Preliminary characterisation of the adenovirus type 40 E1A region

by

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Summary

The enteric adenoviruses were first identified from stool samples of infants with acute gastroenteritis (Flewett *et al.*, 1973). Although enteric adenovirus particles were shed in large numbers from the gut, they were unable to be cultivated on conventional cell lines used to propagate other adenoviruses (Flewett *et al.*, 1973; Madeley *et al.*, 1977; Retter *et al.*, 1979). However, they would grow in 293 cells, a human embryo kidney (HEK) cell line which had been transformed with the Ad5 E1 region (Graham *et al.*, 1977; Takiff *et al.*, 1981), albeit at reduced levels compared to other serotypes. This observation suggested that functions encoded in the E1 region were poorly expressed or of intrinsically lower activity in certain cell types, thereby implicating the E1 region in the restrictive growth properties of the enteric adenoviruses. Previous work had demonstrated that the Ad40 E1B region played a significant role in this phenotype (Mautner *et al.*, 1989; Steinthorsdottir, 1991; Gomes *et al.*, 1992). However, the Ad40 E1A region had also been implicated due to a delay in the onset of Ad40 E1A mRNA expression when compared to Ad5 E1A mRNA expression (Ullah, 1997), and the observation that the Ad40 E1A proteins appeared to be weaker *trans*-activators when compared to the Ad5 E1A proteins (van Loon *et al.*, 1987b; Ishino *et al.*, 1988).

Therefore to investigate the involvement of the E1A region in the restricted growth of Ad40, a preliminary characterisation of sequences which were important for basal and *trans*-activated transcription was undertaken. Comparison of the intact Ad40 E1A promoter with the intact Ad5 E1A promoter in transient transfection assays, revealed that basal transcription from the Ad40 E1A promoter was lowered by approximately 6 fold when compared to basal transcription from the Ad5 E1A promoter. To identify sequences important for basal transcription within the Ad40 E1A promoter, a series of promoter deletions were constructed using *Bal* 31 nuclease digestion, revealing a region between -349 to –140, relative to the cap site at +1, to be important for basal transcription from the Ad40 E1A promoter. Comparison of the Ad40 E1A promoter with the transcription factor database, held at EMBL (Ghosh, 1990), revealed that this region contained a number of possible transcription factor binding sites similarly arranged to the Ad5 E1A promoter. To

map sequences within the region –349 to –140, a series of deletions were constructed which deleted transcription factor binding sites known to be important for basal transcription from the Ad5 E1A promoter. The deletions were characterised in parallel with the intact and *Bal* 31 deletion mutants of the Ad40 E1A promoter by transient transfection assays, which revealed that the Ad40 E1A transcriptional control region contains an enhancer element located between –328 to –235 relative to the Ad40 E1A cap site at +1 similar to that in the Ad5 E1A promoter (Hearing and Shenk, 1983a, 1986; Bruder and Hearing, 1989, 1991).

To investigate whether the Ad40 E1A 249R (equivalent to the Ad5 E1A 13S mRNA which encodes a 289R protein) and 221R (equivalent to the Ad5 E1A 12S mRNA which encodes a 243R protein) proteins were involved in the aberrant expression of the Ad40 E1A region, a cDNA equivalent to the Ad5 E1A 13S mRNA was generated by RT-PCR of Ad40 infected cell extracts, then cloned into a CMV expression plasmid. An Ad40 equivalent to the Ad5 E1A 12S mRNA was not generated by RT-PCR of Ad40 infected cell extracts However,a library of Ad40 E1A specific cDNAs were generated, and four novel Ad40 E1A specific cDNAs were characterised. Comparison of *trans*-activated transcription from the Ad40 E1A promoter by the Ad40 E1A 249R protein and the Ad5 E1A 289R protein, revealed that *trans*-activated transcription from the Ad40 E1A promoter was approximately 6 fold lower in the presence of the Ad40 E1A 249R protein. To map sequences which were important for *trans*-activated transcription from the Ad40 E1A promoter, cells were co-transfected with either the intact or deleted Ad40 E1A promoter and the Ad5 E1A 289R protein or the Ad40 E1A 249R E1A protein, revealing a region between –328 to –235, relative to the Ad40 E1A cap site at +1, to be important for Ad5 E1A 289R activated transcription from the Ad40 E1A promoter. Ad40 E1A 249R activated expression for both the Ad5 and Ad40 E1A promoters was not discussed, due to an equipment (incubator) problem which rendered the experimental data unreliable. Comparison of the ratios of Ad5 E1A 289R activated expression to basal expression within the Ad5 and Ad40 promoter constructs indicated that no single sequence element could be identified which was uniquely important for *trans*-activated expression. Rather those elements which affected the level of basal expression seemed to have an effect on the level of *trans*-activated

expression observed in the presence of the Ad5 E1A 289R protein. Thus the Ad5 E1A 289R protein probably activates gene expression through the basal transcription apparatus, as demonstrated by the Ad5 E1A promoter.

To investigate the number and size of Ad40 E1A proteins, and the interactions of the Ad40 E1A proteins with other cellular and viral proteins during an Ad40 infection *in vitro* and *in vivo*, antisera were generated to conserved region (CR) 3 and the C-terminus of the Ad40 E1A 249R protein. The antibodies to CR3 and the C-terminus of the Ad40 E1A 249R protein reacted with peptide in ELISA but did not efficiently detect Ad40 E1A products in western blots of infected cell extracts or cell extracts overexpressing the Ad40 E1A 249R protein, or in immunoprecipitations of Ad40 infected cells.

In summary, the work in this thesis suggests that in addition to the previously observed defects in the function of the Ad40 E1B region, the failure of Ad40 to propagate in cell lines used to propagate other adenoviruses can in part be attributed to: (i) the intrinsically lower activity of the Ad40 E1A promoter; (ii) aberrant splicing of E1A mRNAs; (iii) intrinsically lower *trans*-activator function of the Ad40 E1A 249R protein. These observations indicate that the Ad40 E1A region is of intrinsically lower activity in tissue culture than the Ad5 E1A region, which would considerably affect progression of Ad40 into the late phase of the infectious cycle.

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Abbreviations

A	adenine
aa	amino acid
ABTS	2,2-Azino-di-[3-ethylbenzthiozoline sulphonate (6)]
Ad	adenovirus
ADP	adenovirus death protein
Adpol	adenovirus DNA polymerase
APS	ammonium persulphate
AR	auxiliary region
ara-C	cytosine arabinoside
ATP	adenosine-5′-triphosphate
bp	base pair
BSA	bovine serum albumin
bZIP	leucine zipper
C	cytosine
C-terminal	carboxy-terminal
cAMP	cyclic adenosine-5′-monophosphate
$CaPO_4$	calcium phosphate
CAR	coxsackie adenovirus receptor
CAT	chloramphenicol acetyl transferase
CBC	cap binding complex
CBP	CREB binding protein
cdk	cyclin-dependent kinases
cDNA	complementary DNA
C/H2	Cys/His-rich region
Ci	Curie
CMV	cytomeglovirus
cpe	cytopathic effect
cPLA2	cytosolic phospholipase A2
cpm	counts per minute
CPSF	cleavage and polyadenylation specificity factor
CR	conserved region
CRE	cAMP-responsive element
CStF	cleavage stimulatory factor
CtBP	C-terminal binding protein
CtIP	C-terminal interacting protein
CTL	cytotoxic T-lymphocyte
δ	standard deviation
Da	Dalton
DBP	DNA binding protein
dCMP	2′-deoxycytidine-5′-monophosphate
ddNTP	dideoxyribonucleoside-5′-triphosphate
DE	downstream element
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2′-deoxyribonucleoside-5′-triphosphate
DPE	downstream promoter element

DTT	dithiothreitol
DW	distilled water
E	early region
E. coli	*Escherichia coli*
EDTA	sodium ethylenediamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
EP	glutamic acid-proline repeats
ER	endoplasmic reticulum
FCS	foetal calf serum
ffu	fluorescent focus unit
FIP	14.7 kDa-interacting protein
FITC	fluorescein isothiocyanate conjugate
FLL	firefly luciferase luminescence
FLLAB	firefly luciferase luminescence assay background
FLL:RLL	ratio of firefly luciferase luminescence to *Renilla* luciferase luminescence
g	gram
G	guanine
GON	group of nine hexons
gp19K	19 kilo-Dalton glycoprotein
h	hour
HAT	histone acetyl-transferase
HDAC1	histone deacetylase 1
HEK	human embryo kidney
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethane-sulphonic acid
hnRNP	heterogeneous ribonucleoprotein particles
HSV	herpes simplex virus
IE	immediate early
i	leader sequence
ITR	inverted terminal repeat
k	kilo
kbp	kilo base pair
kDa	kilo Dalton
l	leftward transcription
l	litre
L	late region
luc	firefly luciferase
luc+	modified firefly luciferase
m	milli
M	molar
μ	mean
μ	micro
mAb	monoclonal antibody
MCS	multiple cloning site
MHC	major histocompatibility complex
mi	mock infected
min	minute(s)
MLP	major late promoter
M-MuLV	Moloney murine leukaemia virus
moi	multiplicity of infection

mRNA	messenger ribonucleic acid
m.u.	map unit
n	number
nm	nanometre
N-terminal	amino-terminal
NCS	newborn calf serum
ND10	nuclear domain 10
NF	nuclear factor
NFAT	nuclear factor of activated T cells
NLS	nuclear localisation signal
NPC	nucleoprotein complex
nt	nucleotide
OD	optical density
ORF	open reading frame
p	precursor
^{32}P	phosphorus-32 radioisotope
p23	cysteine protease
p300	300kDa cellular protein
p/CAF	p300/CBP associated factors
p/CIP	p300/CBP interacting protein
PCR	polymerase chain reaction
pfu	plaque forming unit(s)
p.i.	post-infection
PIC	pre-initiation complex
PKA	protein kinase A
poly A	polyadenylic acid
pp	polypeptide
PP2A	protein phosphatase 2A
pTP	precursor terminal protein
r	rightward transcription
R	residue
Rb	retinoblastoma gene product
RLL	*Renilla* luciferase luminescence
RLLAB	*Renilla* luciferase luminescence assay background
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription – polymerase chain reaction
S	sedimentation coefficient unit
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	seconds
snRNP	small nuclear ribonucleoprotein particles
ss DNA	salmon sperm DNA
SV40	simian virus 40
T	thymine
TAF	TBP-associated factor(s)
TAP	transporter associated with antigen presentation

TBP	TATA-box binding protein
TEMED	tetra-methyl-ethylene-diamine
TLC	thin layer chromatography
TLU	transmitted light units
TNF	tumour necrosis factor
TNFR1	TNF receptor 1
TP	terminal protein
TRAIL R1	TNF-related apoptosis-inducing ligand R1
TRAM	transcriptional adaptor motif
u	unit
U	uridine
USF	upstream stimulating factor
UV	ultra violet
V	volts
VA	virus-associated
vol	volume
VZV	varicella-zoster virus

1. INTRODUCTION

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1.1 The Adenoviridae

1.1.1 Classification

The adenovirus family is divided into two genera, mastadenoviridae and aviadenoviridae. The mastadenoviridae genus includes human, simian, bovine, equine, porcine, ovine and canine viruses. Rowe *et al* (1953) first discovered human adenoviruses in explants of adenoid tissue. To date there have been 49 serotypes of human adenoviruses identified, which are classified into six subgroups (A-F) based upon their immunological properties, oncogenicity in rodents, DNA homologies and morphological properties (table 1.1.1) (Hierholzer *et al.*, 1991). Two further candidate serotypes 50 and 51 have been identified in immunocompromised patients (de Jong *et al.* 1999). The human adenoviruses are pathogens associated with a number of infectious diseases including respiratory, ocular, urinary and gastrointestinal. Adenoviruses are widely used as model systems in molecular biology; their use as important biological tools particularly as vectors in gene therapy has been extensively reported (reviewed in Haddada *et al.*, 1995; Strayer, 1998). In the following sections it will become apparent that much of our current understanding of the human adenoviruses comes from extensive studies with adenovirus (Ad) type 2 and the closely related serotype Ad5.

1.1.2 Morphology

The adenovirus particle is approximately 90 nanometers (nm) in diameter, and consists of a nonenveloped, icosahedral particle (Horne *et al.*, 1959), within which is a linear double strand of deoxyribonucleic acid (DNA) (approximately 20-30x10^6 Dalton (Da)) and at least eleven viral encoded polypeptides, which have been localised (figure 1.1.2) (Burnett, 1997). The capsid consists of 252 capsomers (Horne *et al.*, 1959), 240 of which are hexon capsomers (virion protein II) located on the facets, the other 12 are penton base capsomers (virion protein III) located at the vertices, arranged in a ring-like configuration with a central cavity (Ginsberg *et al.*, 1966). Inserted into each cavity is a fibre protein, which is a trimer of virion protein IV.

Table 1.1.1 Properties of human adenovirus serotypes of subgroups A-F (modified from Wadell *et al*, 1994).

Sub-group	Serotype	Intra-subgroup	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, 50	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- 49, 51	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

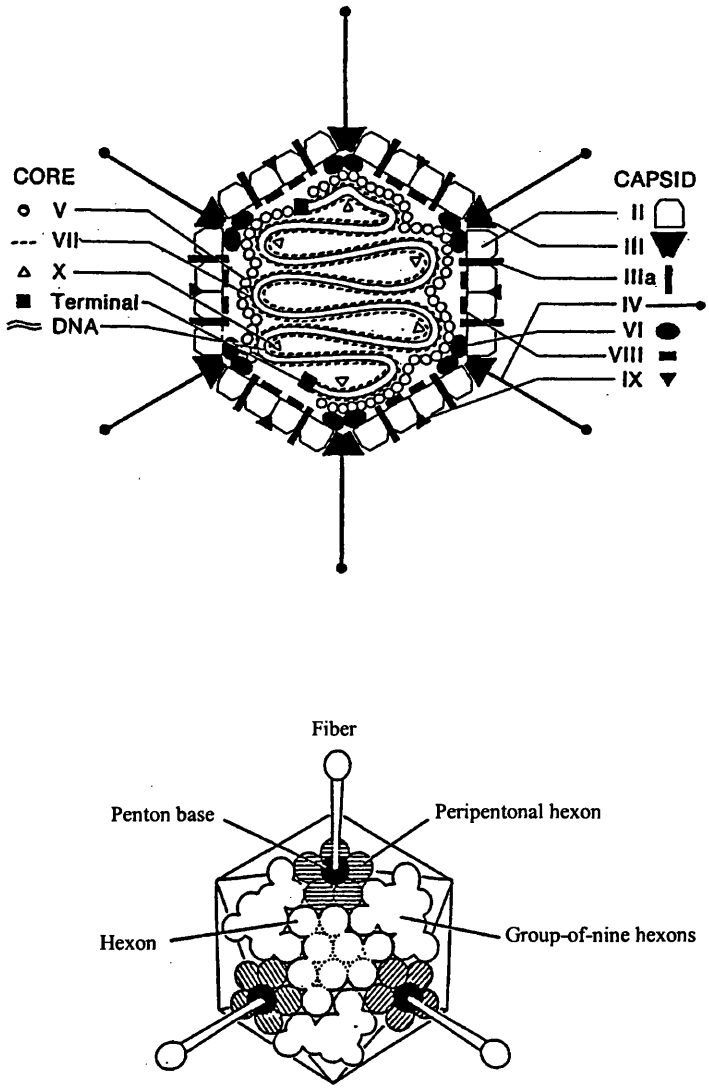


Figure 1.1.2 Structure of the adenovirus particle. **A.** A schematic view of the Adenovirus particle. A schematic view of the adenovirus particle based on current understanding of its polypeptide components and viral DNA. No real section of the icosohedral virion would contain all these components. (reproduced from Stewart and Burnett, 1995). **B.** A diagram of the Icosohedral 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).

Hexon is the major structural component of the virus capsid and consists of a trimer of three identical polypeptide chains of protein II, with a hexagonal base facing the inside and a pyramidal top to the outside of the capsid (Grütter and Franklin, 1974; Roberts, M.M. *et al.*, 1986; Akusjärvi *et al.*, 1984; Athappilly *et al.*, 1994). Hexons are found as either groups of nine hexons (GONs) which make up the facets, or as peripentonal hexons which link the facets i.e. they link the GONs and the pentons. Adjacent facets are linked by protein IIIa spanning the capsid. Polypeptide IX stabilises GONs, whilst protein VI anchors the ring of peripentonal hexons by bridging with the DNA core (van Oostrum and Burnett, 1985; Furcinitti *et al.*, 1989; Stewart *et al.*, 1993; Mathews and Russell, 1995).

The penton capsomer consists of a penton base made up of five subunits, which are arranged in a ring-like structure with a central cavity (van Oostrum and Burnett, 1985; Furcinitti *et al.*, 1989; Stewart *et al.*, 1993). Inserted into the cavity is the trimeric fibre, which consists of three 62 kiloDalton (kDa) subunits (Devaux *et al.*, 1990; Ruigrok *et al.*, 1990; Stouten *et al.*, 1992). The fibre protein consists of an N-terminal region which interacts with the penton base, a shaft region and a C-terminal globular head responsible for binding to the cell surface receptor designated coxsackie and adenovirus receptor (CAR), which binds all adenovirus serotypes except those belonging to subgroup B (Ruigrok *et al.*, 1990; Bergelson *et al.*, 1997; Roelvink *et al.*, 1998). Most human adenoviruses have one fibre gene, although Ad40 and Ad41 have two (Pieniazek *et al.*, 1990). The other minor proteins VIII, X, XI and XII have not been localised within the capsid, however it is likely that they are contained within the capsid.

The DNA core of the virion is condensed with proteins V, VII and μ (a minor component); it is also covalently linked to terminal protein (TP) at each 5′ end of the genome (reviewed in Shenk, 1996). The organisation of the DNA within the capsid is not well understood however, two models have been suggested. The first model suggested the DNA is organised into 12 globular domains (Brown *et al.*, 1975; Newcombe *et al.*, 1984) which fit into the 12 vertices of the icosahedral capsid of the virus. The second model involves winding of the DNA around protein VII in the form of nucleosomes (Corden *et al.*, 1976; Nermut, 1980; Vayda *et al.*, 1983; Chatterjee *et*

al., 1986). Also inside the adenovirus capsid are 10 to 50 copies of the cysteine protease (p23) (Anderson, 1990; Mathews and Russell, 1995; Mangel *et al.*, 1997).

1.1.3 Genome structure

The complete nucleotide sequences for human adenoviruses type 2 (Roberts et al., 1986), type 5 (Chroboczek et al., 1992), type 40 (Davison et al., 1993), type 12 (Sprengel et al., 1994) and type 17 (Zabner et al., 1999) are now available. The organisation of the Ad2 genome is shown in figure 1.1.3. It consists of a linear double stranded DNA molecule (approximately 35 kilo-base pairs (kbp) in size) which is divided into 100 map units (m.u.), and the two strands are denoted r and l for rightward and leftward transcription.

The deoxycytidine at the 5′ end of each strand of the genome is covalently linked via a serine phosphoryl bond to a 55kDa TP (Robinson *et al.*, 1973; Rekosh *et al.*, 1977; Desideiro and Kelly, 1981). The TP is generated late in infection from an 80kDa precursor terminal protein (pTP) (Webster *et al.*, 1993). The ends of the genome contain inverted terminal repeats (ITR) which vary in length (102 to 165bp) depending upon the serotype (Wolfson and Dressler, 1972; Shinagawa and Padmanabhan, 1980; Ishino *et al.*, 1987). The ITRs contain a number of *cis*-acting elements, which are involved in the initiation of viral DNA replication (Tamanoi and Stillman, 1983), as well as a number of *trans*-acting elements capable of acting as upstream enhancers (Yoshida *et al.*, 1995). Several regions within the ITRs are conserved in all serotypes, for example the minimal origin of DNA replication, which is located in the terminal 18bp and contains a 10bp region which is highly conserved amongst all adenoviruses (Stillman *et al.*, 1982; Tamanoi and Stillman, 1983; van Bergen *et al.*, 1983; Challberg and Rawlins, 1984; Lally *et al.*, 1984; Wides *et al.*, 1987; Harris and Hay, 1988). The minimal origin binds a heterodimer of virion proteins, pTP and adenovirus DNA polymerase (Adpol) (Miralles *et al.*, 1989; Chen *et al.*, 1990; Temperley and Hay, 1992). The minimal origin is separated from two host cell protein binding sites by a defined spacer region (Wides *et al.*, 1987); transcription factors which bind to the aforementioned sites stimulate the initiation of replication by different mechanisms (reviewed by Hay *et al.*, 1995; van der Vliet, 1995; de Jong and van der Vliet, 1999).

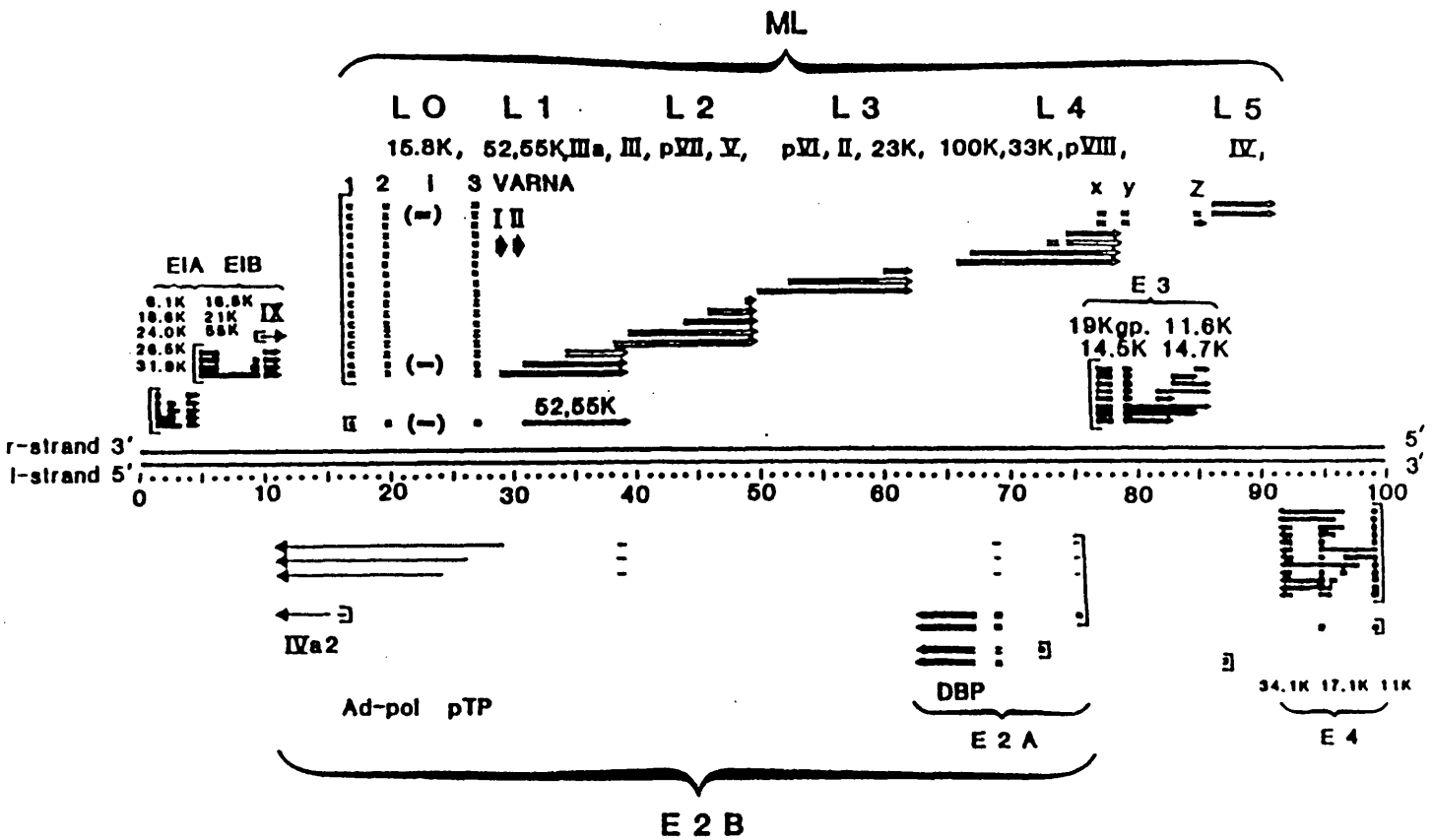


Figure 1.1.3 Organisation of the Ad2 genome. The parallel lines indicate the linear duplex DNA genome, which is divided into 100 map units (m.u.). Rightward and leftward transcription is indicated by r and l, respectively. Vertical brackets indicate position of promoter sites and the arrow heads show 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 Waddell, 1990).

1.1.4 The lytic cycle

1.1.4.1 Viral entry into cells

The adenoviruses enter their host cell via receptor mediated endocytosis (reviewed in Greber, 1998; Nemerow and Stewart, 1999). Initially the adenovirus attaches to the cellular receptor via the fibre protein (Philipson *et al.*, 1968; Henry *et al.*, 1994; Louis *et al.*, 1994). A common receptor, CAR, was reported for coxsackie B viruses and adenoviruses (Bergelson *et al.*, 1997; Tomko *et al.*, 1997) and shown to be a cellular receptor for subgroups A, C, D, E and F (Roelvink *et al.*, 1998). An alternative receptor has also been identified as MHC class I (Hong *et al.*, 1997). Entry is facilitated by the RGD sequence (Stewart *et al.*, 1997) within the penton base binding with $\alpha_v\beta_{3/5}$ integrins (Wickham *et al.*, 1993; Bai *et al.*, 1994), allowing viral endocytosis into clathrin coated pits (Svensson, 1985; Varga *et al.*, 1991; Bai *et al.*, 1993; for detailed review see Nemerow and Stewart, 1999), at which point the fibre protein dissociates (Greber *et al.*, 1993).

The virus particles then penetrate into the cytoplasm via endosomes; the endosomes are eventually lysed releasing viruses into the cytosol (Dales, 1978; Pastan *et al.*, 1986). Lysis of the endosomes is thought to be catalysed by a viral factor which is activated by low pH (Pastan *et al.*, 1986; Wohlfart, 1988; Varga *et al.*, 1990). Stepwise uncoating of the viral particle continues through the cytoplasm (Greber *et al.*, 1993) until the virion reaches the nucleus. Release and dissociation of the viral DNA requires the cysteine protease p23, which is activated by a redox-dependent conformational change, enabling degradation of protein VI thus weakening the cytosolic capsid (Greber *et al.*, 1996; Mangel *et al.*, 1997). Entry of viral DNA and the associated proteins V, VII, TP and μ into the nucleus, requires interaction between the weakened cytosolic capsid and the nuclear pore complexes (NPC) (Greber *et al.*, 1997). The TP p55 is covalently attached to the viral DNA and contains a nuclear localisation signal (NLS), which may function in threading the viral DNA through the NPC (Zhao and Padmanabhan, 1988; Schaack *et al.*, 1990). The viral DNA is then tethered via the TP to the nuclear subregion characterised by the nuclear domain (ND10) antigens (Ishov and Maul, 1996).

1.1.4.2 Transcription

The adenovirus genes are expressed in two distinct phases termed early (E) (E1A, E1B, E2A, E3, E4 and L1) and late (L) (E2B, L1, L2, L3, L4 and L5) separated by the onset of DNA replication. However, several other genes are expressed at intermediate times (ppIX, IVa2, VA1 and VA2 RNA). VA RNAs are transcribed by cellular RNA polymerase III whilst the other adenovirus genes are transcribed by cellular RNA polymerase II (Price and Penman, 1972; Weinmann *et al.*, 1976).

Early Transcription

Early region 1A

The early region 1A (E1A) transcription unit is situated on the r strand between 1.5-4.5 m.u. (figure 1.1.3) (Jones and Shenk, 1979). A common nuclear precursor RNA or primary transcript is differentially spliced to produce five messenger ribonucleic acids (mRNA) known as 13S, 12S, 11S, 10S and 9S mRNA; each mRNA differs in size as a result of the excised intron size, which is determined by different splice donor sites linking to a common splice acceptor site (figure 1.1.4.2). The 13S, 12S, 11S, 10S and 9S mRNAs share common 5′ and 3′ ends (Berk and Sharp, 1978; Chow *et al.*, 1979; Stephen and Harlow, 1989; Ulfendahl *et al.*, 1987). The 13S and 12S mRNAs are predominant in infection producing two proteins of 289 residues (R) and 243R respectively. These proteins differ in an internal 46 amino acid (aa) region that is present only in the 289R protein (Perricaudet *et al.*, 1979). The 289R protein is primarily responsible for *trans*-activation of the E1A region and the other early regions (Jones and Shenk, 1979; Ricciardi *et al.*, 1981; Montell *et al.*, 1982; reviewed by Berk, 1986). The 11S and 10S mRNAs are present late in infection, but are only minor species of mRNA (Stephens and Harlow, 1987). The 9S mRNA is predominant late in infection (Spector *et al.*, 1978; Chow *et al*, 1979). An in depth review of the E1A region is undertaken in section 1.2.

Early region 1B (E1B)

The early region 1B (E1B) transcription unit lies downstream of the E1A region between 4.6-11 m.u.. Transcription from the E1B promoter is maximised by a *cis*-dominant property of the early viral template, which does not allow transcription to

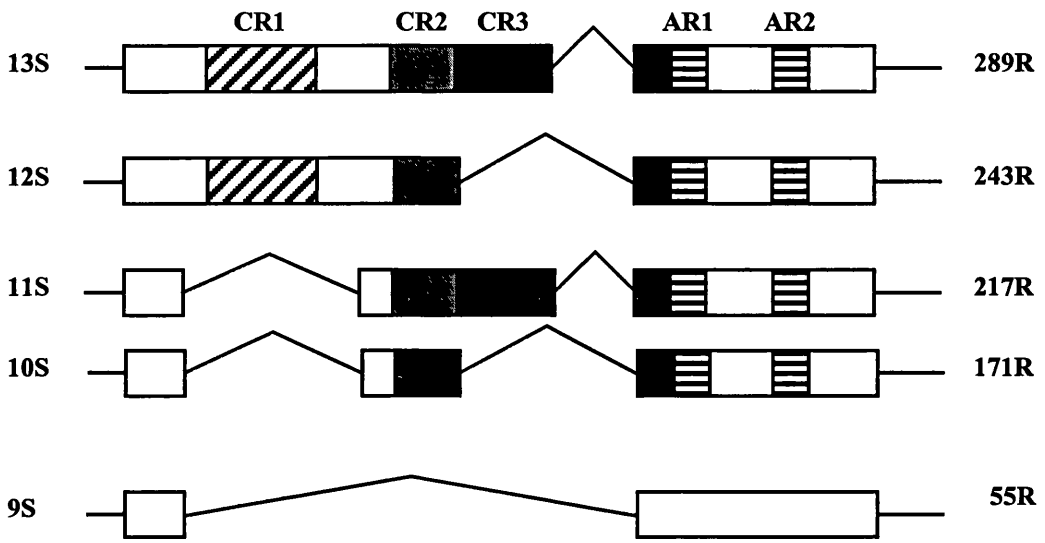


Figure 1.1.4.2 The Ad2 E1A mRNAs and protein products. The mRNA is denoted by black lines; introns are denoted by caret symbols; open reading frames are denoted with boxes; conserved regions 1, 2 and 3 denoted by CR1, CR2 and CR3; and auxiliary regions 1 and 2 denoted by AR1 and AR2. The size of the mRNA is denoted on the left-hand side and protein size indicated on the right-hand side (modified from Jones, 1992).

terminate between the E1A gene and the E1B gene, instead readthrough transcription occurs offering maximum early E1B gene expression (Maxfield and Spector, 1997). A common precursor mRNA is then differentially spliced to produce a 22S, 14.5S, 14S, 13S and 9S mRNA which share common 5′ and 3′ termini (Berk and Sharp, 1978; Pettersson *et al.*, 1983). The 22S mRNA is predominant early in infection and encodes a 19kDa protein using the first AUG and a 55kDa protein using the second AUG (Halbert *et al.*, 1979; Bos *et al.*, 1981; Glenn and Ricciardi, 1988). The 19kDa proteins function is to suppress p53-dependent and p53-independent induced apoptosis during adenovirus infection; correct localisation of the 19kDa protein is essential for this function, and the interaction of the 19kDa protein with lamin A/C may represent a means for nuclear envelope localisation (Rao *et al.*, 1997). The 19kDa protein is also capable of inhibition of tumour necrosis factor (TNF) cytolysis (Gooding *et al.*, 1991). The 55kDa protein is a multifunctional phosphoprotein, which is required for complete oncogenic transformation of rodent cells, viral DNA replication and late viral RNA translation (Harada and Berk, 1999). The 14.5S and 14S mRNAs are minor species which are similar to the 13S mRNA, but have an additional third exon and encode for the 55kDa and 19kDa proteins (Anderson *et al.*, 1984; Virtanen and Pettersson, 1985). The 13S mRNA however, is predominant at intermediate times after infection and also encodes the 19kDa protein (Halbert *et al.*, 1979; Bos *et al.*, 1981). The 9S mRNA is transcribed from a separate promoter within the E1B region at intermediate times after infection and encodes the structural ppIX which is associated with the hexon-hexon interfaces of GONs within the capsid (Furcinitti *et al.*, 1989; Stewart *et al.*, 1993).

Early region 2 (E2)

The early region 2 (E2) transcription unit is situated on the l strand and lies between 75.4-11.3 m.u. (Chow *et al.*, 1979). It differs from other adenovirus transcription units by having two alternative promoter sites, E2A and E2B, which are used at different times in infection (Chow *et al.*, 1979). The E2A promoter is activated by the E1A and E4 ORF6/7 gene products (reviewed in Swaminathan and Thimmapaya, 1995), whilst the E2B promoter is repressed by the E1A gene products (Rossini, 1983). Early in infection E2A transcripts are expressed from a promoter at 75.4 m.u., then terminate using the polyadenylation (poly A) site at 62.4 m.u.; following the early to late phase transition E2B transcripts are expressed from a promoter at 72.2 m.u., then terminate

using the poly A site at 11.3 m.u. (Nevins *et al.*, 1979; Stillman *et al.*, 1981; Glenn and Ricciardi, 1988). The E2A primary transcript is differentially spliced to produce two mRNAs which vary in length by approximately 100 nucleotides (nt) (Berk and Sharp, 1978); both mRNAs encode a 72kDa single stranded DNA binding protein (DBP) (Lewis *et al.*, 1976). DBP is essential for viral DNA replication (section 1.1.4.3) (reviewed in de Jong and van der Vliet, 1999), 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 enhances transcription from the E1A promoter, the E2A promoter and the major late promoter (MLP) (Morin *et al.*, 1989; Chang and Shenk, 1990; Zijderveld *et al.*, 1994; Swaminathan and Thimmapaya, 1995). The E2B primary transcript is differentially spliced to produce three mRNAs (Stillman *et al.*, 1981; Shu *et al.*, 1988), two of which encode an 80kDa pTP and a 140kDa adenovirus DNA polymerase (Adpol). pTP acts as a primer for DNA replication (Smart and Stillman, 1982; Salas, 1991) and is cleaved by p23 late in infection to produce the 55kDa TP (reviewed Challberg and Kelly, 1989; Hay *et al.*, 1995). Adpol functions in the initiation and elongation of viral DNA replication (Stillman *et al.*, 1982; Hay *et al.*, 1995; van der Vliet, 1995).

Early region 3 (E3)

The early region 3 (E3) transcription unit is situated on the r-strand between 76.8-85.9 m.u.. The E3 primary transcript is polyadenylated at one of two poly A sites (E3A or E3B), producing two families of transcripts which differ in their poly A site (Berk and Sharp, 1977, 1978; Chow *et al.*, 1979; Cladaras and Wold, 1985; Cladaras *et al.*, 1985; Wold *et al.*, 1995). The subgroup C adenovirus E3 region is predicted to encode nine proteins (reviewed Wold *et al.*, 1995; Mahr and Gooding, 1999; Wold *et al.*, 1999) of which five have been assigned functions. The E3B proteins interfere with either the host cell functions or the immune response during an adenovirus infection by a number of different routes. The E3 19kDa glycoprotein (gp19K) is an abundant transmembrane protein which binds to certain alleles of the class I major histocompatibility complex (MHC), retaining them within the endoplasmic reticulum (ER) through an ER retention signal (reviewed in Sparer and Gooding, 1998). This has been shown to reduce cytotoxic T-lymphocyte (CTL) recognition of adenovirus-infected cells *in vitro*, however it is unclear if this is the case *in vivo* (reviewed in Ploegh, 1998; Mahr and

Gooding, 1999). The Ad12 genome does not encode gp19K, however it is still able to repress transcription of the class I MHC genes through the E1A gene products. Ad12 can also mediate transcriptional repression of genes involved in antigen presentation, namely the transporter associated with antigen presentation (TAP) genes and MHC-encoded proteasome components (reviewed in Blair and Hall, 1998).

The E3 RIDα/β (heteromeric complex of E3 10.4kDa and 14.5kDa proteins) and 14.7kDa proteins protect adenovirus-infected cells from cytolysis by tumor necrosis factor (TNF). Adenovirus infected cells are sensitised to TNF cytolysis by the E1A proteins through the TNF receptor 1 (TNFR1), Fas and the TNF-related apoptosis-inducing ligand- (TRAIL) R1, (reviewed in Mahr and Gooding, 1999; Wold *et al.*, 1999). The 14.7kDa protein is a nonmembrane protein located in the cytosol and the nucleus of infected cells. TNF cytolysis is circumvented by the 14.7kDa protein binding with cellular proteins such as the 14.7kDa-interacting protein (FIP) -3, FIP-2 and FIP-1 (reviewed in Wold *et al.*, 1999). The RIDα/β proteins are type I integral membrane proteins, located in the plasma membrane, which act as a complex consisting of one RIDβ protein and two RIDα proteins. The RIDα/β protein complex is able to protect cells against TNF-mediated cytolysis by preventing activation of cytosolic phospholipase A2 (cPLA2) by TNF; or Fas-mediated cytolysis by downregulation of Fas at the cell surface (reviewed in Mahr and Gooding, 1999; Wold *et al.*, 1999). The RIDα/β complex also mediates TNF cytolysis by downregulation of E1A gene expression at the level of E1A mRNA translation (Zhang *et al.*, 1994). Other E3 proteins include the 11.6kDa protein, also known as the adenovirus death protein (ADP), a glycoprotein located primarily at the nuclear membrane, which functions to program efficient cell death, that is lysis of infected cells (Scaria *et al.*, 1992; Tollefson *et al.*, 1996). The functions of the 12.5kDa and 6.7kDa proteins are still unknown (reviewed in Wold *et al.*, 1999).

Early region 4 (E4)

The early region 4 (E4) transcription unit lies on the l-stand between 91.3-99.1 m.u. (figure 1.1.3). The E4 promoter is activated by E1A at early times after infection, resulting in an early peak of E4 transcription, followed by a decrease in E4 transcription at intermediate times after infection as a result of E4 promoter inhibition by DBP

(Nevins and Winkler, 1980; Handa *et al.*, 1983; Glenn and Ricciardi, 1988). The E4 primary transcript can produce up to 24 mRNAs by alternative splicing, these are temporally regulated during infection and can produce at least ten proteins (Freyer *et al.*, 1984; Tigges and Raskas, 1984; Virtanen *et al.*, 1984; Ross and Ziff, 1992; Dix and Leppard, 1993). Five of the aforementioned proteins are produced from open reading frames (ORF) colinear with the viral DNA, denoted ORF 1, 2, 3, 4 and 6; and two proteins are created by alternative splicing of the primary transcript, denoted ORF 3/4 and 6/7 (Freyer *et al.*, 1984; Virtanen *et al.*, 1984). Expression of ORF1 has not been demonstrated in most adenovirus infected cells, however it is expressed in Ad9 infected cells, where it cooperates with E1A and E1B to produce mammary tumours in rats (Javier, 1994; reviewed in Leppard, 1997). The ORF2 protein is a soluble cytoplasmic component, which has not been found in association with any other infected cell components (Dix and Leppard, 1995). It is also unclear whether the ORF3/4 protein is expressed, as it has not been detected in Ad5 infected cells (Dix and Leppard, 1993). ORF4 produces a 14kDa protein, which regulates transcription from the E1A and the E4 promoters, by protein dephosphorylation of E1A and c-fos by protein phosphatase (PP) 2A (Bondesson *et al.*, 1996; Whalen *et al.*, 1997; reviewed in Leppard, 1997). It may also induce p53-independent apoptosis in a PP2A-dependent manner (Shtrichman and Kleinberger, 1998). The proteins from ORF3 (11kDa) and ORF6 (34kDa) increase late protein production at the level of mRNA accumulation. Both ORF3 and ORF6 stabilise unprocessed late viral mRNA within the nucleus of the cell, thereby increasing the pool of RNA available for maturation. ORF6 can also cooperate with the E1B 55kDa protein to increase nuclear export of viral mRNA and prevent nuclear export of cellular mRNA. ORF6 contains a nuclear export signal (NES), which is responsible for nucleocytoplasmic shuttling by ORF6 and E1B 55kDa (Weigel and Dobbelstein, 2000). The association of ORF6 and E1B 55kDa results in viral inclusion bodies within the nucleus, which are essential for late viral transcription and RNA processing. On the other hand ORF3 affects distribution of structures essential for transcription and replication, known as ND10 or PODs (Leppard and Everett, 1999). Finally ORF6 has been shown to inactivate p53-dependent apoptosis and cooperate with E1A to transform primary rodent cells (Nevels *et al.*, 1999). ORF6/7 encodes a 19.5kDa protein, which is located in the nucleus of infected cells (Cutt *et al.*, 1987). Two molecules of ORF6/7 stabilise cooperative binding of E2F on the E2F dimeric binding site, within the E2A

promoter (reviewed in Swaminathan and Thimmapaya, 1995; all the E4 proteins are reviewed in detail in Leppard, 1997).

Intermediate Transcription

The transcription unit encoding polypeptide (pp) IX lies within the E1B region (Wilson *et al.*, 1979), and is expressed at intermediate times during infection (Spector *et al.*, 1978). Maximum transcriptional activity from the ppIX transcription unit requires viral DNA replication (Vales and Darnell, 1989), producing an mRNA which is unspliced and 3′ coterminal with the E1B transcripts (Alestrom *et al.*, 1980).

The virus-associated (VA) transcription unit is situated on the r-strand at 30 m.u. and is transcribed by host RNA polymerase III (Weinman *et al.*, 1976; reviewed in Mathews and Shenk, 1991; Mathews, 1995). Most human adenoviruses have two VA genes (VAI and VAII), however adenoviruses in subgroups A, F and some of B have only one gene (Ma and Mathews, 1993; Kidd and Tiemessen, 1993; Ma and Mathews, 1996). The efficiency of late adenoviral protein synthesis is mediated by VAI RNA, which is responsible for repression of the cellular kinase PKR (also known as DAI), allowing viral protein synthesis to proceed (Thimmappaya *et al.*, 1982; Kitajewski *et al.*, 1986; reviewed in Mathews, 1995). VAII RNA can bind to RNA helicase A and NF90 (a component of the heterodimeric nuclear factor of activated T cells (NFAT)), suggesting that VAII RNA may mediate their regulation (Liao *et al.*, 1998).

The IVa2 transcription unit is located on the l-strand between 11.3-16 m.u.. Transcription from the IVa2 transcription unit terminates at the E2B poly A site. The IVa2 protein is found in the nucleus of cells (Lutz and Kedinger; 1996; Lutz *et al.*, 1996), and is required for late phase activation of the MLP (Lutz and Kedinger, 1996), and in assembly intermediates (Winter and D'Halluin, 1991; Hasson *et al.*, 1992). The IVa2 protein also forms complexes with L1 52kDa/55kDa protein complex, which is required to mediate stable association between viral DNA and the empty capsid (Gustin *et al.*, 1996; Gustin and Imperiale, 1998). IVa2 is also able to bind to the adenoviral DNA packaging signal which suggests a role for IVa2 in viral DNA encapsidation (Zhang and Imperiale, 2000).

Late Transcription

The MLP is situated at 16.8 m.u. on the r strand, and is active throughout adenovirus infection. However, a fully functional MLP is required to demonstrate a decrease in early gene expression, and is mediated in *trans* but not *cis* (Fessler and Young, 1998). Transcription from the MLP is increased late in adenovirus infection by two proteins known as DBP and IVa2. DBP enhances binding of upstream stimulating factor (USF) through conformational changes to the binding site (Zijderveld *et al.*, 1994), while IVa2 stimulates late phase activation by co-operation of the downstream promoter element (DPE) with factors bound to upstream elements. IVa2 binds to the DPE either as a homodimeric complex of DEF-B; or as part of a heteromeric complex known as DEF-A, which binds to the DPE1 and the 3′ end of DPE2 (Jansen-Durr *et al.*, 1989; Mondesert *et al.*, 1992; Tribouley *et al.*, 1994; Lutz *et al.*, 1996). At late times in infection five mRNA families (L1, L2, L3, L4 and L5) are transcribed from this promoter (Shaw and Ziff, 1980; Nevins and Wilson, 1981). Each mRNA species contains a common set of three short 5′ leader sequences, known as the tripartite leader, which are joined to different splice acceptor sites (Berget *et al.*, 1977; Chow *et al.*, 1977). The poly A sites L1 and L3 contain *cis*-acting elements that are necessary to elicit a temporal switch in poly A site usage (Gilmartin *et al.*, 1996; Prescott *et al.*, 1997; reviewed in Edwalds-Gilbert *et al.*, 1997; Zhao *et al.*, 1999).

The late region (L1) region is expressed at early and late times during infection (Shaw and Ziff, 1980; Nevins and Wilson, 1981), producing mRNAs during the early phase of viral infection which differ from the major late mRNAs by the presence of an additional leader sequence (i), which is spliced between the second and third exons of the tripartite leader (Akusjärvi and Persson, 1981). The inclusion of the i leader is facilitated by E4 ORF3 (Nordqvist *et al.*, 1994). Transcripts from the L1 region encode three proteins; the 52kDa and the 55kDa proteins are expressed at early and late times in infection, whilst protein IIIa is expressed at late times in infection (Akusjärvi and Persson, 1981; Kreivi and Akusjärvi, 1994). The 52kDa and the 55kDa proteins are required for assembly of virions (Gustin and Imperiale, 1998; reviewed in Greber, 1998), in particular they act as a scaffold mediating stable association of the viral DNA and the empty capsid. The protein IIIa functions as a structural polypeptide linking adjacent facets of hexons by spanning the capsid wall (reviewed in Burnett, 1997). The L2

region encodes four virion proteins, known as precursor (p)III, pVII, pV and pX, which is the precursor for μ (Anderson *et al.*, 1989; Russell and Kemp, 1995). Proteins V, VII and the minor component μ are condensed with the viral DNA (reviewed in Burnett, 1997). The L3 region encodes three virion proteins known as pVI, hexon and p23 (see sections 1.1.2; 1.1.4.4) (reviewed in Burnett, 1997; Mangel *et al.*, 1997). The L4 region encodes three proteins, one of which is structural pVIII, and two non-structural proteins of 33kDa and 100kDa in size. The minor protein pVIII is localised with the capsid, and is most likely to occur inside the capsid. The 33kDa protein has no known function (Oosterom-Dragon and Anderson, 1983), however the 100kDa protein functions to assemble hexon into trimers (Oosterom-Dragon and Ginsberg, 1981; Cepko and Sharp, 1982, 1983), and is necessary for the efficient initiation of translation of late mRNA (Hayes *et al.*, 1990). The L5 region encodes 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 (Chroboczek *et al.*, 1995).

1.1.4.3 DNA replication

DNA replication is initiated at either end of the adenovirus genome, then proceeds unidirectionally by a strand displacement mechanism made up of Type I and Type II replication (reviewed in van der Vliet, 1995; de Jong and van der Vliet, 1999). Type I replication consists of initiation at the end of the terminus using a protein-primer (3′ OH group) and elongation using a single strand as a template and Adpol; the non-template (or displaced) strand is then replicated during Type II replication, for which the origin of replication is a panhandle structure formed as a result of the ITRs base pairing (reviewed in Challberg and Kelly, 1989; Hay and Russell, 1989; Stillman, 1989). Adpol and pTP form a heterodimer (pTP-pol) which recognises the core origin in the ITR, this complex catalyses the formation of pTP-dCMP from which the 3′ OH group originates (Smart and Stillman, 1982).

Initiation of DNA replication is enhanced by two cellular transcription factors nuclear factor (NF) I or CAAT transcription factor, and NFIII or octamer binding protein (for a detailed review see de Jong and van der Vliet, 1999), which bind to two adjacent sites near the core origin in the ITR; this region is known as the auxiliary region. NFI binds as a dimer to its recognition site and interacts with the pTP-Adpol heterodimer (Dekker

et al., 1996), acting to stabilise the initiation complex on the template at the correct positions (Chen *et al.*, 1990; Mul and van der Vliet, 1992; Armentero *et al.*, 1994). NFIII binds to its recognition site stimulating initiation of DNA replication by direct interaction with the pTP-pol complex (Coenjaerts *et al.*, 1994). Initiation of DNA replication is also enhanced by DBP, which increases the binding efficiency of NFI to its recognition site (Cleat and Hay, 1989; Stuvier and van der Vliet, 1990), and lowers the K_m value for the formation of the initiation complex (Mul and van der Vliet, 1992). DBP has helix destabilising properties (Zijderveld and van der Vliet, 1994) which could assist in strand displacement during replication. DBP is also required for elongation, during which it increases the rate of synthesis and processivity of Adpol, and covers the displaced strands (Lindenbaum *et al.*, 1986).

1.1.4.4 Virion assembly

Adenovirus particles are formed during a multistage process (figure 1.1.4.4), the first of which results in the formation of hexon, penton base and fibre capsomers. The hexon trimers are formed in the presence of the L4 100kDa protein, which is thought to avoid assembly errors. The hexon trimer, along with the penton base and fibre are then transported into the nucleus where they are assembled into an empty capsid, composed of hexon, penton base, fibre and the proteins pIIIa, pVI and pVIII (reviewed in D'Halluin, 1995). The empty capsids also contain the L1 52kDa/55kDa heteromeric complex, which is thought to act as a scaffolding protein. The next intermediate is known as the heavy intermediate, as it contains the viral DNA covalently linked to TP. However, it lacks most of the scaffolding proteins and the core proteins V, VII and μ. It is not clear how the DNA is packaged into the empty capsid, however a number of proteins including L1 52kDa/55kDa, IIIa and IVa2 are involved. The young virion contains pIIIa, pVI, pVII, pVIII, pX and pTP, which are cleaved by p23 to form the mature virion (reviewed in D'Halluin, 1995; Mangel *et al.*, 1997; Greber *et al.*, 1998).

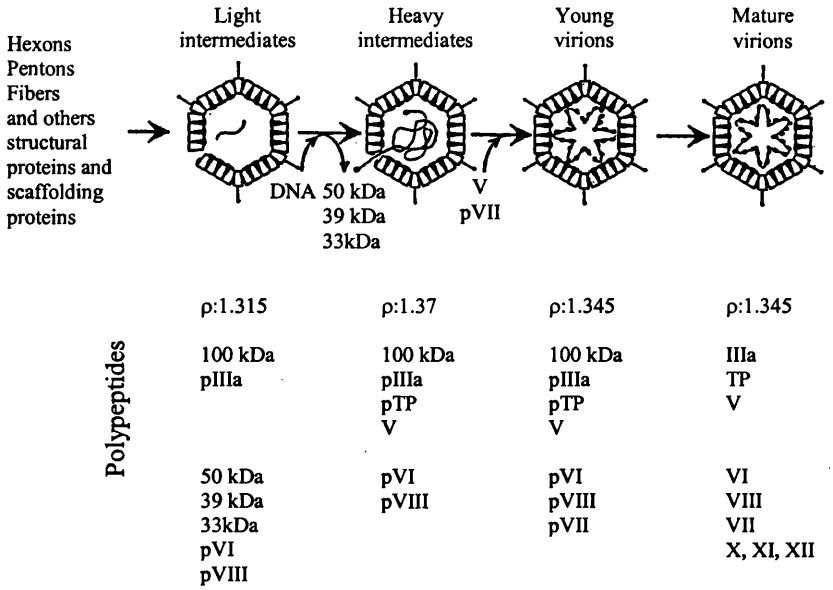


Figure 1.1.4.4 The adenovirus assembly pathway. The four stages of virus assembly; light intermediates, heavy intermediates, young virions and mature virions are shown. The polypeptides only present in some structures are also shown. (modified from D'Halluin, 1995).

1.2 Early Region 1A

The E1A region has been studied extensively, and found to play an important role in the regulation of adenovirus transcription and transformation. This section describes the E1A promoter, E1A gene transcription, the E1A protein products and their functions.

1.2.1 The E1A promoter

The E1A promoter is able to initiate transcription in the absence of any viral factors, however transcription of the remaining early viral genes requires E1A gene expression (Berk *et al.*, 1979; Jones and Shenk, 1979; Nevins, 1981).

Eukaryotic promoters contain a core promoter element and upstream enhancer elements (Roeder, 1991). The E1A promoter, like other eukaryotic promoters, contains a core promoter element and upstream enhancer elements (figure 1.2.1). The E1A promoter is able to initiate RNA polymerase II directed transcription immediately after adenovirus infection through the core promoter and upstream enhancer sequences (sections 1.2.1.1 and 1.2.1.2; reviewed in Yoshida *et al.*, 1995).

1.2.1.1 The E1A core promoter

The core promoter consists of a TATA element, an initiator sequence, and a DPE, which binds TFIID to initiate the assembly of the preinitiation complex (PIC) containing RNA polymerase II and its associated basal transcription factors (Hoey *et al.*, 1990; Verrijzer *et al.*, 1995; Zawel and Reinberg, 1995; Burke and Kadonaga, 1997). The upstream enhancer elements are bound to a variety of cellular transcription factors, which are bridged by coactivators to the basal transcription factors bound at the core promoter, supporting enhancer-dependent transcription (reviewed by Hampsey, 1998). The three dimensional structure of TFIID consists of a horseshoe-like structure, which suggests some conformational flexibility (Brand *et al.*, 1999; Andel *et al.*, 1999). The TFIID complex consists of the TATA-box binding protein (TBP), and several TBP-associated factors (TAFs). TBP is located near the midpoint of the complex, adjoining a 4nm central cavity that most likely accommodates the DNA at the TATA box. Recombinant TBP has been shown to stimulate the initiation of basal transcription *in vitro* as efficiently as the TFIID complex, but it is unable to direct enhancer-dependent

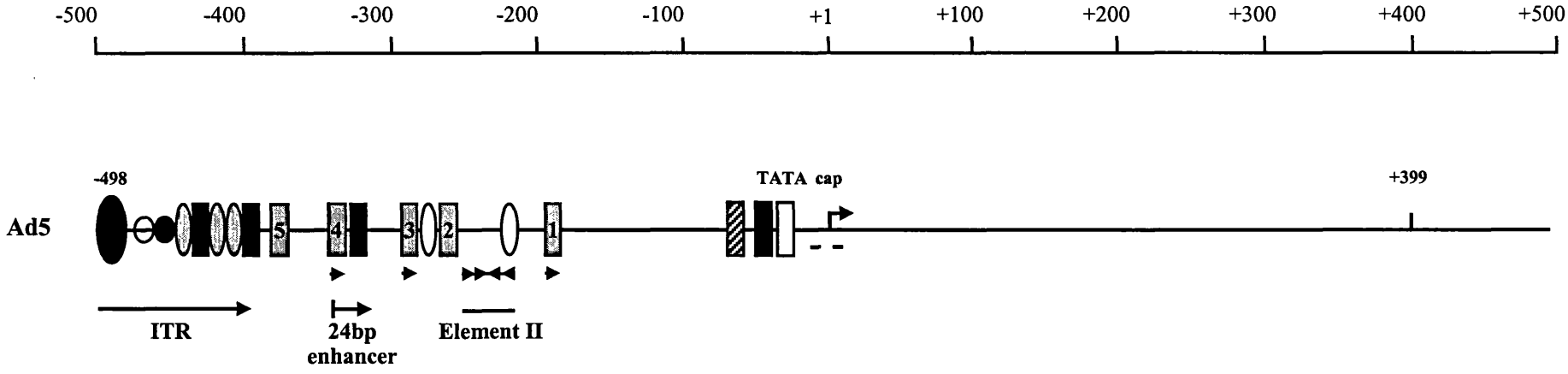


Figure 1.2.1 The adenovirus type 5 early region 1A promoter. Transcription factor binding sites are denoted by: ● denotes ORP-A; ○ denotes NF I; ● denotes NF III; 0 denotes SP1; ■ denotes ATF; ▩ denotes EF1A; ▨ denotes CAAT; 0 denotes E2F; □ denotes TATA; ↱ denotes cap site; ▸ denotes Element I; ▸▸◂◂ denotes Element II; - - denotes hyphenated symmetry around cap site; ⊢→ denotes 24bp enhancer.

transcription as efficiently as TFIID (Kambadur *et al.*, 1990; Meisterernst *et al.*, 1990; Pugh and Tijan, 1990; Dynlacht *et al.*, 1991; Tanese *et al.*, 1991). The functional difference between TFIID and TBP in enhancer-dependent transcription can be explained by an additional set of factors found in association with TBP (approximately 8-12 ranging in size from 250 to 20 kDa), known as TAFs, which mediate signals between upstream enhancer elements and TBP, and between *trans*-activators and TBP (section 1.2.4.2) (Pugh and Tijan, 1990). TAFs not only function as activators of enhancer-dependent transcription, but also act as promoter selectivity factors, and in the case of $TAF_{II}250$, are also able to act enzymically, modifying neighbouring proteins or histones in the chromatin-assembled DNA template and thereby controlling their function (reviewed in Albright and Tijan, 2000). The activity of TFIID also appears to be regulated by inhibitory proteins such as Dr1, which interact with TBP. This may serve as an important regulatory function by keeping genes, which have been removed from inactive chromatin, in a repressed but rapidly inducible state (Inostroza *et al.*, 1992).

