THE REPLICATION OF hAd5 IN MDBK, MDCK, HELA, & L CELLS

THE ABILITY OF HUMAN ADENOVIRUS TYPE 5 TO REPLICATE IN MDBK, MDCK, HELA, & L CELLS

by

GRACE L. MARTINS, B.Sc.

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AUTHOR: Grace L. Martins (McMaster University)

SUPERVISOR: Dr. Ludvik Prevec

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ABSTRACT

Human adenovirus type 5 (hAd5) may be used as an effective mammalian expression vector for foreign genes. In the course of determining the usefulness of hAd5 as a vector in different species, it was observed that hAd5 does not undergo a productive infection in canine kidney cells *in vitro* (unpublished observations), though it produces an immune response *in vivo* (Prevec et al., 1990). Murine fibroblasts (L cells) are semi-permissive for hAd5 replication, with DNA being synthesized at low levels, and the E3 region displaying delayed kinetics for expression when compared to permissive infections of HeLa cells. Bovine kidney cells (MDBK) display a similar rate of hAd5 replication as in HeLa cells, although infectious virus yield is somewhat lower in MDBK cells than in HeLa cells; this may be in part due to a decreased level in late protein synthesis. Canine kidney cells (MDCK) appear non-permissive for hAd5, with early proteins being synthesized, DNA replication occuring at lower levels, and no late proteins being produced. Infection of MDCK cells with Ad5lacZ resulted in similar rates of β-galactosidase expression as in permissive HeLa cells, suggesting that high levels of early protein expression can be achieved without virus replication. Although no late proteins are made, at least one late transcript (penton base) was detected in the cytoplasm of infected MDCK cells, which may suggest a block in translation initiation and/or elongation of late transcripts.

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1. INTRODUCTION

1.1 BACKGROUND ON ADENOVIRUSES

The family Adenoviridae contains two genera, *Mastadenovirus* and *Aviadenovirus*, of which the human adenoviruses are members of the former. There are over 40 distinct antigenic types of adenoviruses that infect humans, causing primarily respiratory, ocular, and enteric diseases. These adenoviruses have not been demonstrated to play a role in any human malignancies (Green et al., 1980); however, tumour induction as a result of human adenovirus infection has been demonstrated in rodents (Trentin, 1962). The human adenoviruses have been subdivided oncogenically into three groups: the highly oncogenic group A viruses that form tumours in rodents at the site of injection within 30 to 90 days, the weakly oncogenic group B viruses where tumour formation requires longer latency (200 to 400 days) and occurs in a smaller proportion of animals (Girardi et al., 1964), and non-oncogenic viruses of groups C, D, and E that are all incapable of tumour induction in injected animals, even after long periods of latency (Freeman et al., 1967). These five sub-groups (A to E) are also characterized according to

hemagglutination properties, viral DNA guanine-cytosine (GC) content, and DNA sequence homology. In addition, there are the tentatively defined subgroups F and G, whose members grow predominantly in cell culture, and are also linked with diarrheal disease. Group C adenoviruses are the most prevalent and account for nearly 95% of the adenoviral infections relating to illness. These adenoviruses infect most children early in life causing mainly mild infections of the upper respiratory tract (Green et al., 1980). Both oncogenic and non-oncogenic adenoviruses can transform cells in culture, although transformation *in vitro* is not necessarily identical to tumor induction *in vivo*. For example, human adenovirus type 5 (hAd5) is considered to be non-oncogenic in humans, though the virus does transform rat embryo cells with low efficiency, *in vitro* (Williams, 1973; Graham et al., 1975). Adenoviruses are studied extensively providing invaluable systems for understanding macromolecular synthesis and its control in higher eukaryotes.

Adenoviruses are non-enveloped, double-stranded, DNA-containing viurses, 65-80 nm in diameter, with regular icosahedral morphology (Horne, 1959). The genomic strand that transcribes mRNA to the right is defined as the *r*-strand, whereas the *l*-strand transcribes mRNA to the left. [Refer to Figure 1 for a model of the adenovirus particle]. The capsid is composed of three major structural proteins, namely hexon (protein II), penton base (protein III) and fiber (protein IV). There are 240 capsomers of hexon per virion, and at each of the 12 vertices of the icosahedron there is a

penton base capsomere from which fiber projects. The fiber particle is likely a trimer of a 62K polypeptide (Pettersson, et. al., 1968). Together, the penton base and fiber are termed penton (Horwitz et al., 1969). Antigenic determinants that are important in the serologic classification of adenoviruses are intrinsic to these structural proteins (Wilcox, 1963). Each virion contains one linear, double-stranded DNA molecule (Green, 1967) covalently linked to a virus-encoded protein at each 5' end (Rekosh, 1977). This 55K-terminal protein is one of two novel features of the viral DNA. The second unique feature is the inverted terminal repetitions of about 100 base pairs. These sequences are thought to allow for the formation of single-stranded circles through "panhandles" produced by reannealing of complementary ends of the DNA strand displaced during replication (Garon et al., 1972; Wolfson & Dressler, 1972) and may be important in DNA replication (Lechner & Kelly, 1977).

1.2 REPLICATION

Infection is the result of a series of events beginning with the attachment of the fiber particles of a virion to a host cell receptor, and ending with the assembly of approximately 10^4 infectious virions per cell (Green & Daesch, 1961). The basic features of viral multiplication are similar for all adenovirus types. Following adsorption to susceptible cells, the virion penetrates the cell either directly through the plasma membrane or by a phagocytotic-like process. Uncoating of the viral DNA occurs in the cytoplasm and the

viral core enters the nucleus where viral replication takes place (Lonberg-Holm & Philipson, 1969).

Adenovirus DNA is transcribed in the nucleus of infected host cells by host cell enzymes, and the RNA is processed and transported into the cytoplasm where it functions as messenger RNA. Translation of viral mRNA occurs on cytoplasmic polyribosomes and is followed by the release of the nacent viral polypeptide chains (Velicer & Ginsberg, 1968). The single polypeptide chains or small complexes are then transported into the nucleus where assembly of mature particles occurs (Velicer & Ginsberg, 1970).

The replicative cycle is temporally divided into early and late phases. Early mRNAs are transcribed from four distinct regions of the hAd 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). By definition, these sequences are transcribed prior to and independent of viral DNA replication; however, many early mRNAs and corresponding proteins, are also synthesized following replication.

Adenovirus gene expression is controlled by transcriptional regulation as well as altering post-transcriptional events including mRNA stability in the cytoplasm. The initiation of mRNA synthesis requires interactions between transcription factors, the RNA polymerase, and control sequences upstream of the genes to be transcribed. These specific sequences are termed promoters and contain various binding sites for transcription factors and enhancer

binding proteins. There are nine different adenoviral promoters that vary greatly in complexity. The E1A promoter functions efficiently during the earliest stages of infection before expression of E1A proteins. These proteins greatly stimulate transcription from other adenoviral promoters, perhaps by inactivating a cellular factor that prevents transcription (Nevins, 1981; Bandara & La Thangue, 1991). Promoters E1B, major late, E2 early, E3, and E4 are activated following E1A expression and transcription occurs during early and late phases of infection. Transcription from promoters IX, IVa_2, and the E2 late promoter occurs following viral DNA replication (Crossland & Raskas, 1983).

Early Region 1

The E1 domain is divided into the E1A and E1B regions. E1A transcripts are defined as immediate early since they are the first transcripts made following infection; the protein encoded by the E1A 13S mRNA is required to activate the transcription of E1B, E2A, E3, and E4 which are termed the delayed early genes (Jones & Shenk, 1979). Expression of some cellular genes such as the β-globin gene (Svensson & Akusjarvi, 1984) can also be stimulated by E1A products. 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 ellicits a response through host proteins. For example, E1A reverses the inhibitory activity of the

E2F-I protein towards DNA-binding of transcription factor E2F (Bagchi et al., 1991). Other functions of the E1A products include the ability to repress transcription induced by certain enhancer sequences such as the SV40, polyoma virus, and Ad2E1A enhancers (Borrelli et al., 1984; Velcich & Ziff, 1985). Although E1A is required to immortalize primary cells *in vitro* (Graham et al., 1974a; Ruley, 1983), it appears that E1B proteins are required to maintain the transformed phenotype *in vivo* (Byrd et al., 1988).

Early Region 2

The three viral polypeptides essential for Ad DNA synthesis are encoded by the E2 domain. Like the E1 region, this domain is separated into two different, non-overlapping transcription units, namely E2A, and E2B. E2A codes for a 72K DNA binding protein (DBP) which binds preferentially to single-stranded DNA (Levine et al., 1974). This protein is required for the elongation reaction in DNA synthesis (Horwitz, 1978). The 72K DBP is also associated with mRNA stability (Babich et al., 1981), repression of E4 transcription (Nevins & Jensen-Winkler, 1980), host range (Klessig & Grodzicker, 1979), and assembly of infectious virus particles (Nicolas et al., 1983). Proteins required for the initiation reaction in DNA replication are encoded by the E2B region (Ikeda et al., 1981) and include the precursor of the 55K adenovirus terminal protein, (pTP) (Stillman et al., 1981), and the 140K adenovirus DNA polymerase (Ad DNA Pol) (Friefeld et al., 1983).

Early Region 3

The E3 region is the only early transcrition unit present downstream of, and in the same orientation as, the major late promoter (on the *r*-strand). This 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). The 19K glycoprotein encoded by the E3 region of hAd2 is localized in the endoplasmic reticulum and has been shown to inhibit glycosylation and transport to the cell surface of human class I histocompatibility antigens (HLA antigens) through binding. This results in a reduction in cytotoxic T-cell response and may allow the infected cell to evade the host's immune response (Burgert et al., 1987).

The 14.7K protein is the second Ad E3 protein to which an activity has been ascribed, and functions to prevent cytolysis by tumour necrosis factor (TNF). TNF inhibits replication of many viruses and causes lysis of cells infected by these viruses to provide an initial defense against acute viral infections (Gooding et al., 1988). In HSV-I infected cells, the actions of TNF and human gamma interferon are synergistic to block a step in replication, following viral entry but prior to or at the transcription of immediate early genes (Feduchi et al., 1989). 14.7K counteracts anti-viral effects of TNF *in vivo*, though the mechanism of action is yet unknown (Wold & Gooding, 1989).

Early Region 4

The hAd5 E4 transcription unit is located between 91 and 100 m.u. on the *l*-strand of the genome. A 34K nuclear protein of the E4 region has been shown to physically associate with the 58K protein of the E1B region (Sarnow et al., 1984) within adenovirus-infected human cells forming a functional complex in lytic infections (Cutt et al., 1987). This complex facilitates the specific accumulation of late viral mRNAs by localizing a nuclear factor essential for movement of RNAs from their site of processing to the nuclear pore for transport to the cytoplasm, to the periphery of the viral replication-transcription centers (Ornelles & Shenk, 1991). Another E4 protein, 19.5K, 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). Functions encoded by the hAd5 E4 region are required for efficient DNA replication, late gene expression, host cell shut-off (Halbert et al., 1985) and virion assembly (Falgout & Ketner, 1987).

Intermediate Regions

Intermediate genes are defined as such as their expression is independent of the E1A function. Although the synthesis of these genes begins early, it is elevated at late times. These genes include those encoding protein IVa_2, and protein IX. Protein IVa_2 is a 50K

protein that is encoded by the *l*-strand of the viral genome and is involved in viral maturation (Persson, 1979). More specifically, it acts as a scaffold protein (one necessary during the formation of an assembled structure but which is absent from the final product) in the formation of the light capsid. Protein IX is a minor capsid component that is essential in packaging full length viral genomes (Ghosh-Choudhury et al., 1987) and appears to confer thermal stability to virions (Colby & Shenk, 1981).

DNA Replication

Transcription beginning at or following DNA replication is defined as late transcription. Expression of late genes is contingent upon viral DNA synthesis though the reasons why remain unclear (Thomas & Mathews, 1980). Adenovirus DNA replication occurs semi-conservatively in the nucleus of the infected host cell. Replication is initiated at either end of the parental genome, and synthesis of the daughter strand results in the displacement of one parental strand. 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. Upon initiation of DNA synthesis, the daughter strands grow by a completely continuous process, in a 5' to 3' direction.

The adenovirus origin of DNA replication contains functionally

distinct domains, A, B, and C. Domain A consists of the first 18 base pairs of the viral genome and is absolutely required for the initiation reaction; however, in the absence of domains B and C, the efficiency of the reaction is only about 3% of the optimal level. Domain B is located between nucleotides 19 and 39 and its presence increases the efficiency of initiation 10-fold. Domain C consists of the region between nucleotides 40 and 51 and increases the efficiency of initiation a further 3-fold (Rosenfeld et al., 1987).

The terminal protein is covalently linked by a phosphodiester bond between the β-OH of a serine residue in the protein and the 5'-OH of the terminal deoxycytidine residue of the DNA (Challberg et al., 1980) and functions as a primer for Ad DNA replication (Rekosh et al., 1977; Lichy et al., 1981). The 140K Ad DNA polymerase may function to catalyze the transfer of dCMP to the pTP during initiation of Ad DNA replication (Lichey et al., 1981; Stillman et al., 1982). In addition to these two viral proteins, efficient initiation of Ad DNA replication *in vitro* requires cellular DNA-binding proteins at the origin of DNA replication.

Nuclear factor I (NF-I) is a cellular protein that binds to a sequence in domain B of the Ad origin of DNA replication and stimulates pTP-dCMP complex formation in the presence of Ad DBP. NF-I also plays a role in the synthesis of DNA chains up to 25% of full-length Ad DNA (Nagata et al., 1982). ORP-A and ORP-C (NF-III) are also two site-specific DNA binding proteins that interact specifically with domains A and C respectively, in the Ad origin and

are also essential in the initiation of DNA replication (Rosenfeld et al., 1987).

Elongation of protein-primed chains is dependent on the adeno-DBP (Ostrove et al., 1983), as well as a cellular protein, nuclear factor II (NF-II). NF-II from HeLa cells co-purifies with a DNA topoisomerase activity and effects synthesis of nacent strands greater than 9 kb in length, but its precise function in Ad DNA chain elongation is not yet clear although evidence lends support to topoisomerase function (Challberg & Kelly, 1989). Thus, synthesis of full-length adenovirus DNA strands *in vitro* requires viral proteins (pTP, DNA pol, and DBP) as well as cellular factors (ORP-A, NF-I, NF-II, and NF-III).

Late Genes

There are five families of late mRNAs (Nevins & Darnell, 1978) transcribed off the *r*-strand, primarily after DNA replication although some exceptions do exist. Each family containing transcripts for more than one protein and is generated after processing long, nuclear, primary transcripts by splicing three non-contiguous leader sequences at the 5' end to sequences containing the message for a single protein (Chow et al., 1977). Messenger RNAs from each class possess a common non-coding sequence at the 3' end (Nevins & Darnell, 1978). [Refer to Figure 2 for the hAd2 transcription map].

The major late promoter of hAd2 is located at 16.45 m.u. and is

responsible for the transcription of the five late mRNA families, L1 to L5 (Shaw & Ziff, 1980). A single member of the L1 gene family containing the tripartite leader plus 29-39 m.u. encoding the 52,55K polypeptide pair is made at low levels prior to DNA replication (Thomas & Mathews, 1980). Although the major late promoter is active within an hour following viral infection to initiate these early transcripts that are processed into the L1 mRNA family, transcription is increased as a result of virus-induced transcription factors interacting with three binding sites in the first intron of the major late promoter transcription unit. In the late phase, elongation is greater and the primary transcript is processed into one of more than 30 possible mRNAs by alternative cleavage, polyadenylation, and alternative RNA splicing (Leong et al., 1990).

Transcription of late messenger RNAs begins soon after the initiation of DNA replication and for the most part, cannot occur in its absence. The L1 gene family codes for two structurally related proteins (52,55K) whose functions are unknown, and protein IIIa which is found associated with hexon (Miller et al., 1980). The L2 gene family encodes three viral structural proteins: protein III (penton), the precursor for protein VII (pVII), and protein V (Miller et al., 1980). The precursor for protein VI (pVI), protein II (hexon), and a 23K protein are encoded by the L3 gene family. pVI is cleaved to form protein VI which is another structural protein. A temperature sensitive hAd2 mutant, ts1 (Hassell and Weber, 1978) mapped within the region encoding the 23K protein (Kruijer et al., 1980) assembles

non-infectious, immature virions containing uncleaved precursor polypeptides pVI, pVII, pVIII, X, XI, and XII (Weber, 1976) suggesting that the 23K protein may be a virus-coded protease (Bhatti & Weber, 1979). The L4 region encodes a 100K protein, a 33K protein and the precursor to protein VIII (pVIII) (Miller et al., 1980). pVIII is cleaved to form protein VIII which is found to be associated with the hexon capsomers in immature virions. The 100K protein appears to be a scaffold protein that initially binds to hexon monomers but is released upon trimer formation. The 100K protein may have functions additional to hexon assembly (Cepko & Sharp, 1983). The function of the 33K protein remains unclear. The L5 gene family of late transcripts contain two major mRNA species that encode the viral structural protein IV (fiber) (Miller et al., 1980). Unlike all other late messages, mRNA from the L5 region may contain non-coding leader sequences x, y, and z, in addition to the common tripartite leader. Although the y-leader is the most prominant additional leader sequence in fiber mRNA, its presence or absence does not appear to influence the translation of fiber mRNA (Dunn et al., 1978).

Virus Associated RNAs

Virus-associated RNAs (VA RNA I and II) are small RNAs with a high degree of secondary structure, and are transcribed from 29 m.u. on the *r*-strand of adenovirus templates by RNA polymerase III of the infected host cell (Soderlund et al., 1976). Both VA RNAs can be

synthesized in the absence of DNA replication, but are also produced at late times of infection. DAI (the double-stranded RNA activated inhibitor) is a protein kinase that phosphorylates the α-subunit of translation initiation factor eIF-2 and leads to a sequence of events that results in inhibition of protein synthesis by blocking polypeptide initiation. At late times of infection, VA RNA I prevents activation of DAI, thereby allowing efficient translation of viral late proteins (Thimmappaya et al., 1982; O'Malley et al., 1989).

Inhibition of Host Protein Synthesis

Following infection, adenoviruses compete with host cells in processes such as DNA synthesis, transcription, processing and transport of mRNAs, protein synthesis, and post-translational modification. Some ways in which adenoviruses subvert the cellular translational machinery in the late phase of infection include high level transcription of viral mRNAs to out-compete cellular mRNAs, changing the intracellular ionic environment to favour translation of viral mRNAs, and inactivation of translation factors (Schneider & Shenk, 1987). The translation of cellular mRNAs in adenovirus-infected cells may be inhibited at two levels. The block in transport of cellular, but not viral, mRNAs from the nucleus to the cytoplasm (Beltz & Flint, 1979) mediated by E1B-55K and E4-34K complex (Halbert et al., 1985; Babiss & Ginsberg, 1984) is not entirely responsible for cellular translation shut-off. There are also mechanisms that facilitate translation of viral mRNAs while

inhibiting cellular translation of pre-existing mRNAs (Schneider & Shenk, 1987). One such mechanism employed by adenoviruses is the inactivation of the cap-binding protein (CBP) which results in inhibition of the CBP complex, a necessary component for cap-dependent mRNA translation (Huang & Schneider, 1991). The CBP complex, also known as eIF-4F, is made up of 3 polypeptides: eIF-4A (protein with ATP-dependent RNA helicase activity), eIF-4E (cap-binding protein), and a large protein (p220). Together, these three proteins function as a cap-dependent, RNA helicase necessary for translation of most capped RNAs (Sonenberg, 1987). Adenoviruses inhibit cellular protein synthesis by underphosphorylating eIF-4E which results in the inactivation of the CBP complex activity (Huang & Schneider, 1991). The adenovirus tripartite leader is found on all late mRNAs, and is required for preferential translation of viral mRNAs at late times during infection (Dolph, et al., 1988). Specifically, the 5' end of the tripartite leader allows efficient mRNA translation independent of a normal cap recognition process (Dolph et al., 1990).

Thus, there are two known methods which presumably occur simultaneously in adenovirus-infected cells, to circumvent host protein synthesis. These are the preferential transport of viral mRNAs from the nucleus to the cytoplasm, and the translation of viral mRNAs over cellular mRNAs through inactivation of the cellular cap-binding protein.

1.3 HOST RANGE

Adenoviruses are wide-spread in nature. Within the *Mastadenovirus* genus exist human, simian, bovine, equine, porcine, ovine, canine, and opossum viruses. The host range of any virus is determined firstly by the specificity of attachment to the cells. This in turn depends on properties of the virion's coat and on the presence of specific receptors on the cell surface. After adsorption, host range may depend on the availability of cellular factors required for viral replication (Lonberg-Holm & Philipson, 1969).

Human adenoviruses seem to mainly infect epithelial cells, and severely inhibit host macromelecular synthesis during lytic infections. Although it appears that adenoviruses establish productive infections in primary gastrointestinal, respiratory, or ocular epithelial cells (Fishaut et al., 1980), primary cultures of various other cells also support viral multiplication, but to much lower yields (Dulbecco, 1980).

Permissivity of Monkey Cells to Human Adenovirus Type 2

The host range of hAd2 can be extended to monkey cells, but only in the presence of Simian Virus 40 (SV40), a papovavirus. Expression of the SV40 large tumor (T) antigen is necessary throughout the infection cycle to permit the unrestrained replication of hAd2 in African green monkey kidney cells (Klessig & Anderson, 1975). More specifically, activity resides in the carboxy-terminal

34 amino acids of T antigen (Cole et al., 1979). This function may be provided by co-infection with SV40 (Rabson, 1964) or by SV40 DNA which is integrated either into the host genome or into the infecting human adenovirus genome (Rapp & Trulock, 1970). A third way of overcoming the block to viral multiplication is through a mutation in the segment of the hAd2 genome encoding the DNA binding protein which can also result in productive infection by the mutant virus (Klessig & Hassell, 1978).

