THE ROLE OF THE E1B GENE PRODUCTS OF ADENOVIRUS SEROTYPE 12 IN LYTIC INFECTION AND TRANSFORMATION

by

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ABSTRACT

It is well documented that two regions of the human adenoviruses, each encoding multiple polypeptides, are responsible for the transforming activity of these viruses and that these genes are essential for the efficient production of virus in permissively infected cells. This study was undertaken to examine the importance of the individual proteins of one of these regions from adenovirus serotype 12 (Ad12), the E1b region, in lytic infection and transformation. Molecular defects have been identified in the smaller protein, the 19K, in two of the cytocidal (cyt) mutants of Ad12. Direct evidence has been obtained demonstrating that the point mutation in the 19K of one of the mutants is sufficient to cause the degradation of DNA in infected KB cells and reduce the transforming activity of the mutant virus and perhaps its tumourigenic potential, which are characteristic phenotypes of the cyt mutants. A mutation was also engineered in the larger, 55K E1b protein and was found to impair viral DNA replication, reduce the expression of the late, structural proteins of the virus and block the inhibition of cellular protein synthesis which is normally observed upon infection of KB cells with the wild type virus. The 55K was also found to be necessary for the efficient expression of the early genes of the virus, particularly the E2b gene, which encodes essential proteins

for viral DNA replication. The multiplicity dependent leakiness of the DNA replication defect of this mutant was exploited to separate the DNA replication defect from the defects in late protein expression and shut off of host protein synthesis. The observation that this mutant exhibited 1% of the transforming activity of the wild type virus but that transformants were fully tumourigenic also separated a transformation function from a tumourigenic function of this protein.

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INTRODUCTION

The proper regulation of cell growth is critical for the normal growth and development of multicellular organisms. At times during the progression of an organism to maturity cells will be required to divide, and at other times they must cease growing and differentiate. One possible consequence of the deregulation of growth is the continued growth and division of cells after they should normally have stopped dividing, which can in some cases lead to the development of cancer. The human adenoviruses have been used as a model system for the study of cancer since it was discovered that hamsters often developed tumours after inoculation with certain serotypes of this virus (Trentin et. al., 1962). This observation initiated the intensive study of the adenoviruses to try to understand how they interrupt the normal regulation of cell growth. As a result of these studies much has been learned about the molecular biology of the virus both in the process of transformation and in the lytic infection of human cells. Adenoviruses are now used as tools to study basic cellular processes, such as the regulation of gene expression, in addition to the study of the regulation of growth.

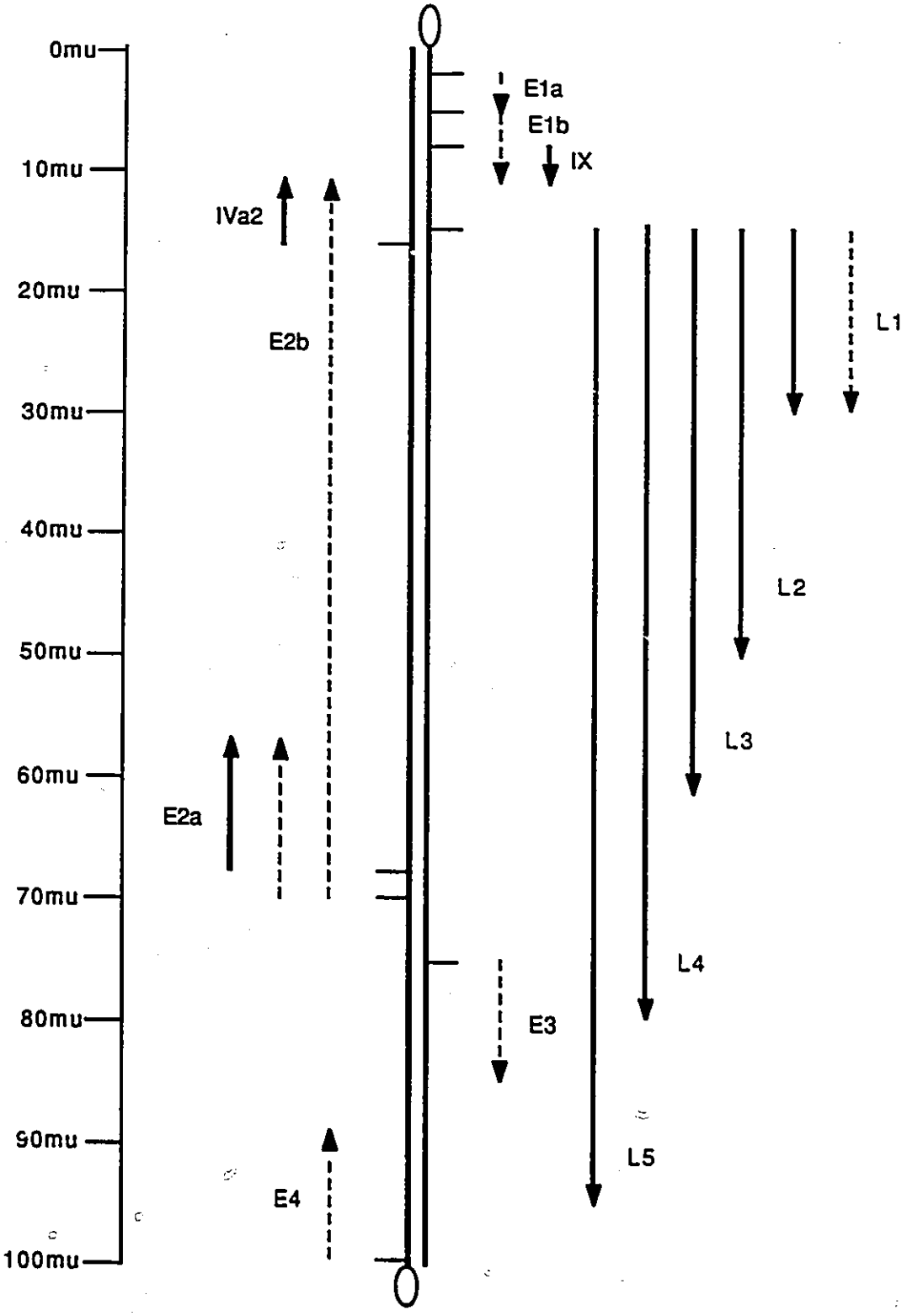
A. Overview of Lytic Infection and Transformation

Adenoviruses are icosahedral viruses containing a single linear duplex molecule of DNA approximately 35,000 base pairs (bp) in length (reviewed in Tooze, 1981; Philipson, 1983). For reference, the genome is divided into 100 map units (mu) of 350 bp each. The genome contains inverted terminal repeats of about 100-200 bp in length (163 bp in Ad12) and the 5′ end of each strand is covalently bound to a protein of molecular weight 55,000 (reviewed in Tooze, 1981; Sussenbach, 1984).

In cells which are permissive for productive infection, the virus executes a program of events culminating in the generation of progeny virus (reviewed in Tooze, 1981; Ginsberg, 1984). Temporally, productive infection is divided into early and late phases, which are demarcated by the onset of viral DNA replication. In the early phase, a subset of the viral genes, encoding a number of proteins required directly or indirectly for viral DNA replication, are expressed (see figure 1-1). In the late phase a second subset of viral genes, which encode predominantly structural components of the virion, are expressed. Progeny virus are then assembled and released.

In nonpermissive cells, adenoviruses can induce changes in growth properties converting the cells from

Figure 1-1. Transcription map of the adenovirus genome. Shown is a schematic illustration of the early (broken lines) and late transcription units (solid lines), which are classified according to the time during infection at which the genes are first expressed. The locations of the viral promoters are indicated by the vertical lines projecting from the schematic genome, and the direction of transcription along the genome by the arrowheads, which are located at the site of polyadenylation of the transcripts. Most of the transcripts diagrammed require the removal of introns to generate the mature mRNA. The most dramatic rearrangements occur in the RNAs from E2b and the major late transcription unit (eg. L1), which are processed by the excision of large introns and the splicing of promoter proximal leader sequence to the main body of the mRNA located closer to the poly A tail. A scale is provided for the approximation of the map unit position (mu) of each gene.


0mu
10mu
20mu
30mu
40mu
50mu
60mu
70mu
80mu
90mu
100mu
E1a
E1b
IX
IVa2
E2b
L1
L2
L3
E2a
L4
E3
L5
E4

normal cells into cancer-like cells, a process called transformation (reviewed in Branton et. al., 1985; Bernards and van der Eb, 1984, van der Eb and Bernards, 1983; Graham, 1984, Mak and Mak, 1986). Cells can also be transformed following the introduction of viral DNA by transfection (Graham and van der Eb, 1973b). Some serotypes of adenovirus, such as Ad12, are also capable of frequently inducing tumours when injected directly into rodents, and hence are classified as highly tumourigenic (Trentin et. al., 1962; Huebner et. al., 1962). Although other serotypes were found to be weakly tumourigenic, such as Ad7 (Huebner et. al., 1965), or even nononcogenic, such as Ad2 and Ad5 (Trentin et. al., 1962; Huebner et. al., 1962), all adenoviruses tested are capable of transforming rodent cells in culture (McBride and Wiener, 1964; Freeman et. al., 1967a,b,c; Gilden et. al., 1968; McAllister et. al., 1969).

Two lines of evidence indicate that the genes responsible for the transforming activity of adenovirus map to the left end of the viral genome. Firstly, studies on the structure of viral sequences integrated into the genomes of transformed cells revealed that the left 14 mu are always present (Gallimore et. al., 1974). Furthermore, a study utilizing DNA transfection demonstrated that approximately the left 5 mu must be intact to retain transforming activity (Graham et. al., 1974a). Subsequently a large number of

studies have demonstrated that restriction fragments of the adenovirus genome can cause transformation and that transforming activity is confined to fragments from the left end. The smallest fragment capable of eliciting the full spectrum of transformed phenotypes is the left 8 mu of the genome (Graham et. al., 1974b; Jochemsen et. al., 1982).

The transforming fragment coincides with one of the regions expressed early in lytic infection, early region 1 (E1), which has been shown to contain two transcription units, E1a and E1b (Wilson et. al., 1979). The smallest fragments exhibiting full transforming activity contain the intact E1a region and the left half of the E1b region.

Although these studies illustrate the central role of E1a and E1b in transformation, other genes also influence transformation in viral mediated assays. This is exemplified by the group N mutants which map outside of the transforming region and are now known to affect the viral DNA polymerase, yet exhibit impaired transforming activity (Williams et. al., 1974; Miller and Williams, 1987).

B. E1a of the Nononcogenic Serotypes Ad2 and Ad5

1. Transcripts and Translation Products

The E1a region (reviewed in Berk, 1986a; Grand, 1987) produces two major mRNAs early in infection, a 12S and

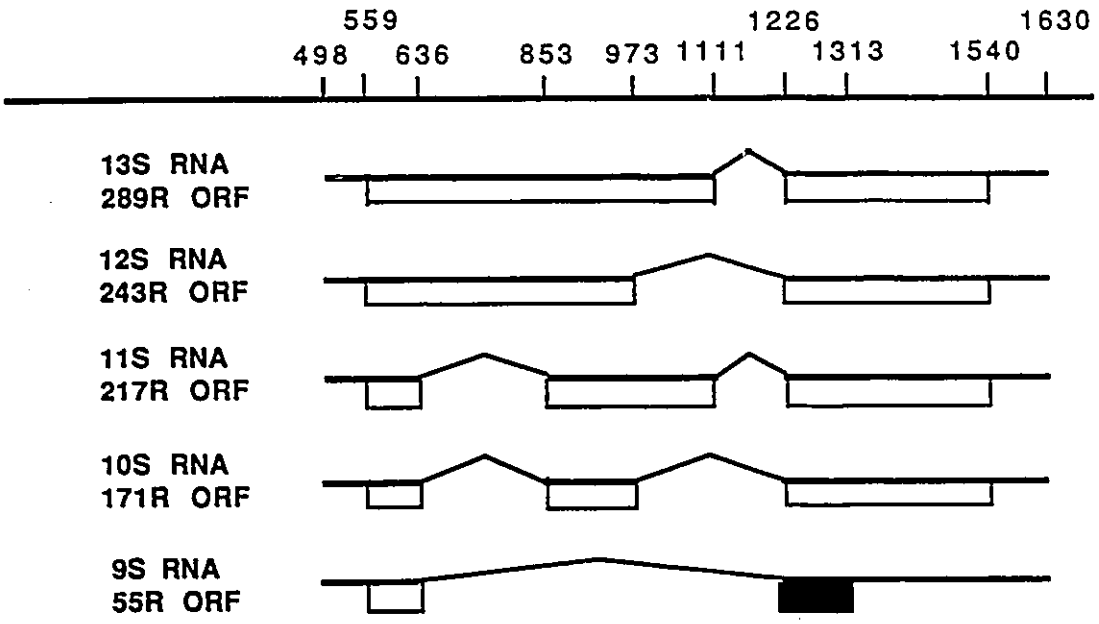
13S mRNA (Berk and Sharp, 1978; Chow et. al., 1979; Kitchingman and Westphal, 1980; Perricaudet et. al., 1979), which share a common 3' exon and differ only in the size of the intron removed (see figure 1-2). A third mRNA, 9S, which accumulates later in infection (Spector et. al., 1978; Chow et. al., 1979), also shares the common 3' exon and has a larger intervening sequence spliced out. There are also two minor transcripts which have a 3 exon structure, the 10S and 11S mRNAs (Stephens and Harlow, 1987; Ulfendahl et. al., 1987). Both contain the first exon of the 9S mRNA and the common 3' exon, with sequences from the 3' end of the first exon of either the 12S or 13S mRNA inserted between them (see figure 1-2).

Each transcript contains a single long open reading frame (Perricaudet et. al., 1979; Ulfendahl et. al., 1987; Stephens and Harlow, 1987). The translation products of each, with the exception of the 9S transcript, are predicted to have common amino and carboxy termini. The proteins encoded by the two major mRNAs are predicted to contain respectively 289 (289R) and 243 amino acids (243R).

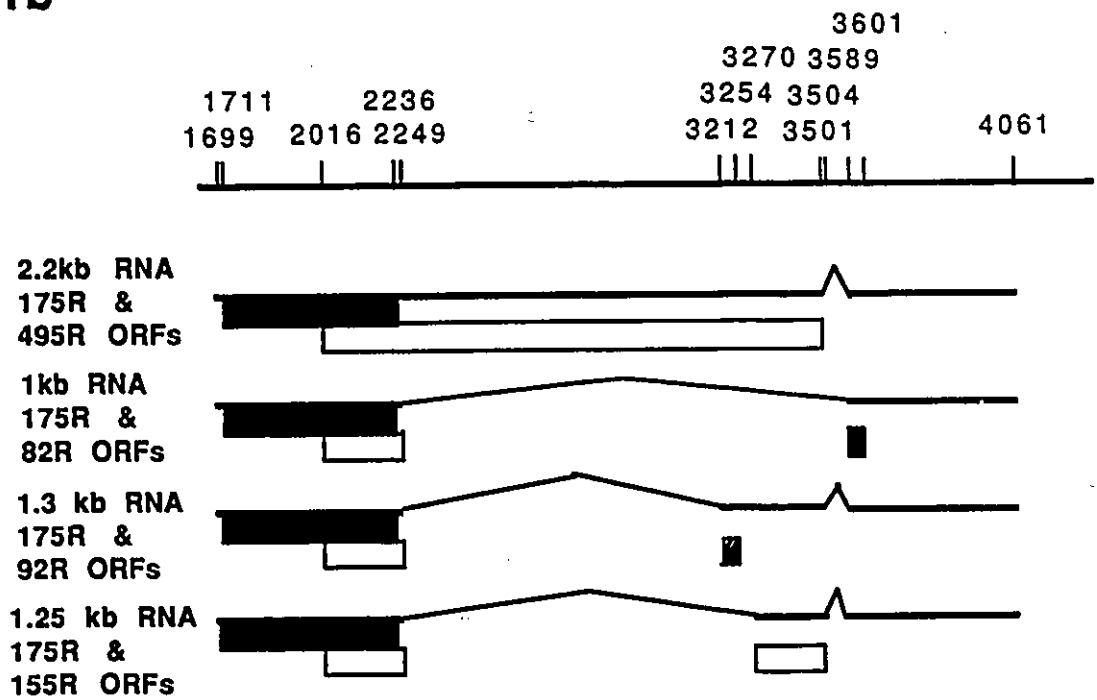
In vitro translation of mRNA selected by hybridization to the E1a region produces a number of highly related polypeptides ranging in size from 35K to 55K (Lewis et. al., 1976; Halbert et. al., 1979; Esche et. al., 1980; Smart et. al., 1981; Halbert and Raskas, 1982). Highly

Figure 1-2. Transcription map of the E1a and E1b genes of Ad2. Horizontal lines represent the mRNAs transcribed from each gene, and the intron sequences which have been spliced out are depicted by the diagonal lines. The boxes denote open reading frames on the mRNAs (open, solid and hatched indicating different reading frames). The nucleotide positions of mRNA cap sites, splice sites and 3′ end sites, as well as the locations of initiation and termination codons of the diagrammed open reading frames are indicated.

E1a


559
1226
1630
498
636
853
973
1111
1313
1540
13S RNA
289R ORF
12S RNA
243R ORF
11S RNA
217R ORF
10S RNA
171R ORF
9S RNA
55R ORF

E1b


3601
3270 3589
3254 3504
1711
2236
1699
2016 2249
3212 3501
4061
2.2kb RNA
175R &
495R ORFs
1kb RNA
175R &
82R ORFs
1.3 kb RNA
175R &
92R ORFs
1.25 kb RNA
175R &
155R ORFs

specific immunological reagents have been used to immunoprecipitate four major, highly related E1a proteins, 45K to 52K in size, and a number of minor E1a proteins from infected cells (Yee et. al., 1983, 1985a; Harlow et. al., 1985). These studies also demonstrated that a subset of the proteins are translated from each of the 12S and 13S mRNAs and confirmed the assignment of the translational reading frame (Halbert et. al., 1979; Esche et. al., 1980; Gaynor et. al., 1982; Rowe et. al., 1983a; Yee et. al., 1985a; Harlow et. al., 1985). Several of the minor E1a proteins have been identified as the translation products of the 10S and 11S mRNAs (Ulfendahl et. al., 1987; Stephen and Harlow, 1987).

The E1a proteins are phosphorylated and localized in the nucleus (Gaynor et. al., 1982; Yee et. al, 1983; Feldman and Nevins, 1983; Rowe et. al., 1983b). Phosphorylation accounts, at least in part, for the heterogeneous nature of the E1a proteins (Dumont et. al., 1989; Smith et. al., 1989) but thus far there is no clear evidence suggesting that phosphorylation is important for E1a function (Dumont et. al., 1989; Smith et. al., 1989; Tsukamoto et. al., 1986). The E1a proteins associate with a number of cellular proteins (Yee and Branton, 1985b; Harlow et. al., 1986), one of which has been shown to be the product of the Rb 1 gene, the susceptibility locus for retinoblastoma (Whyte et. al.,

1988). A second associated protein, 60K, remains unidentified, but has been shown to also form an association with the cell cycle regulatory protein cdc-2 (Giordano et. al., 1989).

2. Functions of the E1a Proteins

The products of the E1a region are required for the efficient growth of virus on human cells (Harrison et. al., 1977; Jones and Shenk, 1979b). The products of the 12S mRNA are dispensable for virus production on growing human cells (Montell et. al., 1982) but are necessary for the efficient production of virus in growth arrested cells (Montell et. al., 1984).

E1a proteins are required for the normal accumulation of viral RNA in infected cells (Berk et. al., 1979; Jones and Shenk, 1979a), and can also act to elevate the expression of the early genes in transient assays (Weeks and Jones, 1983; Leff et. al., 1984). The expression of a few cellular genes has also been reported to be elevated upon infection, an effect ascribed to the translation products of the E1a region (Kao and Nevins, 1983; Stein and Ziff, 1984). This positive regulation by the E1a proteins is primarily mediated by the 13S products (Montell et. al., 1984) and occurs at the transcriptional level (Nevins, 1981; Leff et. al., 1984). Although transactivation is mediated

through viral promoter elements, in most cases it has not been possible to divorce elements for transactivation from basal promoter elements (reviewed in Berk, 1986a,b; Nevins, 1987). The E1a proteins are believed to modulate transcription by RNA polymerase II through cellular transcription factors. As well as regulating transcription by RNA polymerase II, they can also activate the transcription of genes transcribed by RNA polymerase III (Berger and Folk, 1985; Gaynor et. al., 1985; Hoeffler and Roeder, 1985). Although it is agreed that activation occurs through the cellular factor TFIIIC, it is not clear if the E1a polypeptides increase the amount of TFIIIC (Yoshinaga et. al., 1986) or modulate its activity by altering its state of phosphorylation (Hoeffler et. al., 1988). In addition to its role as a positive regulator of transcription, the E1a proteins can also repress transcription from enhancer driven genes in transient assays, a function which is shared by the products of both the 12S and 13S mRNAs (Borrelli et. al., 1984; Velcich and Ziff, 1985; Velcich et. al., 1986).

Analysis of a large number of E1a mutants has demonstrated that this gene is required for the transformation of rodent cells in culture (eg. Graham et. al., 1978; Jones and Shenk, 1979b). Mutations eliminating the expression of the 12S products reduce transforming

activity (Montell et. al., 1984; Winberg and Shenk, 1984; Hurwitz and Chinnadurai, 1985), whereas mutations affecting only the 13S products often cause the production of large numbers of abnormal foci, which lack some of the features of fully transformed cells (Graham et. al., 1978; Ruben et. al., 1982; Montell et. al., 1984; Winberg and Shenk, 1984; Haley et. al., 1984). Thus both products are required for full transformation. Several E1a mutants exhibit a cold sensitive transforming activity and studies with these mutants have demonstrated that a functional 13S product is required for the maintenance of transformation (Ho et. al., 1982; Babiss et. al., 1983; Babiss et. al., 1984a).

The introduction of E1a alone into primary cells fails to induce the full transformation phenotype, but nevertheless, can immortalize these cells (Houweling et. al., 1980). The introduction of E1b (see above) or the ras oncogene is required along with E1a to induce full transformation (Ruley, 1983). The role of E1a in transformation does not seem to be limited to its immortalizing properties, since an E1a function is required, in addition to the ras oncogene, to transform at least one immortal cell line (Franza et. al., 1986).

Two other functions have also been ascribed to E1a. The E1a proteins induce the synthesis of cellular DNA in rodent cells (Braithwaite et. al., 1983; Stabel et. al.,

1985) and induce the release of an epithelial growth factor upon infection of primary BRK cells (Quinlan et. al, 1987).

C. E1b of the Nononcogenic Serotypes Ad2 and Ad5

1. Transcripts and Translation Products

The primary transcript of the E1b region is alternately spliced into two major mRNAs of 2.2 kilobases (kb) and 1 kb in size (Berk and Sharp, 1978; Chow et. al., 1979; Kitchingman and Westphal, 1980; Perricaudet et. al., 1980a)(For a review of E1b see Stillman, 1986; Grand, 1987). The large message is generated by the removal of a small intron located between nucleotides 3504 and 3589, and the smaller mRNA by removal of an intron extending from nucleotides 2249 to 3589 (see figure 1-2). Two other mRNAs have been detected amongst the E1b transcripts of Ad2, each containing 3 exons (Virtanen and Pettersson, 1985). They contain the first and second exons of the E1b 1 kb mRNA, between which are inserted the last 234 or 250 nucleotides of the first exon of the 2.2 kb mRNA (see figure 1-2).

The 2.2 kb mRNA contains two long, partially overlapping open reading frames (Bos et. al., 1981; Gingeras et. al., 1982) (see figure 1-2). Initiation of translation at the first AUG will produce a protein of 175R, whereas use of the second initiation codon, located 317 nucleotides

downstream, will generate a 495R protein. The integrity of the 175R open reading frame is maintained in all the smaller mRNAs, but the other open reading frame is altered due to the removal of the introns. Utilization of the second initiation codon in the smaller mRNAs would result in the translation of 155R, 92R and 82R proteins which are structurally related to the 495R protein (Virtanen and Pettersson, 1985).

The translation products of the E1b region were first identified by the in vitro translation of mRNA that had been selected by hybridization to DNA fragments from the E1b region (Halbert et. al., 1979; Esche et. al., 1980; Lupker et. al., 1981). Subsequently, they have been analyzed by immunoprecipitation using antisera from animals bearing adenovirus induced tumors (Lassam et. al., 1979; Ross et. al., 1980; Gaynor et. al., 1982). These two types of analysis have demonstrated a large E1b protein, reportedly 50K to 65K in size, and one or two small E1b proteins, 15K to 21K in size. The use of highly specific antipeptide antisera (Green et. al., 1983, Yee et. al., 1983; Spindler et. al., 1984), and determination of the primary structure of the amino terminus of one of the smaller translation products (Anderson and Lewis, 1980) has verified that the large E1b protein, henceforth referred to as 58K, is translated from the 495R open reading frame, and

one of the small E1b proteins, henceforth referred to as 19K, from the 175R open reading frame. The other small E1b protein was shown to be structurally related to 58K and has been identified as the translation product of the 155R open reading frame of one of the minor E1b mRNAs (Green et. al., 1979; Lucher et. al., 1984b; Anderson et. al., 1984). Recently two additional 58K related proteins, approximately 19K in size have been identified (Lewis and Anderson, 1987). One is apparently translated from the 92R open reading frame of the second minor E1b mRNA, while the mRNA encoding the other remains to be identified. The translational product of the 82R open reading frame has yet to be observed.

The E1b 58K is a phosphoprotein (Malette et. al., 1983; Sarnow et. al., 1982a,b), and was shown by immunofluorescence and cell fractionation studies to be present in both the nucleus and the cytoplasm (Sarnow et. al., 1982a; Rowe et. al., 1983b; Yee et. al., 1983). The 58K forms a physical complex with two different proteins. It associates with p53, and stabilizes this normally labile cellular protein, in Ad5 transformed cells (Sarnow et. al., 1982b; Zantema et. al., 1985) and with a virally encoded protein from the E4 region, called 34K, in infected human cells (Sarnow et. al., 1984).

The E1b 19K protein is acylated (McGlade et. al., 1987), and a minor fraction has also been reported to be

phosphorylated (McGlade et. al., 1989). Cell fractionation studies have demonstrated that the 19K is membrane associated, and is found in both the nuclear and cytoplasmic fractions (Persson et. al., 1982; Rowe et. al., 1983b). Immunofluorescence studies have localized the 19K primarily to the nuclear envelope, although some cytoplasmic staining was also evident (White et. al., 1984b).

2. Functions of 58K

Almost invariably E1b 58K mutants are defective for plaquing and growth on human cells, except for 58K expressing cell lines which complement the defect (Harrison et. al., 1977; Lassam et. al., 1978; Babiss and Ginsberg, 1984; Barker and Berk, 1987; Bernards et. al., 1986; Logan et. al., 1984). The degree of defectiveness varies, depending upon the conditions used in the experiment, but generally the mutants are reduced 100 to 1000 fold. The 58K mutants of Ad5 have been reported to replicate their DNA to wild type levels (Lassam et. al., 1978; Babiss and Ginsberg, 1984; Logan et. al., 1984).

The reduced ability of 58K mutants to grow in lytically infected human cells can be at least partly explained by a reduction in the production of viral late proteins (Babiss and Ginsberg, 1984). This depression of late protein synthesis is due to a reduction in the amount

of viral late mRNAs in mutant infected cells. Although transcription rates are similar in wild type and mutant infected cells, newly synthesized viral RNA accumulates in the cytoplasm and nucleus at a reduced rate in mutant infected cells (Babiss et. al., 1985; Pilder et. al., 1986; Leppard and Shenk, 1989). Concomitant with the reduced expression of late proteins in mutant infected cells is the continued synthesis of host cellular proteins, which are normally shut off during infection (Babiss and Ginsberg, 1984). This is believed to be the manifestation of a virally induced alteration in RNA metabolism. The cytoplasmic accumulation of newly synthesized cellular mRNA is blocked at late times after infection with wild type virus, but in mutant infected cells, nascent cellular mRNA continues to accumulate in the cytoplasm at the same rate as in uninfected cells (Babiss et. al., 1985; Pilder et. al., 1986). Mutants of early region 4 evince the same phenotype (Halbert et. al., 1985; Weinberg and Ketner, 1986; Sandler and Ketner, 1989) suggesting that the E1b 58K-E4 34K complex functions in mRNA metabolism.

The E1b 58K is required for the viral mediated transformation of rodent cells (Graham et. al., 1978; Ho et. al., 1982; Logan et. al., 1984; Barker and Berk, 1987; Babiss et. al., 1984b; Bernards et. al., 1986) since mutations which impinge upon the 58K coding sequences reduce

the transforming activity of the virus. It has been reported that the 58K requirement can be bypassed by delivering the mutant viral or plasmid DNA into the cells by transfection (Rowe and Graham, 1983; McKinnon et. al., 1982). Others have reported a decrease in the transforming activity of plasmids containing the E1 region if they harbour a 58K defect (Babiss et. al., 1984b; Bernards et. al., 1986; Barker and Berk, 1987). Transformation studies using restriction fragments have demonstrated that the 58K in its entirety is not required for transformation, although it cannot be ruled out that the amino terminus of the protein is required (Graham et. al., 1974b).

3. Functions of 19K

The E1b 19K protein is apparently required for the efficient growth of the virus on human cells since 19K mutants exhibit a 10 to 100 fold reduction in yield or plaque forming ability relative to wild type (Pilder et. al., 1984; Subramanian et. al., 1984a,b; Bernards et. al., 1986). There is, however, one report that the 19K is dispensable for viral growth (Barker and Berk, 1987). The 19K is required to prevent the extensive destruction of infected cells, called the cytocidal cytopathic effect (CPE), and the degradation of both viral and cellular DNA in infected human HeLa or KB cells (Pilder et. al., 1984;

Subramanian et. al., 1984a,b; Takemori et. al., 1984; White et. al., 1984a; Barker and Berk, 1987). The induction of DNA degradation in the absence of 19K has been shown to occur even when only the early genes are expressed, and thus is a function of an early gene product (Pilder et. al., 1984; White et. al., 1984a). Through the use of double mutants, White et. al. (1987) have demonstrated that either the 289R or the 243R E1a product must be intact for the induction of DNA degradation. In addition to the cytocidal CPE and DNA degradation phenotypes, 19K mutants also form larger, clearer plaques than wild type on human cells.

The large plaque, cytocidal, and degradation phenotypes of 19K mutants are separable. The Ad2 mutants lp3 and lp5 both form large plaques (Chinnadurai, 1983) yet neither causes a cytocidal CPE (Subramanian et. al., 1984b) and only lp5 induces DNA degradation (White et. al., 1984a; Subramanian et. al., 1984a).

The E1b 19K has also been reported to regulate gene expression. Mutation of the 19K results in elevated levels of expression of viral genes in infected cells suggesting that 19K acts in a negative fashion to reduce viral gene expression (White et. al., 1986; White et. al., 1988). In transient assays the E1b 19K has been shown to cause an increase in expression from a cotransfected reporter plasmid (Hermann et. al., 1987; Natarajan, 1986; Yoshida et.

al.,1987), and to antagonize the repression of enhancer driven genes by E1a (Yoshida et. al., 1987). The 19K has now been shown to stabilize transfected plasmid DNA, suggesting that the observed elevation of gene expression in transient assays may be due to an increase in the amount of template and not to regulation of the expression of the gene (Hermann and Mathews, 1989). It has also been reported that 19K mutations have no effect on viral gene expression nor on E1a mediated enhancer repression in infected cells (Herbst et. al., 1988). Thus this putative function of 19K remains controversial.

There is a consensus that the 19K is required for the transformation of rodent cells by Ad2 and Ad5 (Chinnadurai, 1983; Babiss et. al., 1984b; Pilder et. al., 1984; Subramanian et. al., 1984b; Takemori et. al., 1984; Bernards et. al., 1986; Barker and Berk, 1987). The defectiveness of different mutants using different cells varies, but transforming activity is typically reduced at least 10 fold relative to wild type, and very often is undetectable. As was the case with 58K mutants, various studies suggest that the 19K is also critical for plasmid mediated transformation (McKinnon et. at., 1982; Barker and Berk, 1987; Chinnadurai, 1983), while others suggest it is less important or even dispensable (Bernards et. al., 1986). While it is clear from transformation studies using

different restriction fragments of the viral genome that part of E1b is required in addition to E1a to fully transform cells, a requirement for the 19k or the amino terminal fragment of the 58K cannot be distinguished (Graham et. al., 1974b).

D. E1a of the Highly Oncogenic Serotype Ad12

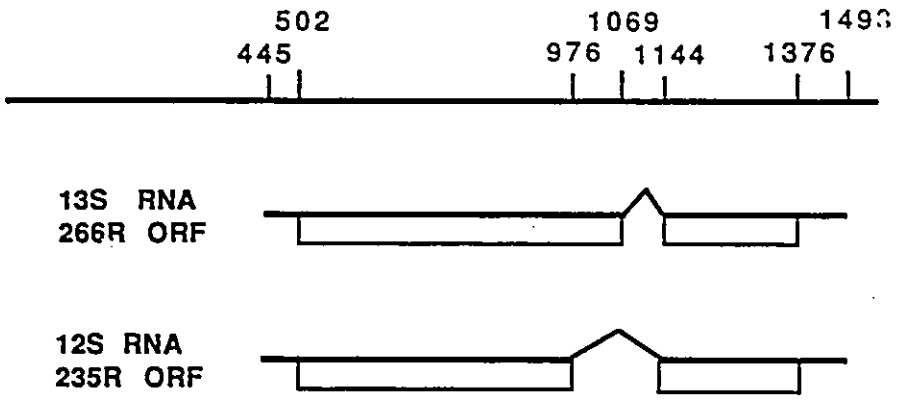
1. Transcripts and Translation Products

The E1a region of Ad12 encodes two mRNAs which are similar in structure to the 12S and 13S mRNAs of Ad2 and Ad5 (Perricaudet et. al., 1980b; Sawada and Fujinaga, 1980; Saito et. al., 1981)(see figure 1-3). Messages comparable to the minor E1a mRNAs seen in Ad2 infected cells have not been described in Ad12 infected cells. Each mRNA contains a single long open reading frame capable of being translated into a protein of 235R, in the case of the small transcript, or 266R (Perricaudet et. al.,1980b).

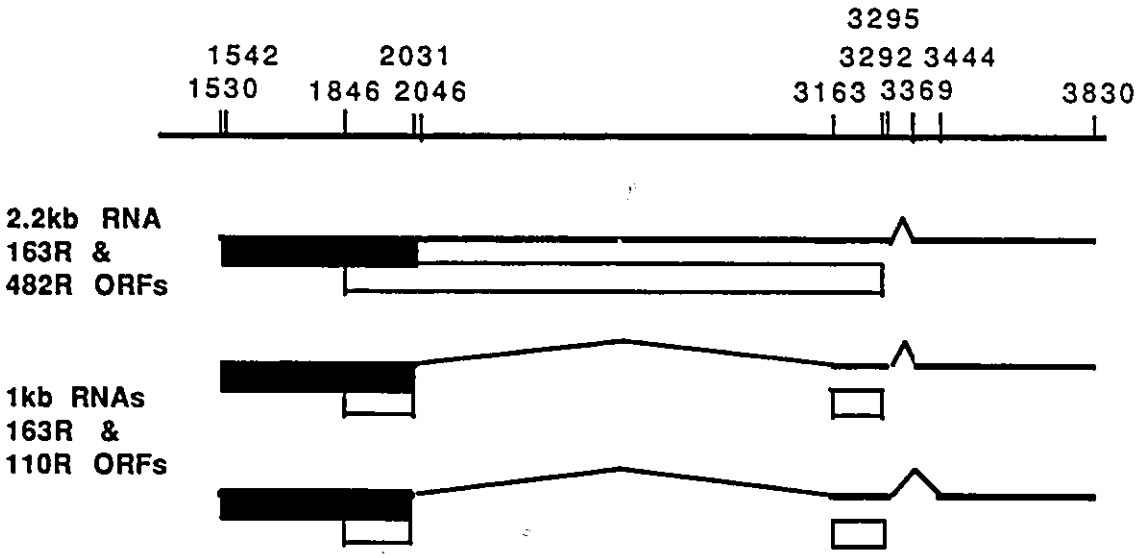
The E1a proteins have been identified by in vitro translation of E1a specific mRNAs, selected by hybridization to fragments of DNA from the E1a region, and by immunoprecipitation from infected cells using highly specific immunological reagents (Segawa et. al, 1980; Lucher et. al., 1984a; Scott et. al., 1984). Each of these studies identified two E1a proteins, which differ in size by about 2

Figure 1-3. Transcription map of the E1a and E1b genes of Ad12. Horizontal lines represent the mRNAs transcribed from each gene, and the intron sequences which have been spliced out are depicted by the diagonal lines. The boxes denote open reading frames on the mRNAs (open and solid indicating different reading frames). The nucleotide positions of mRNA cap sites, splice sites and 3' end sites, as well as the locations of initiation and termination codons of the diagrammed open reading frames are indicated.

E1a


502
445
1069
976
1144
1376
13S RNA
266R ORF
12S RNA
235R ORF

E1b


3295
1542
2031
3292 3444
1530
1846 2046
3163 3369
3830
2.2kb RNA
163R &
482R ORFs
1kb RNAs
163R &
110R ORFs

kilodaltons, although the reported molecular weights range from 35K and 37K to 45K and 47K. The E1a proteins of Ad12 exhibit less heterogeneity in size than the E1a proteins of Ad5.

The E1a proteins of Ad12, similar to those from Ad5, are localized in the nucleus and are phosphorylated (Grand and Gallimore, 1984; Lucher et. al., 1984a; Scott et. al., 1984).

2. Functions of the E1a Proteins

A number of the functions of the E1a proteins of Ad12 are similar to those of the E1a proteins of Ad2 and Ad5. The proteins are required for the efficient production of virus in infected human cells (Ohshima and Shiroki, 1986; Breiding et. al., 1988) and have been shown to enhance the expression of the other viral early genes during infection (Ohshima and Shiroki, 1986). They are also capable of repressing the expression of genes whose transcription is controlled by enhancer elements (Velcich et. al., 1986). A property unique to the E1a proteins of Ad12 is the ability to suppress the expression of the class I major histocompatability antigen (MHC) (Schrier et. al., 1983), which occurs at the level of transcription (Friedman and Ricciardi, 1988; Meijer et. al., 1989). A cellular protein that binds to the 5′ end of the class I MHC gene has been

shown to be present in Ad12 transformants and absent in Ad5 transformed cell lines (Akrill and Blair, 1989). This factor is hypothesized to be responsible for the reduction of gene expression.

The E1a proteins are required for the transformation of rodent cells in culture by Ad12 (Ohshima and Shiroki, 1986; Murphy et. al., 1987; Byrd et. al., 1988). Two mutants have been described in which the defect affects only the translation products of the 13S mRNA. These mutants exhibit a reduction in transforming activity (Ohshima and Shiroki, 1986; Byrd et. al., 1988), in contrast to similar mutants in Ad2 and Ad5, which produce more foci, albeit abnormal, than wild type virus (see section B2).

E1a alone can immortalize primary baby rat kidney cells (Gallimore et. al., 1984), but once again it is believed to be required for more than immortalization in the process of transformation since E1a can induce some of the properties of transformation in an established cell line, which is already immortal (Shiroki et. al., 1979).

The E1a region is also required for the induction of tumours when Ad12 is injected into animals (Murphy et. al., 1987; Byrd et. al., 1988). Furthermore, a property of the E1a genes at least partly accounts for the difference in tumourigenic potential between Ad5 and Ad12. Using recombinant constructs to establish cell lines, it has been

shown that the presence of the Ad12 E1a region correlates with the ability of the cell lines to form tumours in syngeneic animals (Bernards et. al., 1983b; Sawada et. al., 1988). This has been hypothesized to be due to the down modulation of the class I MHC antigen resulting in a more successful evasion of the immune response directed against the transformed cells.

Another E1a function common to both Ad5 and Ad12 products when expressed in rodent cells is the induction of cellular DNA synthesis (Stabel et. al., 1985; Oda et. al., 1986).

E. E1b of the Highly Oncogenic Serotype Ad12

1. Transcripts and Translation Products

The E1b region of Ad12 is transcribed to produce 3 mRNAs, one 2.2 kb in size and similar in structure to the large E1b mRNA of Ad2 and Ad5, and two about 1 kb in size (Virtanen et. al., 1982; Saito et. al., 1983)(see figure 1-3). The two smaller mRNAs are generated by the excision of two introns, one of which is common to both and lies between nucleotides 2046 to 3163. The second intron of one of these mRNAs is located between nucleotides 3295 and 3369, and is common to the 2.2 kb mRNA, whereas the second intron of the other 1 kb mRNA extends from nucleotides 3295 to 3444

(see figure 1-3). The large E1b transcript contains two long, partially overlapping open reading frames encoding proteins of 163R and 482R (Bos et. al., 1981). The two smaller mRNAs also contain the 163R open reading frame and a 110R open reading frame containing the first 67 and last 43 codons of the 482R open reading frame (see figure 1-3).

The proteins encoded by the E1b region of Ad12 have been identified by the translation of hybrid selected mRNA in vitro. A large protein between 50K and 59K in size, and a smaller protein about 19K in size have been consistently reported (Jochemsen et. al., 1980; Esche and Siegmann, 1982; Saito et. al., 1983; Mak and Mak, 1986). In addition to these, proteins of 17K, 15K and 14K have been translated from Ad12 E1b mRNA (Mak and Mak, 1986). These are believed to be authentic E1b translation products since proteins similar in size can be immunoprecipitated from infected cells by antitumour sera which have been shown to react with the proteins translated in vitro (Saito et. al., 1983; Mak and Mak, 1986). The large translation product, which will be referred to as 55K, has been shown to be encoded by the 482R open reading frame by Schughart et. al. (1985) using a highly specific immunological reagent. Recent studies using site directed mutagenesis have demonstrated that the 19K is the translation product of the 163R open reading frame (Edbauer et. al., 1988; Zhang, in preparation). The product

of the 110R open reading frame has not been identified, but the 17K, 15K and 14K E1b products described above remain to be characterized.

The Ad12 E1b 55K is a phosphoprotein and has been found in both the nucleus and cytoplasm (Grand and Gallimore, 1984; Schughart et. al., 1985). In contrast to the E1b 58K of Ad5, no association between the Ad12 55K and p53 can be detected in Ad12 transformed cell lines (Zantema et. al., 1985; Mak et. al., 1988). Nevertheless, these studies demonstrate that the presence of the E1b 55K results in the stabilization of p53.

The E1b 19K protein of Ad12 is acylated, as is the Ad5 19K, however, the biochemical nature of the linkage in each apparently differs. The fatty acid moiety is linked to the Ad12 protein by an ester linkage and to the Ad5 protein by an amide linkage (Grand et. al., 1985; McGlade et. al., 1987). The 19K is associated with membranes and has been identified in both nuclear and cytoplasmic fractions in cell fractionation experiments (Grand and Gallimore, 1984).

2. Functions of 55K

The E1b 55K is required for the efficient production of virus in infected human cells (Shiroki et. al. 1986; Breiding et. al., 1988). This requirement probably reflects the necessity of the E1b 55K for viral DNA replication

(Shiroki et. al., 1986; Breiding et. al., 1988), a function which is apparently not shared by the E1b 58K of Ad5 (see section C2). The 55K is also required for the efficient expression of the viral late proteins (Breiding et. al., 1988) and mRNAs (Shiroki et. al., 1986), as is the 58K of Ad5, but in the case of Ad12 this may be a secondary effect due to the DNA replication defect. The 55K is also necessary for the inhibition of cellular protein expression in infected cells (Breiding et. al., 1988).

The E1b 55K is necessary for the transformation of rodent cells by Ad12 (Shiroki et. al., 1986; Byrd et. al., 1988) but, as with Ad5, there is one report that 55K is not necessary for transformation using plasmid DNA (Shiroki et. al., 1986). Transformation studies using restriction fragments demonstrate that the E1b 55K open reading frame need not be intact for transformation, although all fragments with transforming activity contain at least part of the 55K coding sequences (Jochemsen et. al., 1982).

The induction of tumours by Ad12 upon injection into animals is dependent upon the E1b 55K (Shiroki et. al., 1986; Byrd et. al., 1988). The role of 55K in the tumourigenicity of transformed cell lines has been unresolved since two studies using cells transformed by various E1 fragments have reached opposing conclusions regarding the necessity of 55K, Jochemsen et. al. (1982)

concluding that 55K was required and Shiroki et. al. (1977) concluding that at least the carboyxl half of the protein was dispensable. Tnis discrepancy may be due to the use of different cells in these studies. A recent study on the tumourigenicity of cell lines transformed by a number of 55K mutants of Ad12 has now implicated the 55K in the tumourigenicity of Ad12 transformants (Mak and Mak, in press).

Further evidence for a role for 55K in tumourigenicity comes from comparative studies on Ad12 and Ad5. Analysis of cell lines transformed by various E1a and E1b plasmids of Ad12 and Ad5 has revealed a strict correlation of tumourigenicity in nude mice with the presence of the E1b 55K of Ad12 (Bernards et. al., 1983a). Although similar studies using syngeneic animals has shown that the E1a region of Ad12 is required for tumourigenicity, the frequency of tumour formation is increased if the E1b region of Ad12 is also present (Bernards et. al., 1983b; Sawada et. al., 1988). These latter two studies have not identified which of the E1b products are responsible for this effect.

3. Functions of 19K

The E1b 19K of Ad12 is not required for viral growth in human cells, but otherwise functions in infected human

cells in the same manner as its Ad2 and Ad5 counterparts, mutants exhibiting the large plaque, cytocidal CPE phenotypes in the human cell line A549 and inducing DNA degradation in human KB cells (Edbauer et. al., 1988).

The role of the 19K protein of Ad12 in the process of transformation may be slightly different from that of the 19K proteins of Ad2 and Ad5. The 19K is required for the transformation of 3Y1 cells by Ad12 (Fukui et. al., 1984; Edbauer et. al., 1988), but is apparently dispensable for the transformation of primary baby rat kidney cells (Edbauer et. al., 1988). All of the evidence from studies using Ad2 or Ad5 indicates that the E1b 19K is required for transformation by these viruses (see section C3).

The role of 19K in tumourigenesis is far from clear. Fukui et. al. (1984) suggest that the 19K is required to induce tumours when hamsters are inoculated with Ad12, whereas Edbauer et. al. (1988) report that the 19K is nonessential for Ad12 to induce tumours in rats. This question has also been addressed by assaying the tumourigenicity of cell lines transformed by 19K mutants. Transformants established with an E1 plasmid bearing a 19K defect were shown to be nontumourigenic in nude mice (Bernards et. al., 1983a), yet, lines established by transformation with virus containing the same 19K mutant allele readily form tumours in syngeneic animals (Edbauer

F. The cytocidal Mutants of Ad12

The cytocidal (cyt) mutants of Ad12 were isolated on the basis of plaque morphology, forming large clear plaques on human embryonic kidney cells (Takemori et. al., 1968). In infected human KB cells they induce a cytocidal CPE (Takemori et. al., 1968) and the degradation of DNA (Ezoe et. al., 1981). The mutants are defective for the transformation of rodent cells in culture (Mak and Mak, 1983) and are weakly tumourigenic when inoculated into hamsters (Takemori et. al., 1968). However, rat cell lines established by transformation with cyt mutants generally induce tumours in syngeneic animals as frequently as wild type transformants (Mak and Mak, 1983; Mak et. al., 1984).

Three lines of evidence suggest that the cyt mutants harbour E1b 19K mutations. First, they phenotypically resemble E1b 19K mutants (see sections C3 and E3), and secondly, their DNA degradation phenotype cannot be complemented by E1b mutants of Ad5 (Lai Fatt and Mak, 1982; Subramanian et. al., 1984a,b). Finally, it was observed that several cyt mutants fail to synthesize detectable levels of the E1b 19K protein in infected cells (Mak et. al., 1984)

This study was undertaken to examine the role of the Ad12 E1b region in productively infected cells and in transformation. The approach taken was to identify the mutations responsible for the defects observed in some of the cyt mutants, and to create a 55K mutant to observe the effect of altering this protein product. The two cyt mutants chosen for study were cyt 68 and cyt 62. The mutant cyt 68 is a typical cyt mutant that has been suggested to contain a deletion since no revertants have ever been observed. The mutant cyt 62 is atypical in that transformed cell lines are only weakly tumourigenic, in contrast to cell lines established by other cyt mutants including cyt 68. The 55K coding sequences were mutated by deleting the intervening sequences between two conveniently located restriction sites.

MATERIALS AND METHODS

A. Recombinant DNA Techniques

1. Materials

a. Enzymes. Enzymes were purchased from a variety of manufacturers and were used in accordance with the directions of the supplier. Restriction enzymes were typically used at several units per μg of DNA in the reaction buffer recommended by the manufacturer. Reactions were incubated for a few hours at 37°C, except for the enzymes Bcl I, Acc I and Bst EII which were used at 50°C, 55°C, and 60°C respectively. When necessary, the enzymes were inactivated by heat or removed by phenol extraction.

