Adenovirus Type 40 Host Range in Tissue Culture: Replication and Gene Expression in INT407 Cells

by

Robina R. Ullah

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in The Faculty of Science at the University of Glasgow.

Institute of Virology,
Church Street,
Glasgow,
G11 5JR.

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Summary.

The enteric adenoviruses show a restricted host range in tissue culture. Adenovirus type 40 (Ad40) can not be passaged in conventional human cell lines used for other adenovirus serotypes, but will grow in cells constitutively expressing Ad2 or Ad5 E1B 55K function (KB16 & 293 cells respectively). Preliminary experiments showed that Ad40 could also be propagated in INT407 cells which were not known to express E1 proteins. The aim of the work described here was to characterise the growth of Ad40 in INT407 cells by examining virus replication and gene expression compared to other permissive (KB16) and semi-permissive cell lines (HeLa & A549).

A timecourse of DNA replication confirmed that INT407 cells were permissive for Ad40. The pattern of DNA replication was similar on KB16 and INT407 cells although the yield from KB16 cells was slightly higher than INT407 cells; the lowest yield was from HeLa. In addition INT407 cells could partially complement the growth of an Ad2/5 55K mutant virus. These results suggest that E1B 55K may be complemented or that the requirement for 55K may vary in INT407 cells.

The kinetics of Ad40 growth were studied by fluorescent focus assay using a monoclonal antibody against Ad5 hexon. Ad40 exhibited one hit kinetics on permissive cells (INT407 & KB16) and two hit kinetics on semi-permissive cells (A549 & HeLa). There was a marked difference in the number of productively infected cells between cell types with fewer cells productively infected at 48h p.i. on semi-permissive cell lines.

The entry events of the Ad40 replicative cycle (i.e. attachment and internalisation) were investigated to determine if any defects at this stage might contribute to the Ad40 growth phenotype on the different cell lines. A similar number of Ad40 particles attached and were internalised on INT407 and HeLa cells. These results could not explain why INT407 cells were more permissive for Ad40 growth than HeLa cells. In a parallel

experiment the attachment and internalisation of dl309 was found to be comparable to Ad40 on these cell lines.

In order to explain the Ad40 growth phenotype in the different cell types E1B gene expression was analysed by Northern blotting and S1 nuclease digestion on the different cell lines. E1B 22S mRNA was observed at early times before the onset of DNA replication in INT407 and KB16 cells, with the 14S mRNA predominantly late in infection in these cell types. In HeLa cells E1B mRNA was detected coincident with the onset of DNA replication at much reduced levels. A similar pattern to HeLa was seen on A549 cells. The pattern of E1B transcription on INT407 cells was comparable to that reported previously on KB16 cells which is similar to Ad12 with 14S, 22S and 9S mRNA.

The lower levels of E1B mRNA and later detection in HeLa cells may indicate that the transcription from the E1B promoter is inefficient or absent early in infection in these cells. Even in permissive cells Ad40 E1B mRNA transcription is poor compared to Ad5 which may indicate inefficient promoter usage. Therefore the Ad40 E1B transcriptional regulatory region in particular the Sp1 binding site was studied by gel retardation assay. The ability of Ad40 and Ad2 GC box sequences (Sp1 binding sites) to bind either partially purified Sp1 or proteins from INT407 and HeLa extracts were compared. The Ad40 Sp1 binding site bound the Sp1 transcription factor but with lower affinity than the Ad2.

This thesis is dedicated to my parents,
and to the memory of my loving Nanajan and Nanijan,
M. Hanif and Asia Begum.

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Abbreviations.

A	adenine
Ad	adenovirus
Adpol	adenovirus DNA polymerase
APS	ammonium persulphate
ara-C	cytosine arabinoside
ATP	adenosine-5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
C	cytosine
C-	carboxy
cAMP	cyclic adenosine-5'-monophosphate
CAT	chloramphenicol acetyl transferase
CAV	canine adenovirus
cDNA	complementary DNA
Ci	Curie(s)
cpe	cytopathic effect
cpm	counts per minute
Da	Daltons
DBP	DNA binding protein
dATP	2'-deoxyadenosine-5'-triphosphate
dC	deoxy cytosine
dCMP	2'-deoxycytidine-5'-monophosphate
DEPC	diethyl pyrocarbonate
dI	deoxy inosine
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DTT	dithiothreitol
DW	distilled water
E	early (gene)
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus

EDTA	sodium ethylenediamine tetra-acetic acid
EM	electron microscopy
FCS	foetal calf serum
FITC	fluorescein isothiocyanate conjugated
g	gram(s)
G	guanine
h	hour(s)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HSV	herpes simplex virus
IE	immediate early
Ig	immunoglobulin
ITR	inverted terminal repeat
k	kilo
K	1000-dalton molecular weight
kb	kilobase
l	litre
L	Late (gene)
m	milli
M	molar
μ	micro
mAb	monoclonal antibody
MAV	mouse adenovirus
mi	mock infected
min	minute(s)
ml	millilitre(s)
MLP	major late promoter
mm	millimetre
mM	milliMolar
moi	multiplicity of infection
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
m.u.	map unit
n	nano
N-	amino
nt	nucleotide
OD	optical density
ORF	open reading frame
^{32}P	phosphorous-32 radioisotope

PBS	phosphate buffered saline
pfu	plaque forming unit(s)
p.i.	post-infection
poly-A	polyadenylic acid
pTP	precursor terminal protein
Rb	retinoblastoma gene product
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SAV	simian adenovirus
SDS	sodium dodecyl sulphate
sec	seconds
SV40	simian virus 40
T	thymine
TAF	TBP-associated factor(s)
TAV	tupaia adenovirus
TBP	TATA-box binding protein
TEMED	N, N, N', N'-tetramethylethylene diamine
Tris	tris (hydroxmethyl) aminomethane
tRNA	transfer RNA
UV	ultra violet
V	volts
v	volume
vol	volume
VZV	varicella zoster virus
W	watts
w	weight
wt	wild type

1. INTRODUCTION.

1.1. The Adenoviridae.

1.1.1. Classification.

The Adenoviridae family is divided into two genera, Mastadenovirus and Aviadenovirus. The Mastadenovirus genus includes: human, simian, bovine, equine, porcine, ovine and canine viruses. Human adenoviruses were first discovered by Rowe *et al.*, (1953), in explants of adenoid tissue. There are currently 47 serotypes of human adenoviruses (Hierholzer *et al.*, 1991) and possibly more, with two candidate serotypes identified by Schnurr and Dondero *et al.*, (1993). The human adenoviruses were classified into six subgenera (A-F) based on the following criteria: immunological properties, oncogenicity in rodents, DNA homologies and morphological properties (Table 1.1.1.). Human adenoviruses are associated with a number of infections including respiratory, ocular, urinary and gastrointestinal, although symptoms are rarely fatal. Adenoviruses are widely used as model systems in molecular biology, and their use as important biological tools (reviewed in Horwitz, 1990) particularly as vectors in gene therapy has been extensively reported (reviewed in Haddada *et al.*, 1995). Much of our current understanding of human adenoviruses comes from extensive studies with Ad2 and the closely related Ad5 serotypes and this is reflected in the following sections.

1.1.2. Morphology.

Adenoviruses are nonenveloped, icosahedral particles, approximately 50-80nm in diameter (Horne *et al.*, 1959). The virion has a dense central core comprising of a tightly packed DNA-protein complex (Nermut, 1984) and an outer protein capsid (see Fig 1.1.1a.). The capsid is composed of 252 capsomers, consisting of 240 hexons (virion protein II) on the facets and 12 penton bases (virion protein III) at the vertices (Ginsberg *et al.*, 1966). Fibre (virion protein IV) is noncovalently attached to each penton base.

Table 1.1.1. Properties of Human Adenovirus Serotypes of Subgroups A-F (Modified from Wadell et al., 1994).

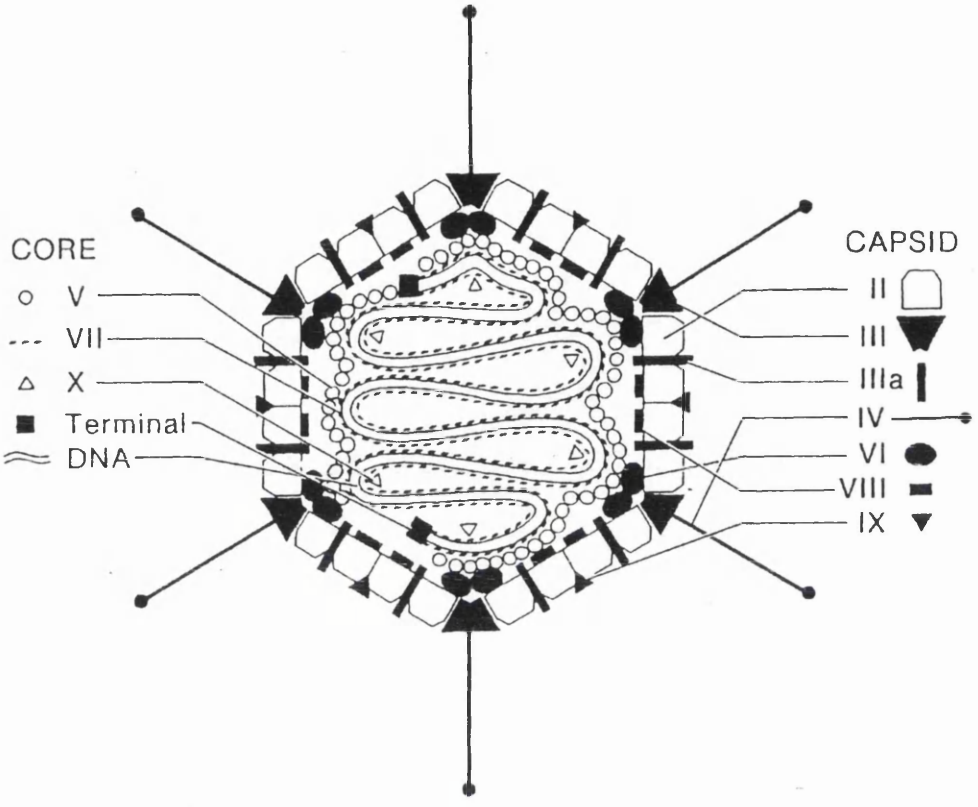
Sub-group	Serotype	DNA Homology % Intra-subgroup	DNA Homology % Inter-subgroup	G+C (%)	Length of Fibres (nm)	Oncogenicity in newborn hamsters	Tropism/ Symptoms
A	12, 18, 31	48-69	8-20	48	28-31	High (tumours in most animals in 4 months)	Cryptic enteric infection
B	3, 7, 11, 14, 16, 21, 34, 35	89-94	9-20	51	9-11	Weak (tumours in few animals in 4-18 months)	Respiratory disease Persistent kidney infection
C	1, 2, 5, 6	99-100	10-16	58	23-31	Nil	Respiratory disease persists in lymphoid tissue
D	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42, 47	94-99	4-17	58	12-13	Nil	Kerato-conjunctivitis
E	4		4-23	58	17	Nil	Conjunctivitis Respiratory disease
F	40, 41	62-69	15-22	51	28-33 (IV-1) 18-20 (IV-2)	Nil	Infantile diarrhoea

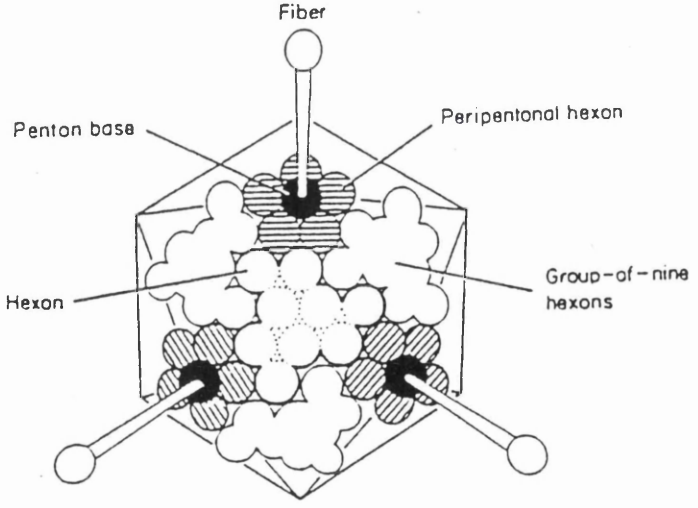
Figure 1.1.1a. A Schematic View of the Adenovirus Particle.

A schematic view of the adenovirus particle based on current understanding of its polypeptide components and viral DNA. No real section of the icosahedral virion would contain all these components. (Reproduced from Stewart and Burnett, 1995).

Figure 1.1.1b. A Diagram of the Icosahedral Adenovirus Capsid.

The 240 copies of the hexon trimer are organised so that 12 lie on each of the 20 facets. The penton complex formed from the penton base and fibre lies at each of the 12 vertices. The central 9 hexons in a facet are cemented together by 12 copies of polypeptide IX. Dissociation of the virion releases the pentons, peripentonal hexons and the planar groups-of-nine hexons. (Reproduced from Burnett et al., 1985).


CORE
V
VII
X
Terminal
DNA
CAPSID
II
III
IIIa
IV
VI
VIII
IX


Fiber
Penton base
Peripentonal hexon
Hexon
Group-of-nine hexons

Hexon is the major structural component of the virus capsid; it is a trimer of three identical polypeptide chains (Grutter and Franklin, 1974; Akusjärvi *et al.*, 1984) and its structure has been solved to 2.9Å resolution by X-ray crystallography (Athappilly *et al.*, 1994). The overall shape of the trimeric hexon molecule consists of a pseudo-hexagonal base with a triangular top. With mild disruption of virions, hexons were subdivided depending on their location in the capsid, into peripentonal hexons or groups of nine hexons (GONs) (see Fig 1.1.1b.). GONs make up the facets with peripentonal hexons the link between the facets and pentons. The minor capsid protein, polypeptide IX was shown to extend along hexon-hexon interfaces of GONs and is involved in stabilising the capsid (van Oostrum and Burnett, 1985; Furcinitti *et al.*, 1989; Stewart *et al.*, 1993). Hexon has a complex array of antigenic determinants, including those with genus, type, intersubgenus and intrasubgenus specificities (Norrby 1969a, b; Norrby and Wadell, 1969). Type-specific determinants of hexon have been demonstrated on the surface of the virion (Willcox and Mautner 1976a, b; Toogood *et al.*, 1992) and group-specific determinants appear to be buried internally (Mautner and Willcox, 1974; Norrby, 1969a; Willcox and Mautner, 1976a, b).

The complex of penton base and fibre polypeptide is called the penton capsomere. The penton base is composed of five subunits (van Oostrum and Burnett, 1985; Furcinitti *et al.*, 1989; Stewart *et al.*, 1993), and the fibre has been shown to comprise of three subunits each of 62K (Devaux *et al.*, 1990; Stouten *et al.*, 1992). The fibre consists of three domains (Green *et al.*, 1983), the amino terminal tail that interacts with the penton base, an intervening shaft region and the carboxy terminus which contains type-specific antigenic determinants and is responsible for binding to the cell surface receptor (Pettersson *et al.*, 1968; Hughes and Mautner, 1973; Devaux *et al.*, 1990; Ruigrok *et al.*, 1990; Louis et al 1994). Most human adenoviruses have one fibre gene with the exception of Ad40 and Ad41 which have two. These fibre genes encode proteins of different shaft length and different knob sequence (Pieniazek *et al.*, 1989; Kidd and Erasmus, 1989; Kidd *et al.*, 1990, 1993; Davison *et al.*, 1993; Yeh *et al.*, 1994).

The virion proteins IIIa, VI, VIII and IX are also associated with the major capsid proteins and are thought to act as "cement proteins" interacting with each other, hexons, pentons and possibly some of the core proteins (Stewart *et al.*, 1993).

The core consists of genomic DNA tightly complexed with two highly basic proteins, virion proteins VII and V, also associated are the virion proteins VI and μ (Russell and Precious, 1982). Protein V also binds penton base and is associated with protein IIIa (Everitt *et al.*, 1975). The organisation of adenovirus DNA inside the capsid is not well understood. It has been suggested that the DNA is organised into 12 globular domains (Brown *et al.*, 1975; Newcombe *et al.*, 1984), which would fit into the 12 vertices of the icosahedral capsid of the virus. A second model, involves the DNA wound around protein VII in the form of nucleosomes (Corden *et al.*, 1976; Chatterjee *et al.*, 1986). Wong and Hsu (1989) suggested the viral DNA is organised into eight supercoiled loops, which are anchored to the centre of the virus core. This model was consistent with the results of Brown *et al.*, (1975) and Newcombe *et al.*, (1984).

1.1.3. Genome Structure.

The adenovirus genome consists of a linear double stranded DNA molecule. The deoxycytidine at the 5' end of each strand is covalently linked to a 55K terminal protein (TP), through a serine phosphoryl bond (Robinson *et al.*, 1973; Rekosh *et al.*, 1977; Desideiro and Kelly, 1981). This protein is generated late in infection by the action of a virus protease (Webster *et al.*, 1993), on an 80K precursor terminal protein (pTP). The ends of the virus genome have an inverted terminal repetition (Garon *et al.*, 1972; Wolfson and Dressler, 1972). The inverted terminal repeats (ITRs) of human adenoviruses vary in length from 102 to 165bp, depending on the serotype (Tamanoi and Stillman, 1983; Shinagawa *et al.*, 1987). Located within the first 51bp of the ITR is the origin of DNA replication. The terminal 18bp represent a minimal origin of DNA replication and contained within this is a 10bp region (nt 9 to 18), which is highly conserved among different adenovirus serotypes (Stillman *et al.*, 1982; Tamanoi and Stillman, 1983; Wides *et al.*, 1987; Harris and Hay, 1988). This minimal origin has been

found to bind a heterodimer of the virion proteins, pTP and adenovirus DNA polymerase (Miralles *et al.*, 1989; Chen *et al.*, 1990; Temperley and Hay, 1992). Separated from the minimal origin by a defined spacer region (Wides *et al.*, 1987), are binding sites for two host cell proteins, these factors stimulate the initiation of replication by different mechanisms (reviewed in Hay *et al.*, 1995; van der Vliet, 1995), and will be discussed in more detail in section 1.1.4.3.

The complete nucleotide sequences for four human adenoviruses are now available: Ad2 (Roberts *et al.*, 1986), Ad5 (Chroboczek *et al.*, 1992), Ad40 (Davison *et al.*, 1993) and Ad12 (Sprengel *et al.*, 1994). The organisation of the Ad2 genome (35,937bp) is shown in Figure 1.1.2. By convention the genome is divided into 100 map units (m.u.) and the two strands denoted r and l for rightward and leftward transcription; genes are expressed from both strands. The viral genes are expressed in two broadly defined phases which are termed early and late, separated by the onset of DNA replication; a few genes are expressed at intermediate times (reviewed in Akusjärvi *et al.*, 1986). E1A genes are the first to be expressed during infection and are sometimes referred to as "immediate early" genes (Berk, 1986). The late genes are transcribed from the same promoter, the major late promoter (MLP). All the major late mRNAs have a common 201nt long 5' leader sequence which consists of three short exons (Berget *et al.*, 1977; Akusjärvi and Pettersson, 1979; Shaw and Ziff, 1989). The replication cycle can therefore really be divided into four stages: Immediate Early, Early, Intermediate and Late.

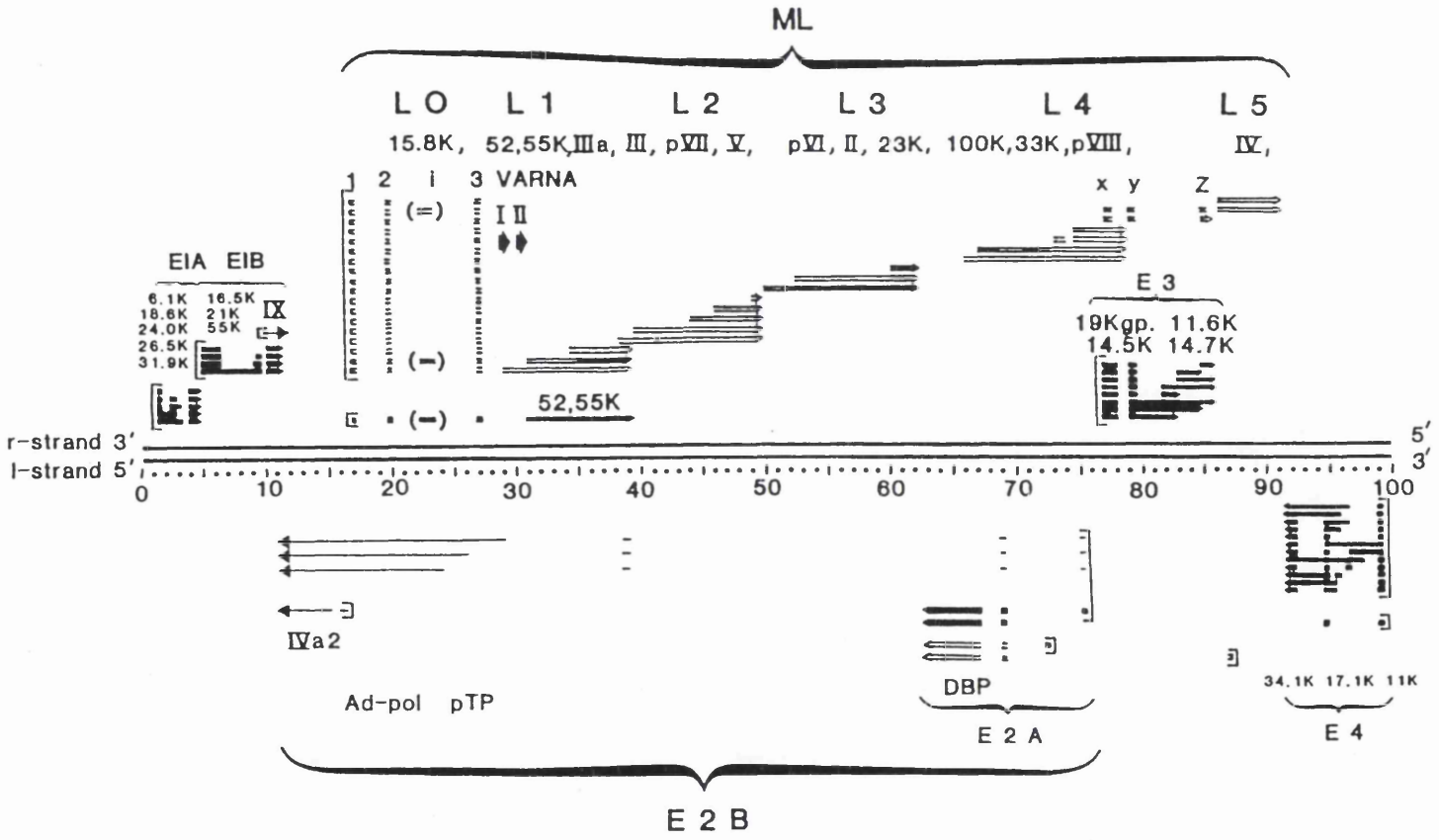
1.1.4. The Lytic Cycle.

1.1.4.1. Viral Entry into Cells.

This section outlines how adenoviruses enter cells, however a more detailed description of attachment and internalisation can be found in section 1.2. Initial attachment of adenoviruses is mediated by the fibre protein (Philipson *et al.*, 1968; Henry *et al.*, 1994; Louis *et al.*, 1994). Although the cell receptor is unknown, some candidate cellular proteins have been shown to bind fibre (Hennache and Boulanger, 1977;

Figure 1.1.2. Organisation of the Ad2 Genome.

The parallel lines indicate the linear duplex DNA genome, which is divided into 100 map units (m.u.). Rightward and leftward transcription is indicated by r and l, respectively. Vertical brackets indicate position of promoter sites and the arrow heads show the location of 3' ends. The split arrows indicate the spliced structure of the mRNAs. Thick lines represent mRNAs expressed early after infection and thin lines mRNAs expressed at intermediate times. The open arrows show sequences present in mRNAs expressed late after infection. Polypeptides assigned to different regions are also shown (Reproduced from Akusjärvi and Wadell, 1993).


ML
L O
L 1
L 2
L 3
L 4
L 5
15.8K, 52,55K,IIIa, III, pVII, V, pVI, II, 23K, 100K,33K,pVIII, IX,
1 2 i 3 VARNA
(=)
(−)
(−)
I II
x y
z
E3
19Kgp. 11.6K
14.5K 14.7K
52,55K
EIA EIB
6.1K 16.5K
18.6K 21K
24.0K 55K
26.5K
31.9K
IX
r-strand 3′
l-strand 5′
5′
3′
0 10 20 30 40 50 60 70 80 90 100
IVa2
Ad-pol pTP
DBP
E 2 A
34.1K 17.1K 11K
E 4
E 2 B

Svensson *et al.*, 1981; Belin and Boulanger, 1993). Adenoviruses from subgroups B and C use different cell receptors for virus attachment (Defer *et al.*, 1990; Di Guilmi *et al.*, 1995; Stevenson *et al.*, 1995; Gall *et al.*, 1996).

After attachment to the fibre receptor, virus particles are rapidly internalised into clathrin-coated pits by receptor-mediated endocytosis (Chardonnet and Dales, 1970; Fitzgerald *et al.*, 1983; Varga *et al.*, 1991). The fibre protein dissociates from the virion particle early in the entry pathway (Greber *et al.*, 1993). In Ad2, internalisation is mediated by the penton base protein binding to specific αv integrins (Wickham *et al.*, 1993; Bai *et al.*, 1993).

Viral particles penetrate into the cytoplasm from endosomes, this process requires a low pH environment as agents which block this environment (in endosomes) also block adenovirus release into the cytoplasm (Blumenthal *et al.*, 1986; Greber *et al.*, 1993). Stepwise uncoating of the viral particle continues through the cytoplasm (Greber *et al.*, 1993). When virions reach the nucleus, the viral core (DNA and virion proteins: V, VII, TP and μ) enters via nuclear pores leaving many of the other virion proteins in the cytoplasm (Morgan *et al.*, 1969; Chardonnet and Dales, 1970; Greber *et al.*, 1993).

1.1.4.2. Transcription.

The adenovirus genes are transcribed from nine initiation sites by cellular RNA polymerase II (Price and Penman, 1972), except for VA (virus associated) RNAs which are transcribed by RNA polymerase III. Transcription occurs in a temporally regulated manner (Nevins *et al.*, 1979; Glenn and Ricciardi, 1988) in the nucleus of the infected cell. Early genes are grouped in six transcription units (E1A, E1B, E2A, E3, E4 and L1 early). The first genes expressed are from the E1A region and are referred to as 'immediate early'. The larger E1A gene product (289R) transactivates the other early transcription units and prepares the cell for efficient viral DNA replication. At intermediate times, close to the onset of DNA replication, intermediate genes are transcribed (ppIX, IVa2, VAI RNA and VAII RNA). Following DNA replication the late genes (L1-L5) are transcribed and these encode mainly viral structural proteins.

Early Transcription

E1A genes are expressed from the r-strand (see Figure 1.1.2) and are situated between 1.5-4.5 m.u. in Ad2 (Jones and Shenk, 1979b). There are five known E1A mRNAs (Berk and Sharp 1978; Chow *et al.*, 1979; Stephens and Harlow 1987; Ulfendahl *et al.*, 1987), named: 13S, 12S, 11S, 10S, and 9S based on their respective sedimentation coefficients. They share 5' and 3' ends but are differentially spliced (see Fig 1.3.1.). The two larger mRNAs, 13S and 12S, are the predominant species early in infection, whilst the 9S mRNA is the most abundant at late stages after infection (Spector *et al.*, 1978; Chow et al 1979). Proteins of 243 and 289 amino acids (243R & 289R) are transcribed from 12S and 13S mRNAs, respectively. These proteins differ by an internal 46 amino acids region, present only in the 289R protein (Perricaudet *et al.*, 1979). The larger protein is primarily responsible for transactivation and induces the transcription of other early regions (Jones and Shenk 1979b; Ricciardi *et al.*, 1981; Montell *et al.*, 1982; reviewed in Berk 1986). The E1A region is described in more detail in section 1.3.

The E1B transcription unit lies downstream of the E1A region on the r-strand between 4.6-11 m.u. (in Ad2). A more detailed description of structure and function from this region can be found in section 1.4. The E1B region produces two major mRNAs 22S and 13S, sharing common 5' and 3' ends (Berk and Sharp 1978); both these mRNAs are derived from a common precursor mRNA by alternative splicing (Pettersson *et al.*, 1983). The 22S mRNA codes for a 19K protein using the first AUG and a 55K protein in a different reading frame via the second AUG, in addition the 13S mRNA also encodes the 19K protein (Bos *et al.*, 1981). In Ad5, 22S mRNA is most abundant at early stages after infection, whereas 13S levels increase at intermediate times (Glenn and Ricciardi, 1988). Two minor mRNAs 14S and 14.5S have also been described, they are similar to 13S mRNA but have an additional third exon (Virtanen and Pettersson, 1985). These mRNAs encode the 19K protein and 55K related proteins (Anderson *et al.*, 1984; Virtanen and Pettersson, 1985).

Along with these mRNAs, a 9S species is transcribed from its own promoter within the E1B region. It encodes the structural polypeptide IX which is associated with

the capsid (Furcinitti *et al.*, 1989; Stewart *et al.*, 1993), and is expressed at intermediate times after infection.

The E2 region is transcribed from the l-strand and lies between 75.4 and 11.3 m.u. (Chow *et al.*, 1979). It varies from other adenovirus transcription units by using two alternative promoter sites, at different times in infection. Transcripts from the E2 region use one of two polyadenylation sites (poly-A), generating two major classes of mRNAs: E2A or E2B mRNAs. Early in infection E2A transcripts are expressed from a promoter at 75.4 m.u. and following the early to late phase transition mRNAs are transcribed from a promoter at 72.2 m.u. (Nevins *et al.*, 1979; Glenn and Ricciardi, 1988). The E2A mRNAs terminate using the poly-A site at 62.4 m.u., whereas E2B mRNAs bypass this poly-A site and terminate at 11.3 m.u. (Stillman *et al.*, 1981).

The E2A gene encodes a 72K single-stranded DNA binding protein (DBP) (Lewis *et al.*, 1976). DBP is essential for viral DNA replication (Rice and Klessig, 1985; Stuvier and van der Vliet, 1990; Mul and van der Vliet, 1993) and is involved in the regulation of viral gene expression (Klessig and Grodzicker, 1979; Babich and Nevins, 1981). DBP also regulates its own synthesis and can enhance transcription from the E1A, E2 (early) and major late promoters (Morin *et al.*, 1989; Zijderveld *et al.*, 1994).

Three mRNAs are transcribed from the E2B region (Stillman *et al.*, 1981; Shu *et al.*, 1988). The proteins pTP and adenovirus DNA polymerase (Adpol) have been assigned to two of these mRNAs, and they are both required for the initiation of adenovirus DNA replication. The 80K protein pTP acts as a primer for DNA replication (Smart and Stillman, 1982; Salas, 1991). This protein is cleaved by the 23K viral protease late in infection, resulting in the 55K terminal protein (TP) (reviewed in Challberg and Kelley, 1989; Hay *et al.*, 1995). The 140K protein Adpol is required for initiation and elongation in DNA replication (see Hay *et al.*, 1995; van der Vliet, 1995 and references therein).

The E3 region is transcribed from the r-strand between 76.8 and 85.9 m.u. This region is not essential for adenovirus replication in tissue culture cells, it is however conserved among adenovirus serotypes (reviewed in Wold *et al.*, 1995) indicating it has an important role *in vivo*. There are approximately nine overlapping mRNAs which arise

by alternative splicing of a common pre-mRNA precursor, and are polyadenylated at one of two sites E3A or E3B. (Chow *et al.*, 1979; see Wold *et al.*, 1995 and references therein). Subgroup C adenoviruses are predicted to encode nine proteins, seven of which have been identified, but functions have been assigned to only five proteins so far (reviewed in Wold *et al.*, 1995; Tollefson *et al.*, 1996).

E3 proteins are involved in modulating the host immune response to adenovirus infection, by a number of different mechanisms. One of these involves the E3 19K glycoprotein (gp19K) an abundant transmembrane protein which is retained in the endoplasmic reticulum (ER) (Pääbo *et al.*, 1987). In the ER gp19K binds to Class I antigens of the major histocompatibility complex (MHC) and thus interferes with their transport to the cell surface (Sester and Burget, 1994). Class I antigens complex with viral peptides at the cell surface and act as a signal for virus- specific cytotoxic T lymphocytes (CTL) to lyse the infected cell. The result of the retention of Class I antigens in the ER is that cells are protected from lysis by adenovirus specific CTL (Burget *et al.*, 1987; Cox *et al.*, 1991).

The second mechanism involves E3 proteins (14.7K, 14.5K and 10.4K), which protect adenovirus infected cells from cytolysis by tumour necrosis factor (TNF). TNF is a cytokine secreted by activated macrophages, which is involved in the inflammatory and immune responses. Adenovirus infected cells are sensitised to TNF cytolysis by E1A (Duerksen-Hughes *et al.*, 1989). Two 'sets' of E3 proteins act independently to inhibit cytolysis induced by TNF: 14.7K which has a more dominant role (Tollefson and Wold, 1988; Horton *et al.*, 1991; Ranheim *et al.*, 1993), and the 10.4K with 14.5K which function as a heterodimer complex protecting some cell lines (in the absence of 14.7K) (Tollefson *et al.*, 1991). E1B 19K protein is another adenovirus protein that prevents TNF cytolysis (Gooding *et al.*, 1991). The 10.4K/14.5K protein complex also downregulates the epidermal growth factor receptor (EGF-R) in adenovirus infected cells (Tollefson *et al.*, 1991), resulting in the internalisation of EGF-R via endosomes and its subsequent degradation. The 10.4K protein alone is able to down regulate the insulin receptor and insulin growth factor I receptor (Kuivinen *et al.*, 1993). In addition, reports suggest that

the 10.4K and 14.5K proteins also downregulate E1A protein levels; this is thought to occur via interference with the translation of E1A mRNA (Zhang *et al.*, 1994).

The 11.6K protein is a glycoprotein which is located largely at the nuclear membrane (Scaria *et al.*, 1992). Recently the 11.6K protein has been shown to be required for efficient cell death or lysis of infected cells and as such the 11.6K protein is also known as the adenovirus death protein (ADP) (Tollefson *et al.*, 1996). The functions of the 6.7K and 12.5K proteins are not known (see Wold *et al.*, 1995 and references therein).

The E4 transcription unit lies on the l-strand between 91.3-99.1 m.u. (see Fig 1.1.1.). Through extensive alternative splicing from a primary transcript, up to 24 mRNAs can be produced, encoding at least ten proteins (Freyer *et al.*, 1984; Tigges and Raskas, 1984; Virtanen *et al.*, 1984). Transcripts from this region are temporally regulated in infection (Tigges and Raskas, 1984; Ross and Ziff, 1992; Dix and Leppard, 1993). The E4 promoter is activated by the E1A proteins and inhibited by the DBP (Handa *et al.*, 1983). The E4 region is predicted to encode seven different proteins; five are produced from ORFs colinear with the viral DNA (ORF1, 2, 3, 4, 6 and 7) and two are created by splicing of the primary transcript (ORF 3/4 and 6/7) (Freyer *et al.*, 1984; Virtanen *et al.*, 1984). Five of these proteins (ORF 2 3, 4, 6 and 6/7) have been detected in Ad5-infected cells, with the later four having functions assigned to them (Sarnow *et al.*, 1982a; Downey *et al.*, 1983; Cutt *et al.*, 1987; Bridge *et al.*, 1993; Dix and Leppard, 1995).

The ORF 3 gene product (11K) is associated with the nuclear matrix (Sarnow *et al.*, 1982a; Downey *et al.*, 1983). Mutations in ORF 3 affect viral DNA replication, late gene expression and shut-off of host cell protein synthesis (Halbert *et al.*, 1985; Bridge and Ketner, 1989; Huang and Hearing, 1989; Ketner *et al.*, 1989).

ORF 6 encodes a 34K protein which can be found physically associated with the E1B 55K protein or uncomplexed (Sarnow *et al.*, 1984; Cutt *et al.*, 1987). Disruption of the E1B 55K/E4 34K complex, resulting in the absence of either or both of these proteins, causes defects in late viral mRNA accumulation and transport, and delays in viral DNA replication (Babiss *et al.*, 1985; Halbert *et al.*, 1985; Pilder *et al.*, 1986; Bridge and

Ketner, 1990). The complex is located in the nucleus of infected cells and is associated with viral inclusions that are thought to be sites of DNA replication and transcription (Ornelles and Shenk, 1991). Transcripts from this region (ORF 6) differ from other E4 mRNAs by the retention of an intron (Dix and Leppard, 1993). ORFs 3 and 6 are required for efficient expression of mRNA from the major late transcription unit. The proteins have redundant activities and expression of one is sufficient for major late mRNA accumulation (Bridge and Ketner, 1989; Huang and Hearing, 1989). They regulate late mRNA accumulation by stimulating constitutive splicing and the two proteins have been shown to have opposite effects on the accumulation of alternatively spliced mRNAs (Nordqvist *et al.*, 1994).

Muller *et al.*, (1992) have shown that the ORF 4 gene product (14K) regulates the phosphorylation of certain viral and cellular proteins. Bridge et al. (1993) found that the proteins expressed from E4 ORF 3, 4 and 6 interact to affect viral DNA replication, with ORF 4 having a negative effect on DNA synthesis.

The E4 ORF 6/7 is generated from the fusion of ORFs 6 and 7 by mRNA splicing (Cutt *et al.*, 1987). This mRNA encodes a 19.5K protein, which is found in the nucleus of infected cells. The 19.5K protein was shown to physically interact with the transcription factor E2F and facilitate the formation of a stable DNA-protein complex on the E2 promoter (reviewed in Nevins, 1995).

Intermediate and Late Transcription

The transcription unit encoding virion polypeptide IX (ppIX) lies within the E1B region (Wilson *et al.*, 1979). The mRNA is transcribed from its own promoter but is 3' coterminal with E1B transcripts (Alestrom *et al.*, 1980); it is the only unspliced adenovirus mRNA. The ppIX gene is referred to as an intermediate gene as it is expressed before other structural proteins are detected (Spector *et al.*, 1978). However viral DNA replication was shown to be required for maximal expression (Vales and Darnell, 1989 and references therein).

The IVa2 gene is located between 11.3 and 16 m.u. and is transcribed from the l-strand; the transcripts share a polyadenylation site with E2B mRNAs. IVa2 is located in

the nucleus (Lutz *et al.*, 1996). The DEF-B protein complex which binds the downstream element (DE) of the MLP was found to comprise of a homodimer of the IVa2 gene product (Tribouley *et al.*, 1994; Lutz *et al.*, 1996 and references therein). The IVa2 protein was also shown to be a component of the DEF-A heteromeric complex which also binds the MLP. The IVa2 protein is required but not sufficient for late phase specific activation of the MLP (Tribouley *et al.*, 1994). Winter and D'Halluin, (1991) have shown that IVa2 is present in assembly intermediates. Recently the L1 52/55K protein has been found in complex with IVa2 (Gustin *et al.*, 1996) and was also found in assembly intermediates (Hasson *et al.*, 1992), whether the interaction of these proteins is required for a step in assembly has still to be determined.

The Ad2 genome encodes the virus-associated (VA)I RNA and VAII RNA each about 160nt long (reviewed in Mathews and Shenk, 1991; Mathews, 1995). They are located at approximately 30 m.u., are transcribed from the r-strand by RNA polymerase III and accumulate to high levels in the cytoplasm of infected cells (Weinman *et al.*, 1976). Most human adenoviruses have two VA genes except for those of subgenera A, F and some of B, which have only one (VAI RNA) (Ma and Mathews, 1993; Kidd and Tiemessen, 1993; Ma and Mathews, 1996). VAI RNA is required for efficient late protein synthesis in infected cells. VAI RNA binds to and inactivates the interferon-induced dsRNA-activated protein kinase (DAI) (Kitajewski *et al.*, 1986). This kinase phosphorylates the protein synthesis initiation factor eIF-2α, which results in the inhibition of protein synthesis. Therefore the inactivation of DAI by VAI RNA counteracts this cellular defence mechanism and allows the synthesis of proteins to proceed (reviewed in Mathews, 1995). VAI RNA is the predominate VA RNA, but VAII RNA has also been shown to suppress DAI activation, albeit less effectively .

At late times after infection, most mRNAs are transcribed from the major late promoter (MLP), which is located at 16.8 m.u. on the r-strand (Shaw and Ziff, 1980). The major late transcription unit (MLTU) consists of a primary transcript alternatively spliced to give 5 families (L1-L5), with each transcript within a family having 3' coterminal ends. All mRNAs from the MLTU are transcribed from the MLP and have a common 201nt 5' leader sequence, the tripartite leader. Although the MLP is active during the early phase

after infection, its activity is strongly increased after the onset of viral DNA replication (Shaw and Ziff, 1980). L1 mRNA is made early in infection; after DNA replication mRNA is expressed from all five families (Shaw and Ziff, 1980).

DBP increases transcripton from the MLP (Zijderveld *et al.*, 1994), by enhancing the binding of USF (cellular protein, upstream stimulating factor) to its recognition site upstream of the MLP. This is thought to occur not by direct binding of USF to DNA, but as a result of structural changes to the binding site induced by DBP.

Sequence elements located downstream of the MLP start site have been identified and shown to be involved in activation of late transcription (Mansour *et al.*, 1986; Leong *et al.*, 1990). Two main sequence elements have been identified (DE1 and DE2) together with the transcription factors which interact with these sites. The heteromeric protein DEF-A binds DE1 and the 3' portion of DE2 (Jansen-Durr *et al.*, 1989; Mondesert *et al.*, 1992), while DEF-B (a homodimer of IVa2) interacts with the 5' part of DE2 (Mondesert *et al.*, 1992; Tribouley *et al.*, 1994). Proteins which bind DE elements cooperate with factors bound to upstream elements in order to achieve maximal MLP activity (Mondesert *et al.*, 1992).

Studies have shown that the polyadenylation (poly-A) sites L1 and L3 contain cis-acting elements necessary to elicit a temporal switch in poly-A site use (Dezazzo *et al.*, 1991). The L1 poly-A site is weak in comparison to L3, differences in processing efficiencies are thought to be one of the factors involved in the early to late MLTU poly-A site processing switch mechanism. Prescott and Falck-Pedersen (1994) have identified a sequence element (A-rich) involved in the different processing efficiencies between the L1 and L3 poly-A sites.

The L1 region encodes three proteins; the 52, 55 kilodalton (52/55K) proteins and the IIIa protein. The 52/55K proteins are produced at early and late times in infection, while IIIa is expressed late (Akusjärvi and Persson, 1981; Kreivi and Akusjärvi, 1994). Protein IIIa is a structural polypeptide (Boudin *et al.*, 1980) associated with hexons. The 52/55K proteins are required for assembly of virions (Hasson *et al.*, 1992). They are present in empty capsids, assembly intermediates and young virions, but not in mature virions. L1 early mRNA differs from other major late mRNAs by the presence of an

additional leader (i) which is spliced between the second and third exons of the tripartite leader (Akusjärvi and Persson, 1981). This i leader inclusion is facilitated by E4 ORF3 (Nordqvist *et al.*, 1994).

L2 region encodes virion proteins pIII, pVII, pV and protein X (precursor for the μ protein) (Anderson *et al.*, 1989; Russell and Kemp, 1995).

The L3 region encodes protein pVI, hexon and the 23K protease. This protease specifically cleaves six virion proteins: pIIIa, pVI, pVII, pVIII, X and pTP (Weber, 1976). The 23K protease cleaves the structural precursors and renders mature virions capable of initiating an infection. The protease is present at approx. 10 copies per virion (Anderson, 1990). Activation of the protease requires interaction with a disulphide linked peptide derived from the C-terminus of protein pVI (Webster *et al.*, 1993; Mathews and Russell, 1995). The mature VI protein has been shown to interact with hexon (Mathews and Russell, 1994). The pVI protein is proteolytically cleaved to mature VI via an intermediate stage iVI. The interaction of VI and hexon increased during each maturation step indicating that each cleavage event was facilitating a stronger interaction.

The L4 region encodes three proteins, a structural protein VIII and two non-structural proteins of 100K and 33K. The 100K protein is involved in the assembly of hexon into trimers (Cepko and Sharp, 1983) and necessary for the efficient initiation of translation of late mRNA (Hayes *et al.*, 1990). Riley and Flint (1993), showed that this process involves binding to RNA.

L5 region codes for the fibre protein. A substantial fraction (30%) of fibre mRNAs, in addition to the tripartite leader, have various combinations of three ancillary leader sequences (x, y and z) (Uhlen *et al.*, 1982).

1.1.4.3. DNA Replication.

Three viral proteins are essential for adenovirus DNA replication: terminal protein precursor (pTP), adenovirus DNA polymerase (Adpol) and DNA-binding protein DBP, which are all encoded by the E2 transcriptional unit (reviewed in Hay *et al.*, 1995; van der Vliet, 1995). DNA replication initiates at either end of the molecule and proceeds unidirectionally by a strand displacement mechanism. In this first stage, initiation and

elongation occur with one strand as template, while the nontemplate strand is displaced as a single strand, this process is termed Type I replication. Subsequently the displaced strand is replicated (Type II replication). The ITRs base pair to form a 'panhandle' structure which serves as an origin for replication for the second strand (reviewed in Challberg and Kelley, 1989; Hay and Russell, 1989; Stillman, 1989). Initiation of adenovirus DNA replication differs from other eukaryotic DNA viruses, but is similar to *Bacillus subtilis* bacteriophage ϕ29; both use a protein as a primer for initiation (reviewed in Salas, 1991). Initiation of replication at either terminus occurs via the formation of a phosphodiester bond between pTP serine residue 580 and the first nucleotide dCMP (Smart and Stillman, 1982). It is the 3' OH group of pTP-dCMP which functions as the protein primer for elongation by Adpol (via strand displacement). Adpol and pTP form a heterodimer (pTP-pol) which recognises the core origin in the ITR, this complex catalyses the formation of pTP-dCMP.

Two cellular transcription factors also function to enhance the efficiency of the initiation of DNA replication, the NFI or CAAT transcription factor and NFIII or octamer binding protein (reviewed in Challberg and Kelley, 1989; Hay *et al.*, 1995; van der Vliet, 1995). These proteins bind to two adjacent sites in the ITR near the core origin, called the auxiliary region and their effect is dependant on the level of pTP-pol complex present (Temperley and Hay, 1992). NFI binds as a dimer to its recognition site (next to the core region) and interacts with the pTP-pol heterodimer (Dekker *et al.*, 1996). This interaction acts to stabilise the initiation complex at the template and correctly positions pTP-pol at the site of initiation (Chen *et al.*, 1990; Mul and van der Vliet, 1992; Armentero *et al.*, 1994). NFIII is a member of the POU family of transcription factors, it stimulates initiation of DNA replication at the octamer element (adjacent to the NFI site in the auxiliary region) and this is thought to occur via a direct interaction between the pTP-pol complex and the octamer element (Coenjaerts *et al.*, 1994).

DBP has a stimulatory effect on initiation and that is to increase the binding of NFI to its recognition site (Cleat and Hay, 1989; Stuvier and van der Vliet, 1990). DBP also stimulates initiation by lowering the Km of the formation of the initiation complex (Mul and van der Vliet, 1993). This protein is absolutely required for elongation, during

which it increases the rate of synthesis and processivity of Adpol and covers the displaced strands (Lindenbaum *et al.*, 1986). It has been shown that DBP has helix destabilising properties (Zijderveld and van der Vliet, 1994) which could assist in strand displacement during replication. The crystal structure of the DNA-binding domain of DBP has been determined (Tucker *et al.*, 1994), and was found to have a 17 amino acid C-terminal extension that hooks onto neighbouring molecules and thus forms a protein chain. Tucker *et al.*, (1994), proposed that single stranded DNA is wound around a protein core of interacting DBP molecules.

1.1.4.4. Virion Assembly.

The first step in the assembly of adenovirus particles is the formation of capsomers from single polypeptides of hexon, penton base and fibre in the cytoplasm. The capsomers are transported to the nucleus where they assemble to form the capsid structure (reviewed in D'Halluin, 1995), a process that involves structural and non structural proteins (see Fig 1.1.3.). The assembly of the virus particle continues through several ordered stages. (see Fig 1.1.3.). Subsequently, DNA enters the capsid, an event which is mediated by a packaging sequence located at the left-hand end of the chromosome (reviewed in Schmid and Hearing, 1995). The encapsidation of the genome is polar and begins with the left end of the viral DNA. The L1 52/55K protein is present in assembly intermediates and is thought to act as a scaffold protein in encapsidation (Hasson *et al.*, 1992). The next stage in assembly involves the entry of the core proteins forming a young virus particle. The final step is the action of the L3 virus coded 23K protease which cleaves six precursor viral proteins (Weber, 1976; Webster *et al.*, 1993), resulting in the formation of an infectious mature virus particle.

