Adenovirus type 40 host range in tissue culture: a study of the E1B region.

BY

Valgerdur Steinthorsdottir

A Thesis Presented for the Degree of Doctor of Philosophy
in the
Faculty of Science, University of Glasgow

Institute of Virology
University of Glasgow

April, 1991.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I am grateful to Professor J.H. Subak-Sharpe for providing research facilities in the Institute of Virology. I would also like to thank my project supervisor Dr. Vivien Mautner for her support and patience during the course of this study and for critical reading of this thesis.

Several members of the Institute have taken time to assist me in various ways, their contribution is gratefully acknowledged. In particular, I would like to thank Nancy Mackay for her invaluable friendship during my time in Lab 304 and beyond. I am also grateful to the crowd that arrived at the Institute on the 1st of October 1987, their companionship through the years has been much appreciated.

A number of people have contributed to making life outside work an enjoyable experience during my stay in Glasgow as well as providing moral support whenever needed. These include amongst others my flatmates/friends Ruth, Janice, Trevor and Ilene, as well as Mark and Jo who introduced me to the Scottish hills.

The friendship of Hrafnhildur Broddadottir and efficiency in dealing with LIN on my behalf is greatly valued.

Finally I would like to thank all my family for being a constant source of support and encouragement in every possible way.

The author was the recipient of an ORS award during the latter two years of this study. Unless otherwise stated, all the results presented in this thesis were obtained by the author's own efforts.

SUMMARY

Growth restriction in tissue culture is a distinct feature of the enteric adenoviruses and they differ in this respect from all other subgroups of human adenoviruses. Ad40 cannot be passaged on Hela cells or primary HEK cells, but will grow in cells expressing the E1 region or only the E1B region of Ad2 or Ad5. The work described here was aimed at understanding the basis for this growth restriction.

In coinfection experiments Ad40 complements an Ad5 E1A mutant and is itself complemented by that mutant as well as Ad5 and Ad12 wild type and E1B 19K mutant viruses. Ad5 and Ad12 E1B 55K mutant viruses are complemented to some extent by Ad40, these mutants however do not complement Ad40 growth on Hela cells.

In order to study the E1B region of Ad40 a transcription map was determined from RNA produced at late times in infected KBa+b cells, using S1 nuclease, primer extension and PCR-cDNA analysis as well as Northern blotting. E1B transcripts corresponding to Ad2 14S, 22S and 9S mRNAs were identified but no 13S mRNA equivalent was detected, a pattern similar to that seen in the Ad12 transcription map. The coding potential for E1B 19K, 55K, and 15K proteins and for ppIX is retained in the Ad40 transcripts. In addition novel E1A-E1B cotranscript counterparts of the 14S and 22S mRNAs were identified. These contain the first 40 codons of the E1A first exon linked to a site 4-5nt downstream of the E1B cap site, retaining all the coding potential of the E1B mRNAs. No new open reading frames are created by the junction, and the E1A ORF terminates with one codon added after the junction. The E1A-E1B junction is unusual in that it does not conform to splice consensus sequences and thus may not be generated by a conventional splicing mechanism. Sequences around the 5' site of the junction bear a certain resemblance to spliced leader sequences utilized in *trans*-splicing in trypanosomes and nematodes.

A timecourse of E1 transcription and DNA replication confirmed that E1B transcripts are not detectable at early times in infection, but appear around the onset of DNA

replication. The E1A-E1B cotranscripts are first seen at the same time as transcripts from the E1B promoter.

Transcription from the Ad40 E1B promoter was analysed in transient transfection assays. Chloramphenicol acetyl transferase (CAT) activity was measured in cell extracts where the CAT gene was expressed under the control of the E1B promoter in the presence or absence of E1A products of Ad5 or Ad40. Expression from the Ad40 E1B promoter was not detectable even in the presence of Ad5 E1A, suggesting that unlike its Ad5 counterpart the Ad40 E1B promoter does not respond to E1A transactivation. This would account for the lack of early E1B gene expression in tissue culture. Moreover, this suggests that in the natural host cells of the virus E1B early gene products may not be needed, or alternatively, that early expression from the Ad40 E1B promoter is dependent on cellular factors present in the natural host cell, but absent in most common tissue culture cell lines.

In this study Ad40 was shown to grow in an intestinal cell line, Int407, almost as well as in KB cells expressing the Ad2 E1 region. This provides a system in which to study Ad40 growth in tissue culture in the absence of complementing viral gene products.

ABBREVIATIONS

A	adenine
Ad	adenovirus
ara C	cytosine arabinoside
ATP	adenosine-5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
C	cytosine
^{14}C	Carbon-14 radioisotope
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CAT	chloramphenicol acetyl transferase
Ci	Curie(s)
CIP	calf intestinal phosphatase
cpe	cytopathic effect
cpm	counts per minute
Da	Daltons
DBP	DNA-binding protein
dATP	2-deoxyadenosine-5'-triphosphate
dCTP	2-deoxycytidine-5'-triphosphate
dGTP	2-deoxyguanosine-5'-triphosphate
dTTP	2-deoxythymidine-5'-triphosphate
dNTP	2-deoxyribonucleoside-5'-triphosphate
ddNTP	2,3-dideoxyribonucleoside-5'-triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
E	early (gene)
E.coli	*Escherichia coli*
EDTA	sodium ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
FCS	foetal calf serum
G	guanine
g	gram(s)
hr	hour(s)
HSV	herpes simplex virus
IE	immediate early

ITR	inverted terminal repeat
k	kilo
K	1000-dalton molecular weight
kb	kilobase
l	litre
L	late (gene)
M	molar
min	minute(s)
ml	millilitre
MLP	major late promoter
mm	millimetre
mM	millimolar
moi	multiplicity of infection
mol	moles
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
N	unspecified nucleotide
n	nano
ng	nanogram
NP40	Nonidet P40
nt	nucleotide
OD	optical density
ORF	open reading frame
^{32}p	phosphorus-32 radioisotope
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
poly A	polyadenylic acid
PrV	pseudorabies virus
pTP	precursor terminal protein
R	purine moiety
RB	retinoblastoma gene product
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
sec	seconds

SV40	simian virus 40
T	thymine
TEMED	n,n,n'n'-tetramethylethylene diamine
Tris	tris(hydroxymethyl)aminoethane
tRNA	transfer ribonucleic acid
UV	ultraviolet
V	volt
v	volume
vol	volume
VZV	varicella zoster virus
w	weight
W	watts
wt	wild type
Y	pyrimidine moiety
µ	micro

1. INTRODUCTION

The work presented in this thesis concerns the host range properties of enteric adenovirus 40 in tissue culture. The growth restriction in cultivated cells is a distinct feature of the enteric adenoviruses (de Jong *et al.*, 1983) and they differ in this respect from all other subgroups of human adenoviruses. Early region 1 of the enteric adenoviruses has been implicated in this host range phenotype since the enteric adenoviruses grow in 293 cells, a cell line transformed with the E1 region of Ad5 (Takiff *et al.*, 1981). The aim of this introduction is to give a brief overview of the Adenoviridae in terms of morphology and the lytic cycle, describe the structure and functions of early regions 1A and 1B in some detail and finally to review current knowledge of the enteric adenoviruses. For general reviews of the adenoviruses see Ginsberg, (1984); Doerfler, (1986).

1.1. The Adenoviridae

The Adenoviridae family has two genera, Mastadenovirus and Aviadenovirus, encompassing adenoviruses occurring in mammals and birds respectively. Over 90 serotypes of Mastadenoviruses have been identified, of which 42 are human serotypes. The first human adenoviruses were discovered by Rowe *et al.* (1953) and Hilleman and Werner (1954) and are now classified into six subgroups, A-F (see table 1), based on immunological, biological, morphological and biochemical criteria. They cause a number of diseases in humans, including respiratory, ocular, urinary and gastrointestinal diseases, and are important human pathogens, although they are not often associated with fatal diseases (White and Fenner, 1986; Horwitz, 1990). As well as being studied as pathogenic agents, much of adenovirus research has focussed on their use as a tool to study eucaryotic molecular biology. A great deal of information has been collected by the use of adenovirus mutants; for an extensive review of adenovirus genetics see Williams, (1986). The complete DNA

Table I: Properties of human adenovirus serotypes of subgroups A-F (modified from Wadell et al., 1987).

Sub-group	Sero-type	DNA homology % Intra-subgroup	DNA homology % Inter-subgroup	G+C (%)	Length of fibres (nm)	Oncogenicity in newborn hamsters	Tropism/ symptoms
A	12, 18, 31	48-69	8-20	48	28-31	High (tumours in most animals in 4 months)	Cryptic enteric infection
B	3, 7, 11, 14, 16, 21, 34, 35	89-94	9-20	51	9-11	Weak (tumours in a few animals in 14-18 months)	Respiratory disease Persistent infection of the kidney
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	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	52	28-33	nil	Infantile diarrhoea

sequence of Ad2 has been determined (Roberts, R.J. *et al.*, 1986) and this serotype and the closely related Ad5 have been most extensively studied. Much of our current understanding of adenoviruses is therefore based on work involving those two serotypes and this is reflected in the following sections.

1.1.1. Morphology

Adenoviruses are non-enveloped icosahedral particles containing linear double stranded DNA of 20-30x10^6 dalton, and several virus encoded polypeptides. The capsid consists of 252 capsomers (Horne *et al.*, 1959). Pentons lie at the 12 vertices and 240 hexons form the facets (Ginsberg *et al.*, 1966).

Hexons contain 3 identical polypeptide chains (protein II) (Grütter and Franklin, 1974; Akusjärvi *et al.*, 1984). The three-dimensional structure of the Ad2 hexon has been determined by X-ray crystallography (Roberts, M.M. *et al.*, 1986); the hexon trimer has a triangular top superimposed on a pseudohexagonal base. Hexons carry type, group, intrasubgroup and intersubgroup antigenic determinants (Norrby, 1969; Norrby and Wadell, 1969). Type-specific antigen determinants have been demonstrated on the virion surface (Norrby, 1969; Willcox and Mautner, 1976) while most group-specific determinants appear to be internal.

Pentons consist of penton base and filamentous projections, the fibres; the penton base is probably a pentamer of protein III and the fibre a trimer of protein IV (van Oostrum and Burnett, 1985), although alternative structures have been suggested (Devaux *et al.*, 1982, 1984; Green *et al.*, 1983). Recent crystallography studies have confirmed the trimeric structure of the fibre and provided further details of the structure (Devaux *et al.*, 1990; Ruigrok *et al.*, 1990). Most Aviadenoviruses have two fibres per penton base while Mastadenoviruses have one; a possible exception is human Ad41 which contains two fibre genes (Pieniazek, N.J. *et al.*, 1990), although it is not yet known if both are

expressed. The fibre contains the sites responsible for interaction of the virus with cellular receptors (Levine and Ginsberg, 1967; Philipson, 1967). It carries type specific antigenic determinants and depending on fibre length can also carry subgroup- and intersubgroup-specific determinants (Pettersson *et al.*, 1968; Pettersson and Höglund, 1969; Norrby, 1968, 1969; Wadell and Norrby, 1969).

In addition to the hexons and pentons, protein IIIa and ppIX also form a part of the outer capsid; protein IIIa has been assigned to the vertex region (Everitt *et al.*, 1973) whereas ppIX is associated with hexons and has a stabilizing effect on the capsid (Colby and Shenk, 1981; Furcinitti *et al.*, 1989). ppIX is essential for the packaging of full length genomes since it is dispensable only for virions containing DNA of less than genomic size (Ghosh-Choudhury *et al.*, 1987).

The core consists of viral DNA tightly bound to protein VII, in association with proteins V, VI and μ (Russell and Precious, 1982). Protein V is also associated with hexon, penton and protein IIIa (Everitt *et al.*, 1975), and Nermut (1979), has suggested that the capsid is lined by a shell of protein V. In addition proteins VI and VIII are associated with hexons (Everitt *et al.*, 1975). Details of the arrangement of DNA and the core proteins within the native nucleoprotein core are not well understood although several models have been discussed. Brown *et al.*, (1975) proposed a structure of 12 large beads associated with the vertices of the icosahedral capsid; this model is supported by ion etching studies (Newcombe *et al.*, 1984). A second model predicts nucleosome-like winding of DNA around protein VII (Corden *et al.*, 1976; Nermut, 1980; Vayda *et al.*, 1983; Chatterjee *et al.*, 1986). Recently Wong and Hsu (1989) have put forward a loop-domain model where the viral DNA is assumed to be organized into eight supercoiled loops and these loops are anchored to the centre of the virus core. This model is consistent with the results of Brown *et al.*, (1975) and Newcombe *et al.*, (1984).

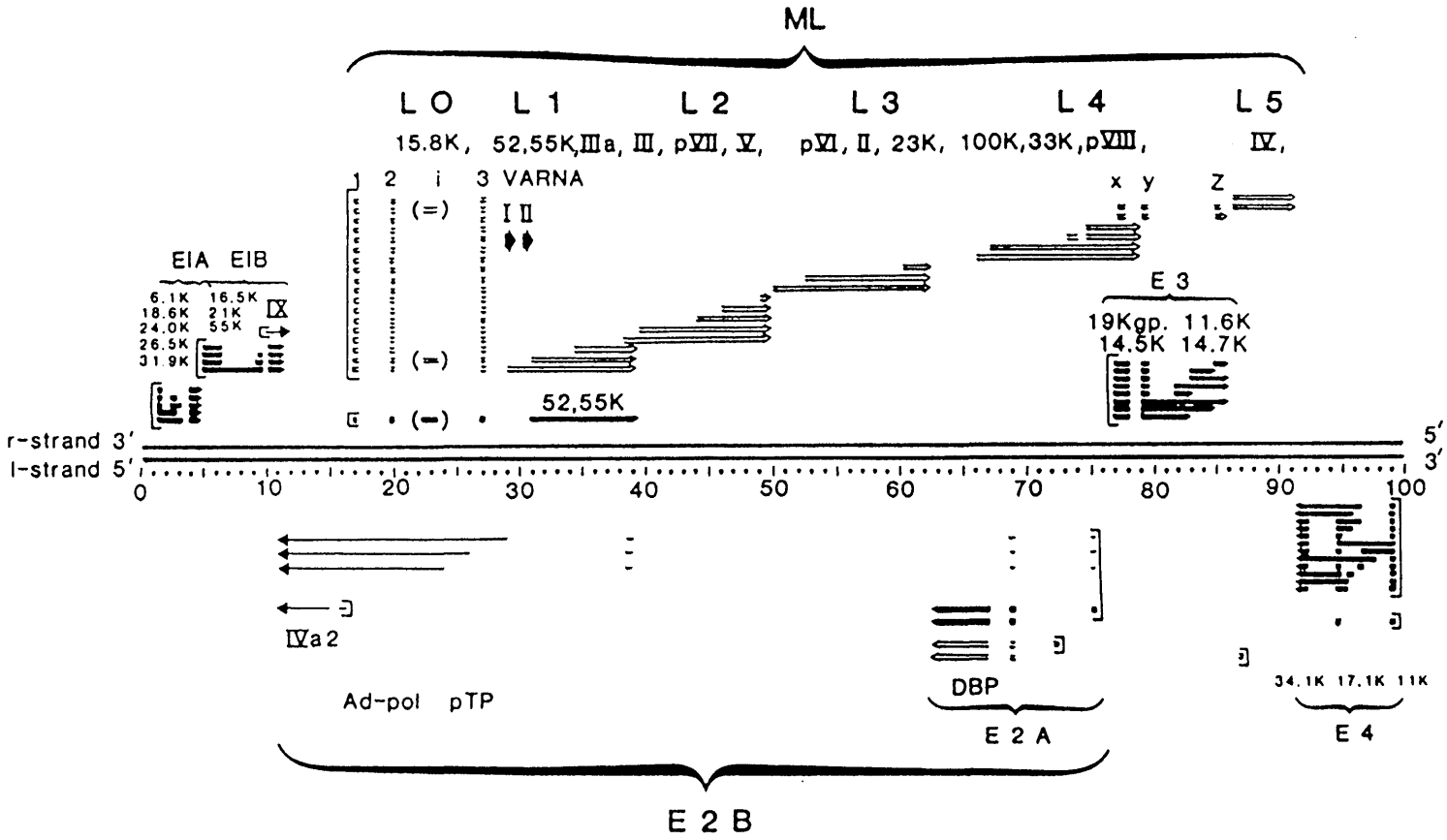
1.1.2. Genome structure

The adenovirus genome is a double stranded linear DNA molecule. A protein of 55Kd molecular weight, terminal protein, is covalently linked to the 5' end of each strand via a serine residue (Robinson et al., 1973; Rekosh *et al.*, 1977; Desideiro and Kelly, 1981). The ends of the genome contain an inverted terminal repetition (ITR) (Garon *et al.*, 1972; Wolfson and Dressler, 1972). The length of the ITR is characteristic of each serotype but several regions within it have been shown to be well conserved. A 10 bp sequence (nt 9-18) is conserved in all serotypes and is essential for initiation of DNA replication (Tamanoi and Stillman, 1983; van Bergen *et al.*, 1983; Challberg and Rawlins 1984; Lally *et al.*, 1984; Harris and Hay, 1988). It has been suggested that this motif is important for the binding of the virus encoded replication proteins to the origin of replication (Rijnders *et al.*, 1983; Kenny and Hurwitz, 1988). Other domains within the ITRs are also required for DNA replication in Ad2 (Guggenheimer *et al.*, 1984; Rawlins *et al.*, 1984; de Vries *et al.*, 1985).

The Ad2 genome is 35,937 nucleotides long. The organization of the Ad2 genome is shown in figure 1. Genes are expressed from both the r-strand (transcribed left to right) and the l-strand (transcribed right to left). Gene expression can be divided into early and late phase, separated by the onset of DNA replication. A subset of genes are expressed at intermediate times. Transcription is initiated from a number of promoters at early and intermediate times. All the late genes are transcribed from the same promoter, the major late promoter (MLP) (Gelinas and Roberts, 1977; Zain and Roberts, 1978). The 5' end of all the late mRNAs consists of a 201 bp tripartite leader sequence derived from 16.8, 19.8 and 26.9 m.u. (Berget *et al.*, 1977; Akusjärvi and Pettersson, 1979).

Figure 1: Organization of the Ad2 genome.

Early (E) and late (L) transcription units are indicated. Arrows show the location of the major RNA species, the arrowhead denotes the direction of transcription. Thick lines illustrate mRNAs expressed early after infection and thin lines mRNAs expressed at intermediate times after infection. Open arrows show sequences present in late mRNA. Polypeptides that have been assigned to the different regions are indicated; see text for further details. (Reproduced from Akusjärvi and Wadell, 1990).


ML
L O
L 1
L 2
L 3
L 4
L 5
15.8K,
52,55K,IIIa, III, pVII, V,
pVI, II, 23K,
100K,33K,pVIII,
IV,
1
2
i
3 VARNA
(=)
I II
x
y
Z
E3
19Kgp. 11.6K
14.5K 14.7K
EIA EIB
6.1K 16.5K
18.6K 21K
24.0K 55K
26.5K
31.9K
IX
52,55K
r-strand 3'
l-strand 5'
5'
3'
0
10
20
30
40
50
60
70
80
90
100
IVa2
Ad-pol pTP
DBP
E2A
34.1K 17.1K 11K
E4
E2B

1.1.3. The lytic cycle

1.1.3.1. Virus entry into cells

Adenoviruses enter cells by receptor-mediated endocytosis (Svensson and Persson, 1984; Svensson, 1985; Pastan *et al.*, 1986). The virus attachment protein is the fibre (Philipson *et al.*, 1968) but the cellular receptor has not yet been identified, although several cellular membrane proteins have been shown to bind fibre (Svensson *et al.*, 1981; Defer *et al.*, 1990). Recent experiments have indicated that different serotypes may use different receptors, since Ad2 and Ad3 do not compete for binding sites (Defer *et al.*, 1990).

Morphological studies have shown that virions are internalized in coated vesicles and delivered to endosomes (Dales, 1978; Pastan *et al.*, 1986). Further studies at the morphological and biochemical level suggest that the viruses are released into the cytosol by lysis of endosomes (Dales, 1978; Pastan *et al.*, 1986). Moreover, it has been suggested that the lytic reaction is catalysed by a viral factor which is activated by low pH (Pastan *et al.*, 1986; Wohlfart, 1988; Varga *et al.*, 1990). Morphological evidence indicates that following exit from endosomes, the virion releases its genome into the nucleus through nuclear pores (Dales, 1978).

1.1.3.2. Transcription

Adenovirus genes are transcribed by the host RNA polymerase II with the exception of two genes, VA1 and VA2 which are transcribed by RNA Pol III (Weinmann *et al.*, 1976). Early genes are transcribed from six regions (E1A, E1B, E2, E3, E4 and L1) of the viral genome, prior to DNA replication (see figure 1). Each of these early regions contains its own promoter (Berk and Sharp, 1977a; Evans *et al.*, 1977) and a number of overlapping mRNAs are processed by differential splicing of the primary transcript from each promoter. Close to the onset of DNA replication, a set of intermediate RNAs (ppIX, IVa2, VA1 and VA2) are transcribed. Finally after the onset of DNA replication the late genes (L1-L5)

are transcribed; however, the L1 region is expressed at both early and late times (Lewis and Mathews, 1980; Miller *et al.*, 1980). Unlike early RNAs, the late transcripts are processed from a common nuclear precursor RNA which is initiated from the major late promoter (MLP) (Ziff and Evans, 1978).

A number of studies of adenovirus gene expression have demonstrated that early transcripts are temporally regulated. The first region to be transcribed is the E1A region (Berk and Sharp 1977a; Jones and Shenk 1979b). The structure and function of this region will be discussed in detail in section 1.2. The E1A region lies near the left end of the genome at 1.5-4.5 m.u. in Ad2. At least five mRNA species are transcribed from the E1A region; they are all generated by differential splicing of a common nuclear precursor RNA (see 1.2.1. figure 2). The 13S and 12S mRNAs are the most abundant species early after infection while at late times, a shift in steady-state levels of the E1A mRNAs occurs, and the 9S mRNA becomes the most abundant species (Spector *et al.*, 1978; Chow *et al.*, 1979). The 13S and 12S mRNAs encode related polypeptides of 289 and 243 amino acids respectively; 46 internal residues are unique to the larger protein (Perricaudet *et al.*, 1979). The 289R polypeptide activates transcription from other early viral promoters (Jones and Shenk, 1979b; Ricciardi *et al.*, 1981; Montell *et al.*, 1982).

Downstream of E1A lies the E1B region (4.6-11 m.u. in Ad2). The structure and function of this region will be described in detail in section 1.3. The E1B region of Ad2 encodes two major overlapping mRNAs, 22S and 13S, which share common 5' and 3' termini but differ in the extent of internal sequences removed by splicing (Berk and Sharp, 1978; see 1.3.1. figure 4). The 13S mRNA encodes a 19-21 kilodalton protein (E1B 19K) and the 22S mRNA encodes the 19K protein using the first AUG and a 55K protein in a different reading frame, starting from the second AUG (Halbert *et al.*, 1979; Bos *et al.*, 1981). Two minor species, 14S and 14.5S, have also been identified, encoding

the 19K protein and polypeptides related to the 55K protein (Virtanen and Pettersson, 1985; Anderson *et al.*, 1984). A 9S mRNA encoding structural polypeptide IX is transcribed from a separate promoter within the E1B region at intermediate times. In Ad5 infected Hela cells the 22S mRNA is detectable at 3.5 hr p.i. and increases in abundance throughout early infection while the 13S mRNA remains at low levels during early infection with an increase at intermediate times (Glenn and Ricciardi, 1988).

The E2 region of Ad2 maps between 75.4 and 11.3 m.u. and is transcribed from the l-strand (see figure 1). It differs from other adenovirus transcription units in using two alternative promoter sites for the initiation of transcription (Chow *et al.*, 1979). The promoter usage is regulated in a temporal manner; transcripts from the E2-early promoter (75.4 m.u.) appear between 2-3 hr p.i. (Nevins *et al.*, 1979; Glenn and Ricciardi, 1988) whereas following the switch from early to late phase the E2-late promoter (72.2 m.u.) is preferentially used. Like other adenovirus early promoters the E2-early promoter is activated by E1A gene products whereas an inhibitory effect on the E2-late promoter is seen in the presence of E1A (Rossini, 1983). In addition to transcriptional regulation by E1A, the E2-early promoter is also activated by an E4 gene product, independently or in synergy with E1A; the E4 ORF 6/7 protein stimulates E2 early gene expression by increasing the DNA binding activity of the cellular transcription factor E2F (Babiss, 1989; Hardy *et al.*, 1989; Reichel *et al.*, 1989; Huang and Hearing, 1989; Neill *et al.*, 1990).

Two major classes of E2 transcripts are generated by the usage of two different polyadenylation sites. The E2A transcripts use a poly A site at 62.4 m.u. while a second set of transcripts, E2B, bypass that signal and extend to a poly A site at 11.3 m.u. (Stillman *et al.*, 1981). Differential splicing within E2A transcripts generates two types of mRNAs varying in length by about 100nt (Berk and Sharp 1978). Both these mRNA species encode the 72K single stranded DNA binding protein (DBP). This multifunctional

polypeptide is located predominantly in the nucleus of infected cells and plays a variety of roles during the infectious cycle. It is essential for viral DNA replication (van der Vliet and Sussenbach, 1975), and has been implicated in the assembly of virus particles (Nicolas *et al.*, 1983). DBP also affects the host range of the virus; DBP mutants have been isolated which multiply efficiently on monkey cells (Klessig, 1977). Monkey cells are non-permissive for wild type adenovirus. This involves aberrant splicing of fibre mRNA; in an abortive infection of monkey cells the fibre mRNA lacks the x and y ancillary leader sequences (Anderson and Klessig, 1984). This block is overcome in these DBP mutants (Klessig and Chow, 1980). The DBP influences the levels to which early viral mRNAs accumulate (Carter and Blanton, 1978a, 1978b). Babich and Nevins (1981) reported a reduction in half life of at least some early mRNAs caused by the DBP, and an inhibitory effect on transcription from the E4 promoter has also been demonstrated (Nevins and Winkler, 1980; Handa *et al.*, 1983). Furthermore, the DBP may autoregulate its own synthesis (Morin *et al.*, 1989) and it has been shown to enhance transcription from the E1A, E2-early and the major late promoters (Chang and Shenk, 1990). The stimulatory effect seen on the MLP led to the suggestion that the DBP may play a role in the activation of this promoter.

The E2B region gives rise to three differentially spliced mRNAs (Stillman *et al.*, 1981; Shu *et al.*, 1988). Protein products have been assigned to two of these mRNAs; they both encode proteins involved in DNA replication. The longest E2B mRNA encompasses the ORF for the 87Kd terminal protein precursor (pTP) (Smart and Stillman, 1982) which serves as a primer for DNA replication and is later cleaved, producing the 55Kd terminal protein. The ORF found in the shortest E2B mRNA encodes a 140Kd DNA polymerase which is essential for adenovirus DNA replication (Stillman *et al.*, 1982).

The E3 region of Ad2 is located between 76.8 and 85.9 m.u. and is transcribed from the r-strand (see figure 1). This region has been shown to be dispensable for viral

growth in tissue culture (Jones and Shenk, 1979a). At least 10 mRNA species are transcribed from the E3 promoter and these fall into two families, differing in the poly A site used (Berk and Sharp, 1977b, 1978; Chow *et al.*, 1979; Cladaras and Wold, 1985; Cladaras *et al.*, 1985). Region E3 is predicted to encode at least nine proteins (Herisse *et al.*, 1980; Herisse and Galibert, 1981; Cladras and Wold, 1985). Six of these proteins have been identified in infected cells (see Wilson-Rawls *et al.*, 1990) and functions are known for three of them. These proteins all interfere with host cell functions or immune response during adenovirus infection.

A glycoprotein, gp19K, encoded by the E3 region, is localized in the membrane of the endoplasmic reticulum where it forms a complex with class I antigens of the major histocompatibility complex and retards their transport to the cell surface (Wold and Gooding, 1989; Gooding and Wold, 1990). This has been shown to prevent cell lysis by adeno-specific cytotoxic T-lymphocytes (Rawle *et al.*, 1989).