Like many other eukaryotic promoters, the E1A core promoter also contains an initiator sequence (which includes the sequence where transcription actually begins, known as the cap site) and a DPE. The Ad2 E1A cap site is located at nucleotide 499 relative to the ITR, and occurs within a hyphenated symmetry (Baker and Ziff, 1981). Sequences between positions –35 to +20 (relative to the cap site) are important for efficient Ad5 E1A transcription *in vitro* and *in vivo*. The E1A TATA box is located within this region, between positions –31 to –24 (relative to the cap site); deletion of the TATA box *in vitro* results in a complete lack of E1A-specific transcripts, whereas *in vivo* the number of E1A-specific transcripts is reduced by 62% (Hearing and Shenk, 1983a). The TATA box deletion mutant *in vivo*, still encodes reduced amounts of E1A-specific mRNAs, which suggests that other sequences within the core promoter direct RNA polymerase II to the transcription initiation site. The DPE (*cis*-acting) is located at +399 (relative to the cap site), and shares homology with the simian virus 40 (SV40) enhancer (Weiher *et al.*, 1983). A single base change at position +399 results in a five-fold reduction in transcription, due to inactivation of the 289R and 243R E1A proteins. The same element also acts in *cis* to reduce E1A mRNA and nuclear RNA concentrations by a factor of ten (Osbourne *et al.*, 1984). These mutations may cause their effect at the

post-transcriptional level. The out of frame mutation of the E1A protein-coding region may cause premature termination in translation and instability of mutant E1A mRNA, resulting in a reduction of E1A mRNA levels (Hearing and Shenk, 1985).

1.2.1.2 Upstream enhancer elements of the E1A promoter

Since the E1A gene should be expressed soon after entry of viral DNA into the nucleus of the cell, it is therefore logical that the E1A gene is preceded by multiple upstream enhancer elements. Four enhancer elements have been identified at the following positions relative to the cap site: -498 to -396 (Hatfield and Hearing, 1991); -343 to -320 (Hen *et al.*, 1983); -305 to -141 (Hearing and Shenk, 1983b); and -188 to -45 (Imperiale *et al*, 1983).

The ITR contains a number of potential transcription factor binding sites (figure 1.2.1.); indeed several laboratories have found intrinsic promoter and enhancer activities within the ITR, although it is not clear whether they are of great functional significance. For example, when the ITR is placed adjacent to the E1A TATA box *in vitro*, transcription from the E1A region increases (Hatfield and Hearing, 1991). Also transcription from the E4 promoter is enhanced by the ITR (Ooyama et al., 1989).

The Ad2 E1A promoter contains a 24bp enhancer element, located between positions -343 and -320 (relative to the cap site) (Hen *et al.*, 1983). This element can stimulate transcription from the MLP when inserted in the direction of transcription, however it is dispensable for efficient transcription within virus-infected cells (Hearing and Shenk, 1983b). The requirement for the 24bp enhancer element is diminished in the presence of the E1A proteins, so it is unclear whether it plays a part in transcription from the E1A promoter before the onset of E1A protein synthesis (Sassone-Corsi *et al.*, 1983). In rodent CREF cells, the 24bp enhancer element also functions as a repressor of the E1A enhancer (Herbst *et al.*, 1990). Repressor function is mediated by a 100-110kDa AP3-like protein called φAP3, which binds to the 24bp enhancer element (Fognani *et al.*, 1993). A second 60 kDa protein isolated from HeLa cells, also binds to the same region within the 24bp enhancer (Barrett *et al.*, 1987). Overlapping the φAP3 binding site are two potential binding sites for the transcription factors ATF (at position -330 to -

323, relative to the cap site), and EF-1A (at position -344 to -338, relative to the cap site) (Lin and Green, 1988; Bruder and Hearing, 1989; Yoshida *et al.*, 1989).

The Ad5 E1A promoter contains a second enhancer region between positions -305 to -141 (relative to the cap site), deletion of which results in a 15 to 20 fold decrease in E1A enhancer-dependent transcription *in vivo* (Hearing and Shenk, 1983b). The conformation of a portion of the E1A enhancer element (from position -301 to -218) has been shown to facilitate transcription from the E1A region. The enhancer adopts a curved structure, which may facilitate E1A gene expression by influencing promoter melting and/or by providing a framework for protein-DNA and/or protein-protein interactions (Ohyama, 1996). Two distinct elements are responsible for E1A enhancer-dependent transcription (element I and II) within this region (Hearing and Shenk, 1986).

The E1A promoter contains two copies of element I, at positions -301 to -289 and -203 to -192 (relative to the cap site) however, this location varies in adenoviruses from subgroups A and B (van Ormondt and Galibert, 1984; Hearing and Shenk, 1986). Element I specifically regulates transcription from the E1A region within virus infected cells. A degenerate copy of element I is located at positions -272 to -263 (relative to the cap site); and two further copies of element I-related sequence lie further upstream. The two copies of element I (sites 1 and 3 figure 1.2.1.), and the three copies of the element I-related sequence (sites 2, 4 and 5 figure 1.2.1.), contain the consensus binding site for EF-1A (Bruder and Hearing, 1989; Yoshida *et al.*, 1989). EF-1A binding is determined by the core nucleotides of each binding site, whereas transcriptional activation is determined by both core and peripheral nucleotides (Bolwig *et al.*, 1992). However, efficient binding of EF-1A at the dimeric binding site (sites 2 and 3) is essential, but not sufficient to activate transcription. EF-1A binds cooperatively at sites 1 and 2, 2 and 3, and 4 and 5; and cooperative binding of EF-1A at site 2 to neighbouring sites 1 and 3 in the E1A enhancer region results in synergistic activation of E1A transcription (Bruder and Hearing, 1991). EF-1A belongs to the *ets* oncogene family, and is composed of two subunits α and β. Binding of both the α and β subunits of EF-1A at the binding site is necessary but not sufficient for transcriptional activation (Bolwig *et al.*, 1992). EF-1Aα contains the ETS domain, which is responsible for site-specific DNA binding, whereas

EF-1Aβ contains four 32-33aa ankyrin repeats in the N-terminal region, and possesses no DNA binding activity. The ETS domain and the ankyrin repeats are required for multi-heterodimerisation of α and β subunits on the dimeric EF-1A binding site which is associated with enhancer activity *in vivo* (Thompson *et al.*, 1991).

Four copies of element II are situated between the two copies of element I (sites 1 and 3, figure 1.2.1), and span approximately 30bp from positions -250 to -218 (relative to the cap site, figure 1.2.1). Element II regulates transcription in *cis* from all early regions, however the factors which interact with element II have not been described. Elements I and II function independently, and neither is required for efficient viral DNA replication (Hearing and Shenk, 1986).

A third enhancer element, an E2F binding site, is repeated at positions -288 to -281 and -225 to -218 (relative to the cap site; figure 1.2.1). The E2F site at -288 to -281 has been shown to stimulate transcription of a heterologous linked gene in an E1A-dependent fashion (Kovesdi *et al.*, 1987). However, in virus-infected HeLa cells, transcription of the E1A gene is not influenced by deletion of the two E2F sites from the enhancer region (Bruder and Hearing, 1989).

A fourth enhancer element has been identified within the E1A promoter, situated at positions -188 to -45, which is able to enhance expression of the E2A 72kDa protein when linked to the E2A gene (Imperiale *et al.*, 1983). However, this enhancer element is unable to enhance transcription from the E1A promoter (Sassone-Corsi *et al.*, 1983), and is dispensable for efficient transcription from the E1A gene in virus-infected cells (Hearing and Shenk, 1983a).

1.2.2 E1A transcription

The E1A primary transcript or pre-mRNA is alternatively spliced to produce a 13S, 12S, 11S, 10S and 9S mRNA species (figure 1.1.4.2); with the exception of the 9S transcript, all transcripts share 5′ and 3′ ends. The E1A primary transcript gives rise to three major mRNAs, the 13S, 12S and 9S mRNAs, by use of three alternative 5′ splice sites (figure 1.1.4.2) (Berk and Sharp, 1978; Chow *et al.*, 1979; Perricaudet *et al.*, 1979).

Two minor mRNAs are also produced from the E1A primary transcript, the 11S and 10S, by the removal of an additional intron i.e. the 9S 5′ splice site is joined to a novel 3′ splice site located upstream of the 12S 5′ splice site (figure 1.1.4.2).

Efficient splicing of the primary transcript requires a 5′ cap structure to enable efficient utilisation of the adjacent 5′ splice site, definition of the first exon, and removal of the first intron (Izaurralde *et al.*, 1994). The primary transcript is spliced in a two-stage reaction (figure 1.2.2.1). In the first stage the primary transcript is cleaved at the 5′ splice site, generating a first exon ribonucleic acid (RNA) species and an intron-second exon RNA species in lariat formation. During the second stage of the splicing reaction the 3′ splice site is cleaved, allowing ligation of the exons to produce a spliced mRNA (Padgett *et al.*, 1984; Ruskin *et al.*, 1984). The splicing reaction takes place in a large macromolecular structure known as the spliceosome (figure 1.2.2.2), which consists of small ribonucleoprotein particles (snRNPs) U1, U2, U4/U6 and U5, and non-snRNPs. The non-snRNPs include a family of proteins known as the SR proteins. The SR proteins are a family of essential splicing factors required for early recognition of splice sites during the spliceosome assembly (Screaton *et al.*, 1995). One such protein has been described for the E1A primary transcript (p54), which is able to recognise and promote the use of the 5′ splice site in the E1A primary transcript in a tissue- and substrate-dependent manner (Zhang and Wu, 1995). The SR protein function can be modified by phosphorylation/dephosphorylation: for example, in late adenovirus infected cells the SR proteins are inactivated as splicing enhancer or repressor proteins by E4ORF4 induced dephosphorylation by PP2A (Kanopka *et al.*, 1998).

The spliceosome assembles on the primary transcript in a stepwise manner, forming four discrete complexes E-A-B-C (figure 1.2.2.2). Complex E is believed to irreversibly define the exon-intron boundaries in the primary transcript, and is formed when U1 snRNP binds to the 5′ splice site; at the same time U2AF binds to the pyrimidine tract between the branch point and the 3′ splice site. Efficient recognition of the 5′ splice site by U1 snRNP is facilitated by the nuclear cap binding complex (CBC) (Lewis *et al.*, 1996). The RS domains of U1 snRNP and U2AF associate with the non-snRNP proteins, forming a bridge between the 5′ and 3′ splice sites allowing functional

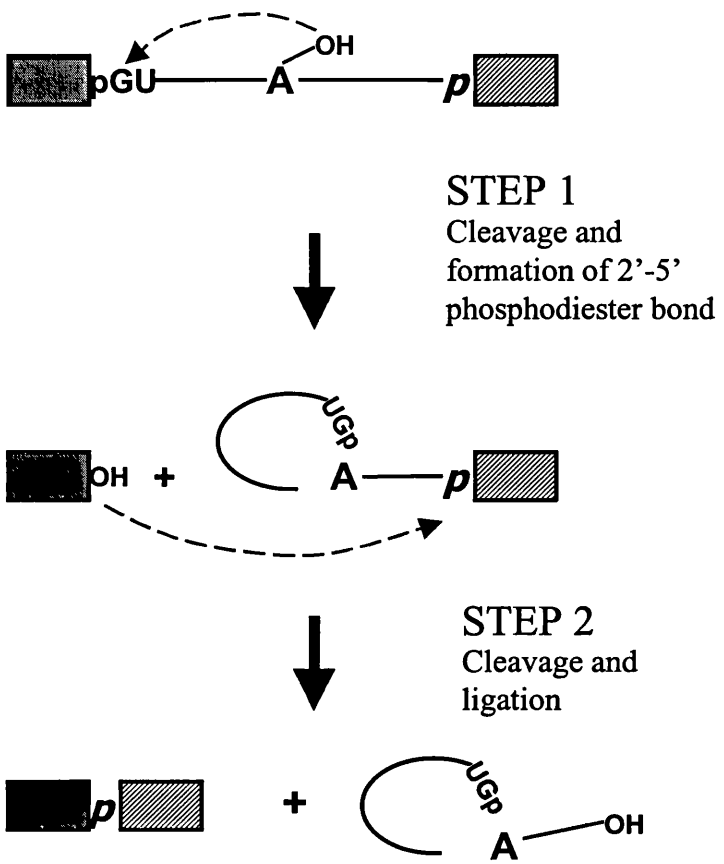


Figure 1.2.2.1 The splicing reaction. Splicing occurs in a two step reaction (section 1.2.2 In step one, the hydroxyl group of the A residue at the branch point site, and **p** is the phosphate of G. In step 2, OH is the hydroxyl group of the last residue of exon 1, and ***p*** is the phosphate group of the first residue of exon 2.). Boxes denote exons 1 and 2; the single line denotes intronic sequences to be removed (reproduced from Green, 1991).

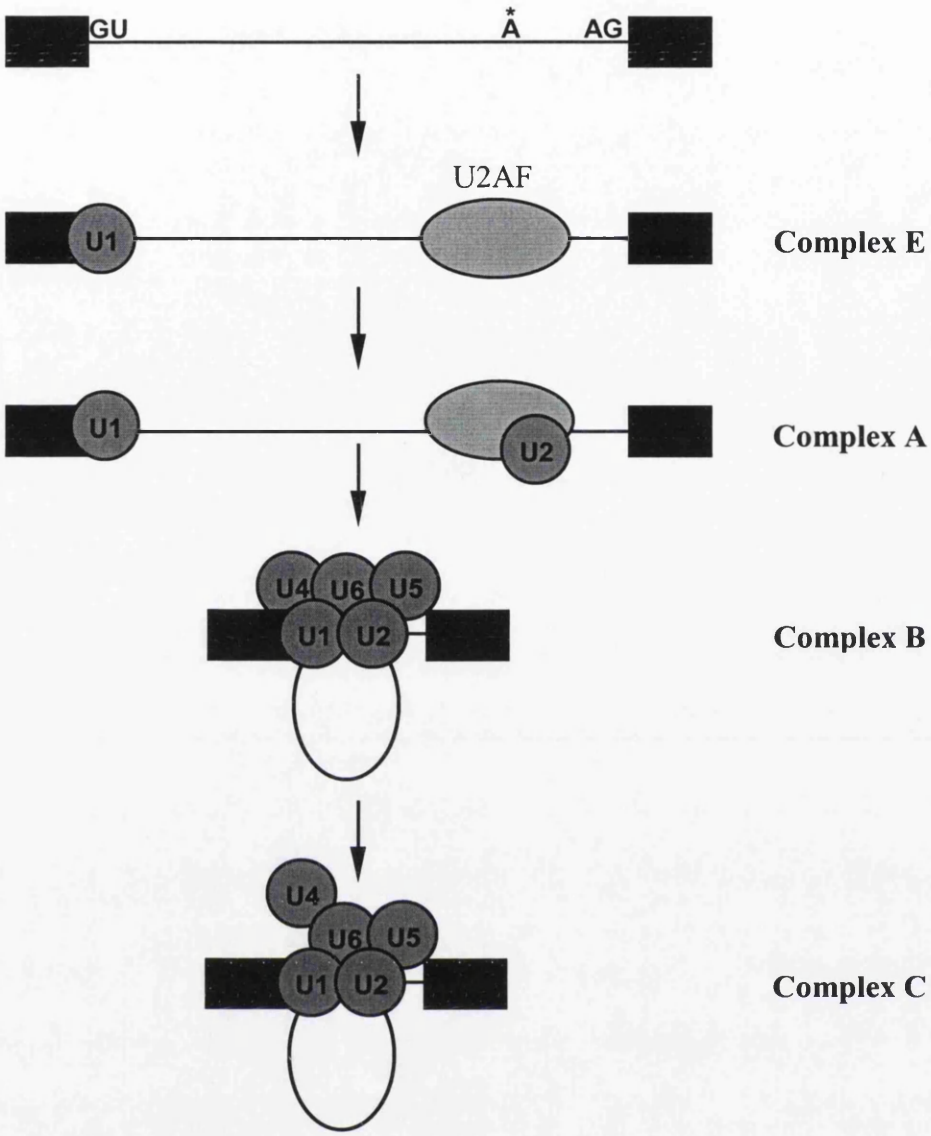


Figure 1.2.2.2 Proteins involved in the mammalian splicing pathway. Two snRNP/pre-mRNA interactions form the basis of the spliceosome. Firstly, U1 snRNP binds to the 5' splice site while at the same time the splicing associated factor U2AF binds to pyrimidine tract between the branch point site (BPS) and the 3' splice site, thus forming complex E. Secondly, U2 snRNP, recruited by U2AF (Gozani & Potashkin, 1998), binds to a region encompassing the BPS. This step irreversibly commits the pre-mRNA to the splicing pathway and forms complex A. Complexes A and E are also called pre-spliceosomes. Thirdly, a pre-existing (U4/U6/U5) particle enters to form complex B, and just prior to splicing a confrontational change occurs that significantly destabilises the association of U4 snRNP with the complex transforming it into complex C. Following splicing, the snRNPs remain associated with the intron, and are recycled. Excised introns are degraded by a phosphodiesterase specific to the lariat structure. GU and AG are the 5' and 3' conserved residues recognised by splicing factors, A is the residue at the branch point site. (reproduced from Green, 1991).

interaction (Wu and Maniatis, 1993; Fu, 1995; Reed, 1996, Manley and Tacke, 1996). The U2 snRNP is then recruited by U2AF, in an ATP-dependent manner, forming complex A, which is believed to irreversibly commit the primary transcript to the splicing pathway (Gozani *et al.*, 1998). A third complex, complex B also known as the mature spliceosome, is formed upon recruitment of U4/U6 and U5 snRNPs. Complex B is transformed into complex C, when a conformational change occurs, which significantly destabilises the association of the U4 snRNP. After the splicing reaction is complete, the snRNPs remain associated with the intron and are recycled, whereas the intron is degraded by a phosphodiesterase specific to the lariat structure. The spliced message is then polyadenylated, a reaction coupled to the splicing reaction *in vivo* (Cooke *et al.*, 1999).

A poly A tail is essential for the survival, transport, stability and translation of most mRNAs. Processing of the 3′ end is a two step process involving endonucleolytic cleavage of the primary transcript, followed by addition of the poly A tail. The core poly A site is made up of a cleavage site, a highly conserved hexanuleotide AAUAAA located approximately 11 to 25 nucleotides upstream of the cleavage site, and a downstream element (DE), which consists of a 6-20 nucleotide guanine (G) + uridine(U) or U-rich sequence approximately 14 to 70 nucleotides downstream of the hexanucleotide AAUAAA. The DE increases the efficiency of 3′ end processing and, as a number of AAUAAA sequences are found within the coding region of the protein, the DE serves to position the polyadenylation apparatus at the correct AAUAAA sequence. Elements upstream of the core poly A site, which improve the efficiency of polyadenylation have been characterised for a number of poly A signals. During 3' end processing a 160kDa cleavage and polyadenylation specificity factor (CPSF) binds to the poly A site by recognition of the hexanucleotide AAUAAA, and a second 64kDa cleavage stimulatory factor (CStF) binds to the DE. For the reaction to proceed the poly A polymerase and at least one other factor are required, at which point cleavage occurs and the poly A tail of approximately 200 residues is added (Imperiale *et al.*, 1995).

The 13S and 12S mRNAs are the major transcripts of the E1A region (Perricaudet *et al.*, 1979), and are first detected at 1.5-2h p.i. (Nevins *et al.*, 1979) and steadily increase to 5h p.i., after which time a steady decrease is observed to 12h p.i. (Glenn and Ricciardi,

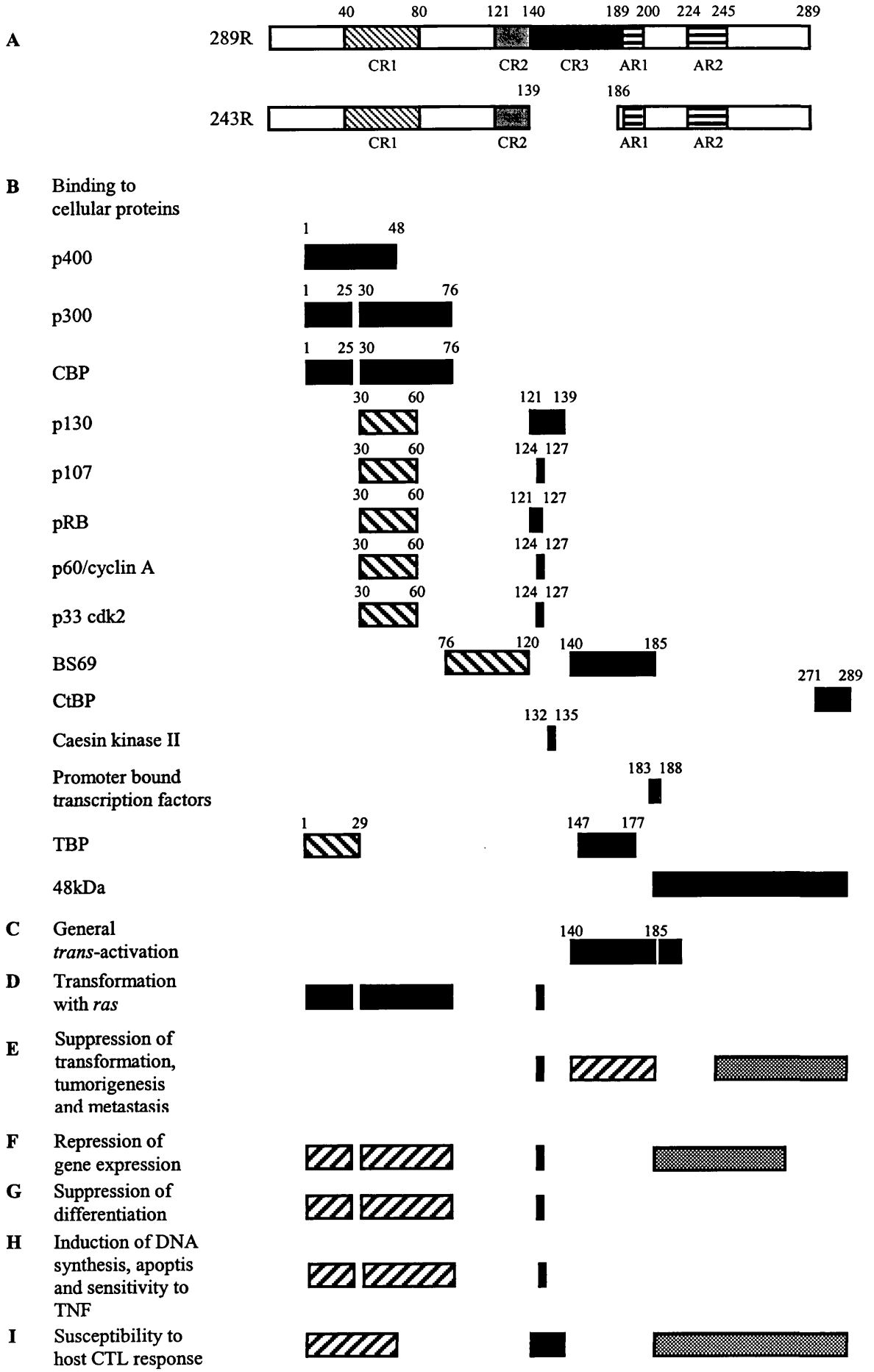
1988). The 9S mRNA is also a major transcript of the E1A region although it is produced at late times after infection (Chow *et al.*, 1979; Wilson and Darnell, 1981; Ulfendhal *et al.*, 1987). The minor transcripts of the E1A region are the 11S and 10S mRNAs, which are detected at late times after infection (Stephens and Harlow, 1987). Considerable efforts have been made to elucidate mechanisms causing a switch between early and late production of E1A transcripts. Viral DNA replication has been shown to play an important part in the switch between early and late E1A transcripts, but in the absence of late protein synthesis the switch is not complete, suggesting that viral proteins possess a regulatory role. (Adam and Babiss, 1991; Larsson *et al.*, 1991). Indeed, E4 ORF3 and E4 ORF6 have analogous biological activities to ASF/SF2 and heterogeneous ribonucleoprotein particles (hnRNP) A1 (Nordqvist *et al.*, 1994), and are therefore believed to regulate E1A alternative splicing *in vivo*. hnRNP A1 and ASF/SF2 (Mayeda and Krainer, 1992) or DSF and ASF/SF2 (Harper and Manley, 1991), are able to modulate 9S splicing *in vitro*. 13S 5′ splice site usage is favoured by a high concentration of ASF/SF2, whereas 9S 5′ splice site usage is favoured by a high concentration of hnRNP A1 or DSF. E4 ORF4 is also able to induce dephosphorylation of the SR proteins by PP2A, thereby exerting regulatory control on alternative splicing (Kanopka *et al.*, 1998).

1.2.3 E1A proteins

E1A proteins are proline-rich phosphoproteins located predominantly in the nucleus of infected cells (Bayley and Mymrk, 1994). They can be phosphorylated by cyclin-dependent kinases (cdk) (Mal *et al.*, 1996), and cyclic adenosine-5′-monophosphate (cAMP) reduces the levels of phosphorylation through the activation of PP2A by the E4 ORF4 protein (Muller *et al.*, 1992; Whalen *et al.*, 1997). The phosphorylation state of the E1A proteins has an effect on its biological function e.g. phosphorylated E1A is more efficient in its association with RB and in disrupting E2F/DP-RB than unphosphorylated E1A (Mal *et al.*, 1996).

The major E1A proteins are the products of the 13S and 12S mRNAs, that is the 289R and 243R proteins respectively (figure 1.2.3). Comparison of the 289R and the 243R proteins from various adenovirus serotypes has identified three highly conserved regions

Figure 1.2.3 Map of the major E1A proteins and the regions required for selected E1A activities. **A.** The 289R and 243R E1A proteins, conserved regions (CR1) 1, 2 and 3, and auxiliary regions (AR) 1 and 2. **B.** Consensus sites for binding to cellular proteins. The hatched regions are of secondary importance. **C.-G.** Regions required for: **C.** general activation of transcription; **D.** transformation with activated *ras* in BRK cells; **E.** suppression of transformation, tumorigenicity and metastasis. The hatched region is required for suppression of transformation of virus. The solid black region is required for suppression transformation, tumorigenicity and metastasis resulting from repression of c-*erb*B2/*neu* transcription. The stippled region in exon 2 is required for suppression of transformation, tumorigenicity and metastasis with activated *ras*. **F.** Repression of gene suppression. Generally the hatched regions are necessary, but for repression of some genes either of the other two regions are required as well. **G.** Suppression of differentiation. Depending on the cells, minimum requirements are the hatched regions; either the hatched or the solid black regions; or all three. **H.** Induction of DNA synthesis, apoptosis and sensitivity to TNF. Minimum requirements are either the two hatched regions or the solid black region. **I.** Susceptibility to the host CTL response. Depending on the haplotype any of the three regions may be different (modified from Mymryk, 1996).


A
289R
40
80
121
140
189
200
224
245
289
CR1
CR2
CR3
AR1
AR2
243R
139
186
CR1
CR2
AR1
AR2
B
Binding to cellular proteins
p400
p300
CBP
p130
p107
pRB
p60/cyclin A
p33 cdk2
BS69
CtBP
Caesin kinase II
Promoter bound transcription factors
TBP
48kDa
C
General trans-activation
D
Transformation with ras
E
Suppression of transformation, tumorigenesis and metastasis
F
Repression of gene expression
G
Suppression of differentiation
H
Induction of DNA synthesis, apoptis and sensitivity to TNF
I
Susceptibility to host CTL response

(CR) within the proteins, designated CR1, CR2 and CR3 (figure 1.2.3) (Kimelman *et al.*, 1985; Moran and Mathews, 1987). CR1 is present in both the 289R and 243R proteins, and is required for transcriptional repression, transformation and induction of DNA synthesis (Lillie *et al.*, 1987; Schneider *et al.*, 1987; Jelsma *et al.*, 1989; reviewed in Jones, 1995). CR2 is present in both the 289R and 243R proteins, and is required for transformation, induction of DNA synthesis but, may be dispensable for transcriptional repression ((Lillie *et al.*, 1986; Moran *et al.*, 1986a; Howe *et al.*, 1990; Stein *et al.*, 1990). CR3 encompasses a 49aa stretch and is present only in the 289R protein, it possesses a potent *trans*-activation function (Shenk and Flint, 1991; Jones, 1992; Bayley and Mymrk, 1994). Protein fusion experiments show that this domain has two separable activities (Lillie and Green, 1989; Martin *et al.*, 1990). The amino terminal (N-terminal) of CR3 contains the transcriptional activation region and the carboxy terminal (C-terminal) contains the promoter-targeting region. Located within the N-terminal of CR3 is a C4 type zinc finger; point mutations of any of the cysteine residues that define this motif eliminate *trans*-activation function (Culp *et al.*, 1988; Martin *et al.*, 1990; Webster and Ricciardi, 1991). The non-conserved C-terminal E1A exon contains two interchangeable elements, designated auxiliary region (AR) 1 and 2. AR1 contains glutamic acid-proline (EP) repeats, the number of which (or the net negative charge) are critical to its function as an enhancer of CR3-mediated *trans*-activation. As it is required for efficient CR3-mediated *trans*-activation it has been suggested that the C-terminal boundary for CR3 should be extended to include AR1 (Bondesson *et al.*, 1992; Strom *et al.*, 1998).

1.2.4 Role of the E1A proteins in the regulation of transcription

The E1A proteins have four main functions: autoregulation of the E1A promoter, *trans*-activation of viral and cellular promoters, transcriptional repression of enhancer containing promoters, and transformation. These functions are described in sections 1.2.4.1, 1.2.4.2, 1.2.4.3 and 1.2.4.4.

1.2.4.1 E1A autoregulation

The 289R and 243R E1A proteins are expressed from the E1A gene at early times after infection (Perricaudet *et al.*, 1979; van Ormondt *et al.*, 1980). In the absence of the E1A

proteins, transcription from the E1A promoter is reduced approximately five fold suggesting a role for the E1A proteins in their own regulation (Montell *et al.*, 1984a; Osborne *et al.*, 1984). Indeed, E1A has been shown to be capable of its own regulation at the level of transcription (Hearing and Shenk, 1985; Tibbetts *et al.*, 1986). The E1A proteins, in particular the 289R protein, are able to *trans*-activate the E1A promoter (Osborne *et al.*, 1984; Hearing and Shenk, 1985), and both the E1A proteins have also been associated with negative regulation of their own promoter at the level of transcription (Tibbetts *et al.*, 1986; Cogan *et al.*, 1992).

1.2.4.2 E1A *trans*-activation

The E1A proteins can *trans*-activate not only their own promoter and enhancer sequences, but also those of other viral (E1B, E2A, E3, E4 and MLP) and cellular (hsp70, c-fos, c-jun and PCNA) genes (reviewed in Jones, 1992, 1995; Flint and Shenk, 1997). The E1A proteins are not thought to bind directly to DNA (Ferguson *et al.*, 1985; Chatterjee *et al.*, 1988), rather they interact with other proteins (through CR1, CR2 and CR3) located at the core promoter, and upstream and/or downstream enhancer elements enabling *trans*-activation.

The E1A proteins can *trans*-activate any promoter containing a TATA motif *in vitro* (Green *et al.*, 1983; Wu *et al.*, 1987; Simon *et al.*, 1988). This may be due in part to the ability of the 289R CR3 to bind to the TBP subunit of TFIID (Horikoshi *et al.*, 1991; Lee *et al.*, 1991); and interact with $TAF_{II}250$ (Geisberg *et al.*, 1995), $TAF_{II}110$ (Mazzarelli *et al.*, 1995) and $TAF_{II}55$ (Chiang and Roeder, 1995). $TAF_{II}250$ possesses a histone acetyl transferase (HAT) activity, which may play a role in allowing access of the basal transcription initiation complex to repressed chromatin (Mizzen *et al.*, 1996). TFIID (of which TBP and TAFs are essential components) plays a central role in the assembly of the PIC, therefore interaction of E1A with the PIC may serve to bridge signals from upstream and downstream enhancer elements with the transcription machinery, as well as to stabilise the PIC. The 35 N-terminal residues of 243R can also bind TBP resulting in *trans*-activation (Sang *et al.*, 1997).

Both the 289R and the 243R proteins have also been shown to relieve transcriptional repression of the TATA motif, albeit that the 243R protein *trans*-activates to a lesser

extent than the 289R protein. The 289R protein relieves p53-mediated transcriptional repression by binding TBP (within which the E1A and p53 binding sites overlap), and displaces p53 from the TBP complex (Liu *et al.*, 1993; Horikoshi *et al*, 1995). The N-terminal segment of the 243R protein is able to relieve Dr1-mediated transcriptional repression by binding Dr1, resulting in the release of TBP from the inhibitory complex (Inostroza *et al.*, 1992; Kraus *et al.*, 1994). In addition to *trans*-activation through the basal promoter, the E1A proteins are also able to *trans*-activate through a number of transcription factors, which bind to sequences within the promoter and upstream and/or downstream promoter elements.

***bZIP* Family**

The *bZIP* family of transcription factors contain a DNA-binding domain, which consists of a cluster of basic amino acids, and a leucine zipper, hence the name *bZIP*. The *bZIP* proteins can form homodimers or heterodimers through the leucine zipper and bind to the cAMP response element (CRE) (Hai and Curran, 1991). Contained within the *bZIP* family are the proteins ATF, AP1 and E4F, all of which interact with E1A to *trans*-activate transcription.

The E1A proteins are able to *trans*-activate ATF-2 either by a direct mechanism involving CR3 disruption of the inhibitory intramolecular interaction between the activation domain and the *bZIP* DNA binding domain, or by an indirect mechanism involving the N-terminal and CR1 interaction with a cellular 300kDa coactivator (p300). The relative contributions of CR1- and CR3-dependent E1A *trans*-activation are dependent upon cell type. CR1-mediated *trans*-activation of the promoter is independent of a direct interaction with E1A, and takes place in a p300-dependent manner (Lee *et al.*, 1996; Mannervik and Akusjärvi, 1997). p300 has been shown to bind to ATF-2 and TBP, which suggests that p300 normally acts as a coactivator, transducing signals between upstream enhancer elements and the basal transcription machinery (Lee *et al.*, 1996; Dallas *et al.*, 1997). p300 also activates transcription through its inherent properties as a HAT (Bannister and Kouzarides, 1996; Ogrysko *et al.*, 1996), and its ability to form complexes with HATs, resulting in relief from chromatin induced transcriptional repression (Yang *et al.*, 1996; reviewed in Howe *et al.*, 1999). E1A binds to p300 (Egan *et al.*, 1988) through sequences overlapping the

HAT binding domain, inducing repressive complexes of chromatin, which prevent transcription from cellular genes, thereby subverting cellular transcription factors for viral *trans*-activation. CREB binding protein (CBP) has also been shown to act as a coactivator in the context of ATF-2 *trans*-activation by E1A (Sano *et al.*, 1998).

CR3-mediated *trans*-activation is a result of a direct interaction of CR3 with the DNA binding domain of ATF-2, relieving inhibitory intramolecular interactions between the activation domain and the *bZIP* DNA binding domain, and it may also result in a positive conformational change within the E1A and ATF-2 activation domains (Liu and Green, 1990; Liu and Green, 1994; Chatton *et al.*, 1994). The CR3 subdomain which interacts with ATF-2 differs from the subdomain which interacts with TBP, which suggests that CR3-dependent *trans*-activation may also involve E1A acting as a coactivator between ATF-2 and the basal transcription machinery (Lee *et al.*, 1991). The N-terminal 96 aa of ATF-2 are also essential for CR3-dependent *trans*-activation, in particular the two threonine residues at positions 69 and 71, although no direct interaction with E1A is evident (Liu and Green, 1994). This region activates ATF-2 via direct phosphorylation of threonines 69 and 71 by an N-terminal bound stress-activated protein kinase (SAPK) which may function to alter the conformation of ATF-2, positively regulating its function (Yen and Hung, 1994; Livingstone *et al.*, 1995). CR1-dependent *trans*-activation is not dependent upon phosphorylation of threonines 69 and 71 (Duyndam *et al.*, 1996).

Another group of proteins belonging to the *bZIP* family are the AP-1 family, which includes members of the Jun and Fos gene families. The AP-1 family becomes transcriptionally active when homodimers of Jun/Jun or Fos/Fos, and heterodimers of Jun/Fos are formed at the AP-1 binding site. AP-1, like ATF-2 can be induced by cAMP and *trans*-activated by E1A (Muller *et al.*, 1989). CR1-dependent *trans*-activation of AP-1 is independent of direct interaction with E1A, but takes place in a CBP-dependent manner (Sano *et al.*, 1998). CBP is a protein of the same family as p300, which is highly related within its biological functions to p300. CBP has been shown to bind to AP-1 and TFIIB (Kwok *et al.*, 1994), which suggests that CBP normally acts as a coactivator, transducing signals between upstream enhancer elements and the basal transcription machinery (Bannister and Kouzarides, 1996). CBP also

activates transcription through its inherent properties as a HAT (Oryzko *et al.*, 1996; Bannister and Kouzarides, 1996), and its ability to form complexes with HATs, including P/CAF, ACTR and SRC-1 (Yang *et al.*, 1996; Chen *et al.*, 1997; Spencer *et al.*, 1997), resulting in relief from chromatin induced transcriptional repression. E1A binds to CBP through sequences overlapping the HAT binding domain, inducing repressive complexes of chromatin, which prevent transcription from cellular genes, subverting cellular transcription factors for viral *trans*-activation (Sano *et al.*, 1998).

The E4F/ATF sequence element contains a binding site for E4F and ATF-2, which mediate E1A-dependent *trans*-activation by different mechanisms depending upon the transcription factor bound. E4F is a 50kDa protein, generated from the N-terminal part of E4F-1, the human homologue of the murine nuclear fragment ϕAP3 (Fernades and Rooney, 1997). The 289R protein regulates phosphorylation of E4F-1 and E4F, leading to an increase in DNA-binding affinity, thereby *trans*-activating the promoter (Raychaudhuri *et al.*, 1989; Bondesson *et al.*, 1992; Fernades and Rooney, 1997). The 289R C-terminal AR1 and AR2 are essential for efficient *trans*-activation of E4F (Bondesson *et al.*, 1992).

E2F Family

The E2F family is composed of two sets of proteins, known as E2F and DP (reviewed in Lam and La Thangue, 1994). E2F-1 heterodimerises with DP-1 to form physiological E2F, which is found in association with the retinoblastoma gene product (Rb), to prevent degradation of E2F by the ubiquitin-proteasome pathway (Helin *et al.*, 1993; Hateboer *et al.*, 1996). E2F binding sites shift from a negative to a positive role in transcription at the commitment point of the cell cycle, a crucial point in G1 that precedes the G1/S transition. Before the commitment point members of the pocket protein family, including Rb, p107 and p130, are found in repressive complexes with E2F (Claudio *et al.*, 1996). When E2F-Rb complexes bind to their target promoter they actively repress transcription; Rb is able to achieve this by recruiting the histone deacetylase (HDAC) 1 (Weintraub *et al.*, 1992, 1995; Ferreira *et al.*, 1998), and by interacting with the C-terminal interacting protein (CtIP)/C-terminal binding protein (CtBP) repressor complex (Meloni *et al.*, 1999). Progression through the commitment point into S phase is triggered by phosphorylation of the pocket proteins by a family of

serine-threonine protein kinases, known as cdks 2, 4 and 6; which hyperphosphorylate the pocket proteins relieving E2F repression by disruption of Rb-associated complexes (Weinberg, 1992; Wolowiec and Ffrench, 1996). CBP/p300 is also hyperphosphorylated by cyclin E-cdk2 at the commitment point, increasing its intrinsic HAT activity, and thereby its ability to activate transcription (Ait-Si-Ali *et al.*, 1998). Therefore cyclin E-cdk2 has a dual role with Rb inactivation by phosphorylation, and CBP HAT activation by phosphorylation. E1A is also able to push the cell cycle through the commitment point into S phase by forming complexes with Rb and CBP/p300 (Ait-Si-Ali *et al.*, 1998).

The E1A proteins are able to relieve transcriptional repression either by CR1 and CR2 disruption of the inhibitory E2F-RB complex (Whyte *et al.*, 1989; Zamanian and La Thangue, 1992), by providing the coactivator CBP (Trouche and Kouzarides, 1996), or by disruption of the CtIP/CtBP inhibitory complex (Meloni *et al.*, 1999). CR1/CR2-dependent *trans*-activation is a two stage process in which CR2 binds to Rb, then CR1 competes for Rb binding to E2F (Raychaudhuri *et al.*, 1990, 1991; Fattaey *et al.*, 1993; Ikeda and Nevins, 1993). E1A has been shown to bind to Rb through CR2 sequences, however it has also been shown to bind Rb and p300 simultaneously, through an LXCXE motif in CR2 which binds Rb, and through the N-terminal of CR1 which binds p300 and CBP (Wang *et al.*, 1995). E1A may act as a carrier for p300 or CBP, providing it to E2F-1 and *trans*-activating E2F-1/DP-1 by direct interaction with the C-terminal domain of E2F-1 (Trouche *et al.*, 1996; Trouche and Kouzarides, 1996), whilst binding to Rb and releasing E2F from repressive Rb complexes. E1A binding to CBP also activates its HAT function, though a conformational change, activating transcription (Ait-Si-Ali *et al.*, 1998). It is possible that the N-terminal and CR1 sequences within E1A, which compete with the Rb proteins (Rb, p107 and p130) for HDAC1, are also linked with CR1/CR2-dependent *trans*-activation, making it a three stage process. The release of HDAC1 from Rb-E2F complexes relieves transcriptional repression from HDAC1 (Trouche *et al.*, 1996; Ferreira *et al.*, 1998).

YY1

The transcription factor YY1 has been shown to mediate transcriptional repression. This is believed to occur by YY1 binding of a repressive cofactor, RPD3, which purifies

with a histone deacetylase activity, which represses transcription. The RPD3 binding site overlaps the E1A binding site (Lewis *et al.*, 1995; Taunton *et al.*, 1996; Yang *et al.*, 1996), suggesting that the E1A N-terminal 35aa displaces the activity of RPD3, thus *trans*-activating the promoter. The 289R protein can *trans*-activate YY1 binding sites through CR3 interaction (Lewis *et al.*, 1995).

1.2.4.3 Transcriptional repression

The E1A proteins can repress the transcription from several viral and cellular genes (reviewed in Jones, 1992; Hagmeyer *et al.*, 1997). A number of elements within the E1A proteins are believed to mediate transcriptional repression, including the N-terminal and CR1 domain (Schneider *et al.*, 1987; Jelsma *et al.*, 1989; Stein *et al.*, 1990), CR2 domain and some second exon sequences (Lillie *et al.*, 1986, 1987; Schneider *et al.*, 1987; Velcich and Ziff, 1988; Jelsma *et al.*, 1989), although in some studies CR2 is shown to be dispensable (Stein *et al.*, 1990). The reason for the difference in the elements used is unclear, but could depend upon the promoter or the cell type studied.

The cellular proteins p300 and CBP are known to function as coactivators interacting with TBP and upstream enhancer sequences (Abraham *et al.*, 1993), translating signals from upstream enhancer sequences to the basal transcription machinery. They have also been shown to bind to the N-terminal and CR1 of E1A (Egan *et al.*, 1988; Whyte *et al.*, 1989; Jelsma *et al.*, 1989; Stein *et al.*, 1990; Moran, 1993). Deletion mutants in the E1A binding site of p300 or CBP loose their ability to drive E1A enhancer-mediated transcriptional repression (Eckner *et al.*, 1994). Therefore a model in which the N-terminal 80 aa of the E1A proteins sequesters p300 or CBP from TFIID complexes and upstream enhancer elements has been proposed (Song *et al.*, 1995).

1.2.4.4 Transformation

Adenoviruses were first shown to be oncogenic when a subcutaneous injection of Ad12 into newborn hamsters led to the development of rapidly growing tumours at the inoculation site (Trentin *et al.*, 1962). The majority of human adenoviruses are non-tumourigenic in hamsters (table 1.1.1.), however it was later shown that all

adenoviruses were able to mediate immortalisation of primary rodent cells (Freeman *et al.*, 1967). The genes responsible for oncogenicity and cell proliferation were located at the left end of the genome and comprised of early region 1 (reviewed in Dyson and Harlow, 1992). The E1A gene products alone were found to immortalise primary cells in tissue culture (Houweling *et al.*, 1980; Ruley, 1983; Cone *et al.*, 1988). However, in cooperation with the adenovirus E1B gene, the activated *ras* gene or the polyoma middle T oncogene, E1A can stably transform cells in culture, which would induce tumours in animals (van den Elsen *et al.*, 1983; Ruley, 1983).

The E1A regions which are necessary for immortalisation and cell proliferation include the nonconserved N-terminal region, CR1 and CR2; whereas CR3 is dispensable (Lillie *et al.*, 1986; Lee *et al.*, 1991). These regions were found to strongly correlate with areas required for interactions with certain cellular proteins (Egan *et al.*, 1988; Whyte *et al.*, 1989; Howe and Bayley, 1992). Indeed out of the three active sites found to be important for immortalisation and cell proliferation in E1A, two were found to bind cellular proteins. The first active site consists of CR2 plus the N-terminal end of CR1, which binds the Rb gene family (Rb, p107 and p130) via the pocket region. The second active site consists of the N-terminal of E1A and the C-terminal of CR1, which binds the phosphoprotein family p300/CBP (reviewed in Moran, 1993; Wang *et al.*, 1995). The third active site consists of a five amino acid NLS at the extreme C-terminal of E1A; efficient nuclear localisation of the 243R protein is essential for cellular transformation. Sequences directly upstream of the NLS, and the protein context of the NLS have also been shown to play a significant role in the efficient localisation of the 243R protein (Douglas and Quinlan, 1996).

The cellular proteins important for immortalisation and cell transformation were identified by co-immunoprecipitation with E1A. Approximately ten proteins were identified, ranging in size from 300kDa to 28kDa (Harlow *et al.*, 1989). A major advance in the understanding of viral oncogenesis came with the realisation that one of the proteins previously identified as an E1A-binding protein was the product of the retinoblastoma tumour suppressor gene (Whyte *et al.*, 1989). The physical interaction of the oncogenic E1A proteins with a key tumour suppressor protein, involved in cell

cycle control, provides a mechanistic view for the oncogenic capacity of the adenoviruses.

The retinoblastoma gene product, also known as Rb or p105, is regulated by phosphorylation i.e. the phosphorylation state of Rb changes through the different phases within the cell cycle (Ludlow *et al.*, 1990). The underphosphorylated form of Rb is thought to be the active form, and it is in this state that Rb can complex with E2F, whilst the hyperphosphorylated form is inactive (Kaelin *et al.*, 1992). Phosphorylation of Rb is controlled by cdks (for example cyclin E/cdk2 kinase, cyclin D/cdk4 kinase and cdk6), whose activity is controlled by association with a protein known as cyclin, and by their own post-translational modifications i.e their phosphorylation and dephosphorylation (Wolowiec and Ffrench, 1996). The S-phase cdks are activated at the end of the G1 phase of the cell cycle, resulting in a switch from an active to an inactive form of Rb, which is no longer able to form repressive complexes with E2F, resulting in the release of free E2F for use in S phase gene transcription. E1A is also capable of pushing the cell cycle through the commitment point into S phase by forming complexes with Rb to release E2F from repressive complexes (section 1.2.3), and by forming complexes with CBP/p300 (Ait-Si-Ali *et al.*, 1998). The E1A proteins bind to Rb and Rb-related proteins through the pocket region, which consists of two regions A and B, separated by a non-conserved spacer region, which is of different lengths in Rb and p107 (Raychaudhuri *et al.*, 1991; Corbeil and Branton, 1994). The phosphorylation of the E1A proteins at Ser-132 also regulates Rb, by enhancing their binding affinity for Rb and Rb-related proteins (Whalen *et al.*, 1996).

Cell protein p107 was first identified by association with E1A and SV40 large T antigen (Dyson and Harlow, 1992), and shares many structural and biochemical similarities with Rb, including a pocket region which binds E2F and E1A (Zhou *et al.*, 1993; Starostik *et al.*, 1996). However, unlike Rb, p107 can form complexes with cyclin A (p60), cyclin E and cdk2 (Faha *et al.*, 1993). p107 functions as a tumour suppressor, which is mediated by inactivation of E2F through p107-repressive complexes. The p107-E2F complex also acts as a general transcriptional repressor when tethered to the promoter via an E2F binding site.

Cell protein p130 is a phosphoprotein located within the nucleus of the cell, which has many similarities with Rb and p107, including a pocket region, which binds E2F and E1A. However, p130 is more closely related to p107 than Rb, as p130 and p107 have similar sized spacers between the pocket region domains A and B (Li *et al.*, 1993). Also like p107, p130 interacts with cyclins A and E, including the pocket region, and is able to bind E2F and E1A (Cobrinik *et al.*, 1993). The E1A viral oncoprotein-associated kinase targets p130, controlling phosphorylation of the pocket proteins and therefore the amount of free E2F during the cell cycle, reaching a peak of activity at the onset of S-phase (Baldi *et al.*, 1995; Mayol *et al.*, 1995).

Simultaneous E1A binding of Rb and p300/CBP is important in modulation of gene expression and oncogenic transformation by E1A (Dyson and Harlow, 1992; Peeper and Zantema, 1993; Bayley and Mymrk, 1994). E1A has been shown to bind to Rb through CR2 sequences, however it has also been shown to bind Rb and p300 simultaneously, through an LXCXE motif in CR2 which binds Rb, and through the N-terminal of CR1 which binds p300 and CBP (Wang *et al.*, 1995). E1A may act as a carrier for p300 or CBP, providing it to E2F-1 and *trans*-activating E2F-1/DP-1 by direct interaction with the C-terminal domain of E2F-1 (Trouche *et al.*, 1996; Trouche and Kouzarides, 1996); whilst binding to Rb and releasing E2F from repressive Rb complexes. By binding to these and other cellular regulatory proteins, E1A is able to alter or inhibit their normal functions within the cell, reprogramming cell growth and differentiation.

Cell protein p300 is able to function both as a coactivator and a transcriptional repressor, as such it has been found in complexes with TBP (Abraham *et al.*, 1993; Eckner *et al.*, 1994; Arany *et al.*, 1995). p300 also has similar functional properties to CBP, which is a coactivator for protein kinase A (PKA) (Lundblad *et al.*, 1995). CBP functions as a coactivator by binding, via a Cys/His-rich region termed C/H2, to a group of *bZIP* proteins, including ATF-2, c-Jun and CREB (Sano *et al.*, 1998). CBP also binds to an RNA helicase A, which is a component of the RNA polymerase II complex, suggesting that CBP functions as a coactivator, by bridging upstream enhancer elements with the basal transcription machinery (Nakajima *et al.*, 1997). CBP/p300 also possesses an intrinsic HAT activity, which is essential for its function as a coactivator. CBP repression is controlled by E1A binding to the C/H3 domain of CBP (a 12 residue

transcriptional adapter motif (TRAM), which contains binding sites for E1A and numerous cellular transcription factors e.g. E2F, TFIIB and p53), thereby inhibiting the acetyltransferase activity. The HAT activities of CBP/p300 are differentially modulated by factors such as p300/CBP interacting protein (p/CIP) and p300/CBP associated factors (p/CAF), which bind to distinct regions within CBP/p300. These interactions are likely to result in differential effects on the coactivator functions of CBP/p300 for different classes of transcription factors (Perissi *et al.*, 1999; O'Connor *et al.*, 1999).

1.3 Enteric Adenoviruses

Viral gastroenteritis in children can be caused by several different pathogens including adenovirus, which is second only to rotavirus as a causal agent (Estes *et al.*, 1983; Uhnoo *et al.*, 1984; Brandt *et al.*, 1985). Human adenoviruses had been suspected of causing gastroenteritis, but it had been difficult to prove, and was hampered by prolonged periods of asymptomatic faecal shedding after adenovirus respiratory tract infections (Fox *et al.*, 1977). The subgroup F adenoviruses were established as casual agents of gastroenteritis by 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). A close correlation has now been established between Ad40 and Ad41, and gastroenteritis (Brandt *et al.*, 1979; de Jong *et al.*, 1983; Yolken *et al.*, 1982; Chiba *et al.*, 1983; Uhnoo *et al.*, 1983, 1984; Leite *et al.*, 1985; Kidd *et al.*, 1986; Kotloff *et al.*, 1989).

1.3.1 Identification and classification

The enteric adenoviruses were first identified from stool samples of infants with acute gastroenteritis (Flewett *et al.*, 1973), and then observed by electron microscope (EM) analysis (Gary *et al.*, 1979; Retter *et al.*, 1979; Takiff and Straus, 1982). The viruses identified in the stool samples of infants with acute gastroenteritis, were unable to be cultivated on conventional cell lines used to propagate other adenoviruses (Flewett *et al.*, 1973; Madeley *et al.*, 1977; Retter *et al.*, 1979). However, they would grow in 293 cells, a human embryo kidney (HEK) cell line which has been transformed with the Ad5 E1 region (Graham *et al.*, 1977; Takiff *et al.*, 1981), albeit at reduced levels compared to other serotypes.

The enteric adenoviruses were shown to be distinct from the other established adenovirus subgroups, by serology and DNA restriction analysis (Jacobsson *et al.*, 1979; Johansson *et al.*, 1980; Kidd and Madeley, 1981). Studies with large numbers of clinical isolates revealed the presence of two enteric adenovirus serotypes, 40 and 41, (de Jong *et al.*, 1983; Uhnoo *et al.*, 1983) which possessed different DNA restriction patterns (Uhnoo *et al.*, 1983; Takiff *et al.*, 1984; Adrian *et al.*, 1986). The two serotypes identified are now classified separately as subgroup F adenoviruses; based upon various

criteria including immunological cross reactivity, DNA homology and size of internal structural polypeptides (van Loon *et al.*, 1985a; Wadell *et al.*, 1987; Hierholzer *et al.*, 1988). Within each serotype a number of variants were observed through restriction enzyme analysis, including eleven variants of Ad40 and twenty-eight variants of Ad41 (Kidd, 1984; Kidd *et al.*, 1984; van der Avoort *et al.*, 1989), for which a classification scheme has been presented (van der Avoort *et al.*, 1989; Kidd *et al.*, 1993).

1.3.2 Epidemiology

Enteric adenoviruses have been detected in stool samples of infants and young children with acute gastroenteritis, in the developed and developing world (reviewed in Uhnoo *et al.*, 1990; Wadell *et al.*, 1994; Mautner *et al.*, 1995). Enteric adenoviruses are reported to be associated with approximately 5 to 20 percent of cases of paediatric diarrhoea (Mautner *et al.*, 1995); being prevalent throughout the year, with little seasonal variation, indicating that they may be endemic (de Jong *et al.*, 1983, 1993; Uhnoo *et al.*, 1984; Brandt *et al.*, 1985; Johansson *et al.*, 1985; Tiemessen *et al.*, 1989; Kotloff *et al.*, 1989).

