CONSTRUCTION AND CHARACTERIZATION OF E1B MUTANTS OF ADENOVIRUS TYPE 12

By

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ABSTRACT

Early region 1 (E1) of the adenovirus (Ad) genome contains two overlapping transcription units, E1A and E1B, which encode proteins essential for productive viral infection of permissive human cells and for transformation of nonpermissive rodent cells.

E1B of Ad12 produces two major proteins of 482 and 163 amino acid residues, termed 482R and 163R (495R and 175R in Ad2, or 496R and 176R in Ad5). Studies with a variety of mutants affecting the 175R, 176R or 163R proteins have indicated that this product is multifunctional and involved in the protection from degradation of viral and cellular DNA and in the determination of the cytopathic phenotype of infected cells. In addition it is required for complete and efficient transformation of cells. However, a recent report in which the mutants with no initiation codon for 163R or with a downstream stop codon in the 163R coding region were found to be capable of transforming primary baby rat kidney cells has seriously questioned the transformation function of 163R. The functions of the E1B 482R protein in Ad12 have also been characterized. This protein is involved in cellular transformation and is required for accumulation of viral late mRNA and proteins while blocking the same processes for cellular mRNAs and proteins. It is also required for tumour induction and plays some role in viral DNA synthesis.

To examine the role of the Ad12 E1B 163R protein more thoroughly, in particular to determine the transforming function of the protein, deletion and point mutations were introduced at various positions in the coding sequence of 163R by oligonucleotide-directed mutagenesis, including a mutant that lacks the 163R AUG initiation codon. The results indicated that many of these mutants yielded unstable 163R-related products, induced DNA degradation and enhanced cytopathic effect (*cyt/deg* phenotype) in infected KB cells, and transformed primary rodent cells at efficiencies significantly lower than wild-type Ad12. Deletion of the final 16 residues at the carboxy terminus did not affect the phenotypes. Alteration of residue 105 reduced transforming efficiency significantly, suggesting that this region of 163R is functionally important. Removal of the initiation codon at nucleotide 1541 blocked production of 163R but resulted in a large increase in the synthesis of the E1B 482R protein and a transforming efficiency similar to that of wild-type Ad12. These results suggested that 163R plays a role in transformation but that normal transforming efficiencies can be obtained in its absence if a sufficient quantity of 482R is produced.

To define the functional role of Ad12 E1B 163R and 482R in viral gene expression, viral mRNA accumulation from infected cells was assessed by Northern blot analysis. The results demonstrated that the E1B 163R protein does not play a crucial role in modulating viral gene expression. However, a 482R mutant lacking residues 114-155 produced greatly reduced levels of both early (E2B and E4) mRNA and late protein and was defective for viral DNA replication. These results indicated that the 482R

protein plays a role in viral early mRNA metabolism resulting in secondary effects on viral DNA replication and viral late protein synthesis.

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

1.1. Adenovirus Oncogenicity

Adenoviruses were the first human viruses shown to be oncogenic. The discovery by Trentin *et al.* (1962) and other researchers (Huebner *et al.*, 1962, 1965; Rabson *et al.*, 1964; Pereira *et al.*, 1965) that human adenovirus serotype 12 (Ad12) and other serotypes were capable of inducing tumours when inoculated into newborn rodents led to the wide spread use of these agents as models to study viral oncology and molecular biology. Adenoviruses have been classified into three groups based on the efficiency of tumour formation and the latent period of tumour development: the highly oncogenic group A (e.g. Ad12, 18 and 31), the weakly oncogenic group B (e.g. Ad3 and 7) and the nononcogenic group C (e.g. Ad2, 5 and 6) (Green, 1970). However all adenoviruses can induce morphological transformation of rat or hamster cells *in vitro* (McBride and Weiner, 1964; Freeman *et al.*, 1967; Casto, 1968; Gilden *et al.*, 1968; McAllister and Macpherson, 1968). Many of the hamster cell lines transformed either by the highly oncogenic or nononcogenic groups are frequently able to cause tumours when injected into newborn hamsters or T cell-deficient nude animals (Cook and Lewis, 1979; Lewis and Cook, 1982), indicating that all adenovirus types tested have some oncogenic

potential. Although the Ad2- or Ad5-transformed rat cells fail to induce tumours, the Ad12-transformed rat cells are still oncogenic even in immunocompetent syngeneic hosts (Gallimore *et al.*, 1972, 1974 and 1977). Thus studies of Ad12 can afford a unique opportunity towards the understanding of the mechanism of transformation and oncogenicity.

1.2. Structure of Adenovirus

Adenoviruses are nonenveloped and have an icosahedrally shaped virion which consists of two major structural complexes, the capsid and the core. The protein-coated capsid consists of 252 capsomeres, 12 pentons and 240 hexons which are named to reflect the number of their neighbours (Ginsberg *et al.*, 1966). Each hexon is a trimer of three molecules of polypeptide IV (120Kd) (Grütter and Franklin, 1974). The finer structural details of hexon, shown by X-ray analysis of Ad2 hexon crystals (reviewed in Roberts *et al.*, 1986), indicated that the hexon consists of a triangular top and a pseudo-hexagonal symmetric base which provides hexon-hexon contact faces. Twelve pentons are located at the vertices and each of them is surrounded by five neighbouring hexons. Each penton contains a penton base and a fiber projecting from the base. Stoichiometric analysis of penton polypeptides has shown that the penton base is composed of five molecules of polypeptide III (85Kd) and the fiber is a trimer of three molecules of polypeptide IV (62Kd) (van Oostrum and Burnett, 1985). Polypeptides VI (24Kd), VIII (13Kd) and IX (12Kd) were found as hexon associated proteins. Polypeptide IIIa (66Kd)

is associated with the penton base and the peripentonal hexon and was therefore proposed as a mediator between them (Everitt *et al.*, 1973; 1975).

Inside the capsid is a nuclear core containing the double-stranded viral DNA and at least three viral proteins. It has been suggested that six molecules of polypeptide VII (18.5Kd) and one molecule of Polypeptide V (48.5Kd) associate with viral DNA to form a nucleosome-like structure. Polypeptide V also binds to a penton base which seems to play a role in packaging virion DNA (Reviewed in Nermut, 1984). Recently, it was reported that the third protein, μ or polypeptide X, is another DNA-binding protein (Anderson *et al.*, 1989).

1.3. Adenovirus Genome

The viral DNA is a linear double-stranded molecule. The size of the molecule varies slightly depending on serotype, 19-22 x 10^6 daltons for the highly oncogenic serotype and 23-24 x 10^6 daltons for the nononcogenic serotypes (Green *et al.*, 1967). Nucleotide sequence data and restriction analysis have suggested that the genomes of Ad12 and Ad5 are about 34,300 and 36,000 base pairs long, respectively, and they are commonly divided into 100 map units (mu). The adenovirus genome has a number of unique features. A virus-coded 55-kd terminal polypeptide (TP) is covalently attached to dCMP at 5' end of the linear genome. This attachment is the first step in adenovirus DNA replication. The adenovirus genome has inverted terminal repetitions (ITR) which can enable denatured single strands of DNA to form a circle with a panhandle structure

by base pairing. This structure has been suggested to play a role in DNA replication (see section 1.5., and reviewed in Sussenbach, 1984; Kelly, 1984).

1.4. Organization of the genome

The organization of the adenovirus genome has been revealed by S1 mapping (Berk and Sharp, 1977a), ultraviolet (UV) mapping (Berk and Sharp, 1977b; Girvitz and Rainbow, 1978), R-loop mapping (Kitchingman *et al.*, 1977), DNA-RNA hybridization (Chow *et al.*, 1977), genetic mapping of mutants (Sambrook *et al.*, 1975), *in vitro* translation of preselected mRNA (Miller *et al.*, 1980), and nucleotide sequence analysis (van Ormondt and Galibert, 1984).

Adenovirus genes are divided into immediate early (E1A), delayed early (E1B, E2A, E2B, E3, and E4), intermediate (IVa2, and IX), and late (L1, L2, L3, L4, and L5) genes. The early genes are transcribed before the onset of viral DNA replication and the late genes are transcribed after DNA replication, whereas the intermediate genes can be expressed early but are most easily detected at late times. It should be mentioned that the L1 transcripts can also be detected early, although in much smaller amounts than found later (Shaw and Ziff, 1980). E1A, E1B, E3, IX and all the late genes are transcribed to the right, or from the *r* DNA strand. E2A, E2B, E4, and IVa2 genes are transcribed to the left, or from the *l* DNA strand. The locations of the genes and their promoters of adenovirus type 2 were determined precisely and are shown in Fig. 1-1 (top): E1A (1.3-4.5 mu), E1B (4.6-11.2 mu), E2A (67.9-61.5 mu), E2B (30.2-11.2 mu),

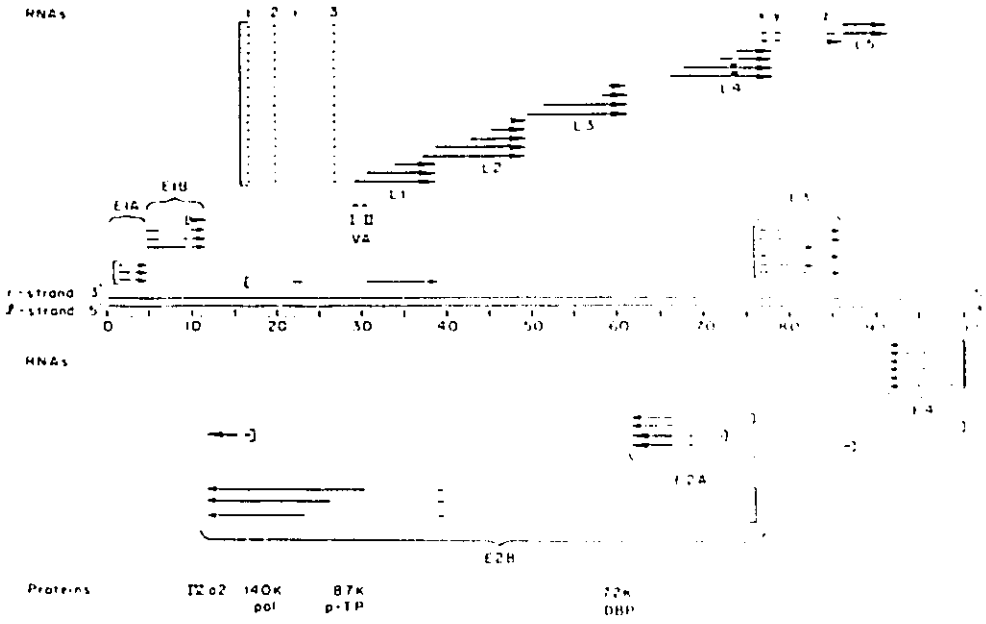
E3 (76.6-86.2 mu), E4 (99.2-91.3 mu), IVa2 (16.1-11.3 mu), IX (9.7-11.2 mu), L1 (31.0-39.3 mu), L2 (39-50 mu), L3 (49.0-61.7 mu), L4 (66.5-77.3 mu) and L5 (86.0-91.3 mu). The adenovirus genome also encodes two small RNAs, virus-associated (VA) RNA I and II. These two RNAs are transcribed from the *r* strand at 28.8-29.5 mu using RNA polymerase III (Reviewed in Sussenbach, 1984).

Approximate locations and coding strands of genes of adenovirus type 12 have been determined and the resulting map is similar to that of adenovirus type 2 (Ortin *et al.*, 1976; Smiley and Mak, 1978). The sizes and more subtle locations of Ad12 early transcripts were obtained by nuclease S1 mapping (Sawada and Fujinaga, 1980; Saito *et al.*, 1981; 1983). The late transcripts were mapped roughly by *in vitro* translation of mRNA selected with different restriction endonuclease fragments of Ad12 DNA (Esche *et al.*, 1984). More recently the nucleotide sequence of the Ad12 genome has been partially determined from 0.0 to 11, 14.1 to 30.6 and 61 to 67 map units (Shinagawa and Padmanabhan, 1980; Sugisaki *et al.*, 1980; Bos *et al.*, 1981; Kimura *et al.*, 1981; Kruijer *et al.*, 1983; Shu *et al.*, 1986). The locations of the genes are: E1A (0.9-4.3 mu), E1B (4.5-11.2 mu), E2A (66.0-61.7 mu), E2B (29.9-11.6 mu), E3 (76.2-85.5 mu), E4 (98.0-91.2 mu), L1 (31-45 mu), L2 (43-54 mu), L3 (52-61.5 mu), L4 (66.0-80 mu), L5 (81-97 mu) [Fig. 1-1 (bottom)], IVa2 (15.1-11.2 mu) and IX (9.8-11.2 mu). Unlike its counterpart, Ad2, the Ad12 genome encodes only VA RNA I (30.0-30.4 mu) (Shu *et al.*, 1986).

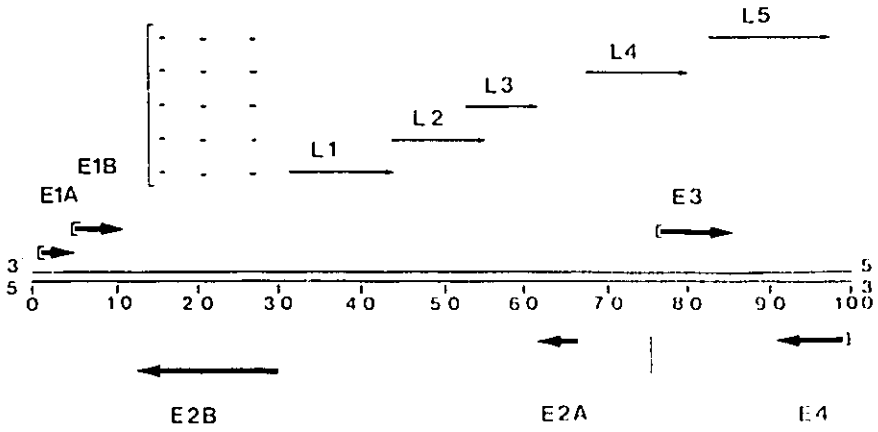
Figure 1-1. Transcriptional maps of adenovirus type 2 (top) and type 12 (bottom).

The double-stranded DNA genome is divided into 100 map units and is transcribed in both rightward and leftward directions from the *r*- and *l*-strands, respectively. The directions of transcription are shown by arrows. The positions of promoters are indicated by capped termini of the arrows, while the 3' polyadenylation sites are represented by arrowheads. The early and the late transcriptional regions are indicated in the figure and E2A and E2B proteins of Ad2 are shown under the E2A and E2B transcriptional regions.

Ad2


RNAs
E1B
E1A
VA
r-strand
l-strand
0
10
20
30
40
50
60
70
80
RNAs
E2A
E2B
Proteins
140K
pol
87K
p-TP
DBP

Ad12


L5
L4
L3
L2
L1
E1B
E1A
E3
3
5
5
3
0
10
20
30
40
50
60
70
80
90
100
E2B
E2A
E4

1.5. Adenovirus Lytic Infection

The lytic cycle of human adenovirus has been studied at high multiplicities of infection in permissive cells to ensure synchronous infection and subsequent biochemical events. The initial process of the infection is the attachment of the fiber of the virus to a specific receptor on the cell membrane (Londberg-Holm and Philipson, 1969). Virus enters the cell by forming a endocytic vesicle or a receptosome (Pastan *et al.*, 1987) and migrates to the nuclear pore in a process involving microtubules (Dales and Chardonnet., 1973). After uncoating, DNA enters the nucleus and leaves the empty capsid behind at the nuclear membrane (Morgan *et al.*, 1969).

The replicative cycle is temporal and the process has been divided into early and late phases according to the synthesis of viral RNAs before and after the onset of viral DNA replication. The notion that early transcription is regulated in a cascade of events is supported by a number of observations. E1A products play a central role in this regulation (reviewed in Flint, 1986). Studies with mutants, *dl*312 and E1A host-range mutants, indicated that E1A is required for the expression of the E1B, E2, E3 and E4 genes (Jones and Shenk, 1979a; Berk *et al.*, 1979). Several investigators have already demonstrated that induction of gene expression results from an E1A-mediated activation of transcription factors such as E2F, E4F and TFIID factors, which bind directly to the Ad promoters (Raychaudhuri *et al.*, 1987; Reichel *et al.*, 1988; Wu and Berk, 1989a, 1989b; Lillie and Green, 1989, reviewed in Flint and Shenk, 1989; Liu and Green, 1990). However, the precise role of E1A proteins in this process is still not clear.

Three early proteins encoded by E2A and E2B are directly involved in viral DNA replication. The precursor to the terminal protein (pTP) plays a unique role in protein-mediated initiation of DNA replication (Stillman, 1981; Lichy *et al.*, 1981). Before priming, this protein is first covalently coupled to dCMP by an adenovirus-coded DNA polymerase (Ad pol), which is closely associated with the pTP (Stillman *et al.*, 1982). This pTP-dCMP complex can then function as a primer to extend a new chain of DNA using one strand of the virus DNA as template starting from either end of the linear DNA molecule (Stillman, 1981; Lichy *et al.*, 1981). Studies with E2A mutants indicated that the DNA-binding-protein (DBP) is involved in the subsequent elongation reaction (Friefeld *et al.*, 1983). Further study demonstrated that DBP binds to DNA template and co-operates with Ad pol in the elongation process. The presence of DBP increased the processivity of Ad pol and permitted the translocation of Ad pol through long stretches of duplex DNA (Lindenbaum *et al.*, 1986). During viral DNA replication, four host-cell polypeptides are involved, nuclear factor I (NFI) for initiation of replication (Nagata *et al.*, 1982), NFII for full elongation of Ad DNA Pol (Nagata *et al.*, 1983), NFIII, and origin recognition protein A (ORP-A) for binding to the origin of replication (Rosenfeld *et al.*, 1987). Adenovirus DNA replication occurs via two processes, type I and type II, representing the synthesis of the daughter molecules from linear ds DNA and circularized ss DNA molecules (reviewed in Kelly, 1984).

DNA synthesis brings the early phase of the viral replicative cycle to an end. The control for the switch from early to late transcription is probably a *cis*-acting function of

the viral DNA (Thomas and Mathews, 1980). In the late phase of the cycle, late mRNAs are transcribed, predominantly from a major late promoter (MLP), from regions between 16.45 and 99 map units. Cleavage and polyadenylation occur at the 3' ends of five families of special late transcripts (reviewed in Flint, 1986). Unlike the early transcripts, late transcripts code for viral structural proteins and some scaffold proteins involved in virion assembly.

Virion assembly occurs in the nucleus. DNA is directed into the capsid through a recognition sequence for DNA packaging. In the last stage of viral morphogenesis, all the precursors are cleaved by a viral-coded protease. The lytic cycles of Ad2 and Ad5 are very efficient and produce approximately 10,000 virions per cell. The cycle for Ad12 is similar, but somewhat longer and less efficient (reviewed in Philipson, 1984).

1.6. Transformation By Adenovirus

In semipermissive and nonpermissive cells derived from rat or hamster, adenovirus can cause morphological transformation (Reviewed in Tooze, 1981). Transformation of human cells can also occur if the infectivity of the viral genome is destroyed. One human cell line was established by transfecting human embryonic kidney cells with sheared adenovirus DNA (Graham *et al.*, 1974). The transformation process is believed to be similar to at least the initial steps of tumour induction because the transformed cells are morphologically similar to cells established from adenovirus-induced tumours and are often capable of inducing tumours which are similar to those

induced directly by viruses (Reviewed in Graham, 1984).

Experiments with primary cultures of cells derived from different tissues of hamsters and rats have shown that the efficiency of transformation is partially determined by the tissue origin of the cells (Mcbride and Wiener, 1964; Yamane and Kusano, 1967; Casto, 1973; Gallimore, 1974). It has been revealed that the amount of virus input also affects the frequency of transformation. The highest transforming efficiency is observed at an optimal virus dose, above which the killing effect of the virus infection reduces such frequency (Gallimore, 1974). Experiments with the DNA replication inhibitor thymidine have shown that an initial round of cellular DNA synthesis seems to be required for the establishment of transformed cells (Casto, 1973). Viral DNA sequences are integrated into cellular chromosomes, however, there is no preferred chromosomal insertion site nor favoured integration site within the viral genome (Reviewed in Tooze, 1981).

Several lines of evidence indicate that the genes responsible for transformation are located at the left end of the viral genome. The only consistent viral sequences found in a variety of transformed rat cells are those from the left 14% of the adenovirus DNA (Gallimore *et al.*, 1974). Moreover, 8% at the left end of the viral genome is sufficient for transformation of rat cells (Graham *et al*, 1974). Subsequently, it has been shown that transforming sequence encodes two sets of early transcripts, E1A and E1B (Wilson *et al.*, 1979).

Although E1A and E1B are central to the process of transformation, it has also

been suggested that other viral sequences may play a role in viral mediated transformation. Group-N mutants are defective for transformation at nonpermissive temperature and mutations were mapped between 18.5 and 22.0 map units, the region which encodes the viral DNA polymerase (Williams, 1975; Galos *et al.*, 1979; Miller and Williams, 1987).

1.7. Early Region 1A (E1A)

1.7.1. E1A of the Nononcogenic Serotypes Ad2 and Ad5

The E1A region encodes two major mRNAs of 13S and 12S expressed in transformed cells and in productively infected cells before viral DNA replication, and three late mRNAs of 9S, 10S and 11S which are detected only late in infection and are believed to play little or no role in transformation. All five mRNAs have common 5' and 3' ends and differ only in the extent of internal splicing. The E1A region has been sequenced and the translation products are known. The products of the 13S and 12S mRNAs are proteins of 289 (289R) and 243 (243R) amino acid residues respectively which are identical except for the additional internal 46 residues in the protein encoded by the 13S mRNA. The products of the 11S and 10S mRNAs are 217R and 171R which are similar to 289R and 243R, respectively, but lack residues 27 to 98. The product of the 9S mRNA is predicted to have common 26 residues at the amino terminus, but to differ at the carboxy terminus because of a change in reading frame (Berk and Sharp, 1978; Spector *et al.*, 1978; Chow *et al.*, 1979; Perricaudet *et al.*, 1979; Kitchingman and

Westphal, 1980; Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987).

E1A proteins have been identified *in vivo* by immunoprecipitation and *in vitro* by translation of hybrid-selected viral mRNAs. *In vitro* translation of the 13S and 12S mRNAs has yielded multiple species of polypeptides with molecular weights in the range from 35K to 58K (Reviewed in Graham, 1984). Analysis of E1A proteins in infected cells using specific antiserum has revealed four major and four minor species. Two major species (52 and 48.5K) are produced by the 13S mRNA, and another two (50 and 45K) by the 12S mRNA (Yee *et al.*, 1983; Rowe *et al.*, 1983a, 1984). All four species represent full-length products of 289R or 243R as determined by partial protein sequencing (Downey *et al.*, 1984). As discussed below, these multiple E1A species could be the results of post-translational modifications. The apparent molecular weights of E1A proteins estimated by migration on SDS-PAGE are all greater than those predicted from the mRNA sequence and are probably caused by post-translational modifications and the inherent high proline content of the proteins. In addition, several late E1A proteins have now been identified as the products of the 9S, 10S and 11S mRNAs (Spector *et al.*, 1980; Winberg and Shenk, 1984; Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987, Trembly *et al.*, 1989).

E1A proteins are phosphorylated. However, the role of this posttranslational modification is unresolved (Harter and Lewis, 1978; Rowe *et al.*, 1983a; Yee *et al.*, 1983; Yee and Branton, 1985a). Phosphorylation occurs at corresponding multiple serine residues on both the 289R and 243R E1A proteins (Tsukamoto *et al.*, 1986; Tremblay

et al., 1988, 1989). It is likely that heterogeneous phosphorylation is largely responsible for the generation of the multiple E1A species (Tsukamoto *et al.*, 1986; Richter *et al.*, 1988; Tremblay *et al.*, 1988, 1989; Dumond *et al.*, 1989, Smith *et al.*, 1989).

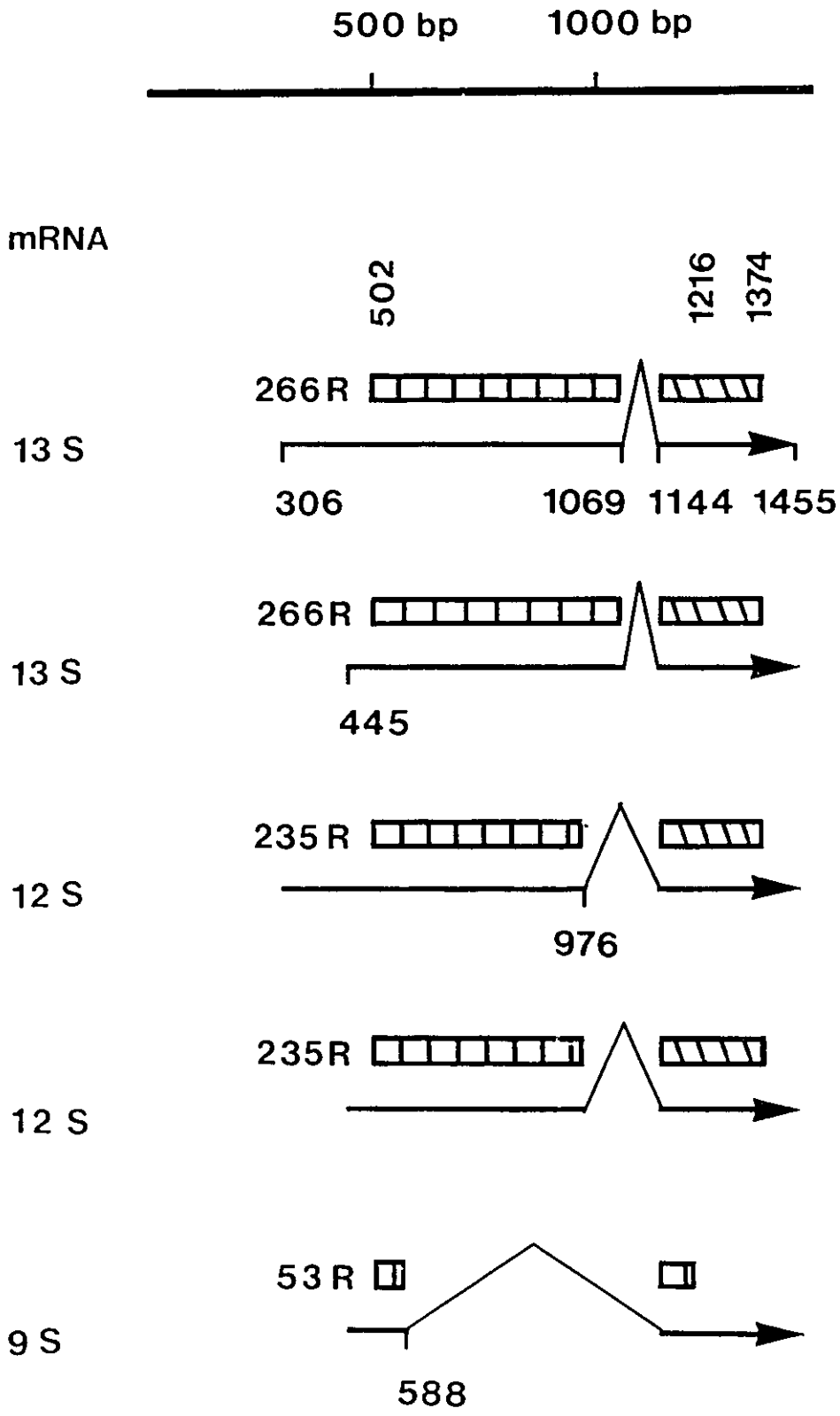
E1A proteins are found in the nucleus of infected cells and are associated with the nuclear matrix or nucleoplasm (Rowe *et al.*, 1983b; Feldman and Nevins, 1983). In infected or transformed cells, E1A proteins form complexes with cellular phosphoproteins of 300K, 107K, and 105K, and with cellular polypeptides of 28-90K (Yee and Branton, 1985b, Harlow *et al.*, 1986; Egan *et al.*, 1987). The 105K protein was shown to be the retinoblastoma susceptibility gene product (Whyte *et al.*, 1988; Egan *et al.*, 1989). Another protein of 60K was identified as a human cyclin A (Pines and Hunter, 1990).

1.7.2. E1A of the Highly Oncogenic Serotype Ad12

The E1A region of Ad12, like its counterpart in Ad2 and Ad5, produces both major mRNAs, 12S and 13S, and a late 9S mRNA in cells. These mRNAs share same 5' and 3' sequence and differ in the size of the introns removed, from nucleotides 1070 to 1143 in 13S, 977 to 1143 in 12S, and 589 to 1143 in 9S. The 12S and 13S mRNAs encode major proteins of 235R and 266R, respectively, which have a common sequence, except for an additional 31 residues in 266R. Two initiation sites for transcription were found for the 12S and 13S mRNAs, however, they do not affect the open reading frames (Perricaudet *et al.*, 1980b; Sawada and Fujinaga, 1980; Saito *et al.*, 1981) (Fig. 1-2). Recently, minor E1A mRNAs of 10S, 9.5S were detected in infected cells, and another of 11S which arise from differential splicing in transformed cells (Brockmann *et al.*,

Figure 1-2. Structure of the E1A region of adenovirus type 12.

The top line represents viral DNA with nucleotide numbers within the E1A region. The thin lines represent the structure of spliced E1A transcripts. The positions of transcription initiation and termination, and of splice donor and acceptor sites are indicated under the mRNAs. Boxes represent the open reading frames, and the different reading frames are distinguished by different shadings. The locations of the first nucleotides in the translation initiation and termination codons are indicated by numbers written vertically. The specific mRNAs and proteins are also indicated.


500 bp
1000 bp
mRNA
502
1216
1374
266 R
13 S
306
1069
1144
1455
266 R
13 S
445
235 R
12 S
976
235 R
12 S
53 R
9 S
588

1990). For the 11S mRNA, the precursor is spliced twice, thus removing nucleotides 589 to 715 and 1170 to 1143. The splice between nucleotides 1170 to 1143 is identical to that used for processing of the 13S mRNA. For the 10S mRNA, the precursor is spliced to remove the two introns, nucleotides 589 to 715 and 977 to 1143, which are used for processing of the 11S and 12S mRNAs, respectively. For the 9.5S mRNA, the precursor is spliced to remove nucleotides 589 to 851 and 977 to 1143. Unlike Ad2 and Ad5 minor mRNAs, Ad12 11S, 10S and 9.5S alter the reading frames from the beginning of their second exons and terminate the reading frames within those exons, resulting in carboxy terminal sequences unrelated to those of the 13S and 12S products (Brockmann *et al.*, 1990).

E1A proteins have been identified *in vivo* by immunoprecipitation of extracts from infected and transformed cells with specific antisera, and *in vitro* by translation of E1A specific mRNAs. Two major E1A polypeptides were detected with apparent molecular weights of 40K or 55K, depending on the gel system used. Again the size is greater than that predicted from the DNA sequence, due presumably to post-translational modifications (Segawa *et al.*, 1980; Saito *et al.*, 1983; Scott *et al.*, 1984; Lucher *et al.*, 1984; Mak and Mak, 1986). With one exception (Esche *et al.*, 1984), most reports revealed that Ad12 E1A proteins are less heterogeneous in size than those of Ad5. A minor species of about 23K was also detected (Mak and Mak, 1986), but its nature is unknown. E1A proteins corresponding to the 11S, 10S and 9.5S mRNAs, with apparent molecular weights of about 10K, have also been reported (Brockmann *et al.*, 1990).

Ad12 E1A proteins are also phosphorylated at the multiple serine sites, both *in vivo* and *in vitro* (Lucher *et al.*, 1984, 1985), and are localized in the nucleus of infected and transformed cells (Shiroki *et al.*, 1980). Both the 235R and 266R products can form complexes with the retinoblastoma protein (Grand *et al.*, 1989).

1.8. Functions of E1A

1.8.1. Functions of Ad2 and Ad5 E1A

E1A proteins are responsible for *trans*-activating transcription of early viral genes (Berk *et al.*, 1979; Jones and Shenk, 1979a; Nevins, 1981), including E1A itself (Osborne *et al.*, 1984) and of some cellular genes, such as those for heat shock protein, β-tubulin and brain creatine kinase (Kao and Nevins, 1983; Stein and Ziff, 1984; Wu *et al.*, 1986; Kaddurah-Daouk *et al.*, 1990). Studies with mutants expressing only 289R or 243R revealed that the former is largely responsible for this *trans*-activating activity (Montell *et al.*, 1984; Winberg and Shenk, 1984). Other work has shown that the unique region of 289R is responsible (Moran *et al.*, 1986; Schneider *et al.*, 1987; Jelsma *et al.*, 1988) and moreover, that it is sufficient for this activity since a synthetic peptide of 49 amino acids comprising the unique region plus three adjacent amino acids was found to possess *trans*-activating activity (Lillie *et al.*, 1987). Despite the central role of the unique region of the 289R protein for *trans*-activation, it seems certain that other regions of the protein also contribute (Lillie and Green, 1989). The synthetic peptide is less efficient than the full length 289R protein in *trans*-activation (Flint and Shenk, 1989), and

a deletion mutant, removing essentially the whole of the N-terminal sequence up to the unique region, impairs *trans*-activating activity (Jelsma *et al.*, 1988). Also, a low level of transcription inducing activity of the 243R protein was detected under certain circumstances (Winberg and Shenk, 1984; Leff *et al.*, 1984; Ferguson *et al.*, 1985; Richter *et al.*, 1985). Moreover, recent reports clearly indicated that regions present in both 243R and 289R are required for E1A *trans*-activation (Bagchi *et al.*, 1990; Kaddurah-Daouk *et al.*, 1990).

E1A is able to activate transcription from promoters newly introduced into cells by either transfection or viral infection. These promoters share no common sequence, suggesting there is no direct interaction between E1A proteins and promoters. Moreover, E1A activates genes transcribed by both RNA polymerase II and polymerase III. The process mediating transcriptional activities by E1A is not known but it seems that E1A products may interact with transcription factors, thus modulating activity of the factors and stabilizing or facilitating the assembly of essential transcription complexes (Reviewed in Berk, 1986a,b; Flint, 1986; Flint and Shenk, 1989; Bagchi *et al.*, 1990; Liu and Green, 1990). A recent report even suggested that the transcription factors may bind to promoter in the absence of E1A proteins and that the transcription complex is then transcriptionally activated by E1A proteins (Schaack *et al.*, 1991).

In addition to its function as a transcriptional activator, E1A proteins can repress the expression of both viral and cellular genes (Borrelli *et al.*, 1984; Hen *et al.*, 1985; Velcich and Ziff, 1985; Velcich *et al.*, 1986; Stein and Ziff, 1987) and such activity

seems to reside equally in the 289R and 243R proteins. E1A-mediated repression affects the enhancer present in the E1A promoter and the expression of the gene encoding the adenovirus DNA-binding protein from an E2 late-phase specific promoter, suggesting that E1A-mediated repression might combine with E1A-mediated *trans*-activation during lytic infection to autoregulate E1A transcription and balance the E2 DBP gene expression (Borrelli *et al.*, 1984, Smith *et al.*, 1985; Guilfoyle *et al.*, 1985). More recently, E1A was found to repress transcription of the collagenase gene by inhibiting activity of a transcription factor AP-1, suggesting a novel mechanism of E1A-mediated repression (Offringa *et al.*, 1990).

While the 289R protein is sufficient for viral replication in human Hela and KB cells, the 243R protein in addition to 289R is required for efficient viral replication in growth-arrested human cells (Montell *et al.*, 1984). In quiescent rodent cells, E1A proteins are involved in inducing synthesis of proliferating cell nuclear antigen (PCNA) and of cellular DNA, production of an epithelial cell growth factor, and mitosis and cell proliferation (Bellett *et al.*, 1985; Stabel *et al.*, 1985; Spindler *et al.*, 1985; Zerler *et al.*, 1987; Quinlan *et al.*, 1987). Studies with various of E1A mutants indicated that the regions involved in the interaction between E1A proteins and cellular phosphoproteins of 105K and 300K or other proteins with similar E1A binding properties are involved in such cell cycle regulation (Moran and Zerler, 1988; Howe *et al.*, 1990; Stein *et al.*, 1990; Pines and Hunter, 1990), suggesting a functional role for such complexes.

The role of E1A products in cellular transformation has been established using

a variety of mutant viruses (Reviewed in Graham, 1984). Ad5 *dl*312 lacks the E1A region and is unable to transform primary rat cells, indicating that E1A is absolutely required for transformation (Jones and Shenk, 1979b). Transfection with E1A can only partially transform cells (Houwelling *et al.*, 1980; Graham *et al.*, 1984). Such cells have the potential to replicate indefinitely, but lack many of the properties typical of fully transformed cells, such as the ability to grow in soft agar, various morphological features, and the ability to grow to high saturation densities. However, E1A can cooperate with E1B or the *ras* oncogene to completely transform primary embryonic cells, thus generating cells with extended proliferative capacity (van der Eb *et al.*, 1979; McKinnon *et al.*, 1982; van den Elsen *et al.*, 1982, Ruley, 1983). These results demonstrated that the function of E1A products in transformation is to immortalize the primary cells. Studies with mutants expressing either 289R or 243R have indicated that both of these proteins are required for full transformation and that a functional 289R product is necessary for maintaining the transformed phenotype (Graham *et al.*, 1978; Ho *et al.*, 1982; Babiss *et al.*, 1983, 1984a; Haley *et al.*, 1984; Montell *et al.*, 1984; Winberg and Shenk, 1984; Hurwitz and Chinnadurai, 1985). Little was known of the molecular mechanism underlying the E1A-mediated cellular transformation until recently when a report demonstrated that the 105K E1A-associated protein is the retinoblastoma protein, the product of the *Rb*-1 gene (designated p105-RB) and known to be a tumour suppressor (Whyte *et al.*, 1988). Therefore, binding of E1A products to the *Rb*-1 protein might result in the functional elimination of this recessive oncogene product, thus

contributing to the transformation process (Egan *et al.*, 1988, 1989; Whyte *et al.*, 1989). Moreover, it seems that binding of E1A products to the 300K and 107K proteins as well is required for this process (Egan *et al.*, 1989; Stein *et al.*, 1990). p105-RB has also been shown to interact with SV40 large T antigen (DeCaprio *et al.*, 1988) and two regions on p105-RB were found to be essential for binding to large T antigen and E1A proteins. These regions overlap with the positions of naturally occurring, inactivating mutations of the *RB* gene (Hu *et al.*, 1990). Significantly, the regions of E1A and large T that are required for RB binding share sequence homologies and are involved in transformation (Figge *et al.*, 1988; Moran, 1988). Similar sequence for binding to p105-RB was also found in the HPV-16 E7 protein (Dyson *et al.*, 1989). These results suggest that DNA tumour virus nuclear oncoproteins transform cells by binding to and inactivating a common target p105-RB.

1.8.2. Functions of Ad12 E1A

Ad12 E1A, like Ad5 E1A, is required for stimulating viral gene expression and viral replication as well as for repressing enhancer-mediated gene expression in lytic infection (Ohshima and Shiroki, 1986; Velcich *et al.*, 1986; Breiding *et al.*, 1988). It also induces cellular DNA synthesis in rodent cells (Stabel *et al.*, 1985; Oda *et al.*, 1986).

Ad12 E1A is also able to down-regulate the expression of MHC class I antigens in transformed rat (Schrier *et al.*, 1983), mouse (Eager *et al.*, 1985), and human cells (Vaessen *et al.*, 1986). Such repression is mediated by the 13S gene product only

(Bernards *et al.*, 1983), and regulated at the level of transcription (Ackrill and Blair, 1989; Friedman and Ricciardi, 1988; Meijer *et al.*, 1989). It seems that the nuclear transcription factor binding to MHC class I gene promoter might be responsible for this regulation (Katoh *et al.*, 1990).

Ad12 E1A alone can immortalize primary rat kidney cells giving rise to partially transformed cells (Shiroki *et al.*, 1979; Gallimore *et al.*, 1984). Mutant with no 235R still transformed rodent cells completely and efficiently, whereas mutant with no 266R was insufficient for transformation, suggesting that the Ad12 and Ad5 larger E1A proteins play different roles in the process of the transformation and with Ad12 only the 266R product is required for this process (Lamberti and Williams, 1990).

