

Permissivity of Human HeLa Cells to Bovine Adenovirus Type 2 (BAV2) Infection

by

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ABSTRACT

Infection of human cells by bovine adenovirus type 2 (BAV2) is abortive. To obtain a better understanding of this phenomenon, and in particular to identify which steps in the viral replicative cycles are altered during this virus-host cells interaction, we have undertaken a detailed study of BAV2 infections of the nonpermissive human HeLa cells. Using autoradiography and ^3H -thymidine-labeled whole virus particles for infection of HeLa cells, we determined that viral attachment appears normal. Furthermore, Southern analysis revealed that internalization and transport to the nucleus occurs in BAV2 infected HeLa cells. To investigate viral DNA synthesis, infectivity assays involving hydroxyurea, a viral DNA synthesis inhibitor, were carried out. The results revealed that BAV2 DNA synthesis does not occur in HeLa cells. Further investigations into viral early gene expression by northern blotting analyses indicated that HeLa cells fail to support expression of E1A. This suggested that abortive infection by BAV2 could be attributed to failure of E1A to express. To test the possibility that the failure to express E1A was due to the inability of the host cell to recognize the E1A promoter, we carried out transient expression transfection experiments using plasmids with the bacterial *lacZ* under the control of either BAV2 or Ad5 E1A promoter. X-gal histochemical assays showed expression of *lacZ* from the Ad5 E1A promoter but no expression of *lacZ* from the BAV2 E1A promoter. This further suggests that the abortive infection by BAV2 could be attributed to failure of E1A to express due to a nonfunctional promoter in human cells. Thus we speculated that abortive infection of HeLa cells by adenoviruses may be averted by providing E1A functions *in trans*. To demonstrate this, we coinfecting HeLa cells with Ad5 and BAV2, reasoning that Ad5 could compensate for E1A deficiency in BAV2. Our results showed that BAV2 DNA synthesis was indeed supported in HeLa cells coinfecting with Ad5 *dIE3* as revealed by Southern analysis. In contrast, coinfection of HeLa cells with BAV2 and Ad5 *dIE1E3* mutant did not support BAV2 DNA synthesis. Interestingly, BAV2 failed to replicate in 293 cells which are constitutively expressing the E1 genes. This could imply that E1 is necessary but not sufficient to avert the failure of BAV2 to undergo productive infection of human cells.

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LIST OF ABBREVIATIONS

μg	microgram
μl	microliter
³² -P	phosphorus-32
Ad	adenovirus
amp	ampicillin
ATP	adenosine triphosphate
BAV	bovine adenovirus
BAV2	bovine adenovirus type 2
bp	base pair
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
CAR	coxsackie adenovirus receptor
CFTR	cystic fibrosis transmembrane regulatory region
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
dH ₂ O	distilled water
dNTP	deoxynucleotide
ds	double-stranded
E	adenovirus early region
E2F	E2 transcription factor
<i>et al.</i>	and co-workers
EtBr	ethidium bromide
EtOH	ethanol
IL	interleukin
ITR	inverted terminal repeat
kb	kilo base-pair
kd	kilo-dalton
L	adenovirus late region
MDBK	Madin-Darby bovine kidney

ml	mililiter
MLP	major late promoter
mM	milimolar
moi	multiplicity of infection
mRNA	messenger RNA
mu	map units
NEB	New England Biolabs
NEN	New England Nuclear
ng	nanogram
nm	nanometer
NFI	nuclear factor one
NFII	nuclear factor two
NFIII	nuclear factor three
OD _{nm}	optical density at wavelength nm
ORF	open reading frame
PBS	phosphate-buffered saline
p.i.	post infection
SSC	saline sodium citrate
TAE	Tris.acetate EDTA
TE	Tris.EDTA
v/v	volume per volume
w/v	weight per volume
Xgal	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside

1. Literature Review

1.1 Scope of review

Adenoviruses (Ads) have been studied extensively since their discovery in 1953 (Rowe *et al.*, 1953). These studies have led to a number of very important discoveries both in adenovirus biology and molecular biology in general. For example, discovery of mRNA splicing was first reported in Ad system (Berget *et al.*, 1977). Furthermore, tumor induction in rodents by Ads has become one of the classical models for studies in oncogenesis (Trentin, 1962). Recently, due to their highly infectious nature and wide tissue tropism, Ads are being utilized as vectors for gene therapy and recombinant vaccine development. Abortive infections of Ads is another area that has been extensively studied for the past 30 years. These studies have contributed not only to the understanding of biology and evolution of Ads but also to the understanding of oncogenesis by DNA viruses and functional consequences of DNA methylation in mammalian system. In addition, studies on abortive infections have great implications in use of Ads as recombinant viral vectors and gene therapy agents.

This review will discuss most of the known abortive infections by Ads focusing the attention on viral and cellular determinants that operate to block Ad at different levels of multiplication cycle. Furthermore, the applications and significance of Ad abortive infections will be explored. However, in order to completely understand Ad abortive infections one must be familiar with the events in productive Ad infection. Hence, classification, biology and multiplication cycle of Ads will be summarized prior to the presentation of Ad abortive infection studies.

1.2 Classification and epidemiology

The family Adenoviridae contains two genera, *Mastadenovirus* and *Aviadenovirus*. While genus *Aviadenovirus* contains Ads that infect only birds, the genus *Mastadenovirus* comprises human, simian, bovine, equine, porcine, ovine, canine, and opossum viruses (Norrby *et al.*, 1976). Even though there is antigenic cross-reactivity among members within each genus due to conserved epitopes located on the hexon protein of the virion, there is no shared specific antigen common to all Ads (reviewed by Shenk, 1996). Recently, a third phylogenetically different group of Ads was formed based on analysis of the viral protease genes. This group includes the avian Egg Drop Syndrome virus -76, bovine Ad type 6, and ovine Ad isolate OAV287 (Harrach *et al.*, 1997).

1.2.1 Human Ads

Human Ads (Ads) were first isolated by Rowe and colleagues in 1953 from tonsils and adenoidal tissue surgically removed from children. Soon afterward, similar viral agents were isolated from feverish military recruits with a variety of respiratory illnesses (Hilleman & Werner, 1954). Currently, there are 49 distinct serological types of Ads that infect humans. These agents can infect and replicate at various sites of the respiratory tract as well as in the eye, gastrointestinal tract, urinary bladder, and liver. Several clinical syndromes have been associated with Ads including acute respiratory disease, pertussislike syndrome, keratoconjunctivitis, acute hemorrhagic cystitis, meningoencephalitis, diarrhea and intussusception. The target population of Ads is primarily children and military recruits (reviewed by Fields, 1996). Recently, these Ads have been shown to cause fatal, respiratory infection (pneumonitis) in adult transplant recipients (Simsir *et al.*, 1998). Although, some Ads experimentally induce tumors in rodents (Trentin, 1962), there is no epidemiologic evidence linking Ads with malignant disease in the human.

Ads are subdivided into 6 subgenera (A-F) primarily based on their ability to agglutinate red blood cells (Hierholzer, 1973; Rosen, 1960). Additional sub-classification of Ads is provided based on viral DNA guanine-cytosine (GC) content, DNA sequence homology and oncogenicity in rodents (*Table 1*).

Table 1. Properties of human Ad serotypes of subgenera A-F (modified from Wadell, 1994).

Subgroup	Hemagglutination groups	Serotype	Oncogenic Animals	potential Cell culture	GC %
A	Little or no agglutination	12, 18, 31	High	+	48-49
B	Complete agglutination of monkey erythrocytes	3, 7, 11, 14, 16, 21, 34, 35	Moderate	+	50-52
C	Partial agglutination of rat erythrocytes	1, 2, 5, 6	Low or none	+	57-59
D	Complete agglutination of rat erythrocytes	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-47	Low or none	+	57-61
E		4	Low or none	+	57-59
F		40, 41	Unknown		

1.2.2 Bovine Ads

Since their first isolation in 1959 (Klein *et al.* 1959) bovine Ads (BAVs) have been isolated from a wide variety of hosts that include cattle, sheep (Belak & Palfi, 1974), free living buffalo (Baber, 1981) and fallow deer (Boros *et al.*, 1985). Currently, there are ten accepted BAV serotypes, differentiated on the basis of serum neutralization and complement fixation tests. The ten serotypes are further subdivided into two subgroups based primarily on differences in their ability to replicate in different cell cultures (Benko *et al.*, 1989), and the lack or presence of complement-fixing antigen common to *Mastadenoviruses* (Bartha, 1969). Subgroup 1 contains

BAVs 1, 2, 3 and 9 that grow in primary bovine testicular or kidney cells as well as in continuous cell lines and share the antigen common to all *Mastadenoviruses*. Subgroup 2 contains serotypes 4, 5, 6, 7, 8 and 10 that do not cross-react with any other mammalian Ad in the complement fixation test, and they can be propagated exclusively in cultures of calf testicular or thyroid cells.

BAVs are associated with respiratory and alimentary tract diseases. Although most infections are subclinical (Matson *et al.*, 1988), occasionally BAVs are associated with more serious diseases such as severe pneumonia, enteritis (Reed *et al.*, 1978) and epizootic haemorrhagic enterocolitis (Adair *et al.*, 1966; Smyth *et al.*, 1996).

Bovine Ad type 2 (BAV2) was first isolated by Klein and coworkers in 1959 from the feces of apparently healthy cattle. BAV2 is subdivided into two subtypes based on restriction endonuclease mapping and hemagglutination test (Belak *et al.* 1974). Subspecies A contain strains of restriction endonuclease patterns similar to the prototype strain No. 19 which hemagglutinate bovine erythrocytes and infect cattle alone. Subspecies B contains strains closely related to the strain ORT-111 originally isolated from sheep which hemagglutinate rat erythrocytes. Besides differences in restriction endonuclease patterns and hemagglutination properties, subspecies A and subspecies B differ in pathogenicity. Apparently, in lambs, the ORT-111 strain is more pathogenic than strain No. 19. The stronger pathogenicity of ORT-111 is demonstrated by the more intensive virus multiplication and shedding, as well as by the more severe histopathological lesions (Rusvai *et al.*, 1992).

1.3 Structure

1.3.1 Capsid and core organization

Ads are non-enveloped DNA viruses composed of an icosahedral protein coat called capsid and an inner DNA protein core (*Figure 1*). The terminology for structural components of adenoviral capsid currently used is as established by Ginsberg *et al.* (1966). The major subunit of the capsid is a spherical structure called capsomere. The virion has 252 capsomeres, of which 240 capsomeres are called hexons since these appear in a position surrounded by six other capsomeres. Pentons, on the other hand, are subunits surrounded by five hexons. Each of 12 pentons present in each virion contain a penton base on the surface of the capsid and a fiber projecting from the base. The fiber is responsible for attachment to cells and for the hemagglutinating activity of the virus. The fiber length varies with subtypes from 10 nm in human Ad3 to 50 nm for some fowl Ads (Norrby, 1969).

Ad core structure is composed of double-stranded DNA and four additional viral proteins not evident in the capsid. The viral DNA is approximately 11 μm in length and has a 55-kd terminal polypeptide covalently linked to each of the 5' ends of the viral genome (reviewed by Horwitz, 1990).

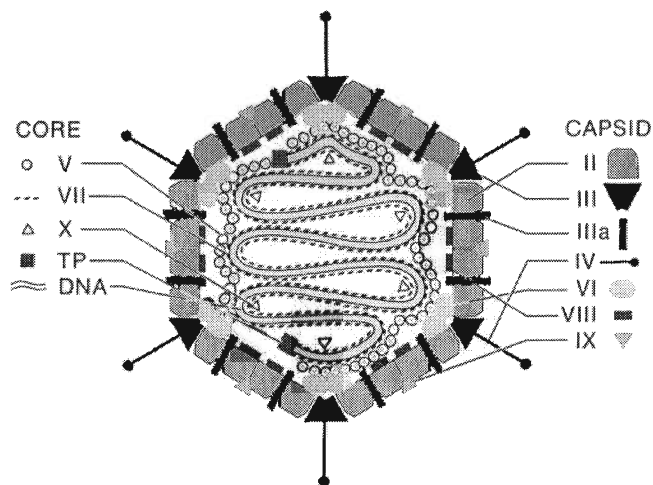


Figure 1. Model of Ad structure (modified from Stewart *et al.*, 1991)

The polypeptide composition of Ads has been determined using Ad2 as the representative virus (Maizel *et al.*, 1968; Everitt *et al.*, 1975). The virion contains at least 11 distinct polypeptide chains separable by electrophoresis on acrylamide gels (*Figure 1*). These proteins are numbered II-X by convention and each had been assigned to a specific viral component, however, the designation for polypeptide I could not be assigned to a single component since the moiety proved to be a mixture of aggregated smaller molecules (Maizel *et al.* 1968). The hexon comprises three tightly associated molecules of 120-kd polypeptide II. The penton base is made of five molecules of polypeptide III (85 kd), while the fiber is a trimer of three polypeptides IV (62 kd) (van Oostrum & Burnett, 1985). The conserved amino terminus of the fiber is attached to the penton base whereas the knob containing the carboxy end projects farthest from the capsid. Polypeptide V (48.5 kd) is a core protein placed immediately internal to the pentons in the vertex regions of the virion. Polypeptides V, VI (24 kd), VII (18.5 kd), VIII (13kd), μ , as well as the 55-kd terminal protein are located within the core of the virion (Hosakawa & Sung, 1976; Russel *et al.*, 1968).

1.3.2 Genome structure and organization

The Ad genome consists of a single linear, double-stranded DNA which is approximately 11 μ m in length and varies in molecular weight from 20 to 24 x 10⁶ Da for different serotypes (Green *et al.*, 1967). The adenoviral DNA has two unique features: (1) it carries a 55kDa terminal protein (TP) (Robinson *et al.*, 1973) that is covalently linked via phosphodiester bond of serine 562 to each 5' end and (2) contains relatively short inverted terminal repeats (ITRs) flanking the coding regions that play a role in replication of the DNA (Arrand & Roberts, 1973). The GC content ranges from 50-60% and is highest for the most oncogenic serotypes (reviewed by Green, 1970).

Most of the adenoviral genomes that has been sequenced and analyzed to date have similar general organization. *Figure 2* shows a transcription and translation map of Ad2. The viral chromosome carries five early transcription units (E1A, E1B, E2, E3 and E4), two delayed early units (IX and IVa2) and one major late unit which is processed to generate five families of late mRNAs (L1 to L5), all of which are transcribed by RNA polymerase II. Early mRNAs are transcribed from four distinct regions of the genome (Sharp *et al.*, 1974), two on the r-strand between 3-14 m.u., and 81-85 m.u., and two on the l-strand between 58.5-70.7 m.u., and 90-94 m.u. (Flint, 1977). Both intermediate and major late units are transcribed by the l-strand of the viral genome (Persson, 1979; Shaw & Ziff, 1980). The transcription and translation of different Ad regions will be discussed in more detail later in section 1.4.

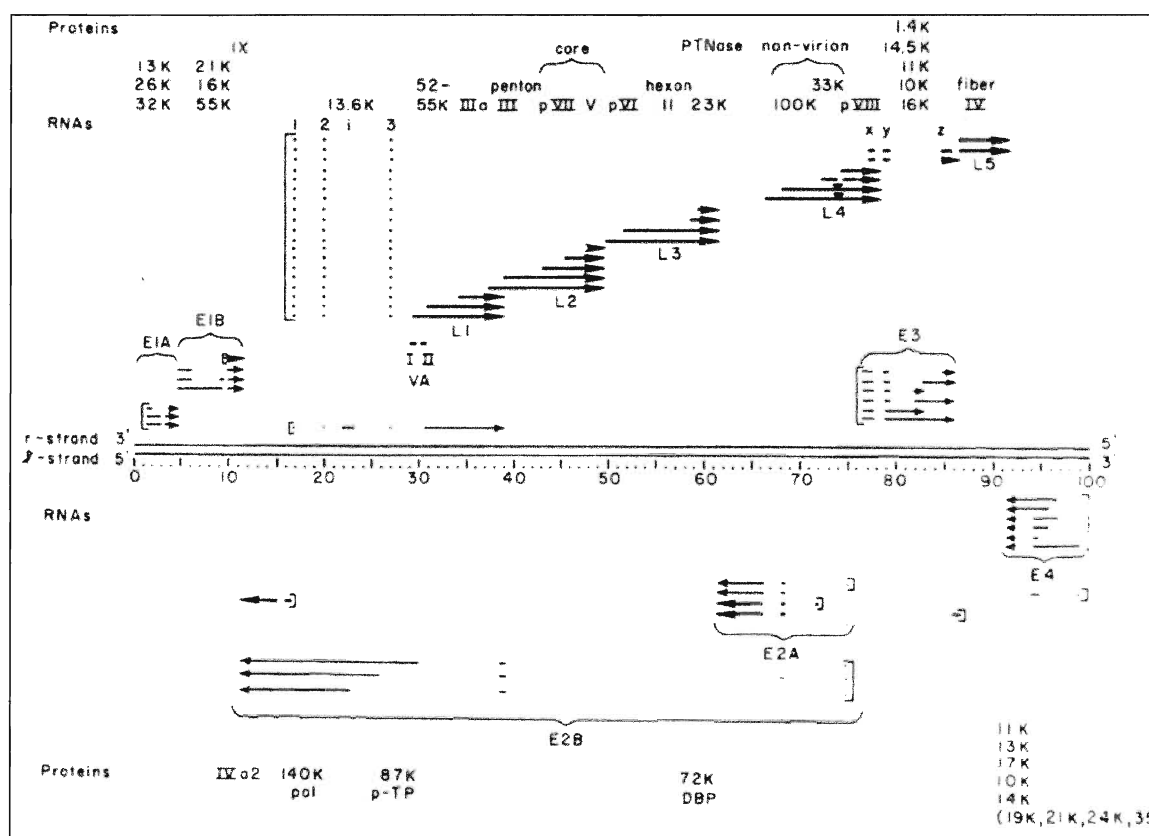


Figure 2. Transcription and translation map of Ad2 (from Horwitz, 1990).

The complete sequence of the BAV2 genome has been deduced and its genetic organization was deduced by comparing it to human Ads (Ojkic, 1997). Thirty one of the BAV2 protein

sequences were identified according to their homology to already known Ad polypeptides. According to these results, the genome organization of BAV2 closely coincides with the genomic organization of human Ads.

1.4 The life cycle of Ads

The Ad infection is a result of a series of events beginning with the attachments of the fiber to a host cell receptor, and ending with the assembly of approximately 10^4 infectious virions per cell. Studies on the Ad life cycle focused mainly on human Ad2 and 5, particularly their infection of HeLa and KB cells. The multiplication cycle is divided by convention into two phases; early and late, with the later commencing with the onset of viral DNA replication (*Figure 3*). Early events include adsorption, penetration, transcription and translation of an early set of genes. The late phase begins at the onset of DNA replication with the expression of late viral genes and assembly of progeny virions. Ad infection has a profound effect on host macromolecular synthesis as depicted in *Figure 3*.

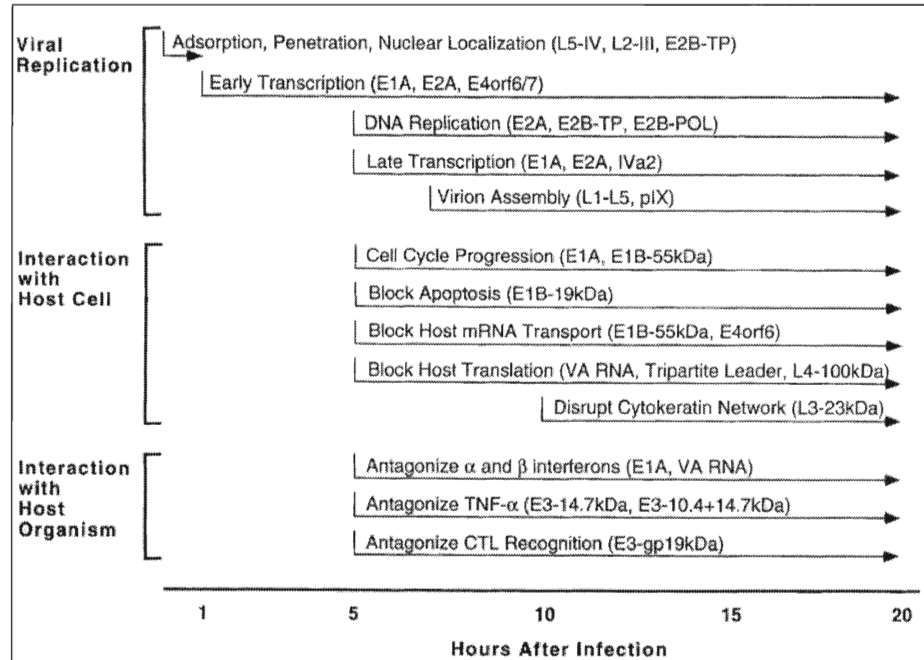


Figure 3. Diagram depicting the major events in the replication cycle of Ad (from Shenk, 1996).

1.4.1 Adsorption and entry

The initial recognition of the plasma membrane and subsequent virus particle attachment is mediated by the trimeric viral fiber protein (Philipson *et al.*, 1968). The C-termini of the fiber polypeptides have been shown to form the distal knob of the protein presumed to bind to the cellular receptor, while the N-termini form a tail by interacting in a non-covalent manner with the pentameric penton base (Devaux *et al.*, 1983). Several observations that the fiber protein length varies among different serotypes (Signas *et al.*, 1985; Kidd *et al.* 1993) and that some Ads contain two fiber genes which provide distinctly different lengths of fiber (Kidd *et al.*, 1993; Gelderblom and Maichle-Lauppe, 1982) have led to suggestions that the fiber length is correlated to the surface density of the cell receptors. Furthermore, the greater fiber length may facilitate attachment if binding to more than one receptor is necessary. The primary receptor CAR for Ad2 and 5 has been recently identified (Bergelson *et al.*, 1997). The same receptor appears to be used for attachment of Coxsackie B viruses. Since the amino acid sequence of the knob region varies among serotypes, it is possible that different receptors may be required for different Ad serotypes.

Internalization of Ad is mediated by a second protein-protein recognition event involving the penton base and specific members of a family of heterodimeric cell surface receptors, termed integrins (Wickham *et al.*, 1993). The interaction occurs through an arg-gly-asp (RGD) sequence present in the penton base polypeptides (Bai *et al.*, 1993). The integrins involved appear to be $\alpha_v\beta_3$ and $\alpha_v\beta_5$ vitronectin-binding integrins (Wickham *et al.*, 1993); however, the recognition of other integrins by the penton base cannot be excluded.

A model for Ad internalization has been proposed (*Figure 4*). Following attachment via the fiber protein and its receptor, Ad internalization occurs by receptor-mediated endocytosis. This process is triggered by the penton-integrin interaction. The precise mechanism by which this

interaction promotes the entry process is yet to be elucidated; however, the activation of a cell signaling molecule phosphoinositide-3-OH Kinase (PI3K) may play an important role in this process. It has been demonstrated recently that PI3K is activated upon Ad interaction with α_v integrins and that this event is required for Ad internalization (Erguang *et al.*, 1997). Once taken up into the cell, the endocytotic vesicle loses the fiber protein and becomes increasingly acidified as it moves to the cell interior. The change in cytosol pH releases the virion into the cytoplasm which is accompanied by the loss of the majority of the pentons, protein IX and, apparently, by proteolysis of protein VI and a sub-population of the hexons (Greber *et al.* 1993). At this stage of infection, Ad resembles Ad cores with some of the hexons from the capsid still associated. The particle then moves to the nuclear pores, where Ad DNA, along with its associated viral core proteins, is deposited into the nucleus (reviewed by Dulbecco & Ginsberg, 1988). Once inside the nucleus, the viral DNA presumably associates with cellular histones (Daniell *et al.*, 1981). Parental Ad chromatin then binds to the nuclear matrix through its terminal protein initiating early transcription (Schaack & Shenk, 1989).

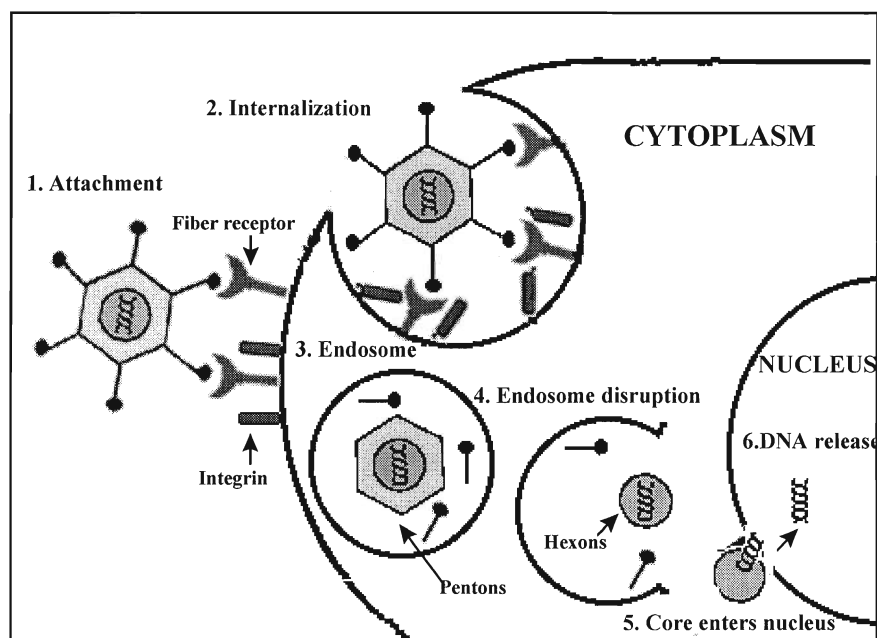


Figure 4. Diagram showing attachment, entry and uncoating of the Ad particle.

1.4.2 Early transcription

As demonstrated by studies on Ad2 and Ad5, expression of the early genes begins with the E1 and proceeds in an ordered sequence through the other early transcription units designated E2, E3 and E4.

The E1 domain is divided into the E1A and E1B regions. Transcription of E1A is controlled by a constitutively active promoter that includes a duplicated enhancer element (Hearing and Shenk, 1986). E1A encodes at least five mRNAs, two early and three late. The two early mRNAs, 13S and 12S, contain identical 5' and 3' ends, differ internally due to differential splicing, and encode for 289- and 243-amino acid protein respectively (reviewed by Shenk, 1996). These products have a number of functions including: 1) trans-activation and repression (Borelli *et al.*, 1984; Velcich & Ziff, 1985) of E1B, E2, E3 and E4 transcription; 2) induction of host cell to enter S phase of the cell cycle (reviewed by Shenk, 1996); 3) induce high-level expression of p53 and apoptosis (Clarke *et al.*, 1993; Querido *et al.*, 1997); and 3) oncogenic transformation of cells in culture (Graham *et al.*, 1974; Ruley, 1983). Unlike certain cellular factors, the E1A gene products do not appear to bind directly to promoter elements (Lillie & Green, 1989), suggesting that E1A alone is insufficient to function as a transcriptional activator, but rather elicits a response through host proteins. The E1A trans-activation occurs as a result of E1A interactions with different cellular transcription factors and regulatory proteins at the TATA motif found 25 to 30 bp upstream of transcriptional initiation sites in many viral and cellular genes. Although E1A is required to immortalize primary cells *in vitro* (Graham *et al.*, 1974; Ruley, 1983), it appears that two E1B proteins are required to maintain the transformed phenotype *in vitro* (Byrd *et al.*, 1988). The Ad E1B-55 kd protein probably blocks apoptosis as a result of its ability to bind to p53 and alter its function (Teodoro and Branton, 1997; Moran,

1993) while the 19 kd protein, in addition to inhibition of E1A-induced apoptosis modulates transcriptional properties of p53 (Sabattini *et al.*, 1995).

The E2 domain is separated into two different, non-overlapping transcription units called E2A, and E2B. The jointly controlled E2A and E2B regions code for proteins that are essential for viral DNA replication. E2A codes for 72kd single-strand DNA-binding protein (DBP) (Levine *et al.*, 1974). Besides its function in the elongation reaction in DNA synthesis (Horwitz, 1978), the 72kd DBP is also associated with mRNA stability (Babich and Nevins, 1981), host range (Klessig & Grodzicker, 1979), repression of E4 transcription (Nevins & Jensen-Winkler, 1980) and assembly of infectious virus particles (Nicolas *et al.*, 1983). E2B codes for two proteins needed for the initiation reaction in DNA replication namely, the 55k Ad terminal protein (pTP) and the 140 kd Ad DNA polymerase (Ad DNA Pol) (Stillman *et al.*, 1981; Friefeld *et al.*, 1983). The functions of the E2 proteins will be discussed in section 1.4.3. E2 contains two promoters; one used early and another late in infection. However, it is not clear why these promoters switch at different times post infection (Swaminathan and Thimmapaya, 1995).

The E3 region appears to be non-essential for viral growth in tissue culture and can be deleted without altering viral replication *in vitro* (Jones & Shenk, 1978). It encodes for proteins that modulate the host response to Ad infection. Polypeptides 14.7 kd, 14.5 kd and 10.4 kd function to prevent cytolysis by tumour necrosis factor (TNF α) (Krajcsi *et al.*, 1996). Furthermore, 10.4 kd and 14.5 k Ads been shown to down-regulate the epidermal growth factor (EGF) receptor in Ad infected cells (Carlin *et al.*, 1989). Another E3 protein (19 kd) interacts with class I MHC antigens and prevents transport of MHC molecules from the rough endoplasmic reticulum to the cell surface (Deryckere *et al.*, 1995). Finally, E3-11.6 kd protein known as Ad death protein (ADP) appears to be necessary for the effective lysis of infected cells (Tolfson *et al.*, 1996).

The E4 regions codes for at least 7 proteins (Dix and Leppard, 1995). Functions encoded by the Ad E4 region are required for efficient DNA replication, late gene expression, host cell shut-off and virion assembly. A 34 kd protein has been shown to form a complex with the 55 kd protein of the E1B region (Sarnow *et al.*, 1984) in the nucleus. This complex is involved in transport of viral late mRNAs to the cytoplasm (Ornelles & Shenk, 1991). In addition, the 34 kd protein promotes transformation of primary rodent epithelial cells (Nevels *et al.*, 1997). The E3-11kd protein binds to the nuclear matrix and is involved in accumulation of late viral mRNA (Sarnow *et al.*, 1982). 19.5 kd E4 protein transactivates the E2 promoter by first activating, along with E1A, the cellular transcription factor E2F, which then binds to two adjacent sites in the early E2 promoter (Hemstrom *et al.*, 1991). The protein encoded by E4-orf4 autoregulates its own transcription by inhibiting Ad E1A-induced activation of E4 transcription (Bondeson *et al.*, 1996).

1.4.3 DNA Replication

The development of an Ad DNA synthesis system *in vitro* allowed for the purification and characterization of the components of the replication system including three viral replication proteins that include 1) DNA binding protein (DBP), 2) preterminal protein (pTP) and 3) the adenoviral DNA polymerase (Ad pol) as well as four cellular factors; nuclear factor I (NFI), II (NFII), III(NFIII), and topoisomerase (reviewed by Shenk, 1996).

According to a novel displacement model for DNA replication (Sussenbach *et al.* 1972; Challberg and Kelly, 1979) Ad DNA synthesis can be divided into initiation and elongation reactions. Initiation begins with the formation of a preinitiation complex. Newly synthesized DBP molecules form a multimeric protein-DNA complex with viral DNA which in turn changes DNA configuration to make the binding sites for NFI and Oct-1 (NFIII) accessible (Stuiver *et al.*, 1992). The pTP-pol complex, NFI and Oct-1(NFIII) then bind to their recognition sequences in

the auxiliary origin of ITR (*Figure 5*) (Mul and Van der Vliet, 1993). Following the assembly of this preinitiation complex, the origin of replication is unwound. The precise mechanisms of this process is not yet identified. Next, initiation occurs when the precursor terminal protein (pTP) covalently linked to the 5' ends of the viral DNA reacts with dCTP to form a pTP-dCMP complex. The pTP-dCMP complex then presents the free 3'-OH group in the initiating cytidyl residue required as a primer for a growing DNA chain (Challberg *et al.*, 1980). Synthesis is initiated at either terminus of the linear DNA; however, only one of the two DNA strands serves as template for synthesis.

Elongation starts after dissociation of DNA from pTP to enable the DNA to pass freely along the polymerase (reviewed by van der Vliet, 1995). Elongation is dependent on the DBP as well as a cellular protein, NFII. DBP facilitates unwinding, enables displacement synthesis, makes the polymerase highly processive presumably by influencing the DNA structure, and protects newly synthesized ssDNA (Zijderveld and van der Vliet, 1994; Monaghan *et al.*, 1994; Lindenbaum *et al.*, 1986; Tsui *et al.*, 1991). The precise function of NFII in Ad DNA chain elongation is not yet clear although evidence lends support to topoisomerase function (Challberg & Kelly, 1989). As elongation proceeds the other parental strand that carries pTP becomes displaced as single strand by the advancing replication fork. The products of replication are a duplex consisting of a daughter and parent strand plus a displaced single strand of DNA (*Figure 6*). The fully displaced parental strand is capable of circularizing by hybridization of the self-complementary terminal sequences, and the resulting double-stranded "panhandles" that are identical to the ends of the double-stranded parental genome may be recognized by the same set of initiation proteins. As a result, the ssDNA can undergo a second round or replication in the same way as the duplex molecule. Ad DNA replication therefore proceeds by type I replication of duplex strands or type II replication of the ssDNA molecules (reviewed by Shenk, 1996).

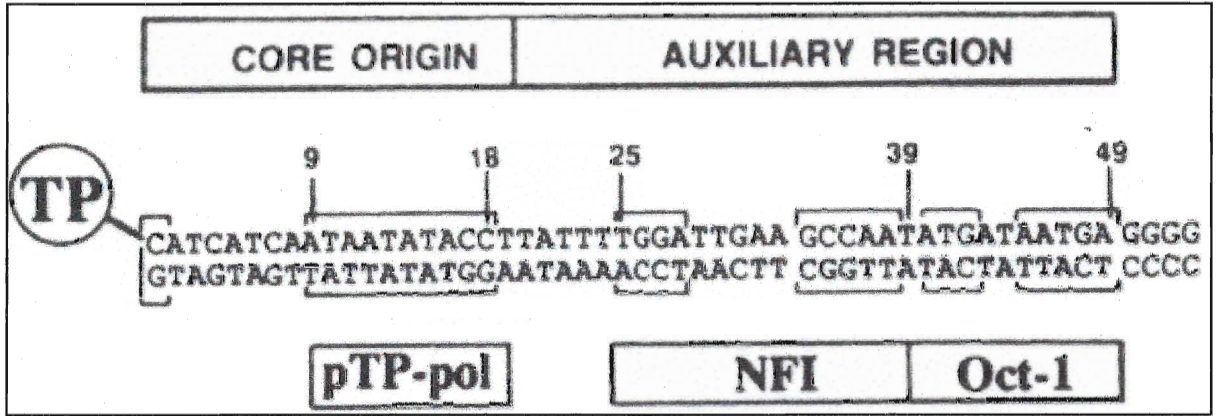


Figure 5. The Ad type 2/5 origin of DNA replication.

