# GENETIC ANALYSIS OF THE TUMORIGENIC PROPERTIES OF HUMAN ADENOVIRUS SEROTYPES 5 AND 12

By

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# TUMORIGENICITY OF HUMAN ADENOVIRUS SEROTYPES 5 AND 12

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# ABSTRACT

Different serotypes of human adenovirus (Ad) have differing oncogenic potentials in rodents. These properties include the resistance of cells transformed or infected by oncogenic serotypes to lysis by natural killer cells, and the suppression of the expression of the class I major histocompatibility complex molecule in oncogenically transformed cells. Two commonly studied Ad serotypes are Ad 5 (non-oncogenic) and Ad 12 (oncogenic). The reasons for their differing oncogenicities are not fully understood.

The purpose of this thesis project was the construction of hybrid Ad 5/Ad 12 early region 1 (E1) plasmids and viruses in order to identify sequences which affect the tumorigenic phenotype. Hybrids were constructed by intra-plasmid homologous recombination in *Escherichia coli*, and rescued into infectious Ad 5 based virus. With one exception, all viruses with hybrid E1A regions were able to replicate efficiently in HeLa cells. The exception, T1036, contained a hybrid E1A transactivation region. Viruses with Ad 12 E1B sequences were impaired for replication in HeLa cells and MH12C2 cells, which express Ad 12 E1 proteins, suggesting that Ad 12 E1B cannot support the replication of the Ad 5 genome.

When tested for their ability to transform primary baby rat kidney cells, hybrid E1 plasmids showed two discreet of transforming efficiencies, based on their sequence compositions. The amino terminus and conserved region 2 of E1A were found

to colocalize with regions involved in transforming efficiency, but no differences were found in the abilities of these sequences to bind to cellular proteins p105Rb, p107, and p300. This suggested that transformation involves functions of E1A in addition to the binding of cellular proteins.

The tumorigenicity of hybrid E1 plasmids was evaluated, and was shown to involve a region of Ad 12 E1A between conserved regions 2 and 3. This region, when introduced into Ad 5 E1A, could impart a low degree of tumorigenicity to the new construct. This suggests that while the region identified plays a role in the oncogenicity of Ad 12, it is not the only factor which influences the differential degrees of oncogenicity between Ad 5 and Ad 12.

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# INTRODUCTION

Adenoviruses (Ad) were initially identified in the 1950s (Rowe *et al.*, 1953; Hillcman and Werner, 1954), and were quickly implicated in the etiology of acute respiratory disease (Ginsberg *et al.*, 1954). However, the observation that some Ad serotypes were able to induce tumors when injected into newborn rodents (Trentin *et al.*, 1962; Huebner *et al.*, 1962) changed the direction of research on adenoviruses. Extensive studies were carried out in subsequent years, to study the mechanisms of tumor induction by adenoviruses, and to assess what role, if any, adenoviruses played in the etiology of human cancer. To date, no report has linked adenoviruses to naturally occurring malignancies of any kind (Gilden *et al.*, 1970; McAllister *et al.*, 1972; Graham, 1984), but the degree of oncogenicity of different Ad serotypes in experimental systems was found to vary, from non-oncogenic to highly oncogenic (reviewed by Huebner, 1967; Trentin *et al.*, 1968; Green, 1970). Despite these varying degrees of oncogenicity, it became clear that all characterized serotypes were able to transform cultured primary cells, inducing many of the morphological and proliferative properties of cancer cells, provided the primary cells were non-permissive or semi-permissive for the growth of the virus (reviewed by Graham, 1984). These observations have inspired an extensive body of research using adenoviruses as a model for oncogenic transformation in general, and

the growth of tumor cells *in vivo*.

## A. Adenovirus Structure

### 1. Virion structure.

Human adenoviruses are icosahedral in shape, and approximately 75 nm in diameter. Each virion contains at least 11 different viral polypeptides and a linear double stranded DNA molecule approximately 36,000 base-pairs (bp) in length (reviewed by Nermut, 1984). The major structural component of the virion capsid is hexon, a protein of 108 kilodaltons (K). Trimers of hexon are associated in planar sheets each containing 9 hexon trimers (groups of nine, or GON), which in combination with unaggregated hexon trimers known as peripentonal hexons, penton base (85K), and fiber (62K) comprise the external shell of the viral capsid. Trimers of penton base molecules occupy each of the 12 vertices of the icosahedron, and a 3 unit fibre associates with each penton base trimer (Nermut, 1984 and references therein). Another component of the external capsid is protein IX (11.5K), which associates with GON hexon trimers at a ratio of 15 molecules of protein IX per GON (Boulanger *et al.*, 1979). Protein IX is not essential for the formation of virus particles (Pereira and Wrigley, 1974; Colby and Shenk, 1981), but viruses lacking this protein exhibit thermolability (Colby and Shenk, 1981) and reduced DNA packaging capacity (Ghosh-Choudhury *et al.*, 1987).

Within the capsid, the viral DNA molecule is non-covalently associated with

protein VII, and less stringently with protein V (reviewed by Nermut, 1984), which organize the viral chromatin into structures analogous to but distinguishable from cellular nucleosomes (Corden *et al.*, 1976; Mirza and Weber, 1982). The adenovirus genome is also linked covalently at its 5' ends to a protein of 55 K referred to as the terminal protein (reviewed by Sussenbach, 1984; Kelly, 1984). The chemical nature of the linkage is a phosphodiester bond between the 5' phosphate of the terminal deoxycytosine residue of the viral chromosome and the hydroxyl group of a serine residue in the terminal protein (Desiderio and Kelly, 1981). The termini of adenovirus DNA comprise inverted terminal repetitions (ITRs) of 100-300 base pairs (bp) in length, with the precise length depending on the serotype. Ad 2 and Ad 5 have ITRs of 103 bp, while Ad 12 has an ITR of 163 bp (Sussenbach, 1984; Kelly, 1984). In addition to the ITRs, the viral packaging signal is required in *cis* for production of infectious virus, and is located approximately 300 base pairs (bp) from the "left" viral terminus of Ad 5 (Hearing *et al.*, 1987).

2. Genetic organization.

Adenovirus transcription occurs in two temporal phases. The first genes which are transcribed after the onset of infection are located in four regions of the genome, and are referred to as early regions 1 through 4 (E1 through E4). Proteins encoded by early genes of adenovirus are required for a variety of functions, including activation of other viral genes and shutoff of host protein synthesis (E1, E4), evasion of host immune responses (E3), and viral DNA replication (E2) (reviewed by Sussenbach, 1984).

Expression of late viral mRNAs from the major late promoter reaches maximal levels only after the onset of viral DNA replication (reviewed by Sharp, 1984). Proteins encoded by the late transcription unit largely constitute the structural components of the virion (see figure 1-1).

## B. The Transforming Region

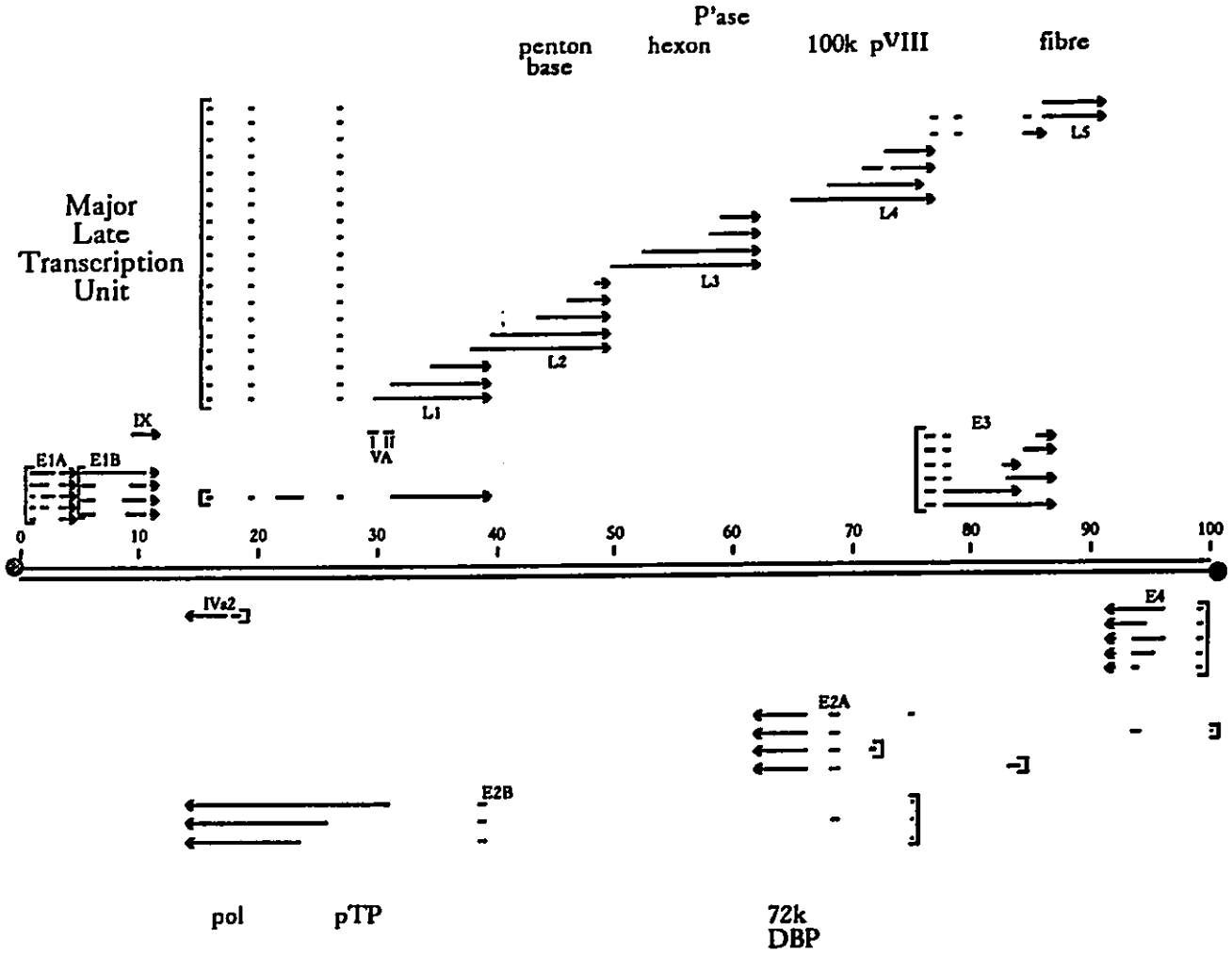
### 1. Identification of E1 as the transforming region.

Early studies which examined the viral DNA content of rat cells transformed by Ad 2 determined that in general only sub-genomic fragments of viral DNA were integrated into the host cell genome (Gallimore *et al.*, 1974; Sharp *et al.*, 1974). Subsequent studies showed that cells transformed by Ad 2 or Ad 5 contained a minimum of the left 12-14% of the viral genome (Gallimore *et al.*, 1974). The location of the transforming region was conclusively mapped by transfection assays with sub-genomic fragments of adenovirus DNA, to determine the minimum fragment size which retained transforming activity. Sheared segments of Ad 5 DNA corresponding to less than 10% of genome size were shown to have transforming activity, and purification of fragments corresponding to the left 10% of the genome identified the transforming region as the E1 region (Graham *et al.*, 1974 a, b; van der Eb *et al.*, 1977), which is now known to consist of two transcription units, E1A and E1B (reviewed by Sharp, 1984).

**Figure 1-1. Adenovirus transcription map.**

A schematic of the adenovirus type 2 genome and the locations of the primary transcripts are shown. A scale above the genome illustration is graded from 0 to 100 map units for reference to the locations of the viral transcripts. The viral genome is indicated as a double stranded molecule, with terminal protein molecules attached to the 5' ends. The E2 transcription unit is transcribed in opposite sense to the major late transcription unit. The late transcripts L1 through L5 are transcribed from a single promoter, but terminate at different locations and are differentially spliced. Most viral transcripts require splicing to generate mature mRNA.

# ADENOVIRUS TRANSCRIPTION MAP



2. The E1A transcription unit.

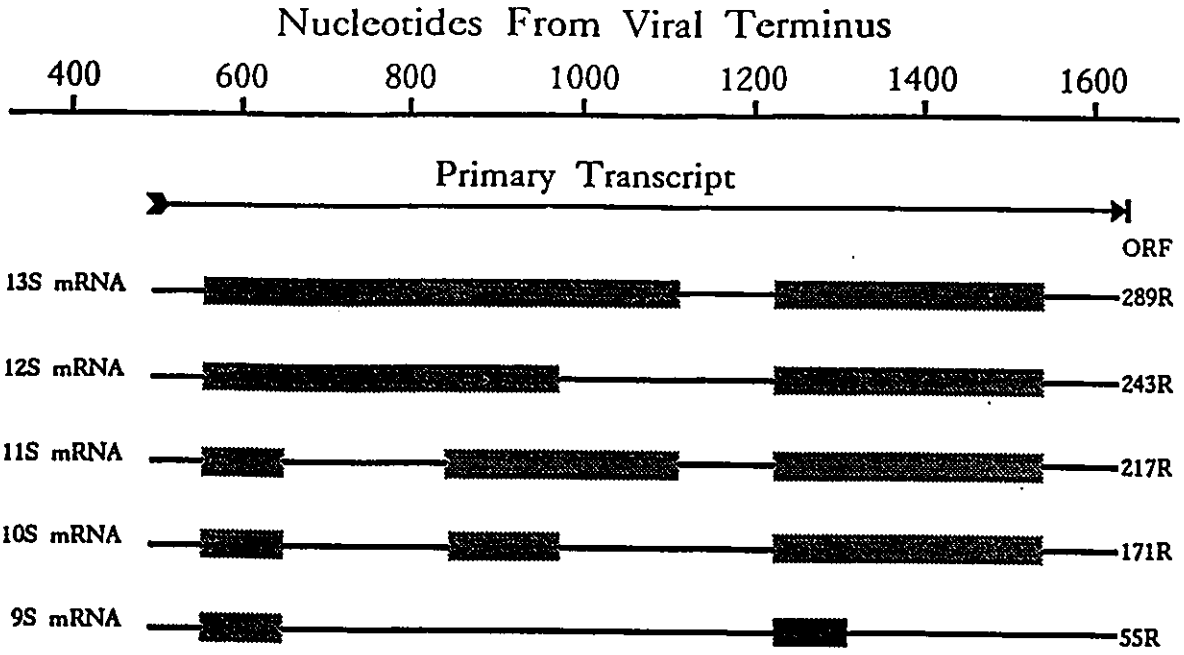
The E1A transcription unit of human adenoviruses (shown in figure 1-2.A) gives rise to two major species of singly spliced mRNA with 12S and 13S sedimentation coefficients, sharing a common 3' splice site but differing in the locations of their in-frame 5' splice sites (Berk and Sharp, 1977, 1978; Chow *et al.*, 1979, Perricaudet *et al.*, 1979, 1980; Kitchingman and Westphal, 1980; Sawada and Fujinaga, 1980; Saito *et al.*, 1981). In Ad 2 or Ad 5, these mRNAs encode proteins of 243 and 289 amino acids respectively (Halbert *et al.*, 1979; Esche *et al.*, 1980; Gaynor *et al.*, 1982; Rowe *et al.*, 1983a; Yee *et al.*, 1983, 1985a; Harlow *et al.*, 1985), while in Ad 12, the corresponding proteins are 235 residues (235R) and 266R (Perricaudet *et al.*, 1980). These alternate forms of E1A differ by an internal stretch of 46 amino acids in Ad 2/Ad 5 and 31 amino acids in Ad 12, which in both cases is unique to the larger protein. Two minor species of mRNA have also been observed for Ad 5 E1A, resulting from additional upstream splices yielding 10S and 11S messages (Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987). A fifth mRNA with a 9S sedimentation coefficient accumulates late in infection, and arises from excision of sequences between the upstream 5' splice site shared by the 10S and 11S messages and the 3' splice site shared by the 12S and 13S messages (Spector *et al.*, 1978; Chow *et al.*, 1979). The resulting second exon is out of frame with respect to the other four messages (reviewed by Sharp, 1984) (See figure 1-2). The 9S, 10S and 11S messages are produced in small quantities, and although it is known that the 10S and 11S mRNAs encode detectable proteins (Stephens and Harlow, 1987; Ulfendahl

**Figure 1-2. Early region 1 of Adenovirus 2.**

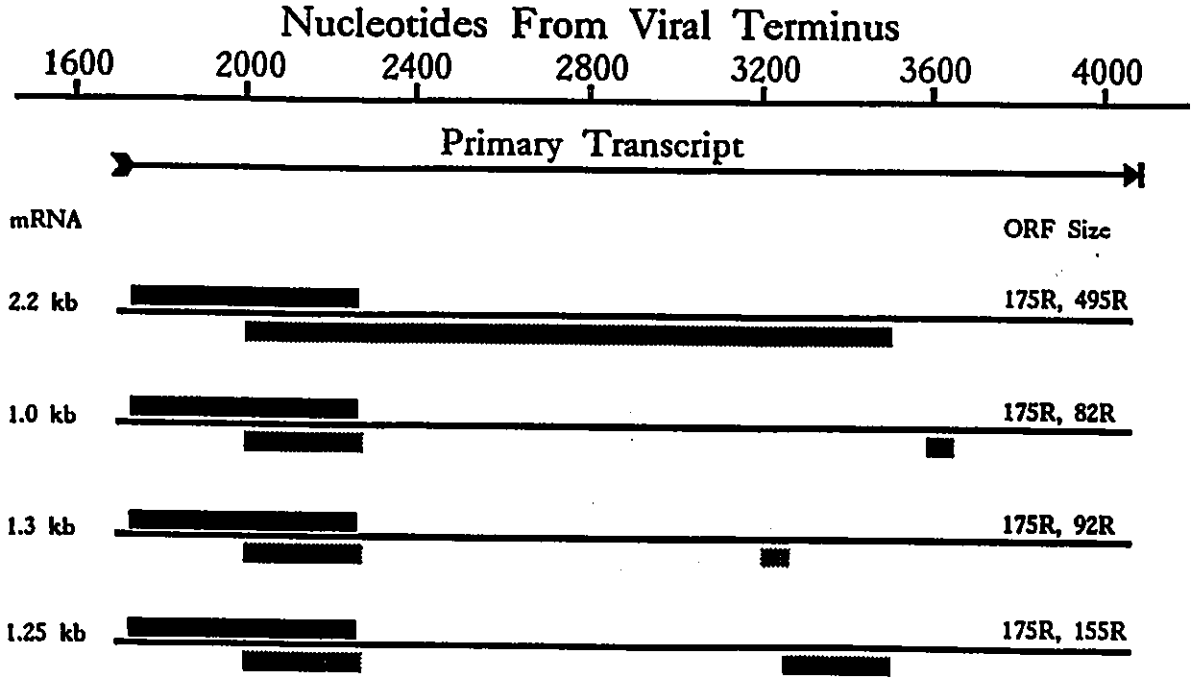
The E1A transcription unit is shown relative to numbered nucleotides on the viral genome. The primary transcript is spliced 5 different ways, generating four in-frame mRNAs and a fifth with a second exon frame shift relative to the other four. The 3' splice acceptor site common to the 13S and 12S mRNAs is also shared by the 9, 10, and 11 S mRNAs, while the 5' splice donor site used by the 9S mRNA is also used by the 10 and 11 S mRNAs. In addition, the 13S and 11S mRNAs share a splice donor site, while the 12S and 10S mRNAs share a distinct splice donor site.

The E1B transcription unit gives rise to a primary transcript which is spliced in four different ways. The 2.2 kb mRNA encodes the two major E1B proteins of 175R and 495R, and while the 175R protein is encoded by all E1B mRNAs, the 495R protein is encoded only by the 2.2 kb mRNA. A 155R protein with identical amino and carboxy termini to the 495R protein is encoded by the 1.25 kb mRNA, while the 1.0 kb mRNA and the 1.3 kb mRNA encode 495R related proteins with serologically distinct carboxy termini. The 175R protein has no homology to any other E1B protein.

# E1A Transcription Map




# E1B Transcription Map



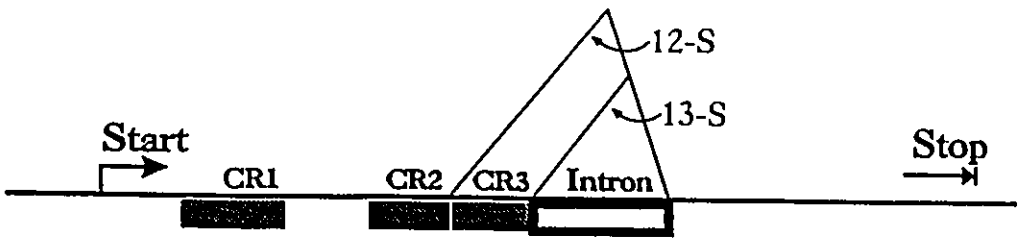
*et al.*, 1987), no protein has been detected *in vivo* corresponding to the 9S mRNA.

The 243R and 289R Ad 5 E1A proteins localize to the nucleus quickly after synthesis, and the carboxy terminus is required for this function (Krippl *et al.*, 1985; Lyons *et al.*, 1987). Two independent estimates of the half-lives of these two proteins are 35 min and 55 min for the 289R protein, 80 min and 90 min for the 243R protein (Spindler and Berk, 1984; Branton and Rowe, 1985, respectively). In part, these half-lives are influenced by the presence of a "turnover signal" in the amino terminus of the protein (Slavicek *et al.*, 1988; Simon and Richter, 1990). The E1A proteins of Ad 5 migrate as multiple, diffuse bands on SDS-polyacrylamide gels due in part to alternate splicing of E1A mRNAs, and to phosphorylation events at serine residues (Gaynor *et al.*, 1982; Yee *et al.*, 1983; Richter *et al.*, 1988; Tremblay *et al.*, 1988, 1989; Dumont *et al.*, 1989; Smith *et al.*, 1989). The Ad 12 E1A proteins appear as two sharp bands on SDS-polyacrylamide gels, but are also localized in the nucleus and are phosphorylated (Segawa *et al.*, 1980; Grand and Gallimore, 1984; Lucher *et al.*, 1984; Scott *et al.*, 1984).

Three regions of the E1A protein are highly conserved between different Ad serotypes, and are referred to as conserved regions 1, 2, and 3 (CR1, CR2, CR3, figure 1-3)(van Ormondt *et al.*, 1980; Kimmelman *et al.*, 1985; Lillie *et al.*, 1986; Moran and Matthews, 1987). CR1 and CR2 are both required for the transforming activity of E1A (discussed in section B.4), while CR3 is required for transcriptional activation of other genes (discussed in section B.3). Structurally, CR3 contains a consensus zinc-finger domain (Berg, 1986; Culp *et al.*, 1988), and interacts with the TATA-box binding

**Figure 1-3. Conserved regions in E1A.**

A schematic of the E1A transcription unit is shown at the top, with the locations of CR1, CR2 and CR3. The amino acid sequences in the conserved regions of several adenovirus serotypes are shown below. The sequences were obtained from Bautista (1989).




# CR1

Ad 5: FEPPTLHELYDLDVTAPEDPNEEAVSQIFPDSVML-AVQEGIDL
Ad 7: FDPPTLHDLYDLEVDGPEDPNEGAVNGFFTDSMLL-AADEGLDI
Ad 12: LYVPSLYELYDLDVESAGEDNNEQAVNEFFPESLILAASEGLFL
SA7: SHNMSLHEMYDLDVTGQEDENEEAVDGVFSDAMLL-AAEEGIEM

# CR2

Ad 5: GPVSMPNLVPEVIDLTCHEAGFPPSDDED
Ad 7: GGKKLPDLGAAEMDLRCYEEGFPPSDDED
Ad 12: GGECMPQLHPEDMDLLCYEMGFPCSDSED
SA7: GGGEMPELQPEEEDLFCYEDGFPPSDSEE

# CR3

Ad 5: EEFVLDYVEHPGHGCRSCHYHRRNTGDPDIMCSLCYMRTCGMFVYSPVSEPEPE
Ad 7: DVFKLDCPELPGHGCKSCEFHRNNTGMEKLLCSLCYMRMHCHFIYSPVSDDE--
Ad 12: EEFQLDHPELPGHNCKSCEHHRNSTGNTDLMCSLCYLRAYNMFIYSPVSDNEPE
SA7: DDFRLDCPSVPGHGCSSCDYHRKTSGCPEILCSLCYLRANSMFIYSPVSDLRCT

Fig 1-3

transcription factor TF-II D (Horikoshi *et al.*, 1991; Lee *et al.*, 1991).

3. The transactivation function of E1A.

The creation of the human 293 cell line, which was transformed by sheared Ad 5 DNA and expresses the Ad 5 E1 proteins (Graham *et al.*, 1977), facilitated studies on the role of E1A in lytic infection. This complementing cell line allowed the propagation of host range *(hr)* mutants and E1 deletion mutants, defective for growth in non-complementing cell lines (Harrison *et al.*, 1977; Jones and Shenk, 1979a). It was determined that mutants with lesions in E1A had impaired transforming activities on primary cells (Graham *et al.*, 1978; Babiss *et al.*, 1983), as well as defective viral growth as a result of impaired viral gene expression. (Harrison *et al.*, 1977; Jones and Shenk, 1979b; Berk *et al.*, 1979; Ricciardi *et al.*, 1981; Glenn and Ricciardi, 1985). E1A function is critical for the accumulation of viral RNA in infected cells (Berk *et al.*, 1979; Jones and Shenk, 1979a). The ability of E1A to activate the expression of both viral and cellular genes is assayed by transient cotransfection of cells with plasmids encoding E1A and other early viral or cellular genes (Weeks and Jones, 1983; Gaynor *et al.*, 1984; Leff *et al.*, 1984; Svensson and Akusjarvi, 1984). Transactivation was subsequently mapped to the 46 internal amino acids present in 289 residue E1A protein, but not in the 243 residue protein (Berk, 1986; Moran *et al.*, 1986; Oshima and Shiroki, 1986; Moran and Matthews, 1987; Schneider *et al.*, 1987; Jelsma *et al.*, 1988; Green *et al.*, 1988; Flint and Shenk, 1989; Bautista *et al.*, 1991). The E1A proteins of Ad 5 and Ad 12 are interchangeable

with respect to supporting viral lytic infection (Williams *et al.*, 1975; Rowe and Graham, 1981; Sawada *et al.*, 1988), suggesting that the mechanism of transactivation is conserved between different serotypes. Evidence suggests that the nature of the mechanism by which E1A transactivates does not involve direct interaction with promoters (Feldman *et al.*, 1982; Ferguson *et al.*, 1985; Kovesdi *et al.*, 1986). Instead, transactivation by E1A is thought to occur by the interaction of E1A with the TATA box binding factor TF-IID, resulting in transcriptional stimulation at the level of the TATA box (Leong *et al.*, 1988; Horikoshi *et al.*, 1991; Lee *et al.*, 1991). A second, recently described mechanism by which E1A transactivates is thought to involve binding of E1A to members of the ATF family of transcription factors, as this binding is sensitive to mutations in the transactivation domain of E1A (Chatton *et al.*, 1993).

In addition to being able to stimulate the expression of other viral genes, E1A stimulates the expression of a large number of cellular genes (Nevins, 1981; Kao and Nevins, 1983; Leff *et al.*, 1984; Stein and Ziff, 1984) by a mechanism also involving transactivation from basal promoter elements (reviewed by Berk, 1986a,b; Nevins, 1987). This activation has been shown to occur at the level of the TATA box for the heat shock protein hsp 70 (Wu *et al.*, 1986; Simon *et al.*, 1988), and *c-fos* (Simon *et al.*, 1990). A distinct mechanism of activation of cellular gene expression by E1A is thought to involve a less ubiquitous transcription factor, E2F. E2F was identified as an E1A-induced cellular transcription factor, which is required for efficient expression of the E2A transcription unit of Ad 2 (Kovesdi *et al.*, 1987). The mechanism of induction was determined to be

post-translational (Nevins *et al.*, 1988; Reichel *et al.*, 1988), involving E1A mediated dissociation of complexes composed of cellular proteins and E2F (Bagchi *et al.*, 1990). The identities of the cellular proteins which complex with E2F are the same as those which co-immunoprecipitate with E1A (discussed in section B.4), suggesting that the activation of E2F by E1A is by the sequestration of inhibitors. In support of this model is the observation that the Rb-E2F complex is an inhibitor of transcription from promoters with E2F binding sites (Weintraub *et al.*, 1992). In addition to the E2A gene, E2F binding sites are present in the promoters of cellular genes such as *N-myc* and *dihydrofolate reductase*, and expression of those genes is activated by E2F in the presence of E1A (Hiebert *et al.*, 1989, 1991).

4. The transforming function of E1A.

As previously indicated, studies on adenovirus transformed rodent cells demonstrated that a minimum of the leftmost 12% of the viral genome was invariably integrated into the host cell genome, a fragment containing early region 1. Analysis of host range mutants with lesions in the E1A gene indicated that disruption of E1A function could result in impaired transforming activity of the host range mutants (Graham *et al.*, 1978; Jones and Shenk, 1979a). The locations of the E1A mutations in host range mutants were within CR3, the region required for transactivation (Glenn and Ricciardi, 1985), suggesting that the transactivation property of E1A is important for viral transforming activity. Subsequent studies established that stable expression of E1A alone could lead to

immortalization of primary cells (Shiroki *et al.*, 1979; Houweling *et al.*, 1980), but full morphological transformation required the cooperation of another oncogene, such as E1B or *ras* (Ruley, 1983). A number of reports indicated that both the products of the 12S and 13S mRNAs were required for full transformation of primary cells by Ad 2 or Ad 5 E1A in conjunction with E1B or *ras* (Haley *et al.*, 1984; Montell *et al.*, 1984; Hurwitz and Chinnadurai, 1985a,b; Moran *et al.*, 1986b), although the 13S mRNA product of Ad 12 E1A has full transforming activity (Lamberti and Williams, 1990). However, the requirement for both the 12S and 13S mRNA products for transformation by Ad 2 and Ad 5 E1A suggests that the transactivation function of E1A is not sufficient for transformation. Further studies on the ability of E1A mutants to transform cells cooperatively with *ras* showed that E1A mutants defective for transactivation nonetheless transformed cells efficiently (Velcich and Ziff, 1988), suggesting that transactivation is not required for transformation by E1A in cooperation with an independently promoted oncogene. This raises the possibility that the transactivation function is necessary only to activate E1B expression in virus mediated assays, or assays involving transfection of E1A plus E1B. By mutational analysis, other regions of E1A important for transformation were identified as the non-conserved amino terminus of the protein, plus CR1 and CR2 (Lillie *et al.*, 1986; Moran *et al.*, 1986a; Kuppuswamy and Chinnadurai, 1987; Subramanian *et al.*, 1988; Whyte *et al.*, 1988; Egan *et al.*, 1989; Jelsma *et al.*, 1989). The functions of CR1 and CR2 in transformation were clarified by co-immunoprecipitation experiments which demonstrated that CR1 and CR2 are sites of interaction between E1A and a number

of cellular proteins. Specifically, a protein of 300K interacts with the amino terminus and CR1 of E1A, while proteins of 60K, 105K, and 130K interact with CR1 and CR2, and a 107K protein interacts with a segment of E1A contained completely within CR2 (Yee and Branton, 1985; Harlow *et al.*, 1986; Egan *et al.*, 1988; Whyte *et al.*, 1989; Giordano *et al.*, 1991). The identity of the 105K protein has been established as the product of the retinoblastoma susceptibility gene (p105Rb) (Whyte *et al.*, 1988), while the 60K protein has been identified as cyclin A (Pines and Hunter, 1990), which associates with $p33^{cdk2}$ in the presence of E1A (Tsai *et al.*, 1991). The 107K protein has recently been identified as a nuclear protein related to p105Rb (Ewen *et al.*, 1991), which is capable of interacting with cyclin A in the absence of E1A (Faha *et al.*, 1992; Ewen *et al.*, 1992). The precise stoichiometry of protein complexes containing E1A, p107, and cyclin A is at present not clear. Although the identity of p300 is not presently known, it has been characterized as a nuclear phosphoprotein whose phosphorylation is increased in mitotic cells (Yaciuk *et al.*, 1991). Mutants in CR1 and CR2 of the Ad 5 E1A protein in which binding to these cellular proteins is diminished or eliminated are impaired for transforming activity (Egan *et al.*, 1988, 1989; Whyte *et al.*, 1989), and it is therefore assumed that interactions between E1A and cellular proteins are essential for the transforming activity of Ad 5, and presumably for that of other serotypes also. One of the consequences of complex formation between E1A and cellular proteins is an increase in free E2F (Bagchi *et al.*, 1990), or a related transcription factor, DRTF1 (Bandara and LaThangue, 1991; Bandara *et al.*, 1991), which exist in independent complexes with p107 (Cao *et al.*, 1992), p105Rb,

and p60 cyclin A (Bagchi *et al.*, 1991; Bandara and LaThangue, 1991; Bandara *et al.*, 1991; Chellappan *et al.*, 1991; Mudryj *et al.*, 1991; Raychaudhuri *et al.*, 1991). At least two cellular genes, *N-myc* and *dihydrofolate reductase*, are transcriptionally stimulated by E2F, an effect which is mediated by E2F binding sites in the 5' flanking regions of these cellular genes (Hiebert *et al.*, 1991). This suggests a mechanism of transformation by E1A involving derepression of E2F activity, leading to transcription of genes involved in cell cycle progression, and consequently cell proliferation. This model is supported by the observation that Rb complexes with E2F during the G1 phase of the cell cycle, resulting in dominant transcriptional repression from promoters which are stimulated by E2F alone (Weintraub *et al.*, 1992). This model does not exclude the possibility that additional activities of E1A and its associated proteins may be important for transformation.

E1A has several additional properties which co-localize with the regions important for its transforming activity, although it is not presently clear whether any such properties are required for transformation. Among these properties are the ability of E1A to repress transcription from various enhancers, the first characterized examples being the enhancers of SV40, polyomavirus, Ad 2 E1A (Borelli *et al.*, 1984; Velcich and Ziff, 1985; Velcich *et al.*, 1986), and the immunoglobulin heavy chain gene (Hen *et al.*, 1985). While early reports attributed the repression function to products of both the 12S and 13S mRNA (Borelli *et al.*, 1984; Velcich and Ziff, 1985; Schneider *et al.*, 1987), subsequent reports have suggested that repression is detectable only with the product of the 12S mRNA (Lillie *et al.*, 1986; Jelsma *et al.*, 1989; Bautista *et al.*, 1991). Identification of

E1A mutants which were able to transactivate but were defective for transformation and repression suggested that repression was involved in transformation (Velcich and Ziff, 1985; Lillie *et al.*, 1986; Schneider *et al.*, 1987). Other reports are in disagreement with this idea, with the description of adenovirus mutants defective for repression but unaffected for transformation (Velcich and Ziff, 1988; Bautista *et al.*, 1991). The repression activity of E1A is thought to map primarily in CR1 (Jelsma *et al.*, 1989; van Dam *et al.*, 1989; Offringa *et al.*, 1990), and correlate with the ability of E1A to bind p300 (Stein *et al.*, 1990). However, other reports have demonstrated defective repression as a result of mutations in CR2 (Lillie *et al.*, 1986), or exon 2 of the E1A protein (Velcich and Ziff, 1988; Bautista *et al.*, 1991). A possible explanation for these observations is that the overall conformation of the E1A protein is important for repression activity, and that while CR1 may contain sequences indispensable for repression, the property is also influenced by sequences elsewhere in the protein (Velcich and Ziff, 1988).

Another property of E1A which may be related to transformation has to do with the natural tropism of adenoviruses for quiescent cells. In contrast to infection of HeLa cells by Ad 5, productive infection of quiescent cells appears to require expression of the 243R E1A protein as well as the 289R protein (Montell *et al.*, 1984; Spindler *et al.*, 1985), suggesting that the E1A protein encoded by the 12S mRNA carries out an important function in quiescent cells. Infection of BRK cells with viruses expressing only the 12S E1A mRNA product results in the induction of host cell DNA synthesis (Berk,

1986a; Kaczmarek *et al.*, 1986), reportedly without the onset of viral DNA replication (Quinlan and Grodzicker, 1987). Ad 12 E1A is also able to induce DNA synthesis when expressed in rat cells (Oda *et al.*, 1986), suggesting that this property is not unique to Ad 5. The region of E1A required for induction of DNA synthesis was initially identified as CR1 (Moran and Zerler, 1988; Smith and Ziff, 1988; Subramanian *et al.*, 1988), and as with repression, DNA synthesis induction appeared to require binding of E1A to p300 (Stein *et al.*, 1990). However, a recent report carried out with deletion mutants of E1A indicates that interaction of the 243R E1A protein with either p105Rb or p300 is sufficient to induce DNA synthesis. Mutations which abolished binding to both cellular proteins were defective in DNA synthesis induction (Howe *et al.*, 1990). The authors suggested the existence of parallel pathways of DNA synthesis induction, mediated by complex formation between E1A and either of p105Rb or p300. Mutant E1A proteins with lesions in CR2 induce DNA synthesis, but not mitosis, resulting in the accumulation of cells in G2 phase with excess DNA content (Berk, 1986a; Zerler *et al.*, 1987; Moran *et al.*, 1988). This suggests that E1A induces DNA synthesis and mitosis by distinct mechanisms.