DNA ligations were done in 20 μl of ligase reaction buffer, either the one specified in Maniatis et. al. (1982) (66mM Tris pH 7.6, 6.6mM $MgCl_2$, 10mM dithiothreitol, and 1mM ATP) or the buffer supplied by the manufacturer. The DNA fragments of interest were present in a 3 to 5 molar excess over the vector, which typically was present at a concentration of 4 to 20 ng per μl. The reaction contained 1 to 2 units of ligase and generally was incubated at 16°C overnight.

Klenow (the large fragment of E. coli DNA polymerase I) was used to fill in the 3' recessed ends generated by cleavage with Eco RII prior to the ligation of an Eco RII fragment to Hinc II digested M13mp18, which has blunt ends at the site of cleavage. 0.25 μg of DNA was incubated in 20 μl of reaction buffer (50mM Tris pH 7.2, 10mM $MgSO_4$, 0.1mM dithiothreitol, and 50 μg per ml BSA) with 1mM of each of dATP, dGTP, and dCTP, and 4.5 units of Klenow at 16°C for 1 hour.

b. Cloning Vectors. All manipulations of cloned DNA were done using one of the following three vectors. The plasmid pBR322 contains the col EI origin of replication and genes encoding products that confer resistance to the antibiotics ampicillin and tetracycline upon the host bacterium (Bolivar et. al., 1977). Most cloning strategies involved insertion of DNA fragments into unique restriction sites within the tetracycline resistance gene. Recombinant plasmids could be identified as those with a functional ampicillin resistance gene, but a defective tetracycline resistance gene due to the insertion of foreign sequences into the middle of the gene.

M13mp18/19 are vectors derived from the single stranded, male specific, E. coli bacteriophage, M13 (Norrander et. al., 1983). These vectors contain a portion

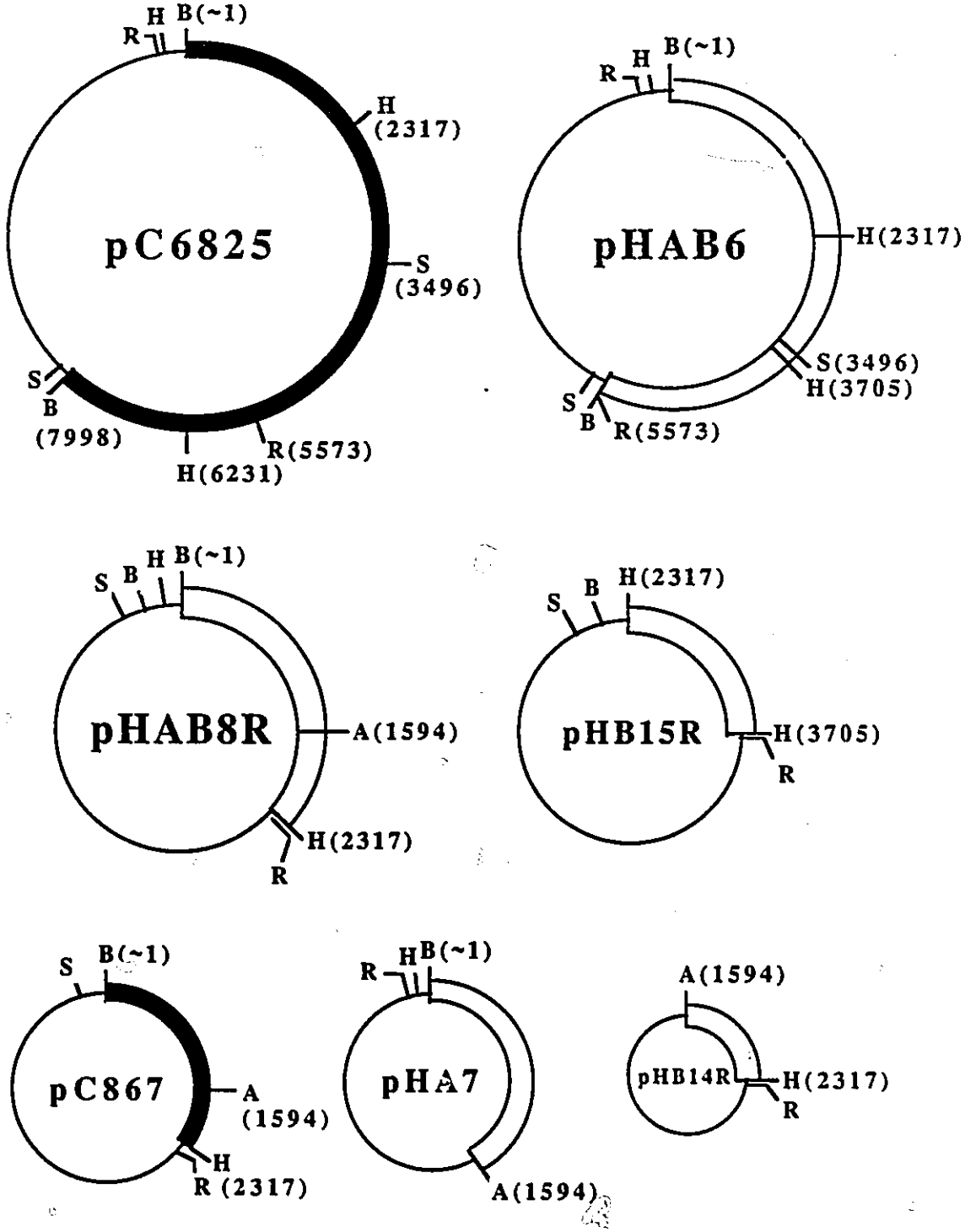
of the lac Z gene, encoding a fragment of beta-galactosidase, inserted into the bacteriophage genome at a site nonessential for viral replication. Within the lac Z gene is a multiple cloning site containing the recognition sites of a variety of restriction enzymes. The successful introduction of foreign DNA into the multiple cloning site can be scored by insertional inactivation of the lac Z gene fragment (Messing, 1983). Beta-galactosidase metabolizes 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (Xgal) to produce a blue product, and inactivation of the lac Z gene fragment yields phage that produce white rather than blue plaques on a lawn of host cells in the presence of Xgal. All recombinant DNA manipulations using these vectors were done with the double stranded replication intermediate of the phage, called the replicative form or RF DNA, which was then introduced into the host cells by transfection and the recombinants propagated as phage.

The plasmids pUC118/119 are chimeric vectors containing portions of both pBR322 and M13mp18/19 (Vieira and Messing, 1987). The pBR322 sequences present contain the col EI origin of replication, which has been modified in the pUC vectors, and the gene for ampicillin resistance. Two different fragments of the M13 vector have been engineered into these pUC vectors. First, the fragment of the lac Z gene and the multiple cloning site have been

introduced into the vector, and secondly, the intergenic region of M13 was introduced. This region contains all the cis acting sequences required for the replication of M13, including the origin of DNA replication and sequences necessary for the assembly of single stranded DNA into mature virus particles. Provision of the proteins required for M13 DNA synthesis results in the synthesis and packaging of single stranded DNA from the pUC vector templates. The pUC vector clones were maintained as plasmid, utilizing the plasmid origin of replication to propagate. Single stranded DNA synthesis and packaging was induced by delivery of the necessary M13 proteins by infection with the M13 derivative, M13K07 (Vieira and Messing, 1987). This phage contains a gene encoding kanamycin resistance providing a means of selecting for infected cells. M13K07 also has an altered origin of replication that renders it less able to compete with a wild type origin for replication and packaging in infected cells. Therefore this procedure generates a high yield of recombinant single stranded DNA.

c. Recombinant Plasmids Containing Ad12 or Mutant DNA Sequences. A number of recombinant plasmids containing adenoviral DNA inserts which were used in this study are illustrated in figure 2-1. They include pHAB6 (the left 5573 bp of wild type Ad12 strain Huie inserted into the Bam

Figure 2-1. Recombinant plasmids used in this study. Some of the plasmids used in this study are illustrated. The boxes indicate adenoviral sequences, open boxes are strain Huie derived and solid boxes are sequences from cyt 68, and the lines represent pBR322 sequences. The positions of recognition sites for the endonucleases Eco RI (R), Hind III (H), Bam HI (B), Sal I (S) and in some cases Acc I (A) are indicated along with the nucleotide position in the viral genome at which the site is located (in brackets). The plasmid pHAB8R has a duplication of the pBR322 sequences extending from the Hind III site to the Bam HI site. In plasmids pC867, pHA7 and pHB14R the viral sequences have been substituted for pBR322 sequences and the Acc I site in the latter two is at nucleotide 2246 of the vector. The plasmid pPAB7R is the same as pHAB6 except its viral sequences are wild type strain P and the insert is in the opposite orientation. The plasmids pC6225 and pC267 are identical to pC6825 and pC867 except that they contain cyt 62 rather than cyt 68 sequences. Plasmid pHA5 is a derivative of pHA7 containing a deletion of a few hundred bp at the Acc I site.


pC6825
R
H
B(~1)
H
(2317)
S
(3496)
S
B
(7998)
H(6231)
R(5573)
pHAB6
R
H
B(~1)
H(2317)
S(3496)
H(3705)
S
B
R(5573)
pHAB8R
S
B
H
B(~1)
A(1594)
H(2317)
R
pHB15R
S
B
H(2317)
H(3705)
R
pC867
S
B(~1)
A
(1594)
H
R (2317)
pHA7
R
H
B(~1)
A(1594)
A(1594)
pHB14R
H(2317)
R

HI site of pBR322) (Mak et. al., 1986) (pHAB7R is similar to pHAB6 except it contains DNA from wild type strain P (R. Lai Fatt, unpublished)), pHA7 (the left 1594 bp of Huie DNA inserted between the Bam HI site and the Acc I site at nucleotide 2246 of pBR322, replacing the vector sequences between those sites) (Mak et. al., 1986), pHAB8R (the left 2317 bp of Huie inserted into the Hind III site of pBR322. As a result of the strategy applied to create this plasmid the pBR322 sequences between the Hind III site and Bam HI sites are duplicated as an inverted repeat.) (Mak et. al., 1986), pHA5 (a derivative of pHA7 in which the viral sequences are resected to approximately 1400 bp) (Mak et. al., 1986), pHB14R (the fragment of Huie extending from nucleotide 1594 to 2317 substituted for the pBR322 sequences between the Acc I site at 2246 and the Hind III site) (S. Mak unpublished), pHB15R (the fragment from nucleotide 2317 to 3706 of Huie inserted into the Hind III site of pBR322) (S. Mak, unpublished), pC6825 and pC6225 (the left 7998 bp of cyt 68 and cyt 62 respectively inserted into the Bam HI site of pBR322) (R. Lai Fatt, unpublished), and pC867 and pC267 (the left 2317 bp of cyt 68 and cyt 62, respectively, inserted between the Bam HI and Hind III sites of pBR322. These plasmids were constructed and analyzed in the same fashion as pP67 (see Results section II)) (M. Schaller unpublished). Some of these plasmids have been shown to

contain small deletions of the sequences normally at the extreme left end of the viral genome (see Results sections I and II), which probably occurred during the original construction of these plasmids. In plasmids pHAB6, pC6825 and pHA7 the insert sequences are oriented such that the conventional numbering systems of the Ad12 and pBR322 maps proceed in the same direction. The other plasmids contain the inserts in the opposite orientation. In all cases, except the joint in pHA5 near nucleotide 1400 of the viral DNA, the junctions between the viral and vector sequences retain the restriction site present at that location in pBR322. References describing the construction of these plasmids are indicated in parentheses.

Three plasmids containing viral DNA inserted into pUC118 were also utilized (S. Y. Zhang, unpublished). Plasmid pH118BCr has the 73 to 59.6 mu Bam HI fragment inserted into the Bam HI site of pUC118, pH118HBr contains the 31.9 to 18.2 mu Hind III fragment inserted into the Hind III site of the vector, and pUC118/E4r has the 100 to 90.2 mu fragment inserted between the Bam HI and Hind III sites of pUC118. The viral sequences at 73 mu, 31.9 mu, and 100 mu are adjacent to the vector sequences which are complementary to the universal primer.

d. Bacteria. A number of strains of E. coli were

used in this study and their genotypes are indicated in table 2-1. GM31 were obtained from the E. coli Genetic Stock Center, Yale University, MV1190 from Dr. C. Harley, and the remainder from Dr. F. Graham. All bacteria were stored at -70°C in 20% glycerol. In cases where the bacteria contained an F' episome, cells from the frozen stock were streaked on M9 plates (1.5% bacto agar containing 42mM Na_2HPO_4, 22mM KH_2PO_4, 8.5mM NaCl, 18.7mM NH_4Cl, 1mM $MgSO_4$, 0.1mM $CaCl_2$, 1mM thiamine HCl and 0.2% glucose) to select for the maintenance of the episome, and a single colony was picked for use (Messing, 1983). Otherwise frozen stocks were used to inoculate cultures.

e. Broth and Plates. Three types of broth were used in these studies: Luria broth (0.5% yeast extract, 1% bacto tryptone, 85mM NaCl and 0.1% glucose) (Maniatis et. al., 1982), YT broth (0.5% yeast extract, 0.8% bacto tryptone and 85mM NaCl) (Messing, 1983) and B broth (1% bacto tryptone and 137mM NaCl) (Messing, 1983). Luria broth was used to grow LE392, GM119, and GM31, YT broth for the growth of JM101, JM103, and JM107, and B broth for the growth of MV1190. These media were used to grow bacteria in liquid culture and also to make agar plates. Luria and B broth agar contained 1.8% bacto agar, whereas YT agar contained 1.5% bacto agar. Plating agar or soft agar, in which

Table 2-1. Strains of E. coli used in this study

Strain	Genotype	Reference
LE392*	F^-, hsdR514($r^- m^+$), supE44, supF58 lacY1 orΔ(lacIZY)6, galK2, galT22 metB1, trpR55, lambda$^-$	Maniatis et. al., 1982; L. Enquist, pers. comm.
GM119	F^-, dam-3, dcm-6, metB1, galk2, galt22,lacY1,tsx-78, supE44, (thi-1, tonA31,mtl-1)?	Arraj and Marinus, 1983
GM31	thr-1, ara-14, leuB6, tonA31, lacY1, tsx-78, supE44, galK2, galT22, lambda$^-$, dcm-6, hisG4, rpsL136, xyl-5, mtl-1, thi-1	Marinus, 1973; Bachmann, pers. comm.
JM103	Δlacpro, thi, strA, supE, end A, sbcB15, hsdR4, F':traD36, proAB, $lacI^q$,ZΔm15	Messing et. al., 1981
JM101	supE, thi, Δ(lac-proAB), F':traD36, proAB, $lacI^q$, ZΔm15	Yanisch-Perron et. al., 1985
JM107	endA 1, gyrA96, thi, hsdR17, supE44, relA 1, lambda$^-$, Δ(lac-proAB), F':traD36, proAB, $lacI^q$,ZΔm15	Yanisch-Perron et. al., 1985
MV1190	Δ(lac-proAB), thi, supE44, (sr1-recA)306::Tn10(tet^r), F':traD36, proAB, $lacI^q$, ZΔm15	Vieira and Messing, 1987

* LE392 is reported to be m^- in Maniatis et. al., 1982, but is in fact m^+ (L. Enquist, personal communication)

bacteria were suspended to produce lawns for the growth of phage, was 0.6% bacto agar made up in YT broth. All broths and agars were sterilized by autoclaving.

Broth and agar were supplemented with 50 to 150 μg per ml of ampicillin or 15 μg per ml of tetracycline to select for bacteria harbouring plasmids bearing genes for antibiotic resistance. Broth used for infections using M13K07 contained 70 μg per ml of kanamycin to select for the drug resistance marker present in this bacteriophage.

Xgal was added to 0.07% in plating agar and to 0.004% in YT or B broth agar, when required, to screen for the presence of an uninterrupted lac Z gene fragment in certain vectors. The product of this gene metabolizes Xgal to form a blue product. Lac Z expression in these cases was induced by the presence of 0.7mM and 0.04mM isopropyl-beta-D-thiogalacto-pyranoside (IPTG) respectively.

2. Transformation of E. coli

The bacteria from a mid-log phase culture were collected and treated as described by Cohen et. al. (1972) (Maniatis et. al. 1982). The cells from about 3.5 ml of the original culture, concentrated to 200 μl of 50mM $CaCl_2$ and 10mM Tris pH 8.0, and approximately 40 ng of ligated DNA (this was the mass of vector), which had been diluted five-fold in TE buffer (10mM Tris pH 8.0, 1mM EDTA), was

used in each transformation reaction. After a 30 minute incubation on ice, the cells were incubated at 42°C for 2 minutes. If plasmid DNA was used to transform the cells, they were diluted in about 1 ml of Luria or YT broth and incubated at 37°C for 30 to 45 minutes to allow the cells to recover before selection with antibiotic. The transformed cells were serially diluted and plated onto the appropriate selection medium and incubated at 37°C overnight. If the RF DNA of M13mp18/19 was used to transform the cells, varying aliquots of cells were removed after heat shocking and mixed with 0.2 ml of plating culture (approximately a 4 hour incubation of a freshly inoculated culture of cells). Three ml of molten plating agar was added to each and the mixture was poured onto YT plates, allowed to harden, and incubated overnight at 37°C (Messing, 1983). The plating agar often contained IPTG and Xgal as described in section 1e.

To score colonies containing plasmids with inactive tetracycline resistance genes, transformed cells were first plated on ampicillin containing plates, then individual colonies were replica plated onto two plates, one containing ampicillin and the other tetracycline. Bacteria which grew on the former but not on the latter were selected for further analysis.

3. DNA Extraction from E. coli

For the preparation of RF DNA to analyze the structure of recombinant M13 phage the procedure described by Messing (1983) was used. Infected bacteria were picked from an isolated plaque and used to inoculate 2 ml of YT broth. After 4 hours at 37°C, 0.2 ml of a mid-log phase bacterial culture was added and the incubation continued for 4 hours more. One and a half ml of cells were collected sterile by centrifugation for 15 seconds in a microcentrifuge. The supernatant was stored at 4°C as a source of recombinant phage, while RF DNA was extracted from the pellet as described by Birnboim and Doly (1979).

Plasmid DNA was extracted by the method of Birnboim and Doly (1979) (Maniatis et. al., 1982). The bacteria from a 1.5 ml overnight culture were pelleted, resuspended in 100 µl of lysozyme solution (10-20 mg lysozyme in 25mM Tris pH 8.0, 10mM EDTA, and 50mM glucose) and incubated on ice for 30 minutes. Two hundred µl of alkaline SDS (0.2M NaOH, 1% SDS) was added and the incubation was continued for 5 to 10 minutes. Then 150 µl of 3M Na acetate pH 4.8 was added and the samples incubated on ice for 1 hour. The white precipitate was removed by centrifugation and the plasmid or RF DNA was recovered from the supernatant by ethanol precipitation. The DNA was resuspended in H_2O or TE buffer.

Large scale extraction of plasmid DNA was achieved

following the same protocol beginning with a 500 ml culture and scaling up the volumes of all the reagents 100 fold. The solution containing the plasmid DNA was made up to 1 gm per ml CsCl and 0.25 gm per ml ethidium bromide, then centrifuged for 48 hours at 110,000g at 15°C (Clewell and Helinski, 1969; Maniatis et. al., 1982). The band of supercoiled DNA was withdrawn through a 23 gauge needle into a syringe, extracted with iso-amyl alcohol saturated with 0.1 X SSC and CsCl, then extensively dialyzed against TE buffer. The final DNA concentration was estimated by absorbance of UV light at a wavelength of 260 nm (assuming 1 OD = 50 μg per ml).

4. Protocols for the Preparation of Single Stranded DNA

a. Growth of M13KO7. M13K07 was streaked onto a YT plate, and 4 ml of soft agar containing 0.5 ml of a saturated culture of JM103 was poured over the plate, which was then incubated at 37°C overnight. Single plaques were picked and used to inoculate YT broth containing 70 μg per ml kanamycin. The culture was incubated at 37°C for 10 to 14 hours and the cells pelleted. The supernatant was titered by plaquing on JM103 and stored at 4°C (Vieira and Messing, 1987).

b. Preparation of Single Stranded pUC118/119. An overnight culture was diluted 1:100 into fresh YT media supplemented with ampicillin and 0.001% thiamine. When this culture reached mid-log phase, the concentration of bacteria was estimated by measuring the optical density (assuming 1 OD = 1 X 10^8 cells per ml), and 2 ml of cells were removed and infected at a multiplicity of 10 plaque forming units per cell. The cells were incubated for 1 hour at 37°C, then 400 µl was removed to 10 ml of fresh broth containing 70 µg per ml of kanamycin. The infected cells were grown for 10 to 12 hours at 37°C, and the cells were pelleted by centrifugation at 16,000g two or three times. The phage were precipitated from the supernatant by addition of one-ninth volume of each of 40% polyethylene glycol and 5M Na acetate and incubation for 30 minutes on ice. The phage were pelleted at 20,000g for 10 minutes and resuspended in 200 µl of TE buffer. The DNA was extracted by two phenol/chloroform (1:1) extractions, one chloroform extraction, one ether extraction, and ethanol precipitation. The precipitate was resuspended in 40 µl of TE buffer and the yield of DNA was estimated by running an aliquot on an agarose gel. This protocol has been described by Vieira and Messing (1987).

c. Preparation of Single Stranded M13 DNA. Thirty ml of YT medium was inoculated with 1 ml of fresh JM103 and 600 µl of the supernatant from a small scale DNA extraction, which contained the bacteriophage. Following a 7 hour incubation at 37°C, the cells were removed by centrifugation at 16,000g for 10 minutes. Virus was precipitated from the supernatant with the addition of one-third volume of 27% polyethylene glycol 6000 and 3.3M NaCl and incubation at 4°C overnight. The virus was then pelleted at 20,000g for 10 minutes and resuspended in 1 ml TE buffer. DNA was obtained by sequential extraction with phenol, phenol/chloroform (1:1), and chloroform and then collected following ethanol precipitation. The concentration was estimated by absorbance of UV light with a wavelength of 260 nm (assuming 1 OD = 40 µg of single stranded DNA per ml). This is essentially as described by Messing (1983).

B. Tissue Culture Techniques

1. Cells and Media

MH12-C2, which is a human embryonic kidney cell line constitutively expressing the E1 region of Ad12 (I. Mak, unpublished), was isolated by I. Mak and grown in monolayers on plastic nunclon dishes (Gibco), in minimal essential medium (MEM) alpha medium (referred to as α MEM)

supplemented with 10% calf serum. Upon confluency, cells were washed with citrate saline (0.134M KCl and 15mM sodium citrate) and lightly trypsinized to remove them from the dish, then plated at a lower dilution onto new plates. KB cells were grown as monolayers on glass bottles in MEM (Eagle) (referred to as F11) + 10% calf serum and were passaged by scraping with a rubber policeman, then diluting into several bottles. Prior to infection, KB cells were expanded in spinner culture in MEM (S-MEM) (Joklik-modified) (referred to as Joklik) containing 5% horse serum, where they were maintained at a density of 2 to 5 X 10^5 cells per ml. Medium 199 (met$^-$) was used for labelling cells with [^{35}S]methionine. All media, with the exception of Joklik's MEM, also contained 50,000 units of penicillin and 50,000 μg of streptomycin per 800 ml of medium. In some cases media were also supplemented with fungizone (to a final concentration of 2.5 μg per ml Amphotericin B). Media (powdered), sera and antibiotics were all purchased from Gibco. Fungizone was purchased from E. R. Squibb and Sons, and trypsin from Difco.

2. Viruses

Two wild type strains of Adenovirus type 12 were employed in these studies, strain Huie (also known as 3569) and strain P (also known as 1131). Two cytocidal mutants of

Ad12-P were analyzed, cyt 68 (6304) and cyt 62 (3820). Ad12-Huie was obtained from M. Green, the other viruses from N. Takemori. The virus pm1542 was constructed by S. Y. Zhang and in700 was a gift of J. Williams. Several chimeric wild type/cyt 62 viruses and a mutant containing an E1b deletion were constructed as described below.

3. Infection Procedures

MH12-C2 cells were infected in monolayer. Medium on the cells was replaced with sufficient Joklik + 1% horse serum to just cover the monolayer, then virus was added and allowed to adsorb for 90 minutes at 37°C. The dishes were tipped periodically to ensure that all the cells remained covered with medium. Following adsorption, the cells were fed with the appropriate medium. KB cells were infected in suspension. Cells were concentrated by centrifugation at 275g for 15 minutes then resuspended in Joklik + 1% horse serum to 10^7 cells per ml. Virus was added and, in the case of small volumes, the cells were rotated on a wheel for 90 minutes at 37°C in 15 ml plastic tubes. For large volumes the cells were constantly agitated using a magnetic stir bar for 90 minutes at 37°C. Following adsorption, cells were plated onto plastic Nunclon dishes in α MEM + 10% calf serum or, in the case of large scale infections for the preparation of virus, were diluted to 3 X 10^5 cells per ml

in Joklik + 5% horse serum and incubated in spinner culture. Generally a multiplicity of 400 viral particles per cell was used.

4. Purification of Virus

Infected MH12-C2 or KB cells were harvested at 48 to 72 hours post infection, pelleted at 400g for 20 minutes and, if the virus was not immediately extracted from the cells, stored at -70°C. Virus was purified by a modification of the procedure developed by Green and Pina (1963). The cell pellet was resuspended in 0.01M Tris pH 8.1 in a volume of 50 ml per 10^9 cells, and the cells were lysed by sonicating for 1 to 2.5 minutes, the precise time determined by microscopic observation. The suspension was then mixed with an equal volume of freon-113 (1,1,2-trichlorotrifluoroethane) (Matheson Gas Products Canada) and homogenized for 1 minute. The phases were separated by centrifugation for 2 minutes at 1100g, and the aqueous phase repeatedly extracted with freon-113 until it appeared almost clear. Thirty-one ml aliquots of the aqueous supernatant were layered onto 7 ml of CsCl of density 1.43g/cc. The virus was spun onto the CsCl cushion by centrifugation at 67,250g for 90 minutes. The band of virus that formed on the cushion was retrieved and the density of the solution adjusted to 1.34. This solution was

centrifuged at 97,600g for 22 to 24 hours, and the visible band of virus which formed in the CsCl gradient was retrieved by piercing the tube with a needle and withdrawing the virus into a syringe. The absorbance of the virus solution at 260 nm was measured and 1 OD taken as equivalent to 4 X 10^{11} viral particles (Mak, 1971). The virus was diluted to a 1 to 2 OD units per ml in 1 X TBS (w/o) (137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 5.6mM glucose and 30mM Tris pH 7.4) and 20% glycerol, then stored at -70°C.

5. Plaque Assay for Determination of Viral Yields

At various times after infection, KB and MH12-C2 cells were harvested by scraping and collected by centrifugation at 340g for 10 minutes. The cells were resuspended in 200 µl of 1 X TBS (w/o) and 20% glycerol and intracellular virus was freed by repeated freezing and thawing. Lysates were stored at -70°C. The amount of virus in each lysate was determined by plaque assay. An aliquot of each lysate, which was determined empirically, was used to infect monolayers of MH12-C2 cells on 60 mm^2 dishes, which were overlaid as described in section C2. Plaques first appeared late in the second week following infection and visible plaques were counted at 14 to 17 days post infection.

C. Construction of Recombinant Viruses

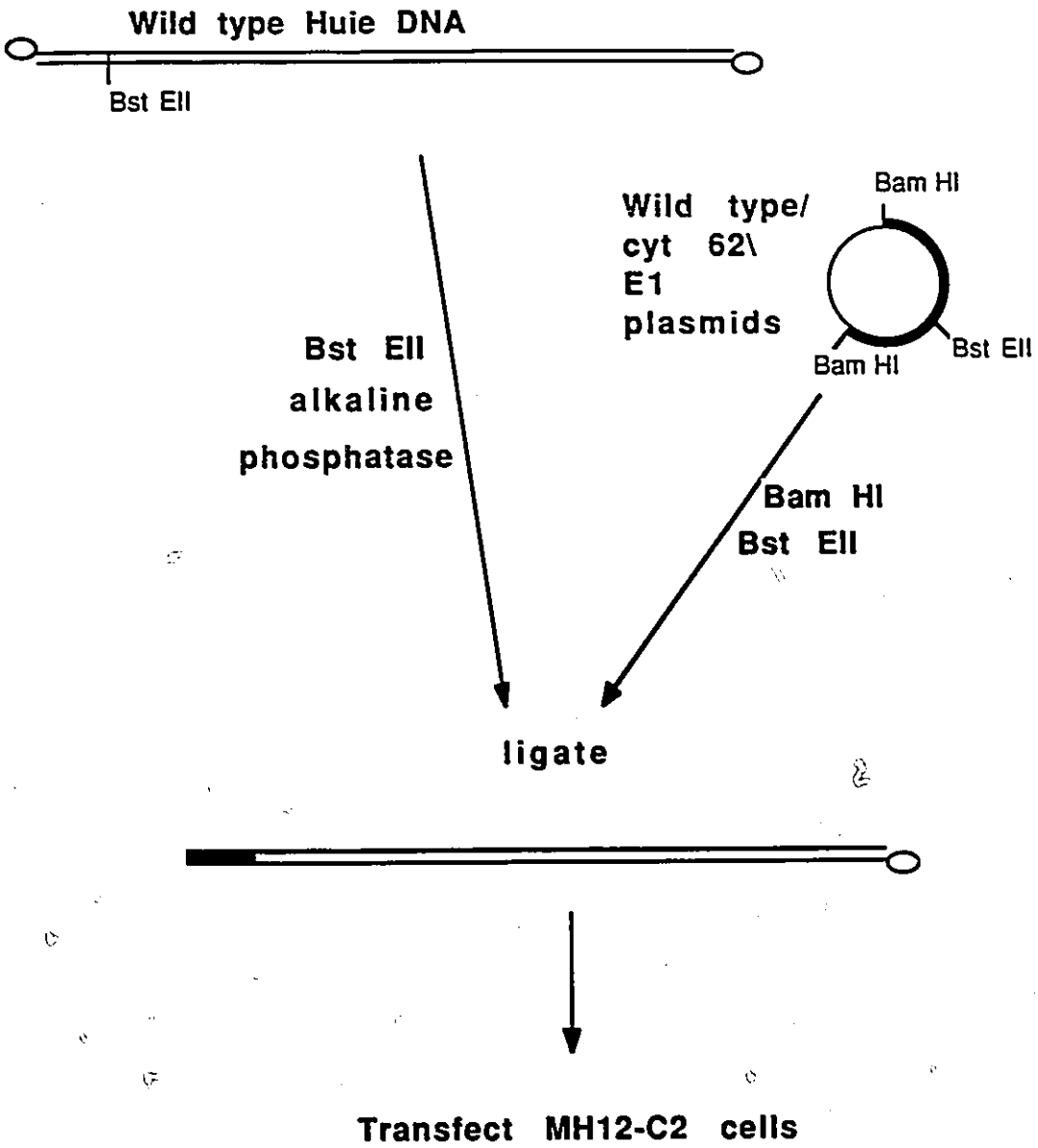
1. Construction of Recombinant Viral DNA Molecules

Mutations or recombinants created in plasmids were engineered into virus by the method originally described by Stow (1981) (see figure 2-2), which has subsequently been used successfully for the rescue of E1 mutants in Ad12 (Fukui et. al., 1984). Eight μg of viral DNA were cleaved with Bst EII, which cuts at a unique site at nucleotide 3443 (9.95 mu), then subjected to a 1 hour incubation with 100 units of bacterial alkaline phosphatase in 10mM Tris pH 8.0 at 65°C. The phosphatase was removed by phenol extraction and the DNA precipitated with ethanol. The pellet was resuspended in H_2O and mixed with a 2 molar excess of plasmid DNA that had been cleaved with Bam HI and Bst EII to liberate most of the E1 sequences from the vector. The volume was made up to 20 μl of ligase reaction buffer and the plasmid and viral DNAs were ligated together. (For ligation conditions see section A1a).

2. Transfection of MH12-C2 Cells

The ligation mixture was introduced into MH12-C2 cells by transfection using the protocol developed by Graham and van der Eb (1973) as modified by Wigler et. al. (1978). Two ml of the $CaPO_4$ solution was prepared, containing 20 μg

Figure 2-2. Strategy for the rescue of chimeric E1 regions into virus. The method originally designed by Stow (1981) was applied. The E1 sequences from recombinant plasmids were released by cleavage with Bam HI and Bst EII and ligated to Bst EII cleaved wild type Huie DNA, which had been treated with alkaline phosphatase. The DNA was transfected onto MH12-C2 cells and plaques isolated.


Wild type Huie DNA
Bst EII
Wild type/
cyt 62\
E1
plasmids
Bam HI
Bam HI
Bst EII
Bst EII
alkaline
phosphatase
Bam HI
Bst EII
ligate
Transfect MH12-C2 cells

of salmon sperm DNA as carrier and the DNA from one entire ligation reaction described in the previous section. One-half ml of this solution was applied to a 60 mm^2 dish of MH12-C2 cells at about 80 to 90% confluency and the cells incubated for a few hours at 37°C. The cells were either immediately overlaid with agar or were fed with α MEM + 10% calf serum, incubated overnight at 37°C, and then overlaid with agar. The overlay medium contained 5 ml of α MEM supplemented with 5% calf serum and 0.9% bacto agar. The cells were overlaid with fresh overlay medium every 6 or 7 days, and plaques, which were typically first seen in the third week following transfection, were picked at the end of the third week of incubation.

3. Growth of Recombinant Viruses and Plaque Purification

Well isolated plaques were selected and the agar above pierced with a sterile pasteur pipette. The holes were washed with 200 μl of 1 X phosphate buffered saline w/o (PBS w/o) (137mM NaCl, 3mM KCl, 8mM Na_2HPO_4, and 1.5mM KH_2PO_4) and 20% glycerol to remove the cells at the bottom of the hole. Intracellular virus was released by freezing and thawing the cells 5 to 6 times, then stored at -70°C. The virus from each plaque was grown on MH12-C2 cells and isolates with the genome structure predicted for recombinant viruses identified by analyzing DNA extracted from infected

cells by Southern blotting. Once identified, recombinant viruses were plaque purified. An aliquot of each viral stock, empirically determined to produce a small number of plaques on a dish, was used to infect monolayers of MH12-C2 cells on 60 mm^2 dishes, which were then overlaid as described in section C2. The process of picking plaques, growing virus and examining the genome structure was then repeated.

D. Transformation and Tumourigenicity Assays

1. Preparation of Primary BRK Cultures

Baby Wistar or Chester Beatty rats less than 10 days old were etherized, rinsed with 70% ethanol, and their kidneys were explanted. The kidneys were washed, finely minced with scissors, then individual cells released by incubation in 20 ml of 1 X citrate saline containing 0.25% trypsin at 37°C for 15 minutes. Trypsin in the single cell suspension was inactivated by adding it to α MEM + 10% fetal calf serum. Undigested tissue was subjected to one or two more trypsinizations and the single cell suspension collected each time. The cells were pelleted at 395g for 10 minutes at room temperature, and resuspended in 50 ml of α MEM + 10% fetal calf serum. The cells were incubated at 37°C for 15 minutes with intermittent shaking, then filtered

through sterilized cheese cloth to remove any large debris. Next, the cells were diluted in α MEM + 10% fetal calf serum and 5 ml aliquots were added to 60 mm^2 dishes. Typically 12 dishes were seeded from each pair of kidneys.

On the following day, at which time the cells were about 80% confluent, the medium was changed, and the cultures were incubated for 4 hours at 37°C prior to infection or transfection. Cells were infected or transfected as outlined in sections B3 and C2, and it was assumed that there were 3 X 10^6 cells per plate for determination of the amount of virus required for infection.

2. Selection and Staining of Foci

Cells were maintained in α MEM + 10% fetal calf serum for 2 days after infection or transfection, then the medium was changed to Joklik + 5% horse serum containing nystatin (Gibco) (Freeman et. al., 1967a; Mak and Mak, 1983). The cells were maintained in Joklik + 5% horse serum, medium being changed every 3 or 4 days. At about 24 days after infection, the cells were washed twice with 5 ml of 1 X phosphate buffered saline (complete) (referred to as PBS) (PBS w/o + 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$). They were fixed for 10 minutes in 5 ml of 1:1 PBS:Carnoy's solution, then fixed for 30 minutes in 5 ml of Carnoy's solution. The fixative was removed and the dishes allowed to dry at least

overnight. Cells were stained for 30 minutes in 5 ml of Giemsa stain, rinsed, then allowed to dry (Mak and Mak, 1983).

3. Tumourigenicity Assay

Well isolated foci, generated by transfection of primary BRK cells (prepared from Chester Beatty rats) with mutant or recombinant plasmids, were sequestered by a stainless steel cloning cylinder. The cells were lightly trypsinized and plated on plastic dishes. Twice weekly three-fourths of the medium was replaced with fresh medium. This procedure was done until the cells grew sufficiently to be subcultured, after which they were subcultured regularly. When a sufficient number of cells were obtained, they were frozen in 10% dimethyl sulphoxide and α MEM + 10% calf serum and stored in liquid nitrogen.

Cells for tumourigenicity assays were harvested by trypsinization, collected by centrifugation at 180g for 10 minutes and resuspended in 1 X PBS to a concentration of 10^7 cells per ml. Syngeneic rats about 3 weeks of age were injected subcutaneously with 10^6 cells, then examined weekly for the appearance of visible tumours (Mak and Mak, 1983).

E. Techniques for the Analysis of DNA

1. Extraction of DNA from Cells in Tissue Culture

Cells were harvested by scraping, pelleted at 340g for 10 minutes, washed 2 to 3 times with 1 X PBS (w/o), then resuspended in 0.35 ml of lysing solution per 8 X 10^6 cells (0.01M Tris pH 7.5 to 8.0, 0.005M EDTA, and 0.1M NaCl). Pronase was added to 1 mg per ml and SDS to a final concentration of 0.5% and this mixture was incubated at 37°C for at least 2 hours, sometimes overnight. For analysis of total cellular DNA, this lysate was extracted with phenol and chloroform, then precipitated with ethanol. The pellet was resuspended in 200 µl of H_2O, and the RNA present was degraded by incubation at 37°C for 30 minutes with 50 µg per ml of RNase. The sample was again extracted with phenol and chloroform and ethanol precipitated. The DNA pellet was resuspended in 1 ml of H_2O and, if the samples were to be analyzed by slot blotting, they were sonicated for 5 minutes, since they were too viscous to pass through the slot blot apparatus. The concentration of DNA in each sample was determined by absorbance of ultra-violet light at 260 nm (assuming that 1 OD = 50 µg of DNA per ml).

For analysis of small molecular weight DNA, the NaCl concentration in the lysate was increased to 1M, and the samples incubated at 4°C for at least 16 hours to

precipitate the high molecular weight DNA (Hirt, 1967). The precipitate was removed by centrifugation at 34,000g for 20 minutes or in a microcentrifuge for 30 minutes. The supernatant, which contained the small molecular weight DNA, was extracted with phenol and chloroform, then precipitated with ethanol. The precipitate was resuspended in a small volume of TE buffer or water and, if the presence of RNA would be detrimental to the analysis, it was degraded by adding RNase to 50 μg per ml and incubating the sample at 37°C for 30 minutes prior to electrophoresis.

2. Gel Electrophoresis of DNA

a. Agarose Gel Electrophoresis. DNA fragments larger than 500 bp were resolved on agarose gels ranging in concentration from 0.8 to 2% (Maniatis et. al., 1982). The gels contained 1 X running buffer and were either run in 1 X Tris borate buffer (TBE) (0.1M Tris, 0.083M boric acid, and 0.001M EDTA) or 1 X Tris acetate buffer (TAE)(0.04M Tris pH 7.8, 0.005M Na acetate, and 0.001M EDTA) (Maniatis et. al., 1982).

Loading buffer (30% glycerol and 0.25% bromophenol blue) (Maniatis et. al., 1982) was added to each sample prior to application to the gel. The gels were run until the dye in the loading buffer migrated at least three-quarters of the length of the gel, a distance of

approximately 14 cm.

b. Polyacrylamide Gel Electrophoresis. DNA fragments smaller than 1 kbp were resolved on 3.5% or 5% polyacrylamide gels of a 29:1 acrylamide to N,N'-methylene-bis-acrylamide (bis) ratio containing 1 X running buffer (Maniatis et. al., 1982). Gel solutions were prepared, degassed, ammonium persulfate added to 0.08% and TEMED to 0.001%, and the gels cast. Loading buffer was added to each sample prior to loading the gel. The gels were run in 1 X TBE or 1 X TAE buffer and electrophoresis was terminated after the dye in the loading buffer had migrated the length of the gel.

Polyacrylamide gels used for the separation of DNA strands were 5% gels of a 50:1 acrylamide to bis ratio. DNA samples were ethanol precipitated, resuspended in 40 ul of 30% DMSO, 1mM EDTA, and 0.5% bromophenol blue, then denatured by heating to 90°C for 2 minutes. The samples were then chilled quickly in an ice bath and immediately loaded onto the gel. Electrophoresis was done in 1 X TBE (Maxam and Gilbert, 1977).

c. Visualization of DNA on gels. Either the solutions used to prepare the gels were made containing 0.5 µg per ml of ethidium bromide or the gels were stained in a

0.5 μg per ml solution of ethidium bromide after electrophoresis. The gel was then observed under UV light to see the DNA (Sharp et. al., 1973). In cases where the DNA was radiolabelled fragments were localized by exposing X-ray film to the gel.

d. Fragment Isolation. i) From Agarose Gels. The DNA fragment of interest was located on the gel, after it was stained with ethidium bromide, by illumination with UV light. An incision was made in front of the band and a single piece of dialysis membrane, backed by a piece of 3MM paper for support, was inserted. Electrophoresis was continued at 70 volts until it could be seen, under UV light, that the band had entirely migrated onto the dialysis membrane. The membrane was then removed from the gel and the DNA was washed onto a piece of parafilm with TBE. The sample was collected and phenol extracted twice, ether extracted and ethanol precipitated. This is a modification of the technique described by Girvitz et. al. (1980).

DNA was also isolated by electroelution. A small piece of gel containing the fragment of interest was cut out, chopped into fine pieces, and placed in the sample well of an electroelution apparatus (International Biotechnologies Inc. (IBI)). The DNA was eluted from the gel and into the buffer in the salt bridge adjacent to the

sample well by electrophoresis. The DNA fragment was recovered from the high salt buffer by precipitation with ethanol (as outlined by IBI).

Gene Clean (Bio 101) was also used to isolate DNA fragments using the protocol recommended by the supplier. A piece of gel containing the fragment was excised, chopped up, and dissolved in approximately 2.5 volumes of the NaI solution. Five μl of glass milk was added to the solution and the sample was incubated on ice for 5 minutes. The silica matrix, and bound DNA, were washed 3 times with New Wash solution, and the DNA eluted from the beads by incubation at 45°C in 25 μl of TE for 3 minutes.

ii) From Polyacrylamide Gels. DNA fragments to be isolated were visualized by UV illumination of ethidium bromide stained gels. A small piece of the gel containing the fragment of interest was cut out, transferred to an eppendorf tube and pulverized using the plunger of a 1 ml syringe. One volume of elution buffer (0.5M ammonium acetate, 1mM EDTA pH 8.0) was mixed with the gel and the sample incubated overnight at 37°C. The polyacrylamide was spun down by microcentrifugation for 10 minutes. The supernatant was removed and 0.5 volumes of elution buffer was added to the gel pellet. This was vortexed, centrifuged again, and the supernatant removed. The supernatants were pooled and passed through a glass wool column then ethanol

precipitated twice (Maxam and Gilbert, 1977).

3. Transfer to and Immobilization of DNA on Membranes

a. Southern Blotting. After electrophoresis the gel, or portion thereof, was soaked in 0.25M HCl for 10 to 30 minutes, then for 30 minutes to 1 hour in 1.5M NaCl and 0.5M NaOH, or 1M NaCl and 0.5M NaOH. It was neutralized by soaking for 30 minutes to 1 hour in 1M Tris pH 8.0 and 1.5M NaCl, or 0.5M Tris pH 7.4 and 1.5M NaCl, then placed inverted onto a pad of 3MM paper that was supported on several plates. A piece of nitrocellulose was cut to the size of the gel, wet in H_2O, soaked for a few minutes in 2 X SSC (1 X = 0.15M NaCl, 0.015M sodium citrate) and placed on the gel. Several pieces of 3MM paper, wet in 2 X SSC, a stack of paper towels and a weight were added to the top of the blot. Transfer of the denatured DNA to the nitrocellulose was allowed to proceed overnight using 10 X SSC as the transfer medium. After transfer the nitrocellulose was soaked in 6 X SSC for 5 minutes, blotted dry and baked at 80°C for 90 to 120 minutes (Southern, 1975; Maniatis et. al., 1982; as outlined by Schleicher and Schuell, Inc.).

b. Electrophoretic Blotting. Double stranded DNA was transferred from polyacrylamide gels to Zeta-Probe

filters using a Bio-Rad transblot apparatus and the protocol recommended by Bio-Rad (Electrophoretic Blotting of DNA to Zeta-Probe Membrane, Bulletin #1232). DNA transfer was accomplished by electrophoresis at 30 volts for 4 hours in 0.5 X Tris borate buffer. Following transfer, the DNA was denatured by setting the filter on a pad of 3MM paper saturated with 0.5M NaOH and 1.5M NaCl for 10 minutes, then neutralized on a pad of 3MM paper saturated with 0.5M Tris pH 7.4 and 1.5M NaCl. The filter was rinsed with 2 X SSC and baked for about 2 hours at 80°C.

c. Slot Blotting. DNA was prepared for application to the slot blot apparatus as outlined by Schleicher and Schuell, Inc. (Transfer and Immobilization of Nucleic Acids to S & S Solid Supports, Schleicher and Schuell, Inc, 1987). The total amount of DNA in each sample was increased to 1 μg by the addition of carrier DNA (sonicated salmon sperm DNA). The samples were denatured in 200 μl of 0.3M NaOH by incubation for 1 hour at 65°C, then after cooling, an equal volume of 2M ammonium acetate pH 7.0 was added to each. Nitrocellulose filters were prepared by wetting in H_2O, then soaking in 1M ammonium acetate pH 7.0. The filter was set up in a slot blot apparatus (Schleicher and Schuell, Inc.), each slot washed with 0.5 ml of 1M ammonium acetate pH 7.0 and the samples were applied under low vacuum. The filter

was blotted dry and baked at 80°C for about 2 hours.

4. Preparation and Use of Radiolabelled Probes

a. Nick Translation. One μg of plasmid DNA was incubated in 50 μl of nick translation buffer (50mM Tris pH 7.2, 10mM $MgSO_4$, 0.1mM dithiothreitol, and 50 μg per ml bovine serum albumin) supplemented with dGTP, dATP, and dTTP (to a final concentration of 20μM each), 100 μCi of [α-^{32}P]dCTP, and 0.01 μg per ml of DNase. The sample was treated with 5 units of E. coli DNA polymerase for 1 hour at 16°C, and the reaction stopped by the addition of EDTA to a final concentration of 25mM (Maniatis et. al., 1982; Rigby et. al., 1977).

b. Primer Extension. All single stranded templates for the preparation of probes contained the DNA of interest inserted next to a site complementary to the universal primer within the vector. One μg of template DNA was mixed with 8 ng of the universal primer in 5 μl of reaction buffer (20mM Tris pH 7.6, 120mM NaCl, and 13.2mM $MgCl_2$) and boiled for 5 minutes. The samples were allowed to cool for at least 45 minutes at room temperature to allow annealing of the primer to the template and dATP, dGTP and dTTP were added to 0.67M. In a final volume of 15 μl containing 2.67mM dithiothreitol and 50 μCi of [α-^{32}P]dCTP, the

reaction was incubated with 5 units of the large fragment of E. coli DNA polymerase (Klenow) for 30 minutes at room temperature, and the reaction terminated by the addition of EDTA to a final concentration of 16mM (Meinkoth and Wahl, 1984).

c. Probe Purification. Unincorporated isotope was separated from the radiolabelled DNA either by centrifugation through a sephadex G50 column at 1600g for 4 minutes as described by Maniatis et. al. (1982), or by purification using Gene Clean (as outlined in section E2di). An aliquot of each sample was diluted in H_2O and the amount of radioactivity present determined by liquid scintillation counting. The DNA was denatured by boiling for 10 minutes either before or after its addition to the hybridization buffer.

d. Hybridization and Washing of Filters. Nitrocellulose filters were incubated in prehybrization solution (50% formamide, 5 X Denhardt's solution (1 X = 200 µg per ml each of ficoll, polyvinylpyrrolidone, and BSA), 0.5% SDS, 5 X SSPE (1 X = 0.18M NaCl, 10mM NaH_2PO_4, and 1mM EDTA), and 100-200 µg per ml of sonicated, denatured salmon sperm DNA) at 42°C for at least a few hours. The solution was then replaced with fresh buffer containing the

denatured, radiolabelled probe and the incubation continued overnight (as outlined by Schleicher and Schuell, Inc.).

