THE DEFECTIVENESS OF THE SUBGROUP F ADENOVIRUSES *IN VITRO*

Caroline Tanya Tiemessen

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ABSTRACT

A distinguishing feature of the human subgroup F adenoviruses, types 40 and 41, is their inability to replicate in cells that permit efficient growth of other adenoviruses. This study was conducted to determine the basis of the viral defect(s) responsible for poor growth *in vitro*.

Cell lines that show different degrees of permissiveness to Ad40 and Ad41 infection were studied for their ability to support a number of key events in the Ad2 life cycle. These events included viral DNA synthesis and packaging, late antigen synthesis, host protein shutoff and viral transcription. The functions were monitored by *in vivo* labelling of DNA with ^{32}P-orthophoshate and ^{3}H-thymidine and by dot blot hybridization, ELISA and a fluorescent focus assay, *in vivo* labelling of proteins with ^{35}S-methionine, and RNA:DNA dot hybridizations and RT-PCR, respectively. Complementation of Ad41 by Ad2 was monitored using a fluorescent focus assay. Transfection assays performed with plasmids containing defined Ad2 early region DNA were monitored for their ability to complement Ad40 and Ad41 in the same way.

The complexity of the growth patterns seen by the subgroup F adenoviruses suggests that defectiveness is a multi-factorial phenomenon, and not easily explained by a single aberrant function. Interferon induction in response to subgroup F adenovirus infection was excluded as a possible factor in limited virus growth in culture. In contrast to Ad2, Ad40 and Ad41 were sensitive to interferon in Chang cells. Both the sensitivity to interferon in Chang cells as well as the growth defect in

HEF cells could be overcome in mixed infection with Ad2. Differences observed in the ability of Ad40 and Ad41 to synthesize DNA in non-permissive cells, and to produce free hexon antigen in epithelial cell lines suggests that there may be replicative defects in subgroup F adenoviruses which are not shared by both serotypes. The block in replication of Ad41 and Ad40 in HEF cells appeared to occur during the early and late phases of the infectious cycle, respectively.

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Caroline Tanya Tiemessen

17th day of August, 1992.

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"The subgroup F adenoviruses" - for daring to be different.

CONTENTS

Page

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER FOUR

COMPLEMENTATION

CHAPTER SEVEN

CONCLUDING REMARKS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Infantile gastroenteritis is a major public health problem worldwide (Guerrant *et al*, 1990). It has been estimated that viral gastroenteritis produces 30-40% of the cases of infectious diarrhoea in the United States (DuPont and Pickering, 1980; Kotloff *et al*, 1988). The most common aetiological agents of viral gastroenteritis are rotaviruses and the subgroup F adenoviruses (types 40 and 41), occuring in 50% and 5-12% of hospitalized cases in temperate climates, respectively (Kapikian *et al*, 1979; Uhnoo *et al*, 1984; Brandt *et al*, 1985; Kotloff *et al*, 1989).

In South Africa, diarrhoeal diseases are a major cause of death in infants and young children (Department of National Health and Population Development, 1989a; b; 1991). In 1986, gastroenteritis was estimated to account for 27.5% of the total number of infant deaths. In the age group 1-4 years the number of deaths was lower, but a higher proportion of deaths resulted from gastroenteritis, namely 32.3%.

Only one study has been conducted to determine the prevalence of the subgroup F adenoviruses in a rural South African setting (Tiemessen *et al*, 1989). Here Third-World conditions prevail. Several factors, including poor sanitation,

contaminated water, and inadequate food hygiene, have been associated with an increased incidence of diarroeal disease (Black, 1984; Guerrant *et al,* 1990). The subgroup F adenoviruses were found to be as prevalent as rotaviruses in this environment and significantly associated with diarrhoea (Tiemessen *et al,* 1989).

In addition to their role in the causation of gastroenteritis in infants and young children, the subgroup F adenoviruses are characteristically defective for growth in cell lines which support the growth of other adenoviruses. They can, however, be propogated in selected cell types, namely 293 cells (Takiff *et al,* 1981; Brown, 1985), Chang conjunctival cells (Kidd and Madeley, 1981; Wigand *et al,* 1983), HT cells (Uhnoo *et al,* 1983), Hep-2 cells and tertiary cynomolgus monkey kidney cells (de Jong *et al,* 1983).

This study was undertaken to investigate the growth restriction of the subgroup F adenoviruses in tissue culture. The aims were (i) to provide the means to develop new and expand upon existing diagnostic methodology that at present is hampered by the inability of these viruses to grow in conventional cell culture systems, (ii) to contribute to the elucidation of the role of these viruses in the gastrointestinal tract, and (iii) to contribute to the general characterization of the subgroup F adenoviruses. A major objective of this study was to try to define their defectiveness (a) by gaining an overview of their behaviour in non-permissive cells (previously ill-defined) and (b) by making a more in-depth analysis of specific aspects of their growth.

The work presented in this thesis has been mainly carried out with Ad41, with Ad40 included for comparison in selected areas of work.

Some of the findings presented in this thesis have been published, or submitted for publication. Manuscripts in preparation are also listed.

TIEMESSEN,C.T., AND KIDD,A.H. (1988). Helper function of adenovirus 2 for adenovirus 41 antigen synthesis in semi-permissive and non-permissive cells. *Arch. Virol.* **103:** 207-218.

TIEMESSEN,C.T., AND KIDD,A.H. (1990). Adenovirus 41 growth in semi-permissive cells shows multiple-hit kinetics. *Arch. Virol.* **110:** 239-245.

TIEMESSEN,C.T., AND KIDD,A.H. (1992). Sensitivity of subgroup F adenoviruses to interferon. *Arch. Virol.* In press.

TIEMESSEN,C.T., AND KIDD,A.H. (1992). Subgroup F adenovirus growth in foetal intestinal organ cultures. Submitted.

TIEMESSEN,C.T., AND KIDD,A.H. (1992). Adenovirus 40 and adenovirus 41 growth *in vitro* : Evidence for multiple defects in the replicative cycle. Submitted.

TIEMESSEN,C.T., AND KIDD,A.H. The subgroup F adenoviruses : Candidates for a defective function. In preparation.

TIEMESSEN,C.T., AND KIDD,A.H. Adenovirus 41 replication blocked at an early stage in HEF cells. In preparation.

TIEMESSEN,C.T., AND KIDD,A.H. Adenovirus sequences hybridize to similar discrete cellular restriction enzyme fragments in human and simian cells. In preparation.

1.2 Literature review

1.2.1 History

Adenoviruses were first discovered in the early 1950s by two groups of workers. Rowe *et al* (1953) recovered infectious virus from surgically removed adenoids of children, while Hilleman and Werner (1954) isolated similar virus agents from military recruits with acute respiratory disease. In 1956 the group name "adenoviruses" (Enders *et al*, 1956) was adopted and in 1976 was given the family status "Adenoviridae" (Norrby *et al*, 1976).

In 1962, adenoviruses were first recognized as potential enteric pathogens (Joncas *et al*, 1962). Adenoviruses detected by electron microscopy in stools from children with diarrhoea could frequently not be grown in cell cultures routinely used for isolation of respiratory adenoviruses (Flewett *et al*, 1975; Schoub *et al*, 1975; White and Stancliffe, 1975; Madeley *et al*, 1977; Retter *et al*, 1979; Richmond *et al*, 1979). Furthermore, adenoviruses that could be propagated were often isolated from stools of asymptomatic children (Moffett *et al*, 1968; Flewett *et al*, 1973). It was then found that only specific serotypes are involved in the causation of gastrointestinal illness (Johansson *et al*, 1980). These viruses have been termed "non-cultivable" (Gary *et al*, 1979), "enteric" (Jacobsson *et al*, 1979), and "fastidious" (Kidd and Madeley, 1981) adenoviruses. De Jong *et al* (1983) studied several fastidious isolates from cases of infantile diarrhoea in the Netherlands and North-West Germany. It was found that these adenoviruses were not related to the 39 known serotypes by neutralization or haemagglutination, and showed two distinct variants. They were designated as serotypes 40 and 41. Further justification for the allocation as two separate types was provided by restriction enzyme analysis (Kidd *et al*, 1983; Kidd, 1984; Kidd *et al*, 1984).

Since the development of specific tests for their identification in clinical

specimens, Ad40 and Ad41 have emerged as a significant cause of gastroenteritis in infants and young children (Uhnoo *et al*, 1984; Kidd *et al*, 1986; Kotloff *et al*, 1989; Tiemessen *et al*, 1989; Kim *et al*, 1990; Cruz *et al*, 1990; Lew *et al*, 1991).

1.2.2 Properties of adenoviruses

1.2.2.1 Classification

Adenoviruses are grouped into 2 genera, one that infects birds (Aviadenovirus) and another that infects mammals (Mastadenovirus). Human adenoviruses are divided into 6 subgroups on the basis of their physical, chemical, and biological properties (Table 1.1). There are at least 47 antigenic types of human adenoviruses (Green *et al*, 1979a; de Jong *et al*, 1983; Wigand *et al*, 1987; Hierholzer *et al*, 1988a; b; Hierholzer *et al*, 1991).

A number of classification systems have been proposed for human adenoviruses. These include serotype grouping on the basis of their ability to agglutinate mammalian red blood cells (Rosen, 1960; Hierholzer, 1973), the molecular weight of virion core proteins (Wadell, 1979), and the presence of virus-specific "T antigens" induced early in infection (Huebner, 1967). In 1979, Green *et al* (a) proposed a system of classification on the basis of DNA homologies using liquid-phase molecular hybridization. This system of grouping correlated well with findings with respect to the base-ratio content of the viral genome, previous haemagglutination groups, properties of virion substructures, T-antigen groups, and epidemiology of human adenoviral infection. At that time 31 human adenoviruses were grouped into 5 subgroups. Since then 47 serotypes of established adenoviruses have been identified.

Table 1.1 Properties of human adenovirus subgroups[a]

Subgroups and types	Length of fibres (nm)	DNA homology[b]	G+C (%)	No. of *Sma*I fragments	MW of internal polypeptides (kDa) V	VI	VII[c]	HA
A 12,18,31	28-31	48-69 (8-20)	47-49	4-5	51.0-51.5 46.5-48.5	25.5-26.0	18	rat[d]
B[e] 3,7,11, 14,16,21 34,35	9-11	89-94 (9-20)	50-52	8-10	53.5-54.5	24	18	monkey
C 1,2,5,6	23-31	99–100 (10-16)	57-59	10-12	48.5	24	18.5	rat[d]
D[f] 8-10,13, 15,17,19, 20,22-30, 32,33, 36-39, 42, 43-47	12-13	95-99 (4-17)	57-60	14-18	50.0-50.5[g]	23.2	18.2	rat
E 4	17	(4-23)	57	16-19	[illegible]	24.5	18	rat[d]
F 40,41	(a) 28-33[h] (b) 18-20[h]*	62-69[i] (15-22)[i]	52	9-12	46.0- 46,48.5	25.5	17.5	rat[j]

[a]Adapted and modified from Wigand and Adrian (1986).
[b]Percent homology within the subgroup; figures in parentheses: homology with other types (Green *et al*, 1979a).
[c]From Wadell (1990).
[d]Incomplete HA pattern.
[e]Types within subgroup B are divided into two clusters of DNA homology based on marked differences in restriction enzyme sites, one cluster including Ad3, Ad7, Ad16, and Ad21, the other Ad11, Ad14, Ad34 and Ad35.
[f]Only DNA restriction and polypeptide analysis have been performed with types 32 to 39. Type 42 has been described by Wigand *et al* (1987). Types 43-47 have been described by Hierholzer *et al* (1988a). Adenovirus types 43 to 47 have only been analysed by DNA restriction enzyme analysis.
[g]Polypeptides V and VI of Ad8 showed apparent molecular weights of 45K and 22K respectively. Polypeptide V of Ad30 showed an apparent molecular weight of 48.5K.
[h]Subgroup F adenoviruses have two fibres (a) and (b). Length of shorter fibre (*) from Kidd *et al* (1992).
[i]DNA homology determinations from van Loon *et al* (1985b).
[j]Agglutination requires incubation at 4°C overnight and selected erythrocytes (de Jong *et al*, 1983).

Adenovirus 40 and Ad41 have been shown to be different from other serotypes with respect to restriction endonuclease analysis of viral DNA, neutralization and haemagglutination inhibition tests, DNA-DNA hybridization (de Jong *et al*, 1983), molecular weights of internal polypeptides (Wadell *et al*, 1980; Wadell, 1990) and their association with gastrointestinal disease (Retter *et al*, 1979; Uhnoo *et al*, 1984). They cannot, however, be distinguished from each other in neutralization tests using 293 cells or Chang cells. Differentiation of Ad40 and Ad41 on the basis of neutralization can only be made when using tertiary cynomolgus monkey kidney cells (de Jong *et al*, 1983). Furthermore, they cannot be distinguished from each other in haemagglutination inhibition (HI) tests (de Jong *et al*, 1983) or by solid phase immune electron microscopy (SPIEM) (Svensson *et al*, 1983). The enteric adenoviruses were initially assigned to two separate subgroups, Ad40 belonging to subgroup F (prototype strain Dugan) and Ad41 belonging to subgroup G (prototype strain Tak) (Uhnoo et al, 1983; de Jong *et al*, 1983; Wadell, 1984). They have since been assigned to a single subgroup F (Wadell *et al*, 1986) and are the only known members to date.

1.2.2.2 Morphology and composition

Adenoviruses are 60-90nm in diameter and the virion consists of a dense central core and an outer icosahedral capsid (Valentine and Pereira, 1965; Ginsberg *et al*, 1966). The capsid is composed of 252 capsomeres, consisting of 12 vertex capsomeres called pentons and 240 nonvertex capsomeres called hexons. The pentons consist of a base and an attached fibre, the length of which varies with viral type (Norrby, 1969). Minor capsid proteins (IIIa, VI, VIII, IX) are found in close association with the pentons and hexons and are thought to function in maintaining virion stability, assist in virion assembly, and associate with core proteins (Maizel *et al*, 1968a; b; Philipson, 1984).

The virus core contains one linear double-stranded DNA molecule with a molecular weight of 20-23 x 10^6 daltons (Green *et al*, 1967; van der Eb *et al*, 1969)

associated with a number of internal core proteins (Maizel *et al*, 1968a; Russell *et al*, 1971; Hosakawa and Sung, 1976; Vayda *et al*, 1983). DNAs of different virus types vary in molecular weight and base composition (viruses within a group share 70%-95% homology, whereas viruses of different groups have only 10%-25% homology). Terminal nucleotide sequences of each DNA strand are inverted repetitions (Wolfson and Dressler, 1972; Garon *et al*, 1972; 1975) and a virus-coded terminal protein is covalently attached to the 5′ end of each strand (Rekosh *et al*, 1977).

Analysis of structural polypeptides using polyacrylamide gel electrophoresis allows the separation of up to 10 polypeptides. The major adenovirus antigens, their relative size, and their location in the virion are shown in Figure 1.1. Antigens associated with the major structural proteins are shown in Table 1.2.

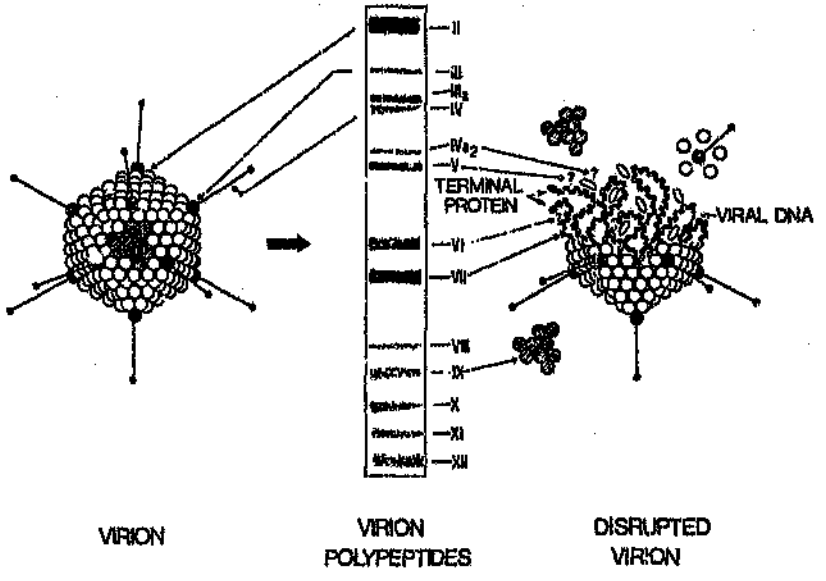


Figure 1.1 Schematic model illustrating the architecture of the adenovirus particle. The SDS-PAGE polypeptide pattern and the tentative locations of some polypeptides in the virus capsid is shown. Reproduced from Pettersson (1984).

Table 1.2 Antigens associated with the major structural proteins[a]

Protein	Corresponding polypeptide	Antigens: Designation	Antigens: Specificity
Hexon	II	α	Group
		?	Inter- and intrasubgroup
		ϵ	Type
Penton base	III	β	Group
			Inter- and intrasubgroup
Fibre	IV	γ	Type
		?	Intersubgroup
		δ	Intrasubgroup
Major core protein	VII	-	Group and type
Minor virion polypeptides	IIIa	-	Group
	IX	-	Group and type

[a]Reproduced from Pettersson (1984).

1.2.2.3 Adenovirus multiplication

1.2.2.3.1 The infectious cycle

Most adenoviruses replicate well in cells of epithelial origin and can be readily propagated on continuous human cell lines such as HeLa, KB, and Hep-2 cells. Adenovirus types 2 and 5 have been most intensively studied and they provide the basis of most of our knowledge of the adenovirus replicative cycle and gene organization. Infection is characterized by a well-ordered series of events beginning with the attachment of an adenovirus particle to a susceptible cell and ending with the assembly of approximately 10^4 infectious Ad2 or Ad5 virions per cell (Ginsberg, 1984). The adenovirus replication cycle (Figure 1.2) is divided into two distinct phases, an early phase and a late phase. The late phase begins with the onset of viral

DNA synthesis and is characterized by shut off of host-cell protein synthesis and the preferential translation of viral mRNA.

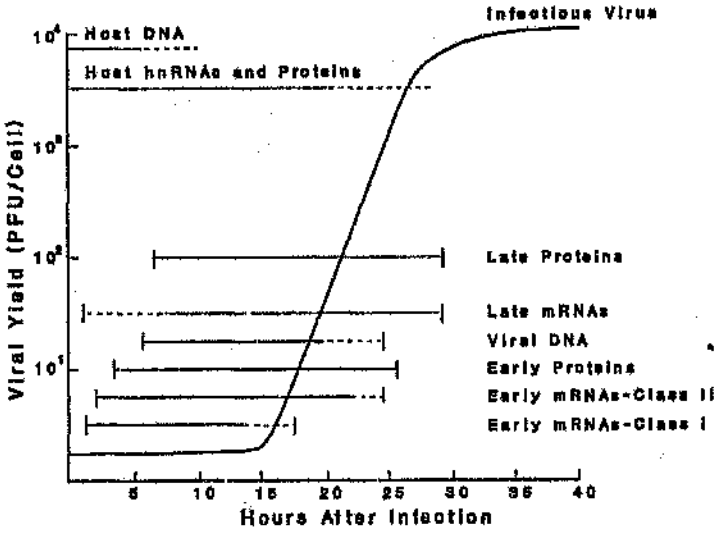


Figure 1.2 Initial cycle of replication of adenovirus type 5, and sequential biosynthetic reactions that are essential for producing viral macromolecules. The effect of viral infection on synthesis of host proteins, RNA, and DNA is also shown. hnRNA: heterologous nuclear RNA; mRNA: messenger RNA; PFU: plaque-forming units. Reproduced from Ginsberg (1984).

(i) Attachment, penetration and uncoating

Adenoviruses attach to specific receptors on the plasma membrane of HeLa cells (Philipson *et al*, 1968; Svensson *et al*, 1981) and KB cells (Philipson *et al*, 1968). The adenovirus fibre mediates this early contact between virus and cell (Levine and Ginsberg, 1967; Philipson *et al*, 1968; Boulanger and Lonberg-Holm, 1981). Following attachment virus is internalized by direct penetration of the plasma membrane (Morgan *et al*, 1969; Brown and Burlingham, 1973) or by receptor–mediated endocytosis (Chardonnet and Dales, 1970; Fitzgerald *et al*, 1983; Svensson and Persson, 1984; Svensson, 1985; Seth *et al*, 1986; Defer *et al*, 1990; Varga *et al*, 1991). Conformational changes occur in the capsid due to a decrease in pH within the endosome resulting in the release of altered virions into the cytoplasm (Seth *et al*,

1984; 1985; 1986). Uncoating of the viral DNA begins immediately after the virions have penetrated into the cytoplasm. The stability of the capsid is reduced by the displacement of pentons and the immediate surrounding hexons and the nucleic acid becomes susceptible to DNase (Philipson *et al*, 1968; Morgan *et al*, 1969). The particles are vectorially transported to the nucleus where final uncoating occurs (Dales and Chardonnet, 1973). The naked viral core enters the nucleus where viral replication takes place (Morgan *et al*, 1969; Chardonnet and Dales, 1972).

(ii) Organization of the genome

A number of conserved features exist in adenoviruses studied to date with respect to the organization of the adenovirus genome and replicative strategy. Transcription maps of Ad3, 7 (Tibbetts, 1977) and Ad12 (Esche *et al*, 1984) are similar to those of Ad2 and Ad5 (Tooze, 1981). An inverted terminal repitition (ITR) of variable length has been found in all adenoviruses studied from mammalian, avian and simian origin (van Ormondt and Galibert, 1984). A common ATAATA sequence is located at the 5′ end of the ITR (Alestrŏm *et al*, 1982; Shinagawa *et al*, 1983). Nucleotide sequence comparisons of Ad5, 7, and 12 reveal various homologous parts (Sambrook *et al*, 1980; van Ormondt and Hesper, 1983). Both ends of the viral genome function as origins for DNA replication (Tolun and Pettersson, 1975; Lechner and Kelly, 1977) and contain a protein, the terminal protein (TP), which is covalently linked to the 5′-end of each DNA strand (Robinson *et al*, 1973; Rekosh *et al*, 1977) via a serine residue (Desideiro and Kelly, 1981).

The entire Ad2 (Roberts *et al*, 1986) and Ad5 (Chroboczek *et al*, 1992) genomes have been sequenced and have lengths of 35,937 and 35,935 nucleotides, respectively. The genome is organized into a number of transcription units and transcription occurs in a leftward direction from one strand, the l-strand, and a rightward direction from the other, the r-strand (Sharp *et al*, 1975). The organization of the Ad2 genome with early and late transcription units is shown in Figure 1.3.

Viral mRNA is synthesized by the cellular DNA-dependent RNA polymerase II. The cellular RNA polymerase III transcribes the viral genes for the two virus-associated (VA) RNA species. Early mRNAs are transcribed from 5 regions of the genome, each with its own early promoter (E1a, E1b, E2, E3 and E4), corresponding to approximately 14% of the r-strand and 13% of the l-strand. Late mRNAs are predominantly encoded in the r-strand. One late mRNA appears to be encoded in the l-strand. Molecular events leading to the generation of specific mRNAs include initiation of transcription at separate promoters, capping of mRNA at the 5′ termini, splicing and polyadenylation of the RNA transcripts at the 3′ termini, regulatory processes resulting in a switch from the early to the late viral genes, as well as regulatory processes that affect the transcription of the host cell genome and selective transport of viral mRNA (Ziff, 1980).

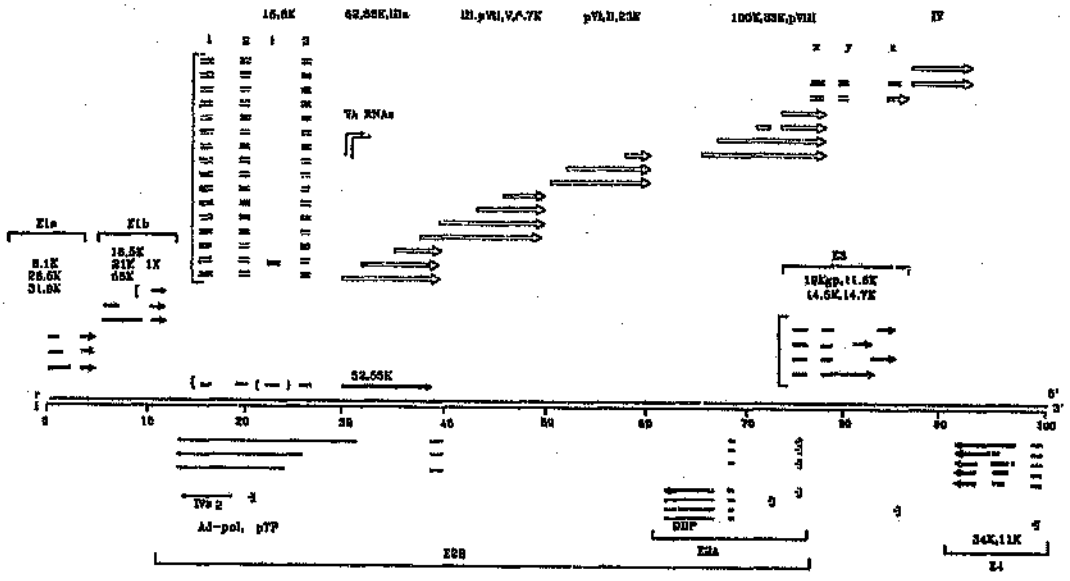


Figure 1.3 Organization of the Ad2 genome. The genome is divided into 100 map units. Arrows show the location of the major RNA species and the direction of transcription. Thick lines illustrate mRNAs expressed early after infection and thin lines mRNAs expressed at intermediate times after infection. Open arrows show sequences present in late mRNA. Polypeptides that have been assigned to the different regions are indicated. Square brackets indicate common promoter sites. Ad-pol: adenovirus polymerase; pTP: precursor terminal protein; DBP: DNA-binding protein. Reproduced from Akusjärvi *et al* (1986).

(iii) Early gene expression

(a) Early region 1

Early region 1 (1.3-11.2 m.u.) at the left end of the viral genome encodes two distinct transcription units, designated E1a (1.3-4.6) and E1b (4.6-11.2). These genes are involved in the regulation of early viral transcription (Berk *et al*, 1979; Jones and Shenk, 1979a; Nevins, 1981) and are responsible for cell transformation (see 1.2.2.4). A third transcription unit, designated pIX, overlaps completely with region E1b (9.8-11.2) and encodes the structural virion polypeptide IX.

Early region 1a (1.3-4.6)

Three major mRNAs are transcribed from the E1a region, a 13S, 12S and 9S species (Berk *et al*, 1979). Two additional mRNAs, a 10S and 11S species, have also been detected (Stephens and Harlow, 1987). All these species, except the 9S mRNA, are translated in the same reading frame. They share 5′ and 3′ termini and differ from each other by the size of the intron which is removed during processing of nuclear RNA.

A number of regulatory sequences are found between the left hand end of the adenovirus genome and the start site of E1a transcription: the terminal repeat, an enhancer sequence and the packaging signal, a CAAT box, and a TATA motif at 30 nucleotides upstream of the cap site (Boulanger and Blair, 1991). The E1a mRNAs have one major cap site located at position 499 (Baker and Ziff, 1981) and multiple minor initiation sites further upstream which are used after the initiation of DNA synthesis (Osborne and Berk, 1983). A *cis*-acting transcriptional control signal is present within the protein coding sequence of E1a (Osborne *et al*, 1984). The E1a enhancer is thought to have two distinct functional domains. Domain I, which contains two repeated sequences around nucleotides -300 and -200, specifically

regulates the E1a unit. Domain II is found between nucleotides -218 and -250 and modulates the activity of all the other transcription units (E1b, E2, E3, and E4) in *cis* (Hearing and Shenk, 1986). The poly(A) addition site for all E1a mRNAs is located at nucleotide 1630 (Perricaudet *et al*, 1979). It is preceded by the hexanucleotide sequence AAUAAA, which is specifically required for the cleavage of the precursor RNA prior to its polyadenylation (Montell *et al*, 1983).

The 13S and 12S mRNAs are the most abundant species early after infection. At late times, the 9S mRNA is preferentially transcribed and becomes the most abundant species (Berk and Sharp, 1978). The polypeptides specified by the 12S and 13S mRNAs are 243 and 289 amino acid residues long, with molecular weights 26.5K and 31.9K, respectively (Perricaudet *et al*, 1979). They contain identical N- and C-terminal ends and differ by 46 internal amino acids. The 9S mRNA is predicted to specify a 55 amino acid protein. The 26 N-terminal amino acids of the 9S polypeptide are common to 12S and 13S polypeptides and the C-terminus contains 29 unique amino acids (Virtanen and Pettersson, 1983). The predicted molecular weight is 6.1K.

The adenovirus E1a proteins function in transcriptional activation, transcriptional repression, the induction of cellular DNA synthesis, and cellular transformation. Region E1a is the first early transcription unit to be expressed and transcripts can be detected within 45 minutes after infection. The 289R, a nuclear phosphoprotein (Ferguson *et al*, 1985; Yee and Branton, 1985) encoded by the E1a 13S mRNA is responsible for the stimulation of early viral transcription.

Early region 1a-dependent activation of transcription from viral promoters is thought to be an indirect process mediated by cellular transcription factors, since the E1a transactivating function can be replaced by that of regulatory genes of unrelated viruses (Feldman *et al*, 1982; Imperiale *et al*, 1983). Furthermore, the E1a 289R E1a protein is not a DNA-binding protein (Ferguson *et al*, 1985). Three cellular

transcription factors have shown increased binding to their cognate DNA sequences, within the control regions of adenovirus early genes, dependent on a functional E1a 289R protein. Transcription factor E2F (Kovesdi *et al*, 1986) binds to the control sequences of the Ad2 E2a gene (Kovesdi *et al*, 1986; SivaRaman and Thimmappaya, 1987) and of the E1a gene itself (Kovesdi *et al*, 1987). The E4 transcription factor (Raychaudhuri *et al*, 1987) recognizes binding sites within the control sequences for E4. The RNA polymerase transcription factor IIIC (TFIIIC) is thought to be involved in E1a 289R activation of the VA RNA genes (Hoeffler and Roeder, 1985; Hoeffler *et al*, 1988; Yoshinaga *et al*, 1986).

The E1a regulatory protein (289R) also induces the synthesis of the cellular heat shock proteins (Nevins, 1982; Kao and Nevins; 1983, Wu *et al*, 1986; Simon *et al*, 1987) and enhance the transcription of cellular β-tubulin genes (Stein and Ziff, 1984) during the early phase of the infectious cycle. The 289R protein can also stimulate expression of rabbit and human β-globin genes (Svensson and Akusjärvi, 1984; Green *et al*, 1983) and rat preproinsulin I gene (Gaynor *et al*, 1984) introduced into cells by transfection or infection. Other genes that have been shown to be responsive to E1a include the rat class I MHC gene which is repressed (Schrier *et al*,1983), and the mouse MHC H-2K gene which is stimulated by E1a (Rosenthal *et al*, 1985).

Three highly conserved domains, termed conserved regions 1,2 and 3 (CR1, CR2, CR3) have been identified, localized and their functions defined in the 289R E1a protein (Lillie *et al*, 1987; Moran and Mathews, 1987). The principal transactivation domain of the 289R protein is contained in the highly acidic stretch of 46 amino acids unique to that protein (Moran and Mathews, 1987; Green *et al*, 1988; Lillie *et al*, 1987). The 243R protein has only the CR1 and CR2 domains. Figure 1.4 shows a summary of the the biological activities mapped to the different conserved regions.

The 243R protein is needed for efficient viral replication in growth-arrested cells (Montell *et al*, 1984b). It may therefore be responsible for inducing cells to progress from G1 to S phase during the cell cycle (Braithwaite *et al*, 1983). The 243R has been shown to function as a transcription repressor (Lillie *et al*, 1986).

No specific function has yet been assigned to the three minor E1a gene products, 55R, 171R, and 217R (Moran *et al*, 1986).

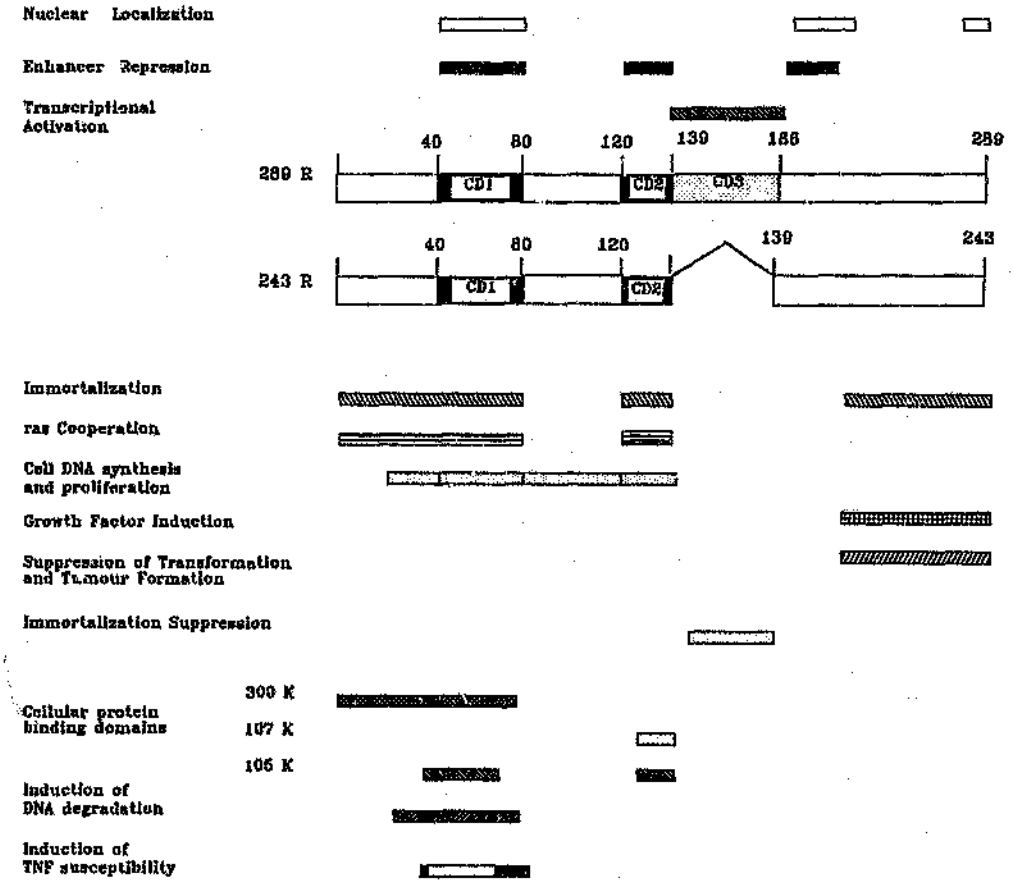


Figure 1.4 Schematic representation showing the two major E1a proteins and indicating the functions mapped to the various domains. Reproduced from Duerksen-Hughes *et al* (1991).

Early region 1b (4.6-11.2)

Region E1b is located immediately adjacent to region E1a and is also transcribed in a rightward direction. Two major overlapping mRNAs (22S and 13S) and two minor mRNAs (14.5S and 14S) are generated by splicing of a common precursor RNA (Berk and Sharp, 1978; Chow *et al*, 1979; Virtanen and Pettersson, 1985). The E1b mRNAs differ from each other by the sizes of the introns that are removed. The 22S mRNA is translated in two different reading frames from two separate initiation codons (Bos *et al*, 1981; Boulanger and Blair, 1991). This polycistronic message thus encodes two different proteins of 55K and 19K. The 13S mRNA species makes only the 19K protein. A 9S mRNA is also transcribed from this region at intermediate times of infection and encodes a structural protein, polypeptide IX (see (iv) intermediate gene expression).

The regulatory region of the E1b transcription unit appears to be a short 60bp eukaryotic polymerase II promoter, consisting of three adjacent elements (Wu *et al*, 1987). The E1b promoter is stimulated 5-10 fold by E1a expression and it is thought that this interaction may be via host cell factors.

The accumulation of E1b mRNAs is subjected to post-transcriptional regulation (Spector *et al*, 1978; Chow *et al*, 1979; Wilson and Darnell, 1981). Early after infection the E1b 22S and 13S mRNAs are present in equal amounts. However, by 20 hours p.i. the accumulation of 13S mRNA exceeds that of 22S mRNA by a factor of approximately 20-fold (Spector *et al*, 1978). This is in part due to a 5- to 10-fold increase in the half-life of the 13S mRNA as compared to the 22S mRNA (Wilson and Darnell, 1981). The stability of the E1b mRNAs is influenced by the E2a-72K DBP (Babich and Nevins, 1981) and at late times, probably by a change in the specificity of the RNA splicing machinery (Montell *et al*, 1984a).

The E1b-55K protein is essential for a complete lytic cycle in HeLa cells. Mutants are defective in accumulation of late viral mRNA in the cytoplasm and in switching off host cell functions (Babiss and Ginsberg, 1984; Pilder *et al*, 1986). Impaired early mRNA and DNA synthesis has also been shown (Fukai *et al*, 1984; Shiroki *et al*, 1986; Byrd *et al*, 1988). In subgroup C adenovirus transformed cells the E1b-55K polypeptide is found complexed with a cellular oncoprotein designated p53 (Sarnow *et al*, 1982; Blair-Zajdel and Blair, 1988). During lytic infection the 55K protein is found in association with the E4-34K (ORF6) polypeptide (Sarnow *et al*, 1984; Cutt *et al*, 1987). Mutants with an altered expression of the E4-34K polypeptide are defective in their inhibition of host protein synthesis (Halbert *et al*, 1985) supporting the idea that an E1b-55K/E4-34K protein complex is involved in the shut off of cellular gene expression late during the infectious cycle. The 55K protein is localized mainly in the nucleus (Rowe *et al*, 1983).

Mutants in the 19K protein allow degradation of host and viral DNA (*deg* phenotype), have a large plaque phenotype (*lp*), and show an enhanced cytopathic effect (*cyt*) (LaiFatt and Mak, 1982; Pilder *et al*, 1984; Subramanian *et al*, 1984; White *et al*, 1984). The 19K protein has recently been implicated in negative regulation of E1a gene expression in the presence of E1a 13S and 12S products (White *et al*, 1986; White and Stillman, 1987). In the absence of the latter products the 19K protein has a positive effect on viral gene expression (Herrmann *et al*, 1987; White *et al*, 1988). It transactivates all the adenovirus early gene regions (Herrmann *et al*, 1987), the cellular heat-shock hsp70 gene (Herrmann *et al*, 1987), enhancer-linked promoters of SV40, polyomavirus, and immunoglobulin heavy-chain enhancers (Yoshida *et al*, 1987), and the adenovirus protein IX gene (Vales and Darnell, 1989). The 19K polypeptide is associated with nuclear and plasma membrane fractions of infected and transformed cells (Persson *et al*, 1982; Rowe *et al*, 1983). The protein has also been found to associate with vimentin-containing intermediate filaments and the nuclear lamina, causing disruption of these structures (White and Cipriani, 1989; 1990). Recently, Gooding *et al* (1991) have reported that the

E1b-19K protein protects human cells from tumor necrosis factor (TNF) lysis when infected with adenovirus mutants that have a deleted E3 region, a function similar to that of the E3-14.7K protein (Gooding *et al*, 1988; Gooding *et al*, 1990).

(b) Early region 2 (11.3-75.4)

The region E2 differs from other transcription units in that there are alternative promoter sites for initiation of transcription (Figure 1.3) (Chow *et al*, 19 /9). A major promoter (E2-E), located at coordinate 75.4, is activated at early times after infection (Nevins *et al*, 1979). A promoter shift occurs at late times, and E2 mRNAs are preferentially transcribed from a promoter located at coordinate 72.2 (E2-L promoter). E1a gene products stimulate expression from E2-E promoter via an upstream enhancer element located within the first 79 nucleotides preceding the E2-E cap site (Imperiale and Nevins, 1984).

Two major classes of transcripts controlled by the same promoter are generated from region E2. The first set of transcripts, designated E2a, extend from the transcription initiation site to a polyadenylation site at coordinate 62.4. Two intron sequences are removed from the RNA precursor generating one principal mRNA (Goldenberg and Raskas, 1979; Weber *et al*, 1980). The second set of transcripts, the E2b mRNAs, use a second polyadenylation site located at coordinate 11.3 (Stillman *et al*, 1981). Three differentially spliced E2b mRNAs accumulate to about 10% of the level of E2a mRNAs (Stillman *et al*, 1981). The three mRNAs share a common set of leader exons from coordinates 76, 68.5, and 39 joined to main bodies beginning at coordinate 30, 26, and 23, respectively.

The E2 transcription unit is activated early in infection as a result of an activation of the cellular transcription factor E2F (reviewed in Nevins, 1991). The products of regions E1a and E4 are necessary for this activation (Babiss, 1989; Reichel *et al*, 1989; Raychaudhuri *et al*, 1990).

The E2 transcription unit encodes three proteins required for viral DNA replication: the 72K DNA binding protein (DBP), the terminal protein precursor (pTP), and the DNA polymerase. An ORF (contained within the longest E2b mRNA) located between coordinates 29.3 and 23.9 encodes the 87K terminal protein precursor (pTP)(Smart and stillman, 1982) which functions as the primer for DNA replication and which is later cleaved to its mature 55K form by a viral-encoded protease (Stillman *et al*, 1981). The shortest E2b mRNA encodes a 140K DNA polymerase required for DNA replication (Stillman *et al*, 1982a). No protein product has been assigned to the third mRNA.

The E2a mRNA codes for the adenovirus DBP. In addition to its replicative function, the DBP also functions in the regulation of gene expression. A functional DBP is necessary for the normal turn off of early mRNA expression (Carter and Blanton, 1978a ;b). The DBP acts by down-regulating E4 transcription (Handa *et al*, 1983) and the stability of E1b mRNA (Lazaridis *et al*, 1988). This protein also influences the host range of the virus. Mutants in DBP have been isolated which allow efficient replication in monkey cells (Klessig, 1977). It has been proposed that the DBP consists of two domains, the amino-terminal domain which functions in host-range determination and the carboxyl-terminal domain involved in DNA replication and DNA and RNA binding (Klein *et al*, 1979; Kruijer *et al*, 1981; Cleghon and Klessig, 1986).

(c) <u>Early region 3</u> (76.8-85.9)

At least 9 E3 mRNAs are transcribed from the r-strand and are generated by differential processing of two major RNA precursors (Bhat and Wold, 1986). These have a common cap site at coordinate 76.8 (Baker and Ziff, 1981) and differ from each other by the position of their 3'-termini. A major polyadenylation site is located at coordinate 85.9 (Stålhandske *et al*, 1983). Two minor polyadeylation sites are located at coordinates 82.9 and 85 (Chow *et al*, 1979). Eight ORFs are present which

could encode proteins larger than 100 amino acids (Hérissé *et al*, 1980; Hérissé and Galibert, 1981). Proteins have been identified corresponding to 6 of the ORFs.

The E3 region has been shown to be dispensable for viral growth in cultured cells (Lewis *et al*, 1974; Thimmappaya *et al*, 1982; Berkner and Sharp, 1983). This transcription unit, however, directs the synthesis of several proteins that appear to be involved in the immune response.

The 19K glycoprotein, a transmembrane protein, is the most abundant viral protein at early times after infection (Wold *et al*, 1985). It can bind class I major histocompatibility (MHC) antigens in the endoplasmic reticulum (Kvist *et al*, 1978; Severinsson and Peterson, 1985) and in so doing inhibits the glycosylation of the antigens and prevents their efficient transport to the cell surface (Andersson *et al*, 1985; Burgert and Kvist, 1985; Severinsson and Peterson, 1985). The reduction of class I antigen expression on the cell surface thus protects the virus-infected cell against cytotoxic T-cell recognition (Burgert and Kvist, 1987; Burgert *et al*, 1987). Two other E3 proteins have interesting properties: the E3-10.4K protein down-regulates the expression of epidermal growth factor receptors on infected cells (Carlin *et al*, 1989), and the E3-14.7K protein prevents lysis of adenovirus infected cells by tumor necrosis factor (TNF) (Gooding *et al*, 1988).

(d) Early region 4 (91.3-99.1)

Region E4 is located at the right hand end of the genome and is transcribed from the l-strand (Sharp *et al*, 1974; Pettersson *et al*, 1976). Transcription from the E4 promoter located at coordinate 99.1 (Baker and Ziff, 1981) is subjected to both positive and negative regulation during the infectious cycle. The E1a 289R protein induces E4 transcription early after infection whereas the E2a 72K-DBP has been shown to down-regulate E4 transcription at intermediate to late times (Nevins and Jensen-Winkler, 1980; Handa *et al*, 1983).

The primary transcript from E4 extends from coordinate 99.1 to 91.3. At least 12 mRNAs are produced by differential splicing (Freyer *et al*, 1984; Virtanen *et al*, 1984). Seven ORFs are predicted from the l-strand nucleotide sequence (Hérissé *et al*, 1981; Gingeras *et al*, 1982). A 14K polypeptide (Downey *et al*, 1983) and a 34K nuclear protein (Sarnow *et al*, 1984) are encoded by ORF 3 and ORF 6, respectively.

The 34K protein forms a complex with the E1b-55K protein (Sarnow *et al*, 1984) and these proteins are thought to function as a unit (Cutt *et al*, 1987; Bridge and Ketner, 1990). Mutants in the E4-34K polypeptide are defective in the synthesis of late mRNAs and proteins (Challberg and Ketner, 1981; Weinberg and Ketner, 1983). Viral DNA replication appears normal but the shut off of host macromolecular synthesis seems to be incomplete. Mutants in the E1b-55K polypeptide are also defective in late mRNA synthesis and in control of cellular gene expression (Babiss and Ginsberg, 1984).

A 19.5K polypeptide is encoded by a fusion between ORFs 6 and 7, created by mRNA splicing (ORF 6/7) (Cutt *et al*, 1987). This nuclear protein is responsible for the transactivation of the E2 promoter (Huang and Hearing, 1989; Marton *et al*, 1990; Neill *et al*, 1990; Raychaudhuri *et al*, 1990). The functions of other E4 proteins are unknown.

(e) <u>The virus-associated RNAs and regulation of late mRNA translation</u>

The genome encodes two low molecular weight RNAs, the virus associated RNA (VA RNA) I and II, each about 160 nocleotides in length (Mathews, 1975; Pettersson and Philipson, 1975). They are transcribed in a rightward direction from two separate promoters around coordinate 30 (Akusjärvi *et al*, 1980) by RNA polymerase III (Weinmann *et al*, 1976). They are capable of forming extensive intramolecular base paired regions (Akusjärvi *et al*, 1980). They are made in approximately equal amounts early in infection, but due to more efficient binding of initiation factors to

the VA RNAI gene (Lassar *et al*, 1983), VA RNAI becomes dominant at late times, concomitant with the shut off of host cell protein synthesis (Söderlund *et al*, 1976; O'Malley *et al*, 1986). The internal control region consists of two regions, the A box and the B box (located at approximately nucleotides 10 to 18 and 54 to 69, respectively, in Ad2 VA RNAI), which serve as the polymerase III promoter (Fowlkes and Shenk, 1980; Guilfoyle and Weinmann, 1981).

Studies of deletion mutants have shown that a virus that lacks the VA RNAI species synthesizes normal amounts of mRNA but is defective in the translation of the late proteins (Thimmappaya *et al*, 1982). A double mutant lacking both VA RNAs grows poorly, producing 5- to 6-fold less virus than the VA RNAI negative mutant (Bhat and Thimmappaya, 1984). Since the virus lacking the VA RNAII species grows like wild type, it is likely that VA RNAII serves a similar function to VA RNAI and probably can partially substitute for it during lytic growth.

VA RNAI limits the activation of a protein kinase, the DAI or P1/eIF-2α kinase, which phophorylates the α-subunit of the translation initiation factor eIF-2 (Kitajewski *et al*, 1986a; Reichel *et al*, 1985). Shut off of protein synthesis in the absence of VA RNAI is due to increased eIF-2α phosphorylation as a result of elevated kinase activity. VA RNAI functions as a general stimulator of translation of both viral and non-viral mRNAs (Svensson and Akusjärvi, 1985). (For review on VA RNA and translation control see Mathews and Shenk, 1991).

(iv) **Intermediate gene expression**

Several regions of the viral genome are transcriptionally active at intermediate times (5-10 hour p.i.). In addition to the early genes two transcription units, encoding polypeptide IX (pIX) and IVa_2, appear to be selectively activated. Both genes are commonly referred to as intermediate genes, since small amounts of their mRNAs can be detected prior to the onset of viral DNA replication (Persson *et al*, 1978; Chow

et al, 1980) . Efficient synthesis of both mRNAs requires viral DNA replication (Crossland and Raskas, 1983).

(a) Polypeptide IX

The synthesis of pIX is controlled by a r-strand specific transcription unit which is located within region E1b (Chow *et al*, 1979; Wilson et al, 1979a). A 9s unspliced mRNA is transcribed from an independent promoter and encodes a structural component of the adenovirus capsid present in groups of nine hexons, which is referred to as polypeptide IX (Boulanger *et al*, 1979). E1b transcription across the IX promoter is responsible for inhibiting IX gene expression early in infection, while the 19K E1b protein activates the IX gene (Vales and Darnell, 1989). The pIX mRNA is translated with the highest efficiency late during infection (Lawrence and Jackson, 1982). Mutant virions lacking this gene are less heat stable than wild type virions (Colby and Shenk, 1981), suggesting the protein plays a role in stabilizing the capsid structure.

(b) Polypeptide IVa_2

The IVa_2 gene is located between 11.3 and 16 m.u. on the Ad2 genome and the mRNA is transcribed from the l-strand (Lewis *et al*, 1977). The polyadenylation site for both the IVa_2 and the E2b mRNAs is located near coordinate 11.3 (Stillman *et al*, 1981). The polypeptide has a predicted molecular weight of 51K. The IVa_2 polypeptide is present in adenovirus assembly intermediates and has been suggested to function as a maturation protein during adenovirus morphogenesis (Persson *et al*, 1979). Winter and D'Halluin (1991), however, have found that IVa_2 is not related to the 50K product found in immature virions suggested to be IVa_2 by Persson *et al* (1979). Since the IVa_2 is present in mature shells, and the IVa_2 gene is distinct from the major late (ML) transcription unit, it has been suggested that a role in addition to one in adenovirus assembly seems likely (Winter and D'Halluin, 1991).

Negative control by the MLP on the IVa_2 promoter has been demonstrated *in vitro* (Natarajan *et al*, 1985; 1987). It has, however, been suggested that the IVa_2 promoter and the MLP do not share elements and that competition for transcription factors is not involved in the divergent regulation (Reach *et al*, 1991). Recent evidence shows that IVa_2 may possess its own set of regulatory elements including its own TFIID binding site and initiator element (Carcamo *et al*, 1990), and a site which binds USF with an affinity lower than that of the UPE (Moncollin *et al*, 1990) (see (vi)(a) The ML transcription unit).

Polypeptide IVa_2 has been shown to have an affinity for viral DNA (Russell and Precious, 1982). It may therefore interact with the viral DNA as it enters the preformed capsid.

(v) <u>DNA replication</u>

Viral DNA replication takes place in the nucleus and marks the beginning of the late phase of viral replication. Initiation occurs at either end of the DNA molecule and proceeds by a mechanism of strand displacement (reviewed by Winnacker, 1978).

The virus-coded, covalently linked terminal protein functions as a primer for initiation of viral DNA synthesis (Challberg *et al*, 1980; 1982; Lichy *et al*, 1981; 1982; Stillman *et al*, 1981). The ITR plays an important role in the initiation of DNA replication (Tamanoi and Stillman, 1983; Challberg and Rawlins, 1984). Nuclear factor I (NFI), a host protein required for the initiation reaction, binds to a sequence located at nucleotides 17-48 of Ad5 (Nagata *et al*, 1983). It has been suggested that by binding to the ITR, NFI unwinds a single stranded region near the termini which then facilitates the binding of the adenovirus polymerase/precursor terminal protein-dCMP (Ad-pol/pTP-dCMP) complex (Nagata *et al*, 1983). DBP, being a single strand specific DNA binding protein, interacts with the displaced DNA strand generated during replication. The DBP is thought to play two roles in DNA synthesis:

as a single stranded DBP that protects the nascent single stranded DNA from nuclease attack and separates it from the replication complex, and as a facilitator of chain elongation through its interaction with another protein (thought to be the adenovirus DNA polymerase) in the replication complex (Lindenbaum *et al*, 1986). A complex of the 80K terminal protein precursor (pTP) and adenovirus 140K DNA polymerase binds to a 10bp core sequence of 9-18 nucleotides (Rijnders *et al*, 1983). This sequence is necessary for pTP-dCMP formation (Tamanoi and Stillman, 1983), a rate-limiting step in the replication of adenoviral DNA (Stillman *et al*, 1982b). This core sequence is highly conserved in human adenoviruses (Tamanoi and Stillman, 1983; Guggenheimer *et al*, 1984).

A model for adenovirus DNA replication is shown in Figure 1.5. The complex of polymerase and pTP, containing a cytidine with a free 3′-OH group, displaces the 5′ end of the parental strand with the attached 55K version of the terminal protein. The 3′-OH end is added to an incoming nucleotide, which pairs to the template strand under direction from DNA polymerase. DNA replication which takes place on a double-stranded DNA is termed type I replication (Lechner and Kelly, 1977). Type II replication takes place on displaced single-stranded DNA. Late in infection, after progeny DNA is packaged into virion particles, the 80K pTP is processed to a mature 55K TP by a virus-coded protease (Challberg and Kelly, 1981; Stillman *et al*, 1981).

Synthesis of full-length adenovirus DNA requires 5 proteins, three viral and two cellular (Table 1.3). The viral proteins are involved in initiation as well as elongation. NFI is required for initiation and partial replication whereas nuclear factor II is only required for elongation (Nagata *et al*, 1982; 1983). It appears that these 5 proteins are sufficient for synthesis of full-length adenovirus DNA (Nagata *et al*, 1983). NFII activity is dependent on the presence of NFI and exhibits topoisomerase I activity (Nagata *et al*, 1983). The requirement for this activity in adenovirus DNA replication suggests that there is a requirement to relieve restaint ahead or behind a replication fork.

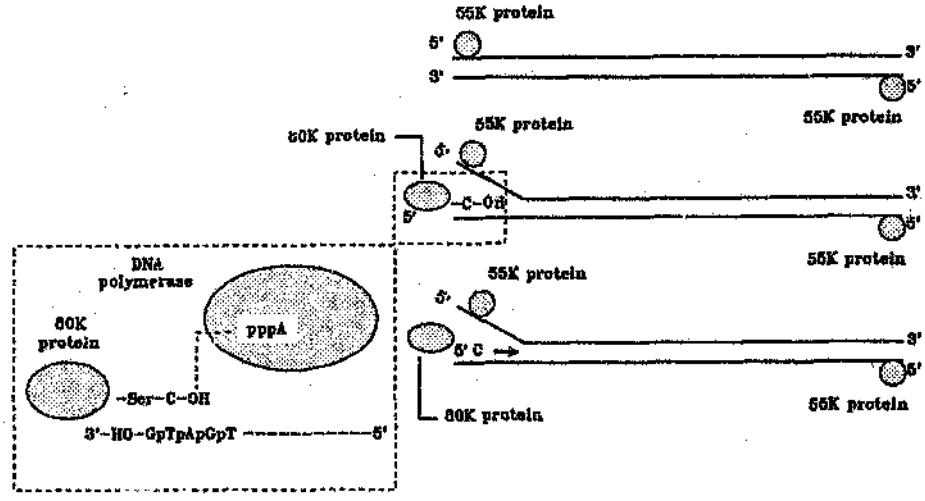


Figure 1.5 Proposed model for adenovirus DNA replication. The 80K pTP displaces the 5′ end of the DNA (which is bound to the 55K TP), and provides CTP to prime synthesis of a new DNA strand. Reproduced from Lewin (1990).

Table 1.3 Proteins required for adenovirus DNA replication[a]

	Apparent MW	Function
Viral proteins		
terminal protein precursor (pTP)	80K	serves as a protein primer
adenovirus DNA polymerase	140K	template-dependent DNA synthesis
adenovirus DNA binding protein	72K	binds ssDNA and interacts with adenovirus DNA polymerase
Cellular proteins		
nuclear factor I	47K	specific binding to origin of replication in ITR
nuclear factor II	<15K	topoisomerase I activity

[a]Reproduced from Tamanoi (1986).

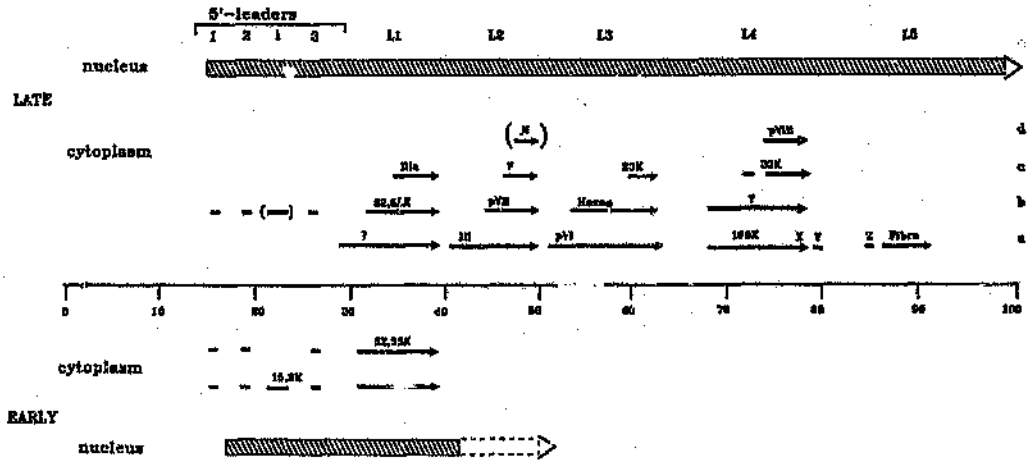


Figure 1.6 Primary transcripts and mRNAs expressed from the major late transcription unit at early and late times after infection. Hatched arrows represent the primary transcripts within the nucleus. Solid arrows represent the mRNA species within the cytoplasm. Proteins are designated by their molecular weights in kilodaltons (K) or by roman numerals (virion components). L1-L5: 5 families of RNAs with coterminal 3'-ends. The 5' tripartite leader (1,2,3) can be joined to at least 15 downstream splice acceptor sites. *i:* intermediate leader. *X,Y,Z:* ancillary leaders. Reproduced from Akusjärvi *et al* (1986).

The MLP is subject to many levels of control. Activation of the MLP can be achieved through an interaction between protein factors which bind to specific regulatory elements (Sawadogo and Roeder, 1985; Meisterernst *et al*, 1990). Alternatively, the MLP can be activated by the transactivating E1a protein (Nevins, 1981; Lewis and Manley, 1985) and DNA replication is required for maximal levels of expression (Thomas and Mathews, 1980).

The onset of DNA replication appears to be the major event governing the transition from early to late gene expression, since unreplicated DNA introduced into adenovirus infected cells in the late phase cannot support late viral gene expression (Thomas and Mathews, 1980). A major difference between early and late times is found in the ML transcription unit (Figure 1.6). At early times the ML transcription unit is active at a level comparable to other early transcription units (Chow *et al*,

1979; Shaw and Ziff, 1980) and transcription terminates near the middle of the genome. At late times transcription continues to a position close to the right terminal end (Shaw and Ziff, 1980; Akusjärvi and Persson, 1981a). Regulation of transcription from the ML promoter therefore involves the termination step and at another level virus-induced changes in the splicing machinery (Akusjärvi and Persson, 1981a; Nevins and Wilson, 1981; Thomas and Mathews, 1980).

(b) Late region 1

The Ad2 L1 mRNAs are characterized by having a common poly(A) addition site at coordinate 39.3. Three major mRNAs, with splice acceptor sites located at coordinate 29.0 (species $L1_a$), 30.7 (species $L1_b$) and 34 (species $L1_c$), have been identified from region L1 (Chow *et al*, 1979; Akusjärvi and Persson, 1981a). No protein product has so far been assigned to species $L1_a$, but the predicted polypeptide has a molecular weight of 8.3K.

The L1 nuclear precursor is the same both early and late after infection. At early times only the $L1_b$ mRNA species accumulates (Chow *et al*, 1979; Akusjärvi and Persson, 1981a) through regulation of splicing and not changes in stability or mRNA transport (Nevins and Wilson, 1981). About half of the early L1 mRNA population contains the normal tripartite leader sequence and encodes two structurally related polypeptides of molecular weights 52K and 55K (Miller *et al*, 1980; Akusjärvi and Persson, 1981a). These products are required for assembly of virus particles (Hasson *et al*, 1989). The other half of the early L1 mRNA population contains an extra 440 nucleotide long leader, derived from coordinate 22.7 (Chow *et al*, 1979; Akusjärvi and Persson, 1981a). This leader, which has been termed the "*i*" leader (intermediate leader), contains an AUG triplet followed by an open translation reading frame (Falvey and Ziff, 1983). This results in the synthesis of a 15.8K polypeptide both *in vitro* and *in vivo* (Akusjärvi and Persson, 1981a; Lewis and Anderson, 1983).

The "*i*" leader is also present in L2, L3, L4, and L5 mRNAs, but at a very low frequency (Akusjärvi and Persson, 1981a).

Species $L1_c$ which becomes the most abundant L1 mRNA late after infection encodes virion polypeptide IIIa (Miller *et al*, 1980; Akusjärvi and Persson, 1981a). This polypeptide which exists in only 60 copies per virion (Everitt *et al*, 1975) is a minor component of the virus particle. It appears to be located in the vertex region of the virion probably acting as a bridge between the pentons and the peripentonal hexons (Everitt *et al*, 1975).

(c) Late region 2

The Ad2 L2 mRNAs are characterized by a common poly(A) addition site at coordinate 50.0. Three major mRNAs with acceptor splice sites at coordinates 39.4 (species $L2_a$), 44.1 (species $L2_b$) and 45.9 (species $L2_c$) have been identified (Chow *et al*, 1980; Aleström *et al*, 1984). Polypeptides III, V and pVII are coded in region L2 (Lewis *et al*, 1977; Miller *et al*, 1980).

Polypeptide III, from $L2_a$ mRNA, has a predicted molecular weight of 63.3. This polypeptide forms the building block of the penton base and associates with the fibre to produce the penton.

Polypeptides V and pVII together constitute the major proteins found in adenovirus chromatin (Maizel *et al*, 1968b). These are encoded by $L2_b$ and $L2_c$ mRNA species, respectively. Polypeptide VII which is tightly bound to the viral DNA is synthesized as a 21.8K precursor (pVII). During the final stages of virus maturation pVII is cleaved by the virus encoded protease to its final size of 19.4K.

Polypeptide V with a molecular weight of 41.6K is less tightly associated with the viral DNA than polypeptide VII. It is thought to form a protein shell around a nucleoprotein particle consisting of polypeptide VII and viral DNA (Nermut, 1979).

A fourth ORF is present in L2 following the terminator for polypeptide V. This ORF could encode an 85 amino acid polypeptide which may prove to be the precursor of the μ polypeptide which is a highly basic, low molecular weight polypeptide that has been found in viral chromatin preparations (Vayda *et al*, 1983).

(d) Late region 3

The Ad2 L3 mRNAs are characterized by a common poly(A) addition site at coordinate 62.4 (Figure 1.6). Three major mRNAs have been identified that code for three well-characterized polypeptides pVI, hexon, and the 23K protease (Lewis *et al*, 1977; Miller *et al*, 1980; Akusjärvi *et al*, 1981).

Polypeptide pVI which is translated from mRNA species $L3_c$ (coordinates 50.1-62.4) consists of 249 amino acids and has a predicted molecular weight of 27K (Akusjärvi and Persson, 1981b). It is processed by proteolytic cleavage to a 24K polypeptide during virion maturation. It is thought to be associated with the hexon capsomer on the inside of the virus particle, perhaps forming a bridge between the viral core and the capsid structure. Polypeptide VI has DNA binding properties (Russell and Precious, 1982) and may thus turn out to be a core protein that is only loosely associated with the viral chromatin (Akusjärvi *et al*, 1986).

The major structural component of the virion, the hexon, is encoded by mRNA species $L3_b$ (coordinates 52.3-62.4). The hexon capsomer is composed of three identical polypeptides, each consisting of 967 amino acids and having a calculated molecular weight of 109K and an apparent molecular weight of 120K (Jörnvall *et al*, 1981).

The 23K polypeptide encoded by mRNA species $L3_c$ (coordinates 60.2-62.4) is the protease responsible for the cleavage of four precursor polypeptides to their mature forms (Bhatti and Weber, 1979; Yeh-Kai *et al*, 1983). These include polypeptides L2-pVIII, L3-pVI, L4-pVIII and E2b-pTP. Polypeptide IIIa may also mature from a slightly larger precursor (Boudin *et al*, 1980). Cleavage by the 23K endopeptidase appears to occur between the Gly-Ala residues in the sequence Met-X-Gly-Gly-Ala/Val (Akusjärvi *et al*, 1986).

(e) Late region 4

The Ad2 L4 mRNAs are characterized by a common poly(A) addition site at coordinate 78.5 (Figure 1.6). Four major mRNAs have been mapped to this region (Chow and Broker, 1978). Three well characterized polypeptides of 100K, 33K and pVIII are encoded.

The 100K polypeptide is most likely encoded by mRNA species $L4_a$ and by analogy with Ad5 mapping studies (Kruijer *et al*, 1982), is located between coordinates 67.0 to 78.5 (Roberts *et al*, 1986). This polypeptide is made abundantly late after infection but is absent in purified virus particles. The L4-100K protein is specifically associated with hexon in infected cells (Cepko and Sharp, 1982; Gambke and Deppert, 1983) and plays an important role in hexon morphogenesis (Oosterom-Dragon and Ginsberg, 1981; Cepko and Sharp, 1983). A functional L4-100K protein is required for the efficient translation of late viral mRNAs (Hayes *et al*, 1990). The 100K polypeptide has the ability to bind RNA (Adam and Dreyfuss, 1987) and consensus sequences corresponding to each of the four subdomains of the common RNA recognition region (Adam *et al*, 1986) have been found in this protein (Hayes *et al*, 1990).