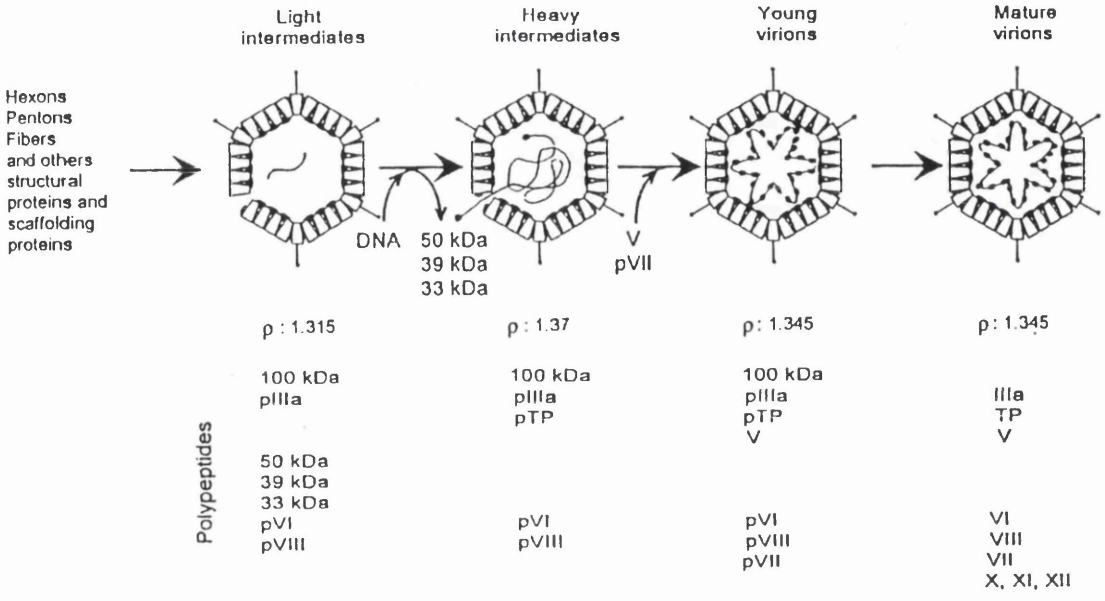


Figure 1.1.3. Adenovirus Assembly Pathway.

The four stages of virus assembly; light intermediates, heavy intermediates, young virions and mature virions are shown (top). The polypeptides only present in some structures are also shown (below). (Modified from D'Halluin, 1995).

1.2. Attachment And Internalisation.

1.2.1. Attachment.

The initial contact between adenovirus and the host cell is mediated by the fibre protein, which binds to cell surface receptors (Philipson *et al.*, 1968). This was shown in competition assays, where pretreatment of cells with purified fibre protein blocked the subsequent attachment of virus (Philipson *et al.*, 1968; Wickham *et al.*, 1993). The cell binding domain resides within the globular head of the fibre molecule at the C-terminus (Philipson *et al.*, 1968; Henry *et al.*, 1994; Louis *et al.*, 1994). The number of adenovirus receptor sites on HeLa and KB cells has been estimated to be approximately 5,000 to 10,000 per cell, and the number of receptor sites for fibre protein are one order of magnitude higher at 10^5 per cell (Philipson *et al.*, 1968; Hughes and Mautner, 1973). This suggested that the virus undergoes multivalent binding at the cell membrane.

The identity of the cellular receptor is unknown at present, although some cellular proteins have been shown to bind fibre (Hennache and Boulanger, 1977; Svensson *et al.*, 1981; Defer *et al.*, 1990; Belin and Boulanger, 1993; Di Guilmi *et al.*, 1995). Hennache and Boulanger (1977) used affinity columns consisting of penton base and fibre such that the C-terminus of the fibre protein was oriented outwards. These authors identified three polypeptides from KB cell membranes that recognised Ad2 fibre (78K, 42K and 34K), furthermore these proteins could inhibit Ad2 attachment to KB cells. Svensson *et al.*, (1981), showed that two glycoproteins of 40K and 42K could be purified from HeLa membranes using Ad2 virions immobilised on wheatgerm agglutinin columns. It is not known if the 42K species corresponds to that previously identified by Hennache and Boulanger (1977). In addition Belin and Boulanger (1993), identified three proteins of 130K, 60K and 44K, from HeLa cell membranes, that bound with Ad2 virions in cross linking experiments. These proteins also bound antibodies to fibronectin and vitronectin receptors, and these authors suggested that extracellular matrix proteins and Ad2 may recognise similar adhesion sequences on the cell membrane. In an overlay protein binding assay Ad3 virus and Ad3 fibre bind a 130K protein from HeLa cells. (Di Guilmi *et al.*, 1995).

Although the cell receptor has not been identified there is evidence that adenoviruses from subgroups B and C use different receptors (Defer *et al.*, 1990; Stevenson *et al.*, 1995). Defer *et al.*, (1990) showed that purified fibres from Ad2 (subgroup C) did not efficiently block the infection of A549 or KB cells by Ad3 (subgroup B). Also, in affinity blotting experiments with immobilised proteins from KB membranes, probing with either Ad2 or Ad3 virus particles revealed different patterns of affinity-labelled proteins from the two viruses. One major protein species of 47K was detected with Ad3, this protein could not be displaced by Ad2 fibre. Many proteins were observed that bound to Ad2 ranging from 15K to 130K. These proteins could be divided into two groups, based on their ability to bind Ad2 fibre. The cellular proteins described by these authors were also thought to be possible cellular receptor sites. Recently, Roelvink *et al.*, (1996) has shown that Ad2 and Ad9 (subgroup D) can use the same cellular fibre receptor, but the binding strategies for attachment are different. Stevenson *et al.*, (1995) found that two adenoviruses (Ad3 and Ad5) representing subgroups B and C, bound different cell receptors and that this interaction occurred via the fibre head domain. Furthermore, using chimeric fibre proteins in which the Ad3 and Ad5 head domains were interchanged resulted in the alteration of receptor specificity (Stevenson *et al.*, 1995). A similar approach was carried out by Krasnykh *et al.*, (1996) where an Ad5 vector was created with chimeric fibres consisting of Ad5 tail and shaft with an Ad3 knob domain. This virus also displayed an altered receptor recognition. Recently the tropism of Ad5 has also been shown to be altered by exchanging the fibre with that of Ad7 (Gall *et al.*, 1996). These modifications may facilitate a more efficient use of adenoviruses in areas such as gene therapy, where specific cell type targeting would be beneficial.

Most of the discussion so far has concentrated on subgroup B and C adenoviruses which have one fibre protein, however subgroup F viruses have two fibre genes encoding proteins of different sequence and shaft length (Kidd and Erasmus, 1989; Pieniazek *et al.*, 1989, 1990; Kidd *et al.*, 1990, 1993; Davison *et al.*, 1993; Yeh *et al.*, 1994). In these viruses a mixture of fibres is present (both long and short) but only one extends from each vertex. In attachment studies by Yeh and Luftig (1991), Ad41 was found to bind to a single cell receptor on Hep-2 cells . The avian CELO adenovirus also has two fibres, but

in contrast to human subgroup F viruses both extend from the same vertex (Laver *et al.*, 1971; Hess *et al.*, 1995).

Early studies showed adenovirus attachment to cells may lead to subsequent interactions with the cell membrane that stimulate virus internalisation. After the initial attachment of virus to cells, there is a clustering of adenovirus cell receptors on the cell surface, which is important in viral entry (Patterson and Russell, 1983; Persson *et al.*, 1983), as reagents that block the clustering of the receptor sites, result in the inhibition of virus uptake.

1.2.2. Internalisation.

1.2.2.1. Penton Base Protein.

The observation that Ad2 attaches to but does not enter some cells, suggested that entry into cells may require another host cell derived factor, as well as the receptor for fibre protein (Silver and Anderson, 1988). Earlier studies had already suggested a possible interaction between the penton base protein and the cell membrane; these studies showed that when exposed to penton base, adherent cells round up and detach from glass or plastic surfaces *in vitro* (Pereira *et al.*, 1958; Rowe *et al.*, 1958).

Penton base sequences are known for a number of serotypes : Ad2 (Roberts *et al.*, 1986), Ad5 (Neumann *et al.*, 1988), Ad12 (Sprengel *et al.*, 1994), Ad40 (Davison *et al.*, 1993), Ad3 (Cuzange *et al.*, 1994), Ad4 (Mathias *et al.*, 1994) and the fowl adenovirus type 10 (FAV-10) (Sheppard and Trist, 1992). Alignment of the amino acid sequences (Cuzange *et al.*, 1994; Mathias *et al.*, 1994), shows that amino and carboxy ends are conserved, however the central region is very variable. All the above serotypes except Ad40 and FAV-10, have a conserved Arg-Gly-Asp (RGD) sequence motif in the variable region. Ad40 differs from other human adenovirus serotypes by having an RGA sequence in place of RGD, however FAV-10 has no RGD sequence at all. The predicted secondary structure of the RGD domain suggests that it consists of a helix-turn-helix with RGD located at an exposed turn at the apex of the two helices (Mathias *et al.*, 1994). The RGD motif is recognised by $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$ and most $\alpha_v\beta$ integrins (reviewed in Hynes, 1992).

Structural studies of Ad2 virions by Stewart *et al.*, (1993) revealed 10Å protrusions on each polypeptide subunit of pentameric capsomer which may correspond to RGD domains. The RGD tripeptide motif is also found in a number of cell adhesion molecules including fibronectin and vitronectin. These extracellular matrix molecules adhere to cell membranes via their RGD sequence, which binds to cell surface receptors, termed integrins (reviewed in Hynes, 1992). Wickham *et al.*, (1993), showed that internalisation of Ad2 is mediated by penton base binding to cell surface αv integrins via the RGD sequence motif.

1.2.2.2. Integrins have a Role in Adenovirus Entry.

Integrins are specific cell surface receptors, they are heterodimers composed of α and β subunits, which are noncovalently associated with each other. There are 15 α subunits and 8 β subunits, which can form some 20 different heterodimers, and the subunit composition dictates ligand-binding properties (Hynes, 1992).

Wickham *et al.*, (1993), showed that a number of human cell lines (A549, HeLa and M21) bound to penton base coated wells, and this binding was significantly reduced by preincubation with an RGD containing peptide. Pretreatment of cells with fibronectin or vitronectin reduced subsequent infection by Ad2, indicating the occupancy of cell surface integrins by matrix proteins. In addition, Ad2 infection was inhibited by preincubation of cells with soluble RGD containing peptides or with recombinant penton base. These results indicated that penton base interaction with cell surface integrins plays an important role in Ad2 infection.

In order to determine the role of specific αv integrins in adenovirus infection two M21 melanoma cell lines were used (Felding-Habermann, 1992). M21-L4 cells express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ cell surface integrins and can attach and spread on immobilised Ad2 penton base protein, whereas M21-L12 cells do not express these integrins. M21-L12 cells were significantly less susceptible to Ad2 infection than M21-L4 cells (Wickham *et al.*, 1993). Also binding of penton base protein to M21-L4 cells could be inhibited by RGD containing peptides. In addition a combination of function-blocking monoclonal antibodies to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ blocked Ad2 penton base attachment to cells. Inhibiting

virus penton base RGD interaction with cells via αv monoclonal antibodies or RGD peptides significantly reduced virus infection. The RGD sequence motif was shown to mediate virus binding to αv integrins by Bai *et al.*, (1993). These authors used a recombinant Ad2 penton base mutant containing the sequence RGE in place of RGD and showed that this mutant failed to support αv integrin mediated cell adhesion. The mutant did not cause cell rounding and in an adhesion assay, cells did not adhere to wells coated with the penton base mutant, also these RGE mutants showed a decreased yield of virus.

Adenovirus attachment to cells is blocked by soluble fibre, but not by penton base protein (Philipson *et al.*, 1968; Wickham *et al.*, 1993). Viral attachment on both M21-L4 (αv+) cells and M21-L12 (αv-) was the same, however internalisation was significantly reduced in M21-L12 cells compared to M21-L4 cells (Wickham *et al.*, 1993). Synthetic RGD peptides, monoclonal antibodies to αv integrins and recombinant penton base, could all be used to block the interaction of αv integrin to virus. All of these inhibitors were shown to have no effect on attachment, although they did inhibit Ad2 internalisation (Wickham *et al.*, 1993). These results showed Ad2 penton base interaction with cell surface integrins was required for efficient viral internalisation, and that virus attachment was an independent and separate event. There is a possibility that other integrins may also be involved in Ad2 internalisation, as M21-L12 cells could be infected albeit at lower levels than M21-L4 cells (approx. 5 to 10 fold less).

1.2.2.3. Role of αv Integrins in Other Adenovirus Serotypes.

The RGD sequence motif is conserved in several human adenovirus serotypes indicating that these viruses may also use αv integrins for entry into host cells (Mathias *et al.*, 1994). Mathias *et al.*, (1994), looked at penton base proteins of different adenoviruses representing subgroups B, C and E (Ad3, Ad2 and Ad4, respectively). The cell lines M21-L12 and M21-L4 were used by these authors to investigate the role of αv integrins. They detected approx. 5 to 10 fold higher levels of infection, with all three serotypes, on M21-L4 cells compared to M21-L12 cells, indicating that αv integrins promote viral infection by different serotypes. Infection by Ad3 and Ad4 could be inhibited with a combination of αv monoclonal antibodies or with RGD containing synthetic peptides, but

not with RGE containing peptides. These results indicate that the RGD sequence motif in Ad3 and Ad4 mediate interaction with αv integrins and this interaction is required for efficient virus infection.

Ad40 does not contain an RGD motif and it is possible that another route of viral entry is used by this virus, however as some non-RGD sequences can also mediate integrin binding (Hynes, 1992), the involvement of cell surface integrins in Ad40 cell entry cannot be ruled out.

1.2.2.4. αv Integrins Enhance Adenovirus Mediated Gene-Delivery.

αv integrins play an important part in adenovirus infection, and can be used to extend the host cell range to monocytes and T lymphocytes. Adenovirus has a restricted ability to infect PBM (peripheral blood monocytes). These cells have only low levels of surface αv integrins and thus are resistant to Ad2 penton base binding. Huang *et al.*, (1995), have shown that by exposing these cells to monocyte-specific growth factors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expression was upregulated on cell membranes. This resulted in increased ability to bind penton base protein and increased susceptibility to adenovirus mediated gene-delivery. These studies could have important significance for the role of adenovirus in gene-delivery as a large part of the host defences involve cells of the immune system (i.e. monocytes and T lymphocytes).

1.2.2.5. Post-Internalisation Events.

Following internalisation adenovirus disrupts cell endosomes, a process requiring the low pH of the endosome and penton base protein (Seth *et al.*, 1985; Mathias *et al.*, 1994; Seth, 1994; Wickham *et al.*, 1994). In Ad2 infection, integrin $\alpha_v\beta_5$ interaction with penton base has also been shown to be required for the release of virus from endosomes (Wickham *et al.*, 1994).

Recently the L3 coded 23K protease has been shown to have a role in virus entry as well as assembly (Cotten and Weber, 1995; Greber *et al.*, 1996). This was shown from studies using inhibitors of the protease which resulted in the inhibition of protein VI degradation and prevention of virus uncoating at the nuclear membrane (Greber et al.

1996). The protease is thought to act in entry by degrading the capsid stabilising protein VI and thus facilitate the dismantling of the virus particle.

The virus follows a stepwise uncoating process in which viral proteins are shed in an ordered fashion resulting in the release of viral DNA into the nucleus via nuclear pore complexes (Greber *et al.*, 1993).

1.3. Early Region 1A.

The E1A region plays an important role in the regulation of adenovirus transcription and in transformation, and has thus been extensively studied. This section describes E1A gene transcription and protein products. The roles of these proteins in different processes will be discussed briefly; more information can be found in the many reviews available (Berk, 1986; Shenk and Flint, 1991; Jones, 1992, 1995; Akusjärvi, 1993; Nevins, 1995).

1.3.1. E1A Transcription.

The E1A sequences for a number of adenovirus serotypes are now known, including those for human adenoviruses Ad2, 4, 5, 7, 12, 40 and 41 (see Davison *et al.*, 1993, and references therein). All E1A mRNAs that have been mapped, have been shown to be very similar, with the exception of mouse adenovirus type-1 (MAV-1) (Ball *et al.*, 1989) which differs in having only one E1A transcript (coterminal with the 3' end of E1B).

There are five mRNAs produced from the Ad2 E1A region: 13S, 12S, 11S, 10S and 9S (see Fig 1.3.1.). All Ad2 E1A mRNAs, with the exception of the 9S transcript, encode proteins which share amino and carboxy terminal ends and are translated in the same reading frame (Virtanen and Pettersson, 1983). The second exon of the 9S mRNA is read in a different reading frame to the 12S or 13S mRNAs. The major transcripts produced from the E1A gene are the 13S and 12S mRNAs (Perricaudet *et al.*, 1979), and the minor transcripts are 11S, 10S and 9S (Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987).

E1A transcripts are the first mRNA expressed in a lytic infection, with 12S and 13S first detected at 1.5-2h (Nevins *et al.*, 1979), the level of these transcripts increase up to 5h p.i. and thereafter gradually decrease to 12h (Glenn and Ricciardi, 1988). The 10S and 11S mRNAs are detected at later times in infection (Stephens and Harlow, 1987) and the 9S mRNA accumulates in the cytoplasm at late stages after infection (Chow *et al.*, 1979; Wilson and Darnell; 1981).

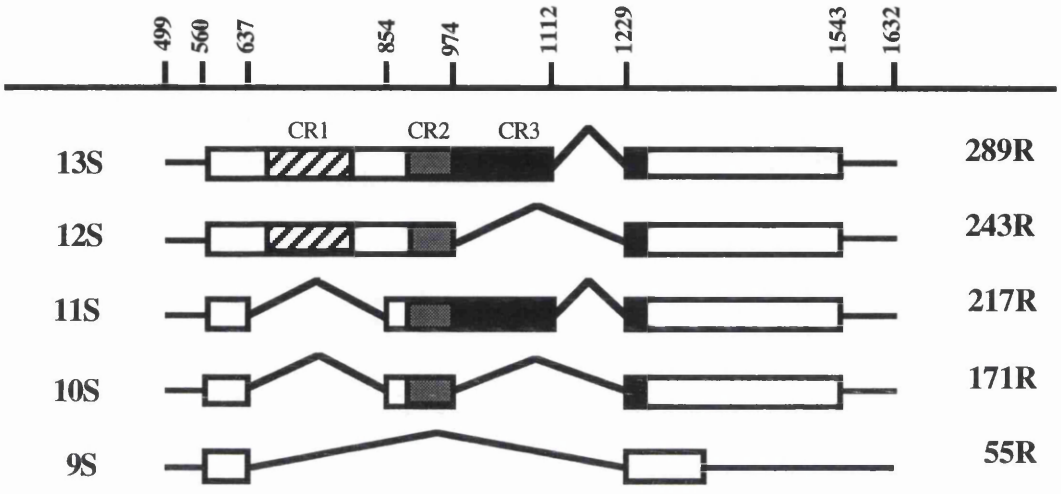


Figure 1.3.1. Ad2 E1A mRNAs and Protein Products .

The mRNAs are indicated by lines, introns by caret symbols and open reading frames by boxes. The three shaded areas represent the three regions of E1A conserved between different adenovirus serotypes. The size of the mRNAs and proteins are indicated. (Modified from Jones, 1992)

The E1A promoter is active in the absence of any viral factors (Nevins, 1981), and consists of a TATA box, ATF binding site and many upstream promoter elements involved in the control of transcription. Three enhancer elements have been identified in the upstream region of E1A. Element I is present in two copies, at least one of which is required to regulate and maintain a high level of E1A gene transcription (Hearing and Shenk, 1986 and references therein). A cellular factor, EF-1A, binds to one binding site on both copies of element I and to three enhancer-proximal sites (Bruder and Hearing, 1991 and references therein). Element II is situated between the two copies of element I and enhances transcription from all early promoters on the viral genome (Hearing and Shenk, 1986). Element III is also present in two copies and contains the binding site for the cellular transcription factor E2F (Kovesdi *et al.*, 1987). The E1A enhancer region is autoregulated by E1A gene products (Tibbetts *et al.*, 1986; Cogan *et al.*, 1992). In the absence of E1A proteins, transcription from the E1A promoter is reduced approximately five fold, which suggested a role for E1A proteins in the regulation of this gene (Montell *et al.*, 1984a; Osbourne *et al.*, 1984). Negative regulation also occurs and is thought to involve the 12S mRNA gene product (Cogan *et al.*, 1992), thus the controlled expression of the gene depends on the relative amounts of E1A proteins present.

1.3.2. E1A Proteins.

E1A proteins are phosphoproteins located predominantly in the nucleus of infected cells (reviewed in Boulanger and Blair, 1991). The major E1A proteins are the products of the 13S and 12S mRNAs, the 289R and 243R proteins respectively (see Fig 1.3.1.). The comparison of E1A regions from various adenovirus serotypes, has identified three highly conserved regions designated CR1, CR2 and CR3 (see Fig 1.3.1) (Kimelman et al 1985, Moran and Mathews, 1987). Figure 1.3.2. shows different regions of the E1A protein that have been found to have an important role in various processes and the binding sites of cellular proteins that are linked to some of these different functions. The CR1 domain present in 289R and 243R proteins is required for transcriptional repression and induction of DNA synthesis (reviewed in Jones, 1995). CR2 is needed for

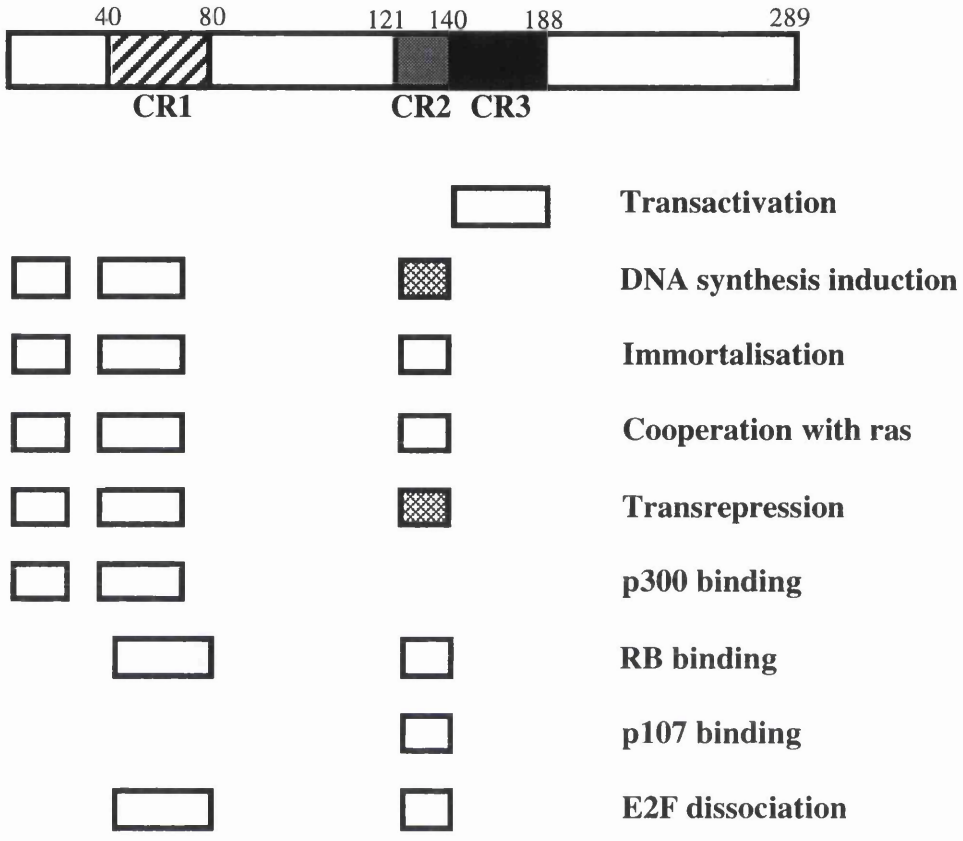


Figure 1.3.2. The Structural and Functional Domains of the E1A Protein.

CR1, CR2 and CR3 refer to the three conserved regions in the E1A protein of different adenovirus serotypes. The regions of the protein that have been shown to be important for the various functions indicated, are depicted by the boxes. The open boxes indicate regions consistently shown to be important, whereas the shaded boxes represent regions shown to be important in some but not all cases. (Modified from Jones, 1992)

transformation and induction of DNA synthesis, but may be dispensable for transcriptional repression (Stein *et al.*, 1990). The CR3 domain is unique to the larger protein and is necessary and sufficient for the transactivation of adenovirus early genes. Protein fusion experiments show that this domain has two separable activities (Lillie and Green, 1989; Martin *et al.*, 1990). The N-terminus of CR3 contains the transcriptional activation region and the C-terminus contains the promoter targeting region. Located within the N-terminus of CR3 is a C4 type zinc finger, point mutations of any of the cysteine residues that defined this motif eliminated transactivation function (Culp *et al.*, 1988; Martin *et al.*, 1990; Webster and Ricciardi, 1991).

1.3.3. Transactivation.

E1A proteins modulate transcription from several viral (E1A, E1B, E2A, E3, E4, MLP and VA) and cellular promoters (including hsp70, *c-fos, c-jun* and PCNA) (for reviews see Berk, 1986; Shenk and Flint, 1991; Jones, 1992, 1995). E1A proteins do not show sequence specific DNA binding (Ferguson *et al.*, 1985; Chatterjee *et al.*, 1988). It now appears transactivation by E1A, occurs indirectly via different cellular components. A number of studies have identified cis-acting elements that are important in E1A dependant transactivation through cellular trans-acting factors. The promoter elements and DNA binding factors important for transcription from a number of adenovirus promoters are shown in Fig 1.3 3. The promoters encompass a wide variety of elements and do not seem to share common sequences. Therefore studies concerning the mechanism of transactivation have concentrated on determining the cellular factors binding to these elements and how the activities of these factors are altered by E1A proteins.

TFIID binds the TATA box sequence and consists of the TATA-box binding protein (TBP) and TBP-associated factors (TAFs) (Zhou *et al.*, 1993). The E1A 289R protein can bind directly to TBP (Horikoshi *et al.*, 1991; Lee *et al.*, 1991), Boyer and Berk (1993) have shown that E1A stimulates transcription by association with TFIID through the CR3 domain. The E1B promoter is one of the simplest found in the

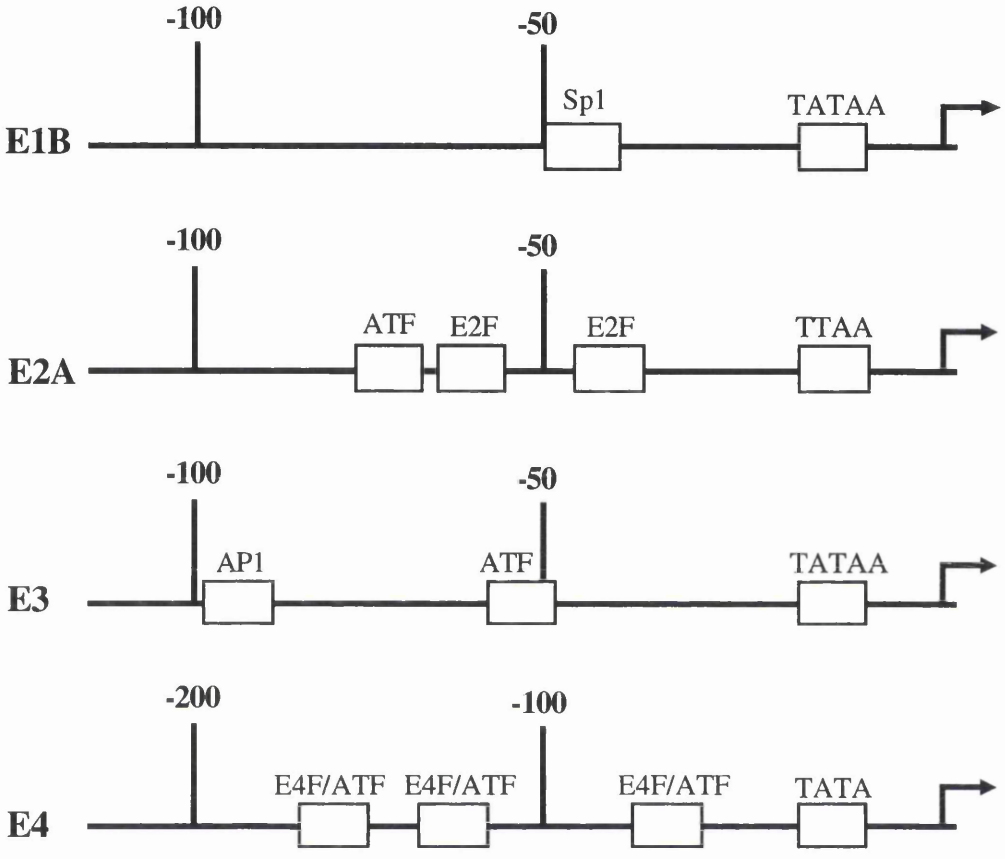


Figure 1.3.3. Diagram of Adenovirus Promoter Elements and Factor Binding Sites.

Adenovirus promoters are represented by the solid horizontal lines and the arrow heads designate the major sites of transcription initiation. The open boxes represent the main promoter elements involved in transcription. Note that the E4 transcriptional control region is on a different scale. (Modified from Jones, 1992)

adenovirus genome, it consists of a TATA box element and a consensus Sp1 binding site (see Fig 1.3.3.) (Wu *et al.*, 1987). Upstream sequences that mediate the effects of E1B transcription in the absence of a functional Sp1 binding site have also been described (Parks *et al.*, 1988: Spector *et al.*, 1993). Mutations in the Sp1 site reduced transcription but E1A transactivation still occurred. However, mutations in the TATA box sequence eliminated E1A protein transactivation (Wu *et al.*, 1987). The TATA element is also important in E1A transactivation of some cellular genes e.g. hsp70 (Simon *et al.*, 1988). Therefore it appears a subset of E1A response promoters primarily require the TATA box for transactivation (Wu *et al.*, 1987; Simon *et al.*, 1988). However, other adenovirus promoters are not as dependent on this element for transactivation by E1A (Berk, 1992). This was illustrated when it was shown that mutations in the TATA box sequence of the E3 promoter did not eliminate transactivation (Garcia *et al.*, 1987). The E2A promoter contains a TATA-like sequence, but similarly mutations in this element do not much alter transactivation by E1A. E1A itself is not wholly dependant on the E1A TATA box motif, as deletion of this element decreases but does not abolish transactivation (Hearing and Shenk, 1985). The 243R protein can also activate through the TATA element, although this activation is weaker than 289R. The 243R protein binds to Dr1 a cellular protein that interacts with TBP (Kraus *et al.*, 1994 and references therein). E1A dissociates the TBP-Dr1 complexes which inhibits transcription (Kraus *et al.*, 1994).

Several of the adenovirus early promoters contain ATF binding sites, which bind the cellular transcription factor ATF (activating transcription factor). In E2A, E3 and E4 these sites are important for both basal and E1A induced transcription (reviewed in Berk, 1986; Shenk and Flint 1991; Jones, 1995). The ATF element is identical to the CRE element (cAMP responsive element) and promoters containing this motif can be transcriptionally activated by increased cAMP levels via the CRE binding protein (CREB). ATF/CRE sites are recognised by a family of related proteins (see Shenk and Flint, 1991), many of the cDNAs encoding ATF/CREB proteins that bind to these elements have been cloned (Chatton *et al.*, 1994, and references therein). Analysis of these proteins showed they belong to the 'bZIP' family, comprising of a basic region at the C-terminus which is adjacent to a leucine zipper motif, these confer sequence-specific

DNA binding and protein dimerisation capabilities. Members of this family have been shown to bind as hetero or homodimers depending on the amino acid composition of the leucine zipper (Hai and Curran, 1991). Initially, ATF-2 was the only member of this family shown to mediate E1A inducible transcriptional activation, whereas other members of the ATF family (ATF-1, ATF-4 and CREB) did not support activation in this system (Liu and Green, 1990). Another member of the ATF family, ATFa, has now also been shown to be responsive to E1A (Chatton *et al.*, 1994). The E1A 289R protein interacts with ATF-2 through its DNA binding motif (bZIP), but the N-terminal 96 amino acids of ATF-2 are also important as deletion of this region destroys E1A activation (Liu and Green, 1994). The ATF-2 and TBP proteins bind to different CR3 domains (Lee *et al.*, 1991). A simple model is proposed in which E1A functions as a coactivator or bridging protein by binding to ATF-2 at an upstream site and TBP, to activate transcription.

Proteins binding to AP-1 (activator protein 1) sites come from a group of related proteins, of the Jun and Fos gene families. They become active transcription factors by forming Jun/Jun homodimers or Jun/Fos heterodimers at target sites. The AP-1 binding site sequence differs from the consensus ATF/CRE site by only one nucleotide. The AP-1 proteins share structural features to ATF/CREB proteins and can also heterodimerise with members of this family (Hai and Curran, 1991). Both the ATF and AP1 sites in the E3 promoter were shown to be crucial for transactivation by E1A *in vivo* (Kornuc *et al.*, 1990).

E1A and E2A promoters contain E2F binding sites (see Fig 1.3.3.) (Manohar *et al.*, 1990). The cellular transcription factor E2F binds two sites in the E2A promoter, and this has been shown to be important for its E1A-induced transcription (reviewed in Nevins, 1995). In addition E2F sites can confer E1A inducibility to an unresponsive promoter (Kovesdi *et al.*, 1987; Yee *et al.*, 1989) and in adenovirus infected cells E2F DNA binding activity was shown to dramatically increase. This suggests a role for the E1A-dependant activation of transcription by E2F. The transcription factor E2F is now known to consist of a group cellular proteins, including E2F-1 and DP-1, that form heterodimers at E2F binding sites (Helin and Harlow, 1994). E2F binds to the two E2F

sites on the E2A promoter, which are inverted with respect to one another. The cooperativity of these elements is abolished if the spacing between the E2F sites is increased. The DNA-protein complex is further stabilised by the cooperative binding of E4 19.5K (see Nevins, 1995 and references therein). E2F forms a complex with Rb (the retinoblastoma gene product), which blocks E2F activation of transcription (Bandara and La Thangue, 1991). E1A activates transcription by binding to Rb and dissociating E2F-Rb complexes, freeing E2F (Whyte *et al.*, 1989; Zamanian and La Thangue, 1992). Interaction with Rb is dependent on sequences within the CR1 and CR2 domains of E1A (Raychaudhuri *et al.*, 1991). Another way the pool of free E2F can be increased is by phosphorylation of Rb. The phosphorylation of Rb prevents the formation of complexes with E2F (Kato *et al.*, 1993), which increases the pool of uncomplexed E2F. Transactivation of the E2A promoter requires four elements; an ATF site, two E2F sites and the TATA-like sequence element; mutation of any one of these elements eliminates transactivation (Manohar *et al.*, 1990). E2F sites however are the main regulators of the E2 promoter, whereas ATF plays a more minor role (reviewed in Akusjärvi, 1993).

The E4F cellular transcription factor binds to three regions in the E4 promoter, two of which overlap ATF binding sites (see Fig 1.3.3.). E4F sites have not been detected in other viral promoters, indicating that these proteins are distinct factors (Raychaudhuri *et al.*, 1989). Mutations that eliminated E4F binding had no effect on ATF binding, indicating that independent pathways were used by these two factors. The E4F sites mediate E1A-responsiveness in the E4 promoter, this was shown when deletion of these upstream sequences resulted in elimination of this response. E1A acts by inducing the phosphorylation of transcription factor E4F, leading to an increase in its DNA-binding activity (Raychaudhuri *et al.*, 1989; Bondesson *et al.*, 1992).

Other adenovirus promoters dependent on E1A activation include those from the major late transcription unit and VA RNA genes. The MLP contains two main promoter elements; the TATA box sequence and an upstream sequence element (UE) (reviewed in Shenk and Flint, 1991), elements downstream have also been shown to have an affect on promoter function (Mansour *et al.*, 1986; Mondesert *et al.*, 1992). Leong *et al.*, (1988) have shown that transcription from the MLP could be stimulated with TFIID-containing

cell extracts. The initiation complex is further stabilised by interactions between TFIID and the factor USF which binds to UE (Carthew *et al.*, 1985; Moncollin *et al.*, 1986). For the MLP E1A transactivation occurs through association with TFIID (Leong *et al.*, 1988). Activation of VA RNA gene transcription requires the transcription factors TFIIIB and TFIIIC. E1A expression increases the amount of transcriptionally active TFIIIC (Hoeffler *et al.*, 1988). TFIIIC then binds directly to a DNA sequence within the internal control region of the genes, thereby allowing TFIIIB and RNA polymerase III to bind (Hoeffler and Roeder, 1985).

1.3.4. Transcriptional Repression.

E1A proteins can also repress the transcription of several viral (both polymerase II and III) and cellular genes (reviewed in Jones, 1992). In many repression studies the N-terminus and CR1 domain were shown to be essential (Stein et al ., 1990 and references therein). However in some studies the CR2 domain and some second exon sequences are also thought to be involved (Lillie *et al.*, 1986; Velcich and Ziff, 1988; Jelsma *et al.*, 1989). The reason for these differences is unclear but could depend on the promoter studied or cells used for the assay. The mechanism is unclear, however it was shown that the binding of a cellular protein p300 to the N-terminus and CR1 domain correlated with E1A enhancer repression (Jelsma *et al.*, 1989; Stein *et al.*, 1990; reviewed in Moran, 1993). Abraham *et al.*, (1993) found p300 interacts with TBP; a model was proposed in which E1A sequestered p300 from TFIID complexes, resulting in transcriptional repression.

1.3.5. Transformation.

Adenoviruses (type 12) were first shown to be oncogenic when it was shown that subcutaneous injection of Ad12 into newborn hamsters lead to the development of rapidly growing tumours at that site (Trentin *et al.*, 1962). The majority of human adenoviruses are non-tumorigenic in hamsters. (see Table 1.1.1.). It was later shown that

all these viruses could transform primary rodent cells (Freeman *et al.*, 1967). The genes responsible for oncogenicity and cell transformation were found to be located at the left end of the genome and comprised the early region 1 (reviewed in Dyson and Harlow, 1992).

The E1A gene products alone can immortalise primary cells in tissue culture (van der Eb *et al.*, 1979; Houweling *et al.*, 1980) but for full transformation cooperation with other oncogenes is required, these include the adenovirus E1B proteins, activated *ras* or polyoma middle T oncogenes (van den Elsen *et al.*, 1983; Ruley, 1983). The E1A nonconserved N-terminus, CR1 and CR2 domains are necessary for cell transformation, whereas the CR3 region is dispensable (Lillie *et al.*, 1986; Lee *et al.*, 1991). The regions important for transformation were found to strongly correlate with areas required for interactions with certain cellular proteins. This suggested that these interactions were important for the transforming function (Egan *et al.*, 1988; Whyte *et al.*, 1989; Howe and Bayley, 1992). Many of these proteins were first identified by co-immunoprecipitation with E1A proteins. Harlow *et al.*, (1986) found approximately ten proteins associated with E1A, and they ranged from 28K to 300K, Some of these have been identified and are described below.

p105 (or Rb) is the retinoblastoma tumour suppressor protein (Whyte *et al.*, 1989). This was the first of the proteins identified which was found to interact with E1A, it has also been found to interact with other virus proteins such as SV40 large T antigen and the papillomavirus E7 oncoprotein (Dyson *et al.*, 1989; Münger *et al.*, 1989). Activity of Rb is regulated by phosphorylation, which changes in different phases of the cell cycle (Ludlow *et al.*, 1990). The unphosphorylated form is thought to be the active form and in this state the protein is able to complex with E2F (Kaelin *et al.*, 1992). The Rb protein binds to E1A via a core binding sequence termed the pocket, which is conserved in Rb and the Rb related protein p107 (Corbeil and Branton, 1994 and references therein). The pocket consists of two regions A and B separated by a nonconserved spacer which is of different lengths in Rb and p107 (Raychaudhuri *et al.*, 1991). Rb binding to E1A requires both CR1 and CR2, but relies largely on CR2. p107 was first identified by association with E1A and SV40 large T antigen (reviewed in

Dyson and Harlow, 1992). This protein shares many structural and biochemical properties with Rb, including binding with E2F. However unlike the Rb protein, p107 can form a complex with cyclin A (p60) and is also found in complexes with cyclin E and cdk2 (Faha *et al.*, 1993). Zhou *et al.*, (1993), have isolated full length p107 cDNA and found that it can inhibit cell proliferation. A function for p107 has yet to be determined. However, owing to its many similarities to the Rb protein it seems likely it may be a tumour suppressor. Another protein that associates with E1A is p130, which shares some features with Rb and p107 proteins. One of these is that it contains the pocket region and has been shown to bind E1A and E2F via this area (Cobrinik *et al.*, 1993). Analysis of p130 cDNA revealed it was more closely related to p107 than Rb, as p130 and p107 have a similar sized spacer between domains A and B in the pocket region (Li *et al.*, 1993). p130 can interact with cyclins A and E, which is reminiscent of p107 interactions.

There are two active sites important for transformation in E1A, the first consists of CR2 plus the N-terminal end of CR1. Proteins that bind here belong to the Rb gene family (Rb, p107 and p130), and binding occurs via the pocket region. The second active site consists of the N-terminus of E1A and the C-terminus of CR1, the phosphoprotein p300 binds this region (for review see Moran, 1993) which has been shown to be responsible for repression (see section 1.3.4). Studies show that p300 is a component of TBP complexes, (Abraham *et al.*, 1993). In addition the sequence of the protein has features associated with a transcriptional adaptor molecule (co-activator) (Eckner *et al.*, 1994). It appears that p300 has similar functional properties to CBP which has been identified as a coactivator for protein kinase A (PKA) (Lundblad *et al.*, 1995). Adding support to the role of p300 as a coactivator, Arany *et al.*, (1995) has shown that CBP and p300 activate transcription when fused to a DNA binding domain. A model suggested for p300 in repression, is that E1A binds to p300 thereby preventing its role as a coactivator and results in the repression of promoters that require this coactivator function.

1.4. Early Region 1B.

1.4.1. Genomic Organisation of the E1B Region.

The Ad2 E1B region lies immediately adjacent to the E1A region, between map units 4.6 and 11.2 (see Fig 1.1.1.). The E1B DNA sequence has been determined for several serotypes including Ad2, 4, 5, 7, 12, 40, 41, CAV-2, MAV-1, SAV-16 and TAV (Bos *et al.*, 1981; Kimura *et al.*, 1981; Gingeras *et al.*, 1982; Dijkema *et al.*, 1982; Dekker *et al.*, 1984; Flügel *et al.*, 1985; Tokunaga *et al.*, 1986; van Loon *et al.*, 1987b; Ishino *et al.*, 1988; Shibata *et al.*, 1989). There are three open reading frames present in this region (see Fig 1.4.1), two of which are overlapping. Comparison of sequences revealed there was considerable homology spread over the region at both the DNA and amino acid levels (van Ormondt and Hesper, 1983; Ishino *et al.*, 1988). This was in contrast to E1A where defined areas were found to be highly conserved (see section 1.3.).

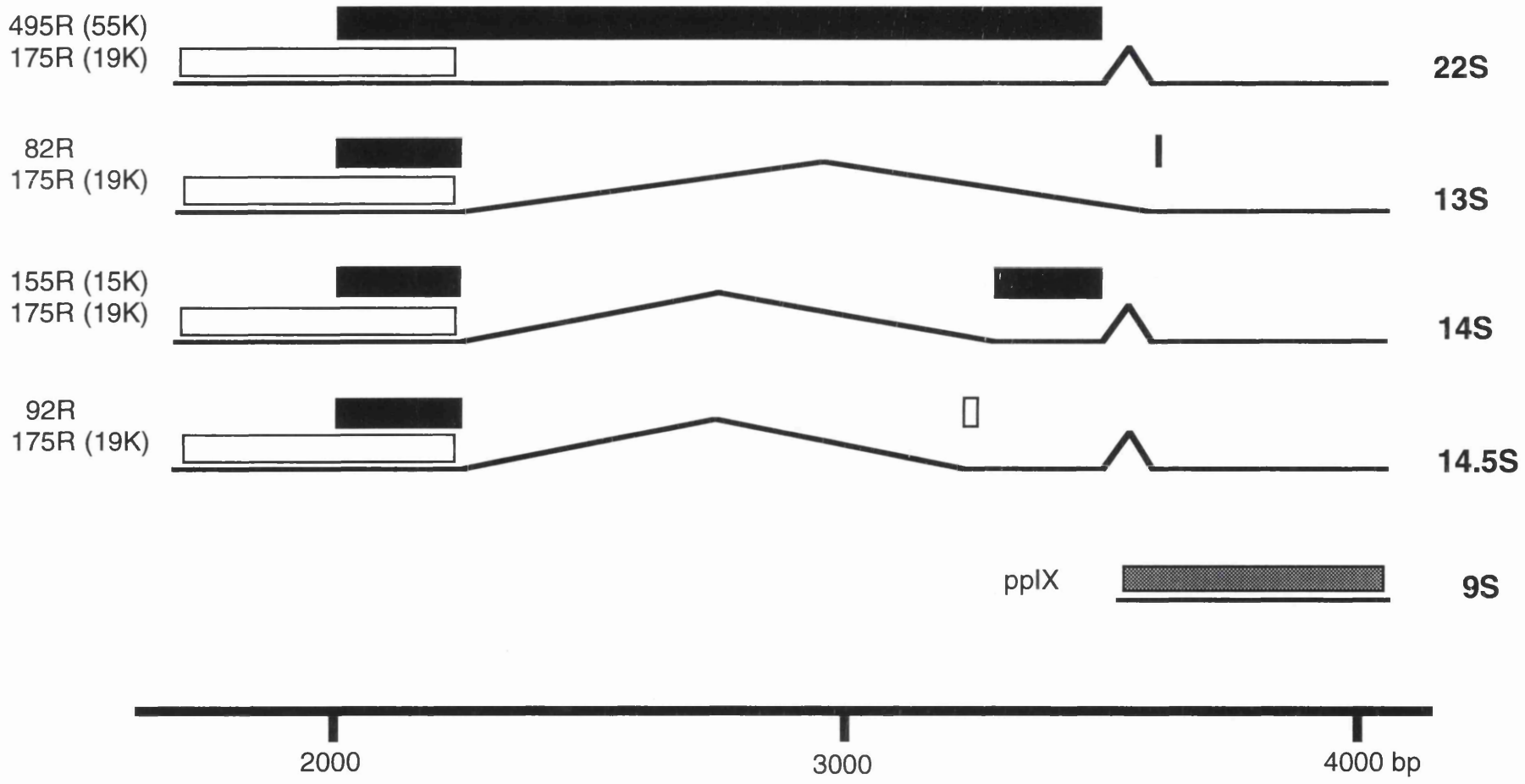
Transcription maps for many adenovirus serotypes are also now known including Ad2, 5, 7, 12, 40, 41 and MAV-1 (see Steinthorsdottir and Mautner, 1991 and references therein; Allard and Wadell, 1992). Figure 1.4.1. shows the Ad2 E1B transcription map and open reading frames. Transcripts made from the Ad2 E1B region are differentially spliced to produce two mRNA species of 22S and 13S, plus two minor species of 14.5S and 14S; all of these have common 5' and 3' termini (Berk and Sharp, 1978; Virtanen and Pettersson, 1985). A 9S mRNA is transcribed from its own promoter and encodes the structural protein ppIX.

Other adenovirus serotypes have similar but not identical organisation to Ad2. In Ad12, no 13S mRNA has been detected (Virtanen *et al.*, 1982) and the 14S transcript which is minor in Ad2, is major in Ad12. The MAV-1 E1B region has only two mRNAs, one corresponds to a 22S mRNA and the other is identical but differs with the presence of a splice which eliminates most of the first open reading frame. A more detailed analysis of the Ad40 and Ad41 E1B transcription has been carried out and will be discussed in detail elsewhere (see section 1.5.), however both were found to be more similar to Ad12 than Ad2 with 22S and 14S the predominant species and no 13S mRNA was detected.

Cotranscript counterparts of E1B mRNAs have been reported, which originate from the E1A promoter and terminate at the 3' end of E1B. They have been detected in

Figure 1.4.1. Transcription Map for the Ad2 E1B Region.

The sedimentation values (S) for mRNAs, and molecular weight (K) are shown, in addition the number of amino acids residues (R) for each protein is indicated. Solid lines denote RNA and introns are indicated by carets; shaded rectangles denote open reading frames.


495R (55K)
175R (19K)
22S
82R
175R (19K)
13S
155R (15K)
175R (19K)
14S
92R
175R (19K)
14.5S
pplX
9S
2000
3000
4000 bp

low amounts in Ad2-, Ad5- and Ad12-infected and-transformed cells (Berk and Sharp, 1978; Kitchingman and Westphal, 1980; Sawada and Fujinaga, 1980; Saito *et al.*, 1983; van den Elsen *et al.*, 1983; Hashimoto *et al.*, 1984). In Ad12, cotranscripts have the same 5' ends and splice sites as the major E1A mRNAs and extend to the 3' end of E1B, with a splice corresponding to the splice in E1B 22S mRNA. The Ad2 cotranscript has fewer than 50bp of the end of E1A linked to a site near the 5' end of E1B, however a detailed analysis of these mRNAs has not been carried out. Cotranscripts have also been detected in Ad40 infected cells (Hashimoto *et al.*, 1991; Steinthorsdottir and Mautner, 1991; Ishida *et al.*, 1994). A more detailed analysis of cotranscript structure with this serotype has been carried out and is described in section 1.5.

1.4.2. E1B Transcription.

Control of the E1B transcriptional unit is complex and occurs on many levels which include initiation of transcription, splicing and mRNA stability.