1.3.3 Pathogenesis

The incubation period for enteric adenovirus infection is 7 to 8 days (Richmond *et al.*, 1979). 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 gastroenteritis lasts longer than other types of viral gastroenteritis, however it produces a milder infection with reduced frequency of vomiting and moderate elevation of temperature. The most prominent feature of the disease is watery diarrhoea, which is followed by 1 to 2 days of vomiting (Uhnoo *et al.*, 1984). The mean duration of diarrhoea in Ad41 (12.2 days) is longer than in Ad40 (8.6 days), however both viruses gave similar symptoms in patients. Other symptoms include low grade fever which lasts two to three days (rare), dehydration, and respiratory tract symptoms which occur in less than 20% of cases (Yolken *et al.*, 1982; Uhnoo *et al.*, 1984). Enteric adenoviruses have rarely been associated with fatal disease (Whitelaw *et al.*, 1977; Johansson *et al.*, 1985), but Ad41 virus particles were isolated from cells in the small intestine of a fatal case of gastroenteritis. On propagation in tissue culture this strain did not appear to be

unusually virulent (Johansson *et al.*, 1985). Nosocomial outbreaks occur (Rodriguez *et al.*, 1985; Kotloff *et al.*, 1989), but spread to adults is not common (Chiba *et al.*, 1983).

1.3.4 The molecular biology of the enteric adenoviruses

The overall DNA homology between Ad40 and Ad41 is 62 to 69 percent (van Loon *et al.*, 1985a), whereas enteric adenovirus identity compared to Ad5 is only 15 to 20 percent. Phylogenetic analysis (the comparison of DNA sequence) reveals that Ad40 and Ad12 are more closely related than was previously shown by DNA hybridisation studies (Bailey and Mautner, 1994; Mautner *et al.*, 1995). This is also observed in the homology within subgroup F, which is similar to that within subgroup A, but is lower than that seen within subgroups B to D (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 nucleotides, having similar genomic organisation to Ad2 and Ad5. The major differences between the Ad40, and the Ad2 and Ad5 genomes, are the presence of two fibre genes, one VA gene and variation within the E3 region of Ad40 (Davison *et al.*, 1993).

1.3.4.1 Early regions

The E1 region of subgroup F adenoviruses has been extensively studied, because it has been implicated in the fastidious growth of the enteric adenoviruses. The E1 sequences of various strains of Ad40 and Ad41 have been determined including: Ad40 Dugan (van Loon *et al.*, 1987a; Davison *et al.*, 1993); Ad40 Sapporo (Ishino *et al.*, 1988); Ad41 Tak (van Loon *et al.*, 1987a); and Ad41 D389 (Allard and Wadell, 1988, 1992). The E1 sequences of both Ad40 strain Dugan and Ad41 strain Tak are 85% identical to each other, and 52% homologous to the Ad5 E1 region (van Loon *et al.*, 1987a). The overall organisation of the E1 region appears to be similar to that of the other adenoviruses. The ITR of adenovirus type 40, type 41 and type 12 are similar in length at 163bp, 163bp and 161bp respectively. However, the first 60-70bp of the adenovirus type 40 genome bears greater sequence homology with adenovirus type 5 than any other adenovirus serotype (Ishino *et al.*, 1987; Shinagawa *et al.*, 1987; Allard and Wadell, 1988; Bailey and Mautner, 1994).

Early region 1A

Ad40 E1A mRNA is first detected approximately 36h p.i. in Ad40 infected cells (Ullah, 1997) compared with approximately 3h p.i. in Ad5 infected cells (Glenn and Ricciardi, 1988). Both Ad40 and Ad41 have the potential to make E1A 13S, 12S and 9S transcripts (figure 1.3.4.1) (van Loon *et al.*, 1987a; Ishino *et al.*, 1988; Allard and Wadell, 1988). However, E1A transcription maps have only been obtained for E1 plasmid-transformed cells, and whilst all three mRNAs (corresponding to the Ad2 13S, 12S and 9S mRNAs) were detected in Ad40 E1 plasmid-transformed cells, only the 13S transcript was detected in Ad41 E1 plasmid-transformed cells (van Loon *et al.*, 1987a). CR1, CR2 and CR3 identified within other adenovirus E1A proteins (Kimelman *et al.*, 1985) have also been identified in Ad40 and Ad41 (van Loon *et al.*, 1987a; Allard and Wadell, 1988; Ishino *et al.*, 1988). The conserved regions of Ad7, Ad12 and SAV-7 share 60% homology to the equivalent Ad5 conserved regions, whilst Ad40 and Ad41 have 51% and 45% homology respectively to the equivalent Ad5 conserved regions (van Loon *et al.*, 1987a). Ad40 CR1 has the least homology, although Ad40 CR2 and CR3 also have low homology with Ad5 compared to other serotypes. CR3 is the *trans*-activating domain of the E1A protein and differences in this sequence may account for the low *trans*-activating function of Ad40 and Ad41 E1A, compared with Ad5 and Ad12 (van Loon *et al.*, 1987a; Ishino *et al.*, 1988).

Early region 1B

The Ad40 and Ad41 E1B transcription maps have been determined in a lytic infection (figure 1.3.4.1; Steinthorsdottir and Mautner, 1991; Allard and Wadell, 1992). Both Ad40 and Ad41 produce transcripts equivalent to the Ad2 14S and 22S mRNA, but no 13S mRNA equivalent was seen, which is the major species in the Ad2 E1B region. The Ad12 E1B region also produces the E1B 14S mRNA as a major transcript, with no detectable 13S mRNA (Virtanen *et al.*, 1982; Virtanen and Pettersson, 1985). In Ad41 an additional small exon is detected in the 14S mRNA which is not observed in Ad40 (Steinthorsdottir and Mautner, 1991; Allard and Wadell, 1992). In addition E1A-E1B co-transcript counterparts of the 14S and 22S mRNAs have been detected in Ad40, these contained the first 40 codons of the E1A region spliced to a site 4-5 nt downstream of the E1B cap site (figure 1.3.4.1). The splice junction is unusual as it does not conform to splice consensus sequences (Steinthorsdottir and Mautner, 1991;

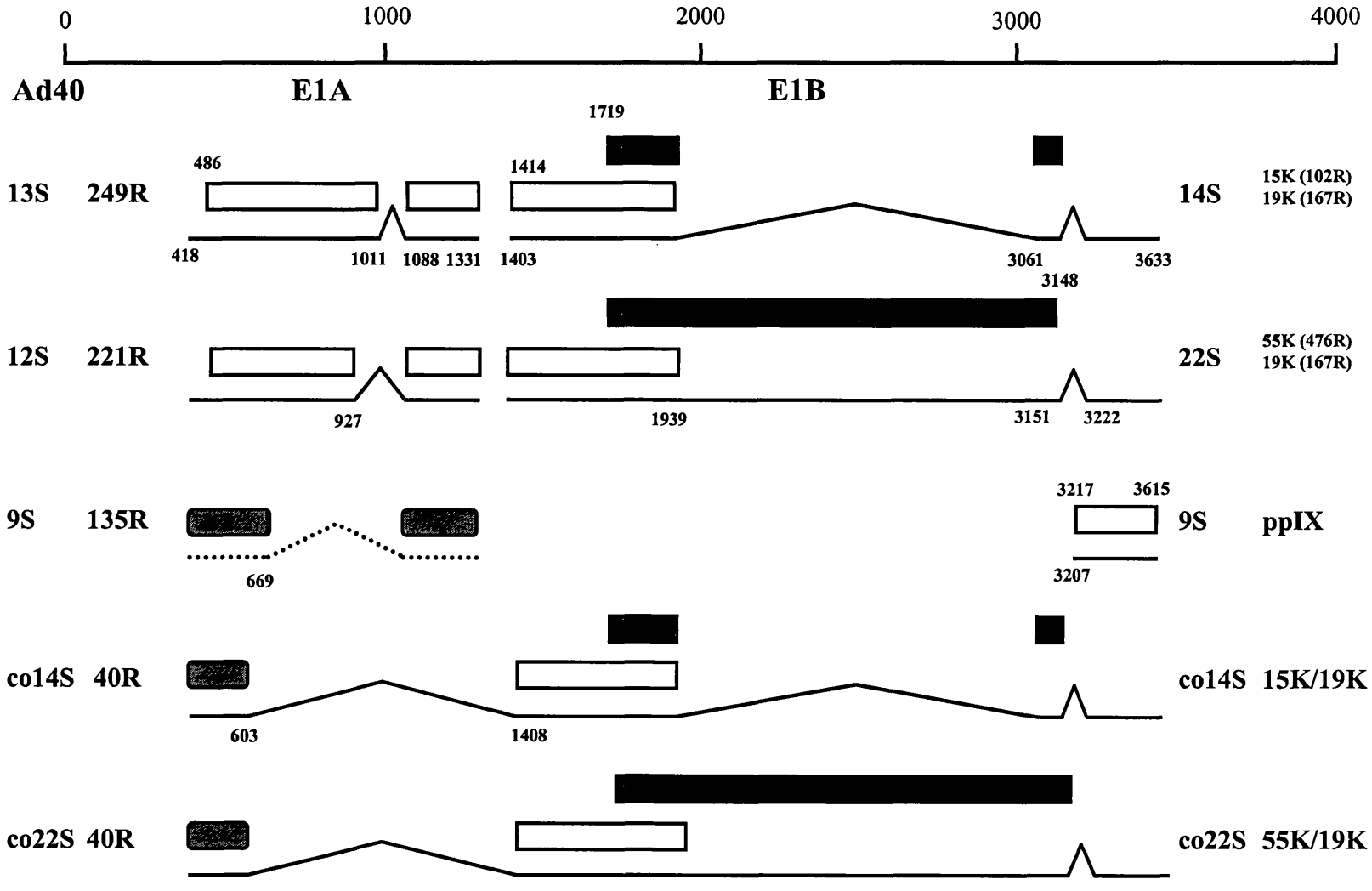


Figure 1.3.4.1 Map of transcription from the Ad40 strain Dugan E1A region. Solid lines denote RNA with introns indicated by carets; filled rectangles are open reading frames; rectangles with rounded corners are proteins which have not been confirmed experimentally (reproduced from Mautner *et al*, 1995).

Ishida *et al.*, 1994). In a productive Ad5 infection, E1B 22S mRNA is produced at early times in infection, and E1B 13S mRNA is produced after the onset of DNA replication (Montell *et al.*, 1984b; Glenn and Ricciardi, 1988). In HeLa cells infected with Ad40, no E1B mRNA is detected early in infection, and only low levels of the 22S and 14S mRNAs are observed after the onset of DNA replication (Mautner *et al.*, 1990; Bailey *et al.*, 1994). However in 293 cells, KB16 and INT407 cells, 22S mRNA is detected before the onset of DNA replication, and the 14S mRNA is first observed at the onset of DNA replication (Bailey *et al.*, 1994). Ad40 E1A and E1B mRNAs have also been detected before the onset of DNA replication in A549 cells (Hashimoto *et al.*, 1991).

In comparisons of the E1B protein coding sequences, the Ad40 E1B ORFs are more similar to Ad12 than to Ad2, and the E1B DNA homology is higher than for E1A (Ishino *et al.*, 1988). Unlike the E1A region there are no conserved regions in E1B, however an alanine rich region near the N-terminal of the 55kDa protein which is present in Ad2 and Ad12, is not found in Ad40. A second central region within the 55kDa protein responsible for p53 binding, transcriptional repression, and important for the transformation activity of Ad2, is moderately conserved in both Ad12 and Ad40 (Yew and Berk, 1992; Yew *et al.*, 1990, 1994). The E1B 19kDa and 55kDa proteins can be detected in 293 and Kba+b cells (Bailey *et al.*, 1993, 1994), whereas 55kDa cannot be detected in HeLa cells (Mautner *et al.*, 1990), and 19kDa can only be detected in HeLa cells at late times during infection (White *et al.*, 1991; Rao *et al.*, 1992).

Early region 2

The E2A gene has been identified in Ad40 and Ad41 (Vos *et al.*, 1988), and encodes for a single stranded DBP, which is smaller in Ad40 (473R) and Ad41 (474R), than in Ad5 (529R). The Ad40 E2B gene encodes two proteins, pTP and Adpol, these share good DNA homology with the Ad2 equivalent proteins (Davison *et al.*, 1993). Less DNA homology is shared between the Ad40 DBP and the Ad2 DBP (Davison *et al.*, 1993). Another 52R exon has been identified upstream of the recognised E2 early and late promoters, between the E3 and fibre regions. Its sequence is highly conserved across all human adenoviruses and canine adenoviruses, which suggests that the region is functionally significant (Davison *et al.*, 1993).

Early region 3

The DNA sequence of the Ad40 E3 region reveals a pattern of ORF and transcriptional signals not unlike Ad2 (Cladras and Wold, 1985), however there are sufficient differences to suggest that a more detailed knowledge of the functions of the encoded genes may further our understanding of the tissue specificity of the enteric viruses. In fact, Ad40 E3 is more closely related to Ad12 than to Ad2, which may not be surprising in view of their shared site of infection. Comparison of the E3 ORFs for Ad40, Ad12, and Ad2 shows the E3B region to be well conserved, whereas there are major differences in the E3A region. The Ad40 E3A region has two large ORFs (276R and 173R) with homologies in Ad12, but unrelated to the E3A 20.1kDa and 20.5kDa proteins found in Ad3 (Signäs *et al.*, 1986). The Ad40 276R sequence bears limited similarity to the mouse interleukin-1 receptor which may be of interest in the light of established relationship between various Ad5 E3 proteins and other components of the immune response. In particular, Ad40 lacks an equivalent of the Ad2 E3A 19kDa glycoprotein, which prevents cytolysis of infected cells by cytotoxic T lymphocytes (Wold and Gooding, 1991). A small ORF can be found out of frame in the 276R sequence which has limited similarity to the Ad5 19kDa glycoprotein, however it is unlikely that this is of functional significance, unless perhaps a 19kDa equivalent is generated by alternative splicing. The 12.5kDa ORF in Ad2 E3A has a homologue in Ad12, which is absent from Ad40. Ad41 possesses three unique E3 ORFs, which may explain why subgroup F adenoviruses differ substantially from other human adenoviruses in their host range (Yeh *et al.*, 1996).

Early region 4

Ad40 complements the Ad2 E4 deletion mutant *dl*808 (which lacks all but ORF1 of the E4 region), indicating that Ad40 has a functional E4 region (Mautner and Mackay, 1991). The Ad40 E4 region contains ORFs corresponding to those identified in Ad2 and Ad12, with the notable exception of ORF1. However, it is known that E4 ORF3 and ORF6 of Ad2 display redundancy in some functions (Bridge and Ketner, 1989; Ketner *et al.*, 1989; Huang and Hearing, 1989a; Weiden and Gisberg, 1994), and this aspect of Ad40 E4 function has not been addressed.

1.3.4.2 Intermediate regions

The human adenoviruses of subgroups A, F and some of B, and the simian adenovirus type 7 (SAV-7) have only one VA RNA gene (Kidd and Tiemessen, 1993; Kidd *et al.*, 1995). Ad40 and Ad41, unlike Ad2 are susceptible to lymphoblastoid interferon in Chang conjunctival cells (Tiemessen and Kidd, 1993); it is unclear if the expression or the function of the VA RNA gene correlates with this susceptibility. Susceptibility could occur through defective expression of the E1A gene, which *trans*-activates the VA RNA gene through the cellular transcription factor TFIIIC, leading to reduced levels of VA RNA, or by aberrant function of VA RNA itself. The E1A proteins are also known to suppress transcription of interferon stimulated cell genes, so impaired E1 activity may act at this level.

1.3.4.3 Late regions

The subgroup F hexon proteins of Ad40 and Ad41 have 88% identity, which is in agreement with that detected between the subgroup C adenoviruses (Kinloch *et al.*, 1984; PringAkerblom and Adrian, 1993). Most differences occur in the loop regions, which are exposed on the surface of the virion (Toogood and Hay, 1988; Toogood *et al.*, 1989), and act as type specific antigenic determinants (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 RGD motif lies within an alanine rich region that is common to Ad2 and Ad5, but is again absent from Ad40, avian adenovirus GAL-10 and Ad12; it is therefore possible that the enteric adenoviruses do not utilise this pathway for virus internalisation (Sheppard and Trist, 1992; Davison *et al.*, 1993). Furthermore there are no other recognised integrin-binding sites within the known penton base sequences (Hynes, 1992). The enteric adenoviruses have two fibre genes (IV-1 and IV-2), which encode proteins with distinct sequences, and different shaft lengths (Kidd and Erasmus, 1989; Pieniazek *et al.*, 1989, 1990c; Kidd *et al.*, 1990, 1993; Davison *et al.*, 1993; Yeh *et al.*, 1994). Phylogeny studies have revealed that the subgroup F fibre genes are more closely related to each other than to other adenovirus fibre genes (Bailey and Mautner, 1994). This could have arisen from gene duplication shortly after divergence of subgroups A and F, but before divergence of serotypes 40 and 41. EM analysis of Ad40 shows that only one type of fibre is

incorporated at each vertex of the capsid (Kidd *et al.*, 1993), in contrast to avian adenoviruses where two fibres of different shaft length are attached to each vertex (Laver *et al.*, 1971). The 100kDa L4 protein is the best conserved of the L4 proteins among the serotypes Ad40, Ad12 and Ad2. The 33kDa protein is also well conserved, and a putative 22kDa polypeptide could be generated if the 33kDa splice is not used (Davison *et al.*, 1993).

1.3.5 Growth properties of enteric adenoviruses in tissue culture

Although Ad40 virus particles are shed in large numbers from the gut they fail to propagate in cells normally used to propagate other human adenoviruses. They will however grow in Chang conjunctival cells (Kidd and Madeley, 1981), Hep-2 and tCMK cells (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), albeit at reduced levels compared to other serotypes. There has been dispute over these results, as some studies have shown that the enteric adenoviruses can be propagated in these cells (Takiff *et al.*, 1981; de Jong *et al.*, 1983), whilst others have not been able to propagate the virus in these cells (Chiba *et al.*, 1983; Uhnoo *et al.*, 1983). For example, Witt and Bousquet (1988) successfully grew Ad40 and Ad41 in Chang conjunctival, 293 and KB cells, but Ad41 would only grow in HeLa cells. Whereas, Pieniazek *et al.* (1990a, b) successfully propagated Ad41 in HeLa, Hep-2 and INT407 cells, but they could not propagate the virus in 293 cells. In addition, one group has reported that the Sapporo strain of Ad40 can produce plaques in A549 cells (Hashimoto *et al.*, 1991). A study of approximately 200 enteric adenovirus isolates suggested that these contrasting observations may result from differences in virus growth due to the virus strain used, the host cell type used, differences in the growth characteristics of the cell line depending upon passage number, and differences in the growth of the same virus strain between laboratories (de Jong *et al.*, 1983). Another factor, which may account for the apparent differences in the growth properties of Ad41 is serum concentration. As growth of Ad41 is restricted in a number of primary cell lines, but can be alleviated by a reduction in the serum concentration (0.2-1%) within the infecting medium. The apparent restriction of Ad41 growth in primary cell lines is thought to be mediated by a factor or factors present in the serum (Pieniazek *et al.*, 1990b).

Within an Ad40 infection in KB and HeLa cells, lower infectious titres of Ad40 compared with Ad41 are found, and when high moi of virus was used no viral DNA replication could be detected. However, Ad40 will grow at a lower moi of input virus (Witt and Bousquet, 1988). This effect may be mediated by defective virus particles, which interfere with infectious non-defective virions. In 293 cells the production of Ad40 virions is 3 to 10 fold lower than in other adenoviruses, and the yield of infectious virus is 100 to 1000 fold lower (Brown, 1985). The majority of 293 cells in a culture can be productively infected, whilst only 10 to 20 percent of HeLa cells are infected, however the uninfected HeLa cells are capable of being infected. For 293 and HeLa cells the intracellular viral yield was comparable, however progeny virus are only released from 293 cells. The yield of viral particles is similar for the enteric adenoviruses compared with Ad5, however the particle to infectivity ratio and the block in release of virus from HeLa cells could be a major factor in the fastidious growth of enteric adenoviruses in tissue culture (Brown *et al.*, 1992).

On the other hand the Ad41 growth restriction in tissue culture appears to be less severe than in Ad40, as a number of cell lines will support Ad41 growth but not Ad40 (de Jong *et al.*, 1983; Uhnoo *et al.*, 1983, 1984; van Loon *et al.*, 1985b). The growth restriction of Ad41 can be complemented by Ad2 in semi-permissive Chang conjunctival cells and non-permissive HEF cells. However, in HEF cells Ad41 interferes with Ad2 replication, the extent of interference depends upon the time for infection of each virus (Tiemessen and Kidd, 1988; 1990). This observation was made with other serotypes, and is mediated by the *trans*-repression function of the E1A proteins (Leite *et al.*, 1986; Tibbetts *et al.*, 1986; Larsen and Tibbetts, 1987). The Ad41 block in replication has been shown to occur within the early phase of the infectious cycle (Tiemessen *et al.*, 1996).

Ad40 can propagate in 293 cells (Takiff *et al.*, 1981), a human embryo kidney (HEK) cell line which has been transformed with the Ad5 E1 region (Graham *et al.*, 1977; Kidd and Madeley, 1981), albeit at reduced levels compared to other serotypes. This observation suggests that the E1 function is poorly expressed, and is therefore implicated in the growth restriction of enteric adenoviruses. To date this has mainly been attributed to the E1B function (Mautner *et al.*, 1995), however the Ad40 E1A

region may also be subject to aberrant expression, as Ad40 E1A mRNA is first detected at approximately 36h p.i. in Ad40 infected cells (Ullah, 1997) compared with approximately 3h p.i. in Ad5 infected cells (Glenn and Ricciardi, 1988). The Ad40 E1A promoter has been shown to have a weaker *cis*-acting potential than the Ad12 E1A promoter (Ishino *et al.*, 1988). The Ad40 and Ad41 E1A proteins have also been shown to be weaker *trans*-activators of viral promoters (Ad2 E4, Ad5 E2 and E3) when compared with the Ad5 or the Ad12 E1A proteins (van Loon *et al.*, 1987b; Ishino *et al.* 1988).

The poor expression of the E1 function has mainly been attributed to E1B, through indirect evidence such as the ability of Ad40 and Ad41 to partially transform BRK cells (van Loon *et al.*, 1985b), and through more direct evidence of propagation of Ad40 in stably transformed KB cell lines which constitutively expressed Ad2 E1A (KB8), E1B (KB18), both E1A and E1B (KB16, from now on referred to as KBa+b) and neither E1A or E1B (KB7) (Babiss *et al.*, 1983); Ad40 was able to grow in cells expressing E1B, but unable to grow in cells expressing E1A alone. The latter observation is supported by coinfection assays on HeLa cells, where there was reciprocal complementation between Ad40 and the Ad5 E1A mutant *dl*312, but not with Ad5 or Ad12 mutants which lacked the E1B region or were defective for the E1B 55kDa protein (Mautner *et al.*, 1989; Steinthorsdottir and Mautner, 1991; Gomes *et al.*, 1992).

To characterise expression of the Ad40 E1B region Ad5/Ad40 recombinants were constructed, to contain the Ad40 E1B coding region in place of the Ad5 E1B coding region in dl309, under the control of either the Ad5 E1B promoter (sub5P) or the Ad40 E1B promoter (sub40P). The yield and growth rate of sub5P and sub40P were reduced in HeLa cells compared with 293 cells, whereas both were able to plaque on 293 cells but not on HeLa cells. These observations suggested that even when the Ad40 E1B region was under the control of the Ad5 E1B promoter, Ad40 E1B gene expression and/or protein function was impaired. Analysis of E1B transcription from sub5P and sub40P revealed a cell-type specific pattern, where the activity of the Ad40 E1B promoter is lower than of the Ad5 E1B promoter in all cell types, and the pattern of E1B RNA splicing and temporal regulation is characteristic of cell type, but the same for each recombinant. These additional observations suggested that not only was the Ad40

E1B promoter important in determining the growth characteristics of Ad40, but also host cell type is important (Bailey *et al.*, 1994).

The Ad40 E1B promoter has been shown to have minimal basal activity, and to be poorly *trans*-activated by the Ad40 E1A proteins, Ad5 E1A proteins and the promiscuous varicella zoster virus (VZV) *trans*-activator p140 (Everett, 1987), when compared with the Ad5 E1B promoter (Steinthorsdottir, 1991). The Ad5 E1B core promoter consists of a TATA box and an Sp1 binding site (Wu *et al.*, 1987), although that upstream sequences are known to modulate its efficient functioning (Parks *et al.*, 1988; Parks and Spector, 1990; Spector *et al.*, 1993). The Ad40 E1B promoter like the Ad5 E1B promoter consists of TATA box and an Sp1 binding site. The Sp1 binding site differs from that found in Ad5, but is identical to the Ad12 Sp1 binding site, it also has a reduced affinity for the Sp1 protein, relative to the Ad5 site, which along with differences in Sp1 binding site core sequence influences Sp1 recognition. Sequences upstream of the Sp1 binding site mediate downregulation of the Ad40 E1B promoter; these sequences contain two protein binding sites which have been implicated in this effect (Mautner *et al.*, 1999).

1.3.6 Aims of the project

The enteric adenovirus type 40 fails to grow in conventional cell lines used to propagate other human adenoviruses, but can be propagated in cells which express the E1 proteins, albeit at reduced levels compared with other adenoviruses. This suggests that the E1 function is poorly expressed, which has mainly been attributed to defects in E1B (Mautner *et al.*, 1995), however E1A expression has also been implicated (Ullah, 1997; van Loon *et al.*, 1987b; Ishino *et al.*, 1988). The aim of the work presented in this thesis was to characterise the Ad40 E1A region, in particular basal and *trans*-activated transcription from the E1A promoter.

2. MATERIALS

2. Materials

2.1 Plasmids

The following plasmids were provided by the acknowledged authors or companies:

pNM80: (Mautner *et al.*, 1989) contains Ad40 E1A sequences from 1-1643 nucleotides.

pLE1A: (Dery *et al.*, 1987) contains Ad5 E1A sequences from 1-1704 nucleotides.

pnull-luc: previously known as pBSKLT-6 (Dr. W. Dietrich-Goetz) contains a firefly (*Photinus pyralis*) luciferase gene upstream of the herpes simplex virus (HSV)-2 immediate early (IE)-5 polyadenylation signal, cloned via *Hind* III and *Bam* HI restriction sites into the multiple cloning site (MCS) of pBluescript (figure 2.1.1).

pHSV2IE5-luc previously known as pKLT-55 (Dr. W. Dietrich-Goetz) contains an HSV-2 IE-5 promoter, inserted via a *Cla* I restriction site into the MCS upstream of the firefly luciferase gene *luc* in pnull-luc (figure 2.1.2).

pGL3-basic: (Promega) contains a modified firefly luciferase gene (*luc*+) upstream of the SV40 late polyadenylation signal (figure 2.1.3). The modifications which distinguish the *luc*+ gene from the native luciferase gene are as follows:

1. The C terminal tri-peptide has been removed to eliminate peroxisome targeting of expressed protein.
2. Codon usage was improved for expression in plant and animal cells.
3. Two potential sites of N-glycosylation were removed.
4. Several DNA sequence changes were made to disrupt extended palindromes, remove internal restriction sites, and eliminate consensus sequences recognised by genetic regulatory binding proteins, thus helping to ensure that the reporter gene itself is unaffected by spurious host transcriptional signals.

pGL3-control: (Promega) contains a SV40 promoter inserted between the MCS and

the modified firefly luciferase gene, and a SV40 enhancer inserted after the SV40 late polyadenylation signal in pGL3-basic (figure 2.1.4).

pRL-null: (Promega) contains a sea pansy (*Renilla reniformis*) luciferase gene upstream of the SV40 late polyadenylation signal (figure 2.1.5).

pRL-SV40: (Promega) contains the SV40 early enhancer and promoter upstream of the sea pansy luciferase gene in pRL-null (figure 2.1.6).

pCMV19K: (White and Cipriani, 1990) encodes the Ad5 E1B 19kDa protein, which is transcribed from the cytomeglovirus (CMV) immediate early promoter, then processed under the control of the SV40 late polyadenylation signal.

pCMV13S: (Morris and Mathews, 1991) encodes the Ad5 13S E1A protein, which is transcribed from the CMV immediate early promoter, then processed under the control of the SV40 late polyadenylation signal.

pCRII: (Invitrogen TA Cloning Kit) contains a MCS inserted into the *LacZ* gene (figure 2.1.7).

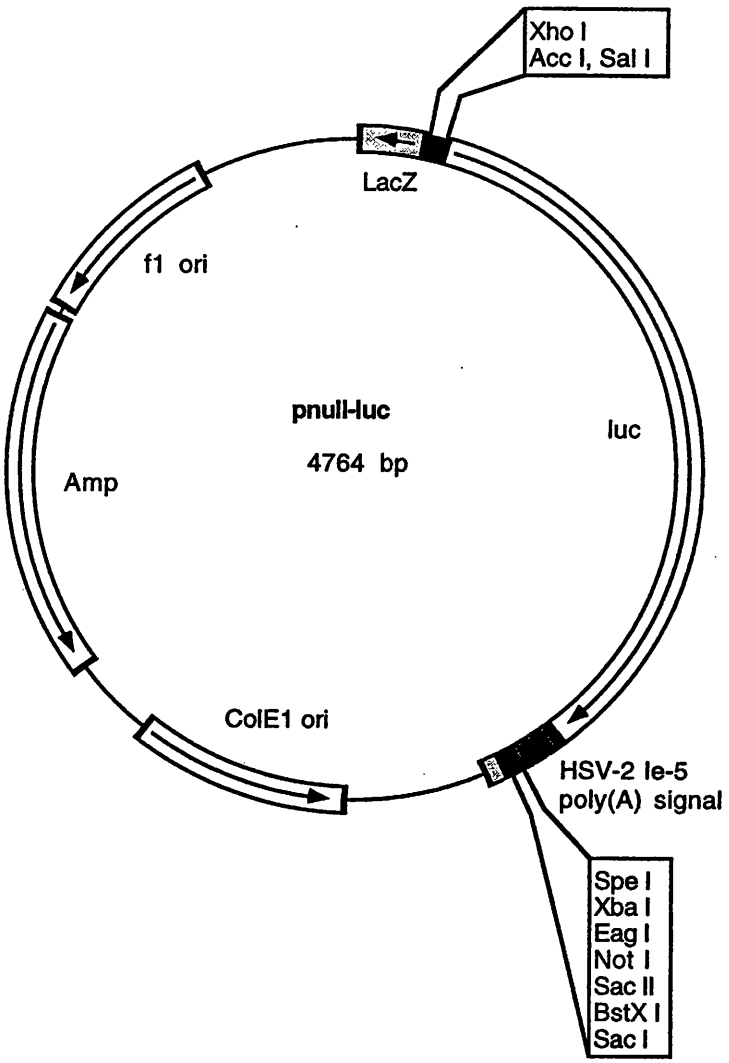


Figure 2.1.1 pnull-luc. Additional description: *luc* denotes a cDNA encoding firefly luciferase; *LacZ* denotes a cDNA encoding the N-terminal peptide fragment of β-galactosidase; *Amp* denotes gene conferring ampicillin resistance in *E. coli*; f1 ori denotes origin of replication derived from filamentous phage; ColE1 ori denotes origin of replication in *E. coli*. Arrows within the *LacZ*, *luc* and *Amp* genes indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis; the arrow in ColE1 ori indicates the direction of dsDNA synthesis.

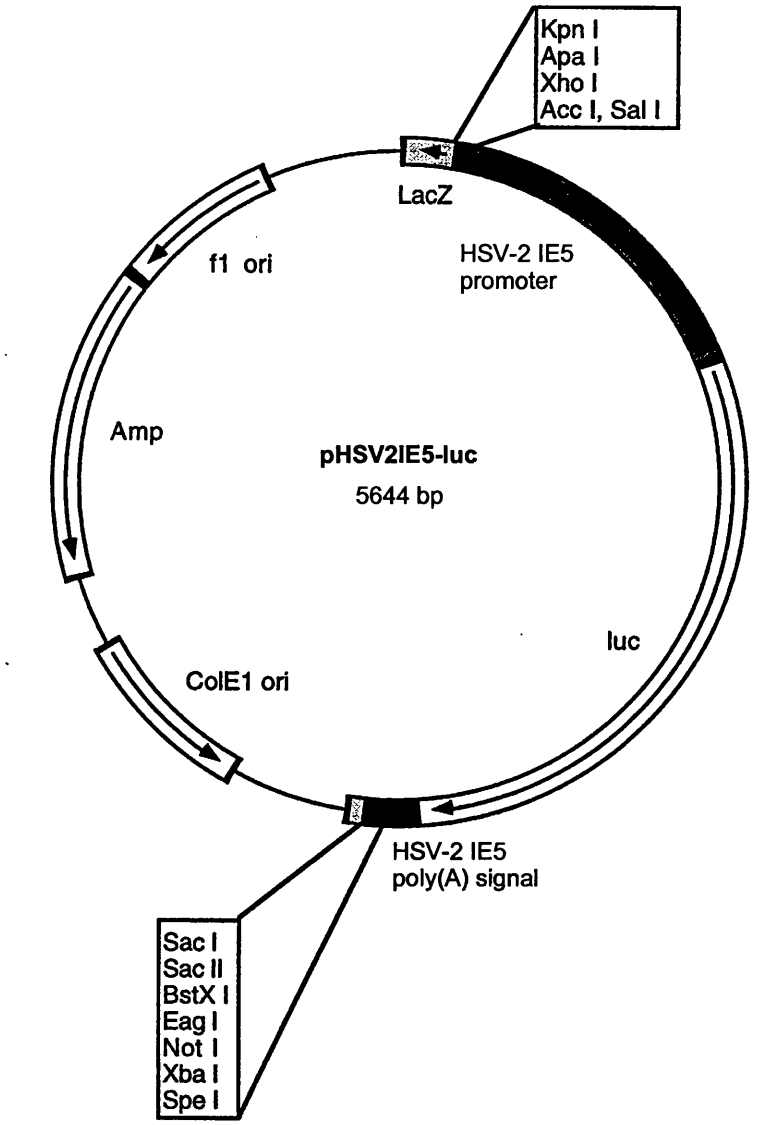


Figure 2.1.2 pHSV2IE5-luc. Additional description: *luc* denotes a cDNA encoding firefly luciferase; *LacZ* denotes a cDNA encoding the N-terminal peptide fragment of β-galactosidase; *Amp* denotes gene conferring ampicillin resistance in *E. coli*; f1 ori denotes origin of replication derived from filamentous phage; ColE1 ori denotes origin of replication in *E. coli*. Arrows within the *LacZ*, *luc* and *Amp* genes indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis; the arrow in ColE1 ori indicates the direction of dsDNA synthesis.

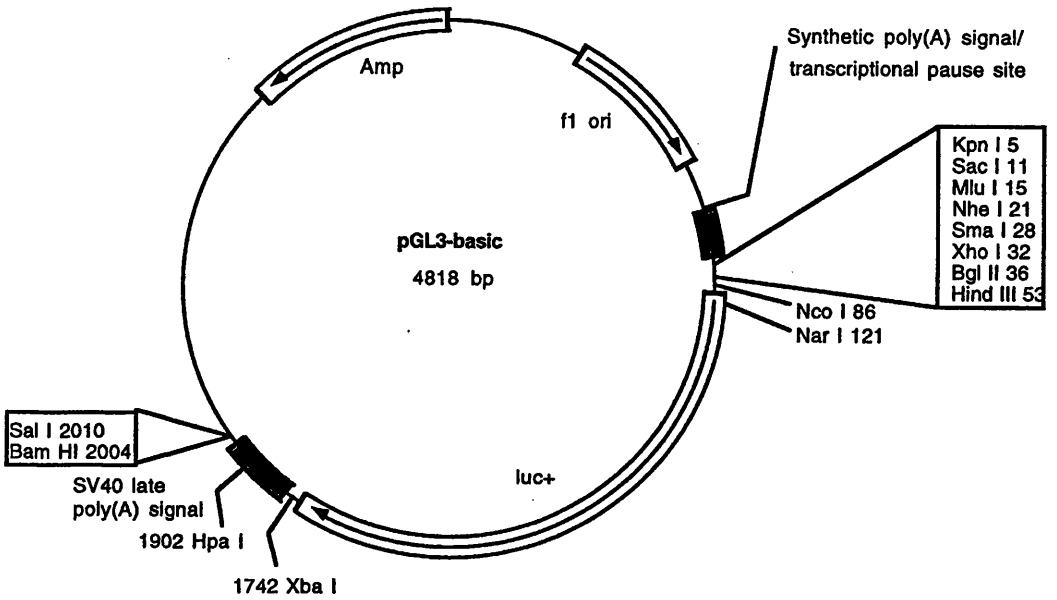


Figure 2.1.3 pGL3-basic. Additional description: *luc+* denotes a cDNA encoding a modified firefly luciferase; *Amp* denotes gene conferring ampicillin resistance in *E. coli*; f1 ori denotes origin of replication derived from filamentous phage. Arrows within the *luc+* and *Amp* genes indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis. Relevant restriction sites are marked with respect to the MCS.

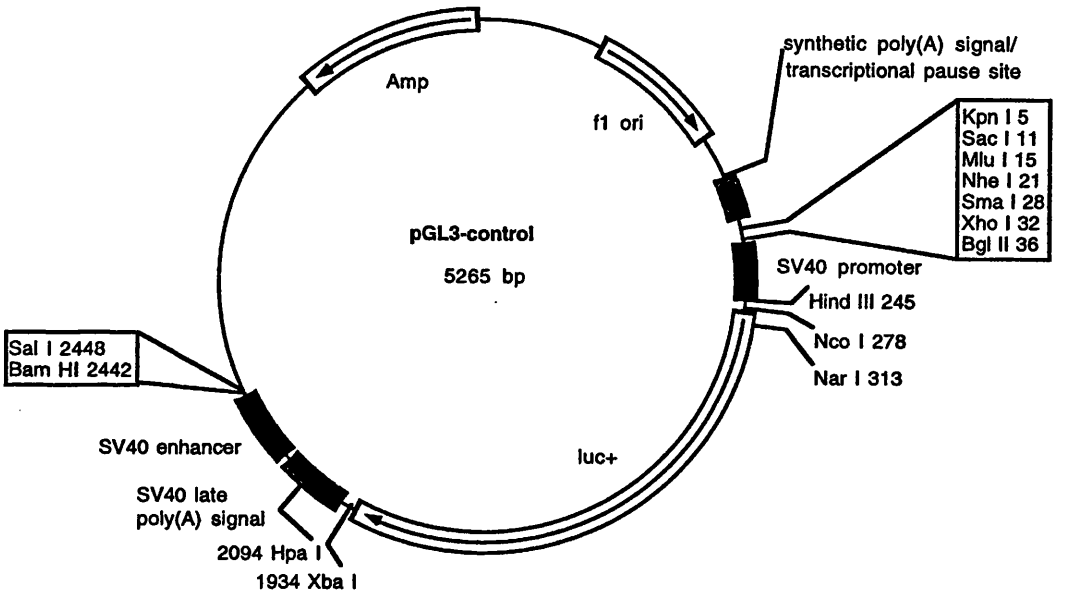


Figure 2.1.4 pGL3-control. Additional description: *luc*+ denotes a cDNA encoding a modified firefly luciferase; *Amp* denotes gene conferring ampicillin resistance in *E. coli*; f1 ori denotes origin of replication derived from filamentous phage. Arrows within the *luc*+ and *Amp* genes indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis. Relevant restriction sites are marked with respect to the MCS.

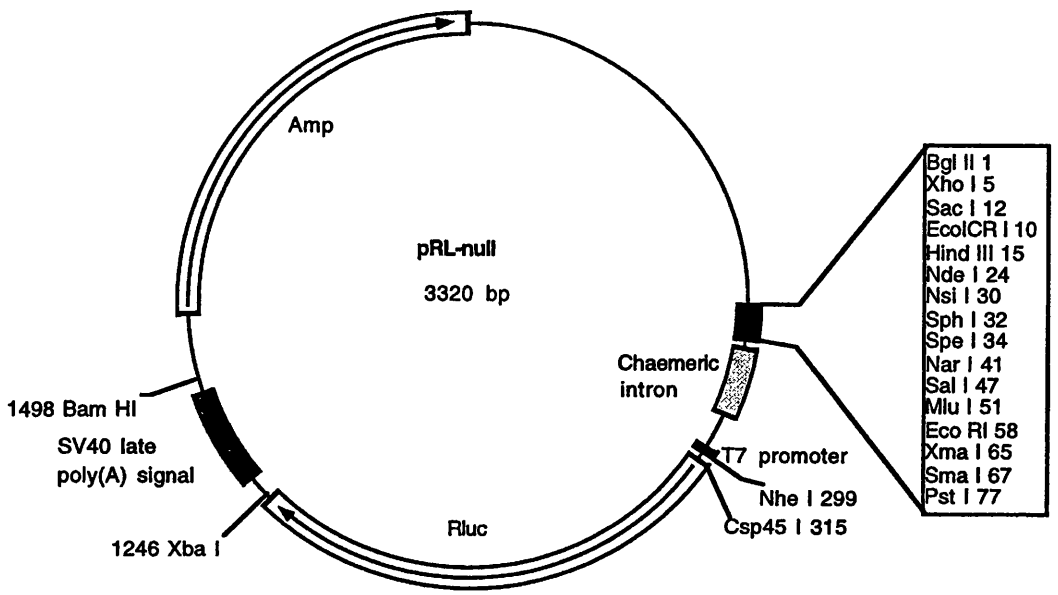


Figure 2.1.5 pRL-null. Additional description: *Rluc* denotes a cDNA encoding *Renilla* (sea pansy) luciferase; *Amp* denotes gene conferring ampicillin resistance in *E.coli*. Arrows within the *Rluc* and *Amp* genes indicate the direction of transcription. Relevant restriction sites are marked with respect to the MCS.

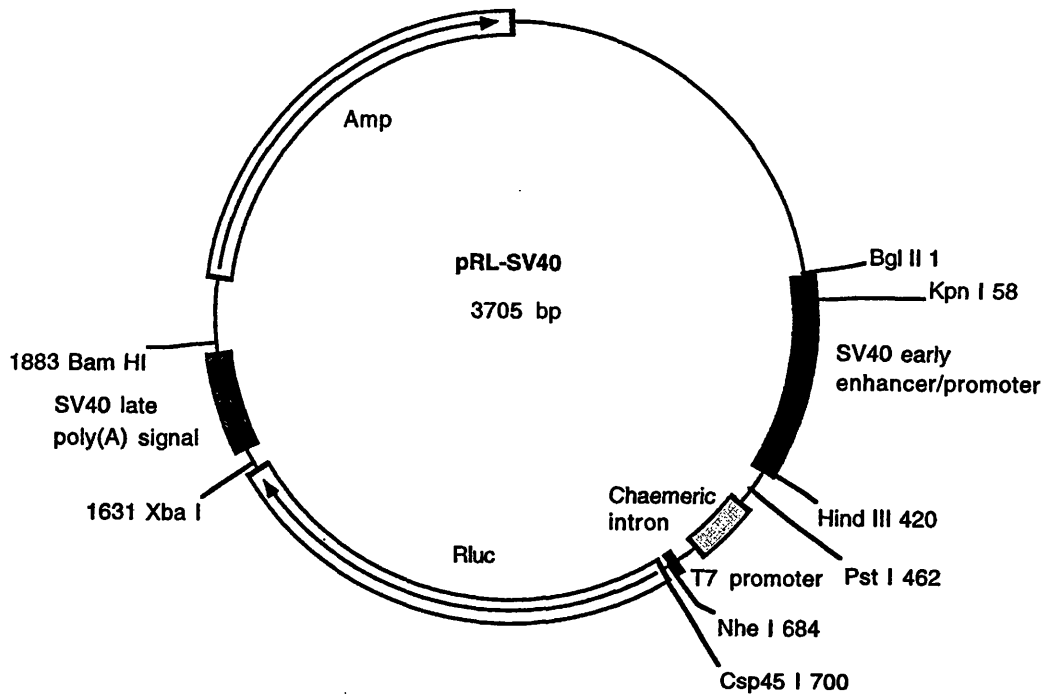


Figure 2.1.6 pRL-SV40. Additional description: *Rluc*+ denotes a cDNA encoding *Renilla* (sea pansy) luciferase; *Amp* denotes gene conferring ampicillin resistance in *E. coli*. Arrows within the *Rluc*+ and *Amp* genes indicate the direction of transcription. Relevant restriction sites are marked with respect to the MCS.

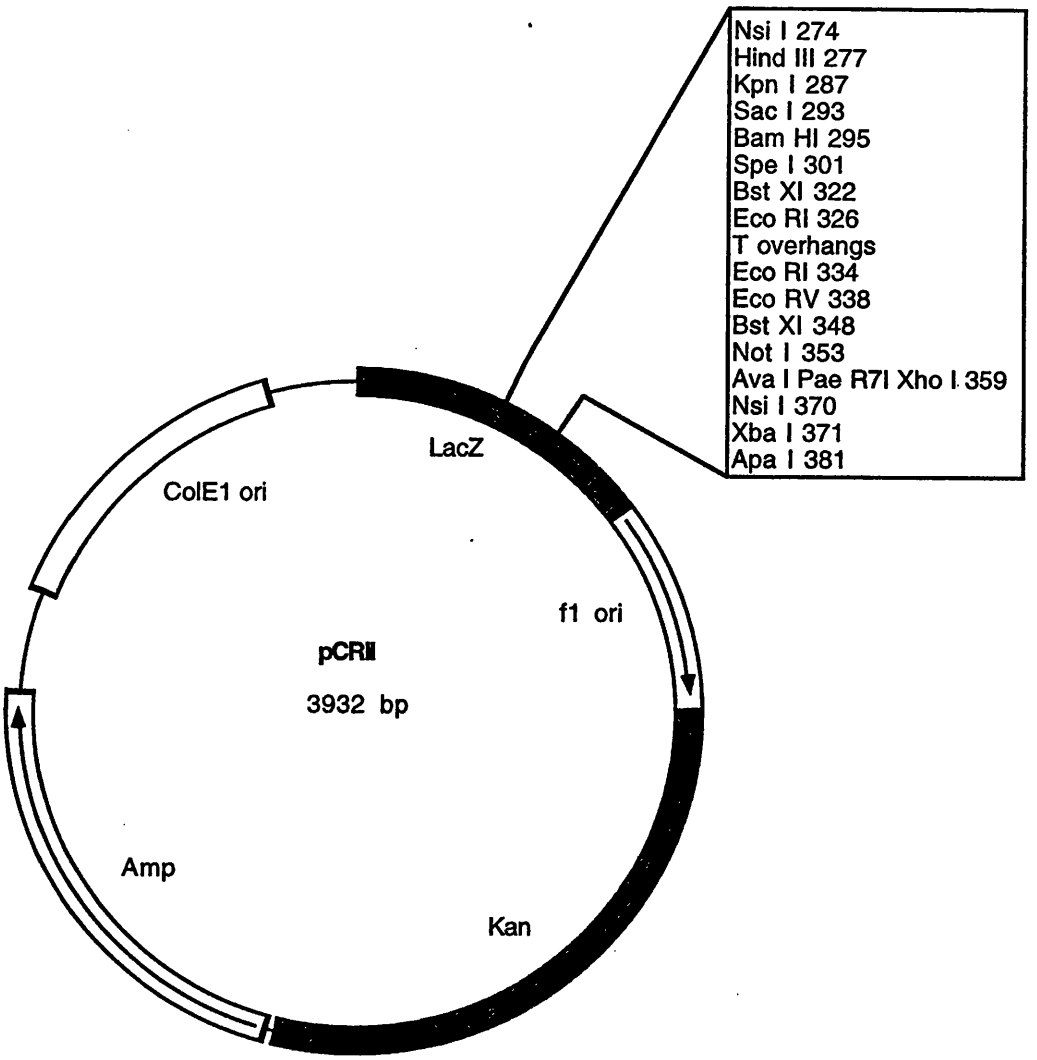


Figure 2.1.7 pCRII. Additional description: *LacZ* denotes a cDNA encoding the N-terminal peptide fragment of β-galactosidase; *Kan* denotes gene conferring kanamycin resistance in *E. coli*; *Amp* denotes gene conferring ampicillin resistance in *E. coli*; f1 ori denotes origin of replication derived from filamentous phage; ColE1 ori denotes origin of replication in *E. coli*. Arrows within the *LacZ*, *Kan* and *Amp* genes indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis.

2.2 Bacteria

The *Escherichia coli* (*E. coli*) strain NM522 [recA$^+$ (supE thi δ(lac-proAB) hsd5 (F′ proAB lacI^q lacZδM15))] was used for maintenance and propagation of pNM80 and pLE1A DNA. The *E. coli* strain DH5α [F′/endA1 hsdR17 ($r_k^-m_k^+$) supE44 thil recA1 gyrA (Nal^r) relA1 Δ(lacZYA-argF)U169 (Φ80dlacΔ(lacZ)M15] was used for maintenance and propagation of pHSV2IE5-luc and pnull-luc based plasmids. The *E. coli* strain JM109 [F′ traD36 proA$^+$ proB$^+$ lacI^q lacZδM15/recA1 endA1 gyrA96 (Nal^r) hsdR17 supE44 relA1 δ(lac-proAB) mrcA] was used for maintenance and propagation of pGL3-control, pRL-SV40, pRL-null and pGL3-basic based plasmids. The *E. coli* strain INVαF′ [endA1 recA1 hsdR17(r^km^{+k}) supE44 λ$^-$ thi-1 gyrA rel a1 Φ80lacZαΔm15Δ(lacZYA-argF) deoR+ F′] was provided with the Invitrogen TA Cloning Kit for the maintenance and propagation of pCRII based plasmids.

2.3 Bacterial culture media

The *E. coli* strains NM522, DH5α and JM109 were routinely grown in L-broth (10gram (g) sodium chloride, 16g bactotryptone, 10g yeast extract in 1 litre (l) distilled water (DW). For the preparation of competent cells (section 3.1.8) 2YT broth (5g sodium chloride, 16g Bactotryptone, 10g yeast extract in 1l of DW) was used. Agar plates were made with 1.5% agar in L-broth. Media was supplemented with 50μg/ml ampicillin to maintain bacteria containing actual and derived plasmids from section 2.1. Screening for recombinant plasmids using the Invitrogen TA Cloning Kit is provided by 25μl 40mg/ml X-Gal in dimethylformamide, which was spread on top of a LB agar plate containing 50μg/ml ampicillin 1hr before plating (recombinant plasmids produce white colonies whereas non-recombinant plasmids produce blue colonies). Colonies of INVαF′ cells containing experimental plasmids were routinely grown in L-broth containing 50μg/ml ampicillin.

2.4 Synthetic oligonucleotides

Oligonucleotides AB34, FS1-5 and the M13 -20 sequencing primer were synthesised in house by Mr. J. McGeehan using a Cruachem Ltd PS250 automated DNA synthesiser. Oligonucleotides Alex1-6 were custom synthesised by Cruachem Ltd.

Table 2.4 Synthetic oligonucleotide primers.

Oligo	Nucleotide	Ad5 or Ad40	Sequence (5′→3′)
AB34	533-512 452-429	Ad5 and Ad40	ggggaga▼agcttCCTCCTCTTTTGAGATGAGCGA *Hind* III
FS1	1-20	Ad5 and Ad40	ggggg▼tcgacCATCATCAATAATATACCTT *Sal* I
FS2	485-504	Ad40	gggg▼aattcg▼gatccATGAGAATGCTGCCGGATTT *Eco* RI *Bam* HI
FS3	1310-1291	Ad40	ggga▼agcttTCAGGAGCACTTGGGGCGCT *Hind* III
FS4	610-627	Ad40	TCTTTTTGATGTGGAGGT
FS5	1201-1184	Ad40	CTGCACGGGATGGTTCTC
Alex1	512-535	Ad40	GGGAACTGGGATGACATGTTCCAG
Alex2	809-832	Ad40	GAAACTGATGAGGCTACAGAGGCG
Alex3	832-809	Ad40	CGCCTCTGTAGCCTCATCAGTTTC
Alex4	929-952	Ad40	GATTTCCATCGGGGCACTAGTGGC
Alex5	1150-1127	Ad40	TGGGGAAAATCAGTGTCCTCAGGC
Alex6	22571- 22548	Ad40	CTGGCTGCAGCTTAAGATCCTCCT

Lower case lettering denotes sequences containing restriction sites, which do not correspond to adenovirus sequences. The symbol ▼ shows the position at which the restriction enzyme cuts. The nucleotide numbers refer to the genome coordinates of Ad5 or Ad40 sequences, which are denoted in upper case lettering.

2.5 Radiochemicals

[α-^{35}S]dATP and [^{35}S]L-methionine were purchased from NEN, whilst [α-^{32}P]dCTP was purchased from Dupont, with the following specific activity:

[α-^{35}S]dATP	1250Ci/mmol (12.5μCurie (Ci)/μl)
[^{35}S]L-methionine	1175Ci/mmol (10μCi/μl)
[α-^{32}P]dCTP	3000Ci/mmol (10μCi/μl)

2.6 Enzymes

All restriction enzymes were obtained from Boehringer Mannheim except for *Aci* I, *Bsm* FI, *Bsl* I and *Hph* I which were obtained from New England Biolabs. Other enzymes were obtained from the following manufacturers:

Amersham:	RNasin
Boehringer Mannheim:	*Bal* 31 nuclease, DNA polymerase I large (Klenow) fragment, proteinase K, T4 DNA ligase
Gibco BRL Life Technologies:	*Taq* DNA polymerase
Promega:	Moloney murine leukaemia virus (M-MuLV) reverse transcriptase, RQ1 RNase free DNase
Sigma:	Lysozyme, RNase A
Stratagene:	*Pfu* DNA polymerase

2.7 Antisera and monoclonal antibodies

The anti-peptide E1B 19kDa antibody (Mautner *et al.*, 1990) which recognises an epitope within the C-terminal region of the E1B 19kDa protein was obtained from Dr V. Mautner.

The anti-Ad5 hexon monoclonal antibody (mAB) (10/5.1) was obtained from Dr I. Sharpe, Central Public Health Laboratory, Colindale.

The anti-Ad5 E1A monoclonal antibody M58 which recognises the Ad5 E1A proteins (Harlow *et al.*, 1985) through an epitope contained within the last 266 amino acids of the 13S E1A protein C-terminal region, was obtained from Pharmingen, San Diego, CA.

Protein A horseradish peroxidase (HRP) conjugate and FITC conjugated to goat anti-mouse immunoglobulin were supplied by Sigma.

2.8 Cells and tissue culture media

293 cells (ATCC N°. CRL-1573, Graham *et al.*, 1977) are an HEK cell line which has been transformed with the Ad5 E1 region. They were grown in Glasgow modified Eagle's medium supplemented with 10% foetal calf serum (FCS).

KBa+b cells (otherwise known as KB16 cells, obtained from the authors of Babiss *et al.*, 1983), are a human, oral, epidermoid carcinoma cell line which has been transformed with the Ad2 E1 region. They were grown in Glasgow modified Eagle's medium supplemented with 5% FCS.

WS HeLa cells (ATCC N°. CCL-2) are a cervical carcinoma cell line. They were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 2.5% FCS, 2.5% newborn calf serum (NCS), 4mM L-glutamine, 100 units/ml penicillin and 100μg/ml streptomycin.

2.9 Viruses

Ad5 strain 75 (obtained originally from Dr H. G. Pereira, NIMR) was propagated in 293 cells.

Ad40 strain Dugan (obtained from the authors of de Jong *et al.*, 1983) was propagated in KBa+b cells to produce a passage 9 stock (p9) which was used throughout.

2.10 Peptides

Peptides corresponding to the Ad40 E1A 13S sequences outlined in table 2.1.10 were synthesised in house by Mrs K. McAulay using a Shimadzu Europa PSSM-8 peptide synthesiser.

Table 2.10 Peptide sequences.