5. Interactions between E1A and the immune system.

While all known human adenovirus serotypes are able to transform primary baby rat kidney (BRK) cells *in vitro*, cells transformed by Ad 2 or Ad 5 are not tumorigenic in syngeneic rats, unless the rats are immunosuppressed (Gallimore, 1972).

In contrast, Ad 12 transformed cells are tumorigenic in immunocompetent syngeneic rats (Raska *et al.*, 1980), suggesting that cells transformed by oncogenic Ad serotypes are tumorigenic because they are able to evade one or more host immune responses which efficiently reject cells transformed by non-oncogenic Ad serotypes. While the nature of the host immune function responsible for the elimination of Ad 5 transformed cells is not clear, experiments involving transformation of cells with combinations of Ad 5 E1A/Ad 12 E1B and vice versa demonstrated that the identity of the E1A gene is the factor which determines whether the transformed cells are tumorigenic in immunocompetent animals (Bernards *et al.*, 1983; Sawada *et al.*, 1988). This indicates that the immunological properties which discriminate between Ad transformed cells with varying degrees of tumorigenicity are controlled by E1A.

One immunological difference between cells transformed or infected by non-oncogenic and oncogenic Ad serotypes is their differential sensitivity to lysis by natural killer (NK) cells. Cells expressing the Ad 2 or Ad5 E1A protein are highly susceptible to lysis by NK cells and activated macrophages, whereas expression of the Ad 12 E1A protein causes no such sensitization (Sawada *et al.*, 1985; Cook *et al.*, 1986, 1987; Kenyon *et al.*, 1991). Furthermore, the introduction of Ad 5 E1A into a tumorigenic hamster sarcoma cell line results in the loss of tumorigenicity concomitant with increased sensitivity to lysis by NK cells (Walker *et al.*, 1991). This implies that the E1A protein of non-oncogenic adenovirus serotypes actively induces non-specific, NK cell mediated cytolysis of cells which express it, while the E1A protein of oncogenic serotypes is

inactive in this respect.

A second difference between cells transformed by the E1 regions of Ad 5 and Ad 12 lies in the levels at which they express class I major histocompatibility complex (MHC) molecules. Class I MHC molecules are essential for antigen specific cytolysis by cytotoxic T lymphocytes (CTL) (Zinkernagel and Doherty, 1974). While class I MHC transcription is transiently elevated following infection of mouse cells with either Ad 5 or Ad 12 (Rosenthal *et al.*, 1985), stable expression of the Ad 12 E1A protein in transformed cell lines leads to transcriptional downregulation of class I MHC genes (Schrier *et al.*, 1983; Mellow *et al.*, 1984; Eager *et al.*, 1985, 1989; Vasavada *et al.*, 1986; Ackrill and Blair, 1988; Friedman and Ricciardi, 1988). This downregulation is promoter specific, as shown by the downregulation of a transgene, the human growth hormone gene under the class I MHC promoter, in transgenic baby mouse kidney cells transformed by Ad 12 (Meijer *et al.*, 1989). Furthermore, this downregulation correlates with the induction of a DNA binding activity to a retinoic acid response element within the class I MHC enhancer (Ge *et al.*, 1992; Kralli *et al.*, 1992). Downregulation of class I MHC expression correlates with reduced levels of CTL killing of Ad 12 transformed cells in *in vitro* assays (Bernards *et al.*, 1983). No reduction in class I MHC expression is seen in cells transformed by Ad 2 or Ad 5, suggesting that tumorigenicity of Ad 12 transformed cells occurs as a consequence of active CTL evasion, mediated by E1A. Since the Ad 5 E1A protein shows no such activity in transformed cells, those cells remain susceptible to cytolysis by CTLs. Both the Ad 5 and Ad 12 E1A proteins are

potentially immunogenic, and can give rise to tumor-specific transplantation immunity, which is mediated by CTLs (Sawada *et al.*, 1986; Bellgrau *et al.*, 1988; Kast *et al.*, 1989). Some studies have quantitated the levels of class I MHC expression in Ad 2 and Ad 5 transformed cells of varying tumorigenicity, and found no correlation between levels of class I MHC expression and the degree of tumorigenicity (Mellow *et al.*, 1984; Haddada *et al.*, 1986), raising the possibility that class I MHC levels may not be a critical factor in determining whether adenovirus transformed cells are tumorigenic. Recently, further questions have been raised about the relevance of class I MHC repression to tumorigenicity, by the observation that the induction of class I MHC expression in Ad 12 transformed mouse cells does not reduce the tumorigenicity of those cells (Soddu and Lewis, 1992).

An immunological property which appears to be shared by the Ad 5 and Ad 12 E1A proteins is their ability to induce sensitivity to cytolysis by tumor necrosis factor (TNF) (Chen *et al.*, 1987). TNF, originally identified as a lymphokine strongly induced by bacterial lipopolysaccharides, has cytotoxic activity against certain tumor cell types (Reviewed by Old, 1985). This sensitivity is overcome in Ad infected human or rodent cells by expression of a 14.7K protein encoded by the E3 region (Gooding *et al.*, 1988), and, in human cells only, by the E1B 19K protein (Hashimoto *et al.*, 1991; Gooding *et al.*, 1991). The region of E1A which is responsible for TNF sensitivity has been defined as the amino terminal portion of CR1, although the location does not correlate precisely with sequences required for transformation and binding to cellular proteins (Duerksen-

Hughes *et al.*, 1991). The induction of TNF sensitivity is independent of cell transformation or even *de novo* protein synthesis, suggesting a post-translational mechanism (Ames *et al.*, 1990; Rodriques *et al.*, 1990). The mechanism by which E1A induces TNF sensitivity is not known, but it is clear that it is distinct from the induction of sensitivity to cytolysis by NK cells (Kenyon *et al.*, 1991). A second mechanism by which Ad infected cells may escape TNF is based on the observation that TNF induces expression of the E3 region (Korner *et al.*, 1992). In addition to the resistance to TNF that is mediated by E3 proteins, products of the E3 region also interfere with E1A expression, thereby removing the cause of TNF induction (Zhang, X. *et al.*, 1992). In addition, the expression of E1A in the absence of E1B induces cell death by cellular DNA degradation in a manner consistent with programmed cell death, or apoptosis. This phenotype can also be blocked by the expression of Bcl-2, a known suppressor of apoptosis (Rao *et al.*, 1992). It is not clear by what mechanism E1A induces apoptosis, and whether it is related to TNF sensitization.

Another property of E1A which is related to host immune responses is the ability of E1A to repress the expression of interferon inducible genes. The course of infection of human cells by Ad 5 is not affected by the addition of interferon, while the replication of vesicular stomatitis virus (VSV) is inhibited. When the same cells are coinfected with Ad 5 and VSV, interferon does not inhibit replication of either virus, and functional E1A is essential for this effect (Anderson and Fennie, 1987). Ad infection induces interferon stimulated gene expression, even in the absence of viral protein

synthesis. Virus particles appear to be responsible for this induction, and synthesis of E1A is required to inhibit these effects (Reich *et al.*, 1988). The mechanism of inhibition of interferon induced gene expression involves the reduction of transcription factor binding activity to interferon stimulated response elements (ISRE), and CR1 of E1A is essential for this response (Ackrill *et al.*, 1991; Kalvakolanu *et al.*, 1991). In this respect, inhibition of ISREs by E1A appears to be indistinguishable from enhancer repression mediated by E1A, although the mechanisms by which these properties arise are unknown.

6. The effect of E1A on metastasis.

Many tumor cells show a preferential attachment affinity for type IV collagen over type I collagen, a characteristic that has been associated with tumorigenicity, invasiveness and metastatic potential (Bober *et al.*, 1987; Murray *et al.*, 1980). Transformed cells expressing Ad 12 E1A share this attachment affinity, whereas those expressing Ad 5 E1A do not (Bober *et al.*, 1988). This would appear to correlate with the tumorigenicity of Ad 12 transformed cells and the corresponding lack of tumorigenicity of Ad 5 transformed cells. However, adenovirus transformed cells are anomalous in that they are neither invasive nor metastatic *in vivo* (Pozzatti *et al.*, 1986). In fact, expression of Ad 2 E1A in metastatic ras transformed rat embryo fibroblasts tends to reduce the degree of metastasis of those cells (Garbisa *et al.*, 1987). Decreased metastasis of cells expressing E1A does not require the 13S mRNA product, as it was found that the introduction of a cDNA encoding the 12S mRNA of Ad 2 E1A into metastatic cells

resulted in a strong decrease in metastasis (Pozzatti *et al.*, 1988a,b). This correlates with the observation that the levels of secreted metalloproteases such as type IV collagenase, interstitial collagenase, stromelysin, and urokinase plasminogen activator are all reduced in cells expressing E1A (Garbisa *et al.*, 1987; Offringa *et al.*, 1988). It has not been determined whether this activity of E1A is related to its enhancer repression function, but this is a reasonable possibility.

7. The E1B transcription unit.

In addition to the host range mutants previously described, whose lesions localized to CR3 of the E1A protein, a second complementation group of mutants were isolated in early studies on Ad 5 replication (Harrison *et al.*, 1977; Lassam *et al.*, 1978). This second class of host range mutants mapped between the left 4.5% and 11.0% of the viral genome, corresponding to the E1B transcription unit (Frost and Williams, 1978; Galos *et al.*, 1980). As shown in figure 1-2.B, E1B gives rise to two predominant mRNA products, 2.2 and 1.0 kilobases in size (Berk and Sharp, 1978; Chow *et al.*, 1979; Kitchingman and Westphal, 1980; Virtanen *et al.*, 1982; Saito *et al.*, 1983 reviewed by Stillman, 1986; Grand, 1987). The larger mRNA contains two open reading frames, which encode two unrelated gene products of molecular weights 19K and 58K in Ad 5, 19K and 55K in Ad 12. The carboxy terminus of the 19K protein is encoded by sequences which overlap those encoding the amino terminus of the 58K/55K protein (Bos *et al.*, 1981; Gingeras *et al.*, 1982). Several additional 58K related proteins are synthesized from

mRNAs generated by alternate splicing patterns (Virtanen and Petterson, 1985; Lewis and Anderson, 1987). The 58K protein is a phosphoprotein, localized to both the nucleus and cytoplasm (Sarnow *et al.*, 1982a,b; Malette *et al.*, 1983; Rowe *et al.*, 1983b; Yee *et al.*, 1983; Grand and Gallimore, 1984; Schughart *et al.*, 1985). In an infected cell the 58K protein associates with and extends the half-life of the cellular protein p53, this complex localizing to discrete portions of the cytoplasm (Sarnow *et al.*, 1982b; Zantema *et al.*, 1985). The Ad 12 55K protein does not show association with p53, although the presence of 55K does extend the half-life of p53 (Zantema *et al.*, 1985; Mak *et al.*, 1988). The sequences of the Ad 5 58K protein which appear to be required for association with p53 are between residues 224 and 354 (Kao *et al.*, 1990), which does not correlate with the requirement for efficient virus replication and transformation of cloned rat embryo fibroblasts by virus (Yew *et al.*, 1990). In addition to p53, Ad 5 58K associates with a 34K protein encoded by early region 4 (Sarnow *et al.*, 1984). Expression of the Ad 5 58K protein and Ad 12 55K protein respectively, is required for efficient virus replication (Harrison *et al.*, 1977; Lassam *et al.*, 1978; Shiroki *et al.*, 1986; Brieding *et al.*, 1988), and accumulation of late viral mRNA (Babiss *et al.*, 1985; Pilder *et al.*, 1986; Stillman, 1986). The complex of the E1B 58K protein and the E4 34K protein is essential for the shutoff of host cell protein synthesis late in infection (Babiss and Ginsberg, 1984; Shiroki *et al.*, 1986; Stillman, 1986). This complex localizes to the periphery of inclusion bodies in the nucleus of infected cells (Ornelles and Shenk, 1991), although the functional relevance of this is not clear. It has been demonstrated that 58K is not tightly associated

with nuclear pores (Smiley *et al.*, 1990), suggesting that it is not a direct mediator of mRNA transport. instead, it is thought to play a role in RNA metabolism after transcription but before transport (Leppard and Shenk, 1989). Expression of 58K/55K is required for cell transformation by virions (Graham *et al.*, 1978; Ho *et al.*, 1982; Babiss *et al.*, 1984b; Logan *et al.*, 1984; Bernards *et al.*, 1986; Pilder *et al.*, 1986; Shiroki *et al.*, 1986; Barker and Berk, 1987; Byrd *et al.*, 1988), and for tumor induction by Ad 12 (Shiroki *et al.*, 1986; Byrd *et al.*, 1988). It is however possible to transform cells by transfection with viral or plasmid DNA containing mutant 58K/55K, or with DNA fragments containing incomplete E1B transcription units (McKinnon *et al.*, 1982; Rowe and Graham, 1983; Rowe *et al.*, 1984; Shiroki *et al.*, 1986) suggesting that transformation by virions differs from transformation by DNA transfection in its requirement for viral functions, and since mutations in 58K/55K result in phenotypes of a pleiotropic nature, the viral functions could be numerous. As with transformation by DNA fragments, it appears that all the sequences of 55K required for tumorigenicity of Ad 12 transformed cells lie in the amino terminal half of the protein (Shiroki *et al.*, 1977; Mak and Mak, 1990).

The 19K protein of both Ad 5 and Ad 12 is acylated (Grand *et al.*, 1985; McGlade *et al.*, 1987), associates with the plasma membrane, and localizes to both the nucleus and cytoplasm (Persson *et al.*, 1982; Rowe *et al.*, 1983b; Grand and Gallimore, 1984, White *et al.*, 1984b). Viruses expressing a defective E1B 19K protein exhibit a cytocidal phenotype, and induce DNA degradation in infected human cells (Pilder *et al.*,

1984; Subramanian *et al.*, 1984a,b; Takemori *et al.*, 1984, White *et al.*, 1984a; Barker and Berk, 1987; Edbauer *et al.*, 1988; Zhang, S., *et al.*, 1992). Constitutive expression of the Ad 2 19K protein in a cell line can complement the cyt defect of Ad 2 and Ad 12 E1B mutants (Kumai *et al.*, 1989). While expression of the 19K protein is important for virus transformation of certain cell types, it does not appear to be essential for transformation of primary cells, or for tumor induction by Ad 12 virions (Edbauer *et al.*, 1988). Other functions of the 19K protein include alteration of the organization of cytoskeletal intermediate filaments, accompanied by augmented anchorage independent growth (White and Cipriani, 1990), resistance to the cytotoxic effects of tumor necrosis factor induced by E1A in human cells, but not mouse cells (Gooding *et al.*, 1991; Hashimoto *et al.*, 1991), and resistance to E1A induced apoptosis (Rao *et al.*, 1992).

While E1A alone can immortalize cells, full morphological transformation also requires the expression of E1B (Shiroki *et al.*, 1977; Graham *et al.*, 1978; Schrier *et al.*, 1979; McKinnon *et al.*, 1982, van den Elsen *et al.*, 1983). In DNA transfection assays, it appears that either E1B protein is capable of complementing E1A for transformation, although the efficiency at which transformants appear increases if both are present (Bernards *et al.*, 1986; McLorie *et al.*, 1991).

## C. Tumor Induction By Adenoviruses

Following the demonstration that Ad 12 could induce tumors when injected into newborn hamsters (Huebner *et al.*, 1962; Trentin *et al.*, 1962), extensive studies were undertaken to characterize the oncogenic properties of different adenovirus serotypes. Initial efforts were aimed at determining whether adenoviruses played any causal role in human cancer, the results of which proved negative (Gilden *et al.*, 1970; McAllister *et al.*, 1972). To date, there have been no reports linking human cancer with any known adenovirus, and with the increasing body of knowledge on human cancer, it is becoming progressively less likely that any adenovirus serotype is the transforming agent in a human neoplasia. The possibility of a "hit-and-run" mechanism of transformation has not been excluded. Many viruses are able to induce random chromosomal damage in cells they infect (Nichols, 1983), and Ad 12 induces site-specific chromosome damage (most frequently but not exclusively at band 17q21-22) (Stich *et al.*, 1964; zur Hausen, 1967; McDougall, 1971; McDougall *et al.*, 1974). This function was mapped to the E1 region (Durnam *et al.*, 1986), and is dependent on expression of the E1B 55K protein (Schramayr *et al.*, 1990). Given this data, it is conceivable that defective Ad 12 particles which are nonetheless capable of expressing the E1 region could cause genetic damage in an abortive infection, leading to translocations and activation of cellular proto-oncogenes. There is currently no evidence to suggest that this actually occurs.

Human adenoviruses have been classified into subgroups, based on a number

of criteria, one of which is their degree of oncogenicity, as was originally assayed in hamsters (Trentin *et al.*, 1968; Green, 1970). Group A includes highly oncogenic serotypes, including Ad 12, Ad 18, and Ad 31. Group B consists of numerous weakly oncogenic serotypes, examples of which are Ad 3, and Ad 7. Group C is the largest group, consisting of non-oncogenic serotypes, the best studied examples being Ad 2 and Ad 5. Additional subgroups of Ads, based on criteria other than oncogenicity, may have oncogenic members. An example is Ad 9, which is non-oncogenic in hamsters, but is able to induce estrogen-dependent mammary tumors at high frequency in rats (Ankerst *et al.*, 1974; Jonsson and Ankerst, 1977; Ankerst and Jonsson, 1989; Javier *et al.*, 1991). In addition to the human adenovirus serotypes, a large number of non-human adenoviruses are also able to induce tumors in newborn hamsters, and often other rodents also (Ishibashi and Yasue, 1984).

Transformation of primary cells with only the E1 region of Ad 12 gives rise to cell lines which are then tumorigenic in syngeneic rats (Raška *et al.*, 1980; Jochemsen *et al.*, 1982). However, when the E1 region of Ad 5 is replaced with that of Ad 12, the resulting virus is not oncogenic, even though it may express E1 proteins in infected cells, and transform cells *in vitro*, which are then tumorigenic in syngeneic animals (Bernards *et al.*, 1984; Sawada *et al.*, 1988; Jelinek and Graham, unpublished). This suggests that some other property of Ad 12 is involved in viral oncogenicity, or alternately, that a property of Ad 5 which localizes outside the E1 region prevents oncogenicity.

## D. Analysis Of E1 Functions

### 1. Mutational analysis.

Genetic analysis of eukaryotic proteins has traditionally been carried out by alteration or deletion of sequences throughout the gene encoding a protein of interest, correlated with the loss or reduction of a measurable phenotype. The loss of a phenotype following directed mutagenesis is often due directly to the loss of sequences specifying that phenotype, but mutations may also alter the structure or stability of an entire protein to the extent that properties unrelated to the altered sequences are lost or reduced. Examples of this are evident with the E1B 55K protein of Ad 2 (Yew *et al.*, 1990), and Ad 12 (Schaller *et al.*, unpublished), whose levels of expression can be reduced by mutations in various regions of the coding sequences.

### 2. Analysis of chimeric proteins.

In some studies, chimeric proteins have been constructed by splicing together gene segments with common restriction sites to analyze differences between related proteins (Jochemsen *et al.*, 1984). When feasible, this approach can offer significant advantages over mutational analysis, as it affords the possibility of correlating the

acquisition of properties by a hybrid protein with the presence of sequences from the parent protein which shares those properties, providing strong evidence that the property being assayed is specified by the sequences in question. A limitation of this approach has been the need for conveniently located restriction sites or complex PCR strategies to create genes encoding chimeric proteins.

## E. Purpose

The purpose of this project was the construction and characterization of a series of hybrid Ad 5/Ad 12 E1 regions, in order to define the region of the Ad 12 E1A protein which is essential for tumorigenicity of transformed cells. Preliminary studies of this type have been carried out by others (Jochemsen *et al.*, 1984; Sawada *et al.*, 1988), and the results obtained suggest that the first exon of Ad 12 E1A in a hybrid plasmid is sufficient to cause suppression of class I MHC expression in transformed cells. However, it is not clear whether this downregulation of class I MHC expression is responsible for the tumorigenicity of adenovirus transformed cells, and whether it is related to the sensitivity of cells expressing Ad 5 E1A to lysis by NK cells. The hybrid construction and tumorigenicity studies described in this thesis, correlated with results of immunological characterizations, should help to determine which immunological properties of E1A are relevant to tumorigenicity, and perhaps will assist in determining the mechanisms by which these immunological properties arise.

Hybrid E1 genes were constructed using a technique involving homologous recombination *in vivo*, between tandem repeats of Ad 12 and Ad 5 E1 sequences on a single plasmid. This technique allows the construction of large numbers of hybrids, each arising from a crossover in a region of patch homology.

# MATERIALS AND METHODS

## A. Bacteria

### 1. Bacterial strains.

Among the *E. coli* strains used in this study for purposes of routine cloning were HMS174 (*recA1 hsdR rif*) (Campbell *et al.*, 1978), DH5 (*recA1 endA1 thi-1 hsdR17 supE44 gyrA relA1* $Nal^r$), and DH5α (same as DH5, *Δ(lacZYA-argF)* U169 *780dlacZΔM15*). Hybrid E1 plasmids were generated in strain LE392 (*hsdR514*($r_K^- m_K^+$) *supE44 supF58 lacY galK2 galT22 metB1 trp55 mcrA*). To isolate non-methylated plasmids, strain GM48 (*dam*$^-$ *dcm*$^-$) was used (Murray *et al.*, 1977).

### 2. Manipulation of bacteria.

Bacteria were grown in Luria-Bertani (LB) broth (10 g/l bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.4) at 37°C. Cultures from frozen stocks or isolated antibiotic resistant colonies were grown in 5 ml LB broth overnight in sterile test tubes with constant shaking. For DNA extraction, each 500 ml culture was inoculated with 1 ml of overnight culture, and incubated at 37°C for approximately 18 hrs in 2 l flasks with constant shaking. To select for bacteria containing plasmids with ampicillin resistance markers, broth was supplemented with 100 μg/ml ampicillin. Samples of overnight cultures were stored in broth supplemented with 20% glycerol, at -80°C.

3. Transformation of bacteria.

Several different techniques to transform *E. coli* were used during this study, as more efficient and convenient techniques became available. i). Initially, log phase cultures were chilled on ice and concentrated from approximately 100 ml of culture to a 2.0 ml suspension of bacterial cells in 50 mM $CaCl_2$, 10 mM Tris pH 8.0 (Cohen *et al.*, 1972). 200 µl aliquots of these bacteria were combined with DNA (when feasible, quantities were within the range of 10-100 ng of DNA) and incubated on ice for approximately 30 minutes. Bacterial cells were then heat shocked at 42°C for 90-120 sec. and briefly placed back on ice. These cells were diluted with LB to 2.0 ml, incubated at 37°C for up to 1 hr, and plated on LB plates (LB broth, 1.5% bacto-agar, 200 µg/ml ampicillin). ii). For higher transformation efficiencies, the method of Hanahan (Maniatis *et al.*, 1982, p254) was adopted for bacterial transformation. iii). For convenience, frozen competent were prepared as follows: *E. coli* strain DH5 cells were pelleted from log-phase cultures, washed in 10 mM PIPES pH 6.8, 10 mM RbCl (Solution A), pelleted, resuspended in 10 mM PIPES pH 6.8, 10 mM RbCl, 75 mM $CaCl_2$ (Solution B), pelleted again, and finally resuspended in a modified solution B containing 15% glycerol. These cells were frozen in 0.5 ml aliquots in liquid nitrogen, and transformed similarly to the manner described in part i. iv). Cells were prepared for electroporation by chilling 1 l of log-phase *E. coli* strain DH5, pelleting the cells, followed by two washes with 100 ml of 10% glycerol, and resuspension in 3.0 ml of 10% glycerol. This suspension was aliquoted into 100 µl samples, and frozen in a liquid nitrogen bath. Cells were stored at -80°C for

extended periods. Upon thawing, 50 µl aliquots of bacteria were each placed in a 0.2 cm-electrode gap electroporation cuvette (BioRad) along with approximately 10 ng of DNA and electroporated at 2.25 kV, 200 Ω, 25 µFD. This suspension was diluted in 1.0 ml of LB broth, further serial dilutions were prepared as appropriate, and the cells were plated as described above. In general, electroporation yielded the most transformants per µg of DNA.

4. Extraction of DNA from *E. coli.*

A modification of the alkaline lysis method described by Birnboim and Doly (1979) was used to extract DNA from *E. coli.* To screen colonies of *E. coli* for the presence of recombinant plasmids, 5.0 ml cultures of antibiotic resistant bacteria were grown overnight, at which time 1.5 ml from each culture was transferred to a polypropylene microcentrifuge tube (Eppendorf). These bacteria were pelleted, and resuspended in 100 µl of 25 mM Tris pH 8.0, 50 mM glucose, 10 mM ethylene-diamine tetraacetic acid (EDTA), 5 mg/ml lysozyme. After 20 min on ice, 200 µl of 0.2 M NaOH, 1% sodium dodecyl sulfate (SDS) was added to each tube, mixed, and left on ice for another 10 min. Finally, 150 µl of 3.0 M sodium acetate pH 4.8 was added to the samples, and left on ice for 45 min. The precipitate was discarded, and DNA was precipitated from the supernatants by the addition of 1 ml ethanol. The DNA was de-salted by re-suspension in 50 mM ammonium acetate, and a second ethanol precipitation. The pellets were then washed a final time in ethanol and dried thoroughly. Finally, the

samples were re-suspended in 100 µl of 10 mM Tris pH 8.0, 0.5 mM EDTA (TE buffer). Aliquots of 5 to 10 µl were used for restriction analysis.

For large scale extractions of DNA, overnight 500 ml cultures were grown, pelleted, and lysed by a scaled-up version of the lysis protocol described above. Upon resuspension of the DNA in 50 mM ammonium acetate, 2.0 ml of pronase solution (described in section F.2) was added to 8.0 ml of DNA/50 mM ammonium acetate solution. This was incubated at 37°C for 1 hr, and precipitated with ethanol. The sample was allowed to dry partially, and resuspended in 50 mM Tris pH 8.0, 10 mM EDTA. The extract was then supplemented with 1 g/ml of CsCl, 0.25 g/ml ethidium bromide, and centrifuged for 16 hrs at 55,000 rpm in a Beckman vTi 65.1 rotor. The band of higher density, corresponding to supercoiled plasmids, was collected with an 18G needle attached to a 3 ml disposable syringe. The ethidium bromide was removed from this solution by three extractions with 2 volumes of 1-propanol saturated with water and CsCl. The sample was then diluted from an original volume of under 1 ml to 4 ml with 50 mM ammonium acetate, and precipitated with ethanol. The pellet was dried and dissolved in TE buffer.

## B. Plasmids

### 1. Cloning vectors.

Both pBR322 and pUC19 were used in this study. pBR322 contains the col E1 origin of replication and the genes for resistance to ampicillin and tetracycline (Bolivar *et al.*, 1977). pUC19 contains a modified col E1 origin of replication, a fragment of the *lac Z* gene, and a polylinker of unique restriction sites interrupting *lac Z* coding sequences but not disrupting the reading frame (Viera and Messing, 1987).

### 2. Adenovirus type 5 plasmids.

Several plasmids containing cloned Ad 5 sequences were used in this study (shown in figure 2-1). pXC1 (McKinnon et.al., 1982) contains the leftmost 16.0% (*Xho* I-C fragment) of the Ad 5 genome in pBR322, with the left terminal viral sequences linked to the vector *Bam* HI site, and the Ad 5 *Xho* I site fused to the *Sal* 1 site of pBR322, resulting in the elimination of both restriction sites. A derivative plasmid, pXC38, contains a deletion of pBR322 sequences between the *Bam* HI and *Hind* III sites. A *Bam* HI linker was inserted into the *Hpa* II site of pXC38 at position 548, to form pXC0548 (Bautista *et al.*, 1991). For the purposes of this study, both pXC38 and pXC0548 were linearized at their unique vector *Eco* RI sites, the overhangs filled in with the Klenow fragment of DNA polymerase 1 (referred to as Klenow) and re-ligated, resulting in the elimination of the *Eco* RI restriction site. In later experiments, pXC38 was

further linearized at its pBR322 derived *Cla* I restriction site, treated with Klenow and religated to eliminate the *Cla* I site. For rescue of hybrid E1 sequences into infectious Ad 5 dl309, pJM17 (McGrory *et al.*, 1988) was used. This plasmid contains all of dl309 as a circle with the viral ITRs joined covalently head to head, and an insert of a pBR322 derivative, pBRX, in the unique *Xba* I site at 3.8 map units of the dl309 genome. When transfected into 293 cells, pJM17 presumably replicates as a linear viral chromosome, but the prohibitive size of the viral DNA molecule (4.4 kb larger than the wt genome) precludes its packaging into infectious virions. Homologous recombination with a cotransfected E1 plasmid results in a genome of packageable size, thus providing a relatively efficient selection mechanism for the rescue of altered E1 sequences.

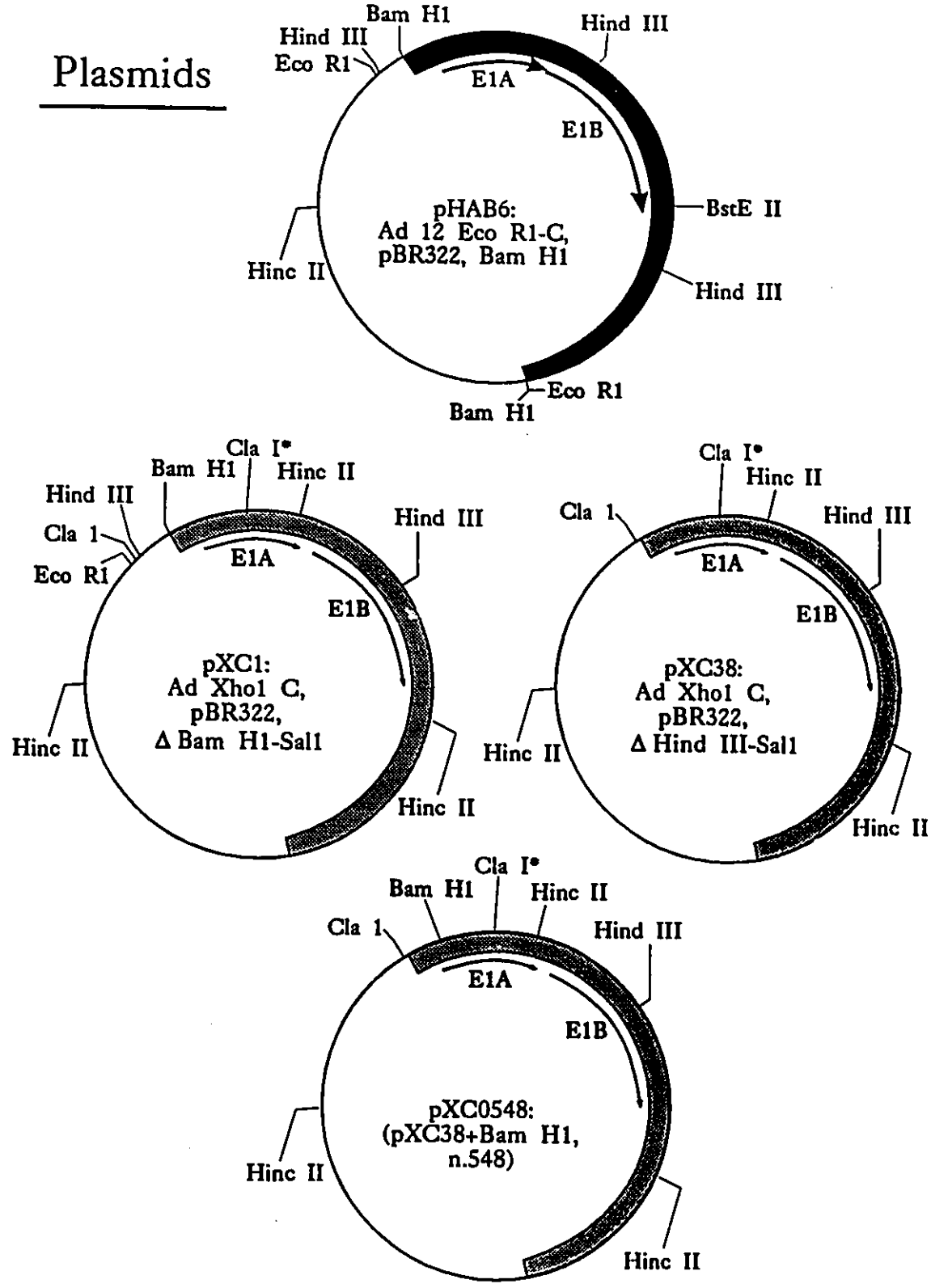
3. Adenovirus 12 plasmids.

The E1 region (leftmost 16%) of Ad 12 (Huie strain) is contained in pHAB6 (Mak *et al.*, 1986) as a cloned viral *Eco* RI-C fragment in the *Bam* HI site of pBR322, and was obtained from S. Mak (figure 2-1). The orientation of the insert is analogous to that of pXC 1 and pXC38, with the Ad 12 E1 sequences oriented clockwise relative to the conventional representation of pBR322. pHAB13 was derived from pHAB6 by deletion of E1A sequences. It contains the E1B transcription unit, and was obtained from S. Mak.

**Figure 2-1. E1 Plasmids used.**

Shown are the plasmids encoding early region 1 of Ad 5 and Ad 12. pHAB6 contains the *Eco* RI C-fragment of Huie strain Ad 12 (described in text), while pXC1 contains the *Xho* I C-fragment of Ad 5. Two derivatives of pXC1 are pXC38 (deletion of pBR322 sequences between *Bam* HI and *Hind* III sites), and pXC0548 (pXC38 with *Bam* HI site at position 548 of E1 sequences) (Bautista and Graham, 1989). Both pXC38 and pXC0548 were linearized with *Eco* RI and repaired with Klenow prior to re-ligation, to eliminate the pBR322 *Eco* RI site. **Cla* I site resistant to digestion due to methylation.

# Plasmids


Bam H1
Hind III
Eco R1
E1A
Hind III
E1B
BstE II
pHAB6:
Ad 12 Eco R1-C,
pBR322, Bam H1
Hinc II
Hind III
Eco R1
Bam H1
Cla I*
Bam H1
Hinc II
Hind III
Cla 1
Eco R1
E1A
Hind III
E1B
pXC1:
Ad Xho1 C,
pBR322,
Δ Bam H1-Sal1
Hinc II
Hinc II
Cla I*
Hinc II
Cla 1
E1A
Hind III
E1B
pXC38:
Ad Xho1 C,
pBR322,
Δ Hind III-Sal1
Hinc II
Hinc II
Cla I*
Bam H1
Hinc II
Cla 1
Hind III
E1A
E1B
pXC0548:
(pXC38+Bam H1,
n.548)
Hinc II
Hinc II

4. Generation of hybrid E1 plasmids.

Plasmids containing tandem repeats of Ad 12 and Ad 5 E1 sequences were grown in *E. coli* LE 392 as 5 ml cultures for 48 hours, to permit homologous recombination. Following this, 1 l cultures were inoculated, and DNA was extracted. The DNA was digested to completion with enzymes whose recognition sites were predicted to be deleted as a result of homologous recombination events. The digests were then used to transform bacteria *de novo*. Potential recombinants were screened by restriction analysis on agarose gels, to eliminate plasmids with sizes not compatible with the predicted size of homologous recombinants. Crossovers were mapped to E1A or E1B by further restriction analysis, and the putative hybrid E1A plasmids were further screened by DNA dot-blot hybridizations to localize the crossover site, and ultimately by DNA sequence analysis.

## C. DNA Manipulation Techniques

1. Synthetic oligonucleotides.

All synthetic oligonucleotides used in this study were synthesized by the central facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

2. Restriction enzymes.

Most restriction digests were carried out for 1-2 hr at 37°C in a standard

buffer of 10 mM Tris pH 7.5, 50 mM KCl, 8 mM $MgCl_2$ using approximately 2U of enzyme per μg of DNA for analytical purposes. Digests with *Kpn* I and *Sma* I were done in buffer supplied by the manufacturer. For cloning purposes, larger quantities of DNA were digested for 4-5 hr using approximately 0.5U of enzyme per μg of DNA.

3. Ligations.

Ligation of DNA fragments was carried out at 14°C for 12-18 hr, or at room temperature for 0.5-2.0 hr in ligation buffer (20 mM Tris pH 7.6, 10 mM MgCl2, 10 mM DTT, 0.5 mM ATP), with 1U of T4 DNA ligase. To ligate synthetic oligonucleotides to digested plasmid DNA, the synthetic oligonucleotides were phosphorylated, and in some cases the plasmid DNA was dephosphorylated.

4. Repair/Proofreading of DNA termini.

In order to ligate DNA molecules with incompatible termini, the DNA was first treated with the Klenow fragment of DNA polymerase I (referred to as Klenow), resulting in blunt ends. Incubations were carried out with 5U of Klenow for one hr at room temperature in nick translation buffer (50 mM Tris pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT, 50 μg/mL BSA fraction V, 0.25 mM dNTPs). Following the reaction, DNA was precipitated with ethanol and resuspended in TE buffer prior to ligation.