After hybridization, filters were washed twice in 1 X SSC and 0.1% SDS for 5 minutes at room temperature, then twice in 0.1 X SSC and 0.1% SDS for 15 minutes each at 42 to 50°C. The filter was blotted dry and exposed to X-ray film (as outlined by Schleicher and Schuell, Inc.).

Zeta-Probe filters were incubated overnight at 68°C in 1.5 X SSPE, 1% SDS, 0.5% blotto (Carnation instant skim milk powder) and 0.5 mg per ml of sonicated, denatured salmon sperm DNA. Fresh buffer containing the denatured probe was used to replace the old buffer and the filter was again incubated at 68°C overnight. After hybridization the filter was rinsed in 2 X SSC and 0.1% SDS, then serially washed for 15 minutes at room temperature in each of 2 X SSC/0.1% SDS, 0.5 X SSC/0.1% SDS, 0.1 X SSC/0.1% SDS, then washed for 30 minutes at 50°C in 0.1 X SSC and 1% SDS. The filter was blotted dry and exposed to X-ray film (Nucleic Acid Hybridizations with DNA Bound to Zeta-Probe Membrane, Bio-Rad Bulletin #1234).

5. DNA Sequencing

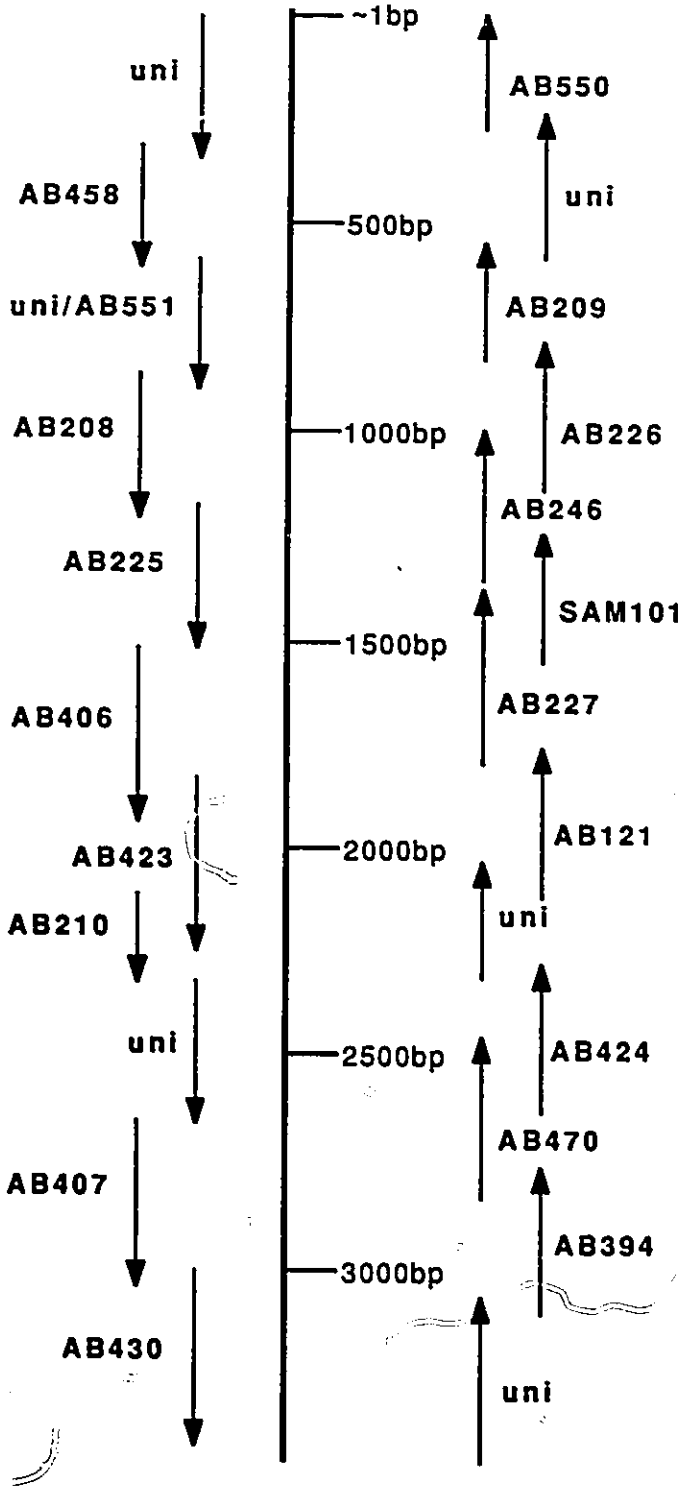
a. Maxam and Gilbert Technique. i) Radiolabelling DNA. DNA fragments to be labelled at their 5′ end were first treated with bacterial alkaline phosphatase in 20 μl

of 10mM Tris pH 8.0 at 65°C for 1 hour. The volume was increased to 100 µl with STE buffer (10mM Tris pH 8.0, 1mM EDTA, and 0.1M NaCl) and the sample spun through a sephadex G50 column (Maniatis et. al., 1982). The sample was then extracted with phenol, extracted with ether and precipitated with ethanol. The fragments were then labelled with 50 µCi [γ-^{32}P]ATP in 30 µl of kinase buffer (70mM Tris pH 7.6, 10mM $MgCl_2$, 100 mM KCl, and 5mM dTT) for 1 hour at 37°C. Following labelling the DNA was spun through a second sephadex G50 column and ethanol precipitated. It was denatured and run on a polyacrylamide gel as described in section E2b, and each strand was isolated from the gel as in section E2dii.

ii) Sequencing Reactions. The nucleotide sequence was determined as outlined originally by Maxam and Gilbert (1977) except that the formic acid reaction used in cleavage following deoxyadenosine was stopped with hydrazine stop buffer.

b. Chain Termination Technique. Nucleotide sequences were determined by the method of Sanger et. al. (1977) using a Sequenase kit from United States Biochemicals and a battery of oligonucleotides synthesized by the Institute of Molecular Biology and Biotechnology, McMaster University (see figure 2-3).

Figure 2-3. Strategy for sequencing the E1 region of Ad12. A number of oligonucleotide primers were synthesized at the central facility of the Institute of Molecular Biology and Biotechnology, McMaster University, and used to prime sequencing reactions using the dideoxy chain termination technique (Sanger et. al., 1977). The arrows represent segments of DNA, the sequence of which was determined using the indicated primer. The nucleotide sequences from the left end to nucleotide 3496 were determined using this strategy.


~1bp
uni
AB458
uni/AB551
AB208
AB225
AB406
AB423
AB210
uni
AB407
AB430
AB550
uni
500bp
AB209
AB226
1000bp
AB246
SAM101
1500bp
AB227
AB121
2000bp
uni
AB424
2500bp
AB470
AB394
3000bp
uni

c. Sequencing Gels. Sequencing reactions were run on 6% or 8% polyacryamide gels with a 19:1 ratio of acrylamide to bis containing 7M urea and 1 X TBE.

F. Techniques for the Analysis of RNA

1. Extraction and Purification of RNA from Cells in Culture

Cells (6 X 10^7) were harvested and pelleted at 340g for 10 minutes, washed with 1 X PBS (w/o), and lysed by dissolution in 5 ml of GIT solution (4M guanidine isothiocyanate, 0.5% N-laurlyl sarcosine, 25mM sodium citrate pH 7, and 0.1M beta-mercaptoethanol) and vortexing for 2 minutes (Chirgwin et. al., 1979). Samples were sometimes frozen at -70°C at this stage. After the high molecular weight DNA was fragmented by sonication for 45 to 90 seconds, the lysates were layered onto 5 ml of 5.7M CsCl and the RNA pelleted by centrifugation at 136,000g for 21 hours (Glisin et. al.,1974). Due to its density the DNA forms a band in the CsCl solution. The solution was removed from the tube and the RNA pellet was resuspended in 400 µl of 1% SDS. Sodium acetate pH 7 was added to 0.3M then the RNA was precipitated with ethanol twice. The pellet was resuspended in 100 µl of H_2O and the concentration of RNA was determined by measuring the absorbance of the solution at 260 nm (assuming 1 OD = 40 µg of RNA per ml). The

samples were examined for intactness by running an aliquot on an RNA denaturing gel (see below) and staining with ethidium bromide to visualize the two ribosomal RNA bands.

When working with RNA great care was taken to prevent degradation of the RNA. All glassware was baked at 250°C for at least 4 hours prior to use and wherever possible plastic pipettes were used. All solutions, with the exception of those containing Tris, were treated with diethylpyrocarbonate (DEPC) (final concentration of 0.1%) overnight, then autoclaved to inactivate any RNases present.

2. Northern Analysis

a. Gel Electrophoresis and Blotting. RNA was analyzed by northern blotting as described by Maniatis et. al. (1982). Ten μg of each sample were denatured by heating at 55°C for 15 minutes in 20 μl of 1 X gel running buffer, 2.2M formaldehyde, and 50% formamide. Two μl of loading buffer (50% glycerol, 1mM EDTA, and 0.4% bromophenol blue) were added to each sample, which was then loaded onto a 1.4% agarose gel containing 1 X running buffer and 2.2M formaldehyde (Lehrach et. al.,1977). The gels were run in 1 X running buffer (20mM morpholinopropanesulfonic acid (MOPS), 5mM NaAc pH 7, and 0.1mM EDTA pH 8) (Goldberg, 1980) at 75 volts for 3 to 4 hours. The formaldehyde was removed from the gel by passage through 3 changes of H_2O (10 to 15

minutes each), then a standard blot was set up (see section E3a). The nitrocellulose was wet in H_2O, then soaked in 20 X SSC prior to use. The RNA was allowed to transfer for about 24 hours using 20 X SSC as the transfer medium. The RNA was fixed to the filter by baking at 80°C for 90 minutes to 2 hours.

b. Hybridization and Washing Filters. The filter was incubated at 42°C in prehybridization buffer (50% formamide, 5 X Denhardt's solution, 0.5% SDS, 100 μg per ml sonicated, denatured, salmon sperm DNA, 5 X SSPE) for several hours. This solution was then replaced with hybridization solution (50% formamide, 2 X Denhardt's solution, 0.1% SDS, 100 μg per ml sonicated, denatured salmon sperm DNA, 5 X SSPE, 10% dextran sulfate, and denatured, radiolabelled probe) and the incubation continued at 42°C overnight. The buffer containing the probe was removed and the filter washed twice in 2 X SSC and 0.1% SDS for 10 minutes at room temperature, twice in 0.1 X SSC and 0.1% SDS for 10 minutes each at room temperature, then twice in 0.1 X SSC and 0.1% SDS for 30 minutes each at 50 to 55°C. Hybridization and washing conditions were as recommended by Schleicher and Schuell, Inc. The filter was blotted dry and exposed to X-ray film.

c. Staining with Methylene Blue. Following the exposure of X-ray film to them, the filters were soaked in 5% acetic acid for 15 minutes at room temperature, then in 0.5M NaAc pH 5.2 containing 0.04% methylene blue for a further 10 minutes. They were rinsed in H_2O for 5 to 10 minutes and photographed using a polaroid camera (Maniatis et. al., 1982).

G. Techniques for the Analysis of Proteins

1. Materials

a. Sera. All sera used in these studies were extracted from rats bearing tumours induced by injection of adenovirus transformed cell lines. The anti-tumour sera 702-C1, AB6a-C3, and A7R-C4 have been previously characterized (Mak et. al., 1984; I. Mak, unpublished). The first two recognize both E1a and E1b proteins, whereas the third recognizes only the E1a proteins. The anti-tumour serum dl55-1 was obtained from rats that developed tumours following the injection of cell lines transformed by the plasmid pH6dl-1 (which contains a deletion in the E1b 55K coding sequences), and has been shown to recognize both the E1a and E1b proteins (M. Schaller, unpublished).

b. Protein A Beads. Three-tenths gm of beads were dissolved in 8 ml of Schweizer's buffer (10% glycerol, 0.1M Tris pH 8.0, 0.1M NaCl, 5mM KCl, 1mM $CaCl_2$, and 0.5mM $MgCl_2$) containing 1% NP-40 and allowed to swell overnight at 4°C. The beads were pelleted for 10 minutes at 235g, then resuspended in 11 ml of Schweizer's buffer + 1% NP-40.

2. Preparation of Radiolabelled Cell Lysates

a. Labelling Cells in Culture. Media on infected cells was replaced with sufficient medium 199 (met^-) to cover the monolayer. If the cells were labelled after they had begun to detach, cells floating in the medium were collected by centrifugation at about 170g for 10 minutes, resuspended in a minimal volume of medium 199 (met^-), and returned to their dish of origin. In some experiments cells were incubated for 30 minutes in medium 199 (met^-) prior to the addition of label, to deplete intracellular methionine before labelling. Cells were labelled with either [^{35}S]methionine (Amersham) or a mixture of [^{35}S]methionine and [^{35}S]cysteine (^{35}S-trans from ICN), typically for 2 to 4 hours at 37°C. To label the unstable mutant proteins, cells were labelled for 30 minutes or 1 hour. In pulse-chase experiments, cells were washed with medium 199 (met^-) following the pulse label, then re-fed with medium 199 (met^-) which had been supplemented with L-methionine to 39 μg per

ml and incubated at 37°C for the duration of the chase. Under the conditions employed, there was at least a 2900 molar excess of cold methionine over the labelled methionine during the chase.

b. Preparation of Cell Lysates. Cells were harvested by scraping, then pelleted at 340g for 10 minutes. The pellet was washed 2 to 3 times with 1 X PBS (w/o), then resuspended in 100 to 200 µl of Schweizer's buffer containing 1% Nonidet P-40 (BRL) (for 3-8 X 10^6 cells). In the experiments designed to detect the unstable 55K protein from dl1201 infected cells, this suspension also contained 1% aprotinin (Sigma) and 0.2mM phenymethylsulfonyl fluoride (PMSF) (Sigma) to inhibit protease activity. Cytoplasmic lysates were prepared by incubation on ice for approximately 15 minutes, followed by removal of nuclei by centrifugation for 10 minutes at 1600g (Schaffhausen et. al., 1978; Mak and Mak, 1983). Alternatively, total cell lysates were prepared by sonicating the suspended cells for 90 seconds and removing cellular debris by centrifugation in a microcentrifuge. Observation of the sonicated lysates microscopically confirmed the destruction of the cells.

The amount of radioactivity in each lysate was determined by precipitation with trichloro-acetic acid (TCA). Five and 10 µl aliquots were taken from each lysate,

the proteins precipitated in 4 ml of 10% TCA, and the precipitate collected on 0.45 micron nitrocellulose filters by suction. The filters were baked for 15 minutes at 80°C, then immersed in toluene containing omnifluor (New England Nuclear), and counted in a scintillation counter.

To analyze cell lysates directly by SDS PAGE, an aliquot of each was taken, mixed with loading buffer (0.0625M Tris pH 6.8, 2% SDS, 10% glycerol, 5% beta-mercaptoethanol, and 0.001% bromophenol blue) (Laemli, 1970), and boiled for 3 minutes prior to loading on the gel.

3. Immunoprecipitation

A portion of each lysate corresponding to either an equal number of cells or an equal number of TCA precipitable counts was subjected to immunoprecipitation. If necessary the volume was adjusted with Schweizer's buffer + 1% NP-40 to make the volume of each sample equal. Between 20 and 30 µl of anti-tumour serum was added to each lysate, as well as 0.2 to 0.3 ml of protein A sepharose beads (Pharmacia) (Schaffhausen et. al., 1978; Mak and Mak, 1983). The immunoprecipitations were rotated for at least 2 hours at 4°C, more often for 4 hours to overnight. The beads were pelleted for 10 minutes at 180g or for 2 minutes at 260g, washed 3 times in LiCl buffer (0.1M Tris pH 8.2, 0.2M LiCl, and 0.14M beta-mercaptoethanol), and collected by

centrifugation after each wash. Finally, the pellet was resuspended in 40 µl of loading buffer, boiled for 3 minutes and the beads pelleted for 1 minute in a microcentrifuge prior to loading on the gel.

4. Analysis by Gel Electrophoresis

a. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE). Polyacrylamide gels were made from a 30% stock of acrylamide of 37.5:1 acrylamide to bis ratio. Twelve and a half per cent running gels in 0.375M Tris pH 8.8 and 0.1% SDS were prepared with 3% stacking gels containing 0.125M Tris pH 6.8 and 0.1% SDS. The running buffer for these gels was 0.025M Tris, 0.192M glycine, and 0.1% SDS. This gel system was originally described by Laemli (1970). Generally, these gels were run at 70 volts for 15 hours.

b. Fluorography. Following electrophoresis, the stacking gels were trimmed off, and the running gel was soaked for 30 minutes in a solution of 47% methanol and 1.14M acetic acid. The gel was transferred through two 30 minute washes with dimethyl sulfoxide (DMSO), then soaked for at least 3 hours in 23% PPO (2',5'-diphenyloxazole) made up in DMSO. The PPO was precipitated in the gel by soaking it for 30 minutes in H_2O. The gel was dried over a steaming water bath while under vacuum, and exposed to X-ray film

(Bonner and Laskey, 1974).

RESULTS

I. IDENTIFICATION OF AN E1B 19K DELETION IN cyt 68

The Ad12 mutant cyt 68 is a typical cytocidal mutant, exhibiting the cytocidal CPE, and DNA degradation phenotypes in infected human KB cells, as well as a reduction in the capacity to transform primary BRK cells in culture (Takemori et. al, 1968; Ezoe et. al., 1981; Mak and Mak, 1983). The mutations responsible for the DNA degradation phenotype in some cyt mutants have been mapped to the E1b region (Lai Fatt and Mak, 1982), in some cases to the coding sequences of the 19K protein encoded by E1b (Subramanian et. al., 1984a), by complementation with mutants of adenovirus types 2 and 5. It has been demonstrated that cyt 68 fails to produce the E1b 19K protein in either infected KB cells or in cell lines established by transformation by this mutant (Mak et. al., 1984). These data suggest that there is a lesion in cyt 68 residing within the 19K coding sequences. This mutant has been studied for a number of years, and has never been observed to revert to wild type, leading to the postulation that it may contain a deletion. Mutant cyt 68 was analyzed

by restriction enzyme analysis to localize the suspected deletion within the E1b region and further analyzed by nucleotide sequencing to precisely define the defect.

A. Restriction Enzyme Analysis of Cloned Viral DNA

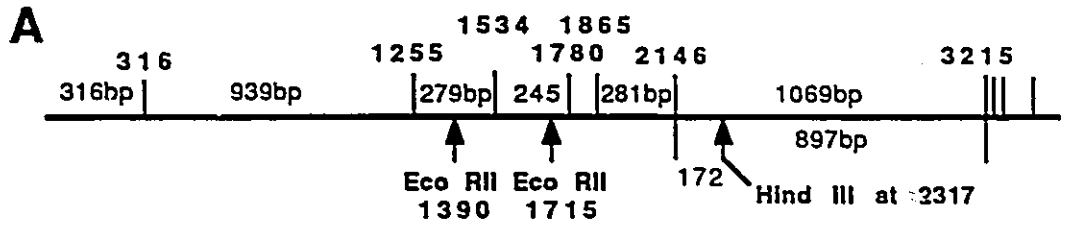
Mutant and wild type DNA were subjected to restriction enzyme analysis in an attempt to identify the putative deletion as a restriction fragment length polymorphism (RFLP) between cyt 68 and wild type. The published nucleotide sequence of the E1 region of adenovirus type 12 (Fujinaga et. al.,1979; Kimura et. al., 1981; Bos et. al., 1981) was surveyed for restriction sites and several restriction endonucleases that cleaved the E1 region frequently were chosen to use in the analysis. Since cyt 68 is derived from wild type strain P, and the published sequences are of wild type strain Huie, DNA from both wild type strains and cyt 68 was analyzed.

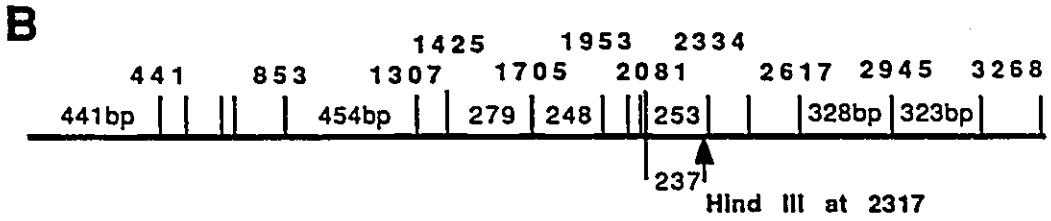
Initially, the analysis was done on the recombinant plasmids pHAB6, pPAB7R and pC6825, which are derivatives of pBR322 containing the E1 region of wild type Huie, wild type P and cyt 68 respectively (see figure 2-1). The reported sequences of Ad12 extend only as far as nucleotide 3814, therefore the restriction pattern generated by cleavage of sequences outside this region could not be predicted. To

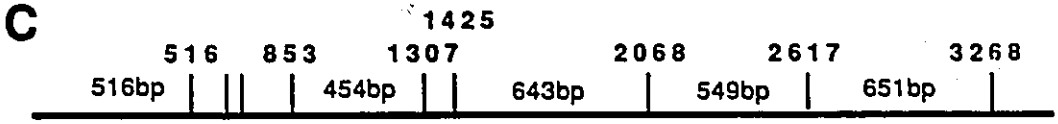
alleviate this problem, and to further simplify the restriction pattern by removal of the pBR322 sequences, either the Bam HI-Sal I fragment containing the left 3496 bp of the viral DNA or the Bam HI-Hind III fragment containing the left 2317 bp of the viral DNA was isolated following agarose gel electrophoresis. The purified fragment was then restricted with Dde I or Hae III and Hinf I, and the resulting fragments were resolved by polyacrylamide gel electrophoresis.

The Dde I restriction map of the E1 region of Ad12 is shown in figure 3-1A, and the pattern of fragments seen after cleavage of the left 2317 bp fragment of pHAB6 is shown in figure 3-2A. As predicted, six fragments were generated and five of the six migrated with the mobility predicted from the restriction map when compared to a Hinf I digest of pBR322, which was used as a size marker. The sixth fragment, which is the left most fragment on the viral genome was predicted to be 316 bp in size but electrophoretically it migrated like a fragment of 455 bp (denoted fragment X). The nucleotide sequencing results obtained in this study (see sections IE1, IE2 and IIE) similarly predict that the Dde I fragment from the terminus of the genome contained in the plasmids pPAB7R, pC6225 and pC6825 should be 316 bp. Furthermore, in every analysis the restriction fragment corresponding to the left terminus of

Figure 3-1. Restriction maps of the left 3496 bp of Ad12 strain Huie. The horizontal lines represent the DNA fragment extending from nucleotide 1 to 3496. The location of recognition sites for the endonucleases Dde I (A), Hinf I (C), and Hae III (D) are indicated as vertical bars. Panel B contains a Hinf I/Hae III map and is a compilation of the maps in panels C and D. A number of the restriction sites are identified by the nucleotide at which cleavage occurs (bold) and the sizes of the larger fragments are indicated in bp. The single Hind III site within this 3496 bp fragment and two of the Eco RII sites are also indicated in panel A. Cleavage with a combination of Dde I and Hind III generates two fragments 897 bp and 172 bp in place of the 1069 bp fragment produced by cleavage with Dde I alone (A). Cleavage with Hind III also splits the 253 bp Hinf I/Hae III fragment into a 237 bp and 16 bp fragment (B).


A
316
1534 1865
1255
1780 2146
3215
316bp
939bp
279bp
245
281bp
1069bp
897bp
Eco RII Eco RII
1390 1715
172
Hind III at 2317


B
1425
1953 2334
441
853
1307
1705
2081
2617 2945 3268
441bp
454bp
279
248
253
328bp
323bp
237
Hind III at 2317


C
1425
516
853
1307
2068
2617
3268
516bp
454bp
643bp
549bp
651bp

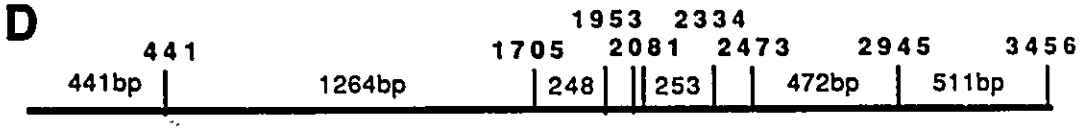
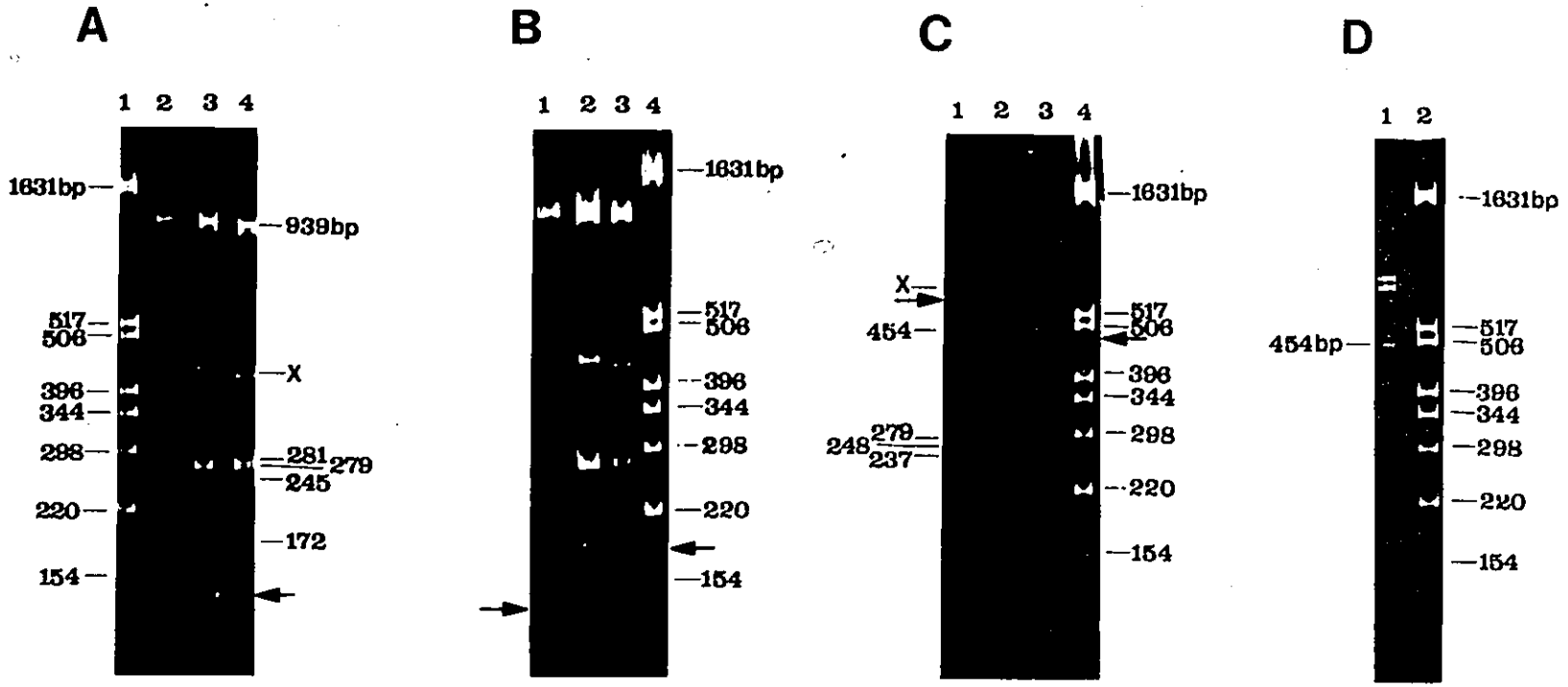

D
1953 2334
441
1705
2081
2473
2945
3456
441bp
1264bp
248
253
472bp
511bp

Figure 3-2. Restriction analysis of DNA fragments isolated from recombinant plasmids. DNA fragments containing nucleotides ~1 to 2317 (panel A, C and D) or nucleotides ~1 to 3496 (panel B) were cleaved with Dde I (A and B), Hinf I (D) or a combination of Hinf I and Hae III (C) and the fragments resolved on a 5% polyacrylamide gel. In all cases pBR322 cleaved with Hinf I was used as a size marker. **A.** 1) marker, 2) pC6825, 3) pPAB7R, 4) pHAB6. The sizes of the pBR322 marker fragments and the predicted sizes of the fragments from pHAB6 (see figure 3-1a) are indicated in bp. The fragment designated X was predicted to be 316 bp in length but migrated as a fragment of 455 bp. **B.** 1) pC6825, 2) pPAB7R, 3) pHAB6, 4) marker. The predicted sizes of the pHAB6 fragments are as in panel A except that the 172 bp fragment is replaced by a 1069 bp fragment (see figure 3-1a). Note the absence of the 245 bp predicted fragment in the pPAB7R and pC6825 samples, which respectively contain novel fragments of 172 bp (indicated by arrow on right) and 150 bp (indicated by arrow on left and arrow in panel A). **C.** 1) pHAB6, 2) pPAB7R, 3) pC6825, 4) marker. The predicted sizes of the pHAB6 fragments are indicated (see figure 3-1b). Fragment X was predicted to be 441 bp but migrated as if it contained 615 bp. The 279 bp

and 248 bp fragments were absent in pPAB7R and pC6825, being replaced by a novel 560 bp (indicated by arrow on left) and 460 bp (indicated by arrow on right) fragment respectively. These analyses identify RFLP's distinguishing strains Huie and P as well as cyt 68 from strain P DNA. Both polymorphisms map to the 5' end of the E1b 19K coding sequences (see figure 3-1). D. 1) pHAB6, 2) marker. The 454 bp fragment indicated in pHAB6 was approximately the same size as the fragment labelled 454 bp in pHAB6 in C indicating that the fragment in panel C was the 454 bp predicted fragment, which is common to the Hinf I and Hinf I/Hae III digests of strain Huie (see figure 3-1b and c).


A
1 2 3 4
1631bp
939bp
517
506
X
396
344
298
281
279
245
220
172
154
B
1 2 3 4
1631bp
517
506
396
344
298
220
154
C
1 2 3 4
1631bp
X
454
517
506
396
344
298
279
248
237
220
154
D
1 2
1631bp
454bp
517
506
396
344
298
220
154

the viral genome electrophoretically migrated slower than predicted. It was presumed that the terminal fragment migrated anomalously due to the formation of secondary structure.

The restriction pattern of the fragment of pPAB7R was similar to that of the pHAB6 fragment, except that the 245 bp fragment seen in pHAB6 was replaced by a fragment which comigrated with the 172 bp fragment common to both plasmids (figure 3-2A, lane 3). There was also a difference in the mobility of fragment X, which probably was the result of the deletion of some sequences from the left terminus of the viral genome during the construction of the plasmid pHAB6, since determination of the nucleotide sequence of a subclone derived from pPAB7R revealed the terminus to be intact.

The restriction pattern generated by Dde I cleavage of the cyt 68 DNA fragment was similar to that of its wild type parent with the exception that it contained a single fragment 172 bp in size, in contrast to the pPAB7R doublet, and an additional unique fragment about 150 bp in size (figure 3-2A, lane 2, arrow indicating the 150 bp fragment). There was also a difference in the mobility of fragment X which will be shown below to be due to the deletion of some nucleotides from the sequences from the left terminus, which probably occurred during construction of this plasmid.

Although this analysis had identified RFLPs in strain P and cyt 68 DNA, the location of the cyt 68 RFLP couldn't be determined since the 150 bp fragment in cyt 68 DNA could have been derived from either the 172 bp fragment unique to the pHAB7R fragment or the 172 bp fragment common to the pPAB7R and pHAB6 fragments. The common fragment lies between nucleotides 2145 and 2317 and was generated by cleavage with Dde I and Hind III. This fragment was eliminated by repeating the Dde I analysis on Bam HI-Sal I fragments extending from the left end to nucleotide 3496. Since Hind III was not used in this analysis, the 172 bp fragment seen in the previous analysis was predicted to be replaced by a fragment 1069 bp in length (see figure 3-1A).

As predicted, there was no 172 bp fragment generated by the cleavage of the ~1 to 3496 bp fragment of pHAB6 (see figure 3-2B). The Dde I restriction pattern of the 3496 bp fragments of pHAB6, pPAB7R and pC6825 contained unique fragments of 245 bp (lane 3), 172 bp (lane 2, indicated by arrow on the right) and 150 bp (lane 1, indicated by arrow on the left) respectively. The simplest interpretation is that a mutation contained in pPAB7R caused a reduction in size of the 245 bp fragment from pHAB6 to 172 bp and a mutation in pC6825 further reduced this fragment to 150 bp. The 245 bp fragment generated from pHAB6 lies between nucleotides 1534 and 1780 and contains the coding sequences

for the amino terminus of E1b 19K. This observation suggested that if cyt 68 contained a large E1b deletion it was located near the 5′ end of the gene.

If the RFLPs observed upon cleavage with Dde I were due to deletions, similar RFLPs should be seen using other restriction enzymes, and the polymorphisms should map to the same place within the E1 region. Therefore this analysis was repeated using different restriction endonucleases. Restriction of the left 2317 bp fragment of pHAB6 with Hinf I and Hae III was predicted to produce twelve fragments (see figure 3-1B), five of which could readily be resolved under the electrophoretic conditions employed. These five fragments were predicted to be 454, 441, 279, 248 and 237 bp in size. The actual pattern obtained was a triplet of bands about 280, 270 and 260 bp in size, a single band at 460 bp and a single larger band about 615 bp in size (see figure 3-2C, lane 1). The observed 460 bp fragment was shown to correspond to the 454 bp predicted fragment by digesting the left 2317 bp fragment of pHAB6 with Hinf I alone. Since the predicted 454 bp fragment is flanked by Hinf I sites, the same size fragment should be generated upon cleavage with Hinf I alone and with the combination of Hinf I and Hae III (see figure 3-1B and C). Hinf I digestion yielded a fragment that was the same size as the 460 bp fragment generated by the double digestion (see figure 3-2D, lane 1),

which demonstrated that this fragment did correspond to the predicted 454 bp fragment. The 615 bp fragment seen in the Hae III and Hinf I double digestion (designated X in figure 3-2C) must therefore correspond to the terminal fragment of the genome, which was predicted to be 441 bp in size, but apparently migrated anomalously.

When the pPAB7R fragment was doubly digested with Hinf I and Hae III, the 280 and 270 bp fragments were absent, being replaced by a single, larger fragment, 560 bp in size, which was approximately the size of the sum of the sizes of these two fragments (see figure 3-2C, lane 2, indicated by arrow on left). Since the predicted 279 and 248 bp fragments are contiguous in the viral genome, this result could be explained by the loss of the Hae III site at nucleotide 1705 (see figure 3-1B). This change was most likely due to a point mutation, rather than a deletion, since there was no obvious loss of genetic material. The only other difference in the restriction patterns of the fragments of pHAB6 and pPAB7R was a slight difference in electrophoretic mobility of the fragments from the left terminus of the viral genome (denoted X) as was observed in the analysis with Dde I.

Restriction of DNA from pC6825 with Hae III and Hinf I produced a pattern of fragments in which the 560 bp fragment seen in pPAB7R was absent. The pattern generated

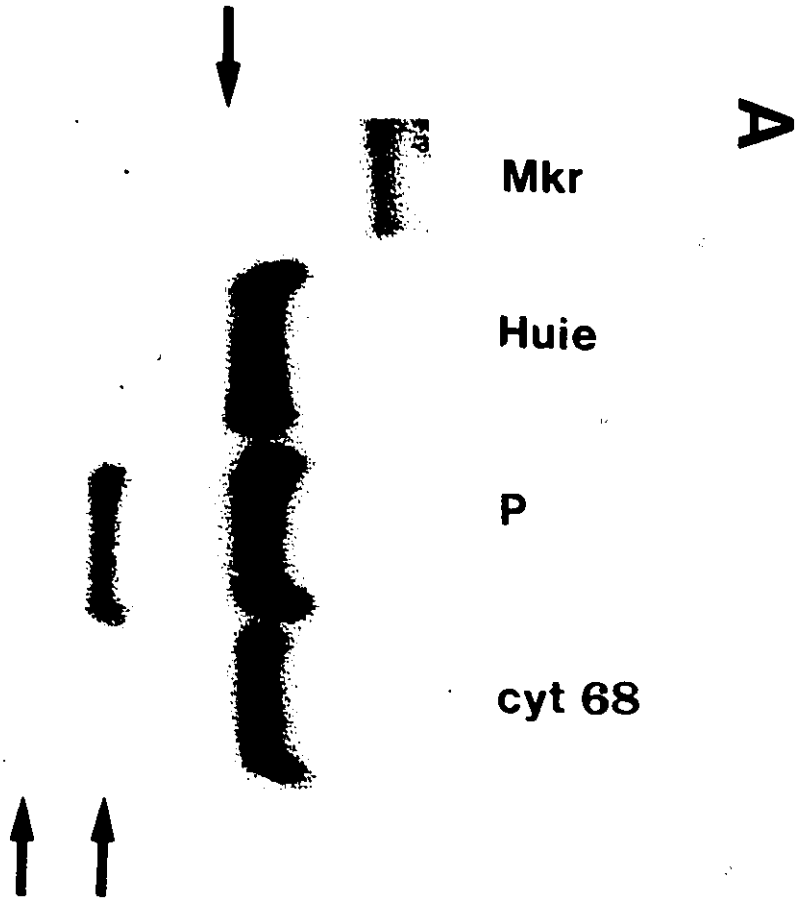
by cleavage of pC6825 in turn contained a novel band approximately 460 bp in size (see figure 3-2C, lane 3, indicated by arrow on the right). The other pC6825 fragments comigrated with the fragments from pPAB7R, except for the fragment from the left end of the viral genome, which migrated with a slightly increased mobility due to a deletion which probably occurred during the construction of pC6825 (see below). The simplest interpretation of these observations is that pC6825 contains a mutation within the fragment corresponding to the 560 bp pPAB7R fragment, and that this mutation is the cause of the alteration in electrophoretic mobility of the pC6825 fragment. This 560 bp pPAB7R fragment, which is the fusion product of pHAB6 fragments predicted to be 279 and 248 bp in size, is located at the 5' end of the E1b region, and the polymorphic Dde I fragment described above is contained entirely within the 560 bp pPAB7R fragment (see figure 3-1A and B).

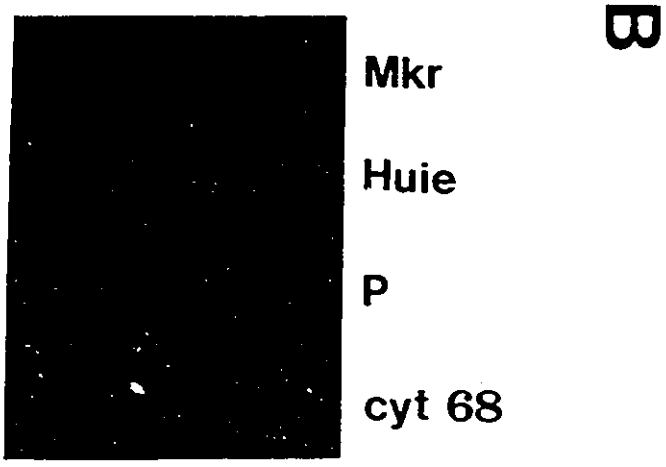
Restriction enzyme analysis using two different enzymes has revealed that the molecularly cloned DNA from the two wild type strains of Ad12 can be distinguished by RFLPs, as can the DNA of cyt 68 and its parent. In each case the polymorphisms map to the 5' end of E1b suggesting that the hypothesized cyt 68 deletion might reside in the sequences encoding the amino terminus of the E1b 19K protein.

B. Restriction Enzyme Analysis of Viral Genomic DNA

The restriction enzyme analyses were repeated using viral DNA, that had been extracted from purified virions, to ensure that the structure of the cloned DNA accurately reflected the structure of the viral genomes. Viral DNA was restricted with Dde I, electrophoresced on a 5% polyacrylamide gel, then using a Hinf I digested pBR322 size marker as a guide, a small piece of the gel containing fragments from about 400 bp to less than 100 bp in size was cut out. The double stranded DNA from this gel piece was electrophoretically transferred to a Zeta-Probe membrane, and fragments from part of the E1b region were detected by hybridization with radioactively labelled pHB14R (containing nucleotides 1594 to 2317 of Ad12). The 344 bp fragment of the pBR322 marker could also be detected by hybridization to the radiolabelled vector sequences from pHB14R. As expected, only two Huie fragments were detected (see figure 3-3A), and they were predicted to be 281 and 245 bp in size (see figure 3-1A). The 245 bp wild type strain Huie fragment was absent in wild type strain P, which contained a unique smaller fragment. Mutant cyt 68 contained neither of these fragments, but contained its own novel fragment smaller in size than the unique strain P fragment. It was

Figure 3-3. Restriction analysis of viral DNA. A. DNA was digested with Dde I, the fragments separated on a 5% polyacrylamide gel, and electrophoretically transferred to a Zeta-Probe membrane. Fragments from the E1b region were detected by hybridization with radiolabelled pHB14R (containing nucleotides 1594-2317 of Ad12). The 245 bp Huie fragment (indicated by arrow on left) was not present in either P or cyt 68 DNA, both of which contained a smaller unique fragment (indicated by arrows on right). The 344 bp pBR322 marker fragment was also detected with this probe. **B.** Viral DNA was restricted with Hae III, the fragments resolved on a 2% agarose gel, denatured and transferred to nitrocellulose by the method of Southern (1977). E1 fragments were detected with nick translated pHAB8R (containing the left 2317 bp of Ad12). The marker was pUC19 cleaved with Hinf I and the fragment detected with the probe was 1.4 kbp in size. A single Huie fragment smaller than the 1.4 kbp marker fragment was detected. The major P fragment was greater than 1.4 kbp and the cyt 68 fragment migrated faster than the P fragment but was still larger than 1.4 kbp. These observations are consistent with those seen in the analysis of the plasmid DNA (see figure 3-2).


A
Mkr
Huie
P
cyt 68


B
Mkr
Huie
P
cyt 68

concluded that the Dde I polymorphisms observed in cloned viral DNA were also present in the viral genomes.

Viral DNA was also analyzed by restriction with Hae III. DNA was restricted, run on a 2% agarose gel, and using a Hind III digested viral DNA size marker, the piece of gel containing fragments from 1 to 2 kbp in size was cut out. The DNA was denatured, transferred to nitrocellulose and detected by hybridization with radiolabelled pHAB8R (containing the left 2317 nucleotides of Ad12). In wild type strain Huie, a single fragment smaller than the 1.4 kbp marker fragment was detected, as anticipated since its predicted size was 1.27 kbp. The major fragment detected in strain P DNA was larger than the 1.4 kbp marker fragment (see figure 3-3B), which is consistent with the loss of the Hae III site at nucleotide 1705 and consequent fusion of 248 bp more to the 1.27 kbp Hae III fragment of Huie DNA. The minor fragment in strain P DNA comigrated with the diagnostic strain Huie fragment and may be due to the contamination of the stock of P DNA with some Huie DNA. A single cyt 68 fragment was detected and migrated with increased mobility relative to its wild type counterpart, but was still larger than the 1.4 kbp marker fragment (see figure 3-3B). These observations suggested that the polymorphisms seen in the Hinf I and Hae III analysis of plasmid DNA were also present in the viral genomic DNA.

These results demonstrated that the structure of the plasmid DNA was a faithful reproduction of the structure of the viral genome and supported the contention that cyt 68 may contain a deletion at the 5' end of the E1b region.

C. Preliminary Sequencing Analysis

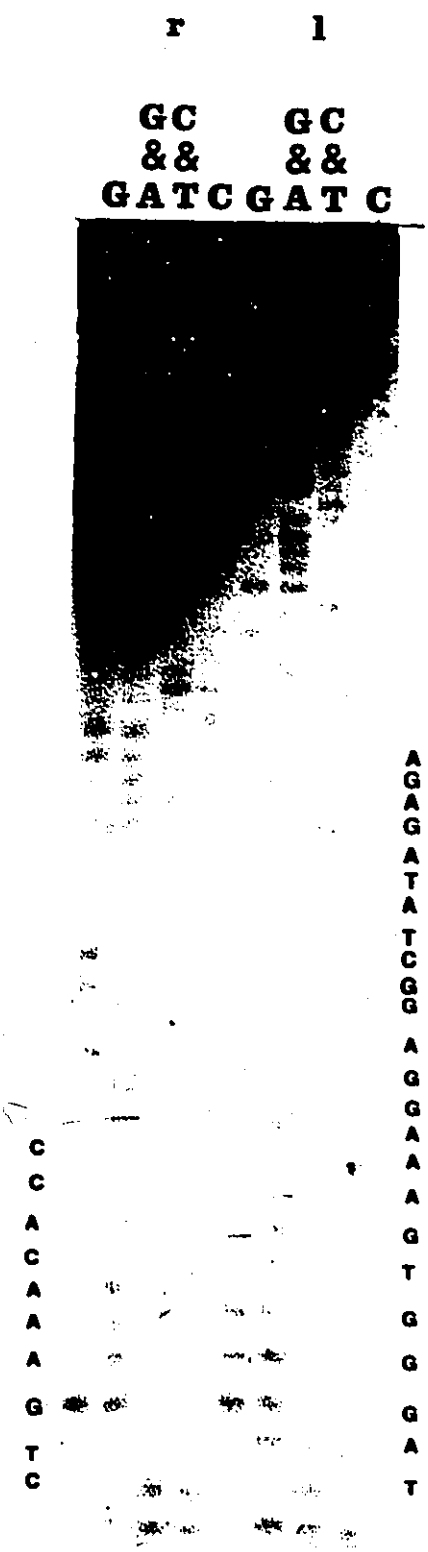
The nucleotide sequences of the cyt 68 Dde I fragment bearing the potential deletion and its corresponding fragment from wild type strain P were partly determined using the chemical modification procedure (Maxam and Gilbert, 1977). The plasmids pPAB7R and pC6825 were restricted with Dde I, and the fragments of interest were isolated from a polyacrylamide gel. The fragments were end labelled, denatured and the two strands of each separated on a polyacrylamide gel and isolated. The nucleotide sequence of a part of each strand was then determined.

The sequence of one strand could be read from nucleotide 1762 to 1719 and the mutant and wild type sequences matched exactly (see strand r in figure 3-4). Approximately 45 bases could also be read from the other strand, but the mutant and wild type sequences differed (see strand l in figure 3-4). The first nucleotide that could be read from strain P was nucleotide 1624, whereas the cyt 68 sequence began at nucleotide 1663. Furthermore, the strain

Figure 3-4. Preliminary nucleotide sequencing of cyt 68. An autoradiogram of a nucleotide sequencing gel of the Dde I fragment of pPAB7R and pC6825 which showed increased mobility in restriction analyses is shown. The Dde I fragments were isolated from a polyacrylamide gel, 5' end labelled, the strands separated and isolated following polyacrylamide gel electrophoresis. Then the individual strands were sequenced using the technique of Maxam and Gilbert (1977). The sequences of the r stransds were identical as far as was determined (from nucleotide 1762 to 1719). The l strands, however, differed in sequence, the cyt 68 sequences beginning about 40 bp downstream of the strain P sequence, which itself began about 70 bp further downstream than predicted from the Ad 12 strain Huie sequence. This analysis mapped the strain P and cyt 68 polymorphisms to the left end of the Dde I fragment.

Ad12

cyt 68



P sequences began about 70 bp further downstream than predicted from the published sequence of strain Huie. Although the mutations responsible for the Dde I polymorphisms in wild type P and cyt 68 could not be identified since the sequences at the extreme 5' end of each strand were not determined, it could be concluded that they were located at the left, or promoter proximal end of the Dde I fragments. A number of differences between the nucleotide sequence of strain P and the published sequence of strain Huie were observed and will be described below.