Binding sites for precursor TP-DNA polymerase (pTP-pol) in the core origin and for nuclear factor I (NFI) and NFIII/Oct-1 in the auxiliary region are included in the Figure (from Van der Vliet, 1995).

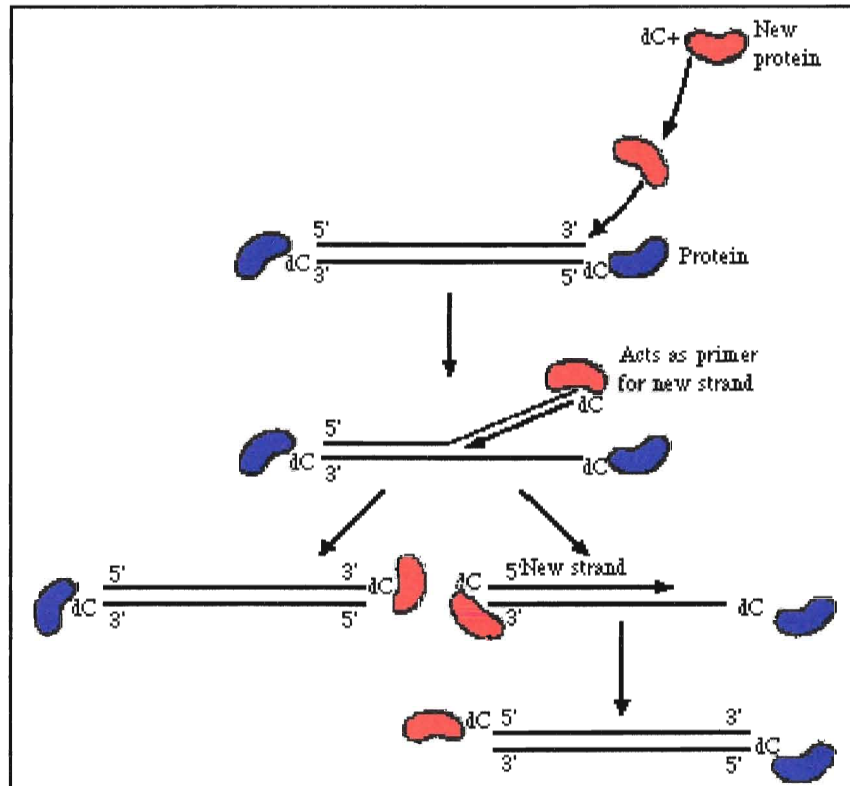


Figure 6. General outline of the first round of Ad DNA replication (from Shenk, 1996).

1.4.4 Late transcription

There are five families of late mRNAs transcribed at the onset of DNA replication. Transcription starts from the major late promoter (MLP) and generates a single large transcription unit whose length is approximately 29,000 bp. This transcript is processed by differential poly(A) site utilization and splicing to generate at least 18 distinct mRNAs (*Figure 2*). Based on the utilization of common poly(A) these monocistronic mRNAs are grouped into five families, termed L1 to L5 (Chow *et al.*, 1977; reviewed by Shenk, 1996). All the late adenoviral messenger RNAs carry identical tripartite leader sequence (Nevins & Darnell, 1978). Following splicing, mRNAs are transported from the nucleus to the cytoplasm and associated with ribosomes (reviewed by Shenk, 1996). The cellular transcription continues during late viral transcription, however, its accumulation in the cytoplasm is blocked. The block is mediated by the complex formed by the E1B-55 kd and the E4-34 kd polypeptides (Sarnow *et al.*, 1984). Although it is not precisely understood how this complex inhibits transport of cellular mRNAs, it has been suggested that it relocates a cellular factor required to transport cellular mRNAs from their site of synthesis to the nuclear pore (Ornelles and Shenk, 1991).

Late viral mRNAs encode both structural and non-structural proteins. The L1 mRNA codes for two structurally related proteins, 52 kd and 55 kd whose functions are unknown, and a hexon protein IIIa. The L2 mRNA encodes three structural proteins: protein III (penton), the precursor for protein VII, and protein V. The L3 gene family encodes the precursor for protein VI, protein II (hexon) and a 23 kd protein. The L4 region encodes a structural precursor to protein VII (hexon), a 33 kd protein and a non structural 100 kd protein (Miller *et al.*, 1980). A 100 kd late protein functions as a scaffold protein for the assembly of the hexon trimers (Cepko & Sharp, 1983). Finally, the L5 gene family encodes the viral structural protein IV (Fiber) (Miller *et al.*, 1980).

Virus-associated RNAs (VA RNA I and II) are also produced at late times of infection; however, they can be synthesized in the absence of DNA replication. These RNAs are transcribed in large amounts from 29 m.u. on the r-strand of Ad templates by cellular RNA polymerase III (Soderlund *et al.*, 1976; Celma *et al.*, 1977; Mathews, 1975; Weinmann *et al.*, 1976). VA RNA I prevents activation of the double-stranded RNA activated inhibitor which phosphorylates the α -subunit of translation initiation factor eIF-2. Hence, these RNAs are required for efficient translation of viral proteins (Thimmappaya *et al.*, 1982; O'Malley *et al.*, 1989).

Virus assembly begins with formation of capsomers of pentons and hexons in the cytoplasm. From there on, virus assembly proceeds in the nucleus through a series of intermediates that encapsidate viral nucleoprotein (consisting of DNA molecules, TP, pV, pVII and μ) and subsequently undergo proteolytic processing to form the final mature particles (reviewed by Shenk, 1996).

1.5 Host range

Host range of Ads is first determined by the ability of Ad to attach to the host cell. However, this is not the only criterion that determines the host range of Ads which also depends on the appropriate intracellular interactions of both viral and cellular factors (Lonberg-Holm & Philipson, 1969). Depending on the extent of Ad multiplication in different cell lines, Ad infection can be divided in three categories, namely, permissive, semi-permissive and non-permissive infections. Permissive or productive infections are those in which progeny yield is maximum. Although semi-permissive infections lead to new viral progeny, the yield is greatly reduced as compared to productive infection. Finally, non-permissive or abortive infections are those in which the virus does not undergo a complete multiplication cycle due to different intracellular blocks.

In general, Ads are species specific and can replicate efficiently in cells derived from their own species. For example, most Ads (*Table 2*), with an exception of enteric Ads, grow best in primary human embryonic kidney (HEK) and continuous malignant human epidermoid carcinoma cells lines such as cervical (HeLa), laryngeal (Hep-2) and oral (KB) cells (reviewed by Horwitz, 1996). The 293 and 549 cell lines appear to be good hosts for most Ads including enteric Ads (Ad40 and Ad41) (Brown *et al.*, 1992; Hashimoto *et al.*, 1991). The ability of Ads to grow in various human cell lines varies among serotypes and is suggested to reflect *in vivo* tissue-specific susceptibility. For instance, Ad5 is capable of replicating in the cultured human fetal skeletal myoblasts such as the precursor myoblasts and the multinucleated myotubes (Kohtz *et al.*, 1991). Furthermore, Ad2, which is pathogenic for certain stratified squamous epithelial including those of upper respiratory tract and oropharynx, proceeds through a complete multiplication cycle when used to infect cultured keratinocytes from oropharyngeal sites (Aneskievich & Taichman, 1988). Infection of cultured human lymphoid cells by Ad type 2 and 5 is also productive (Lavery *et al.*, 1987).

Not all human Ads replicate efficiently in all cultured human cell lines. Many of these infections are either semipermissive or abortive (*Table 2*). For example, enteric Ads in particular Ad40 and Ad41, grow very poorly in HeLa, KB and HeP-2 cell lines (Pieniazek *et al.*, 1990). Furthermore, Ad12 infections of human melanoma cells Nki-4 and human cervical carcinoma cells C4/1 exhibit a late block (Schwarz *et al.*, 1982; Kruczek *et al.*, 1981). Ad2 abortively infects a number of cell lines including stratified cultures of human epidermal keratinocytes, suprabasal differentiated cells (Aneskievich & Taichman, 1988) and human peripheral blood lymphocytes (Horwath *et al.*, 1986). Also, freshly isolated peripheral blood lymphocytes (PBL) are abortively infected by Ad2 or Ad5 (Schranz *et al.*, 1979).

Although Ads are species specific the events of cross species infections have been documented both in animal models and in cell culture. Human Ads, for example, produce pulmonary disease in cotton rats similar to that of man. Moreover, human Ad5 was found to infect New Zealand rabbits, chimpanzees and hamsters (reviewed by Horwitz, 1996). Several additional human adenoviral serotypes extend their host range to the New Zealand rabbits. These include Ad1, 2, 5 and 6 (Romanowski *et al.*, 1998). In cell culture studies, human Ad5 and Ad2 were found to replicate semipermissively in baby hamster kidney cells (BHK21) (reviewed by Doerfler, 1994). While human Ad5 can efficiently replicate in bovine kidney cells (MDBK) (Martins, 1995), its replication in mouse cell lines BALB/c 3T3 cells is restricted resulting in at least 1000-fold lower virus burst compared to human cells (Blair *et al.*, 1989). A number of abortive infections of human Ads in cell lines of nonhuman origin has been documented. These include the infection of BHK21 by Ad12 (reviewed by Doerfler, 1994), African monkey cell lines by Ad2 (Anderson and Klessig, 1982), canine kidney cells (MDCK) by Ad5 (Martins, 1995), hamster embryo cultures (HamE) by Ad3,-7,-12 or -31 (Shimojo and Yamashita, 1968) and chinese hamster ovary (CHO) cells by Ad2 (Eggerding and Pierce, 1986). Most of the abortive infections mentioned above will be further discussed in section 1.6.

Productive infections of human cell lines by animal Ads have not been documented. To date only one semipermissive infection of human cells by animal Ads has been characterized, namely, the infection of HeLa cells by mouse Ad F1. HeLa cells infected with F1 produce at least 2000 times less virus than permissive mouse 3T3 cells. The defect in virion production is linked to a dramatic reduction in the synthesis of AdF1 structural proteins, in particular hexon (Antoine, 1982). Two abortive infections of human cell lines by animal Ads have been reported. These are canine Ad type 2 (CAV-2) infection of human 293, HeLa and KB cells, and ovine Ad (OAV) infection of a range of human cell types, including lung and foreskin fibroblasts as well as liver,

prostate, breast, colon, and retinal lines. These infections will be discussed in more detail in section on Abortive Infections.

	PRODUCTIVE		SEMIPERMISSIVE		ABORTIVE	
ORIGIN	Cell line	Ad serotype	Cell line	Ad serotype	Cell line	Ad serotype
Human	HeLa, HEK, HeP-2 and KB	all except Ad40/41	HeLa, HEK, HeP-2 and KB	Ad41	Human peripheral blood lymphocytes	Ad2
	293	all				
	A549	all				
	Fetal skeletal myoblasts	Ad5				
	Oropharyngeal keratinocytes	Ad2				
	Human lymphoid cells	Ad2/Ad5				
Nonhuman	Mardin-Darbin bovine kidney (MDBK)	Ad5	Baby hamster kidney (BHK21)	Ad2/Ad5	Baby hamster kidney (BHK21)	Ad12
			Mouse BALB/c 3T3	Ad5	Monkey (CV1)	Ad2
					Mardin-Darbin Canine kidney (MDCK)	Ad5
					Hamster embryo (HamE)	Ad3/7/12/31
				Chinese hamster ovary (CHO)	Ad2	

Table 2: Permissive, semi-permissive and non-permissive infections of various cell lines by Ads.

1.6 Abortive infections

1.6.1 Early block

1.6.1.1 Ad12 infection of baby hamster cells (BHK21)

Human Ad12 infection of BHK21 cells is abortive. When hamster cells growing in monolayer cultures are infected with Ad12 at low or at high multiplicity, there is a steady decline of infectious virus with time after infection. BHK21 cells continue to shed Ad12 particles into the medium for at least 68 hours post infection. Since, the Ad12 titer does not increase at any time after inoculation, Ad12 does not complete its multiplication cycle in this cell line (Doerfler, 1970).

Binding, entry and movement of Ad12 to the nucleus appear normal in BHK21 cells. Independent studies on the efficiency of Ad12 binding to hamster cells showed that approximately 0.03% to 22.5 % of prelabeled virus inoculum remained associated with BHK21 cells (Doerfler and Lundholm, 1970; Zur Hausen and Sokol, 1969). The wide range observed is probably due to differences in virus stocks used. Ad12 virions are readily detected by electron microscopy in the cytoplasm. Approximately, 43% of cell-associated Ad12 DNA can be found in the nuclear fraction of BHK21 cells 2 h after infection. This is in close agreement with results obtained in productive infection of Ad12 in KB cells (Doerfler et al., 1972). However, the amount of viral DNA in BHK21 nuclei decreases steadily with time after infection, and with increasing passage number of infected cells (Fanning and Doerfler, 1976).

Early Ad12 mRNA is synthesized from the same early regions expressed during productive infection. Although, most of the early Ad12 gene products can be synthesized *in vitro* when RNA from Ad12-infected hamster cells is used in cell-free translation experiments (Esche, et al., 1979; Raska and Strohl, 1972), there are apparent defects in early expression. For example, a 34

kd protein encoded in the E1A region of Ad12 DNA cannot be translated. It is not known, whether this is due to failure of the corresponding mRNA to be expressed or to defects in a translation step (Esche *et al.*, 1979). Moreover, the amount of Ad12 E1A tumor (T) antigen in BHK cells is approximately 10-15 % of that synthesized in KB cells as revealed by the use of anti-peptide antibodies. Also, E1A T antigen synthesis in BHK cells is delayed and starts 12 hour post infection as opposed to 6 hr in KB cells. The synthesis of the E1B proteins is also decreased in this system. In particular, the synthesis of E1B 163R antigen is only 5-10% of that detected in KB extracts (Lucher, 1990). The decreased levels of E1A and E1B proteins are not due to differences in protein turnover, since E1A protein stability as determined by pulse-chase immunoprecipitation is very similar in infected KB and BHK cells (Lucher, 1990). Reduced E1 protein synthesis could be attributed to the defects in associated mRNAs, since slot blot northern analysis indicates that E1A and E1B total RNA concentrations are reduced in abortive infection (reviewed by Lucher, 1995). It is not known, however, whether this reduction is a result of decreased transcription or increased RNA turnover in BHK cells.

The levels of early E2 proteins expression are also affected during Ad12 infection of BHK21 cells. The concentration of a DNA binding protein (DBP), for instance, is 120-fold lower in extracts from infected hamster cells when compared to infected permissive KB cells. This difference is not a consequence of replication of viral DNA during productive infection, since this difference is detected in the presence of a DNA synthesis inhibitor, hydroxyurea. *In vitro* studies show that DBP isolated from abortive infection maintains the ability to bind the denatured DNA-cellulose. In addition, BHK297-C131 cells (Ad5-transformed carrying the left 18.7% of the Ad5 genome and express E1 proteins constitutively) that complement the Ad 12 DNA replication defect also stimulate accumulation of the DNA-binding protein even when the cells are treated with hydroxyurea. Thus, the low concentration of DBP in BHK21 may interfere

with DNA replication. In contrast, the expression of other E2 products that are necessary for a successful Ad DNA synthesis, Adp1 and pTP, appear to be normal in BHK cells (Lucher, Khuntirat et al., 1992). To date, nothing is known about the synthesis of Ad12 E3 or E4 proteins during Ad12 infection of BHK cells. However, since E3 genes are not required for viral multiplication in culture, any defect in E3 expression may be irrelevant. On the other hand, it would be of interest to investigate the level of expression of E4 proteins in abortive infection, since these are involved in regulating transcription and DNA replication.

BHK21 cells do not allow replication of Ad12 DNA during abortive infection by this virus. However, crude extracts of BHK cells abortively infected with Ad12 support *in vitro* formation of the Ad12 pTP-cCMP initiation complex. The synthesis of the Ad12 pTP-dCMP initiation complex by BHK extracts is two- to five-fold less than when crude infected human (KB) cell extracts are used in the reaction. Combining infected KB cytoplasmic and uninfected BHK nuclear extracts in the reaction suggests that the reduced reaction rate is not a consequence of the amount of Ad12 pTP and DNA polymerase since it appears to be normal, nor the presence of an inhibitor in BHK cells. Furthermore, the considerably reduced DBP concentration in BHK21 cytoplasmic extract does not affect the reaction. Rather, the decreased level of pTP-dCMP complex is probably due to a lesser ability of hamster nuclear extracts to support the initiation reaction (Lucher, 1990; Cowrira et al., 1991).

The effects of nuclear factors in this abortive infection have been investigated (Schiedner and Doerfler, 1996). The binding affinity of NFI factor of BHK21 cells to the *ori* of Ad12 DNA appears to be normal. However, NFIII of BHK21 cells has a lower affinity for the *ori* of Ad12 DNA than for the *ori* of Ad2 DNA and the levels of NFIII in BHK21 cells are markedly reduced compared with the levels in permissive human KB cells or complementing BHK297-C131 hamster cells. These deficiencies are possibly contributing factors for the abortive infection of

BHK21 hamster cells with Ad12. The lack of sufficient levels of NFIII in BHK21 cells is also consistent with the decreased replication capacity of Ad2 in hamster compared with human cell lines (Schiedner *et al.*, 1996).

Transcription of late Ad12 genes in infected hamster cells is defective. Both L1 and VA RNA are not detected as revealed by Northern blotting and hybridization experiments (Juttermann *et al.*, 1989). Ad12-specific L1 and VA RNAs are not synthesized even after the Ad2-transformed cell line BHK-Ad2E1 is infected with Ad12. This implies that the available E1A viral functions by themselves are obviously not sufficient for the transcription of L1 and VA RNAs.

The late transcriptional defect of Ad12 DNA in hamster cells has been attributed to the viral MLP. The MLP promoter is not functional in Ad12-infected BHK21 cells or Ad5-transformed hamster cells (Weyer and Doerfler, 1985). There is evidence for the presence of a mitigator element in the first intron following the Ad12 MLP. Removal of this sequence from the Ad12 MLP-CAT plasmid increases the basal activity of the plasmid in uninfected BHK21 and HeLa during transient expression (Zock and Doerfler, 1990). More importantly, in the absence of the mitigator, the Ad12 MLP can be activated in BHK21 by Ad12 infection as well as by Ad2 infection, using the authentic transcription initiation site. In conclusion, the mitigator somehow blocks proper interaction among Ad12 proteins, BHK21 transcription proteins, and Ad12 DNA to support efficient transcription of the MLP. The Ad2 MLP does not appear to have an analogous mitigator element.

Complementation experiments have demonstrated the importance of the E1 region in the mechanism of Ad12 abortive infection. Ad12 DNA replication is supported in BHK21 cells when coinfecting with either Ad2 or Ad5 (Klimkait and Doerfler, 1985). Ad12, however, does not yield viral progeny in these coinfection experiments. In the Ad5-transformed cell line BHK21-C131, both Ad12 L1 and VA RNA segments are stably transcribed (Jutterman *et al.*,

1989), however, these functions fail to be expressed in the Ad2-transformed hamster cell line BHK-Ad2E1. This suggests the involvement of cellular functions in abortive infection. Coinfection of baby hamster cells with Ad12 and Ad5 *dl312* (E1A-minus) or *dl313* (E1B-minus) mutants shows the relative contributions of E1A and E1B to Ad5 complementation (Doerfler and Klimkait, 1987). Ad5E1A does not complement Ad12 DNA replication in BHK21, whereas Ad5E1B does. Nevertheless, hamster cell lines transformed by and expressing the Ad5 E1B region alone do not support Ad12 DNA replication. These data also suggest that the concerted action of more than one group of early Ad5 functions is essential for Ad12 complementation in hamster cells. During complementation, the Ad5E1 may act directly or indirectly on the Ad12 genome. Directly, it can substitute for Ad12 E1 proteins. Ad12 recombinants in which the E1A and/or E1B are replaced with Ad5 E1A and/or E1B are viable in human cells (Sawada *et al.*, 1988), confirming that Ad5 E1A and E1B proteins can act directly on Ad12 DNA. Indirectly, Ad5E1 may render the hamster environment suitable for the proper interaction of the Ad12 E1 proteins with the viral DNA and the required cellular components. To date, there is no clear evidence of such action.

Early after infection of hamster cells with Ad12, integration of Ad12 in cellular DNA has been observed (Doerfler, 1970). Cellular specific DNA sequence for Ad DNA integration has not been found (Doerfler *et al.*, 1983); however, Ad DNA exhibits a preponderance in insertional recombination at sites of cellular transcriptional activity (Gahlmann *et al.*, 1982; Schulz *et al.*, 1987). The significance of DNA integration for abortive infection is not known.

1.6.1.2 Ad 40 infection of HeLa cells

The fastidious enteric Ad type 40 is found in stool specimens of infants and young children showing symptoms of gastroenteritis. Ad40 differs from viruses of the other Ad serotypes with respect to its ability to grow in tissue culture. While, most human Ads grow well in HeLa cells, HeLa cells are considered to be nonpermissive for the replication of Ad40 (de Jong *et al.*, 1983).

Ad40 can be successfully propagated in 293 cells that constitutively express E1 proteins of Ad5 and KB-derived cell KB18 and KB6 that express E1B proteins only. Upon infection of KB18 which expresses E1A only, Ad40 does not grow successfully (Mautner *et al.*, 1990). It could be surmised from these experiments, that the early defect in Ad40 infection of HeLa cells involves the E1B region but not the E1A region.

Mutants of Ad2 and Ad12 with lesions in E1B 55K or 19K protein have been used to further underline the requirements for Ad40 growth in HeLa cells. Complementation is achieved by E1B 55K mutant alone, but not 19K alone (Mautner *et al.* 1990). These results show how Ad40 behaves as a DNA replication-negative E1B 55K mutant and emphasize the regulatory role of the E1B 55K protein in DNA replication.

The failure of the Ad40 to replicate well in HeLa cells is attributed to the control of E1B gene transcription early in infection. Ad40 E1B mRNA is not detectable in cytoplasmic RNA isolated at early times following infection of either nonpermissive HeLa or permissive KB16 cells (Mautner *et al.* 1990). Since E1 RNA is not found to accumulate in the nucleus at early times following infection, the defect is not likely to be in RNA transport. At late times postinfection, however, both the 22s mRNA encoding 55K and 19K proteins, and the 13s mRNAs encoding the 19K protein, are both easily detectable by northern blot analysis in KB16 cells. However, only 19K proteins are detected in KB16 cells by anti-peptide antisera at late times postinfection. The lack of early Ad40 E1B mRNA even during complementation implies that complementation in

293, KB16 is most likely due to substitution of the heterologous E1B for the Ad40 E1B early during infection.

1.6.1.3 Ad41 infection of human embryo fibroblasts (HEF)

Ad40 can successfully propagate in A549 cells which do not have E1B genes (Hashimoto *et al.*, 1991). E1B mRNAs are detectable in A549 cells early in infection as revealed by dot blot analysis. These results suggest the presence of cellular factors in A549 cells that can enhance Ad40 E1B gene expression and/or can help Ad40 DNA replication in the absence of the Ad E1B 55K protein. This further supports the evidence that Ad40 cannot propagate in HeLa cells because of the failure in E1B gene expression at early times postinfection.

In similar manners to its counterpart Ad40, Ad41 cannot be propagated in cell lines that permit the efficient replication of other human Ads. In one of those cell lines, namely, human embryo fibroblasts (HEF), Ad41 undergoes abortive infection due to early block in viral DNA synthesis (Tiemessen & Kid, 1988). Upon coinfection with permissive Ad2, Ad41 DNA synthesis is repaired in HEF cells (Tiemessen *et al.*, 1996). The analysis of Ad41-specific transcripts produced following infection of HEF cells revealed that the only gene region to be transcribed is that of E1 (0-12 m.u.). These results suggest that the E1 region is not capable of transactivating expression of other early genes. Further analysis is required to determine whether this block is due to E1A and/or E1B-specific processes (Tiemessen *et al.*, 1988).

1.6.1.4 Ovine and canine Ad infection of human cells.

Ovine Ad (OAV), which is phylogenetically distinct from the *Mastadenoviruses* and *Aviadenoviruses* undergoes abortive infection in a range of human cell types including lung and foreskin fibroblasts as well as liver, prostate, breast, colon, and retinal lines (Khatri *et al.*, 1997). OAV is unable to attach and internalize only in two cell lines, LNCaP (prostate cancer) and 293.

The attachment and internalization of OAV does not account for the abortive infection in the rest of the human cells tested. This is surprising since OAV has a fiber protein with a unique cell binding domain and a penton protein that lacks the integrin-binding Arg-Gly-Asp motif known to facilitate entry by human Ads. The intracellular block is early at the level of viral DNA synthesis in these cell lines. OAV promoter activity in various human cell types was assayed by RT-PCR. Early promoter activity was detectable in some, but not all cell types. For example, early promoters on the left and right-hand ends (LHP1, LHP2, RH) of the genome as well as those for E2 and E4 were all active in MRC-5 (human lung fibroblasts). None of the promoters analyzed was active in HepG2 cells (liver carcinoma) and only the LHP2 (identical to E1A/E1B region of other Ads) promoter was active in HT29 cells (human colon carcinoma). In addition, major late promoter activity was not detectable in any cell types. The lack of DNA replication and MLP function suggests that a critical transition from early to late gene expression does not occur during abortive infection by OAV (Khatri *et al.*, 1997).

Infection of HeLa, KB and 293 cells by canine Ad type 2 (CAV-2) is nonpermissive. Viral DNA synthesis does not occur at 10 moi and even at high moi the level of DNA replication is hardly detectable. Moreover, CAV-2 is not able to generate new viral particles at any moi tested in these cells. Further studies are needed to determine which viral transcripts are impaired in CAV2-human cell system (Klonjkowski *et al.*, 1997).

1.6.2 Late block

1.6.2.1 Ad2 infection of monkey cells

Ad2 infection of CV 1 monkey cells is an example of host restriction during the late phase of infection. Synthesis of early mRNA species from all five major early regions (E1A, E1B, E2, E3 and E4) appears normal in this system. Nevertheless, transcription of late virus-associated RNA is greatly affected. Although production of VA RNA is identical in productive and abortive infections of monkey cells, synthesis of VA is significantly greater in HeLa cells than in CV1 cells (Anderson *et al.*, 1985). There are at least three factors believed to contribute to the reduced concentration of late VA, in this virus-cell interaction. Firstly, the rate of RNA transcription from the Ad2 MLP is reduced four- to ten-fold and correlates with reductions in steady-state levels of cytoplasmic RNA. Secondly, reduction in rates of nuclear RNA synthesis could be attributed in part to premature termination of transcription from the major late promoter. Although, transcription from the major late promoter of Ad2 prematurely terminates in both monkey and human cells, it is greatly enhanced in abortive infections of monkey cells (Johnston, *et al.*, 1985). Thirdly, Ad infection of monkey cells results in a more efficient attenuation of RNA initiated 182-188 nucleotides downstream from the MLP transcription start site (Seiberg *et al.*, 1989). Interestingly, Ad with mutations in DBP that binds to the attenuated RNA relieve this restriction. The detailed mechanism of the role of DBP in abortive infection of monkey cells is still unclear.

Synthesis of fiber protein in CV1 cells abortively infected with Ad2 is at least 100-fold less than the synthesis of fiber protein in CV1 cells productively infected with a host range mutant of Ad2 (*Ad2_{hdr400}*). However, the amount of fiber mRNA present in the cytoplasm of abortively infected CV1 cells is only five- to ten-fold less than that in productively infected CV1 cells (Anderson and Klessig, 1983). The reduction in fiber protein synthesis is most likely due to

translational defects. In productive infection, 10-25% of the fiber mRNA contain one of the ancillary leaders *x*, *y*, or *z*, in addition to the common tripartite leader found in all late mRNA. In abortive infection little or no *x* or *y* leader is found on fiber mRNA. The absence of the *y* leader decreases translation initiation twofold in abortively infected cells, but the elongation rate during translation of all fiber mRNA is decreased about threefold, a reduction that is specific for fiber (Silverman & Klessig, 1989). The late block to Ad2 multiplication in monkey cells is repaired by coinfection with SV40 or by infection of SV40 large T antigen-expressing monkey cells (Klessig, 1984). Upon coinfection with SV40, normal levels of Ad2 late proteins are synthesized due to effects on late mRNA metabolism and Ad2 produces new viral progeny (Seiberg *et al.*, 1989).

1.6.3 Conditional Abortive Infections

1.6.3.1 Ad41 infection of WI-38 cells

Ad41 cannot be cultivated in primary human cell cultures due to blocked DNA replication. Initially, it was suggested that the lack of growth of Ad41 in human primary cells is the result of a defect in the Ad41 E1A region (Takiff and Straus, 1982). However, Pieniazek *et al.* (1990) demonstrated Ad41 DNA replication in human diploid lung fibroblasts (WI-38) when the serum concentration in the medium was dropped from 5%-10% down to 0.5%-1%, suggesting the presence of an inhibitory serum factor. The serum inhibitory effect seems limited to primary cells because Ad41 replication in 293 cells is not affected by differences in serum concentrations. Furthermore, this effect appears specific only for Ad41, since conventional Ad5, grows well in both 1 and 10% FBS. The mechanism of serum inhibition does not involve conditional defects in either E1A or E1B activity, since Ad41 can overcome the multiplication defects of coinfecting Ad5 *dl312* and *dl313* in the presence of 10% serum. In addition, Ad41 DNA replication remains defective in 10% serum during coinfection with *dl312* or *dl313*. The nature of the serum

inhibitor is not known, nor it is known why it affects only Ad41 multiplication in primary cells but no continuous cell lines.

1.6.3.2 Ad2 infection of human continuous lymphoid cell lines

Ad2 infection of human continuous T cell line MOLT-3 and a B cell line Raji is another example of conditional abortive infection (Silver and Anderson, 1988). Almost all MOLT-3 and Raji cells exposed to Ad2 bind virus, but no more than 5% of the cells show evidence of infection by DBP or hexon immunofluorescence. Most of the absorbed virus remain at the cell surface. In synchronized cell populations, the percentage of cells positive for DBP immunofluorescence and negative for surface virion capping correlate very well with the percentage of mitotic cells. Since receptor capping is suppressed during mitosis (Bourguignon *et al.*, 1983) only mitotic lymphoid cells appear to be susceptible to Ad2 infection. In conclusion, the block in resting human lymphoid cells is very early, disrupting internalization of the bound Ad2, and conditional, being restored at mitosis.

1.7 Applications of abortive infection

Studies on Ad abortive infections can provide us with many answers about the biology of Ads and viral-cellular factors interactions required for successful Ad multiplication. For example, the extensively studied abortive infection of BHK21 cells by Ad12 offers insights into the roles of E1 proteins in infection and the limitations of viral replication due to functional restrictions on a viral promoter. In addition, as exemplified by Ad40 infection of HeLa cells, the roles of E1B proteins in regulating viral DNA replication may be uncovered. Understanding the late block in Ad2 infection of African monkey cells may provide some insights in transcription attenuation, translation and involvement of DBP in Ad infection. Also, conditional abortive infection of WI-38 cells by Ad 41 may identify serum factors affecting growth of Ads in cell culture. Moreover,

identification of intracellular blocks in cross-species infections provides an opportunity to study the evolution of Ads. Analysis of abortive infections is however not only restricted to understanding Ad biology; it can be also used to study biology of mammalian system. For example, integration of Ad12 during abortive infection of BHK21 cells is an attractive model for investigating mechanisms of foreign DNA integration in mammalian cells, functional consequences of DNA methylation in eukaryotic system and oncogenicity by DNA viruses.

Abortive infection is also important in considering the use of recombinant Ads as vaccine and gene delivery vectors. The following sections will review the current strategies and limitations in adenoviral gene therapy and vaccine development, and discuss the ways in which these limitations can be partially resolved by analysis and application of nonpermissive infections.

1.7.1 Vaccine development

Ads have been shown to be excellent candidates for constructing recombinant viral vaccines (Berkner, 1988; Graham, 1990; Graham & Prevec, 1992). Most of the research on use of Ads as vaccines has been primarily focused on the use of human Ad type 5 and type 2 being the most extensively studied Ads. To generate recombinant viruses, foreign genes are inserted into the Ad genome at either E1 or E3 regions (Haj-Ahmad & Graham, 1986; Ghosh-Choudhury *et al.*, 1987). Since E3 appears to be non-essential for viral multiplication in both cell culture (reviewed by Tooze *et al.*, 1981) and animal models (reviewed by Ginsberg *et al.*, 1989), this region is used for foreign gene insertion to generate replication-competent recombinant viruses for use as live recombinant virus vaccines. Many antigenic proteins have been cloned into Ad vectors including hepatitis B surface or core antigen (Morin *et al.*, 1987), polyoma middle T antigen (Bernker *et al.*, 1987), HIV-1 env, gag, or p24 proteins (Dewar *et al.*, 1989), glycoproteins such as the gB from herpes simplex virus (McDermott *et al.*, 1989), the respiratory syncytial F protein (Collins *et al.*, 1990) and glycoprotein D from rabies virus (Prevec *et al.*, 1990). Many of these vectors

have proven to be effective in conferring both humoral and cellular immunity against above mentioned pathogens in experimental animals.

1.7.1 Gene therapy

The genome structure and biology of human Ads offer a number of advantages for use as recombinant vectors in gene therapy. These viruses can be easily manipulated and generated in high titers for *in vivo* gene therapy but do not integrate into the genome of infected cells (reviewed by Shenk, 1996). Moreover, human Ads can infect a wide variety of dividing and quiescent cells from various organs and are able to accommodate relatively large foreign inserts. Most current recombinant vectors are generated by inserting the therapeutic genes in place of the essential E1 genes. Such vectors are therefore replication-deficient in the host but can be produced at high titers using appropriate complementing cell lines such as 293 (reviewed by Douglas and Curiel, 1997). Various recombinant Ad vectors have been produced for the development and evaluation of novel gene therapeutics to potentially treat inherited or acquired diseases. For instance, a recombinant Ad5 carrying the human cystic fibrosis transmembrane conductance regulatory gene (CFTR) has been undergoing evaluation in phase I trial that target cancer and cystic fibrosis (Crystal *et al.*, 1994; Yang *et al.*, 1994). Likewise, Ad vectors expressing the dystrophy gene are being evaluated for the treatment of neuromuscular diseases such as Duchenne Muscular Dystrophy (Haeker, 1996). Injection of these vectors into mice lacking dystrophy gene (mdx) mice induced a correction of the defective phenotype (Acsadi *et al.*, 1996; Floyd *et al.*, 1998). Hemophilia and hypercholesterolemia are also considered for treatment with Ad mediated *in vivo* gene therapy (Yao *et al.*, 1997). Moreover, Ads were used to derive the murine or human leptin gene for treatment of obesity in the obese (ob/ob) mouse model. This treatment is promising since the rate of weight loss and percentage satiety were significantly reduced in treated mice. Another disease, mucopolysaccharidosis type VII (Sly

syndrome) caused by inherited deficiency of the lysosomal enzyme β -glucuronidase is evaluated for treatment with Ad gene therapy in a mouse model (Ohashi *et al.*, 1996).

