteins with λ protein phosphatase. Both phosphatase-treated p300 proteins migrated as doublets exhibiting altered mobilities relative to the p300 species immunoprecipitated from DP5-2 and 12-1 cells. Furthermore, following phosphatase treatment, the p300 mobilities were indistinguishable whether the proteins were derived from cells expressing Ad5 E1A or Ad12 E1A. This suggested that differences in mobility of p300 from Ad12 E1A- or hybrid E1A-transformed rat cells was probably due entirely to differences in phosphorylation.

To study the basis of this gel mobility shift further, we labelled DP5-2 and 12-1 cells with [^{32}P]-orthophosphate in an attempt to generate two-dimensional tryptic map and phosphoaminoacid analyses of the p300 proteins present in Ad5 E1A- and Ad12 E1A-transformants. Several attempts to generate adequately labelled and well resolved tryptic patterns proved unsuccessful despite using as much as 50 mCi of ^{32}P per sample. This analysis was complicated both by the inability to incorporate ^{32}P efficiently into p300 and failure of all but a small fraction of labelled peptides to sufficiently resolve in two dimensions (data not shown). However, sufficient incorporation of ^{32}P into the p300 proteins present in the Ad5 E1A- and Ad12 E1A-transformed rat cells was obtained to permit phosphoamino acid analyses (Fig. 5B). Densitometric analysis of the intensity of ^{32}P incorporation into the serine and threonine profiles shown in Fig. 5B suggested that the ratio of serine:threonine phosphorylation was similar in p300 proteins from Ad5 E1A (3.2:1) and Ad12 E1A- (3.6:1) transformed rat cells. Incorporation into phosphotyrosine residues was not observed. Finally, no differences in the phosphoamino acid profile were detected whether p300 was derived from the Ad5 E1-transformant (DP5-2) or the Ad12 E1-transformant (12-1).

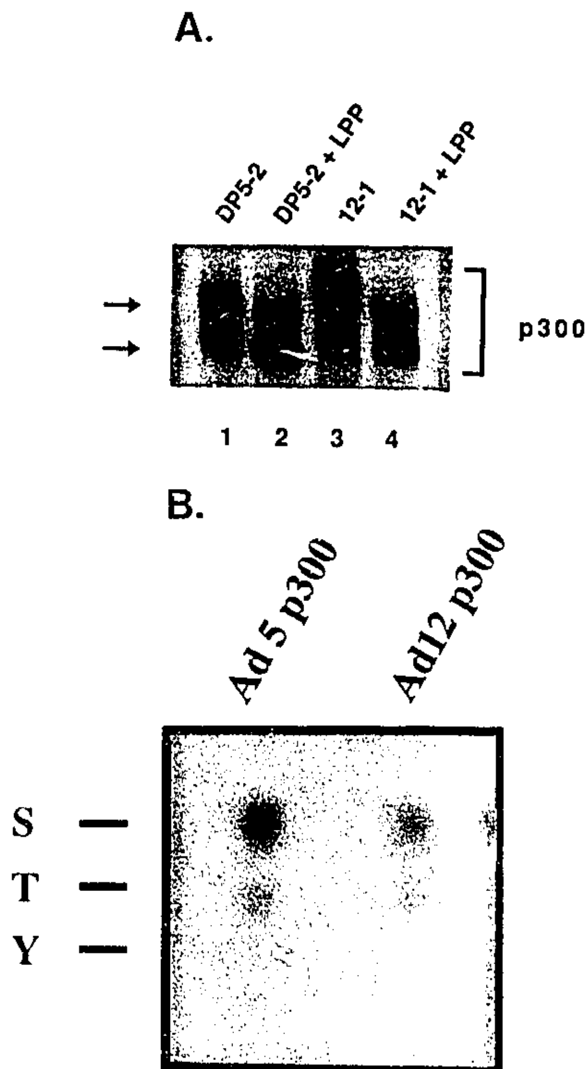

Figure 5. p300 is differentially phosphorylated in Ad12 E1A- compared to Ad5 E1A-transformed cells. (A) α-p300 polyclonal serum was used to immunoprecipitate p300 from the DP5-2 (Ad5 E1A-transformed cell line; lane 1) and 12-1 (Ad12 E1A-transformed cell line; lane 3) cell lines. Lanes 2 and 4 show the result of treating p300 immunoprecipitates from DP5-2 and 12-1 cells with λ protein phosphatase respectively. The doublet bands seen after λ protein phosphatase treatment are indicated by two arrows to the left of the gel; (B) Phosphoamino acid profiles of p300 proteins from DP5-2 (Ad5 p300) and 12-1 (Ad12 p300) cells. The serine, threonine, and tyrosine amino acid residues (shown to the left of the gel) are represented by the letters S, T and Y respectively.

Because our studies of p300 phosphorylation to this point were conducted using a selection of Ad5 E1A- and Ad12 E1A-transformed rat cells, it was important to determine the effect of E1A on the p300 proteins in additional Ad5 E1A- and Ad12 E1A-transformed rat lines as well as in Ad5 E1A- and Ad12 E1A-transformed human lines (Fig. 6). p300 proteins from these cells were immunoprecipitated using α-p300 polyclonal antiserum. Shown in the left part of Fig. 6 are analyses of p300 proteins from rat cells transformed by Ad5 E1A genes [lanes 1 (DP5-2), 3 (5-M) and 5 (DP5-1)] and by Ad12 E1A genes [lanes 2 (12-1), 4 (702-C2), 6 (HABaC1) and 7 (HABaC2)]. As was the case with the p300

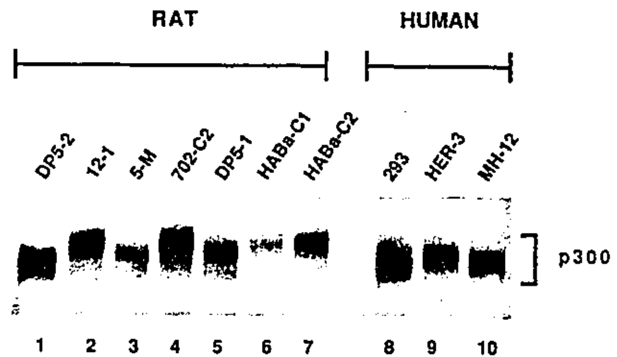

Figure 6. p300 immunoprecipitations from rat and human cells transformed by Ad5 and Ad12 E1A genes. Additional rat cells transformed by Ad5 E1A [lanes 1 (DP5-2), 3 (5-M) and 5 (DP5-1)] and Ad12 E1A genes [lanes 2 (12-1), 4 (702-C2), 6 (HABaC1) and 7 (HABaC2)] are shown on the left, while human cells transformed by Ad5 E1A genes (lane 8 (293) and Ad12 E1A genes (lanes 9 (HER-3) and 10 (MH-12)) are shown on the right. Samples were immunoprecipitated with α-p300 polyclonal serum, analyzed on a 20 cm long 7% SDS-polyacrylamide gel and visualized by autofluoro-radiography.

protein from 12-1 cells, the p300 proteins from 702-C2, HABaC1 and HABaC2 cells also demonstrated a decreased electrophoretic mobility. Human cells transformed by Ad5 E1A genes [lane 8 (293)] and Ad12 E1A genes [lanes 9 (HER-3) and 10 (MH-12)] are shown to the right of the figure. It is apparent that the decrease in mobility of p300 proteins in Ad12 E1A- relative to Ad5 E1A-transformants occurs to a greater extent in rat than in human cells and moreover, any change in the mobility of p300 for the human cells was too small to be determined with certainty.

Discussion

Previously, using rat cells transformed by hybrid Ad5/12 E1A genes, we identified at least two regions of Ad12 E1A which influenced tumorigenicity: amino terminal residues up to amino acid 83 and a region consisting of amino acids 108-147 or 108-193 (20). In this report, we have begun to explore the mechanisms by which these regions influence tumorigenicity by examining their affect on cell surface MHC class I expression and on the interaction between Ad12 E1A and cellular proteins.

While our data suggest that hybrid Ad5/12 E1A, Ad5 E1A and Ad12 E1A proteins associated with cellular proteins with similar affinity, closer examination revealed that the electrophoretic mobility of p300 in SDS-polyacrylamide gels decreased as the Ad12 E1A content in the series of hybrid Ad5/12 E1A-transformants increased. Whether antisera to E1A or p300 were used to immunoprecipitate p300, maximum retardation of p300 mobility was observed when the two regions of Ad12 E1A implicated in conferring tumorigenicity were co-expressed in the same strongly tumorigenic cell lines transformed by the hybrid Ad5/12 E1A constructs (plus Ad12 E1B), p975, p1036 and p1227 as well as in the strongly tumorigenic Ad12 E1-transformant, 12-1. Although one-dimensional electrophoresis did not allow the precise number of p300 forms in Ad12 E1A-transformed rat

cells to be determined, the existence of multiple forms is likely since two-dimensional gel analysis of HeLa cells at different phases of the cell cycle has revealed 4-7 forms of p300 (50). The fact that mobility differences in Ad12 E1A-associated p300 proteins (Ad12 p300) were resolvable in the 300 kilodalton range of an SDS-polyacrylamide gel suggests that a significant post-translational modification may have occurred which was different in cells transformed by Ad12 E1A versus Ad5 E1A.