Ad12 E1A is necessary but not sufficient for oncogenicity. Studies with Ad12 E1A mutants have shown that 266R is involved in inducing tumours when inoculated into newborn animals (Murphy *et al.*, 1987; Byrd *et al.*, 1988; Lamberti and Williams, 1990). Moreover, recombinant viruses containing Ad5 E1A and Ad12 E1B are still non-tumorigenic and cells transformed by these recombinants fail to induce tumours in new born syngeneic rats, indicating that Ad12 E1A is at least one of the factors contributing to the oncogenicity of Ad12 (Bernards *et al.*, 1983b; Sawada *et al.*, 1988). Since Ad12 E1A proteins also specifically suppresses the expression of (MHC) class I antigens, as described above, it has been suggested that MHC suppression mediated by Ad12 E1A at least partly accounts for increased tumorigenicity of Ad12. In fact there is a strong correlation between the level of MHC expression and the oncogenic potential of

transformed cells. Ad12 transformed rodent cells, with reduced MHC expression, are highly tumorigenic in the syngeneic host. The cells lines derived from the tumours continually expressed MHC at reduced level (Bernards *et al.*, 1983a; Tanka *et al.*, 1985; Eager *et al.*, 1985). Furthermore, highly oncogenic Ad12 transformed mouse cells could be rendered nontumourigenic by introducing MHC class I gene into the cells (Tanka *et al.*, 1985) or by increasing the level of endogenous class I gene expression after treatment with interferon (Hayashi *et al.*, 1985).

1.9. Early Region 1B (E1B)

1.9.1. E1B of the Nononcogenic Serotypes Ad2 and Ad5

The E1B region produces two major mRNAs of 22S and 13S. These two mRNAs share the same 5' and 3' sequences, but are differentially spliced. E1B also produces at least two minor mRNAs of 14.5S and 14S, which also have common 5' and 3' exons, but differ in internal splicing. The 22S mRNA has two open reading frames coding for two major E1B products of 495R and 175R in Ad2, or 496R and 176R in Ad5. These two proteins overlap in coding sequence but are translated from different reading frames utilizing two different initiation codons. The 13S, 14S, 14.5S transcripts code for the same 175R (176R) polypeptide and are also predicted to encode three other 495R (496R)-related proteins, 82R, 155R and 92R in Ad2, or 84R, 156R and 93R in Ad5. These three proteins share amino-terminal sequences with 495R (496R), but have their own unique carboxy termini, except for 155R (156R) which has the same carboxy terminus

as 495R (496R). However, the functions of these minor proteins, if any, are still unknown (Berk and Sharp, 1978, Chow *et al.*, 1979; Perricaudet *et al.*, 1980a; Kitchingman and Westphal, 1980; Bos *et al.*, 1981; Gingeras *et al.*, 1982; 1984; Virtanen and Pettersson, 1985). The E1B region also encodes a 9S mRNA with its own promoter. This mRNA is produced late in infection and codes for virion structural protein IX which is unlikely to play a role in transformation (Alestrom *et al.*, 1980).

Using antibodies to immunoprecipitate extracts of infected and transformed cells or using *in vitro* translation of selected E1B mRNAs, E1B proteins have been detected with apparent molecular weights of 53K to 65 K and 15K to 21K (Harter and Lewis, 1978; Green *et al.*, 1979, 1982, Halbert *et al.*, 1979; Lassam *et al.*, 1979a,b; Schrier *et al.*, 1979; Ross *et al.*, 1980; Jochemsen *et al.*, 1981). By using antisera direct against synthetic peptides or E1B fusion protein, and by N terminal partial sequencing of a smaller polypeptide, it has been shown that the larger protein corresponds to 495R (or 496R) and the small one to 175R (176R) (Anderson and Lewis, 1980; Yee *et al.*, 1983; Green *et al.*, 1983; Lucher *et al.*, 1984; Spindler *et al.*, 1984). These two major E1B products are often termed the 55K and 19K proteins. To avoid confusion, I will refer to them as 495R (496R) and 175R (176R). A number of other E1B proteins, ranging in size from 14K to 25K, have been detected in infected cells. Recently, these polypeptides have been identified as E1B 495R (496R)-related proteins by peptide mapping and sequencing and by immunoprecipitation with peptide-specific antisera targeted to either the amino or carboxy terminus of the proteins (Green *et al.*, 1979; Anderson *et al*, 1984;

Lucher *et al.*, 1984; Lewis and Anderson 1987; S. Brown, D. Takayesu and P. E. Branton, manuscript in preparation).

The E1B 495R (496R) protein is phosphorylated and phosphorylation probably occurs at two serine and one threonine residues (Levinson and Levine, 1977; Malette *et al.*, 1983). This protein forms a physical complex with an anti-oncogene product, p53, in transformed cells (Sarnow *et al.*, 1982b), and associates with a virally encoded E4 34K protein in productively infected cells (Sarnow *et al.*, 1984). Although the 495R (496R) protein has been found to be localized in the nucleus and cytoplasm in both infected and transformed cells (Sarnow *et al.*, 1982a; Rowe *et al.*, 1983b; Yee *et al.*, 1983; Zantema *et al.*, 1985a), it is specifically associated with nuclear viral inclusions when the E4 34K protein is present (Ornelles and Shenk, 1991).

The E1B 175R (176R) protein is acylated (McGlade *et al.*, 1987) and also phosphorylated at a low level in infected cells (McGlade *et al.*, 1989). It is possible that acylation plays a role in its association with membranes (Persson *et al.*, 1982; Rowe *et al.*, 1983b). Early after infection, the 175R (176R) protein is associated with cytoplasmic membranes, whereas at late times, it is found on the inner and outer nuclear membranes with some associated with the nuclear lamina (White *et al.*, 1984a).

1.9.2. E1B of the Highly Oncogenic Serotype Ad12

The E1B region of Ad12 produces two major early mRNAs, 22S and 13S, and one late mRNA of 9S, which share 5' and 3' sequences but are differentially spliced. The 22S mRNA encodes two major products of 482R and 163R in different reading

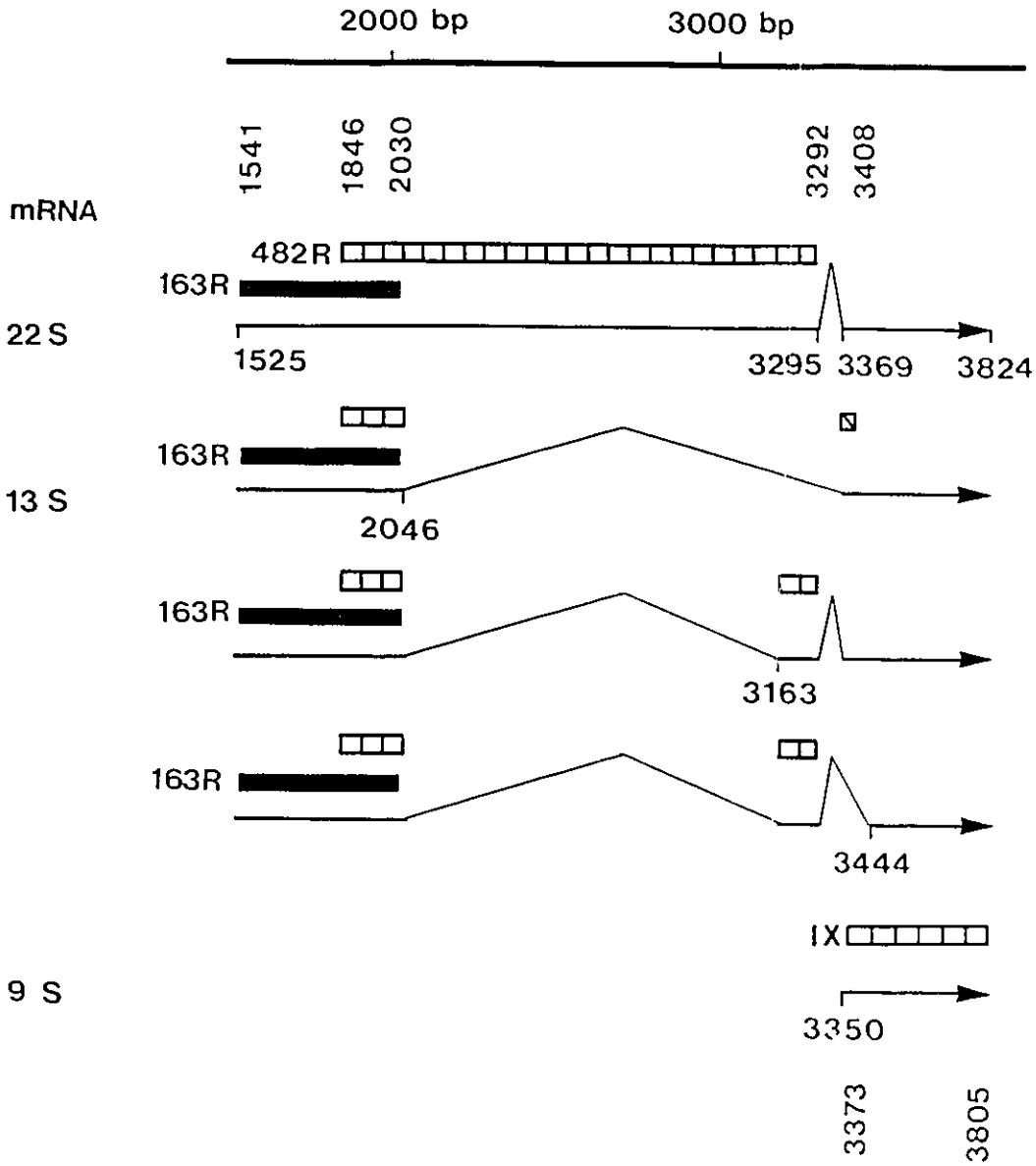
frames using two separate initiation codons, 163R at nucleotide 1541 and 482R at nucleotide 1846. The 13S mRNA of Ad12 also produces 163R and, as in Ad2, is predicted to encode a 482R-related protein using the same initiation codon as 482R. In addition, the E1B region encodes several minor mRNAs which encode 163R and other minor polypeptides related to the 482R protein (Fig. 1-3) (Bos *et al.*, 1981; Saito *et al.*, 1983; van Ormondt and Galibert 1984).

The Ad12 E1B proteins have been identified primarily by *in vitro* translation of hybrid-selected E1B mRNAs which yields two major proteins of 55K and 19K (Saito *et al.*, 1983; Esche *et al.*, 1984; Mak and Mak, 1986). Using tumour antisera derived from tumours induced by E1 transformed cells, 55K appears to correspond to 482R, and 19K to 163R. This has been confirmed partly by using antibodies directed against a synthetic peptide corresponding to the predicted 482R sequence (Schughart *et al.*, 1985). Studies with site-directed mutants, which selectively eliminate AUG codons for 163R open reading frame, have demonstrated that the mutants failed to produce 19K (Edbauer *et al.*, 1988; Zhang *et al.*, manuscript in preparation). Three other E1B proteins of 17K, 15K and 14K were also detected by *in vitro* translation of selected E1B mRNAs (Mak and Mak, 1986) however, their identities have not been characterized. Hereafter, the two Ad12 E1B major proteins will be referred to as 482R and 163R.

The Ad12 E1B 482R protein is phosphorylated at serine and threonine residues in infected and transformed cells (Schughart *et al.*, 1985). Approximately equal amounts of the 482R protein were detected in the cytoplasm and nucleus in infected cells,

Figure 1-3. Structure of the E1B region of adenovirus type 12.

The top line represents viral DNA with nucleotide numbers within the E1B region. The thin lines represent the structure of spliced E1B transcripts. The positions of transcription initiation and termination, and of splice donor and acceptor sites are indicated under the mRNAs. Boxes represent the open reading frames and different reading frames are distinguished by different shadings. The locations of the first nucleotides in the translation initiation and termination codons are indicated by numbers written vertically. The specific mRNAs and two major E1B proteins are also indicated.


2000 bp
3000 bp
1541
1846
2030
3292
3408
mRNA
482R
163R
22 S
1525
3295
3369
3824
163R
13 S
2046
163R
3163
163R
3444
IX
9 S
3350
3373
3805

however, only a small fraction of the protein was found in the nucleus in transformed cells (Grand and Gallimore, 1984; Schughart *et al.*, 1985). Although physical association between the Ad12 482R and p53 proteins has not been demonstrated, there seems to be an interaction between these two proteins, because the half life of the p53 protein is increased in the presence of the 482R protein (Zantema *et al.*, 1985b; Mak *et al.*, 1988)

The Ad12 E1B 163R is acylated and associated with cell membranes (Grand and Gallimore, 1984; Grand *et al.*, 1985). 163R has also been found on the surface of cells, suggesting it can function as an integral membrane protein (Föhring *et al.*, 1983; Smith *et al.*, 1989).

1.10. Functions of E1B

1.10.1. Functions of Ad2 and Ad5 E1B 495R (496R) Proteins

The E1B 495R (496R) protein is required for productive infection of human cells (Harrison *et al.*, 1977; Lassam *et al.*, 1978; Babiss and Ginsberg, 1984; Logan *et al.*, 1984; Bernards *et al.*, 1986; Barker and Berk, 1987). Mutants with disruptions in the 495R (496R) protein are defective in the accumulation of normal levels of viral late mRNA and protein in the cytoplasm of infected cells, resulting in a reduction in virus yield. Since DNA replication, transcription rates and steady-state nuclear RNA species of mutants are normal, it was concluded that reduced levels of cytoplasmic mRNAs resulted from a defect in the transport or in the stabilization of viral transcripts in the

cytoplasm. In contrast to the wild type virus, mutants transport and accumulate cellular mRNAs continuously, suggesting that the E1B 495R (496R) protein is also involved in host cell shutoff (Babiss and Ginsberg, 1984; Babiss *et al.*, 1985; Pilder *et al.*, 1986). Recently, the function of the 495R (496R) protein in the metabolism of viral and cellular mRNAs was defined more precisely to an intranuclear step, after transcription and prior to translocation across the nuclear envelope (Leppard and Shenk, 1989). Moreover, studies with the E4 mutants indicate that physical association between the E1B 495R (496R) protein and E4 34K protein is required for this process (Halbert *et al.*, 1985; Weinberg and Ketner, 1986; Sandler and Ketner, 1989; Bridge and Ketner, 1990).

The E1B 495R (496R) protein is involved in cellular transformation. It was reported that mutants affecting the 495R (496R) coding region are defective for virion-mediated cellular transformation (Graham *et al.*, 1978; Ho *et al.*, 1982; Logan *et al.*, 1984; Babiss *et al.*, 1984b; Bernards *et al.*, 1986; Barker and Berk, 1987). Although mutations in the 3' region of E1B 495R (496R) have no effect on DNA-mediated transformation (McKinnon *et al.*, 1982; Rowe and Graham, 1983), in agreement with results obtained by transfection with a DNA fragment lacking the right end of E1B 495R (496R) gene (van der Eb *et al.*, 1977), some studies indicated that deletion or C-terminal truncation mutants and a mutant lacking the 495R (496R) AUG initiation codon induce cellular transformation less efficiently than wild type virus in DNA-mediated assays (Babiss *et al.*, 1984b; Bernards *et al.*, 1986; Barker and Berk, 1987; McLorie *et al.*, 1991). Moreover, transfection with an expression construct lacking the gene for 495R

(496R) yields transformants at lower efficiency (White and Cipriani, 1990), and a double mutant, preventing synthesis of the 495R (496R) protein, reduces transformation efficiency dramatically in both virion- and DNA-mediated assays (Barker and Berk, 1987). These results indicated that the 495R (496R) protein is necessary for transformation by both virus infection and viral DNA transfection, although the protein needs not be completely intact, especially in DNA-mediated assay. Since 495R (496R) can associate with the tumour suppressor p53 (Sarnow *et al.*, 1982), it is possible that 495R (496R) transforms cells by eliminating such recessive oncogene product. Again the large T antigen of SV40 and the E6 protein of human papillomavirus can also form such complex with p53 (Lane and Crawford, 1979; Linzer and levine, 1979; Werness *et al.*, 1990), providing further evidence that DNA tumour viruses participate in transformation through similar cellular pathways.

1.10.2. Functions of Ad12 E1B 482R Protein

The Ad 12 E1B 482R protein is required for virus growth in human cells since mutants affecting the 482R coding region rarely produce infectious virus (Shiroki *et al.*, 1986; Breiding *et al.*, 1988; Mak and Mak, 1990). These mutants are also defective in production of viral late proteins (Breiding *et al.*, 1988) and accumulation of viral mRNA (Shiroki *et al.*, 1986). In contrast to the Ad5 and Ad2 495R (496R) mutants, Ad12 482R mutants are defective in efficient viral DNA replication, suggesting that all the phenotypes described above might result from secondary effects due to a defect in DNA replication (Shiroki *et al.*, 1986; Breiding *et al.*, 1988; Mak and Mak, 1990). In infected

human embryonic kidney cells, this protein is responsible for the induction of specific and random chromosome damage (Schramayr *et al.*, 1990).

Ad12 E1B 482R seems to be required for transformation of primary rodent cells, as mutants affecting 482R transform these cells very poorly (Byrd *et al.*, 1988; Mak and Mak, 1990). Again, the coding sequences of the 482R gene need not be completely intact (Jochemsen *et al.*, 1982). Shiroki *et al.* (1977, 1986) have suggested that Ad12 E1B 482R is required for the transformation of rat 3Y1 cells using a virion-mediated assay, but not by DNA-mediated assay. Edbauer *et al.* (1988), however, demonstrated that Ad12 E1B 482R was not required for transforming rat 3Y1 cells even in a virion-mediated assay. The apparent discrepancy between two methods of transformation could be due to different requirements for the integrity of 482R, and the different transformation results with rat 3Y1 cells by two different groups might be due to the different 482R mutants and experimental conditions employed.

Ad12 E1B 482R, like E1A, is involved in tumour induction following virus injection (Shiroki *et al.*, 1986; Byrd *et al.*, 1988), and is also a factor contributing to the difference in oncogenic potential between Ad12 and Ad5 transformed cell lines in T cell-deficient nude animals (Bernards *et al.*, 1983c). Rat cell lines established by transformation using mutants defective in 482R are non-tumorigenic when inoculated into animals (Mak and Mak, 1990). Therefore, Ad12 E1B 482R protein appears to be involved in both the initiation and the maintenance of the oncogenicity. Similar apparently clear-cut results were not observed with baby rat kidney and rat 3Y1 cells

transformed by a DNA fragment lacking the coding sequence for intact 482R, suggesting that different cell types may have different tumorigenic potentials after transformation (Shiroki *et al.*, 1977; Jochemson *et al.*, 1982).

1.10.3. Functions of Ad2 and Ad5 E1B 175R (176R) Proteins

The E1B 175R (176R) mutants of Ad5 and Ad2 have been shown to be 10 to 100-fold less efficient for virus growth in human KB and Hela cells (Pilder *et al.*, 1984; Subramanian *et al.*, 1984a, White *et al.*, 1984b; Bernards *et al.*, 1986). However, two other studies found no difference in growth in Hela cells (Barker and Berk, 1987; McLorie *et al.*, 1991). In WI38 cells, such mutants have a substantial growth advantage over the wild-type virus (White *et al.*, 1986), indicating a host range phenotype for the 175R (176R) mutant virus replication in human cells. Mutants of 175R (176R) induce enhanced cytopathic effects in human cells, which result in extensive cellular destruction and usually large clear plaques, and also cause a dramatic degradation of both cellular and viral DNA in KB and Hela cells. Thus they have been designated as possessing *cyt* (cytocidal), *lp* (larger plaques) and *deg* (degradation) phenotypes (Chinnadurai, 1983; Pilder *et al.*, 1984; Subramanian *et al.*, 1984a,b; Takemori *et al.*, 1984; White *et al.*, 1984b; Barker and Berk, 1987). Using double mutants, it has been shown that expression of the 175R (176R) mutant phenotypes is dependent on the expression of either E1A 289R or 243R proteins (White *et al.*, 1987, 1991), and that the phenotypes are host range (White *et al.*, 1986). In the absence of E3 proteins, the 175R (176R) protein is required to protect adenovirus-infected human cells against tumour necrosis

factor (TGF) cytosis, whereas in the presence of E3 it is dispensable (Gooding *et al.*, 1991), suggesting the difference between TNF cytosis and *cyt* phenotype.

The role of the 175R (176R) protein in regulating gene expression remains controversial. Examination of viral gene expression in 175R (176R) mutant-infected human cells suggested that the 175R (176R) protein functions to repress viral gene expression in the presence of E1A proteins, but to activate viral gene expression in the absence of E1A (White *et al.*, 1986, 1988). However, one group reported that the mutant viruses display normal rates of viral gene expression in the absence of the functional 175R (176R) protein, suggesting that 175R (176R) is not involved in gene regulation (Herbst *et al.*, 1988). In contrast, results obtained in transient assays have suggested that the 175R (176R) protein may increase expression from several cotransfected cellular and viral genes (Natarajan *et al.*, 1986; Herrmann *et al.*, 1987; Yoshida *et al.*, 1987) and in fact it has been proposed that effects on transcription result from a 175R (176R)-induced stabilization of transfected plasmid DNA (Herrmann and Mathews, 1989).

The E1B 175R (176R) protein is required for efficient transformation of rodent cells. Viruses containing the mutations within E1B 175R (176R) coding region induce cellular transformation at an efficiency more than 10-fold lower than that of the wild-type virus (Chinnadurai, 1983; Babiss *et al.*, 1984b; Pilder *et al.*, 1984; Subramanian *et al.*, 1984a; Takemori *et al.*, 1984; white *et al.*, 1984b; Bernards *et al.*, 1986; Barker and Berk, 1987). In assays involving transfection of DNA from plasmids containing

mutations in 175R (176R), transforming efficiency was also found to be significantly less than with wild-type virus DNA (Chinnadurai, 1983; Bernards *et al.*, 1986; Barker and Berk, 1987). In recent studies using expression vectors or mutants to express the two major E1B proteins separately, the data suggested that the E1B 175R (176R) and 495R (496R) proteins function via independent pathways in transformation (White and Cipriani, 1990; McLorie *et al.*, 1991). Furthermore, the former authors suggested that the role of 175R (176R) may be to induce perturbations in intermediate filaments.

Among the various 175R (176R) mutants, *lp*3 and *lp*5 were found to produce large plaques and to fail to transform rodent cells efficiently (Chinnadurai, 1983). However, neither mutant induced cytocidal phenotypes (Subramanian *et al*, 1984a) and only *lp*5 caused DNA degradation (Subramanian *et al*, 1984b, White *et al.*, 1984b), suggesting that the various 175R (176R) mutant phenotypes are separable (Subramanian and Chinnadurai, 1986).

1.10.4. Functions of Ad12 E1B 163R Protein

The first suggestion on the function of the 163R protein came from the phenotype induced by a series of Ad12 *cyt* mutants: production of large clear plaques, extensive cellular destruction in both KB and HEK cells (Takemori *et al.*, 1968) and degradation of DNA in infected human KB cells (Ezoe *et al.*, 1981). Complementation assays mapped the mutations to the E1B region (Lai Fatt and Mak, 1982) and sequence analysis located a 107 base pair out-of-frame deletion in the region of the genome encoding the 163R protein in one of the mutants, *cyt*68, indicating that a mutation in the 163R protein

was responsible for the *cyt* and *deg* phenotypes (M. Schaller, personal communication). Recently, Ad12 E1B 163R mutants were isolated directly, and they displayed the *cyt*, *lp* and *deg* phenotypes. However, these mutants were able to grow as efficiently as wild-type virus in infected human cells, indicating that the E1B 163R protein is not involved in viral replication (Fukui *et al.*, 1984; Edbauer *et al.*, 1988). Unlike Ad5 and Ad2 E1B 175R (176R) proteins, Ad12 E1B 163R is not required to protect DNA from degradation in transfected cells. Moreover, it can stimulate expression of beta interferon by activating tandemly repeated hexamer sequences within the promoter (Shiroki *et al.*, 1990).

Ad12 *cyt* mutants are inefficient in transforming primary baby rat kidney cells (Mak and Mak, 1983), and Ad12 insertion and truncation mutants deficient in synthesis of the 163R protein are defective for transformation of rat 3Y1 cells (Fukui *et al.*, 1984). Therefore the Ad12 E1B 163R protein is involved in transformation of rodent cells.

However, mutants in which either the initiation codon for 163R protein had been eliminated or a downstream stop codon introduced were, unlike Ad12 *cyt* and Ad5 or Ad2 175R (176R) mutants, found to be capable of transforming primary BRK cells at wild-type efficiency, although they were defective in transforming 3Y1 cells (Edbauer *et al.*, 1988).

Transformation-defective *cyt* mutants are non-tumorigenic when inoculated into hamsters (Takemori *et al.*, 1968; Fukui *et al.*, 1984), but cells transformed by these mutants, including *cyt*68 which does not produce the 163R protein, are highly

tumorigenic (Mak *et al.*, 1984). Therefore the Ad12 E1B 163R is involved in the initiation of tumour induction, but it seems not to be required for the maintenance of the tumorigenic state (Mak and Mak, 1986). Since two transformation-positive *cyt* mutants lacking the 163R AUG initiation codon or possessing a downstream stop codon are tumorigenic following virus injection (Edbauer *et al.*, 1988), it seems that the function of the 163R protein is not absolutely necessary in initiation of tumour induction (Edbauser *et al.*, 1988).

1.11. Project Description

To examine the role of the Ad12 E1B 163R protein more thoroughly, in particular to determine the transforming function of the 163R protein, I have constructed a series of mutants by oligonucleotide-directed mutagenesis with lesions at various positions across the 163R coding sequence, including a mutant that lacks the AUG initiation codon for 163R. The results indicated that many of these mutants yielded unstable 163R-related products, induced DNA degradation and enhanced cytopathic effect (*cyt/deg* phenotype) in infected KB cells, and transformed primary rodent cells at efficiencies significantly lower than wild-type Ad12. Elimination of the initiation codon for 163R resulted in overexpression of the E1B-482R product and a normal transforming efficiency. These results suggested that 163R plays a role in transformation but that normal transforming efficiencies can be obtained in its absence if a sufficient quantity of 482R is produced.

To define the functional role of Ad12 E1B 163R and 482R in viral gene expression, viral mRNA accumulation from infected cells was assayed by Northern blot analysis. The results demonstrated that the E1B 163R protein does not play a crucial role in modulating viral gene expression. However, a 482R mutant lacking residues 114-155 produced greatly reduced levels of both early (E2B and E4) mRNAs and late proteins, and was defective for viral DNA replication, indicating that the 482R protein plays a role in viral early mRNA metabolism resulting in secondary effects in DNA replication and late protein synthesis.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Recombinant DNA Techniques

2.1.1. Enzymes

Restriction enzymes: Restriction enzymes were obtained from a variety of commercial suppliers. Reactions were carried out in buffer supplied by the manufacturers or prepared according to Maniatis *et al.* (1982). Restriction enzymes were added to the reaction in sufficient amounts (2-5 units/μg DNA) to ensure complete digestion and the mixtures were incubated at 37°C, except for *Bst*E II which was at 60°C for 2-3 hours. Multiple-enzyme digestion was performed with two or more enzymes simultaneously if the requirements of the reaction buffers and incubation temperatures were the same. Otherwise, such reactions were done separately, with low salt buffer first. When necessary the enzymes were removed by phenol/chloroform extraction followed by ethanol precipitation in the presence of carrier tRNA if the amount of DNA was less than 10 μg.

T4 DNA ligase: DNA ligations were carried out in 20 μl of reaction buffer either supplied by the manufacturer [50 mM Tris (pH 7.6) containing 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol (DTT), and 5% PEG-8000] or prepared according to Maniatis

et al. (1982) [66 mM Tris (pH 7.6) containing 6.6 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP] with 0.1 unit of T4 DNA ligase and 10 ng of vector DNA plus the appropriate DNA fragment at a 3 molar excess to the vector.

T4 polynucleotide kinase: T4 polynucleotide kinase was used to phosphorylate the 5' ends of DNA fragments (single-stranded synthetic oligonucleotides in these studies) in the presence of ATP or to label the oligonucleotides in the presence of [γ-^{32}P]ATP (NEN, 3000 Ci/mmole, 10 mCi/ml). Typically, 200 pmol of oligonucleotide were phosphorylated in 20 μl of kinase buffer [100 mM Tris (pH 8.0) containing 10 mM $MgCl_2$, and 10 mM DTT] with 4.5 units of T4 polynucleotide kinase and 0.5 mM ATP at 37°C for 45 minutes. The reaction was stopped by heating at 65°C for 10 minutes. Alternatively, 20 pmol of oligonucleotide was labelled in 20 μl kinase buffer with 4.5 units of T4 polynucleotide kinase in the presence of 20-50 μCi of [γ-^{32}P]ATP. The labelled oligonucleotides were separated from unincorporated [γ-^{32}P]ATP using a DEAE-cellulose (Whatman DE-52) column. The column was equilibrated with TE buffer containing 0.1 M NaCl. The free [-^{32}P]ATP was washed out with 0.3 M NaCl and the labelled oligonucleotide was collected by washing the column with 1 M NaCl (Zoller and Smith, 1984). An aliquot of the labelled oligonucleotide was diluted in water and the amount of radioactivity determined by liquid scintillation counting.

Alkaline phosphatase: Calf intestinal alkaline phosphatase (CIP) was used to remove the free 5' phosphates in DNA fragments to prevent self-ligation. In this report, 50 μg of *Bst*E II digested Ad12 DNA was dephosphorylated with 10 units of CIP in 50

μl of 50 mM Tris (pH 8.0) containing 0.1 mM EDTA at 37°C for 30 minutes. The reaction was stopped by heating at 68°C for 15 minutes in 100 mM Tris (pH 8.0) containing 1 mM NaCl, 10 mM EDTA, and 0.5% SDS. DNA was extracted with phenol and chloroform, and was precipitated by ethanol (Maniatis *et al.*, 1982).

DNA polymerase I and DNase I: To label double stranded DNA, 1 μg of DNA was nicked with 0.5 ng of DNase I and nick-translated for 60 minutes at 16°C with 5 units of *E. coli* DNA polymerase I in 50 μl of nick-translation buffer [50 mM Tris (pH 7.2) containing 10 mM $MgSO_4$, 0.1 mM DTT, and 50 μg/ml bovine serum albumin (BSA) and supplemented with 20 μM dATP, dTTP and dGTP, and 100 μCi of [α-^{32}P]dCTP (ICN, 5000 Ci/mmol, 10 mCi/ml). The reaction was stopped by addition of 2 μl of 0.5 M EDTA and the labelled DNA separated from unincorporated [α-^{32}P]dCTP by centrifugation through a Sephadex G-50 column (Maniatis *et al.*, 1982).

Klenow fragment of DNA polymerase I: The Klenow fragment of DNA polymerase I, consisting of a single polypeptide chain produced by cleavage of intact DNA pol I, was used to extend the DNA chain in making DNA probes, oligonucleotide-directed mutagenesis and DNA sequencing as described below. To label DNA, 1 μg of template DNA was mixed with 8 ng of M13 universal primer in 10 μl of reaction buffer [10 mM Tris (pH 7.6) containing 60 mM NaCl and 6 mM $MgCl_2$] and boiled for 5 minutes. After cooling for 45 minutes at room temperature, the DNA was extended at room temperature for 30 minutes using 1 unit of Klenow fragment in a final volume of 20 μl containing 0.5 M dATP, dTTP and dGTP, 50 μCi of [α-^{32}P]dCTP, and 3 mM

DTT (Meinkoth and Wahl, 1984). The reaction was stopped by adding EDTA and the probe purified on a Sephadex G-50 column (Maniatis *et al.*, 1982).

2.1.2. Cloning Vectors

pBR322 has one ColEI replication origin, two selective markers, ampicillin and tetracycline resistance, and a variety of single recognition sites for different restriction enzymes, which are useful for the insertion of foreign DNA fragments (Bolivar *et al.*, 1977).

M13mp18 derived from ssDNA M13 bacteriophage contains a portion of the *lac* Z (β-galactosidase) gene plus an upstream regulatory gene. A polylinker cloning site was inserted into the *lac* Z gene for cloning purposes. In the presence of the inducer, e.g. isopropyl-β-D-thiogalactosidase (IPTG), a fragment of the β-galactosidase will be produced from the M13mp18 vector and therefore display intra-allelic (α) complementation with a defective form of β-galactosidase encoded by the host, resulting in conversion of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) to a blue product (Messing, 1983).

pUC118, a recombinant between pBR322 and M13mp18, possesses both replication origins, selective markers (*amp*^{r} and *lac* Z), and *cis* elements for packaging from the parental vectors. Therefore it can be maintained as a dsDNA plasmid, or replicated and packaged as a ssDNA phage in the presence of the helper phage, M13K07, which provides the necessary M13 proteins (Vieira and Messing, 1987).

2.1.3. Plasmids

pHAB6 is a pBR322-derived plasmid in which a fragment of the Ad12 (Huie) sequences, approximately 5573 bp from the left end, were inserted into the *Bam*H I site of pBR322 (R. B. Lai Fatt, unpublished). **pHAB80** was made by inserting a portion of the Ad12 E1 region extending from the 0 to 8.2 mu (the left 2809 bp fragment) into the *Bam*H I site of pBR322 (M. Schaller, unpublished). **pHA5** has the Ad12 E1A region, approximately 1400 bp from the left end, substituted for the pBR322 sequences between the *Acc* I and the *Bam*H I sites (Mak *et al.*, 1986). **pHB14R** and **pHB15R** contain the E1B sequence of strain Huie extending from nucleotide 1594 to 2371 and 2317 to 3706 inserted between the *Acc* I and the *Hin*d III sites and into the *Hin*d III sites, respectively, of pBR322 (S. Mak, unpublished). **pUC118/E4r** has the E4 region, extending from the 100 (*Bam*H I) to 90.2 (*Hin*d III) mu, substituted for the sequence in the polylinker of pUC118 between *Hin*d III and *Bam*H I sites (U. Sankar, unpublished). **pHMαA-1** has a full-length cDNA for human skeletal α-actin cloned between the *Hin*d III and *Pvu* II sites of pBR322 and can hybridize to all actin sequences under moderate hybridization conditions (Gunning *et al.*, 1983).

Other plasmids are described in detail in Chapter 3.

2.1.4. Bacteria

Three strains of *Escherichia coli* were used as hosts in various cloning experiments. They are **LE392** (F^- *hsdR514* (r_k^-,m_k^-) *supE44 supF58 lacY1* or Δ(*lacIZY*)6 *galK2 galT22 metB1 trpR55*), **JM103** (Δ(*lac pro*) *thi strA supE endA sbcB hsdR*$^-$ F'*traD36 proAB*$^+$ *lacI*^{q} *Z*Δ*M15*), and **MV1184** (Δ(*lac-proAB*) (*srl-recA*)*306::Tn10*(*tet*^{r}) (

ϕ80*lacZ*Δ*m15*) F'*traD36 proAB*$^+$ *lacI*^{q} *Z*Δ*M15*). All bacteria were maintained on the surface of agar media at 4°C for temporary storage or in 20% glycerol at -70°C for long-term storage. To recover bacteria from frozen stocks, cells were inoculated into a small volume of growth medium, or streaked on M9 (see below) plates to select for colonies containing F' episomes which were then inoculated into a growth medium.

Three types of media were used in these studies. M9 minimal medium was prepared by mixing 1 M $MgSO_4.7H_2O$, 1% vitamin B1, 10 mM $CaCl_2$, 20% glucose and M9 salt solution (Na_2HPO_4, KH_2PO_4, NaCl and NH_4Cl) together to a final concentration of 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$, 1 mM vitamin B1, 0.1 mM $CaCl_2$, and 0.2% glucose. M9 plates were then prepared by adding bacto agar to the medium at a final concentration of 1.5%. YT broth (0.5% yeast extract, 0.8% bacto tryptone, and 85 mM NaCl) was used for the growth of JM103 and MV1184, and Luria broth (0.5% yeast extract, 1% bacto tryptone, 85 mM NaCl, and 0.1% glucose) for LE392. To make agar plates, bacto agar was added to the liquid medium at a final concentration of 1.5% for the YT plate or 1.8% for the LB plate. To make top agar for bacteriophage plaquing, bacto agar was added to the YT broth at 0.6%.

Ampicillin was used at a final concentration of 50 μg/ml to select for the bacteria harbouring plasmids with ampicillin resistance. Kanamycin at a final concentration of 70 μg/ml was used to select bacteria infected with M13K07 which has kanamycin resistant marker.

2.1.5. Transformation of *E. coli*

Preparation of competent cells: 1.0 ml of overnight bacterial culture was inoculated into 100 ml of broth and incubated until it reached mid-log phase as determined by optical density (Maniatis *et al.*, 1982). After chilling the culture on ice for 10 minutes, the cells were pelleted, and resuspended in half of the original culture volume of 50 mM $CaCl_2$ and 10 mM Tris (pH 8.0). After incubating on ice for 15 minutes, the cells were collected again by centrifugation and resuspended in 4 ml of 50 mM $CaCl_2$ and 10 mM Tris pH (8.0).

Transformation of competent cells: 40 ng of ligated DNA was added to about 0.2 ml of a competent bacterial suspension. After incubation on ice of 30 minutes for absorption of the DNA, the cells were heat-shocked at 42°C for 2 minutes.

If pBR322 recombinant DNA was used, the cells were incubated in 1.0 ml of broth at 37°C for 1 hour to allow them to recover and to express antibiotic resistance. After a serial dilution, the transformed cells were spread onto plates containing the appropriated antibiotic, ampicillin in these studies, and they were incubated at 37°C overnight. Single colonies were picked up for further analysis (Maniatis *et al.*, 1982).

If the pUC118 recombinant was used, cells were treated in a similar fashion except that IPTG and Xgal were added into the ampicillin plate to select for white recombinant colonies (Vieira and Messing, 1987).

If RF DNA of the M13 recombinant was used, varying amounts of transformed cells were mixed with 0.2 ml of plating bacteria, a four hour culture of a fresh

inoculation. Then 3 ml of top agar together with IPTG and Xgal was added to the cells and the mixture poured onto YT plates. After hardening of the top agar, the plates were incubated at 37°C overnight and white recombinant plaques were picked for further analysis (Messing *et al.*, 1983).

2.2. Oligonucleotide-directed Mutagenesis

2.2.1. Mutagenic Primers

All mutagenic primers were synthetic oligonucleotides harbouring base conversions or deletions near the middle of the primers (see Table 3-1) and were purchased from the Central Facility of the Institute for Molecular Biology Biotechnology, McMaster University. The primers were dissolved in the 0.1 mM Tris (pH 8.0) buffer and stored at -20°C.

2.2.2. Oligonucleotide-directed Mutagenesis

Oligonucleotide-directed mutagenesis was performed by using the method described by Zoller and Smith (1984) with minor modifications. 1.4 pmol of recombinant M13mp18 ssDNA template containing the Ad12 E1 sequence was mixed with 20 pmol of phosphorylated mutagenic primer(s) and 0.7 pmol of *Bam*H I- and *Hin*d III-digested M13mp18 dsDNA in 10 μl of annealing buffer [20 mM Tris (pH 7.5) containing 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT]. After boiling for 5 minutes, the mixture was cooled down to room temperature. DNA was then extended and ligated at 15°C overnight in 20 μl of 20 mM Tris (pH 7.5) containing 10 mM $MgCl_2$, 25 mM

NaCl, 10 mM DTT, 0.5 mM dNTPs, and 0.5 mM ATP with 3 units of T4 DNA ligase and 2.5 units of Klenow fragment of DNA polymerase I. After dilution with water the DNA was ready for transformation.

2.3. Tissue Culture Techniques

2.3.1. Cells

2.3.1.1. Human KB cells: Human KB cells were maintained as monolayers at 37°C in a humid atmosphere of 5% CO_2 in minimal essential medium (MEM) (GIBCO 410-1100) (referred to as F11 medium) containing 10% calf serum. Confluent monolayers were subcultured by scraping cells from the glass surface and seeding aliquots to new cultures. Prior to infection, KB cells were expanded in suspension culture in Joklik's modified MEM (GIBCO 410-1300) supplemented with 5% horse serum with constant stirring, and maintained at a density of 2.5 to 5 x 10^5 cells/ml by periodic dilution with fresh medium. Except for Joklik's modified medium, all the media used in these studies contained 50 units/ml penicillin and 50 μg/ml streptomycin.

2.3.1.2. Human MH12-C2 cells: Human MH12-C2 cells, a line of human embryonic kidney cells transformed by a DNA fragment of Ad12 (strain Huie) and expressing E1A and E1B proteins (Mak and Mak, 1990), were maintained at 37°C supplemented with 5% CO_2 as monolayers in α-MEM (GIBCO 410-2000) containing 10% calf serum. Confluent monolayer of cells were washed with citrate saline (0.134 M KCl and 15 mM sodium citrate) and treated with 0.125% Bacto-trypsin (GIBCO) in

citrate saline. The detached cells were seeded into fresh plastic dishes.