A 14.7Kd E3 protein has also been identified in infected cells (Persson et al., 1978; Tollefson and Wold, 1988; Wang *et al.*, 1988). This protein has been shown to prevent lysis of adeno-infected cells by tumor necrosis factor (TNF) and may mitigate the anti-viral effects of TNF *in vivo* (Gooding *et al.*, 1988). TNF is an immunoregulatory protein which is secreted by activated monocytes and macrophages and which has antiviral properties (Beutler and Cerami, 1989); expression of adenovirus E1A renders cells sensitive to cytolysis by TNF (Duerksen-Hughes *et al.*, 1989); this effect is cell type dependent (Vanhaesebroeck *et al.*, 1990).

The E3-14.4Kd protein, identified by Tollefson *et al.*, (1990a) is a membrane protein which stimulates endosome-mediated internalization and degradation of the receptor for epidermal growth factor (Carlin *et al.*, 1989). Three additional E3 proteins, 11.6Kd, 14.5Kd and 6.7Kd, have been identified in infected cells but not as yet assigned functions (Wold *et al.*, 1984; Tollefson *et al.*, 1990b; Wilson-Rawls *et al.*, 1990).

The right end of the genome contains the E4 region (91.3-99.1 m.u. in Ad2) which is transcribed from the l-strand (see figure 1). In addition to transcriptional control by E1A gene products, the E4 promoter is down-regulated by the 72K DBP (Nevins and Winkler, 1980; Handa *et al.*, 1983), resulting in a dramatic decrease in transcription at intermediate times after infection following an initial early peak (Glenn and Ricciardi, 1988). A complex family of overlapping E4 mRNAs is generated during viral infection by alternative splicing of a primary transcript (Freyer *et al.*, 1984; Tigges and Raskas, 1984; Virtanen *et al.*, 1984), twelve of which have been analysed by cDNA cloning and sequencing. Analysis of the E4 DNA and cDNA sequences suggests that at least seven different polypeptides can be produced from this region; three E4 products have been immunologically identified.

The product of ORF 3 is an 11.5Kd protein (Sarnow *et al.*, 1982; Downey *et al.*, 1983). This polypeptide affects viral DNA replication, late protein synthesis, the shut-off of host cell protein synthesis and the production of infectious virus, but is non-essential in the presence of a functional E4 ORF 6 gene product (Halbert *et al.*, 1985; Hemström *et al.*, 1988; Bridge and Ketner, 1989; Huang and Hearing, 1989b).

The gene product of ORF 6 is a 34Kd protein (Challberg and Ketner, 1981) which in infected cells is found physically associated with the E1B 55K protein (Sarnow *et al.*, 1984) as well as in a free form (Cutt *et al.*, 1987). Mutagenesis studies of individual E4 ORFs have identified the 34K protein as the only essential E4 gene product within infected cells (Halbert *et al.*, 1985; Hemström *et al.*, 1988). However, a mutant virus lacking all E4 coding sequences is much more defective for viral growth than an ORF6 mutant virus, suggesting that other E4 products may compensate for the lack of the 34Kd protein (Halbert *et al.*, 1985). Studies using double mutants have demonstrated that E4 ORF6 and ORF3 are individually sufficient for normal late protein synthesis although the ORF3 product appears to

be slightly less effective then the ORF6 product (Bridge and Ketner, 1989; Huang and Hearing, 1989b). This has led to the conclusion that the 11.5Kd and 34Kd proteins act in parallel in late protein synthesis. One of the roles of the E4 gene products is to stabilize late viral mRNAs in the nucleus (Sandler and Ketner, 1989). The 34K/55K complex acts either in the transport of late mRNAs or stabilization of late mRNAs in the cytoplasm and may act in parallel with the 11.5Kd protein in the accumulation of late viral mRNA in the cytoplasm (see Bridge and Ketner, 1990).

E4 ORF 6/7 is created by mRNA splicing. A 19.5Kd polypeptide has been identified as the product of this gene (Cutt *et al.*, 1987). This protein stimulates the DNA binding activity of the cellular transcription factor E2F by a direct complex formation between the 19.5K protein and E2F (Huang and Hearing, 1989a; Neill *et al.*, 1990); E2F exerts positive control on transcription from the E1A (Hearing and Shenk, 1983, 1986) and the E2-early promoters (Babiss, 1989; Reichel *et al.*, 1989).

The gene for structural polypeptide IX is contained within the E1B region. The promoter lies in E1B intron sequences and ppIX coding sequences are in the E1B non-translated region; the mRNA is 3' coterminal with E1B mRNAs (Aleström *et al.*, 1980). This is the only adenovirus mRNA that is not spliced. Transcription is initiated from the ppIX gene promoter at intermediate times after infection; there is controversy in the literature as to whether DNA replication is necessary and sufficient to activate this promoter (Matsui *et al.*, 1986; Venkatesh and Chinnadurai, 1987) or if the promoter is inactive at early times due to E1B transcription through the promoter (Vales and Darnell, 1989).

The gene for polypeptide IVa2 is transcribed from a promoter at 16.2 m.u. on the l-strand, generating a spliced mRNA which is 3' coterminal with E2B transcripts. The 50Kd protein product is a major component of virus assembly intermediates and has been proposed to play a role in virus maturation (Persson *et al.*, 1979).

Two small RNAs, termed virus associated RNAs (VA1 and VA2), are transcribed from two separate promoters near 30 m.u. on the r-strand by RNA polymerase III (Weinman *et al.*, 1976). The VA RNAs accumulate in large amounts late after infection and are required for efficient late protein synthesis (Thimmappaya *et al.*, 1982). VA RNA binds to DAI, a cellular protein kinase (Katze *et al.*, 1987), thereby preventing phosphorylation and inactivation of protein synthesis initiation factor elF-2 (see O'Malley *et al.*, 1989). VA RNA contains two domains, one that promotes binding to DAI and another that interferes with DAI activation (Mellits *et al.*, 1990).

The major late promoter (MLP) is located at 16.8 m.u. on the r-strand of the Ad2 genome. At late times in infection five mRNA families, L1-L5, are transcribed from this promoter, differing in the poly-adenylation site used (Shaw and Ziff, 1980; Nevins and Wilson; 1981). Each mRNA species contains a common set of three short 5'-leader sequences, the tripartite leader, joined to different splice acceptor sites (Berget *et al.*, 1977; Chow *et al.*, 1977). During early infection the L1 mRNAs are expressed from the MLP but following DNA replication the L2-L5 mRNAs are also made (Nevins and Wilson, 1981; Shaw and Ziff, 1980). The molecular mechanism behind the switch between early and late transcription pattern from the MLP is not well understood. Transcription is strongly stimulated after the onset of DNA replication (Shaw and Ziff, 1980; Grass *et al.*, 1987), possibly due to the particular state of the newly replicated DNA (Thomas and Mathews, 1980). Jansen-Durr *et al.*, (1989b) have identified a major late promoter element within the first intron of the late transcript, essential for replication dependent activation of the MLP, and a cellular protein, downstream element factor, which binds to a sequence motif within this element, after the onset of DNA replication in infected cells. Furthermore, the 72K DBP has been shown to stimulate transcription from the MLP, suggesting a possible role in the activation of this promoter (Chang and Shenk, 1990).

Region L1 encodes one structural polypeptide, protein IIIa, as well as the 52/55Kd proteins which have been shown to play a role in the assembly of infectious virus particles (Hasson *et al.*, 1989). The other late regions mostly encode virus structural proteins. In addition the L3 region encodes a protease responsible for the proteolytic cleavage of many viral polypeptides including the structural proteins VI, VII and VIII (Bhatti and Weber, 1979). The protease is present in virus particles at about 10 polypeptides per virion, primarily associated with the virion core (Anderson *et al.*, 1990). Two non-structural proteins are encoded by the L4 region, a 100Kd protein which is involved in assembly of hexon capsomers (Oosterom-Dragon and Ginsberg, 1981; Cepko and Sharp, 1982, 1983) and required for the efficient translation of late mRNA (Hayes *et al.*, 1990), and an unrelated 33Kd protein of unknown function (Oosterom-Dragon and Anderson, 1983).

1.1.3.3. DNA replication

The virus lytic cycle is divided into early and late phase by the onset of DNA replication. The viral proteins essential for replication, the terminal protein precursor (pTP), the adenovirus DNA polymerase (AdPol), and the DNA binding protein (DBP), are all expressed at early times from the E2 region. For a review of adenovirus DNA replication see Challberg and Kelly, 1989).

A number of studies have shown that adenovirus DNA replication occurs in two stages. In the initial stage replication begins at one end of the genome and elongation takes place using one parental strand as template and displacing the other strand. The second stage involves the replication of the displaced single strand. The mechanism of replication is unique among eucaryotic DNA viruses but has a parallel in the bacteriophage ø29 (Salas, 1983). The precise order of events in the initiation of replication has not yet been resolved. The initiation reaction requires the interaction between the pTP and AdPol and two cellular

nuclear factors, NFI and NFIII, which have been shown to bind to sequences within the ITR (Nagata *et al.*, 1983; de Vries *et al.*, 1985; Jones *et al.*, 1987). Binding of NFI in the presence of pTP-AdPol complex stimulates the binding of dCTP to the pTP (Nagata *et al.*, 1983); this C residue becomes the first residue in the new DNA chain and is complementary to the G residue at the 3' end (Challberg *et al.*, 1980). It is thought that the synthesis of a strand complementary to the displaced DNA strand occurs following a circularization of the single stranded template by annealing of the ITRs (Daniell, 1976; Stow, 1982; Hay *et al.*, 1984). The resulting "panhandle" structure resembles the terminus of the duplex adenovirus genome and is presumably recognized by the same initiation machinery that operates in the first stage of replication (Challberg and Kelly, 1989).

1.1.3.4. Assembly of virions

The details of adenovirus assembly are not well understood. The initial step appears to be the formation of the capsomers, hexon, penton base and fibre, from their monomeric forms in the cytoplasm and the assembly of empty capsids; viral DNA and core proteins are then inserted into the capsid, either together or separately in the nucleus (see Philipson, 1984). An alternative mechanism has been suggested by Weber *et al.*, (1985) whereby virion assembly is linked to DNA replication and capsid units assemble around replicating DNA. A *cis* acting packaging domain, essential for complete virion assembly, has been identified near the left end of the Ad5 genome; this domain can function in either orientation and near either end of the viral genome (Hearing *et al.*, 1987). This region is composed of at least five distinct, functionally redundant, sequence elements four of which contain an AT-rich repeated sequence motif (Gräble and Hearing, 1990). These authors have suggested two models for the function of this packaging domain. One model is that DNA packaging protein(s) may directly recognize and bind to those sequences thereby facilitating

the packaging of the viral genome; the other model suggests that the AT-rich sequences may introduce bends in the DNA and that a packaging protein(s) recognizes the overall structure of the DNA molecule. Virus packaging proteins have not so far been identified. In addition to structural proteins some non-structural viral proteins are known to affect virion assembly. The IVa2 protein is present in assembly intermediates and it has been suggested that this polypeptide may interact with viral DNA when it enters the capsid (Pettersson, 1984). The L1 52/55Kd proteins have been shown to be required for virion assembly; they may function late in the assembly process since intermediates representing capsids associated with the left end of the genome have been isolated from cells infected with a virus defective for these proteins (Hasson *et al.*, 1989).

1.2. Early region 1A

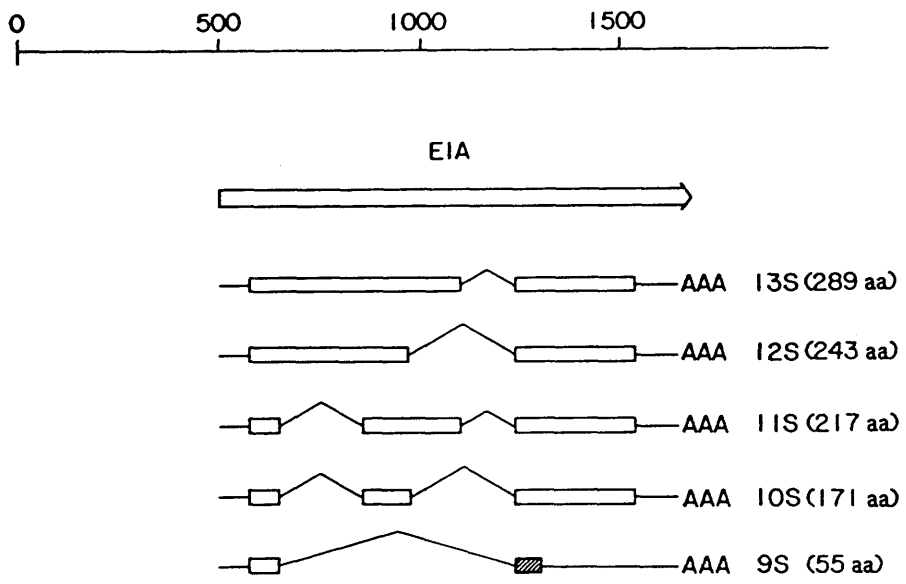
Early region 1A (E1A) of adenoviruses has been extensively studied for a long time. Two aspects of E1A protein functions have mainly led to a great interest in this gene region; the role of E1A in gene regulation and E1A involvement in cell transformation. The main emphasis here will be on the role of E1A in regulation of gene expression during the lytic cycle.

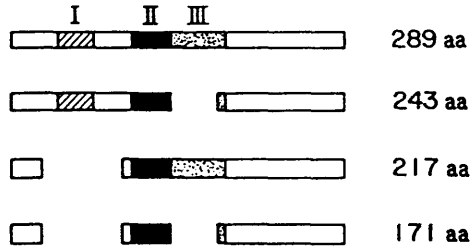
1.2.1. E1A transcription

The E1A DNA sequence has been determined for many human serotypes and other non-human adenoviruses (Ad2, Gingeras *et al.*, 1982; Ad4, Tokunaga *et al.*, 1986; for a comparison of Ad5, Ad7 and Ad12, see van Ormondt *et al.*, 1980; Ad40, van Loon *et al.*, 1987b; Ishino *et al.*, 1988; Ad41, van Loon *et al.*, 1987b; Allard and Wadell, 1988; Simian adenovirus 7 (SAV-7), Kimelman *et al.*, 1985; Tupaia adenovirus, Brinckmann *et al.*, 1983; mouse adenovirus 1 (MAV-1), Ball *et al.*, 1988). Comparison of sequences of different adenoviruses has revealed three conserved domains within the E1A coding sequences (Kimelman *et al.*, 1985; Moran and Mathews, 1987). Furthermore, these conserved regions are partly reflected in the E1A splicing patterns (see figure 2). E1A transcripts have been mapped for a number of different serotypes and are remarkably similar (Ad2, Perricaudet *et al.*, 1979; Berk and Sharp, 1978; Chow *et al.*, 1979; Baker and Ziff, 1981; Virtanen and Pettersson, 1983; Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987; Ad7, Dijkema *et al.*, 1980; Ad12, Sawada and Fujinaga, 1980; Saito *et al.*, 1981; Brockmann *et al.*, 1990; Ad40 and Ad41, van Loon *et al.*, 1987b; SAV-7, Kimelman *et al.*, 1985; MAV-1, Ball *et al.*, 1989); the mouse virus differs from the other serotypes in having only one E1A mRNA, 3' coterminal with E1B mRNAs. Figure 2 shows the structure of Ad2 E1A mRNAs, which have been most extensively studied. The three major mRNA species, 13S, 12S and 9S have been identified from most serotypes studied so far. The mRNAs share common 5' and 3' termini but differ in the size of the excised introns as a

Figure 2: Structure of the Ad2 E1A mRNAs and open reading frames.

Sedimentation values (S) for mRNAs and amino acid numbers for protein products are indicated. An alternative splice acceptor site is used to generate the 9S RNA which changes the open reading frame (hatched box) relative to other proteins. The lower panel shows a schematic representation of the domains of sequence conservation in the E1A proteins. The line at the top represents the Ad2 genome (bp). See text for details. (Reproduced from Nevins, 1989).


0
500
1000
1500
E1A
AAA 13S (289 aa)
AAA 12S (243 aa)
AAA 11S (217 aa)
AAA 10S (171 aa)
AAA 9S (55 aa)


I
II
III
289 aa
243 aa
217 aa
171 aa

result of linking different splice donor sites to a common splice acceptor site. The 12S and 13S mRNAs are translated in the same reading frame but their products differ by a block of 46 amino acids found only in the 13S product. Although the same splice acceptor site is used in the 9S mRNA, because of the pattern of the splice junction the second exon is read in a different reading frame to the 12S and 13S mRNAs (Virtanen and Pettersson, 1983). Two additional minor mRNA species have been identified in Ad2. These 11S and 10S mRNAs contain splice junctions identical to those used in the 13S and 12S mRNAs respectively; in addition the 11S and 10S mRNAs share a splice junction where the splice donor site used in the 9S mRNA is linked to a unique splice acceptor site (see figure 2) (Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987).

The E1A region is the first region to be transcribed during a viral infection (Nevins *et al.*, 1979). At 1.5-2 hr p.i. the 12S and 13S mRNAs are detectable in the cytoplasm, the steady state levels of these transcripts peak at 5 hr p.i. and gradually decline through 12 hr p.i. (Glenn and Ricciardi, 1988). Transcription from the E1A promoter reaches maximal rate at 3 hr p.i. and that rate is maintained through the onset of DNA replication at 6 hr p.i. (Nevins *et al.*, 1979). The E1A 9S mRNA accumulates in the cytoplasm at late times in infection (Chow *et al.*, 1979; Wilson and Darnell, 1981). Similarly, the minor 10S and 11S mRNAs increase in abundance late in infection (Stephens and Harlow, 1987).

The E1A promoter is active in the absence of any viral factors (Nevins, 1981). This promoter consists of a TATA box and an ATF binding site; several upstream elements are also involved in regulation of transcription (see figure 3B for protein binding sites). The insertion of E1A upstream promoter sequences either upstream or downstream of another early gene confers this basal activity onto that promoter, indicating that these E1A sequences possess enhancer properties (Imperiale *et al.*, 1983). Three distinct enhancer elements have been identified in E1A upstream regions in

Ad5. Element I is present in two copies and specifically regulates E1A transcription *in vivo* (Hearing and Shenk, 1983, 1986). A cellular factor, EF-1A, has been shown to bind to these sites *in vitro* (Bruder and Hearing, 1989). Element II is located between the two copies of element I and enhances transcription from all early regions on the chromosome in *cis* (Hearing and Shenk, 1986). The third element, a binding site for the transcription factor E2F, is also present in two copies (Kovesdi *et al.*, 1987). These sites may mediate autoregulation of E1A gene expression (Tibbetts *et al.*, 1986) since the E2F binding site from the E1A enhancer region can confer E1A inducibility on a heterologous promoter (Kovesdi *et al.*, 1987) and the DNA binding activity of E2F is stimulated by E1A (Raychaudhuri *et al.*, 1990); this E1A dependent activation may involve phosphorylation (Bagchi *et al.*, 1989). Transcription from the E1A promoter is reduced around 5 fold in the absence of E1A proteins as compared to wild type indicating that E1A gene products have a role in regulating their own expression (Montell *et al.*, 1984; Osborne *et al.*, 1984). E1A upstream sequences may include other sites involved in transcriptional regulation; Yoshida *et al.*, (1989) have described 21 binding sites of Hela cell nuclear proteins in the E1A upstream region. These include sites that overlap the cap site, TATA box and the enhancer elements described previously as well as sites constituting the origin of DNA replication. In addition to upstream sequences affecting E1A transcription, Osborne *et al.*, (1984) identified a sequence element within E1A coding sequences which enhances E1A transcription.

1.2.2. E1A protein products

E1A polypeptides are phosphoproteins, predominantly localized in the nucleus of infected cells (Harlow *et al.*, 1985; Tsukamoto *et al.*, 1986; Schmitt *et al.*, 1987; White *et al.*, 1988b). They play a variety of roles during a lytic infection and are central to the process of transformation

by adenovirus (for reviews see Berk, 1986a; Grand, 1987). Specific functions have been assigned to three regions within E1A which are well conserved between different serotypes.

A schematic diagram of E1A protein products is shown in figure 2. The presence of conserved regions (CR) I-III is indicated. The protein encoded by the 9S mRNA does not include any conserved regions and has not been assigned a function. CR I, which is present in the 289 residue (R) protein encoded by the 13S mRNA and the 243R product of the 12S mRNA, is required for transcriptional repression, transformation and induction of DNA synthesis (Lillie *et al.*, 1987; Schneider *et al.*, 1987; Jelsma *et al.*, 1989), but is dispensable for viral growth on Hela cells (Smith and Ziff, 1988). CR II is present in all but the smallest E1A protein; this region is required for transformation (Lillie *et al.*, 1986; Moran *et al.*, 1986a) as well as induction of DNA synthesis (Howe *et al.*, 1990), but may be dispensable for transcriptional repression (Stein *et al.*, 1990). CR III is unique to the 289R protein and is essential for the transactivating function of E1A (Moran *et al.*, 1986b; Lillie *et al.*, 1987; Jelsma *et al.*, 1988), but dispensable for transformation and transcriptional repression (Zerler *et al.*, 1986; Velcich and Ziff, 1985). The conserved regions of E1A appear to represent distinct functional domains which may act independently of other parts of the protein; a 49 amino acid peptide including CR III efficiently activates an E1A inducible viral promoter upon coinjection into Hela cells (Lillie *et al.*, 1987).

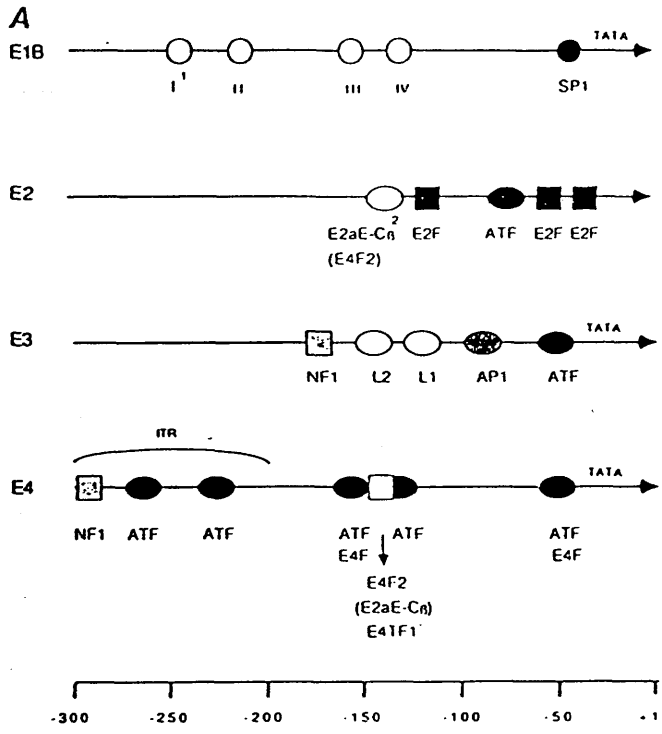
1.2.3. Role of E1A proteins in regulation of transcription

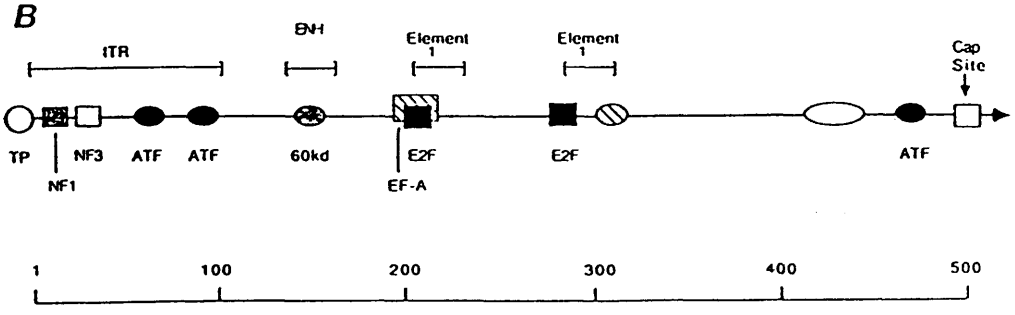
E1A protein products regulate transcription from a number of viral and cellular promoters (for reviews of E1A trans-activation see Berk, 1986b; Jones *et al.*, 1988; Nevins, 1989). In addition to the transactivating functions of the 289R protein, E1A products can also repress transcription from various enhancer-containing promoters (Borelli *et al.*,

1984; Velcich and Ziff, 1985). The mechanism of E1A transactivation appears to be indirect, that is not involving an interaction between E1A proteins and specific DNA sequences within the activated promoter. A variety of studies indicate that E1A mediates transcriptional regulation through cellular components. Activation of E1A dependent promoters can be mediated by another viral transactivating factor, the pseudorabies virus immediate early protein (Imperiale *et al.*, 1983; Wu and Berk, 1988), as well as in a cell type specific manner (Imperiale *et al.*, 1984). Transcription of the gene for the cellular heat shock protein (hsp70) is activated in the presence of E1A (Nevins, 1982). F9 teratoma stem cells, which constitutively express hsp70, also allow early adenovirus gene expression in the absence of E1A; upon differentiation induced by retinoic acid early viral gene expression requires the E1A gene product and there is a loss of hsp70 gene expression (Imperiale *et al.*, 1984). A great deal of effort from various laboratories has over the last few years led to the identification of a number of *cis* acting promoter sequences and *trans* acting cellular factors involved in E1A dependent promoter activation. Figure 3 shows a summary of the DNA-protein interactions within the upstream promoter regions of the adenovirus early genes.

The Ad2 E1B promoter (see figure 3A) contains two elements essential for full activity *in vivo*, a binding site for the Sp1 transcription factor (GC box) (Dynan and Tjian, 1983) and a TATA box (Wu *et al.*, 1987). Studies on other serotypes including the closely related Ad5 indicate that other upstream elements may be important for promoter activity (Bos and ten Wolde-Kraamwinkel, 1983; Parks *et al.*, 1988; Parks and Spector, 1990). The organization of the E1B promoter will be described in detail in section 1.3.1. Mutations in the TATA box sequence but not the GC box affect activation of the E1B promoter by E1A, thus implicating the TATA box transcription factor (TFIID) in the process (Wu *et al.*, 1987). However, Pei and Berk (1989) have shown that when the E4 TATA box is substituted for the E1B TATA box,

Figure 3: A) DNA-protein interactions within the upstream promoter region of the adenovirus early genes. The position of the binding sites and names of the factors are indicated. The arrowhead denotes the direction of transcription. The scale represents position relative to each cap site (+1).
Footnotes: [1] Footprints protected by factors I-IV are as follows: I is TGGGCGTCGC, which may bind Sp1; II is GCTGTGGAATGT, which is identical to the GTII motif of the SV40 enhancer; III is CCCCAGGC, which may bind AP2; IV is TGTGGTTA. [2] This factor contains the same binding site as the E4F2 factor that binds at about -150 on the E4 promoter.
B) DNA-protein interactions within the upstream region of the E1A gene. The scale represents the DNA genome (bp). (Modified from Jones *et al.*, 1988).


A
E1B
TATA
I
II
III
IV
SP1
E2
E2aE-C
E2F
ATF
E2F
E2F
(E4F2)
E3
TATA
NF1
L2
L1
AP1
ATF
ITR
E4
TATA
NF1
ATF
ATF
ATF
E4F
ATF
E4F
ATF
E4F
E4F2
(E2aE-C)
E4F1
-300
-250
-200
-150
-100
-50
+1


B
ITR
ENH
Element 1
Element 1
Cap Site
TP
NF3
ATF
ATF
60kd
E2F
E2F
ATF
NF1
EF-A
1
100
200
300
400
500

the promoter construct no longer responds to E1A activation indicating specific sequence requirements for an E1A responsive TATA box. In the absence of upstream sequences the presence of the E4 TATA box is not sufficient for E1A induction of the E4 promoter (Gilardi and Perricaudet, 1984; 1986).