The block to multiplication of human adenoviruses in monkey cells presents a convenient system in which to study the mechanisms by eukaryotic cells to control the expression of incoming viral genes as well as perhaps that of their own genes. Although early viral transcription and translation, along with the onset and rate of viral DNA synthesis, appear to be normal in abortive infections, the yield of infectious virus is reduced by a factor of at least 500 (Anderson & Klessig, 1984). Levels of late proteins are reduced 2 to 10 fold compared to productive infections, in most cases corresponding to the lower levels of late mRNA synthesis. Furthermore, at least one late protein, fiber, is reduced more than 100 fold although the steady-state level of its mRNA is reduced by a factor of only 5 to 10 (Anderson & Klessig, 1984).

Permissivity of Rodent Cells to Human Adenoviruses

Human adenoviruses vary considerably in their ability to cause tumours in rodents. The oncogenic Class A or Class B adenoviruses

result in abortive infections of rodent cells (Shimojo & Yamashita, 1968). Adenovirus type 12 (Class A) was shown to be the first human virus with oncogenic properties when injection of the virus into newborn hamsters induced tumor formation (Trentin, 1962). Infection of baby hamster kidney (BHK21) cells with hAd12 *in vitro*, is a model system of abortive virus infection that may lead to transformation (Strohl, 1967). The hA12 genome or a portion thereof, is able to become covalently linked to the DNA of BHK-21 cells (Doerfler, 1969). Genes involved for cell transformation are contained in the leftmost part of the viral *r*-strand (Graham et al., 1975). Cellular DNA synthesis is uninhibited in hAd12 infected BHK21 cells. The morphologically altered cells produce the hAd12 specific tumor antigen, whereas normal fibroblastic BHK21 cells do not (Strohl, 1967). While hAd12 can infect BHK21 cells, viral DNA replication does not occur (Doerfler, 1969), and viral capsid proteins cannot be detected (Pope, 1964). The viral RNA sequences present in hAd12-infected BHK-21 cells correspond to sequences transcribed exclusively early in productive infections (Raska & Strohl, 1972). It has been shown that the virus-associated (VA) RNA and L1 genes of hAd12 DNA are not transcribed in hamster cells. (Juttermann, et al., 1989). Furthermore, the nucleotide sequence between +320 and +352 (relative to nucleotide +1 as the transcriptional initiation site) in the major late promoter has been implicated as a causative agent in the block of Ad12 in hamster cells, as well as reducing the promoter activity in human cells (Zock & Doerfler, 1990).

Adenovirus type 3 is a Class B virus that also produces abortive infections in hamster cells. No viral DNA is synthesized and no infectious virus is produced. As early hAd3 RNA can be detected in association with polysomes in BHK21 cells, it is unlikely that the block is at the level of early transcription (Groff & Daniell, 1981).

Unlike the oncogenic adenoviruses from Classes A and B, Class C adenoviruses (such as types 2 and 5) can initiate viral DNA synthesis and produce infectious virus in hamster cells (Williams, 1973). However, infection of rodent cells by hAd2 results in a semi-permissive virus-host cell interaction where viral replication is restricted and virus production is decreased by at least 3 logs (Gallimore, 1974; Tremblay et al., 1985). Viral DNA synthesis does occur in various rodent cells, but only low levels of late structural proteins are detected (Eggerding, 1986). Abnormalities in viral mRNA biosynthesis or mRNA translation may contribute to the reduction in viral protein accumulation in infected rodent cells.

1.4 ADENOVIRUS VECTORS

The molecular biology of hAd5 is extensively known with the genomic structure well defined (Flint, 1977). Up to 2.7 kb can be deleted from the E3 region (A.J. Bett, unpublished results) as this region appears to be non-essential for viral multiplication *in vitro* (Tooze, 1981). An additional 3.2 kb (unpublished results) may be deleted from the E1 region, as long as these functions are provided

in trans. As an additional 2 kb can be packaged into virions, foreign sequences up to 7.9 kb may be inserted into adenoviruses to generate a viable, conditional, helper-independent vector (Berkner, 1988).

The large coding capacity, and the genetic and physical stability of recombinants make adenoviruses useful to successfully express foreign proteins in mammalian cells. The major late promoter located at map position 16.5 and the tripartite leader sequence present at the 5' termini of the late mRNAs are responsible for the abundant expression of late viral proteins. Incorporating these two components in vector design has resulted in vectors capable of expressing moderate to high levels of many foreign proteins such as the hepatitis B virus surface antigen (Morin et al., 1987), polyoma middle T antigen, and dihydrofolate reductase (Berkner et al., 1987). Adenovirus vectors have also been used in the expression of herpes simplex virus type 1 (HSV-1) glycoprotein B (Johnson et al., 1988), and the vesicular stomatitis virus (VSV) glycoprotein (Schneider et al., 1989). Viral vectors that express foreign antigens *in vivo* are useful in studying immunological effects, and these vectors may allow the development of effective recombinant vaccines. An adenovirus vector expressing HSV-1gB has proven effective in conferring protection to mice against a subsequent lethal challenge with HSV (McDermott et al., 1989). Similarly, recombinant adenoviruses carrying the hepatitis B virus surface antigen are able to induce a significant antibody response in hamsters (Morin et al., 1987). An infectious adenovirus vector

(AdG12) have been constructed to express the vesicular stomatitis virus glycoprotein gene in bovine, canine, and murine cells in culture AdG12 was constructed to express the VSV glycoprotein under the HSV-1 TK promoter and the Tk polyadenylation region, in parallel to the E3 transcription unit (Schneider et al., 1989) Mice, calves, piglets, and dogs all produce a good immune response, even though murine and canine cells are respectively, semi-permissive and non-permissive for hAd5 replication in tissue culture (Prevec et al , 1989). A hAd5 recombinant, AdRG1, expressing the rabies glycoprotein gene in the E3 region between the SV40 early promoter and the SV40 polyadenylation site in the same orientation as the E3 transcription unit has also proven highly effective in producing good levels of rabies-neutralizing antibodies in dogs and mice, as well as protecting mice against lethal intracerebral challenge with rabies virus (Prevec et al., 1990) However, it is interesting to note that this same recombinant virus does not undergo a productive infection in canine kidney cells. The purpose of the following experiments was to examine hAd5 infections in various cell types with respect to various macromolecular events

FIGURE 1: MODEL OF AN ADENOVIRUS PARTICLE

The apparent architectural relationships between the structural proteins (roman numerals) and the nucleoprotein core are shown. The capsid consists of hexon (II), penton base (III), fiber (IV), and the hexon-associated proteins (IIIa, VI, VIII, and IX). Proteins V and VII are core proteins associated with the viral DNA, and the 55K protein is covalently linked to the 5' end of the DNA (Modified from Dulbecco & Ginsberg, 1980).

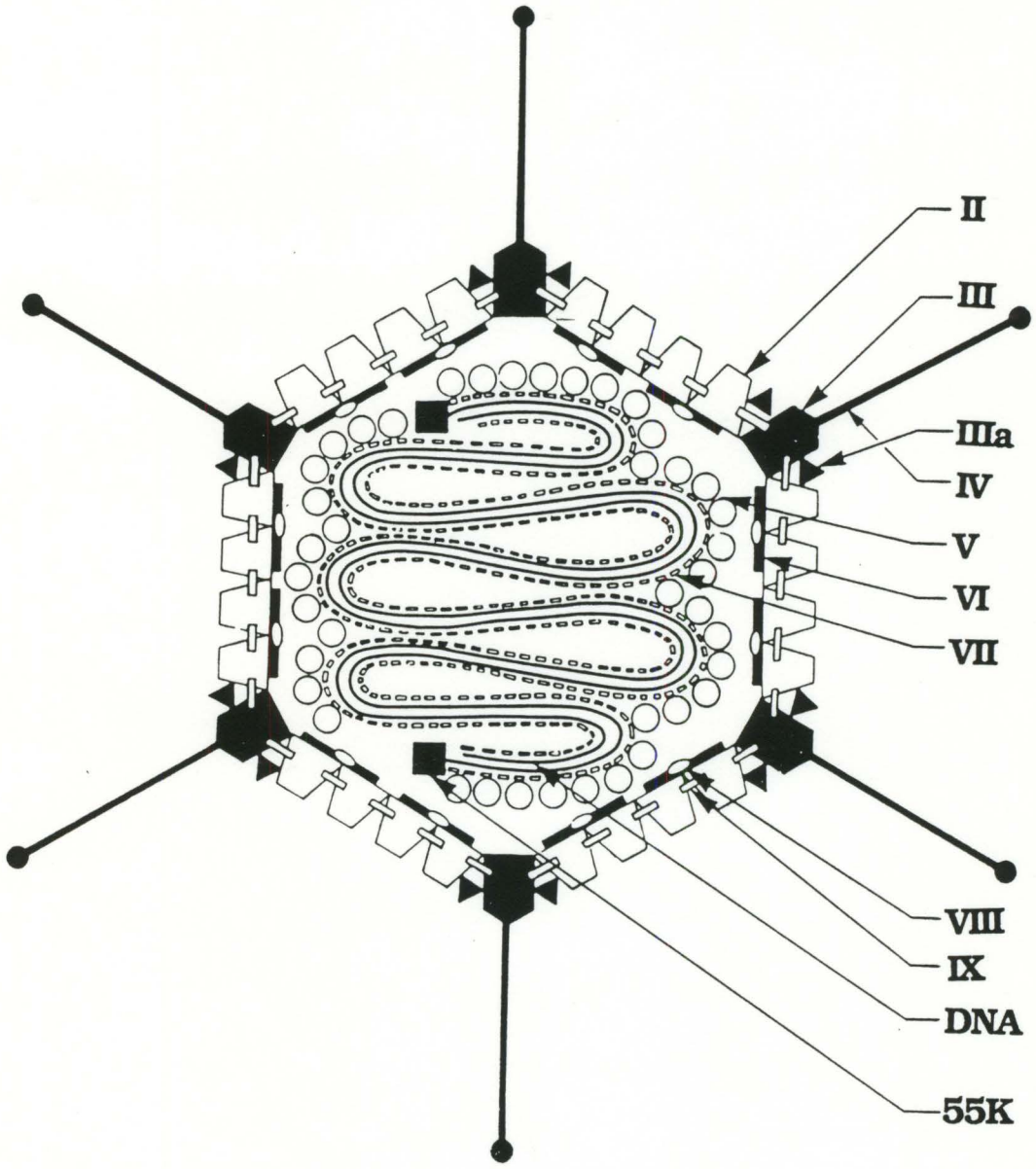
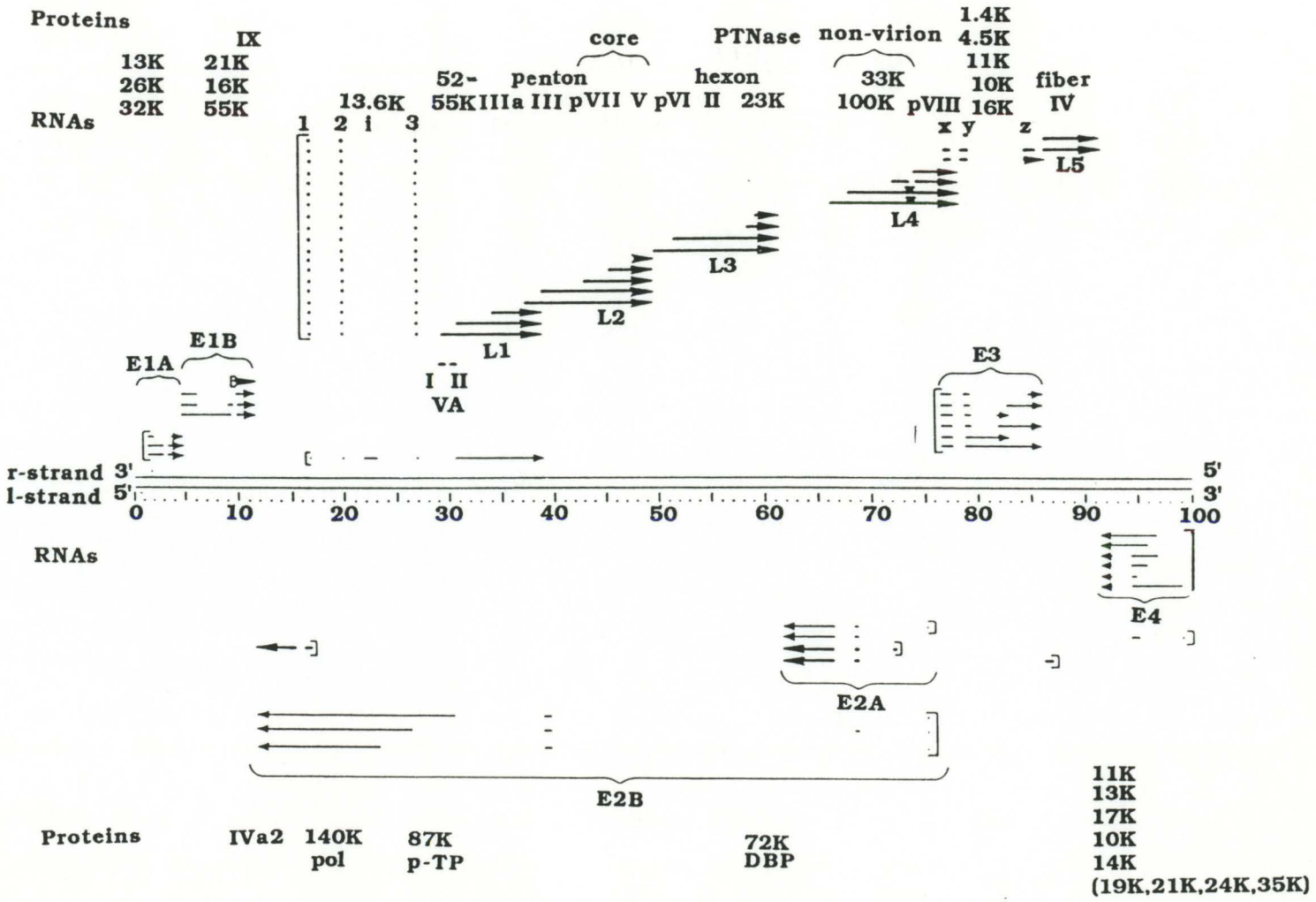

II
III
IIIa
IV
V
VI
VII
VIII
IX
DNA
55K

FIGURE 2: TRANSCRIPTION AND TRANSLATION MAP OF ADENOVIRUS TYPE 2

The early mRNAs are designated E and are transcribed by both strands. All late mRNAs, designated L, originate at 16.3 m.u. and contain the tripartite leader, designated 1, 2, and 3; some of the late mRNAs also contain a fourth leader segment, i.

The virion structural components are indicated by roman numerals, while the polypeptides designated in kilodaltons refer to non-structural virus-coded translation products (Modified from Horwitz, 1990).


Proteins
IX
13K 21K
26K 16K
32K 55K
RNAs
13.6K
1 2 i 3
52- 55K
core
penton IIIa III pVII V pVI
hexon II 23K
PTNase
non-virion
33K 100K pVIII
1.4K
4.5K
11K
10K 16K
fiber IV
x y z
L5
L4
L3
L2
L1
E1A
E1B
I II VA
E3
r-strand 3'
l-strand 5'
5'
3'
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A
E2B
Proteins
IVa2
140K pol
87K p-TP
72K DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

2. MATERIALS & METHODS

2.1 CELL LINES

The cell types used in these studies were HeLa (human cervical carcinoma), MDBK (Maden Darby Bovine Kidney), MDCK (Maden Darby Canine Kidney), L (murine fibroblast) and 293 (human embryonic kidney transformed with DNA from human adenovirus type 5 (Graham et al., 1977)).

293 cells were maintained as monolayers in plastic tissue culture dishes (Falcon^R, Corning^R, and Nunclon^R, Delta) containing minimal essential medium F-11 (MEM F-11) supplemented with 10% newborn calf serum (v/v) (Gibco^R Laboratories), 100 units/ml Penicillin and 100 μg/ml Streptomycin (Gibco^R Laboratories). Monolayers were dispersed by treatment with a solution of Versene (1.4 M NaCl, 6.8 mM EDTA, 26 mM KCl, 15 mM KH_2PO_4, 15 mM Na_2HPO_4, 0.2% Glucose (w/v)).

The remaining cell types were maintained as monolayers in α-MEM media supplemented with 10% newborn calf serum (v/v), 100 units/ml Penicillin and 100 μg/ml Streptomycin. Monolayers were

dispersed by treatment with a solution containing 0.5% Trypsin, and 5.3 mM EDTA (Gibco® Laboratories).

2.2 VIRUS TITRATIONS

To determine viral growth in various cell types, infected cell extracts underwent seven freeze/thaw cycles, and dilutions were made to infect confluent monolayers of 293 cells in 60 mm tissue culture dishes. Following an adsorption period of one hour, monolayers were overlaid with solution containing 50% 2X F-II media (v/v) supplemented with 10% newborn calf serum (v/v), 200 units/ml Penicillin and 200 μg/ml Streptomycin, and 0.5% melted agarose (w/v) at 45°C. Monolayers were incubated at 37°C until plaques were visible (four to five days), and cells were fixed using Carnoy's fixative (methanol:acetic acid, 3:1).

2.3 VIRAL DNA MANIPULATIONS

Extraction of Viral DNA from Infected Cells

(Modification of the protocol described by Hirt, 1967)

Infected monolayers were washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.6 mM KCl, 8.3 mM Na_2HPO_4, 1.5 mM

KH_2PO_4, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$), and 0.4 ml of Pronase/SDS (sodium dodecyl sulfate) (10 mM Tris (pH 7.4), 10 mM EDTA, 0.5% SDS (w/v), 0.25% pronase (w/v), pre-digested by incubating at 37°C for 30 minutes, and at 56°C for 30 minutes) was added to each dish. Cells were incubated at 37°C for at least 5 hours, harvested, and the cell extracts were poured into microfuge tubes. 100 µl of 5 M NaCl was added to each tube, and the solutions were centrifuged at 13,000 rpm for 30 minutes at 4°C to precipitate chromosomal DNA. Viral DNA was purified from the supernatant by extracting once with an equal volume of phenol saturated with 1 M Tris (pH 8.0), once with an equal volume of phenol:chloroform (phenol:chloroform: isoamyl alcohol, 25:24:1 (v/v/v)), and once with an equal volume of chloroform (chloroform:isoamyl alcohol, 24:1 (v/v)). The aqueous phase was transferred to a new microfuge tube and DNA was precipitated by adding 2 volumes of ice cold 95% ethanol, and incubating at -20°C for 30 minutes, followed by centrifugation at 13,000 rpm for 30 minutes at 4°C. The DNA pellet was washed with 70% ethanol, dried, and resuspended in 50 µl H_20.

Inhibition of Viral DNA Synthesis

Cytosine arabinoside (ara-c) was added to culture medium after virus adsorption to a concentration of 20 µg/ml.

2.4 CLONING PROCEDURES

Large Scale Plasmid DNA Preparations

Following inoculation with a 1 ml bacterial overnight culture, 750 ml Luria Broth (LB) (1% bactotryptone (w/v), 1% NaCl (w/v), 0.5% yeast extract (w/v)) containing 50 μg/ml ampicillin was incubated overnight at 37°C with vigorous shaking (300 cycles/min on a rotary shaker). Bacteria were harvested by centrifuging at 4,000 rpm for 15 minutes at 4°C in a Sorvall GS3 rotor and washed in 100 ml of ice-cold STE buffer (0.1 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)). Bacterial cells were collected by centrifuging at 4,000 rpm for 15 minutes at 4°C and cells were resuspended in 10 ml of a solution containing 50 mM glucose, 25 mM Tris (pH 8.0), and 10 mM EDTA (pH 8.0). Cell walls were digested with the addition of 2 ml of a freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris (pH 8.0)), 40 ml of a solution of 0.2 N NaOH and 1% SDS (w/v). The contents were mixed thoroughly by gently inverting the closed centrifuge bottle several times, and the mixture was then incubated at room temperature for 5 to 10 minutes. Chromosomal DNA, high molecular weight RNA and proteins were precipitated by the addition of 20 ml of an ice-cold solution 3 M with respect to potassium and 5 M with respect to acetate, which was then mixed and incubated on ice for 10 minutes. The bacterial lysate was

centrifuged at 4,000 rpm for 15 minutes at 4°C and the supernatant was filtered through four layers of cheesecloth. Isopropanol (0.6 volumes) was added to the supernatant, mixed and incubated for 10 minutes at room temperature and the nucleic acids were recovered by centrifuging at 5,000 rpm for 15 minutes at room temperature. The pellet was washed in 70% ethanol and then dissolved in 3 ml of TE (pH 8.0). Plasmid DNA was further purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient according to the protocol described by Maniatis, 1989.

Small Scale Plasmid DNA Preparations

Single bacterial colonies were transferred into 2 ml of LB containing the appropriate antibiotic (50 μg Ampicillin/ml) in loosely capped tubes and incubated overnight at 37°C with vigorous shaking. Bacteria was pelleted by centrifuging 1.5 ml of the culture in microfuge tubes at 12,000 rpm for 30 seconds and the medium was removed by aspiration. Pellets was resuspended in 100 μl of lysosyme buffer (50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0)) and incubated on ice for 10 minutes. The cells were lysed in 200 μl alkaline SDS at room temperature for 15 minutes, and 150 μl of 3 M potassium acetate (pH 4.8) was added to the lysate. Following incubation on ice for 20 minutes, the tubes were centrifuged for 5 minutes and the supernatant was decanted into

fresh microfuge tubes. DNA was precipitated with 95% ethanol, washed in 70% ethanol, dried, and resuspended in 50 µl H_2O.

Digesting DNA with Restriction Enzymes

Digestions were carried out in buffers supplied by restriction enzyme manufacturers (Gibco® BRL or Boehringer Mannheim). Purified DNA was digested with 3 units of enzyme per µg of DNA at 37°C for at least 2 hours. Reactions were stopped by phenol/ chloroform extraction or gel loading buffer (0.25% bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v), 15% Ficoll (type 400: Pharmacia) in H_2O) if the DNA was to be analyzed directly on a gel.