2.3.1.3. Primary baby rat kidney (BRK) cells: Primary baby rat kidney cells were prepared from 7- to 10-day-old Wistar or Chester Beatty rats. Kidneys were explanted from the etherized rats and they were well washed with citrate saline and finely minced before incubation in 20 ml of 0.25% trypsin in citrate saline at 37°C for 15 minutes. Released individual cells were transferred to the α-MEM containing 10% fetal calf serum to inactivate trypsin. Undigested tissue was subjected to trypsinization repeatedly until all the cells were released. The cells were collected by centrifugation at 300 g for 10 minutes at room temperature and resuspended in 50 ml of fresh medium. After an incubation at 37°C for 15 minutes, the cells were filtered through cheese cloth to remove the viscous large debris. The cells were then seeded into 60-mm plastic dishes (12 dishes per pair of kidneys) and cultured at 37°C overnight in α-MEM containing 10% fetal calf serum. The cell monolayer reached 80% confluence (approximately 2 x 10^6 cells/dish) and the medium was changed 4 hours prior to infection.

2.3.2. Viruses

Wild type strain of adenovirus type 12 (strain Huie, M8) was isolated from a single plaque of Ad12-Huie obtained from M. Green. The viruses, *pm*1852, *dl*17 and *dl*42, were constructed by I. Mak (Mak and Mak, 1990) and virus *in*700 was provided by J. Williams (Edbauer *et al.*, 1988). Ad12 163R mutants were constructed as described in this thesis.

2.3.3. Virus Infection

KB cells in suspension culture were concentrated by centrifugation at 300 g for 10 minutes and resuspended in Joklik's modified MEM supplemented with 1% horse serum to a concentration of 1 x 10^7 cells per ml. Cells were infected at a multiplicity of infection of 400 virions per cell except when specified otherwise, and absorption was carried out for 90 minutes at 37°C while gently agitating with a stir bar or by rotating on a rolling wheel at 48 rpm. For co-infections, viruses were pre-mixed and then added to the cells. The cells were then cultured in α-MEM containing 10% calf serum on plastic dishes or in Joklik's modified MEM supplemented with 5% horse serum in suspension culture at a density of 3 x 10^5 cells per ml for virus propagation.

MH12-C2 cells were infected in monolayer at a multiplicity of infection of 400 virions per cell in Joklik's modified medium containing 1% horse serum which just covered the monolayer. After absorption at 37°C for 90 minutes with tipping each 15 minutes, the cells were fed with α-MEM containing 10% calf serum.

2.3.4. Virus Preparation

Except for preparation of wild type virus which was done in KB cells, all mutant virus stocks were propagated in MH12-C2 cells. After 48 to 72 hours after infection, the cells were harvested by centrifugation at 400 g for 15 minutes and resuspended in 10 mM Tris (pH 8.1) at 2 x 10^7 cells per ml. The cells were then lysed by sonication for 1 minute and the extent of lysis was checked by microscopic observation. After mixing with an equal volume of freon-113 (1,1,2-trichlorotrifluoroethane), the suspension was homogenized at 4°C for 1 minutes and then subjected to centrifugation for 2 minutes at

1100 g. The aqueous phase was removed and extracted with freon-113 again until it became clear. Viruses were concentrated from the aqueous phase by sedimentation at 67,250 g for 90 minutes onto a CsCl cushion of a density of 1.44 g/ml. The virus band was removed and the density of the suspension adjusted to 1.34 g/ml. After isopycnic banding at 97,600 g for 24 hours, the virus band was collected from the CsCl gradient and the concentration of the virus solution was estimated by measuring the absorbance of the solution at 260 nm with one OD corresponding to 4×10^{11} virions/ml (Green and Pina, 1963). All viruses were diluted in virus storage buffer, 1 x TBS [30 mM Tris (pH 7.4) containing 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 5.6 mM glucose] plus 25% glycerol, and stored at -70°C.

2.3.5. Plaque Assay to Determine Virus Yields in Infected KB Cells

At various times after infection, infected KB cells were harvested, and separated from the medium by centrifugation at 400 g for 10 minutes. The cells were resuspended in virus storage buffer and intracellular viruses were released by freezing and thawing 5 times. An aliquot of medium was also collected and diluted in virus storage buffer as a source of extracellular viruses. The virus titre was determined by plaque assays in which MH12-C2 cells were infected with an aliquot of the virus suspension. After absorption, the cells were cultured in α-MEM supplemented with 10% calf serum for 6 hours and overlaid with 5 ml of 0.9% bacto agar in α-MEM containing 5% calf serum. After six days the dishes were overlaid again and after 15 days after infection, the visible plaques were counted.

2.4. Recombinant Virus Techniques

2.4.1. Ligation of the Mutated E1 Sequence With Parental Viral DNA

5 μg of DNA from the pHAB recombinants harbouring various mutations were digested with *Bam*H I and *Bst*E II. 10 μg of Ad12 (Strain Huie) DNA were digested with *Bst*E II, which only cut Ad12 DNA once at nucleotide 3443 (Fukui *et al.*, 1984), and then dephosphorylated with CIP. They were then mixed and ligated with 10 units of T4 DNA ligase as described before (see 2.1.1).

2.4.2. Transfection of MH12-C2 Cells

The ligated Ad12 DNA molecules were introduced into MH12-C2 cells by transfection as described by Graham *et al.* (1980). 15 μg of ligation mixture DNA was mixed with 10 μg of carrier salmon sperm DNA in 1 ml of TE buffer (pH 7.1) containing 250 mM $CaCl_2$. The DNA solution was then added to 1 ml of 2 x HEPES buffered saline (prepared by dissolving 8.0 g of NaCl, 0.37 g of KCl, 0.1 g of Na_2HPO_4, 5.0 g of HEPES and 1.0 g of glucose in 500 ml of H_2O, pH 7.2) while bubbles were introduced from the bottom of tube. After 30 minutes the DNA precipitate was added to 6 dishes (60 mm) of MH12-C2 cells at about 80-90% confluence. After incubating at 37°C for 6 hours, the cells were overlaid with bacto agar in α-MEM as described before (see 2.3.5). Plaques formed in about three weeks and were picked for preparation of viral stocks.

2.4.3. Plaque Purification

Individual plaques were picked with a Pasteur pipette and resuspended in 200 μl

of virus storage buffer. After freezing and thawing 5 times, half of the virus solution was used to grow the virus for screening the mutant by dot-blot hybridization and restriction analysis (see below). The other half was diluted and used to infected MH12-C2 for further plaque purification (see 2.3.5). Each of the mutants was plaque purified three times.

2.5. Preparation and Analysis of DNA

2.5.1. DNA Preparation

2.5.1.1. Isolation of plasmid DNA: Plasmid DNA was extracted using the alkaline SDS lysis described by Birnboim and Doly (1979). A 1.5 ml of bacteria overnight culture was pelleted using a bench-top microcentrifuge (Eppendorf) at 15,000 rpm for 1 minute and resuspended in 100 μl of lysozyme solution [25 mM Tris (pH 8.0) containing 10 mM EDTA, 50 mM glucose, and 10 mg/ml lysozyme]. The resuspended pellet was incubated on ice for 30 minutes and then 200 μl of alkaline SDS (0.2 N NaOH, and 1% SDS) was added and mixed gently by inverting the tube. After an incubation on ice for 5 minutes, the mixture was neutralized by adding 150 μl of 3M Na acetate (pH 4.8) and mixed by inverting the tube quickly. After a further 1 hour on ice, the cellular DNA was removed by centrifugation and plasmid DNA was recovered from the supernatant by ethanol precipitation which was repeated once. The DNA pellet was dried, resuspended in double-distilled water or TE buffer.

2.5.1.2. Isolation of RF DNA: After incubating bacteria picked from a single

plaque in 2 ml of YT broth for 4 hours at 37°C, the culture was inoculated with 0.2 ml of a mid-log phase bacterial culture and incubated for a further 4 hours. The bacteria were pelleted and RF DNA was isolated by the method described above, while the supernatant was stored at 4°C as a source of recombinant phage for infection or for small scale ssDNA isolation.

2.5.1.3. Large scale purification of plasmid or RF DNA: DNA was isolated from 500 ml of culture as described above. CsCl and ethidium bromide were added into the DNA solution to the final concentrations of 1 g/ml and 0.25 g/ml, respectively. After centrifugation for 36 hours at 55,000 rpm at 15°C in a Beckman Type-50 or Type-65 rotor, the supercoiled DNA was collected through a 21 gauge needle into a tube. Ethidium bromide was removed by extracting with iso-amyl alcohol saturated with water, and CsCl removed by dialysis against several changes of TE buffer (Maniatis *et al.*, 1982).

2.5.1.4. Isolation of single stranded M13 or recombinant M13 DNA: Single stranded M13 DNA was isolated by the method described by Messing (1983). The phages were aggregated from 300 μl of the phage supernatant by adding one-third volume of 27% polyethylene glycol (PEG) 6000 and 3.3 M NaCl. After an incubation at 4°C overnight, the phages were pelleted at 20,000 g for 10 minutes and resuspended in TE buffer. DNA was extracted once with buffered phenol and once with ether. After precipitation with ethanol, DNA was dissolved in H_2O and was ready for screening by dot-blot hybridization. Large scale isolation of ssDNA was achieved by inoculating

thirty ml of YT medium with 1 ml of overnight JM103 culture and 600 μl of the phage supernatant. After a 7 hour incubation at 37°C, the cells were removed by centrifugation at 16,000 g for 10 minutes. Supernatants were subjected to precipitation by PEG 6000. DNA was extracted once with phenol, phenol/chloroform saturated with 0.1 M Tris (pH 8.0) and twice with chloroform and ether. After precipitation with ethanol, DNA was ready as a template for mutagenesis and sequencing.

2.5.1.5. Isolation of single stranded pUC118 DNA: YT broth containing ampicillin and 0.001% thiamine was inoculated at 1:100 dilution with an overnight culture of bacteria harbouring pUC118. After an incubation to mid-log phase, 2 ml of cells were removed and infected with phage M13K07 at a multiplicity of 10 plaque forming units per cell, assuming that 1 O.D. culture is equivalent to 1 x 10^8 cells per ml. After an incubation for 1 hour at 37°C, 400 μl of the culture were transferred to 10 ml of fresh YT broth containing 70 μg/ml kanamycin and further incubated for 12 hours (Vieira and Messing, 1987). Cells were pelleted, and supernatants were removed and precipitated with PEG 6000 and then DNA was isolated as described above.

To preparing M13K07 stock for superinfection as described above, M13K07 was streaked onto a YT plate and covered with 4 ml of soft agar containing 0.5 ml of saturated JM103 culture. After an incubation overnight at 37°C, single plaques were picked and YT broth containing 70 μg/ml kanamycin were inoculated with the plaques. After incubating the culture for 14 hours, the cells were pelleted and the supernatant was titrated on JM103 plate.

2.5.1.6. Isolation of virion DNA: The viruses collected from CsCl banding (see section 2.3.4) were dialysed against 10 mM Tris (pH 8.1) at 4°C to remove CsCl and incubated at 37°C for 1 hour with 1% SDS and 0.8 mg/ml pronase in 150 mM sodium phosphate (pH 6.0) containing 7.5 mM EDTA. After extracting the virus suspensions three times with phenol saturated with 50 mM sodium phosphate (pH 6.8), the aqueous phase was removed and dialysed against 10 mM Tris (pH 7.5). The concentration of DNA was estimated by measuring the absorbance of the DNA solution at 260 nm with one OD corresponding to 50 μg of DNA per ml.

2.5.1.7. Isolation of viral DNA from infected cells: Infected cells at various times after infection were collected, washed three times with 1 x phosphate buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4), resuspended in lysing buffer [10 mM Tris (pH 7.9) containing 5 mM EDTA, 100 mM NaCl, 0.5% SDS, and 1 mg/ml pronase] and incubated at 37°C for 2 to 4 hours.

For analysis of small molecular weight DNA, the concentration of NaCl in the lysate was adjusted to 1 M and the suspension put at 4°C overnight. Cellular DNA was selectively removed by centrifugation at 15,000 rpm in a microcentrifuge (Eppendorf) at 4°C for 10 minutes (Hirt, 1967). An aliquot of the supernatant containing small molecular weight DNA was removed and subjected to RNase digestion followed by gel electrophoresis for the DNA degradation assay. Alternatively, the supernatant was digested with restriction enzyme before gel electrophoresis.

For analysis of virus DNA replication, the lysate was sonicated for 30-120

seconds and after phenol extraction and ethanol precipitation, DNA was dissolved in TE buffer and digested with RNase. Then DNA was extracted twice with phenol, phenol/chloroform saturated with 0.1 M Tris (pH 8.0) and chloroform. After precipitation by ethanol, DNA was dissolved in H_2O and the amount measured as described in section 2.5.1.6.

2.5.2. Agarose Gel Electrophoresis of DNA

2.5.2.1. Electrophoresis of DNA: 1% agarose gels were prepared in Tris-borate EDTA (TBE) buffer (0.1 M Tris, 0.083 M boric acid, and 0.001 M EDTA), according to Maniatis *et al.* (1982). DNA in 6.7% sucrose containing 4% bromophenol blue was applied and electrophoresis was carried out at 1.0 V/cm.

2.5.2.2. Visualizing DNA in the gels: The fluorescent dye ethidium bromide was used to visualize DNA under UV-irradiation (260 nm and 360 nm). Ethidium bromide (0.5 μg/ml) was either added into the gels and the running buffer or into water to stain the gels after electrophoresis (Maniatis *et al.*, 1982).

2.5.2.3. Isolation of DNA fragments: Digested DNA fragments were separated by gel electrophoresis. DNA bands were stained with ethidium bromide (0.5 μg/ml) in water and visualized under weak UV light. The desired band was removed, chopped into pieces, and placed in the electroelution apparatus (IBI) according to the manufacturer's instructions. DNA was eluted into a salt cushion (7.5 M ammonium acetate containing 0.01% bromophenol blue). The neutralized DNA molecules were removed from the salt cushion by two ethanol precipitation and then dissolved in distilled water.

2.5.3. DNA Sequencing

2.5.3.1. Sanger Technique: DNA sequencing was performed by the chain termination procedure described by Sanger *et al.* (1977) using a sequence kit from BRL.

2.5.3.2. Sequencing gels: The glass plates were pretreated as follows. The short plate was treated with bind-silane solution (0.3% [v/v] 3-(trimethoxysilyl) propyl methacrylate [Aldrich]and 0.3% [v/v] glacial acetic acid prepared in absolute ethanol) to facilitate binding of polyacrylamide gels to the plate. The longer plate was treated with repel-silane (3% [v/v] dichloro-dimethyl silane [Kodak] prepared in 1,1,1-trichloro-ethane [Caledon]) to prevent binding of the gels. Gels containing 6% or 8% polyacrylamide with an acrylamide to N,N'-methylene-bis-acrylamide (bis) ratio of 19:1 and 7 M urea were prepared in 1 x TBE buffer (see 2.5.2.1). Ammonium persulfate was added to 0.1% and TEMED to 0.03%. The solution was polymerized between the pretreated glass plates.

2.5.3.3. Electrophoresis: The sequencing gels were run on an IBI sequencing apparatus with an aluminum thermal plate to distribute heat evenly during electrophoresis. The gels were pre-heated by running at 45 V/cm for 1 hour. 5 μl of reaction mixture together with 9 μl of loading buffer (0.1% bromophenol blue and 0.15 xylene cyanol in formamide) was boiled, and an aliquot loaded on the gel and run at 50 V/cm for a few hours. After electrophoresis, the gels were fixed in 10% glacial acetic acid, dried and exposed to a Kodak X-ray film.

2.5.4. DNA Hybridization

2.5.4.1. Dot-Blot hybridization: dsDNA, linearized, boiled and cooled down on ice, or ssDNA was spotted directly onto a nitrocellulose filter and then baked at 80°C for at least half an hour. The filter was prehybridized in 6 x SSC (1 x = 0.15 M NaCl and 0.015 M sodium citrate), 10 x Denhardt's solution (0.02% BSA, Polyvinylpyrolidone and Ficoll) and 0.2% SDS at 67°C for 1 hour. Then the filter was hybridized with labelled mutagenic oligonucleotide (see Table 3-1) in 6 x SSC and 10 x Denhardt's solution at room temperature for 1 hour. After washing with 6 x SSC at room temperature and at higher temperatures, the filter was exposed to X-ray film.

2.5.4.2. Slot-Blot hybridization: Equivalent amounts of DNA, adjusted by the addition of carrier DNA (sonicated salmon sperm DNA or calf thymus DNA), was denatured in 0.3 M NaOH at 65°C for 1 hour. After neutralizing with ten volumes of 1 M ammonium acetate (pH 7.0), the DNA was blotted onto a nitrocellulose filter using a slot blot apparatus (Schleicher and Schuell, Inc). The filter was then baked as described above and incubated in prehybridization solution [50% formamide, 5 x Denhardt's solution, 0.1% SDS, 5 x SSPE (0.9 M NaCl, 50 mM NaH_2PO_4 and 5 mM EDTA, pH 6.8), and 100 μg/ml carrier DNA] at 42°C for a few hours. The filter was then hybridized with probes in the same fresh solution at 42°C overnight. After washing twice in 1 x SSC and 0.1% SDS for 5 minutes at room temperature, the filter was washed twice in 0.1 x SSC and 0.1% SDS at 74°C for 30 minutes and exposed to X-ray film.

2.5.4.3. Quantitation of radioactivity hybridized to DNA: The amount of

radioactivity present in hybridized DNA was measured by scanning the autoradiograms with a Laser Densitometer (LKB 2222-020 Ultrascan XL). The relative intensity of the blot was converted to an absorption curve and then the relative area of the peak under the curve, representing the amount of specific DNA, was integrated and presented as an absolute unit (AU*mm) which was then plotted versus the corresponding DNA concentration. For each sample, one value was calculated from the curve and normalized based on 5 mg of total DNA. The level of DNA synthesis over various times after infection was then obtained.

2.6. Preparation and Analysis of RNA

2.6.1. Isolation of RNA From Infected Cells

2.6.1.1. Separation of the cytoplasmic fraction from nuclei: Infected cells were harvested, washed three times with 1 x PBS and resuspended at 10^7 cells per ml in NP40 lysis buffer [10 mM Tris (pH 7.4) containing 10 mM NaCl, 3 mM $MgCl_2$, and 0.7% NP40]. After 10 minutes on ice with constant shaking, this mixture was centrifuged at 500 g for 5 minutes, and the suspension was removed and used for isolation of cytoplasmic RNA. The nuclear pellet was washed with lysis buffer for one or two times and used for isolation of nuclear RNA (Greenberg and Ziff, 1984). Guanidine isothiocyanate solution was added to the cytoplasmic and nuclear fractions to a final concentration of 4 M guanidine isothiocyanate, 0.025 M sodium acetate (pH 6), and 0.1 M β-mercaptoethanol. At this stage, the cytoplasmic suspension and nuclear

lysate could be frozen at -70°C.

2.6.1.2. Purification of RNA: The suspension or lysate was layered onto 5 ml of 5.7 M CsCl in 0.025 M sodium acetate (pH 6) and sedimented at 174,000 g at 20°C for 21 hours. The RNA pellet was dissolved in 0.3 M sodium acetate (pH 6) and precipitated by ethanol after which it was resuspended in water, incubated at 65°C for 15 minutes with pipetting to dissolve the RNA completely, and finally diluted to 2 μg/μl (assuming 1 OD = 45 μg/ml) (Davis *et al.*, 1986).

2.6.1.3. Glassware and plasticware: Sterile disposable RNase-free plasticware was used wherever possible. Glassware was pretreated by baking at 250°C for 4 hours or more. All the solutions (except those containing Tris) and water were treated with 0.1% diethylpyrocarbonate for at least 12 hours and autoclaved to inhibit RNase (Maniatis *et al.*, 1982).

2.6.2. Gel Electrophoresis of RNA

RNA was separated electrophoretically on formaldehyde gels according to Maniatis *et al.* (1982). 5 μg of RNA was denatured by heating at 55°C for 15 minutes in 20 μl of 50% formamide, 2.2 M formaldehyde, and 1 x gel-running buffer [0.2 M morpholinopropanesulfonic acid (pH 6.8) containing 0.05 M sodium acetate, and 0.001 M EDTA]. After adding 2 μl of loading buffer (50% glycerol, 0.001 M EDTA, 0.4% bromophenol blue, and 0.4% xylene cyanol) to the RNA solution, 20 μl of the sample were applied to 1.4% agarose gel prepared in 1 x running buffer and 2.2 M formaldehyde. pBR322 DNA digested with *Rsa* I was used as molecular weight marker.

Electrophoresis was performed at 2 V/cm for 3 hours.

2.6.3. Northern-blot Hybridization

The gels were soaked for 30 minutes in two changes of 20 x SSC to remove the formaldehyde and put onto a piece of Whatman 3MM paper draped into 20 x SSC transfer buffer. A piece of nitrocellulose filter soaked in 20 x SSC was then place on the gels. Two pieces of 3MM paper, one stack of paper towels and a weight were added on top of the filter. Transfer of RNA to the filter proceeded overnight by capillary action (Davis *et al.*, 1986). The filter was then baked at 80°C for half an hour or longer, prehybridized and hybridized with the appropriate probe under conditions described above (section 2.5.4). The filter was washed twice in 2 x SSC and 0.1% SDS at room temperature, and twice in 0.1 x SSC and 0.1% SDS at 60°C. In the case of the actin probe, the last two washes were performed in 0.5 x SSC and 0.1% SDS at 65°C according to Gunning *et al.* (1983). Filter was dried and exposed to X-ray film.

2.7. Preparation and Analysis of Protein

2.7.1. Immunoprecipitation

2.7.1.1. Preparation of labelled cell lysates: At the appropriate times after infection cells were labelled with 200 μCi of [^{35}S]methionine (ICN Corp., Irvine, CA; specific activity, 1142 Ci/mmol) in 2 ml of medium 199 lacking methionine (met) in 60 mm dishes at 37°C for either 4, 1 or half an hour. Cells were harvested, washed three times with 1 x PBS and then resuspended in 200 μl of Schweizer's buffer [100 mM Tris

(pH 8.0) containing 100 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM KCl, 10% glycerol, and 1% Nonidet P-40] (Schaffhausen *et al.*, 1978; Mak and Mak, 1983). After 10 minutes on ice, cytoplasmic lysates were prepared by removing nuclei through centrifugation at 400 g for 10 minutes. Alternatively, cells were sonicated for 2 minutes in RIPA buffer [10 mM Tris (pH 7.4) containing 1% deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 1% aprotinin, 0.25 mM phenylmethylsulfonyl fluoride] to prepare total cell lysates, and cellular debris was removed by centrifugation in a microcentrifuge.

The amount of radioactivity in each lysate was determined by trichloro-acetic acid (TCA) precipitation. 5 or 10 μl of lysate supplemented with 100 μg of carrier BSA protein was precipitated with 10% cold TCA and the precipitates were collected on nitrocellulose filters by suction. The filters were baked and the radioactivity counted in a scintillation counter (Beckman) in toluene containing omnifluor. Samples containing equivalent acid-precipitable counts were either directly boiled for 5 minutes in loading buffer [0.0625 M Tris (pH 6.8) containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue] (Laemli, 1970) prior to gel electrophoresis, or processed by immunoprecipitation.

2.7.1.2. Immunoprecipitation: The anti-tumour sera AB6a-C3 and A7Ra-C4, which recognize Ad12 E1 and E1A proteins (Mak and Mak, 1990), respectively, normal rat serum and anti-Ad12 virion serum were used in these studies. In some experiments, lysates were denatured by boiling in 0.5% SDS for 5 minutes before

immunoprecipitation. Typically, 20 μl of antiserum and 350 μl of protein A beads (0.3 g of protein A beads dissolved in Schweizer's buffer at a total volume of 11 ml) were mixed with about 10 μl of lysate and the mixture was mixed by rotation overnight at 4°C. The beads were then pelleted for 2 minutes at 100 g and the supernatant was either discarded or used for sequential immunoprecipitation. After washing with LiCl buffer [100 mM Tris (pH 8.2) containing 0.2 M LiCl, and 0.14 M β-mercaptoethanol] three times, the beads were resuspended and boiled in 45 μl of loading buffer prior to gel electrophoresis.

2.7.1.3. SDS polyacrylamide gel electrophoresis (SDS-PAGE): Discontinuous gels were prepared containing a 12.5% polyacrylamide separating gel in 0.375 M Tris (pH 8.8) containing 0.1% SDS, and a stacking gel in 0.125 M Tris (pH 6.8) containing 0.1% SDS. Usually a ratio of 37.5:1 acrylamide to bis-acrylamide was used (Laemli, 1970) although in some cases a ratio of 30:0.2 was employed. Protein samples were loaded in the gel and run in a buffer of 0.025 M Tris, 0.192 M glycine, and 0.1% SDS at 2.5 V/cm for 16 hours.

2.7.1.4. Processing of gels: After fixing gels in 47% methanol and 1.14 M acetic acid for 30 minutes, it was dehydrated in two changes of dimethyl sulfoxide (DMSO) with shaking for 30 minutes, and soaked in 23% PPO (2',5'-diphenyloxazole) prepared in DMSO for 3 hours. After the PPO was precipitated by soaking the gels in water for 30 minutes, they were dried and exposed to X-ray film.

2.7.1.5. Quantitation of protein: The amount of protein was determined by

estimating the intensity of the protein band on the autoradiographs using a Laser Densitometer (see section 2.5.4.3). Alternatively, serial twofold dilutions of lysate were made and subjected to immunoprecipitation, and proteins were separated on SDS-PAGE. The intensity was compared and semi-quantative results were achieved.

2.7.2. Analysis by Western Blotting

Cell lysates were prepared and analyzed by SDS-PAGE as described above. Proteins were transferred onto a piece of nitrocellulose filter by electroblotting at 14 V constant voltage for 4 hours at 4°C in electroblotting buffer [20 mM Tris (pH 8.0) containing 150 mM glycine, and 20% methanol] using a Bio-Rad transblot apparatus. After transfer, the filter was incubated in blocking buffer [5% (w/v) milk powder, 0.01% antifoam A, 0.05% Tween 20, and 0.01% thimerosol] at room temperature for 45 minutes and then for 90 minutes in blocking buffer containing Ab6a-C3 E1 antiserum (1:1000 dilution). After washing with the same buffer for four times, the filter was incubated for 60 minutes with anti-rat goat IgG linked to alkaline phosphatase (BioCan Scientific Inc., 1:5000 dilution). Then the filter was washed with two changes of the blocking buffer and two changes of borate buffer (15 mM boric acid, pH 9.5). The color reaction was developed by incubating the filter for 2 minutes in the reaction buffer [15 mM boric acid (pH 9.5) containing 0.01% p-nitro blue tetrozoleun chloride, 0.5% 5-bromo-4-chloro-3-indolyl phosphate, and 4 mM $MgCl_2$] (Blake *et al.*, 1984).

2.8. Cellular Transformation Assay

BRK cells prepared as described in section 2.3.1.3 were infected as monolayers (see section 2.3.3) with viruses at several multiplicities of infection. After 2 days in α-MEM containing 10% fetal calf serum, the cells were maintained in Joklik's modified MEM plus 5% horse serum which was replenished every 3-4 days. At 23-25 days the cells were washed twice with 1 x PBS (complete) (PBS plus 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$), and fixed in PBS (complete) and Carnoy's solution (1:1) for 10 minutes, and in Carnoy's solution for 30 minutes. After staining cells with Giemsa for 30 minutes, the transformed cell colonies were counted (Mak and Mak, 1983).

2.9. A Computer Program for Displaying the Hydropathic Profile of Protein

The hydropathic profiles of the E1B 163R, 175R and 178R proteins of adenovirus types A, B and C were determined as described by Kyte and Doolittle (1982), based on the hydrophilic and hydrophobic properties of each of the 20 amino acid side-chains. The average hydropathy value was determined over a range of 9 residues. The sequences of the proteins were predicted from the published DNA sequence (van Ormondt and Galibert, 1984).

CHAPTER THREE

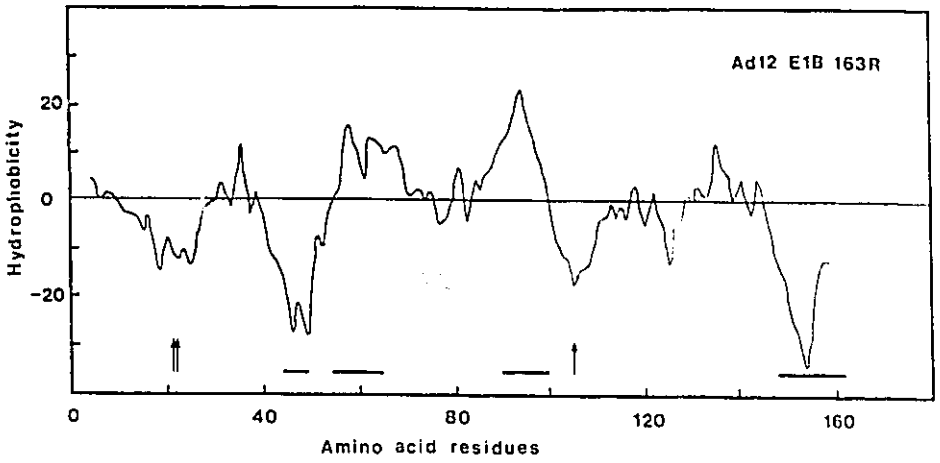
RESULTS: CLONING OF Ad12 DNA SEQUENCES AND CONSTRUCTION OF AD12 E1B 163R MUTANTS

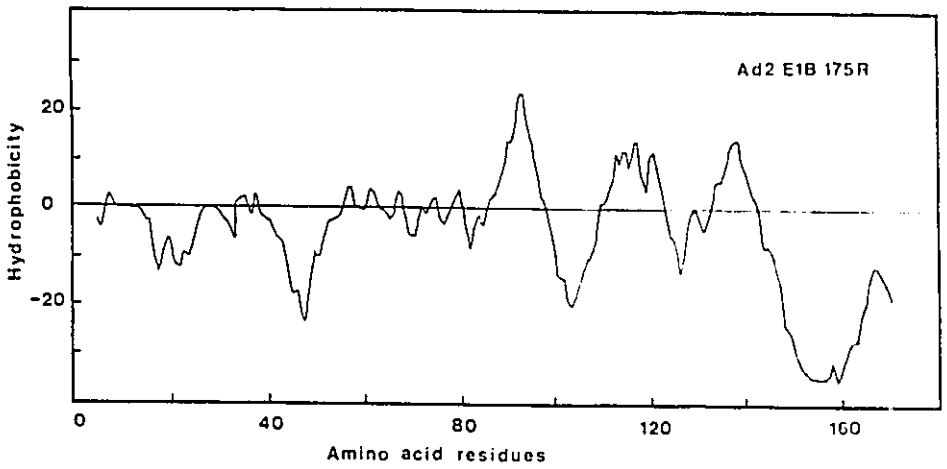
3.1. Analysis of the Hydropathic Profile of Adenovirus E1B 163R

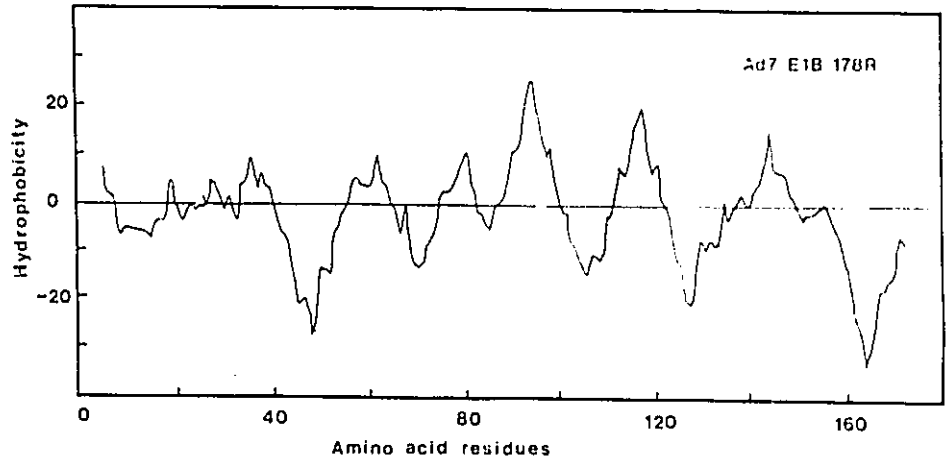
The E1B 163R protein is multifunctional. One approach to identify functional domains is to make a series of mutations along the coding sequence and then to study the biological effects using mutant viruses. To determine potential sites for mutagenesis a hydropathic profile of 163R was produced and is shown in Figure 3-1. Hydrophilic amino acid sequences, representing more exposed parts of the protein, have been given negative values and hydrophobic regions, representing interior regions, positive values. The 163R, 175R and 178R proteins of Ad12, Ad2 and Ad7, respectively, share conserved sequences in several areas (van Ormondt and Galibert, 1984) and their hydropathic profiles are very similar, especially those of Ad12 and Ad2. In the case of hydrophobic domains, all three proteins share one common hydrophobic region near residue 90. Sites for mutagenesis were therefore selected to eliminate or affect conserved areas within hydrophilic and the hydrophobic regions.

Figure 3-1. Hydropathic profiles of the E1B 175R, 178R and 163R protein of adenovirus type 2, 7 and 12.

The hydropathic profiles were plotted according to the method of Kyte and Doolittle (1982) using a span of nine amino acid residues. Hydrophobic regions are positive, hydrophilic regions are negative. In the profile of Ad12 E1B 163R, mutations resulting in exchanges of residues are indicated by arrows and mutations resulting in deletions of residues by bars.


Ad12 E1B 163R
Hydrophobicity
20
0
-20
0
40
80
120
160
Amino acid residues


Ad2 E1B 175R
Hydrophobicity
20
0
-20
0
40
80
120
160
Amino acid residues


Ad7 E1B 178R
Hydrophobicity
20
0
-20
0
40
80
120
160
Amino acid residues

3.2. Mutation Sites in the Ad12 E1B 163R Protein

Mutations were generated in 163R coding region of plasmid pMAB67 (see section 3.3) using oligonucleotide-directed mutagenesis (see section 3.4). The mutated 163R sequences were then rescued into Ad12 genome by co-transfection (see section 3.6), and thus eight mutants were generated. Mutants *dl*1670, *dl*1700 and *dl*1808 contain deletions in highly conserved hydrophobic and hydrophilic regions across the 163R coding sequence which eliminate residues 44-49, 54-65 and 90-100, respectively (see Fig. 3-2). A double mutant, *dl*1700/1808 was also produced. All of these changes are upstream of the overlapping coding sequence for the E1B 482R protein. Three point mutants contain missense mutations which affect hydrophilic regions of 163R but have no effect on the amino acid coding sequence for 482R. *pm*1602 alters conserved Ser^{21}-Lys^{22} to Leu-Ile, *pm*1854 changes conserved Glu^{105} to Val, and *pm*1983 introduces a termination codon following residue 147, thus eliminating the last 16 amino acids at the carboxyl terminus. In addition, one 163R null mutant, *pm*1542 was produced in which the initiation codon for 163R had been eliminated.

3.3. Construction of Recombinant M13 Phage, pMAB67, for Mutagenesis

The left-most 2317 bp fragment of Ad12, *Bam*H I-*Hin*d III (0 to 6.7 mu), containing the E1B 163R sequence was isolated from pHAB80 and inserted into a single-stranded DNA vector to create a recombinant, designated pMAB67 (Fig. 3-3). After transforming *E. coli* JM103 with recombinant plasmid DNA in the presence of X-gal

Figure 3-2. Positions of mutations in the 163R coding sequence.

The thick lines represent the DNA sequence across the E1B 163R coding region. The positions of the nucleotides involved in mutations are indicated above the lines and the locations of mutated or deleted amino acid residues under the lines.

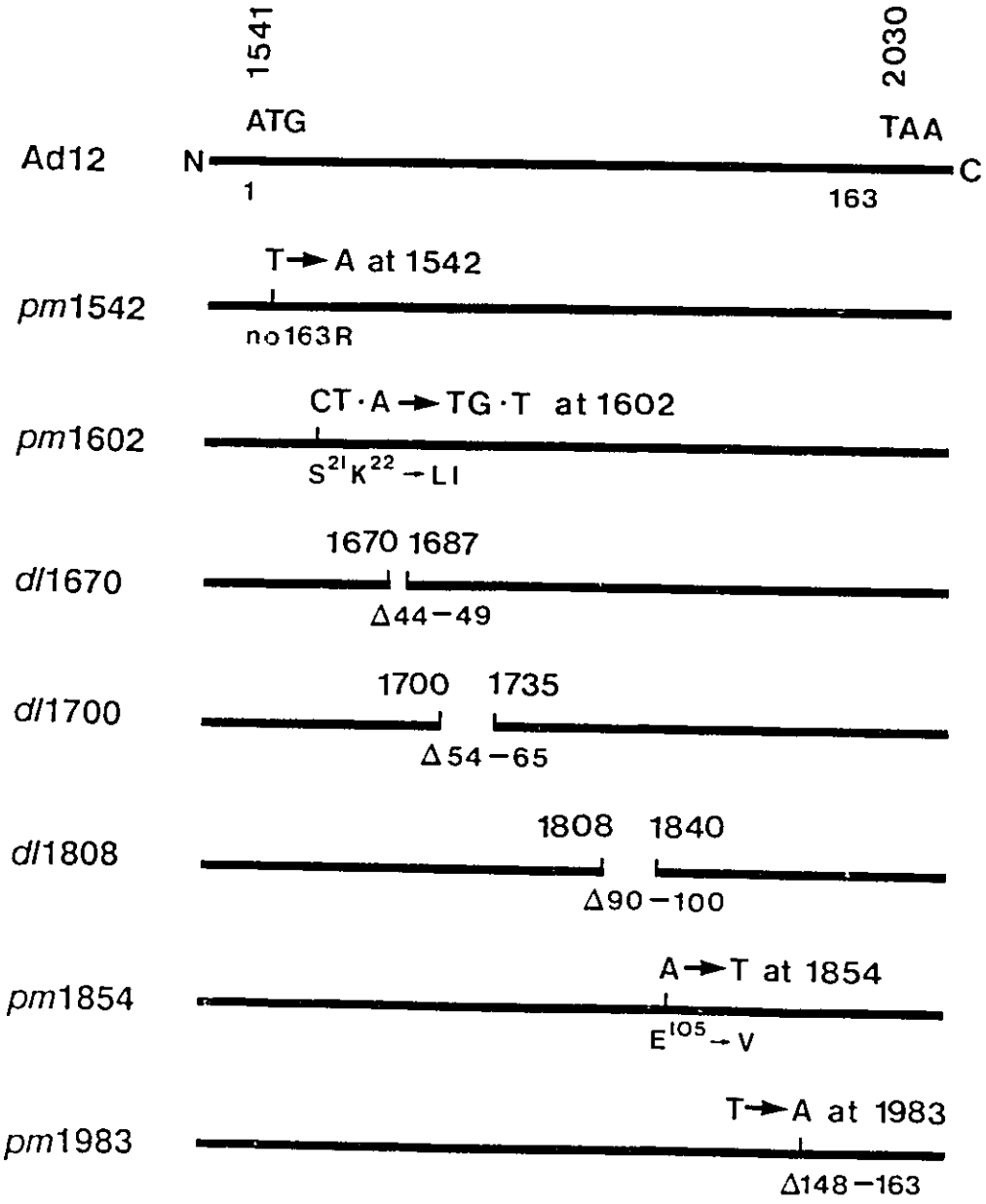
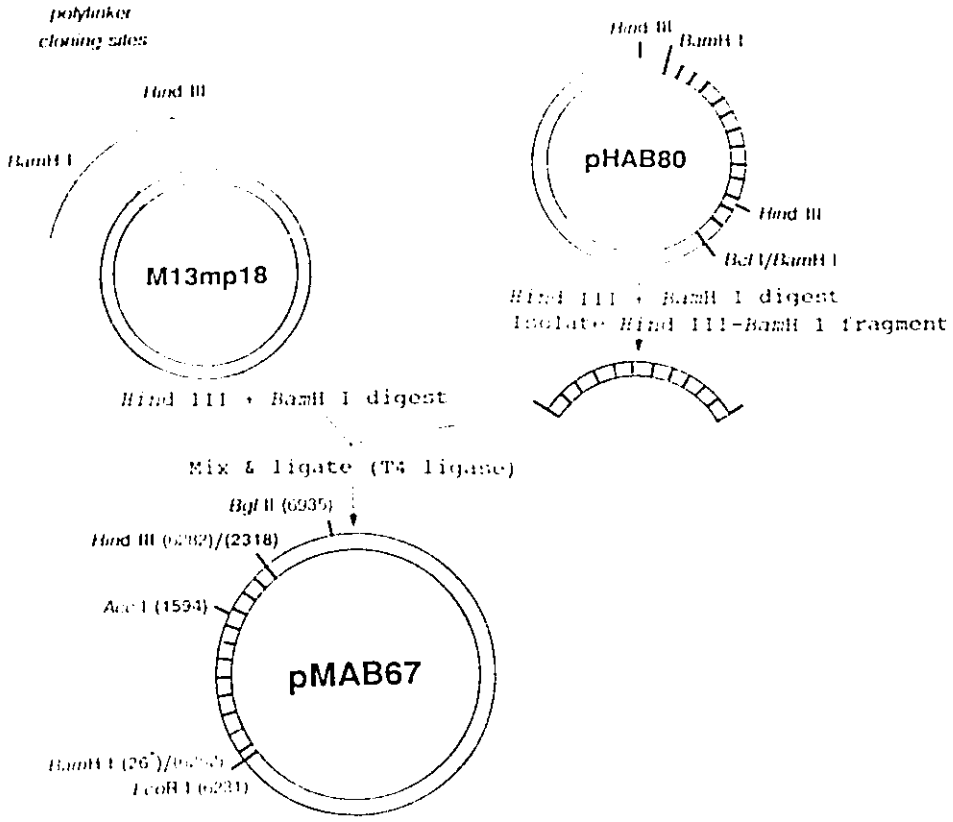
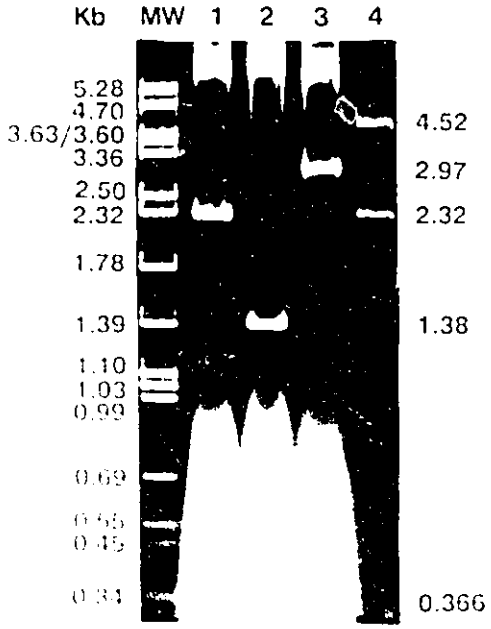

1541
2030
ATG
TAA
Ad12
N
C
1
163
T→A at 1542
pm1542
no163R
CT·A→TG·T at 1602
pm1602
S21K22→LI
1670 1687
dl1670
Δ44–49
1700 1735
dl1700
Δ54–65
1808 1840
dl1808
Δ90–100
A→T at 1854
pm1854
E105→V
T→A at 1983
pm1983
Δ148–163

Figure 3-3. Strategy for the construction of recombinant M13 phage, pMAB67.