Despite all enthusiasm about the use of Ads in gene therapy, the current gene therapy protocols have revealed several limitations which seriously impair the success of genetic treatment with these recombinant viruses. Expression of therapeutic genes appear to be transient, lasting only three or four weeks (Yang *et al.*, 1995; Yang *et al.*, 1996). The short-lived *in vivo* expression of the transgene is related to the induction of innate and specific host immune response against the Ad antigen. Worgall and coworkers (1997) were the first to seriously address the involvement of innate immunity in the elimination of the Ad vectors. They reported that 90% of the recombinant viral genome is eliminated from the liver 24 hours after intravenous injection of the vector. This elimination is probably generated by the action of nonspecific liver macrophages (Kupfer cells) since the transient depletion of these cells partially enhanced both the persistence of the vectors and delayed their clearance. Despite the deletion of E1, the first generation Ad vectors were shown to still express late virus genes coding for structural proteins (Yang *et al.*, 1995; Yang *et al.*, 1996). This will generate cellular immune responses since newly synthesized proteins are presented by MHC class I molecules to CD8⁺ cells, which are activated to form cytotoxic T lymphocytes. The cytotoxic T cells destroy the vector-infected cells, resulting in loss of expression of the therapeutic gene and in inflammation. Also, CD4⁺ helper cells and B cells are activated by MHC class II presented proteins from the input virus, leading to production of neutralizing antibodies following primary exposure of the vector. This prevents the efficient readministration of the vector by binding to the virus and blocking its cellular entry (reviewed by Douglas *et al.*, 1998).

The current strategies to diminish clearing of Ad vectors by immune system include use of immunosuppressants such as FK506 (Lockmuller *et al.*, 1996) and use of monoclonal antibodies

such as anti-CD4 that block either the T cell receptor or costimulation pathways necessary for T lymphocytes activation (Yang *et al.*, 1996). In addition, in order to prevent expression of late viral proteins, an isogenic E3-deleted Ad vectors defective in E1 and E2A or E1 and E4 (Lusky *et al.*, 1998) were engineered. However, even in the absence of transgenes, the progressive deletion of the Ad genome does not extend the *in vivo* persistence of transduced cells and does not reduce antiviral immune responses.

1.7.2 Advantages of non-permissive vectors

The restricted host range of Ads can be advantageous in developing recombinant viral vaccines for a number of reasons. Firstly, the use of semi-permissive or non-permissive Ads as recombinant vaccines could provide means for decreasing or even eliminating adverse clinical effects when permissive viruses are used as vaccines. For example, a recombinant vaccine against rabies has been constructed by engineering Ad5 vector to carry the pseudorabies virus glycoprotein D. This vaccine has been tested in foxes and skunks (Charlton *et al.*, 1992). Although the Ad rabies vaccine replicated very poorly in these animals, the protective antibodies were generated within 4 weeks, without the need for boosters and redundant obvious pathology. The same vaccine has also proven highly effective in producing good levels of rabies-neutralizing antibodies in dogs and mice (Prevec *et al.*, 1990). In addition to Ad rabies vaccine, Ad7 recombinant vector carrying multiple hepatitis B antigens has been constructed and used in dogs (Ye *et al.*, 1991). The Ad hepatitis B vaccine was capable of eliciting antibody response to all antigens included in the delivery vector (Ye *et al.*, 1991). Recently, canine Ad type 2 (Klonjowski *et al.*, 1997), ovine Ad (Khatri *et al.*, 1997) and bovine Ad type 3 (Mittal *et al.*, 1995; Mittal *et al.*, 1996) have been considered as potential vaccine vectors in animals and humans. Since, these animal viruses are not known to be pathogenic in humans it would be

advantageous to use them as recombinant vaccine vectors. However, the capability of such animal vectors to confer immunity in humans still needs to be investigated.

Another potential application of Ad host restriction in recombinant viral vaccine development is in the use of nonpermissive Ads as subsequent boosters. Most current Ad vectors are made from Ad2 and Ad5 (Horwitz *et al.*, 1990). Patients who are seropositive for Ad2 and -5 (and other subsequent human-derived adenoviral vectors) could be excluded from benefiting from their potential use. The nonpermissive vectors would be more advantageous in these cases because they are nonpathogenic and because the host presumably does not have a preexisting immunity against nonpermissive agents. This was demonstrated by Klonjowski *et al.* (1997) who studied the potential use of canine Ad type 2 (CAV2) as a recombinant viral vaccine and gene therapy agent. The researchers assayed sera containing “exceptionally” high titers of Ad5 neutralizing antibodies for CAV neutralizing antibodies. At the lowest dilution tested (1/50), five of the seven samples tested were not able to significantly inhibit CAV2-induced plaques on permissive cells. The same five samples, on the other hand, were able to inhibit >95% of the recombinant Ad5 derived vector. In addition to the problem in preexisting immunity against Ad vectors, the initial immunization with these Ads produces antibodies against the Ad surface proteins that contain neutralizing epitopes. Hence, it is usually not possible to re-administer the same serotype and expect sufficient expression of the cloned insert (Yang *et al.*, 1995). The second Ad serotype carrying the identical foreign gene, to which the host is not immune, is then used as a booster. Hence, the antigenic diversity of a large number of nonpermissive Ads provides an extended pool of virus to be used as subsequent boosters.

1.8 Objectives of the present study

Our lab is interested in the potential use of bovine Ads (BAVs) as recombinant viral vaccines and gene therapy vehicles. Although foreign genetic material can be easily engineered into bovine Ads, many factors must be considered in determining their usefulness as viral vectors. These include host range, stability of the vector, site of replication and mode of replication. The development of bovine Ad vector entails a precise understanding of the state of the BAV genome in the infected human cells. The purpose of the present study is to determine the degree of permissivity of human HeLa cells for BAV2 growth and to identify what aspects of the viral replicative cycles are altered. In the work described in this thesis, we characterized BAV2 infected HeLa cells with respect to:

1. Viral attachment and internalization
2. Viral DNA synthesis
3. Early viral transcription
4. Activity of E1A promoter
5. Complementation of BAV2 defects in HeLa cells by coinfection with human Ad5
6. Persistence and stability of viral DNA

2. MATERIALS AND METHODS

2.1 Recombinant DNA techniques

2.1.1 Bacterial strain

2.1.1.1 Preparation of competent bacteria

Escherichia coli (*E. coli*) strain DH5- α (Gibco BRL) was used as host in various cloning experiments described in this study. Bacterial cultures were grown in Luria broth (LB per liter: 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, NaOH to pH 7.4). Cells were stored in liquid medium at 4°C for temporary storage, or in 15% glycerol at -70°C for extended periods of time.

An overnight inoculum of DH5- α was grown in 500 ml of LB broth at 37°C until the OD₅₅₀ was between 0.45-0.55. The culture was then transferred into 50cc tubes (50 ml/tube) and centrifuged @ 3500 rpm for 10 minutes at 4°C (IECCentra-8R centrifuge). The supernatant was discarded and 25 ml of sterile transformation buffer (75 mM CaCl₂ and 5mM Tris-HCl, pH7.5) was added to each tube. The tubes were incubated on ice overnight to make competent cells. The cells were collected again by centrifugation @ 3500 rpm for 10 minutes at 4°C. Supernatant was discarded and 1 ml of transformation buffer was added to each tube. Resuspended cells were frozen in 15% glycerol and stored in 100 μ l aliquots in small Eppendorf tubes at -70°C.

2.1.2 Plasmids

Several plasmids that were used in the present study were obtained from several sources (Table 3). Plasmids constructed in this study are described in detail in the Results section.

Table 3. List of plasmids used for cloning and other purposes in this study

<i>Plasmid Name</i>	<i>Reference</i>
<i>pCMV-β</i>	Clontech
<i>pdlE1-E</i>	FitzGerald, L. (1995)
<i>pBB12</i>	Salmon, K. (1994)
<i>cBAV-10</i>	Ojkic, D. (1998)
<i>pE1AlacZ</i>	Bautista, D.S. (1989)
<i>pJM17</i>	McGrory, WJ <i>et al.</i> (1988)

2.1.3 Enzymes

All enzymes used for cloning purposes were obtained from New England Biolabs (NEB) and were stored in 50% glycerol at -20°C.

2.1.3.1 Restriction Enzymes

A number of restriction enzymes was used in this study. Restriction enzyme digests were carried out as suggested by manufacturer of the enzymes. Reaction volume and the amount of enzyme varied depending on the DNA purity, DNA concentration and type of enzyme used. Generally, digestions were carried out at 37°C for 2 hours or more. Restriction enzymes were inactivated by heat inactivation or phenol extraction depending on the type of the enzyme used.

2.1.3.2 Klenow Fragment of DNA polymerase I

Klenow Fragment of DNA polymerase I was used to fill-in 3' recessed ends produced by some restriction enzymes. The typical reaction mixture contained DNA at a concentration of 50 µg/ml, in one of the four standard NEB buffers (1X) supplemented with 33 µM each dNTP and 1

unit of Klenow per μg DNA. The reaction was carried out at room temperature for 15 minutes. Klenow was inactivated by incubation at 75°C for 20 minutes.

2.1.3.3 Alkaline Phosphatase

Alkaline phosphatase (Calf Intestinal - CIP) removes 5' and 3' phosphoryl groups from nucleic acids and it was used in cloning procedures to prevent the self-ligation of vector DNA. The reaction mixture contained vector DNA at a concentration of $0.5\mu\text{g}/10\mu\text{l}$ in one of the four standard NEB buffers and 1.0 unit CIP per pmol DNA ends. The reaction was incubated at 37°C for 60 minutes. CIP was inactivated by phenol extraction and the DNA was recovered by alcohol precipitation.

2.1.3.4 T4 DNA Ligase

T4 DNA ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA. This enzyme was used for joining blunt-end and/or cohesive-end termini of vector and DNA fragments that were used in this study. Ligation was carried out in $20\mu\text{l}$ volume with appropriate ratio of vector:fragment ratio, 1X reaction buffer (50 mM Tris-HCl, 10 mM MgCl_2 , 10mM dithiothreitol, 1 mM ATP, $25\mu\text{g}/\mu\text{l}$ bovine serum albumin pH=7.5) and 100 units of T4 DNA ligase. The reaction was incubated at 16°C overnight.

2.1.4 Transformation of competent bacteria

About $100\mu\text{l}$ of a competent bacterial suspension was used for transformation with ligation reactions not exceeding $20\mu\text{l}$ volumes. When frozen cells were used, the cells were thawed on ice. DNA was added to the competent cells and incubated on ice for 30 minutes. To facilitate uptake of DNA, cells were heat-shocked at 42°C for 45 seconds and immediately chilled on ice

for 3 minutes. Then, 900 μ l of SOC medium (2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) was added to the cells and incubated in 37°C water bath shaker for 45-60 minutes to allow expression of antibiotic resistance genes. Aliquots were plated on LB agar plates that contained ampicillin and X-gal. The plates were then incubated upside down at 37°C overnight.

2.1.5 Selection

LB agar was made by adding 1.8% agar (Difco) to LB broth and autoclaving to sterilize. After the agar was cooled down to 50°C, 50 μ g/ μ l of ampicillin and 60 μ g/ μ l of Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was added. Agar was poured onto petri dishes (30 ml/dish), solidified and stored at 4°C.

2.1.6 Small-scale plasmid DNA preparations

Small-scale plasmid DNA preparations were carried out by the modified alkaline SDS lysis technique of Birnboim and Doly (1978). Individual colonies were picked with a sterile toothpick and were used to inoculate tubes containing 2 ml of LB broth and ampicillin. The tubes were incubated in a 37°C water bath shaker overnight. Then, 1.5 ml of overnight cultures were centrifuged for 30 seconds in a microcentrifuge @ 12,000 rpm (Sorval® MC12V). The supernatant was removed by vacuum aspiration and the pellet was then resuspended in 100 μ l lysing buffer (10mM EDTA, 50mM glucose, 25mM Tris-HCl and 2mg/ml lysozyme, pH8.0). The tube was incubated for 10 minutes at room temperature then 200 μ l of alkaline SDS (1% SDS and 0.2N NaOH, mixed fresh from concentrated stocks of SDS and NaOH) was added and mixed gently by inverting the tube several times. After incubation at room temperature for 5 minutes the mixture was neutralized by adding 150 μ l of 3M sodium acetate (pH4.8) which precipitates cellular components and denatured macromolecules. The sample was placed on ice

for 15 minutes and then centrifuged in a microcentrifuge @ 12,000 rpm for 5 minutes. The supernatant was transferred to a fresh tube to which two volumes of cold 95% ethanol was added. After incubation at -20°C for 10 minutes the mixture was centrifuged at 12,000rpm for 5 minutes. The ethanol was removed by aspiration and the nucleic acid pellet at the bottom of the tube was dissolved in 200 µl of autoclaved distilled water. DNA was precipitated for a second time by adding 500 µl of cold 95% ethanol and again immediately centrifuged. The pellet was dried for 5-10 minutes at 37°C and dissolved in 100 µl of autoclaved TE buffer (10mM Tris-HCl, 1mM EDTA; pH8.0) and stored at 4°C or -20°C.

2.1.7 Large-scale plasmid DNA preparations

Large-scale plasmid DNA preparations were carried out by using Promega Maxi-Prep Wizard DNA purification kit. A 500 ml overnight culture was pelleted by centrifugation at 5,000 g for 10 minutes at room temperature (Beckman Avanti™ J-25). The resulting cell pellet was resuspended in 15 ml of cell resuspension solution (50 mM Tris-HCl, 10 mM EDTA, 100mg/ml RNase, pH7.5). The cells were lysed by adding 15 ml of cell lysis solution (0.2 M NaOH, 1% SDS). The solution was mixed gently by inverting the tubes. After the solution became clear and viscous 15 ml of neutralization solution (1.32 M potassium acetate pH4.8) was added, mixed and centrifuged at 14,000g for 15 minutes at room temperature. The supernatant was filtered through filter paper (Whatman #1) and then transferred to a centrifuge bottle. Then, 0.5 volume of isopropanol was added, mixed by inversion and centrifuged at 14,000g for 15 minutes at room temperature. The supernatant was discarded and the DNA pellet resuspended in 2ml of TE buffer. In order to purify the plasmid DNA, 10 ml of Wizard Maxipreps DNA Purification Resin was added to the DNA solution, mixed and transferred into a Maxicolumn. Vacuum was applied to pull the resin/DNA mix into the column. 25 ml of column wash solution (80 mM potassium acetate, 8.3 mM Tris-HCl [pH7.5], 40 mM EDTA in 55% ethanol) and 5 ml of 80% ethanol were

passed through the column respectively. The column was centrifuged at 1,300g for 5 minutes at room temperature to remove ethanol and column wash from the DNA/resin mix. Then, 1.5 ml of preheated (65°-70°C) TE buffer was added to the column and incubated for 1 minute. The DNA was eluted by centrifugation at 1,300g for 5 minutes. In order to completely remove the resin, the eluate was filtered through a 0.2 mm filter into a clean tube. Plasmid DNA preparations were stored at 4°C.

2.1.8 Purification of DNA fragments from agarose gels using GeneClean

GeneClean was carried out according to the supplier's recommendations (Bio 101 Inc). Restriction enzyme digested DNA fragments were separated on low melting agarose gels. The desired DNA band was excised from an ethidium bromide-stained agarose gel with a razor blade. The gel slice was then transferred to a plastic tube to which 3 volumes of NaI stock solution was added. The tube was placed in a 45°-55°C water bath and incubated for about 5 minutes or until the agarose gel was completely dissolved. 5 µl of GLASSMILK suspension was added to solution containing 5µg of less of DNA and incubated on ice for 5 minutes. The tube was mixed every 1-2 minutes to keep GLASSMILK in suspension. The silica matrix with the bound DNA was centrifuged in a microcentrifuge for 5 seconds. The supernatant was aspirated off and the pellet was washed by adding 300 µl of NEW WASH. The tube was centrifuged for 5 seconds and the pellet was washed with NEW WASH two more times (Sorval® MC12V). To elute the DNA from GLASSMILK the washed pellet was resuspended in 20 µl of TE buffer or water. After 5 minutes of incubation at 45°-55°C, the tube was centrifuged for 50 seconds and the supernatant was placed to a new tube. Eluted DNA was kept at 4°C or -20°C.

2.2 Cell culture

2.2.1 Cell lines

HeLa is the first aneuploid, epithelial-like cell line to be derived from human tissue and maintained continuously by serial cell culture. It was isolated from a carcinoma of the cervix of a 31 year old Negro female (Grey *et al.* 1952). Human papillomavirus types 16 and 18 sequences are integrated in HeLa genome (Yee *et al.*, 1985; Pater *et al.*, 1985). Since its origin, HeLa has been one of the most widely studied cell lines. It is used in studies on cytotoxicity, cell biology, transformation, bacterial invasiveness, tumorigenicity and virology. In addition HeLa cells are used for antitumour testing.

MDBK (Mardin & Darby bovine kidney cells) were derived from a kidney of an apparently normal adult steer (Mardin *et al.*, 1958).

293 cell line is a permanent line of primary human embryonal kidney transformed by sheared human Ad type 5 DNA. The cells are particularly sensitive to human Ad, are highly permissive for Ad DNA, and contain and express E1 genes of Ad5 (Graham, 1977). The line has been used in the isolation of transformation defective host range mutants of Ad5 and is excellent for titrating human Ads.

2.2.2 Propagation and subcultivation

Cell lines were grown on modified Eagle medium (MEM) supplemented with 10% donor bovine sera or fetal calf sera (Cansera, Rexdale, Ontario), 0.29 mg/ml L-glutamine, 0.225% sodium bicarbonate and antibiotics [10,000 units/ml penicillin, 25mg/ml amphotericin B and 10,000 mg streptomycin (Gibco BRL)].

All the cells were passaged twice a week when 80-90% confluency was reached. The medium was removed by aspiration and the cells were washed with phosphate buffered saline

once (137 mM NaCl, 2.6 mM KCl, 8.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.5 mM MgCl₂, 0.7 mM CaCl₂). 2 ml of versene (1.4M NaCl, 8.8 mM EDTA, 26 mM KCl, 15mM KH₂PO₄, 15 mM Na₂HPO₄, 0.2% glucose (w/v)) and 0.5 ml of trypsin (Gibco, BRL) were added to the 120x60 mm dish and incubated at 37°C until the cells in culture partially detached from the bottom of the dish. After tapping the side of the dish to assist in complete detachment, cells were immediately suspended in the solution. Cells were then transferred to a sterile 25cc tube and centrifuged @ 1,000 rpm for 5 minutes (IEC Centra-8R Centrifuge). The supernatant containing the proteolytic enzyme solution was aspirated, and finally proper dilutions were made to be distributed to fresh petri dishes containing 15 ml of medium supplemented with appropriate serum and antibiotics.

2.2.3 Cell freezing and thawing

Cell freezing was carried out as outlined by Freshney (1994). Cells were grown up to late log phase, detached, counted and centrifuged @ 1000 rpm for 5 minutes (IEC Centra-8R Centrifuge). Cells were then resuspended at approximately 5×10^6 - 2×10^7 cells/ml in fetal bovine or donor serum containing 10% of dimethyl sulfoxide (DMSO). Cell suspensions were dispensed into 1-2-ml prelabeled plastic ampules with screw caps. These were then placed at -70°C for up to 1 week after which they were transferred to liquid nitrogen.

Cells were thawed by incubating in a 37°C waterbath. When thawed, they were transferred to a 120x60 mm plate containing medium supplemented with 10% serum. To remove toxic DMSO, the medium was changed as soon as cells attached to the plate.

2.3 Virus culture

2.3.1 Preparation of virus stock

Type of adenoviruses used in this study are shown in *Table 4*. Virus stocks were prepared as outlined by Murray (1991). Confluent cell monolayer in 120x60 mm plates was infected with virus at moi of 0.1-1.0 PFU/cell. The plates were then placed in 37°C incubator for 60 minutes to allow for virus adsorption. Unadsorbed virus was aspirated, MEM was added, and the plates were incubated at 37°C. Upon completion of cytopathic effect cells were scraped off the plates and centrifuged for 10 minutes @ 1,500 rpm (IEC Centra-8R Centrifuge). Supernatant was removed and the cell pellet was resuspended in 0.5 ml PBS²⁺ + 10% glycerol/plate. Cells were alternatively frozen in liquid nitrogen and thawed in a 37°C water bath three times and centrifuged again @ 1,500 rpm for 10 minutes. Supernatants containing concentrated adenovirus were stored at -70°C.

Table 4. Adenovirus serotypes used in this study and cell lines adopted for their propagation

<i>Adenovirus serotype</i>	<i>Cell line for Ad growth</i>
<i>Bovine adenovirus type 2 (BAV2)</i>	<i>MDBK</i>
<i>Human adenovirus type 5 (Ad5dlE3)</i>	<i>HeLa</i>
<i>Ad5 E1 deletion mutant (Ad5dlE1E3)</i>	<i>293</i>

2.3.2 Plaque assay

A modified plaque assay (Murray, 1991) was used to determine the number of infectious Ad particles. MDBK and HeLa cells were plated in two 6-well plates (35x10 mm) at a density of 10^5 cells/ml or 3×10^5 cells/ well and placed at 37°C in the CO₂ incubator for 24 hours or until the cell monolayers reached 80-90% confluency. Serial virus dilutions (10^{-5} - 10^{-8}) were prepared in PBS²⁺ in a total volume of 1 ml. The medium in the 6 well plates was aspirated and the cells were washed 2 times with sterile 1xPBS (1 ml/wash). Then, 0.2 ml of each dilution was added to triplicate wells of each plate. Plates were placed at 37°C in the CO₂ incubator for 1 hour to allow for virus adsorption. To prevent monolayer desiccation and for even virus distribution plates were tilted at 10 minute intervals. Unadsorbed virus was then aspirated off and the cells were washed with 1XPBS. Then, 3 ml of overlay containing one volume of 2xMEM supplemented with 1.2 mM MgCl₂ and 4% serum, and one volume of 1.2% noble agar) was added to each well. Following agar solidification the plates were incubated at 37°C.

When plaques became visible the monolayer was fixed by adding 1ml/well of 10% formalin for 30 minutes. The overlay was then removed and the cells washed with tap water. The cell monolayer was stained by adding 1 ml/well of 1% crystal violet for 30 minutes. The excess crystal violet was removed and plaques counted.

In order to estimate the virus titer the dilution with a good number of discrete countable plaques (20-80) was selected. The mean number of plaques for the three monolayers at that dilution was multiplied by the reciprocal of the dilution and the reciprocal of the volume added. The value was expressed as number of plaque forming units (pfu) per ml.

2.4 Viral DNA and RNA manipulations

2.4.1 Extraction of viral and cellular DNA

First, the medium was removed by aspiration and the plates were washed with 1 X PBS. Cells were then trypsinized and washed three times with 1XPBS. Cells were lysed by the addition of a solution containing 0.01 M Tris-HCl pH7.5, 0.1 M EDTA and 0.4% SDS. Cells were then transferred to a 15cc tube, proteinase K was added at 100 µg/ml and incubated at 37°C overnight. The DNA was then extracted with phenol, 1:1 phenol-chloroform and chloroform, respectively. DNA was precipitated by the addition of 0.3 M sodium acetate (pH7.8) and 2 volumes of 95% ethanol. Finally, DNA was washed with 70% ethanol and resuspended in an appropriate volume of TE buffer.

Approximate DNA concentration was determined by UV absorption at 260 nm. However, since cellular DNA preparations could contain a considerable degree of RNA contamination the absolute DNA concentration was measured by the diphenylamine reaction.

2.4.2 Extraction of nuclear DNA

Extraction of nuclear DNA was carried out as outlined by Stott (1991). First, the cells were washed in 1XPBS and harvested with trypsin. Then, cells were pelleted by spinning for 5 minutes at 1000-1500 rpm in a bench-top centrifuge (Sorval® MC12V) and resuspended in 20 ml ice-cold nuclear isolation buffer (10 mM Tris-HCl pH7.4; 10 mM NaCl; 5mM MgCl₂; 1mM dithiothreitol; 0.5% Nonidet P-40). The resuspended cells were then incubated on ice for 10 minutes. This step causes lysis of the plasma membrane, leaving the nucleus intact. The nuclei were pelleted by centrifuging at 2,000 rpm at 0°C for 5 minutes (IEC Centra-8R Centrifuge). Nuclei were then washed two to three times in ice-cold nuclear isolation buffer by resuspending.

gently and centrifuging as outlined above. DNA was then isolated from prepared nuclei by the same procedure outlined in section 2.4.1.

2.4.3 Diphenylamine Assay

A modified diphenylamine assay (Giles and Myers, 1965) was used to determine the absolute concentration of DNA stocks. Standard curve was established by a simultaneous assay with known amounts of herring sperm DNA. Therefore, a set of standard solutions containing 0 - 10µg of herring sperm DNA, and approximately 1-5µg of unknown samples (estimated by measuring OD₂₆₀) were diluted in double distilled water to a final volume of 100 µl in 1.5 ml Eppendorf tube. Immediately after, the following reagents were added sequentially: 100 µl of 20 % perchloric acid (v/v in ddH₂O), 200 µl of 4% diphenylamine (w/v in glacial acetic acid) and 20 µl of 0.16 % acetaldehyde (v/v in ddH₂O). The tubes were vortexed and incubated at room temperature overnight until a blue colour in the mixture developed. The absorbance of the samples was determined at wavelength 595 nm using a Hitachi U-2000 spectrophotometer.

2.4.4 Restriction enzyme digestion

Cellular DNA isolated from infected cells was cleaved with the *EcoRI* restriction endonuclease. The reaction mixture was performed in a 50-65 µl volume. Digestions were carried out according to the enzyme supplier's recommendations with the following modifications when digesting viral and cellular DNA: 1.) a two- to threefold excess of enzyme was used in each reaction, 2) 4mM of spermidine was added to each mixture to increase the efficiency of digestion and 3) digestions were incubated at 37°C overnight.

2.4.5 Gel electrophoresis

Gel electrophoresis was carried out using TAE buffer (40mM Tris acetate [pH7.9], 5mM sodium acetate, and 1 mM Na₂EDTA). Agarose gel was made by mixing 1% of agarose (BRL) with TAE buffer containing 0.25 µg/ml w/v of ethidium bromide and boiled until completely dissolved. The solution was poured on a gel tray after it was cooled down to 50°C. Then, 10% of FLG stopper (50% sucrose, 10mM EDTA, 1% SDS, 0.1% bromophenol blue) was added to DNA which was then added to the solidified gel placed in the TAE buffer. Viral and cellular fragments were separated at 20V for 24 hours. λ phage DNA *Hind*III fragments or BRL's 1-kb linear DNA ladder markers were electrophoresed as size standards.

Following electrophoresis gel was visualized under UV light. A clear plastic ruler backed with masking tape was placed on the side of the gel, and a photograph was taken using a digital camera.

2.4.6 Southern blotting

A modified Southern transfer technique (Southern, 1975) was used to immobilize DNA fragments from electrophoresis gels to nitrocellulose membrane. The gel was incubated in 0.25 N HCl for 15 minutes at room temperature. Gel was then soaked in 0.4N NaOH-0.6M NaCl for 30 minutes and in 1.5M NaCl-0.5M Tris-HCl, pH 7.5 for 1 hour.

A Southern transfer apparatus was made by emerging a sponge into a tray or glass dish filled with 10 X SSC (3M NaCl, 0.3M sodium citrate). The gel was placed on 2 layers of blotting paper on the surface of the sponge. A nitrocellulose membrane (GeneScreenPlus) which was soaked in 10X SSC for 15 minutes was placed on top of the gel and all edges were covered with Saran Wrap. Then, 4-5 pieces of dry filter paper, 2-3 inch stack of absorbent paper towels and

about 500 g of weight was placed over the membrane. After the transfer, membrane was washed in 6XSSC, air dried and UV crosslinked.

2.4.7 Nick translation and hybridization technique

Viral and plasmid DNA were labeled by the method of nick-translation, using a commercial nick-translation system (PROMEGA). Reaction mixture contained 0.5-1 µg of purified template DNA, 5 µl 10X nick-translation buffer, 5 µl nucleotide mix (300mM stock), 7 µl a³²P-dATP (35 mCi), 5 µl nick translation enzyme mix and nuclease-free water to 50 µl. The reaction was incubated at 15°C for 1 hour. The entire reaction mixture was used in one hybridization reaction.

Prehybridization and hybridization were carried out at 65°C (Southern blots) or at 62°C (Northern blots) in a Hybaid hybridization oven. The solution used in prehybridization and hybridization steps contained 1M NaCl, 1%SDS and 10% Dextran sulfate. The membrane was prehybridized at 65°C or 62°C for 15-30 minutes. Hybridization was performed overnight in a hybridization solution supplemented with 100 µg denatured Herring sperm DNA and labeled probe. The membrane was then washed in 2xSSC(w/v) (10 minutes, room temperature), 2xSSC and 0.1% SDS (w/v) (1 hour, 65°C or 62°C) and finally with 0.1xSSC at room temperature. The membrane was then placed on a piece of 3MM Whatman paper, in an X-ray cassette containing a Cronex intensifying screen, covered with Saran wrap and exposed to Kodak X-OMAT X-ray film for the appropriate period of time.

2.4.8 Extraction of viral & cellular RNA

Infected cell monolayers in 120x60 mm plates were washed three times in PBS. Cells were lysed by adding 5.2 ml of TRIZOL LS Reagent (Gibco BRL) and incubating for 5 minutes. Following transfer to 15cc tube, 1.4 ml of chloroform was added to the homogenate, mixed and incubated at room temperature for 15 minutes. The homogenate was then centrifuged @ 3,000

rpm (IEC Centra-8R Centrifuge) for 15 minutes and the supernatant containing RNA was transferred to a fresh tube. Supernatant was then mixed with 3.5 ml of isopropyl alcohol, incubated at room temperature for 10 minutes and centrifuged for 10 minutes at 3,000g. The RNA pellet was washed once with 75% ethanol, centrifuged for 5 minutes, dried and resuspended in an appropriate volume of DEPC-treated water.

RNA preparations were quantitated by measuring the optical density at 260 nm and 280 nm. Samples with an OD_{260/280} ratio < 1.7 were reextracted and precipitated since they contained high content of proteins. The quality of RNA preparations was determined by visualizing 18s and 28s bands on agarose gels.

2.4.9 Northern blot and hybridization

Total RNA mixed with 10% of bromophenol blue was separated by electrophoresis in a 0.9% agarose-0.1 M sodium phosphate gel @ 50.V for 5 hours. Sodium phosphate buffer (pH6.8) was made as a 1M solution containing 46.3 ml of 1M Na₂HPO₄ and 53.7 ml of 1M NaH₂PO₄ solutions.

The RNA was transferred to a nitrocellulose membrane as described in Southern analysis but without pretreatment of the gel in 0.25 N HCl, 0.4N NaOH/0.6 M NaCl and 1.5 M NaCl/0.6 M Tris-HCl.

2.5 Emulsion autoradiography

2.5.1 In vivo labeling of BAV2

Confluent MDBK cell monolayer in 120x60 mm plates was infected with virus at moi of 0.1-1.0 PFU/cell. The cells were incubated at 37°C for 60 minutes. The medium was then added to the infected cell monolayer and again incubated at 37°C. At 6 hr p.i. the medium was removed and replaced by medium containing 50μCi of [³H] thymidine/5 ml. After completion of

cytopathic effect, the virus was harvested and concentrated as described previously in section 2.3.1.

2.5.2 Infection of cells with [³H] labeled virus

Cells were grown overnight directly on pre-cleaned slides which were placed in 120x60mm plates. The medium was then aspirated and the plate was washed once with 1xPBS. The cell monolayer was infected with [³H]BAV2 at 10 moi. Slides were taken out from the plates at various times post infection and washed 3 times with 1xPBS to remove unabsorbed virus.

2.5.3 Fixation of infected cells and autoradiography

The infected cells were fixed by immersion in formalin fixation buffer (0.9 g NaCl, 5 ml formaldehyde per 100 ml of water; pH7.4) for 4-24 hours. Following fixation the slides were rinsed in water and dehydrated by successive washing (1 minute each) in the graded ethanol series (50%, 70%, 95% & 100%). Slides were then washed in xylene for 1 minute and air dried for about 15 minutes.

In a dark room, under a safe light, slides were dipped in NTB2 nuclear track emulsion (Kodak) diluted 1:1 with water. The slides were allowed to air-dry in the dark for 1-2 hours after which they were placed in a box which contained desiccant. The box was wrapped with 2 layers of aluminum foil and placed at 4°C. The slides were exposed for up to 1 week.

After exposure, the slides were removed from the box and allowed to equilibrate to room temperature. The slides were developed by immersion in developing solution (Kodak GBX) for 2-4 minutes, stop bath (water) for 30 seconds and fixative (Kodak GBX) for 4 minutes. Following fixation the slides were rinsed for 15 minutes in running tap water and allowed to air dry.

2.5.4 Counterstaining of slides

Slides were immersed in hematoxylin solution (Sigma) for 1 minute and then washed in 0.1XSSC for 1 minute and 2X SSC for 15 minutes. The slides were then dehydrated through graded ethanol solutions (59%, 70%, 95% and 100%) for 1 minute each. Following immersion in eosin solution (Sigma) for 1 minute the slides were washed three times in 95% and once in 100% ethanol for 1 minute each. The slides were then soaked in xylene, two times for ten minutes each and mounted in Permount gelatin.

The slides were viewed under a light microscope (Leitz, LABORLUX 11) under 1000x magnification (oil immersion). The pictures were taken by 35 mm Kodak camera mounted on the microscope.

2.6 PCR procedures

2.6.1 Primers and reaction conditions for PCR

Primers used in this study were designed by the Cprimer software (Bristol G., and R. Andersen). The following criteria were used in primer design: 1) primers were 20-28 nucleotides in length, 2) primers contained 50% G+C content, 3) primer pairs have closely matched melting temperature (T_m) of 60-65°C. Primers were synthesized by Procyon Biopharma Inc. (London, Ontario).

A typical PCR reaction was carried out in 50 μ l volume and contained template DNA , 1-2 units Taq DNA polymerase (MBI Fermentas), 5 μ l of 10X buffer (100 mM Tris-HCl [pH8.8], 500 mM KCl, 0.8% v/v Nonidet P40), 0.2 mM of each dNTP , 1 μ M of each primer and 1-3 mM $MgCl_2$ which was optimized for each set of primers.

2.6.2 Temperature cycling

PCR was carried out in the Single Block Easy Cycler (Ericomp Inc.). Samples were first denatured for 5 minutes at 94°C. Template DNA was amplified in 35 PCR cycles each consisting of 45 seconds denaturation at 94°C, annealing for 1 minute @ 65°C and extension for 2 minutes at 72°C. Following the completion of PCR cycles, the final extension was carried out at 72°C for 10 minutes.