Peptide	Amino acids	Sequence
1051C	1-15 (N-terminal)	$(MRMLPDFFTGNWDDMGGGG)_4 K_2KA$
1051D	162-176 (CR3)	$(CALCYMRLTGHCIYSGGGG)_4 K_2KA$
1051E	236-249 (C-terminal)	$(DEPLNLSLKRPKGGGG)_4 K_2KA$

2.11 Solutions

ABTS substrate:	0.5mg/ml 2, 2-Azino-di-[3-ethylbenzthiozoline sulphonate (6)] (ABTS), 0.0003% H_2O_2 in citrate phosphate buffer
10X Agarose gel loading buffer:	1X TBE, 1% sodium dodecyl sulphate (SDS), 50% glycerol, 1mg/ml bromophenol blue
10X *Bal* 31 nuclease buffer:	0.5M Tris.HCl, 100mM $MgCl_2$, 100mM $CaCl_2$, 6M NaCl, 0.25mg/ml bovine serum albumin (BSA), pH 8
3X Boiling mix:	29% SGB, 6% SDS, 2M β-mercaptoethanol, 29% glycerol, 1mg/ml bromophenol blue
Chase mix:	0.25mM dTTP, dCTP, dGTP and dATP
Citrate phosphate buffer:	100mM citric acid, 100mM Na_2HPO_4, pH 4.2
50X Denhardt's solution:	1% polyvinylpyrrolidone, 1% BSA, 1% ficol
DNase I buffer:	40mM Tris.HCl, 10mM NaCl, 6mM $MgCl_2$, 10mM $CaCl_2$, pH 7.9
dT-0:	1µl 5mM dTTP, 20µl 5mM dCTP, 20µl 5mM 7-deaza dGTP, 50µl 10X TE, 370µl DW
dC-0:	20µl 5mM dTTP, 1µl 5mM dCTP, 20µl 5mM 7-deaza dGTP, 50µl 10X TE, 370µl DW
dG-0:	20µl 5mM dTTP, 20µl 5mM dCTP, 1µl 5mM 7-deaza dGTP, 50µl 10X TE, 370µl DW
dA-0:	20µl 5mM dTTP, 20µl 5mM dCTP, 20µl 5mM 7-deaza dGTP, 50µl 10X TE, 540µl DW
Extraction buffer:	250mM NaCl, 50mM HEPES, 0.1% NP-40, pH 7.0
Formamide dye mix:	10mM EDTA, 1mg/ml xylene cyanol FF, 1mg/ml bromophenol blue in formamide
Gel soak I:	1.5M NaCl, 0.5M NaOH
Gel soak II:	1.5M NaCl, 0.5M Tris.HCl, 1mM sodium ethylenediamine tetra-acetic acid (EDTA), pH 7.2

2X HBS:	280mM NaCl, 50mM N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid (HEPES), 1.5mM Na_2HPO_4, pH to 7.12 with NaOH
Phosphate buffered saline (PBSa):	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.2
PBS-complete:	PBSa plus 0.001% $CaCl_2.2H_2O$, 0.001% $MgCl_2.6H_2O$
Pre-hybridisation solution:	5X SSC, 5X Denhardt's solution, 0.5% SDS, 20µg/ml salmon sperm (ss) DNA
RGB (resolving gel buffer):	1.5M Tris.HCl, 0.4% SDS, pH 8.9
RNA lysis buffer:	150mM NaCl, 0.65% NP40, 10mM Tris.HCl pH7.9
10X Sequencing buffer:	100mM Tris.HCl, 100mM $MgCl_2$, pH 8.5
T sequencing mix:	500µl dT-0, 500µl 600µM ddTTP
C sequencing mix:	500µl dC-0, 105µl 140µM ddCTP
G sequencing mix:	500µl dG-0, 155µl 200µM ddGTP
A sequencing mix:	500µl dA-0, 250µl 140µM ddATP
SGB (stacking gel buffer):	0.5M Tris.HCl, 0.4% SDS, pH 6.7
Solution I:	50mM glucose, 25mM Tris.HCl, 10mM EDTA, pH8
Solution II:	0.2M NaOH, 1% SDS
Solution III:	5M Potassium acetate, pH 4.8 with acetic acid
20X SSC:	3M NaCl, 0.3M tri-sodium citrate
STET:	8% sucrose, 5% Triton X-100, 50mM EDTA, 50mM Tris.HCl, pH 8.0
Stripping buffer:	100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris.HCl, pH 6.7
Tank buffer:	0.05M Tris.HCl, 0.05M glycine, 0.1% SDS
20X TBE:	2.5M Tris.HCl, 800mM boric acid, 54mM EDTA
TBS:	0.02M Tris.HCl, 0.5M NaCl, pH 7.5.
TE:	10mM Tris.HCl, 1mM EDTA, pH 8.0

Towbin (blotting buffer):	25mM Tris.HCl, 192mM glycine, 20% methanol, pH 8.3
TTBS:	TBS plus 0.05% Tween 20
Versene:	0.6mM EDTA in PBS(A), 0.002% phenol red
Wash buffer:	PBS plus 0.3% Tween 20

2.12 Chemicals and reagents

All chemicals and reagents were purchased from BDH Chemicals UK or Sigma Chemical Company, unless otherwise stated below.

Beecham Research:	Ampicillin
Bio-Rad:	Ammonium persulphate (APS), gelatin, N, N, N′, N′ Tetra-methyl-ethylene-diamine (TEMED), Bradford reagent.
Boehringer Mannheim:	Tris.HCl
Difco:	Agar, bactotryptone, yeast extract
Fisons:	Acetone
Fluka:	Formamide
Gibco BRL Life Technologies:	FCS, L-glutamine, NCS, penicillin, streptomycin
JT Baker:	Acetic acid, isopropanol, methanol
Melford Laboratories Ltd:	Caesium chloride
National Diagnostics:	30% Acrylamide (2.5% cross-linker)
New England Biolabs:	*Sal* I linkers
Pharmacia:	dNTPs, ddNTPs
Prolabo:	Boric acid, chloroform, glycerol
Scotlab:	Acrylamide: N, N′-methylene-bis-acrylamide 19:1
UKC Chemical Laboratories:	Citifluor

3. METHODS

3. Methods

Unless otherwise stated the method is fully referenced in "Current Protocols in Molecular Biology" (Ausubel *et al.*, 1995).

3.1 Manipulation of DNA

3.1.1 Purification of synthetic oligonucleotides

Synthetic oligonucleotides were synthesised in house by Mr. J. McGeehan on a Cruachem Ltd PS250 automated DNA synthesiser, using 200μM synthesis columns. To remove the oligonucleotide from the column, 5ml disposable syringes were attached to both ends of the column with one containing 1.5ml ammonium hydroxide (0.88 specific gravity), and the solution was pushed through the column in 200μl aliquots with 20min incubation between each fresh addition. After incubation with the final aliquot, the 1.5ml solution was pushed backwards and forwards through the column 4-5 times to mix the aliquots thoroughly. The eluted oligonucleotide solution was incubated in a tightly sealed tube for 5hr at 55°C to remove protecting groups. Following lyophilisation, the oligonucleotide was dissolved in 200μl DW. An equal volume of formamide plus 10μl formamide dyes were added before boiling for 5min prior to electrophoresis on a 12% denaturing polyacrylamide gel (section 3.1.4.2).

3.1.2 Polymerase chain reaction (PCR)

PCR was used to amplify specific fragments from either a DNA template using *Pfu* DNA polymerase, or from a cDNA template using *Taq* DNA polymerase. *Pfu* DNA polymerase shows high fidelity during amplification, therefore it was used to reduce the likelihood of aberrations during PCR amplification. *Taq* DNA polymerase, on the other hand, was used specifically for the construction of expression plasmids containing Ad40 E1A cDNAs using the pCRII cloning kit (Invitrogen).

3.1.2.1 PCR using *Pfu* DNA polymerase

PCR reactions were set up in 50μl reaction volumes of 1X *Pfu* DNA polymerase buffer (supplied with the enzyme) containing 100ng template DNA, 5μM each primer, 250μM each dNTP and 2.5 units (U) *Pfu* DNA polymerase; which was overlaid with 50μl light

mineral oil to prevent evaporation during cycling. Following optimisation of the PCR reaction, cycling was carried out using a Cambio thermal cycler, where the cycles comprised 1 cycle at [94°Cx7min, 55°Cx3min, 72°Cx2min], 30 cycles at [94°Cx2min, 55°Cx1min, 72°Cx2min] and 1 cycle at [72°Cx5min].

3.1.2.2 PCR using *Taq* DNA polymerase

PCR reactions were set up in 50µl reaction volumes of 1X *Taq* DNA polymerase buffer (supplied with the enzyme) containing template cDNA, 1µM each primer, 250µM each dNTP, 1.5mM $MgCl_2$ and 2.5U *Taq* DNA polymerase; which was overlaid with 50µl light mineral oil. Template cDNA consisted of 5µl of a reverse transcription reaction containing an unquantified amount of cDNA, which was made from cytoplasmic RNA as in sections 3.2.1 and 3.2.2. Following optimisation of the PCR reaction, cycling was carried out using a Cambio thermal cycler, where the cycles comprised 1 cycle at [95°Cx5min, 70°Cx1min, 72°Cx3min] then 50 cycles at [95°Cx1min, 70°Cx1min, 72°Cx5min].

3.1.3 Digestion of DNA

3.1.3.1 *Bal* 31 nuclease digestion of DNA

Bal 31 nuclease digestion of restriction enzyme digested plasmid DNA (50µg) was carried out at 37°C for 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60min using 0.75U of pre-warmed enzyme in 200µl 1X *Bal* 31 nuclease buffer. To terminate *Bal* 31 nuclease digestion 0.5µl 0.5M EDTA was added to each 15µl aliquot of the reaction mix, which was immediately phenol:chloroform extracted. The DNA was then ethanol precipitated and resuspended in 20µl DW, before restriction enzyme analysis of a 2µl aliquot.

3.1.3.2 Restriction enzyme digestion of DNA

Restriction enzyme digestion of DNA was carried out at 37°C (or the temperature specified by the supplier) in 20µl reaction volumes of 1X buffer (supplied with the enzyme) containing 1U of enzyme per µg of DNA to be digested in an hour. The buffer system used most frequently was the Boehringer Mannheim A, B, H, L, M system. On all other occasions New England Biolabs NEBuffer 1, 2, 3, 4 system was used. Usually, 0.5µg DNA was digested for diagnostic analysis, and 5-20µg for isolation of specific

restriction fragments. Samples (2µl) of plasmid DNA prepared by the miniprep method (see section 3.1.9) were digested in the presence of 500µg/ml RNase A.

3.1.4 Electrophoretic separation and purification of DNA fragments

3.1.4.1 Non-denaturing agarose gels

DNA fragments produced by *Bal* 31 nuclease digestion, PCR or restriction enzyme digestion were resolved by non-denaturing agarose gel electrophoresis. Horizontal slab gels approximately 0.5cm thick were utilised and samples, including appropriate size markers, were loaded following the addition of 2µl agarose gel loading buffer. Fragments greater than 100bp were separated on 0.5-2% agarose gels, run in 1X TBE at 150 Volts (V) on a Gibco BRL 11-14 gel electrophoresis kit. Following electrophoresis the gel was stained with 1µg/ml ethidium bromide, then the DNA was visualised under short wave ultraviolet (UV) light on an UV Products Inc. transilluminator (long wave UV light for preparative gels). Photography was carried out using the Appligene Imager.

3.1.4.2 Denaturing polyacrylamide gels

Vertical denaturing polyacrylamide (acrylamide:N, N'-methylene-bis-acrylamide 19:1) gels containing 7M urea and 1X TBE, were used to resolve the products of DNA sequencing reactions and to purify synthetic oligonucleotides. Polymerisation was initiated by adding 0.01vol 10% APS and 0.001vol TEMED. DNA sequencing gels containing 6% or 8% acrylamide were run at 60W in a Gibco BRL S2 sequencing kit for varying lengths of time depending upon the sequence to be visualised. Gels were then vacuum dried on a Bio-Rad S83 gel dryer and exposed to Kodak X-OMAT S film for 1-3 days, before developing in a Kodak X-OMAT ME-3 processor. Gels for purifying oligonucleotides contained 12% acrylamide and were run at 250V, in a kit manufactured in house, until the bromophenol blue marker was approximately ¾ way down the gel. Gels were then transferred to cling film on top of a thin layer chromatography (TLC) plate, and the oligonucleotides could be visualised at 254nm as dark shadows against a fluorescing TLC plate. Bands containing the oligonucleotides were cut out of the gel, crushed and covered with 200µl DW. The oligonucleotides were then eluted overnight at room temperature (RT), precipitated twice with ethanol, then washed once with 70% ethanol before resuspending in an appropriate volume of DW. The concentration of the

oligonucleotide was determined by measuring the optical density (OD) at 260nm on a Beckman DU-62 spectrophotometer, this value was then used in the following calculation:

Concentration (μM) = (OD_{260} x dilution factor)/extinction coefficient

[Extinction coefficient = (number (n)A x 15 200) + (nT x 8 400)+ (nC x 7 050)+ (nG x 12 010]

3.1.5 DNA purification from agarose gels

DNA fragments of the correct size were excised from gels under long wave UV light on a UV Products Inc. transilluminator, and the DNA recovered using the commercial GENECLEAN II kit (BIO101 Inc., La Jolla, CA). The kit contains a silica matrix, which binds DNA in the presence of high concentrations of sodium iodide. The gel slice was weighed to estimate its volume and cut into approximately 2mm cubes to facilitate gel dissolution in 4.5 volumes (vol) sodium iodide solution plus 0.5vol TBE modifier, at 55°C for 5min. The silica matrix was then added to the solution (5μl for up to 5μg DNA then an additional 1μl for every 0.5μg above 5μg), which was mixed by inversion before incubating on ice for 5min. Following a 5sec centrifugation at 13,000 revolutions per minute (rpm) on a MSE microcentaur, the silica matrix pellet was washed three times in 10-50vol NEW wash. The DNA was eluted from the matrix into 20μl DW by incubating at 55°C for 5min. The matrix was pelleted and the supernatant containing the DNA transferred to a fresh tube.

3.1.6 Production of blunt ended DNA fragments

Fragments generated by enzymes which produced 5′ or 3′ overhangs were blunt ended at 37°C for 30min in a 20μl reaction volume of 1X M buffer containing 50μM of each dNTP and 2U of DNA polymerase I large (Klenow) fragment.

3.1.7 DNA ligation

Suitably prepared insert and vector fragments in a 4:1 ratio (i.e. 200ng : 50ng), were ligated at RT overnight in a 20μl reaction volume of 1X T4 ligase buffer (supplied with the enzyme) containing 2U of T4 DNA ligase. A 10μl aliquot of the reaction was then transformed into an appropriate strain of competent *E. coli* cells.

3.1.8 Preparation and transformation of competent *E. coli* cells

The appropriate *E. coli* cells were streaked onto a LB agar plate and incubated at 37°C overnight. A single colony was picked and used to inoculate 10ml 2YT, which was incubated at 37°C in a shaking incubator for 2-3hr until the OD_{450} was approximately 0.3. The cells were pelleted by centrifugation at 3,000rpm, 4°C for 5min in a Beckman GPR centrifuge, resuspended in 0.5ml ice cold 0.1M $MgCl_2$ and repelleted at 3,000rpm, 4°C for 2min. The pellet was resuspended in 0.5ml ice cold 0.1M $CaCl_2$, then incubated on ice for 30min.

Approximately 10ng of plasmid or ½ (10µl) of a ligation reaction was added to a 100µl aliquot of freshly prepared competent cells, then incubated on ice for 20min. The cells were heat shocked at 42°C for 1min, then added to 0.5ml L-broth prior to incubation at 37°C for 30min. A 50µl aliquot of cells or the entire transformation mix was then plated onto a LB agar plate containing 50µg/ml ampicillin and incubated at 37°C overnight.

3.1.9 Small scale plasmid DNA preparation

Miniprep DNA for the analysis of bacterial colonies was prepared by alkaline lysis. A single bacterial colony was picked from a LB agar plate containing 50µg/ml ampicillin and used to inoculate 5ml L-broth, which was incubated in a shaking incubator at 37°C overnight. A 1.5ml aliquot of the culture was centrifuged (in a MSE microcentaur) at 13,000 rpm, RT for 5 min, then the pellet was resuspended in 100µl solution I and incubated at RT for 5 min. A 150µl aliquot of freshly prepared solution II was then added and the reaction was incubated at RT for 5 min before the addition of 150µl solution III and incubation at RT for a further 5 min. Cell debris was pelleted by centrifugation at 13,000 rpm, RT for 5 min and the supernatant was transferred to a fresh tube where it was phenol:chloroform extracted and ethanol precipitated. The pellet was then resuspended in 1µl 10mg/ml RNase A plus 19µl TE.

3.1.10 Large scale plasmid DNA preparation

E. coli containing the desired plasmid was streaked (from a glycerol stock or from a single colony picked from an agar plate) or spread (from an aliquot of newly transformed competent cells) onto a LB agar plate containing 50µg/ml ampicillin then

incubated at 37°C overnight. A single colony was picked and used to inoculate 350ml L-broth containing 50μg/ml ampicillin, which was incubated in a shaking incubator at 37°C overnight.

Plasmid DNA was prepared from bacteria according to the method of Holmes and Quigley (1991), and the bacterial lysate was purified further on a caesium chloride/ethidium bromide gradient to yield supercoiled plasmid DNA. Bacterial cultures were centrifuged at 5,000 rpm, RT for 8min in a Sorvall GS3 rotor, and the pellets resuspended in 20ml STET plus 2ml freshly prepared 10mg/ml lysozyme in STET. The mixture was brought to the boil over a Bunsen flame and placed in a boiling water bath for 45sec, before pelleting cell debris by centrifugation in a Sorvall SS34 rotor at 18,000 rpm, RT for 50min. Nucleic acids were precipitated from the supernatant by the addition of 0.9 volumes of isopropanol, and pelleted by centrifugation in a Beckman GPR centrifuge at 3,000 rpm, RT for 5min. The pellet was resuspended in 1x TE to give a volume 5.5ml, then 6.2g caesium chloride and 0.2ml 10mg/ml ethidium bromide were added. The samples were incubated on ice for 20min prior to centrifugation at 3,000 rpm, 4°C for 10min. The supernatant was transferred to a Dupont 03945 crimp-seal centrifuge tube using a syringe, then centrifuged at 45,000 rpm, 15°C for 16-18hr in a Sorvall TV865 vertical rotor. The lower band (which contains supercoiled plasmid DNA) was removed using a syringe, taking care to avoid contamination with chromosomal DNA from the upper band. The ethidium bromide was removed from the plasmid solution by 2-3 extractions with TE saturated butan-1-ol, and the caesium chloride was removed by dialysing against 1X TE at RT for 2hr. Following this the solution was treated with 100μg/ml RNase A at 65°C for 1hr, then 100μg/ml proteinase K plus 0.1% SDS at 37°C for 1hr. The plasmid DNA was cleaned by phenol:chloroform extraction, and concentrated by ethanol precipitation. The pellet was resuspended in 400μl 0.3M sodium acetate pH 5.5 then ethanol precipitated again. The final pellet was washed in 70% ethanol and resuspended in an appropriate volume of DW before storage at ⁻20°C. The plasmid concentration was determined by measuring the absorbance at 260nm on a Beckman DU-62 spectrophotometer, assuming $1A_{260}$=50μg/ml.

3.1.11 Dideoxy sequencing of DNA

Plasmid DNA was sequenced by the dideoxy nucleotide chain termination method as described by Sanger *et al.* (1977). Miniprep DNA (10µl i.e. half a miniprep sample) was denatured at RT for 10min in a 20µl reaction volume containing 0.4M sodium hydroxide. The denatured DNA was then precipitated on ice for 10min by the addition of 6µl 3M sodium acetate pH 4.5, 14µl DW and 120µl ethanol, then pelleted by centrifugation in a MSE microcentaur at 13,000rpm, RT for 10min. The pellet was then washed with 70% ethanol and resuspended in 8µl DW.

The primer was annealed to the DNA template at 37°C for 20min in a 10µl reaction volume of 1X sequencing buffer containing 5pmol of primer. Meanwhile four chain termination mixes were made up (for 6 samples: Labelling mix = 20µCi [α-^{35}S] dATP + 6µl 11.8µM dATP. Chain termination mix = 12µl T, C, G or A sequencing mix + 2µl labelling mix), and 2µl aliquots of each dispensed into one of four round bottomed wells on a 96 well microtitre plate. After the annealing reaction was completed, 2units of *E. coli* DNA polymerase I large (Klenow) fragment were added and 2µl aliquots dispensed into the 4 wells containing the chain termination mixes, the plate was then incubated at 37°C for 15min. Following this any newly synthesised strands which had not been terminated by incorporation of a dideoxy nucleotide were extended into high molecular weight products by adding 2µl chase mix which was incubated at 37°C for 30min. The reactions were stopped by the addition of 2µl formamide dyes. The samples were boiled for 1min prior to electrophoresis on a denaturing polyacrylamide gel (section 3.1.4.2).

3.1.12 Southern blotting

Products from PCR reactions (section 3.1.2.2) were separated on agarose gels (section 3.1.4.1). The gels were incubated at RT, whilst shaking, in Gel Soak I for 45min, then Gel Soak II for 45min. The gel was then placed face down onto a wick of Whatman 3mm paper (soaked in 10X SSC and descending into a 10X SSC reservoir), then a Hybond N+ filter (pre-soaked in 2X SSC), two pre-soaked sheets of Whatman 3mm paper, a stack of absorbent paper towels and a light weight were placed on top of the gel. Care was taken to eliminate air bubbles between layers to prevent uneven

transfer of DNA from the gel to the filter. After transfer at RT overnight, the filter was briefly rinsed in 2X SSC before denaturing the DNA by placing the filter (DNA side up) on Whatman 3mm paper which had been pre-soaked in 0.4N sodium hydroxide for 20min, after which time the filter was rinsed in 2X SSC.

Hybond N+ filters were pre-hybridised for 3-4hr at 65°C in pre-hybridisation solution, then the probe DNA (10^7-10^8 counts per minute (cpm), see below) was added to the mix, and incubation continued overnight at 65°C. The filter was washed twice in 2X SSC plus 0.1% SDS for 30min at RT and then twice in 0.1X SSC plus 0.1% SDS for 30min at RT. The filter was air dried and exposed to Kodak X-OMAT S film.

Plasmid DNA probes were prepared using the Megaprime system from Amersham. Approximately 25ng of DNA was mixed with 5µl Megaprime primer (Amersham) in a final volume of 20µl, then placed in a boiling water bath for 5min. The mixture was cooled and 10µl Megaprime buffer, 50µCi [α-^{32}P]dCTP and 4-5U DNA polymerase I large (Klenow) fragment were added, bringing the reaction volume to 50µl, and the mix was incubated at 37°C for 20min. The reaction was stopped by the addition of 5µl 0.2M EDTA. Bromophenol blue was added to colour the mix which was then loaded onto a Pharmacia G-50 Sephadex nick column which had been equilibrated with 1X TE containing 0.1% SDS. Fractions (approximately 0.25ml) were collected from the column, then those containing the highest counts in the initial peak were pooled and used as probe. The probe was denatured at RT for 10min with a 1/20vol of 5N sodium hydroxide, then neutralised by adding a 1/20vol of 5M hydrochloric acid.

3.2 Manipulation of RNA

3.2.1 Preparation of cytoplasmic RNA

Cytoplasmic RNA was prepared from 60mm plates of Ad40 infected (5ffu/cell) 293 cells as described by Ausubel *et al.* (1995). Reagents used in this protocol were made up in DEPC treated DW, and the entire protocol was performed at 4°C to minimise any RNase activity. The cell sheet was harvested into the medium at various time points post-infection (p.i.) (outlined in results figure legends), then transferred to a falcon tube where the cells were washed twice with ice cold PBS. The cell pellet was resuspended, by vortexing, in 200µl RNA lysis buffer and centrifuged in an Ole Dich Instrument Makers 157.MP microfuge at 20,000rpm, 4°C for 30sec. SDS was added to the cytoplasm to a final concentration of 0.2%, then the RNA was immediately phenol:chloroform extracted twice and ethanol precipitated. To remove any DNA present in the sample the pellet was incubated in 100µl 1X DNase buffer containing 1U RNase free DNase at 37°C for 30min. The sample was then phenol:chloroform extracted, ethanol precipitated, and washed with 70% ethanol. The pellet was resuspended in a suitable volume of DEPC treated DW.

3.2.2 Reverse transcription of RNA

RNA was reverse transcribed to make a complementary DNA (cDNA) template for use in PCR. The primer was annealed to the RNA template (prepared as in section 3.2.1) at 65°C for 15min in a 42µl reaction volume of 1X M-MuLV reverse transcriptase buffer (supplied with the enzyme) containing 100pmol of primer and 150µM each of dATP, dCTP, dGTP and dTTP. To half the annealing reaction, 100µg/ml BSA, 20U of RNasin and 50U M-MuLV reverse transcriptase were added, and to the other half of the sample 100µg/ml BSA plus 20U of RNasin were added (the later reaction acted as a negative control determining whether or not DNase I treatment had been effective). The samples were incubated at 37°C for 2hr, then phenol:chloroform extracted, ethanol precipitated and the pellet resuspended in 50µl DW. Typically a 5µl aliquot was used in a 50µl PCR amplification using *Taq* DNA polymerase (section 3.1.2.2).

3.3 Manipulation of proteins

3.3.1 Preparation of protein extracts from transfected or infected cells

Protein extracts were prepared from 60mm plates of WS HeLa cells which had either been transfected with 10μg plasmid DNA or infected with Ad5 (5pfu/cell) or Ad40 (5ffu/cell). Cells were harvested at the desired time point by removing the medium, washing the cell sheet twice with PBS, then incubating for 5min at RT with 400μl 1X boiling mix. The cell lysate was then transferred to an Eppendorf tube and stored at -20°C.

3.3.2 Preparation of protein extracts from [^{35}S]L-methionine labelled cells

[^{35}S]L-methionine labelled cells were prepared from 100mm plates of WS HeLa cells which had been infected with Ad5 (5pfu/cell) or Ad40 (5ffu/cell), then blocked with cytosine arabinoside (Ara C, 20μg/ml Ara C 12 hourly, section 3.5.3) to allow early viral proteins to accumulate. At 42hr post infection (p.i.) the cells were labelled at 37°C for 2hr with 100μCi [^{35}S]L-methionine in methionine free medium. The cells were then scraped into the medium and transferred to a 15ml Falcon tube where they were washed twice with ice cold PBS, then resuspended in 200μl extraction buffer minus 0.1% NP-40. The cells were lysed by sonication in a Kerry sonibath at RT for 30-60sec, 0.1% NP-40 was added then the cell debris pelleted by centrifugation in an Ole Dich Instrument Makers 157.MP microfuge at 13,000rpm, 4°C for 10min. An aliquot of the supernatant was stored at $^{-}$70°C for later analysis by electrophoresis on a SDS polyacrylamide gel (SDS-PAGE) section 3.3.4), the remainder was transferred to a fresh tube to be used in immunoprecipitation.

3.3.3 Immunoprecipitation

Proteins were immunoprecipitated by a modification of the method used by Stephens and Harlow (1987). The supernatant prepared in section 3.3.2 was pre-cleared (end over end mixing) at 4°C for 1hr with 30μl 50% protein A sepharose (in extraction buffer), then pelleted by centrifugation in an Ole Dich Instrument Makers 157.MP microfuge at 13,000rpm, 4°C for 1min. The supernatant was split into two equal amounts then

incubated (end over end) at 4°C for 3hr with 20µl pre-immune or immune serum. Upon addition of 60µl of protein A sepharose the reactions were incubated for a further 1hr at 4°C. The protein A sepharose was washed 3X with 200µl extraction buffer to remove any non-specifically bound proteins. Specifically bound proteins were eluted into 20µl 1X boiling mix by boiling for 5min, before being separated by SDS polyacrylamide electrophoresis (SDS-PAGE section 3.3.4).

3.3.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Proteins were resolved on polyacrylamide gels using the Bio-Rad mini-protean II apparatus. A 10% resolving gel mix (3.33ml 30% acrylamide (2.5% cross-linker), 2.5ml RGB, 4.17ml DW, 80µl 10% APS, 8µl TEMED) was poured into the gel former apparatus, then overlaid with butanol and allowed to set. The butanol was washed away with water, and the resolving gel overlaid with the stacking gel mix (0.4ml 30% acrylamide (2.5% cross-linker), 0.6ml SGB, 1.4ml DW, 20µl 10% APS, 3µl TEMED) before inserting the comb and allowing the gel to set at RT. Protein samples in 1X boiling mix were boiled for 2min prior to loading. Gels were run in 1X tank buffer at 100V until the dye front reached the bottom, then the gels were transferred to nitrocellulose membranes for western blot analysis (section 3.3.5).

3.3.5 Western blot analysis of proteins

Proteins resolved on SDS-PAGE mini-gels (section 3.3.4) were transferred to a nitrocellulose membrane in a Bio-Rad mini-transblot cell. A blotting sandwich was set up such that the gel was in contact with a sheet of nitrocellulose membrane (Schleicher and Schuell Inc.), which in turn were sandwiched between Whatman 3mm paper and sponges (contained within the kit), all of which had been pre-soaked in Towbin buffer. Transfer of proteins from the gel to the nitrocellulose was carried out in Towbin buffer at 0.25mA for 3h.

Nitrocellulose membranes were blocked by incubation at 37°C for 2X 30min in 100ml 3% gelatin in 1X TBS, whilst shaking. They were then washed 2X 5min at RT in TTBS before incubation overnight at RT (whilst shaking) with the first antibody diluted appropriately in 20ml 1% gelatin in TTBS. Following this the blots were washed at RT

5X 5min in TTBS, then incubated at RT for 90min in 20ml 1/1000 dilution of protein A HRP conjugate (diluted in 1% gelatin in TTBS), before being washed again at RT 3X 5min in TTBS. Proteins were detected using the Amersham enhanced chemiluminescence (ECL) system; the two reagents in the system were mixed in equal amounts then added to the filter, which was incubated at RT for 1min, then wrapped in cling film and exposed to Kodak X-OMAT S film. Blots were stripped at 55°C for 1hr in stripping buffer before being blocked, and reprobed with a second antibody.

3.4 Tissue culture

3.4.1 Growth of cells

Mammalian cells were passaged in sterile, 175cm^2 plastic flasks in the appropriate medium (section 2. 8), and incubated at 37°C in a humidified incubator under 5% CO_2. Confluent monolayers were harvested by washing twice with versene for 293 and KBa+b cells, or twice with trypsin/versene (1:4) for WS HeLa cells, then resuspended in 10ml of the appropriate medium. For continual passage 293 cells were split at a 1:4 dilution, KBa+b cells were split at a 1:6 dilution, and WS HeLa cells were split at a 1:10 dilution every 3-4 days. Plates (60mm) were seeded at 1.5x10^6 cells/plate for 293 and KBa+b cells, and at 1x10^6 cells/plate for WS HeLa cells to obtain approximately 70% confluency the next day.

3.4.2 Electroporation

A 250µl aliquot of serum free medium containing 1x10^7 WS HeLa cells/ml was incubated on ice for 10min with 20µg plasmid DNA. Meanwhile, the Invitrogen electroporator II was connected to a power supply set at 300V, 25mA, 25W, with the unit settings at 1000µF, ∞ Ω. The unit was charged for 2min and the cells were pulsed according to the manufacturers instructions; the cells were then incubated at RT for 10min. The sample was then inoculated into 5ml medium on a 60mm plate and incubated at 37°C, 5% CO_2 for 48hr, at which point the cells were harvested according to section 3.3.1.

3.4.3 Calcium phosphate mediated transfection

WS HeLa cells were seeded at 1x10^6 cells/60mm plate in 5ml medium then incubated at 37°C 5% CO_2 overnight until cells were approximately 70% confluent; 2-4hr prior to the addition of the transfection mix the medium on the plates was replaced. Diluted plasmid DNA for transfection was standardised by spectrophotometric analysis at 260nm and 280nm, and by agarose gel electrophoresis. The transfection mix contained the appropriate amount of plasmid DNA made up in 140µl with DW. A 20µl aliquot of 2M calcium chloride followed by 160µl 2X HBS were then added dropwise to the DNA whilst vortexing and incubated at RT for 30min. The transfection mix was added to the medium on the plate while gently swirling, then the plate was incubated at 37°C, 5%

CO_2 for 6hr. The medium containing the transfection mix was removed, the plate washed once with 5ml PBS, and then 5ml fresh medium was added. The plates were reincubated until 48hr had passed from the time when the transfection mix was initially added, at which point the cells were harvested. Cells were harvested using either the luciferase reporter assay system (Promega) for transfections of plasmids which contained the firefly luciferase gene (section 3.4.3.1), or the dual-luciferase reporter assay system (Promega) for dual plasmid transfections of plasmids containing the firefly and the sea pansy luciferase gene (section 3.4.3.2).

3.4.3.1 Harvest of cells using the luciferase reporter assay system

Cells were harvested by passive lysis using the luciferase assay system from Promega. The growth medium was removed from the plate and the cells were washed twice with 2ml PBS (making sure to remove as much of the final PBS wash as possible), then incubated with 400µl reporter lysis buffer at RT for 15min. To ensure the release of all the luciferase enzyme from the cells the samples were freeze thawed once, then vortexed for 10-15sec before pelleting the cellular debris in an MSE microcentaur at 13,000rpm, RT for 15sec. The supernatant was transferred to a fresh tube, and immediately assayed for luciferase activity (section 3.4.4.1) and protein concentration (section 3.4.5).

3.4.3.2 Harvest of cells using the dual-luciferase reporter assay system

Cells were harvested by active lysis using the dual-luciferase reporter assay system from Promega. The growth medium was removed from the plate and the cells washed once with 2ml PBS, making sure to remove as much of the PBS wash as possible. The cells were then harvested into 400µl 1X passive lysis buffer using a rubber policeman. The accumulated lysate was pipetted up and down several times to obtain a homogenous suspension, from which an aliquot (20µl) was taken and tested for luciferase activity (section 3.4.4.2).

3.4.4 Luciferase assay

3.4.4.1 Luciferase assay using the luciferase reporter assay system

Cells harvested by passive lysis (section 3.4.3.1) were assayed for firefly luciferase activity using the luciferase assay system (with reporter lysis buffer) from Promega. A

20µl aliquot of cell extract was added to 100µl pre-dispensed luciferase assay reagent, and immediately read on a Biotrace Ltd M3 single sample luminometer, which had been programmed to have a two second pre-read delay, followed by a ten second measurement period for luciferase activity (transmitted light units (TLU)).

3.4.4.2 Luciferase assay using the dual-luciferase reporter assay system

Cells harvested by active lysis (section 3.4.3.2) were assayed for firefly and sea pansy luciferase activity using the dual-luciferase reporter assay system from Promega. A 20µl aliquot of cell extract was added to 100µl pre-dispensed luciferase assay reagent II, and firefly luciferase activity read using the Biotrace Ltd M3 single sample luminometer, which had been programmed to have a two second pre-read delay, followed by a ten second measurement period for luciferase activity (transmitted light units (TLU)). Sea pansy luciferase activity was then read using the same luminometer following the addition of 100µl Stop & Glo reagent.

3.4.5 Protein assay

Protein concentration was assayed according to the method of Bradford (1976). A 10µl aliquot of passive lysis cell extract (section 3.4.3.1) was added to 1ml Bradford reagent, incubated at RT for 20min, then the OD_{595} was determined using a Beckman DU-62 spectrophotometer. To determine the protein concentration of the samples a standard curve was created using BSA standards of 0, 0.2, 0.4, 0.6, 0.8 and 1mg/ml.

3.5 Maintenance and growth of virus

3.5.1 Growth of virus seed stocks

Ad5 and Ad40 seed stocks were prepared by respectively infecting 10x60mm plates (approximately 80% confluent) of 293 or KBa+b cells with 0.1 plaque forming units (pfu)/cell or 5 fluorescence forming units (ffu)/cell. The virus was allowed to adsorb to the cells for 1hr at 37°C, 5% CO_2 before 5ml Eagles A+B plus 2% (Ad5) or 0.5% (Ad40) FCS was added to each plate then incubated at 37°C, 5% CO_2 for 3-5 days until a cytopathic effect (cpe) was visible. Cells were scraped into the medium with a rubber policeman, pelleted in a Beckman GPR centrifuge at 2,000rpm, 4°C for 15min and resuspended in 0.25ml Tris saline. Virus particles were released from the cells by three rounds of freeze/thawing. The cellular debris was pelleted at 2,000rpm, 4°C for 10min. The supernatant containing the virus particles was stored at $^{-}$70°C.

3.5.2 Virus titration

3.5.2.1 Adenovirus type 5

Seed stocks were titrated on 293 cells to determine the pfu/ml (Williams, 1970). Two 60mm plates of 293 cells (approximately 80% confluent) were infected with 10^{-6} to 10^{-9} serial dilutions of Ad5. The virus was allowed to adsorb to the cells for 1hr at 37°C, 5% CO_2, before 4ml titration medium (100ml 1.3X Eagles, 35ml Eagles B without phenol red, 3.2ml FCS, 1.5ml 1.25M magnesium chloride, 40ml noble agar) was added to each plate and allowed to set. The plates were incubated at 37°C, 5% CO_2 for 4 days, at which point a further 2ml titration medium was added, and the plates re-incubated for 2 days. The number of plaques/plate were counted then used to calculate the seed stocks pfu/ml.

3.5.2.2 Adenovirus type 40

Ad40 does not form plaques under any known conditions, therefore the titre of Ad40 was estimated by the fluorescent focus assay. KBa+b cells were seeded on 13mm glass coverslips in a 24 well Linbro plate at $0.5x10^5$ cells/well in 1ml medium, then incubated at 37°C, 5% CO_2 overnight. Dilutions of the Ad40 seed stock (20µl 1/10, 1/20 and 1/40 dilution) were allowed to adsorb to the cells for 1hr at 37°C, 5% CO_2, before the addition of 1ml Eagles A+B plus 2% FCS, and re-incubation of cells for a further 47hr.

The cells were then washed twice with ice cold PBS before being fixed at RT for 10min with a 3:1 mix of ice cold methanol and acetone. The cells were washed twice with ice cold PBS, then incubated at RT for 30min with aliquots (20µl 1/20 dilution in PBS) of primary antibody (Ad5 anti-hexon monoclonal antibody (10/5.1), section 2.7). The cells were washed three times with ice cold PBS, then incubated at RT for 30min with 20µl 1/40 dilution of goat anti mouse fluorescein isothiocyanate conjugate (FITC) (secondary antibody). After a further three washes with PBS the coverslips were mounted on glass slides with 10µl Citifluor, and single cells infected with Ad40 visualised as bright green spots as opposed to uninfected cells which were appear as dull red spots under a Nikon MICROPHOT-SA fluorescence microscope.

3.5.3 Virus infections

Ad5 and Ad40 infections were set up at 5pfu/cell for Ad5 or 5ffu/cell for Ad40 on 293 or WS HeLa cells. The virus was allowed to adsorb to the cells for 1hr at 37°C, 5% CO_2. 5ml Eagles A+B plus 2% FCS was then added to each plate which was incubated at 37°C, 5% CO_2 for various times before harvesting (sections 3.2.1, 3.3.1 or 3.3.2). The addition of 20µg/ml Ara C to the medium every 12hr blocked DNA replication therefore allowing overexpression of adenovirus early proteins.

3.6 Antibodies

3.6.1 Production of antipeptide antibodies

Duplicate rabbits were injected by the intramuscular route with 50μg peptide in 0.5ml PBS plus 0.5ml Freund's complete adjuvant; subsequent boosts were made with 50μg peptide in 0.5ml PBS plus 0.5ml Freund's incomplete adjuvant. Test bleeds were taken two weeks after each boost, and the antiserum was tested for antibodies to its homologous peptide by enzyme linked immunosorbent assay (ELISA section 3.6.2).

3.6.2 Enzyme Linked Immunosorbent Assay (ELISA)

Rabbit test bleeds were allowed to clot at 4°C overnight, then the serum was transferred to a 15ml falcon tube and centrifuged in a Beckman GPR centrifuge at 2,000rpm, 4°C for 5min. The serum was transferred to a fresh tube and heat inactivated at 56°C for 30min; 1ml aliquots were stored at -20°C.

The antiserum was tested against 100ng or 1μg (in 50μl PBS) of its homologous peptide, which had been adsorbed at 37°C overnight onto an Immulon I ELISA plate; excess peptide was removed by washing four times with wash buffer. To reduce non-specific background the plate was blocked for 1hr at 37°C with 100μl/well PBS plus 2% BSA, then washed four times with wash buffer. The antiserum was allowed to react with its homologous peptide by incubating 50μl/well of neat, 1/10 or 1/100 dilution of the antiserum (diluted with PBS plus 2% FCS) at 37°C for 1hr; unbound antibodies were then removed by washing four times with wash buffer. Any antibodies which had bound to the immobilised peptide were detected by incubating each well with 50μl of a 1:500 dilution of Protein A HRP conjugate (diluted with PBS plus 2% FCS) for 1hr at RT. The system was then washed free of any unbound Protein A HRP conjugate, and examined for bound enzyme by incubating each well with 100μl ABTS substrate at RT for 15min. The products of enzyme cleavage were assayed at 405nm on a Titertek multiskan plus plate reader.

4. RESULTS

4. Results

Introduction

The aim of this project was to characterise the adenovirus type 40 early region 1A. Previously it had been shown that Ad40 virus particles were shed in large numbers from the gut of infected individuals, but failed to propagate in cells such as HEK, KB, HeLa, HEP-2 and WI-38, which are normally used to propagate other human adenovirus serotypes in tissue culture. Ad40 will however grow in 293 cells (Takiff *et al.*, 1981; Kidd and Madeley, 1981), a human embryo kidney (HEK) cell line which has been transformed with the Ad5 E1 region (Graham *et al.*, 1977), albeit at reduced levels compared to other serotypes. This observation suggests that the E1 functions are poorly expressed or of intrinsically lower activity in certain cell types. Previous work had demonstrated that the E1B region plays a significant role in this phenotype (Mautner *et al.*, 1989; Steinthorsdottir and Mautner, 1991; Gomes *et al.*, 1992), however the Ad40 E1A region had also been implicated, due to a delay in the onset of Ad40 E1A mRNA expression (Ullah, 1997) when compared with Ad5 E1A mRNA expression (Glenn and Ricciardi, 1988). The Ad40 E1A proteins were also shown to be weaker *trans*-activators than the Ad5 E1A equivalent proteins (van Loon *et al.*, 1987b; Ishino *et al.*, 1988). Taken together these data suggest that the Ad40 E1A region may be subject to aberrant expression in tissue culture, which would considerably affect progression of Ad40 into the late phase of the infectious cycle.

4.1 Preliminary characterisation of basal transcription from the Ad40 E1A promoter

Introduction

The aberrant expression of the Ad40 E1 region (Takiff *et al.*, 1981; Kidd and Madeley, 1981) had mainly been attributed to E1B (Mautner *et al.*, 1989; Steinthorsdottir and Mautner, 1991; Gomes *et al.*, 1992) however, E1A was also implicated (Ullah, 1997; van Loon *et al.*, 1987b; Ishino *et al.*, 1988). Ad40 E1A mRNA was expressed 36h p.i. (Ullah, 1997) compared with Ad5 E1A mRNA expression at 1.5-2h p.i.. The Ad40 E1A proteins were demonstrated as weaker *trans*-activators of the Ad2 E4 promoter (van Loon *et al.*, 1987b) and the Ad5 E2 and E3 promoters (Ishino *et al.*, 1988) compared with the Ad5 E1A promoters in plasmid transformed cells. Taken together these data suggest the Ad40 E1A region is either subject to aberrant expression or of intrinsically lower activity. Therefore, to investigate the involvement of the E1A region in the restricted growth of Ad40, a preliminary characterisation of sequences which were important for basal transcription (section 4.1) was undertaken. This was achieved in a plasmid background rather than a viral background as Ad40 is unable to form plaques in tissue culture (de Jong *et al.*, 1983), making it difficult to isolate and characterise promoter deletions within the virus.

The E1A promoter sequences of Ad5 (van Ormondt *et al.*, 1978) and Ad40 (van Loon *et al.*, 1987a) had previously been determined, allowing comparison of the Ad5 E1A upstream enhancer and core promoter sequences (Yoshida *et al.*, 1995), with the Ad40 E1A promoter (figure 4.1). To identify possible transcription factor binding sites within the Ad40 E1A promoter, sequences from the ITR (nucleotide 1) to the core promoter (as identified in Ad5 (Hearing and Shenk, 1983a); nucleotide 452) were compared with the transcription factor database held at EMBL (using a single mismatch algorithm; Ghosh, 1990). This revealed a number of possible transcription factor binding sites within the Ad40 E1A promoter (figure 4.1). The known Ad5 E1A core promoter and upstream enhancer sequences (Yoshida *et al.*, 1995) were then compared with the proposed map of the Ad40 E1A promoter (figure 4.1). This provided a basis for the design of a series

of deletion mutants, which would be used to characterise sequences important for basal transcription from the Ad40 E1A promoter.

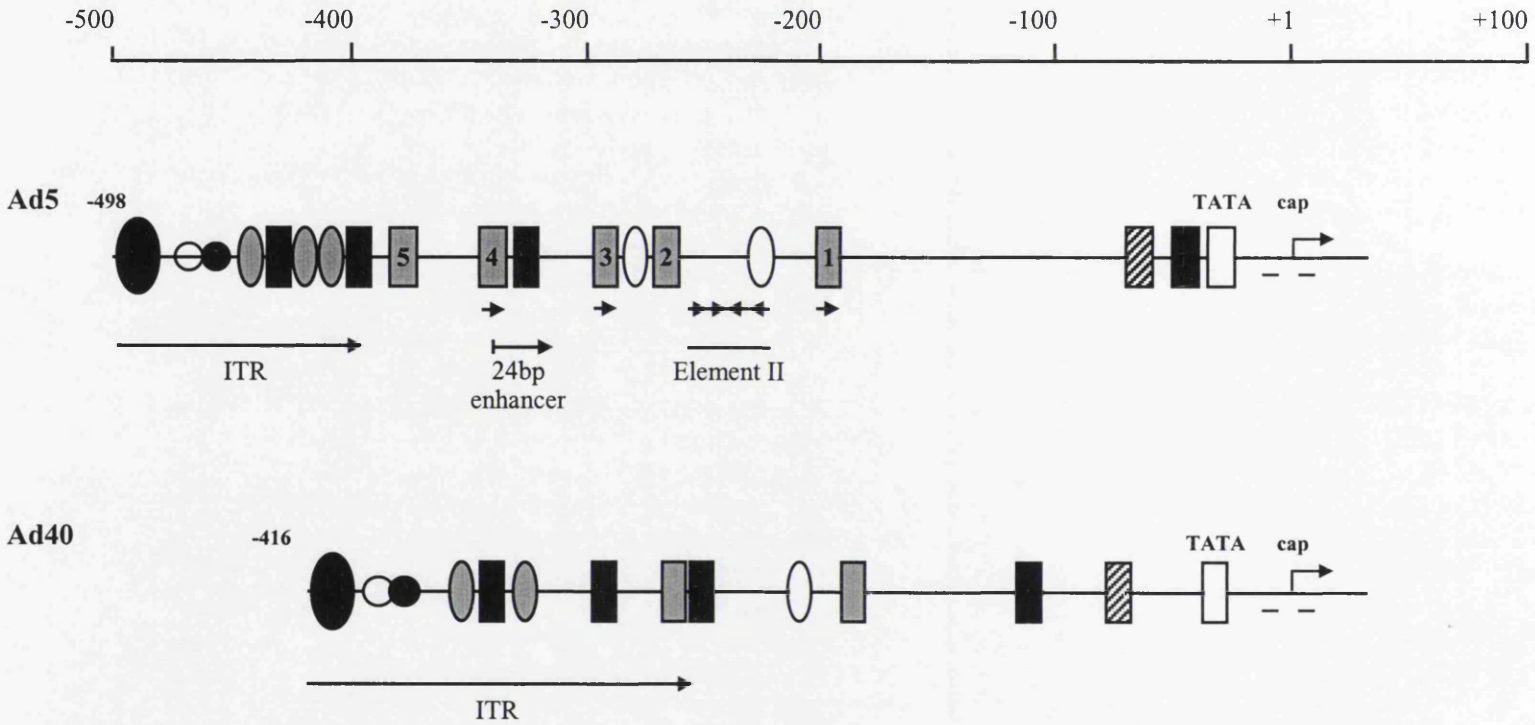


Figure 4.1 Comparison of the Ad5 E1A promoter with the Ad40 E1A promoter. Transcription factor binding sites within the Ad40 E1A promoter were predicted by comparison of the Ad40 sequence (1-452bp) with the transcription factor database held at EMBL (using a single mismatch algorithm). Transcription factor binding sites are indicated by: ● denotes ORP-A; ○ denotes NF I; ● denotes NF III; 0 denotes SP1; ■ denotes ATF; ▯ denotes EF-1A; ▨ denotes CAAT; 0 denotes E2F; ▯ denotes TATA; → denotes Element I; ⋈ denotes Element II; ⟼ denotes 24bp enhancer; ⌐→ denotes cap site; – – denotes hyphenated symmetry around cap site.

4.1.1 Construction of plasmids containing the Ad5 or the Ad40 E1A promoters

To characterise basal transcription from the Ad40 E1A promoter with the Ad5 E1A promoter, sequences encompassing the Ad5 and Ad40 E1A promoters were cloned into a luciferase reporter vector, pnull-luc (figure 2.1.1), then analysed in WS HeLa cells by transient transfection assays. The luciferase gene was chosen as the reporter, as previous transient transfection assays which had used the more traditional chloramphenicol acetyl transferase (CAT) reporter, under the control of the E1B promoter, resulted in *CAT* expression levels which were close to background, making analysis of the promoter difficult (Mautner *et al.*, 1999).

The Ad5 (1-533bp) and Ad40 (1-452bp) E1A promoters were generated by PCR of 100ng pLE1A (section 2.1) or pNM80 (section 2.1) respectively, using *Pfu* DNA polymerase (section 3.1.2.1) and primers FS1 and AB34 (table 2.4). The primers FS1 and AB34 contained *Sal* I and *Hind* III restriction sites respectively, a design which allowed directional cloning of the promoters into pnull-luc (figure 2.1.1). Plasmids containing the cloned Ad5 (pAd5-luc) and Ad40 (pAd40-luc) E1A promoters were identified by restriction analysis of miniprep DNA with *Sal* I and *Hind* III, and then sequenced using the M13 -20 sequencing primer to confirm the absence of mutations which may have arisen during the PCR. Large scale plasmid preparations of pAd5-luc and pAd40-luc, along with a positive (pHSV2IE5-luc; figure 2.1.2) and negative (pnull-luc; figure 2.1.1) control, were then prepared for use in transient transfection assays.

4.1.2 Transient transfection of pnull plasmids containing the Ad5 and the Ad40 E1A promoters

To characterise basal transcription from the Ad40 E1A promoter with the Ad5 E1A promoter, cells were transiently transfected with a calcium phosphate ($CaPO_4$) precipitate containing plasmids with the Ad5 and the Ad40 E1A promoters, in parallel with a positive and a negative control. Before transient transfection of the aforementioned sequence elements, background levels of firefly luciferase luminescence were calibrated for the luciferase reporter assay system, and conditions for $CaPO_4$ mediated transient transfection in WS HeLa cells were optimised.

Background levels of firefly luciferase luminescence result from instrumentation and sample tubes. The electronic design of the luminometer can greatly affect its measurable level of background signal, for example many luminometers do not read zero in the absence of a luminescent sample. Background signals can also be attributed to sample tubes, which may result from static electricity (particular when polystyrene tubes are used) or from phosphorescence, therefore handling and storage of tubes aimed to minimise static buildup. Samples were also handled away from sunlight and very bright lights before luminescence measurements were taken. To obtain the background signal contributed by the instrument and sample tube, 10x30mm plates of non-transfected WS HeLa cells (seeded at $0.5x10^6$ cells/plate) were harvested using the luciferase reporter assay system (section 3.4.3.1), then 5µl aliquots of cell extract were assayed for firefly luciferase luminescence (section 3.4.4.1) and protein content (section 3.4.5). A series of protein concentration standards (0mg, 0.2mg, 0.4mg, 0.6mg, 0.8mg, 1.0mg, 1.2mg and 1.4mg) were also assayed to calibrate the concentration of protein within each experimental sample. The firefly luciferase luminescence values (transmitted light units (TLU)) and the protein content (µg/µl) values for each sample were tabulated (see columns 2 and 3 in Table 4.1.2.1). The firefly luciferase luminescence values were then expressed as transmitted light units per µg of protein (TLU/µg) in order to compare the background values between samples (see column 4 in Table 4.1.2.1). The mean (µ) transmitted light units per µg of protein was calculated across ten samples of non-transfected cells, and tabulated (see column 5 in Table 4.1.2.1). In subsequent experiments the mean firefly luciferase luminescence assay

Table 4.1.2.1 Firefly luciferase luminescence assay background due to sample tube and instrumentation, using the luciferase reporter assay.

Sample	FLL[a] (TLU/μl)	Protein concentration (μg/μl)	TLU/μg	μ[b] (TLU/μg)
Non-transfected cells	6.4	0.91	7.0	10
Non-transfected cells	6.2	0.78	7.9	
Non-transfected cells	6.2	0.70	8.6	
Non-transfected cells	6.4	0.84	7.6	
Non-transfected cells	7.0	0.48	15	
Non-transfected cells	8.0	0.87	9.2	
Non-transfected cells	8.2	0.76	11	
Non-transfected cells	7.8	0.76	10	
Non-transfected cells	7.0	0.65	11	
Non-transfected cells	6.5	0.58	11	

a FLL denotes firefly luciferase luminescence

b μ denotes mean

background (10 TLU/μg) was subtracted from each firefly luciferase luminescence value per μg of protein (TLU/μg), in transient transfection assays with pnull-luc and derivatives.

The conditions for $CaPO_4$-mediated transient transfection were standardised with respect to: the total amount of DNA transfected 10μg (5μg pHSV2IE5-luc:5μg carrier (salmon sperm) DNA), 20μg (5μg pHSV2IE5-luc:15μg carrier DNA) or 40μg (5μg pHSV2IE5-luc:35μg carrier DNA); the incubation period of the $CaPO_4$ precipitate with the cells (6h or 16h); whether a glycerol shock was required to enhance transfection; and the ratio of plasmid to carrier DNA (1μg:9μg, 2μg:8μg, 3μg:7μg, 4μg:6μg, 5μg:5μg, 6μg:4μg, 7μg:3μg, 8μg:2μg, 9μg:1μg or 10μg:0μg; pHSV2IE5-luc:carrier DNA). The plasmid used in each transfection, was the positive control pHSV2IE5-luc (figure 2.1.2), which contains the HSV-2 IE-5 promoter upstream of the luciferase gene in pnull-luc, and the carrier DNA was salmon sperm DNA. The experiment was set up with duplicate 30mm plates of WS HeLa cells (seeded at $0.5x10^6$ cells/plate), which were transiently transfected with independently derived $CaPO_4$ precipitates. Cells were harvested using the luciferase reporter assay system (section 3.4.3.1), then 5μl aliquots of cell extract were assayed for firefly luciferase luminescence (section 3.4.4.1) and protein content (section 3.4.5), to calculate the firefly luciferase luminescence (TLU) per μg protein. The standard conditions for $CaPO_4$-mediated transient transfection in WS HeLa cells were determined as: 10μg of total DNA, of which 5μg was plasmid DNA and 5μg was carrier DNA, a 6h exposure to the $CaPO_4$ precipitate with no glycerol shock. These standard conditions were used for all $CaPO_4$-mediated transient transfections in WS HeLa cells using the pnull-luc plasmid and its derivatives.