To this end, we studied the basis of the gel mobility shift exhibited by Ad12 p300 in greater detail through protein phosphatase treatment and attempts to generate ^{32}P-labelled tryptic peptide map and phosphoamino acid profiles. Treatment of immune complexes with λ protein phosphatase suggested that Ad12 p300 was differentially phosphorylated relative to that of Ad5 p300. At least two distinct species of p300 were well resolved following dephosphorylation, but migrated predominantly as a single broad unresolved band prior to phosphatase treatment. Despite several attempts using as much as 50 mCi of [^{32}P] orthophosphate, we were not successful in obtaining sufficiently labelled p300 proteins for resolution of phosphopeptides by two-dimensional tryptic peptide maps. Moreover, the inability to adequately resolve peptides in two-dimensions, likely due to their large size and/or insolubility, further complicated our efforts (data not shown). Phosphoamino acid profiles of Ad5 and Ad12 E1A-associated p300 proteins revealed that both proteins were comparably phosphorylated on serine and threonine residues. Our data not only suggest that Ad12 p300 is differentially phosphorylated, but that phosphorylation of Ad12 p300 is the major cause of its gel mobility shift relative to Ad5 p300. Whether this shift is due to hyperphosphorylation as opposed to phosphorylation at novel sites, could not be concluded from these studies. Although we did not attempt to identify the kinase responsible for phosphorylation of p300, evidence derived from *in vitro* kinase studies suggests that the $p33^{cdk2}$ kinase which indirectly associates with E1A, or the $p34^{cdc2}$ kinase are capable of efficiently phosphorylating p300 (5). Interestingly, this study also demonstrated that the two major Ad5 E1A proteins inhibit phosphorylation of p300, a finding which may suggest that Ad5 E1A and Ad12 E1A may differ in ability to affect kinase activity directed at p300. The possibility that Ad12 E1A, unlike Ad5 E1A has lost the ability to inactivate a phosphatase can not be excluded.

Before determining the role that a differentially phosphorylated p300 protein may play in conferring tumorigenicity to Ad12 E1A-transformed cells, current knowledge of p300 function must be considered. A number of properties of p300 suggest that it is involved in regulation of gene expression. It is a nuclear phosphoprotein, present in a variety of cell types (human, rat, monkey, mouse and mink), exists in various phosphorylated forms in HeLa and 293 (Ad5 E1-transformed human kidney line) cells and is actively phosphorylated in all phases of the cell cycle (50). p300 has not only been shown to possess DNA-binding activity with specific affinity for enhancer motifs (including MHC class I H2TF1 enhancer motif) (34), but has been identified as a component of TATA-binding protein (TBP) complexes (1). Recently, the sequence of p300, derived from cloned cDNA, was shown to contain a so-called bromodomain which is characteristic of transcriptional activators such as the TBP-associated factor, $TAF_{II}250$/CCG1 (13). Experiments using this cDNA not only revealed that p300 could overcome repression of the SV40 transcriptional enhancer by E1A, but suggested that p300 participates in activating this enhancer (13). Moreover, on the basis of sequence similarity, p300 and the CREB-associated CBP nuclear protein, have been proposed to belong to a conserved family of transcriptional co-activators (4). That an E1A/p300 complex may be involved in repression of gene expression is supported by several reports indicating that E1A repression of a variety of viral and tissue specific promoters is dependent on interaction of E1A proteins with p300 (23,34,41,47,48).

Based on evidence above, we suggest that the E1A-p300 complex present in Ad12 E1-transformed cells may be functionally altered in transcriptional regulation compared to its counterpart in Ad5 E1-transformants. This hypothesis is consistent with previous findings from our laboratory which suggest that transformation of primary rodent cells by E1A may not be simply due to sequestration of cellular proteins involved in transcription, but rather that an E1A-cellular protein complex may play a distinct functional role which may differ between Ad serotypes (20,22). The Ad12 E1A gene contains a spacer region between CR2 and CR3 which is not only absent from Ad5 E1A, but which has previously been proposed to influence tumorigenicity (20,42). This 20 amino acid Ad12 E1A spacer region features a stretch of consecutive alanine residues which is characteristic of other transcriptional repressors (20,42). Consequently, it was suggested that this region may repress transcription of cellular genes, such as the class I MHC gene, in a manner which may influence tumorigenicity. In this report, we showed that this region is contained within a region which causes down-regulation of cell surface MHC class I expression. Moreover, we have found that the two Ad12 E1A regions thought to influence tumorigenicity (20), also participate in down-regulation of MHC class I expression.

That an Ad12 E1A-p300 complex may play a role in transcriptional regulation is not without precedent since the amino terminus of Ad12 E1A, which binds p300, appears to mediate repression of class I MHC H-2K^b through the H2TF1 binding motif (26). Moreover, recent evidence from the same laboratory suggests that the Ad12 E1A-p300 protein complex participates in the process of MHC class I down-regulation through interactions with nuclear proteins which bind to a (CAA)n repeated element in the negative regulatory domain of the H-2K^b enhancer (K. Yokoyama, personal communication). Since MHC class I down regulation is a strategy that many persistent and tumorigenic viruses such as Ad12 employ to evade lysis by MHC class I restricted cytotoxic T lymphocytes (35), it is intriguing that differential phosphorylation of the Ad12 E1A-associated p300 protein may play a role in this process.

The possibility that tumorigenicity of Ad-transformed rodent cells requires down-regulation of MHC class I expression is a topic of great debate (17,30). In fact, it has been reported that transfection of an MHC class I gene into a tumorigenic Ad12 E1-transformed BALB/c mouse line (expressing low but recognizable levels of cell surface class I

antigens) enhances rather than abrogates tumorigenicity (40). Failure to find a strict correlation between tumorigenicity and reduced class I expression is likely attributable to differences among the species and strains of transformed cells and host animals, as well as to the failure of Ad12 E1A to consistently down-regulate MHC class I levels below a threshold required for CTL recognition (38,39; this report and Pereira *et al*, submitted). While the Ad12 E1A-p300 complex may mediate MHC class I down-regulation at the level of transcription, this event may not be the sole factor influencing tumorigenicity. Therefore, we suggest that changes in phosphorylation of the Ad E1A-associated p300 protein may alter its activity in transcriptional regulation in a manner which may not only affect cell cycle progression, but may help to explain phenotypic differences (e.g. tumorigenicity) between Ad5 E1- and Ad12 E1-transformed rat cells.

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Contributions to Pereira et al., 1994:

(1) Performed E1A co-immunoprecipitation studies to determine whether the hybrid Ad5/12 E1A proteins differed in their interactions with cellular proteins in a manner which may have correlated with tumorigenicity.

(2) Using anti-p300 antibody in immunoprecipition studies and protein phosphatase studies I compared the relative migration of p300 in the hybrid E1A lines as well as in a variety of rat and human cells transformed by the E1 regions of Ad5 or Ad12.

Conclusions:

In this report, again using the hybrid Ad5/12 E1A- (plus Ad12 E1B) transformants, expression of the same two Ad12 E1A regions involved in tumorigenesis not only correlated with down-regulation of cell surface MHC class I expression, but coincided with the presence of an altered form of the E1A-associated cellular protein, p300, detected as a more slowly migrating species in SDS-polyacrylamide gels. The decreased electrophoretic mobility of p300 from hybrid Ad5/12 E1A- and Ad12 E1A-transformants could be abolished by protein phosphatase treatment suggesting that the change in mobility resulted from differential phosphorylation of p300.

Unpublished Data Related To Pereira et al., 1994

To determine whether the Ad12 E1A-associated p300 protein was hyperphosphorylated with respect to the Ad5 E1A-associated p300 protein, I labelled DP5-2 (Ad5 E1-transformed) and 12-1 (Ad12 E1-transformed) rat cells with [^{32}P]-orthophosphate in an attempt to generate two-dimensional tryptic maps of the p300 proteins in collaboration with Tomas Jelinek (University of Virginia). Several attempts to generate adequately labelled and well resolved tryptic patterns proved unsuccessful despite using as

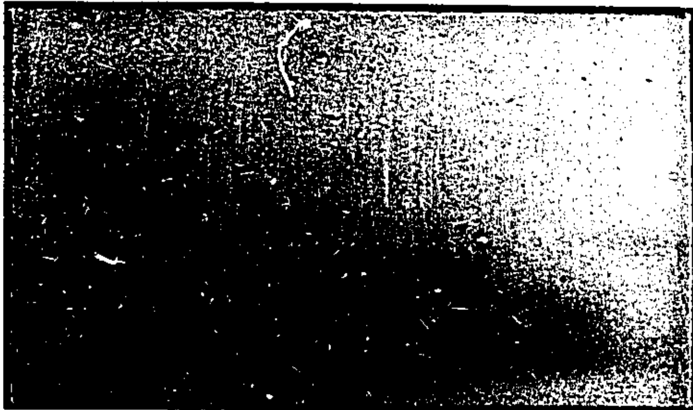
much as 50 mCi of ^{32}P per sample. The analysis was complicated both by the inability to incorporate ^{32}P efficiently into p300 proteins and failure of all but a small fraction of labelled peptides to sufficiently resolve in two dimensions. Fig. 9 represents the best attempt to generate a well resolved and adequately labelled two-dimensional tryptic phosphopeptide maps for the p300 proteins from rat cells transformed by the E1 region of Ad5 and Ad12. In comparison to the Ad5 E1A-associated p300, the Ad12 E1A-associated p300 could not be conclusively shown to be hyperphosphorylated.