To prepare samples for electrophoresis, 1/10 of the PCR product from each reaction was mixed with stopper and loaded on 1% agarose gels. Occasionally, the agarose gel electrophoresis was followed by Southern blotting and hybridization of a specific probe to allow the detection of a given PCR product in a background of high nonspecific amplification. This was described in sections 2.3.4 and 2.3.6.

2.7 DNA Transfection

2.7.1 Lipofectamine transfection

DNA transfections using lipofectAMINE reagent were carried out according to the supplier's recommendations (Gibco, BRL). HeLa and 293 cells were plated in two 6-well plates (35x10 mm) at density of 10^5 cells/ml or 3×10^5 cells/ well and placed at 37°C in the CO₂ incubator for 12 hours. For each well 1 µg of DNA was mixed with serum-free medium to a final volume of 100 µl. In another tube, 2.5 µl of lipofectAMINE was diluted in 100 µl of serum-free medium. The two solutions were then combined and the mixture was incubated at room temperature for 30 minutes. Immediately prior to transfection, the cells were washed three times with serum free medium. Then, 800 µl of serum-free medium was added to the tubes containing DNA-liposomes complexes and this mixture was added onto the washed cell monolayer. Cells were then incubated at 37°C and 4 hours post transfection 2ml of medium with

20% serum was added to each well without removing the transfection mixture. The cells were assayed for lacZ activity 24 hours post transfection by X-gal-based histochemical assay.

2.7.2 Histochemical assay for lacZ activity

This procedure was carried out as outlined by MacGregor *et al.* (1991). The medium from the cell monolayer was twice aspirated and rinsed gently and thoroughly with PBS. The cells were then overlaid with 4% paraformaldehyde fixative (pH7.4) and incubated at room temperature for 5 minutes. The fixative was then aspirated and rinsed 3 times gently with 1XPBS. The fixed cells were then overlaid with X-gal stain (35mM $K_3Fe(CN)_6$, 35mM $K_4Fe(CN)_6$, 1mM $MgCl_2$, in PBS; pH7.4 supplemented with 1mg/ml of X-gal) and incubated at 37°C until a blue color developed. The total number of blue cells was counted under 40X magnification on an inverted microscope (Telaval 3; Carl Zeiss, West Germany).

2.8 Statistical analysis

Mann-Whitney analyses were performed using SigmaStat™, version 1.0.

3. RESULTS

3.1 BAV2 replication in HeLa cells

Ad permissive infections produce morphological changes in infected cells commonly known as cytopathic effects (CPEs). In order to examine if BAV2 infection of HeLa cells is permissive, we investigated the ability of BAV2 to produce cytopathic effects in this system. This experiment involved infecting HeLa and MDBK cells with BAV2 at moi's of 1, 10, 100 and 1000 and observing the appearance of cytopathic effect (CPE) by inverted microscopy. Infected cells from one of these experiments were photographed (*Figures 7 and 8*). Over a period of 96 hours no CPEs characteristic of Ad infection were observed on HeLa cells infected with BAV2 as cell morphology and appearance remained similar to control noninfected cells (*Figure 8*). Permissive MDBK cells were used as a positive control in this experiment. As expected, CPEs occurred 12 hours p.i. in permissive MDBK cells and was complete 48 hours p.i. (*Figure 7*).

While Ad permissive infections produce CPEs, many semipermissive infections, in which progeny yields are reduced, do not result in CPEs. Hence, to determine whether BAV2 infects HeLa cells semipermissively or abortively, we compared the production of BAV2 progeny in HeLa cells to MDBK cells by plaque assay. In this experiment, cells infected with BAV2 at 10 PFU/cell were harvested at 1, 12, 24, 48 and 96 hours p.i.. The infected cells were then subjected to several freeze/thaw cycles to release intracellular virus and infectious virus yields in these extracts were titered on MDBK cells. The results are presented in *Figure 9*. As expected, the infectious virus was observed by 12 hours p.i. in MDBK cells and increased exponentially reaching a maximum at 48 hours p.i.. No viral replication was apparent in HeLa cells suggesting that BAV2 undergoes abortive infection in this system.

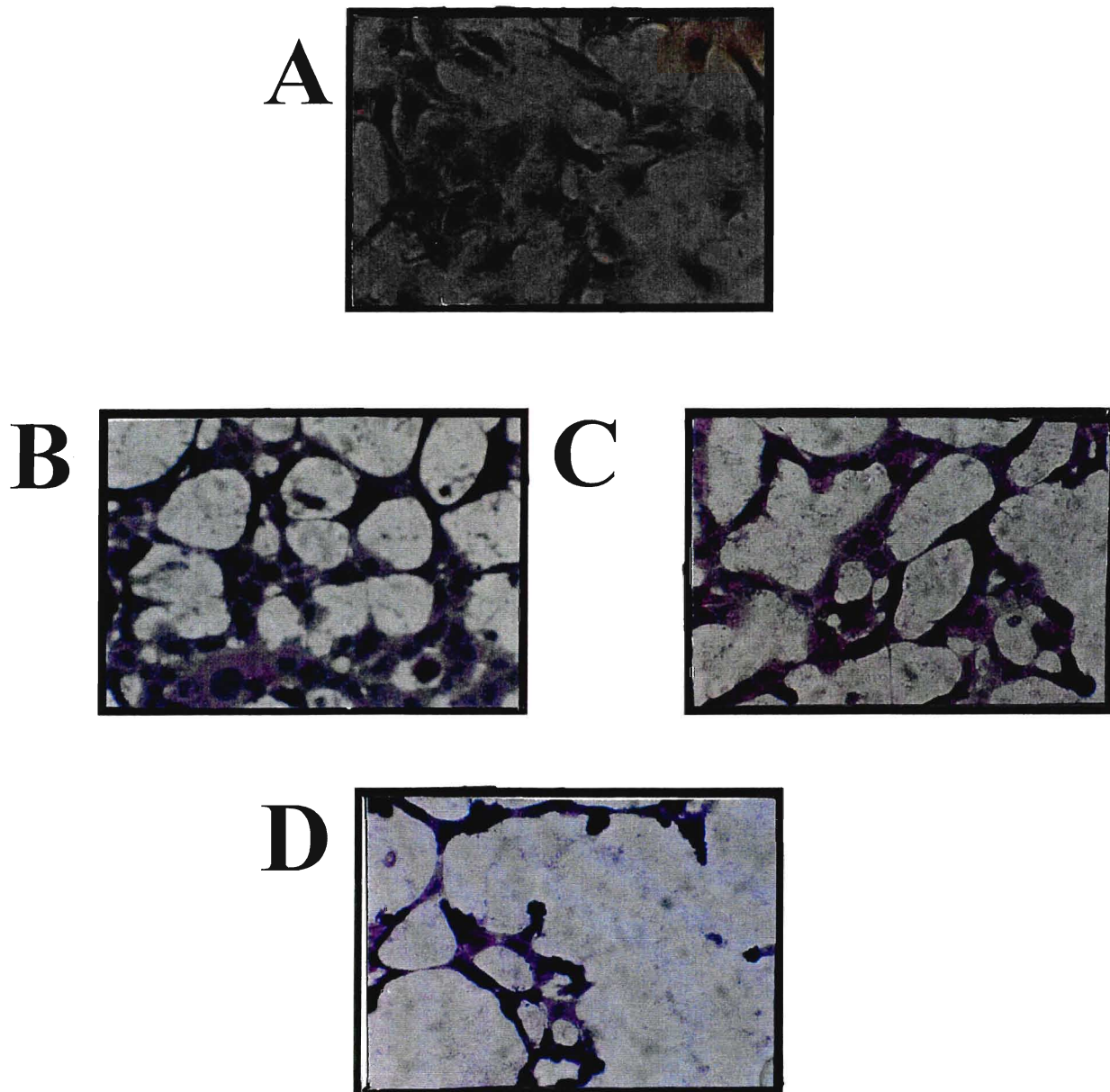


Figure 7: Cytopathic effects in BAV2 infected MDBK cells

Cells were grown on slides overnight and infected at 10 moi. At indicated times p.i. slides were taken out, fixed with formaldehyde and stained with hemotoxylin and eosin. Cytopathic effects were monitored by inverted microscopy. Magnification, 450X.

- A)* control (mock infected) MDBK cells
- B)* 12 hours p.i.
- C)* 24 hours p.i.
- D)* 48 hours p.i.

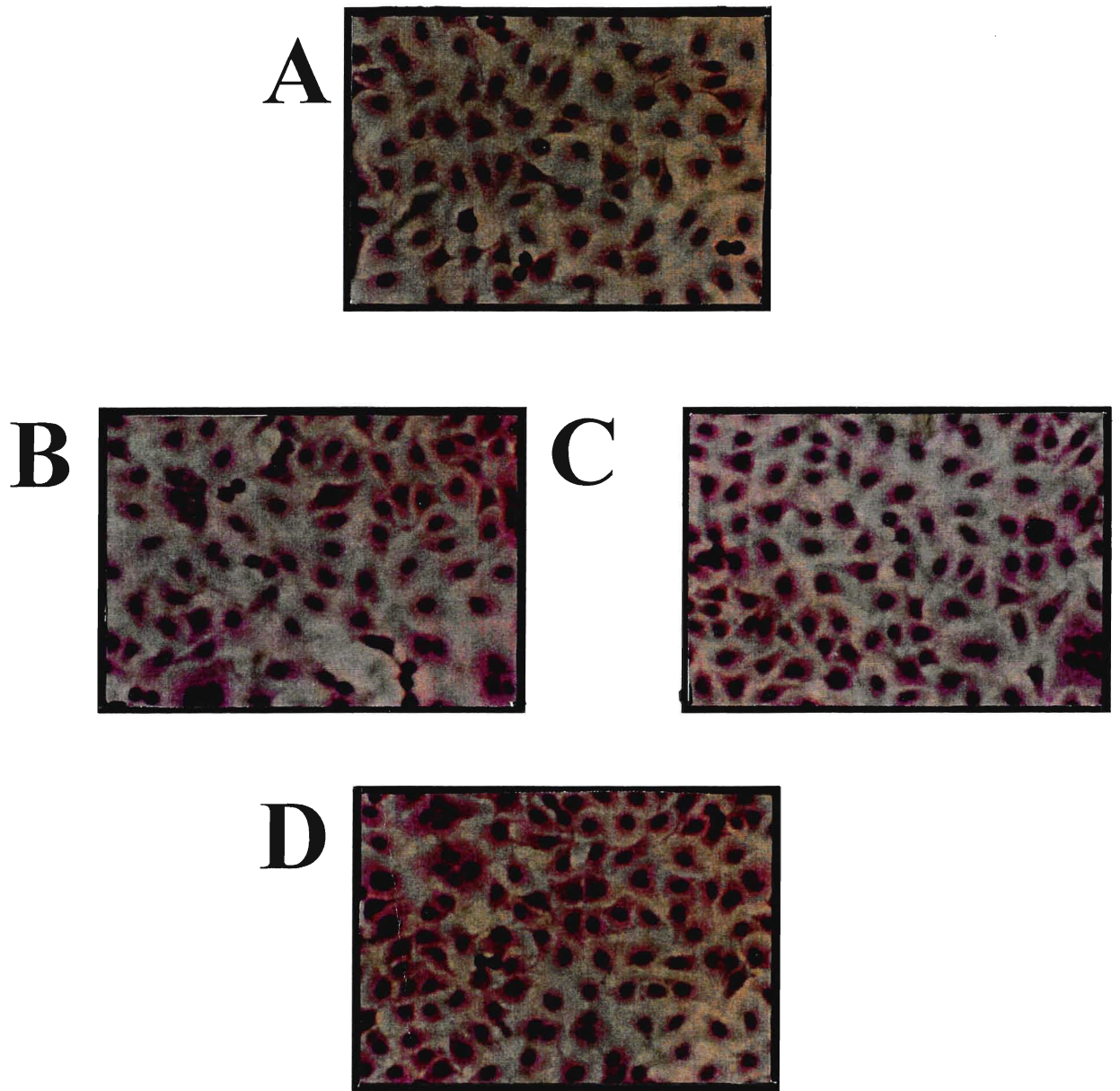


Figure 8: Absence of cytopathic effects in BAV2 infected HeLa cells

Cells were grown on slides overnight and infected at 10 moi. At indicated times p.i. slides were taken out, fixed with formaldehyde and stained with hemotoxylin and eosin. Cytopathic effects were monitored by inverted microscopy. Magnification, 450X.

- A)* control (mock infected) HeLa cells
- B)* 12 hours p.i.
- C)* 24 hours p.i.
- D)* 96 hours p.i.

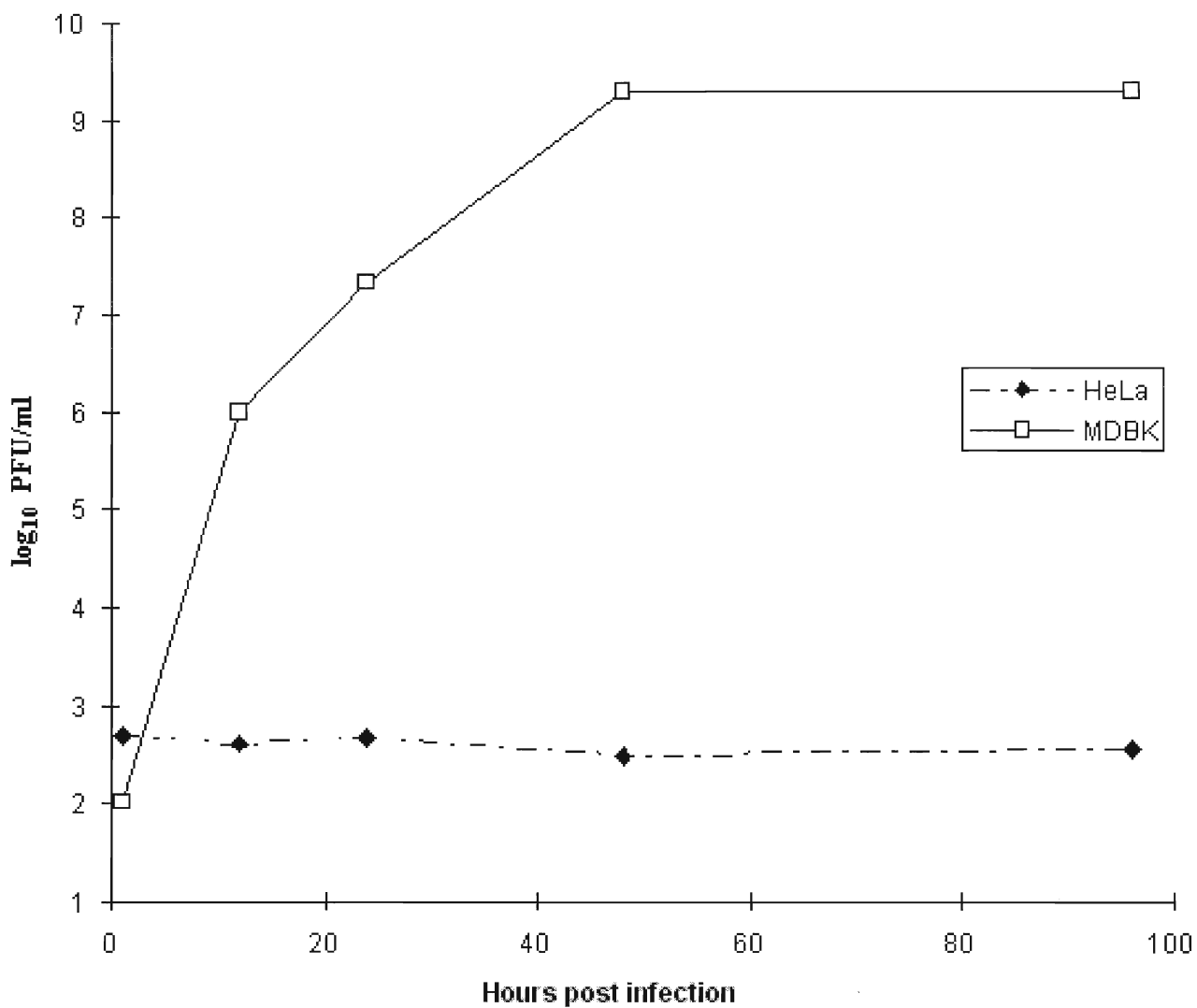


Figure 9. Kinetics of BAV2 replication in MDBK and HeLa cells.

At the indicated times, the cells were scraped into 1ml of PBS²⁺, sonicated, and titrated by plaque assay on MDBK cell monolayers as described in the *Materials and Methods*.

3.2 BAV2 attachment to HeLa cells

As in most virus-host cell systems, the infectivity of Ads depends on the availability of specific cell receptors. If these receptors are absent, Ad fails to internalize. Therefore, the first test in determining the block of BAV2 multiplication in HeLa cells was to study the ability of BAV2 to attach to HeLa cells. This was approached by plating MDBK and HeLa cells on slides and infecting them with ^3H labeled BAV2. Slides were taken out 1hr p.i. and fixed in formaldehyde as outlined in *Materials and Methods*. Following fixation, slides were dipped in nuclear track emulsion and exposed for 1 week. Slides were then developed, fixed and stained with hematoxylin and eosin. The slides were examined under a microscope at 1000X magnification as outlined in *Materials and Methods*. Cells were photographed (*Figure 10*) and randomly screened for silver grains. The analysis revealed a considerable number of silver grains in the area surrounding the cells. Therefore, a comparison was made between grain counts over a given cell to those in background relative to the area of each cell. The results are depicted in histograms shown in *Figure 11*. The number of silver grains associated with both HeLa and MDBK cells was significantly higher than the relative area background count (Mann-Whitney Rank Sum Test; $p=0.05$). These results suggested that BAV2 attaches to both HeLa and MDBK cells.

To compare the relative amount of absorbed virus between HeLa and MDBK cells, number of silver grains associated with cells was corrected by subtracting the silver grain number over cells from that in the background. The results shown in *Figure 12* revealed that MDBK cells had a higher number of adsorbed BAV2 particles (Mann-Whitney Rank Sum Test; $p=0.05$).

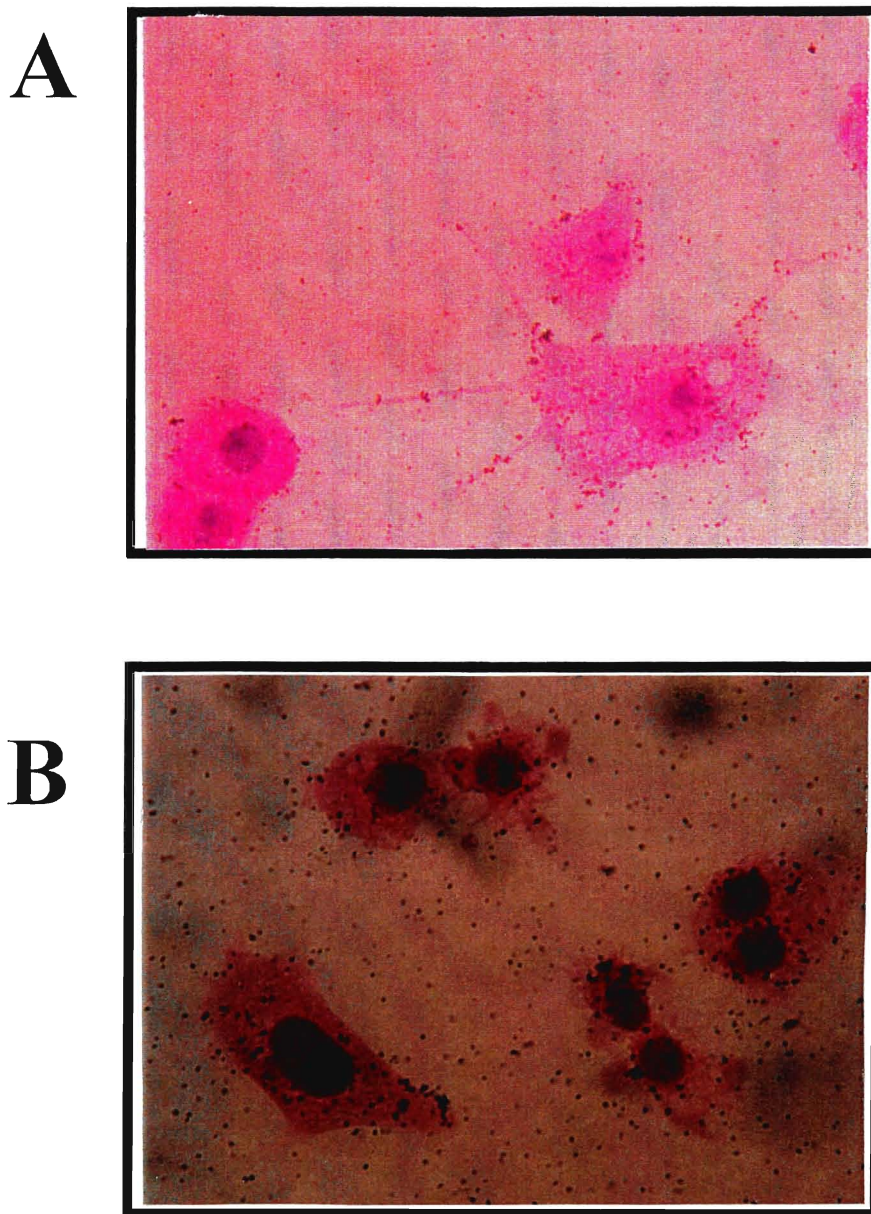


Figure 10: Adsorption of BAV2 particles to HeLa and MDBK cells

Cells were infected with ^3H labeled BAV2 at 10 moi and analyzed by emulsion autoradiography as discussed in the text. Magnification is 2000X.

A) MDBK cells

B) HeLa cells

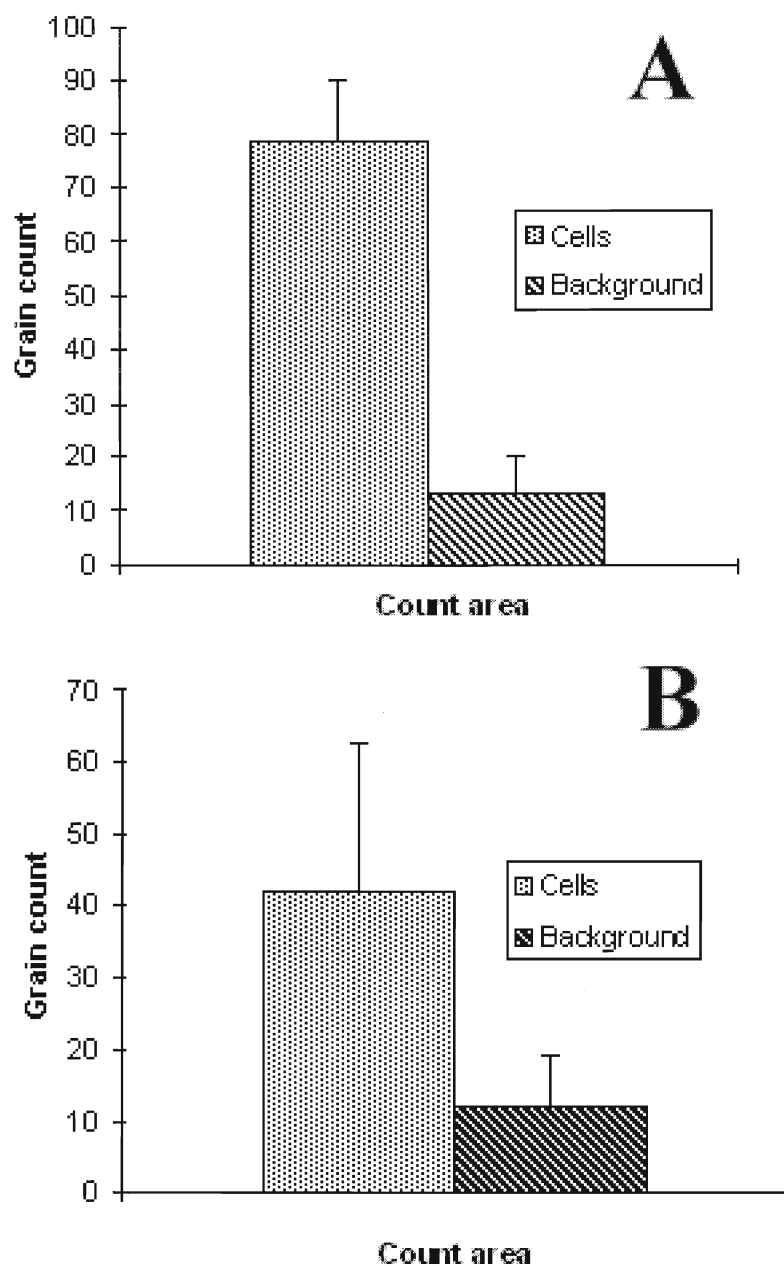


Figure 11: Distribution of silver grains

The silver grains were counted as indicated in the text. The grain counts are based on the evaluation of 100 cells (two independent experiments). Error bars denote one standard deviation.

A) MDBK cells

B) HeLa cells

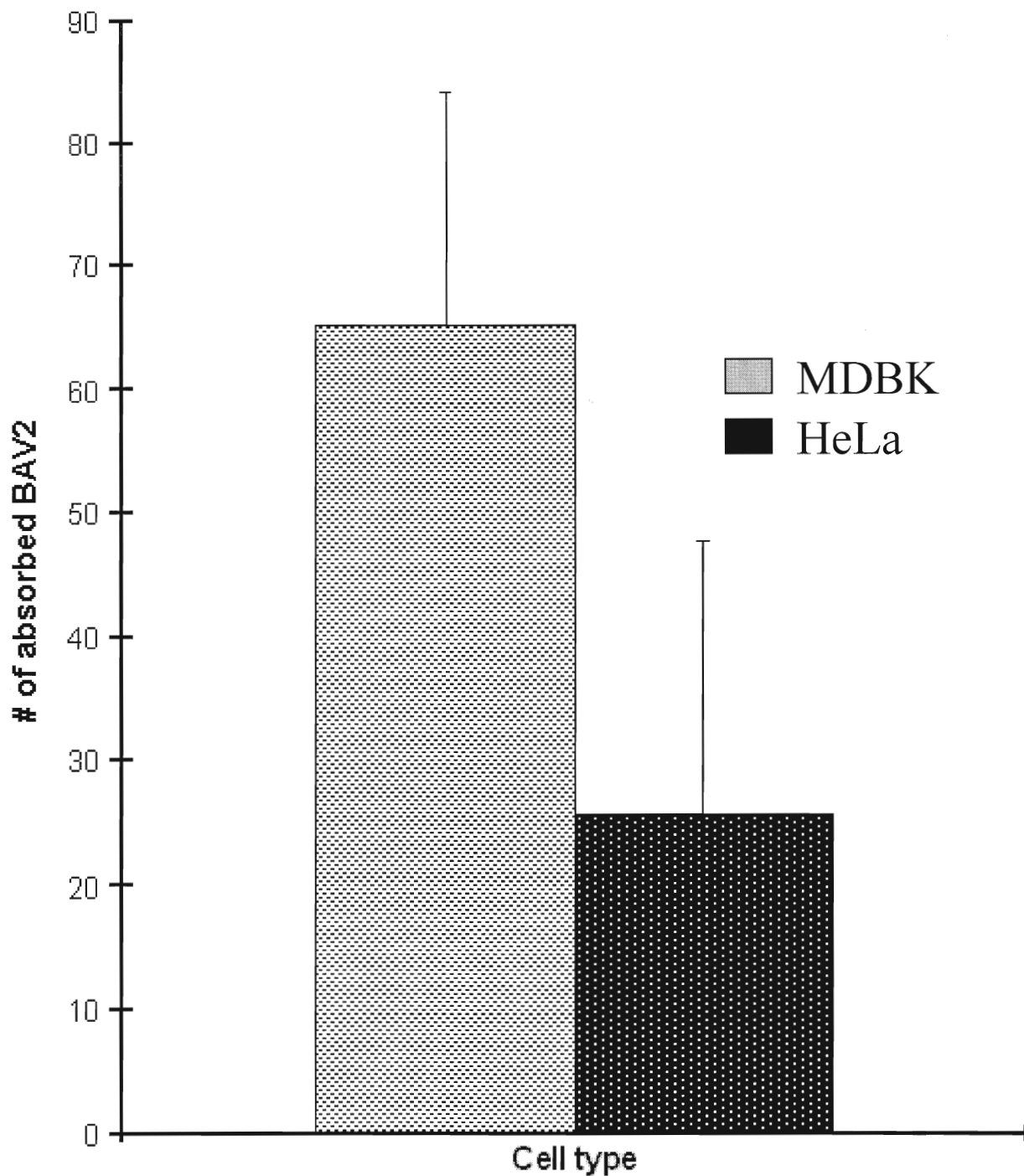


Figure 12: Adsorption of BAV2 to HeLa and MDBK cells

Histogram showing the relative number of BAV2 particles attached to HeLa and MDBK cells expressed as grain counts normalized by subtracting silver grain number over cells from that over the relative surrounding area. Error bars denote one standard deviation.

3.3 BAV2 internalization in HeLa cells

The next step in Ad life cycle involves the internalization of Ad by endocytosis. Since, the failure of Ad to internalize would result in an abortive infection, it was of interest to study the internalization of BAV2 in HeLa cells. In this experiment, HeLa cells were infected with BAV2 at 10 moi and 24 hours p.i. the cells were trypsinized and washed 2 and 4 times with PBS to remove the adsorbed virus. The total genomic DNA was then isolated, digested with *Eco*RI, fractionated on 1% agarose gels and analyzed by Southern Blotting using ³²P labeled intact BAV2 DNA (*Figure 13*) as probe. The results are shown in *Figure 14*. Complete BAV2 DNA sequences were present in HeLa cells following trypsin treatment indicating BAV2 internalization in HeLa cells (*Figure 14A; lanes 2 and 3*). Amount of BAV2 DNA slightly decreased following trypsin treatment (*Figure 14A; lanes 2 and 3*) suggesting that not all adsorbed virus internalized 24 hours p.i.. The densitometric analysis revealed that about 80% of adsorbed BAV2 particles internalized in HeLa cells 24 hours p.i. (*Figure 14B*).

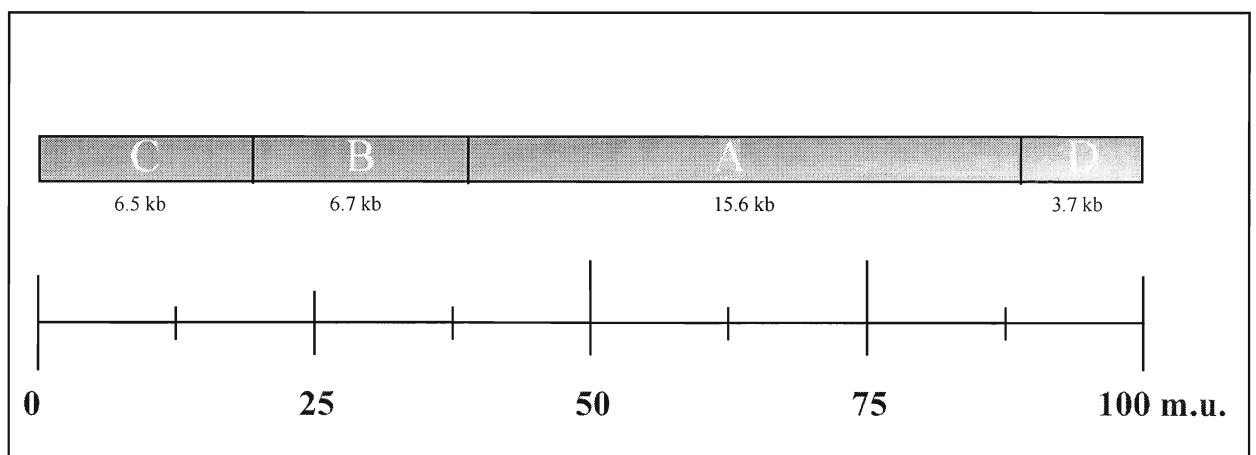


Figure 13: The *Eco*RI restriction map of BAV2 and a length scale in map units

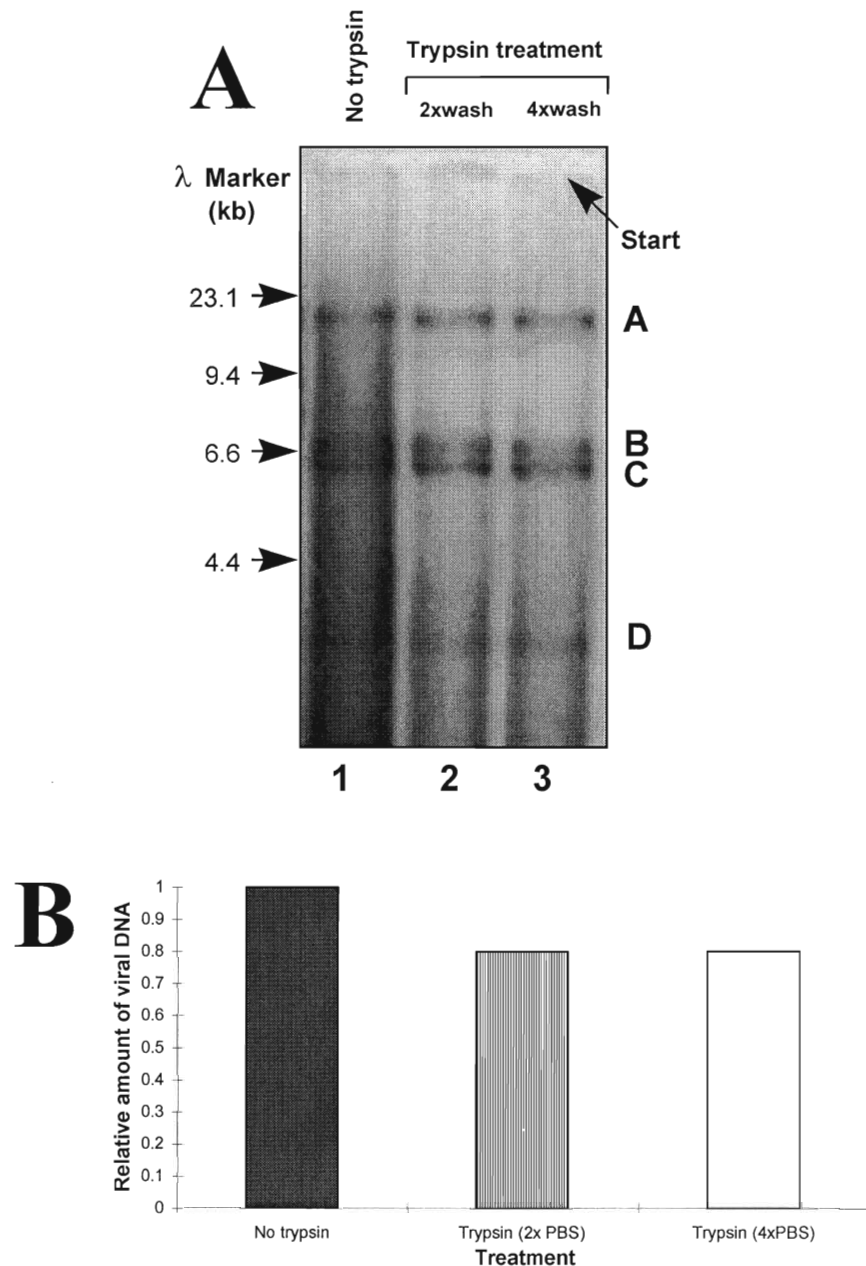


Figure 14: Internalization of BAV2 in HeLa cells

A) Representative autoradiogram showing the effect of trypsin on BAV2 infection of HeLa cells. The details of the experiment are described in text above. The total genomic DNA was isolated from infected HeLa cells and analyzed by Southern blotting as outlined in *Materials and Methods*. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the Figure show BAV2 DNA fragments cleaved with *EcoRI* (see *Figure 13*). *HindIII* fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of the Figure.