Top: The *Bam*H I-*Hin*d III fragment of Ad12 E1 region, 0-6.7 mu, was inserted into M13mp18 to create recombinant pMAB67. *Bottom*: Restriction enzyme analysis of recombinant pMAB67. MW, molecular weight marker [*Hin*d III-digested Ad12 (Huie) DNA]; 1, *Bam*H I- and *Hin*d III-digested pMAB67 DNA; 2, *Acc* I- and *Bgl* II-digested pMAB67 DNA; 3, *Bgl* II- and *Eco*R I-digested pMAB67 DNA; 4, *Bam*H I- and *Hin*d III-digested pHAB80 DNA. Sizes of the fragments are indicated in kilobase pairs.


polylinker
cloning sites
Hind III
BamH I
M13mp18
Hind III
BamH I
pHAB80
Hind III
Bcl I/BamH I
Hind III + BamH I digest
Isolate Hind III-BamH I fragment
Hind III + BamH I digest
Mix & ligate (T4 ligase)
Bgl II (6935)
Hind III (2318)
Acc I (1594)
pMAB67
BamH I (26)
EcoR I (6231)


Kb
MW
1
2
3
4
5.28
4.70
3.63/3.60
3.36
2.50
2.32
1.78
1.39
1.10
1.03
0.99
0.69
0.45
0.31
4.52
2.97
2.32
1.38
0.366

and IPTG, white plaques were picked and DNA extracted from amplified cultures. Recombinant DNA was analyzed by restriction enzyme digestion. The successful recombinant with the Ad12 E1 insertion (0-6.7 mu) displayed a diagnostic restriction pattern as shown in Fig. 3-3 (bottom). *Bam*H I and *Hin*d III double-digestion gave rise to two fragments of 7.25 and 2.32 kb in size (lane 1) while *Acc* I and *Bgl* II generated two bands of 8.8 and 1.38 kb (lane 2) and *Eco*R I and *Bgl* II produced two fragments of 6.55 and 2.97 kb (lane 3). The apparent smear near the bottom of the gel was due to the presence of RNA in DNA preparations (lane 1 to 3). The wide bands on the top of the gel were probably caused by overloaded DNA. Single-stranded DNA was prepared as the template for subsequent oligonucleotide-directed mutagenesis.

3.4. Oligonucleotide-directed Mutagenesis in the Ad12 E1B 163R Coding Region

The process of oligonucleotide-directed mutagenesis has been summarized in Fig. 3-4. Template DNA was mixed and annealed with a 5' phosphorylated mutagenic primer (Table 3-1) and *Hin*d III- and *Bam*H I-digested M13mp18 as a second primer. The annealed DNA was subjected to extension and ligation reactions and then transformed into *E. coli* to produce white plaques. To produce the double mutation *dl*1700/1808, two corresponding mutagenic primers were used in the reaction. Successfully mutated recombinants were identified as described below in section 3.7, and DNA was isolated for further subcloning. Overall eight pMAB recombinants were generated and designated as pMAB1542, pMAB1602, pMAB1670, pMAB1700, pMAB1808, pMAB1700/1808,

Figure 3-4. Strategy for oligonucleotide-directed mutagenesis.

The procedure is described in the text. ssDNA of pMAB67 was used as a template. The mutagenic primer #86 (see Table 3-1) and the pMAB1542 recombinant are used as examples.

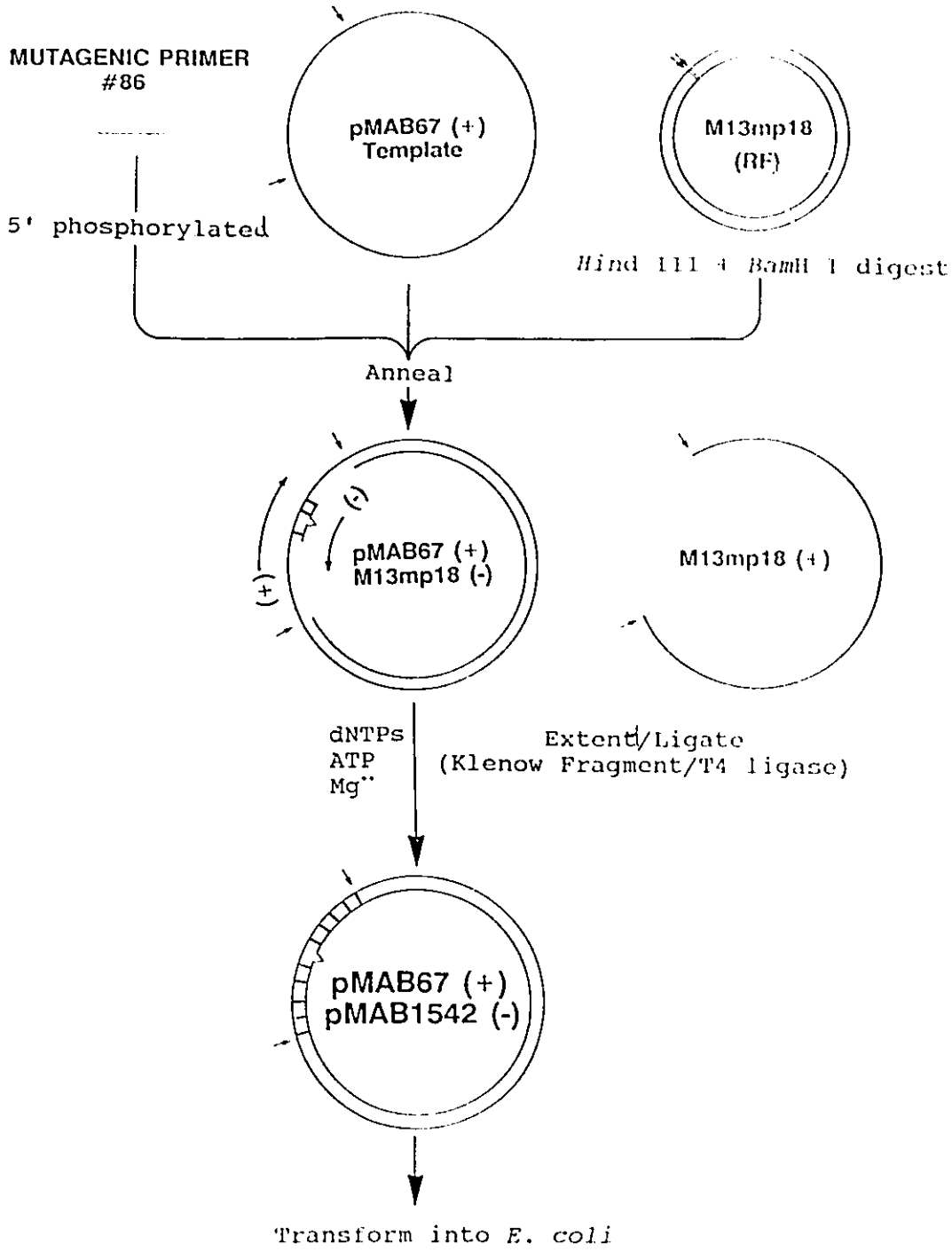

MUTAGENIC PRIMER
#86
5' phosphorylated
pMAB67 (+)
Template
M13mp18
(RF)
Hind III + BamH I digest
Anneal
pMAB67 (+)
M13mp18 (-)
M13mp18 (+)
dNTPs
ATP
Mg++
Extend/Ligate
(Klenow Fragment/T4 ligase)
pMAB67 (+)
pMAB1542 (-)
Transform into E. coli

Table 3-1. List of mutagenic primers

Primer	Sequence[1]	Recombinants[2]		Mutant
#86	5'TTCCAACTCCTTTACTAAG3'	pMAB1542	pHAB1542	*pm*1542
#52	5'GAAGTGTTTATCAAGGTATACT3'	pMAB1602	pHAB1602	*pm*1602
#96	5'TTTTCAAATTC•CACCCTATTT3'	pMAB1670	pHAB1670	*dl*1670
#31	5'TAACAAAGGTC•GTTTTCAAAT3'	pMAB1700	pHAB1700	*dl*1700
#32	5'CTCCATTTATC•CGTTCGTCCC3'	pMAB1808	pHAB1808	*dl*1808
#31+#32		pMAB1700/1808	pHAB1700/1808	*dl*1700/1808
#29	5'GGTGGGATTTCACGCTCCATTT3'	pMAB1854	pHAB1854	*pm*1854
#30	5'TCTCCTCTTGCTACGTCGGCAG3'	pMAB1983	pHAB1983	*pm*1983

[1]Underlined letters represent converted bases within Ad12 163R coding region and the dots within the sequences indicate deleted sequences.
[2]The pMAB recombinants represent M13mp18 recombinants containing Ad12 E1 region from 0 to 6.7 mu and the pHAB recombinants represent pBR322 recombinants containing Ad12 E1 region from 0 to 16 mu.

pMAB1854 and pMAB1983.

3.5. Subcloning DNA Fragments Containing Mutated E1B 163R Coding Sequences Into pHAB6

To facilitate manipulation during rescue of mutated regions into virus, the *Kpn* I-*Hin*d III fragment (1.5-6.7 mu, nucleotides 588 to 2317) encompassing the mutation was isolated and then subcloned into pHAB6 which contains the entire Ad12 E1 region

and has a *Bst*E II restriction site at nucleotide 3443th which is unique in the viral genome. DNA fragments were isolated from either pHAB6 or one of pMAB recombinants and ligated together, then used for transformation of *E. coli* LE392 (Fig. 3-5). The pHAB recombinants were isolated (pHAB1542, pHAB1602, pHAB1670, pHAB1700, pHAB1808, pHAB1700/1808, pHAB1854 and pHAB1983) and DNA was prepared.

3.6. Rescue of the Mutated E1B 163R Gene Into Virus

Utilizing the unique *Bst*E II restriction site at nucleotide 3443 of the Ad12 viral genome, the left 3443 bp fragment harbouring mutated Ad12 E1B 163R gene was excised from pHAB plasmids with *Bst*E II and *Bam*H I and ligated with the remainder of the dephosphorylated viral genomic DNA which was then transfected into MH12-C2 cells (Fig. 3-6). After 3 weeks virus plaques formed and mutants were identified (see section 3.7) and amplified.

3.7. Identification of Successful Recombinants and Mutants

3.7.1. Screening and DNA sequencing of M13 recombinants: After oligonucleotide-directed mutagenesis, dot-blot hybridization was used to identify the mutated recombinant DNA. The single-stranded recombinant DNA was spotted onto a nitrocellulose filter and labelled mutagenic primer was used as a probe. At room temperature, the primer was able to bind to wild type DNA as well as mutated DNA.

Figure 3-5. Strategy for subcloning DNA sequences containing mutations into pHAB6.

The *Kpn* I-*Hin*d III (1.5-6.7 mu) fragment was isolated from one of the pMAB recombinants and then inserted into pHAB6 as indicated. pMAB1542 is used as an example.

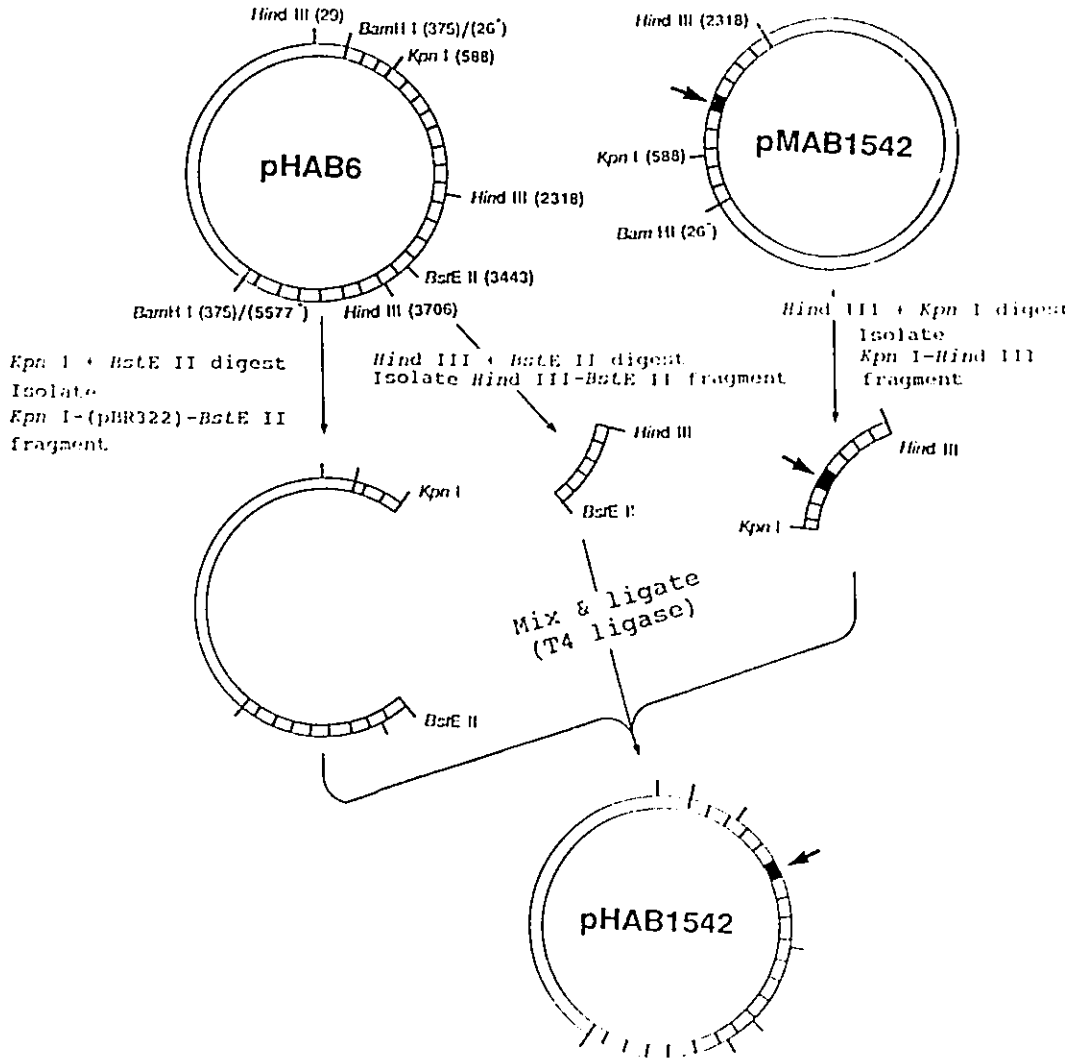
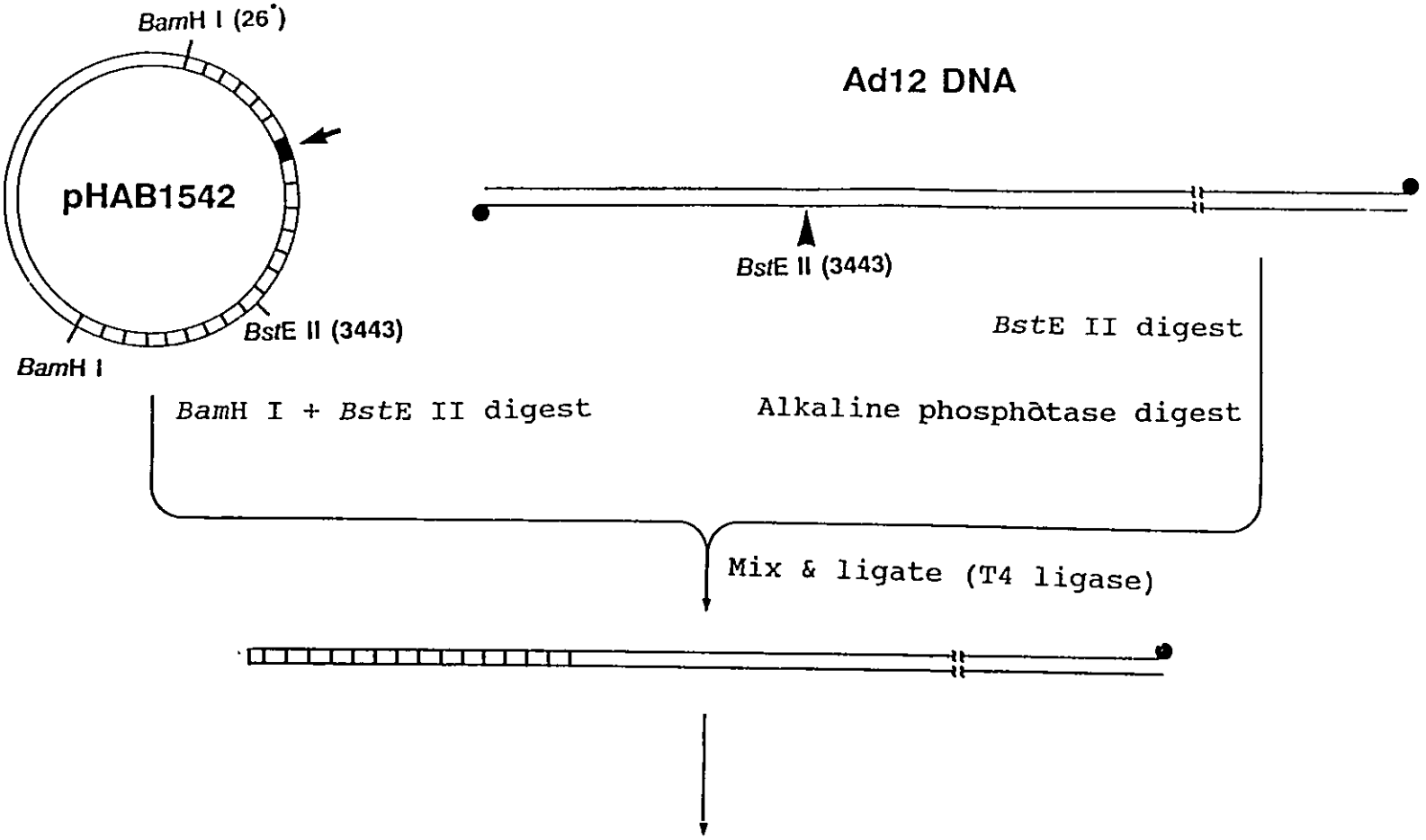

Hind III (29)
BamH I (375)/(26)
Kpn I (588)
pHAB6
Hind III (2318)
BstE II (3443)
BamH I (375)/(5577)
Hind III (3706)
pMAB1542
Hind III (2318)
Kpn I (588)
Bam HI (26)
Kpn I + BstE II digest
Isolate
Kpn I-(pBR322)-BstE II
fragment.
Hind III + BstE II digest
Isolate Hind III-BstE II fragment
Hind III + Kpn I digest
Isolate
Kpn I-Hind III
fragment
Hind III
BstE II
Kpn I
Hind III
Kpn I
Mix & ligate
(T4 ligase)
BstE II
pHAB1542

Figure 3-6. Strategy for rescuing mutated E1B 163R genes into virus.

Viral DNA was digested with *Bst*E II and dephosphorylated. Plasmid DNA was released with *Bam*H I and *Bst*E II. DNA fragments were then ligated before transfecting into MH12-C2 cells. pHAB1542 is used as an example.


BamH I (26*)
pHAB1542
BstE II (3443)
BamH I
Ad12 DNA
BstE II (3443)
BstE II digest
BamH I + BstE II digest
Alkaline phosphatase digest
Mix & ligate (T4 ligase)
Transfect into MH12-C2 cells

After washing the filter at higher temperature, however, the difference in binding affinity between wild type and mutated DNAs became obvious, and allowed identification of mutant sequences by the presence of strong hybridization signals (Fig. 3-7, top). In the case of the double deletion, mutant pMAB1700/1808, a successful recombinant was identified by its ability to hybridize to both corresponding primers. The optimal temperatures were different for different primers and have been indicated in Fig. 3-7 (right side).

Because recombinant M13 DNA is not stable and often yields deletions or rearrangements of inserted DNA during replication, it was necessary to examine the size of the inserted regions after transformation. Thus dsDNA was isolated from each positive plaque (indicated by an arrow, see Fig. 3-7) and digested with *Bam*H I and *Hind* III. The fragment containing a point mutation gave rise to a band of about 2.3 kb and that containing a deletion a band close to 2.3 kb (Fig. 3-7, bottom). The minor bands smaller than 2.3 kb might be due to deletion occured in a small percentage of recombinants pMAB1854 and pMAB1700/1808.

To further eliminate any possible unexpected alteration which was not detected by restriction analysis, the E1 region encompassing the *Kpn* I-*Hind* III (nucleotides 588-2317) DNA fragment was sequenced . The sequencing primers have been listed in Table 3-2. Except for M13 universal primer which has complementary sequences in M13mp18 near *Hind* III site, all these primers have sequences complementary to the Ad12 E1 region and the locations have been illustrated (see table 3-2). They were capable of

Figure 3-7. Dot-blot hybridization (top) and restriction analysis of pMAB recombinant DNA (bottom).

Top: Recombinant ssDNAs were isolated and spotted onto a nitrocellulose membrane. After hybridization with the primer indicated to the left, the membrane was washed at the indicated temperatures. The positive recombinants containing mutations were identified at higher wash temperature and those pointed by arrows were used for further subcloning (see Fig. 3-5). The numbers inside the brackets refer to the ratio of positive recombinants over total samples analyzed. Td represents the melting temperature of the primer calculated by the formula of Td=2 x Number of A/T + 4 x Number of G/C. *Bottom*: In the restriction analysis, *Bam*H I- and *Hin*d III-digested recombinant DNA was analyzed by gel electrophoresis. The size of the *Bam*H I-*Hin*d III fragment is indicated as 2.3 Kb. MW, molecular weight marker (see Fig. 3-3).

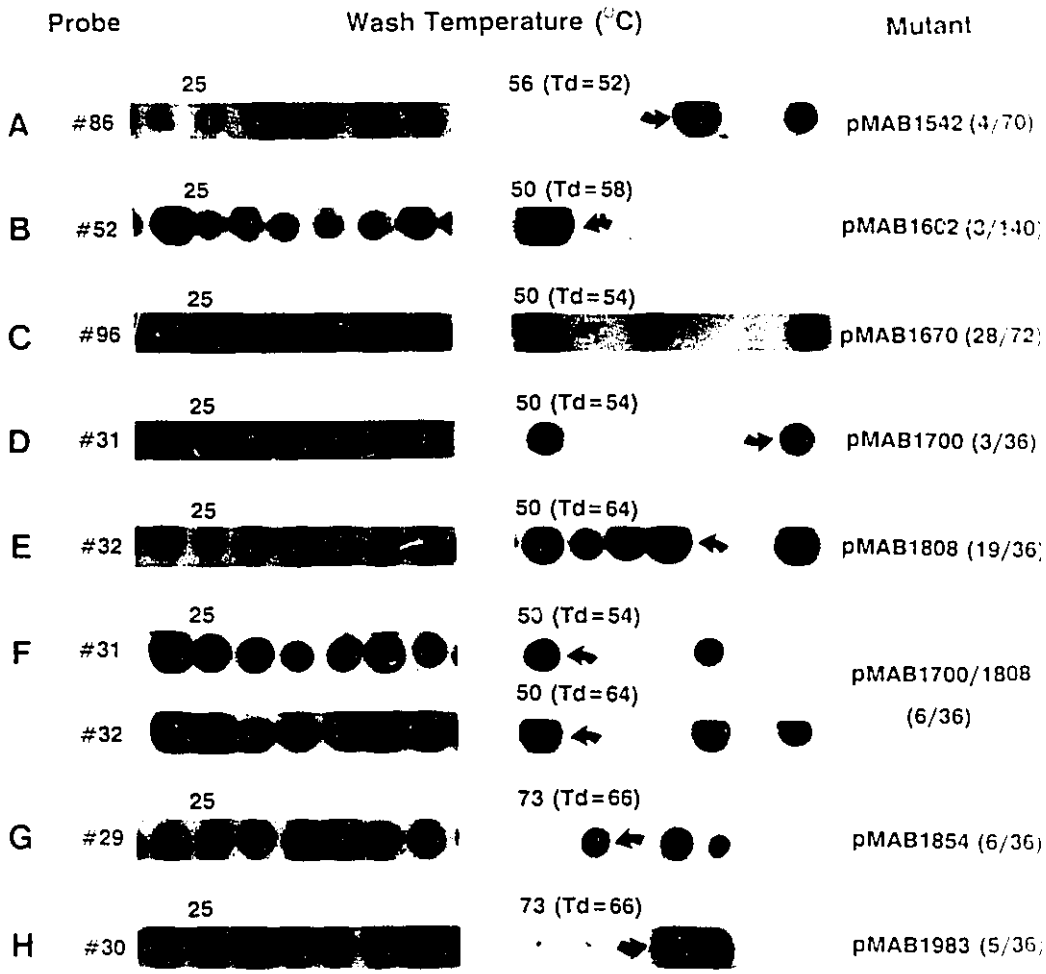

Probe
Wash Temperature (°C)
Mutant
A
#86
25
56 (Td=52)
pMAB1542 (4/70)
B
#52
25
50 (Td=58)
pMAB1602 (3/140)
C
#96
25
50 (Td=54)
pMAB1670 (28/72)
D
#31
25
50 (Td=54)
pMAB1700 (3/36)
E
#32
25
50 (Td=64)
pMAB1808 (19/36)
F
#31
25
50 (Td=54)
#32
50 (Td=64)
pMAB1700/1808
(6/36)
G
#29
25
73 (Td=66)
pMAB1854 (6/36)
H
#30
25
73 (Td=66)
pMAB1983 (5/36)
MW
pMAB1542
MW
pMAB1670
2.3 Kb
MW
pMAB1602
pMAB1854
pMAB1983
pMAB1700
pMAB1808
pMAB1700/1808

Table 3-2. List of sequencing primers

Primer	Sequence	Location[1]
AB209	5'TCTTCCGAATCGCTACA3'	863-847
AB226	5'TATCGGAAACAGGACCT3'	1158-1142
AB246	5'TATTTTATTGCCCAGGT3'	1405-1389
#86	5'TTCCAACTCCTTTACTAAG3'	1552-1534
SAM101	5'GGTATACTGCTAGAGCTGGCG3'	1600-1580
#29	5'GGTGGGATTTCACGCTCCATTT3'	1865-1844
#30	5'TCTCCTCTTGCTACGTCGGCAG3'	1994-1973
AB121	5'CATCAGATTAACAGTTAG3'	2172-2155
Universal	M13 Universal primer	

[1]The numbers represent the positions of nucleotides on the Ad12 genome.

priming sequencing leftwards in the viral genome (see Figure 1.1) and producing overlapping sequencing regions from nucleotides 588 to 2318 in the E1 region. Only the expected mutations were found in all cases (Fig. 3-8).

3.7.2. Screening and restriction analysis of recombinant DNA subcloned in pHAB6: The *Kpn* I-*Hind* III fragments containing the various mutations were subcloned into pHAB6 (see section 3.5) and recombinants were first screened by dot-blot hybridization using pHAB6 as a control (Fig. 3-9).

Successful recombinants were also characterized by restriction enzyme digestion

Figure 3-8. DNA sequencing of pMAB recombinant.

DNA from nucleotides 588 to 2318 was sequenced and the regions near mutations are illustrated. The black dots indicate the locations of either a base conversion or a deletion, which are also indicated by arrows or triangles in the sequence at the side of the autoradiograms.

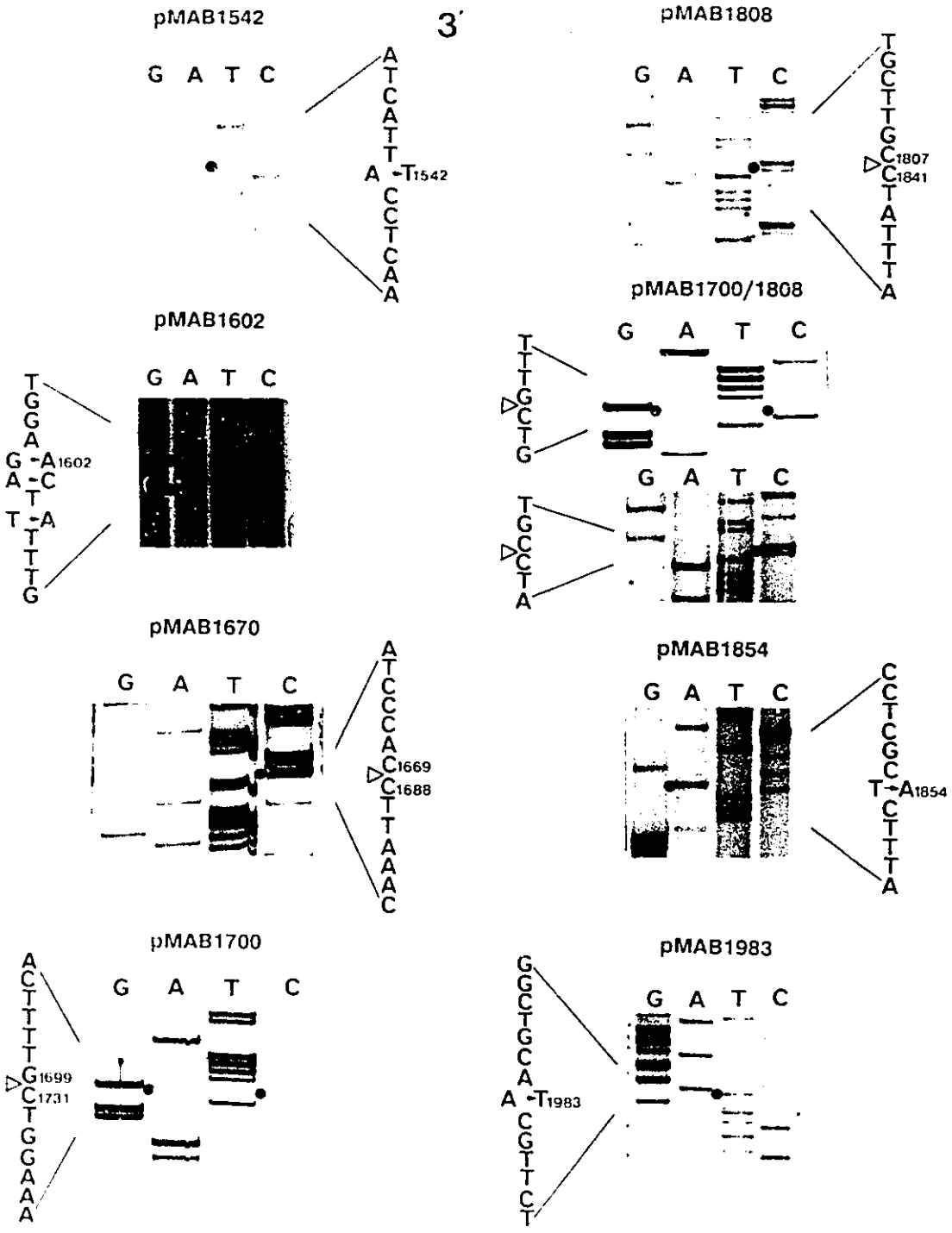
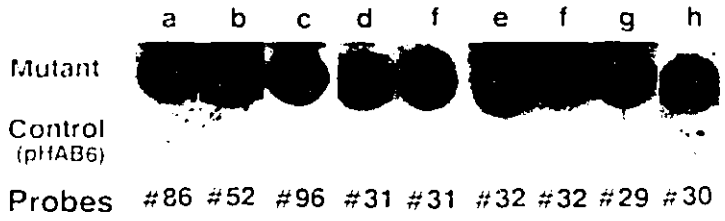
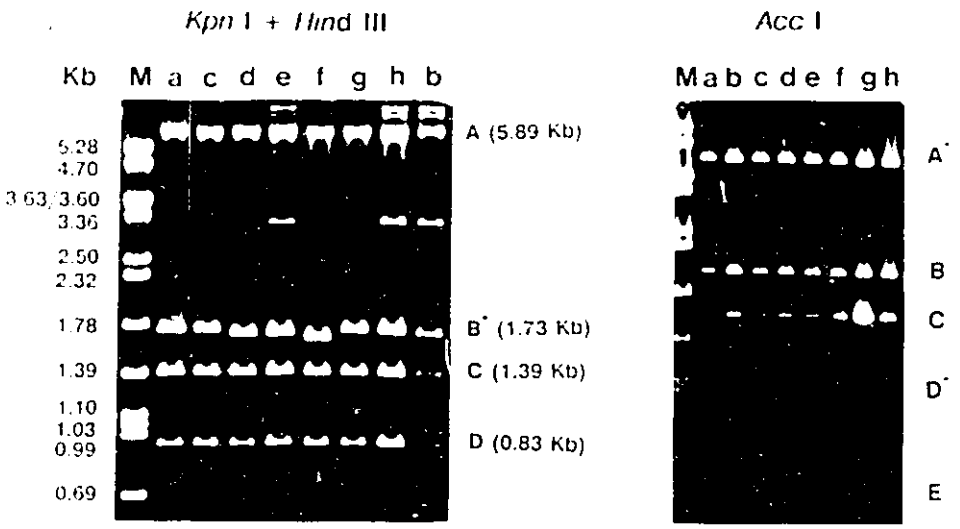
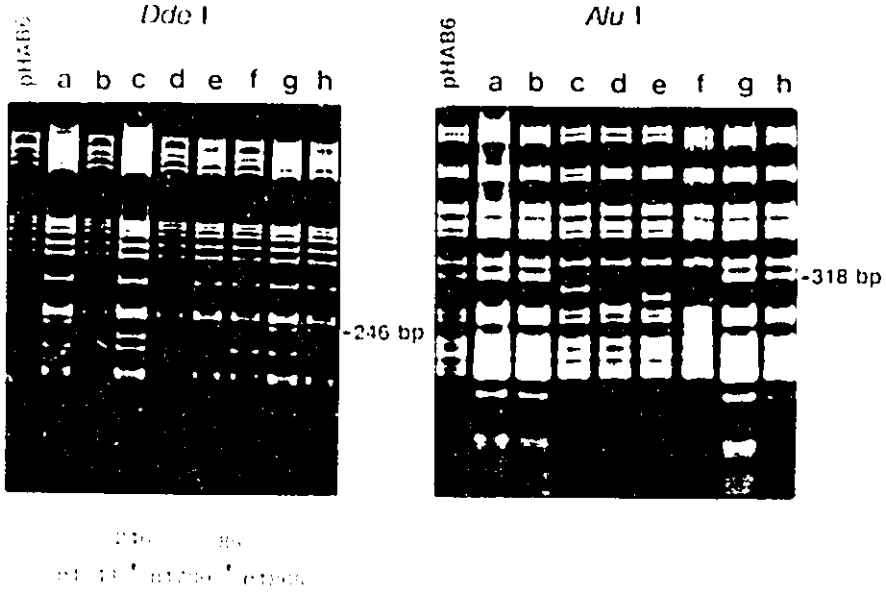

pMAB1542
3'
G A T C
ATCATT
A →T1542
CCTCAA
pMAB1808
G A T C
TGCTTGC1807
C1841
TATTTA
pMAB1602
G A T C
TGGA
G →A1602
A →C
T
T →A
TTTTG
pMAB1700/1808
G A T C
TTGCTG
G A T C
TGCCTA
pMAB1670
G A T C
ATCCCAC1669
C1688
TTAAAC
pMAB1854
G A T C
CCTCGC
T →A1854
CTTTA
pMAB1700
G A T C
ACTTTTTG1699
C1731
TGGAAA
pMAB1983
G A T C
GGCTGCA
A →T1983
CGTTCT
5'

Figure 3-9. Dot-blot hybridization (top) and restriction analysis of pHAB recombinant DNA (bottom).

Top: Recombinant DNA was isolated, linearized and spotted on a nitrocellulose membrane. pHAB6 was used as a negative control and probes have been indicated. *Bottom*: In the restriction analysis, the sizes of the molecular markers (same as in Fig. 3-3) are shown to the left and the digested fragments from the recombinants are shown to the right with *Kpn* I + *Hin*d III. With *Acc* I, the fragments are indicated by capital letters to the right. With *Dde* I and *Alu* I, the fragments containing deletions are shown to the right (see text). M, molecular weight marker; a, pHAB1542; b, pHAB1602; c, pHAB1670; d, pHAB1700; e, pHAB1808; f, pHAB1700/1808; g, pHAB1854; h, pHAB1983.


a b c d f e f g h
Mutant
Control
(pHAB6)
Probes #86 #52 #96 #31 #31 #32 #32 #29 #30


Kpn I + Hind III
Kb M a c d e f g h b
5.28
4.70
3.63 3.60
3.36
2.50
2.32
1.78
1.39
1.10
1.03
0.99
0.69
A (5.89 Kb)
B* (1.73 Kb)
C (1.39 Kb)
D (0.83 Kb)
Acc I
M a b c d e f g h
A*
B
C
D*
E


Dde I
pHAB6
a b c d e f g h
~246 bp
Alu I
pHAB6
a b c d e f g h
~318 bp

and the expected restriction patterns were produced (Fig. 3-9). *Hin*d III and *Kpn* I generated four bands in which band B contains the mutation. Point mutations did not alter the migration rate (lanes a, b, g and h), but deletions caused faster migration of band B (lanes d, e and f). pHAB1670 contains a smaller deletion which did not cause much difference in the migration of band B (lane c). A few extra bands were produced which were probably due to incomplete digestions. *Acc* I produced five bands, and faster migration due to deletions was seen for fragment D (lanes c, d, e and f). *Dde* I cut the plasmid frequently and two of the fragments, 246 bp and 85 bp, encompass the deletions. The 85 bp fragment was probably too small and likely ran out of the gel. An altered 246 bp from pHAB1700 and pHAB1700/1808 was absent at the position of the *wt* 246bp fragment and likely co-migrated with other bands (lanes d and f). pHAB1670 had clearly displayed the presence of a deletions through faster gel migration (lane c). *Alu* I also cut the plasmid frequently and revealed the expected deletion patterns in the 318 bp fragment (lanes c, d, e and f). Except for these fragments, all the other fragments appeared identical to those found in wild-type.