In Ad5, 22S mRNA is detected early in infection between 3-3.5 h p.i. and increases in abundance up to 9h, after which levels are constant through to 12h. The 13S transcript is detected at low levels until 8h p.i. and then increases in abundance; at 12h p.i. levels are similar to 22S. After this time 22S gradually decreases and at late times in infection the major transcript becomes the 13S mRNA (Glenn and Ricciardi, 1988). Minor E1B species were not studied by these authors, however Virtanen and Pettersson (1985) analysed the pattern for 14S and 14.5S mRNAs. They found that at 8h p.i. the 14S transcript was approx. 20 fold more abundant than 14.5S mRNA, while at late times in infection both species were detected in similar amounts.

Changes in the abundance of cytoplasmic E1B mRNAs is partly due to the relative changes in the half-life of 13S mRNA compared to 22S mRNA (Wilson and Darnell, 1981). Stability of E1B mRNAs is influenced by DBP (Babich and Nevins, 1981) which causes the rapid turnover of mRNA. These authors suggested that at late times 13S mRNA accumulates as it is no longer destabilised by DBP and 22S is thought not to accumulate as it is degraded by an unknown mechanism. Another factor that may

account for the differences in accumulation of E1B mRNA, is the change in specificity of the RNA splicing mechanisms. Montell *et al.*, (1984b) found that there is an increased propensity to use the 13S 5' splice site at late times in infection.

1.4.3. E1B Proteins.

The E1B 22S mRNA codes for two proteins (see Fig 1.4.1) from different overlapping reading frames (Bos *et al.*, 1981). The 19K protein is produced using the first AUG and comprises 175 amino acid residues (175R), the 55K protein (495R) is produced from the second AUG in a different reading frame (Perricaudet *et al.*, 1979; Bos *et al.*, 1981; Gingeras *et al.*, 1982). The 13S mRNA encodes the 19K protein, and a smaller species (82R) related to the 55K protein. 14S and 14.5S mRNAs encode proteins of 92R and 155R (15K), respectively (Green *et al.*, 1982; Lucher *et al.*, 1984; Lewis and Anderson; 1987). Both the 55K and 19K proteins will be discussed in more detail see sections 1.4.5. and 1.4.6.

1.4.4. E1B Promoter.

Unlike other early adenovirus genes (see Fig. 1.3.3.), E1B has a relatively simple promoter located within 50bp of the cap site (Wu *et al.*, 1987). It consists of a TATA box (TFIID binding site) and a GC box (Sp1 binding site). These motifs are present in the E1B transcriptional control region of all the adenovirus serotypes sequenced (see Fig 4.6.1.).

In the Ad2 E1B promoter the Sp1 binding site is situated -48 to -39bp and the TATA box -30 to -23bp with respect to the cap site (Wu *et al.*, 1987; Schmidt *et al.*, 1989). The GC box is not necessary for transactivation by E1A, which occurs through the TATA box (Wu *et al.*, 1987), but does act to stimulate transcription and deletion of this element reduces E1B transcription by approx. 7 fold. The spacing between the two elements was shown to be critical in Ad5 (Wu and Berk, 1988a). Increasing the distance between the two elements by insertion of additional sequences resulted in a decrease in

transcription (Wu and Berk 1988a; Segal and Berk, 1991). A 30bp separation decreases transcription levels such that the stimulatory effect of the GC box is eliminated. In addition to the proximal promoter elements described above upstream sequences have also been reported to play a role in E1B transcription (Parks *et al.*, 1988; Spector *et al.*, 1993). Four protein binding sites (I-IV) were identified in the region -250 to -120bp (with respect to the cap site) in DNase I footprint assays using KB cell nuclear extracts. These sites were found to stimulate transcription in *cis*, but only in the absence of the GC box (Parks *et al.*, 1988: Spector *et al.*, 1993). An additional site (V) which is situated adjacent to the E1B GC box has also been reported (Spector *et al.*, 1993). However other studies have reported that this distal region (-250 to -125) makes only a modest contribution to E1B transcription *in vivo* (Wu *et al.*, 1987; Wu and Berk, 1988b).

The transcription factor Sp1 activates transcription from both viral and cellular genes which have a GC box sequence (reviewed in Kadonaga *et al.*, 1986; Courey and Tjian, 1992). A comparison of a large number of binding sites for Sp1 (Kadonaga *et al.*, 1986) led to a consensus recognition sequence (5' G/T GGGCGG G/A G/A C/T 3'). Most Sp1 binding sites do not differ from the consensus by more than one or two bases, with the central residues highly conserved. Purified Sp1 appears on a SDS-PAGE gel as 2 bands of 95K and 105K. Analysis has shown that this heterogeneity is due to posttranslational modifications of a single polypeptide (Kadonaga *et al.*, 1987; Jackson *et al.*, 1990). The two forms of Sp1 arise by differential phosphorylation. Jackson *et al.*, (1990) showed that Sp1 is multiply phosphorylated by a kinase that requires the presence of DNA. The kinase was later identified as DNA-dependent protein kinase (DNA-PK) (Gottlieb and Jackson, 1993 and references therein). Sp1 has several O-linked N-acetyl glucosamine residues and these appear to enhance the stimulatory effect of Sp1 to activate transcription *in vitro* (see Courey and Tjian, 1992 and references therein). Sp1 can be purified from HeLa cells (Briggs *et al.*, 1986), and its cDNA has been cloned and sequenced (Kadonaga *et al.*, 1987). Analysis of Sp1 structure and function in mutagenesis studies revealed discrete functional domains within the protein. The carboxy terminal 168 amino acids comprise the DNA-binding domain which consists of three zinc fingers

(Cys-2His-2) that bind to the GC box. The amino terminus contains several regions that mediate transcriptional activation, two of these are glutamine-rich.

Sp1 activates transcription in a synergistic manner when more than one GC box is present, at proximal and distal sites (Courey et al 1989; Pascal and Tjian, 1991). This synergy involves direct contact between Sp1 molecules bound at two sites, with looping out of the intervening DNA. (Pascal and Tjian, 1991). A DNA-binding deficient form of Sp1 can interact with DNA-binding competent forms of the protein to stimulate transcription synergistically; this phenomenon is known as superactivation. Both these observations suggest that Sp1 binds to DNA and then activates the basic transcriptional apparatus through protein-protein interactions. *In vitro* studies show that Sp1 communicates with TFIID through a coactivator TAF protein, TAF110 (Pugh and Tjian, 1990; Hoey *et al.*, 1993).

1.4.5. The E1B 19K Protein.

The 19 kilodalton (19K) protein is located in the nuclear and cytoplasmic membranes of infected (Ad2 & Ad12) and transformed (Ad12) cells (Persson *et al.*, 1982; Grand and Gallimore, 1984; White *et al.*, 1984a; Smith *et al.*, 1989; Mitchison *et al.*, 1990) and has been found to associate with intermediate filaments (White and Cipriani, 1989, 1990). Immunofluorescence studies show that 19K is present in the cytoplasm early in infection and accumulates in the nucleus late in infection. The 19K protein is post-translationally modified; it is acylated with palmitate and myristate residues (McGlade *et al.*, 1987). Acylation may have a role in the membrane association of this protein. Another modification is that a subfraction of molecules are phosphorylated (at Ser-164), however this has not shown to be essential for the function of the protein (McGlade *et al.*, 1989). Mutants defective in the synthesis of the 19K protein have various phenotypes; these include an enhanced cytopathic effect in human cells (*cyt* phenotype) (Pilder *et al.*, 1984; Subramanian *et al.*, 1984; Takemori *et al.*, 1984) and the formation of large plaques (*lp* phenotype) in KB, HeLa and A549 cells (Chinnadurai *et al.*, 1983). In many cell lines, some of these mutants also elicit a DNA degradation

phenotype (*deg*), with extensive degradation of cellular and viral DNA, (Ezoe *et al.*, 1981; Lai Fatt and Mak, 1982; Pilder *et al.*, 1984; Subramanian *et al.*, 1984; White *et al.*, 1984b) resulting in a reduced yield of virus (Subramanian *et al.*, 1984). 19K is therefore thought to have an important role in the virus replication cycle and is necessary for stabilising viral and cellular DNAs (reviewed in Stillman, 1986; Boulanger and Blair, 1991).

In a number of studies 19K (loss of function) mutants were shown to be defective for complete transformation and the 19K protein required for oncogenic transformation (see Boulanger and Blair, 1991, and references therein; McLorie *et al.*, 1991). DNA transfection studies show that either E1B 19K or 55K proteins can cooperate with E1A to induce transformation (at reduced levels) by independent but additive pathways (White and Cipriani, 1990; McLorie *et al.*, 1991). In contrast, others have found that the 19K protein is not required for transformation (Zhang *et al.*, 1992a; Telling and Williams, 1993; Telling *et al.*, 1994). There is no clear explanation for the differences observed among these studies. Zhang et al (1992a) proposed these differences (for Ad12) could be due to an overexpression of 55K protein observed with a 19K mutant in which the 19K start codon is removed. Elimination of this site, which normally suppresses induction at the downstream 55K start codon, leads to overexpression of the 55K protein and efficient transformation of BRK cells. However no overexpression is detected with comparable Ad5 mutants and transformation efficiencies with these mutants is low (White and Cipriani, 1990; McLorie *et al.*, 1991).

Adenovirus early gene expression triggers apoptosis or programmed cell death which is the process by which a cell can actively commit suicide under tightly controlled circumstances. Apoptosis has an important role in normal mammalian development and abnormal regulation can lead to neoplastic transformation, in addition it is a defence against cancer and removes virally infected cells (for reviews see White, 1994, 1995). The effect of apoptosis in adenovirus was first shown with E1B 19K loss of function mutants, which displayed *cyt* and *deg* phenotypes, also DNA fragmentation. These properties were also shown to be characteristics associated with apoptosis (see White, 1995, and references therein). The DNA fragmentation characteristic of apoptosis is the

digestion of cellular DNA into nucleosome sized pieces, indicating that DNA is cleaved between nucleosomes in chromatin (Wyllie *et al.*, 1980). A pattern of nucleosomal DNA fragmentation more associated with apoptosis was observed with a 19K temperature sensitive mutant (Rao *et al.*, 1992).

Studies with 19K defective mutants suggested that this protein acted to suppress apoptosis and another viral protein was the trigger for apoptosis (White and Stillman, 1987). The induction of apoptosis by adenovirus was mapped to the E1A gene by constructing and analysing recombinant adenoviruses with mutations in various genes in the background of an E1B 19K mutation. When E1A was not expressed, this led to an absence of the effects of apoptosis and suggested that E1A was the factor that induced apoptosis (White and Stillman, 1987; White *et al.*, 1991).

Apoptosis contributes to the transformation process; this was shown with studies using BRK cells (White *et al.*, 1992). When E1A alone was expressed, foci formation was observed but not sustained. However coexpression of E1A with a second cooperating oncogene, E1B, allowed growth of transformed foci to be sustained. This indicated that E1B proteins contributed to transformation by suppressing cell death (Rao *et al.*, 1992; White *et al.*, 1992). Similarly a known inhibitor of apoptosis, the Bcl-2 protein, when coexpressed with E1A decreases cell death and increases transformation efficiencies (Rao *et al.*, 1992). The 19K protein shows structural and functional homology to Bcl-2, indicating it might be a member of the Bcl-2 family. Both the E1B proteins 19K and 55K inhibit E1A induced apoptosis (Rao *et al.*, 1992). The 55K protein binds to and inhibits the function of p53 (tumour suppressor) in transformed cells (Yew and Berk, 1992, Yew *et al.*, 1994). As 55K expression also inhibits apoptosis in transformation studies, this indicates that inactivation of p53 may prevent apoptosis. The E1B 19K protein is a more potent inhibitor of apoptosis than 55K in transformed cells (Rao *et al.*, 1992). The consequence of the induction of apoptosis by E1A during infection and in the absence of 19K is the premature death of the host cell, which can substantially impair virus yield (Pilder *et al.*, 1984; Subramanian *et al.*, 1984). The suggestion that the 19K protein acts at a common step in the apoptosis pathway came from the ability of 19K to inhibit

apoptosis induced by TNF-α and Fas antigen, and also with the drug cisplatin (Gooding *et al.*, 1991; White *et al.*, 1992; Subramanian, 1993; Chiou *et al.*, 1994).

There are several lines of evidence indicating that p53 has a role in E1A-induced apoptosis: 55K can bind to and inhibit p53 which will also suppress apoptosis (Sarnow *et al.*, 1982b; Yew *et al.*, 1994); E1A expression produces p53 accumulation (Lowe and Ruley, 1993; Chiou *et al.*, 1994); dominant negative mutant forms of p53 suppress induction of apoptosis by E1A (Debbas and White, 1993) while returning p53 to wild type results in apoptosis. This suggested that p53 was necessary for the induction of apoptosis by E1A with p53 perhaps acting as a molecular switch for E1A induced apoptosis. The 19K and Bcl-2 proteins were later shown to inhibit the induction of apoptosis by p53 (Debbas and White, 1993; Chiou *et al.*, 1994). Using BRK cell lines transformed with E1A which contained a temperature sensitive p53 mutant, introduction of either the 19K gene or bcl-2 gene prevented apoptotic cell death with wild type p53 (Debbas and White, 1993). p53 can induce either apoptosis or growth arrest depending on the physiological conditions (reviewed in White, 1994, 1995). 19K (and Bcl-2) can inhibit p53-mediated apoptosis with p53-mediated growth arrest left intact (Chiou *et al.*, 1994). This suggests that apoptosis and growth arrest activities of p53 are separable.

p53 is a transcription factor which can repress or transactivate genes (reviewed in Zambetti and Levine, 1993). Sabbatini *et al.*, (1995) have shown that 19K alleviates p53 mediated transcriptional repression but does not affect p53 mediated transactivation. These authors suggest that p53 could induce apoptosis via a generalised transrepression mechanism and 19K inhibits this by modulating the transrepression function of p53.

The precise mechanism of how E1B inhibits apoptosis is not known yet. However studies have been carried out to identify cellular proteins that bind E1B 19K in an attempt to understand the molecular basis of this inhibition (Farrow *et al.*, 1995; Han *et al.*, 1996 and references therein). A few proteins were found to bind both 19K and Bcl-2, one of these is Bax which is p53-inducible (Han *et al.*, 1996). Interaction of Bax with Bcl-2 prevents the inhibition of apoptosis and interaction with 19K similarly blocks the ability of 19K to inhibit apoptosis (Chen *et al.*, 1996). Chen *et al.*, (1996) have recently shown that 19K does not interact with the Bad protein, but Bcl-2 will bind it. Bad, is a cellular

protein that regulates the Bcl-2/Bax interaction and restores susceptibility to continue down the apoptosis pathway. Another cellular protein that binds 19K is Bak, a member of the Bcl-2 family (Farrow *et al.*, 1995).

1.4.6. The E1B 55K Protein and Related Polypeptides.

The Ad2 22S mRNA encodes the 55 kilodalton (55K or 495R) protein (see Fig 1.4.1 and section 1.4.1). In addition there are 55K related proteins (82R, 92R and 155R or 15K) which are encoded by the smaller mRNA species (see Fig 1.4.1) (for reviews see Stillman, 1986; Boulanger and Blair, 1991).

A number of studies have shown that the 55K protein is phosphorylated at both serine and threonine residues (Malette *et al.*, 1983; Spindler *et al.*, 1984; Schughurt *et al.*, 1985). The Ad5 15K (153R) protein was also shown to be phosphorylated, and both the 84R and 93R proteins were found to be either unphosphorylated or phosphorylated at levels below detection (Takayesu *et al.*, 1994). Experiments by Teodoro *et al.*, (1995), in which phosphorylation sites were mutated, indicated that C-terminal phosphorylation sites may be important for transformation

The 55K protein is found both in the nucleus and cytoplasm of infected cells, although it is predominantly located within the nucleus (Sarnow *et al.*, 1984; Ornelles and Shenk, 1991). In the cytoplasm it is found in a diffuse pattern and in an intensely stained fibrous body adjacent to the nucleus (Ornelles and Shenk, 1991). In the nucleus the protein is observed in a granular diffuse pattern and also in local concentrations both within and near the periphery of virus-specific inclusion bodies. Different locations may reflect different functional forms and different interactions of proteins.

The 55K protein is found in complex with the E4 34K protein in infected cells (Sarnow *et al.*, 1984; Cutt *et al.*, 1987; Leppard and Shenk, 1989); both proteins are also detected uncomplexed (Cutt *et al.*, 1987; Smiley *et al.*, 1990). The E1B 55K/ E4 34K complex is present in nuclei of infected cells in association with the nuclear viral inclusion bodies which are thought to be the sites of viral DNA synthesis and transcription (Moyne *et al.*, 1978; Wolgemuth and Hsu; 1981; Chaly and Chen, 1993).

Association of the 55K protein with nuclear pore complexes was studied by Smiley *et al.*, (1990); no stable complex was detected although a portion of 55K was fractionated with the pore complex lamina. They suggested that 55K may affect an early step in RNA transport as opposed to modulating the function of nuclear pore complexes. This was in agreement with Ornelles and Shenk, (1991) who proposed that the 55K/34K complex associates with and sequesters a nuclear factor to the periphery of inclusion bodies, which is necessary for the transport of mRNAs from their site of processing in the nucleus to the nuclear pores. Indeed, nuclear factors that may be involved have been identified in studies using *in vitro* transport systems (Moffet and Webb, 1983; Subramaniam *et al.*, 1990). In adenovirus subgroup C transformed cells the 55K protein is present in complex with the cellular protein p53 (Sarnow *et al.*, 1982b; Zantema *et al.*, 1985a; Kao *et al.*, 1990).

The 55K protein is necessary for the productive infection of most cell types and virus yield is severely impaired in the absence of wild type 55K protein. Several virus mutant studies have shown that the 55K protein is essential for the accumulation of late viral mRNAs in the cytoplasm, late viral protein synthesis and shutoff of host metabolism (Babiss and Ginsberg, 1984; Babiss *et al.*, 1985; Halbert *et al.*, 1985; Bernards *et al.*, 1986; Pilder et al 1986; Williams *et al.*, 1986; Barker and Berk, 1987; Leppard and Shenk, 1989; Bridge and Ketner, 1990; McLorie *et al.*, 1991). Mutants in the E4 ORF6 gene which result in the absence of a functional 34K protein, also display a similar phenotype (Halbert *et al.*, 1985; Weinberg and Ketner, 1986; Cutt *et al.*, 1987; Bridge and Ketner, 1990). Double mutants (defective for both proteins) have no more severe phenotype than single mutants with either mutation (Cutt *et al.*, 1987; Bridge and Ketner, 1990). This indicates that the 55K/34K complex is important for the function of the 55K protein.

In Ad5 the absence of 55K, viral DNA replication and transcription of late genes is normal and this suggests that the 55K protein regulates gene expression post transcriptionally, either by facilitating viral mRNA transport from the nucleus or by stabilising the mRNA as it enters the cytoplasm (Babiss and Ginsberg, 1984; Babiss *et al.*, 1985; Halbert *et al.*, 1985; Pilder *et al.*, 1986; Leppard and Shenk, 1989; Sandler and Ketner, 1989; Bridge and Ketner, 1990). Leppard and Shenk (1989) more precisely

defined this by showing that the 55K protein acts in the nucleus on viral RNA. These authors monitored the movement of newly synthesised mRNA through a series of biochemically defined nuclear subfractions. Cells infected with a 55K defective virus failed to accumulate viral late transcripts in a nuclear compartment. RNA which had left the nuclear matrix accumulated in this compartment before interacting with the nuclear envelope. Furthermore the absence of either 55K or 34K or both proteins, resulted in a reduction of late viral mRNA in the nucleus in addition to defective nuclear mRNA transport (Sandler and Ketner, 1989; Bridge and Ketner, 1990). This agrees with the view that the 55K/34K complex facilitates viral RNA metabolism at an intranuclear step. This could occur by preserving the pool and stability of fully processed mRNA before translocation to the cytoplasm or even facilitating the translocation step itself (Ornelles and Shenk, 1991). Leppard (1993) looked at the extent late mRNA was dependent on 55K for accumulation in the cytoplasm, by studying the cytoplasmic levels of individual early and late mRNAs in a wild type infection and with a 55K mutant. All of the transcripts from the MLTU showed dependence on 55K, although the level of dependence varied among the differentially spliced products. The longer transcripts showed the greatest dependence on 55K. This was in agreement with the model that 55K facilitates the movement of mature viral mRNA from the nucleus to the cytoplasm. The localisation of 55K (and E4 34K) has recently been studied in the absence of other adenovirus proteins in yeast cells and transfection studies (Liang *et al.*, 1995: Goodrum *et al.*, 1996). In these studies 55K is predominantly cytoplasmic, as it is in adenovirus-transformed cells (Zantema *et al.*, 1985a, b; van den Heuvel *et al.*, 1993). Liang *et al.*, (1995) overcame cytoplasmic restriction of the 55K protein in yeast cells by attaching a nuclear localisation signal to the protein. In transfection studies the E4 34K protein directed the 55K protein to the nucleus in primate cells, but not rodent or most mouse cell lines (Goodrum *et al.*, 1996). These authors suggest that a primate factor may be involved in the interaction of 55K and 34K proteins.

Most of the knowledge about 55K has come from studies with group C adenoviruses. The Ad12 equivalent is the 54K (482R) protein and it is also required for productive infection, late protein synthesis and accumulation of viral mRNA (Shiroki *et*

al., 1986; Brieding *et al.*, 1988; Mak and Mak, 1990). However in contrast to subgroup C viruses the 54K protein is also necessary for viral DNA replication (Zhang *et al.*, 1992b). With a 54K (loss of function) mutant (Zhang *et al.*, 1992b) both viral DNA replication and late viral protein synthesis was defective. Levels of E1A transcripts were normal in this study but the accumulation of E2B and E4 mRNAs both at early and late times were severely reduced. This suggests that the need for 55K in Ad12 DNA replication could be related to its role in the metabolism of E2B and E4 transcripts, which are required for viral DNA synthesis.

There were also differences observed in the host range phenotype of adenovirus group C (Ad5) and group A (Ad12) viruses with 55K/54K mutations. Ad5 mutants that do not grow efficiently on HeLa cells, will grow to WT levels on HEK cells (Harrison *et al.*, 1977), whereas Ad12 mutants do not grow efficiently on HEK cells (Brieding *et al.*, 1988).

The role of 55K in transformation and virus replication were shown to be separable in mutational studies (Mak and Mak, 1990; Yew *et al.*, 1990). Studies with group A and C adenoviruses have shown that the larger E1B protein is required for complete transformation of rodent cells *in vitro* (Logan *et al.*, 1984; Bernards *et al.*, 1986; Barker and Berk, 1987; Byrd *et al.*, 1988). 55K was shown to be required in both viral and plasmid DNA-mediated transformation, as mutations in this protein abolished transformation by these means (Bernards *et al.*, 1986; Barker and Berk, 1987; White and Cipriani, 1990; McLorie *et al.*, 1991; Rao *et al.*, 1992; Zhang *et al.*, 1992a). 55K can function independently of 19K, but transformation efficiency is increased when both are expressed (McLorie *et al.*, 1991). The 55K protein may derive its transforming activity from interactions with and inactivation of the cellular p53 tumour suppressor (Sarnow *et al.*, 1982b; Kao *et al.*, 1990; Yew and Berk, 1992).

In Ad5 transformed cells, p53 is associated with the 55K protein and sequesters it into a filamentous cytoplasmic complex (Sarnow *et al.*, 1982b; Zantema *et al.*, 1985a, b). It was proposed this sequestration could be a possible mechanism of how 55K inactivates p53. In studies with Ad12 transformed cells, initially no association between p53 and 55K was detected although p53 was present in the nucleus and inhibition of p53 was still

detected (Zantema *et al.*, 1985b; Yew and Berk, 1992). Grand *et al.*, (1994) have shown the Ad12 54K protein can be found in complex with p53, albeit in small amounts using a different experimental protocol, indicating that the mechanism for inhibition of p53 function in Ad12 may be similar to Ad5. Indeed, both the Ad5 and Ad12 proteins (55K/54K) contribute equally well to transformation of primary cells in culture (Bernards *et al.*, 1982). This is supported by the observation that the half life of p53 is strongly increased in Ad5 and Ad12 transformed cells (Zantema *et al.*, 1985b), and that both proteins (55K and 54K) are able to inhibit p53-mediated transcriptional activation (Yew and Berk, 1992).

Another possible mechanism as to how p53 function is inhibited by the larger E1B protein, is that it (55K) interferes with the ability of p53 to bind its DNA recognition sequence. Kao *et al.*, (1990) showed that 55K binds to the activation domain of p53 and thus may block it from interacting with possible targets. However a 55K mutant which bound p53 like wild type was defective in transformation and inhibition of p53 mediated transcriptional activation (Yew and Berk, 1992), suggesting 55K could play a larger part than simply blocking the p53 activation domain. Yew and Berk, (1994), have shown that 55K acts as a direct transcriptional repressor. The binding of 55K to p53 does not interfere with the ability of p53 to bind DNA. Furthermore a fusion of 55K to the Gal4 DNA binding domain can function as a transcriptional repressor. These results indicate 55K contributes to adenovirus transformation by functioning as a direct transcriptional repressor targeted to p53 activated genes.

1.5. Enteric Adenoviruses.

1.5.1. Identification and Classification.

Enteric adenoviruses comprise serotypes Ad40 and Ad41, belonging to subgroup F adenoviruses (for reviews see Albert, 1986; Wadell *et al.*, 1994; Uhnoo *et al.*, 1990; Mautner *et al.*, 1995; Tiemessen and Kidd, 1995). These viruses were first observed in several studies by EM analysis of stool samples from infants with acute gastroenteritis. Approximately 10^{11} virus particles were detected per gram of sample (Gary *et al.*, 1979; Retter *et al.*, 1979; Takiff and Straus, 1982), these virus particles could not be cultivated on conventional cell lines used for the propagation of other adenoviruses (Flewett *et al.*, 1975; Madeley *et al.*, 1977; Retter *et al.*, 1979), but could grow in 293 cells (Graham *et al.*, 1977; Takiff *et al.*, 1981). These viruses were referred to as uncultivatable, fastidious or enteric adenoviruses (Jacobsson *et al.*, 1979; Retter *et al.*, 1979; Kidd and Madeley, 1981). The enteric adenoviruses were shown to be distinct from the established adenoviruses by serology and DNA restriction analysis (Jacobsson *et al.*, 1979; Johansson *et al.*, 1980). Studies on large numbers of clinical isolates revealed the presence of two enteric adenovirus serotypes (Ad40 and Ad41) (de Jong *et al.*, 1983; Uhnoo *et al.*, 1983) with different restriction patterns (Uhnoo *et al.*, 1983; Takiff *et al.*, 1984; Adrian *et al.*, 1986). These two serotypes are now classified separately as subgroup F adenoviruses; this was based on various criteria including immunological cross reactivity, DNA homology and size of internal structural polypeptides (van Loon *et al.*, 1985b; Hierholzer *et al.*, 1988). A number of Ad41 and Ad40 DNA variants have been observed through restriction enzyme analysis (Kidd *et al.*, 1984; van der Avoort *et al.*, 1989). van der Avoort *et al.*, (1989) described 11 Ad40 DNA variants and 24 Ad41 DNA variants, this has now been extended to 28 (cited by Tiemessen and Kidd, 1995).

Human adenoviruses had been suspected of causing gastroenteritis, this was difficult to prove and was hampered by the prolonged period of asymptomatic faecal shedding after respiratory tract infection observed with some adenoviruses (Fox *et al.*, 1977). Subgroup F adenoviruses were established as causal agents of gastroenteritis in epidemiological and clinical studies (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).

Furthermore some studies show that enteric adenoviruses are second only to rotaviruses as the most common cause of infantile viral gastroenteritis (Estes *et al.*, 1983; Uhnoo *et al.*, 1984; Brandt *et al.*, 1985; Kotloff *et al.*, 1989). Subgroup A adenoviruses (Ad12, 18 & 31) have also been associated with diarrhoea in immunocompromised adults (Hierholzer, 1992).

1.5.2. Epidemiology.

In both developed and developing countries enteric adenoviruses have been detected in stool samples of infants and young children with acute gastroenteritis (reviewed in Uhnoo *et al.*, 1990; Wadell *et al.*, 1994; Mautner *et al.*, 1995). In many studies from around the world (see Mautner *et al.*, 1995, and references therein), enteric adenoviruses are reported to be associated with approximately 5 to 20 percent of paediatric diarrhoea. Enteric adenovirus gastroenteritis is endemic and appears to have no seasonality (Uhnoo *et al.*, 1984; Brandt *et al.*, 1985; Johansson *et al.*, 1985; Kotloff *et al.*, 1989). Initially both enteric adenovirus serotypes were isolated with equal frequency (de Jong *et al.*, 1983; Johansson *et al.*, 1985), however more recently the incidence of Ad40 as a cause of gastroenteritis was found to have fallen dramatically, while Ad41 has increased and is now the predominant serotype (de Jong *et al.*, 1983; Brown *et al.*, 1990; Shinozaki *et al.*, 1991).

1.5.3. Pathogenesis.

The clinical characteristics of enteric adenovirus infection are described in detail elsewhere (see Uhnoo *et al.*, 1984, 1990). The incubation period is 8 to 10 days (Richmond *et al.*, 1979), considerably longer than the 1 to 3 days seen with rotavirus. Nosocomial outbreaks occur (Rodriguez *et al.*, 1985; Kotloff et al 1989), but spread to adults is not common (Chiba *et al.*, 1983). Illness typically lasts 5 to 12 days (Uhnoo *et al.*, 1984; Kotloff *et al.*, 1989) but can occasionally last for more than two weeks. Enteric adenoviral diarrhoea lasts longer than other types of viral gastroenteritis. The most

prominent feature of the disease is a watery diarrhoea which is followed by 1 to 2 days of vomiting (Uhnoo *et al.*, 1984). Various other symptoms include low grade fever which lasts 2 to 3 days and dehydration (Yolken *et al.*, 1982; Uhnoo *et al.*, 1984). Respiratory tract symptoms have been reported, but are less common. Although the mean duration of diarrhoea was longer in Ad41 (12.2 days) than Ad40 (8.6 days), both viruses gave similar symptoms in patients. Studies show that enteric adenoviruses cause a more protracted but milder infection than do rotavirus, with reduced frequency of vomiting and moderate elevation of fever. In addition, enteric adenoviruses have been associated with fatal disease (Whitelaw *et al.*, 1977; Johansson *et al.*, 1985); Ad41 virus particles were isolated from cells in the small intestine of a fatal case of gastroenteritis.

1.5.4. The Molecular Biology of the Enteric Adenoviruses.

The overall DNA homology, determined by liquid phase hybridisation, between Ad40 and Ad41 is 62 to 69 percent (van Loon *et al.*, 1985b), whereas enteric adenovirus identity compared to Ad5 is 15 to 20 percent. Homology within subgroup F is similar to that within subgroup A, but lower than seen within subgroups B to E (see Table 1.1.1.). The complete nucleotide sequence of Ad40 strain Dugan has been reported (Davison *et al.*, 1993) and is comprised of 34,214 nt. It has a similar genomic organisation to Ad2 and Ad5, with a few distinct differences, namely the presence of two fibre genes, one VA gene and variation in the E3 region. The Ad12 genome has also recently been completed (Sprengel *et al.*, 1994) and is 34,125nt long, closer in length to Ad40 than Ad2 at 35,937nt.

Two novel protein coding regions were identified by Davison *et al.*, (1993) in Ad40, one orientated rightward within the L4 33K protein coding region, the other a leftward 52R exon located between regions E3 and L5. These authors found this region highly conserved among human serotypes and propose it may be functionally significant.

Phylogenetical analysis among adenoviruses by comparison of DNA sequence revealed that Ad40 and Ad12 are more closely related than was previously shown by DNA hybridisation studies (Bailey and Mautner, 1994). Comparison of enteric

adenoviruses to other adenoviruses is based on DNA homology and phylogeny comparisons of protein-coding regions (see Mautner *et al.*, 1995, and references therein).

1.5.4.1. Early Regions.

The ITRs of Ad40, Ad41 and Ad12 are of similar length at 163, 163 and 161bp respectively. The Ad40 sequence shows more sequence homology to Ad5 than to any of the other adenovirus serotypes, especially in the first 60-70bp (Ishino *et al.*, 1987; Shinagawa *et al.*, 1987; Allard and Wadell, 1988; Bailey and Mautner, 1994).

Extensive study of the E1 region of subgroup F adenoviruses has been carried out primarily as it is implicated in the fastidious growth of these viruses. van Loon *et al.*, (1987b) first determined the E1 sequences of both Ad40 and Ad41. These authors found they were 85% identical to each other and were 52% homologous to the Ad5 E1 region. E1 DNA sequences have now been determined for different strains of both enteric adenovirus serotypes: Ad40 Dugan (van Loon *et al.*, 1987b; Davison *et al.*, 1993), Ad40 Sapporo (Ishino *et al.*, 1988;), Ad41 Tak (van Loon *et al.*, 1987b) and Ad41 D389 (Allard and Wadell, 1988, 1992). The overall organisation of the E1 region appears to be similar to that of other adenoviruses and ORFs have been identified which correspond to the E1A and E1B regions of other serotypes. E1A conserved regions (CR1 to CR3) have been identified in Ad40 and Ad41 (van Loon *et al.*, 1987b; Allard and Wadell, 1988; Ishino *et al.*, 1988;). The conserved regions of Ad7, Ad12 and SAV-7 share 60% homology to equivalent Ad5 regions while Ad40 and Ad41 have 51% and 45% homology to Ad5 conserved regions, respectively (van Loon *et al.*, 1987b). CR1 is the least well conserved, although CR2 and CR3 also have low homology with Ad5 compared to other serotypes. In comparisons of E1B protein -coding sequences, both Ad40 E1B ORFs (19K & 55K) are more similar to Ad12 than to Ad2 (Ishino *et al.*, 1988). Although no conserved regions have been identified in E1B, an alanine rich region near the N-terminal of the 55K protein present in Ad2 and Ad12 is not found in Ad40, but a central domain in 55K that binds p53 and is important for the transformation

activity of Ad2 is moderately conserved in both Ad12 and Ad40 (Yew and Berk, 1992; Yew *et al.*, 1990, 1994).

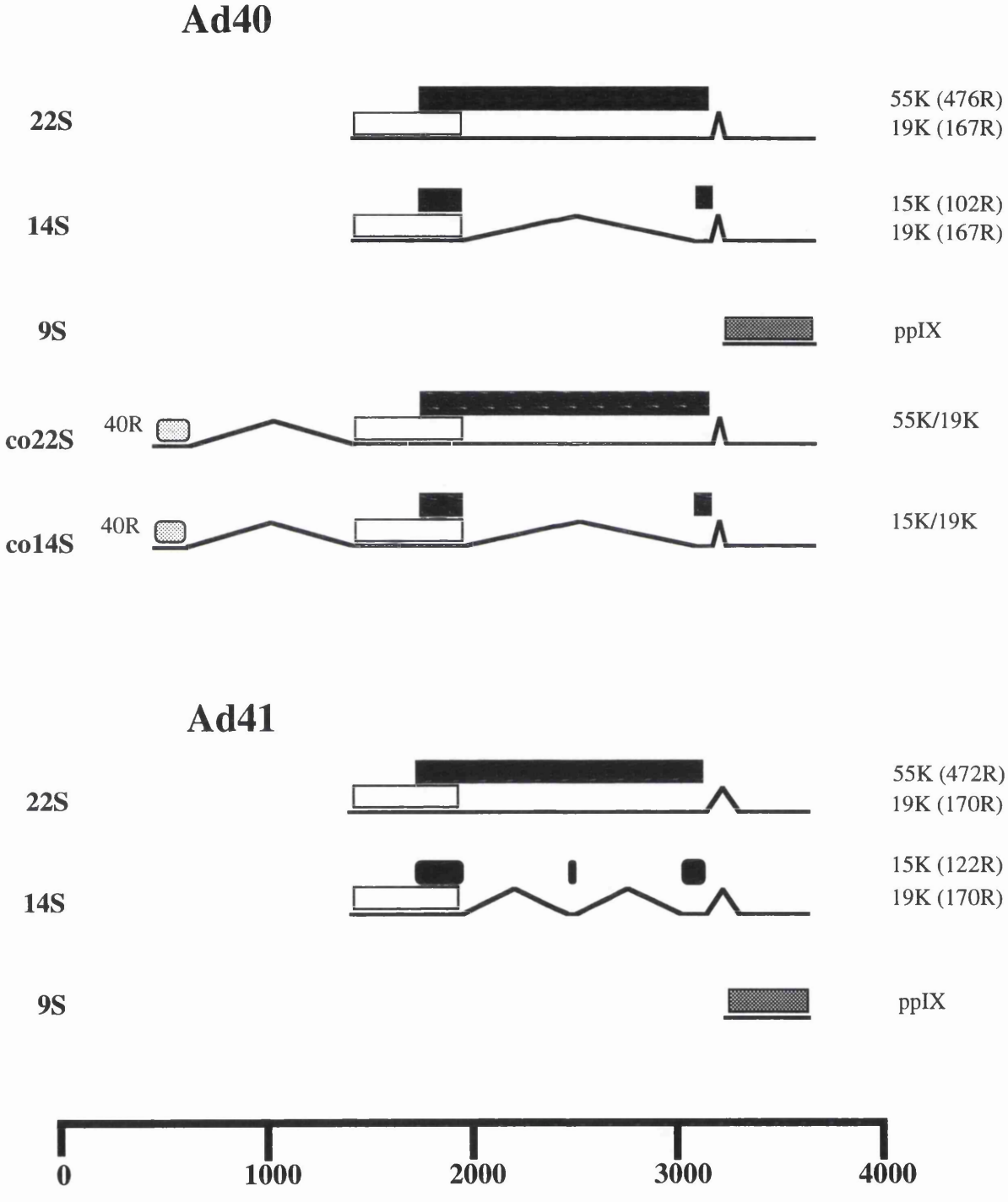
E1 transcription maps for Ad40 and Ad41 have been determined in cells transformed with Ad40 and Ad41 E1 plasmids (van Loon *et al.*, 1985a, 1987b). For Ad40 the three E1A mRNA species corresponding to Ad2 9S, 12S and 13S mRNAs were detected. However no Ad40 E1B mRNA was detected in these cells (van Loon *et al.*, 1985a). For Ad41 transformed cells only the E1A 13S transcript and E1B 22S mRNA were detected. Both the Ad40 and Ad41 E1B transcription maps have been determined in a lytic infection (Steinthorsdottir and Mautner, 1991; Allard and Wadell, 1992) (see Figure 1.5.1). Both viruses have transcripts equivalent to the Ad2 14S and 22S mRNA but lack 13S mRNA which is the major species in the Ad2 E1B region. The Ad12 E1B transcription map also has the E1B 14S mRNA as a major transcript with no 13S detected (Virtanen *et al.*, 1982; Virtanen and Pettersson, 1985). In addition E1A-E1B cotranscript counterparts of the 14S and 22S mRNAs were detected in Ad40, which contained the first 40 codons of the E1A region spliced to a site 4-5nt downstream of the E1B cap site. The splice junction is unusual in that it does not conform to splice consensus sequences (Steinthorsdottir and Mautner, 1991; Ishida *et al.*, 1994). In Ad41 an additional small exon is detected in the 14S mRNA that is not observed in Ad40 (Allard and Wadell, 1992).

The E2A gene (encoding the DBP) has been identified in Ad40 and Ad41 (Vos *et al.*, 1988). The enteric adenoviruses have smaller DBPs (Ad40 is 473R and Ad41 is 474R) compared to Ad5 (529R). The Ad40 pTP compared to the Ad2 pTP and the Ad40 Adpol protein compared to Ad2 Adpol, share good DNA homology. In contrast less DNA homology was detected between the Ad40 DBP and the Ad2 DBP (Davison *et al.*, 1993).

The Ad40 (Davison *et al.*, 1993) and Ad41 (Yeh *et al.*, 1996) E3 sequences are almost identical. The E3 ORFs from Ad40, 12 and 2 were compared by Mautner *et al.*, (1995), these authors found the E3B region moderately conserved. The Ad40 E3B proteins 90R, 107R and 122R had respectively 41%, 33% and 53% amino acid identity, with their Ad2 counterparts. However there were significant differences in the E3A region between these serotypes. Furthermore, in Ad40 no E3A 19K glycoprotein

Figure 1.5.1. Transcription Maps for Ad40 (Dugan) and Ad41 (Tak) E1B Regions.

Solid lines denote RNA and introns are indicated by carets; filled rectangles are open reading frames; rectangles with rounded corners are proteins that have not yet been confirmed experimentally. (Modified from Mautner et al., 1995)


Ad40
22S
55K (476R)
19K (167R)
14S
15K (102R)
19K (167R)
9S
ppIX
co22S
40R
55K/19K
co14S
40R
15K/19K
Ad41
22S
55K (472R)
19K (170R)
14S
15K (122R)
19K (170R)
9S
ppIX
0
1000
2000
3000
4000

equivalent to the one seen in Ad2 was present. In addition, Ad40 can encode two proteins of 173R and 276R that share no homology to the other adenovirus proteins; these were also present in the Ad41 E3 region (Yeh *et al.*, 1996).

The Ad40 E4 region contains ORFs equivalent to those in Ad2 and Ad12, except for ORF1 which it lacks (Davison *et al.*, 1993). However Ad40 can complement the Ad2 E4 mutant dl808 which lacks ORF1 and this indicates that the Ad40 E4 region is functional (Mautner and Mackay, 1991).

1.5.4.2. Late Regions.

The enteric adenoviruses contain two fibre genes, which encode proteins of different shaft length and have distinct sequences (Kidd and Erasmus, 1989; Pieniazek *et al.*, 1989, 1990a; Kidd *et al.*, 1990, 1993; Davison *et al.*, 1993; Yeh *et al.*, 1994). Phylogeny studies revealed that the subgroup F two fibre genes (IV-1 and IV-2) are more closely related to each other (i.e. Ad40 IV-1 to Ad41 IV-1 and Ad40 IV-2 to Ad41 IV-2) than to other adenovirus fibre genes (Bailey and Mautner, 1994). These authors propose this could have arisen through gene duplication shortly after subgroups A and F diverged, but before the separation of serotypes Ad40 and Ad41. EM analysis show only one type of fibre is incorporated at each vertex of the capsid (Kidd *et al.*, 1993). This is in contrast to avian adenoviruses, where two fibres (of different length) are attached to each vertex (Laver *et al.*, 1971).

The 100K L4 protein is the best conserved of the L4 proteins among the serotypes Ad40, 12 and 2. The 33K protein is well conserved; also present is a putative 22K polypeptide that could be generated if the 33K splice is not used (Davison *et al.*, 1993).

The hexon proteins of Ad40 and Ad41 have 88% identity (Toogood and Hay, 1988; Toogood *et al.*, 1989). This level of sequence homology was in agreement with that detected between subgroup C adenoviruses (Kinloch *et al.*, 1984; PringAkerblom and Adrian , 1993). However there are differences detected in the variable loop regions which are on the surface of the virion. It has been shown that these regions contain type-specific determinants (Toogood *et al.*, 1989; Toogood *et al.*, 1992; Crompton *et al.*, 1994).

The Ad40 penton base protein is 81% homologous to Ad12 (Sprengel *et al.*, 1994), but unlike other adenovirus penton bases (Ad2, 3, 5 and 12) which have an RGD motif, Ad40 has an RGA motif (Davison et al 1993).

The human adenoviruses of subgroups A, F and some of B, together with simian adenovirus type 7 (SAV-7) have only one VA gene (Kidd and Tiemessen, 1993; Kidd *et al.*, 1995). In a study of 12 monkey adenovirus genomes Kidd *et al.*, (1995) found they also possess only one VA RNA gene which had good DNA homology with the VA RNA gene from adenovirus subgroups A and F. Ad40 and Ad41 are susceptible to lymphoblastoid interferon in Chang conjunctival cells (Tiemessen and Kidd, 1993), unlike Ad2 which is resistant. It is unclear if the function or expression of the VA RNA gene correlates with this susceptibility. This could occur through defective expression of the E1A gene which transactivates the VA gene through the cellular transcription factor TFIIIC.

1.5.5. Growth Properties of Enteric Adenoviruses in Culture.

The enteric adenoviruses cannot be grown in most cell lines that are used for the routine isolation of adenoviruses. However they can be propagated to a limited extent in: Chang conjunctival cells (Kidd and Madeley, 1981); Hep-2 and tertiary cynomolgus monkey kidney cells (tCMK) (de Jong *et al.*, 1983); 293 cells (Takiff *et al.*, 1981; de Jong *et al.*, 1983); HT-29 cells (Uhnoo *et al.*, 1983) and HRT-18 cells (Gomes *et al.*, 1992). 293 cells are HEK cells that have been immortalised by integration of DNA from the Ad5 early region 1 (Graham *et al.*, 1977). Although some studies have shown Ad40 can be propagated in these cells (Takiff *et al.*, 1981; de Jong *et al.*, 1983), others have not (Chiba *et al.*, 1983; Uhnoo *et al.*, 1983). de Jong *et al.*, (1983) carried out a detailed study of some 200 isolates from subgroup F adenoviruses in various cell lines. These authors reported there were differences in virus growth due to the virus strain used, host cell type used, different batches of the same cell line and differences in the growth of the same virus strain between laboratories. This may explain the contrasting results obtained in different laboratories regarding the growth of enteric adenoviruses in various cell lines.

For instance, Witt and Bousquet (1988), successfully grew Ad40 and Ad41 in Chang conjunctival, 293 and KB cells, but only Ad41 could grow in HeLa cells. In contrast, Pieniazek *et al.*, (1990a, b), could not propagate Ad41 in 293 cells but were successful with HeLa, Hep-2 and INT407 cells. In addition, Perron-Henry *et al.*, (1988) report that not all strains of Ad41 can grow in Hep-2 cells. Hashimoto *et al.*, (1991) have reported that the Sapporo strain of Ad40 can produce plaques in A549 cells (derived from lung cell carcinoma). Ad40 and Ad41 can also grow efficiently in the human hepatocellular carcinoma cell line PLC/PRF/5 and reportedly Ad41 can produce plaques in this cell line (Grabow *et al.*, 1992).

Witt and Bousquet (1988) detected lower infectious titres with Ad40 compared to Ad41 in KB and HeLa cells. When high moi of virus were used, no viral replication was detected, however Ad40 did grow at lower moi. A possible explanation for this may be the presence of defective virus particles and their consequent interference with infectious nondefective virions. In addition, Brown (1985) has reported that the production of Ad40 virions is 3 to 10 fold lower in 293 cells than that observed for other adenoviruses, whereas the yield of infectious virus is 100 to 1000 fold lower. Brown *et al.*, (1992) have conducted studies on the propagation of Ad40 and Ad41 in 293 and HeLa cells. These authors demonstrated that most 293 cells in a culture could be productively infected, while only 10 to 20 percent of the HeLa cell culture were infected. However the uninfected HeLa cells were capable of being infected. The intracellular viral yield was comparable for the two cell lines, however progeny virus was released from 293 but not HeLa cells. Although the yield of viral particles was similar for the enteric adenoviruses compared to Ad5, the particle to infectivity ratios were 100 to 1000 fold greater for Ad40. It was proposed that the high particle to infectivity ratio and the block in release of virus from HeLa cells could be a major factor in the fastidious growth of these viruses in tissue culture. Indeed Tiemessen and Kidd (1994) showed that different strains of Ad40 and Ad41 display differing growth characteristics at several stages in the viral replication cycle and in various cell lines. This suggests that the poor growth of enteric adenoviruses in cell culture is multifactorial.

Another factor influencing Ad41 propagation is serum concentration. Ad41 growth restriction on a number of primary cell lines can be alleviated by reduced serum concentrations (0.2-1%) in the infecting medium (Pieniazek *et al.,* 1990b), these authors suggest that a factor(s) present in the serum is responsible for the effect. This serum inhibition was not observed in continuous cell lines (Hep-2 or 293 cells).

It appears that the Ad41 growth defect is less severe than Ad40 in cell culture, as demonstrated by the number of cell lines that support Ad41 growth but not Ad40 (de Jong et al 1983; Uhnoo et al, 1983, 1984; van Loon et al 1985b). Studies by Tiemessen and Kidd (1988, 1990) were focused on the growth restriction of Ad41 in tissue culture. These authors have shown that Ad41 can be complemented by Ad2 in semi-permissive Chang conjunctival cells and non-permissive HEF cells. In addition, interference by Ad41 on Ad2 replication in HEF cells was observed, with the extent of interference depending on the time of infection of each virus (Tiemessen and Kidd, 1988). This is also detected with other adenovirus serotypes and is attributed to the trans-repression function of E1A proteins (Leite et al 1986; Tibbetts et al 1986; Larson and Tibbetts, 1987).

The pattern of Ad41 growth kinetics was analysed by Tiemessen and Kidd (1990). A pattern of one-hit kinetics was observed with Ad41 on 293 cells, where one infectious virus particle was capable of infecting a cell. In a double infection of Ad2 and Ad41 on Chang conjunctival or HEF cells, a pattern of 2-hit kinetics was observed. However with a single Ad41 infection on Chang conjunctival cells 4 to 5 infectious virus particles were required to productively infect a cell. A possible explanation for this pattern of replication, is that there is a requirement for a certain product(s) which is produced in limiting amounts, thus infection by a number of genomes may be needed to attain a critical level, in order for the progression of viral replication in semi-permissive cells.