Considerable evidence exists suggesting that p300 plays a role in regulating transcription. Since one of these studies reported that p300 possessed intrinsic DNA-binding activity with specific affinity for enhancer motifs such as those which bind NF-κB, I decided to test the possibility that the differentially phosphorylated form of p300 present in Ad12 E1-transformants could competitively inhibit the binding of NF-κB to the MHC class I R1 element thereby accounting for the decreased levels of cell surface class I molecules. For this purpose, standard electrophoretic mobility shift assays (EMSAs) were conducted using synthetic MHC class I R1 oligonucleotides and whole cell extracts of DP5-2 and 12-1 cells (Fig. 10). To determine whether p300 was present in cellular protein-R1 complexes, efforts to supershift these complexes with α-p300 antiserum were attempted. p300 was not present in cellular protein-R1 complexes in either DP5-2 or 12-1 cells. The failure of p300 to be detected in a complex with the R1 (NF-κB binding) oligonucleotide may be genuine, however, since these complexes were resolved in non-denaturing gels, it is likely that given its large size, a p300/R1 complex may not have penetrated the polyacrylamide gel matrix.

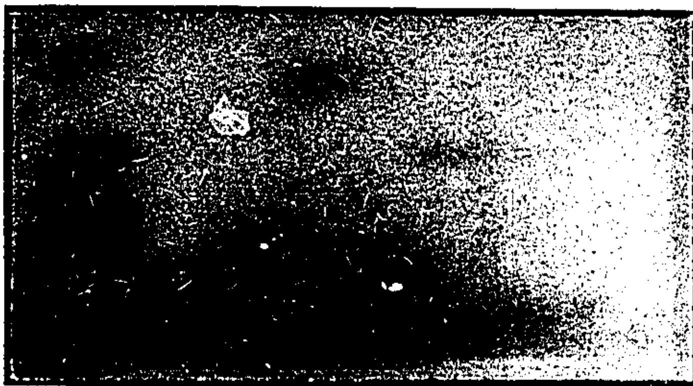
Figure 9: Two-dimensional tryptic phosphopeptide maps of the E1A-associated p300 protein derived from Ad5 E1- (A) and Ad12 E1- (B) transformed Hooded Lister rat cells.

Phosphopeptides (prepared as described in the Materials and Methods section) were spotted at the "origin" of a thin layer chromatography cellulose plate and electrophoresed in the first horizontal dimension from the anode ("+") to the cathode ("-"). Liquid chromatography allowed the separation of phosphopeptides in the second vertical dimension.

A)



B)



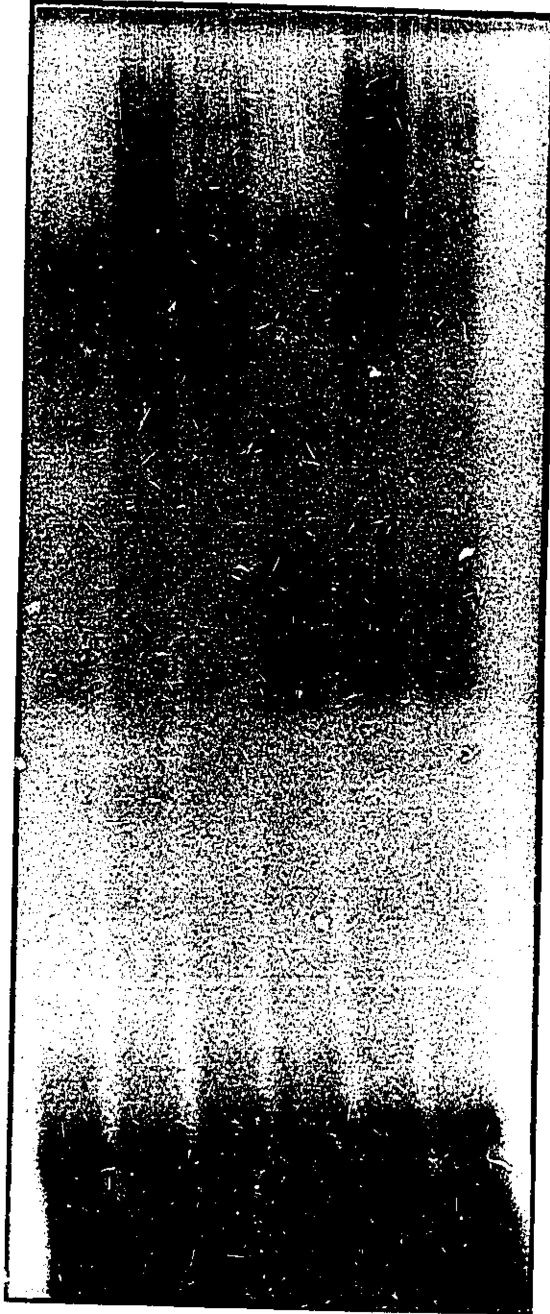
+ -

Origin

Figure 10: Electrophoretic mobility shift assays (EMSAs) using Ad5 E1- and Ad12 E1-transformed Hooded Lister rat cells.

EMSAs were performed using whole cell extracts and synthetic oligonucleotides representing the NF-κB binding site in the R1 element of the H2-K^b MHC class I enhancer. Lanes 1 and 4 represent cellular protein-R1 complexes in DP5-2 (Ad5 E1-transformant) and 12-1 (Ad12 E1-transformant) cells respectively. Lanes 3 and 6 show the same respective complexes incubated with α-p300 antiserum in an attempt to identify p300 as a component of the cellular protein-R1 complexes. Pre-immune serum (PIS) was also incubated with DP5-2 (lane 2) and 12-1 (lane 5) whole cell extract cellular protein/R1 mixtures.

DP5-2 DP5-2 + PIS DP5-2 + α-p300 12-1 12-1 + PIS 12-1 + α-p300



1 2 3 4 5 6

DISCUSSION

During the course of this research, a series of previously described hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed rat cells (hybrids) were characterized to obtain a better understanding of the basis for the different tumorigenic capacities of rodent cells transformed by the E1 regions of Ad5 and Ad12. Regions of Ad12 E1A mediating tumorigenesis were mapped and their ability to serve as CTL epitopes and affect MHC class I expression was examined (results summarized in Fig. 11).

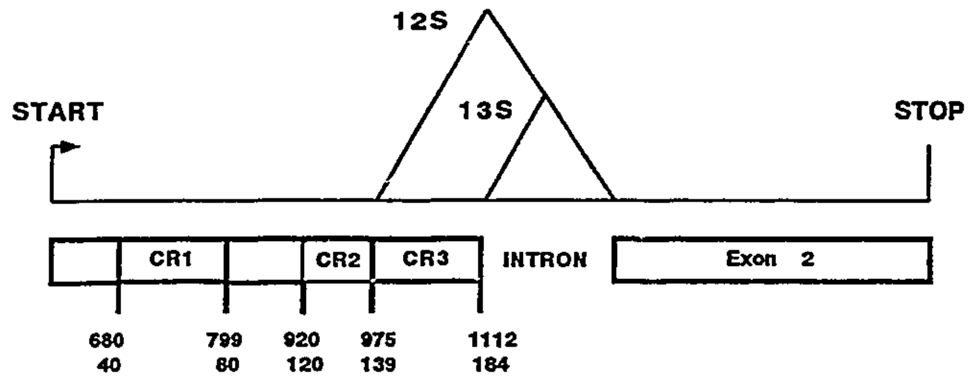
a) Mapping Ad12 E1A Regions Which Mediate Tumorigenesis

At least two regions within the first exon of Ad12 E1A, excluding CR3 (transactivation domain), were identified which mediate tumorigenicity: residues 105-144 and the amino terminal 105 amino acids. A stretch of 20 amino acids (124-144; EQDENGMAHVSASAAAAAADRER) between CR2 and CR3 are present within Ad12 E1A residues 105-144 yet absent from corresponding Ad5 E1A sequences. The possibility that this region contributes to Ad12 E1A-mediated tumorigenesis is supported by the fact that it bears >55% homology to the corresponding region of the highly oncogenic simian Ad7 E1A protein. Intriguingly, a feature of this region is the presence of several consecutive alanine residues which appear to be characteristic of some transcriptional repressors.

b) Effect of Ad12 E1A Regions on MHC Class I Expression

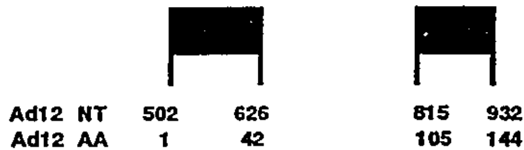
Expression of the Ad12 E1A regions involved in tumorigenesis was found to correlate with reduced cell surface MHC class I levels observed in Ad12 E1-transformed cells. Despite the fact that a reduction in class I expression is thought to allow these cells to

Figure 11: Schematic denoting functional regions in the first exon encoded sequences of Ad5 and Ad12 E1A proteins.
Ad5 and Ad12 E1A protein regions are shown as open and closed boxes respectively. The open reading frame of Ad5 E1A and locations of conserved regions (CRs) are shown atop. Ad12 E1A regions involved in tumor induction (A) and down-regulation of cell surface MHC class I expression (B). Ad5 E1A regions (1 and 2) potentially encoding CTL epitopes (C).



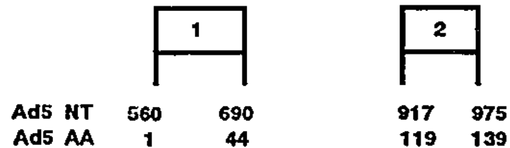
(A)

Ad12 E1A Regions Mediating Tumorigenicity.



(B)

Ad12 E1A Regions Correlating With Down-Regulation of MHC Class I Expression.