5. Dephosphorylation of DNA termini.

In cases when it became necessary to prevent vector recircularization in ligation reactions, the DNA molecules were treated with calf intestinal alkaline phosphatase to remove the phosphate groups from the 5' termini of DNA. The reactions were carried out in the buffer supplied by the manufacturer (Boehringer Manheim), at 37°C for 1 hr. Following this, the enzyme was inactivated in 10mM ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) at 65°C for 30 min and extracted with phenol-chloroform (1:1). The DNA was then precipitated with ethanol prior to further use.

6. Phosphorylation of synthetic oligonucleotides.

To label the 5' end of synthetic oligonucleotides with $^{32}P$, $2.85x10^{-11}$ mol of oligomer was incubated at 37°C in the presence of 100 μCi of γ-$^{32}P$ ATP, 30U of T4 kinase (purchased from BRL), 50 mM Tris pH 7.6, 10mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 5 mM EDTA for 1 hr. To phosphorylate synthetic oligonucleotides with non-radioactive phosphate, a similar procedure was employed, with the modification that the γ-$^{32}P$ ATP was replaced by 0.05 mM ATP. For certain applications, radiolabelled oligomers were purified from unincorporated γ-$^{32}P$ ATP by elution through a column containing Sephadex G50 beads in TE buffer. Radioactivity was monitored by Geiger counter, and the first peak eluting from the column was collected for use.

7. Agarose gel electrophoresis.

Restriction digests of plasmids were electrophoresed on 1% agarose gels in TAE buffer (40 mM Tris, pH 7.9, 5 mM sodium acetate, 1 mM EDTA), containing 15 µg/L ethidium bromide. DNA fragments were gel-purified from 0.5%-1.5% (depending on fragment size) agarose gels in TBE buffer (100 mM Tris, 80 mM boric acid, 2 mM EDTA, 15 ug/mL ethidium bromide), by electro-elution onto dialysis membrane, a modification of the technique described by Girvitz *et al.* (1980), followed by extraction with phenol-chloroform and ethanol precipitation. All agarose gels were of a horizontal slab-gel format. Unless otherwise indicated, the standard molecular weight marker used for agarose gel electrophoresis was a *Hind* III digest of DNA extracted from wild-type Ad 5. The fragment sizes are as follows: **A**: 8010 bp; **B**: 5665 bp; **C**: 5324 bp; **D**: 4597 bp; **E**: 3437 bp; **F**: 2937 bp; **G**: 2804 bp; **H**: 2081 bp; **I**: 1005 bp; **J**: 75 bp.

8. Polyacrylamide gel electrophoresis of DNA.

DNA fragments of less than 1000 base pairs (bp) were purified from polyacrylamide gels of varying concentrations, but with constant 30:1 ratios of acrylamide:N,N'-methylene-bis-acrylamide (bis). Gels were stained with ethidium bromide, as described in section C.7, and the fragments of interest were excised. The polyacrylamide containing the fragment of interest was crushed, and the DNA was eluted into a buffer consisting of 500 mM ammonium acetate, 10 mM Mg acetate, 1 mM EDTA, and 0.1% SDS. The solution was passed through a spin-column containing siliconized

glass wool, and the eluted solution was precipitated with ethanol.

9. Polymerase chain reaction.

Segments of DNA were synthesized by the polymerase chain reaction in some cases, to introduce restriction sites into DNA at certain locations. The Gene Amp kit supplied by Perkin-Elmer-Cetus was used for these purposes, along with the Amplitaq (DNA polymerase from *Thermus aquaticus*) enzyme. Conditions were varied based on individual primers and templates. Thermal cycling was carried out in a Perkin-Elmer-Cetus thermal cycler.

10. DNA Sequence Analysis.

Sequencing was done using the Sequenase kit (United States Biochemical) according to the instructions provided by the supplier. This technique relies on a modified form of bacteriophage T7 DNA polymerase to incorporate dideoxynucleotide triphosphate chain terminators (Sanger *et al.*, 1977). Nascent polynucleotides were labelled by incorporation of $\alpha$-$^{32}$P dATP. Electrophoretic analysis of sequencing reactions was performed on 7% polyacrylamide gels containing a 30:1 ratio of acrylamide:bis, dissolved in freshly made TBE, and 40% urea. Polymerization reactions were catalyzed by 0.1% ammonium persulfate (APS), and 40µl/100ml of TEMED. Prior to loading, sequencing gels were pre-run at 1800V for 1 hr. Aliquots of 3µl per lane were then loaded in 0.02%

bromophenol blue, 0.02% xylene cyanol, and electrophoresed at 2200V. Following electrophoresis, the gels were fixed in 5% methanol, 10% acetic acid, dried and subjected to autoradiography on Kodak XAR-5 film.

11. DNA dot blot hybridizations.

Approximately 100 ng of DNA was heat denatured, manually applied to nitrocellulose and allowed to air-dry at room temperature. The filters were then baked at 80°C for 2 hr, and pre-hybridized at room temperature for 3 hr in 6X saline sodium citrate (SSC; 1X is 0.15 M NaCl, 0.015 M sodium citrate), 10X Denhardt's solution (2g/l ficoll, 2g/l polyvinyl pirrolidone, 2 g/l bovine serum albumin) and 0.2% SDS. Labelled oligonucleotides were hybridized to the blot in the same solution for 12 hr at 37°C, followed by three washes of 45 min in 6X SSC at 37°C. Filters were air-dried at 37°C for 2 hr and exposed to Kodak XAR-5 film at -70°C with an intensifying screen for 1 hr.

## D. Protein Analysis

1. Antibodies and antisera.

Anti-tumor sera were used during this study. To obtain these sera, tumor bearing rats were anaesthetised with di-ethyl ether, and bled by cardiac puncture. Blood was pooled from tumor-bearing littermates injected with the same cell line, allowed to clot overnight at 4°C, and pelleted at 2,000 rpm. The supernatant was further cleared by

centrifugation at 15,000 rpm in eppendorf microcentrifuge tubes, and finally by filtration through a 0.2 μm filter. The serum was stored at -80°C until use.

Monoclonal antibodies were also used in this study. M73 (Harlow *et al.*, 1985; Oncogene Science) is specific for an epitope in the carboxy terminal region of the Ad 2/Ad 5 E1A protein. PAB419 is a monoclonal antibody specific for SV40 large T-antigen (Harlow *et al.*, 1981).

2. Protein A sepharose beads.

0.5 g aliquots of protein A sepharose beads (Pharmacia) were suspended in 20 ml of E1A buffer, consisting of 50 mM N,N'-hydroxyethylpiperazine-ethanesulfonic acid (HEPES), pH 7.1, 250 mM NaCl, 0.1% Nonidet P-40, as previously described (Harlow *et al.*, 1986). The beads were allowed to swell for at least 24 hrs, then pelleted and resuspended in 3 ml of the same buffer.

3. *In vivo* protein labelling.

HeLa cells infected with recombinant viruses were pre-labelled with medium 199, lacking methionine but supplemented with L-glutamine, and Pen/Strep ($Met^-$ medium; see section E for a description of media and additives) from 10 to 11 hrs post-infection, and then incubated with fresh $Met^-$ medium with 250 μCi of $^{35}$S-Translabel (containing radiolabelled Met, Cys, purchased from ICN) from 11 to 12 hrs post-infection. Following this, cells were washed with ice-cold $PBS^{++}$ prior to lysis. Care was taken to

remove all $PBS^{++}$ from the dishes.

4. Cell lysis and immunoprecipitation.

0.5 ml of ice-cold E1A buffer (section D.2), supplemented with the protease inhibitor aprotinin and phenyl-methyl-sulfonyl-fluoride (PMSF), was added to each infected and labelled 60 mm dish of cells, and maintained on ice for 30 min. The cells were then scraped from the dish and collected in Eppendorf tubes. These tubes were spun for 15 min at 15,000 rpm in a microcentrifuge located in a 4°C cold room. The supernatant was then decanted into eppendorf tubes containing 50 µl of protein A sepharose beads, and the appropriate antibody. The samples were rotated for various amounts of time at 4°C, and pelleted. The protein A sepharose beads were then washed with E1A buffer several times, each cycle consisting of resuspension, varying amounts of agitation, and pelleting. Supernatants were aspirated with a kinked 23-G needle. After the final wash, the protein A beads were resuspended in SDS-PAGE loading buffer (40 mg/ml urea, 0.1 M Tris pH 6.8, 3.3% SDS, 27% glycerol, 7% β-mercaptoethanol, 0.1 mg/ml bromophenol blue), and boiled for 2 min prior to loading on gels.

5. Electrophoresis of proteins.

SDS-polyacrylamide gels were prepared by the method of Laemmli (1970), with a ratio of acrylamide:bis of 37.5:1. The concentration of acrylamide in gels varied inversely with the molecular weights of proteins which were analyzed. Running gels

contained (in addition to polyacrylamide) 0.375 M Tris pH 8.8, 0.1% SDS, while stacking gels contained 3% polyacrylamide, with 0.125 M Tris pH 6.8, and 0.1% SDS. The electrophoresis buffer in this system was one of 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. The most commonly used markers were $^{14}$C-labelled markers (Amersham), with the following molecular weights: A: 200K, B: 97.4K, C: 69K, D: 46K, E: 30K, F: 21.5K, G: 14.3K.

6. Autofluororadiography of protein gels.

Following electrophoresis, protein gels were washed twice in dimethyl sulfoxide (DMSO), for 10 min in each case, with gentle agitation. The gels were then soaked in 22% 2',5'-diphenyloxazole (PPO) dissolved in DMSO. This step was also carried out with gentle agitation, for 1 hr. To precipitate the PPO in the gel, it was rinsed with distilled water for approximately 5 min. At this point, the stacking gel was detached from the running gel and dried under vacuum with a Bio Rad gel drier at a constant temperature of 60°C for 1 hr. The dried gels were exposed to X-ray film at -80°C for varying lengths of time (Bonner and Laskey, 1974).

7. Transfer of proteins to filters.

Following electrophoresis, the stacking gel was detached from the running gel, which was itself trimmed of sections where no samples were located. The gel was then placed in transfer buffer (20 mM Tris, 150 mM glycine, pH 8.0, 20% methanol).

Meanwhile, nylon membrane (Immobilon P, purchased from Millipore) was cut to the size of the gel, and soaked for 5 min in methanol, then water, and finally transfer buffer. The membrane was then placed on the gel, and all air bubbles were eliminated. Several pieces of Whatman 3mm paper were soaked in transfer buffer, and placed on either side of the gel-membrane "sandwich". This complex was placed in a transfer tank (BioRad), held together by a plastic holder, and the tank was filled with transfer buffer. The tank was placed in a 4°C cold room, connected to a power supply with the gel facing the negative electrode, and the membrane facing the positive electrode. Current was applied to the tank for 18 hr, at a constant voltage of 15 V. Following transfer, the gel was stained in 10% acetic acid, 25% 2-propanol, 0.05% Coomassie blue for 5 min, and destained in 10% acetic acid.

8. Western blots.

Filters were immersed in 5% skim milk powder dissolved in PBS, and agitated for 2 hr. Following this, the filters were agitated with the same solution but containing diluted antibody. With monoclonal antibodies at a concentration of 1 mg/ml, a typical dilution factor was 1:1,000, whereas for tumor sera, the sera were diluted between 1:300 and 1:500. Incubations were for 1 hr at room temperature, or overnight at 4°C. Stronger signals were generally observed following overnight incubations. The filters were rinsed in phosphate buffered saline (PBS) three times for 10 min each time, with agitation. A secondary antibody solution was then applied to the blots. This solution was a mixture

of biotinylated sheep antisera directed against rat or mouse immunoglobulin (depending on the species origin of the primary antibody), and streptavidin conjugated to horseradish peroxidase (purchased from Amersham). This solution was kept in contact with the blots for 1 hr with agitation, followed by three washes with PBS. Blots were stored in PBS for periods as long as several weeks, and could be probed with alternate antibodies, provided the novel bands had distinct molecular weights.

9. Detection of proteins by enhanced chemiluminescence (ECL).

Blots in PBS were picked up with Millipore foreceps and placed face-up on a sheet of Saran Wrap, and covered with ECL solution (Amersham) for 1 min (ECL solution is a mixture of luminol and hydrogen peroxide, which in the presence of horseradish peroxidase results in the oxidation of luminol, and thereby excitation of electrons. Decay to the ground state results in the emission of light). The blots were then placed face-down on a second sheet of Saran Wrap, covered with a piece of overhead transparency, and placed in a film cassette. The blots were exposed to X-ray film for varying lengths of time, from 1 min to 60 min.

## E. Culturing of Cells and Viruses

1. Cells and Reagents.

Human 293 cells are an embryonic kidney cell line transformed by Ad 5 DNA

(Graham *et al.*, 1977) that constitutively express all E1 proteins necessary for the growth of Ad 5. These cells were grown in monolayers on plastic dishes (Nunclon, distributed by GIBCO), in F-11 minimum essential medium (MEM), supplemented with 10% newborn bovine serum (NBS). Confluent cells were passaged by two washes with lukewarm citric saline solution (15mM sodium citrate, 130mM KCl), then left in citric saline for up to 10 min, or until they lifted off the plates. For transfections and plaque assays, 293 cells were seeded in 60mm plastic plates (Corning), in α-MEM supplemented with 10% NBS.

HeLa cells were maintained in plastic dishes in α-MEM supplemented with 10% newborn bovine serum. Confluent monolayers were washed once with lukewarm citric saline, and treated with trypsin until they lifted off the plates. New plates were seeded with cells at a ratio of approximately 1:5. For plaque assays, HeLa cells were seeded in 60 mm dishes at a density of approximately $10^6$ cells per dish, one day prior to infection.

The Ad 12 transformed human embryonic kidney cell line MH12-C2 was obtained from S. Mak. Its growth properties are very similar to 293 cells, and it was grown in exactly the same manner as 293s.

BHK 21 cells are cloned from a culture of baby hamster kidney cells, with fibroblastic morphology, and were maintained in α-MEM supplemented with 10% newborn bovine serum. These cells were passaged at a ratio of 1:10, by one wash with citric saline followed by trypsinization.

Cultures of primary baby rat kidney cells (BRK) were established from 6-day old hooded lister rats by mincing of kidneys followed by trypsinization. The resulting suspension was filtered through sterile cheesecloth, and cells were pelleted, and resuspended in α-MEM (GIBCO) supplemented with antibiotics, and 10% fetal bovine serum. Cells were transfected by the calcium phosphate technique (Graham and van der Eb, 1973; Wigler *et al.*, 1978) when approximately 60% confluent. After an additional 3 days post transfection, the cultures were maintained in Joklik's medium with 5% horse serum. In some experiments, primary BRK cells were prepared and concentrated to $10^8$ cells per ml in α-MEM with 10% FBS and 5 mM BES pH 7.1 (Sigma), electroporated in 0.25 ml aliquots, and then plated on 60 mm dishes. Selection for transformed cells expressing E1A and E1B was effected by incubation in Joklik's medium (GIBCO) supplemented with antibiotics and 5% horse serum. After 3-4 weeks, transformed colonies were isolated from separate 60 mm dishes with the use of stainless steel cloning cylinders, and established as cell lines, first in α-MEM with 10% fetal bovine serum, and subsequently in α-MEM with 10% newborn bovine serum. Samples from each cell line were concentrated in fetal bovine serum with 10% dimethyl sulfoxide (DMSO), and frozen in liquid nitrogen for long-term storage.

All media were purchased from GIBCO, in powdered form. In addition to the media listed above, medium 199 (met$^-$) was used for labelling cells with $^{35}$S-methionine. Media were supplemented with 1x Pen/Strep solution (25,000 U penicillin, 25,000 μg streptomycin per 400 ml media). In the case of BRK cells, media was supplemented with

2x Pen/Strep solution, and with 2.5 μg per ml of amphotericin B (fungizone). Pen/Strep was purchased from GIBCO, and fungizone from E. R. Squibb. Serum used in this study was purchased from GIBCO and from Bocknek. Bottles containing 500 ml of serum were thawed and subject to 56°C heat treatment for 30 min to inactivate complement.

2. Viruses.

Wild-type Ad 5 used in this study was described by Harrison *et al.* (1977). Ad 5 mutants often used include dl309 (lacking all but one Xba 1 restriction site, and containing a deletion/substitution in early region 3), and dl312 (lacking E1A) (Jones and Shenk, 1979a), obtained from T. Shenk, and ΔE1 (Gluzman *et al.*, 1982), obtained from J. Hassell. Wild type Ad 12, Huie strain was obtained from S. Mak. Recombinant viruses used in this study are described in section F.

Viral stocks were stored at -80°C in phosphate buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$), supplemented with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ ($PBS^{++}$), and 10% glycerol.

3. Propagation of Viruses.

Plaques appearing on 60 mm dishes of 293 cells were isolated by punching plugs of media/agarose over the area of the plaque with a sterile Pasteur pipette, and crushing them in small glass vials containing $PBS^{++}$ and 10% glycerol. These plaque isolates were used to infect confluent 60mm dishes of 293 cells, which were allowed to

proceed to full cytopathic effect (CPE). The media from these dishes were used to infect 150 mm dishes of 293 cells. When CPE was complete, cells were scraped from the dishes with sterile silicone scrapers and pelleted in 50 mL polypropylene centrifuge tubes at 2 000 rpm. Cell pellets containing high concentrations of virus were resuspended in $PBS^{++}$ with 10% glycerol, and frozen and thawed three times before use. Virus stocks were stored at -80°C for extended periods of time.

4. Titration of viral stocks.

To determine the infectious titres of viral stocks, confluent 60mm dishes of 293 cells were infected with serial dilutions of virus in $PBS^{++}$ at room temperature for 1 hr. Following this absorption period, the infected cells were overlaid with 10 ml per dish of MEM (F-11) supplemented with 5% horse serum, 1x Pen/Strep, fungizone, and 0.5% agarose, which had been equilibrated to 37°C and mixed with the media. The overlay was allowed to solidify at room temperature for 1 hr, and the cells were placed in an incubator for two weeks. At that time, plaques were counted by visual examination of the plates, and titres were estimated based on the average of duplicate dishes at the dilution which yielded the maximum number of countable plaques.

5. Plaque assays.

HeLa cell monolayers were infected and overlaid in a manner similar to that described for 293 cells in section A-4, and incubated for two weeks, at which time the

dishes were overlaid with a further 5 ml of overlay containing 0.03 mg/ml of neutral red vital dye (GIBCO). White plaques were visualized against the red background after another 3 days, and titres were estimated as described above. MH12-C2 cells were infected and analyzed in exactly the same manner as 293 cells.

6. Virus growth assays on BHK 21 cells.

Confluent 60mm dishes of BHK 21 cells were infected with $10^5$ plaque forming units of virus, and maintained in liquid medium for five days. At that point, the cells were washed with $PBS^{++}$, then scraped from the dishes and suspended in $PBS^{++}$ and 10% glycerol. These suspensions were subject to three cycles of freezing and thawing, and titrated on 293 cells.

7. Host shutoff assays.

HeLa cells were infected with different viruses at multiplicities ranging from 10 to 100 plaque forming units per cell. At 48 hr post-infection, medium was replaced with medium lacking in methionine, and the cells were incubated for 1 hr. The cells were then labelled with 5 μCi of $^{35}$S-methionine per 60 mm dish for 30 min, and lysed with 0.5 ml SDS-PAGE loading buffer (section D.4). The lysate was invariably viscous after harvesting, and was passaged several times through a 26-G needle to shear cellular chromatin. 40 μl of this lysate was loaded on a 10% SDS-polyacrylamide gel and electrophoresed. The gel was then processed as described above.

## F. Construction Of Recombinant Viruses

### 1. Transfections.

60 mm plates of 70% confluent 293 cells were transfected by the calcium phosphate technique (Graham and van der Eb, 1973). Precipitate was formed with salmon sperm or sheared hamster carrier DNA (5 μg per 60 mm dish), a hybrid Ad 5/Ad 12 E1 plasmid, and pJM 17 (McGrory *et al.*, 1987, see figure 2-2). 10 μg of pJM 17 and hybrid E1 plasmid was added to each 60 mm dish, and the precipitate was left on the cells approximately 5 hrs. The medium was then aspirated, and the cells were overlaid as described in section A-4. Plaques began to appear after 5 days, and were picked after 7 days.

### 2. Analysis of recombinant viral DNA.

Plaque isolates derived from transfections were used to infect confluent 60 mm dishes of 293 cells. When these dishes reached complete CPE, they were lysed in 10 mM Tris pH 7.4, 1 mM EDTA, 0.4% SDS, and 0.5 mg/ml pronase (mixture of non-specific proteolytic enzymes). Digestion with pronase was allowed to proceed overnight at 37°C. The samples were then extracted with phenol-chloroform while vortexing vigorously to preferentially shear cellular DNA, and the DNA was precipitated with

ethanol. Restriction analysis on agarose gels was then used to determine whether the plaque isolate had the correct genotype.

3. Plaque purification.

When the restriction pattern obtained from a plaque isolate corresponded to that expected for a recombinant virus, the original plaque isolate was serially diluted and used to infect 60 mm dishes of 293 cells. Those plates were overlaid, and plaques were allowed to form. Well isolated plaques were isolated and this process was repeated a further time. This is subsequently referred to as plaque purification.

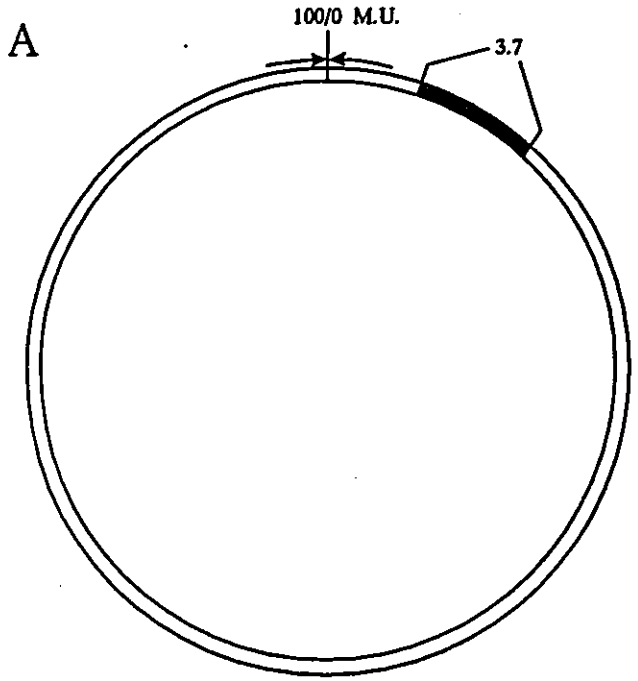
## G. Transformation and Tumorigenicity

1. Transformation of BRK cells.

For transformation of BRK cells by the calcium phosphate technique, salmon-sperm DNA was used as a carrier. The calcium-DNA precipitate was placed on 60% confluent primary BRK cell cultures, and incubated for approximately 16 hours before the medium was replaced. To transform BRK cells by electroporation, the cells were prepared in the conventional manner by trypsinization of 6-day old rat kidneys, then pelleted at 2 000 rpm for 5 minutes in a Beckman GPR centrifuge, and resuspended in a small volume of α-MEM with 10% fetal bovine serum and 5 mM BES to a cell concentration of approximately $10^8$ cells per mL. 250 μL of this cell suspension was aliquoted into Bio-

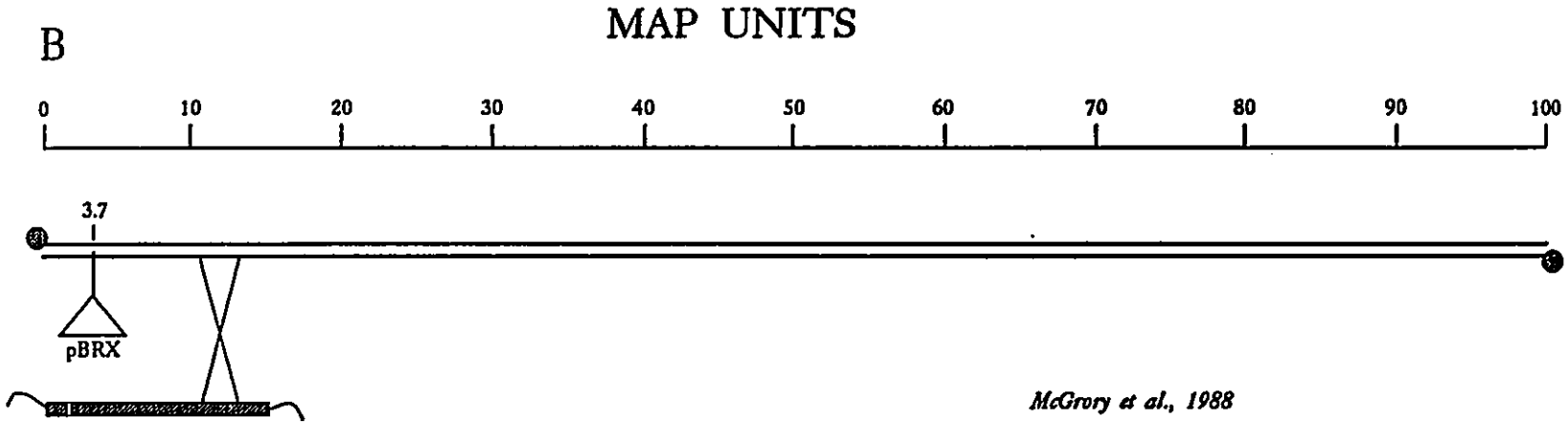
**Figure 2-2. pJM17.**

pJM17, shown at right, contains the entire Ad 5 dl309 genome in a circle with the viral termini joined in an inverse orientation, and an insert of pBRX (a 4.4 kb pBR322 derivative with an *Xba* I site added) in the unique *Xba* I site of the viral genome. Following transfection into 293 cells, the viral DNA linearizes and replicates but cannot be packaged because the total size exceeds the packaging capacity of adenovirus capsids. Also shown is a recombination event between pJM17 and an E1 plasmid bearing a lesion. This type of event can give rise to a genome of a smaller size which can be packaged. Since 293 cells can support the growth of viruses with defective E1 regions, this technique can rescue E1 mutations into infectious virus.




A. Transfection of pJM17 results in DNA replication, but not encapsidation, due to the pBRX insert.

B. Recombination with a cotransfected E1 plasmid bearing a lesion results in encapsidation of the smaller, recombinant viral genome.




*McGrory et al., 1988*

Rad electroporation cuvettes (0.4 cm electrode gap) containing appropriate amounts of DNA, and electroporated at 220V, 960 μFD by a Bio-Rad Gene Pulser. The cell suspension was incubated at room temperature for a further 10 minutes before being removed from the cuvettes, diluted in α-MEM, and plated on 4x60 mm plates from each cuvette (E. White, personal communication).

The following day, the medium was replaced with new α-MEM, supplemented with 10% FBS, 2x Pen/Strep and fungizone. On approximately the third day after transfection, or when the cells became confluent, the medium was changed to Joklik's medium, supplemented with antibiotics and 5% horse serum. Medium was changed twice weekly until transformed colonies became visible. To quantitate the transforming activity of recombinant plasmids, dishes transfected with that plasmid were placed in 75% methanol, 25% acetic acid for 30 min, to fix the cells, then dried, and stained with 5% Giemsa in PBS for 30 min, rinsed, and dried (Mak and Mak, 1983).

2. Tumorigenicity assays.

Selected cell lines were injected sub-cutaneously, mid-dorsally into syngeneic Hooded-Lister rats. When newborn rats were injected, the litter of rats as well as the mother and the cage were doused liberally with talcum powder to cover up any odours foreign to the mother. Rats were monitored for tumors for a period of as long as six months. However, with few exceptions, tumors generally appeared by approximately 4 months if at all.

3. Injection of viruses into newborn rodents.

Newborn hamsters or rats (less than 1 full day old) were injected subcutaneously with $10^8$ PFU of virus, and treated as described in section G-2 above.

# RESULTS

## Rationale

The E1 regions of both Ad 5 and Ad 12 are critical for transformation of primary cells, but in spite of the fact that proteins encoded by the E1 genes of the 2 serotypes are very similar, cells transformed by these adenoviruses display significant phenotypic differences. Taking advantage of the conserved structures, and to a lesser degree, the sequence conservation between the Ad 5 and Ad 12 E1 regions, an attractive approach to analyze the differences between cells transformed by Ad 5 and Ad 12 is the construction and analysis of hybrid proteins. If successful, this approach would help to map the properties which differ between Ad 5 and Ad 12. Preliminary work of this kind has previously been undertaken by constructing hybrid E1A genes using common restriction sites (Jochemsen *et al.*, 1984). However, splicing fragments generated by preexisting restriction sites cannot generate the number of hybrid E1 regions necessary for precise mapping of biological properties. Instead, homologous recombination in *E. coli* was employed to generate multiple hybrids with crossovers in regions of homology.

## A. Approaches To Hybrid Construction

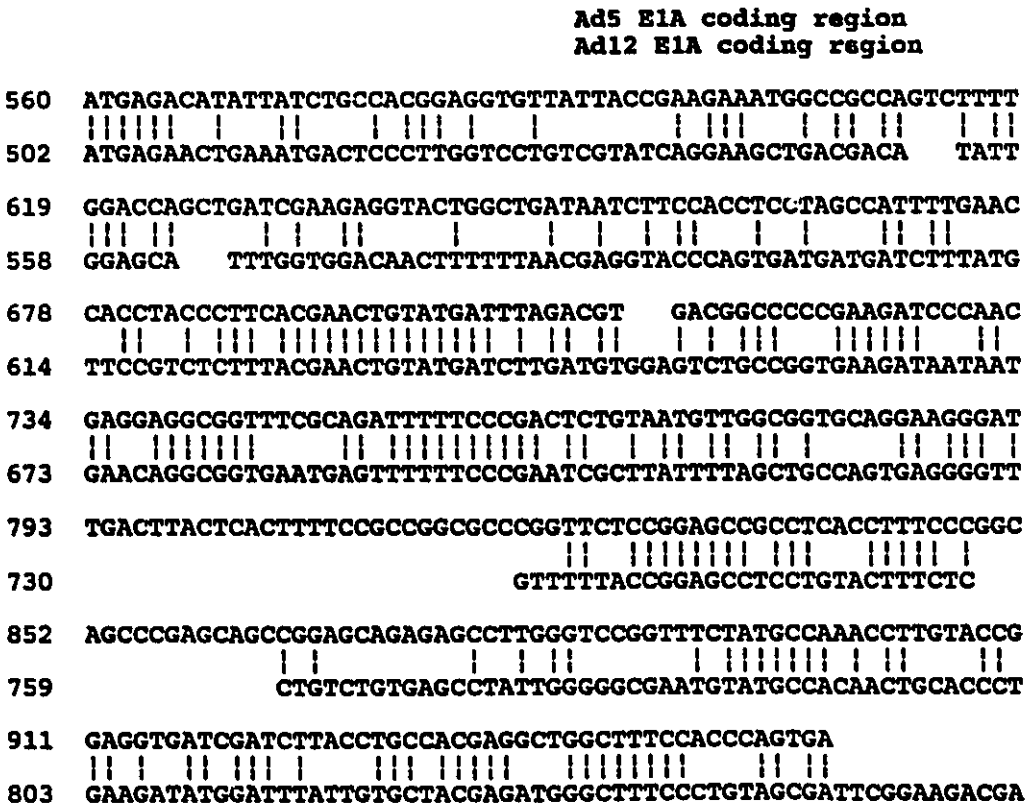
An alignment of Ad 12 and Ad 5 E1A DNA sequences (figure 3-1) indicates a homology of approximately 50%, with typical patch homologies on the order of ten base pairs in length. It was not clear whether this degree of homology would readily allow the isolation of multiple recombinants, so several approaches were employed in attempts to generate recombinant E1 regions.

### 1. Transfection of 293 cells with pJM17 and pHAB6.

In an attempt to isolate viruses containing hybrid E1 regions by recombination, human 293 cells were co-transfected with pJM17 and pHAB6. pJM17 contains the Ad 5 dl309 genome, with an insert of 4.4 kb in the E1 region, and as mentioned previously, is too large to package into a viral capsid (figure 2-2). Crossovers between pJM17 and cotransfected E1 plasmids result in smaller genomes which can be packaged (McGrory *et al.*, 1988). Plaques which arose were picked, and 38 were expanded and analyzed. Figure 3-2.A shows an example of the types of genomes which were isolated. The viral DNA in lane 3 has an abnormally large *Hind* III-G fragment, relative to the wild-type Ad 5 in lane 1. This fragment corresponds to the left-terminal genome fragment, which, in pJM17, contains the 4.4 kb insert. This restriction pattern suggests that a portion of the left terminal sequences, potentially part of the 4.4 kb insert, were spontaneously deleted from this genome, resulting in a smaller, packageable genome.

## Figure 3-1. E1A region sequence alignment.

E1A regions of Ad 5 and Ad 12 were aligned by Beckmann Microgenie software, and converted to Wordperfect format. The aligments were then edited, as Microgenie does not consider the presence of open reading frames when aligning DNA sequences. Gaps were therefore normalized in size to multiples of three, with the exception of the non-coding region of the intron. Furthermore, the software does not detect all conserved regions, so those were aligned manually.