D. Identification of a 107 bp Deletion in cyt 68

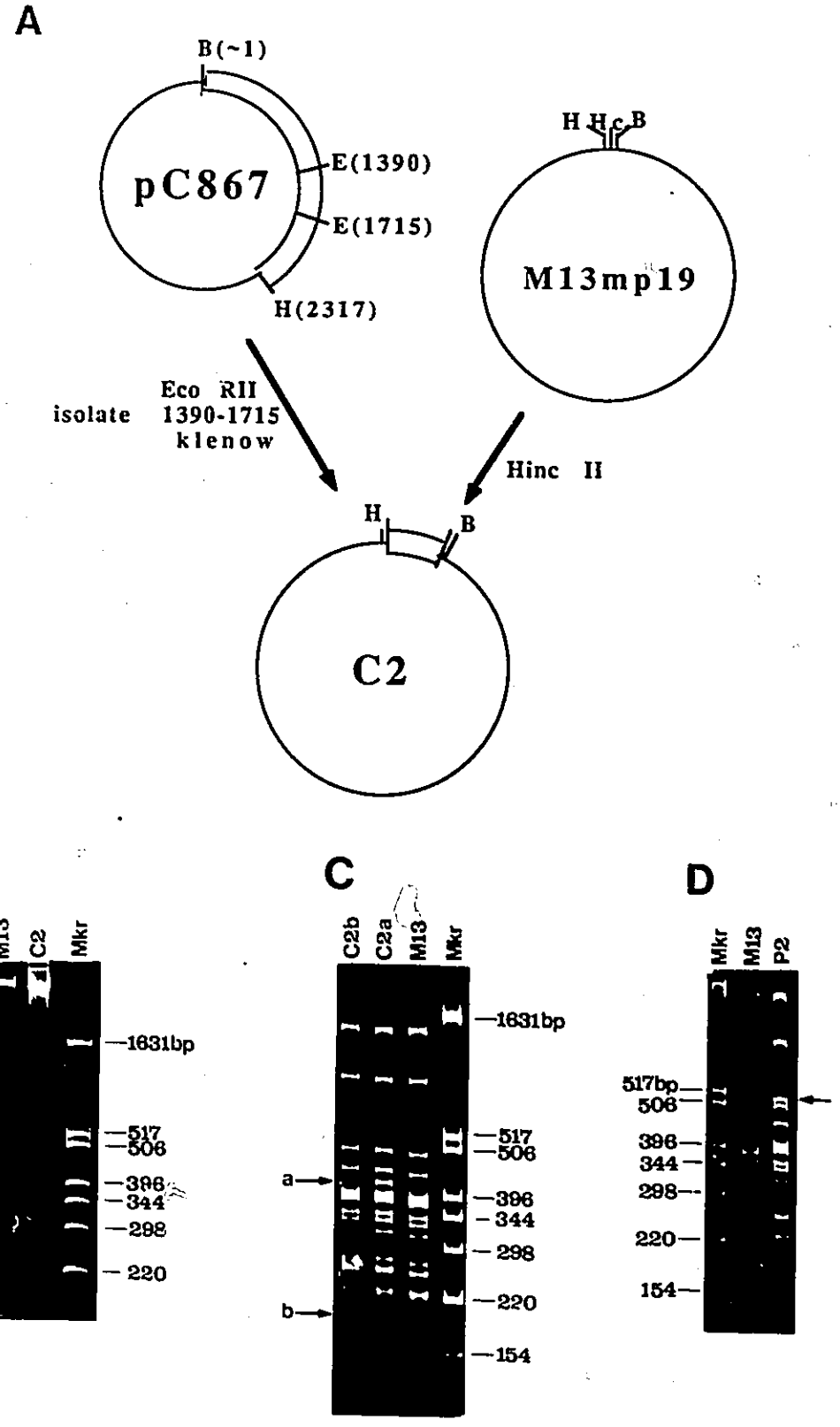
Although the sequence analysis of the Dde I fragments failed to define the mutations responsible for the observed polymorphisms, it demonstrated that they were located at the left end of the fragments. This placed them within the 324 bp Eco RII fragment extending from nucleotide 1390 to 1715 (see figure 3-1A). The 324 bp Eco RII fragment from wild type strain P and the corresponding fragment from cyt 68 were subcloned into a single stranded M13 vector for sequencing by the dideoxy chain termination technique described by Sanger (1977).

The restriction enzyme Eco RII does not cleave DNA if the sequences recognized by the enzyme are methylated by

the dcm methylase of E. coli. Therefore the plasmids used to subclone the Eco RII fragments were transferred to GM31, which is a dcm$^-$ strain of E. coli, and unmethylated DNA was prepared.

The plasmids pP67 and pC867 were digested with Eco RII and the 324 bp fragment from pP67 and its counterpart in pC867, a fragment about 230 bp in length, were isolated after resolution on a polyacrylamide gel. The multiple cloning site of the vector used, M13mp19, is devoid of Eco RII sites and of unique sites for enzymes that generate cohesive ends compatible for ligation with Eco RII. Therefore prior to insertion into M13mp19, the ends of the isolated Eco RII fragments were made blunt using the large fragment of DNA polymerase, then ligated into the unique Hinc II site present in M13mp19 (as shown for the cyt 68 fragment in figure 3-5A). The ligated DNA was introduced into JM103 by transfection and phage containing inserts were identified as those that formed white plaques on a lawn of JM103 in the presence of Xgal and IPTG. Recombinants of the correct structure were identified by restriction analysis. Bam HI and Hind III, which cut at sites flanking the Hinc II site, were used to demonstrate the presence of an insert of the appropriate size (clone C2 is shown as representative in figure 3-5B). The orientation of the insert in the recombinant phage was determined by cleavage with Hinf I.

Figure 3-5. Construction of M13 recombinants for sequencing. **A.** The strategy for cloning the M13 recombinant C2 is shown. The position of restriction sites for Hind III (H), Eco RII (E), Bam HI (B) and Hinc II (Hc) are indicated. The number by each site denotes its nucleotide position in the viral genome. The same strategy was applied to construct the wild type P counterpart of C2, called P2, using pP67 and M13mp19. **B.** RF DNA was digested with Bam HI and Hind III and the fragments resolved on a 5% polyacrylamide gel. Two fragments were observed from C2, a large vector sized fragment and a small fragment approximately the size predicted for the C2 insert. Similar results were obtained with P2. **C.** RF DNA was digested with Hinf I and analyzed as in B. The two C2 clones shown, C2a and C2b, contained all the fragments seen in a Hinf I digestion of M13 and a single additional fragment (arrows a and b). The different patterns exhibited by C2a and C2b suggested that they had the insert in opposite orientations. **D.** P2 clones were analyzed as in C and all contained the 500 bp fragment indicated by the arrow, in addition to the fragments common to M13. Since all the clones exhibited the same restriction pattern it appeared that they all contained the insert in the same orientation.

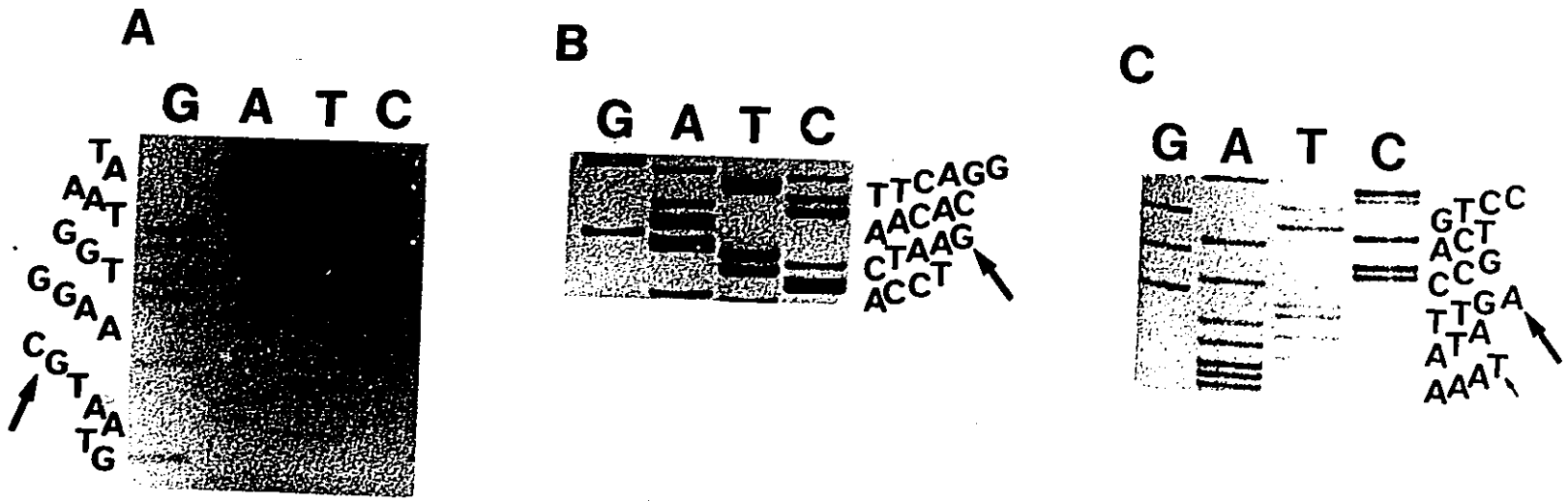

A
B(~1)
pC867
E(1390)
E(1715)
H(2317)
H H c B
M13mp19
Eco RII
isolate 1390-1715
klenow
Hinc II
H
B
C2
B
M13
C2
Mkr
1631bp
517
506
396
344
298
220
C
C2b
C2a
M13
Mkr
a
b
1631bp
517
506
396
344
298
220
154
D
Mkr
M13
P2
517bp
508
396
344
298
220
154

Although restriction of M13mp19 with Hinf I produces a complex pattern of fragments, a Hinf I site asymmetrically located within the insert should yield DNA fragments that are diagnostic for the orientation of the insert. The Hinf I restriction pattern of each recombinant was compared to that of M13mp19 to identify the diagnostic fragment in each, which was unique to the recombinant. The Hinf I restriction pattern of one recombinant with a cyt 68 insert contained a single fragment of about 200 bp, in addition to the fragments common to M13mp19 (indicated by arrow b in figure 3-5C, lane C2b). A second predicted diagnostic fragment was obscured by the presence of vector fragments. Other cyt 68 containing recombinants exhibited all of the vector fragments and an additional fragment 410 bp in size, which was 100 bp smaller than predicted for the mutant DNA if the fragment was in the opposite orientation (indicated by arrow a in figure 3-5C, lane C2a). A second predicted diagnostic fragment was run off the gel. Despite the difference between the predicted and observed size of one of the diagnostic fragments, the difference in restriction patterns suggested that one clone had the insert in one orientation and others had the insert in the opposite orientation. Five recombinants containing the 324 bp Eco RII fragment of wild type strain P were isolated, all of which displayed the same unique Hinf I fragment of 515 bp, which again was 100 bp

smaller than predicted if the insert was in a particular orientation (indicated by arrow in figure 3-5D). The similarity of restriction patterns of the recombinants suggested that all had the insert in the same orientation. A representative phage of each construct was plaque purified, single stranded DNA prepared from infected cells and the nucleotide sequence determined by the dideoxy technique using the universal primer to prime the synthesis reaction.

Comparison of the nucleotide sequence of wild type strain P with the published sequence of wild type strain Huie revealed a number of nucleotide polymorphisms, one of which explained the altered Dde I restriction pattern observed in wild type strain P. A point mutation at nucleotide 1606 (see figure 3-6B) created a new Dde I site which resulted in the cleavage of the 245 bp Dde I fragment seen in wild type strain Huie into a 173 bp fragment, which was observed, and a 72 bp fragment, which was not detected due to its small size. This nucleotide substitution had no effect upon the amino acid sequence of the E1b 19K. Another point mutation resulted in the conserved substitution of arginine in wild type strain P for the lysine in strain Huie at amino acid 29 of the E1b 19K. Since the wild type strain P recombinants were only of one orientation, and the sequencing reactions run at the time were incapable of

Figure 3-6. Autoradiograms of selected sequencing gels. **A.** The sequence of cyt 68 demonstrating the E1b 19K deletion. The G and C indicated by the arrow were separated by 107 bp in wild type strain P. The G is nucleotide 1543 in wild type P and the C is nucleotide 1651. **B.** The sequence of wild type strain P contains a new Dde I site. The arrow denotes nucleotide 1606, which is an A in wild type Huie. Conversion to a G created a Dde I site (CTAAG). **C.** The sequence of wild type strain P has lost a Hae III site. The A specified by the arrow is nucleotide 1706. The conversion of the G in Huie to the A in strain P destroys a Hae III site (GGCC). The smaller arrow designates nucleotide 1699, which is a C in the Huie sequence. The transition of the C to T is a silent mutation.


A
G A T C
B
G A T C
C
G A T C

resolving 300 bp, it was not possible to determine the sequence of the entire insert. The mutation resulting in the altered Hae III restriction pattern in strain P was inferred to be a point mutation since the nucleotide sequence of the cyt 68 mutant, which was derived from strain P, contained a substitution at nucleotide 1706 which destroyed the Hae III site at that position. This nucleotide substitution was later confirmed by sequencing cloned wild type strain P DNA (see below).

The only difference between the sequence of cyt 68 and its wild type parent was the loss of 107 bp beginning with the second codon within the 19K coding region (see figure 3-6A). This deletion causes a shift in the 19K reading frame, precluding the synthesis of any protein product resembling 19K. This lesion accounts for the observed absence of 19K from cyt 68 infected and transformed cells (Mak et. al., 1984). A relevant point mutation was also observed distinguishing cyt 68 from the published sequence of strain Huie. This was a substitution at nucleotide 1706, which would result in the alteration of an alanine residue to threonine at amino acid 56 of the 19K protein, if it was produced. This same mutation eliminates the Hae III site at 1706 consistent with the results of the restriction enzyme analysis presented above.

The deletion in E1b 19K could entirely explain the

observed phenotype of cyt 68, based upon the evidence in the literature at that time. Numerous laboratories have created E1b 19K mutants in Ad2, Ad5 and Ad12, and most report that the mutant viruses induce DNA degradation during the lytic infection of human cells and exhibit a reduced ability to transform cells in various assays. (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). These are two of the characteristic phenotypes of cyt 68 (Takemori et. al., 1968; Ezoe et. al., 1981).

Since that time it has been reported that the E1b 19K is not required for the transformation of primary BRK cells by Ad12 (Edbauer et.al., 1988). This conclusion is based upon the study of two mutants which are unable to synthesize any 19K product. One, pm700, contains a point mutation that eliminates the initiation codon, and the second, in700, has a frame shift due to a 2 bp insertion and can consequently encode only the first 20 amino acids of the normal 19K protein fused to 17 missense residues. A comparable initiation codon mutant, pm1542, was independently created and studied by Shi Yun Zhang in our laboratory. All three viruses induced DNA degradation in infected cells, however, all had transforming activity that matched or exceeded that of wild type virus when assayed using primary BRK cells (Edbauer et.al., 1988; Zhang in

preparation). Based on this evidence, cyt 68 would be predicted to be fully transformation competent if the failure to synthesize 19K was its sole defect. This implied that cyt 68 might contain a second defect, in addition to its 19K deletion, that contributed to its transformation defectiveness. A likely location for this putative mutation was in the E1 region. Therefore the nucleotide sequence of the entire E1 region of cyt 68 was determined.

E. Sequencing Analysis of The E1 Region

1. Analysis of Strain P

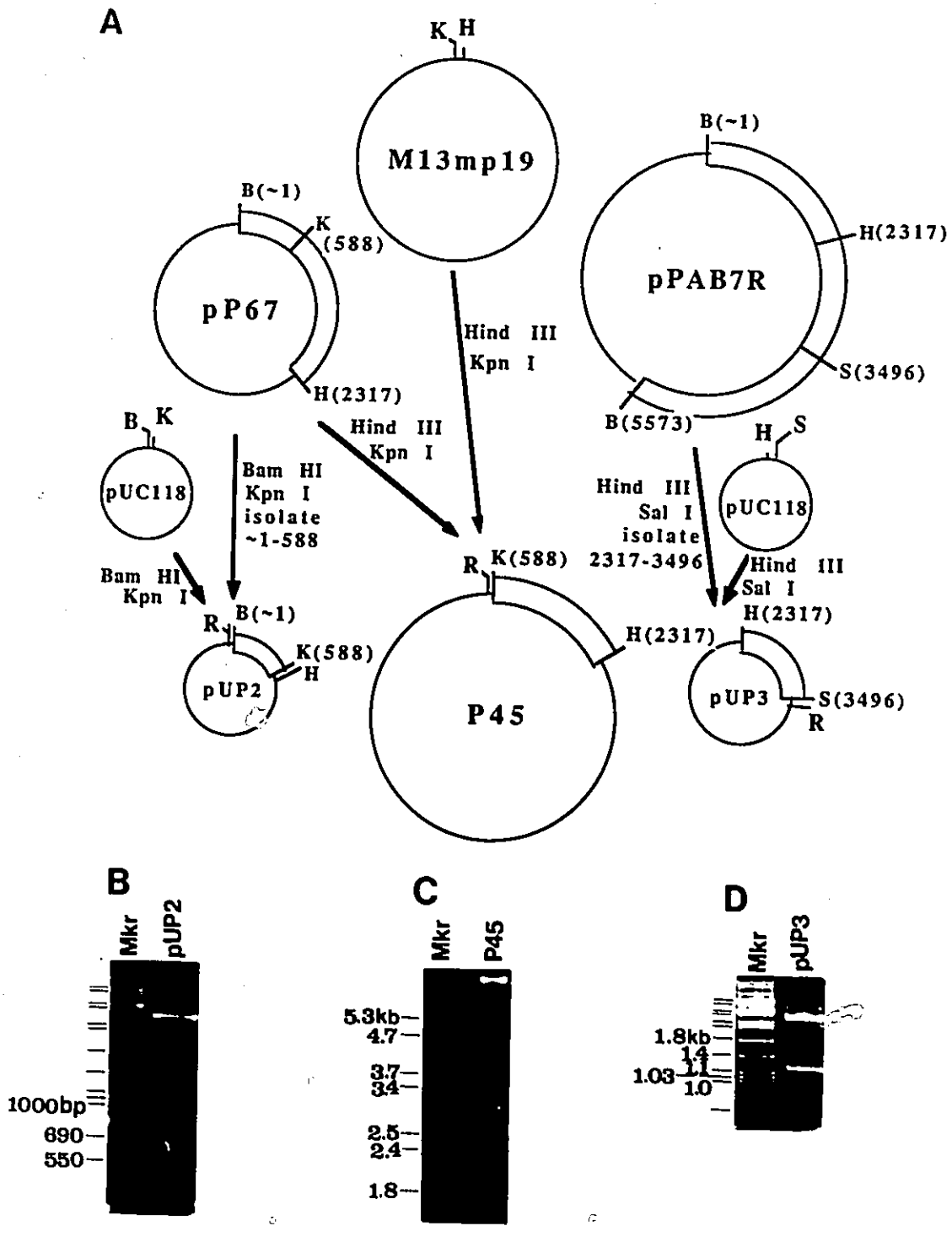
Since cyt 68 is derived from the wild type P strain of Ad12, the sequence of the E1 region of wild type strain P was determined to provide a normal control with which to compare the cyt 68 sequence. Three fragments of P DNA spanning the E1 region were subcloned into single stranded vectors and the nucleotide sequence determined by the dideoxy technique.

The fragment extending from nucleotide 588 to 2317 was cloned into M13mp18 and M13mp19. This fragment was isolated from an agarose gel prior to its insertion into M13mp18, but for cloning into M13mp19 pP67 was simply cleaved with Hind III and Kpn I to free the fragment of interest, then mixed and ligated with identically cut vector

(see figure 3-7A). JM107 cells were transfected with the mixture and recombinant phage isolated from white plaques on JM107 lawns. The left 588 bp fragment of P was isolated from a 3.5% polyacrylamide gel after cleavage of pP67 with Bam HI and Kpn I, then inserted between the Bam HI and Kpn I sites of the double stranded vectors pUC118 and pUC119 (see figure 3-7A). The Hind III-Sal I fragment extending from nucleotide 2317 to 3496 was cut out of pPAB7R, isolated from an agarose gel and ligated with pUC118 or pUC119 that had been restricted with Hind III and Sal I (see figure 3-7A). In these latter two experiments E. coli MV1190 were transformed and bacteria harbouring recombinant plasmids were identified as white colonies when plated onto YT plates containing IPTG and Xgal.

The double stranded RF DNA from white plaques and plasmid DNA from white colonies were extracted and analyzed by restriction to screen for successful constructs. All were digested with Hind III and Eco RI, which cut the vector at each end of the multiple cloning site, thus releasing any inserted DNA. Restricted DNA was run on agarose gels and recombinants of the correct structure identified by the presence of a 0.6 kbp (the left 588bp fragment), a 1.7 kbp (the fragment from nucleotide 588 to 2317), or a 1.2 kbp (the 2317 to 3496 bp fragment) insert fragment and either a 7.2 kbp (M13) or 3.2 kbp (pUC) vector fragment (shown for

Figure 3-7. Construction of recombinants for sequencing strain P DNA. A. The strategy for the construction of three recombinant plasmids/phage for determination of the nucleotide sequence of the E1 region of strain P is shown. The restriction sites for Hind III (H), Kpn I (K), Bam HI (B), Eco RI (R), and Sal I (S) and their nucleotide position on the viral genome are indicated. Recombinants designed to allow sequencing of the other strand of DNA were similarly constructed utilizing pUC119 and M13mp18 and this same strategy was used to generate recombinants for determining the nucleotide sequence of the E1 region of cyt 62 (see section IIE2). **B to D.** Plasmid and RF DNA from recombinants was restricted with Hind III and Eco RI, and the fragments were resolved on 1% agarose gels. The marker in each case was Hind III digested wild type Huie DNA. The recombinants pUP-2, P45, and pUP-3 contained a fragment of 588 bp, 1.7 kbp and 1.2 kbp respectively, which were the expected sizes of the inserts, in addition to a fragment the size of the vector. Identical results were obtained upon analysis of the complementary constructs pUP1, p1-7 and pUP4.


A
M13mp19
K H
pP67
B(~1)
K
(588)
H(2317)
pPAB7R
B(~1)
H(2317)
S(3496)
B(5573)
Hind III
Kpn I
Hind III
Kpn I
pUC118
B K
Bam HI
Kpn I
isolate
~1-588
Hind III
Sal I
isolate
2317-3496
pUC118
H S
Bam HI
Kpn I
Hind III
Sal I
R K(588)
H(2317)
P45
R B(~1)
K(588)
H
pUP2
H(2317)
pUP3
S(3496)
R
B
Mkr
pUP2
1000bp
690
550
C
Mkr
P45
5.3kb
4.7
3.7
3.4
2.5
2.4
1.8
D
Mkr
pUP3
1.8kb
1.4
1.1
1.03
1.0

pUP-2, P45 and pUP-3 in figure 3-7B to D). Successful recombinants were plaque purified (M13 clones) or streaked (pUC clones), rescreened and single stranded DNA prepared from each.

A battery of oligonucleotides was used to prime nucleotide sequencing reactions using the dideoxy technique (see figure 2-3). The nucleotide sequence of both strands of the entire E1 regulatory and coding sequences was determined. Analysis of the sequence of wild type strain P revealed differences from the wild type Huie sequences at forty sites within E1a and E1b (see figure 3-9). With the exception of two deletions and two insertions, the differences were due to nucleotide substitutions. Despite the variability at the nucleotide level, only eight amino acid differences existed between the E1 proteins of the two strains, two of which were in E1a. An A to G transition at nucleotide 835 converted amino acid 112 of the E1a proteins from methionine to valine. The deletion of 3 base pairs at position 1280/1282 resulted in the replacement of an arginine and cysteine in Huie with a serine in P. This arginine in Huie is amino acid 235 of the E1a 13S product (amino acid 204 of the translation product of the E1a 12S mRNA).

The E1b 19K contained 3 amino acid substitutions. The conservative change of arginine to lysine occurred at

amino acid 29, due to a G to A mutation at nucleotide 1626 (see figure 3-6C). The G to A transition at nucleotide 1706 had two consequences. First, it destroyed the Hae III site that was observed to be changed using restriction analysis, and secondly, it converted amino acid 56 to threonine from an alanine. Finally a T to C transition at nucleotide 1745 converted tyrosine 69 in Huie to histidine.

Similarly, the E1b 55K had three substitutions due to point mutations at nucleotides 1910, 2104 and 2755. The first changed a glutamic acid to an alanine at amino acid 22, and the latter two converted aspartic acid residues to asparagine residues at amino acids 87 and 304 respectively. These mutations have no effect upon the 19K. The remainder of the polymorphisms between Huie and P did not alter the sequences of the encoded proteins, nor any known regulatory sequences. However, they can serve as useful markers to distinguish the two strains of virus.

2. Analysis of cyt 68

To subclone the E1 region of cyt 68 for sequencing, pC6825 was cleaved with Bam HI and Sal I and the fragments separated on a 1% agarose gel. The Bam HI-Sal I fragment containing the left 3496 bp was isolated from the gel using Gene Clean and inserted between the Bam HI and Sal I sites

of pUC118 and pUC119 (see figure 3-8A). E. coli JM103 were transformed and plasmid DNA isolated from colonies that were white when grown on plates containing Xgal. The DNA was analyzed by restriction with Bam HI and Hind III, which generated the three expected fragments, the 3.2 kbp vector fragment and two fragments 2.3 kbp and 1.2 kbp in size from the insert (shown for p68/118 in figure 3-8D). Successful constructs were streaked and rescreened, then single stranded DNA was prepared following infection of the cells with the helper phage M13K07. The fragment cloned into pUC119 seemed refractory to the induction of single stranded DNA, since all attempts at preparation apparently yielded only the single stranded DNA of the helper phage. This problem was surmounted by splitting the insert into two and cloning into the appropriate vector. This was attained by cleaving pC6825 with either Bam HI and Hind III or Hind III and Sal I and isolating the left 2317 nucleotide Bam HI-Hind III fragment and the Hind III-Sal I fragment containing nucleotides 2317 to 3496 by the Gene Clean protocol following agarose gel electrophoresis. The former was ligated to pUC119 and the latter to pUC118 suitably cleaved to accept the insert (see figure 3-8A). The recombinant DNA was introduced into JM103, screened on YT plates with Xgal, and recombinants of the desired structure identified by restriction with Eco RI and Hind III to liberate the insert.

Figure 3-8. Construction of recombinants for sequencing cyt 68 DNA. A. The cloning strategy applied to create plasmids for sequencing cyt 68 is shown. The location of recognition sites for the enzymes Hind III (H), Bam HI (B), Eco RI (R) and Sal I (S) are indicated along with the nucleotide position of each on the viral genome. The left 2317 bp fragment, the left 3496 bp fragment or the nucleotide 2317 to 3496 fragment of pC6825 were isolated from an agarose gel and inserted into pUC118 or pUC119. The DNA was introduced into JM103 by transfection. **B to D.** Restriction analysis of the recombinants. Plasmid DNA was extracted from E. coli and p68BH9 and p68HS8 were cleaved with Eco RI and Hind III, and p68/118 was cut with Bam HI and Hind III. The fragments were run on 1% agarose gels and observed after EtBr staining using UV illumination. The marker in each was wild type Huie DNA cleaved with Hind III. Based upon the restriction patterns produced, it was concluded that these recombinants contained the desired inserts. Plasmids p68BH9 and p68HS8 each contained the 3.2 kbp vector fragment and a single insert fragment 2.3 and 1.2 kbp in size respectively, as predicted. Plasmid p68/118 contained the 3.2 kbp vector fragment and the two fragments 2.3 kbp and 1.2 kbp in size which were predicted from the insert.

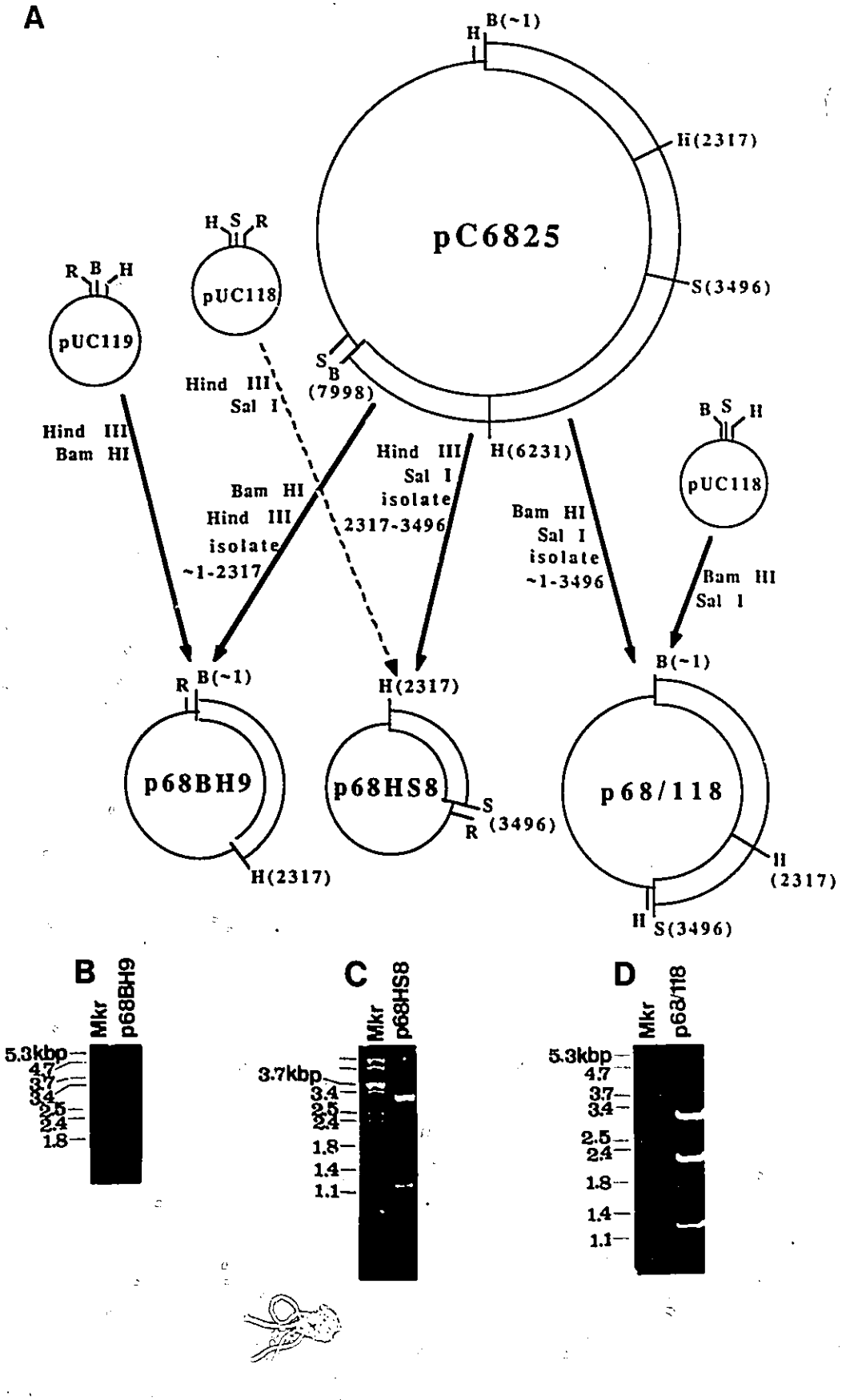
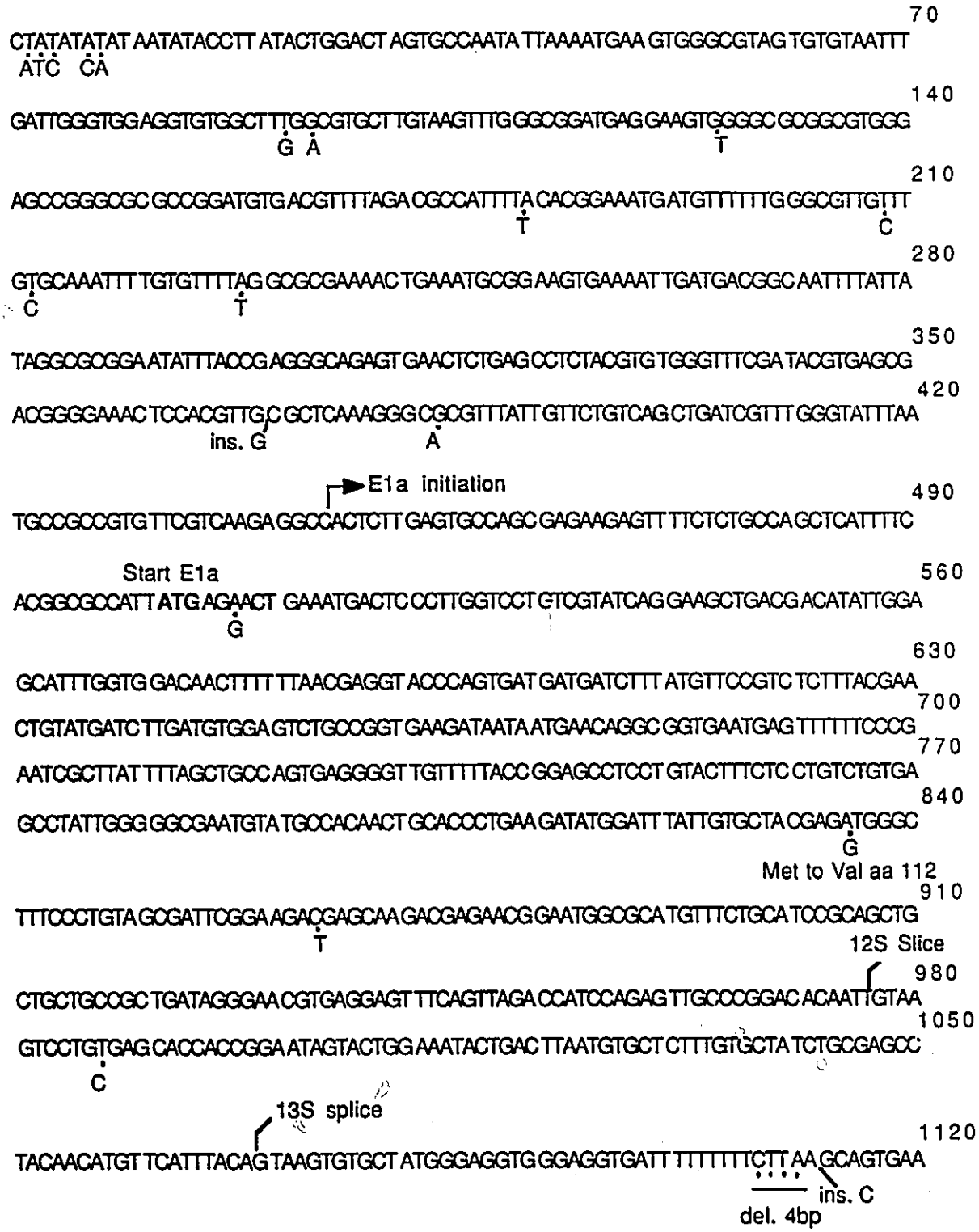
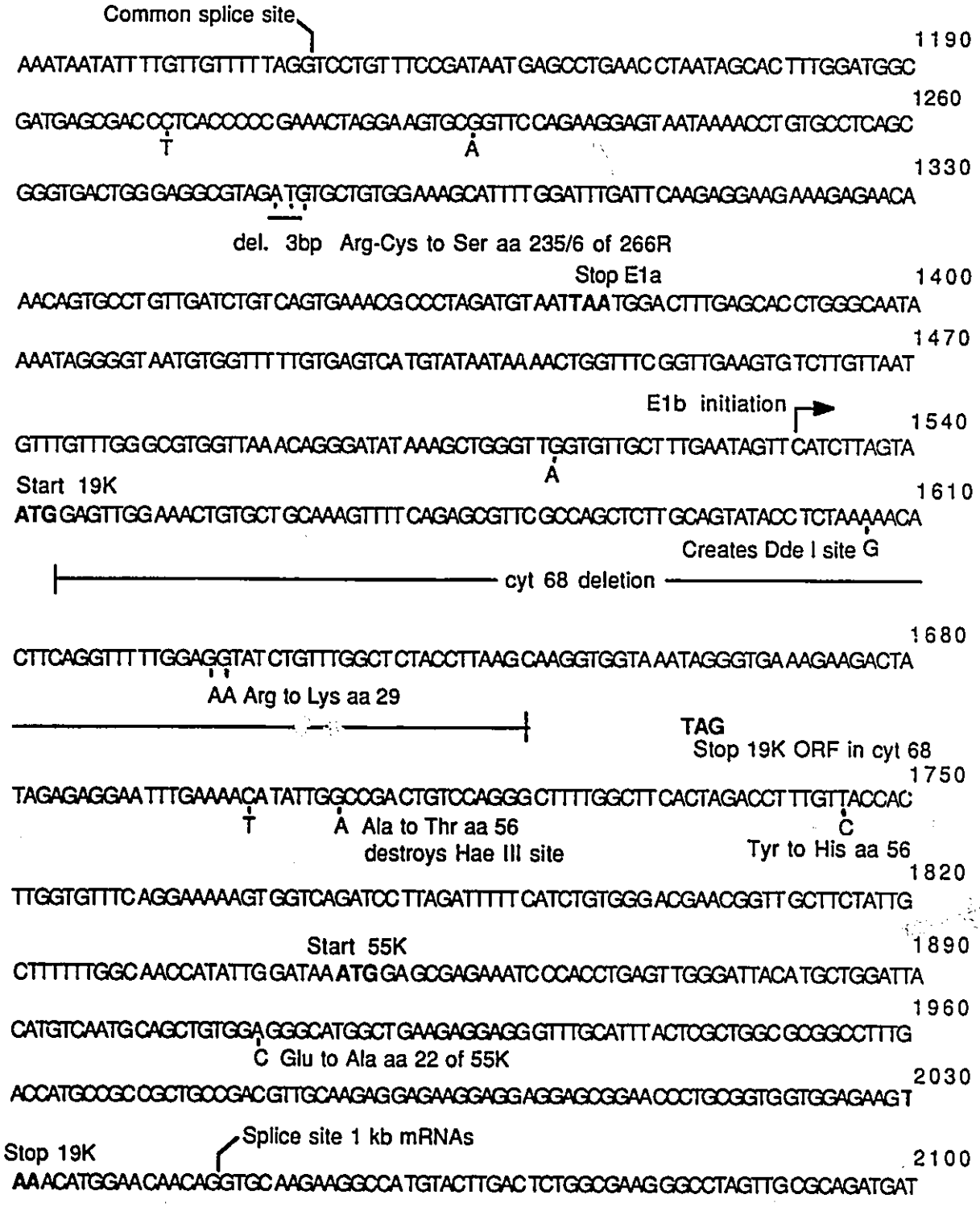

A
pC6825
B(~1)
H
H(2317)
S(3496)
pUC119
R B H
pUC118
H S R
S B (7998)
Hind III
Sal I
Hind III
Bam HI
Bam HI
Hind III
isolate
~1-2317
Hind III
Sal I
isolate
2317-3496
H(6231)
Bam HI
Sal I
isolate
~1-3496
pUC118
B S H
Bam HI
Sal I
p68BH9
R B(~1)
H(2317)
p68HS8
H(2317)
S
R (3496)
p68/118
B(~1)
H
(2317)
H S(3496)
B
Mkr
p68BH9
5.3kbp
4.7
3.7
3.4
2.5
2.4
1.8
C
Mkr
p68HS8
3.7kbp
3.4
2.5
2.4
1.8
1.4
1.1
D
Mkr
p68/118
5.3kbp
4.7
3.7
3.4
2.5
2.4
1.8
1.4
1.1

Figure 3-9. Comparison of the nucleotide sequence of region E1 from strains Huie and P. Shown is the nucleotide sequence of strain Huie from nucleotide 1 to 3360 as determined by Fujinaga et. al. (1979), Kimura et. al. (1981) and Bos et. al. (1981). Above the sequence the positions of transcriptional start sites and splice sites are indicated. Initiation and termination codons of the E1a and E1b open reading frames are printed in bold and indicated by text above the sequence. Nucleotide differences seen in strain P and their consequence with respect to amino acid coding and in certain cases recognition by endonucleases are indicated below the strain Huie sequence. Also shown below the printed sequence is the deletion found in cyt 68. The nucleotide sequence from position 3360 to 3496 is identical in the two strains.


70
CTATATATAT AATATACCTT ATACTGGACT AGTGCCAATA TTAAAATGAA GTGGGCGTAG TGTGTAATTT
ATC CA
140
GATTGGGTGG AGGTGTGGCT TGGCGTGCTT TGTAAGTTTG GGCGGATGAG GAAGTGGGGC GCGGCGTGGG
G A T
210
AGCCGGGCGC GCCGGATGTG ACGTTTTAGA CGCCATTTTA CACGGAAATG ATGTTTTTTG GGCGTTGTTT
T C
280
GTGCAAATTT TGTGTTTTAG GCGCGAAAAC TGAAATGCGG AAGTGAAAAT TGATGACGGC AATTTTATTA
C T
350
TAGGCGCGGA ATATTTACCG AGGGCAGAGT GAACTCTGAG CCTCTACGTG TGGGTTTCGA TACGTGAGCG
420
ACGGGGAAAC TCCACGTTGC GCTCAAAGGG CGCGTTTATT GTTCTGTCAG CTGATCGTTT GGGTATTTAA
ins. G A
E1a initiation
490
TGCCGCCGTG TTCGTCAAGA GGCCACTCTT GAGTGCCAGC GAGAAGAGTT TTCTCTGCCA GCTCATTTTC
Start E1a
560
ACGGCGCCATT ATG AGAACT GAAATGACTC CCTTGGTCCT GTCGTATCAG GAAGCTGACG ACATATTGGA
G
630
GCATTTGGTG GACAACTTTT TTAACGAGGT ACCCAGTGAT GATGATCTTT ATGTTCCGTC TCTTTACGAA
700
CTGTATGATC TTGATGTGGA GTCTGCCGGT GAAGATAATA ATGAACAGGC GGTGAATGAG TTTTTTCCCG
770
AATCGCTTAT TTTAGCTGCC AGTGAGGGGT TGTTTTTACC GGAGCCTCCT GTACTTTCTC CTGTCTGTGA
840
GCCTATTGGG GGCGAATGTA TGCCACAACT GCACCCTGAA GATATGGATT TATTGTGCTA CGAGATGGGC
G
Met to Val aa 112
910
TTTCCCTGTA GCGATTCGGA AGACGAGCAA GACGAGAACG GAATGGCGCA TGTTTCTGCA TCCGCAGCTG
T
12S Slice
980
CTGCTGCCGC TGATAGGGAA CGTGAGGAGT TTCAGTTAGA CCATCCAGAG TTGCCCGGAC ACAATTGTAA
1050
GTCCTGTGAG CACCACCGGA ATAGTACTGG AAATACTGAC TTAATGTGCT CTTTGTGCTA TCTGCGAGCC
C
13S splice
1120
TACAACATGT TCATTTACAG TAAGTGTGCT ATGGGAGGTG GGAGGTGATT TTTTTTTCTT AA GCAGTGAA
ins. C
del. 4bp


Common splice site
1190
AAATAATATT TTGTTGTTTT TAGGTCCTGT TTCCGATAAT GAGCCTGAAC CTAATAGCAC TTTGGATGGC
1260
GATGAGCGAC CCTCACCCCC GAAACTAGGA AGTGCGGTTC CAGAAGGAGT AATAAAACCT GTGCCTCAGC
T
A
1330
GGGTGACTGG GAGGCGTAGA TGTGCTGTGG AAAGCATTTT GGATTTGATT CAAGAGGAAG AAAGAGAACA
del. 3bp Arg-Cys to Ser aa 235/6 of 266R
Stop E1a
1400
AACAGTGCCT GTTGATCTGT CAGTGAAACG CCCTAGATGT AATTAATGGA CTTTGAGCAC CTGGGCAATA
1470
AAATAGGGGT AATGTGGTTT TTGTGAGTCA TGTATAATAA AACTGGTTTC GGTTGAAGTG TCTTGTTAAT
E1b initiation
1540
GTTTGTTTGG GCGTGGTTAA ACAGGGATAT AAAGCTGGGT TGGTGTTGCT TTGAATAGTT CATCTTAGTA
A
Start 19K
1610
ATG GAGTTGG AAACTGTGCT GCAAAGTTTT CAGAGCGTTC GCCAGCTCTT GCAGTATACC TCTAAAAACA
Creates Dde I site G
cyt 68 deletion
1680
CTTCAGGTTT TTGGAGGTAT CTGTTTGGCT CTACCTTAAG CAAGGTGGTA AATAGGGTGA AAGAAGACTA
AA Arg to Lys aa 29
TAG
Stop 19K ORF in cyt 68
1750
TAGAGAGGAA TTTGAAAACA TATTGGCCGA CTGTCCAGGG CTTTTGGCTT CACTAGACCT TTGTTACCAC
T
A Ala to Thr aa 56
destroys Hae III site
C
Tyr to His aa 56
1820
TTGGTGTTTC AGGAAAAAGT GGTCAGATCC TTAGATTTTT CATCTGTGGG ACGAACGGTT GCTTCTATTG
Start 55K
1890
CTTTTTTGGC AACCATATTG GATAA ATG GA GCGAGAAATC CCACCTGAGT TGGGATTACA TGCTGGATTA
1960
CATGTCAATG CAGCTGTGGA GGGCATGGCT GAAGAGGAGG GTTTGCATTT ACTCGCTGGC GCGGCCTTTG
C Glu to Ala aa 22 of 55K
2030
ACCATGCCGC CGCTGCCGAC GTTGCAAGAG GAGAAGGAGG AGGAGCGGAA CCCTGCGGTG GTGGAGAAGT
Splice site 1 kb mRNAs
Stop 19K
2100
AA ACATGGAA CAACAGGTGC AAGAAGGCCA TGTACTTGAC TCTGGCGAAG GGCCTAGTTG CGCAGATGAT


2170
AGAGATAAGC AGGAAAAAAA AGAAAGTTTA AAGGAAGCTG CTGTTCTTAG TAGGCTAACT GTTAATCTGA
A Asp to Asn aa 87 of 55K
2240
TGTCCCGCCC GCGTTTGGAA ACTGTATATT GGCAGGAGTT GCAGGATGAA TTTCAGCGGG GTGATATGCA
T
A
2310
TTTACAGTAC AAATACAGTT TTGAACAATT AAAAACCCAC TGGTTAGAGC CATGGGAGGA TATGGAGTGT
G
2380
GCTATTAAAG CTTTTGCTAA ATTGGCCTTA CGTCCTGATT GTAGCTACAG AATTACTAAA ACAGTAACCA
2450
TTACTTCATG CGCCTATATT ATAGGTAACG GGGCAATAGT TGAGGTAGAT ACAAGCGACA GAGTTGCTTT
T
2520
TAGATGTCGA ATGCAGGGTA TGGGCCCAGG GGTGGTGGGT TTGGATGGAA TTACATTTAT AAATGTTAGG
2590
TTTGCTGGAG ATAAGTTTAA AGGCATTATG TTCGAAGCTA ATACCTGTCT TGTCTTGCAT GGTGTTTACT
2660
TTCTTAACTT TAGTAACATT TGTGTAGAGT CTTGGAATAA GGTTTCTGCT AGGGGCTGTA CTTTTTATGG
2730
ATGTTGGAAG GGTTTGGTGG GTAGACCAAA AAGTAAACTG TCTGTAAAAA AGTGTTTGTT TGAAAAATGT
2800
GTACTTGCTT TAATTGTAGA GGGGGATGCA CATATTAGGC ATAATGCAGC TTCAGAAAAT GCCTGTTTTG
G destroys Rsa I site
A Asp to Asn aa 304
2870
TATTATTGAA GGGAATGGCT ATTTTAAAGC ATAATATGGT TTGTGGGGTG TCTGATCAAA CTATGCGACG
2940
TTTTGTTACC TGTGCTGATG GAAATTGTCA TACCTTAAAA ACTGTTCATA TTGTGAGCCA CAGTAGACAT
3010
TGTTGGCCTG TATGTGATCA TAACATGTTT ATGCGCTGTA CCATACATTT AGGCTTAAGG CGGGGTATGT
3080
TTAGACCTTC CCAATGTAAC TTCAGCCACT CAAACATTAT GCTGGAACCT GAAGTGTTTT CTAGAGTGTG
3150
TTTAAATGGG GTATTTGATT TATCTGTGGA ATTATGTAAG GTTATAAGAT ATAATGATGA TACTCGACAT
Splice site for 1 kb mRNAs
3220
CGTTGCCGAC AGTGTGAGTG TGGTAGCAGT CATCTAGAAC TTCGTCCCAT TGTGCTAAAT GTAACTGAGG
C
3290
AGCTGAGAAG TGACCACCTT ACCCTGTCTT GCCTGCGGAC TGACTATGAG TCAAGTGATG AAGACGACAA
Stop 55K
Common splice site
3360
CTGAGGTAAG TGGGTGGAGC TAGGTGGGAT TATAAAAGGC TGGAAGTCAA CTAAAAATTG TTTTTGTTCT
T

The diagnostic insert fragments were 2317 and 1179 bp in length (see figure 3-8B and C). Recombinants were purified by streaking, and single stranded preparations of DNA produced.

The sequence of both strands of most of the E1 region of cyt 68 was determined by the dideoxy nucleotide procedure using the primers diagrammed in figure 2-3. The end of the cyt 68 fragment at the Bam HI site differed from the wild type end, in that it began at nucleotide 43, whereas the wild type end began at nucleotide 1. This presumably was a consequence of the strategy applied to clone the terminal fragment of the viral genome, which involved the resection of the end to aid in removal of the terminal protein (Mak et.al., 1986). With the exception of the E1b 19K deletion described above, no difference was seen between cyt 68 and its wild type parent. Therefore the transformation defect observed in cyt 68 was not due to the presence of a second mutation within the transforming region of the virus.