The E1 region was implicated in the growth restriction of enteric adenovirus from studies by Takiff *et al.,* (1981), in which these viruses were successfully propagated on 293 cells. Both Ad40 and Ad41 E1A proteins are able to induce expression from the Ad2 E4 promoter in transient transfection assays, but the level of transactivation is a great deal lower than that detected with the Ad5 E1A protein (van Loon *et al.,* 1987a). Ishino *et al.,* (1988) also demonstrated that the Ad40 E1A protein is a weaker transactivator than either

Ad5 or Ad12 E1A. In addition these authors found that Ad40 E1A has a weaker *cis*-acting activity than Ad12. More studies implicate the E1B gene as a major factor in the poor growth of subgroup F adenoviruses. Both Ad40 and Ad41 only partially transform BRK cells, but in Ad40 transformed cells no E1B mRNA is detected (van Loon *et al.*, 1987b). The importance of the E1B region in the growth restriction of Ad40 was clearly demonstrated by Mautner *et al.*, (1989). These authors used stably transformed KB cell lines constitutively expressing E1A only (KB8), E1B only (KB18), both E1A and E1B (KB16) and neither E1A or E1B (KB7) (Babiss *et al.*, 1983). Ad40 could not grow in cells expressing E1A alone, but could grow in cells containing E1B (Mautner *et al.*, 1989; Gomes *et al.*, 1992).

In HeLa cells infected with Ad40, no E1B mRNA is detected early in infection and only low amounts of 22S and 14S mRNA are observed after the onset of DNA replication (Mautner *et al.*, 1990; Bailey *et al.*, 1994; this thesis). However in 293, KB16 and INT407 cells, 22S mRNA is detected before the onset of DNA replication and 14S is first observed at the same time as DNA synthesis (Bailey *et al.*, 1994; this thesis). Hashimoto *et al.*, (1991) have reported Ad40 E1A and E1B mRNAs before the onset of DNA synthesis in A549 cells. Initially the 55K protein was not observed in permissive Ad40 infected cells (Mautner *et al.*, 1990). Subsequent studies have shown that the 55K protein can be detected in Ad40 infected KB16 and 293 cells both at early and late times, using antiserum to the 15K protein expressed in bacteria (Bailey *et al.*, 1993). The 19K protein is also produced in Ad40 infected cells; in HeLa cells at late times and, both at early and late times in 293 and KB16 cells.

Expression of the Ad40 E1B region has been studied in more detail using Ad5/Ad40 recombinants which have the Ad40 E1B region in place of the Ad5 E1B region in dl309, either under the control of the Ad5 E1B promoter (sub5P) or Ad40 promoter (sub40P) (Bailey *et al.*, 1994). Both recombinants could plaque on 293 cells but not on HeLa cells, also the yield and growth rate of recombinants was reduced in HeLa cells compared to 293 and KB16 cells. The expression from the Ad40 E1B region was impaired even under the control of the Ad5 promoter. Both recombinants showed a comparable pattern of E1B mRNA splicing and temporal regulation, but this was cell

type specific. The results show that the E1B promoter is important for determining the growth characteristics of Ad40, but the host cell type is also important. In further studies on the Ad40 E1B promoter (see Mautner *et al.*, 1995 for review), it was shown to be poorly transactivated in transient transfection assays by Ad40 E1A, Ad5 E1A and VZV p140. Compared to the Ad5 E1B promoter, very low activity was detected and was shown to be host cell type specific with higher levels detected in 293 and intermediate in INT407 cells.

1.5.6. Aims of Project.

The fastidious enteric adenovirus type 40 grows poorly in conventional cell lines used to grow other human adenoviruses, but can be propagated in cells which express E1 proteins. This growth restriction has been attributed to defects in E1B gene expression. The aim of the work presented in this thesis was to investigate the host range phenotype of Ad40 in tissue culture, primarily using INT407 cells which were not known to express E1 proteins in comparisons with other permissive and semi-permissive cell lines. Two main areas investigated were the growth properties of the virus and early region E1 gene expression. A timecourse of Ad40 DNA replication on the different cell lines was compared. Viral infectivity was examined by fluorescent focus assay with the different cell types in order to determine if there were differences in the number of productively infected cells. Infection of permissive and semi-permissive cells was further characterised in attachment and internalisation assays. The E1B transcription map had previously been determined using RNA obtained from cells with endogenous E1 proteins. Therefore the pattern of E1B transcription was analysed on INT407 cells in order to determine if there were any differences in E1B splicing patterns with this cell line. In addition, the Ad40 E1B promoter was examined by looking at protein binding at the putative Sp1 binding site.

2. MATERIALS.

2.1. Tissue Culture.

2.1.1. Tissue Cells.

KB16 are human nasopharyngeal derived cells, expressing the Ad2 E1 region (Babiss *et al.*, 1983). These cells were routinely used for the growth of Ad40 seed stocks. They will herein be referred to as KBa+b cells.

HeLa are human cervical carcinoma cells. The current laboratory stock, originally obtained from Flow laboratories, was used for virus infection and nuclear extract preparation.

293 are human embryonic kidney cells derived by transformation with the Ad5 E1 region (Graham *et al.*, 1977). They were used for virus infection and for the propagation of adenovirus mutants.

INT407 were described as human embryonic intestinal cells (Henele and Deinhardt, 1957) and obtained from the European Collection of Animal Cell Culture (ECACC). Subsequently these cells were found to be HeLa cell derivatives (Gilbert *et al.*, 1990). These cells were used for virus infection and nuclear extract preparation.

A549 are human lung carcinoma cells (Giard *et al.*, 1973) originally obtained from the ECACC, and were used for virus infections.

CV-1 are African green monkey kidney cells (Jensen *et al.*, 1964, Kit *et al.*, 1965) and were kindly donated by Dr A. Davison. They were used to prepare Sp1 protein.

Sf21 are Spodoptera frugiperda cells (Brown and Faulkner, 1977) and were used for the growth of AcNPV^{fib}FL582.

2.1.2. Tissue Culture Media.

HeLa, INT407, A549 and CV-1 cells were propagated in Dulbeccos modified Eagles medium (DMEM), without sodium pyruvate, with 4500mg/L glucose (Gibco).

The medium was supplemented with 5% FCS, 4mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin.

KBa+b and 293 cells were propagated in Glasgow modified medium (GMEM) supplemented with 5% FCS.

Sf21 cells were grown in TC100 medium (Gibco) which was supplemented with 5% FCS, 100units/ml penicillin and 100µg/ml streptomycin.

2.2. Viruses.

The adenovirus type 40 (**Ad40**) strain Dugan was used in this study; it was originally obtained from Dr J. de Jong (de Jong *et al.*, 1983). KBa+b cells were used to propagate the virus (see 2.1.1.). Most experiments were carried out using passage 9 stocks of the virus, but for the Immunofluorescence experiments passage 10 was used.

dl309 (Jones and Shenk, 1979a) is derived from Ad5 but lacks Xba I sites at 29, 79 and 85 mu, also the region from 83 to 85mu is deleted. dl309 does not produce E3 14.7K, 14.5K, and 10.4K proteins.

dl312 (Jones and Shenk., 1979a; Ross *et al.*, 1980) is derived from dl309 and contains an additional deletion between nt 448-1349. This results in an absence of E1A proteins.

pm1722 (Barker and Berk, 1987) is derived from dl309 and the E1 region of Ad2; it contains a G to A mutation at nt 1722 introducing a stop codon. pm1722 does not make the E1B 19K protein.

dl1520 (Barker and Berk., 1987) is also derived from dl309 and Ad2 E1 sequences. It has a C to T mutation at nt 2022, changing an arginine to a stop codon and the region from 2496 to 3323 nt is deleted. The E1B 55K and 15K proteins are not made in this virus.

AcNPV^{fib}FL582 (Novelli and Boulanger, 1991); this recombinant baculovirus was a kind gift from Dr P. A. Boulanger. The virus AcNPV^{fib}FL582 contains the gene for full length Ad2 fibre.

v-Sp1 is a recombinant vaccinia virus which overexpresses wild-type Sp1 protein (Jackson *et al*, 1990). This virus was a kind gift from Dr S. P. Jackson.

2.3. Bacteria.

2.3.1. Bacterial Strain.

Plasmids were propagated in the *E.coli* strain K12 NM522 [supE, thi, δ(lac-proAB), hsd5, (F', proAB, lacI, lacZδM15)], (Gough and Murray, 1983).

2.3.2. Bacterial Culture Media.

Bacterial cultures were routinely grown in suspension at 37°C in L-broth [177mM NaCl, 10g/L Difco bactopeptone, 5g/L yeast extract (pH7.5 prior to sterilisation)].The medium was supplemented with ampicillin (50μg/ml) and chloramphenicol (200μg/ml) where appropriate. Plates were made with L-broth agar containing 1.5% (w/v) agar and the appropriate antibiotic.

2.4. Plasmids.

Clones were grown from seed stocks supplied by Dr V. Mautner unless otherwise stated.

The following plasmids were derived from pAA12 (van Loon *et al.*, 1985a, 1987b) which contain the *Cla* I-B fragment of Ad40 (nt 1-3933). Regions described below were subcloned into pTZ18 vectors (Mautner *et al.*, 1989, 1990).

pNM82 has the whole of the *Cla* I-B fragment, comprising the E1 region.

pNM83 contains the Ad40 E1A region from nt 1-1211.

The following plasmids were derived from the *Bgl* II fragment of Ad2 and subcloned into pTZ18 vectors (Mautner *et al.*, 1990).

pNM90 contains the Ad2 E1 region from nt 1-1569.

pNM92 contains the Ad2 E1 region from nt 1569-3322.

The plasmid **pAB106** was kindly donated by Dr A. Bailey. It was constructed from a PCR product cloned into the pET3a vector (Bailey *et al..*, 1993) and contains the Ad40 E1B region from nt 1719-3149.

2.5. Oligonucleotides.

The oligonucleotides used in this study were synthesised on a PS-250 Cruachem DNA synthesiser. Oligonucleotides are listed below :

RTR3 Ad40 E1 region, nt 1345-1373.

5' GTGTTTATTTCTTGGGCGTGTTTGTGGG 3'

RTR4 Complementary strand to RTR3.

5' CCCACAAACACGCCCAAGAAATAAACAC 3'

RTR5 Ad2 E1B nt -59 to -31 with respect to the Ad2 E1B cap site.

5' CGTGTTAAATGGGGCGGGGCTTAAAGGG 3'

RTR6 Complementary strand to RTR5.

5' CCCTTTAAGCCCCGCCCCATTTAACACG 3'

RTR11 Derived from RTR3, underlined nt are T to G or C mutations.

5' GTGTTTATTTCTGGGGCGGGGCTGTGGG 3'

RTR12 Derived from RTR4, underlined nt are A to G or C mutations. It is complementary to RTR11.

5' CCCACAGCCCCGCCCCAGAAATAAACAC 3'

OCT27 HSV-1 IE gene 2 promoter octamer/TAATGARAT motif, nt -158 to -139 with respect to the IE2 cap site (Bailey and Thompson, 1992).

5' CGATATGCTAATTAAATACAT 3'

OCT28 Complementary strand to OCT27.

5' ATGTATTTAATTAGCATATCG 3'

AB42 Ad40 E1B region, nt 3110-3049, lower case nt are non Ad40 E1 sequences.

5' CGACATCATCTGGTGGTCCGTAACGCAGCTGCTCGGTCA CGTTCAGAGTTGCTGGATATAACCgctacg 3'

2.6. Enzymes.

Restriction enzymes were obtained from Boehringer Mannheim or Bethesda Research Laboratories (BRL).

Boehringer Mannheim also supplied S1 nuclease.

New England Biolabs supplied T4 Polynucleotide Kinase.

The following were obtained from Sigma: Lysozyme, Proteinase K, RNase A and subtilisin.

RQ1 RNase free DNase I was obtained from Promega.

DNA polymerase I / DNase I mix was obtained from Pharmacia.

2.7. Antibodies.

10/5.1 is an Ad5 anti-Hexon mAb, it was a kind gift from Dr I. R. Sharp (Laboratory of Microbiological Reagents, Central Public Health Laboratory, 61 Colindale Avenue, London.).

The Goat anti-mouse antibody used in the immunofluorescence experiments was an anti-mouse IgG obtained from Nordic Immunochemicals.

2.8. Chemical Reagents.

All chemicals were of analytical grade or higher and were supplied by BDH Chemicals and Sigma Chemical Company, except for the following:

Ammonium persulphate (APS) and **N, N, N', N'-Tetra methylenediamine (TEMED)** - BioRad Laboratories, California

Caesium chloride - Melford Laboratories Ltd, Suffolk

Ecoscint - Nuclear Medical Electronic Systems and Services Ltd, Livingstone

Ethanol - Hayman Ltd, Essex

Ficoll 400 and **Deoxynucleotide triphosphates (dNTPs)** - Pharmacia LKB Biotechnology, Milton Keynes.

Formamide and **Formldehyde** - Fluka Chemicals Ltd, Dorset.

Acetic acid, Acetone, Chloroform, Hydrochloric acid (HCl), Isopropanol and **Methanol** - Prolabo, Manchester.

Glycerol - May and Baker Ltd, Essex.

Trichloro-trifluro-ethane (Arcton) - ICI, Cheshire.

2.9. Radiochemicals.

All radiochemicals used in this study were supplied by Amersham International PLC. The specific activities were 3,000 Ci/mmol for [α -^{32}P]-dNTPs, 60 Ci/mmol for [^{3}H] - thymidine and 5000 Ci/mmol [γ -^{32}P]-ATP.

2.10. Solutions.

3 x aqueous hybridisation mix: 3M NaCl, 0.5M HEPES pH7.5, 1mM EDTA pH8.0.

Bradford reagent: 0.1% (w/v) Coomassie brilliant blue G, 4.75% (v/v) ethanol, 8.5% (v/v) phosphoric acid.

Buffer A: 10mM HEPES pH7.9, 1.5mm $MgCl_2$, 10mM KCl, 0.5mM phenylmethylsulfonyl fluoride (PMSF), 0.5mM DTT.

Buffer C: 20mM HEPES pH7.9, 25% (v/v) glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF.

Cell adhesion buffer: DMEM supplemented with 2mM $MgCl_2$, 1% (w/v) BSA, 20mM HEPES pH7.9.

50 x Denhardts solution: 1% (w/v) ficoll, 1% (w/v) BSA fraction V, 1% (w/v) polyvinylpyrollidone.

5 x DNase I buffer: 200mM Tris-HCl pH7.5, 30mM $MgCl_2$, 50mM NaCl.

EB buffer: 0.5% (w/v) SDS, 100mM NaCl, 50mM Tris-HCl pH7.5.

Formamide dye mix: 98% (v/v) formamide, 10mm EDTA pH8.0, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue.

2 x HBS: 280mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4, [pH the whole solution to 7.12].

1 x HBSE: 1xHBS, 20mM EDTA.

High salt buffer (HS): 50mM Tris-HCl pH7.5, 0.42M KCl, 20% (v/v) glycerol, 10% (w/v) sucrose, 5mM $MgCl_2$, 0.1mm EDTA, 1mM PMSF, 1mM sodium metabisulphite, 2mM DTT.

Hirt buffer: 10mm Tris-HCl pH7.9, 10mM EDTA, 0.6% (w/v) SDS.

10 x Kinase buffer: 50mM Tris-HCl pH7.6, 10mM $MgCl_2$, 0.1mM EDTA, 0.1mM spermidine, 0.5mM DTT.

Loading dye mix: 50% glycerol, 1mM EDTA pH8, 0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue.

10 x MOPS: 180mM MOPS [3-(n-Morpholino)-propanesulphonic acid] pH7.0, 50mM sodium acetate, 10mM EDTA.

Phosphate buffered saline (PBS):

PBSA: 170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4 and buffered to pH 7.2.

PBSB: 6.8mM $CaCl_2$.

PBSC: 4.8mM $MgCl_2$.

PBS: 8 parts PBSA : 1 part PBSB : 1 part PBSC.

Phenol/Chloroform: phenol equilibrated with TE was mixed in equal volumes with chloroform.

4 x RM: 0.4mg/ml (w/v) BSA, 25mM HEPES pH7.4, 2mM DTT, 8mM $MgCl_2$.

RNA lysis buffer: 150mM NaCl, 10mM Tris-HCl pH7.9, 0.65% NP40.

2 x S1 buffer: 0.56M NaCl, 100mM sodium acetate pH4.5, 9mM $ZnSO_4$.

Sample buffer: 50% (w/v) sucrose, 100mM EDTA, 0.2% (w/v) bromophenol blue.

Solution I: 50mM glucose, 10mm EDTA, 25mM Tris-HCl pH8.

Solution II: 0.2M NaOH, 1% (w/v) SDS.

Solution III: 3M potassium acetate, 11.5% (v/v) glacial acetic acid.

20 x SSC: 3M NaCl, 0.3M tri-sodium citrate pH7.0.

20 x SSPE: 3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA pH7.4.

Sucrose solution: 250mm sucrose, 2mm $MgCl_2$, 50mM Tris-HCl pH8.0.

TBE: 90mM Tris, 90mM boric acid, 1mM EDTA pH8.3.

TE: 10mM Tris-HCl pH8.0, 1mM EDTA.

Tris-saline (TS): 140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml glucose, 25mM Tris-HCl pH7.4, 0.001% (w/v) phenol red, 100 units/ml penicillin, 100 µg/ml streptomycin.

Triton solution: 0.5% (v/v) Triton X-100, 62.5mM EDTA pH8.0, 50mM Tris-HCl pH8.0.

Trypsin: 0.25% (w/v) trypsin (Difco) dissolved in Tris-saline.

Urea buffer: 7M urea, 0.35M NaCl, 0.01M Tris-HCl pH7.4, 0.01M EDTA, 1% (w/v) SDS.

Versene: 0.6mM EDTA in PBSA containing 0.0015% (w/v) phenol red.

Z' buffer: 25mM HEPES pH7.6, 12.5mM $MgCl_2$, 20% (v/v) glycerol, 0.1% NP40, 10mM $ZnSO_4$, 1mM DTT, 0.1M KCl.

3. METHODS.

3.1. Tissue Culture.

Most cell types were grown as monolayers at 37°C in an atmosphere of 95% air and 5% CO_2. They were grown to 80-90% confluency in 80cm² or 175cm² flasks (Nunclon) and routinely passaged every 3-4 days. Cells were harvested by washing twice in 12ml of a 5:1 versene:trypsin mix and resuspended in fresh medium. This suspension was used to seed subsequent monolayers.

Sf21 cells were passaged every 3-4 days in 175cm² flasks at 28°C in air. Confluent monolayers were dislodged by vigorous shaking into 10ml of medium and this suspension was used to seed subsequent monolayers.

For storage, cells were resuspended at approximately 2.5 x 10^6 cells/ml in either 40% medium, 40% FCS and 20% glycerol or 10% DMSO, 70% medium and 20% FCS. Aliquots were frozen slowly to -140°C. For recovery, cells were thawed rapidly and resuspended in growth medium.

3.2. Virus Preparation.

3.2.1. Virus Stocks.

Ad40 seed stocks were prepared by infecting subconfluent monolayers of KBa+b cells grown on 50mm plates. Plates were inoculated with 100µl of a 1/10 dilution of virus in TS. Virus adsorption was carried out at 37°C for 1h, after which medium (GMEM + 0.5% FCS) was added. Incubation was continued at 37°C, until a good cpe was apparent (3-5 days). Cells were harvested by scraping into the overlaying medium and transferred to a 15ml falcon tube. Samples were centrifuged at 2,000rpm for 10min in a refrigerated centrifuge. The supernatant was discarded and the cell pellet resuspended in TS, at 0.25ml/plate. Samples were subjected to three freeze thaw cycles (-70°C to

37°C). The cell debris was pelleted as above and the supernatant containing the virus stock was transferred to a fresh vial; virus stocks were stored at -20°C and -70°C. Ad40 virus yield was estimated by restriction digest analysis of viral DNA.

Ad40 does not plaque on any of the permissive cell lines used in this laboratory, although the virus has been reported to plaque on A549 cells (Hashimoto *et al.*, 1991). This cell line was also tried, but no plaques were observed even after attempts to enhance plaque formation. Differences in virus strain and different laboratory cell stocks may have accounted for this. In the absence of a suitable quantitation method for Ad40, virus yield was estimated by restriction digest analysis of viral DNA.

Adenovirus mutant seed stocks were prepared in a similar fashion to Ad40. Virus was inoculated onto 293 cells at 0.1pfu/cell. Cells were harvested 3-5 days postinfection as above and titrated by plaque assay on 293 cells (Williams, 1970).

v-Sp1 (see 3.18.).

AcNPV ^{nb}FL582 (see 3.15.).

3.2.2. Density-Gradient Purified Virus.

Crude virus stocks were grown as described (see 3.2.1.). *n*-butanol was added to virus preparations to give a final concentration of 1% (v/v) and incubated on ice for 1h with frequent mixing. The suspension was spun at 500g for 20min at 4°C. The supernatant containing virus was removed and purified on a glycerol/caesium chloride gradient. The gradient was made up of 2ml 40% glycerol in 10mM Tris-HCl pH7.9 at the top followed by 3ml of 0.32g/ml caesium chloride in 10mM Tris-HCl pH7.9 and then 2ml of 0.418g/ml caesium chloride in 10mM Tris-HCl pH7.9, finally the virus supernatant was carefully layered on top. Tubes were spun at 25,000rpm for 1.5h. The opalescent virus band was collected and dialysed against TE at 4°C overnight. The virus was aliquoted and stored at -70°C.

3.2.3. Labelled Virus.

dl309 - 293 cells were grown on a 140mm plate ($1x10^7$ cells) and infected at 50 pfu/cell. Adsorption was carried out at 37°C for 1h and cells overlaid with infecting medium (GMEM + 2% FCS). 2.5 mCi of tritiated thymidine was added to the overlaying medium at 12h postinfection and incubation continued at 37°C. The virus was harvested 3 days post infection and purified as described (see 3.2.2.). The specific activity of the virus was 2.8×10^{-5} cpm/particle.

Ad40 - KBa+b cells were grown on a 140mm plate ($1x10^7$ cells) and infected with a 1/5 dilution of Ad40. Virus was adsorbed for 1h at 37°C and then overlaid with infecting medium (GMEM + 0.5% FCS). 2.5 mCi of tritiated thymidine was added to the overlaying medium at 24h postinfection. The virus was harvested at 5 days postinfection and purified as described (see 3.2.2.). The specific activity of the virus was 4.3×10^{-6} cpm/particle.

Virus particle concentration (above) was estimated by the Bradford assay (Bradford, 1976), using a molecular wt of $1.75x10^8$ daltons for adenovirus (Green *et al.*, 1967).

3.3. Extraction of Nucleic Acids.

3.3.1. Extraction of Viral DNA.

Viral DNA was prepared by incubating virus suspensions with 100µg/ml of Proteinase K and 0.1% (w/v) of SDS at 37°C for 2-3h. An equal volume of phenol/chloroform was added, mixed, then centrifuged at 12,000rpm for 2min in a microfuge. This was followed by a chloroform extraction. The DNA-containing upper aqueous phase was concentrated by the addition of 2.5 volumes of ethanol, 0.1 volumes of 3M sodium acetate pH5.2, with 1µg of tRNA added as a carrier. Samples were incubated at -70°C for 30 min or -20°C overnight. DNA was pelleted by centrifugation at 12,000rpm for 10min in a microfuge and resuspended in DW.

3.3.2. Extraction of Intranuclear Viral DNA.

Nuclei were isolated as described (see 3.3.3.) and intranuclear DNA was extracted by a modified Hirt procedure (Hirt *et al.*, 1967; Hay *et al* ., 1984).The nuclear pellet was resuspended in 100µl TE, after which 600µl of Hirt buffer was added and incubated at RT for 10 min. This was followed by the addition of 150µl of 5M NaCl, with vials inverted to mix and incubated at 4°C overnight. Samples were spun free of cell debris at 20,000g for 30 min in a refrigerated microfuge. The DNA supernatant was incubated with 0.1mg/ml RNase A for 1h at 37°C, followed by the addition of 0.1mg/ml Proteinase K for 3h at 37°C. A phenol/chloroform followed by a chloroform extraction was then carried out. The DNA was precipitated at -20°C overnight with an equal volume of isopropanol and 0.1 volumes of 3M sodium acetate pH5.2. The DNA was pelleted and resuspended in either TE or DW and stored at -20°C.

3.3.3. Preparation of Cytoplasmic RNA.

Cytoplasmic RNA was extracted (Sharp *et al.*, 1974) from cells grown on 50mm plates. Monolayers were washed twice with cold PBS, the cells from two plates were pooled by scraping into 1ml PBS and centrifuged at 5,000rpm in a benchtop microfuge for 30 sec. The cell pellet was resuspended in 200µl ice-cold RNA lysis buffer and centrifuged at 5,000rpm for 40 secs. The nuclear pellet was frozen at -70°C and stored for subsequent extraction of intranuclear DNA (see 3.3.2.). The supernatant containing cytoplasmic RNA was transferred to a fresh vial containing 200µl urea buffer at RT, and two successive phenol/chloroform and chloroform extractions were carried out to remove proteins. The RNA was precipitated by the addition of 0.1 volumes of 10M ammonium acetate and 2 volumes of ethanol. Tubes were incubated at -20°C overnight. The RNA was pelleted by centrifugation at 12,000rpm for 10min and washed with 70% ethanol. The RNA was either DNase I treated (see 3.4.) or resuspended in 100µl DEPC treated water, then 200µl ethanol was added and the RNA stored in this mix at -20°C. Before use an aliquot of the RNA mix was removed and precipitated by the addition of 0.1 volumes

of 10M ammonium acetate and incubated for 30min at -70°C. RNA was pelleted at 15,000g for 10min at 4°C and redissolved in DEPC treated water.

3.4. DNase I Treatment of RNA.

Cytoplasmic RNA pellets (see 3.3.3.) were resuspended in 1xDNase I buffer and 2 units of RQ1 RNase free DNase I was added. Samples were incubated at 37°C for 30min after which they were phenol/chloroform followed by chloroform extracted. The RNA was precipitated at -20°C overnight with 2 volumes of ethanol and 0.1 volumes of 10M ammonium acetate. The RNA was pelleted at 15,000g for 10min at 4°C and redissolved in 100µl DEPC treated water, 200µl of ethanol was then added. The RNA was stored in this mix until use (see 3.3.3.).

3.5. Quantitation of Nucleic Acids.

Quantitation of nucleic acids was carried out by measuring optical density (OD) at 260nm and 280nm on a Beckman DU-62 spectrophotometer. Readings at 260nm allowed concentrations to be calculated using conversion factors for 1 OD unit at 260nm. For single stranded DNA this was 20µg/ml, for RNA 40µg/ml and for double stranded DNA this corresponds to 50µg/ml.

The ratio between 260 and 280nm gave an estimate of the purity of the nucleic acid. Pure preparations of DNA have a ratio of 1.8 and pure preparations of RNA have a ratio of 2.

The molarity of oligonucleotides was determined using the following formula, when E_M (molar extinction coefficient) is the optical density of a 1M solution of the oligomer at 260nm (Tullis *et al.*, 1989).

$$OD/E_M$$

[where E_M = A(16,000) + G(12,000) + C(7,000) + T(9,600)]

3.6. Electrophoretic Separation of Nucleic Acids.

3.6.1. Agarose 'Midi' Gels.

DNA was analysed by preparing 100ml horizontal 0.8-2% agarose gels (140mm x 110mm x 7mm) containing 0.5µg/ml ethidium bromide in 1xTBE. Electrophoresis was carried out with gels submerged and run at 150V for 2h in 1xTBE. DNA samples had 0.2 volumes of sample buffer added prior to loading.

3.6.2. Formaldehyde Agarose Gels.

RNA was prepared for Northern blotting (see 3.12.) by electrophoresis on denaturing formaldehyde agarose gels (140mm x 110mm x 7mm). The gel was prepared by boiling 1.2g of agarose in 87ml DEPC treated water. The agarose was cooled to 55°C, then 10ml of 10xMOPS and 3ml of 37% formaldehyde was added, thoroughly mixed and the gel poured immediately. RNA samples were prepared by dissolving RNA pellets in 6µl DEPC treated water followed by the addition of 2.5µl 10xMOPS, 12.5µl formamide and 4.5µl 37% formaldehyde. Samples were mixed gently and incubated at 55°C for 15min, 5µl of loading dye mix was added prior to loading. Gels were run submerged in 1xMOPS and electrophoresis carried out at 70 volts for 2.5h in a fumehood.

3.6.3. Non-denaturing Polyacrylamide Gels.

Gel retardation assays (see 3.19.) and the purification of double stranded radiolabelled oligonucleotides (see 3.10.2.) were carried out using vertical gels (260mm x 160mm x 2mm) with the appropriate concentration of acrylamide in 0.5xTBE. Acrylamide gels were prepared from a stock solution of 30% acrylamide (29% acrylamide and 1% N-N' methylene bisacrylamide), with 0.5xTBE. Gels were polymerised by the addition of 0.2ml of 25% APS and 30µl TEMED. Samples for gel retardation assays were run without loading dye mix but double stranded DNA samples

had 0.2 volumes of loading dye mix added prior to loading. Electrophoresis was carried out at 200V for 2-2.5h.

3.6.4. Denaturing Urea/Polyacrylamide Gels.

Gels for S1 analysis (see 3.13.) were carried out using vertical gels (450mm x 230mm x 0.35mm) with the appropriate concentration of acrylamide in 1xTBE. Gels for purification of oligonucleotides (see 3.10.1) were carried out in vertical gels (260mm x 160mm x 2mm) with 15 or 20% acrylamide in 1xTBE. Gels were made from a stock solution of 20% acrylamide and 1% N-N' methylene bisacrylamide with 7M urea in 1xTBE. Polymerisation was carried out by the addition of 0.25ml of 25% APS and 40µl TEMED. Electrophoresis was carried out with a prerun (40W for S1 gels and 200V for purification of oligonucleotides on gels) for 1h prior to samples being loaded. The samples were denatured by adding 0.2-1 volumes of formamide dye mix, boiling for 5min and rapid cooling on ice. Samples were run for 1-2h in 1xTBE. The gels for S1 analysis were dried under vacuum, then exposed to film at -70°C for autoradiography.

3.7. Electroelution of DNA from Polyacrylamide Gels.

Polyacrylamide gels used for purification of radiolabelled single or double stranded oligonucleotides were exposed to autoradiography film for 2 min. Bands of interest were cut out and electroelution performed in a Schleicher and Schuell BIOTRAP apparatus. Electroelution was carried out in 0.25xTBE at 200V for 2h into a 200µl volume trap in the biotrap. Electroeluted DNA was stored in this 200µl volume at -20°C.

3.8. Preparation of Plasmid DNA.

3.8.1. Small Scale Preparation of Plasmid DNA.

A single colony was picked using a sterile toothpick into 5ml L-broth with 50µg/ml ampicillin and incubated overnight in an 37°C orbital shaker. 1.5ml was

removed and pelleted at 12,000rpm for 20secs in a benchtop microfuge. The pellet was resuspended in 100µl of Solution I containing 1mg/ml fresh lysozyme and incubated at RT for 5 min. This was followed by the addition of 150µl of fresh Solution II and after mixing a further 5 min incubation at RT. 150µl of Solution III was added, tubes were inverted to mix and incubated for 5min at RT. Samples were pelleted at 12,000rpm for 5min in a benchtop microcentrifuge. The DNA-containing supernatant was transferred to a fresh vial and phenol/chloroform extracted. DNA was precipitated by adding 2 volumes of ethanol and centrifuged at 12,000rpm for 5min. Pellets were washed in 70% ethanol and resuspended in 40µl DW. DNA was analysed by restriction enzyme digestion, in the presence of 20µg/ml of RNase A.

3.8.2. Large Scale Preparation of Plasmid DNA.

A 10ml L-broth culture containing 50µg/ml ampicillin was grown up overnight in a 37°C orbital incubator. The overnight culture was added to 350ml L-broth containing ampicillin and shaken at 37°C until OD_{630} was between 0.6-0.8. Chloramphenicol was added to a final concentration of 200µg/ml and incubation continued overnight. The culture was pelleted by centrifugation at 7,000rpm for 10min and resuspended in 2ml sucrose solution with fresh lysozyme added to a final concentration of 4mg/ml. After incubating at RT for 30min, 3.2ml Triton solution and 800µl 0.25M EDTA pH8.0 were added, mixed, and left for a further 15min at RT. The cell debris was removed by centrifugation at 17,000rpm for 40min at 4°C in a Sorvall SS34 rotor. Plasmid DNA was purified by equilibrium centrifugation in a caesium chloride gradient. For each 6ml sample, the gradient consisted of 3.5ml of DNA sample, 250µl of 10mg/ml ethidium bromide and 4.5g caesium chloride, the final solution was made up to 9.3g with TE. The gradients were centrifuged at 40,000rpm for 18h at 18°C in a Dupont ultracentrifuge using a TV865 rotor. The lower band of the gradient consisting of closed circular plasmid DNA was collected. Ethidium bromide was removed by adding an equal volume of isopropanol, mixing, allowing the two layers to separate and discarding the top isopropanol layer. This was repeated twice or until the bottom DNA containing layer was

no longer pink. Caesium chloride was removed from the DNA solution by dialysing against TE for 18h at 4°C. DNA was incubated with RNase A to a final concentration of 100μg/ml for 1h at 37°C followed by Proteinase K at 100μg/ml and 1% SDS for 2h at 37°C. Finally the DNA was extracted once with phenol/chloroform and twice with chloroform, before being ethanol precipitated at -20°C overnight (with 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate pH5.2). DNA was pelleted at 12,000rpm for 10min and resuspended in DW.

3.9. Radioactive Labelling of DNA.

3.9.1. Internal Labelling of DNA by Nick Translation.

0.5-1μg of plasmid DNA was radiolabelled by the addition of 25μCi of two α[^{32}P]-dNTPs, 20μM of the other two dNTPs and 2 units of DNA polymerase I /DNase I enzyme mix. After a 1h incubation at 16°C, unincorporated nucleotides were removed by fractionation on a 5ml G-50 Sephadex column. Sephadex beads (Pharmacia) were swollen overnight in DW. Columns were prepared in plastic 5ml pipettes with glass wool plugs and equilibrated with 10ml TE. To determine percentage incorporation of radiolabel, 1μl from labelled DNA, (loaded onto a Sephadex G-50 column) was spotted onto glass fibre filter paper discs (Whatman GF/C, 2.1cm diameter) and dried. After elution through the column, the first four 250μl peak fractions were pooled and 10μl dotted onto a filter and dried. To remove unincorporated nucleotides from this filter only, it was washed twice in 10% (w/v) TCA for 5min and twice in 5% (w/v) TCA for 5min, followed by a 5min ethanol wash. The filter was then dried. Both filters were counted in a scintillation counter. Comparison between the two determined percentage incorporation. Probes generally had a specific activity of 1x10^{7}-1x10^{8}cpm/μg.

3.9.2. 5' End Labelling of Oligonucleotides.

To 100-200ng of oligonucleotide was added 50 μCi of [γ -^{32}P]-ATP and 20 units of T4 polynucleotide kinase in 1xKinase buffer. Incubation was carried out at 37°C for

2h. The DNA was phenol/chloroform and chloroform extracted followed by ethanol precipitation with 2 volumes of ethanol, 0.1 volumes of 3M sodium acetate pH5.2 and 3µl of 1µg/µl tRNA at -20°C overnight. After pelleting for 10min at 12,000rpm in a benchtop microfuge, the DNA was resuspended in DW.

3.10. Manipulation of Oligonucleotides.

3.10.1. Purification of Oligonucleotides.

Synthetic oligonucleotides were synthesised, on a Cruachem PS-250 DNA synthesiser, on columns. The DNA was eluted from the column by incubating in 2.5ml of concentrated ammonia solution for 2-2.5h at RT. The oligonucleotides were deprotected by incubating the oligonucleotide containing ammonia solution at 55°C for 5h, after which the solution was split into three aliquots and lyophilised. Pellets were resuspended in 90% formamide, heated at 95°C for 3min before being applied to a 15 or 20% denaturing polyacrylamide gel containing 7M urea and run in 1xTBE (see 3.6.4.). 3µl of formamide dye mix was loaded in a separate well to act as a migration marker. The gels were electrophoresed at 200V until the bromophenol blue had migrated two thirds of the way down the gel. The DNA was visualised by placing a TLC plate underneath the gel and viewed with an angled shortwave UV lamp. The oligonucleotide appeared as a dark band against a uniform green fluorescent background. The band containing the full length oligonucleotide was excised and eluted overnight at RT into 1ml of TE. The DNA supernatant was filtered into a fresh vial and ethanol precipitated, with two volumes of ethanol and 0.1 volumes of 3M sodium acetate pH5.2. The DNA was pelleted at 12,000rpm for 10min in a benchtop microfuge and resuspended in DW.

3.10.2. Annealing Complementary Oligonucleotides.

Equivalent amounts of each oligonucleotide were added together in 50µl of DW. Oligonucleotides were thoroughly denatured by the addition of 2µl of 5M NaOH, samples were mixed and incubated at 37°C for 30min. The NaOH was neutralised and

DNA precipitated by the sequential addition of 5µl of 3M sodium acetate pH5.2, 100µl of ethanol and 2µl of 1µg/µl tRNA. Samples were precipitated at -70°C for 15min before centrifugation at 12,000rpm for 10min. DNA pellets were air dried, resuspended in 20µl DW and annealed at 37°C for 30min. Double stranded oligonucleotides were stored at -20°C.

Where one strand was radiolabelled, as for gel retardation probes, annealed oligonucleotides were run on non-denaturing polyacrylamide gels (see 3.6.3.) to check for annealing efficiency. Probes were purified by electroeluting (see 3.7.) into 200µl 0.25xTBE and subsequently stored at -20°C.

3.11. DNA Slot Blot Analysis.

Samples of DNA were boiled for 5min in a 200µl volume of 6xSSC and rapidly cooled on ice to denature. Nylon membranes (Hybond-N from Amersham) were presoaked for 5min in DW before soaking in 6xSSC for 5min. Membranes were placed in the slot blot apparatus (Schleicher and Scheull). A vacuum was applied, and the DNA samples were loaded into the slots. The wells were washed once with 6xSSC. Membranes were then air dried for 10-15min and the DNA irreversibly bound by exposing to shortwave UV for 3.5min. DNA hybridisation was carried out by prehybridising and hybridising membranes in 50% formamide, 5xSSC, 1xDenhardts and 100µg/ml salmon sperm DNA at 42°C in a shaking water bath. The membranes were prehybridised for 4h after which the prehybridisation mix was replaced by the hybridisation mix which also contained denatured radiolabelled probe. Hybridisation was carried out at 42°C overnight. Membranes were washed at RT twice in 2xSSC, 0.1% SDS for 30min and twice in 0.1xSSC, 0.1%SDS for 30min. Finally membranes were washed briefly in DW before autoradiography on flashed film with an intensifying screen at -70°C.

3.12. Northern Blot Analysis.

RNA samples were run on formaldehyde gels (see 3.6.2.) until the bromophenol blue dye was approximately two thirds of the way down the gel. The gel was washed four times in DW, for 5min each wash, at RT to remove formaldehyde. The gel was equilibrated in 10xSSC for 1h at RT. The RNA was blotted onto nylon membrane (Hybond-N) by capillary blot with 20xSSC. Nylon membrane was cut to the size of the gel, as were two pieces of filter paper; a wick was also made of filter paper. This was soaked in 20xSSC and placed over a glass plate bridging the transfer dish such that the ends of the wick touched the bottom of the dish. Air bubbles were removed and the gel placed on top of the wick, the nylon membrane equilibrated in 10xSSC was carefully placed over the gel. Finally the two pieces of filter paper and a two inch thick pile of paper towels were added and a weight applied on top. The capillary transfer was left overnight at RT. The membrane was carefully removed and the RNA crosslinked by exposing to UV for 30 seconds in a Stratagene stratalinker. Membranes were prehybridised and hybridised in 50% formamide, 5xSSPE, 1% SDS, 5xDenhardts solution and 0.5µg/ml salmon sperm DNA. Prehybridisation was carried out at 42°C for 2h, after which the solution was replaced with hybridisation mix containing denatured probe and hybridised overnight at 42°C. Membranes were washed at 42°C once with 2xSSPE, 0.1%SDS for 15min, followed by 0.5xSSPE, 0.1% SDS for 10min and finally once in 0.1xSSPE, 0.1%SDS for 10min. Membranes were briefly washed in DW and exposed to flashed film with an intensifying screen at -70°C.

Membranes were stripped of probe by either boiling a solution of 0.1% (w/v) SDS, which was poured onto the membrane and allowed to cool to RT. The second method was to incubate membranes at 65°C for 2h in 5mm Tris-HCl pH8, 2mm EDTA and 0.1xDenhardts solution. Membranes were autoradiographed to check probe had been removed. The filter was then prehybridised and hybridised with new probe.

3.13. S1 Nuclease Digestion.

S1 nuclease digestion was carried out essentially as described (Ausubel *et al.*, 1992). RNA pellets (15μg) to be digested were air dried and resuspended in 30μl of 1xaqueous hybridisation mix containing approximately 2ng of a 5' labelled oligonucleotide probe. Samples were denatured at 75°C for 10min and rapidly transferred to 55°C for hybridisation overnight. Tubes were spun briefly and 300μl of S1 digestion mix was added, containing 4μg of salmon sperm DNA and 400units of S1 nuclease in 1xS1 buffer; digestion was carried out at 37°C for 1.5h. The reaction was stopped by the addition of 6μl of 0.25M EDTA (pH8), and 2μl 1mg/ml tRNA with 700μl of ethanol was added. Protected fragments were precipitated at -70°C for 30min then pelleted at 12,000rpm for 10min and washed with 70% ethanol. They were resuspended in 3μl of 0.1M NaOH and 3μl of formamide dye mix was added. The samples were denatured by boiling before being loaded on a denaturing polyacrylamide gel (see 3.6.4.). Gels were electrophoresed for 1-1.5h at 40W and dried under vacuum before autoradiography at -70°C.

3.14. Indirect Immunofluorescence.

Coverslips were prepared for cells by boiling in 500ml DW with a few drops of Decon. They were then washed for 30min with DW and stored overnight in ethanol; before being dried between two pieces of filter paper and sterilised (by autoclaving) in glass petri dishes.

Cells were grown on circular coverslips (16mm in diameter) in 50mm plates at 37°C. The cells were infected when just confluent, with various dilutions of Ad40 in TS, each coverslip was infected with 20μl of virus dilution. Coverslips were incubated at 37°C for 1h before medium was added and further incubated at 37°C. Cells were fixed at 48h postinfection. Cells were washed twice with cold PBS and fixed by adding a 3:1 mix

of ice-cold methanol:acetone, followed by incubation at RT for 10min. The methanol mix was removed and cells were washed twice with PBS. Coverslips were either stored under PBS at 4°C for a few days or stained immediately.

The primary antibody used was an Ad5 anti-hexon mAb (10/5.1) which was diluted 1/100 in PBS and spun at 12,000rpm for 2min. Coverslips were incubated with 20µl of the antibody dilution for 30min at RT. The coverslips were washed in PBS four times. The second antibody was goat antimouse IgG fluorescein isothiocyanate conjugate (Nordic Immunochemicals) diluted 1/100 in PBS, filtered through an 0.45µm filter and spun at 12,000rpm for 2min. 20µl was layered onto the coverslips and cells were incubated at RT for 30min. The cells were washed 7 times in PBS and once in DW. Coverslips were mounted on microscope slides in a 1:1 mix of glycerol:PBSA. The coverslips were sealed with nail varnish and the cells examined on a Nikon Microphot-SA fluorescence microscope using the blue filter at wavelength 480nm for fluorescein.

3.15. Preparation of Crude Ad2 Fibre.

$5x10^7$ Sf21 cells were infected with 1pfu/cell of AcNPV^{fib}FL582 (Novelli and Boulanger, 1991). Virus was adsorbed for 1h at 28°C before the addition of medium and incubation continued at 28°C. Cells were harvested 72h postinfection. Virus was extracted by five freeze thaw cycles (-70°C to 37°C) and pelleted free of debris at 2,000rpm for 10min. An equal volume of arcton was added to the virus containing supernatant and vortexed for 1min, after which the mix was spun at 2,000rpm for 10min. The top layer of crude Ad2 fibre was stored in aliquots at -70°C.

Maintenance of AcNPV^{fib}FL582 and preparation of crude Ad2 fibre were kindly carried out by G. Brown.

3.16. Attachment and Internalisation Assay.

Cells were grown to confluency in linbro wells (2cm^2 surface area) at 37°C. The amount of attached virus particles was determined by incubating cells at 4°C with tritium labelled virus particles (see 3.2.3.). Cells were precooled at 4°C for 30min; before infection cells were counted (on extra wells plated) and virus dilutions were adjusted accordingly. 40µl of labelled virus (diluted in TS) was then added and incubation continued for 2h at 4°C, with wells shaken every 15min. Cells were washed with cold 1xHBS three times followed by the addition of 200µl of cell adhesion buffer (warmed to 37°C). Cells were warmed to 37°C and incubated for various times at this temperature. To distinguish attached from internalised virus half the wells were treated with the protease subtilisin. Cells were washed twice in cold 1xHBSE, after which 200µl of either 1xHBSE or 1xHBSE containing 2mg/ml subtilisin was added (both sets of buffers were warmed to 37°C); incubation was continued at 37°C for 30min. Finally the cells were scraped into a 1.5ml vial and washed twice with 400µl cold cell adhesion buffer. 100µl EB buffer was added to cell pellets, samples were vortexed and incubated at RT for 10min. 10ml of ecoscint was added to each sample and thoroughly mixed, and the tritium counts per minute were counted in a scintillation counter.

For competition experiments cells were preincubated with either ten fold excess unlabelled banded Ad40 virus particles or 8µg of crude Ad2 fibre (see 3.15.); both competitors were diluted in TS and pretreated at 56°C for 30min. Cells in linbro wells were cooled to 4°C for 30min, and competitors added (in 40µl volumes) to half the wells and 40µl of TS added to the remainder; incubation was continued for 1h with frequent shaking. Cells were washed twice with cold 1xHBS and labelled particles added at 4°C. After a 2h incubation at 4°C the cells were washed three times with cold 1xHBS and pelleted. The pellets were dissolved in 100µl of EB buffer and processed as above.

For attachment experiments, cells were precooled to 4°C for 30min, various dilutions of labelled virus particles (40µl in TS) were added and incubation continued for

2h at 4°C (with frequent shaking). Cells were washed three times with cold 1xHBS and pelleted. The pellets were dissolved in 100µl of elution buffer and processed as above.

3.17. Preparation of Nuclear Extracts.

Nuclear cell extracts were prepared using the modified Dignam procedure (Dignam *et al.*, 1983). Cells were grown on 140mm plates to confluency. Plates were washed with 10ml ice cold PBS and cells scraped into 5ml ice cold PBS. Cells were pooled and pelleted by centrifugation in a Dupont table top centrifuge at 4°C for 5min at 2,000rpm. Cell pellets were resuspended in two pellet volumes of buffer A and incubated on ice for 20min. Samples were mixed by pipetting up and down, and incubation on ice continued for a further 10min. Cells were then lysed by 15 strokes in a dounce homogeniser. Nuclei were pelleted by centrifugation at 6,000rpm in a benchtop microcentrifuge for 2min at 4°C. The cytoplasmic fraction was removed and the nuclei packed down again by centrifugation at 15,000g in a microcentrifuge for 15min at 4°C. Any remaining supernatant was removed and the nuclei were resuspended in two cell volumes of buffer C. Samples were incubated on ice for 20min, and mixed every 5min by pipetting up and down. Suspensions were pelleted free of nuclear debris by centrifugation at 15,000g for 15min. The supernatant was aliquoted and stored at -70°C.

Protein concentrations were determined by the Bradford protein assay (Bradford, 1976) using known amounts of BSA for a standard curve. 1ml of Bradford reagent was added to samples, mixed and incubated for 20min at RT. The absorbance was then measured at 595nm.

3.18. WGA Affinity Chromatography of Sp1.

3.18.1. Preparation of Sp1 Extracts.

CV-1 cells ($5x10^7$ cells) were infected with the vaccinia virus recombinant vSp1 at 10pfu/cell. Virus was adsorbed at 37°C for 1.5h and then medium was added. At 26h

postinfection cells were harvested by washing twice with cold PBS and scraping into PBS. The cells were pelleted at 2,000rpm for 10min at 4°C. The pellet was resuspended in 2ml of HS buffer and spun free of cell debris at 42,000rpm for 1h at 4°C. The supernatant was subjected to wheat-germ agglutinin (WGA) affinity chromatography (see 3.18.2.).

Maintenance of vSp1 and preparation of Sp1 extracts were kindly carried out by A. Rinaldi.

3.18.2. WGA Affinity Chromatography.

Sp1 was purified by WGA affinity chromatography (Jackson and Tjian, 1989). The column was made from WGA-agarose resin (Vector laboratories). The packed column volume was 0.4ml, and was equilibrated with 2ml of HS. Cell extracts were loaded onto the column in 2ml of HS buffer (see 3.18.1.) and the column washed with 2ml of HS buffer followed by 4ml of Z' buffer. Glycosylated proteins were eluted in five 0.4ml fractions with 2ml of 0.3M N-acetylglucosamine (NAcGlc) in Z' buffer. The fractions were pooled, made up to 50% glycerol with respect to Z' buffer and stored in aliquots at -70°C.