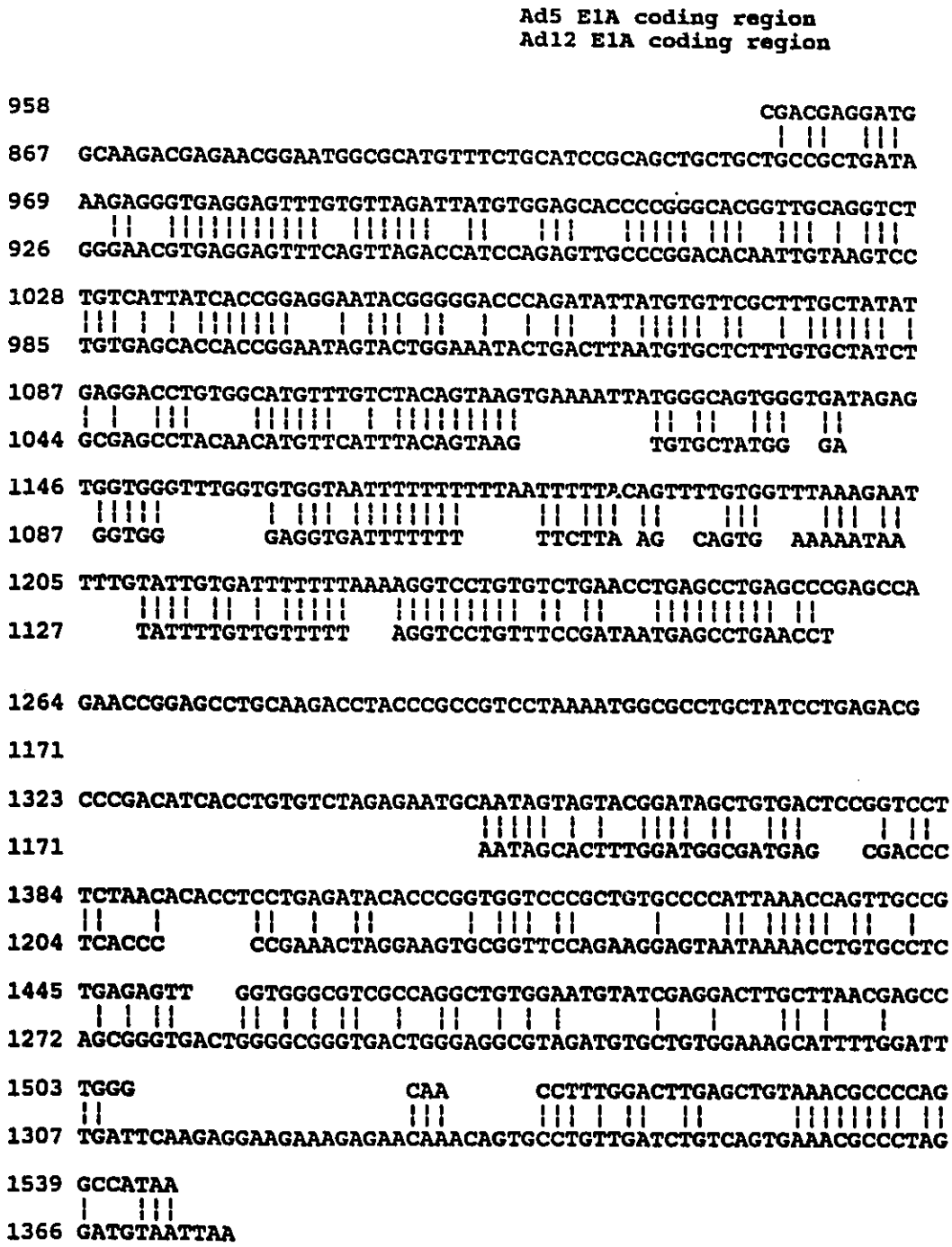
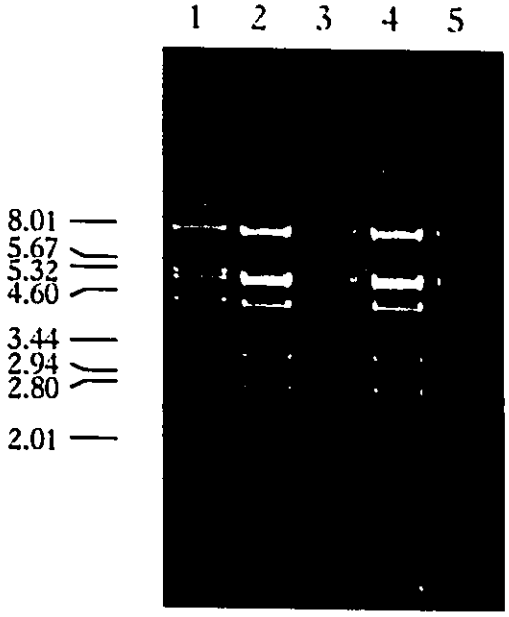

Ad5 E1A coding region
Ad12 E1A coding region
958 CGACGAGGATG
867 GCAAGACGAGAACGGAATGGCGCATGTTTCTGCATCCGCAGCTGCTGCTGCCGCTGATA
969 AAGAGGGTGAGGAGTTTGTGTTAGATTATGTGGAGCACCCCGGGCACGGTTGCAGGTCT
926 GGGAACGTGAGGAGTTTCAGTTAGACCATCCAGAGTTGCCCGGACACAATTGTAAGTCC
1028 TGTCATTATCACCGGAGGAATACGGGGGACCCAGATATTATGTGTTCGCTTTGCTATAT
985 TGTGAGCACCACCGGAATAGTACTGGAAATACTGACTTAATGTGCTCTTTGTGCTATCT
1087 GAGGACCTGTGGCATGTTTGTCTACAGTAAGTGAAAATTATGGGCAGTGGGTGATAGAG
1044 GCGAGCCTACAACATGTTCATTTACAGTAAG TGTGCTATGG GA
1146 TGGTGGGTTTGGTGTGGTAATTTTTTTTTTAATTTTTACAGTTTTGTGGTTTAAAGAAT
1087 GGTGG GAGGTGATTTTTTT TTCTTA AG CAGTG AAAAATAA
1205 TTTGTATTGTGATTTTTTTAAAAGGTCCTGTGTCTGAACCTGAGCCTGAGCCCGAGCCA
1127 TATTTTGTTGTTTTT AGGTCCTGTTTCCGATAATGAGCCTGAACCT
1264 GAACCGGAGCCTGCAAGACCTACCCGCCGTCCTAAAATGGCGCCTGCTATCCTGAGACG
1171
1323 CCCGACATCACCTGTGTCTAGAGAATGCAATAGTAGTACGGATAGCTGTGACTCCGGTCCT
1171 AATAGCACTTTGGATGGCGATGAG CGACCC
1384 TCTAACACACCTCCTGAGATACACCCGGTGGTCCCGCTGTGCCCCATTAAACCAGTTGCCG
1204 TCACCC CCGAAACTAGGAAGTGCGGTTCCAGAAGGAGTAATAAAACCTGTGCCTC
1445 TGAGAGTT GGTGGGCGTCGCCAGGCTGTGGAATGTATCGAGGACTTGCTTAACGAGCC
1272 AGCGGGTGACTGGGGCGGGTGACTGGGAGGCGTAGATGTGCTGTGGAAAGCATTTTGGATT
1503 TGGG CAA CCTTTGGACTTGAGCTGTAAACGCCCCAG
1307 TGATTCAAGAGGAAGAAAGAGAACAAACAGTGCCTGTTGATCTGTCAGTGAAACGCCCTAG
1539 GCCATAA
1366 GATGTAATTAA

**Figure 3-2. Cotransfection of pJM17 and pHAB6.**

A. Plaques isolated following transfection of 293 cells were expanded and subject to restriction analysis. Lane 1 is a *Hind* III digest of Ad 5 DNA (marker), while lanes 2 through 5 are plaque isolates. Lanes 2, 4, and 5 represent wild-type restriction patterns, while lane 3 has a G-fragment with altered mobility.

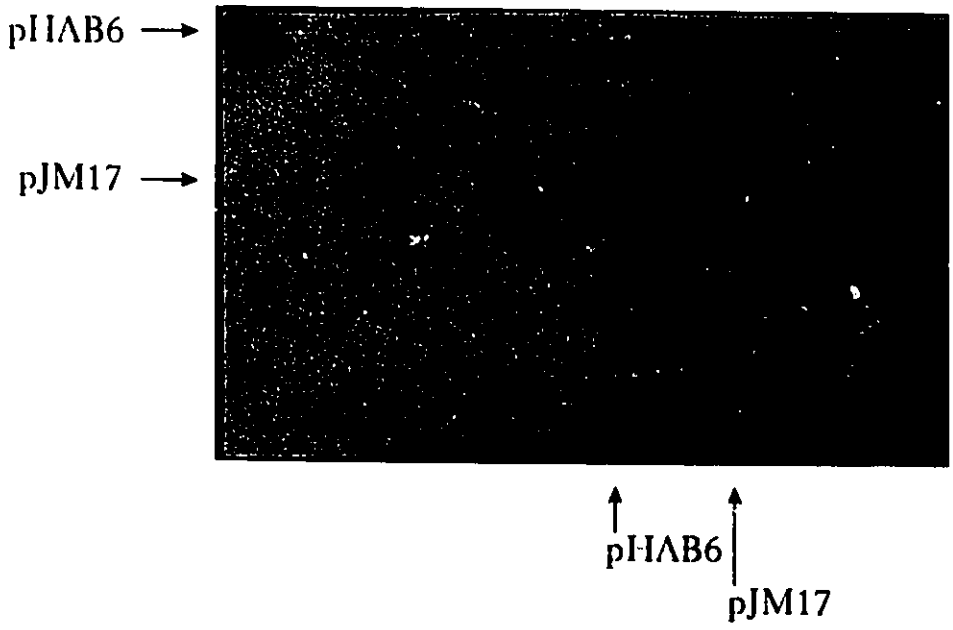
B. To analyze plaques with wild-type restriction patterns for the presence of Ad 12 sequences, DNA from each plaque isolate was blotted to nitrocellulose and probed with radiolabelled oligonucleotide as shown. The positions of control samples, pHAB6 and pJM17 (each applied to two locations) are shown. In addition, 38 viral plaque isolates were probed, but no signal was observed.

A




B

AB278: 5' CCGAAACTAGGAAGTGCG 3'



genome. Lanes 2, 4, and 5 represent restriction patterns indistinguishable from wild-type Ad 5, with the exception that the B-fragment is smaller than wild-type Ad 5. This is because pJM17 is derived from the deletion-substitution mutant dl309, which has a net deletion of DNA in the E3 region, and therefore has a smaller than wild-type *Hind* III B-fragment. These genomes therefore could represent recombinant viruses containing Ad 12 sequences from the left terminus to the crossover site, which would be located to the right of the 4.4 kb insert in pJM17 (n1339) and left of the Ad 5 E1 *Hind* III site (n2318). Alternatively, these genomes could represent viruses identical to dl309, resulting from spontaneous excision of the 4.4 kb insert. To discriminate between these possibilities, an oligonucleotide was synthesized (AB278) which was complementary to the Ad 12 sequences which are analogous to Ad 5 sequences to the left of position 1339 (shown in figure 3-2.B). DNA extracted from 293 cells infected with each plaque isolate was applied to nitrocellulose and subjected to DNA dot-blot hybridization with radiolabelled AB278. The result, shown in figure 3-2.B, indicate that the only positive signal was seen with pHAB6. This suggests that the isolated viruses do not contain Ad 12 sequences, and are rearrangements of pJM17. In fact, the ITRs of Ad 5 and Ad 12 are not highly conserved, and it would probably require a double recombination event between pJM17 and pHAB6 to generate a viable virus containing Ad 12 E1A and the Ad 5 viral terminus. The likelihood of such an occurrence is probably low, and this approach was not pursued further. Instead, intramolecular recombination in bacteria was employed to make hybrid E1 regions, a system which is both economical and rapid.

2. Construction of pTJ1.

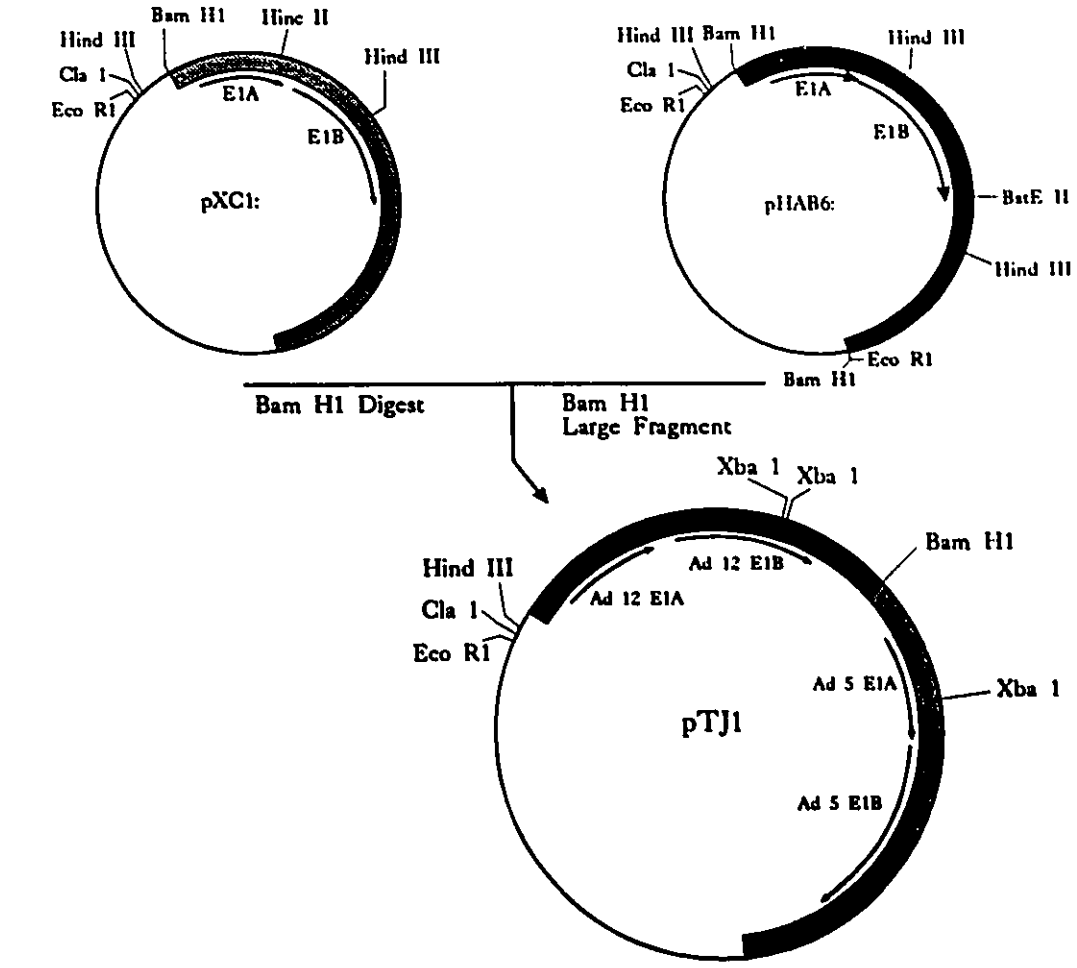
To generate hybrid E1 plasmids by intra-plasmid homologous recombination, a parental plasmid containing the E1 regions of Ad 12 and Ad 5 in tandem (pTJ1) was constructed by excising and purifying the Ad 12 E1 region from pHAB6 as a *Bam* HI fragment, and inserting it into the unique *Bam* HI site at the left terminus of the Ad 5 E1 insert in pXC1 (figure 3-3). The orientation of the insert was confirmed by restriction analysis with *Hind* III. In the forward orientation (figure 3-3, lane 2) the predicted sizes of the B and C fragments are 4.8 kb and 2.6 kb respectively, whereas in the reverse orientation (figure 3-3, lane 5) the B and C fragments have predicted sizes of 5.1 kb and 2.1 kb. The clone in lane 2 was used for further work.

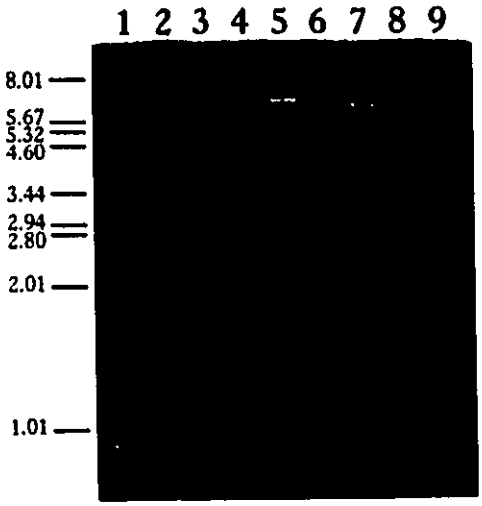
3. Generation of hybrid E1 plasmids.

To promote homologous recombination, the plasmid pTJ1, containing Ad 12 E1 in tandem with Ad 5 E1, was grown in *E. coli* strain LE 392 (Rec $A^+$), from which DNA was extracted, and digested to completion with *Xba* I, cleaving Ad 12 sequences at positions 3070 and 3183, and Ad 5 at position 1339 (figure 3-3). Using this DNA to transform *E. coli de novo* enriches for pre-existing plasmids which have recombined *in vivo*, and lack *Xba* I sites as a result of a crossover left of position 3070 of Ad 12 and right of position 1339 of Ad 5. Such plasmids are resistant to linearization by *Xba* I, and transform *E. coli* more efficiently than linearized parental plasmids. Of three clones obtained in this way, one appeared to have the size expected for a homologous

**Figure 3-3. Construction of pTJ1.**

The Bam H1 fragment containing Ad 12 E1 sequences was excised from the plasmid pHAB6 and purified, then ligated into the unique *Bam* HI site of pXC1. To distinguish between the two orientations of the insert, clones containing the insert were analyzed by *Hind* III digestion. In the orientation which puts Ad 12 sequences in tandem with Ad 5 sequences, the junction fragment of Ad 12 E1 and Ad 5 E1 gives rise to a fragment of 4.8 kb (B-fragment, lanes 2, 3, and 4). In the reverse orientation, the predicted size is 5.1 kb (B-fragment, lane 5). The other diagnostic fragment is the *Hind* III fragment spanning sequences from the pBR322 *Hind* III site to the first Ad 12 *Hind* III site. In the forward orientation, the fragment size is 2.6 kb (C-fragment, lanes 2, 3, 4) and in the reverse orientation, 2.1 kb (C-fragment, lane 5).


Hind III
Bam H1
Hinc II
Hind III
Cla 1
Eco R1
E1A
E1B
pXC1:
Hind III Bam H1
Hind III
Cla 1
Eco R1
E1A
E1B
pHAB6:
BstE II
Hind III
Eco R1
Bam H1
Bam H1 Digest
Bam H1
Large Fragment
Xba 1
Xba 1
Bam H1
Hind III
Ad 12 E1B
Ad 12 E1A
Cla 1
Eco R1
Ad 5 E1A
Xba 1
pTJ1
Ad 5 E1B


1 2 3 4 5 6 7 8 9
8.01
5.67
5.32
4.60
3.44
2.94
2.80
2.01
1.01

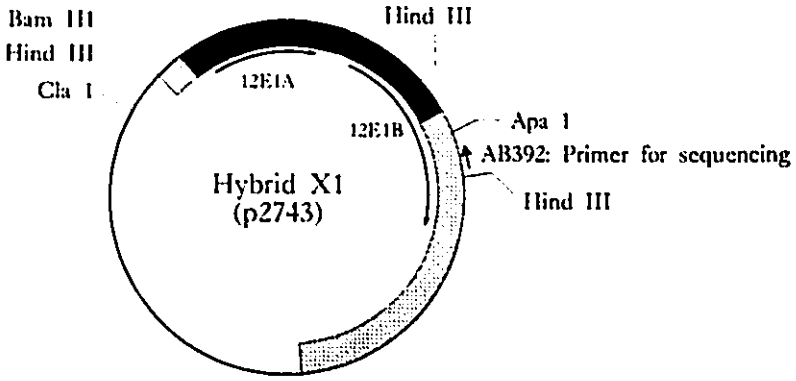
recombinant. The clone, designated X1, was characterized first using restriction analysis and was found to contain an *Apa* 1 site normally present in Ad 12 at position 2472, but to lack an *Asu* II site normally present at position 2551 of Ad 12. It was concluded that a crossover event had occurred in the intervening stretch of 81 base pairs. The corresponding region of Ad 5 sequences is nt 2690-2770. In order to sequence this region, an oligonucleotide complementary to the coding strand of Ad 5 was synthesized, with its 3' end at position 2811 (AB392). The sequence spanning the crossover site, giving rise to a hybrid E1B 55K/58K protein, is shown in figure 3-4. A homologous recombination event had occurred in a region of only four base pairs of perfect match, adjacent to a larger region of 19/22 matches. The leftmost Ad 5 nucleotide in this plasmid (in fact, a portion of the patch homology, common to both Ad 5 and Ad 12) was nt 2743, and therefore this plasmid is referred to as p2743. This system of nomenclature is used throughout this study. The isolation of p2743 implied that the sequence homology requirements for homologous recombination in *E. coli* were not overly stringent and that it should be possible to generate many hybrid E1 plasmids for further characterization.

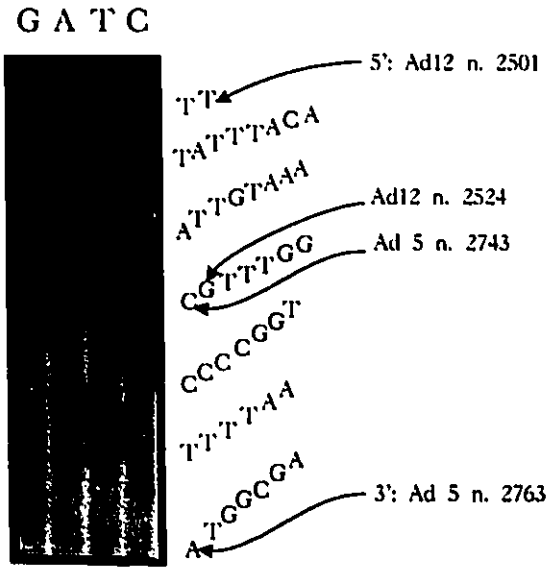
4. Construction of pTJ4.

The next objective was to design a plasmid which could be used to generate hybrid E1A genes suitable for rescue into infectious virus by cotransfection with pJM17. It is believed that an important intermediate in the replication of adenovirus DNA is a

**Figure 3-4. Sequence of X1.**

The map of the putative hybrid plasmid X1 is shown at top, indicating the presence of two *Hind* III sites (one each from Ad 5 and Ad 12), and an *Apa* I site at Ad 12 position 2472. The plasmid lacks the Ad 12 *Asu* II site normally at position 2551, suggesting that the crossover maps to the intervening 79 bp. This region was sequenced with AB392 as a primer, as described in Materials and Methods. The precise location of the crossover (bottom) is at Ad 12 position 2524, and Ad 5 position 2743.




Ad 12: CATTTATAAATGTTAGGTTTGCTGGAGATAAGT

Ad 5: TTATTATGAATGTAAGGTTTACTGGCCCCAATT

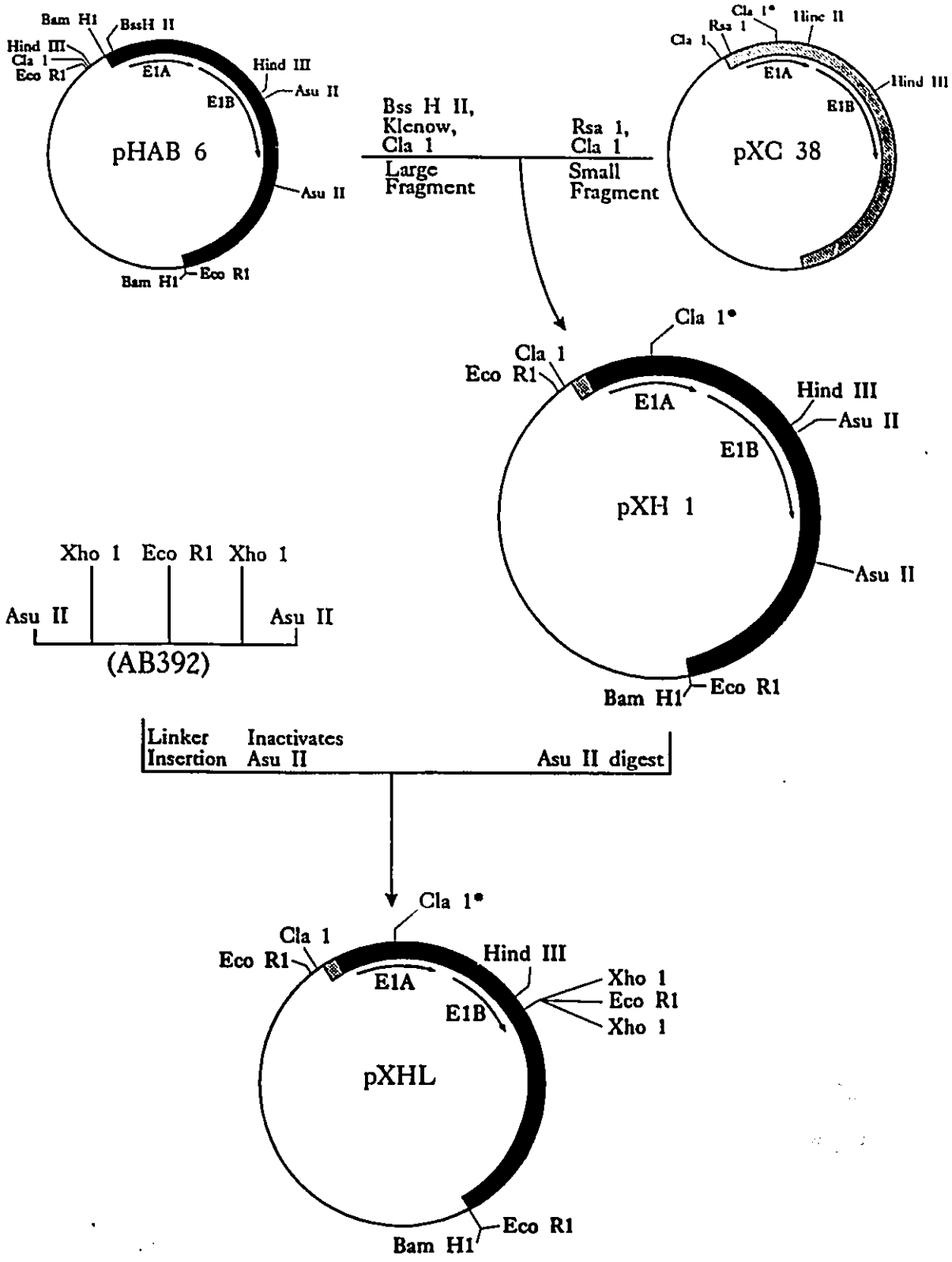
Crossover site

"panhandle" structure formed by annealing the two inverted terminal repeats (ITRs) of a displaced single strand of viral DNA (Sussenbach, 1984). Therefore in order to generate viable hybrid Ad 5 viruses with Ad 12 E1 sequences, it was decided to attach Ad 5 ITR sequences to the left terminus of the Ad 12 sequences to facilitate viral replication. Figure 3-5 shows the construction of pXH1, which contains E1 of Ad 12, but lacks sequences left of the *Bss* HII site (nt 147). In place of these sequences is the ITR-containing 193 bp left-terminal *Rsa* I fragment of Ad 5 DNA. This plasmid was cleaved with *Asu* II at positions 2551 and 3648 of Ad 12 and a linker containing an *Eco* RI site flanked by two *Xho* I sites (AB392; self-complementary) was inserted in place of the 1097bp *Asu* II fragment of Ad 12 (figure 3-5). Designated pXHL, this plasmid was cut with *Cla* I and *Bam* HI at the extremes of the viral DNA fragment, and the excised segment was inserted into a derivative of pXC0548 (linearized at *Eco* RI, blunt-ended with Klenow, re-ligated) digested with *Cla* I in its pBR322 sequences and *Bam* HI at position 548 of the Ad 5 sequences (figure 3-6). [The resulting plasmid (pTJ 3) contains Ad 12 E1 with the 193 bp terminal segment of the Ad 5 and a linker in place of sequences between positions 2551 and 3648, fused to Ad 5 E1 from nt 548 to 5788.] To reduce its overall size, pTJ3 was cleaved with *Eco* RI in the linker and just left of the *Bam* HI site that flanks the Ad 12 insert, and re-ligated, deleting the intervening sequences. The final product was pTJ4 (figure 3-5.B) which contains the Ad 5 ITR, all of Ad 12 E1A and approximately one half of the Ad 12 E1B coding sequences in tandem with the coding sequences of Ad 5 E1 but separated by unique restriction sites *Xho* I,

**Figure 3-5. Generation of pXHL.**

pHAB6 was digested with *Bss* H II, the ends were made blunt using Klenow, and the plasmid was further digested with *Cla* I, excising the terminal 147 bp of the viral genome plus 328 bp of vector sequences. In place of these sequences, the terminal 193 bp of the Ad 5 genome were inserted, from the *Rsa* I site of pXC38 to the pBR322 Cla 1 site. The resulting plasmid, pXH1, was digested with *Asu* II at Ad 12 nucleotide positions 2551 and 3648, removing a large part of the E1B coding sequences, and a linker was inserted at this location, which eliminated the *Asu* II site and introduced an *Eco* RI site flanked by two *Xho* 1 sites (pXHL).

**Cla* I site resistant to digestion due to methylation.


Bam H1
BssH II
Hind III
Cla 1
Eco R1
E1A
Hind III
Asu II
E1B
pHAB 6
Asu II
Bam H1
Eco R1
Bss H II,
Klenow,
Cla 1
Large
Fragment
Rsa 1,
Cla 1
Small
Fragment
Cla 1*
Rsa 1
Cla 1
Hinc II
E1A
Hind III
E1B
pXC 38
Cla 1*
Cla 1
Eco R1
E1A
Hind III
Asu II
E1B
pXH 1
Asu II
Bam H1
Eco R1
Xho 1
Eco R1
Xho 1
Asu II
Asu II
(AB392)
Linker
Insertion
Inactivates
Asu II
Asu II digest
Cla 1*
Cla 1
Eco R1
Hind III
E1A
Xho 1
Eco R1
Xho 1
E1B
pXHL
Bam H1
Eco R1

*Eco* RI and *Bam* HI. Restriction digests confirming the identity of pJT4 are shown in figure 3-6. The plasmid contains unique *Eco* RI and *Xho* I sites (lanes 3, 4), and gives rise to a B fragment of approximately 2.5 kb when digested with *Hind* III (lane 5), or with *Cla* I and *Bam* HI (lane 2). The structure of this plasmid was confirmed by analysis with several other restriction enzymes, and it was then used to generate hybrid E1A plasmids.

5. Isolation of E1A hybrids.

DNA of pTJ 4 extracted from *E.coli* strain LE 392 was digested to completion with *Xho* I, *Bam* HI and *Eco* RI, and used to transform *E. coli* (strategy shown in figure 3-7). DNA from the resulting colonies was analyzed by digestion with *Hind* III to screen out parental plasmids (containing two *Hind* III fragments) that escaped the selection (figure 3-8.A, lanes 2-14, 17, 18, 22-24) and plasmids of a size inconsistent with that of potential hybrids (figure 3-8.A, lane 20). The plasmids in Figure 3-8.A, lanes 15, 16, 19, and 25 were then characterized with respect to the location of crossovers in E1A or E1B by restriction analysis with *Hinc* II (figure 3-8.B), which cleaves just downstream of the Ad 5 E1A stop codon. Therefore, a plasmid with Ad 5 E1A sequences is expected to contain three *Hinc* II sites, generating bands of 5.3 kb, 2.3 kb and 1.9 kb (figure 3-8.B, lanes 3, 8, 9). When crossovers occur in E1B, only two *Hinc* II sites are present in the plasmid, resulting in bands of 5.3 kb and 4.4 kb (figure 3-8.B, lanes 2, 4, 5, 7, 10). This stage of screening also allowed the further

**Figure 3-6. Construction of pTJ4.**

pXHL was digested with *Bam* HI and *Cla* I, and the large fragment (containing Ad sequences) was inserted into pXC0548 also digested with *Bam* HI and *Cla* I. The resulting plasmid, pTJ3, was reduced in size by digestion with *Eco* RI and re-ligation, resulting in the elimination of non-E1 Ad 12 sequences (pTJ4). The structure of pTJ4 was confirmed by digestion with *Cla* I and *Bam* HI (lane 2), *Eco* RI (lane 3), *Xho* I (lane 4), and *Hind* III (lane 5). The plasmid contains two *Hind* III sites, one *Xho* I site, one *Eco* RI site, and one each of *Cla* I and *Bam* HI.

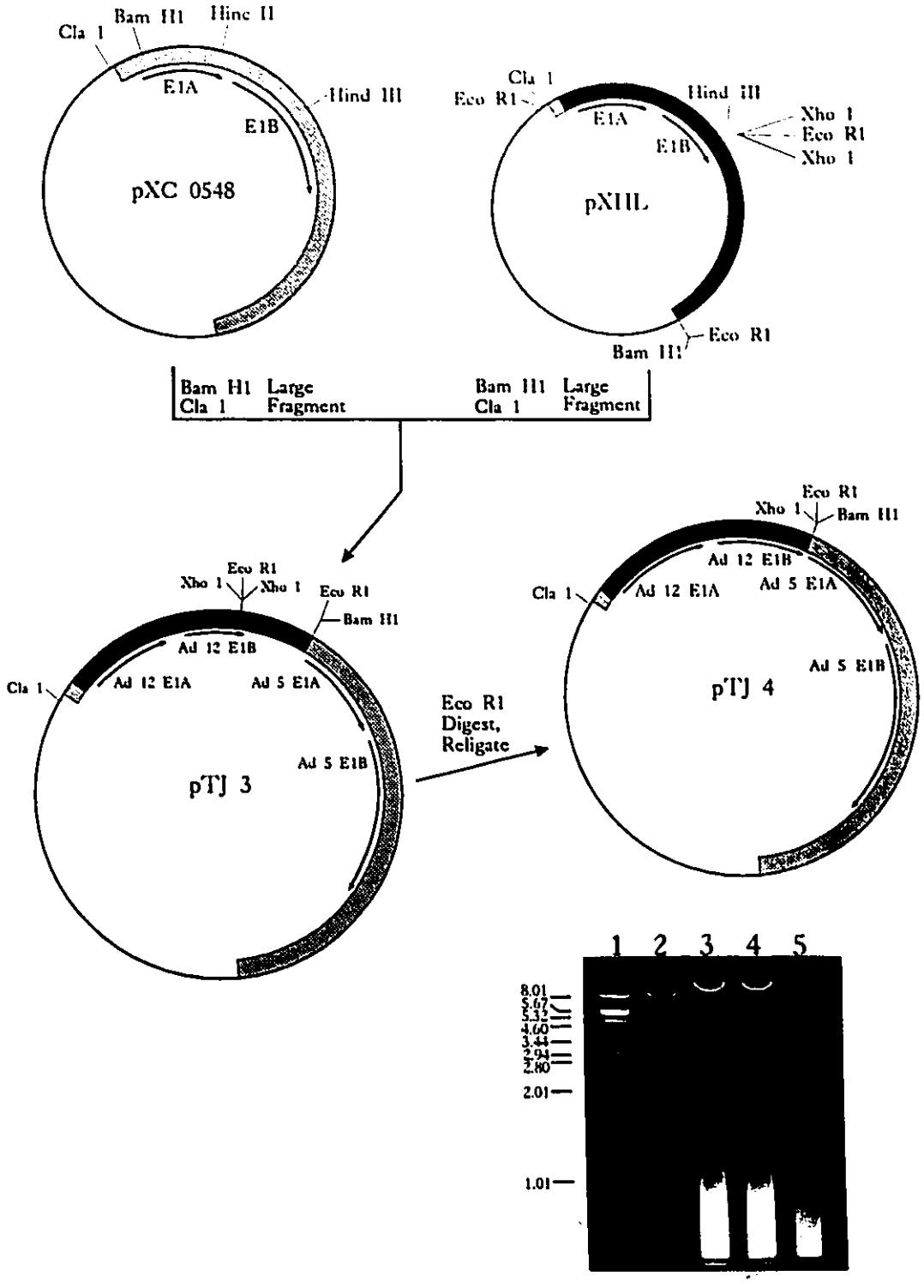

Cla 1
Bam HI
Hinc II
E1A
E1B
Hind III
pXC 0548
Cla 1
Eco R1
Hind III
E1A
E1B
Xho 1
Eco R1
Xho 1
pXIIL
Eco R1
Bam HI
Bam H1 Large
Cla 1 Fragment
Bam III Large
Cla 1 Fragment
Eco R1
Xho 1
Xho 1
Eco R1
Bam H1
Ad 12 E1B
Cla 1
Ad 12 E1A
Ad 5 E1A
Ad 5 E1B
pTJ 3
Eco R1
Digest,
Religate
Eco R1
Xho 1
Bam H1
Ad 12 E1B
Cla 1
Ad 12 E1A
Ad 5 E1A
Ad 5 E1B
pTJ 4
1 2 3 4 5
8.01
5.67
5.32
4.60
3.44
2.94
2.80
2.01
1.01

**Figure 3-7. Intraplasmid homologous recombination.**

A. pTJ4 was grown in *E.coli* strain LE 392, which is *Rec* $A^+$. B. In these bacteria, plasmids are able to undergo intramolecular recombination between sequences of partial homology aligned as direct repeats, resulting in the formation of two topologically distinct closed circles, and the loss of sequences distal to the antibiotic resistance marker and origin of replication. C. The resulting plasmid contains a crossover in a region of homology between the two parental sequences, and lacks the restriction sites and other sequences originally present between the crossover sites. In a crossover which creates a hybrid E1 region, the deletion spans the unique *Xho* I, *Eco* RI and *Bam* HI sites. Digestion of plasmid DNA extracted from these bacteria with *Xho* I, *Eco* RI and *Bam* HI results in the linearization of unrecombined parental species (A), but not of species which lack the restriction sites. Transformation of *E. coli* strain DH 5 with this digest enriched for plasmids which remained circular, predominantly ones which had undergone recombination. Clones were selected in this way, and following an initial screening step by restriction analysis (figure 3-8), and DNA dot-blot hybridization (figure 3-9).