(C)

Ad5 E1A Regions Encoding CTL Epitopes.

evade lysis by class I-restricted $CD8^+$ CTLs, all the hybrids, regardless of their oncogenic potential and with the exception of three tumorigenic transformants (975, 1036, and 1227), were found to express sufficient cell surface class I levels for recognition and lysis by allogeneic CTLs. Our data are therefore in agreement with previous studies that failed to find a strict correlation between class I expression, susceptibility to allogeneic CTLs and tumorigenicity among Ad5 E1- and Ad12 E1-transformants (Mellow et al., 1984; Haddada et al., 1986; Shemesh *et al.*, 1991; Shemesh and Ehrlich, 1993). In fact, results from one study suggests that transfection of class I genes into already tumorigenic Ad12-transformed BALB/c mouse cells enhanced rather than abrogated tumorigenicity (Soddu and Lewis, 1992). It should be noted that differences in class I levels among various transformants were not the result of clonal variation following transformation by the hybrid Ad5/12 E1A and Ad12 E1B proteins since two or more independently derived cell lines transformed by the same E1 sequences displayed similar class I levels. Moreover, differences in class I levels between clones could not be attributed to differing levels of E1A expression since comparable E1A levels were detected by Western blot analysis in all transformed cell lines.

c) Ability of Ad E1A Proteins to Serve as CTL Epitopes

Taken together, the relationship between class I down-regulation, susceptibility to allo-CTLs and tumorigenicity suggests that factors in addition to reduced class I expression contribute to the tumorigenic capacities of Ad5 E1- and Ad12 E1-transformed cells. For example, cells transformed by Ad2 or Ad5 E1A are known to be susceptible to NK cells. When the sensitivity of the hybrids was measured, however, susceptibility did not correlate with tumorigenicity and was observed only by transformants expressing insufficient levels of cell surface class I molecules for recognition by allogeneic CTLs. This observation is in agreement with other reports suggesting that cell surface class I expression can interfere with NK cell recognition/lysis (Storkus *et al.*, 1989; Ljunggren and Karre, 1990; Kaufman *et al.*, 1992; Pena and Solona, 1992). Another factor which may contribute to the

tumorigenicity of Ad E1-transformed cells is whether their E1A products serve as CTL epitopes. When used to immunize syngeneic Hooded Lister rats (MHC haplotype C), Ad12 E1-transformed cells (12-1 cells) were unable to generate syngeneic Ad12 E1-specific CTLs despite expressing sufficient cell surface class I levels for recognition by allo-CTLs. It should be noted that Ad12 E1-specific syngeneic CTLs have been generated following *in vitro* stimulation of spleen cells isolated from tumor-bearing Hooded Lister rats injected with Ad12 E1-transformed cells (Raska *et al.*, 1980). However, the percentage specific lysis of target cells, at an effector:target ratio of 400, ranged from approximately 8-30% and was associated with standard errors as great as 15%. This range of lysis is comparable to the lysis observed for the 12-1 line (12%) at a far lower effector:target ratio of 50. Thus, it seems unlikely that Ad12 E1A encodes CTL epitopes in conjunction with class I antigens. The possibility that the inability of these cells to generate CTLs is due to down-regulation of peptide transporter genes, a recent finding in Ad12 transformed cells, cannot be excluded (Rotem-Yehudar *et al.*, 1994). The susceptibility of the hybrids to syngeneic Ad5 E1-specific CTLs, suggested that Ad5 E1A encodes CTL epitopes in two regions: the amino terminal 44 amino acids (region 1) and residues 119-139 (region 2). These are in excellent agreement with previous results which identified CTL epitopes between nucleotides 625-810 (aa 21-83) and 916-974 (aa 119-138) (Routes *et al.*, 1991). Independent expression of either region 1 (present in the tumorigenic p917-975- and p917-1227-transformants) or region 2 (present in the non-tumorigenic p690-, p753-, and p827-transformants) resulted in approximately 40% lysis by these CTLs, suggesting that the epitopes encoded by these regions are equivalent in their ability to elicit CTL lysis *in vitro*. That transformants, containing either region 1 or 2, demonstrate similar sensitivities to lysis by syngeneic Ad5 E1-specific CTLs *in vitro* yet differ in their ability to induce tumors *in vivo* may suggest that the Ad5 E1A epitope encoded by region 2 is dominant to the region 1 epitope in eliciting an *in vivo* CTL response. That a hierarchy

exists among peptides encoded by the same protein for induction of a response by T-cells is not without precedent (for review see Sercarz *et al.*, 1993).

d) Molecular Basis of Ad12 E1A-Mediated MHC Class I Repression

Although MHC class I expression did not strictly correlate with the oncogenic potential of the hybrids, it remains an important factor contributing to tumorigenicity. Thus, the molecular basis of class I down-regulation in Ad12 E1- versus Ad5 E1-transformed cells was investigated. In Ad12 E1-transformed cells, down-regulation of class I expression occurs primarily at the transcriptional level and is mediated by cellular proteins such as NF-κB (inducer) and COUP-TF (repressor) that regulate class I expression by binding the R1 and R2 class I enhancer elements respectively. The binding activities of NF-κB and COUP-TF are respectively decreased and increased in Ad12 E1- versus Ad5 E1-transformed cells. In collaboration with the laboratory of R. P. Ricciardi (University of Pennsylvania), the hybrids were used to show that the same first exon regions of Ad12 E1A involved in tumorigenesis and class I down-regulation also mediate the differential binding activities of NF-κB and COUP-TF in Ad12 E1-transformed rat cells.

At least two possible explanations may account for how sequences within the first exon of Ad12 E1A mediate the down-regulation of NF-κB binding to R1 in Ad12 transformed cells. One explanation is that nuclear translocation of NF-κB is blocked as a result of being retained in the cytoplasm by association with IκBs (Beg & Baldwin, 1993; reviewed by Miyamoto & Verma, 1995). However, this does not appear to be the case since the nuclei of Ad12 transformed rat cells contain levels of NF-κB1-p50 (p50) and RelA (p65) which are similar to those seen in Ad5 transformed rat cells, where decreased NF-κB binding activity is not observed. These results suggest a second possibility, in which a nuclear inhibitor is responsible for blocking the binding of NF-κB to R1 in Ad12 transformed rat cells. Interestingly, a nuclear inhibitor has also been postulated to block NF-κB binding to the HIV-LTR in THP-1 monocytic cells, negatively regulating NF-κB

activity and therefore restricting HIV-1 gene expression (Raziuddin *et al.*, 1991). The existence of nuclear inhibitors of NF-κB might serve as one mechanism by which certain viruses block NF-κB function. In any case, it is unclear how the sequences within the first exon of Ad12 E1A are able to mediate the differential binding activities of NF-κB and COUP-TF. Future studies should not only reveal whether these E1A-mediated effects are regulated independently, but whether the DNA binding activities of COUP-TF and NF-κB are influenced by their potential to directly interact with one another. In this context, it is interesting that a glucocorticoid nuclear hormone receptor has been implicated in repressing the ability of NF-κB to activate gene expression by preventing its binding (Ray & Prefontaine, 1994; Mukaida *et al.*, 1994; Scheinman *et al.*, 1995).

During the course of this study, other evidence was proposed suggesting that diminished NF-κB binding activity in Ad12 E1-transformed rat cells is due to an interference in the processing of NF-κB1-p105 to NF-κB1-p50, resulting in significantly reduced levels of NF-κB1-p50 (Schouten *et al.*, 1995). This is in contradiction with my evidence which clearly shows that NF-κB1-p50 and NF-κB1-p105 (precursor of NF-κB1-p50) are present at similar levels in Ad12 E1- and Ad5 E1-transformed rat cells. Moreover, the notion that processing of NF-κB1-p105 to NF-κB1-p50 is defective in Ad12 E1 transformed rat cells is not consistent with the finding that steady-state levels of NF-κB1-p50 are comparable in nuclear and whole cell extracts prepared from Ad12 E1- and Ad5 E1-transformed rat cells. While the discrepancy between our data and the findings of Schouten *et al.* seem unexpected in light of the fact that the E1 transformed cells examined in both cases were derived from primary rat kidney cells, it may be explained on the basis of differences in the specificity of the anti-NF-κB1-p50 antibodies used. The specificity of the 1157 anti-NF-κB1-p50 antibody, used here, was confirmed using cell extracts from NF-κB1-p50 knock-out mice.

e) Do Ad12 E1A-Associated Proteins Contribute to Tumorigenesis ?

Differences in the tumorigenicity of Ad5 E1- and Ad12 E1-transformants may be explained by differences in the ability of their E1A proteins to associate with cellular proteins. Although E1A co-immunoprecipitation studies revealed that the affinity of the common Ad5 E1A-associated cellular proteins p300, p107, and p105 was similar regardless of whether E1A proteins were hybrid or wild type, a slower migrating form(s) of p300 (in SDS-polyacrylamide gels) was observed in hybrid Ad5/12 E1A- (plus Ad12 E1B) transformed cells. Through protein phosphatase treatment of immune complexes, it was determined that the Ad12 E1A-associated p300 protein (Ad12 p300) was differentially phosphorylated compared to that of Ad5 p300. Whether Ad12 p300 was hyper-phosphorylated compared to Ad5 p300 could not be determined despite several attempts to generate adequately resolved two-dimensional tryptic phosphopeptide maps using as much as 50 mCi of [^{32}P] orthophosphate. Although the kinase responsible for phosphorylation of p300 proteins was not determined, evidence derived from *in vitro* kinase studies suggests that the p33^{cdk2} kinase which indirectly associates with E1A, or the p34^{cdc2} kinase, are capable of efficiently phosphorylating p300 (Banerjee et al., 1994). Interestingly, Banerjee and colleagues also demonstrated that the two major Ad5 E1A proteins inhibit phosphorylation of p300, a finding which may suggest that Ad5 E1A and Ad12 E1A differ in their ability to affect kinase activity directed at p300. That Ad12 E1A, unlike Ad5 E1A, has lost the ability to inactivate a phosphatase can not be excluded.