It has been proposed that mRNA species $L4_c$ (coordinates 73-78.5) encodes the non-structural 33K polypeptide (Oosterom-Dragon and Anderson, 1983). This

mRNA is unique among late mRNAs in that it contains a splice within the coding sequence (Oosterom-Dragon and Anderson, 1983). No function has been assigned to the 33K polypeptide but its intracellular location suggests that it may be a nuclear protein (Gambke and Deppert, 1981).

Polypeptide VIII, a minor component of the virion, is most likely translated from mRNA species $L4_d$ (coordinates 75-78.5). It is synthesized as a 27K precursor which is cleaved at maturity to a 13K polypeptide by the L3-23K endopeptidase (Maizel *et al*, 1968b; Anderson *et al*, 1973).

(f) Late region 5

The Ad2 L5 mRNAs are characterized by a poly(A) site at coordinate 91.3. A single polypeptide is encoded within region L5, the fibre protein (Lewis *et al*, 1977; Miller *et al*, 1980). The fibre mRNA (coordinates 86.3-91.3) differs from other late mRNAs in that one mRNA body can be connected to a number of different 5'-leader sequences (Figure 1.6). In addition to the normal tripartite leader a substantial fraction of the fibre mRNA population (approximately 30%) has been shown to be connected to the ancillary *x*, *y* and *z* leaders or the *"i"* leader in various combinations (Chow and Broker, 1978; Zain *et al*, 1979). There is an indication that *x* and *y* leaders may be important for efficient translation of fibre mRNA *in vivo* (Anderson and Klessig, 1984). The mRNA codes for ... amino acid residues that constitute a protein with a theoretical molecular weight of 61.9K. The apparent molecular weight of the fibre protein is 62K (Gingeras *et al*, 1982; Sussenbach, 1984). The fibre functions in attachment to the cellular receptor.

(vii) Virus assembly

During the late phase of adenovirus replication host cell protein synthesis is almost totally shut off (Ginsberg *et al*, 1967) and the infected cells synthesize large quantities

of viral structural proteins and viral DNA. Most of the viral polypeptides synthesized during the late phase of the productive infection are rapidly released from polyribosomes and transported to the nucleus within 3-6 minutes (Horwitz *et al*, 1969; Velicer and Ginsberg, 1970). During this short interval the monomeric structural polypeptides of the hexon (polypeptide II), the penton base (polypeptide III), and the fibre (polypeptide IV) assemble into multimeric proteins (Velicer and Ginsberg, 1970). Large pools of structural subunits are present in the nuclei of infected cells of which only a small fraction is incorporated in virus particles. It has been estimated that only 1-5% of the fibres and penton bases and 20-30% of hexon trimers are incorporated into virions (White *et al*, 1969; Everitt *et al*, 1971) and only 10–20% of viral DNA enters virus particles (Green, 1962; Green *et al*, 1970; Philipson and Lindberg, 1974).

Hexons are assembled into groups of nine (nonamers) which together form the triangular surfaces of the icosahedron. Polypeptide IX may associate with the nonamers as a cementing substance (Everitt *et al*, 1975). The vertices of the icosahedron contain polypeptides III, IIIa, and IV. A tentative diagram of the assembly pathway is shown in Figure 1.7 (Philipson, 1979). Capsomeres self-assemble into empty shell capsids in the nucleus. Viral DNA is then inserted into the empty capsids via a specific recognition sequence located within the left-hand 400 nucleotides of the virus genome (Hammarskjöld and Winberg, 1980; Hearing and Shenk, 1983).

Three polypeptides are associated with the cores, polypeptides V, VII (Maizel *et al*, 1968a; Russell *et al*, 1971; Vayda *et al*, 1983), and the μ protein (Hosakawa and Sung, 1976). The young virions that are not infectious lack polypeptide IVa_2 and the 32K and 40K scaffolding proteins. Virion maturation requires proteolytic cleavage of precursor proteins (Weber, 1976). The mature particle is then stable, infectious, and resistant to nucleases.

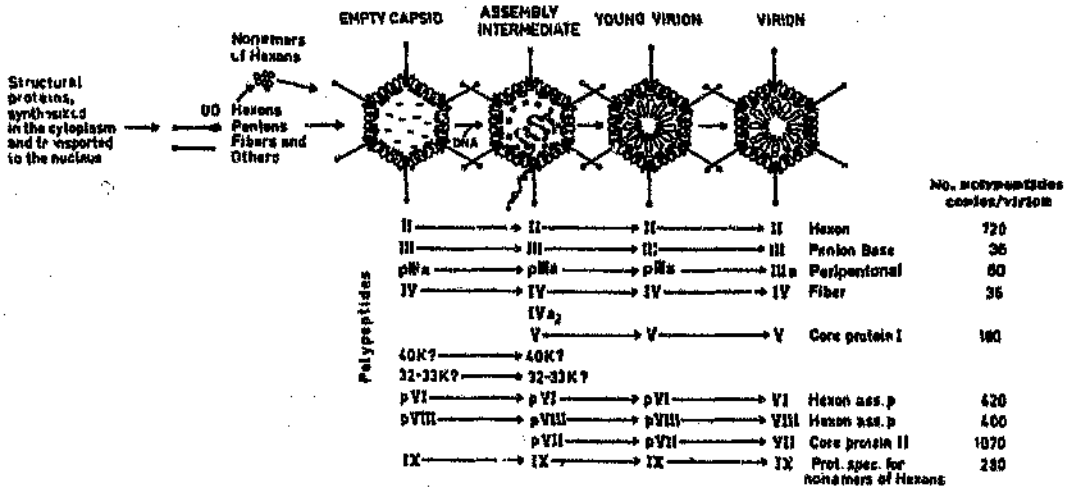


Figure 1.7 Tentative assembly pathway for adenoviruses. The structural proteins are synthesized in the cytoplasm and rapidly transported to the nucleus, probably in the form of a trimer for the main capsid units: hexon, penton base, and fibre. From nonamers of hexon, empty capsids are possibly formed by a process of self-assembly. The main assembly intermediates contain varying portions of DNA or varying amounts of core proteins, depending on the procedure used to extract the particles from the cell. The next step is the young virions, in which several polypeptides are present in a precursor form, followed by the final step involving proteolytic cleavage of at least five polypeptides in the virion structure. The four stages of virus assembly are indicated at the top and the polypetides present in each structure below; the numbers of copies of the polypeptides per virion are indicated at right below. Reproduced from Philipson (1984). Original reference : Philipson (1979).

1.2.2.3.2 Virus-cell interactions

The interaction between adenovirus and host cell may result in a permissive, semi-permissive, or non-permissive infection. Adenovirus entry into most cells of human origin leads to lytic infection, resulting in the production of infectious virus.

In permissive infections of human cells, the cytopathic effect of adenoviruses is usually characterized by marked rounding and aggregation of infected cells in grape-like clusters. Rounded intranuclear inclusions can be clearly seen in cells

infected with Ad3, Ad4, and Ad7. Crystalline arrays of virus particles can also be seen in the nucleus. In cells infected with Ad5 or Ad6, large bar-shaped eosinophilic crystals are formed. Infected cells do not lyse and less than 1% of the total virus is in the culture medium at times when the maximal viral titre is attained. Approximately 7000 particles are produced per infected cell. Most particles remain within the cell after the cycle is complete and the cell is dead (Dulbecco and Ginsberg, 1980; Jawetz *et al*, 1987).

Cellular changes may also be brought about by the accumulation of viral structural proteins. Externally applied penton causes rounding and clumping of cells and detachment. For this reason the penton has also been termed toxin or cell-detaching factor. The toxic activity resides in the penton base. The toxic action of purified fibres on cells results from a block in the synthesis of DNA, RNA, and protein and the cessation of cell division. The fibre also affects cells in such a way that they cannot support the multiplication of related or unrelated viruses (Dulbecco and Ginsberg, 1980).

The replication of human adenoviruses in most cultured cells of non-human origin is incomplete or abortive. Infection of monkey or rodent cells by Ad2 results in a semi-permissive interaction, characterized by restricted viral replication and a substantial reduction in virus production (Eron *et al*, 1975; Thomasset and Chardonnet, 1980; Paraskeva *et al*, 1982; Eggerding and Pierce, 1986). In adenovirus infected monkey cells this block can be overcome by coinfection with simian virus 40 (SV40) (Klessig, 1984). In non-permissive infections some early genes may be expressed with the restriction generally affecting the efficiency of viral DNA replication and late gene expression. Abortive infections may lead to cell transformation as a result of the expression of early gene products (Graham, 1984) (see section 1.2.2.4). Infection of hamster cells by subgroup A (Ad12) and subgroup B (Ad3) adenoviruses is non-permissive (Doerfler and Lundholm, 1970). Integration

of virus-specific DNA has been reported both in productive (Schick *et al*, 1976) and in non-productive (Doerfler, 1970) infections.

Another consequence of virus-cell interactions is the induction of interferon (IFN) in response to infection. IFNs are glycoproteins secreted by virus infected cells which promote the establishment of an antiviral state in uninfected cells. They are classified into three antigenically distinct types, α, β, and γ. α- and β-IFNs can be induced in a variety of cells by viruses, bacteria, and double stranded RNA (dsRNA). γ-IFNs are induced in lymphoid cells by mitogens and antigens to which cells have been sensitized (Stewart, 1979; Lengyel, 1982; Sen, 1984).

Human adenoviruses are known to induce IFN in non-permissive chick embryo fibroblast (CEF) cells (Tarodi *et al*, 1977; Toth *et al*, 1983). No IFN induction has been demonstrated in adenovirus infected human, monkey, mouse, and hamster cells (Toth *et al*, 1987). Adenoviruses are generally weak inducers of IFN in CEF cells. The IFN response is triggered by the adenovirus capsid or some component thereof (Toth *et al*, 1987; Reich *et al*, 1988). Reich *et al* (1988) found that although transcription of IFN-stimulated genes is induced in HeLa cells by adenovirus particles, it is suppressed by E1a gene products. The E1a 12S mRNA product was responsible for the suppressive activity.

The highly oncogenic subgroup A adenoviruses (Ad12, Ad18, Ad31), however, are potent inducers of IFN in CEF cells. Here the induction is UV-sensitive and empty particles are incapable of eliciting an IFN response. Studies with mutant and recombinant adenoviruses have shown the 13S mRNA product of Ad12 early region E1a to be sufficient for inducing IFN in CEF cells (Toth *et al*, 1987).

The antiviral effect of IFN depends on active cellular RNA and protein synthesis. After IFN binds to its specific cellular receptor, a set of cellular genes, termed IFN-stimulated genes, are transcriptionally induced. The antiviral state takes

several hours to develop and disappears within 24 to 48 hours. Among the enzymes induced by IFNs are two dsRNA-dependent ones, the 2-5A polymerase and a protein kinase (P1 kinase or dsRNA activated inhibitor (DAI)). The polymerase synthesizes 2′-5′ oligoadenylates from ATP. These oligonucleotides activate RNase L, a latent endoribonuclease, that degrades viral as well as rRNA and mRNA. The P1 kinase phosphorylates and thereby impairs the activity of the initiation factor eIF-2, thus inhibiting protein synthesis. (Reviewed in Baglioni, 1979; Lengyel, 1982; McMahon and Kerr, 1983; Sen, 1984; Staeheli, 1990).

Adenoviruses have limited susceptibility to human IFN (Gallagher and Khoobyarian, 1972; Stewart, 1979). However, adenoviruses have been shown to be susceptible to recombinant human γ-IFN but not recombinant α- and β-IFN, indicating differences in the mechanism of antiviral action (Mistchenko and Falcoff, 1987; Mistchenko *et al*, 1987; 1989). Two adenovirus products appear to be involved in virus defense against the action of IFN, VA-RNAI (Kitajewski *et al*, 1986 a; b; Munemitsu *et al*, 1986) and E1a (Anderson and Fennie, 1987; Reich *et al*, 1988).

1.2.2.3.3 Virus interactions

The coexistence of related and unrelated viruses can result in a number of interactions depending on the growth characteristics of either infecting virus. Such interactions include complementation, interference, recombination, genetic reactivation, and phenotypic mixing.

Complementation can be defined as the provision of a function specified by the genome of one virus that enables a second virus to replicate. In mixed infection an enhanced yield of one virus is often accompanied by a depressed yield of the other. This phenomenon is termed interference. One-hit kinetics applies when only one virus particle is required to infect a cell productively. It is based on the one-particle theory (Luria, 1940; Dulbecco and Vogt, 1954) which assumes a linear

relationship between relative virus concentration and infectivity count. Thus each focus of infection would be initiated by a single virus particle, not divisable by dilution. This theory has been dealt with in several early reviews (Isaacs, 1957; Dougherty, 1964) and has been established for many viruses (Cooper, 1961), including adenoviruses (Kjellen, 1961; Tytell *et al*, 1962). There are several examples of virus infectivity assays that follow two-hit rather than one-hit kinetics (Rowe, 1967). In such cases, two types of virus particle infect a single cell so that either one or both viruses may replicate. Examples include adeno-associated viruses which replicate only in cells infected by an adenovirus (Atchison *et al*, 1965; Blacklow *et al*, 1967; Richardson and Westphal, 1981) or herpesvirus (Young and Mayor, 1979), and defective PARA SV40-Ad7 hybrid particles which require complementation by Ad7 in African green monkey kidney cells (Boeye *et al*, 1966). Similar phenomena occur among Retroviridae (Hanafusa *et al*, 1963; 1964; Hartley and Rowe, 1966).

Human adenovirus infection of monkey kidney cells is abortive. Replication can be achieved by coinfection with SV40. The resultant hybrids consist of some SV40 sequences covalently linked to adenovirus DNA. Two types of hybrids have been identified, a defective adenovirus-SV40 genome in an adenovirus capsid and a nondefective adenovirus type 2 carrying a portion of the SV40 genome (Kiessig, 1984). These hybrids have proved useful for genetic analyses but are not of any known medical importance.

The expression of the SV40 large tumour (T) antigen is essential for adenovirus replication in monkey cells (Cole *et al*, 1979). Host range mutants of Ad2 (Ad2*hr*400) which replicate efficiently in monkey cells contain mutations that map to the amino-terminal portion of the 72K DNA binding protein (Klessig and Hasselt, 1978). In mixed infection with SV40, adenovirus can help expression of SV40 genes or inhibit SV40 DNA replication (Klessig 1984).

Adeno–associated viruses (AAV) are defective parvoviruses that fail to replicate in the absence of either a helper adenovirus or herpesvirus. A number of adenoviral early functions have been implicated in the helper activity, E1a, E1b, E2a, E4 (Richardson and Westphal, 1983), and VA RNAI (Janik et al, 1981). The subgroup F adenoviruses have been shown to support AAV growth, the helper effect, however, being very much reduced in comparison to Ad5 (Takiff and Straus, 1982).

There are various examples of complementation among adenoviruses of different types, including wild type and mutant viruses (Mak, 1969; Williams *et al*, 1975; Rowe and Graham, 1981; Williams *et al*, 1981; McDonough and Rekosh, 1982; McDougall and Mautner, 1987). Mutant virus studies have been widely used for the functional identification of specific gene products (Young *et al*, 1984; Williams, 1986). Dominance of one serotype over another in coinfection of simian and human cells has been reported (Delsert and D'Halluin, 1984). This was due to an E1a gene product acting as a repressor (Leite *et al*, 1986).

Complementation has also been shown between human and simian adenoviruses. Simian adenoviruses help replication of human adenoviruses in GMK cells (Altstein and Dodonova, 1968). A special case of complementation, termed nongenetic recombination, has been found to occur in cells simultaneously infected with heat-inactivated Ad1 or Ad6 and UV-irradiated Ad8 (Béládi *et al*, 1970). The mechanism of rescue of heat-inactivated Ad1 and Ad6 was not further elucidated.

Recombination occurs within a serotype and between closely related serotypes of adenovirus (Williams *et al*, 1974; 1975: Mautner and Boursnell, 1983). There is generally no recombination between members of different subgroups even though gene products have similar functions (Williams *et al*, 1975; McDonough and Rekosh, 1982). It has been found that crossover sites occur only in regions of high DNA sequence homology (Boursnell and Mautner, 1981; Mautner and Boursnell, 1983; Mautner and Mackay, 1984).

Phenotypic mixing has been reported to occur between human serotypes (Norrby and Gollmar, 1971; Williams *et al*, 1975) and between human and simian adenoviruses in simian cells (Altstein and Dodonova, 1968). This involves production of heteropolymer hexon capsomers containing polypeptides derived from viruses of two different serotypes, as well as capsids containing only capsomers of one or other parent (Norrby and Gollmar, 1977). The frequency of occurrence of mixed capsids was found to be much lower in mixed infections of serotypes belonging to different subgroups compared to serotypes within a subgroup (Norrby and Gollmar, 1977).

1.2.2.4 Animal susceptibility and transformation of cells

Human adenoviruses have been widely used as models for studying cellular transformation. The E1a and E1b regions together comprise the adenovirus transforming region (Gallimore *et al*, 1974; Graham *et al*, 1974). The E1a region alone is sufficient to immortalize primary rodent cells (Houweling *et al*, 1980). The resulting cells do not possess a fully transformed phenotype and are non-tumorigenic. Complete transformation requires the expression of E1a in cooperation with E1b (Houweling *et al*, 1980; van den Elsen *et al*, 1983). Several other oncogenes, including an activated H-*ras* oncogene isolated from T24 bladder carcinoma cells (T24 Ha-*ras*) and the polyoma middle T antigen (*pmt*), can also complement E1a by substituting for E1b in transformation assays (Ruley, 1983; Zerler *et al*, 1986). On the other hand oncogene products of members of the *myc* gene family, the polyoma large T antigen gene, or the p53 oncogene can also cooperate with the *ras* oncogene to transform cells, suggesting functional similarities with E1a (Land *et al*, 1983; Ruley, 1983; Eliyahu *et al*, 1984; Yancopoulos *et al*, 1985).

The transforming activity of the 289R and 243R E1a proteins, in cooperation with E1b or an activated *ras* gene, has been mapped to conserved regions CR1 and CR2 (Zerler *et al*, 1986; Moran and Mathews, 1987; Velcich and Ziff, 1988; Whyte *et al*, 1988a) (see Figure 1.4). The E1a oncoproteins have been shown to associate

with host cell proteins, p300 and p107, which may play a role in E1a-*ras* transformation (Whyte *et al*, 1988b; 1989). The E1a proteins have also been found to be physically associated with the p105-*RB* (retinoblastoma susceptibility gene) anti-oncogene product (Whyte *et al*, 1988a). The regions of E1a proteins required for binding to these proteins have been mapped (Whyte *et al*, 1989) (Figure 1.4).

The 55K and 19K products of the E1b region are both required for transformation in cooperation with E1a (Senear and Lewis, 1986; Barker and Berk, 1987). In Ad5-transformed cells the E1b-55K protein forms a complex with the cellular anti-oncogene product p53 (Sarnow *et al*, 1982). This, however, does not occur with the Ad12 E1b-55K protein. The 19K protein is essential for transformation by adenoviruses or plasmid DNAs but does not appear to be required for transformation or viral oncogenicity of Ad12 (Edbauer *et al*, 1988). It has been suggested that the anchorage-independent growth of adenovirus-transformed cells may be as a result of disruption of the vimentin-containing intermediate filament network induced by the 19K protein (White and Cipriani, 1989; 1990).

Not all adenovirus serotypes can induce tumours. They have been classed according to oncogenic potential as either nononcogenic (subgroup C), weakly oncogenic (subgroup B), or highly oncogenic (subgroup A) (Huebner, 1967; Trentin *et al*, 1968; Green, 1970). The increased tumorigenicity of Ad12 transformants in syngeneic rats is associated with very low levels of major class I histocompatibility antigens and the mRNAs encoding them (Bernards *et al*, 1983; Schrier *et al*, 1983). It has been proposed that cytotoxic T lymphocytes (CTLs) may reject Ad2- and Ad5-transformed cells and that Ad12-transformed cells escape immune surveillance by CTLs (Bernards *et al*, 1983). It has however been found that cell lines transformed by Ad2, though expressing a high level of class I MHC antigen, are tumorigenic for immunocompetent animals (Haddada *et al*, 1986; 1988).

The transforming capacity of Ad40 and Ad41 has been studied by transfection of primary baby rat kidney (BRK) cells with plasmids containing the left terminal regions of their genomes (van Loon *et al*, 1985a). The DNA regions of both viruses were able to transform BRK cells. Upon injection into nude athymic mice these cells did not cause tumours. Takiff and Straus (1982) have reported that Ad40 and Ad41 virions are not able to transform primary hamster cells *in vitro*. Adenovirus 40 and Ad41 are also nononcogenic in baby hamsters (Wadell *et al*, 1980; de Jong *et al*, 1983). This property makes them comparable to the subgroup C adenoviruses which do not induce tumours in rodents.

Cousin *et al* (1991) found the ability of Ad40 and Ad41 E1a plus E1b genes to transform BRK cells to be considerably lower than that of the corresponding genes of Ad5 and Ad12. The E1a genes of Ad40 and Ad41 could cooperate with an activated *ras* oncogene for full transformation. Only the Ad41 E1b gene could be complemented by the E1a gene of Ad5 or Ad12 for cell transformation, reflecting a difference in the cooperation of Ad40 and Ad41 with Ad5 E1a products. The conserved region 1 of Ad41 E1a was found to be responsible for inefficient transformation.

MHC class I antigen expression on subgroup F adenovirus-transformed cells was similar to the low level of Ad12-transformed cells (Cousin *et al*, 1991). The Ad40- and Ad41-transformed cells, however, did not appear to be tumorigenic in syngeneic rats.

1.2.2.5 Adenovirus infections in humans

1.2.2.5.1 Pathogenesis, clinical findings and epidemiology

Adenoviruses infect epithelial cells of mucous membranes, the cornea, and other organ systems (Jawetz *et al*, 1987). They can be isolated during acute illness and may persist for long periods. Types 1,2,5, and 6 can be isolated in explants from surgically removed adenoids or tonsils of most children by growing the epithelium *in vitro*.

Most human adenoviruses grow in intestinal epithelium after ingestion but usually do not produce symptoms or lesions (Jawetz *et al*, 1987). Shedding of low-numbered serotypes by children can persist for many months (Kidd *et al*, 1982), which renders any pathogenic role difficult to prove, and unlikely. However, at the acute stage of gastrointestinal disease, subgroup F adenoviruses are excreted in large quantities of up to 10^{11} particles per gram of stool (Gary *et al*, 1979; Retter *et al*, 1979). This suggests active multiplication in the gastrointestinal tract. Whitelaw *et al* (1977) observed crystalline arrays of virus particles in the small intestinal mucosa in a fatal case of adenovirus gastroenteritis. The adenovirus was later typed as Ad41 (Kidd, 1984). Petric *et al* (1982) looked at the possible enteric adenovirus multiplication in the respiratory tract. No evidence for this could be demonstrated despite respiratory symptoms. A subgroup F adenovirus has however been isolated from a respiratory specimen from a young child with gastroenteritis (Jeffries *et al*, 1988).

(i) Respiratory diseases and other syndromes

The description of clinical illnesses associated with human adenoviruses has been reviewed by Straus (1984). The epidemiological features of these syndromes are dealt with in the same review.

Respiratory diseases associated with adenoviruses include the following: undifferentiated acute respiratory disease, pharyngoconjunctival fever, nonstreptococcal exudative pharyngitis, pneumonia, and pertussis syndrome (Jawetz *et al*, 1987).

About 5% of acute respiratory disease (ARD) in young children is due to adenoviruses. Adenovirus types 1,2,5, and 6 are most commonly involved. Acute respiratory disease of military recruits has been caused mainly by types 4 and 7 under conditions of fatigue and crowding (Dudding *et al*, 1973). Adenoviruses are associated with only 0.3% to 3% of respiratory illnesses in civilian adult populations (Grayston *et al*, 1958; Evans, 1967).

Adenovirus types 3 and 7 frequently cause pharyngoconjunctival fever outbreaks in which conjunctivitis is a predominant symtom (Bell *et al*, 1955; Buchta, 1974). Similar sporadic cases involve types 1,2,5,6,37 and many others (Jawetz *et al*, 1987).

Eye infections caused by adenoviruses include milder forms of ocular involvement such as "swimming pool conjunctivitis" (Fukumi *et al*, 1958) and self-limited follicular conjunctivitis (Bell *et al*, 1960) and the more serious epidemic keratoconjunctivitis also known as "shipyard eye" (Huebner, 1959). The milder forms are caused by types 3,7 and many others, with types 8,19, and 37 implicated in the more serious syndrome.

Other conditions associated with adenoviruses include acute haemorrhagic cystitis, cervicitis, urethritis, aseptic meningitis, meningoencephalitis, encephalitis, and myocarditis (White and Fenner, 1986). Adenoviruses have been reported to be serious pathogens in immunocompromised individuals and specific serotypes of adenovirus, types 43 to 47, have to date only been isolated from patients with acquired immunodeficiency syndrome (AIDS) (hierholzer *et al*, 1988a).

(ii) Gastrointestinal disease

Adenovirus types 40 and 41 are aetiologically associated with infantile gastroenteritis (Gary *et al*, 1979; Retter *et al*, 1979; Yolken *et al*, 1982; Uhnoo *et al*, 1984; Kidd *et al*, 1986; Madeley, 1986; Tiemessen *et al*, 1989; Kotloff *et al*, 1989; Cruz *et al*, 1990; Kim *et al*, 1990; Lew *et al*, 1991). Another emerging diarrhoea-causing agent is the subgroup A adenovirus, Ad31 (Adrian *et al*, 1987). Schmidt *et al* (1983) analysed WHO reports on adenovirus isloations over a 10-year period (1967-1976) and found the subgroup A adenoviruses to be strongly associated with the intestinal tract and with gastroenteritis. The three members of this subgroup (Ad12, Ad18, Ad31) were, however, not analysed individually. Other gastrointestinal disorders reported to be associated with adenoviruses include mesenteric adenitis, intussusception, and appendicitis (White and Fenner, 1986).

Seroepidemiological studies have shown that the subgroup F adenoviruses are widespread in both developed and developing countries. Kidd *et al* (1983) found neutralizing antibodies to the subgroup F adenoviruses in more than one-third of the sera analysed from the U.K., New Zealand, Hong Kong and Gambia. The proportion of sera positive for neutralizing antibodies increased with increasing age. This was also shown in a study of serum samples obtained from individuals of various ages in the Tokyo area (Shinozaki *et al*, 1987). There was, however, a low seropositivity of serum samples from people aged over 70 years. Whether this is due to reduced exposure by this group to these viruses or a decrease in pre-existing subgroup F adenovirus antibodies remains to be established. Seven of 100 cord sera obtained from Kuwait had subgroup F specific antibodies (Kidd *et al*, 1983). Of serum samples from newborns not older than two weeks, 15% contained subgroup F antibodies (Shinozaki *et al*, 1987). These findings demonstrated the existence of passively transferred neutralizing antibodies to Ad40 and Ad41. It remains to be shown if these antibodies can confer protection during infancy.

The subgroup F adenoviruses have been associated with two to 22% of cases of endemic pediatric diarrhoea in studies from Asia, Africa, Europe, North and South America (Uhnoo *et al*, 1984; Leite *et al*, 1985; Kidd et al, 1986; Hermann *et al*, 1988; Kotloff *et al*, 1989; Tiemessen *et al*, 1989; Cruz *et al*, 1990; Kim *et al*, 1990). Most infections caused by these viruses occur in children aged two years and under. Up to 2% of children without diarrhoea were shown to be asymptomatically infected (Uhnoo *et al*, 1984; Kotloff *et al*, 1989; Kim *et al*, 1990). These viruses have also been associated with a number of outbreaks (Flewett *et al*, 1975; Whitelaw *et al*, 1977; Richmond *et al*, 1979; Chiba *et al*, 1983) and are presumably spread by the faecal-oral route.

Diarrhoea can be associated with symptoms such as fever, vomiting or respiratory symptoms. In one study 21% of children presented with upper respiratory symptoms of tonsillitis, pharyngitis, otitis, coryza and cough (Uhnoo *et al*, 1984). Respiratory involvement has also been reported in other studies (Flewett *et al*, 1975; Yolken *et al*, 1982; Chiba *et al*, 1983).

The incubation period is approximately 7-10 days, with the duration of diarrhoea one to two weeks (Richmond et al, 1979; Uhnoo *et al*, 1984). Prolonged diarrhoea has been associated with Ad41 infection (Uhnoo *et al*, 1984). Fatal cases of enteric adenovirus gastroenteritis have been reported by Whitelaw *et al* (1977) and Retter *et al* (1979).

Endemic gastroenteritis associated with Ad40 and Ad41 occurs throughout the year with no marked seasonal variation in most localities studied (Uhnoo *et al*, 1984; Brandt *et al*, 1985; Hermann *et al*, 1988; Kim *et al*, 1990). In South Africa, however, subgroup F adenoviruses occurred more frequently in the summer months (Kidd *et al*, 1986; Tiemessen *et al*, 1989) when bacterial infections are most common (Freiman *et al*, 1977). This was in contrast to the seasonal occurrence of rotavirus which peaks in the winter months (Steele *et al*, 1986; Tiemessen *et al*, 1989).

1.2.2.5.2 Laboratory diagnosis

Established adenoviruses are isolated by inoculation of permissive human cell lines with clinical specimens. Continuous cell lines such as Hep-2, HEK and HeLa cells are commonly used. Typical cytopathic effects occur within two to 20 days after inoculation. Clinical specimens include throat, conjunctival, and rectal swabs, stool and urine specimens. Isolates can be identified as adenoviruses using fluorescent antibody to detect group-specific antigens. Serotypes can be further identified using neutralization and haemagglutination inhibition tests (Jawetz *et al*, 1987). Other methods of detection include immune electron microscopy (Luton, 1973; Leite *et al*, 1985), radioimmunoassays (Scott *et al*, 1975), counterimmunoelectrophoresis (Hierholzer and Barme, 1974), and enzyme-linked immunosorbent assays (Harmon *et al*, 1979; Mortensson-Egnund and Kjeldsberg, 1986).

Adenovirus 40 and Ad41 have been detected by presumptive methods which are based on the presence of large numbers of adenovirus particles in stool as detected by EM and the inability to culture the adenovirus in cell types that support the growth of other adenoviruses (Brandt *et al*, 1984). Growth of the fastidious agent in, for example, 293 cells then provides further confirmation as to the presence of a subgroup F adenovirus.

More definitive techniques for their identification include those which detect subgroup- or type-specific DNA sequences or viral antigens. Techniques such as DNA restriction endonuclease analysis (Brown *et al*, 1984; Kidd, 1984; Kidd *et al*, 1984; Brandt *et al*, 1985; Buitenwerf *et al*, 1985; van der Avoort *et al*, 1989), DNA hybridization (Chiba *et al*, 1983; Allard *et al*, 1985; Kidd *et al*, 1985; Stålhandske *et al*, 1985; Takiff *et al*, 1985; Niel *et al*, 1986), the Polymerase Chain Reaction (Allard *et al*, 1990), ELISA (Johansson *et al*, 1980, 1985; Anderson *et al*, 1983; Uhnoo *et al*, 1984; Herrmann *et al*, 1987; Singh-Naz *et al*, 1988), immune electron microscopy (Leite *et al*, 1985; Wood and Bailey, 1987), counter immunoelectro-

osmophoresis (Jacobsson *et al*, 1979), indirect immunoflourescence (Witt and Bousquet, 1988), and four layer radioimmunoassay (Halonen *et al*, 1980; Vesikari *et al*, 1981) can be used to determine the presence of subgroup F adenoviruses. A presumptive diagnosis of a subgroup F adenovirus in stool should be confirmed by further characterization as to the subgroup or type using a more definitive method.

1.2.3 Growth characteristics of the subgroup F adenoviruses

The subgroup F adenoviruses cannot be grown in most cell culture systems that support the growth of adenoviruses from subgroups A to E, namely, KB, HeLa, human amnion, and human embryo kidney cells. Adenovirus 40 and Ad41 have been propagated in Chang conjunctival cells (Kidd and Madeley, 1981), Hep-2 and tertiary cynomolgus monkey kidney cells (de Jong *et al*, 1983), HT-29 cells (Uhnoo *et al*, 1983), and 293 cells (Brown *et al*, 1984; Brown, 1985; Takiff *et al*, 1981). 293 cells contain integrated early region Ad5 DNA (Graham *et al*, 1977) and have been particularly useful for the growth of Ad41 (van Loon *et al*, 1985b) rather than Ad40 (Chiba *et al*, 1983; Uhnoo *et al*, 1983; 1984; Brown, 1985; van Loon *et al*, 1985b).

Differences in the susceptibility to virus growth in different batches of the same cell line, variations in the growth of different strains, and differences in the growth of identical strains between laboratories has been reported (de Jong *et al*, 1983). Adenovirus 41 has been shown to grow in a variety of primary cell lines under culture conditions employing low serum concentrations of 0.2 to 1% (Pieniazek *et al*, 1990b). It was suggested that Ad41 replication in primary cells is inhibited due to some unknown serum factor or factors.

Recently, a rapid loss of Ad41 infectivity with passage in 293 cells has been demonstrated (Pieniazek *et al*, 1990a). The core protein V was found to be lacking in complete virus particles, due possibly to a defect in virus assembly. In contrast, HEp-2 cells allowed the continuous propagation of Ad41 (Pieniazek *et al*, 1990a).

There has however been a report where not all initial isolates of Ad41 were able to grow in these cells (Perron-Henry *et al*, 1988).

Brown (1985) has shown that while the production of Ad40 virions is 3- to 10-fold lower than that observed for other adenoviruses, the yield of infectious virus is 100- to 1000-fold less. Witt and Bousquet (1988) have demonstrated low levels of infectious virions and hexon protein produced in cells infected with Ad40 when compared to Ad41.

Babiss *et al* (1983) stably transformed KB cells with a recombinant plasmid containing the prokaryotic gene, xanthine-guanine phosphoribosyl transferase (XGPRT), and the Ad2 *XhoIC* fragment. KB cell lines that constitutively expressed E1a only, E1b only, both E1a and E1b, and neither E1a or E1b were described. Mautner *et al* (1989) could only cultivate Ad40 in the E1b-containing cell lines and not in cells expressing E1a alone. Furthermore, Ad40 was found to complement an Ad5 E1a mutant (Ad5 *dl*312) but not one with a defect in region E1b (Ad5 *dl*313). Complementation of Ad40 was found only with Ad5 *dl*312. A mutant virus defective for the E1b-19K function could complement Ad40 growth, whereas an E1b-55K mutant could not.

The expression of Ad40 E1b mRNAs in permissive cells is only detected after the onset of DNA synthesis and no accumulation of these mRNAs occurs in the presence of a drug that blocks DNA synthesis (Mautner *et al*, 1990). A 55K product could not be detected in these cells and there was no host protein shutoff. The E1b-55K protein and E4-34K protein (ORF 6) are known to form a complex and function in the shut off of host protein synthesis and transport of late viral mRNAs from the nucleus to the cytoplasm. Mautner and Mackay (1991) tested the functions of the E4-34K and the E4-11K ORF 3 products of Ad40 using E4 mutants and found that they do not play a role in growth restriction.

It has recently been confirmed that an E1b function in the KB18 cell line complements Ad40 growth (Hashimoto *et al*, 1991). These authors have further demonstrated efficient growth and plaque production of Ad40 and Ad41 using a human lung carcinoma cell line, A549 cells. Adenovirus 40-specific E1a and E1b mRNAs have been detected before the onset of DNA synthesis in A549 cells (Hashimoto *et al*, 1991), unlike immediate early gene expression in KB and KB18 cells (Mautner *et al*, 1990; Hashimoto *et al*, 1991). A human hepatocellular carcinoma cell line (PLC/PRF/5) has recently been shown to be highly permissive for Ad40 and Ad41 growth (Grabow *et al*, 1992).

Transformed Rhesus monkey kidney cells that express Ad5 and Ad3 E1a proteins, and an Ad5-transformed cell line expressing the E1b-19K protein in addition to the E1a proteins, support the growth of Ad40 but not of Ad41 (Nascimento *et al*, 1990).

1.2.4 Molecular characterization of the subgroup F adenoviruses

Physical maps of Ad40 (Takiff *et al*, 1984; Allard *et al*, 1985; van der Avoort *et al*, 1989) and Ad41 (Allard *et al*, 1985; van der Avoort *et al*, 1989) genomic DNA have been constructed. Restriction enzyme sites for Ad40 were mapped using *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Sma*I, and *Xho*I (Takiff *et al*, 1984; van der Avoort *et al*, 1989). Maps for *Eco*RI, *Sal*I, *Cla*I, *Bcl*I, *Bst*EII, and *Pvu*I digestion of Ad40 DNA were constructed by van Loon *et al* (1985b). Restriction enzyme sites for Ad41 were mapped using *Bam*HI (Takiff *et al*, 1984; Allard *et al*, 1985; van der Avoort *et al*, 1989) *Eco*RI (Takiff *et al*, 1984; Allard *et al*, 1985; van Loon *et al*, 1985b; van der Avoort *et al*, 1989), *Hpa*I, *Nru*I, *Pvu*I, *Sal*I (Allard *et al*, 1985), *Hind*III, *Kpn*I, *Sma*I (Takiff *et al*, 1984; van der Avoort *et al*, 1989), *Xho*I (Takiff *et al*, 1984; van Loon *et al*, 1985b; van der Avoort *et al*, 1989), *Cla*I, *Sal*I (van Loon *et al*, 1985b) and *Pst*I (van der Avoort *et al*, 1989). The total length of the DNA was estimated to be 34.0kbp for Ad40 and 34.7kbp for Ad41 (van Loon *et al*, 1985b). An average

estimated length of 35kbp was obtained for both Ad40 and Ad41 DNA by van der Avoort *et al*, (1989).

DNA homology studies have shown that, overall, Ad40 and Ad41 are 62-69% homologous to each other. The overall homology between DNA from both types and Ad5 DNA is 15-22% (see Table 1.1 for comparitive homologies within and between adenovirus subgroups). The sites of least homology were the left terminal 8% and the right terminal 27% of the genome (van Loon *et al*, 1985b), approximate map unit coordinates 2.9 to 11.3 and 75 to 100 (Takiff *et al*, 1984).

Restriction enzyme analysis has revealed the existence of a number of DNA variants of types 40 and 41 (Kidd, 1984; Kidd *et al*, 1984; van der Avoort *et al*, 1989). With the use of 9 restriction enzymes for Ad40 and 10 for Ad41, van der Avoort *et al* (1989) have grouped 48 strains of Ad40 and 128 strains of Ad41 according to genetic relatedness. Eleven Ad40 DNA variants (D1-D11) and 24 Ad41 DNA variants (D1-D24) have been described.

A number of Ad40 and Ad41 early and late genes and portions of genes have been characterized (van Loon *et al*, 1987b; Allard and Wadell, 1988; Ishino *et al*, 1988; Toogood and Hay, 1988; Vos *et al*, 1988; Kidd and Erasmus, 1989; Pieniazek *et al*, 1989a; b; Toogood *et al*, 1989; Luftig *et al*, 1990; Pieniazek *et al*, 1990). In addition the nucleotide sequence of the Ad40 inverted terminal repeat (ITR) has been determined (Ishino *et al*, 1987).

There are two distinct regions of the adenovirus ITR sequences, region I and region II. Region I is highly conserved among human adenoviruses and contains the binding sites for pTP and NFI (Guggenheimer *et al*, 1984). Region II has a much lower A+T content than region I and tends to have more divergent sequences among human adenoviruses. The ITR regions of Ad40 and Ad5 show extensive homology and are more closely related than any other pairwise comparisons including Ad4,

Ad7, and Ad12 (Ishino *et al*, 1987). The ITR region of Ad40 (Ishino *et al*, 1987) and Ad41 (Allard and Wadell, 1988) is 163 nucleotides in length compared to 103 nucleotides for Ad2 and Ad5 (van Ormondt and Galibert, 1984).

Some conserved binding sites for viral and cellular factors involved in viral DNA replication, which are found in all adenovirus types investigated (Sussenbach, 1984; Kelly, 1984; Pruyn *et al*, 1986) are also present in Ad40 and Ad41 DNA (van Loon *et al*, 1987b).

The E1a region of Ad41 (van Loon *et al*, 1987b; Allard and Wadell, 1988) and the E1a and E1b region of Ad40 (van Loon *et al*, 1987b; Ishino *et al*, 1988) have been sequenced and compared with known adenoviral sequences. The overall organization of these gene regions appears similar to that of other adenoviruses. Some differences in possible strategic sequences were however noted. A 30bp region between two enhancers in Ad5 has only 60% homology with the corresponding region of Ad40 and Ad41 (van Loon *et al*, 1987b). This region in Ad5 (Hearing and Shenk, 1986) but not in Ad3 (Robinson and Tibbetts, 1984) is essential for the transcription of other early genes. An encapsidation signal of about 30 nucleotides (consensus sequence TATTTR$(N)_3$RG$(N)_4$RNYTYTGA; Brinkman *et al*, 1983) is not present in Ad40 and Ad41 (van Loon *et al*, 1987b). Adenovirus 40 and Ad41 lack an enhancer (Hen *et al*, 1983) in a region corresponding to a stretch of 33bp between 102 and 135bp upstream from the cap site in the Ad12 E1b region (van Loon *et al*, 1987b). In Ad12 this enhancer region, starting at position 1411 (van Ormondt and Hesper, 1983), was shown to be essential for transcription of E1b under control of the E1a gene product (Bos and Ten Wolde-Kraamwinkel, 1983). It has been suggested by van Loon *et al* (1987b) that the above differences do not explain the fastidious growth of the subgroup F adenoviruses.

The Ad40 E1a gene region codes for 221R and 249R proteins (Ishino *et al*, 1988). In Ad41, putative proteins of 222R and 251R predicted to be coded for by the

12S and 13S Ad41 E1a mRNA species, respectively (Allard and Wadell, 1988). The Ad40 E1b gene region is deduced to code for protein products of 166R and 475R (Ishino *et al*, 1988). No significant differences were found between E1b proteins of Ad41 and Ad5 (van Loon *et al*, 1987b). The homology between E1b polypeptides of Ad40 (Ishino *et al*, 1988; van Loon *et al*, 1987b) and Ad41 (van Loon *et al*, 1987b) and Ad5 is higher than that of the E1a proteins, namely 45%.

Allard and Wadell (1988) compared the conserved sequences of the predicted Ad41 13S mRNA product (251R) with the corresponding sequences of Ad4, Ad5, Ad7, and Ad12. Amino acid homology was high in CR2 with one residue in the Ad41 sequence different out of 8. The amino acid conservation of CR1 and CR3 was less, with 10 of 16 conserved residues and 18 of 23 conserved residues shared by Ad41, respectively. The conserved regions of the Ad40 and Ad41 large E1a proteins was only 51% and 45% homologous to the corresponding Ad5 regions, respectively (van Loon *et al*, 1987b). Conserved region 1 of Ad40 was found to be only 37% homologous to the corresponding region of Ad5 (Ad7 and Ad12 are 63% and 54% homologous to Ad5, respectively) (Ishino *et al*, 1988).

A reduced transactivating function of Ad40 (Ishino *et al*, 1988; van Loon *et al*, 1987a) and Ad41 (van Loon *et al*, 1987a) E1a products has been shown in comparison with the E1a products of other serotypes using chloramphenicol acetyltransferase (CAT) assays. In addition, the Ad40 E1a promoter has little *cis*-acting activity in rat 3Y1 cells, as determined by the level of CAT expression (Ishino *et al*, 1988).

An ORF within the E1b transcription unit corresponding to the structural protein IX found in other adenoviruses (see section 1.2.2.3.1 (iv)) was also found in Ad40 and Ad41 (van Loon *et al*, 1987b). Unlike other adenoviruses, this ORF starts in the intron of the larger mRNA. The Ad40 and Ad41 IX proteins share 53% homology with the IX protein of Ad5. Part of the ORF that encodes the intermediate

gene protein IVa_2 was also sequenced (van Loon *et al*, 1987b). The C-terminal domains of the Ad40 and Ad41 IVa_2 proteins were 74% homologous to the Ad5 and Ad7 protein.

The gene encoding the Ad40 and Ad41 DBP and the 23K protease have been characterized (Vos *et al*, 1988). From the nucleotide sequences it was determined that the DBPs were the smallest DBPs characterized so far. The Ad40 DBP has 473 amino acid residues and Ad41 has one additional residue as opposed to the 529R protein of Ad5. Their small size is due to the small amino-terminal domains of 119R for Ad40 and 120R for Ad41. The corresponding Ad5 domain has 173 amino acid residues. The carboxy-terminal domains of DBPs have a number of highly conserved regions (Kruijer *et al*, 1983; Quinn and Kitchingman, 1984; Kitchingman, 1985) and four strongly conserved domains have been identified in the DBP of Ad40 and Ad41 (Vos *et al*, 1988).

The 23K proteases of both Ad40 and Ad41 show a strong homology to the Ad2 and Ad5 proteins (Vos *et al*, 1988). A portion of the hexon and 100K protein were also sequenced (Vos *et al*, 1988). The N-terminal part of the 100K proteins of Ad40 and Ad41 show some deletions when compared to Ad2 and Ad5. Recently Slemenda *et al* (1990) sequenced the genes for the 100K and 33K proteins encoded by the L4 transcription unit. A 777R ORF coding for the 100K protein could be identified. It was predicted from donor and acceptor splice sequences that an intron of 178 nucleotides could be removed from 33K protein gene transcripts (Slemenda *et al*, 1990), as has been found for Ad2 (Oosterom-Dragon and Anderson, 1983).

The Ad41 hexon-associated precursor, pVIII (Pieniazek *et al*, 1989b), has 80% amino acid homology with the Ad2 pVIII (Hérissé *et al*, 1980). As for Ad2, Ad3, and Ad5 (Cladaras and Wold, 1985; Signäs *et al*, 1986), the promoter of the Ad41 E3 region is located within the pVIII gene (Pieniazek *et al*, 1989b). However, no ORF was located downstream from the pVIII gene that could code for the E3-12.4K protein (Pieniazek *et al*, 1989b).

The genes encoding the hexon polypeptides of Ad40 and Ad41 have been sequenced (Toogood and Hay, 1988; Toogood *et al*, 1989). The Ad40 and Ad41 polypeptides share 88% homology in amino acid composition. Differences in sequence occur in two main regions and these differences are consistent with those between Ad2 and Ad5 (subgroup C). These areas of the trimeric protein may therefore represent type-specific determinants (Toogood *et al*, 1989). The variable regions of the hexon are located in the l_1, l_2, and l_4 loops (predicted from X-ray analysis of Ad2 hexons) that form the surface of the virion. A highly acidic region of 32 amino acids, present in the l_1 loop of Ad2 and Ad5, is absent in Ad40 and Ad41 (Toogood *et al*, 1989).

The genes coding for the Ad40 (Kidd and Erasmus, 1989) and Ad41 (Pieniazek *et al*, 1989a; 1990; Kidd *et al*, 1990) fibre polypeptide have been characterized. There is a high degree of homology between the 59K Ad40 and 60.5K Ad41 fibre proteins, except in the shaft region (Kidd and Erasmus, 1989; Pieniazek *et al*, 1989a). The shaft domain of the Ad41 fibre has 22 15-amino acid repeat motifs (Pieniazek *et al*, 1989a; Kidd *et al*, 1990) and Ad40 has 21 (Kidd and Erasmus, 1989). A study of various Ad40 and Ad41 DNA variants has indicated that the 15-amino acid difference in polypeptide length, due to an additional repeat residue in Ad41, is a type specific difference among the subgroup F adenoviruses, with the exception of two uncommon isolates of Ad41 which had a block deletion different to that of Ad40 (Kidd *et al*, 1990).

An additional ORF of 387 amino acids upstream from the Ad41 fibre gene, coding for a 41.4K protein, has been located whose general organization shows a high degree of similarity to other known fibre proteins (Pieniazek *et al*, 1990). A typical shaft domain of 12 15-residue repeat motifs has been found. The knob domain is about 15% shorter than for Ad2, Ad5, and Ad41. The possible existence of two fibres of different lengths in Ad41 resembles that found in avian adenoviruses (Laver *et al*, 1971).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cells

293 cells (human embryonic kidney cells transformed with Ad5 E1 region DNA) and Chang conjunctival cells were obtained from the American Type Culture Collection (ATCC No. CRL 1573 and CCL 20.2, respectively). A semi-continuous line of human embryo fibroblasts (HEF cells) was derived from primary cultures of foetal lung tissue. The following cells were all obtained from ATCC: HeLa (human epitheloid carcinoma, No. CCL 2), CV-1 (African green monkey kidney, No. CCL 70), vero (African green monkey kidney, No CCL 81), BHK-21 (baby Syrian/Golden hamster kidney, CRL 6282 CCL 70), MDCK (Madin-Darby canine kidney, No. CCL 34), MDBK (Madin-Darby bovine kidney, No. CRL 6071), B95-8 (EBV-transformed marmoset leukocytes, No CRL 1612) and RK13 (rabbit kidney, No. CCL 37) cells. Peer cells (lymphoblastoid cell line) were obtained from Dr. Z. Zakay-Rones, Jerusalem, Israel. Mouse embryonic fibroblasts (Clone 1 NIH-3T3 cells) were from the National Institute of Health, Maryland.

Cells were grown as monolayers in 150cm^2 flasks in Eagle's MEM (EMEM) containing 10% FCS. At confluency they were reseeded either into 63.61cm^2 (9cm dishes), 25cm^2 flasks, 24 well dishes or onto 22 x 22mm sterile glass coverslips in 9.6cm^2 dishes. Cells in dishes were grown in Leibovitz' L15 medium with 10% FCS

for 24 hours before infection. After inoculation, monolayers were maintained in L15 with 2% FCS.

For IFN assay, vero cells were grown as monolayers in $75cm^2$ flasks in EMEM with 10% FCS. When confluent, 100μl of cells (4 x 10^4 cells/ml) was added to the wells of a microplate. Cells were incubated at 37°C until confluent (24 hours) before the addition of samples to be assayed for IFN activity.

Cultures of intestinal segments from foetuses 8-15 weeks old were prepared and maintained as described by Kidd and Madeley (1981), after Dolin *et al* (1970). After removal of the intestine from the foetus followed by washing in phosphate buffered saline (PBS) with antibiotics, segments 2mm in length were cut with fine scissors. These tube-like segments were then cut once to yield square pieces of intestine. The pieces were placed villus-side up on cross-hatched scratches made in 40mm dishes with a scalpel blade. Four segments were placed in each dish with 1.5ml L15 containing 0.2% w/v BSA. Cultures were infected with virus 24 hours after preparation and the medium replaced every two days.

2.2 Virus

Prototype Ad2, strain adenoid 6, originally obtained from the National Institutes of Health, Maryland (NIAID 202), was passaged twice in human embryo kidney cells and twice in Chang cells. Adenovirus 40 strain Hovi-X, an isolate from Finland (Kidd *et al*, 1984), was passaged 5 times in tertiary cynomolgus monkey kidney cells and three times in Chang cells. Adenovirus 41 strain 26341-77, a Glasgow isolate (Kidd, 1984; Kidd *et al*, 1983), was passaged four times in Chang cells and three times in 293 cells. These were used as stock virus preparations for all infections. For Ad40 two stock preparations were used, one which had a titre of one log less than the other (used in IFN studies).

Adenovirus 40 strains Hovi-X and Dugan, representing genomic variants D1 and D4 respectively (van der Avoort *et al*, 1989), and Ad41 strains Tak and 26341-77, representing genomic variants D1 and D2 respectively (van der Avoort *et al*, 1989), have been described previously (de Jong *et al*, 1983; Kidd *et al*, 1984).

Prototype strains of other adenovirus serotypes, representing subgroups A to E, were supplied by Dr.R.Wigand, Homburg, FDR.

Sindbis virus, originally from Dr.J.Desmyter (Leuven, Belgium), was passaged in MK and HEF cells prior to receipt (Lyons *et al*, 1982).

2.3 Viral DNAs

DNA from Ad40 variants D2, D5, D9 and D11, and from Ad41 variants D6, D10, D12, D13, D14, D15, D16, D18, D22 and D24 (van der Avoort *et al*, 1989) was kindly donated by Dr.H.van der Avoort, Eilthoven, The Netherlands.

2.4 Plasmids

The 6.1 kilobase pair (kbp) *Eco*RI fragment *B* of Ad41, and the 6.1kbp *Eco*RI fragment *C* of Ad40 (both spanning map units (m.u.) 74 to 92) were cloned in pSP64 or pSP65 (Kidd and Erasmus, 1989; Kidd *et al*, 1990). Similarly, a 700bp fragment of Ad40, spanning m.u. 87.5 to 89.5 which includes the target sequence for PCR within the fibre gene, was cloned in *Sma*I cut pSP64 (Kidd, unpublished).

Inserts N26 (Ad40 *Pst*I fragment *H*, 1.8 kbp) and M9 (Ad41 *Pst*I fragment *B*, 6.5kbp) in pBR322 have been described previously (Kidd et al, 1985).

Plasmids containing the left terminal 11% of the Ad40 genome (pAd40-*ClaIB*) and the left terminal 12% of the Ad41 genome (pAd41-*ClaID*) were kindly provided by Dr.J.S.Sussenbach, Utrecht, the Netherlands (van Loon *et al*, 1985a).

Plasmids pUC18-*Bam*HI*F* and pML2-*Bam*HI*D* containing the E1a and E1b regions respectively, constructed using DNA from the Sapporo strain of Ad40 (Ishino *et al*, 1988), were obtained from Dr.K.Fujinaga, Sapporo, Japan.

2.5 Primers

Oligodeoxyribonucleotides of defined sequence were synthesized by the Biochemistry Department, University of Cape Town using a Beckman DNA synthesizer.

2.6 Stool Specimens

Specimens were prepared as 10% v/v suspensions in phosphate buffered saline (PBS) and clarified at 3000rpm (Sorvall rotor H1000B) at 4°C for 30 minutes.

2.7 Bacteria

The following bacterial reference cultures were supplied by the Anaerobic Unit of the South African Institute for Medical Research, Johannesburg: *Fusobacterium mortiferum, Fusobacterium symbiosum, Clostridium bifermentans, Clostridium malenominatum, Clostridium perfringens, Bacteroides distasonis, Bacteroides thetaiotaomicron, Bacteroides fragilis, Bacteroides ouatus, Peptostreptococcus asaccharolyticus, Peptostreptococcus prevo III.* To extract DNA from broth cultures, the bacteria were centrifuged for one minute in a 1.5 ml microcentrifuge tube. The cells were resuspended in 300μl of 25mM Tris pH 8.0, 50mM glucose, 10mM EDTA containing 5mg/ml lysozyme and left to stand at room temperature for 30 minutes. DNA was then extracted from the solution as described in 2.22.

2.8 Interferon

Human lymphoblastoid Interferon (HuIFN) and mock IFN, produced in Namalwa cells was kindly supplied by Dr.S.F.Lyons. The IFN produced in Namalwa cells consists of a mixture of IFN-α (leukocyte) and IFN-β (fibroblast), with IFN-α as the predominant type (Interferon Nomenclature, 1980). Mock IFN was produced in the same way as IFN except for the omission of IFN induction by infection with Sendai virus. The concentration of the stock HuIFN was 16,000U/ml (Lyons *et al*, 1982). Dilutions of IFN were made in EMEM with 2% FCS for radioimmunoassays (100,50,25,10, and 5U/ml) and in L15 medium with 2% FCS for indirect immunofluorescent focus assays (100 and 10U/ml).

Antibodies

Polyclonal antiserum from horse directed against Ad5 hexon was used as adenovirus group-specific antiserum. Polyclonal antibody raised against Ad3 in guinea pigs was provided by Dr.A.H.Kidd (Kidd and Madeley, 1981).

Monoclonal antibodies raised against CsCl-purified Ad41 virions were obtained from Dr.A.H.Kidd (Kidd and Blackburn, unpublished). Two monoclonal antibodies specific for Ad40 and Ad41 (subgroup F) by immunofluorescence were further characterized (MAb-4 and MAb-5) (see 4.2.1). Dr.J.C.de Jong (Bilthoven, The Netherlands) kindly donated monoclonal antibodies MA3-20, specific for Ad40 and MA5-15 specific for Ad41.

Polyclonal anti-Sindbis antiserum, prepared as immune ascitic fluid in mice was supplied by Dr.M.Crespi (Lyons *et al*, 1982).

2.10 Virus Infections

Using indirect immunofluorescence, stock Ad2 preparations were titrated separately on Chang and HEF cells, and stock Ad40 and Ad41 preparations were titrated on Chang cells. For all infections, virus dilutions were made in PBS. Monolayers of cells were washed once with PBS and virus adsorbed for 2 hours at 35°C. One hundred microlitres of inoculum was used for cells in 24 well plates, 300μl for 9.6 cm^2 dishes and 500μl for 25cm^2 flasks. The cells were then washed three times with PBS and incubated with maintenance medium for appropriate periods of time.

Foetal intestinal organ cultures were infected by adding 10 or 100μl volumes of virus to the growth medium and incubating with gentle rocking at 35°C overnight. The inoculum medium was then replaced with fresh maintenance medium. Fluids removed were stored at -20°C.

2.11 Virus concentration

Virus preparations grown in 150cm^2 flasks were clarified at 3000rpm (Sorvall rotor H1000B) for 20 minutes at 4°C. The supernatant was clarified a second time for 60 minutes. PEG 6000 (50% w/v) was added to the supernatant to a final concentration of 10% v/v. The virus was allowed to precipitate for 90 minutes at 4°C with moderate stirring. Virus was recovered by centrifugation at 3000rpm (Sorvall rotor H1000B) for 30 minutes at 4°C. DNA extracted from PEG-precipitated virus was used as a source of virus DNA free of cell DNA.

2.12 Adenovirus purification

Infected culture supernatants were extracted with an equal volume Freon 113 in a blender. The supernatants were clarified for 20 minutes at 3000rpm (Sorvall rotor H1000B) at 4°C. The virus was concentrated by centrifugation onto a CsCl cushion

(refractive index (R.I.) of 1.372) at 24,000rpm for 60 minutes in a Beckman SW40 Ti rotor. The interface material was then pooled and centrifuged in CsCl of R.I. 1.366 at 26,500rpm for 20 hours at 4°C. The virus band was removed and purified on a second CsCl gradient as described. The virus material was dialyzed against 50mM Tris pH 7.4. Purified virus was used as a source of pure virus DNA.

2.13 Isotyping of mouse monoclonal antibodies

A kit using typing sticks (Amersham) was used to isotype mouse monoclonal antibodies. This was done according to the manufacturer's specifications. The typing sticks consist of nitrocellulose membrane which have goat anti-mouse antibodies specific for the class (IgA and IgM), the IgG subclass (G1, G2a, G2b and G3) and light chain type (K and λ) as well as a positive control. MAb samples were incubated with the typing stick for 15 minutes at room temperature. All steps of the procedure require agitation. Antibody preparations were diluted in the wash buffer TBS-T pH 7.6 (20mM Tris base, 137mM NaCl, 0.38% v/v 1M HCl, 0.1% v/v Tween-20). After two 5 minute washes the typing sticks were then incubated for 15 minutes at room temperature with peroxidase labelled sheep anti-mouse antibody. The typing sticks were washed as above and then incubated in substrate solution for 15 minutes at room temperature. The final washes were in three changes of distilled water.

2.14 ELISA

Wells of microplates were each coated with 100μl of hyperimmune anti-adenovirus 5 hexon serum diluted 1:5,000 in carbonate buffer pH 9.6 (0.15M Na_2CO_3, 0.35M $NaHCO_3$). After 24 hours at 4°C, plates were washed three times for 10 minutes with PBS-Tween 20 (0.05% v/v). One hundred microlitres of culture fluid from infected and uninfected cells was added to duplicate wells. The plates were incubated at 37°C for 60 minutes, washed thoroughly as before and 100μl of a 1:1,600 dilution of guinea pig anti-adenovirus 3 serum added. After incubation at 37°C for 60 minutes,

the plates were washed and 100μl of a 1:400 dilution of peroxidase conjugated rabbit anti-guinea pig serum (Dako, Denmark) added and the plates incubated at 37°C for 60 minutes. After washing, 200μl of substrate solution (0.04% o-phenylamine diamine, 0.7% di-sodium tetraborate, 0.36% succinic acid, 0.08% urea hydrogen peroxide [all w/v]) was added. The reaction was stopped after 15 minutes in the dark at room temperature by adding 50μl of a 3N HCl solution. The absorbance was read at 492nm.

For the determination of monoclonal antibody specificity, the ELISA included a blocking step (100μl of 1% BSA diluted in PBS and incubated at 37°C for 60 minutes) after addition of virus preparations to the wells. After washing, 100μl of test antibody preparation was added to duplicate wells, and incubated at 37°C for 60 minutes. Plates were washed as before and 100μl of peroxidase conjugated rabbit anti-mouse serum added and treated further as described above.

2.15 Indirect immunofluorescence

At 44 hours post-infection (p.i.) coverslips were washed with PBS and fixed in 100% acetone for 10 minutes at room temperature. Coverslips were treated with the appropriate adenovirus antibody (diluted in PBS) for 60 minutes at 35°C then given three 10 minute washes in PBS. The appropriate anti-species conjugate diluted in PBS was added for 60 minutes at 35°C and the coverslips washed as before. Coverslips were then mounted on glass slides with PBS:glycerol (1:1) and viewed with a Nikon Labophot fluorescent microscope using a 20x objective lens.

For Ad41 fluorescent cell counts in single and mixed infections, MAb-4 or MAb-5 was used as first antibody (1/500), with fluorescein isothiocyanate conjugated rabbit anti-mouse immunoglobulin (Dako, Denmark) as second antibody (1/80). Monoclonal antibodies MA3-20 and MA5-15 were used to distinguish Ad40 and Ad41

fluorescence in mixed infections of the two viruses, respectively. The fluorescent conjugate used was as above.

For Ad2 fluorescent cell counts in single infection and total fluorescent cell counts in mixed infections, anti-adenovirus hexon group-specific polyclonal horse antiserum was used as first antibody (1/200), with a fluorescein isothiocyanate conjugated rabbit anti-horse IgG (Nordic Biochemicals, The Netherlands) as second antibody (1/100).

2.16 Radioimmunoassay

Spent tissue culture medium was removed from confluent vero cells grown in a microplate. Samples to be tested for the presence of IFN were clarified at 3000rpm for 30 minutes at 4°C and the supernatant checked for possible residual virus by indirect immunofluorescence following ultracentrifugation (40,000rpm, 4°C, SW40 rotor). One hundred microlitres of each sample, including diluted IFN control samples, was added to duplicate wells. The plate was incubated at 37°C in a CO_2 incubator for 24 hours.

The medium was removed and the cells washed with EMEM. Sindbis virus was added to each well at a dilution of 0.5×10^{-2} in EMEM (100μl). Cell and antibody control wells each received 100μl of EMEM. The virus was adsorbed for one hour at 37°C in a CO_2 incubator. After the removal of the virus, 100μl of EMEM with 2% FCS was added to each well and incubated as before. Cells were checked for CPE after overnight incubation. The medium was removed and cells washed three times with warm wash buffer (0.05% v/v Tween 20, 0.02% w/v sodium azide, 0.1% w/v BSA, 1xPBS) and the plate air-dried for 30 minutes.

A volume of 200μl of ice-cold methanol was added to each well and the plate held at -20°C for 10 minutes. The methanol was removed, the plate air-dried, washed

twice with wash buffer and incubated with 200μl of wash buffer per well. The wash buffer was removed and 50μl of anti-Sindbis antiserum (1/30) diluted in wash buffer, added. Cell control wells received 50μl of wash buffer.

After removing the antiserum and washing cells three times with wash buffer, 100μl of ^{125}I-protein A was added to each well. The radiolabel was diluted to 30,000cpm/100μl in wash buffer. The plate was incubated at 37°C for 60 minutes and then washed 4 times with wash buffer. Cells were solubilized with 100μl 0.1N NaOH and 0.1% w/v SDS (100μl/well) for 60 minutes at 37°C. Fifty microlitres of solubilized material was transferred to plastic tubes and samples counted in a Packard 5160 gamma-counter.

2.17 *In vivo* labelling of proteins with ^{35}S-methionine

Cells growing in 25cm^2 flasks were washed twice with 5ml methionine-free, L-glutamine-free MEM (-MET MEM) and 1ml labelling mixture added (-MET MEM containing 2% dialysed FCS, 292.3 μg/ml L-glutamine, and a final concentration of 20μCi/ml ^{35}S-methionine [20nmol]). After an appropriate labelling period the medium was removed and the cells were washed twice with PBS and collected by scraping with a rubber policeman. They were pelleted at 1000rpm (Sorvall rotor H1000B) for 10 minutes at 4°C and resuspended in 100μl PAGE sample buffer.

2.18 *In vitro* translation

Specific mRNAs were hybrid selected by hybridization to and elution from nitrocellulose discs containing either 40μg of pSPT18-Ad2 *SmaIA* or 40μg of pSPT18-Ad2 *BclID*. Hybridizations were carried out as described in 2.32. The RNA was eluted for 2 minutes at 100°C in 1ml sterile water containing 5μg carrier yeast tRNA. This was repeated and the eluates were pooled and separated from contaminating DNA by oligo-dT chromatography (see 2.30.2).

The mRNAs were translated in an Amersham rabbit reticulocyte lysate system. The translation reaction mixtures contained 40μl message-dependent lysate, 7μl 15mCi/ml L-[^{35}S]-methionine and 3μl mRNA and were incubated at 30°C for 90 minutes. Incorporation of radiolabel into polypeptides was determined after 60 minutes of incubation.

2.19 Polyacrylamide gel electrophoresis

Samples to be electrophoresed were incubated at 95°C for 5 minutes and 20μl volumes loaded on 12% polyacrylamide gels (see Appendix B). Electrophoresis was carried out for 4 hours at 50mA constant current. Separated proteins were fixed (see Appendix B) for 30 minutes and the gels dried at 80°C for 2 hours in a Hoefer gel dryer. The gels were autoradiographed for 24-48 hours using Amersham MP X-ray film.