B) Histogram representing the relative amount of viral DNA estimated by densitometry scanning of autoradiograms of DNA fragments. The optical density values were normalized to a value of 1 designated for sample in lane 1.

3.4 BAV2 transport to the nuclei of HeLa cell

To further examine BAV2 abortive infection of HeLa cells the fate of BAV2 DNA was followed. This was approached by infecting HeLa cells with BAV2 at 10 moi and harvesting trypsinized infected cells at 1 and 6 hour postinfection. The total genomic DNA was then isolated from half of the harvested cells, and the other half for nuclear DNA extraction. The DNA was analyzed as described above. The results (*Figure 15*) showed that no BAV2 DNA copies were detected in HeLa nuclei 1 hour p.i. (*Figure 15A; lane 2*). However, viral DNA was clearly detectable in the nuclear fraction 6 hours p.i. (*Figure 15A; lane 4*). Densitometric analysis showed that approximately 35% of total BAV2 entered the nucleus 6 hours p.i. (*Figure 15B*). There was an unexpected increase in the copy number of total BAV2 DNA 6 hour p.i. (*Figure 15A; lane 3*). This increase could be attributed to the delay in BAV2 internalization.

3.5 BAV2 DNA synthesis in HeLa cells

The previous experiment showed that BAV2 DNA had a 35% entry rate into the nuclei of infected cells. This, however, did not indicate whether the detected DNA was from input virus or newly synthesized. To better understand the phenomenon, infection experiments involving hydroxyurea (HU), a viral DNA synthesis inhibitor, were carried out. Permissive MDBK (positive control) and non permissive HeLa cells were infected at 10 moi and treated either with no drug or with 10mM HU. The total genomic DNA was isolated at various times p.i., and analyzed as described above. The amount of BAV2 DNA increased over time in MDBK cells not undergoing hydroxyurea treatment (*Figure 16A; lanes 1, 2, and 4*). Furthermore, hydroxyurea treatment inhibited BAV2 DNA synthesis in MDBK cells (*Figure 16B and 16A; lanes 3 and 5*). There was no apparent change in viral DNA levels in BAV2 infected HeLa cells in the presence of HU suggesting that viral DNA synthesis of BAV2 does not occur in HeLa cells (*Figure 17*).

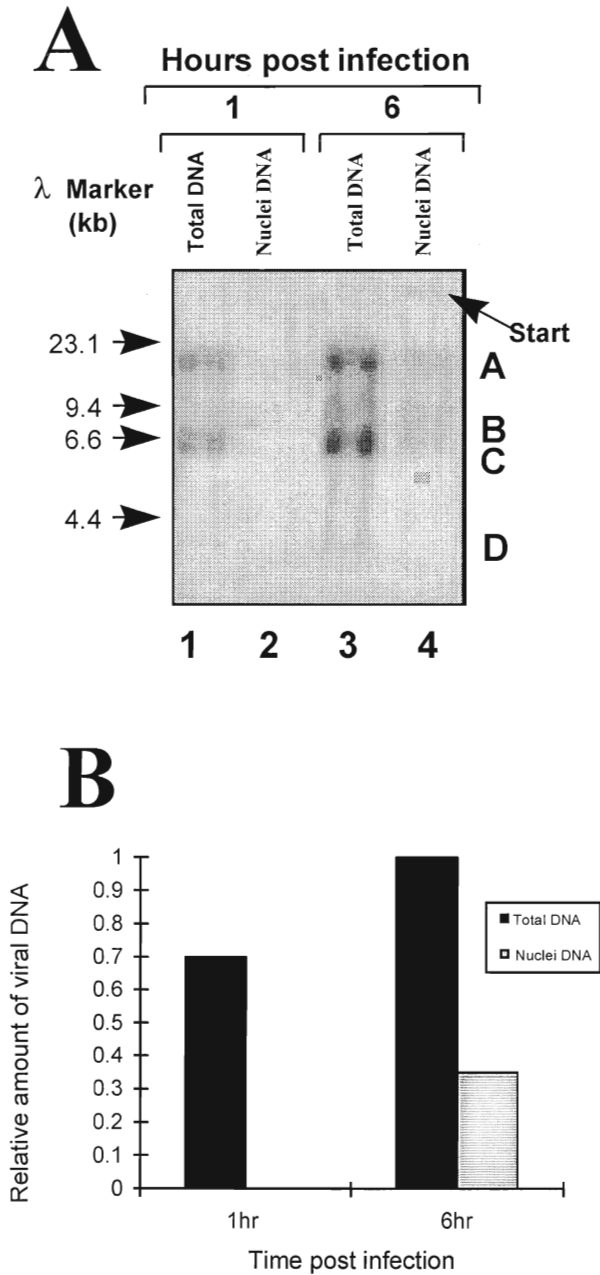


Figure 15: Presence of BAV2 DNA in nuclei of infected HeLa cells.

A) Restriction endonuclease analysis of BAV2 DNA isolated from whole HeLa cells and nuclei of HeLa cells 1 hour and 6 hour p.i. as revealed by the cleavage with *EcoRI*, by Blotting and Autoradiography. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the Figure show BAV2 DNA fragments cleaved with *EcoRI* (see Figure 13). *HindIII* fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of the Figure.

B) Histogram showing the relative amount of viral DNA expressed in arbitrary units representing the ratio between the intensity of each lane to the intensity of the lane 3.

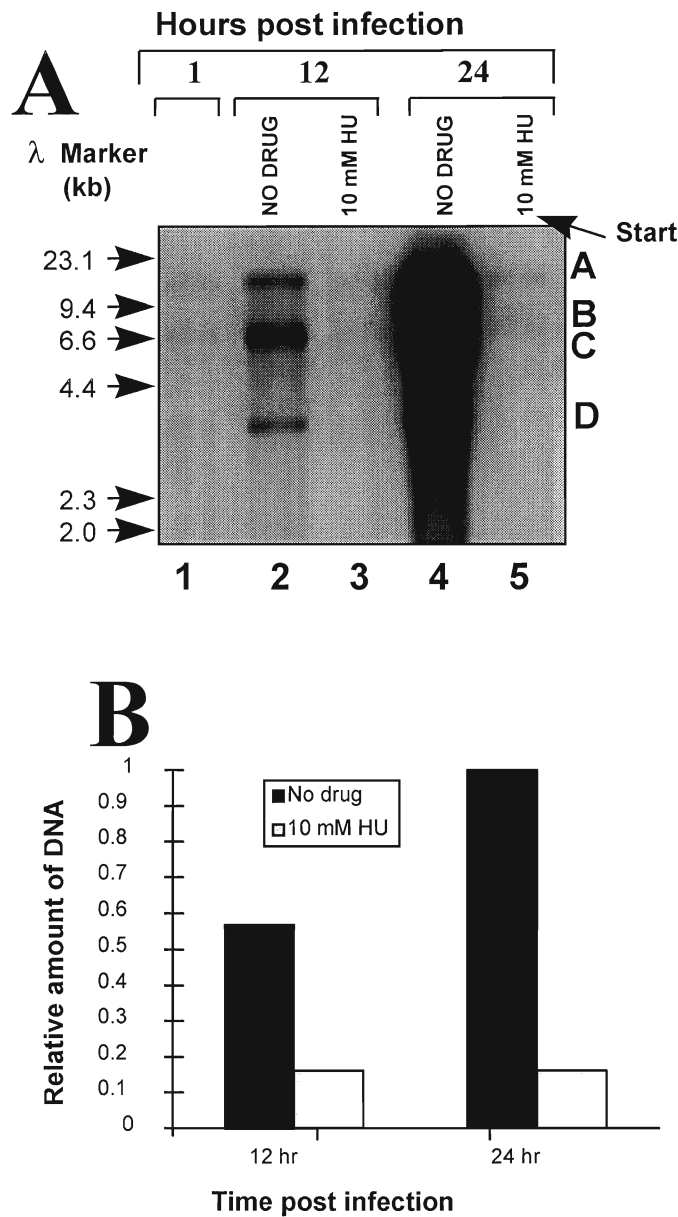


Figure 16: BAV2 DNA synthesis in MDBK cells

A) Restriction endonuclease analysis of BAV2 DNA isolated from MDBK cells treated with no drug or 10 mM hydroxyurea as revealed by the cleavage with *EcoRI*, by Blotting and Autoradiography. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the Figure show BAV2 DNA fragments cleaved with *EcoRI* (see Figure 13). *HindIII* fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of the Figure.

B) Histogram showing the relative amount of viral DNA expressed in arbitrary units representing the ratio between the intensity of all lanes to the intensity of lane 4.

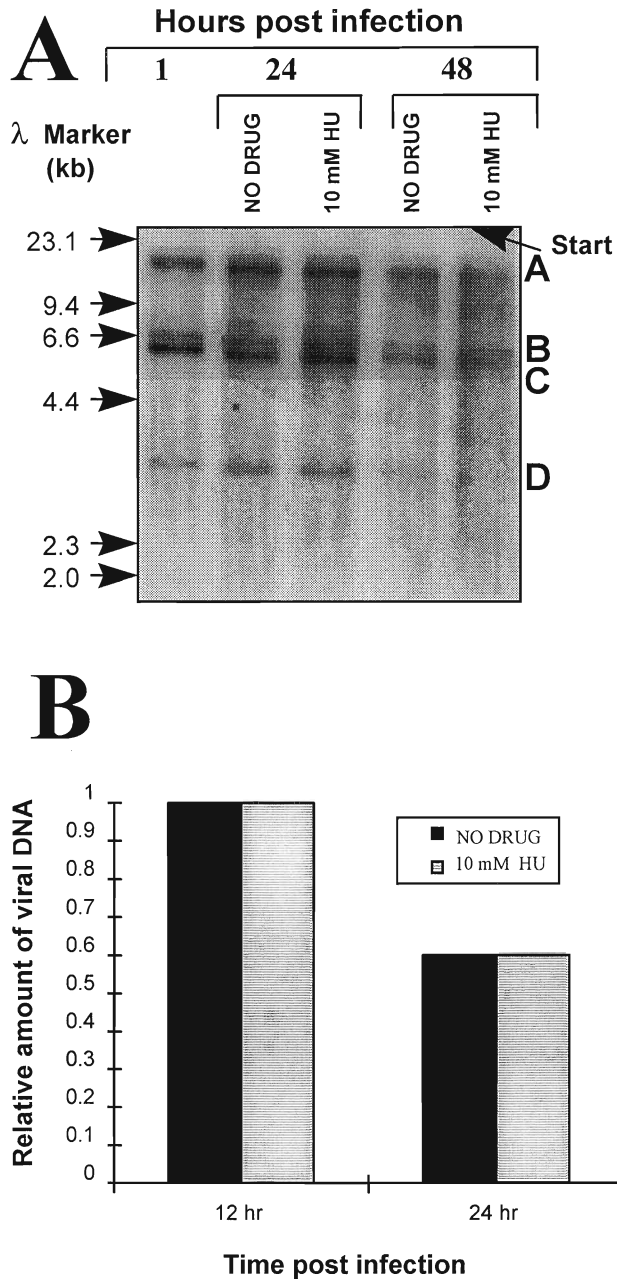


Figure 17: Absence of BAV2 DNA synthesis in HeLa cells.

A) Restriction endonuclease analysis of BAV2 DNA isolated from HeLa cells treated with no drug or 10 mM hydroxyurea as revealed by the cleavage with *EcoRI*, by Blotting and Autoradiography. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the Figure show BAV2 DNA fragments cleaved with *EcoRI* (see Figure 13). *HindIII* fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of the Figure.

B) Histogram showing the relative amount of viral DNA expressed in arbitrary units representing the ratio between the intensity of all lanes to the intensity of lane 3.

3.6 BAV2 E1A transcription in HeLa cells

The abortive block in BAV2 infection of HeLa cells is at an early stage since BAV2 DNA synthesis is defective in HeLa cells. One hypothesis that might explain the defect in viral DNA synthesis is that expression of at least one of the early proteins is affected. Since the E1A products are needed for efficient transcription of the other viral early genes, transcription of BAV2 E1A in HeLa cells was investigated.

Permissive MDBK and nonpermissive HeLa cells were infected with BAV2 at 10 moi. Total RNA was isolated 6 and 24 hours p.i. and subjected to agarose gel electrophoresis. The gel was transferred to a nitrocellulose membrane which was then probed with ^{32}P labeled nt 50-1092 BAV2 fragment isolated from BAV2 E1 containing plasmid pBB12 by *HpaI/SnaBI* digest (Figure 18). Figure 19 revealed the presence of E1A RNA species in infected MDBK cells 6 hours (Figure 19A; lane 1) and 24 hours p.i. (Figure 19B; lane 1). No E1A specific RNA was detected in the BAV2 infected HeLa cells 6 hours (Figure 19A; lane 3) or 24 hours p.i. (Figure 19B; lane 3).

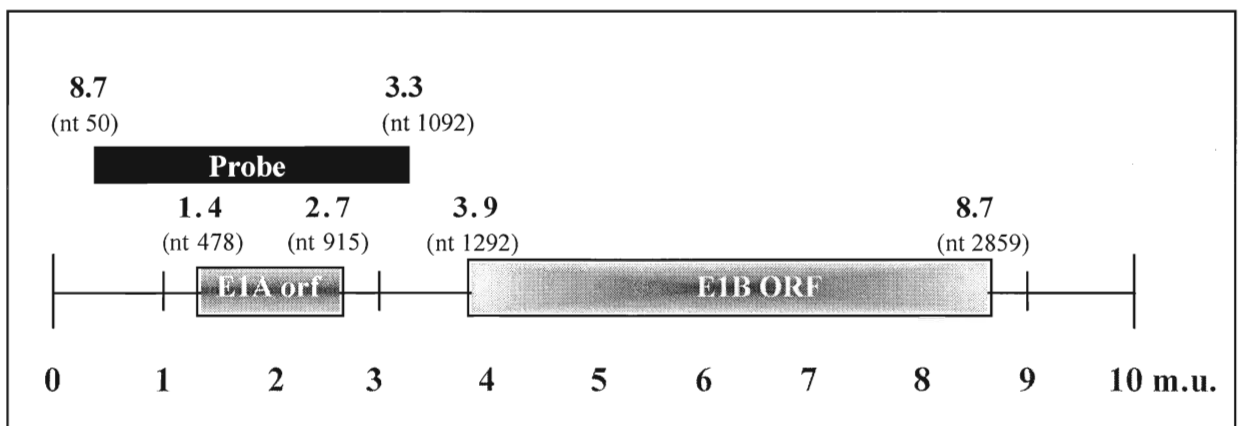


Figure 18: Position of ^{32}P -labeled pBB12 *HpaI/SnaBI* fragment used as a probe shown relative to the E1A and E1B ORFs of BAV2.

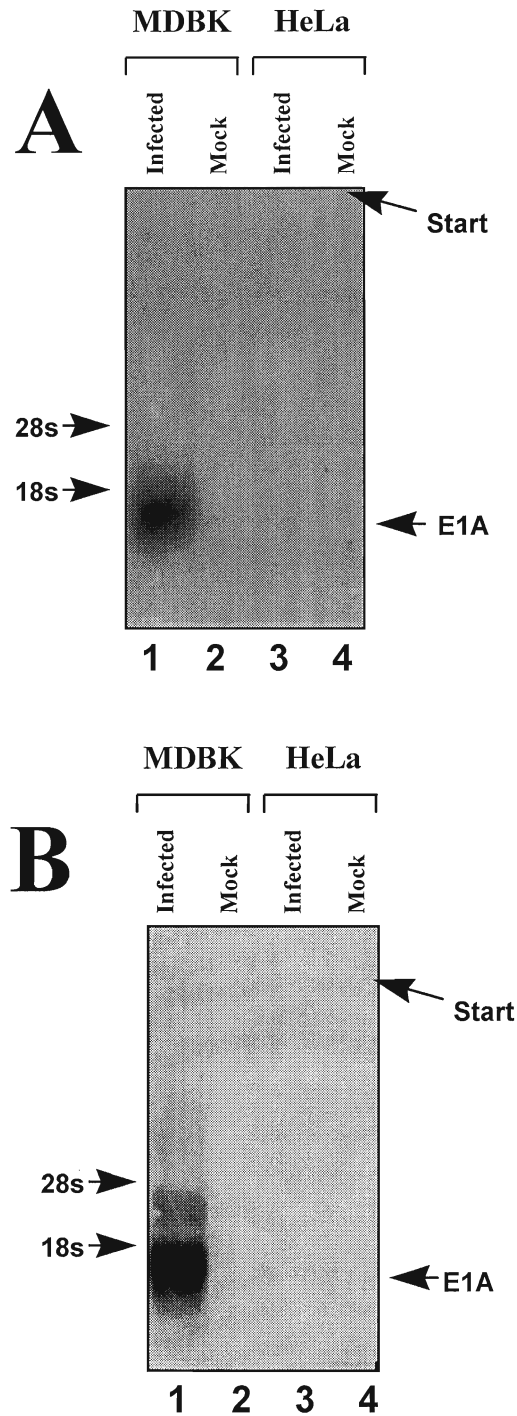


Figure 19: Expression of BAV2 E1A region in infected cells

Northern blot analysis of total RNA isolated from noninfected and BAV2 infected MDBK and HeLa cells **A)** 6 hours p.i. and **B)** 24 hours p.i. Details of the experiment are outlined in the text and in *Materials and Methods*. Each lane contains 10 μ g of total RNA. Positions of 18s and 28s bands as well as predicted position of E1A transcripts are indicated on the left end of the gel.

3.7 The activity of BAV2 E1A promoter in HeLa cells

3.7.1 Plan of experiments

The previous experiments showed that BAV2 E1A is not expressed in HeLa cells, which explains why BAV2 DNA cannot replicate in these cells. We then asked the question why E1A is not expressed in this system. One hypothesis that might explain the defect in viral E1A transcription is that E1A promoter is not recognized in HeLa cells. This hypothesis was investigated by directly testing for the activity of the E1A promoter of BAV2 in HeLa cells using *lacZ* as a reporter gene. In these experiments, BAV2 E1A promoter was fused with *lacZ* and in transient expression assays its activity was tested in HeLa cells. The experiment was controlled against the E1A promoter of Ad5 fused to *lacZ* (pE1A*lacZ*) and a promoterless *lacZ* plasmid (pNPlacZ).

3.7.2 Construction of pB2*lacZ*

A plasmid that contains the reporter gene β -galactosidase coding sequence under the control of BAV2 E1A promoter was constructed in this study to assess the innate activity of BAV2 E1A promoter in HeLa cells. According to a recent study, the TATA box of the BAV2 E1A promoter resides at nt 337 (Salmon et al., 1995). This region is conveniently demarcated by the restriction site *EagI* located at nt 363. In the plasmid pE1E1-E, a linker containing a *XhoI* site and others was introduced in the same *EagI* site. This plasmid was therefore the source of the E1A promoter for the construction of the reporter plasmid (Figure 20). The resulting plasmid, referred to as pB2*lacZ*, hence contained nt 1-363 of BAV2 DNA that served to control the expression of *lacZ*. Furthermore, the plasmid also contained the SV40 polyA signal downstream of *lacZ*.

3.7.3 Construction of pNPlacZ

Plasmid, pNPlacZ containing β -galactosidase coding sequence under no promoter was used as a negative control plasmid in this study. The construction of this plasmid was quite simple and involved excising BAV2 E1A promoter sequence from pB2lacZ. DNA sequence analysis of pB2lacZ revealed two convenient restriction sites for removing BAV2 E1A promoter from pB2lacZ; *Sna*BI at nt 50 and *Xho*I at nt 383. *Figure 23* illustrates the cloning strategy.

3.7.4 Comparison of the activities of Ad5dIE3 and BAV2 E1A promoters in HeLa cells

To compare the activities of Ad5 and BAV2 E1A promoters in HeLa cells, HeLa cells were transfected with expression plasmids pB2lacZ, pE1AlacZ and pNPlacZ as outlined in *Materials and Methods*. The activities of the promoters were measured as percentage of HeLa cells expressing *lacZ*. The results are represented in a histogram shown in *Figure 25* and as photographs of transfected HeLa cells in *Figure 26*. Transfection of HeLa cells with pE1AlacZ resulted in 1.4% of cells expressing *lacZ* (*Figure 25*). However, no cells expressing *lacZ* were observed after transfection with either pNPlacZ or pB2lacZ (*Figures 25 and 26*).

3.7.5 The effects of BAV2 or Ad5dIE3 infection on activity of Ad5dIE3 and BAV2 E1A promoters in HeLa cells

Although the previous section demonstrated that *lacZ* under BAV2 E1A promoter control did not express in HeLa cells, suggesting that BAV2 E1A promoter does not function in these cells, still we have not excluded the possibility that perhaps the BAV2 E1A-*lacZ* construct used was defective to start with. To eliminate this possibility the expression of pB2lacZ in permissive MDBK cells was tested. Thus, MDBK cells were transfected with four different plasmids; pCMVlacZ, pNPlacZ, pE1AlacZ and pB2lacZ. Results showed that even upon transfection with

lacZ under a strong promoter such as CMV on average one in million cells expressed *lacZ*. Hence, it was not surprising that no cells expressing *lacZ* were observed after transfection with either pN*PlacZ* or pB2*lacZ*.

Considering that the above experiments were not conclusive in demonstrating that the lack of expression of pB2*lacZ* in HeLa cells was not due to the defective construct, we needed to conduct a different test. Since, number of adenoviral proteins can act as transactivators, it was hypothesized that the BAV2 E1A promoter could be activated upon Ad infection. If the Ad infection resulted in no *lacZ* expression we could conclude that pB2*lacZ* was either not properly constructed or the BAV2 region (0-363 bp) does not include all necessary promoter factors. However, if pB2*lacZ* was transactivated by some viral functions, then it would be possible to implicate that human cell functions are deficient in the recognition of the BAV2 E1A promoter. In these experiments, HeLa cells were infected with either BAV2 or Ad5*dIE3* at 10 moi, 20 hours after transfection with various *lacZ* plasmids. The cells were tested for *lacZ* expression 4 hours p.i.. The results are shown in *Figures 27, 28, and 29*. After Ad5*dIE3* infection of HeLa cells transfected with control plasmid pE1A*lacZ*, the percentage of cells expressing *lacZ* increased by 43% (*Figure 27*) (Mann-Whitney Rank Sum Test; $p=0.05$). There was no significant difference in the pE1A*lacZ* expression between noninfected or BAV2 infected HeLa cells (*Figure 27*). Results further revealed that the pB2*lacZ* construct was inactive in both uninfected and BAV2-infected HeLa cells. However, upon Ad5*dIE3* infection of HeLa cells transfected with pB2*lacZ*, approximately 0.5% of transfected cells expressed *lacZ*. This suggests that Ad5*dIE3* was capable of activating the BAV2 E1A promoter.

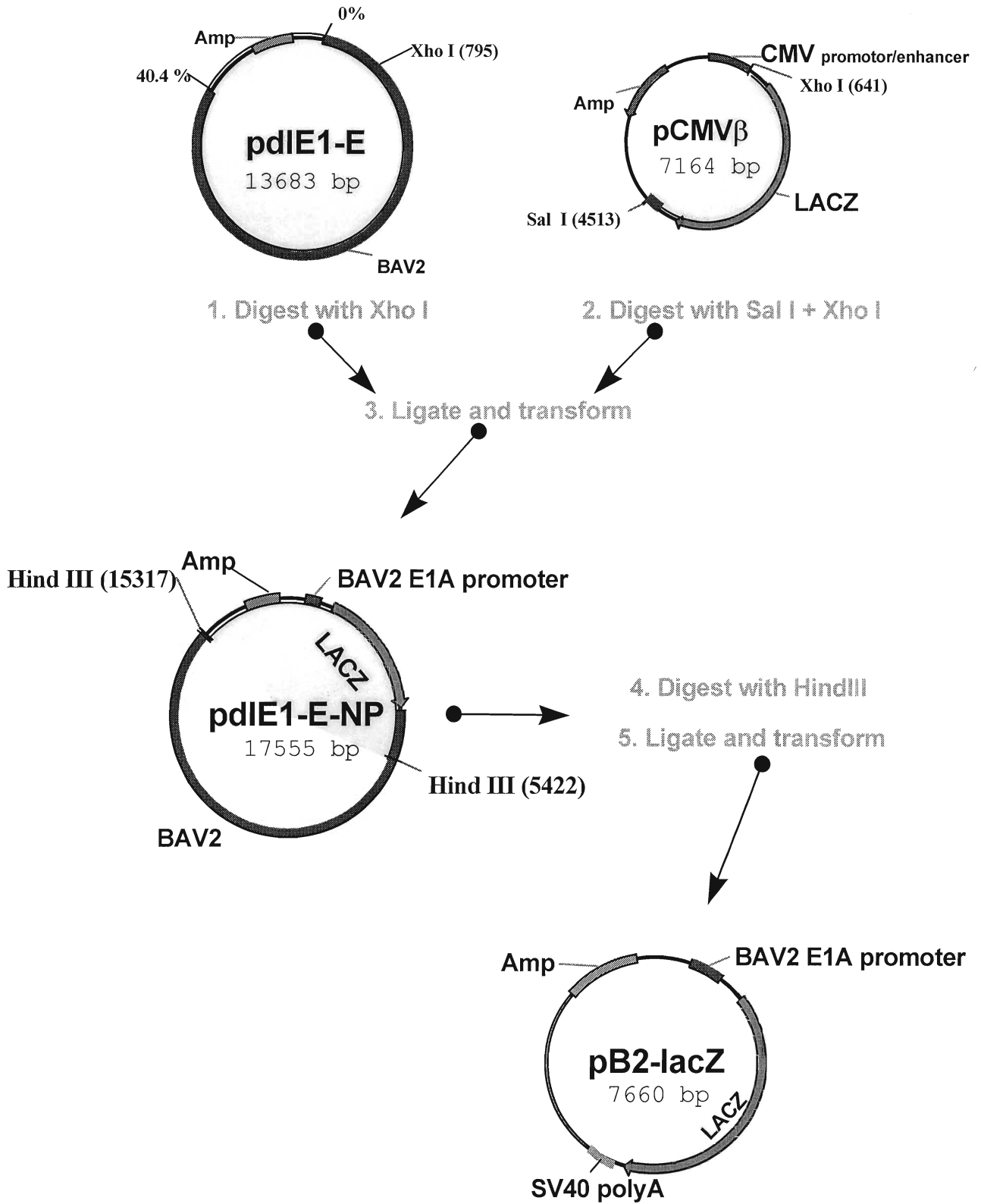


Figure 20: Schematic diagram of pB2lacZ construction.

The first step in cloning of pB2*lacZ* involved construction of the intermediate plasmid pdIE1-E-N. The source of the BAV2 E1A promoter was the plasmid pdIE1-E which contained 0-40% of BAV2 genome with a deletion in E1(1.1-8.2%). This plasmid was cleaved at the XhoI site (1.2% in the BAV2 sequence) and the ends were treated with alkaline phosphatase. Next, the XhoI/SalI fragment of pCMV β -gal that contains the complete beta galactosidase coding sequence and SV40 polyadenylation signal was gel-purified and cloned into the XhoI site of pdIE1-E. The resulting plasmid, pdIE1-E-N contained a BAV2 DNA fragment from 0-40.4% with *lacZ* gene in place of deleted E1 and under the control of BAV2 E1A promoter. This plasmid was confirmed by digestion with several indicative restriction enzymes, followed by separation of the fragments on a 0.9% agarose gel (*Figure 21*).

The second step in cloning of pB2*lacZ* involved deleting BAV2 sequence (10.5-40.4%) from pdIE1-E-N. This was accomplished by digesting pdIE1-E-N with *HindIII* and filling the ends with Klenow polymerase. The plasmid was then religated and used to transform DH5 α *E. coli*. The restriction enzyme analysis confirmed integrity of the resulting plasmid pB2*lacZ* which contained the prokaryotic gene for *lacZ* with the E1A promoter at the 5' site and SV40-derived polyadenylation sequences at the 3' end to facilitate translation of the message in eukaryotic cells (*Figure 22*).

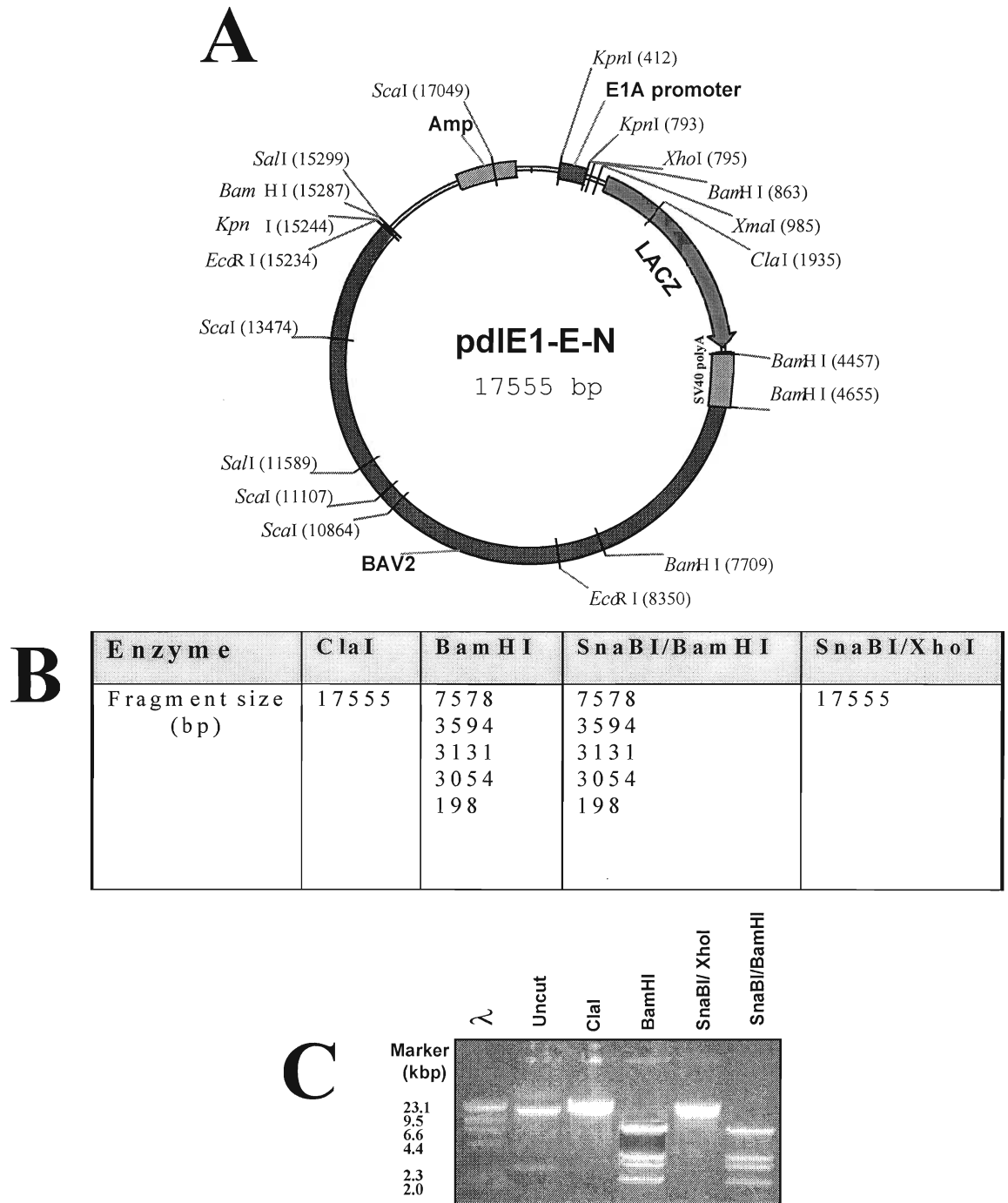


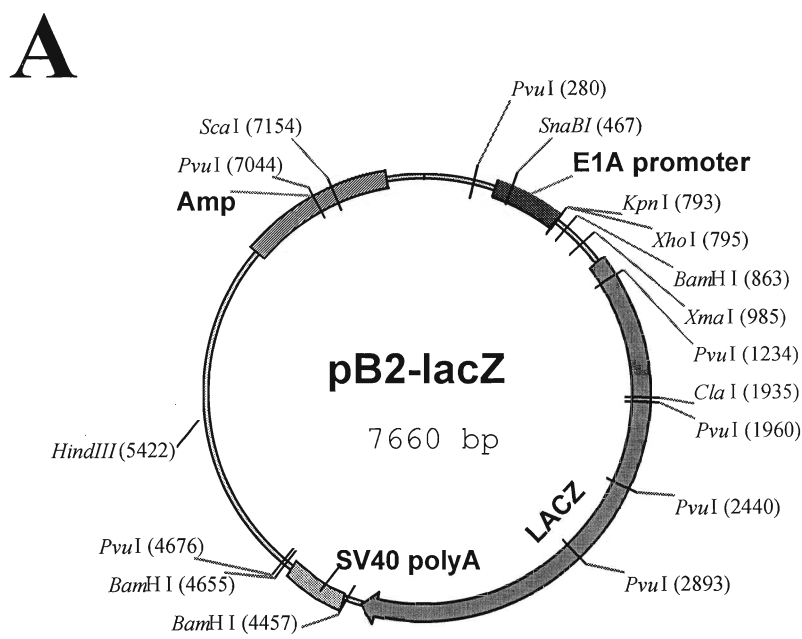
Figure 21: Restriction map and restriction endonuclease enzyme analysis of pdIE1-E-N

A) Restriction map of pdIE1EN was deduced from the DNA sequence analysis using VectorNTI.

B) Restriction enzyme analysis of pdIE1EN.

C) Restriction enzyme digest of pdIE1EN. Plasmid was confirmed by digestion with several indicative restriction enzymes, followed by separation of the fragments on a 0.9% agarose gel.

Amp, ampicillin resistance gene; **LacZ**, beta galactosidase coding sequence; **CMV**, cytomegalo virus promoter; **SV40** poly A, simian virus 40 polyadenylation signal.