Gel Purification of DNA Fragments Using GeneClean™ (Bio/Can Scientific Inc.)

The fragment to be purified was excised from the agarose gel and placed in a microfuge tube, to which 2.5 to 3 volumes of 6 M NaI solution was added. The agarose was dissolved by a 55°C incubation for 5 to 10 minutes, cooled on ice, and 5 µl of Glassmilk suspension (specially prepared suspension of silica matrix in H_2O) was added to the solution. The Glassmilk/DNA complex was pelleted by centrifuging for 5 seconds, and the supernatant was removed. The pellet was washed 3 times in NEW wash (50% ethanol (v/v), 50% of a

solution containing 20 mM Tris (pH 7.4), 1 mM EDTA, 100 mM NaCl) (v/v) stored at -20°C, and the DNA was eluted into 20 µl of H_2O.

Ligations

Plasmid and foreign DNAs were digested with the appropriate enzymes which were then inactivated by heat or phenol/chloroform extraction. No more than 0.5 µg vector DNA was added to an equimolar amount of foreign DNA in a sterile microfuge tube and incubated for 2 to 4 hours at 16°C, with bacteriophage T4 DNA ligase buffer (20 mM Tris (pH 7.6), 5 mM $MgCl_2$, 5 mM dithiothreitol, 50 µg/ml BSA (bovine serum albumin)), 1 unit T4 DNA ligase, and 0.5 mM ATP in a total volume of 10 µl. 1 µl of the ligation reaction was used to transform competent *E. coli* cells.

Transformation of *E. coli* by Calcium Chloride

To prepare competent cells for transformation by the calcium chloride technique, 100 ml of LB media was inoculated with 1 ml DH5-α overnight culture and $MgCl_2$ was added to a final concentration of 10 mM. Cells were grown at 37°C with constant agitation until a OD_{550} reading of 0.5 was reached. Cells were chilled on ice for 10 minutes, pelleted by centrifugation and

resuspended in an ice cold solution of 50 mM $CaCl_2$ and 10 mM Tris (pH 8.0). Following a 15 minute incubation period on ice, cells were again pelleted and resuspended in 3.5 ml of the $CaCl_2$·Tris solution. For the actual transformation, 400 ng of DNA were added to 200 µl of the cell solution, incubated on ice for 30 minutes and heat shocked at 42°C for 2 minutes. 1 ml of LB with appropriate antibiotic was added and the culture was incubated at 37°C for the appropriate amount of time before plating.

Transformation of *E. Coli* by High-Voltage Electroporation (Gene Pulser®, Bio-Rad Laboratories)

To prepare competent cells for electroporation, DH5-α cells were grown in LB to an ABS_{600} of 0.5, washed twice in ice cold H_20, washed and resuspended in 10% glycerol before freezing. 1 to 2 µl of DNA from the ligation mixture was added to 55 µl of the cell suspension and transferred to a cold Gene Pulse cuvette. The Gene Pulser apparatus was set at 25 µF and 2.25 kV; the Pulse Controller was set to 200 ohms. The cuvette containing the cell suspension was placed in the Gene Pulser apparatus chamber, and following exposure to one pulse, 1 ml of SOC media (2% bacto-tryptone (w/v), 0.5% bacto-yeast extract (w/v), 0.05% NaCl (w/v), 2.5 mM KCl, 0.01 M $MgCl_2$ (pH 7.0)) was immediately added to the cells. Cells were grown for 1 to 2 hours at 37°C with agitation and then plated on appropriate antibiotic resistant LB plates.

Identification and Analysis of Recombinants

Plates were made with media containing 0.004% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (w/v), 0.1% agar (w/v), and the appropriate antibiotic. Bacteria were plated and incubated at 37°C for at least 12 hours. Colonies containing active β-galactosidase were blue whilst colonies harbouring recombinant plasmids appeared white.

The structure of recombinant plasmids was determined by restriction enzyme analysis of small scale plasmid DNA preparations from a random selection of recombinant bacterial colonies.

2.5 β-GALACTOSIDASE ASSAY

Monolayers were infected with Ad5lacZ (lacZ gene of *E. coli* inserted in the parallel orientation of the hAd5 E3 region) at a MOI of 5 to 10, harvested at various times post-infection, and assayed for β-galactosidase activity.

Media was removed, each monolayer was washed with PBS and cells were harvested by scraping in PBS with a rubber policeman. Following centrifugation at 1,000 rpm for 10 minutes, each cell pellet was resuspended in 200 to 300 μl of 250 mM Tris (pH 7.8), 1 mM PMSF (phenylmethylsulfonylfluoride) and 0.5% NP-40 (Nonidet

P-40) (v/v), and vortexed for one minute; extracts were then transferred to microfuge tubes and put on a rotating platform for 20 minutes at 4°C. After cell extracts were centrifuged for 10 minutes at 4°C, 20 µl of cell extract supernatant was added to 350 µl of 10mM KCl, 1 mM $MgSO_4$, 100 mM sodium phosphate, 50 mM β-mercaptoethanol (pH 7.5), and solutions were pre-incubated at 37°C for 5 minutes. 132 µl of o-nitrophenyl β-D-galactopyranoside (4 g/l in 100 mM sodium phosphate (pH 7.5)) was added and the incubation was continued at 37°C for an additional hour.

The reaction was terminated by addition of 172 µl of 1 M Na_2CO_3, and the optical density of a 1:10 dilutions was measured at 420 nm.

2.6 ANALYSIS OF VIRAL PROTEINS

Metabolic Labelling

Cell monolayers in 60 mm plastic tissue culture dishes were infected with hAd5 at an MOI of 10 to 20. At appropriate times following infection, media was removed and cells were washed with 199 Met^- media (media lacking methionine) containing no serum. Proteins were labelled by the addition of 1 ml of fresh, pre-warmed (37°C) 199 Met^- media (media supplemented with newborn calf

serum, penicillin and streptomycin, and 50 to 100 μCi [^{35}S] methionine). Cells were incubated for one to two hours at 37°C.

Immunoprecipitation

To harvest cells, media was removed and monolayers were washed 3 times with PBS. Cells from each dish were collected in 5 ml of PBS using a rubber policeman and pelleted by centrifuging at 1,000 rpm for 10 minutes. PBS was discarded, cells were resuspended in 1.1 ml RIPA buffer (0.05 M Tris (pH 7.2), 0.15 M NaCl, 0.1% SDS (w/v), 1% Sodium Deoxycholate (w/v), 1% Triton-X-100 (v/v)) and transferred to microfuge tubes. Extracts were incubated on ice for 20 minutes, vortexed for 2 minutes and centrifuged for 30 minutes at 4°C.

For each immunoprecipitation, 500 μl of supernatant was incubated with 100 μl of a suspension of protein A sepharose beads (15% dry protein A sepharose beads (w/v), 85% RIPA buffer (v/v)) and the appropriate amount of antibody; samples were gently rotated for at least 6 hours at 4°C. The beads were pelleted and washed 4 to 6 times in RIPA buffer. Proteins were denatured by resuspending the beads in 50 μl of 2X sample buffer (50 mM Tris (pH 7.2), 2% SDS (w/v), 5% β-mercaptoethanol (v/v), 10% glycerol (v/v), 0.1% bromophenol blue (w/v)) and heating to 100°C for 3 minutes. After pelleting the beads, proteins from 20 μl of supernatant were

resolved on 9% SDS polyacrylamide gels (w/v).

Protein A Sepharose beads (Pharmacia Inc.) were prepared by swelling 500 mg of beads in 25 ml of RIPA buffer on a rotating platform at 4°C. The beads were washed 3 times by centrifugation at 1,500 rpm for 5 minutes and resuspending each time in RIPA buffer. The final pellet was resuspended in 3.5 ml of RIPA buffer and stored at 4°C.

Various antibody solutions of different specificities and concentrations were used. The hAd5 proteins were immuno-precipitated with an hAd5 polyclonal rabbit serum (10 µl per reaction). The hAd5 72K DNA binding product was immuno-precipitated with serum obtained from P. Branton (McGill University, Montreal, PQ) (5 µl per reaction). The hAd5 protein II (hexon) was immunoprecipitated with a monoclonal antibody obtained from J. Williams (Carnegie-Mellon University, Pittsburgh, PA) (5 µl per reaction). The following ascites fluids were obtained from A. Wandeler (Animal Diseases Research Institute, Nepean, ON): 21E7-4-5, 1C5-5-2, and 22B3-5-11. These were classified as anti-hexon, anti-fiber, and anti-hexon/anti-fiber respectively, and 10 µl of each ascites fluid were used per reaction.

SDS Polyacrylamide Gel Electrophoresis

Acrylamide solutions were diluted to the appropriate concentration from a stock solution containing 30% acrylamide (w/v) and 0.9% N,N'-Methylenebisacrylamide (w/v). Resolving gels consisting of 9% acrylamide (w/v) in 0.37 M Tris (pH 8.8), 0.1% SDS (w/v) and 0.1% glycerol (v/v) were polymerized by the addition of 0.1% ammonium persulfate (w/v) and 0.35 µl/ml of N,N,N',N'-Tetramethlyethylenediamine (TEMED). Stacking gels consisting of 5.6% acrylamide (w/v) in 0.12 M Tris (pH 6.8), 0.1% SDS (w/v) and 0.05% glycerol (v/v) were polymerized using 0.06% ammonium persulfate (w/v) and 0.72 µl/ml TEMED. Electrophoresis was carried out in tris-glycine electrophoresis tank buffer (0.025 M Tris, 0.2 M Glycine, 0.1% SDS (w/v)) until the bromophenol blue reached the bottom of the gel.

Fluorography

Gels were dehydrated by soaking in two changes of dimethyl sulfoxide (DMSO), impregnated with 2,5-diphenyloxazole (PPO) by soaking in a 20% PPO (w/v) solution in DMSO, washed free of DMSO by soaking in water for 30 minutes and dried. Gels were then exposed to either Kodak X-Omat RP or X-Omat AR film at -70^{o}C.

2.7 EXTRACTION OF MESSENGER RNA FROM EUKARYOTIC CELLS

Preparation of Cytoplasmic RNA

Infected cell monolayers in 150 mm dishes were harvested and washed in ice cold PBS. Cytoplasmic RNA was extracted by resuspending the cell pellet in 375 µl ice cold RNA extraction buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.6), 0.5% NP-40 (v/v), 1 mM dithiothreitol, 1,000 units/ml placental RNAase inhibitor (Boehringer Mannheim, 799017)) and centrifuging at 12,000 rpm for 90 seconds at 4°C in a microfuge tube. The supernatant fluid was transferred to a clean microfuge tube containing 4 µl of 20% SDS (w/v), proteinase digestion buffer (0.2 M Tris (pH 8.0), 25 mM EDTA (pH 8.0), 0.3 M NaCl, 2% SDS (w/v)) was added, and the mixture was vortexed. Proteinase K was added to a final concentration of 50 µg/ml and the solution was mixed well and incubated for 30 minutes at 37°C. Proteins were removed by extracting once with an equal volume of phenol:chloroform. Organic and aqueous phases were separated by centrifugation at 4,000 rpm for 10 minutes at room temperature in a Beckman GPR centrifuge. The aqueous phase was transferred to a fresh microfuge tube and 400 µl of ice-cold isopropanol was added, mixed and chilled for 30 minutes on ice to precipitate the RNA. The RNA was collected by centrifugation at 12,000 rpm for 10 minutes at 4°C, and the pellet was washed with

70% ethanol. To remove any DNA that may have been present, the pellet was redissolved in a small volume of 50 mM Tris (pH 7.8), 1 mM EDTA (pH 8.0). $MgCl_2$ and dithiothreitol were added to final concentrations of 10 mM and 0.1 mM, respectively, and placental RNAase inhibitor was added to a final concentration of 100 units/ml. RNAase-free pancreatic DNAase I (Pharmacia, LKB Biotechnology, 27-0514) was added to a final concentration of 2 μg/ml and samples were incubated for 60 minutes at 37°C. EDTA and SDS were added to final concentrations of 10 mM and 0.2% (w/v), respectively and the solution was extracted once with an equal volume of phenol: chloroform. After centrifugation, 3 M sodium acetate (pH 5.2) was added to the aqueous phase to a final concentration of 0.3 M, and RNA was precipitated using ice-cold ethanol.

Preparation of Total RNA

Infected cell monolayers in 150 mm dishes were harvested and washed 3 times in ice cold PBS. Cells were pelleted by centrifuging at 2,000 rpm for 10 minutes. 2.5 ml of a guanidinium salt solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.1 M β-mercaptoethanol) was added to the cell pellet. The solution was vortexed and then layered onto 1.8 ml of a 5.7 M CsCl solution in a Beckman Ultraclear ultracentrifuge tube and centrifuged in a Beckman SW 50.1 rotor at 35,000 rpm for about 18 hours. The

supernatant was removed and the pellet was resuspended in 300 µl H_2O. NaCl was added to a final concentration of 0.1 to 0.2 M. RNA was precipitated by adding 2 volumes of 95% ethanol and incubated at -20°C for 1 hour. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 500 µl H_2O.

Purified RNA preparations were quantitated by measuring the optical density of a 1:10 dilution at 260 nm.

2.8 SOUTHERN HYBRIDIZATION

(Modification of the protocol described by Southern, 1975)

Transfer

Following gel electrophoresis, the 1% agarose gel was uv irradiated for 3 minutes and the DNA was denatured by soaking the gel in a solution that was 0.5M with respect to NaOH, and 1.5 M with respect to NaCl, for 1 hour. The gel was neutralized by soaking in two changes of 2 M Tris (pH 8.0)/3 M NaCl (1:1) for 45 minutes and 30 minutes. The DNA was transferred from the gel to GeneScreen *Plus* ™(Dupont, NEF-976) hybridization transfer membrane by capillary transfer, and the DNA was fixed to the membrane by uv cross-linking.

Prehybridization/Hybridization

A blocking agent consisting of 6X SSC, 5X Denhardt's reagent (50X Denhardt's reagent contains 5 g Ficoll (Type 400, Pharmacia), 5 g polyvinylpyrrolidone, 5 g BSA (Fraction V; Sigma) made up with H_20 to 500 ml), 0.5% SDS (w/v), and 100 µg/ml denatured, fragmented salmon sperm DNA (prepared using the protocol described by Maniatis, 1989) was used. 0.2 ml of prehybridization solution was used for each square centimeter of nylon membrane, and prehybridization was carried out at 68°C for 1 to 2 hours.

Double-stranded radiolabeled DNA probes were denatured by boiling for 5 minutes followed by incubating on ice, before being added to the prehybridization solution, and hybridization was carried out at 68°C for at least 8 hours.

Blots were washed in 2X SSC and 0.5% SDS (w/v) (5 minutes, room temperature), 2X SSC and 0.1% SDS (w/v) (15 minutes, room temperature with gentle agitation), 0.1X SSC and 0.5% SDS (w/v) (30 minutes, 37°C with gentle agitation), 0.1X SSC and 0.5% SDS (w/v) (30 minutes, 68°C with gentle agitation) and finally with 0.1X SSC at room temperature before being wrapped in Saran Wrap and exposing to x-ray film at -70°C for the appropriate period of time.

2.9 SLOT HYBRIDIZATION OF RNA

(Adapted from Maniatis, 1989)

The final solution of each RNA sample contained 50% formamide (v/v), 7% formaldehyde (v/v), and 1X SSC. A filtration manifold by Schleicher and Schuell was used to deposit the RNA onto GeneScreen *Plus* ™ membrane in a given order. The RNA was fixed onto the membrane by uv cross-linking and probed as described for DNA hybridization.

2.10 PROBE PREPARATION

Nick Translation

(Adapted from Maniatis, 1989)

0.5 µg DNA was incubated with 2.5 µl of 10X nick-translation buffer (0.5 M Tris (pH 7.5), 0.1 M $MgSO_4$, 1 mM dithiothreitol, 500 µg/ml BSA (Fraction V; Sigma)), 20 nmol each of dTTP, dCTP, and dGTP, 16 pmol of [α-^{32}P]dATP in a total volume of 21.5 µl. The mixture was chilled to 0°C and 2.5 µl of DNAase I (Pharmacia, 10 ng/ml) was added followed by 2.5 units of *E. coli* DNA polymerase I. The reaction was incubated for 60 minutes at 16°C and stopped with 1 µl of 0.5 M EDTA (pH 8.0). Unincorporated dNTPs were separated from radiolabeled DNA by spun-column chromatography.

Probe Preparation Using Sequenase®

The Sequenase® Version 2.0 kit was purchased from United States Biochemical Corporation. A modification of the sequencing reaction protocols outlined in the kit was followed to generate probes, but the reactions were not terminated with dideoxy-nucleotides. Oligodeoxynucleotide primers for probe preparation were synthesized by the Institute for Molecular Biology and Biotechnology, McMaster University, with an Applied Biosystems automated DNA synthesizer. Briefly, the appropriate primer (0.5 pmol) was annealed to the template DNA (0.5 to 1.0 pmol) by adding to 2 µl 5X Annealing Buffer (200 mM Tris (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl) and bringing the total volume up to 10 µl with H_20. The capped tube was warmed to 65°C for 2 minutes, and the mixture was allowed to cool slowly to room temperature over a period of 30 minutes. The labeling reaction was initiated by adding the following to the annealed template-primer: 1 µl DTT (0.1 M), 2 µl labeling nucleotide mix (containing 1.5 µM dGTP, 1.5 µM dCTP, and 1.5 µM dTTP), 5 µCi [α-^{32}P] dATP, and 3 units Sequenase® Version 2.0. Contents were mixed thoroughly and incubated for 5 minutes at room temperature. Radiolabeled DNA was separated from the unincorporated dNTPs by spun-column chromatography.

Synthesis of RNA Probes for Slot Blots

(Adapted from Maniatis, 1989)

Template DNA was prepared by digestion with the appropriate restriction enzyme and purified by phenol:chloroform extraction followed by ethanol precipitation. The reaction began with the addition of the following in the order given, and incubating for 1-2 hours at 37°C: 0.4 µl RNAase-free H_2O, 0.2 pmol template DNA (in a volume no more than 1 µl), 0.1 µl 1 M dithiothreitol, 1.0 µl of a solution containing rATP, rCTP, and rGTP, each at 5 mM, 1.0 µl of 10X transcription buffer (400 mM Tris, pH 7.5, 60 mM $MgCl_2$, 20 mM spermidine HCl, and 50 mM NaCl), 10 units placental RNAase inhibitor (Boehringer Mannheim, 50,000 units/ml), 0.5 µl bovine serum albumin (Fraction V; Sigma), 5.0 µl [α-^{32}P] rUTP, and 1.0 µl of T7 bacteriophage DNA-dependent RNA polymerase.

Template DNA was digested by adding 1 µl RNAase-free pancreatic DNAase I (Pharmacia LKB Biotechnology, 10,000 units/ml) and incubating at 37°C for 15 minutes . Following the addition of 100 µl of RNAase-free H_2O, the RNA was purified by extracting with phenol:chloroform, and precipitating with 95% ethanol.

Synthesis of RNA Probes for RNAase Protection Analysis

Template DNA was digested at a restriction site appropriate to terminate transcription of the riboprobe, run on a 1% agarose gel (w/v), and gel purified using the GeneClean^{TM} protocol (Bio/Can Scientific, Inc.). The following reagents were added: 10.0 µl [α-^{32}P] rUTP, 4 µl 5X transcription buffer (200 mM Tris (pH 8.2), 30 mM $MgCl_2$, 20 mM spermidine), no more than 0.5 pmol of gel template DNA (in a volume of 2.0 µl or less), 2.0 µl of a solution containing rATP, rCTP, and rGTP (1:1:1 mix of 10 mM stock of each), 1 µl RNAsin (40,000 units/ml, Boehringer Mannheim, human placental RNAase inhibitor), and 1 µl RNA polymerase--either T3 or T7 RNA pol (40,000 units/ml, isolated from HB101). The solution was mixed and incubated at 37°C. After 20 minutes, another 1 µl RNA polymerase was added, and the reaction was incubated for an additional 30 minutes. Template DNA was digested with the addition of 2 µl of RNAase-free pancreatic DNAase I (Pharmacia LKB Biotechnology, 10,000 units/ml) and incubating at 37°C for 10 minutes. RNA was precipitated with the addition of 50 µl H_20, 10 µl of 3 M sodium acetate (pH 5.2), 20 µg yeast tRNA, and 250 µl of 95% ethanol, incubating at -70°C for 15 minutes, and centrifuging at 13,000 rpm for 15 minutes at 4°C. The pellet was resuspended in H_20, and percent incorporation was determined.

A 1:10 dilution of the probe was made, of which 5.0 µl was blotted onto Whatman DE81 ion exhange paper in duplicate. One

sample was washed with 0.5 M Na_2HPO_4, rinsed with H_20, and dried with 95% ethanol. The washed and unwashed samples were counted in scintillation cocktail to determine the percent incorporation.

2.11 SPUN-COLUMN CHROMATOGRAPHY

Columns were prepared by plugging the bottom of a 1 ml disposable syringe with a small amount of sterile glass wool and filling with Sephadex G-50 equilibrated in 1X TEN buffer (10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), and 100 mM NaCl). The syringe was inserted into a 15 ml disposable plastic tube and centrifuged at 1600 rpm for 4 minutes at room temperature. More resin was added and re-centrifuged until the column was packed to a fixed volume of approximately 0.9 ml. The column was washed 3 times with 1X TEN buffer and then placed in a fresh disposable tube containing a decapped microfuge tube. The DNA sample was added to the column in a total volume of 0.1 ml and eluted DNA (excluding unincorporated radiolabeled dNTPs or other small components) was collected by centrifuging at 1600 g for 4 minutes at room temperature.