The possibility that the differentially phosphorylated Ad12 p300 protein may contribute to the tumorigenic capacity of Ad12 E1-transformed cells by modulating MHC class I expression is likely since expression of the Ad12 E1A regions implicated in tumorigenesis, class I down-regulation, and differential binding of NF-κB and COUP-TF to the class I enhancer, correlate with differential phosphorylation of p300. Since a significant body of data now exists implicating a role for p300 in transcriptional regulation, namely that p300 can bind DNA with affinity for sequences related to enhancer elements

recognized by NF-κB, the possibility that Ad12 p300 interferes with class I transcription directly by competitively inhibiting binding of NF-κB to the class I enhancer, was tested in electrophoetic mobility shift assays with inconclusive results. Since this work was conducted, other evidence from the laboratory of Yokoyama has implicated the Ad12 p300 protein in down-regulation of class I repression: 1) the amino terminus of Ad12 E1A, which binds p300, may mediate repression of class I MHC H-2K^b through the R1 element (Katoh et al., 1990); 2) the Ad12 E1A-p300 protein complex participates in the process of MHC class I down-regulation through interactions with nuclear proteins that bind to a (CAA)n repeated element in a negative regulatory domain of the H-2K^b enhancer (K. Yokoyama, personal communication). In the future, efforts aimed at determining whether p300 associates with NF-κB and whether this interaction differs in Ad5 E1- and Ad12 E1-transformants may shed light on the mechanisms which govern the differential oncogenic potentials of these cells.

APPENDIX

Attempts to Construct an Infectious Ad12 Genomic Plasmid

Introduction

During the process of Ad replication inverted terminal repeats (ITRs), present at both ends of the genome, serve as origins for viral DNA replication which proceeds via a semi-conservative mechanism (see Horwitz, 1990). A model has been proposed explaining the manner in which the displaced DNA strand is duplicated (see Tooze, 1980; Kelly, 1984). Replication of the displaced DNA strand is thought to initiate following its circularization due to hybridization of ITR sequences which has been proposed to generate a panhandle structure. Circularization of the Ad genome has been observed in Ad5-infected HeLa and primary BRK cells where a significant number of linear double stranded DNA molecules are covalently closed (Ruben et al., 1983). Subsequently, it was shown that these circles could not only be cloned as plasmids which replicate in bacteria, but that they could generate infectious virus following transfection of human cells (Graham, 1984).

Since their discovery, these infectious Ad5 genomic plasmids have allowed the creation of many recombinant viruses carrying mutations of E1 and E3 as well as foreign genes which were rescued by homologous recombination between Ad5 genomic plasmid sequences and Ad5 shuttle plasmid sequences harboring mutations and foreign genes. Using this tecnnology a large number of recombinant Ads have been generated and widely applied to vaccine, expression, and gene therapy protocols (for review a review dealing with the construction and characterization of Ad vectors, see Berkner, 1992; Hitt et al., 1994, 1995; Graham and Prevec, 1995).

Objective

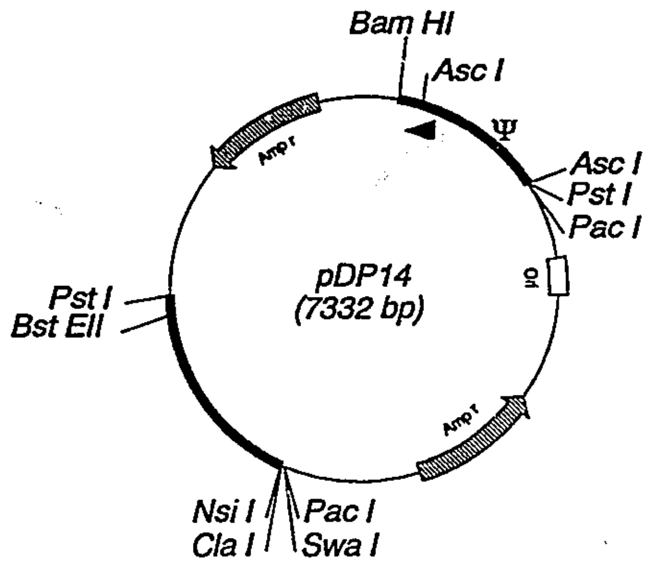
Once regions of Ad12 E1A mediating tumorigenesis were identified, the affect of these regions on the ability of the Ad12 virus to influence tumorigenicity would require the creation of mutant Ad12 viruses. One way to accomplish this would be by digesting the viral genome once near the left end and ligating the large fragment generated to plasmid DNA containing mutated E1A sequences (Stow, 1981). To considerably simplify this process, I attempted to create an Ad12 genomic plasmid. If successful, this plasmid, like its Ad5 counterpart, could be used not only to rescue genomic mutations, but to generate recombinant viruses expressing foreign genes.

Experimental

Creating an infectious Ad12 genomic plasmid capable of replicating in bacteria required the construction of a recombinant Ad, designated Ad12 Amp^r, harboring an ampicillin resistance (Amp^r) gene and bacterial origin of replication (Ori). This task was accomplished by creating pDP14, an Ad12 E1-encoding plasmid in which nucleotides (nts) 972-2236 were replaced with pDP13, a pUC19 derived plasmid containing an Amp^r gene, Ori, and a unique Asc I restriction enzyme site. By virtue of a Bst EII (Ad12 nt 3443) site, which is unique to the Ad12 genome, and a Bam HI site corresponding to the extreme left end of the Ad12 genome, this Bam HI/BstEII fragment was liberated from pDP14 and ligated to the large BstEII Ad12 viral DNA fragment, thereby creating a recombinant viral DNA molecule containing an Amp^r gene and Ori (Fig. 12, step 1). Since the Amp^r gene and Ori substitute for E1 sequences (including E1A) which are essential for viral replication, Ad12 Amp^r was propagated in human HER3 cells which constitutively express Ad12 E1. 293 cells, which constitutively express Ad5 E1, were also used to propagate Ad12 Amp^r since Ad5 E1A complements Ad12 E1A function and vice versa. Next, primary BRK cells were infected by Ad12 Amp^r to generate covalently closed circular viral

Figure 12: Strategy to create an infectious Ad12 genomic plasmid using primary BRK cells.

This strategy involves 3 steps (numbered). Viral DNA sequences are depicted as closed lines. Arrow heads represent the inverted terminal repeats (ITRs) present at the ends of the Ad12 genome and the Ψ symbolizes the location of Ad12 viral packaging sequences. Open and hatched boxes, present in plasmid and viral DNA molecules, represent bacterial origin of replication (Ori) and ampicillin resistance (Amp^r) gene sequences respectively.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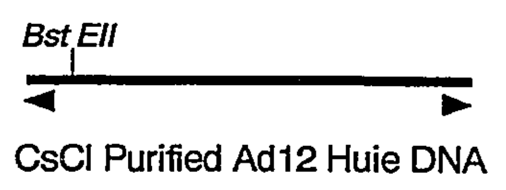
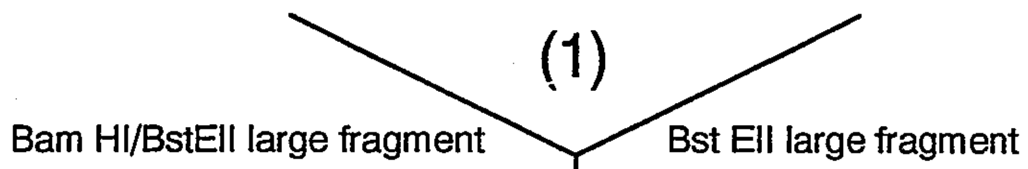
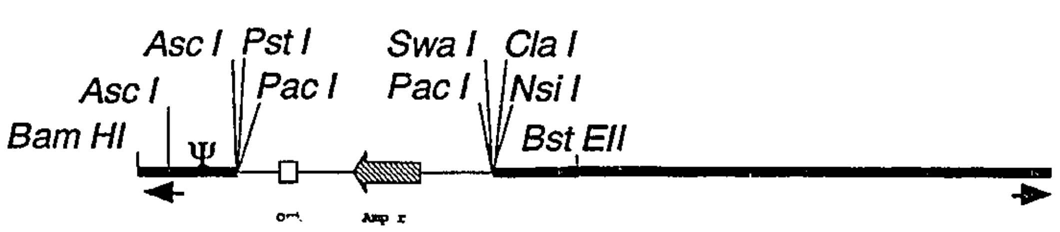
Asc I
Ψ
Asc I
Pst I
Pac I
ori
pDP14
(7332 bp)
Pst I
Bst EII
Amp r
Amp r
Nsi I
Cla I
Pac I
Swa I