To characterise basal transcription from the Ad40 E1A promoter (pAd40-luc) compared with the Ad5 E1A promoter (pAd5-luc), cells were transiently transfected with a calcium phosphate ($CaPO_4$) precipitate containing the aforementioned plasmids, in parallel with a positive (pHSV2IE5-luc; figure 2.1.2) and a negative (pnull-luc; figure 2.1.1) control. The negative control used in each experiment was pnull-luc, which contains a firefly luciferase gene upstream of the HSV-2 IE-5 polyadenylation signal (figure 2.1.1). Each experiment was set up with duplicate 30mm plates of WS HeLa cells (seeded at $0.5x10^6$ cells/plate), which were transiently transfected with

independently derived $CaPO_4$ precipitates (section 3.4.3), in two independent experiments. Cells were harvested using the luciferase reporter assay system (section 3.4.3.1), then 5µl aliquots of cell extract were assayed for firefly luciferase luminescence (section 3.4.4.1) and protein content (section 3.4.5). The tabulated values of firefly luciferase luminescence (TLU/µl) and protein content (µg/µl) (see columns 3 and 4 in Table 4.1.2.2) were used to calibrate the firefly luciferase luminescence per µg of protein (TLU/µg), which was corrected for firefly luciferase luminescence assay background (TLU/µg) (see column 5 and 6 in Table 4.1.2.2). To determine the average number of transmitted light units per µg of protein within each duplicate, the mean was calculated (μ_1, μ_2) by summing the corrected transmitted light units per µg protein (see column 6 in Table 4.1.2.2) then dividing by the number of samples (n) within each independent experiment (see column 7 in Table 4.1.2.2). To determine the range of the observations made within each duplicate, the standard deviation was calculated by summing the squares of the differences between the corrected transmitted light units per µg protein (see column 6 in Table 4.1.2.2) and the duplicate mean (µ1 or µ2, see column 7 Table 4.1.2.2), dividing by (n-1), and then taking the square root (see column 8 Table 4.1.2.2). The mean (µ, see column 9 Table 4.1.2.2) across the two independent experiments was calculated in the same manner as each of the duplicate means (µ1, µ2) however, the standard deviation across the two independent experiments was calculated using the following formula:

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

δ=standard deviation for duplicates one and two

δ_1=standard deviation duplicate one

δ_2=standard deviation duplicate two

The methods used to calculate the mean and standard deviations, for the replicates within each independent experiment, assumed a normal distribution for a large number of replicates. However, it should be noted that only a small number of replicates were actually made and therefore the mean (µ) and standard deviation (δ) obtained are estimated values. In addition, although comparisons within a single experiment are

valid, there may be problems in comparing between experiments. For example, measurement of ratios of *Renilla* to firefly luciferase between experiments might show an inherent variability due to differences in the activity or stability of the different substrates. For this reason, interpretation and discussion of the data focuses on the major qualitative trends seen within single experiments. In almost all instances (and unless discussed otherwise) the same general trends were consistently observed between experiments.

Figure 4.1.2 shows the mean (μ) and standard deviation (δ) values for firefly luciferase luminescence per μg of protein, for the Ad5 and the Ad40 E1A promoters, in parallel with the positive control which contains the HSV2 IE5 promoter and the negative control which is promoterless. It is immediately apparent that the negative control which does not contain a promoter, expresses greater levels of firefly luciferase than the Ad40 E1A promoter. The high levels of transcription from the negative control were thought to be mediated by cryptic promoter sequences lying upstream of the luciferase gene (Levitt *et al.*, 1989; Enriquez-Harris *et al.*, 1991). Cloning of the Ad40 E1A promoter upstream of the firefly luciferase may have disrupted the cryptic promoter sequences. These results demonstrate that the negative control is no longer appropriate for studies of basal transcription from the Ad40 E1A promoter. An alternative luciferase reporter system was therefore sought, in which a polyadenylation signal was situated directly upstream of the firefly luciferase gene, preventing readthrough transcription from cryptic promoter elements.

Table 4.1.2.2 Transient transfection of pAd5-luc and pAd40-luc

Duplicate	Sample	FLL[a] (TLU/μl)	Protein concentration (μg/μl)	TLU/μg protein	Corrected[b] TLU/μg	μ_1[c] μ_2	δ_1[d] δ_2	μ[e]	δ[f]
1	Ad5 E1A promoter	11	0.22	50	40	45	6.4	32	15
	Ad5 E1A promoter	20	0.34	59	49				
2	Ad5 E1A promoter	11	0.79	14	3.9	18	20		
	Ad5 E1A promoter	20	0.48	42	32				
1	Ad40 E1A promoter	5.9	0.49	12	2.0	1.7	0.42	0.85	0.30
	Ad40 E1A promoter	4.2	0.37	11	1.4				
2	Ad40 E1A promoter	7.0	0.81	8.6	0	0	0		
	Ad40 E1A promoter	7.2	0.91	7.9	0				
1	Positive control	84	0.50	168	158	157	2.1	121	43
	Positive control	86	0.52	165	155				
2	Positive control	93	0.68	137	127	84	61		
	Positive control	43	0.85	51	41				
1	Negative control	10	0.43	23	13	9.5	5.0	6.2	3.6
	Negative control	9.6	0.60	16	6.0				
2	Negative control	8.4	0.63	13	3.3	2.8	0.71		
	Negative control	8.0	0.65	12	2.3				

a FLL denotes firefly luciferase luminescence (TLU)

b The assay background value was 10 TLU/μg

c μ_1 denotes mean of duplicate one

d δ_1 denotes standard deviation of duplicate one

e μ denotes mean of duplicate one (μ_1) and two (μ_2)

f δ denotes standard deviation of duplicate one (δ_1) and two (δ_2), which was calculated using the formula described in section 4.1.2

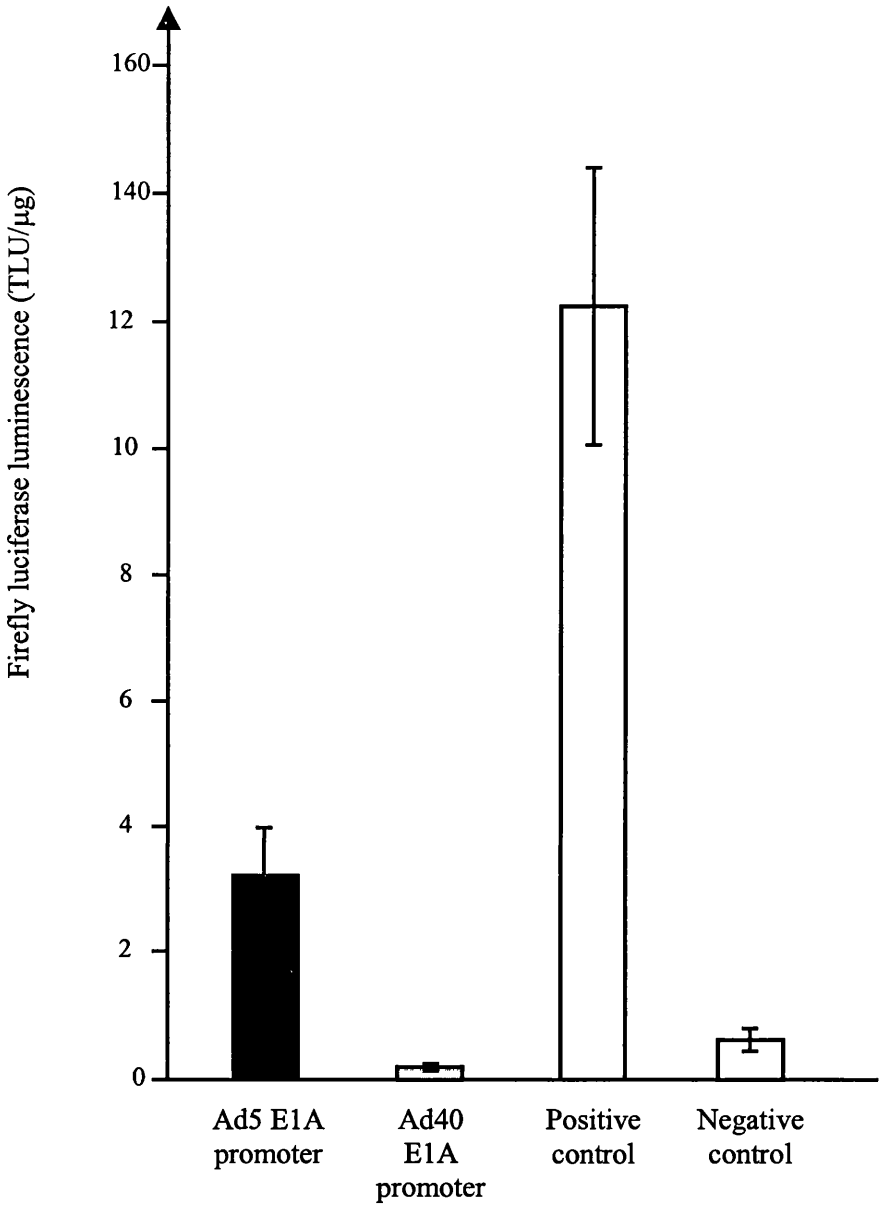


Figure 4.1.2 Transient transfection of pnull plasmids containing the Ad5 or the Ad40 E1A promoters. Two independent experiments were set up with duplicate 30mm plates of WS HeLa cells (seeded at $0.5x10^6$ cells/plate), which were transfected with independently derived calcium phosphate precipitates (section 3.4.3) containing 5µg of experimental DNA in parallel with a positive and negative control. The positive control contained the HSV2 IE5 promoter (pHSV2IE5-luc; figure 2.1.2), whilst the negative control (pnull-luc; figure 2.1.1) was promoterless. The cells were harvested using the luciferase reporter assay system (section 3.4.3.1), then 5µl aliquots were assayed for firefly luciferase luminescence (section 3.4.4.1) and protein content (section 3.4.5) to determine the TLU/µg protein. The µ and δ (capped lines) values for the TLU/µg protein are shown for the Ad5 and the Ad40 E1A promoter, along with the positive and negative control.

4.1.3 Subcloning of the Ad5 and the Ad40 E1A promoters into pGL3-basic

An alternative luciferase reporter system was sought to prevent readthrough transcription from upstream cryptic promoter sequences (Levitt *et al.*, 1989; Enriquez-Harris *et al.*, 1991). The reporter system identified was known as the pGL3 system (Promega), which contained two plasmids, one of which facilitated cloning of experimental promoters into a luciferase reporter system, and acted as a negative control in transient transfections (pGL3-basic; figure 2.1.3); whilst the second (pGL3-control; figure 2.1.4) was used as a positive control in transient transfections. pGL3-basic contains a synthetic polyadenylation signal and transcriptional pause site directly upstream of the multiple cloning site (MCS), downstream of the MCS is a modified firefly luciferase gene (*luc*+), which is processed under the control of the SV40 late polyadenylation signal (figure 2.1.3). pGL3-control contains the SV40 promoter inserted between the MCS and the modified luciferase gene (*luc*+), and a SV40 enhancer inserted downstream of the late polyadenylation signal (figure 2.1.4). The intact Ad5 and Ad40 E1A promoters were subsequently cloned into pGL3-basic, then analysed by transient transfection to establish differences in basal transcription from the Ad40 E1A promoter compared with the Ad5 E1A promoter.

The Ad5 and Ad40 E1A promoter fragments were generated by restriction digest of pAd5-luc and pAd40-luc, respectively with *Sal* I and *Hind* III, the promoter fragments were then ligated into pGL3-basic via *Xho* I and *Hind* III restriction sites in the MCS (figure 2.1.3). Candidate clones were then identified by restriction digest of miniprep DNA with *Sac* I and *Hind* III, prior to large-scale preparation of pGL3-Ad5, pGL3-Ad40, along with a positive (pGL3-promoter) and a negative (pGL3-basic) control for use in transient transfection assays.

4.1.4 Analysis of basal transcription from the Ad5 and the Ad40 E1A promoters

To characterise basal transcription from the Ad40 E1A promoter compared with the Ad5 E1A promoter, the promoter fragments had been cloned from the redundant pnull system into the pGL3 system. These plasmids were then assayed in transiently transfected WS HeLa cells, in parallel with a positive and negative control. Before transient transfection of the aforementioned plasmids could take place, a number of controls (described in the text below) were calibrated and the conditions for transient transfection were optimised using the pGL3 system.

Background levels of firefly luciferase luminescence in the dual luciferase reporter assay are also mediated by instrumentation and sample tubes (section 4.1.2). To obtain the background signal contributed by the instrument and sample tube within the dual luciferase reporter system, 10x60mm plates of non-transfected WS HeLa cells (seeded at $1x10^6$ cells/plate) were harvested (section 3.4.3.2), then 20µl aliquots of cell extract were assayed for firefly luciferase luminescence (section 3.4.4.2) measured in transmitted light units (TLU)). The firefly luciferase luminescence values (TLU) were tabulated (see column 2 samples 1-10 in Table 4.1.4.1). The mean (µ) firefly luciferase luminescence assay background due to instrumentation and sample tube, was calculated across ten samples of non-transfected cells (see column 3 in Table 4.1.4.1) to obtain a statistically significant value. In subsequent experiments the mean firefly luciferase luminescence assay background (FLLAB) was subtracted from each firefly luciferase luminescence value (TLU). Subsequent transient transfection experiments included a single plate of non-transfected WS HeLa cells, which were assayed for firefly luciferase activity to establish that they approximated the control sample value (see column 3 in Table 4.1.4.1).

Conditions for $CaPO_4$-mediated transient transfection in WS HeLa cells were optimised, with respect to the total amount of DNA transfected (optimum = 10µg), the incubation period of the $CaPO_4$ precipitate with the cells (optimum = 6h) and whether a glycerol shock was required to enhance transfection (optimum = not required) in section 4.1.2. The ratio of plasmid DNA:carrier DNA (0.5µg:9.5g, 1µg:9µg, 2µg:8µg, 5µg:5µg, or

Table 4.1.4.1 Firefly luciferase luminescence assay background due to sample tube and instrumentation, using the dual-luciferase reporter assay

Sample	FLL[a] (TLU)										FLLAB[b] (TLU)
	1	2	3	4	5	6	7	8	9	10	
Non-transfected cells	26	25	24	26	23	25	23	27	25	25	25

a FLL denotes firefly luciferase luminescence

b FLLAB denotes firefly luciferase luminescence assay background, which was calculated by taking the mean of samples 1-10

Table 4.1.4.2 *Renilla* luciferase luminescence assay background due to instrumentation, sample tube and auto-luminescence of coelenterazine

Sample	RLL[a] (TLU)										RLLAB[b] (TLU)
	1	2	3	4	5	6	7	8	9	10	
Non-transfected cells	26	24	23	26	22	26	23	27	25	25	25

a RLL denotes *Renilla* luciferase luminescence

b RLLAB denotes *Renilla* luciferase luminescence assay background, which was calculated by taking the mean of samples 1-10

10μg:0μg; pGL3-Ad5:salmon sperm DNA, pGL3-Ad40:salmon:sperm DNA and pGL3-control:salmon sperm DNA) was then optimised for WS HeLa cells. Each experiment was set up with duplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were transiently transfected with independently derived $CaPO_4$ precipitates. Cells were harvested using the luciferase dual reporter assay system (section 3.4.3.2), then 20μl aliquots of cell extract were assayed for firefly luciferase luminescence (section 3.4.4.2) and protein concentration section 3.4.5). The optimum ratio of plasmid:carrier DNA was determined as 10μg plasmid DNA:0μg carrier DNA, for pGL3-Ad5, pGL3-Ad40 and pGL3-control.

The addition of an internal control, pRL-SV40 (figure 2.1.6), into the transfection protocol provided a single tube dual luciferase reporter system, which allowed normalisation of results within a single experiment. The co-reporter plasmid, pRL-SV40, contains a SV40 early enhancer/promoter region inserted upstream of the *Renilla* (sea pansy) luciferase gene, which is processed under the control of the SV40 late polyadenylation signal (figure 2.1.6); the negative control for the co-reporter pRL-SV40, is pRL-null which contains a sea pansy luciferase gene upstream of the SV40 polyadenylation signal (figure 2.1.5). *Renilla* luciferase luminescence (like firefly luciferase luminescence) is subject to background signals, which can arise from three possible sources, including instrument and sample tube, auto-luminescence of coelenterazine caused by non-enzymatic oxidation of the coelenterazine in solution, and residual luminescence from the firefly luciferase reaction. To obtain the value of the *Renilla* luciferase luminescence background signal, contributed by the instrument and sample tube, and auto-luminescence of coelenterazine, 10x60mm plates of non-transfected WS HeLa cells (seeded at $1x10^6$ cells/plate) were harvested using the dual-luciferase reporter assay system (section 3.4.3.2), then 20μl aliquots were assayed for *Renilla* luciferase luminescence (section 3.4.4.2). The mean (μ) was determined across ten samples to obtain a statistically significant value for *Renilla* luciferase luminescence assay background (RLLAB), contributed by instrument and sample tube, and auto-luminescence of coelenterazine; this value was subtracted from each *Renilla* luciferase luminescence value henceforth (see column 3 in Table 4.1.4.2). Subsequent transient transfection experiments included a single plate of non-transfected WS HeLa

cells, which were assayed for *Renilla* luciferase activity to establish that they approximated the control sample value (see column 3 in Table 4.1.4.2).

Renilla luciferase luminescence is subject to background signals from residual firefly luciferase luminescence, which are variable depending upon the levels of firefly luciferase gene expression. Therefore, it was important to verify that the maximum expected level of firefly luciferase luminescence did not yield significant residual luminescence, which may have impacted upon the accurate measurement of *Renilla* luciferase luminescence. To verify that the maximum expected level of firefly luciferase luminescence from the positive control (pGL3-control) did not yield significant residual luminescence, 10x60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate) were transiently transfected with independently derived $CaPO_4$ precipitates containing 10μg pGL3-control (section 3.4.3). Cells were harvested using the dual-luciferase reporter assay system (section 3.4.3.2), then 20μl aliquots of cell extract were assayed for firefly luciferase luminescence then *Renilla* luciferase luminescence (section 3.4.4.2). The sample values were tabulated, then the mean firefly (FLLAB) and *Renilla* luciferase luminescence assay background (RLLAB) values were subtracted from the respective sample values (see Table 4.1.4.3). The background contributed by residual firefly luciferase luminescence was either negligible or zero in relation to *Renilla* luciferase luminescence assay background (RLLAB), therefore residual firefly luciferase luminescence did not need to be taken into account within future experiments.

Before commencing transient transfection analyses with the internal control, pRL-SV40, it is important to realise that *trans* effects between promoters in co-transfected plasmids can potentially affect reporter gene expression (Farr and Roman, 1991). The occurrence and magnitude of *trans* effects depends upon several factors, including the combination and activities of regulatory enhancer and promoter elements within the co-transfected vectors, the ratio of experimental to co-reporter DNA, and the cell type. To ensure independent expression from the experimental (pGL3-Ad5, pGL3-Ad40 and pGL3-control) and the co-reporter (pRL-SV40) plasmids, the optimum ratio of experimental to co-reporter DNA (10:1, 20:1 or 50:1) was determined. Duplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate) were transfected with independently derived $CaPO_4$ precipitates containing 10μg pGL3-Ad5, pGL3-Ad40 or

Table 4.1.4.3 *Renilla* luciferase luminescence assay background contributed by residual firefly luciferase luminescence

Plasmid	Sample 1				Sample 2				Sample 3				Sample 4				Sample 5			
	FLL[a] (TLU)	FLL – FLLAB[b] (TLU)	RLL[c] (TLU)	RLL – RLLAB[d] (TLU)	FLL (TLU)	FLL - FLLAB (TLU)	RLL (TLU)	RLL - RLLAB (TLU)	FLL (TLU)	FLL - FLLAB (TLU)	RLL (TLU)	RLL - RLLAB (TLU)	FLL (TLU)	FLL - FLLAB (TLU)	RLL (TLU)	RLL - RLLAB (TLU)	FLL (TLU)	FLL - FLLAB (TLU)	RLL (TLU)	RLL - RLLAB (TLU)
Positive control[e]	8215	8190	26	1	52962	52937	28	3	22829	22804	24	0	6825	6800	25	0	15121	14996	26	1
Plasmid	Sample 6				Sample 7				Sample 8				Sample 9				Sample 10			
	FLL (TLU)	FLL - FLLAB (TLU)	RLL (TLU)	RLL - RLLAB (TLU)	FLL (TLU)	FLL - FLLAB (TLU)	RLL (TLU)	RLL - RLLAB (TLU)	FLL (TLU)	FLL - FLLAB (TLU)	RLL (TLU)	RLL - RLLAB (TLU)	FLL (TLU)	FLL - FLLAB (TLU)	RLL (TLU)	RLL - RLLAB (TLU)	FLL (TLU)	FLL - FLLAB (TLU)	RLL (TLU)	RLL - RLLAB (TLU)
Positive control[e]	4116	3991	25	0	65198	65173	27	2	46610	26585	24	0	14487	14462	25	0	43727	43702	25	0

a FLL denotes firefly luciferase luminescence

b FLLAB denotes firefly luciferase luminescence assay background

c RLL denotes *Renilla* luciferase luminescence

d RLLAB denotes *Renilla* luciferase luminescence assay background

e Positive control denotes pGL3-control (figure 2.1.4)

pGL3-control to 1μg, 0.5μg or 200ng pRL-SV40 (section 3.4.3). The optimum ratio of experimental to co-reporter DNA for pGL3-Ad5, pGL3-Ad40 and pGL3-control was determined as 10μg experimental to 200ng co-reporter DNA i.e. a ratio of 50:1.

Basal transcription from the Ad40 E1A promoter (pGL3-Ad40) compared with the Ad5 E1A promoter (pGL3-Ad5) was established by transiently co-transfecting WS Hela cells with a $CaPO_4$ precipitate containing the aforementioned plasmids, in parallel with a positive (pGL3-control; figure 2.1.4) and negative (pGL3-basic; figure 2.1.3) control. The negative control used in each experiment was pGL3-basic, which contains a modified firefly luciferase gene (*luc+*) upstream of the late SV40 polyadenylation signal (figure 2.1.3). Each experiment was set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were transiently transfected with independently derived $CaPO_4$ precipitates, in two independent experiments (section 3.4.3). Cells were harvested using the dual-luciferase reporter assay system (section 3.4.3.2), then 20μl aliquots of cell extract were assayed for firefly luciferase luminescence and *Renilla* luciferase luminescence (section 3.4.4.2). The tabulated values of firefly luciferase luminescence (TLU) and *Renilla* luciferase luminescence (TLU) (see columns 3 and 5 in Table 4.1.4.4) were corrected for firefly luciferase luminescence assay background (FLLAB) and the *Renilla* luciferase luminescence assay background (RLLAB) respectively (see columns 4 and 6 in Table 4.1.4.4). Samples within a single experiment were then normalised with respect to each other by calculating the ratio of firefly luciferase luminescence to *Renilla* luciferase luminescence (see column 7 in Table 4.1.4.4), by dividing the corrected firefly luciferase luminescence value by the corrected *Renilla* luciferase luminescence value (see columns 4 and 6 in Table 4.1.4.4). The mean (μ_1, μ_2) ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) and standard deviation (δ_1, δ_2) for each triplicate were calculated (see columns 8 and 9 in Table 4.1.4.4). The δ (see column 11 in Table 4.1.4.4) value was calculated using the formula expressed in section 4.1.2. Table 4.1.4.4 shows the standard deviation (δ) between the two independent experiments is acceptable. However if the FLL:RLL ratio for the first triplicate containing the Ad5 E1A promoter (μ_1 is 21, taken from column 7 in Table 4.1.4.4) is divided by the FLL:RLL ratio for the first triplicate containing the Ad40 E1A promoter (μ_2 is 3.2, taken from column 7 in Table 4.1.4.4),

giving a value of 6.6, then compared with the equivalent figure for triplicate two whose value is 6.5, we can see that the co-reporter system provides a controlled system between independent experiments.

Figure 4.1.4 shows the mean (μ) and standard deviation (δ) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) across two independent experiments, for the Ad5 and the Ad40 E1A promoters, in parallel with the positive control which contains the SV40 promoter and the negative control which is promoterless. Expression from the Ad40 E1A promoter is significantly lower when compared with expression from the Ad5 E1A promoter. The numerical value for the difference in basal transcription from the Ad40 E1A promoter compared with the Ad5 E1A promoter, was calculated by dividing the mean (μ) value for the Ad5 E1A promoter by the mean (μ) value for the Ad40 E1A promoter (see column 10 in Table 4.1.4.4). Basal transcription from the Ad40 E1A promoter in WS HeLa cells was lowered by a factor of approximately 6.5 when compared with basal transcription from the Ad5 E1A promoter.

Table 4.1.4.4 Transient transfection of pGL3-Ad5 and pGL3-Ad40

Triplicate	Sample	FLL[a] (TLU)	FLL - FLLAB[b] (TLU)	RLL[c] (TLU)	RLL - RLLAB[d] (TLU)	FLL/ RLL	μ_1[e] μ_2	δ_1[f] δ_2	μ[g]	δ[h]
1	Ad5 E1A promoter	23893	23958	1473	1448	17	21	3	17	2.1
	Ad5 E1A promoter	21329	21304	993	968	22				
	Ad5 E1A promoter	22563	22538	1013	988	23				
2	Ad5 E1A promoter	11563	11538	891	866	13	13	0		
	Ad5 E1A promoter	10988	10963	852	827	13				
	Ad5 E1A promoter	11256	11231	869	844	13				
1	Ad40 E1A promoter	8421	8396	2650	2625	3.2	3.2	0	2.6	0.14
	Ad40 E1A promoter	8285	8260	2581	2556	3.2				
	Ad40 E1A promoter	8293	8268	2610	2585	3.2				
2	Ad40 E1A promoter	3589	3564	1994	1969	1.8	2.0	0.2		
	Ad40 E1A promoter	3911	3886	1956	1931	2.0				
	Ad40 E1A promoter	4112	4087	1869	1844	2.2				
1	Positive control	15121	15096	1164	1139	13	12	1.0	8.1	0.72
	Positive control	4116	4091	404	379	11				
	Positive control	15523	15498	1264	1239	13				
2	Positive control	6593	6568	1648	1623	4.1	4.2	0.2		
	Positive control	4566	4541	1062	1037	4.4				
	Positive control	5463	5438	1366	1341	4.1				

Triplicate	Sample	FLL[a] (TLU)	FLL - FLLAB[b] (TLU)	RLL[c] (TLU)	RLL - RLLAB[d] (TLU)	FLL/ RLL	μ_1[e] μ_2	δ_1[f] δ_2	μ[g]	δ[h]
1	Negative control	73	48	4403	4378	0.01	0.01	0	0.01	0
	Negative control	31	6	2646	2621	0.01				
	Negative control	51	26	3454	3429	0.01				
2	Negative control	45	20	2563	2538	0.01	0.01	0		
	Negative control	61	36	3765	3740	0.01				
	Negative control	51	26	3112	3087	0.01				

a FLL denotes firefly luciferase luminescence

b FLLAB denotes firefly luciferase luminescence assay background

c RLL denotes Renilla luciferase luminescence

d RLLAB denotes Renilla luciferase luminescence assay background

e μ_1 denotes mean of triplicate 1

f δ_1 denotes standard deviation of triplicate 1

g μ denotes the mean of triplicate 1 (μ_1) and two (μ_2)

h δ denotes the standard deviation of triplicate one (δ_1) and two (δ_2), which was calculated using the formula described in section 4.1.2

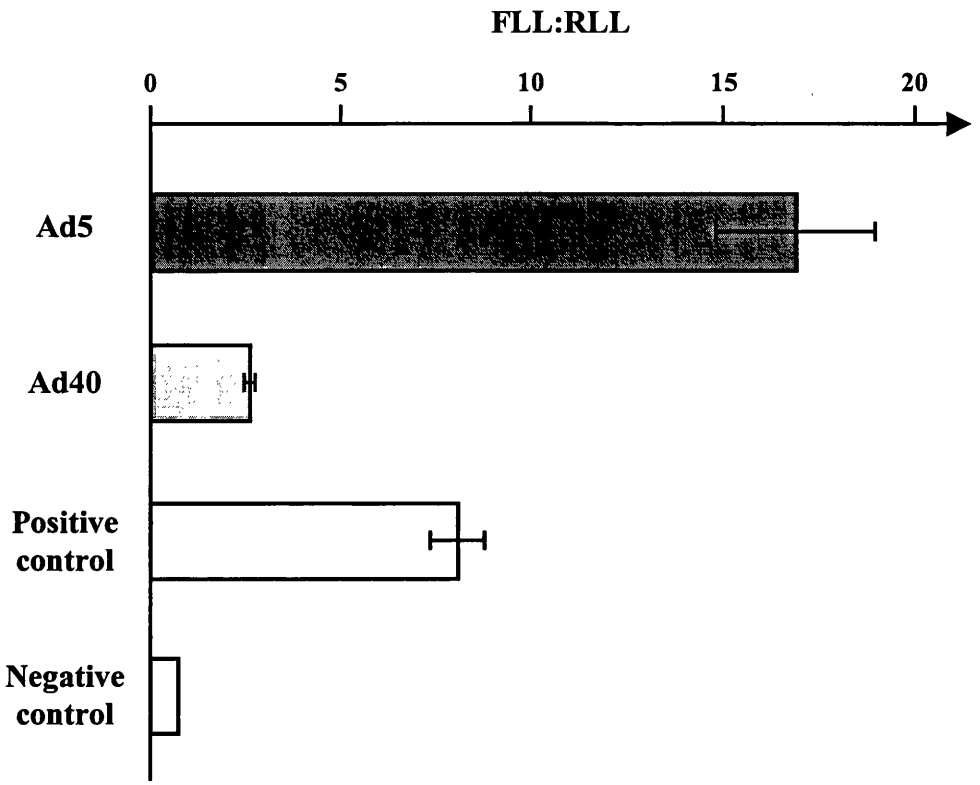


Figure 4.1.4. Analysis of the Ad5 and the Ad40 E1A promoters. Two independent experiments were set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were transfected with independently derived calcium phosphate precipitates containing 10μg of experimental DNA plus 200ng pRL-SV40, in parallel with a positive and negative control (section 3.4.3). The positive control pGL3-control (figure 2.1.4) contained the SV40 promoter, whilst the negative control pGL3-basic (figure 2.1.3) was promoterless. pRL-SV40 (figure 2.1.6) contains the *Renilla* luciferase gene under the control of the SV40 promoter, which acts as a co-reporter for the assay system. The cells were harvested using the dual luciferase reporter assay system (section 3.4.3.2), then 20μl aliquots were assayed for firefly luciferase luminescence and *Renilla* luciferase luminescence (section 3.4.3.2). The μ and δ (capped lines) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) are shown for the Ad5 and Ad40 E1A promoters, along with a positive and negative control.

4.1.5 Construction of *Bal* 31 deletions of the Ad5 and Ad40 E1A promoters

Transient transfection analyses had revealed that basal transcription from the Ad40 E1A promoter was significantly reduced (6.5 ± 0.04 fold) when compared with the Ad5 E1A promoter. In order to delineate the sequences important in basal transcription from the Ad40 E1A promoter, a series of *Bal* 31 deletion mutants were constructed for use in transient transfection assays. A second series of *Bal* 31 deletion mutants were also constructed for the Ad5 E1A promoter, allowing comparison of sequences known to be important for basal transcription in the Ad5 E1A promoter, with sequences in the Ad40 E1A promoter.

Deletions of the Ad5 and the Ad40 E1A promoters were generated by *Bal* 31 nuclease digestion of 50μg *Sal* I digested pAd5-luc and pAd40-luc for 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min. To confirm that a range of fragments between 1-533bp for the Ad5 E1A promoter, and 1-452bp for the Ad40 E1A promoter had been generated, a sixth of each aliquot was digested with *Hind* III, and visualised on an ethidium bromide stained agarose gel. The aliquots were pooled, and then cloned via *Sal* I (using *Sal* I linkers) and *Hind* III restriction sites into pnull-luc (figure 2.1.1). Plasmids containing *Bal* 31 deletions of the Ad5 and the Ad40 E1A promoters were identified by restriction analysis of miniprep DNA with *Sal* I and *Hind* III, then sequenced using the M13 -20 sequencing primer to confirm the length of each deletion mutant (figure 4.1.5). *Bal* 31 deletion mutants were chosen to approximately divide the Ad40 E1A promoter into four (pAd40-luc.349, pAd40-luc.268, pAd40-luc.140), however the smallest deletion (pAd40-luc.37) containing the predicted TATA box was not generated, therefore it was constructed by restriction digest of the Ad40 E1A promoter (section 4.1.7). *Bal* 31 deletions of the Ad5 E1A promoter (pAd5-luc.428, pAd5-luc.321, pAd5-luc.171, pAd5-luc.42) were selected to correspond with the predicted transcription factor binding sites contained within the *Bal* 31 deletions of the Ad40 E1A promoter.

The *Bal* 31 deletion mutants of the Ad5 and the Ad40 E1A promoter had been cloned into pnull-luc before the system was found to be inappropriate (section 4.1.2), therefore

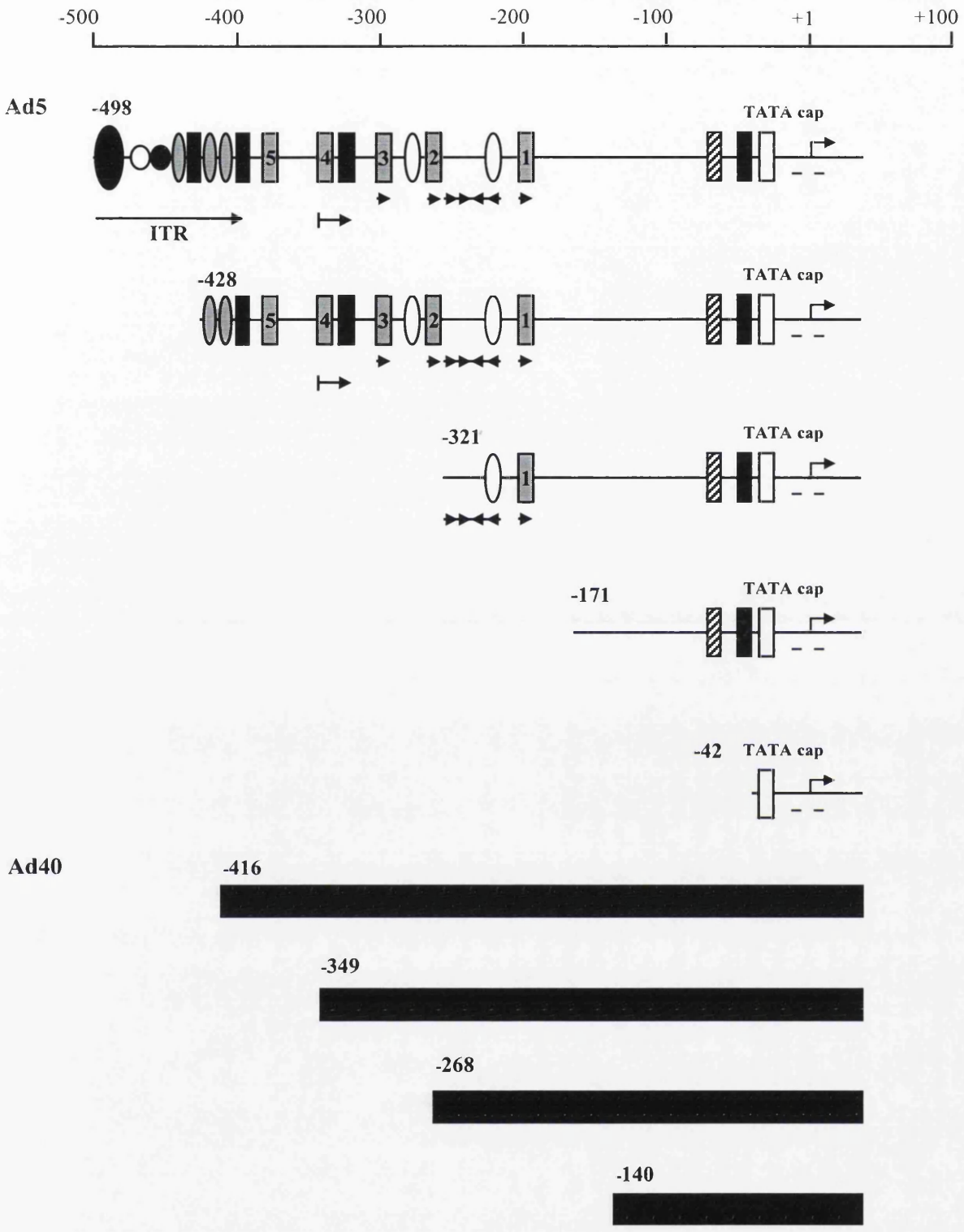


Figure 4.1.5 The intact and *Bal* 31 deleted Ad5 and Ad40 E1A promoters. Transcription factor binding sites within the intact and deleted Ad5 E1A promoter are indicated by: ● denotes ORP-A; ○ denotes NF I; ● denotes NF III; ◊ denotes SP1; ■ denotes ATF; ▯ denotes EF-1A; ◊ denotes E2F; ► denotes Element I; ►◄ denotes Element II; ⟼ denotes 24bp enhancer; ▨ denotes CAAT; ▯ denotes TATA; ↱ denotes cap site; - - denotes hyphenated symmetry around cap site.

deletions were subcloned into pGL3-basic. The Ad5 and the Ad40 E1A promoter fragments were generated by restriction digest of pAd5-luc.428, pAd5-luc.321, pAd5-luc.171, pAd5-luc.42, pAd40-luc.349, pAd40-luc.268 and pAd40-luc.140 with *Sal* I and *Hind* III, the promoter fragments were then ligated into pGL3-basic via *Xho* I and *Hind* III restriction sites in the MCS (figure 2.1.3). Candidate clones were identified by restriction digest of miniprep DNA with *Sac* I and *Hind* III, prior to large-scale preparation of pAd5-luc.428, pAd5-luc.321, pAd5-luc.171, pAd5-luc.42, pAd40-luc.349, pAd40-luc.268, pAd40-luc.140 and pGL3-Ad40.37 for use in transient co-transfection assays.

4.1.6 Analysis of basal transcription from the intact and *Bal*31 deleted Ad5 and Ad40 E1A promoters

To characterise sequences important for basal transcription from the Ad40 E1A promoter compared with the Ad5 E1A promoter, cells were transiently transfected with a $CaPO_4$ precipitate containing either the intact Ad5 (pGL3-Ad5) or Ad40 (pGL3-Ad40) E1A promoters, or the *Bal* 31 deleted Ad5 or Ad40 E1A promoters (pGL3-Ad5.428, pGL3-Ad5.321, pGL3-Ad5.171, pGL3-Ad5.42, pGL3-Ad40.349, pGL3-Ad40.268, pGL3-Ad40.140, pGL3-Ad40.37), in parallel with a positive (pGL3-control; figure 2.1.4) and a negative (pGL3-basic; figure 2.1.3) control. The experiment was set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were transiently transfected with independently derived $CaPO_4$ precipitates (section 3.4.3). Cells were harvested using the dual-luciferase reporter assay system (section 3.4.3.2), then 20µl aliquots of cell extract were assayed for firefly luciferase luminescence then Renilla luciferase luminescence (section 3.4.4.2). These values were tabulated (see columns 3 and 4 in Table 4.1.6) then used to calculate the mean (µ) and standard deviation (δ) for each sample, across two independent experiments (see columns 10 and 11 in Table 4.1.6).

Figure 4.1.6 A shows a diagrammatic representation of the intact and *Bal* 31 deleted Ad5 and Ad40 E1A promoters, whilst B shows the mean (µ) and standard deviation (δ) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) across two independent experiments, for the corresponding promoter construct in A. Basal transcription from the intact Ad40 E1A promoter compared with the intact Ad5 E1A promoter is lowered by a factor of six, as in figure 4.1.4. Subsequent deletion of sequences from the ITR to the TATA box in both the Ad5 and the Ad40 E1A promoters resulted in an almost identical pattern of expression (figure 4.1.6). Expression from the intact Ad5 E1A promoter increased by an approximated 1.7 fold with the deletion of sequences from -498 to -428 (relative to the cap site), upon deletion of sequences from -428 to -321 an approximated 5.5 fold decrease in expression was observed. Basal transcription from the Ad5 E1A promoter then steadily decreased with the subsequent deletion of sequences from -321 to -171

Table 4.1.6 Transient transfection of the intact and *Bal* 31 deleted Ad5 and Ad40 E1A promoters

Triplicate	Sample	FLL [a] (TLU)	FLL - FLLAB [b] (TLU)	RLL [c] (TLU)	RLL - RLLAB [d] (TLU)	FLL/ RLL	μ_1 [e] μ_2	δ_1 [f] δ_2	μ [g]	δ [h]
1	Ad5 E1A promoter	1738	1713	822	797	2.2	2.1	0.13	7.2	1.1
	Ad5 E1A promoter	2696	2671	1290	1265	2.1				
	Ad5 E1A promoter	1132	1107	610	585	1.9				
2	Ad5 E1A promoter	2705	2680	211	186	14	12	1.6		
	Ad5 E1A promoter	3191	3166	319	294	11				
	Ad5 E1A promoter	3521	3496	329	304	12				
1	Ad5 del.428	12001	11976	1806	1781	6.7	6.1	0.49	12	2.4
	Ad5 del.428	3475	3450	652	627	5.5				
	Ad5 del.428	18516	18491	3051	3026	6.1				
2	Ad5 del.428	10110	10085	607	582	17	18	3.3		
	Ad5 del.428	4883	4858	353	328	14				
	Ad5 del.428	9187	9162	431	406	22				
1	Ad5 del.321	4681	4656	2185	2160	2.2	2.1	0.25	2.2	0.32
	Ad5 del.321	5151	5126	2192	2167	2.4				
	Ad5 del.321	2405	2380	1370	1345	1.8				
2	Ad5 del.321	410	385	200	175	2.2	2.3	0.37		
	Ad5 del.321	639	614	247	222	2.8				
	Ad5 del.321	396	371	222	197	1.9				
1	Ad5 del.171	882	857	1054	1029	0.83	0.66	0.12	0.71	0.10
	Ad5 del.171	493	468	895	870	0.54				
	Ad5 del.171	1147	1122	1871	1846	0.61				
2	Ad5 del.171	76	51	91	66	0.77	0.76	0.08		
	Ad5 del.171	80	55	90	65	0.85				
	Ad5 del.171	104	79	147	122	0.65				
1	Ad5 del.42	97	72	1614	1589	0.05	0.05	0.01	0.06	0.01
	Ad5 del.42	119	94	1726	1701	0.06				
	Ad5 del.42	191	166	3336	3311	0.05				
2	Ad5 del.42	30	5	98	73	0.07	0.07	0.01		
	Ad5 del.42	30	5	111	86	0.06				
	Ad5 del.42	30	5	81	56	0.09				

Triplicate	Sample	FLL [a] (TLU)	FLL - FLLAB [b] (TLU)	RLL [c] (TLU)	RLL - RLLAB [d] (TLU)	FLL/ RLL	μ_1 [e] μ_2	δ_1 [f] δ_2	μ [g]	δ [h]
1	Ad40 E1A promoter	1638	1613	1387	1362	1.2	1.2	0.06	1.2	0.15
	Ad40 E1A promoter	10956	10931	8638	8613	1.3				
	Ad40 E1A promoter	4005	3980	3313	3288	1.2				
2	Ad40 E1A promoter	87	62	91	66	0.94	1.2	0.20		
	Ad40 E1A promoter	147	122	114	89	1.4				
	Ad40 E1A promoter	319	294	261	236	1.3				
1	Ad40 del.349	5670	5645	3806	3781	1.5	1.4	0.1	1.9	0.12
	Ad40 del.349	977	952	654	629	1.5				
	Ad40 del.349	1317	1292	1014	989	1.3				
2	Ad40 del.349	675	650	300	275	2.4	2.3	0.14		
	Ad40 del.349	778	753	336	311	2.4				
	Ad40 del.349	946	921	466	441	2.1				
1	Ad40 del.268	2280	2255	3827	3802	0.59	0.63	0.04	0.58	0.04
	Ad40 del.268	3912	3887	5705	5680	0.68				
	Ad40 del.268	5820	5795	9189	9164	0.63				
2	Ad40 del.268	168	143	284	259	0.55	0.53	0.03		
	Ad40 del.268	96	71	171	146	0.49				
	Ad40 del.268	133	108	223	198	0.55				
1	Ad40 del.140	76	51	887	862	0.06	0.09	0.04	0.10	0.03
	Ad40 del.140	145	120	1774	1749	0.07				
	Ad40 del.140	81	56	414	389	0.14				
2	Ad40 del.140	44	19	180	155	0.12	0.10	0.02		
	Ad40 del.140	36	11	169	144	0.08				
	Ad40 del.140	42	17	178	153	0.11				
1	Ad40 del.37	57	32	2661	2636	0.01	0.01	0	0.05	0.01
	Ad40 del.37	54	29	2335	2310	0.01				
	Ad40 del.37	39	14	1331	1306	0.01				
2	Ad40 del.37	39	14	233	208	0.07	0.08	0.01		
	Ad40 del.37	40	15	221	196	0.08				
	Ad40 del.37	41	16	201	176	0.09				

Triplicate	Sample	FLL [a] (TLU)	FLL - FLLAB [b] (TLU)	RLL [c] (TLU)	RLL - RLLAB [d] (TLU)	FLL/ RLL	μ_1 [e] μ_2	δ_1 [f] δ_2	μ [g]	δ [h]
1	Positive Control	11512	11487	923	898	12	13	0.6	14	0.6
	Positive Control	55602	55577	4205	4180	13				
	Positive control	22921	22896	1756	1731	13				
2	Positive control	1733	1708	141	116	14	14	0.6		
	Positive control	547	522	63	38	13				
	Positive control	893	868	85	60	14				
1	Negative control	34	9	910	885	0.01	0.01	0	0.03	0.01
	Negative control	30	5	833	808	0.01				
	Negative control	34	9	1777	1752	0.01				
2	Negative control	30	5	220	195	0.03	0.04	0.01		
	Negative control	28	3	109	184	0.04				
	Negative control	31	6	154	129	0.05				

a FLL denotes firefly luciferase luminescence

b FLLAB denotes firefly luciferase luminescence assay background

c RLL denotes *Renilla* luciferase luminescence

d RLLAB denotes *Renilla* luciferase luminescence assay background

e μ_1 denotes mean of duplicate one

f δ_1 denotes standard deviation of duplicate one

g μ denotes mean of duplicate one (μ_1) and two (μ_2)

h δ denotes standard deviation of duplicate one (δ_1) and two (δ_2), which was calculated using the formula described in section 4.1.2

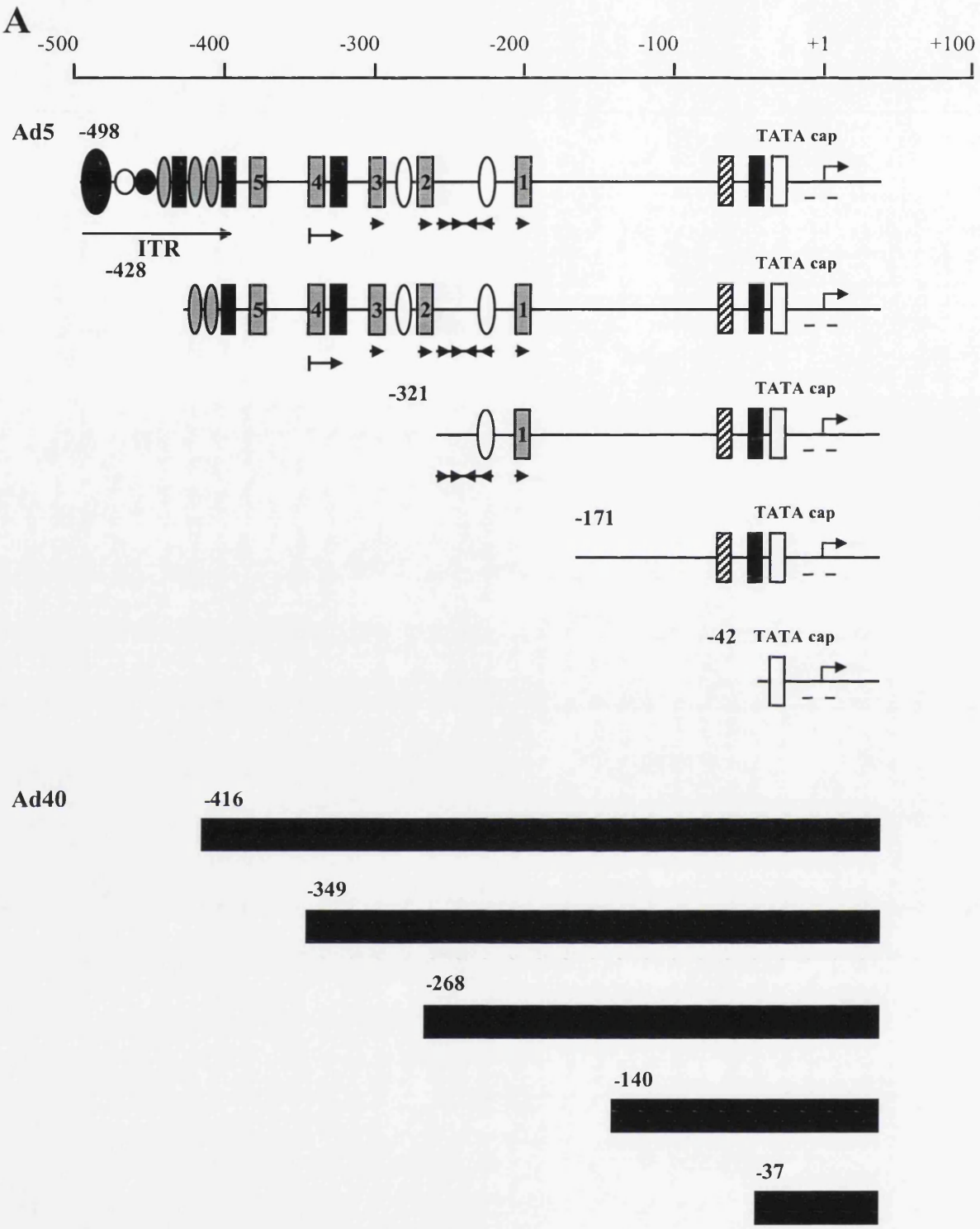
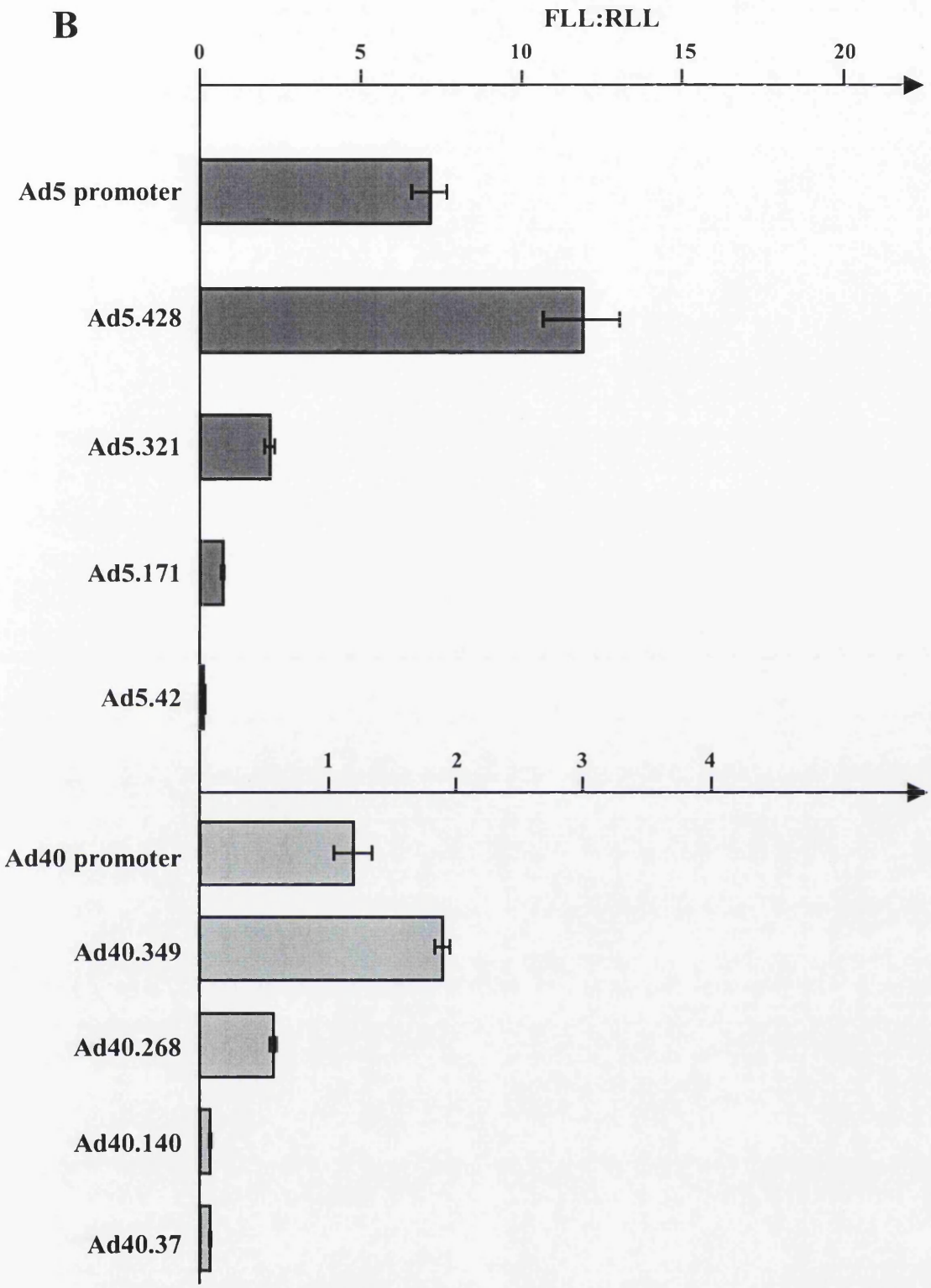


Figure 4.1.6 Analysis of the intact and *Bal* 31 deleted Ad5 or Ad40 E1A promoters. Two independent experiments were set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were transfected with independently derived calcium phosphate precipitates containing 10μg experimental DNA plus 200ng pRL-SV40, in parallel with a positive and negative control. The positive control contained the SV40 promoter, whilst the negative control was promoterless. The cells were harvested using the dual luciferase reporter assay system, then were assayed for firefly luciferase luminescence (TLU) and *Renilla* luciferase luminescence (TLU). **A.** The Ad5 and the Ad40 E1A full



length and *Bal* 31 deleted promoters. Transcription factor binding sites are indicated by: ⬮ denotes ORP-A; ○ denotes NF I; ● denotes NF III; ⬯ denotes SP1; ■ denotes ATF; ▯ denotes EF-1A; ⬯ denotes E2F; ➤ denotes Element I; ➤➤ denotes Element II; ⟼ denotes 24bp enhancer; ▨ denotes CAAT; ▯ denotes TATA; ⮡ denotes cap site; – – denotes hyphenated symmetry around cap site. **B.** The μ and δ (capped lines) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) are shown for the intact and *Bal* 31 deleted Ad5 and Ad40 E1A promoters.

(which mediated an approximated 3.1 fold decrease) and -171 to -42 (which mediated an approximated 12 fold decrease).

The Ad40 E1A promoter followed the same pattern of expression as the Ad5 E1A promoter, where deletion of sequences in the ITR resulted in an increase in basal transcription, followed by a decrease in basal transcription mediated by subsequent deletion of sequences to the TATA box (figure 4.1.6). Deletion of sequences from -416 to -349 (relative to the cap site) mediated an approximated 1.6 fold increase in basal transcription from the Ad40 E1A promoter, followed by an approximated 3.3 fold decrease in expression with the deletion of sequences from -349 to -268. The subsequent deletion of sequences from -268 to -140 resulted in basal transcription from the Ad40 E1A promoter close to background. The TATA box alone was unable to support basal transcription from the Ad40 E1A promoter.

Summary

Basal transcription from the intact Ad40 E1A promoter was decreased by a factor of 6 when compared to the Ad5 E1A promoter. Deletion of sequences from -498 to -428 in Ad5 and from -416 to -349 in Ad40 resulted in increased expression from both promoters. Subsequent deletion of sequences from -428 to -171 in Ad5 and from -349 to -140 in Ad40 resulted in levels of basal transcription from both promoters being reduced close to background, showing that sequences from -349 to -140 within the Ad40 E1A promoter are important for basal transcription. Finally, both the Ad5 and the Ad40 TATA boxes alone were unable to support basal transcription.

4.1.7 Construction of specific deletions within the Ad40 E1A promoter

Transient transfection analyses had revealed that a region between -349 to -140 (relative to the cap site) was important for basal transcription from the Ad40 E1A promoter. Comparison of the Ad40 E1A promoter with the transcription factor database, held at EMBL (Ghosh, 1990), revealed that this region contained a number of possible transcription factor binding sites (figure 4.1), which would impose a similar structure to the Ad5 E1A promoter. To map sequences within the region -349 to -140, a series of deletions were constructed which deleted transcription factor binding sites known to be important in basal transcription from the Ad5 E1A promoter (figure 4.1.7). The deletions were characterised in parallel with the intact and *Bal* 31 deletion mutants of the Ad40 E1A promoter, by transient transfection assays.