The E2 early promoter has been extensively studied in terms of activation of transcription (see figure 3A). Several elements within this promoter can respond to E1A transactivation. The E2 promoter contains a TATA-like element (TAGA) (Zajckowski *et al.*, 1985) which is involved in E1A induction *in vivo* (Manohar *et al.*, 1990). Site directed mutagenesis studies have identified three upstream promoter elements, two copies of a binding site for the transcription factor E2F and one ATF binding site (Loeken and Brady, 1989). An additional E2F binding site is located further upstream, outside the minimal promoter region, alongside a binding site for a factor termed E2aE-Cβ (Jalinot *et al.*, 1988). A cellular factor Cα, distinct from the E2F factor, has been reported to bind to sequences overlapping the upstream and downstream E2F binding sites and may be involved in E1A induction (Jansen-Durr *et al.*, 1990). Several lines of evidence indicate that the E2F factor plays a critical role in E1A-dependent regulation of transcription of the E2 early promoter. A segment of the E2 promoter containing the two E2F binding sites can confer E1A inducibility onto a heterologous promoter (Kovesdi *et al.*, 1987; Yee *et al.*, 1989) indicating a role for E2F in E1A response. Furthermore, purified E2F protein specifically activates transcription from promoters containing E2F binding sites *in vitro* (Yee *et al.*, 1989). The level of E2F binding activity increases during virus infection and increased binding stimulates E2A gene expression (Babiss, 1989). This increased binding activity is dependent on the presence of the E1A 289R protein and appears to occur in the absence of protein synthesis, leading to the suggestion of regulation by posttranslational modification (Reichel *et al.*, 1988). This may involve phosphorylation of E2F (Bagchi

et al., 1989) possibly by an E1A induced cellular kinase. E1A dependent modification leads to the cooperative formation of stable complexes between two E2F molecules and their binding sites *in vitro*, but does not affect E2F binding to a single binding site (Jansen-Durr *et al.*, 1989a). In addition to E1A, an E4 gene product is also important for E2F activation and stimulation of E2 transcription (Babiss, 1989; Hardy *et al.*, 1989; Reichel *et al.*, 1989). The E4 19.5K protein stimulates stable E2F binding to DNA by a direct complex formation between the 19.5K protein and E2F (Huang and Hearing, 1989a; Neill *et al.*, 1990; Raychaudhuri *et al.*, 1990). A scheme for combined action of E1A and E4 in the activation of E2F has been proposed by Raychaudhuri *et al.*, (1990). They suggest that E1A acts to increase the levels of functional E2F independent of E4 functions, possibly by stimulating a cellular kinase or inactivating a phosphatase; E4 stimulation of stable promoter binding of functional E2F could involve an alteration of E2F as a result of E4-protein interaction or alternatively the E4 protein may dimerize, bringing together the bound E2F molecules. This model accounts for independent activation seen in the presence of either the E1A 289R protein or the E4 19.5K protein and predicts the much greater effect seen when both are present. However, this contradicts the finding by Jansen-Durr *et al.*, (1989a) that E1A does not affect the binding of E2F to a single site.

The ATF binding site in the E2 promoter is important for both uninduced and E1A induced promoter activity although the promoter still responds to E1A in the absence of this element (Imperiale *et al.*, 1985). A family of related cellular polypeptides interact with an ATF binding site (Hai *et al.*, 1988) and these bind to and mediate the transcription of both E1A- and cyclic AMP (cAMP) inducible promoters (Lee *et al.*, 1987; Lin and Green, 1988; Roesler *et al.*, 1988; Hardy and Shenk, 1988; Pei and Berk, 1989; Muchardt *et al.*, 1990). Analysis of cDNA clones encoding factors binding to ATF binding sites and cAMP responsive

elements (CRE) has revealed a family of cellular transcription factors that possess homologous DNA binding domains (Hoeffler *et al.*, 1988; Hai *et al.*, 1989), and are capable of forming DNA binding heterodimers (Hai *et al.*, 1989). Dimerization involves a leucine zipper structure originally proposed by Landschulz *et al.*, (1988). Liu and Green (1990) have suggested that E1A dependent activation of transcription through an ATF binding site may involve an interaction between E1A and a DNA bound ATF factor. They used the retinoblastoma susceptibility gene product (RB), which binds to E1A gene products (Whyte *et al.*, 1988a), to demonstrate that when the E1A gene product is brought into the vicinity of a promoter, transcription from that promoter is activated. A vector expressing a Gal4-RB fusion protein including the Gal4 DNA binding domain and the RB E1A binding domain was cotransfected with an E1A expression vector and a reporter plasmid with Gal4 binding sites in the promoter. This resulted in transcriptional activity which was not seen in the absence of the Gal4 binding sites or E1A. Similarly the Gal4 polypeptide without RB did not support this activity. In a parallel experiment they demonstrated that a GAL-4-ATF-2 fusion protein similarly mediated E1A activation, probably by bringing the 289R E1A protein to the promoter. The ATF factors may be functionally distinguishable since another factor, ATF-1, did not support E1A response in this type of experiment (Liu and Green, 1990). Furthermore, ATF-2 is not inducible by cAMP (Liu and Green, 1990), while another ATF factor, CREB, does respond to cAMP activation (Hoeffler *et al.*, 1988; Gonzales *et al.*, 1989; Gonzales and Montminy, 1989). ATF-2 appears not to activate transcription in the absence of E1A (Liu and Green, 1990) so it is not clear if induction of transcription through an ATF binding site in the absence of E1A (Imperiale *et al.*, 1985) is mediated through another ATF factor. Liu and Green, (1990) propose that ATF-2 has a role in transcriptional activation in uninfected cells; they suggest a possible activation of ATF-2 by modification such as phosphorylation. It is already known that CREB is activated by phosphory-

lation (Gonzales and Montminy, 1989). Alternatively a cellular counterpart of E1A may mediate transcriptional activation by ATF-2 (Liu and Green, 1990).

Several promoter elements have been mapped to the E3 promoter (see figure 3). Three of these, the TATA box, ATF site and AP1 site are important for both basal and E1A induced gene expression (Garcia *et al.*, 1987; Simon *et al.*, 1988; Kornuc *et al.*, 1990). Factors L1 and L2 are lymphoid cell specific (see Jones *et al.*, 1988); binding of nuclear factor I (NFI) has little effect on either basal or E1A induced gene expression (Garcia *et al.*, 1987; Kornuc *et al.*, 1990). A number of TATA box elements, including the E1B promoter, the cellular heat shock promoter (hsp70) and HIV LTR, as well as the E3 promoter element, are important for E1A activation of transcription (Wu *et al.*, 1987; Simon *et al.*, 1988; Kliewer *et al.*, 1989). However, unlike the other TATA elements, mutation or deletion of the E3 TATA box affects basal and induced transcription levels *in vitro* only but not *in vivo* (Kornuc *et al.*, 1990).

The ATF and AP1 binding sites are critical for E1A induction of the E3 promoter *in vivo*; the concentration and DNA binding activity of these factors appears not to be altered in the presence of E1A (Kornuc *et al.*, 1990). Possible mechanisms for E1A dependent activation of transcription through an ATF binding site were discussed above. An AP1 binding site is similar although not identical to an ATF site (Angel *et al.*, 1987; Lee *et al.*, 1987b, 1987c), and some factors can bind to both sites with different specificity (Hai *et al.*, 1989). A family of transcription factors, including the *c-jun* and *c-fos* gene products interact with an AP1 binding site (Bohmann *et al.*, 1987; Angel *et al.*, 1988; Bos *et al.*, 1988; Zerial *et al.*, 1989; Hirai *et al.*, 1989). A functional DNA binding domain requires the formation of protein dimers that can be composed of either homologous or heterologous *jun* and *fos* subunits (Rauscher *et al.*, 1988; Turner and Tjian, 1989; Zerial *et al.*, 1989). Like the ATF factors, dimerization of AP1 factors involves a leucine zipper motif (Landschutz *et*

al., 1988; Sassone-Corci *et al.*, 1988; Turner and Tjian, 1989; Gentz *et al.*, 1989). The role of an AP1 binding site in regulation of transcription may involve competition for DNA binding by the various combinations of AP1 dimers since different factors have different biological properties (Chiu *et al.*, 1989; SchÜtte *et al.*, 1989; Bohmann and Tjian, 1989). Recent studies indicate that a cellular inhibitor may be involved in the regulation of *c-jun* transcriptional activity (Baichwal and Tjian, 1990). These authors suggest that AP1 may communicate with the general transcriptional machinery through a coactivator (Lewin, 1990; Pugh and Tjian, 1990) and that activation may be regulated by the inhibitor and coactivator competing for the same binding site. However, the mechanism by which E1A regulates transcription through an AP1 binding site remains unsolved.

DNA-protein interactions in the E4 upstream promoter region are shown in figure 3. The E4 gene is transcribed from the right hand end of the genome so upstream regions include the ITR, similar to the E1A promoter at the other end of the genome. The ITR includes two ATF binding sites and an NFI binding site, these sequences can be removed without much effect on expression from the E4 promoter when other promoter elements are intact (Gilardi and Perricaudet, 1986; Hanaka *et al.*, 1987). It has been suggested that the repression of E4 transcription by the E2 DBP (Nevins and Winkler, 1980; Handa *et al.*, 1983), may result from interference of the DBP with factors bound to the ITR (Nevins, 1989). Downstream of the ITR the E4 promoter contains three copies of an ATF binding site that is critical for transcription and E1A-dependent *trans* activation (Lee and Green, 1987). Several protein factors have been shown to interact with this element (Raychaudhuri *et al.*, 1987; Lee *et al.*, 1987a; Lee and Green, 1987; Cortes *et al.*, 1988; Leza and Hearing, 1988, 1989). ATF is in fact a family of transcription factors (see above). A factor termed EIVF interacts with this site and similar to ATF the characteristics and amount of DNA binding activity appears to be unaffected by adenovirus infection (Cortes *et al.*, 1988). The relation-

ship of this factor to ATF is not clear. Another factor that interacts with the ATF binding site, E4F, shows an increase in DNA binding activity after adenovirus infection, dependent on E1A function (Raychaudhuri *et al.*, 1987). This regulation of DNA binding activity involves phosphorylation (Raychaudhuri *et al.*, 1989), suggesting a possible parallel with E1A regulation of E2F binding activity (Bagchi *et al.*, 1989). E4F may be a member of the ATF family since its DNA binding pattern is similar to that of ATF-3 (Rooney *et al.*, 1990). In addition, two other factors have been reported to bind to the E4 enhancer region. E4F2 binds to a site identical to the E2aE-Cβ site described by Jalinot *et al.*, (1988) and another factor, E4TFI was reported to bind in the same region (see Jones *et al.*, 1988). Lee *et al.*, (1989) have shown that there is not a simple correlation between the presence of an ATF site and E1A or cAMP inducibility; ATF sites differ among themselves in a way that probably involves flanking sequences. The E4 promoter may therefore be regulated in a complex manner involving several, possibly related, *trans* acting factors and promoter elements with different protein binding specificities. Wada *et al.*, (1991) have recently described different DNA binding properties and transcriptional activation of ATF homodimers and heterodimers binding to the E4 promoter.

Early transcription from the major late promoter is dependent on E1A (Nevins, 1981). The promoter region involved in E1A activation has been mapped between nt -66 and +33 relative to the MLP cap site (Lewis and Manley, 1985; Jansen-Durr *et al.*, 1988). This region overlaps the TATA box region which is identical to the E1A inducible E1B and hsp70 TATA elements (Wu *et al.*, 1987; Simon *et al.*, 1988) thus implicating the TATA-binding factor TFIID in the activation of the MLP by E1A. Furthermore, Leong *et al.*, (1988) have shown by using fractionated cell extracts that the fraction including TFIID stimulates transcription from the MLP several fold higher in infected cell extracts than extracts from mock infected cells.

E1A dependent activation of transcription of viral genes

extends to the RNA polymerase III transcribed VA RNA genes (Berger and Folk, 1985; Gaynor *et al.*, 1985; Hoeffler and Roeder, 1985; Yoshinaga *et al.*, 1986). Stimulation of VA RNA transcription by E1A involves the transcription factor TFIIIC (Yoshinaga *et al.*, 1986), which recognizes and binds to the internal promoter element of polymerase-III transcribed genes (Lassar *et al.*, 1983; Fuhrman *et al.*, 1984). Expression of E1A may lead to an increase in phosphorylation of TFIIIC and thereby enhance functional complex formation at the promoter site (Hoeffler *et al.*, 1988b).

Although the role of E1A in activation of transcription has been extensively studied the mechanism involved is by no means clear. However, a complex picture is emerging; E1A *trans*-activation may be obtained by several pathways involving a number of *cis* acting sequences and *trans* acting factors. The picture is further complicated by the finding that several factors with different biological activities can bind to the same E1A responsive promoter elements. There is evidence to suggest that in some cases the effect of E1A is stimulation of DNA binding activity, which may involve a change in phosphorylation of a transcription factor. A direct interaction between E1A and DNA-bound factors has been suggested in other cases, but so far there is no direct evidence for such interaction.

1.2.4. Enhancer repression by E1A proteins

Transcriptional repression may be an important mechanism for the regulation of eucaryotic gene expression (for a review see Levine and Manley, 1989). E1A protein products can repress transcription dependent on either viral or cellular enhancer sequences (Borelli *et al.*, 1984; Hen *et al.*, 1985; Velich and Ziff, 1985; Hen *et al.*, 1986; Stein and Ziff, 1987; Stein and Whelan, 1989), including the E1A enhancer (Tibbets *et al.*, 1986). Extensive mutational analysis has revealed that the repression function is distinct from E1A transactivation (Borelli *et al.*, 1984; Velich and Ziff, 1985; Schneider *et al.*, 1987). There is

some controversy over the role of conserved region II in transcriptional repression; mutations within this region have been found to have marked effect (Lillie *et al.*, 1986; Velich and Ziff, 1988) or little or no effect (Kuppuswamy and Chinnadurai, 1987; Schneider *et al.*, 1987; Jelsma *et al.*, 1989; van Dam *et al.*, 1989; Rochette-Egly *et al.*, 1990; Stein *et al.*, 1990) on repression activity. Sequences within CRI are essential for repression (Lillie *et al.*, 1987; Schneider *et al.*, 1987; Jelsma *et al.*, 1989; Stein *et al.*, 1990). In addition, sequences N-terminal to CRI are required for transcriptional repression functions (Jelsma *et al.*, 1989; Stein *et al.*, 1990). There is strong correlation between enhancer repression activity and binding of a 300Kd cellular protein whose association with E1A requires sequences within the N-terminus (Egan *et al.*, 1988; Whyte *et al.*, 1989), suggesting a possible role for this protein-protein interaction in enhancer repression (Jelsma *et al.*, 1989; Stein *et al.*, 1990). The 300Kd protein has not been identified and its function is unknown.

1.2.5. Other functions of the E1A proteins

One of the features of the E1A proteins is their involvement in oncogenic transformation (for reviews see Berk, 1986a; Mak and Mak, 1986). E1A products can immortalize primary rodent cells in culture and cooperate with other oncogenes, such as the E1B gene products or an activated *ras* gene, to produce fully transformed cells.

E1A proteins form stable complexes with three major cellular polypeptides with molecular weight around 105Kd, 107Kd and 300Kd (Yee and Branton, 1985; Harlow *et al.*, 1986). Recent studies have shown that the binding sites for these cellular proteins lie within the region of E1A needed to cooperate with an activated *ras* gene for transformation, suggesting that these proteins may play a key role in cellular transformation (Whyte *et al.*, 1988b; Egan *et al.*, 1988; Whyte *et al.*, 1989; Jelsma *et al.*, 1989). The 105Kd protein (p105-RB) is the product of the retinoblastoma sus-

ceptibility gene (Whyte *et al.*, 1988a, Egan *et al.*, 1989), an anti-oncogene that like other anti-oncogenes is thought to act in a pathway that restricts cell proliferation (for review see Klein, 1988; Hansen and Cavenee, 1988). p105-RB has also been shown to complex with the SV40 large T antigen (DeCaprio *et al.*, 1988; Ludlow *et al.*, 1989). Mutants of T antigen (DeCaprio *et al.*, 1988) as well as E1A (Egan *et al.*, 1988; Jelsma *et al.*, 1989; Whyte *et al.*, 1989; Egan *et al.*, 1989) that fail to bind p105-RB have drastically reduced transforming properties, leading to the suggestion that complex formation inactivates p105-RB, thereby permitting the uninhibited growth associated with oncogenically transformed cells.

This association is not the only event required for E1A mediated transformation since E1A products that bind p105-RB but fail to bind the 300Kd protein are also defective for transformation (Egan *et al.*, 1988; Jelsma *et al.*, 1989; Whyte *et al.*, 1989; Stein *et al.*, 1990), indicating a possible role for the 300Kd protein in growth control as well. Furthermore, Stein *et* al., (1990) have shown that the ability of E1A to induce DNA synthesis in quiescent cells (Spindler *et al.*, 1985) and repress enhancer-stimulated transcription (see 1.2.4.) colocalize with N-terminal transforming functions and correlate closely with binding of the 300Kd protein. The binding of either the 300Kd protein or p105-RB appears to be sufficient for the induction of DNA synthesis (Howe *et al.*, 1990) while binding of both is needed for transformation (Egan *et al.*, 1988; Jelsma *et al.*, 1989). This suggests that the pathways by which p105-RB and the 300Kd protein participate in the control of cell growth are more complex than those that control the onset of DNA synthesis (Howe *et al.*, 1990).

The role of the 107Kd protein is unknown; this protein and p105-RB interact with overlapping sequences within CRII (Egan *et al.*, 1988; Whyte *et al.*, 1989) but the two polypeptides appear to be unrelated (Dyson *et al.*, 1989). The 107Kd protein also forms protein complexes with the large T antigens of SV40 and JC virus and may represent a

further common component in transformation by E1A and large T antigen (Dyson *et al.*, 1989).

The transforming regions of the E1A proteins may be involved in induction of expression of some cellular proteins (Zerler *et al.*, 1987; Simon *et al.*, 1987; Kaddurah-Daouk *et al.*, 1990). The significance of this for the role of E1A in oncogenesis is not clear but Kaddurah-Daouk *et al.*, (1990) have suggested that the induction of an enzyme for cellular energy metabolism, by E1A, may be related to metabolic events that take place after oncogenic activation.

1.3. Early region 1B

Early region 1B (E1B) together with the E1A region is involved in transformation by adenoviruses and this has led to a great interest in this gene complex. However, E1B gene products also have interesting properties in a lytic infection and this section will focus mainly on E1B in the lytic cycle.

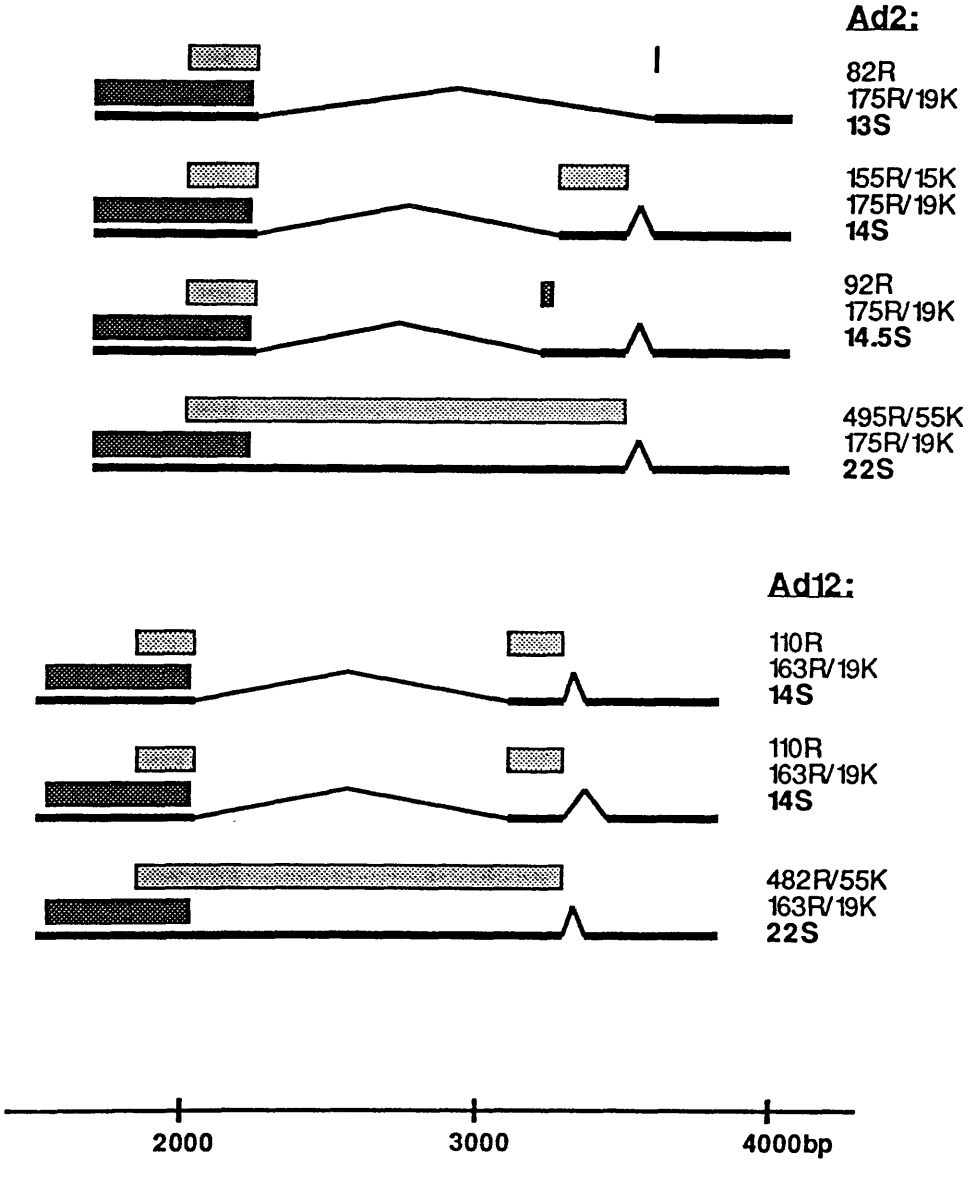
1.3.1. General organization of the E1B region

The E1B region is located immediately downstream of the E1A region. The E1B DNA sequence is known for a number of serotypes (Ad2, Gingeras *et al.*, 1982; Ad5, Bos *et al.*, 1981; Ad7, Dijkema *et al.*, 1982; Ad12, Kimura *et al.*, 1981; Bos *et al.*, 1981; Ad40, van Loon *et al.*, 1987b; Ishino *et al.*, 1988; Ad41, van Loon *et al.*, 1987b; MAV-1, Ball *et al.*, 1988; 1989; Tupaia adenovirus, Flügel *et al.*, 1985). There is considerable sequence homology between the different serotypes both on the DNA and amino acid level (van Ormondt and Hesper, 1983; Ishino *et al.*, 1988). Unlike the E1A region, this homology is not limited to specific regions but is spread over most parts of the open reading frames. Two large open reading frames are apparent from the DNA sequences (figure 4).

Transcription maps from the E1B region have been determined for several serotypes (Ad2, Berk and Sharp, 1978; Chow *et al.*, 1979; Perricaudet *et al.*, 1980; Virtanen and Petersson, 1985; Ad5, Bos *et al.*, 1981; Ad7, Yoshida and Fujinaga, 1980; Dijkema *et al.*, 1982; Ad12, Bos *et al.*, 1981; Virtanen *et al.*, 1982; for a comparison of Ad5, 7 and 12 see van Ormondt and Hesper, 1983; Ad41 (transformed cells), van Loon *et al.*, 1987b; MAV-1, Ball *et al.*, 1989). Figure 4 shows the transcription maps and open reading frames for Ad2 and Ad12. The two major E1B transcripts in Ad2 are 22S and 13S coterminal mRNAs; they use different splice donor sites but share a splice acceptor site. Two minor species, 14S and 14.5S include the splice junction used in the 22S mRNA and in addition have the 13S splice

Figure 4: Structure of the Ad2 and Ad12 E1B mRNAs and open reading frames.

Sedimentation values (S) for Ad2 mRNAs and molecular weight (K) of Ad2 polypeptides are shown and the same nomenclature used for the Ad12 counterparts. Number of amino acid residues (R) for each protein product is indicated. Shades of hatched boxes denote different reading frames.


Ad2:
82R
175R/19K
13S
155R/15K
175R/19K
14S
92R
175R/19K
14.5S
495R/55K
175R/19K
22S
Ad12:
110R
163R/19K
14S
110R
163R/19K
14S
482R/55K
163R/19K
22S
2000
3000
4000bp

donor site linked to different splice acceptors; the 14S splice is in frame but the 14.5S splice changes the reading frame. A 9S mRNA encoding polypeptide IX is transcribed from a separate promoter within the E1B region. Ad12 also has a 22S mRNA and two transcripts corresponding to the Ad2 14S mRNA, differing only in the use of different splice acceptor sites in the 3' untranslated region, but no 13S mRNA has been identified.

The other human serotypes have not been as extensively studied but appear to have a similar organization. The MAV-1 E1B region differs in only having two large transcripts, one corresponding to a 22S mRNA and another identical but with a splice removing most of the first open reading frame.

In addition to E1B transcripts from the E1B promoter, several authors have reported E1A-E1B cotranscripts which encode E1B polypeptides. These have been detected in cells lytically infected or transformed with Ad2 (Berk and Sharp, 1978; Kitchingman and Westphal, 1980; Hashimoto *et al.*, 1984), Ad5 (van den Elsen *et al.*, 1983) or Ad12 (Sawada and Fujinaga, 1980; Saito *et al.*, 1983). The cotranscripts are relatively low in abundance, they have not been extensively analysed and cDNA sequences have not been determined. However, the Ad12 cotranscripts are reported to have 5' ends and splice sites identical to those of the major E1A mRNAs and to extend to the 3' end of E1B, with a splice corresponding to the splice in E1B 22S mRNA. Berk and Sharp (1978), describe an Ad2 transcript consisting of fewer than 50bp from the 5' end of E1A linked to a site near the 5' end of E1B, resulting in a 22S type E1A-E1B cotranscript, but the precise structure of the E1A-E1B junction was not analysed. Hashimoto *et al.*, (1984) report *in vitro* translation of Ad2 E1A proteins from E1B-selected transcripts, indicating a different structure for E1A-E1B cotranscripts. They suggest that potential splice donor and acceptor sequences, spanning the E1A-E1B junction, may be utilized in these cotranscripts, but the usage of these splice sites has not so far been demonstrated.

E1B mRNAs encode two major polypeptides in different

reading frames, a 19Kd protein (19K) encoded by the first ORF and a 55Kd protein (55K) by the second ORF (Bos *et al.*, 1981; Anderson *et al.*, 1984) (see figure 4). The first ORF is present in all the E1B mRNA species while the whole of the second ORF is only present in the 22S mRNA. Polypeptides related to the 55K protein are encoded by the smaller E1B mRNAs (Green *et al.*, 1982; Lucher *et al.*, 1984; Lewis and Anderson, 1987). The Ad2 13S and 14.5S mRNAs encode 82 residue (R) and 92R products respectively, both comprising the amino terminus of the 55K protein linked to a different ORF after the splice junction in the mRNA (Lewis and Anderson, 1987). Similarly, the 14S mRNA encodes a 155R (15K) protein which consists of the amino- and carboxy-termini of the 55K protein (Lewis and Anderson, 1987).

1.3.2. E1B transcription

E1B gene expression appears to be controlled in a complex manner on several different levels including regulation of initiation of transcription, splicing and mRNA stability. At early times in infection (3.5-9 hr p.i.) the 22S mRNA accumulates preferentially in the cytoplasm, while the 13S mRNA remains at low levels until 8 hr p.i. when it begins to increase in abundance, attaining a level comparable to that of the 22S mRNA by 12 hr p.i. (Glenn and Ricciardi, 1988). The minor 14S and 14.5S mRNA species were not analysed in this experiment but Virtanen and Pettersson (1985) have shown that these are also differentially regulated. At 8 hr p.i. the 14.5S mRNA is 20 fold less abundant than the 14S mRNA while at late times they are present in approximately equal amounts. The differential accumulation of mRNA species from the same primary transcript is influenced by a change in relative cytoplasmic stability (Wilson and Darnell, 1981): The 72K DBP has been reported to cause rapid turnover of early mRNA (Babich and Nevins, 1981). These authors suggest that at late times in infection the 13S E1B mRNA accumulates since it is no longer destabilized by the DBP, but the 22S mRNA fails to accumulate because of

specific degradation mediated by an unknown late viral function. However, the relative abundance of E1B mRNAs may also be regulated by a different mechanism. Montell *et al.*, (1984b) have shown that the relative concentrations of the 13S to 22S nuclear mRNAs increased at late times during infection. Furthermore, an infection with a viral mutant with defect in the 13S splice donor site results in the use of cryptic splice donor sites near the original 13S site, specifically at a late time in infection. This evidence suggests that the temporal increase in the relative abundance of the 13S mRNA is at least partly due to an increased tendency to use the 13S splice donor site at late times in infection.