3.19. Gel Retardation Assay.

Probes for gel retardation assays were prepared by 5' labelling oligonucleotides (see 3.9.2.) which were subsequently annealed with their unlabelled complementary strands (see 3.10.2.), and purified (see 3.7.) on non-denaturing polyacrylamide gels.

Binding reactions were performed in a 20µl mix containing 1xRM, 1-2µg poly (dI-dC).poly (dI-dC), approximately 1ng of probe, 5µg of nuclear extract or 0.5µg Sp1 protein purified by WGA affinity chromatography (see 3.18.) and a total of 4µl buffer C. Samples were incubated at 25°C for 30min then loaded directly onto a 3.5% non-denaturing polyacrylamide gel (see 3.6.3.). 4µl loading dye mix was run in an adjacent

well as a marker. Electrophoresis was carried out in 0.5xTBE at 180V for 2.5h. Gels were dried under vacuum, and exposed to film at -70°C.

In competition experiments, annealed unlabelled oligonucleotides were added to extracts (with all buffers present) and incubated for 15min at 25°C, after which probe was then added to the samples, followed by a 30min incubation at 25°C. The samples were then processed as above.

4. RESULTS.

Introduction.

The overall aim of this thesis was to characterise Ad40 growth in the permissive cell line INT407.

Ad40 grows poorly on conventional human cell lines, such as HeLa and KB, which are used to grow non-fastidious adenovirus serotypes (Takiff and Straus, 1982; Mautner., *et al* 1989). Takiff and Straus (1982) found it was an early replicative block that was responsible for the poor growth of Ad40 (and Ad41). This block was overcome by growing in cells which constitutively express the Ad5 or Ad2 E1 regions; these are 293 and KBa+b cells lines respectively (Takiff *et al.,* 1981, de Jong *et al.,* 1983; Mautner *et al.,* 1989). In contrast, some workers have had variable success growing Ad40 on 293 cells (Chiba *et al.,* 1983; Uhnoo *et al.,* 1983). This may have arisen due to differences in virus isolates used. Alternatively, Ad40 can grow in KBb cells which express only the Ad2 E1B region (Mautner *et al.,* 1989). This suggested that the Ad40 E1B region was defective. Complementation assays conducted *in trans* with helper viruses, supplying the Ad2 or Ad12 E1B 19K or 55K functions (Mautner *et al.,* 1989; Gomes *et al.,* 1992), indicated that expression or function of the Ad40 E1B 55K protein was defective. a

As it is uncertain how Ad40 gene expression and DNA replication may be affected by the endogenous E1 proteins present in KBa+b and 293 cells, a better cell line to use would be of intestinal origin, which supports virus growth but does not express adenovirus viral gene proteins. Some continuous cell lines, which are permissive for enteric adenoviruses but do not express adenovirus proteins have been reported (Kidd and Madeley, 1981; Pieniazek *et al.,* 1990a; Hashimoto *et al.,* 1991; Steinthorsdottir 1991; Gomes *et al.,* 1992). Steinthorsdottir (1991) found that the INT407 cell line (described in the ATCC as an embryonic intestinal cell line) could support Ad40 growth. The virion DNA yields on INT407 cells were 70% of that in KBa+b cells, while for HeLa the level was only 17%. This experiment indicated that Ad40 could grow in tissue culture without

complementing viral gene products and there was an increase of permissiveness from HeLa through INT407 to KBa+b cells.

INT407 cells were permissive for Ad40 growth and were not known to express adenovirus proteins. INT407 cells have subsequently been shown to be a HeLa cell derivative (Gilbert *et al.*, 1990). However there is a clear difference in the permissivity for Ad40 between INT407 and HeLa cells. Therefore this study was undertaken to characterise the growth of Ad40 in INT407 cells in comparison to other permissive (KBa+b, 293) and semi-permissive cell lines (HeLa, A549).

4.1. Timecourse of Viral DNA Replication in Ad40 Infected Permissive and Semi-Permissive Cells.

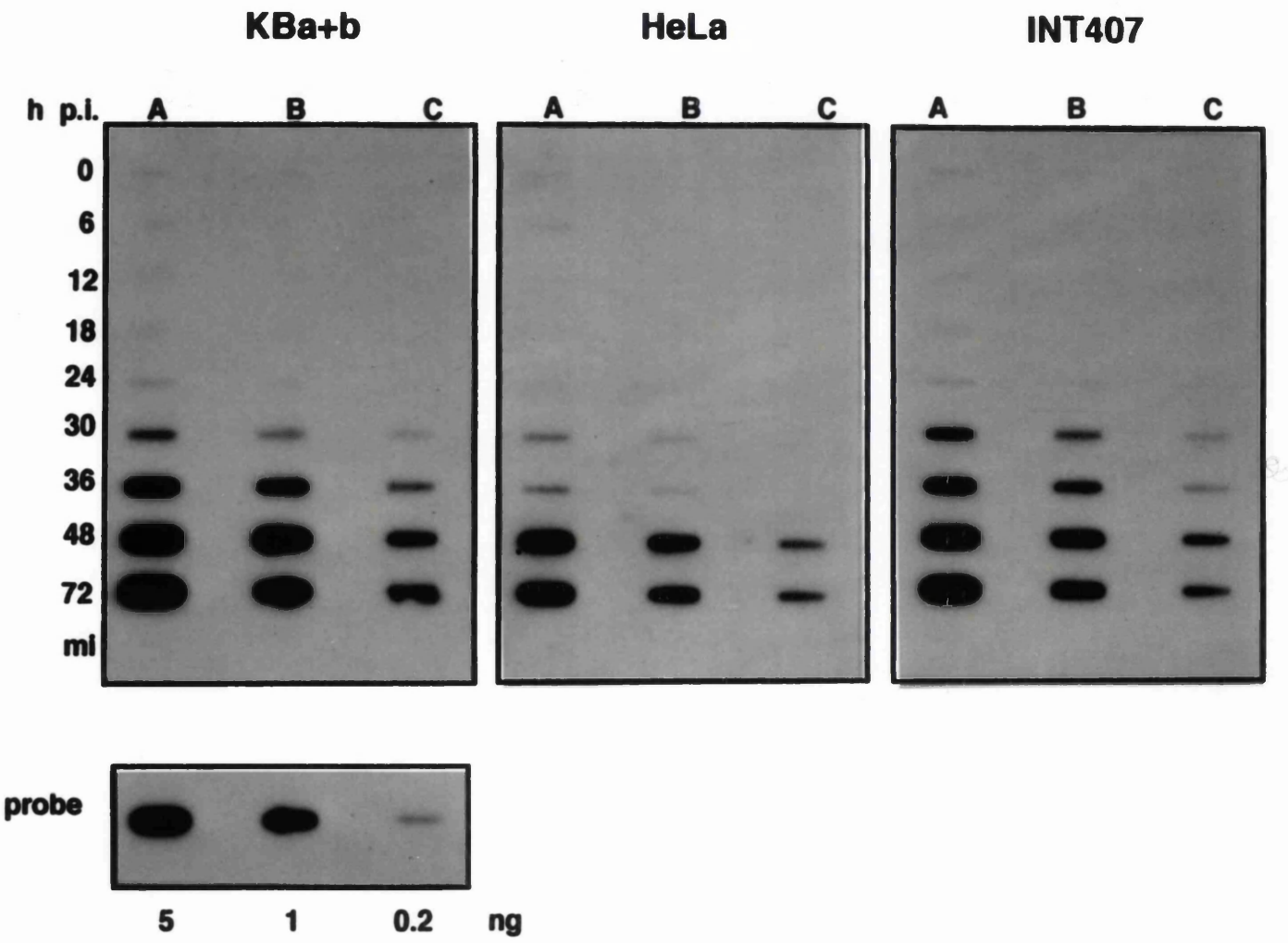
A detailed comparison of Ad40 DNA replication in INT407, KBa+b and HeLa cells was conducted. The following experiment was carried out in order to elucidate the time of onset of DNA replication and the overall yield of Ad40 virion DNA on KBa+b cells (which complement the Ad40 E1B 55K defect) in comparison with INT407 cells (which are not known to express adenovirus proteins).

KBa+b, HeLa and INT407 cells were grown on 50mm plates and infected with 100µl of a 1/10 dilution of Ad40 p9 (in TS). After incubation at 37°C, two plates were pooled and harvested at various times for each cell type. Viral DNA was extracted as described (see 3.3.1.) and dilutions of DNA were applied to nylon membranes using a slot blot apparatus (see 3.11.). Membranes were probed with nick translated plasmid containing the Ad40 E1 region (pNM82). The membranes were exposed to autoradiography film (Figure 4.1.1.) and quantitated using phosphorimager technology (Figure 4.1.2.).

The pattern of virion DNA is shown in Figure 4.1.1. and is first detected in small quantities at 24h for both KBa+b and INT407 cells, after which there is a steady increase in DNA replication up to 72h post infection. A greater level of DNA replication is detected on INT407 compared to KBa+b cells at 30h. At subsequent times the level observed on KBa+b cells is greater than on INT407 cells and this difference is more marked as time goes on, as shown in Fig 4.1.2. For HeLa cells, Ad40 replication was just detectable at 30 and 36h p.i., an increase in DNA is detected later between 36 to 42h post infection. At 72h the amount of Ad40 replication on HeLa cells was approximately 4-fold lower than that seen on KBa+b cells. In Fig 4.1.2., the pattern of virion DNA replication on both INT407 and KBa+b cells is shown to be very similar up to 36h, after which there is a dramatic increase in KBa+b infected cells. Levels of virion DNA on INT407 cells are intermediate between KBa+b and HeLa, but the pattern is the same as KBa+b, with no plateau observed. In HeLa infected cells very small amounts of replication are detected

Figure 4.1.1. Timecourse of Virion DNA Replication on Ad40 Infected KBa+b, HeLa and INT407 Cells.

KBa+b, HeLa and INT407 cells were infected with 100µl of a 1/10 dilution of Ad40 p9 stock (in TS). Virus was harvested at the times indicated p.i., and viral DNA extracted. Dilutions (A is 1/10, B is 1/25 and C is 1/100) were applied to nylon membranes using a slot blot and probed with a nick translated plasmid containing the Ad40 E1 region (pNM82). The experiment was conducted three times with a similar outcome, a typical result is shown. The top three panels show a timecourse of Ad40 DNA replication on infected KBa+b, HeLa and INT407 cells. The bottom left hand panel shows the plasmid control.


KBa+b
HeLa
INT407
h p.i.
A
B
C
0
6
12
18
24
30
36
48
72
mi
probe
5
1
0.2
ng

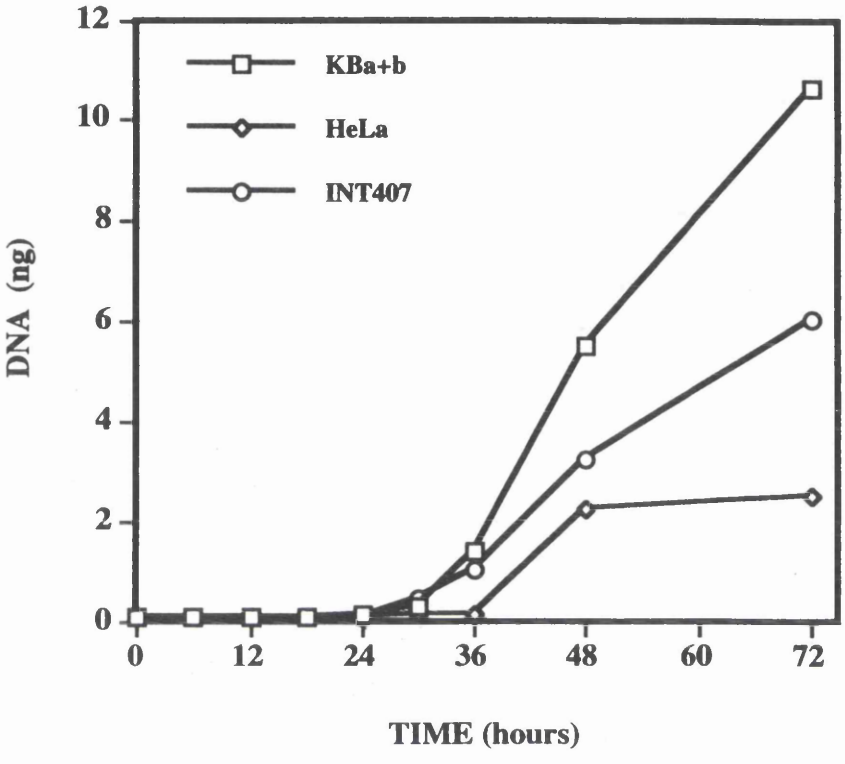


Figure 4.1.2. Quantitative Analysis of Ad40 DNA in Various Cell Lines.

Membranes were scanned on a Phosphorimager and quantitated with a Imagequant software package (Molecular Dynamics). The amount of DNA in ng was calculated by comparing the 5ng value of Ad40 E1 plasmid standard (see Fig 4.1.1.) to Ad40 DNA from the timecourse. The values above correspond to the 1/10 dilution (see Fig 4.1.1.).

until 36h, after which there is a sharp increase to 48h, thereafter there is an apparent plateau.

4.1.1. Summary.

A similar pattern of virus replication is observed on KBa+b and INT407 cells. This experiment indicates that Ad40 can grow in cells not known to express E1 proteins. The growth of Ad40 on INT407 cells is reduced compared to KBa+b cells at 72h post infection. However these cells are clearly more permissive than HeLa cells in terms of the onset of replication and the overall yield of virus replication.

4.2. Replication of Ad5 and Adenovirus Early Region E1 Mutants on Permissive and Semi-Permissive Cells.

The following experiments were undertaken in order to investigate the growth of other adenovirus serotypes on the INT407 cell line. If INT407 cells could support the growth of Ad40 which had an E1B 55K defect, the question was asked if adenovirus mutants defective in E1B 55K function could also be complemented by INT407 cells.

The A549 cell line which was reported to be permissive for Ad40 (Hashimoto *et al.*, 1991), but not known to express E1 proteins, was also included. A549 cells were shown to be permissive for Ad40 by Steinthorsdottir (1991). However this author found the level of virus replication was intermediate between INT407 and HeLa cells.

The first experiment was carried out with Ad5/2 E1 mutants. The Ad5 mutant dl309 was isolated by Jones and Shenk (1979a). This virus lacks three of the four Xba I sites present in wt Ad5, and in addition a large portion of the E3 region is deleted (83 to 85 mu). However dl309 grows as well as wt in tissue culture and was used as a model for wt virus in these studies. The two E1B mutants used (Barker and Berk, 1987) were constructed from dl309 and Ad2 E1 sequences. The E1B mutant pm1722 contains the Ad2 E1B region in a dl309 background. The E1B sequences have a G to A point mutation at nt 1722, introducing a stop codon, which prevents expression of the E1B 19K protein. This mutant replicates as well as wt in HeLa cells, but displays *cyt* and *deg* phenotypes characterised by extreme cytopathic effect and degradation of cellular DNA, respectively. The E1B 55K mutant, dl1520, consists of Ad2 E1A and E1B in a dl309 background. The E1B sequences are altered with a C to T point mutation at nt 2022, which generates a stop codon. In addition, the region from 2496 to 3323nt is deleted, thus the E1B 55K and related proteins are not made, but the 19K open reading frame is unaffected. Virus replication is defective in HeLa cells with this mutant, but can be partially overcome at high moi.

Ad40 was used at a 1/10 dilution, and the Ad5/2 viruses were used at a moi of 3pfu/cell. The four cell lines used in this experiment were: KBa+b, HeLa, INT407 and A549. Cells were grown on 50mm plates, infected with 100µl of virus dilutions (in TS)

and harvested at 72h post infection. Virus was released by three rounds of freeze thawing and the virion DNA extracted as described (see 3.3.1.). Dilutions of the DNA were applied to nylon membranes. The membranes were probed with a mix of Ad2 E1A- and E1B-containing plasmids (for the Ad5/2 mutant viruses) or with a plasmid which contained the whole of the Ad40 E1 region. The resulting autoradiographs were quantitated by Hoeffer scanning densitometry. Probes had previously been tested for lack of cross-reaction between Ad5 and Ad40 sequences by Steinthorsdottir (1991).

The autoradiographs are shown in Figure 4.2.1. The wt virus dl309 grows on all cell types, with similar overall levels observed on INT407 and A549 cells. Virus replication in KBa+b cells is 25% less than the level detected on A549 and INT407 cells. In HeLa cells, a lower DNA yield is observed, approximately 50% less than the level detected in INT407 and A549 cells. The 19K mutant pm1722 replication is similar to wt (dl309) on A549, but lower in HeLa cells. However, very low yields are obtained in KBa+b and INT407 cells; from previous reports higher DNA levels of pm1722 were expected on KBa+b cells (Steinthorsdottir, 1991). Extreme cytopathic effects had been observed on both these cell types at 72h post infection. This may account for the lower levels seen on these cell lines, particularly if cells had lysed, releasing virus into the surrounding medium. This virus would not have been harvested, as virion packaged DNA was prepared from cell-associated virus.

Wild type virus grew equally well on INT407 and A549 cells, but the 55K mutant dl1520 grew better on INT407 than on A549, however even on INT407 the yield is lower relative to wt. The amount of dl1520 virion DNA from KBa+b cells is reduced compared to that described previously (Mautner *et al.*, 1989). The highest level observed is on INT407 cells (with 40% of wt levels). On A549, dl1520 replication is 70% of the level seen on INT407 cells. In contrast very little complementation is observed for dl1520 on HeLa cells, as expected (Barker and Berk, 1987; Mautner. *et al.*, 1989).

For Ad40 infected cells, the amount of DNA detected is 5-fold and 3-fold lower on HeLa and A549 cells (respectively), compared to KBa+b cells. These results were comparable to those found previously (see 4.1.) and reported elsewhere (Steinthorsdottir, 1991).

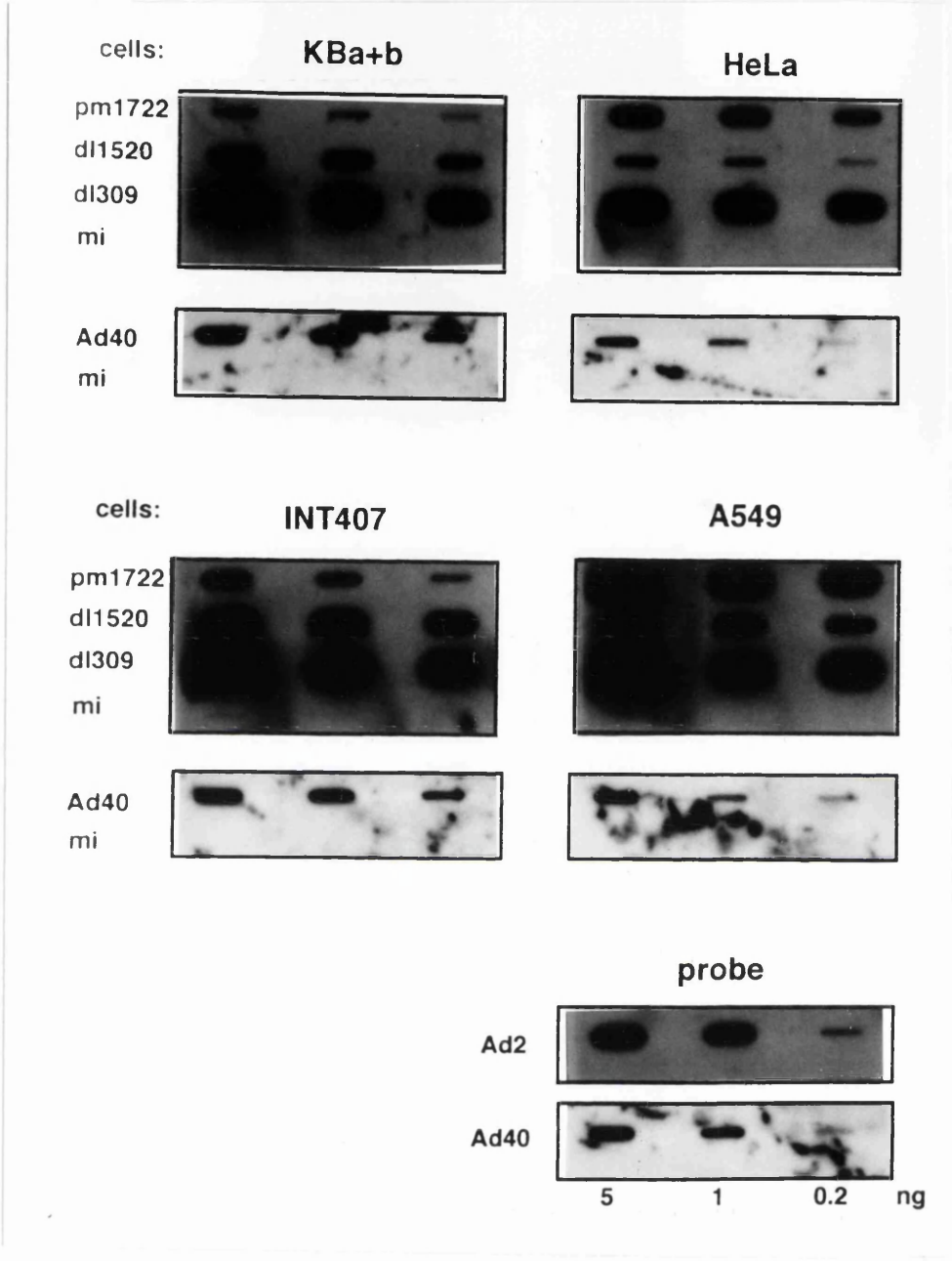


Figure 4.2.1. Infection of Ad5/2 Mutants on KBa+b, HeLa, INT407 and A549 Cells.

Cells were infected with 3pfu/cell of the Ad5/2 mutants and 100µl of a 1/10 dilution of p9 Ad40 (in TS). Virus was harvested at 72h p.i. and virion DNA extracted. The following dilutions were applied to nylon membranes; 1/40, 3/400 and 1/400. Membranes were probed with nick translated plasmids containing either a mix of Ad2 E1A and E1B (pNM90 and pNM92) for the Ad5/2 mutants or the Ad40 E1 region (pNM82) for Ad40 sequences. The top eight panels show the yield of virus on the four cell types and the two lower bottom right hand panels show plasmid controls used.

The experiment was repeated with a lower moi; in order to see if the low level of virus DNA detected on KBa+b and INT407 cells with the pm1722 mutant was a result of the extreme cytopathic effect observed. In addition, it would be possible to investigate if the complementation obtained with dl1520 on INT407 and A549 cells was due to the virus being used at a high moi, as has been previously reported for this mutant (Barker and Berk, 1987).

The Ad5 E1A mutant dl312 was also included in this experiment. dl312 was constructed by Jones and Shenk (1979a) and it contains a large deletion in the E1A region of dl309. It is defective for growth on HeLa cells, but the defect can be complemented by growing on 293 cells. This virus was included in order to determine if adenovirus E1A mutants could be complemented on INT407 and A549 cells.

The resulting autoradiographs are shown in Figure 4.2.2. and the quantitation by densitometric analysis in Figure 4.2.3. At 1pfu/cell, the level of virus replication detected for dl309 on INT407 is lower than the level observed on KBa+b cells. The level of viral DNA replication detected on HeLa and A549 cells is similar, but is reduced (approx. 3 fold) compared to KBa+b. Near wild type (dl309) levels of pm1722 are observed on most cell lines. The amount of pm1722 viral DNA is similar on INT407 and KBa+b cells, but in comparison virus replication was reduced on HeLa and A549 cells. Thus the lower levels of pm1722 DNA replication (compared to wt) detected for INT407 and KBa+b cells in Figure 4.2.1. was probably due to the higher moi used and extreme cytopathic effect observed. dl1520 was complemented most on KBa+b and INT407 cells, and moderately on A549 cells (see Figure 4.2.3.). In addition, dl1520 replication is reduced compared to wt, on these cell lines. This mutant is defective for replication on HeLa cells, as described previously.

The pattern of Ad40 DNA replication seen in Figure 4.2.2. was similar to that described previously, with highest levels of Ad40 DNA replication detected on KBa+b and lowest on HeLa cells with intermediate levels on INT407 and A549 cells i.e. KBa+b > INT407 > A549 > HeLa.

In KBa+b cells, the E1B 55K defect in dl1520 is complemented by endogenous E1B proteins. However this mutant also grew in INT407 and A549 cells, which are not

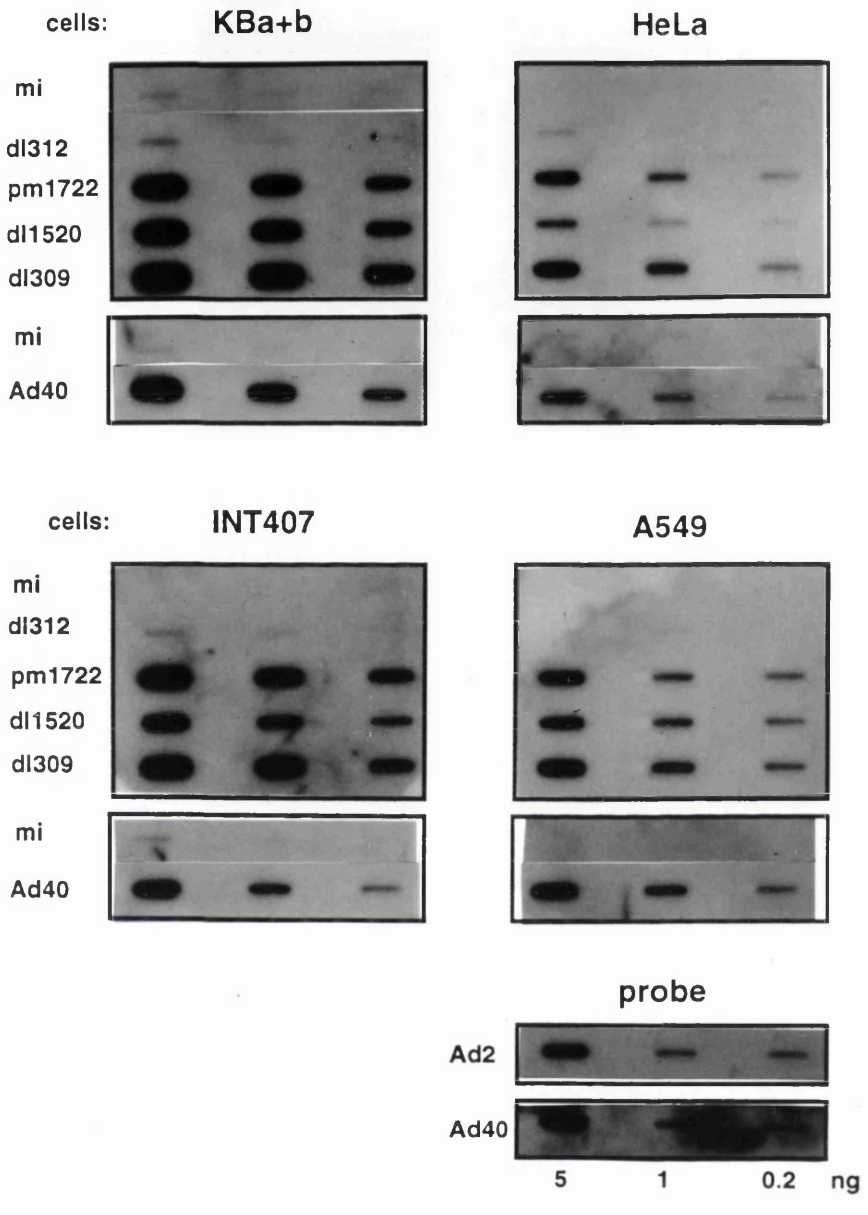


Figure 4.2.2. Infection of Ad5/2 Mutants on KBa+b , HeLa, INT407 and A549 Cells at a Low moi.

Cells were infected with 1pfu/cell of the Ad5/2 mutants and 100µl of a 1/10 dilution of p9 Ad40 (in TS). Cells were harvested at 72h p.i. and virion DNA extracted. The following dilutions were applied to nylon membranes; 1/40, 3/400 and 1/400. The membranes were probed with nick translated plasmids, either a mix of pNM90 and pNM92 (containing the Ad2 E1A and E1B region) or pNM82 (containing the Ad40 E1 region). The top eight panels show virus replication on the four cell types and the bottom right hand panel shows plasmid controls used.

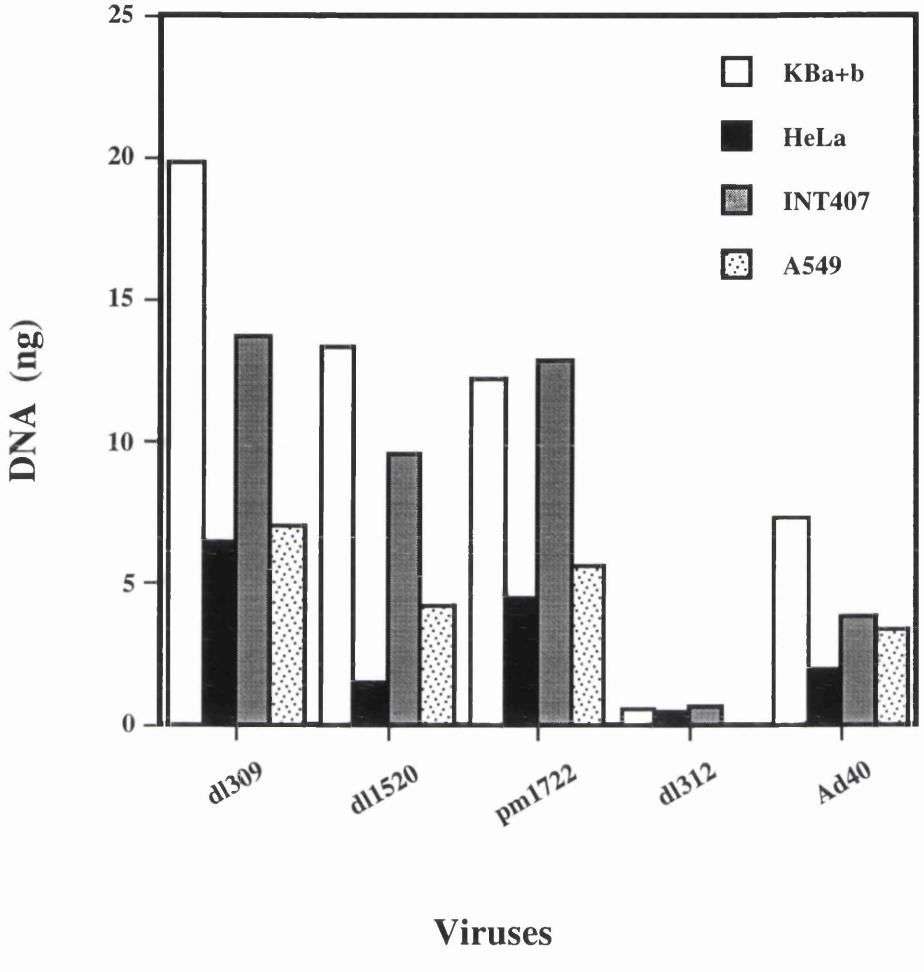


Figure 4.2.3. Quantitation of Ad5/2 and Ad40 Virion DNA from Various Cell Lines.

Autoradiographs (Fig 4.2.2.) were quantitated by densitometric analysis (Hoeffer). The amount of DNA in ng was calculated by comparing the 5ng values of the adenovirus E1 plasmid standards (see Fig 4.2.3.) to virion DNA at 72h post infection. The values above correspond to the 1/40 dilution (see Fig 4.2.3.).

4.3. Growth Kinetics of Ad40 on Permissive and Semi-Permissive Cells.

Tiemessen and Kidd (1988) employed an immunofluorescent assay to investigate the complementation and interference effects of Ad2 and Ad41 separately and in mixed infections. In non-permissive human embryo fibroblasts (HEF) cells, Ad41 late antigen synthesis was only detected in coinfections, with Ad2 providing a helper function. Ad41 replication in semi-permissive Chang conjunctival cells was enhanced by Ad2, and this complementation was dependent on the input concentration of the virus. In addition there was interference by Ad41 on Ad2 replication in HEF cells. The extent of interference depended on the relative time of infection by each serotype. The complementation of Ad41 by Ad2, in HEF cells, suggested that Ad41 was completely dependant on Ad2 helper function in these cells. These workers used this assay to monitor the number of Ad41 infected cells at various dilutions (Tiemessen and Kidd, 1990). Ad41 infection of Chang conjunctival cells followed multiple-hit kinetics (i.e. many virions were required to infect a cell), while in Ad41/Ad2 coinfections in HEF cells, Ad41 showed two-hit kinetics (i.e. two virus particles were needed to infect a cell). On the permissive 293 cell line, fluorescent cell counts for Ad41 were directly proportional to input concentration, which showed infectivity of one-hit kinetics. A possible explanation for the multiple hit pattern seen in Chang conjunctival cells , may have been due to the limiting concentration of an essential product. Many infecting genomes may overcome this limitation and lead to increased numbers of cells being productively infected, as the concentration of input virus increased. The requirement for this product is probably cell type dependent as single infections on HEF and Chang conjunctival cells are non-permissive and semi-permissive respectively.

The work in this section described the growth kinetics of Ad40 in cells showing different degrees of permissiveness. These experiments were conducted in order to see if differences in infectivity could explain the pattern of DNA replication previously observed between cell types (see 4.1.).

4.3.1. Investigation of the Growth Kinetics of Ad40 on Permissive and Semi-Permissive Cell Lines Using Indirect Immunofluorescence.

A similar approach was used as described by Kidd and Tiemessen (1988, 1990). Ad41 late antigen synthesis was monitored by indirect immunofluorescence using an Ad41 mAb. The four cell types studied were KBa+b, HeLa, INT407 and A549 cells. The method is described in detail elsewhere (see 3.14.) and briefly outlined here. Cells were grown on coverslips; when subconfluent they were infected with various dilutions of Ad40 in TS. The virus stock had a titre of $5.6x10^6$ FFU/ml, on KBa+b cells. Cells were fixed at 48h p.i. and incubated with Ad5 anti-hexon mAb at a 1/100 dilution (in PBS). The second antibody (Goat anti-mouse FITC) was added at a 1/100 dilution (in PBS). Coverslips were mounted and examined using either the x20 or x10 objective on a Nikon Microphot-SA fluorescence microscope.

The raw data is shown in Table 4.3.1., and is expressed as focus forming units (FFU) per field, where one FFU represents a single infected fluorescent cell. A graticule with a grid (10mm x 10mm) was fitted into the right hand eye piece of the microscope; one field counted corresponded to the area of the graticule. Ten fields were counted for each coverslip and four replicate coverslips were counted per dilution for each cell type. Coverslips with KBa+b and INT407 infected cells were counted using the x20 objective, as there were a higher number of fluorescent cells, whereas the x10 was employed for A549 and HeLa cells, which had fewer fluorescent cells.

Table 4.3.1. shows the mean FFU/field and the standard error of the mean (SEM) for different moi of Ad40 infected cells. On KBa+b cells the number of FFU/field observed decreases proportionally compared to Ad40 input concentration i.e. the count for the 1/20 dilution was 40.2 FFU/field, for the 1/40 and 1/80 dilutions the values were 22.52 and 11.60 respectively; showing an approximately two fold decrease at each two fold reduction in dilution. Thus the FFU/field appears to be directly proportional to the virus input concentration. The greatest number of FFU/field are observed on KBa+b cells at each input concentration, compared to the other cell lines. Ad40 FFU/field on INT407 cells have the second highest counts and also appear to decrease proportionally to Ad40 input, although the correlation is not as close as observed on KBa+b cells. For both HeLa

Table 4.3.1. Ad40 Late Antigen Synthesis at 48h p.i. on KBa+b, INT407, HeLa and A549 Cell Lines.

Ad40 input concentration (Dilution)	Ad40 Fluorescence mean FFU/Field [a] ± SEM			
	x 20 [b]		x 10 [b]	
	KBa+b	INT407	HeLa	A549
1/20	40.20 ± 3.13	32.02 ± 2.97	31.60 ± 4.97	10.87 ± 2.82
1/40	22.52 ± 2.10	17.45 ± 2.85	13.05 ± 2.52	5.62 ± 2.48
1/60	17.40 ± 1.94	13.50 ± 2.36	5.60 ± 1.26	1.87 ± 0.85
1/80	11.60 ± 1.37	6.65 ± 1.59	4.85 ± 1.29	1.50 ± 0.78
1/100	6.20 ± 1.28	5.02 ± 1.53	1.95 ± 1.22	0.87 ± 0.72

a this value is the mean of 40 fields counted over 4 replicate coverslips (10 fields counted/coverslip).

b Cells were counted using the x20 or x10 objectives.

and A549 cells much lower numbers of FFU/field are detected, the numbers decrease as input concentration is reduced and the relationship does not appear to be directly proportional.

Fluorescent counts were expressed as FFU/10^6 cells (see Table 4.3.2.) which took into account the magnification and the differences in the total number of cells, between cell types. The results expressed as FFU/10^6 cells are shown in Table 4.3.2. with the Ad40 input concentration, expressed as FFU/ml. To determine the total number of cells per coverslip, 20 fields were counted over four replicate coverslips (5 fields/coverslip), with the x40 objective, using the bright field path of the microscope.

The greatest levels of Ad40 late antigen synthesis are detected at every Ad40 input concentration on KBa+b cells. The number of cells productively infected on INT407 are comparable to those seen on KBa+b cells. Ad40 fluorescent cell counts are approx. reduced by 7 and 16 fold on HeLa and A549 cells, respectively compared to KBa+b cells, at the 1/20 dilution.

In order to examine if Ad40, infected cells with one or two hit kinetics, the log values of Ad40 fluorescence and log Ad40 input concentration were calculated and plotted in Figure 4.3.1., the lines of best fit are shown and were generated from these data points using Linear Regression analysis (see below).

The question was asked how the data from this experiment differed from one and two hit kinetics. One hit kinetics is when a single virus particle is sufficient to infect a cell productively (Dulbecco and Vogt, 1953) and for two hit kinetics two virus particles are required. Theoretical data points were calculated for one and two hit kinetics. These data points were calculated assuming that each particle has an equal chance of infecting a cell following one or two hit kinetics and that infection of individual cells occurs independently. An arbitrary value was used for the 1/20 dilution ($5x10^5$ FFU/10^6 cells for both lines) and subsequent dilutions were calculated based on one or two hit kinetics. The results are expressed as log FFU/10^6 cells and are shown in graphical form in Figure 4.3.2 , with best fit lines.

Table 4.3.2. Ad40 FFU on Permissive and Semi-Permissive Cells at 48h p.i., Expressed as FFU/10^6 Cells.

Ad40 input concentration		Ad40 Fluorescence FFU/10^6 cells (x 10^5)			
Dilution	FFU/ml (x10^5)	KBa+b	INT407	HeLa	A549
1/20	2.850	2.525	1.827	0.349	0.140
1/40	1.425	1.410	0.999	0.144	0.073
1/60	0.950	1.100	0.768	0.062	0.024
1/80	0.712	0.730	0.379	0.054	0.020
1/100	0.570	0.390	0.285	0.022	0.014

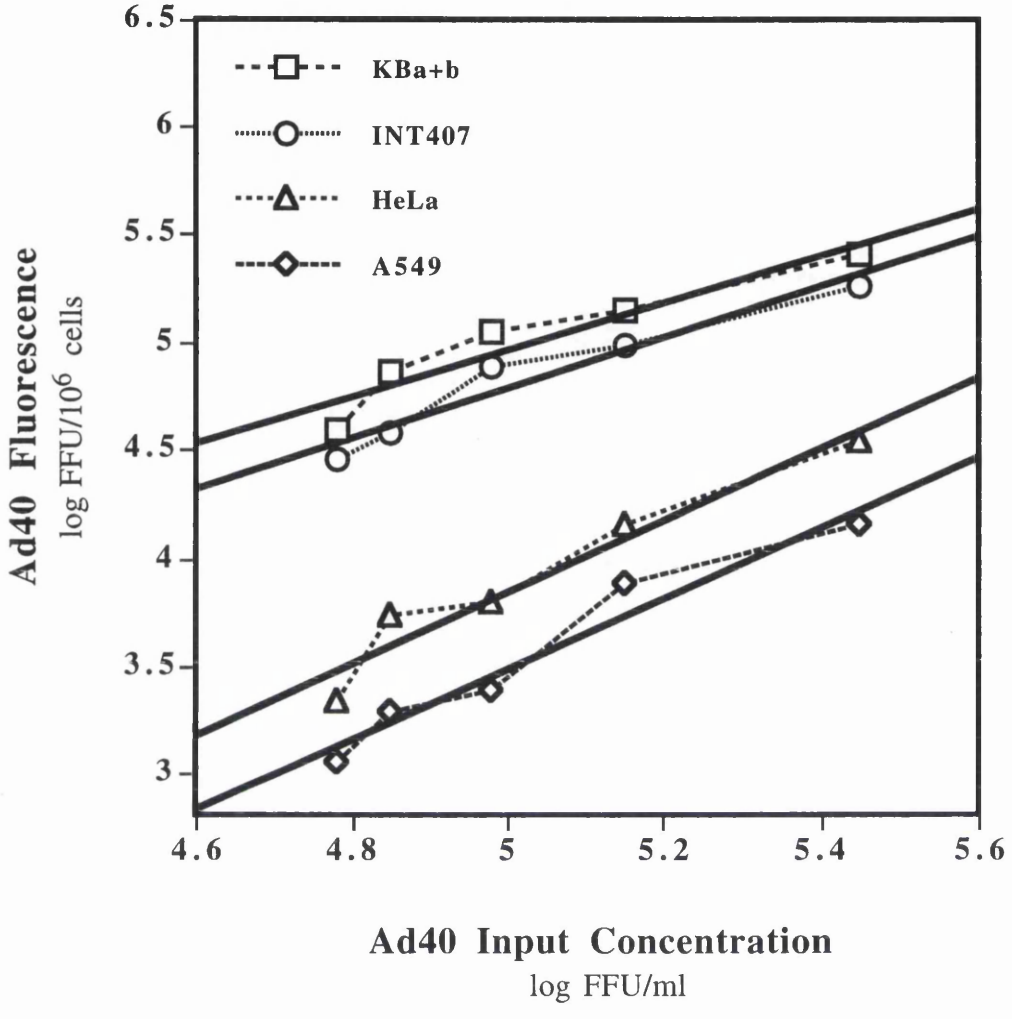


Figure 4.3.1. Ad40 Fluorescent Cell Counts for Permissive and Semi-Permissive Cell Lines, at Different Input Concentrations.

Ad40 fluorescent cell counts from Table 4.3.2. were expressed as log FFU/10^6 cells, these values were plotted against Ad40 input concentration log FFU/ml for each of the four cell types: KBa+b, INT407, HeLa and A549. The lines of best fit are also shown (solid black lines).

The lines of best fit (see Figures 4.3.1. & 4.3.2.) were generated using the following Linear Regression equation (Minitab statistical package) and the data points shown in Fig 4.3.1.:

$$y = \beta_o + \beta x + \varepsilon$$

β_o = y intercept of the line.

β = slope of the line of best fit.

ε = is the residual or the distance between the y value of the data point and the corresponding value of y that lies on the line of best fit at the same x value for the data point i.e. the predicted y value.

The β_o, β, se(β) and R-sq values from this analysis are shown in Table 4.3.3. In order to determine if the experimental data followed one or two hit kinetics, the slopes of the best fit lines for this data (Fig 4.3.1.) were compared in turn to the slopes for one or two hit kinetics (Fig 4.3.2.) using a *t* confidence interval test (Tytell *et al.*, 1962; Altman, 1991; Personal communication Dr B. Tarsney). This statistical test estimates if the slopes of two lines differ from each other, by assessing what the 95% confidence interval of the slope is when one slope is subtracted from the other. If the resulting interval contains zero the conclusion is that the slopes are not significantly different from each other, as in theory the two lines could have the same slope. However if the interval does not contain zero the conclusion is that the slopes are significantly different from each other. The equation to calculate the 95% confidence interval for the slopes of two lines is:

$$\beta_1 - \beta_2 \pm t_{0.05}\sqrt{[se(\beta_1)]^2 + [se(\beta_2)]^2}$$

β_1 = slope of best fit line 1.

β_2 = slope of best fit line 2.

se(β_1) = standard error of the slope β_1.

se(β_2) = standard error of the slope β_2.

$t_{0.05}$ = is a constant and corresponds to the 95% confidence limits for the *t* distribution.

For this analysis the degrees of freedom (def) are 6 and represent the 5 data points for each line minus the two regression coefficients (which are the y intercept and the

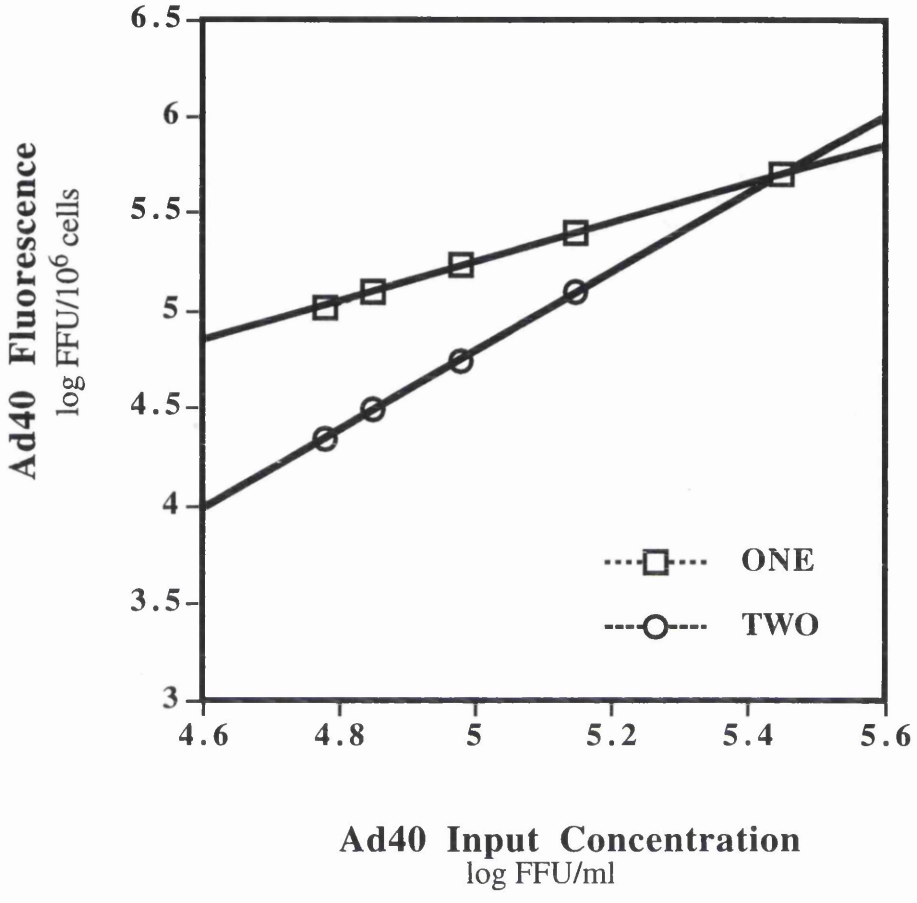


Figure 4.3.2. Theoretical Lines for One and Two Hit Kinetics.

Theoretical one and two hit lines were determined using an arbitrary value for the 1/20 dilution (5 x 10^5 FFU/10^6 cells), subsequent dilutions were calculated based on one and two hit kinetics. The log values for FFU/106 cells and Ad40 input concentration log FFU/ml are shown. The lines of best fit are also shown (solid black lines).

Table 4.3.3. Linear Regression Analysis of Lines of Best Fit.

Line of Best Fit for:	Slope of line (β)	se(β)	y-intercept (β_o)	R-Sq (%)
One Hit Kinetics	1.0044	0.2255	0.0032	100.0
Two Hit Kinetics	2.0027	0.0180	0.0180	100.0
KBa+b cells	1.1401	0.1662	-0.7260	94.0
INT407 cells	1.1573	0.1547	-0.9988	94.9
HeLa cells	1.6115	0.2013	-4.5630	95.5
A549 cells	1.6385	0.2053	-4.3450	95.5

se (β) = is the standard error of the slope β.

R-Sq = is the coefficient of determination and indicates the percentage of the variation in the data explained by the straight line relationship.

slope of the line) for each line, thus def =[(n-2) + (n-2)] (Personal communication Dr B. Tarsney). The $t_{0.05}$ value corresponds to 2.477 for 6 def (see t distribution tables in Altman, 1991). The 95% confidence intervals were calculated using the data from Table 4.3.3. and are shown in Table 4.3.4.

The analysis revealed that the slopes of the best fit lines for KBa+b and INT407 cells are not significantly different from the one hit slope, but are significantly different from the two hit line. In contrast the slopes for HeLa and A549 cells are not significantly different from the two hit slope, but are significantly different from the one hit slope.

4.3.2. Particle to Infectivity Ratio.

In addition the data from this experiment was used to derive Ad40 titres on each of the cell lines and thus compare particle to infectivity ratios for cell types. These are tabulated in Table 4.3.5. Titres of Ad40 on KBa+b and INT407 cells are similar but a lower titer is detected on HeLa cells, with the lowest titer detected on A549 cells. The Ad40 titer on A549 cells was approximately 15 fold less than that obtained with KBa+b cells.