2.12 RNAase PROTECTION ANALYSIS

Each RNA sample was in a volume of 20 µl. 1.0 µl of riboprobe was added to a solution of 1X Pipes (Piperazine-N,N'-bis[2-ethane-sulfonic acid]) buffer (10X Pipes buffer consists of 400 mM Pipes, 4 M NaCl, 10 mM EDTA (pH8.0)), 50% formaldehyde (v/v), and 30% RNA sample; the mixture was denatured at 85°C followed by hybridization at 50°C for at least 12 hours. As a control for probe self-protection, 20 µg of yeast tRNA was used in place of test RNA. Following hybridization of the probe to the mRNA, single-stranded RNAs were digested by adding 300 µl RNAase digestion buffer containing 40 µg/ml RNAase A and 1,000 units/ml RNAase T_1 (Sigma, Grade V) in RNAase buffer (10 mM Tris (pH 7.5), 1 mM EDTA (pH 8.0), 300 mM NaCl), and incubating at 37°C for 15 minutes, followed by a 10 minute incubation at room temperature. The RNAase was digested by the addition of 10 µl of Proteinase K (10µg/µl) and 20 µl of 10% SDS to each sample, and incubating at 37°C for 15 to 30 minutes. 300 µl of phenol/chloroform was added to each sample which was then vortexed and centrifuged at 13,000 rpm for at least 5 minutes. The aqueous phase was removed to a fresh microfuge tube containing 30 µg tRNA as a carrier for precipitation, and 1.0 ml 95% ethanol. The protected RNA was precipitated by incubating at -70°C for at least 20 minutes and pelleted by centrifuging at 13,000 rpm for 30 minutes at 4°C. The supernatant was removed and pellets dried before resuspending in

10 µl of 10X RNAase protection loading buffer made up with H_20, 3:1 (10X loading buffer consists of 50% glycerol (v/v), 1X TAE buffer, 0.25% bromophenol blue (w/v), and 0.25% xylene cyanol (w/v)). RNA was denatured by incubating at 100°C for 4 minutes, and immediately putting on ice. Samples were electrophoresed on a 6% denaturing polyacrylamide gel until the bromophenol blue reached the bottom. The gel was dried, and exposed against Kodak X-Omat AR film at -70°C.

3. RESULTS

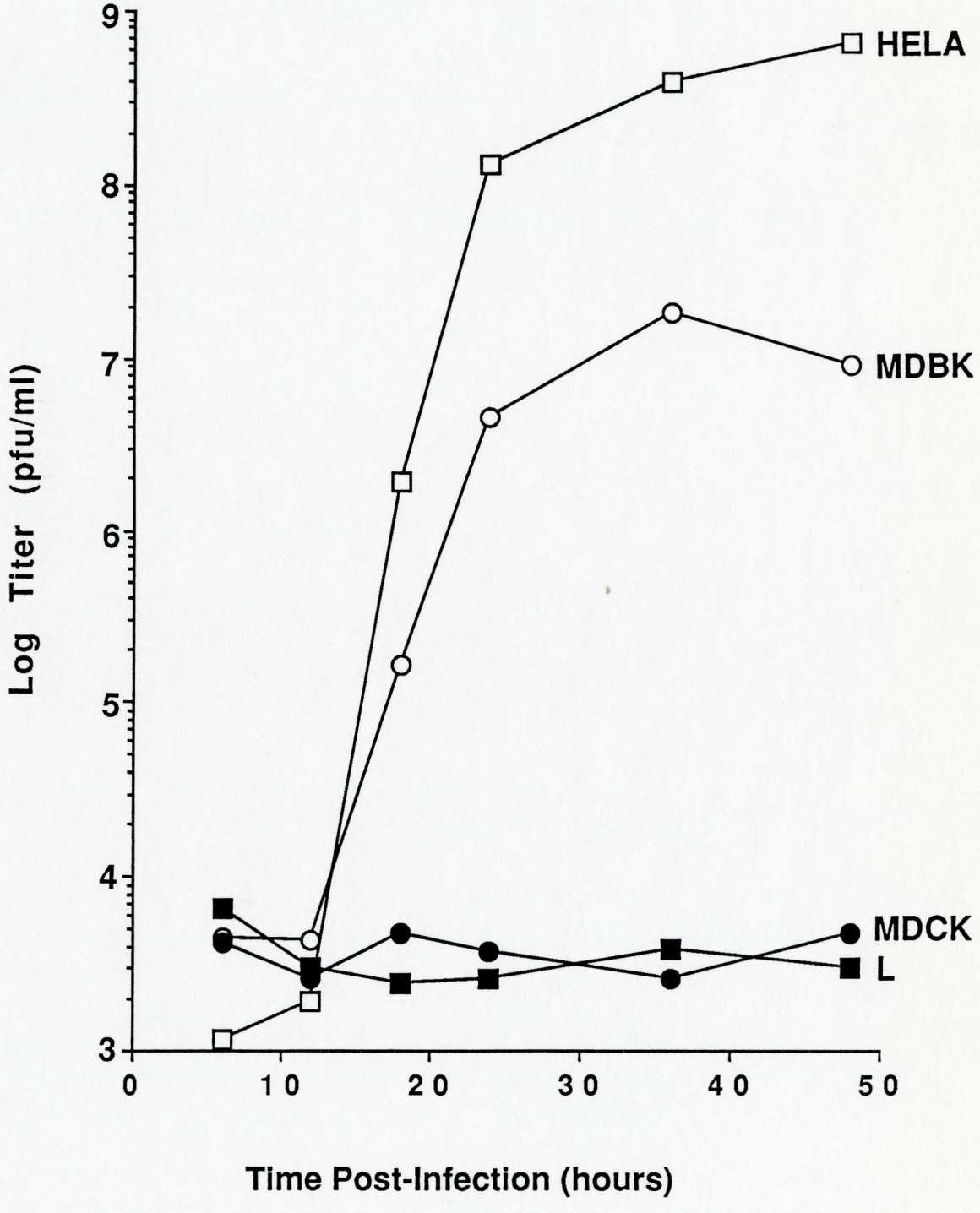
3.1 ADENOVIRUS TYPE 5 REPLICATION IN MDBK, MDCK, HELA, AND L CELLS

In order to examine the host range of hAd5, MDBK, MDCK, HeLa and L cells were infected at an MOI of 10, and harvested by freezing. The infected cells were subjected to several freeze/thaw cycles to release intracellular virus and infectious virus yields in these extracts were titered on 293 cells. The results are shown in Figure 3. Ad5 is a human virus and as expected, replicated in HeLa cells. MDBK cells also proved to be permissive for Ad5 replication, although to a lesser extent than in HeLa cells. Infectious virus titers in MDBK and HeLa cells were observed by 12 hours post-infection, and increased at an exponential rate reaching a maximum at 36 hours in MDBK cells. In HeLa cells, infectious virus continued to be produced to at least 48 hours, with the final yield of infectious virus at least 1.2 logs above that observed in MDBK cells. No viral replication was observed in MDCK and L cells during the

FIGURE 3: ADENOVIRUS TYPE 5 REPLICATION IN MDBK, MDCK, HELA, AND L CELLS

Cells were infected with hAd5wt at a MOI of 10 and harvested at 6, 12, 18, 24, 36, and 48 hours post-infection. Cell extracts underwent several freeze/thaw cycles to release all intracellular virus, and virus was titered on 293 cells as described in the Materials and Methods.

ADENOVIRUS TYPE 5 REPLICATION IN MDBK, MDCK, HELA, & L CELLS



first 48 hours of infection. In the case of the L cells which may be semi-permissive for hAd5 infection, this may simply be due to the delayed growth kinetics (Gallimore, 1974; Tremblay et al., 1985). However, there seems to be a block in hAd5 replication in MDCK cells.

3.2 ADENOVIRUS TYPE 5 DNA SYNTHESIS IN MDBK, MDCK, HELA, AND L CELLS

In order to determine whether the replicative block in MDCK cells was at the level of DNA replication, Southern blot analyses were performed on MDBK, MDCK, HeLa, and L cells infected with hAd5 at an MOI of 10. Viral DNA was extracted from cells harvested at various times post-infection and the DNA concentration normalized to cell number. The DNA was digested with *Hind III* and fragments were separated on a 1% agarose gel by gel electrophoresis. Following transfer of the DNA from agarose to nitrocellulose, the filter was probed with [^{32}P]-labelled pFGdX1 (1.28 x 10^8 cpm/μg), which is the hAd5wt *BamHI* B fragment (59.5 to 100 m.u.) minus the hAd5wt *XbaI* D fragment (78.5 to 84.7 m.u.) cloned into the *BamHI* site of pBR322 (Haj-Ahmad and Graham, 1986) and viral sequences were detected by autoradiography.

Figures 4.1, 4.2, and 4.3 represent different times of film exposure to the same filter. Figure 4.1 is a 2.3 hour exposure and shows that a detectable level of viral DNA is present in MDBK cells

FIGURES 4.1, 4.2, & 4.3:

ADENOVIRUS TYPE 5 DNA SYNTHESIS IN MDBK, MDCK, HELA, AND L CELLS

Cells were infected with hAd5wt at a MOI of 10, harvested at 6, 12, 24, 36, and 48 hours post-infection, and viral DNA was extracted using a modification of the protocol described by Hirt (1967). Samples were digested with *HindIII*, separated by agarose gel electrophoresis, and transferred onto nitrocellulose by a modification of the protocol described by Southern (1975).

Viral DNA was probed using [^{32}P]-labelled pFGdX1 which is the hAd5wt *BamHI* B fragment (59.5 to 100 m.u.) minus the hAd5wt *XbaI* D fragment (78.5 to 84.7 m.u.) cloned into the *BamHI* site of pBR322 (Haj-Ahmad and Graham, 1986). The mock lanes represent DNA extracted from cells treated with PBS^{++}. Panels A, B, C, and D represent mock and hAd5-infected MDBK, MDCK, HeLa, and L cells respectively.

Figure 4.1: 2 hour, 20 minute exposure
Figure 4.2: 28 hour exposure
Figure 4.3: 5 day exposure

FIGURE 4.1:

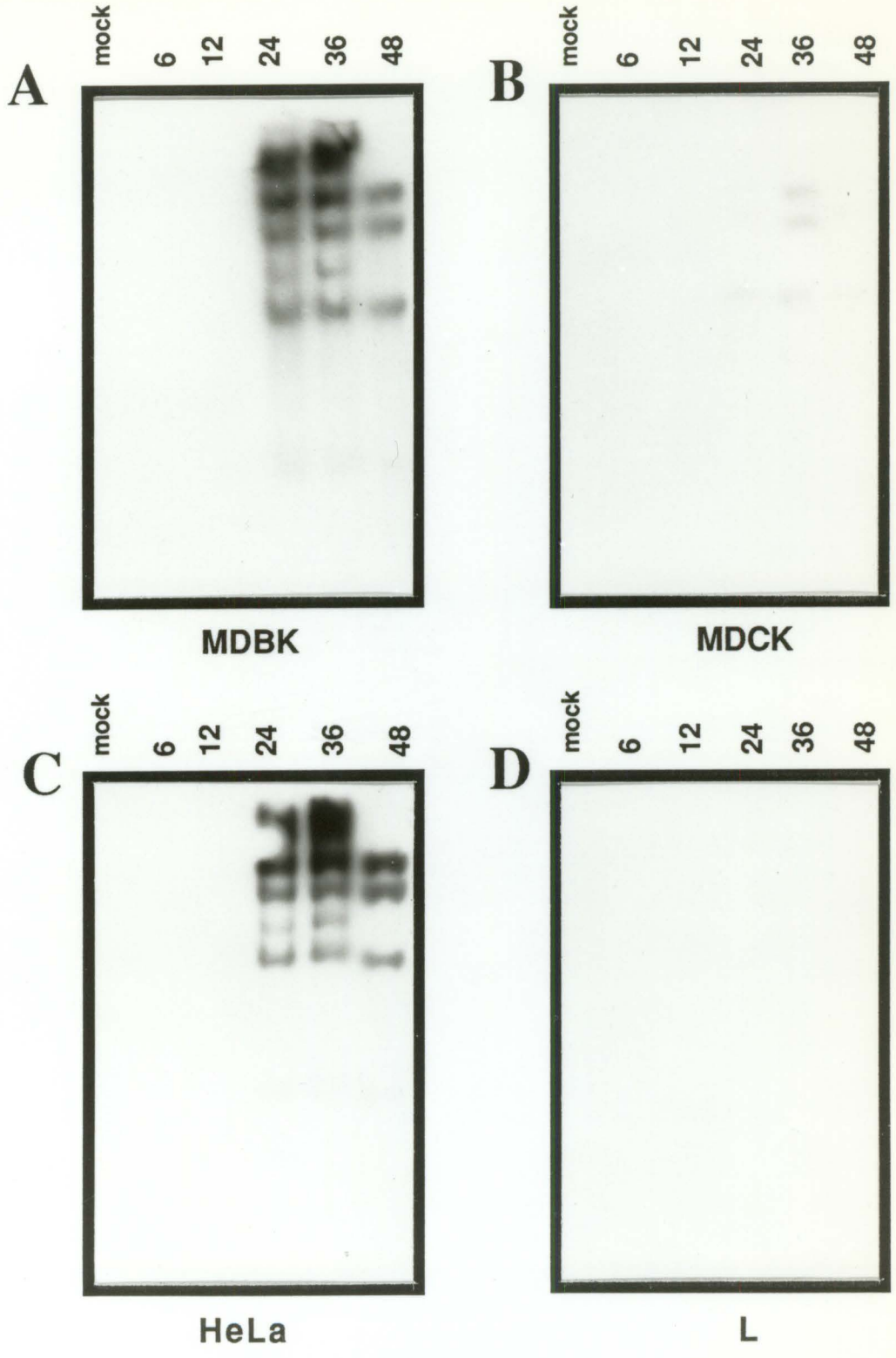

A
mock
6
12
24
36
48
MDBK
B
mock
6
12
24
36
48
MDCK
C
mock
6
12
24
36
48
HeLa
D
mock
6
12
24
36
48
L

FIGURE 4.2:

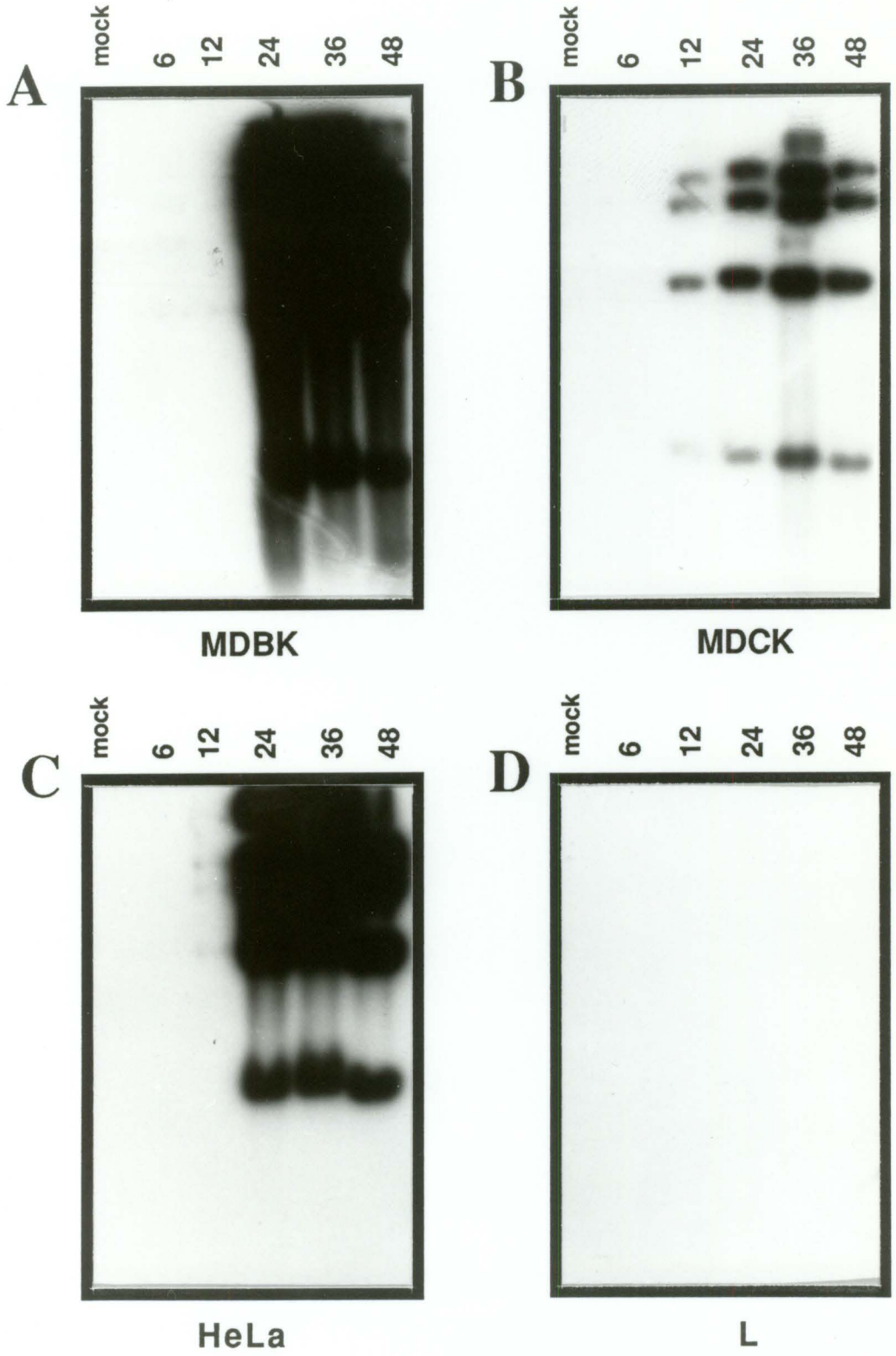
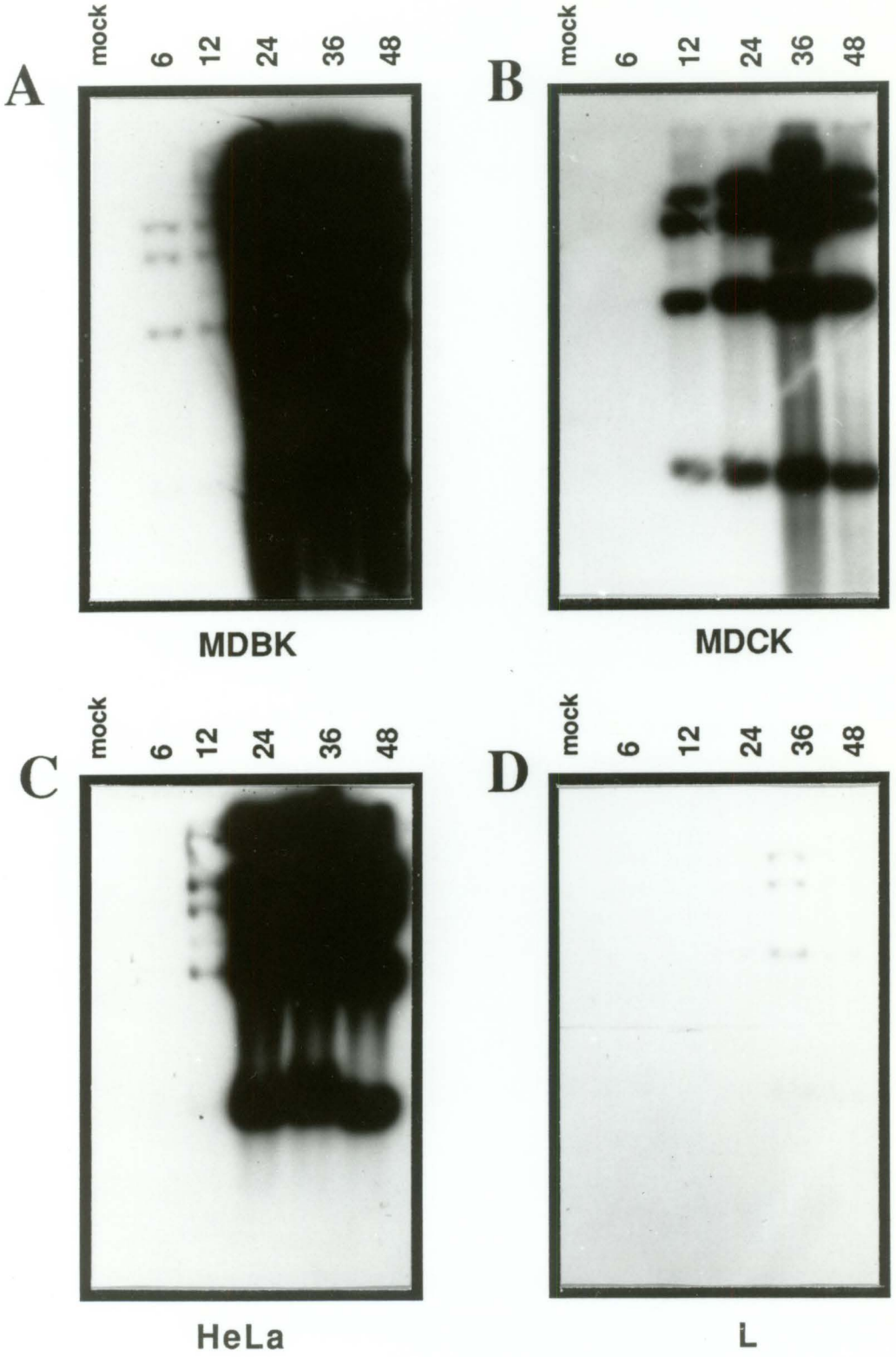

A
mock
6
12
24
36
48
MDBK
B
mock
6
12
24
36
48
MDCK
C
mock
6
12
24
36
48
HeLa
D
mock
6
12
24
36
48
L

FIGURE 4.3:


A
mock
6
12
24
36
48
MDBK
B
mock
6
12
24
36
48
MDCK
C
mock
6
12
24
36
48
HeLa
D
mock
6
12
24
36
48
L

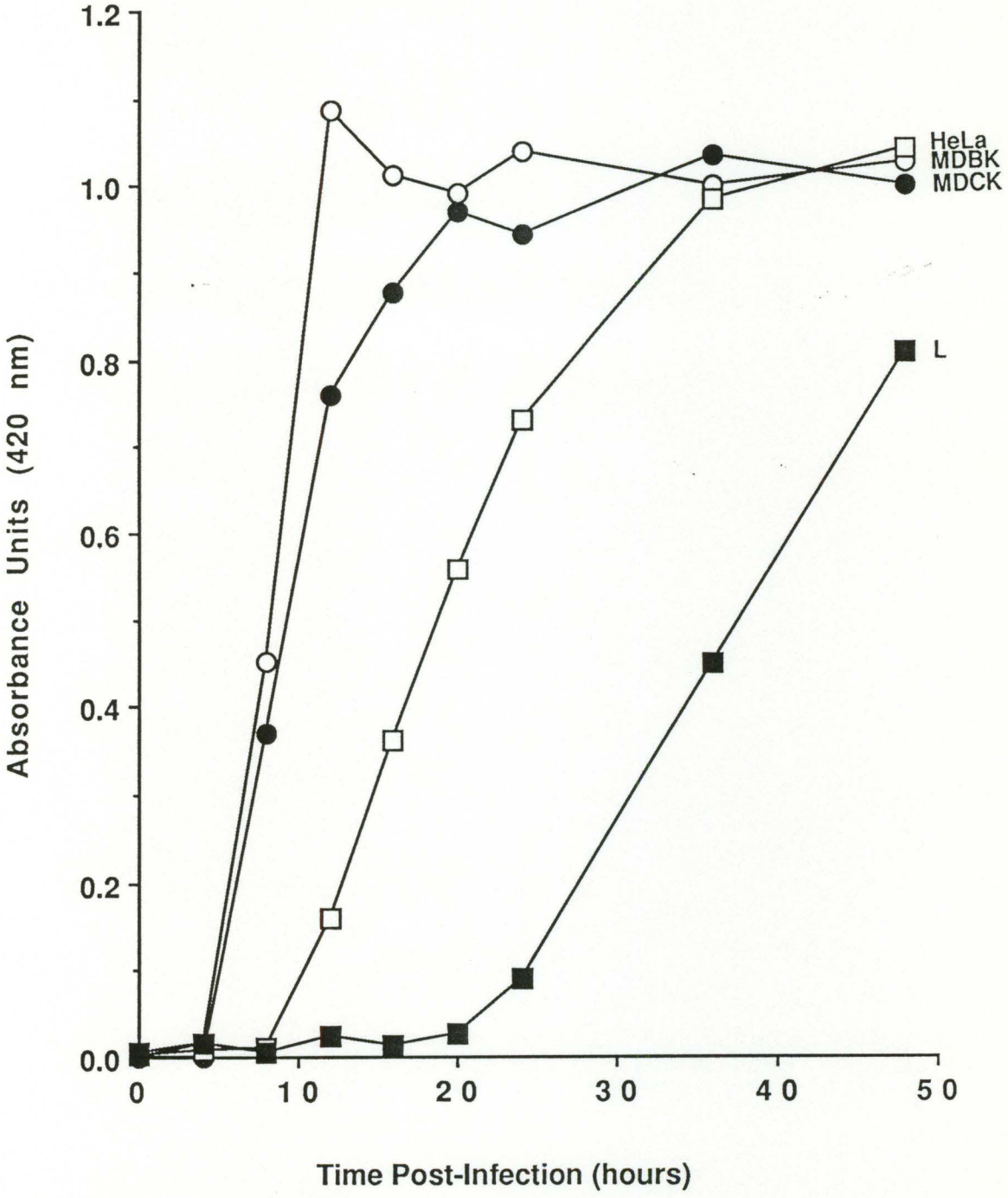
3.3 EXPRESSION OF ADENOVIRUS TYPE 5 EARLY PROTEINS IN MDBK, MDCK, HELA, AND L CELLS

Ad5lacZ contains the *E. coli* lacZ gene inserted into the E3 region of hAd5 along with the SV40 promoter, enhancer and polyadenylation sequences. Expression in host cells can be studied in a semi-quantitative manner by assaying for β-galactosidase expression by the Ad5lacZ vector. To determine the extent of early gene expression in MDBK, MDCK, HeLa, and L cells, monolayers were infected with Ad5lacZ at an MOI of 5, harvested at various times post-infection and cell extracts were assayed for β-galactosidase (β-gal) activity. Activity was measured by the amount of o-nitrophenol hydrolyzed from o-nitrophenyl β-D-galactopyranoside upon cleavage by β-galactosidase. This reaction produces a colour change which is quantitated by absorption at 420 nm. The results are depicted in Figure 5. β-galactosidase activity was detected at 8 hours post-infection in MDBK, and MDCK cells, and increased linearly to a maximum value at 20 hours. Expression in HeLa cells arose later, between 8 and 12 hours post-infection, and plateaued at approximately 48 hours post-infection while expression in L cells is even further retarded with activity first apparent about 24 hours post-infection and still increasing by 48 hours. As β-gal expression occurred later in HeLa cells, compared to the expression in MDBK and MDCK cells, β-galactosidase expression may also be a late function driven by the major late promoter in HeLa cells.

FIGURE 5: β-GALACTOSIDASE EXPRESSION IN MDBK, MDCK, HELA, & L CELLS

Cells were infected with Ad5lacZ (*E. coli* lacZ gene inserted into the E3 region of hAd5 along with the SV40 promoter, enhancer and polyadenylation sequences) at a MOI of 5, and harvested at 4, 8, 12, 16, 20, 24, 36, and 48 hours post-infection. Cell extracts were assayed for β–galactosidase activity as described in the Materials and Methods.

β-galactosidase Expression in MDBK, MDCK, HeLa and L Cells

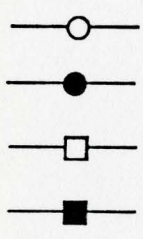


To determine the effects of inhibition of viral DNA synthesis on β-gal expression in MDBK and HeLa cells, monolayers were infected with Ad5lacZ, both in the presence and in the absence of ara-c (inhibitor of DNA synthesis) and cell extracts were assayed for β-gal activity. Activity was first apparent in MDBK cells between 6 and 8 hours post-infection, and increased linearly until about 12 hours post-infection, where it leveled off (Figure 6). There was no discernible difference in β-gal expression in MDBK cells, in the presence or absence of ara-c indicating that expression is solely an early event. β-gal expression was delayed in HeLa cells, compared to MDBK cells, with activity first appearing between 8 and 10 hours post-infection. HeLa cells grown in the absence of ara-c express β-gal to levels significantly higher those expressed by HeLa cells grown in the presence of ara-c. This suggests that by 20 hours post-infection, approximately one half of the β-gal activity in HeLa cells is due to expression from the late promoter.

To confirm that DNA synthesis was sufficiently inhibited by ara-c, viral DNA was prepared from Ad5lacZ infected cultures of MDBK and HeLa cells grown in the presence and absence of ara-c, digested with *HindIII*, separated on a 1% agarose gel by gel electrophoresis, and transferred onto nitrocellulose where radio-labelled portions of the hAd5 genome were used as a probe for viral sequences. Figure 7 shows a 24 hour exposure of the filter. As seen in panel A, viral DNA was detected by 12 hours post-infection in MDBK cells grown in the absence of ara-c, whereas viral DNA in

FIGURE 6: EFFECT OF AraC ON β-GALACTOSIDASE EXPRESSION IN MDBK AND HELA CELLS

Cells were infected with Ad5lacZ at a MOI of 10, and harvested at 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours post-infection A second set of dishes were treated with ara-c to a final concentration of 20 mg/ml and harvested at the same times Cell extracts were assayed for β-galactosidase activity as described in the Materials and Methods This figure is a pooled agerage of results obtained from two experiments



Effect of AraC on β-galactosidase Expression in MDBK & HeLa Cells

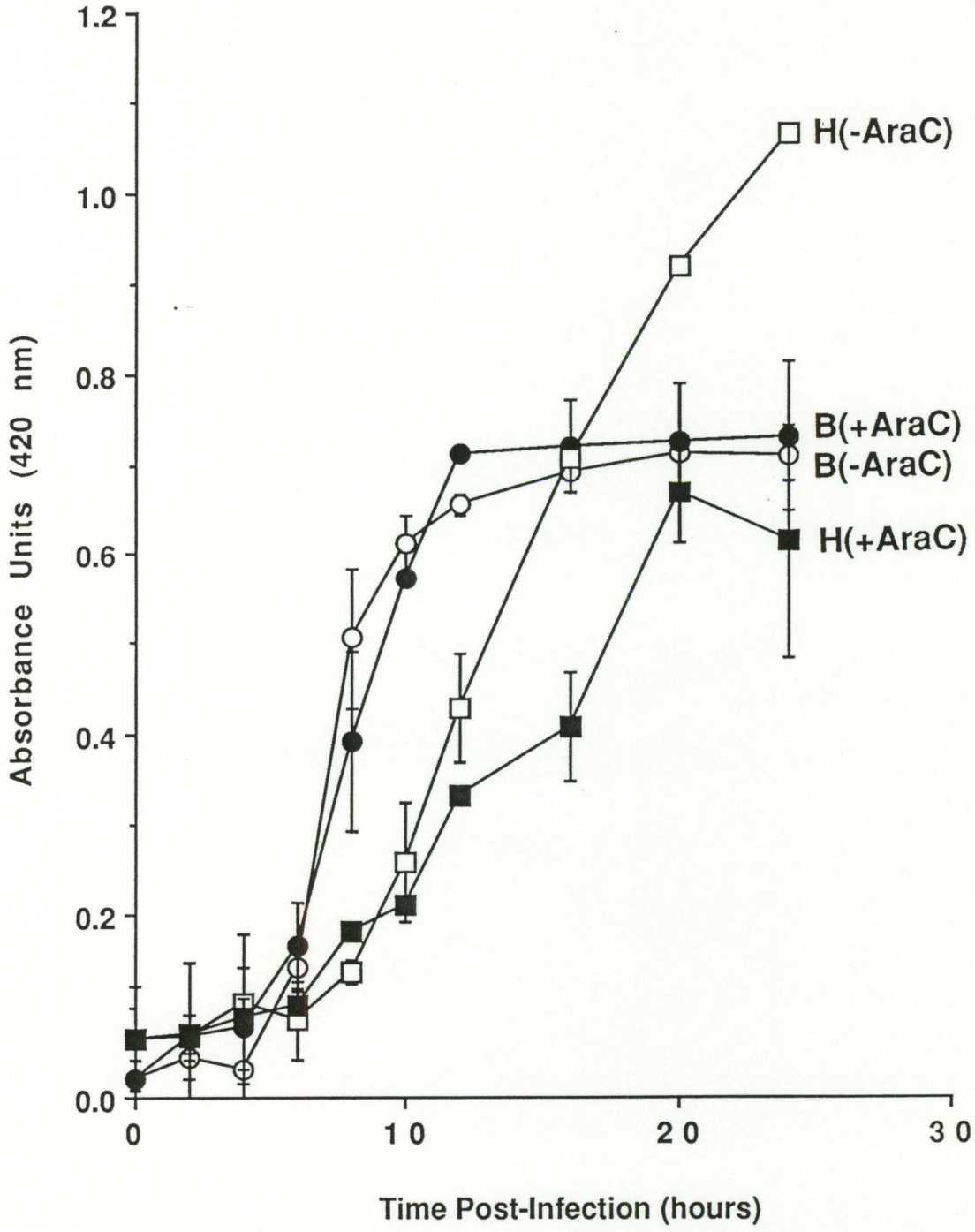


FIGURE 7: EFFECT OF AraC ON Ad5lacZ DNA SYNTHESIS IN MDBK AND HELA CELLS

To confirm that ara-c was sufficiently inhibiting DNA synthesis when looking at the effects of ara-c on β-galactosidase expression in MDBK and HeLa cells (refer to Figure 6), a duplicate set of dishes were infected with Ad5lacZ at a MOI of 10, grown in the presence and absence of ara-c, and harvested at 4, 12, and 24 hours post-infection.

Viral DNA was extracted using a modification of the protocol described by Hirt (1967), digested with *HindIII*, and separated by agarose gel electrophoresis. DNA fragments were transferred onto nitrocellulose using a modification of Southern's protocol (1975), and probed with [^{32}P]-labelled pFGdX1. Panel A shows DNA from hAd5-infected MDBK cells, while panel B shows DNA from hAd5-infected HeLa cells. hAd5wt DNA was digested with *HindIII* and run as a marker.

A
MDBK
Ad5 wt
4
4
12
12
24
24
4
4
12
12
24
24
+Ara C
Ad5 wt
B
HeLa
Ad5 wt
4
4
12
12
24
24
4
4
12
12
24
24
+Ara C
Ad5 wt

infected HeLa cells can only be detected by 24 hours post-infection (panel B). Neither cell type allows for the synthesis of detectable levels of viral DNA in the presence of ara-c. A longer exposure of the same filter confirms this observation (results not shown).

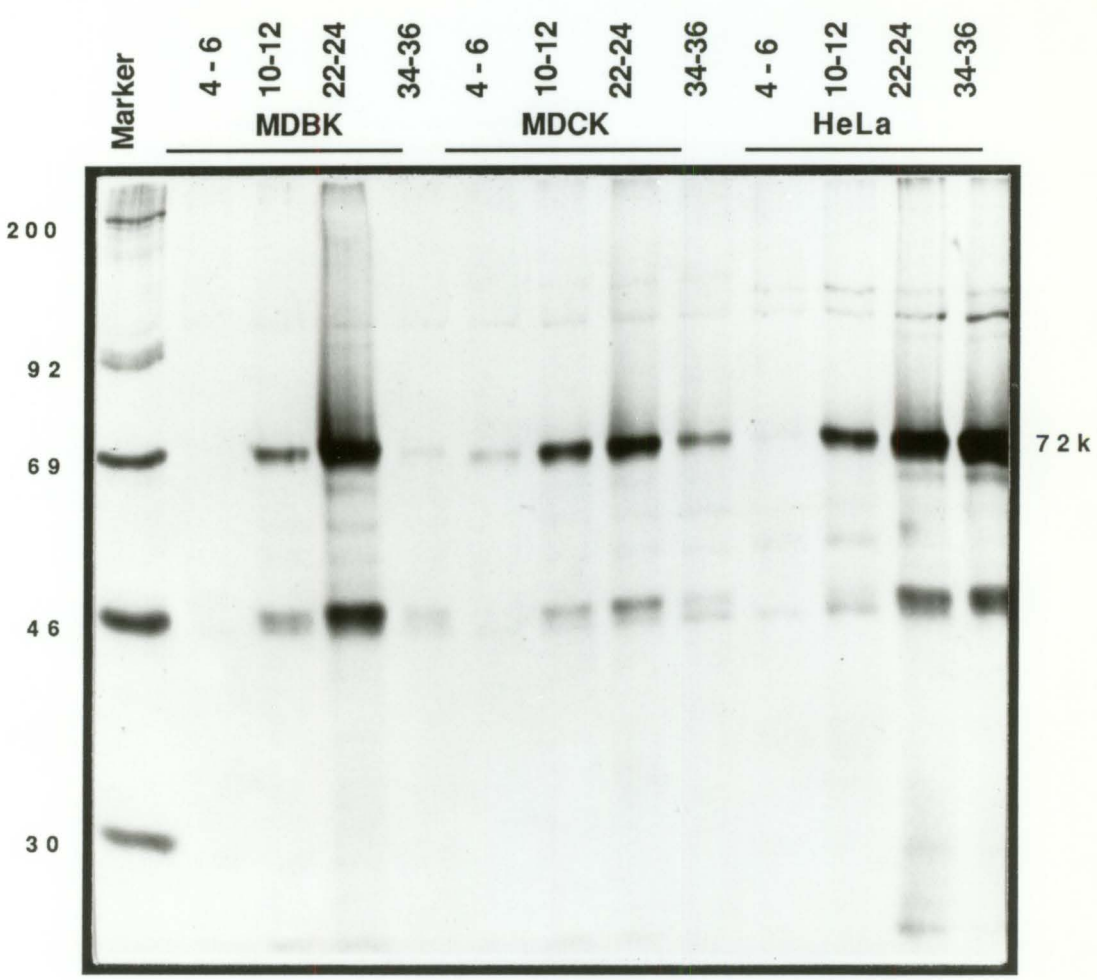
The accumulation of the 72K DNA binding protein, encoded in the E2A transcription block was also examined (Figure 8). [^{35}S] methionine labelled extracts from infected MDBK, MDCK, and HeLa cells were immunoprecipitated with anti-72K immune serum and proteins were resolved on a 9% SDS polyacrylamide gel. A band running slightly behind the 69K protein marker likely represents the 72K DBP, and was present by 12 hours post-infection in all cell types. In MDBK and MDCK cells, this band increased in intensity by 24 hours, before decreasing at 36 hours post-infection. This is consistent with observation that transcription of E2 slowly declines during the late phase of infection (Nevins, 1987). HeLa cells exhibited an increase in intensity up to 36 hours post-infection. In all cases, there is a lower band, migrating approximately at 45K, whose intensity corresponds to the intensity of the upper 72K band, suggesting that the 45K protein is the 72K degradation product. Lower levels of 72K are present in MDBK and MDCK cells, than in HeLa, perhaps indicating a partial block in translation of early proteins.

β-galactosidase is expressed in Ad5lacZ infected MDCK cells, as is the E2 72K protein (in hAd5 infected cells). This indicates that

FIGURE 8: EXPRESSION OF ADENOVIRUS TYPE 5 72K DNA BINDING PROTEIN IN MDBK, MDCK, AND HELA CELLS

Cells were infected with hAd5wt and labelled with [^{35}S] methionine (100 μCi/60 mm dish) for the indicated time intervals. One half of each cell extract was immuno-precipitated with anti-72K serum, and the other half of the same extract was immunoprecipitated with anti-hexon serum (refer to Figure 10); proteins were resolved on a 9% SDS polyacrylamide gel. The 72K DBP is indicated on the right of the gel; the smaller band migrating approximately as a 45K protein probably represents the 44K carboxy-terminal proteolytic breakdown product of the DBP frequently observed in infected cell extracts (Rosenwirth et al., 1975).

Marker proteins are shown in the left lane, with the size of the proteins indicitated in kilodaltons.


Marker
4 - 6
10-12
22-24
34-36
4 - 6
10-12
22-24
34-36
4 - 6
10-12
22-24
34-36
MDBK
MDCK
HeLa
200
92
69
46
30
72k

the block in hAd5 replication in MDCK cells is not solely at the level of early translation.

3.4 EXPRESSION OF ADENOVIRUS TYPE 5 LATE STRUCTURAL PROTEINS IN MDBK, MDCK, AND HELA CELLS

Next, hAd5 structural protein-synthesis in hAd5 infected MDBK, MDCK and HeLa cells was examined. Infected cells were labelled with [^{35}S] methionine for one to two hours before harvesting, and hAd5 proteins were resolved on a 9% SDS polyacrylamide gel following immunoprecipitation with rabbit anti-hAd5 serum (Figure 9). The 72K DNA-binding protein, along with the 44K degradation product, is present in all cell types by 6 hours post-infection, increases up to at least 12 hours, and then appears to decrease. The three bands running at approximately 108K, 85K and 62K correspond to hexon, penton and fiber respectively, and are present in MDBK and HeLa cells 12 hours post-infection, though at lower levels in MDBK cells. No bands other than the 72K DBP and the putative breakdown product were present at any time point in MDCK cells.

The translation of hexon in infected MDBK, MDCK and HeLa cells was examined by immunoprecipitating labelled cell extracts with monoclonal antibody directed against hexon (Figure 10). A distinct 108K band was visualized in MDBK cells 12 hours post-infection, increased by 24 hours and then decreases by 36 hours post-infection.

FIGURE 9: EXPRESSION OF ADENOVIRUS TYPE 5 PROTEINS IN MDBK, MDCK, AND HELA CELLS

Cells were infected with hAd5wt and labelled with [^{35}S] methionine (50 μCi/60 mm dish) for the indicated time intervals. Cell extracts were immuno-precipitated with anti-hAd5 rabbit serum and proteins were resolved on a 9% SDS polyacrylamide gel. Structural proteins are indicated on the right. The band running slightly ahead of what is identified as penton is likely the 72K DNA binding protein of the E2 region. The smaller band migrating approximately as a 45K protein probably represents the 44K carboxy-terminal proteolytic breakdown product of the DBP frequently observed in infected cell extracts (Rosenwirth et al., 1975).

Marker proteins are shown in the left lane, with the size of the proteins indicitated in kilodaltons.