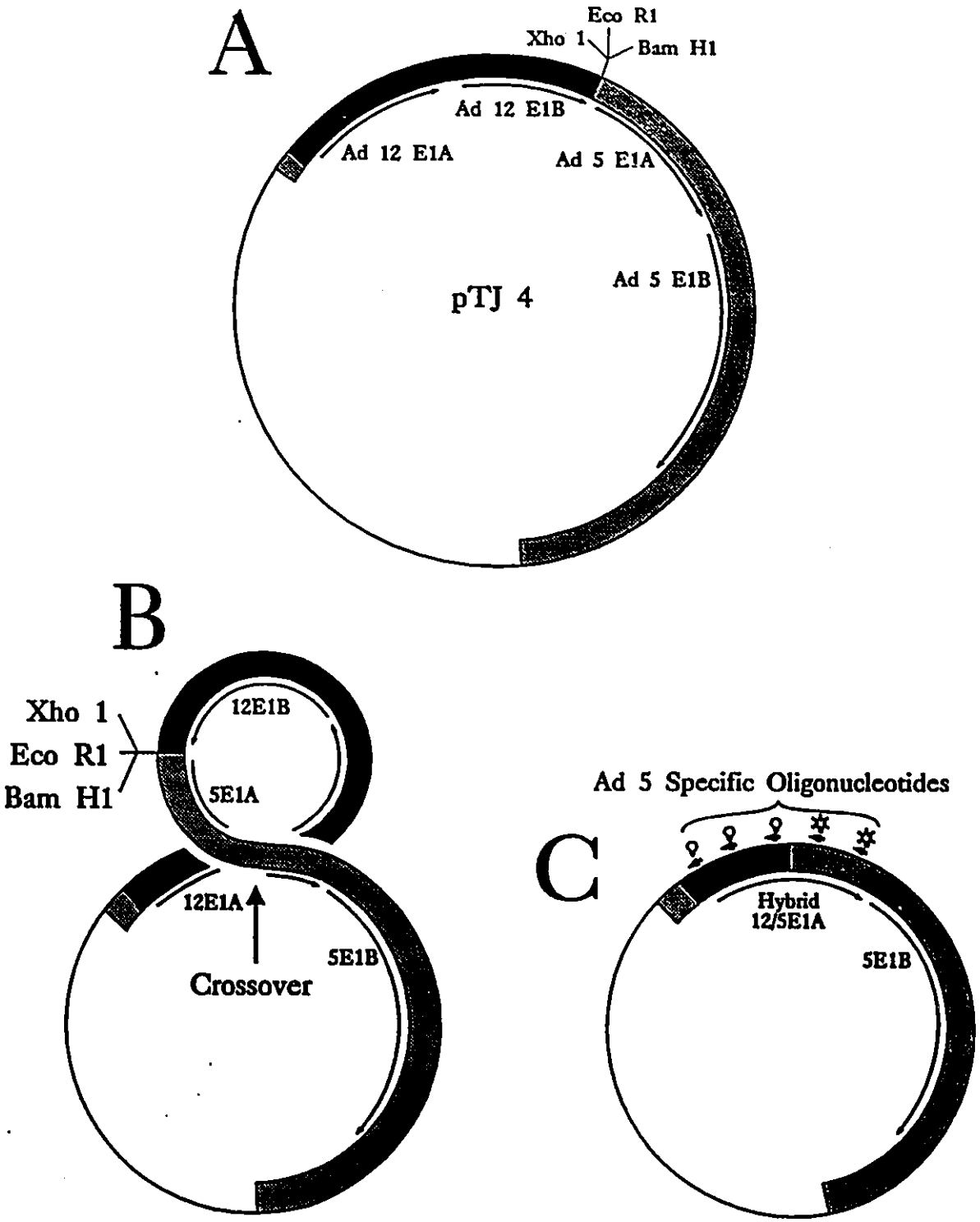
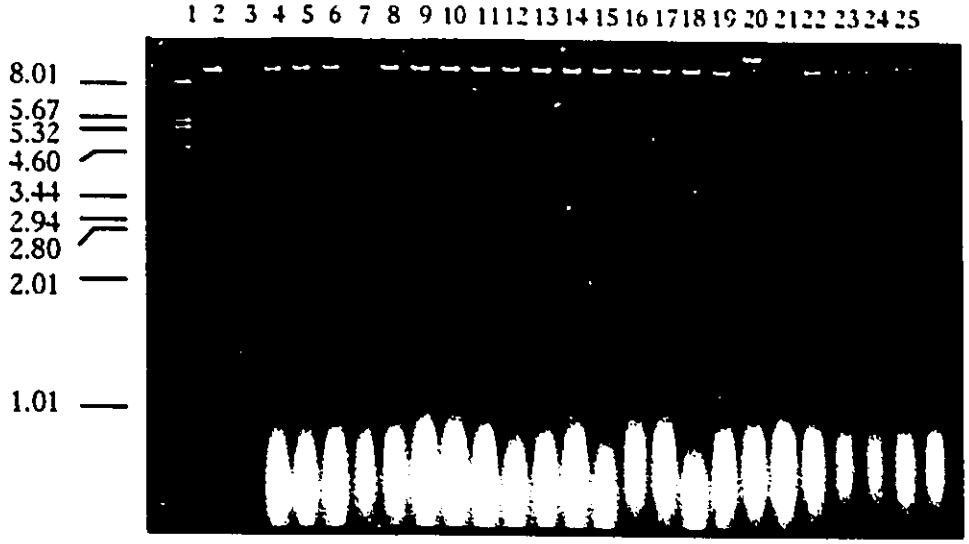

A
Eco R1
Xho 1
Bam H1
Ad 12 E1B
Ad 12 E1A
Ad 5 E1A
Ad 5 E1B
pTJ 4
B
Xho 1
Eco R1
Bam H1
12E1B
5E1A
12E1A
5E1B
Crossover
Ad 5 Specific Oligonucleotides
C
Hybrid
12/5E1A
5E1B

**Figure 3-8. Screening of putative hybrids.**

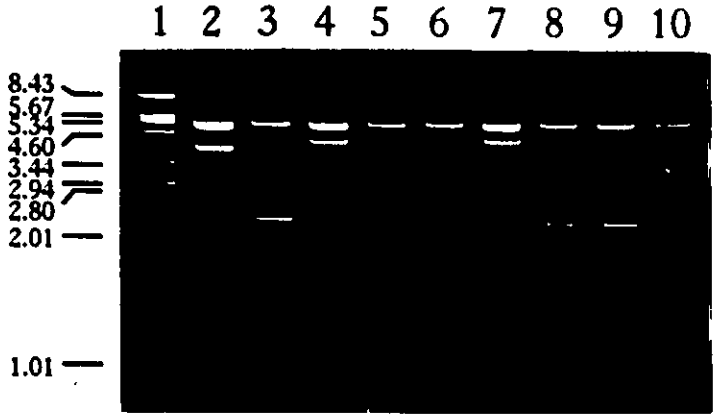
A. All clones were subject to analysis by digestion with *Hind* III. The parental plasmid, pTJ4 gives rise to two fragments when digested with *Hind* III (figure 3-6). The vast majority of clones shown here have restriction patterns matching pTJ4, although this varied between experiments. These plasmids were discarded. Also eliminated were the samples in lanes 15 and 16, due to the presence of an extra *Hind* III fragment of approximately 1.2 kb. The sample in lane 20 was judged too large to be a potential homologous recombinant. The samples in lanes 19 and 25 were chosen for further study.

B. Samples of clones which were selected from the preliminary screening were digested with *Hinc* II, to localize the crossover site to E1A or E1B. Lanes 2, 4, 5, 7, and 10 show fragments of 5.3 kb and 4.4 kb, and thus lack the Ad 5 *Hinc* II site at position 1572. This suggests that analogous Ad 12 sequences are present at this region, and therefore, that the crossovers localize to E1B. Lanes 3, 8, and 9 show three bands, of 5.3 kb, 2.3 kb, and 1.9 kb, consistent with the presence of the *Hinc* II site at position 1572. This suggests that these clones contain crossovers in E1A. The sample in lane 6 was discarded, as the C-fragment is too small to represent a homologous recombination event.

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
8.01
5.67
5.32
4.60
3.44
2.94
2.80
2.01
1.01

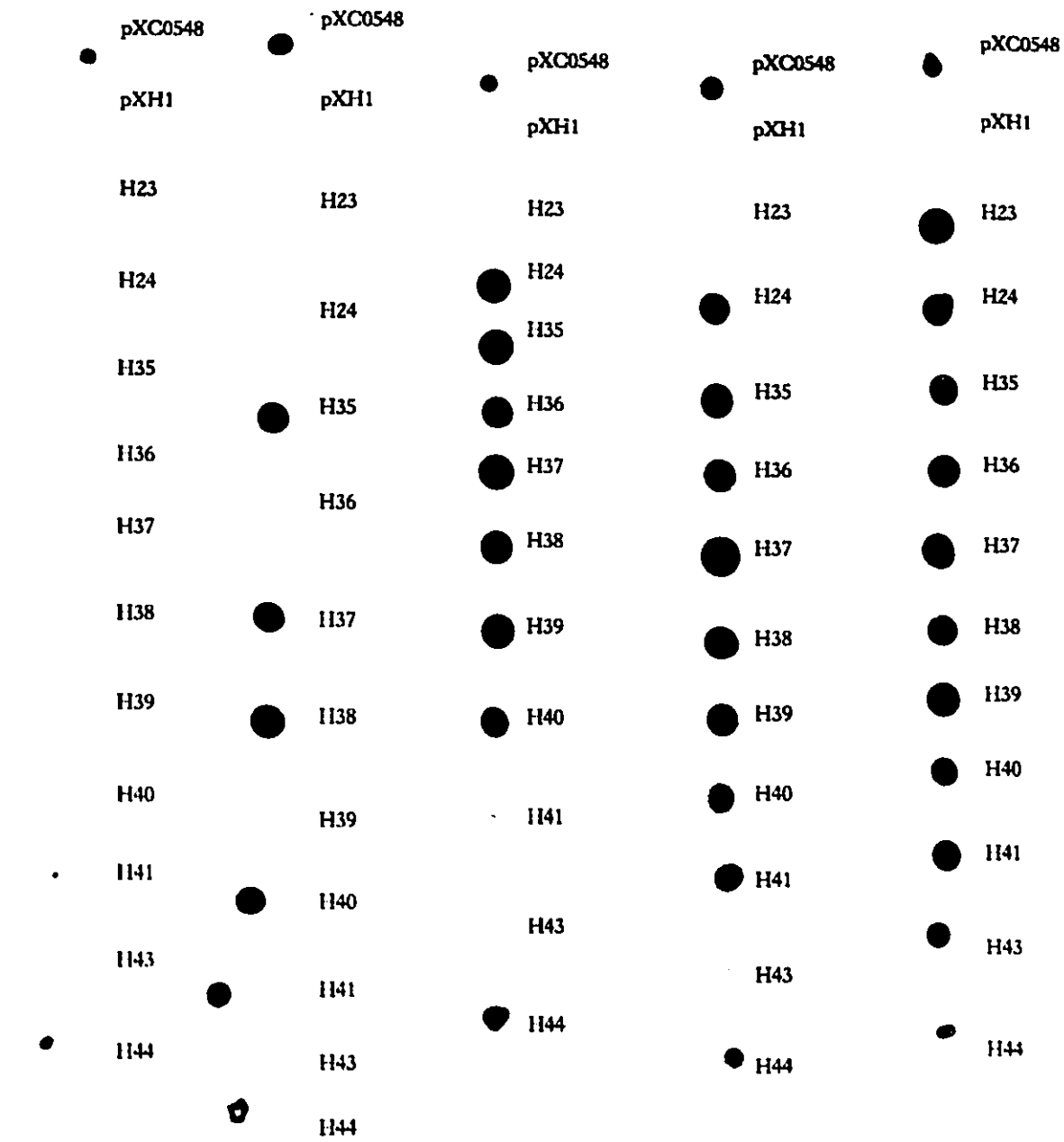
B


1 2 3 4 5 6 7 8 9 10
8.43
5.67
5.34
4.60
3.44
2.94
2.80
2.01
1.01

characterization of the sizes of fragments containing putative crossover sites, and elimination of those of incorrect size (cf. figure 3-8.B, lane 6). The above screening procedures only characterized plasmids for restriction sites in Ad 5 sequences, so the presence of Ad 12 E1A sequences in recombinants was confirmed using *Kpn* I, which cleaves Ad 5 sequences at position 2048 and Ad 12 at position 588. Hybrid plasmids which reached this stage of screening all had a diagnostic 1.5 kb *Kpn* I-B fragment. Figure 3-7.C shows an homologous recombination event in E1A resulting in a hybrid plasmid containing two *Kpn* I sites. This stage of screening was carried out because recombinant plasmids of a size approximately equal to that of pXC38 were chosen as putative hybrids, not excluding the possibility that pXC38, a ubiquitous plasmid in the laboratory, could be isolated as a contaminant. The sites of recombination were then further localized by DNA dot-blot hybridizations. Putative hybrid plasmid DNA was applied to five strips of nitrocellulose and probed with five oligomers complementary to sequences at various locations in Ad 5 E1A. In any given hybrid plasmid, Ad 5 sequences extend rightward from the point of the crossover. Using dot-blot hybridizations, the region of the crossover can be localized between the last oligomer which hybridizes to give a signal and the first to show no signal or a strongly reduced signal. In figure 3-9, putative hybrids H24, H36, and H39 all hybridize strongly to oligomers corresponding to positions 1563, 1311, and 1118, but do not hybridize to oligomers at positions 887 or 634. The interpretation of this result is that crossovers occurred between positions 1118 and 887. Other crossovers were similarly localized with respect to Ad 5 sequences, and finally,

**Figure 3-9. Screening crossovers by dot-blots.**

Approximately 100 ng of each putative hybrid DNA was heat denatured, applied to five separate strips of nitrocellulose, and hybridized to each of five radiolabelled oligonucleotides complementary to the coding strand of Ad 5 E1A. The locations of the 3' ends of the oligomers relative to the Ad 5 genome, are shown at the top. From right to left, the point at which the signal for any given clone disappears or greatly diminishes was interpreted as the interval in which a crossover had occurred. For example, H24, H36, and H39 all show a dropoff in signal between positions 1118 and 887, suggesting that they contain crossovers in that region.


AB417(634)
AB413(887)
AB414(1089)
AB415(1311)
AB416(1563)
pXC0548
pXH1
H23
H24
H35
H36
H37
H38
H39
H40
H41
H43
H44

dideoxy-nucleotide DNA sequence analysis primed by the particular oligomer which was the last to give a signal (eg. for H24 - position 1118) was used to map the exact locations of crossovers. The sequences of hybrid plasmids flanking the crossover sites are shown in figure 3-10. In three cases, a perfect match of 7bp was sufficient to permit recombination (figure 3-10; p738, p1036, p1461), while in other cases 4 perfect matches were sufficient when flanked by longer homologous stretches (cf. figure 3-4; p2743). This indicates that recombination is not restricted to regions of high sequence homology. In one case (figure 3-10; p1461) a recombinant was isolated consisting of all of Ad 12 E1A plus the initial portion of Ad 12 E1B fused to the last portion of Ad 5 E1A. This does not represent a true homologous recombinant but rather was the result of a crossover in a region of 7 bp of match between two unrelated proteins, not flanked by other patch homologies, and is therefore in all likelihood fortuitous. The relative compositions of the E1A hybrids with respect to Ad 5 and Ad 12 E1A sequences are shown in figure 3-11. Five crossovers map to exon 1 of E1A, and one is at the common major splice acceptor site (p1227). In the first exon, two crossovers are in CR1 (p690, p753), one is between CR1 and CR2 (p827), one maps at the left border of CR3 (p975) and one in CR3 (p1036). The E1A regions of Ad 5 and Ad 12 are not perfectly colinear with respect to the locations of patch homologies, and therefore the predicted sizes of the hybrid E1A proteins are not constant and not identical to E1A of either parent. The precise amino acid sequences of all hybrid E1A proteins can be deduced from figure 3-12, which shows an amino acid sequence alignment between Ad 5 and Ad 12 E1A, with the residues at

**Figure 3-10. DNA sequences across crossover sites.**

Alignments of the Ad 12 and Ad 5 sequences at the sites of crossovers are shown, with the residues common to both parental serotypes and particular hybrids indicated in bold. Vertical lines indicate sites of identity. The lowest degree of homology which is still sufficient to facilitate recombination is 7 bp, as seen with p1036 and p1461, although flanking homology is significantly higher in p1036.

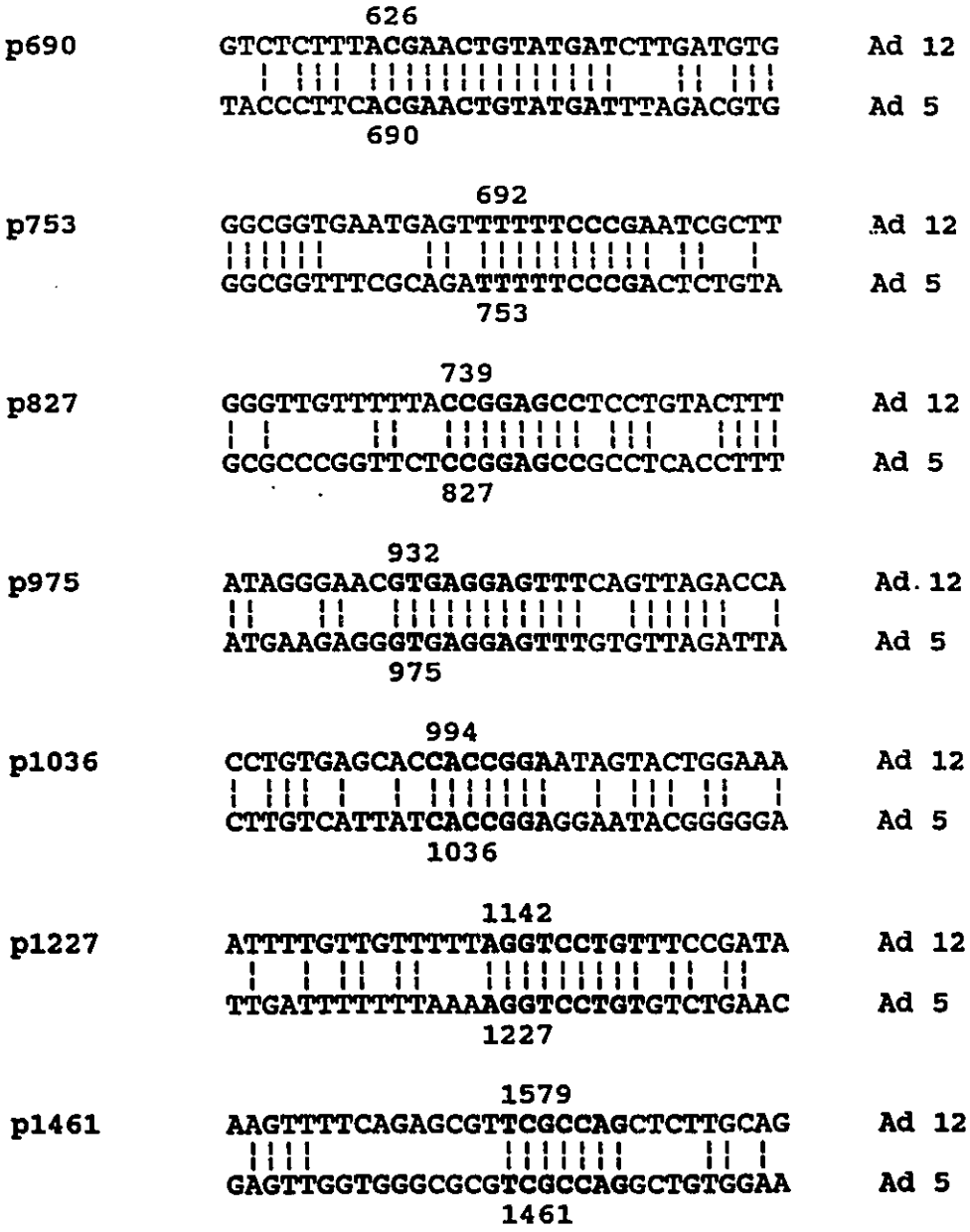

626
p690
GTCTCTTTACGAACTGTATGATCTTGATGTG
Ad 12
TACCCTTCACGAACTGTATGATTTAGACGTG
Ad 5
690
692
p753
GGCGGTGAATGAGTTTTTTCCCGAATCGCTT
Ad 12
GGCGGTTTCGCAGATTTTTCCCGACTCTGTA
Ad 5
753
739
p827
GGGTTGTTTTTACCGGAGCCTCCTGTACTTT
Ad 12
GCGCCCGGTTCTCCGGAGCCGCCTCACCTTT
Ad 5
827
932
p975
ATAGGGAACGTGAGGAGTTTCAGTTAGACCA
Ad 12
ATGAAGAGGGTGAGGAGTTTGTGTTAGATTA
Ad 5
975
994
p1036
CCTGTGAGCACCACCGGAATAGTACTGGAAA
Ad 12
CTTGTCATTATCACCGGAGGAATACGGGGGA
Ad 5
1036
1142
p1227
ATTTTGTTGTTTTTAGGTCCTGTTTCCGATA
Ad 12
TTGATTTTTTTAAAAGGTCCTGTGTCTGAAC
Ad 5
1227
1579
p1461
AAGTTTTCAGAGCGTTCGCCAGCTCTTGCAG
Ad 12
GAGTTGGTGGGCGCGTCGCCAGGCTGTGGAA
Ad 5
1461

**Figure 3-11. Structures of hybrid E1A plasmids.**

The constructs isolated are diagrammed in alignment with the structure of Ad 5 E1A. The precise locations of crossovers are indicated beneath each construct at the junctions. All constructs contain the leftmost 353 bp of Ad 5, encompassing the viral inverted terminal repetition, and the enhancer/packaging region (see section B.2). To this are attached Ad 12 sequences from nucleotide 290, encompassing the E1A promoter, transcriptional start site and the amino terminus of the E1A protein. At the indicated site, a crossover event has taken place, and Ad 5 sequences are present from that point to 16% of the genome length. Of note is the fact that p975 contains neither the Ad 5 nor Ad 12 12 S splice donor site. p1227 contains a crossover at the common major splice acceptor site, and thereby encodes the first exon of the Ad 12 E1A protein joined to the second exon of the Ad 5 E1A protein. p1461 arose from a crossover in a region of fortuitous homology, not corresponding to any alignment of E1A sequences. It contains the entire Ad 12 E1A coding region, the untranscribed region between E1A and E1B and the amino terminus of the Ad 12 E1B 19 kDa protein coding region, joined to the carboxy terminus of the Ad 5 E1A coding region, and an intact Ad 5 E1B region.

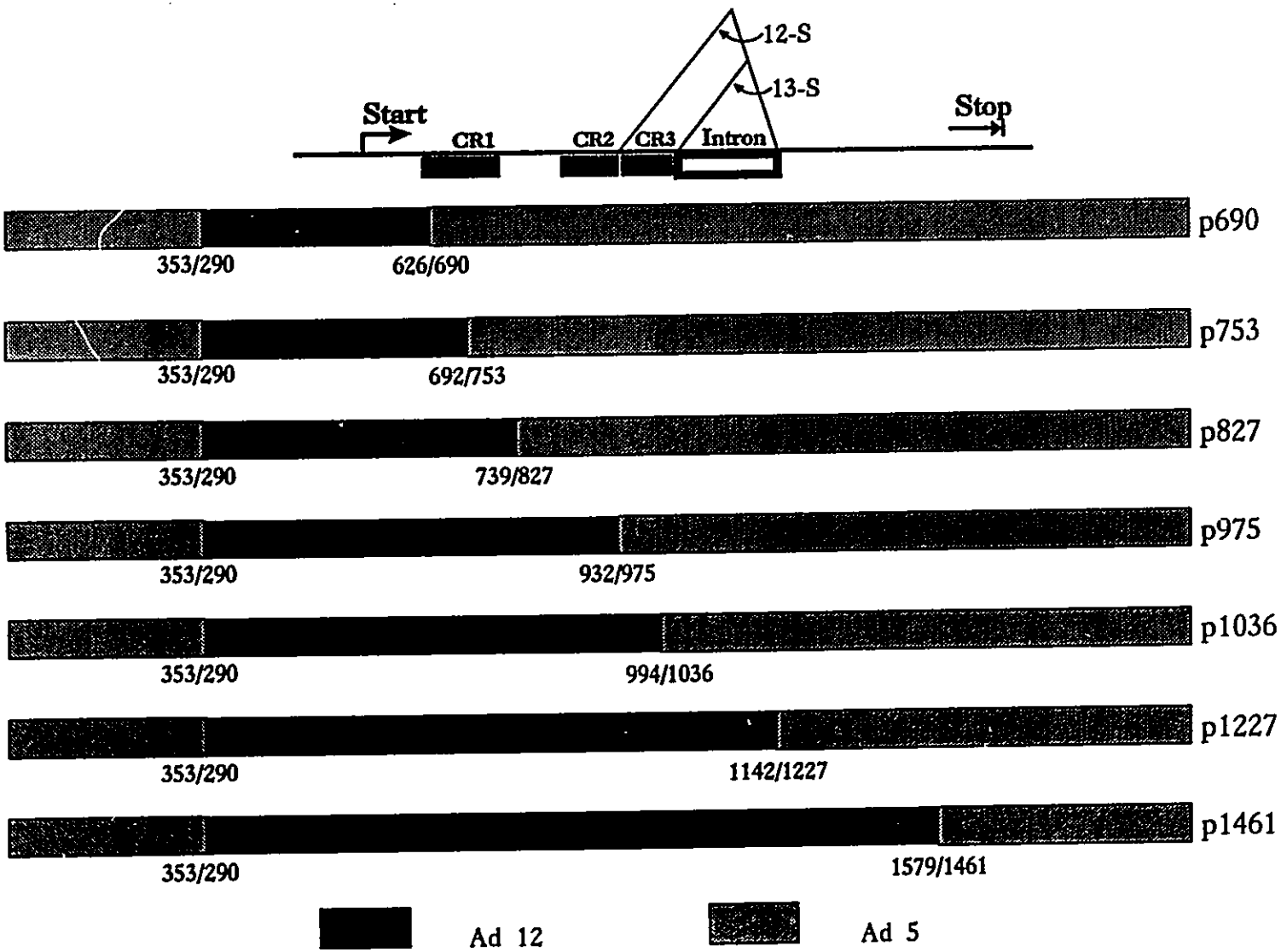

12-S
13-S
Start
Stop
CR1
CR2
CR3
Intron
p690
353/290
626/690
p753
353/290
692/753
p827
353/290
739/827
p975
353/290
932/975
p1036
353/290
994/1036
p1227
353/290
1142/1227
p1461
353/290
1579/1461
Ad 12
Ad 5

**Figure 3-12. Amino acid sequence of E1A hybrids.**

The amino acid sequences of Ad 12 and Ad 5 E1A were aligned manually to reflect regions of maximum conservation. Residues at crossover sites common to both serotypes and each particular hybrid are indicated in bold, and the identity of the hybrids is shown beneath the alignment at each crossover site.


Residue
Serotype
1 MRTEMTPLVLSYQEA-----DDILEHLVDNFFNEVPSDDDLYVPSLYELYDLD Ad 12
1 MRHIICHGGVITEEMAASLLDQLIEEVLAD--N-LPPPSHFEPPTLHELYDLD Ad 5
p690
CR1
49 VESAGEDNNEQAVNEFFPESLILAASEGLFL---------PEPPVLS-----P Ad 12
51 V-TAPEDPNEEAVSQIFPDSVMLAVQEGIDLLTFPPAPGSPEPPHLSRQPEQP Ad 5
p753
p827
CR2
88 VCEPIGGECMPQLHPEDMDLLCYEMGFPCSDSEDEQDENGMAHVSASAAAAAA Ad 12
103 EQRALGPVSMPNLVPEVIDLTCHEAGFPPSDDEDEEG---------- Ad 5
CR3
141 DRERREEFQLDHPELPGHNCKSCEHHRNSTGNTDLMCSLCYLRAYNMFIYSPVS Ad 12
140 ----EEFVLDYVEHPGHGCRSCHYHRRNTGDPDIMCSLCYMRTCGMFVYSPVS Ad 5
p975
p1036
p1227
194 DPEPEP---------- Ad 12
189 EPEPEPEPEPEPARPTRRPKMAPAILRRPTSPVSRECNSSTDSCDSGPSNTPP Ad 5
220 SAVPEGVIKPVPQR-VT-GRRRCAVESILDLIQEEEREQTVPVDLSVKRPRCN Ad 12
242 EIHPVVFLCPIKPVAVRVGGRRQAVECIEDLLNEPG--Q--PLDLSCKRPRP- Ad 5

crossover sites, common to individual hybrids indicated in bold.

## B. Rescue Of Hybrid Plasmids Into Virus

### 1. Transfection of 293 cells with hybrid plasmids and pJM17.

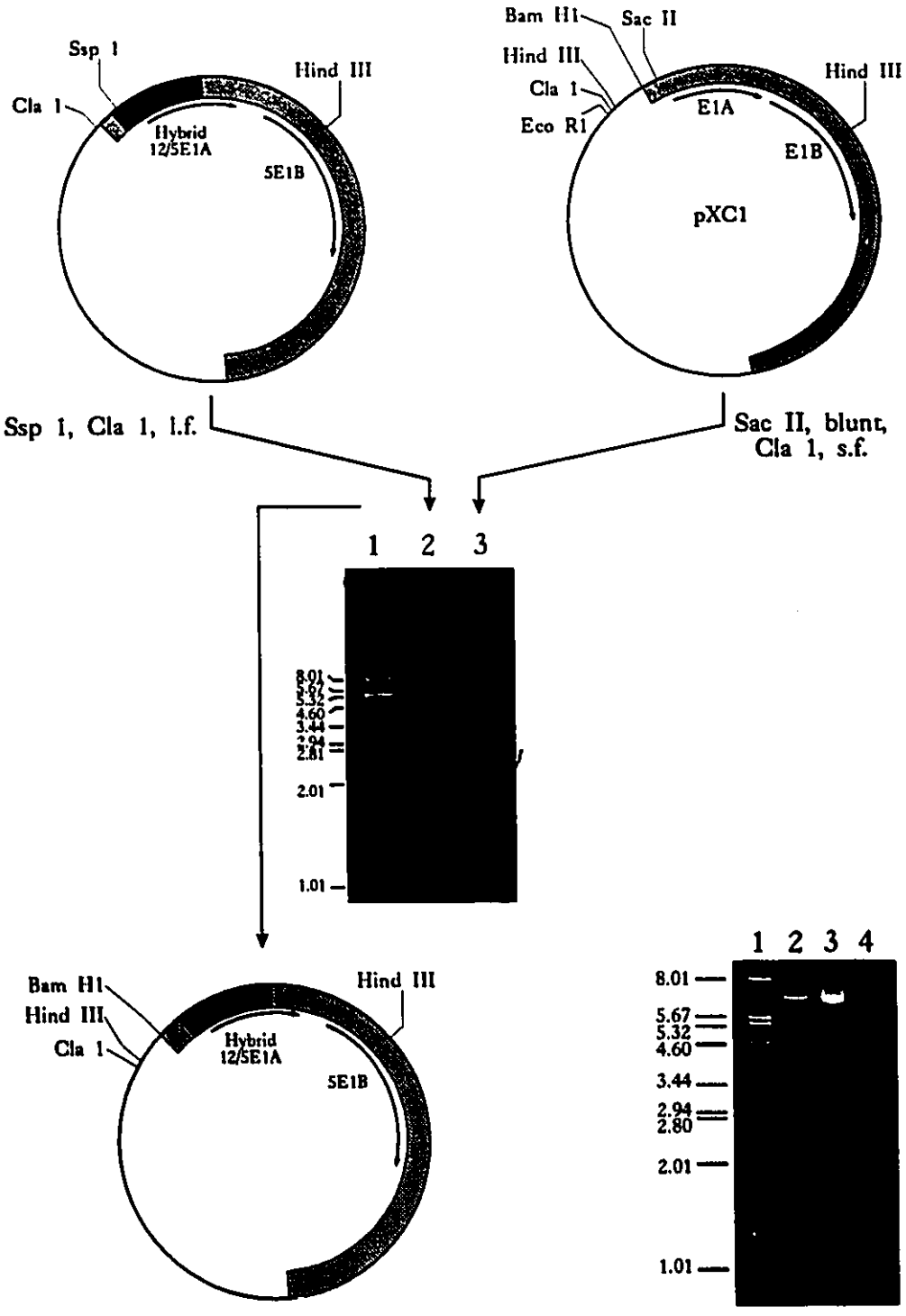
Several hybrid plasmids were independently co-transfected with pJM 17, and despite multiple independent attempts, no recombinant viruses were isolated among the plaques which were analyzed. Upon examination, it was discovered that two *cis*-acting elements which are normally required for efficient packaging of Ad 5 DNA (Hearing *et al.*, 1987) were not well conserved in corresponding Ad 12 sequences. It is likely therefore that if recombinant viral genomes arose in transfected cells, they would not be packaged into capsids. Therefore, in order to rescue the hybrid E1 plasmids into infectious Ad 5 virions, it was necessary to modify the left terminal sequences of each individual hybrid plasmid to include the Ad 5 specific packaging signal.

### 2. Reconstruction of the left terminal sequences of hybrid plasmids.

It was necessary to replace the terminal sequences of all hybrid plasmids with segments of Ad 5 DNA including the packaging signal and ITR, to facilitate viral packaging as well as genome replication. As outlined in figure 3-13, hybrid plasmids were cleaved at position 290 of Ad 12 sequences with *Ssp* I and in pBR322 sequences with *Cla* I (figure 13-3.A, lane 2). This terminal *Cla* I-*Ssp* I fragment of each hybrid plasmid was

**Figure 3-13. Replacement of left-terminal sequences of each hybrid plasmid.**

A. Each hybrid E1A plasmid was cleaved with *Cla* I and *Ssp* I, removing a small section of non-coding left terminal sequences (example of purified fragment, lane 2, middle of page). pXC1 was cleaved with *Sac* II, treated with the Klenow polymerase, and cleaved again with *Cla* I, and the small fragment was purified (lane 3). B. These two fragments were ligated together, and the resulting clones were screened by digestion with *Bam* HI and *Hind* III, which give rise to a fragment identical in size to the Ad 5 *Hind* III-G fragment (lane 4). In lane 2 of part B, a slightly larger fragment was observed. Since it was not clear how this arose, the clone was discarded.


Ssp I
Cla I
Hind III
Hybrid 12/5E1A
5E1B
Ssp I, Cla I, l.f.
Bam HI
Sac II
Hind III
Cla I
Eco RI
E1A
E1B
pXC1
Hind III
Sac II, blunt, Cla I, s.f.
1 2 3
8.01
5.67
5.32
4.60
3.44
2.94
2.80
2.01
1.01
Bam HI
Hind III
Cla I
Hybrid 12/5E1A
5E1B
Hind III
1 2 3 4
8.01
5.67
5.32
4.60
3.44
2.94
2.80
2.01
1.01

replaced with the Ad 5 left terminal *Cla* I-*Sac* II (blunt) fragment (figure 3-13.A, lane 3). The resulting plasmids contain *Bam* HI sites at the junctions between Ad sequences and pBR322 sequences, and when digested with *Bam* HI and *Hind* III give rise to a restriction fragment of approximately 2.8 kb (figure 13-3.B, lane 4), similar in mobility to the Ad 5 *Hind* III G-fragment present in the marker lane (figure 13-3.B, lane 1). Variations of this proceedure were carried out for all E1A hybrids as well as p2743 (hybrid E1B 55K/58K coding region). The structures of hybrid E1A regions shown in figure 3-11 in fact correspond to the versions with the left-terminal Ad 5 *Sac* II fragment described here. All subsequent characterizations were carried out with these altered constructs.

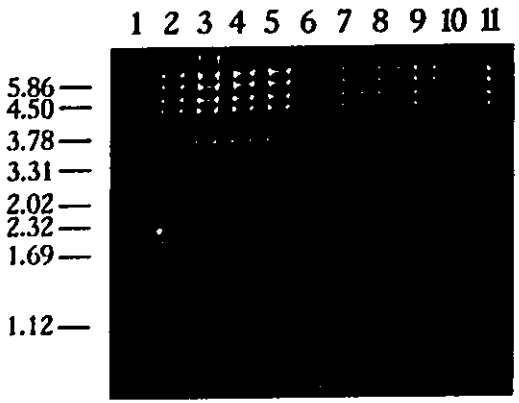
3. Rescue of hybrid plasmids.

293 cells were co-transfected with pJM17 and the hybrid E1 plasmids with altered left-terminal sequences, and viral DNA from plaque isolates was analyzed by restriction digestion. Recombinant viruses were identified by their *Kpn* I restriction patterns, since the site at position 588 of Ad 12, present in all hybrids, was diagnostic for Ad 12 sequences. In figure 3-14.A, the *Kpn* I-H fragment of 2.0 kb corresponding to the left terminal fragment of Ad 5 DNA (figure 3-14.B) is absent for certain plaque isolates, and is replaced by a fragment of approximately 1.5 kb (figure 3-14.A, lanes 4, 6, 8, 11). Plaque isolates with this restriction pattern were considered recombinant, and were digested with *Hind* III to confirm the viral DNA structure. In lanes 5, 7, and 10, the H-fragment is sub-molar in concentration. This probably represents a mixed plaque isolate.

**Figure 3-14. Construction of recombinant virus.**

Plaques isolated following transfection of hybrid plasmids (shown here is p827) and pJM17 were analyzed by digestion with *Kpn* I. The *Kpn* I map of wild-type Ad 5 (lane 1) is shown, with the genome localization of each fragment. All hybrid E1 plasmids contained the Ad 12 *Kpn* I site at position 588. As a result, rescue of these plasmids into virus would result in the shortening of the *Kpn* I-H fragment from 2.0 kb to 1.5 kb. This type of restriction pattern is seen in lanes 4, 6, 8, and 11. The viral DNA was subsequently checked with *Hind* III before plaque purification.