1.3.3. The E1B promoter

Like the other adenovirus early promoters the E1B promoter responds to transcriptional activation by E1A. The basic Ad2 E1B promoter is relatively simple, consisting only of a TATA box and a binding site for the cellular transcription factor Sp1 (Wu *et al.*, 1987). These elements are conserved in other adenovirus serotypes (see figure 20, section 4.3.). Studies involving Ad12 have suggested that sequences upstream of these promoter elements may also be important for gene expression (Bos and ten Wolde-Kraamwinkel, 1983). In the Ad5 E1B promoter four protein binding sites have been identified upstream of the basic promoter (see figure 3 and figure legend for sequences of binding sites) (Parks *et al.*, 1988). Site IV, which resembles sequences found in analogous positions in other serotypes, is located downstream of the E1A ORF. Sites I, II and III are within E1A coding sequences; similar sequences have been identified in control regions of various viral and cellular genes. Parks *et al.*, (1988) showed that in an *in vitro* analysis increased transcriptional activity was seen in the presence of these upstream sequences only when the Sp1 binding site was absent. Wu *et al.*, (1987) found that deletion of sequences upstream of the basic

promoter had little effect on E1B transcription *in vivo*, suggesting that these upstream elements are not important for E1B transcription. However, the possibility remains that these protein binding sites may be of importance under conditions not reflected in those experiments and perhaps different regulatory elements are important in different cell types.

The Sp1 binding site (Kadonaga *et al.*, 1986) in the E1B promoter is essential for efficient transcription from that promoter (Wu and Berk, 1987; Parks *et al.*, 1988). However, mutations of this site do not interfere with transactivation by the E1A protein or by the pseudorabies virus immediate early (IE) protein (Wu and Berk, 1987; 1988a). Transcription factor Sp1 is a sequence specific DNA binding protein; it enhances transcription from several viral and cellular promoters that include at least one GC box (Kadonaga *et al.*, 1986). Sp1 binds to some but not all DNA sequences that contain the GGGCGG hexanacleotide, affinity is affected by flanking sequences; Sp1 binding sites are functional in either orientation (Kadonaga *et al.*, 1986). cDNA clones encoding this factor have been isolated and the DNA sequence determined (Kadonaga *et al.*, 1987; 1988). The protein binds to DNA via zinc finger motifs (Kadonaga *et al.*, 1987; 1988). Glutamine rich domains in the protein, outside the DNA binding region, are responsible for mediating transcriptional activation (Courey and Tjian, 1988). Sp1 molecules activate transcription synergistically and the synergy appears to be at the level of transcriptional activation rather than DNA binding; that is, the protein-protein interaction appears to aid promoter activation by directly increasing the rate at which the general transcription machinary initiates transcription, rather than by stabilizing the protein-DNA complex (Courey *et al.*, 1989). Pugh and Tjian (1990) have suggested that Sp1 may activate transcription by interacting with the general transcription apparatus, possibly TFIID, through specific coactivators. However, such coactivators have not so far been identified. Altered spacing between the Sp1 site and TATA box in the E1B

promoter affects transcriptional activation by Sp1 (Wu and Berk, 1988b). This is consistent with protein-protein interaction between Sp1 and TFIID playing a role in transcriptional activation. Sp1 binding does not affect the DNA binding activity of TFIID but instead appears to act by increasing the number of productive transcription complexes, possibly by increasing the binding activity of some other factor in the initiation complex (Schmidt *et al.*, 1989).

E1A transactivation of E1B is mediated through the TATA box and is not affected by Sp1 binding; the E1B TATA element is also a target for activation by the pseudorabies virus IE protein (Wu *et al.*, 1987; Wu and Berk, 1988a,b). This suggests that both these transactivating proteins can increase expression from the E1B promoter by stimulating the activity of the TATA binding factor TFIID. Studies with partially purified pseudorabies virus IE protein have shown that this protein stimulates transcription *in vitro* by increasing the efficiency or rate of TFIID binding to the DNA template to form the preinitiation complex (Abmayr *et al.*, 1988). Leong *et al.*, (1988) have shown that the fraction including TFIID from adenovirus infected cell extracts has higher transcriptional activity than the parallel mock infected extract, consistent with the idea that E1A stimulates TFIID activity. The mechanism of such stimulation is still not well understood.

1.3.4. The 19K protein

The smaller E1B ORF is translated into a 19 kilodalton polypeptide (19K) which is located in the nuclear envelope and cytoplasmic membranes of infected and transformed cells (White *et al.*, 1984a; Mitchison *et al.*, 1990). Posttranslational acylation may play a role in membrane association of the protein (McGlade *et al.*, 1987). A subset of the protein molecules are phosphorylated but this appears to be nonessential for the function of the protein as shown by site-directed mutagenesis studies (McGlade *et al.*, 1989).

Several phenotypes have been associated with defects in

19K expression. E1B 19K mutants show a host range phenotype for virus replication in human cells; replication in Hela cells is unaffected while replication in KB cells and WI38 cells is defective and enhanced respectively (White *et al.*, 1986; White and Stillman, 1987). Mutations can also cause enhanced cytopathic effect (*cyt*) in infected Hela and KB cells (Takemori *et al.*, 1968, 1984; Pilder *et al.*, 1984; Subramanian *et al.*, 1984b; White *et al.*, 1984b). This phenotype is also associated with large plaque morphology (*lp*) (Chinnadurai, 1983; Subramanian *et al.*, 1984b). Furthermore, 19K mutants cause extensive degradation of viral and cellular DNA (*deg*) in most cell lines (D'Halluin *et al.*, 1979; Ezoe *et al.*, 1981; Lai Fatt and Mak, 1982; Pilder *et al.*, 1984; Subramanian *et al.*, 1984a; White *et al.*, 1984b); therefore a major function of the 19K protein appears to be to protect or stabilize viral and cellular DNA during infection. These phenotypes are all dependent on the expression of E1A 12S or 13S gene products, and cellular factors may also be involved since the phenotype is host cell dependent (White and Stillman, 1987). Mutants in the 19K protein are defective for virion and DNA mediated transformation (Chinnadurai, 1983; Babiss *et al.*, 1984; Fukui *et al.*, 1984; White *et al.*, 1984b; Barker and Berk, 1987; Herbst *et al.*, 1988). The 19K protein also modulates viral gene expression. In the presence of E1A 13S or 12S gene products it appears to have a negative effect on viral gene expression (White *et al.*, 1986; White and Stillman, 1987) while in the absence of E1A the 19K protein has a positive effect on the expression of viral genes (Herrmann *et al.*, 1987; White *et al.*, 1988a; Herrmann and Mathews, 1989). In cotransfection experiments, the presence of the 19K protein causes an increase in the level of gene expression from plasmids encoding viral or cellular proteins (Herrmann *et al.*, 1987; Vales and Darnell, 1989; Herrmann and Mathews, 1989; Shiroki *et al.*, 1990). The 19K protein has been shown to activate transcription indirectly by increasing the stability of plasmid and viral DNA (Herrmann and Mathews, 1989). This is consistent with the fact that the 19K

protein is known to protect viral and chromosomal DNA from degradation during viral infection. The association of the 19K protein with the nuclear lamina has been implicated in this function since this localization is disrupted in cells infected with *deg* mutants (White *et al.*, 1984a), leading to the suggestion that DNA stability is increased by altering its structure or distribution (Herrmann and Mathews, 1989). The 19K protein alters the structure and organization of the nuclear lamina and the intermediate filament network (White and Cipriani, 1989, 1990) and this may compartmentalize DNA or protect it from degradation by some unknown mechanism (Herrmann and Mathews, 1989). Disruption of the organization of the intermediate filaments by the 19K protein has also been associated with the transformed cell phenotype (White and Cipriani, 1989, 1990). These authors have suggested that this interference may promote transformation by modulating signal transduction, cell-cell interaction or chromatin structure.

1.3.5. The 55K protein and related polypeptides

The large E1B ORF is translated into a 55 kilodalton protein (55K) from the 22S mRNA (Bos *et al.*, 1981; Anderson *et al.*, 1984). Related proteins have been identified from Ad2 infected cells and *in vitro* translated mRNA (Anderson *et al.*, 1984; Lewis and Anderson, 1987). An 82R protein is a product of the 13S mRNA; it comprises the amino terminal 78 codons of the large ORF. A 155R (15K) protein, amino- and carboxy-coterminal with the 55K protein is a product of the 14S mRNA. These polypeptides have not been assigned a function and they are dispensable for virus growth and transformation in the presence of the 55K protein (Montell *et al.*, 1984b). A 92R predicted product of the 14.5S mRNA, sharing the amino-terminus with the 55K protein, has not so far been identified.

The 55K protein is modified by phosphorylation on threonine and serine residues (Malette *et al.*, 1983; Spindler *et al.*, 1984; Schughart *et al.*, 1985). The impor-

tance of this modification to the biological function of the protein is not known. Virus replication in most cell types is severely affected in the absence of a functional 55K protein. The E1B 55K protein plays a critical role in the selective expression of viral genes; unlike wild type adenovirus, viruses carrying mutations that impair production of the 55K protein do not induce inhibition of cellular protein synthesis or selective entry of viral mRNA into the cytoplasm (Babiss and Ginsberg, 1984; Babiss *et al.*, 1985; Pilder *et al.*, 1986; Williams *et al.*, 1986; Barker and Berk, 1987). A similar phenotype is observed with viruses defective for expression of the 34K protein encoded by ORF 6 of the E4 region (see 1.1.2.2.) (Halbert *et al.*, 1985; Weinberg and Ketner, 1986; Cutt *et al.*, 1987). Sarnow *et al.*, (1984) have shown that the 55K protein and the E4 34K protein form a complex in infected cells and they and others (Pilder *et al.*, 1986) have suggested that this complex is a functional unit involved in the mRNA accumulation process. Further evidence for this comes from studies using double mutants, lacking the ability to synthesize both proteins. Such double mutants have no more severe phenotype than single mutants carrying either mutation (Cutt *et al.*, 1987; Bridge and Ketner, 1990).

In the absence of a functional 55K protein, no primary defect in transcription rate of late viral genes is seen, and their RNAs are polyadenylated and spliced within the nucleus (Pilder *et al.*, 1986). Furthermore, these authors showed that a cDNA copy of a late gene produced unspliced mRNAs that failed to accumulate in the cytoplasm of E1B 55K mutant infected cells, confirming that the defect is not at the level of mRNA splicing. This led to the conclusion that the 55K protein regulates gene expression post-transcriptionally, either at the level of RNA transport or by stabilizing viral mRNA upon entry into the cytoplasm. Leppard and Shenk (1989) looked at the site of action of the 55K protein by monitoring the movement of newly synthesized mRNA through a series of biochemically defined cellular subfractions. They found that in cells infected with a 55K deficient

virus, late viral mRNA failed to accumulate efficiently within a specific nuclear compartment, where RNA was normally seen to accumulate after leaving the nuclear matrix and before associating with the nuclear envelope. This demonstrates that the 55K protein acts within the nucleus.

The absence of either or both of the 34K E4 protein and the 55K protein results in a reduction of late viral mRNA in the nucleus as well as a defect in the transport of this nuclear mRNA (Sandler and Ketner, 1989; Bridge and Ketner, 1990). This is consistent with the idea that the 55K/34K complex facilitates viral RNA metabolism at an intranuclear step, possibly by maintaining the stability of the pool of processed RNA molecules prior to translocation or perhaps facilitating the translocation step itself (Ornelles and Shenk, 1991).

Although the 55K protein and E4 34K protein are found as a complex in infected cells (Sarnow *et al.*, 1984), large quantities of free proteins have also been detected (Cutt *et al.*, 1987; Smiley *et al.*, 1990). Biochemical and immunochemical studies have shown that the 55K protein is present at several locations in infected cells. By immunofluorescent staining it is found in a diffused pattern within the cytoplasm and in an intensely stained fibrous body adjacent to the nucleus, while in the nucleus it is present in a granular, diffuse distribution and in local concentrations within and about the periphery of viral inclusion bodies (Ornelles and Shenk, 1991). These bodies have been shown to be the site of viral DNA synthesis and accumulation (Moyne *et al.*, 1978; Brigati *et al.*, 1983; Voelkerding and Klessig, 1986) and may also be the sites of late viral RNA synthesis (Moyne *et al.*, 1978; Wolgemuth and Hsu, 1981). Smiley *et al.*, (1990) examined the association of the 55K protein with nuclear pore complexes. They found that although some of the nuclear 55K protein fractionated with the pore complex-lamina the protein does not form a stable complex with these organelles during the late phase of infection. This led them to conclude that the role of the 55K protein was not to modify the function of nuclear pore complexes but rather to

affect some earlier step in the RNA transport process. This is consistent with the model for 55K function proposed by Ornelles and Shenk (1991). They suggest that the 55K/34K protein complex interacts with, and draws to the periphery of viral replication-transcription centres, a nuclear factor required for movement of mRNAs from their site of processing to the nuclear pores for transport to the cytoplasm. This would account for the ability of the complex to inhibit accumulation of RNAs transcribed from cellular locations as well as stimulating accumulation of viral transcripts. Studies using *in vitro* transport systems have identified nuclear factors which could play such a role in RNA transport (Moffett and Webb, 1983; Subramaniam *et al.*, 1990).

Much of our current understanding of the functions of the E1B 55K protein comes from studies on viral mutants (see Williams, 1986 for a review; Barker and Berk, 1987; Byrd *et al.*, 1988; Breiding *et al.*, 1988; Mak and Mak, 1990; Yew *et al.*, 1990; Kao *et al.*, 1990). Those studies have involved mutant viruses of subgroup A (Ad12) and C (Ad2, Ad5). While these mutants share the phenotype of a defect in late viral mRNA transport and lack of host cell protein synthesis shut off, some differences have been revealed between the two subgroups. Ad12 mutant viruses defective for the 55K E1B protein function are severely impaired for viral DNA synthesis (Shiroki *et al.*, 1986; Breiding *et al.*, 1988; Mak and Mak, 1990). This is in contrast with subgroup C where the 55K protein is not required for viral DNA replication (Lassam *et al.*, 1978; Babiss and Ginsberg, 1984; Williams *et al.*, 1986). A further difference is seen in the host range characteristics of Ad12 and Ad5 E1B 55K mutants. While Ad5 mutants with a growth defect on Hela cells grow to wild type levels on primary human embryo kidney cells (Harrison *et al.*, 1977), Ad12 mutants do not grow efficiently upon these cells (Breiding *et al.*, 1988). The basis for this apparent difference in function between those two closely related polypeptides is not known.

Like other adenovirus E1 gene products the 55K protein

has been implicated in cellular transformation by adenoviruses. Transformation by viral infection appears to require the entire E1 region including the 55K protein coding sequences (Babiss *et al.*, 1984; Bernards *et al.*, 1986; Barker and Berk, 1987; Byrd *et al.*, 1988). However, efficient DNA-mediated transformation using viral DNA carrying mutations in the 55K ORF has been reported, suggesting that the 55K protein is non-essential for DNA-mediated transformation (Rowe and Graham, 1983; Babiss *et al.*, 1984). Others have found that mutations in the 55K protein decrease or abolish transformation by plasmid or viral DNA (Bernards *et al.*, 1986; Barker and Berk, 1987). Studies on E1B 55K mutants are complicated by the fact that due to the overlap in the 55K and 19K E1B ORFs many of the mutants retain the ability to express a truncated protein from the 55K ORF. However, a mutant virus, dl1520, constructed by Barker and Berk (1987), which does not express any truncated products from the 55K ORF or 55K related polypeptides, is unable to transform cells by infection or DNA transfection.

The role of the 55K protein in transformation has not been elucidated. The Ad5 55K protein is known to interact with a cellular protein p53 (Sarnow *et al.*, 1982b) a potential tumor suppressor gene product, and it has been suggested that this interaction inactivates p53 function (see Levine, 1990 for review). However, in Ad12 transformed cells p53 is not associated with the E1B 55K protein (Zantema *et al.*, 1985) suggesting that this interaction is not necessary for the transformation process. Furthermore, mutational analysis revealed no correlation between the ability of Ad5 55K protein to bind p53 *in vitro* and transformation efficiency (Yew *et al.*, 1990; Kao *et al.*, 1990). Recently van den Heuvel *et al.*, (1990) have shown that complex formation between p53 and the Ad5 E1B 55K protein reduces oncogenicity of transformed cells. This is consistent with Ad12 transformed cells being more oncogenic than Ad5 transformed cells.

Mutational analysis of the Ad2 and Ad12 55K proteins have shown that while large parts of the 55K are needed for DNA-

(Ad12) and virus-replication and transformation function, the two functions can be separated by mutation (Mak and Mak, 1990; Yew *et al.*, 1990). This indicates that the role of the 55K protein in replication and transformation involves two different biological functions. However, the precise mechanism by which the 55K protein provides those functions awaits further studies.

1.4. The 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). Although several adenovirus serotypes can be isolated from diarrhoeal stools most earlier epidemiological studies generally found as many adenovirus isolates in the stools of controls as from those with diarrhoea (Ramos-Alvares and Sabin, 1958). However, more recent studies have shown a close correlation between two distinct serotypes, designated Ad40 and Ad41 (Uhnoo *et al.*, 1983; de Jong *et al.*, 1983), and gastroenteritis (Brandt *et al.*, 1979; 1985; Yolken *et al.*, 1982; Chiba *et al.*, 1983; Uhnoo *et al.*, 1984; Leite *et al.*, 1985; Kidd *et al.*, 1986; Kotloff *et al.*, 1989). Accordingly those two serotypes are commonly referred to as the enteric adenoviruses (Jacobsson *et al.*, 1979; Retter *et al.*, 1979). For a review of the enteric adenoviruses see Albert (1986).

1.4.1. Identification and classification

The enteric adenoviruses were first detected by electron microscopy on stool specimens from infants with acute gastroenteritis (Flewett *et al.*, 1975). It was found that they were excreted in large numbers but failed to grow in tissue culture. Further studies showed that while enteric adenoviruses did not grow in cells routinely used for the propagation of other adenoviruses such as human embryo kidney cells (HEK), KB, Hela, Hep-2 and WI-38, they grew readily in 293 cells (Takiff *et al.*, 1981), HEK cells transformed with the Ad5 E1 region (Graham *et al.*, 1977). Kidd and Madeley (1981) showed that some but not all enteric adenovirus specimens could be passaged in Chang conjunctiva cells. Morover, their serological data (Johansson *et al.*, 1980; Kidd and Madeley, 1981) indicated that irrespective of infectivity in Chang cell cultures, different isolates of enteric adenoviruses were antigenically related and distinct from the (then) 35 established adenovirus serotypes. The

presence of two distinct serotypes of enteric adenoviruses was revealed by studies on large numbers of clinical isolates (Uhnoo *et al.*, 1983; de Jong *et al.*, 1983). The two types were indistinguishable in heamagglutination inhibition tests but neutralization tests distinguished between them. This data was further supported by restriction endonuclease analysis showing different DNA restriction patterns for the two new serotypes, Ad40 and Ad41 (Uhnoo *et al.*, 1983; Takiff *et al.*, 1984). Those two serotypes are now classified as a separate subgroup, F, based on several criteria such as cross-reactivity in immunological studies, DNA homology and size of internal structural polypeptides (van Loon *et al.*, 1985b; Wadell *et al.*, 1987; Hierholzer *et al.*, 1988).

1.4.2. Epidemiology

Enteric adenoviruses have been detected in the stools of young children and infants with acute gastroenteritis in several studies conducted in industrialized and developing countries. These viruses are prevalent throughout the year with little seasonal variations, indicating that they may be endemic (de Jong *et al.*, 1983; Uhnoo *et al.*, 1984; Brandt *et al.*, 1985; Johansson *et al.*, 1985; Tiemessen *et al.*, 1989). In two one year studies conducted in Sweden, enteric adenoviruses were detected in 7-8% of children with diarrhoea (Uhnoo *et al.*, 1984; Johansson *et al.*, 1985). Two similar studies in South Africa found enteric adenoviruses in 6.5% and 13.2% of patients, although in the latter study it was the only pathogen in 6.2% of cases (Kidd *et al.*, 1986; Tiemessen *et al.*, 1989). A seven year study in the USA detected adenoviruses in feces of 5.1% of infants with acute gastroenteritis and concluded that most of them were causally associated with the disease (Brandt *et al.*, 1985). This rate of detection of enteric adenoviruses associated with disease (5-13%) suggests an important role in viral gastroenteritis second only to rotaviruses. Moreover, isolation of enteric adenoviruses in as much as 52% of hospitalized children with diarrhoea has been reported in a short

study in the USA (Yolken *et al.*, 1982). A recent one year study in a rural community in Guatemala monitored fecal excretion of Ad40 and Ad41 and rotaviruses in 194 children under the age of 3, as well as studying children hospitalized because of diarrhoea (Cruz *et al.*, 1990). Half of the hospitalized children shed rotaviruses while over 30% shed Ad40 or Ad41. In the community, 22% of children excreted Ad40 or Ad41 whereas 10% shed rotaviruses. Asymptomatic infections were found with both adenoviruses and rotaviruses. Up to four Ad40 or Ad41 infections were observed in some children. Of 385 episodes of diarrhoea studied, over 20% lasted more than 2 weeks; 25% of these were associated with Ad40 or Ad41, 6% with rotaviruses. In a community where 25% of mortality in preschool children is caused by diarrhoea or dehydration (Cruz *et al.*, 1990) this suggests a great clinical importance for the enteric adenoviruses as well as rotaviruses.

Ad40 and Ad41 have been isolated with approximately equal frequency in most studies which have distinguished between the two (de Jong *et al.*, 1983; Uhnoo *et al.*, 1982; Johansson *et al.*, 1985). They appear to cause disease mainly in infants and young children, with median age of patients reported as 7-9 months (Brandt *et al.*, 1985; de Jong *et al.*, 1983).

1.4.3. Pathogenesis

Clinical features of enteric adenovirus infection were studied by Uhnoo *et al.*, (1984; 1986). After an incubation period of seven to eight days, infected children had a clinically moderate disease with diarrhoea and vomiting. Elevated temperatures were rare and less than 20% of patients had respiratory symptoms. The two serotypes caused similar symptoms although Ad41 infected children had more protracted diarrhoea (mean 12.2 days) than those infected with Ad40 (mean 8.6 days). These studies showed that the enteric adenoviruses cause a more prolonged but milder infection with a lower frequency of vomiting and elevated

temperatures than did rotaviruses. A study in the USA has shown that dehydration is as likely to develop in children with enteric adenovirus diarrhoea as among those with rotavirus diarrhoea (Kotloff *et al.*, 1989). Moreover, enteric adenoviruses have been associated with a fatal disease. Ad41 virus particles were isolated from small intestinal mucosal cells in a fatal case of adenovirus gastroenteritis (Whitelaw *et al.*, 1977; Johansson *et al.*, 1985). Upon propagation in tissue culture this strain did not appear to be unusually virulent (Johansson *et al.*, 1985).

1.4.4. Propagation of Ad40 and Ad41 in tissue culture

A remarkable feature of the enteric adenoviruses is their growth restriction in cultured human cell lines, hence their late discovery as important pathogenic agents. The difficulty in culturing clinical isolates of these viruses *in vitro* is not due to low virus concentrations since up to 10^{11} virus particles can be detected per gram of diarrhoeal stool (Gary *et al.*, 1979; Retter *et al.*, 1979; Takiff and Straus, 1982). Isolates of enteric adenoviruses do not replicate in human embryonic kidney cells (HEK) (Kidd and Madeley, 1981; Takiff *et al.*, 1981; Takiff and Straus, 1982; de Jong *et al.*, 1983), which are routinely used for the isolation of other adenoviruses. Takiff *et al.*, (1981) reported efficient growth of enteric adenovirus isolates in 293 cells, HEK cells transformed with the Ad5 E1 region, and suggested that the early Ad5 gene functions expressed in those cells might be providing helper functions to permit virus replication. However, while successful growth of Ad40 in 293 cells has been reported (de Jong *et al.*, 1983; Brown *et al.*, 1984), others have failed to detect Ad40 replication in these cells (Chiba *et al.*, 1983; Uhnoo *et al.*, 1983). This may reflect differences between virus isolates or variations between laboratories.

A detailed study on the growth characteristics of around 200 isolates of enteric adenoviruses in different cell lines was conducted by de Jong *et al.*, (1983). They report

difficulties in growing both Ad40 and Ad41 and although both serotypes propagated in 293 cells and tertiary cynomologus monkey kidney cells (tCMK) they found in general that growth depended on virus strain, cell type, subline and batch and other unknown factors. This may explain conflicting results obtained by others attempting to propagate enteric adenoviruses in several cell lines. Uhnoo *et al.*, (1983) used clinical isolates to infect A549, HT29 and 293 cells and found that while Ad41 isolates grew in HT29 and 293 cells all the cell lines were nonpermissive for Ad40. Different results were obtained by Witt and Bousquet (1988) investigating the growth properties of purified Ad40 and Ad41 virions. Both serotypes were infectious for A549 and 293 cells as well as Chang conjunctiva and KB cells whereas Hela cells were only permissive for Ad41. Virus harvest of all permissive cells was infectious on A549 cells. These authors suggest that the increased host range of enteric adenoviruses seen in this study compared to previous studies may reflect the difference between using purified virions as opposed to clinical material. They propose that other viruses present in virus isolates may partly explain the host cell restriction of enteric adenoviruses from clinical isolates. However, this would not account for the differences in host range between the enteric and other adenoviruses in clinical specimens. Although Witt and Bousquet observed virus replication with both Ad40 and Ad41, infectious titers obtained, as determined by an indirect immunofluorescent assay, were relatively low for both serotypes and Ad40 in particular, in all the cell lines. This indicates that these cell lines are only semipermissive for the enteric adenoviruses and their propagation is restricted in tissue culture compared to an *in vivo* infection.

An interesting observation reported by Witt and Bousquet (1988) is that when high concentrations of Ad40 were used in an infection, virus replication was not observed, while at a lower multiplicity of infection Ad40 did replicate in the same cell lines. This might suggest the presence of defective virus particles in the virus stock, interfering

with infection by nondefective virus particles. Indeed it has been shown that while the production of Ad40 virions is 3-10-fold lower that that observed for other adenoviruses, the yield of infectious virus is 100-1000-fold less (Brown, 1985). However, this inhibitory effect of using high input of Ad40 virions may be cell type specific since Witt and Bousquet did not see such inhibition in KB cells and similarly this effect has not been detected in KB derived cell lines (Nancy Mackay, personal communication).

The ability of the enteric adenoviruses to propagate in 293 cells (Takiff *et al.*, 1981) implicated the E1 region in the growth restriction of these viruses. Further studies led several authors to suggest that a malfunction of the E1A region was at least partly responsible for the host range phenotype. Although both Ad40 and Ad41 E1A gene products are capable of inducing expression from the Ad2 E4 promoter in transient transfection assays, the level of transactivation is considerably lower than by the Ad5 E1A gene product (van Loon *et al.*, 1987a). Similarly, Ishino *et al.*, (1988) found that the Ad40 E1A gene product is a weaker transactivator than either Ad5 or Ad12 E1A; they also demonstrated that the Ad40 E1A gene has a weaker *cis*-acting promoter than Ad12. Other evidence suggests a lack of E1B functions in the enteric adenoviruses. Plasmids containing the E1 region of either Ad40 or Ad41 are capable of transforming baby rat kidney cells (van Loon *et al.*, 1985a) whereas these serotypes are not capable of virion mediated transformation (Takiff and Straus, 1982). Similar phenotypes have been reported for E1B 55K mutant viruses (Rowe and Graham, 1983; Babiss *et al.*, 1984; see section 1.3.5.). More direct evidence for the involvement of the E1B region in the growth restriction of Ad40 comes from studies by Mautner *et al.*, (1989). By using a series of KB cell lines expressing either one or both of the Ad2 E1A and E1B regions it was shown that Ad40 grows equally well on cells expressing the whole of the E1 region and cells expressing only the E1B region, as determined by the yield of virion DNA. Cells expressing only E1A gene products are no more permissive

than KB cells without any adenovirus sequences. This indicates that a lack of E1B but not E1A functions may be involved in the growth restriction of Ad40. This is supported by the finding that in coinfection assays there is reciprocal complementation between Ad40 and the Ad5 E1A mutant dl312 while complementation of Ad40 by mutants lacking the E1B region or defective for the E1B 55K protein is hardly detectable (Mautner *et al.*, 1989; this thesis).