Deletions of the Ad40 E1A promoter were generated by restriction digest of 50μg pGL3-Ad40with *Hph* I, *Aci* I, *Bgl* I, *Bsm* FI or *Bsl* I, digests were then separated on an ethidium bromide stained agarose gel, and DNA fragments containing the specific deletions of the Ad40 E1A promoter identified. The excised bands containing the Ad40 E1A promoter deletions were cleaned using a Geneclean II kit (Bio101 Inc., La Jolla, CA), before being digested with *Hind* III, and cloned into the MCS of pGL3-basic via *Sma* I and *Hind* III restriction sites (figure 2.1.3). Plasmids containing specific deletions of the Ad40 E1A promoter were identified by restriction analysis of miniprep DNA with *Sac* I and *Hind* III, prior to large-scale preparation of pGL3-Ad40.328, pGL3-Ad40.235, pGL3-Ad40.209, pGL3-Ad40.166 for use in transient co-transfection assays.

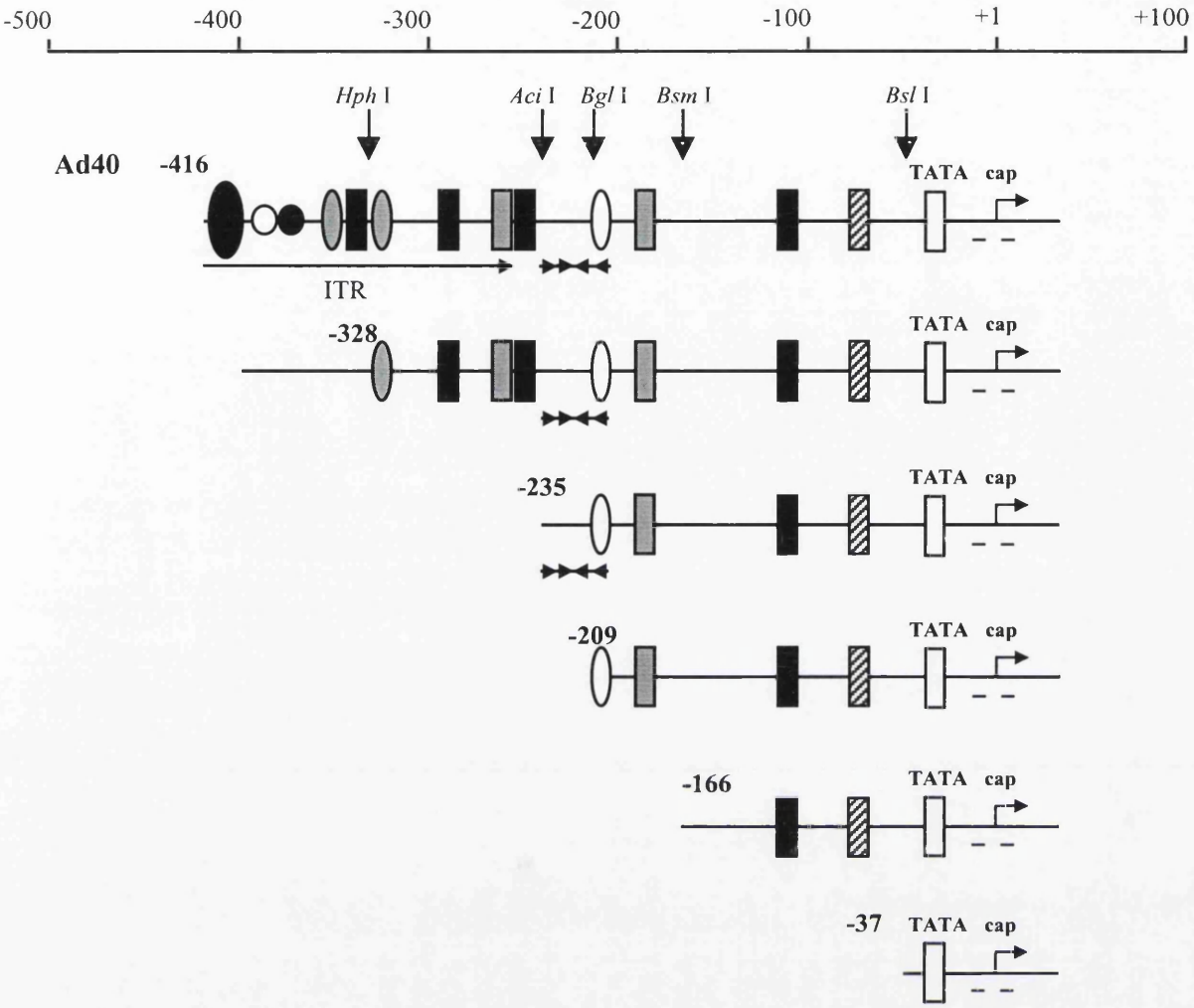


Figure 4.1.7 Specific deletion mutants of the Ad40 E1A promoter. Sequence data for the deleted Ad40 E1A promoter is available in appendix 1. Transcription factor binding sites are indicated by: ● denotes ORP-A; ○ denotes NF I; ● denotes NF III; ▯ denotes SP1; ■ denotes ATF; ▯ denotes EF-1A; ▯ denotes E2F; ▶◀ denotes Element II-like sequence; ▨ denotes CAAT; ▯ denotes TATA; ↱ denotes cap site; - - denotes hyphenated symmetry around cap site.

4.1.8 Analysis of basal transcription from the intact and deleted Ad40 E1A promoter

To map sequences between -349 to -140 (relative to the cap site) which were important for basal transcription from the Ad40 E1A promoter, WS HeLa cells were transiently co-transfected with a $CaPO_4$ precipitate containing either the intact Ad40 (pGL3-Ad40) E1A promoter construct, or the deleted Ad40 E1A promoter constructs (pGL3-Ad40.349, pGL3-Ad40.328, pGL3-Ad40.268, pGL3-Ad40.235, pGL3-Ad40.209, pGL3-Ad40.166, pGL3-Ad40.140 or pGL3-Ad40.37), in parallel with a positive (pGL3-control) and a negative (pGL3-basic) control. The experiment was set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were transiently transfected with independently derived $CaPO_4$ precipitates (section 3.4.3). Cells were harvested using the dual-luciferase reporter assay system (section 3.4.3.2), then 20µl aliquots of cell extract were assayed for firefly luciferase luminescence then *Renilla* luciferase luminescence (section 3.4.4.2). Sample firefly luciferase luminescence (FLL) and *Renilla* luciferase luminescence values were tabulated (see columns 2 and 4 in Table 4.1.8) then used to calculate the mean (µ) and standard deviation (δ) for each sample (see columns 7 and 8 in Table 4.1.8; see section 4.1.4).

Figure 4.1.8 A shows a diagrammatic representation of the intact and deleted Ad40 E1A promoter, whilst B shows the mean (µ) and standard deviation (δ) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) for the intact and deleted Ad40 E1A promoter. Expression from the intact Ad40 E1A promoter increased by a factor of 1.9 upon deletion of sequences from -416 to -349, then deletion of sequences from -349 to -328 mediated a further increase in expression by a factor of 1.3. Deletion of sequences from -328 to -268 mediated the largest decrease in expression, giving an approximated 5 fold decrease in expression from the Ad40 E1A promoter. Deletion of sequences from -268 to -235 resulted in an approximate 2 fold decrease in expression, to a level which remained unchanged upon the deletion of further sequences from -235 to -37.

Table 4.1.8 Transient transfection of the intact and deleted Ad40 E1A promoter

Sample	FLL[a] (TLU)	FLL - FLLAB[b] (TLU)	RLL[c] (TLU)	RLL - RLLAB[d] (TLU)	FLL/ RLL	μ[e]	δ[f]
Ad40 E1A promoter	87	62	91	66	0.94	1.2	0.2
Ad40 E1A promoter	147	122	114	89	1.4		
Ad40 E1A promoter	319	294	261	236	1.3		
Ad40 del.349	675	650	300	275	2.4	2.3	0.14
Ad40 del.349	778	752	336	311	2.4		
Ad40 del.349	946	921	466	441	2.1		
Ad40 del.328	1630	1605	581	556	2.9	3.0	0.49
Ad40 del.328	1002	977	294	269	3.6		
Ad40 del.328	658	633	287	262	2.4		
Ad40 del.268	168	143	284	259	0.6	0.6	0.06
Ad40 del.268	96	71	171	146	0.5		
Ad40 del.268	133	108	223	198	0.6		
Ad40 del.235	51	26	183	158	0.16	0.13	0.02
Ad40 del.235	43	18	181	156	0.12		
Ad40 del.235	46	21	203	178	0.12		
Ad40 del.209	34	9	175	150	0.06	0.08	0.02
Ad40 del.209	38	13	151	126	0.10		
Ad40 del.209	42	17	232	207	0.08		
Ad40 del.166	40	15	429	202	0.04	0.10	0.05
Ad40 del.166	56	31	268	243	0.13		
Ad40 del.166	99	74	564	539	0.14		
Ad40 del.140	44	19	180	155	0.12	0.10	0.02
Ad40 del.140	36	11	169	144	0.08		
Ad40 del.140	42	17	178	153	0.11		
Ad40 del.37	39	14	233	208	0.07	0.08	0.01
Ad40 del.37	40	15	221	196	0.08		
Ad40 del.37	41	16	201	176	0.09		
Positive control	1733	1708	141	116	14	14	0.6
Positive control	547	522	63	38	13		
Positive control	893	868	85	60	14		
Negative control	30	5	220	195	0.03	0.04	0.01
Negative control	28	3	109	184	0.04		
Negative control	31	6	154	129	0.05		

[a] FLL denotes firefly luciferase luminescence

[b] FLLAB denotes firefly luciferase luminescence assay background

[c] RLL denotes *Renilla* luciferase luminescence

[d] RLLAB denotes *Renilla* luciferase luminescence assay background

[e] μ denotes mean

[f] δ denotes standard deviation

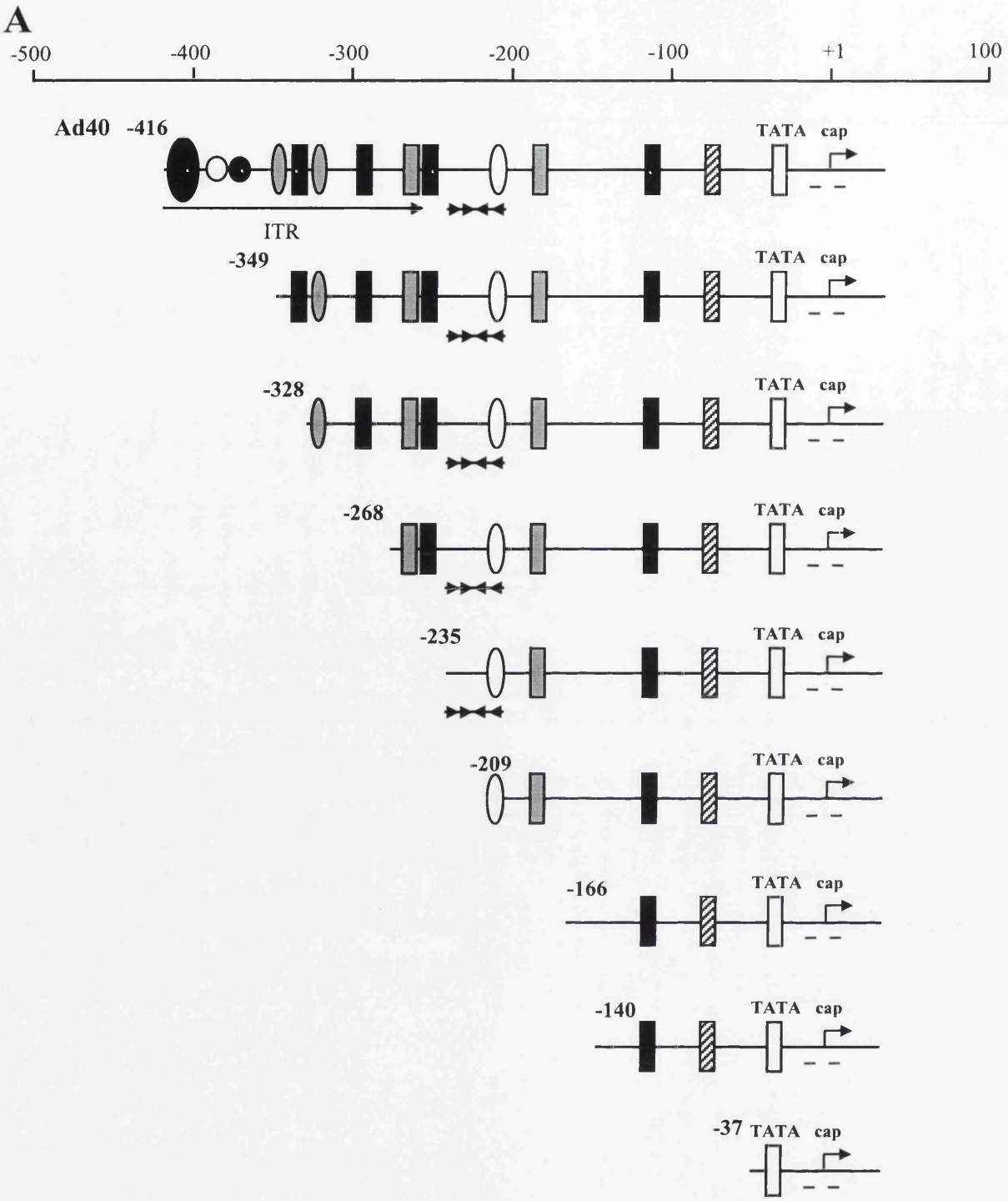
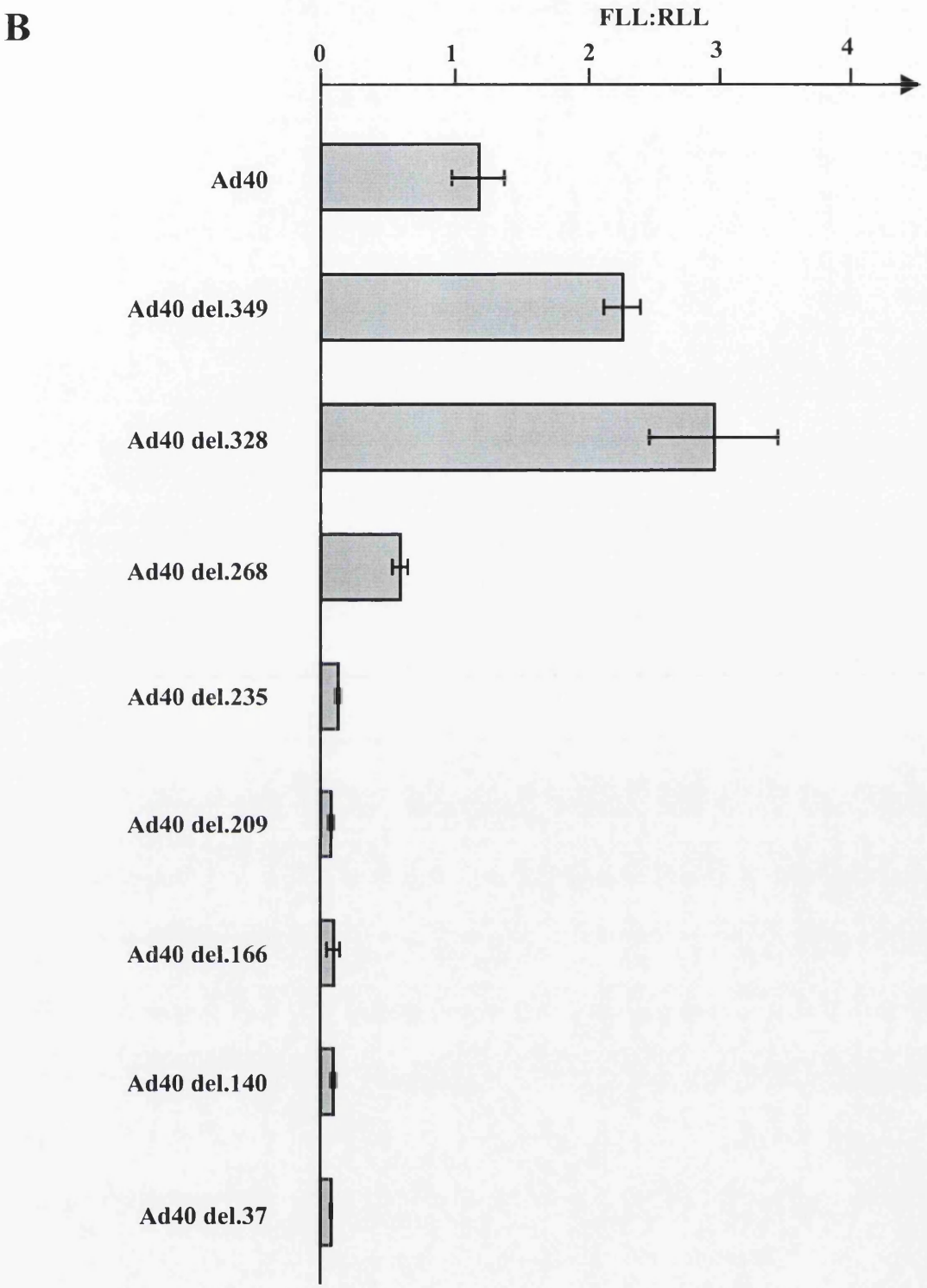


Figure 4.1.8 Analysis of the intact and deleted Ad40 E1A promoter. The experiment was set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were transfected with independently derived calcium phosphate precipitates containing 10μg experimental DNA plus 200ng pRL-SV40, in parallel with a positive and negative control. The positive control contained the SV40 promoter, whilst the negative control was promoterless. The cells were harvested by using the dual-luciferase reporter assay system, then 20μl aliquots were assayed for firefly luciferase luminescence (TLU) then *Renilla* luciferase luminescence (TLU). **A.** The intact and *Bal* 31 deleted Ad5 E1A and Ad40



E1A promoters. Transcription factor binding sites are indicated by: ● denotes ORP-A; ○ denotes NF I; ● denotes NF III; ▯ denotes SP1; ■ denotes ATF; ▯ denotes EF-1A; ▯ denotes E2F; ▨ denotes CAAT; ⟼ denotes 24bp enhancer; ⋈ denotes an Element II-like sequence; ☐ denotes TATA; ⌐ denotes cap site; - - denotes hyphenated symmetry around cap site. **B.** The μ and δ (capped lines) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence are shown for the intact and *Bal* 31 deleted Ad5 and Ad40 E1A promoters.

Summary

Basal transcription from the intact Ad40 E1A promoter was concordant with observations made in section 4.1.4 and 4.1.6. Deletion of sequences from -416 to -328 in the Ad40 E1A promoter mediated an overall increase in the expression by a factor of 2.5. Deletion of sequences from -328 to -268 and from -268 to -235 resulted in a promoter construct, which decreased expression of the firefly luciferase gene by a factor of 23, resulting in a promoter unable to support basal transcription. We can therefore conclude that sequences important for basal transcription from the Ad40 E1A promoter lie between -328 to -235.

4.2 Preliminary characterisation of *trans*-activation by the Ad40 E1A 249R protein.

Introduction

The Ad5 289R and 243R E1A proteins are expressed from the E1A gene at early times after infection (Perricaudet *et al.*, 1979; van Ormondt *et al.*, 1980). In the absence of the E1A proteins, transcription from the E1A promoter is reduced approximately five fold suggesting a role for the E1A proteins in their own regulation (Montell *et al.*, 1984a; Osborne *et al.*, 1984). Indeed, E1A has been shown to be capable of its own regulation at the level of transcription (Hearing and Shenk, 1985; Tibbetts *et al.*, 1986). The E1A proteins, in particular the 289R protein, are able to *trans*-activate the E1A promoter (Osborne *et al.*, 1984; Hearing and Shenk, 1985), and both the E1A proteins have also been associated with negative regulation of their own promoter (Tibbetts *et al.*, 1986; Cogan *et al.*, 1992).

Previous studies of the adenovirus type 40 E1A protein products revealed that *trans*-activation of the Ad2 E4 promoter by the Ad40 E1A proteins was significantly reduced when compared with *trans*-activation by the Ad5 E1A proteins (van Loon *et al.*, 1987b). The *trans*-activation of the Ad5 E2A and E3 promoters by the Ad40 E1A proteins were also significantly reduced when compared with *trans*-activation by the Ad5 and the Ad12 E1A proteins (Ishino *et al.*, 1988). Taken together these results suggest that the Ad40 E1A proteins may well be implicated in the aberrant expression of E1A, and therefore the growth restriction of Ad40. To investigate the involvement of the Ad40 E1A proteins in the restricted growth of Ad40, *trans*-activation of the Ad40 E1A promoter by the Ad40 E1A proteins was compared with *trans*-activation of the Ad5 E1A promoter by the Ad5 E1A proteins. The sequences important in *trans*-activation of the Ad40 E1A promoter were then mapped by transient transfection analysis.

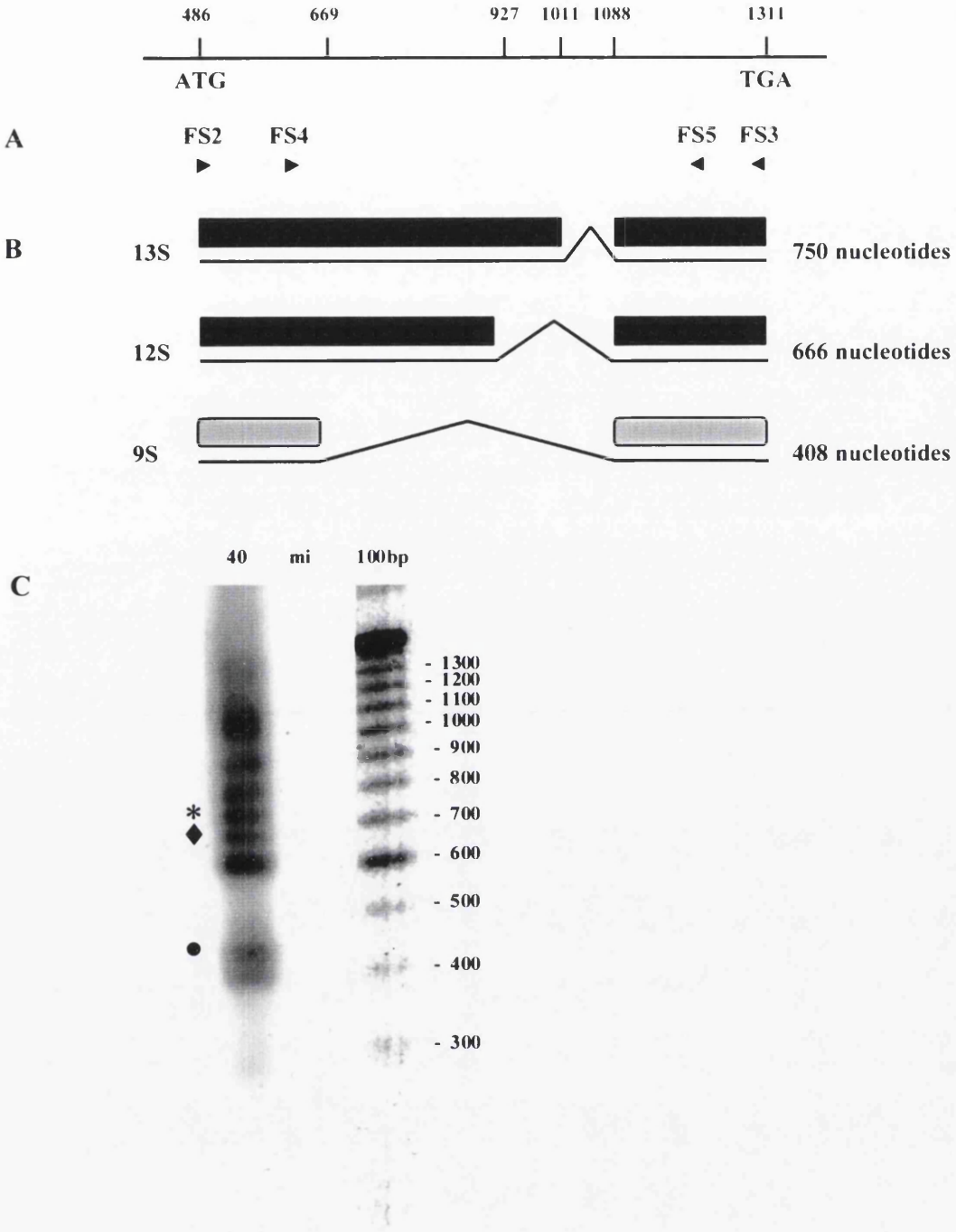
4.2.1 Construction of a CMV expression plasmid containing the Ad40 E1A 13S equivalent cDNA

To investigate the involvement of the Ad40 E1A proteins in the aberrant expression of the Ad40 E1A region, the Ad40 E1A equivalent 13S and 12S protein coding sequences were cloned into a CMV expression plasmid. The CMV expression plasmid used was pCMV19K (White and Cipriani, 1990), which had previously been modified to construct plasmids which expressed the Ad5 289R and 243R E1A proteins (Morris and Mathews, 1991). Expression plasmids containing either the Ad5 or the Ad40 E1A protein coding sequences were co-transfected with their homologous promoters to compare *trans*-activation.

A library of Ad40 E1A protein coding sequences was generated to retrieve the Ad40 E1A equivalent 13S and 12S protein coding sequences. The library was generated by reverse transcription-polymerase chain reaction (RT-PCR) of cytoplasmic RNA harvested 42h p.i. from 293 cells infected with 5ffu/cell Ad40 and blocked with 20μg/ml araC 12 hourly (section 3.2.1, 3.2.2 and 3.5.3). 100pmol of FS3 was used to prime the RT reaction, whilst 1μM FS2 was used to prime the PCR reaction using *Taq* DNA polymerase (Table 2.4; figure 4.2.1.1). The primers used in the RT-PCR (FS2 and FS3) were designed to generate protein coding sequences (from the E1A start codon at nucleotide 486, to the E1A stop codon at nucleotide 1311) of the Ad40 E1A region, allowing efficient expression of the Ad40 E1A protein coding sequences (in place of 19K in pCMV19K) by the CMV promoter, which would be processed under the control of the SV40 poly A signal. Figure 4.2.1.1 A illustrates the position of the primers FS2 and FS3 in relation to the Ad40 E1A sequence. FS2 and FS3 were also designed to contain *Eco* RI and *Hind* III restriction sites respectively, facilitating directional cloning of the Ad40 E1A protein coding sequences into the CMV expression plasmid.

Figure 4.2.1.1 B illustrates the open reading frames contained within the Ad40 E1A equivalent 13S, 12S and probable 9S mRNAs. The predicted size of the Ad40 E1A equivalent 13S, 12S and 9S protein coding sequences, which would be generated by RT-PCR are indicated to the right of the Ad40 E1A equivalent 13S, 12S and 9S mRNAs. Figure 4.2.1.1 C shows the Southern blot analysis of RT-PCR products

Figure 4.2.1.1 Construction of expression plasmids containing the Ad40 E1A protein coding sequences. Two 60mm plates of 293 cells were infected with 5ffu/cell Ad40 or mock infected with (tris saline) then blocked with 20μg/μl ara C 12-hourly. Cytoplasmic RNA from Ad40 infected and mock infected cells was reverse transcribed with 100pmol FS3, before being used to set up PCR reactions with 1μM FS2 and *Taq* DNA polymerase. A tenth of the RT-PCR reactions were then analysed by southern blot using pNM80 as a probe (section 2.1). The cDNAs generated were cloned into pCRII (section 2.1) and characterised by dideoxy-sequencing with FS2 and FS3, then FS4 and FS5. **A.** Denotes position of primers used in RT-PCR of the Ad40 E1A protein coding sequences (FS2 and FS3), and in sequencing (FS4 and FS5) of cDNAs generated. **B.** Denotes the open reading frames of the Ad40 E1A equivalent 13S, 12S and 9S mRNAs, marked with the predicted sizes of cDNAs which should be generated during RT-PCR (right-hand side). Solid lines denote RNA with introns indicated by carets; filled rectangles are open reading frames; rectangles with rounded corners are proteins, which have not yet been confirmed experimentally. **C.** Southern blot analysis of the Ad40 E1A cDNAs generated by RT-PCR. 40 denotes Ad40 E1A specific cDNAs; mi denotes RT-PCR of mock infected cytoplasmic RNA; 100bp denotes 100bp DNA ladder (Gibco BRL Life Technologies); * denotes predicted Ad40 E1A equivalent 13S cDNA; ♦ denotes predicted Ad40 E1A equivalent 12S cDNA product; • denotes predicted Ad40 E1A equivalent 9S cDNA product.

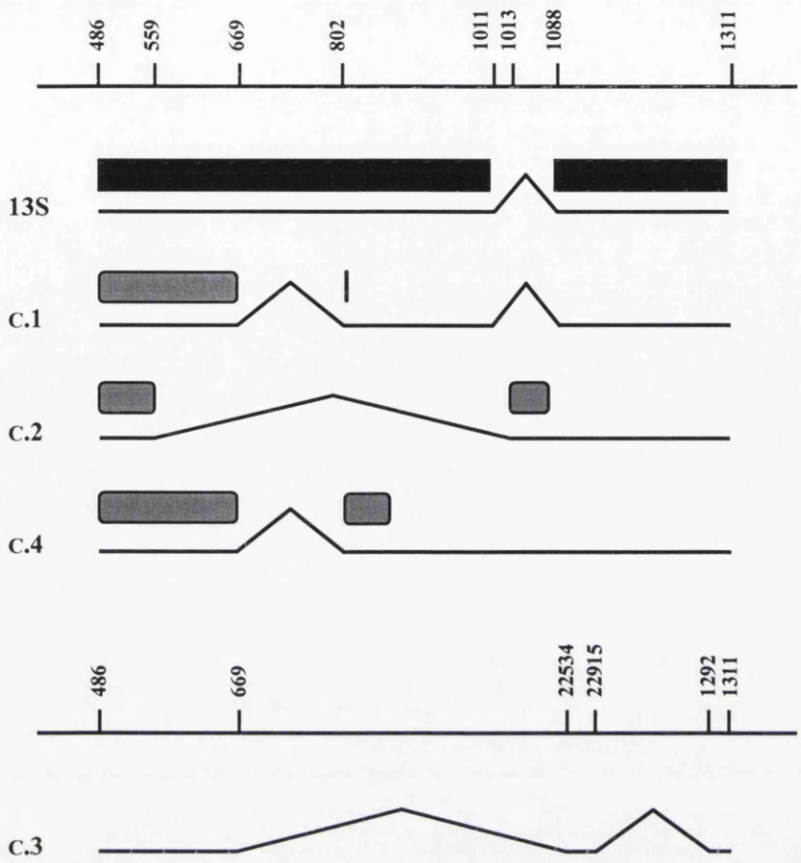

486
669
927
1011
1088
1311
ATG
TGA
A
FS2
FS4
FS5
FS3
B
13S
750 nucleotides
12S
666 nucleotides
9S
408 nucleotides
C
40
mi
100bp
- 1300
- 1200
- 1100
- 1000
- 900
- 800
- 700
- 600
- 500
- 400
- 300

generated from Ad40 infected and mock infected 293 cells (see lanes marked 40, for Ad40 infected 293 cells; and m.i., for mock infected 293 cells). The RT-PCR products from Ad40 infected 293 cells (lane marked 40 in figure 4.2.1.1 C) contained eight distinct cDNA species which were Ad40 E1A specific. Comparison of the eight cDNA species (in lane marked 40 in figure 4.2.1.1 C) with the predicted sizes of the Ad40 E1A 13S, 12S and 9S protein coding sequences (see figure 4.2.1.1 B), revealed three cDNAs which corresponded to the predicted size of the 13S (indicated by * in figure 4.2.1.1 C), 12S (indicated by ♦ in figure 4.2.1.1 C) and 9S (indicated by • in figure 4.2.1.1 C) cDNAs.

The RT-PCR products were then cloned into pCRII (figure 2.1.7) to maximise ligation efficiency, as only small quantities of products were available. The pCRII cloning vector is able to maximise ligation efficiency by utilising the A' overhang generated by *Taq* DNA polymerase during the PCR reaction. Plasmids containing the Ad40 E1A cDNAs were identified by restriction analysis of miniprep DNA with *Hind* III and *Eco* RI. Clones containing Ad40 E1A cDNAs were sequenced using primers FS2 and FS3 to confirm the absence of mutations which may have arisen during the PCR, and also to characterise the cDNAs produced *in vivo*, in particular the position of introns, and the splice donor and splice acceptor sites providing a map of E1A mRNAs. Preliminary sequencing data was used to design a second set of primers, FS4 (nucleotides 610-627) and FS5 (1201-1184) (Table 2. 4; indicated in figure 4.2.1.1 A), which were used to complete the characterisation of the cloned cDNAs. The primer FS4 was also used to screen clones for the Ad40 E1A 12S splice junction.

Figure 4.2.1.2 A illustrates the Ad40 E1A mRNAs which were isolated and characterised, whilst figure 4.2.1.2 B denotes the size of the cloned cDNA and the splice donor and splice acceptor sites. The splice donor and acceptor sites were defined on the basis of a matrix mean model which was derived from all known mammalian splice site signals held at GenBank up to that time (Stephens and Schneider, 1992). The Ad40 13S cDNA is denoted by a black line, with the intron indicated as a caret spanning nucleotides 1011 to 1088 (see figure 4.2.1.2 A). The cloned product size was as the predicted 749bp (see column 2 in figure 4.2.1.2 B), which contained a consensus splice donor site (TACA/gtaa, known henceforth as the 13S splice donor) and a consensus

A



B

Clone	Cloned product size (bp)	Splice donor site	Splice acceptor site
13S	749	**TACA/gtaa**	**ttag/GTCC**
c.1	617	**GATG/gtat** **TACA/gtaa**	**ctag/CGAT** **ttag/GTCC**
c.2	372	**ATGT/gttt**	cagt/AAGT
c.3	585	**GATG/gtat** AACC/cgcg	**ctag/GCGA** ttaa/AGCG
c.4	693	**GATG/gtat**	**ctag/CGAT**

Figure 4.2.1.2 Analysis of the Ad40 E1A specific cDNAs. **A.** Solid lines denote RNA with introns indicated by carets; filled rectangles are open reading frames; rectangles with rounded corners are proteins, which have not been confirmed experimentally. **B.** Table of sequences at the slice donor and splice acceptor site. Bold type denotes consensus splice donor or splice acceptor sites; normal type denotes non-consensus splice donor or splice acceptor sites.

splice acceptor site (ttag/GTCC, known henceforth as the 13S splice acceptor site) (see columns 3 and 4 in figure 4.2.1.2 B). The Ad40 cDNA clone 1 was 617bp in length, containing two introns from 669 to 802 nucleotides and from 1011 to 1088 nucleotides (figure 4.2.1.2 A). The consensus splice donor sequence at nucleotide 669 (GATG/gtat; known henceforth as the 9S splice donor site) (see column 3 in figure 4.2.1.2 B) splices to a consensus sequence (ctag/CGAT) at nucleotide 802. Clone 1 then utilises the consensus 13S splice donor and acceptor sites at nucleotides 1011 and 1088 respectively (see columns 3 and 4 figure 4.2.1.2 B). The Ad40 cDNA clone 2 was 372bp in length, and contained one intron spanning nucleotides 559 to 1013 (figure 4.2.1.2 A). The concensus splice donor sequence at nucleotide 559 (ATGT/gttt) (see columns 3 and 4 figure 4.2.1.2 B) splices to a non-consensus splice acceptor at position 1013 (cagt/AAGT) (see columns 3 and 4 figure 4.2.1.2 B). The Ad40 cDNA clone 3 was 585bp in length, containing two introns, which spanned from nucleotides 669 to 22, 534 and 22, 915 to 1292 (figure 4.2.1.2 A). This is remarkable as clone 3 utilises the predicted 9S splice donor at position 669, splicing to a consensus splice acceptor (ctag/GCGA) at position 22, 534 in the L4 100kDa exon (see columns 3 and 4 figure 4.2.1.2 B). The second splice donor site is a non-consensus sequence (AACC/cgcg) located at 22, 915 which splices to a non-consensus splice acceptor site (ttaa/AGCG) within the E1A region at position 1292 (see columns 3 and 4 figure 4.2.1.2 B). Finally, the Ad40 cDNA clone 4 was 693bp, containing one intron spanning nucleotides 669 to 802 (figure 4.2.1.2 A). Clone 4 utilises the predicted 9S splice donor at position 669, splicing to a consensus splice acceptor at position 802 (ctag/CGAT) (see columns 3 and 4 figure 4.2.1.2 B).

The Ad40 E1A 13S cDNA which was characterised by dideoxy-sequencing was then substituted for the E1B 19kDa protein coding sequences in the expression vector pCMV19K (White and Cipriani, 1990). The Ad40 E1A 13S cDNA was generated by restriction digest of pCRII-Ad4013S with *Eco* RI and *Hind* III, the cDNA fragment was then ligated into pCMV19K via *Eco* RI and *Hind* III restriction sites which substituted the E1B 19kDa protein coding sequences. Candidate clones were then identified by restriction digest of miniprep DNA with *Eco* RI and *Hind* III, prior to large-scale preparation of pCMVAd4013S and pCMV13S (which contains the Ad5 13S mRNA

under the control of the CMV promoter; Morris and Mathews, 1991) for use in transient co-transfection assays (section 4.2.3).

Summary

RT-PCR of Ad40 infected cells generated a library of Ad40 E1A specific cDNAs, which contained eight distinct cDNA species ranging from approximately 400bp to 1100bp in length. Dideoxy-sequencing of a number of cloned cDNAs identified the Ad40 E1A equivalent 13S cDNA, however the Ad40 E1A equivalent 12S and 9S cDNAs were not characterised. Several novel cDNA clones were identified by dideoxy-sequencing, three (c.1, c.3 and c.4) of which utilise the 9S splice donor at nucleotide 669 to produce three distinct mRNAs. Clones 1 and 4 also use the same non-consensus splice acceptor at nucleotide 802 producing two distinct mRNAs. The most remarkable cDNA isolated is the cDNA clone 3 which splices to the L4 100kDa exon, then splices a second time into sequences containing the E1A region.

4.2.2 Qualitative analysis of the Ad40 E1A mRNAs in Ad40 infected cells.

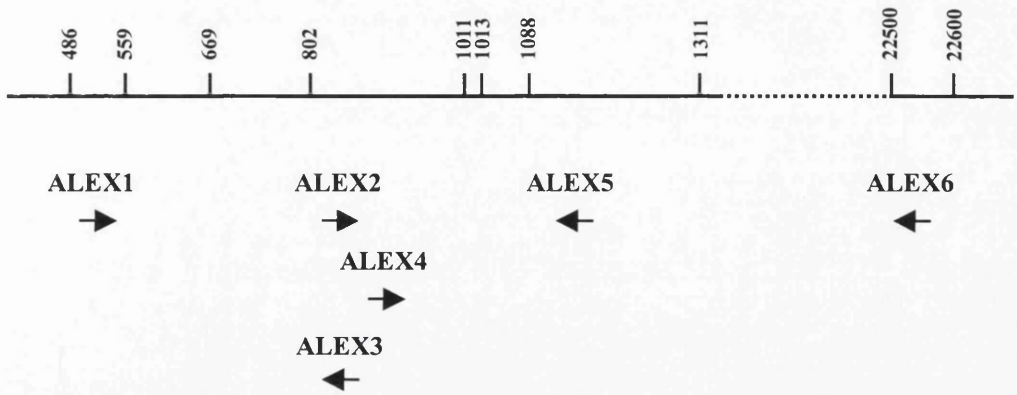
In section 4.2.1 the Ad40 E1A equivalent 13S cDNA and four novel Ad40 E1A cDNAs had been generated by RT-PCR (figure 4.2.1.1), then characterised by dideoxy-sequencing (figure 4.2.2.2). To establish the status of the Ad40 E1A equivalent 13S mRNA and the four novel Ad40 E1A mRNAs in a productive Ad40 infection, primers were designed to span the splice junction/s contained within the aforementioned mRNAs, these would generate specific sized cDNA products in RT-PCR. The Ad40 E1A equivalent 12S mRNA was not detected in section 4.2.1 therefore, to establish whether the Ad40 12S mRNA was indeed present in a productive Ad40 infection, primers were designed to span the 12S splice junction.

The primers which would be used in the RT-PCR of Ad40 infected cells, known as ALEX1-6, were designed to generate specific cDNA products which spanned the splice junction or junctions of the Ad40 E1A equivalent 13S mRNA, the four novel Ad40 E1A mRNAs and the Ad40 E1A equivalent 12S mRNA (figure 4.2.2). Figure 4.2.2 A and table 2.4, illustrates the position and direction of the primers ALEX 1-6 in relation to the Ad40 E1A sequence. Figure 4.2.2 B indicates the primer pairs used in RT-PCR of Ad40 infected cells (see column 2 in figure 4.2.2 B), to detect the specific splice junction/s contained within the cloned Ad40 E1A cDNAs and the Ad40 E1A equivalent 12S mRNA. Also indicated are the predicted sizes of cDNA and DNA products, which would be generated by RT-PCR or PCR of a plasmid containing the Ad40 E1A sequences respectively (see columns 3 and 4 in figure 4.2.2 B).

Specific cDNA fragments were generated for the Ad40 E1A 13S equivalent mRNA, the Ad40 E1A equivalent 12S mRNA and the Ad40 E1A mRNA clones one to four by RT-PCR of cytoplasmic RNA harvested 42h p.i. from 293 cells infected with 5ffu/cell Ad40 and blocked with araC (section 3.2.1, 3.2.2 and 3.5.3). Combinations of primers used in each RT-PCR reaction are outlined in figure 4.2.2 B, 100pmol of primer was used within the RT reaction (primer for RT is denoted in bold type in column 2 of figure 4.2.2 B), whilst 1μM primer was used in PCR with *Taq* DNA polymerase (primer for PCR is denoted in normal type in column 2 of figure 4.2.2 B). A tenth of the RT-PCR

Figure 4.2.2 Qualitative analysis of Ad40 E1A mRNAs in Ad40 infected cells. Two 60mm plates of 293 cells were infected with 5 ffu/cell Ad40 or mock infected with tris saline, then blocked with ara C 12 hourly. Cytoplasmic RNA was harvested 42h p.i. to set up RT-PCR reactions with 100*p*mol of the RT primer and 1μM of the PCR primer, in parallel with a positive PCR control (pNM80, section 2.1) and a negative PCR control. A tenth of the products along with a 100bp DNA ladder (Gibco BRL Life Technologies) and a 123bp DNA ladder (Gibco BRL Life Technologies) were visualised on an ethidium bromide stained gel. **A.** Position of primers used for RT-PCR. Arrow denotes direction of primer. **B.** Table of predicted DNA and cDNA sizes with different combinations of primers spanning the intron sequences of each clone. Normal type denotes primer used in RT reaction; bold type denotes primer used in PCR. **C.** Agarose gel analysis of RT-PCR products from Ad40 infected and mock infected cells. 13S denotes Ad40 13S E1A equivalent cDNA fragment; 12S denotes Ad40 E1A equivalent 12S cDNA fragment; c.1 denotes Ad40 E1A clone 1 cDNA fragment; c.2 denotes Ad40 E1A clone 2 cDNA fragment; c.3 denotes Ad40 E1A clone 3 cDNA fragment; c.4 denotes Ad40 E1A clone 4 cDNA fragment; 40 denotes Ad40 infected cells; mi denotes mock infected cells; +ve denotes positive PCR control; -ve denotes negative PCR control.

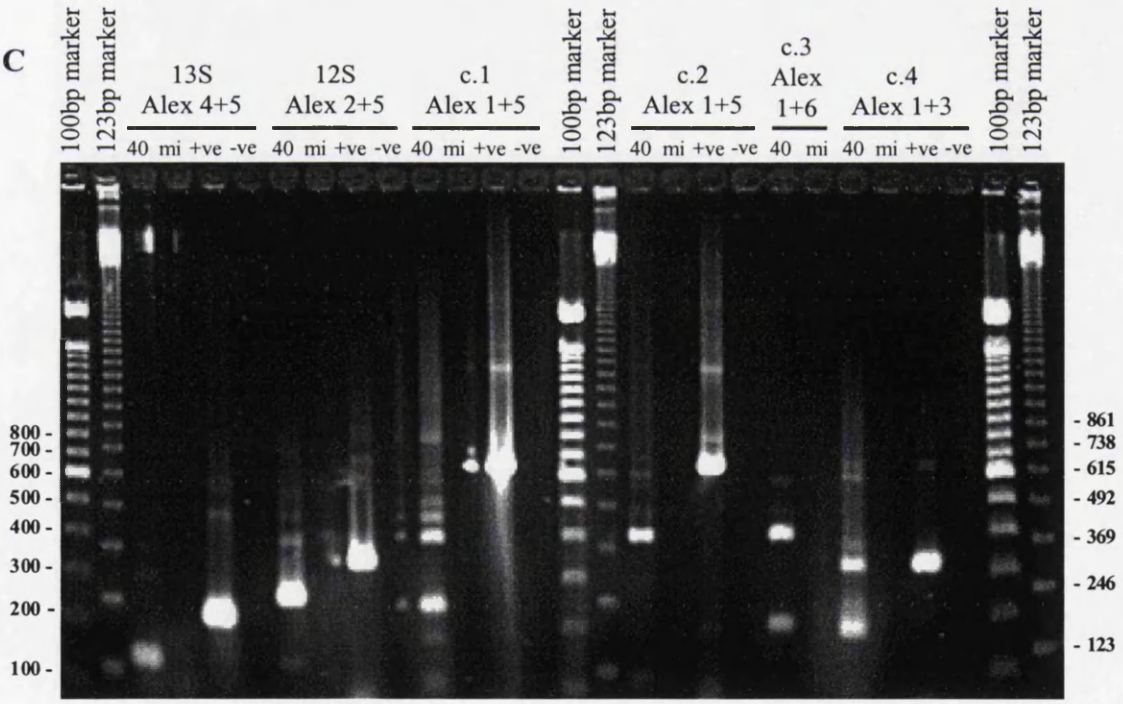
A



B

Clone	Primers	Predicted DNA size (bp)	Predicted cDNA size (bp)
13S	**ALEX4**, ALEX5	222	146
12S	**ALEX2**, ALEX5	342	182
c.1	**ALEX1**, ALEX5	639	431
c.2	**ALEX1**, ALEX5	639	186
c.3	**ALEX1**, ALEX6	-	196
c.4	**ALEX1**, ALEX3	323	189

C



products were then visualised on an ethidium bromide stained agarose gel (see figure 4.2.2 C).

Figure 4.2.2 C shows the RT-PCR products generated from Ad40 infected (denoted 40) and mock infected 293 cells (denoted mi), in parallel with a positive (denoted by +ve) and negative (denoted –ve) PCR control. The primers used in each RT-PCR or PCR are indicated above the lane markings. Figure 4.2.2 B indicates the size of the specific cDNA or DNA fragments generated for Ad40 E1A equivalent 13S and 12S mRNAs, clone one, clone two, clone three and clone four. Figure 4.2.2 C indicates that a product of approximately 140bp was produced for primers, which spanned the Ad40 E1A equivalent 13S cDNA, suggesting that the 13S cDNA is present early in Ad40 infection. However, a product of approximately 250bp in size, was produced when primers, which span the Ad40 E1A equivalent 12S splice junction were used, suggesting that the Ad40 E1A 12S mRNA is either a minor species of Ad40 E1A mRNA at early times in infection or is absent from Ad40 infected 293 cells. The cDNA clones one and four were predicted to produce cDNA products of 431bp and 189bp respectively, indeed the RT-PCR products contain bands which approximate the predicted sizes, suggesting that clones one and four are present in a productive Ad40 infection. A number of alternatively sized RT-PCR products are observed in tracks containing clone one and four, which would suggest that a number of Ad40 E1A mRNAs are produced which splice between 512 to 1150 nucleotides and 512 to 832 nucleotides respectively. The cDNA clones two and three are predicted to produce cDNA fragments of 186bp and 196bp respectively however, neither product was observed. This suggests that the Ad40 E1A cDNA clones two and three are either minor species of Ad40 E1A mRNA at early time in infection, or they may be absent from Ad40 infections in 293 cells, or the cDNA clones may have been unusual by-products of the RT-PCR.

The Ad40 E1A 12S equivalent cDNA fragment, of predicted size 182bp, was not generated by RT-PCR of Ad40 infected 293 cells. This could possibly have resulted from RNA degradation therefore, the PCR reaction was repeated using the RT reaction which generated the Ad40 E1A equivalent 13S cDNA fragment and 1μM ALEX2, in parallel with a positive and a negative PCR control. A tenth of the PCR products were then visualised on an ethidium bromide stained agarose gel. The Ad40 E1A equivalent

12S cDNA fragment of predicted size was not observed, which suggested that the previous observations made were not subject to experimental error, but were in fact real.

Summary

Primers designed to produce specific cDNA fragments spanning the Ad40 E1A equivalent 13S and 12S splice junctions, and spanning the Ad40 E1A cDNA clones one to four, were used in RT-PCR of Ad40 infected 293 cells. The Ad40 E1A 13S mRNA and the Ad40 E1A clones one and four were present at early times in Ad40 infected cells. Clones one and four are of particular interest as they produce a number of uniquely sized cDNA fragments by RT-PCR. The Ad40 E1A 12S mRNA and the Ad40 E1A clones two and three were not observed at early times in Ad40 infected cells.

4.2.3 Analysis of *trans*-activation by the Ad5 E1A 289R protein and the Ad40 E1A 249R protein upon their respective promoters

Previous studies of the adenovirus type 40 E1A protein products, had revealed that the *trans*-activation of the Ad2 E4 promoter, the Ad5 E2A promoter and the Ad5 E3 promoter by the Ad40 E1A proteins was significantly lower when compared with *trans*-activation by the Ad5 E1A proteins (van Loon *et al.*, 1987b; Ishino *et al.*, 1988). These results suggested that the Ad40 E1A proteins may well be implicated in the aberrant expression of E1A, and therefore the growth restriction of Ad40. To investigate the involvement of the Ad40 E1A proteins in their own aberrant expression, the Ad40 E1A equivalent 13S protein coding sequences were cloned into a CMV expression plasmid (section 4.2.1). The Ad40 E1A equivalent 12S protein coding sequences were not detected in Ad40 infected 293 cells (section 4.2.1; 4.2.2). To compare *trans*-activation by the Ad5 E1A 289R protein on the Ad5 E1A promoter with *trans*-activation by the Ad40 E1A equivalent 289R protein (known henceforth as the Ad40 E1A 249R protein) on the Ad40 E1A promoter, cells were co-transfected with the appropriate plasmids.

Before transient transfection of the aforementioned plasmids could take place, the conditions for transient transfection were optimised for the CMV expression vectors, pCMV13S and pCMVAd4013S. These co-transfections also established whether or not the CMV expression plasmid encoding the Ad40 E1A 249R protein produced functional protein. Conditions for $CaPO_4$-mediated transient transfection in WS HeLa cells were optimised, with respect to the total amount of DNA transfected (optimum = 10μg), the incubation period of the $CaPO_4$ precipitate with the cells (optimum = 6h) and whether a glycerol shock was required to enhance transfection (optimum = not required) in section 4.1.2. The ratio of plasmid DNA to carrier DNA (10μg:0μg) had also been optimised for WS HeLa cells in section 4.1.4. The ratio of the experimental reporter plasmid to expression plasmid (10μg pGL3-Ad5 or pGL3-Ad40 to 0.5μg, 1μg, 2μg or 5μg pCMVAd4013S) was optimised in WS HeLa cells. Each experiment was set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were transiently transfected with independently derived $CaPO_4$ precipitates (section 3.4.3).

Cells were harvested using the dual luciferase reporter assay system (section 3.4.3.2), then 20µl aliquots of cell extract were assayed for firefly luciferase luminscence and *Renilla* luciferase luminescence (section 3.4.4.2). Sample firefly luciferase luminescence (FLL) and *Renilla* luciferase luminescence values were tabulated (see columns 2 and 4 in Table 4.2.3.1) then used to calculate the mean (µ) and standard deviation (δ) for each sample (see columns 7 and 8 in Table 4.2.3.1; see section 4.1.4).

Figure 4.2.3.1 A and B show the mean (µ) and standard deviation (δ) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) for the Ad5 E1A promoter *trans*-activated by increasing amounts of the Ad5 E1A 289R protein, and the Ad40 E1A promoter *trans*-activated by increasing amounts of the Ad40 E1A 249R protein. Expression from the Ad5 E1A promoter increased steadily with the addition of 0.5µg, 1µg, 2µg and 5µg expression plasmid pCMV13S, which contains the Ad5 E1A 13S cDNA. Expression from the Ad40 E1A promoter also increased steadily with the addition of 0.5µg, 1µg, 2µg and 5µg expression plasmid pCMVAd4013S, which contains the Ad40 E1A 13S cDNA. The dose dependent effect of both the Ad5 E1A 289R protein and the Ad40 E1A 249R protein demonstrated that the corresponding expression plasmids pCMV13S and pCMVAd4013S express functional protein. The optimum ratio of experimental reporter plasmid to expression plasmid was 10µg pGL3-Ad5/pGL3-Ad40 to 5µg pCMV13S/pCMVAd4013S in WS HeLa cells. The two to one ratio of experimental reporter plasmid to expression plasmid was used in all subsequent co-transfections.

To establish whether *trans*-activation by the Ad5 E1A 289R protein (pCMV13S) and the Ad40 E1A 249R protein (pCMVAd4013S) were comparable, expression vectors containing the aforementioned protein coding sequences were transiently co-transfected with the Ad5 E1A promoter (pGL3-Ad5) or the Ad40 E1A promoter (pGL3-Ad40), in parallel with a positive (pGL3-control) and a negative (pGL3-basic) control. Each experiment was set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were transiently transfected with independently derived $CaPO_4$ precipitates (section 3.4.3). Cells were harvested using the dual-luciferase reporter assay system (section 3.4.3.2), then 20µl aliquots of cell extract were assayed for firefly luciferase luminescence and *Renilla* luciferase luminescence (section 3.4.4.2). Sample

Table 4.2.3.1 Transient co-transfection with increasing amounts of the Ad5 E1A 289R or the Ad40 E1A 249R protein with their corresponding promoters.