Bst EII
CsCl Purified Ad12 Huie DNA


(1)
Bam HI/BstEII large fragment
Bst EII large fragment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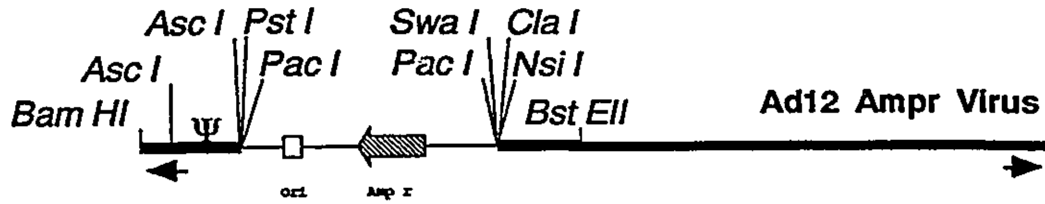
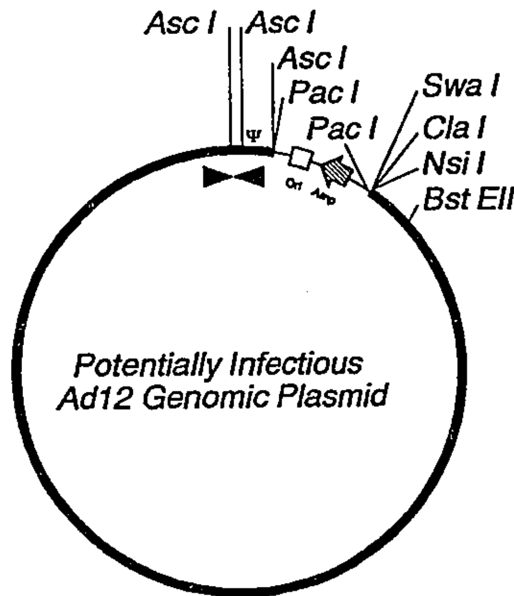
Pst I
Swa I
Cla I
Asc I
Pac I
Pac I
Nsi I
Bam HI
Ψ
Bst EII



(2) Infect Primary BRK Cells to Circularize Genome



(3)

Transform Bacteria With Ad12 Ampr-Infected BRK DNA
Containing Potentially Infectious Ad12 Genomic Plasmid

DNA molecules (Fig. 12, step 2). Transformation of bacteria with DNA isolated from infected BRK cells resulted in colonies derived from cells transformed by these covalently closed circular viral DNA molecules since they carried an Amp^r gene and Ori (Fig. 12, step 3). Due to their relatively large size (approximately 36 000 bp) these Ad12 genomic plasmids are prone to rearrangements. Therefore, to determine whether colonies were transformed by intact Ad12 genomic plasmids, plasmid DNA was screened by digestion with PvuII which cleaves the Ad12 genome many times. Following this round of screening, potential candidates were screened by restriction with Asc I which yields a 292 bp fragment representing intact ITRs. The integrity of the ITRs is crucial in determining whether these Ad12 genomic plasmids will be infectious following transfection of complementing HER3 or 293 cells. In total, 308 colonies were obtained following transformation of bacteria with Ad12 Amp^r infected BRK DNA, 30 potential Ad12 genomic plasmids existed after screening with PvuII and 7 remained following Asc I screening (see Table 5). To accurately assess the integrity of the ITRs, the ITR junctions of these 7 clones were sequenced. pPSG.1 (Fig 13B) exhibited the most nearly intact ITR junction but had 90 bp missing from the left ITR and 7 bp from the right. In addition, a 7 and 10 bp duplication/insertion was present between the omitted ITR sequences. That pPSG.1 was not infectious following transfection of HER3 and 293 cells was not unexpected since studies using Ad5 genomic plasmids have demonstrated that nearly intact ITRs (lacking no more than about 15-20 bp from one end and 2 to 3 bp from the other end) are required for infectivity (Graham, 1984; A. Bett, personal communication).

Because attempts to generate an infectious Ad12 genomic plasmid were unsuccessful following the above strategy, a second strategy was devised to replace the ITR junction present in pPSG.1 with a pFG140-like ITR junction, thereby creating the hybrid junction which would ultimately be present in the infectious Ad12 genomic plasmid, pIG12 (Fig. 13E). The pFG140-like ITR junction was chosen since pFG140 is a stable and highly infectious Ad5 genomic plasmid (Graham, 1984). The creation of pIG12 from

Table 5: **Ad12 genomic plasmids screened for intact ITR junctions following transformation of bacteria with DNA isolated from Ad12 Amp^r-infected BRK cells.**

Experiment‡	Colonies	PvuII Clones*	Asc I Clones*
1	25	3	0
2	88	15	5
3	6	1	0
4	36	1	0
5	24	0	0
6	11	3	0
7	21	2	1
8	25	0	0
9	12	0	0
10	13	2	0
11	47	3	1
Totals:	308	30	7

‡ Eleven experiments were conducted in which the DH5-α and SURE strains of *E. coli* were transformed with DNA isolated from Ad12 Amp^r infected BRK cells.

* Colonies were screened with the Pvu II and Asc I restriction enzymes to respectively determine whether Ad12 genomic clones were free from rearrangement and carrying potentially intact ITR junctions.

Figure 13: ITR sequences from viral genomes and genomic plasmids of Ad5 and Ad12.

Sequence of the ITRs present at the ends of the Ad12 and Ad5 viral DNA molecules are shown in **A** and **C** respectively. **B** and **D** respectively depict the sequences of the ITR junctions present in the non-infectious Ad12 genomic plasmid, pPSG.1, and the infectious Ad5 genomic plasmid, pFG140. Section **E** illustrates the ITR junction of pIG12, a potentially infectious Ad12 genomic plasmid. The ITR junction present in pIG12 contains ITR sequences from pFG140 (not underlined) placed in a background of Ad12 ITR nucleotides (underlined). A Cla I restriction site is shown in bold type.

```
                 -30         -20         -10                          10          20          30

A)  Ad12     ACTAGTCCAGTATAAGGTATATTATTGATGATG  CATCATCAATAATATACCTTATACTGGACTAGT
             TGATCAGGTCATATTCCATATAATAACTACTAC  GTAGTAGTTATTATATGGAATATGACCTGATCA

B)  pPSG.1   ACTAGTCCAGTATAAGGTATATTAAA ATTATATATATTATTA (DELETION TO +90)
             TGATCAGGTCATATTCCATATAATAA TAATATATATAATAAT
                                  ------>  <-------
                                  ========>        ========>

C)  Ad5      TTCAATCCAAAATAAGGTATATTATTGATGATG  CATCATCAATAATATACCTTATTTTGGATTGAA
             AAGTTAGGTTTTATTCCATATAATAACTACTAC  GTAGTAGTTATTATATGGAATAAAACCTAACTT

D)  pFG140   TTCAATCCAAAATAAGGTATATT                CATCAATAATATACCTTATTTTGGATTGAA
             AAGTTAGGTTTTATTCCATATAA                GTAGTTATTATATGGAATAAAACCTAACTT

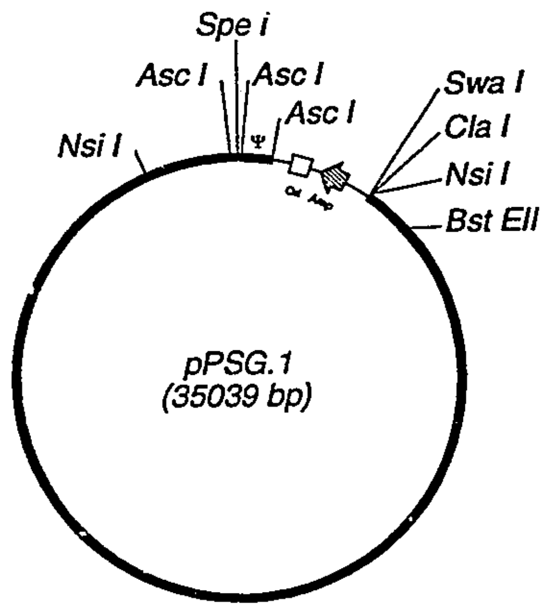
E)  pIG12    ACTAGTCCAGTATAAGGTATATTATCGAT          CATCAATAATATACCTTATACTGGACTAGT
             TGATCAGGTCATATTCCATATAATAGCTA          GTAGTTATTATATGGAATAAGACCTGATCA

                 -30         -20                              10          20          30
```