2.20 Radioimmunoprecipitation assay

^{35}S-methionine labelled cells were harvested by low speed centrifugation (1000rpm in a Sorvall rotor H1000B) and washed twice with PBS. Ten microlitres of 50μl was removed to serve as a control for the protein profile of each preparation. The remaining 40μl of cells was resuspended in 5ml of NP40-lysis buffer (0.5% w/v NP40, 150mM NaCl, 5mM EDTA, 50mM Tris-Cl, 1mg/ml BSA). Cells were vortexed well and centrifuged at 3000rpm at 4°C for 10 minutes to pellet nuclei. This step was repeated to ensure the removal of nuclei. Fifty microlitres of 10% SDS and 10μl of test serum was added to the supernatant and incubated with occasional shaking for 4 hours at room temperature. One millilitre of protein A-sepharose beads (CL-4B, 10mg/ml in NP40-lysis buffer) was added to each sample and incubated overnight at room temperature with gentle tilting. The beads were washed 5 times with the above buffer and finally resuspended in 100μl of PAGE sample buffer (see Appendix B). PAGE was carried out as described in 2.19.

2.21 *In vivo* labelling of DNA

2.21.1 Phosphorus-32

Cells growing in 25cm^2 flasks were washed twice with phosphate-free, L-glutamine-free MEM and 1ml labelling mixture added (phosphate-free MEM containing 2% dialysed FCS, 292.3μg/ml L-glutamine, and a final concentration of 20μCi/ml ^{32}P-orthophosphate). After three to 5 days, cells were washed twice with PBS and harvested by scraping with a rubber policeman. They were pelleted by centrifugation at 1000rpm (Sorval rotor H1000B) for 10 minutes at 4°C.

2.21.2 ^{3}H-thymidine

Cells growing in 25cm^2 flasks were washed twice with PBS and 1ml labelling mixture added (MEM containing 2% dialysed FCS and a final concentration of 20μCi/ml ^{3}H−thymidine). Labelling was carried out for an appropriate period of time and the cells harvested as described above.

2.21.2.1 Alkaline sucrose gradient centrifugation

Cell pellets were resuspended in 100μl lysis buffer (0.5M NaOH, 50mM EDTA). Lysates were loaded on 5-20% alkaline sucrose gradients (in 0.3M NaOH, 0.7M NaCl, 1mM EDTA) and allowed to stand at 4°C for 16 hours. The gradients were spun at 34,500rpm for 5 hours at 4°C in a SW40 rotor. Four hundred microlitre fractions were collected from the bottom of the tubes, 2.5 volumes of ice-cold 20% TCA added and a 100μl aliquot spotted on nitrocellulose filters. After two 10% TCA washes and one wash in 95% ethanol, the filters were air dried and radioactivity counted in the presence of 5ml scintillation fluid.

2.22 DNA Extractions

Cells were resuspended in TE buffer pH 7.4 (10mM Tris-Cl, 1mM EDTA) and pelleted at 1000rpm (Sorvall rotor H1000B) for 10 minutes at 4°C. The cells were then transferred in a small volume of buffer to an eppendorf reaction vessel containing 300μl 100mM Tris-Cl, pH 7.4, 1% w/v sarkosyl, 100mM 2-mercaptoethanol, 10mM EDTA, and 27% sucrose. Proteinase K was added to a final concentration of 250μg/ml, and the reaction incubated for 2-3 hours at 37°C. Protein was extracted twice using phenol saturated with TE buffer. The aqueous phase was then extracted once with phenol-chloroform (1:1 of TE-saturated phenol and chloroform-isoamylalcohol) and once with chloroform-isoamylalcohol (24:1 v/v). Sodium acetate was added before the final chloroform-isoamylalcohol extraction to a final concentration of 0.5M. Two to three volumes of absolute ethanol were added and the DNA allowed to precipitate at -70°C for 60 minutes or -20°C overnight. The DNA was pelleted in a micro-centrifuge at 10,000g for 10 minutes, washed with 70% ethanol, vacuum dried and resuspended in TE buffer pH 7.4.

2.23 Restriction enzyme analysis

Restriction enzyme digestions were carried out with 4 units of enzyme per μg of DNA at 37°C for 1-3 hours in recommended buffers supplied with the enzymes. Fourty units of enzyme was used for every μg of DNA for digestions of cellular DNAs. The products were analysed on 0.8 or 1% agarose gels.

2.24 *Bal*31 exonuclease digestions

Virus DNA extracted from PEG-precipitated virus was used for all *Bal*31 digestions. Digestions were carried out at 30°C in the buffer supplied with the enzyme and 2.5 units of *Bal*31 per reaction. An aliquot was removed at specific times and added to 3.5μl of 0.5M EDTA, pH 8.0, to stop the reaction. The DNA was then extracted

with phenol-chloroform and precipitated with absolute ethanol. The DNA was analysed further using the restriction enzyme *Pst*1.

2.25 Agarose gel electrophoresis

Agarose gels were made up in TAE electrophoresis buffer (see Appendix B) containing 1μg/ml ethidium bromide. Twenty microlitres of each sample in loading buffer (see Appendix B) was applied. Electrophoresis was performed at 19V for 16 hours or 50V for 5 hours. Gels containing ^{32}P-labelled DNAs were dried at 80°C for 2 hours in a Hoefer gel dryer and autoradiographed for 5 hours using Amersham βmax X-ray film.

2.26 Southern blotting

After electrophoretic separation of DNA fragments, the gels were treated with a solution of 0.2M NaOH and 0.6M NaCl for 40 minutes at room temperature. The gels were then washed and the single-stranded DNA electrophoretically transferred to either nitrocellulose (Hybond™-C) or nylon membrane (Hybond™-N). After blotting at 1.2A for 60 minutes in 25mM sodium phosphate pH 6.5, the membranes were equilibrated with 2xSSC (0.3M NaCl, 0.03M sodium citrate) for 20 minutes. The DNA was fixed to nitrocellulose membranes by baking at 80°C for 2 hours and to nylon membranes by treating with medium wavelength UV light (302nm) for 3 minutes.

2.27 DNA Dot blots

One hundred microlitres of DNA in TE buffer was denatured by adding 12μl of 1M NaOH and 12μl of 3M NaCl and heating at 95°C for 10 minutes. Samples were cooled on ice and then added to 100μl of 2M ammonium acetate in the wells of a Bio-Rad dot apparatus prefitted with nitrocellulose or nylon membrane. Vacuum was

applied and the liquid suctioned through. Membranes were washed twice in 1M ammonium acetate, equilibrated with 6xSSC (0.9M NaCl, 0.09M sodium citrate), and allowed to air dry. DNA was fixed to membranes as described above (2.26).

2.28 Nick translation of DNA

DNA was labelled with ^{32}P by nick translation (Rigby *et al*, 1977) using an Amersham nick translation kit. DNA (100ng) was incubated with 30μCi [$\alpha^{32}P$]dCTP (80pmol), 20μM dATP, dGTP and dTTP and 5U of DNA polymerase I and 100pg of DNase I in a total volume of 25μl. The reaction was allowed to proceed at 16°C for 75 minutes. ^{32}P-labelled DNA was separated from unincorporated nucleotides by fractionation on Sephadex G-50 columns. Specific activities were determined by transferring a small volume of labelled DNA to a vial with 5ml Aquagel and counting in a LKB counter. Specific activities of approximately 10^7 to 10^8 cpm/μg of DNA were generally obtained.

2.29 DNA:DNA hybridizations

All pre- and post-washes of membranes were carried out at 65°C. Prewashes were as follows: (i) 6xSSC (0.9M NaCl, 0.09M sodium citrate), 30 minutes, (ii) 6xSSC with 5x Denhardt's solution (0.1% w/v polyvinylpyrolidone, 0.1% w/v ficoll, 0.1% w/v BSA fraction V), 30 minutes, (iii) 6xSSC with 5x Denhardt's, 0.5% w/v SDS, 100μg/ml herring sperm DNA denatured at 95°C for 10 minutes. Hybridization of nick-translated probe DNAs was carried out overnight in 6xSSC, 5x Denhardt's, 0.5% w/v SDS, 10mM EDTA, and 100μg/ml herring sperm DNA. The probe and herring sperm DNA were denatured at 95°C for 10 minutes before addition. Post washes were as follows: (i) one 5 minute and three 40 minute washes in 2xSSC and 0.5% w/v SDS, (ii) one 5 minute and two 60 minute washes in 0.1xSSC and 0.5% w/v SDS. For less stringent conditions, the low salt washes were omitted. The membranes were dried and autoradiographed for 5 to 24 hours with Amersham MP

X-ray film. For re-use of DNA blots the probe was removed by boiling in a solution of 0.1% w/v SDS for 60 minutes.

2.30 Isolation of cytoplasmic RNA and mRNA

2.30.1 Fractionation of cells and cytoplasmic RNA isolation

Cells were fractionated using the method of McGrogan and Raskas (1977). They were harvested by scraping, washed in PBS and pelleted at 1000rpm (Sorvall rotor H1000B) for 10 minutes at 4°C. They were then resuspended in isotonic buffer (0.15M NaCl, 10mM Tris-Cl pH 7.5, 1.5mM $MgCl_2$, 0.65% v/v Nonidet-P40), vortexed and placed on ice for 10 minutes. The nuclei were removed by two centrifugations at 3000rpm for 15 minutes at 4°C. EDTA and SDS were added to the supernatant to a final concentration of 2mM and 0.2% w/v, respectively. The RNA was then purified by two phenol-chloroform and one chloroform-isoamylalcohol extractions. The RNA was precipitated in the presence of 0.1 volume 3M sodium acetate pH 5.2.

2.30.2 Oligo-dT selection of mRNA

The mRNA was isolated from total RNA by oligo-dT column chromatography as follows. The RNA pellet was resuspended in 300μl H_2O and held at 65°C for 5 minutes. An equal volume of 2x low salt binding buffer (1x: 10mM Tris-Cl pH 7.5, 100mM NaCl, 1mM EDTA, 0.1% w/v SDS) was added, the reaction cooled to room temperature and applied to an oligo-dT column. One gram of oligo-dT was used for every 10mg of RNA or 200 A_{260} units of total RNA. The column was equilibrated with 10 column volumes of high salt binding buffer (as above with 500mM NaCl instead of 100mM) before applying the RNA sample. The flow through was collected and incubated at 65°C for 5 minutes, cooled to room temperature and again applied to the column. The poly(A)$^+$ RNA was eluted by washing the column with 8 column

volumes (400μl) of high salt NaCl binding buffer and 4 column volumes of low salt binding buffer. The elution was monitored by reading samples at A_{260}. Poly(A)$^+$ RNA was eluted from the column with 3 volumes of elution buffer (10mM Tris-Cl pH 7.5, 1mM EDTA, 0.1% w/v SDS), 0.1 volume 3M sodium acetate added and the RNA precipitated with 2.5 volumes absolute ethanol.

2.31 RNA dot blots

Cytoplasmic or Poly(A) selected RNA was resuspended in 100μl water and denatured at 60°C for 15 minutes after the addition of 60μl 20xSSPE (see Appendix B) and 40μl formaldehyde. The 200μl sample was then applied to nylon membrane in a Bio-Rad dot apparatus using suction, the wells washed with 6xSSPE, the membrane air-dried and the RNA fixed to the membrane by exposure for 3 minutes to medium wavelength UV light (302nm) for 3 minutes.

2.32 DNA:RNA hybridizations

RNA blots were prewashed for four hours at 42°C with hybridization solution (5xSSPE, 50% v/v formamide, 5x Denhardt's, 0.5% v/v SDS, 10% v/v dextran sulphate, 100μg/ml herring sperm DNA). The DNA probe and herring sperm DNA were denatured at 95°C for 10 minutes before addition to fresh hybridization solution. Hybridization was carried out overnight at 42°C. Post washes were as follows: (i) two 15 minute washes at 42°C in 5xSSPE and (ii) two 30 minute washes at 65°C in 1xSSPE and 0.1% w/v SDS. The membranes were dried and autoradiographed with Amersham MP X-ray film. For re-use of RNA blots the probe was removed by washing for 60 minutes at 65°C in 5mM Tris-Cl pH 8.0, 2mM EDTA and 0.1x Denhardt's solution.

2.33 Reverse transcription

Reactions were performed in a total volume of 100μl, in PCR amplification buffer (see 2.34) also containing 44mM KCl, 3.75% v/v DMSO, 1 unit RNasin, 500μM of all four deoxynucleotides, and primer K403 added to a final concentration of 124nmol/ml. Twenty-five units of reverse transcriptase was added and samples incubated at 42°C for 2.5 hours.

2.34 Polymerase chain reaction.

Reactions were performed in 1.8 ml reaction vessels in a total volume of 100μl, in a buffer (pH 8.8 at 25°C) consisting of 67mM Tris, 16.6mM $(NH_4)_2SO_4$, 6.7mM $MgCl_2$, and 10mM 2-mercaptoethanol. Bovine serum albumin was added to a final concentration of 170μg/ml. Deoxynucleotides (dATP, dCTP, dGTP and dTTP) were each added to 1mM (final). Primers K402 and K403 were each added to a final concentration of 124nmol/ml.

Templates consisting of cellular, bacterial, or reference virus DNAs were added to a final concentration of 10μg/ml. One unit of *Thermus aquaticus (Taq)* polymerase was added and the mixture was overlaid with 120 μl liquid paraffin. Cycling consisted of one minute at 95°C to denature the DNA, followed by 1.75 minutes at 60°C for primer annealing and 2.5 minutes at 70°C for DNA synthesis. Following this elongation step, the DNA was immediately returned to 95°C to denature.

After the DNA synthesis step of the 30th cycle, 10μl of the reaction mixture was added to 2μl DNA loading buffer (see Appendix B). The mixture was loaded into the wells of a 4% w/v composite agarose gel consisting of 3% Nu-Sieve and 1% SeaKem GTG agarose. Electrophoresis was performed at 10V/cm for 90 min or 1.2 V/cm overnight, in TBE electrophoresis buffer (see Appendix B). The 125bp

fragment of *Hind*III-digested phage lambda DNA was used as a size reference in all tests.

To confirm the identity of an amplified product further, 10μl of the reaction mixture was digested with 1 unit of an appropriate restriction enzyme. In all cases, the amplification buffer proved suitable for this purpose. After digestion for 2 hours at 37°C, the product was electrophoresed alongside its undigested counterpart.

2.35 Molecular cloning of Ad2 DNA restriction fragments

2.35.1 Plasmid preparation

2.35.1.1 Restriction endonuclease digestion

Plasmids pBR322 and pSPT18/19 were used for cloning purposes. The pSPT18 and pSPT19 plasmids contain the SP6 and T7 phage transcription promoter sequences on opposite ends of a multiple cloning site. Restriction enzyme digestions were carried out as described in 2.23.

2.35.1.2 Alkaline phosphatase treatment of linearized plasmids

The terminal 5′ phosphates were removed from the linear vector DNA according to the method for blunt ends or recessed 5′ ends as follows (Maniatis *et al*, 1982). After digestion of the plasmid, the DNA was extracted once with phenol-chloroform and once with chloroform-isoamylalcohol and precipitated with ethanol. The DNA was then dissolved in 10mM Tris-Cl pH 8.0. Calf intestinal alkaline phosphatase (CIP) was added to the reaction at 0.01 units per μg of plasmid DNA in CIP buffer (final concentration: 0.05M Tris-Cl pH 9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine). The reaction was incubated at 37°C for 15 minutes and 56°C for 15 minutes. A further aliquot of CIP was added and the incubations repeated at the two

temperatures. To a reaction volume of 30μl, 50μl of H_2O, 9μl of 10xSTE (100mM Tris-Cl pH 8.0, 1M NaCl, 10mM EDTA) and 5μl 10% w/v SDS were added and incubated for 15 minutes at 68°C to inactivate the enzyme. The DNA preparation was extracted twice with phenol-chloroform and twice with chloroform before precipitation with ethanol.

2.35.2 Preparation of Ad2 DNA restriction fragments

2.35.2.1 Restriction enzyme digestion

Purified Ad2 DNA was cleaved with appropriate restriction enzymes as described in 2.23.

2.35.2.2 Removal of the adenovirus terminal protein

For cloning of adenovirus terminal fragments the 5′ terminal protein was removed by one of two methods: 1. Piperidine treatment, or 2. A combination of enzyme reactions that result in the final removal of the terminal protein by cleavage with S1 nuclease of single stranded DNA attached to the terminal protein.

2.35.2.2.1. Piperidine treatment

Pronase-treated, restriction enzyme digested adenovirus DNA was treated with 0.5M piperidine for 2 hours at 37°C (Tamanoi and Stillman, 1982). The piperidine was then removed by multiple lyophilizations. DNA is denatured in the presence of piperidine and therefore requires reannealing. The DNA fragments were reannealed in 10mM Tris-Cl pH 8.0, 1mM EDTA, 1M NaCl at 65°C for 4 hours. The completion of the annealing reaction was checked by agarose gel electrophoresis.

2.35.2.2.2. Enzymatic treatment

(i) Pronase digestion

A stock solution of pronase (20mg/ml in H_2O) was pretreated for 2 hours at 37°C. Adenovirus DNA was incubated with 1mg/ml of this pronase in a 0.01M Tris-Cl pH 7.8, 0.01M EDTA, and 0.5% w/v SDS reaction buffer for 60 minutes at 37°C. The preparation was extracted once with phenol-chloroform, once with chloroform and precipitated with ethanol.

(ii) T4 DNA polymerase 3′ to 5′ exonuclease reaction

The pronase-digested DNA was resuspended in 1x T4 DNA polymerase buffer (50mM Tris-Cl pH 8.5, 15mM $(NH_4)_2SO_4$, 7mM $MgCl_2$, 0.1mM EDTA, 10mM 2-mercaptoethanol, 2μg/ml BSA) and 1.5μl of a 2mM solution of each of dATP and dTTP added. T4 DNA polymerase was added at a concentration of 1.5U/μg of DNA and the reaction allowed to proceed at 37°C for 10 minutes. The enzyme was removed by phenol-chloroform and chloroform extractions as before. The DNA was precipitated with ethanol.

(iii) S1 nuclease reaction

DNA treated with T4-DNA polymerase was resuspended in 1x S1 nuclease buffer, pH 7.0 (0.2M NaCl, 0.05M sodium acetate, 1mM $ZnSO_4$, 5% w/v glycerol) at a concentration of 10μg/ml and 0.25U/ml S1 nuclease added (according to van Loon *et al*, 1985a). The reaction was incubated at 37°C for 20 minutes. Extractions and DNA precipitations were as before.

2.35.2.3 Conversion of fragments with protruding 5′ ends to blunt ends

S1 nuclease treated DNA was resuspended in 1x nick-translation buffer (0.05M Tris-Cl pH 7.2, 0.01M $MgSO_4$, 0.1mM dithiothreitol, 50μg/ml BSA) with 1μl of each of 2mM solution of all four dNTPs added. Two units of the Klenow fragment of DNA polymerase I was added per microgram of DNA. DNA was extracted and precipitated as in section 2.22.

2.35.2.4 Purification of DNA fragments from agarose

Adenovirus 2 restriction enzyme fragments were separated on 1% w/v agarose gels and bands of interest purified using one of the following methods: A. Electroelution, or B. Extraction with glass powder (Geneclean™).

2.35.2.4.1 Electroelution

DNA fragments were electroeluted at 80V from agarose gels into troughs cut into the slab gels. Electrophoresis buffer was removed from the troughs every 30 seconds and replaced with fresh buffer. The DNA was recovered by extracting the samples with 2−butanol to reduce the volume, then one chloroform extraction and precipitation with ethanol.

2.35.2.4.2 Geneclean method

DNA fragments were purified according to the manufacturer's instructions, with a few modifications. Following restriction enzyme digestion and separation of DNA fragments by electrophoresis, the desired DNA bands were excised, sliced finely with a scalpel and placed at -20°C until frozen. Samples were then thawed and saturated NaI solution added. The freezing step aids the dissolving of the agarose at this stage. The samples were incubated at 55°C for 5 minutes. One microlitre of glassmilk

(silica matrix in water) was added for every 0.5μg DNA, mixed and allowed to stand at room temperature for 5 minutes. The silica matrix with bound DNA was pelleted in a micro-centrifuge and washed three times in ice-cold NEW™(Ethanol, NaCl, H_20). The silica pellet was then vacuum dried. DNA was eluted from the silica by resuspending in 10μl of TE, incubating at 55°C for 3 minutes, pelleting the silica and pooling the supernatants from two further elutions.

2.35.3 DNA ligation procedure

Plasmid DNA was linearized with the appropriate restriction enzyme and treated with CIP. Ratios of plasmid:insert were determined to ensure the same number of DNA ends in the ligation reaction. Ligations were carried out in a final volume of 20μl for 16 hours at 21°C in a ligation buffer (1x: 50mM Tris-Cl pH 7.4, 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP, 100μg/ml BSA). One unit of T4 DNA ligase was used in reactions involving cohesive ends. Blunt-end ligations were performed with 5-10 units of enzyme and a 4-fold higher quantity of DNA than in the above reactions.

2.35.4 Preparation of competent *E.coli* cells

An overnight culture of *E.coli* strain MC1061 was used to inoculate 150ml of L broth (see Appendix B). The cells were grown with shaking at 37°C for 4 hours. The culture was chilled on ice for 10 minutes and centrifuged at 4000g for 5 minutes at 4°C. The cells were resuspended in 10ml of an ice-cold solution of 50mM $CaCl_2$ and 10mM Tris-Cl pH 8.0. The cells were dispensed in 500μl aliquots and stored at 4°C for 24 hours and then transferred to -70°C for storage.

2.35.5 Bacterial transformation

Ligation reaction mixtures were mixed with 40μl of competent *E.coli* cells and placed on ice for 20 minutes. The cells were incubated at 37°C for 5 minutes, 400μl of preheated LB added and incubated for a further 30 minutes at 37°C. The cells were then plated onto LA plates containing ampicillin (50μg/ml) and incubated at 37°C overnight.

2.35.6 Selection of recombinant bacterial colonies

2.35.6.1 Bacterial colony hybridizations

2.35.6.1.1 Labelling of DNA with digoxigenin-11 dUTP

The protocol followed was the standard experimental procedure recommended by Boehringer Mannheim for non-radioactive DNA labelling and detection of target DNA. Briefly, 3μg of linearized, purified DNA was denatured (10 minutes at 95°C) and chilled quickly on ice for 3 minutes. Two microlitres of hexanucleotide mix, 2 μl of dNTP labelling mixture and 1μl of Klenow enzyme (2 units) were added to the DNA and the reaction volume made up to 20μl with sterile H_2O. The reaction was allowed to proceed for 20 hours at 37°C. Two microlitres of 0.2M EDTA, pH 8.0, was added and the DNA precipitated with 75μl ethanol and 2.5μl 4M LiCl. The DNA was resuspended in 50μl 10mM Tris-Cl pH 8.0, 1mM EDTA.

2.35.6.1.2 Hybridization of labelled DNA to bacterial colonies *in situ*

Individual colonies were picked with sterile toothpicks and transferred from transformation plates to gridded nitrocellulose membranes placed on LA plates containing ampicillin (50μg/ml) and replica plates without nitrocellulose. The plates were incubated at 37°C overnight and replica plates stored at 4°C. The colonies on

the nitrocellulose were lysed (10% w/v SDS), the DNA denatured (0.5M NaOH, 1.5M NaCl) and neutralized (1.5M NaCl, 0.5M Tris-Cl pH 8.0) before baking the nitrocellulose filters for 2 hours at 80°C.

Filters were prehybridized in 15ml hybridization solution (5xSSC, 0.1% w/v N-lauroylsarcosine, 0.02% w/v SDS, 1% w/v blocking reagent; dissolved at 70°C for 1 hour) at 68°C for 60 minutes. This step was repeated to remove excess colony debris. Hybridization mixture was added containing 50-100ng freshly denatured DIG-labelled DNA and incubated overnight with shaking. Filters were washed twice for 5 minutes at room temperature with 40ml 2xSSC, 0.1% w/v SDS and twice for 15 minutes at 68°C with 0.1xSSC, 0.1% w/v SDS.

2.35.6.1.3 Detection of probe DNA - target DNA hybrids

Filters were washed briefly in buffer 1 (100mM Tris-Cl pH 7.5, 150mM NaCl) followed by a 30 minute incubation in buffer 2 (buffer 1 with 0.5% w/v blocking reagent added). All reactions were carried out at room temperature with shaking. After washing with buffer 1, alkaline phosphatase conjugated sheep anti-DIG diluted to 150mU/ml in buffer 1 was added and incubated for 30 minutes. The filters were washed twice for 15 minutes with buffer 1 and equilibrated in buffer 3 (100mM Tris-Cl pH 9.5, 100mM NaCl, 50mM $MgCl_2$). The colour solution (NTB and X-phosphate solution supplied in kit) was added and the filters incubated in the dark for up to one day depending on the strength of the signal. The reaction was stopped by adding 10mM Tris-Cl pH 8.0, 1mM EDTA.

2.35.6.2 Small scale plasmid preparations

Recombinant colonies were picked from stored replica plates and grown at 37°C overnight in 5ml LB with ampicillin (50μg/ml). A small volume was removed and DNA extracted by the alkaline lysis method (Maniatis *et al*, 1982). Bacterial cells

were pelleted and resuspended in GET solution (see Appendix B). After standing for 5 minutes at room temperature a solution of 0.2N NaOH and 1% w/v SDS was added and the contents mixed by inverting the tube rapidly a few times. The tubes were placed on ice for 5 minutes. An ice-cold solution of potassium acetate pH 4.8 was added (see Appendix B), the tubes vortexed gently in an inverted position and stored on ice for 5 minutes. The debris was pelleted for 5 minutes at room temperature. The supernatant was extracted with an equal volume of phenol-chloroform and precipitated with 2 volumes of ethanol. Plasmid preparations were treated with DNase-free RNase (20μg/ml) at 37°C for 15 minutes. A 10μl aliquot was removed, digested with the appropriate restriction enzyme and analysed by gel electrophoresis.

2.35.7 Plasmid amplification for large scale isolation

Plates containing LA and ampicillin (50μg/ml) were streaked from glycerol cultures stored at -70°C. After overnight incubation at 37°C, a single colony was picked and transferred to 5ml LB with antibiotic and incubated at 37°C with shaking. One hundred microlitres of culture was transferred to 100ml LB with antibiotic and allowed to shake at 37°C overnight. The addition of chloramphenicol was omitted as higher yields of plasmid were obtained without.

2.35.8 Large scale isolation and purification of plasmid DNA

Bacterial cells from 2.35.7 were harvested by centrifugation at 4000g for 15 minutes at 4°C. They were washed twice in ice-cold STE (see Appendix B).

The pellets were resuspended in 2ml GET solution (see Appendix B) containing 5mg/ml lysozyme and allowed to stand at room temperature for 5 minutes. Four millilitres of a solution of 0.2N NaOH and 1% w/v SDS was added, the sample mixed by inversion and allowed to stand on ice until clear. The sample was again

mixed by inversion after the addition of 3ml potassium acetate pH 4.8 (see Appendix B). The contents were allowed to stand on ice for 10 minutes followed by the removal of cell debris by centrifugation at 15,000rpm for 40 minutes in a Sorvall SS34 rotor.

The supernatant was transferred to Beckman polyallomer tubes and 0.6 volumes of isopropanol added. The contents were mixed by inversion and the nucleic acid allowed to precipitate at room temperature for 30 minutes. The DNA was pelleted at 10,000rpm for 30 minutes at room temperature in a Beckman SW40 rotor. The pellets were washed with 70% ethanol, vacuum dried and resuspended in 1ml TE.

Plasmids were purified according to Maniatis *et al* (1982; p93). To 8ml of TE pH 7.4, 8.55g of CsCl was added and allowed to dissolve. One millilitre of plasmid preparation and 900μl of EtBr (10mg/ml) was added and the remaining tube volume made up with TE pH 7.4, giving a final density of 1.55g/ml (R.I.= 1.3860). The tubes were centrifuged at 35,000rpm for 48 hours at 18°C. The band corresponding to the closed circular plasmid DNA was removed from the side of the tube under medium wavelength UV light (302nm) using a #21 hypodermic needle. The EtBr was removed by 4 extractions with isoamylalcohol-saturated 1-butanol (1:3). The aqueous phase was dialyzed against several changes of TE pH 7.4. The DNA was then precipitated with 2.5 volumes ethanol and 1/10 volume 3M sodium acetate. The concentrations of plasmid DNA preparations were determined spectrophotometrically at 260nm and small aliquots stored at -70°C.

2.36 Transfection of cells with Ad2 early DNA regions

2.36.1 DNA

For transfections, plasmids containing inserts corresponding to Ad2 early regions were used. Plasmid pSPT18 without insert, pMAM-neo (a eukaryotic expression vector) or Ad2 DNA was used as control.

2.36.2 Transfection methods

2.36.2.1 Calcium phosphate transfection

DNA pellets were resuspended in 450μl of sterile water and 50μl of a 2.5M solution of $CaCl_2$ added. The amount of DNA varied from 6 to 16μg depending on the size of the dish used and number of replicates. The DNA-$CaCl_2$ solution was added dropwise to 500μl of 2x HeBS with constant agitation, followed by vortexing for 5 seconds. The precipitate was allowed to stand at room temperature for 20 minutes. The precipitate was then added to the monolayer of cells and gently swirled. Cells were incubated for 6 hours, the medium removed and cells shocked with 250-670μl 10% v/v glycerol (made up in L15 medium) for 3 minutes at room temperature. One to two millilitres of PBS was added to the glycerol solution on the cells, mixed and removed and the cells were washed two more times in PBS. Growth medium was added and the cells incubated at 37°C.

2.36.2.2 DEAE-dextran transfection

DNA pellets were resuspended in TBS (40μl for each transfection), the plates washed with PBS and fresh growth medium (with 10% FCS) added. The DNA solution was added to 10mg/ml DEAE-dextran in TBS (80μl per transfection) prewarmed to 37°C. A volume of 120μl DEAE-dextran was added dropwise to each plate and the plate

swirled gently. Plates were incubated at 37°C for 4 hours, the DEAE-dextran solution removed and the cells shocked with 10% v/v DMSO in PBS for one minute at room temperature. The DMSO was removed, the cells washed with PBS and growth medium added before incubation at 37°C.

2.36.2.3 Protoplast fusion

This method was carried out according to Pollard *et al* (1984; p326-327). One hundred millilitres of bacterial cells containing the plasmid of interest were pelleted at 3000g for 15 minutes. The pellets were washed twice in 50ml 10mM Tris-Cl pH 8.0 and resuspended in 15ml 20% w/v sucrose in 0.1M Tris-Cl pH 8.0 to give 10 OD_{600} units/ml. The suspension was transferred to a conical flask and warmed to 37°C in a waterbath. Lysozyme was added to 100μg/ml and stirred for 12 minutes. The suspension was removed from the waterbath and 1 volume of EDTA added to 10 volumes cells added dropwise over 3 minutes. After 10 minutes at 37°C the preparation of protoplasts was complete. These were diluted with 3 volumes L15 medium (without serum) containing 7% w/v sucrose, 10mM $MgCl_2$, and 0.2μg/ml DNase I and incubated for 10 minutes at 37°C. The medium was removed from cells in 60mm tissue culture dishes (50-60% confluent) and 5ml of the protoplast preparation added. The protoplasts were centrifuged onto the monolayers in one litre buckets of a Sorvall RC-3 centrifuge at 3000rpm for 5 minutes. The resulting clear layer was removed and 1ml PEG 1000 added dropwise to the cell/protoplast layer with constant rocking. After 90 seconds 5ml of TBS was added and aspirated, and the cells washed three times with 4ml TBS. Growth medium containing 100μg/ml kanamycin was added and incubated at 37°C.

2.36.2.4 Scrape loading

This method was carried out as described by Fecheimer *et al* (1987). Culture medium was removed from a 90mm culture dish of near confluent cells and the cells washed

with 5ml loading solution (10mM KH_2PO_4, 1mg/ml glucose, 0.1mM EDTA, 140mM NaCl, pH 7.2). Ten micrograms of DNA in 1ml loading solution was added to the cells. These were loaded with DNA by scraping them from the dish with a rubber policeman and allowed to stand for 10 minutes. Growth medium was added and the cells a wed to settle in a new 90mm dish.

2.36.2.5 DOTMA transfection

Cells were grown in 25cm^2 flasks or on acetylated sterile glass coverslips (see Appendix B) until they were 60-80% confluent. In general, 4μg of DNA was used for transfections in 25cm^2 flasks and 1.5μg for coverslip cultures. These values were adjusted to ensure transfection with identical copy numbers of plasmids containing Ad2 inserts. For 25cm^2 flasks, 40μl of DOTMA reagent (Boehringer Mannheim) was added to 1.5ml MEM and 4μg of DNA diluted separately in 1.5ml MEM. The two preparations were then mixed and added to the monolayers after removing the old medium. The cells were incubated at 37°C for 5-6 hours, the medium removed and replaced with fresh MEM with 10% w/v FCS. After incubation for a further 16 hours at 37°C, the medium was again replaced fresh growth medium.

2.36.3 Detection of transfected DIG-labelled DNA

Reconstituted fluorescein conjugated sheep anti-DIG antibody (200μg/ml) was diluted 1:10 in PBS, 0.5% w/v BSA, 1% w/v blocking reagent, pH 7.4. Transfected cells on coverslips were fixed in 100% acetone for 10 minutes at room temperature. Three hundred microlitres of diluted anti-DIG fluorescein was added to each coverslip and incubated at 37°C in a humidified chamber. After three 10 minute washes in PBS, the coverslips were mounted on glass slides with PBS:glycerol (1:1) and viewed using a fluorescence microscope.

CHAPTER THREE

VIRUS INFECTIVITY

3.1 Introduction

The infectivity of a virus for a particular cell type can be defined as its ability not only to enter the cell and uncoat its nucleic acid, but also to utilize host cell biosynthetic machinery effectively for its replication. The outcome of an infection therefore depends on the type of cell and the serotype involved. Cells described as permissive cells are productively infected and the viral life cycle ends in the accumulation of infectious particles. A semi-permissive infection is characterized by restricted replication and the production of fewer new virus particles from infected cells. The term non-permissive infection usually refers to abortive virus replication after entry, so that no progeny virions are produced. Semi-permissive and non-permissive infections often occur when a virus infects cells from a species other than its normal host (Doerfler and Lundholm, 1970; Eron *et al*, 1975; Thomasset and Chardonnet, 1980; Paraskeva *et al*, 1982; Eggerding and Pierce, 1986).

The subgroup F adenoviruses grow poorly in most cell types that are used to propagate other human adenovirus types (de Jong *et al*, 1983). There are varied reports in the literature concerning the ability of these viruses to replicate in different cell lines such as 293 cells (Takiff *et al*, 1981; Brown *et al*, 1984; Chiba *et al*, 1983;

Uhnoo *et al*, 1983; Pieniazek *et al*, 1990) and KB cells (Witt and Bousquet, 1988; Mautner *et al*, 1989). It has been suggested that permissive Ad40 and Ad41 infection of the 293 cell line results from its expression of Ad5 E1 products, which might complement a defective subgroup F adenovirus E1 function (Takiff *et al*, 1981). The E1a regions of Ad40 and Ad41 have been shown to have a relatively poor capacity to transactivate other early genes (van Loon *et al*, 1987a, Ishino *et al*, 1988). There is also evidence from complementation studies using Ad5 E1b products that E1b function may also be compromised, at least in Ad40 (Mautner *et al*, 1989).

The aims of this study were to characterize subgroup F adenovirus infectivity in cells that are permissive, semi-permissive and non-permissive for these viruses. Various biosynthetic events such as transcription, DNA synthesis, and late antigen synthesis were monitored in infected cells in order to provide a better understanding of their growth restriction *in vitro*. Virus growth was also monitored using an *in vitro* foetal intestinal organ culture model with the hope that this would provide some insight into subgroup F adenovirus growth *in vivo*.

3.2 Results

3.2.1 Growth in different cell types

3.2.1.1 Virus titration

Stock preparations of Ad40 grown in Chang cells and Ad41 grown in 293 cells were titrated separately on Chang and HEF cells by immunofluorescence using subgroup F specific monoclonal antibody (MAb-5). No late antigen synthesis was detected in HEF cells, whereas appreciable counts (2.4 x 10^4 FFU/ml) were detected for both viruses in Chang cells. Both stock preparations gave 1000-fold less counts than the stock Ad2 preparation in Chang cells. Thus, HEF cells were considered to be non-permissive for Ad40 and Ad41 late antigen synthesis, and Chang cells to be semi-permissive.

3.2.1.2 Growth in permissive cells

Cells that display varying degrees of permissiveness for the subgroup F adenoviruses were monitored for their ability to support both the intracellular accumulation and release of virus into the medium. A group-specific ELISA that detects free hexon antigen was used to assess virus growth over time.

293 cells were seeded in 24-well dishes and each well infected with 2.4 x 10^4 FFU/ml of virus. After 2 hours adsorption at 35°C, the cells were washed with PBS and maintenance medium added. For intracellular free hexon group antigen determinations, the medium was removed and the cells scraped off with a rubber policeman in an equal volume of fresh maintenance medium. The cells were frozen and thawed three times and clarified to remove cellular debris. Extracellular free hexon group antigen determinations were made directly on clarified tissue culture medium removed from infected cells. The total produced at any one time was

considered to be the sum of the intracellular and extracellular hexon antigen determinations.

Figure 3.1 shows a comparison of intracellular and extracellular virus antigen with cells harvested at various times after infection for Ad2, Ad40 and Ad41 in 293 cells. The intracellular accumulation of Ad2 hexon rose sharply between days two to four, reached a plateau over the next two days and then increased again between days 6 to 7 (Figure 3.1A). Extracellular Ad2 hexon rose very gradually during the period when hexon was accumulating intracellularly (days two to four). This was followed by a large increase in extracellular hexon levels over the next two days corresponding to the intracellular plateau phase.

Intracellular hexon accumulation rose sharply between days one to three for Ad40 (Figure 3.1B) and days one to two for Ad41 (Figure 3.1C). Between days four and 5, intracellular Ad40 hexon levels decreased followed by a second cycle of accumulation after day 5. Extracellular Ad40 and Ad41 hexon levels in the medium rose gradually over the first four days with a tendency to plateau thereafter. The accumulation of Ad41 hexon intracellularly remained constant after the second day.

Figure 3.2 shows a comparison of Ad2, Ad40 and Ad41 free hexon antigen detection intracellularly (A) and extracellularly (B) in 293 cells. Adenovirus 2 and Ad41 showed comparable levels of free hexon both intracellularly and extracellularly, with free hexon accumulating more rapidly in Ad41 infected 293 cells. Adenovirus 40, by comparison, appeared to grow poorly in these cells.

3.2.1.3 Growth in semi-permissive cells

Chang cells were seeded in 24-well dishes, infected and the culture fluids harvested as described for 293 cells. Free intracellular and extracellular hexon group antigen was determined as before.

Adenovirus 2 hexon accumulated intracellularly over four days and declined over the next three days (Figure 3.3A). Extracellular hexon levels reached a peak at four days p.i. with a more gradual decline thereafter.

Adenovirus 40 hexon levels rose sharply within the cells between days one and two and declined thereafter (Figure 3.3B). Hexon levels in the medium increased significantly between days three and four and then reached a plateau.

Intracellular Ad41 hexon levels increased between days two and three followed by a decrease between days three and four coinciding with an increase in the amount of hexon released into the medium (Figure 3.3C). The amount of extracellular hexon remained constant for the next two days with a further increase in intracellular hexon levels during this time.

The pattern of intracellular hexon accumulation over time was similar for the three viruses with differences mainly in the amounts produced (Figure 3.4A). Under the conditions of infection used for Ad2, hexon was released into the medium after two days of infection and reached a peak at day four with a decline in the appearance of extracellular hexon in the medium over the next two days (Figure 3.4B). Adenovirus 40 and Ad41 hexon antigens were released into the medium only after three days of infection with their peak at day four as for Ad2. There was no further accumulation of hexon in the medium over the next two days of infection.

In contrast to Ad40 and Ad41, Ad2 grew very efficiently in Chang cells. Adenovirus 40 appeared to grow better than Ad41 in these cells.

3.2.1.4 Growth in non-permissive cells

HEF cells were infected and assayed for intracellular and extracellular hexon group antigen as described for 293 cells.

The accumulation of Ad2 hexon in HEF cells was delayed when compared to that in Chang cells (between days three and four), with antigen accumulating in the medium from day three to 6 (Figure 3.5A). There was a decrease in levels of intracellular hexon between days four and 6, followed by a further accumulation after day 6.

There was no detectable accumulation of Ad40 or Ad41 hexon antigen in the medium or within cells over the 6 and 7 day period of infection, respectively (Figure 3.5B and C).

A comparison of intracellular hexon antigen accumulation over time for Ad2, Ad40 and Ad41 in HEF cells is shown in Figure 3.6A. Adenovirus 2 growth was characterized by an accumulation and release phase followed by another accumulation phase. Adenovirus 40 and Ad41 showed no detectable hexon accumulation.

A comparison of the amount of hexon antigen released into the medium over time is shown in Figure 3.6B. Only Ad2 showed an increase in the amount of hexon released over time. The values obtained for Ad40 and Ad41 at all times after infection did not differ significantly from those obtained at each time for the uninfected controls. These results are consistent with the absence of late antigens in Ad40 and Ad41 infected HEF cells as detected by indirect immunofluorescence (3.2.1.1).

3.2.1.5 Growth in foetal intestinal organ culture

To determine the ability of the subgroup F adenoviruses to grow in cells which they infect *in vivo*, an *in vitro* intestinal organ culture system was employed. Segments of human foetal intestine were placed villus-side up in scored plastic dishes containing Leibovitz (L15) medium supplemented with 0.2% BSA. (If cultures have FCS added to the medium, fibroblasts grow outwards from the tissue and become the

predominant cell type). Maintenance medium was replaced every 24 hours for the first two days and then every second day. The survival time of the cultures was variable and appeared to depend on the condition and age of the aborted foetus which ranged from 8 to 15 weeks. For virus infections, the spent maintenance medium was removed after 24 hours and maintenance medium containing the virus inoculum was added. After a further 24 hours incubation the inoculum was removed and replaced with fresh medium. The culture fluids were harvested at two-day intervals after addition of the virus inoculum unless otherwise stated, and stored at −20°C. Virus growth in the organ cultures was monitored using the ELISA that detects free hexon antigen. All optical density values obtained were averaged from three replicate cultures.

Figure 3.7 shows the hexon levels in the medium from the intestinal cells of a 15 week-old foetus at the indicated times for Ad2, Ad40 and Ad41. Since medium replacement every two days is essential to the survival of the cultures, the values on the graphs represent the accumulation of antigen between two time points and not from the time of inoculum removal. The fluid removed from the cultures at day one p.i. contains unadsorbed inoculum virus. The Ad2 input concentration was 10 times greater than that of Ad40 and Ad41 (namely, 10^4 FFU/dish or 6.7×10^3 FFU/ml) in this experiment. Adenovirus 2 growth increased over time with a substantial increase in release of virus between time points taken. Adenovirus 40 and Ad41 growth, however, showed a low, but more or less constant output of virus over the two day accumulation periods up to 8 days.

The results obtained under the same conditions over a longer period of time were similar with intestinal tissue from two foetuses, one aged 8 weeks (Figure 3.8A) and the other 12 weeks (Figure 3.8B). The rate of Ad2 hexon release into the medium increased rapidly over the first 10 days and then reached a plateau. The amount of hexon antigen of Ad40 and Ad41 released into the medium was questionable over the first 13 days of infection, and increased slightly when allowed to accumulate for 8

days up to 21 days after infection. The tissue did not receive fresh maintenance medium over this period of time and may not have been metabolizing optimally. The virus growth over this period of time may therefore have been compromised. Furthermore, the cells may have been at a stage where they could no longer support more virus growth as a result of cell damage due to infection.

To ensure that good Ad2 growth was not due to the its higher input concentration when compared to that of Ad40 and Ad41, cultures were infected with identical concentrations of virus (10^3 FFU/dish or 6.7×10^2 FFU/ml). The results are shown in Figure 3.9A and 3.9B. The intestinal tissue was obtained from 12-week old twins. The results were as before with Ad2 showing a marked increase over time and Ad40 and Ad41 having a low and fairly constant release of free hexon antigen.

The effect of trypsin on virus growth (using hexon release as indicator) was determined by inclusion in the maintenance medium at a concentration of 0.5μg/ml. Figure 3.10 illustrates the effect of trypsin on the growth of Ad2 (A), Ad40 (B), Ad41 (C), and FB585 (D), an Ad41 variant inoculated directly as stool extract. FB585 was included to determine if any differences could be detected due to virus passage in tissue culture as opposed to infection with virus directly from stool. The hexon release of FB585 was no different to that of the tissue culture passaged viruses. Trypsin had no effect on Ad2 growth as determined by hexon release. The presence of trypsin in the medium resulted in enhanced Ad40, Ad41, and FB585 growth. This was especially evident for the FB585 stool extract virus. However, upon repitition with intestinal tissue from another foetus, the addition of trypsin did not have any effect. That is, there was no difference in the optical density values obtained from medium removed from trypsin treated and untreated tissue over the same time period.

The results showed that Ad40 and Ad41 growth, monitored through group-specific hexon antigen production, was poor in comparison to that of Ad2 in foetal intestinal organ cultures.

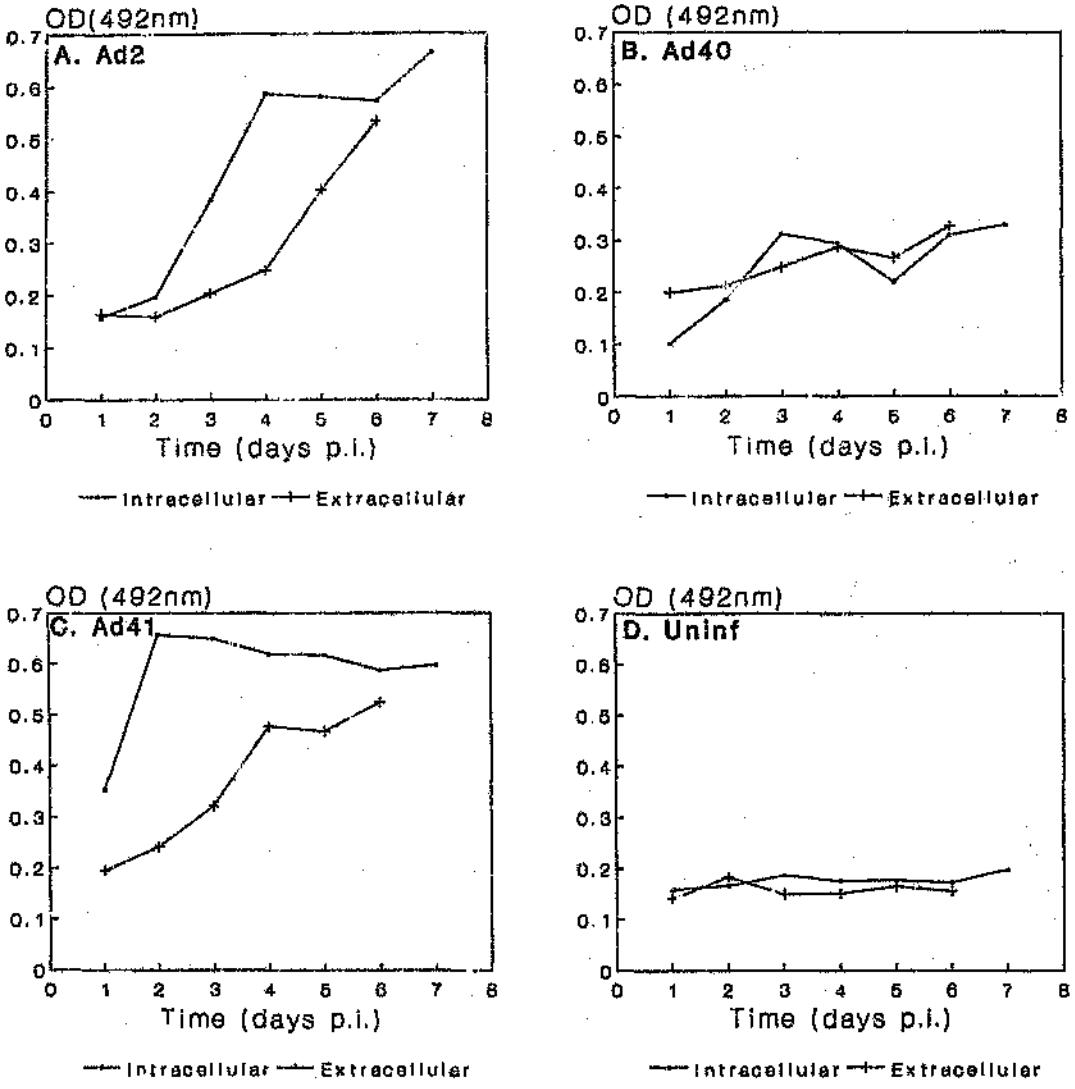


Figure 3.1 Comparison of intracellular and extracellular hexon levels at various times after infection of 293 cells. Cells in 24-well dishes were infected with 2.4 x 10^5 FFU/well of Ad2 (A), Ad40 (B), and Ad41 (C). The uninfected control received PBS (D). Intracellular and extracellular hexon accumulation was monitored by indirect ELISA. Optical density values were the average of two replicates.

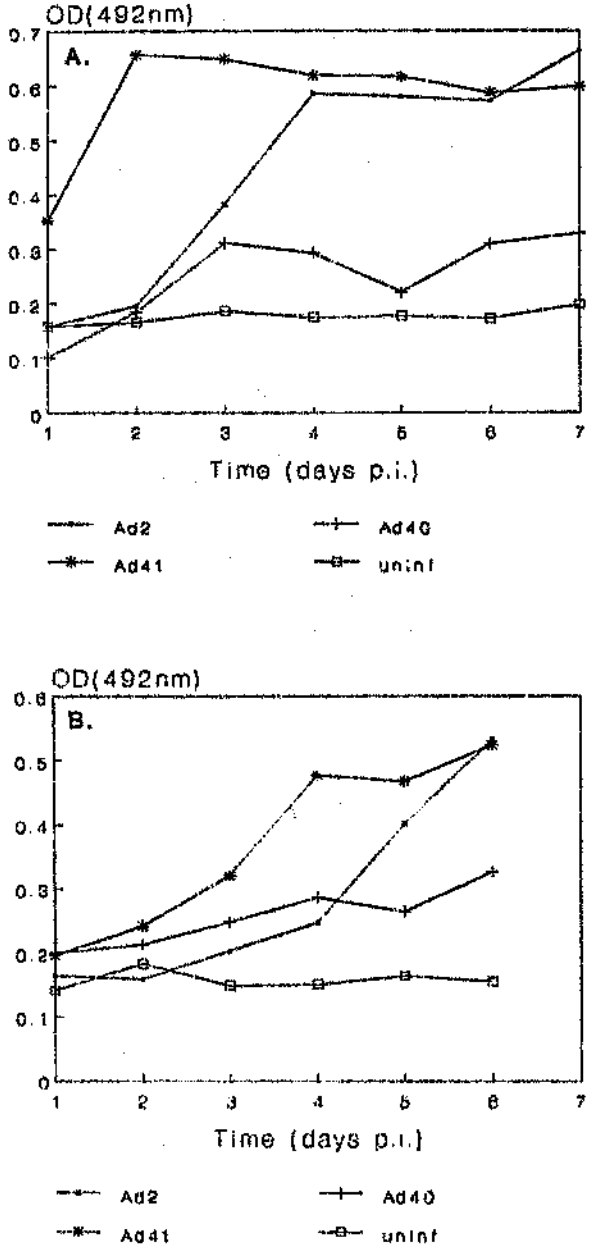


Figure 3.2 Comparison of Ad2, Ad40, and Ad41 hexon accumulation within infected 293 cells (A) and of hexon released into the medium (B) over time.

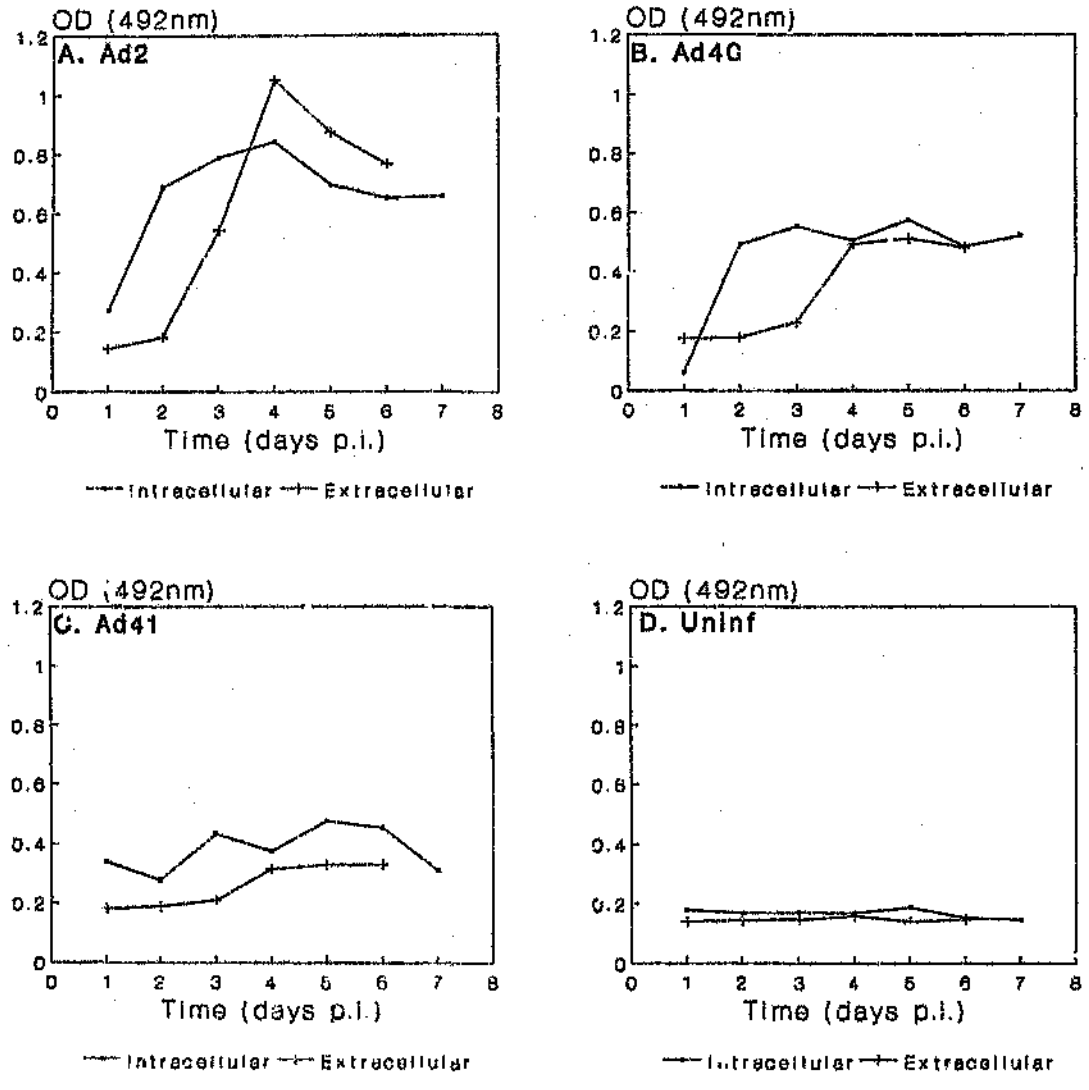


Figure 3.3 Comparison of intracellular and extracellular hexon levels at various times after infection of Chang cells. Cells in 24-well dishes were infected with 2.4 x 10^3 FFU/well of Ad2 (A), Ad40 (B), or Ad41 (C). The uninfected control received PBS (D). Intracellular and extracellular hexon accumulation was monitored by indirect ELISA.

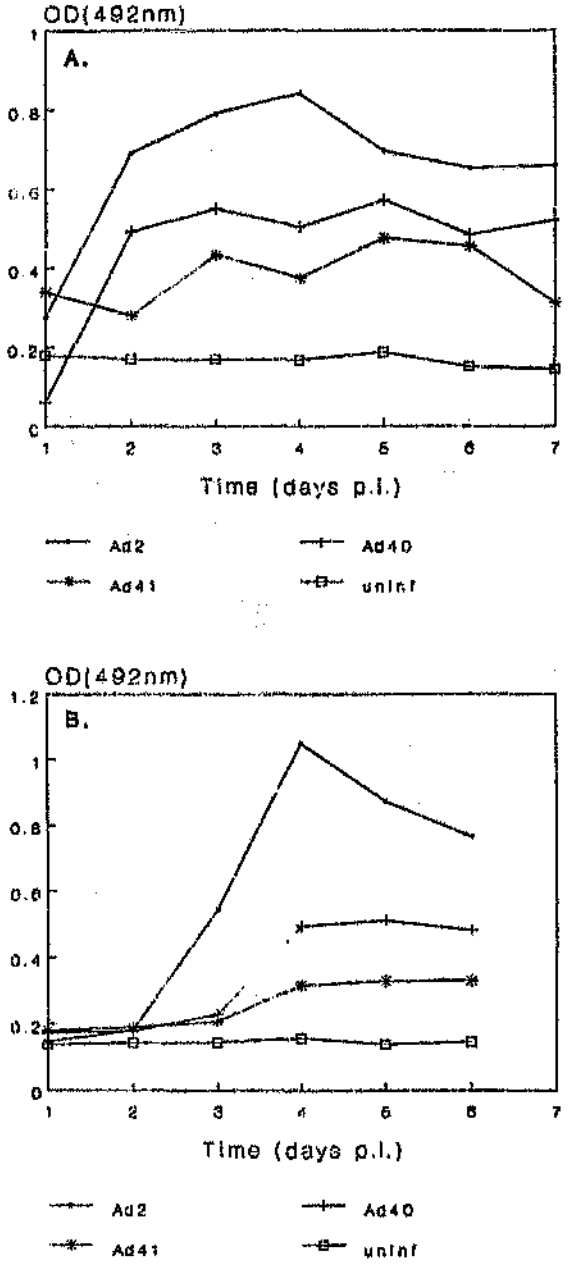


Figure 3.4 Comparison of Ad2, Ad40, and Ad41 hexon accumulation within infected Chang cells (A) and of hexon released into the medium (B) over time.

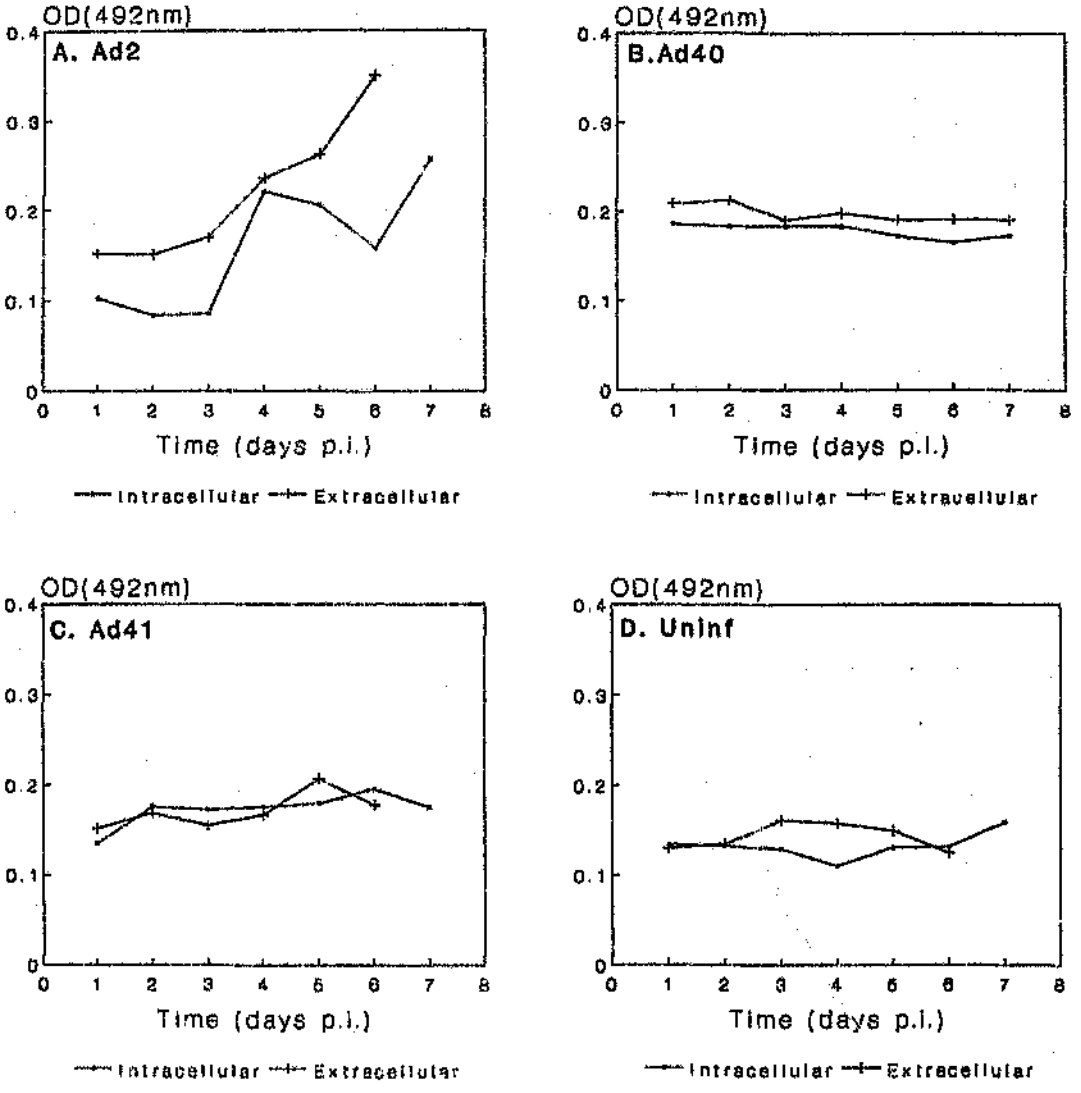
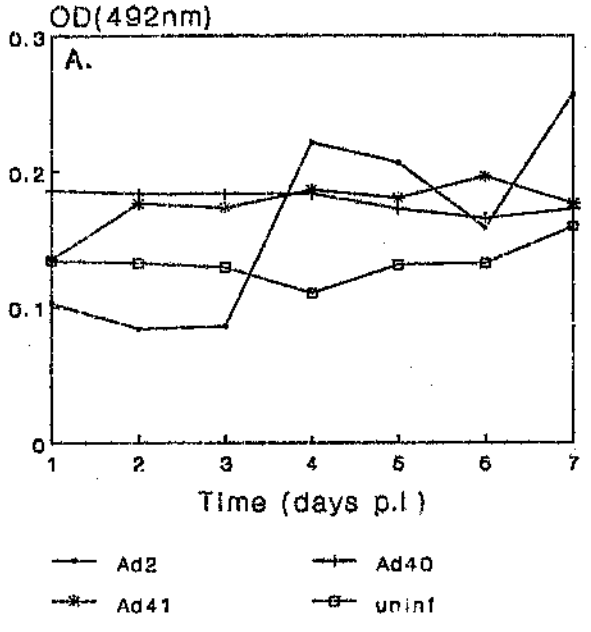


Figure 3.5 Comparison of intracellular and extracellular hexon levels at various times after infection of HEF cells. Cells in 24-well dishes were infected with 2.4 x 10^3 FFU/well of Ad2 (A), Ad40 (B), or Ad41 (C). The uninfected control received PBS (D). Intracellular and extracellular hexon accumulation was monitored by indirect ELISA.



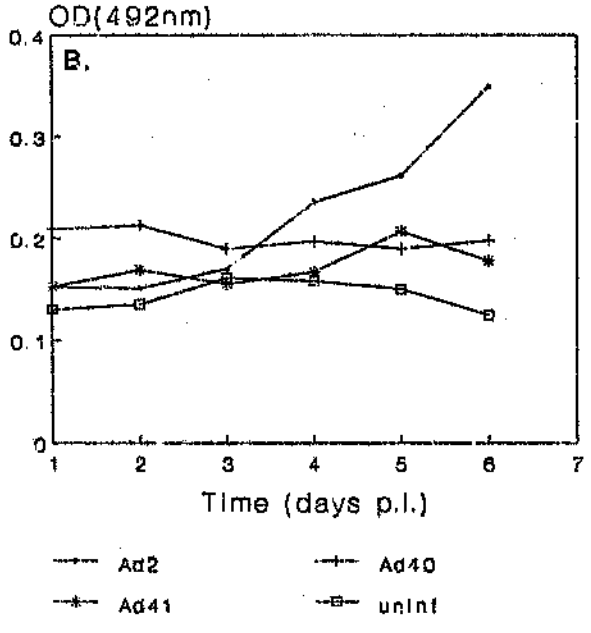


Figure 3.6 Comparison of Ad2, Ad40, and Ad41 hexon accumulation within infected HEF cells (A) and of hexon released into the medium (B) over time.

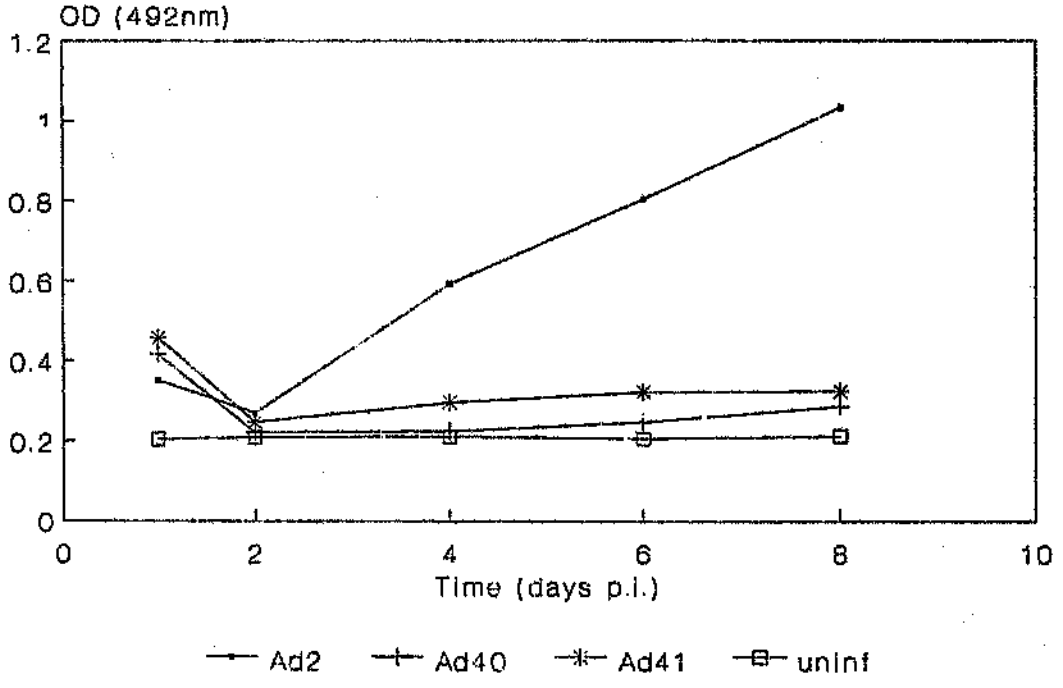


Figure 3.7 Accumulation of adenovirus hexon over time in medium from infected foetal intestinal organ cultures. Triplicate cultures were infected with 10^4 FFU/dish of Ad2, 10^3 FFU/dish of Ad40 or Ad41, or mock-infected with PBS. Fluids were harvested at indicated times and hexon levels monitored using an indirect ELISA. Optical densities were determined at 492nm.