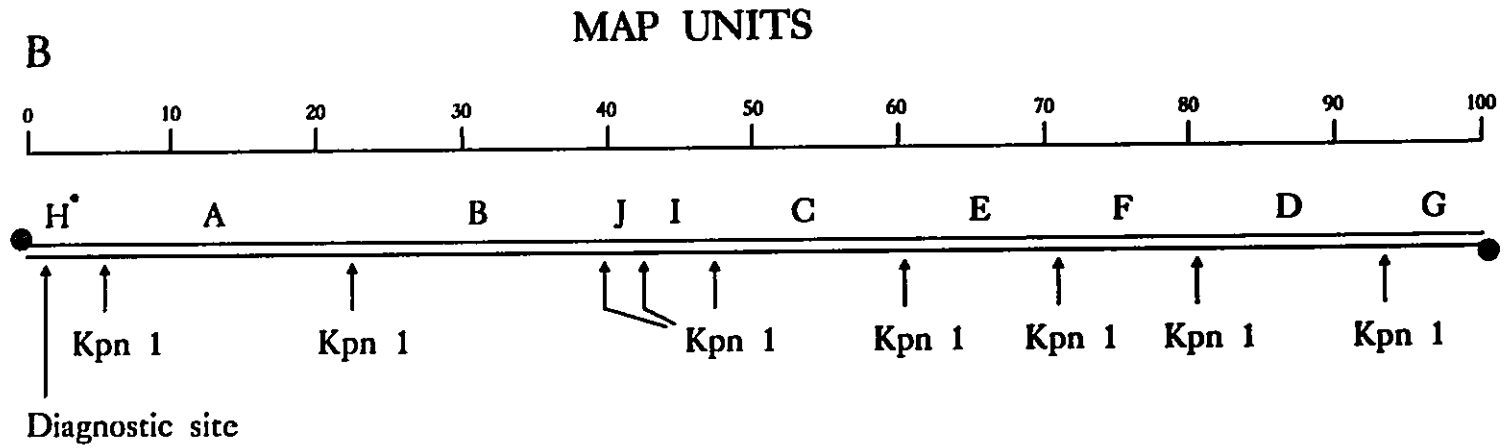
A




Lanes 4, 6, 8, 11: H-fragment lost

New fragment, 1.5 kb

B



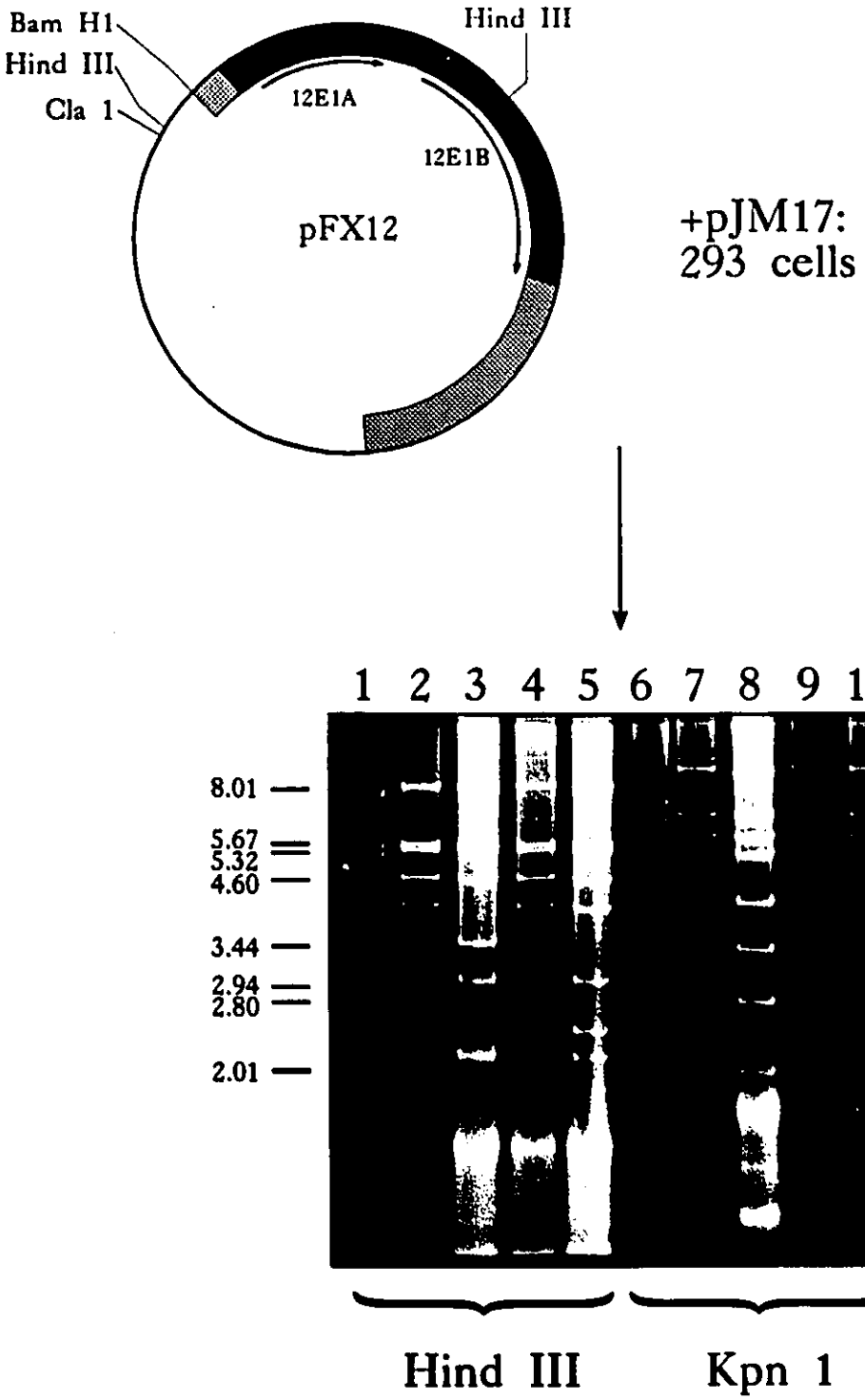
To isolate pure plaques, all recombinant viruses were subjected to two cycles of plaque purification.

4. Generation of recombinant Ad 5 expressing Ad 12 E1.

It was anticipated that it would be useful to be able to compare the properties of recombinant viruses expressing various portions of the Ad 12 E1 region with those of a virus expressing all of Ad 12 E1. To construct an E1 plasmid which could give rise to such a virus, pHAB6 was digested with *Kpn* I and *Cla* I, removing the left terminal 588 bp. This fragment was replaced with the corresponding fragment derived from p2743, which contained the left-terminal Ad 5 *Sac* II fragment in place of the left-terminal Ad 12 *Ssp* I fragment (cf. Fig. 3-13), resulting in an Ad 12 E1 plasmid with left-terminal Ad 5 sequences to facilitate replication of recombinant virus. To promote recombination between the Ad 12 E1 plasmid and pJM17, this plasmid was digested with *Cla* I and *Bst* EII, *Cla* I cutting at the pBR322 *Cla* I site, and *Bst* EII at a single site in Ad 12 E1, just 3' of the E1B transcription unit. The excised fragment was inserted into pXC1 which had been digested with *Cla* I and *Bgl* II, *Cla* I again cutting in pBR322 sequences, and *Bgl* II cutting in the E1B transcription unit, prior to the transcription termination site, but after the translation termination codon. The resulting plasmid, which contains the intact Ad 12 E1 region flanked by Ad 5 sequences, is referred to as pFX12 (figure 3-15). The results of rescue experiments of pFX12 into virus are also shown in figure 3-15, containing *Hind* III and *Kpn* I digests of plaque isolates obtained following co-transfection of pFX12 and

## Figure 3-15. Construction of T12E1.

A virus containing the Ad 12 E1 region in an Ad 5 background was constructed by first constructing pFX12, which contains left terminal Ad 5 sequences attached to Ad 12 E1A just like all hybrid plasmids, but at the downstream terminus of Ad 12 E1B coding sequences the plasmid has approximately 2.5 kb of Ad 5 sequences corresponding to the transcription termination region of the E1B region, the protein IX transcription unit, and sequences from there to the *Xho* I site. This plasmid was co-transfected with pJM17 into 293 cells, and plaques were analyzed as described in Fig. 14. The *Hind* III patterns of plaque isolates in lanes 2, 4, and 5 correspond to the predicted structure of T12E1, with the leftmost *Hind* III site closer to the viral terminus, resulting in a smaller G-fragment and a larger E-fragment. This is confirmed by digestion with *Kpn* I, which shows loss of the H-fragment, and a larger than normal A-fragment (lanes 7, 9, 10), as the *Kpn* I site normally at position 2048 in Ad 5 is lost.


Bam H1
Hind III
Cla 1
Hind III
12E1A
12E1B
pFX12
+pJM17:
293 cells
1 2 3 4 5 6 7 8 9 10
8.01
5.67
5.32
4.60
3.44
2.94
2.80
2.01
Hind III
Kpn 1

pJM17. Lanes 2, 4, and 5 show a *Hind* III E-fragment with larger than wild-type size and a *Hind* III G-fragment of smaller than wild-type size. This is consistent with the rescue of pFX12, because the Ad 12 derived *Hind* III site is closer to the viral terminus than the Ad 5 derived site it replaces. These structures were confirmed by digestion with *Kpn* I, which generates a larger than wild-type *Kpn* I A-fragment, and no *Kpn* I H-fragment in lanes 7, 9, and 10. This is consistent with the loss of the Ad 5 *Kpn* I site at position 2048. The virus was plaque purified twice, and named T12E1.

## C. Analysis Of Recombinant Viruses

### 1. Titration of Viruses.

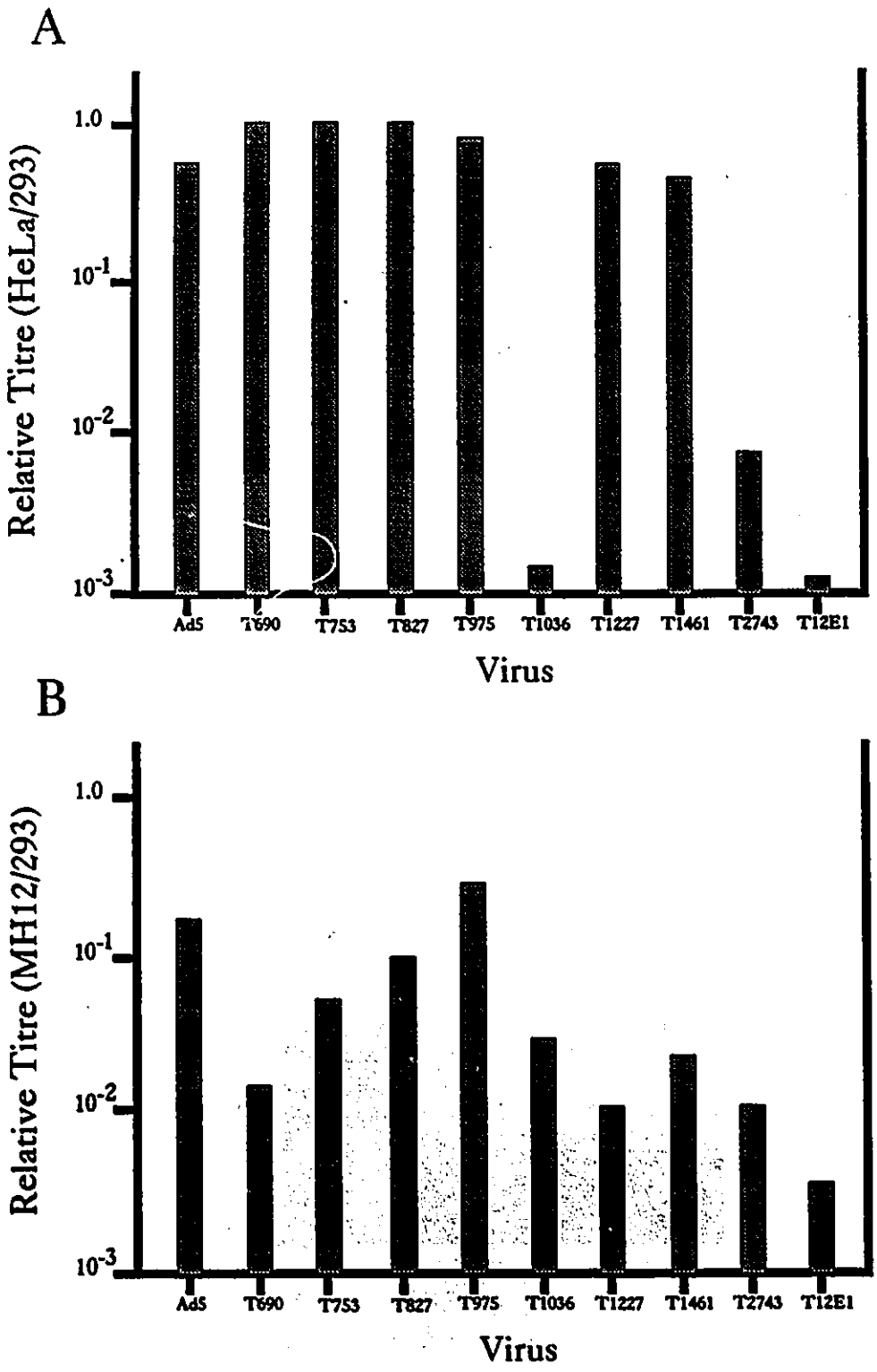
Recombinant viruses were grown on 293 cells, and infectious titres were determined by plaque titrations on 293 cells. Plaque assays were also carried out with recombinant viral stocks on HeLa cells, to determine whether the E1 proteins expressed by recombinant viruses could facilitate viral replication in a non-complementing cell line. Previous work had indicated that Ad 12 E1A is capable of functionally substituting for Ad 5 E1A to support replication of the Ad 5 genome (Williams *et al.*, 1975; Rowe and Graham, 1981; Sawada *et al.*, 1988), and this assay would confirm that the recombinant viruses make stable and functional proteins by virtue of their ability to support Ad 5 growth. The results, as shown in figure 3-16.A, indicate that of the E1A hybrids, only T1036 was deficient for growth on HeLa cells. This virus encodes an E1A protein with

a crossover in the transactivation domain, CR3. Since it is known that transactivation is the only function of E1A required for virus replication in HeLa cells, these results suggest that the hybrid transactivation domain encoded in T1036 is non-functional. Both T2743 and T12E1 showed host-range phenotypes. Since the defect shown by T12E1, which expresses an intact Ad 12 E1B region, was more severe than that seen with T2743, it is likely that the Ad 12 E1B region cannot functionally replace Ad 5 E1B to support Ad 5 replication. To examine this question further, viruses were tested for plaquing efficiency on MH12C2 cells, which express the E1 region of Ad 12. Several conclusions can be drawn from the results, shown in figure 3-16.B. First, no virus, even wt Ad 5, replicated as efficiently on MH12C2 cells as on 293 cells, although the experimental variations in these experiments were much larger than plaque titrations on HeLa or 293 cells. On average, titres were 10- to 20-fold lower on MH12C2 cells than on 293 cells. Possible explanations for these observations will be presented in the Discussion. Second, T1036, which presumably has a defective transactivation function, replicated no worse on MH12C2 cells than other E1A hybrids or Ad 5. While the other hybrid E1A viruses replicated less efficiently on MH12C2 cells than on 293 or HeLa cells, the titres of T1036 on MH12C2 cells were higher than on HeLa cells (figure 3-16.A and .B). This improvement of the plaquing efficiency of T1036 is consistent with the ability of Ad 12 E1A (expressed by MH12C2 cells) to substitute for Ad 5 E1A to support virus growth. Third, T12E1 forms plaques as poorly on MH12C2 cells as on HeLa cells, confirming that the presence of Ad 12 E1 proteins supplied by the MH12C2 cells do not overcome

**Figure 3-16. Titration of recombinant viruses on HeLa, MH12C2 cells.**

A. HeLa cells were infected with serial dilutions of viral stocks which had been previously titrated on 293 cells. Dishes were overlaid, and after three weeks, stained with neutral red. Plaques were counted, and the average of duplicate dishes was used to estimate the titre of the original stock. This value was divided by the estimated titre on 293 cells, and expressed as a ratio as shown at right.

B. MH12C2 cells were infected and overlaid as described above. After three weeks, plaques were visualized and counted. The titres were estimated, and expressed as ratios with titres observed on 293 cells.


A
Relative Titre (HeLa/293)
1.0
$10^{-1}$
$10^{-2}$
$10^{-3}$
Ad5
T690
T753
T827
T975
T1036
T1227
T1461
T2743
T12E1
Virus
B
Relative Titre (MH12/293)
1.0
$10^{-1}$
$10^{-2}$
$10^{-3}$
Ad5
T690
T753
T827
T975
T1036
T1227
T1461
T2743
T12E1
Virus

the defective nature of this virus which encodes those same proteins.

2. Analysis of hybrid E1A proteins.

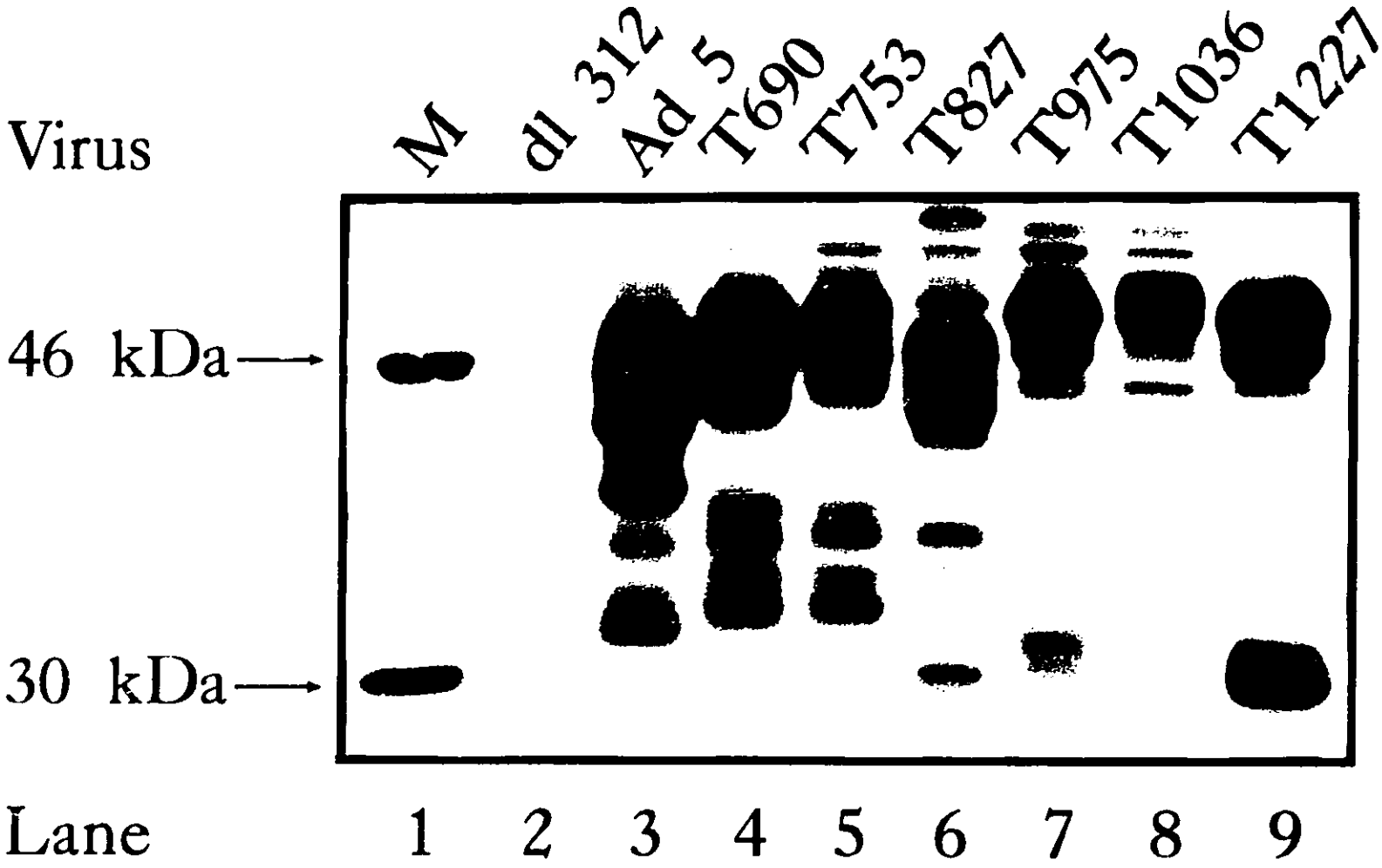
The fact that most hybrid viruses were able to replicate efficiently on HeLa cells suggested that they expressed functional E1A proteins. To confirm this, and to determine their levels of protein expression, HeLa cells were infected with each virus with the exception of T1461 (containing the entire Ad 12 E1A coding region) at a multiplicity of 30 pfu/cell, labelled with $^{35}$S-methionine, and cell extracts were immunoprecipitated with the M73 monoclonal antibody (Harlow *et al.*, 1985; specific for the carboxy terminal region of the Ad 2/Ad 5 E1A protein). Since all the hybrid E1A proteins contained at least the second exon of Ad 5 E1A, they were all expected to be immunoprecipitable with M73. The results (figure 3-17) show that all the hybrid E1A viruses produced immunoprecipitable E1A proteins, although the amounts made in cells infected by T1036 were reduced.

3. Characterization of the host shutoff property.

To further characterize the hybrid E1 viruses, and to determine the reasons why viruses expressing Ad 12 E1B did not replicate efficiently on HeLa cells, the ability of these viruses to shut off host cell protein synthesis was assayed in HeLa cells. Cells were infected at a multiplicity of 50 pfu/cell for 48 hr, then pulse labelled with 5 μCi $^{35}$S-

**Figure 3-17. Immunoprecipitation of E1A from infected cells.**

Immunoprecipitation of hybrid E1A proteins. HeLa cells were infected at a multiplicity of 30 PFU per cell, and labelled 10 hours later with 0.25 mCi of $^{35}$S-methionine. Cell lysis and immunoprecipitation was carried out as described in Materials and Methods. The M73 monoclonal antibody was used to immunoprecipitate E1A proteins.


Virus
M
dl 312
Ad 5
T690
T753
T827
T975
T1036
T1227
46 kDa
30 kDa
Lane
1
2
3
4
5
6
7
8
9

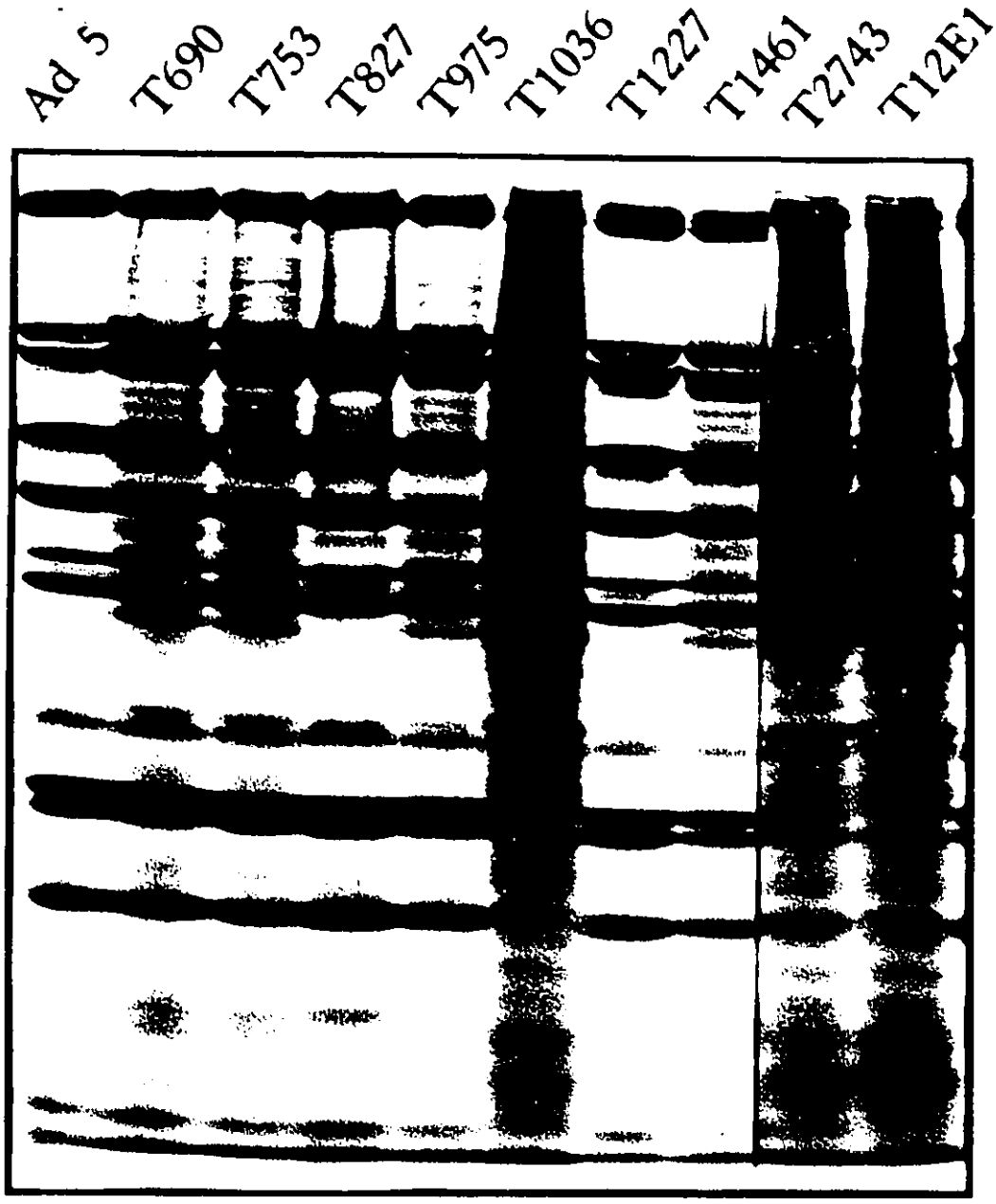
methionine per 60 mm dish for 30 min, and electrophoresed on a 10% SDS-polyacrylamide gel. Discreet protein bands corresponding predominantly to viral proteins are visible when shutoff occurs, and a smear presumably containing a mixture of cellular and viral proteins is seen when shutoff is defective, as shown in figure 3-18. T1036 (lane 6) shows defective shutoff, which is most likely attributable to defective activation by the hybrid E1A protein of the Ad 5 E1B and E4 transcription units, which are directly involved in the shutoff of host protein synthesis. T2743 (figure 3-18, lane 9) and T12E1 (figure 3-18, lane 10) also show defective host shutoff, suggesting that neither the hybrid E1B 55K/58K protein encoded by T2743, nor the Ad 12 E1B 55K protein encoded by T12E1 are capable of efficiently inducing host shutoff in cells infected with Ad 5 genomes. These results are consistent with at least one other report (Sawada *et al.*, 1988). The authors suggested that since host shutoff is dependent on the products of both E1B and E4, it is likely that the interactions between Ad 5 E1B and E4 proteins are serotype specific, with heterogeneous combinations of E1B and E4 either failing to interact, or interacting to form non-functional complexes.

4. Analysis of E1A-associated cellular proteins.

The cellular proteins which coimmunoprecipitate with E1A of Ad 5 have been extensively characterized (see Introduction). However, while the regions of E1A that bind these cellular proteins are largely conserved in Ad 12, studies of interactions between Ad 12 E1A and these cellular proteins have not been reported. To examine whether

**Figure 3-18. Host shutoff by recombinant viruses.**

HeLa cells were infected for 48 hr, and labelled for 30 min with 5 μCi of $^{35}$S-methionine per 60 mm dish. Lysates were run on a 10% SDS-polyacrylamide gel and autofluororadiographed. Incomplete shutoff of host cell protein synthesis is seen in lanes 6, 9, and 10.

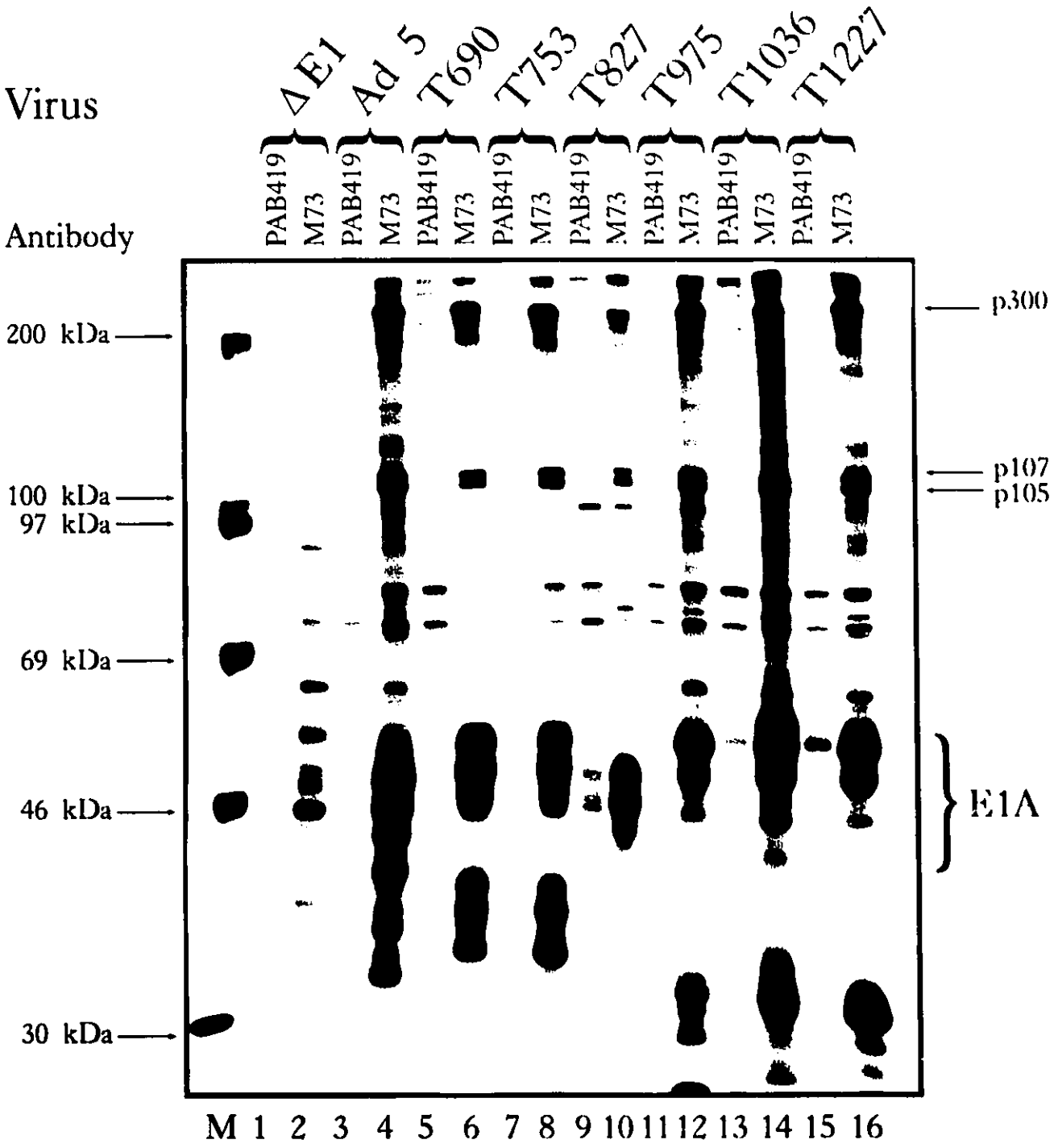



Lane 1 2 3 4 5 6 7 8 9 10

differences exist in the binding avidities of Ad 5 and Ad 12 E1A protein sequences to cellular proteins, HeLa cells were infected at a multiplicity of 100 pfu/cell with recombinant viruses encoding hybrid E1A proteins, and labelled protein complexes were subsequently immunoprecipitated under non-dissociating conditions (Harlow *et al.*, 1986) (figure 3-19). Since the M73 monoclonal antibody is specific for proteins containing an Ad 5 carboxy terminus, the E1A proteins encoded by viruses T690, T753, T827, T975, T1036 and T1227 were all immunoprecipitable (figure 3-17). Although it was not possible to immunoprecipitate Ad 12 E1A with M73, it was possible to examine the cellular proteins which coimmunoprecipitate with hybrid E1A proteins encoded by varying amounts of the first exon of Ad 12 E1A, which contains the sequences expected to bind to ceilular proteins. The results obtained with hybrid T1227, which encodes all of exon 1 of Ad 12 E1A, would therefore likely reflect the ability of the intact Ad 12 E1A protein to bind to cellular proteins. Comparison of the lanes representing Ad 5 (figure 3-19, lane 4) and T1227 (figure 3-19, lane 16) indicates that p105, p107 and p300 all associate with E1A equally well whether their binding sites are derived from Ad 5 or Ad 12. The virus T1036 expressed normal amounts of E1A protein when cells were infected at high multiplicity, and the 1036 E1A protein also associated with cellular proteins as efficiently as did the other hybrid E1A products. Coimmunoprecipitated p60 $^{cyclin\ A}$ was not detected in these experiments, as it co-migrates with E1A, and becomes clearly detectable only when mutant forms of E1A with accelerated electrophoretic mobilities are immunoprecipitated (Howe and Bayley, 1992). Densitometric analysis of the

**Figure 3-19. Coimmunoprecipitation of E1A and associated cellular proteins.**

HeLa cells were infected with viruses expressing hybrid E1A proteins and appropriate controls at a multiplicity of 100 pfu/cell, cells were labelled at 10 hr post-infection with 0.25 mCi of $^{35}$S-methionine, and proteins were analyzed as described (Harlow *et al.*, 1986). M73 is a monoclonal antibody specific for the carboxy terminus of Ad 5 E1A, while PAB 419, a monoclonal antibody specific for SV40 large T antigen, was used as a negative control. Molecular weight marker sizes are given at the left side of the autofluororadiogram, while the identities of given protein bands (E1A, p105, p107, and p300) are noted along the right side. No additional specific bands could be positively identified.


Virus
ΔE1
Ad 5
T690
T753
T827
T975
T1036
T1227
Antibody
PAB419
M73
200 kDa
100 kDa
97 kDa
69 kDa
46 kDa
30 kDa
p300
p107
p105
E1A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

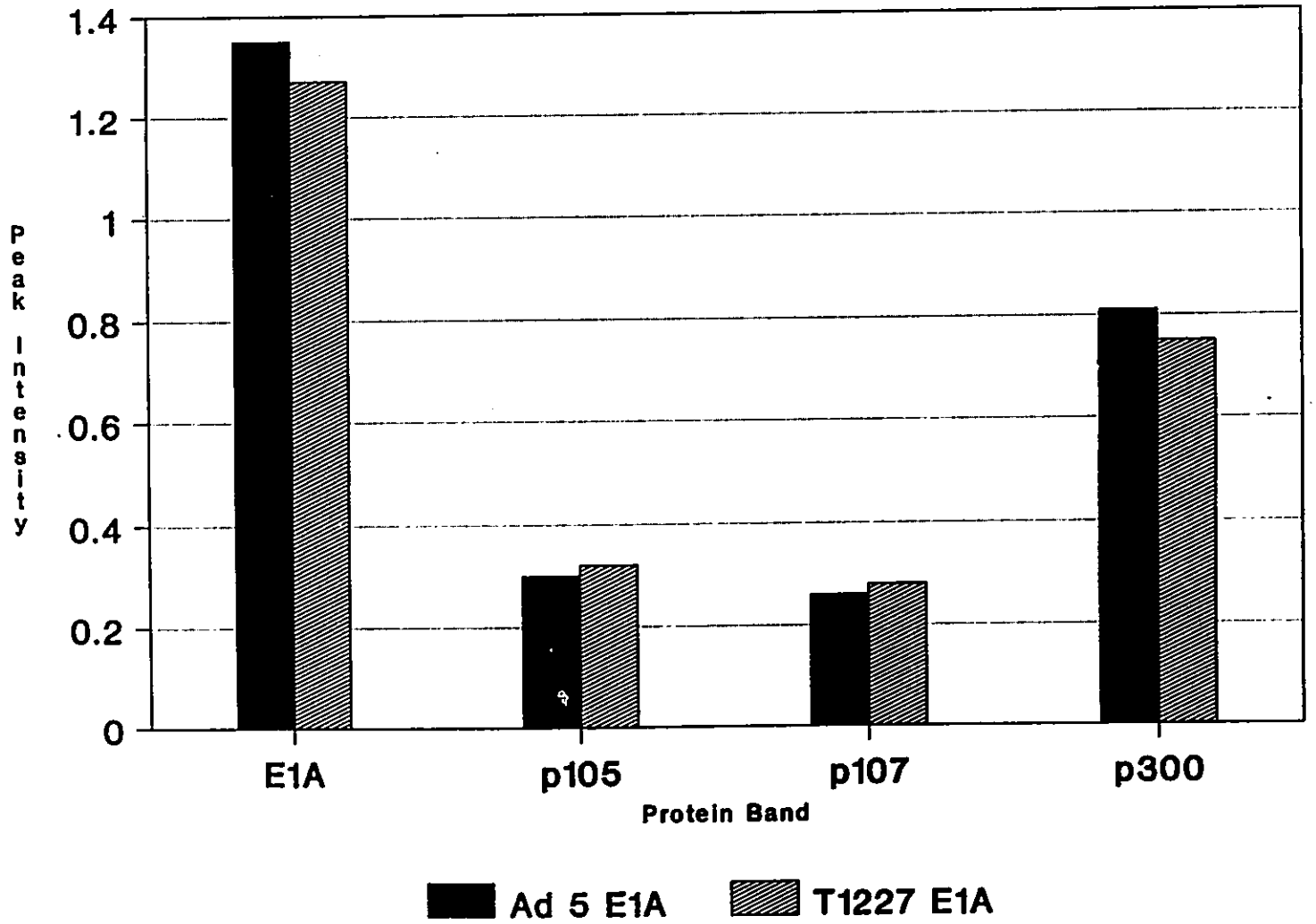
autofluororadiogram in figure 3-19 confirmed that the intensities of the bands corresponding to Ad 5 E1A and its associated proteins were indistinguishable from their counterparts in the T1227 lane (figure 3-20), indicating that the binding of E1A to these proteins is not significantly different, and is unlikely to account for phenotypic differences.