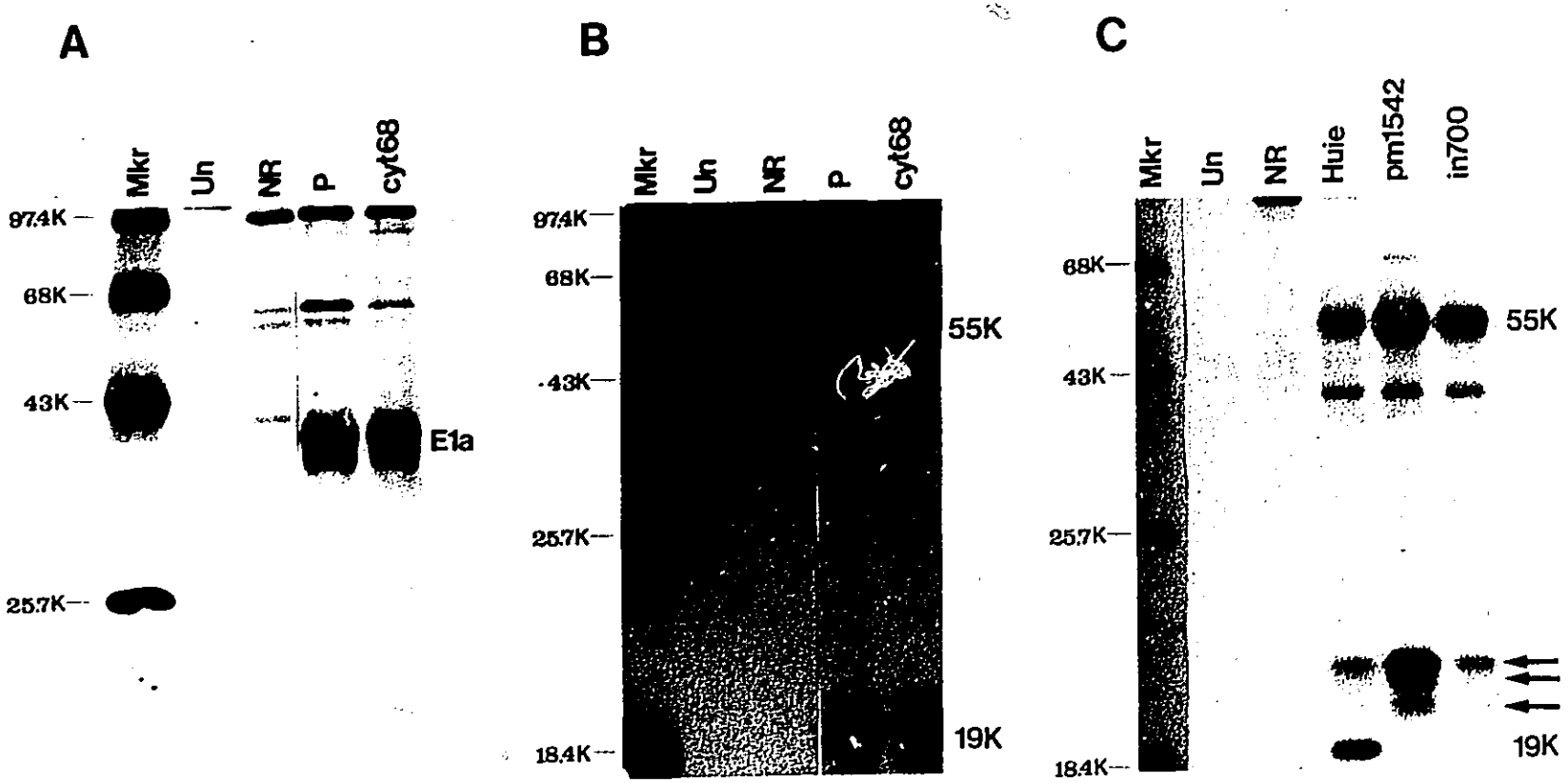
F. Expression of the E1 Proteins in cyt 68 Infected Cells

The failure of the nucleotide sequencing experiments to identify a second mutation in E1 may be interpreted to mean that cyt 68 contains a second defect outside the E1

region, that nevertheless causes a reduction in transformation by the virus. However, the discrepancy between the transformation results using cyt 68 and pm700, pm1542, and in700 could be explained if these 19K mutations had differential secondary effects upon the expression of the other E1 transforming proteins. This hypothesis was suggested by the observation of Shi Yun Zhang that elimination of the 19K initiation codon in pm1542 resulted in an elevation in the synthesis of the E1b 55K protein (in preparation). This appears to be the result of removing a competing initiation codon from a site on the E1b transcript 5' to the start codon for 55K, and may compensate for the complete loss of 19K in transformation. For this reason, the production of the E1a proteins and E1b 55K in KB cells infected with cyt 68 was measured.

Cells were infected with 400 virus particles per cell and the proteins synthesized at 22 hours post infection were labelled with [^{35}S]methionine for two hours. Cytoplasmic lysates were prepared and the E1 proteins present were immunoprecipitated using anti-tumour sera and analyzed on SDS polyacrylamide gels. The E1b 55K was examined by immunoprecipitation using the AB6a-C3 anti serum, and the synthesis of the E1b 55K was found to be comparable in cyt 68 and wild type infected cells (see figure 3-10B). Similarly, analysis of the synthesis of the

Figure 3-10. E1 protein expression in cyt 68 infected KB cells. Cells were infected with 400 particles per cell and at 22 hours post infection the cells were labelled for 2 hours with [^{35}S]methionine in medium 199 (met$^-$). Cytoplasmic lysates were prepared and the E1 proteins were immunoprecipitated from equal TCA precipitable cpm using the anti-tumour sera A7R-C4 (in A) or AB6a-C3 (in B and C). Included as controls were immunoprecipitations from uninfected KB cells and immunoprecipitations using normal rat serum (NR) instead of the anti-tumour sera. Strain P and cyt 68 were observed to express similar amounts of E1a (A) and E1b 55K (B) proteins. The mutant pm1542 expressed about ten times more 55K than wild type strain Huie, and in700 about twice as much (C). These observations demonstrated that the cyt 68 deletion didn't alter the expression of the other E1 proteins, and that the in700 mutation had only a small effect upon the expression of the E1b 55K. The proteins expressed by strain Huie derived viruses qualitatively differed from those of the strain P derivatives in that they contained three proteins between 19.5K and 20.5K in size (indicated by arrows in C).


A
Mkr
Un
NR
P
cyt68
97.4K
68K
43K
25.7K
E1a
B
Mkr
Un
NR
P
cyt68
97.4K
68K
43K
25.7K
18.4K
55K
19K
C
Mkr
Un
NR
Huie
pm1542
in700
68K
43K
25.7K
18.4K
55K
19K

E1a proteins with the anti serum A7R-C4 revealed no difference between wild type and cyt 68 (see figure 3-10A). The E1b 19K deletion in cyt 68, therefore had no obvious secondary effect altering the expression of the other proteins of the virus that have been implicated in transformation.

As described by Zhang (in preparation), pm1542 expressed considerably more E1b 55K than wild type virus, an estimated 10 times more (by densitometry) (see figure 3-10C). However, in 700, which synthesizes no 19K but exhibits wild type transforming activity (Edbauer et.al., 1988), expressed levels of 55K that were much closer to wild type levels and were estimated densitometrically to be only 2 to 3 times more than wild type (see figure 3-10C). Therefore the transformation competence of the 19K null mutants cannot be correlated with a large increase in the expression of the E1b 55K.

There was one qualitative difference between the proteins precipitated from different lysates by AB6a-C3 in these experiments. The mutants pm1542 and in700, as well as wild type Huie, produced two to three proteins of about 19.5K to 20.5K in size (indicated by arrows in figure 3-10C) which were absent in cyt 68 and wild type P infected cells (see figure 3-10B). The cyt 68 immunoprecipitate contained a heterogeneous smear of material in the region of migration

of the E1b 19K. In one experiment this material was resolved into two bands approximately 19K and 18.5K in size (The E1b 19K protein from strain P migrated with an intermediate mobility). These may represent truncated versions of the 20.5K and 19.5K proteins. Closer examination of the E1b 19K in wild type strain P revealed an underlying heterogeneous material similar to, although much less intense than, that in cyt 68 (see figure 3-10B, fourth lane). Thus this qualitative difference may be due to the wild type strain of origin and not specifically pertain to cyt 68. Preliminary experiments revealed that this material was also seen in other strain P derived cyt mutants, most notably the transformation competent cyt 61 (data not shown).

The results of these experiments suggested that the transformation defect of cyt 68 is not due to a secondary effect of the E1b deletion, namely the alteration of the expression of the other transforming proteins.

II. IDENTIFICATION OF AN E1B 19K MUTATION IN cyt 62

Cyt 62 exhibits the cytocidal CPE, DNA degradation and transformation defective phenotypes of most other cyt

mutants but is atypical in that cyt 62 transformed cells are only weakly tumourigenic when injected into syngeneic animals (Mak et.al., 1984). This study was undertaken to identify any mutations within the transforming region of cyt 62 which could be responsible for the observed phenotypes.

Initially, the identification of the defect(s) in cyt 62 was approached using the same strategy applied to map the 19K deletion in cyt 68. However, no differences in restriction patterns could be detected between cyt 62 and wild type DNA. The alternate strategy chosen to localize the cyt 62 defect(s) within the E1 region was to create chimeric viruses, in which portions of the cyt 62 genome were substituted for sequences in the wild type genome. The phenotypes of the chimerae were examined to determine which fragment of cyt 62 conferred mutant characteristics onto the chimeric viruses. The construction of the chimerae was accomplished by manipulating cloned viral DNA fragments to create chimeric E1 regions cloned into pBR322, which were then rescued into virus.

A. Construction of Wild Type/cyt 62 Chimeric Viruses

The E1 region was subdivided into three pieces using the Acc I site at nucleotide 1594 and the Hind III site at nucleotide 2317. The left 1594 bp fragment contained the

entire E1a region, the E1b promoter, and the first 17 codons of the E1b 19K protein. The fragment from nucleotide 1594 to 2317 contained the remainder of the coding sequences for 19K and the first 154 codons of the E1b 55K protein, while the carboxy terminal 300 amino acids of 55K was encoded in the fragment extending from nucleotide 2317 onwards (see figure 4-1).

Three recombinant plasmids containing the left 2317 bp of viral DNA were constructed. The first, pP67, contained wild type strain P DNA and was generated by inserting the terminal 2317 bp Bam HI-Hind III fragment from pPAB7R between the Bam HI and Hind III sites of pBR322 (see figure 4-2a). The other two contained chimeric wild type P/cyt 62 DNA and the strategy used to generate pCAPB, containing the left 1594 bp of cyt 62, is diagrammed in figure 4-3A. The left 2.3 kbp Bam HI-Hind III fragments of pPAB7R and pC6225 were isolated following agarose gel electrophoresis, then cleaved in two using Acc I which cut at nucleotide 1594. The two mutant fragments were separated by agarose gel electrophoresis, then isolated as were the two wild type fragments. The left 1594 bp fragment from cyt 62 was mixed and ligated with the fragment containing nucleotides 1594 to 2317 from wild type, and pBR322 which had been cleaved with Bam HI and Hind III. The complementary construct, pPACB, which contained the 1594 to

Figure 4-1. Strategy for the construction of wild type/cyt 62 recombinants. The E1 region was divided into three using the Acc I site at nucleotide 1594 and the Hind III site at nucleotide 2317. Wild type/cyt 62 recombinants were created by substituting various cyt 62 fragments for the equivalent wild type sequences. The predicted structures of the recombinants are diagrammed, the open boxes representing wild type sequences and the solid boxes cyt 62 sequences. In plasmid form the entire recombinant fragments shown were inserted between the Bam HI and Eco RI sites of pBR322. The chimeric viruses generated contained the recombinant sequences shown to the left of the Bst EII site at nucleotide 3443 fused to the remainder of the wild type genome.

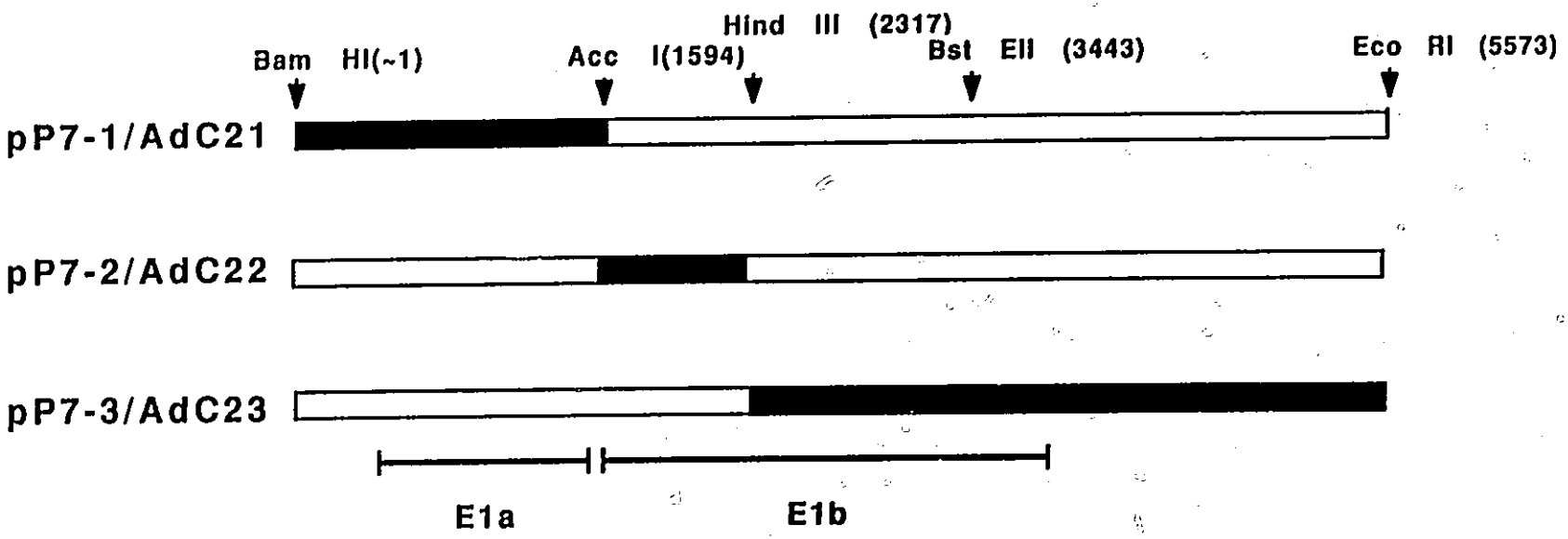

Bam HI(~1)
Acc I(1594)
Hind III (2317)
Bst EII (3443)
Eco RI (5573)
pP7-1/AdC21
pP7-2/AdC22
pP7-3/AdC23
E1a
E1b

Figure 4-2. Construction of the recombinant plasmid pP67. A. The strategy used to construct pP67 is shown. The sites for cleavage with Hind III (H), Bam HI (B) and Kpn I (K) and their nucleotide position on the viral genome are indicated. The fragment between nucleotides -1 and 2317 from pPAB7R was isolated from an agarose gel and inserted between the Bam HI and Hind III sites of pBR322 and the DNA used to transform LE392. **B.** Restriction enzyme analysis of pP67 using Bam HI and Hind III. Plasmid DNA was extracted from E. coli, digested and run on a 1% agarose gel. The size marker was Hind III digested Ad12 DNA. As predicted two fragments were produced, the 4 kbp vector fragment and a 2.4 kbp insert fragment. Identical results were obtained when plasmids pCAPB and pPACB were similarly analyzed. **C.** Restriction enzyme analysis of pP67 using Kpn I and Hind III. Plasmid DNA was digested and run on a 1% agarose gel. The size marker was Hind III digested Ad12 DNA. Two fragments were observed, a 1.7 kbp fragment and a 4.6 kbp fragment, which are the sizes predicted for a plasmid of the correct structure. Identical results were obtained when plasmids pCAPB and pPACB were similarly analyzed. By these analyses pP67 appeared to be of the expected structure.

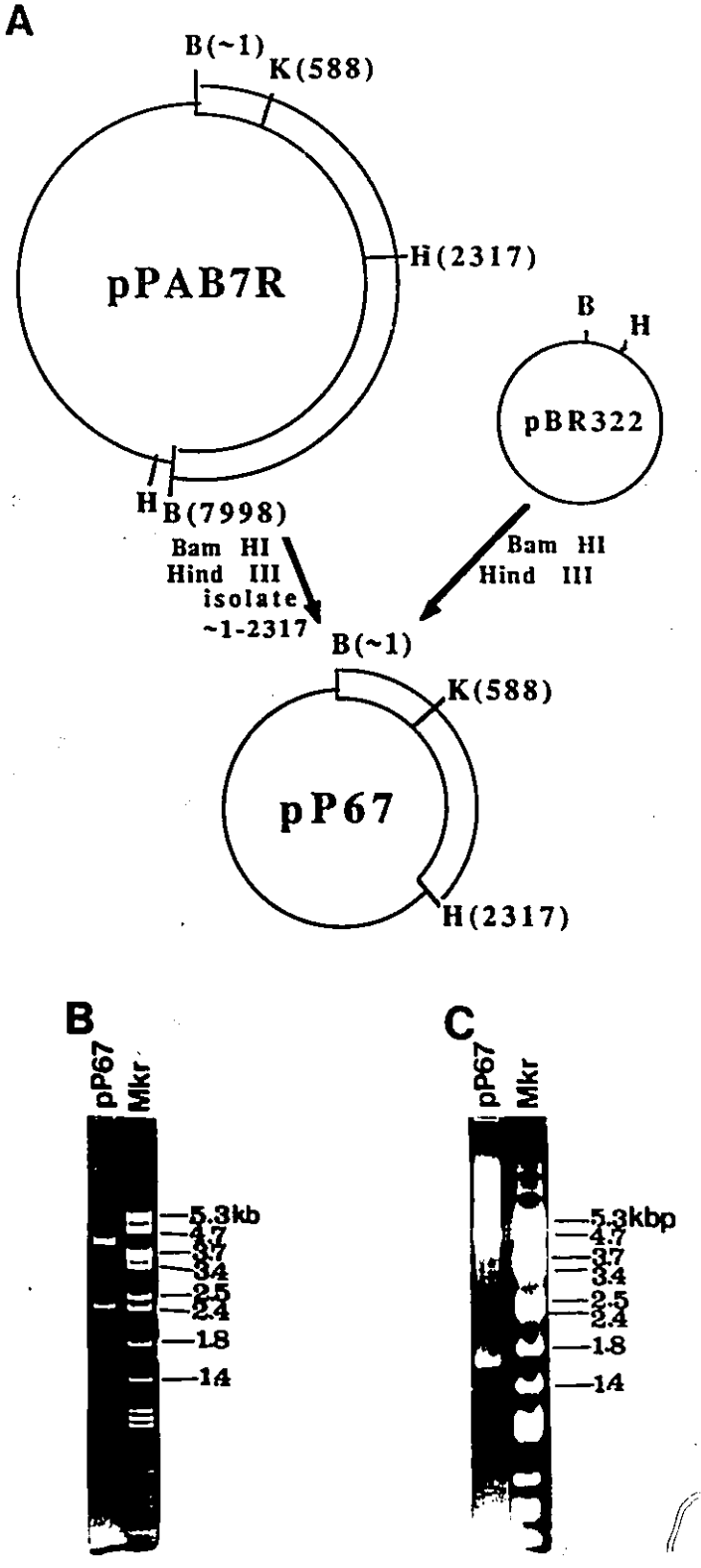
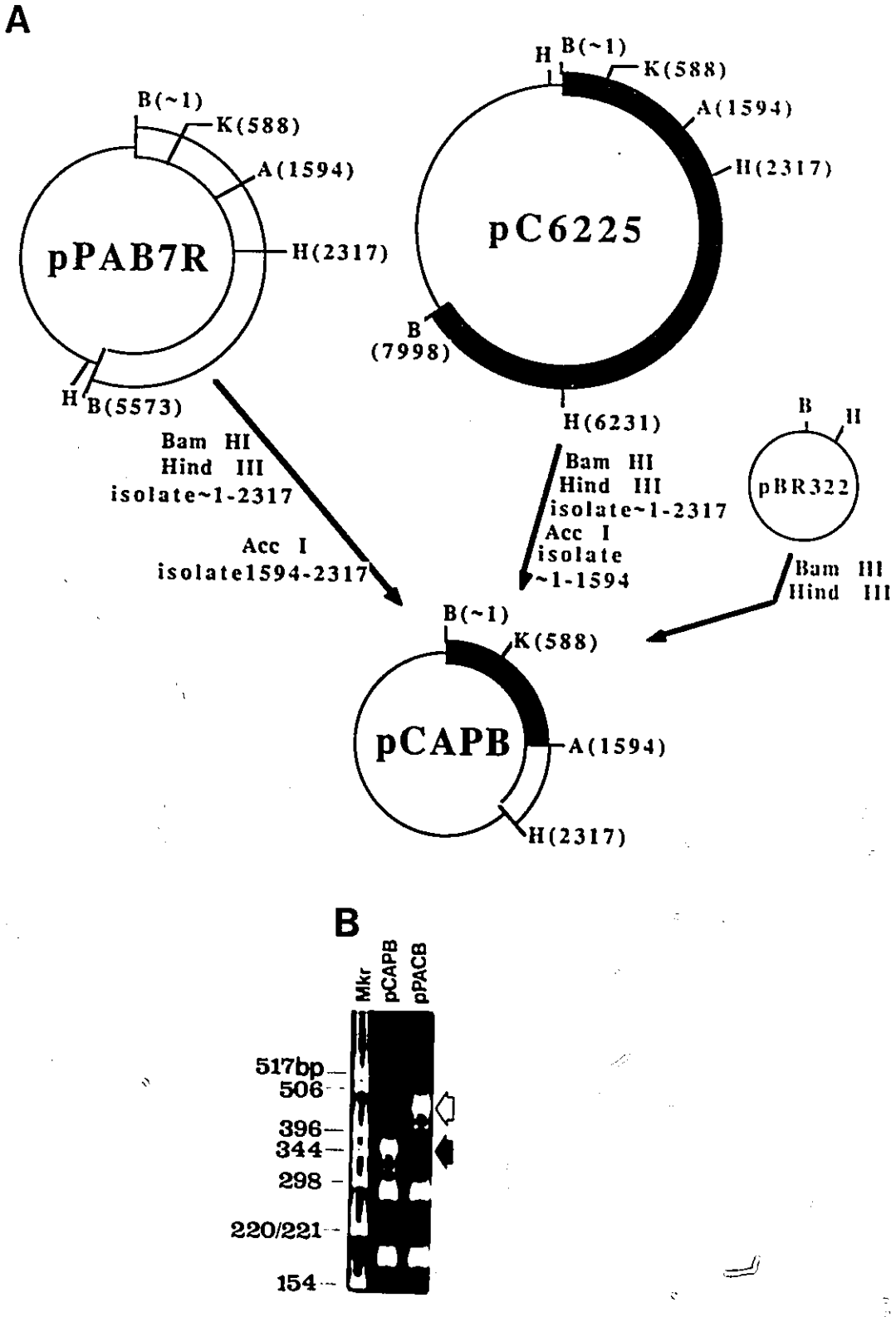

A
B(~1)
K(588)
pPAB7R
H(2317)
H
B(7998)
B
H
pBR322
Bam HI
Hind III
isolate
~1-2317
Bam HI
Hind III
B(~1)
K(588)
pP67
H(2317)
B
pP67
Mkr
5.3kb
4.7
3.7
3.4
2.5
2.4
1.8
1.4
C
pP67
Mkr
5.3kbp
4.7
3.7
3.4
2.5
2.4
1.8
1.4

Figure 4-3. Construction of chimeric wild type P/cyt 62 plasmids. **A.** The strategy used to construct pCAPB is shown. Restriction sites and their nucleotide positions are shown for Hind III (H), Bam HI (B), Acc I (A) and Kpn I (K). The black boxes indicate cyt 62 sequences and the open boxes wild type strain P sequences. The complementary construct pPACB was constructed in a similar fashion and the structure of pCAPB and pPACB confirmed as in figure 4-2B and C. **B.** The nucleotide -1 to 2317 bp fragments of pCAPB and pPACB were isolated from an agarose gel, restricted with Dde I and Acc I, then run on a 5% polyacrylamide gel. The marker was Hinf I cleaved pBR322. Plasmid pCAPB contained a novel fragment 360 bp in size (indicated by solid arrow) and pPACB a unique 445 bp fragment (indicated by open arrow). These fragments are diagnostic for the left terminus of the cloned cyt 62 and P DNA respectively which differ in size, as did the terminal fragments of cloned cyt 68 and P DNA. This verifies the strain of origin of the E1a fragment in each of these plasmids.


A
pPAB7R
B(~1)
K(588)
A(1594)
H(2317)
H
B(5573)
pC6225
H
B(~1)
K(588)
A(1594)
H(2317)
B
(7998)
H(6231)
Bam HI
Hind III
isolate~1-2317
Acc I
isolate1594-2317
Bam HI
Hind III
isolate~1-2317
Acc I
isolate
~1-1594
pBR322
B
H
Bam HI
Hind III
pCAPB
B(~1)
K(588)
A(1594)
H(2317)
B
Mkr
pCAPB
pPACB
517bp
506
396
344
298
220/221
154

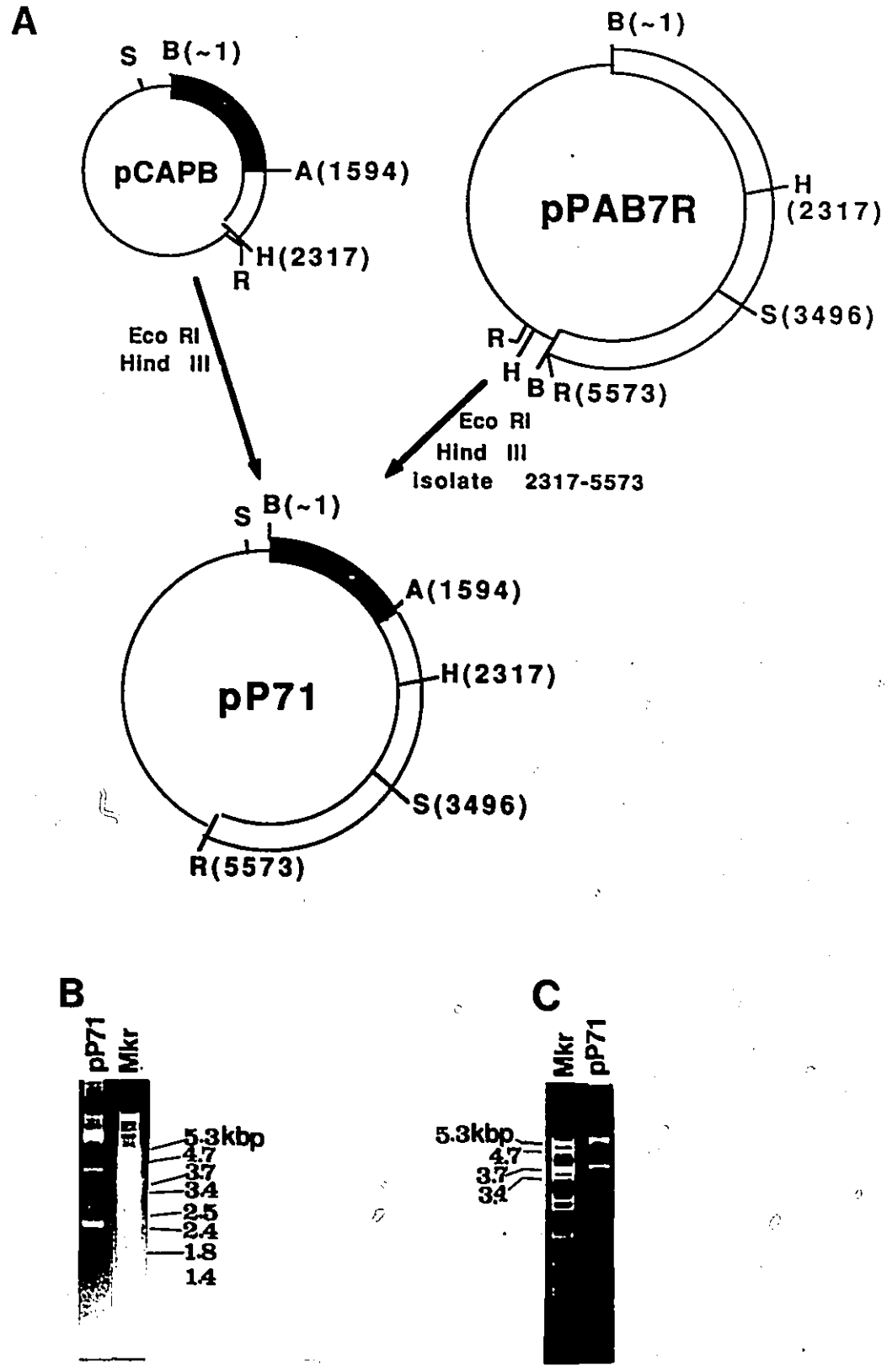
2317 bp fragment from cyt 62, was constructed in a similar manner. The ligated DNA in each case was used to transform LE392 to ampicillin resistance and colonies transformed by recombinant plasmids were identified as tetracycline sensitive. Plasmid DNA was extracted and analyzed by restriction with Hind III and Bam HI. Successful constructs were identified by the presence of the two predicted fragments, the 4 kbp vector fragment and a 2.4 kbp insert fragment (as shown for pP67 in figure 4-2B). The structure of each was confirmed by cleavage with Hind III and Kpn I which generated fragments 4.6 and 1.7 kbp in size, as predicted (shown for pP67 in figure 4-2C). The plasmids pCAPB and pPACB were further analyzed to confirm the origin of the left 1594 bp fragment in each. The left terminal fragment of cloned P and cyt 62 DNA could be distinguished by their different electrophoretic mobility (data not shown). The increased mobility of the cloned cyt 62 DNA was due to a deletion of 83 bp from the left terminus of the viral sequences (see section IIE) which presumably arose during the construction of pC6225 analogous to the loss of 43 bp from the left end of the cloned cyt 68 DNA. The insert from pCAPB and pPACB were excised, isolated from an agarose gel and restricted with Dde I and Acc I. The left terminal Dde I fragment of the insert from pCAPB was about 360 bp in size (indicated by solid arrow in figure 4-3B) and

that of pPACB was approximately 445 bp in size (indicated by open arrow in figure 4-3B), which are the sizes previously observed for the terminal fragments in pC6225 and pPAB7R respectively. These results confirmed that the E1a region in pCAPB was from cyt 62, and the E1a region of pPACB was from strain P.

In the second stage of the construction of the chimeric plasmids, the Hind III-Eco RI fragment containing nucleotides 2317 to 5579 from pPAB7R was isolated from a gel, then inserted between the unique Hind III and Eco RI sites in pPACB and pCAPB (see figure 4-4A). Similarly the equivalent fragment from pC6225 was inserted between the Hind III and Eco RI sites of pP67. Plasmid DNA was extracted from ampicillin resistant LE392 transformants and subjected to restriction enzyme analysis to determine its structure. Cleavage with Bam HI and Hind III yielded the two diagnostic fragments, one large fragment predicted to be 7.3 kbp and a second 2.4 kbp fragment (shown for the pCAPB derivative in figure 4-4B). The structure of the recombinants was confirmed by restriction with Sal I which generated fragments of 5.8 and 3.8 kbp in length (as shown for the pCAPB derivative in figure 4-4C).

Through these manipulations, three chimeric plasmids containing portions of cyt 62 replacing wild type sequences were created: pP7-1, containing the left 1594 nucleotides of

Figure 4-4. Construction of plasmids pP7-1, pP7-2 and pP7-3. A. The strategy used to insert the nucleotide 2317 to 5573 fragment from wild type P into pCAPB is shown. Restriction sites for Eco RI (R), Hind III (H), Acc I (A), Bam HI (B) and Sal I (S) are indicated as well as their viral map positions. The same strategy was applied to insert the nucleotide 2317 to 5573 wild type P fragment into pPACB, to generate plasmid pP7-2 and to insert the 2317 to 5573 bp fragment from pC6225 into pP67 to create the plasmid pP7-3. **B.** Plasmid DNA was digested with Bam HI and Hind III and run on a 1% agarose gel. The marker was Hind III digested Ad12 DNA. Two fragments were observed and as predicted one was a very large vector sized fragment and the other was 2.4 kbp. **C.** Plasmid DNA was digested with Sal I and run on a 1% agarose gel and Hind III digested Ad12 DNA was used as a marker. Two fragments were observed, one 3.8 kbp and the other about 5.8 kbp, which are approximately the sizes predicted. Identical results were obtained from similar analyses of pP7-2 and pP7-3.


A
S
B(~1)
pCAPB
A(1594)
H(2317)
R
Eco RI
Hind III
B(~1)
pPAB7R
H
(2317)
S(3496)
R
H
B
R(5573)
Eco RI
Hind III
Isolate 2317-5573
S
B(~1)
A(1594)
pP71
H(2317)
S(3496)
R(5573)
B
pP71
Mkr
5.3kbp
4.7
3.7
3.4
2.5
2.4
1.8
1.4
C
Mkr
pP71
5.3kbp
4.7
3.7
3.4

cyt 62, pP7-2, containing nucleotides 1594 to 2317 of cyt 62, and pP7-3, containing nucleotides 2317 to 5579 of cyt 62 (see figure 4-1).

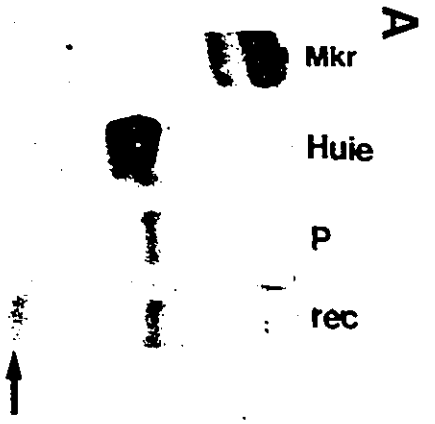
The method chosen to rescue these recombinants into virus was direct ligation of the cloned E1 regions to the remainder of the wild type genome (Stow, 1981) (see figure 2-2). Some of the polymorphisms between strains Huie and P that were described in section I were utilized to identify successfully rescued constructs. Mutant cyt 62 is derived from strain P, and the wild type sequences used to create the chimeric E1 plasmids were also strain P in origin. By using wild type Huie genomic DNA in the rescue experiments, rescued constructs could be identified as viruses containing strain P E1 regions and strain Huie sequences throughout the rest of the genome.

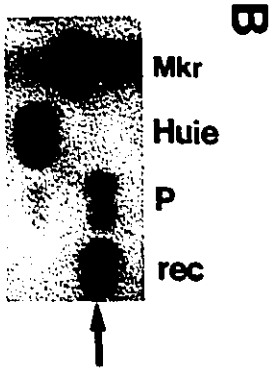
The plasmids pP7-1, pP7-2 and pP7-3 were cleaved with Bam HI and Bst EII, to liberate the left 3443 nucleotide (9.95 mu) fragment. Strain Huie genomic DNA was restricted with Bst EII which split it into two fragments, one 0 to 9.95 mu, and the other 9.95 to 100 mu. The genomic DNA was then treated with alkaline phosphatase to remove the phosphate from the 5′ ends generated by Bst EII cleavage, the 5′ ends of the genome being blocked by the remnants of the terminal protein, thereby rendering the original E1 fragment unligatable to the large genomic fragment. The

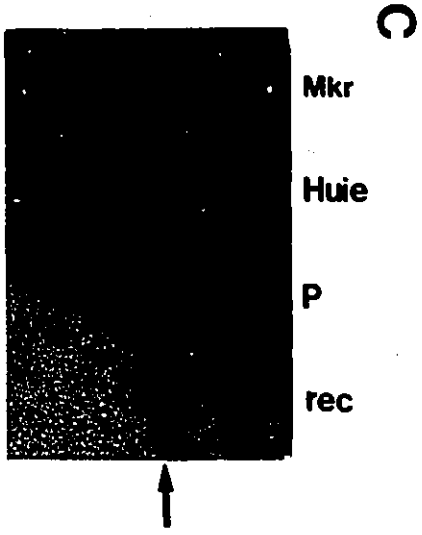
plasmid DNA was mixed with and ligated to the viral DNA, then introduced into MH12-C2 cells by transfection. Transfected adenovirus DNA is infectious and can therefore produce virus (Graham and van der Eb, 1973a). However, the successful ligation of the plasmid E1 sequences to the large genomic fragment was a prerequisite for the generation of virus, since sequences near the left terminus must be intact for viral DNA to be infectious (Stow, 1982). Plaques were picked and the virus from each grown on MH12-C2 cells. DNA was extracted from infected cells by the procedure outlined by Hirt (1967) (also in White et. al., 1984a) and the structure of the viral DNA determined by restriction enzyme analysis.

Initially, each virus was screened for the presence of the novel strain P Dde I site at nucleotide 1604. The fragment diagnostic for strain P was 173 bp in length, whereas its Huie counterpart was 245 bp long. DNA was digested with Dde I and separated on a polyacrylamide gel. The double stranded DNA was then electrophoretically transferred to Zeta-Probe filters and fragments from the E1 region detected by hybridization to radiolabelled pHAB8R (containing the left 2317 nucleotides of Ad12). The restriction pattern of a pP7-1 derived virus is shown as representative in figure 4-5A (the fragment diagnostic for P is indicated by an arrow). The viruses from these plaques

Figure 4-5. Analysis of the structure of the E1 region of recombinant viruses. DNA was extracted from infected cells by the method of Hirt (1967), digested and analyzed. **A.** Dde I digested DNA was resolved on a 5% polyacrylamide gel, electrophoretically transferred to Zeta-Probe and the fragments of interest detected by hybridization to radiolabelled pHB14R (containing nucleotides 1594 to 2317 of Ad12). The recombinant virus AdC21 (rec) contained the P strain diagnostic fragment (indicated by arrow). **B.** Hae III digested DNA was run on a 2% agarose gel, denatured and transferred to nitrocellulose by the method of Southern. The fragment of interest was detected by hybridization to nick translated pHA7 (containing nucleotide -1 to 1594 of Ad12). The AdC22 fragment that was detected comigrated with the strain P diagnostic fragment (compare lanes P and rec). The marker was pUC19 cut with Hinf I and the fragment present was 1400 bp (mkr). **C.** Rsa I digested DNA was analyzed as in A, except the blot was probed with radiolabelled pHB15R (containing nucleotides 2317 to 3705 of Ad12). The recombinant AdC22 (lane rec) contained the fragment that was diagnostic for strain P (compare lanes Huie and P). Identical results were obtained for the analyses of AdC21, AdC22 and AdC23.


A
Mkr
Huie
P
rec


B
Mkr
Huie
P
rec


C
Mkr
Huie
P
rec

were also examined for a second diagnostic strain P polymorphism, the loss of the Hae III site at nucleotide 1706 resulting in the fusion of two strain Huie fragments 1264 and 248 bp in size to generate a fragment 1512 bp in length in strain P. DNA was restricted with Hae III, resolved on a 2% agarose gel, denatured, transferred to nitrocellulose by the method of Southern, and probed with pHAB8R. The analysis of a pP7-2 derived virus is shown as representative in figure 4-5B (the P strain diagnostic fragment is shown by an arrow). In both analyses the viruses shown contained the characteristic P fragment and it was concluded that these viruses contained at least a portion of E1 from strain P.

Seven plaques were screened from the rescue of pP7-3 and five exhibited only the diagnostic P fragment. The two others were apparently a mixture of the successful construct and intact strain Huie, since DNA fragments characteristic for both the Huie and P strains of virus were present. Three of the five plaques screened from the rescue of pP7-2 contained only the P diagnostic fragment. One other was a mixed plaque, and the last appeared to contain only wild type strain Huie. Only two plaques were isolated from two attempts at rescuing pP7-1 and each appeared as a mixed plaque. One of these isolates was plaque purified once and rescreened with Dde I to identify a pure recombinant from

the original mixed plaque. Successfully constructed viruses were called AdC21 (from pP7-1), AdC22 (from pP7-2) and AdC23 (from pP7-3).

Successful constructs were screened for another polymorphism, the loss of the Rsa I site at nucleotide 2731. Strain Huie contains a 247 bp fragment that is converted into a 330 bp fragment in strain P due to the loss of this Rsa I site. DNA was cut with Rsa I, then run on a polyacrylamide gel and electrophoretically transferred to a Zeta Probe membrane and the sequences of interest were detected by probing with radiolabelled pHB15R (containing nucleotides 2317 to 3705 of Ad12). The recombinant AdC21 is shown as representative in figure 4-5C and contained the diagnostic P fragment (indicated by arrow) demonstrating that strain P sequences extended at least as far as nucleotide 2733 in these viruses.

The structure of the rest of the genome of recombinant viruses containing P strain E1 regions was examined by restriction with Hind III, alone or in concert with Bam HI or Xho I, followed by agarose gel electrophoresis. AdC21 is shown as representative in figure 4-6A, AdC22 in 4-6B and AdC23 in 4-6C. It can be seen that the restriction pattern of the recombinants matched the wild type strain Huie pattern exactly. Identical results were obtained when each virus was subjected to each analysis.

Figure 4-6. Analysis of the structure of the genomes of recombinant viruses. DNA was extracted from infected cells by the method of Hirt (1967), then cleaved with Hind III (A), Hind III and Bam HI (B) or Hind III and Xho I (C), and the fragments resolved on 1% agarose gels. In each panel wild type strain Huie DNA is compared to the DNA of a recombinant virus (rec lanes), either AdC21 (A), AdC22 (B) or AdC23 (C). The restriction patterns of each of the recombinant genomes was identical to the patterns produced for the strain Huie genome. Identical results were obtained for each of the three recombinant viruses in each analysis. These analyses demonstrated that the structure of these viruses was the same as strain Huie, outside of the E1 region.

A
Huie
rec
B
Huie
rec
C
Huie
rec

This result confirmed that each virus contained strain Huie sequences throughout the rest of the genome and that there had been no detectable rearrangements of the genomes during their manipulation. There was one exceptional isolate of AdC22 which contained a Hind III fragment which migrated differently than the corresponding fragment in strain Huie (data not shown) and this isolate was discarded.

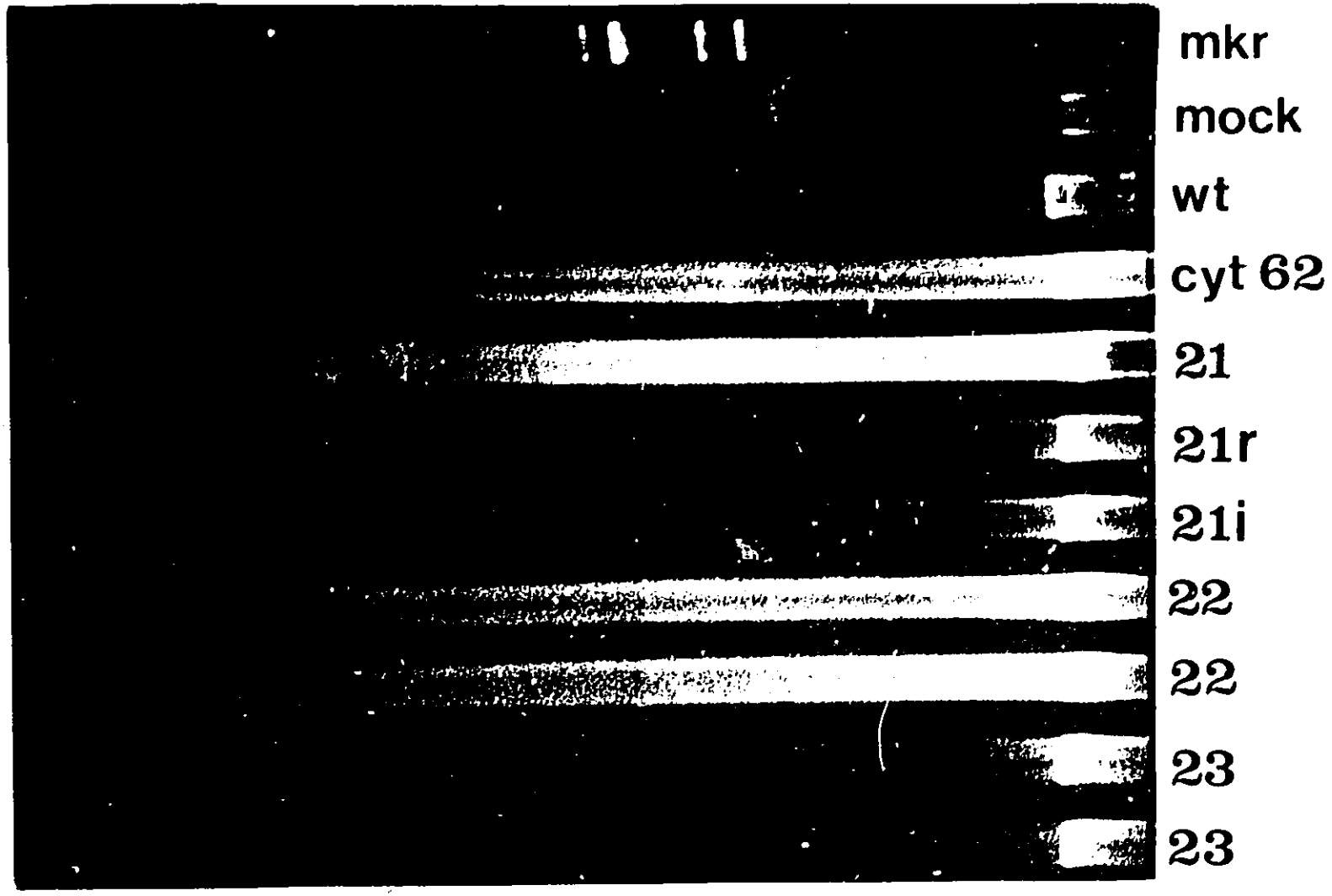
The plaque purified isolate of AdC21 and one isolate each of AdC22 and AdC23, which appeared to be pure by Southern analysis were grown up and purified by banding in CsCl. These stocks of virus were used for some preliminary experiments and as a starting point for plaque purification. AdC21 was plaque purified twice more and AdC22 and AdC23 purified thrice. The structure of the purified virus was determined by restriction enzyme analysis using Dde I, Hae III, Hind III, Hind III and Bam HI, and Hind III and Xho I. In addition, a second isolate of each construct was selected and plaque purified, if it appeared to be a mixed plaque, in order to obtain an apparently pure recombinant virus. These viruses were then plaque purified an additional time and a stock of CsCl banded virus prepared. In the case of AdC21, virus was also reisolated and purified from the original plaque used to grow up the first isolate. To avoid confusion the original isolate of AdC21 will be designated AdC21, the reisolate from the same original plaque AdC21r

and the independent isolate AdC21i. These purifications were monitored by analysis using Hae III and Hind III. All experiments conducted using the original, unplaqued stocks were repeated using the thrice purified stocks and the independent isolates of each construct.

B. AdC22 Induces DNA Degradation in Infected KB Cells

Each of the chimeric viruses was assessed for its ability to induce the degradation of DNA in infected KB cells to determine if any of the cyt 62 DNA fragments could confer the mutant phenotype upon the chimera. Cells were infected at 400 particles per cell and the integrity of intracellular DNA was ascertained between 40 and 48 hours post infection. DNA was extracted by the method of Hirt (1967) (as applied in White et. al., 1984a) and analyzed by agarose gel electrophoresis. The gels were stained with ethidium bromide, then photographed under ultraviolet illumination. As can be seen in figure 4-7, degradation occurred neither in uninfected cells, nor in cells infected with wild type virus. However, in samples from cyt 62 infected cells, there was clearly a long smear of DNA throughout the lane, indicative of DNA degradation. There also appeared to be more DNA in this sample than in the uninfected or wt infected cells which was probably due to

Figure 4-7. DNA degradation analysis. KB cells were infected with 400 particles per cell, and DNA was extracted at 48 hours post infection by the method of Hirt (1967). Equal aliquots were run on a 0.8% agarose gel, and the gel stained with EtBr then observed under UV. The marker was Ad12 DNA cleaved with Hind III. DNA degradation was detected by the presence of a large amount of fragmented viral and cellular DNA which ran as a smear on the gel. In addition to the wild type and cyt 62 infected controls the recombinant viruses AdC21 (21), AdC21r (21r), AdC21i (21i) and two independent isolates each of AdC22 (22) and AdC23 (23) were analyzed. AdC21 and AdC22 degraded DNA, as did the cyt 62 control. AdC21r, AdC21i and AdC23 failed to induce the degradation of DNA. The origin of the band present in the cyt 62 and AdC21 samples, migrating between the two upper bands of the marker, was unclear but it was not adenoviral in origin (see text). These results demonstrated that AdC22, which contained nucleotides 1594 to 2317 of cyt 62, had acquired the DNA degradation phenotype of that virus.


mkr
mock
wt
cyt 62
21
21r
21i
22
22
23
23

the fragmentation of cellular DNA and its subsequent extraction along with the viral DNA.