The growth defect of Ad40 in tissue culture is more severe than that of Ad41 as seen by the lack of Ad40 growth in several cell lines that are permissive for Ad41 (de Jong *et al.*, 1983; Uhnoo *et al.*, 1983; 1984; van Loon *et al.*, 1985b). It is therefore possible that the basis for the host range may differ between the two serotypes. Several recent studies have focused on the growth restriction of Ad41 in tissue culture. Tiemessen and Kidd (1988) assayed for complementation of Ad41 growth by Ad2 on the semi-permissive Chang conjunctiva cells (Kidd and Madeley, 1981) and non-permissive human embryo fibroblasts (HEF) (de Jong *et al.*, 1983). They determined the effect of Ad2 infection, before, after or together with Ad41, on late antigen production. Ad2 provided a helper function for Ad41 in HEF cells and the complementation was most effective when cells were preinfected with Ad2. In Chang conjunctiva cells, Ad41 late antigen synthesis was enhanced in the presence of Ad2; the degree of complementation was dependent on Ad2 input concentration in both cell types. Although these results show that Ad41 propagation in non-permissive and semi-permissive cells is enhanced in the presence of Ad2 they do not give any indication as to which gene products may be involved in the complementation. In double infection experiments on HEF cells interference of Ad41 on Ad2 replication was detected, dependent on the relative time of infection by each serotype (Tiemessen and Kidd, 1988). This is consistent with a hierarchy of dominance between different adenovirus serotypes which has been attributed to the transcriptional repression function of E1A gene products (Leite *et al.*, 1986; Tibbetts *et al.*, 1986; Larsen and

Tibbetts, 1987).

Further studies on the growth kinetics of Ad41 on HEF and Chang conjunctiva cells in the absence and presence of Ad2 and on 293 cells, were performed by Tiemessen and Kidd, (1990). Several different patterns emerged. Namely, on 293 cells Ad41 showed infectivity with one-hit kinetics, i.e. infectivity was directly proportional to input virus concentration, while in Ad2-Ad41 double infections on HEF and Chang conjunctiva cells the infectivity followed a pattern of two-hit kinetics. An Ad41 single infection on the semi-permissive Chang conjunctiva cells showed multiple-hit kinetics. This indicates that while in 293 cells a single Ad41 particle is capable of a productive infection, in Chang conjunctiva cells several virus particles are needed to infect a cell productively. As a way of explaining this finding Tiemessen and Kidd (1990) propose that the virus stock contains a homogeneous population of virus particles, each particle having an equal probability of initiating infection but a low probability of continuing infection to late antigen synthesis, perhaps due to a limiting concentration of some essential product. This limitation would then be overcome by multiple infecting genomes, the possibility of a successful cellular infection increasing markedly with increasing concentration of input virus. According to this model the limiting product would most likely be an E1 gene product since the need for a multiple infection is overcome in 293 cells, expressing the Ad5 E1 region, as well as in a coinfection with Ad2. Furthermore, the need for this product would have to be cell type dependent since HEF cells are non-permissive for Ad41 in the absence of a complementing Ad2 infection while Chang conjunctiva cells are semi-permissive for Ad41.

It is not clear if this model could apply to the growth properties of Ad40 since it has been reported that a high multiplicity of Ad40 infection can inhibit virus replication in some cell lines (Witt and Bousquet, 1988; see above). Moreover, this may be an over-simplification of the basis for Ad41 growth restriction. Pieniazek, D. *et al.*, (1990a)

have recently reported that in their hands Ad41 loses infectivity rapidly upon passaging on 293 cells, while propagation on HEp-2, Int407 and Hela cells is successful. Comparison of yields of complete and incomplete Ad41 particles showed that in 293 cells 62% of virus particles were incomplete and in HEp-2 cells incomplete particles made up 40% of the total virus particles. This implies that Ad41 virus stocks include a high proportion of defective virus particles and is in contrast to the prediction by Tiemessen and Kidd (1990) that Ad41 virus stocks are homogeneous. The loss of Ad41 infectivity upon passaging in 293 cells was found to be related to a deficiency of the core protein V in virus particles, probably due to a defect in the assembly of Ad41 virions in 293 cells (Pieniazek, D. *et al.*, 1990a). The basis for this selective loss of infectivity is not clear and contradicts previous reports where Ad41 has successfully been passaged in 293 cells (de Jong *et al.*, 1983; Uhnoo *et al.*, 1983).

One of the factors that can influence Ad41 propagation is serum concentration. The growth restriction of Ad41 on HEK cells, previously reported to be non-permissive (Takiff *et al.*, 1981; Kidd and Madeley, 1981), and other primary cell lines, can be overcome by keeping a low serum concentration (0.2-1%) in the infection medium (Pieniazek, D. *et al.*, 1990b). Although this results in accumulation of viral DNA and virus particles similar to that seen in permissive cells, no CPE is seen on the human primary cells. The lack of CPE suggests that the primary cells produce lower amounts of penton protein since appearance of CPE is linked to the amount of penton present in the infected cells (Pettersson, 1984). The serum effect was found exclusively in primary cells and not seen for continuous cell lines such as HEp-2 and 293 cells. In primary cells the accumulation of Ad5 DNA is not related to serum concentration (Pieniazek, D. *et al.*, 1990b). Thus it appears that some factor present in serum may selectively inhibit Ad41 DNA replication in primary cells, possibly by affecting interaction between viral and cellular factors in those cells. Another example of a

selective replicative block in a particular cell type is that of Ad12 in primary hamster cells which are permissive for Ad2 and Ad5 replication (Klimkait and Doerfler, 1987). Pieniazek, D. *et al.*, (1990b) suggest that this growth restriction of Ad41 and Ad12 in certain primary cells may be related to the fact that both serotypes infect cells of the gastrointestinal tract *in vivo* and may as such have specific growth requirements. It is of interest that the replicative block of Ad12 in primary hamster cells can be overcome in a double infection with Ad2 or Ad5 and in cells expressing the Ad2 or Ad5 E1 region (Klimkait and Doerfler, 1985). Moreover the E1A mutant dl312 provides helper functions for Ad12 DNA replication in a double infection whereas the E1B mutant dl313 does not (Klimkait and Doerfler, 1987). This is analogous to the complementation of Ad40 growth on KB cell lines and by E1A but not E1B mutant viruses (Mautner *et al.*, 1989) and further implicates the E1B region in the host range of adenoviruses.

1.4.5. The molecular biology of the enteric adenoviruses

Detailed studies of adenovirus serotypes 2 and 5 and to a lesser extent Ad12 have created a vast amount of information some of which may apply to adenoviruses in general. As a result, a comparison with those well characterized serotypes is a useful approch to studying the enteric adenoviruses. Conversely, such a comparison can add to the perception of general adenovirus biology.

The overall DNA homology, as determined by liquid phase hybridization, is 62-69% between Ad40 and Ad41, while the DNA from both serotypes is 15-22% homologous to Ad2 DNA (van Loon *et al.*, 1985b). The homology within subgroup F is lower than seen within subgroups B-E but similar to subgroup A; the intersubgroup homology is in the same range as seen for other subgroups (see table 1). A direct DNA sequence comparison has also been used to study the relationship between the enteric adenoviruses and other serotypes. The DNA sequence of the Ad40 ITR shows a closer relation to that

of Ad5 than to viruses of any of the other subgroups, but it is one of the longest of the known ITRs of human adenoviruses and resembles subgroup A in that respect (Ishino *et al.*, 1987). The nucleotide sequence of several genes is also known for Ad40 (E1 region, van Loon *et al.*, 1987b; Ishino *et al.*, 1988; DBP and 23K protease, Vos *et al.*, 1988; fibre, Kidd and Erasmus, 1989; hexon, Toogood *et al.*, 1989) and Ad41 (E1 region, van Loon *et al.*, 1987b; Allard and Wadell, 1988; DBP and 23K protease, Vos *et al.*, 1988; 100K and 33K proteins, Slemenda *et al.*, 1990; pVIII and the E3 promoter, Pieniazek *et al.*, 1989b; fibre, Pieniazek, N.J. *et al.*, 1989a, 1990; Kidd *et al.*, 1990; hexon, Toogood and Hay, 1988).

According to the DNA sequence data available so far the genome organization of the enteric adenoviruses corresponds to that of other human adenoviruses. However, an intriguing difference is that the Ad41 genome contains two adjacent fibre genes (Pieniazek, N.J. *et al.*, 1989a, 1990). This may be analogous to avian adenoviruses which have two fibre proteins of different size (Laver *et al.*, 1971). The corresponding Ad40 sequence has not been analysed in detail although the DNA sequence of one fibre gene has been determined (Kidd and Erasmus, 1989). The predicted Ad40 and Ad41 fibre polypeptides fit the secondary structure model predicted by Green *et al.*, (1983) which proposes a shaft made up of pseudorepeat units approximately 15 amino acids long. Like Ad5 and Ad2, one of the Ad41 fibre proteins has 22 such repeats whereas the other one has 12 (Pieniazek, N.J. *et al.*, 1989a, 1990; Kidd *et al.*, 1990). Ad40 has a pattern of 21 repeats in the shaft region (Kidd and Erasmus, 1989). The Ad40 fibre gene is highly homologous (95.6%) to the larger of the Ad41 fibre genes, excluding a 45bp region present only in the Ad41 gene which accounts for one 15 amino acid repeat (Kidd *et al.*, 1990). The overall homology with Ad2 fibre is only 33% on the amino acid level although regions of higher homology can be identified. The fibre is responsible for cellular attachment of the virus and it is interesting to speculate that differences in host cell

specificity may be reflected in the divergence of the fibres. However, the replicative block of the enteric adenoviruses in tissue culture is after entry into cells (Takiff and Straus, 1982), thus making this an unlikely explanation for the growth restriction of these viruses in cells permissive for Ad2.

Analysis of hexon sequences reveals 88% overall amino acid sequence homology between Ad40 and Ad41 (Toogood *et al.*, 1989). Regions of low homology correspond to regions that are variable between Ad2 and Ad5 indicating that those are areas of type specific antigen determinants as previously suggested by Kinloch *et al.*, (1984). A comparison between these four serotypes also reveals other regions, conserved within a subgroup but variable between subgroups, probably representing group specific antigens (Toogood *et al.*, 1989). The hexon amino acid sequence is 77% homologous between Ad41 and Ad2 (Toogood and Hay, 1988), which is considerably higher conservation than seen for the fibre. This may reflect more structural constraints on hexon sequences.

Vos *et al.*, (1988) determined the nucleotide sequence of the DBP gene of Ad40 and Ad41 as well as the 23K protease gene and parts of the hexon and 100K protein genes. They found that the overall sequence organization in that region is extremely compact and contains large deletions with respect to Ad2, both within promoters and protein coding sequences. The amino acid sequence of the 23K protease is very well conserved between the two serotypes (90%) whereas a homology of 72% for the DBP is only slightly higher than between either serotype and Ad12 (69%).

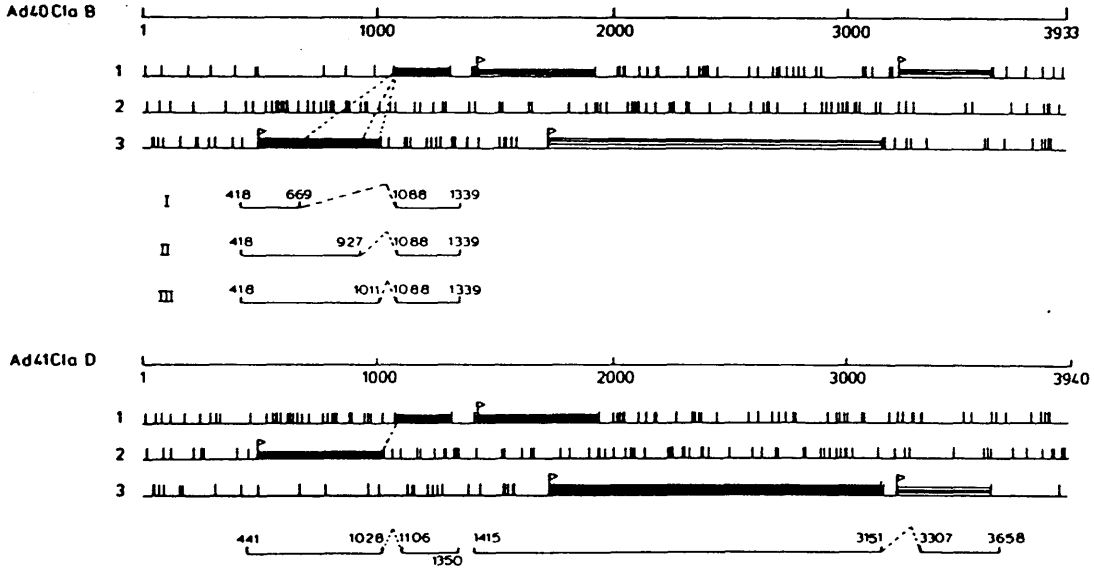
The E1 region of the enteric adenoviruses has been most extensively studied, partly because it has been implicated in the fastidious growth of these viruses. The DNA sequence of this region of both serotypes was determined by van Loon *et al.*, (1987b) and found to be 85% homologous to each other and to share 52% homology with the corresponding Ad5 region. The organization of the region is similar to that found for other serotypes and strategic sequences essental for the

propagation of all human adenoviruses are present in both serotypes. Open reading frames were identified corresponding to the E1A and E1B regions of other serotypes. Comparable results were obtained by Ishino *et al.*, (1988) who sequenced the E1 region of a different strain of Ad40 and Allard and Wadell (1988) who analysed the E1A region of a different Ad41 strain. Ishino *et al.*, (1988) compared the amino acid sequence of E1A proteins and E1B 19K and 55K proteins to that of the corresponding Ad2 and Ad12 polypeptides. While Ad40 E1A proteins share a similar homology with Ad2 and Ad12 (40% and 39% respectively) both the E1B 19K and 55K proteins share a higher homology with Ad12 (48% and 53% respectively) than with Ad2 (44% and 47% respectively).

For RNA analysis, rat cells transformed with Ad40 or Ad41 E1 containing plasmids were used as a source of total RNA because of the inability to propagate the fastidious viruses in conventional cell lines (van Loon *et al.*, 1987b). The E1 transcription maps as determined by S1 analysis are shown in figure 5. Three Ad40 E1A mRNA species were detected, corresponding to the Ad2 9S, 12S and 13S mRNAs. However, although the intact Ad40 E1B region was present in those cells (van Loon *et al.*, 1985a) no E1B mRNA was detected either by S1 analysis or Northern blotting. In Ad41 transformed cells an E1A 13S mRNA was identified as well as an E1B transcript corresponding to the Ad2 22S mRNA. Van Loon *et al.*, (1987b) postulate that the absence of Ad40 E1B mRNA may be due to low transactivation of the E1B promoter by Ad40 E1A and that this may be the basis for the replicative block of Ad40 in tissue culture. The same argument may apply to Ad41 but the presence of multiple copies of E1 sequences in transformed cells may overcome this low expression level. These authors (van Loon *et al.*, 1987a) and others (Ishino *et al.*, 1988) have shown that the Ad40 and Ad41 E1A products are weaker transactivators than the Ad5 E1A products (see 1.4.4.). However, efficient growth of Ad40 in KB cells expressing the Ad2 E1B region and not in the parallel cell line expressing the E1A region (Mautner *et*

Figure 5: Virus specific mRNAs present in cells transformed with the Ad40 or Ad41 E1 region.

Transcripts were mapped by S1 nuclease analysis. The mRNAs transcribed from the Ad40 E1A region are indicated by roman numerals. The dashed carets represent intron sequences. Start and stop (bars) codons and ORFs have been denoted. ORFs in mRNAs transcribed from the viral sequences are indicated by black boxes, while ORFs in the E1B region of Ad40 and the polypeptide IX coding region of Ad41 are indicated by striped boxes. Scales are in nt. (Reproduced from van Loon *et al.*, 1987b).


Ad40Cla B
1
1000
2000
3000
3933
1
2
3
I
418
669
1088
1339
II
418
927
1088
1339
III
418
1011
1088
1339
Ad41Cla D
1
1000
2000
3000
3940
1
2
3
441
1028
1106
1350
1415
3151
3307
3658

al., 1989) disputes this conclusion since the presence of Ad2 E1A would be expected to complement for low transactivation by Ad40 E1A.

The three conserved regions within the E1A region (Kimelman *et al.*, 1985; see 1.2.2.) have also been identified in Ad40 and Ad41 (van Loon *et al.*, 1987b; Ishino *et al.*, 1988; Allard and Wadell, 1988). However, while the homology of the conserved regions of Ad7, Ad12 and SAV-7 with the corresponding Ad5 regions is over 60%, the Ad40 and Ad41 conserved regions share only 51% and 45% homology with Ad5 respectively (van Loon *et al.*, 1987b). CR I is least well conserved (Allard and Wadell, 1988; Ishino *et al.*, 1988) but CR II and CR III also share lower homology with Ad5 than do other serotypes. CR III 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 to E1A products of Ad5 and Ad12. Although this may partly explain the restrictive growth of the enteric adenoviruses in tissue culture other factors are likely to be involved as well. A comparison of E1B protein sequences shows that both Ad40 E1B ORFs share a higher homology with Ad5 and particularly Ad12 than the coresponding E1A ORFs (Ishino *et al.*, 1988). No strictly conserved domains have been identified in the E1B proteins so the relevance of any sequence differences are difficult to assess. Studies on other adenoviruses have shown that mutations in both the 19K and 55K E1B proteins affect the host range of those viruses (see 1.3.4. and 1.3.5.) making this region a likely candidate for having a role in the host range of the enteric adenoviruses.

1.4.6. The aims of the project

The aim of the work presented in this thesis was to investigate the molecular basis for the growth restriction of enteric adenovirus 40 in tissue culture. This virus is severely restricted for growth in most tissue culture systems. Successful propagation of the virus in KB cell

lines expressing the E1 region or only the E1B region of Ad2 facilitated *in vitro* studies of the virus which had previously been limited. This also implicated the E1B region of Ad40 in the fastidious growth. Initial studies using complementation assays with Ad5/2 and Ad12 E1 virus mutants were consistent with a lack of Ad40 E1B function. A study of the Ad40 E1B region was thus undertaken with the aim of understanding the basis for the apparant E1B deficiency. This involved the analysis of E1B transcription and detailed transcription mapping in order to determine if the kinetics of E1B transcription or splicing patterns of E1B transcripts differed from that of other serotypes. Moreover, the activity of the E1B promoter and response to E1A trans-activation was investigated. In addition several cell lines were tested for their ability to support Ad40 replication in the absence of a complementing adenovirus function in order to provide a system more analogous to the *in vivo* growth of the virus.

2. MATERIALS

2.1. Viruses

Adenovirus 40 strain Dugan was obtained from Dr. J. de Jong as a low passage seed stock (de Jong *et al.*, 1983). It was propagated in KB16 cells (see 2.2.); preliminary experiments were done using passage 3-5 virus stocks while later experiments used p9 of the virus.

Table 2 lists Ad5, Ad2 and Ad12 E1 mutants used. The mutations in dl309 are also present in the Ad5 E1 mutants and Ad2 E1B mutants. Ad12 mutant viruses and wild type were obtained from Dr. Phil Gallimore (Breiding *et al.*, 1988; Byrd *et al.*, 1988; Edbauer *et al.*, 1988):

Table 2

Type	Mutant	Mutation	Protein	Reference
Ad5	dl309	Lacks XbaI sites at 29 and 79 mu. 83-85 mu. deleted	E3 proteins deleted	(1)
Ad5	dl312	nt 448-1349 deleted	E1A proteins not made	(1),(2)
Ad5	dl313	nt 1334-3639 deleted	E1B and ppIX not made, E1A truncated	(1),(2)
Ad2/5	pm1722	G→A at nt 1722	E1B 19K not made, 55K ORF not affected	(3)
Ad2/5	dl1520	C→T at nt 2022 nt 2496-3323 deleted	E1B 55K&15K not made, 19K ORF not affected	(3)
Ad12	hr700	G→A at nt 949	E1A 12S and 13S products Asp→Asn in CR III	(4)
Ad12	in602	Insertion of 8bp at nt 2247, frameshift.	E1B 55K truncated	(4)
Ad12	dl620	nt 2127-2823 deleted inframe.	E1B 55K altered	(4)
Ad12	pm700	ATG→ATC at nt 1543	E1B 19K not made	(5)

(1) Jones and Shenk (1979).
(2) Ross *et al.*, (1980).
(3) Barker and Berk (1987).
(4) Breiding *et al.*, (1988).
(5) Edbauer *et al.*, (1988).

2.2. Cells

- KB16 cells, expressing Ad2 E1A and E1B products (Babiss *et al.*, 1983) were used for the propagation of Ad40. They will be referred to here as KBa+b cells.
- 293 cells, expressing Ad5 E1A and E1B products (Graham *et al.*, 1977) were used for the propagation of Ad2/5 and 12 E1 mutants.
- Hela cells, a current laboratory stock was used for virus infections.
- WS Hela cells (originally from Dr. W. Schaffner, Zurich), were used for transient assays.
- Tera-2 C13: cloned human teratoma cells (Thompson *et al.*, 1984), obtained from Dr. R. Everett.
- EJ: Human bladder carcinoma cells, obtained from J. Lang, the Beatson Institute.
- HT29: Human colon adenocarcinoma cells, obtained from Dr. C. Parasceva, Bristol.
- Human pancreatic carcinoma cells were obtained from A. Grant, St. Georges Hospital (Grant *et al.*, 1979).
- A549: Human lung carcinoma cells, obtained from the European Collection of Animal Cell Cultures (ECACC).
- Int407: Human embryonic intestinal cells, epithelial, obtained from the ECACC.
- A431: Human squamous carcinoma cells, obtained from Dr. J. Jaukroger, Glasgow Vet. School.

2.3. Bacteria

The following strains of *E. coli* K12 were used:

- DH1 [recA1, endA1, thi-1, hsdR17, supE44, gyrA96, (Nal^r), relA1] (Hanahan 1983), was used as a host for CAT gene plasmid constructs.
- GM48 [dam-6, dam-3, galT22, dral4, lacyl, thr-1, leu-6, tonA31, tsxA78, supE44], (Marinus, 1973), was used as a host to prepare unmethylated plasmid DNA.
- JM109 [F', traD36, $proA^+$, $proB^+$, $lacI^q$, lacZδM15/recA1, endA1, gyrA96, (Nal^r), hsdR17, supE44, relA1, δ(lac-proAB), mrcA], (Yanisch Perron *et al.*, 1985), was used as

a host for all other plasmids throughout this work.

- NM522 [recA$^+$ (supE, thi, δ(lac-proAB), hsd5, {F', proAB, lacI^q, lacZδM15})], (Gough and Murray, 1983), was the host strain used for M13 bacteriophage.

2.4. Cloning vectors

- pUC8 (Vieira and Messing, 1982) was used for cloning of double stranded cDNA.

- M13 bacteriophage vectors (Messing and Vieira, 1982) were used for the production of single stranded DNA for sequencing. PCR amplified cDNA was cloned into the double stranded replicative form of M13mp8 (obtained from A. Dolan).

2.5. Plasmids

The following plasmid constructs were generously provided by N. Mackay; the Ad40 E1 plasmids were all derived from pAA12 (van Loon *et al.*, 1985) which consists of the ClaIB fragment of Ad40 (nt. 1-3933), by subcloning into pTZ18 vectors (Mautner *et al.*, 1989, 1990):

- pNM80: Ad40 E1A, nt 1-1643
- pNM81: Ad40 E1B, nt 1643-3933
- pNM82: Ad40 E1, nt 1-3933
- pNM83: Ad40 E1A, nt 1-1211
- pNM86: Ad40 E1B 3'end, nt 2857-3928
- pNM87: Ad40 E1B 5'end, nt 1643-2857
- pNM90: Ad2 E1A, nt 1-1569
- pNM92: Ad2 E1B, nt 1569-3322
- pAsc10.3: Ad12 E1 (SalIC fragment) was obtained from Dr. P.J. Byrd (Byrd *et al.*, 1982).

The following plasmids were a gift from Dr. C. Herrmann, Cold Spring Harbor:

- pLE1A: Ad5 E1A, nt 1-1704 in pAT153 vector (Dery *et al.*, 1987)
- pMT13S: $^{Ad5}_{\wedge}$E1A 13S cDNA sequences under the control of the mouse metallothionein-1 promoter in pAT153 vector (Zerler *et*

al., 1986)

- pE1B-CAT: Ad5 E1B promoter, nt 1336-1702, upstream of the CAT gene in a pXf3 derived plasmid vector (Dery *et al.*, 1987).

2.6. Oligonucleotides

The oligonucleotides described below were used in this study. They all include Ad40 DNA sequences and were made by reference to the DNA sequence published by van Loon *et al.* (1987). The nucleotide numbers refer to that sequence; R and L refer to the rightward and leftward reading strands; lower case sequences are linker sequences including cloning sites. Oligonucleotides were synthesised by Dr. J. McLauchlan on a Biosearch 8600 DNA Synthesiser by trityl-on synthesis and purified on OPC cartridges.

Table 3

Oligo	nt.	str.	Sequence
VS1	1548-1528	L	5'CTCCTTAGCTCTATAGATTAC3'
VS4	3287-3267	L	5'CGGGTTGTCAGATATGGGCTA3'
VS5	3380-3400	L	5'CCGACGATCCAATAGTGGCGT3'
VS6	3000-3020	R	5'GAGTCAAAGGTTCGTTGTCGC3'
VS9	1368-1385	R	5'ctgcagctcgaGTGGGTATATAAGCAGGT3'
VS10	1352-1369	R	5'ctgcagctcgagTTTCTTGGGCGTGTTTGT3'
VS11	1298-1315	R	5'ctgcagctcgagCCAAGTGCTCCTGAGATC3'
VS12	1056-1074	R	5'ctgcagctcgagAACAAGAGTTAACGACTT3'
VS13	1411-1395	L	5'ctgcagaagcttGAATAAACTAACACTAA3'
VM40	567-586	R	5'TTCCCTGAACCTTCTGAGGC3'
RU3	1719-1736	R	5'gggctgcagatctATGGAGCGCCCAAACTCA3'
RU4	3129-3149	L	5'gggctgcagatctTTAATCCTCATCGCTGGATTC3'

2.7. Reagents

Most chemicals were purchased from BDH Chemicals, UK or

Sigma Chemical Co. and were of analytical grade. Otherwise, Bio-Rad Laboratories supplied ammonium persulphate and TEMED; Koch-Light Laboratories supplied acrylamide and boric acid; Melford Laboratories supplied caesium chloride; Amersham supplied Hybond-N nylon membranes, and Zeta probe blotting membranes were obtained from Bio-Rad. Ultrapure dNTPs, T7 DNA sequencing kit and Oligolabelling kit were purchased from Pharmacia. DOTMA (transfection reagent) was supplied by Boehringer-Mannheim; RNasin (ribonuclease inhibitor) was purchased from Promega; RNA ladder (molecular weight marker) and Diethylpyrocarbonate (DEPC) were purchased from Bethesda Research Laboratories (BRL). Wacker Chemical Co. supplied Wacker Silane GF38; NuSieve GTG agarose was supplied by FMC Bioproducts; Formamide and formaldehyde were supplied by Fluka; Methylmercury was from Alpha Products (W. Germany). TMPD and Xylenes were supplied by Aldrich Chemical Co. OPC cartridges were purchased from Applied Biosystems.

2.8. Radiochemicals

Radioisotopes were supplied by Amersham at the following specific activities:

- 5'[α-^{32}P]dNTPs, 3000 Ci/mmol (10µCi/µl)
- 5'[γ-^{32}P]ATP, 5000 Ci/mmol (10µCi/µl)
- ^{14}C Chloramphenicol, 45 mCi/mmol, was obtained from NEN DuPont

2.9. Enzymes

Restriction enzymes were supplied by Bethesda Research Laboratories (BRL), Boehringer-Mannheim and Northumbrian Biologicals Ltd. (NBL). Klenow fragment DNA polymerase (LFP), PolI DNA polymerase, S1 nuclease, T4 ligase and MMLV reverse transcriptase were obtained from BRL; AMV reverse transcriptase was from Life Sciences; RNase free DNase was purchased from Promega; AmpliTaq DNA polymerase was obtained from Perkin-Elmer Cetus; Calf Intestinal Phosphatase (CIP)

was supplied by Boehringer-Mannheim and T4 kinase by NBL.

2.10. Tissue culture media

Most cell types were grown in Dulbecco's modified eagle medium (DMEM), without sodium pyruvate, with 4500 mg/l glucose (Gibco). Medium was supplemented with 100IU/ml penicillin, 100 µg/ml streptomycin, 4mM L-glutamine and 5% foetal calf serum (FCS) for Hela cells and KBa+b cells or 10% FCS for other cell types.

WS Hela cells were grown in DMEM, with sodium pyruvate, with 1000mg/l glucose, supplemented with 100IU/ml penicillin, 100 µg/ml streptomycin, 4mM L-glutamine and 5% FCS.