3.7.3. Screening and restriction analysis of mutants: Dot-blot hybridization was used to screen for the mutants. As expected the positive mutants displayed much more intense signals (Fig. 3-10). Two of the positive plaques from each mutant were purified three times and screened repeatedly by dot-blot hybridization. The last purified samples were also subjected to *Hin*d III digestion. The result from one of each of two plaque samples is shown in Figure 3-11. The overall patterns of mutant DNA from

Figure 3-10. Dot-blot hybridization of mutant virus DNA.

Viral DNA was isolated from infected MH12-C3 cells and spotted onto a nitrocellulose filter. After hybridization with appropriate primers, the filter was washed at lower and then at higher temperatures. The positive mutants were identified through their ability to hybridize the probes at higher washing temperature and those pointed by arrows were used to prepare virus stocks. To the left are shown the probes and to the right are shown the mutants.

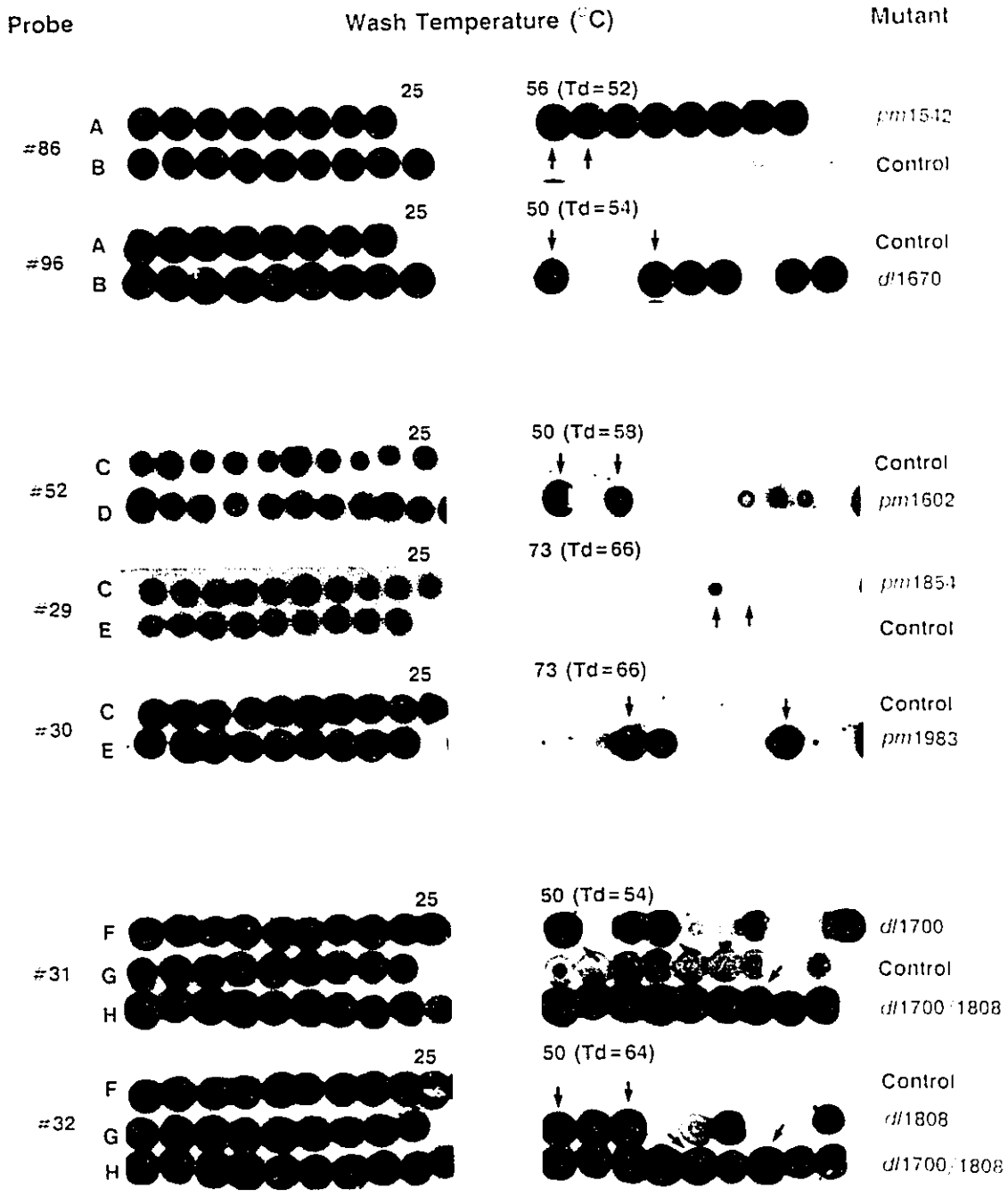
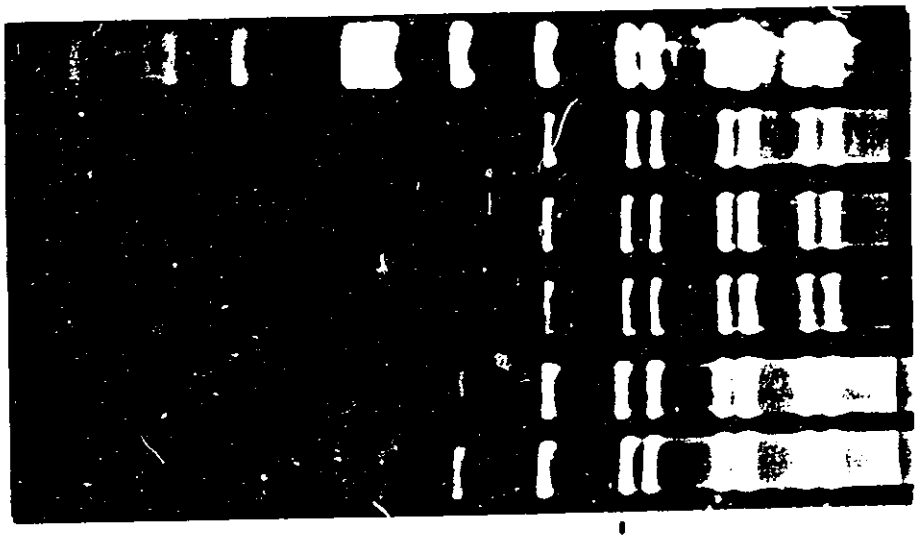

Probe
Wash Temperature (°C)
Mutant
#86
A
B
25
56 (Td=52)
pm1542
Control
#96
A
B
25
50 (Td=54)
Control
dl1670
#52
C
D
25
50 (Td=58)
Control
pm1602
#29
C
E
25
73 (Td=66)
pm1854
Control
#30
C
E
25
73 (Td=66)
Control
pm1983
#31
F
G
H
25
50 (Td=54)
dl1700
Control
dl1700/1808
#32
F
G
H
25
50 (Td=64)
Control
dl1808
dl1700/1808

Figure 3-11. Restriction analysis of mutant virus DNA.

Viral DNA was isolated from infected MH12- C3 cells, digested with *Hin*d III and subjected to gel electrophoresis. Mutation is located inside G fragment.


Ad12
pm1542
pm1854
dl1670


Ad12
pm1602
dl1700
dl1808
dl1700/1808
pm1983

G

infected cells and of wild type virus DNA from purified virions were comparable, and the mutations were located in band G. The intensities of some bands near the bottom of the gels were not sufficient in this exposure to clearly identify some fragments, but these were observed upon longer exposure (not shown).

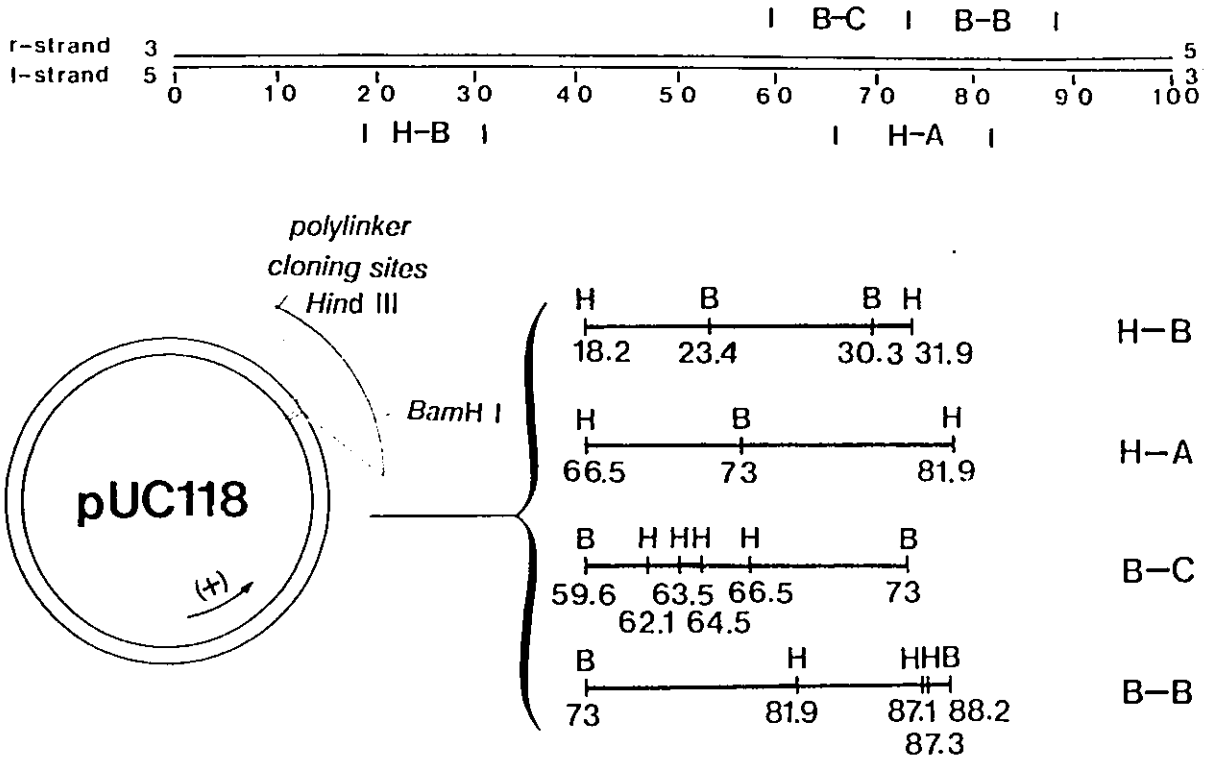
3.8. Cloning Ad12 DNA Sequences Containing E2A, E2B, E3 and L4 Regions

Ad12 DNA fragments containing either the E2A, E2B, E3 or L4 regions were isolated and cloned into pUC118. As shown in Figure 1-1 (bottom) and Figure 3-12, the *Hin*d III-A fragment (66.5-81.9 mu, 5.3 kb) contains part of the E3 and L4 regions in the *l* strand. The *Hin*d III-B fragment (18.2-31.9, 4.7 kb) encompasses most of the E2B region in the *r* strand. In addition the *Bam*H I-B fragment (73-88.2 mu, 5.2 kb) contains most of the E3 region and probably part of L4 in the *l* strand. The *Bam*H I-C fragment (59.6-73 mu, 4.6kb) encompasses part of L4 in the *l* strand and part of E2A in the *r* strand. Therefore E2A-, E2B-, E3- and L4-specific probes could be created by inserting viral DNA with the desired orientation into pUC118, which could be grown as a "plus" single-stranded recombinant DNA. The four viral DNA fragments described above were each isolated and inserted into either *Hin*d III- or *Bam*H I-digested pUC118 (Fig. 3-12). The recombinants were designated as pUC118 plus the restriction fragment and viral strand retaining in ssDNA (+): pUC118HA-r, pUC118HA-l, pUC118HB-r, pUC118HB-l, pUC118BB-r, pUC118BB-l, pUC118BC-r and pUC118BC-l.

dsDNA from the recombinants was screened by restriction enzyme analysis with

Figure 3-12. Physical maps of *Bam*H I-B, C and *Hin*d III-A, B fragments of Ad12 (Huie).

The positions of *Bam*H I-B, C (B-B, B-C) and *Hin*d III-A, B (H-A, H-B) fragments on the Ad12 viral genome are indicated an the top. Within the *Bam*H I or *Hin*d III fragments the recognition sites of *Hin*d III (H) or *Bam*H I (B) are indicated at the bottom. The numbers represent the map units. The isolated fragments of B-B, B-C, H-A and H-B digest were inserted into either *Bam*H I or *Hin*d III sites in pUC118 with one of two possible orientations. The orientation of positive strand of pUC118 is indicated by an arrow on the plasmid.


r-strand 3 5
l-strand 5 3
0 10 20 30 40 50 60 70 80 90 100
B-C
B-B
H-B
H-A
polylinker
cloning sites
Hind III
BamH I
pUC118
(+)
H B B H
18.2 23.4 30.3 31.9
H-B
H B H
66.5 73 81.9
H-A
B H HH H B
59.6 63.5 66.5 73
62.1 64.5
B-C
B H HHB
73 81.9 87.1 88.2
87.3
B-B

either *Bam*H I or *Hin*d III. The size of the expected fragments have been listed in Table 3-3 and diagnostic fragment size specifying orientation of the insertion have been presented in bold. For some of the recombinants, the diagnostic restriction patterns are illustrated in Figure 3-13. For Northern blot analysis described below in chapter 6, pUC118BC-r was used to prepare the probe for E2A, pUC118HB-r for E2B, pUC118BB-l for E3 and pUC118BC-l for L4.

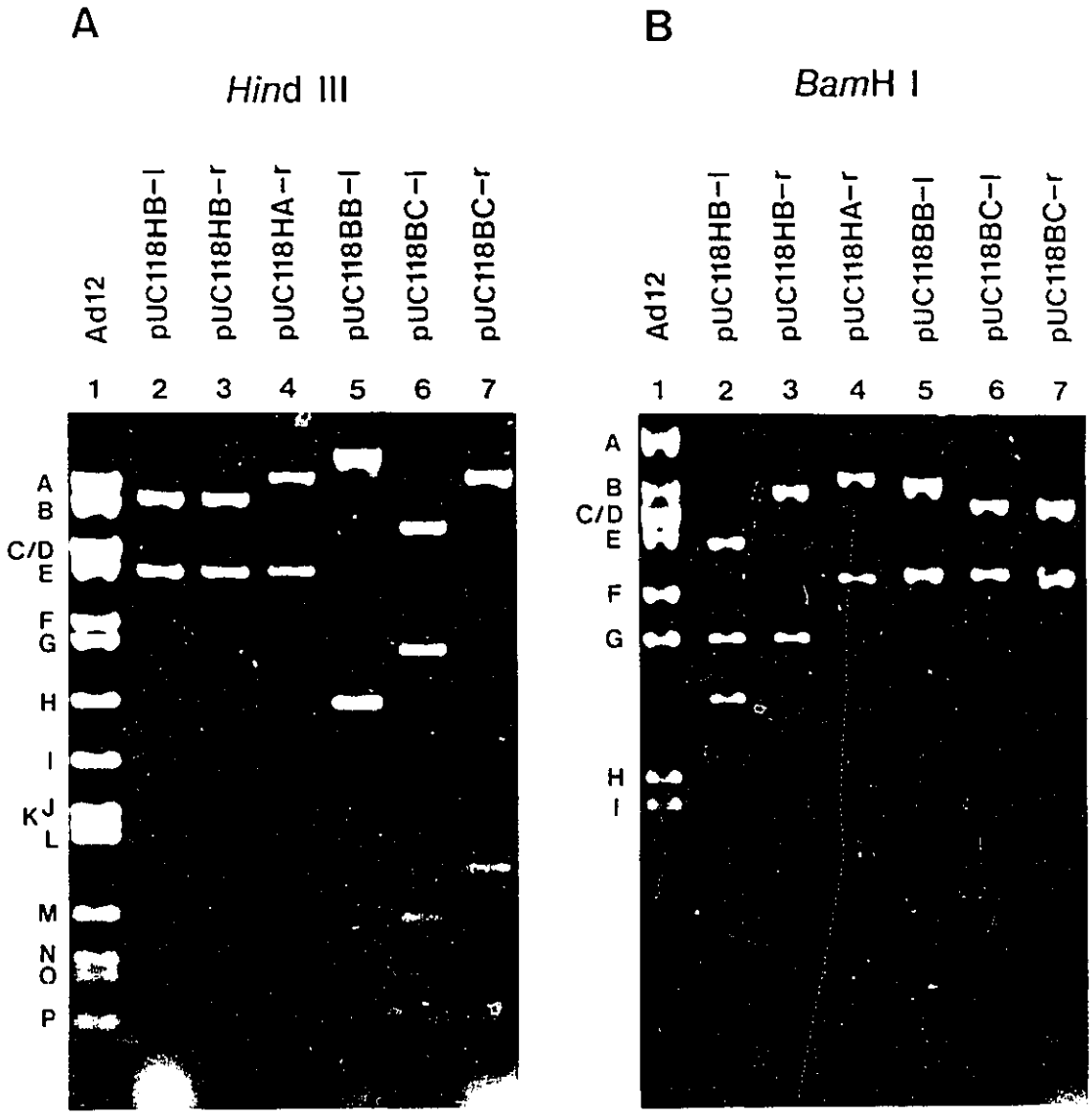
Table 3-3: Restriction analysis of recombinants by *Hin*d III and *Bam*H I

Recombinant	*Bam*H I	*Hin*d III
pUC118HA-l	**2.24, 6.16**	3.16, 5.24
pUC118HA-r	**3.06, 5.34**	3.16, 5.24
pUC118HB-l	**1.80**, 2.31, **3.71**	3.16, 4.66
pUC118HB-r	**0.61**, 2.31, **4.89**	3.16, 4.66
pUC118BB-l	3.16, 5.17	0.07, **0.34**, 1.77, **6.16**
pUC118BB-r	3.16, 5.17	0.07, 1.77, **3.06**, **3.44**
pUC118BC-l	3.16, 4.56	0.34, 0.48, 0.68, **2.24**, **3.98**
pUC118BC-r	3.16, 4.56	0.34, 0.48, 0.68, **0.88**, **5.34**

The numbers represent the sizes (kb) of the fragments produced by restriction enzyme digestion. Each pair of the recombinants represent the same fragments inserted in different orientations. The numbers written in bold represent the diagnostic fragments.

Figure 3-13. Restriction analysis of recombinants.

dsDNA was isolated and subjected to either *Bam*H I or *Hin*d III digestion. The designations of the plasmids are defined in the text. The size of the fragment was determined by comparison to the molecular markers. *Hin*d III-digested Ad12 (Huie) DNA is shown in lane 1 of Fig. A. A, 5.28 Kb; B, 4.70 Kb; C/D, 3.63/3.60 Kb; E, 3.36 Kb; F, 2.50 Kb; G, 2.32 Kb; H, 1.78 Kb; I, 1.39 Kb; J, 1.10 Kb; K, 1.03 Kb; L, 0.99 Kb; M, 0.69 Kb; N, 0.55 Kb; O, 0.45 Kb; P, 0.34 Kb. *Bam*H I-digested Ad12 (Huie) DNA is shown in lane 1 of Fig. B. A, 7.98 Kb; B, 5.18 Kb; C/D, 4.57/4.50 Kb; E, 4.02 Kb; F, 2.86 Kb; G, 2.35 Kb; H, 1.26 Kb; I, 1.12 Kb.


A
Hind III
Ad12
pUC118HB-l
pUC118HB-r
pUC118HA-r
pUC118BB-l
pUC118BC-l
pUC118BC-r
1 2 3 4 5 6 7
A
B
C/D
E
F
G
H
I
K
J
L
M
N
O
P
B
BamH I
Ad12
pUC118HB-l
pUC118HB-r
pUC118HA-r
pUC118BB-l
pUC118BC-l
pUC118BC-r
1 2 3 4 5 6 7
A
B
C/D
E
F
G
H
I

CHAPTER FOUR

RESULTS: PHENOTYPES OF THE E1B 163R MUTANTS

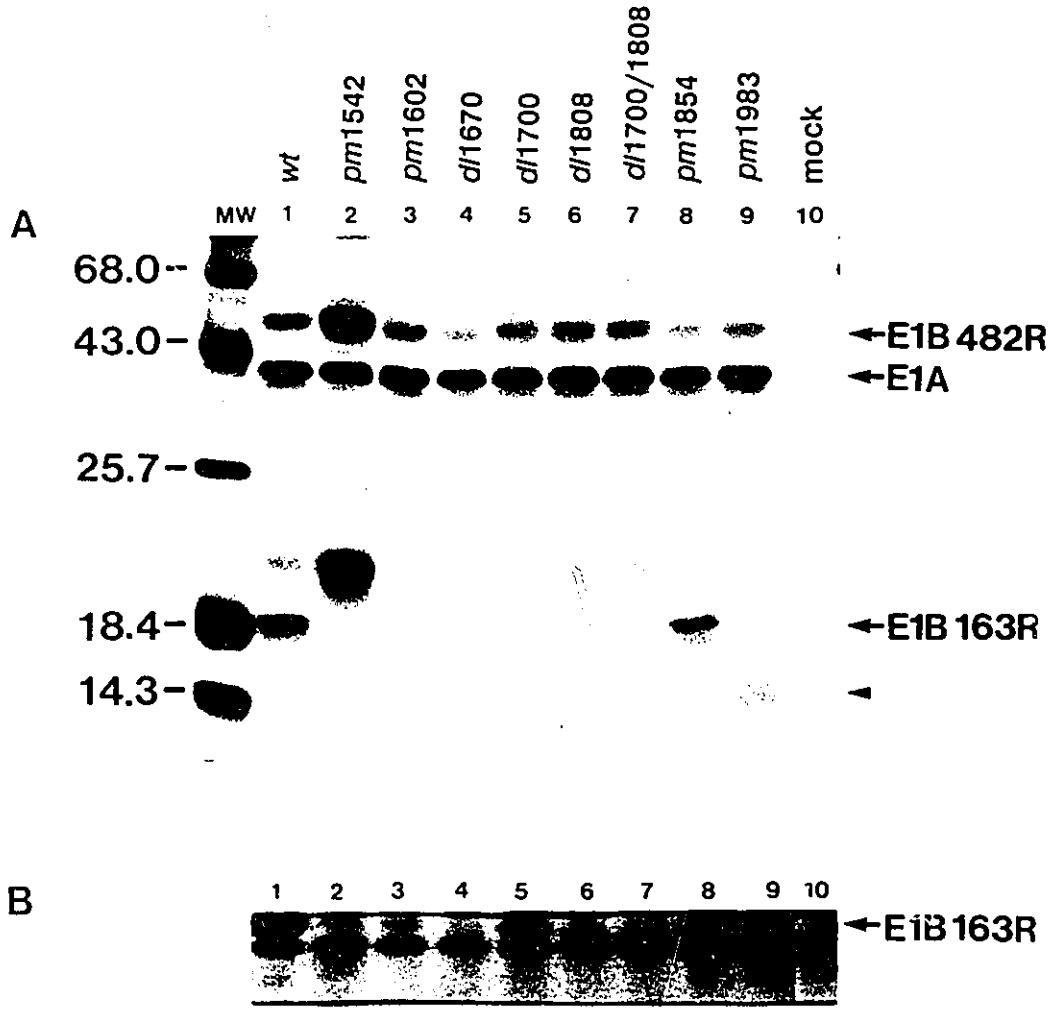
4.1. Synthesis of E1 Proteins By Mutant and *wt* Ad12.

To test the ability of the present series of Ad12 mutants to synthesize stable 163R-related proteins, extracts from infected KB cells labelled with [^{35}S]methionine for one hour were immunoprecipitated with tumour serum AB6a which recognizes Ad12 E1A and E1B products, and the precipitates were analyzed by SDS-PAGE. Fig. 4-1A shows an autoradiograph of such a gel, and Fig 4-1B, from a separate experiment, an overexposure of a portion of the gel containing 163R.

With *wt* Ad12, the major E1B products migrated with an apparent molecular weight of 55,000 and 19,000 daltons, while E1A proteins appeared as two closely-migrating species at about 41,000 daltons (lane 1). The amount of the 163R protein detected in mutant-infected cells varied considerably (see Fig. 4-1B). As expected, *pm*1542 synthesized no detectable 163R (lane 2). With *pm*1602 and *dl*1670, 163R-related products were detected either in very low amounts or not at all (lanes 3 and 4, respectively). However, mutants *dl*1700, *dl*1808 and *dl*1700/1808 (lanes 5, 6 and 7) all synthesized proteins of lower apparent molecular weight than 19,000 dalton, at levels considerably lower than those observed with *wt* Ad12 (lane 1). The decreased amounts

Figure 4-1. Analysis of E1 proteins synthesized by mutant and *wt* Ad12.

KB cells infected with 163R mutants or *wt* Ad12 virus at a multiplicity of infection of 400 virions per cell were labelled with [^{35}S]methionine from 20-21 h after infection. Cell extracts were immunoprecipitated using serum AB6a and then analyzed by SDS-PAGE. A. Two day exposure of autoradiogram. B. Two week exposure of autoradiogram showing the region of the gel containing 163R. Lanes: MW, ^{14}C-labelled molecular weight marker; 1, *wt*; 2, *pm*1542; 3, *pm*1602; 4, *dl*1670; 5, *dl*1700; 6, *dl*1808; 7, *dl*1700/1808; 8, *pm*1854; 9, *pm*1983; 10, mock-infected cells. The positions of E1A and E1B proteins are indicated at the right of the figure (arrows) as is that of the truncated 163R-related product of *pm*1983 (arrowhead).


A
MW
1
2
3
4
5
6
7
8
9
10
wt
pm1542
pm1602
dl1670
dl1700
dl1808
dl1700/1808
pm1854
pm1983
mock
68.0
43.0
25.7
18.4
14.3
E1B 482R
E1A
E1B 163R
B
1
2
3
4
5
6
7
8
9
10
E1B 163R

of 163R found with these mutants (and with *pm*1602 and *dl*1670) appeared to result from decreased stability of the altered products because higher levels could not be detected even after a 4-hour labelling period (data not shown, also see fig. 5-5, lanes 3 and 4). With *pm*1854, 163R was detected at high levels (lane 8) while *pm*1983 gave rise to a stable truncated form of this product (lane 9). The anomalous migration rate of the truncated product in *pm*1983 was not directly related to the size of the truncated peptides, but rather may have resulted from removal of acidic and proline residues, as found previously by Egan *et al* (1988) with E1A deletion mutants. With all infected cells, a protein below 19,000 daltons was detected (Fig. 4-1B, lanes 1 to 9). It is likely to be a cellular protein since it was also detected in mock-infected cells (lane 10). Alternatively, it might be a minor E1b protein as described by Mak and Mak (1986), which co-migrated with the cellular protein (lane 10).

All of these mutants synthesized E1A products and E1B-482R, usually at levels comparable to *wt* Ad12. Surprisingly, the level of 482R expressed in *pm*1542-infected cells was about 8-fold higher than that observed with *wt* Ad12 or any of the other mutants, as determined by comparison of intensities of the protein bands in an autoradiogram containing serial dilutions of the precipitates (see chapter 5). Such was also the case for another protein of an apparent molecular weight of 22,000 daltons. This species probably represented the 482R-related product of the 13S mRNA (see chapter 5) which has been found to be produced at high levels by Ad2 (Anderson *et al.*, 1984; Lewis and Anderson, 1987) and Ad5 (S. Brown, D. Takayesu and P. E. Branton,

manuscript in preparation).

4.2. DNA Degradation

It has been well documented that the 163R protein is important for protection of both viral and cellular DNA, however little is known about the regions of 163R involved in this function. The applicability of the present series of mutants to address this question was complicated by the apparent instability of the altered 163R products. Nevertheless, we examined migration on agarose gels of low molecular weight DNA isolated from cells at various times after infection. Fig. 4-2 shows that with mock- and *wt* Ad12-infected cells, DNA migrated as a single band near the top of the gel. With *pm*1542, *pm*1602, *dl*1670, *dl*1700, *dl*1808 and *dl*1700/1808, all of which produce unstable 163R-related products, DNA degradation was apparent by 25 h p.i., as evidenced by the presence of a continuous smear of DNA throughout the gels. With *pm*1854 which contains a point mutation at Glu^{105} and with *pm*1983 which eliminates the last 16 residues, no such degradation was observed even at late times after infection (Fig. 4-2). Such was also the case when the multiplicity of infection was increased to 1600 virions per cell (Fig. 4-3), indicating that DNA degradation was not due to the acceleration of the viral replication cycle. While these data did not identify a specific domain involved in the *deg* phenotype, they indicated that this function likely resides in the first 100 amino terminal residues of 163R.

Figure 4-2. Analysis of DNA degradation I.

Cells were infected with 163R mutants or *wt* Ad12 at a multiplicity of infection of 400 virions per cell or they were mock-infected. Low-molecular-weight DNA was extracted at various times post-infection (h p.i.) (indicated at the top of each panel) and analyzed on agarose gels.

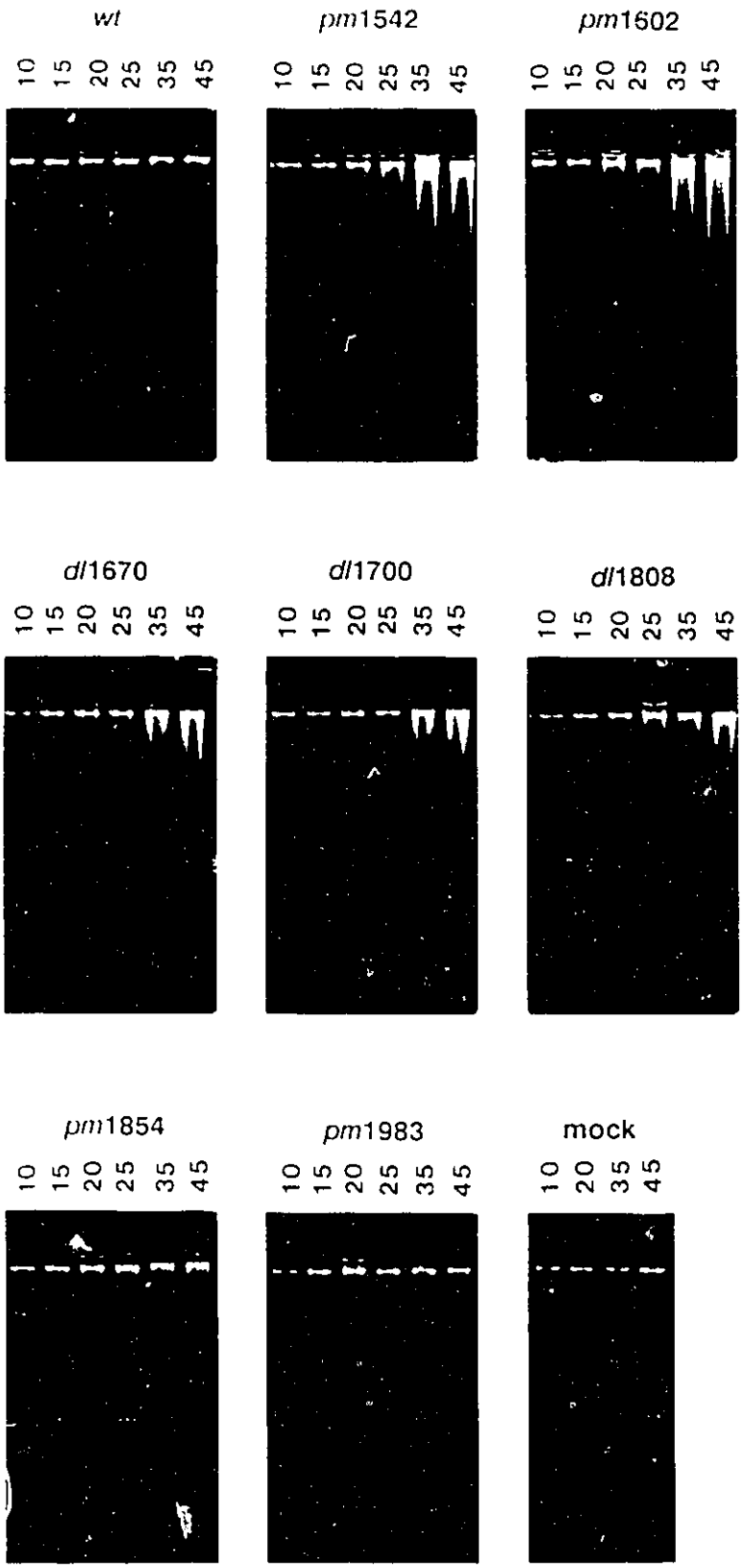
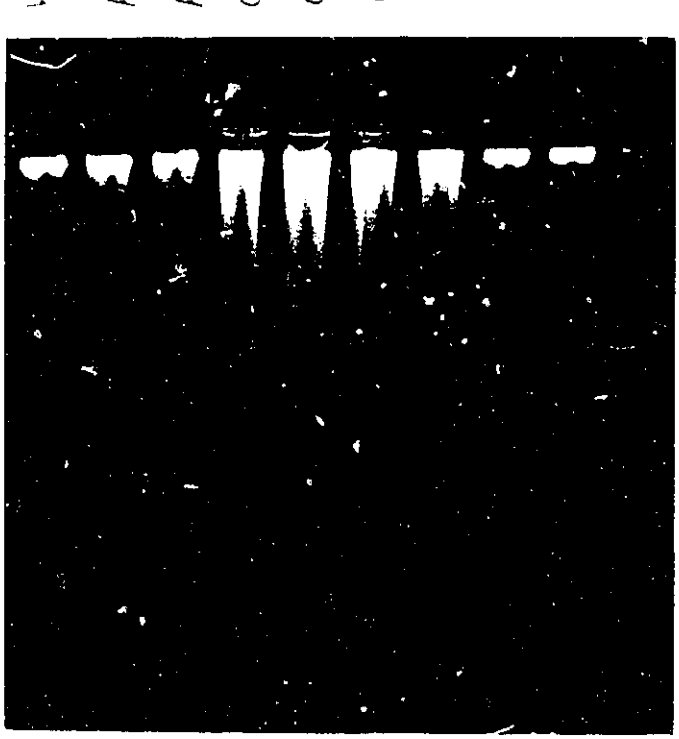

wt
10 15 20 25 35 45
pm1542
10 15 20 25 35 45
pm1602
10 15 20 25 35 45
dl1670
10 15 20 25 35 45
dl1700
10 15 20 25 35 45
dl1808
10 15 20 25 35 45
pm1854
10 15 20 25 35 45
pm1983
10 15 20 25 35 45
mock
10 20 35 45

Figure 4-3. Analysis of DNA degradation II.

Cells were infected with 163R mutants or *wt* Ad12 at a multiplicity of infection of 1600 virions per cell or they were mock-infected. Low-molecular-weight DNA was extracted at 48 hours after infection and analyzed on agarose gel.



1600 virions per cell

4.3. Induction of the *cyt* Phenotype and Viral Replication.

The *cyt* phenotype is characterized by the appearance of large plaques, extensive disruption of cellular morphology, and enhanced detachment of infected cells (Takemori *et al.*, 1968, 1984; Chinnadurai, 1983; Subramanian *et al.*, 1984a; White *et al.*, 1984). The ability of our mutants to induce this phenotype was analyzed by microscopic examination of infected KB cells. All of the mutants that exhibited the *deg* phenotype yielded cells that, by 25 h p.i., were highly condensed, disrupted, and largely detached from the plate, as is typical of the *cyt* phenotype. However, with *pm*1854, *pm*1983 and *wt* Ad12, cells retained a round and intact morphology even after they began to detach later during infection (data not shown).

The infectivity of some mutants was determined by measuring plaque-forming ability of mutants on MH12-C2 cells. An aliquot of diluted virus stock was used to infect MH12-C2 monolayers directly and the number of plaques were counted. As expected, the ability of plaque formation of mutants *pm*1542, *dl*1670, *dl*1808, and *pm*1983, and *wt* viruses is very comparable (Table 4-1). Since 1 OD virus suspension corresponds to 4×10^{11} virions/ml (Green and Pina, 1963), 1 plaque forming unit (PFU) is equivalent to approximately 85 virions [$4 \times 10^{11} \div (4.60 + 5.18 + 4.98 + 3.93 + 4.67)/5 \times 10^{9}$]. To analyze the ability of the present series of mutants to replicate in KB cells, the yields of intracellular and extracellular progeny virions at various times after infection were determined by plaque assay on MH12-C2 cells. Generally, there was no significant difference in virus titers produced by mutant and *wt* Ad12, although the titers

Table 4-1. Infectiviy of 163R mutants and *wt* Ad12

Virus	Infectivity (PFU/ml)
wt	$4.60 \pm 0.47 \times 10^9$
*pm*1542	$5.18 \pm 0.30 \times 10^9$
*dl*1670	$4.98 \pm 0.34 \times 10^9$
*dl*1808	$3.93 \pm 0.32 \times 10^9$
*pm*1983	$4.67 \pm 1.56 \times 10^9$

Virus stocks (1 OD/ml) were diluted at dilution factors of 4×10^6 and 4×10^7, and an aliquot of virus suspension was used to infect MH12-C2 cells. Three dishes were tested for each dilution. The data were obtained from the plates with number of plaques between 10 to 200.

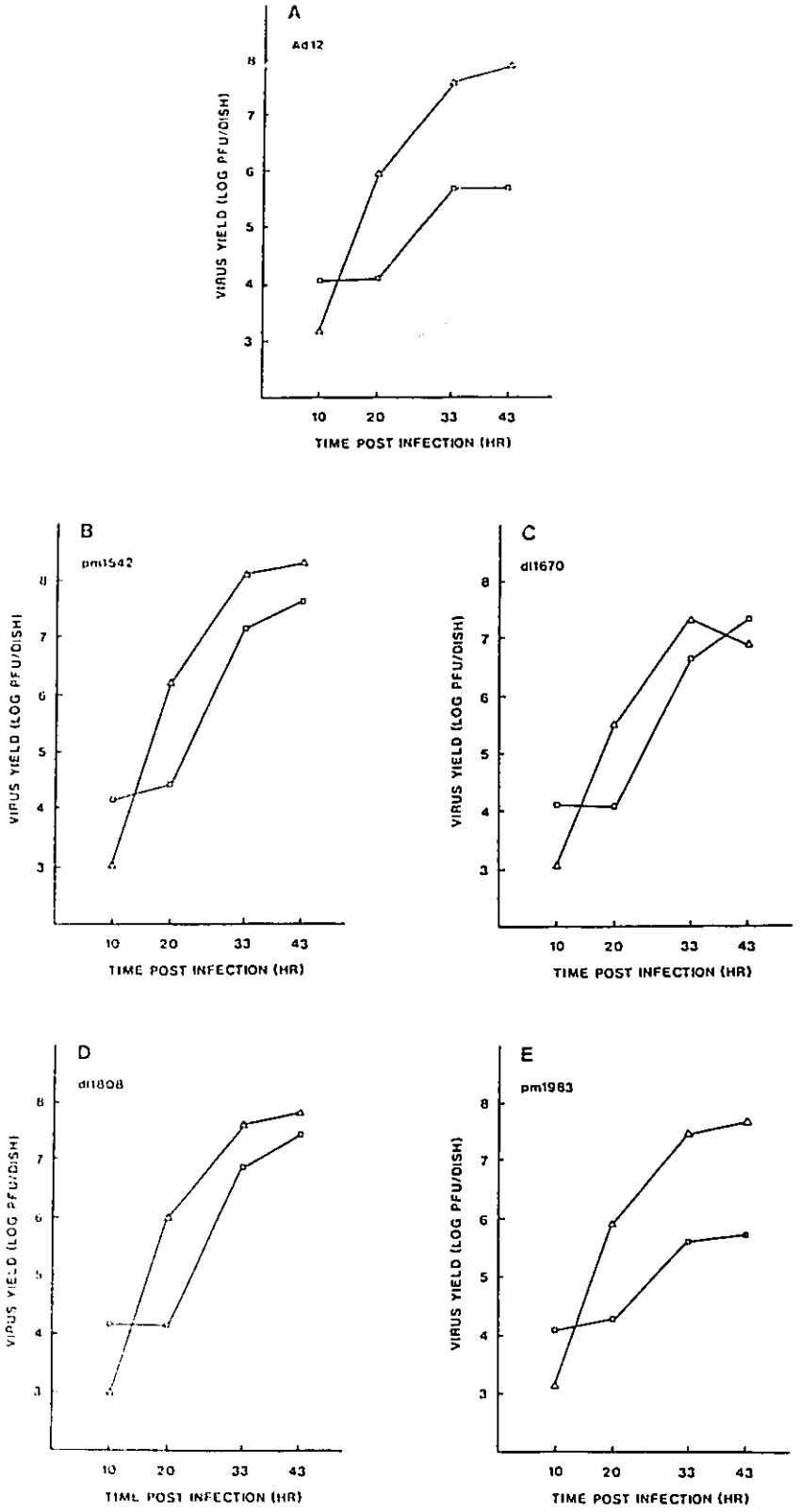
Table 4-2. Total virus yields from KB cells infected with 163R mutant and *wt* Ad12.