4. Tumorigenicity of viruses.

As mentioned previously, the original classification of adenovirus serotypes was on the basis of tumor induction in newborn hamsters. Since the E1 region alone is sufficient to induce tumors in animals injected with transformed cell lines, newborn hamsters were injected with $10^8$ pfu of Ad 5, Ad 12, or T12E1, to determine whether the E1 region of Ad 12 could induce tumors when delivered into cells in the context of the Ad 5 genome. Of 20 hamsters injected with Ad 12, 5 died in infancy, 5/15 died from multiple tumors within 40 days, and 7 more within 52 days. The remaining three showed no signs of tumors for the duration of the assay (6 months). Of the hamsters injected with Ad 5 (12 hamsters) and T12E1 (20 hamsters), none showed any signs of tumors for 6 months. These results are consistent with the findings of Bernards *et al.*, 1984, and Sawada *et al.*, 1988, who reported no tumors in hamsters or rats following injection of newborns with viruses similar to T12E1. To verify that the lack of tumorigenicity was not due to permissivity of hamster cells for T12E1 replication, 60 mm dishes of BHK21 cells were infected with $10^5$ pfu/dish of Ad 5, Ad 12 or T12E1. The infections were allowed

**Figure 3-20. Densitometric quantitation of coimmunoprecipitated cellular proteins.**

Densitometry was carried out on bands corresponding to E1A, p105, p107 and p300 in the autofluororadiogram in figure 3-19. Relative amounts of protein are represented by the maximum intensity of the band corresponding to each species.




PEAK HEIGHTS REFLECT FILM DARKNESS

to proceed for 5 days, during which no cytopathic effect was observed. At 5 days post-infection, the cells were scraped from the plates, and subjected to three cycles of freeze-thaw. The extracts were then titrated on 293 cells. The results, shown below, indicate that while Ad 5 is able to replicate on BHK21 cells, the final titre being 1000-fold higher than the input, neither Ad 12 or T12E1 could replicate on this cell line.

| VIRUS | Ad 5 | Ad 12 | T12E1 |
|---|---|---|---|
| TITRE | $10^8$ pfu | $10^2$ pfu | $10^3$ pfu |

## D. Transformation Of Primary Cells

### 1. Quantitation of transforming activity of hybrid E1 plasmids.

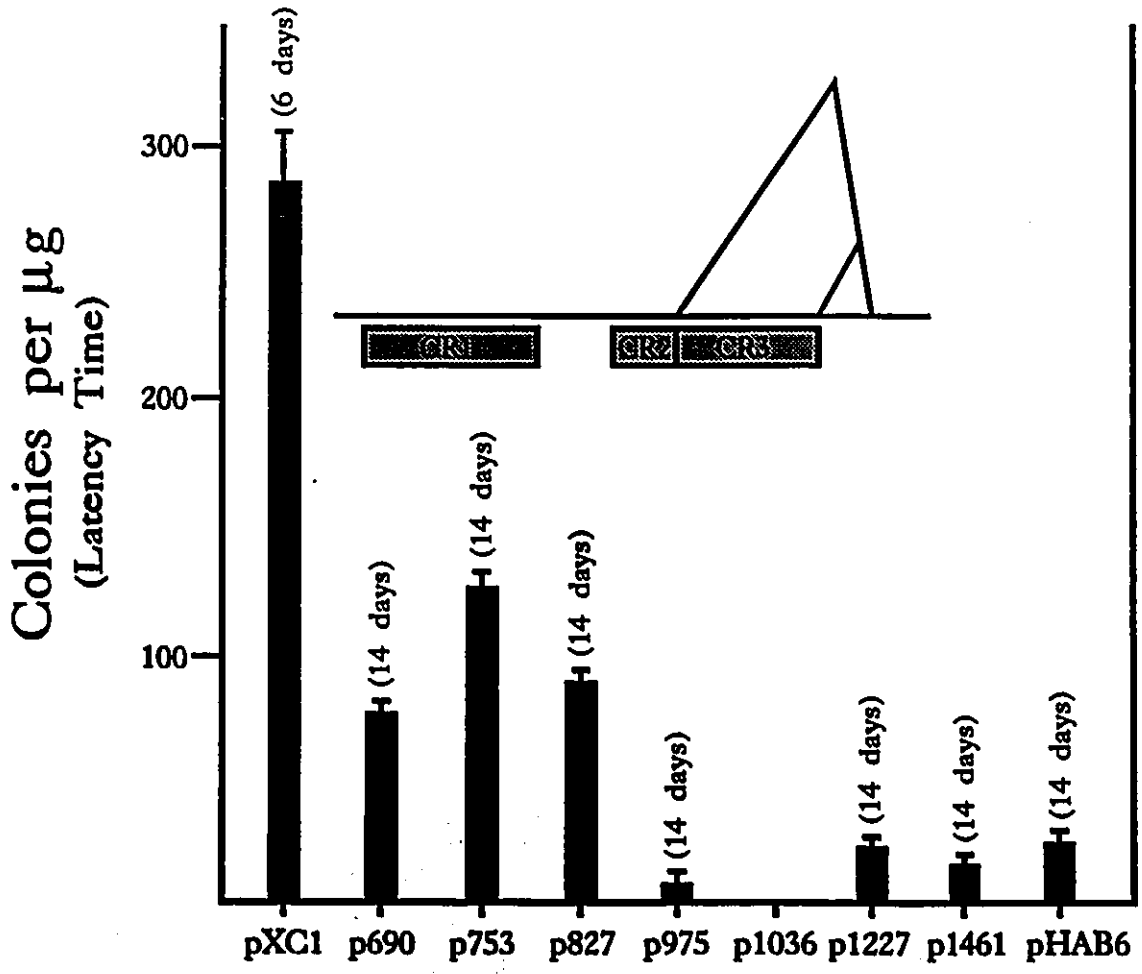
As previously discussed, one phenotypic difference between the E1 regions of Ad5 and Ad12 is the frequency at which such cloned E1 regions give rise to transformed colonies. To determine which regions of the E1 protein influence the frequency of transformation, hybrid E1 plasmids were used to electroporate primary BRK cells.

Plasmid DNA was purified on cesium chloride gradients, and concentrations were determined by diphenyl-amine reactions specific for deoxyribose. The DNA

concentrations were then diluted to 0.5 mg/mL as a standard. The efficiency at which hybrid E1 plasmids were able to transform was then quantitated by electroporation of primary BRK cell with the plasmid DNA. Dose response curves were plotted, and the linear portion of the curve was used to determine the efficiency of colony formation (figure 3-21). pXC1 gave rise to transformed colonies at an efficiency of approximately 280 colonies per μg of transfected plasmid, with the first visible transformed colonies appearing at 6 days post-transfection. Hybrids with crossovers to the left of CR 2 (690, 753, 827) transformed BRK cells at efficiencies ranging between 50 and 100 colonies/μg, with colonies typically appearing between 10 and 14 days post-transfection. In contrast, hybrids p975, p1227, and p1461 as well as pHAB6 transformed BRK cells at efficiencies not exceeding 15 colonies/μg, with colonies appearing after a time interval similar to that seen with amino terminal crossovers. The plasmids p2743 and pFX12 were also analyzed, and had transforming efficiencies and latency times very similar to pHAB6. These results suggest that the differential transforming efficiency of Ad 5 and Ad 12 E1A is influenced by two regions of E1A, the first located in the amino terminus, and the second extending from the right border of CR1 to the right border of CR2. The time of appearance of transformed colonies appeared to be dictated primarily by the amino terminal region of the E1A protein. No transformed colonies were observed with p1036, but since the virus T1036 behaved like a transactivation defective host-range mutant, the probable cause of defective transformation is the inability to transactivate the Ad 5 E1B transcription unit, consistent with other reports (Ho *et al.*, 1982; Babiss *et al.*, 1984; Byrd *et al.*, 1988;

**Figure 3-21. Transforming activity of hybrid E1 plasmids.**

Primary baby rat kidney cells were prepared by trypsinization of kidneys from 6-day old rats, and the cells were electroporated with varying amounts of hybrid E1 plasmids. Cultures were fixed and stained at two weeks post-transfection, and colonies were counted. Dose response curves were generated from four points for each hybrid, each point representing the average of four 60 mm dishes. Values shown were determined from the linear portions of the dose-response curves, and error bars indicate standard error. The latent period for colony appearance is indicated in brackets above each bar, while the amino acid content of each hybrid is indicated beneath the histogram. A schematic of the E1A gene is superimposed on the histogram. The drawing is not to scale; instead its purpose is to indicate by alignment the location in E1A of the crossover in each hybrid.




| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ad 12 Residues | - | 1-49 | 1-66 | 1-83 | 1-147 | 1-166 | 1-193 | 1-266 | E1A+ E1B |
| Ad 5 Residues | 1-289 | 45- | 66- | 90- | 141- | 160- | 184- | - | - |

Bautista *et al.*, 1991). As described in section D.2, the E1A sequences alone from this plasmid were cotransfected with a plasmid containing the Ad 12 E1B transcription unit, giving rise to a a single transformed colony which was expanded to establish a fully transformed cell line expressing E1 proteins. This suggests that the ability of the p1036 hybrid E1A protein to maintain the transformed state was not compromised.

2. Establishment of Cell Lines.

The primary purpose of establishing transformed cell lines was to compare the tumorigenicity of lines transformed by various E1A hybrids in syngeneic rats. The hybrid E1 plasmids as, originally constructed, contained varying amounts of Ad 12 E1 sequence from the beginning of the E1A transcription unit to the specific crossover point. Besides pFX12 and p2743, which contained all or part of Ad 12 E1B, all the other hybrid plasmids encoded the Ad 5 E1B transcription unit. Previous work has indicated that while the tumorigenicity of adenovirus transformed cells in immunocompetent animals is influenced primarily by the identity of the E1A transcription unit, the E1B transcription unit is also relevant. Specifically, Ad 12 E1B mediates a higher degree of tumorigenicity in a shorter time period than Ad 5 E1B. To study the role of E1A in tumorigenicity, it was decided to transform cells with combinations of a hybrid E1A gene and the Ad 12 E1B transcription unit in order to subsequently be able to identify tumorigenic cell lines within a short time, and to increase the significance of negative results. To this end, all hybrid plasmids with crossovers in E1A were digested at positions 1770, 3641, and 5644

with *Sac* I (figure 3-22) and re-ligated, resulting in the deletion of all but the first 120 nucleotides of the E1B transcription unit. The resulting clones were screened by digestion with *Hind* III, which cleaves the resulting plasmid at a single site to generate a fragment of 6.0 kb (figure 3-22, lanes 2, 6).

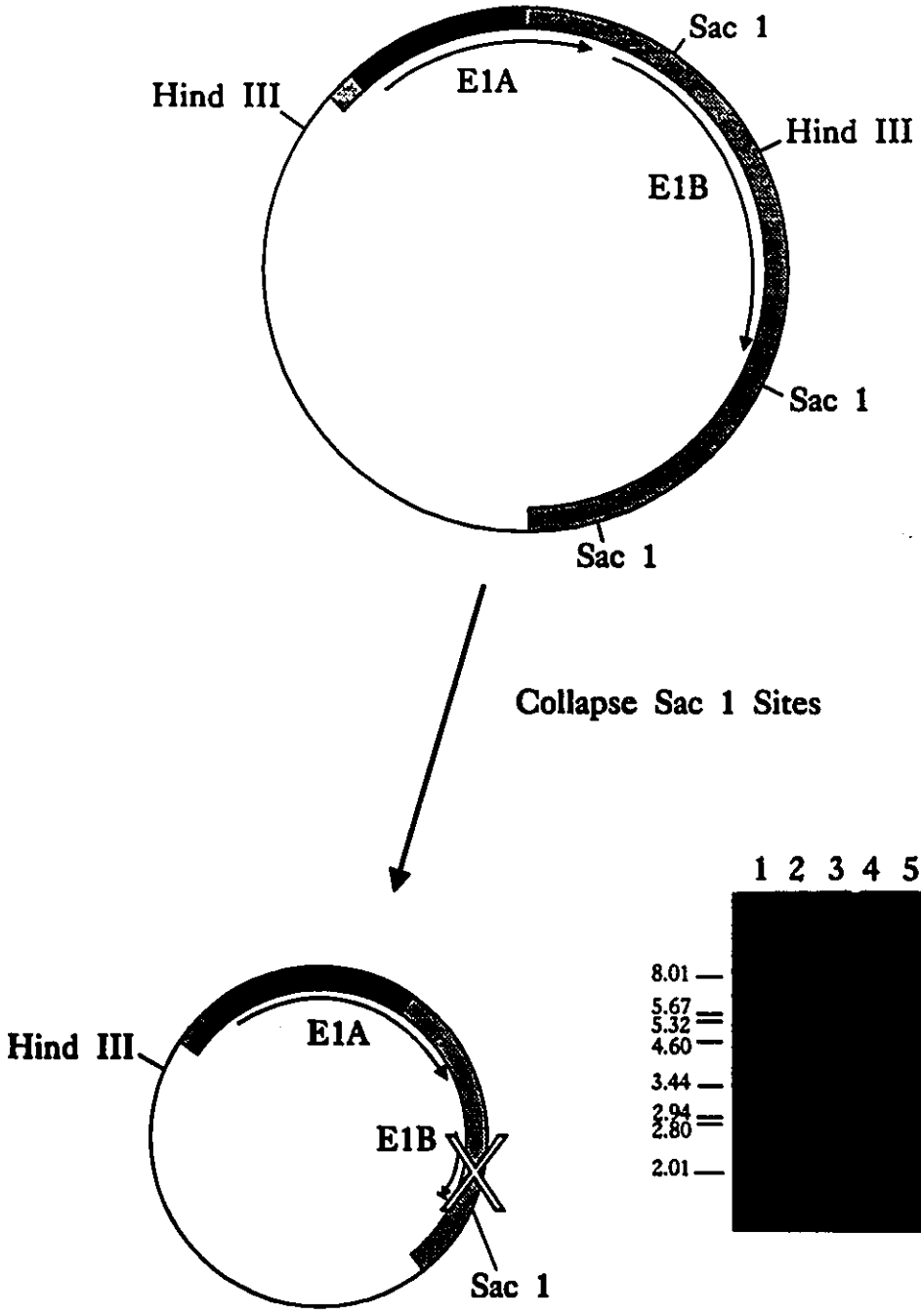
Primary BRK cells were electroporated with hybrid E1A plasmids lacking E1B sequences and pHAB13, a derivative of pHAB6 which has a deletion of E1A sequences to nucleotide 1385 (S. Mak, unpublished), but encodes an intact Ad 12 E1B transcription unit. Following 4 to 6 weeks in the transfected dish, individual colonies of transformed cells were isolated by the use of a sterile stainless steel cloning cylinder, and trypsinized. The cells were grown in 25 $cm^2$ flasks until confluent, then expanded into larger flasks and ultimately 150 mm plates. At certain times during the establishment of cell lines, particularly when seeded at a low density, cells grew in isolated patches which often reached a high level of confluence. When this occurred, the cells were trypsinized and re-seeded at a ratio of 1:1 to spread cells more evenly and optimize growth conditions. Once sufficient numbers of cells were obtained, samples were frozen in fetal bovine serum and 10% DMSO, and stored in liquid nitrogen.

3. Characterization of transformed cell lines.

Protein samples from each transformed cell line were subjected to electrophoresis and Western blotting, to verify that the cells expressed various E1 proteins. The blots were probed with the M73 monoclonal antibody to examine E1A

## Figure 3-22. Deletion of E1B From Hybrid Plasmids.

Hybrid E1 plasmids were digested with *Sac* I, and re-ligated. The purpose of this is the elimination of E1B sequences between the *Sac* I sites at positions 1770 and 5644. To screen for clones which had lost E1B sequences, plasmids were analyzed by *Hind* III digestion, which yields one fragment of approximately 6 kb (lanes 2, 6). Other restriction patterns indicate deletion of a *Sac* I fragment not encompassing E1B sequences (lane 7), or deletions of the fragment containing E1B, but retention of non-E1 sequences between positions 3641 and 5644 (lanes 3, 4, 5).


Sac 1
Hind III
E1A
Hind III
E1B
Sac 1
Sac 1
Collapse Sac 1 Sites
1 2 3 4 5 6 7
8.01
5.67
5.32
4.60
3.44
2.94
2.80
2.01
Hind III
E1A
E1B
Sac 1

expression, and with serum from tumor bearing rats to examine E1B expression. As shown in figure 3-23.A, E1A expression was easily detected in cells transformed by the Ad 5 E1 region, as well as in cells transformed by hybrid E1A plasmids p827, p975, p1036, and p1227. Reduced amounts of E1A were detected in extracts from cells transformed by p690 and p753, which could reflect reduced stabilities of these E1A proteins in rat cells, or reduced levels of expression. No protein was detected in the lane corresponding to 1461B12C1, which is expected because p1461 encodes Ad 12 E1A, which is not recognized by the M73 monoclonal antibody.

To examine E1B expression, the same blot which was probed with M73 was washed and re-probed with serum from rats bearing tumors induced by the Ad 12 E1 transformed cell line, 12-1. Figure 3-23.B shows this result, in which it is evident that the signal corresponding to E1A proteins has not been eliminated. However, novel bands of higher molecular weight corresponding to the Ad 12 E1B 55k protein are clearly visible, and absent from cells transformed by Ad 5 E1 and the control cell line (1mT), which is a Rat-2 cell derivative, transformed by polyomavirus middle T antigen (Jelinek and Hassell, 1992).

## E. Tumorigenicity Assays On Transformed Cells

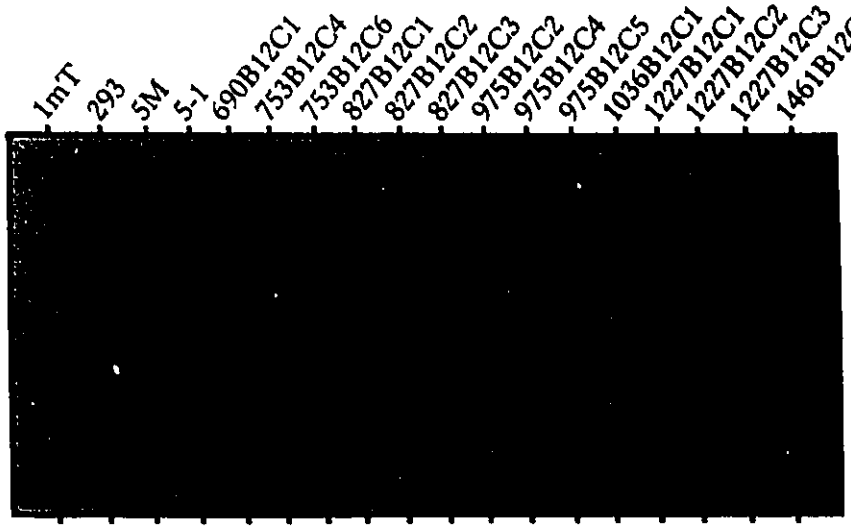
To determine which hybrid E1 plasmids could give rise to tumorigenic cell lines, selected transformed cell lines were assayed for tumorigenicity in syngeneic rats.

## Figure 3-23. Western Analysis of Transformed Cells.

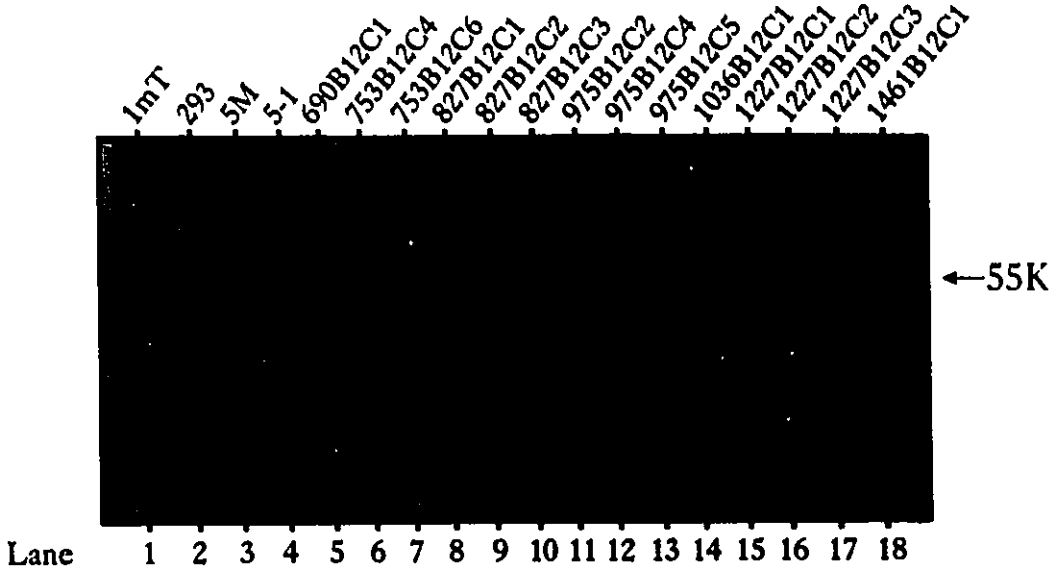
Filters were probed first with the M73 monoclonal antibody against the carboxy terminus of Ad 5 E1A (A). The polyomavirus middle-T transformed negative control, 1mT, showed no signal, nor did the cell line transformed by p1461, encoding an Ad 12 E1A protein. The cell lines 690B12C1 and 753B12C4 also showed no signal. Ad 5 E1A was readily visible in the lanes corresponding to 293 cells, and the rat cell lines transformed by Ad 5 E1, 5M, and 5-1. E1A proteins were visible in the remainder of the cell lines, although the transfer appeared to be imperfect over the area of E1A in the 753B12C6 lane.

The same filters were probed with 12-1 serum, raised against Ad 12 transformed BRK cells. A faint band was visible in the 1mT lane, which did not correspond in size to any bands in other lanes. The residual E1A signals were still visible in many lanes, but two clear bands appeared in the 1461B12C1 lane, corresponding to E1A (lower), and the E1B 55K protein (upper). The latter band was also visible in all lanes but the control, the Ad 5 transformed cell lines, 975B12C2, and 1227B12C3. The last two cell lines have been assayed in two separate experiments, and in both cases lacked detectable E1B 55K protein.

A


1mT
293
5M
5-1
690B12C1
753B12C4
753B12C6
827B12C1
827B12C2
827B12C3
975B12C2
975B12C4
975B12C5
1036B12C1
1227B12C1
1227B12C2
1227B12C3
1461B12C1
Lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

B


1mT
293
5M
5-1
690B12C1
753B12C4
753B12C6
827B12C1
827B12C2
827B12C3
975B12C2
975B12C4
975B12C5
1036B12C1
1227B12C1
1227B12C2
1227B12C3
1461B12C1
←55K
Lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

## E. Tumorigenicity Assays On Transformed Cells

To determine which hybrid E1 plasmids could give rise to tumorigenic cell lines, selected transformed cell lines were assayed for tumorigenicity in syngeneic rats. Cell lines were collected from 150 mm plates following a minimum number of passages in culture (usually less than 10), in an attempt to minimize the time during which cells could adapt to or be influenced by the cell culture environment. Tumorigenicity assays generally used newborn rats, but certain cell lines were also assayed on weanling rats. The results of assays with newborn rats are summarized in Table 3-1, and a typical tumor-bearing rat is shown in figure 3-24. Cell lines transformed by p827 E1A plus Ad 12 E1B were not tumorigenic in three separate experiments, even at an input dose of $10^8$ cells per rat. In two instances, small tumors arose at the site of injection after 4 months, but both tumors regressed, and did not recur. These rats were sacrificed at the age of 8 months, and bled by cardiac puncture to obtain serum. The rats were then dissected, to examine tissues at the site of injection. No signs of tumors or vascularized bodies were noted. These results are interpreted as indicating that p827 either does not contain Ad 12 sequences necessary for tumorigenicity, or alternatively, this hybrid could contain Ad 5 sequences which preclude tumorigenicity. Clonal variation was seen with cells transformed by p975 plus Ad 12 E1B. Cell line 975B12C2 did not produce any tumors within 6 months. However, cell line 975B12C4 was highly tumorigenic, giving rise to tumors within 2 months. Lastly, cell line 975B12C5 was weakly tumorigenic, giving rise

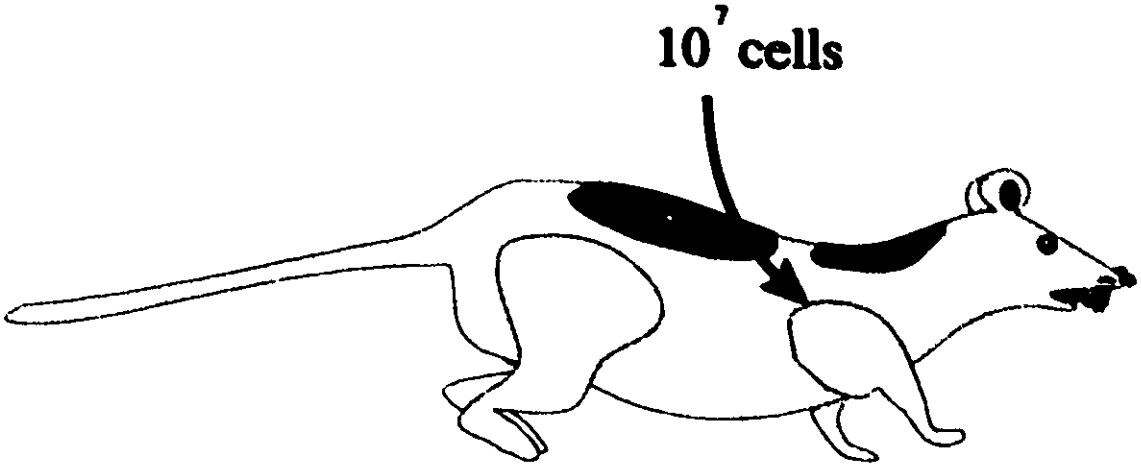
TABLE 3-1. Tumorigenicity of cell lines transformed by hybrid E1A plasmids plus Ad12 E1B.

| | Time (Months) | | | | | |
|---|---|---|---|---|---|---|
| Cell Line[a] | 1 Month | 2 | 3 | 4 | 5 | 6 |
| 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 |

[a]Each row represents the injection of a cell line into a single litter of newborn rats at $10^7$ cells per rat.
[b]This litter was injected with $10^8$ cells per rat.

## Figure 3-24. A Tumor Bearing Rat.

The rat shown here was injected as a newborn with cell line 1036B12C1, and at 5 months of age etherized, bled by cardiac puncture, and photographed. The tumor on the back of the rat was entirely subcutaneous, with no sign of metastasis. Tumors were not permitted to grow beyond this size.


10^7 cells

to only one tumor in each of two separate experiments. Several additional rats developed what appeared to be small tumors, which subsequently receded. Following etherization and cardiac puncture, one of the tumor bearing rats injected with 975B12C5 was dissected, revealing a readily detectable disc-shaped tumor approximately 15 mm in diameter and 3 mm in thickness. Cell lines transformed by p1227 were tumorigenic in three separate experiments, showing clonal variation only in the time of tumor appearance (1227B12C2 induced tumors slowly, but 5/5 rats eventually developed tumors). As was mentioned in section D.1, p1036 gave rise to a single transformed colony, from which a cell line expressing E1 proteins was established. The cell line 1036B12C1 was tumorigenic, indicating that the transactivation property of E1A is not required for tumorigenicity. These results suggest that hybrid E1A proteins with Ad 12 sequences from the amino terminus to the region between CR1 and CR2 are unable to give rise to tumorigenic cells, while those containing Ad 12 sequences at least to the left border of CR3 are tumorigenic (sequences shown in figure 3-12).

In addition to assaying cell lines for tumorigenicity in newborn rats, several cell lines were injected into weanlings, to determine whether rats which are more immunologically mature can reject cells which are tumorigenic in newborns. Summarized in Table 3-2, the results indicate that for the cell lines tested, there was concurrence with the results observed in newborn rats. The cell lines 2743-1 and 2743-2 were not tested in newborns, but were fully tumorigenic in weanling rats, indicating that the properties of E1 required for tumorigenicity are not encoded within the carboxy half of the E1B 55K

TABLE 3-2. Tumorigenicity of transformed cell lines on weanling rats.

| | Time (Months) | | | |
|---|---|---|---|---|
| Cell Line[a] | 1 | 2 | 3 | 4 |
| 1227B12C1 | 0/7 | 3/7 | 4/7 | 4/7 |
| 2743-1 | 0/12 | 1/12 | 1/12 | 1/12 |
| 2743-2 | 0/10 | 5/10 | 5/10 | 5/10 |
| 12-1 | 0/10 | 0/10 | 6/10 | 3/10 |
| 12-2 | 2/10 | 1/10 | 2/10 | 3/10 |

[a]Weanling rats (3 weeks) were injected with $10^7$ cells per rat.

protein.

## F. Construction Of Additional Hybrids

### 1. Rationale.

The results presented above indicate that a region of the Ad 12 E1A protein surrounding CR2 is important for tumorigenicity. This raises the question of whether this region is the sole factor determining the oncogenicity of different E1A proteins. In particular, the region of the Ad 12 E1A protein located between CR2 and CR3, has no counterpart in Ad 5 E1A. To assess the role of this region in tumorigenicity, CR2 of Ad 5 E1A was replaced with the corresponding region of Ad 12 E1A, containing CR2 and the region between CR2 and CR3. To create such plasmids, it was necessary to use the polymerase chain reaction to amplify specific fragments with engineered restriction sites. Since the existing hybrid E1A plasmids p975 and p1227 contained crossovers downstream of the region of interest, the crossover sites in these plasmids were used as the rightmost borders of Ad 12 sequences in second generation hybrids. Although p975 appeared to contain the minimum amount of Ad 12 E1A necessary for tumorigenicity, two cell lines transformed by this plasmid were not highly oncogenic. This could suggest that sequences to the right of the crossover site in p975 also play some role in the tumorigenic property. For this reason, p1227 was also used for these experiments, since it contains all of CR3 of Ad 12 E1A, and all cell lines transformed by it were tumorigenic.

2. The use of PCR to construct new hybrids.

The Ad 5 E1A gene contains a unique *Cla* I restriction site, which is methylated by the *dam* methylase in most strains of bacteria. The location of the restriction site is at nucleotide 917 (ATCGAT, figure 3-1), which encodes amino acid residues 120 and 121 (Ile, Asp), at the left border of CR2 (figure 3-12). Ad 12 has Met and Asp residues at the analogous positions (residues 105, 106), but no restriction site.

```
                GluValIleAspLeuThrCysHisGluAla
Ad 5:  911      GAGGTGATCGATCTTACCTGCCACGAGGCT
                || |  || ||| |    ||| |||||
Ad 12: 803      GAAGATATGGATTTATTGTGCTACGAGATG
                GluAspMetAspLeuLeuCysTyrGluMet
```

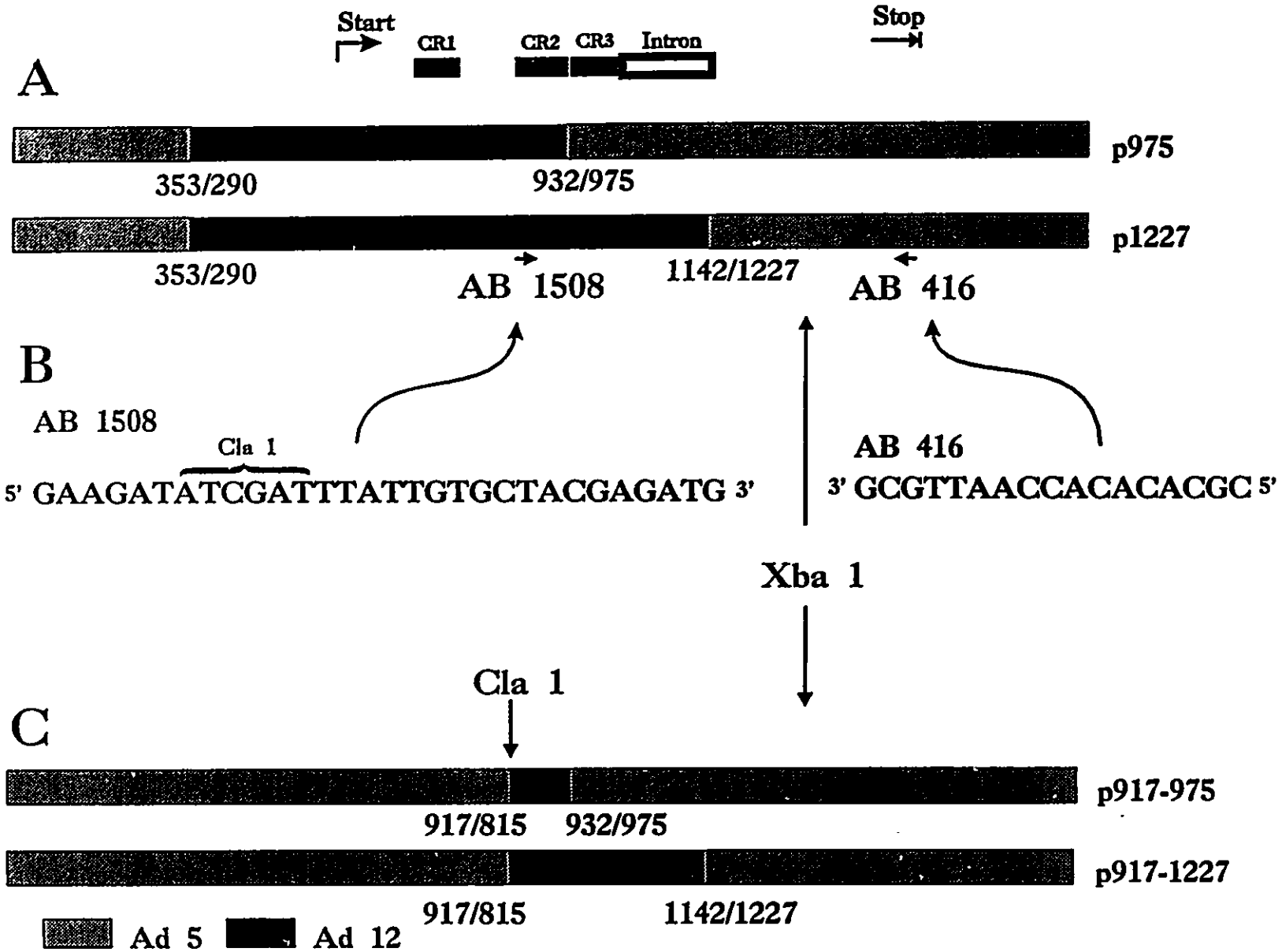
To generate an Ad 12 E1A PCR fragment with a *Cla* I site at this position, an oligonucleotide (AB1508, figure 3-25.B) was synthesized which was identical to the coding strand of Ad 12 at this region with the exception that it encoded a *Cla* I site near its 5' end. This oligonucleotide, and AB416 (complementary to the Ad 5 E1A coding strand, figure 3-25.B) were used in two separate PCR reactions, to amplify the fragments of p975 and p1227 DNA containing the crossovers (figure 3-25.A). The resulting fragments were cloned in pUC19 as blunt-ended fragments, and sequenced. The *Cla* I-*Xba* I fragments of each plasmid were then excised, purified, and ligated to a *Cla* I-*Xba* I digest of a pXC38 derivative (with pBR322 *Cla* I site eliminated) which had been grown in the *dam*⁻ *E. coli* strain GM48 so that the Ad 5 E1A *Cla* I site at position 917 would not be methylated. The resulting constructs are shown in figure 3-25.C. p917-975 contains

## Figure 3-25. Construction of New Hyrid E1A Genes.

A. The hybrid E1A plasmids p975 and p1227 were linearized and used as templates in PCR reactions.

B. The primers for PCR. AB1508 was nearly identical to 30 base pairs of the coding strand of Ad 12 E1A overlapping the left border of CR2. A *Cla* I site was introduced into this primer, resulting in 2 mismatches. AB416, previously used for dot blot and sequence analysis of hybrid E1A plasmids, is complementary to the Ad 5 E1A coding strand in the second exon of the protein. With this combination of primers, only hybrid E1A plasmids p975, p1036 and p1227 can give rise to a PCR product. The amplified products were treated with the Klenow fragment of pol I, and cloned into pUC19 as blunt-end inserts.

C. pXC38 was linearized with *Cla* I, treated with the Klenow fragment of pol I, and re-ligated. The resulting plasmid, which no longer carried a *Cla* I site in the vector, was purified from *dam*$^-$, *dcm*$^-$ bacteria, GM48. It was then digested with *Cla* I and *Xba* I, and the large fragment was ligated to the hybrid E1A *Cla* I-*Xba* I fragments from the PCR generated recombinants. The resulting plasmids, p917-975, and p917-1227, contain Ad 12 sequences from the beginning of CR2, as defined by the position of the *Cla* I site in Ad 5 E1A, to the crossover point of the original hybrid.