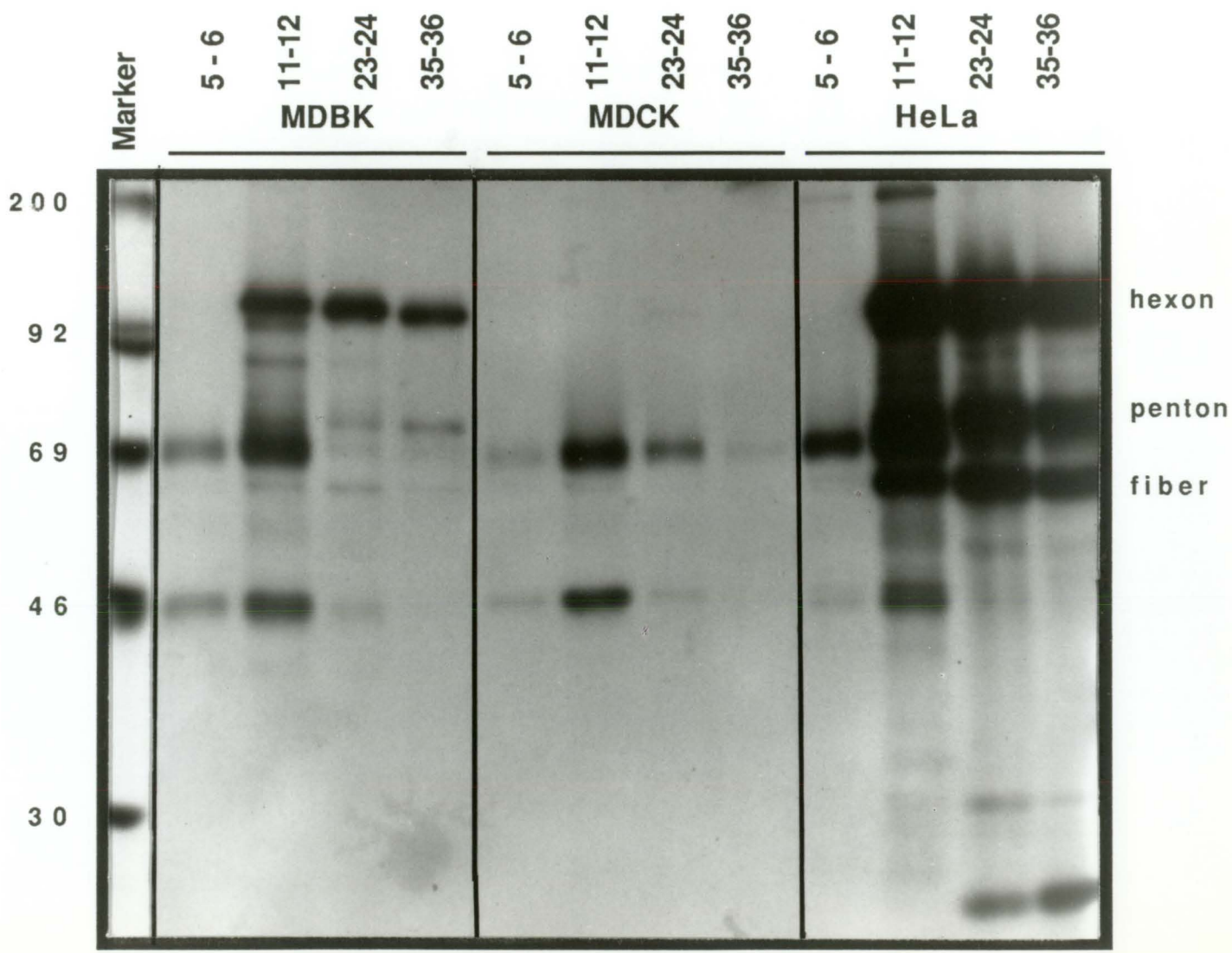
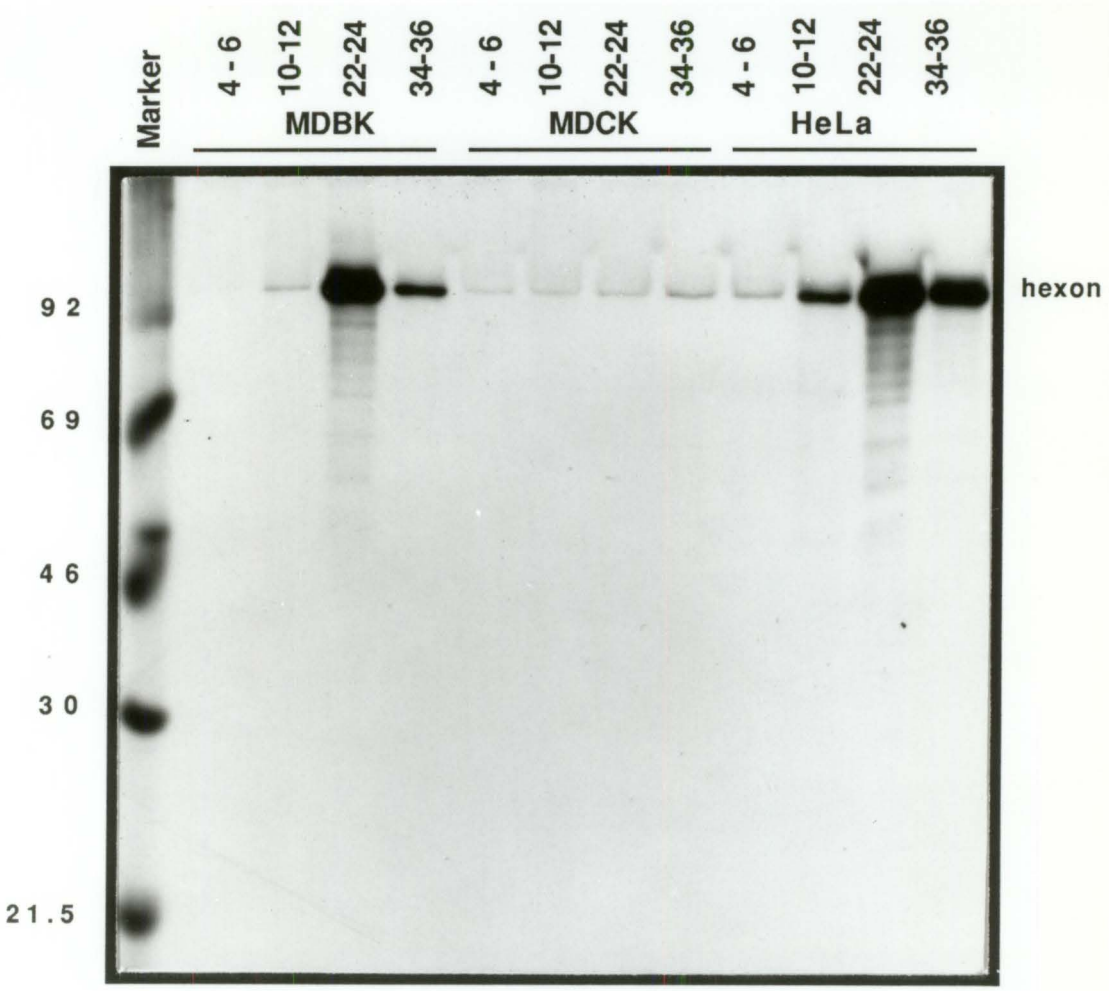

Marker
5 - 6
11-12
23-24
35-36
MDBK
5 - 6
11-12
23-24
35-36
MDCK
5 - 6
11-12
23-24
35-36
HeLa
200
92
69
46
30
hexon
penton
fiber

FIGURE 10: EXPRESSION OF ADENOVIRUS TYPE 5 PROTEIN II (HEXON) IN MDBK, MDCK, AND HELA CELLS

Cells were infected with hAd5wt and labelled with [^{35}S] methionine (100 μCi/60 mm dish) for the indicated time intervals. One half of each cell extract was immunoprecipitated with monoclonal anti-hexon antibodies, and the other half of the same extract was immunoprecipitated with anti-72K serum (refer to Figure 8); proteins were resolved on a 9% SDS polyacrylamide gel. Hexon is indicated on the right of the gel.

Marker proteins are shown in the left lane, with the size of the proteins indicitated in kilodaltons.

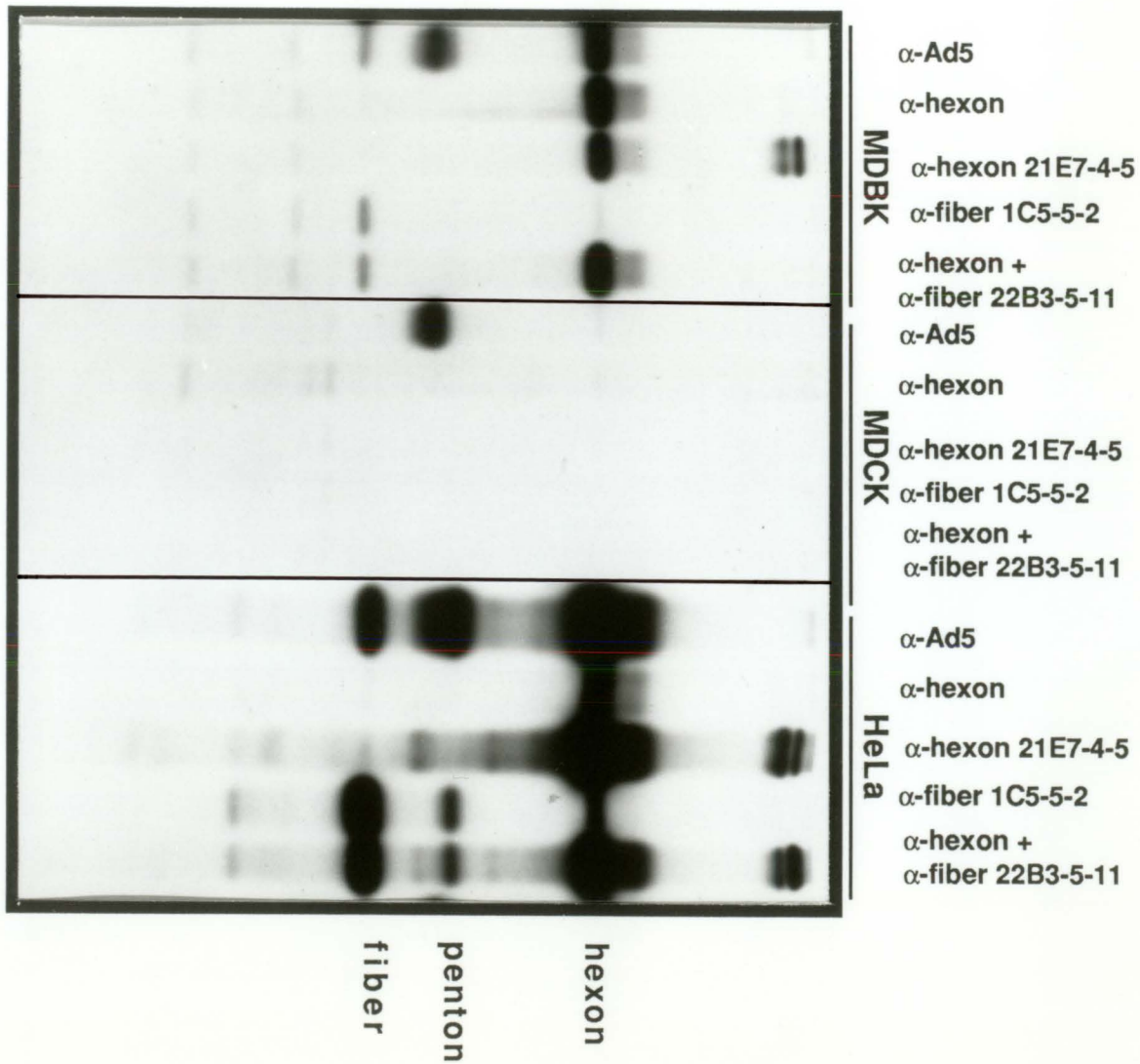

Marker
4 - 6
10-12
22-24
34-36
MDBK
4 - 6
10-12
22-24
34-36
MDCK
4 - 6
10-12
22-24
34-36
HeLa
92
69
46
30
21.5
hexon

The same pattern was observed in infected HeLa cells, but the bands were more intense. There were no detectable levels of hexon in infected MDCK cells. The absence of hexon in MDCK cells was not due to lack of infection, as half of each cell extract was also immunoprecipitated with anti-72K serum which brought down 72K in all three cell types along with the 72K degradation product.

A number of mouse monoclonal anti-adenovirus cell lines were produced by Dr. A. Wandeler at Animal Diseases Research Institute in Ottawa. He provided us with ascites fluids produced by these lines and the monoclones were characterized in our laboratory using immunoprecipitation procedures. The anti-hAd5 lines used in this thesis were classified as follows: 21E7-4-5 (anti-hexon), 1C5-5-2 (anti-fiber), and 22B3-5-11 (anti-hexon and anti-fiber). Figure 11 shows the autoradiogram of proteins immunoprecipitated from hAd5 infected cells, with hAd5 polyclonal rabbit serum, anti-hexon monoclonal antibodies and the monoclonal ascites fluids. The hAd5 polyclonal serum immunoprecipitated the structural proteins, hexon, penton, and fiber, as well as the 72K DNA binding protein in hAd5 infected MDBK and HeLa cells. Hexon was produced in MDBK and HeLa cells as seen in the immunoprecipitation with anti-hexon monoclonal antibody. Ascites fluid from cell line 21E7-4-5 immunoprecipitated hexon in MDBK and HeLa cells. Similarly, ascites fluid from line 1C5-5-2 immunoprecipitated fiber, and line 22B3-5-11 immunoprecipitated hexon and fiber, in both, MDBK and HeLa cells. Neither of these late structural proteins were immuno-

FIGURE 11: EXPRESSION OF ADENOVIRUS TYPE 5 PROTEIN II (HEXON) AND PROTEIN IV (FIBER) IN MDBK, MDCK, AND HELA CELLS

Cells were infected with hAd5wt and labelled with [^{35}S] methionine (500 μCi/100 mm dish) for 4 hours and harvested at 26 hours post-infection. Samples of each cell extract were immunoprecipitated with anti-hAd5 rabbit serum, monoclonal anti-hexon antibodies, and ascites fluids 21E7-4-5, 1C5-5-2, and 22B3-5-11 corresponding to anti-hexon, anti-fiber, and anti-hexon/fiber respectively; proteins were resolved on a 9% SDS polyacrylamide gel. Structural proteins are indicated on the right with the 72K DBP migrating slightly ahead of penton.


α-Ad5
α-hexon
α-hexon 21E7-4-5
α-fiber 1C5-5-2
α-hexon +
α-fiber 22B3-5-11
MDBK
α-Ad5
α-hexon
α-hexon 21E7-4-5
α-fiber 1C5-5-2
α-hexon +
α-fiber 22B3-5-11
MDCK
α-Ad5
α-hexon
α-hexon 21E7-4-5
α-fiber 1C5-5-2
α-hexon +
α-fiber 22B3-5-11
HeLa
fiber
penton
hexon

precipitated from MDCK cells with the monoclonal ascites fluids. Only the E2 72K DBP appeared to be synthesized in MDCK cells.

Thus, the block to hAd5 replication in MDCK cells appears to occur either at or before the level of late translation but after early protein synthesis and DNA replication

3.5 TRANSCRIPTION OF ADENOVIRUS TYPE 5 mRNA IN MDCK AND HELA CELLS

At this point, it was known that hAd5 was not replicated in MDCK cells although viral DNA was synthesized Early proteins were translated in MDCK cells, but no late proteins appeared to be produced In order to ascertain whether the block in viral replication in MDCK cells was at the level of late transcription or RNA transport, total and cytoplasmic RNA was extracted from hAd5 infected MDCK, and HeLa cells, and the presence of late messenger RNA for penton base protein was probed

The construction of plasmids to generate riboprobes for penton base in the sense and anti-sense orientation is shown in Figure 12. To ensure that the riboprobes hybridized to RNA only, the extracted RNA was treated with RNAase-free DNAaseI, and slot blotted onto a nylon membrane. The probe made anti-sense to penton message detected increasing amounts of RNA in hAd5 infected HeLa cells at later times post-infection, indicating that penton mRNA is being synthesized (see panel B of Figure 13, blot 1) The validity of this

result lies in the fact that the sense probe bound with approximately equal intensities to RNAs from HeLa cells at all times post-infection This indicates that the probe may be binding to cellular mRNA in HeLa cells (panel B of Figure 13, blot 2), although the sense probe was constructed using the hAd5 fragment from 37.5 m u to 50 1 m u and contains intron regions of E2B transcripts (refer to Figures 12 and 2) Slot blot analysis of RNA extracted from hAd5 infected MDCK cells indicates that if penton message is indeed transcribed, it is at very low levels (panel A of Figure 13, blot 1) In order to more accurately demonstrate the presence or absence of penton mRNA in hAd5 infected MDCK cells, an RNAase protection analysis was carried out, using a portion of the hAd5 genome from 45.3 m u to 45 9 m u to generate a riboprobe for detection of penton transcripts (for probe construction, refer to Figure 14)

The results of the RNAase protection analysis to detect the transcription of penton base in hAd5 infected MDCK and HeLa cells are shown in Figure 15 Plasmids to generate riboprobes specific for hexon mRNA and fiber mRNA were constructed, but technical difficulty was experienced in carrying out the RNAase protection analysis (refer to Figures 16 and 17)

Fragments of penton message are protected in cytoplasmic as well as nuclear RNA preparations of hAd5 infected MDCK cells 24 hours post-infection Message is present in the nucleus and cytoplasm of infected HeLa cells 12 hours post-infection, and at levels higher than in MDCK cells No message is present in either

mock-infected cell type Therefore, the block to hAd5 replication in MDCK cells is not at the level of penton transcription or transport of penton mRNA into the cytoplasm

FIGURE 12: CONSTRUCTION OF PLASMIDS TO GENERATE RIBOPROBES FOR PENTON BASE mRNA

The hAd5 *HindIII* D fragment (37.5 to 50.1 m.u.) was cut out of the pBR322 derivative and inserted into the *HindIII* site of pTZ18R. Orientation of the insert was determined by digesting with *SmaI*. p18D1 contains the *HindIII* D fragment in the right to left orientation and p18D2 contains the same fragment in the left to right orientation. p18D1 was shortened by digesting with *BamHI* (unique to the multiple cloning region of the parent plasmid), and *BglII* (site at 45.3 m.u. within the hAd5 *HindIII* D fragment), and ligating the identical 5' overhang sequences, thereby destroying the *BamHI* and *BglII* sites. The new plasmid, p18D1dBB, contains the portion of the hAd5 genome from 45.3 m.u. to 37.5 m.u.

The open arrow represents the T7 promoter.

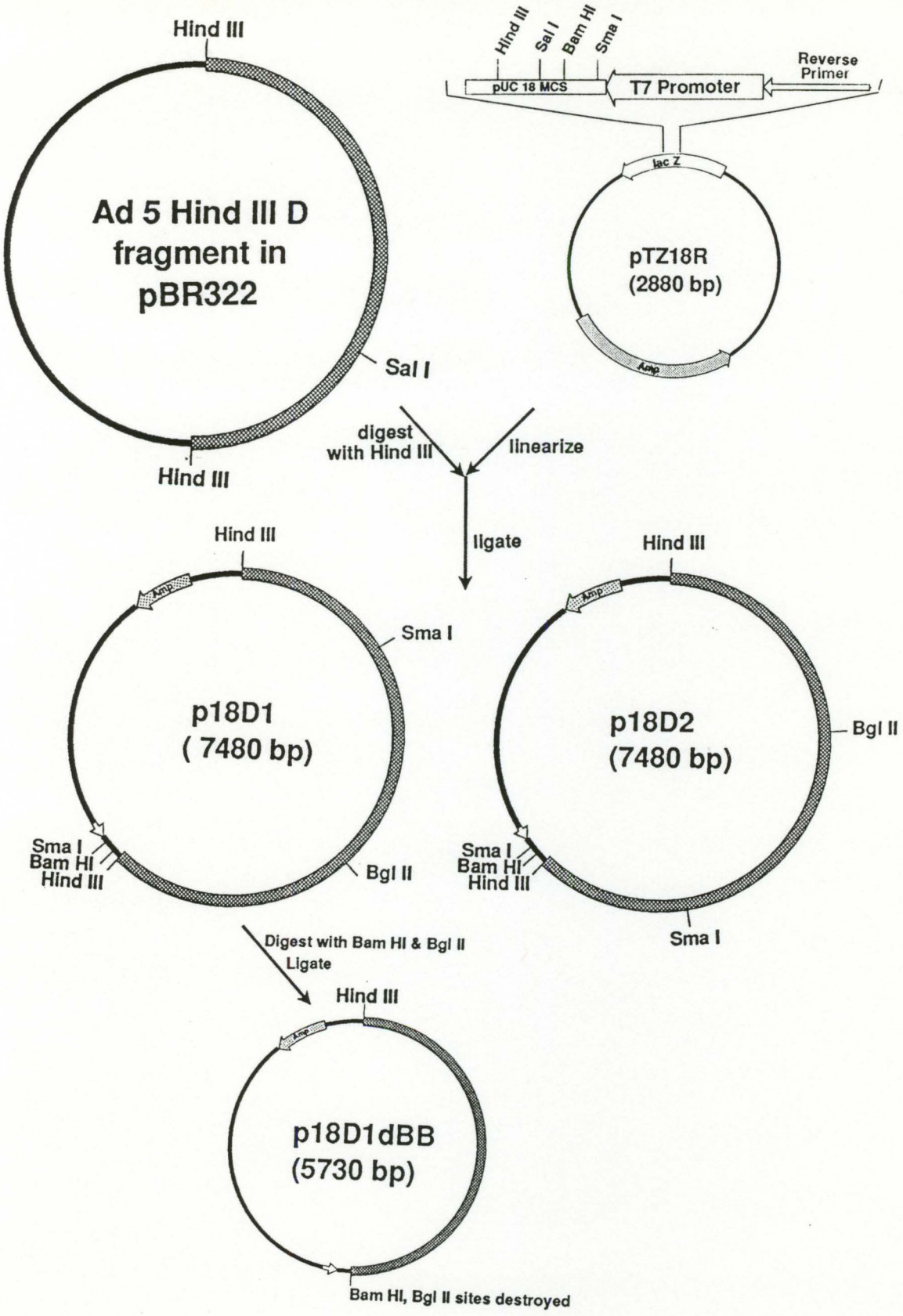
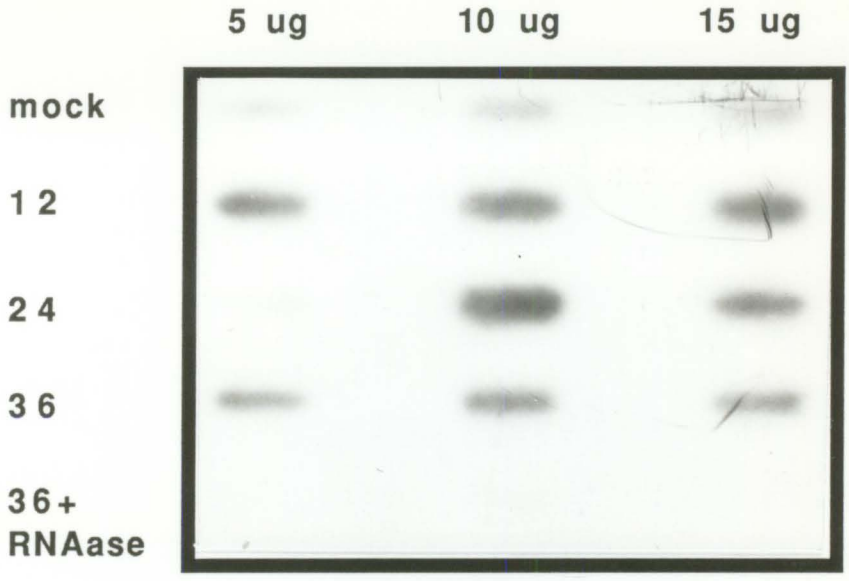

Hind III
Ad 5 Hind III D
fragment in
pBR322
Sal I
Hind III
Hind III
Sal I
Bam HI
Sma I
pUC 18 MCS
T7 Promoter
Reverse
Primer
lac Z
pTZ18R
(2880 bp)
Amp
digest
with Hind III
linearize
ligate
Hind III
Amp
Sma I
p18D1
(7480 bp)
Sma I
Bam HI
Hind III
Bgl II
Hind III
Amp
p18D2
(7480 bp)
Bgl II
Sma I
Bam HI
Hind III
Sma I
Digest with Bam HI & Bgl II
Ligate
Hind III
p18D1dBB
(5730 bp)
Bam HI, Bgl II sites destroyed

FIGURE 13: TRANSCRIPTION OF ADENOVIRUS TYPE 5 PENTON BASE IN MDCK, AND HELA CELLS

Cytoplasmic RNA was extracted from hAd5 infected MDCK, and HeLa cells at 12, 24, and 36 hours post-infection, quantitated and blotted onto a nylon membrane as described in the Materials and Methods. Panel A of Blot 1 contains RNA from mock and hAd5-infected MDCK cells, while panel B shows RNA from mock and hAd5-infected HeLa cells. The slots labelled Mock contain RNA from cells treated with PBS^{++}. As a negative control (and to determine non-specificity of binding), 10 µg of the 36 hour RNA sample was treated with RNAase A and blotted.