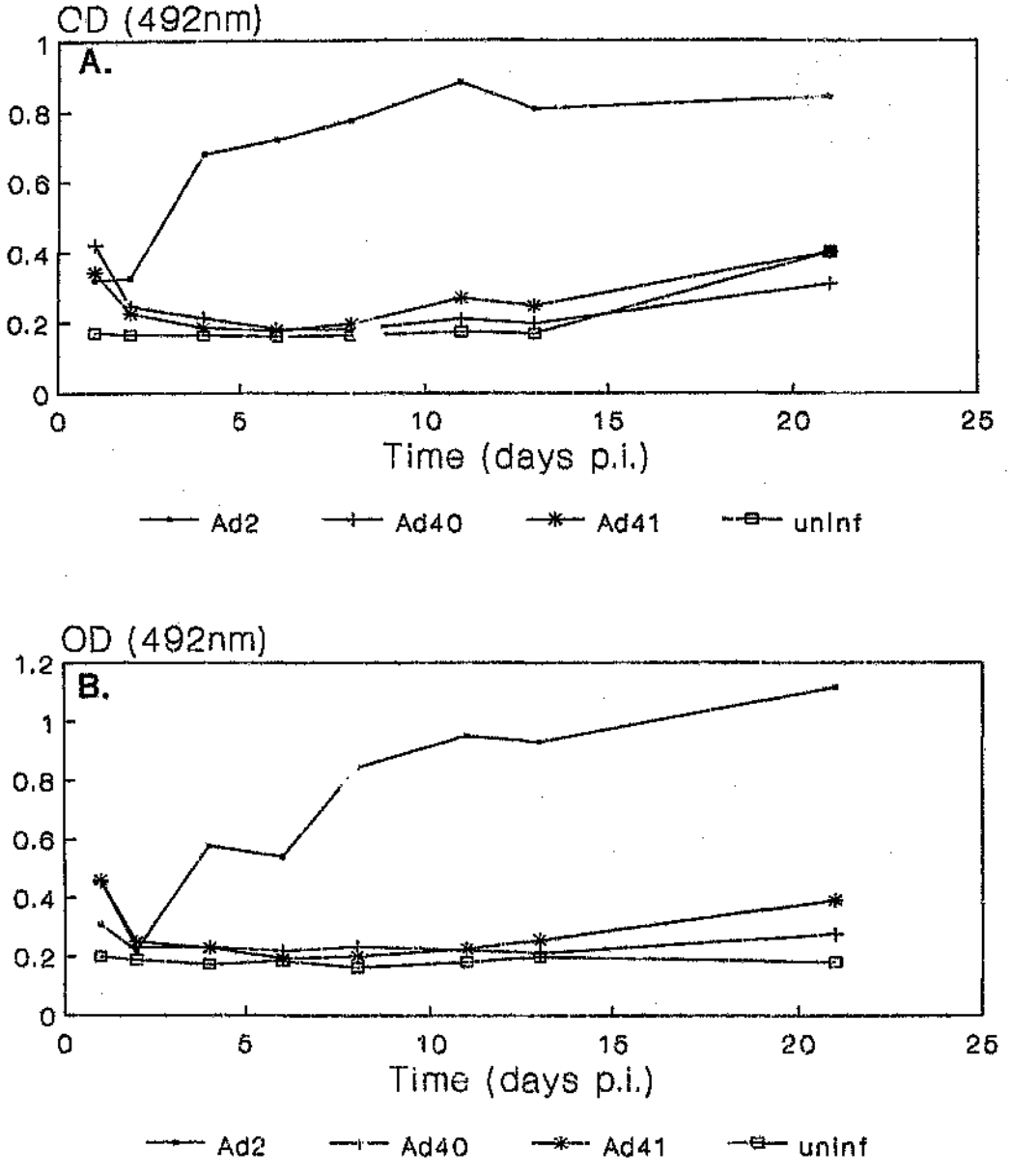


Figure 3.8 Accumulation of adenovirus hexon over time in medium from foetal intestinal organ cultures prepared from intestines of foetuses of different age. A. 8 weeks old. B. 12 weeks old. Triplicate cultures were infected with 10^6 FFU/dish of Ad2 and 10^3 FFU/dish for Ad40 and Ad41. Uninfected controls received PBS.

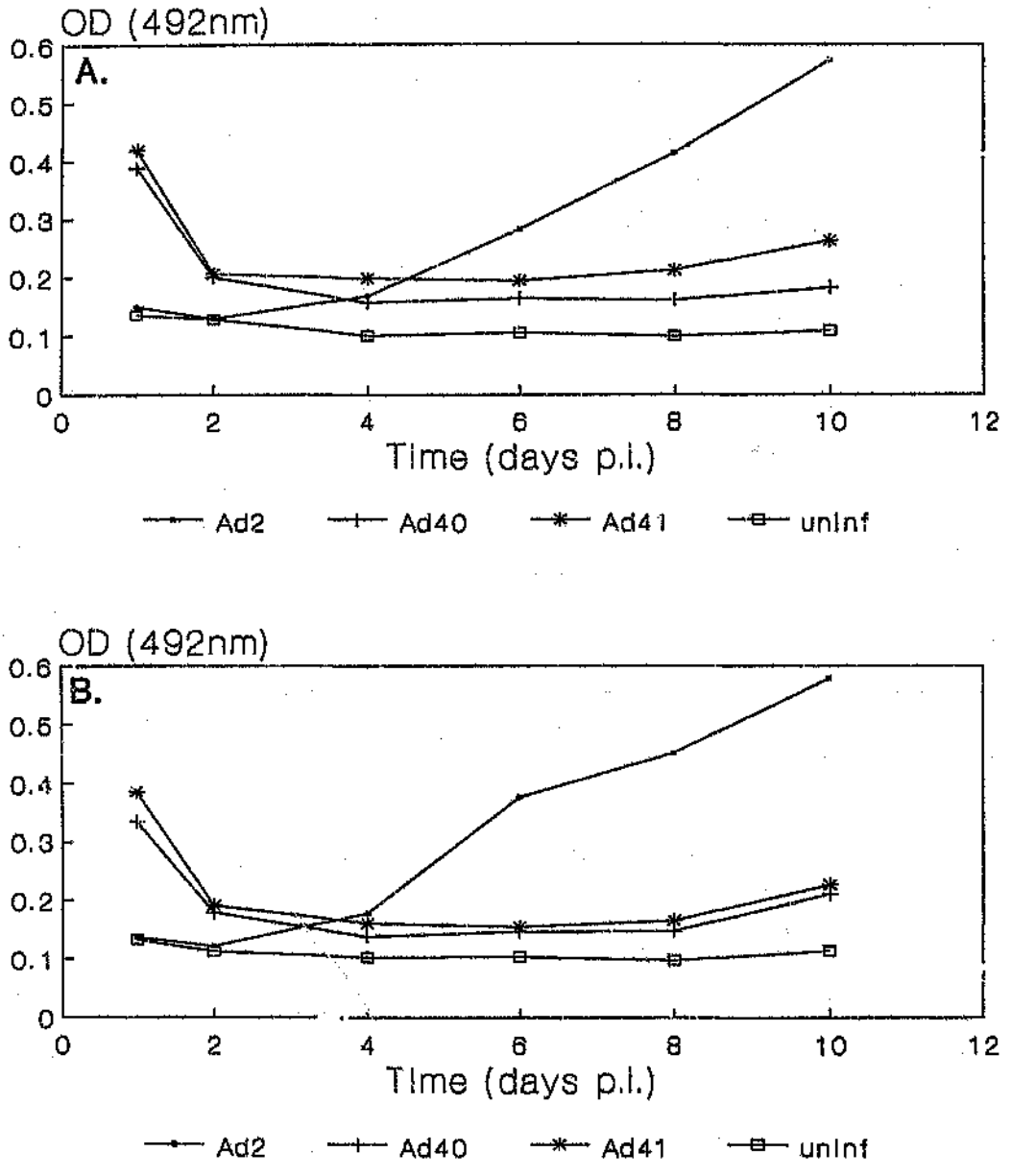


Figure 3.9 Accumulation of adenovirus hexon over time in medium from foetal intestinal organ cultures prepared from twin foetuses, 12 weeks of age. A. Twin 1. B. Twin 2. Triplicate cultures were infected with Ad2, Ad40, and Ad41 at a concentration of 10^3 FFU/dish, fluids harvested at indicated times and optical densities determined by ELISA.

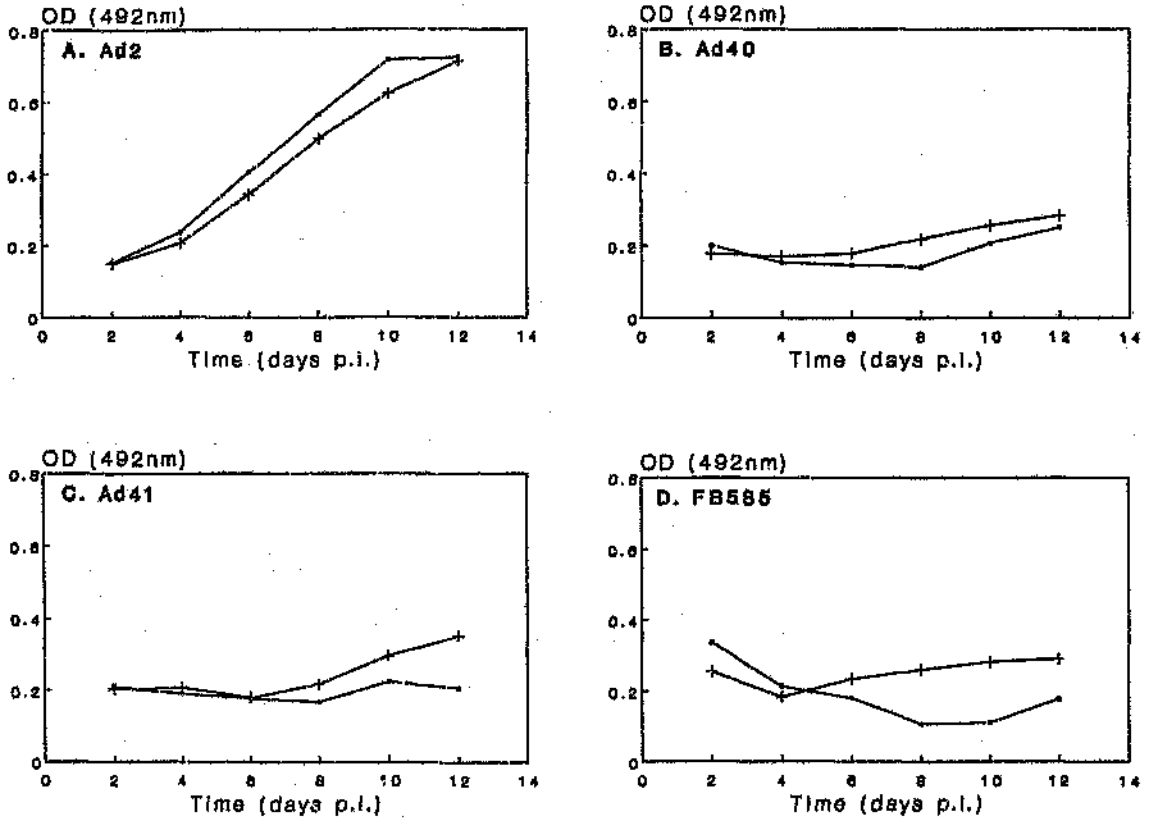


Figure 3.10 Effect of trypsin on hexon accumulation in medium from foetal intestinal organ cultures. Two sets of triplicate cultures were infected with Ad2 (A), Ad40 (B), and Ad41 (C) at concentrations of 10^3 FFU/dish, and 50μl of Ad41 FB585 (D) clarified stool extract. One set had medium with trypsin (0.5μg/ml) added from the time of culture preparation and for the duration of infection. Fluids were harvested at indicated times and hexon accumulation monitored by ELISA.

3.2.2 Kinetics of infection

A fluorescent focus assay was employed to investigate the relationship between virus concentration and infectivity. Adenovirus 41 late antigen synthesis was monitored by indirect immunofluorescence using a subgroup F specific mouse monoclonal antibody. Fluorescent cell counts were averaged from the results of 4 replicate coverslips. Differences between counts in single infection and corresponding counts in coinfection were evaluated using the Wilcoxon signed rank sums test (see Appendix D). Slopes were compared by linear regression analysis (Armitage, 1971).

A stock preparation of Ad41 at 2.4 x 10^4 FFU/ml was diluted serially and titrated by immunofluorescence in Chang cells, which are semi-permissive for this virus. Beyond a 1/2 dilution, there were virtually no fluorescent cell counts. A 1/8 dilution would have been expected to give one quarter of the counts measured at a 1/2 dilution, but the reduction determined at 44 hours p.i. in two separate experiments was 400-fold (Table 3.1). The slopes of logarithm of fluorescent cell counts against logarithm of input concentration did not differ significantly from the slope predicted for one-hit kinetics ($p < 0.05$).

The drastic reduction in infectivity when the inoculum was diluted beyond 1/4 suggested an infection with multiple-hit kinetics. If a number of particles (n) rather than one is required to initiate an infectious centre or fluorescent cell, the number of infected foci should decrease with the nth power of the inoculum dilution (where $n > 1$)(Luria *et al*, 1978). In this case, the actual reduction in infectivity over a 4-fold dilution was 400-fold (4^n), thus n, the number of particles required to productively infect a Chang cell, was calculated to be 4.32.

Coinfections of Chang cells with Ad41 and Ad2 were performed to determine if the presence of Ad2 as helper (see Chapter Four) could result in efficient Ad41 infection of one-hit character. Three Ad2 concentrations were used, and each of these

was kept constant for a range of Ad41 input concentrations (Table 3.1), which were 1/2, 1/4, 1/8, and 1/10 dilutions of stock Ad41. A significant difference was noted between the slopes of the dose-response graph of Ad41 in single infection and those obtained for coinfection with an Ad2 input concentration of 1 x 10^3 and 3 x 10^4 FFU/ml (Figure 3.11). The presence of Ad2 at either concentration had the effect of altering the Ad41 dose response graph to a slope approaching that expected for two-hit kinetics. Although Ad41 counts at each Ad41 dilution were significantly elevated at the higher Ad2 concentration, the slopes did not differ significantly from each other. This is in agreement with the finding that the degree of complementation is dependent on Ad2 concentration (see Chapter Four).

Table 3.1 Promotion of Ad41 late antigen synthesis in Chang cells by Ad2 at different input concentrations of either virus[a]

Ad2 input concentration (FFU/ml)	Ad41 fluorescence (FFU/10^6 cells)			
	Ad41 input concentration (FFU/ml)			
	1.2 x 10^4	6 x 10^3	3 x 10^3	2.4 x 10^3
0[b]	637.99 ± 162.78	32.34 ± 14.79	1.59 ± 2.23	0
1 x 10^3	949.63 ± 143.58	368.95 ± 174.09	56.12 ± 31.78	13.93 ± 11.13
0[b]	977.49 ± 370.68	91.54 ± 38.40	2.39 ± 2.05	0.39 ± 0.79
3 x 10^4	15,960.60 ± 2,556.80	1,947.02 ± 257.66	753.81 ± 84.59	36.62 ± 5.02
3 x 10^5	1 594.79 ± 838.67	58.11 ± 26.84	13.13 ± 12.89	18.71 ± 19.03

[a]Cells were coinfected simultaneously.
[b]Two separate experiments, each with controls of Ad41 in single infection.

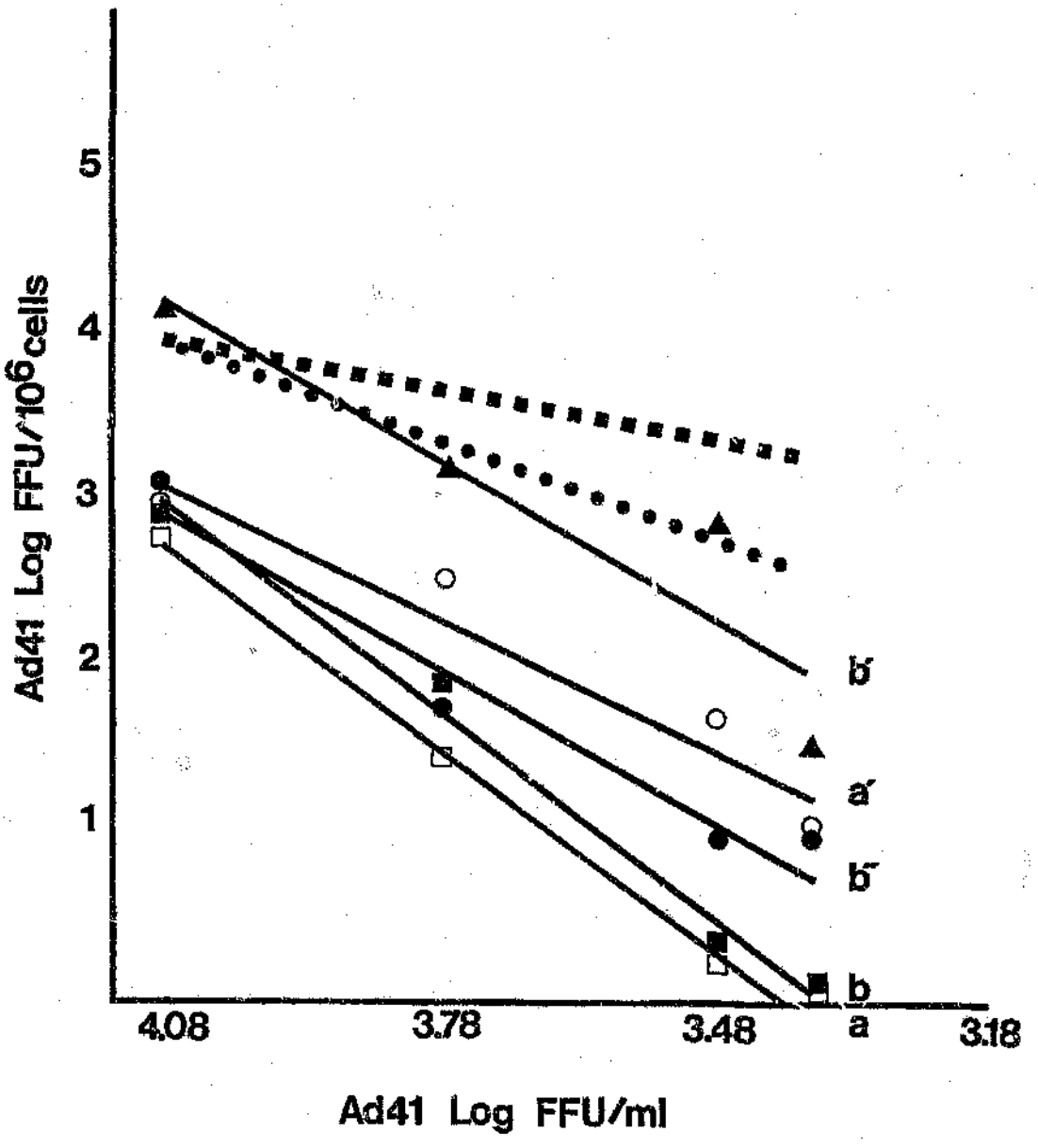


Figure 3.11 Relationship between Ad41 fluorescent cell counts and Ad41 input dose in single infection (a,□; b,■) and in coinfection of Chang cells with Ad2. For coinfections of Ad41 and Ad2, input concentrations of 1 x 10^3 FFU/ml (a',○), 3 x 10^4 FFU/ml (b',▲), and 3 x 10^5 FFU/ml (b'',●)were used. Experiment 1: a,a'; experiment 2: b,b',b''. Theoretical one-hit slope ■ ■ ■. Theoretical two-hit slope ● ● ●. Theoretical slopes were determined mathematically by choosing an arbitary fluorescent cell count value for the 1/2 dilution (1.2 x 10^4 FFU/ml) and calculating the values for subsequent dilutions assuming one- and two-hit kinetics.

A further increase in the Ad2 concentration in coinfection resulted in a dose-response graph similar in slope to those for lower input concentrations of Ad2, but Ad41 fluorescent cell counts at all Ad41 dilutions were substantially reduced. This may be explained by the high Ad2 concentration somehow interfering with Ad41 replication, and complementing it at the same time by another mechanism, the positive and negative effects nullifying each other. It is equally possible that such a high inoculum of Ad2 produced sufficient loss of infected cells by 44 hours p.i. as to give an underestimate of the true number of cells expressing late Ad41 antigens.

Unlike Chang cells, HEF cells are non-permissive for Ad41 growth unless pre- or co-infected with another adenovirus (see Chapter Four). Based on the above results, HEF cells were preinfected with Ad2 10 hours before infection with Ad41 to determine if the helper effect of Ad2 in these cells was sufficient to result in an Ad41 infection of one-hit character. The input concentrations of each virus are given in Table 3.2. The slope of the graph of mixed Ad41 and Ad2 infection of HEF cells (Figure 3.12) did not differ significantly from the slopes for coinfections in Chang cells, and was similar to that of the theoretical two-hit slope. Since Ad41 does not grow in HEF cells, the conversion from no growth to two-hit kinetics was solely dependent on the presence of Ad2.

293 cells, a line of human embryo kidney cells transformed with Ad5 E1 region DNA (Graham *et al*, 1977) were infected with concentrations of Ad41 as indicated in Table 3.3. These were 1/20, 1/40, 1/80, and 1/100 dilutions of stock Ad41. In contrast with the results in Chang cells, fluorescent counts decreased in proportion to the dilution factor. The dose response slope was not significantly different from the theoretical one-hit slope ($p < 0.05$), but differed from the theoretical two-hit slope ($p \geq 0.05$) (Figure 3.13), indicating that a single Ad41 particle is capable of initiating a fluorescent focus.

Table 3.2 The effect of mixed Ad41 and Ad2 infections on late antigen synthesis in HEF cells, at different input concentrations of Ad41

Ad2[a] input concentration[b] (FFU/ml)	Ad41 influorescence (FFU/10^6 cells)			
	Ad41 input concentration[b] (FFU/ml)			
	1.2×10^4	6×10^3	3×10^3	2.4×10^3
0	0	0	0	0
3×10^4	1,394.67 ± 880.23	685.40 ± 617.70	74.44 ± 34.44	68.82 ± 53.93

[a]HEF cells were preinfected with Ad2 10 hours before Ad41.
[b]All virus concentrations were determined by titration on Chang cells using indirect immunofluorescence.

Table 3.3 The effect of varying Ad41 input concentrations on late antigen synthesis in 293 cells

Ad41 fluorescence (FFU/10^6 cells)			
Ad41 input concentration[a] (FFU/ml)			
1.2×10^3	6×10^2	3×10^2	2.4×10^2
268.65 ± 33.15	143.61 ± 15.68	67.16 ± 10.76	22.86 ± 5.59

[a]Ad41 concentrations were determined by titration on Chang cells using indirect immunofluorescence.

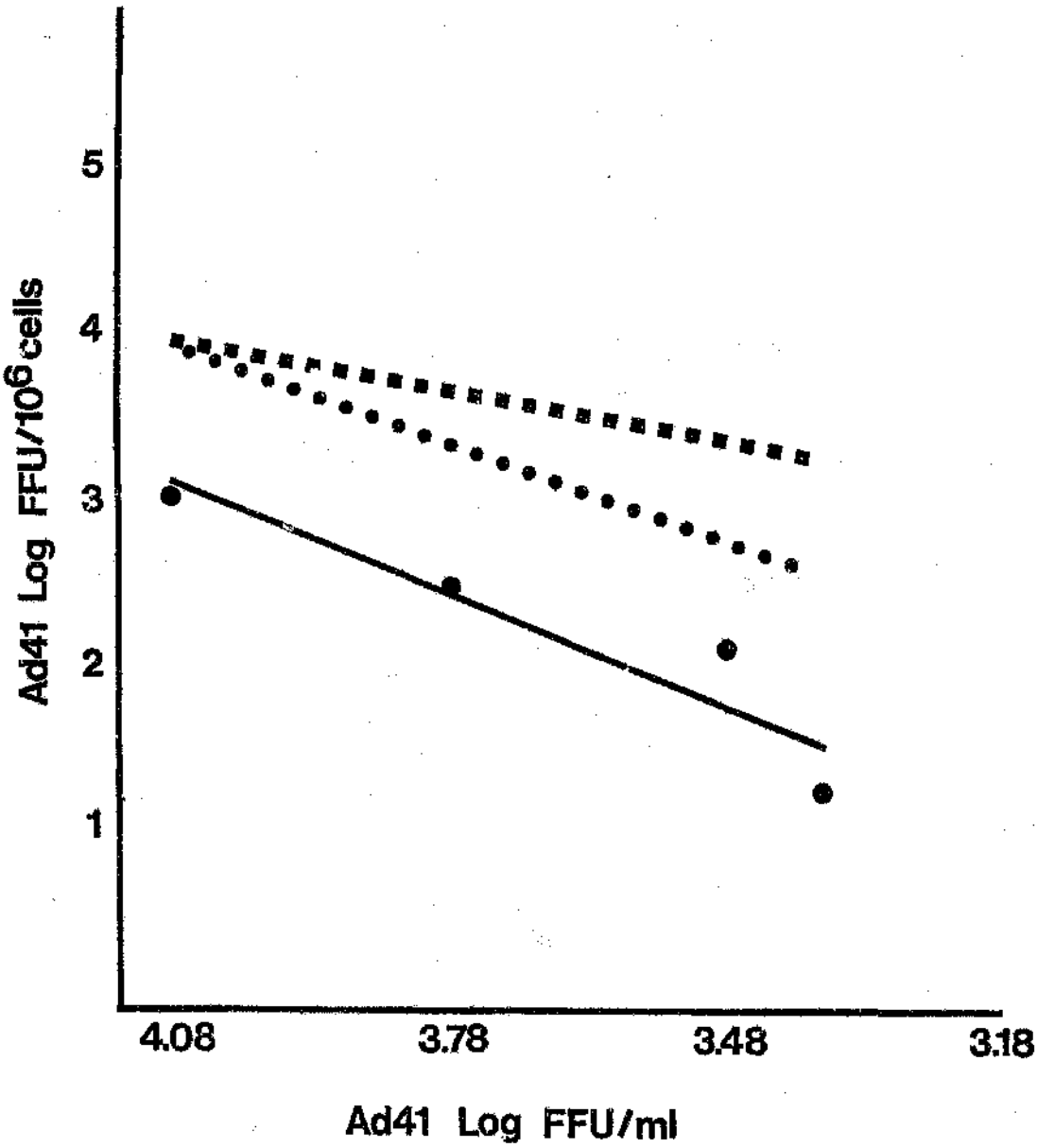


Figure 3.12 Relationship between Ad41 fluorescent cell counts and Ad41 input dose in coinfection of HEF cells with Ad2 and Ad41 (●). Input concentrations were determined in Chang cells. Theoretical one-hit slope ■ ■ ■. Theoretical two-hit slope ● ● ●.

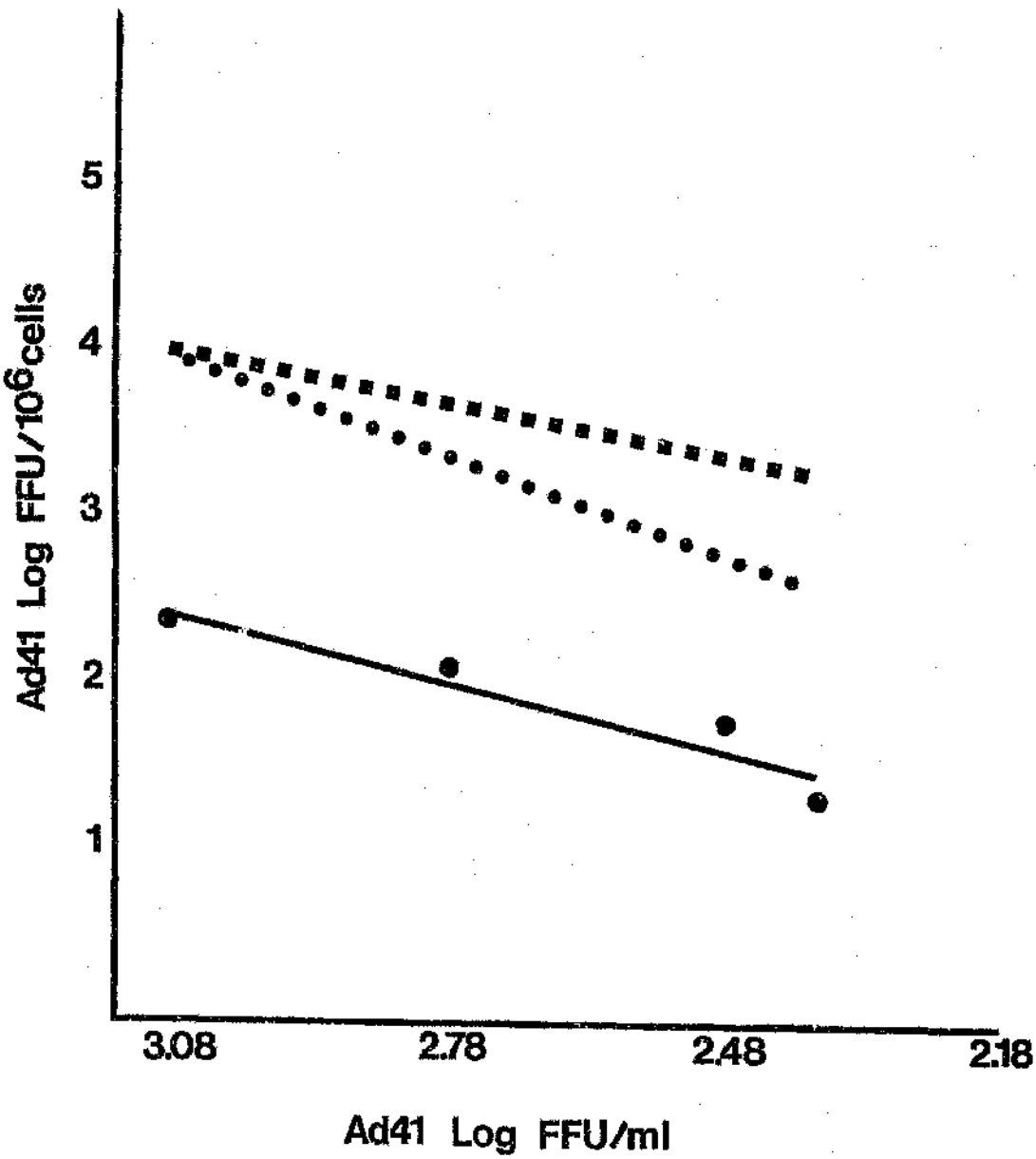


Figure 3.13 Relationship between Ad41 fluorescent cell counts and Ad41 input dose in single infection of 293 cells (●). Theoretical one-hit slope ■ ■ ■. Theoretical two-hit slope ● ● ●. Theoretical slopes were determined mathematically by choosing an arbitary fluorescent cell count value for the 1/20 dilution (1.2×10^3 FFU/ml) and calculating the values for subsequent dilutions assuming one- and two-hit kinetics.

3.2.3 Detection of virus DNA synthesis

3.2.3.1 Detection of newly synthesized viral DNA

^{32}P-labelling of newly synthesized DNA *in vivo* Infected cells in 25cm^2 flasks were labelled with of ^{32}P-orthophosphate 6 hours after infection and harvested when CPE became apparent or when they became detached due to toxicity. DNA was then extracted and digested with restriction endonucleases *Eco*RI and *Xho*I. The products were separated on 1% agarose gels. Figure 3.14A shows the resultant characteristic DNA profiles of Ad2 and Ad41 in 293 cells. The DNA doublet evident in the *Eco*RI digested DNA of uninfected 293 cell DNA was most likely due to a contaminant which did not interfere with virus replication. Viral DNA synthesis was further demonstrated in Chang cells (Figure 3.14B). Adenovirus 41 DNA synthesis could not be detected in HEF cells (Figure 3.14C).

3.2.3.2 Time course of DNA detection in semi-permissive and non-permissive cells

Adenovirus 2, Ad40 and Ad41 infected Chang and HEF cells were harvested at the times indicated in Figure 3.15. For the detection of packaged DNA the cells were frozen and thawed four times, treated with DNase and the DNA extracted from the virions. Packaged and unpackaged DNAs were dotted on nylon membranes and Ad40 and Ad41 DNAs hybridized with ^{32}P-labelled Ad40 derived probe, N26*H*. The DNA region contained within this plasmid also hybridizes to the corresponding region of Ad41 DNA, although not as strongly as with Ad40 DNA and can therefore be used to detect both types. A ^{32}P-labelled plasmid containing the E2a gene of Ad2 (pSPT18-Ad2 *Sma*L4) was used to probe Ad2 dot blots.

A comparison of DNA synthesized and packaged over time for Ad2 (A), Ad40 (B) and Ad41 (C) is shown in Figure 3.15. As expected, DNA replication and

packaging of Ad2 DNA in Chang cells over time was much more efficient than in HEF cells. Approximately 10% of the Ad2 DNA synthesized was packaged in both cell types.

In Chang cells, Ad40 and Ad41 DNA synthesis could be detected from day two onwards. The relative amount of Ad40 DNA packaged in Chang cells was approximately 1%. Packaged DNA was first detected three days after infection, increased over the next two days and then declined. Adenovirus 41 DNA synthesis and packaged DNA reached a peak from days two to four and declined thereafter. Packaged DNA could be detected earlier and over a longer period of time compared to that of Ad40. The proportion of Ad41 DNA that ultimately becomes packaged was also less than that for Ad2 and was estimated to be about 1% of the total Ad41 DNA synthesized.

No DNA was synthesized or packaged into virions in HEF cells infected with Ad41. In contrast, Ad40 DNA was synthesized in these cells but the DNA was apparently not packaged. It can be argued that the amount of DNA packaged may have been below the level of detection if similar relative proportions of DNA incorporation into virions obtained with Chang cells is expected for HEF cells. However, longer exposure of Ad40 dot blots showed an increased signal for intracellular DNA but did not show any evidence of DNA packaged into virions.

3.2.3.3 Determination of DNA integrity in infected Chang and HEF cells

^{3}H-thymidine labelling of viral DNAs *in vivo* Cells in 25cm^2 flasks were infected with 500μl 2.4 x 10^4 FFU/ml of Ad2, Ad40 and Ad41. Infected Chang and HEF cells were labelled as described in Figure 3.16 and 3.17, respectively. The samples were treated and loaded on 5-20% alkaline sucrose gradients as described in section 2.21.2.1.

The results obtained for incorporation of ^{3}H-thymidine into newly synthesized viral DNA in Chang cells is shown in Figure 3.16. Adenovirus 2 infected cells were included as a control for comparison. In Chang cells, a discrete peak of labelled Ad40 DNA in the region of the gradient close to Ad2 DNA could not be detected. There was however labelled DNA corresponding to the smaller size classes of DNA from fractions close to the top of the gradient. This appeared to be a result of viral DNA degradation rather than cell DNA as seen by comparing incorporation of label into uninfected cell DNA. Adenovirus 41 showed two discrete populations of DNA, one that sedimented close to unit length (34S) Ad2 DNA and another that sedimented in a lower sucrose density fraction. Neither of these two DNA populations were detected when cells were labelled for 8 hours at 24 hours p.i.

Adenovirus 40 and Ad41 infected HEF cells were labelled with ^{3}H-thymidine at three and 6 days p.i. for a period of three days. This was to allow for incorporation of radiolabel into slowly replicating viral DNA. Lysate from Ad2 infected Chang cells from the above analysis was used as a control for size comparison in zone sedimentations. The results are shown in Figure 3.17. No newly synthesized DNA could be detected in Ad41 infected HEF cells, confirming the findings in 3.2.3.1 and 3.2.3.2. In Ad40 infected HEF cells, on the other hand, incorporation of radiolabel into a wide range of DNA size classes occurred from day three to 6 p.i., with the most abundant labelled DNA in fractions 11 to 17. When infected cells were labelled from days 6 to 9 p.i. there was a decrease in the uptake of radiolabel into DNA and a shift in the more abundant size class to higher molecular weight forms, corresponding to fractions 5 to 9. Consistent with this, dot blot hybridization of Ad40 DNA (Figure 3.15C) showed that virus specific DNA could be detected mainly between days three to 5 and appears to decline thereafter. This may be a result of degradation of DNA by nucleases with time.

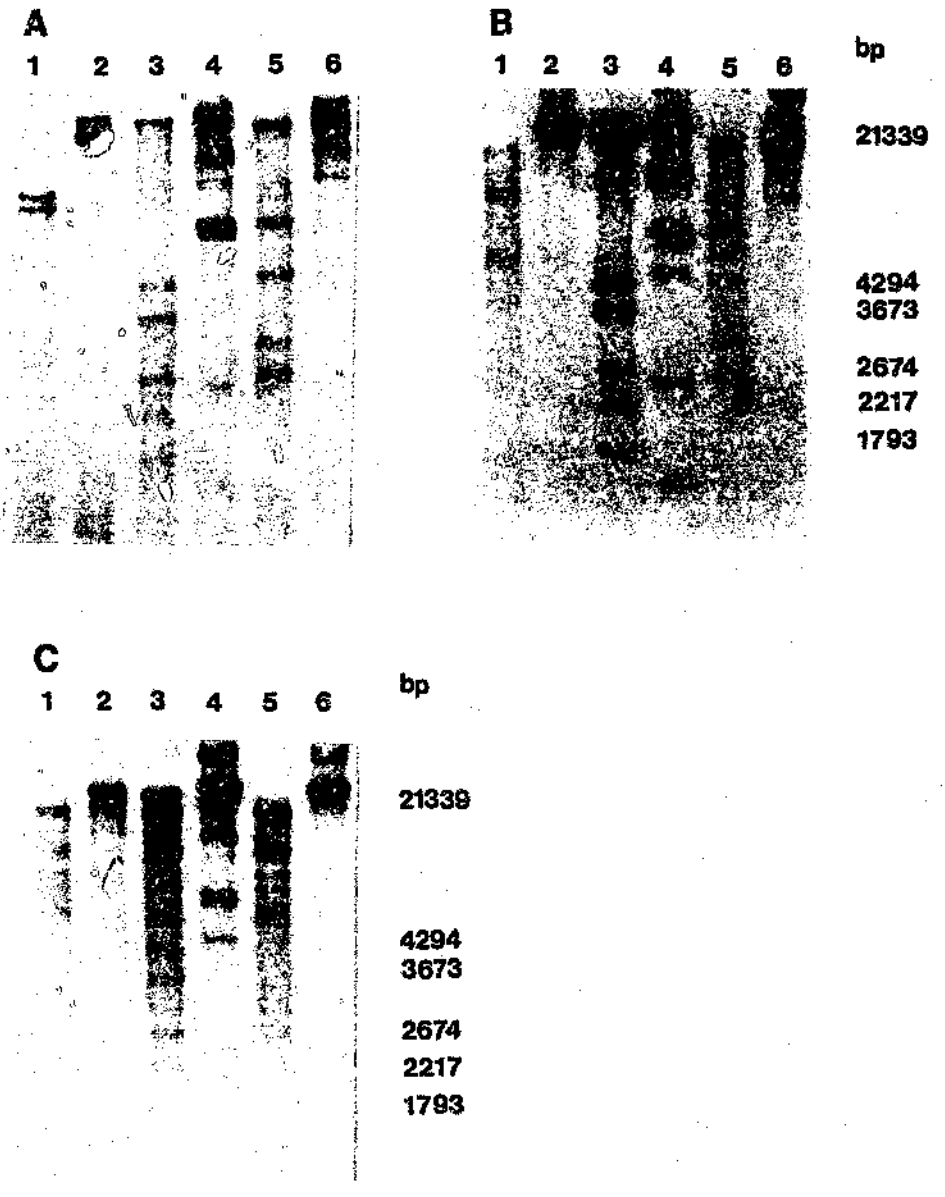


Figure 3.14 Autoradiograph of restriction enzyme profiles of Ad2 and Ad41 DNA in 293 (A), Chang (B), and HEF (C) cells. DNA was labelled *in vivo* with ^{32}P-orthophosphate, extracted and digested with restriction enzymes *Eco*RI and *Xho*I. The products were separated on a 1% agarose gel and the gel dried under vacuum. Lane 1. Uninfected DNA digested with *Eco*RI. Lane 2. Uninfected DNA digested with *Xho*I. Lane 3. Ad2 infected cell DNA digested with *Eco*RI. Lane 4. Ad2 infected cell DNA digested with *Xho*I. Lane 5. Ad41 infected cell DNA digested with *Eco*RI. Lane 6. Ad41 infected cell DNA digested with *Xho*I. Sizes (bp) of Ad2 *Eco*RI fragments are indicated.

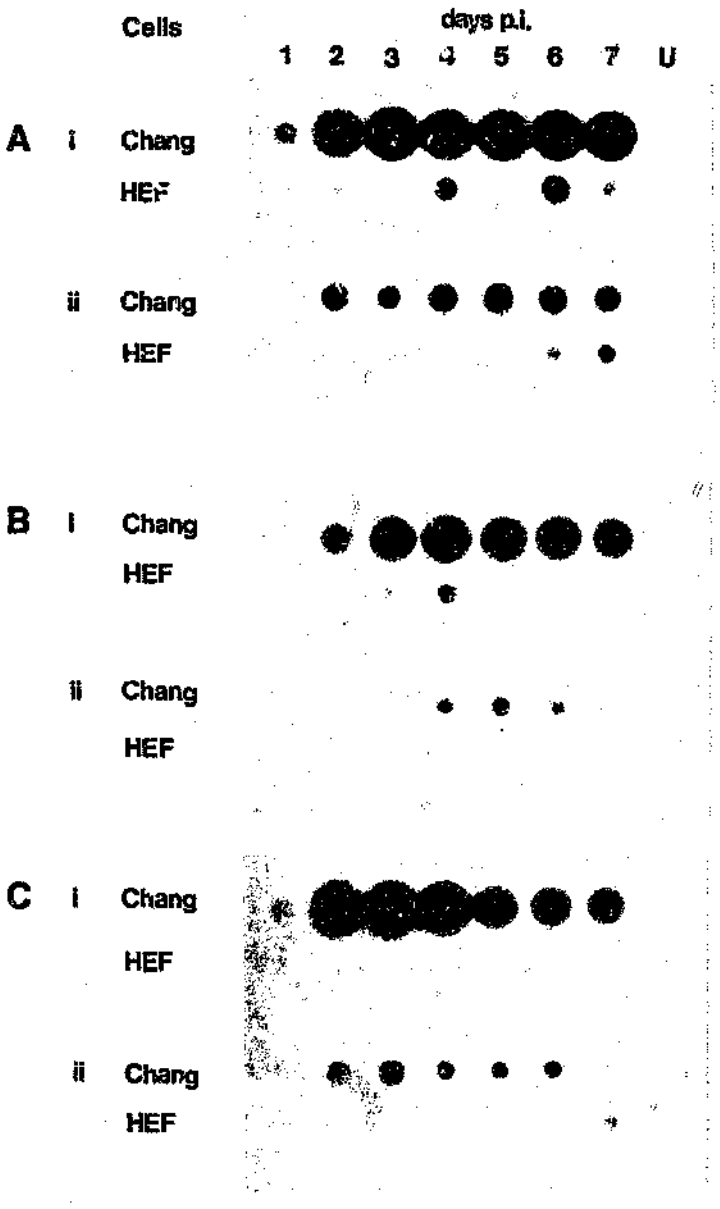


Figure 3.15 Dot blot hybridizations showing the time course of DNA synthesis (i) and packaging of viral DNA (ii) in Ad2 (A), Ad40 (B), and Ad41 (C) infected Chang and HEF cells. DNA was extracted from infected cells at the indicated times (days), dotted on nylon membranes and Ad2 blots hybridized to pSPT18-Ad2 *Sma*I*A* and Ad40 and Ad41 blots hybridized to the Ad40 derived probe, N26*H*. Autoradiographs were developed after 5 hours (Ad2 and Ad40) and 24 hours (Ad41). The sample dotted in C(ii) D7 p.i. (HEF cells) was incorrectly placed and corresponds to Ad41 DNA p.. kaged in Chang cells at D7 p.i. Most of the Ad41 DNA remained upon transferral of the sample to the correct position. U. Uninfected control.

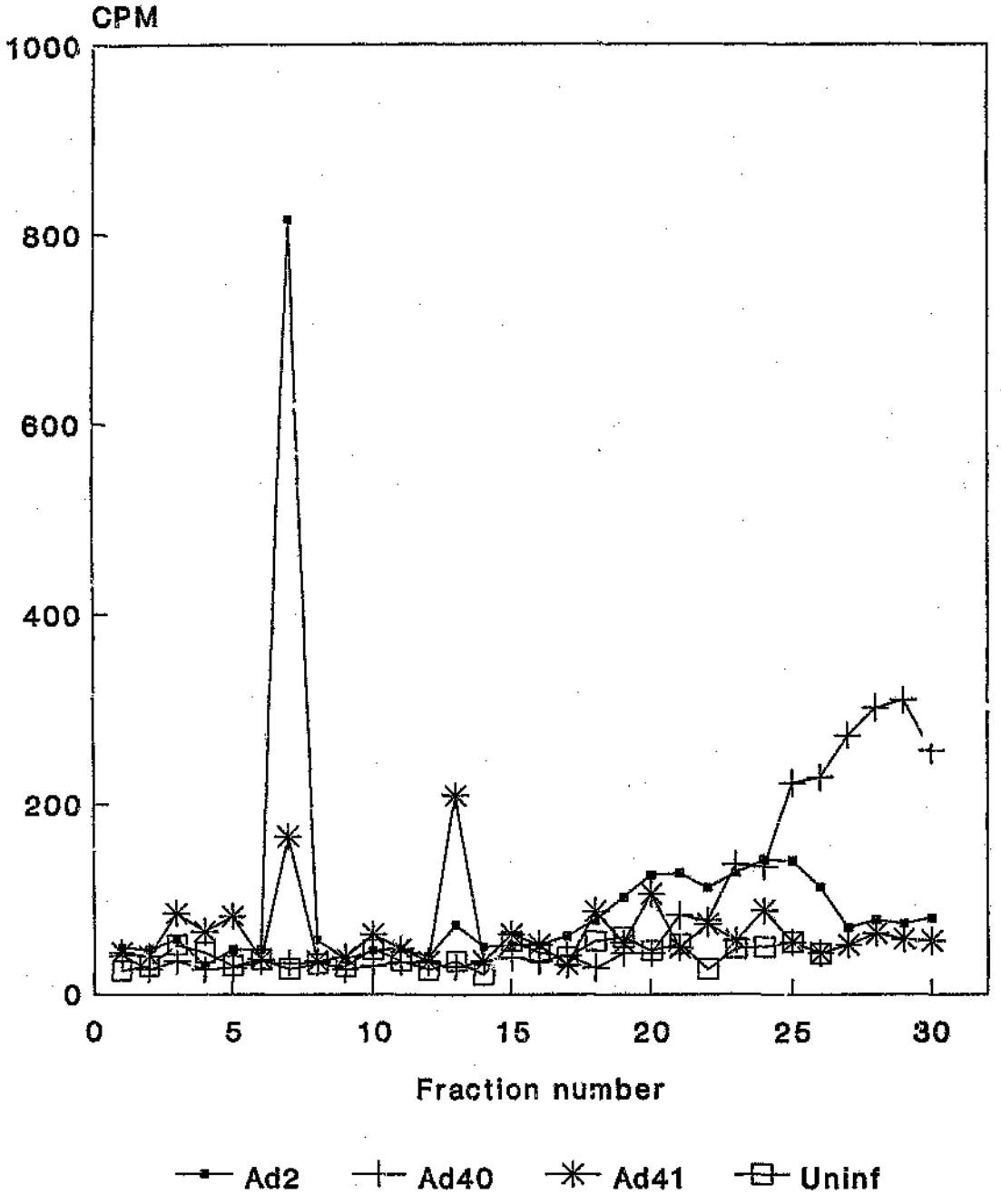


Figure 3.16 Alkaline sucrose gradient centrifugation of ^{3}H-thymidine labelled viral DNAs in Chang cells. Cells were labelled 48 hours after infection for 8 hours, lysed and spun on 5-20% alkaline sucrose gradients at 34,500rpm at 4°C for 5 hours in a SW40 rotor. Fractions were collected, dotted on nitrocellulose and counted in a liquid scintillation counter.

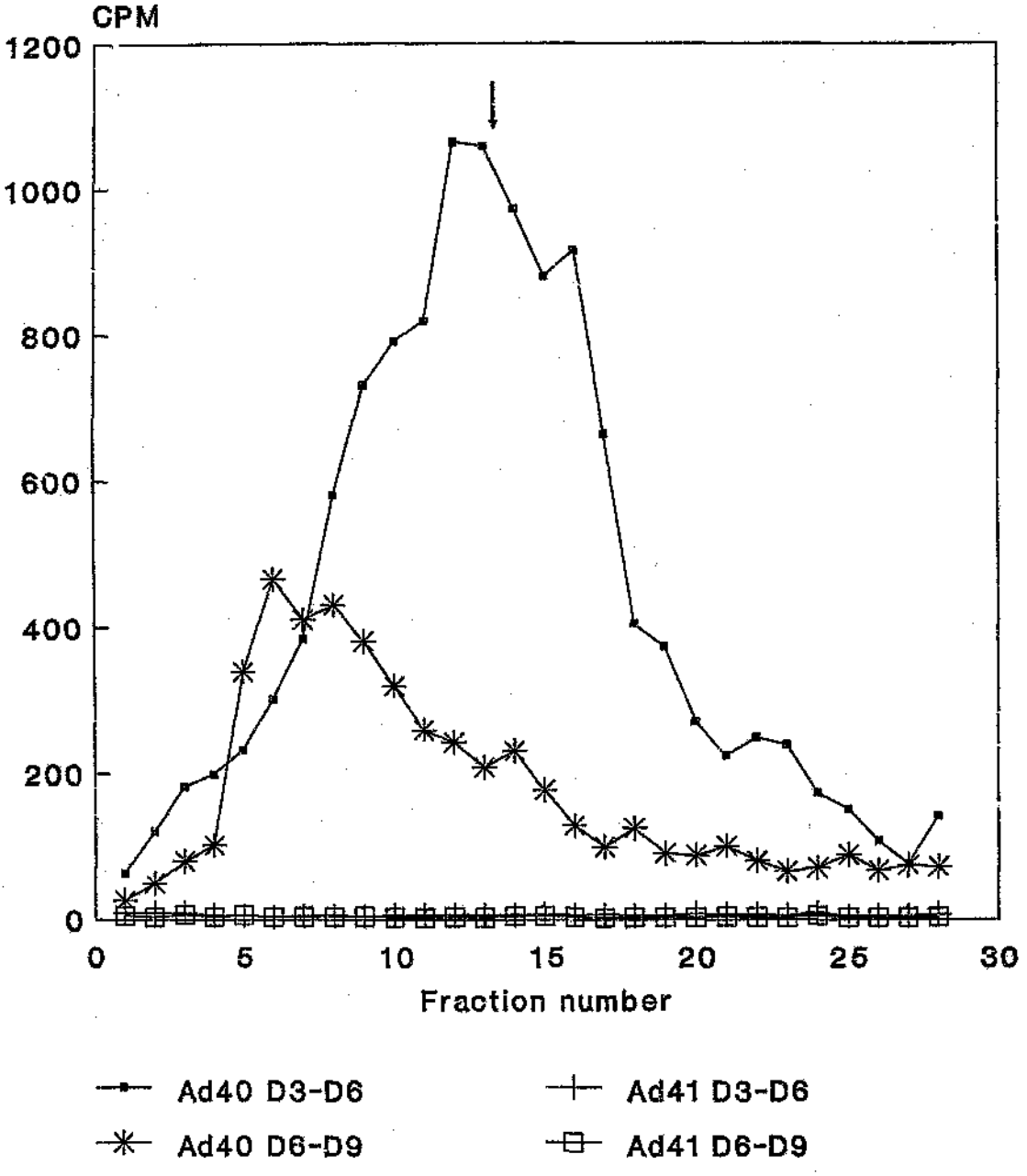


Figure 3.17 Alkaline sucrose gradient centrifugation of ^{3}H-thymidine labelled Ad40 and Ad41 DNAs in HEF cells. Cells were labelled for 3 days at day three (D3-D6) and day 6 (D6-D9) p.i., lysed and spun on 5-20% alkaline sucrose gradients at 34,500rpm at 4°C for 5 hours in a SW40 rotor. Lysate of Ad2 infected Chang cells was included as a marker. Fractions were collected, dotted on nitrocellulose and counted in a liquid scintillation counter. The position of the Ad2 marker DNA is indicated with an arrow.

3.2.4 Detection of host protein shutoff

The ability of Ad40 and Ad41 to shut off host protein synthesis was determined in 293 and Chang cells. Cells in 25cm^2 flasks were infected with 2.4 x 10^4 FFU/ml of virus and labelled with ^{35}S-methionine for one, 8 or 20 hours at specific time intervals. Detection of shut off of host protein synthesis was found to be unaffected by the time allowed for incorporation of ^{35}S-methionine. The results for infected Chang cells labelled *in vivo* for one hour at 24 hour intervals are shown in Figure 3.18. The Ad2 proteins can be clearly seen, but no Ad40 and Ad41 proteins could be detected over the time period tested. When the labelling time was increased to 20 hours and a larger dose applied for Ad40 and Ad41, only Ad41 hexon protein in 293 cells could be detected at both input doses. Adenovirus 2 proteins could be detected in both cell types but in the absence of host protein shutoff (Figure 3.19). The time selected for the addition of the label in this case was too early. Longer labelling times could also contribute to an increased background of cellular proteins. The same experiment was repeated but with a labelling period of 8 hours beginning at 48 hours after infection (Figure 3.20). Adenovirus 2 and Ad41 proteins were detected in 293 cells with no evidence of efficient host protein shutoff. Adenovirus 40 proteins were not detected in either cell type. No Ad41 proteins were seen in Chang cells.

To clarify the time- and dose- dependent parameters of host protein synthesis, Chang cells were infected with different concentrations of Ad2 and labelled for 20 hours at 24 hour intervals over a 5 day period. At the highest concentration of Ad2 used (2.4 x 10^6 FFU/ml), host protein shutoff was evident within the first 24 hours (Figure 3.21). This concentration corresponds to a m.o.i. of 1FFU/cell determined in Chang cells at 44 hours p.i. At an input concentration of one log less, host protein shutoff began after 48 hours of infection and continued to completion over four days. The progression of host protein shutoff with time can be clearly seen with the two cellular proteins (arrows). A further one log decrease in Ad2 input concentration resulted in a reduction in host proteins only at day 5, with incomplete inhibition. At

an Ad2 input concentration of 2.4 x 10^3 FFU/ml no inhibition of host protein synthesis could be detected over the time period tested. The level of cellular protein synthesis was as for control uninfected Chang cells. The highest input concentration of Ad40 and Ad41 that could be used as inoculum is comparable to 2.4 x 10^4 FFU/ml of Ad2. At this concentration of Ad2 complete inhibition of host protein synthesis did not occur. Since multiplicities of infection likely to show this effect cannot be attained with Ad40 and Ad41, it cannot be shown conclusively that these viruses do not inhibit cellular protein synthesis. However, since Ad41 did not appear to shut off host protein synthesis in permissive 293 cells, even although prominent virus bands could be seen, it seems improbable that shutoff would occur if greater concentrations of virus inoculum were attainable.

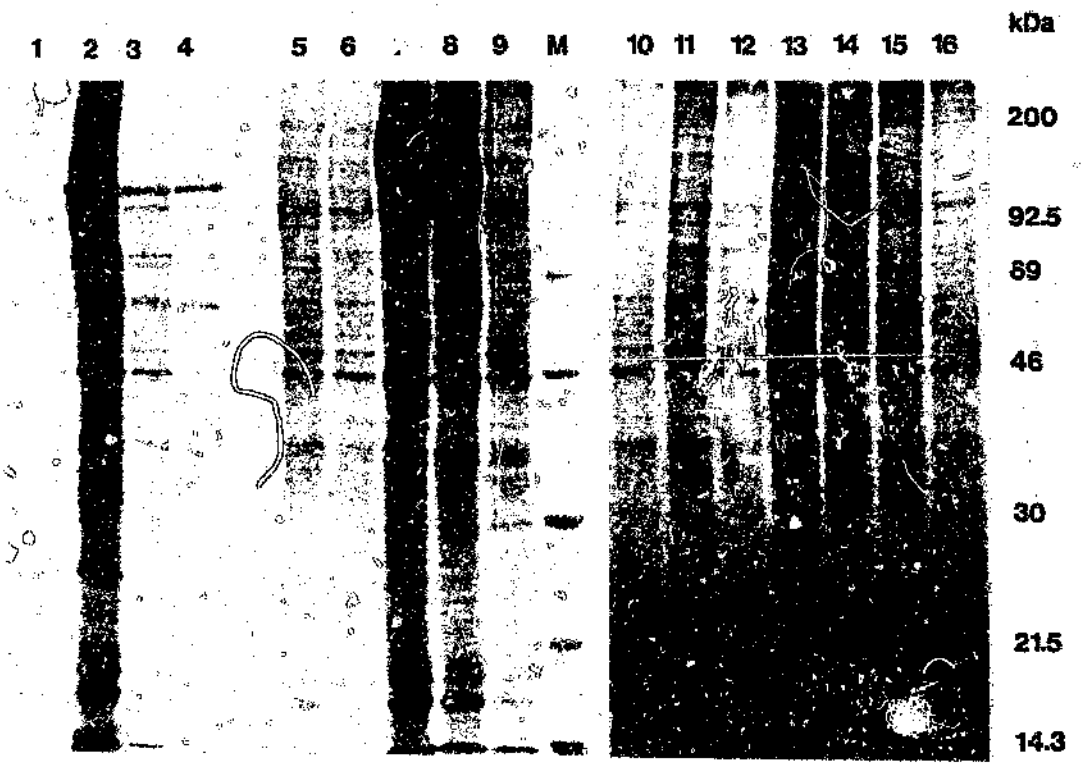


Figure 3.18 Autoradiograph of ^{35}S-methionine labelled proteins of Ad2, Ad40 and Ad41 infected Chang cells. Infected cells were labelled with ^{35}S-methionine for one hour at 24 hour intervals. Cells were harvested and 20μl of cell lysate loaded into the wells of a 12% polyacrylamide gel. Lane M. ^{14}C-marker. Lanes 1-4. Ad2, days 1-4 respectively. Lane 5 and 16. Uninfected cell proteins. Lanes 6-9. Ad40, days 1,2,3,6 respectively. Lanes 10-15. Ad41, days 1-6 respectively. Molecular weights of marker proteins (kDa) are indicated.

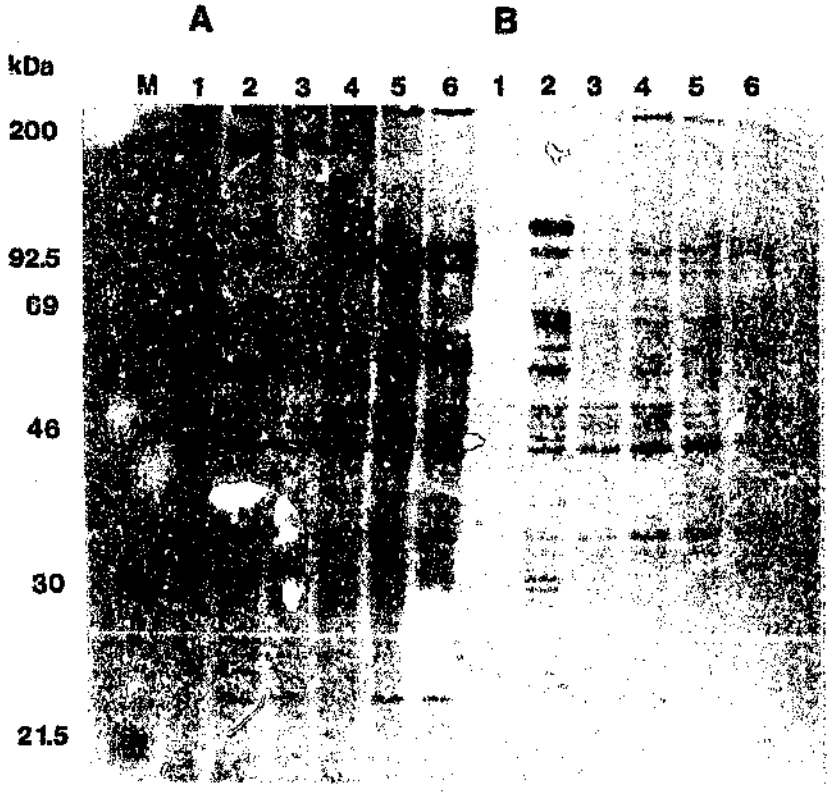


Figure 3.19 Autoradiograph of ^{35}S-methionine labelled proteins of Ad2, Ad40 and Ad41 infected 293 (A) and Chang (B) cells. Proteins were labelled *in vivo* for 20 hours with ^{35}S-methionine at 24 hours p.i. Twenty microlitres of cell lysate was added to the wells of a 12% polyacrylamide gel. Lane M. ^{14}C-marker. Lane 1. Uninfected cell proteins. Lane 2. Ad2, input concentration 2.4 x 10^5 FFU/ml. Lanes 3-4. Ad40, input concentration 2.4 x 10^4 FFU/ml : 1.2 x10^4 FFU and 2.4 x 10^4 FFU respectively. Lanes 5-6. Ad41, input concentration 2.4 x 10^4 FFU/ml : 1.2 x 10^4 FFU and 2.4 x 10^4 FFU respectively. Molecular weights of marker proteins (kDa) are indicated.

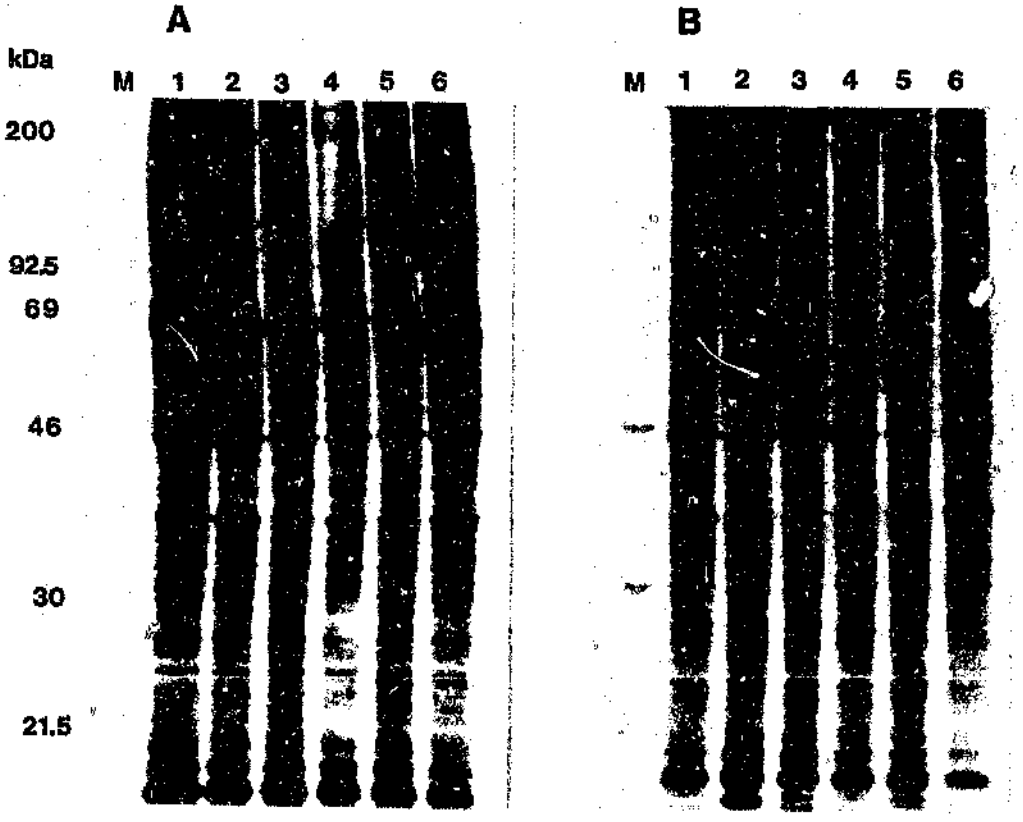


Figure 3.20 Autoradiograph of ^{35}S-methionine labelled proteins of Ad2, Ad40 and Ad41 infected 293 (A) and Chang (B) cells. Proteins were labelled for 8 hours with ^{35}S-methionine at 48 hours p.i. Cell lysates were electrophoresed on 12% polyacrylamide gels. Lane M. ^{14}C-marker. Lane 1. Uninfected cell proteins. Lane 2. Ad2, input concentration 2.4 x 10^5 FFU/ml. Lanes 3-4. Ad40, input concentration 2.4 x 10^4 FFU/ml. Lanes 5-6. Ad41, input concentration 2.4 x 10^4 FFU/ml. Molecular weights of marker proteins (kDa) are indicated.