Pancreatic carcinoma cells were grown in Ham's F-12 medium (Flow Laboratories), supplemented with 100IU/ml penicillin, 100µg/ml streptomycin, 4mM L-glutamine and 10% FCS.

2.11. Bacterial culture media

NM522 cells were grown in 2YT broth, all other strains were grown in L-broth. Media was supplemented with ampicillin (50µg/ml) and chloramphenicol (200µg/ml) where appropriate. Agar plates contained 1.5% (w/v) agar in L-broth.

- L-broth: 177mM NaCl, 10g/l Difco Bactopeptone, 5g/l yeast extract (pH7.5 prior to sterilization).
- 2YT broth: 85mM NaCl, 10g/l Difco Bactotryptone, 10g/l yeast extract.
- Top agar: 1% (w/v) agar in water.

2.12. Solutions

- 10xBlunt end kinase buffer: 500mM Tris-HCl pH9.5, 100mM $MgCl_2$, 50mM DTT, 50% glycerol
- 50xDenhardt's solution: 1% (w/v) BSA, 1% (w/v) Ficoll, 1% (w/v) Polyvinyl pyrollidine
- 10xEM buffer: 50mM Sodium borate, 100mM Sodium sulphate, 10mM EDTA, 150mM Boric acid

- Formaldehyde loading buffer: 50% glycerol, 1mM EDTA pH8.0, 0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanol
- Formamide dyes: 98% (v/v) formamide, 2% (v/v) 0.5M EDTA pH8.0, 0.1% (w/v) Xylene cyanol, 0.1% (w/v) Bromophenol blue
- GSCN buffer: 4M Guanidinium thiocyanate, 5mg/ml Sarcosine, 0.1M β-mercaptoethanol, 0.33% (v/v) Antifoam A, 50mM Tris-HCl pH7.5, 2mM EDTA pH8.0
- Hirt buffer: 10mM Tris-HCl pH7.9, 10mM EDTA, 0.6% (w/v) SDS
- Hybridization buffer: 80% (v/v) formamide, 400mM NaCl, 40mM Pipes pH6.4, 1mM EDTA
- 5xKinase buffer: 350mM Tris-HCl pH7.5, 50mM $MgCl_2$, 25mM DTT
- 5xLigase buffer: 250mM Tris-HCl pH7.6, 50mM $MgCl_2$, 5mM DTT, 5mM ATP, 25% (w/v) polyethylene glycol-8000
- Lysis buffer: 50mM Tris-HCl pH8.0, 100mM NaCl, 5mM $MgCl_2$, 0.5% (v/v) Nonidet P-40
- 10xMOPS: 180mM MOPS [3-(N-morpholino)-propanesulfonic acid] pH7.0, 50mM sodium acetate, 10mM EDTA pH8.0
- 10xNT buffer: 500mM Tris-HCl pH7.8, 50mM $MgCl_2$, 10mM DTT
- PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4 (pH ~7.3),.
- 10xPCR buffer: 100mM Tris-HCl pH8.3, 500mM KCl, 15mM $MgCl_2$, 0.01% (w/v) gelatin
- Phenol/chloroform: 1:1 mixture of phenol equilibrated in TE, and chloroform
- Potassium acetate solution: 3M Potassium acetate, 11.5% (v/v) Acetic acid
- 10xRT buffer: 500mM Tris-HCl pH8.0, 50mM $MgCl_2$, 50mM DTT, 500mM KCl, 500 µg/ml BSA
- S1 buffer: 250mM NaCl, 30mM Sodium acetate pH4.6, 1mM $ZnSO_4$
- Sample buffer: 50% (w/v) Sucrose, 0.1M EDTA pH8.0, 0.2% (w/v) Bromophenol blue
- SOB: 2% (w/v) Bactotryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$

- SOC: 20mM glucose in SOB
- 20xSSC: 3M NaCl, 0.3M tri-Sodium citrate (pH7.0)
- 20xSSPE: 3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA (pH7.4)
- TBE: 90mM Tris, 90mM Boric acid, 1mM EDTA (pH8.3)
- TE: 10mM Tris-HCl pH8.0, 1mM EDTA
- TEN: 100mM NaCl, 50mM Tris-HCl pH7.5, 10mM EDTA
- TFB: 10mM MES, 100mM RbCl, 45mM $MnCl_2$, 10mM $CaCl_2$, 3mM hexamine cobaltic chloride
- TS: 140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/l glucose, 0.001% (w/v) phenol red, 25mM Tris-HCl pH7.4, 100U/ml penicillin, 100µg/ml streptomycin
- Versene: 0.6mM EDTA in PBS, 0.002% (w/v) phenol red

3. METHODS

3.1. Eucaryotic cell culture

Cells were routinely passaged at 80% confluency in 80cm^2 or 175cm^2 flasks (Nunclon). They were harvested by washing twice in versene, resuspended in fresh medium and incubated in an atmosphere of 95% air/5% CO_2 at 37°C. For storage, cells were resuspended at approximately 10^7 cells/ml in storage medium (70% growth medium, 10% glycerol, 20% FCS), and aliquots frozen slowly to -140°C. For recovery, cells were thawed at 37°C and resuspended in growth medium.

3.2. Virus preparation

Ad40 seed stocks were prepared by infecting subconfluent monolayers of KBa+b cells on 50mm plates with 0.1ml of a 1/10 - 1/40 dilution of virus stock in TS. Virus was allowed to absorb for 1 hr at 37°C before the addition of infection medium (5ml DMEM/0.5% FCS per plate). Plates were incubated 3-5 days at 37°C until cpe was apparent. Cells were harvested by scraping into the medium, pelleted at 1,000rpm/5 min in an MSE coolspin centrifuge and resuspended in 0.2ml TS/plate. Virus was released by three rounds of freezing and thawing (-70°C/37°C), followed by centrifugation (2,000rpm/10 min) to remove cell debris. The supernatant, containing the virus, was stored at -20°C. Seed stocks of other serotypes were prepared in the same way by infecting 293 cells with 0.1pfu/cell; stocks were titrated by a plaque assay on 293 cells (Williams, 1970). Ad40 does not form plaques under any conditions tested so far; yield of Ad40 virus stocks was estimated by restriction digest analysis of viral DNA.

3.3. Preparation of viral DNA

DNA was prepared from crude harvested virus by incubating virus suspension 2 hr/37°C in the presence of Proteinase K at 100µg/ml and 0.1% (w/v) SDS. The reaction mix was then

extracted with an equal volume of phenol/chloroform, followed by a chloroform extraction. Finally, the DNA was concentrated by ethanol precipitation in the presence of 0.3M sodium acetate pH5.5 and 1µg tRNA carrier.

3.4. Preparation of whole cell RNA

Total RNA was harvested by a modification of the guanidinium thiocyanate method (Chomczynski *et al.*, 1987), from cell monolayers on 50mm plates. Medium was removed and cell monolayers lysed directly by adding 200µl GSCN buffer onto the plates. The harvest was transferred to Eppendorf tubes and sequentially 1/10 vol. water saturated phenol and 1/5 vol. chloroform/isoamyl alcohol (49:1) were added to the cell lysate with thorough mixing after the addition of each reagent. The final mixture was incubated on ice for 15 min and centrifuged 12,000rpm/10 min. The aqueous phase was precipitated with an equal volume of isopropanol 1 hr/-20°C, centrifuged, the pellet resuspended in GSCN buffer and the isopropanol precipitation repeated. After centrifugation the pellet was resuspended in 0.3M sodium acetate pH7.0 and ethanol precipitated. Prior to analysis the RNA was pelleted by centrifugation 12,000rpm/10 min and resuspended in water. The concentration of RNA was determined by spectrophotometry (see 3.7.).

3.5. Preparation of cytoplasmic RNA

Cytoplasmic RNA was isolated from cell monolayers on 50mm plates as described (Ausubel *et al.*, 1987). Reagents were either treated with DEPC or made up in DEPC treated water to prevent RNase activity, and cells and reagents were kept on ice. Monolayers were washed three times with PBS, cells harvested into 1ml PBS, the harvest from 3 plates pooled, pelleted by centrifuging 1,000rpm/5 min/4°C (Sorvall benchtop centrifuge) and resuspended in 375µl lysis buffer (see 2.12.). After incubation on ice for 5 min nuclei were pelleted 12.000rpm/2 min/4°C and frozen at -70°C. The

supernatant was made 0.2% in SDS and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1) prior to ethanol precipitation. Before analysis, the RNA was pelleted by centrifugation 12,000rpm/15 min/4°C, washed with 75% ethanol/25% 0.1M sodium acetate and resuspended in DEPC treated water. RNA concentration was determined by spectrophotometry.

3.6. Preparation of intranuclear viral DNA

Nuclei were isolated as described above (see 3.5) and DNA was extracted by a modification of the Hirt procedure (Hirt 1967). The nuclear pellet was resuspended in 40µl TE, 20 volumes Hirt buffer added and incubated 10 min at room temperature, 1/5 vol 5M NaCl was added, mixed and left on ice at 4°C overnight. Lysed nuclei were spun 15,000rpm/30 min in SS34 rotor and the supernatant incubated with 0.1 mg/ml RNase A for 1 hr/37°C and 0.1 mg/ml Proteinase K for 3 hrs/37°C. The lysate was subsequently extracted with phenol/chloroform and chloroform and concentrated by ethanol precipitation.

3.7. Quantitation of nucleic acids

The concentration of nucleic acids was determined by the optical density (OD) at 260nm using a Beckman DU-62 spectrophotometer.

Values used:

OD_{260} of 1 = 50µg/ml - for double stranded DNA
40µg/ml - for RNA
20µg/ml - for oligonucleotides

The purity of a nucleic acid preparation was assessed from the ratio of absorbance at 260nm/280nm, a pure DNA preparation having a ratio of 1.8 and a pure RNA sample having a ratio of 2.

The molar concentration of oligonucleotides was determined directly as OD_{260}/E_M (molar extinction coefficient)

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

E_M is the optical density of a 1M solution of the oligomer determined at 260nm (Tullis *et al.*, 1989).

3.8. Radioactive labelling of DNA

3.8.1. Nick translation:

100-200µg plasmid DNA was radiolabelled (Rigby et al., 1977) in 1xNT buffer with 40µg/ml gelatin, two dNTPs at 40mM and 25µCi [α^{32}P]dNTP of the other two. The reaction mix was incubated at 37°C for 10 min with DNaseI (0.2µg/ml) and subsequently 2U *E. coli* DNA PolI was added and incubated at 14°C/2 hrs. Labelled DNA was separated from unincorporated nucleotides by fractionation on a 5ml G-50 Sephadex column, after the addition of 1/5 vol sample buffer. Probes prepared by this method generally had a specific activity of $1x10^7$-$1x10^8$ cpm/µg.

3.8.2. 5'end labelling:

In order to prepare 5'end labelled probes for S1 mapping, the appropriate restriction fragments were isolated and 20pmol DNA termini treated with 0.5U calf intestinal phosphatase (CIP) in 100µl final volume of 20mM Tris-HCl pH8.0, 1mM $MgCl_2$ and 1mM $ZnSO_4$ at 37°C/30 min. The phosphatase was removed by phenol/chloroform and chloroform extraction, the sample ethanol precipitated and resuspended in water at 1pmol/µl. A labelling reaction contained 15µl of 1xkinase buffer, 100µCi [γ-^{32}P]ATP, 1pmol DNA and 5U T4 polynucleotide kinase. After incubation, 37°C/45 min, the reaction mix was ethanol precipitated with 1µg tRNA carrier in the presence of 2M ammonium acetate, and reprecipitated in 0.3M sodium acetate. The final pellet was washed with 70% ethanol and resuspended in 20µl H_2O.

Oligonucleotides were 5'end labelled in the same way using 25µCi [γ-^{32}P]ATP/25ng oligonucleotide, without a prior phosphatase treatment. Alternatively, 10pmol oligonucleotide was 5'end labelled in 20µl 1xblunt end buffer, 50µCi

[γ-^{32}P]ATP and 10U T4 kinase at 37°C/2 hrs. The reaction was precipitated in the presence of 4M ammonium acetate for 30 min on ice and pelleted at 12,000rpm/20 min. The pellet was washed in 70% ethanol and resuspended in water.

3.8.3. 3'end labelling:

DNA was 3' end labelled by the method of Ausubel *et al.*, (1987). Restriction fragments with overhanging 5'ends were isolated and 3'end labelled for use as probes in S1 mapping. For each restriction site to be labelled, the base complementary to the first unpaired base was added on using Klenow fragment DNA polymerase. The 10µl reaction mix included 200ng DNA restriction fragment, 50mM Tris-HCl pH8.0, 10mM $MgCl_2$, 50mM NaCl, 40µCi of the appropriate [α-^{32}P]dNTP and 1U Klenow. After incubation 15 min/30°C the reaction was stopped by adding 1µl 0.5M EDTA and precipitated as described for 5' end labelled fragments.

3.8.4. Random priming:

An oligolabelling kit from Pharmacia was used to make specific probes by the random priming method (Feinberg and Vogelstein, 1983). Restriction fragments (50ng), isolated from NuSieve agarose gels (see 3.10), were denatured by boiling and flash cooling and incubated with the reagent mix provided, 50µCi [α-^{32}P]dCTP and 1U Klenow in 50µl at 37°C/1 hr. The reaction mix was made up to 100µl in TE, loaded onto a 1ml G-50 spun column and centrifuged at 2.000rpm/10 min. The flowthrough, containing labelled probe was collected, while unincorporated ^{32}PdCTP remained on the column. The specific activity of probes prepared by this method was generally around $1x10^8$ cpm/µg.

3.9. Gel electrophoresis

3.9.1. Agarose gels:

For the separation of DNA fragments agarose gels were run in BRL gel electrophoresis kits at 150 volts, submerged in

1xTBE buffer at room temperature. A 100ml gel slab contained 0.8 - 2% (w/v) agarose, 1xTBE buffer and 0.5µg/ml ethidium bromide. One tenth volume of sample buffer was added to samples prior to electrophoresis. For the isolation of DNA fragments NuSieve GTG agarose was used at a concentration of 2 -4% (w/v).

Formaldehyde gels for the separation of RNA contained 1.2% agarose in 1xMOPS and 3% (v/v) of 37% formaldehyde. RNA samples in 50% formamide, 17.5% (v/v) 37% formaldehyde and 1xMOPS were incubated at 55°C/15 min and 1/5 vol formaldehyde loading buffer added to each sample prior to loading on gel. Gels were run submerged in 1xMOPS at 120 volts for 2½ hrs in a fume hood.

Methyl mercury gels for RNA analysis contained 1% agarose and 5mM methyl mercury in 100ml 1xEM buffer. Prior to electrophoreses, samples were mixed with an equal volume of 2x RNA sample buffer (20% glycerol, 0.05% (w/v) bromophenol blue, in 2xEM buffer) and a 1/10 vol of 0.1M methyl mercury. Gels were run in a fume hood, nonsubmerged at 80 volts with 1xEM buffer recycled from the cathode to the anode.

3.9.2. Denaturing acrylamide gels:

For separation of DNA fragments from S1 analysis and primer extension assays 5% gels were made from a 30% stock of 29:1 acrylamide / N,N'-methylene-bis-acrylamide, with 7M urea in 1xTBE buffer. They were polymerized by adding TEMED (0.7µl/ml) and 1/150 volume 10% ammonium persulphate (APS).

For DNA sequence analysis 6% gels were made from a 40% stock of 19:1 acrylamide / bis-acrylamide, with 9M urea in 0.5xTBE buffer. They were polymerized by the addition of TEMED (1.7µl/ml) and 1/600 volume 25% APS.

The gels (0.35mm x 230mm x 450mm) were prerun at 40W for 1 hr prior to loading samples. Samples were denatured by boiling in 0.3-1x formamide dyes and run at 40W in 1x or 0.5xTBE for the appropriate length of time. After electrophoresis, gels were either fixed in 10% acetic acid/45% methanol for 30 min and dried for 2 hrs/80°C or wet gels were exposed directely to XS film at -70°C for autoradi-

ography. For drying gels one gel plate was pretreated with 0.1% (v/v) Waker silane GF38/0.3% (v/v) acetic acid in ethanol to allow adhesion of the gel to the plate.

3.10. Recovery of DNA from agarose gels

Agarose gels containing 0.5 µg/µl ethidium bromide were briefly exposed to long wave UV light to visualize DNA and a small slice containing the DNA fragment of interest cut out of the gel. The DNA was electroeluted onto dialysis membrane in 0.5xTBE buffer for 2 hrs at 100 volts, recovered by washing the membrane in buffer and concentrated by ethanol precipitation. DNA was retrieved from NuSieve GTG agarose gel slices by hot phenol extraction. 2x (v/w) TE was added to the gel slice and melted at 65°C/10 min. One volume phenol at 65°C was added, vortexed 1min and spun 12,000rpm/5 min. Phenol extraction was repeated, followed by a chloroform extraction and ethanol precipitation.

3.11. Cloning of DNA fragments

DNA fragments made by the polymerase chain reaction (see 3.18.) were used for cloning. Where restriction sites were included in the primers comprising the ends of the DNA fragments, these were used as cloning sites. Fragments without restriction sites were cloned by blunt end ligation.

3.11.1. PCR products without restriction sites were cloned into M13mp8 RF for sequencing. DNA fragments were isolated from a NuSieve agarose gel by hot phenol extraction (see 3.10. above), kinased in 1xkinase buffer, 1mM ATP and 20U T4 kinase for 1 hr/37°C, phenol/chloroform and chloroform extracted and ethanol precipitated. The M13 vector was digested with SmaI in the presence of CIP. A 10µl ligation mix consisted of 1xligase buffer, 1U T4 ligase, 20ng SmaI/CIP treated M13 and approximately 25ng PCR product (1/10 of isolated fragment). The reaction was incubated at 15°C overnight and 5µl used to transform NM522 bacteria.

3.11.2. PCR products containing PstI cloning sites were cloned into pUC8. Fragments were isolated as before and digested with PstI, and pUC8 was digested with PstI in the presence of CIP. Samples were extracted with phenol/-chloroform and chloroform and ethanol precipitated after restriction digest. Ligations were carried out as above with a 20µl ligation mix containing 1/5 of isolated PCR product and 20ng PstI/CIP treated pUC8, and the total 20µl was used to transform JM109 bacteria.

3.11.3. PCR products containing HindIII and XhoI restriction sites were cloned into the HindIII and XhoI sites of pE1B-CAT (see 4.3.1.). The plasmid was digested with HindIII and XhoI in the presence of CIP to remove the original insert, and the backbone isolated from NuSieve agarose gel as described above. The PCR reaction mix was diluted 1 in 5 in 1x restriction-buffer b (Boehringer-Mannheim), digested with HindIII and XhoI, phenol/chloroform extracted and ethanol precipitated. Ligations were carried out as before in 20µl 1xligase buffer with 1U T4 ligase, 1/10 PCR product and 100ng E1B-CAT backbone. Half of the ligation mix was used to transform DH1 cells.

3.12. Transformation of *E. coli* with plasmid DNA

3.12.1. Calcium chloride method (Cohen *et al.*, 1973):

An overnight culture of bacteria (1ml) was diluted into 50ml L-broth and shaken at 37°C until an OD_{630} of 0.3-0.4 had been reached. The bacteria were then pelleted at 2,000rpm/10 min/4°C, resuspended in 50mls ice cold 30mM $CaCl_2$, pelleted, resuspended again in 50mls 30mM $CaCl_2$ and left on ice for 20 min. After further centrifugation the pellet was resuspended in 1ml 30mM $CaCl_2$. A ligation mix (or 100ng plasmid DNA) was incubated with 200µl cell suspension on ice for 30 min. Cells were heat shocked at 42°C/2 min and incubated shaking at 37°C/1-2 hrs after the

addition of 800µl L-broth. The bacteria were then plated out on L-broth agar plates (containing ampicillin where appropriate) and incubated overnight at 37°C.

3.12.2. Hanahan method (Hanahan, 1983):

50µl overnight culture of bacteria was incubated with 6.5ml SOB at 37°C until an OD_{630} of 0.4 was reached. After standing on ice for 15 min the bacteria were pelleted at 2,500rpm/12.5 min/4°C, resuspended in 2ml TFB, on ice for 15 min and the centrifugation repeated. The pellet was taken up in 0.5ml TFB with sequential addition of 17.5µl DMSO (5 min on ice), 17.5µl 2.25M DTT in 40mM MES pH6.2, (10 min on ice), 17.5µl DMSO, (5 min on ice). A ligation mix (or 100ng plasmid DNA) was incubated with 200µl cell suspension on ice/30 min, heat shocked 42°C/90 sec, incubated 37°C/1 hr after the addition of 800µl SOC and plated out as before.

3.13. Small scale plasmid DNA preparation

Miniprep DNA for the analysis of bacterial colonies was prepared by alkaline lysis. A single bacterial colony in 5ml L-broth was allowed to grow to saturation. 1.5ml cell suspension was centrifuged 12,000rpm/20 sec in a microfuge, pellet resuspended in 100µl TE at room temperature for 5 min, followed by the addition of 200µl 0.2N NaOH/1% SDS and incubation on ice for 5 min. After the addition of 150µl potassium acetate solution (see 2.12) the mix was vortexed for 2 sec, placed on ice for 5 min and centrifuged 12,000rpm/1 min to pellet cell debris and chromosomal DNA. Plasmid DNA was precipitated by adding 0.9ml 100% ethanol to the supernatant and incubating for 2 min at room temperature. After centrifugation 12,000rpm/1 min the pellet was washed with 70% ethanol, resuspended in 20µl TE and 5µl used for further analysis by restriction enzyme digest.

3.14. Large scale preparation and purification of plasmid DNA

A 10ml overnight culture of bacteria was diluted to 400ml in L-broth containing the appropriate antibiotic and incubated shaking at 37°C until the OD_{630} was between 0.6 and 0.8. An aliquot was taken at this stage for storage as glycerol stock. A glycerol stock was made by diluting an aliquot of culture 1:1 in 60% L-broth/40% glycerol, and stored at -70°C. To the rest of the culture chloramphenicol was added to a final concentration of 200µg/ml and the culture incubated shaking overnight. The cell suspension was pelleted (Sorvall GS3 rotor) 7,000rpm/10 min, drained and resuspended in 2ml sucrose solution (250mM sucrose, 2mM $MgCl_2$, 50mM Tris-HCl pH8.0) and lysozyme added to 4µg/ml final concentration. After leaving at room temperature for 30 min, 800µl of 0.25M EDTA pH8.0 and 3.2ml Triton solution (0.5% Triton, 62,5mM EDTA pH8.0, 50mM Tris-HCl pH8.0) were added and left at room temperature for a further 15 min. The suspension was centrifuged 17,000rpm/40 min/4°C (Sorvall SS34 rotor) and the supernatant subjected to equilibrium centrifugation on a caesium chloride gradient. A 6ml sample consisted of 3.5ml DNA supernatant, 250µl ethidium bromide (10mg/ml) and 4.5g $CsCl_2$, made up to 9.3g in TE. After centrifugation at 40.000rpm/18 hr/15°C (Dupont TV865 rotor), the lower band on the gradient, containing the banded plasmid DNA, was isolated and extracted with isopropanol to remove ethidium bromide, dialysed vs TE for 2 hr/4°C and incubated with RNase A (100µg/ml) 1 hr/37°C and Proteinase K (100µg/ml)/1% SDS, 1 hr/37°C. After one phenol/chloroform and two chloroform extractions the DNA was concentrated by ethanol precipitation.

3.15. Transformation of bacteria with M13 phage

For the propagation and isolation of M13 recombinant phage M13 replicative form (RF) was introduced into NM522 cells by the method of Bankier *et al.*, 1987. A single colony of bacteria was incubated in 100ml 2YT broth until

OD_{630} reached 0.3-0.4, when cells where pelleted at 2,000rpm/10 min/4°C and resuspended in 2.5ml TFB. The suspension was left on ice for 15 min followed by the sequential addition of 100µl DMSO (5 min on ice), 100µl 2.25M DTT/0.04M potassium acetate pH6.0 (10 min on ice) and 100µl DMSO (5 min on ice). The ligation mix was incubated with 200µl cell suspension on ice for 45 min, heat shocked at 42°C/3 min, mixed with 3ml top agar and plated onto L-broth agar plates. Top agar included 50µl X-gal (2% 5-bromo-4-chloro-3-indolyl-β-galactoside in DMF) and 50µl IPTG (2.5% isopropyl-β-D-thiogalactopyranoside in H_2O) for identification of recombinant M13 by colour. Cells containing self ligated M13 will produce β-galactosidase which in the presence of IPTG hydrolyses X-gal thus producing blue plaques, while plaques containing recombinant M13 remain colourless. After incubation overnight, white plaques were picked for further analysis.

3.16. Preparation of single stranded DNA from M13 recombinant phage

White plaques were picked into 1.5ml of diluted NM522 overnight culture (1/100 in 2YT) and shaken vigorously at 37°C/5½ hr. Cells were pelleted at 12,000rpm/5 min and 0.2ml of 20% (w/v) polyethylene glycol/2.5M NaCl added to the supernatant to precipitate phage. After leaving 30 min at room temperature, and spinning 12,000rpm/5 min, the pellet was resuspended in TE, extracted with phenol and ethanol precipitated in the presence of 0.4M sodium acetate. The DNA pellet was washed with 70% ethanol and finally dissolved in 50µl 0.2xTE, and 4µl used for a sequencing reaction.

3.17. DNA sequencing

DNA was sequenced by the dideoxy-chain termination method (Sanger *et al.*, 1977) using a T7 DNA polymerase sequencing kit from Pharmacia according to the producers standard

protocol. Plasmid DNA was denatured prior to sequence analysis by incubating 5 min at room temperature with 0.2M NaOH in TE, followed by ethanol precipitation in the presence of 2M ammonium acetate. Denatured plasmid DNA (2µg) or single stranded M13 DNA (see above) was incubated with 1-10 pmol of the appropriate primer at 37°C/20 min in annealing buffer. Subsequently, labelling mix (including dGTP, dCTP and dTTP), 5µCi [α-^{32}P]dATP and 3U T7 polymerase were added to the reaction and left 3-5 min at room temperature. 4.5µl aliquots were then added to four 2.5µl stop solutions, each including one ddNTP, and reaction allowed to continue at 37°C/5 min. After the addition of 5µl formamide dye mix the reactions were denatured by boiling for 1 min and 4µl analysed on 6% denaturing acrylamide gels.

3.18. Polymerase chain reaction (PCR)

3.18.1. Automated PCR (Saiki *et al.*, 1985, Mullis *et al.*, 1986, Mullis and Faloona, 1987) was used to amplify specific fragments from a DNA template. A pair of template specific primers are extended using a thermostable DNA polymerase (Saiki *et al.*, 1988) in successive rounds of template denaturation, annealing of primers and extension, resulting in a theoretical 10^6-10^7 fold amplification of a DNA fragment. A typical 50µl reaction mix consisted of 200µM dNTPs, 1U AmpliTaq DNA polymerase, 0.2µM each primer and template DNA (various amounts, see results) in 1xPCR buffer. In a 1.5ml eppendorf tube the reaction mix was overlaid with 50µl liquid paraffin and amplification performed on a Cambio thermal cycler. Initial denaturation at 94°C/5-7 min was followed by 30 cycles of 94°C denaturation, annealing of primers at the appropriate temperature and 72°C extension. Reaction times were varied (see results), annealing temperature was usually 5-10°C below the T_M of the primers, where T_M = 2[AT] + 4[GC]. Prior to analysis the paraffin was removed by chloroform extraction and subsequently 1/5 of

a reaction mix was run on a 1.5-2% agarose gel with 123 ladder (BRL) as a size marker.

3.18.2. cDNA-PCR

A combination of cDNA synthesis and PCR amplification was used for the analysis of splice junctions in cytoplasmic RNA essentially as described by Kawasaki and Wang, (1989). Prior to cDNA synthesis cytoplasmic RNA was treated with RNase free DNase, extracted twice with phenol/chloroform, once with chloroform and ethanol precipitated in order to remove any residual DNA that might act as a template in a subsequent PCR reaction. 5µg cytoplasmic RNA were incubated with 1µM "downstream" primer, 1mM each dNTP, 10U RNasin and 10U AMV reverse transcriptase in 10µl 1xPCR buffer, for 10 min at room temperature followed by 15-30 min/42°C. The reaction mix was boiled 5 min, quickly chilled on ice and half or all of the reaction mix used for a PCR reaction. A 50µl PCR mix included 5-10µl cDNA reaction mix, 0.2µM "upstream" primer, an additional 0.2µM of downstream primer and 1U AmpliTaq polymerase in 1xPCR buffer, and amplification was performed as described above (3.18.1.). A cDNA-PCR reaction without RNA template and a PCR reaction on DNA template were performed in parallel as controls. All reagents were made up and aliquoted using positive displacement pipettes and kept separate from PCR products in order to avoid contaminating the reaction mix with amplified DNA template.