B

Enzyme	HindIII	BamHI	XmaI	SnaBI
Fragment size (bp)	7660	3868 3594 198	7660	7660

C

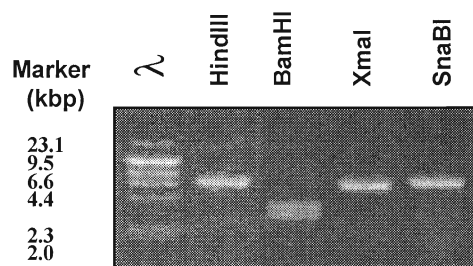


Figure 22. Restriction map and restriction endonuclease enzyme analysis of pB2lacZ.

A) Restriction map of pB2lacZ was deduced from the DNA sequence analysis using VectorNTI.

B) Restriction enzyme analysis of pB2lacZ

C) Restriction enzyme digest of pB2lacZ. Plasmid was confirmed by digestion with several indicative restriction enzymes, followed by separation of the fragments on a 0.9% agarose gel. **Amp**, ampicillin resistance gene; **LacZ**, beta galactosidase coding sequence; **CMV**, cytomegalo virus promoter; **SV40** poly A, simian virus 40 polyadenylation signal.

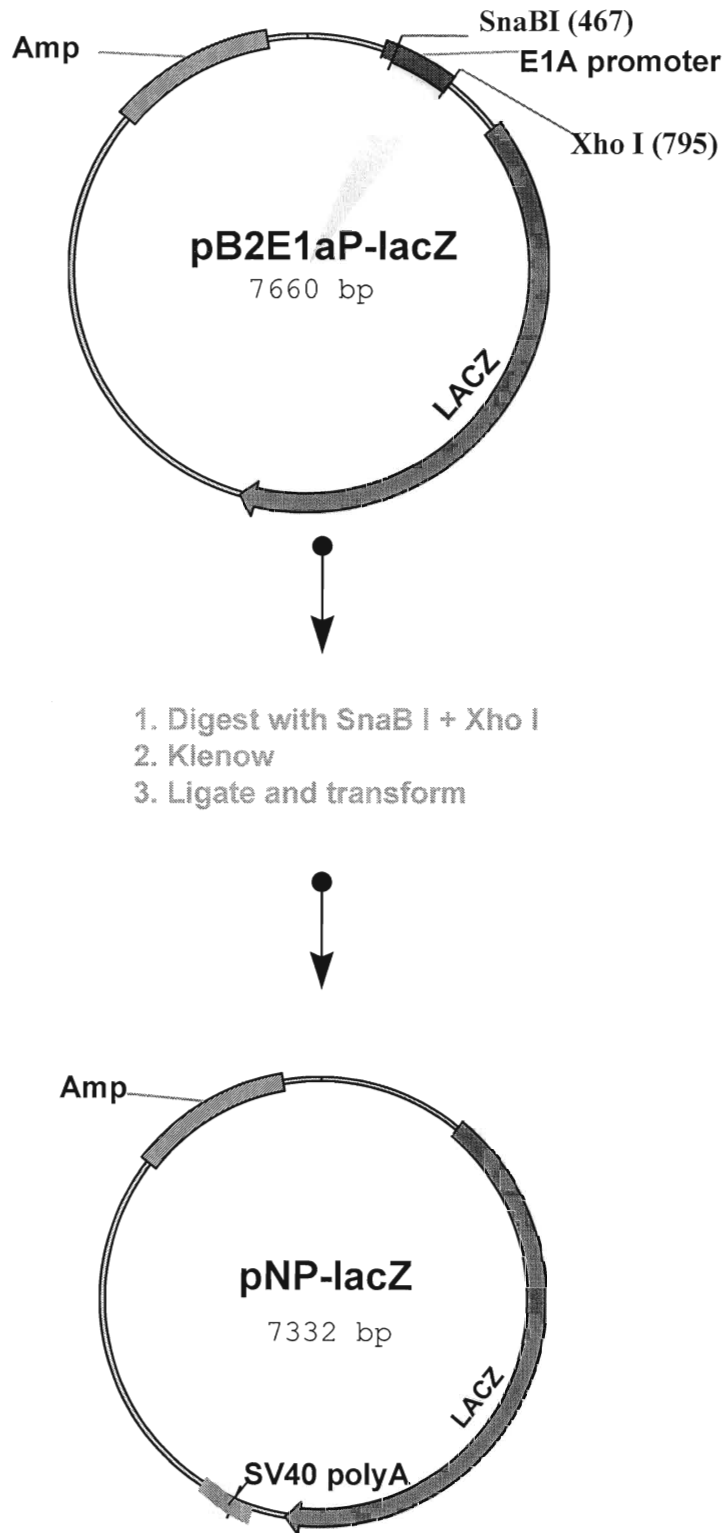


Figure 23. Schematic diagram of pNP*lacZ* construction.

In order to construct a control plasmid pNP*lacZ*, pB2*lacZ* was digested with *Sna*BI and *Xho*I restriction enzymes, positions 0.1-1.1% in BAV2. Next, the ends were made blunt using Klenow polymerase and religated.

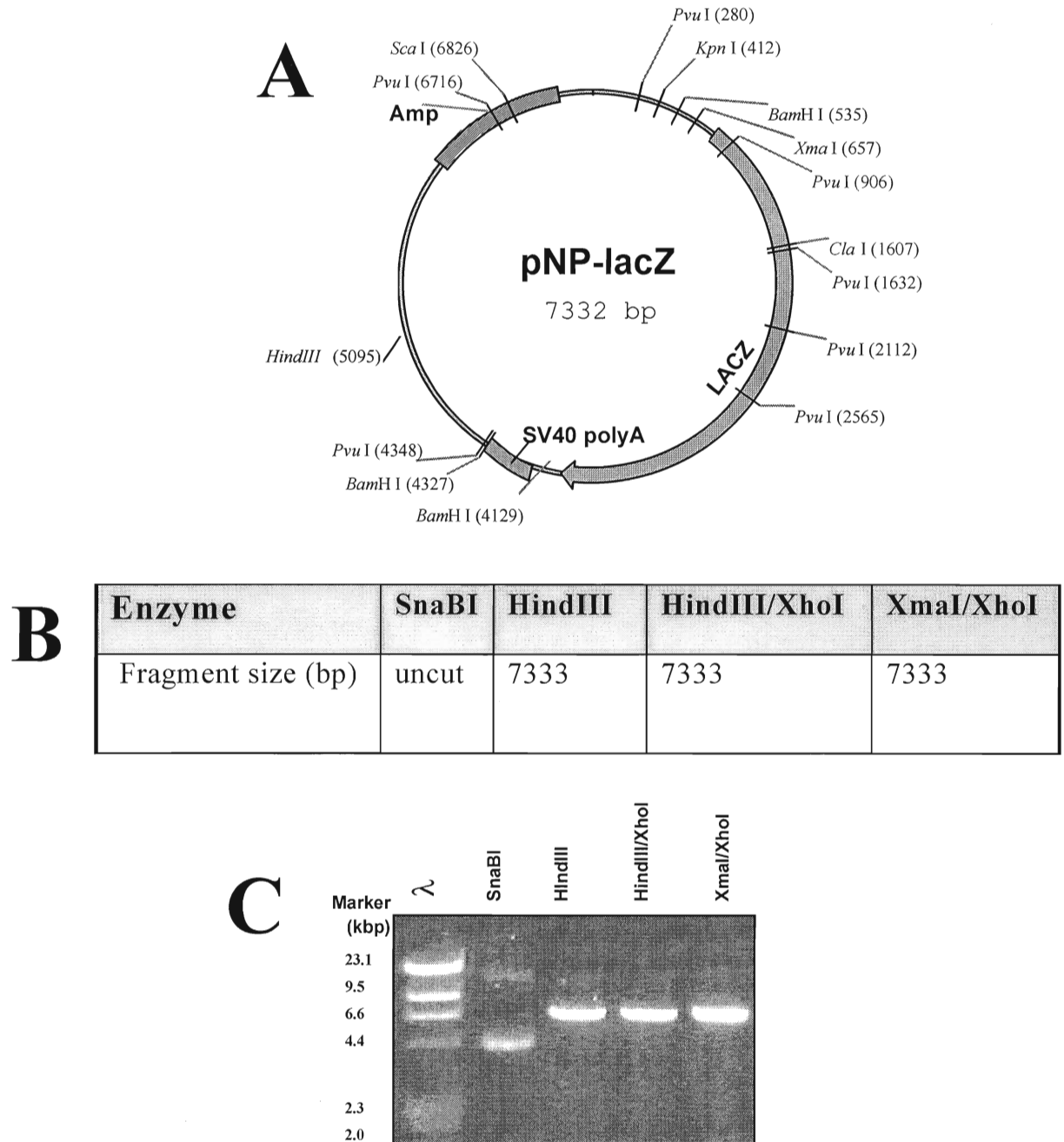


Figure 24: Restriction map and restriction endonuclease enzyme analysis of pNP $lacZ$.

A) Restriction map of pNP $lacZ$ was deduced from the DNA sequence analysis using VectorNTI.

B) Restriction enzyme analysis of pNP $lacZ$

C) Restriction enzyme digest of pNP $lacZ$. Plasmid was confirmed by digestion with several indicative restriction enzymes, followed by separation of the fragments on a 0.9% agarose gel. **Amp**, ampicillin resistance gene; **LacZ**, beta galactosidase coding sequence; **CMV**, cytomegalovirus promoter; **SV40** poly A, simian virus 40 polyadenylation signal.

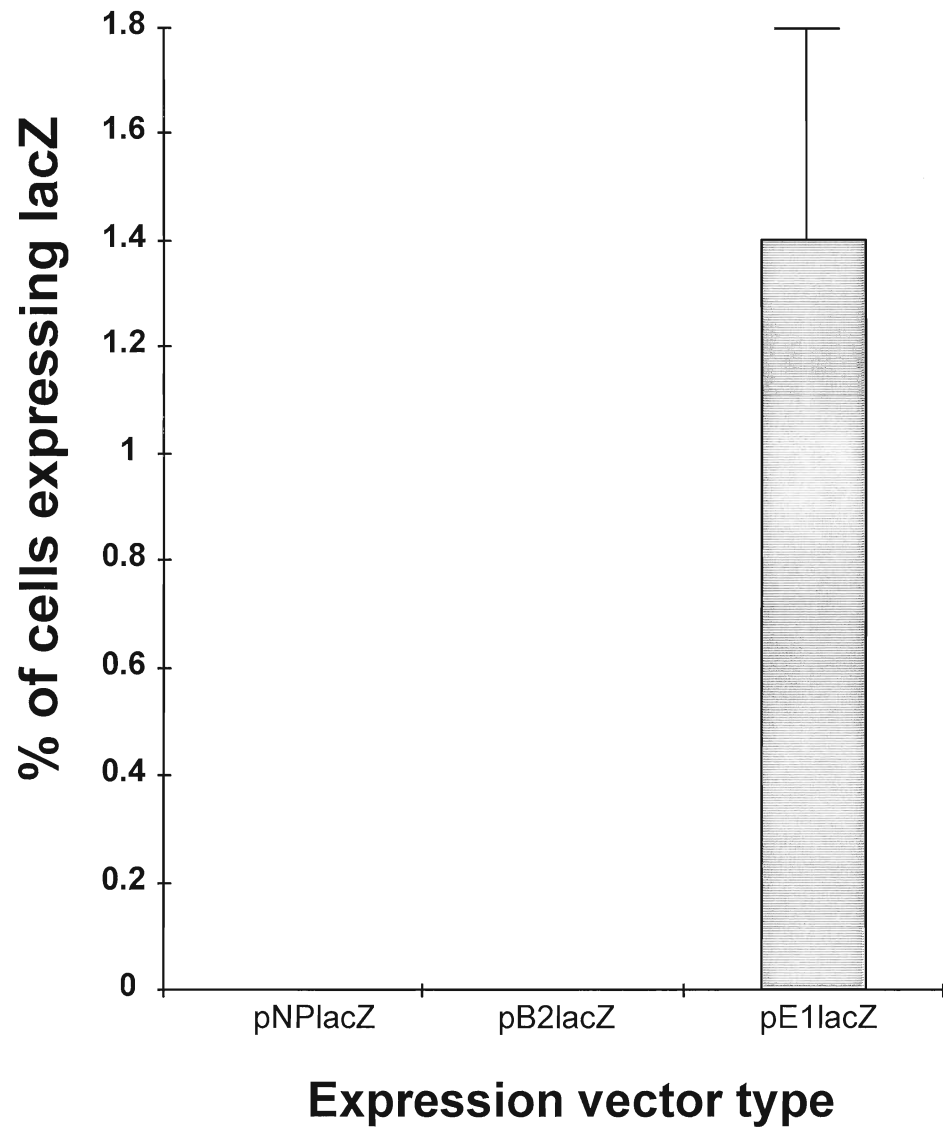
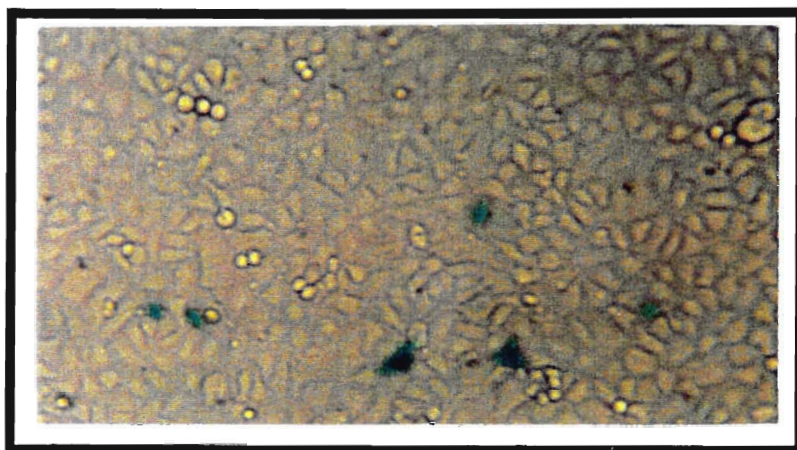


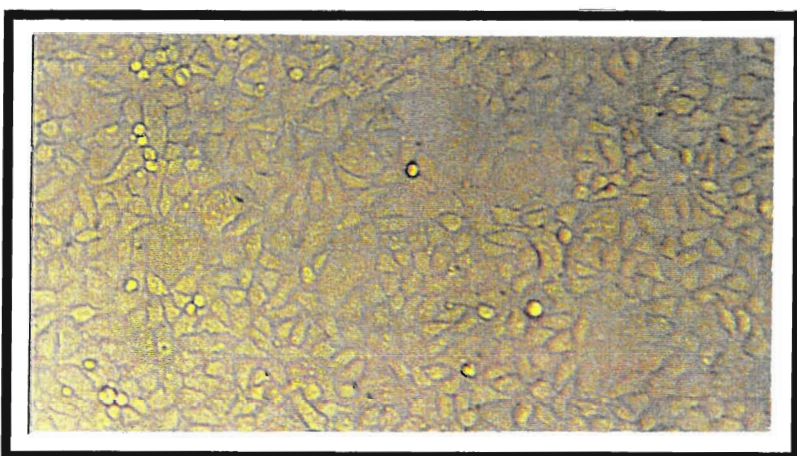
Figure 25. Analysis of Ad5 and BAV2 E1A promoter activity in HeLa cells.

The graph indicates the percentage of HeLa cells expressing *lacZ* 24 hours after transfection. Each value represents the average of two experiments, in which two dishes were used per experiment. The error bars denote one standard deviation.

A



B



C

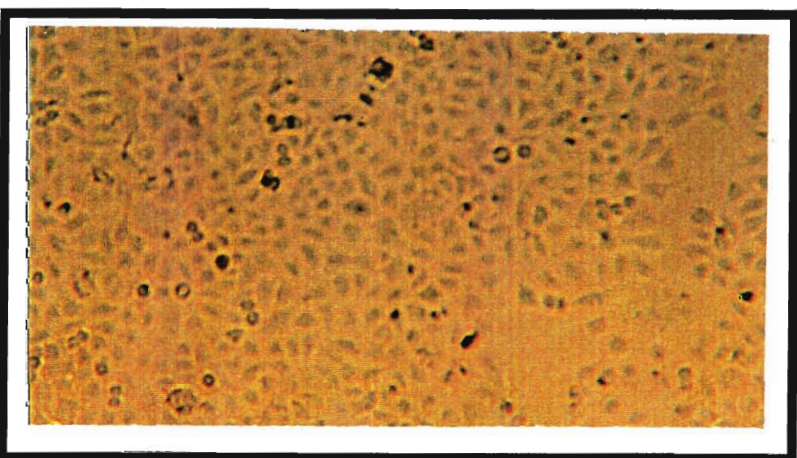


Figure 26 : Photographs of HeLa cells transfected with various *lacZ* plasmids (100X).
A) pE1*lacZ* B) pB2*lacZ* and C) pNF *lacZ*.

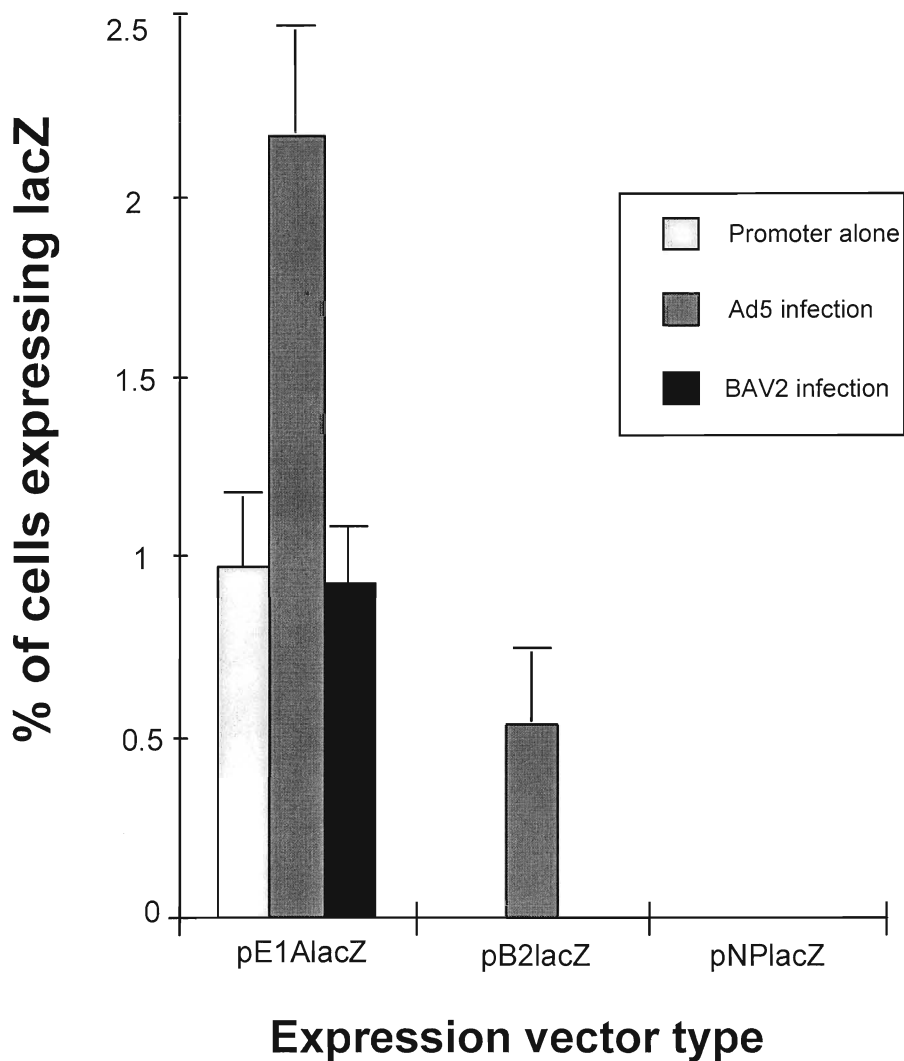


Figure 27. Analysis of Ad5 and BAV2 E1A promoter activity in infected and noninfected HeLa cells.

The graph indicates the percentage of HeLa cells expressing *lacZ* 24 hours after transfection. When Ad infected cells were used, cells were assayed 4 hours p.i. (see text for details). Each value represents the average of two experiments, in which two dishes were used per experiment. Error bars represent one standard deviation.

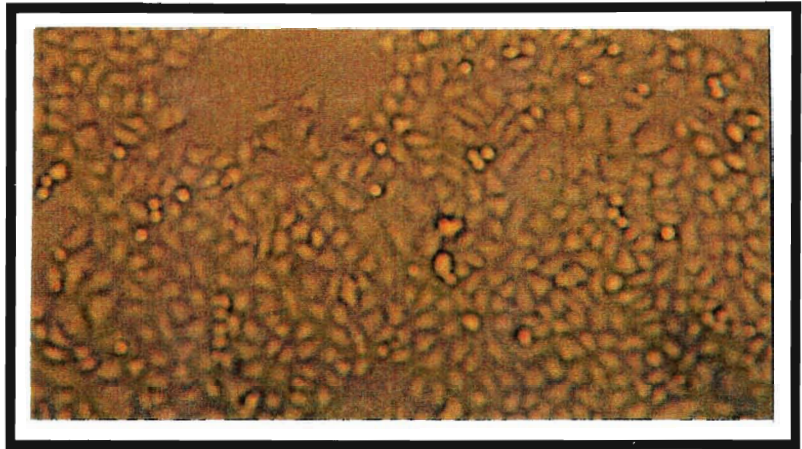
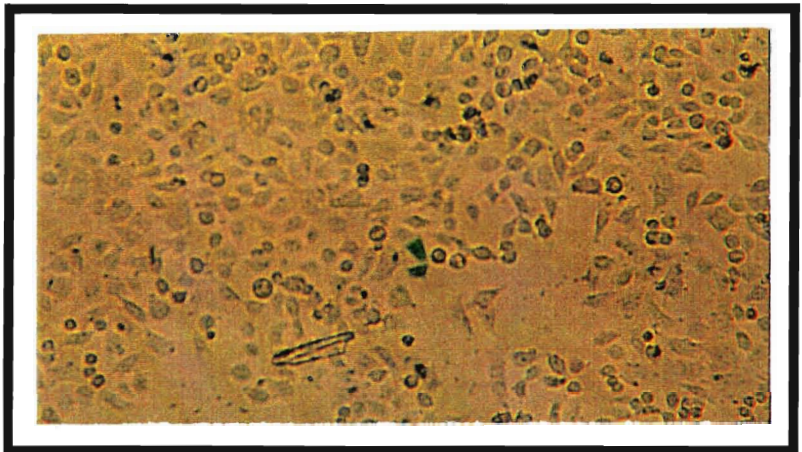
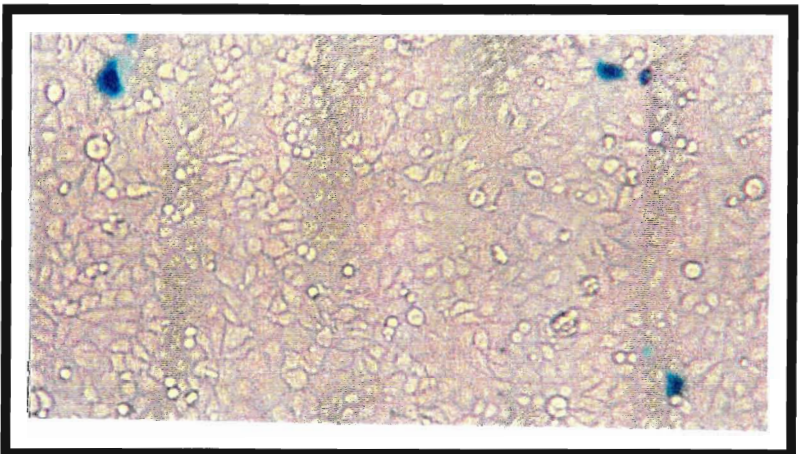
A**B****C**

Figure 28 :Photographs of Ad5 infected HeLa cells transfected with various *lacZ* plasmids (100X).

A) pNPlacZ **B)** pB2lacZ and **C)** pE1AlacZ.

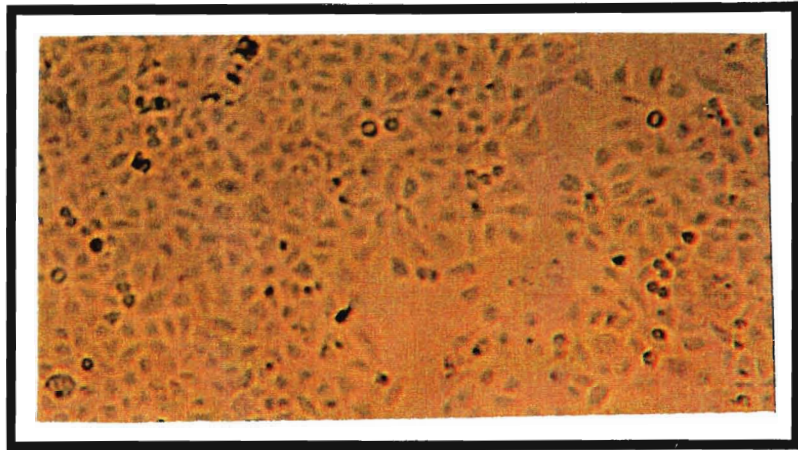
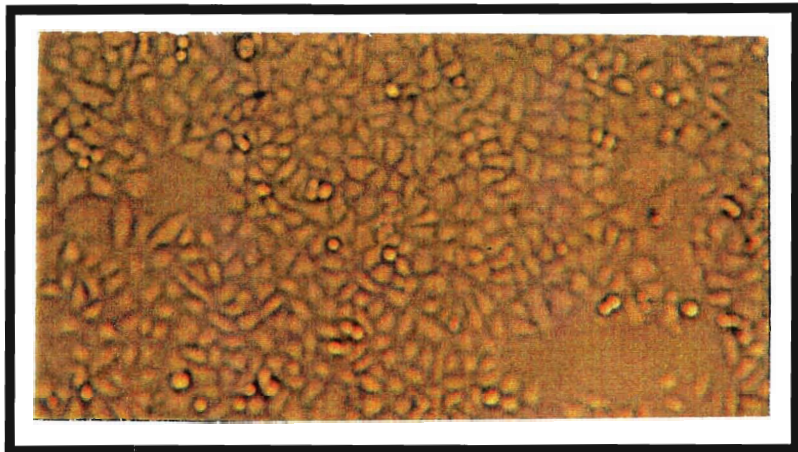
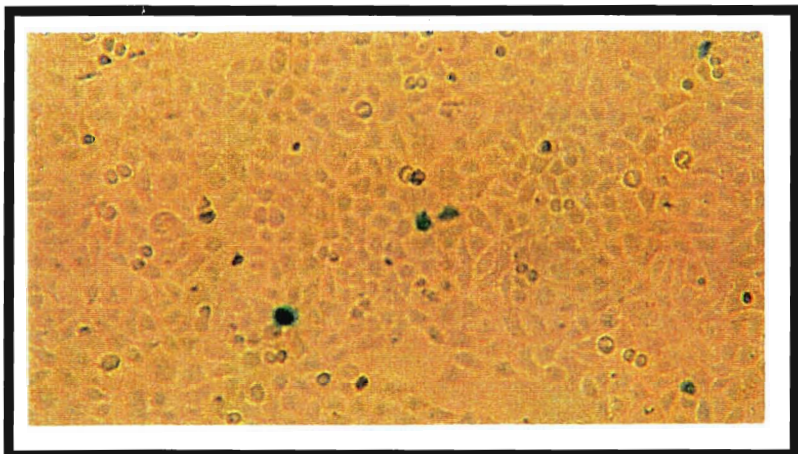
A**B****C**

Figure 29 :Photographs of BAV2 infected HeLa cells transfected with various *lacZ* plasmids (100X).

A) pNPlacZ B) pB2lacZ and C) pE1AlacZ.

3.8 Complementation of impaired BAV2 functions in HeLa cells by Ad5dIE3

It is evident from the preceding sections that BAV2 E1A region was not expressed in HeLa cells, likely because of the ineffectiveness of human cellular factors to activate the E1A promoter. Since at least 2 E1A products are required during the early phase of infection, it is likely that the block in E1A transcription in HeLa cells constitute the initial and principal reason for its failure to infect human cells. An interesting possibility might be that when the block in E1A expression is removed, then BAV2 might replicate in HeLa cells. To gain a better insight into this phenomenon, coinfection experiments between human Ad5 and BAV2 in HeLa cells were carried out.

3.8.1 BAV2 E1A transcription

First, to determine whether the defective BAV2 E1A expression in HeLa cells could be complemented by Ad5dIE3, northern analysis of the total RNA isolated from infected MDBK and HeLa cells was carried out. MDBK and HeLa cells were infected with BAV2 alone, Ad5dIE3 alone or doubly infected with BAV2/Ad5dIE3 at moi of 10, and the total RNA was extracted 12 hours p.i.. Total RNA was analyzed by Northern Blotting with ³²P labeled E1A probe (*Figure 18*). The results in *Figure 30* show that BAV2 E1A specific RNA was detected in MDBK cells infected with BAV2 alone (*lane 6*) and upon coinfection with Ad5dIE3 (*lane 5*). As expected, the E1A probe did not hybridize to total RNA isolated from BAV2 infected HeLa cells (*Figure 30; lane 2*); however, BAV2 E1A specific RNA was detected in HeLa cells doubly-infected with BAV2 and Ad5dIE3 (*Figure 30; lane 3*). The data clearly demonstrated that Ad5dIE3 functions could complement the defect in BAV2 E1A transcription in HeLa cells.

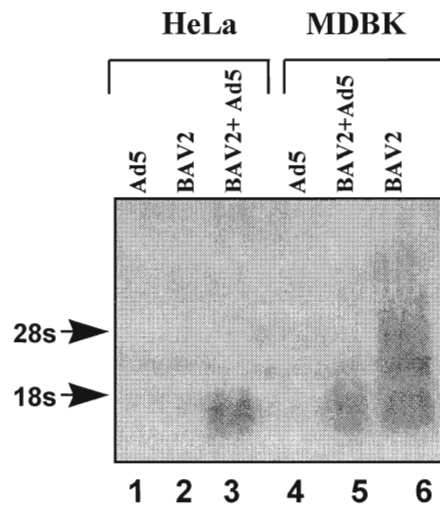


Figure 30: Transcription of BAV2 E1A region in Ad5dlE3/BAV2 doubly-infected HeLa cells.

Northern blot analysis was carried out on total RNA isolated from BAV2, Ad5dlE3 and Ad5dlE3/BAV2 infected MDBK and HeLa cells. Details of the experiment are outlined in the text and in *Materials and Methods*. Each lane contains 10 μ g of total RNA. Positions of 18s and 28s bands are indicated on the left end of the gel.

3.8.2 BAV2 DNA synthesis

We then determined whether BAV2 DNA replication occurs in HeLa cells upon coinfection with permissive Ad5dlE3. Thus, HeLa cells were infected with BAV2 alone, and doubly-infected with Ad5dlE3 and BAV2. At various times p.i., as indicated in *Figures 32* and *33*, the total DNA was extracted and cut with *EcoRI*, and fragments were separated by electrophoresis on a 1% agarose gel. After blotting, the Ad5dlE3- and BAV2- specific fragments were detected by hybridization to 32 P-labeled pJM17 which contains the complete Ad5dlE3 (*Figure 31*) or BAV2 DNA followed by autoradiography. The results are presented in *Figures 32* and *33*. As previously reported, there was no evidence for the replication of BAV2 in HeLa cells as the BAV2 DNA copy number remained constant for 72 hours. However, BAV2 DNA replication was apparent in HeLa cells coinfecting with Ad5dlE3. Both BAV2 and Ad5dlE3 DNA replication

showed the same kinetics in mixed infection (*Figure 34*). By about 12 hours p.i., BAV2 and Ad5*dIE3* DNA copy number increased by 35% and continued to increase reaching the maximum at 48 hours p.i. The apparent reduction in viral DNA at 72 hours most likely represents loss of cells at this time, rather than viral DNA degradation. Comparison of kinetics of Ad5*dIE3* DNA replication in HeLa cells coinfecting with BAV2 and HeLa cells infected with Ad5*dIE3* alone revealed some differences. In HeLa cells infected with Ad5*dIE3* alone viral DNA synthesis reached half its maximum in 7 hours as compared to 15 hours in mixed infection (*Figure 34*). The slower rate of Ad5*dIE3* DNA replication in mixed infection could be due to competition between Ad5*dIE3* and BAV2 over limited cell resources needed for viral DNA replication.

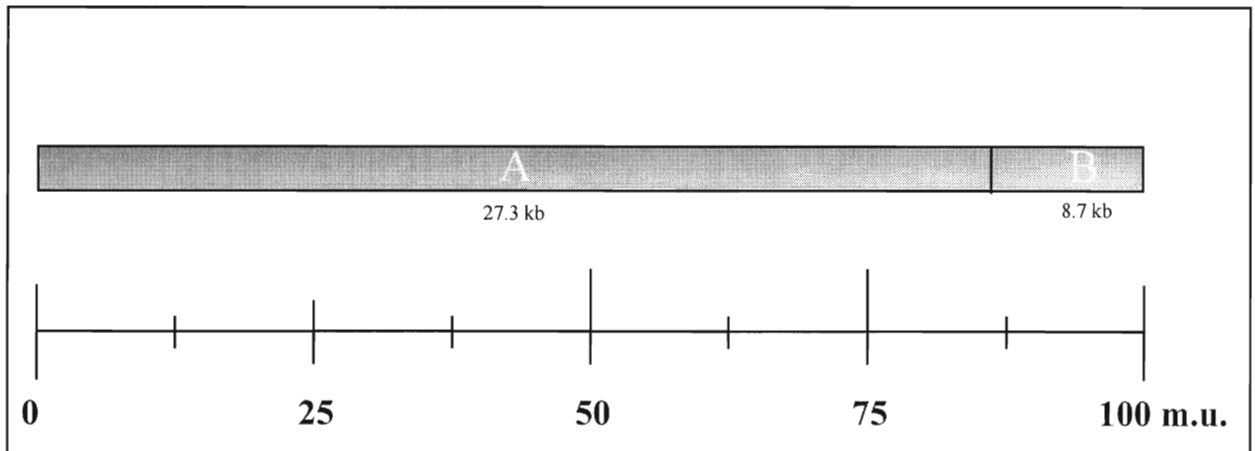


Figure 31: The *EcoRI* restriction map of Ad5*dIE3* and a length scale in map units (m.u.).