	PFU per dish			
Virus	10 h	20 h	33 h	43 h
wt	$1.3 \pm 0.4 \times 10^4$	$9.1 \pm 0.4 \times 10^5$	$4.5 \pm 0.3 \times 10^7$	$8.9 \pm 1.7 \times 10^7$
*pm*1542	$1.6 \pm 0.4 \times 10^4$	$1.7 \pm 0.4 \times 10^6$	$1.5 \pm 0.4 \times 10^8$	$2.5 \pm 0.3 \times 10^8$
*dl*1670	$1.4 \pm 0.3 \times 10^4$	$3.2 \pm 1.1 \times 10^5$	$2.7 \pm 0.8 \times 10^7$	$3.1 \pm 0.6 \times 10^7$
*dl*1808	$1.6 \pm 0.2 \times 10^4$	$1.1 \pm 0.4 \times 10^6$	$6.2 \pm 0.9 \times 10^7$	$9.8 \pm 1.8 \times 10^7$
*pm*1983	$1.4 \pm 0.2 \times 10^4$	$8.7 \pm 1.9 \times 10^5$	$3.4 \pm 0.4 \times 10^7$	$5.3 \pm 2.0 \times 10^7$

Total virus yield was derived by adding intracellular and extracellular virus yields together. Each value represents an average virus titre. At each indicated time after infection, serial tenfold dilutions were prepared and three consecutive dilutions were used for plaque assay. Three dishes were tested for each dilution. The data were those obtained using dilutions yielding number of plaques between 10 to 200.

Figure 4-4. Growth kinetics of mutant and *wt* viruses.

KB cells were infected with 163R mutants or *wt* Ad12 at a multiplicity of infection of 400 virions per cell. At various times after infection. samples of infected cells and culture media were harvested and intracellular and extracellular virus yields were determined by plaque assay on MH12-C2 cells. A, *wt*; B, *pm*1542; C, *dl*1670; D, *dl*1808; E, *pm*1983. Symbols: , intracellular viruses; , extracellular viruses.


A
Ad12
B
pm1542
C
dl1670
D
dl1808
E
pm1983
VIRUS YIELD (LOG PFU/DISH)
TIME POST INFECTION (HR)
10
20
33
43
3
4
5
6
7
8

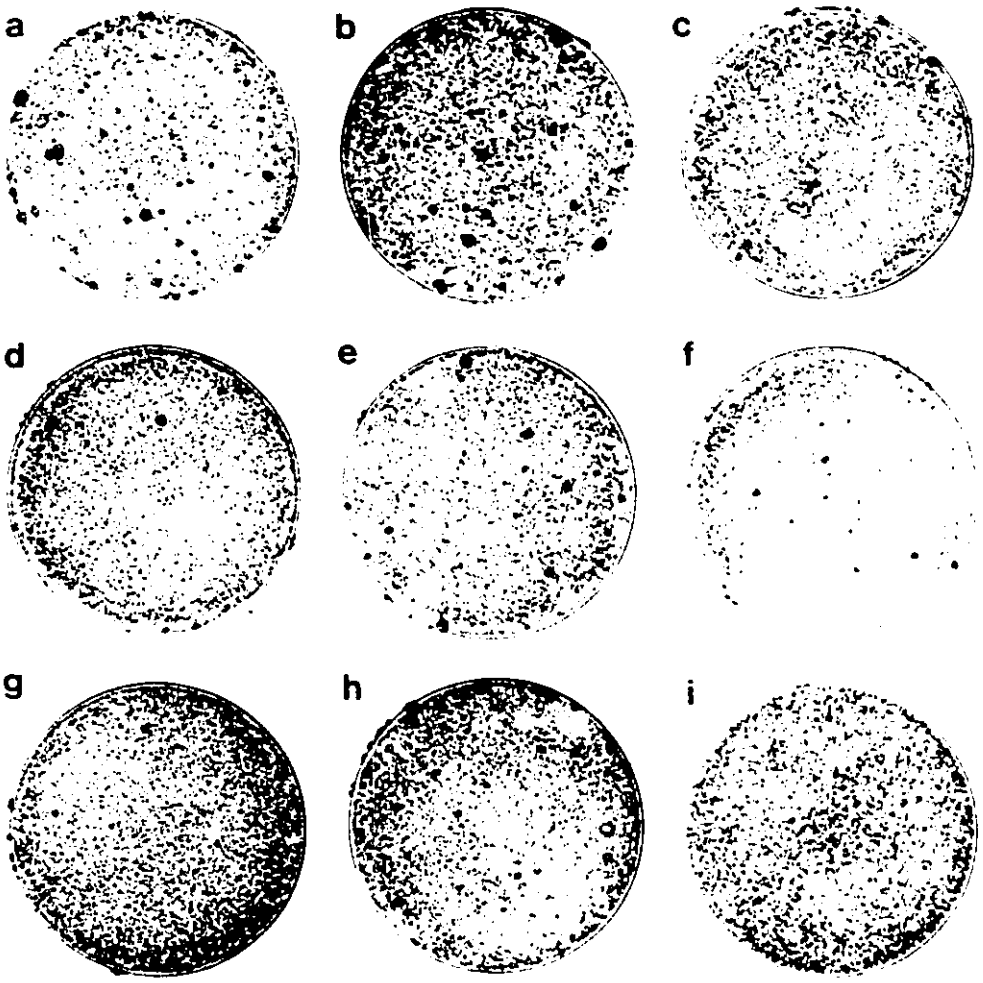
of *dl*1670 were two- to three-fold less than *wt*, and with *pm*1542 they were two- to three-fold higher (Table 4-2). These results confirmed that 163R is not absolutely essential for virus replication. However release of virus was much more efficient with the *cyt/deg* mutants *pm*1542, *dl*1670 and *dl*1808 since the yield of extracellular *cyt/deg* mutant viruses was considerably higher (Figs. 4-4B, C and D, respectively). With *pm*1983 which exhibits a *wt* phenotype, virus growth was comparable to that of *wt* Ad12 (Fig. 4-4A).

4.4. Cellular Transformation by Mutant and *wt* Ad12 Viruses.

Previous studies with Ad12 UV-induced *cyt* mutants (Lai Fatt and Mak, 1982; Mak and Mak, 1983; Mak *et al.*, 1984) and with Ad12 163R, Ad2 175R and Ad5 176R mutants (Chinnadurai, 1983; Babiss *et al.*, 1984b; Fukui *et al.*, 1984; Pilder *et al.*, 1984; Subramanian *et al.*, 1984a; Takemori *et al.*, 1984; White *et al.*, 1984b; Bernards *et al.*, 1986; Barker and Berk, 1987) had suggested a role for 163R in oncogenic transformation. However, results of a recent study seriously questioned the involvement of this Ad12 protein in the transformation of primary rat cells (Edbauer *et al.*, 1988). To examine this question further, BRK cells were infected at various multiplicities with *wt* or mutant Ad12 viruses and following three weeks in culture the number of transformed cell foci was counted. A series of dishes containing transformed cell foci are shown in Fig. 4-5. Mock-infected cells exhibited no foci and virus-infected cultures produced darkly stained foci which varied in number. Table 4-3 shows the results

Figure 4-5. Transformation of primary baby rat kidney (BRK) cells.

BRK cells were infected as monolayers at various multiplicities with mutant or *wt* Ad12 viruses. After 2 days in α-MEM containing 10% fetal calf serum, the cells were maintained in Joklik's modified MEM plus 5% horse serum which was replenished every 3-4 days. At 23-25 days the cells were fixed and stained with Giemsa for 30 minutes. Transformation results of one set of the transformed dishes with a multiplicity of infection of 500 virions per cell are shown, illustrating a relative efficiency of transformation.



a *wt* b *pm*1542 c *pm*1602

d *dl*1670 e *dl*1700 f *dl*1808

g *pm*1854 h *pm*1983 i mock

in two similar studies. With *wt* virus, the number of foci increased continuously with
in two similar studies. With *wt* virus, the number of foci increased continuously with
virus input up to about 500 particles per cell, after which increasing virus dose actually led to a decline in focus formation, presumably due to cell killing at high virus concentrations. With mutants containing defects in the first 100 amino acid residues of 163R (*pm*1602, *dl*1670, *dl*1700, *dl*1808 and *dl*1700/1808), a highly reproducible reduction in the numbers of transformants was observed. Even at optimal virus doses transformation was in all cases less than 40% of *wt* Ad12. Because most of these mutants produced unstable 163R products it was not possible to resolve the functional contributions of the regions of 163R affected by these mutations. These results did, however, strongly suggest that 163R may contribute to efficient Ad12 transformation. Mutant *pm*1854, which alters conserved residue 105, also yielded reduced numbers of transformants. These data suggested that a region of 163R containing this residue must be functionally important for transformation as this mutant produced a stable product. Mutant *pm*1983, which encodes a 163R protein lacking the carboxy terminal 16 amino acids, yielded transformants at efficiencies similar to *wt*, suggesting that this region is not required for this process. Of considerable interest were results obtained with *pm*1542 in which the initiation codon for 163R had been altered. Surprisingly, this mutant, which produces no detectable 163R-related products, transformed at an efficiency quite similar to that of *wt* Ad12. As discussed in chapter 5, I believe that such result might be explained by the observed overexpression of the E1B 482R product.

Table 4-3. Transformation of BRK cells by Ad12 E1B 163R mutants I.

Virus	AVERAGE NUMBER OF FOCI/DISH (% *wt*)*						
	Multiplicity of infection (Virions/cell)						
	50	125	250	500	1000	2000	4000
wt	31(100)	76(100)	108(100)	116(100)	107(100)	103(100)	300)
*pm*1542	26(84)	63(83)	93(86)	111(96)	73(68)	56(54)	17(57)
*pm*1602	4(13)	23(30)	42(38)	35(30)	20(19)	15(15)	6(20)
*dl*1670	4(13)	11(14)	19(18)	16(14)	8(7)	3(3)	2(7)
*dl*1700	3(10)	22(29)	35(32)	35(30)	29(27)	12(12)	9(30)
*dl*1808	3(10)	14(18)	21(19)	24(21)	15(14)	9(9)	3(10)
*dl*1700/1808	4(13)	20(26)	22(20)	24(21)	15(14)	11(11)	4(13)
*pm*1854	4(13)	27(36)	38(35)	41(35)	33(31)	28(27)	11(37)
*pm*1983	23(74)	48(63)	84(78)	91(78)	102(95)	87(84)	29(97)

*Baby rat kidney cells were infected at various multiplicities with *wt* or mutant Ad12. Each value represents the average number of foci obtained from four dishes of infected cells. The data presented were derived from a single experiment, however, comparable efficiencies of transformation with the various mutants were obtained in three separate experiments.

CHAPTER FIVE

RESULTS: BIOCHEMICAL ANALYSIS OF MUTANT *pm*1542

5.1. *pm*1542 Overproduces the E1B 482R Protein Consistently

The results described in Chapter 4 characterizing the phenotypes of Ad12 163R mutants showed that mutations affecting the stabilities of 163R-related products induced the *deg/cyt* phenotype in infected KB cells and yielded fewer primary rat cell transformants. *pm*1542, however, transformed primary rat cells at an efficiency similar to *wt* Ad12 in spite of the fact that it induced the *deg/cyt* phenotype. Of some interest was the fact that only *pm*1542 overexpressed the E1B 482R protein. To investigate the possible relationship between this overexpression and transformation, extracts from Ad12- or *pm*1542-infected cells labelled for 30 minutes at various times after infection were immunoprecipitated with antiserum AB6a. Fig. 5-1 shows that with *wt* Ad12, 163R was detected by 10 hours after infection (lane 4) and was readily observed thereafter (lanes 7, 10 and 13). With *pm*1542, no detectable 163R was produced (lanes 5, 8, 11 and 14). However the E1B 482R protein was detected at significantly higher levels with *pm*1542 than with *wt* Ad12 (lanes 2, 5, 8, 11 and 14). Using serial twofold dilutions of lysate from *pm*1542-infected cells (Fig. 5-2, lanes b to e), it was estimated that *pm*1542 overproduced 482R by about 8-fold relative to *wt* (lanes a and e). This difference was

Figure 5-1. Comparison of E1 proteins synthesized in KB cells infected with wt or pm1542 viruses.

KB cells were infected at a multiplicity of infection of 400 virions per cell and at the indicated times after infection labelled with [^{35}S]methionine for 30 minutes. Cell lysates were subjected to immunoprecipitation with E1 antiserum AB6a-C3 and analyzed by SDS-PAGE. E1A and E1B proteins are indicated at the right of the figure. [^{14}C]-labelled molecular weight (MW) markers are in kilodaltons (Kd).

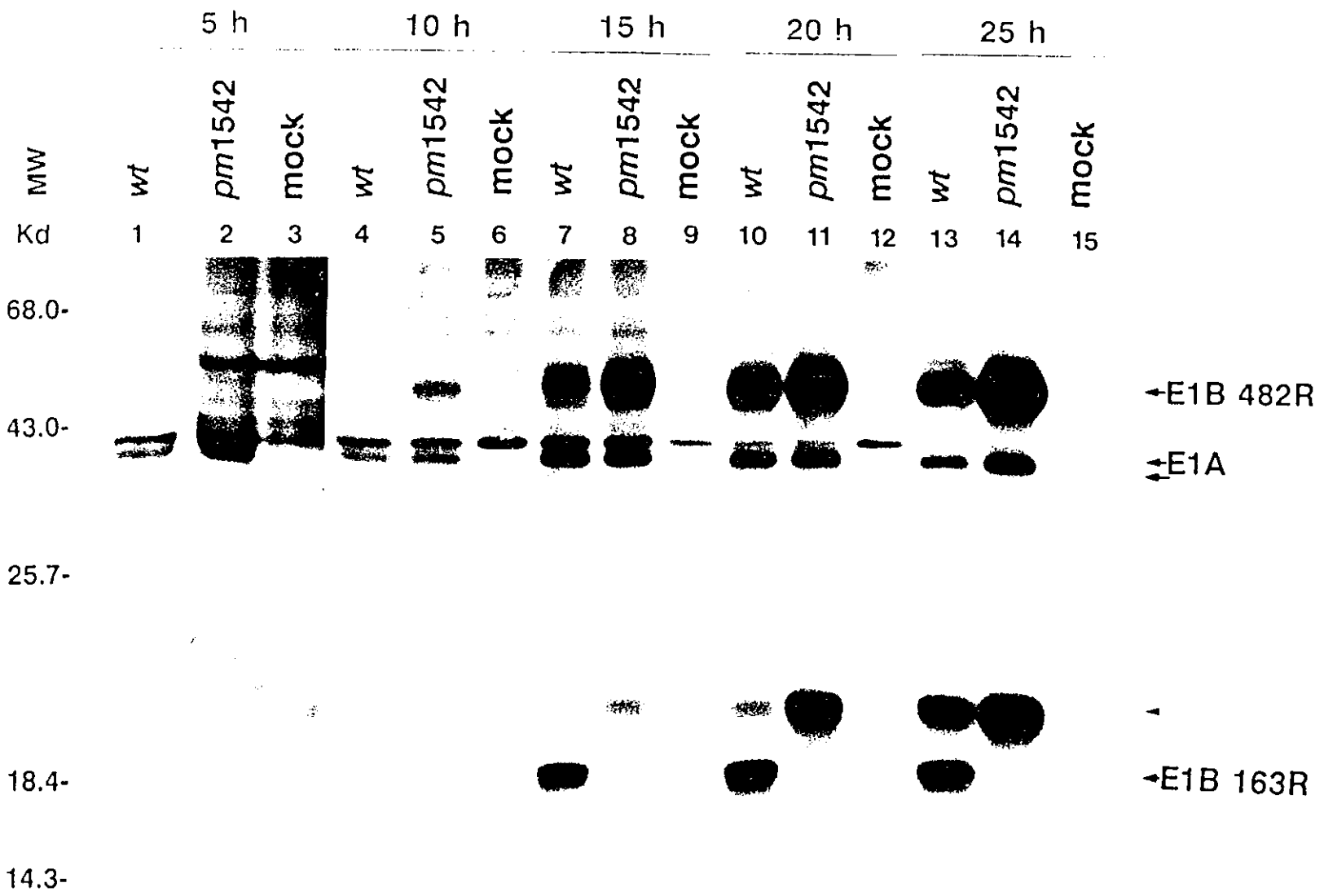
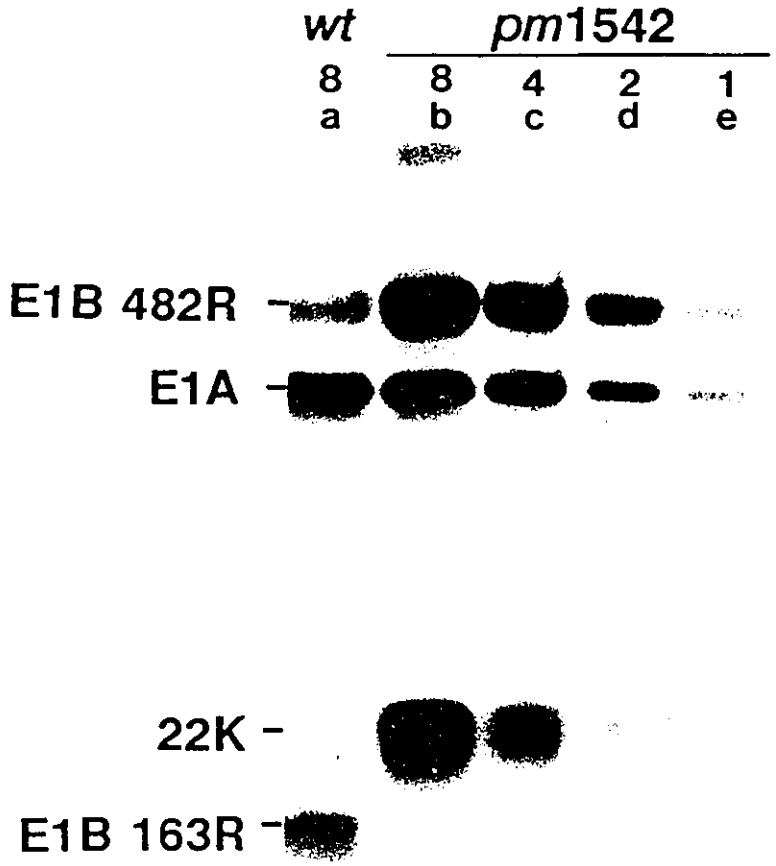

5 h
10 h
15 h
20 h
25 h
MW
Kd
wt
pm1542
mock
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
68.0-
43.0-
25.7-
18.4-
14.3-
E1B 482R
E1A
E1B 163R

Figure 5-2. Comparison of the levels of the E1 proteins produced with *pm*1542 and *wt*.

Cell lysates were prepared as described in the legend to Fig. 5-1. Lysates of serial twofold dilutions were made with *pm*1542 and immunoprecipitated. The numbers at the top of the figure indicate the relative amounts of lysates. The E1A and E1B proteins are indicated on the left side.


wt
pm1542
8
8
4
2
1
a
b
c
d
e
E1B 482R
E1A
22K
E1B 163R

apparent at early times and remained consistent even at later times (Fig. 5-1, and data not shown), suggesting that it was not due to a delay in virus replication in *wt*-infected cells. Analysis of whole cell extracts also indicated increased level of 482R with *pm*1542, suggesting that the difference was not due to changes in the distribution of 482R in the cytoplasm (see Fig. 5-8, bottom, lane 4 and 5). It was not due to a shortened half-life of 482R in the presence of 163R either, because 163R supplied *in trans* could not reduce the level of 482R in *pm*1542-infected cells (see section 5.3). Similar increase was also observed for another protein with an apparent molecular weight of 22,000 (22K) (Fig. 5-1, lanes 7, 8, 10, 11, 13, and 14). This protein appears to be a 482R-related protein (see below), almost certainly the product of the 13S E1B mRNA that shares amino terminal sequences with 482R. This 22K protein was detected after 15 hours postinfection and thus its expression correlated with the expression pattern of the 13S E1B mRNA (see below). Interestingly, the levels of the E1A proteins produced in mutant- and wt-infected cells did not show any significant difference over the period examined (Fig. 5-1), suggesting that the increased level of 482R is not regulated through the level of E1A.

5.2. *pm*1542 and *wt* Viruses Accumulate E1B mRNA to Similar Levels

To determine whether the overproduction of the 482R protein in *pm*1542-infected cells resulted from elevated levels of E1B 22S mRNA, cytoplasmic mRNA was isolated from infected cells at various times after infection and equal amounts of total RNA were

subjected to Northern analysis. In Fig. 5-3, 22S mRNA was detected early after infection and accumulated to a higher level at late times. The levels of the 22S mRNA with *wt* and *pm*1542 were quite similar at 10, 20 and 30 hours after infection (lanes 1 vs 2, 6 vs 7 and 11 vs 12). Therefore the overproduction of the 482R protein appeared to be regulated at the translational rather than the transcriptional level. With a *deg* mutant, *dl*1808 (lanes 4, 9 and 14), the levels of E1B mRNA were also comparable to *wt* (lanes 1, 6 and 11). Another *deg* mutant, *dl*1670, produced 2 to 3-fold less E1B mRNAs than *wt* Ad12 (lanes 8 and 13), which might be the reason for the slightly lower level of 482R found in *dl*1670-infected cells (see Fig. 3-1, lane 4). This mutant will be discussed further in Chapter 6. It should also be noted that appearances of the 13S E1B mRNA (not present at 10 hours p.i. and first detected at 20 hours p.i.) parallelled those of the 22K protein described in Fig. 5-1.

5.3. Overproduction of the E1B 482R Protein Is Not Due to the Absence of the E1B 163R Protein.

The studies just described with *pm*1542 indicated that overproduction of 482R appeared to be a translational effect. Two models of activation have been considered. The first proposes that 163R normally reduces production of 482R *in trans* and in the absence of the former, the latter is synthesized at higher levels. The second proposes that overproduction of 482R is a *cis* effect relating to the absence of the 163R AUG initiation codon which normally suppresses the downstream AUG codon for 482R.

Figure 5-3. Northern blot analysis of accumulation of E1B mRNAs in infected KB cells.

KB cells were infected as described in the legend to Fig. 5-1. At the indicated times after infection, cytoplasmic RNA was isolated and equal amounts of total RNA were separated on formaldehyde agarose gels. After transfer to a nitrocellulose membrane, the blots were hybridized to E1B specific probes pHB14R and pHB15R (see section 2.1.3). The E1B mRNAs are indicated at the right and the positions of *Rsa* II-digested pBR322 fragments used as molecular weight (MW) markers at the left in kilobases (kb).

10 h
20 h
30 h
MW
kb
wt
pm1542
dl1670
dl1808
mock
2.12
1.57
0.68
22S
13S
9S

Through analyses of sequences around the E1B initiation codons according to the model of Kozac (1986), it is apparent that the context around the 163R AUG initiation codon is optimal, with a purine base at position -3 and a guanine base at position +4 (Fig. 5-4). Therefore most ribosomes should very efficiently commence translation at the 163R AUG initiation codon and proceed to the first in-frame stop codon on the mRNA which is downstream of the 482R AUG initiation codon. The effect of such a highly efficient initiation process would be to suppress translation of 482R. With *pm*1542, all of the ribosomes should initiate translation at the 482R AUG initiation codon because that for 163R is no longer present.

To test the first model directly, complementation studies were carried out in which the 163R protein was provided *in trans*. KB cells were infected with *wt* Ad12, *pm*1542, *dl*1670, *dl*1808, *dl*17 [an Ad12 mutant which contains a deletion of residues 80-96 of 482R but which produces a normal 163R product (Mak and Mak, 1990)], or a combination, as indicated below. At 20 hours after infection, cells were labelled with [^{35}S]methionine for 4 hours and lysates were subjected to immunoprecipitation and electrophoresis. Fig. 5-5A shows that *pm*1542 again overproduced 482R relative to *wt* Ad12, *dl*1670 or *dl*1808 (lanes 1-4). E1A was barely detectable in these experiments due to its short half-life and the long labelling period employed. With *dl*17, E1B 163R was evident as was an altered form of 482R (*ie.* 465R) which migrated slightly faster than *wt* 482R (lane 5). The levels of these proteins, however, were elevated (lane 5 vs 1). The reason for such elevation is still unknown. It might be due to the fact that the mutant

Figure 5-4. Schematic representation of the sequences around initiation codons of the E1B proteins.

The optimal context for translational initiation is shown on the top of the figure with the numbers representing the positions of the bases, considering the position of the first nucleotide of AUG as +1. Purine (Pu) base (at position -3) and AUG codons are highlighted. Dots inside the sequence represent any one of four bases. The sequences around the E1B initiation codons of *wt* Ad2/5 and Ad12 were adapted from van Ormondt and Galibert (1984) and the sequence of *pm*700 and *in*700 from Edbauer *et al.* (1988).

		-3 +1 +4
Optimal context		**Pu**..**AUG**G (preferably with A in position -3)
Ad12	22S mRNA	------**G**..**AUG**G----------U..**AUG**G----------
		start 163R start 482R
Ad2/5	22S mRNA	------C..**AUG**G----------U..**AUG**G----------
*pm*1542	22S mRNA	------**G**..**AA**GG----------U..**AUG**G----------
*pm*700	22S mRNA	------**G**..**AU**CG----------U..**AUG**G----------
*in*700	22S mRNA	------**G**..**AUG**G-----TAA-------U..**AUG**G----------

was more progressive in viral replication (Mak and Mak, 1990). When cells were co-infected with pre-mixed *dl*17 and *pm*1542 or other 163R mutants (lanes 6-9), both the 482R and 465R E1B species were observed. In spite of the fact that functional 163R protein was supplied by *dl*17 *in trans*, overproduction of the 482R protein was still evident with *pm*1542. Again the 22K protein was also overproduced (lane 7). Complementation was clearly successful in this experiment because DNA degradation, which was observed with *deg* mutants (Fig. 5-5B, lanes 2-4), was suppressed in co-infected cells (Fig. 5-5B, lanes 7-9).

To study further the overproduction of 482R, complementation assays were performed using *dl*42, another 482R mutant which produces a 440R protein lacking residues 114-155. As described by Mak and Mak (1990), *dl*42 is defective in viral DNA replication, exhibiting a shortage of viral DNA templates and insufficient transcription and translation. Fig. 5-6 shows that with *dl*42 E1A and E1B proteins were barely detectable at 20 hours post-infection (lane 4). However, when cells were co-infected with pre-mixed *dl*42 and *wt* Ad12 or *pm*1542 viruses, the 482R supplied *in trans* enabled *dl*42 virus to replicate successfully, resulting in comparable amounts of E1B 163R and 440R produced from the *dl*42 template (lane 6). The appearance of adequate amounts of 163R and 440R in co-infected cells thus could serve as an excellent control for complementation. With cells co-infected with *dl*42 and *wt* or *dl*42 and *pm*1542 viruses, at input multiplicities of infection of 400 virions per cell for each virus, overproduction of 482R relative to 440R was observed with *dl*42/*pm*1542 (lane 6) but not with *dl*42/*wt*

Figure 5-5. Complementation assay I.

KB cells were infected at a multiplicity of infection of 400 virions per cell or co-infected as indicated at a multiplicity of infection of 400+400 virions per cell. A. At 20 hours after infection cells were labelled with [^{35}S]methionine for 4 hours. Cell lysates were immunoprecipitated with E1 antiserum AB6a and proteins were separated by SDS-PAGE. E1B proteins are indicated on the right side of the figure. Molecular weight (MW) marker is in kilodaltons (Kd) on the left side. B. DNA preparations from infected cells were analyzed by agarose gel electrophoresis.

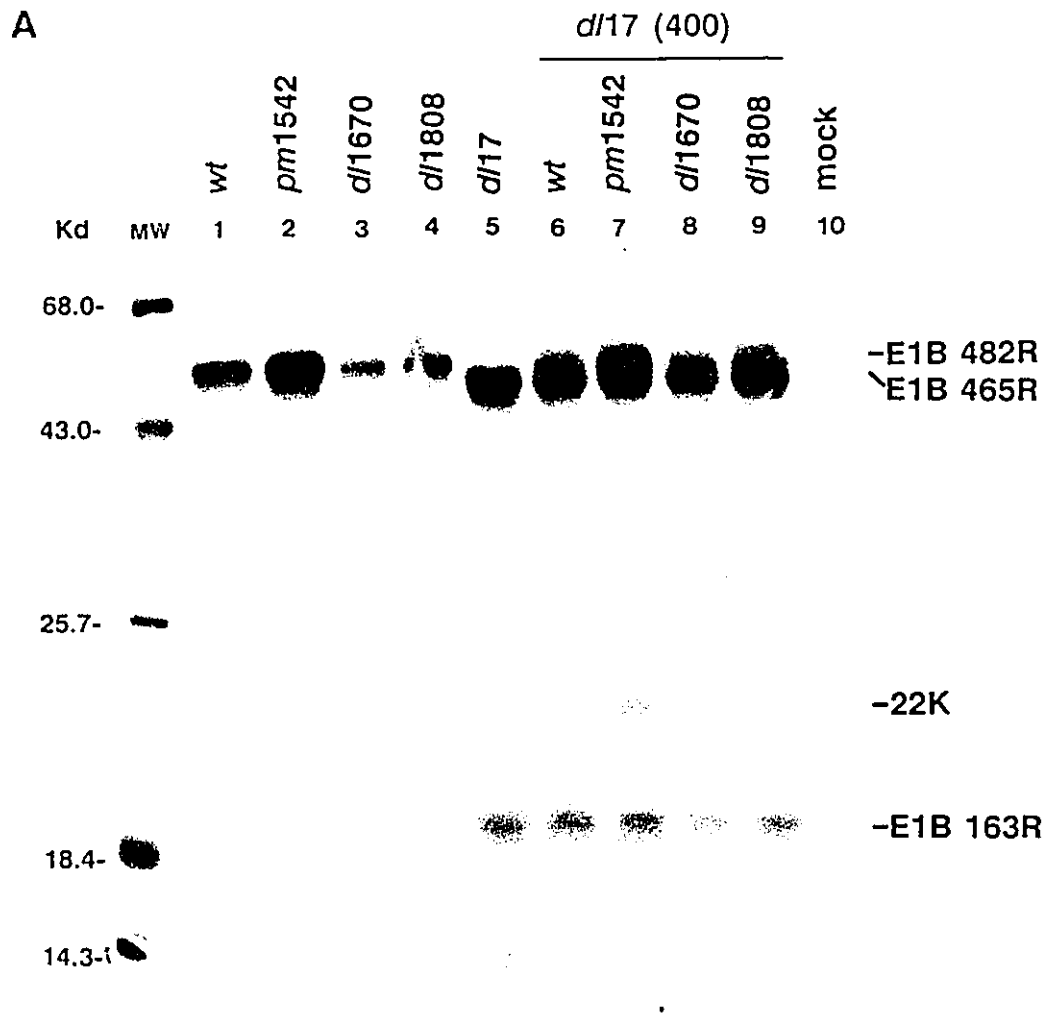
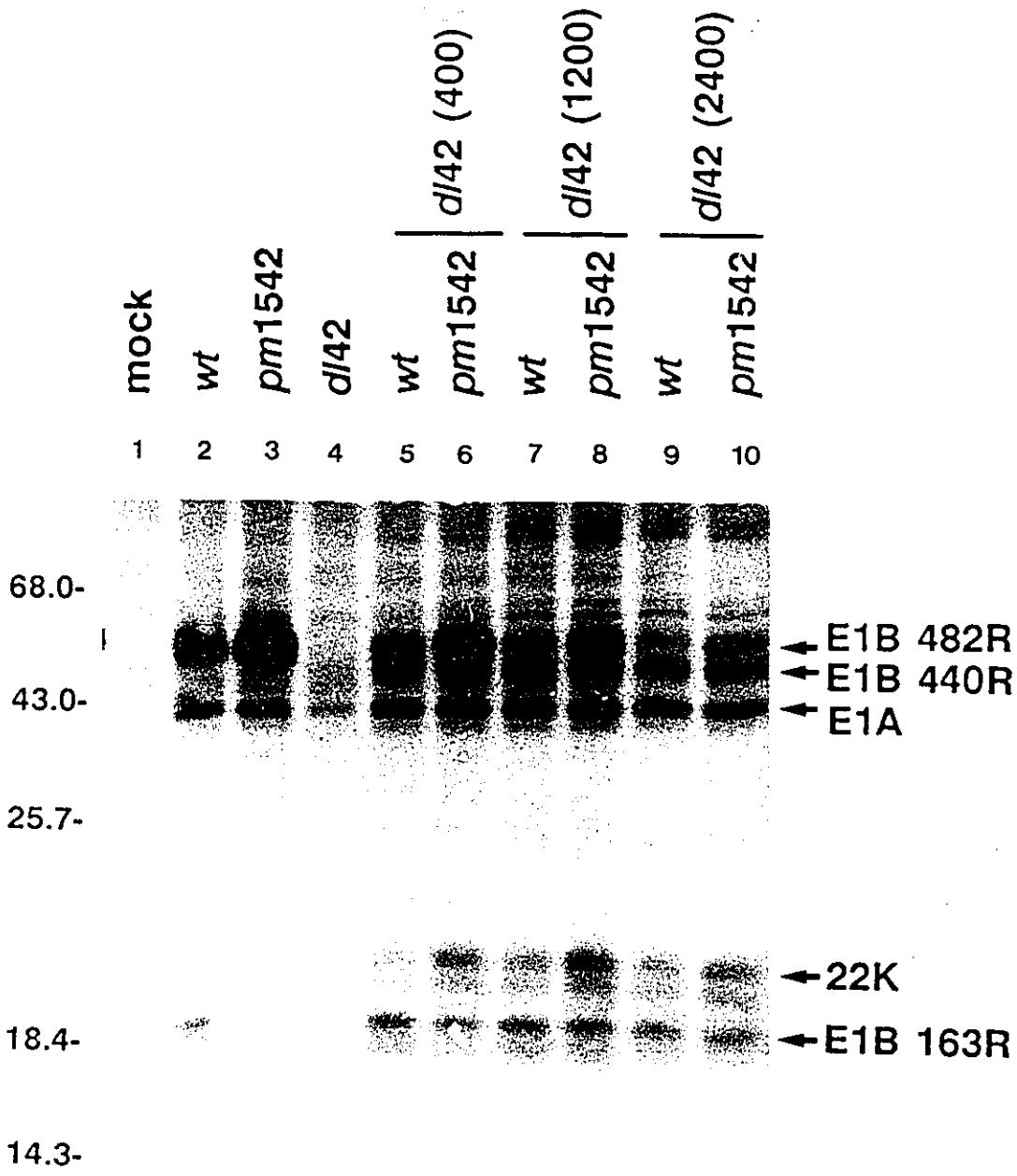

A
dl17 (400)
wt
pm1542
dl1670
dl1808
dl17
wt
pm1542
dl1670
dl1808
mock
Kd
MW
1
2
3
4
5
6
7
8
9
10
68.0-
43.0-
25.7-
18.4-
14.3-
-E1B 482R
-E1B 465R
-22K
-E1B 163R
B
1 2 3 4 5 6 7 8 9 10

Figure 5-6. Complementation assay II.

KB cells were infected at a multiplicity of infection of 400 virions per cell or co-infected as indicted at multiplicities of infection of 400+400, 400+1200 and 400+2400. At 20 hours after infection, cells were labelled with [^{35}S]methionine for one hour. Cell lysates were prepared and analyzed as described in the legend to Fig. 5-1.


mock
wt
pm1542
dl42
wt
pm1542
wt
pm1542
wt
pm1542
dl42 (400)
dl42 (1200)
dl42 (2400)
1
2
3
4
5
6
7
8
9
10
68.0-
43.0-
25.7-
18.4-
14.3-
E1B 482R
E1B 440R
E1A
22K
E1B 163R

(lane 5). To study the effect further, the multiplicity of infection of *dl*42 was increased to 1200 or 2400 virions per cell, leaving that of *wt* or *pm*1542 at 400. Again a similar specific overproduction of 482R was observed with *dl*42/*pm*1542 only (lanes 8 and 10). Although the general level of the 482R decreased at a relatively high input of *dl*42 (lanes 7-10), the extent of overproduction of 482R (*ie.* the relative amount of 482R between *dl*42/*pm*1542 and *dl*42/*wt*) remained comparable (lanes 5 vs. 6, 7 vs. 8 and 9 vs. 10). It should be mentioned that overproduction of 482R was also observed in *pm*1542-infected MH12-C2 cells (data not shown) which produce the 163R protein constitutively (Mak and Mak, 1990).

These data all suggested that lack of the 163R AUG codon rather than the absence of 163R in *pm*1542 might be responsible for the overproduction of the 482R protein. Such might also be the case for the 22K protein which, as described above and in Section 5-6, is probably also initiated from the 482R AUG initiation codon downstream. The AUG initiation codons for the 175R and 176R proteins of Ad2 and Ad5 are not present in an optimal context (see Fig. 5-4) and thus it would be predicted that no upstream suppression of 495R (496R) translation should occur. In fact it has been shown that elimination of the 175R (176R) initiation codon in Ad2 or Ad5 does not result in overproduction of the 495R (496R) protein (Barker and Berk, 1987; McLorie *et al.*, 1991).

5.4. *in*700 Also Overproduces E1B 482R Protein

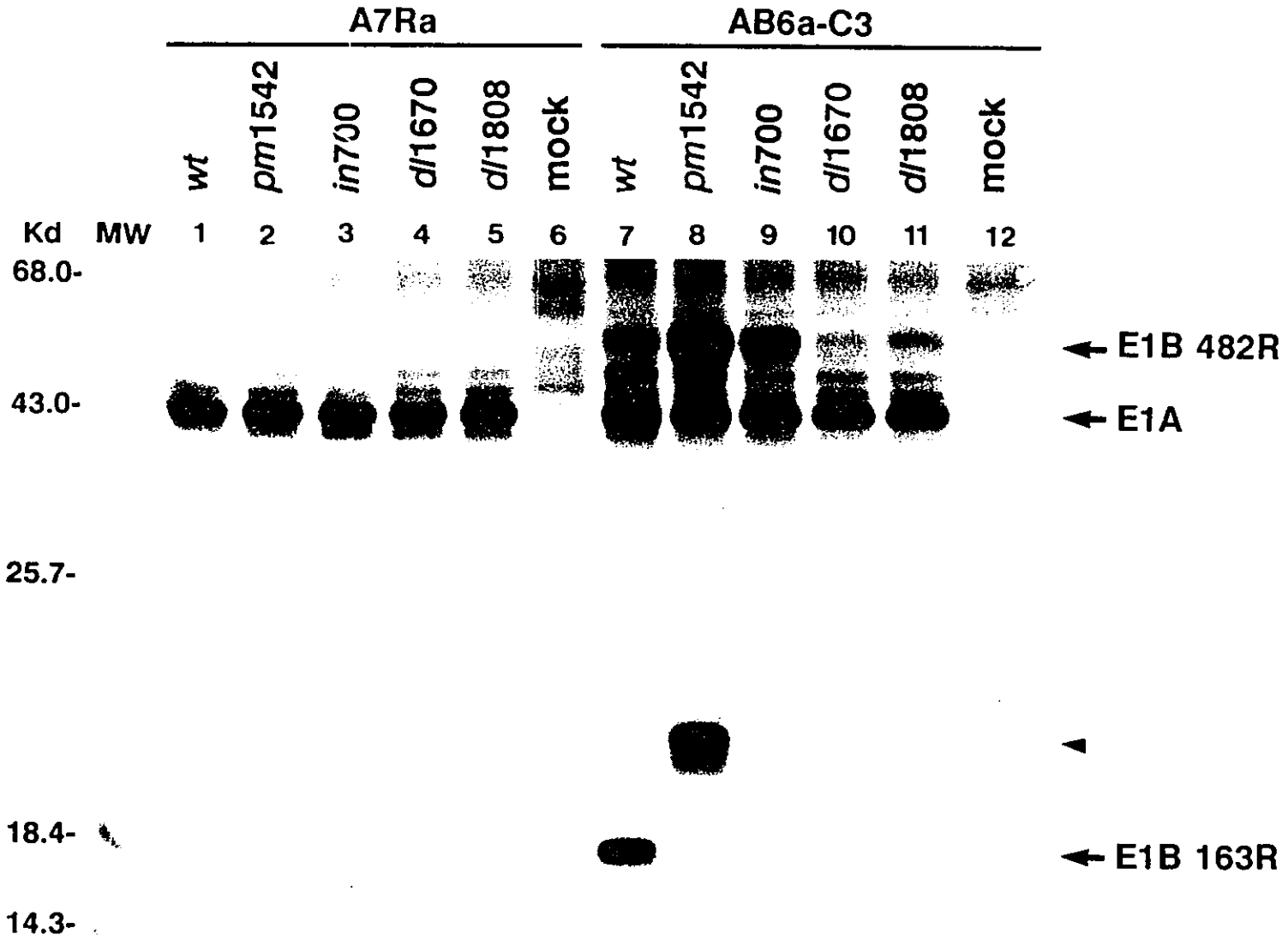
So far, two other Ad12 163R mutants, *pm*700 and *in*700, have been reported to transform BRK cells at normal efficiency (Edbauer *et al.*, 1988) and some studies were carried out on them to determine their level of production of 482R products. It is reasonable to predict that *pm*700, which is equivalent to *pm*1542 in that it lacks the AUG codon for 163R (see Fig. 5-4), should overproduce 482R. In addition, *in*700, which contains the 163R AUG codon but has a stop codon just downstream (see Fig. 5-4), could also overproduce 482R, perhaps by increasing the overall loading efficiency of ribosomes to the 482R AUG codon through reinitiation. To test this, *in*700-infected cells were labelled at 20 h p.i. and cell extracts were precipitated with E1A specific antiserum A7Ra (Mak and Mak, 1990) or E1 specific antiserum AB6a. Fig. 5-7 shows that *in*700 synthesized E1A products at levels comparable to *wt* Ad12 and other 163R mutants (lanes 1 to 5). It also overproduced the E1B 482R protein relative to *wt* and other 163R mutants, but the increase was less than that observed with *pm*1542 (lanes 7 to 11). In this particular experiment, the 22K protein was not intensively labled with *wt* and mutants, except for that with *pm*1542 (lanes 7 to 11). Therefore, slightly increased level of 22K with *in*700 might not be easily detected.