Both isolates of AdC22 induced DNA degradation upon infection of KB cells (see figure 4-7, lanes 22), whereas neither isolate of AdC23 was capable of causing the degradation of DNA in KB cells (see figure 4-7, lanes 23). The results with AdC21 were somewhat inconclusive. Analysis using the original isolate demonstrated that AdC21 could induce DNA degradation (see figure 4-7, lane 21). However, examination of the second isolate, AdC21i, and the reisolate of the first virus, AdC21r, revealed no evidence for DNA degradation in infected cells (see figure 4-7, lanes 21r and 21i).

The cyt 62 and AdC21 samples also contained a band that migrated with a mobility between that of the first and second DNA fragments of the size marker. The origin of this DNA fragment was unclear but it was apparently not adenoviral in origin since it did not hydridize to radiolabelled Ad12 DNA when similar samples were subjected to Southern analysis (data not shown). Its presence did not correlate with DNA degradation since the band could not be detected in the AdC22 samples, which exhibited DNA degradation (see figure 4-7) and lysates from cells infected with one of the unplaqued stocks of AdC23 also contained this DNA fragment yet did not exhibit DNA degradation (data

not shown).

These results demonstrated that there was a mutation in cyt 62 between nucleotides 1594 and 2317, that could cause the degradation of DNA in infected cells. The most probable explanation for the discrepancy amongst the AdC21 isolates is that the virus that degraded DNA had incurred a mutation during its isolation or growth, since a virus reisolated from the same plaque, and a second, independently isolated virus were both phenotypically normal. Therefore it was unlikely that there was a mutation in the left 1594 nucleotide fragment of cyt 62 that could induce DNA degradation.

C. AdC22 is Transformation Defective

The capability of each chimeric virus to transform primary BRK cells was determined to identify which, if any, of the cyt 62 DNA fragments could render the chimera transformation defective, as is cyt 62. Primary BRK cultures were prepared and within 24 hours the cells were infected with varying amounts of virus. Two days post infection the medium was changed to Joklik's medium containing 5% horse serum to select for the growth of transformed cells. Cells were maintained in this medium, with changes of medium twice weekly, for approximately 24

days after infection, at which time the cells were washed, fixed in Carnoy's solution and stained with Giemsa. Visible foci were then counted.

There was a large variation in the absolute number of foci formed between experiments, but in all cases the number of foci induced by cyt 62 infection was reduced relative to the number obtained with wild type virus. Typically this reduction was of the order of ten fold, which is comparable to values reported in the literature (Mak and Mak, 1983), and the maximal number of foci per dish generated by cyt 62 ranged from one third to one thirtieth the number induced by wild type.

The chimeric virus, AdC22, clearly exhibited a reduced transforming ability when compared to wild type virus (see figure 4-8). In almost all cases the number of foci formed following infection with AdC22 was not significantly different from the number seen with cyt 62, and in the one case where the chimera transformed better than the mutant, it still fell short of wild type transforming levels by a factor of three (for statistical analysis of the transformation results shown in figure 4-8 see appendix I). Although there was some variability in the transforming activity of AdC23, this virus could transform BRK cells as efficiently as wild type (see figure 4-8B). In the instances where it transformed less efficiently (see

Figure 4-8. Transforming activity of the recombinant viruses. Primary BRK cells were prepared and infected with various amounts of virus. After 24 days of selection in Joklik's + 5% horse serum, the foci were fixed, stained and counted. The results plotted in A and B are from different experiments and are statistically analyzed in appendix I. In both experiments AdC22 exhibited a transforming activity similar to cyt 62. Although AdC23 transformed at a reduced efficiency relative to wild type in experiment A, it still had significantly better activity than cyt 62. The first isolate of AdC21 had a transforming activity characteristic of the mutant cyt 62 (A) but the reisolate, AdC21r, and the independent isolate, AdC21i, both exhibited wild type transforming activity. These results demonstrated that AdC22 was defective for transformation, similar to cyt 62, and that AdC23, AdC21i and AdC21r all had approximately wild type levels of transforming activity.

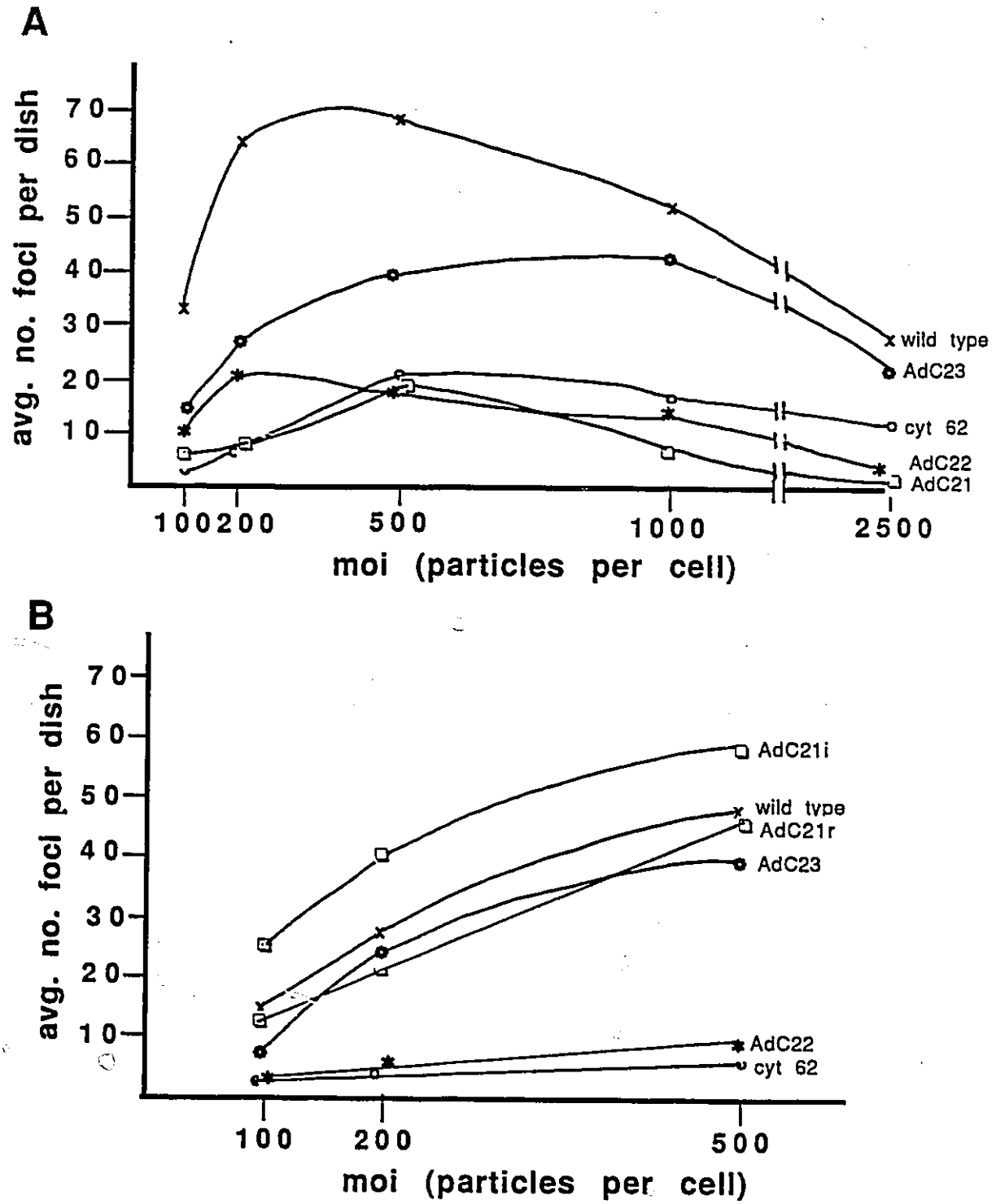

A
avg. no. foci per dish
70
60
50
40
30
20
10
100200
500
1000
2500
moi (particles per cell)
wild type
AdC23
cyt 62
AdC22
AdC21
B
avg. no. foci per dish
70
60
50
40
30
20
10
100
200
500
moi (particles per cell)
AdC21i
wild type
AdC21r
AdC23
AdC22
cyt 62

figure 4-8A), its activity was still significantly higher than that of cyt 62. The results of the transformation experiments using AdC21 were again incongruent. The original isolate of AdC21 transformed like cyt 62 (see figure 4-8A). The subsequent reisolate, AdC21r, and the independent isolate, AdC21i, both transformed substantially better than cyt 62, typically exhibiting wild type levels of transforming activity (see figure 4-8B).

These results showed that a mutation lying between nucleotides 1594 and 2317 of cyt 62 could cause a reduction in the transforming ability of an otherwise wild type virus comparable to the observed reduction in cyt 62. Viruses containing the region from nucleotide 2317 to 3443 of cyt 62 appeared to be nearly wild type in their ability to function in transformation. Although the results using various isolates of AdC21 were in disagreement, the left 1594 bp fragment of cyt 62 was probably wild type in nature with respect to transformation, as argued above (see section IIB).

D. Tumourigenic Potential of pP7-2 Transformed Cells

Perhaps the most interesting phenotype of cyt 62 is its reduced ability to induce tumour formation when either virus, or transformed cells are injected into animals. In

order to localize the lesion responsible for this defect within the cyt 62 E1 region, transformed cell lines were established by transfecting primary BRK cells with pP7-1, pP7-2 and pP7-3. Foci were selected by growth in Joklik's medium containing 5% horse serum, and isolated foci were picked and grown up into cell lines. The oncogenicity of four transformed cell lines established by each of the chimeric plasmids was determined by subcutaneous injection into syngeneic animals. One million cells of each were injected into each weanling rat and the rats observed for the development of visible tumours.

Two cell lines established by transformation with pP7-2, i.e. L72-2 and L72-4, were only weakly oncogenic, forming tumours in one and two of eight animals respectively (see table 4-1). A third line, L72-3, induced tumours in five of eight animals injected. The cell line L72-1 formed visible tumours in five of seven animals injected with a mean latency of 11.5 weeks. The two other animals became moribund at 12.5 and 18.5 weeks, and upon sacrifice and necropsy enormous tumours were found in the chest cavity of each. Although these tumours were not analyzed, they presumably were virally induced tumours that arose in the chest cavity due to injection deeper into the animal than intended. Since this cell line behaved so differently from the other lines established by transformation with pP7-2,

Table 4-1. Tumourigenicity of Cell Lines Transformed By pP7-1, pP7-2 and pP7-3

Cell Line	Fraction of rats with tumours	Latent Period (weeks)
L71-1	6/8	11.5
L71-2	2/7	40.5
L71-3	5/5	10
L71-4	7/8	7
L72-1 #1	5/7*	12.5
#2	2/7	18
L72-2	1/8	18
L72-3	5/8	13
L72-4	2/8	10
L73-1	6/8	11
L73-3	8/8	11
L73-4	8/9	15
L73-5	7/8	6

10^6 cells were injected subcutaneously into weanling rats (3-4 wks.) and they were observed weekly. The latent period is defined as the time required for the appearance of the tumour in 50% of the maximum number of tumour-bearing animals.

* The other two animals became moribund and died exhibiting no obvious tumours. Autopsy revealed tumours in the lungs.

this analysis was repeated, using freshly thawed L72-1. In contrast to the first experiment, only two of seven animals developed tumours over a 7.5 month period, and these arose with a mean latency of 18 weeks. In total, approximately 45% of all animals injected with pP7-2 transformed cell lines developed tumours.

Three of four cell lines transformed with pP7-1 were highly tumourigenic, tumours being induced in at least 75% of the animals injected (see table 4-1). The remaining line, L71-2 was poorly oncogenic, inducing two tumours in seven animals, and the tumours developed with an extremely long latency of about 40.5 weeks. Seventy-two per cent of all animals injected with pP7-1 transformed lines developed tumours.

All four pP7-3 transformed lines were highly tumourigenic (see table 4-1). In each case at least 75% of the animals injected developed tumours, and tumours arose with mean latencies ranging from 6.5 to 15 weeks. Approximately 88% of all animals injected with pP7-3 transformed cell lines developed tumours.

These results were analyzed statistically (see appendix II) and there was no difference in the tumourigenic potential of the pP7-1 and pP7-3 transformed cell lines, nor between the pP7-1 and pP7-2 transformants. However, the pP7-2 transformed cell lines were significantly less

tumourigenic than the pP7-3 transformants. It must be noted that there was a large variation in the tumourigenic potential of individual cell lines, which may impair the accuracy of this analysis.

From these results it appeared that the left 1594 bp of cyt 62 and the fragment from nucleotide 2317 to 3443 functioned normally in imparting tumourigenic potential to transformed cells. The case for the oncogenicity of pP7-2 transformed cell lines was less clear cut. Clearly the construct was capable of conferring tumourigenic potential upon transformants since each cell line exhibited some degree of tumourigenicity. However, the frequency of tumour formation was slightly reduced in pP7-2 transformants. This may reflect the existence of a mutation between nucleotides 1594 and 2317 in cyt 62 that fails to abrogate the ability of the E1 genes to change normal cells into tumourigenic cells, but reduces its potency.

E. Nucleotide Sequence of E1 of cyt 62

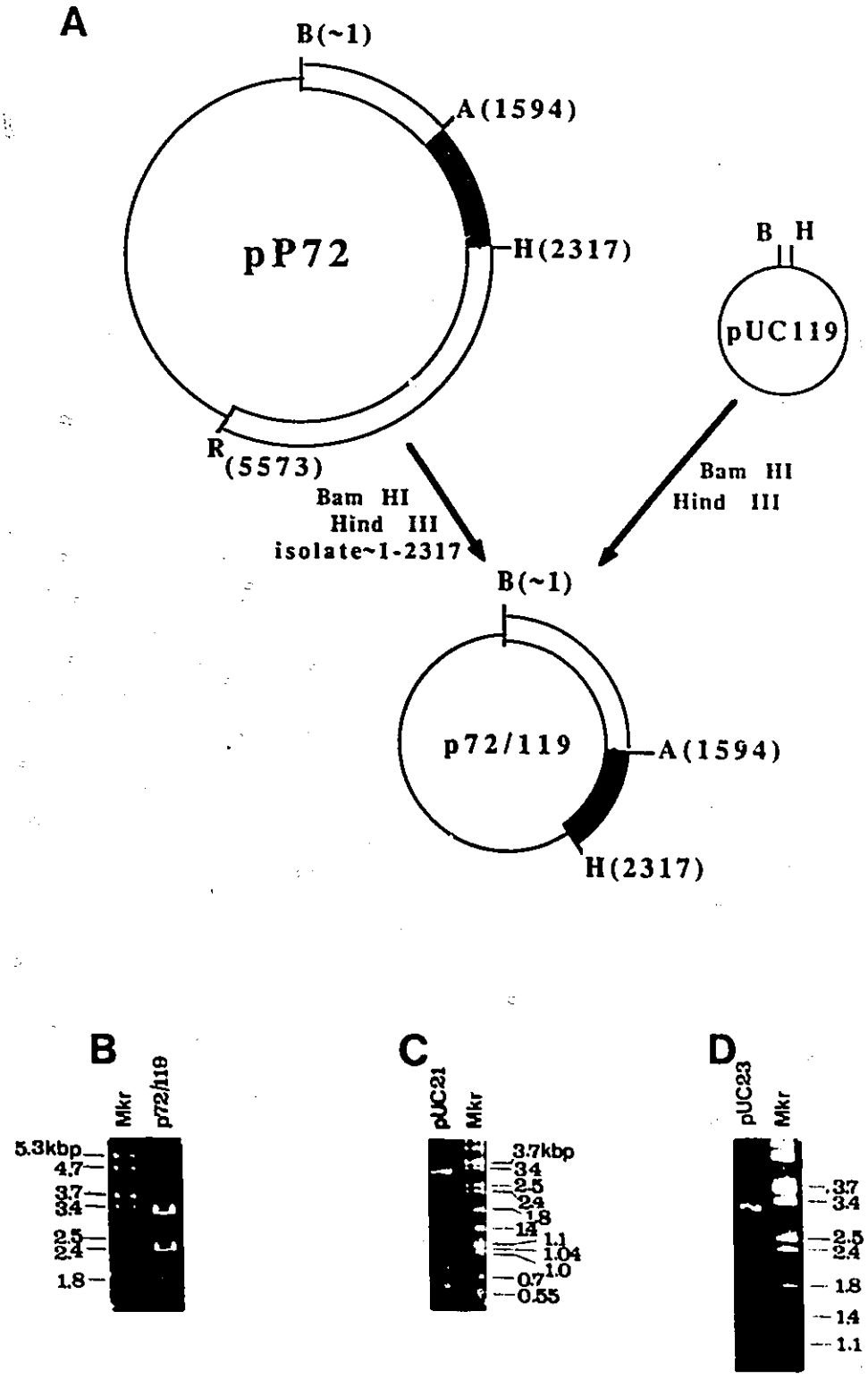
The nucleotide sequence of the E1 region of cyt 62 was determined using the dideoxy termination technique following its subcloning into single stranded vectors suitable for sequencing. Of particular interest was the sequence between nucleotides 1594 and 2317 since this

fragment from cyt 62 could confer the degradation phenotype upon chimeric viruses, and reduce the transformation capability. It also appeared to slightly reduce the oncogenic potential of cell lines transformed by the chimeric E1 plasmids. The nucleotide sequence of the left 1594 bp fragment of cyt 62 was also determined to assess whether it was mutant or wild type in nature, an issue incompletely resolved by analysis of AdC21.

Fragments of cyt 62 DNA were first subcloned into single stranded vectors. The plasmid pC6225 was cleaved with Bam HI and Kpn I to release the 588 bp fragment containing the E1a 5' regulatory sequences and the first 29 codons of E1a. This fragment was isolated from a 3.5% polyacrylamide gel and inserted between the Bam HI and Kpn I sites of pUC118 and pUC119 (as diagrammed in figure 3-7A). Plasmid pC6225 was also digested with Hind III and Sal I to cut out the 2317 to 3496 bp fragment, which was isolated following agarose gel electrophoresis and inserted between the Hind III and Sal I sites of pUC118 and pUC119 (as in figure 3-7A). The left 2317 nucleotide fragment from pC6225 was also inserted into pUC119 using a strategy similar to the one outlined in figure 4-9A. The construct complementary to this clone, that would allow sequencing of the opposite strand, was constructed using pP7-1 and pP7-2. This strategy allowed the verification that any nucleotide

Figure 4-9. Construction of recombinant plasmids for sequencing. **A.** The strategy for the construction of a recombinant plasmid for sequencing the E1 region of cyt 62 is shown. Restriction sites for Eco RI (R), Hind III (H), and Bam HI (B) are shown along with their nucleotide positions on the viral genome. The same strategy was applied to subclone the -1 to 2317 bp fragment of pP7-1 into pUC119 and the equivalent fragment of pC6225 into pUC118. **B.** Plasmid DNA digested with Hind III and Bam HI and the fragments resolved on a 1% agarose gel. The marker was Hind III digested Ad12 DNA. As predicted two fragments were seen, the 3.2 kbp vector fragment and the 2.4 kbp insert fragment. Identical results were seen with p71/119 and p62/118. **C.** Restriction enzyme analysis of pUC2-1. This plasmid, containing the -1 to 588 bp fragment of cyt 62 DNA inserted into pUC118 was constructed using the strategy outlined for the construction of pUP-2 in figure 3-7. Here, plasmid DNA was cleaved with Hind III and Eco RI and run on a 1% agarose gel. As predicted two fragments were seen, the 3.2 kbp vector and the insert, about 0.6 kbp in size. Identical results were obtained for the complementary construct which was built using pUC119. **D.** Restriction enzyme analysis of pUC2-3. Plasmid pUC2-3 was

constructed as diagrammed for pUP-3 in figure 3-7 and contained the nucleotide 2317 to 3496 fragment of cyt 62 inserted into pUC118. Plasmid DNA was analyzed as in C and the two predicted bands, 3.2 and 1.2 kbp in size were seen. Identical results were obtained for a similar construct in which the fragment was inserted into pUC119.


A
B(~1)
A(1594)
pP72
H(2317)
R(5573)
B H
pUC119
Bam HI
Hind III
isolate~1-2317
Bam HI
Hind III
B(~1)
p72/119
A(1594)
H(2317)
B
Mkr
p72/h19
5.3kbp
4.7
3.7
3.4
2.5
2.4
1.8
C
pUC21
Mkr
3.7kbp
3.4
2.5
2.4
1.8
1.4
1.1
1.04
1.0
0.7
0.55
D
pUC23
Mkr
3.7
3.4
2.5
2.4
1.8
1.4
1.1

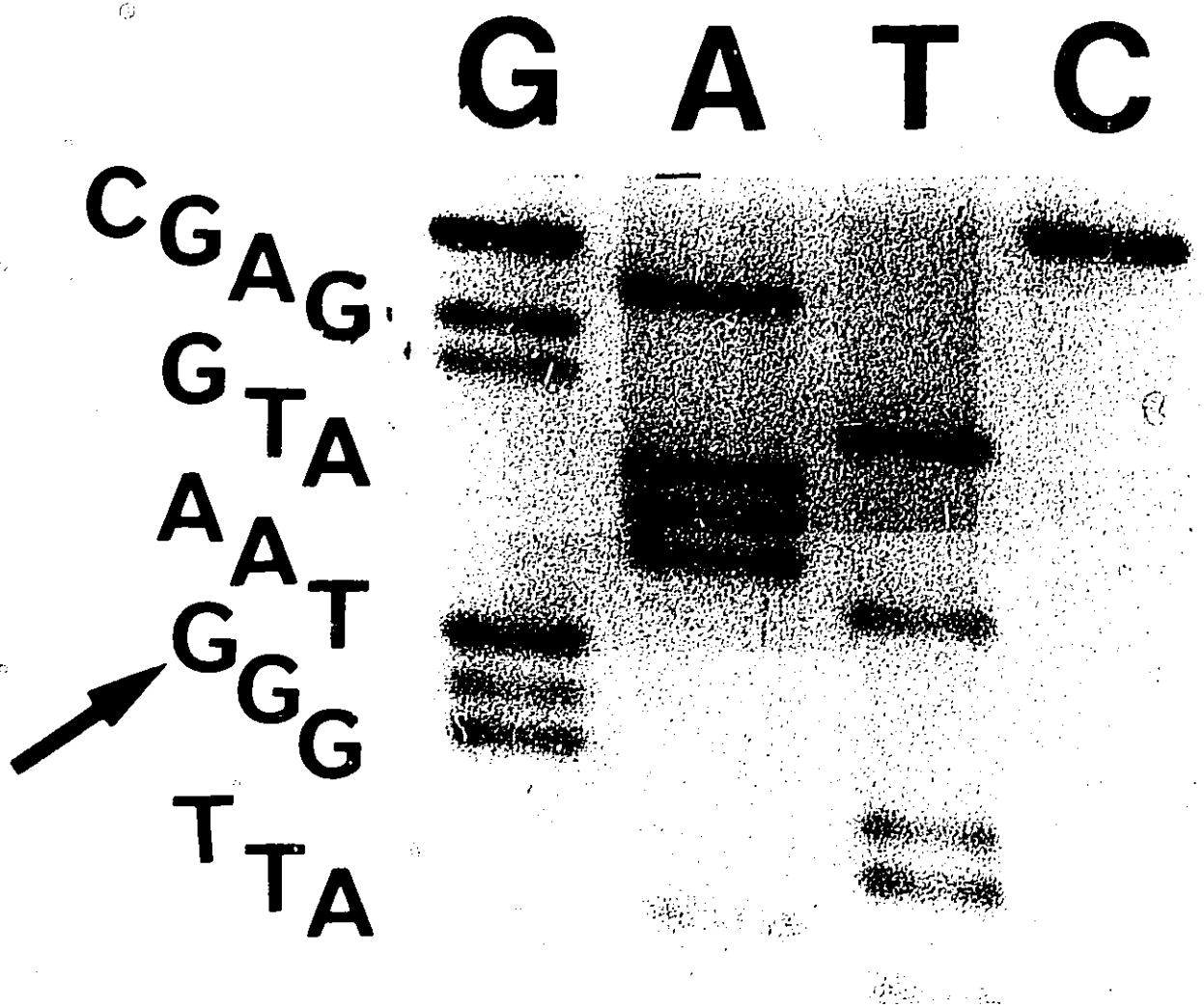
changes seen in cyt 62 were also faithfully delivered into the chimeric plasmids prior to rescue into virus. The left 2317 nucleotide fragments from pP7-1 and pP7-2 were cut from the vector sequences using Bam HI and Hind III, isolated from an agarose gel and then inserted between the Bam HI and Hind III sites of pUC119 (see figure 4-9A). The ligated DNAs were used to transform MV1190 to ampicillin resistance and recombinant plasmids were identified as those producing white colonies in the presence of Xgal due to the insertional inactivation of the portion of the lacZ gene present in the vector. Plasmid DNA was prepared from cells bearing recombinant plasmids, then analyzed by endonuclease digestion using a combination of Hind III and either Eco RI or Bam HI to cleave the vector from the insert. The samples were run on agarose gels and successful constructs were identified by the presence of diagnostic vector and insert fragments. The vector fragment was 3.2 kbp in size, and inserts were, as predicted, about 2.4 kbp (the left 2317 bp fragment), 0.6 kbp (the left 588 bp fragment), and 1.2 kbp (the nucleotide 2317 to 3496 fragment) in size (shown for the constructs p72/119, pUC2-1 and pUC2-3 in figure 4-9B to D). Constructs of the correct structure were purified by streaking, rescreened using the same strategy as above, then used to prepare single stranded template for sequencing.

Using the primers diagrammed in figure 2-3, the

nucleotide sequence of both strands of the E1 region of cyt 62 was determined. The only significant difference between the mutant sequence and wild type (strain P) was a single point mutation, the substitution of a G for A at nucleotide 1842 (5.3 m.u.) (see figure 4-10). This results in the substitution of a glycine for the aspartic acid at amino acid 101 of the wild type E1b 19K protein. This mutation lies outside of the E1b 55K open reading frame. The particular clone of cyt 62 that was sequenced also contained a deletion of 82 bp from the extreme left end of the viral DNA and an insertion of 8 bp between the vector and viral sequences. The deletion probably occurred during the construction of the parent plasmid which involved the resection of the 3′ ends of the viral genome, then digestion with a single strand specific nuclease to remove the terminal protein covalently attached to the 5′ end of each strand. This terminal deletion was inconsequential since restriction analysis revealed that the terminus was intact in cyt 62 genomic DNA, and that these sequences were restored upon the rescue of AdC21 into virus (data not shown).

It was concluded that the single mutation at position 1842 was apparently sufficient to cause the DNA degradation phenotype, a reduction in the efficiency of transformation by virus, and a slight decline in the

Figure 4-10. Autoradiogram of nucleotide sequence of cyt 62. The G designated by the arrow was an A in the wild type sequence. This substitution was at nucleotide 1842 and resulted in the substitution of a glycine for aspartic acid at amino acid 101 of the 19K protein.


G A T C
CGAG
GTA
AAT
GGG
TTA

tumourigenic potential of the E1 region as witnessed by the analysis of AdC22 and pP7-2.

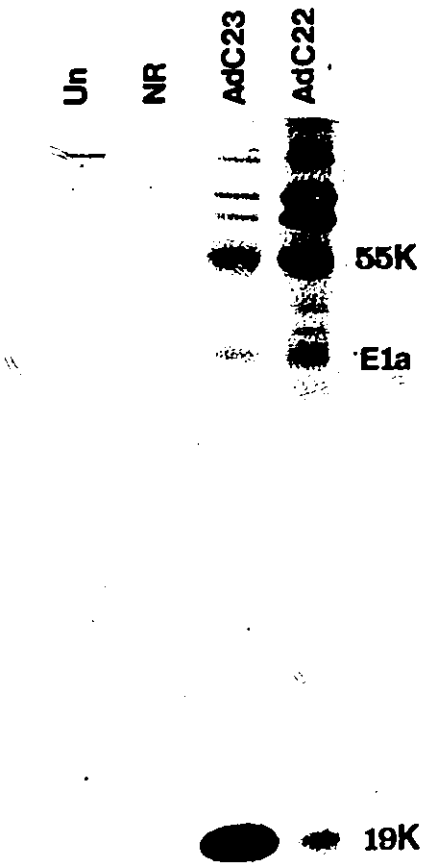
F. AdC22 Encodes an Unstable E1b 19K Protein

The synthesis of the mutant protein was examined in infected KB cells to evaluate the effect of the substitution upon the expression of the protein. Cells were infected with AdC23 or AdC22 at 400 particles per cell, and were incubated with [^{35}S]methionine in 199 met$^-$ medium for 2 hours at 22 hours post infection to label proteins synthesized during that time. Cytoplasmic lysates were prepared and the E1 proteins were immunoprecipitated from equivalent TCA precipitable cpm of each supernatant using various anti-tumour sera. The immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis and the radiolabelled proteins visualized by fluorography. The E1b 19K protein was readily detectable in lysates from AdC23 infected cells, but was drastically reduced in the AdC22 samples (see figure 4-11a). The synthesis of the E1b 55K and E1a proteins was not diminished in cells infected with AdC22.

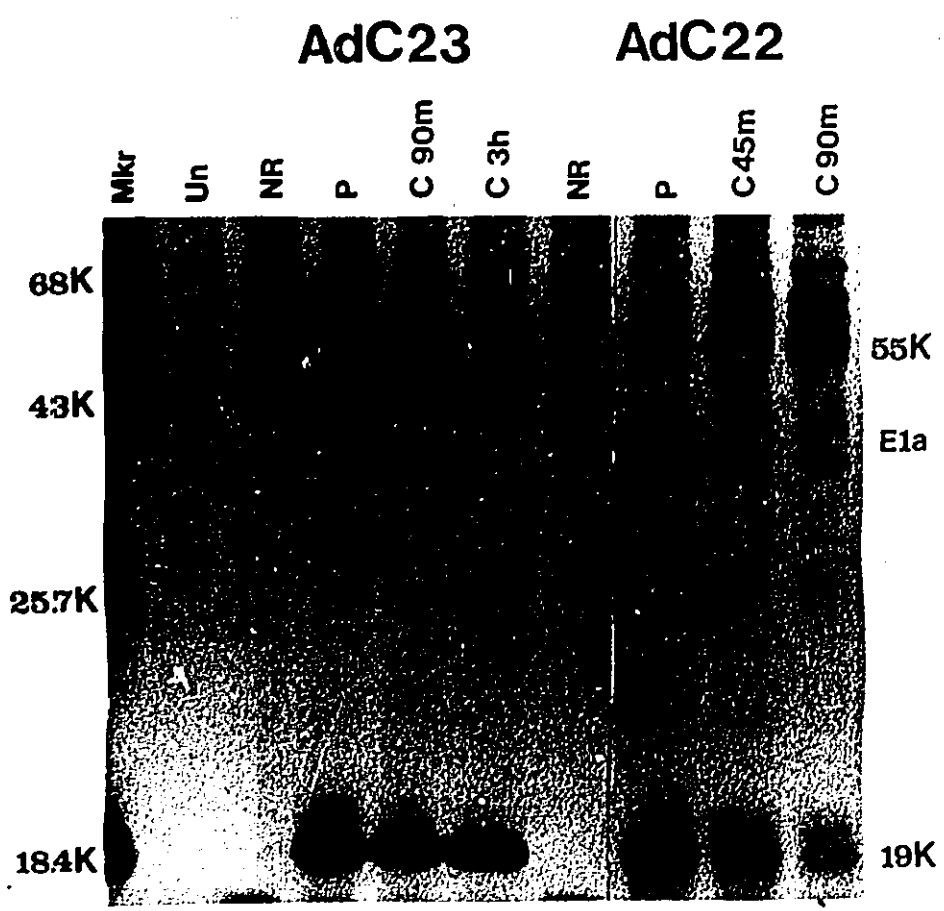
The most plausible explanation for the reduced expression of E1b 19K in AdC22 infected cells was the destabilization of the mutant protein due to the substitution at residue 101. This hypothesis was tested by

Figure 4-11. E1 protein synthesis in AdC22 infected KB cells. A. Cells were infected with 400 particles of AdC23 or AdC22 per cell and labelled with [^{35}S]methionine in medium 199 (met$^-$) for 2 hours at 22 hours post infection. Cytoplasmic lysates were prepared and the E1 proteins immunoprecipitated with the anti-tumour serum AB6a-C3. Control precipitations were done on uninfected lysates (Un) and on infected lysates with normal rat serum (NR). AdC22 synthesized amounts of the 55K and E1a proteins comparable to AdC23 but produced very little of the E1b 19K protein. **B.** Cells were infected as in A, then incubated for 30 minutes in medium 199 (met$^-$) at 22 hours post infection, then labelled with [^{35}S]methionine for one hour. Samples were harvested at this time (lanes P), or washed and incubated in medium 199 (met$^-$) supplemented with L-methionine for 45 minutes (45m), 90 minutes (90m) or 3 hours (3h) prior to harvest. Cytoplasmic lysates were prepared and the E1 proteins immunoprecipitated with the anti serum dl55-1. In contrast to the E1b 19K protein in AdC23 infected cells, the AdC22 encoded 19K was unstable since the amount of the protein immunoprecipitated was diminished after 45 minute and 90 minute chase periods.

A


Un
NR
AdC23
AdC22
55K
E1a
19K

B


AdC23
AdC22
Mkr
Un
NR
P
C 90m
C 3h
NR
P
C45m
C 90m
68K
43K
25.7K
18.4K
55K
E1a
19K

pulse labelling infected cells with [^{35}S]methionine in 199 met$^-$ medium for 1 hour at 22 hours post infection, then chasing for various lengths of time in medium 199 (met$^-$) supplemented wth L-methionine. Cytoplasmic lysates were prepared and equivalent TCA precipitable counts from each lysate were immunoprecipitated with the dl55-1 antiserum. Proteins were resolved by SDS polyacrylamide gel electrophoresis. After the one hour pulse, the E1b 19K could be immunoprecipitated from AdC23 infected cell lysates (see figure 4-11B, AdC23 lane P) and after a 3 hour chase the intensity of the 19K band was undiminished (lane C3h). The amount of 19K immunoprecipitated from AdC22 infected cells was readily detectable following the one hour pulse (see figure 4-11b, AdC22 lane P) but in contrast to the protein from AdC23 infected cells, the 19K immunoprecipitated from AdC22 infected cells rapidly diminished during 45 minute and 90 minute chase periods (lanes C45m and C90m). The half life of the mutant protein was less than 45 minutes whereas the half life of the wild type protein exceeded 3 hours.

Therefore the point mutation in the E1b 19K of AdC22 destabilized the protein and the observed phenotypes may be a consequence of the mutation itself, or of the reduced amount of the protein present.

III. CONSTRUCTION AND ANALYSIS OF AN E1B 55K DELETION MUTANT

The E1b 55K protein, which is the other characterized protein of the E1b region of Ad12 and its counterpart in Ad2 and Ad5, 58K, have been documented to fulfill a number of functions in lytically infected cells and in transformation. It seems possible, in a multi-functional protein the size of the E1b 55K, that different functions might be localized in discrete regions or domains within the protein. However, there is very little evidence relating the different functions of the protein to its primary structure. This analysis was undertaken to determine the importance of the carboxyl region of the protein in a number of functions ascribed to the 55K protein.

The approach used was to create a small deletion in the gene and then evaluate the effect of deleting a portion of the molecule upon the function of the protein. A survey of the nucleotide sequence of the E1b region of Adenovirus type 12 revealed several pairs of restriction sites which could be utilized to create a series of nested, in-frame deletions within the coding sequences of the 55K if the intervening DNA was deleted. The smallest deletion in the series was created, since a large carboxyl deletion in 55K

had already been shown to completely impair the functions of 55K (Shiroki et. al., 1986).

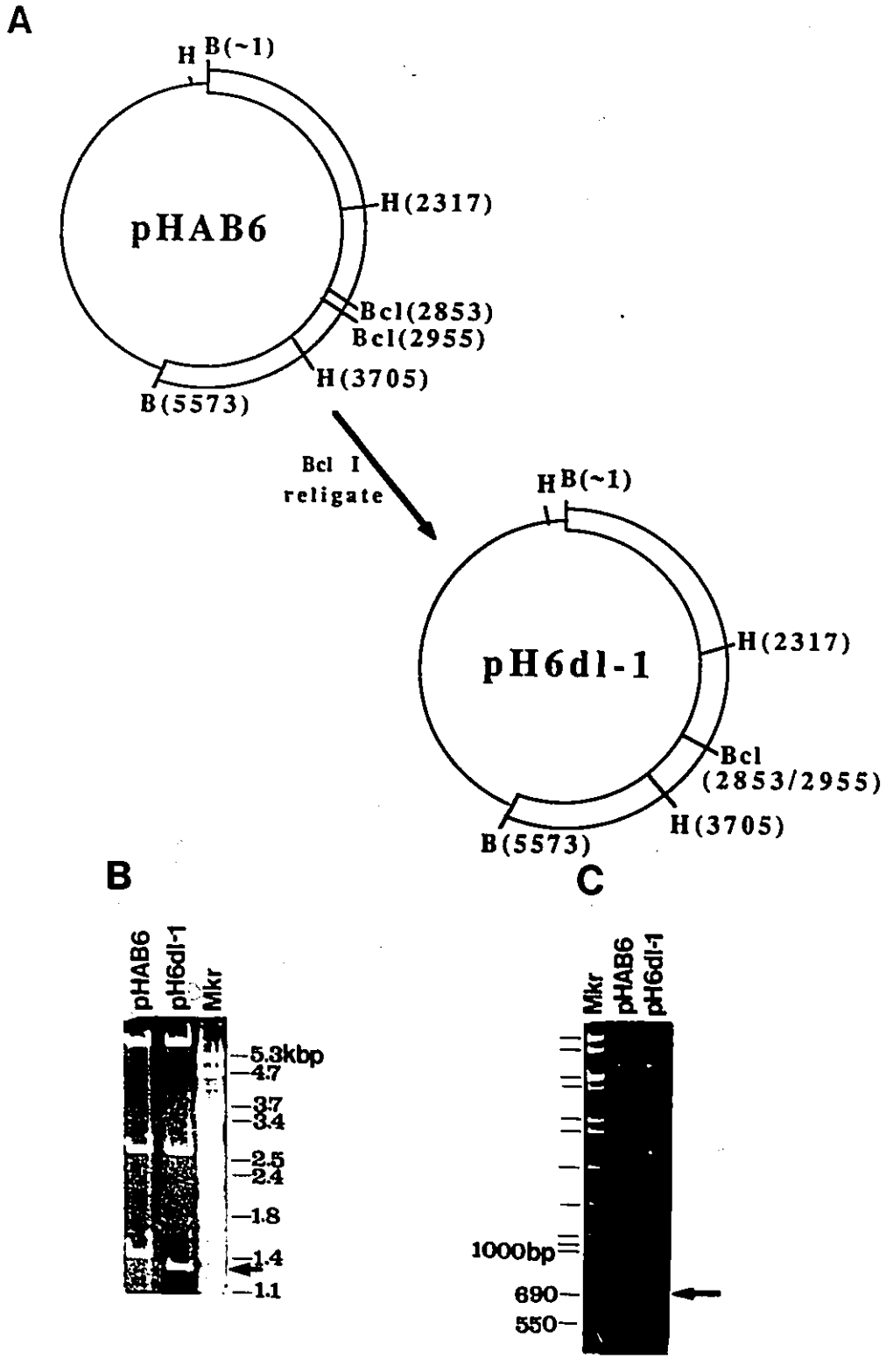
A. Deletion of 102 bp of the Coding Sequences of 55K

A 102 base pair deletion was engineered in the E1b region by manipulation of the recombinant plasmid pHAB6, which contains the left 5573 bp of Ad12 strain Huie inserted into the Bam HI site of pBR322. The plasmid pHAB6 has only two Bcl I sites, one at nucleotide 2833 of the adenovirus sequence and the second at nucleotide 2955. Since the restriction enzyme Bcl I fails to cleave DNA that has been methylated by the dam methylase of E. coli, pHAB6 was prepared from the GM119 strain of E. coli, which is dam$^-$.

Unmethylated pHAB6 was restricted with Bcl I, then religated resulting in the deletion of the 102 base pairs between the two Bcl I sites, an in-frame deletion within the coding sequences of 55K (see figure 5-1A). E. coli LE392 were transformed with the ligated DNA, and transformants were identified by selection for growth on plates containing ampicillin. Plasmid DNA was extracted from a number of colonies and subjected to restriction enzyme analysis to identify derivatives of pHAB6 containing the desired deletion.

Restriction of pHAB6 with Hind III produced three

Figure 5-1. Construction of recombinant plasmid pH6dl-1. A. Strategy used for the construction of pH6dl-1 is shown. Restriction sites and their nucleotide positions on the viral genome are indicated for Bam HI (B), Hind III (H) and Bcl I (Bcl). **B.** Plasmid DNA was digested with Hind III and run on a 1% agarose gel. The marker was Hind III digested Ad12 DNA. Comparison of the restriction patterns of pHAB6 and pH6dl-1 revealed that the smallest fragment migrated faster in pH6dl-1 (indicated by arrow), as predicted by the deletion of 102 bp from this fragment. **C.** DNA was digested with Acc I, then analyzed as in B. The restriction patterns of pHAB6 and pH6dl-1 differ only in the mobility of a single fragment. In pHAB6 there was a 564 bp fragment which was absent in pH6dl-1, which in turn contained a unique fragment about 700 bp in size (indicated by arrow). The deletion that was introduced removed one Acc I site from pH6dl-1 and shortened the predicted fusion fragment by 102 bp. Therefore it was predicted that the 564 bp fragment of pHAB6 should be replaced by a 714 bp fragment in pH6dl-1, which was observed.


A
H
B(~1)
pHAB6
H(2317)
Bcl(2853)
Bcl(2955)
H(3705)
B(5573)
Bcl I
religate
pH6dl-1
Bcl
(2853/2955)
B
C
pHAB6
pH6dl-1
Mkr
5.3kbp
4.7
3.7
3.4
2.5
2.4
1.8
1.4
1.1
1000bp
690
550

fragments that could be resolved on a 1% agarose gel, a 5.9, a 2.7 and a 1.4 kbp fragment. As a result of the deletion introduced in the new construct, the 1.4 kbp fragment in pHAB6 was reduced to 1.3 kbp in size. As seen in figure 5-1B, recombinant plasmids containing the smaller DNA fragment (indicated by arrow) were generated. Ten out of fifteen plasmids screened were found to exhibit the 1.3 kbp diagnostic fragment, one of which was selected for further studies and named pH6d1-1.

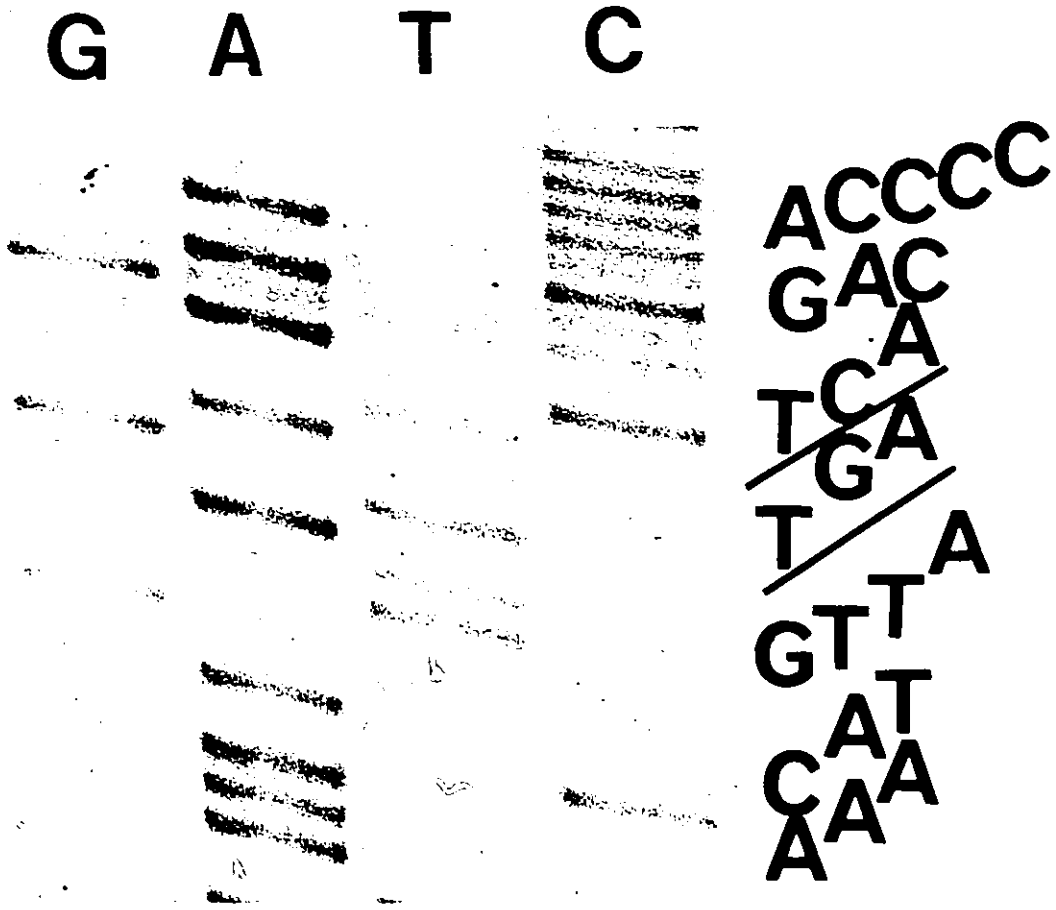
The presence of the deletion was confirmed by restriction with Acc I, which is predicted to cleave pHAB6 to generate seven fragments of 4.1, 2.0, 1.6, 1.1, 0.56, 0.33 and 0.25 kp in length. The pattern observed when pHAB6 was digested with Acc I was the same as predicted although the two smallest fragments were run off the gel (see figure 5-1C). Cleavage of pH6d1-1 with Acc I produced a pattern identical to the pHAB6 pattern except that the 0.56 kbp fragment in pHAB6 was replaced by a 0.7 kbp fragment (indicated by arrow in figure 5-1C). This alteration in mobility was the result of the deletion of the sequences between the two Bcl I sites which contained an Acc I site at nucleotide 2933. Consequently, the 0.56 kbp and 0.25 kbp fragments were fused, and 102 bp was deleted from the fusion product due to the loss of sequences between the two Bcl I sites. It was concluded from these two analyses that

pH6dl-1 contained a deletion of approximately the size and in the location predicted. A successful construct was selected and a large scale preparation of banded plasmid was prepared. The size of the deletion was confirmed to be 102 bp, and the 55K open reading frame conserved by directly sequencing the double stranded plasmid preparation of pH6dl-1 using an oligonucleotide primer synthesized by the Institute of Molecular Biology and Biotechnology at McMaster and the technique described by Sanger et. al. (1977) (see figure 5-2).

The deletion in pH6dl-1 was rescued into virus as illustrated in figure 2-2. Plaques first became visible in the third week following transfection and a number were subsequently picked, and stored at -70°C in 1 X phosphate buffered saline and 20% glycerol. Virus from each plaque was grown on MH12-C2 cells to produce a crude stock of virus and to obtain viral DNA to analyze for the presence of the E1b deletion. Viral DNA was isolated from infected cells using a Hirt extraction and examined by restriction enzyme analysis to identify recombinant viruses containing the E1b deletion.

A small percentage of each DNA sample was restricted with Hind III and the fragments resolved by agarose gel electrophoresis. The deletion was contained entirely within the Hind III I fragment of Ad12 and this fragment was

Figure 5-2. Autoradiogram of nucleotide sequence of pH6dl-1. Banded plasmid DNA was subjected to nucleotide sequencing using primer AB394 (see figure 2-3) and the technique developed by Sanger et. al. (1977). A portion of the autoradiogram is shown and the underlined sequence is the Bcl I site in pH6dl-1. Sequences prior to the Bcl I site extended as far as nucleotide 2961 and the sequences following the site began at 2852. Therefore the 102 bp between 2853 and 2955 were successfully removed and the 55K open reading frame maintained.


G A T C
ACCCC
GAC
A
TC
A
G
T
A
GTT
TA
AA
A

predicted to migrate with increased mobility in the mutant, as was exhibited in the plasmid analysis. All of the plaques isolated were either mixtures of wild type and mutant viruses, their DNA containing both the Ad12 Hind III I fragment and the predicted deletion fragment, or appeared to be pure wild type virus.

Several mixed plaques were plaque purified and viral DNA was once again prepared and screened by restriction with Hind III. A restriction map of Ad12 showing the location of the Hind III sites is shown in figure 5-3. Two out of six plaques screened apparently contained only the mutant virus, since their DNA contained the diagnostic deletion fragment (fragment I′ in dl1201 lane of figure 5-4A), and did not contain the Hind III I fragment. Otherwise the restriction patterns of wild type and the mutant were identical. The structure of the viral genome was also analyzed by Bam HI and Hind III double digestion and Xho I and Hind III double digestion to confirm that the structure of the chromosome was grossly the same as wild type except for the engineered deletion. Restriction maps are shown in figure 5-3 and the fragment containing the deletion, the Hind III-Bam HI and the Hind III-Xho I K fragments, are indicated by the arrows. With the exception of these fragments (designated K′ in the dl1201 lanes of figure 5-4B and C), the restriction patterns of Ad12 strain Huie DNA and dl1201 DNA were

Figure 5-3. Restriction maps of Ad12. The location of recognition sites for the endonucleases Hind III (A), Hind III and Bam HI (B) and Hind III and Xho I (C) and the map unit positions of some of the sites are indicated. The fragments generated are designated alphabetically beginning with the largest fragment. The arrows indicate the fragments containing the Bcl I deletion.