The particle count of the Ad40 stocks used were kindly carried out by Mr J. Aitken and for this experiment was $2x10^{10}$ particles/ml. The number of particles present was divided by the titer to give particle to FFU ratios. Greater particle to infectivity ratios were detected for the semi-permissive cell lines than the permissive cells, with the lowest ratio observed on KBa+b cells. The ratios were comparable to particle to FFU ratios previously reported by Mautner *et al.*, (1990), these authors reported a ratio of $x10^3$ on KBa+b cells.

4.3.3. Summary.

These results show a marked difference in the number of productively infected cells between different cell types. The number of cells infected with Ad40 at each dilution from KBa+b and INT407 cells was much greater than from A549 and HeLa

Table 4.3.4. The 95% Confidence Intervals of Lines of Best Fit.

Lines of Best Fit		95 % Confidence Interval	P	Significance
1	2			
One Hit	KBa+b	0.2710, -0.5424	P > 0.05	NS
One Hit	INT407	0.2254, -0.5312	P > 0.05	NS
One Hit	HeLa	-0.1321, -1.1369	P < 0.05	S
One Hit	A549	-0.1147, -1.1033	P < 0.05	S
Two Hit	KBa+b	1.2720, 0.4534	P < 0.05	S
Two Hit	INT407	1.2271, 0.4637	P < 0.05	S
Two Hit	HeLa	0.8685, -0.1401	P > 0.05	NS
Two Hit	A549	0.8857, -0.1033	P > 0.05	NS

P = Probability

NS = Not Significant; the interval does contain zero therefore conclude slopes are not significantly different.

S = Significant; the interval does not include zero therefore conclude that slopes are significantly different.

Table 4.3.5. Titres and Particle-to-FFU Ratios for Ad40 on KBa+b, INT407, HeLa and A549 Cell Lines.

Cells	Titre (FFU/ml)	Particle:FFU
KBa+b	5.345×10^6	3.7×10^3:1
INT407	4.208×10^6	4.7×10^3:1
HeLa	9.050×10^5	2.2×10^4:1
A549	3.465×10^5	5.8×10^4:1

cells. Regression analysis of the slopes of the best fit lines when virus yield was plotted against virus input show that the KBa+b and INT407 slopes are not significantly different from a theoretical slope for one hit kinetics. Moreover the HeLa and A549 slopes are not significantly different from a theoretical slope for two hit kinetics.

Fewer cells were infected on HeLa and A549 cells, showing they may be defective in their ability to support the growth of Ad40. One possibility for this difference could be that lower numbers of particles attach and possibly enter HeLa and A549 cells compared to KBa+b and INT407 cells (see next section).

4.4. Attachment and Internalisation of Ad40 and dl309 on INT407 and HeLa Cells.

Viral attachment to and internalisation in cells has been extensively studied for adenovirus subgroups B and C (see section 1.2.) (Philipson *et al.*, 1968; Chardonnet and Dales, 1970; Defer *et al.*, 1990; Wickham *et al.*, 1993). The fibre protein, which extends from the penton base, mediates the initial attachment of adenovirus to cells. Following attachment to cells the virus is internalised by receptor-mediated endocytosis (Varga *et al.*, 1991). In Ad2 the virus binds to cell surface α_V integrins via an RGD sequence (Arg-Gly-Asp) which is present in the penton base protein. Wickham *et al.*, (1993) found that the interaction of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins with penton base promoted efficient uptake of virus into cell endosomes. These authors used antibody blocking assays and two melanoma cell lines, one possessing αv integrins and another deficient in these receptors. Their studies demonstrated that in Ad2, binding of penton base to integrins is needed for efficient internalisation, but not for attachment where interaction of the virion with a cell receptor is required. Mathias *et al.*, (1994) found other adenovirus serotypes (Ad3 and Ad4) also use αv integrins for infection. Ad40 does not have an RGD motif, instead an RGA motif is present. It is therefore possible that Ad40 may not use the pathway described for Ad2.

A possible explanation for the difference in the number of cells productively infected with Ad40, between permissive and semi-permissive cells, (see section 4.3.) may be due to differences in cell attachment and/or internalisation of the virus. Therefore the following assays were conducted in order to look at the possible differences in attachment and internalisation of Ad40 on INT407 and HeLa cells. In addition these assays were also carried out with dl309, to compare attachment and internalisation with Ad40 on these cell lines.

4.4.1. Attachment of Ad40 and dl309 to INT407 and HeLa Cells.

The method is described in detail elsewhere (see 3.16.). Briefly, cells were grown in linbro wells and various dilutions (40µl in TS) of tritium labelled virus (see 3.2.3.) were added to cells that had been cooled for 30min at 4°C. Studies have shown that at this temperature virus does not internalise but can bind cells efficiently (Fitzgerald *et al.*, 1983; Varga *et al.*, 1991; Wickham *et al.*, 1993), therefore this experiment was conducted at 4°C to ensure that none of the virus was internalised. Assays were carried out in duplicate for each dilution. The multiplicity of infection in this section corresponds to the number of labelled virus particles added to cells. Different multiplicities were used for Ad40 and dl309 which reflected their relative specific activities (see 3.2.3.). The intention had been to label and use virus of the same specific activities, however when the viruses were labelled the specific activity of Ad40 was found to be lower than dl309 (see 3.2.3.). Preliminary experiments were conducted using the same moi for both Ad40 and dl309. For Ad40, the level of cpm (counts per minute) detected bound to cells was only a few fold greater than background levels, whereas for dl309 counts were dramatically higher. Therefore a higher range was used for Ad40 in order to increase cpm detected. Attachment was carried out for 2h at 4°C, after which the cells were washed and pelleted. The pellets were dissolved in EB buffer and scintillation fluid was added, samples were finally counted in a scintillation counter.

The tritium counts per minute for each duplicate were tabulated along with the error (δ_1 δ_2) (e.g. see columns 2 and 3 in Table 4.4.1.). The error was calculated by the scintillation counter as a percentage of the counts for each sample which were then converted into cpm, these represented the standard deviations for each of the duplicates (δ_1 and δ_2 for each duplicate). The background values were not subtracted, but are shown in notes attached to each table.

This cpm data was expressed as particles/cell (e.g. see columns 4 and 5 in Table 4.4.1.) in order to compare experiments carried out on each cell line. Samples containing known numbers of labelled virus particles were also counted in each assay, and were used as a standard with which the number of particles was calculated for each sample in the assay. In addition extra linbro wells were seeded with cells which were counted for each

experiment, this data along with the number of particles per sample was then used to calculate particles/cell. The experiments in this section do not however take into account any loss of cells due to washing and assumes that the number of cells remain the same throughout the assay (i.e. compared to the number that was counted at the start of the assay).

The mean number of particles bound (particles/cell) and standard deviation (δ) of the two duplicates was calculated and tabulated (e.g. see columns 6 and 7 in Table 4.4.1.). The δ value was calculated using the following formula (Personal communication from Dr J. Love and Dr G. Murray).

$$\delta = \sqrt{\frac{\delta_1^2 + \delta_2^2}{2}}$$

Where :

δ_1 = is the error from duplicate one.

δ_2 = is the error from duplicate two.

δ = is the combined standard deviation from duplicates one and two.

The cell lines used for these experiments were INT407 and HeLa cells. In preliminary experiments KBa+b cells had also been included, but these monolayers were not as robust as INT407 and HeLa cells; even with gentle washing, KBa+b cell, monolayers detached after two washes, therefore further experiments were carried out with INT407 and HeLa cells only.

4.4.1.1. Attachment of Ad40 to INT407 and HeLa Cells.

The first experiment was carried out with labelled Ad40, at moi of 1000, 3000, 5000 and 7000 particles per cell, with both INT407 and HeLa cells. The cpm data, particles/cell, mean and δ values are tabulated in Tables 4.4.1. and 4.4.2. The mean number of particles bound and δ values were also represented in graphical form and are shown in Figure 4.4.1.

Table 4.4.1. Tritium Labelled Ad40 Attachment on INT407 Cells.

Input moi	cpm Bound [a]	Error δ1, δ2 (cpm)	Particles Bound [b] (part/cell)	Error δ1, δ2 (part/cell)	Mean Particles Bound (part/cell)	δ [c] (part/cell)
1000	53, 68	15, 16	61, 78	17, 18	69	17
3000	184, 214	27, 29	196, 228	29, 31	212	30
5000	367, 321	38, 36	479, 419	50, 47	449	48
7000	446, 399	42, 40	609, 545	57, 54	577	55

Table 4.4.2. Tritium Labelled Ad40 Attachment on HeLa Cells.

Input moi	cpm Bound [a]	Error δ1, δ2 (cpm)	Particles Bound [b] (part/cell)	Error δ1, δ2 (part/cell)	Mean Particles Bound (part/cell)	δ [c] (part/cell)
1000	89, 82	19, 18	102, 94	22, 21	98	21
3000	323, 343	36, 37	345, 368	38, 39	356	38
5000	398, 504	40, 45	520, 659	52, 59	589	56
7000	444, 662	42, 51	606, 904	57, 69	755	63

a The background value was 13 cpm.

b part/cell denotes particles/cell.

c The δ value was calculated from δ_1 and δ_2 using the formula described in section 4.4.1.

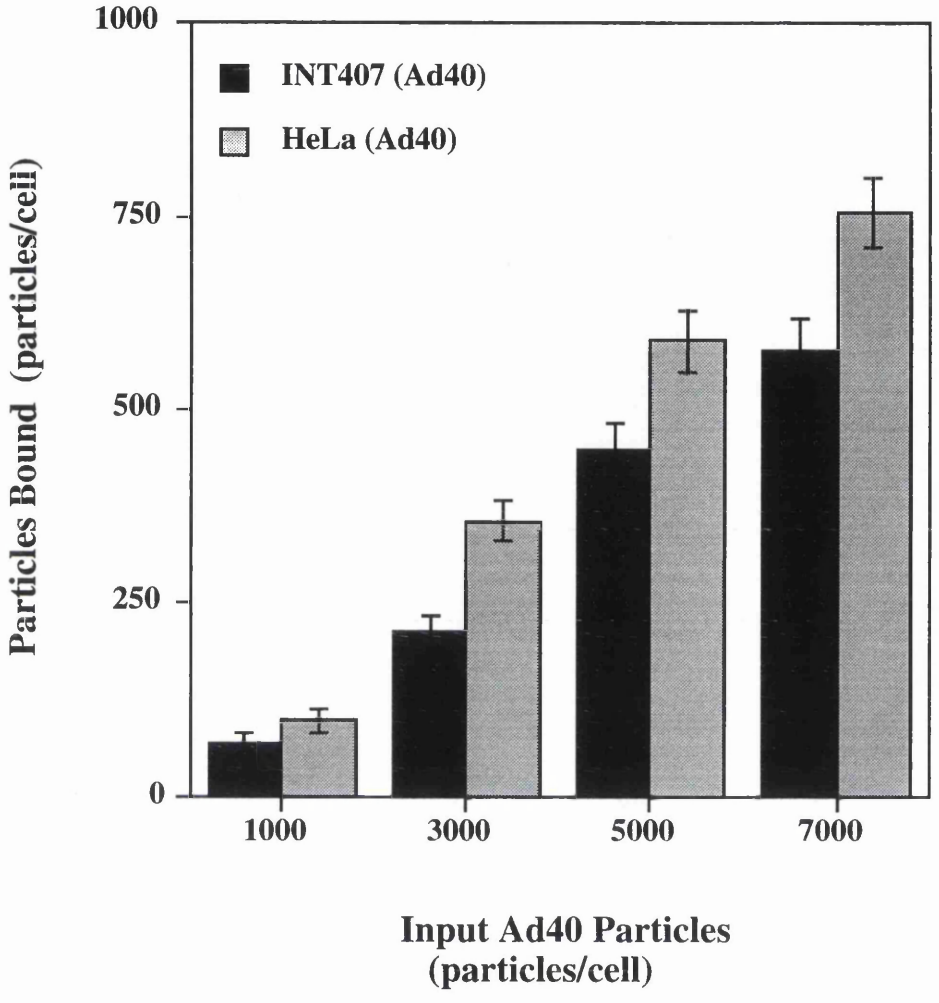


Figure 4.4.1. Analysis of Ad40 Attachment to INT407 and HeLa Cells.

Cells grown in linbro wells were cooled for 30min at 4°C, after which 40µl of labelled virus was added (in TS), the input moi used are shown above. Incubation was carried out at 4°C for 2h. Cells were then washed three times with cold 1xHBS and dissolved in 100µl of EB buffer. Ecoscint was added to each sample and counted in a scintillation counter. The mean and δ (capped lines) values of Ad40 particles that bound to INT407 and HeLa cells are shown.

The results show that approximately 7 to 9 % of input Ad40 particles attached to INT407 cells and, 10 to 12% bound to HeLa cells. At each moi a greater number of particles bound to HeLa cells than INT407 cells. In addition, Ad40 cell receptor sites were not saturated, as no plateau of attached virus is observed over the range of input multiplicities, with both HeLa and INT407 cells. Higher moi were not used in this study, as there would have been insufficient virus to complete the experiments. A moi of 7000 particles/cell was used for Ad40 in subsequent experiments.

4.4.1.2. Attachment of dl309 Virus to INT407 and HeLa Cells.

Attachment of dl309 on INT407 and HeLa cells was looked at, to examine if there were any differences in attachment of another adenovirus serotype on these cells. The method was as stated previously, but the input moi used for labelled dl309 were 500, 1000, 2000 and 3000 particles/cell. The results are shown in Tables 4.4.3. and 4.4.4. with the cpm data, particles/cell, mean and δ values. The number of mean particles bound were plotted on a graph and are shown with δ values in Figure 4.4.2.

Over the range of input multiplicities approximately 7 to 10% of labelled dl309 attached to INT407 cells and 5.5 to 7% bound to HeLa cells. In three of the input concentrations used, more dl309 particles attached to INT407 cells than to HeLa cells, this was the opposite of the pattern detected with Ad40 in the previous section. Again no plateau is observed over the various moi, indicating that the receptor sites for the virus were not saturated. Previous studies with Ad2 (Philipson *et al.*, 1968) found the number of receptor sites on HeLa cells to be approximately 10^4 sites. Higher moi were not looked at in this study and the moi used for labelled dl309 in subsequent experiments was 3000 particles/cell.

Initially it was also hoped to compare these results to the Ad40 results. However the viruses were found to have different specific activities (see 3.2.3.). Therefore different input multiplicities were used and direct comparison could only be made with the 1000 and 3000 moi which were included in both experiments and this is shown in Fig 4.4.3. More dl309 virus attached to INT407 cells than Ad40 virus at both input moi, whereas on HeLa cells more Ad40 bound to cells than dl309.

Table 4.4.3: Tritium Labelled dl309 Attachment on INT407 Cells.

Input moi	cpm Bound [a]	Error δ1, δ2 (cpm)	Particles Bound [b] (part/cell)	Error δ1, δ2 (part/cell)	Mean Particles Bound (part/cell)	δ [c] (part/cell)
500	156, 137	25, 23	46, 41	7, 7	43	7
1000	220, 224	30, 30	78, 80	11, 11	79	11
2000	483, 518	44, 45	139, 149	13, 13	144	13
3000	1644, 1186	81, 69	343, 247	17, 14	295	16

Table 4.4.4. Tritium Labelled dl309 Attachment on HeLa Cells.

Input moi	cpm Bound [a]	Error δ1, δ2 (cpm)	Particles Bound [b] (part/cell)	Error δ1, δ2 (part/cell)	Mean Particles Bound (part/cell)	δ [c] (part/cell)
500	109, 77	21, 17	32, 33	6, 5	27	5
1000	178, 201	27, 28	63, 72	10, 10	67	10
2000	540, 449	46, 42	155, 129	13, 12	142	12
3000	1034, 937	64, 61	216, 195	13, 13	205	13

a The background value was 13 cpm.

b part/cell denotes particles/cell.

c The δ value was calculated from δ_1 and δ_2 using the formula described in section 4.4.1.

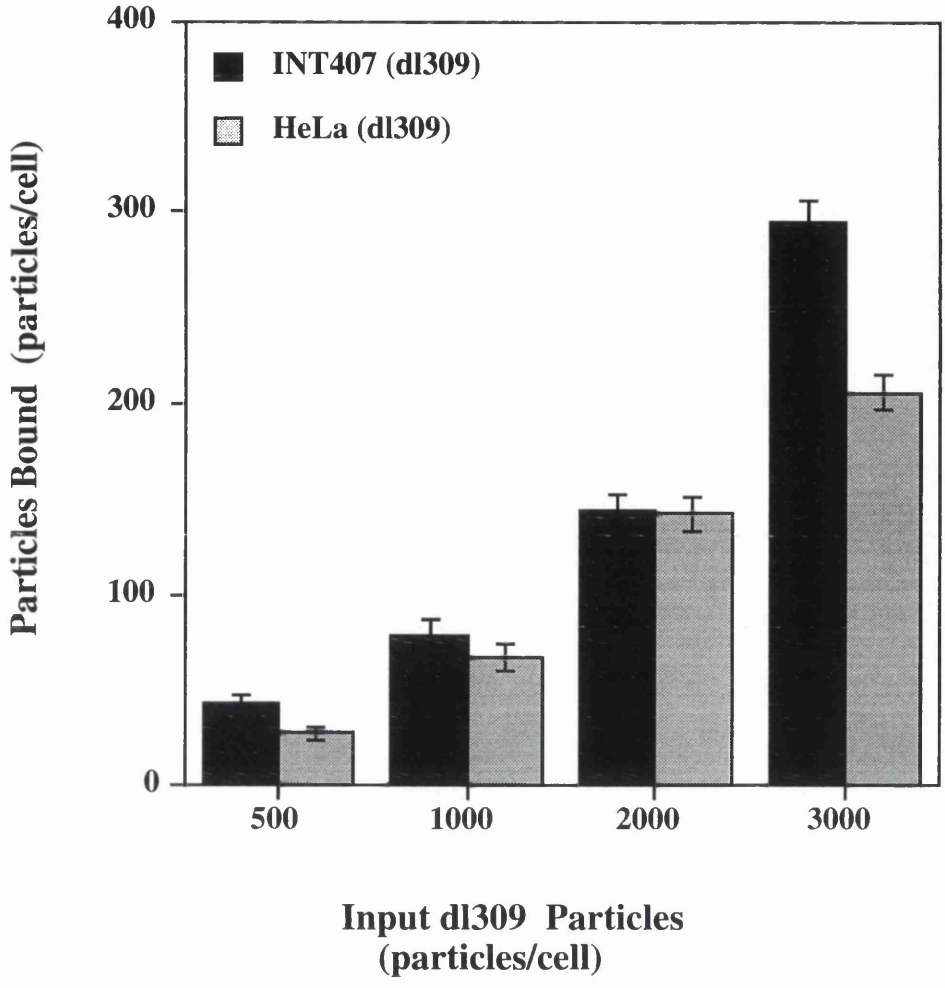


Figure 4.4.2. Analysis of dl309 Attachment to INT407 and HeLa Cells.

Cells grown in linbro wells were cooled for 30min at 4°C, after which 40µl of labelled virus was added (in TS), the input moi used are shown above. Incubation was carried out at 4°C for 2h. Cells were then washed three times with cold 1xHBS and dissolved in 100µl of EB buffer. Ecoscint was added to each sample and counted in a scintillation counter. The mean and δ (capped lines) values of dl309 particles that bound to INT407 and HeLa cells are shown.

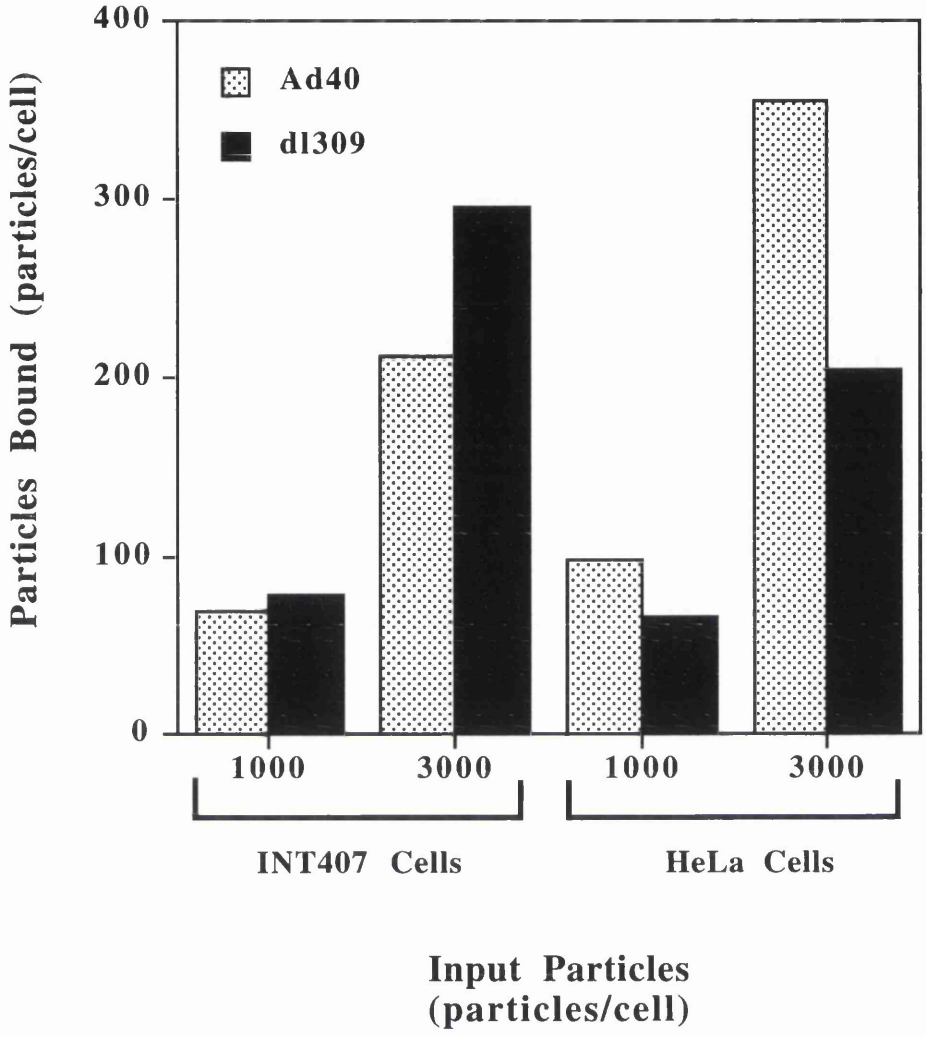


Figure 4.4.3. Comparison of Ad40 and dl309 Virus Attachment.

The mean number of virus particles bound to INT407 and HeLa cells at input multiplicities of 1000 and 3000 particles/cell for Ad40 and dl309 viruses are shown above.

4.4.1.3. Competition of Attached Virus with Ad40 Virus or Ad2 Fibre.

Competition experiments were carried out in order to look at the specificity of attachment (see 3.16.). Cells grown in linbro wells (cooled to 4°C for 30min) were preincubated with competitor (40µl in TS) to block receptor sites. Half the wells received competitor while the other half received 40µl of TS. After 1h at 4°C, cells were washed and labelled virus was added to all cells, the incubation was then continued for 2h at 4°C. The cells were washed, pelleted and processed as described (see 3.16.). The tritium counts per minute data was used to calculate particles/cell, mean and δ values as described previously.

The competitor used for tritium labelled Ad40 was a ten fold excess of unlabelled banded Ad40 virus. Labelled virus was used at a moi of 7000 particles/cell. The results for competition experiments with INT407 and HeLa cells are shown in Table 4.4.5., in addition mean and δ values are represented in graphical form in Fig 4.4.4. Assays were carried out twice for each cell line and within each assay each sample was done in duplicate. The first set of experiments denoted by INT/1 for INT407 cells and HeLa/1 for HeLa cells were carried out concurrently with attachment assays (see 4.4.1.1.). The second set of experiments are denoted by INT/2 and HeLa/2.

The results show that attached Ad40 virus is inhibited by approximately 80 percent on both cell lines, using a ten fold excess of unlabelled virus as a competitor.

The competitor used for dl309 was 8µg of Ad2 fibre (in 40µl of TS), which was a crude preparation of baculovirus expressed recombinant protein. The assay was carried out as previously described (see 3.16.) with labelled virus used at a moi of 3000 particles/cell. Experiment 1 on both cell lines was carried out concurrently with attachment assays (see section 4.4.1.). The cpm data, particles/cell, mean and δ values are shown in Table 4.4.6., also the mean and δ values are shown on a graph shown in Figure 4.4.5.

With Ad2 fibre as a competitor, the amount of dl309 that bound to both cell lines was inhibited by some 95 to 97%, indicating that dl309 binding is specifically inhibited.

Table 4.4.5. Competition of Labelled Ad40 with a 10-fold Excess of Unlabelled Virus.

Cells [a] /Expt No	Comp [b]	cpm Bound [c]	Error δ_1, δ_2 (cpm)	Particles Bound [d] (part/cell)	Error δ_1, δ_2 (part/cell)	Mean Particles Bound (part/cell)	δ (part/cell)
INT/1	-	446, 399	42, 40	609, 545	57, 54	**577**	**55**
INT/1	+	89, 78	19, 18	121, 106	26, 24	**113**	**25**
INT/2	-	244, 315	31, 35	479, 617	61, 67	**548**	**64**
INT/2	+	47, 56	14, 15	92, 110	27, 29	**101**	**28**
HeLa/1	-	444, 662	42, 51	606, 904	57, 69	**755**	**63**
HeLa/1	+	91, 101	19, 20	124, 138	26, 27	**131**	**26**
HeLa/2	-	364, 384	38, 39	713, 753	75, 77	**733**	**76**
HeLa/2	+	82, 86	18, 18	161, 169	35, 35	**165**	**35**

a INT represents INT407 cells.

b Denotes the presence or absence of a ten fold excess of unlabelled virus as competitor.

c The background value for experiment 1 was 13cpm and 21cpm for experiment 2.

d part/cell denotes particles/cell

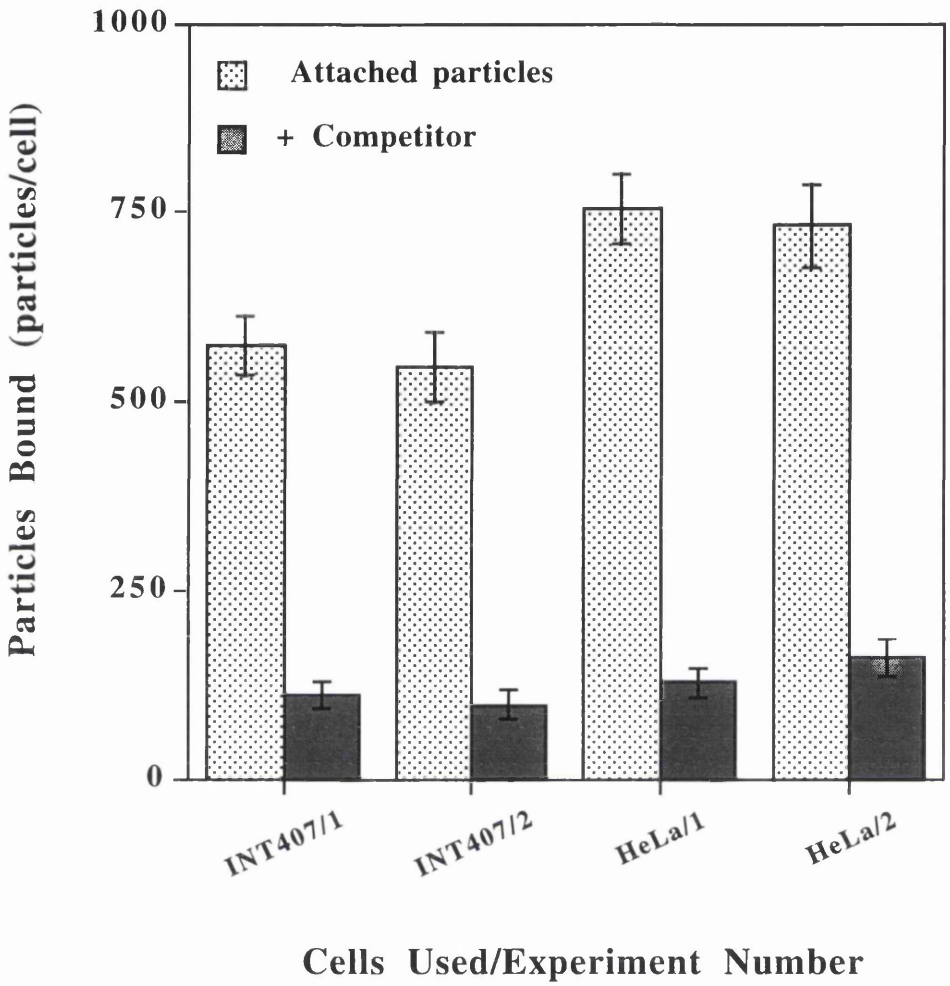


Figure 4.4.4. Attachment of Labelled Ad40 Virus is Inhibited by a Ten Fold Excess of Unlabelled Virus.

Cells were grown in linbro wells, half the wells were preincubated with a ten fold excess of unlabelled Ad40 (40µl in TS) and the other half with 40µl of TS. After 1h at 4°C the cells were washed twice with 1xHBS. All cells were infected with labelled virus (40µl in TS) at 7000 particles/cell and incubated for 2h at 4°C, after which they were washed three times with cold 1xHBS and pelleted. The pellet was dissolved in 100µl of EB buffer and 10ml of ecoscint was added, samples were counted in a scintillation counter. The mean number of particles bound (particles/cell) with δ values (capped lines) are shown.

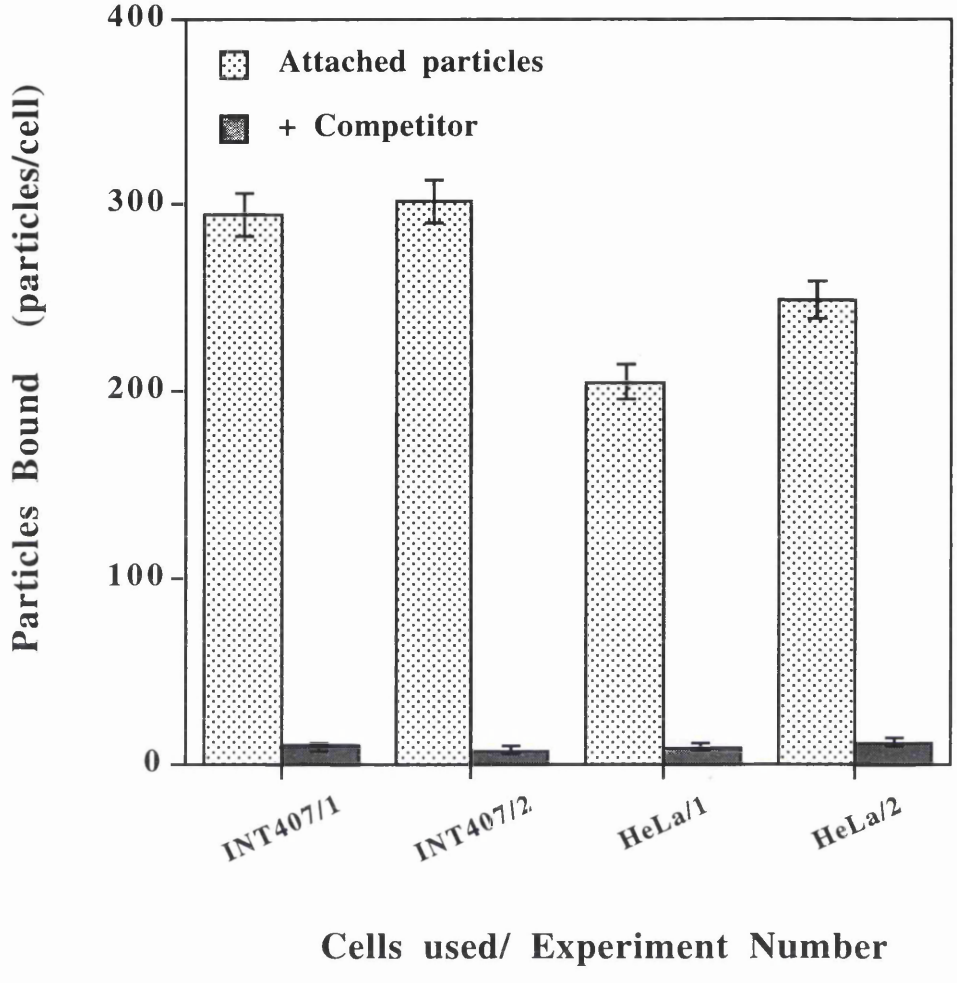


Figure 4.4.5. Competition of dl309 Virus with Ad2 Fibre.

Cells were grown in linbro wells, half the wells were preincubated with 8μg of Ad2 fibre (40μl in TS) and the other half with 40μl of TS. After 1h at 4 ^{0}C the cells were washed twice with 1xHBS. All cells were infected with labelled virus (40μl in TS) at 3000 particles/cell and incubated for 2h at 4^{0}C, after which they were washed three times with cold 1xHBS and pelleted. The pellet was dissolved in 100μl of EB buffer and 10ml of ecoscint was added, samples were counted in a scintillation counter. The mean number of particles bound (particles/cell) with δ values (capped lines) are shown.

4.4.1.4. Summary.

The percentage of Ad40 and dl309 particles binding to INT407 and HeLa cells was similar, with approximately 7 to 12 percent of Ad40 input particles attaching to cells compared to 6 to 10 percent of dl309 particles. Overall on both cell types viral attachment was inhibited with unlabelled competitor, indicating that attachment was specific. However these results do not explain the differences observed between permissive and semi-permissive cells in section 4.3. (i.e. the number of cells going on to productive infection). Indeed slightly more Ad40 particles attached to HeLa cells than to INT407 cells, therefore the amount of virus internalised was investigated (see next section).

4.4.2. Analysis of Adenovirus Internalisation.

The amount of virus that was internalised in cells was examined, in order to see if there were differences between cell types. The internalisation assay is described in detail elsewhere (see 3.16.), but briefly cells (in linbro wells) were precooled to 4°C for 30min before infection. Tritium labelled virus (40µl in TS) was added and cells incubated for 2h at 4°C. Cells were washed to remove unbound virus and after cell adhesion buffer was added, internalisation was initiated by warming to 37°C for various times. Half the wells were treated with the protease subtilisin (Sigma), in order to distinguish attached from internalised virus. Attached virus is removed by treatment with subtilisin, whereas internalised virus is protease resistant and is not affected (Philipson *et al.*, 1968). Samples not protease treated represent both attached and internalised virus, except for the 0min timepoint which is not incubated at 37°C and therefore represents attached virus only. At various times cells were washed and either 1xHBSE or 1X HBSE containing subtilisin was added. Incubation was continued for 30min at 37°C, after which cells were washed with cell adhesion buffer and pelleted. Samples were processed as described previously (see 3.16.). Each sample was carried out in duplicate for the timepoints 0, 15, 30 and 45min and every assay was carried out twice for each virus on the two cell types.

Experiments marked experiment 1 in each set were conducted by G. Brown, all other experiments (experiment 2) were the authors own efforts.

4.4.2.1. Internalisation of Ad40 in INT407 and HeLa cells.

The input moi used for Ad40 was 7000 particles/cell. The results (represented as particles/cell) are shown in Tables 4.4.7. and 4.4.8., for both experiments 1 and 2. The mean values were also plotted on graphs with δ values in Figures 4.4.6. and 4.4.7.

The mean total number of virus particles (attached and internalised) show some variability between experiments 1 and 2 with both cell types. This may be due to the fact that they were carried out by different people and reflect different washing intensities. The number of particles binding to INT407 and HeLa cells and not internalised is represented by the 0min timepoint. Higher numbers of Ad40 particles are observed binding to INT407 cells than had previously been detected in section 4.4.1. However the results from subtilisin treated samples are more consistent than untreated samples and there appears to be a gradual increase in the number of particles/cell in subtilisin treated samples from 0 to 45min (see Fig 4.4.6. and Fig 4.4.7.). This indicates that virus is being internalised.

Originally the intention had been to pool experiments 1 and 2, however as results from experiment 1 showed more variability on HeLa cells, only the experiment 2 data was used to look at the percentage of internalisation between cell types. The percentage of virus that was internalised was calculated, by subtracting the mean particles/cell for the 0min timepoint of subtilisin treated samples from the subsequent timepoints (15, 30, 45min) of subtilisin treated and untreated samples. The treated samples were then represented as a percentage of the total number of virus particles (untreated samples) at each timepoint (15, 30, 45min). These values are shown in Table 4.4.9a.

For Ad40, the percentage of particles internalised is similar up to 30min on both cell types, after which on INT407 cells the percentage of virus only increases slightly to 21%. On HeLa cells there is a larger increase up to 45min with 31% of virus internalised.

Table 4.4.7. Internalisation of Ad40 in INT407 Cells.

Subtilisin Treatment	Time (min)	Particles Bound (part/cell) [a]	Error δ_1, δ_2 (part/cell)	Mean Particles Bound (part/cell)	δ (part/cell) [b]
Experiment 1					
-	0	433, 449	50, 51	**441**	**50**
-	15	356, 508	47, 54	**432**	**51**
-	30	447, 522	51, 55	**484**	**53**
-	45	710, 515	64, 54	**612**	**59**
+	0	138, 100	28, 23	**119**	**26**
+	15	155, 124	31, 26	**139**	**29**
+	30	158, 167	31, 31	**162**	**31**
+	45	204, 284	35, 41	**244**	**38**
Experiment 2					
-	0	727, 799	72, 75	**763**	**73**
-	15	685, 788	70, 75	**736**	**72**
-	30	766, 690	73, 73	**728**	**73**
-	45	788, 752	75, 73	**770**	**74**
+	0	148, 148	32, 32	**148**	**32**
+	15	196, 228	38, 39	**212**	**38**
+	30	255, 261	43, 43	**258**	**43**
+	45	219, 343	39, 50	**281**	**45**

a part/cell denotes particles/cell

b The δ value was calculated using the formula in section 4.4.1.

Table 4.4.8. Internalisation of Ad40 in HeLa Cells.

Subtilisin Treatment	Time (min)	Particles Bound (part/cell) [a]	Error δ_1, δ_2 (part/cell)	Mean Particles Bound (part/cell)	δ (part/cell) [b]
Experiment 1					
-	0	513, 552	55, 57	532	56
-	15	567, 474	58, 52	520	55
-	30	534, 624	57, 61	579	59
-	45	943, 922	75, 75	932	75
+	0	108, 113	25, 25	110	25
+	15	183, 227	33, 37	205	35
+	30	200, 188	34, 33	194	33
+	45	281, 204	40, 34	242	37
Experiment 2					
-	0	708, 700	74, 74	704	74
-	15	687, 680	74, 74	683	74
-	30	770, 713	78, 76	741	77
-	45	869, 767	84, 78	818	81
+	0	127, 135	32, 32	131	32
+	15	135, 233	32, 42	184	37
+	30	287, 265	48, 46	276	47
+	45	295, 390	48, 56	342	52

a part/cell denotes particles/cell

b The δ value was calculated using the formula in section 4.4.1.

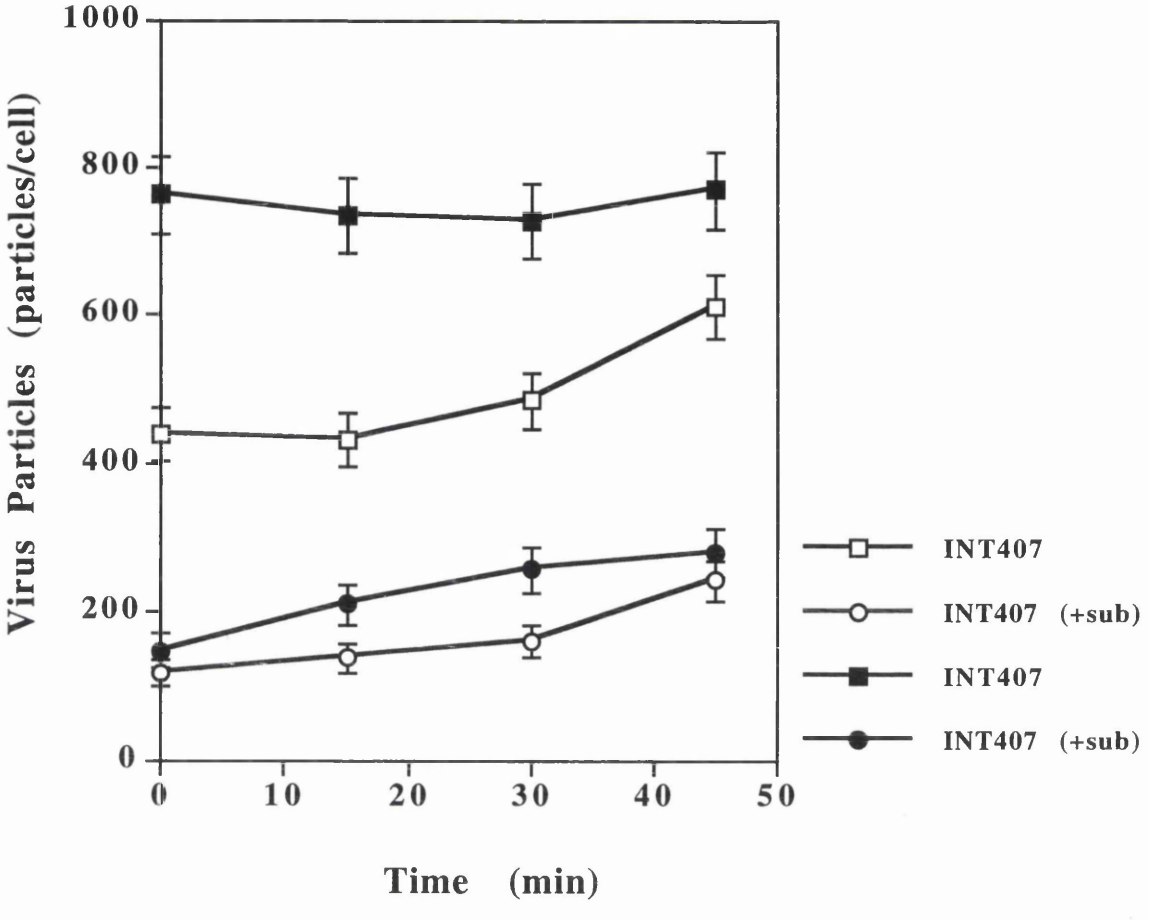


Figure 4.4.6. Ad40 Internalisation in INT407 Cells.

Cells grown in linbro wells were cooled for 30min at 4°C, then infected with 7000 particles/cell of labelled Ad40 virus (40µl in TS) and incubated for 2h at 4°C. Cells were washed three times with 1xHBS and cell adhesion buffer added to all wells, after which cells were warmed to 37°C and incubated for various times. At each timepoint four wells were washed twice with 1xHBS. To two wells 200µl of 1xHBSE was added while the other two received 200µl of 1xHBSE containing 2mg/ml of subtilisin. These four wells were incubated for 30min at 37°C. Cells were then scraped into 1.5ml vials and washed twice with cell adhesion buffer before being pelleted. The pellet was dissolved in 100µl of elution buffer and 10ml ecoscint was added, samples were counted in a scintillation counter. The mean number of particles (particles/cell) are shown with δ values (capped lines). The unfilled symbols represent results from experiment 1 and filled symbols represent results from experiment 2.

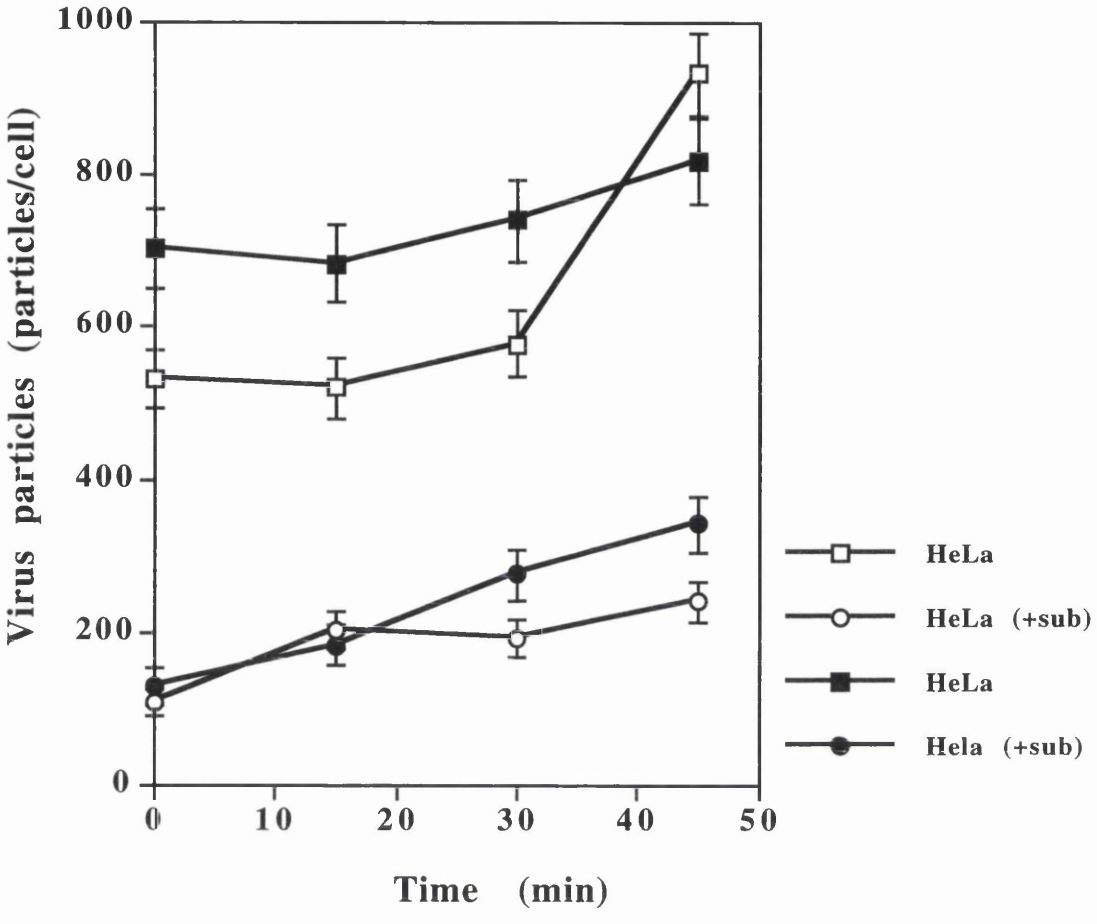


Figure 4.4.7. Ad40 Internalisation in HeLa Cells.

Cells grown in linbro wells were cooled for 30min at 4°C, then infected with 7000 particles/cell of labelled Ad40 virus (40µl in TS) and incubated for 2h at 4°C. Cells were washed three times with 1xHBS and cell adhesion buffer added to all wells, after which cells were warmed to 37°C and incubated for various times. At each timepoint four wells were washed twice with 1xHBS. To two wells 200µl of 1xHBSE was added while the other two received 200µl of 1xHBSE containing 2mg/ml of subtilisin. These four wells were incubated for 30min at 37°C. Cells were then scraped into 1.5ml vials and washed twice with cell adhesion buffer before being pelleted. The pellet was dissolved in 100µl of elution buffer and 10ml ecoscint was added, samples were counted in a scintillation counter. The mean number of particles (particles/cell) are shown with δ values (capped lines). The unfilled symbols represent results from experiment 1 and filled symbols represent results from experiment 2.

Table 4.4.9a. Internalisation of Ad40 in INT407 and HeLa Cells.

Cells	% Internalisation at various Times (min)		
	15	30	45
INT407	11	19	21
HeLa	10	24	31

Table 4.4.9b. Internalisation of dl309 in INT407 and HeLa Cells

Cells	% Internalisation at various Times (min)		
	15	30	45
INT407	9	21	33
HeLa	9	19	41

4.4.2.2. Internalisation of dl309 in INT407 and HeLa Cells.

The next experiment was carried out to examine the amount of dl309 that was internalised in INT407 and HeLa cells. The input moi used was 3000 particles/cell. The counts per minute were converted to particles/cell and are shown in Tables 4.4.10. and 4.4.11., together with the mean and δ values, which are also represented in graphical form in Figure 4.4.8. and 4.4.9.

Results from both experiment 1 and 2 on HeLa cells and experiment 2 on INT407 cells show a similar pattern and only differ in the number of particles that were bound. In these experiments the pattern of viral internalisation is the same with a gentle increase of virus particles observed up to 45min. Thus the overall pattern for dl309 is similar to that observed with Ad40.

The data from experiment 1 on INT407 cells was variable, therefore only the data from experiment 2 was used to look at the percentage of virus that had been internalised on HeLa and INT407 cells. This was calculated in the same way as described for Ad40 in the previous section, and results are shown in Table 4.4.9b.

More dl309 is internalised in HeLa cells compared to INT407, however the overall pattern of internalisation detected over time is the same. At 45min the amount internalised was some 33 to 41% in INT407 and HeLa cells, respectively and this compared to the 31% level in Ad40 infected HeLa cells at 45min cells in Table 4.4.9a.