5.5. Correlation Between Overproduction of the E1B 482R Protein and Transforming Efficiency

The data presented in Chapter 4 indicated that mutants defective in the production of 163R transformed BRK cells at reduced efficiency. However, with *pm*1542 which

Figure 5-7. Comparison of the levels of 482R protein produced in mutant-infected cells.

KB cells were infected at a multiplicity of infection of 400 virions per cell and at 20 hours after infection labelled as described in the legend to Fig. 5-1. Cell lysates were immunoprecipitated with either E1A antiserum A7Ra or E1 antiserum AB6a and proteins were separated by SDS-PAGE.


A7Ra
AB6a-C3
wt
pm1542
in700
dl1670
dl1808
mock
wt
pm1542
in700
dl1670
dl1808
mock
Kd MW
1 2 3 4 5 6 7 8 9 10 11 12
68.0-
43.0-
25.7-
18.4-
14.3-
E1B 482R
E1A
E1B 163R

produces no functional 163R but overexpresses 482R, the transforming efficiency was comparable to that of *wt* virus. Since the increase in 482R products with *in*700 was less than that found with *pm*1542, we anticipated that *in*700 might transform BRK at an efficiency in between that of *pm*1542 and other 163R mutants. Table 5-1 shows that the numbers of transformed cells obtained with *wt*- and *pm*1542-infected BRK cells were comparable at various levels of virus input, although the number of foci declined more with *pm*1542 at higher virus levels. With *in*700, the number of foci was always lower than with *pm*1542, averaging about 50 % of that obtained with *pm*1542, but clearly higher than that seen with other 163R mutants, *dl*1670 and *dl*1808. These data further suggested that overproduction of the 482R protein can compensate for the transforming function of 163R.

5.6. Characterization of 22K Protein

A 22K protein was originally detected in virus-infected cells using AB6a-C3 E1 antiserum and it was overproduced by mutant *pm*1542 (Fig. 5-8, left panel). Since non-immune serum failed to precipitate this species (Fig. 5-8, right panel), it was first assumed to be the 23K E1A protein previously reported by Mak and Mak (1986). However, using E1A antiserum A7Ra and a gel system containing a lower concentration of crosslinker (see Chapter 2, section 2.7.1.3), as employed previously by Mak and Mak (1986), 22K was shown not to be a E1A product. As shown in Fig. 5-8 (bottom), a total cell lysate was prepared at 20 hours after infection in RIPA buffer, it was precipitated

Table 5-1. Transformation of BRK cells by Ad12 E1B 163R mutants II

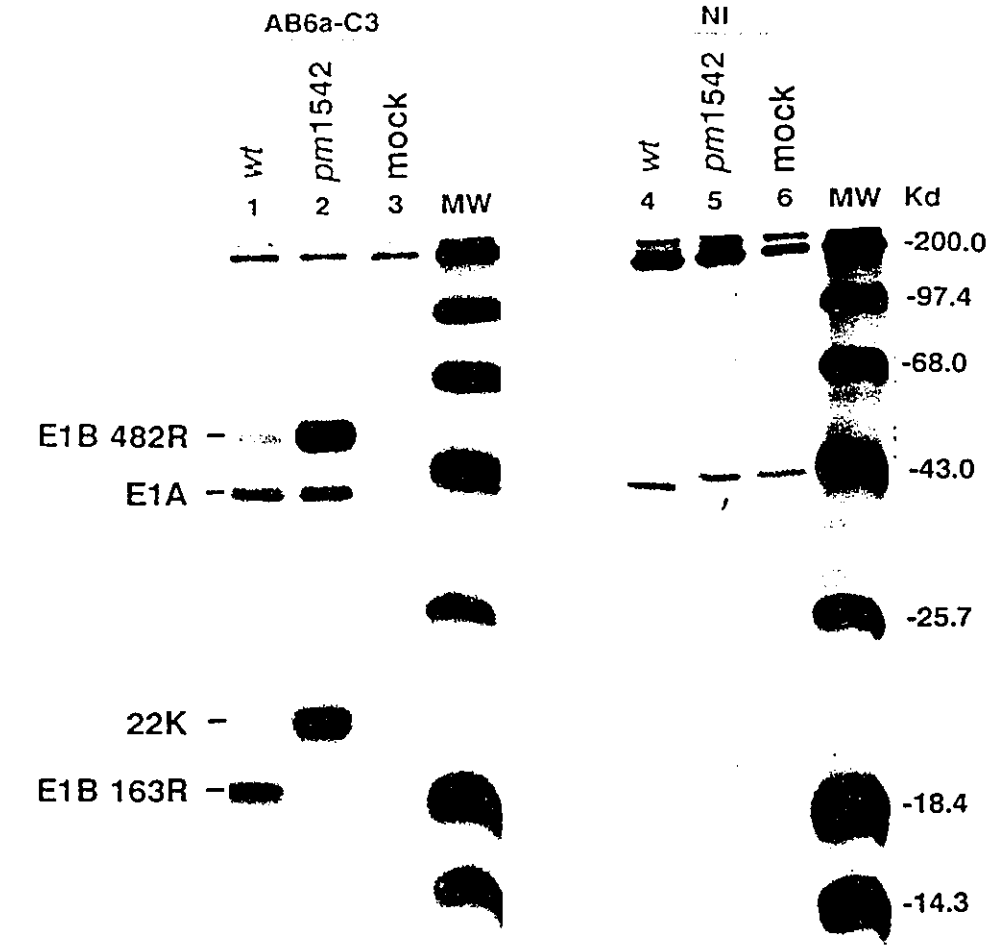
Virus	Average number of foci/dish (% *wt*) Multiplicity of infection (Virions/cell)						
	62.5	125	250	500	1000	2000	4000
wt	20(100)	37(100)	56(100)	46(100)	43(100)	44(100)	3(00)
*pm*1542	16(82)	34(92)	48(86)	47(101)	28(67)	28(63)	2(70)
*in*700	10(49)	16(43)	17(29)	12(25)	8(18)	11(25)	ND
*dl*1670	4(22)	4(11)	5(8)	6(14)	1(3)	0(0)	3(8)
*dl*1808	8(41)	8(22)	6(11)	11(24)	4(8)	4(9)	6(20)

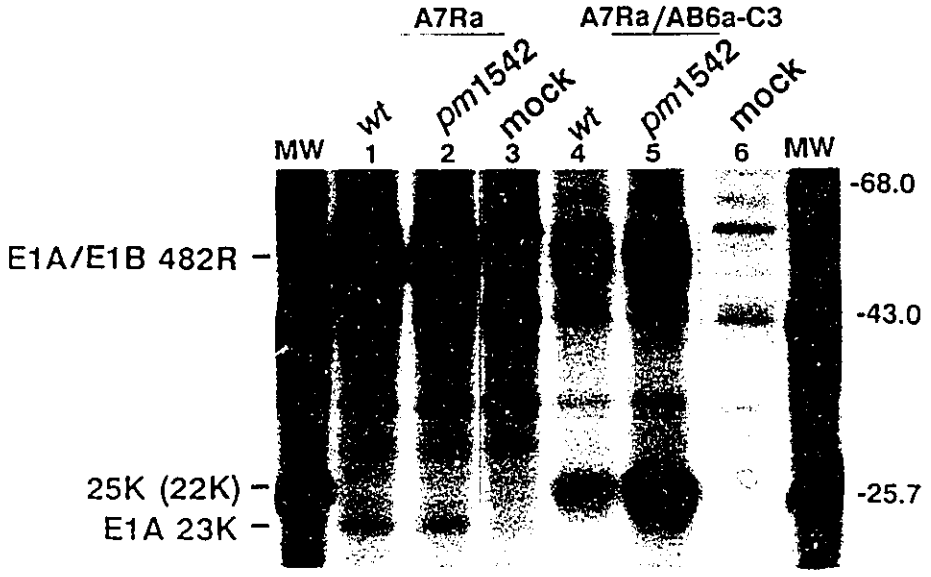
Baby rat kidney cells were infected at various multiplicities with *wt* or mutant Ad12. Each value represents the average number of foci obtained from four dishes of infected cells. The data were derived from a single experiment and comparable data were also obtained in other separate experiments.
ND stands for "Not Determined".

with E1A antiserum and then the E1A-depleted supernatant was further precipitated with AB6a-C3. Although the E1A 23K protein was precipitated by A7Ra (Fig. 5-8, bottom, lanes 1 and 2), another protein of 25K was also detected in the E1A-depleted lysate using AB6a-C3 serum (lanes 4 and 5). Moreover, it was shown that, as in the case of 482R, *pm*1542 also overproduced this protein (lane 5 vs 4), suggesting that the 25K and 22K species were the same protein which migrated differently in different gel systems. Since

Figure 5-8. Analysis of 22K protein with E1A and E1 antisera.

KB cells infected with *pm*1542 or *wt* Ad12 at a multiplicity of infection of 400 virions per cell or mock-infected were labelled with [^{35}S]methionine for 1 hour at 20 hours after infection and total cell lysates were prepared by sonication in RIPA buffer. After boiling in 0.5% SDS for 5 minutes, the lysates were immunoprecipitated with E1A (A7Ra-C4), E1 (AB6a-C3) or non-immune (NI) sera. Proteins were separated by SDS-PAGE. The 25K (22K) and the E1A 23K proteins were indicated at the left.

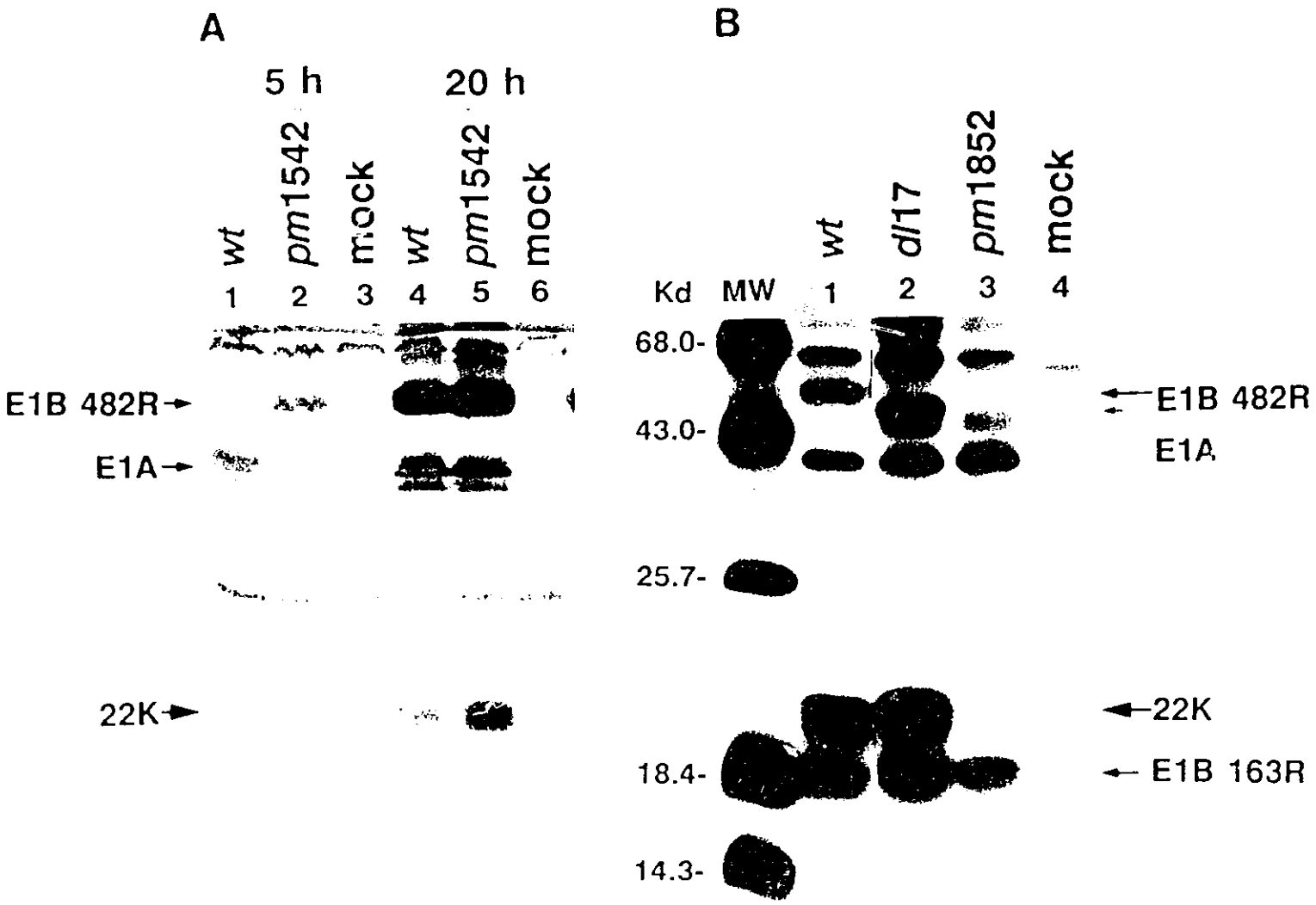

AB6a-C3
NI
wt
pm1542
mock
1
2
3
MW
4
5
6
MW
Kd
-200.0
-97.4
-68.0
-43.0
-25.7
-18.4
-14.3
E1B 482R
E1A
22K
E1B 163R


A7Ra
A7Ra/AB6a-C3
MW
wt
pm1542
mock
1
2
3
4
5
6
MW
-68.0
-43.0
-25.7
E1A/E1B 482R
25K (22K)
E1A 23K

the 22K protein was also overproduced in complementation assays (see Fig. 5-5 and 5-6), it is likely that the 22K species is either a 482R-related protein, or a 482R-associated protein which, as in the case of the E4 protein described in Ad2 (Sarnow *et al.*, 1984), co-precipitates with 482R. However, Western blot analysis revealed that the 22K protein was recognized by AB6a-C3 serum (Fig. 5-9A, lanes 4 and 5), indicating that it was not present as a result of immunoprecipitate. Genetic evidence also strongly suggested that it represented a 482R-related protein. Two 482R mutants, *dl*17 and *pm*1852, were used. *dl*17 contains a deletion in an intron of the E1B region which does not affect production of 482R-related proteins, while *pm*1852 has a stop codon which replaces the third amino acid residues of the 482R polypeptide and related proteins. Neither of these two mutants was found to be defective for viral DNA replication and virus growth (Mak and Mak, 1990). In addition early and late viral gene expressions were not affected (see next chapter). Precipitation of cell lysates using AB6a-C3 serum prepared at 20 hours from cells infected with *dl*17, yielded an altered 482R protein which migrated at 50K (Fig. 5-9B, lane 2). Surprisingly, one protein of apparent molecular weight less than 50K was also observed with *pm*1852 (Fig. 5-9B, lane 3). This protein probably resulted from translational reinitiation at an AUG codon downstream giving rise to an unstable product as described by Mak and Mak (1990). Such a reinitiated product is probably functional to a certain extent, resulting in normal viral replication. The 22K protein was observed with *wt* and *dl*17, but not with *pm*1852 (Fig. 5-9B, lanes 1-3), indicating that the stop codon three amino acids downstream from the AUG codons of 482R and related

Figure 5-9. Analysis of 22K protein with Western blot (A) or with Ad12 E1B 482R mutants.

A. KB cells were infected with *pm*1542 or *wt* Ad12 at a multiplicity of infection of 400 virions per cell and cell lysates were prepared at the indicated times after infection. Proteins were separated by SDS-PAGE and transferred to a piece of nitrocellulose membrane. After incubation with E1 antiserum AB6a-C3, the blots were further reacted with anti-rat goat IgG linked to alkaline phosphatase. The colour reaction was achieved by incubating the membrane in the reaction buffer (see section 2.7.2). B. KB Cells were infected with the E1B 482R mutant or *wt* Ad12 at a multiplicity of infection of 400 virions per cell and labelled for 1 hour at 20 hours after infection. Cell lysates were prepared as described in the legend to Fig. 5-1, immunoprecipitated with AB6a-C3 and subjected to gel electrophoresis.


A
5 h
20 h
wt
pm1542
mock
wt
pm1542
mock
1
2
3
4
5
6
E1B 482R
E1A
22K
B
Kd
MW
wt
dl17
pm1852
mock
1
2
3
4
68.0-
43.0-
25.7-
18.4-
14.3-
E1B 482R
E1A
22K
E1B 163R

proteins blocks translation of both the 482R and the 22K proteins and suggesting that the 22K protein might be the 482R-related protein.

CHAPTER SIX

RESULTS: FUNCTIONAL ROLE OF AD12 E1B PROTEINS

6.1. The Role of the E1B 163R Protein in Viral mRNA Accumulation

The function of the adenovirus E1B 175R (176R) protein in gene regulation remains controversial. Studies using transient assays revealed that it exerts a positive effect on the expression of viral genes (Natarajan *et al.*, 1986; Herrmann *et al.*, 1987; Yoshida *et al.*, 1987), but this effect has been proposed to result from the secondary effect of stabilization of transfected plasmid DNA mediated by this protein (Herrmann and Mathews, 1989). Following virus infection, however, this protein was reported to negatively modulate viral gene expression (White *et al.*, 1986) in the presence of the E1A protein, or to positively modulate viral gene expression in the absence of the E1A protein. However, another group reported that 175R has no effect on viral gene expression (Herbst *et al.*, 1988). To define the functional role of Ad12 E1B 163R in accumulation of viral mRNAs, northern blot analysis was employed.

Cytoplasmic mRNAs were isolated from *wt*- and mutant-infected KB cells either before DNA degradation (20 hours after infection) or after DNA degradation (30 hours after infection) and equivalent amounts of total RNA were subjected to northern analysis. At the earlier times, no obvious difference in E1A, E1B, E2A and E4 mRNA

accumulation was observed between *wt* and 163R mutant viruses, although *dl*1670 produced less of these early mRNAs (Fig. 6-1). More extensive studies were carried out with *wt* Ad12 and mutants *pm*1542, *dl*1670 and *dl*1808. Fig. 6-2 shows that with *pm*1542, the steady-state levels of viral mRNAs from E1A, E1B, E2A, E2B, E4 and L4 at 20 hours after infection were comparable to those observed with *wt* Ad12 (lanes a and b). With *dl*1670, a two- to three-fold decrease in the accumulation of viral mRNAs was observed except for E1A mRNA (lane c) which probably differs due to the fact that E1A was produced early after infection. With *dl*1808, no obvious difference in mRNA accumulation was observed as a consequence of this mutation in 163R (lane d). At 30 hours after infection, the accumulation of viral mRNA with 163R mutants was decreased as a consequence of DNA degradation which reduced the amount of viral DNA template for transcription (lanes f-i). Nevertheless the relative level of mRNA accumulation was lowest with *dl*1670. As a control an actin probe was used and the results showed that at 20 hours after infection, the accumulation of actin mRNA was comparable with all those mutants (see Fig. 6-2: Actin, lanes a-e). At 30 hours postinfection, the level of actin was reduced with infected samples (lanes f-i), resulting from the host shutoff (lanes f-i) and DNA degradation (lanes g-i).

6.2. The Role of the E1B 163R Protein in Viral DNA Replication

In order to further assess the results from northern blot analysis, the amount of viral DNA synthesized in mutant- and wild type-infected KB cells was examined. Total

Figure 6-1. Northern analysis of total RNA from KB cells infected with 163R mutant or *wt* Ad12.

Cells were infected at a multiplicity of infection of 400 virions per cell and equal amounts of total RNA isolated at 20 hours after infection were subjected to electrophoresis on formaldehyde-agarose gels and transferred to nitrocellulose membranes. The membranes were hybridized to the probe specific for the E1A, E1B, E2A or E4 regions of Ad12. E1A and E1B probes were generated by nick-translation of pHA5, and pHB14R and pHB15R respectively (see section 2.1.3). E2A and E4 probes were generated by primer extension of single stranded DNA templates from pUC118BC-r (see section 3.8) and pUC118/E4r (see section 2.1.3) respectively. Lanes a to i are described in the figure. The three bars at the left represent the sizes of molecules of *Rsa* I-digested pBR322.

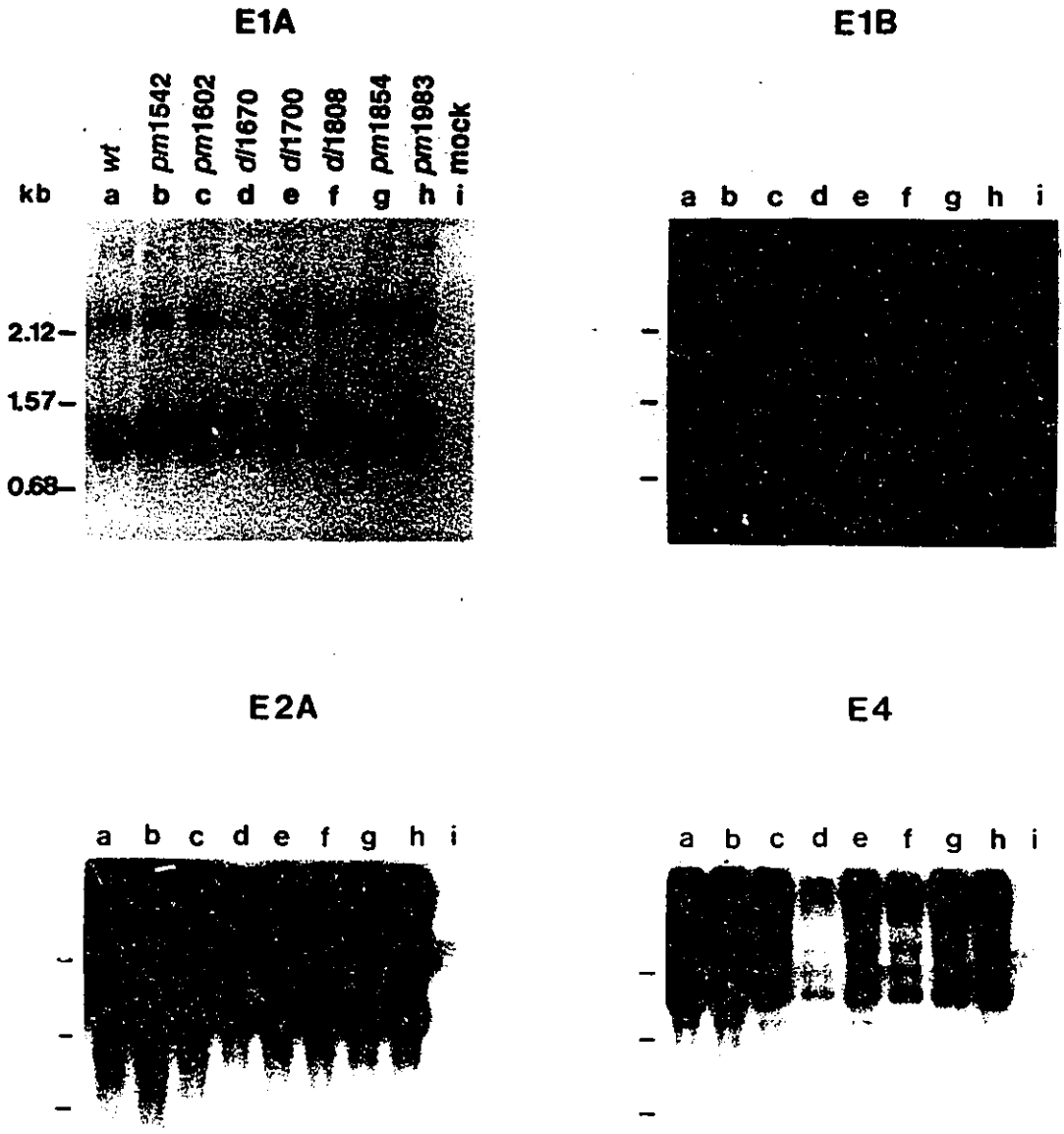
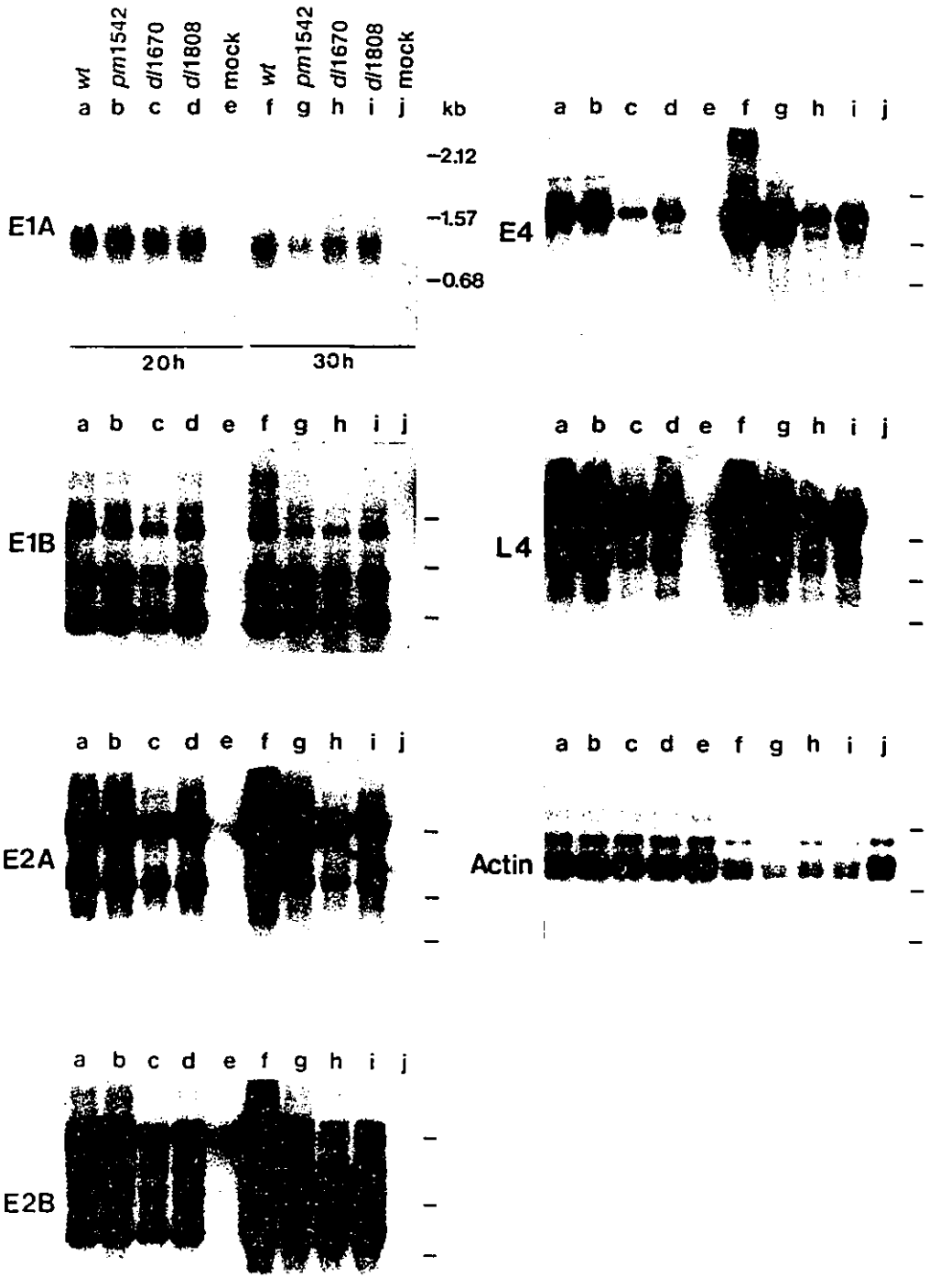

E1A
wt
pm1542
pm1602
dl1670
dl1700
dl1808
pm1854
pm1983
mock
kb
a b c d e f g h i
2.12
1.57
0.68
E1B
a b c d e f g h i
E2A
a b c d e f g h i
E4
a b c d e f g h i

Figure 6-2. Northern analysis of cytoplasmic RNA from KB cells infected with 163R mutant or *wt* Ad12.

Cells were infected as described in the legend to Fig. 6-1 and equal amounts of cytoplasmic RNA isolated at the indicated times after infection were subjected to Northern analysis. The RNA blots were hybridized to the probes specific for the E1A, E1B, E2A, E2B, E4 or L4 regions of Ad12, or for human actin. E1A, E1B, E2A and E4 probes were prepared as described in the legend to Fig. 6-1. E2B and L4 probes were generated by primer extension of single stranded DNA templates from pUC118HB-r and pUC118BC-l (see section 3.8) respectively. Human actin probe was generated by nick translation of pHMαA-1 (see section 2.1.3). Lanes a to j are defined in the figure.


wt
pm1542
dl1670
dl1808
mock
wt
pm1542
dl1670
dl1808
mock
a b c d e f g h i j
kb
-2.12
-1.57
-0.68
E1A
20h
30h
E4
E1B
L4
E2A
Actin
E2B

DNA was extracted from the cells at the indicated times after infection. The amount of viral DNA was determined by hybridizing total DNA blotted on the filter with viral DNA probe and then quantitating by densitometer scanning (Fig. 6-3). At 5 and 10 hours after infection, DNA replication had not occurred and very little difference in accumulation of DNA for *wt* Ad12 and mutants was evident. DNA replication was detected from 15 to 25 hours after infection and a slight difference in accumulation of DNA was observed (Fig. 6-4). However only about a 30% increase was detected with *pm*1542 and a 30% decrease was found with *dl*1670. With *dl*1808, no difference in DNA accumulation was evident relative to *wt* Ad12 (Fig. 6-3). After 25 hours of infection, the accumulation of DNA decreased with mutants (Fig. 6-3, 6-4), probably as a result of extensive DNA degradation. A summary of the time course of DNA replication has been illustrated in Fig. 6-4.

6.3. The Role of the E1B 482R Protein in Late Protein Accumulation

Adenovirus E1B 495R (496R) protein is involved in facilitating transport and accumulation of viral late mRNAs while blocking the same processes for cellular mRNAs, thus resulting in efficient translation of viral late proteins and shutoff of host protein synthesis (Babiss and Ginsberg, 1984; Babiss *et al.*, 1985; Pilder *et al.*, 1986). Ad12 E1B 482R, unlike its counterpart in type 2 and 5, is required for viral DNA replication, which probably results in secondary effects on late mRNA and protein accumulation (Shiroki *et al.*, 1986; Breiding *et al.*, 1988; Mak and Mak, 1990). To

Figure 6-3. Viral DNA synthesis in KB cells infected with 163R mutant or *wt* Ad12.

Cells were infected as described in the legend to Fig. 6-1 and total DNA was isolated at the indicated times after infection. Serial two-fold diluted DNA preparations were blotted onto a nitrocellulose membrane and probed with labelled viral DNA. The relative amounts of viral DNA were determined by measuring the intensities of the blots (see section 2.5.4.3) which were then plotted versus the corresponding DNA concentration.

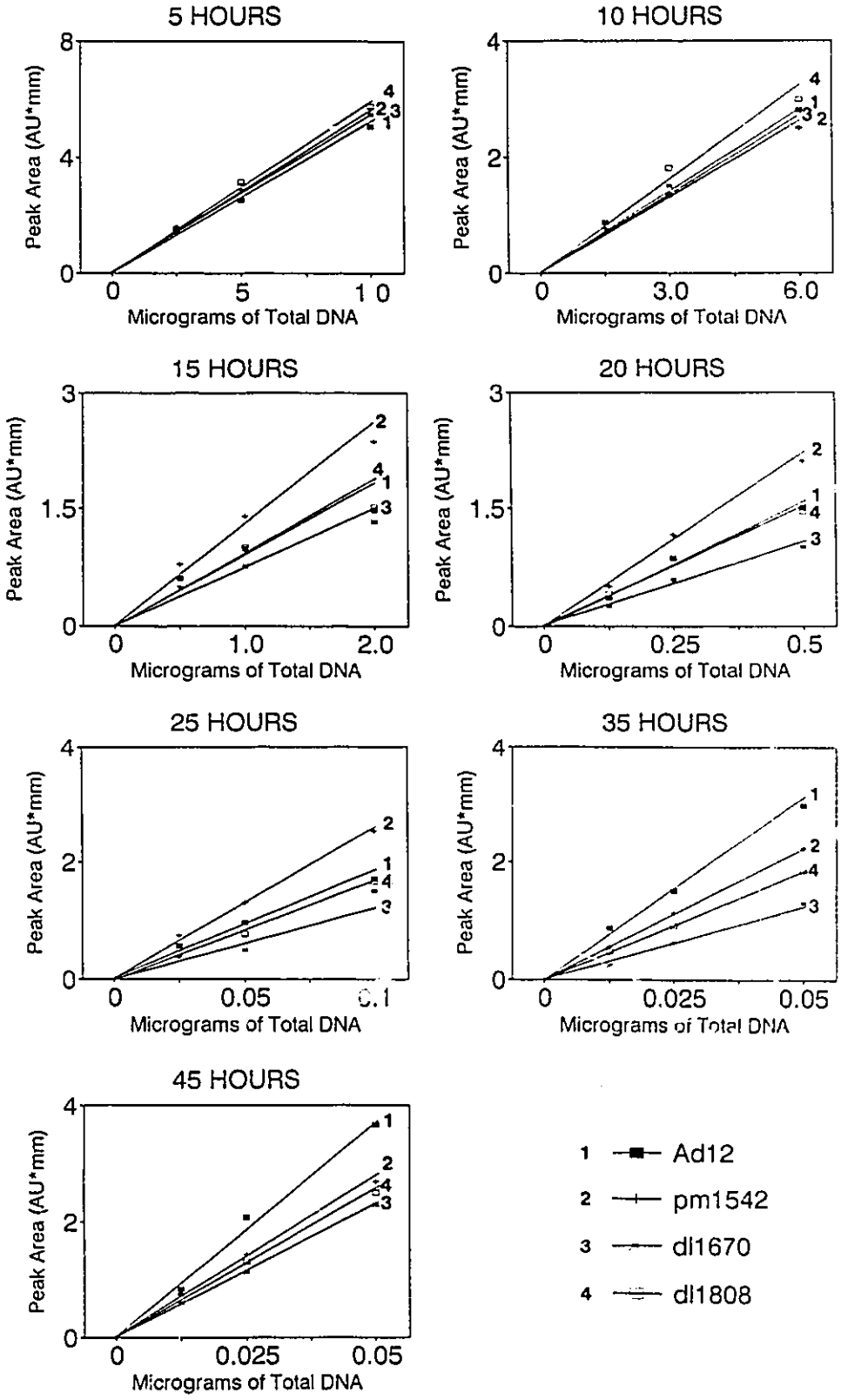
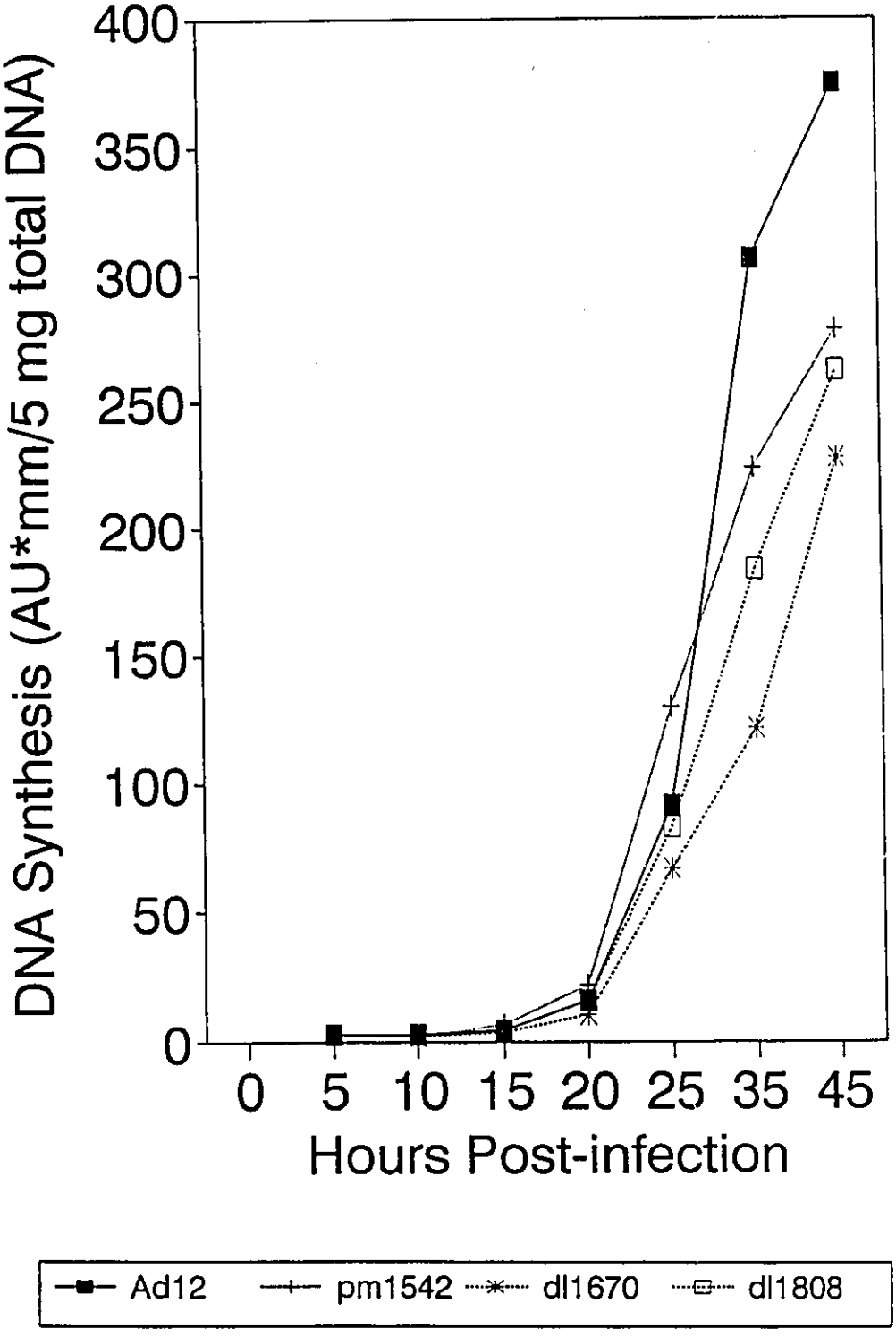

5 HOURS
Peak Area (AU*mm)
Micrograms of Total DNA
10 HOURS
15 HOURS
20 HOURS
25 HOURS
35 HOURS
45 HOURS
1 Ad12
2 pm1542
3 dl1670
4 dl1808

Figure 6-4. Accumulation of viral DNA in infected KB cells.