Sample	FLL[a] (TLU)	FLL-FLLAB[b] (TLU)	RLL[c] (TLU)	RLL-RLLAB[d] (TLU)	FLL/ RLL	μ^e	δ^f
Ad5 E1A promoter	2128	2103	923	898	2.3	2.5	0.36
Ad5 E1A promoter	2999	2974	1024	999	3.0		
Ad5 E1A promoter	1875	1850	875	850	2.2		
Ad5 E1A + 0.5µg 13S	5885	5860	359	334	18	21	2.1
Ad5 E1A + 0.5µg 13S	5626	5601	298	273	21		
Ad5 E1A + 0.5µg 13S	38431	38406	1672	1647	23		
Ad5 E1A + 1µg 13S	21268	21243	1057	1032	21	25	3.9
Ad5 E1A + 1µg 13S	22709	22684	790	765	30		
Ad5 E1A + 1µg 13S	6141	6116	295	270	23		
Ad5 E1A + 2µg 13S	13270	13245	422	397	33	32	2.6
Ad5 E1A + 2µg 13S	7383	7358	239	214	34		
Ad5 E1A + 2µg 13S	13393	13368	503	478	28		
Ad5 E1A + 5µg 13S	1656	1631	57	32	51	43	6.1
Ad5 E1A + 5µg 13S	3267	3242	115	90	36		
Ad5 E1A + 5µg 13S	5057	5032	142	117	43		
Ad40 E1A promoter	273	248	1067	1042	0.24	0.24	0.02
Ad40 E1A promoter	252	227	870	845	0.27		
Ad40 E1A promoter	498	473	2291	2266	0.21		
Ad40 E1A + 0.5µg 13S	381	356	882	857	0.42	0.47	0.04
Ad40 E1A + 0.5µg 13S	445	420	827	802	0.52		
Ad40 E1A + 0.5µg 13S	300	275	593	568	0.48		
Ad40 E1A + 1µg 13S	330	305	657	632	0.48	0.48	0.06
Ad40 E1A + 1µg 13S	141	116	306	281	0.41		
Ad40 E1A + 1µg 13S	321	296	550	525	0.56		
Ad40 E1A + 2µg 13S	535	510	956	931	0.55	0.56	0.04
Ad40 E1A + 2µg 13S	408	383	782	757	0.51		
Ad40 E1A + 2µg 13S	216	191	336	311	0.61		
Ad40 E1A + 5µg 13S	145	120	259	234	0.51	0.54	0.04
Ad40 E1A + 5µg 13S	203	178	373	348	0.51		
Ad40 E1A + 5µg 13S	207	182	331	306	0.59		

Sample	FLL[a] (TLU)	FLL-FLLAB[b] (TLU)	RLL[c] (TLU)	RLL-RLLAB[d] (TLU)	FLL/ RLL	μ^e	δ^f
Positive control	26951	26926	4610	4585	5.9	5.0	0.67
Positive control	8551	8526	1875	1850	4.6		
Positive control	7020	6995	1629	1604	4.4		
Negative control	36	11	2603	2578	0	0	0
Negative control	38	13	3252	3227	0		
Negative control	26	1	1518	1493	0		

a FLL denotes firefly luciferase luminescence

b FLLAB denotes firefly luciferase luminescence assay background

c RLL denotes *Renilla* luciferase luminescence

d RLLAB denotes *Renilla* luciferase luminescence assay background

e μ denotes mean

f δ denotes standard deviation

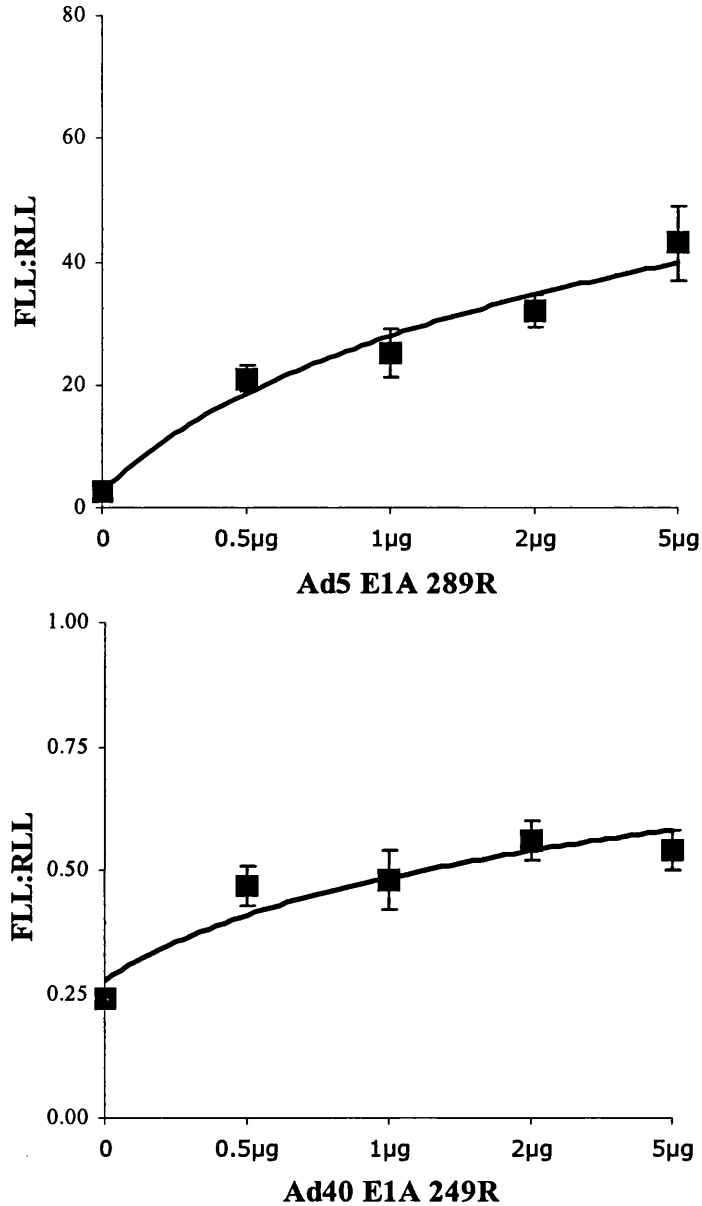


Figure 4.2.3.1 *Trans*-activation of the Ad5 and the Ad40 E1A promoters with increasing amounts of the Ad5 E1A 289R protein and the Ad40 E1A 249R protein respectively. The experiment was set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were co-transfected with independently derived calcium phosphate precipitates containing 10µg Ad5 or Ad40 promoters with either 0.5µg, 1µg, 2µg or 5µg Ad5 E1A 13S cDNA or Ad40 E1A equivalent 13S cDNA, in parallel with a positive and negative control (section 3.4.3). The positive control (pGL3-control; figure 2.1.4) contained the SV40 promoter, whilst the negative control (pGL3-basic; figure 2.1.3) was promoterless. The cells were harvested using the dual luciferase reporter assay system (section 3.4.3.2), then 20µl aliquots were assayed for firefly luciferase luminescence (TLU) then *Renilla* luciferase luminescence (section 3.4.4.2). The µ (■) and δ (capped lines) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) are shown for the Ad5 and the Ad40 E1A promoters with increasing amounts of the Ad5 E1A 289R protein and the Ad40 E1A 249R protein respectively.

firefly luciferase luminescence (FLL) and *Renilla* luciferase luminescence (RLL) values were tabulated (see columns 2 and 4 in Table 4.2.3.2) then used to calculate the mean (μ) and standard deviation (δ) for each sample (see columns 7 and 8 in Table 4.2.3.2; see section 4.1.4).

Figure 4.2.3.2 shows the mean (μ) and standard deviation (δ) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) for the Ad5 E1A promoter and the Ad40 E1A promoter *trans*-activated by the Ad5 E1A 289R protein and the Ad40 E1A 249R protein. The Ad5 E1A 289R protein *trans*-activates the Ad5 E1A promoter by a factor of 10.4, it is also able to *trans*-activate the Ad40 E1A promoter by a factor of 40, which gives an expression level comparable to Ad5. The Ad40 E1A 249R protein *trans*-activates the Ad40 E1A promoter by a factor of 1.7 fold, but is unable to *trans*-activate the Ad5 E1A promoter. *Trans*-activation from the Ad40 E1A promoter by the Ad40 E1A 249R protein was activated 6.2 fold less when compared to *trans*-activation of the Ad5 E1A promoter by the Ad5 E1A 289R protein; this value was calculated by dividing the ratio of *trans*-activation from the Ad5 E1A promoter by the Ad5 E1A 289R protein, by the ratio of *trans*-activation from the Ad40 E1A promoter by the Ad40 E1A 249R protein.

Summary

The Ad5 E1A 289R protein is a strong *trans*-activator, which is able to *trans*-activate both the Ad5 and the Ad40 E1A promoters to similar expression levels. On the other hand the Ad40 E1A 249R protein is an extremely weak *trans*-activator, which is able to *trans*-activate its own promoter but unable to *trans*-activate the Ad5 E1A promoter. Overall *trans*-activation by the Ad40 E1A 249R protein on the Ad40 E1A promoter is approximately 6.2 fold less than *trans*-activation by the Ad5 E1A 289R protein on the Ad5 E1A promoter.

Table 4.2.3.2 Transient co-transfection of the intact Ad5 or Ad40 E1A promoters with either the Ad5 E1A 289R protein or the Ad40 E1A 249R protein.

Sample	FLL[a] (TLU)	FLL-FLLAB[b] (TLU)	RLL[c] (TLU)	RLL-RLLAB[d] (TLU)	FLL/ RLL	μ[e]	δ[f]
Ad5 E1A promoter	1765	1740	138	113	15	15	1.7
Ad5 E1A promoter	3035	3010	203	178	17		
Ad5 E1A promoter	3062	3037	247	222	14		
+Ad5 13S	48966	48941	307	282	174	156	15
+Ad5 13S	62925	62900	429	404	156		
+Ad5 13S	65144	65119	498	473	137		
+ Ad40 13S	19639	19614	1173	1148	17	16	1.0
+ Ad40 13S	14205	14180	834	809	17		
+ Ad40 13S	4256	4231	311	286	15		
Ad40 E1A promoter	197	172	82	57	3.0	3.4	0.26
Ad40 E1A promoter	186	161	71	46	3.5		
Ad40 E1A promoter	202	177	74	49	3.6		
+Ad5 13S	11773	11748	104	79	149	137	27
+Ad5 13S	15667	15642	183	158	99		
+Ad5 13S	1656	1631	35	10	163		
+ Ad40 13S	1552	1527	317	292	5.2	5.7	0.41
+ Ad40 13S	4329	4304	721	696	6.2		
+ Ad40 13S	114	89	41	16	5.6		
Positive control	229	204	53	28	7.3	7.4	1.4
Positive control	171	146	41	16	9.1		
Positive control	224	199	60	35	5.7		
Negative control	25	0	1190	1165	0.0	0.0	0.0
Negative control	24	0	960	935	0.0		
Negative control	26	1	1369	1344	0.0		

a FLL denotes firefly luciferase luminescence

b FLLAB denotes firefly luciferase luminescence array background

c RLL denotes *Renilla* luciferase luminescence

d RLLAB denotes *Renilla* luciferase luminescence array background

e μ denotes mean

f δ denotes standard deviation

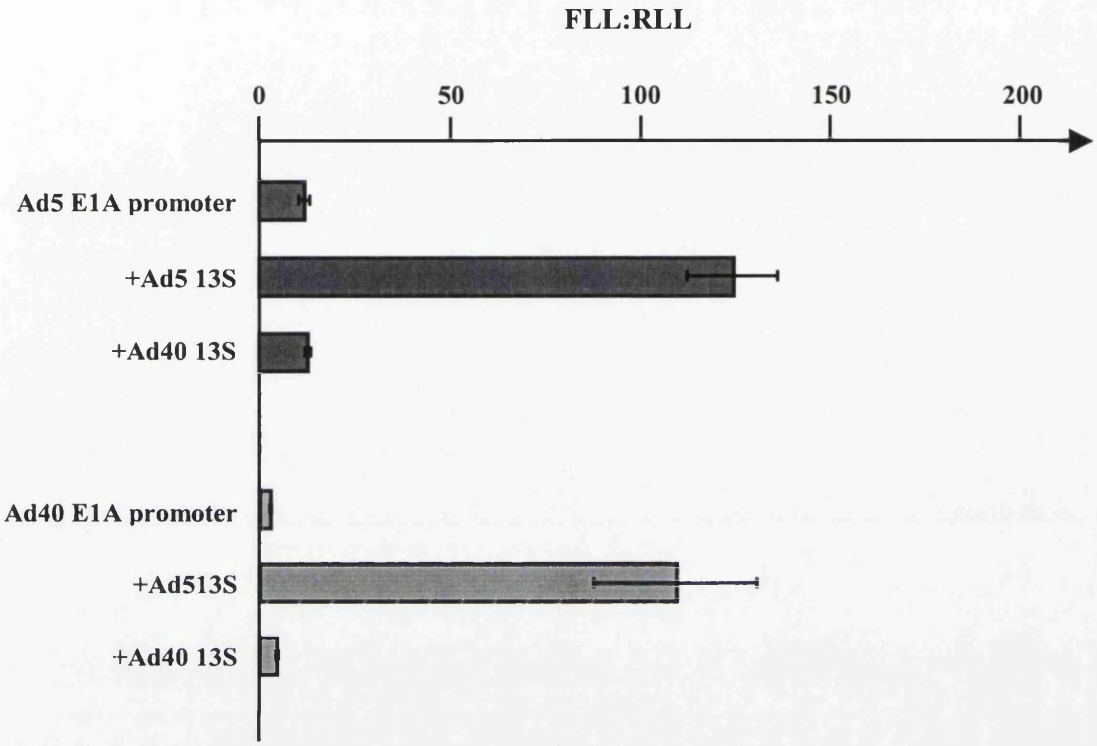


Figure 4.2.3.2 Analysis of *trans*-activation by the Ad5 E1A 289R protein and the Ad40 E1A 249R protein on the Ad5 and the Ad40 E1A promoters. The experiment was set up with triplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were co-transfected with independently derived calcium phosphate precipitates containing 5μg Ad5 or Ad40 E1A promoters with either 5μg Ad5 E1A 13S cDNA or Ad40 E1A 13S equivalent cDNA, in parallel with a positive (pGL3-control; figure 2.1.4) and negative control (pGL3-basic; figure 2.1.3). The positive control contained the SV40 promoter whilst the negative control was promoterless. The cells were harvested by using the dual-luciferase reporter assay system (section 3.4.3.2), then 20μl aliquots were assayed for firefly luciferase luminescence then *Renilla* luciferase luminescence (section 3.4.4.2). The μ and δ (capped lines) values are shown for the Ad5 and the Ad40 E1A promoters with the Ad5 E1A 289R protein and the Ad40 E1A 249R protein.

4.2.4 Analysis of *trans*-activation by the Ad5 E1A 289R protein and the Ad40 E1A 249R protein upon the intact and deleted Ad5 and Ad40 E1A promoters

The Ad40 E1A 249R protein is a weak *trans*-activator of Ad40 E1A transcription when compared with *trans*-activation of the Ad5 E1A promoter by the Ad5 E1A 289R protein. Suggesting that *trans*-activation of the Ad40 E1A promoter may be implicated in the growth restriction of Ad40. To map sequences which were important for *trans*-activation from the Ad40 E1A promoter, WS HeLa cells were transiently co-transfected with a $CaPO_4$ precipitate containing the intact or deleted Ad5 or Ad40 E1A promoters (section 4.1.5 and 4.1.7) with expression vectors containing either the Ad5 E1A 13S coding sequences (pCMV13S) or the Ad40 equivalent E1A 13S protein coding sequences (pCMVAd4013S), in parallel with a positive (pGL3-control; figure 2.1.4) and a negative (pGL3-basic; figure 2.1.3) control. The experiment was set up with duplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), transiently co-transfected with independently derived $CaPO_4$ precipitates (section 3.4.3). Cells were harvested using the dual-luciferase reporter assay system (section 3.4.3.2), then 20µl aliquots of cell extract were assayed for firefly luciferase luminescence and *Renilla* luciferase luminescence (section 3.4.4.2). Sample firefly luciferase luminescence (FLL) and *Renilla* luciferase luminescence (RLL) values were tabulated (see columns 2 and 4 in Table 4.2.4) then used to calculate the mean (µ) and standard deviation (δ) for each sample (see columns 7 and 8 in Table 4.2.4; see section 4.1.4).

Figure 4.2.4 A shows a diagrammatic representation of the intact and deleted Ad5 and Ad40 E1A promoters used in the co-transfection, whilst B shows the mean (µ) and standard deviation (δ) values for the ratio of firefly luciferase luminescence (FLL) to *Renilla* luciferase luminescence (RLL) for the intact and deleted Ad5 and Ad40 E1A promoters *trans*-activated by the Ad5 289R E1A protein. The equivalent Ad5 and Ad40 E1A promoters *trans*-activated by the Ad40 E1A 249R protein were not obtained as an error occurred within the incubator, rendering the co-transfections null and void. Figure 4.2.4 B illustrates that the overall expression levels within this experiment were adversely affected by the error which occurred within the incubator during the experiment however, the results were still valid from this half of the experiment as the

Table 4.2.4 Transient co-transfection of the intact and deleted Ad5 or Ad40 E1A promoters with the Ad5 E1A 289R protein

Sample	FLL[a] (TLU)	FLL-FLLAB[b] (TLU)	RLL[c] (TLU)	RLL-RLLAB[d] (TLU)	FLL/RLL	μ^e	δ^f
Ad5 E1A promoter	205	180	44	19	9.5	10	0.8
Ad5 E1A promoter	209	184	41	16	11		
Ad5 E1A promoter +13S	1325	1300	43	18	72	97	25
Ad5 E1A promoter +13S	1494	1469	37	12	122		
Ad5 del.428 + 13S	8192	8167	131	106	77	81	3.0
Ad5 del.428 + 13S	5547	5522	91	66	84		
Ad5 del.321 + 13S	231	206	37	12	17	13	4.1
Ad5 del.321 + 13S	174	149	40	15	9.9		
Ad5 del.171 + 13S	209	184	43	18	10	12	1.6
Ad5 del.171 + 13S	204	179	39	14	13		
Ad5 del.42 + 13S	39	14	37	12	1.2	0.85	0.35
Ad5 del.42 + 13S	35	10	45	20	0.50		
Ad40 E1A promoter	128	103	51	26	4.0	2.3	1.7
Ad40 E1A promoter	40	15	52	27	0.56		
Ad40 E1A promoter + 13S	56	31	29	4	7.8	7.4	0.4
Ad40 E1A promoter + 13S	95	70	35	10	7.0		
Ad40 del.349 + 13S	972	947	66	41	23	24	1.0
Ad40 del.349 + 13S	1627	1602	89	64	25		
Ad40 del.328 + 13S	646	621	54	29	21	24	3.0
Ad40 del.328 + 13S	505	480	43	18	27		
Ad40 del.268 + 13S	3474	3449	278	253	14	15	0.7
Ad40 del.268 + 13S	581	556	61	36	15		
Ad40 del.235 + 13S	39	14	42	17	0.82	0.91	0.09
Ad40 del.235 + 13S	34	9	34	9	1.00		
Ad40 del.209 + 13S	30	5	43	18	0.28	0.39	0.11
Ad40 del.209 + 13S	37	12	49	24	0.50		
Ad40 del.166 + 13S	38	13	42	17	0.76	0.55	0.22
Ad40 del.166 + 13S	30	5	40	15	0.33		
Ad40 del.140 + 13S	66	41	112	87	0.47	0.50	0.03
Ad40 del.140 + 13S	69	44	108	83	0.53		

Sample	FLL[a] (TLU)	FLL-FLLAB[b] (TLU)	RLL[c] (TLU)	RLL-RLLAB[d] (TLU)	FLL/RLL	μ[e]	δ[f]
Ad40 del.37 + 13S	35	10	54	29	0.34	0.48	0.14
Ad40 del.37 + 13S	36	11	43	18	0.61		
Positive control	254	229	52	27	8.5	8.6	0.1
Positive control	276	251	54	29	8.7		
Negative control	25	0	659	634	0.00	0.0	0.0
Negative control	26	1	635	610	0.00		

a FLL denotes firefly luciferase luminescence

b FLLAB denotes firefly luciferase luminescence assay background

c RLL denotes *Renilla* luciferase luminescence

d RLLAB denotes *Renilla* luciferase luminescence assay background

e μ denotes mean

f δ denotes standard deviation

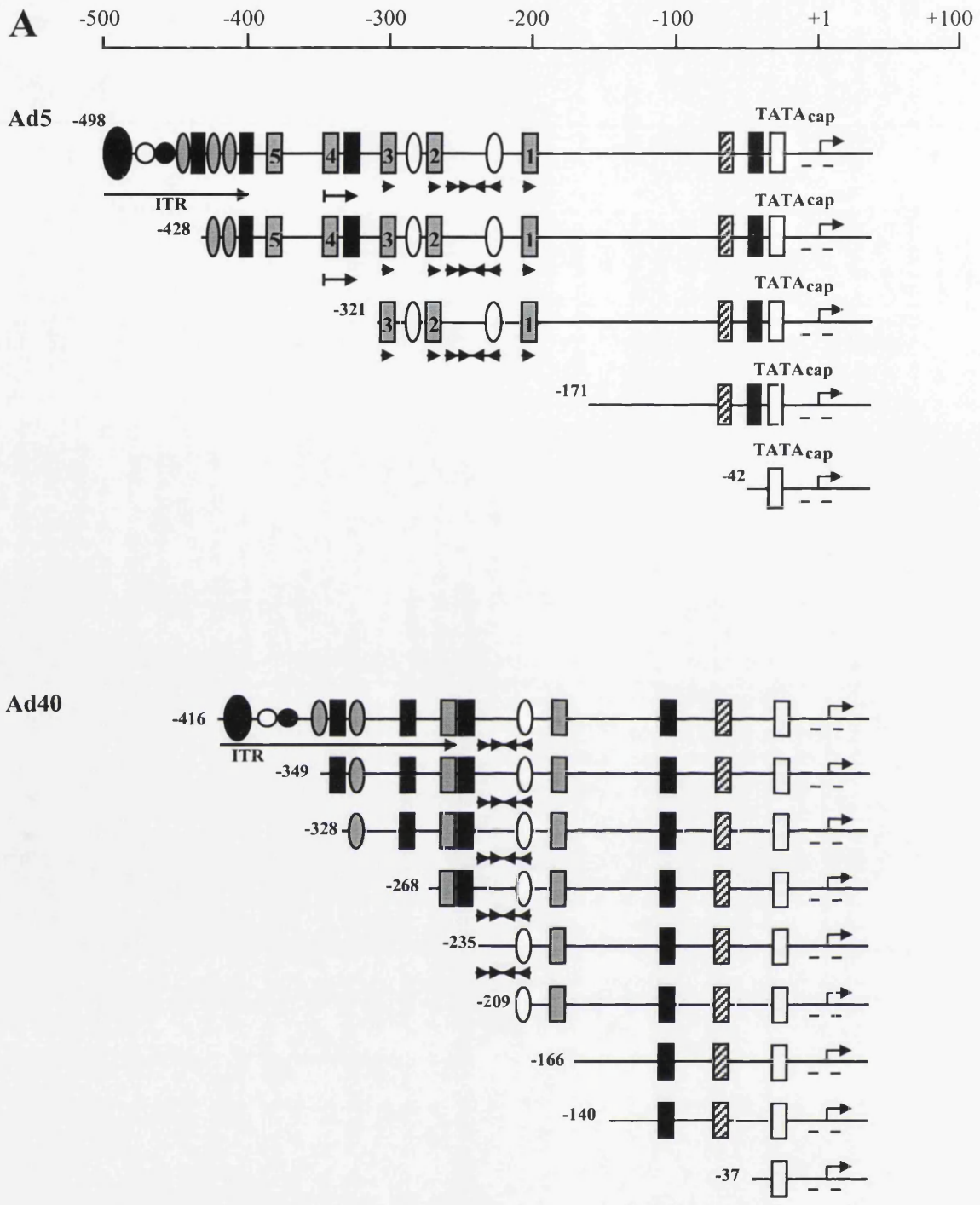
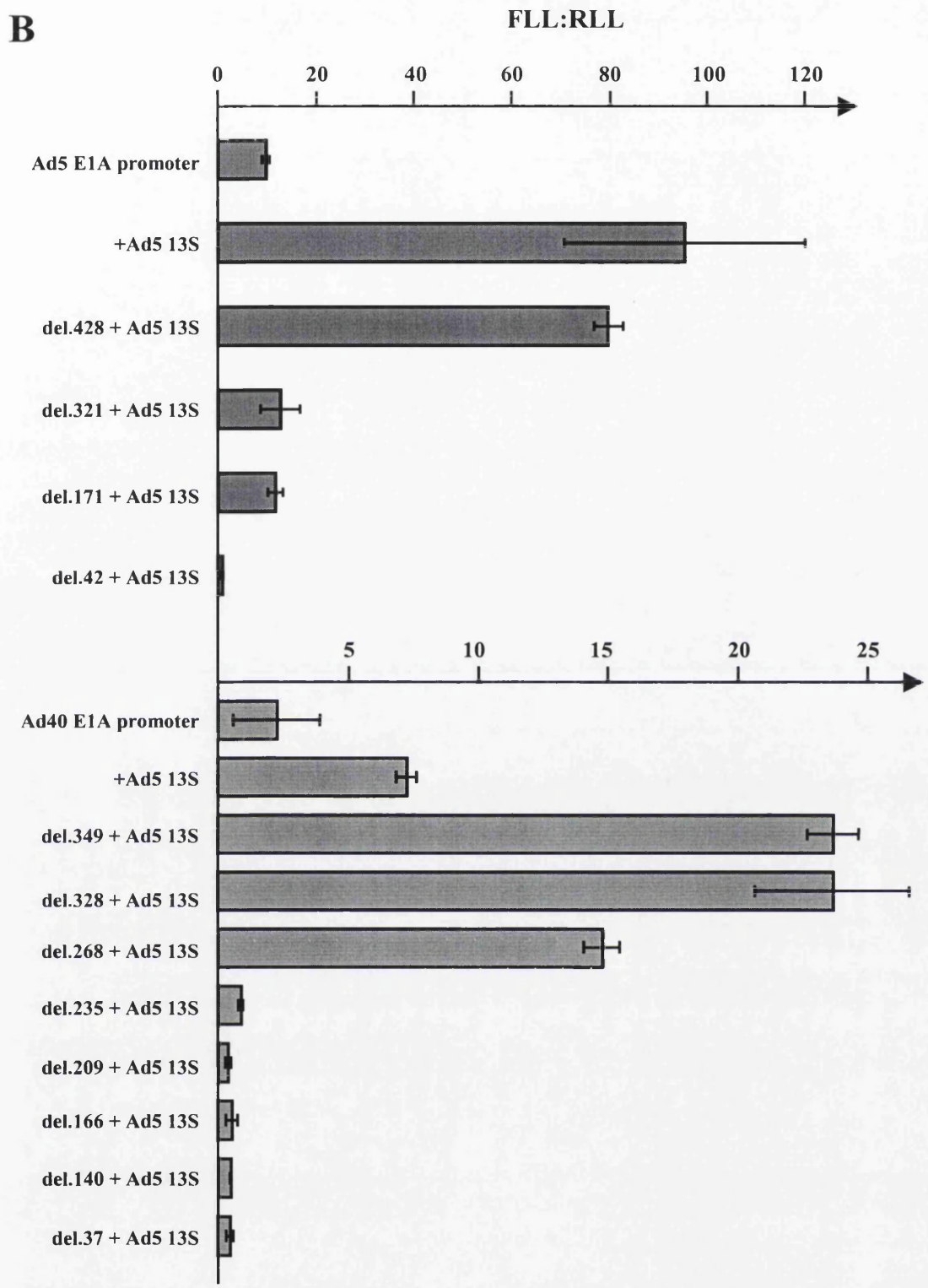


Figure 4.2.4 Analysis of *trans*-activation by the Ad5 E1A 289R protein on the intact and deleted Ad5 and the Ad40 E1A promoters. The experiment was set up with duplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate), which were co-transfected with independently derived calcium phosphate precipitates (section 3.4.3) containing 5µg intact or deleted Ad5 or Ad40 E1A promoters with 5µg Ad5 E1A 289R protein, in parallel with a positive and negative control. The positive control contained the SV40 promoter (pGL3-control; figure 2.1.4), whilst the negative control (pGL3-basic; figure 2.1.3) was promoterless. The cells were harvested by using the dual-luciferase reporter assay system (section 3.4.3.2), then 20µl aliquots were assayed for firefly luciferase



luminescence then *Renilla* luciferase luminescence (section 3.4.4.2). **A.** The Ad5 and the Ad40 E1A full length and deleted promoters. **B.** The μ and δ (capped lines) values are shown for the intact and deleted Ad5 and Ad40 E1A promoters with/without the Ad5 E1A 289R protein. Transcription factor binding sites are indicated by: ⬮ denotes ORP-A; ● denotes NF I; ○ denotes NF III; ⬯ denotes SP1; ■ denotes ATF; ▯ denotes EF-1A; ○ denotes E2F; ▨ denotes CAAT; ▶ denotes Element I; ▶◀ denotes Element II in the Ad5 sequence and an Element II-like sequence in the Ad40 sequence; ⟼ denotes 24bp enhancer; ☐ denotes TATA; ↱ denotes the cap site; - - denotes the hyphenated symmetry around the cap site.

Ad5 E1A 289R protein was a strong *trans*-activator of transcription. Basal transcription from the Ad40 E1A promoter when compared with the Ad5 E1A promoter is reduced by approximately the same levels as observed in previous experiments in sections 4.1 and 4.2. The Ad5 E1A 289R protein *trans*-activated both the intact Ad5 and the intact Ad40 E1A promoters however, not to the same level as was previously demonstrated (Figure 4.2.3.2). This may have been related to the equipment problem mentioned above, and given more time it would have been beneficial to repeat these experiments.

Deletions within the Ad5 and the Ad40 E1A promoter demonstrated similar patterns of expression. Deletion of sequences from -498 to -428 (relative to the cap site) in the Ad5 E1A promoter resulted in a 1.2 fold decrease in *trans*-activated expression however, the decrease is within the range for standard deviation and therefore is not significant. The largest decrease in *trans*-activated expression within the Ad5 E1A promoter came with the deletion of sequences from -428 to -321, and resulted in a decrease by a factor of 6.2. Deletion of sequences from -321 to -171 did not affect *trans*-activated expression from the Ad5 E1A promoter, the final decrease in *trans*-activated expression resulted from deletion of sequences from -171 to -42, with an approximated value of 14 fold.

Deletion of sequences within the Ad40 E1A promoter demonstrated a similar pattern of expression to that observed within the Ad5 E1A promoter i.e. deletion of sequences resulted in a sequential decrease in *trans*-activated transcription from the Ad40 E1A promoter. There was however one difference to the pattern of expression observed within the deletion analysis of the Ad40 E1A promoter, namely deletion of sequences from -416 to -349 relative to the Ad40 E1A cap site, effected an increase in *trans*-activated levels of expression from the Ad40 E1A promoter. Deletion of sequences from -349 to -328 mediated a slight decrease in *trans*-activated expression. Deletion of sequences from -328 to -268 and -268 to -235 reduced *trans*-activated expression to the level observed from the Ad40 E1A TATA box deletion construct (del.37). The decrease in *trans*-activated expression within these sequences was mediated in two stages, with the deletion of -328 to -268 resulting in a 17 fold decrease, whilst deletion of the region -268 to -235 resulted in a- 2.3 fold decrease. Overall the deletion of sequences from the Ad40 E1A promoter in the region of -328 to -235 mediated a decrease in *trans*-activated

expression by a factor of 39. Subsequent deletion of sequences from -235 to -37 had no real effect on *trans*-activated expression from the Ad40 E1A promoter.

Summary

The Ad5 E1A 289R protein *trans*-activated both the intact Ad5 E1A promoter and the intact Ad40 E1A promoter however, this was not consistent with the previous experiment (section 4.2.3) where the Ad5 E1A 289R protein was shown to *trans*-activate the Ad40 E1A promoter to the same level as the Ad5 E1A promoter (Table 4.2.3.2; figure 4.2.3.2). This may be accounted for by the equipment filure mentioned above. Deletions within the Ad5 and the Ad40 E1A promoters demonstrated similar patterns of expression. Deletion of sequences from the ITR to the TATA box in the Ad5 E1A promoter resulted in a decrease in *trans*-activated expression with each deletion, the greatest decrease being mediated by a region between -428 to -321. *Trans*-activation of the Ad40 E1A promoter deletion constructs also mediated a sequential decrease in *trans*-activated expression from the ITR to the TATA box. The region from -328 to -235 mediating the greatest decrease in *trans*-activated expression by the Ad5 E1A 289R protein.

4.3 Characterisation of branched chain anti-peptide antibodies directed against the Ad40 E1A proteins

Introduction

The Ad5 E1A region has been shown to produce a number of proteins (figure 1.1.4.2). The major Ad5 E1A proteins are the 289R and 243R proteins, which contain three highly conserved regions, designated CR1, CR2 and CR3 (Kimelman *et al.*, 1985; Moran and Mathews, 1987). CR1 is required for transcriptional repression, transformation and induction of DNA synthesis (Lillie *et al.*, 1987; Schneider *et al.*, 1987; Jelsma *et al.*, 1989; reviewed in Jones, 1995). CR2 is required for transformation and induction of DNA synthesis, but may be dispensable for transcriptional repression ((Lillie *et al.*, 1986; Moran *et al.*, 1986a; Howe *et al.*, 1990; Stein *et al.*, 1990). CR3 possesses a potent *trans*-activation function (Shenk and Flint, 1991; Jones, 1992; Bayley and Mymrk, 1994). The non-conserved C-terminal region of E1A is also required as an enhancer of CR3-mediated *trans*-activation (Bondesson *et al.*, 1992; Strom *et al.*, 1998). The functions of the E1A proteins have been well documented, and include their abilities for autoregulation, *trans*-activation, *trans*-repression and transformation (section 1.2). The E1A proteins ability to mediate these functions is dependent upon their interaction with a number of viral and cellular proteins (see section 1.2.3, in particular figure 1.2.3; reviewed in Mymrk, 1996).

Expression from the Ad40 E1A region in plasmid transformed cells results in a 249R protein and a 221R protein equivalent to the Ad5 E1A 289R and 243R proteins (van Loon *et al.*, 1987a). The mRNA encoding the 249R protein was demonstrated in Ad40 infected cells, however the 221R protein was not demonstrated at early times in Ad40 infected cells (section 4.2.2). The Ad40 E1A 135R protein has also been predicted but has not been demonstrated experimentally (van Loon *et al.*, 1987a). These results suggest that perhaps like the Ad5 E1A region the Ad40 E1A region encodes a number of proteins. To determine the number and size of the respective Ad40 E1A proteins, infected cell extracts were analysed in western blot experiments with anti-peptide antibodies raised against the Ad40 E1A 249R protein.

4.3.1 Production of branched chain anti-peptide antibodies directed against the N-terminal, CR3 and C-terminal regions of the Ad40 E1A 249R protein

To investigate the number and size of Ad40 E1A proteins and the interactions with other cellular and viral proteins during an Ad40 infection *in vitro and in vivo*, antisera were generated to the Ad40 E1A proteins. Two of these sera would be expected to contain antibodies which recognise the N-terminus and the C-terminus sequences, which would detect both the 249R and the 221R E1A proteins, while the third would recognise CR3, and therefore would be specific to the 249R protein.

Ad40 E1A specific anti-peptide antibodies were generated using branched-chain peptides to the first 15 amino acids of the N-terminus (peptide denoted 1051C), CR3 between amino acids 162 to 176 (peptide denoted 1051D) and the last 12 amino acids of the C-terminus of the Ad40 E1A 249R protein (peptide denoted 1051E) (see Table 2.10 for details of the peptide sequences used). The branched-chain peptides 1051C, 1051D and 1051E were synthesised in house by Mrs K. McAulay using a Shimadzu Europa PSSM-8 peptide synthesiser, then each peptide was used to raise antibodies in pairs of rabbits. Peptides corresponding to the N-terminus, CR3 and the C-terminus of the Ad40 E1A 249R protein were initially injected at 50μg 1051C, 1051D or 1051E in 0.5ml Freund's complete adjuvant, then subsequent boosts were made with 50μg of peptide in 0.5ml Freund's incomplete adjuvant. Before the rabbits were boosted, test bleeds were taken from each rabbit to establish whether or not the rabbits were raising antibodies to the peptide, and to establish whether subsequent boosts were required, and finally to decide when the rabbits should be bled out. The test bleeds were processed, then tested undiluted or diluted at concentrations of 1 in 10, 1 in 100, 1 in 1, 000 and 1 in 10, 000, against 100ng and 1μg of the appropriate peptide in ELISA assays.

Figures 4.3.1 illustrates the immune response of rabbits 228 and 229 to the N-terminus branched-chain peptide 1051C, rabbits 230 and 231 to the CR3 branched-chain peptide 1051D and rabbits 232 and 233 to the C-terminus branched-chain peptide 1051E, at antibody dilutions of 1 in10, 1 in 100, 1in 1, 000 and 1 in 10, 000, across six test bleeds. Rabbits 228 and 229 did not appear to raise antibodies to the Ad40 E1A 249R

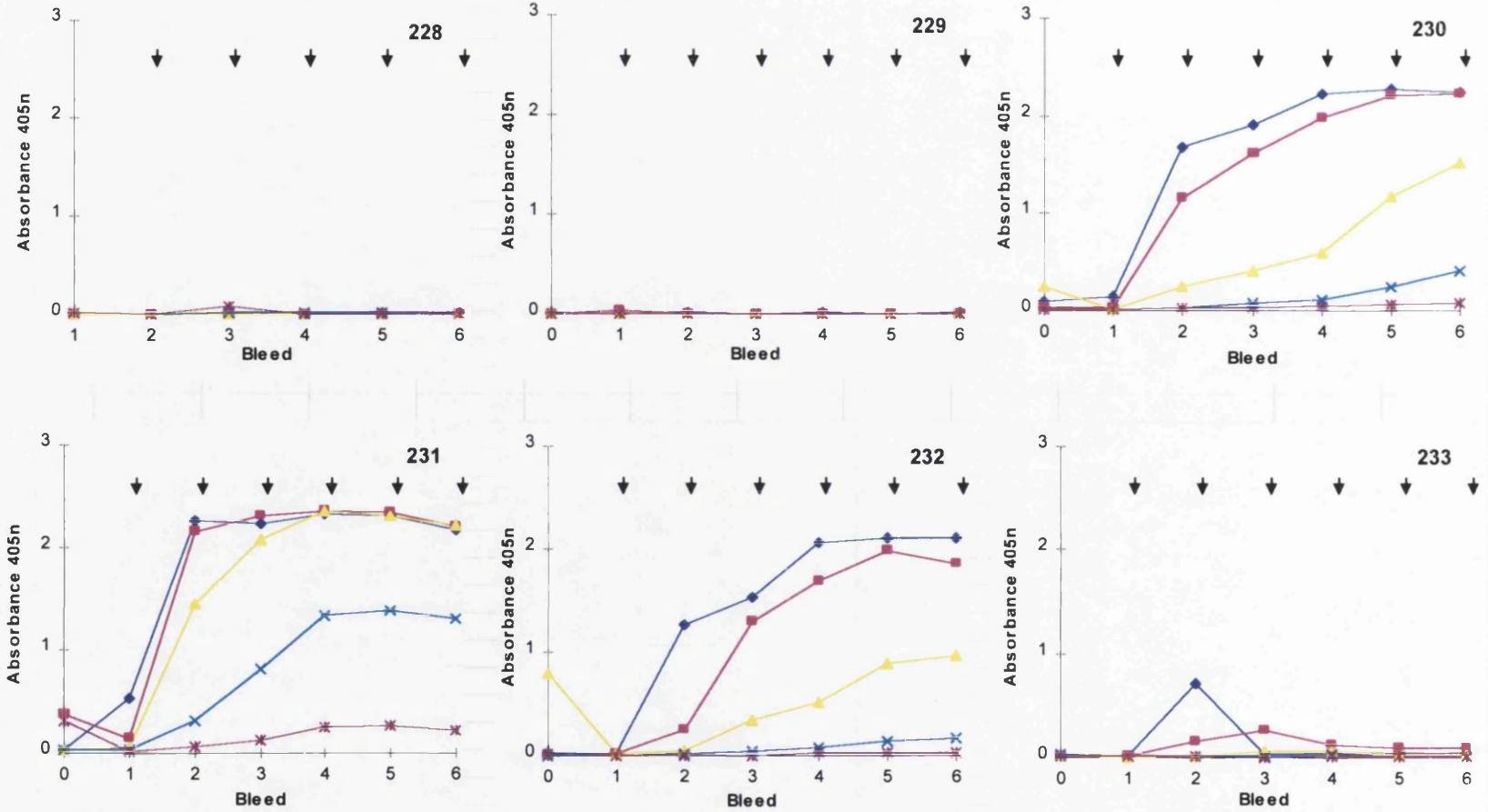


Figure 4.3.1 Immune response of rabbits to specific branched chain peptides to the N-terminal, CR3 and C-terminal of the Ad40 E1A 249R protein. Pairs of rabbits (228-229, 230-231, 232-233) were initially injected with 50μg peptide Ad40 E1A N-terminal (1051C), CR3 (1051D) and C-terminal (1051E) specific branched-chain peptide in 0.5ml Freunds complete adjuvant, subsequent boosts were made in Freunds incomplete adjuvant. Test bleeds were performed between each boost and tested against 1μg corresponding peptide in ELISA. Additional description: ♦ neat; ■ $^{1}/_{10}$; Δ $^{1}/_{100}$; x $^{1}/_{1\,000}$; * $^{1}/_{10\,000;}$ ↓ denotes boost.

N-terminus branched-chain peptide 1051C. However, antibodies were produced in rabbits 230 and 231 against the Ad40 E1A 249R CR3 branched-chain peptide 1051D, and in rabbit 232 against the Ad40 E1A 249R C-terminus branched-chain peptide. Rabbits 230 and 232 produced antibodies, which increased in titre over the immunisation period and appeared to plateau by the sixth test bleed, whereas rabbit 231 raised antibodies by the second test bleed and the titre remained constant over the immunisation period. Rabbit 233 did not produce antibodies, which recognised the C-terminus branched-chain peptide 1051E.

Summary

Rabbits 230 and 231 raised antibodies against the Ad40 E1A 249R CR3 sequences, whilst rabbit 232 raised antibodies against the Ad40 E1A 249R C-terminus. An antibody was not raised against the Ad40 E1A 249R N-terminus. The antibodies generated were subsequently tested in Western blot analysis and immunoprecipitations.

4.3.2 Western blot analysis of Ad5 and Ad40 infected cells

Antibodies raised against the Ad40 E1A 249R CR3 and the C-terminus were used in western blot analysis of Ad40 infected cells to characterise the antibodies generated and determine the number and size of Ad40 E1A proteins which are produced during an Ad40 infection. To characterise the anti-peptide antibodies in western blot analysis, duplicate 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate) were infected with 5pfu/cell Ad5 WT, 5ffu/cell Ad40 (strain Dugan) or mock infected, then blocked with 20 μg/ml araC 12 hourly (section 3.5.3), which prevented DNA replication, allowing accumulation of the early proteins in both Ad5 and Ad40 infected cells. Duplicate plates were harvested 42h p.i., then one tenth of the whole cell extract was analysed by western blot analysis (section 3.3.4; section 3.3.5) using either an Ad5 E1A monoclonal antibody (denoted M58) to detect Ad5 E1A proteins, or an Ad40 E1A 249R CR3 anti-peptide antibody (denoted as 230 or 231) or an Ad40 E1A 249R C-terminus anti-peptide antibody (denoted 232).

Figure 4.3.2 shows the Western blot analysis of Ad5 and Ad40 infected cells probed with the Ad5 E1A 13S C-terminus monoclonal antibody M58, the Ad40 E1A 249R CR3 and C-terminus anti-peptide antibodies or the Ad40 E1B 19kDa C-terminus antibody (Mautner *et al.*, 1990). The track probed with Ad5 E1A 13S C-terminus monoclonal antibody M58 contains bands which correspond with the expected size of the Ad5 E1A proteins in the infected cell extract, but the track with the uninfected cell extract did not. However, the tracks containing Ad40 infected cell extracts probed with the Ad40 E1A 249R CR3 and C-terminus anti-peptide antibodies, when compared with mock infected cell extracts contained no infection-specific bands, suggesting that either the antibodies were not functional within the western blot or that there had been no Ad40 infection. To establish whether or not an Ad40 infection had taken place the blot was stripped, then reprobed with the Ad40 E1B 19kDa C-terminus antibody. A band was observed between the 21.5kDa and 14.3kDa rainbow markers in the Ad40 infected cells, which corresponded with the predicted size of the E1B 19kDa protein. This suggests that a productive Ad40 infection had in fact occurred, although the amount of the E1B 19kDa polypeptide was apparently very low. Therefore the Ad40 infected samples used in these experiments may have come from an inefficient infection, and the affinities of the antibodies raised against the Ad40 E1A proteins may have been

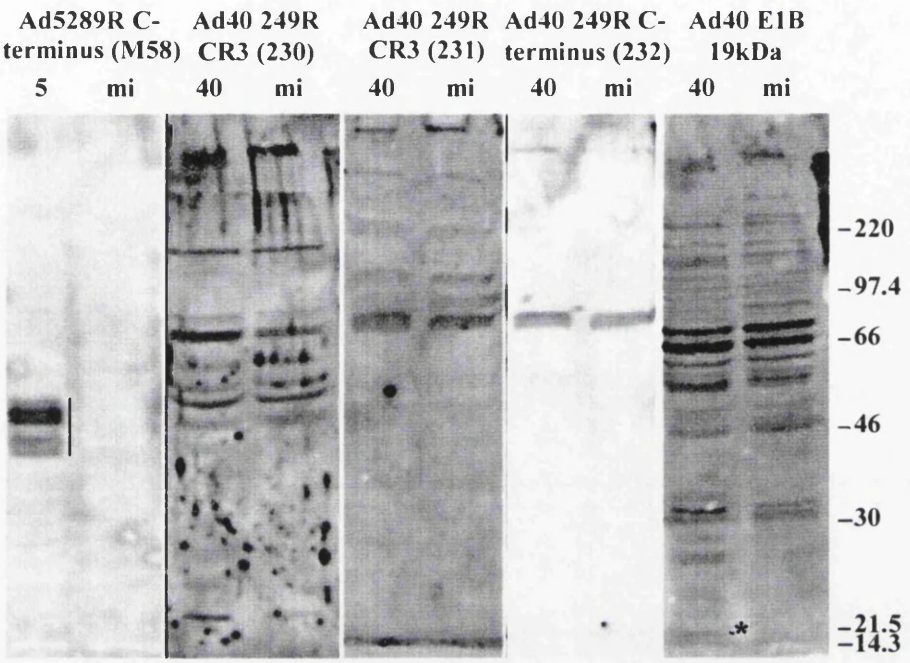


Figure 4.3.2 Western blot analysis of Ad5 and Ad40 infected cells. WS HeLa cells were infected with Ad5 (5pfu/cell), Ad40 (5ffu/cell) or mock infected (tris saline), and DNA replication blocked with 20μg/ml araC every 12h (section 3.5.3). Cells were harvested into 1X boiling mix 42h p.i. (section 3.3.1), then a tenth of the whole cell extract was separated, in parallel with high molecular weight rainbow markers (Amersham Life Science) on a 12% polyacrylamide gel (section 3.3.4). Proteins resolved on the gel were then transferred to nitrocellulose, which was probed with an Ad5 E1A 289R C-terminus monoclonal antibody M58, Ad40 E1A 289R CR3 anti-peptide antibodies 230 and 231, and an Ad40 E1A 289R C-terminus anti-peptide antibody 232; bands were visualised with ECL system (Amersham Life Science) (section 3.3.5). The blot was then stripped and reprobed with an Ad40 E1B 19kDa C-terminus antibody (Mautner *et al.*, 1990). Line denotes the position of the Ad5 E1A proteins; star denotes the position of the Ad40 E1B 19kDa protein.

insufficient to detect the low amounts of viral proteins in these extracts. Unfortunately, time constraints did not allow further analysis using different infected cell samples.

Summary

Bands in the region of 46kDa were detected in Ad5 infected cell extracts, which correspond with the Ad5 E1A proteins. However, no bands were detected in Ad40 infected cell extracts. Although the Ad40 infection was productive very little E1B 19kDa protein was detectable, suggesting that the apparent lack of Ad40 E1A proteins may be because they are present below the level of detection. To eliminate this possibility it would be necessary to repeat the western blot experiments using cell extracts expressing high levels of Ad5 and Ad40 E1A proteins.

4.3.3 Western blot analysis of the Ad5 E1A 289R protein and the Ad40 E1A 249R protein in transfected cells.

The Ad40 E1A 249R CR3 and C-terminus anti-peptide antibodies had been unable to detect Ad40 E1A proteins in infected cells, which was likely to have been due to the poor expression of Ad40 E1A in the infected cell samples used. In an attempt to establish whether or not the low level of Ad40 E1A proteins in infected cells was an issue, 60mm plates of WS HeLa cells (seeded at $1x10^6$ cells/plate) were transiently transfected with independently derived calcium phosphate precipitates (section 3.4.3) containing 10μg pCMV13S, pCMVAd4013S or pUC9 (which acted as a negative control). Cells were harvested 48h after transfection (section 3.3.1), then one tenth of the resultant whole cell extract was analysed by western blotting (section 3.3.4; section 3.3.5) using the Ad5 E1A monoclonal antibody M58, anti-Ad40 E1A CR3 antibodies 230 and 231, or an anti-Ad40 E1A C-terminus antibody 232.

Figure 4.3.3 shows the western blot analysis of WS HeLa cells transfected with the Ad5 E1A 13S cDNA and the Ad40 E1A equivalent 13S cDNA probed with the Ad5 E1A 13S C-terminus monoclonal antibody M58, the Ad40 E1A 249R CR3 anti-peptide antibody or the Ad40 E1A 249R C-terminus anti-peptide antibody. The track probed with Ad5 E1A 13S C-terminus monoclonal antibody M58 contains several bands, which range in size from approximately 54kDa to 43kDa; these correspond to the expected sizes of the Ad5 E1A proteins. However, cells which were transfected with expression plasmid containing the Ad40 E1A 13S cDNA and probed with either the Ad40 E1A 249R CR3 anti-peptide antibody (230 and 231) or the Ad40 E1A 249R C-terminus anti-peptide antibody (232) appeared to contain no bands which differed from those present in extracts from mock transfected cells.

Summary

Several bands were detected in Ad5 transfected cell extracts which ranged in size from approximately 54kDa to 43kDa, corresponding to the pattern of Ad5 E1A proteins observed in section 4.3.2. However, no bands were detected in Ad40 transfected cell extracts. This raises two questions: firstly, do the anti-peptide antibodies recognise the Ad40 E1A proteins, and secondly is the Ad40 E1A equivalent 13S cDNA being

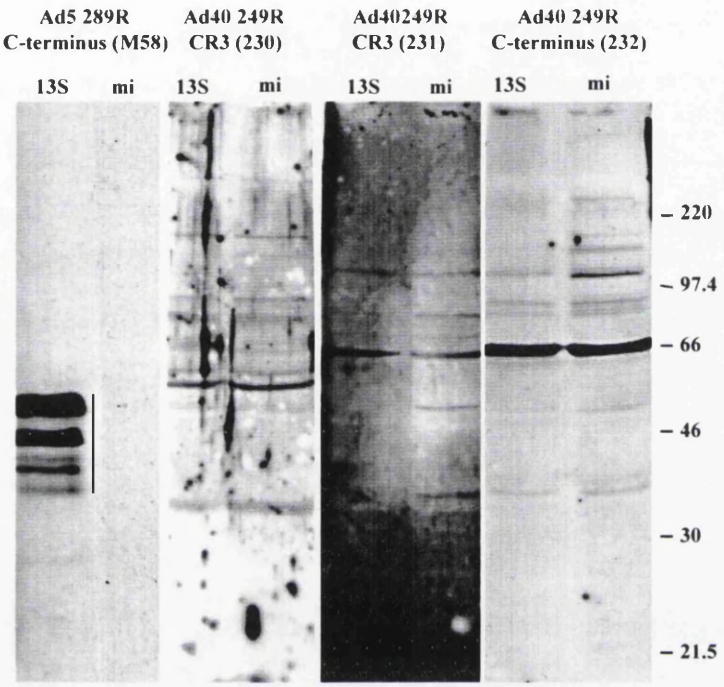


Figure 4.3.3 Western blot analysis of overexpressed Ad5 E1A 289R protein and Ad40 E1A 249R protein. WS HeLa cells were transfected with 10 μg pCMV13S, pCMVAd4013S or pUC9 (section 3.4.3), then harvested into 1X boiling mix (section 3.3.1). A tenth of whole cell extract was separated, in parallel with high molecular weight rainbow markers (Amersham Life Science) on a 12% polyacrylamide gel (section 3.3.4). Proteins which were resolved were transferred to nitrocellulose, which was probed with an Ad5 E1A 289R C-terminus monoclonal antibody (M58), anti-peptide Ad40 E1A 249R CR3 antibodies (230 or 231) and an anti-peptide Ad40 E1A 249R C-terminus antibody (232); bands were visualised with ECL system (Amersham Life Science; section 3.3.5). Dot denotes the position of the Ad5 E1A 289R protein.

correctly expressed from the pCMV expression plasmid. The pCMVAd4013S plasmid appears to express a functional Ad40 E1A 249R protein, as demonstrated by the enhanced promoter activity in transfection analysis described in section 4.2.3. It is likely that the protein is expressed and functional since comparison of the *trans*-activation achieved by the Ad5 E1A 289R and the Ad40 E1A 249R proteins on their respective promoters is comparable with that determined by other groups (van Loon *et al.*, 1987b; Ishino *et al.*, 1988), and the effect is dose dependent (section 4.2.3). It is therefore more likely that the anti-peptide antibodies are unable to recognise efficiently the Ad40 E1A proteins.

4.3.4 Immunoprecipitation of E1A proteins from Ad5 and Ad40 infected cells

It is possible that rabbit antibodies, even those raised against branched peptide antigens, could recognise conformational epitopes which could be lost from the denatured proteins present in western blotting experiments. Such conformation-specific epitopes would be maintained in native proteins in crude cell extracts. To test this possibility, the anti-peptide antibodies to the Ad40 E1A CR3 (230 and 231) and to the Ad40 C-terminus were used in immunoprecipitation experiments. WS HeLa cells (seeded at $1x10^6$ cells per 100mm plate) were infected with Ad5 WT (5pfu/cell), Ad40 (5ffu/cell) or mock infected (Tris saline), labelled for 2h at 42h p.i. with 100 μCi [^{35}S]L-methionine (section 3.3.2), then harvested into high salt extraction buffer. Aliquots were then pre-cleared before being incubated with pre-immune (pre-bleed of rabbit) or immune serum (either Ad5 E1A 13S C-terminus monoclonal M58, anti-Ad40 E1A 13S CR3 (230 or 231) or anti-Ad40 E1A 13S C-terminus (232)) before being processed as described in section 3.3.3. Immune complexes were then separated on a 12% polyacrylamide gel (section 3.3.4), and the radiolabelled proteins were visualised by autoradiography on Kodak X-OMAT S film.

Figure 4.3.4 shows immunoprecipitations of Ad5 and Ad40 infected cells using the Ad5 E1A 13S C-terminus monoclonal antibody M58, the Ad40 E1A 249R CR3 and C-terminus anti-peptide antibodies or the Ad40 E1A C-terminus antibody. The track containing Ad5 infected cell proteins immunoprecipitated with Ad5 E1A 13S C-terminus monoclonal antibody M58 contains several bands ranging in size from approximately 43kDa to 50kDa, which correspond with the expected size and pattern of Ad5 E1A proteins previously observed in the infected cell extract. However, the tracks containing Ad40 infected cell proteins precipitated with the Ad40 E1A 249R CR3 or C-terminus anti-peptide antibodies showed no bands specific to Ad40 infection, suggesting that either the same detection problem had arisen as in the western blots or that the anti-peptide antibodies were unable to detect folded Ad40 E1A proteins in crude cell extracts.

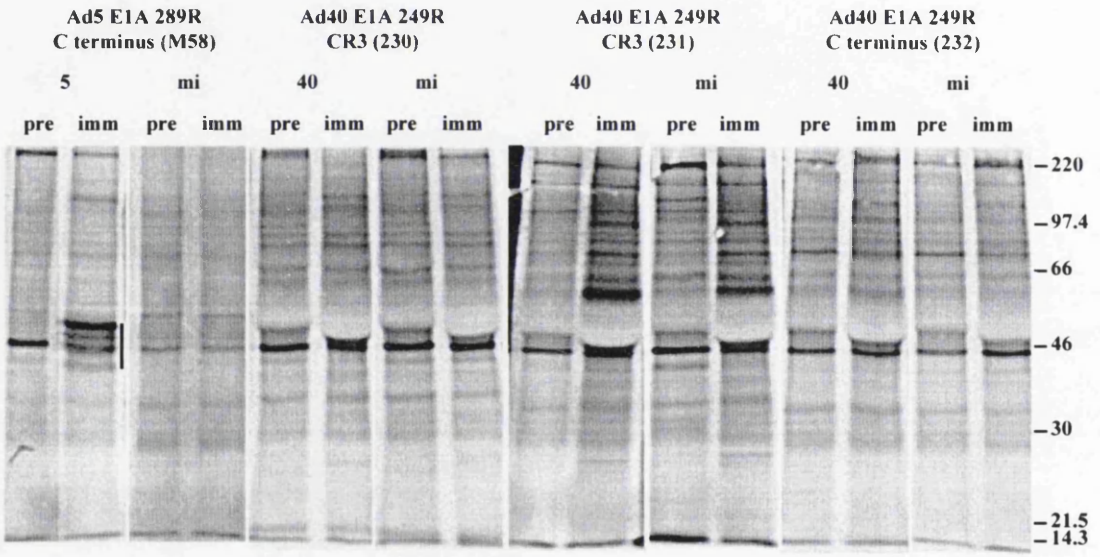


Figure 4.3.4 Immunoprecipitation of E1A proteins from Ad5 and Ad40 infected cells. WS HeLa cells were infected with either Ad5 (5 pfu/cell), Ad40 (5 ffu/cell) or mock infected with tris saline (section 3.5.3), labelled for 2h at 42h p.i. with [^{35}S]L-methionine, then harvested into high salt detergent extraction buffer (section 3.3.2). Aliquots were pre-cleared before being incubated with pre-immune (pre-bleed of rabbit) or immune serum (either Ad5 E1A 289R C-terminus monoclonal antibody M58, Ad40 E1A 249R CR3 anti-peptide antibodies 230 and 231, or Ad40 E1A 249R C-terminus anti-peptide antibody 232 before being processed (section 3.3.3). Immune complexes, in parallel with high molecular weight rainbow markers (Amersham Life Science) were separated on a 12% polyacrylamide gel (section 3.3.4). Line denotes the position of the Ad5 E1A protein complexes.