4.4.2.3. Summary.

A similar pattern of internalisation was observed for dl309 particles in INT407 and HeLa cells and this was comparable to the amount of Ad40 virus internalised in HeLa cells. A larger number of Ad40 virus particles were internalised in HeLa cells than INT407 cells, this was surprising given the previous results from sections 4.1. and 4.3., thus differences between these cell types is probably not due to the number of particles that attach or are internalised in these cell lines. The pattern of E1 transcription was therefore investigated in order to see if this could account for the increased permissivity of INT407 cells over HeLa cells (see next section).

Table 4.4.10. Internalisation of dl309 in INT407 Cells.

Subtilisin Treatment	Time (min)	Particles Bound (part/cell) [a]	Error δ_1, δ_2 (part/cell)	Mean Particles Bound (part/cell)	δ (part/cell) [b]
Experiment 1					
-	0	323, 343	12, 12	333	12
-	15	256, 236	10, 10	246	10
-	30	190, 210	9, 9	200	9
-	45	207, 256	9, 10	231	9
+	0	73, 110	5, 7	91	6
+	15	167, 198	8, 9	182	8
+	30	99, 126	6, 7	112	6
+	45	117, 112	7, 7	114	7
Experiment 2					
-	0	270, 278	15, 15	274	15
-	15	251, 242	14, 14	246	14
-	30	256, 263	14, 15	259	14
-	45	244, 338	14, 16	291	15
+	0	52, 61	6, 7	56	6
+	15	66, 81	7, 8	73	7
+	30	94, 103	9, 9	98	9
+	45	120, 146	10, 11	133	10

a part/cell denotes particles/cell

b The δ value was calculated using the formula in section 4.4.1.

Table 4.4.11. Internalisation of dl309 in HeLa Cells.

Subtilisin Treatment	Time (min)	Particles Bound (part/cell) [a]	Error $\delta 1, \delta 2$ (part/cell)	Mean Particles Bound (part/cell)	δ (part/cell) [b]
Experiment 1					
-	0	299, 307	20, 21	303	20
-	15	289, 283	20, 20	286	20
-	30	324, 288	21, 20	306	20
-	45	277, 295	19, 20	286	19
+	0	77, 77	10, 10	77	10
+	15	86, 87	11, 11	86	11
+	30	102, 110	12, 12	106	12
+	45	142, 159	14, 15	150	14
Experiment 2					
-	0	291, 372	13, 15	331	14
-	15	385, 304	15, 14	344	14
-	30	350, 330	15, 14	340	14
-	45	319, 316	14, 14	317	14
+	0	139, 134	9, 9	136	9
+	15	163, 148	10, 10	155	10
+	30	175, 175	10, 10	175	10
+	45	211, 212	11, 11	211	11

a part/cell denotes particles/cell

b The δ value was calculated using the formula in section 4.4.1.

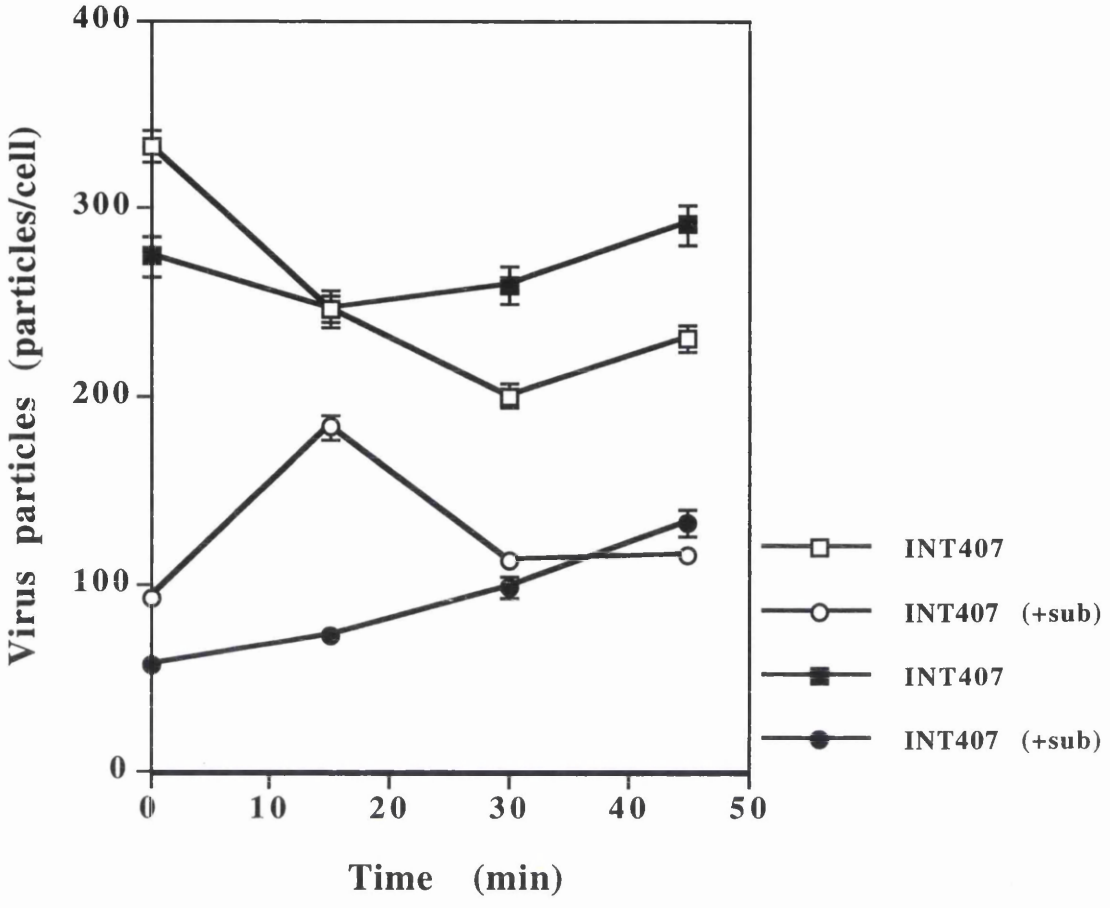


Figure 4.4.8. dl309 Internalisation in INT407 Cells.

Cells grown in linbro wells were cooled for 30min at 4°C, then infected with 3000 particles/cell of labelled dl309 virus (40µl in TS) and incubated for 2h at 4°C. Cells were washed three times with 1xHBS and cell adhesion buffer added to all wells, after which cells were warmed to 37°C and incubated for various times. At each timepoint four wells were washed twice with 1xHBS. To two wells 200µl of 1xHBSE was added while the other two received 200µl of 1xHBSE containing 2mg/ml of subtilisin. These four wells were incubated for 30min at 37°C. Cells were then scraped into 1.5ml vials and washed twice with cell adhesion buffer before being pelleted. The pellet was dissolved in 100µl of elution buffer and 10ml ecoscint was added, samples were counted in a scintillation counter. The mean number of particles (particles/cell) are shown with δ values (capped lines). The unfilled symbols represent results from experiment 1 and filled symbols represent results from experiment 2.

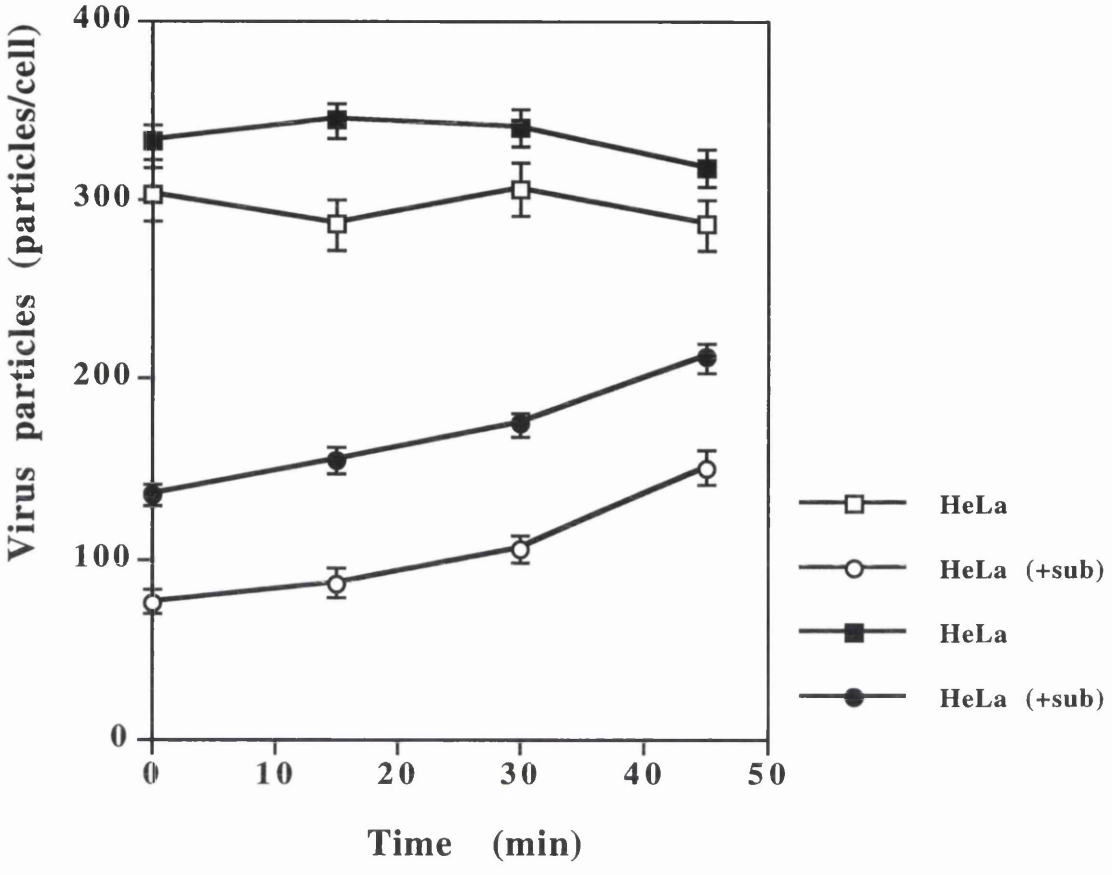


Figure 4.4.9. dl309 Internalisation in HeLa Cells.

Cells grown in linbro wells were cooled for 30min at 4°C, then infected with 3000 particles/cell of labelled dl309 virus (40µl in TS) and incubated for 2h at 4°C. Cells were washed three times with 1xHBS and cell adhesion buffer added to all wells, after which cells were warmed to 37°C and incubated for various times. At each timepoint four wells were washed twice with 1xHBS. To two wells 200µl of 1xHBSE was added while the other two received 200µl of 1xHBSE containing 2mg/ml of subtilisin. These four wells were incubated for 30min at 37°C. Cells were then scraped into 1.5ml vials and washed twice with cell adhesion buffer before being pelleted. The pellet was dissolved in 100µl of elution buffer and 10ml ecoscint was added, samples were counted in a scintillation counter. The mean number of particles (particles/cell) are shown with δ values (capped lines). The unfilled symbols represent results from experiment 1 and filled symbols represent results from experiment 2.

4.5. Analysis of E1 Transcription in Ad40 Infected Cells.

Study of the Ad40 DNA sequence shows that the E1 region has a similar organisation of transcriptional units and ORFs to that of other human serotypes (van Loon *et al.*, 1987b; Ishino *et al.*, 1988; Davison *et al.*, 1993). Transcripts for the Ad40 and Ad41 E1 regions were mapped by van Loon *et al.*, (1987b), using RNA obtained from BRK cells transformed with an Ad40 E1 containing plasmid. In Ad40 infected cells, these authors identified E1A transcripts corresponding in size to the Ad2 9S, 12S and 13S mRNAs, but no E1B mRNA was detected. For Ad41 infected cells, only 13S E1A and 22S E1B transcripts were present. Steinthorsdottir and Mautner (1991) determined the Ad40 E1B transcription map by S1 mapping and primer extension analysis of RNA obtained at late times from infected KBa+b cells. E1B transcripts equivalent to the Ad2 14S (encoding 19K and 15K), 22S (encoding 19K and 55K) and 9S (encoding ppIX) mRNAs were identified, but there was no transcript equivalent to E1B 13S mRNA. A similar pattern is seen in Ad12 (Virtanen *et al.*, 1982; Saito *et al.*, 1983), where no 13S message is detected and the predominant E1B species at late times is 14S mRNA. Steinthorsdottir and Mautner (1991) also identified novel cotranscript counterparts of the Ad40 E1B 14S and 22S mRNAs. These consisted of the first 40 codons of the E1A region spliced to a site 4-5nt downstream of the E1B capsite. This splice junction did not contain consensus splice sequences and thus may not be generated by conventional splicing mechanisms. Allard and Wadell (1992) have determined the Ad41 E1B transcription map from a lytic infection. They found a similar pattern to Ad40 with 22S, 14S and 9S mRNA detected, but no 13S transcript. However no E1A-E1B cotranscript counterparts were found in Ad41, but an extra exon in the 14S mRNA was detected which is not present in Ad40. Mautner *et al.*, (1990) found that in KBa+b cells, Ad40 E1B transcripts could be detected, but only after the onset of DNA replication. In HeLa cells, Ad40 E1B mRNA was detected at low levels, after the onset of DNA replication. Subsequently Bailey *et al.*, (1993) have shown that the E1B 55K protein (&19K) is indeed expressed in KBa+b cells.

The following experiments were conducted to see if there were any differences in the pattern of E1 transcription in infected INT407 cells compared to KBa+b and HeLa cells. In order to look at the transcripts produced over time in infected cells, a timecourse of mRNA was analysed by Northern blotting. In addition E1B mRNA was examined by S1 nuclease analysis in the presence and absence of DNA replication.

4.5.1. A Timecourse of Ad40 E1 Transcription and DNA Replication in Infected Cells.

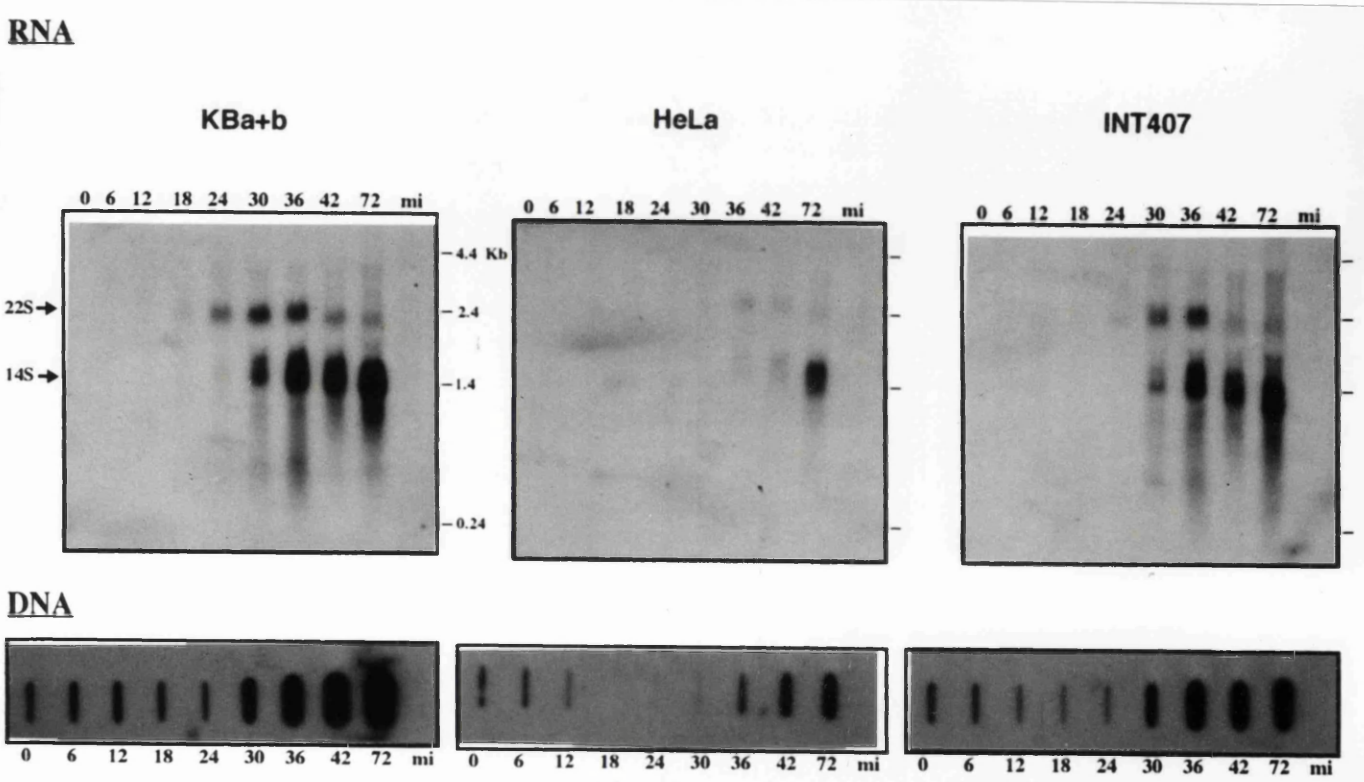
The pattern of E1 transcription was investigated by Northern blot analysis (see 3.12.) of cytoplasmic RNA from infected KBa+b, HeLa and INT407 cells, harvested at various times post infection. Cells grown on 50mm plates were infected with 100µl of a 1/10 dilution of Ad40 p9 (in TS). After incubation at 37°C, two plates were pooled and harvested for each cell type. Cytoplasmic RNA was extracted (see 3.3.3.) and separated on 1.2% agarose/ formaldehyde gels (one third harvest of a 50mm plate/lane). The RNA was then blotted onto nylon membranes (Hybond-N) and probed with a nick translated plasmid containing the Ad40 E1B 55K region (pAB106). Following exposure to autoradiography film, the blots were stripped of probe (see 3.12.) and reprobed with a nick translated plasmid containing Ad40 E1A sequences (pNM83). To examine the efficiency of RNA transfer, stripped membranes were also probed with a nick translated plasmid containing the mouse γ actin pseudogene (Leader *et al.*, 1987), similar levels of actin mRNA were observed for the three cell lines (not shown). An RNA marker (BRL) was run in conjunction with samples to determine sizes of mRNA. In parallel, to the Northern blot analysis, DNA was recovered from mock infected and infected cell nuclei (see 3.3.2.) and applied to nylon membranes on a slot blot (one twentieth harvest of a 50mm plate/slot). Membranes were then probed with a nick translated plasmid containing the Ad40 E1 region (pNM82).

The pattern of E1B transcription and DNA replication is shown in Figure 4.5.1. In infected KBa+b cells low levels of 22S E1B mRNA are first detected at 18h p.i. preceding the onset of DNA replication which was observed at 24-30h. 14S mRNA was

Figure 4.5.1. A Timecourse of E1B Transcription and DNA Replication in KBa+b, HeLa and INT407 Cells.

Cells grown on 50mm plates were infected with 100µl of a 1/10 dilution of Ad40. Cytoplasmic RNA was then harvested from cells at the times indicated. The RNA was run on 1.2% agarose/formaldehyde gels (1/3 harvest of a 50mm plate/lane) and blotted onto nylon membranes. An RNA marker (BRL) was also run on gels (not shown). Membranes were probed with nick translated plasmid containing the Ad40 E1B 55K region (pAB106). The Northern blots, shown in the upper part of the figure, were exposed to autoradiography film for 5 days.

In parallel, DNA was extracted from cell nuclei and applied to nylon membranes using a slot blot (1/20 harvest of a 50mm plates/slot). Membranes were probed with a nick translated plasmid containing the Ad40 E1 region (pNM82). These blots are shown in the lower part of the figure and were exposed to autoradiography film for 18h.


RNA
KBa+b
HeLa
INT407
0 6 12 18 24 30 36 42 72 mi
22S
14S
4.4 Kb
2.4
1.4
0.24
DNA
0 6 12 18 24 30 36 42 72 mi

not detected until 24h p.i., coinciding with the onset of DNA replication. This pattern is similar to Ad5 (Glenn and Ricciardi, 1988) with 22S mRNA predominant at early times in infection. However in Ad5 13S mRNA accumulates after the onset of DNA replication whereas here 14S mRNA is the main E1B transcript at late times in infection. No E1B mRNA is detected at early times in Ad40 infected HeLa cells, confirming previous reports (Mautner *et al.*, 1990; Steinthorsdottir, 1991). The appearance of mRNA is concurrent with the onset of DNA replication (30-36h p.i.) with small amounts of 22S and 14S mRNAs first detected at 36h and a dramatic increase in 14S at 72h p.i. For Ad40 infected INT407 cells, the pattern of E1B mRNA is similar to that seen in KBa+b cells, but delayed by approximately 6h. A small amount of 22S is first detected at 24h p.i. before the onset of DNA replication between 24-30h, thereafter it increases up to 36h, but from 42h to 72h much reduced amounts are observed. The 14S mRNA is first detected at 30h and becomes the predominant mRNA late in infection in INT407 cells. The level of DNA replication increases up to 36h, but lower levels are detected at later timepoints compared to KBa+b cells.

Figure 4.5.2. shows the results when the Northern blots were probed with E1A sequences. The 12S and 13S transcripts are indistinguishable in KBa+b infected cells but are clearly visible, especially, at lower exposures in INT407 infected cells (not shown). In INT407 and KBa+b infected cells, E1A mRNA is detected in low amounts at the same times that E1B transcripts were first detected (see above). For both these cell types there is a steady increase in E1A mRNA up to 72h. In HeLa cells, Ad40 E1A mRNA is first detected at 36h, coincident with the onset of DNA replication, and lower levels are detected than for the other two cell types. In this experiment both E1A and E1B mRNA appear at the same time for each cell type. However from this study the possibility that E1A mRNA is made in very low amounts before E1B cannot be ruled out at present, as times between 12-18h were not investigated.

4.5.1.1. Summary.

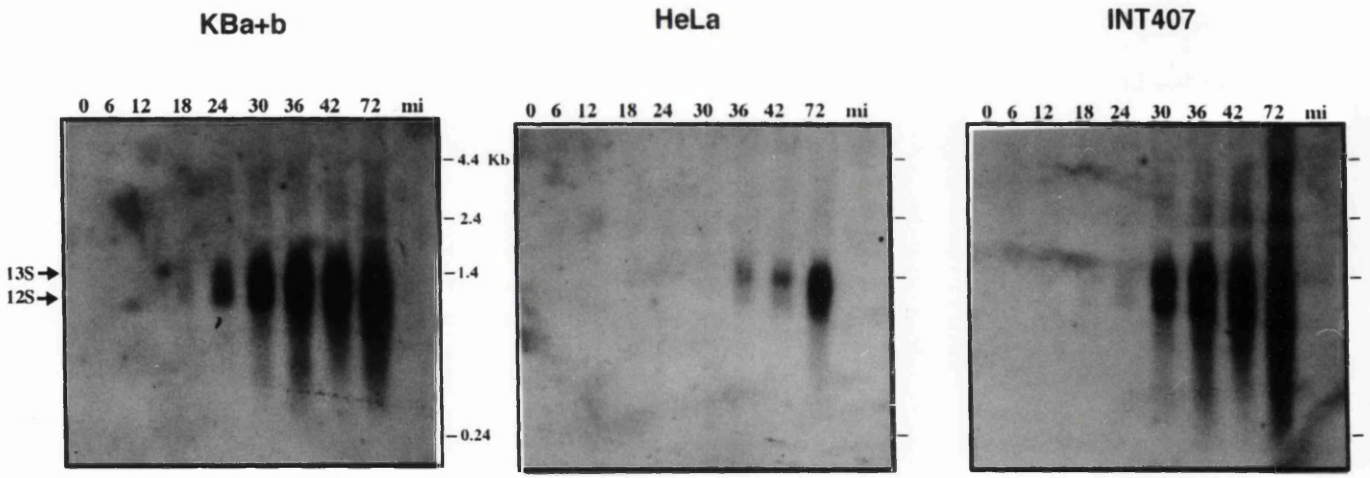
These experiments showed for the first time that Ad40 E1 mRNAs were made at early times in infection, in both KBa+b and INT407 cells. Both E1B 22S mRNA and

Figure 4.5.2. A Timecourse of E1A Transcription and DNA Replication in KBa+b, HeLa and INT407 Cells.

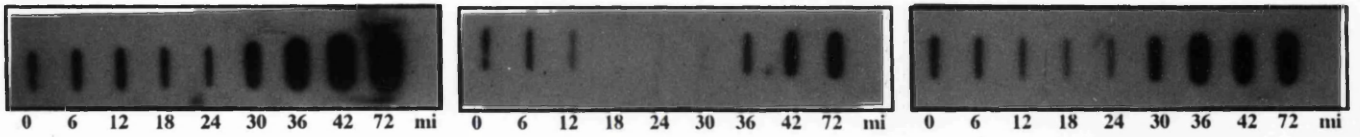
Northern blots from Figure 4.5.1. were stripped of probe and reprobed with a nick translated plasmid containing the E1A region (pNM83). RNA blots were exposed for 5 days to autoradiography film.

DNA blots are as in Figure 4.5.1., and show the onset of DNA replication compared to E1A transcription.

RNA


KBa+b
HeLa
INT407
0 6 12 18 24 30 36 42 72 mi
4.4 Kb
2.4
1.4
0.24
13S
12S

DNA


0 6 12 18 24 30 36 42 72 mi

E1A transcripts were detected before the onset of DNA replication. The pattern and level of mRNA produced in INT407 infected cells was more comparable to that seen in KBa+b, than to HeLa cells. In HeLa cells mRNA was detected coincident with the onset of DNA replication and at much lower levels compared to the other two cell types. This pattern was consistent with previous reports of Ad40 infected HeLa cells (Mautner *et al.*, 1990; Steinthorsdottir, 1991).

The probe (nick translated plasmid pAB106) used for the detection of E1B mRNA (in Figure 4.5.1.), contained the whole of the E1B 55K region and was uniformly labelled. This made the probe more sensitive to 22S than to 14S mRNA, as more radioactive signal hybridised to the longer transcript. Thus comparison between these mRNAs could not be made easily. To circumvent this, E1B transcription was further investigated by S1 nuclease protection (see next section).

4.5.2. Analysis of E1B mRNA Produced in the Presence and Absence of DNA Replication.

From Northern blot analysis it appeared that low amounts of E1B mRNA were produced before the onset of DNA replication in KBa+b and INT407 cells. Therefore the following experiment was conducted to confirm that E1B transcripts were indeed made before the onset of DNA replication. The experiment involved investigating E1B transcription by S1 analysis in the presence and absence of DNA replication. S1 analysis was used for this experiment, as it is a sensitive technique that determines the amount of a specific RNA in a sample and thus allows comparison of mRNAs. It is also a technique which is frequently used to map the 5' end of a transcript. In S1 nuclease analysis, end labelled single stranded DNA probe is hybridised to a measured amount of mRNA. Unhybridised RNA and DNA is digested away with enzymes and the resulting protected fragments analysed by PAGE.

In addition to KBa+b, HeLa and INT407 cells; A549 cells were included in this study to compare with HeLa as another semi-permissive cell line. 293 cells were also included; experiments conducted by Bailey *et al.*, (1994) found these cells to be more

permissive for Ad40 than KBa+b cells, it was therefore of interest as another permissive cell line. In brief, the experiment was as follows; cells grown on 50mm plates were infected with a 100µl of a 1/10 dilution of Ad40 p9 and then incubated at 37°C. Infected and mock infected cells were grown in the absence or presence of cytosine arabinoside (ara-C) which inhibits DNA replication. This inhibitor was first added to plates after adsorption, to a final concentration of 40µg/ml, and replenished every 12h thereafter. Cells were harvested at various timepoints for each cell type, when cytoplasmic RNA (see 3.3.3.) and nuclear DNA (see 3.3.2.) were extracted. The timepoints were chosen with reference to the northern blotting experiment described above, to look for early and late transcripts. Cytoplasmic RNA was treated with DNase I (see 3.4.) to remove possible DNA contamination and then hybridised to 2ng of probe 5'-labelled with [^{32}P] ATP. The probe used to detect Ad40 E1B 22S and 14S mRNA was a synthetic single stranded oligonucleotide which spanned the Ad40 14S splice acceptor site (oligo AB42 3049-3110nt). At the 3' end, 6nt were synthesised which were not of adenovirus origin, these were added in order to distinguish undigested probe from mRNA that bound to all of the probe Ad40 sequences (i.e. 22S). Panel A in Figure 4.5.4. shows the region spanned by the probe and the expected sizes of protected fragments for each species. A marker (not shown) comprising of labelled oligonucleotides of known sizes was run alongside the samples, to identify sizes of protected fragments. S1 analysis was carried out as described (see 3.13.) with 15µg of cytoplasmic RNA. In parallel, DNA was recovered from cell nuclei (see 3.3.2.) and applied to nylon membranes using a slot blot (one twentieth harvest of a 50mm plate/slot). Membranes were probed with nick translated plasmid pAB106 containing the Ad40 E1B 55K region.

The DNA slot blots are shown in Fig 4.5.3. In the presence of ara-C, DNA replication from all cell types is inhibited. The timepoints span the expected onset of DNA replication in all the cell types with early and late timepoints. The onset of DNA replication for 293 and KBa+b cell lines is between 12-24h and for INT407 and A549 cells is 24-30h. Interestingly the onset of DNA replication in HeLa cells is between 24-30h; earlier than observed in section 4.5.1.

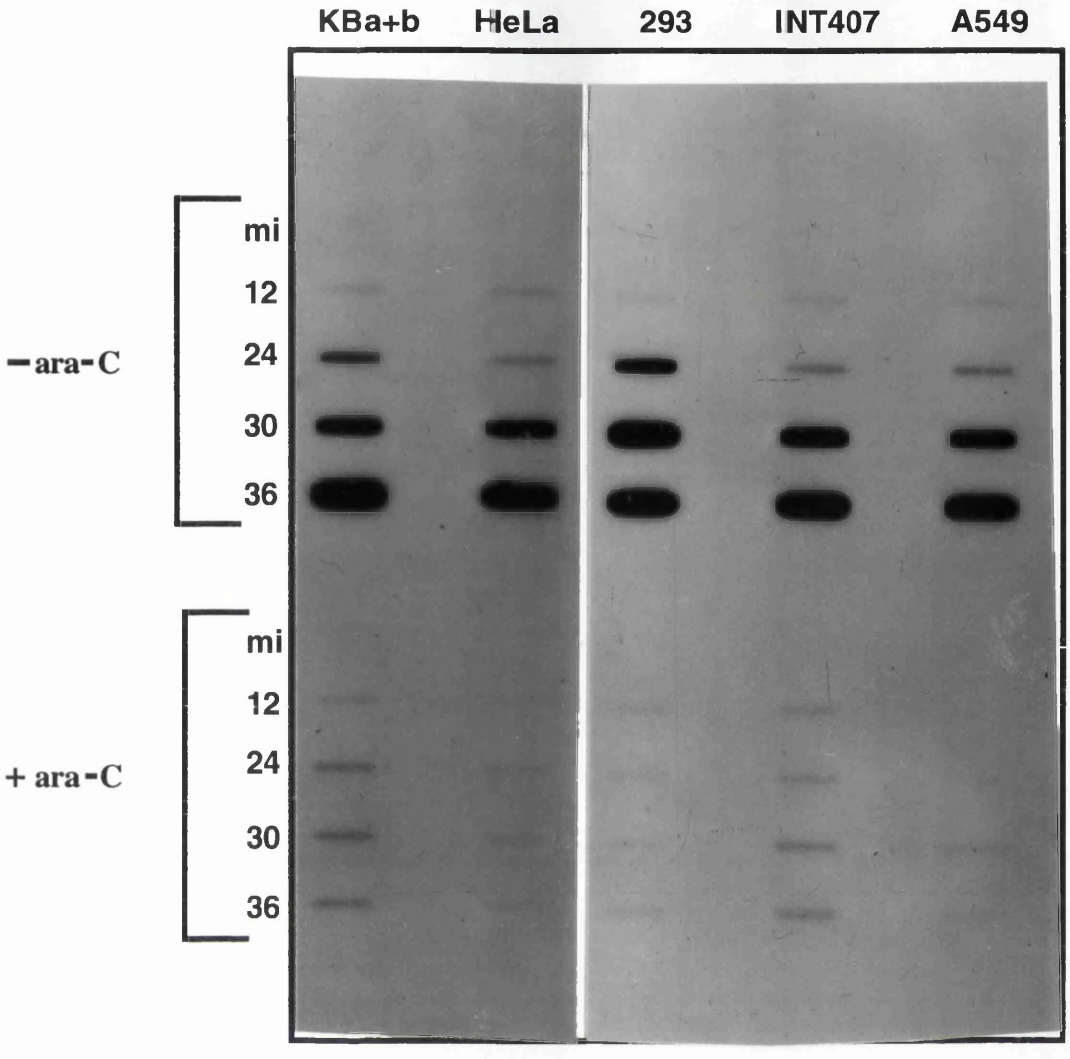


Figure 4.5.3. Inhibition of DNA Replication by ara-C in Ad40 Infected Cells.

Cells grown on 50mm plates were infected with a 100µl of a 1/10 dilution of Ad40 p9, at the times indicated post infection. To inhibit DNA replication, half the plates were treated (after adsorption) with 120µg of cytosine arabinoside (ara-C) which was added to plates every 12h thereafter. Cytoplasmic RNA was harvested and analysed by S1 nuclease protection (see Figure 4.5.4.). DNA was extracted from cell nuclei and applied to nylon membranes using a slot blot (1/20 harvest of a 50mm plate/slot), blots were probed with a nick translated plasmid containing the Ad40 E1B 55K region (pAB106). The DNA blots were exposed to autoradiography film for 24h.

The pattern of E1B transcription in the presence and absence of DNA replication is shown in Panel B of Figure 4.5.4. In 293 cells, in the absence of ara-C, E1B 22S mRNA is first detected at 12h, 6h before 14S. For KBa+b cells no mRNA is detected until 24h and then both messages are detected. In these cells 22S and 14S levels are at their most abundant at 36h p.i., as was found in section 4.5.1. In INT407 cells, 22S is first seen at 24h and 14S at 30h. For both INT407 and 293 cells 22S increases steadily up to 36h, 14S increases in a similar way but is more abundant in 293 cells than INT407. In A549 and HeLa cells, overall much lower levels of 14S are detected and they are first observed at 30h. Lower levels of 22S mRNA are detected in A549 and HeLa cells, compared to the other cell lines especially at the 36h timepoint.

In the presence of ara-C in A549 and HeLa cells, no mRNA corresponding to 14S and 22S is detected. For the more permissive cell lines, a low amount of 22S mRNA is detected from 12 to 36h in 293 cells and 24 to 36h for both INT407 and KBa+b cells. A more dramatic decrease is seen with 14S mRNA, with extremely low amounts detected in the presence of ara-C in all cell types.

The fact that some probe was detected in all samples (Fig 4.5.4.), indicated that S1 digestion was incomplete. In addition, certain samples were smeared and also appeared undigested (i.e. The samples were- in the absence of ara-C; 24h INT407, mi and 12h HeLa; in the presence of ara-C; mi HeLa, 24h 293 and 12h A549 samples). These samples were repeated: for the mi and 12h samples no E1B mRNA was detected and for the two 24h samples similar amounts of 22S were detected as for the equivalent 30h timepoints.

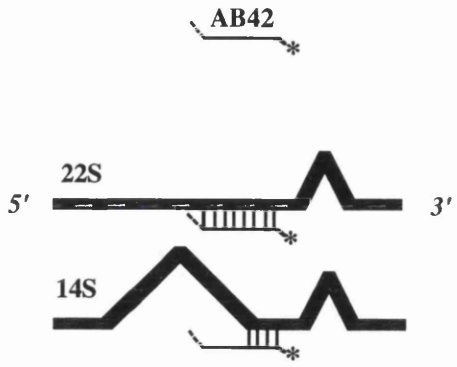
A laddering effect was detected in all gels and again indicated incomplete digestion by S1 nuclease. To optimise digestion conditions a control using tRNA instead of mRNA, was hybridised to the probe. Although complete digestion was achieved with the control, using these same conditions with the samples above still led to the laddering effect, even with mi mRNA. Alternatively this laddering could be an indication that the RNA was degraded. As there was insufficient RNA to run on a gel this was not tested further.

Figure 4.5.4. S1 Analysis of Ad40 E1B Transcription in the Absence and Presence of ara-C in Permissive and Semi-Permissive Cells.

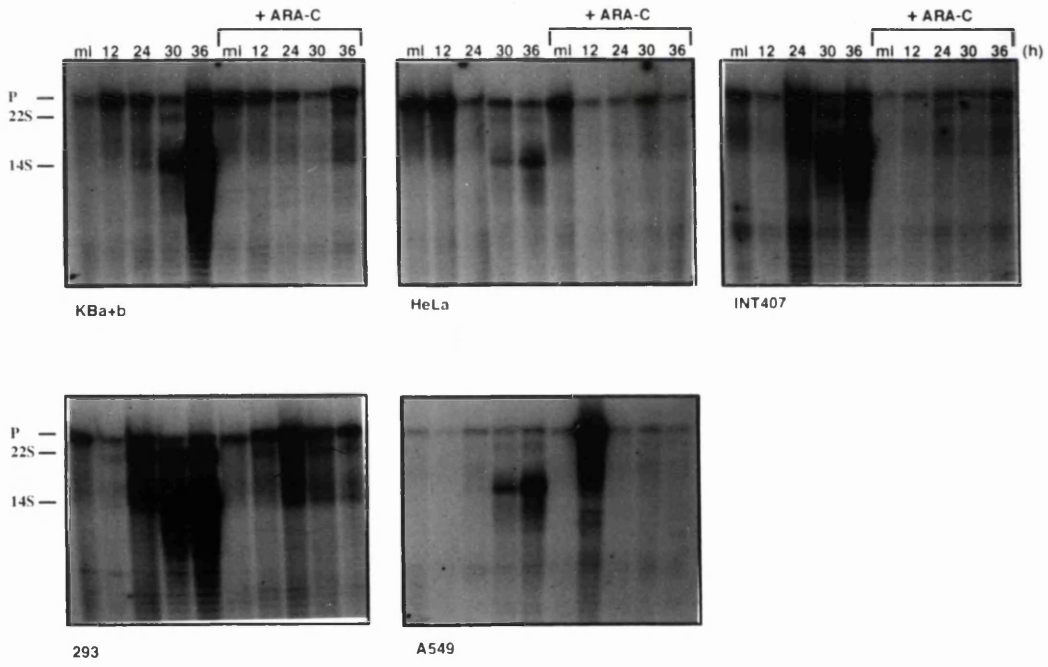
Panel A- The oligonucleotide AB42 spans the Ad40 14S splice acceptor site (3049-3110nt) and contains an additional 6nt of non adenoviral sequence at the 3' end (represented by the dashed line). Expected sizes of protected fragments for 22S and 14S mRNA are shown.

Panel B- Ad40 infected cells were grown in the absence and presence of ara-C for the times indicated. Cytoplasmic RNA was extracted and 15μg hybridised to 2ng of a 5' labelled oligonucleotide (AB42) probe. Hybridisation was carried out at 55°C overnight, unhybridised DNA was removed by digestion with S1 nuclease. Resulting protected fragments were run on 15% denaturing polyacrylamide gels, which were dried and exposed to autoradiography film for 3 days. A DNA marker was also run on gels (not shown).

A


AB42
Full length probe - 68nt
22S
5′
3′
Protected fragment- 62nt
14S
Protected fragment - 50nt

B


+ ARA-C
mi 12 24 30 36 mi 12 24 30 36
(h)
P
22S
14S
KBa+b
HeLa
INT407
293
A549

Interpretation was complicated by the appearance of the gel, with complete digestion of the probe variable both among cell types and across the timecourse and this in turn made quantitation difficult. However quantitation was attempted for the 36h timepoint using phosphorimage technology and is shown in Table 4.5.1.

The level of 14S is dramatically reduced in the presence of ara-C with an approximate 13-16 fold reduction observed in KBa+b, INT407 and 293 cell lines. No E1B mRNA is detected for both HeLa and A549 cells in the presence of ara-C at 36h. In the absence of ara-C the 22S transcript is most abundant in 293 cells, at levels approx. 3 fold greater than seen in KBa+b and INT407 cells, while only a fraction of this amount is observed for HeLa and A549 cells. The level of 22S is the same for both INT407 and KBa+b in the absence of ara-C, and in the presence of ara-C comparable levels of 22S mRNA are observed in KBa+b, INT407 and 293 cells. The amount of 22S is also reduced in the presence of ara-C in these cell lines with an approximate 3 fold reduction in KBa+b and INT407 cells and an 8 fold difference in 293 cells.

4.5.3. Summary.

Ad40 DNA replication was inhibited by the treatment of ara-C. In the absence of DNA replication E1B 22S mRNA could be detected in small amounts in 293, KBa+b and INT407 cells, but was not detected in HeLa or A549 cells. The level of 14S mRNA was dramatically reduced in the presence of ara-C. These results confirmed that E1B mRNA is made early in infection, albeit in small amounts, in the absence of DNA replication but only in permissive cells. The more permissive cell lines (KBa+b, INT407 and 293) show similar amounts of E1B mRNA in the absence of DNA replication, while no E1B mRNA is detected in HeLa and A549 cells. In the presence of DNA replication, E1B mRNA is most abundant in 293 cells, followed by KBa+b and then INT407 cells. In comparison, only small amounts of E1B mRNA were detected in HeLa and A549 cells. There was a large difference in the amount of E1B mRNA that was observed between the permissive and semi-permissive cell lines. Experiments were therefore carried out (see next section)

Table 4.5.1. Comparison of E1B mRNA Levels at 36h p.i. in Ad40 Infected Cells in the Presence and Absence of ara-C.

Cells	E1B mRNA			
	- ara-C		+ ara-C	
	22S	14S	22S	14S
KBa+b	1041 [a]	8575	406	642
HeLa	46	509	nd [b]	nd
INT407	1193	6964	386	501
293	3290	10749	429	681
A549	138	1930	nd	nd

a these values were determined by scanning membranes on a phosphorimager and quantitated using Imagequant.

b nd denotes not detected.

to investigate the E1B promoter function in permissive and semi-permissive cells, in order to see if this could explain the differences in expression of E1B transcripts.

4.6. Analysis of the Ad40 E1B Promoter.

E1B mRNA are observed before the onset of DNA replication in both INT407 and KBa+b cells, while in HeLa cells they are detected concurrent with the start of DNA replication. These results suggest that in HeLa cells the E1B promoter may be inactive at early times in infection.

The Ad2 (and Ad5) promoter is relatively simple consisting of a TATA box, (binding site for the TATA-binding protein or TBP) and a GC box sequence (binding site for the transcription factor Sp1) (Wu *et al.*, 1987; Parks *et al.*, 1988). The GC box sequence is important for increasing the level of transcription above the basal level and an approximate 6 fold reduction in E1B mRNA production was observed with partial or complete deletion of the Sp1 binding site (Wu *et al.*, 1987). This sequence element was found not to be involved in transactivation by E1A, as only mutations in the TATA box affected E1A transactivation (Wu *et al.*, 1987). Parks *et al.*, (1988) have identified four protein binding sites upstream of the Ad5 E1B promoter and suggested that these could be involved in stimulating transcription in the absence of a functional GC box.

Figure 4.6.1. shows sequence comparison between the Ad40 E1B promoter and those of other adenovirus serotypes. A high degree of conservation is observed for both the TATA and GC boxes. The TATA box shows the highest degree of conservation and core sequences are identical, with the exception of 2 nucleotides in Ad12. GC box sequences show more variability, but the Ad2 and Ad5 sequences are identical to the high affinity Sp1 binding site reported by Kadonaga *et al.*, (1988). All the other GC box sequences approximate to the consensus sequence 5' (G/T)GGGCGG(G/A)$_2$(C/T) 3', with only slight variations from this consensus. The GC box sequence for Ad12 is most similar to the putative Sp1 binding site for Ad40, with only 1 nucleotide difference between them. The spacing between the TATA and GC box sequences is identical for Ad40, 41 and 7 E1B promoters at 6nt, whereas this spacing is 8nt for both Ad2 and Ad5, and 10nt for Ad12. Wu and Berk, (1988b) found that the Ad2 E1B promoter was constrained; increasing the distance between the two sequence elements led to a decrease in the level of transcription equivalent to the deletion of the Sp1 binding site. In Ad5, a

Ad2	CGTGTTAAAT	**GGGGCGGGGC**	TTAAAGGG..	**TATATAA**TGC	GCCG
Ad5	CGTGTTAAAT	**GGGGCGGGGC**	TTAAAGGG..	**TATATAA**TGC	GCCG
Ad4	TTATGACTCA	**GGGGAGGGGA**	CTTTGGG...	**TATATAA**GCA	CGTG
Ad7	TATTACTTCT	**TGGGTGGGGT**	CTTGGA....	**TATATAA**GTA	GGAG
Ad12	AATGTTTGTT	**TGGGCGTGGT**	TAAACAGGGA	**TATAAAG**CAT	GGTT
Ad40	GTTTATTTCT	**TGGGCGTGTT**	TGTGGG....	**TATATAA**GCA	GGTA
Ad41	TTTTATTTCT	**TGGGTGTGTC**	CGCGGG....	**TATATAA**TGC	GCGG
		GC BOX		**TATA BOX**	

Figure 4.6.1. Alignment of Adenovirus E1B Promoter Sequences.

Adenovirus E1B promoters sequences from serotypes 2, 5, 4, 7, 12, 40 and 41 were extracted from the EMBL database and aligned with respect to the GC and TATA boxes (shown here in bold).

decrease in spacing of 4nt also resulted in an impaired promoter (Personal communication A. Berk). It may therefore be likely that a decreased spacing, as described above for the Ad40 E1B promoter could also affect the level of E1B transcription.

4.6.1. Analysis of the Ad40 and Ad2 GC Box Sequences by Gel Retardation Assay.

The aims of the following experiments were to determine if the Ad40 E1B GC box sequence could indeed bind Sp1 protein, and if so to examine if there are differences in binding between the Ad2 Sp1 binding site and the Ad40 GC box. In addition the differences between INT407 and HeLa cell extracts in terms of protein binding to the Ad40 and Ad2 GC box sequences were investigated, in order to see if the differences in the E1B mRNA pattern were related to the amount of protein binding to the E1B GC box and hence affect promoter activity.

The nuclear extracts used were from HeLa and INT407 cells and these were prepared as described in section 3.17. In addition, Sp1 protein which had been partially purified by WGA affinity chromatography (see 3.18.), was also used in experiments to look at Sp1 binding to the two GC box sequences. All oligonucleotides sequences used are described in section 2.5. Two double stranded probes were used encompassing the Ad40 GC box (oligos RTR3&RTR4) and the Ad2 GC box (oligos RTR5&RTR6), both probes were 28bp long. The probes were designed to exclude the TATA box sequence as a large number of proteins in cell extracts have been reported to bind this site therefore inclusion of this site may have cloaked protein binding to the GC box sequence and adjacent sequences. Gel retardation assays were performed as described in section 3.19. For binding reactions, 1ng of probe was used with either 5µg of nuclear extract or 0.5µg of Sp1 partially purified extract. These reactions were performed at 25°C for 30min and retarded complexes separated from free unbound probe on 3.5% non denaturing gels.

The first experiment was conducted with all three extracts described above (i.e. INT407, HeLa and Sp1) and examined the ability of the two probes and hence the two GC boxes to bind with protein from the extracts. The results of this experiment are shown

in Figure 4.6.2. Both the Ad40 and Ad2 probes formed a single DNA-protein complex with the Sp1 extract. The major retarded complex (RC), from both INT407 and HeLa extracts, has a similar mobility to the complex formed with the Sp1 extract. The amount of complex (RC), is least with the INT407 extract whichever probe is used. In addition to the major complex a few minor bands which have a higher mobility, are also observed with the INT407 and HeLa extracts, but not with partially purified Sp1. The Ad40 probe was labelled to a higher specific activity than the Ad2 probe, as observed from the difference in intensity of free probe (FP) in tracks without extract.

The amounts of the major retarded complex and free probe were quantitated by scanning gels using a phosphorimager (Molecular Dynamics) and analysing scans with Imagequant software, this data is shown in Table 4.6.1.

The Ad40 value for FP, in the sample without extract (which represented the amount of input probe), is approx. two fold higher than the RC+FP values for the other samples which contained Ad40 probe. This showed that a proportion of the probe is being lost, this could have arisen by pipetting errors and/or the presence of phosphatases in extracts, indeed smearing was observed in lanes (see Fig 4.6.2.).

Looking at the fraction of probe bound as a percentage of the total input probe takes into account the specific activities of the probes (see Table 4.6.1.). The greatest percentage of probe bound is 80% with Ad2 probe and Sp1 extract. Comparable levels of binding are also observed with Ad2 probe and HeLa extract (70%) and the least amount of binding with this probe is observed with INT407 nuclear extract. With the Ad40 probe the highest binding is detected with the HeLa extract (36%), whereas in comparison the percentage of probe bound to protein in the Sp1 extract is approximately 3 fold less. Only 4.5% of Ad40 probe binds with the INT407 extract, and this is the lowest percentage detected.

In summary this preliminary experiment indicates that the Ad40 probe binds less protein than the Ad2 GC box probe, in addition less probe is bound with INT407 than HeLa extracts. Only one complex is detected with the partially purified Sp1 extract and this is most likely to be Sp1 protein.