The amounts of viral DNA at indicated times after infection were determined as described in Fig. 6-3, and normalized to 5 mg of total DNA. Data are plotted versus times after infection.


DNA Synthesis (AU*mm/5 mg total DNA)
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
35
45
Hours Post-infection
Ad12
pm1542
dl1670
dl1808

examine the role of the Ad12 482R protein in detail, three transformation defective mutants were studied. *pm*1852 has a stop codon at the third amino acid of the protein, but it may synthesize a protein by reinitiation downstream at 24th and/or 65th amino acid from the normal AUG codon, and is neither defective for viral DNA replication nor for growth in human cells. *dl*17 contains a deletion of amino acid residues 80-96, and is normal for lytical infection but defective for tumorigenicity. *dl*42 has a deletion of residues 114-155 and is defective in viral DNA replication (Mak and Mak, 1990).

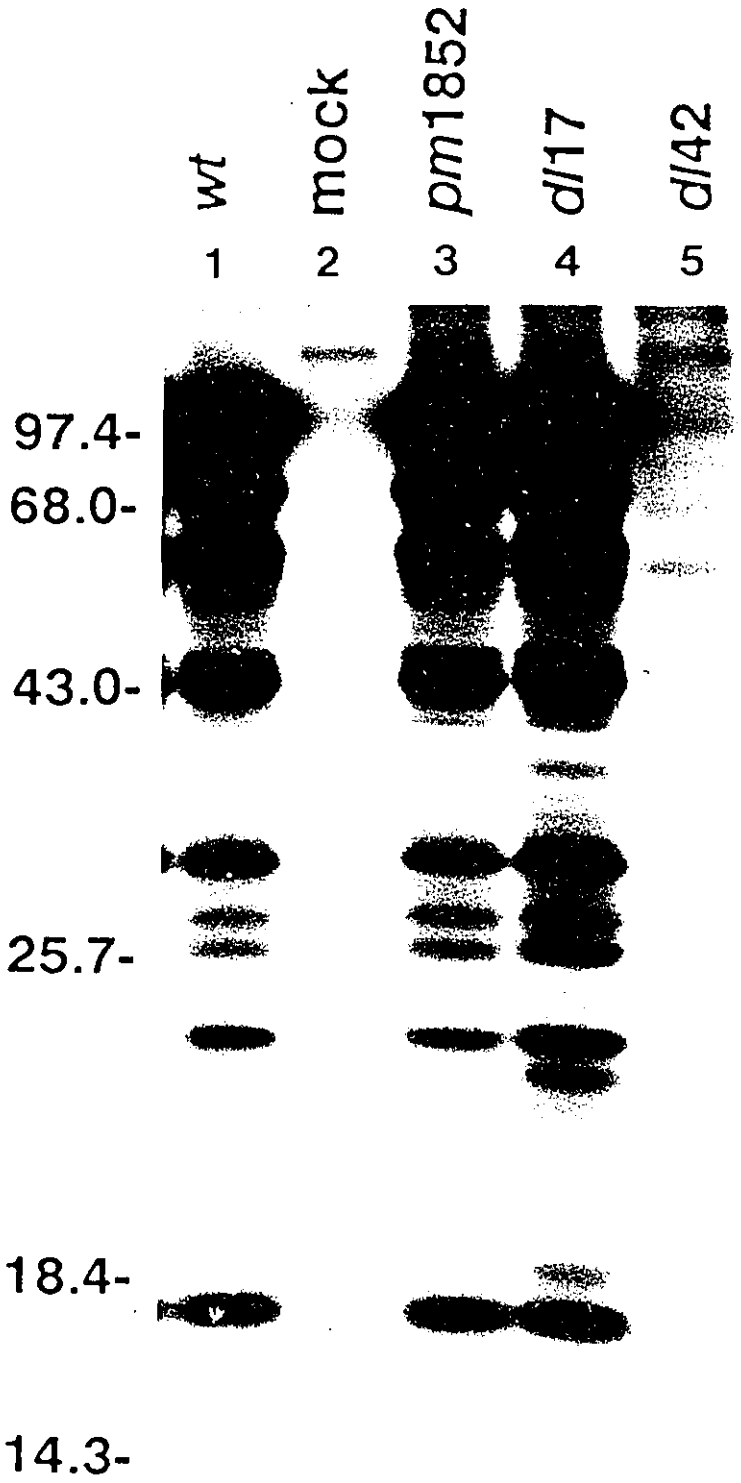
In the present studies, the effect of the mutations in viral late protein accumulation has been examined. KB cells infected with *wt* or mutant virus were labelled with [^{35}S]methionine at 40 hours after infection and lysates were immunoprecipitated with antiserum against Ad12 virions. The pattern of late protein synthesis observed with *wt* Ad12 is shown in Fig. 6-5 (lane 1). With mutants *pm*1852 and *dl*17, the relative amounts and patterns of late proteins were comparable to *wt* (lanes 3 and 4), suggesting that N-terminal sequences (*pm*1852) and residues 80-96 (*dl*17) are dispensable for lytic infection of KB cells. However, *dl*42 exhibited very little virus-specific late proteins (lane 5), suggesting that residues 114-155 of 482R is directly or indirectly involved in production of late proteins.

6.4. The Role of the E1B 482R Protein in Accumulation of Early mRNA

Because *dl*42 was found to be defective in DNA replication (Mak and Mak, 1990), it was necessary to determine whether the expression of the early genes involved

Figure 6-5. Viral late protein synthesis in KB cells infected with 482R mutant or *wt* Ad12.

Cells were infected at a multiplicity of infection of 400 virions per cell and labelled for 1 hour at 40 hours after infection. Cell lysates were precipitated by antiserum against Ad12 virion. The numbers indicate the size of protein markers in kilodaltons.


wt
mock
pm1852
dl17
dl42
1
2
3
4
5
97.4-
68.0-
43.0-
25.7-
18.4-
14.3-

in adenovirus DNA replication was altered. The accumulation of viral early mRNAs in both cytoplasmic and nuclear fractions of infected KB cells at an early time after infection was analyzed by Northern blot analysis (Fig. 6-6). At 6 hours after infection, cytoplasmic mRNAs from the E1A, E1B, E2A and E3 regions were detected and the accumulation levels of mRNAs were similar in both *wt* Ad12- and *dl*42-infected cells (Fig. 6-6, left panel at 6). At 9 hours after infection, the quantities of the E1A and E3 mRNAs remained unchanged while those of E1B and E2A mRNAs seemed to be slightly increased in *wt* Ad12-infected cells (left panel at 9). At 12 hours after infection, the amount of E1A mRNA was unchanged but the quantities of E1B, E2A and E3 mRNAs were greatly increased. More interestingly, cytoplasmic mRNAs from the E2B and E4 regions were first observed in *wt* Ad12-infected cells at 12 hours after infection (left panel at 12). The overall time course of viral early mRNA accumulation in the nuclear fraction was more or less the same as that in the cytoplasm except that corresponding mRNAs tended to appear early in the former (Fig. 6-6, right panel), indicating that the difference in viral mRNA accumulation was not due to a defect in transport of mRNAs from the nucleus to the cytoplasm. An actin probe was also used as a control and the accumulation of actin mRNA was comparable with *dl*42 and *wt* in both cytoplasm and nuclei although it was reduced in nuclei of infected cells (Fig. 6-6, actin).

More quantitative analysis was achieved by making serial dilutions of RNA from *wt* Ad12-infected cells, which were then subjected to Northern analysis together with RNA from *dl*42-infected cells (Fig. 6-7A). The data indicated that E1A mRNA from

Figure 6-6. Northern analysis of RNA from KB cells infected with 482R mutant or *wt* Ad12.

Cells were infected at a multiplicity of infection of 400 virions per cell and equal amounts of cytoplasmic and nuclear RNAs were subjected to electrophoresis on formaldehyde-agarose gels and transferred to nitrocellulose membranes. The membranes were hybridized to the probes specific for the E1A, E1B, E2A, E2B, E3 or E4 regions of Ad12 or for human actin. The sizes of molecules of *Rsa* I-digested pBR322 are indicated at the left. The numbers at the top indicate the times after infection (hours post-infection). Cytoplasmic and nuclear RNAs were indicated by C and N respectively.

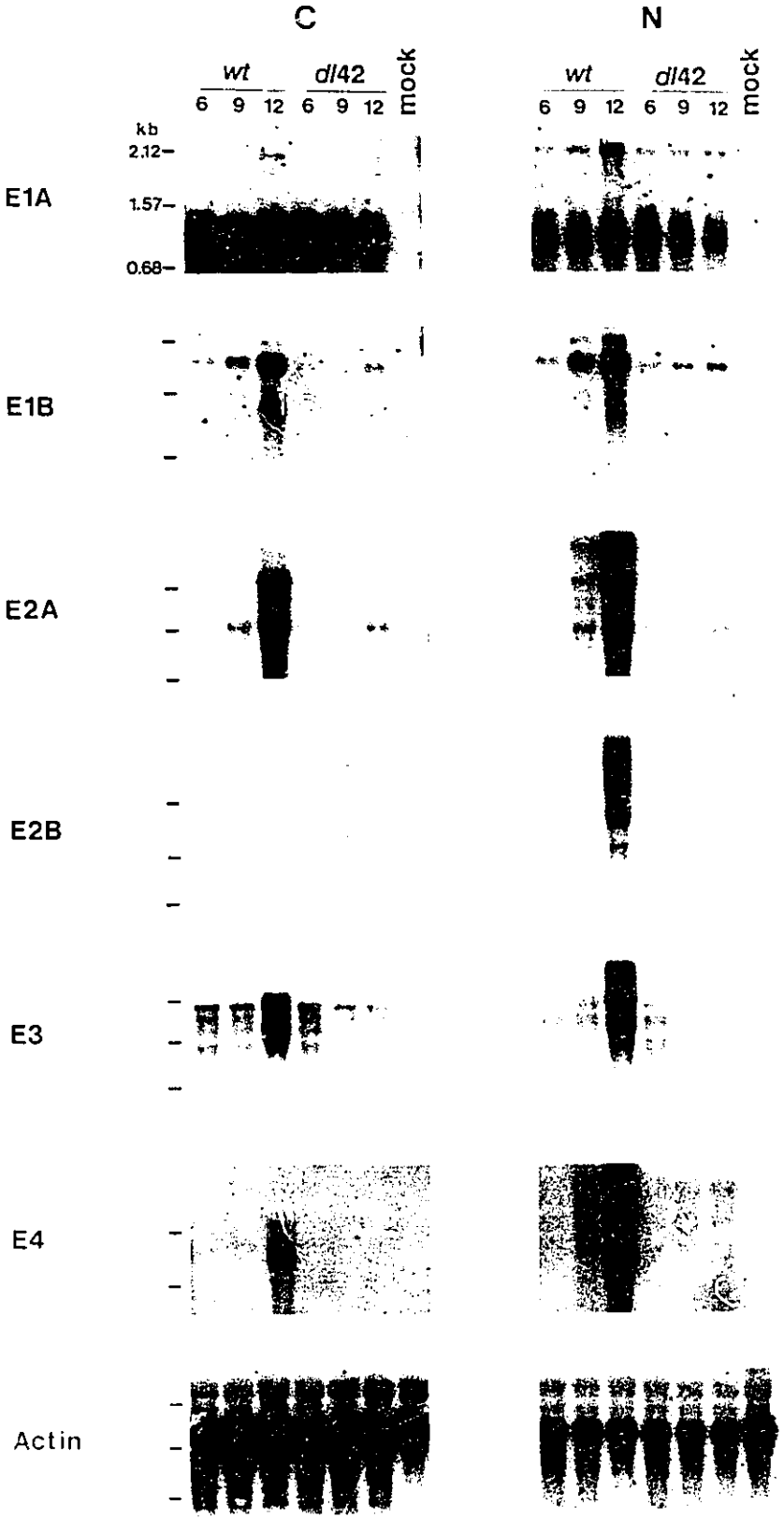
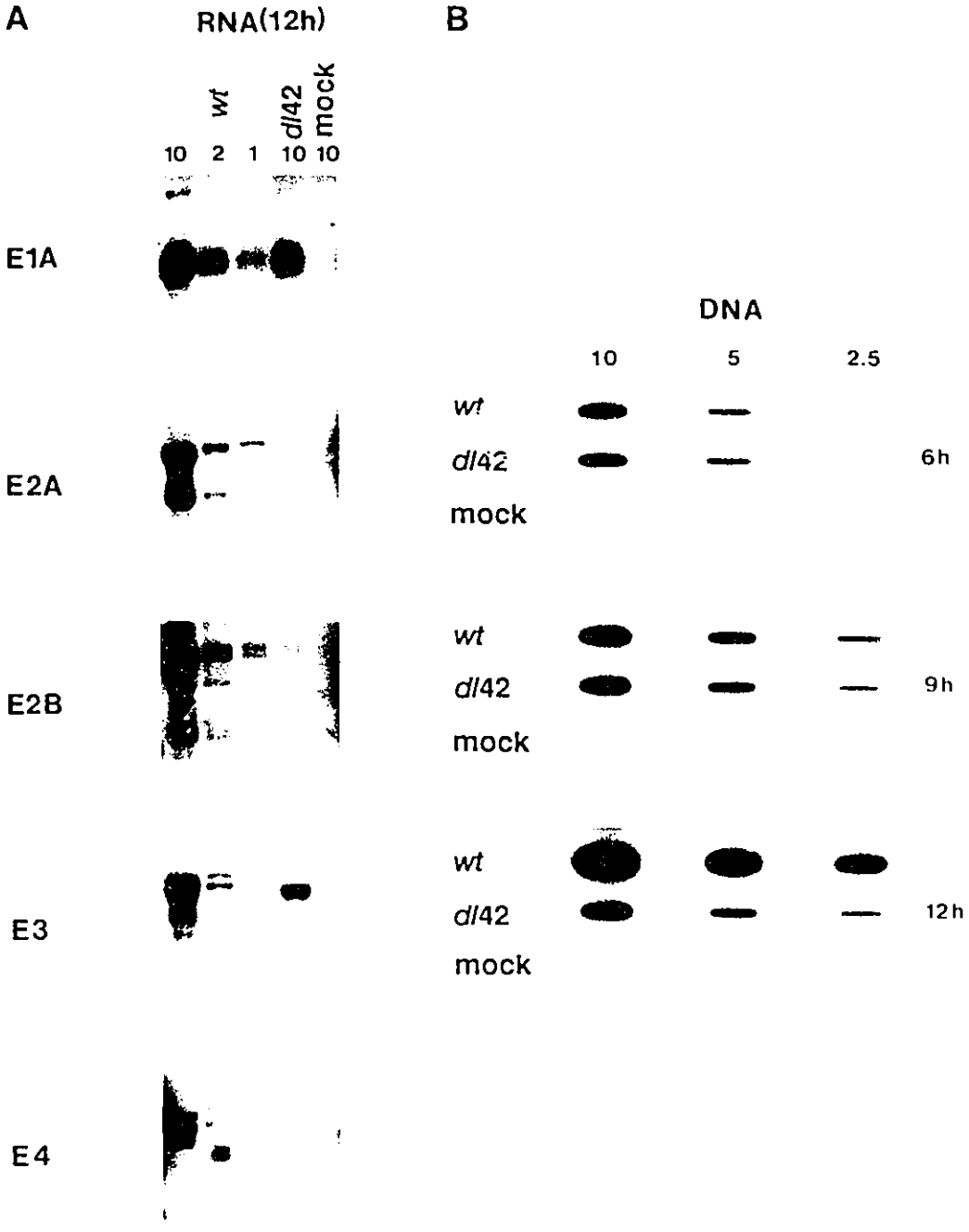

C
N
wt
dl42
mock
6 9 12 6 9 12
kb
2.12
1.57
0.68
E1A
E1B
E2A
E2B
E3
E4
Actin

Figure 5-7. Quantitation of viral RNA (A) and DNA (B) in infected KB cells.

A. Cytoplasmic RNA isolated at 12 hours after infection was subjected to Northern analysis with the probes specific for the E1A, E2A, E2B, E3 or E4 regions of Ad12. After serial dilutions, the amount of RNA from *wt*-infected cells was compared to that from *dl*42-infected cells. The numbers indicate total amounts (μg) of RNA in the lanes. B. Viral DNA from cells infected with *dl*42 or *wt* was blotted onto a nitrocellulose membrane and probed with Ad12 DNA. The numbers at the top indicate the total amounts (μg) of DNA blotted. The time after infection are indicated on the right.

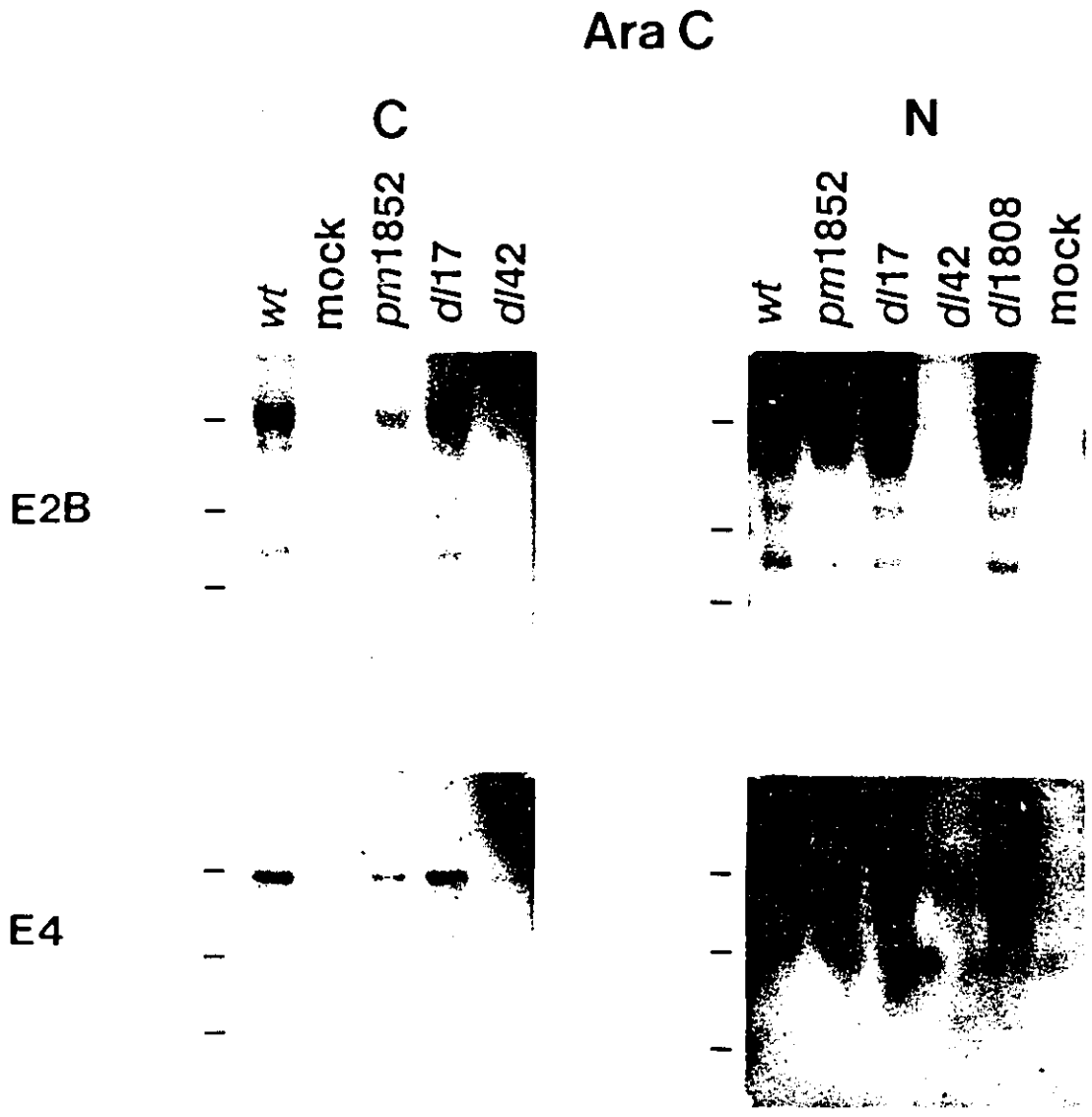

A
RNA(12h)
wt
dl42
mock
10
2
1
10
10
E1A
E2A
E2B
E3
E4
B
DNA
10
5
2.5
wt
dl42
mock
6h
wt
dl42
mock
9h
wt
dl42
mock
12h

*dl*42 at 12 hours after infection accumulated at a level comparable to that of *wt* (10 vs 10). The level of E2A mRNA was about five times lower with *dl*42 than with *wt* (2 vs 10). The top band in Fig. 6-7A (E2A) was probably E2B mRNA. The levels of E2B and E4 mRNAs were at least twenty times lower with *dl*42 than with *wt* (1 vs 10). The level of E3 mRNA was about two to three times lower with *dl*42 than with *wt* (10 and 2 vs 10). A parallel study to monitor the amount of DNA template was also performed and indicated that the level of DNA template was four times lower with *dl*42 than with *wt* at 12 hours after infection (Fig. 6-7B). Taking the level of DNA template into consideration, it was clear that only the accumulation of the E2B and E4 mRNAs were significantly affected (see discussion in Chapter 7).

To eliminate the interference caused by DNA replication, mRNAs isolated from infected cells in the presence of the DNA replication inhibitor 1-β-D-arabinofuranosylcytosine hydrochloride (ara C) were prepared and analyzed by Northern blot. Fig. 6-8 shows that accumulation of both E2B and E4 mRNAs was greatly reduced in *dl*42-infected cells, confirming the role of the 482R protein in the metabolism of viral E2B and E4 mRNAs. It was noticed that the mRNA pattern of E4 was changed in the presence of ara C (Fig. 6-8 vs 6-7) but the reason is unknown.

Figure 6-8. Northern analysis of RNA from KB cells infected with 482R mutant or *wt* Ad12 in the presence of ara C.

Cells were infected at a multiplicity of infection of 400 virions per cell and at 4 hours after infection, ara C was added to a concentration at 40 μg/ml. At 12 hours after infection, the medium was replaced by fresh medium containing ara C. At 20 hours after infection, cells were collected and RNA was analyzed by Northern analysis. C and N represent the cytoplasmic and nuclear RNAs respectively.


Ara C
C
N
wt
mock
pm1852
dl17
dl42
wt
pm1852
dl17
dl42
dl1808
mock
E2B
E4

CHAPTER SEVEN

DISCUSSION

Since the Ad12 *cyt* mutants were isolated by Takemori *et al.* (1968), a variety of mutants in the adenovirus E1B coding region have been generated, and the E1B 495R (496R) and 175R (176R) proteins in Ad2 (Ad5) and the E1B 482R and 163R proteins in Ad12 have been studied extensively. Studies with mutants affecting the 175R (176R) and 163R proteins have indicated that these polypeptides are multifunctional and involved in the protection of viral and cellular DNA from degradation and in the determination of the cytopathic phenotype of infected cells. In addition each is required for complete and efficient transformation of cells (Takemori *et al.*, 1968; Ezoe *et al.*, 1981; Lai Fatt and Mak, 1982; Chinnadurai, 1983; Mak and Mak, 1983; Babiss *et al.*, 1984b; Fukui *et al.*, 1984; Pilder *et al.*, 1984; Subramanian *et al.*, 1984a,b; Takemori *et al.*, 1984; White *et al.*, 1984b; Bernards *et al.*, 1986; Barker and Berk, 1987; Edbauer *et al.*, 1988; White and Cipriani, 1990). Although many mutants have been described and characterized, few studies have been carried out to identify functional regions of this protein. Moreover, recent studies with Ad12 mutants severely questioned the role of Ad12 E1B 163R protein in cellular transformation (Edbauer *et al.*, 1988). Other studies have now questioned the role of 175R (176R) in gene regulation (White *et al.*, 1986, 1988; Herbst *et al.*, 1988).

Thus further studies using additional mutants seemed to be necessary to evaluate the role of 163R in cellular transformation, to look for the possible functional regions, and to analyze its importance in gene regulation.

The functions of the E1B 495R (496R) protein in Ad2 (Ad5) and the comparable E1B 482R protein in Ad12 have been well characterized. It is involved in cellular transformation and is required for accumulation of viral late mRNA and proteins while blocking the same processes for cellular mRNAs and proteins (Graham *et al.*, 1978; Ho *et al.*, 1982; Logan *et al.*, 1984; Babiss and Ginsberg, 1984; Babiss *et al.*, 1984b; Babiss *et al.*, 1985; Pilder *et al.*, 1986; Bernards *et al.*, 1986; Barker and Berk, 1987). In addition, the E1B 482R protein of Ad12 has two unique functions. It is required for tumour induction and for viral DNA synthesis. (Shiroki *et al.*, 1986; Breiding *et al.*, 1988; Mak and Mak, 1990). The regions critical for these functions have been partly defined. It seems that amino acid residues 113 and beyond are required for viral DNA synthesis in human cells (Shiroki *et al.*, 1986; Breiding *et al.*, 1988; Mak and Mak, 1990), while any defects in this protein may affect the transforming function of 482R. At least residues 80-96 are involved in tumorigenicity (Mak and Mak, 1990). However, questions concerning the role in viral DNA synthesis have not been addressed.

In the present studies, a series of mutations have been produced across the coding sequence which affect various hydrophilic or hydrophobic regions of the 163R protein. These mutants have been characterized and used to analyze the structure and functions of this protein. Viruses with mutations in the 482R coding region were also

characterized.

All the mutants were constructed by oligonucleotide-directed mutagenesis. Dot-blot hybridization was used to identify the mutated recombinant through construction of mutants in which the labelled mutagenic primer could only be preferentially retained by its corresponding DNA sequence in the mutant at higher temperatures. The hybridization results clearly demonstrated that dot-blot hybridization is valid in screening these mutants because none of the mutagenic primers were retained non-specifically by control DNA at higher washing temperatures (see Fig. 3-9 and 3-10). Since recombinant M13 DNA is unstable and causes alterations in inserted DNA during replication, the inserted E1 regions were cut out and the sizes verified on agarose gels in primary screens. To further eliminate any possible alterations which were undetected by dot-blot hybridization and restriction analysis, DNA sequencing was carried out over the E1 region of *Kpn* I-*Hin*d III fragment (nucleotides 588-2318) to be subcloned into pHAB6 (see Fig. 3-5). As expected there were no additional alterations. During the processes of subcloning and rescue of DNA sequences containing mutated 163R coding regions into plasmid pHAB6 or virus genome, appropriate restriction analyses (see Fig. 3-9 and 3-11) were carried out to make certain there were no extra alterations such as the formation of concatemers, etc. Overall, the screening strategies were adequate, leading to successful construction of several mutants.

Analysis of the altered protein products of these mutants indicated that in-frame deletions and point mutations in sequences encoding the first hundred or so amino acids of 163R yielded products that were, to varying degrees, unstable (see Fig. 4-1). It was possible that in this and previous studies the pleiotropic effects of mutations were not due to amino acid changes in functionally important domains, but rather to the presence of reduced levels of 175R, 176R and 163R in Ad2, Ad5 and Ad12, respectively. All of the mutants that yielded unstable products exhibited the *cyt/deg* phenotype typical of mutants which produce no 163R at all. Conversely, changes in the carboxy terminal region yielded stable products and a normal replication phenotype (see Fig. 4-2). Thus at present it is only possible to conclude that the carboxy terminus is not involved in this effect on the lytic cycle of Ad12. The mechanism by which defects in 163R produce the *cyt/deg* phenotype is still unresolved.

Regardless of the positions of mutations or stabilities of the altered 163R proteins, growth of all mutant viruses proceeded at an efficiency comparable to *wt* Ad12. Statistically only a 50% difference was observed with some mutants (see Table 4-2). These data confirmed that 163R is not absolutely necessary for Ad12 replication in human KB cells (Fukui *et al.*, 1984; Edbauer *et al.*, 1988). Some mutants exhibited the *cyt* phenotype characteristic of defects in 163R and in these cases significantly higher levels of extracellular virus were produced (see Fig. 4-4), presumably due to enhanced cytolysis and release of progeny which is probably the direct reason leading to large size

plaques.

Several studies have implicated both the 163R and 482R E1B products are involved in adenovirus transformation (Takemori *et al.*, 1968; Chinnadurai, 1983; Mak and Mak, 1983; Babiss *et al.*, 1984b; Fukui *et al.*, 1984; Pilder *et al.*, 1984; Subramanian *et al.*, 1984a,b: Takemori *et al.*, 1984; White *et al.*, 1984b; Bernards *et al.*, 1986; Barker and Berk, 1987; Edbauer *et al.*, 1988; White and Cipriani, 1990; McLorie *et al.*, 1991). The present report indicated that mutations in the amino terminal region of the first one hundred amino acids of 163R resulted in transformation of primary BRK cells at a significantly lower efficiency than with *wt* virus (see Table 4-3). Because of the production of unstable products by these mutants it was unclear what regions in this portion of 163R are functionally important for this process. This reduction in transforming activity was quite similar to that observed previously with Ad5 mutants in which synthesis of this E1B product had been completely blocked (White and Cipriani, 1990; McLorie *et al.*, 1991). However, the data differed from those of other studies with Ad12 or Ad5 which suggested that the product corresponding to 163R was entirely dispensable (Edbauser *et al.*, 1988) or necessary (Barker and Berk, 1987) for transformation. In addition, mutant *pm*1542, which produced no detectable 163R, transformed at high efficiency (see Table 4-3). The present results did indicate clearly that the last 16 residues at the carboxy terminus were not necessary for transformation. However, with mutant *pm*1854, in which residue 105 had been altered, a stable 163R

protein was produced and, while *wt* for replication, it appeared to transform at a reduced efficiency. These data suggested that a region of 163R containing residue 105 may be functionally important for transformation but may have no significance in 163R-dependent DNA protection.

Of some importance was the apparent discrepancy between our results and those of Edbauer *et al.* (1988). Using mutants *pm*700, in which the initiation codon for 163R had been eliminated, and *in*700, in which a stop codon had been introduced downstream, these authors observed transformation of primary rodent cells at high efficiency, although both mutants were defective when the established cell line 3Y1 was employed. In our studies, all mutants that yielded unstable 163R products transformed BRK cells inefficiently, however, with mutant *pm*1542 in which the initiation codon had been eliminated, a high level transformation was observed. How might these data be reconciled? In both studies it was unlikely that transformation resulted from the production of a 163R-related product from an internal initiation site as deletions in the amino terminal region have been found to produce highly unstable products and the sequences surrounding internal AUG codons are not compatible with efficient translation initiation. It seems more likely that the efficient transforming activity observed with *pm*1542 and by Edbauer *et al.* (1988) could have resulted from changes in the level of expression of the E1B 482R protein (see below).

While examining the possible relationship between gene regulation and cellular

transformation, it was surprising to find that only *pm*1542 overproduced 482R. None of the other 163R mutants overproduced 482R in spite of the fact that some of them made an unstable or undetectable altered 163R product. Moreover, the level of E1A protein was unchanged (see Fig. 4-1 and Fig. 5-1). Clearly, this overproduction of 482R with *pm*1542 was not E1A-dependent and thus was totally different from the overexpression of early genes seen with 175R mutants described by White *et al.* (1988). Northern analysis of E1B mRNA indicated that the overproduction was regulated somehow at the translational level (see Fig. 5-3). The reason for this observation may lie in the fact that the initiation codon for 482R lies downstream of that for 163R, within the 22S mRNA. Considering the scanning model for translation initiation (Kozac, 1986, 1989), the presence of the 163R initiation codon, would suppress initiation of 482R at the internal site. Thus elimination of the 163R initiation site could result in increased expression of 482R. In fact, complementation assays suggested that overproduction of 482R was a *cis* effect due to the elimination of the 163R initiation codon rather than a *trans* effect caused by the absence of functional 163R protein (see Fig. 5-5 and Fig. 5-6).

Present results with the 163R mutants could be interpreted as follows. With mutations that alter the stability of 163R dramatically, the functions of the protein are effectively eliminated, resulting in defective transformation as well as the *deg/cyt* phenotype. With mutations that eliminate the initiation codon of 163R (eg. *pm*1542),

functions mediated by 163R are also eliminated, but a secondary effect of the mutation is the overproduction of the 482R transforming protein, which may result in a compensation for the lack of the 163R transformation function. However, the 163R function involved in protection of DNA and induction of wild-type cytopathic effect could not be compensated for by elevated levels of 482R. I believe that a similar process permits *pm*700 to transform BRK cells efficiently. However, the reason that *pm*700 is defective in the transformation of established 3Y1 cells could lie in the fact that 482R is not required for or is not sufficient for the transformation of these cells, as reported by Edbauer *et al.* (1988). Therefore the overproduction of 482R would not compensate for the lack of 163R in transformation of 3Y1 cells.

With Ad 5 or Ad 2, the suboptimal context around the initiation codon for the products corresponding to 163R (*i.e.* 176R and 175R) would not suppress downstream initiation of the larger E1B protein (496R and 495R) efficiently (Kozac, 1986). As expected, the level of 495R (496R) in Ad2 (Ad5) was found to be unchanged by elimination of the 175R (176R) initiation codon, and transformation by this mutant occurred at low efficiency (Barker and Berk, 1987; McLorie *et al.*, 1991).

Since *in*700 is a transformation positive mutant, as described by Edbauer *et al.* (1988). I would also expect it to overproduce 482R. The mutation in *in*700, an in-frame stop codon in between the 163R AUG initiation codon and that for 482R downstream may lead to the overproduction of 482R through ribosome reinitiation following

translation of the upstream minicistron. However, the efficiency of reinitiation is not easy to predict (Kozac, 1986). The present studies revealed that with *in*700, 482R was overproduced by about two to three times relative to *wt* Ad12 (see Fig. 5-7). Considering the numerous examples of transformation due to the dosage-dependant activation of oncogenes, it seemed reasonable that even this level of overproduction of 482R by *in*700 could yield somewhat higher levels of transformation but perhaps not as high as with *pm*1542 which overproduces 482R to an even greater extent. Table 5-1 demonstrated that in fact this relation between transforming ability and the level of synthesis of E1B protein did appear to exist.

The present study also revealed a new protein with a molecular weight of about 22,000 daltons. It was also overproduced with *pm*1542. This protein probably represents an E1B 482R-related product (see Chapter 5, section 5-7). Equivalent proteins have been reported with Ad2 and Ad5 (Anderson *et al.*, 1984; Lewis and Anderson, 1987; S. Brown, D. Takayesu and P. E. Branton, manuscript in preparation), but the functions of these minor E1B protein are unknown. The E1B 13S mRNA and its E1B 482R-related proteins have been detected in infected cells late after infection, but not in transformed rodent (Saito *et al.*, 1983; Mak and Mak, 1983), or human cells (S. Mak, unpublished data). Thus it is unlikely that such products play essential role in transformation. These proteins may be produced at later times simply to balance the levels of E1B products by reducing the level of 22S message through splicing. Therefore

it seems unlikely that overproduction of the 22K protein could be responsible for the increased transforming efficiency of *pm*1542.

Many studies have suggested that adenovirus E1B 175R is involved in modulating viral or cellular gene expression. In studies using transient expression assays, it was suggested that 175R stimulates gene expression indirectly by stabilizing transfected plasmid DNA (Herrmann and Mathews, 1989). In studies using virus-infected cells, 175R was seen to repress E1A-dependant gene expression (White *et al.*, 1986 and 1988). Recently, however, it was reported that 175R had no effect in modulating the level of viral mRNA following virus infection (Herbst *et al.*, 1988), suggesting that it is not involved in the control of gene expression at the transcriptional level. To reveal the role of the 163R protein in gene expression in Ad12, the accumulation of viral mRNA was examined before and after the appearance of the *deg/cyt* phenotype. Most 163R mutants accumulated viral mRNA to a level comparable to that of *wt*, except for *dl*1670 which produced somewhat reduced levels of viral mRNA (see Fig. 6-1 and Fig. 6-2). While monitoring the level of the viral DNA template in a parallel experiment, it was observed that DNA templates were slightly higher with *pm*1542, but were lower with *dl*1670 (see Fig. 6-3). Because the difference was so little, it appeared that 163R does not play a major role in gene modulation, if any. Alternatively, 163R may play a minor role in mRNA accumulation. The absence of functional 163R with *dl*1670, for example, might result in a 30% reduction in DNA replication. Then the effect on transcription, with

another 30% reduction, could result in a 50% (70% x 70% = 49%) reduction in mRNA accumulation. However, with *pm*1542, the overexpression of 482R, which may stimulate viral DNA synthesis slightly, resulted in a 30% increase. Such an increase might compensate for the reduction in mRNA transcription caused by the absence of 163R, as described with *dl*1670. The present study did not explain why the virus yield was slightly higher with *pm*1542, but lower with *dl*1670 (see Table 4-1). Presumably the viral DNA at 20 hours postinfection, and before DNA degradation, might be engaged in virus assembly. Therefore the difference in the amount of viral DNA could thus partly affect the virus yield.

Previous studies already had indicated that 482R is involved in cellular transformation and tumour induction in rodents, and is required for viral DNA replication and late mRNA and protein accumulation. To determine the possible relations between gene regulation and these functions, three transformation defective 482R mutants were isolated (Mak and Mak, 1990) and viral proteins and mRNA synthesis was examined. As expected, with *pm*1852 and *dl*17, viral late proteins were synthesized at *wt* levels. However, with *dl*42, late proteins were hardly detected (see Fig. 6-5). Further studies examined the level of early mRNA accumulation (see Fig. 6-6), and found that E1A was expressed predominantly at 6 hours postinfection and the level of E1A mRNA did not change significantly, either with *wt* or *dl*42. These data suggested that E1A is expressed early and that the mRNAs are fairly stable. E2A and E3 might be expressed as early as

E1B. However, E2B and E4 were clearly expressed later than E1B. With *dl*42, the levels of E2B and E4 were clearly lower than those seen with *wt*. Quantitative results (see Fig. 6-7) indicated that the level of E2A mRNA at 12 hours postinfection with *dl*42 was about 5 times lower than that with *wt* virus, which probably resulted from the lower level of DNA template. E3 mRNA with *dl*42 was relatively abundant early but was about two times lower than *wt* at 12 hours postinfection. These effects may at least partly result from the lower amount of the DNA template. However, E2B and E4 mRNAs with *dl*42 accumulated to a level at least twenty times lower with *wt*. Such a difference was also observed when ara C was present during the infection. These results indicated that 482R plays a role in the accumulation of the products of E2B (DNA polymerase and viral terminal protein) and E4. Therefore, the absence of a functional 482R product may result in defects in viral DNA replication due to the absence of adequate amounts of E2B and possibly E4 products. These defects in viral DNA replication could cause secondary effects on the accumulation of viral late mRNAs and proteins. Since *dl*42 was not defective for tumour induction (Mak and Mak, 1990), it is clear that modulation of early mRNA levels is not involved in this process. Since all three mutants were defective for cellular transformation, it appeared that the ability to modulate early mRNA levels was not sufficient for transformation. Probably the modulation of early mRNAs by 482R may not be involved in transformation at all.

The present data have revealed the role of Ad12 E1B proteins in modulating the

level of other viral early gene expression. These data, and results obtained with Ad5 (reviewed by Branton *et al.*, 1985; White and Cipriani, 1990; McLorie *et al.*, 1991), suggested that in cooperation with products of E1A, 482R (or 496R in Ad5) can transform cells in the absence of 163R. Overexpression of Ad12 482R seemed to eliminate the need for 163R for high efficiency of transformation. The primacy of the former in transformation by Ad12 and Ad5 appears to differ. With Ad5, transformants were produced at similar reduced efficiency (25-50%) in the presence of either 496R or 176R (McLorie *et al.*, 1991), whereas with Ad12, mutants that failed to produce functional 482R transformed at extremely low efficiency (less than 10%) in both virus- and plasmid DNA-mediated assays (Mak and Mak, 1990). The 482R and 163R E1B products could function in a common pathway or by completely separate biochemical processes, but regardless of the precise mechanism, these products appear to act inefficiently. Efficient transformation appears to result only if both are expressed or, as in the case in the present study, if 482R is present at high levels. Whether or not overexpression of 163R in the absence of 482R would have similar effects is unclear. Analysis of this question and the precise biochemical roles for each of these E1B products is awaiting for further investigations.

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