Summary

The Ad5 E1A proteins were immunoprecipitated with the Ad5 E1A 13S C-terminus monoclonal antibody M58, but the Ad40 E1A proteins could not be precipitated with either the Ad40 E1A CR3 anti-peptide antibodies 230 or 231, or the Ad40 E1A C-terminus anti-peptide antibody 232. It can be concluded that the branched chain anti-peptide antibodies produced using the CR3 and C-terminus regions of the Ad40 249R protein were functional against peptide in ELISA, but they were not sufficiently active in western blots of infected cell extracts, cell extracts overexpressing the Ad40 E1A 249R protein, or in immunoprecipitations of Ad40 infected cells, using the extracts that were available for these studies.

5. DISCUSSION

5. Discussion

Introduction

The enteric adenoviruses were first identified from the stool samples of infants with acute gastroenteritis (Flewett *et al*, 1973). Although enteric adenovirus particles are shed in large numbers from the gut, they fail to propagate in cells such as HEK, KB, HeLa, HEP-2 and WI-38, which are normally used to propagate other adenovirus serotypes in tissue culture (Madeley *et al.*, 1977; Retter *et al.*, 1979). They will however propagate in 293 cells, a human embryo kidney (HEK) cell line which has been transformed with the Ad5 E1 region (Graham *et al.*, 1977; Takiff *et al.*, 1981), albeit at reduced levels compared with other serotypes. This observation suggests that the E1 function is poorly expressed or of intrinsically lower activity in certain cell types, therefore implicating the E1 region in the restrictive growth properties of the enteric adenoviruses. Previous work had demonstrated that the Ad40 E1B region played a significant role in this phenotype (Mautner *et al.*, 1989; Steinthorsdottir, 1991; Gomes *et al.*, 1992), however the Ad40 E1A region was also implicated. The E1A region was implicated for two reasons firstly, a delay in the onset of Ad40 E1A mRNA expression was observed compared to Ad5 E1A mRNA expression (Ullah, 1997); and secondly, the Ad40 E1A proteins were observed as weaker *trans*-activators when compared to the Ad5 E1A proteins (van Loon *et al.*, 1987b; Ishino *et al.*, 1988). Taken together these observations suggested that the Ad40 E1A region is of intrinsically lower activity in tissue culture, which would considerably affect progression of Ad40 into the late phase of the infectious cycle. A preliminary characterisation of the Ad40 E1A region involved analysis of basal transcription (sections 4.1 and 5.1) and *trans*-activated transcription from the Ad40 E1A promoter (sections 4.2 and 5.3) compared to the Ad5 E1A promoter. A preliminary map of Ad40 E1A cDNAs was also determined (sections 4.2 and 5.2).

5.1 Analysis of basal transcription from the Ad40 E1A promoter

The aberrant expression of the Ad40 E1 region (Takiff *et al.*, 1981; Kidd and Madeley, 1981) had mainly been attributed to E1B (Mautner *et al.*, 1989; Steinthorsdottir and Mautner, 1991; Gomes *et al.*, 1992) however, E1A was also implicated (Ullah, 1997; van Loon *et al.*, 1987b; Ishino *et al.*, 1988). Ad40 E1A mRNA was expressed 36h p.i. (Ullah, 1997) compared with Ad5 E1A mRNA expression at 1.5-2h p.i.. These data suggest that the Ad40 E1A region is either aberrantly expressed or of intrinsically lower activity when compared to Ad5 in tissue culture. Therefore, to investigate the involvement of the E1A region in the restricted growth of Ad40, a preliminary characterisation of sequences important for basal transcription (section 4.1) was undertaken.

The E1A promoter sequences of Ad5 (van Ormondt *et al.*, 1978) and Ad40 (van Loon *et al.*, 1987a) had previously been determined, allowing comparison of the Ad5 E1A upstream enhancer and core promoter sequences (Yoshida *et al.*, 1995), with the Ad40 E1A promoter (figure 5.1). To identify possible transcription factor binding sites within the Ad40 E1A promoter, sequences from the ITR (nucleotide 1) to the core promoter (as identified in Ad5 (Hearing and Shenk, 1983a); nucleotide 452) were compared with the transcription factor database held at EMBL (using a single mismatch algorithm; Ghosh, 1990). This revealed a number of possible transcription factor binding sites within the Ad40 E1A promoter (figure 5.1). The known Ad5 E1A core promoter and upstream enhancer sequences (Yoshida *et al.*, 1995) were then compared with the proposed map of the Ad40 E1A promoter (figure 5.1). This revealed a number of potential similarities and differences between the Ad5 and the Ad40 E1A promoters. Sequences within the ITRs were relatively well conserved between the promoters, with Ad40 containing predicted ORPA, NFI, NFIII, two Sp1 and two ATF binding sites conserved in the same relative order as Ad5 (Hatfield and Hearing, 1991). Ad40 was also predicted to map an enhancer region like Ad5, comprising of two distinct elements (Hearing and Shenk, 1986; Bruder and Hearing, 1989, 1991). Two copies of element I or EF-1A binding sites (sites 1 and 2 in figure 5.1), and three EF-1A-like sequences are

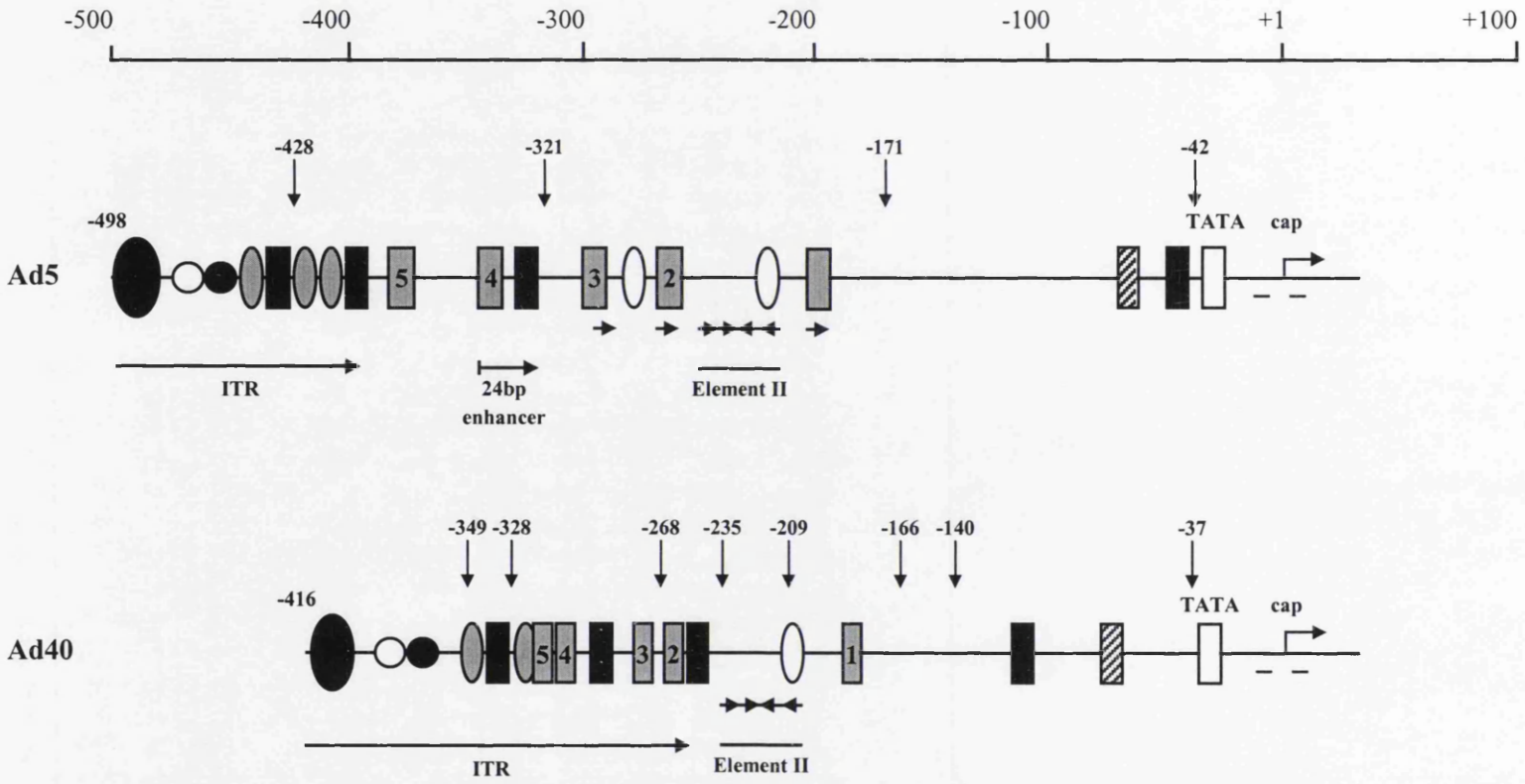


Figure 5.1 Comparison of the intact and deleted Ad5 and Ad40 E1A promoters. Transcription factor binding sites are indicated by: ↓ denotes deletion point; ⬮ denotes ORP-A; ○ denotes NF I; ● denotes NF III; 0 denotes SP1; ■ denotes ATF; ▯ denotes EF1A; ▨ denotes E4F1; 0 denotes E2F; ▯ denotes TATA; → denotes Element I; ⟿ denotes Element II in the Ad5 sequence and an Element II-like sequence in the Ad40 sequence; ⟼ denotes 24bp enhancer; ⌐→ denotes cap site; – – denotes hyphenated symmetry around cap site.

predicted (sites 3, 4 and 5 in figure 5.1). The three EF-1A-like elements were not obvious from initial comparisons with the transcription factor database held at EMBL, therefore the sequence was re-analysed using TRANSFAC (http://transfac.gbf.de; Quandt *et al.*, 1995), which identified three binding sites for Elk 1, Elk 1 and GABP respectively (sites 5, 4 and 3 respectively, figure 5.1), members of the *ets* oncogene family (Wasylyck, 1993).

An element II-like sequence was also identified within the Ad40 E1A promoter, which had 75% homology to the Ad5 element II sequence. One copy of element III (the E2F binding site) and two ATF binding sites are also predicted, although the ATF sites are not conserved in the same relative order between the promoters. The core promoter is well conserved between Ad5 and Ad40, containing a CAAT homology, TATA box and a cap site within a hyphenated symmetry (Hearing and Shenk, 1983a). The CAAT homology strongly resembles the Ad5 E1A CAAT homology (Hearing and Shenk, 1983a). Situated downstream of the CAAT homology lies the Ad40 E1A TATA homology showing one nucleotide difference from the Ad5 TATA homology and four nucleotide difference from the TATA homology (Benoist *et al.*, 1980). This may be significant as mutational analysis of the TATA homology within the adenovirus MLP conferred differences to transcription (Hoopes *et al.*, 1998). A cap site for Ad40 E1A mRNAs was identified at nucleotide position 418 from the left end of the viral genome (Hearing and Shenk, 1983a). The cap site occurs within a hyphenated symmetry which strongly resembles the Ad5 E1A cap sequence (Hearing and Shenk, 1983a).

The intact Ad5 and Ad40 E1A promoters were cloned upstream of a modified firefly luciferase gene then analysed by transient transfection in HeLa cells. The mean and standard deviation were then calculated. The methods used to calculate the mean and standard deviations, for the replicates within each independent experiment, assumed a normal distribution for a large number of replicates. However, it should be noted that only a small number of replicates were actually made and therefore the mean (μ) and standard deviation (δ) obtained are estimated values. In addition, although comparisons within a single experiment are valid, there may be problems in comparing between experiments. For example, measurement of ratios of *Renilla* to firefly luciferase between experiments might show an inherent variability due to differences in the activity or

stability of the different substrates. For this reason, interpretation and discussion of the data focuses upon the major qualitative trends seen within single experiments. In almost all instances (and unless discussed otherwise) the same general trends were consistently observed between experiments. Baring this in mind the analysis of the Ad5 and the Ad40 E1A promoters revealed that basal transcription from the Ad40 E1A promoter was lowered by approximately 6.5 fold when compared with expression from the Ad5 E1A promoter (section 4.1.4), demonstrating that expression from the Ad40 E1A promoter is indeed of intrinsically lower activity when compared to the Ad5 E1A promoter. To map sequences important for basal transcription from the Ad40 E1A promoter, a series of *Bal* 31 deletions were constructed for the Ad5 and the Ad40 E1A promoters.

Transient transfection of the intact and *Bal* 31 deleted Ad40 E1A promoters compared with the equivalent Ad5 E1A promoter constructs revealed an almost identical pattern of expression between the two promoters, albeit at reduced levels from the Ad40 E1A promoter. Interestingly, deletions of sequences within the ITR from -498 to -428 relative to the Ad5 E1A cap site, and from -416 to -349 relative to the Ad40 E1A cap site, resulted in increased expression levels from both promoters. This was unexpected, as previously the Ad5 ITR had demonstrated intrinsic promoter and enhancer activities when the Ad5 ITR was placed adjacent to the E1A TATA box (Hatfield and Hearing, 1991). The simplest model, which would account for the increase in transcription from both the Ad5 and Ad40 E1A promoters, would be that the sequences deleted from each promoter contained a binding site for a transcriptional repressor. However, no such sequences have previously been demonstrated within the Ad5 E1A promoter.

Deletion of sequences from -428 to -321 in the Ad5 E1A promoter resulted in a decrease in expression levels by a factor of 5.5, this was the largest decrease observed within the Ad5 E1A promoter. This observation was expected as the region from -428 to -321 contains two copies of an element I-related sequence (EF-1A sites 4 and 5), deletion of which effects a decrease in Ad5 E1A mRNA levels (Bruder and Hearing, 1991). The region -428 to -321 also contains three Sp1 binding sites, three ATF binding sites and a 24bp enhancer. These sites have been demonstrated to increase transcriptional activity, although it is not clear the extent of the role they play within the context of basal transcription from the E1A promoter (Sassone-Corsi *et al.*, 1983; Jones *et al.*, 1988;

Hatfield and Hearing, 1991). An E2F binding site (also described as element III) is also contained within the deleted region -428 to -321, however this will not be discussed further within this section as basal transcription from the E1A transcriptional control region is unaffected by deletion of either or both copies of element III (Bruder and Hearing, 1989).

Further deletion of sequences from -321 to -171 within the Ad5 E1A promoter resulted in an approximated 3-fold decrease in expression. This region contains two EF-1A binding sites and an EF-1A-related sequence, which demonstrate co-operative binding of EF-1A resulting in synergistic activation of E1A transcription (Bruder and Hearing, 1991). Nested between the two EF-1A elements is a second control region known as element II, which regulates transcription from all early regions within the viral genome (Hearing and Shenk, 1986). Finally, deletion of sequences from -171 to -42, which included the CAAT homology and an ATF binding site, rendered the Ad5 E1A promoter inactive. Previous studies had found that partial deletions within this region had no effect on transcription *in vitro* or *in vivo,* but in this study the deletions were more extensive (Hearing and Shenk, 1983a; Jones *et al.*, 1987).

Transient transfection of the intact and *Bal* 31 deleted Ad40 E1A promoter constructs revealed that apart from the initial increase in transcription which resulted from the deletion of sequences from -416 to -349, the series of deletions from -349 to -37 resulted in decreasing expression levels, which were comparable with the pattern of expression observed within the *Bal* 31 deleted Ad5 E1A promoter. Sequences from -349 to -140 mediated the greatest decrease in expression from the Ad40 E1A promoter. To further map sequences important for basal transcription, a second series of Ad40 E1A promoter deletions were constructed (section 4.1.7).

Transient transfection analysis of the intact and deleted Ad40 E1A promoter revealed a further 1.3 fold increase in expression with the deletion of sequences from -349 to -328, giving an overall increase in expression by a factor of 2.5 with the deletion of sequences from -416 to -328. Further deletion of sequences from -328 to -268 mediated a 5-fold decrease in expression. This region is predicted to contain three EF-1A-related sequences, which would bind alternative members of the *ets* oncogene family known as

Elk 1, Elk 1 and GABP (sites 5, 4 and 3 respectively, figure 5.1). The *ets* family of transcription factors comprises of seven subfamilies including Ets1 and Ets2, Erg, GABP, PEA3, Elk, Elf and PU.1, which show tissue specificity between themselves and the cofactors which regulate their biological function (reviewed in Wasylyk *et al.*, 1993). This suggests a role for transcription factor tissue specificity within the Ad40 E1A promoter. If the EF-1A-related sequences were functional within the Ad40 E1A promoter, deletion of the region -328 to -268 would be expected to mediate a decrease in expression levels as observed within the Ad5 E1A promoter deletions (Bruder and Hearing, 1991).

Deletion of the region -268 to -235 mediated a further 4.6-fold decrease in expression rendering the promoter inactive. This region is predicted to contain an ATF binding site, one copy of element I and an element II-related sequence. The element II-related sequence shows 75% homology to the Ad5 element II, deletion of which mediates a decrease in Ad5 E1A mRNA expression (Hearing and Shenk, 1986). Subsequent deletion of sequences from -235 to -209, -209 to -166, -166 to -140 and -140 to -37, which contained one copy of element I, one copy of element II, an ATF binding site and the CAAT homology, did not affect basal transcription from the Ad40 E1A promoter. The deletion construct containing only the Ad40 E1A TATA box was unable to support basal transcription. Therefore it was concluded that the Ad40 E1A enhancer region maps to sequences within -328 to -235 relative to the Ad40 E1A cap site at +1. Alternative mechanisms for basal transcription from the Ad40 E1A promoter *in vivo* can not be ruled out, as sequence comparison of the Ad40 E1A promoter with TRANSFAC (Quandt *et al.*, 1995) revealed that the Ad40 E1A promoter contains motifs related to the binding sites of gut-specific transcription factors from the HNF and HFH families within the region -209 to -37. It is therefore possible that Ad40 has evolved to utilise gut specific transcription factors, and therefore it would be interesting to analyse basal transcription from the intact and deleted promoter constructs in the context of gut biopsy samples.

5.2 Preliminary characterisation of the Ad40 E1A cDNA map

To characterise *trans*-activated expression from the Ad40 E1A promoter an expression plasmid containing the Ad40 E1A equivalent 13S protein coding sequence was constructed. The 12S mRNA protein coding sequences were not isolated from Ad40 infected 293 cells blocked with ara C. Ara C blocked DNA replication therefore allowing only early viral proteins to accumulate. It is therefore possible that the Ad40 E1A 221R protein is only present at late times in infection. Previously the 12S mRNA was reported in plasmid transformed cells (van Loon *et al.*, 1987a). A number of other Ad40 E1A specific cDNAs were also characterised from the early stage of Ad40 infected 293 cells. These included the Ad40 cDNA clones one to four, which are described in section 4.2.1. Repeat RT-PCR of Ad40 infected 293 cells revealed that clones one and four were produced during an Ad40 infection. This was surprising as clones one and four appear to encode truncated products or proteins unrelated to the previously characterised E1A region proteins. Furthermore, if these cDNAs arose from aberrantly spliced mRNAs, it would be expected that they might be more common in the heterogeneous nuclear mRNA fraction and not efficiently exported to the cytoplasm.

The failure to find a cDNA expressing the Ad40 12S E1A message and the presence of a family of apparently aberrant E1A mRNAs which express non-functional truncated E1A proteins suggests a further significant reason for the failure of Ad40 to grow well in cultured cells. It is possible that the correct splicing of Ad40 primary transcripts is influenced by factors present in different cell types. Differential splicing of transcripts is a well established regulatory mechanism, so it is possible that Ad40 has evolved to utilise the splicing machinery present in gut cells. If so, it would be of great interest to analyse the pattern of Ad40 E1A mRNA transcripts in biopsy samples of infected human tissue.

5.3 Analysis of *trans*-activation within the Ad40 E1A promoter

The aberrant expression of the E1 region (Takiff *et al.*, 1981; Kidd and Madeley, 1981) had in part been attributed to the delayed expression of the Ad40 E1A mRNAs (Ullah, 1997) and the intrinsically lower activity observed in basal transcription from the Ad40 E1A promoter (section 4.1 and 5.1). Previous studies of the Ad40 E1A protein products had also revealed that the *trans*-activation of the Ad2 E4 promoter by the Ad40 E1A proteins was significantly reduced when compared with *trans*-activation by the Ad5 E1A proteins (van Loon *et al.*, 1987b). The *trans*-activation of the Ad5 E2A and E3 promoters by the Ad40 E1A proteins was also significantly reduced when compared with *trans*-activation by the Ad5 and the Ad12 E1A proteins (Ishino *et al.*, 1988). As the Ad40 E1A proteins are weaker *trans*-activators of other early adenovirus promoters they are also likely to be weaker *trans*-activators of their own promoter, which would contribute to the aberrant expression of the E1 region. To investigate the involvement of the Ad40 E1A proteins in the aberrant expression of Ad40, the Ad40 E1A equivalent 13S protein coding sequences were cloned into a CMV expression plasmid (section 4.2.1.). The Ad40 E1A equivalent 12S protein coding sequences were not isolated from Ad40 infected 293 cells (sections 4.2.1; 4.2.2) therefore, *trans*-activation was only analysed with reference to the Ad40 E1A 249R protein. The Ad5 and the Ad40 E1A promoters were co-transfected with expression vectors encoding either the Ad5 E1A 289R protein or the Ad40 E1A 249R protein.

The Ad5 E1A 289R protein *trans*-activates the Ad5 E1A promoter by a factor of 10.4, whereas the Ad40 E1A 249R protein *trans*-activates the Ad40 E1A promoter by a factor of 1.7. Therefore, *trans*-activation by the Ad40 E1A 249R protein is lowered by a factor of 6.2 when compared to *trans*-activation by the Ad5 E1A 289R protein. Published data revealed that *trans*-activation of the Ad2 E4 promoter by the Ad40 E1A proteins was lowered by an approximated factor of 4.6 when compared to *trans*-activation by the Ad5 E1A proteins (van Loon *et al* 1987b). *Trans*-activation of the Ad5 E2A and E3 promoters by the Ad40 E1A proteins was also lowered when compared to *trans*-activation by the Ad5 E1A proteins. However, this data was not quantified therefore we

can only conclude that the overall reduction in expression was comparable with previously published data (Ishino *et al.*, 1988). The published data are concordant with the data produced in section 4.2, any differences are likely to arise from the differences in E1A proteins produced within the co-transfections i.e. the Ad40 E1A 249R protein *trans*-activated the Ad40 E1A promoter (section 4.2) whereas the Ad40 E1A 249R and 221R proteins *trans*-activated the Ad2 E4 promoter (van Loon *et al*, 1987b).

Interestingly, the Ad5 E1A 289R protein was able to *trans*-activate the Ad40 E1A promoter by a factor of 40, which brought firefly luciferase expression levels from the Ad40 E1A promoter to levels measured within the *trans*-activated Ad5 E1A promoter. This suggests that the Ad40 E1A promoter contains transcription factor binding sites necessary for *trans*-activation by the Ad5 E1A 289R protein. In the light of sequence analysis of the Ad40 E1A promoter, which predicts an E2F binding site, four ATF binding sites and the TATA box this is reasonable. The reciprocal is not true for *trans*-activation of the Ad5 E1A promoter by the Ad40 E1A 249R protein, suggesting that either the Ad40 E1A 249R protein is aberrantly expressed or *trans*-activates via different mechanisms. Section 4.2.3 established that functional Ad40 E1A 249R protein was produced *in vitro* from the CMV expression vector, albeit a weaker *trans*-activator than the equivalent Ad5 E1A 289R protein. In principle there could be a difference between functionality of the Ad40 E1A 249R protein *in vitro* and *in vivo*. This principle was demonstrated by the expression of the Ad12 E1A proteins from bacterial cells, whereby the conditions for protein formation were shown to affect protein function (Grand *et al.*, 1998). Post-translational modification is an established regulatory mechanism of E1A protein function, so it is possible that Ad40 has evolved to utilise the post-translational modification system present in gut cells. If so, it would be of great interest to analyse *trans*-activation of the Ad40 E1A promoter by the 249R protein in biopsy samples of infected human tissue.

To map sequences which are important in *trans*-activation from the Ad40 E1A promoter, cells were co-transfected with the reporter constructs containing intact or deleted Ad5 and Ad40 E1A promoters along with CMV expression vectors containing either the Ad5 E1A 13S protein coding sequences or the Ad40 E1A equivalent 13S protein coding sequences. Table 5.3 illustrates the mean (μ) value obtained for basal

Table 5.3 Basal and *trans*- activation of the Ad5 and the Ad40 E1A promoters.

Sample	Basal expression [a]	*Trans*-activated expression [b]	Fold activation of expression by the Ad5 E1A 289R protein [c]
Ad5 E1A promoter	7.2	97	12.5
Ad5 E1A promoter del.428	12	81	6.7
Ad5 E1A promoter del.321	2.2	13	5.9
Ad5 E1A promoter del.171	0.71	12	16.9
Ad5 E1A promoter del.42	0.06	0.85	14.2
Ad40 E1A promoter	1.2	7.4	6.2
Ad40 E1A promoter del.349	2.3	24	10.4
Ad40 E1A promoter del.328	3.0	24	8
Ad40 E1A promoter del.268	0.6	15	25.0
Ad40 E1A promoter del.235	0.13	0.91	7.0
Ad40 E1A promoter del.209	0.08	0.39	4.9
Ad40 E1A promoter del.166	0.10	0.55	5.5
Ad40 E1A promoter del.140	0.10	0.50	5.0
Ad40 E1A promoter del.37	0.08	0.48	6.0

[a] Basal expression denotes the mean (μ) calculated in Table 4.1.6 and Table 4.1.8

[b] *Trans*-activated expression denotes the mean (μ) calculated in Table 4.2.4

[c] Fold activation of expression by the Ad5 E1A 289R protein was calculated by dividing the *trans*-activated expression (column 3) by the basal expression (column 2).

expression from the intact and deleted Ad5 and Ad40 E1A promoters (see column 2 Table 5.3); the mean (μ) value obtained for *trans*-activated expression from the intact and deleted Ad5 and Ad40 E1A promoters by the Ad5 E1A 289R protein (see column 3 Table 5.3); and the ratio of *trans*-activated to basal activity (column 4 Table 5.3). *Trans*-activated expression of the Ad5 and Ad40 E1A promoters by the Ad40 E1A 249R protein are not discussed, due to an equipment (incubator) problem which rendered the experimental data unreliable.

Previously the Ad5 E1A 289R protein was shown to *trans*-activate the Ad40 E1A promoter to the same level as the Ad5 E1A promoter (Table 4.2.3.2; Figure 4.2.3.2) however, this was not the case within this experiment (Table 5.3). This may have been related to the equipment problem mentioned above and given more time it would have been beneficial to repeat these experiments

Deletions within the Ad5 and the Ad40 E1A promoter demonstrated similar patterns of expression. Deletion of sequences from -498 to -428, -428 to -321, -321 to -171 and -171 to -42 relative to the Ad5 E1A cap site resulted in a sequential decrease in *trans*-activated expression from the Ad5 E1A promoter. The largest decreases in *trans*-activated expression from the Ad5 E1A promoter were effected by sequences contained within the regions -428 to -321 and -171 to -42 relative to the Ad5 E1A cap site, which contained two copies of ATF and Sp1 transcription factor binding sites, and a single copy of the ATF transcription factor binding site respectively. Previously *trans*-activation by the Ad5 E1A 289R protein was demonstrated to involve both ATF and Sp1 (reviewed in Flint and Shenk, 1997; see section 1.2). It was therefore not surprising that deletion of sequences corresponding to Sp1 and ATF transcription factor binding sites would cause a decrease in *trans*-activated expression from the Ad5 E1A promoter. Deletion of sequences from -321 to -171 had little effect on *trans*-activated expression, which was surprising as this region contains two copies of the E2F transcription factor binding site, which is known to be involved in *trans*-activation by the Ad5 E1A 289R protein (reviewed in Flint and Shenk, 1997). Finally, expression from the Ad5 E1A promoter construct containing the TATA box alone (del.42) was not abolished, expression probably resulting from *trans*-activation of the TATA box through TBP and/or through a number of TAFs which are components of a larger

complex known as TFIID (reviewed in section 1.2).

Deletion of sequences within the Ad40 E1A promoter demonstrated a similar pattern of expression to that observed within the Ad5 E1A promoter i.e. deletion of sequences from -349 to -328, -328 to -268, -268 to -235, -235 to -209, -209 to -166, -166 to -140 and -140 to -37 relative to the Ad40 E1A cap site resulted in a sequential decrease in *trans*-activated expression from the Ad40 E1A promoter. There was however one difference to the pattern of expression observed within the deletion analysis of the Ad40 E1A promoter, namely deletion of sequences from -416 to -349 relative to the Ad40 E1A cap site effected an increase in *trans*-activated levels of expression from the Ad40 E1A promoter. This result should, however, be interpreted with caution since previously the Ad5 E1A 289R protein had *trans*-activated the Ad40 E1A promoter to the same level as the *trans*-activated Ad5 E1A promoter (section 4.2.3). It is therefore possible that the *trans*-activated level of expression observed with the intact Ad40 E1A promoter in this experiment is an aberrantly low value, possibly associated with the equipment problems. In order to confirm whether this is the case the experiment needs to be repeated.

The largest decrease in *trans*-activated expression from the Ad40 E1A promoter was effected by sequences contained within the region -268 to -235, which are predicted to contain a single copy of the ATF transcription factor binding site. Deletion of sequences from -328 to -209, which are predicted to contain two ATF and one Sp1 transcription factor binding sites, reduced *trans*-activated expression to the level observed from the Ad40 E1A TATA box deletion construct. Sequences contained within the region -209 to -37 did not appear to be important for *trans*-activation by the Ad5 E1A 289R protein, suggesting that the predicted ATF and E2F transcription factor binding sites are not important. Finally the del.37 construct was *trans*-activated by the Ad5 E1A 289R protein presumably through the TATA box (reviewed in section 1.2). From comparisons of the ratios of *trans*-activated to basal expression for both the Ad5 and Ad40 promoter constructs (table 5.3) no single sequence element can be identified which is uniquely important for *trans*-activation. Rather, those elements which affect the level of basal expression seem also to have an effect on the level of *trans*-activated expression observed in the presence of the Ad5 E1A 289R protein. Thus the Ad5 E1A

289R protein probably activates gene expression through an effect on the basal transcription apparatus. It would be interesting to analyse *trans*-activated expression of the Ad40 E1A promoter constructs by the Ad40 E1A 249R protein in WS HeLa cells and biopsy samples of gut tissue to map sequences important for *trans*-activation and to allow comparison with the Ad5 E1A 289R protein.

In summary, the work in this thesis suggests that, in addition to the previously observed defects in E1B region function, the failure of Ad40 to propagate in HeLa and other similar cultured cell lines can in part be attributed to: (i) intrinsically poor activity of the Ad40 E1A promoter region; (ii) apparently aberrant splicing of E1A mRNAs; (iii) intrinsically low activity of the Ad40 major E1A *trans*-activator protein encoded by the 13S E1A mRNA. Each of these factors may be modulated by the environment in different cell types, and future studies could seek to determine differences in E1A promoter- and E1A protein-binding factors between cultured cells and gut tissue.

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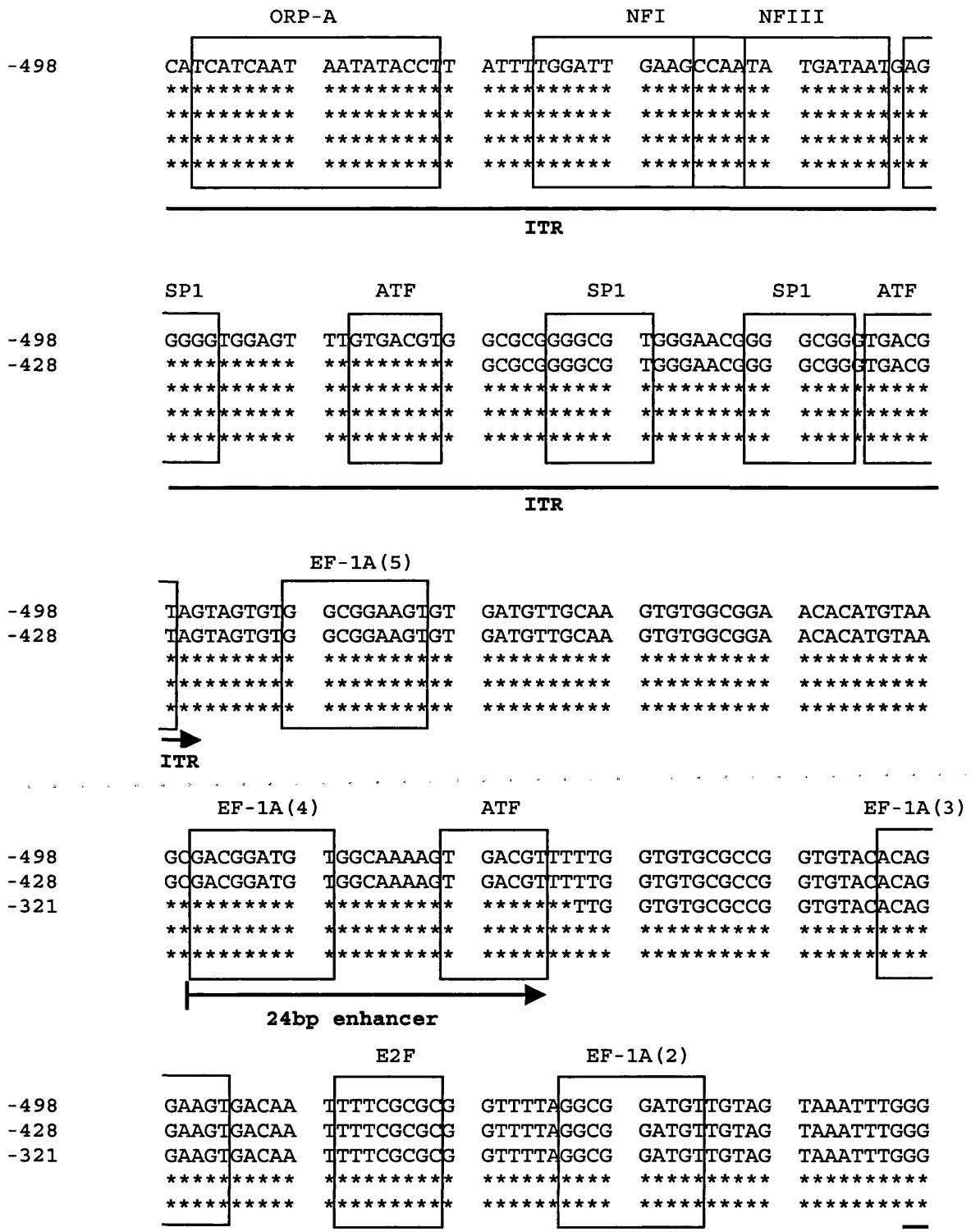
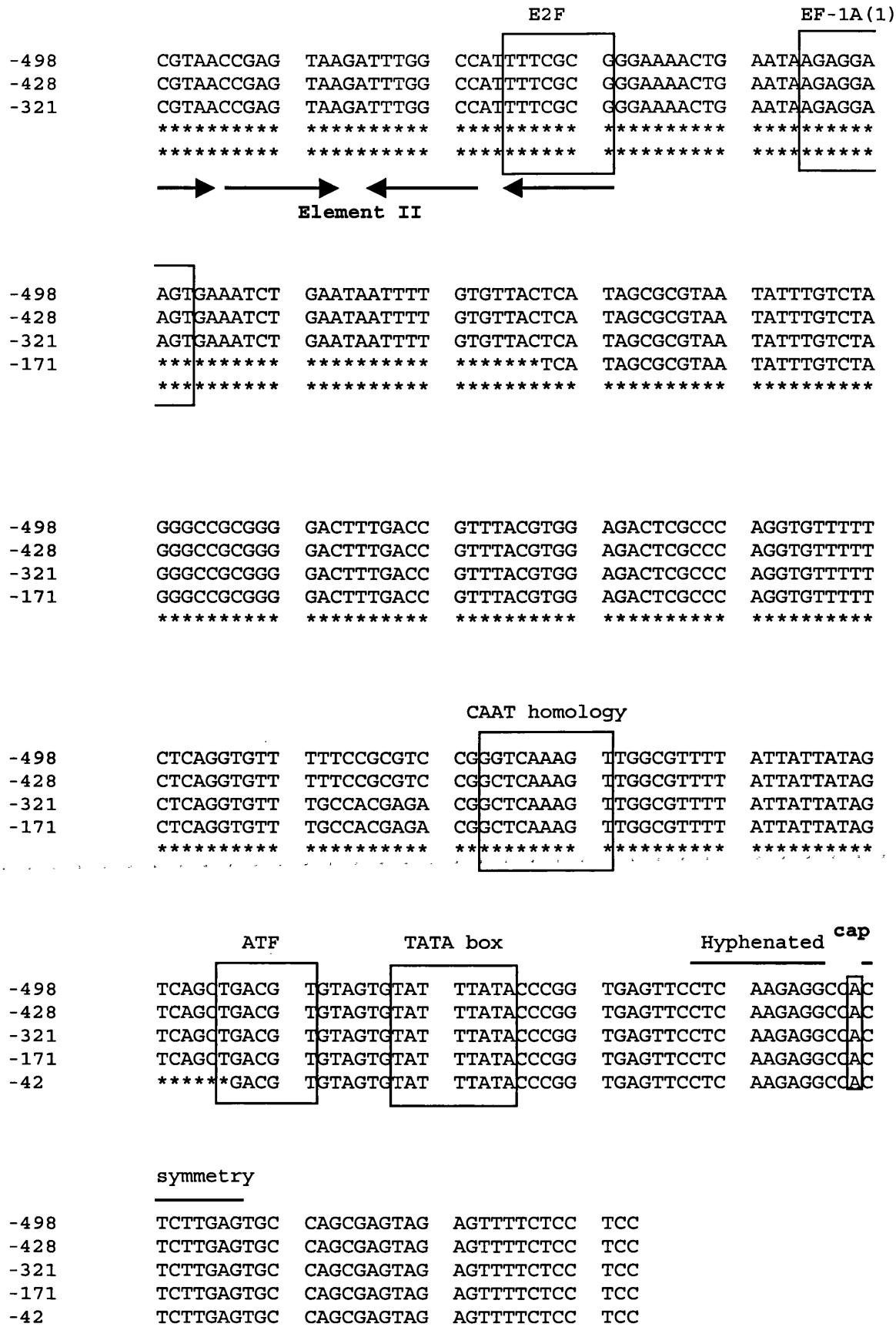
APPENDIX 1

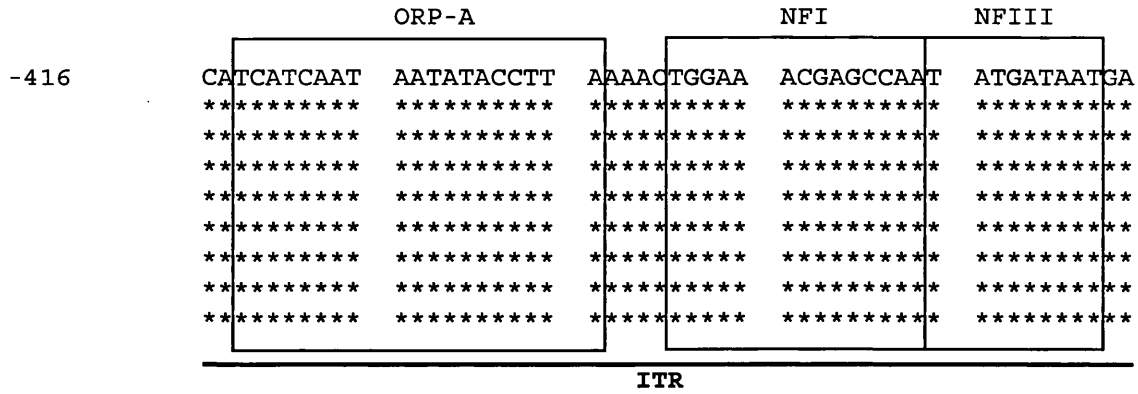
Appendix 1 - Sequence of the intact and deleted Ad5 E1A promoters

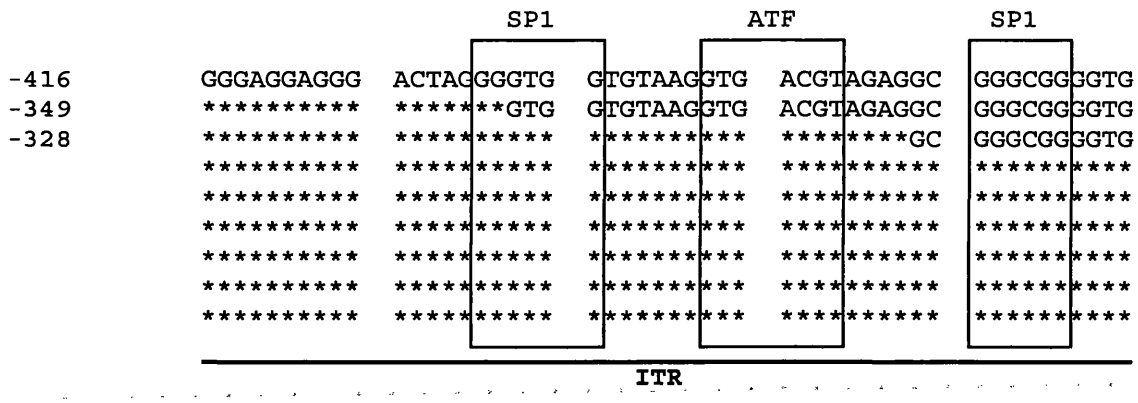


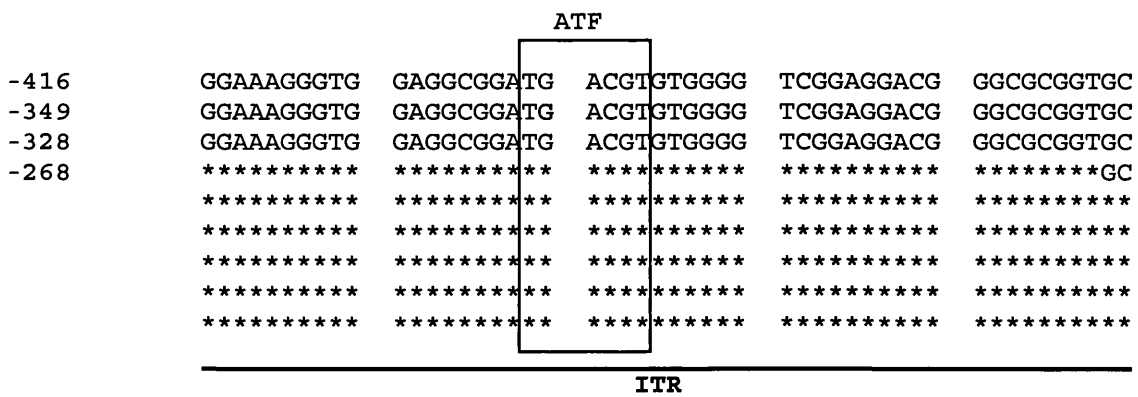

E2F
EF-1A(1)
-498 CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAAACTG AATAAGAGGA
-428 CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAAACTG AATAAGAGGA
-321 CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAAACTG AATAAGAGGA
Element II
-498 AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA
-428 AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA
-321 AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA
-171 TCA TAGCGCGTAA TATTTGTCTA
-498 GGGCCGCGGG GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT
-428 GGGCCGCGGG GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT
-321 GGGCCGCGGG GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT
-171 GGGCCGCGGG GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT
CAAT homology
-498 CTCAGGTGTT TTTCCGCGTC CGGGTCAAAG TTGGCGTTTT ATTATTATAG
-428 CTCAGGTGTT TTTCCGCGTC CGGCTCAAAG TTGGCGTTTT ATTATTATAG
-321 CTCAGGTGTT TGCCACGAGA CGGCTCAAAG TTGGCGTTTT ATTATTATAG
-171 CTCAGGTGTT TGCCACGAGA CGGCTCAAAG TTGGCGTTTT ATTATTATAG
ATF
TATA box
Hyphenated
cap
-498 TCAGCTGACG TGTAGTGTAT TTATACCCGG TGAGTTCCTC AAGAGGCCAC
-428 TCAGCTGACG TGTAGTGTAT TTATACCCGG TGAGTTCCTC AAGAGGCCAC
-321 TCAGCTGACG TGTAGTGTAT TTATACCCGG TGAGTTCCTC AAGAGGCCAC
-171 TCAGCTGACG TGTAGTGTAT TTATACCCGG TGAGTTCCTC AAGAGGCCAC
-42 GACG TGTAGTGTAT TTATACCCGG TGAGTTCCTC AAGAGGCCAC
symmetry
-498 TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCC
-428 TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCC
-321 TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCC
-171 TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCC
-42 TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCC

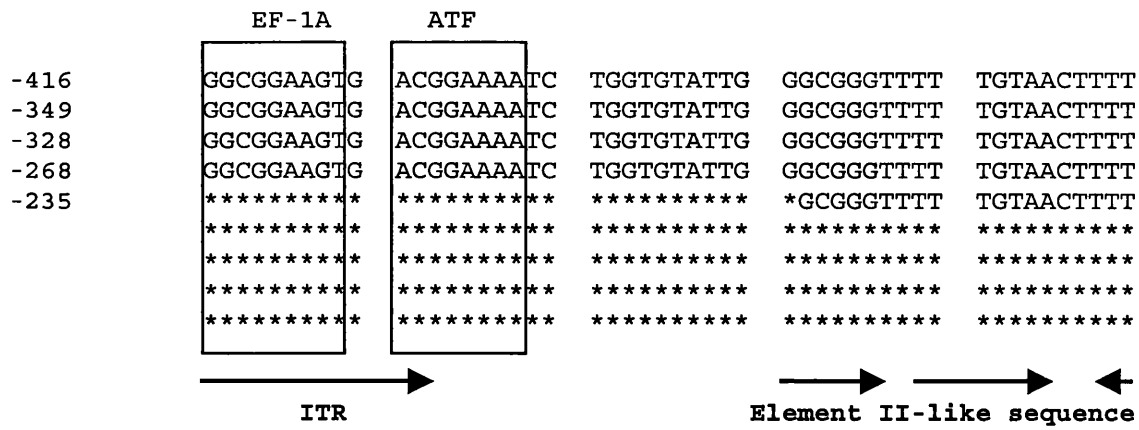
APPENDIX 2

Appendix 2 - Sequence of the intact and deleted Ad40 E1A promoters








EF-1A
ATF
-416 GGCGGAAGTG ACGGAAAATC TGGTGTATTG GGCGGGTTTT TGTAACTTTT
-349 GGCGGAAGTG ACGGAAAATC TGGTGTATTG GGCGGGTTTT TGTAACTTTT
-328 GGCGGAAGTG ACGGAAAATC TGGTGTATTG GGCGGGTTTT TGTAACTTTT
-268 GGCGGAAGTG ACGGAAAATC TGGTGTATTG GGCGGGTTTT TGTAACTTTT
-235 ********** ********** ********** *GCGGGTTTT TGTAACTTTT
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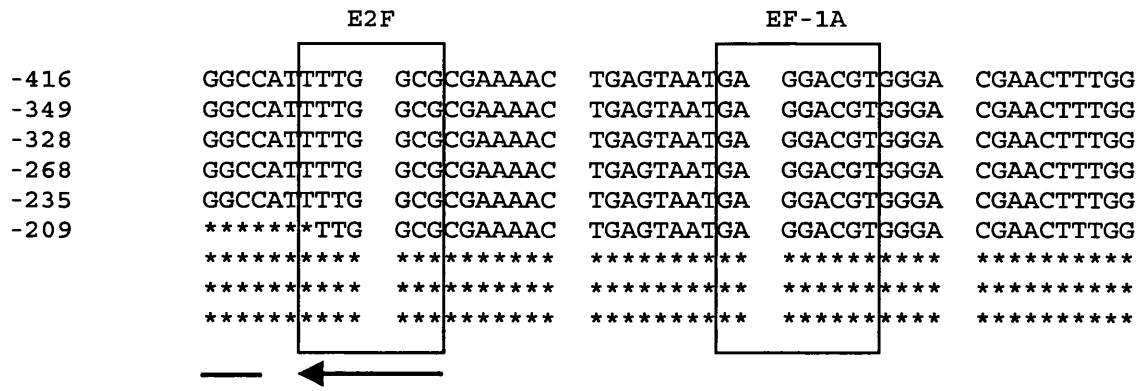
ITR
Element II-like sequence


E2F
EF-1A
-416 GGCCATTTTG GCGCGAAAAC TGAGTAATGA GGACGTGGGA CGAACTTTGG
-349 GGCCATTTTG GCGCGAAAAC TGAGTAATGA GGACGTGGGA CGAACTTTGG
-328 GGCCATTTTG GCGCGAAAAC TGAGTAATGA GGACGTGGGA CGAACTTTGG
-268 GGCCATTTTG GCGCGAAAAC TGAGTAATGA GGACGTGGGA CGAACTTTGG
-235 GGCCATTTTG GCGCGAAAAC TGAGTAATGA GGACGTGGGA CGAACTTTGG
-209 *******TTG GCGCGAAAAC TGAGTAATGA GGACGTGGGA CGAACTTTGG
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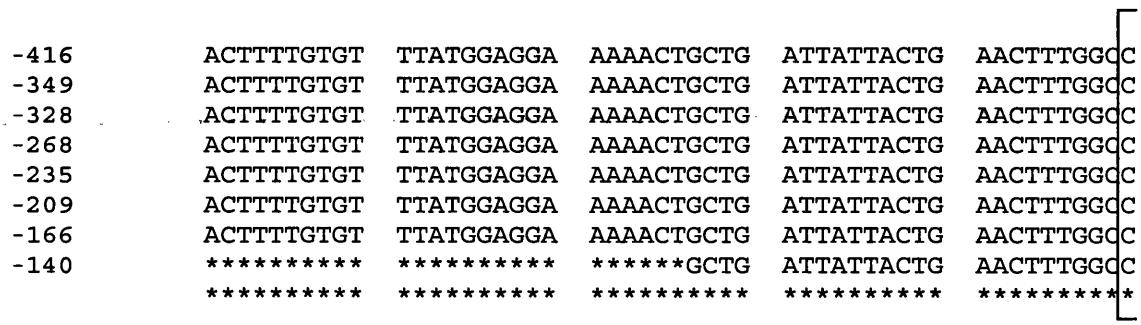
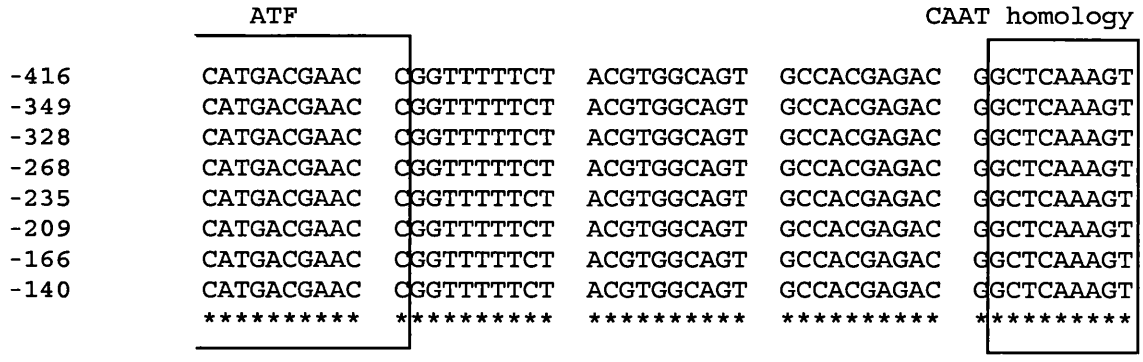

-416 ACTTTTGTGT TTATGGAGGA AAAACTGCTG ATTATTACTG AACTTTGGCC
-349 ACTTTTGTGT TTATGGAGGA AAAACTGCTG ATTATTACTG AACTTTGGCC
-328 ACTTTTGTGT TTATGGAGGA AAAACTGCTG ATTATTACTG AACTTTGGCC
-268 ACTTTTGTGT TTATGGAGGA AAAACTGCTG ATTATTACTG AACTTTGGCC
-235 ACTTTTGTGT TTATGGAGGA AAAACTGCTG ATTATTACTG AACTTTGGCC
-209 ACTTTTGTGT TTATGGAGGA AAAACTGCTG ATTATTACTG AACTTTGGCC
-166 ACTTTTGTGT TTATGGAGGA AAAACTGCTG ATTATTACTG AACTTTGGCC
-140 ********** ********** ******GCTG ATTATTACTG AACTTTGGCC
********** ********** ********** ********** **********


ATF
CAAT homology
-416 CATGACGAAC CGGTTTTTCT ACGTGGCAGT GCCACGAGAC GGCTCAAAGT
-349 CATGACGAAC CGGTTTTTCT ACGTGGCAGT GCCACGAGAC GGCTCAAAGT
-328 CATGACGAAC CGGTTTTTCT ACGTGGCAGT GCCACGAGAC GGCTCAAAGT
-268 CATGACGAAC CGGTTTTTCT ACGTGGCAGT GCCACGAGAC GGCTCAAAGT
-235 CATGACGAAC CGGTTTTTCT ACGTGGCAGT GCCACGAGAC GGCTCAAAGT
-209 CATGACGAAC CGGTTTTTCT ACGTGGCAGT GCCACGAGAC GGCTCAAAGT
-166 CATGACGAAC CGGTTTTTCT ACGTGGCAGT GCCACGAGAC GGCTCAAAGT
-140 CATGACGAAC CGGTTTTTCT ACGTGGCAGT GCCACGAGAC GGCTCAAAGT
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TATA box

-416	CCTAATTTTT	TATTGTGTGC	TCAGCCCGTT	TGAGGCTATT	TAAACACAGC
-349	CCTAATTTTT	TATTGTGTGC	TCAGCCCGTT	TGAGGCTATT	TAAACACAGC
-328	CCTAATTTTT	TATTGTGTGC	TCAGCCCGTT	TGAGGCTATT	TAAACACAGC
-268	CCTAATTTTT	TATTGTGTGC	TCAGCCCGTT	TGAGGCTATT	TAAACACAGC
-235	CCTAATTTTT	TATTGTGTGC	TCAGCCCGTT	TGAGGCTATT	TAAACACAGC
-209	CCTAATTTTT	TATTGTGTGC	TCAGCCCGTT	TGAGGCTATT	TAAACACAGC
-166	CCTAATTTTT	TATTGTGTGC	TCAGCCCGTT	TGAGGCTATT	TAAACACAGC
-140	CCTAATTTTT	TATTGTGTGC	TCAGCCCGTT	TGAGGCTATT	TAAACACAGC
-37	**********	**********	*********T	TGAGGCTATT	TAAACACAGC

cap

Hyphenated symmetry

-416	CAGAACATCA	AGAGGCCACT	CTTGAGTGCG	AGCGAGTAGA	GTTTTCTCCT
-349	CAGAACATCA	AGAGGCCACT	CTTGAGTGCG	AGCGAGTAGA	GTTTTCTCCT
-328	CAGAACATCA	AGAGGCCACT	CTTGAGTGCG	AGCGAGTAGA	GTTTTCTCCT
-268	CAGAACATCA	AGAGGCCACT	CTTGAGTGCG	AGCGAGTAGA	GTTTTCTCCT
-235	CAGAACATCA	AGAGGCCACT	CTTGAGTGCG	AGCGAGTAGA	GTTTTCTCCT
-209	CAGAACATCA	AGAGGCCACT	CTTGAGTGCG	AGCGAGTAGA	GTTTTCTCCT
-166	CAGAACATCA	AGAGGCCACT	CTTGAGTGCG	AGCGAGTAGA	GTTTTCTCCT
-140	CAGAACATCA	AGAGGCCACT	CTTGAGTGCG	AGCGAGTAGA	GTTTTCTCCT
-37	CAGAACATCA	AGAGGCCACT	CTTGAGTGCG	AGCGAGTAGA	GTTTTCTCCT

-416	CC
-349	CC
-328	CC
-268	CC
-235	CC
-209	CC
-166	CC
-140	CC
-37	CC