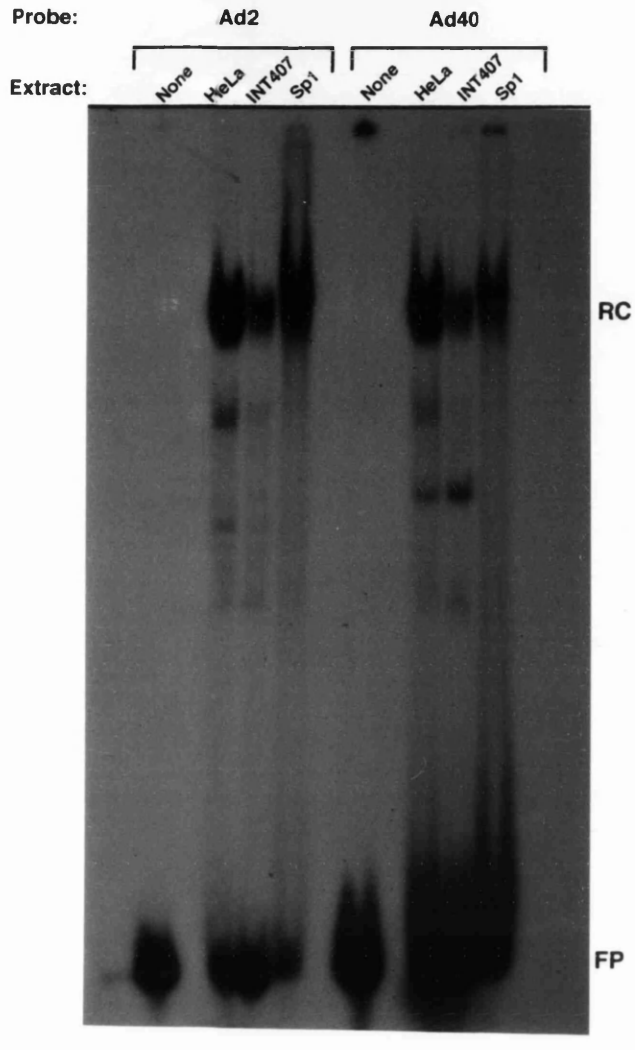


Figure 4.6.2. Gel Retardation Analysis of Ad2 and Ad40 GC Box Sequences with Different Extracts.

Extracts were incubated with an end-labelled probe encompassing either the Ad40 GC box or the Ad2 GC box sequence. Binding was carried out at 25°C for 30min with approx 1ng of probe and either 5µg of nuclear extract (HeLa or INT407) or 0.5µg of partially purified Sp1 extract. Free and bound probes were separated by electrophoresis on non-denaturing 3.5% acrylamide gels. Complexes were visualised by autoradiography, and are shown above. **RC** and **FP** represent major retarded complex and free unbound probe, respectively.

Table 4.6.1. Quantitation of Ad2 and Ad40 GC Box Binding to Different Cell Extracts.

Probe	Extract	RC [a]	FP [a]	RC + FP	RC/RC+FP x100 (%) [b]
Ad2	None	nd [c]	1,472,427	1,472,427	0.0
	HeLa	674,803	288,588	963,391	70.0
	INT407	124,259	926,704	1,050,963	12.0
	Sp1	609,233	147,230	756,463	80.0
Ad40	None	nd [c]	4,318,274	4,318,274	0.0
	HeLa	572,019	1,028,327	1,600,346	36.0
	INT407	107,846	2,305,895	2,413,741	4.5
	Sp1	255,271	1,901,308	2,156,579	12.0

a these numbers represent pixel values which were determined by performing volume integration with Imagequant software.

b this represents the fraction of probe bound expressed as a percentage of the total.

c nd, not detected.

4.6.2. Gel Retardation Competition Assays of Ad40 and Ad2 GC Box Sequences.

The specificity of the Ad40 and Ad2 GC box sequence probes binding to protein was tested in competition binding assays. Oligonucleotides comprising the probes (Ad2 and Ad40) above were used as competitors, but in an unlabelled form. In addition a hybrid competitor (Ad40m4) consisting of the core Ad2 GC box sequence with Ad40 flanking sequences (oligos RTR11&12) was included. A nonspecific competitor consisting of the octamer/TAATGARAT motif (Oct 1) from the upstream promoter region of the HSV-1 IE gene 2 was also used and comprised of the oligos OCT27 and OCT28.

Competition experiments are outlined in more detail in section 3.19., but briefly the competitor DNAs were added to extracts for 15min at 25°C and after which probe was added, samples incubated for a further 30min at 25°C. Competitior DNAs corresponding to the Ad2, Ad40 and Ad40m4 GC box sequences were used at 50, 100 and 200 fold molar excess. The nonspecific competitor Oct 1 was used at 100 fold molar excess.

Competition binding assays using the Ad2 sequence as the labelled probe with INT407, HeLa and Sp1 extracts are shown in Figure 4.6.3. Using the homologous competitor (Ad2), complex formation (RC) is significantly reduced with all three extracts. The Ad40 sequence is the least effective competitor, but the amount of protein bound to probe decreased with the Ad40m4 DNA sequence i.e. less RC was detected. The hybrid competitor is more effective than Ad40, but Ad2 is still the best competitor.

A number of minor complexes were again observed with the INT407 and HeLa extracts. The competition indicates some of these minor bands are specific and others are non specific. These minor bands are not included in the quantitative analysis. The major retarded complex (RC) and free unbound probe (FP) were quantitated as described (see 4.6.1.) and the fraction of probe bound is shown in Table 4.6.2.

The fraction of probe bound by the Ad2 GC box sequence in the absence of competitor was almost identical to that detected previously (see Table 4.6.1) for each of the cell extracts. The values with the non specific competitor (Oct 1) are comparable to

Figure 4.6.3. Competition of DNA-Protein Binding with the Ad2 Probe.

Gel retardation assays were carried out as described previously in section 3.19. Either 5µg of nuclear extract (HeLa or INT407) or 0.5µg of Sp1 extract was incubated with 1ng of end-labelled Ad2 probe. Competitor DNAs were added to extract (with all buffers) and incubated for 15min at 25°C. The competitors Ad40, Ad2 and Ad40m4 were used at 50, 100 and 200 fold molar excess. Non-specific competitor Oct 1 was used at 100 fold molar excess. Probe was then added and samples incubated for a further 30min at 25°C. Bound and free probes were then separated by electrophoresis on non-denaturing 3.5% acrylamide gels. Complexes were visualised by autoradiography, and are shown above. **RC** and **FP** represent major retarded complex and free unbound probe, respectively. **N** represents samples with no extract and no competitor i.e. probe and buffers alone. Samples with **H**, **I** and **S** had no competitors added i.e. probe (and buffers) with corresponding extract.

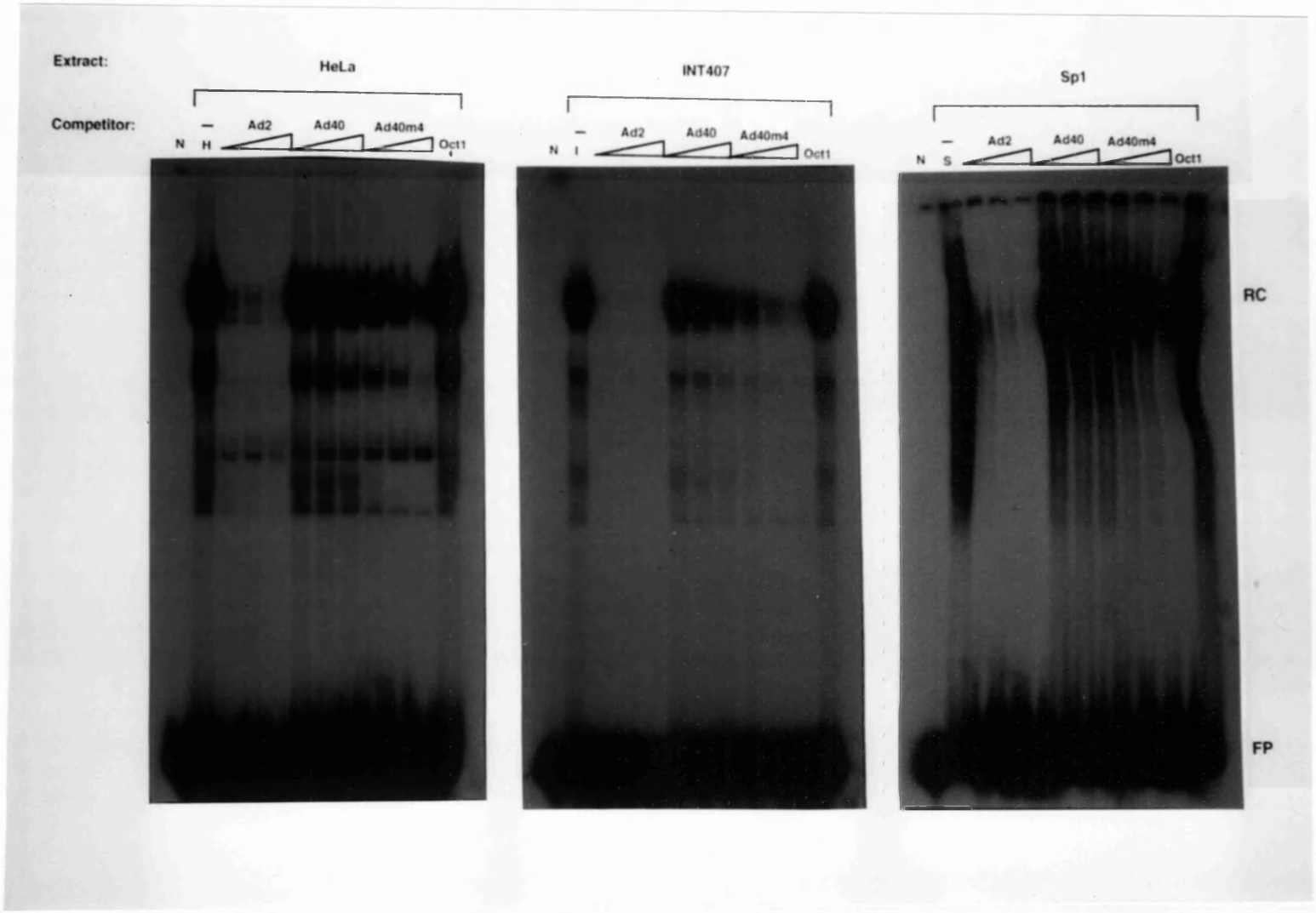

Extract:
HeLa
INT407
Sp1
Competitor:
–
Ad2
Ad40
Ad40m4
N
H
I
S
Oct1
RC
FP

Table 4.6.2. The Quantitation of Ad2 Labelled Probe Binding to Different Cell Extracts in Competition Assays.

Competitor (molar excess)	Extract [a]		
	HeLa	INT407	Sp1
None	67.90	12.30	78.80
Ad2 (x50)	10.60	0.10	3.00
Ad2 (x100)	0.60	0.05	1.10
Ad2 (x200)	0.60	0.04	0.70
Ad40 (x50)	35.90	6.30	56.30
Ad40 (x100)	23.30	5.60	49.70
Ad40 (x200)	14.50	2.70	35.40
Ad40m4 (x50)	12.60	1.90	22.90
Ad40m4 (x100)	7.80	0.80	12.40
Ad40m4 (x200)	2.40	0.40	8.00
Oct 1 (x100)	56.50	11.60	81.50

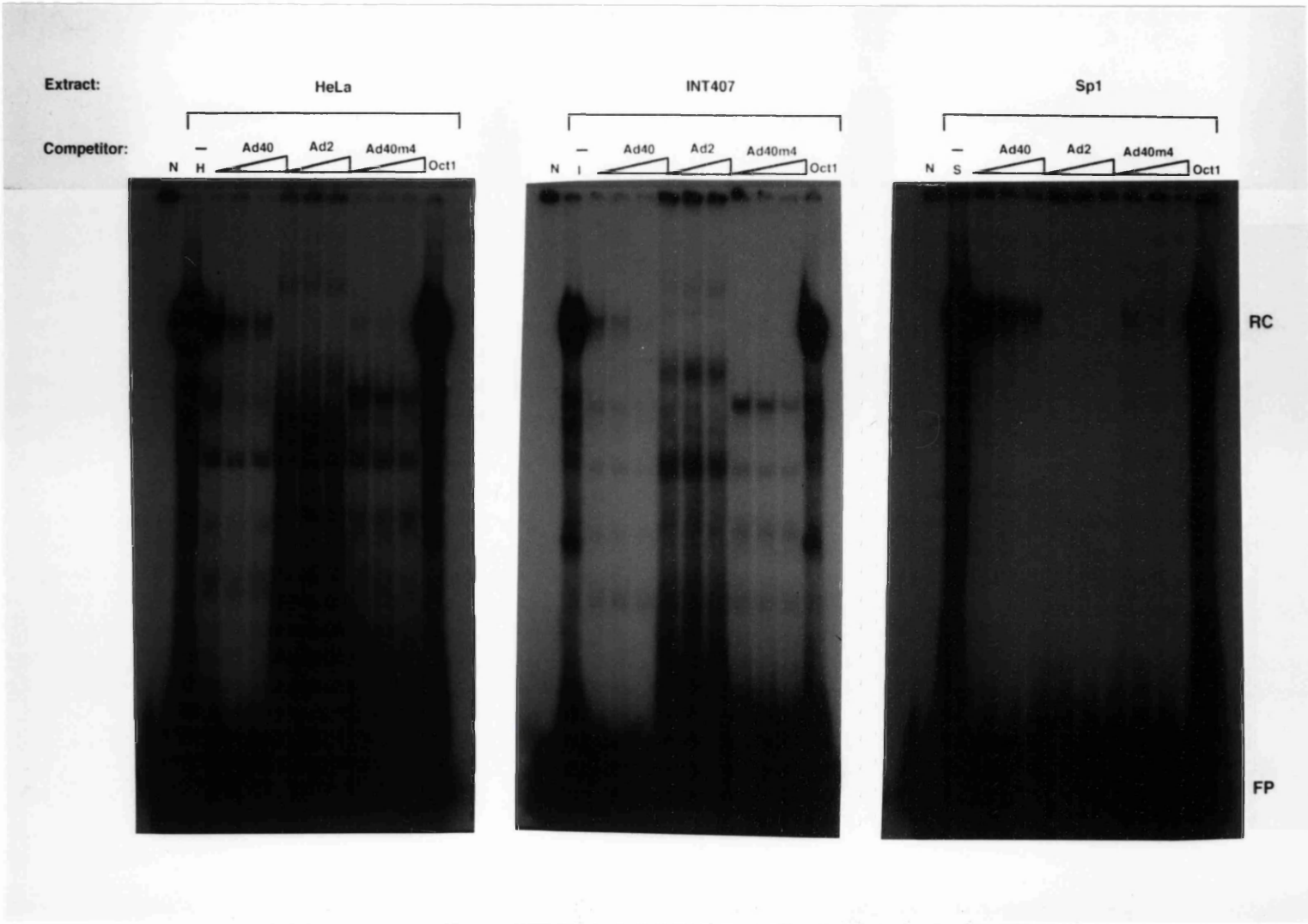
a values represent the fraction of probe bound to protein expressed as a percentage of the total radioactive signal i.e. RC/RC+FP x100.

values without competitor added, confirming that binding is specific. Relative affinities for this protein-DNA interaction were investigated using different concentrations of competitor DNAs. There is an approximate 5 fold difference between INT407 and HeLa extracts in the amount of probe that bound protein, although a similar pattern of competition is observed. When Ad2 competitor DNA is used at 100 or 200 fold molar excess with HeLa extract, probe binding is reduced to 0.6%, whereas using the Ad40 competitor at 200 fold molar excess only reduces binding to 14.5 %, while the Ad40m4 competitor at 200x excess gives an intermediate value of 2.4%. This implies that the Ad40 flanking sequences have an affect on probe binding to protein. A similar overall hierarchy is seen with both INT407 and Sp1 extracts; Ad2 is the best competitor for Ad2 probe, next is Ad40m4 and the poorest is the Ad40 competitor. For the nonspecific competitor, Oct 1, the major retarded complex detected with both INT407 and Sp1 extracts appears to be specific, as there is little difference with or without Oct 1. However with HeLa extract there is a small reduction in the amount of RC detected with Oct 1, this could be due to an excess of DNA added or indicate that the complex is non specific. As this small reduction is only a fraction of that observed with the other competitors, the RC is most likely a specific complex.

Similar competition binding assays were carried out using the Ad40 GC box as probe with the extracts and competitors described above, the gels are shown in Figure 4.6.4. Minor bands are again detected with HeLa and INT407 extracts. Using the Ad40 competitor with HeLa and Sp1 extracts, a similar pattern of competition is observed where there is a reduction in the amount of RC observed over the three concentrations of competitor. However small amounts of complex are still detected even with the x200 concentration. With the INT407 extract and Ad40 competitor there is a more dramatic decrease in complex and no RC is observed with 200 fold molar excess of competitor. Using Ad2 as a competitor, no RC is detected with all three extracts even at 50 fold molar excess. With the hybrid competitor Ad40m4 and the HeLa and INT407 extracts a pattern more similar to the Ad2 competitor is observed for RC with no complex detected even at the highest concentration. However the pattern of minor bands more resembles the Ad40 competitor. Small amounts of RC are detected with the Sp1 extract and Ad40m4

Figure 4.6.4. Competition of DNA-Protein Binding with the Ad40 Probe.

Gel retardation assays were carried out as described previously in section 3.19. Either 5μg of nuclear extract (HeLa or INT407) or 0.5μg of Sp1 extract was incubated with 1ng of end-labelled Ad40 probe. Competitor DNAs were added to extract (with all buffers) and incubated for 15min at 25°C. The competitors Ad40, Ad2 and Ad40m4 were used at 50, 100 and 200 fold molar excess. Non-specific competitor Oct 1 was used at 100 fold molar excess. Probe was then added and samples incubated for a further 30min at 25°C. Bound and free probes were then separated by electrophoresis on non-denaturing 3.5% acrylamide gels. Complexes were visualised by autoradiography, and are shown above. **RC** and **FP** represent major retarded complex and free unbound probe, respectively. **N** represents samples with no extract and no competitor i.e. probe and buffers alone. Samples with **H**, **I** and **S** had no competitors added i.e. probe (and buffers) with corresponding extract.


Extract:
HeLa
INT407
Sp1
Competitor:
–
Ad40
Ad2
Ad40m4
N
H
I
S
Oct1
RC
FP

competitor at x50 and x100 fold molar excess but not at x200. The major retarded complex (RC) and free probe (FP) were quantitated as described previously (see section 4.6.1.) and the percentage of probe bound is shown in Table 4.6.3.

Approximately equivalent levels of Ad40 probe bound are observed with HeLa and Sp1 extracts when there is no competitor present. There is a 6 to 7 fold less difference with the INT407 extract compared to the other two extracts. The percentage ratio of probe bound with the INT407 extract (without competitors) was the same as that observed previously (see Table 4.6.1.). However there is some variability in the amount of RC detected with the HeLa and Sp1 extracts compared to the previous experiment (see Table 4.6.1.). The pattern of competition is very similar for HeLa and Sp1 extracts, with all competitors very effective in reducing the fraction of probe bound. The INT407 extract bound only 4.5% of probe and all competitors were very effective in reducing this fraction.

Overall competitors even at highest dilutions (50x molar excess) were very effective at reducing binding to 2% or less. To measure differences in affinity it would be necessary to use lower levels of competitors (e.g. x5, x10 etc.). For the non-specific competitor Oct 1, no difference is observed with INT407 extract and Ad40 probe indicating that this binding is specific. For HeLa and Sp1 extracts a reduction in complex is detected with Oct 1, however this is very small compared to the effect of the other competitors used, and the RC is most likely specific.

4.6.3. Summary.

Less RC was detected with the Ad40 GC box than the Ad2 GC box for all three extracts and with INT407 extract less probe was bound compared to HeLa and Sp1 extracts. The major retarded complex is most likely formed with Sp1 protein as the mobility of RC corresponded to the only complex detected with the Sp1 extract. The RC observed with Ad2 GC box probe was efficiently competed with unlabelled Ad2 GC box sequence but less so by unlabelled Ad40 GC sequence. The hybrid sequence Ad40m4 was an intermediate competitor indicating that the sequences flanking the Ad40 GC box

Table 4.6.3. The Quantitation of Ad40 Labelled Probe Binding to Different Cell Extracts in Competition Assays.

Competitor (molar excess)	Extract [a]		
	HeLa	INT407	Sp1
None	24.80	4.50	25.30
Ad40 (x50)	1.70	0.20	2.10
Ad40 (x100)	0.40	0.10	0.80
Ad40 (x200)	0.20	0.05	0.50
Ad2 (x50)	0.20	0.10	0.80
Ad2 (x100)	0.20	0.10	0.80
Ad2 (x200)	0.10	0.10	0.05
Ad40m4 (x50)	0.20	0.06	0.20
Ad40m4 (x100)	0.10	0.05	0.10
Ad40m4 (x200)	0.30	0.07	0.01
Oct 1 (x100)	19.90	4.60	17.70

a values represent the fraction of probe bound to protein expressed as a percentage of the total radioactive signal i.e. RC/RC+FP x100.

may also have an affect on protein binding. With Ad40 probe, the Ad2 and Ad40m4 competitors were very effective and reduced binding to below 1% with all three extracts. The Ad40 competitor was also effective at reducing the amount of RC observed and binding was reduced to 2% or less at x50 fold molar excess.

5. DISCUSSION.

5.1. INT407 Cells are Permissive for Ad40.

The E1 region was first implicated with the fastidious growth of the enteric adenoviruses when it was shown that these viruses grew on 293 cells which are HEK cells transformed with the Ad5 E1 region (Takiff *et al.*, 1981). The E1B region was later shown to be specifically involved, as Ad40 could grow in cell lines expressing the Ad2 E1B region (KBb, KBa+b cells), but not the E1A region (KBa) alone (Mautner *et al.*, 1989). Furthermore complementation assays with adenovirus E1B mutants demonstrated that it was the expression or function of the Ad40 E1B 55K protein that was impaired (Mautner *et al.*, 1989). Thus far, permissive cell lines used to successfully propagate Ad40 were transformed with either the E1B or E1 regions of adenovirus (293, KBb, KBa+b); what effect these endogenous proteins had in Ad40 gene expression and DNA replication was unknown. At this time, in a preliminary experiment, the INT407 cell line was shown to support Ad40 growth, albeit at reduced levels compared to KBa+b cells (Steinthorsdottir, 1991). Therefore a detailed timecourse of Ad40 DNA replication on INT407, KBa+b and the semi-permissive cell line HeLa was undertaken (section 4.1.). This experiment revealed that the time of onset and yield of DNA was similar on KBa+b and INT407 cells, although the DNA yield from KBa+b cells was higher than INT407 cells. In HeLa cells, the onset of DNA replication was later and the final virus yield much lower than the other two cell lines. This confirmed that Ad40 could grow in cells which had not been transformed with the E1 region and were not known to express adenovirus proteins. The eclipse phase prior to DNA replication for Ad40 (see Fig 4.1.1.) on both KBa+b and INT407 cells lasts at least 24h, and these results are comparable to Mautner *et al.*, (1989) where the eclipse phase for Ad40 was determined to be of similar duration. On HeLa cells the eclipse phase for the virus was 6h longer than for both KBa+b and INT407

cells. In comparison the eclipse phase of Ad5 grown on KBa+b cells lasts approx. 8h (Mautner *et al.*, 1989), and demonstrates that Ad40 grows more slowly than Ad5 even on the permissive cells lines KBa+b and INT407.

Two possible explanations why INT407 cells are able to support the growth of Ad40 are that either E1B 55K is complemented by a cellular factor in INT407 which has E1B-like properties or the requirement for 55K may vary in these cells. INT407 cells could support the growth of Ad40, therefore some adenovirus mutants were investigated to determine if this cell line could complement the growth of E1B adenovirus mutants or whether the effect was specific to Ad40. INT407 complemented the Ad5/2 E1B 19K mutant pm1722 to wild type (dl309) levels and partially complemented the Ad5/2 E1B 55K mutant dl1520 (Fig 4.2.3.). This implies that the mechanism by which the Ad40 E1B 55K defect is overcome in INT407 cells may also be used for other adenovirus 55K mutants, although some virus specificity is exhibited i.e. dl1520 was only partially complemented.

Other researchers have also reported the growth of enteric adenoviruses on cell lines which have no endogenous E1 complementary proteins. Gomes *et al.*, (1992) have shown that Ad40 (Dugan) can be propagated on the HRT-18 cell line which is derived from human rectal adenocarcinoma. In these cells Ad40 grew better than the E1B 55K mutant dl1520 from analyses of Hirt extracted DNAs; this was similar to the pattern observed in section 4.2. where dl1520 is partially complemented in INT407 cells. Interestingly in coinfections with Ad40 and dl1520 in HRT 18 or 293 cells (Gomes *et al.*, 1992) no DNA was detected for both viruses. This was not in agreement with Mautner *et al.*, (1989) in which DNA was detected by slot blot hybridisation for both viruses in a coinfection assay conducted on HeLa cells. Hashimoto *et al.*, (1991) has shown the A549 (derived from lung carcinoma) cell line can support Ad40 (Sapporo strain) growth. These authors also reported the production of plaques on these cell lines at 18 days post infection. During work carried out for this thesis a plaque assay using A549 cells and Ad40 (Dugan strain) was conducted but no plaques were observed, even with the same experimental conditions as those used by Hashimoto *et al.*, (1991). Indeed Grabow *et al.*, (1993) using A549 cells in a plaque assay with Ad40 (Hovi-X strain) also failed to obtain

plaques. The A549 cell line was also used by Steinthorsdottir *et al.*, (1991) to look at Ad40 (Dugan strain) growth in comparison to the INT407 and KBa+b cell line It was shown to be less permissive than INT407 cells which were in turn less permissive than KBa+b cells. In contrast Hashimoto *et al.*, (1991) reported that A549 cells are more permissive for Ad40 than KBb cells. As Mautner *et al.*, (1990) have shown that KBa+b and KBb cells are equally permissive for Ad40 growth, it is not known if differences between these results are affected by the presence of E1A in KBa+b cells. Different strains of Ad40 are used in these studies and different laboratory conditions may well account for some of the differences in these results as has been well documented by de Jong *et al.*, (1983). In this thesis (sections 4.2., 4.3. and 4.5.), A549 is also shown to be less permissive for Ad40 than INT407 cells particularly in terms of the growth of the virus and E1 transcription.

Other cell lines which can support Ad40 and Ad41 with variable degrees of success are: colonic carcinoma cell line CaCo-2 (Pinto *et al.*, 1994); Chang conjunctival cells (Kidd and Madeley, 1981); Hep-2 cells (Pieniazek *et al.*, 1990a); PLC/PRF/5 hepatoma cell line (Grabow *et al.*, 1992); primary rhesus monkey Kidney cells transformed with Ad3 or Ad5 E1 sequences (Nascimento *et al.*, 1990). Ad40 grows in some of these cell lines, but it is uncertain if similar functions to INT407 cells are supplied or if there is something else in these cells that complements Ad40. Ad40 growth in these cell lines warrants further investigation.

5.2. Ad40 Growth in Permissive Cells Shows One Hit Kinetics.

It was shown that the yield of Ad40 obtained from INT407 cells was considerably greater than that on HeLa cells. Explanations to account for this difference were sought, primarily through viral infectivity assays. The work described in section 4.3. was an attempt to define the growth kinetics of Ad40 in cells showing different degrees of permissiveness. An indirect fluorescence focus assay was employed to monitor Ad40 infected cells in relation to virus dilution. In KBa+b and INT407 cells a pattern of one hit kinetics was obtained, while in A549 and HeLa cells a two hit pattern was observed.

A one hit pattern has been described for many viruses (for review see Cooper, 1961), although in most of these studies a plaque assay was employed to examine virus infectivity. This pattern of infection has been described for many adenoviruses; Ad4 and Ad5 on HeLa cells (Kjellén, 1961), also for Ad1 and Ad7 on Grivet monkey kidney cells (Tytell *et al.*, 1962) and Ad41 on the permissive cell line 293 (Tiemessen and Kidd, 1990). An example of another virus that displays the one hit pattern is that of poliomyelitis virus (Dulbecco and Vogt, 1953).

A pattern where two virus particles are required to infect a cell has also been described. The most notable example is that of adeno-associated viruses; these viruses only replicate in cells that are infected by an adenovirus or herpesvirus (Atchison *et al.*, 1965; Blacklow *et al.*, 1967). Another example is the defective SV40-Ad7 hybrid particle which is complemented by Ad7 in African green monkey kidney cells (Boeyé *et al.*, 1966). In the examples where two particles are required, the particles are distinct and one particle appears to have the role of a helper. HeLa and A549 cells on the other hand are infected by two Ad40 particles and no helper appears to be present. Tiemessen and Kidd (1990) showed that Ad41 exhibits a two hit pattern in coinfections with Ad2 and a multiple hit pattern in single infections on Chang conjunctival cells. A possible explanation put forward by these authors for the multiple hit pattern was that multiple infecting genomes were required to overcome a limiting concentration of some essential product in these cells. For HeLa and A549 cells it is not clear if the requirement of two

Ad40 genomes overcomes an essential but scarce product in these cells, which may be present in INT407 cells.

In the absence of a reliable plaque assay with Ad40, the enumeration of virus infectivity was carried out using the data from the indirect fluorescent focus assay in order to compare Ad40 titres on different cell lines. Titres obtained (see Table 4.3.5.) were comparable to those previously reported by Gomes *et al.*, (1992) (2.4 x 10^5 FFU/ml) on HeLa cells and the permissive HRT-18 cell line (1.4 x 10^6 FFU/ml). The particle to infectivity ratios for Ad40 are high; 1:10^3 for KBa+b & INT407 and 1:10^4 for HeLa & A549 cells (from Table 4.3.5.). Brown *et al.*, (1992) found that the particle to infectious unit (IU) ratios for Ad40 (where infectivity was measured by endpoint dilution method) were between x10^3 to x10^6 on HeLa cells and x10^4 to x10^5 for 293 cells. Moreover these authors showed that a similar yield of Ad5, Ad40 and Ad41 particles was produced from infected cells, but the particle to infectious unit ratios were approx. 100-1000 fold less for Ad40 and Ad41. This ratio is high and one possible contributor could be the poor growth of these viruses in cell culture. A higher particle to infectivity ratio compared to other adenoviruses is also an indication of the presence of defective virus particles, probably resulting from a failure at some stage in the lytic cycle. It was apparent from these studies, as well as others that complementation of E1B function only partially corrected the defective growth phenotype of Ad40. Therefore an investigation of the early events during the Ad40 replicative cycle (i.e. attachment and internalisation) was initiated to determine if any defects at this stage might contribute to the growth phenotype of Ad40.

5.3. Ad40 Entry into Cells.

The first event in adenovirus entry into cells is virus to cell recognition. For adenovirus types 2 and 5 this has been shown to occur via attachment of the distal part of the fibre protein 'knob', (Philipson *et al.*, 1968; Defer *et al.*, 1990; Henry *et al.*, 1994; Louis *et al.*, 1994), followed by penton base interaction with cell surface integrin proteins (Wickham *et al.*, 1993) which is essential for internalisation of the virus.

The amount of Ad40 virus that attached to HeLa and INT407 cells was examined (see 4.4.1.), to investigate why the number of HeLa cells infected with Ad40 is much reduced compared to INT407 cells (see 4.3.). In this study approximately 10% of the viral input attaches to cells and of this bound virus 21-31% is actually internalised. This is consistent with findings by Brown *et al.*, (1992) who found that 10% of HeLa cells can support a lytic cycle. This may explain the high particle to infectivity ratio observed for Ad40 (Table 4.3.5.), although the molecular mechanism behind it is unknown. However this did not explain the difference in Ad40 permissivity between INT407 and HeLa cells, as slightly more Ad40 particles attach to HeLa cells than to INT407 cells. Was there a difference in the amount of virus that was taken up by the two cell types? In order to address this, internalisation of Ad40 was investigated in INT407 and HeLa cells (see 4.4.2.). More virus is internalised in HeLa cells than INT407 cells. This again can not account for the difference observed in the later onset of DNA replication (see 4.1.) and reduced yield of virus in HeLa cells (see 4.3.) compared to INT407 cells.

In a parallel experiment attachment and internalisation of dl309 was examined on INT407 and HeLa cells; approximately 10% of virus attaches to both these cell types and of this 33-41% of bound virus is internalised. The amount of Ad40 virus that attached and internalised in HeLa cells was comparable to dl309. In this study the percentage of bound virus that was internalised was considerably less compared to other studies for Ad2; 60% in Wickham *et al.*, (1993) and 50% in Varga *et al.*, (1991). In both these studies the cell cultures used were in suspension as opposed to monolayers used in this study. This may in part account for the reduced amount of dl309 that was internalised compared to these studies, but Greber *et al.*, (1993) have observed 80% of attached Ad2 virus internalised in

KB monolayers. It is unclear what could account for the differences in the amount of internalisation. However it should be noted that virus receptor sites for both dl309 and Ad40 on HeLa and INT407 cells are not saturated, as no plateau is observed (section 4.1.1.). Philipson *et al.*, (1968) reported that there are 10^4 Ad2 receptors on HeLa cells, in this study $3x10^3$ particles/cell for dl309 and $7x10^3$ particles/cell for Ad40 were used, which would be insufficient to saturate receptor sites. Presumably with a higher moi an increase in internalisation (& attachment) may be seen. Whether this approaches the amount reported for Ad2 remains to be determined.

The attachment of dl309 was significantly inhibited by Ad2 fibre indicating that attachment is mediated by the fibre protein, and is consistent with previous reports for adenovirus (Philipson *et al.*, 1968). Internalisation of Ad2, 3, 4 and 12 is mediated by penton base which binds to cell surface αv integrin proteins via their RGD sequence (Bai *et al.*, 1993; Wickham *et al.*, 1993; Mathias *et al.*, 1994). However Ad40 does not have an RGD sequence. Instead an RGA motif is present, and it is unclear whether Ad40 also uses this mechanism or whether another mechanism is involved. Another virus which has no RGD sequence is FAV-10 (Sheppard and Trist, 1992).

Other viruses that possess an RGD sequence include foot-and-mouth disease virus and coxsackievirus A9. The foot and mouth disease virus RGD motif present on a capsid protein can bind $\alpha_V\beta_3$ integrins on monkey cells and is essential for cell attachment (Berinstein *et al.*, 1995 and references therein). Deletion of the RGD sequence renders the virus non infectious and unable to bind cells. Coxsackievirus A9 (CAV-9) can also bind $\alpha_V\beta_3$ integrins via an RGD sequence present on capsid protein VP1, this interaction mediates virus attachment to cells (Roivainen *et al.*, 1994). Hughes *et al.*, (1995) have shown that CAV-9 mutants lacking the RGD motif can bind cells and are infectious. These results indicate that CAV-9 can use an RGD-independent pathway for entry into cells. Ad40 may also use an RGD-independent pathway that does not involve integrin binding, but some integrins can bind non-RGD sequences i.e. $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_X\beta_2$ (Hynes, 1992). Therefore an integrin-mediated internalisation step cannot be fully ruled out for Ad40.

Ad40 (and Ad41) contains two types of fibre, one type extending from each vertex of the capsid, how this relates to attachment is not clear. Avian adenovirus CELO also has two types of fibre, but in contrast to enteric adenoviruses, both types of fibre protrude from each vertex (Laver *et al.*, 1971; Hess *et al.*, 1995). Whether the two Ad40 fibres bind to the same cell receptor or two different receptors one for attachment and one for internalisation is not known. The lack of homology between the two head domains of adenovirus subgroup F fibres (Kidd *et al.*, 1993) may indicate that these viruses bind to two different receptors.

In this study attachment and internalisation of Ad40 was comparable to dl309, indicating that the Ad40 entry process was as efficient as that of dl309, which has one type of fibre. The amount of Ad40 virus internalised in HeLa cells was higher than in INT407 and was not a determinant as to why INT407 cells are more permissive than HeLa cells. There could be a failure at some other stage in the lytic cycle and the next stage investigated was E1 gene expression.

5.4. Ad40 E1 Gene Expression.

In this study the major mRNAs from the Ad40 E1B region correspond to the Ad2 22S and 14S mRNAs (see Fig 4.5.1.). This was consistent with the species of mRNA produced from the Ad40 E1B promoter characterised by Steinthorsdottir and Mautner, (1991). Both in terms of appearance and abundance of mRNA, the pattern of E1B gene expression in INT407 cells more closely follows the pattern in KBa+b cells than HeLa cells. However the amount of E1B mRNA produced in Ad40 is reported to be 5 fold less than in Ad5 at a modest moi (Mautner *et al.,* 1990). In this study E1B 22S mRNA is observed at early times, before the onset of DNA replication, in KBa+b and INT407 cells, albeit at low levels. This is the first time E1B mRNA has been detected before the onset of DNA replication and is contrary to previous reports, where no Ad40 mRNA was seen at early times (Mautner *et al.,* 1990; Steinthorsdottir, 1991). Possible explanations to account for this difference may be from the use of a higher titre virus in this study compared to Mautner *et al.,* (1990) or a different extraction method for mRNA compared to Steinthorsdottir, (1991) or quite possibly a combination of these factors. In HeLa cells, mRNA is detected coincident with the onset of DNA replication, however as both mRNA and DNA first appear at the same time, it is unclear if there is an increase in either E1B mRNA or DNA replication first. E1A is also observed before the onset of DNA replication in permissive cells but not in semi permissive cells. It is not clear if E1A is produced before E1B as times between 12-18h were not investigated. However for both Ad40 E1A and E1B regions mRNA is delayed compared to Ad5 (Ad5 E1A is detected at 2h p.i. and Ad5 E1B is seen at 3.5h) (Glenn and Ricciardi, 1988). Ad40 E1A has been shown to have a low transactivation activity which could explain its late detection. In addition the Ad40 E1A promoter has been shown to be weak compared with Ad5 and Ad12 E1A (Ishino *et al.,* 1988). The low transactivation and promoter activity may play a role in the replication of the virus and thus contribute to a poor viral yield. However the later detection of Ad40 E1A is unlikely to account for the differences in E1B gene expression observed among cell types, as Ad40 could not be complemented in cells which express adenovirus E1A gene products, and could complement a Ad2 E1A mutant

indicating there was no gross defect in this region (Mautner *et al.*, 1989: Hashimoto *et al.*, 1991).

To confirm that E1B mRNA was detected at early times, infection was carried out in the presence and absence of cytosine arabinoside (ara-C), which inhibits DNA replication, allowing the accumulation of early mRNA. The mRNA was analysed by S1 nuclease protection (Fig 4.5.4.). The 22S transcript is detected in the presence of ara-C in 293, KBa+b and INT407 cells. The amount of 22S mRNA was reduced in the presence of ara-C (Table 4.5.1.) (approx. 2-3 fold in INT407 and KBa+b cells, and 7.6 fold in 293 cells at 36h p.i.), but the reduction in 14S was considerably greater at approx. 13-16 fold less for these cell types. This is consistent with reports by Bailey *et al.*, (1993, 1994) where the 55K protein is detected in approx. similar levels in the presence and absence of ara C in 293 and KBa+b cells, but the expression of the 19K protein is inhibited in the presence of ara C. Furthermore this correlates with the low levels of 19K protein detected at early times in infection (Bailey *et al.*, 1993) and implies that for efficient 19K expression progression into the later stages of infection is needed. For HeLa and A549 cells infected with Ad40, E1B mRNA is not detected when DNA replication is inhibited by ara-C. A possible explanation for this is that the E1B region is only transcribed on newly replicated DNA. This would be consistent with the detection of DNA and RNA at approx. 36h and the increased replication thereafter (Fig 4.5.1.). Alternatively E1B transcription is inefficient in these cells and as such not observed until after the onset of DNA replication after which an increase in template results in a detectable transcription level.

In Ad12 the pattern of E1B gene expression is similar to Ad40 with a 14S transcript detected by cDNA cloning (Virtanen *et al.*, 1982), and no 13S mRNA is observed. The amino acid sequence of Ad40 E1B proteins are more closely related to Ad12 than Ad2 (Ishino *et al.*, 1988). Moreover Ad40 and Ad12 display similar DNA replication profiles with onset occurring between 20-30h p.i. (Brieding *et al.*, 1988; Mautner *et al.*, 1990) compared with 10-12h for Ad5.

The 55K protein is not essential for DNA replication in Ad5 (Babiss and Ginsberg, 1984), but it has been found to be necessary for Ad12 DNA replication

(Shiroki *et al.*, 1986; Brieding *et al.*, 1988; Zhang *et al.*, 1992b). It appears that the ability of Ad40 to replicate in the permissive cell line may correlate with the expression of 55K at early times, which in turn most likely effects the efficiency of DNA replication. Consistent with this idea is the finding that semi-permissive cells require more than one infectious particle to infect a cell, and it may be that there is a need for a minimum amount of the E1B 55K protein for DNA replication to proceed. Therefore the presence of more genomes may make up for poor E1B gene expression.

5.5. The Ad40 E1B Promoter.

A possible explanation for the lack of Ad40 early E1B mRNA and protein (Bailey *et al.*, 1993, 1994) in semi permissive cells, is that transcription from the E1B promoter is inefficient or absent early in infection. Even in permissive cells Ad40 E1B mRNA transcription is poor compared to Ad5 (Mautner *et al.*, 1990) which may indicate inefficient promoter usage.

The activity of the Ad40 E1B promoter was compared to Ad5 by Bailey *et al.*, (1994). Two recombinant viruses were used for this study and were constructed from Ad5 (dl309) with the Ad40 E1B region in place of the Ad5 equivalent under the control of either the Ad40 (sub40P) or Ad5 (sub5P) promoter. This enabled study of the expression from the Ad40 E1B region in a well characterised adenovirus system. In 293 and KBa+b cells, expression from the Ad40 promoter as measured at the RNA level was respectively 10 and 20 fold lower compared to the Ad5 promoter, in HeLa cells a 80 fold reduction was detected. Results indicated that the Ad40 E1B promoter was less active than the Ad5 E1B promoter. The pattern of E1B mRNA processing was the same for both recombinants with the 14S mRNA as the predominant species. The amount of E1B mRNA was much less in sub40P infected cells and the low expression in HeLa cells paralleled Ad40 E1B expression in these cells. This suggested the activity of the Ad40 E1B promoter was a likely cause for the poor expression of E1B mRNA in HeLa cells (see section 4.5.). Furthermore CAT assays were conducted where the CAT gene replaced the Ad40 E1B coding region in the recombinants sub40P and sub5P (Mautner *et al.*, 1995). Results confirmed previous studies with the Ad40 E1B promoter showing significantly lower activity than the Ad5 E1B promoter. In addition the amount of activity of the E1B promoter was shown to vary depending on the cell type used; with the highest activity in 293, intermediate in INT407 and lowest activity in HeLa cells. This mirrored the levels of Ad40 E1B mRNA and virus yields among these cell types in this study.

What could account for the lower promoter activity?. Differences in E1B gene expression between Ad40 and other serotypes are not obvious from direct comparison of the sequence of promoter elements (see Fig 4.6.1.). In Ad5, the E1B promoter is

transactivated by E1A which acts through the TATA, while the Sp1 binding site is essential for efficient transcription from the E1B promoter but does not affect E1A-mediated transactivation (Wu *et al.*, 1987; Parks *et al.*, 1988). The TATA box sequence is identical to Ad2/5 and various other TATAs (Fig 4.6.1.). The Ad40 E1B promoter was shown to respond to the VZV 140K protein which acts through the TATA box (Steinthorsdottir, 1991) although the response is weaker compared to Ad5 E1B promoter. This indicates that the TATA box sequence is not a likely cause of the low promoter activity. It was not clear if the GC box in the Ad40 E1B promoter was a functional Sp1 binding site. Did weak binding of Sp1 to the site result in lower activity of the promoter. Indeed mutations in the Sp1 binding site in the Ad2 E1B promoter do result in a decrease in activity (Wu *et al.*, 1987).

In section 4.6. it was shown in gel retardation assays that the Ad40 GC box sequence will bind recombinant Sp1 protein (Jackson *et al.*, 1990), but less efficiently than the Ad2 (Ad5) GC box sequence, and that the flanking sequences as well as the GC box core sequence have an influence on Sp1 binding. To look at the differences between permissive and semi permissive cells, INT407 and HeLa extracts were used. In INT407 extracts less protein (Sp1) bound both GC box sequences than from HeLa extracts. The amount of Sp1 in cell extracts was not quantitated, and it is not certain if there are decreased levels of this protein in INT407 cells. Whether this was a contributory factor in the amount of Sp1-DNA complexes observed with INT407 cells is unclear.

Alternatively there may be other transcription factors that complement Sp1 or replace Sp1 in INT407 cells. Sp1 related proteins have been reported (i.e. Sp2, Sp3 & Sp4) (Hagen *et al.*, 1992), the existence of which indicates that Sp1 is a member of a multigene family of transcriptional regulatory proteins. One of these proteins, Sp3, can have an activating or inhibitory role in transcription (Dennig *et al.*, 1996 and references therein). Looking at the Ad40 promoter, does the fact that more Sp1 bind from HeLa extracts actually have a negative regulatory effect. The spacing between the TATA box and Sp1 binding site in Ad40 is reduced compared to Ad2 (Ad5). How this affects binding to TAF110 and thus transcriptional activation is unknown, but a possibility that the decreased spacing may lead to an altered interaction of Sp1 with TAF110 and TBP,

compared with Ad2 cannot be over looked. The spacing of the Sp1 binding site and TATA box is important in the Ad2 E1B promoter (Wu and Berk, 1988a; Segal and Berk, 1991). Increasing the spacing by 30bp is equivalent to the deletion of the Sp1 site, and insertion of a half turn of DNA helix is more detrimental than a full turn. These results indicate that the position and spacing of Sp1 and TBP are important for interaction. In Ad2 a reduction in the spacing between these promoter elements has also been reported to reduce E1B transcription (Personal communication A. Berk). In this respect the reduced spacing in Ad40 may also be important. However it must be noted that the Ad7 E1B promoter has a similar spacing between its promoter elements (see Fig 4.6.1.) and does not appear to have a gross defect in this region.

5.6. Future.

The detailed characterisation of Ad40 has only been possible since the discovery of cell lines such as KBa+b that the virus can grow in efficiently. This has now been extended to include INT407 cells which are not known to have endogenous adenovirus proteins but are permissive for the virus. The work presented in this thesis and by other workers have shown there are still many unanswered questions and several lines of interesting study.

Previously the comparison of infectivity among different serotypes compared to Ad40 could not be looked at easily because Ad40 did not plaque. Most comparison was done by comparing DNA yields. However, the indirect immunofluorescent assay could be extended to compare the infectivity of other adenovirus serotypes in comparison to Ad40 using the cell lines detailed in this study, and thus compare particle to infectivity ratios.

In this study monolayers were used in the examination of viral entry. However spinner cultures could be used to increased the numbers of particles attaching to cells. Indeed initial experiments carried out with spinner HeLa cells increased the amount of attached virus 2 fold. Therefore extending this assay with spinner cultures of INT407 cells would be advantageous in an attempt to increase the amount of virus internalised.

The amount of Ad40 that is internalised and/or attached is similar in both HeLa and INT407 cells and appears to be comparable to dl309. However Ad40 does not contain the RGD motif which has been found to be important for cell entry in many other adenovirus serotypes. The question of whether integrins are involved in Ad40 entry into cells or is an alternate pathway used remains to be answered. Also what role do the two types of fibres present on Ad40 have in attachment and do they have different receptors. Does this relate to the number of Ad40 receptors on INT407 cells, which in turn also needs to be determined. Characterisation of the proteins that bind the Ad40 fibres, could also be explored. Are there differences in the number and types of cellular proteins that bind?.

Studies on E1B gene expression in this thesis and elsewhere would indicate that Ad40 needs the E1B 22S mRNA and hence the 55K protein to be expressed early for efficient growth. The ability of Ad40 to replicate in some cells lines and not others may depend on the extent to which 55K can be expressed at early times. It is predicted that cells in which E1B mRNA can be expressed early would be suitable hosts for replication of Ad40 in tissue culture. This could account for the reported growth of Ad40 in some cell lines not expressing endogenous E1 proteins. Although the 22S mRNA was detected in infected INT407 cells, the presence of 55K has still to be confirmed. The question of whether the Ad40 55K protein is actually functional is another area requiring investigation.

Ad12 55K mutants resemble Ad40 more than Ad5 55K mutants, and it may therefore be useful to compare Ad12 to Ad40 as well as Ad5 in future investigations. An area not addressed in this thesis is that of the presence of cotranscripts-counterparts previously detected in Ad40 infected KBa+b cells. What is the function of these mRNAs and their importance in viral replication?.

Why does less Sp1 bind the Ad40 GC box with INT407 extracts than HeLa? By binding to HeLa is the Sp1 protein acting in an inhibitory capacity by masking a site for TAFs or the general transcription machinery and thus if less binds in INT407 does this lead to increased promoter activity? The actual amount of Sp1 still remains to be determined in INT407 cells. In addition further analysis of the whole Ad40 promoter by DNase I analysis has yet to be carried out.

In this thesis and the literature many different cell lines have been used to characterise Ad40 with varying degrees of success. The permissive cell lines presented in this thesis have been very successful in detecting, propagating and studying Ad40, but yields of virus have never achieved the levels of viral growth seen for other adenovirus subgroups. A cell system which is more like the natural host environment (i.e. an intestinal cell line) would be a better way to explore the growth requirements of Ad40, ideally producing higher levels of the virus for study and comparison with other serotypes.

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