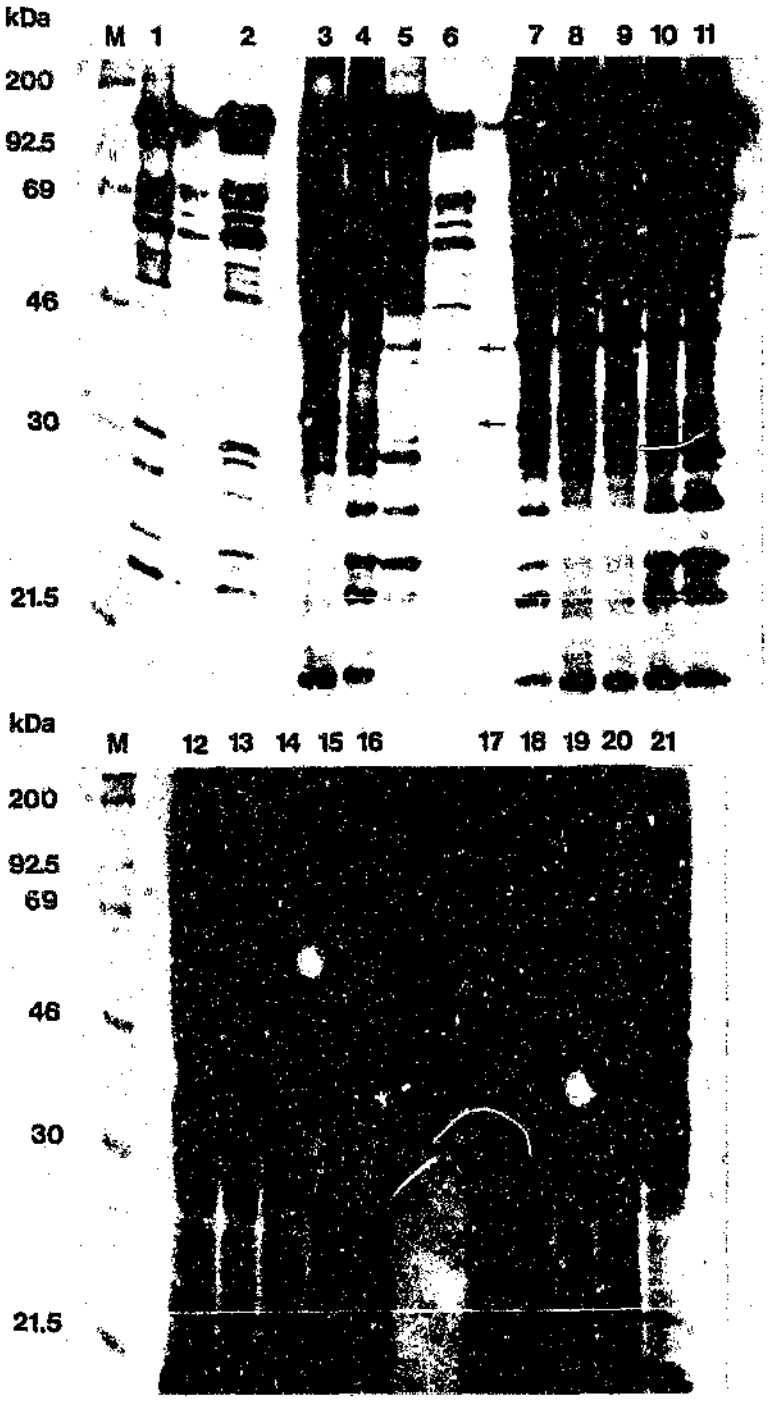


Figure 3.21 Autoradiograph showing the effect of different input concentrations and time of addition of label on host protein shutoff in Ad2 infected Chang cells. Cells were infected with 2.4 x 10^6 FFU/ml (Lanes 1-2) days 1 and 2, respectively, 2.4 x 10^5 FFU/ml (Lanes 3-6) days 1-4, respectively, 2.4 x 10^4 FFU/ml (Lanes 7-11) days 1-5, respectively, and 2.4 x 10^3 FFU/ml (Lanes 12-16) days 1-5, respectively, of Ad2. Uninfected cells (Lanes 17-21) days 1-5, respectively, received PBS. ^{35}S-methionine was added at 24 hour intervals and labelling continued for 16 hours. Twenty microlitres of cell lysate was loaded into the wells of a 12% polyacrylamide gel. Molecular weights of marker proteins (kDa) are indicated.

3.2.5 Detection and typing of subgroup F adenovirus DNA by polymerase chain reaction (PCR)

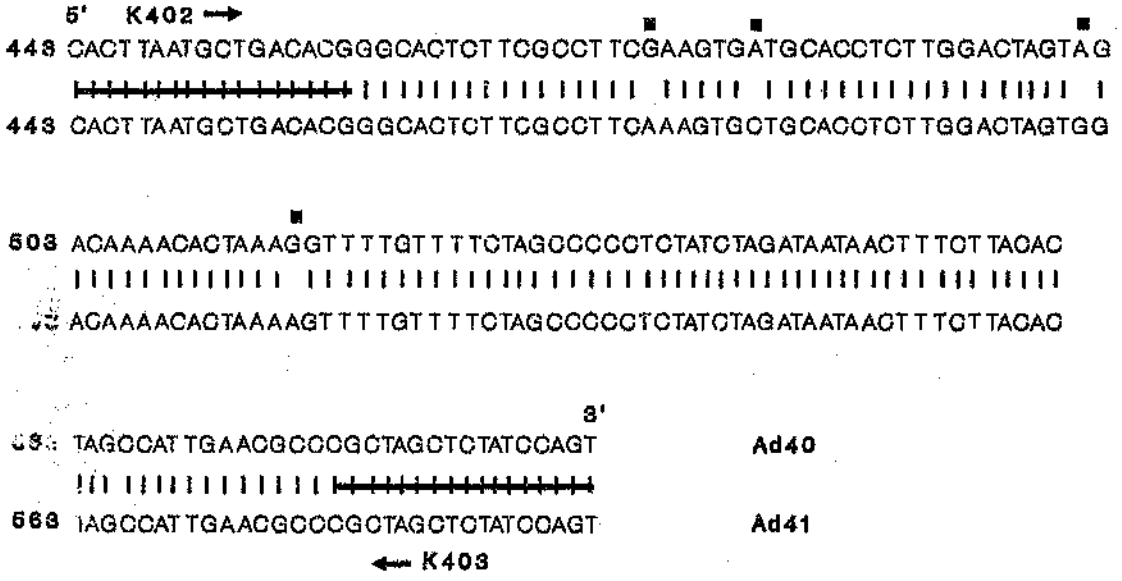
3.2.5.1 Target sequence and primers.

The PCR test was evaluated for both the specific detection and typing of subgroup F adenovirus DNA, as well as for the detection of specific mRNAs in Ad41 infected cell cultures. The PCR was based on sequences of the 1641-base Ad40 fibre gene (Kidd and Erasmus, 1989). The chosen target sequence was 152bp long, covering base positions 443 to 594, inclusive (Figure 3.22). The target sequence for amplification was defined by two 17-mer primers, K402 and K403, representing bases 443 to 459 of the r-strand and bases 578 to 594 on the l-strand, respectively. The 152bp sequence of Ad40 thus defined has 44.9% homology with the corresponding region of the Ad2 fibre gene, whereas it has 97.4% homology with its Ad41 counterpart, with only four base mismatches (Kidd *et al*, 1990).

Within the target sequences of both Ad40 and Ad41, there are common restriction enzyme sites for *Xba*I (1 site), *Spe*I (1 site) and *Mnl*I (2 sites). The Ad40 sequence alone has single sites for *Acc*I, *Asu*II, *Sfa*I and *Taq*I, whereas the Ad41 sequence alone has a single site for *Bbv*I.

3.2.5.2 Ad40 and Ad41 DNA as templates.

Plasmids containing Ad40 or Ad41 DNA spanning m.u. 74 to 92, which contain the fibre gene and hence the 152bp target sequence, gave products of the size predicted whereas plasmid without insert gave no product (data not shown). DNA from several variants of Ad40 and Ad41 also gave a single reaction product of the same size. In total, 8 of the 11 known DNA variants of Ad40, including the commonest variant D1, and 17 of the 24 known DNA variants of Ad41, including the commonest variant D2 (van der Avoort et al, 1989), were tested and found to yield a reaction product with electrophoretic mobility consistent with the expected size of 152bp.



K402	5' CAC TTA ATG CTG ACA CG 3'
K403	5' ACT GGA TAG AGC TAG CG 3'

Figure 3.22 Comparison of the 152bp target sequences within the fibre genes of Ad40 and Ad41. The regions from which the primers are derived are shown by continuous lines. The numbers at the left of the figure refer to base positions within the fibre gene. (Kidd and Erasmus, 1989; Kidd *et al*, 1990). Single base mismatches (■). Primer sequences (5′ to 3′) are shown below.

The reaction products from the representative variants of Ad40 and Ad41 were then treated in separate reactions with restriction enzymes *Xba*I, *Spe*I and *Acc*I. *Xba*I would be expected to digest the 152bp amplification products of both Ad40 and Ad41 into 98bp and 54bp (Table 3.4). The results of electrophoresis were consistent with this prediction (Figure 3.23). Similarly, *Spe*I digested all Ad40- and Ad41- derived reaction products into two products of the sizes predicted from sequencing (Table 3.4; Figure 3.23). As predicted, *Acc*I digested Ad40-derived DNA into products of 94bp and 58bp, but did not digest Ad41-derived DNA. Thus, digestion of the PCR product with *Acc*I allowed typing of the template DNA from which it was derived as either Ad40 or Ad41.

Table 3.4 Restriction enzyme digestion products of the Ad40 and Ad41 152bp target sequence predicted from sequencing (Kidd and Erasmus, 1989; Kidd *et al*, 1990)

	Product sizes (bp)	
Restriction enzyme	Ad40	Ad41
*Xba*I	54	54
	98	98
*Spe*I	53	53
	99	99
*Acc*I	58	152
	94	-

DNA was extracted directly from stool suspensions and subjected to *Taq* polymerase-directed DNA amplification. Twenty stool specimens containing viable Ad40 (10 specimens) and Ad41 (10 specimens) particles as determined by cell culture and restriction enzyme digestion of DNA extracted from infected cells, were positive in amplification tests. These reaction products were digested with enzymes *Xba*I and *Acc*I. There was a 100% correlation in results obtained by restriction enzyme typing and typing by *Acc*I digestion of amplified products.

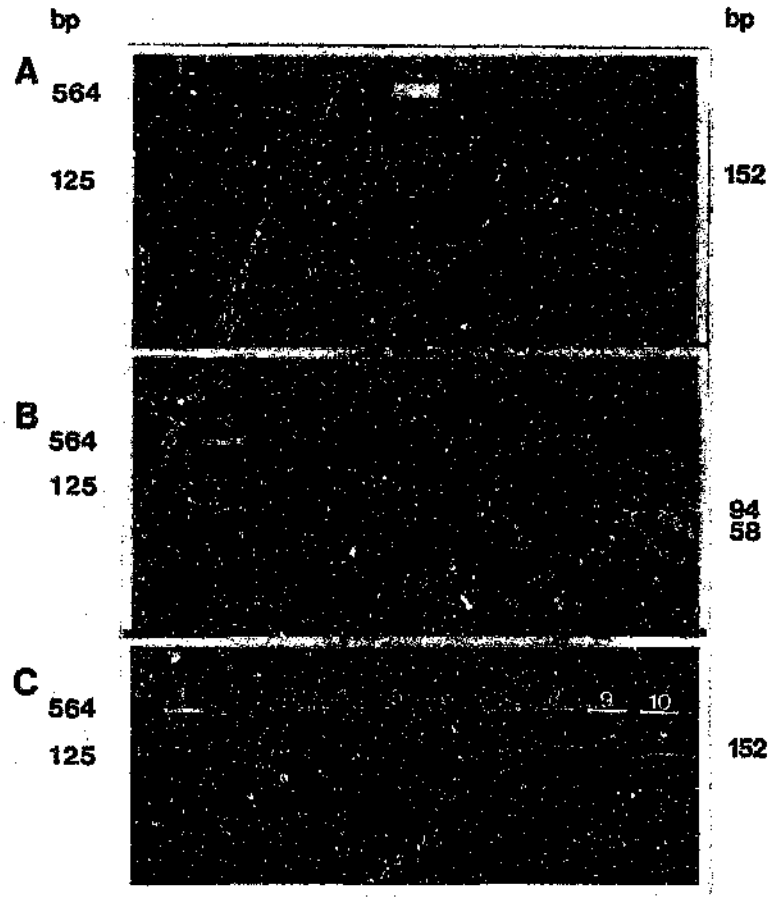


Figure 3.23 Restriction enzyme typing of Ad40 and Ad41 target sequence.
Ten μl volumes of Ad40 and Ad41 reaction products were digested with restriction enzymes in amplification buffer and loaded on a 4% agarose gel.

A. Lane 1. Undigested Ad40 PCR product. Lanes 2-4. Ad40 PCR product digested with *Xba*I, *Spe*I and *Acc*I respectively. Lane 5. Lambda DNA digested with *Hind*III (size marker). Lane 6. Undigested Ad41 PCR product. Lanes 7-9. Ad41 PCR product digested with *Xba*I, *Spe*I and *Acc*I respectively.

B. PCR products from Ad40 variants digested with *Acc*I. Lane 1. Lambda DNA digested with *Hind*III. Lane 2. Dugan (variant D4). Lane 3. FB273 (D7). Lane 4. N5234 (D6). Lane 5. AM55 (D7). Lane 6. NA95 (undefined Ad40 isolate from Johannesburg). Lane 7. FB205 (D8). Lane 8. Hovi-X (D1).

C. Reaction products from Ad41 variants digested with *Acc*I. Lane 1. Lambda DNA digested with *Hind*III. Lane 2. Tak (variant D1). Lane 3. 26341-77 (D2). Lane 4. FB311 (D20). Lane 5. FB276 (D18). Lane 6. N7798 (undefined Ad41 isolate from Canada). Lane 7. N7761 (D8). Lane 8. FB171 (D18). Lane 9. FB143 (D19). Lane 10. FB585 (D17). Sizes (bp) of lambda *Hind*III fragments are indicated at the left and PCR product sizes at the right of the figure. The 125bp marker fragment in B and C is not visible as a result of double photographic reproduction.

3.2.5.3 Specificity of the test.

DNA from prototype strains of Ad18 and Ad31 (representing subgroup A), Ad3, Ad7 and Ad16 (representing subgroup B), Ad1, Ad2, Ad5 and Ad6 (representing subgroup C) and Ad8, Ad15 and Ad37 (representing subgroup D) were tested individually as templates for the PCR reaction at a concentration of approximately 10μg/ml. All DNAs were negative except one preparation of Ad31 DNA, which was consistently positive. DNA from a different source of Ad31, with a different passage history, was found to be negative and it was concluded that the prototype Ad31 preparation had probably been contaminated with subgroup F adenovirus DNA.

DNAs from uninfected cultures of HeLa, Chang, 293, HEF and Peer cells, all of human origin, were negative in the test. In addition, DNA derived from foetal human intestine was negative. However, two cell lines of simian origin were consistently positive. DNAs from 11 different species of anaerobic bacteria which commonly colonise the human gut were negative in the test.

The identity of amplified target sequences was confirmed by hybridization of electroeluted 152bp DNA derived from Ad40 strain 85-9305 (variant D11) and Ad41 strain 82-20257 (variant D18) as ^{32}P-labelled probe to Southern blots of Ad40- and Ad41- derived DNA digested with *Sma*I, *Xho*I and *Eco*RI, Ad2 DNA digested with *Eco*RI, and plasmids carrying some or all of the fibre gene of Ad40 and Ad41. The target sequence hybridized to *Sma*I fragment *B*, *Xho*I fragment *B* and *Eco*RI fragment *C* of Ad40 (Figure 3.24) and *Sma*I fragment *A*, *Xho*I fragment *B* and *Eco*RI fragment *B* of Ad41, which all cover the position of the fibre gene (map units 87 to 92 approximately) (Takiff et al, 1984; Kidd and Erasmus, 1989; van der Avoort *et al*, 1989). There was no detectable hybridization of the PCR product to Ad2 DNA.

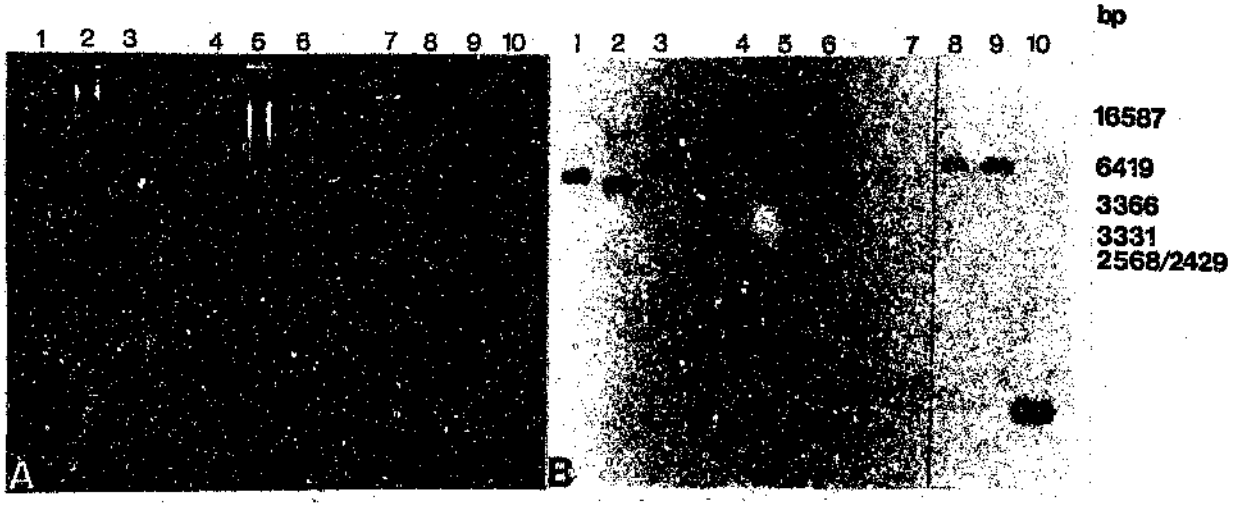


Figure 3.24 Specificity of the 152bp target sequence.

A. Restriction enzyme digests of adenovirus DNA and plasmids containing adenovirus DNA were loaded on 1% agarose gels. Lanes 1-3. Ad40 strain Dugan DNA (variant D4) digested with *Sma*I, *Xho*I and *Eco*RI respectively. Lanes 4-6. Ad41 strain 26341-77 DNA (variant D2) digested with *Sma*I, *Xho*I and *Eco*RI respectively. Lane 7. Ad2 DNA digested with *Eco*RI. Lane 8. Plasmid pSP65 containing the *Eco*RI *C* fragment of Ad40 digested with *Eco*RI. Lane 9. Plasmid pSP64 containing the *Eco*RI *B* fragment of Ad41 digested with *Eco*RI. Lane 10. Plasmid pSP64 containing a 700bp fragment with the target sequence digested with *Eco*RI and *Hind*III. Sizes (bp) of Ad41 *Eco*RI fragments are indicated.

B. Southern blot of panel A probed with the 152bp PCR product derived from Ad40 strain 85-9308 (variant D11).

3.2.5.4 Detection of target sequences in infected cell DNAs

Permissive cells (293), semi-permissive cells (Chang) and non-permissive cells (HEF) infected with 2.4 x 10^3 FFU of Ad41 were harvested at various days after infection and the DNA extracted. Detection of Ad41 DNA by PCR preceded any cytopathic effects in semi-permissive Chang cells (Table 3.5). In non-permissive cells, Ad41 was detected by PCR from day two after infection and for two further passages of the infected cells, whereas no cytopathic effects were evident at any stage.

Table 3.5 Comparison of Ad41 detection by PCR and by CPE in three different cell types

	Day harvested	Amplification	CPE
293 cells			
infected	4	+	+
	5	+	+
uninfected	4	-	-
Chang cells			
infected	3	+	-
	5	+	+
	6	+	+
uninfected	6	-	-
HEF cells			
infected	1	-	-
	2	+	-
	3	+	-
	4	+	-
P1 p.i.[a]		+	-
P2 p.i.[a]		+	-
uninfected	4	-	-

[a]HEF cells were infected and passaged 24 hours later. Upon confluency, one flask of cells was harvested for DNA extraction (P1 p.i.), the other was passaged again and DNA extracted when confluent (P2 p.i.).

3.2.6 Detection of virus DNA expression

3.2.6.1 mRNA

3.2.6.1.1 Detection of fibre gene expression

To determine if Ad41 infection of HEF cells progresses to a late stage in infection, a RT-PCR method was employed to detect late gene expression. Expression of the subgroup F adenovirus fibre gene was selected as a marker for transcription from the (presumed) major late promoter.

Messenger RNAs were isolated at 12 hour intervals from 293, Chang and HEF cells infected with Ad41. The procedure is schematically shown in Figure 3.25. Cells were fractionated and cytoplasmic RNA extracted. After selection of mRNA by two rounds of oligo-dT chromatography, the mRNA was precipitated and lyophilized. One of two preparations was treated with RNase-free DNase before the reverse transcription reaction to ensure the removal of p[illegible]ible contaminating DNA. Negative PCR reactions of these confirmed the purity of the mRNA preparations. The results were identical for DNase treated and untreated mRNAs, indicating the adequate purification of mRNA with the methods employed.

The reverse transcription reaction was carried out using the K403 primer (Figure 3.22) which binds specifically to fibre mRNA. The protocol was simplified by the use of PCR buffer in both the reverse transcriptase and PCR reactions. The presence of fibre mRNA in infected cells was identified as a positive reaction upon amplification of the 152bp target sequence using primers K402 and K403. The specificity of the reaction was confirmed, and the sensitivity of detection enhanced by hybridizing Southern blots of the electrophoresed amplification products with a radioactive probe containing the Ad41 fibre gene.

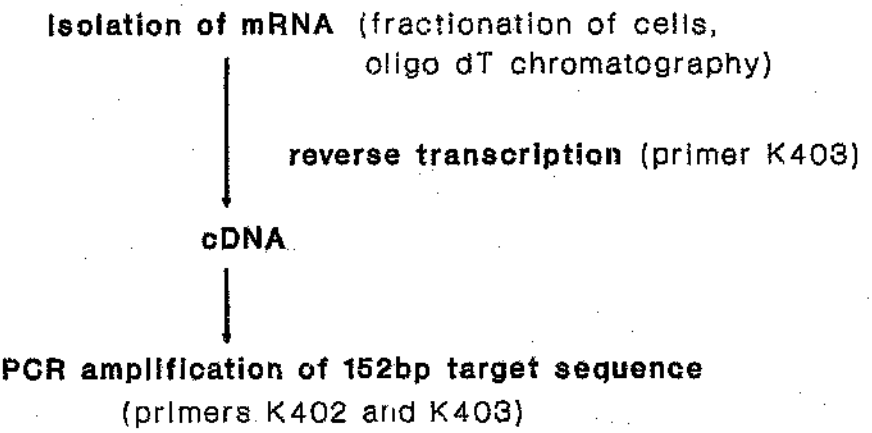


Figure 3.25 Scheme for detection of Ad41 fibre gene expression at the RNA level. Messenger RNA was isolated and purified. First strand synthesis was accomplished using the downstream oligonucleotide, K403 and AMV reverse transcriptase. Amplification of a 152bp target sequence using primers specific for the subgroup F adenovirus fibre gene (K402 and K403) would indicate the presence of fibre mRNA in infected cells.

The results obtained with infected 293, Chang and HEF cells are shown in Figure 3.26. Fibre mRNAs were first detected in 293 cells between 12 and 24 hours after infection and continued to be expressed until after 48 hours. Detection was optimum at 36 hours p.i. In Chang cells, fibre mRNAs were present from 12 hours onward, with maximum detection at 60 to 72 hours after infection, followed by a subsequent decline in detection. No 152bp product could be detected at any time in infected HEF cells by visualization of EtBr-stained gels under UV light or after hybridization of a Southern blot of the gel with the Ad41 fibre DNA probe. The positive DNA control is highly over exposed under these conditions of detection (Figure 3.26C).

Figure 3.26 Detection of fibre gene expression in Ad41 infected 293, Chang, and HEF cells using RT-PCR. Cells were infected with Ad41, mRNA isolated at the indicated times and the mRNAs reverse transcribed using primer K403. Amplification of Ad41 fibre cDNA in 293 (A), Chang (B), and HEF cells (C) was carried out using primers K402 and K403. The products were electrophoresed on 1.5 agarose gels, transferred to nylon membrane, and hybridized with radiolabelled pSP64-Ad41 *Eco*RI*B*. The presence of the specific 152bp target sequence indicates expression of the Ad41 fibre gene. Lanes 1-7. Messenger RNA isolated at 12, 24, 36, 48, 60, 72, and 84 hours, respectively. Lane 8. Uninfected control. Lane 9 (A and C),10 (B). Amplification of Ad41 FB311 DNA. Arrows indicate position of PCR products.

3.2.6.1.2 RNA dot blots

(i) Mapping of cloned Ad40 and Ad41 restriction enzyme fragments

In order to detect Ad41 specific mRNAs in infected cells it was necessary to first map cloned Ad40 and Ad41 *Pst*I fragments for their use as probes in hybridizations. Seven of 11 *Pst*I fragments from Ad40 strain N5911 and 8 of 11 *Pst*I fragments from Ad41 strain M4550 had been cloned in pBR322 (Kidd *et al*, 1985). These strains share identical *Pst*I profiles with Ad40 Hovi-X and Ad41 26341-77, respectively. The estimated sizes and corresponding map unit values of Ad40 Hovi-X and Ad41 26341-77 *Pst*I fragments are shown in Table 3.6. The total length of Ad40 DNA was determined to be 34,160.81 ± 204.62bp and that of Ad41 DNA 35,288.06 ± 298.54bp. Published values have been determined by the summation of fragment lengths from viral DNA digested with a number of different restriction enzymes giving a value of 34,000bp for Ad40 and 34,700bp for Ad41 in one study (van Loon *et al*, 1985b) and 35,000bp for both Ad40 and Ad41 in another (van der Avoort et al, 1989).

Single and double restriction endonuclease digestions were carried out using Ad2, Ad40 Hovi-X, and Ad41 26341-77 DNA. Table 3.7 shows the restriction enzymes used to digest each virus DNA. Fragments were separated on 1% agarose gels, transferred to nylon membrane and the DNA UV cross-linked. Southern blots were hybridized with ^{32}P-labelled *Pst*I fragment-containing plasmids, autoradiographed and analysed. The maximum limits of cloned Ad40 and Ad41 fragments were determined in relation to available restriction enzyme maps (see Appendix E) (Table 3.8). Larger *Pst*I fragments were more accurately mapped by excising insert DNAs from plasmids and digesting them with enzymes that cut within the maximum map unit limits deduced from DNA:DNA hybridizations. The restriction enzymes used and the sizes of the resultant products are shown in Table 3.9. Figure 3.27 shows the maximum limits of Ad40 (A) and Ad41 (B) *Pst*I fragments in relation to map unit

coordinates of their respective physical maps determined by DNA:DNA hybridizations and digestion of large insert DNAs with restriction enzymes.

Terminal fragments were identified using *Bal*31 nuclease digestion of Ad40 and Ad41 DNA, followed by restriction enzyme cleavage with *Pst*I. *Bal*31 has a potent double-strand exonuclease activity which shortens both strands of linear duplex DNA molecules from both termini in a time-dependent fashion. Upon digestion with *Pst*I, progressive disappearance of *Pst*I fragments could be seen from either end of the genome, allowing evaluation of the order of fragments from the ends. The left terminal fragments were also identified by hybridizing Southern blots of Ad40 and Ad41 *Pst*I profiles with plasmids containing the E1 regions of Ad40 and Ad41 (pAd40 and pAd41 which contain the *ClaIB* and *ClaID* fragments inserted into pBR322, respectively). This was confirmed using plasmids containing the E1a and the E1b genes of Ad40, pUC18-*Bam*HI*F* and pML2-*Bam*HI*D*, respectively.

Figure 3.28 illustrates the *Bal*31 exonuclease analysis carried out with Ad40. Purified Ad40 DNA was treated with *Bal*31 exonuclease and the reaction stopped at the times indicated in the figure. The DNA samples were digested with *Pst*I, the fragments separated by agarose electrophoresis and transferred to nylon membrane. The blots were hybridized with whole viral DNA (Figure 3.28A). *Pst*I fragment *K* disappeared first and would therefore be either at the right or left terminal end. Fragments *C* and *F* disappeared at the same time and are therefore located at opposite ends of the genome. Hybridization with pAd40-*ClaIB* placed fragments *A*, *F* and *K* at the left hand end of the genome (Figure 3.28B). Fragment *C* is therefore the rightmost fragment on the physical map. Plasmid pUC18-*Bam*HI*F* hybridized with fragment *F* and *K* (Figure 3.28C), while pML2-*Bam*HI*D* hybridized with fragment *A* and *F* (Figure 3.28D). The order of *Pst*I fragments at the left hand end is therefore first *K*, followed by fragment *F* and then *A*.

In order to align the Ad40 and Ad41 *Pst*I maps in relation to each other, Southern blots of the *Bal*31 exonuclease digestions were used for hybridizations with plasmids described in Table 3.10. This allowed the identification of homologous Ad40 and Ad41 *Pst*I fragments and confirmation of location of the fragments.

The deduced *Pst*I maps for Ad40 and Ad41 are shown in Figure 3.29. Ad41 *Pst*I fragments *K* and *J* could not be clearly resolved upon separation by electrophoresis and due to their small size may have been lost upon transfer to nylon membrane. A positive hybridization reaction would have been expected with ^{32}P-labelled pSP64-Ad41 *Eco*RI*B* as the insert DNA spans coordinates 74 to 92. As a result of their small size and the degree of error in determining fragment sizes the exact position of these fragments could not be determined by deduction from the location of adjoining fragments. The positions of these fragments on the physical map were therefore reproduced from the published *Pst*I map for Ad41 strain Tak (van der Avoort *et al*, 1989), the *Pst*I profile of which is identical to that of strain 26341-77 and M4550. There were, however, differences in the location of certain fragments when the *Pst*I map deduced for Ad41 strain 26341-77 was compared to that determined for Ad41 strain Tak by van der Avoort *et al* (1989). Fragments *A* and *B* are interchanged as are fragments *F* and *G* (see Appendix E for comparison). The differences are not likely to be due to strain differences as the sizes of *Pst*I fragments *A*, *B*, *F*, and *G* are identical in the three restriction enzyme profile patterns found and map to the same location with all Ad41 strains analysed (van der Avoort *et al*, 1989). Since fragments *A* and *B* and fragments *F* and *G* are similar in size it is likely that the discrepancy may be due to this. To ensure that the plasmids used were not incorrectly marked they were checked by digestion with *Pst*I and separation of the products on 0.8% agarose gels. The sizes correlated with the assigned letter for both comparisons confirming that, together with the other approaches used, the approximate location of the *Pst*I sites on the Ad41 genome.

The *Pst*I maps deduced for Ad40 and Ad41, in common with map comparisons of other restriction enzymes, are notably unalike. This is a peculiarity of the subgroup F adenoviruses as other subgroups have types with similar restriction enzyme profiles. The former proposed classification of Ad40 and Ad41 in different subgroups was based upon the dissimilarity of their genomes (Uhnoo *et al*, 1983).

From the well characterized transcription map of Ad2 (see Figure 1.3) and the alignment of Ad40 and Ad41 fragments to restriction maps of Ad2 by DNA:DNA hybridization (Table 3.7), tentative Ad40 and Ad41 transcription units were assigned to specific fragments. By convention, a transcription unit is defined as those sequences spanning the genome from the site of initiation of transcription to the site of termination of transcription (Berget *et al*, 1977; Wilson *et al*, 1979b). A transcription unit was, however, assigned to a specific fragment even if only a small portion was likely to be present. The E2a and E2b transcription units were considered to be the coordinate regions that encompass the main bodies of their corresponding mRNAs. Plasmids that are likely to (Ad40) and do (Ad41) contain the VA RNA gene sequences (Kidd and Tiemessen, manuscript in preparation) are also indicated. The descriptions appear in Table 3.11 together with other Ad40- and Ad41- DNA containing plasmids.

Table 3.6 Estimated sizes of Ad40 Hovi-X and Ad41 26341-77 *Pst*I DNA fragments

*Pst*I fragment	size (bp)[a]	m.u.[b]
Adenovirus 40 DNA[c]		
*A**	6309.57	18.47
*B**	5956.62	17.44
*C**	5100.00	14.92
D	4451.00	13.03
*E**	3162.28	9.26
F	2818.38	8.25
G	2401.28	7.03
*H**	1778.28	5.20
*I**	945.00	2.77
*J**	760.40	2.23
K	478.00	1.40
Adenovirus 41 DNA[d]		
*A**	6606.93	18.73
*B**	6456.54	18.30
C	5011.87	14.20
*D**	4799.00	13.59
*E**	4466.80	12.66
*F**	2371.37	6.72
*G**	2317.39	6.57
*H**	1445.44	4.10
*I**	1000.00	2.83
J	530.88	1.50
K	281.84	0.80

[a]Fragment sizes were determined from standard curves of *Hind*III or *Hind*III + *Eco*RI lambda DNA fragments of known size as a function of mobility (mm) within 1% agarose gels. Values are the average of 4 independent determinations.
[b]Map unit (m.u.) values were calculated for each fragment by dividing the size (bp) of the fragment by the total size (bp) of the genome and multiplying by 100. Summation of the values of all the *Pst*I fragments therefore gives a total of 100 m.u.
[c]The length of Ad40 DNA was estimated to be 34,160.81 ± 204.62 bp. Fragments cloned from Ad40 strain N5911 are indicated (*).
[d]The length of Ad41 DNA was estimated to be 35,288.06 ± 298.54bp. Fragments cloned from Ad41 strain M4550 are indicated (*).

Table 3.7 Restriction enzymes used to digest Ad2, Ad40, and Ad41 DNA for Southern transfers

DNA		
Adenovirus 2	Adenovirus 40	Adenovirus 41
Single restriction enzyme digestions[a]		
*Bam*HI	*Bam*HI	*Bam*HI
*Eco*RI	*Eco*RI	*Eco*RI
*Hind*III	*Hind*III	*Hind*III
*Sma*I	*Sma*I	*Sma*I
*Xho*I	*Xho*I	*Xho*I
*Xba*I	*Pst*I	*Pst*I
*Hpa*I	*Kpn*I	*Hpa*I
	*Sal*I	*Sal*I
	*Cla*I	*Cla*I
		*Kpn*I
Double restriction enzyme digestions[b]		
		*Sal*I+*Cla*I
		*Eco*RI+*Bam*HI
		*Xho*I+*Cla*I
		*Kpn*I+*Bam*HI
		*Eco*RI+*Hpa*I

[a]Virus DNA was digested according to the manufacturer's recommendations for each restriction enzyme.
[b]Digestions were carried out in a buffer compatible with both enzymes.

Table 3.8 Maximum map unit limits of cloned Ad40 and Ad41 *Pst*I fragments

Plasmids containing *Pst*I fragments[a]	Maximum limits of restriction fragment[b] (m.u.)
Adenovirus 40 DNA	
N27*A*	0 - 26.0
N4*B*	38.7 - 65.1
N17*C*	83.5 - 100
N8*E*	25.9 - 41.4
N26*H*	68.9 - 78.7
N9*I*	73.3 - 78.7
N22*J*	25.9 - 38.8[c]
Adenovirus 41 DNA	
M51*A*	33.6 - 57.3
M34*B*	12.9 - 32.5
M25*D*	62.0 - 73.5
M31*E*	82.5 - 100
M2*F*	72.8 - 83.6
M50*G*	53.6 - 61.0
M25*H*	80.0 - 90.1
M30*I*	29.0 - 35.0

[a]N plasmid designations apply to Ad40 *Pst*I fragments, and M to Ad41.
[b]Restriction enzyme coordinates were determined from results of DNA:DNA hybridizations in relation to restriction enzyme maps of Ad40 and Ad41 DNA.
[c]The *Sma*I profile was not included in the analysis as small fragments mapping to this region of the Ad40 Hovi-X genome could not be resolved in Southern blot hybridizations.

Table 3.9 Restriction enzyme digestion of Ad40 and Ad41 *Pst*I fragments to determine location of fragments on the corresponding physical maps

DNA	enzyme[a]	size[b](bp)	m.u.[c]
*Pst*I fragments[d]			
N27*A*	*Cla*I	5623.41	16.46
	*Sma*I	2770.44	8.11
		2049.65	6.00
		1496.24	4.38
	*Hind*III†	3981.07	11.65
		1496.23	4.38
	*Bam*HI	3907.99	11.44
		2435.67	7.13
N4*B*	*Sma*I	3682.54	10.78
		1496.24	4.38
	*Kpn*I*	5827.83	17.06
	*Hind*III†	1412.54	4.13
N17*C*	*Sma*I	4714.19	13.80
	*Kpn*I*	5011.87	14.67
	*Hind*III†	2238.72	6.55
N8*E*	*Sma*I	1571.97	4.60
N26*H*	*Kpn*I*	1739.44	5.10
M51*A*	*Bam*HI*	6415.37	18.18
	*Eco*RI	5720.19	16.21
	*Xho*I	3609.97	10.23
		2865.39	8.12
M34*B*	*Sma*I	3034.77	8.60
		2540.74	7.20
M25*D*	*Hind*III	4047.54	11.47
	*Eco*RI	4047.54	11.47
M31*E*	*Xho*I*	4305.14	12.20
	*Hind*III	1678.80	4.76
	*Eco*RI	2706.59	7.67
		1789.10	5.07
M25*H*	*Hind*III*	1404.46	3.98

[a]Enzymes that do not appear to cut within the fragment (*).
Sizes of Ad40 DNA products obtained with *Hind*III did not correlate with results obtained with other enzymes (†).
[b]Sizes are shown of only those fragments that could be accurately resolved by gel electrophoresis.
[c]Map unit values were determined as described in Table 3.6.
[d]N and M denote *Pst*I fragments isolated from Ad40 and Ad41 DNA-containing plasmids, respectively.

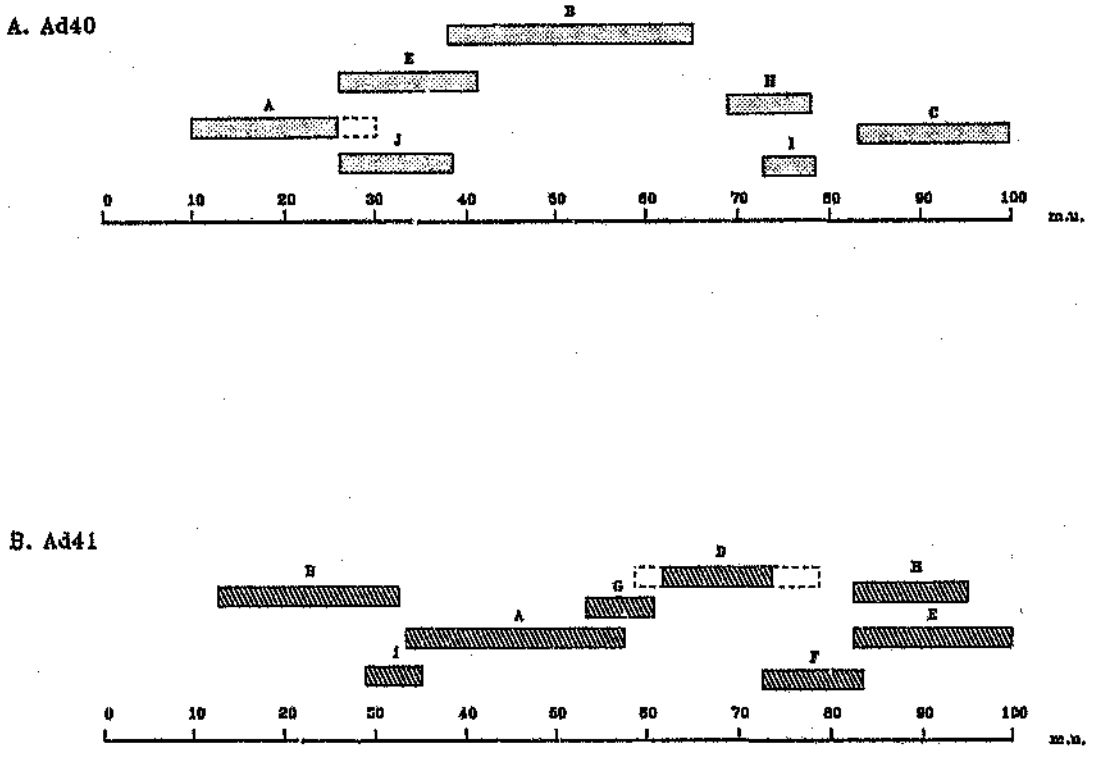


Figure 3.27 Maximum limits of Ad40 (A) and Ad41 (B) *Pst*I-cloned fragments determined by DNA:DNA hybridization and restriction enzyme digestion of plasmid inserts. Virus DNA was digested with restriction enzymes listed in Table 3.7. Isolated insert DNAs were digested with enzymes as outlined in Table 3.9. Fragments were separated on 1% agarose gels. For DNA:DNA hybridizations, DNA was transferred to nylon membrane and cross-linked by UV irradiation. Hybridizations were carried out with ^{32}P-labelled plasmids containing Ad40 and Ad41 *Pst*I fragments. The m.u. coordinate limits were determined from available restriction enzyme maps (see Appendix E). Solid lines represent maximum m.u. limits. Broken lines indicate expected extensions either to the right and/or left of determined limits.

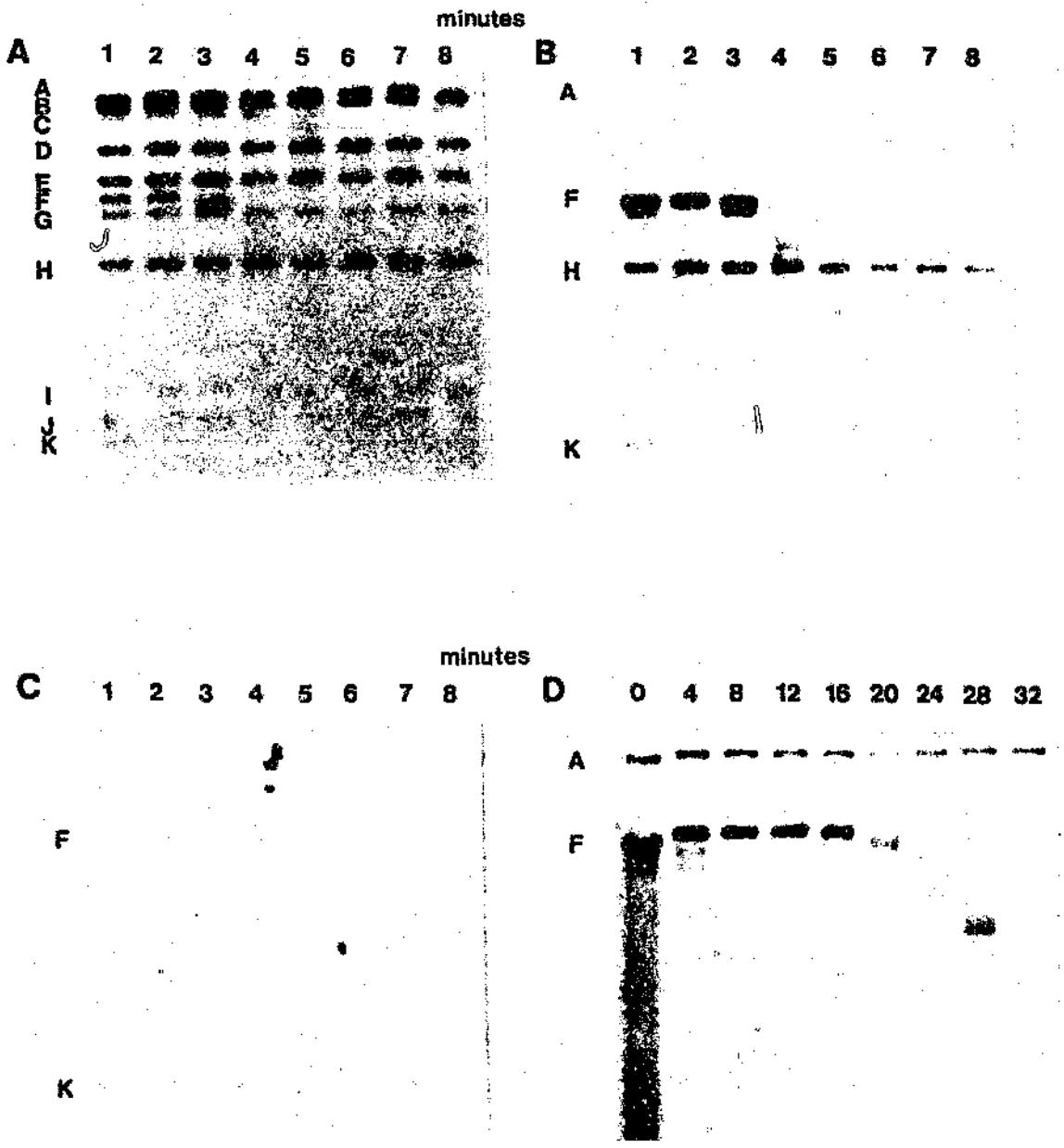


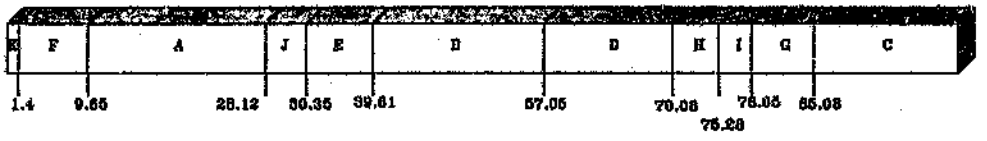
Figure 3.28 Electropherograms showing the ordered removal of Ad40 *Pst*I fragments from the genome termini using exonuclease *Bal*31. Adenovirus 40 DNA was digested with *Bal*31 nuclease for the indicated time intervals (minutes) followed by *Pst*I digestion and electrophoresis on 1% agarose gels. Southern blots were hybridized with whole Ad40 DNA (A), pAd40-*ClaIB*+N26*H* (B), pUC18-*Bam*HI*F* (C), and pML2-*Bam*HI*D* (D).

Table 3.10 Hybridization of Ad40 and Ad41 DNA-containing plasmids to Ad40 and Ad41 *Pst*I profiles[a]

Plasmid	Restriction enzyme fragment	Hybridization to *Pst*I fragment
		Adenovirus 41
N27	*PstIA*	*B,C*
N4	*PstIB*	*A*
N17	*PstIC*	*E,H*
N8	*PstIE*	*A,I*
N26	*PstIH*	*D*
N9	*PstII*	*F,D*
N22	*PstIJ*	*B,I*
pAd40-*ClaIB*	*ClaIB*	*C*
pUC18-*Bam*HI*F*	*Bam*HI*F*	*C*
pML2-*Bam*HI*D*	*Bam*HI*D*	*B,C*
pSP64-*Eco*RI*B*	*Eco*RI*B*	*E,F,H*
		Adenovirus 40
M51	*PstIA*	*B,D*
M34	*PstIB*	*A,J*
M25	*PstID+H*	*D,H,I+C,G*
M31	*PstIE*	*C*
M2	*PstIF*	*G*
M50	*PstIG*	*D*
M30	*PstII*	*E*
pAd41-*ClaID*	*ClaID*	*A,F,K*
pUC18-*Bam*HI*F*	*Bam*HI*F*	*F,K*
pML2-*Bam*HI*D*	*Bam*HI*D*	*A,F*
pSP64-*Eco*RI*B*	*Eco*RI*B*	*C,G*

[a]Southern blots of *Bal*31 exonuclease treated, *Pst*I digested Ad40 and Ad41 DNA were used in hybridizations.

A. Ad40



B. Ad41

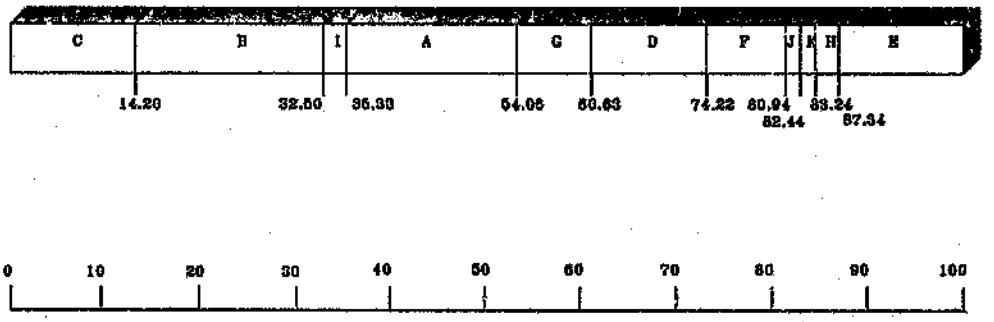


Figure 3.29 *Pst*I restriction enzyme maps deduced for Ad40 (A) and Ad41 (B) from DNA:DNA hybridizations, restriction enzyme digestion of *Pst*I fragments, and *Bal*31 exonuclease digestions. Map positions of Ad41 *Pst*I fragments *J* and *K* were taken from van der Avoort *et al* (1989).

Table 3.11 Description of Ad40 and Ad41 *Pst*I fragment-containing plasmids and other Ad40- and Ad41- derived plasmids

Plasmid[a]	Restriction enzyme fragment	m.u.[b] coordinates	Size[c] (bp)	Assigned transcripton unit[d]
Adenovirus 40 DNA[e]				
N27	*PstIA*	9.65-28.12	6309.57	IVa_2,E2b
N4	*PstIB*	39.61-57.05	5956.62	L2,L3
N17	*PstIC*	85.08-100	5100.00	L5,E4
N8	*PstIE*	30.35-39.61	3162.28	L1
N26	*PstIH*	70.08-75.28	1778.28	L4
N9	*PstII*	75.28-78.05	945.00	L4,E3
N22	*PstIJ*	28.12-30.35	760.40	VA RNA[g]
pAd40-*ClaIB*	*ClaIB*	0-11	3740	E1*
pUC18-*Bam*HI*F*	*Bam*HI*F*	0-5.50	1870	E1a*
pML2-*Bam*HI*D*	*Bam*HI*D*	5.50-13.80	2822	E1b*
Adenovirus 41 DNA[f]				
M51	*PstIA*	35.33-54.06	6606.93	L1,L2,L3
M34	*PstIB*	14.20-32.50	6456,54	VA RNA E2b,IVa_2,L1
M25	*PstID*	60.63-74.22	4799.00	L4,E2a
M31	*PstIE*	87.34-100	4466.80	L5,E4
M2	*PstIF*	74.22-80.94	2371.37	L4,E3
M50	*PstIG*	54.06-60.63	2317.39	L3
M25	*PstIH*	83.24-87.34	1445.44	E3
M30	*PstII*	32.50-35.33	1000.00	L1
pAd41-*ClaID*	*ClaID*	0-12.00	4164	E1*
pSP64-*Eco*RI*B*	*Eco*RI*B*	74.00-92.00	6246	L4,E3,L5*

[a]N plasmid designations apply to Ad40 *Pst*I fragments, and M to Ad41. Other plasmids are named according to plasmid vector and restriction fragment inserted.
[b]The Ad40 and Ad41 physical maps are divided into 100 map units (m.u.). The values are either known (*) or were determined according to location and size (N and M plasmids).
[c]Fragment sizes were determined from standard curves of *Hind*III and *Hind*III+*Eco*RI generated lambda DNA fragments of known size as a function of mobility.
[d]Transcription units were assigned to *Pst*I fragments by alignment with the Ad2 transcription map (Broker *et al*, 1984). Other plasmids contain sequenced DNA regions (*) (van Loon *et al*, 1985a; 1987b; Allard and Wadell, 1988; Ishino *et al*, 1988; Kidd *et al*, 1990).
[e]Adenovirus 40 DNA has an estimated length of 34,000bp (van Loon *et al*, 1985b). *Pst*I fragment coordinates were calculated using the genome size determined in this study, namely, 34,160.81bp.
[f]Adenovirus 41 DNA has an estimated length of 34,700bp (van Loon *et al*, 1985b). *Pst*I fragment coordinates were calculated using the genome size determined in this study, namely, 35,288.06bp.
[g]Tentative position based on map position in Ad41.

(ii) DNA:RNA hybridizations

Since no late gene expression could be demonstrated in Ad41 infected HEF cells using RT-PCR, mRNAs were isolated at 12 hour intervals after Ad41 infection of 293, Chang and HEF cells and RNA dot blots prepared. Each dot represented the mRNA isolated from 6.7 x 10^6 infected cells. The dot blots were hybridized with plasmids representing early and late adenovirus regions (Table 3.12). The following plasmids were used to probe the mRNA dot blots: pUC18-*Bam*HI*F*, pML2-*Bam*HI*D*, pAd41-*ClaID*, M34*B*, N8*E*, M51*A*, M50*G*, M25*D*+*H*, M2*F*, and M31*E*.

When the blot was probed with pUC18-*Bam*HI*F*, an Ad40 E1a gene-containing plasmid, there was no reaction. A poor hybridization signal with high background was obtained with pML2-*Bam*HI*D*, which contains the Ad40 E1b gene. These probes would be expected to give a weaker reaction compared to plasmids containing homotypic DNA. Similarly, plasmid N8*E* to which the Ad40 L1 transcription unit was assigned, did not react with the blot. Less stringent conditions resulted in higher backgrounds and difficulty in determining weak reactions.

Results obtained with dot blot hybridizations using Ad41 DNA-derived plasmids are shown in Table 3.12. All the plasmids used in hybridizations reacted with varying strength to mRNA isolated only at 36 hours p.i. from Ad41 infected 293 cells. A plasmid containing the entire E1 gene of Ad41 (pAd41-*ClaID*) reacted strongly (Figure 3.30A). Interestingly, these mRNAs were present and accumulated over a much longer time period in Chang cells, from 24 hours through to 84 hours p.i. Messenger RNAs hybridizing to the same region in infected HEF cells were detected at 36 hours and to a lesser extent 48 hours p.i. No other transcripts were detected in HEF cells.

In infected Chang cells transcripts that hybridized to plasmid M34*B* were detected at 60 hours p.i. (Figure 3.30B), a region that would be expected to detect

IVa_2 mRNA and E2b mRNAs. This plasmid also contains the VA RNA gene sequences of Ad41 (Kidd and Tiemessen, manuscript in preparation) but since mRNAs were selectively purified from cytoplasmic RNAs double-stranded VA RNA was excluded. Plasmid M51*A* which contains part of the L1 and L3 and the whole L2 tentative transcription units showed an accumulation of transcripts that hybridize to this region from 48 to 84 hours p.i. (Figure 3.30C). Plasmids M50*G*, M25*D+H* and M2*F* all detected mRNA at 60 hours p.i. that hybridized to assigned regions L3, E2a and L4, and E3 and L4, respectively. Plasmid pSP64-*Eco*RI*B* contains DNA from coordinates 74 to 92 and encompasses part of the L4 region as well as the E3 and L5 region of Ad41. Hybridization was obtained from 24 hours p.i. through to 84 hours after infection (Figure 3.30D). The reaction was weaker at times 36 and 48 hours compared to 24, 60, 72 and 84 hours after infection. The plasmid which contains the E4 region of Ad41, M31*E*, did not hybridize to mRNA isolated from infected Chang cells at any of the indicated times.

A comparison of Ad41 DNA regions that showed hybridization to dotted mRNAs isolated from permissive (293), semi-permissive (Chang) and non-permissive (HEF) cells is schematically shown in Figure 3.31.

Table 3.12 Detection of Ad41 specific mRNAs in infected 293, Chang and HEF cells by DNA:RNA dot hybridizations

	Intensity of reaction[a]							
	Time (hours)[b]							
Cells	12	24	36	48	60	72	84	U[c]
pAd41-*ClaID*[d]								
293	-	-	>4+	-	-	-	-	-
Chang	1+	1+	2+	3+	4+	4+	4+	-
HEF	-	1+	±*	-	-	-	-	-
M34*B*/M50*G*/M25*D*+*H*/M2*F*[d]								
293	-	-	1+	-	-	-	-	-
Chang	-	-	-	-	±*	-	-	-
HEF	-	-	-	-	-	-	-	-
M51*A*[d]								
293	-	-	4+	-	-	-	-	-
Chang	-	-	-	±*	2+	2+	2+	-
HEF	-	-	-	-	-	-	-	-
PSP64-Ad41*EcoRIB*[d]								
293	-	-	4+	-	-	-	-	-
Chang	-	±*	±*	±*	2+	2+	2+	-
HEF	-	-	-	-	-	-	-	-
M31*E*[d]								
293	-	-	1+	-	-	-	-	-
Chang	-	-	-	-	-	-	-	-
HEF	-	-	-	-	-	-	-	-

[a]The intensity of the hybridization reaction was determined visually from the autoradiograph. Reactions rated as ±(*) are visible on autoradiographs but are not visible upon photographic reproduction (Figure 3.30).
[b]Messenger RNAs were isolated at the indicated times.
[c]Uninfected.
[d]Ad41-derived plasmids.

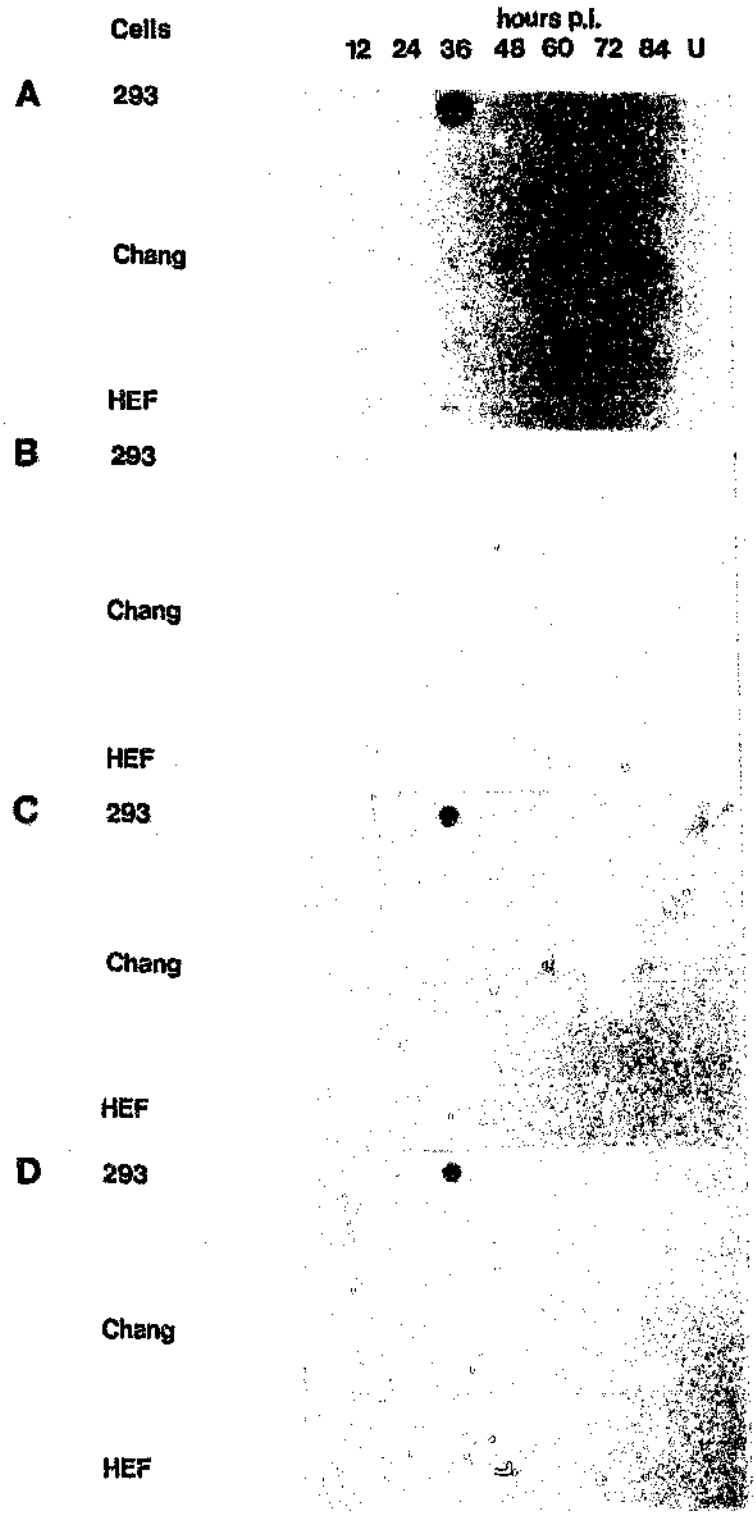
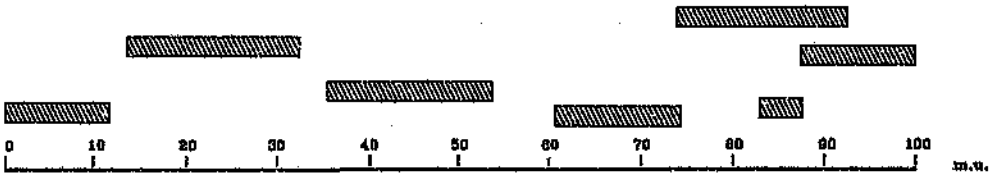


Figure 3.30 Autoradiographs of DNA:RNA dot hybridizations performed with Ad41 DNA-containing plasmids. A. pAd41-*ClaID*. B. M34*B*. C. M51*A*. D. pSP64-Ad41 *Eco*RI*B*. Messenger RNAs were isolated at the indicated times (hours p.i.). U. Uninfected control. Autoradiographs were exposed for 24 to 48 hours.

A. 293 cells



B. Chang cells

C. HEF cells

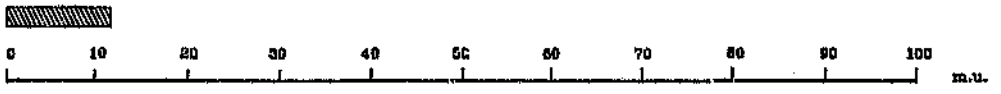


Figure 3.31 Comparison of Ad41 specific transcripts detected in 293 (A), Chang (B), and HEF (C) cells. Restriction enzyme fragments showing hybridization to dotted mRNAs are shown by shaded boxes corresponding to the map unit (m.u.) lengths of each fragment (see Table 3.11).

3.3 Discussion

In an attempt to better understand the basis of the growth restriction of the subgroup F adenoviruses in most human cell lines, a number of stages of the replication cycle were investigated. It has been established previously that virus attachment, penetration and uncoating are unlikely to play a role in their restricted growth in cell culture (Gary *et al,* 1979). This was further supported by the detection of some expression of early gene functions in non-permissive cells (Takiff *et al,* 1981). The purpose of this study was to analyse events beyond the uncoating of the virus genome in order to distinguish the function or functions that may contribute to reduced infectivity of the subgroup F adenoviruses *in vitro.*

Adenovirus growth in cells permissive (293), semi-permissive (Chang), and non-permissive (HEF) for subgroup F adenovirus growth was monitored over time using an indirect ELISA test that detects hexon antigen. To allow for a direct comparison, all conditions of infection were identical with respect to number of cells inoculated, input virus concentration (as determined in Chang cells), and time of harvesting tissue culture fluids and cells. Since the input concentration of virus was low, the relative amount of hexon released into the medium and accumulation of hexon antigen intracellularly was determined over 6 and 7 days, respectively.

Adenovirus hexons carry a group-specific antigen (α) that is oriented towards the interior of the virus particle (Norrby, 1969) and therefore unaccessible to group-specific antisera. Homotypic antibodies have been shown to attach to the surface of virions (Smith *et al,* 1965; Norrby *et al,* 1969). The group-specific antiserum used in the ELISA test therefore allowed only for the definite detection of free hexon antigen and not necessarily of whole virus. For the purpose of this study it was assumed that free hexon antigen production reflects the relative abundance of whole virus produced within and released from infected cells, that is, that the particle : free hexon ratios are similar for Ad2, Ad40 and Ad41 in each cell type and are

representative of their relative growth capabilities. This assumption is thus based on efficiency of assembly being comparable. A comparison of type -, subgroup - and group--based ELISA yielded similar results when used to determine hexon antigen levels in cultures infected either with Ad40 or Ad41 (data not shown) thereby validating this assumption.

Adenovirus 41 appeared to grow best in 293 cells and attained intracellular and extracellular hexon levels comparable to those of Ad2, and at an earlier time. It is noteworthy that Ad41 hexon accumulated very rapidly intracellularly, within a 24 hour period, with no further accumulation thereafter. The stock Ad41 preparation had been passaged once in 293 cells prior to use in infections. The ELISA test detects the total amount of hexon present but gives no indication of the proportion of infectious virus. The loss of infectivity of Ad41 observed with passage in 293 cells by Pieniazek et al (1990) was therefore not detected here. It is possible that the number of complete virus particles formed may remain high upon initial passage in 293 cells with the relative proportion of infectious particles decreasing with each passage. Adenovirus 40 appeared to grow poorly in 293 cells. This is in agreement with reports of difficulties in propagating it in 293 cells (Chiba *et al*, 1983; Uhnoo *et al*, 1983; Brown, 1985; van Loon *et al*, 1985b).

Chang cells were apparently more permissive for Ad40 growth than 293 cells and both these cell types should be considered semi-permissive for Ad40. Adenovirus 41 did not appear to grow as well in Chang cells as Ad40. Adenovirus 2 in turn grew very efficiently in these cells.

Adenovirus 40 and Ad41 infection of HEF cells did not result in any detectable hexon production. Adenovirus 2 growth was reduced in comparison to 293 and Chang cells. The higher optical density values obtained with extracellular hexon as opposed to intracellular hexon was unexpected but was consistent upon repetition. This may be due to substantial amounts of adsorbed virus leaking off into the medium

over time. This could be related to the predilection adenoviruses have for infecting epithelial cells as opposed to fibroblasts, perhaps due to a cell type related restriction at the stage of attachment and/or penetration.

An in-vitro foetal intestinal organ culture model was employed to assess the growth capabilities of the subgroup F adenoviruses in intestinal cells. Growth was monitored over time using the same indirect ELISA. Assuming that free hexon antigen production mirrors whole virus production in subgroup F adenoviruses, the results showed that the subgroup F adenoviruses do not grow as well as Ad2 in foetal intestinal cells, further demonstrating their fastidious nature. Their poor growth was not likely to be due to virus changes that may occur as a result of prior passage in cell culture, as a stool extract containing Ad41 strain FB585 also grew poorly. Since intestinal tissue from a number of different foetuses was used in this study and results were consistent throughout, poor growth could not be attributed to variations in tissue from different foetuses. One difference was, however, noted in that addition of trypsin to the growth medium resulted in enhanced growth of cell culture passaged Ad40 and Ad41, as well as of Ad41 strain FB585 inoculated as stool extract, in the intestinal tissue of one foetus but not of another.