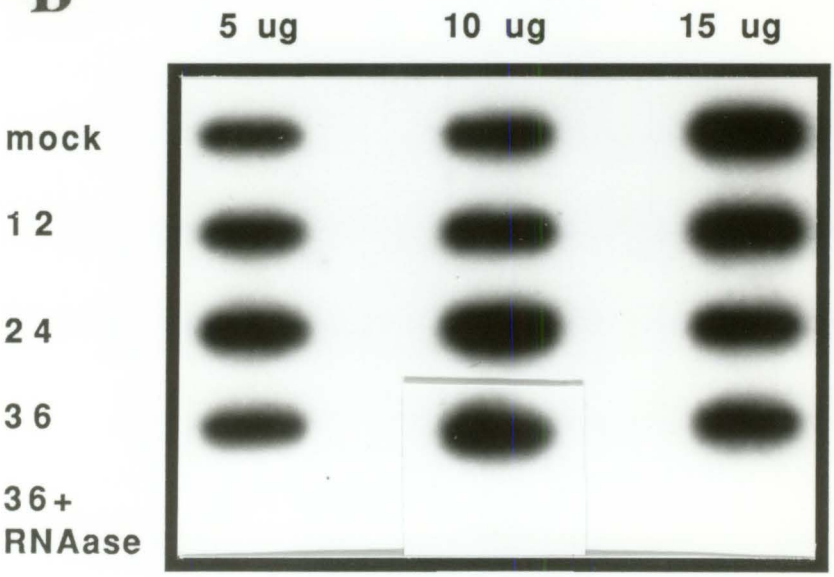
RNA was probed with a [^{32}P]-labelled riboprobe generated from p18D1dBB (construction depicted in Figure 12). This probe was specific for penton base mRNA. Blot 2 is an autoradiogram of an RNA blot probed with a [^{32}P]-labelled penton sense riboprobe, and is used to assess the amount of non-specific hybridization. Panels A and B are as described above.

2 A



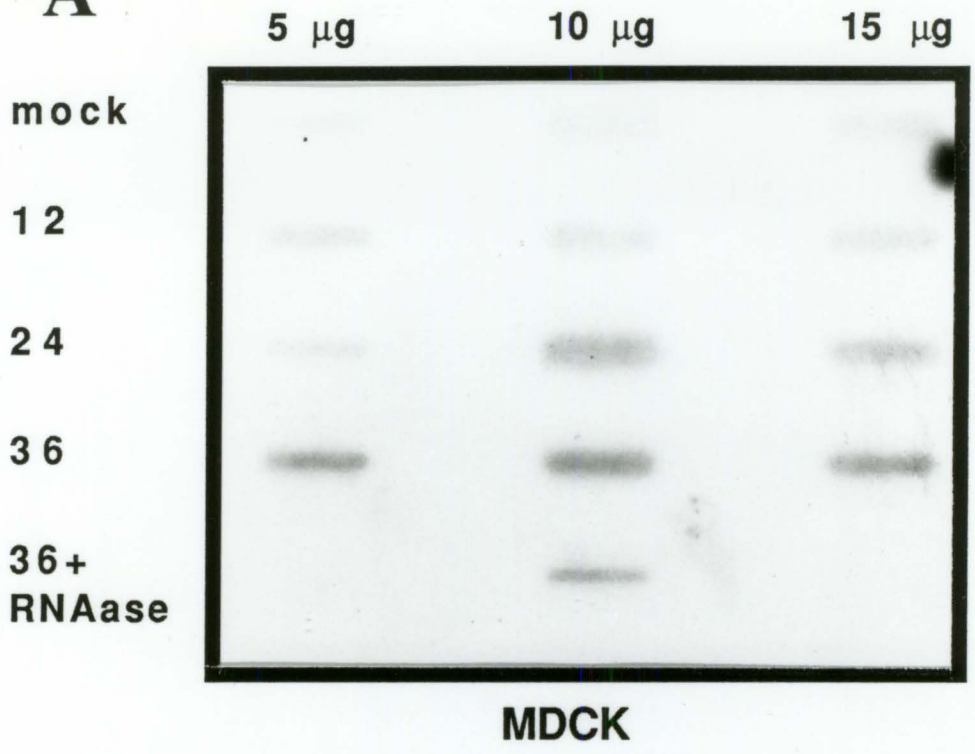
MDCK

B



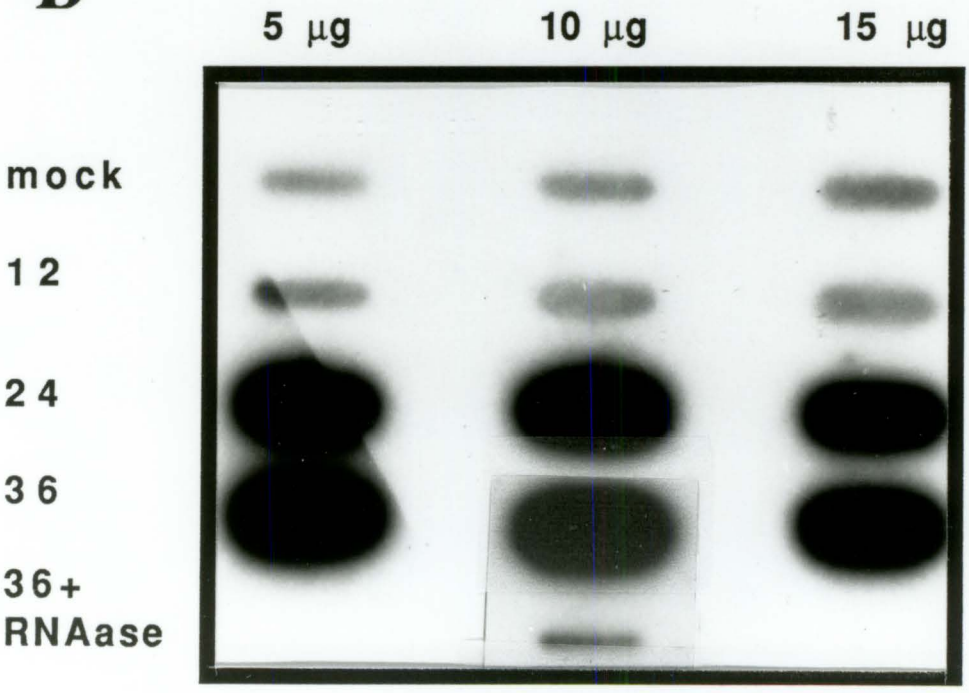
HeLa

1 A



MDCK

B



HeLa

FIGURE 14: PLASMID CONSTRUCTION TO GENERATE A RIBOPROBE TO DETECT PENTON BASE mRNA BY RNAase PROTECTION ANALYSIS

The hAd5 fragment from 45.3 m u to 45.9 m.u was excised from p18D2 (for construction of this plasmid, refer to Figure 12), by digesting with *BglII* and *SalI* pBluescript SK (+/-) Phagemid contains the T3 and T7 promoters, and was cut with *BamHI* and *SalI* to allow the insertion of the hAd5 fragment. The T3 promoter of the resulting plasmid, pBlueD2SB, allowed for the synthesis of a probe to detect penton base Transcripts were terminated by linearizing pBlueD2SB with *XbaI*

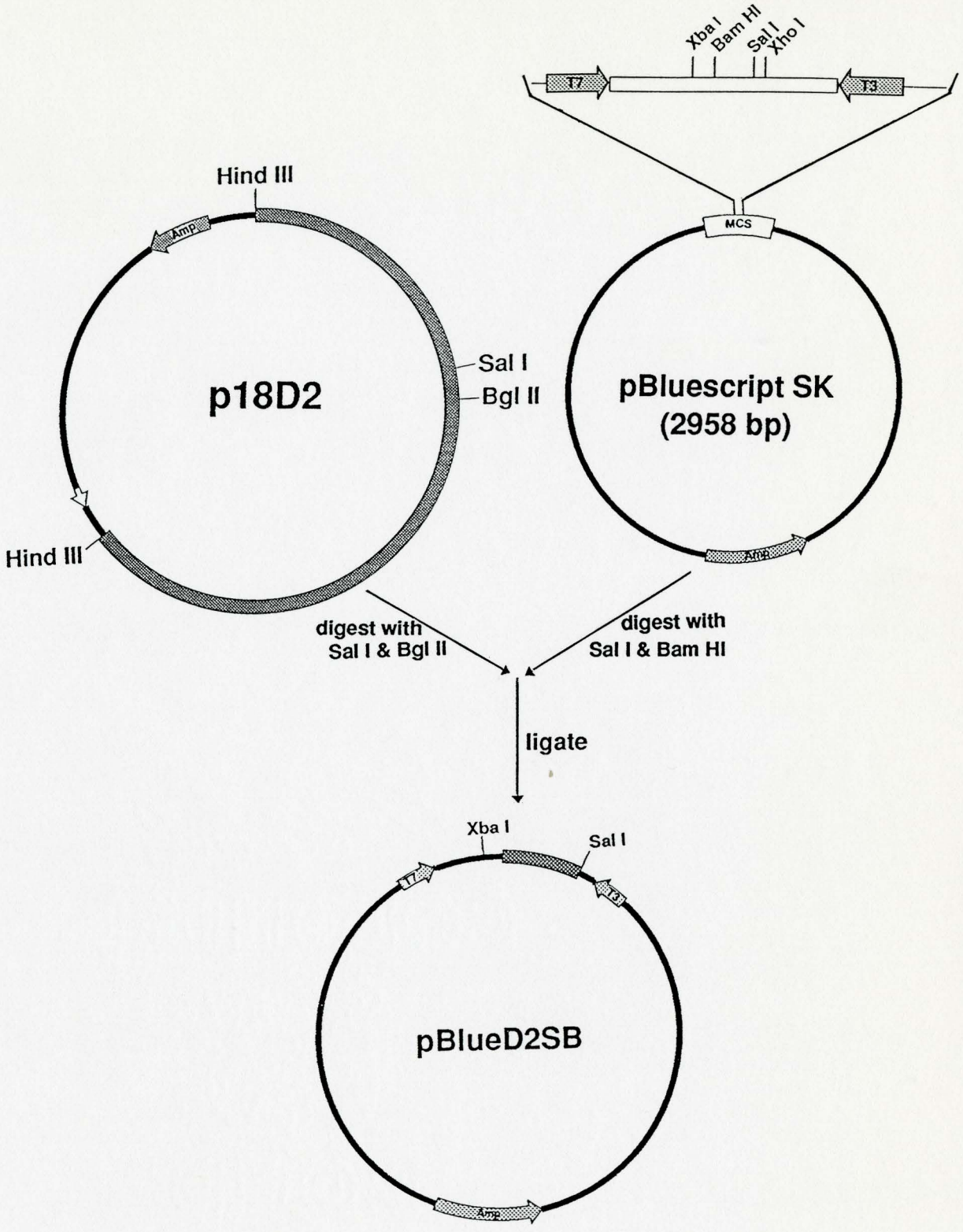

Xba I
Bam HI
Sal I
Xho I
T7
T3
MCS
Hind III
Amp
p18D2
Sal I
Bgl II
Hind III
pBluescript SK
(2958 bp)
Amp
digest with
Sal I & Bgl II
digest with
Sal I & Bam HI
ligate
Xba I
Sal I
T7
T3
pBlueD2SB
Amp

FIGURE 15: RNAase PROTECTION ANALYSIS OF PENTON BASE mRNA

Cytoplasmic as well as total RNA was extracted from mock infected and hAd5 infected MDCK and HeLa cells, 12, 24, and 36 hours post-infection. 10 μg of each RNA sample was hybridized to a riboprobe for penton base Lane 1 containing undigested probe alone, represents 1/53 the amout of probe used in the remaining lanes Lane 2 contains unprotected probe digested with RNAase A and RNAase T_1 In MDCK cells, penton is first detected in the cytoplasm by 24 hours post-infection. Detection is earlier in HeLa cells, by 12 hours at least, and to higher levels

FIGURE 15: RNAase PROTECTION ANALYSIS OF PENTON BASE mRNA

Cytoplasmic as well as total RNA was extracted from mock infected and hAd5 infected MDCK and HeLa cells, 12, 24, and 36 hours post-infection. 10 μg of each RNA sample was hybridized to a riboprobe for penton base Lane 1 containing undigested probe alone, represents 1/53 the amout of probe used in the remaining lanes Lane 2 contains unprotected probe digested with RNAase A and RNAase T_1 In MDCK cells, penton is first detected in the cytoplasm by 24 hours post-infection. Detection is earlier in HeLa cells, by 12 hours at least, and to higher levels

FIGURE 16: PLASMID CONSTRUCTION TO GENERATE A RIBOPROBE TO DETECT HEXON mRNA BY RNAase PROTECTION ANALYSIS

The hAd5 fragment from 59.5 to 61.3 m.u. was excised from the pBR322 derivative containing the hAd5 *HindIII* A fragment, by *BamHI* and *KpnI* digestion. pBluescript SK (+/-) Phagemid contains the T3 and T7 promoters, and was cut with *BamHI* and *KpnI* to allow the insertion of the hAd5 fragment. The T3 promoter of the resulting plasmid, pBlueABK, allowed for the synthesis of a probe to detect hexon. Transcripts were terminated by linearizing pBlueABK with *XbaI*.

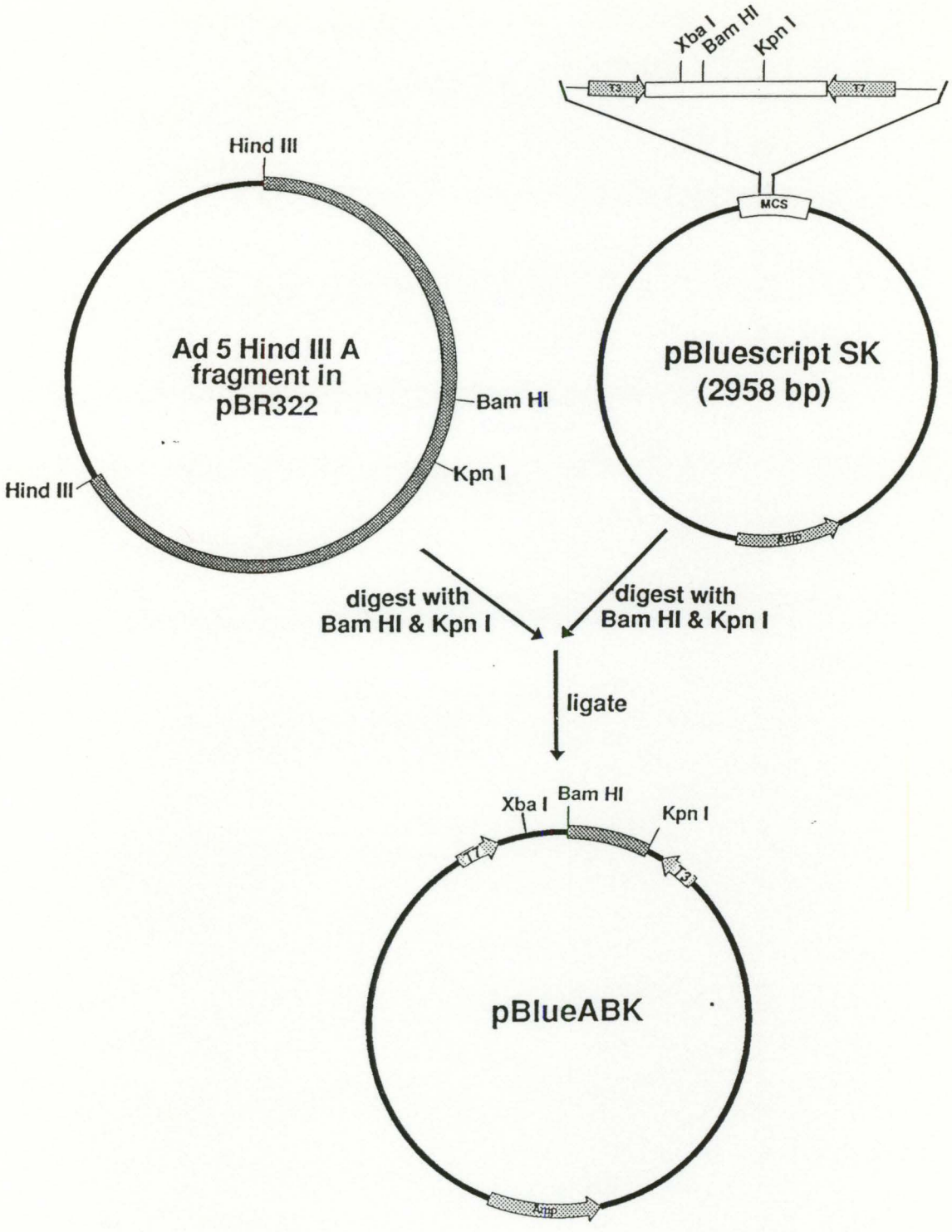
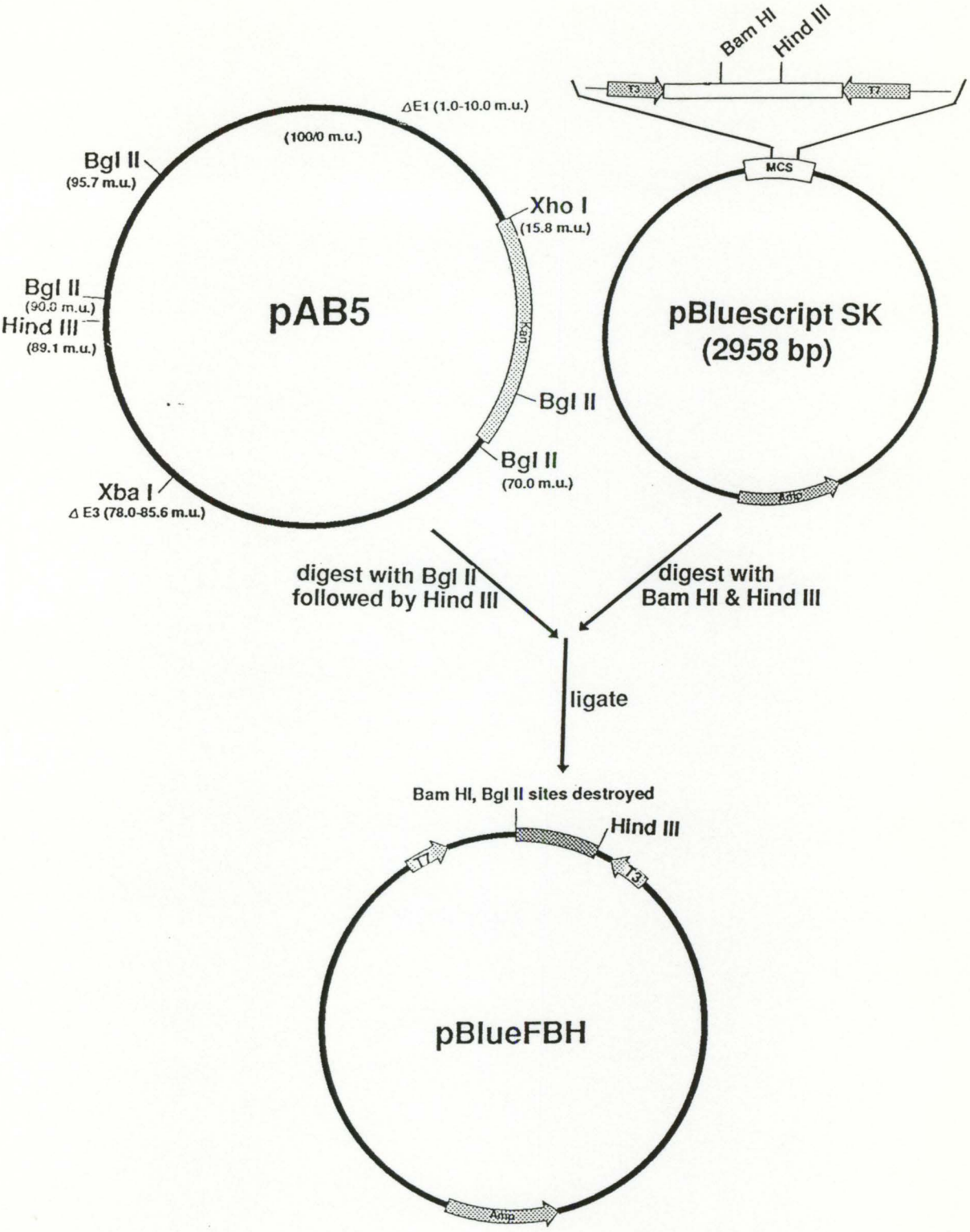

Xba I
Bam HI
Kpn I
T3
T7
MCS
Hind III
Ad 5 Hind III A
fragment in
pBR322
Bam HI
Kpn I
Hind III
pBluescript SK
(2958 bp)
digest with
Bam HI & Kpn I
digest with
Bam HI & Kpn I
ligate
Xba I
Bam HI
Kpn I
T7
T3
pBlueABK

FIGURE 17: PLASMID CONSTRUCTION TO GENERATE A RIBOPROBE TO DETECT FIBER mRNA BY RNAase PROTECTION ANALYSIS

The *BglII* fragment from 70.0 to 90.0 m.u. excluding a deletion in the E3 region (78.0 to 85.6 m.u.) was excised from plasmid pAB5, and gel purified. The gel purified fragment was further digested with *Hind III* and the portion of the hAd5 genome from 89.1 to 90.0 m.u. was inserted into pBluescript SK (+/-) Phagemid. The T7 promoter of the resulting plasmid, pBlueFBH, allowed for the synthesis of a probe to detect fiber. Transcripts were terminated by linearizing pBlueFBH with *Hind III*.