A
Start
CR1
CR2
CR3
Intron
Stop
p975
353/290
932/975
p1227
353/290
AB 1508
1142/1227
AB 416
B
AB 1508
Cla 1
5' GAAGATATCGATTTATTGTGCTACGAGATG 3'
AB 416
3' GCGTTAACCACACACGC 5'
Xba 1
Cla 1
C
p917-975
917/815
932/975
p917-1227
917/815
1142/1227
Ad 5
Ad 12

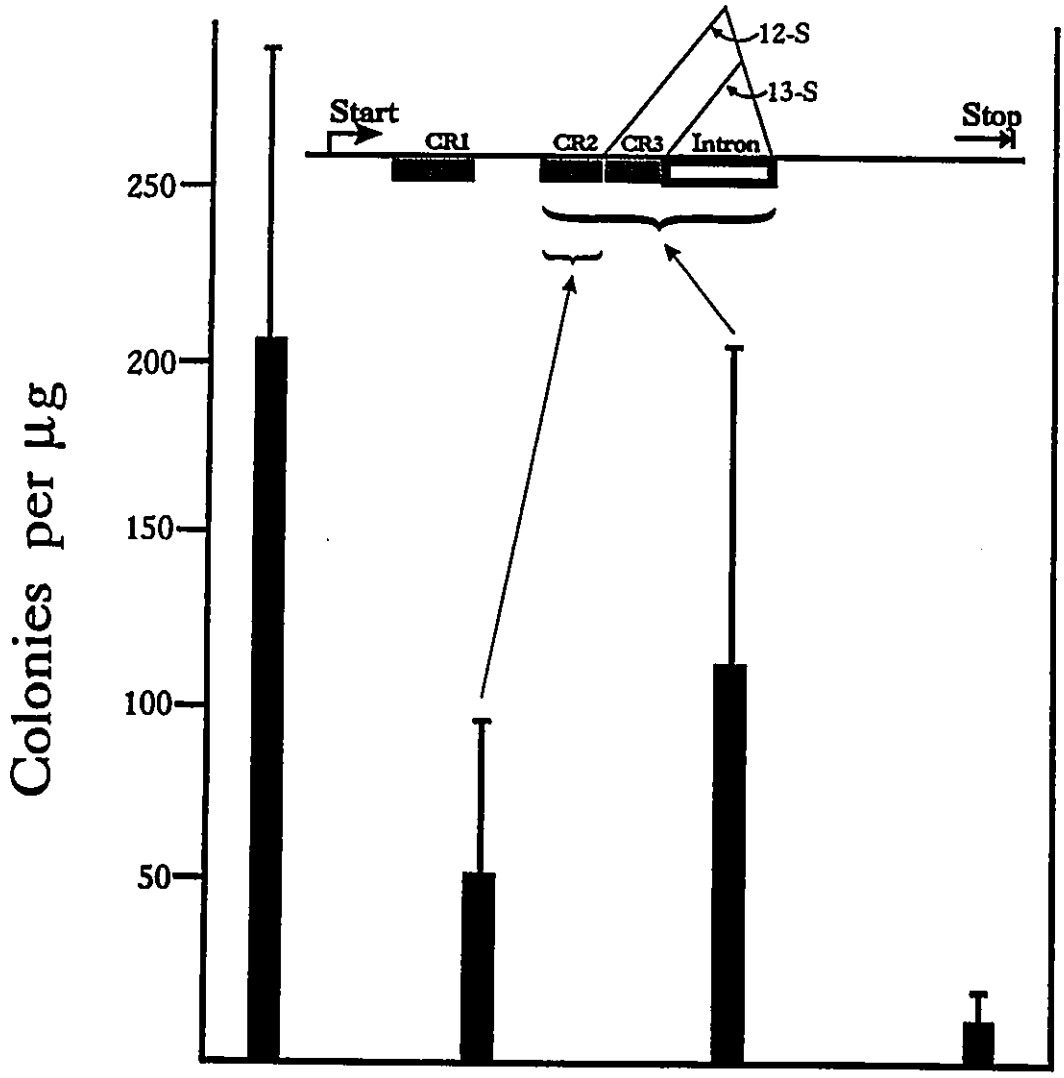
Ad 12 CR2 and the region between CR2 and CR3, in place of Ad 5 CR2. The second construct, p917-1227, contains Ad 12 CR2 through CR3 in place of the corresponding Ad 5 sequences. The locations of the Ad 5/Ad 12 junctions are shown in figure 3-25.C, and are defined by the site at which AB1508 was engineered to contain a *Cla* I site, and the crossover sites of the template hybrid E1A plasmids p975 or p1227.

3. Characterization of the transforming activities of the new hybrid E1A plasmids.

In previously described transformation assays, regions which corresponded roughly to CR2 and the amino terminus appeared to be important in determining the transforming efficiencies of hybrid E1A plasmids, with p690, p753 and p827 (Ad 5 CR2) transforming cells 7-10 fold more efficiently than p975, p1227, and pHAB6. Since both p917-975 and p917-1227 contain CR2 of Ad 12 E1A, but Ad 5 E1A through the leftmost portion of exon 1, quantitation of the transforming activities of these plasmids would indicate whether CR2 actually played a significant role in determining the transforming efficiencies of Ad 5 and Ad 12. As described previously, primary BRK cells were electroporated with each plasmid containing a hybrid E1A and Ad 5 E1B, and transformed colonies were counted after two weeks. The results, summarized in figure 3-26, revealed no significant difference in the abilities of p917-1227 and pXC1 to transform BRK cells. p917-975 was slightly less efficient at transforming cells, a characteristic which was shared by the hybrid p975, when compared to p1227 and pHAB6. Again, this could be due to the lack of a 12S mRNA product, which is important for transformation

## Figure 3-26. Transforming Efficiency of Second Generation Hybrid E1A Plasmids.

The results of two separate experiments were analyzed and are presented as described in the legend accompanying Fig. 3-21. This histogram shows the transforming efficiency of each second generation hybrid E1A plasmid, the standard error, and the sequence composition of each hybrid.




| | pXC1 | p917-975 | p917-1227 | pHAB6 |
|---|---|---|---|---|
| Ad 12 Residues | - | 107-147 | 107-193 | 1-266 |
| Ad 5 Residues | 1-289 | 1-121, 141- | 1-121, 184- | - |

(Haley *et al.*, 1984; Montell *et al.*, 1984; Hurwitz and Chinnadurai, 1985a,b; Moran *et al.*, 1986b; Lamberti and Williams, 1990). These results suggest that the differences in transforming efficiency between the group of p690, p753, p827, and the group of p975, p1227, p1461, and pHAB6 are not due to differences in CR2, but rather to the region between CR1 and CR2. While the latency time for transformation was approximately 14 days for the previously characterized hybrid E1A plasmids as well as for Ad 12, both p917-975 and p917-1227 had latency times of approximately 6 days, indistinguishable from Ad 5. This suggests that the latency time is determined by a region of E1A to the left of CR2. This is in agreement with the conclusion reached from transformation assays on the first group of hybrid E1A plasmids, which indicated that the presence of even a small portion of the first exon of Ad 12 E1A (p690) resulted in a delayed time of appearance of transformed colonies.

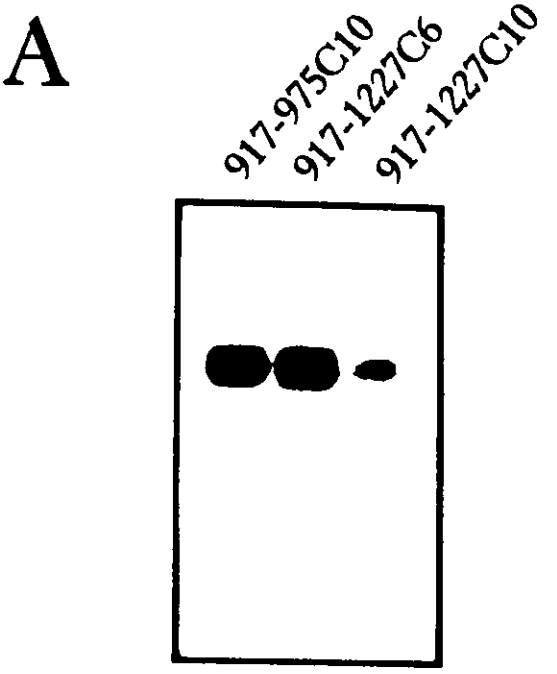
4. Establishment of cell lines transformed by p917-975 and p917-1227.

The E1B regions of both new hybrid E1A plasmids were deleted, and BRK cells were electroporated with these plasmids plus pHAB13, as described in section D.2. Cell lines were established, and assayed for protein expression by Western blot analysis. As seen in Fig. 3-27, all three cell lines expressed E1A, detectable by M73 monoclonal antibody (figure 3-27.A). To detect E1B proteins, this blot was probed with 12-1 anti-tumor serum, directed against the Ad 12 E1 region (figure 3-27.B). Three cell lines transformed by the Ad 12 E1 region were also probed with this serum, to confirm its

## Figure 3-27. Western Analysis of Cell Lines Transformed by Second Generation Hybrid E1A Plasmids.

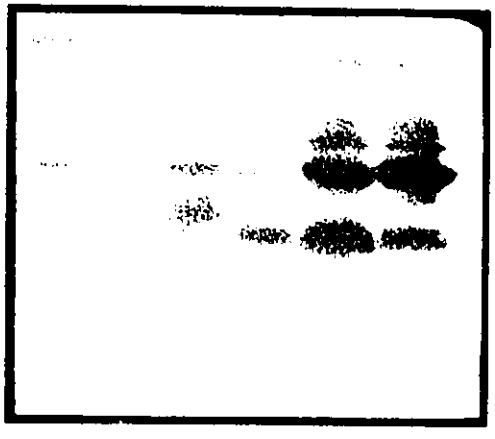
These assays were carried out simultaneously with those shown in figure 3-23, under the same conditions with the same controls. When probed with M73, the cell lines transformed by the new hybrid E1A plasmids all showed bands corresponding to E1A (A). When these blots were re-probed with 12-1 serum, a band appeared which corresponds to the Ad 12 E1B 55K protein. In addition, three cell lines transformed by the Ad 12 E1 region were included in these assays, and showed reactivity against E1A and E1B.

A




Lane 1 2 3

B




Lane 1 2 3 4 5 6

specificity (figure 3-27.B, lanes 4-6). While the bands corresponding to residual E1A signal were still present, new bands were clearly visible, corresponding in size to the E1B 55K band in the Ad 12 transformed cell lines (figure 3-27.B).

5. Tumorigenicity of new cell lines.

Newborn rats were injected ($10^7$ cells each) with cell lines transformed by p917-975 and p917-1227 plus pHAB13. The results, shown in Table 3-3, indicate that these cells were weakly tumorigenic. The region of Ad12 E1A introduced into Ad5 E1A in these plasmids was therefore not sufficient to confer a high degree of tumorigenicity to transformed cells, suggesting that while the newly introduced region of Ad 12 E1A may be important for tumorigenicity, one or more additional regions of the E1A protein are likely to be involved in the tumorigenicity of transformed cells.

TABLE 3-3. Tumorigenicity of cell lines transformed by p917-975 and p917-1227.

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

# DISCUSSION

## A. Analysis Of Hybrid Proteins

Genetic analysis of eukaryotic proteins has traditionally been carried out by alteration or deletion of sequences throughout the gene encoding a protein of interest, correlated with the loss or reduction of a measurable phenotype. Loss of a phenotype following directed mutagenesis is often due directly to the loss of sequences specifying that phenotype, but mutations may also alter the structure or stability of an entire protein to the extent that properties not specifically encoded by the altered sequences may be lost or reduced. Examples of this are evident with the E1B 55K protein of Ad 2 (Yew *et al.*, 1990), and Ad 12 (Schaller *et al.*, unpublished), whose levels of expression can be reduced by mutations in various regions of the coding sequences.

In some studies, chimeric proteins have been constructed by splicing together gene segments with common restriction sites to analyze differences between related proteins, as in the case of chimeric E1A proteins containing exon 1 of Ad 12 E1A linked to exon 2 of Ad 5 E1A and vice versa (Jochemsen *et al.*, 1984). When feasible, this approach can offer significant advantages over mutational analysis, as it affords the possibility of correlating the acquisition of properties by a hybrid protein with the

presence of sequences from the parent protein which shares those properties, providing strong evidence that the property being assayed is specified by the sequences in question. A limitation of this approach has been the need for conveniently located restriction sites or complex PCR strategies to create genes encoding chimeric proteins.

This study has utilized a technique involving homologous recombination in *E. coli* to generate chimeric E1A proteins, without the need for splicing together of common restriction fragments or use of PCR. Similar techniques have been used to construct hybrid α1/α2 interferon genes (Weber and Weissmann, 1983) and to analyze subtilisin proteases where protein expression can be assayed directly in bacteria bearing the recombinant plasmid (Gray *et al.*, 1986). Both these studies created hybrids between proteins with approximately 80% homology at the DNA level, but this study demonstrates the feasibility of this technique to construct hybrids between less-related genes, Ad 5 and Ad 12 E1A being approximately 40% homologous at the DNA level. Chimeric E1A genes were generated by crossovers in regions of patch homology as short as 7bp throughout the coding sequences of the Ad 12 and Ad 5 E1A genes. It is not clear whether any seven random nucleotides are sufficient or whether certain sequences serve as preferential substrates for recombination. The low stringency requirements for recombination indicate that this type of approach can successfully generate a large number of hybrid plasmids to map properties with a reasonably high degree of precision. The localization of crossovers in regions of partial sequence homology in most cases generated in-frame junctions of similar amino acid coding sequences. This makes it likely that the resulting chimeric proteins are not altered in terms of their secondary and tertiary structures.

However, since each crossover represents a unique juxtaposition of partially dissimilar sequences, individual hybrids can be non-functional in some respects (discussed later). Two hybrids were isolated with crossovers in conserved region 1, where the homology between Ad 5 and Ad 12 is fairly high. There is little homology in the region separating conserved regions 1 and 2, with the exception of one patch homology corresponding to the crossover site in p827. The low degree of homology between even closely related proteins in certain regions will undoubtedly limit the usefulness of this technique to study properties localizing to these regions. However, when justified by available evidence, specifically designed chimeric genes can be generated by introduction of restriction sites with PCR and splicing together of the resulting fragments, as was done in the construction of p917-975 and p917-1227.

## B. Characterization Of Recombinant Viruses

Attempts to isolate recombinant viruses directly by transfection of 293 cells with pJM17 and pHAB6 were not successful. The formation of an infectious virus derived from pJM17 and containing Ad12 E1 sequences would require Ad5 sequences at the left terminus to facilitate packaging and DNA replication in the context of the Ad5 genome, and such a construct could only arise from two recombination events between Ad5 and Ad12 DNA. While recombination between regions of limited homology appears to occur frequently in *E. coli*, it is probably less common in human cells. Subsequent attempts to

isolate hybrid viruses by cotransfection of pJM17 and pT2743 (Ad 12 left end, Ad 5 sequences at the right half of the E1 region) were also unsuccessful. Only when the Ad 5 ITR and packaging signal were added to the hybrid E1 plasmids was it possible to generate recombinant viruses. This indicates that if a crossover were to occur between identical Ad 5 sequences in pJM17 and pT2743, then the packaging signal of Ad 12 could not substitute for that of Ad 5, suggesting that the packaging machinery of Ad 5 is serotype specific.

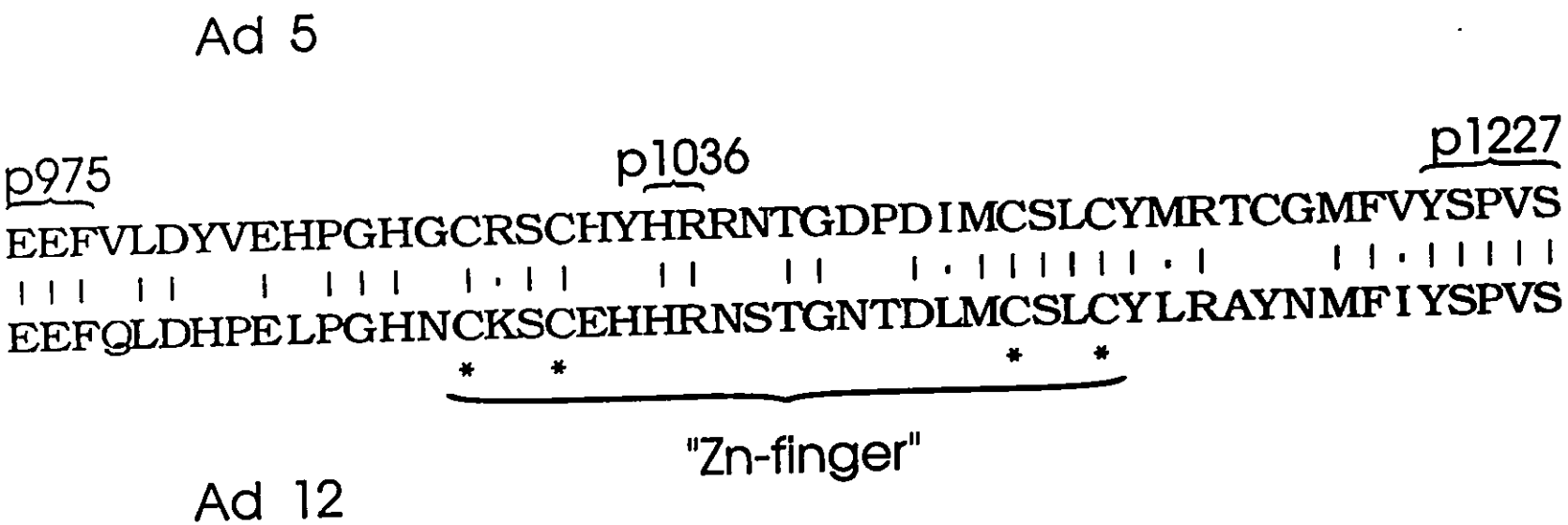
In terms of the ability of the hybrid E1A proteins to activate viral gene transcription, as assayed by viral replication on HeLa cells, all but one construct produced a functional protein. The exception was T1036, which has a hybrid CR3 and expressed a protein which conferred to the virus the characteristics of a transactivation defective host-range mutant. This virus expressed lower than normal levels of E1A when cells were infected at lower multiplicities. In light of the host range phenotype of this virus, it is likely that the decreased amounts of protein were a consequence of the failure to transactivate early viral gene expression, including E1A. A second possibility is that the hybrid E1A protein produced by T1036 is less stable than those produced by other viruses. Several lines of evidence suggest that this is not the case. First, it is known that greatly reduced quantities of E1A do not result in a host range phenotype on HeLa cells, while mutations in CR3 of E1A do (Hitt and Graham, 1990). Furthermore, defective E1A mutants express lower than normal levels of E1A protein (Hitt and Graham, 1990). For these reasons, it is unlikely that the reduced quantities of the E1A protein produced by T1036 would alone result in a host range phenotype. Second, infection of cells with

T1036 at higher multiplicities, whereupon E1A was less important for viral gene expression, resulted in a normal level of E1A expression. Lastly, the T1036 E1A protein was expressed at levels comparable to those of other hybrid E1A proteins in transformed cell lines, suggesting that protein stability is not the issue. These data suggest that the nature of the defect in T1036 is the transactivation function of E1A. This was a somewhat surprising result, since sequence alignment (figure 4-1) indicates that the homology between Ad 5 and Ad 12 is very high in CR 3. Furthermore, a 2 amino acid insertion into the Ad 5 E1A transactivation region at the same site as the crossover in p1036 had no effect on the ability of the mutant E1A protein to transactivate (Bautista *et al.*, 1991). The fact that a hybrid protein with a chimeric domain in this highly homologous region was unable to transactivate suggests that CR 3 is sensitive to subtle alterations in structure, since hybrids with crossovers to the left or right of CR 3 were fully active. Furthermore, the precise location of the crossover in p1036 is in the middle of the putative zinc finger region (Berg, 1986; Culp *et al.*, 1988). If the conformation of this region is critical for efficient function, then slight alterations imposed by the combination of two heterologous halves of this domain could account for defective transactivation by the p1036 E1A protein.

Viruses containing all (T12E1) or part (T2743) of Ad 12 E1B were defective for plaquing on HeLa cells, consistent with the results of others suggesting that Ad 12 E1B is not able to support the replication of Ad 5 in the absence of Ad 5 E1B (Sawada *et al.*, 1988). Since T12E1 replicates less efficiently than T2743, while both contain the Ad 12 E1B 19K coding region, it is probable that the defect in these viruses is mediated

## Figure 4-1. Sequence Alignment in CR3.

The location of the putative zinc finger is indicated below the alignment, with the locations of the cysteine pairs (*). Residues identical in Ad 5 and Ad 12 E1A are indicated by longer vertical lines, and similar residues by shorter vertical lines. Residues at the crossover sites in p975, p1036 and p1227 are indicated above the alignment.


Ad 5
p975
p1036
p1227
EEFVLDYVEHPGHGCRSCHYHRRNTGDPDIMCSLCYMRTCGMFVYSPVS
EEFQLDHPELPGHNCKSCEHHRNSTGNTDLMCSLCYLRAYNMFIYSPVS
"Zn-finger"
Ad 12

by the 55K protein, which is derived exclusively from Ad 12 in T12E1, but is an Ad 12/Ad 5 hybrid protein in T2743.

When titrated on MH12C2 cells, all viruses except Ad 12 grew less efficiently than on 293 cells, although the plaquing efficiency of T1036 was in line with that of other viruses, and in fact more efficient than on HeLa cells. This result is consistent with numerous reports which showed that Ad 12 E1A is able to complement Ad 5 E1A for supporting virus growth (Williams *et al.*, 1975; Rowe and Graham, 1981; Sawada *et al.*, 1988). However, neither T2743 or T12E1 were able to form plaques efficiently on HeLa cells, which do not complement viral functions, and MH12C2 cells, where functional Ad 12 E1B proteins are provided in *trans*. This indicates that the cause of the impaired replication of these viruses is the inability of the Ad 12 E1B proteins to support the replication of Ad 5, rather than any defects in the E1B proteins made by these viruses. On both HeLa and MH12C2 cells, T12E1 was less efficient at plaque formation than T2743, and the plaques appeared after a significantly longer period of time. The implication of this result is that the E1B proteins encoded by T2743 are better at supporting the replication of Ad 5 than are intact Ad 12 E1B proteins, and therefore that the hybrid E1B 55K protein encoded by T2743 is partially functional. The reduced plaquing efficiency of all Ad 5 based viruses on MH12C2 cells compared with 293 cells suggests that one or more of the E1 proteins expressed by MH12C2 cells interferes with Ad 5 replication. Based on the inability of the E1B 55K protein of Ad 12 to support Ad 5 replication, one is led to speculate that the presence of the 55K protein in MH12C2 cells interferes with Ad 5 replication.

One of the critical functions of the E1B 58K protein in virus replication is the shutoff of host cell protein synthesis, in concert with the E4 34K protein. In light of the fact that viruses containing the Ad 12 E1B 55K coding region or part thereof were defective for plaquing on HeLa cells, it was possible that the cause of the defect was incomplete host shutoff. The host shutoff function was therefore assayed, and found to be defective in T1036, T2743 and T12E1. In the case of T1036, the defective transactivation of this virus suggests that the E1B and E4 transcription units are probably not transcriptionally activated, resulting in defective host shutoff. T2743 and T12E1 express the Ad 12 E1A gene, which was shown in T1461 to support the replication of the Ad 5 genome, in combination with the Ad 5 E1B transcription unit. This suggests that the cause of defective host shutoff seen with T2743 and T12E1 lies in the E1B transcription unit. Furthermore, since the 55K protein is known to be important for host shutoff, it is possible that the Ad 12 55K protein in T12E1 and the hybrid 55K/58K protein in T2743 are unable to interact with the Ad 5 E4 34K protein to shut off host cell protein synthesis. Others have reached a similar conclusion following titration of Ad 5 based viruses containing the Ad 12 E1 region or Ad 12 E1B with Ad 5 E1A (Sawada *et al.*, 1988).

In tumorigenicity assays on hamsters, neither T12E1 nor Ad 5 were able to induce tumors, while Ad 12 induced tumors very efficiently. This is in agreement with two other studies on viruses similar to T12E1, which were not tumorigenic in hamsters or rats (Bernards *et al.*,1984, Sawada *et al.*, 1988). Since transformed cells containing only the E1 region of Ad 12 are tumorigenic (Raška *et al.*, 1980; Jochemsen *et al.*, 1982;

this report), other viral functions are not critical for tumor formation. This suggests that a property of the Ad 5 genome besides those mapping to E1 interferes with the induction of tumors *in vivo*. The precise nature of that property is not clear, but since cell lines transformed *in vitro* by viruses such as T12E1 are tumorigenic, even if they contain a large portion of the Ad 5 genome (Sawada *et al.*, 1988), it is possible that these viruses are cleared from animals by a host immune mechanism before transformation can occur.

## C. Transforming Activity of Hybrid E1A Proteins

Previous work with chimeric E1A proteins has indicated that the different transforming efficiencies of Ad 5 and Ad 12 E1 plasmids are mediated by the first exon of E1A (Jochemsen *et al.*, 1984). By constructing hybrid E1A proteins with crossovers throughout the coding region, the studies described in this thesis have more precisely mapped this difference. Transformation assays with plasmids encoding hybrid E1A and Ad 5 E1B proteins identified two regions which determine serotype-specific levels of transforming efficiency. Compared to Ad 12, Ad 5 E1 was able to transform BRK cells at a relatively high efficiency, and colonies appeared after a brief incubation (6 days). Plasmids encoding hybrid E1A proteins with all or part of CR 1 of Ad 12 E1A transformed cells at a slightly reduced efficiency, and with a longer latency (10-14 days). When the Ad 12 E1A content was increased to include sequences from the amino terminus up to and including CR 2, the transforming efficiency dropped further, to levels

seen with pHAB6 and p2743, containing all of Ad 12 E1A (approximately 20 fold less than those with Ad 5 E1). Further increases in the amount of Ad 12 E1A in the hybrid protein did not affect the transforming properties. The plasmids p917-975 and p917-1227 transformed cells at an efficiency approximating Ad 5 E1A, even though both these plasmids encode Ad 12 E1A CR2. This suggests that CR2, which is critical for the transforming activity of both E1A proteins, is not involved in the differential transforming efficiency of Ad 5 and Ad 12 E1A. This is further supported by the observation that the cellular proteins whose interaction with CR2 of Ad 5 E1A is critical for transformation, interact equally well with CR2 of Ad 12 E1A. This suggests that sequences to the left of CR2 are in fact responsible for the differences in transforming efficiency between Ad 5 E1A and Ad 12 E1A. The second generation hybrid E1A plasmids also had latency times comparable with Ad 5 E1A. Since the initial hybrids from p690 to p1461 all had lengthy latency times, this suggests that sequences near the amino terminus of the E1A protein mediate this effect. With the exception of T1036, recombinant viruses containing hybrid proteins expressed similar levels of E1A in HeLa cells (figure 3-17), suggesting that differences in transforming efficiency were not attributable to altered levels of E1A expression. In fact, others have shown that decreases in Ad 5 E1A expression can actually augment transforming efficiency in virus-mediated assays (Adami and Babiss, 1990).

Transformation by E1 could take place in two phases: The first involving uptake and expression of large quantities of plasmid DNA by the cell, resulting in initiation of transformation. The second phase would involve occasional integration events of E1 sequences into the cell genome, facilitating long term stable expression of E1

proteins to maintain the transformed phenotype. Since established cell lines transformed by Ad 5 or Ad 12 grow at comparable rates, both doubling approximately every 2 days (unpublished observations), it is likely that both serotypes adequately fulfil the requirements for maintenance of the transformed phenotype. This implies that the efficiencies at which E1A proteins from various serotypes transform primary cells, as well as the times at which transformants appear may be due to functional differences manifested at early stages of transformation, although the reasons for these differences remain unclear.

The major mechanism by which E1A mediated transformation is thought to occur involves complex formation between E1A proteins and cellular proteins which regulate cell proliferation, functionally inactivating the repressors and consequently derepressing pathways which lead to cell cycle progression (Green, 1989). This model, which assumes that inactivation of growth repressors is the primary mechanism by which E1A transforms cells, predicts that E1A of adenovirus serotypes such as Ad 12, which transform cells less efficiently than Ad 5 would bind less stringently to one or more of these cellular proteins. The results presented here indicate that at least for p105, p107 and p300, this is not the case. It is possible that there is a difference in the ability of Ad 5 and Ad 12 E1A to bind to $p60^{cyclin\ A}$, whose electrophoretic mobility was too similar to that of hybrid E1A proteins to permit its detection. However, the sequences of Ad 5 E1A which appear to be required for binding of $p60^{cyclin\ A}$ overlap with those known to be required for binding to p105 and p107 (Giordano *et al.*, 1991; Howe and Bayley, 1992), suggesting that similar levels of $p60^{cyclin\ A}$ might bind E1A of Ad 5 and Ad 12. However,

the region corresponding to the binding sites for several of these proteins, CR2, does not appear to be involved in the differential transforming efficiency between Ad 5 and Ad 12 E1A, suggesting that proteins which interact with E1A in this region are not involved in the differential transforming efficiency between the two serotypes. An alternate possibility is that Ad 5 E1A binds to an uncharacterized cellular protein more tightly than Ad 12 E1A. It is also possible that the mechanism by which E1A transforms cells is not exclusively the sequestration of cellular proteins, but that complexes of E1A and cellular proteins could assume a novel function, resulting in cell proliferation. The function of such a complex could be influenced by sequences of E1A other than those which bind cellular proteins, and could therefore differ between adenovirus serotypes. This is consistent with the lack of detectable differences in the abilities of Ad 5 and Ad 12 E1A protein segments to bind to cellular proteins, and the fact that CR2 does not appear to mediate differences in transforming efficiency between Ad 5 and Ad 12 E1A.

## D. Tumorigenicity of Transformed Cell Lines

In a number of experiments, tumors which appeared on rats subsequently receded. Two factors were probably involved in this. First, small tumors are difficult to discern due to the thickness of the fur on the rats. It is possible that certain rats were incorrectly diagnosed as having small tumors, when in fact they merely had irregularities in the skin or fur at the site of injection. In general, this was probably not a common

occurrence, as extensive efforts were made to verify that lumps appearing under the skin of rats were not irregularities or knots of fur. However, at a certain size, probably on the order of 3-4 mm, tumors became unmistakable, and could be palpated. In several instances, tumors of this size or slightly larger eventually receded. The most likely explanation for this is that immunological recognition of the expressed E1 proteins, and rejection of the tumor cells occurred after the tumor had grown to a substantial size. If such delayed immune recognition was taking place, then it was possible that the practice of injecting newborn rats with transformed cells was leading to some degree of immunological tolerance, potentially resulting in tumorigenicity of cells injected into newborn rats which would be non-tumorigenic in weanlings. To rule this out, weanling rats were also tested for tumorigenicity, and the results obtained from these experiments were in agreement with those from newborn rats, suggesting that mechanisms of immunological tolerance, if they occurred, did not affect tumorigenicity assays.

Hybrid E1A proteins which gave rise to tumorigenic cell lines were those encoded by p975, p1036, p1227, and of course, Ad12 E1A. Included in the region of Ad12 E1A which is present in the oncogenic p975 but absent from the non-oncogenic p827 is 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. 4-2). Examination of the sequences for a number of adenovirus serotypes indicated that this region, characterized by the presence of a monotonous stretch of alanine residues, has no true counterpart in other human adenoviruses for which the sequence is known, but bears

a close resemblance to the analogous region in the simian adenovirus SA7 (Fig. 4-2). SA7 is also a very oncogenic adenovirus, and this homology, together with the results presented here, suggests that the region between CR2 and CR3, which is unique to these two unrelated oncogenic adenoviruses, plays a role in their oncogenicity. When this region as well as CR2 of Ad12 E1A was introduced into Ad5 E1A (p917-975, p917-1227), the resulting transformed cells were only weakly oncogenic. Therefore, while the region between CR2 and CR3 may be involved in tumorigenicity, other regions of E1A may also influence the tumorigenic phenotype. The differential tumorigenic potentials of cells transformed by Ad5 and Ad12 may therefore be attributable to several functions of 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 (Bernards *et al.*, 1983; Sawada *et al.*, 1988). In addition, the present study provides evidence suggesting that at least two regions of E1A are involved in tumorigenicity. While the nature of the functions attributable to these regions is not clear at present, it is possible that individual functions of both the Ad5 and Ad12 E1A proteins influence the tumorigenic phenotype. It is very likely that Ad5 E1A has an anti-tumorigenic function, consistent with the tumorigenicity in nude mice but not syngeneic immunocompetent animals of cells expressing the Ad5 E1A (Bernards *et al.*, 1983), and with the decreased tumorigenicity of transformed cell lines transfected with it (Walker *et al.*, 1991). It is possible therefore, that the low degree of tumorigenicity of cell lines transformed by p917-1227 is due to a combination of effects arising from the presence of the previously described Ad12 motif, which positively influences tumorigenicity, with

the simultaneous presence of a function of Ad5 E1A which lessens the degree of tumorigenicity. It may therefore be possible to generate constructs with higher degrees of tumorigenicity by the elimination of the appropriate Ad5 E1A sequences.

The mechanism by which the region of Ad12 E1A between CR2 and CR3 might influence tumorigenicity is not clear, but this region has features which suggest that it is involved in transcriptional regulation. It is adjacent to the transactivation domain of the Ad12 E1A protein, raising the possibility that it may interact with or function as part of this domain. Secondly, several proteins with transcriptional repressor activity have been characterized in which that repression function maps to a stretch of alanine residues, similar to that seen between CR2 and CR3 of Ad12 and SA7 E1A (Licht *et al.*, 1990; see Fig. 4-2). A previously characterized transcriptional repression activity of Ad12 which may influence tumorigenicity is class I MHC repression, mediated by the induction of a retinoic acid response element repressor (Ge *et al.*, 1992; Kralli *et al.*, 1992). Further characterizations will determine whether induction of this repressor is mediated by Ad12 E1A sequences in the region between CR2 and CR3, and whether this repression is involved in the tumorigenicity of Ad12 transformed cells.

In addition to the properties of Ad12 E1A which positively influence tumorigenicity, a function of Ad5 E1A probably limits the extent to which cells can form tumors, as reflected in the weakly oncogenic cell lines transformed by p917-1227. Since cells transformed by p975, p1036, and p1227 are tumorigenic, the second exon of the Ad5 E1A protein, which is shared by all these constructs, appears not to be involved in this function. Rather, the region of Ad5 E1A which downregulates or represses tumorigenicity

```
          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
```

**Figure 4-2. The region between CR2 and CR3.**

The sequences of various adenovirus E1A proteins were aligned (Bautista, 1989), and this figure has adopted a segment of that alignment, corresponding to the last 3 residues of CR2, the regions between CR2 and CR3, and the first 3 residues of CR3. Gaps have been introduced in an effort to maximize the number of matches.

probably maps between the amino terminus and the left border of CR2 of Ad 5 E1A, sequences which are present in both p917-971 and p917-1227. A likely function mapping within this region is the induction of susceptibility to lysis by natural killer cells, which has been shown to limit the tumorigenic potential of cells expressing Ad 5 E1A (Walker *et al.*, 1991). This would be consistent with the observed low levels of tumorigenicity of cells transformed by p917-1227. In particular, partial cytotoxic immunological activity directed against these cells would be consistent with their slow rates of tumor formation in rats. These results suggest that the cells which eventually comprised the tumor had acquired a secondary phenotypic change which facilitated their development into tumors. The specific nature of that phenotypic change could be clarified by an immunological characterization of cell lines explanted from tumors in these rats, to determine if they differ from the parental cell lines with respect to their sensitivity to lysis by NK cells.

## E. Concluding Remarks.

This thesis has described the construction of a series of hybrid Ad 12/ Ad 5 E1 regions by use of a technique which obviates the need for specific stragegies to generate chimeric proteins, and which results in a high proportion of hybrid proteins which contain junctions in regions of nucleotide and amino acid similarity. This homology at crossover sites results in most, but not all cases, in proteins which are functional with respect to supporting viral growth, cell transformation, and tumorigenicity.

Analysis of the hybrid E1 regions described has revealed several observations.

First, replication of the Ad 5 genome in HeLa cells is efficient when recombinant viruses containing portions of the Ad 12 E1 region contain Ad 5 E1B, and a functional E1A protein of Ad 5 or Ad 12 origin. The hybrid E1A proteins were all able to transform primary baby rat kidney cells, although with widely varying efficiencies (near zero with p1036). Regions of the E1A protein involved in these varying transformation efficiencies appeared to be the amino terminus plus a region between CR1 and CR2. Finally, the ability of transformed cell lines to induce tumors in newborn rats was found to depend on the presence of an Ad 12 E1A region just amino-terminal to CR3, although this region alone could not confer a high degree of tumorigenicity to Ad 5 E1A, suggesting that other regions of E1A also influence tumorigenicity.

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