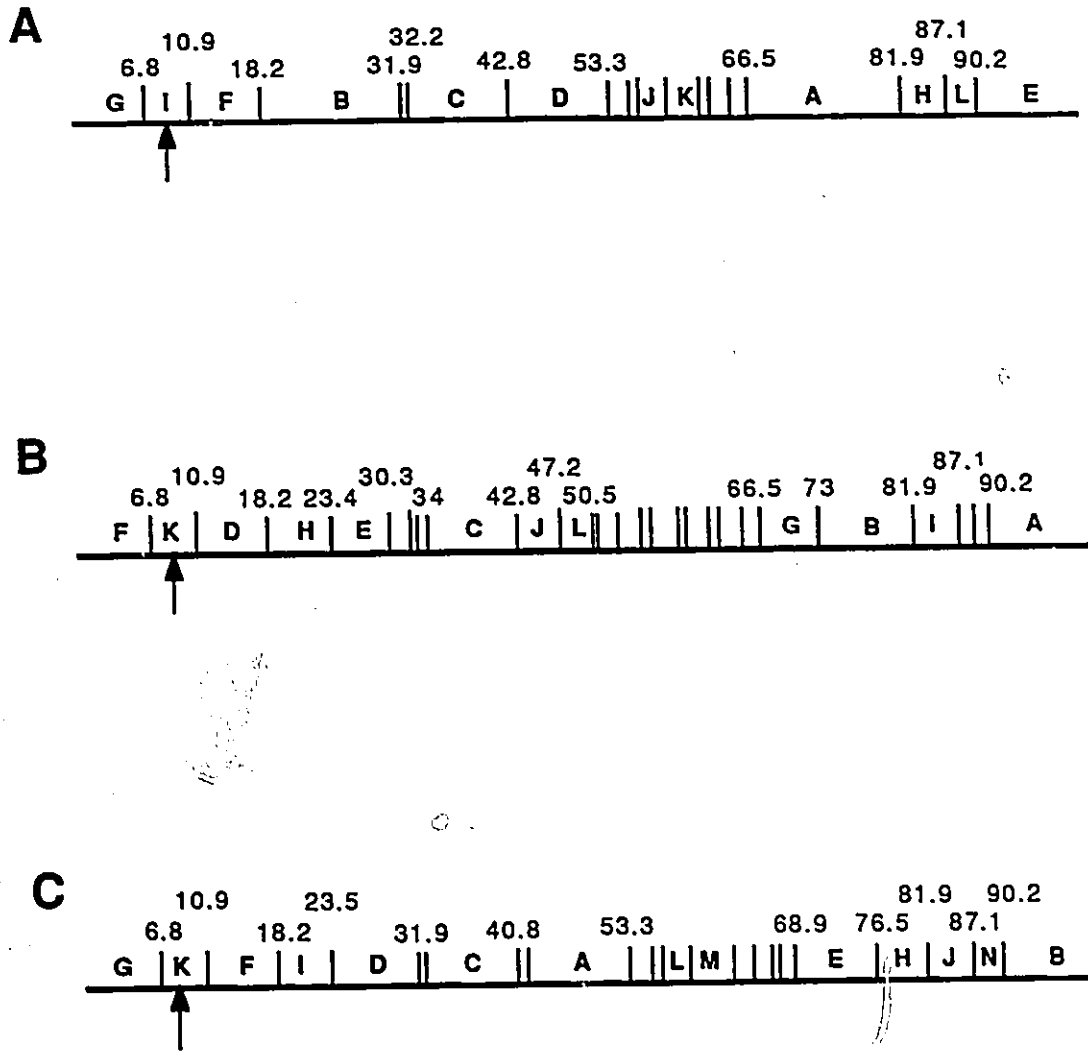
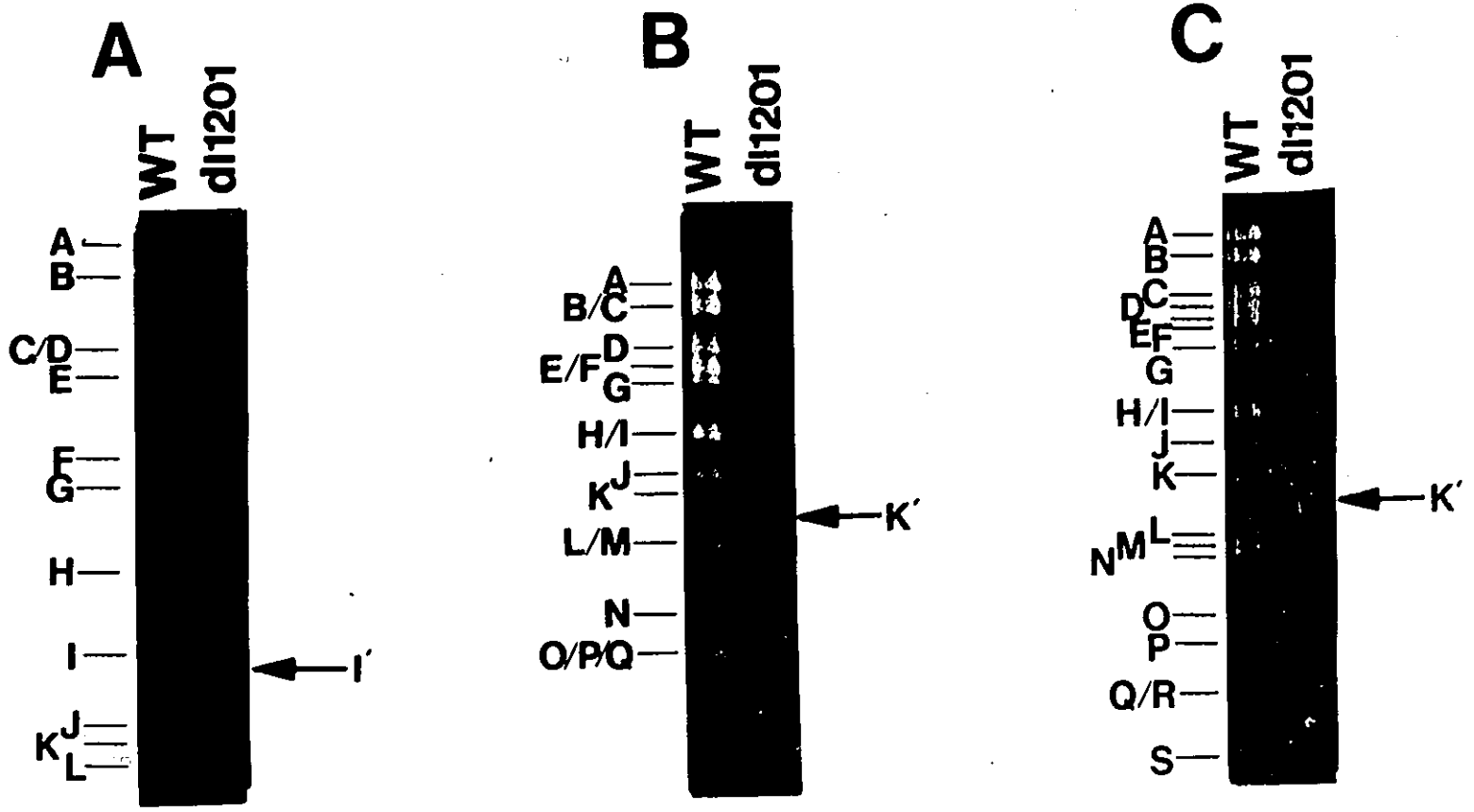

A
10.9
6.8
18.2
32.2
31.9
42.8
53.3
66.5
87.1
81.9
90.2
G I F B C D J K A H L E
B
10.9
6.8
18.2
23.4
30.3
34
47.2
42.8
50.5
66.5
73
87.1
81.9
90.2
F K D H E C J L G B I A
C
10.9
6.8
23.5
18.2
31.9
40.8
53.3
68.9
76.5
81.9
87.1
90.2
G K F I D C A L M E H J N B

Figure 5-4. Analysis of the genome structure of dl1201. DNA was extracted from infected cells by the method of Hirt (1967), restricted with Hind III (A), Hind III and Bam HI (B) or Hind III and Xho I (C), and run on 1% agarose gels. The bands are designated alphabetically and correspond to the fragments illustrated in figure 5-3. In each case the restriction pattern of dl1201 was the same as the pattern from wild type, except for the fragment containing the Bcl I deletion (denoted I′ and K′) which migrated faster than the equivalent wild type fragment (I and K). Therefore dl1201 contained the E1b 55K deletion but otherwise appeared wild type in structure.


A
WT
dl1201
A
B
C/D
E
F
G
H
I
I′
K
J
L
B
WT
dl1201
A
B/C
E/F
D
G
H/I
K
J
K′
L/M
N
O/P/Q
C
WT
dl1201
A
B
D
C
E
F
G
H/I
J
K
K′
N
M
L
O
P
Q/R
S

indistinguishable. The results of these analyses demonstrated that the deletion engineered into the plasmid pH6d1-1 had been successfully introduced into the viral genome, and that the resultant virus, dl1201, had not suffered any other obvious alteration that could be observed by the restriction enzyme analyses conducted. One plaque was chosen and grown on MH12-C2 cells to produce a stock of virus and some initial experiments were performed using this stock. This stock was also used as the initial point for two further rounds of plaque purification of the mutant virus. The final product was again screened by restriction to verify the mutant nature of the viral genome and this was used to grow a stock of virus. In addition, an apparently pure mutant virus was purified from an independently isolated mixed plaque. This virus was plaque purified once more, rescreened by restriction, then grown into a virus stock. All experiments done using the original stock of dl1201 were subsequently repeated using both the thrice plaque purified stock and the independently isolated construct.

B. The 55K Mutation Impairs Virus Growth in KB Cells

During preliminary experiments with this mutant virus it became apparent that the normal adenovirus

cytopathic effect (CPE) failed to develop upon infection of human KB cells with dl1201. At a time in infection when wild type virus had induced extensive rounding of the infected cells, the dl1201 infected cells resembled uninfected cells in their morphology. The mutant infected cells were very flat, even flatter than uninfected cells and appeared normal for a few days, then progressively degenerated until they lifted from the dish at about 5 days post infection. However, the dl1201 infected cells were unlike uninfected cells in that they ceased to divide. The failure to develop normal CPE in infected cells indicated that dl1201 was unlike wild type virus and raised the possibility that the mutant was unable to replicate on KB cells.

The yield of mutant virus grown on MH12-C2 or KB cells was determined to further test the ability of the mutant to grow in KB cells. Cells were infected at a multiplicity of 400 particles per cell and harvested at various times post infection. The cells from a $60mm^2$ dish (2×10^6 cells) were resuspended in 0.2 ml of 1 X tris buffered saline and 20% glycerol and stored at $-70^{\circ}C$. Intracellular virus was liberated by freezing and thawing the cells and the amount of virus in each lysate was quantified by plaque assay.

As can be seen in table 5-1, the yield of virus from

Table 5-1. Growth of Ad12 and dl1201 in MH12-C2 and KB Cells

		Time post infection				
		24h	48h	72h	96h	120h
Expt. #1	MH12-C2					
	Ad12	9×10^7	5.6×10^8	3.9×10^8	-	-
	dl1201	2.7×10^8	3.8×10^8	7.1×10^8		
	KB					
	Ad12	3.9×10^6	8.9×10^8	2×10^9	-	-
	dl1201	$>8 \times 10^4$	$>8 \times 10^4$	$>8 \times 10^4$	6×10^3	4.2×10^3
Expt. #2	MH12-C2					
	Ad12	-	7×10^9	-	-	-
	dl1201	-	5.4×10^9	-	-	-
	KB					
	Ad12	-	6×10^9	-	-	-
	dl1201	-	1×10^3	-	1×10^3	-
	dl1201 (@3600p)	-	1.6×10^5	-	7.3×10^4	-

Cells were infected at 400 or 3600 (@3600p) particles per cell and at the time indicated intracellular virus was freed and titered on MH12-C2 cells. The value shown is PFU/dish.

dl1201 infected MH12-C2 cells differed by no more than a factor of 3 from the yield from wild type infected MH12-C2 cells during the course of a three day infection. In striking contrast to this result, the yield from dl1201 infected KB cells was reduced by at least a factor of 10^5 compared to the yield of wild type virus. This reduction in yield was not simply due to delayed kinetics of virus production since the mutant yield never exceeded this level even if the infection was allowed to proceed for five days. The results of a single preliminary experiment suggested that the mutant was slightly leaky for growth on KB cells, since increasing the multiplicity of infection resulted in a disproportionate increase in the yield of mutant virus. However, the yield was still about four orders of magnitude below wild type yields. Therefore the deletion introduced into the E1b 55K created a host range mutant, capable of growing when the defect was complemented in MH12-C2 cells but unable to grow in KB cells. This indicated that the E1b 55K plays a critical role in the replication of the virus in KB cells under the conditions employed in this experiment. Similar conclusions have been drawn from analysis using other 55K mutants of Ad12 (Shiroki et.al., 1986; Breiding et.al., 1988) and mutants of the equivalent protein, 58K, from serotypes 2 and 5 (Harrison et.al., 1977; Lassam et.al., 1978; Babiss & Ginsberg, 1984; Loga et.al., 1984;

Benards et.al., 1986; Barker & Berk, 1987).

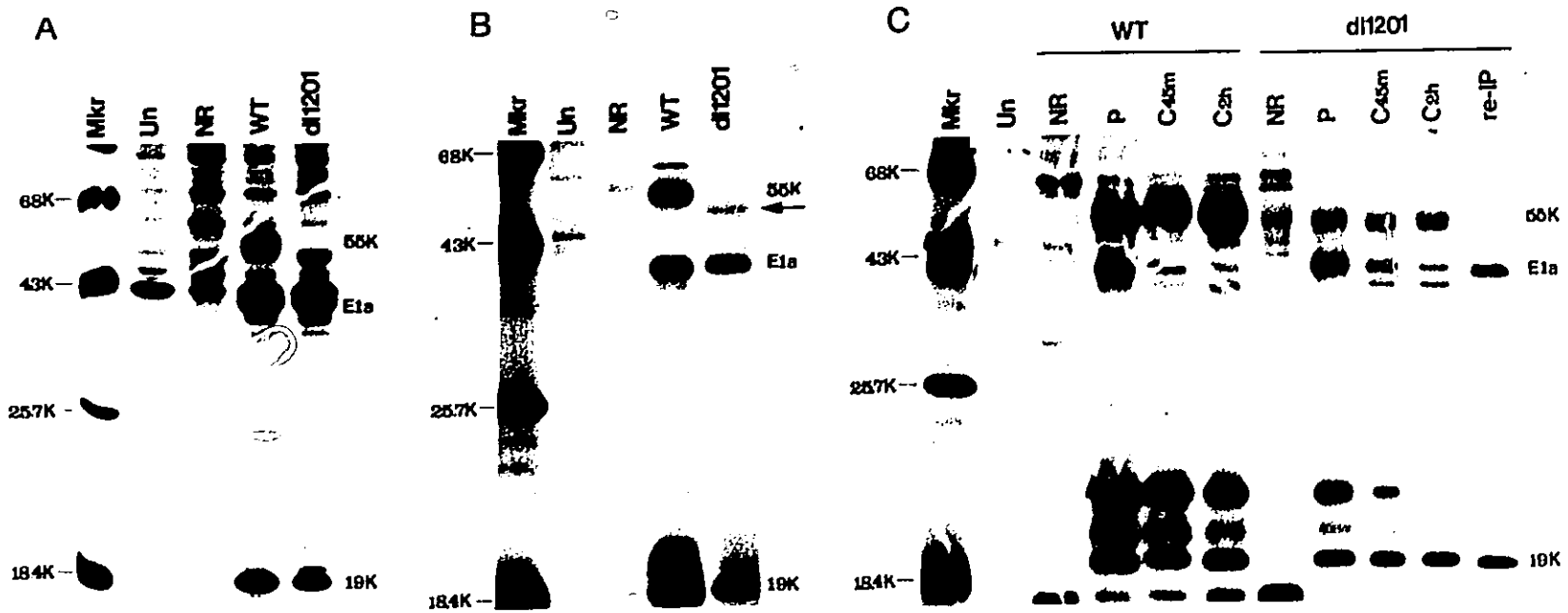
C. Identification of the Mutant Protein Product

The synthesis of the E1 proteins in dl1201 infected cells was examined to determine if the mutant 55K protein was made. Infected cells were labelled for 2 or 4 hours with [^{35}S] methionine at 7 or 24 hours post infection, harvested, and either cytoplasmic or total cell lysates were prepared. The E1 proteins were immunoprecipitated with a variety of anti-tumour sera, and the immunoprecipitates were analyzed on 12.5% SDS polyacrylamide gels. The synthesis of the E1a and E1b 19K proteins was apparent, but the presence of the mutant 55K could not conclusively be demonstrated in either infected 293 cells or infected KB cells (data not shown). Since the mutant protein might be unstable due to the alteration of the wild type primary structure, KB cells infected with 400 particles per cell were labelled for 30 minutes at nine and one half hours post infection, a time preceding the onset of viral DNA replication in wild type infections using the conditions employed (Mak and Green, 1968; data not shown). The E1 proteins were immunoprecipitated from cytoplasmic lysates using AB6a-C3 and analyzed by SDS-PAGE. The E1a and E1b 19K proteins could easily be detected, but no mutant 55K could

conclusively be identified (see figure 5-5A). Although there was a protein immunoprecipitated from dl1201 infected cells that migrated slighly faster than the 55K from wild type infected cells it was concluded that it was not the mutant 55K since a protein with similar mobility could be precipitated from dl1201 infected lysates with normal rat serum, and from uninfected cells with AB6a-C3. The mutant protein was finally detected by infecting KB cells with 2500 particles per cell and labelling with [^{35}S]methionine for 30 minutes at 22 hours post infection. Immunoprecipitates from dl1201 infected cells contained a novel protein whose molecular weight was slightly smaller than the E1b 55K from wild type infected cells (indicated by arrow in figure 5-5B). The amount of this protein synthesized was substantially reduced relative to the amount of 55K produced in a wild type infection.

An experiment was done to test whether the reduction in the amount of the mutant 55K detected was due to its instability. The stability of this protein was compared to that of the wild type 55K by labelling infected cells for 30 minutes then washing and incubating the labelled cells in medium 199 (met^-) supplemented with L-methionine for 45 minutes or 2 hours prior to harvesting and lysing. The mutant 55K did not have a shortened half life since most of the labelled protein could still be immunoprecipitated after

Figure 5-5. E1 protein expression in mutant infected KB cells. A. Cells were infected with 400 particles of wild type or dl1201 per cell and labelled with [^{35}S]methionine for 30 minutes in medium 199 (met$^-$) at nine and a half hours post infection. Cytoplasmic lysates were prepared and the E1 proteins immunoprecipitated with the anti-tumour serum AB6a-C3. Uninfected cell lysates (Un) were similarly precipitated and some infected cell lysates were precipitated with normal rat serum (NR). No E1b 55K could be detected in dl1201 infected cells. B. Cells infected with 2500 virus particles per cell were incubated for 30 minutes in medium 199 (met$^-$) at 22 hours post infection, then labelled for 30 minutes with [^{35}S] methionine and E1 protein expression analyzed as in A. A small amount of a protein migrating slightly faster than the wild type 55K could be seen in dl1201 infected cell lysates (indicated by arrow) and was presumed to be the mutant 55K. C. Cells were infected and labelled as in B. Samples were harvested after labelling (lanes P), or washed and incubated in medium 199 (met$^-$) supplemented with L-methionine for 45 minutes (C45m) or 2 hours (C2h) prior to harvest. The E1 proteins were analyzed as in A. The mutant 55K appeared as stable as the wild type protein.


A
Mkr
Un
NR
WT
dl1201
68K
43K
25.7K
18.4K
55K
E1a
19K
B
Mkr
Un
NR
WT
dl1201
68K
43K
25.7K
18.4K
55K
E1a
19K
C
WT
dl1201
Mkr
Un
NR
P
C45m
C2h
NR
P
C45m
C2h
re-IP
68K
43K
25.7K
18.4K
55K
E1a
19K

a 2 hour chase (see figure 5-5C). Furthermore the antibody apparently recognized the altered 55K efficiently since re-immunoprecipitation of the supernatant from the first immunoprecipitation failed to precipitate much more of the altered 55K (lane reIP in figure 5-5C). These experiments suggested that the mutant 55K was synthesized in dl1201 infected cells, although the amount of protein detected was substantially reduced relative to wild type. The reason for the reduction in the amount of mutant 55K detected was not determined, but was not due to the destabilization of the protein, nor apparently due to its inefficient recognition by the antibody.

D. dl1201 is Defective for Viral DNA Replication

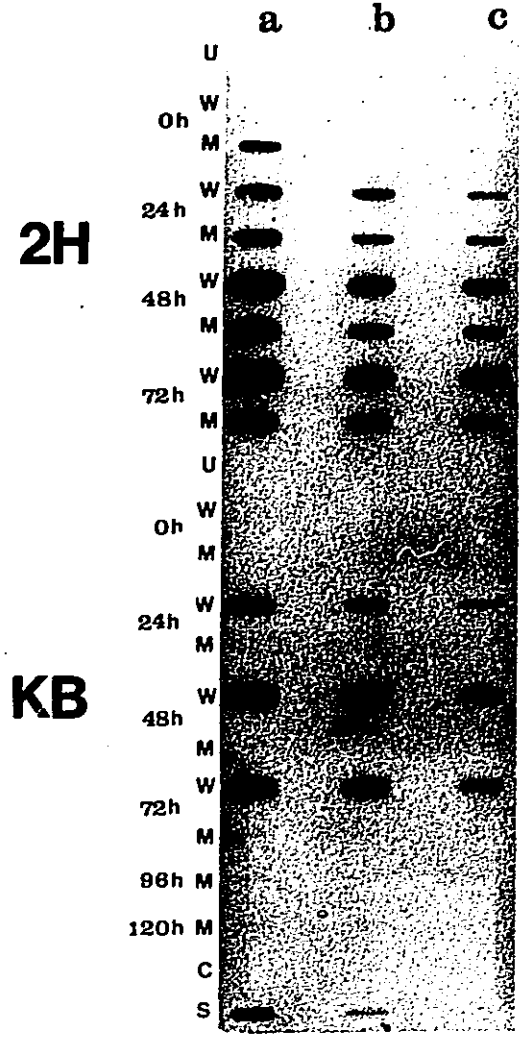
The results presented in section IIIB demonstrated that the 55K mutation in dl1201 compromised the ability of the virus to replicate in KB cells. Other 55K mutants of Ad12, which also fail to replicate on some human cell lines, have been documented to be defective for the replication of viral DNA (Shiroki et. al., 1986; Breiding et. al., 1988). Therefore the ability of the mutant dl1201 to support viral DNA replication in KB cells was tested using two assays. DNA was isolated from infected cells by the method Hirt (1967), digested with restriction enzymes, resolved by

agarose gel electrophoresis and the presence of viral DNA determined by inspection of the gel for the characteristic restriction pattern of viral DNA. Alternatively, total cellular DNA was isolated, sonicated, and quantitated by OD. An equal amount of DNA from each lysate was applied to a nitrocellulose filter using a slot blot apparatus and the amount of viral DNA present determined by hybridization with a radiolabelled Ad12 viral DNA probe. The results from both assays were similar.

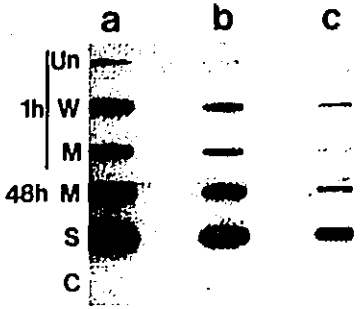
Figure 5-6A illustrates the results of an experiment to examine the accumulation of viral DNA in MH12-C2 cells and KB cells during the course of an infection. Samples harvested immediately after adsorption of the virus contained levels of viral DNA that were undetectable in this blot, but which could be seen in these samples if a greater amount of DNA was applied to the slot (see figure 5-6B). Viral DNA replication occurred in both wild type and dl1201 infected MH12-C2 cells, as was evident by the increase in the amount of radiolabelled DNA that hybridized to the 24 hour and 48 hour post infection samples. In KB cells, the accumulation of DNA in wild type infected cells was similar to that seen in MH12-C2 cells, but, there was no detectable viral DNA in dl1201 infected KB cells at any time examined (see figure 5-6A). The failure to detect DNA in mutant infected cells was not due to altered kinetics of DNA

Figure 5-6. Viral DNA replication in mutant infected cells. A. MH12-C2 (2H) or KB cells were infected with 400 viral particles per cell. At the times indicated total cellular DNA was extracted from wild type (W) or dl1201 infected cells (M). Forty ng (column a), 20 ng (column b) or 10 ng (column c) was mixed with sonicated salmon sperm DNA to bring the total DNA to 100 ng, then applied to nitrocellulose using a slot blot apparatus. Viral DNA was detected by hybridization to radiolabelled wild type Huie DNA. Neither salmon sperm DNA (C) nor DNA from uninfected cells (U) hybridized to the probe. Viral DNA was seen to accumulate in the samples from both wild type and mutant infected MH12-C2 cells (2H), but only the wild type infected KB cells exhibited an accumulation of viral DNA with time. A slight accumulation in the amount of viral DNA in dl1201 infected cells could be seen if more DNA was applied to the filter (panel B contains 3 μg, 1 μg or 0.33 μg of cellular DNA per slot). The standard (S) contains 1 ng, 0.2 ng and 0.04 ng (A) or 1.5, 0.5 and 0.17 ng (B) of viral DNA per slot.

A



B

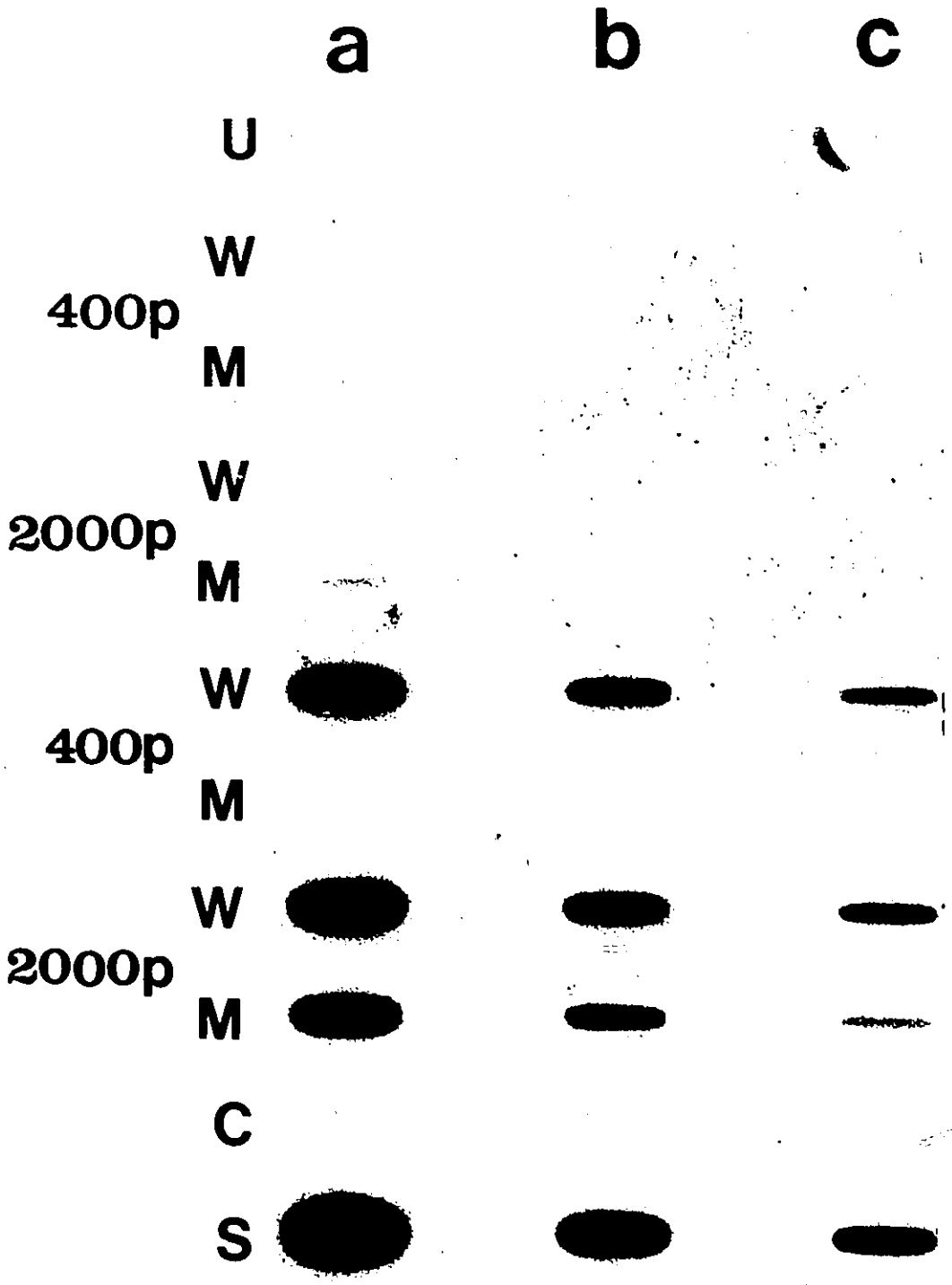


replication, since cells were assayed up to five days post infection, the time at which they completely detached from the dish. If enough DNA was added to the slot to allow the detection of input DNA, the amount of viral DNA in dl1201 KB infected cells could be seen to slightly increase with time, but this invariably corresponded to a few fold increase over the input DNA (see figure 5-6B). These results demonstrated that this virus was severely compromised in its ability to synthesize viral DNA in KB cells when compared to the wild type virus.

The DNA replication defect in dl1201 could be at least partially overcome, if KB cells were infected using a higher multiplicity of infection. By increasing the amount of virus applied to the cells by a factor of five, viral DNA could easily be seen to accumulate in dl1201 infected cells, and by 48 hours post infection accumulation was reduced by no more than a factor of three when compared to wild type infected cells (estimated by densitometry) (see figure 5-7).

Therefore the E1b 55K is required for normal viral DNA replication in human KB cells, at least at low multiplicities of infection. This result is very different from the reports on Ad5 mutants bearing E1b 58K mutations. These host range mutants are capable of replicating their DNA in the less permissive host cell and the block in virus production lies in a later event in the viral cycle (Lassam

Figure 5-7. Multiplicity dependent leakiness of the dl1201 DNA replication defect. KB cells were infected with 400 particles (400P) or 2000 particles (2000P) of wild type virus or dl1201 per cell. At 1 hour (top) or 48 hours post infection (bottom) total cellular DNA was extracted and analyzed as in figure 5-6. Column a contains 90 ng, column b contains 30 ng and column c 10 ng of DNA from wild type infected (W) or dl1201 infected cells (M). All samples contained a total of 1 μg of DNA (made up with salmon sperm DNA). Also shown is DNA from uninfected cells (U), salmon sperm DNA (C) and a DNA standard (S). The standard contained 1.5 ng, 0.5 ng and 0.17 ng per slot. Whereas no viral DNA accumulated by 48 hours post infection in KB cells infected with 400 particles of dl1201 per cell, viral DNA could be seen to accumulate in cells infected with 2000 particles of dl1201 per cell. It was estimated that the viral DNA that accumulated in cells infected with dl1201 at the higher multiplicity was at least 35% of that which accumulated in wild type infected cells.


a
b
c
U
W
400p
M
W
2000p
M
W
400p
M
W
2000p
M
C
S

et. al., 1978; Babiss and Ginsberg, 1984; Logan et. al., 1984). The dl1201 result is, however, in agreement with the results from two other studies on the function of E1b using adenovirus type 12 (Shiroki et. al., 1986; Breiding et. al., 1988).

E. dl1201 is Defective for Early Gene Expression

The failure of the mutant dl1201 to replicate its DNA in infected KB cells could be indicative of a role for 55K in the process of DNA synthesis or it could reflect some other defect early in the viral lytic cycle which prevents normal viral DNA replication. The early events that occur during infection were therefore examined to address this issue. Prior to DNA replication, at least four viral genes that are required for efficient viral DNA synthesis are expressed. The expression of these genes was examined in wild type or dl1201 infected KB cells.

Under the conditions of infection used in these experiments, viral DNA replication has been reported to commence at approximately 12 hours post infection (Mak and Green, 1968). These kinetics have been confirmed by extracting total cellular DNA from wild type infected cells at various times following infection and assaying for viral DNA by slot hybridization using a radiolabelled viral DNA

probe (data not shown). Gene expression in wild type and dl1201 infected KB cells was therefore compared at 10 hours post infection, prior to the onset of DNA replication in wild type infected cells, to determine if early events are comparable between the two infections.

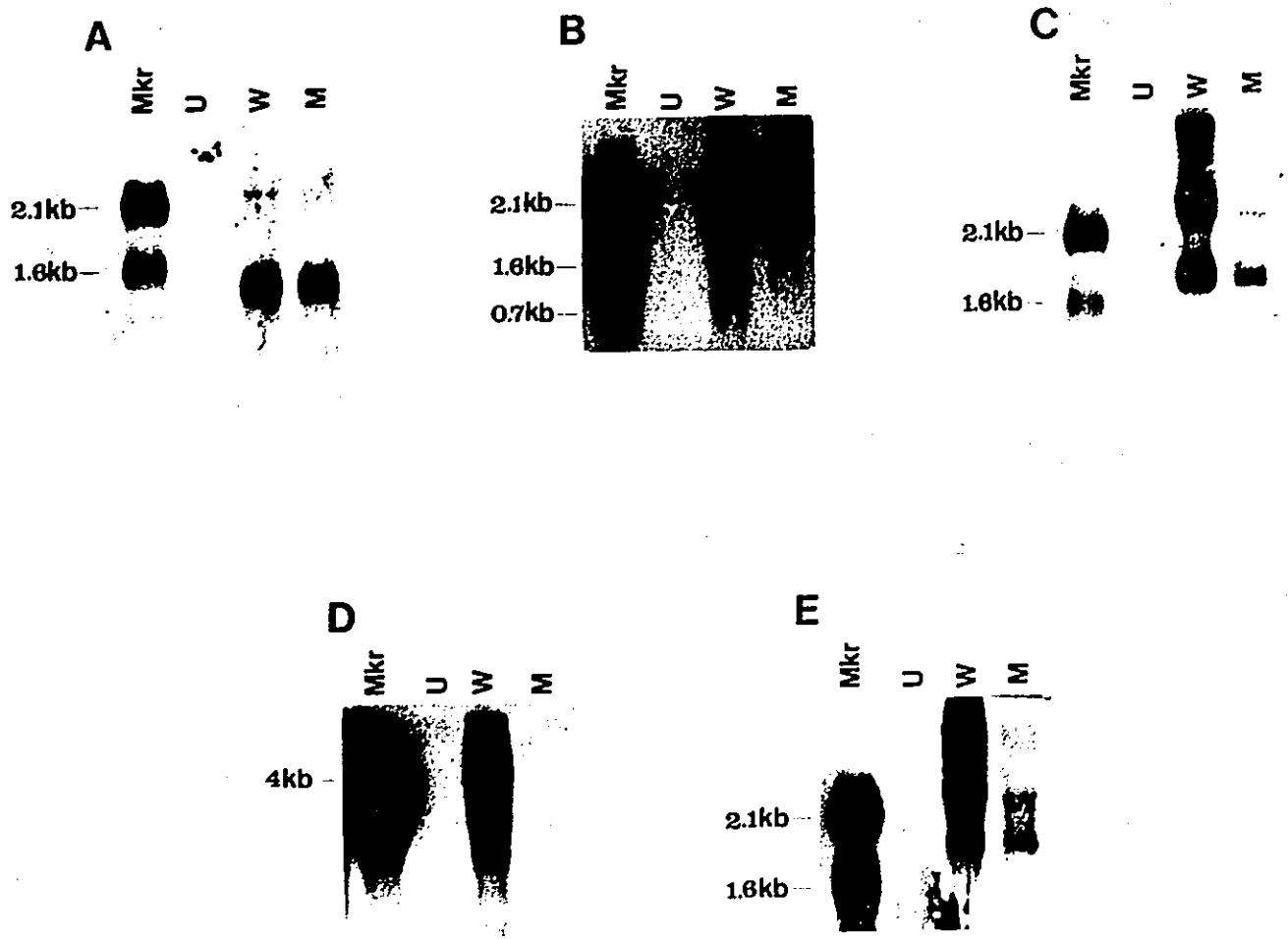
Total cellular RNA was extracted by lysing the cells in guanidinium isothiocyanate solution and then purified by centrifugation through a 5.7M CsCl cushion. The concentration of each sample was determined by OD, and 10 μg was denatured and subjected to electrophoresis on a 1.4% agarose gel containing 2.2M formaldehyde and transferred to nitrocellulose. The mRNA from each of five early genes was visualized by hybridization to radioactively labelled probes. The plasmid pHA5 (containing approximately the left 1400 bp of Ad12) was used to detect RNA from the E1a region and a mixture of pHB14R (containing nucleotides 1594 to 2317 of Ad12) and pHB15R (containing nucleotides 2317 to 3706 of Ad12) was used to detect E1b transcripts. These plasmids contain viral DNA inserted into pBR322 and were radiolabelled by nick translation. With the exception of the E1 region, both strands of the adenovirus are utilized as transcription templates (see figure 1-1). Therefore transcripts from the other early genes were detected with single stranded probes using the recombinants pH118BCr (73.0 to 59.6 mu), pH118HBr (31.9 to 18.2 mu) and pUC118/E4r (100

to 90.2 mu) to prepare probes for the E2a, E2b and E4 mRNAs respectively. These constructs contain the viral DNA inserted into the plasmid vector pUC118. Probes were synthesized by primer extension using the purified single stranded DNA as template and the universal primer, which anneals adjacent to the adenoviral DNA inserted into the vector, as the primer. Ribosomal RNA was used as an internal control to ensure that equivalent amounts of RNA were loaded and that transfer occurred evenly. After exposure of the filters to X-ray film, they were stained with methylene blue to visualize the rRNA.

Qualitatively, there was no difference in the expression of the early genes between wild type and mutant infected cells. A single band approximately 1 kb in length was detected using the E1a probe (see figure 5-8A). The E1a region of Ad12 is transcribed to produce two major messages 885 and 978 nucleotides in length (Saito et. al., 1981), and the single band that was detected probably contained both mRNAs from E1a, which simply were not resolved on this gel system.

Two E1b RNAs were detected, one major RNA about 2 kb in length and a second, slightly larger RNA which was present in lower amounts (see figure 5-8B). Early in infection only a single E1b transcript 2.2 kb in size has been reported (Saito et. al., 1983). Presumably the major

Figure 5-8. Early gene expression in dl1201 infected KB cells. Cells were infected with 400 particles per cell, and at 10 hours post infection total cellular RNA was extracted. Ten μg of RNA from wild type infected (W), dl1201 infected (M) or uninfected (U) cells was analyzed by northern blotting. **A.** E1a mRNA was detected with pHA5 (containing nucleotide -1 to 1400 of Ad12). **B.** E1b mRNA was detected with pHB14R (containing nucleotides 1594 to 2317 of Ad12) and pHB15R (containing nucleotides 2317 to 3705 of Ad12). E2a (in C), E2b (in D) and E4 (in E) RNAs were detected with probes generated by primer extension of single stranded template DNA from pH118BCr (containing 73 to 59.6 mu of Ad12), pH118HBr (containing 31.9 to 18.2 mu of Ad12) and pUC118/E4r (containing 100 to 90.2 mu of Ad12) respectively. The marker in A to C and E was Rsa I digested pBR322. In D the marker was Eco RI digested Huie DNA. Although there were no qualitative differences between RNAs from wild type and mutant infected cells, the amount of E1a RNA was slightly reduced, the amount of the E1b, E2a and E4 RNAs reduced a few fold and the amount of E2b RNA severely reduced in dl1201 infected cells. This demonstrated that the 55K was important for the regulation of gene expression, particularly of the E2b gene.


A
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2.1kb
1.6kb

band seen was the Ad12 2.2 kb mRNA, and the slightly larger, minor band may have been the unspliced E1b precursor. The gels used were apparently incapable of resolving the wild type and mutant E1b transcripts, which differed in size by 102 nucleotides.

The E2a probe hybridized to a number of bands (see figure 5-8C), the major band being approximately 1700 nucleotides in length, which is the reported size of the Ad12 E2a mRNA (Saito et. al., 1981). There was another RNA about 2 kb in length, the intensity of which varied with the preparation of radiolabelled probe, and several very large RNAs. Once again the 2 kb transcript may have been an unspliced precursor RNA, and the large RNAs may have been transcripts from the E2b region of the virus which could also hybridize with the E2a probe due to a 5' leader common to both the E2a and E2b mRNAs.

Using the E2b probe, a number of transcripts were detected (see figure 5-8D), including a very wide band of RNA, between 4 and 8 kb in length, which was sometimes resolved into several distinct bands. The messages encoded by the E2b region of Ad12 have yet to be described, but by analogy to Ad5, these may represent species comparable to the 4.5, 5.5, and 7.5 kb E2b mRNAs expressed by that virus (Stillman et. al., 1981). There were several smaller RNAs detected with the E2b probe, the origins of which were

unclear. These included RNAs at 2.5 kb, 1.5 kb, 1 kb and a very small RNA which migrated far below the 600 nucleotide fragment in the pBR322 marker.

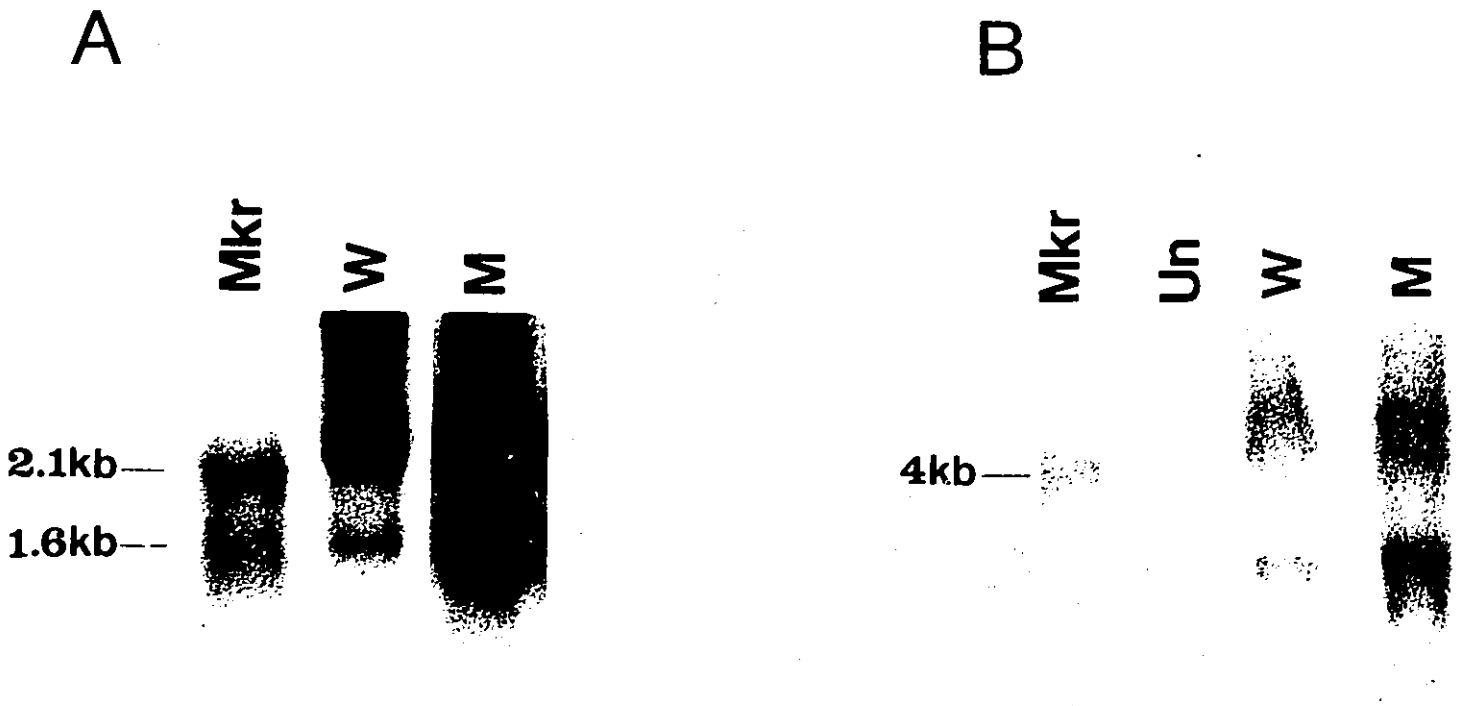
The E4 probe similarly detected a number of RNAs (see figure 5-8E), the major signal being a diffuse band which migrated much slower than the 2.1 kb fragment in the marker. Its origin was unclear since no mRNA of this size has been described from the E4 region. There were two RNAs migrating with similar mobility at approximately 2.1 kb, and fainter bands at about 1.8 kb and 1.5 kb in size, all of which are the sizes of previously described Ad12 E4 mRNAs (Saito et. al., 1981). There are two other reported E4 transcripts that have not been detected in this study, mRNAs of 1100 and 770 nucleotides.

Although there were no qualitative differences in early gene expression, wild type and dl1201 differed quantitatively. The level of E1a expression was slightly reduced in mutant infected cells, estimated by densitometry to be 50 to 80% of the wild type level, and all the other early regions tested showed a greater reduction in accumulation of RNA in mutant infected cells. The relative amount of the E1b, E2a and E4 RNAs in mutant and wild type infected KB cells was approximated by densitometric analysis of autoradiograms and found to be reduced 2 to 4 fold in mutant infected cells. There was a more drastic reduction

in the amount of RNA detected with the E2b probe in dl1201 infected cells. While the amount of the high molecular weight RNA, postulated to be the E2b transcripts, was very easily detected in wild type infected cells, the amount present in dl1201 infected cells was virtually undetectable, and estimated to be less than 15% of the wild type level by densitometry (less than 5% in the autoradiogram shown in figure 5-8D). The accumulation of the very small RNA that hybridized to this same probe was unaffected by the E1b 55K mutation. Therefore the mutation of E1b 55K led to a reduction in the expression of most of the early genes and a severe reduction in the accumulation of E2b mRNA. This suggests that 55K plays a role in regulating gene expression, most notably of the E2b gene.

The DNA replication defect exhibited by dl1201 was leaky, and nearly wild type levels of viral DNA accumulation could be seen in mutant infected cells if the multiplicity of infection was increased (see section IIID). Examination of early events in KB cells infected with dl1201 at high multiplicity revealed that mRNA accumulation from all the early regions, including E2b, was substantially increased. In fact expression generally matched or exceeded the level of expression seen in cells infected with wild type virus at the lower multiplicity (shown for the E2a and E2b RNAs in figure 5-9). Therefore there was a correlation between the

Figure 5-9. Early gene expression in KB cells infected at high multiplicity with dl1201. KB cells were infected with 400 particles of wild type virus or 2000 particles of dl1201 per cell and total cellular RNA was extracted at 10 hours post infection and analyzed as in figure 5-8. E2a RNA was detected with a single stranded probe synthesized using pH118BCr (containing 73 to 59.6 mu of Ad12) as template (A). The marker was Rsa I digested pBR322. E2b RNA was detected with a single stranded probe synthesized using pH118HBr (containing 31.9 to 18.2 mu of Ad12) as template (B). The marker was Eco RI digested Ad12 DNA. The amount of E2a and E2b RNA in KB cells infected with dl1201 at 2000 particles per cell (M) equalled or exceeded the amount in KB cells infected with 400 particles of wild type virus per cell (W). Therefore, under conditions where dl1201 replicated its DNA it also efficiently expressed the E2a and E2b genes.


A
Mkr
W
M
2.1kb
1.6kb
B
Mkr
Un
W
M
4kb

reduction of gene expression, particularly the expression of E2b, and the failure to replicate viral DNA in dl1201 infected KB cells.

The differences in levels of expression were not due to differences in the amount of DNA template present in mutant and wild type infected cells. Total cellular DNA was extracted at the same time as RNA, and the amount of viral DNA present determined by slot hybridization. There was no apparent difference in the amount of viral DNA in wild type or dl1201 infected cells (data not shown).