Bam HI
Hind III
T3
T7
MCS
ΔE1 (1.0-10.0 m.u.)
(100/0 m.u.)
Bgl II
(95.7 m.u.)
Xho I
(15.8 m.u.)
Bgl II
(90.0 m.u.)
Hind III
(89.1 m.u.)
pAB5
Kan
Bgl II
Bgl II
(70.0 m.u.)
Xba I
Δ E3 (78.0-85.6 m.u.)
pBluescript SK
(2958 bp)
Amp
digest with Bgl II
followed by Hind III
digest with
Bam HI & Hind III
ligate
Bam HI, Bgl II sites destroyed
Hind III
T7
T3
pBlueFBH
Amp

4. DISCUSSION

To date, many foreign genes have been inserted into human adenovirus type 5, including the β-galactosidase gene of *E. coli*, the rabies glycoprotein gene (Prevec et al , 1990), and the vesicular stomatitis virus glycoprotein gene (Schneider et al , 1989) Incorporating the major late promoter and leader sequences into vector design has resulted in vectors that are able to express high levels of foreign proteins It is of considerable benefit to consider adenoviruses for their possible role as vectors in vaccine development. Although genetic material may be engineered into almost any virus, many considerations must be made in determining the vector of choice Factors to be considered include host range, stability of the vector, site of replication, and mode of replication (Graham et al , 1988) In order to use recombinants as effective vaccines, it is important to characterize the ability of the vector to replicate in various hosts In the course of determining the usefulness of human adenovirus vector in different species, it was observed that Ad5 does not multiply in canine cells although it acts as an effective vaccine vector in dogs (Prevec et al , 1989, Prevec et al , 1990) The purpose of these experiments was to look at the

macromolecular events in alternate hosts following hAd5 infection, using murine fibroblasts (L cells), bovine kidney cells (MDBK), and canine kidney cells (MDCK)

According to the growth curves in Figure 3, it appears that L cells are not permissive for hAd5 replication However, viral DNA is synthesized in L cells though at very low levels compared to HeLa cells (Figure 4.3) As β-galactosidase activity was also observed in L cells (Figure 5), it seems likely that early proteins are expressed, though there is a long delay in time of appearance when compared to HeLa cells.

Although further experiments need to be done to determine the permissivity of L cells for hAd5 replication, the delayed kinetics of β-gal activity (Figure 5), and the low DNA accumulation (Figure 4.3) lend support to the idea that L cells are semi-permissive for hAd5 infection Studies have shown mouse (3T3-Swiss) cells to be semi-permissive for hAd2 infection, with very low levels of infectious virus released The E1A 45-54K proteins, the E1B 58K protein, fiber, and hexon are not detected at all in hAd2 infected cells and the E1B 21K protein is made in low amounts, late in infection, however, the rate of viral DNA synthesis appears to be normal (Eggerding & Pierce, 1986) Similarly, replication of hAd5 in mouse cells is reported to be poor (Tremblay et al , 1985)

It is known that human adenovirus type 12 (hAd12) fails to replicate in Syrian hamster cells (BHK21) (Doerfler, 1969, Juttermann et al , 1989) However, these cells are permissive for

hAd2 growth (Gallimore, 1974; Tremblay et al., 1985). Most early functions are present in hAd12, and it was observed that the replication block was due, at least in part, to the absence of transcription of VA RNAs as well as the L1 RNAs (Juttermann et al., 1989). Host cell functions are required in addition to the E1A products, to activate transcription of these hAd12-specific RNAs. It would appear that BHK21 cells do not provide these functions necessary for hAd12 transcription, but do for hAd2 replication. It is possible that the replication of hAd5 in L cells is similarly affected by limiting quantities of particular host cell factors required for virus replication.

In contrast to the murine system, replication of hAd5 in MDBK cells follows a time course similar to that in HeLa cells (Figure 3). The lower levels of late structural proteins (especially penton base and fiber) in MDBK cells (Figures 9, 10, and 11) correspond to a reduced yield of infectious virus (Figure 3), although levels of DNA accumulation are similar in both cell types (Figure 4.1). The decreased amounts of late proteins could result from a decrease in transcription levels or late protein translation. Analysis of viral late transcripts would elucidate whether or not the decreased levels of late proteins were a result of limiting amounts of required specific transcription factors. Alternatively, it is also possible that transcription factors in MDBK cells bind less efficiently to the hAd5 promoter sequences than do human transcription factors.

In contrast to the permissive MDBK system, MDCK cells appear to be completely non-permissive for hAd5 virus production (Figure 3) Viral DNA accumulates in infected MDCK cells although there is an approximate 5 fold reduction, compared to total viral DNA in HeLa cells (Figure 4.2) The fact that the synthesis of β-galactosidase, driven presumably from the E3 promoter in the AdlacZ vector, is comparable in MDCK cells and the permissive lines would suggest that the block to replication is not at adsorption or penetration Although β-gal expression in MDCK cells is similar to that in HeLa cells (Figure 5), it appears that the E2-72K DBP is synthesized at levels somewhat lower than that in HeLa cells (Figure 8) This might suggest that there is some differential effect on the level of early gene transcription or protein synthesis in MDCK cells

None of the major late structural proteins are synthesized in hAd5-infected MDCK cells (Figures 9, 10, and 11) The major block to hAd5 replication in MDCK cells would therefore seem to be at or before the translation of late proteins though clearly earlier functions, such as DNA replication, are also affected to some extent.

There are many potential barriers to hAd5 replication in MDCK cells. The 58K protein of the E1B region is required in conjunction with the E4 34K protein to facilitate late cytoplasmic mRNA accumulation (Ornelles & Shenk, 1991) Ad40, an enteric adenovirus of subgroup F, is non-permissive in HeLa cells where only traces of E1B- and pplX-specific mRNAs are detected (Mautner et al , 1990) It appears that expression of the adenovirus E1B genes is important

in defining permissivity MDCK cells show no significant adenovirus-induced inhibition of host cell protein synthesis during the late phase of infection (Prevec et al , 1989) This could be explained by the absence of either the E1B 55K and/or the E4 34K polypeptides as these proteins do exist in a complex (Sarnow et al , 1984) that functions to shut off host cell metabolism (Halbert et al , 1985) Since only 72K DBP and β-gal were examined, it is not possible to draw conclusions regarding the E1B or E4 gene products

The fact that DNA synthesis in MDCK cells begins at the same time as in HeLa and MDBK cells and with comparable initial rates (Figure 4.3, R Spessot, unpublished observations) suggests that most of the initial requirements for DNA replication are met in the MDCK cell line The failure of MDCK cells to attain final DNA synthesis levels seen in the permissive lines could be due to increased DNA degradation or insufficient amounts of proteins needed to sustain maximal rates of DNA synthesis The decreased amount in MDCK cells of E2A 72K DBP, a protein required in the elongation reaction of DNA synthesis (Horwitz, 1978), could help explain this result. In any case, it is clear that some hAd5 DNA copies are available for late transcription in MDCK cells Penton base mRNA is transcribed in MDCK cells and was detected in the cytoplasm though at reduced levels compared to HeLa cells (Figure 15) As the same level of mRNA was present in total as well as cytoplasmic RNA, it is unlikely that the replication block is at the level of RNA transport. Plasmids to generate riboprobes specific for

hexon mRNA and fiber mRNA were constructed, but technical difficulty was experienced in carrying out the RNAase protection analysis.

The fact that penton mRNA was detected in the cytoplasm of infected MDCK cells, suggests that the block to penton production lies either at the level of translation initiation or elongation. A number of possible sites and mechanisms by which translation may be affected are known. Some of these are briefly considered below.

Adenoviruses are known to functionally inactivate the cellular cap-binding protein (CBP) complex necessary for cap-dependant mRNA translation (Huang & Schneider, 1991). The tripartite leader sequence, present on all late viral transcripts, is thought to have very little secondary structure and allows translation of non-capped mRNAs free of CBP complex (eIF-4F) activity (Dolph et al., 1990). As previously mentioned, MDCK cells show no inhibition of host cell protein synthesis during the late phase of infection (Prevec et al., 1989), possibly because of an inability to inactivate the CBP complex. Capped mRNAs may be translated much more efficiently than viral transcripts with the tripartite leader, resulting in unsuccessful competition between the virus and the host cell for translation factors.

The hAd5 L4 100K non-structural protein is required for efficient translation of late viral mRNAs. In addition, this protein plays an essential role in hexon maturation. The L4 100K protein seems to preferentially bind to cytoplasmic viral mRNA to somehow

increases the efficiency of translation initiation (Hayes et al., 1990). Therefore, the synthesis of this particular late protein is also critical to viral replication in adenovirus-infected MDCK cells.

VA RNAs are transcribed by RNA polymerase III and accumulate to very high levels in the later phases of adenovirus infection. These small, highly folded RNAs function to allow translation of late viral mRNAs (Thimmappaya et al., 1982) by sequestering the cellular DAI, and thereby preventing dsRNA-activated inhibition of translation. It is possible that VA RNAs are either not synthesized, or are non-functional in MDCK cells. Perhaps other host cell functions are also necessary for efficient transcription of these RNAs. In either case, a block in VA RNA synthesis would result in the inhibition of the translation of late viral mRNAs.

It was also observed that neither hexon nor fiber proteins were synthesized in MDCK cells. It would be interesting to determine whether or not these transcripts are present in the nucleus and the cytoplasm of infected cells. Once again, if these transcripts are present in the cytoplasm, the inhibition of protein synthesis would likely be at the level of translation initiation or translation elongation.

Perhaps the best studied model of non-permissive infection comparable to that seen in MDCK cells in hAd2 infection of CV-1 cells (African green monkey kidney cells). These cells undergo abortive hAd2 infections (Silverman et al., 1989) in which early viral functions and DNA synthesis appear normal, but the yield of

infectious virus is reduced at least 500 fold as compared to productive infections (Anderson & Klessig, 1984). Late transcription is reduced and in most cases, corresponds to an equivalent reduction in translation of late proteins. However, protein IV (fiber) is reduced more than 100 fold although its steady-state mRNA level is reduced by only a factor of 5 to 10.

Several pieces of evidence exist to show that this reduced level of fiber is not a property of the translation machinery of the cell (Silverman et al., 1989). When cell-free translation lysates prepared from CV-1 cells were compared to those prepared from HeLa cells, using translatable hAd2 mRNA prepared from a productive infection of human cells by hAd2, both lysates synthesized the same set and amounts of viral proteins, including fiber (Quinlan & Klessig, 1982). Monkey-human hybrid clones reconstructed from infected human karyoplasts and monkey cytoplasts express fiber whereas cells reconstructed from infected monkey karyoplasts and human cytoplasts did not. This indicated that the block to human adenovirus replication in monkey cells involves a nuclear event and that the translational apparatus of monkey cells is competent to translate functional fiber mRNA (Zorn & Anderson, 1981). Rather, in abortive infections of monkey cells, there is a marked deficiency of fiber messages containing the x or y ancillary leader sequences as opposed to fiber messages in productive infections (Anderson & Klessig, 1984). Work by Silverman and co-workers (1989) suggests that components other

than the adenovirus tripartite leader, namely the x or y ancillary leader sequences, may be required for efficient translation of fiber mRNA.

To determine whether a similar process may be occuring in hAd5-infected MDCK cells, the 5' end of fiber messages from infected cells could be analyzed by primer extension As the sizes of the leader sequences are known (Anderson & Klessig, 1984), it would be possible to determine whether altered splicing of fiber message correlates with decreased synthesis of fiber in MDCK cells.

While the specific block to hAd5 replication in MDCK cells has not been defined, it is clear that genes inserted into hAd5 vectors under the control of the E3 promoter can be expressed to high levels in these cells Whether this level of early expression, without virus replication, is sufficient to achieve the levels of immunity developed in dogs using the hAd5-rabies vector (Prevec et al , 1990), or whether replication of hAd5 does occur n some cells in the animal, can only be answered by *in vivo* studies

REFERENCES

Anderson, K P , and D F Klessig 1984 Altered mRNA splicing in monkey cells abortively infected with human adenovirus may be responsible for inefficient synthesis of the virion fiber polypeptide Proc. Nat. Acad Sci USA 81.4023-4027

Aneskievich, B.J , and L.B Taichman 1988 Evidence for two points of restriction in the expression of adenovirus type 2 in cultured epidermal keratinocytes J Virol 62.4365-4368

Arrand, J R., and R.J Roberts 1979 The nucleotide sequence at the termini of adenovirus 2 DNA J Mol Biol 128.577-594

Babich, A., and J R Nevins 1981 The stability of early mRNA is controlled by the viral 72kD DNA binding protein Cell 26:371-379

Babbis, L.E., and H S Ginsberg 1984. Adenovirus type 5 early region 1B gene product is required for efficient shutoff of host protein synthesis J Virol 50.202-212

Bagchi, S., R Weinmann, and P Raychaudhuri 1991 The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F Cell 65 1063-1072

Bandara, L.R , and N B. La Thangue 1991 Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor Nature 351.494-497

Beltz, G.A., and S.J Flint. 1979 Inhibition of HeLa cell protein synthesis during adenovirus infection restriction of cellular messenger RNA sequences to the nucleus J Mol Biol 131:353-373

Berk, A.J., F. Lee, T. Harrison, J. Williams, and P.A. Sharp. 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell 17:935-944.

Berkner, K.L. 1988. Development of adenovirus vectors for the expression of heterologous genes. BioTechniques 6:616-629.

Birnboim, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nuc. Acid Res. 7:1513-1523.

Bhatti, A.R., and J. Weber. 1979. Protease of adenovirus type 2: Partial characterization. Virology 96:478-485.

Borrelli, E., R. Hen, and P. Chambon. 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. Nature 312:608-612.

Burgert, H.G., J.L. Maryanski, and S. Kvist. 1987. "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. Proc. Nat. Acad. Sci. USA 84:1356-1360.

Byrd, P.J., R.J.A. Grand, D. Breiding, J.F. Williams, and P.H. Gallimore. 1988. Host range mutants of adenovirus type 12 E1 defective for lytic infection, transformation, and oncogenicity. Virology 163:155-165.

Cepko, C.L., and P.A. Sharp. 1983. Analysis of Ad 5 hexon and 100k ts mutants using conformation-specific monoclonal antibodies. Virology 129:137-154.

Challberg, M.D., S.V. Desiderio, and T.J. Kelly, Jr. 1980. Adenovirus DNA replication *in vitro*: Characterization of a protein covalently linked to nacent DNA strands. Proc. Nat. Acad. Sci. USA 77:5105-5109.

Challberg, M.D., and T.J. Kelly. 1989. Animal virus DNA replication. Annu. Rev. Biochem. 58:671-717.

Chow, L.T., R.E. Gelinas, T.R. Broker, and R.J. Roberts. 1977. An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. Cell 12:1-8.

Colby, W.W., and T. Shenk. 1981. Adenovirus type 5 virions can be assembled *in vivo* in the absence of detectable polypeptide IX. J. Virol. 39:977-980.

Cole, C.N., L.V. Crawford, and P. Berg. 1979. Simian virus 40 mutants with deletions at the 3' end of the early region are defective in adenovirus helper function. J. Virol. 30:683-391.

Crossland, L.D., and H.J. Raskas. 1983. Identification of adenovirus genes that require template replication for expression. J. Virol. 46:737-748.

Cutt, J.R., T. Shenk, and P. Hearing. 1987. Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. J. Virol. 61:543-552.

Dennis, D., and J.R. Smiley. 1984. Transactivation of a late herpes simplex virus promoter. Mol. Cell. Biol. 4:544-551.

Doerfler, W. 1969. Nonproductive infection of baby hamster kidney cells (BHK21) with adenovirus type 12. Virology 38:587-606.

Dolph, P.J., V. Racaniello, A. Villamarin, F. Palladino, and R.J. Schneider. 1988. The adenovirus tripartite leader may eliminate the requirement for cap-binding protein complex during translation initiation. J. Virol. 62:2059-2066.

Dolph, P.J., J. Huang, and R.J. Schneider. 1990. Translation by the adenovirus tripartite leader: Elements which determine independence from cap-binding protein complex. J. Virol. 64:2669-2667.

Dulbecco, R., and H. Ginsberg. 1980. *Virology*. Harper & Row, Publishers, Inc., Philadelphia. p. 1048-1059.

Dunn, A.R., M.B. Mathews, L.T. Chow, and J. Sambrook. 1978. A supplementary adenoviral leader sequence and its role in messenger translation. Cell 15:511-526.

Eggerding, F.A., and W.C. Pierce. 1986. Molecular biology of adenovirus type 2 semipermissive infections. Virology 148:97-113.

Eron, L., H. Westphal, and G. Khoury. 1975. Post-transcriptional restriction of human adenovirus expression in monkey cells. J. Virol. 15:1256-1261.

Falgout, B., and G. Ketner. 1987. Adenovirus early region 4 is required for efficient virus particle assembly. J. Virol. 61:3759-3768.

Falgout, B., and G. Ketner. 1988. Characterization of adenovirus particles made by deletion mutants lacking the fiber gene. J. Virol. 62:622-625.

Feduchi, E., M.A. Alonso, and L. Carrasco. 1989. Human gamma interferon and tumor necrosis factor exert a synergistic blockade on the replication of herpes simplex virus. J. Virol. 63:1354-1359.

Fishaut, M., D. Tubergen, and K. McIntosh. 1980. Medical progress: cellular responses to respiratory viruses with particular reference to children with disorders of cell mediated immunity. J. Pediatr. 96:179.

Flint, J. 1977. The topography and transcription of the adenovirus genome. Cell 10:153-166.

Freeman, A.E., P.H. Black, E.A. Vanderpool, P.H. Henry, J.B. Austin, and R.J. Huebner. 1967. Transformation of primary rat embryo cells by adenovirus type 2. Proc. Nat. Acad. Sci. USA 58:1205-1212.

Friefeld, B.R., J.H. Lichy, J. Hurwitz, and M.S. Hurwitz. 1983. Evidence for an altered adenovirus DNA polymerase in cells infected with the mutant H5ts149. Proc. Nat. Acad. Sci. USA 80:1589-1593.

Garon, C.F., K.W. Berry, and J.A. Rose. 1972. A unique form of terminal redundancy in adenovirus DNA molecules. Proc. Nat. Acad. Sci. USA 69:2391-2395.

Ghosh-Choudhury, G.,Y. Haj-Ahmad, and F.L. Graham. 1987. Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. EMBO J. 6:1733-1739.

Girardi, A.J., M.R. Hilleman, and R.E. Zwickey. 1964. Tests in hamsters for oncogenic quality of ordinary viruses including adenovirus type 7. Proc. Soc. Exp. Biol. Med. 115:1141-1150.

Gooding, L.R., L.W. Elmore, A.E. Tollefson, H.A. Brady, and W.S.M. Wold. 1988. A 14,700 mw protein from the E3 region of adenovirus inhibits cytolysis by tumour necrosis factor. Cell 53:341-346.

Graham, F.L., A.J. van der Eb, and H.L. Heijneker. 1974a. Size and location of the transforming regions of human Ad5 DNA. Nature 251:687.

Graham, F.L., P.J. Abrahams, C. Mulder, H.L. Heijneker, S.O. Warnaar, F.A.J. deVries, W. Fiers, and A.J. van der Eb. 1975. Studies on *in vitro* transformation by DNA and DNA fragments of human adenoviruses and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. 39:637-650.

Graham, F.L., J. Smiley, W.C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36:59-72.

Green, M., and G.E. Daesch. 1961. Biochemical studies on adenovirus multiplication. II. Kinetics of nucleic acid and protein synthesis in suspension cultures. Virology 13:169-176.

Green, M., and M. Pina. 1963. Biochemical studies on adenovirus multiplication. IV. Isolation, purification, and chemical analysis of adenovirus. Virology 20:199-207.