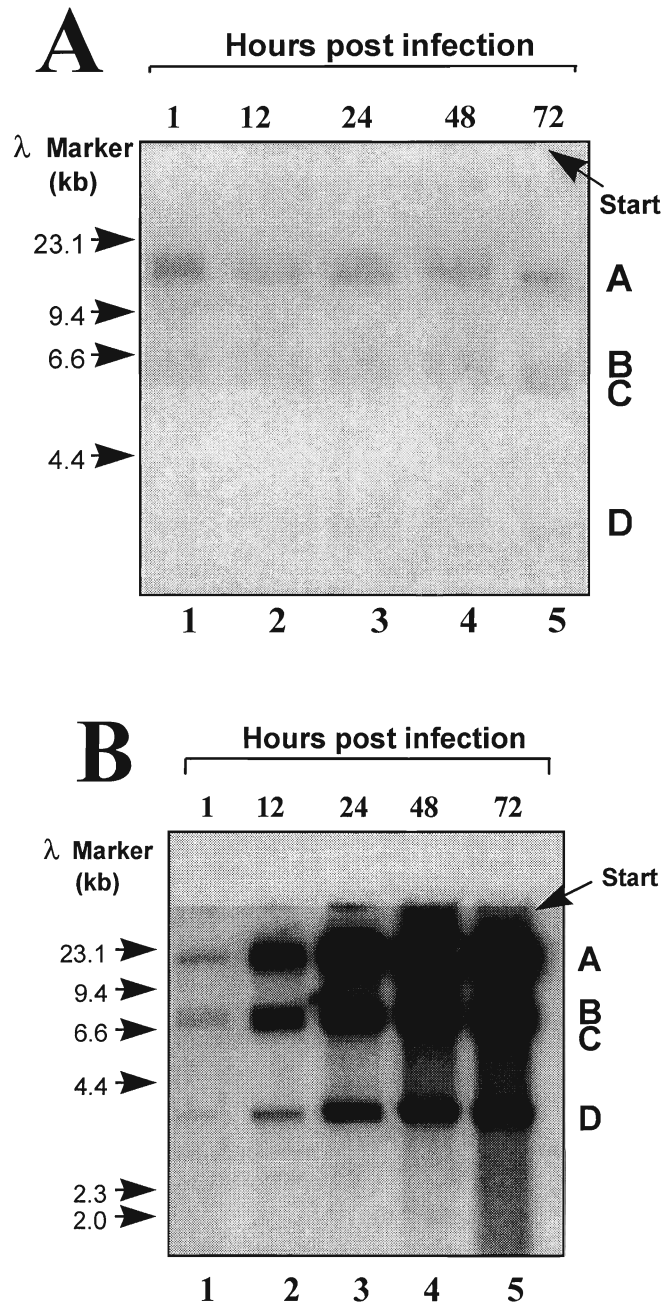


Figure 32: Complementation of the replication defect of BAV2 DNA in human HeLa cells by coinfection with Ad5dlE3.

Autoradiograms showing restriction endonuclease analysis of BAV2 DNA isolated from HeLa cells infected with **A**) BAV2 **B**) Ad5dlE3/BAV2. Membranes A and B were probed simultaneously with the same probe and the autoradiograms were exposed for the same period of time. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the figure show BAV2 DNA fragments cleaved with *EcoRI* (see Figure 13). *HindIII* fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of the figure.

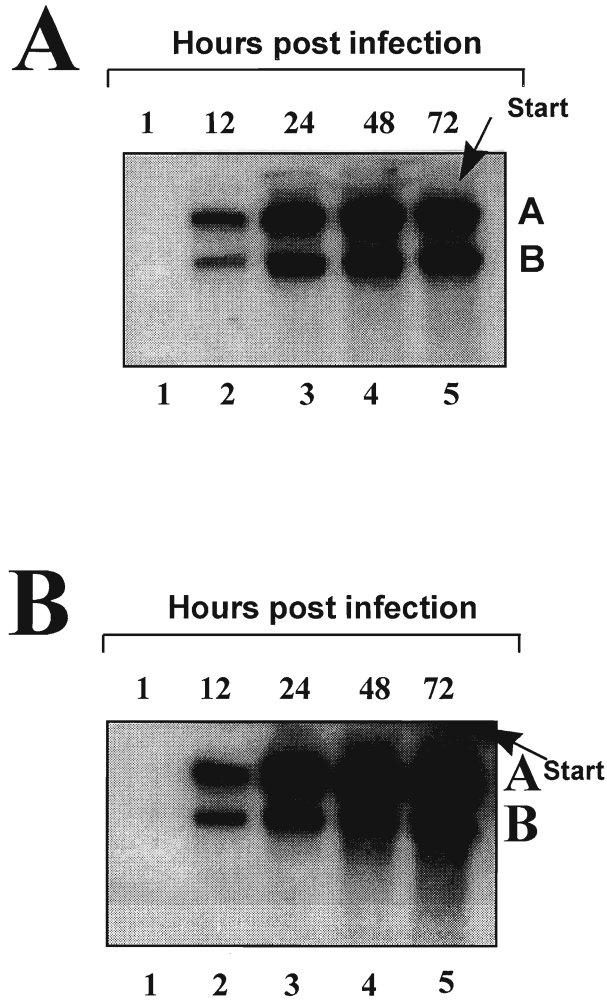


Figure 33: Replication of Ad5 DNA in human HeLa cells upon infection with Ad5dlE3 alone and coinfection with BAV2

Autoradiograms showing restriction endonuclease analysis of Ad5 DNA isolated from HeLa cells infected with **A)** Ad5dlE3 **B)** Ad5dlE3/BAV2. Membranes A and B were probed simultaneously with the same probe and the autoradiograms were exposed for the same period of time. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-B on the right side of the figure show Ad5dlE3 DNA fragments cleaved with *EcoRI* (see Figure 13).

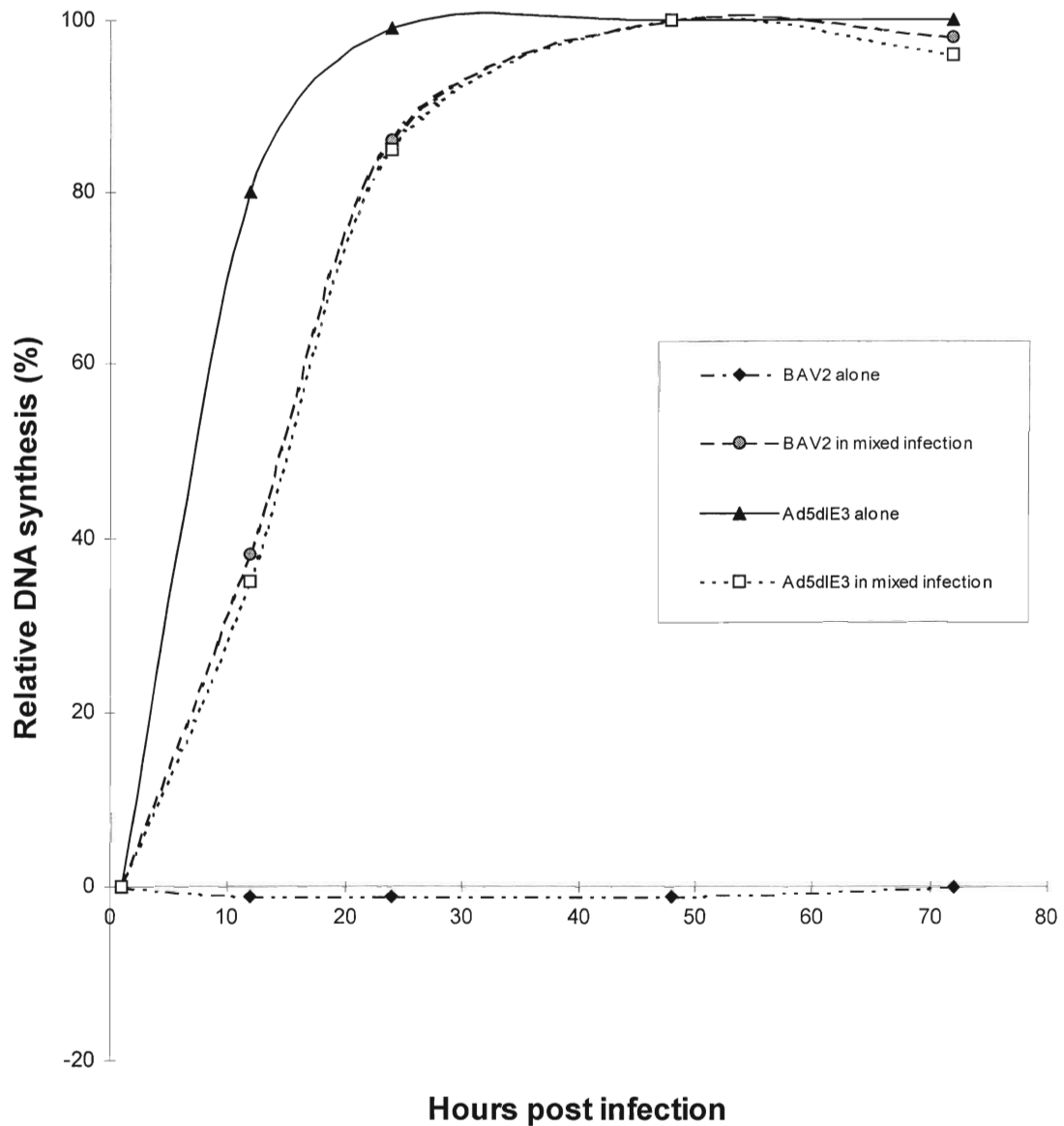


Figure 34: Kinetics of BAV2 and Ad5dlE3 DNA replication in HeLa cells

The level of BAV2 and Ad5 DNA replication was estimated by densitometry scanning of autoradiograms of DNA fragments (*Figures 32 and 33*). The intensity of lane 1 hr p.i. was subtracted from the intensity value of other lanes on the gel. The intensity of each lane was then normalized to an arbitrary percentage with the highest intensity lane being 100%.

To further show that the increase in BAV2 DNA copies in doubly-infected BAV2 and *Ad5dlE3* HeLa cells was due to viral DNA synthesis, an experiment involving hydroxyurea was carried out. In this experiment, HeLa cells simultaneously infected with BAV2 and *Ad5dlE3* were treated with no drug or with 10 mM HU previously shown to inhibit BAV2 DNA synthesis in permissive MDBK cells (*Figure 35*). The total genomic DNA was isolated at different times p.i. as indicated in *Figure 35*. Southern analysis was carried out as discussed above. Hydroxyurea inhibited BAV2 DNA synthesis at 12 hours p.i. (*Figure 35; lane 3*) in HeLa cells coinfecting with *Ad5dlE3* suggesting that BAV2 DNA synthesis takes place in this system.

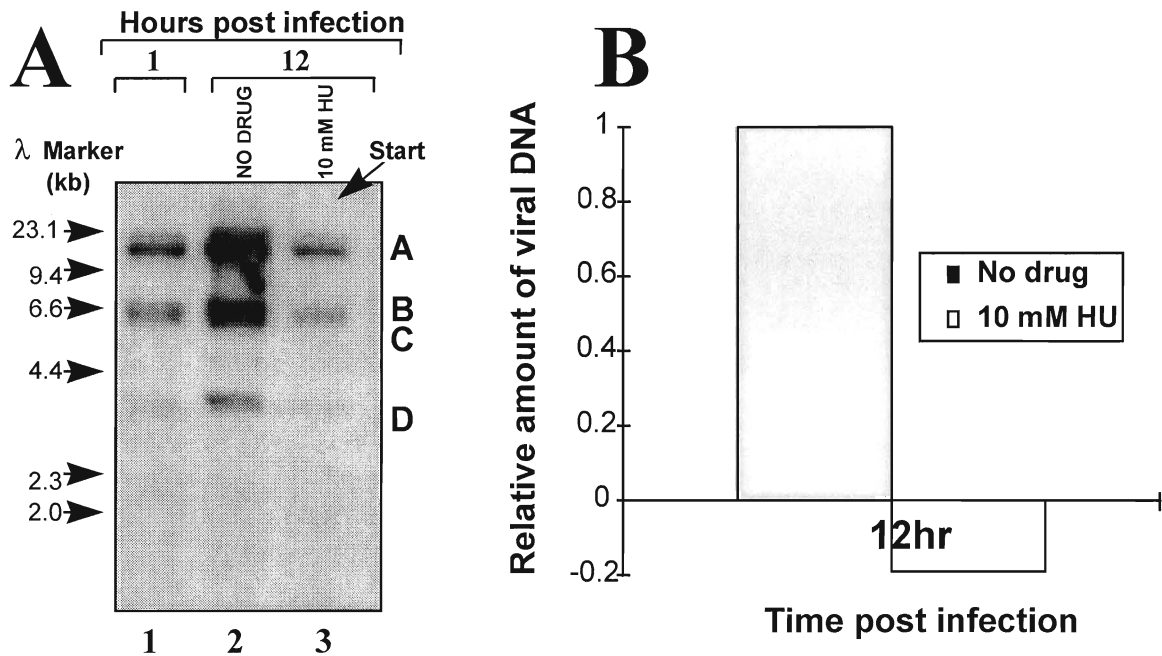


Figure 35: BAV2 DNA synthesis in HeLa cells doubly-infected with BAV2 and *Ad5dlE3*

A) Autoradiogram showing restriction endonuclease analysis of BAV2 DNA isolated from doubly-infected HeLa cells treated with no drug or 10 mM hydroxyurea. Details of the experiment are outlined in the text. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the figure show BAV2 DNA fragments cleaved with *EcoRI* (see *Figure 13*). *HindIII* fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of the figure.

B) The bands were analyzed by densitometry scanning. The intensity of lane 1 was subtracted from the intensity value of other lanes on the gel. The intensity of each lane was then normalized to an arbitrary value of 1 designated for lane 2.

3.8.3 BAV2 multiplication

To determine the extent to which *Ad5dIE3* complements defective BAV2 infection of HeLa cells, the possible synthesis of BAV2 virions in double-infected HeLa cells was investigated. Since MDBK cells are permissive to *Ad5dIE3* infection the production of BAV2 virions could not be tested by titration on MDBK cell layers. Hence, another test was used. In this experiment, HeLa cells were infected with *Ad5dIE3* alone and doubly infected with *Ad5dIE3* and BAV2. Also, MDBK cells were infected with BAV2 serving as a positive control. After completion of cytopathic effect (72 hours p.i.) infected cells were centrifuged at 6000 rpm for 10 minutes. Following centrifugation, supernatant was spun down at 24,000 rpm for 90 minutes to collect newly produced virions released by lysed cells. The DNA was then extracted from virions as described in *Materials and methods*, digested with XbaI, separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with ³²P labeled BAV2 DNA and ³²P labeled pJM17. The results (*Figure 36*) demonstrated that BAV2 virions replicated in MDBK cells as expected for a permissive system (*Figure 36A; lane 1*). In contrast, in HeLa cells doubly infected with *Ad5dIE3* and BAV2, there was no evidence for the presence of newly produced BAV2 virions (*Figure 36A; lane 3*). On the other hand, *Ad5dIE3* virions were produced in both HeLa cells infected only with *Ad5dIE3* and those coinfecting with BAV2 (*Figure 36B; lanes 2 and 3*). Overall, these experiments clearly indicate that *Ad5* complementation of BAV2 defects in HeLa cells was such that BAV2 DNA synthesis occurred but no new virion particles were packaged.

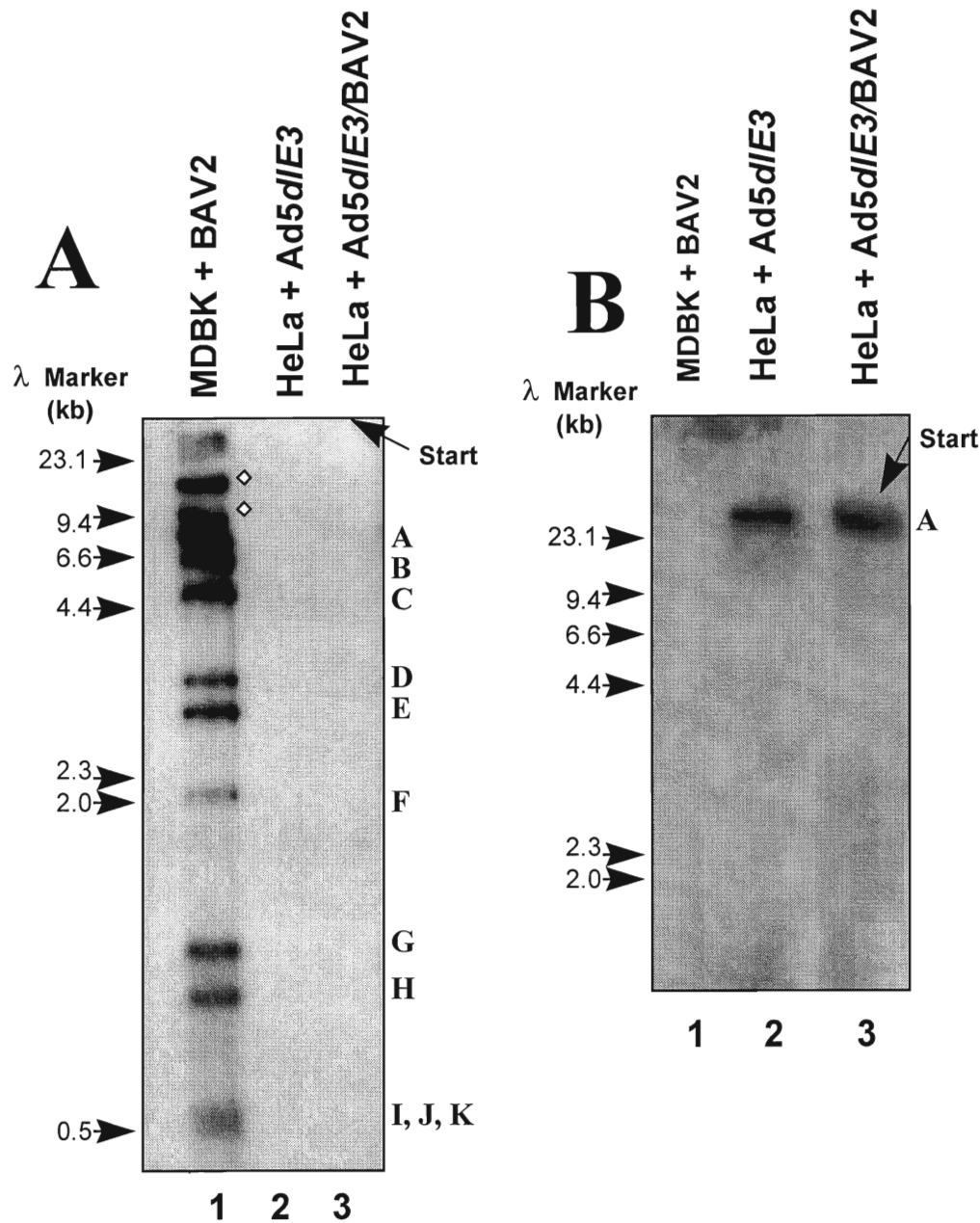


Figure 36: Absence of newly produced BAV2 virions in HeLa cells coinfecting with *Ad5dlE3*.

HeLa cells were infected with *Ad5dlE3* alone or *Ad5dlE3/BAV2* and the production of new A) BAV2 virions and B) *Ad5dlE3* virions was determined as described in the text. BAV2 infected MDBK cells served as a positive control. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. Letters on the right side of both *Figures* show BAV2 and *Ad5dlE3* DNA fragments cleaved with *Xba*I. *Hind*III fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of both *Figures*. \diamond denotes partially digested BAV2 DNA fragments.

3.9 Complementation of BAV2 DNA replication in HeLa cells by functions in the E1 region of Ad5 DNA

Our previous experiments clearly demonstrated that BAV2 DNA replication and E1A transcription which are totally defective in HeLa cells can be elicited by Ad5 functions. Next, we needed to examine which Ad5 functions are complementing BAV2 defects in HeLa cells. Number of studies on abortive infections of adenoviruses have shown that the complementing functions reside mostly in the E1 region of the Ad5 genome (Klimkait *et al.*, 1987; Klimkait *et al.*, 1985; Mautner *et al.*, 1990; see literature review in this thesis). Hence, the complementing function of Ad5 E1A region in BAV2/Ad5 infected HeLa cells was examined. In this experiment, HeLa cells were doubly-infected with BAV2 and a deletion mutant of Ad5 named Ad5dlE1E3 which carried deletions in the E1 region. The total genomic DNA was isolated at indicated times post infection (*Figure 37*) and the BAV2 DNA replication was then investigated by standard blotting techniques. According to the results presented in *Figure 37*, Ad5dlE1E3 failed to complement BAV2 DNA replication. In conclusion, the Ad5 function operative in complementing BAV2 DNA replication was located in the E1 region of Ad5.

3.10 BAV2 DNA replication in 293 cells

Although our results revealed that E1 region of Ad5 DNA is responsible for complementing BAV2 DNA replication in doubly-infected HeLa cells it is not known, however, whether the E1 region is exclusively responsible for complementation. In order to answer this question, BAV2 DNA replication was investigated in 293 cells which constitutively express E1 region of Ad5. Thus, 293 cells were infected with BAV2 and doubly-infected with BAV2/Ad5dlE1E3 at 10 moi and the genomic DNA was isolated 1, 24, 48 and 72 hours p.i. Southern analysis (*Figure 38A*) revealed no increase in BAV2 DNA copy number in BAV2 infected 293 cells over 48 hours p.i. suggesting that BAV2 failed to replicate its DNA in 293 cells. However, BAV2 DNA copy

number in doubly-infected 293 cells increased 24 hours p.i. and continued to increase reaching the maximum at 48 hours p.i. (*Figure 38B*). These results imply that E1 is necessary but not sufficient to avert the failure of BAV2 to undergo DNA replication in HeLa cells.

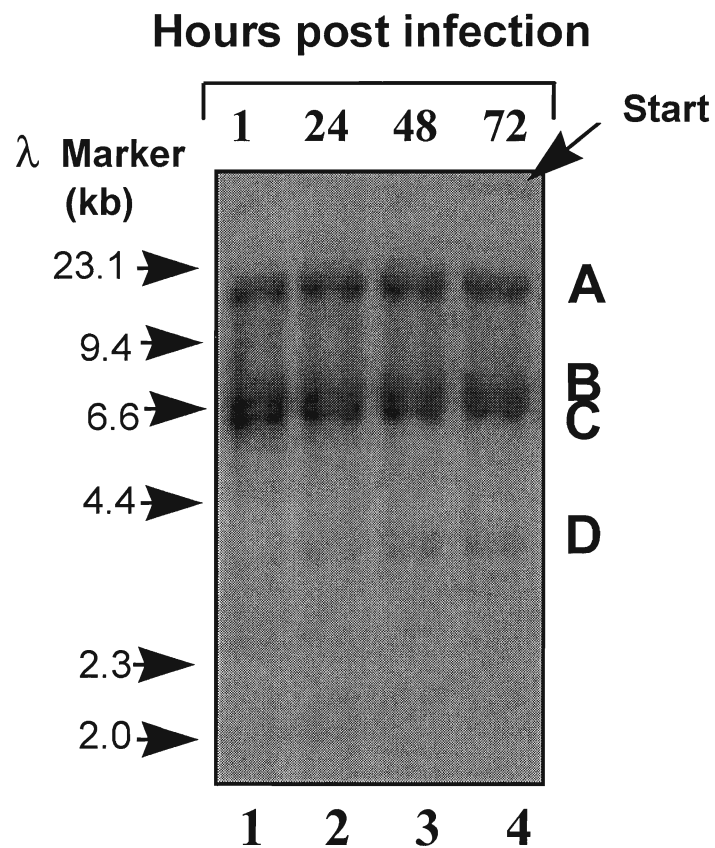


Figure 37: Absence of BAV2 DNA synthesis in HeLa cells coinfecting with Ad5dIE1E3.

Autoradiogram showing restriction endonuclease analysis of BAV2 DNA isolated from 293 cells infected with BAV2. Details of the experiment are outlined in the text above. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the figure show BAV2 DNA fragments cleaved with *EcoRI* (see *Figure 13*). *HindIII* fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of the figure.

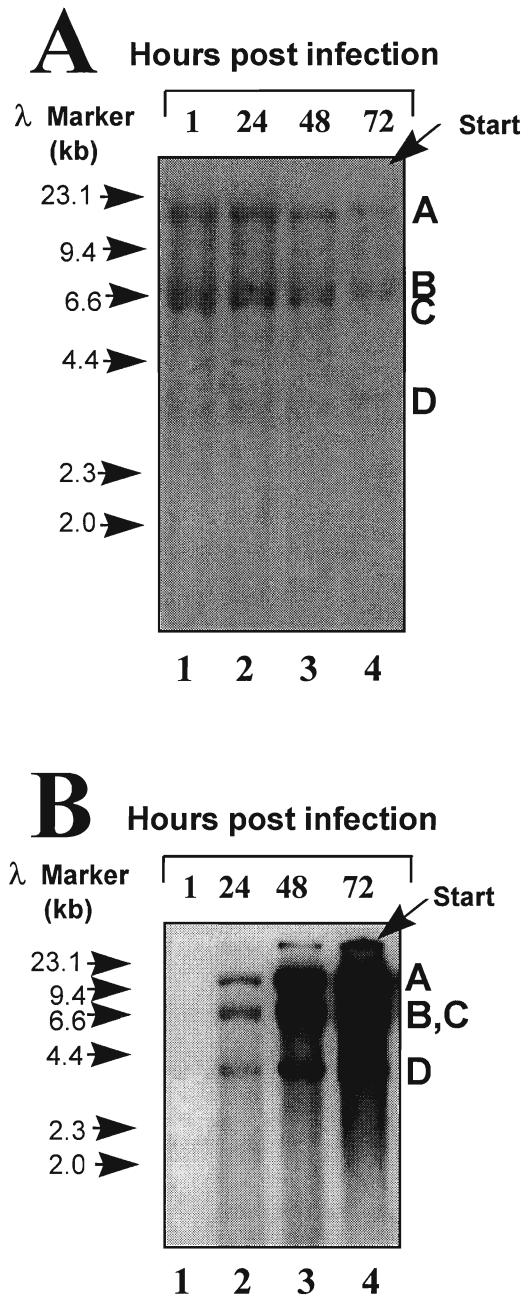


Figure 38: BAV2 DNA replication in 293 cells.

Autoradiograms showing restriction endonuclease analysis of BAV2 DNA isolated from 293 cells infected with **A)** BAV2 **B)** Ad5*dlE1E3*/BAV2. Details of the experiment are outlined in the text above. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the figure show BAV2 DNA fragments cleaved with *Eco*RI (see *Figure 13*). *Hind*III fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of the figure.

3.11 Persistence of BAV2 genome in infected HeLa cells prior to passaging

Our previous experiments clearly demonstrated that BAV2 DNA replication is not supported by HeLa cells, however, the viral DNA was still present in infected HeLa nuclei. Hence, it was of interest to examine how long this DNA persisted in HeLa cells. Thus, HeLa cells were infected with BAV2 at 10 moi and harvested at 1, 12, 24, 48, 72 and 96 hours p.i.. The total genomic DNA was prepared from infected cells, digested with *EcoRI*, and analyzed as outlined above. *Figure 39* shows that BAV2 genome was present in an intact form in HeLa cells over a period of 96 hours. Results further revealed that there was no discernible increase in viral DNA copy number in HeLa cells over a period of 96 hours. This suggests the absence of viral DNA synthesis in HeLa cells and is in agreement with hydroxyurea experiments discussed earlier. There was a decrease in viral DNA copy number starting 24 hours p.i. (*Figure 39A; lane 3*), however, from that point on there was no apparent decrease in BAV2 DNA copy number.

3.12 Persistence of BAV2 genome in infected HeLa cells as a result of passaging

Since, BAV2 DNA did not replicate in HeLa cells one could assume that it would eventually get lost with subsequent cell passaging. To study the effect of passaging on BAV2 DNA persistence and stability in HeLa cells, infected cells from the above experiment which were passaged every four days starting at 96 hours p.i. and the total genomic DNA was extracted after 1st, 3rd, 5th and 7th passage. DNA analysis was carried out as described above. BAV2 sequences were no longer detectable by high stringency hybridization after the first passage of infected HeLa or Chang Liver cells (*Figure 40; lane 3*).

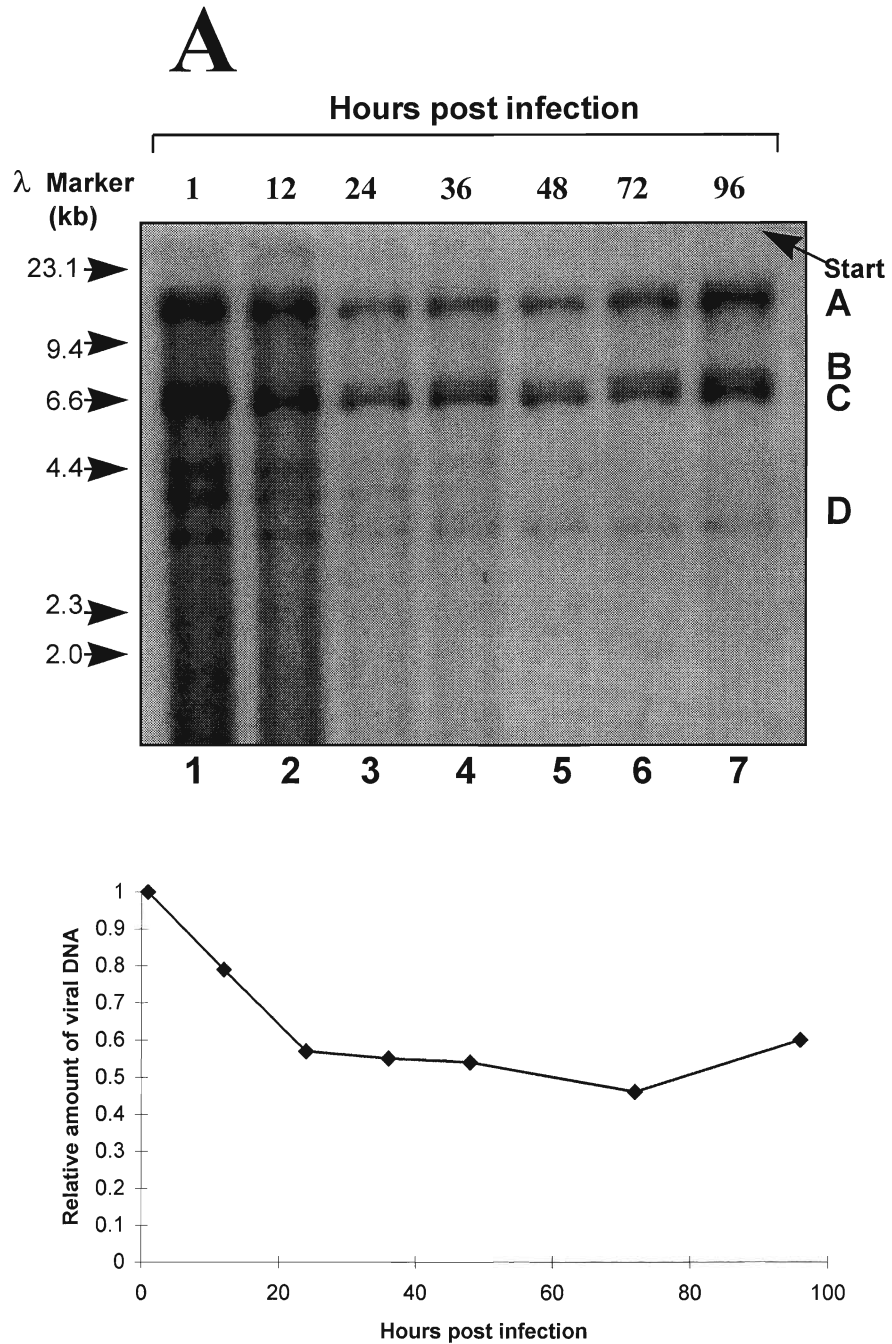


Figure 39. Persistence of BAV2 DNA in HeLa cells

A) Autoradiogram showing restriction endonuclease analysis of BAV2 DNA isolated from infected HeLa cells. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the figure show BAV2 DNA fragments cleaved with *EcoRI* (see Figure 13). *HindIII* fragments of λ phage DNA were co-electrophoresed as size markers and their positions are indicated to the left of the figure.

B) Histogram showing the relative amount of viral DNA expressed in arbitrary units representing the ratio between the intensity of all lanes to the intensity of lane 4.

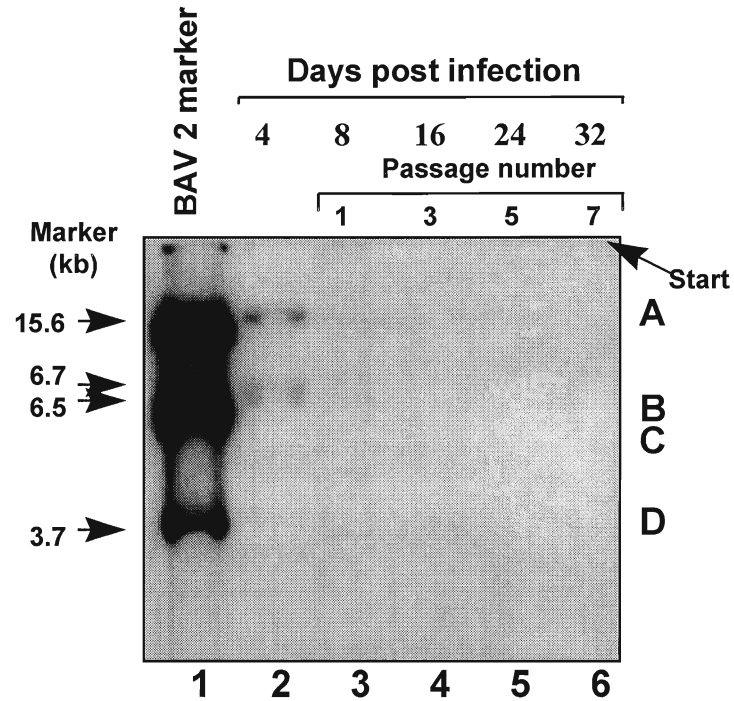


Figure 40: Effect of passage number on persistence of BAV2 genome in HeLa cells.

A) Autoradiogram showing restriction endonuclease analysis of BAV2 DNA isolated from infected HeLa cells. Details are explained in the text. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. A-D on the right side of the figure show BAV2 DNA fragments cleaved with *EcoRI* (see Figure 13). *EcoRI* fragments of BAV2 DNA (lane 1) were co-electrophoresed as size markers and their positions are indicated to the left of the figure.

3.13 Persistence of E1A region in passaged HeLa cells

Since, southern blot analysis has limited sensitivity in detecting very low levels of copies per cell, DNA from passaged infected HeLa cells was further analyzed by PCR. In this experiment, BAV2 E1A region (Figure 41) was amplified in 1 μ g of infected HeLa DNA extracted at various passages. Amplified DNA was subjected to agarose gel electrophoresis. Figure 42A shows the

results of the amplification. The amplified fragments were ~920 bp and were found in BAV2 control DNA and in HeLa DNA passaged up to 5 times.

To further identify the amplified products as BAV2 E1A region, the same gel was transferred to a nitrocellulose membrane and probed with BAV2 E1A *HpaI/SnaBI* fragment isolated from pBB12 (*Figure 42B*). This analysis confirmed the presence of BAV2 E1A region in cells passaged up to 5 times (*Figure 42B; lanes 4-6*). However, BAV2 E1A region disappeared after 7th passage of infected HeLa cells (*Figure 42B; lane 7*).