The question then arises as to what factors *in vivo* promote infection to such a degree as to result in the production of large numbers of Ad40 or Ad41 virion particles. This is particularly perplexing since, in contrast to Ad40 and Ad41, Ad2 can be persistently shed in faeces for long periods of time with no overt disease but has the ability, as shown here, to propagate well in primary intestinal cells.

The foetal intestinal organ culture model used in this study probably provides the closest parallel to conditions *in vivo* except for the absence of a functional mucosal immune system and the presence of normal gut contents. Factors *in vivo* that can affect the outcome of enteric infection include gastrointestinal defense mechanisms such as gastric acidity, normal microflora, motility, mucous, and

humoral and cellular immunity (Guerrant, 1983). The organ culture model does, however, provide the means to study infection of these cells without the effects of these extraneous factors. It is important to remember that even though these cultures are immediate descendants from the organism, they may undergo some changes that may not be representative of the original cell phenotype *in vivo*. Such changes usually occur with established cells during the process of adaptation to continuous culture and are not considered to occur with primary cells.

The restricted growth of Ad40 and Ad41 in these cultures may be a result of differentiation-related processes that occur *in vivo* which were perhaps not duplicated in the in−vitro organ culture system. For example, the presence of a cellular factor with E1a-like activity in mouse F9 teratocarcinoma stem cells precludes the need for adenovirus E1a gene expression for early viral gene expression (Imperiale *et al,* 1984). This factor is lost upon differentiation of these cells and the requirement for E1a for early gene expression is restored. In contrast to adenoviruses, papovaviruses and retroviruses do not replicate in undifferentiated embryonic carcinoma cells (Segal and Khoury, 1979; Linnenbach *et al,* 1980; Stewart *et al,* 1982) but the block to viral gene expression is relieved by the process of cell differentiation. Human cytomegalovirus has also been reported to replicate in differentiated but not undifferentiated cells (Ibanez *et al,* 1991). In a similar way, efficient Ad40 and Ad41 replication *in vivo* could be dependent on the state of differentiation of the cell, this governing the presence of, or the critical concentration of a specific cellular factor.

Various extraneous factors could play a role in the potentiation of subgroup F adenovirus infection *in vivo* in the absence of a specific complementing cellular factor. In the following chapter it is shown that Ad2 provides a helper function for Ad41 growth *in vitro*. It is therefore possible that Ad2 and perhaps other adenoviruses could promote subgroup F adenovirus growth in coinfections *in vivo*. However, if this mechanism were entirely responsible for their potentiation then other adenoviruses

would be isolated more frequently from stool samples together with the subgroup F adenoviruses.

The pathology of subgroup F adenovirus enteritis may not be dependent on their growth capabilities in intestinal cells *per se* but rather as a result of their ability to avoid immune surveillance upon infection of these cells. This would then allow the slow accumulation of virus particles within the cells, giving rise to the liberation of large numbers of particles at the acute stage of infection. The difference between Ad2 and the subgroup F adenoviruses in their ability to cause gastrointestinal disease might then in some way be related to the interplay of their respective pathogenic genes and the immune system. Consistent with this possibility is the finding that there are distinct differences in the E3 coding region of Ad41 in comparison to that of other adenoviruses (Luftig *et al,* 1990). The E3 gene region is known to code for proteins involved in the interaction of the virus and the immune system. The best characterized protein, a 19K glycoprotein, binds major histocompatibility complex (MHC) class I antigens and in this way blocks their cell surface expression thereby preventing recognition of infected cells by cytotoxic T-cells (Burgert *et al,* 1987). Adenoviruses from subgroups B, C, D, and E have all been shown to modulate MHC class I expression via the E3-19K protein (Pääbo *et al,* 1986). With subgroup A adenoviruses, however, down-regulation of class I gene expression is induced by a mechanism that involves modulation by E1a (Bernards *et al,* 1983). Recently it has been found that the Ad41 E3 region does not code for a protein resembling the 19K proteins of other adenoviruses (Luftig *et al,* 1990). Assuming that the Ad12 E1a immune modulatory function is distinct from the E1a function responsible for augmenting viral replication (which is defective in Ad40 and Ad41), it is not unreasonable to suggest that the subgroup F adenoviruses may utilize a similar mechanism to that of subgroup A adenoviruses for down-regulating class I antigens. The common feature of tropism for the gastrointestinal tract of both these adenovirus subgroups and a definite (subgroup F) (Brandt *et al,* 1985; Uhnoo *et al,* 1984) and emerging (subgroup A) (Adrian *et al,* 1987) association with pediatric diarrhoeal

illness suggests that they may be subject to the same immune pressures and may have evolved similar mechanisms to ensure their survival.

The Ad41 E3 region has been shown to contain an ORF that has only 50% homology to the Ad2 14.7K protein which prevents lysis of adenovirus-infected cells by tumour necrosis factor (TNF) (Kraftig *et al*, 1990). In the absence of an E3 region the E1b-19K protein protects cells from TNF lysis (Gooding *et al*, 1988; 1990). Adenoviruses therefore seem to have alternative means of overcoming specific immune functions. Adenovirus 40 and Ad41 may possess some as yet unidentified protein that may play an important role in evasion of the mucosal immune system.

In semi-permissive Chang cells, infections with high concentrations of Ad40 and Ad41 result in appreciable fluorescent cell counts. However, upon inoculum dilution the decrease is much greater than expected. This phenomenon was analysed for Ad41 and it was found that infection of Chang cells did not comply with one-hit kinetics. One-hit kinetics implies that one infectious virus particle is required to productively infect a cell. Using statistical linear regression analysis it was apparent that Ad41 infection of Chang cells is multi-hit in character and it was calculated that approximately 4 to 5 infectious Ad41 particles are required to render a cell infected. This explains the partial growth restriction of Ad41 in these cells as well as difficulties in isolating virus from clinical specimens. This phenomenon highlights the importance of avoiding as far as possible the dilution of virus prior to inoculation.

The presence of Ad2 in coinfection of Chang cells with Ad41 altered the dose response graph to that expected for two-hit kinetics. In non-permissive HEF cells, Ad41 showed infectivity with two-hit kinetics in the presence of Ad2. Since Ad41 does not grow in HEF cells, the conversion from no-growth to two-hit kinetics was solely dependent on the presence of Ad2. Type 41 infectivity was seen to be directly proportional to input concentration (one-hit) only in 293 cells. The increased

efficiency of Ad41 replication in these cells may have been directly or indirectly due to the expression of Ad5 E1 products, as suggested earlier (Ta[illegible] *et al,* 1981).

The apparent multiple-hit requirement for Ad41 infection of Chang cells has more than one possible explanation. The virus population may consist of both defective and non-defective Ad41 particles, the multiplication of the defective particles being dependent on standard Ad41. DNA analysis of Ad41 infected Chang cells using alkaline sucrose gradient centrifugation revealed two discrete populations of DNA, one that sedimented close to that of unit length Ad2 DNA and one that sedimented in a lower density fraction. These could represent DNA from standard and defective particles, respectively, but requires further analysis. However, virus defectiveness that can be overcome by a helper virus is usually associated with a two-hit rather than a multiple-hit dose response (Blacklow *et al,* 1967; Young and Mayor, 1979). A more likely explanation is one that assumes a homogeneous population of virus particles, each particle having an equal probability of initiating infection, but a low probability of continuing infection to late antigen synthesis, perhaps due to a limiting concentration of some essential product. This limitation could then be overcome by multiple infecting genomes, the possibility of a successful cellular infection increasing markedly with increasing concentrations of input virus.

The above hypothesis correlates well with the recent finding that E1a products of Ad41 (van Loon *et al,* 1987a) and Ad40 (van Loon *et al,* 1987a; Ishino *et al, 1988)* have a reduced transactivating function when compared to those of other serotypes. In addition, there is little *cis*-acting activity of the Ad40 E1a promoter (Ishino *et al,* 1988). It has been recently found that the level of E1a expression does not affect Ad5 growth in cell culture (Hitt and Graham, 1990). This may mean that the subgroup F adenovirus growth restriction cannot be due to lack of E1a activity. On the other hand infection of a specific cell type may necessitate certain levels of E1a expression for efficient growth, the dependence being related to cell type. The E1b-55K function of Ad40 has been shown to be responsible for inefficient growth

in cell culture (Mautner *et al*, 1989). The E1 products from several infecting genomes within the one cell may have a cumulative effect, perhaps reaching sufficient activity to remove growth restriction in Chang cells, but not attaining the critical concentration necessary for infection of HEF cells. The two-hit dose response graphs for Ad41 in HEF cells are entirely compatible with this mechanism if one assumes that cells infected with Ad2 no longer have a growth restriction caused by limited adenovirus products.

Takiff and Straus (1982) suggested that the block in viral replication occurs at an early stage. The transition from the early phase of the adenovirus infectious cycle to the late phase is marked by the onset of viral DNA synthesis. This provides an ideal marker for determining whether the restriction occurs at an early or late stage. In this work, the *de novo* synthesis of Ad41 DNA was analysed using *in vivo* labelling with ^{32}P-orthophosphate followed by restriction enzyme digestion of extracted DNA. This was carried out in 293, Chang and HEF cells and Ad2 was included for comparison. Unlike Ad41 infection of permissive and semi-permissive cells, no newly synthesized DNA could be detected in Ad41 infected HEF cells.

Of the progeny genomes synthesized in adenovirus infected cells, only 10-20% are packaged to form progeny viral particles (Green, 1962; Green *et al*, 1970; Philipson and Lindberg, 1974). In order to determine the ability of Ad40 and Ad41 to synthesize and package their DNA in semi-permissive and non-permissive cells, intracellular and packaged DNA was compared at 24 hour intervals by dotting extracted DNAs and hybridizing with a subgroup F specific ^{32}P-labelled probe. In semi-permissive Chang cells Ad40 and Ad41 packaged DNA represented less than 10% of their respective intracellular DNA yields. Results obtained with Ad2 DNAs probed with a plasmid containing the E2a gene of Ad2, included as a control, were in keeping with a figure of 10-20%. A distinct difference was noted between Ad40 and Ad41 DNA synthesis and subsequent packaging of genomes in non-permissive cells. Adenovirus 40 intracellular DNA synthesis could be detected but no evidence

of packaging of the DNA into virion particles. Unlike Ad40, no Ad41 DNA synthesis could be detected. It therefore appears likely that the Ad41 replicative defect resides in an early function or functions. Since the adenovirus late phase of replication is marked by the onset of DNA replication, it is clear that Ad40 has the ability to progress to at least this stage albeit not with great efficiency. A defective early function in addition to a later replicative block can therefore not be excluded in the case of Ad40. It is also possible that the later defect may be a result of the early defect rather than being two mutually exclusive events.

Newly synthesized Ad40 DNA in HEF cells was not of one size class and appeared to exist in many size classes as shown in zone sedimentations. This level of incorporation could only be detected when long labelling periods were used, possibly due to a slow replication rate. Since fractions were not further analysed for the proportion of the counts that hybridize specifically to Ad40, incorporation of radiolabel mostly into cellular DNA could not be ruled out. If this was so it could be a result of the Ad40 infection itself, as no incorporation of radiolabel into cellular DNA could be detected under identical conditions in Ad41 infected HEF cells.

The ability of the subgroup F adenoviruses to shut off host protein synthesis was studied in permissive and semi-permissive cells. By comparison with Ad2 input multiplicities used to demonstrate effective host protein shutoff, it was found that concentrations of Ad40 and Ad41 required to show such an effect could not be attained. It could therefore not be shown conclusively that these viruses do not inhibit cellular protein synthesis. It is important to note that virus titrations were determined on Chang cells and not on 293 cells and as a result of the multiple-hit kinetics phenomenon already described for Ad41 infection of Chang cells, may be subject to an underestimation of the true number of infectious particles. Since Ad41 did not seem to shut off host protein synthesis in permissive 293 cells, even though prominent viral protein bands could be detected, efficient shut off of host protein synthesis seems an unlikely event even in these cells. Mautner *et al* (1989) found that the

E1b-55K function of Ad40 is deficient in non-permissive cells. The Ad5 E1b-55K protein functions in the accumulation of late viral mRNAs and in the switching off of host cell functions (Babiss and Ginsberg, 1984; Pilder *et al*, 1986). KB cells that provide this function in *trans* supported Ad40 growth but no host protein shutoff could be demonstrated (Mautner *et al*, 1990). Some function in addition to the Ad40 E1b-55K protein must therefore be involved. Such a function necessary for host protein shutoff could either be provided by the fibre antigen which has been shown to inhibit cellular macromolecular synthesis (DNA, RNA and protein) (Levine and Ginsberg, 1967), or, as has been recently suggested, the VA-RNAI and DAI (double-stranded RNA activated inhibitor) kinase may play a role in both the selective translation of viral mRNA and shut off of host protein synthesis late during infection (O'Malley *et al*, 1989).

In addition to studies on DNA synthesis, another approach was taken to determine if Ad41 infection of non-permissive cells progressed to a late stage in infection. A RT-PCR method was employed to detect virus late gene expression. Expression of the subgroup F adenovirus fibre gene was selected as a marker for a late stage in infection. In this assay the detection of the specific 152bp target sequence would indicate the expression of the Ad41 long fibre gene. Fibre mRNAs could be detected at earlier times in permissive 293 cells, were present over a much longer time period in semi-permissive Chang cells, and could not be detected in non-permissive HEF cells. These results were in agreement with results from DNA synthesis in Ad41 infected cells confirming that the Ad41 block in replication occurs before DNA replication. Considering the fact that Ad40 does replicate its DNA at least to some extent in these cells, it would be interesting to determine if fibre mRNA can be detected in infected HEF cells.

The extent of transcription in Ad41 infected 293, Chang, and HEF cells was further monitored by DNA:RNA hybridizations using cloned Ad40 and Ad41 fragments representing different regions of the genome. Cloned *Pst*I-fragments were

mapped in relation to known restriction enzyme maps and assigned tentative transcription units. The viral r- and l-strands of the DNA probes were not separated and therefore could not distinguish between mRNAs specifically transcribed from one or other strand. In addition, as a result of poly(A) RNA selection on oligo-dT columns, the production of VA RNA was not monitored.

The temporal distribution of all mRNAs was distinctly different between 293, Chang and HEF cells, showing a similar time span of appearance to fibre mRNAs as detected by RT-PCR. Adenovirus 41 transcription in 293 cells was limited to a time period of about 12 hours although RT-PCR being the more sensitive technique detected fibre-specific transcripts at an additional time point. This rapid transcription rate corroborated well with the marked increase in the number of Ad41 hexons within infected cells between 24 and 48 hours after infection. It is important to note that due to practical considerations, the input multiplicities of virus were different between the two analyses, with cells used for virus growth determinations receiving a 7.9 times larger inoculum (0.019 FFU/cell versus 0.0024 FFU/cell for transcription studies). This could alter the temporal appearance of certain events in relation to each other. Determination of the progression of transcription in these cells would require isolation of mRNAs at closer time points between 24 and 48 hours after infection using the same conditions of infection.

Of the DNA regions tested, only one region hybridized with mRNAs from Ad41 infected HEF cells, the E1 gene region (0-12.0 m.u.). This region encodes the E1a and E1b proteins produced early after infection as well as at intermediate times, *viz.* the structural polypeptide IX. Transcripts from this region were first detected 36 hours after infection, decreased over the next 12 hours and were not detected beyond this time. It is unlikely that transcription from a gene for a structural protein would be detected in the absence of detectable transcription from other early genes, even though small amounts of polypeptide IX transcripts can normally be detected before DNA synthesis (Persson *et al*, 1978; Chow *et al* 1980). From the results it appears

that the major restriction to Ad41 growth in HEF cells involves the E1 gene region. Further analysis is required to determine whether this block is due to E1a- and/or E1b- specific processes.

In Chang cells, on the other hand, E1-specific transcripts accumulated gradually over time from approximately 24 hours through to 84 hours after infection. This was not unexpected as transcripts from adenovirus E1a region (13S and 12S mRNAs) are known to persist throughout infection, whereas the 9S mRNA is made preferentially at late times after infection (Chow *et al*, 1979; Esche *et al*, 1980; Spector *et al*, 1980; Virtanen and Pettersson, 1983). The results obtained with a plasmid, pSP64-*Eco*RI*B*, which spans E3, L5 and part of the L4 transcription unit may have been a result of initial expression of E3-specific transcripts followed by late transcription from regions L4 and L5. Most of the plasmids used in this study contained at least one late region and it was therefore difficult to distinguish early from late transcription. The two *Pst*I fragments *D* and *H* contained within plasmid M25 were not used individually and therefore did not discriminate between E2a and L4, and E3 transcription, respectively. Transcripts from one or all of these regions were, however, detected only at 60 hours after infection. Plasmid M34 contains a *Pst*I fragment that spans region E2b which encodes the 140K DNA polymerase and the pTP, the transcription unit coding for the IVa_2 protein which is produced at intermediate times, and a small part of the L1 transcription unit. Transcripts complementary to some or all of these regions were also detected at 60 hours p.i. It may be significant that no transcripts were detected that hybridized to the E4 region of Ad41 in infected Chang cells. By comparison with mRNA from 293 cells which presented a weak hybridization signal, a reduced level rather than the absence of E4 gene expression seems likely but requires confirmation. It may be that levels of transcription from most early transcription units are below the level of detection. If this is so, low levels of early transcription may be as a direct result of abberant functioning of the E1a gene, an important function of which is the activation of transcription from all other early promoters.

Only low levels of late transcription could be detected in Chang cells. This was unexpected as adenoviral RNA synthesis at late times can account for 20-30% of the total RNA synthesis in the cell (Lucas and Ginsberg, 1971). Messenger RNAs from Ad2 early regions reach a steady-state level of approximately 500-1000 per cell, while a late mRNA typically reaches a steady-state level of 2000-5000 copies per cell (Flint and Sharp, 1976). This is further amplified to 20,000-50,000 copies per cell at later times after infection. A reduced level of late transcription is consistent with lower production of hexon antigens both intracellularly and extracellularly when compared to that in Ad2 infected Chang cells. This may in part be due to fewer template DNAs as a result of less efficient DNA synthesis relative to that of Ad2. Levels of late transcripts also appeared to be low in Ad41 infected 293 cells even though intracellular hexon levels were comparable with those of Ad2

The transcription work described here served as a preliminary study to provide some indication of the temporal distribution of transcripts from various regions of the Ad41 genome in cells that show varied permissiveness for Ad41 growth. There is a need, however, to distinguish more clearly between early and late gene transcription. This can be achieved with plasmids that contain for example an early region mapping to the l-strand and late regions to the r-strand. Single-stranded probes can be made from these plasmids that will specifically detect either l-strand or r-strand transcripts. However, most of the plasmids used in this study require some modification in order to contain one transcription unit only. A more detailed approach would involve the isolation of specific size transcripts at specific times and annealing of these to separated strands of discrete DNA regions (Flint *et al*, 1976). Alternatively, the determination of relative transcription rates *in vivo* from viral initiation sites could be determined by pulse-labelling with ^{3}H-uridine at different times after infection (Nevins *et al*, 1979). From the results shown here, marked differences might be expected in viral transcription rates between the different cell types. The RT-PCR method, used here for Ad41 fibre gene expression and in another study for mapping of Ad40 E1b

transcripts (Steinthorsdottir and Mautner, 1991) can readily be applied to transcription studies of regions of known sequence.

Since the subgroup F adenoviruses display such varied responses when infecting different host cells, and in addition, show type-related differences in these interactions, they provide an excellent model for studying viral and cellular functions essential to adenovirus propagation *in vitro* and *in vivo*.

CHAPTER FOUR

COMPLEMENTATION

4.1 Introduction

It has been postulated that a block early in virus replication prevents the efficient growth of the subgroup F adenoviruses (Takiff and Straus, 1982). This was based on a number of observations. Only a few isolated subgroup F adenovirus infected KB and HeLa cells showed detectable virion proteins when viewed by immunofluorescent microscopy and DNA synthesis was highly restricted in HeLa cells. Subgroup F adenoviruses have the ability to help AAV, a defective parvovirus, although not as efficiently as Ad5 (Takiff and Straus, 1982). This finding indicated that at least some early functions are expressed.

Human adenovirus infection of monkey cells is abortive with the block in replication occurring late in infection. Coinfection with SV40 results in productive adenovirus infection (Klessig, 1984). It was found, however, that SV40 cannot complement subgroup F adenovirus growth in monkey cells (Takiff and Straus, 1982).

It has been suggested that 293 cells overcome an early replicative block in subgroup F adenovirus replication by supplying a gene product derived from the integrated sequences of the Ad5 E1 region (Takiff *et al,* 1981; Takiff and Straus,

1982). If this is truly complementation in *trans*, it could be expected that coinfection of non-transformed cells with a subgroup F adenovirus and another serotype would emulate the helper effect of 293 cells on Ad41 growth.

The aims of this chapter were to investigate the effect of Ad2 infection on Ad41 late antigen production in cells which are normally either semi-permissive or non-permissive for Ad41 growth. On the basis of the results obtained the study was extended to further investigate the nature of the subgroup F adenovirus defective function or functions.

4.2 Results

4.2.1 Characterization of monoclonal antibodies

The specificity of anti-Ad41 monoclonal antibody reactions was determined using indirect immunofluorescence and ELISA. The results are shown in Table 4.1. Monoclonal antibodies MAb-4 and MAb-5 (produced in Johannesburg) were specific for subgroup F adenoviruses and did not react with Ad2. Monoclonal antibody MA3-20 (from the Netherlands) reacted only with Ad40 and MA5-15 reacted only with Ad41. An adenovirus group-specific polyclonal antibody which reacted with all three viruses was included for comparison.

Table 4.1 Specificity of antibody reactions determined by indirect immunofluorescence and ELISA

Antibody	Virus		
	Ad2	Ad40	Ad41
Group-specific anti-Ad5[a]	+	+	+
MAb-4	-	+	+
MAb-5	-	+	+
MA3-20	-	+	-
MA5-15	-	-	+

[a]Polyclonal antiserum.

The monoclonal antibodies were then isotyped to determine the class, subclass and light chain type. This was carried out to determine if any of the antibodies were of the IgG1 subclass. Antibodies of this subclass require coating of protein-A beads before immunoprecipitations can be performed. The results are shown in Table 4.2.

Monoclonal antibodies MAb-4 and MA5-15 are of subclass IgG2a, and MAb-5 of subclass IgG2b. Monoclonal antibody MA3-20, however, reacted with three

antibody subclasses, although more strongly with the IgG2a subclass, even when 5% milk powder was included in the reaction as blocking agent. This may be due to high concentrations of monoclonal antibody showing low levels of cross-reactivity with the goat antibodies on the typing stick, the presence of host antibodies in ascitic fluid, or a mixture of antibodies requiring further cloning of hybridoma cells. Unfortunately, culture supernatants were not available for this antibody, so it was not possible to distinguish between these possibilities.

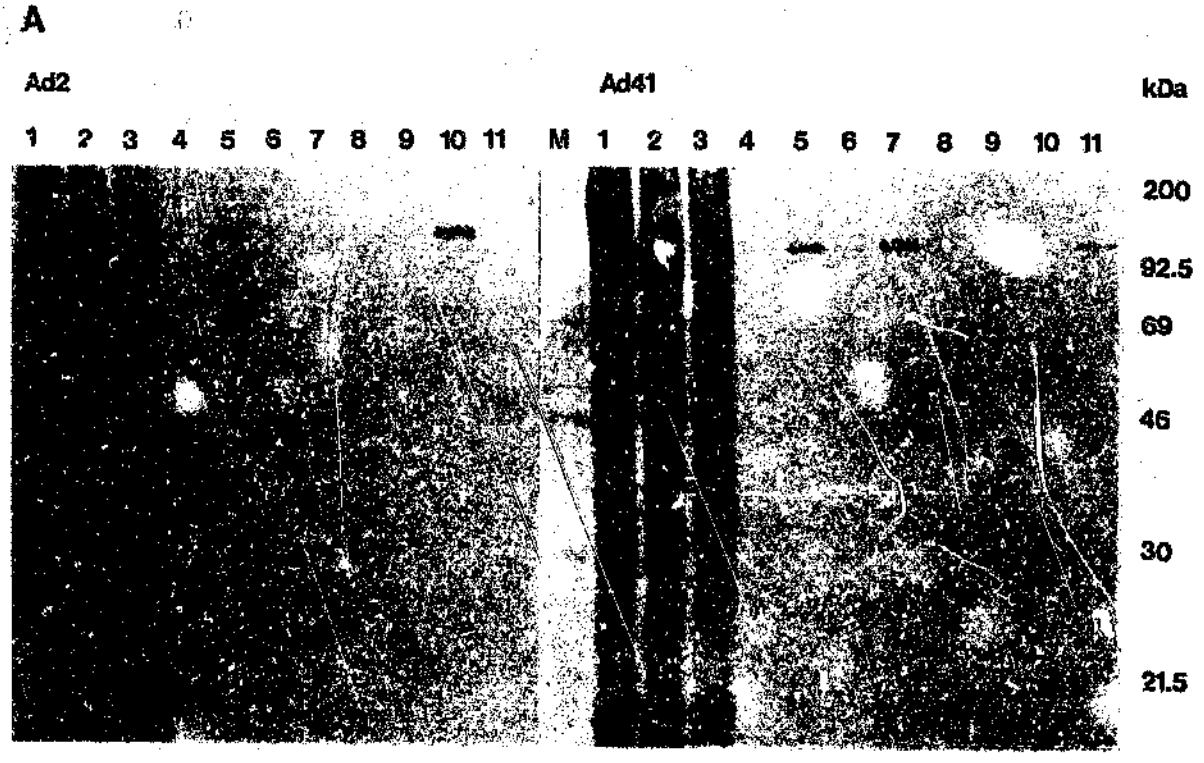
Table 4.2 Isotyping of mouse monoclonal antibodies

MAb	Reaction on typing stick[a]								
	IgA	IgM	IgG1	IgG2a	IgG2b	IgG3	K	λ	control[b]
MAb-4	-	-	-	+	-	-	+	-	+
MAb-5	-	-	-	-	+	-	+	-	+
MA3-20	-	-	+	+	+	-	+	-	+
MA5-15	-	-	-	+	-	-	+	-	+

[a]The isotype is determined by the appearance of two purple dots on nitrocellulose sticks, one corresponds to the antibody class (IgM or IgA) or subclass (IgG) and one to the antibody light chain (K or λ).
[b]mouse antibody control.

To identify the specific viral proteins that the monoclonal antibodies react with as well as confirm their specificity, infected cell proteins were labelled *in vivo* with ^{35}S-methionine. Radioimmunoprecipitations were performed as described in section 2.20. Labelled proteins from uninfected cells and radioimmunoprecipitations using polyclonal antibodies raised against adenovirus hexon were used as controls. Monoclonal antibodies MAb-4 and MAb-5 reacted specifically with Ad41 but not with Ad2 (Figure 4.1A). Adenovirus 40 was not included in this comparison. Monoclonal antibodies MA3-20 and MA5-15 reacted with Ad40 and Ad41, respectively (Figure.4.1B). Control group-specific antiserum reacted with Ad2 hexon (123K), Ad40 hexon (97K) (not shown) and Ad41 hexon (109.4K). Molecular weights of proteins were determined from standard curves of known molecular weights of ^{14}C-labelled marker proteins as a function of distance migrated within each gel.

Monoclonal antibodies MAb-4, MA3-20, and MA5-15 all precipitated hexon antigen. Monoclonal antibody MAb-5 precipitated a 61K protein of Ad41, most likely the IIIa or fibre protein and would be expected to precipitate the same protein of Ad40. The Ad40 and Ad41 large fibre protein molecular weights have been determined from sequencing studies as 59K and 60.5K respectively (Kidd and Erasmus, 1989, Kidd *et al*, 1990). A comparison of fluorescent cell counts of Ad41 infected Chang cells on 4 replicate coverslips showed that there was no significant difference in counts between the different antibodies at various times tested (data not shown).



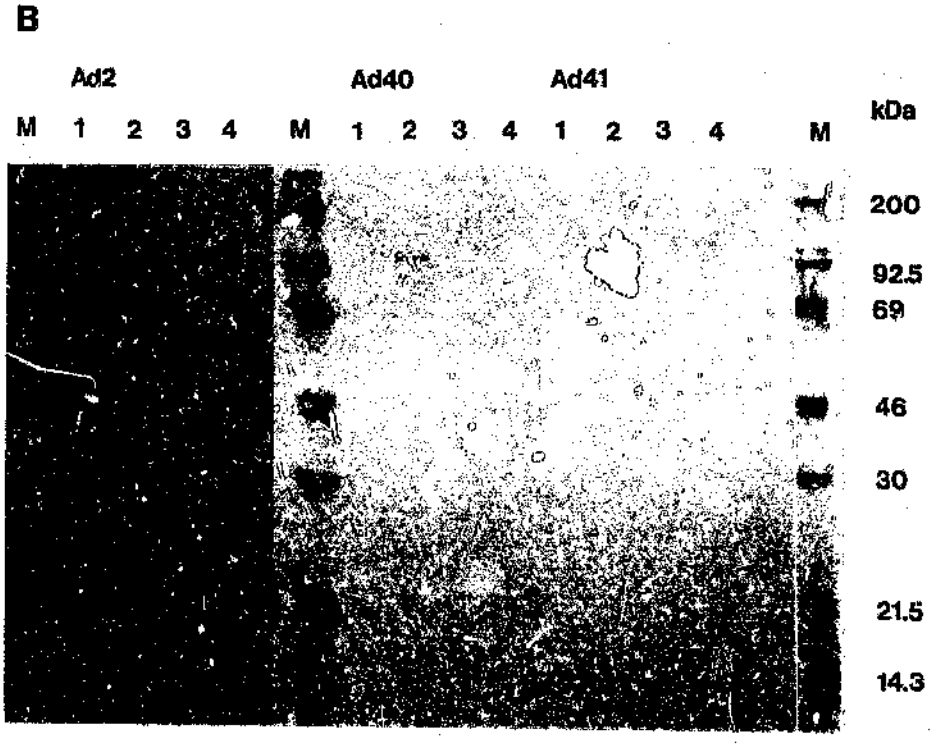


Figure 4.1 Immunoprecipitation of ^{35}S-labelled proteins of Ad2 and Ad40 infected Chang cells, and Ad41 infected 293 cells with polyclonal and monoclonal antibodies.

A. Reactivity of MAbs raised against Ad41. Lane 1. control uninfected cells. Lanes 2-3. control infected cells. Lanes 4,6,8,10. R.I.P.A. of labelled uninfected cells with group-specific anti-Ad5 antiserum, MAb-4, MAb-5, group-specific anti-Ad3 antiserum, respectively. Lanes 5,7,9,11. R.I.P.A. of labelled infected cells with group-specific anti-Ad5 antiserum, MAb-4, MAb-5, group-specific anti-Ad3 antiserum, respectively. Lanes 10 and 11 of Ad2 were interchanged.

B. Reactivity of Ad40 and Ad41 specific MAbs. Lanes 1,3. R.I.P.A. of labelled uninfected cells with MA3-20 and MA5-15, respectively. Lanes 2,4. R.I.P.A. of labelled infected cells with MA3-20 and MA5-15, respectively. Lane M. ^{14}C-labelled protein molecular weight markers (14,300 - 200,000 kDa range).

4.2.2 Virus complementation

4.2.2.1 Complementation of Ad41 by Ad2 in semi-permissive cells

To determine if Ad2 could enhance Ad41 late antigen synthesis in Chang cells, cells were infected with Ad41 alone, or coinfected with Ad2 and Ad41 at varying input ratios of viable particles (Table 4.3). The Ad41 input concentration was kept constant and at its highest in all coinfections, with 1 log differences in Ad2 input concentration. Equal volumes of appropriate virus dilutions for coinfections were mixed prior to inoculation. The input Ad41 concentration was, therefore, a 1 in 2 dilution of stock virus (1.2×10^4 FFU/ml). Coverslips were treated for the detection of cells with Ad41 late antigens using subgroup F specific monoclonal antibody.

Table 4.3 Complementation of Ad41 late antigen synthesis by Ad2 in Chang conjunctival cells

Input ratio of viable particles* Ad41: Ad2	Ad41 fluorescence	
	FFU/10^6 cells	% of total cells
1:10	25,606.92 ± 4,290.97	2.56
1:1	4,290.44 ± 491.48	0.43
1:0.1	1,672.00 ± 204 86	0.17
1:0.01	1,677.57 ± 98.39	0.17
1:0.00	1,997.56 ± 199.29	0.20

*Cells were coinfected with 1.2×10^4 FFU/ml of Ad41 and 1.4×10^5 FFU/ml, 1.4×10^4 FFU/ml, 1.4×10^3 FFU/ml, and 1.4×10^2 FFU/ml of Ad2, corresponding to input ratios (Ad41:Ad2) of 1:10, 1:1, 1:0.1, and 1:0.01, respectively.

The lower Ad2 concentrations tested in coinfection had no effect on Ad41 fluorescent cell counts (Figure 4.2), the number of viable Ad2 particles required to initiate a significant helper effect being greater than 1.4×10^3 FFU/ml. This concentration corresponds to approximately 0.05% of cells infected with Ad2. Ad41 fluorescent cell counts were elevated from 0.2% of cells fluorescing to 2.56% with the highest concentration of Ad2 used in coinfection, a 12.8-fold increase.

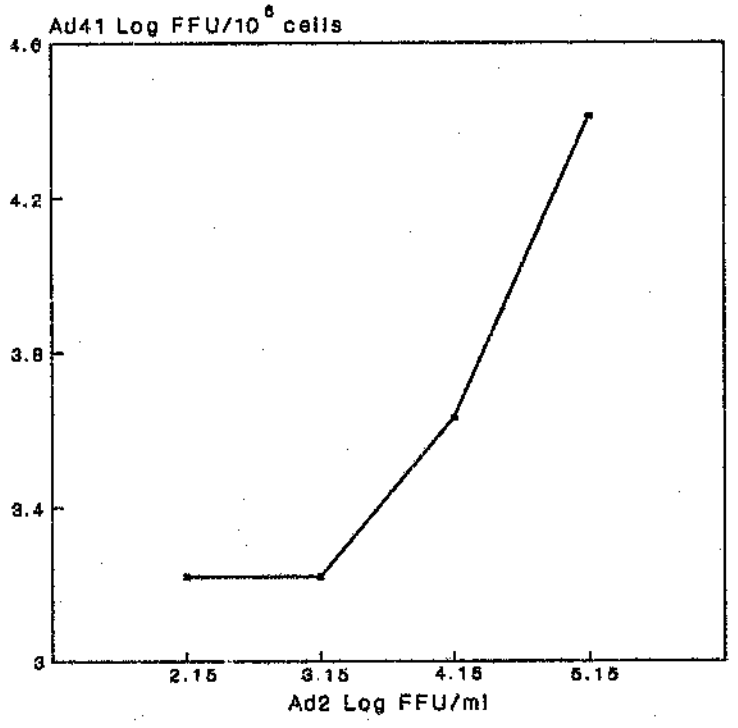


Figure 4.2 Complementation of Ad41 late antigen synthesis by Ad2 in Chang conjunctival cells. Cells were coinfected with Ad41 and Ad2 at concentrations and input ratios described in Table 4.3.

4.2.2.2 Complementation of Ad41 by Ad2 in non-permissive cells

Complementation of Ad41 by Ad2 in HEF cells was demonstrated at the level of DNA synthesis (Figure 4.3). This was shown by incorporation of radioactive phosphate into newly synthesized viral DNA in coinfections of cells followed by restriction endonuclease typing. Adenovirus 41 infection of HEF cells showed no evidence of DNA synthesis. With the enzymes *Xho*I and *Eco*RI a mixture of both Ad2 and Ad41 DNA profiles were detected in mixed infections. The presence of Ad41 in coinfection with Ad2 results in reduced Ad2 DNA synthesis when compared to Ad2 DNA synthesis in single infection.

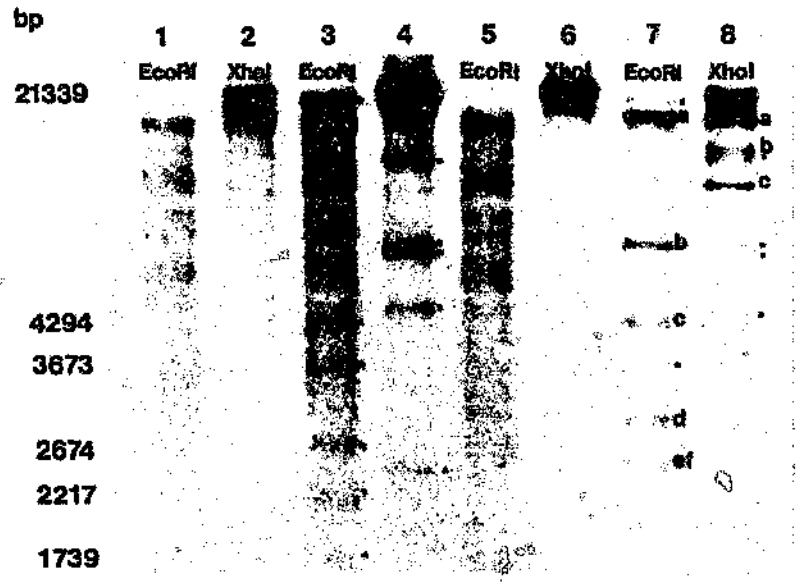


Figure 4.3 Autoradiograph showing virus DNAs labelled *in vivo* with ^{32}P-orthophosphate in HEF cells. DNAs extracted from uninfected cells (lanes 1-2), cells infected with Ad2 (lanes 3-4), Ad41 (lanes 5-6), and coinfected with Ad41 and Ad2 (lanes 7-8) were digested with restriction enzymes *Eco*RI and *Xho*I, respectively, and electrophoresed on a 1% w/v agarose gel. The gel was dried and autoradiographed for 3 hours. Sizes (bp) of Ad2 *Eco*RI fragments are indicated. Lane 3. Ad2 *Eco*RI (*A-F*). Lane 4. Ad2 *Xho*I (*A-G*) fragments. Ad2 fragment *XhoIG* is not visible. Lane 7. Ad41 *Eco*RI (*a-f*). Lane 8. Ad41 *Xho*I (*a-c*) fragments. Ad2 fragments are shown by dots, and Ad41 fragments by letters. The Ad2 fragments visible in coinfection with Ad41 are shown.

Coinfections of Ad41 and Ad2 were performed in HEF cells to determine if the presence of Ad2 could promote Ad41 replication to the stage of late antigen

synthesis. HEF cells were infected with Ad41 alone and with mixtures of Ad41 and Ad2 at the input viable particle ratios indicated in Table 4.4. The concentration of Ad41 was once again kept constant and at its highest in coinfection, with 1 log differences in Ad2 input concentration. Infections and Ad41 fluorescent cell count determinations were as for Chang cells.

There was an increase in Ad41 fluorescent cell counts with increasing Ad2 concentration in coinfection (Figure 4.4). At the highest concentration of Ad2 tested (0.1% of cells infected with Ad2), 0.01% of cells were positive for Ad41 fluorescence. These counts were 17.8-fold less than Ad41 counts for single infection of Chang cells. Adenovirus 41 fluorescent focus counts were proportional to the input Ad2 concentration : increasing the Ad2 concentration by a factor of 10 resulted in a 1 log increase in Ad41 fluorescent cell counts. Linear regression analysis of the composite data indicated a minimum required Ad2 concentration of 86.78 FFU/ml as determined in Chang cells, and 15.43 FFU/ml as determined in HEF cells, necessary to initiate a detectable helper effect in coinfections of HEF cells.

Table 4.4 Complementation of Ad41 late antigen synthesis by Ad2 in human embryonic fibroblasts

Input ratio of viable particles[a] Ad41:Ad2	Ad41 fluorescence	
	FFU/10^6 cells	% of total cells (x 10^{-4})
1:10	112.36 ± 54.83	112.4
1:1	11.24 ± 4.58	11.2
1:0.1	7.02 ± 5.38	7.0
1:0.01	1.40 ± 2.80	1.4
1:0.00	NONE	-

[a]Cells were coinfected with 1.2 x 10^4 FFU/ml of Ad41 and 1.4 x 10^5 FFU/ml, 1.4 x 10^4 FFU/ml, 1.4 x 10^3 FFU/ml, and 1.4 x 10^2 FFU/ml of Ad2, corresponding to input ratios (Ad41:Ad2) of 1:10, 1:1, 1:0.1, and 1: 0.01, respectively. These titres were determined in Chang cells. The Ad2 titres as determined in HEF cells were 2.5 x 10^4 FFU/ml, 2.5 x 10^3 FFU/ml, 2.5 x 10^2 FFU/ml, and 2.5 x 10^1 FFU/ml, respectively.

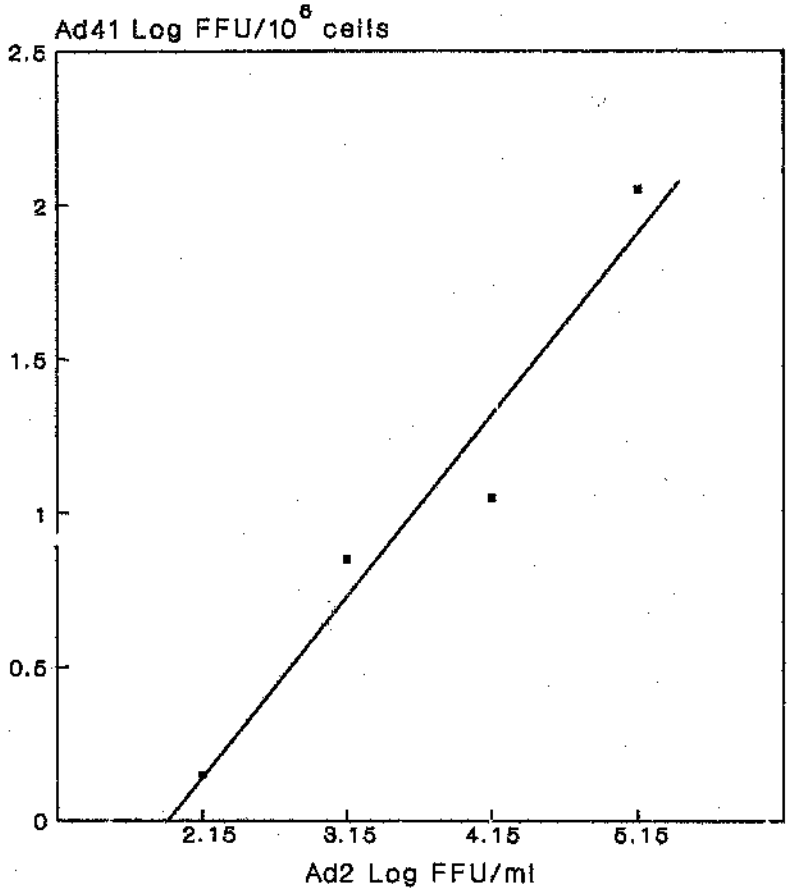


Figure 4.4 Complementation of Ad41 late antigen synthesis by Ad2 in HEF cells. Cells were coinfected with Ad41 and Ad2 at concentrations and input ratios described in Table 4.4.

4.2.2.2.1 Effect of preinfection, coinfection, and post-infection with Ad2 on Ad41 late antigen synthesis in HEF cells

To determine the effect of the time of Ad2 infection on Ad41 late antigen synthesis, monolayers of HEF cells were: (i) preinfected with Ad2 10 hours before Ad41

superinfection, (ii) coinfected with Ad2 and Ad41, and (iii) infected with Ad2 10 hours after Ad41 infection. Virus was adsorbed at the relevant times, cultures were incubated for 44 hours and the coverslips were fixed. Time zero was taken to be the end of the Ad41 adsorption period and all other times were considered in relation to this time. Adenovirus 41 fluorescent counts were determined using the subgroup F specific monoclonal antibody.

The effect of the two viruses on each other in mixed infections, with varying time of Ad2 infection in relation to that of Ad41, is shown in Table 4.5 and schematically represented in Figure 4.5. Adenovirus 41 synthesis was helped by the presence of Ad2 if Ad2 was applied 10 hours before, during, of 10 hours after Ad41 infection of HEF cells. The Ad41 fluorescent cell counts for coinfection and post-infection with Ad2 did not differ significantly from each other. There was, however, a significant increase in Ad41 fluorescent cell counts when Ad2 was applied 10 hours before Ad41, indicating an even greater helper effect afforded by Ad2 under these conditions.

In a separate experiment HEF cells were preinfected with Ad2 at varying times prior to Ad41 superinfection, and coinfected with Ad2 and Ad41 (Table 4.6), in order to determine if the provision of the Ad2 helper effect for Ad41 could be related to the time of Ad2 preinfection. Coverslip cultures of HEF cells were infected with Ad2 either 20, 10, 6, or 3 hours before Ad41 infection, or coinfected with Ad41. A constant input ratio of 1:10 (Ad41:Ad2) was used. Cells were incubated for 44 hours from the time of Ad41 infection and fixed and treated with subgroup F specific monoclonal antibody. There was no significant difference in Ad41 fluorescent cell counts whether Ad2 was applied with Ad41, or up to 6 hours before Ad41. Between Ad2 preinfection times of 10 and 6 hours, there was a 14.82-fold increase in Ad41 fluorescent cell counts. A more gradual increase of 3.75-fold was noted resulting from preinfection times between 20 and 10 hours.

Table 4.5 Effect of preinfection, coinfection, and post-infection by Ad2 on Ad41 late antigen synthesis in HEF cells

Time of infection with Ad2[a]	Fluorescence (FFU/10^6 cells)		
	Ad2 in single infection[b]	Total in mixed infection[b]	Ad41 in mixed infection[c]
Preinfection	24,957.97 ± 2,577.76	14,200.90 ± 2,140.40	964.89 ± 91.91
Coinfection	6,130.64 ± 1,373.71	1,790.74 ± 204.10	113.76 ± 37.64
Post-infection	998.60 ± 254.61	1,860.96 ± 247.30	50.56 ± 26.35

[a]Times of Ad2 infection are shown in relation to that of Ad41 : cells were preinfected with Ad2 10 hours prior to, coinfected with, and post-infected 10 hours after Ad41 infection. The input ratio of viable particles was 1:10 (Ad41:Ad2).
[b]Cells treated with group-specific polyclonal antibody.
[c]Cells treated with subgroup F specific monoclonal antibody.

Table 4.6 Effect of varying times of Ad2 preinfection on Ad41 late antigen synthesis in HEF cells

Ad2 preinfection time (hours)	Ad41 fluorescence	
	FFU/10^6 cells	% of total cells (x 10^{-3})
20	5,235.98 ± 923.37	526.60
10	1,394.67 ± 880.23	139.47
6	94.10 ± 14.78	9.41
3	43.54 ± 19.67	4.35
0 (coinfection)	47.75 ± 11.69	4.78

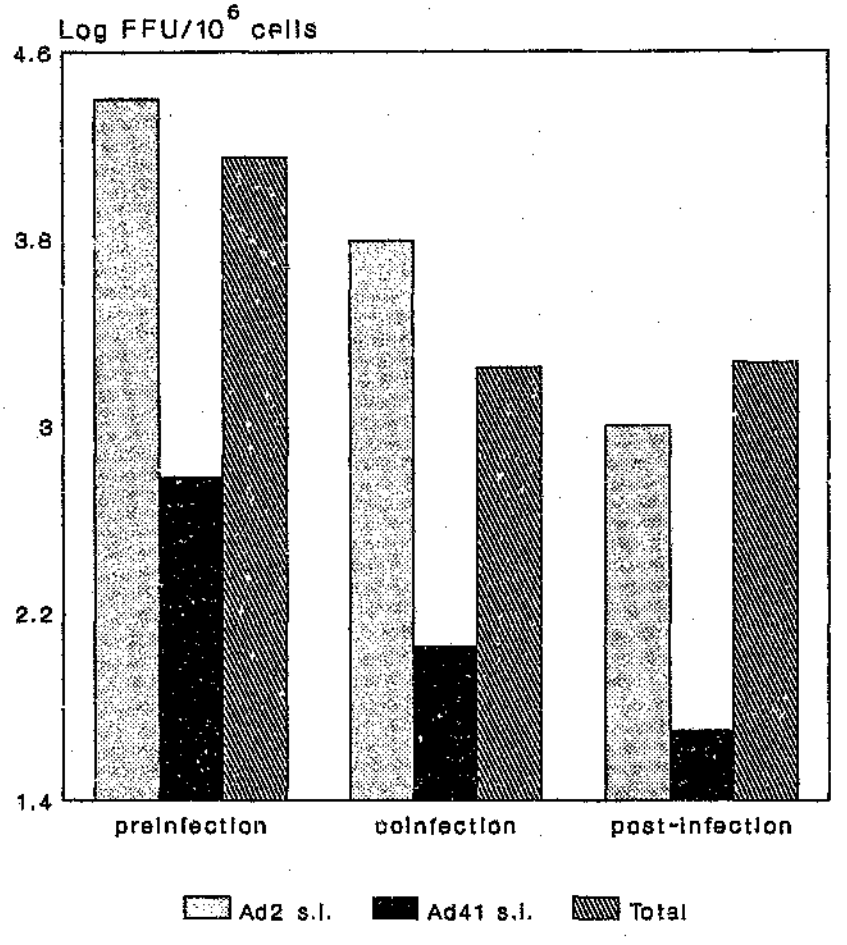


Figure 4.5 Histogram showing the effect of preinfection, coinfection, and post-infection of Ad2 on Ad41 late antigen synthesis in HEF cells. Cells were infected with 1.2 x 10^4 FFU/ml of Ad41, and 1.4 x 10^5 FFU/ml of Ad2 applied 10 hours before, with, and 10 hours after Ad41. The input ratio of viable particles was 1:10 (Ad41:Ad2). Bar heights represent Log FFU/10^6 cells for Ad2 in single infection (s.i.), and total and Ad41 fluorescence in mixed infection as a function of time of Ad2 infection.

4.2.2.3 Coinfections of Ad40 and Ad41 in non-permissive cells

To determine if Ad40 and Ad41 had the ability to complement each other in non-permissive cells, cells were coinfected with Ad40 and Ad41 at concentrations and input ratios described in Table 4.7. Cells were fixed at 44 hours after infection and separate coverslips treated with Ad40 and Ad41 specific monoclonal antibody. No complementation was detected of either Ad40 or Ad41 at any of the input concentrations used in coinfections. The results were the same when coinfections were continued for a further 5 days.

Table 4.7 Coinfections of Ad40 and Ad41 in human embryonic fibroblasts

Input ratio of viable particles[a] Ad40:Ad41	Fluorescence	
	Ad40[b]	Ad41[c]
1:1	-	-
1:0.5	-	-
1:0.25	-	-
1:0	-	-
0.5:1	-	-
0.25:1	-	-
0:1	-	-

[a]Cells were coinfected with 1.2 x 10^4 FFU/ml of Ad40 and 1.2 x 10^4 FFU/ml, 6 x 10^3 FFU/ml, and 3 x 10^3 FFU/ml of Ad41, corresponding to input ratios (Ad40:Ad41) of 1:1, 1:0.5, and 1:0.25, respectively; and 1.2 x 10^4 FFU/ml of Ad41 and 6 x 10^3 FFU/ml, and 3 x 10^3 FFU/ml of Ad40, corresponding to input ratios (Ad40:Ad41) of 0.5:1, and 0.25:1, respectively.
[b]Cells treated with Ad40 specific monoclonal antibody (MA3-20).
[c]Cells treated with Ad41 specific monoclonal antibody (MA5-15).

4.2.3 Virus interference

In addition to monitoring the effect of Ad2 on Ad41 in mixed infections, the effect of Ad41 on Ad2 was also monitored. Adenovirus 2 fluorescent cell counts in single infection and total fluorescent cell counts were determined using group-specific polyclonal antibody. Since HEF cells are non-permissive for Ad41 and on no occasion did Ad41 in single infection give fluorescent foci, each cell showing positive fluorescence in mixed infection would therefore be infected with helper Ad2. Thus, if Ad2 fluorescent cell counts detected by the group-specific antiserum in mixed infection were equivalent to the total number of fluorescent cells detected by the same antibody in single infection, this would indicate that Ad41 had no effect on Ad2 late antigen synthesis.

Comparisons of Ad2 fluorescent cell counts in single infections and total fluorescent cell counts in mixed infections of Ad41 and Ad2 showed significant differences for all times of Ad2 infection (Table 4.5, Figure 4.5). When HEF cells were preinfected with Ad2, or coinfected with Ad2 and Ad41, Ad41 interfered with Ad2 as shown by a reduction of total counts compared to Ad2 counts in single infection. The opposite occurred when Ad2 was applied 10 hours after Ad41, in that Ad2 counts were enhanced by the presence of Ad41.

4.2.4 Complementation by transfected viral sequences

4.2.4.1 Molecular cloning of Ad2 restriction enzyme fragments

Adenovirus 2 DNA restriction enzyme fragments were cloned into plasmids pSPT18/19 and pBR322 according to the methods outlined in section 2.35. Fragments were either blunt-end ligated into *Sma*I digested plasmids or inserted into plasmids cut with enzymes that generate compatible sticky ends. Adenovirus DNA has a 55K protein attached to the 5′ end of each DNA strand. Proteinase K treatment of DNA is not sufficient to remove this protein and a resistant peptide remains. Cloning of terminal fragments requires the removal of this peptide (Berkner and Sharp, 1982).

Two methods were employed to separate the terminal protein from the DNA: (i) piperidine treatment and (ii) enzymatic treatment of the DNA-protein complex. The steps in the latter procedure are schematically represented in Figure 4.6. DNA was first incubated in the presence of pronase to ensure adequate digestion of the TP, even though DNA preparations had already been treated with proteinase K at the extraction stage. After removal of the enzyme and precipitation of the DNA, T4 DNA polymerase was added in the presence of dATP and dTTP. This allows the specific removal of G or C bases in the absence of dGTP and dCTP due to the 3′ to 5′ exonuclease activity of T4 DNA polymerase. In this instance, the 3′ G base was removed. The enzyme would not proceed further due to the presence of dTTP and dATP in the reaction. The enzyme S1 nuclease is a single-strand specific endonuclease which cleaves DNA to release 5′-mono- and 5′-oligonucleotides. Here it was used to remove the pronase-resistant 5′ TP peptide by nicking the DNA between the unpaired 5′ C base and the adjoining paired nucleotide.

The terminal restriction fragments were then treated with the Klenow fragment of DNA polymerase I to ensure blunt ends on either side of the fragment. The fragment was ligated using T4 DNA ligase to a *Sma*I digested plasmid previously treated with CIP to prevent circularization.

Competent bacterial cells were transformed with ligated plasmid-Ad2 preparations and colonies containing Ad2 DNA selected by *in situ* colony hybridizations with DIG-labelled Ad2 DNA (section 2.35.6). Plasmid DNAs prepared from these colonies were digested with restriction enzymes to confirm the presence of cloned fragments (Figure 4.7A). Further confirmation was provided by hybridization of a Southern blot of the digested plasmids with ^{32}P-labelled Ad2 DNA (Figure 4.7B).

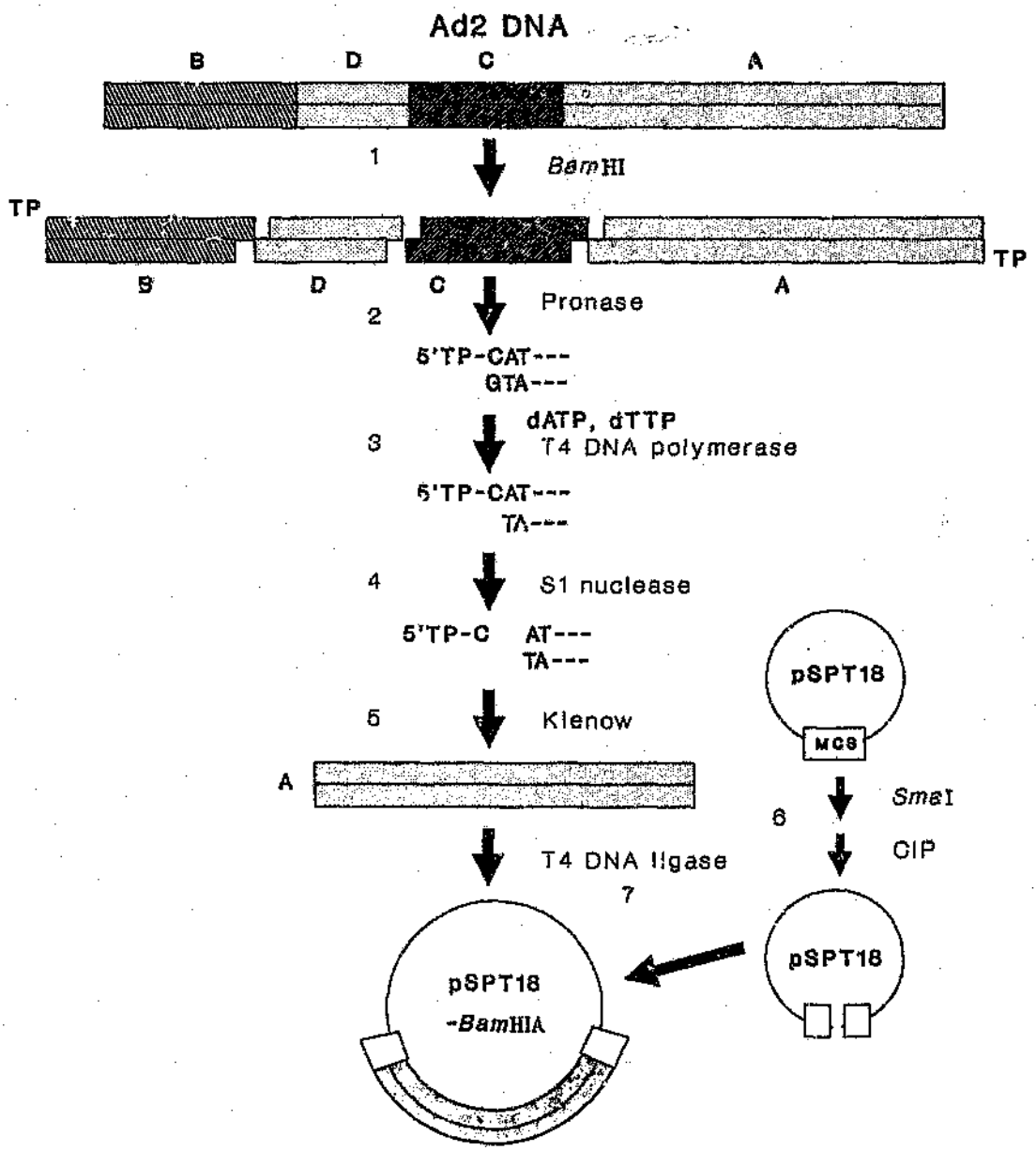


Figure 4.6 Procedure for cloning of adenovirus terminal fragments. 1. Digestion of DNA with restriction enzyme. 2. Pronase treatment - a resistant TP peptide remains. 3. T4 DNA polymerase 3′ to 5′ exonuclease reaction - removal of the first 3′ nucleotide (G) in the presence of dATP and dTTP. 4. S1 nuclease reaction - removal of the TP-C from the first paired nucleotide. 5. Klenow fragment of DNA polymerase 1 - to convert DNA ends to blunt ends. 6. *Sma*I digested, CIP treated plasmid. 7. Ligation of blunt-ended restriction fragment to *Sma*I digested plasmid. MCS: multiple cloning site.

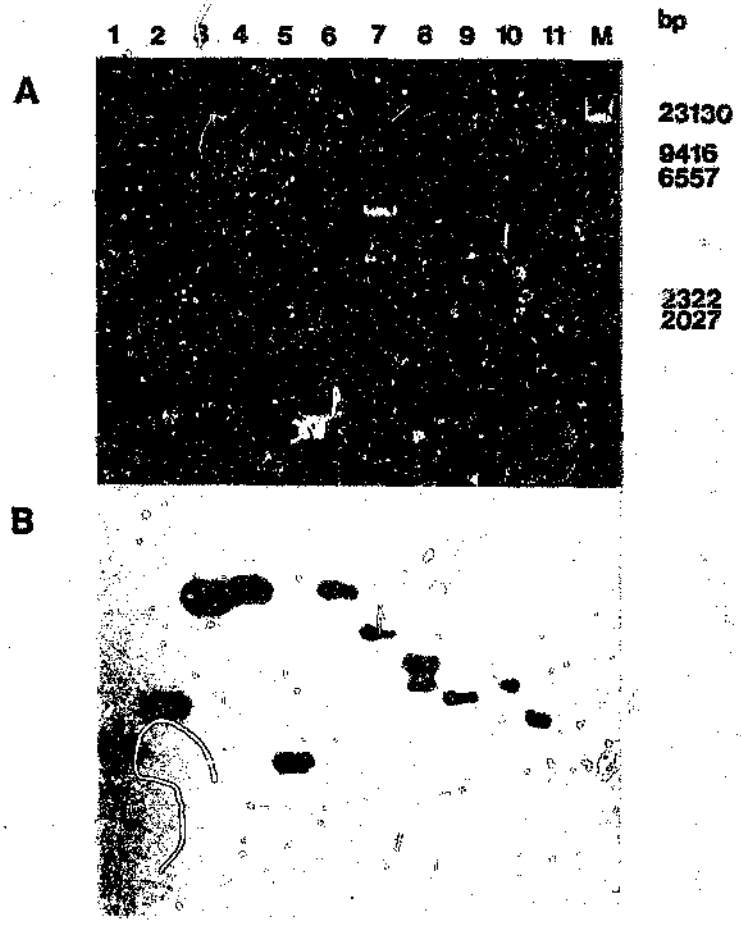


Figure 4.7 Restriction enzyme digestion of Ad2 plasmid constructs.

A. Agarose gel electrophoresis of restriction enzyme digested plasmid constructs. Lane 1. pSPT18-Ad2 *BalII* cut with *Eco*RI and *Hind*III. Lane 2. pSPT18-Ad2 *Hpa*I-*Bcl*I cut with *Eco*RI and *Xba*I. Lane 3. pSPT18-Ad2 *SmaIA* cut with *Sma*I. Lane 4. pSPT18-Ad2 *KpnIA* cut with *Kpn*I. Lane 5. pSPT18-Ad2 *Eco*RI*C* cut with *Eco*RI and *Xba*I. Lane 6. pSPT18-Ad2 *HpaIC* cut with *Eco*RI and *Xba*I. Lane 7. pSPT18-Ad2 *HpaID* cut with *Sac*I and *Bam*HI. Lane 8. pSPT18-Ad2 *BclID* cut with *Sac*I and *Sal*I. Lane 9. pSPT18-Ad2 *Bam*HI*A* cut with *Sal*I and *Xmn*I. The latter enzyme cuts at m.u. positions 82.69 and 89.79 within the *Bam*HI*A* fragment. Lane 10. pSPT18-Ad2 *SmaIE* cut with *Sma*I. Lane 11. pSPT18-Ad2 *SmaIG* cut with *Sma*I. Lane M. Lambda DNA digested with *Hind*III. One microgram of plasmid was digested at 37°C for 2 hours with the appropriate restriction endonuclease(s). Samples were loaded on a 1% w/v agarose gel. The sizes (bp) of the marker DNA fragments are indicated.

B. Southern blot of panel A after hybridization with ^{32}P-labelled Ad2 DNA.

4.2.4.2 Description of cloned Ad2 early genes

The Ad2 restriction enzyme fragments cloned and the resulting plasmid constructs are described in Table 4.8. The viral origin and genomic location of the Ad2 inserts was confirmed by labelling plasmids with ^{32}P and hybridizing them to Southern blots of Ad2 DNA digested with restriction enzymes *Eco*RI, *Hind*III, *Bam*HI, *Sma*I, *Hpa*I, *Xho*I, and *Xba*I. All recombinant plasmids reacted with the expected restriction fragments of the 7 enzymes as determined from Ad2 physical maps (see Appendix E). The confirmed locations of Ad2 cloned fragments are shown in Figure 4.8.

All attempts to clone the Ad2 E1 region as a *BclIC* fragment from 0 to 11.29 m.u. and the E1a gene alone as an *Xmn*I fragment from 0 to 6.29 m.u. were unsuccessful. Plasmid pSPT18-Ad2 *BalII*, however, contains the coding sequences for region E1a and contains the following regulatory elements: the 'TATA' like sequence approximately 30 nucleotides upstream from the major cap site which is essential to transcription, a CAAT box, and one of the repeated sequences in the E1a enhancer region around position -200. The E1a region-containing plasmid in this study therefore would be expected to have reduced activity as a result of the absence of one of the repeat sequences at -300 and a deletion of 22 nucleotides in domain II (Hearing and Shenk, 1986).

The E1b regulatory region consists of three adjacent elements (Wu *et al*, 1987; Berk, 1986; Kadonaga *et al*, 1986) all of which are present within the *Hpa*I-*Bcl*I (4.37-11.29 m.u.) fragment of Ad2. The *SmaIA* fragment of Ad2 contains the sequences known to be essential for uninduced and E1a-induced expression from the E2a early promoter (Imperiale and Nevins, 1984; Kingston *et al*, 1984; Imperiale *et al*, 1985). The *KpnIA* fragment of Ad2 contains the sequences required for uninduced and E1a-induced transcription of E3 (Jones, 1986). Three elements found to be important for E4 transcription (Handa and Sharp, 1984; Gilardi and Perricaudet, 1984) are all present in the *Eco*RIC fragment of Ad2.

In addition to the regulatory sequences discussed above, each cloned gene contains the signals for post-transcriptional processing of the primary transcript. These include signals for capping of mRNA at the 5′ end, the removal of internal sequences by RNA splicing and polyadenylation at the 3′ end.