F. Synthesis of Viral Late and Cellular Proteins

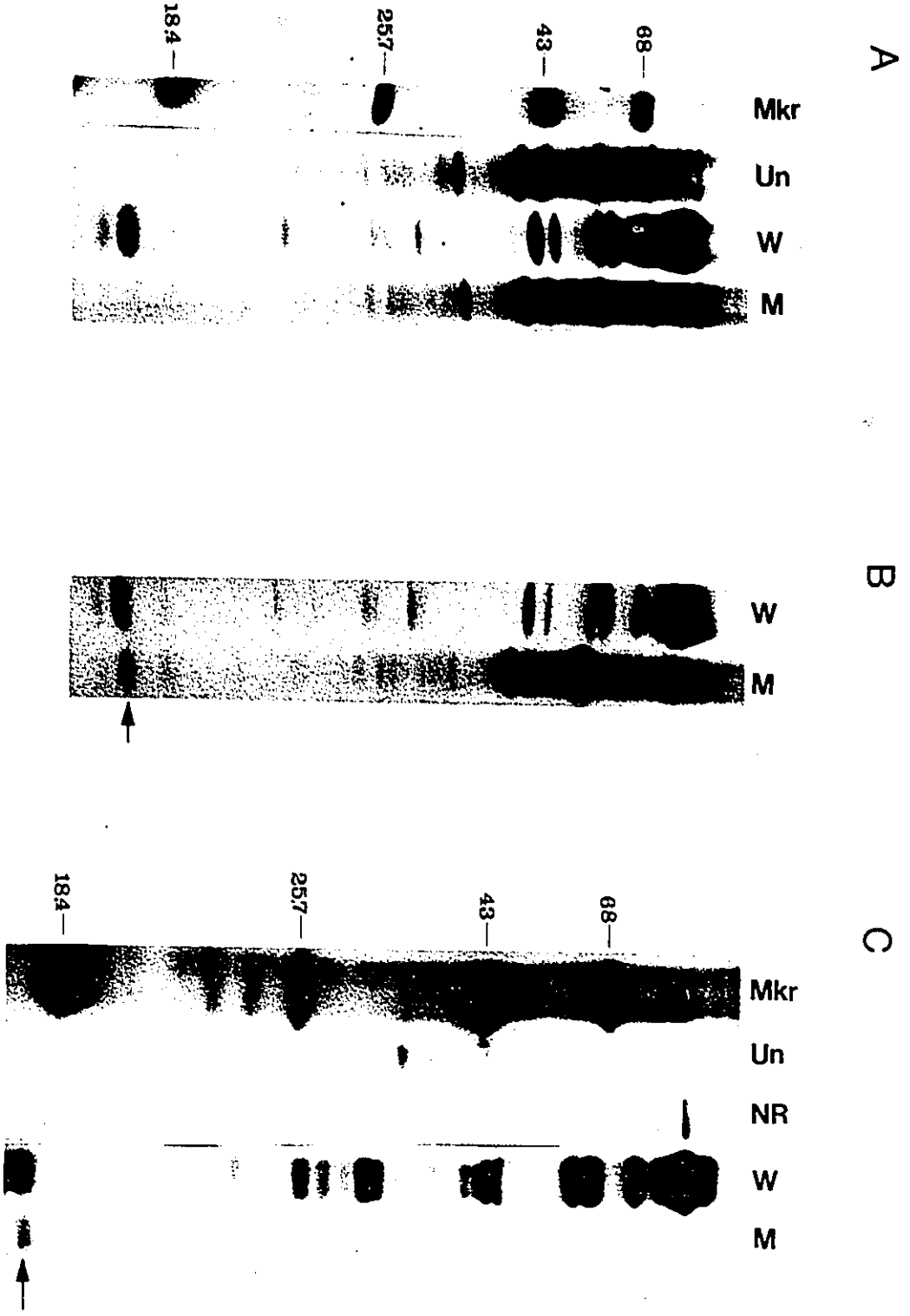
E1b 55K mutants of Ad12, and 58K mutants of Ad5 are reportedly defective for the shut off of host protein synthesis which normally occurs in adenovirus infected cells (Breiding et. al., 1988; Babiss and Ginsberg, 1984). The synthesis of proteins late after the infection of KB cells with dl1201 was examined to determine if this virus shared this phenotype with these mutants. KB cells infected with dl1201 at 400 particles per cell were labelled with [^{35}S]methionine in 199 medium (met$^-$) for 2 hours prior to harvest. Cells were harvested at 36, 48 or 72 hours post infection and cytoplasmic lysates prepared.

The amount of [^{35}S]methionine incorporated into

protein was estimated by measuring TCA precipitable radioactivity in each lysate. The counts incorporated into protein in Ad12 infected lysates were always reduced relative to uninfected lysates, in most cases by a factor of two or three. The total number of TCA precipitable counts in dl1201 infected lysates rarely deviated by more than a factor of two in either direction from the number of counts detected in uninfected lysates. This result indicated that there was little difference in the amount of total protein synthesis that occurred in uninfected and dl1201 infected cells, although the proteins synthesized could have been either viral or cellular in origin. This was determined by characterization of the proteins synthesized by SDS PAGE. Equal TCA precipitable counts from each lysate were run on a 12.5% gel and the proteins visualized by fluorography.

A number of distinct protein bands amidst a dark background could be seen in the uninfected cell lysates (see figure 5-10A). Presumably the background was isotope that had been incorporated into proteins that could not be resolved into distinct bands. The Ad12 infected cell lysates were distinguishable from uninfected cell lysates by the presence of novel protein bands and the absence of many of the bands and the dark background seen in the uninfected cell lysates (see figure 5-10A). The proteins observed in the Ad12 infected cell lysates were shown to be viral

Figure 5-10. Total protein synthesis in mutant infected KB cells. **A.** Cells were infected with 400 particles per cell and labelled with [^{35}S]methionine in medium 199 (met$^-$) for 2 hours at 51 hours post infection. Cytoplasmic lysates were prepared and equal TCA precipitable cpm were analyzed directly on a 12.5% SDS polyacrylamide gel. Shown are lysates from uninfected (U), wild type infected (W) and dl1201 infected cells (M). **B.** As in A, except that the cells were infected with 2000 particles per cell. The pattern of proteins synthesized in dl1201 infected cells was similar to the pattern in uninfected cells demonstrating that dl1201 failed to shut off host protein synthesis and did not synthesize substantial amounts of most of the viral late proteins even at high multiplicities of infection. **C.** Equal volumes of the samples shown in B were immunoprecipitated with an anti serum raised against Ad12 virions. The recognition of the major proteins seen in wild type infected lysates (see panel B lane W) by this anti-serum demonstrated their viral origin. The only protein precipitated from dl1201 infected cells that was a 16K protein. Therefore with the exception of the 16K protein, little synthesis of the viral late proteins was detected in dl1201 infected cells.


A
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proteins by immunoprecipitation with an anti serum raised against Ad12 virions (see figure 5-10C). These observations demonstrated the supplantation of cellular protein synthesis by the synthesis of viral proteins in Ad12 infected cells.

Analysis of dl1201 infected cell lysates revealed a pattern similar to that seen in uninfected cells (see figure 5-10A). There were differences, for example the prominent protein of molecular weight 54,000 which was seen only in the mutant infected lysates, but most of the cellular protein bands and the dark background seen in uninfected cell lysates were also present in the dl1201 infected cell lysates. The presence of a full complement of newly synthesized cellular proteins, and the comparable level of incorporation of [^{35}S]methionine into protein in dl1201 infected and uninfected cells, demonstrated that dl1201 was unable to inhibit cellular protein synthesis. It was not surprising that the synthesis of viral late proteins was not detected since viral DNA replication did not occur in dl1201 infected cells at the multiplicity of infection used in this experiment.

It was possible that the failure of dl1201 to shut off host protein synthesis was an indirect effect secondary to its defect in viral DNA synthesis. The multiplicity dependent leakiness for DNA replication in dl1201 infected cells was exploited to test if the protein synthesis

phenotype was linked to the DNA replication defect. Cells were infected with 2000 viral particles per cell, then analyzed for protein synthesis at 48 hours post infection as described above. The number of TCA precipitable counts in these lysates was reduced to 40 to 60% of those in lysates from cells infected with dl1201 at 400 particles per cell. When analyzed by SDS-PAGE, the dl1201 infected cell lysates could be seen to contain most, but not all, of the cellular protein bands seen in uninfected cell lysates, and a dark background (see figure 5-10B). Therefore dl1201 was defective for the shut off of host protein synthesis even under conditions where the accumulation of viral DNA approached the levels seen in wild type infected cells. Thus, this phenotype was not an indirect effect due to the failure of dl1201 to accumulate sufficient viral DNA. The mutant infected cell lysates did contain several other bands in addition to those that comigrated with proteins in the uninfected cell lysates, the most obvious of which were about 54K and 16K in size. Although the origin of the 54K was uncertain, the 16K protein comigrated with a protein in the Ad12 infected cell lysates and could be immunoprecipitated with an anti serum directed against Ad12 virions (see figure 5-10C), which suggested that it was viral protein. The 16K protein remains to be identified, but based upon its molecular weight, it may be protein IX.

The expression of the majority of the viral late proteins was severely reduced in dl1201 infected cells, if they were expressed at all. This concomitant failure to shut off host protein synthesis and to synthesize normal amounts of viral late proteins has been reported for other 55K mutants of Ad12 and E1b 58K mutants of Ad5 (Breiding et. al., 1988; Babiss and Ginsberg, 1984).

G. dl1201 HAS AN ALTERED ONCOGENIC POTENTIAL

The oncogenic potential of dl1201 was determined using two assays, transformation of primary BRK cells in tissue culture, and a tumourigenicity assay, in which transformed cell lines were injected into rats and their ability to grow into tumours assessed. Primary BRK cell cultures were prepared and the ability of dl1201 to transform these cells measured by infection with virus at various multiplicities of infection, then scoring for foci that survived and grew during approximately 24 days of selection in Joklik +5% horse serum. Foci were visualized by fixation in Carnoy's fixative and staining with Giemsa.

In all experiments, no foci were observed on uninfected dishes. The number of foci generated by infection with wild type virus was dose dependent, increasing as the amount of input virus was increased, until

a maximum was reached. Further increases in the amount of virus applied resulted in a reduction in the number of foci scored. This is the typical dose response seen with adenovirus transformation. Although some variability in the actual number of foci generated was seen, the mutant virus was always greatly reduced in its ability to transform primary BRK cells in culture. Typically, as seen in table 5-2, dl1201 transformed at about 1% the efficiency of wild type virus. The mutation of 55K, therefore severely compromised the ability of the virus to transform primary BRK cells in culture.

Due to the extreme defectiveness of dl1201 in transformation assays in culture, it seemed likely that it would also exhibit a defect in tumourigenicity if a tumourigenicity assay employing virus was used. For this reason the tumourigenic potential of cell lines established by transformation by dl1201 was determined. Cell lines were established by transfecting primary BRK cells with the plasmid pH6dl-1, which contained the nucleotide ~1 to 5573 bp fragment of Ad12 DNA into which the dl1201 defect was engineered. Transformed cells were selected as described above, foci picked, and expanded into cell lines. One million cells from four independant lines were injected subcutaneously into syngeneic, weanling rats. The animals were observed weekly for the development of visible tumours

Table 5-2. Transformation Efficiency of dl1201

		Viral particles per cell					
		100	200	250	500	1000	2500
Expt # 1	Ad12	-	8.0(6)	-	13.0(7)	26.2(5)	-
	dl1201	-	0(5)	-	0(6)	0.17(6)	-
Expt. # 2	Ad12	114.5(2)	-	-	142.0(2)	122.0(1)	52.6(5)
	dl1201	0.33(3)	-	0(1)	1.6(5)	1.5(4)	0.4(5)

Transformation efficiency is indicated as average number of foci per dish. The number of dishes counted is in brackets.

near the site of injection.

As demonstrated in table 5-3, 75% of the cell lines tested induced tumours in almost all of the animals injected. This indicated that the mutation in the construct does not impede the viral induced changes occurring in transformed cells that result in the acquisition of tumourigenic potential. The mutation may not be inconsequential with respect to tumourigenicity. This experiment was run concurrently with the tumourigenicity experiments described in section IID and after their initial appearance, the tumours induced by pH6dl-1 transformants grew slower than those induced by any other cell line tested. Furthermore, in two instances nodules near the site of injection, which were scored as tumours for a considerable period of time, later disappeared, presumably regressing. This observation suggested that the 55K mutation may have a subtle effect upon tumourigenicity, reducing the growth rate following the initial establishment of the tumour.

These results indicated that the mutation in 55K impaired the transforming ability of dl1201, had little effect upon the tumourigenicity of cell lines once established by transformation by the mutant, as judged by the frequency of tumour formation, but may alter the growth properties of the tumours which form.

Table 5-3. Tumourigenicity of Cells Transformed by pH6dl-1

Cell Line	Fraction of rats with tumours	Latent Period (weeks)
Ldl55-1	6/8*	6
Ldl55-2	7/8*	6
Ldl55-3	4/8	7.5
Ldl55-5	7/8	9

10^6 cells were injected subcutaneously into weanling rats (3-4 wks.) and they were observed weekly. The latent period is defined as the time required for the appearance of the tumour in 50% of the maximum number of tumour-bearing animals.

* One tumour apparently regressed.

DISCUSSION

This study was undertaken to examine the role of the E1b region of Ad12 in lytic infection and transformation by linking the phenotypic traits of mutant viruses with molecular defects found in the E1b region. Two of the cytocidal mutants of Ad12, which have been phenotypically characterized and were believed to contain defects in one of the E1b proteins, the 19K, were analyzed to identify the lesions responsible for the observed phenotypes. The function of the other E1b protein, the 55K, was examined by creating and characterizing a 55K mutant to establish the consequences of mutating the sequences encoding this protein. The results demonstrated a requirement for both proteins for the normal progression of the lytic cycle and for transformation.

A. The Function of 19K in Lytic Infection and Transformation

The cyt mutants have been reported to form large plaques on human cells, cause a cytocidal CPE and induce DNA degradation in infected cells (Takemori et. al., 1968; Ezoe et. al., 1981). These viruses have also been demonstrated

to transform primary BRK cells less efficiently than wild type virus and to be only weakly tumourigenic when injected into animals (Mak and Mak, 1983; Takemori et. al., 1968). Cell lines established by transformation with some of the cyt mutants, cyt 68 included, display the full oncogenic potential of wild type transformants, whereas cells transformed by others, notably cyt 62, exhibit a reduced incidence of tumour formation when injected into syngeneic rats (Mak and Mak, 1983; Mak et. al., 1984).

The genome of the mutant cyt 68 was subjected to restriction enzyme analysis and nucleotide sequencing to identify an E1b defect. A defect in cyt 62 was mapped to the portion of the E1b region encoding the 19K protein by the creation of chimeric viruses in which fragments of the E1 region from cyt 62 were substituted for the corresponding sequences in a wild type genome and the phenotypes of the resulting constructs compared to those of cyt 62. During this analysis there was a discrepancy regarding the phenotype of one of the chimeric constructs. Three isolates of AdC21, containing the left 1594 bp of cyt 62, were obtained and the phenotypes of the isolates differed. The first isolate, AdC21, exhibited the mutant phenotype, whereas the others, AdC21r and AdC21i, were wild type. The mutant isolate and one of the subsequent isolates, AdC21r,

both originated from the same plaque and both viruses had the same genomic structure as determined by restriction analysis. The discrepancy in phenotypes between the two could be explained by the acquisition of a mutation in the first virus during its isolation. This mutation may have arisen during the manipulation of DNA in vitro and existed as a variant in the original plaque which was subsequently isolated upon plaque purification or it may have occurred during the purification and growth of AdC21. A less likely alternative is that a mutation common to both could have reverted during the growth of the re-isolate, AdC21r. It appears that the wild type behaviour of AdC21r was not due to the reversion of a mutation since an independent isolate, AdC21i, also exhibited the wild type phenotype. Determination of the nucleotide sequence of the E1 region of cyt 62 revealed no differences from wild type in the left 1594 bp. These results predict that the phenotype of an authentic AdC21 chimera, containing the left 1594 bp of cyt 62 should be wild type. Preliminary experiments revealed that the mutant isolate of AdC21 and AdC22 failed to complement for the DNA degradation phenotype, suggesting that this isolate of AdC21 may have a mutation in the E1b 19K coding sequence.

1. Identification of Mutations in Two cyt Mutants of Ad12

In this study molecular defects have been identified within the E1b region of both cyt 62 and cyt 68. The E1b 19K open reading frame of cyt 68 contains a 107 bp deletion which removes codons 2 through 37 and shifts the reading frame of the remaining sequences accounting for the observed failure of the mutant to produce the 19K protein (Mak et. al., 1984). The E1b 19K gene of cyt 62 was found to differ from wild type by a single point mutation, the transition of nucleotide 1842 from A to G. This mutation results in the substitution of a glycine for the wild type aspartic acid at residue 101 of the 19K protein and resulted in the destabilization of the protein. This is perhaps not surprising, since this substitution alters the charge and size of side chain present at this position, a consequence of which could be an alteration of structure and increased turnover. The analysis of cyt 62 has failed to provide information relating the function of 19K to its structure, since the phenotypes observed could be attributed either to the mutation of a region critical for function or to the reduced levels of accumulated 19K due to its instability.

In each mutant the observed E1b 19K defect did not affect sequences encoding the E1b 55K protein and was the only mutation found within the transforming region of the

mutant viruses. However, these studies cannot rule out the existence of other mutations outside the E1 region which could potentially contribute to the mutant phenotypes. It would be possible to determine if these potential mutations exist by changing nucleotide 1842 of cyt 62 back to the wild type sequence and restoring the 19K sequence which were deleted from cyt 68 then testing to see if these viruses also revert phenotypically.

2. Role of the 19K Protein in Lytic Infection.

The cytocidal and DNA degradation phenotypes of the cyt mutants can be complemented by wild type Ad2 and Ad5, but not by mutants containing E1b 19K defects (Subramanian et. al., 1984a,b; Lai Fatt and Mak, 1982), suggesting that the observed cyt 68 deletion and cyt 62 point mutation are responsible for these phenotypes. There is more conclusive evidence that the point mutation in cyt 62 is responsible for the cytocidal and degradation phenotypes of the mutant. Substitution of the sequences from nucleotide 1594 to 2317 from cyt 62 for the corresponding sequences of the wild type genome produced a virus, AdC22, which exhibited the cytocidal and degradation phenotypes. This chimeric virus was created by ligating a recombinant wild type strain P/cyt 62 E1 region to the remainder of a wild type strain Huie

genome, which allowed the identification of the chimera by the presence of strain P E1 region RFLPs in an otherwise strain Huie genome. The replacement of the strain Huie E1 region with one from wild type strain P was not responsible for the mutant phenotypes exhibited by AdC22, since other chimeric viruses with similar genomic structures, AdC21r, AdC21i and AdC23, were phenotypically wild type. Therefore the mutant phenotypes observed were due to the introduction of the mutant sequences, which differed from wild type only by the mutation at nucleotide 1842, into the virus.

These data demonstrate a requirement for the E1b 19K for the protection of both viral and cellular DNA from degradation in lytically infected cells and for the prevention of the extensive cellular destruction seen in mutant infected cells. These functions have previously been linked to the E1b 19K protein from the analysis of a number of E1b 19K mutants of Ad2, Ad5 and Ad12 (Chinnadurai, 1983; 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, in preparation).

3. Role of the E1b 19K in Transformation

The transformation defect of cyt 62 can be attributed to the 19K mutation at nucleotide 1842 since the

introduction of this mutation into an otherwise wild type genome generated a virus, AdC22, which exhibited the transformation defect observed in cyt 62. Whether or not the 19K deletion is responsible for the transformation defectiveness of cyt 68 is debatable, since there is no experimental evidence addressing the importance of the deletion for this phenotype. Three other mutants of Ad12 that are incapable of synthesizing the 19K product exhibit wild type transforming activities on primary BRK cells (Edbauer et. al., 1988; Zhang, in preparation). From these studies, the 19K appears to be dispensable for transformation, and therefore, the lack of 19K production by cyt 68 can not simply explain the transformation defect of the virus.

Although the mutants cyt 68, pm1542 (and pm700) and in700 all fail to synthesize the 19K protein, they differ in the pattern of protein expression seen in infected KB cells. Mutant pm1542 overproduces the E1b 55K ten fold, whereas cyt 68 expresses the same amount of 55K as wild type. The increased expression of 55K in pm1542 infection is believed to result from the removal of a competing initiation codon from a site on the E1b transcript 5′ to the start codon for 55K. The elevated levels of 55K have been hypothesized to compensate for the absence of 19K facilitating wild type

frequencies of transformation (Zhang, in preparation). However, a second 19K mutant, in700, which is incapable of producing a 19K product exhibits a transforming activity similar to wild type (Edbauer et. al., 1988) yet was observed to express only about twice as much 55K as wild type. Therefore the overexpression of the E1b 55K apparently does not account for the difference in transforming activities of cyt 68 and these other 19K mutants, unless a small change in the level of 55K expression can dramatically alter the efficiency of transformation. A second difference in protein expression was the absence, or altered mobility, in cyt 68 infections of several proteins of about 19.5K to 20.5K, which were prominently seen in pm1542 and in700 infections. This difference is due to the strain of origin of these mutants since these proteins were also seen in cells infected with the wild type parent of pm1542 and in700 (strain Huie) but not in cells infected with strain P (the parental strain of cyt 68). Since the transforming activity of the two wild type strains are comparable, this qualitative difference in protein expression alone does not account for the dissimilarity in transforming activity of cyt 68 and the other two mutants. The combinatorial effect of the cyt 68 deletion and alteration of the 19.5 to 20.5K proteins upon

transformation has not been assessed.

This study has focussed upon the transforming region of cyt 68, and thus fails to address the occurrence of lesions outside of E1 that, in addition to the E1b deletion, could contribute to the observed phenotypes. Therefore the transformation defect of cyt 68 could also be attributed to a second mutation that resides outside of E1, yet alters transforming activity. This putative mutation could be located in E2b for example, a region which has been documented to contain mutations rendering certain mutants of Ad5 transformation defective (Williams et. al., 1974; Miller and Williams, 1987). This hypothesis could readily be tested by repairing the 19K defect in cyt 68 and examining the phenotype of the resultant virus to see if it regained the wild type phenotype.

Two studies have now demonstrated that wild type levels of transformation can occur in the absence of the E1b 19K protein (Edbauer et. al., 1988; Zhang, in preparation). These results conflict with the data presented here and in one other study (Zhang, in preparation), in which transformation defects are linked to the production of altered E1b 19K products. Therefore it appears that the E1b 19K may be involved in transformation, although this becomes obvious only upon the analysis of certain mutants. Several

hypotheses could be advanced to explain the allele specific differences in transforming activity. Missense mutants could interfere with the normal transformation process, or alternatively, a compensatory secondary effect of the mutations in the null mutants, eg. overexpression of the E1b 55K in pm1542, may obviate the requirement of the 19K for transformation. The studies done here on the 19K mutant in700 apparently rule out the overexpression of 55K as a compensatory event since this virus only exhibited a two fold increase above wild type levels of expression, yet transformed like wild type (Edbauer et. al., 1988). This hypothesis could be more rigorously tested by placing the 55K coding sequences under the control of an inducible promoter and replacing the entire E1b region of a wild type virus with this 55K construct. This design will allow the determination of transforming activity in the absence of the E1b 19K and under conditions allowing the controlled variation of 55K expression. Thus it could be determined whether transforming activity correlated with 55K expression. The hypothesis that mutant 19K proteins interfere with transformation might be addressed by complementation experiments, since one prediction of this hypothesis is that missense mutants should inhibit the transforming activity of the 19K null mutants.

4. Role of the 19K in Tumourigenicity

The mutants cyt 62 and cyt 68 are only weakly oncogenic when injected into hamsters (Takemori et. al., 1968) whereas the 19K null mutants pm700 and in700 are highly tumourigenic in rats (Edbauer et. al., 1988). The disparity between the tumourigenicity of the cyt mutants and the other mutants may simply reflect the different transforming activities observed in tissue culture. Inefficient transformation in culture may translate into inefficient establishment of oncogenic cells in the animal, an event which must precede tumour formation. Therefore the tumourigenic defect reported for the cyt mutants may be due to the same mutation that renders the virus transformation defective. There is evidence supporting this hypothesis from tumourigenicity studies utilizing pm700, in700 and cyt 68 transformed cell lines. Lines established by transformation with each of these mutants are highly tumourigenic, suggesting that if the transformation defect of cyt 68 can be overcome this mutant has the oncogenic potency of pm700 and in700. Furthermore, the results from the studies of these mutants demonstrate that the 19K protein is not absolutely required to impart tumourigenic potential on rat cells upon transformation by virus (Edbauer

et. al., 1988; Mak et. al., 1984). In contrast, cyt 62 transformed cell lines exhibit reduced tumourigenicity (Mak et. al., 1984) suggesting that the virus is impaired in a function required for inducing oncogenicity in transformed cells in addition to its transformation defect.

The tumourigenicity experiments conducted in this study suggested that the 19K mutation of cyt 62 may cause some reduction in the tumourigenic potential of transformants, but not the magnitude in reduction previously observed (Mak et. al., 1984). The reason for the difference is difficult to pinpoint since the previous study used cell lines transformed by virus and this study utilized plasmid transformants. It could be due to differences in activity of the E1 region in viral and plasmid transformants due for example to different levels of expression. It could also be due to the presence of additional viral sequences in viral mediated transformants. Their presence may impose additional requirements for full oncogenicity, requirements which cannot be fulfilled by cyt 62.

The modest reduction in tumourigenic potential of cells transformed by an E1 plasmid containing the 19K point mutation of cyt 62 may be indicative of a role for 19K in tumourigenicity, which is contrary to the conclusions drawn from the studies of cyt 68, pm700 and in700 (Mak et. al.,

1984; Edbauer et. al., 1988). This discrepancy may be explained by the fact that the other studies used virally transformed cells and this study employed cells transformed by a plasmid containing the mutant E1 region. These results may indicate that the 19K may be required for full tumourigenicity in cell lines transformed by E1 plasmids, but not for the tumourigenicity of viral transformants, an issue which could be resolved by repeating these analyses using both alleles in plasmid and virus form to establish lines, then assessing their tumourigenicity. There is some evidence in the literature supporting this hypothesis. Two groups have independently created the same E1b 19K mutant and one reports that cell lines transformed by an E1 plasmid containing the mutation are nontumourigenic (Bernards et. al., 1983a) whereas the other group reports that cell lines transformed by the mutant virus are fully tumourigenic (Edbauer et. al, 1988).

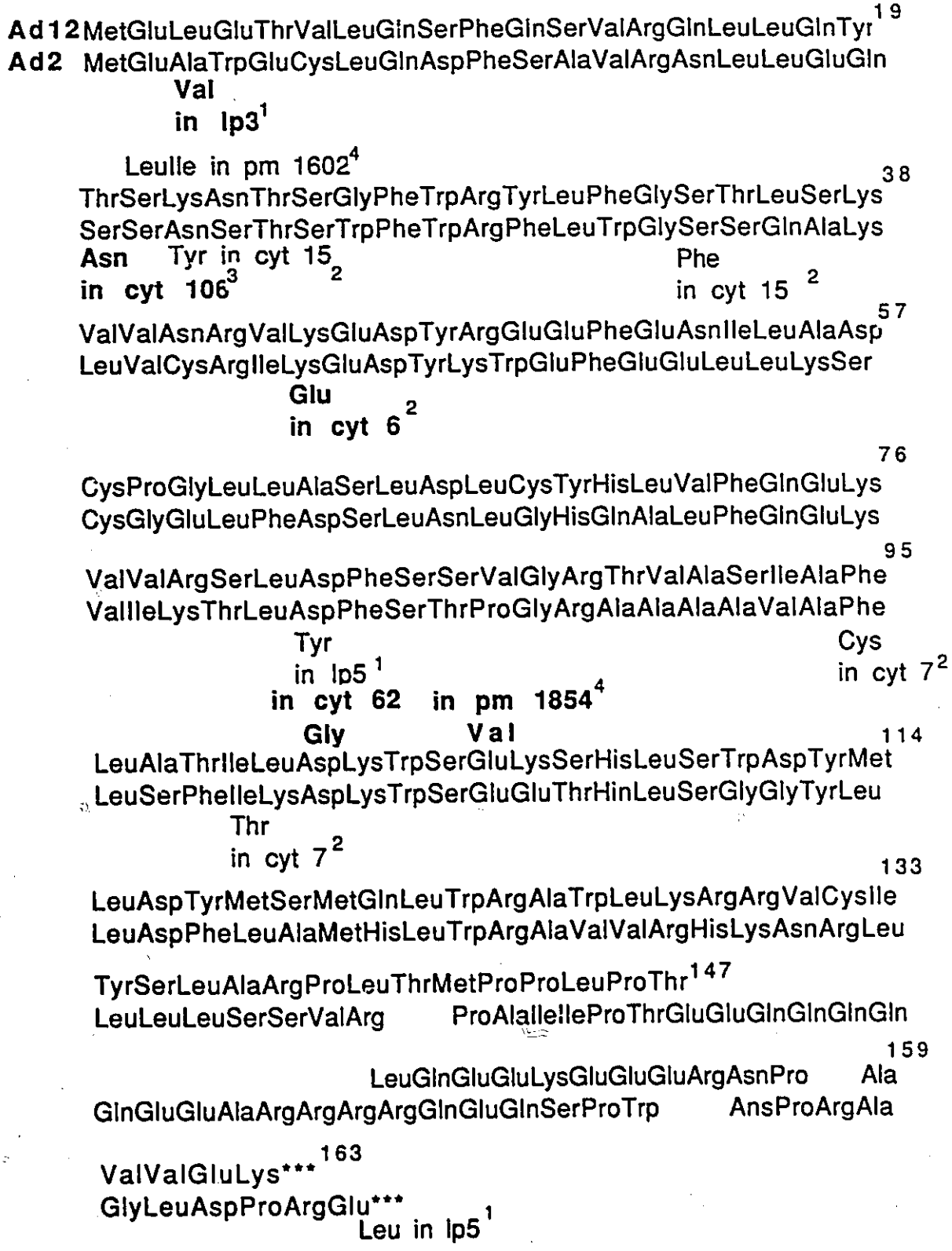
Alternatively, there may be a real difference in the tumourigenic potential of a 19K missense mutant and a 19K deletion mutant just as there appears to be a difference between the two in transforming activity. Analysis of the tumourigenic potential of cell lines transformed by other 19K missense mutants might provide corroborative evidence for this hypothesis. As hypothesized in the previous

section, the contradictory data using different mutants could be reconciled if altered 19K products could interfere with tumourigenicity, or if there was a secondary effect of the elimination of 19K expression in the null mutants which compensated for the absence of 19K. These two alternatives could be distinguished by introducing a missense 19K mutant into tumourigenic cell lines which had been transformed by cyt 68. If the altered 19K could interfere with tumourigenicity, the oncogenic potential of these cell lines may then be reduced.

5. Comparison of E1b 19K Mutants of Ad2 and Ad12

To date seven 19K mutants of Ad2 and Ad12 containing one or two missense mutations have been isolated (Chinnadurai, 1983; Takemori et. al., 1984; White et. al., 1984a). Four contain mutations within the first 44 codons, and the remainder are localized between codons 82 and 101, although lp5 also has an altered termination codon resulting in the extension of the translation product in addition to its residue 82 substitution (see figure 6-1). A number of additional 19K missense mutants of Ad12 have been engineered, and two of them contain substitutions within these two regions (Zhang, in preparation). Protein data is available for six of these nine mutants, cyt 62 inclusive

Figure 6-1. E1b 19K mutants of Ad2 and Ad12. The amino acid sequence of Ad12 (top) and Ad2 (bottom) are aligned as in Bos et. al. (1981). Above and below are the substitutions found in selected 19K mutants of Ad12 and Ad2 respectively. Only mutants containing either one or two missence mutations are shown. Mutants containing single substitutions are shown in bold.



1. Chinnadurai et. al., 1983
2. Takemori et. al., 1984
3. White et. al., 1984a
4. Zhang, in preparation

and only two express wild type levels of the mutant protein. These are pm1854, which contains a glutamic acid to valine substitution at codon 105 of the Ad12 19K (Zhang, in preparation), and lp3, which contains a valine for alanine substitution at amino acid 3 of the Ad2 protein. These two viruses are the least defective of the mutant viruses, lp3 exhibiting only the lp phenotype and a transformation defect (Chinnadurai, 1983; Subramanian et. al., 1984a,b; White et. al., 1984a) and pm1854 being virtually wild type in phenotype (Zhang, in preparation). It is interesting that these mutations are localized within two regions of the coding sequences, but it is presently unclear whether they represent regions necessary for particular functions since their phenotypes could also be attributed to the reduction in the expression of the 19K protein.

B. The Function of 55K in Lytic Infection and Transformation

The E1b 55K of Ad12 and 58K of Ad5 are required for the efficient production of virus in some human cells and for transformation (Shiroki et. al., 1986; Breiding et. al., 1988; Byrd et. al., 1988; Harrison et. al., 1977; Graham et. al., 1978; Lassam et. al., 1978; Ho et. al., 1982; Babiss and Ginsberg, 1984; Babiss et. al., 1984; Logan et. al.,

1984; Bernards et. al., 1986; Barker and Berk, 1987). During lytic infection the 55K of Ad12 is necessary for viral DNA replication (Shiroki et. al., 1986; Breiding et. al., 1988), and the 58K of Ad5 has been documented to enhance the expression of the late proteins and inhibit the expression of cellular proteins by regulating mRNA transport and/or stability (Babiss et. al., 1985; Pilder et. al., 1986; Leppard and Shenk, 1989). In this study a 55K mutant, dl1201, which contained a 102 bp deletion in the sequences encoding the carboxyl third of the protein, was created in an attempt to separate some of the functions of 55K.

1. dl1201 Expresses Reduced Levels of the Mutant Protein

Human KB cells infected with the mutant dl1201 failed to synthesize the normal E1b 55K protein, but a protein with slightly increased mobility was seen. This was presumed to be the mutant 55K protein since its size was similar to the wild type 55K, it was only seen in dl1201 infected cells, and it was immunoprecipitated by an anti-tumour serum which contained antibodies that recognize the E1b 55K and not by a control serum. The amount of this protein that was detected was dramatically reduced relative to the amount of 55K protein seen in wild type infections. The basis for this reduction is unresolved but may involve

more than one mechanism. Protein instability cannot account for the magnitude of reduction seen. Mutant dl1201 exhibited a reduced accumulation of E1b mRNA at least at early times after infection, and it also exhibited a reduction in the accumulation of viral DNA even under conditions allowing replication to occur. These two factors may contribute to the diminished expression of the mutant protein. The conditions applied to immunoprecipitate the mutant protein resulted in the precipitation of all the 55K protein that was recognized by the anti-tumour serum. However, it is possible that different conformational isomers of 55K exist and that the mutation abolishes the recognition of a sub-population of the mutant protein by the anti-tumour serum. It is also possible that the mutation alters the biochemical properties of the protein resulting in a non-quantitative extraction of the mutant protein from infected cells. The disparity in the amount of 55K immunoprecipitated from wild type and dl1201 infected cells was also observed using total cell lysates, suggesting that the reduced detectability of the mutant protein was not due to its altered compartmentalization within the cell. Finally it is also possible that the mutation results in a reduction in the translation of the 55K product.

2. Requirement of 55K for Viral Growth and DNA Replication

The mutant dl1201 is a host range mutant growing as well as wild type on MH12-C2 cells, which can complement the defect, but virtually unable to grow on human KB cells. A single preliminary experiment suggests some degree of leakiness for growth on KB cells at high multiplicities of infection, but even so the yield was four orders of magnitude below wild type yields.

The failure of the mutant to grow on KB cells at low moi's can be attributed to its failure to replicate its DNA. This phenotype is shared by other 55K mutants of Ad12 (Shiroki et. al., 1986; Breiding et. al., 1988; Mak and Mak, in press) but not by 58K mutants of Ad5 (Lassam et. al., 1978; Babiss and Ginsberg, 1984; Logan et. al., 1984). The replication defect of dl1201 can be overcome by increasing the multiplicity of infection. Other 55K mutants of Ad12 have not been tested for multiplicity dependent leakiness, however, some were assessed for DNA replication in cells infected with 20 PFU per cell, which is approximately equivalent to 2000 particles per cell. Since dl1201 can replicate its DNA at this multiplicity of infection but the other 55K mutants remain defective for DNA replication under these conditions of infection it would appear that the leakiness of dl1201 was due to the increased levels of the

mutant 55K protein, which had at least partial activity, rather than the circumvention of the requirement for 55K at high multiplicities of infection.

3. The 55K Regulates Early Gene Expression

Viral mRNA accumulation was reduced early in dl1201 infections suggesting that the 55K may play some role in modulating gene expression. The 55K was most critical for the expression of E2b. This region encodes the viral DNA polymerase and pre-terminal protein which is involved in the initiation of viral DNA replication. In light of the critical nature of these proteins in DNA replication, the observed reduction in E2b expression is the probable cause of the DNA replication defect of this virus. One other study has addressed the expression of viral genes in Ad12 55K mutant infected cells and found little difference from wild type, however, the expression of the E2b region was not examined (Shiroki et. al., 1986).

The differential regulation of the E2a and E2b transcripts is intriguing since both are part of the same transcription unit (Chow et. al., 1979; Kitchingman and Westphal, 1980; Stillman et. al., 1981). In Ad2, E2a transcription is driven from two different promoters, at least one of which is also used for the transcription of

E2b. Common leader sequences are spliced to the main body of the E2a transcript, at about 67 to 62 mu, or to one of several bodies of the E2b transcripts, between about 30 and 11 mu. There are also two sites for polyadenylation, one following each of the E2a and E2b specific sequences. The E2a transcript in Ad12 is structurally similar to that of Ad2 (Saito et. al., 1981), but the structure of the E2b transcript has not been analyzed. Several long open reading frames have been identified in the region of Ad12 corresponding to the E2b region of Ad2 and there is sequence similarity between these open reading frames and those of the DNA polymerase and the preterminal protein in Ad2 (Shu et. al., 1986). Therefore the organization of the E2a and E2b regions of Ad12 appears similar to those of Ad2. Given the complexity of this transcription unit, its regulation may be quite sophisticated. The mechanism by which 55K regulates gene expression has not been addressed.

4. The 55K and Protein Expression in Infected Cells

Mutant dl1201 infected cells exhibited a dramatic reduction in the expression of viral late proteins. Similar defects have been observed in 58K mutants of Ad5 and 55K mutants of Ad12 (Babiss and Ginsberg, 1984; Breiding et. al., 1988) and there are two other reports that a 58K and a

55K mutant accumulate reduced amounts of late mRNAs (Logan et. al., 1984; Shiroki et. al., 1986). In the cases of the other 55K mutants of Ad12, and KB cells infected at a low multiplicity of infection with dl1201, the reduction of late gene expression may be a consequence of the failure of viral DNA replication to occur. However, even at a high multiplicity of infection, under conditions where viral DNA replication does occur, dl1201 was defective for the synthesis of viral late proteins, with few exceptions, most notably a 16K protein which might be protein IX. Thus, the E1b 55K of Ad12 is required for the efficient expression of late proteins in addition to its function in DNA replication. The observation that a protein similar to protein IX was expressed in dl1201 infected cells and the other late proteins were not might imply that 55K regulates the expression of only the viral late proteins encoded in regions L1 through L5, which are transcribed from the major late promoter (see figure 1-1). Finally this reduction in the expression of the late proteins could account for the host range phenotype of dl1201 at high multiplicity of infection which was observed in a preliminary experiment.

Concomitant with the reduced expression of viral late proteins, dl1201 failed to shut off host cell protein synthesis during infection. A similar phenotype is

exhibited by 58K and 55K mutants of Ad5 and Ad12 (Babiss and Ginsberg, 1984; Breiding et. al., 1988). Again, this phenotype in the Ad12 mutants could be secondary to the defect in viral DNA replication. Mutant dl1201 also failed to shut off host protein synthesis in cells infected at a high multiplicity of infection, demonstrating that the necessity of 55K for the inhibition of cellular protein synthesis is independant of its role in viral DNA replication.

The mechanism by which the Ad12 55K depresses host cell protein synthesis and enhances viral late protein synthesis has not been addressed, but by analogy with Ad5, the protein may function by altering the stability or transport of cellular and viral mRNAs (Babiss et. al., 1985; Pilder et. al., 1986; Leppard and Shenk, 1989).

5. Role of the 55K in Transformation and Tumourigenicity

Mutant dl1201 was defective for viral mediated transformation, a result in agreement with other studies involving 58K mutants of Ad2 and Ad5 (Graham et. al., 1978; Babiss et. al., 1984b; Barker and Berk, 1987) and 55K mutants of Ad12 (Byrd et. al., 1988). The transformation defect of dl1201 was more severe than reported for a number of other 55K and 58K mutants, which exhibit a 4 to 5 fold

reduction in activity (Logan et. al., 1984; Bernards et. al., 1986; Shiroki et. al., 1986). In some cases, but not all, the difference in the severity of the transformation defectiveness of these mutants can be explained by the use of different cells in the transformation experiments.

Once cell lines were established by transformation with an E1 containing plasmid bearing the dl1201 deletion, they exhibited the full tumourigenic potential seen in wild type transformants with respect to the frequency of tumour formation, although these tumours may have a reduced growth potential. Recently the analysis of a mutant of Ad12 bearing a defect near the amino terminus of 55K has demonstrated that 55K is required to confer oncogenic potential upon transformed cell lines (Mak and Mak, in press). Therefore the analysis of dl1201 has at least defined a region of the 55K that is dispensable for the formation of tumours by plasmid transformants. There is one other report in which the oncogenicity of cell lines transformed with a 55K mutant is addressed. Cell lines transformed with an E1 plasmid containing a large carboxyl terminal deletion in 55K are nontumourigenic (Shiroki et. al., 1986). It is not clear whether the difference between this result and that obtained with dl1201 are due to the size of the deletion or to the use of different cells and

animals in the two experiments. Interestingly, a mutant virus bearing the same large carboxyl deletion in 55K generates fully tumourigenic cell lines (Shiroki et. al., 1986), underscoring the idea that viral and plasmid transformed cell lines are different. Several other studies have concluded that the carboxyl terminus of 55K is not required for tumourigenicity based upon the analysis of rat cells transformed by subfragments of the E1 region (Shiroki et. al., 1977), and that a similar region of the 58K of Ad5 is nonessential for the tumourigenicity of transformed hamster cells (Rowe et. al., 1984).

C. Concluding Remarks

The study of the two cyt mutants has revealed an allele specific effect of the E1b 19K of Ad12 in transformation and perhaps in tumourigenicity. Whereas 19K null mutants exhibit wild type transforming activity and tumourigenic potential (Edbauer et. al., 1988; Zhang, in preparation), the results presented here show that a 19K point mutation can reduce the transforming activity of Ad12 and marginally reduce the tumourigenicity of cells transformed by E1 plasmids bearing this mutation. The disparity in these results remains to be resolved, but the

analysis of cyt 62 demonstrated that, at least under some circumstances, the 19K can be involved in adenoviral transformation.

The analysis of the 55K mutant has clarified two points regarding its function during lytic infection. First dl1201 expressed reduced levels of the viral late proteins and failed to shut off host protein synthesis, as reported for certain other 55K mutants, which all exhibit a defect in viral DNA replication. The study of dl1201 has succeeded in separating the viral DNA replication defect from the defect in the expression of viral late proteins and the repression of cellular protein synthesis. Secondly, evidence has been obtained that the 55K regulates gene expression, most notably of E2b, suggesting that the role of 55K in viral DNA replication may be to enhance the production of critical components of the DNA replication machinery by activating E2b expression.

The study of mutants dl1201 and cyt 68, which are both transformation defective yet generate cell lines that can form tumours as frequently as wild type transformants, has separated functions which are important for transformation from the process of tumour formation. This illustrates that the transformation of cells in tissue culture and tumourigenesis are distinct events, and as such,

there may be limitations upon the conclusions which can be drawn from model systems employing transformation in culture regarding oncogenesis.

The observed difference in tumourigenic potential between cells transformed by the cyt 62 virus (Mak et. al., 1984) and by E1 containing plasmids harbouring the cyt 62 19K mutation raises the issue that there may be different requirements for the formation of tumours by viral and plasmid mediated transformants, an idea for which there is support in the literature (Bernards et. al., 1983a; Edbauer et. al., 1988; Shiroki et. al., 1986). Therefore studies employing viral transformants may not be comparable to studies using plasmid transformants and caution must be excercised in the interpretation and comparison of these results.

The data presented here suggests that both of the major E1b proteins of Ad12 may be required for the transformation of primary cells in culture and other studies have implicated the products of the E1a region as important for transformation (see introduction). It appears that transformation by adenovirus is complex, involving a number of proteins and presumably a number of distinct functions are required to overcome the normal regulatory mechanisms of the cell leading to the transformed state. In this regard

adenoviral transformation is similar to the development of cancer which is believed to involve multiple events, the culmination of which is the deregulation of growth.

APPENDIX I

Statistical analysis of the transforming activity of the wild type/cyt 62 chimerae.

For Expt. # 1 **Transforming Activity**

Viral particles per cell

	100	250	500	1000	2500
Ad12	33.7	64.3	68.2	52.8	28.7
	(8.1,3)	(6.2,6)	(6.9,6)	(7.7,6)	(5.1,3)
cyt 62	2	7.2	21	17.2	12
	(0,3)	(2.7,6)	(4.2,6)	(3.4,6)	(4.4,3)
AdC21	5	7.7	19	7.5	2.3
	(2,3)	(3.4,6)	(4.5,6)	(2,6)	(2.5,3)
AdC22	11.3	23.6	18.5	16	3.7
	(6,3)	(5.9,5)	(6,6)	(5.5,4)	(2.1,3)
AdC23	15	25.6	39.5	42	22
	(8,3)	(5.3,5)	(3.5,4)	(4.9,5)	(1 dish

Transforming activity is indicated as average number of foci per dish (standard deviation, number of dishes).

Transforming activities were compared for each pair of viruses at each multiplicity of infection used using Student's t test.

For Expt. #1 **t Values**

Viral particles per cell

	100	250	500	1000	2500
cyt 62 vs:					
AdC21	2.6(4)	0.28(10)	-0.79(10)	-6.0(10)	-3.3(4)

At the first 3 mois there is no significant difference.

AdC22	2.68(4)	6.13(9)	-0.84(10)	-0.43(8)	-2.94(4)

At mois of 100, 500 and 1000 there is no significant difference.

AdC23	2.81(4)	7.47(9)	7.25(8)	9.91(9)

At all mois AdC23 transforms significantly better than cyt 62.

Ad12 vs:				
AdC23	2.83(4)	10.98(9)	9.09(10)	2.7(9)

At all mois AdC23 is significantly different than Ad12.

The t values are indicated with the degrees of freedom in brackets.

For Expt. #2 **Transforming Activity**

Viral particles per cell

	100	250	500
Ad12	15.75(6.3,4)	27.6(6.3,5)	48.4(5,5)
cyt 62	1.0(1,5)	3.25(3.2,4)	7.6(2.7,5)
AdC21r	13.6(5.8,5)	22.5(2.4,4)	47.0(3.7,5)
AdC21i	26.0(4,5)	41.3(3.1,4)	60.0(10,5)
AdC22	2.2(1.3,5)	5.0(3.3,5)	10.0(4.2,2)
AdC23	8.0(2.7,5)	23.75(3.8,4)	>40

Transforming activity is indicated as average number of foci per dish (standard deviation, number of dishes).

Transforming activities were compared for each pair of viruses at each multiplicity of infectection used using Student's t test.

For Expt. # 2 **t Values**

Viral particles per cell

	100	250	500
AdC21r vs Ad12	0.53(7)	1.5(7)	0.503(8)

There is no significant difference at any moi.

AdC21i vs Ad12	-2.99(7)	-3.95(7)	-2.28(8)

There is no significant difference at an moi of 500 but at the two lower mois AdC21i transformed significantly better than Ad12.

AdC22 vs cyt 62	1.63(8)	0.8(7)	0.92(5)

There is no significant difference at any moi.

AdC23 vs Ad12	2.51(7)	1.07(7)

At an moi of 250 there was no significant difference.

The t values are indicated with the degrees of freedom in brackets.

Appendix II

Statistical analysis of the tumourigenicity of cells lines established by transformation by the chimeric plasmids pP7-1, pP7-2 and pP7-3.

Fractional Occurence of Tumours Observed

pP7-1 transformants - 0.75 0.29 1 0.875

Avg = 0.73+/-0.31

pP7-2 transformants - 1(a) 0.125 0.625 0.25 0.29

Avg = 0.45+/-0.36

pP7-3 transformants - 0.75 1 0.89 0,875

Avg = 0.88+/-0.1

a - This line produced visible tumours in 5/7 animals in the experiment, and the other two animals developed tumours in the chest cavity. For this analysis all the animals were considered to have tumours.

t Values

pP7-1 vs pP7-3 t = 0.92 with 6 degrees of freedom

There is no significant difference.

pP7-1 vs pP7-2 t = 1.199 with 7 degrees of freedom

There is no significant difference.

pP7-2 vs pP7-3 t = 2.25 with 7 degrees of freedom

These two plasmids have significantly different activity.

It must be cautioned that there is a large variation

in the frequency of tumour formation by individual cell lines and that this variation may prevent accurate statistical analysis.

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