3.19. S1 mapping

S1 nuclease analysis was carried out by a modification of the method of Berk and Sharp (1977). Restriction fragments were isolated by electroelution (see 3.10.) and 3' or 5' end labelled (see 3.8) for use as probes. The probes are listed in table 4. Both strands on each double stranded fragment will be labelled. Approximately $5x10^4$ cpm probe was freeze dried with 50µg total or cytoplasmic RNA from mock infected or Ad40 infected KBa+b cells. The pellet was resuspended in

20µl hybridization buffer, denatured at 68°C/10 min and transferred directly to 55°C/3 hr. Samples were diluted tenfold in ice cold S1 buffer including 166U S1 nuclease (BRL) and incubated 42°C/30 min. After ethanol precipitation, protected fragments were resuspended in formamide dye mix, denatured by boiling and analysed on 5% acrylamide gels using 3' end labelled HpaII fragments of pBR322 as molecular weight markers.

Table 4. Restriction fragments used for S1 analysis

Size	Restr. frag.	Nt.	End label	Source
579bp	HpaI-BclI	1064-1647	5'	pNM82
1022bp	BamHI-EcoRI	1849-2857	3'	pNM87
475bp	AvaI-PstI	2938-3410	5' or 3'	pNM86
565bp	TaqI-TaqI	3366-3690	3'	pNM86

3.20. Primer extension analysis

Primer extension anaysis of RNA from mock infected or Ad40 infected cells was performed by the method described by Ausubel *et al.*, (1987). Oligonucleotides were 5' end labelled and approximately 1ng annealed to 50µg of total RNA at 30°C overnight in 30µl hybridization buffer. The following day samples were ethanol precipitated and resuspended in reaction mix. A 12.5µl reaction mix including 10U AMV reverse transcriptase contained 0.5mM dNTPs and 25U RNasin (ribonuclease inhibitor) in 1x RT buffer. Alternatively, Moloney Murine Leukemia Virus (MMLV) reverse transcriptase was used according to the manufacturer's specifications. A 20µl reaction mix including 200U MMLV reverse transcriptase was 10mM DTT and 0.5mM dNTPs in 1x buffer (50mM Tris-HCl pH8.3, 75mM KCl, 3mM $MgCl_2$). Primer extension was carried out at 42°C/1-1.5 hr and stopped by the addition of 0.5M EDTA followed by treatment with 0.1mg/ml RNase A at 37°C for 30 min. After increasing the volume to 125µl with 2.5M ammonium acetate, samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated. Extended products were analysed on 5% denaturing acrylamide gels with 3' end labelled pBR322 HpaII fragments as markers,

or on 6% gels alongside the appropriate sequencing ladders (see results).

3.21. Northern blot analysis

3.21.1 Methyl mercury gels

RNA samples were run on methyl mercury gels as described (see 3.9.1.) and directly after electrophoresis the RNA was blotted onto a Hybond-N membrane in 20xSSC overnight. After fixing the blot by exposing to short wave UV light for 4 min, prehybridization (3-5 hr) and hybridization (overnight) were carried out in 50% formamide, 5xSSPE, 5xDenhardt's solution, 0.5% SDS and 0.1mg/ml denatured salmon sperm DNA, by incubating at 42°C in a shaking waterbath. Denatured DNA probe, labelled by the random priming method, was included in the hybridization mix. Blots were washed at room temperature in 2xSSC/0.1% SDS for 2x30 min and 0.1xSSC-/0.1%SDS for 2x30 min and exposed to flashed film with intensifying screen at -70°C.

3.21.2. Formaldehyde gels

Following electrophoresis (see 3.9.1.), formaldehyde gels were equilibrated in 0.5xTBE for 2x10 min and electroblotted onto Zeta Probe membrane in 0.5xTBE using a Mini Trans-blot (Bio-Rad) at 80V/1 hr. The membrane was dried at 80°C under vacuum and prehybridized and hybridized as described above in 50% formamide, 4xSSPE, 1%SDS, 5xDenhardt's and 0.5mg/ml salmon sperm DNA, with nick translated probe included in the hybridization mix. Blots were washed at room temperature for 15 min in each of the following solutions: 2xSSC/0.1% SDS; 0.5xSSC/0.1% SDS; 0.1xSSC/0.1% SDS, and exposed to flashed film with intensifying screen.

3.22. Slot blot / Dot blot analysis

DNA was applied directly to Hybond-N using a dot blot or a slot blot (Schleicher and Schuell) apparatus. Samples

were either denatured with 1 vol 1M NaOH (10 min/room temperature) and neutralized with 2 vol 2M ammonium acetate, or by boiling for 3 min in 6xSSC and cooling on ice. Membrane was presoaked in 1M ammonium acetate or 6xSSC and samples applied under vacuum and the wells washed through with 200µl presoak solution. Filters were air dried and fixed by exposing to short wave UV light for 4 min, and prehybridized and hybridized in 50% formamide, 5xSSC, 1xDenhardt's and 100µg/ml salmon sperm DNA at 42°C. A ^{32}P labelled DNA probe was included in the hybridization mix. After hybridization blots were washed at room temperature in 2xSSC/0.1% SDS for 2x30 min and 0.1xSSC/0.1% SDS for 2x30 min and exposed to flashed film with intensifying screen at -70°C. DNA concentrations were determined from the autoradiographs by densitometric analysis using the data system for the Hoefer GS-360 scanning densitometer. Within the ranges used there was a linear relationship between the amount of DNA present and the area under the peak corresponding to the DNA slot/-dot on the autoradiograph.

3.23. Transfection of DNA into eucaryotic cells

Plasmid DNA was introduced into WS Hela cells for the detection of gene expression from a CAT reporter plasmid by CAT assays. WS Hela cells were seeded at a low density on 50mm plates and the next day the subconfluent monolayers were transfected using DOTMA (N-[1-(2,3-Dioleyloxy)propyl]-N,N,N-trimethylammonium chloride), a liposome mediated transfection-reagent. For each transfection assay, 4µg of the CAT reporter plasmid were cotransfected with 4µg of inducer plasmid or pUC8. Plasmid DNA was mixed with 1.5ml tissue culture medium and separately 40µl DOTMA was added to 1.5ml medium. The two solutions were mixed, added to drained cell plates and the cells incubated at 37°C/6 hr. Transfection medium was then removed by suction, replaced by fresh growth medium and incubated for a further 48 hr. The cells were harvested into 3ml TEN after washing once in PBS, pelleted at 2,000rpm/1 min/4°C, drained and resuspended in

75µl 0.25M Tris-HCl pH8.0. After sonication cell extracts were cleared by centrifugation 12,000rpm/2 min and the supernatant used directly for CAT-assays or stored at -20°C.

3.24. CAT assays

Cell extracts from transfected cells were assayed for chloramphenicol acetyl transferase (CAT) activity by the method of Seed and Sheen (1988). This assay exploits the relatively low specificity of CAT for the acyl donor, which allows butyryl CoA to be substituted for acetyl CoA. The greater hydrophobicity of butyrylated chloramphenicol allows an effective discrimination between free and acylated chloramphenicol by a simple phase extraction (Seed and Sheen, 1988). A reaction mix contained 25µl cell extract (see above), 1µl 25mM butyryl CoA, 0.5µl [^{14}C]chloramphenicol stock and 18.5µl H_2O. After incubation at 37°C/1 hr the reaction mix was extracted with 180µl of TMPD (tetramethylpentadecan)/Xylenes 2:1, followed by centrifugation for 1 min in a microfuge. Subsequently, 150µl of the organic (top) phase was removed to a scintillation vial and counted by scintillation counting in 4ml ecoscint as a measure of the CAT enzyme activity in each cell extract.

4. RESULTS

The aim of the work described in this thesis was to investigate the properties involved in the growth restriction of Ad40 in tissue culture as compared to other adenovirus serotypes. It has been shown previously that Ad40 can grow on 293 cells, expressing the E1 region of Ad5 (Takiff *et al.*, 1981; de Jong *et al.*, 1983), although others have failed to detect Ad40 replication in those cells (Chiba *et al.*, 1983; Uhnoo *et al.*, 1983). In this laboratory it was established that Ad40 will grow on KB cell lines expressing the Ad2 E1 region or the E1B region alone but not on cells expressing only the E1A region (Mautner *et al.*, 1989). This suggests that the growth restriction of Ad40 in tissue culture can be overcome by supplying Ad2 E1B gene products *in trans* but not by providing only the Ad2 E1A gene products. These results indicate that the low transactivating function of Ad40 E1A gene products and the weak *cis*-activity of the E1A promoter (van Loon *et al.*, 1987a; Ishino *et al.*, 1988) are not a major cause of the restrictive growth of Ad40 in tissue culture. Moreover, this implies that one or more Ad40 E1B gene products are not expressed in a functional form in KB cells or alternatively they are functionally different from their Ad2 counterparts. Studies on other adenovirus serotypes have shown that mutations in either the E1B 19K or the 55K proteins can affect the host range of these viruses. Hence the Ad40 E1B region is a potential candidate for having a role in the host range of the virus. In order to better understand the involvement of the E1B region in the restricted growth a study of the Ad40 E1B region was undertaken.

4.1. Virus complementation

The ability of Ad40 to complement Ad2/5 and Ad12 E1 mutant viruses was tested using a coinfection assay. This was done in order to see if Ad40 could provide functions lacking in viruses defective for E1A or E1B protein func-

tions, thus indicating the presence or absence of those functions in Ad40. Further, if coinfection with wild type virus enhances Ad40 replication, this assay should indicate which E1 mutations affect the ability of those serotypes to complement Ad40 growth.

4.1.1. Coinfection of Ad5/2 E1 mutants with Ad40:

Ad2/5 mutant viruses used in this study are described in table 2 (section 2.1.). Ad5 mutants dl309, dl312 and dl313 were isolated by Jones and Shenk (1979). Briefly, dl309 is an Ad5 virus that lacks three XbaI sites and has a large deletion within the E3 region, but grows as well as wild type in tissue culture. This is the background for a large deletion in the E1A region in dl312 and a deletion of the E1B region in dl313; both viruses are defective for growth on Hela cells but complement each other in a double infection (Jones and Shenk, 1979). Gene specific E1B mutants were constructed by Barker and Berk (1987) by recombining altered Ad2 E1B sequences into a dl309 background. A mutant virus, pm1722 specifically lacking the E1B 19K product was created by introducing a stop codon after the first 3 residues of the 19K ORF. This mutant replicates as well as wild type in Hela cells. The mutant dl1520 specifically lacking the E1B 55K protein and related products has a point mutation creating a stop codon after the second residue of the 55K ORF but not affecting the overlapping 19K ORF, and a 827bp deletion downstream of the 19K ORF. Replication of this virus is defective on Hela cells but like dl312 and dl313 the defect is less marked at a high multiplicity of infection.

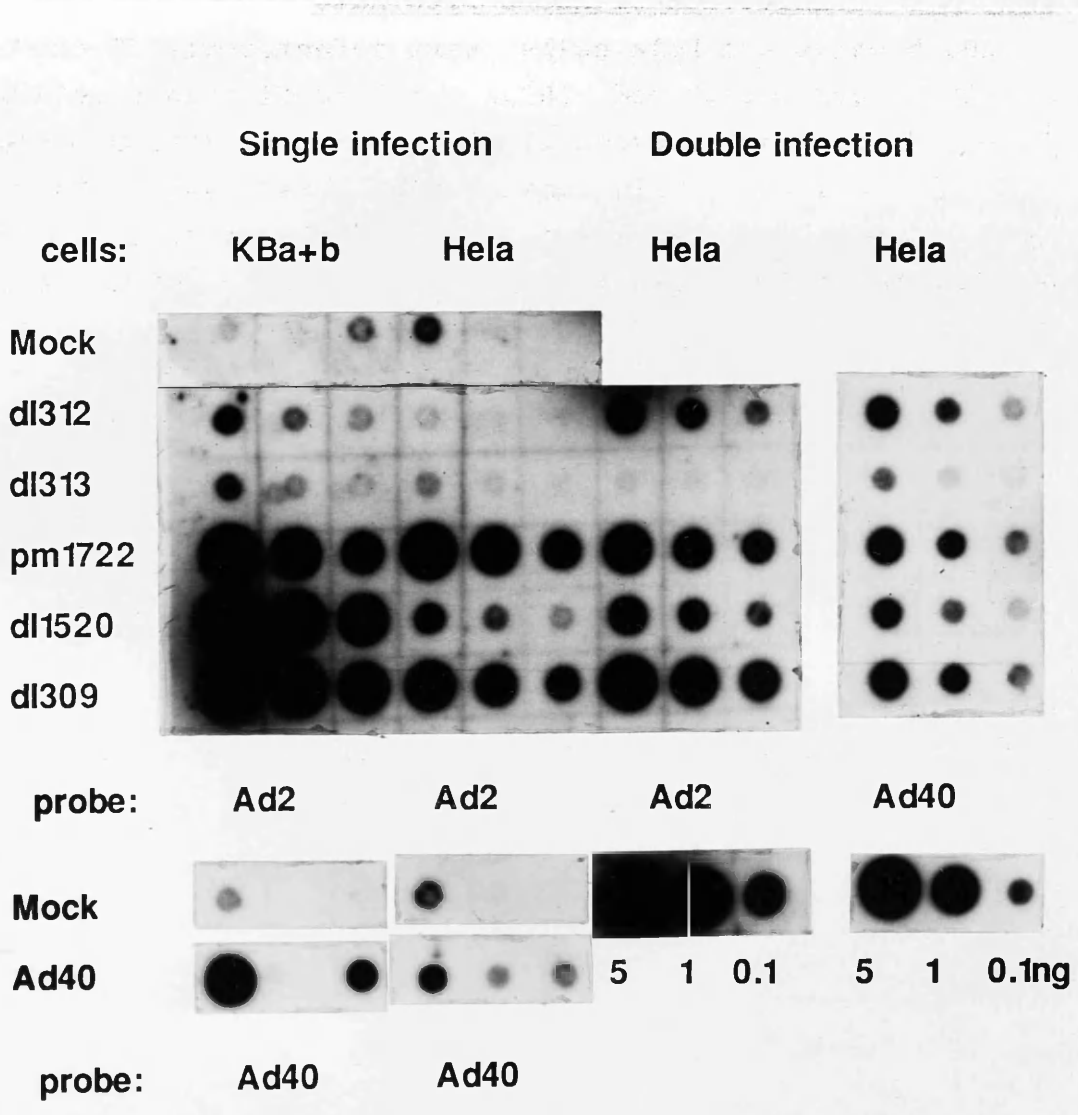
In coinfection assays a p3 stock of Ad40 was used diluted 1/10 in TS in the absence of a suitable quantitation method for Ad40. The virus does not form plaques on KBa+b cell or 293 cells and attempts to enhance plaque formation have so far been unsuccessful. Ad5/2 mutant viruses were used at a multiplicity of 3pfu/cell. Single and double infections were used to assess reciprocal complementation between each

of these mutant viruses and Ad40. Hela cells on 50mm plates were infected with 100µl virus dilution in a single infection and 100µl of each virus in double infections. Single infections on KBa+b cells were performed in parallel. Cells were harvested at 72 hr.p.i., virus released by freezing and thawing and virion DNA extracted as described (see 3.2. and 3.3.). DNA obtained by this method resides within virus particles since DNaseI treatment prior to DNA extraction does not alter the yield (Mautner *et al.*, 1989). Dilutions of virion DNA were applied in duplicate to Hybond-N on a dot blot apparatus and probed with a mixture of E1A and E1B containing plasmids of the appropriate serotype (pNM80/pNM81 and pNM90/pNM92 for Ad40 and Ad5/2 respectively). Autoradiographs were quantitated by densitometry.

The autoradiographs are shown in figure 6. In the absence of Ad40, dl312 and dl313 DNA was not detectable in Hela cell extracts. However, in the presence of Ad40, dl312 DNA was detected at the highest dilution, indicating a substantial increase in the level of virion DNA, whereas dl313 DNA was not detected. Moreover, coinfection with dl312 resulted in a 2-fold increase in Ad40 yield while in the presence of dl313 the Ad40 yield was even less than for a single infection. The E1B 55K mutant dl1520 replicated poorly on Hela cells but the yield of virion DNA was increased around 2-fold in the presence of Ad40. However, the coinfection with dl1520 only resulted in a 30% increase in the yield of Ad40 compared to a single infection. Both dl309 and pm1722 replicated efficiently in a single infection in Hela cells as expected. In the double infection the yield of pm1722 was somewhat reduced while nearly a 2-fold increase was seen in dl309 virion DNA. Both viruses however, increased the yield of Ad40 around 3-fold in a double infection relative to a single Ad40 infection. While the mutation in dl1520 was efficiently complemented in KBa+b cells, dl312 and dl313 grew poorly in those cells. Better yields of those mutants were obtained from 293 cells which were used for preparation of virus stocks; this finding was also noted by Nancy Mackay (personal communication). The

Figure 6: Coinfection of Ad5/2 with Ad40

KBa+b cells and Hela cells were infected with 3 pfu/cell of Ad5 or Ad2 mutant and 100µl of 1:10 diluted p3 stock of Ad40. Virus was harvested at 72 hr p.i. and virion packaged DNA was extracted. Dilutions (1/40, 3/400 and 1/400) were applied to nylon filters and probed with a mixture of E1A and E1B containing plasmids (100ng of each) of the appropriate serotype (pNM80/pNM81 and pNM90/pNM92 for Ad40 and Ad5/2 respectively). The top left panel shows the yield of Ad5/2 viruses in a single infection on KBa+b cells and Hela cells. The yield of Ad5/2 viruses and Ad40 in a double infection on Hela cells is shown on the right. The lower panel on the left shows the yield of Ad40 in a single infection on KBa+b cells and Hela cells. Plasmid controls are shown on the right. The lack of cross-reaction between Ad40 and Ad5 sequences was established by probing dl309 with the Ad40 probe and Ad40 with the Ad5 probe (not shown).


Single infection
Double infection
cells:
KBa+b
Hela
Hela
Hela
Mock
dl312
dl313
pm1722
dl1520
dl309
probe:
Ad2
Ad2
Ad2
Ad40
Mock
Ad40
5
1
0.1
5
1
0.1ng
probe:
Ad40
Ad40

basis for this difference is not known.

4.1.2. Coinfection of Ad12 E1 mutants with Ad40:

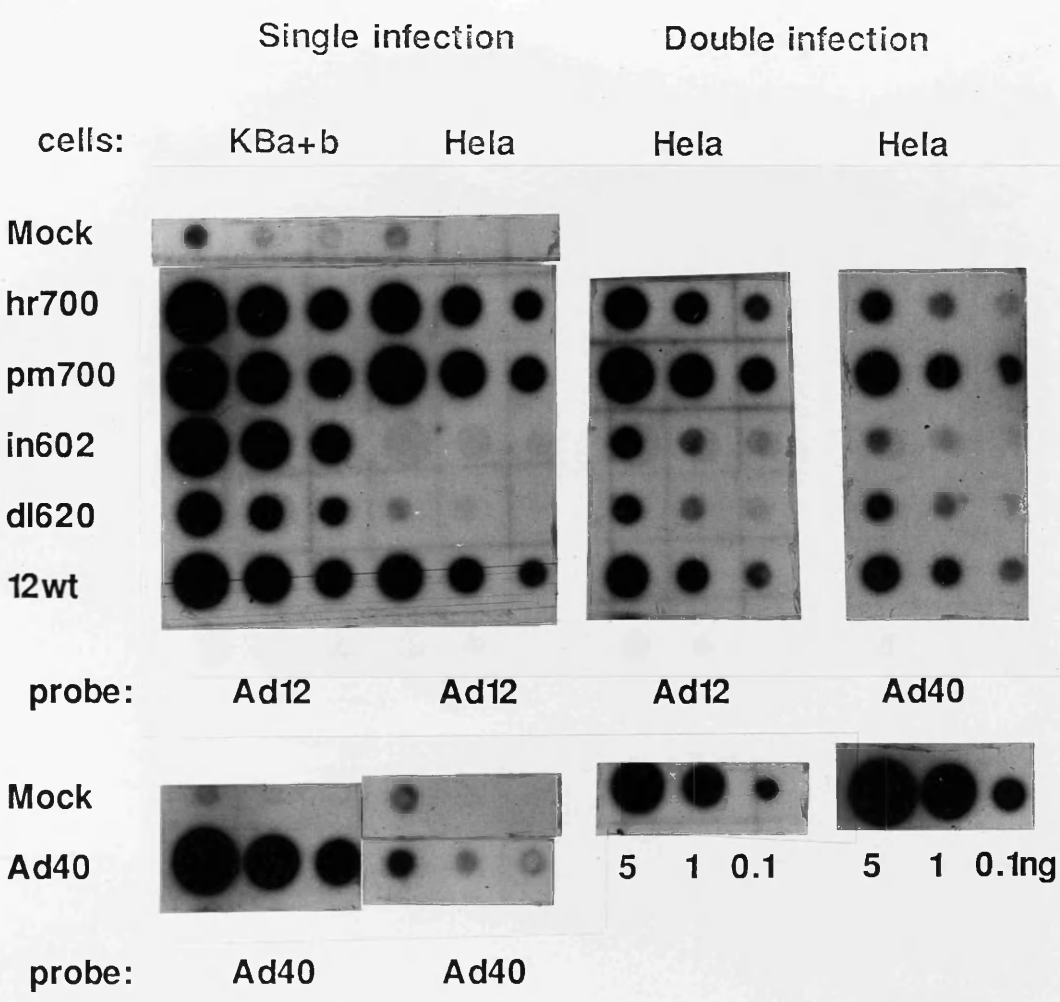
The Ad12 mutant viruses used in this study are described in table 2 (section 2.1.). Gene specific E1 mutants of Ad12 were constructed by Breiding *et al.*, (1988) and Edbauer *et al.*, (1988). The E1A mutant hr700 has a point mutation resulting in altered amino acid sequence in conserved region 3 of the 266R and 235R E1A proteins. Replication of this virus is restricted in Hela cells (Breiding *et al.*, 1988). A point mutation in pm700 changes the AUG initiation codon of the E1B 19K ORF to AUC. In in602 a frameshift in the E1B 55K ORF leads to a termination codon after 148 residues, and dl620 has a 696bp in frame deletion in the 55K ORF. Both viruses retain the coding potential for the 19K E1B protein and they are defective for growth on Hela cells (Breiding *et al.*, 1988).

Single and double infections on Hela cells were carried out as described above and single infections on KBa+b cells were carried out in the same way. Dilutions of virion DNA were analysed on dot blots by probing with an Ad40 E1 containing plasmid (pNM82) or an Ad12 E1 containing plasmid (pAsc10.3).

The results are shown in figure 7. The efficient growth of the E1A mutant hr700 in Hela cells was unexpected. A contamination of the virus stock by wild type virus or a reversion of the point mutation can not be excluded as an explanation. A reduced yield was seen when coinfected with Ad40, while the yield of Ad40 was increased nearly 2-fold compared to a single infection. The two E1B 55K mutants (in602 and dl620) did not replicate on Hela cells in a single infection, but in the presence of Ad40, both were detectable. They had little effect on the Ad40 yield, although a slight decrease was seen in the presence of in602 relative to a single Ad40 infection. Replication of pm700 and 12wt was not affected by the presence of Ad40 but they increased the yield of Ad40 6- and 4-fold respectively in

Figure 7: Coinfection of Ad12 with Ad40

KBa+b cells and Hela cells were infected with 3pfu/cell of Ad12 viruses and 100µl of 1:10 diluted Ad40 p4 stock. Virus was harvested at 72 hr p.i. and virion DNA applied to dot blots (diluted 1/40, 3/400 and 1/400) and probed with E1 containing plasmids (pNM82 and pAsc10.3) of Ad40 and Ad12. The top left panel shows the yield of Ad12 viruses in a single infection on KBa+b cells and Hela cells. The yield of Ad12 viruses and Ad40 in a double infection on Hela cells is shown on the right. The lower panel on the left shows the yield of Ad40 in a single infection on KBa+b cells and Hela cells. Plasmid controls are shown on the right. The lack of cross-reaction between Ad40 and Ad12 sequences was established by probing Ad40 with the Ad12 probe and Ad12wt with the Ad40 probe (not shown).


Single infection
Double infection
cells:
KBa+b
Hela
Hela
Hela
Mock
hr700
pm700
in602
dl620
12wt
probe:
Ad12
Ad12
Ad12
Ad40
Mock
Ad40
5 1 0.1
5 1 0.1ng
probe:
Ad40
Ad40

the double infection.

4.1.3. Summary

The results described above show that Ad40 growth on Hela cells can be complemented by coinfecting with Ad2/5 or Ad12 viruses that replicate well on Hela cells. Furthermore, Ad40 and dl312, an Ad5 E1A mutant which does not grow on Hela cells in a single infection, can complement each other, resulting in increased replication of both viruses on this cell line. On the other hand, Ad40 does not complement dl313, an E1B negative virus. All the E1B 55K mutants show an increased yield in a double infection relative to a single infection but interestingly this effect is one sided in all cases, since the yield of Ad40 is not increased.

These results are consistent with the observation that Ad40 can grow on cells expressing the E1B region of Ad2 (Mautner *et al.*, 1989) and suggest that Ad40 expresses a functional E1A protein capable of acting *in trans* to complement an Ad5 E1A mutant. The results also indicate that expression of the E1B 55K protein is essential for complementation of Ad40 since the mutant viruses that do not increase the yield of Ad40 in a double infection are the E1B negative virus (dl313) and the E1B 55K mutants of both Ad5/2 and Ad12. However, Ad40 may not be completely devoid of functional E1B 55K protein since it can provide some helper function for the 55K mutants. Alternatively, an increase in the levels of some other viral products in the presence of Ad40 may account for the increased replication of mutant viruses in double infections. This would be analogous to the permissive growth of many E1 mutant viruses at a high multiplicity of infection and could apply to both E1A and E1B mutant viruses. However, the complementation of Ad40 by an E1A mutant (dl312) but not by any of the E1B mutant viruses implies that the growth restriction of Ad40 in Hela cells is related to the lack of an E1B 55K protein function. The E1B 19K protein is not essential in tissue culture and the 19K mutant viruses grow like wild type, so these

experiments do not address the question whether or not Ad40 has a functional E1B 19K protein.

4.2. Analysis of RNA transcribed from the E1 region of Ad40

The DNA sequence of the E1 region of Ad40 shows similar organization of transcription signals and open reading frames to that of other human serotypes (van Loon *et al.*, 1987b, Ishino *et al.*, 1988; see figure 5). Van Loon *et al.* used baby rat kidney cells transformed with an Ad40 E1 containing plasmid as a source of E1 RNA in Northern blotting and S1 mapping experiments. They identified 9S, 12S and 13S E1A mRNAs but did not detect any E1B transcripts despite the fact that the intact E1B region was present in those cells (van Loon *et al.*, 1985a). However, Ad40 E1 transcripts have not previously been identified from a lytic infection. If transcription of the E1B region in infected cells is as incompetent as it appears to be in transformed cells this could account for the E1B 55K deficiency of Ad40, suggested by the virus complementation experiments described above and by complementing cell line experiments (Mautner *et al.*, 1989), In order to address this question, Ad40 E1 transcription in Ad40 infected KBa+b cells was investigated in several different ways. A timecourse of E1 transcription was studied by Northern blotting and after establishing the presence of E1B mRNAs the structure of these transcripts was analysed in detail by nuclease protection and primer extension assays, PCR analysis and partial cDNA sequencing and by Northern blotting.