Table 4.5 Characterization of Ad2 plasmid constructs

Ad2 region (m.u.)[a]	Ad2 DNA restriction fragments (m.u.)[a]	Plasmid constructs[b]
A. Ad2 early gene regions[c]		
E1a (1.3-4.6)	*BalI*[d]*I* (0.75-5.75)	pBR322-*Bal*I-Ad2 *BalII* pSPT18-*Sma*I-Ad2 *BalII*
E1b (4.6-11.2)	*Hpa*I[d]-*Bcl*I (4.37-11.29)	pSPT18-*Sma*I/*Bam*HI-Ad2 *Hpa*I-*Bcl*I[e]
E2a (62.4-75.4)	*Sma*I[d]*A* (56.82-76.08)	pSPT18-*Sma*I-Ad2 *SmaIA*
E3 (76.8-85.9)	*KpnIA* (72.03-93.49)	pSPT19-*Kpn*I-Ad2 *KpnIA*
E4 (91.3-99.1)	*Eco*RIC[f] (89.78-100)	pSPT18-*Sma*I-Ad2 *Eco*RIC
B. Other cloned fragments[g]		
E1b	*Hpa*I[d]*C* (4.37-25.80)	pSPT18-*Sma*I-Ad2 *HpaIC*
E3 del (76.80-85.04) E4 del (98.30-99.10)	*Hpa*I[d]*D* (85.04-98.30)	pSPT18-*Sma*I-Ad2 *HpaID*
L5 (86.00-91.30)	*BclID* (81.90-91.52)	pSPT18-*Bam*HI-Ad2 *BclID*[e]
E2a,E3,E4,L5, L4 (66.50-77.30)	*Bam*HIA[h] (60.12-100)	pSPT18-*Sma*I-Ad2 *Bam*HI*A*
E1a del (1.30-2.80) E1b del (10.92-11.2)	*Sma*I[d]*E* (2.80-10.94)	pSPT18-*Sma*I-Ad2 *SmaIE*
E4 del (91.30-92.09) (98.40-99.10)	*Sma*I[d]*G* (92.09-98.40)	pSPT18-*Sma*I-Ad2 *SmaIG*

[a]Map unit (m.u.) values according to Broker *et al* (1984).
The physical map of Ad2 is divided into 100 m.u., each m.u. being equivalent to 359.37 nucleotide pairs. (See Appendix E)
[b]Plasmid constructs are named according to the plasmid vector used, the restriction enzyme used to cleave the plasmid, and the Ad2 restriction enzyme fragment inserted into the vector, respectively. For future referral to plasmids the restriction enzyme used to cleave the plasmid will be ommitted.
[d]The restriction enzymes *BalI*, *HpaI*, and *SmaI* generate blunt DNA ends. Any blunt-ended fragment can be inserted into a plasmid with blunt ends.
[e]The restriction enzyme *Bam*HI generates compatible ends with *BclI*.
[f]The Ad2 TP was removed by treatment with piperidine and blunt ends generated with the use of Klenow enzyme.
[g]Fragments that contain various defined deletions, coding regions of two late genes, a region containing more than one gene, and one with 5.2kbp in addition to the E1b gene.
[h]The Ad2 TP was removed as described in Figure 4.6.

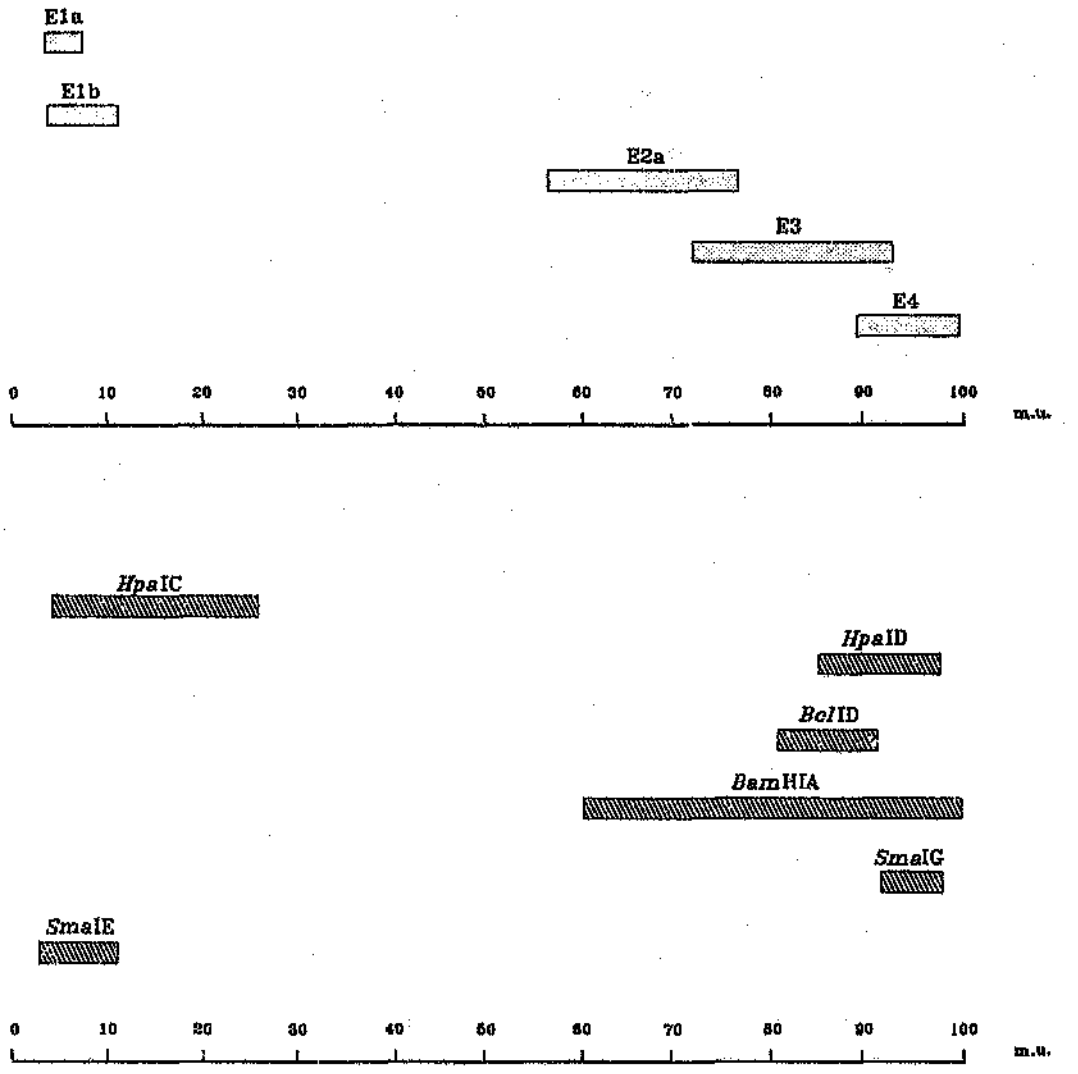


Figure 4.8 Location of Ad2 cloned DNA fragments on the physical map of Ad2 (0-100 m.u.). Adenovirus 2-derived fragments were mapped by DNA:DNA hybridizations to 7 restriction enzyme profiles of Ad2. Early region restriction enzyme fragments are named according to the early genes encoded by them.

4.2.4.3 Comparison of transfection methods

DNA was extracted from duplicate cultures of HEF cells in 9cm dishes transfected with pSPT18-Ad2 *BalII* using 5 different methods of transfection (see 2.36.2). Before lysis, the cells were treated with DNase (1mg/ml) to ensure that only intracellular transfected DNA would be detected. DNAs were dotted on nitrocellulose membranes and hybridized with the same plasmid labelled by nick translation with ^{32}P. When dot blots were exposed to X-ray film for one hour following several high stringency washes, only the DEAE-dextran and DOTMA transfection methods showed strong reactions (Figure 4.9) Under these conditions the plasmid controls, pSPT18 and pSPT18-Ad2 *BalII*, could detect 100pg of plasmid DNA. From the intensity of the dots it was determined that of the 10μg plasmid DNA used to tranfect 5 x 10^5 cells, 100pg and 1ng of plasmid DNA was detected in DEAE-dextran and DOTMA transfected cells, respectively. This corresponds to 9.23 x 10^{10} and 1.85 x 10^{11} copies per 5 x 10^5 cells, or 1.85 x 10^5 and 3.7 x 10^5 copies per cell, respectively. On the basis of these results, the DOTMA transfection method was selected for further use.

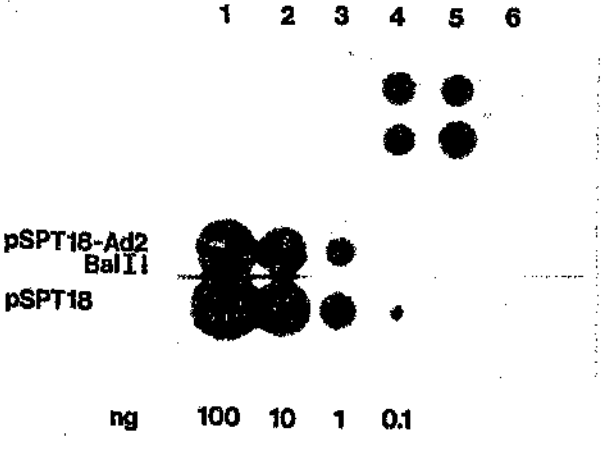


Figure 4.9 Hybridization of ^{32}P-labelled pSPT18-Ad2 *BalII* to DNA from cells transfected with pSPT18-Ad2 *BalII* using different methods. 1. Calcium phosphate transfection. 2. Scrape loading. 3. Protoplast fusion. 4. DEAE dextran transfection. 5. DOTMA lipofection. 6. mock-transfection.

4.2.4.4 DOTMA lipofection

HEF cells in 25cm^2 flasks were transfected with 4μg of Ad2 DNA, DNase treated and DNA extracted three and 6 days after transfection. DNA from transfected cells and 100ng control Ad2 DNA was digested with restriction enzymes *Eco*RI and *Xho*I and fragments separated on a 1% agarose gel. The DNA was transferred to nitrocellulose membrane and hybridized with ^{32}P-labelled Ad2 DNA. Figure 4.10 shows the results obtained after a one week autoradiographic exposure. Very faint bands could be seen three days after transfection. The absence of any detectable hybridization 6 days after transfection may be due to degradation of DNA with time.

To confirm these results, the above experiment was repeated with a large plasmid (8 p), pMAM-neo, which contains two *Eco*RI sites and one *Xho*I site. One microgram of digested plasmid was used as control. Transfected cells were harvested at day three, DNA extracted and digested as above. The fragments were separated on a 1% agarose gel, the DNA transferred to nitrocellulose membrane and hybridized with ^{32}P-labelled pMAM-neo. DNA bands of the correct size could be detected (Figure 4.11).

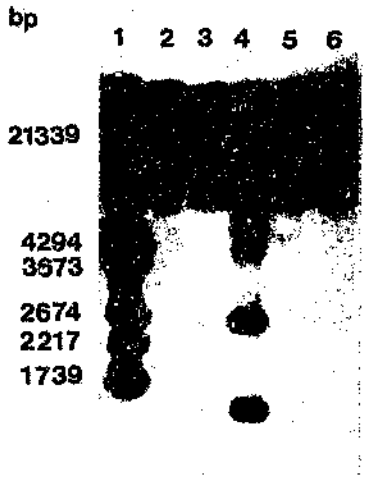


Figure 4.10 Detection of Ad2 DNA in transfected HEF cells. One hundred nanograms of control Ad2 DNA was digested with *Eco*RI (lane 1) and *Xho*I (lane 4). Cells transfected with Ad2 were harvested at day three (lanes 2 and 5) and day 6 (lanes 3 and 6), DNase treated and DNA extracted. DNAs were digested with *Eco*RI (lanes 2 and 3) and *Xho*I (lanes 5 and 6). DNA fragments were separated on 1% agarose gels, transferred to nitrocellulose membrane and hybridized with ^{32}P-labelled Ad2 DNA. The X-ray was developed after an exposure of one week.

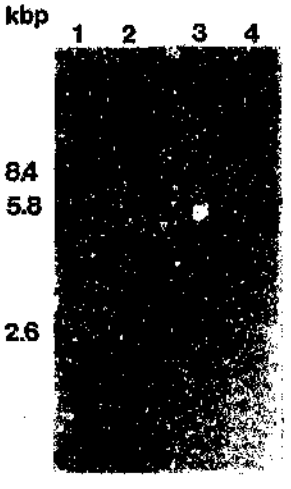


Figure 4.11 Detection of pMAM-neo DNA in transfected HEF cells. DNA was extracted from transfected cells and digested with *Eco*RI (lane 2) and *Xho*I (lane 4). One microgram of control pMAM-neo DNA was digested with *Eco*RI (lane 1) and *Xho*I (lane 3) giving two bands of 5.8kbp and 2.6kbp and a band of 8.4kbp, respectively. A Southern blot of DNA fragments separated on 1% agarose gels was hybridized with ^{32}P-labelled pMAM-neo. The X-ray was developed after an exposure of one week.

4.2.4.5 Superinfection of Ad2 early region transfected HEF cells

Cells on coverslips in 9.6cm^2 dishes were transfected in duplicate with individual plasmids and plasmid combinations as described in Table 4.9. Adenovirus 2 DNA and pSPT18 DNA were used as positive and negative controls, respectively. Since the immediate early gene E1a is required for the expression of the delayed early genes (E1b, E2a, E3, E4), the latter plasmids transfected on their own would not be expected to be functional. These would therefore serve as negative controls for the E1a transactivating function.

Table 4.9 Plasmid transfections of HEF cells

DNA[a]	concentration (μg/ml)[b]
pSPT18	1.0
Ad2 DNA	3.33
E1a	1.0
E1b	1.0
E2a	1.0
E3	1.0
E4	1.0
E1a+E1b+E2a+E3+E4	5.0
E1a+E1b+E2a+E3	4.0
E1a+E1b+E2a	3.0
E1a+E1b	2.0

[a]Plasmids containing Ad2 DNA are designated as the insert gene. Constructs are described in Table 4.8.
[b]The concentration is expressed as the amount of DNA in the medium before the addition of one volume diluted DOTMA reagent. The concentration of DNA in the mixture added to the cells was therefore half of the values shown above.

Cells were infected two days after DNA transfections and infections allowed to proceed for 7 days. Cells on coverslips were then fixed with acetone and treated with subgroup F specific monoclonal antibody and fluorescein isothiocyanate conjugated rabbit anti-mouse immunoglobulin. One of the two Ad2 control coverslips was reacted with group-specific antiserum to see if Ad2 late antigens were synthesized within the time allowed. Adenovirus DNA is infectious and transfections can

therefore lead to viable virus production. There was no evidence of this occuring with Ad2 under the experimental conditions used. Furthermore, neither Ad2 DNA or any of the plasmid combinations had the ability to complement Ad40 or Ad41 growth as determined by the detection of late antigen by indirect immunofluorescence.

Another approach was taken using the same conditions as described in Table 4.9, except that cells were transfected one hour after virus adsorption. If DNA degradaton occurs with time, delivery of the DNA close to the time of infection may be preferable. From the results of mixed infections of Ad41 with Ad2 (Figure 4.5), preinfection of HEF cells with Ad41 resulted in enhanced Ad2 fluorescent cell counts. Infection of cells with Ad40 and Ad41 before transfection with Ad2 DNA could improve the chance of a complementing event by an early function(s) acting in *trans* with Ad2 DNA regions. It has recently been shown that differential expression of the E1a region of adenoviruses has little effect on growth in tissue culture (Hitt and Graham, 1990). If the subgroup F adenovirus E1a gene is expressed in HEF cells to some degree, it is possible that it could substitute for the transfected Ad2 E1a plasmid if it were inadequately expressed or rendered nonfunctional as a result of DNA degradation. As before, no fluorescent cells were detected in any of the transfection assays.

4.2.4.6 Efficiency of uptake of DNA by different cell types

To test the possibility that the assay may not be sensitive enough, the efficiency of uptake of DNA by three different cell types including HEF cells was compared. This was determined by transfecting cells on duplicate coverslips with 2μg of DIG-labelled pMAM-neo DNA, fixing cells after 48 hours and enumerating the percentage of cells fluorescing after treating cells with anti-DIG-fluorescein. Table 4.10 shows a comparison of transfection efficiencies obtained with 293 cells, Chang cells, and HEF cells. 293 cells were efficiently transfected with approximately 90% of cells taking up the DNA. Thirty percent of Chang cells fluoresced with the anti-DIG-fluorescein.

HEF cells showed an efficiency of transfection of less than 10%. Therefore, the sensitivity of the assay in HEF cells may in part be responsible for no detectable complementation.

Table 4.10 Efficiency of uptake of DNA by 293, Chang, and HEF cells using DOTMA transfection

	DIG fluorescence[a]
Cell type	Estimated % of total cells
293	90
Chang	30
HEF	<10

[a]Cells on duplicate coverslips were transfected with DIG-labelled pMAM-neo DNA, fixed and stained with anti-DIG-fluorescein.

4.2.4.7 Superinfection of HEF cells transfected with Ad2 cytoplasmic RNA

The expression of Ad2 DNA and Ad2 DNA-containing plasmids may in some way be compromised in the transfection assays. To determine if the provision of nucleic acid already in an expressed form (mRNA) can complement Ad40 and Ad41 late antigen synthesis, HEF cells were transfected with early and late Ad2 cytoplasmic RNA.

Chang cells in 150cm^2 flasks were infected with 1.4 x 10^7 FFU/ml of Ad2. One flask received cycloheximide at a concentration of 25μg/ml in L15 containing 2% FCS directly after infection and infection allowed to proceed for 9 hours. Cycloheximide inhibits the synthesis of early proteins thereby inhibiting viral DNA synthesis and allowing the accumulation of early viral mRNAs. The other infection was continued for 22 hours in L15 with 2% FCS. Here late mRNAs would be expected to predominate.

Cytoplasmic RNA was isolated as described in section 2.30.1. Dot blots of these RNAs were prepared (section 2.31) and hybridized with early and late DNA region-containing plasmids to confirm the presence of specific transcripts. The plasmid containing the Ad2 E2a gene and part of the L3 and L4 transcription units (pSPT18-Ad2 *SmaIA*) hybridized to early and late cytoplasmic RNAs and pSPT18-Ad2 *BclID* which contains region L5 hybridized only with late cytoplasmic RNAs. The plasmids did not hybridize to cytoplasmic RNA isolated from uninfected cells.

Coverslip cultures of HEF cells were infected with Ad40 and Ad41 24 hours before transfection with Ad2 early and late cytoplasmic RNA preparations (30μg/9.6cm^2 dish or 30μg/8 x 10^5 cells). With lipofection using the DOTMA reagent the same principles hold for DNA and RNA and therefore a similar protocol was employed for the transfection assay. Infections were allowed to proceed for a further 7 days, fixed and stained for late antigen detection. No fluorescent cells were seen in mock-transfected cultures, cultures transfected with uninfected cytoplasmic RNA or those transfected with Ad2 early and late cytoplasmic RNAs.

To determine if these cytoplasmic RNAs were in fact functional, mRNAs were purified using oligo-dT chromatography and tested for viral protein expression *in vitro* in rabbit reticulocyte lysates (section 2.18). Messenger RNAs coding for the 72K DBP and the Ad2 hexon antigen, and the fibre protein were isolated by hybrid selection using pSPT18-Ad2 *SmaIA* (m.u. 56.82 - 76.08) and pSPT18-Ad2 *BclID* (m.u. 81.90 - 91.52) immobilized on nitrocellulose filters, respectively. Incorporation of ^{35}S-methionine into proteins synthesized from hybrid selected mRNAs from uninfected cells and early and late viral mRNAs, as well as the BMV mRNA control was determined after 60 minutes. In contrast to the BMV mRNA control, there was no detectable incorporation of ^{35}S-methionine into protein for either of the viral mRNA preparations. Furthermore, there was no difference in radioactive counts obtained with mRNAs from uninfected and infected cells.

4.3 Discussion

Virus coinfection studies were conducted to determine whether the presence of an adenovirus of another serotype can emulate the helper effect on Ad41 replication apparent in 293 cells, which is most likely due to the expression of E1 products (Takiff *et al,* 1981; Takiff and Straus, 1982). Cells were chosen which were known to support the growth of subgroup F adenoviruses to a limited degree (Chang conjunctival cultures) (Kidd and Madeley, 1981; Wigand *et al,* 1983; Ujfalusi *et al,* 1984), or not at all (HEF cultures) (de Jong *et al,* 1983). Adenovirus 41 late antigen synthesis in the presence and absence of Ad2 was monitored exclusively using a subgroup F specific monoclonal antibody and the fluorescence test was rendered as sensitive as possible with respect to complementation effects by infection of a large number of cells (6 x 10^5 cells/coverslip for Chang cells and 2 x 10^5 cells/coverslip for HEF cells). The results indicate that Ad2 is capable of complementing the growth of Ad41 in a semi-permissive cell line, and of supporting its replication (at least to the stage of late antigen synthesis) in cells which are normally totally refractory to growth. The same was found for Ad40 (data not shown). Complementation of Ad41 by Ad2 could also be demonstrated in HEF cells at the level of DNA synthesis.

The method of monitoring the effect of Ad2 on Ad41 late antigen synthesis using a monoclonal antibody has the advantage of allowing the analysis of large numbers of cells and therefore valid statistical analyses are possible when comparing treatments. The main disadvantage of monitoring complementation by the extent of Ad41 late antigen synthesis is that the mechanism of complementation cannot be deduced, nor can the complementation events be mapped properly in time.

There have been several other reports of members of different adenovirus subgroups complementing each other for some necessary functions (Mak, 1969; Williams *et al,* 1975; Rowe and Graham, 1981; Williams *et al,* 1981; McDonough and Rekosh, 1982; McDougall and Mautner, 1987). Delsert and D'Halluin (1984)

have shown a hierarchy of dominance between serotypes of different adenovirus subgroups in simian and human cells. This has been attributed to an E1a gene product acting as a repressor (Leite *et al,* 1986). In addition to complementation between Ad41 and Ad2 in this study, interference between the two serotypes has also been demonstrated, the outcome of mixed infection being dependent on factors such as input dose, the cell type coinfected and the relative time of infection by each serotype.

Certain serotypes of adenovirus, notably Ad1 and Ad2 can infect intestines of children for long periods (Fox *et al,* 1977; Kidd *et al,* 1982). In the light of the complementation results obtained with Ad2, coinfection of intestinal cells with other serotypes *in vivo* may promote the replication of subgroup F adenoviruses.

The effects of Ad40 and Ad41 on each other in coinfection of non-permissive cells was monitored using type specific monoclonal antibodies in the fluorescent focus assay. If Ad40 and Ad41 are defective in different functions, they could theoretically complement each other in *trans* for their respective defective functions. If, on the other hand, they have identical defective functions, then no complementation would be expected in non-permissive cells. In HEF cells no complementation could be demonstrated in coinfections, indicating the likelihood that they share similar defective functions.

It is possible, however, that the defective functions do not map to the same genomic region but that their respective functions are not adequately expressed to allow the detection of complementation. The finding that Ad40 can to some degree replicate its DNA in HEF cells but Ad41 cannot suggests the presence of a late and early block in replication, respectively. There are differences displayed in their growth capabilities in various transformed cell lines (Chiba *et al,* 1983; Uhnoo *et al,* 1983; 1984; Brown, 1985; van Loon *et al,* 1986b; Nascimento *et al,* 1990; Pieniazek *et al,* 1990a) and in their ability to transform various cell types (van Loon *et al,*

1985a; Cousin *et al*, 1991) that would indicate at least some difference in expression of E1 gene products. Their growth, even in so-called permissive cells that constitutively express the Ad5 or Ad2 E1 gene region, does not match that of established adenovirus serotypes. It is therefore possible that some other function in addition to a possible E1 defect may be involved in the subgroup F adenovirus growth restriction in tissue culture.

In an attempt to further analyse the defective function or functions operative in Ad40 and Ad41 infected HEF cells, a transient transfection assay was employed to monitor complementation of Ad40 and Ad41 late antigen synthesis by specific Ad2 genes. It was decided to test selected genes of Ad2 (E1a, E1b, E2a, E3, E4) that are expressed early in the infectious cycle and which are known to be capable of being expressed by the host cell machinery relatively independently of other viral functions.

No complementation could be detected by the provision of the Ad2 early genes individually or in any of the plasmid combinations either before or after subgroup F adenovirus infection of HEF cells. Whole Ad2 genome, unexpectedly, did not complement Ad40 and Ad41 growth in these cells and no Ad2 late antigens could be detected if whole Ad2 DNA was transfected alone, indicating either poor infectivity of the DNA or a problem relating to sensitivity of the assay. The Ad2 DNA which had been proteinase K treated in the DNA extraction procedure would be expected to contain only a proteinase K resistant peptide at its 5′ end. The presence of a TP greatly increases the infectivity of adenovirus DNA (Sharp *et al*, 1976). The relative infectivity of adenovirus is reduced at least 100-fold if the terminal protein is removed by proteolysis.

Adenovirus cytoplasmic RNAs delivered to HEF cells by lipofection followed by superinfection with Ad40 or Ad41 did not result in late antigen synthesis of either virus. The rationale here was to supply cells directly with expressed virus specific mRNAs thereby bypassing transcription from Ad2 genes in plasmids, an event which

may not have been optimal. These mRNAs in reaching the cytoplasm could theoretically provide templates for translation of viral proteins and in this way exert their effect on the Ad40 or Ad41 genomes. The absence of any detectable complementation could have been a result of degradation of Ad2 RNA by cellular RNases upon entry into the cells or due to the mRNAs being non-functional prior to entry. It was not possible to express proteins in rabbit reticulocyte lysates using hybrid-selected, oligo-dT purified mRNAs as templates. This may have been in part due to loss or degradation of mRNA in the selection procedure itself. In other studies complementation of AAV growth by specific Ad2 mRNAs has been accomplished by microinjection into the cytoplasm of cells (Richardson *et al*, 1980; Richardson and Westphal, 1983; 1984). RNA transfections have also been successfully carried out using calcium phosphate-mediated uptake in studies of RNA processing and ribonucleoprotein assembly (Kleinschmidt and Pederson, 1990).

A number of explanations could account for the lack of success of the transient transfection assays:

(i) Choice of recipient cells. It has been shown that transfected DNA is stabilized in the nuclei of 293 cells to a much greater extent than in other cell lines (Alwine, 1985). The comparison of DIG-labelled DNA taken up by 293, Chang and HEF cells showed cell specific differences in anti-DIG fluorescence that are consistent with a much greater stability of transfected DNA in 293 cells. The uptake of DNA in HEF cells was detected in less than 10% of cells as compared to 90% in 293 cells. Assuming that the plasmid DNA taken up in HEF cells was not totally degraded one would still expect complementation to be detected in this proportion of cells. Fibrobiasts are generally considered refractory to most methods of transfection (Goldstein *et al*, 1989), but unavoidably had to be used here.

(ii) Choice of transfection method. Lipofection using the DOTMA reagent showed more efficient uptake of DNA than other methods tested in HEF cells. Cationic

liposomes have the ability to transfer DNA into the cell through fusion of the liposome with the cell membrane. The cationic lipid (DOTMA) forms positively charged liposomes that interact spontaneously with DNA or RNA to form complexes (Felgner *et al*, 1987). Transfection efficiencies have been shown to be one or more orders of magnitude higher than other methods of transfection for some cells (Felgner *et al*, 1987). In this study the efficiency was monitored with respect to uptake of DNA and not gene expression. The percentage of cells that took up the DNA by liposome-mediated transfection varied for different cell types and indicated overall a very high efficiency of uptake. It may be possible, however, that the transfected DNA/RNA could remain associated with the cell membrane and not be available for further expression in the cell. Alternatively, degradation of DNA may be so extensive that only a very small proportion of nucleic acid is ultimately functional. Groneberg *et al*, (1975) in a study of the uptake of Ad2 DNA (34S) in KB cells have shown that of 3-9% of Ad2 DNA that became associated with KB cells, 70% reached the nucleus and about 50% of that in the cytoplasm and nucleus was cleaved into fragments sedimenting at 19-23S.

(iii) Transient expression of plasmids. Assuming delivery of the genes to the cells was accomplished, the next crucial step for the assay would be the adequate expression of the transfected gene. This is dependent on a number of factors, including the presence of elements responsible for efficient transcription initiation, regulation of early gene expression at the level of transcription, post-transcription and translation.

The expression of the E1a gene is dependent on *cis*-acting transcriptional enhancers. The absence of certain enhancer sequences in the E1a plasmid used in this study was clearly a reason for reduced E1a activity and may have been entirely responsible for the negative results obtained with plasmid transfections. By analogy with deletion studies carried out by Hearing and Shenk (1986) some degree of expression would, however, be expected. Ideally, plasmid constructs should have been tested using a reporter system such as the CAT gene to monitor gene expression.

The E1a gene of Ad2 codes for a 289R protein that has a *trans*-acting function on the promoters of other early gene regions. This is thought to be mediated through cellular transcription factors (for review see Nevins, 1991). The Ad2 E1a proteins themselves or proteins translated from the other early regions would be expected to act in *trans* by providing a gene function that is absent in Ad40 or Ad41 infected HEF cells either directly or indirectly through altering some host function. Adenovirus 40 and Ad41 E1a gene products may be expressed to some extent in these cells. These products could conceivably also transactivate the Ad2 plasmids containing the other early genes and thereby permit complementation of their replication in *trans* by these plasmids. However, this may not be very likely for two reasons. Firstly, the Ad40 (van Loon *et al,* 1987a; Ishino *et al,* 1988) and Ad41 (van Loon *et al,* 1987a) E1a transactivating activity is reduced compared to that of other adenoviruses in *in vitro* assays. Secondly, the Ad40 E1a promoter has low *cis*-acting activity in rat 3Y1 cells (Ishino *et al,* 1988).

The relative strength of core elements in the upsteam region of the E1a gene may also be related to the cell type in which expressed as has been shown for the early gene of polyoma virus (Herbomel *et al,* 1984). In other words, the transcription factors present in the cell must be able to efficiently recognize the enhancer sequence. It is possible that cells of species other the host cell type of the virus would be deficient in this regard. It has also been shown that the choice of plasmid in which the E1a gene is inserted can affect E1a gene expression. For example, plasmid pBR322 contains a sequence element that has a *cis*-acting negative effect on the expression of a cloned adenovirus E1 gene in transient transfection assays (Leite *et al,* 1989).

Another important consideration is the finding that translation of plasmid-derived mRNA may be inefficient due to DAI (dsRNA activated inhibitor) kinase activation in transfected cells (Akusjärvi *et al,* 1987; Kaufman and Murtha, 1987). Protein synthesis is then inhibited through the phosphorylation of the initiation

factor eIF-2α by the kinase. The adenovirus VA RNAI binds and inhibits the activation of DAI kinase thereby preventing the inhibition of protein synthesis (Kitajewski *et al,* 1986a; Munemitsu *et al,* 1986). Since the Ad2 VA RNAI was not produced by any of the plasmid constructs and since the subgroup F adenovirus VA RNAI gene may be defective in this function (see Chapter Five), inhibition of viral protein synthesis would not be circumvented in transfected cells.

Since whole Ad2 DNA could not complement Ad40 or Ad41 late antigen synthesis in HEF cells, the sensitivity of the assay may have been below the level of detection. Alternatively, viral genomic and plasmid DNA may have been inadequately expressed. Complementation may, however, have taken place to some degree at the transcriptional level. This would not have been detected, as the assay monitored progression of the virus replication cycle to a stage of late antigen synthesis. If this were the case, one or more of these genes may act to promote the subgroup F adenovirus infectious cycle in HEF cells to some further stage. An additional function or functions would then be required to permit progression to viral late antigen synthesis. Alternatively, the helper function afforded by Ad2 in mixed infection with Ad41 may be related to infection with virus as opposed to viral DNA.

In conclusion, involvement of any of the Ad2 early genes in complementation of Ad40 or Ad41 late antigen synthesis can not be excluded on the basis of the results obtained with the transfection assay and requires further evaluation.

CHAPTER FIVE

INTERFERON INDUCTION AND SENSITIVITY

5.1 Introduction

Unlike the subgroup A adenoviruses, members of subgroups B to E are weak inducers of interferon (IFN) in CEF cells. The IFN response is triggered by the capsid or some capsid component (Toth *et al,* 1987; Reich *et al,* 1988). No adenovirus has yet been shown to induce IFN in human cells (Toth *et al,* 1987), although induction of transcription of IFN-stimulated genes in HeLa cells does occur with subsequent suppression by the E1a 12S mRNA product (Reich *et al,* 1988). The Ad12 E1a 13S mRNA alone has been shown to be sufficient for potent IFN induction in CEF cells (Toth *et al,* 1987).

Adenoviruses are considered relatively insensitive to the antiviral effects of IFN (Gallagher and Khoobyarian, 1972; Stewart, 1979) although differences in susceptibility have been noted with different recombinant types (Mistchenko *et al,* 1987; Mistchenko and Falcoff, 1987; Mistchenko *et al,* 1989).

It is possible that the subgroup F adenovirus growth restriction seen in cell cultures may be due to the induction of IFN in response to infection. The aims of this study, therefore, were to determine if IFN can be induced in Chang conjunctival cells

in response to subgroup F adenovirus infection, and to compare the IFN sensitivity of these viruses to that of Ad2 in these cells. Since the subgroup F adenoviruses are defective for growth in most human cells it is possible that the defective function(s) could play some role in their interaction with IFN-treated cells. Furthermore, since other adenoviruses can function in the reversal of the IFN-induced antiviral state (Feduchi and Carrasco, 1987; Anderson and Fennie, 1987), it was thought that Ad2 may have the ability to abrogate any effect of IFN on Ad40 and Ad41 in mixed infections of cells.

5.2 Results

5.2.1 Interferon induction

An indirect radioimmunoassay was used to determine if IFN is produced in response to adenovirus infection of HEF and Chang cells. In this assay, the sensitivity of Sindbis virus to IFN in IFN-pretreated vero cells was assessed by inhibition of CPE. Fluids from single infections of Ad2, Ad40 and Ad41, coinfections of Ad2 with either Ad40 or Ad41 and from uninfected cultures were collected 44 hours after infection and ultracentrifuged to remove virus. The supernatants were then checked for residual virus by indirect immunofluorescence. Supernatants, undiluted and at 1/5 dilution (Table 5.1) were assayed for IFN activity. All wells containing cells treated with the test supernatants as well as the virus control wells showed CPE. A concentration of 5U/ml of IFN also failed to inhibit CPE. Figure 5.1 shows the IFN dose response graph for the microplate assay. The results for test samples are shown in Table 5.1.

The end point of detection, defined as that concentration of IFN which results in 50% inhibition of virus CPE, was 50U/ml, corresponding to a detection end point of 5U of IFN per well.

All the counts obtained for the test samples were similar to those of the virus controls without IFN pretreatment. Counts for supernatants of uninfected and infected cells did not differ from each other. It was clear from these results that the adenovirus tested either singly or in combination did not induce detectable IFN in either HEF or Chang cell cultures.

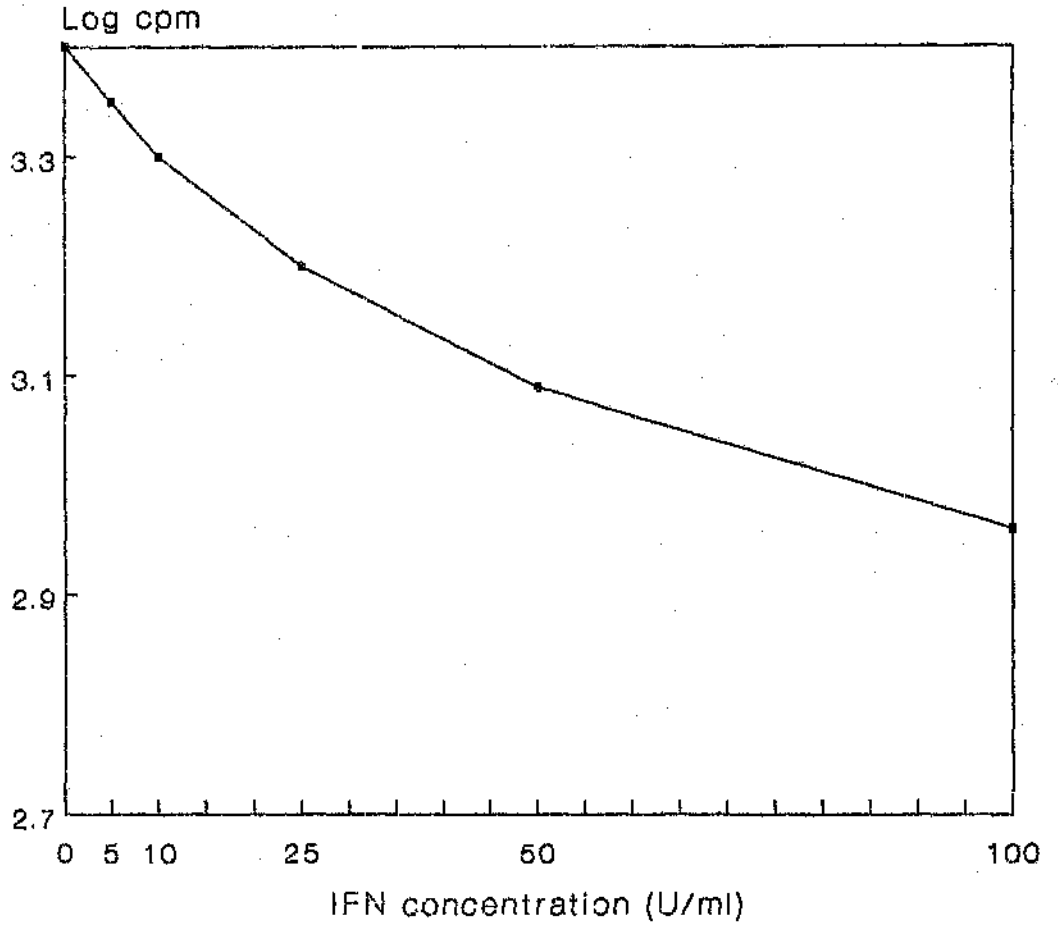


Figure 5.1 IFN dose response graph for Sindbis virus. Vero cells in microplates were treated with 5, 10, 25, 50, and 100 U/ml of lymphoblastoid IFN before infection with Sindbis virus. Counts obtained in the absence of IFN are also shown (virus control).

Table 5.1 Indirect radioimmunoassay for detection of IFN in adenovirus infected HEF and Chang cell cultures

		cpm[a]	
Cells	Virus	Undiluted[b]	1/5[b]
HEF	uninfected	1991 ± 647.71	2211 ± 62.22
	Ad2	2416 ± 33.94	2577 ± 190.21
	Ad40	2532 ± 134.35	2496 ± 367.69
	Ad41	2369 ± 180.38	2429 ± 212.64
	Ad2 + Ad40	2496 ± 113.14	2519 ± 168.89
	Ad2 + Ad41	2444 ± 271.61	2518 ± 215.45
Chang	uninfected	2431 ± 166.40	2565 ± 193.24
	Ad2	2280 ± 148.49	2392 ± 342.16
	Ad40	2153 ± 311.13	2437 ± 379.01
	Ad41	2164 ± 113.14	2364 ± 168.89
	Ad2 + Ad40	2513 ± 242.51	2534 ± 264.23
	Ad2 + Ad41	2432 ± 66.47	2338 ± 350.01

[a] ^{125}I counts per minute. Values are the average of duplicate counts.
[b] Culture fluids were tested undiluted and diluted 1/5 in MEM with 2% FCS.

5.2.2 Interferon sensitivity

Chang cells growing on coverslips were pretreated for 24 hours with 10 and 100U/ml of lymphoblastoid IFN followed by infection with concentrations of adenovirus shown in Table 5.2. Similarly infected cells were treated with IFN directly after infection. A sample of mock IFN was tested to ensure that results obtained were specifically due to IFN and not another agent. As there was no difference in the results between mock IFN and maintenance medium, the latter was used as negative control throughout.

The stock Ad40 preparation had a titre 10 times less than that of Ad41. A low Ad2 concentration comparable to that of Ad40 was used. This served as a control to ensure that any effects of IFN on Ad40 could not be related to a low input titre of virus. The coverslips were fixed 44 hours after infection and cells expressing hexon antigen detected by indirect immunofluorescence. The significance of the effects noted was determined using the Wilcoxon signed-ranks matched-pairs test [$\alpha = 0.005$] (see Appendix D).

Post-infection treatment of cells with IFN had no significant effect on any of the adenovirus infections (Table 5.2). Pretreatment of cells with IFN resulted in a significant decrease in numbers of cells expressing hexon antigens of Ad40 and Ad41, but not of Ad2. The decrease in fluorescent cell counts for Ad40 was by a factor of 14.93 for 10U/ml IFN and a factor of 20.35 for 100U/ml, and for Ad41 4.86 and 7.65 respectively. The higher concentration of IFN did not reduce the counts proportionately and significantly when compared with the lower concentration (Figure 5.2).

To determine the effect of Ad2 infection on Ad40 and Ad41 sensitivity to IFN, cells were pretreated with 100U/ml of IFN for 24 hours before Ad40 and Ad41 infection (T_o-24). Adenovirus 2 was applied to cells 36 hours before (T_o-36), or

together with Ad40 and Ad41 (T_o). Cells preinfected with Ad2 for 36 hours were fixed after a further 12 hours of mixed infection (T_o+12). Since the complementing ability of Ad2 appears more enhanced the earlier the preinfection time (see 4.2.2.2.1), the 12 hour p.i. (relative to time of subgroup F adenovirus inoculation) time point was chosen so as to obtain countable fluorescent cell values for Ad40 and Ad41. Cultures simultaneously infected with Ad2 and Ad40, or Ad2 and Ad41 were fixed 44 hours after infection.

The fluorescent cell counts obtained for Ad2 single infections of Chang cells for the two different times are shown in Table 5.3. Interferon had no effect on Ad2 fluorescent cell counts when compared to untreated controls. Therefore, it was unlikely that any effect of IFN on Ad40 or Ad41 in mixed infection with Ad2 would be due to an effect of IFN on Ad2.

The use of subgroup F adenovirus specific anti-hexon antiserum allowed differentiation of Ad40 or Ad41 hexon production from Ad2 hexon production in mixed infection. The effect of Ad2 on Ad40 and Ad41 IFN sensitivity is shown in Table 5.4, and graphically represented in Figure 5.3. The sensitivity of Ad40 to IFN was not significantly overcome by the presence of Ad2 at two different input concentrations used in coinfections, nor when Ad2 at the lower concentration was applied 36 hours before Ad40. The sensitivity was, however, not as marked when cells were preinfected with Ad2. The Ad40 fluorescent cell counts decreased by a factor of 7.18 as a result of IFN treatment when cells were coinfected with 1.4×10^4 FFU/ml of Ad2. This was greater than the difference noted between IFN-treated and untreated Ad40 infected cultures, a factor of 4.5. A one log higher concentration of Ad2 resulted in a decrease in Ad40 fluorescent cell counts by a factor of 3.4. The decrease caused by IFN was by a factor of 1.49 when Ad2 was applied to cells 36 hours before Ad40. The sensitivity of Ad40 to IFN, therefore, appeared less in cultures preinfected with Ad2 than in cultures infected simultaneously with Ad2 and Ad40.

Fluorescent cell counts in Ad41 infected Chang cells were decreased by a factor of 2.82 due to IFN. The presence of Ad2 at a concentration of 1.4 x 10^4 FFU/ml resulted in a decrease in fluorescent cell counts due to IFN by a factor of 3.43. The one log higher concentration of Ad2 resulted in a decrease by a factor of 5.46. There was no difference in Ad41 fluorescent cell counts between IFN-treated and untreated cultures when Ad2 was applied 36 hours before Ad41.

Table 5.2 Sensitivity of Ad2, Ad40 and Ad41 to IFN treatment of Chang cells

	Fluorescence (FFU/10^6 cells)[a]				
	Treatment[b]				
Virus input concentration (FFU/ml)	No IFN	pretreatment		p.i. treatment	
		10U/ml	100U/ml	10U/ml	100U/ml
Ad2 1.4 x 10^3	81.19± 8.72	55.72± 5.67	54.92± 20.24	46.17± 10.48	47.76± 9.28
Ad40 1.2 x 10^3	89.15± 6.88	5.97± 0.80	4.38± 1.52	49.75± 9.13	57.71± 3.98
Ad41 1.2 x 10^4	1285.14± 337.23	264.67± 90.35	167.96± 86.74	1264.05± 174.95	1172.91± 153.25

[a]Adenovirus 2 infected cells were treated with group-specific polyclonal antibody, Ad40 and Ad41 with subgroup F specific monoclonal antibody.
[b]Cells were either treated with IFN 24 hours before infection, or IFN applied directly after infection for the duration of infection. IFN was used at a concentration of 10U/ml and 100U/ml. Medium without IFN was L15 with 2% FCS.

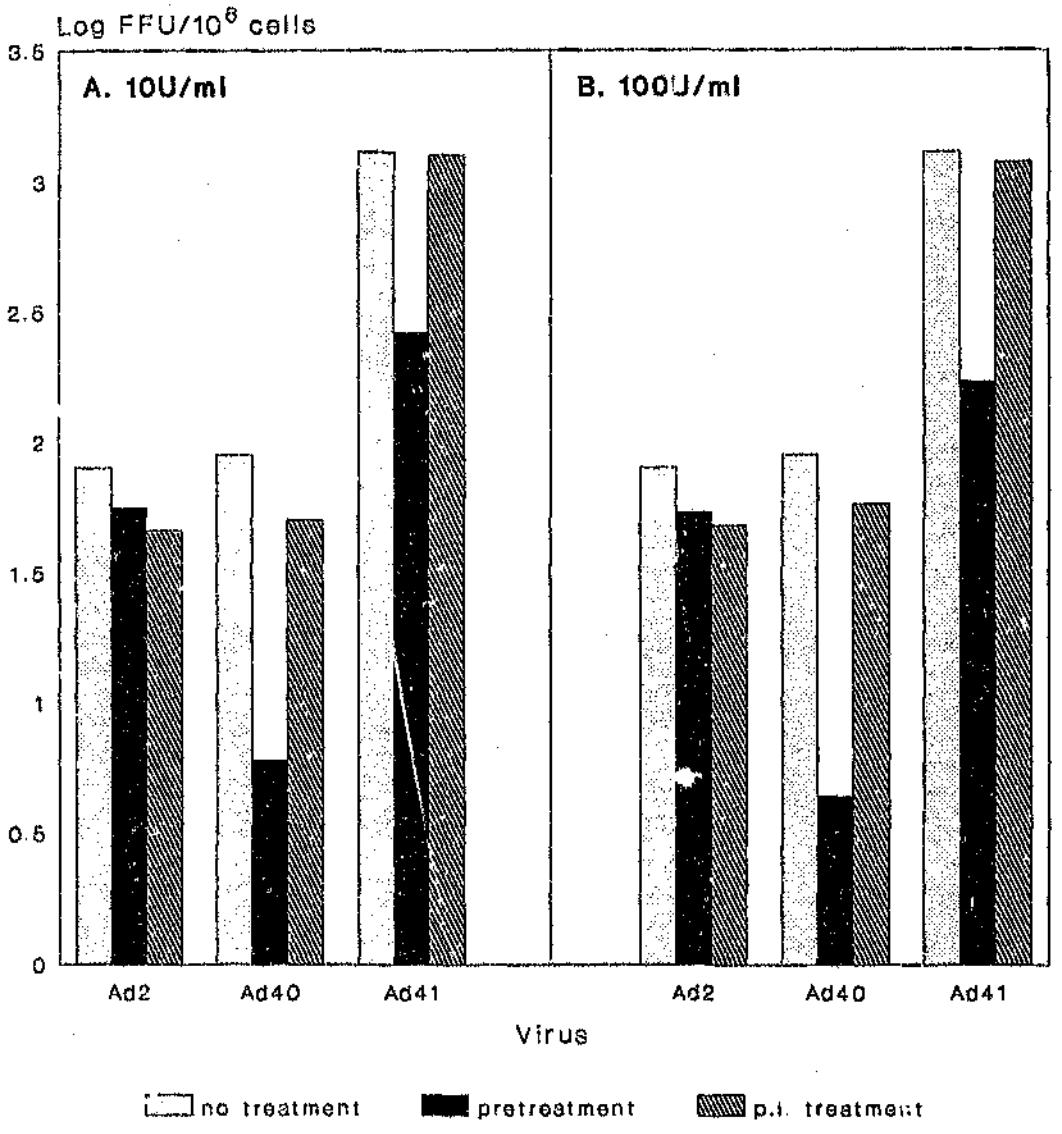


Figure 5.2 Histogram showing the effect of pretreatment and post-infection treatment with IFN on adenovirus infection of Chang cells. Cells were infected with Ad2, Ad40, and Ad41 at concentrations described in Table 5.2. A. IFN concentration 10U/ml. B. IFN concentration 100U/ml.

Table 5.3 Determination of Ad2 sensitivity to IFN treatment in single infections of Chang cells

Time of infection with Ad2[b]	Ad2 input concentration (FFU/ml)	IFN (100U/ml)[a] -IFN	IFN (100U/ml)[a] +IFN
Ad2 fluorescence (FFU/10^6 cells)[c]			
T_0	1.4×10^5	8393.07 ± 1466.21	8007.36 ± 831.63
	1.4×10^4	824.26 ± 43.05	820.28 ± 127.52
T_0-36	1.4×10^4	4808.64 ± 227.53	4729.04 ± 236.85

[a]IFN was diluted in maintenance medium.
[b]Time zero (T_0) corresponds with the time used for coinfection of Ad2 with a subgroup F adenovirus. Cells were also infected 36 hours before (T_0-36) the time used for coinfections.
[c]Cells treated with group-specific polyclonal antibody.

Table 5.4 IFN sensitivity of Ad40 and Ad41 infections of Chang cells in the presence of Ad2

Infection	Input ratio of viable particles[b] Ad40/41:Ad2	IFN (100U/ml)[a] -IFN	+IFN
Ad40 fluorescence (FFU/10^6 cells)[c]			
single[d]	1:0	189.85 ± 52.66	44.18 ± 15.89
preinfection[e]	1:10	212.93 ± 20.03	142.88 ± 18.49
coinfection[f]	1:100	2833.76 ± 365.98	833.81 ± 43.45
	1:10	1687.12 ± 94.06	234.82 ± 30.33
Ad41 fluorescence (FFU/10^6 cells)[c]			
single[d]	1:0	746.25 ± 57.28	264.67 ± 24.09
preinfection[e]	1:1	1311.81 ± 106.71	1152.61 ± 181.99
coinfection[f]	1:10	14786.00 ± 1412.08	2707.75 ± 223.20
	1:1	2553.57 ± 234.34	743.86 ± 248.60

[a]IFN was diluted in maintenance medium and applied 24 hours before infection with Ad40 or Ad41 (T_0-24).
[b]Cells were coinfected with 1.2×10^3 FFU/ml of Ad40 or 1.2×10^4 FFU/ml of Ad41 and 1.4×10^5 FFU/ml of Ad2 (Ad40/41:Ad2 ratios 1:100 and 1:10, respectively) or with 1.4×10^4 FFU/ml of Ad2 (Ad40/Ad41 ratios 1:10 and 1:1, respectively).
[c]Cells treated with subgroup F specific monoclonal antibody.
[d]Cells were singly infected with 1.2×10^3 FFU/ml of Ad40 or 1.2×10^4 FFU/ml of Ad41.
[e]Cells were infected with 1.4×10^4 FFU/ml of Ad2 36 hours before infection with Ad40 or Ad41 (T_0-36) and fixed after a further 12 hours (T_0+12).
[f]Virus mixtures (Ad40 with Ad2 and Ad41 with Ad2) corresponding to the indicated ratios were applied at time zero (T_0).

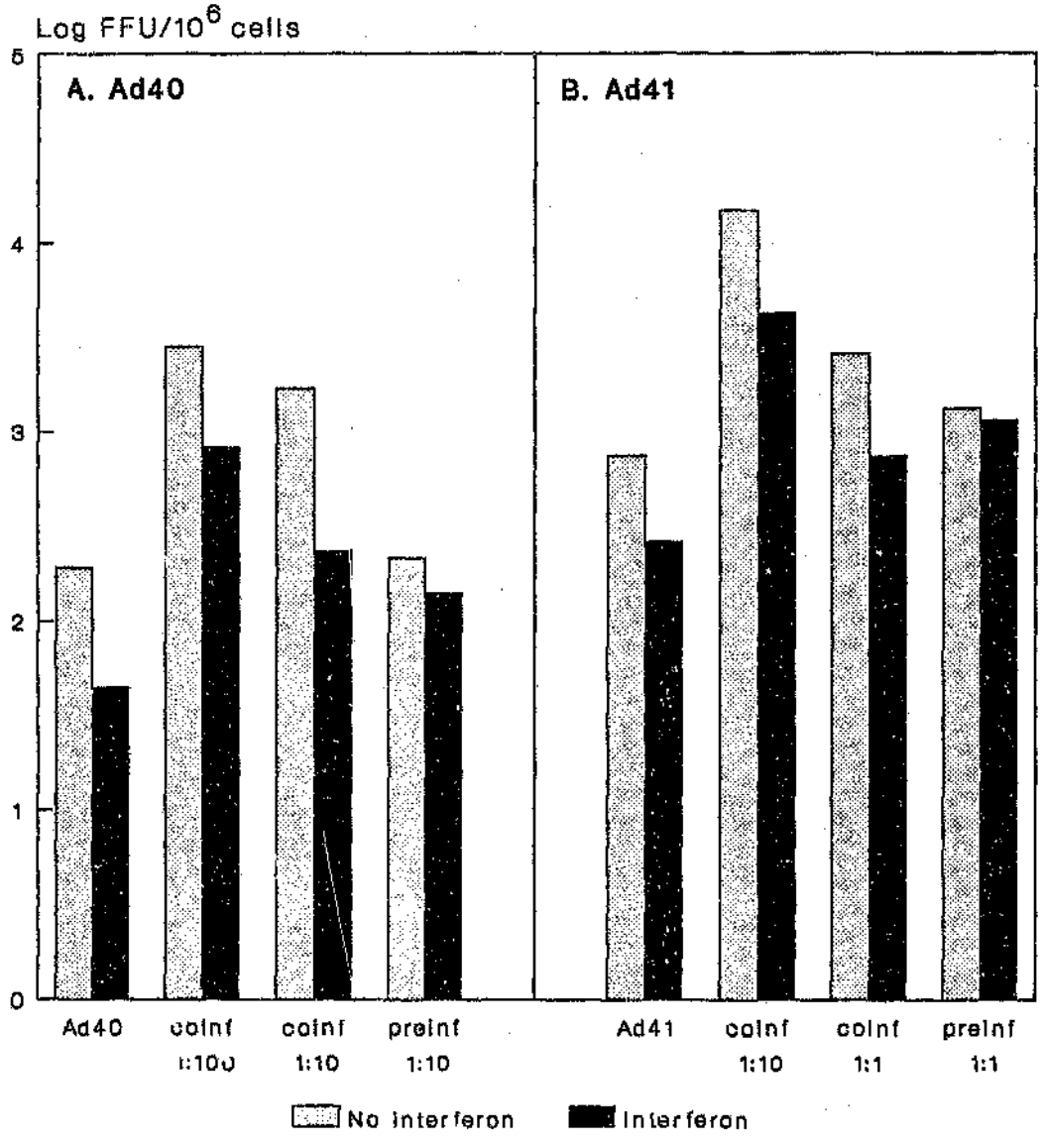


Figure 5.3 Histogram showing the effect of IFN on Ad40 and Ad41 in mixed infections with Ad2 in Chang cells. A. Ad40 Log FFU/10^6 cells. B. Ad41 Log FFU/10^6 cells. Cells were treated with IFN 24 hours before subgroup F adenovirus infection. Cells were infected with input ratios of viable particles described in Table 5.4. Cells were coinfected with Ad2 and either Ad40 or Ad41 or Ad2 applied 36 hours before. Coinfected coverslips were fixed after 44 hours. Cultures preinfected with Ad2 were fixed 48 hours after Ad2 infection, i.e. after 12 hours of mixed infection.

5.3 Discussion

The susceptibility of the subgroup F adenoviruses to IFN and the possible induction of IFN by Ad40 and Ad41 in non-permissive and semi-permissive cell cultures was investigated in this study. Induction of interferon was tested using an indirect radioimmunoassay which uses the IFN sensitivity of Sindbis virus to quantitate interferon in test samples (Lyons *et al,* 1982). Chang cells and HEF cells did not produce detectable IFN spontaneously or in response to infection by any of the adenoviruses. Thus, IFN induction in response to subgroup F adenovirus infection is not a likely reason for the poor growth of these viruses in either cell type.

IFN sensitivity was determined by comparing adenovirus specific fluorescent cell counts in IFN-pretreated and untreated infected cultures. Adenovirus 40 and Ad41 were found to be sensitive to IFN in Chang cells, whereas Ad2 was not. The sensitivity of subgroup F adenoviruses to IFN, albeit modest, is unusual since adenoviruses in general are not susceptible to its antiviral effects.

It is possible that the inordinately high IFN sensitivity of the subgroup F adenoviruses may be related in some way to their defectiveness *in vitro*. For example, the same genetic defect may give rise to both phenomena. It is apparent that Ad40 and Ad41 behave similarly with respect to IFN sensitivity in Chang cells. Since inhibition of Ad40 and Ad41 replication by IFN was not absolute, it seems likely that expression of viral functions known to be responsible for suppressing the antiviral activity of IFN in other adenoviruses is not entirely absent, but limited.

Adenovirus type 2 has been shown to complement replication of subgroup F adenoviruses in Chang cells (see Chapter Four). To test the possibility that a function required by Ad40 and Ad41 to overcome the antiviral effects of IFN could also be provided by Ad2, mixed infections were performed in IFN-treated Chang cells. The addition of Ad2 to cells before pretreatment with IFN resulted in the partial and

complete abrogation of Ad40 and Ad41 IFN sensitivity, respectively. The former result may have been due to the lower input concentration of Ad40 compared to Ad41.

The apparent reversal of the IFN-induced antiviral state by Ad2 in Chang cells appears to be a time-dependent phenomenon, since the effect was not seen when cells were simultaneously infected with Ad2 and either Ad40 or Ad41, but rather when Ad2 was applied 36 hours before (and [illegible] before IFN pretreatment of cells). It was not determined whether Ad2 infection prior to IFN treatment of cells was necessary to inhibit the effect of IFN.

These results are similar to those of another study where preinfection with Ad5 36 or 48 hours before resulted in the abrogation of vesicular stomatitis virus (VSV) sensitivity to IFN, but coinfection of the viruses did not (Anderson and Fennie, 1987). Further, it was found that Ad5 infection 12 hours after or at the time of IFN treatment also reduced the sensitivity of VSV to IFN. Therefore, the time-dependence of the IFN abrogation activity by Ad5 was related to the relative time of infection by the sensitive virus as opposed to the time of IFN pretreatment. This implies that reversal of IFN-induced inhibition of viral replication is dependent on expression of the "helper" virus before subsequent infection with the IFN-sensitive virus. In addition, the inhibition by Ad5, vaccinia virus, and HSV-1 of IFN-induced sensitivity of poliovirus and VSV has also been shown (Thacore and Youngner, 1973; Paez and Esteban, 1984; Feduchi and Carrasco, 1987).

The immunofluorescence system used in this study allows the determination of total proportion of cells which have proceeded to late antigen production at any time point, and as such is a sensitive means of monitoring inhibition of growth. A more molecular approach will be required to investigate the reason for IFN sensitivity.

Two adenovirus gene products have been implicated in the suppression of the IFN-induced antiviral state, E1a and VA RNAI. The VA RNAs are small transcripts synthesized by RNA polymerase III at low levels early in infection (Akusjärvi *et al*, 1986; Mathews and Shenk, 1991). The rate of synthesis of VA RNAI is markedly increased after DNA replication and becomes the most abundant viral RNA late in infection. VA RNAI is required for efficient translation of host cell and viral mRNAs late after infection. It has been shown to prevent the activation of the IFN-induced dsRNA-dependent protein kinase, thereby preventing the phosphorylation of initiation factor eIF-2 and thus allowing translation of proteins (Kitajewski *et al*, 1986a; Munemitsu *et al*, 1986). The E1a proteins suppress transcription of IFN-stimulated genes (Reich *et al*, 1988; Gutch and Reich, 1991).

The subgroup F adenoviruses have a partially defective *cis*-acting and *trans*-acting E1a function (van Loon *et al*, 1987a; Ishino *et al*, 1988). The level of E1a expression in Ad5 has recently been shown not to affect virus growth in cell culture (Hitt and Graham, 1990). The reduced activity of the subgroup F adenovirus E1a gene region may therefore not be involved in virus growth restriction *in vitro*. If, however, the suppression of transcription of IFN-stimulated genes by E1a is concentration dependent, the reduced activities of subgroup F adenovirus E1a genes may account for the inability of these viruses to fully overcome the antiviral effects of IFN.

The E1a gene also plays a role in enhanced transcription of class III genes (Hoeffler and Roeder, 1985). This gene could therefore affect VA RNAI levels. VA gene expression has not been observed during infection of HeLa cells in Ad5 E1a deletion mutants (Jones and Shenk, 1979a). Reduced expression of the VA RNAI, modulated through E1a, may therefore be responsible for the sensitivity to IFN seen in the subgroup F adenoviruses. This could lead to some phosphorylation of eIF-2 and therefore inhibition of translation.

Another possibility that cannot be excluded is an abberant VA RNAI function in subgroup F adenovirus infected cells independent of E1a function. A mutant Ad5 virus (*dl*331) that does not produce the VA RNAI species grows poorly in cell culture (Flint, 1986). Since the subgroup F adenoviruses display poor growth in most cell cultures a defective or ill-adapted VA RNAI is not an unlikely candidate for both their growth characteristics as well as their partial sensitivity to IFN.

The Ad2 suppression of the inhibitory effect of IFN on subgroup F adenovirus replication is consistent with the provision of a modulatory function such as VA RNAI level.

CHAPTER SIX

VIRUS PERSISTENCE AND INTEGRATION

6.1 Introduction

Abortive virus infections often result in a covalent association of viral and cell DNA, that is, the viral DNA becomes integrated (Doerfler, 1977). Integration has also been found in cells productively infected with adenovirus (Burger and Doerfler, 1974; Schick *et al,* 1976). However, the only cells that adenoviruses consistently transform, albeit at low frequency, are those that are either semi-permissive or non-permissive for viral growth. Hybridization studies have shown that hamster cells transformed by adenoviruses contain 90-100% of the viral genome (May *et al,* 1975; Fanning and Doerfler, 1976; Green *et al,* 1976). Adenovirus-transformed rat cells, on the other hand, contain viral sequences corresponding to 10-90% of the genome (Galiimore *et al,* 1974; Sharp *et al,* 1974; Flint *et al,* 1976). The only sequences common to all these cell lines comprise the left-hand 14% of the adenovirus genome containing the transforming genes (Graham *et al,* 1974).

Several reports have shown that the transforming genes of human adenoviruses appear to share homology with nucleotide sequences from normal human cells (Jones *et al,* 1979; Arrand *et al,* 1983; Braithwaite *et al,* 1984; 1986). The existence of an E1a-like cellular gene has also been described (Braithwaite *et al,* 1986).

Viral genome integration into cellular DNA may provide an explanation for the deficient gene expression of the subgroup F adenoviruses in human cells resulting in semi-permissive and abortive infections. This study was carried out in an attempt to provide some preliminary data on the subject of possible subgroup F adenovirus integration in a semi-permissive and non-permissive cell type. In addition, a number of uninfected cell lines were analysed for the presence of adenovirus specific sequences to determine if cellular homologues of adenovirus genes may exist.

6.2 Results

6.2.1 Search for viral sequences in "uninfected" cellular DNAs

Various cells (Table 6.1) of human and non-human origin were grown to confluency in 75cm^2 culture flasks, harvested by scraping and the DNA extracted. Foetal intestinal organ cultures were harvested after 48 hours of growth and the DNA extracted. DNA from the human cell types, and also vero (monkey) and BHK-21 (hamster) cells was then digested with *Bam*HI, *Sma*I, *Xba*I, *Eco*RI, *Hind*III, and *Eco*RI + *Hind*III. The remaining non-human cell DNAs were digested only with *Hind*III and *Hind*III + *Eco*RI. Ten micrograms of each digest was loaded onto 0.8% agarose gels. One microgram of lambda DNA digested with *Hind*III served as a molecular weight marker. Following electrophoresis the DNAs were transferred to nylon membranes and fixed by UV exposure. Hybridizations were then carried out using nick-translated DNA probes corresponding to various regions of the adenovirus genome. ^{32}P-labelled lamoda DNA was added to probe mixtures and adjusted where necessary to prevent overexposure on longer autoradiographs. Table 6.2 lists the probes used to detect nucleotide sequence similarity in cellular DNAs.

The results of all probes showing positive hybridization to cellular restriction fragments are shown in Table 6.3. The intensity of the hybridization reaction was rated as either weak (+), moderate (++), or strong (+++) taking the time of autoradiographic exposure into consideration. Of all the DNAs from the non-human cell lines, only vero cell DNA reacted positively with some of the probes.

Table 6.1 Human and non-human cell types tested for hybridization of their DNA to adenovirus DNA

Cell type	
Designation	Origin
Human	
293	human embryonic kidney, transformed with Ad5 E1 DNA
Chang	human conjunctival cells
intestinal organ culture	human foetus intestine
HEF	human embryo lung fibroblasts
Non-human	
vero	African green monkey kidney
BHK-21	baby Syrian/Golden hamster kidney
MDCK	Madin-Darby canine kidney
MDBK	Madin-Darby bovine kidney
B95-8	EBV-transformed marmoset leukocytes
Clone 1 NIH-3T3	mouse embryonic fibroblasts
RK13	rabbit kidney

Table 6.2 Restriction enzyme fragments and plasmids used to probe cell DNAs[a]

Adenovirus 2	Adenovirus 40	Adenovirus 41
pSPT18-Ad2-*BalII* (0.75-5.75)	*ClaIB*[b] (0-11.0)	pSP64-Ad41-*EcoRIB* (74.0-92.0)
pSPT18-Ad2-*HpaI-BclI* (4.37-11.29)		M51*A* (35.33-54.06)
pSPT18-Ad2-*SmaIA* (56.82-76.08)		M34*B* (14.20-32.50)
HpaID[b] (85.04-98.30)		M25*D+H* (60.63-74.22; 83.24-87.34)

[a]See Table 3.11 and Table 4.8 for description of plasmids. Map unit coordinates of viral DNA fragments appear in parentheses.
[b]Fragments were excised from the plasmids using the appropriate restriction enzymes and purified from agarose after electrophoresis using a silica solution.