Figure 41: PCR primers used to amplify BAV2 E1A region

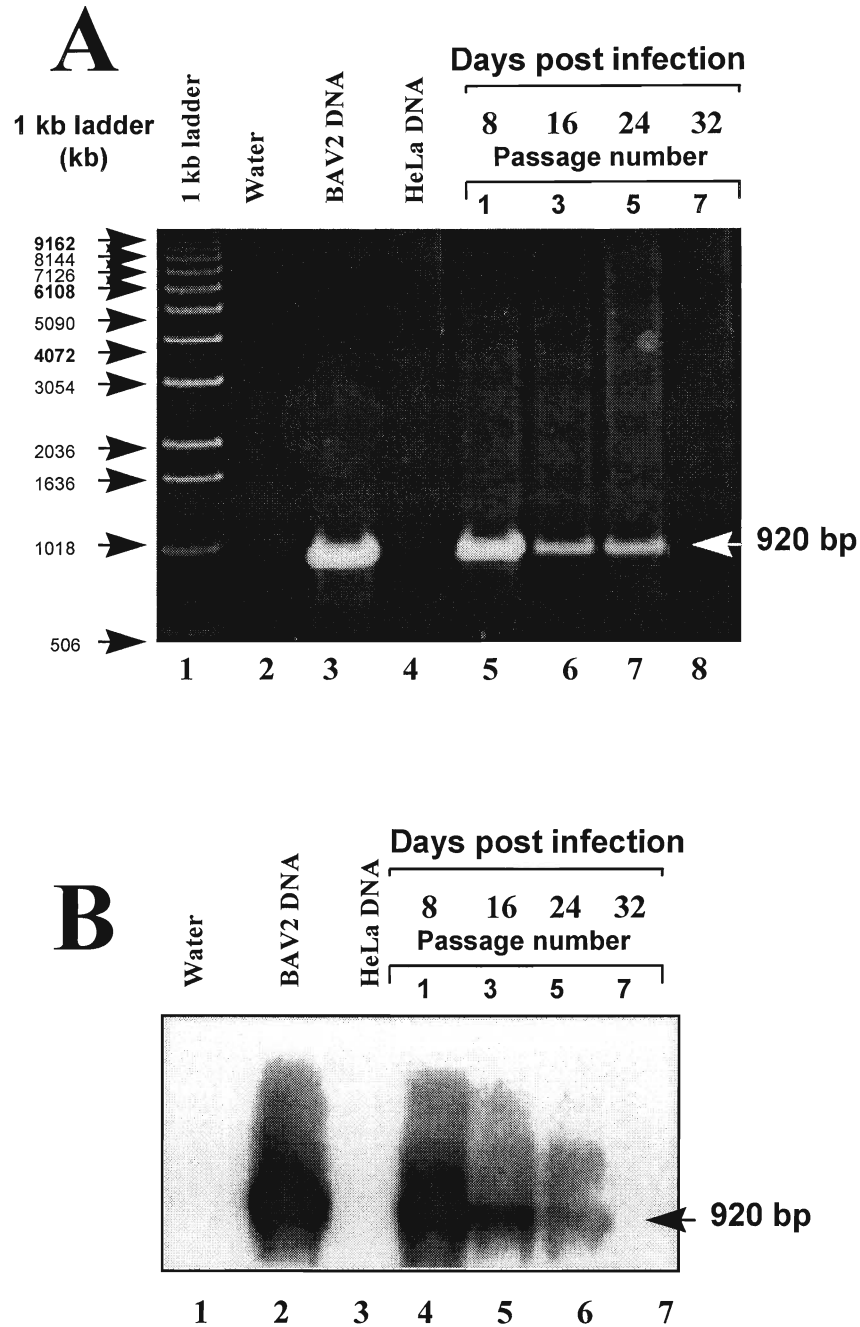


Figure 42: Persistence of BAV2 E1A region in passaged HeLa cells

A) Photograph of the ethidium bromide stained agarose gel showing amplified BAV2 E1A region (478-1398 bp) in 1µg of infected HeLa DNA isolated at various passages. The origin and type of DNA electrophoresed in each slot is described by the respective abbreviations on top of each lane. Three controls (1ng of BAV2 DNA, water and 1µg of noninfected HeLa DNA) were included. Size marker (1 kb ladder) was co-electrophoresed and the positions or fragments are indicated to the left of the figure.

B) Autoradiogram of gel A. PCR products were probed with ³²P labeled 50-1092 BAV2 E1A fragment. Legend is same as above.

4. DISCUSSION and CONCLUSIONS

Adenoviruses are among the most extensively examined gene delivery vectors for human gene therapy and vaccine development. Most of the Ad vectors constructed for human use are based on human Ad2 and Ad5 serotypes. This generation of adenoviral vectors, however, suffers from a number of important limitations preventing the realization of their full potential. One limitation is the existence of natural immunity against human Ads in the majority of the human population, thus greatly impairing the administration of Ad-based vectors. Another problem lies in Ad5's ability to cause minor respiratory illnesses making it less useful in immunocompromised recipients. Furthermore, expression of late proteins by Ad5 vectors induces host cellular immune response resulting in the loss of transgene expression. These limitations have led several investigators to seriously consider development of nonpermissive adenovirus vectors. OAV (Khatri *et al.*, 1997), CAV2 (Klonjkowski *et al.*, 1997) and BAV3 (Mittal *et al.*, 1995) are currently being studied as potential vectors for use in human gene therapy and vaccine development.

Similarly, this laboratory is interested in developing nonpermissive BAV2 based vector for the same purpose. Previous studies on BAV2 vector focused mainly on genetic engineering of BAV2. However, to use BAV2 in humans other factors must be considered including BAV2 host range. Thus, the present study focused on the host range of BAV2 by determining the extent of BAV2 multiplication in HeLa cells.

According to the results depicted in *Figures 7 and 8*, HeLa cells appeared to be nonpermissive for BAV2 growth. No detectable amount of infectious virus production was observed in BAV2-infected HeLa cells during 96 hours of infection. These findings indicated that the cycle's eclipse period might extend beyond 96 hours or simply that the virus completely fails to replicate. In

fails to replicate. In addition, we showed that the block to virus replication could not be overcome by increasing the moi, suggesting that viral gene dosage does not affect BAV2 replicative events in HeLa cells. These results are not surprising since adenoviruses are highly species-specific. Although rare, heterologous infections with human Ads have been documented in both cell culture studies and animal models (Romanowski *et al.*, 1998; Martins, 1995). Interestingly, however, studies investigating the host range of non-human adenoviruses revealed that most of these Ads do not grow in human cell lines. In particular, CAV2 and OAV were found to undergo abortive infection in a number of human cell lines (Khatri *et al.*, 1997; Klonjowski *et al.*, 1997).

To determine where the block(s) to replication occurred, the abortive infection of human HeLa cells with BAV2 was analyzed in considerable detail. Since Ad DNA replication marks the end of early transcription and the beginning of late transcription it provides an ideal marker for determining whether the restriction in replication occurs at an early or late step. The ability of BAV2 to undergo DNA synthesis in HeLa cells was determined by experiments using hydroxyurea to block formation of endogenous replication complexes. The results shown in *Figure 17* indicated that BAV2 DNA replication did not occur in non-permissive HeLa cells, suggesting the presence of an early block in BAV2 DNA replicative cycle. A number of early events in adenovirus life cycle could contribute to the defect in DNA replication. These include attachment, internalization, transport to the nuclei and early gene expression. Hence, all aspects of the BAV2 replicative cycle were studied in detail.

The cellular permissiveness and tissue tropism of Ads depends largely on the first step in adenovirus infection: viral attachment. Adenovirus attachment occurs through the interaction of the viral fiber-knob protein and high-affinity cellular receptors, for which a candidate has recently been identified for human Ads (CAR) (Bergelson *et al.*, 1997).

The expression of these high-affinity receptors are strongly correlated with the susceptibility of the cell to infection (Pickles *et al.*, 1998). Thus, it was of interest to determine whether BAV2 abortive infection of HeLa cells is perhaps the result of the defects in BAV2 attachment to HeLa cells. To this end, we used radiolabeled virus particles to demonstrate that BAV2 successfully attaches to the surface of HeLa cells. Interestingly, however, the number of attached BAV2 was significantly lower in HeLa cells as compared to permissive MDBK cells (*Figures 10, 11 & 12*). The explanation of the reduced number of attached BAV2 particles on HeLa cells depends largely on the mode of BAV2 attachment which can be either through fiber-receptor interaction or non specific fiber independent route. Since these experiments were not designed to reveal the mode of BAV2 attachment to HeLa cells several possible explanations are nonetheless proposed to explain the reduced number of BAV2 particles on HeLa cell surface keeping in mind both modes of BAV2 attachment to HeLa cells.

First, if we assume that HeLa cells do not have high affinity receptors specific for BAV2 then the attachment of BAV2 fiber to HeLa cells might occur via other receptors, such as receptors for human Ad. It has been reported, however, that the cell attachment domains in adenovirus fibers varies considerably among different adenovirus subgroups, suggesting that the binding to receptors specific to other Ad subgroups is unlikely (reviewed by Chroboczek *et al.*, 1995). However, the ultimate test for this hypothesis would be a competition assay in which BAV2 attachment to HeLa cells is measured following infection of HeLa cells with an excess of unlabeled human Ad. If BAV2 and human Ad compete for the same receptor BAV2 attachment to HeLa cells will be competed against by increasing amounts of human Ad particles.

Alternatively, BAV2 attachment to human HeLa cells could be mediated by some nonspecific processes not involving fiber receptor. This nonspecific attachment of adenoviruses has been previously documented (Pickles *et al.*, 1998). For example, Ad5 attaches via nonspecific

interactions to both human tracheobronchial epithelial cells (PD) and rat tracheal epithelium (PD) cells. The majority of Ad5 attached to the human PD culture surface is associated with the abundant glycocalyx present on the microvilli, suggesting that the glycocalyx itself was binding Ad5 by a fiber-knob-independent mechanism (Pickles *et al.*, 1998). If BAV2 indeed attaches nonspecifically to HeLa cells then the affinity between BAV2 virions and some nonspecific HeLa cell surface receptors will not be expected to be as high as might be found naturally on MDBK cell surface. Hence, it is possible that such nonspecifically attached BAV2 virions could be removed by nondetergent washing with PBS in our experiments. This perhaps explain the reduced number of BAV2 particles associated with HeLa cells surface.

Finally, it is possible that BAV2 attached to HeLa cells via an interaction between fiber and high-affinity receptors. It has been shown recently that OAV successfully adsorbs to a number of human cell lines despite the differences in Ad5 and OAV fiber domains (Khatri *et al.*, 1997). These results revealed that OAV attachment to human HeLa cells is not a consequence of nonspecific processes but the presence of OAV receptor on human cells. Hence, it is possible that some receptors on human cells recognized nonhuman Ad fiber. Correspondingly, HeLa cells could express some receptors that are recognized by BAV2 fiber domain. If this is the case, a reduced number of attached BAV2 particles in HeLa cells could be explained by the difference in the expression of primary receptors for BAV2 between HeLa and MDBK cells. In another scenario, perhaps the mode of fiber interaction with receptors on HeLa cells surface is different from those on MDBK cells. In other words, if BAV2 attachment to high specific receptors on HeLa cells is multivalent then the apparent number of virus sites is less than the number of receptors. This could be tested by comparing receptor number to the number of bound virus.

A second step in adenovirus infection involves internalization mediated by an interaction between the Arg-Gly-Asp (RGD) motif in a loop of the penton base and certain classes of

integrins (Bai *et al.*, 1993; Wickman *et al.*, 1993). Internalization of Ads can also occur through some nonspecific interactions such as pinocytosis as was demonstrated in Ad5 of human and rat PD cells (Pickles *et al.*, 1998). This nonspecific internalization, however, is not as efficient as the entry mediated by penton-integrin interactions. Our analyses revealed that only 80% of attached BAV2 internalized HeLa cells 24 hour post infection (*Figure 14*). Thus, it is possible that BAV2 internalized in HeLa cells by some nonspecific processes, especially if the attachment was mediated by some fiber independent interactions. If indeed BAV2 internalized through penton base-integrin interaction, the remaining portion of virus that failed to internalize could be explained by irreversible binding. Irreversible binding would take place if some viral capsid proteins interact with membrane proteins (belonging or not to the viral receptor) with a high affinity such that the resulting complex cannot dissociate under physiological conditions, then irreversibly bound virions would not be delivered to the cytoplasm but stay sequestered in the plasma membrane component (Belin *et al.*, 1993). This was documented in infection of HeLa cells by Ad2 (Belin *et al.*, 1993).

Experiments on BAV2 transport to the nuclei of HeLa cells showed that 35% of the total BAV2 entered the nuclei of HeLa cells 6 hour post-infection (*Figure 15*). In contrast to adenovirus productive infections in which the transport in the nucleus is completed within 2 hours post infection (reviewed by Shenk, 1996), not all internalized BAV2 particles reached the nuclei of HeLa cells within 6 hours. Since we did not investigate the accumulation of BAV2 in HeLa nuclei over longer period of time we cannot conclude with certainty that the transport to nuclei is delayed in this system. One could speculate that the low percentage of nuclei associated BAV2 particles is a result of one or more defects in nuclei transport.

Transport of Ads to nuclei of infected cells is preceded by a number of critical events such as sequential disassembly of virion coat proteins, acidification and rupture of the endosome which

triggers Ad penetration in the cytoplasm. Defects in any of these steps would seriously impair successful Ad transport to the nucleus. The results in this study clearly showed that a modest percentage of BAV2 was successfully internalized in HeLa cells. However, neither the precise location nor the physical integrity of BAV2 particles was demonstrated. In other words, we could not determine whether all BAV2 particles present in endosomes penetrated the cytoplasm of HeLa cells and whether or not uncoating of all internalized BAV2 particles occurred within the endosome in the same fashion as that for human Ads. It was shown previously that under conditions which inhibit uncoating, virions tend to accumulate in intracellular vesicles (Shenk, 1996). Hence, if uncoating of BAV2 was inhibited in HeLa cells then most of the internalized virus would remain associated with endosomes and may not reach the nuclei. Furthermore, although the precise nature of the signal directing the delivery of Ad genome to the nucleus is not known, several studies have suggested that some protein primary structure motifs such as nuclei localization signals (NLS) of some viral proteins could play a major role. The major candidates for the role of directing the adenovirus DNA to the nucleus following uncoating are the pTP and protein V since they were found to contain a sequence similar to nuclear localization signal (reviewed by Shenk *et al.*, 1995). Hence, if some BAV2 viral DNA was not associated with pTP or protein V as a result of impaired uncoating process it would not be successfully transported to the nucleus of HeLa cells. Further experimentation on the uncoating process of BAV2 in HeLa cells needs to be conducted in order to answer these questions. For the purposes of the present study, however, the finding that BAV2 in fact penetrates the nuclei of HeLa cells is significant.

Our experiments on BAV2 E1A transcription clearly demonstrated that HeLa cells did not support BAV2 E1A transcription (*Figure 19*). Since E1A products act as a master regulator of viral gene expression without which the viral cycle cannot proceed successfully, these findings help explain why no DNA replication was ever observed.

To answer why BAV2 E1A is not expressed in HeLa cells, we investigated the activity of BAV2 E1A promoter in HeLa cells. According to our results BAV2 E1A promoter cannot be activated in HeLa cells, presumably, because host factors do not interact positively with this promoter (*Figures 25 & 26*). Upon co-infection with Ad5, however, this promoter is activated (*Figures 27 & 28*). Although it is not clear which viral functions are responsible for the activation of the BAV2 E1A promoter, we speculate that E1 functions of Ad5 play an important role. That is, human Ad5 E1A functions could trigger the otherwise inactive BAV2 E1A promoter in HeLa. This is not without a precedent since studies on Ad12 abortive infection of BHK21 cells have shown that the MLP, which is not active in hamster cells, can be activated upon infection with permissive Ad2 (Weyer and Doerfler, 1985). They have also demonstrated that E1 functions of Ad2 have the capacity to activate the MLP of Ad12 DNA. Although E1 proteins are most likely to be involved in the activation of BAV2 E1A promoter the involvement of other viral functions in this activation cannot be ruled out. In addition, it is not known whether these viral functions directly activate the BAV2 promoter, or whether they interact somehow with cellular factors which in turn activate the BAV2 E1A promoter. What this study found is that the BAV2 E1A promoter could just be one other promoter known to respond to Ad5 E1A activation.

Studies on the defects of Ad12 MLP promoter in hamster cells found a mitigator element in the first intron following the Ad12 MLP. The mitigator somehow blocks proper interaction among Ad12 proteins, BHK21 protein, and Ad12 DNA to support efficient transcription of the MLP (Zock and Doerfler, 1990). It would be of interest to determine whether there are some sequences in BAV2 E1A promoter that affect its activity in HeLa cells such as found for Ad12 MLP.

Our approach to studying the BAV2 E1A promoter involved the lacZ reporter gene with BAV2 E1A promoter as its regulatory element. This approach had an advantage in that it allowed the study to focus on the sequences in question. The disadvantage, however, is that it proved difficult to demonstrate that the complete E1A promoter was present in the construct. Sequence analysis of the E1 region (Salmon *et al.*, 1995) suggested the presence of E1A's TATA box at nt 337. A more recent computer program (SignalScan) suggested the presence of another TATA box further downstream. Since our reporter gene construct contained only the first TATA box, but not the second, it might have an incomplete E1A promoter. Without a function test, either TATA box could be correct. Future experimentations could resolve the issue.

We have determined that the block in BAV2 E1A transcription in HeLa cells is the reason for BAV2's failure to undergo viral DNA synthesis. Our prediction that BAV2 might undergo its viral DNA synthesis in HeLa cells if the block in E1A expression is removed was confirmed by coinfection experiments involving permissive Ad5. It was shown by these experiments that BAV2 E1A transcription took place in doubly-infected HeLa cells (*Figure 30*). This was not surprising since we have already shown that Ad5 could activate BAV2 E1A promoter. BAV2 DNA synthesis in HeLa cells could also be complemented by Ad5 infection (*Figures 31 and 32*). As revealed by densitometric analysis there were not obvious differences in amount of viral DNA replicated between Ad5 and BAV2 in doubly-infected HeLa cells. We do not know, however, if all of the early functions of BAV were expressed in this system or whether BAV2 used some Ad5 proteins for successful viral DNA replication. For example, it would be interesting to study whether Ad5 DNA polymerase could bind to BAV2 *ori* and initiate BAV2 DNA replication. Comparison of Ad5 and BAV2 binding sites for precursor TP-DNA polymerase (pTP-pol) revealed very close similarity (*Figure 43*). Indeed the two sequences differ only in nucleotide at position 18 (residue C in Ad5 *ori*; residue G in BAV2 *ori*). Although it would be possible for

Ad5 polymerase to bind to BAV2 ori, all other interactions required for the initiation of Ad replication must still be considered. we still have to keep in mind all other interactions required for the initiation of Ad replication. For example, since successful initiation of Ad replication depends largely on DNA polymerase interacting with pTP it would be of interest to study whether Ad5 DNA polymerase is capable of interacting with BAV2 TP. To answer all these questions one must determine if BAV2 expresses its E2 proteins in mixed infection.

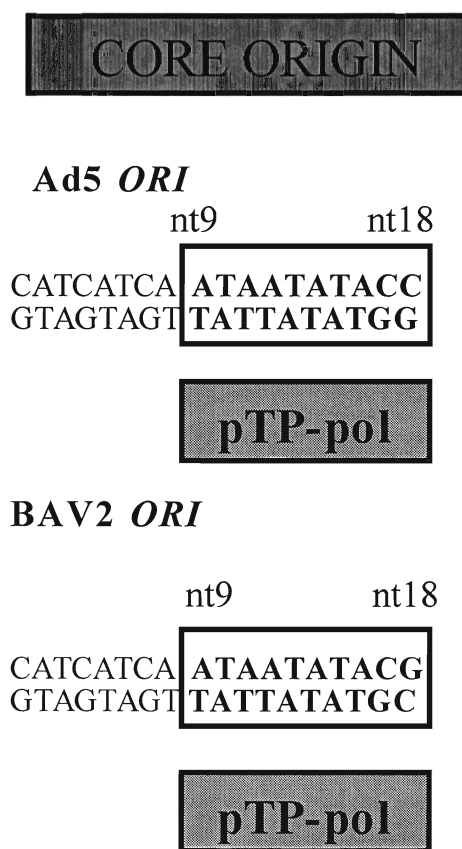


Figure 43: Comparison of Ad5 and BAV2 binding sites for precursor TP-DNA pol

The Ad5/Ad2 sequence of ORI was taken from Van der Vliet (1995), the BAV2 sequence was taken from Ojkic *et al.* (1998).

The E1 region of Ad5 DNA was shown to play an important role in complementation of impaired BAV2 functions (*Figure 37*). However, it was also shown that 293 cells were not capable of complementing BAV2 DNA replication suggesting that the concerted action of more

than one group of Ad5 functions was essential for BAV2 complementation in HeLa cells (*Figure 38*). Although our analyses revealed no BAV2 DNA replication upon infection of 293 cells we cannot conclude with certainty that this was due mainly to the failure of Ad5 E1 to complement BAV2 impaired functions. First, we have not assessed BAV2 infection of 293 cells in terms of attachment, internalization and transport to the nuclei. We cannot conclude that BAV2 behaves in the same fashion upon infection of 293 and HeLa cells since these two cell lines are of different origin. As indicated before, Ad attachment depends on the presence of specific receptors on the cell surface. Since cells of different origin express different sets of membrane proteins not all cells are susceptible to adenovirus infection.

In spite of complementing activity of Ad5 functions on BAV2 DNA replication, intact BAV2 virions were not present. The question on whether this is due to impaired synthesis of structural proteins or assembly remains unresolved (*Figure 36*).

Summaries of events resulting from BAV2 infection of permissive MDBK cells and nonpermissive HeLa cells as well as events resulting from BAV2 infection of Ad5 infected HeLa cells are presented in a schematic diagrams in *Figures 44, 45 and 46*. The unresolved questions and potential focuses of future experimentations are also presented in these diagrams.

If bovine adenovirus will be used in human gene therapy and vaccine development, its interaction with human cells must be fully understood. This study took the initial steps and found that BAV2 undergoes abortive infection in human HeLa cells with an early block in DNA replication. In this abortive infection, BAV2 successfully penetrated HeLa cells and nuclear transport was evident. However, viral DNA synthesis, early transcription and virus production were not evident in this infection. Many questions still need to be answered before applying BAV2 vector in human gene therapy. If BAV2 behaves the same way in all human cell lines as it does in HeLa cells then the use of BAV2 for human gene therapy and vaccine development looks

very promising for number of reasons. First, since BAV2 does not replicate in human cells it is very safe for human use. Second, it is unlikely that humans have preexisting immunity against BAV2 which makes it an ideal vaccine booster. Third, BAV2 vector would successfully deliver the foreign genes to human cell nuclei. Fourth, BAV2 infection of human cells does not express any of its proteins due to defects in E1A transcription. Hence, if BAV2 vector was used *in vivo* human gene therapy the risk of the generation of cellular and humoral immune response against the vector would be greatly reduced if not eliminated.

Several disadvantages of BAV2 vectors in human gene therapy are apparent and include the possibility that BAV2 would not be able to target all human cells. However, recent studies on human adenovirus vectors have demonstrated that it is possible to alter the capsid fiber protein by genetic engineering to direct gene delivery specifically to certain target cell types (reviewed by Douglas *et al.*, 1997). Perhaps the same technology could be applied to BAV2 vectors to render them more efficient in targeting human cells.

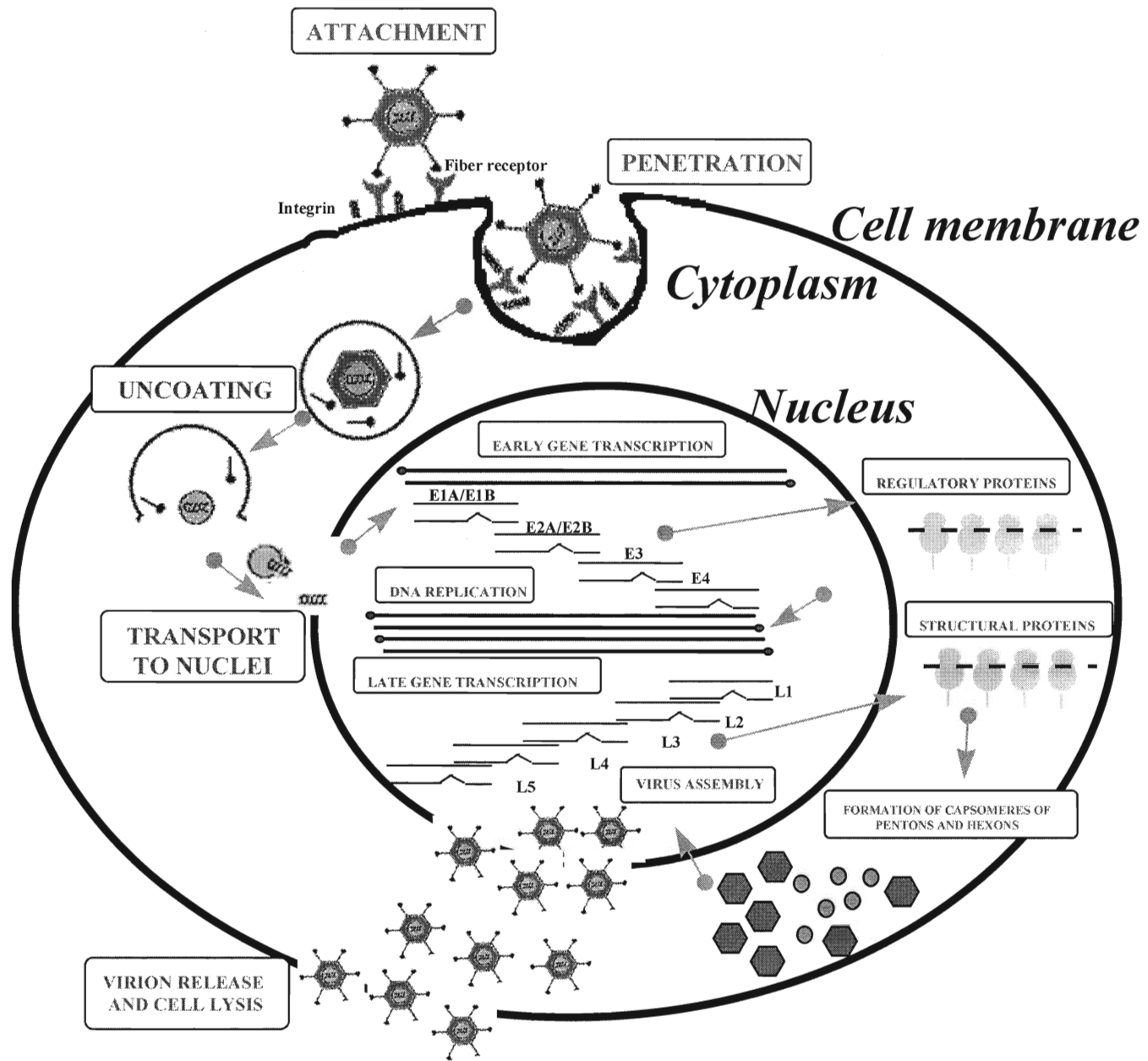


Figure 44. Schematic presentation of the major events in the replication cycle of BAV2 in permissive MDBK cells.

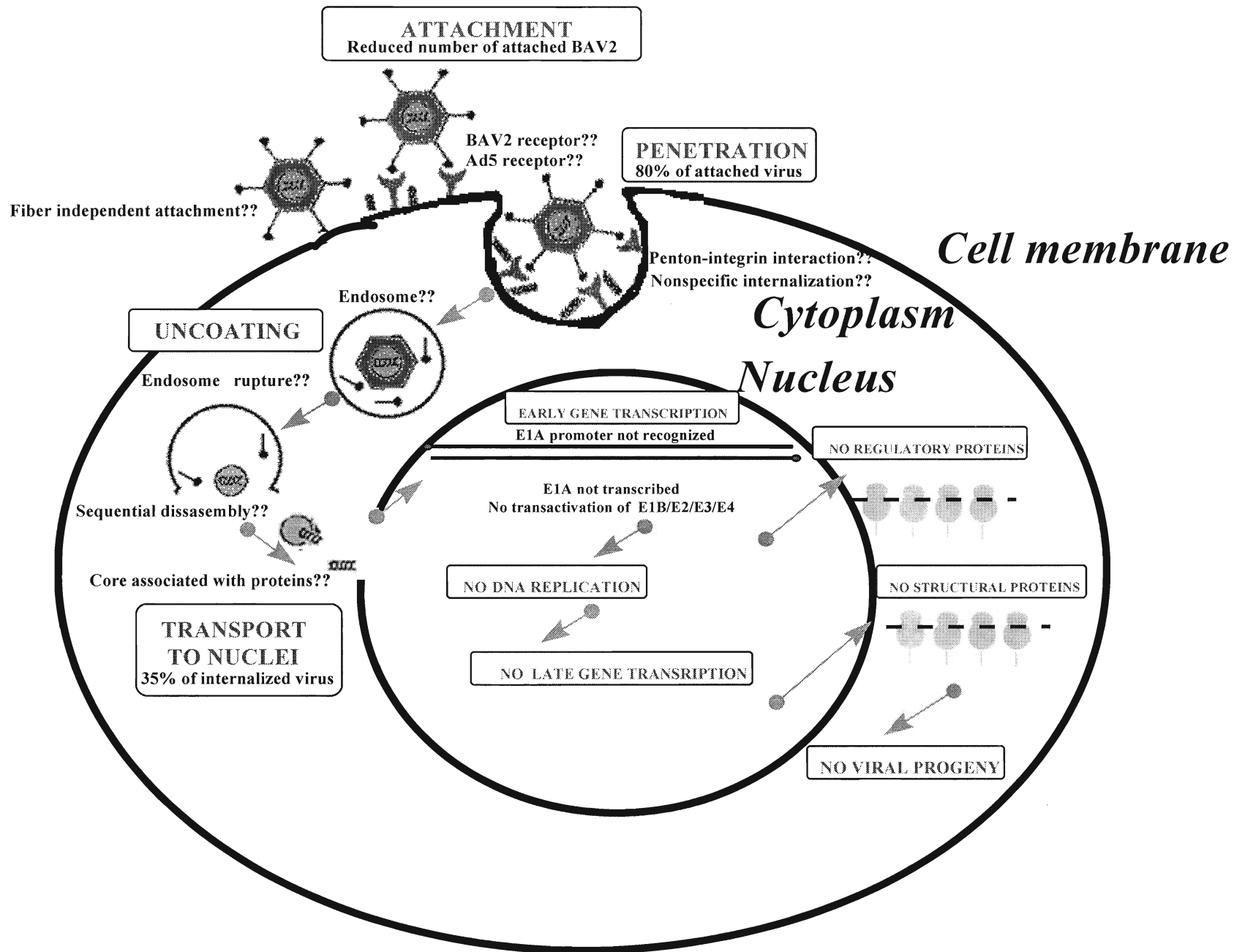


Figure 45. Schematic presentation of the major events in the replication cycle of BAV2 in nonpermissive HeLa cells.

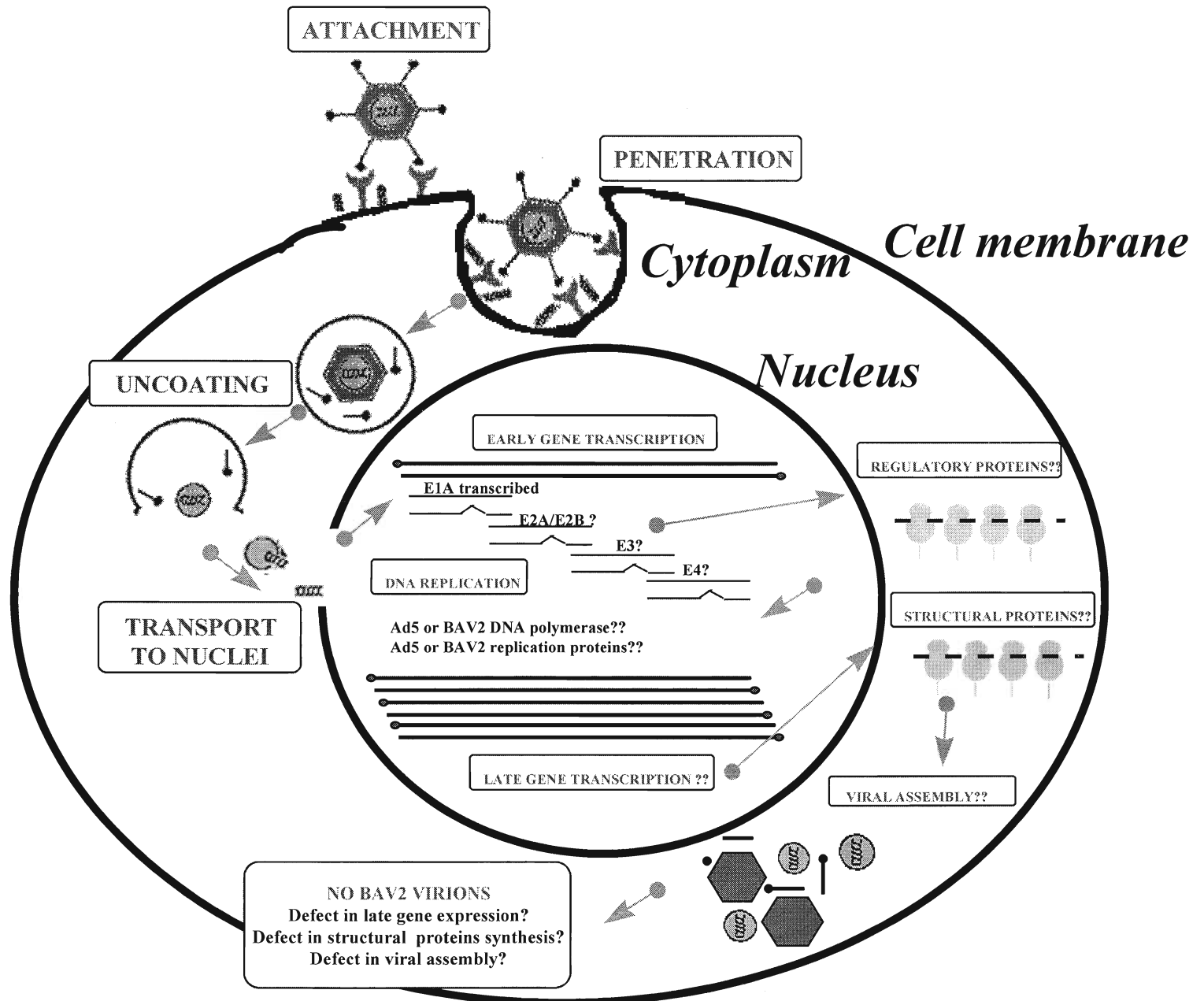


Figure 46. Schematic presentation of the major events in the replication cycle of BAV2 in nonpermissive HeLa cells upon coinfection with Ad5.

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