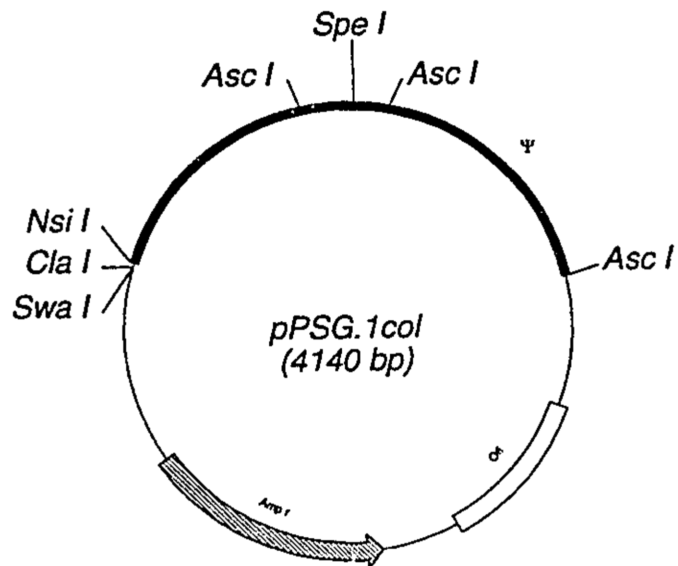
pPSG.1 was divided into 7 steps (Fig 14). First, a collapsed more manageable version of pPSG.1, pPSG.1col was constructed following an Nsi I digestion/re-ligation. Second, pS2 was constructed when a Spe I/Asc I fragment corresponding to nt 28-146 was excised from pPSG.1col, purified, and ligated to the large Spe I/Asc I fragment of pS1, a pNEB193 derived plasmid carrying an Spe I restricition enzyme site. Third, synthetic DNA oligonucleotides, representing a pFG140-like ITR junction between two Spe I sites, were generated, annealed, and inserted into the SpeI site present in pS2, resulting in pS2J. Fourth, the kanamycin resistance (Kan^r) gene present in pABS.3 was liberated after restriction by Swa I and ligated to the cleaved Swa I site present in pS2J within the pFG140-like ITR junction sequences to give rise to pS2JK. Fifth, the pS2JK Spe I Kan^r fragment was inserted into the Spe I site of pPSG.1col to generate p12JK1. Sixth, p12JK2 was created when the large Spe I/Pst I fragment of pS2, which contains a majority of the right Ad12 ITR, was joined to the Pst I, partial Spe I fragment of p12JK1 which carries a Kan^r gene followed by the left 972 nucleotides of Ad12. Seventh, a p12JK1 partial Asc I fragment harboring Ad12 nt -146 to 972 (encompassing the pFG140-like ITR junction divided by a Kan^r gene) was ligated to the large Asc I fragment of pPSG.1, thereby creating pPSG.2. Finally, pIG12, a pPSG.2 plasmid lacking the Kan^r gene but possessing a pFG140-like ITR junction should have been generated when the two largest ClaI fragments of pPSG.2 (each possessing an ITR at the Cla I site interface) were ligated to one another. Out of 80 transformed bacterial colonies obtained following step number 8 of the above strategy, none of the 28 potential candidates contained the ITRs facing one another despite the fact that the *E. coli* SURE strain, which can accomodate the replication of plasmids possessing unstable ITR structures, was used in transformations. When the large Bst EII/Cla I fragment of pPSG.2 was directionally ligated to a Bst EII, partial Cla I fragment of pPSG.2 (harboring the Ampr gene, Ori, and left pFG140-like ITR) and used to transform SURE cells, no colonies were obtained despite several attempts. If pIG12 could have been generated and found to be infectious in 293 and HER3 cells, the final step

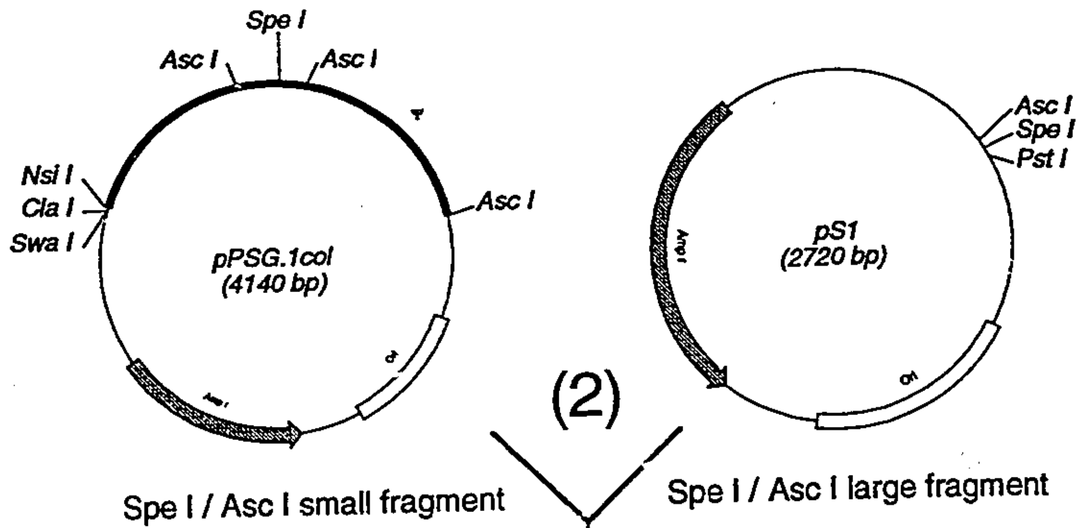
Figure 14: Strategy to create pIG12, an Ad12 genomic plasmid carrying a pFG140-like ITR junction.

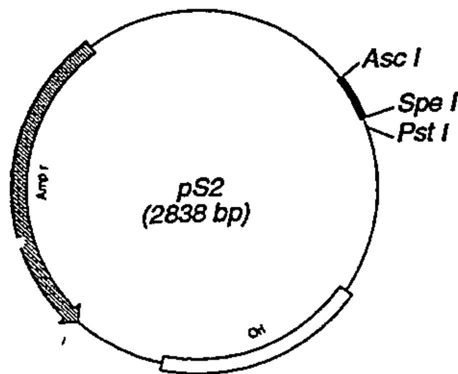
This strategy involves 8 steps (numbered) aimed at modifying the non-infectious Ad12 genomic plasmid, pPSG.1, by creating a synthetic pFG140-like ITR junction. In theory, the Ad12 genomic plasmid resulting from this strategy, pIG12, should be capable of generating virus following transfection of 293 or HER3 cells. Viral DNA sequences are depicted as closed lines. Open, hatched, and bricked boxes represent the bacterial origin of replication (Ori) and ampicillin resistance (Amp^r) and kanamycin resistance gene sequences respectively. The Ψ symbol shows the location of Ad12 viral packaging sequences.



(1) Complete Nsi I Digestion, Ligation

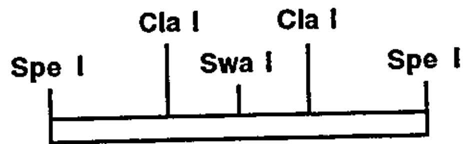


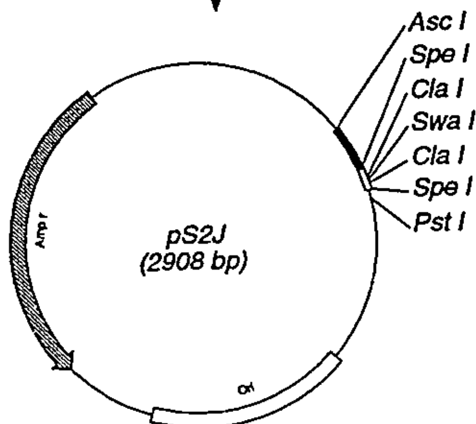


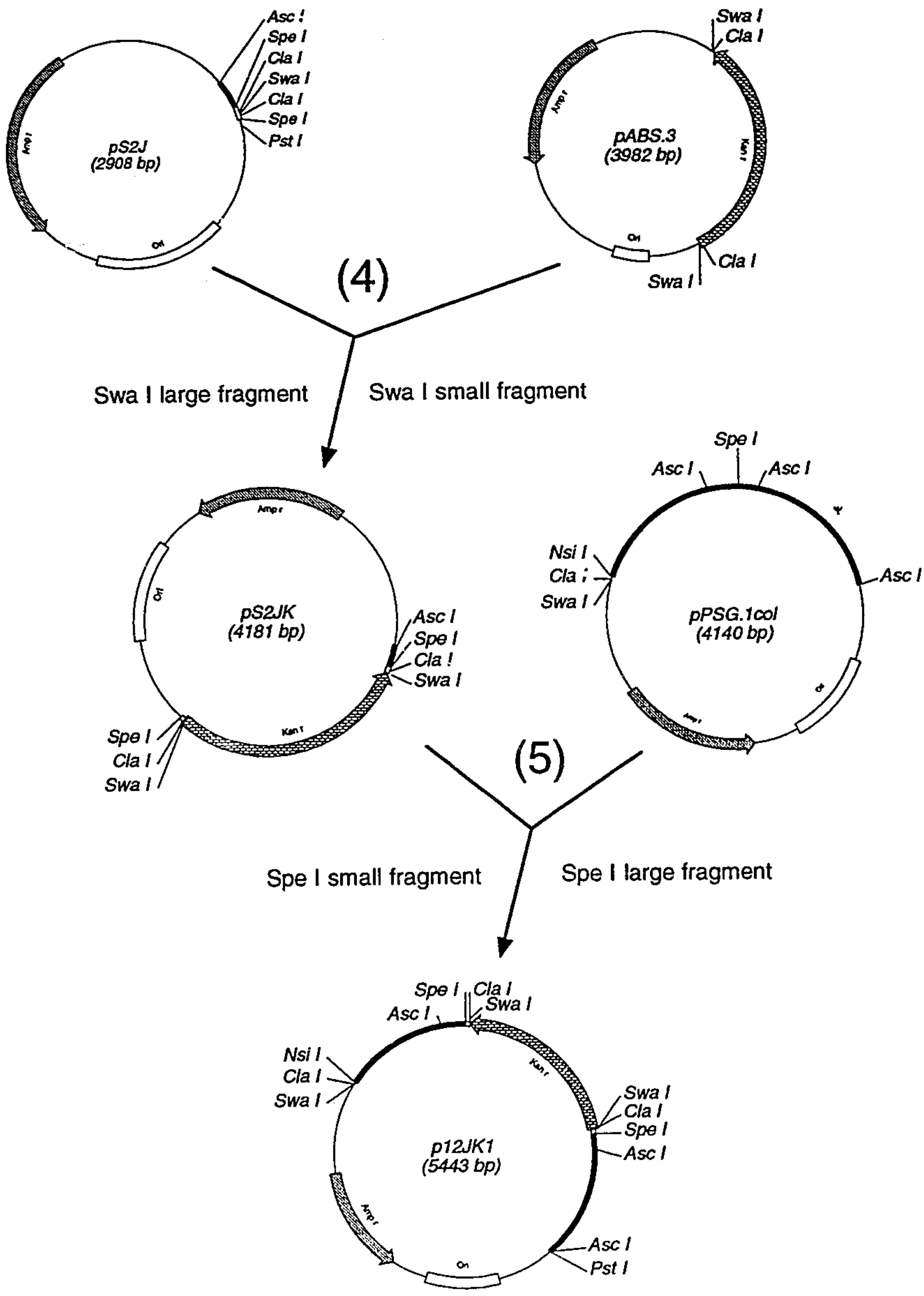


Spe I Digestion and Ligation of Synthetic pFG140-Like Junction:

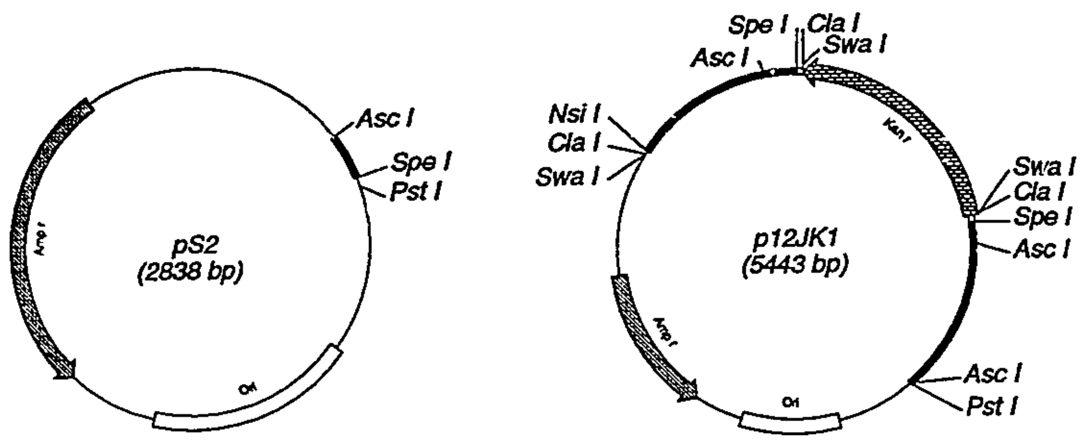
(3)







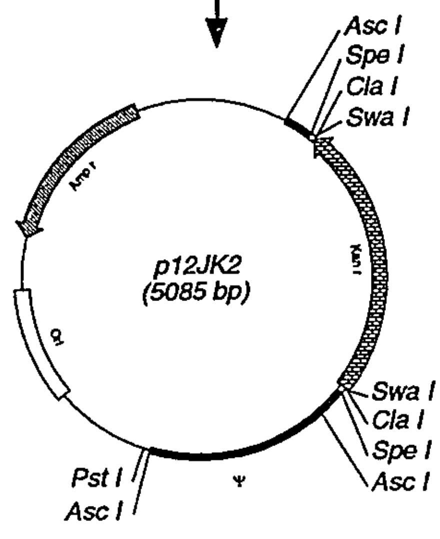
Asc I
Spe I
Cla I
Swa I
Cla I
Spe I
Pst I
pS2J
(2908 bp)
Swa I
Cla I
pABS.3
(3982 bp)
Cla I
Swa I
(4)
Swa I large fragment
Swa I small fragment
pS2JK
(4181 bp)
Asc I
Spe I
Cla I
Swa I
Spe I
Cla I
Swa I
Spe I
Asc I
Asc I
Nsi I
Cla I
Swa I
Asc I
pPSG.1col
(4140 bp)
(5)
Spe I small fragment
Spe I large fragment
Spe I
Cla I
Swa I
Asc I
Nsi I
Cla I
Swa I
Swa I
Cla I
Spe I
Asc I
p12JK1
(5443 bp)
Asc I
Pst I

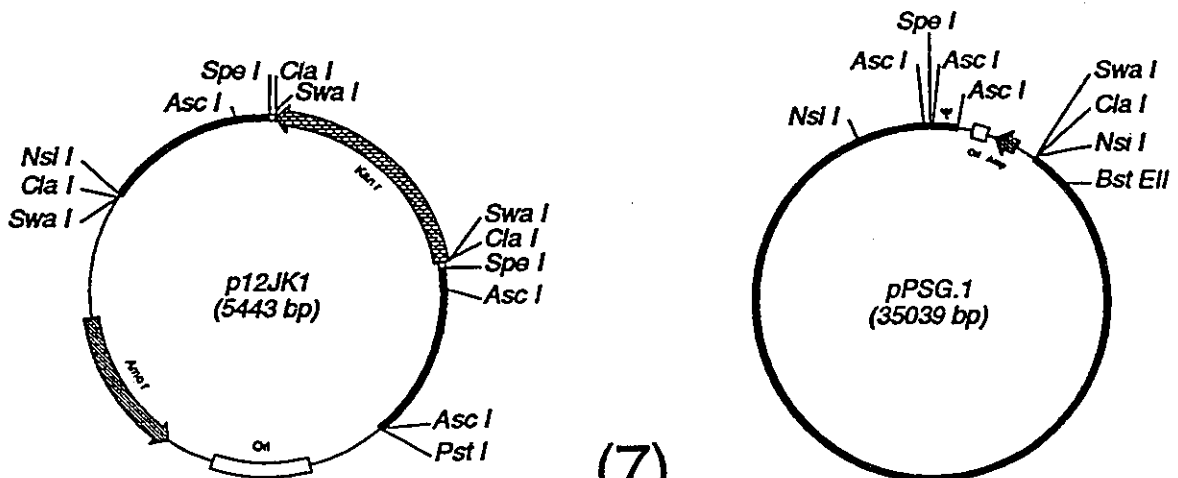


(6)

Spe I / Pst I large fragment

Pst I, Partial Spe I fragment

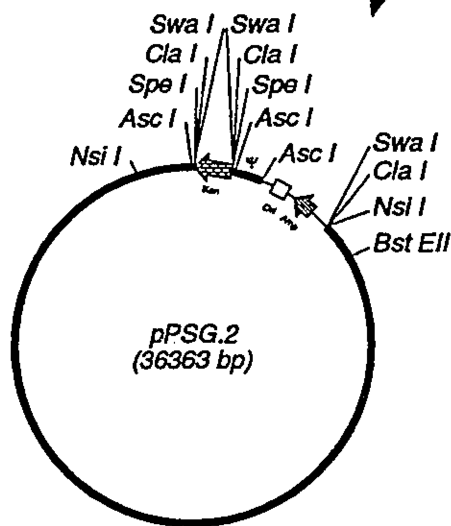


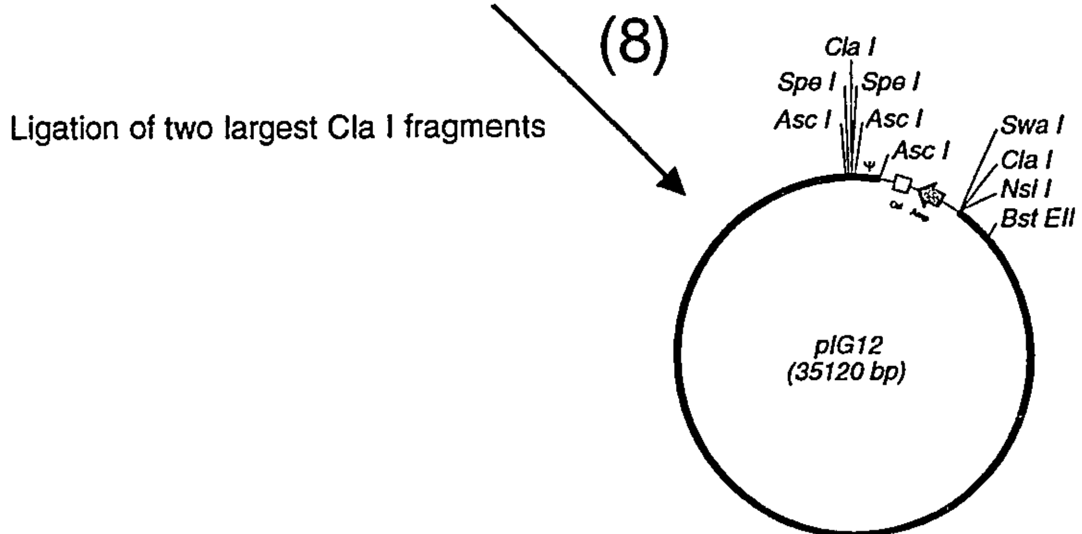


(7)

Partial Asc I fragment

Complete Asc I large fragment





in the strategy would have been to create a non-infectious genomic plasmid following removal of the Asc I fragment (nt 146-972) harboring the Ad12 viral packaging sequence (Ψ). Once constructed this plasmid would have been tested for its ability to rescue mutations of E1A as well as foreign genes following cotransfection and homologous recombination of 293 or HER3 cells. The resulting recombinant viruses could have been used in protein expression and biochemistry studies as well as for vaccine and perhaps gene therapy protocols.

Conclusions

Attempts to generate infectious Ad12 genomic plasmids were unsuccessful. Although circularization of the Ad12 genome did occur in Ad12 Amp^r-infected BRK cells, some of a large number of genomic plasmids (308) screened following transformation of bacteria (DH5-α and SURE strains of *E. coli*) with DNA isolated from these cells did not possess ITR junctions sufficiently intact to ensure infectivity. The apparent lack of stability of the Ad12 ITR in bacteria may be due to its size (163 bp) which is among the largest of human serotypes. Interestingly, the Ad5 ITR junction, which is 103 bp in length, is stable in infectious Ad5 genomic plasmids when only a few bp are deleted from one or both ends of the viral genome.

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