Table 6.3 Relative intensity of hybridization of adenovirus probes to discrete cellular restriction enzyme fragments[a]

DNA probe[b]	Cell type				
	293	Chang	intestinal	HEF	Vero
pSPT18-Ad2 *BalII*	+ (7D)	+ (7D)	++ (5D)	++ (5D)	+ (22D)
Ad40 *ClaIB*	+ (1D)	+ (1D)	+++ (1D)	+ (1D)	ND
pSPT18-Ad2 *HpaI-BclI*	- (22D)	- (22D)	- (22D)	- (22D)	- (22D)
Ad2 *HpaID*	- (22D)	- (22D)	- (22D)	- (22D)	- (22D)
pSPT18-Ad2 *SmaIA*	- (22D)	- (22D)	- (22D)	- (22D)	- (22D)
pSP64-Ad41 *EcoRIB*	- (5D)	- (5D)	+ (5D)	+ (5D)	+++ (5D)
M51*A*	ND	ND	+ (9D)	+ (9D)	ND
M34*B*	ND	ND	+ (9D)	+ (9D)	ND
M25*D+H*	ND	ND	- (22D)	- (22D)	ND

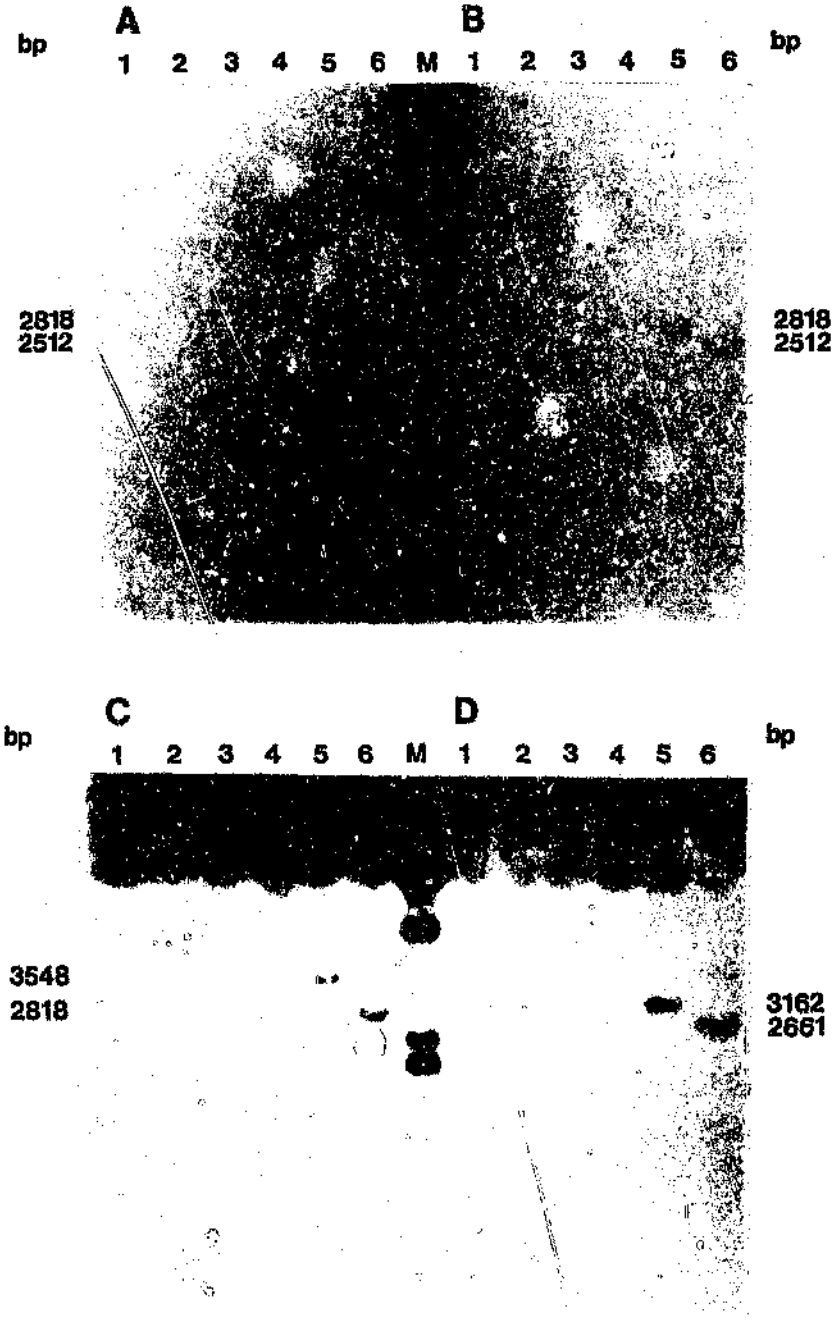
[a]Positive hybridization reactions with different probes appeared to be localized to the same *Hind*III and *Hind*III-*Eco*RI cellular restriction enzyme fragments.
[b]See Table 3.11 and Table 4.8 for description of plasmids.
ND: not done.
Numbers within parentheses indicate the time of autoradiographic exposure (days).

Discrete cellular restriction enzyme fragments that hybridize to Ad2 E1a plasmid were detected in the human cells as well as the vero cell line (Figure 6.1). Their sizes were determined and found to differ between cell types. In 293 and Chang cell DNA a *Hind*III fragment of 2818bp and a *Hind*III-*Eco*RI fragment of 2512bp were detected. The latter fragment is not an *Eco*RI fragment as a comigrating fragment would then have been present in the *Eco*RI lane. Foetal intestinal and HEF cell DNA contained larger *Hind*III and *Hind*III-*Eco*RI fragments of 3548bp and 3162bp, and 2818bp and 2661bp, respectively. The same plasmid hybridized to similar restriction enzyme fragments in vero cells. The results obtained for the human cells were further confirmed by hybridization of the same blots with the *ClaIB* fragment of Ad40 which contains the entire E1 region (Figure 6.2). Here the Ad40 E1 region showed a much stronger hybridization to cellular bands of foetal intestinal cell DNA than those of other human DNAs. No discrete Ad2 E1b-like sequences or sequences that hybridize to a region between coordinates 56.82 and 76.08 on the Ad2 genome could be detected in any of the cell DNAs.

No hybridization reaction with any of the cell DNAs was obtained using the *HpaID* fragment of Ad2 as a probe. This fragment encompasses part of the E3 and L5 transcription units. However, a plasmid with the *Eco*RI*B* fragment of Ad41 gave a very strong hybridization signal in *Hind*III and *Hind*III + *Eco*RI digests of vero cell DNA, a weak reaction in foetal intestinal and HEF cell DNA, and no detectable reaction in 293 and Chang cell DNA (Figure 6.3).

A PCR reaction using subgroup F adenovirus specific primers K402 and K403 from the same region as the above probe confirmed the vero cell DNA result, yielding a 152bp product that could not be digested with the restriction enzyme *Acc*I. This tentatively suggests an Ad41 specific PCR product as opposed to Ad40 on the basis of *Acc*I digestion (see section 3.2.5.2). The same primers did not bind to any of the human cell DNAs under the same conditions used.

The *PstLA* (M34) and *B* (M51) fragments of Ad41 showed weak hybridization to the same cellular bands as for other probes whereas *PstI* fragments *D* and *H* did not (M25). The location of these fragments in relation to the Ad41 genome are shown in Figure 3.29.



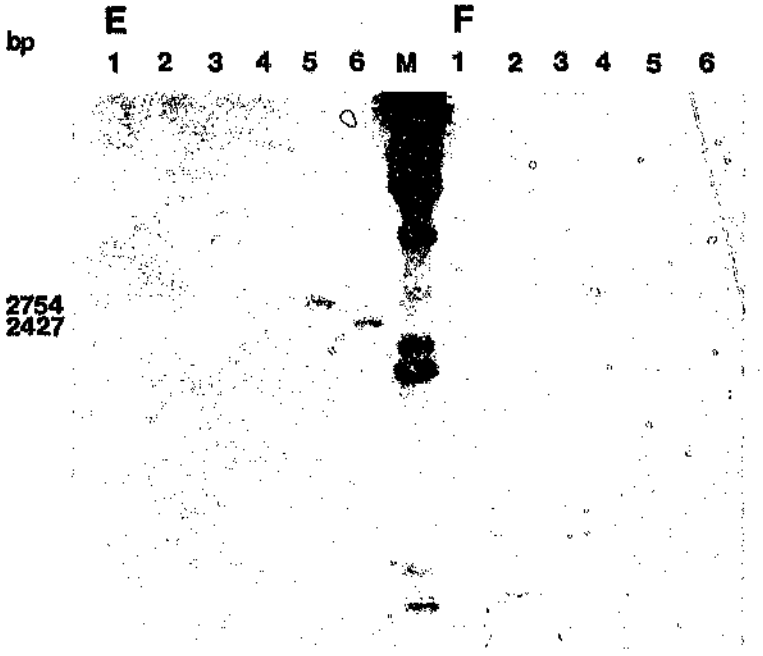


Figure 6.1 Hybridization of pSPT18-Ad2 *BalII* to Southern blots of restriction enzyme digested cell DNAs. DNA from 293 (A), Chang (B), foetal intestinal (C), HEF (D), vero (E), and BHK-21 (F) cells were digested with restriction enzymes: Lane 1. *Bam*HI Lane 2. *Sma*I Lane 3. *Xba*I Lane 4. *Eco*RI Lane 5. *Hind*III Lane 6. *Hind*III + *Eco*RI. Lane M. Lambda DNA digested with *Hind*III. Autoradiographs were exposed for 7 days (A and B), 5 days (C and D) and 22 days (E and F). Sizes (bp) of discrete cellular bands showing hybridization to probe are indicated.

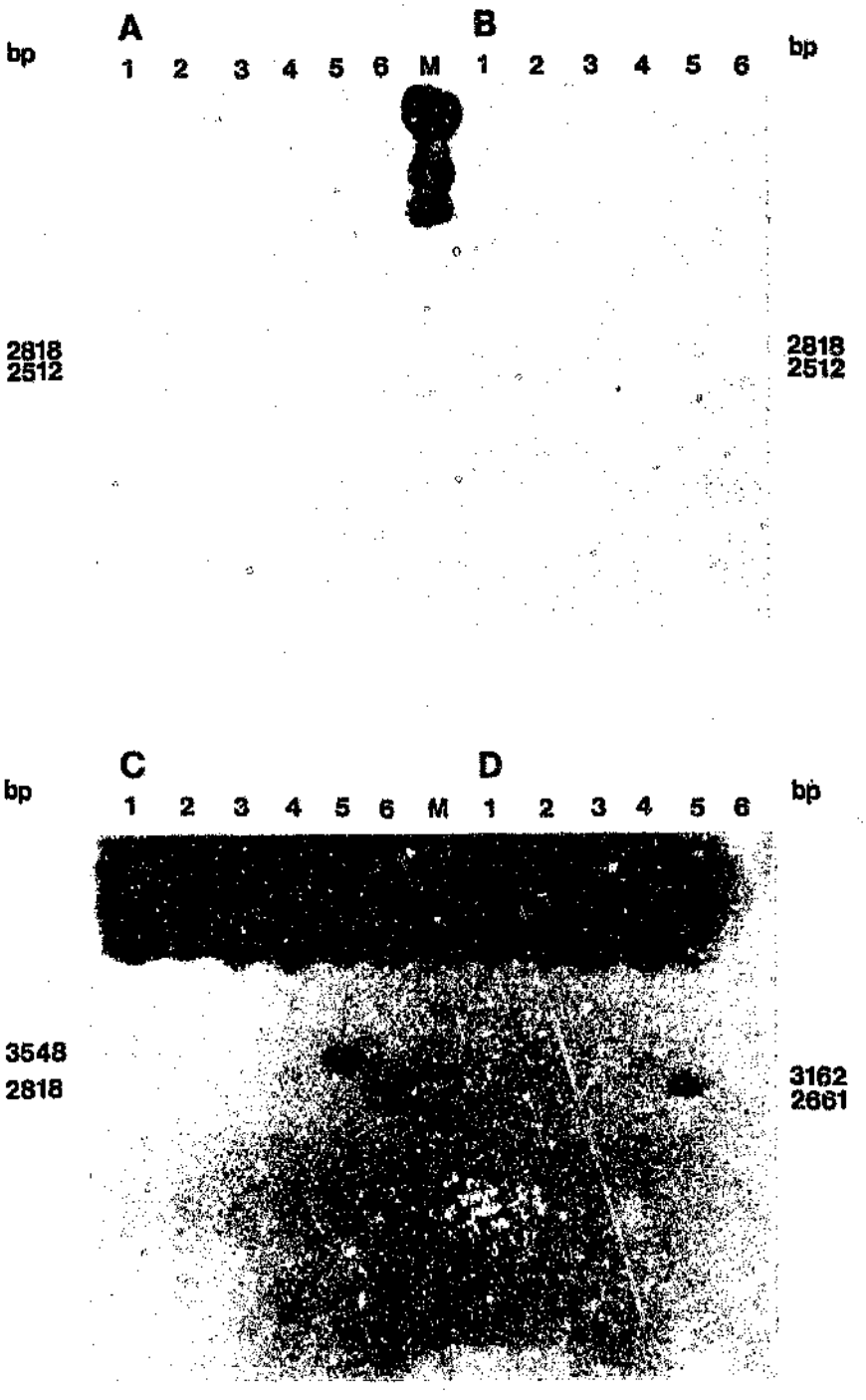


Figure 6.2 Hybridization of the Ad40 *ClaIB* fragment to Southern blots of restriction enzyme digested cell DNAs. DNA from 293 (A), Chang (B), foetal intestinal (C), and HEF (D) cells were digested with restriction enzymes: Lane 1. *Bam*HI Lane 2. *Sma*I Lane 3. *Xba*I Lane 4. *Eco*RI Lane 5. *Hind*III Lane 6. *Hind*III + *Eco*RI. Lane M. Lambda DNA digested with *Hind*III. Autoradiographs were exposed for 24 hours. Sizes (bp) of discrete cellular bands showing hybridization to probe are indicated.

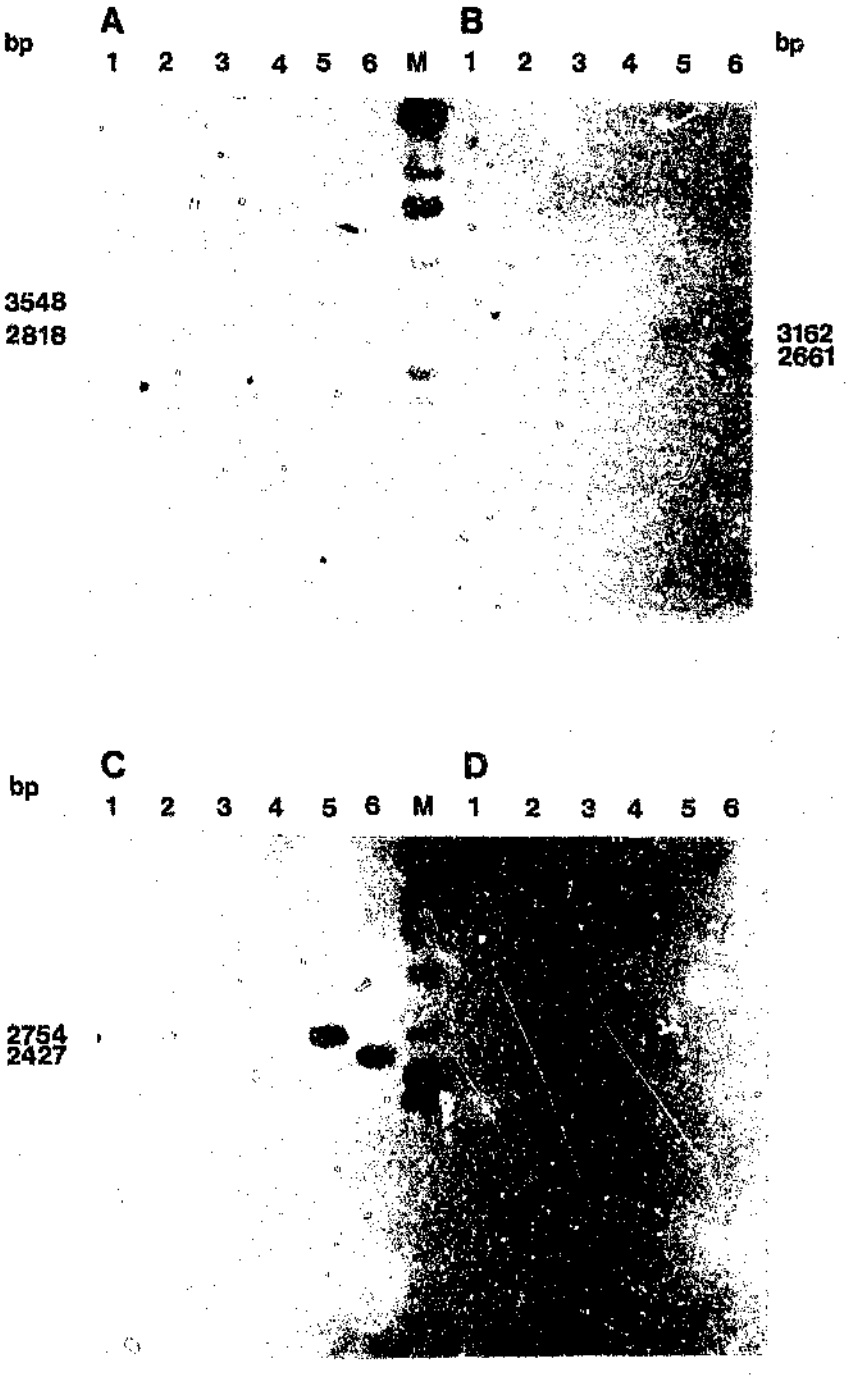


Figure 6.3 Hybridization of pSP64-Ad41 *EcoRIB* to Southern blots of restriction enzyme digested cell DNAs. DNA from foetal intestinal (A), HEF (B), vero (C), and BHK-21 (D) cells were digested with restriction enzymes: Lane 1. *Bam*HI Lane 2. *Sma*I Lane 3. *Xba*I Lane 4. *Eco*RI Lane 5. *Hind*III Lane 6. *Hind*III + *Eco*RI. Lane M. Lambda DNA digested with *Hind*III. Autoradiographs were exposed for 5 days. Sizes (bp) of discrete cellular bands showing hybridization to probe are indicated.

6.2.2 Search for viral sequences in cellular DNAs of previously infected cells

Chang cells and HEF cells in 75cm^2 flasks were infected with 1.2 x 10^4 FFU/ml of either Ad40 or Ad41. Duplicate cultures of infected and uninfected cells were passaged upon confluency. After the first passage maintenance medium was replaced with growth medium containing 10% FCS. Survival of the cultures was dependent on the permissiveness of the cells for Ad40 and Ad41. Infected Chang cells could not be cultivated beyond one passage due to progressive CPE over this time. However, HEF cells infected with Ad41 were viable over 6 passages, while those infected with Ad40 survived for only three passages. The stage prior to loss of the culture was marked by a CPE-like affect on the cells. Surviving cells did not recover upon continued maintenance. Uninfected HEF cells in this experiment did not survive beyond 6 passages, so cell death could not be attributed to the presence of Ad41. DNA was extracted from one of the flasks at each passage and the other one was used to propagate the cells further.

Passage zero (P0) DNAs for infected and uninfected DNAs were digested with *Bam*HI, *Eco*RI, *Hind*III, and *Sma*I. The resultant fragments were separated on 1% agarose gels and transferred and fixed to nylon membranes. The blots were hybridized with homologous viral DNA isolated from CsCl-purified Ad40 and Ad41 particles. Virus specific DNA fragments were detected only in Ad40 infected HEF cells (Figure 6.4).

DNA from all 6 passages for Ad41 and three passages for Ad40 were digested with *Eco*RI, separated on 1% agarose gels and transferred to nylon. These were hybridized with pSP64-Ad41 *Eco*RI*B*. Viral bands could be seen on EtBr-stained agarose gels only for Ad40 P3 DNA (Figure 6.5A). All four Ad40 *Eco*RI fragments were present. Hybridization with the Ad41 plasmid containing fibre DNA revealed viral DNA in all three passages of Ad40 and a weak reaction in P1 DNA in Ad41 infected HEF cells (Figure 6.5B). The autoradiograph was overexposed to allow for

the detection of possible weak reactions in the later passages of Ad41 infected HEF cells. The resolution was therefore compromised for strong hybridization signals, with the radioactive probe cross-reacting with *Eco*RI fragments in addition to the *Eco*RI fragment *C* of Ad40 which contains the fibre gene. It was, however, clear from the results that there was an increase in the amount of Ad40 DNA with passage.

The DNAs were also tested for the presence of specific viral DNA at each passage using PCR with primers K40 and K403. DNA from all passages of infected and uninfected HEF cells gave positive and negative results by PCR, respectively. Positive reactions were confirmed by type specific *Acc*I cleavage and hybridization with pSP64-Ad41 *Eco*RI*B* (data not shown).

The synthesis of late viral antigens was monitored at each cell passage using indirect immunofluorescence. No late antigens were detected in Ad41 infected HEF cells at any of the passages. Adenovirus 40 fluorescent cells were detected at a rate of less than one in 10^5 cells and there was no apparent increase in the number of fluorescent cells with passage. As the DNA was seen to undergo replication in Ad40 infected HEF cells, the lack of hexon antigen suggests a late replicative block in these cells.

Since all four *Eco*RI fragments of Ad40 could be detected in DNA from passaged HEF cells (Figure 6.5), the entire genome was present, either in an episomal or an integrated form. The latter possibility is less likely in view of the fact that all Ad40 fragments were of the predicted lengths for genomic DNA. To further investigate this whole genome persistence (episomal or integrated) in these cells, superinfections of cells were carried out using Ad2 as helper virus. It has already been shown that Ad2 can provide a helper function in these cells for Ad41 growth (see Chapter Four). Adenovirus 2 could conceivably reactivate or rescue inactive Ad40 and Ad41 genomes in HEF cells. Adenovirus 40 and Ad41 infected HEF cells were superinfected with Ad2 (2.4×10^5 FFU/ml; 0.1 FFU/cell) three days after the

7th passage (P7) when cells were approximately 70% confluent. DNA was extracted from a control flask of Ad40 and Ad41 infected cells at P7 p.i. and digested with *Eco*RI. As before all four Ad40 *Eco*RI fragments could be detected but no Ad41 DNA. The cells were grown over several passages to reduce the likelihood of inoculum virus being complemented by Ad2. Control flasks received PBS. After 44 hours cells were harvested and spot slides prepared for the detection of late viral antigens by indirect immunofluorescence. Adenovirus 2 infections were monitored using a group-specific polyclonal antiserum to ensure good growth. Passaged Ad40 and Ad41 infected cells superinfected with Ad2 and those not infected with Ad2 were treated with a subgroup F specific monoclonal antibody. No reactivation of subgroup F adenovirus growth in HEF cells could be detected due to the presence of Ad2 (Table 6.4).

A higher concentration of Ad2 (2.4 x 10^6 FFU/ml; 1 FFU/cell) was used in the same experiment to ensure infection of every cell and therefore a greater probability of detecting rescue in a small number of cells. This resulted in more enhanced CPE in HEF cells but no significant detection of subgroup F adenovirus late antigens.

In this experiment HEF cells infected with Ad40 and Ad41 were successfully passaged 7 times over a period of 49 days continuous culture. Adenovirus 40 infected cells underwent a similar stage of apparent virus-induced cell destruction as described for the previous experiment but this time surviving cells were able to grow to confluency. A similar crisis phase occurred a second time, after a further 20 days of growth. Infected cells from the 8th passage were frozen down.

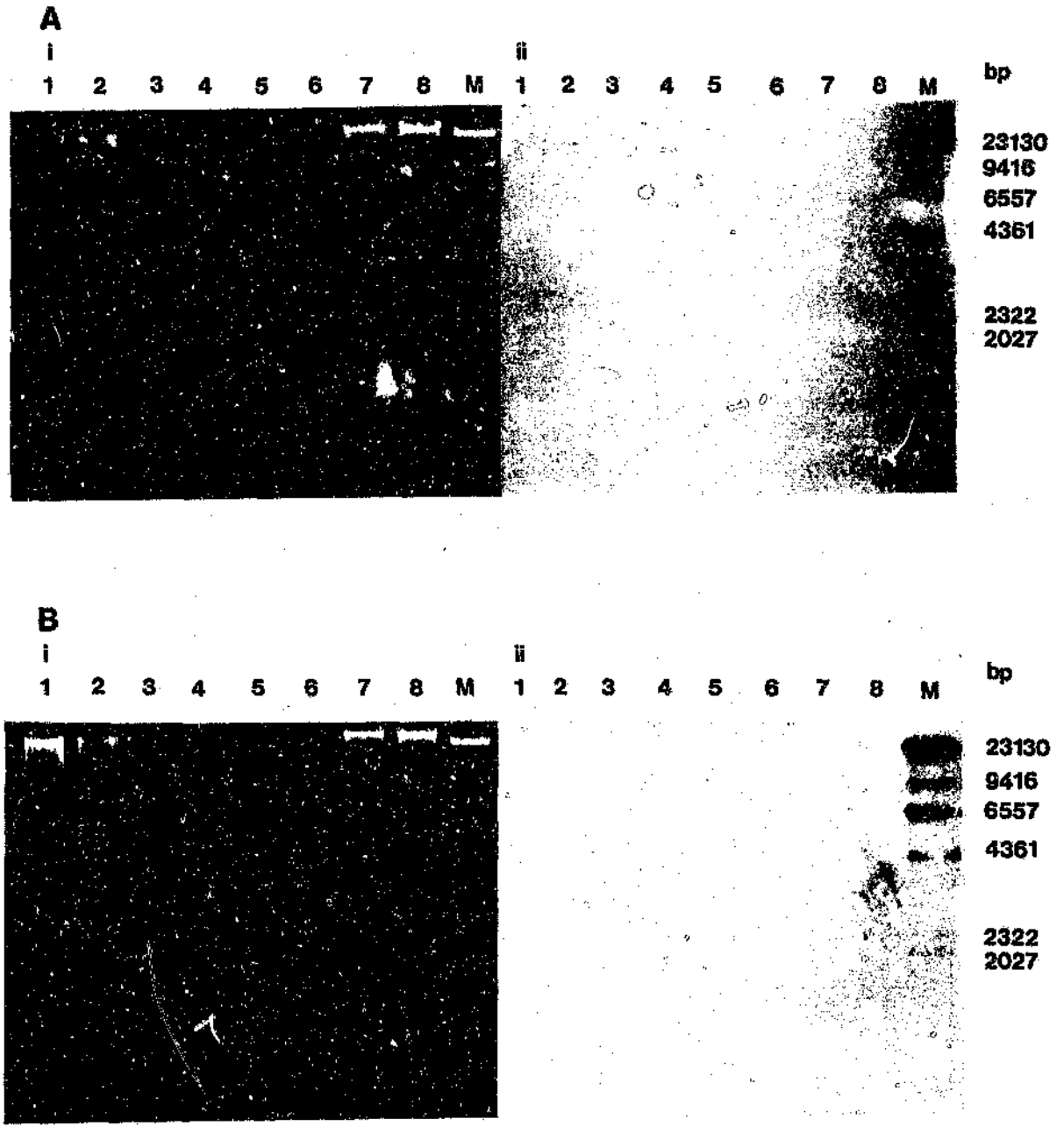


Figure 6.4 Detection of adenovirus specific restriction enzyme fragments in DNA from Ad40 (A) and Ad41 (B) infected HEF cells at passage zero (P0). DNA was digested with *Bam*HI (lanes 1-2), *Eco*RI (lanes 3-4), *Hind*III (lanes 5-6), and *Sma*I (lanes 7-8) and 10μg of DNA loaded into wells of 1% agarose gels. Lanes 1,3,5,7. Uninfected cell DNAs. Lanes 2,4,6,8. Infected cell DNAs. Lane M. Lambda DNA digested with *Hind*III. Sizes (bp) of digested marker DNA are indicated. (i) EtBr-stained agarose gel. (ii) Autoradiograph of Southern blot of the gel in (i) hybridized with purified Ad40 (A) or Ad41 (B) DNA.

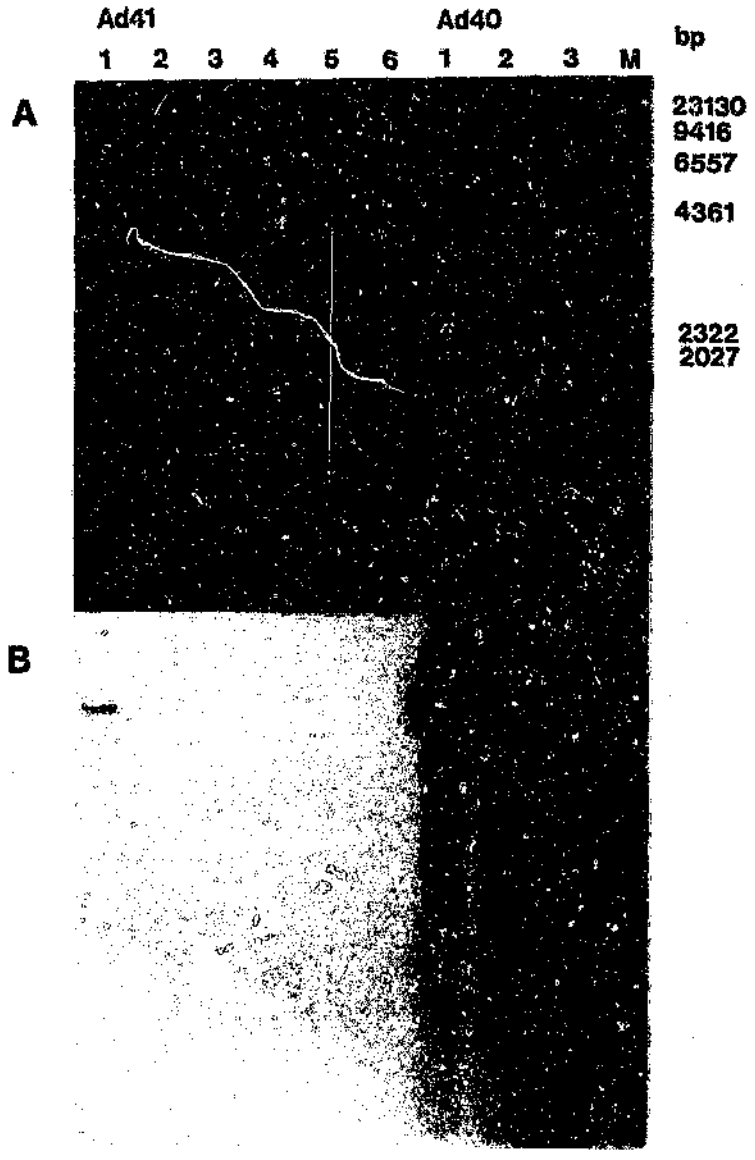


Figure 6.5 Detection of adenovirus specific restriction enzyme fragments in DNA from Ad40 and Ad41 infected HEF cells at different passages post-infection. DNA was extracted from infected cells at P1 (lane 1), P2 (lane 2), P3 (lane 3), P4 (lane 4), P5 (lane 5), P6 (lane 6). Lane M. Lambda DNA digested with *Hind*III. A. EtBr-stained 0.8% agarose gel. B. Autoradiograph of Southern blot in (A) hybridized with pSP64-Ad41 *Eco*RI*B*. Sizes (bp) of *Hind*III digested lambda DNA are indicated.

Table 6.4 Detection of Ad40 and Ad41 late antigens in HEF cells grown over 7 passages and superinfected with Ad2

Ad2 infection FFU/cell[b]	% P7 cells fluorescing[a]		
	uninfected	Ad40	Ad41
Group-specific antibody[a]			
0.0	0		
0.1	60		
1.0	80		
Subgroup F specific antibody[a]			
0.0	0	0.0001	0
0.1	0	0.001-0.002	0
1.0	0	0.001-0.002	0

[a]For detection of Ad2 and subgroup F adenovirus fluorescence in passage 7 (P7) fibroblasts, cells were stained with group-specific polyclonal antibody directed against Ad5 hexon and MAb-5, respectively (see section 4.2.1).
[b]Ad2 m.o.i. (FFU/cell) was determined in Chang cells, and corresponded to concentrations of 2.4 x 10^5 FFU/ml (0.1 FFU/cell) and 2.4 x 10^6 FFU/ml (1.0 FFU/cell) of Ad2.

6.3 Discussion

A search for the presence of virus-like sequences in cell DNAs was initially conducted to determine if cellular E1a-like sequences existed. The existence of these sequences has been suggested from direct (Braithwaite *et al,* 1986) and indirect evidence (Kao *et al,* 1985). A cellular homologue of the adenovirus E1a gene could conceivably substitute for a defective subgroup F adenovirus E1a gene function in the infected host cell. Plasmids containing early region 1 DNA showed varying degrees of hybridization to discrete cellular DNA bands ranging from about 2.4kbp to 3.5kbp in length depending on the cell type and restriction enzymes used. The results were identical for hybridization with the whole E1 region or E1a alone.

No sequences homologous to the E1b region of Ad2 were detected in any of the cell lines. This was unexpected in the case of 293 cells since they have integrated Ad5 E1 DNA (Graham *et al,* 1977). Adenovirus 2 and Ad5 share a high degree of homology throughout their sequences (Tooze, 1981; Chroboczek *et al,* 1992) and therefore poor homology could not be an explanation. Restriction enzymes used for digesting the cellular DNA included *Bam*HI and *Eco*RI which do not recognize sites within Ad5 E1 region DNA. An analysis of the arrangement of Ad5 DNA sequences in 293 cells by Aiello *et al,* (1979) revealed two *Bam*HI bands and two smaller *Eco*RI bands all of sizes intermediate between the *A* (23,130bp) and *B* (9,416bp) fragments of *Hind*III cleaved lambda DNA. These high molecular weight cellular fragments, however, could not be detected in 293 cell DNA in the present work. Since the probes were labelled to high specific activity and longer exposures gave the same results, a possible explanation could either be inefficient electrophoretic transfer of larger DNA fragments or the loss of these cellular restriction enzyme sites that flank the viral sequences with continuous passage of the cells *in vitro*. It is also possible that the E1 region itself may have been modified over a large number of passages and may have acquired or lost restriction enzyme sites. The constitutive expression of the Ad5 E1 integrated region in these cells requires confirmation by hybridizing

radiolabelled nuclear RNA of 293 cells to restriction profiles of Ad5 DNA, a procedure not limited by the choice of cellular restriction enzyme sites. This highlights the fact that regions of homology between cellular and viral DNA may easily be overlooked on the basis of restriction enzyme choice and that a negative result does not necessarily mean the absence of homologous sequences.

Regions of homology between disparate regions of the adenovirus genome and cellular DNA all apparently mapped to the same cellular restriction enzyme fragments measuring not more than one tenth of the adenovirus genome length. Other studies have shown that adenoviral sequences share homology with cellular nucleotide sequences (Jones *et al*, 1979; Arrand *et al*, 1983; Braithwaite *et al*, 1984; 1986). Jones et al (1979) found that apparently normal human tissues contain RNA that hybridizes with four regions of the Ad2 genome. These were contained within the limits of coordinates 0-7.5, 26-32.15, 37-58.5, and 70.7-75.9 m.u. on the Ad2 genome. Unlike Ad2, Ad12 showed no appreciable hybridization with tissue RNA. RNA from gorilla organs, but not from chickens, displayed similar homology to Ad2, confirming the hybridization map deduced for human tissue RNA. In contrast, RNA from cell lines gave faint reactions in hybridization tests. Early adenovirus antigens could also be detected in tissue cells but not in cells maintained in culture. Horvath *et al* (1986) suggested, however, that these findings may have been due to the presence of peripheral blood lymphocytes carrying persistent adenovirus genomes in these tissues.

Braithwaite *et al* (1984) have shown that the Ad2 E1a, E1b and E4 genes hybridize with a plasmid containing human repetitive sequences. This finding is not inconsistent with the results presented above. The hybridizing region within the E1a and E1b genes was further defined as a stretch of 200bp between 3.8% and 4.4% at the 3′ end of E1a and about 150bp at the 3′ end of E1b (Braithwaite *et al*, 1986). It was found that both of these regions hybridize with 2.35kbp *Bam*HI-*Sma*I fragment

of the DNA probe containing the cellular repeat sequences even though E1a and E1b do not share any homology.

The presence of cellular sequences that hybridize with a DNA probe containing the fibre gene of Ad41 in foetal intestinal, HEF and vero cells, but not in 293 and Chang cells may reflect differences in copy number of related sequences from different individuals or in different cell types. Using subgroup F specific primers in a PCR reaction showed that at least vero cells contain sequences highly homologous to subgroup F adenovirus fibre DNA. The sequence may be more closely related to Ad41 fibre DNA than that of Ad40 as determined by the absence of an *Acc*I restriction enzyme site in the vero cell PCR product. Although no such sequences were evident in human cells using PCR (despite positive hybridization of foetal intestinal and HEF cells with the fibre DNA probe), the high stringency of the PCR reaction may have excluded sequences containing one or more mismatched bases, especially if present at the 3′-terminal end of a primer. Certain mismatches at or near the 3′-terminal of PCR primers have been shown to affect primer stability and the efficiency of primer-extension by DNA polymerase (Petruska *et al*, 1988), the reduction in product yield being dependent on the type of base mismatch (Kwok *et al*, 1990).

The existence of subgroup F adenovirus sequences from the fibre gene in simian cells may have some evolutionary significance. Another unusual feature of the subgroup F adenovirus fibre region is the existence of two fibre genes (Pieniazek *et al*, 1990) coding for two fibres (Kidd *et al*, 1992), a situation which has only been described for avian adenoviruses (Laver *et al*, 1971). The presence of two fibres in itself implies that the subgroup F adenoviruses may have evolved mechanisms in the course of natural infection that may be advantageous for attachment and entry into gut epithelial cells. Strong hybridization of the subgroup F adenovirus E1 and fibre genes to simian cell sequences further suggests the possible exchange of sequences between virus and a cell type at some stage in evolution, although the cells are no longer host

to these viruses. However these viruses may have evolved, the strategies employed by them must have some selective advantage in the human host.

Adenovirus 40 and Ad41 infected HEF cells were monitored over a number of passages for DNA synthesis and late antigen production. No DNA was synthesized or late antigens produced in Ad41 infected HEF cells at any passage. These results were in agreement with previous results showing a block in replication prior to DNA synthesis. In contrast, Ad40 DNA persisted in passaged HEF cells and these persistently infected cells were characterized by the following: (i) The first stage was marked by a CPE-like effect on the cells, at which time the cells either died or recovered upon continued maintenance, (ii) The absence of detectable late antigen synthesis. Cells showing late antigens occurred at a frequency of about 1 in 10^5 cells with no apparent increase in number with passage, (iii) The presence of the entire genome probably episomally and its amplification over passage, and (iv) Resistance to rescue of Ad40 replication by Ad2, an event which readily occurs in unpassaged Ad40 infected HEF cells. These results further provided evidence for a late block in Ad40 replication in these cells.

Low grade genomic DNA amplification with cell turn-over may be a means of ensuring persistence by overcoming elimination by cellular nucleases. In addition, limited expression of some genes and the absence of others would be expected and may be regulated in some specific way. A mechanism such as gene methylation (for review see Doerfler, 1991 and references therein) could account for controlled switching on and off of gene functions. Adenovirus DNA within the virus particle is not methylated (Günthert *et al*, 1976) but integrated DNA becomes methylated in specific patterns (Sutter *et al*, 1978; Kruczek and Doerfler, 1982). This raises the question as to whether episomal adenovirus DNA could be methylated and in this way regulate specific gene expression by long-term promoter inactivation. Promoter-inactivation as a result of DNA methylation can be partly overcome by *cis*-acting or *trans*-acting factors such as the strong enhancer in the immediate early gene of human

cytomegalovirus (Knebel-Mörsdorf *et al*, 1988) or the 289R transactivating E1a protein of Ad2 (Langner *et al*, 1986; Weisshaar *et al*, 1988). The inability of Ad2 to rescue the viral genome in these cells does not necessarily mean that this is true for the *in vivo* situation in the cell type in which they may persist. Likewise, the finding that Ad41 cannot persist in HEF cells does not mean that this cannot occur *in vivo*.

Adenovirus 2 has been shown to provide a helper function for both Ad40 and Ad41 late antigen synthesis in mixed infections of HEF cells (see Chapter Four). The presence of Ad2 did not result in reactivation of the replication cycle of either Ad40 or Ad41 as determined by the presence of subgroup F specific late antigens using indirect immunofluorescence. The DNA was therefore not in a form that could be rescued by Ad2 in a similar way to mixed infections carried out in the absence of cell passaging. If the DNA was episomal and intact Ad2 could theoretically, on the basis of previous complementation results, promote the synthesis of late antigens. The likelihood of episomal DNA being degraded over a number of cell passages is high unless it exists in some protected form. In Ad40 infected HEF cells the DNA may be integrated in such a way that genetic reactivation by a superinfecting virus would be extremely rare if not impossible. It is unlikely that different fragments of Ad40 DNA occupy different cellular sites as one would expect a change in migration of the fragments containing viral sequences due to the presence of adjoining cellular regions and not the type specific *Eco*RI profile seen. It is however possible that sequences containing cellular and viral sequences could not be detected above the background cellular DNA smear in EtBr-stained gels or when cellular DNA was probed with just a portion of the genome.

The subgroup C adenoviruses readily form latent infections of lymphoid tissues, especially tonsils. In addition to free adenoviral DNA molecules corresponding to the entire genome, other virus DNA-containing bands representing possible integrated viral sequences have been detected in tonsil cells, peripheral blood lymphocytes and lymphoblastoid cell lines (Green *et al*, 1979b; Horvath *et al*, 1986).

Only one in 10 cord blood samples were positive for adenovirus DNA (Horvath *et al,* 1986). On the basis of these results it was suggested by Horvath *et al* (1986) that infections in childhood with the subgroup C adenoviruses result in the persistence of the viral genome in circulating lymphocytes. In a recent study, Chu *et al* (1992) have shown that a block in replication of Ad5 at the late phase results in persistent infection of human monocyte hybridoma cell lines. Results obtained in this study, as exemplified by Ad40 infection of HEF cells, are consistent with a role for a late replicative block in adenovirus persistence.

The subgroup F adenoviruses may persist in a similar manner in some population of cells in the gastrointestinal tract, perhaps in cells that they do not replicate in. In this way these viruses may have developed the means to avoid elimination by the host immune reponse. From the results it is evident that there is a distinct difference between the ability of Ad40 and Ad41 to persist in HEF cells, most likely a function of their respective replicative capabilities in the host cell.

CHAPTER SEVEN

CONCLUDING REMARKS

7.1 Candidates for a defective growth function *in vitro*

The interaction of subgroup F adenoviruses with human cells *in vitro* presents an interesting example of the varied outcome of a virus infection in relation to the permissiveness of the host cell. This study was an attempt to define and establish the basis of the growth restriction of these viruses *in vitro*. Table 7.1 summarizes the phenotypic traits assigned to Ad40 and Ad41 based on findings from this work. Candidate functions that may contribute to a specific phenotype are discussed.

One major problem that deserves consideration is the distinction between direct and indirect effects of a specific defect in a system where many gene functions interact to regulate gene expression. A complex phenotype can be generated and interpretation in terms of these traits can be difficult and often misleading. For example, in HEF cells at least one Ad41 defect clearly occurs at a very early stage and the phenotype may be governed exclusively by this initial aberrant function(s). On the other hand, in Chang cells Ad41 replication is not as restricted and progresses beyond the replicative block apparent in HEF cells. This same defect may be operational to some extent in Chang cells and could theoretically give rise to further inefficiencies later in the replication cycle. This could best be described as "a house

of cards effect" caused by an early function that is less debilitating in Chang cells and therefore gives rise to more detectable abberations than in HEF cells. In addition, certain functions may be more severely affected by an earlier defect than others. Alternatively, different defects may be the result of mutually exclusive events.

7.1.1 A role for early region 1 functions

The characteristic growth defect of the subgroup F adenoviruses in cell culture is reminiscent of that of adenoviruses that display an altered host-range, the so-called host-range mutants of the subgroup C adenoviruses with defects in region E1 (Harrison *et al,* 1977; Jones and Shenk, 1979b; Young *et al,* 1984). These mutants can be propagated on 293 cells which provide a helper function for growth through expression of the integrated Ad5 E1 region (Graham *et al,* 1977; Aiello *et al,* 1979). KB and HeLa cells are less permissive for replication of these E1 mutants.

Sequencing studies (van Loon *et al,* 1987b; Allard and Wadell, 1988; Ishino *et al,* 1988) show that the overall organization of the E1a and E1b gene regions appears similar to that of other adenoviruses. However, the E1a products of Ad40 (Ishino *et al,* 1988; van Loon *et al,* 1987a) and Ad41 (van Loon *et al,* 1987a) have a reduced capacity to transactivate other early genes. Transcription of the E1a gene itself is controlled by a number of *cis*-acting elements (Hearing and Shenk, 1983; Hen *et al,* 1983; Imperiale *et al,* 1983). The *cis*-acting activity of the Ad40 E1a promoter in rat 3Y1 cells was also found to be deficient (Ishino *et al,* 1988). Evidence from complementation studies has also implicated a defective E1b-55K function, at least in Ad40 (Mautner *et al,* 1989). Even in permissive cells the Ad40 E1b-55K protein equivalent is not expressed (Mautner *et al,* 1990).

A number of findings in this study lend support to a role for defective E1a and/or E1b functioning in the subgroup F adenovirus growth restriction *in vitro*.

Table 7.1 Phenotypic characteristics of Ad40 and Ad41 in cell culture

Stage in replication	Phenotypic trait	Remarks
Late antigen production[a]	Growth permissiveness	
	293 cells	
	permissive (Ad41)	Ad41 infectivity showed one-hit kinetics
	semi-permissive (Ad40)	
	Chang cells	
	semi-permissive (Ad40 and Ad41)	Ad41 infectivity showed multiple-hit kinetics Ad41 late antigen synthesis potentiated by Ad2
	sensitivity to IFN (Ad40 and Ad41)	Ad40 and Ad41 partial and complete sensitivity to IFN, respectively, was overcome by preinfection with Ad2 A defective E1a and/or VA RNAI may be responsible
	HEF cells	
	non-permissive (Ad40 and Ad41)	Ad2 provided a helper function for Ad41 late antigen synthesis Ad41 interfered with Ad2 replication Ad41 promoted Ad2 late antigen synthesis if applied to cells before Ad2 Ad40 and Ad41 did not complement each other
	Foetal intestinal organ cultures	
	Ad40 and Ad41 grew poorly compared to Ad2	
DNA synthesis and packaging into virions[b]	Chang cells	
	DNA accumulation was reduced compared to that of Ad2 (Ad40 and Ad41)	Adenoviruses normally package 10-20% of DNA synthesized
	Approximately 1% of DNA synthesized was packaged into virions (Ad40 and Ad41)	

Stage in replication	Phenotypic trait	Remarks
	HEF cells	
	Ad40 DNA replication but no evidence of packaging	Late block in replication No discrete size class of DNA detected
	Persistence and amplification of Ad40 genome over passage in infected cells	Episomal DNA: all four *Eco*RI fragments of predicted size detected Infection could not be reactivated by Ad2
	Ad41 defective for DNA replication	Early block in replication Defect could be overcome by coinfection with Ad2 Ad41 interfered with Ad2 DNA synthesis
Host protein shutoff[c]	Not detected in 293 or Chang cells (Ad40 and Ad41)	Multiplicities required to show host protein shutoff in Ad2 could not be obtained for Ad40 and Ad41, therefore not conclusively shown
Transcription[d]	Chang cells Ad41 transcripts accumulated gradually and were detected at later times when compared to 293 cells	
	No detectable E4 transcription	May have been below level of detection
	HEF cells Only Ad41 transcripts mapping to 0-12 m.u. were detected	Early block in replication
	No detectable Ad41 late gene expression	

[a]Monitored by group-specific ELISA and a fluorescent focus assay using subgroup F specific monoclonal antibodies.
[b]Monitored by alkaline gradient analysis of ^{3}H-thymidine labelled viral DNAs, ^{32}P-orthophosphate labelling of viral DNA *in vivo* and restriction enzyme analysis, and DNA:DNA hybridizations of dot blots and Southern blots.
[c]Monitored by ^{35}S-methionine labelling of proteins *in vivo*.
[d]Monitored by DNA:RNA hybridizations and RT-PCR.

Firstly, productive and semi-productive growth of Ad41 and Ad40 in 293 cells, respectively, is most likely due to the provision of E1 functions in *trans*. With Ad41 one infectious virus particle was found to be sufficient to productively infect a 293 cell. However, reports of loss of infectivity upon passage of Ad41 in 293 cells (Pieniazek *et al*, 1990a) and poor growth of Ad40 in these cells (Chiba *et al*, 1983; Uhnoo *et al*, 1983; Brown, 1985; van Loon *et al*, 1985b) suggests that the provision of E1 functions alone is not sufficient to overcome growth restriction.

Secondly, only transcripts that map to the E1 region (0-12 m.u.) were detected in Ad41 infected non-permissive (HEF) cells. This indicates a block either at the start of delayed early transcription most likely due to inefficient transactivation of other early promoters by E1a products and/or a defect in E1b. It has been shown with prototype E1a mutants that the requirement for the E1a transactivating protein is not absolute and transcription rates from the early promoters gradually increase over an extended period of time (Nevins, 1981; Gaynor and Berk, 1983). Since no other Ad41 transcripts were detected even at later times in HEF cells, an E1 function in addition to deficient transactivation is likely to be involved. Here, the failure to produce early cytoplasmic RNAs seems to be due to a failure to transcribe the appropriate regions. Since transcription of all early regions requires a functional E1a it seems most likely that this gene is defective for transactivation in HEF cells.

The accumulation of transcripts from the Ad41 E1 region (0-12 m.u.) in semi-permissive cells was gradual and could be detected throughout infection. In general, transcription from all regions was very much delayed compared to that found in 293 cells. This could be explained in part by reduced transactivation of other early promoters by E1a products and therefore longer periods of time required for accumulating critical concentrations of certain products. In keeping with this it was found that 4 to 5 infectious virus particles are required to productively infect a Chang cell. Collaboration between a number of particles may therefore be necessary to attain critical levels of a certain product(s) to allow progression of the infectious cycle.

A defective E1a and/or the VA RNAI gene may be responsible for susceptibility of the subgroup F adenoviruses to lymphoblastoid interferon in Chang cells. Since defects in the individual functions do affect growth *in vitro,* inadequate functioning of either one or both of these genes may give rise to the Ad40 and Ad41 phenotypic traits seen in Chang cells. In the absence of a functional VA RNAI gene the cellular DAI kinase is activated (Schneider *et al*, 1985). This results in the phosphorylation of the translation initiation factor eIF-2α and the subsequent inhibition of protein synthesis. It would be interesting to test whether the growth defect in part may be due to the inadequate functioning of the VA RNAI gene by infecting a cell line that expresses a mutant eIF-2α (Davies *et al*, 1989) with the subgroup F adenoviruses. The mutant factor cannot be phosphorylated and in this way precludes the need for a functional VA RNAI gene in adenovirus infection. Although this particular cell line was derived from 293 cells, its use may provide some indication of whether infection can be enhanced by the presence of a mutant eIF-2α. The E1a 289-amino acid protein is thought to stimulate VA RNAI gene expression by modifying the cellular transcription factor TFIIIC (Hoefler *et al*, 1988). Reduced transactivation via this factor by the E1a protein may in this way indirectly affect VA RNA I activity.

7.1.2 DNA replication and packaging into virus particles

In Chang cells, Ad40 and Ad41 DNA synthesis was less efficient than that of Ad2. In Ad2, the E2 transcription unit encodes the proteins required for adenovirus DNA replication. E4 gene products play an important role, together with E1a products in the activation of the cellular transcription factor E2F (Babiss, 1989; Reichel *et al*, 1989; Raychaudhuri *et al*, 1990), which in turn activates the E2 transcription unit (reviewed in Nevins, 1991). It may be significant in this regard that no gene transcripts were detected in Ad41 infected Chang cells that hybridize to the region between 87.34 and 100 m.u., a region containing the tentative E4 transcription unit. Transcripts from this region were detected in 293 cells. This may be a result of

reduced transactivation of the E4 promoter by E1a products or a short half-life of E4 messages and therefore a failure to detect these. Alternatively, products of the E2 transcription unit itself or cellular factors necessary for DNA replication may be incapacitated. The latter possibility could be tested in a soluble *in vitro* DNA replication system (Challberg and Kelly, 1979). Unlike Ad41, Ad40 has the ability to replicate its DNA in HEF cells and to further sustain its genome over a number of passages. Further analysis of this phenomenon could reveal the specific viral-encoded function(s) responsible for this notable difference between the replicative abilities of Ad40 and Ad41 in HEF cells.

The proportion of Ad40 and Ad41 DNA synthesized in Chang cells that was packaged into virions was very much less than that found for Ad2. This may be a result of an earlier block affecting some or all subsequent functions as already mentioned, or due to a function associated specifically with packaging of DNA. Both Ad40 and Ad41 do not contain an encapsidation signal (consensus sequence: Brinkmann *et al*, 1983) at the left-hand end of the genome as found in other adenoviruses (Hammarskjöld and Winberg, 1980; Hearing and Shenk, 1983). DNA incorporation into capsids, however, is not absolutely dependent on the packaging sequence and other factors are thought to be involved (Kosturko and Vanech, 1986). Adenovirus 40 synthesized its DNA in HEF cells but no DNA was packaged into virions, implicating a late block in replication. Further delineation of the events leading to this abortive infection awaits results of future studies on the regulation of gene expression and DNA synthesis in Ad40 infected HEF cells.

Taken together, there are distinct differences in the capacity of Ad40 and Ad41 to replicate their DNA that imply, (i) the presence of a necessary cellular factor(s) or the critical concentration of such a factor(s) which is dependent on the cell type (e.g. Chang versus HEF cells), and (ii) a virus-dependent function(s) that is operational in Ad40 and not in Ad41 infected HEF cells.

It is interesting that most E1b-55K mutants synthesize DNA normally (Esche, 1986). E4 mutants that carry large deletions affecting most or all of the E4 ORFs have large DNA replication defects (Halbert *et al*, 1985; Bridge and Ketner, 1989; Weinberg and Ketner, 1986). It has been shown that Ad40 can complement the E4 defect in one such deletion mutant (Weinberg and Ketner, 1986) in coinfections of HeLa and vero cells, attaining levels comparable to wild-type Ad2 (Mautner and Mackay, 1991). A recent study with Ad5 E1b/E4 double mutants has suggested that the E1b-55K and E4-34K complex acts in parallel with the E4 ORF 3 product (116R protein) to permit normal DNA synthesis in infected cells (Bridge and Ketner, 1990). Neither of the mutant parents has been reported to have a notable DNA defect (Babiss and Ginsberg, 1984; Bridge and Ketner, 1989). The ability of Ad40 to replicate its DNA in HEF cells supports a defect in E1b-55K but not E4. In Ad41 infected HEF cells it would appear from preliminary transcription studies that a stage where such a E4 defect may become apparent is not reached.

7.1.3 Host protein shutoff

The late phase of adenovirus infection is marked by inhibition of host cell protein synthesis and preferential translation of viral mRNAs. No shut off of host protein synthesis could be detected in Chang or 293 cells, even though prominent viral bands could be detected in the latter. This has also been found in KB cells which complement the growth of Ad40 (Mautner *et al*, 1990). This suggests that the E1b-55K protein constitutively produced in 293 cells and KB18 or KB16 cells cannot complement Ad40 or Ad41 for inhibition of host protein shutoff.

A product of the Ad2 E4 region, the 34K protein, forms a complex with the E1b-55K protein during infection (Sarnow *et al*, 1984). Defects in either of these functions results in similar phenotypic characteristics, namely defective synthesis of viral late mRNAs and proteins and inefficient shut off of host macromolecular synthesis (Challberg and Ketner, 1981; Weinberg and Ketner, 1983; Babiss and

Ginsberg, 1984). It has been shown that Ad40 can complement the E4 defect in an Ad2 deletion mutant in coinfections of HeLa and Vero cells (Mautner and Mackay, 1991). Other functions known to inhibit macromolecular synthesis include the fibre antigen (Levine and Ginsberg, 1967), and both the VA RNAI and DAI kinase as suggested recently by O'Malley *et al* (1989). The subgroup F adenoviruses are unique amongst human adenoviruses in having two fibre genes (Pieniazek *et al*, 1990; Kidd *et al*, 1992) which are known to be expressed, at least in Ad40 (Kidd *et al*, 1992). However the actual levels of expression of both fibre proteins have not been assessed. O'Malley *et al* (1989) have proposed that the protein synthetic machinery in adenovirus infected cells is divided into two isolated compartments, one containing VA RNAI and viral mRNA and the second containing cellular (and perhaps viral early) mRNA species but no VA RNAI. This would account for a role both in the selective translation of viral mRNAs and shut off of host protein synthesis by VA RNAI and DAI.

7.1.4 Cellular factors and their role in viral replication

Any explanation of the defectiveness of subgroup F adenoviruses has to take into account the fact that some cells not deliberately transformed with adenovirus sequences can support their replication to some extent, and others (notably HEF cells) are totally refractory to growth. Cells such as Chang conjunctiva and tertiary monkey kidney possibly provide some necessary viral function(s) or a cellular counterpart of some viral product. Alternatively, the cellular function expressed might derepress a viral product. In either case the growth permissiveness of a cell type would then be governed by the degree of expression of such a function.

The permissive state or the degree of permissiveness of a cell for a particular virus can be altered by the presence of another infecting virus with the ability to provide a helper function for growth, either directly by providing a necessary viral function in *trans*, or indirectly by modifying some cellular factor. Such a change in

permissiveness of Chang and HEF cells to Ad41 infection was shown to occur in the presence of Ad2.

Recently, a number of cellular transcription factors have been described that are required for the expression of various adenovirus genes. Some require E1a gene expression for their activation. These include TFIIIC which stimulates VA RNAI gene expression (Hoeffler *et al*, 1988) and TFIID (TATA-binding protein) which is believed to be involved in E1a modulation of E1b expression (Wu *et al*, 1987). One factor that does not require E1a expression for its activation is the ATF-E4F factor, which appears to be required for E1a, E2a, E3, and E4 expression (SivaRaman *et al*, 1986; DeVaux and Kedinger, 1987; Hurst and Jones, 1987; Lee and Green, 1987). Transcription factor E2F has already been mentioned (7.1.2). Cells non-permissive for Ad40 and Ad41 may have limiting concentrations of some such endogenous transcription factor(s). Since reduced transactivation by E1a has been shown for these viruses, the inability of the E1a transactivating product to adequately modify a preexisting cellular factor may be the most crucial step in initiation of transcription from a specific promoter(s). In this regard, the Ad40 E1a gene may not be as incapacitated as that of Ad41 in HEF cells. In certain cell types the E1a gene function may not be as necessary as in others. Adenovirus 5 E1b mutants have been shown to grow in primary embryo kidney cells while being defective for growth in continuous cell lines (Harrison *et al*, 1977; Bernards *et al*, 1986).

The state of differentiation of a cell has been shown to affect the presence of certain cellular factors. For example, undifferentiated F9 teratocarcinoma stem cells, which can transcribe the Ad5 E2 promoter in the absence of E1a, contain an E2F factor that binds to the E2 promoter elements. Upon differentiation, this E2F binding activity is lost together with a decrease in the ability to transcribe the E2 early promoter in the absence of E1a (Reichel *et al*, 1987). Imperiale *et al* (1984) have shown that cells that have high uninduced levels of expression of the heat shock gene allow the expression of early adenovirus genes in the absence of the E1a gene

product. The constitutive expression of heat shock protein in F9 stem cells and loss of this expression upon differentiation also correlates with early gene expression in the absence of, or requiring the presence of E1a, respectively.

A cellular activity which functionally substitutes for E1a in transactivation of both the E2a early promoter and E1b promoter has recently been found in HepG2 cells, a human hepatocellular carcinoma cell line (Spergel and Chen-Kiang, 1991). These cells supported productive infection of E1a deletion mutant virus and full complementation of infection upon induction by interleukin-6 (IL-6). This is in contrast to the situation in mouse teratocarcinoma cells where the E2F activity is lost upon differentiation (Reichel *et al*, 1987). The cellular factor present in HepG2 cells that can substitute for E1a in transactivation has recently been identified as NF-IL6, an IL-6 regulated human nuclear factor (Spergel *et al*, 1992). Adenovirus 40 and Ad41 have recently been shown to grow well in PLC/PRF/5 cells, another hepatocellular carcinoma cell line (Grabow *et al*, 1992). Assuming defective E1a functioning in these cells it is very likely that the presence of a similar factor in PLC/PRF/5 cells may complement Ad40 and Ad41 growth.

Intracellular second messenger molecules, such as cyclic AMP (cAMP), are synthesized in response to the binding of peptide hormones to cell surface receptors (Jungmann and Russell, 1977). Hormonal responses which result include transcriptional changes in gene expression (Buchler *et al*, 1988; Grove *et al*, 1987). Increased intracellular levels of cAMP induces the expression of a number of cellular genes (Roesler *et al*, 1988). The cellular transcription factor, ATF, binds to viral early promoter regions of E1a, E2a, and E4 and cAMP response elements (CRE) of cellular genes (Jones *et al*, 1988). Expression from viral promoters can be induced by cAMP (Leza and Hearing, 1989). Induction by E1a and cAMP were shown to act via independent mechanisms. This provides a further example of how expression of certain viral genes can be induced by an external stimulus in the absence of E1a

transactivation. This may be particularly important in the natural host cell of the subgroup F adenoviruses.

A defective VA RNAI gene may in part be responsible for the subgroup F adenovirus sensitivity to IFN. Activated DAI kinase in KB cells infected with the *dl*331 mutant (defective in VA RNAI expression) is insufficient to severely inhibit protein synthesis of the mutant which grows to wild-type levels (Kitajewski *et al*, 1986b). Thus, a defective VA RNAI gene would go unnoticed in these cells. On the other hand, this mutant grows very poorly in 293 cells as a result of the rapid appearance of active kinase and the subsequent inhibition of viral protein synthesis. This further demonstrates distinct cell-type related differences in response to adenovirus infection.

The restricted growth of the subgroup F adenoviruses may lie in their inability to respond to, or utilize the transcription factors that are available in different cell types. This lack of growth versatility in human cell lines is reminiscent of the growth phenotypes displayed by other human adenoviruses when infecting cells of other species.

It is apparent that the growth defectiveness of the subgroup F adenoviruses cannot easily be explained by one deficient function and appears to be a multi-factorial phenomenon. Further support for this has come from a recent study by Brown *et al* (1992) who found that factors such as the block in release of progeny virus and a high particle-to-infectious unit ratio contribute to poor growth of Ad40 and Ad41 in cell culture. These authors concluded that separate blockages exist in subgroup F adenovirus replication which is in keeping with the observations and conclusions of this thesis.

7.2 The question of subgroup F adenovirus persistence

It is possible that as with adenoviruses associated with respiratory disease, the subgroup F adenoviruses may infect the individual at an early age and then persist for life. Seroepidemiological studies have shown that these viruses are widespread (Kidd *et al*, 1983). If the subgroup F adenoviruses do persist in some cell population in the gastrointestinal tract, it might be expected that certain genes may be involved in maintaining the genome in a state that either results in the continuous production of small amounts of virus (persistence) or a form that is harboured and can be reactivated by certain factors (latency).

These viruses have been associated with diarrhoeal disease in infants and young children, but appear not to be associated with disease in adults (Richmond *et al*, 1979; Chiba *et al*, 1983). It is not known whether these viruses are reactivated *in vivo* after initial infection. One study has, however, addressed the question of prolonged and intermittent shedding of adenoviruses in stool using a sensitive two-step PCR method for detection (Allard *et al*, 1992). General primers that could detect representatives of all adenovirus subgroups and primers specific for subgroup F adenoviruses were used. Interestingly, the subgroup F adenoviruses were not detected in healthy children or adults or in adults with diarrhoea, whereas adenoviruses in general were frequently detected in these groups. The type specific PCR assay developed in the present study may prove useful in assessing subgroup F adenovirus shedding in rural populations in South Africa where these viruses have been shown to be as prevalent as rotaviruses (Tiemessen *et al*, 1989). The sensitivity of this test, however, largely precludes its use in identifying a subgroup F adenovirus as the causal agent of diarrhoea purely on the basis of a positive PCR test.

The subgroup F adenoviruses pose an interesting challenge with respect to studies on molecular aspects of disease. Since the molecular basis of differences in pathogenicity between adenoviruses causing respiratory and other syndromes and

adenoviruses that are tropic for cells of the gastrointestinal tract is unknown, there is a need for research in this area. One interesting prospect involves the finding that the subgroup F adenoviruses code for a second fibre gene, the implications of which are important with respect to (i) virus attachment and entry strategy, and (ii) usage of coding capacity in a portion of the genome hitherto associated with early (E3) function. In addition, the strategies employed by these viruses in their interaction with the mucosal immune system may prove interesting.

Work in this study has demonstrated the potential for whole genome persistence in cells that the virus fails to infect productively. By definition, this virus-cell interaction could not be defined by the term latency as this implies reversible non-productive infection. Infection could not be reactivated by Ad2, which is normally capable of complementing Ad40 to a stage of late antigen synthesis. The involvement of other factors in reactivation cannot however be excluded. Further study of the difference in the ability of Ad40 and Ad41 to sustain their genomes in this model may allow some insight into the mechanisms employed by Ad40 in the establishment and maintenance of this unusual virus-cell interaction. Findings with other adenovirus serotypes show the presence of episomal DNA and possible integrated sequences in the lymphoid tissue and peripheral blood lymphocytes from apparently normal individuals (Green *et al*, 1979; Horvath *et al*, 1986), a situation not unlike Ad40 genome persistence described here. The Ad40-HEF cell interaction may thus provide a system in which this phenomenon could be studied. This may conceivably allow the identification of factors or specific genes that may be involved in maintaining a reservoir of apparently episomal DNA for long periods of time and its possible role in adenovirus persistence.

The events leading to gastrointestinal disease as a consequence of subgroup F adenovirus infection of intestinal mucosal cells requires evaluation in a model system that maintains the host cell in its normal environment. This would greatly aid in the

elucidation of the pathophysiology, the unique biology, and the immunology of subgroup F adenovirus infection *in vivo*.

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