4.2.1. Timecourse of E1 transcription

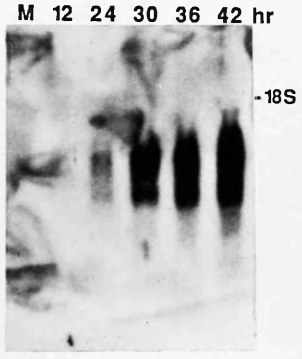
RNA transcripts mapping to the E1 region were analysed by Northern blotting of cytoplasmic RNA from Ad40 infected KBa+b cells harvested at various times after infection. Cells on 50mm plates were infected with 100µl of a 1/10 dilution of Ad40 p9. Following incubation at 37°C two plates were harvested at each timepoint and cytoplasmic RNA extracted as described (see 3.5.). Duplicate samples were run on 1.2% agarose/formaldehyde gels and blotted onto Zeta probe membranes. Blots were probed with pNM83 and pNM81 for

E1A and E1B respectively. In figure 8A E1A mRNAs are first seen at 24 hr p.i., and they increase in abundance up to 42 hr p.i. The higher band corresponds to E1A 12S and 13S mRNAs which would not be separated under these conditions, and the lower band corresponds to the 9S mRNA. This is consistent with the E1A transcripts mapped by van Loon *et al.*, (1987b; figure 5). In figure 8B bands detected by the E1B probe can first be seen at 24 hr p.i. albeit at a low intensity, and they increase in abundance up to 42 hr p.i. On a longer exposure all the bands seen at 30 hr p.i. are seen clearly at 24 hr p.i. but none of them are seen at 12 hr p.i. Three major bands are apparent on this gel. The probe, pNM81, extends beyond the 3' end of the E1B region and the top band is most likely a transcript from the IVa2 region downstream of E1B (see Mautner et al., 1990; section 4.2.2.1.v.). From analogy with other adenoviruses a 22S mRNA would also be expected but the presence of such a band may be obscured by the IVa2 mRNA. The middle band could correspond to a 13-14S E1B mRNA and the third band is most likely the 9S mRNA for polypeptide IX. It is notable that the late ppIX mRNA appears at the same time as the presumed early 13-14S mRNA although the possibility that the 13-14S mRNA is transcribed first cannot be ruled out at this stage in the absence of a timepoint between 12 and 24 hr p.i. The late appearance of the early transcripts was somewhat unexpected. However, the relationship between early transcription and DNA replication was not determined in this experiment. Mautner *et al.*, (1990) found that E1B mRNA was not detected until after the onset of DNA replication (see also 4.2.3.). Moreover, they showed that in the presence of a cytosine arabinoside block of DNA replication E1B transcripts were not detectable.

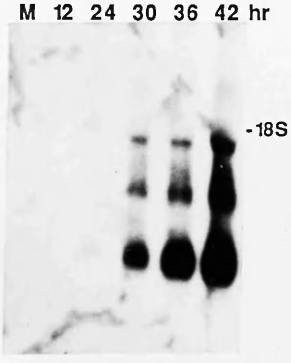
Figure 8: A timecourse of E1 transcription in KBa+b cells

Cytoplasmic RNA was harvested from mock infected and Ad40 (p9) infected KBa+b cells at the times indicated post infection. Duplicate samples were separated on 1.2% agarose/formaldehyde gels and blotted onto Zeta probe membranes. The harvest from one 50mm plate was loaded per lane. The migration of the 18S ribosomal RNA is indicated. A) E1A RNA. The blot was probed with a nick translated Ad40 E1A containing plasmid, pNM83 (nt 1-1211). B) E1B RNA. The blot was probed with a nick translated Ad40 E1B containing plasmid, pNM81 (nt 1643-3933). Blots were exposed for 3 days.

A



B



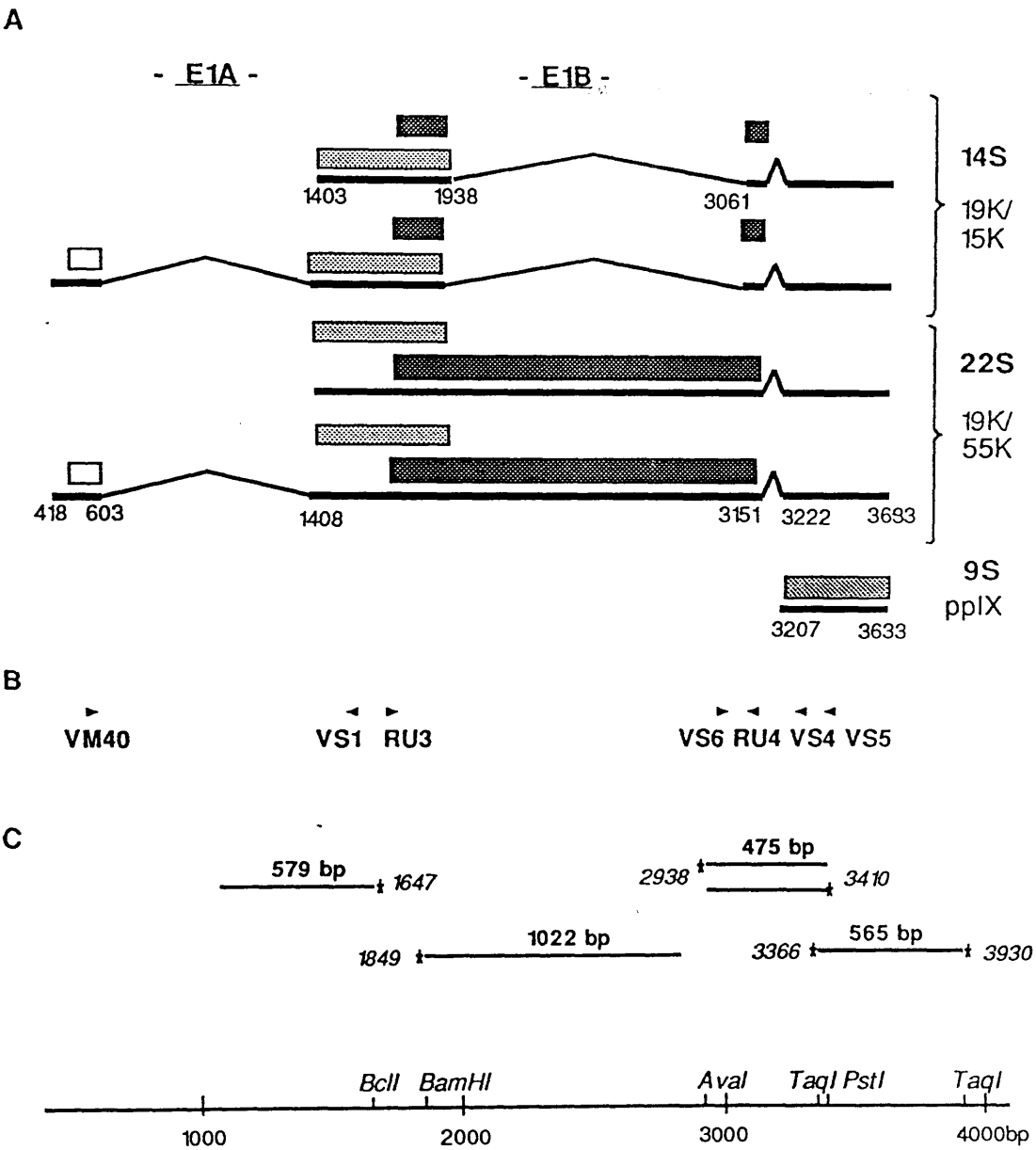
4.2.2. Mapping of E1B mRNA

In order to determine the protein coding capacity of E1B mRNAs a transcription map of the Ad40 E1B region was obtained using RNA from Ad40 infected KBa+b cells harvested at 42 hours post infection. This timepoint was chosen with reference to the Northern blotting experiment described above, where all E1B mRNAs appear to increase in abundance at least up to 42 hr p.i. Those Northern blots and the following mapping experiments all used RNA from cells infected with Ad40 p9. Initial experiments were performed using a p4 stock of Ad40. Although E1B RNA was detectable on Northern blots it was low in abundance compared to a p9 infection; S1 mapping experiments on that material were not successful most likely due to the low abundance. The p9 virus was more concentrated than the p4 stock (as seen from a comparison of virion DNA), hence using the p9 stock at a 1/10 dilution resulted in a higher multiplicity of infection which could account for the increase in E1B transcription. However, in the absence of a plaque assay or other reliable titration method a comparison between different virus stocks is not feasible.

Several methods were applied to obtain a transcription map of the E1B region. S1 nuclease mapping and primer extension analysis were used to locate 5' ends, 3' ends and splice sites. Restriction fragments and oligonucleotides used in this analysis were chosen by reference to the DNA sequence and the mRNA structure predicted from analogy with other adenovirus serotypes. Further details of mRNA structure were determined by PCR mapping, partial cDNA sequencing and Northern blotting. Figure 9A shows the transcription map obtained by the above methods. Also shown are locations of oligonucleotides used for primer extension and PCR analysis (figure 9B), and end labelled restriction fragments used in S1 analysis (figure 9C). Nucleotide numbers indicate transcription initiation sites, splice sites and polyadenylation sites. Open reading frames were predicted from the published sequence (van Loon *et al.*, 1987b; Ishino *et al.*, 1988) and from the mRNA structure.

Figure 9: Ad40 E1B transcription map.

The transcription map was determined by S1 nuclease mapping, primer extension analysis, PCR mapping partial cDNA sequencing and Northern blotting. See text for details. A) mRNA structure and location of open reading frames. Exons are represented by bold lines, and carets indicate introns. Hatched boxes denote ORFs for the predicted E1B proteins and polypeptide IX. The 15K ORF is composed of the first 73 and last 29 amino acids of the 55K ORF. A 41 residue E1A ORF is indicated by an open box on the E1A-E1B cotranscripts. Positions of 5' ends, 3' ends and junctions are indicated. RNA and protein sizes are designated by analogy with Ad2. B) Oligonucleotides used in primer extension and PCR analysis. The oligonucleotide sequences are shown in table 3. C) Restriction endonuclease fragments used in S1 analysis. The bold number refers to the fragment size and the number in italics to the position of the label. Only labelling of L strand is indicated except for the 565bp fragment where 3' end labelling of both strands is indicated. 579 is a HpaI-BclI fragment from pNM82, 1022 is a BamHI-EcoRI fragment of pNM87, 475 is an AvaI-PstI fragment from pNM86 and 565 is a TaqI fragment of pNM86. The line at the bottom represents the DNA genome; end labelled restriction sites are indicated.


A
- E1A -
- E1B -
1403
1938
3061
14S
19K/
15K
22S
19K/
55K
418
603
1408
3151
3222
3693
9S
pplX
3207
3633
B
VM40
VS1
RU3
VS6
RU4
VS4
VS5
C
579 bp
1647
475 bp
2938
3410
1849
1022 bp
3366
565 bp
3930
BclI
BamHI
AvaI
TaqI
PstI
TaqI
1000
2000
3000
4000bp

4.2.2.1 S1 nuclease analysis and primer extension assays

S1 nuclease analysis was performed by the method of Berk and Sharp (1977) as described (section 3.19). Briefly, double stranded restriction fragments (see figure 9C and table 4) were either 5' or 3' end labelled and after denaturation allowed to hybridize to total or cytoplasmic RNA from Ad40 infected or mock infected cells. Following digestion of single stranded DNA with S1 nuclease, protected DNA fragments were analysed on a 5% acrylamide gel. Primer extension analysis (section 3.20) used 5' end labelled oligonucleotides (see figure 9B and table 3) which after hybridization to total RNA were extended by reverse transcription. The products were analysed on acrylamide gels as above.

i) 5' end of E1B mRNA

In order to determine the 5' end of E1B mRNAs, a 579 nt fragment (figure 9C) 5' end labelled at nt 1647 was used in S1 nuclease assay on infected and mock infected cell extracts (figure 10). Protected fragments of 170 and 240 nt were obtained from infected cell extracts, which correspond to 5' ends or splice acceptor sites at nts 1480 and 1408 approximately. From the nucleotide sequence and by analogy with Ad2 the site at 1408 is likely to be the cap site. Primer extension analysis was used to determine the precise location of the cap site: A primer (VS1) 5' labelled at 1548 was extended using RNA template from mock infected or infected cells (figure 11A, lanes 1 and 2). Unique bands of 137 and 142 nt were obtained, and these were precisely located on the genome by running alongside a sequencing ladder (figure 11B). This identifies a T residue at nt 1412 and an A residue at nt 1403 as 5' ends. A 5' end around nt 1480, a possibility raised by the S1 analysis, would have resulted in a band of 60-70 nt, but nothing was seen in this region of the gel (figure 11A, lane 2). The possibility of a splice acceptor site around nt 1480 can not be excluded;

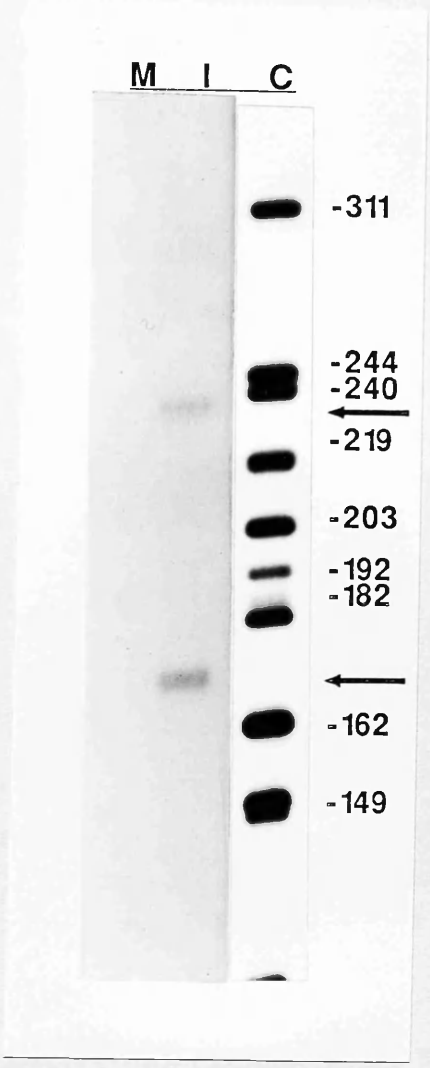
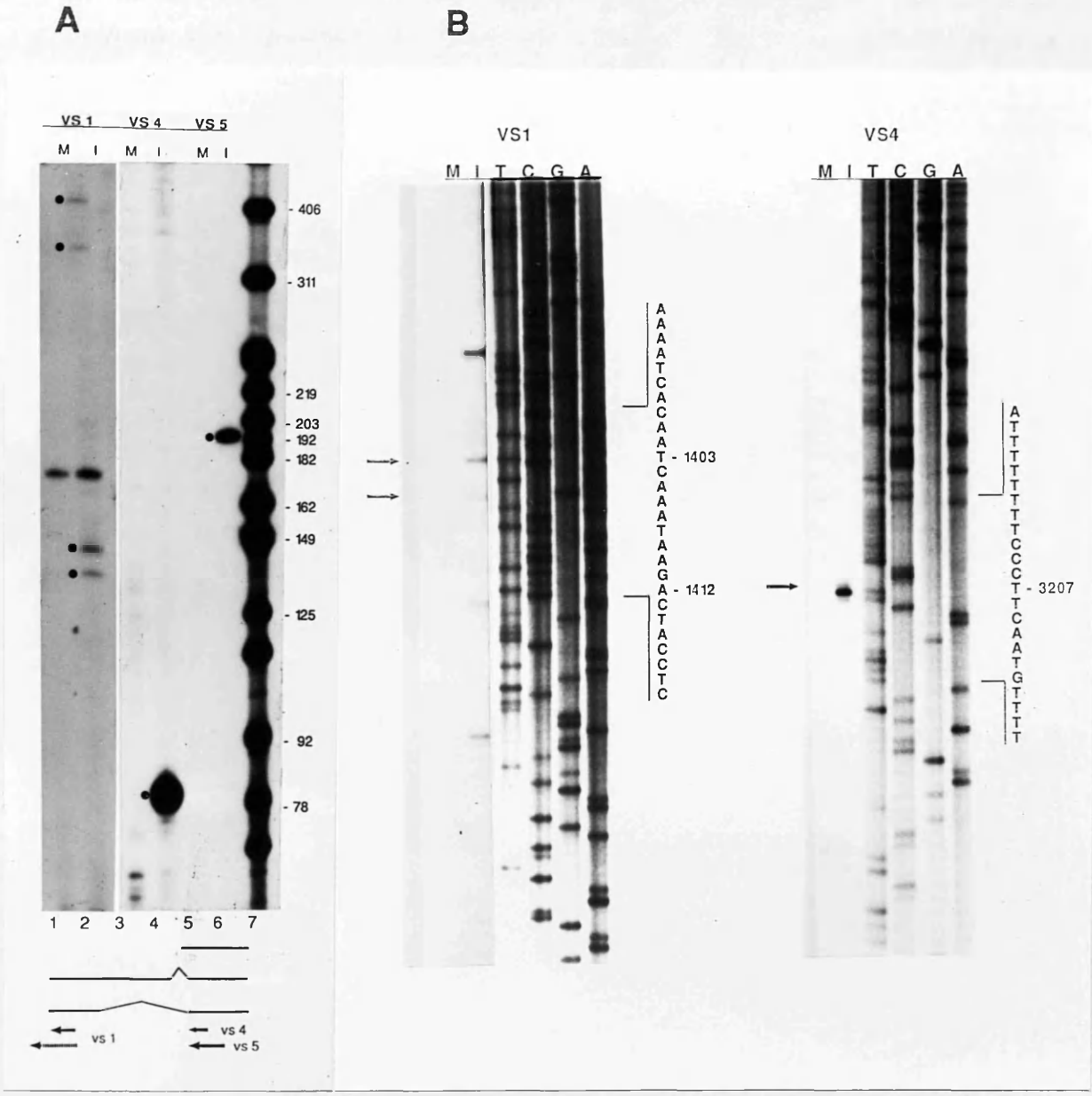


Figure 10: S1 nuclease analysis of the 5'end of E1B mRNA

A restriction fragment spanning nt 1064-1647 of Ad40 was 5'end labelled and used in S1 nuclease assay on whole cell RNA obtained from Ad40 infected (I) and mock-infected (M) KBa+b cells at 42 hr p.i. Hybridization and S1 nuclease digestion were done at 55° and 42° respectively. The molecular weight marker (C) is a HpaII digest of pBR322, the size of the fragments (bp) is indicated. Arrows show the positions of protected fragments obtained.

Figure 11: Primer extension analysis of E1B and ppIX mRNAs. Ad40 infected and mock infected KBa+b cells were harvested at 42hr p.i. Hybridization of oligonucleotide primers and reverse transcription were performed at 30° and 42° respectively. A) Primer VS1, 5' end labelled at nt 1548 was used to analyse E1B mRNA 5' end (lanes 1 and 2). Primers VS4 and VS5, 5' end labelled at nt 3287 and 3400 respectively, were used to analyse ppIX mRNA 5' end (lanes 3-6). Reactions used AMV reverse transcriptase. Samples were run on 5% denaturing acrylamide gels. A HpaII digest of pBR322 was used as molecular weight marker (lane 7). The filled circles mark unique products from infected cells. The diagram indicates predicted mRNA structure, location of primers and extended products. B) Primers VS1 and VS4 were extended using MMLV reverse transcriptase and run on 6% denaturing acrylamide gels. Sequencing reactions using the same primers on a pNM82 plasmid template were run alongside. Arrows point to the bands seen in A here precisely located alongside sequencing ladders. Other bands seen with VS1 in lane I were consistently obtained on mock infected cell extracts as well using the MMLV reverse transcriptase (not shown) although not seen on this gel; the top band corresponds to the band seen at 180 in lanes 1 and 2 in A. For precise location and sequence of oligonucleotide primers, see table 3.


A
B
VS 1
VS 4
VS 5
M I M I M I
406
311
219
203
192
182
162
149
125
92
78
1 2 3 4 5 6 7
VS 1
VS 4
VS 5
VS1
M I T C G A
AAAATCACAATCAAAATAAGACTACCTC
1403
1412
VS4
M I T C G A
ATTTTTTTTCCCTTCAATGTTTT
3207

the sequence CTTCTGCCAG/A (nt 1472-1482) may have such a role. There were indeed bands of approximately 330 nt and 420 nt, obtained by extension of primer VS1 (figure 11A lane 2) which suggests that some E1B transcripts may extend beyond the predicted E1B 5' end. This possibility was addressed by cDNA analysis (see 4.2.2.2.). A 175 nt fragment was obtained from mock infected as well as infected cell RNA (figure 11A lanes 1 and 2). This most likely results from the primer annealing to cellular RNA, possibly endogenous Ad2 mRNA.

ii) Splice donor site 1

A splice donor site corresponding to the Ad2 E1B 13S splice donor site was mapped by S1 analysis using a 1022 nt fragment 3' labelled at nt 1849 as probe (figure 9C). A ladder of protected fragments was obtained, the largest one corresponding to a 90 nt protected fragment (figure 12) locates a splice donor site at nt 1938, where there is a splice donor consensus sequence (AGG/GTACTA, nt 1936-1944). No other splice donor sites were detected using this probe in the region between nt 1849 and 2870. The ladders of bands in the figure (seen on a lower exposure) can be explained as resulting from heterogeneous 3' labelling of the BamHI site in the probe. A likely reason for the heterogeneity is as follows. The BamHI site has the sequence 5'GATCCA/$_T$G/$_c$T/$_A$G/$_c$**C/$_G$**A/$_T$G/$_c$.., (letters in subscripts denote the 3' end). The only dNTP added to the labelling reaction was [α^{32}p]dGTP. In some cases the 3'-5' exonuclease activity of the Klenow polymerase may degrade the double stranded DNA back to the **C/$_G$** (bold) and add a labelled G in that position. These molecules may then have a variable number of bases added back to the 3' end. In addition, other molecules will have labelled G paired directly to the single stranded C residues and again these may have a variable number of bases added to the 3' end. This could account for the two sets of ladders where the sequence indicates a single splice donor site. No convenient alternative restriction site was available to make a different probe for the detection of this splice

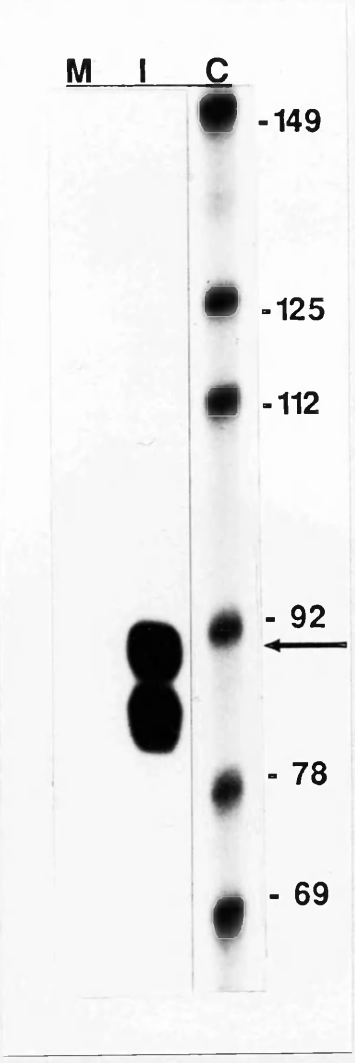


Figure 12: S1 nuclease analysis of splice donor site 1

A restriction fragment spanning nt 1849-2857 of Ad40 was 3'end labelled and used in S1 nuclease assay on cytoplasmic RNA obtained from Ad40 infected (I) and mock-infected (M) KBa+b cells at 42 hr p.i. Hybridization and S1 nuclease digestion were done at 55° and 42° respectively. The molecular weight marker (C) is a HpaII digest of pBR322, the size of the fragments (bp) is indicated. The arrow indicates the size of the protected fragment obtained. The multiple bands are the result of a heterogeneous 3'end labelling of the probe (see text).

donor site by S1 analysis and another fragment including the same BamHI site also produced the same ladders. The use of this site was independently confirmed by cDNA sequencing (see 4.2.2.2.).

iii) Splice donor site 2

A second splice donor site, corresponding to the Ad2 E1B 22S splice donor site, was mapped using a 475 nt fragment 3' labelled at nt 2938 as a probe in S1 nuclease assay (figure 9C). In figure 13 a 214 nt band corresponds to a splice donor site at nt 3151 which is immediately after the termination signal of the large open reading frame (TAAGG/GTAAGG, nt 3147-3157). The two smaller bands in figure 13, 147 and 151 nt, map to a region which includes a potential splice donor consensus sequence (nt 3085-3089). However, attempts to identify a use of this potential site were not successful (see 4.2.2.2.). In addition faint bands of unknown origin were seen on the gel between 170-190 nt.

iv) Splice acceptor site and 5' end of ppIX mRNA

In order to look for a splice acceptor site and the 5' end of polypeptide IX mRNA, the same 475 nt fragment was 5' end labelled at nt 3410 and used in S1 analysis. This resulted in two protected fragments, 204 and 189 nt long (figure 14) corresponding to ends at nt 3207 and 3222 respectively. Primer extension analysis resolved that there is a 5' end at nt 3207; figure 11A, lanes 4 and 6, show extended products from VS4 and VS5 5' labelled at nt 3287 and 3400 respectively. A 194 nt band (lane 6) and a heterogeneous (see below) 86-87 nt band (lane 4) identify a 5' end at nt 3207-8. This was further confirmed by running primer extended products against a sequencing ladder (figure 11B) demonstrating the major 5' end at nt 3207. According to the DNA sequence, the 3222 site is a splice acceptor (AAGTTACAAAATGAG/TG, nt 3207-3223). This is analogous to the situation in Ad2 where the cap site for polypeptide IX mRNA is located within the intron of the E1B mRNAs. Primers VS4 and VS5 would also be expected to be extended to the 5'

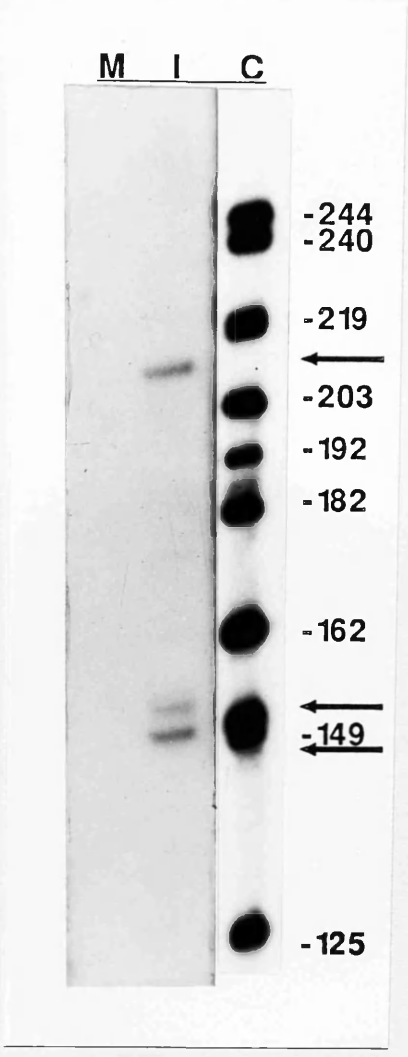


Figure 13: S1 nuclease analysis of splice donor site 2

A restriction fragment spanning nt 2938-3410 of Ad40 was 3'end labelled and used in S1 nuclease assay on whole cell RNA obtained from Ad40 infected (I) and mock-infected (M) KBa+b cells at 42 hr p.i. Hybridization and S1 nuclease digestion were done at 55° and 42° respectively. The molecular weight marker (C) is a HpaII digest of pBR322, the size of the fragments (bp) is indicated. Arrows show the positions of protected fragments obtained.

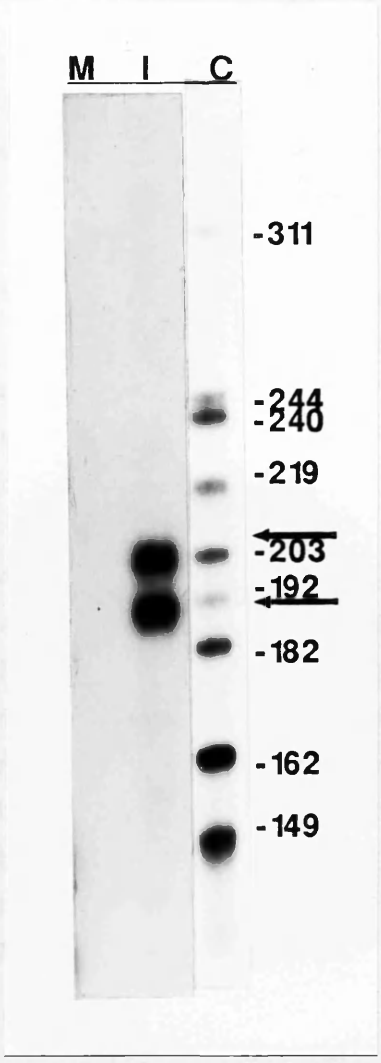


Figure 14: S1 nuclease analysis of a splice acceptor site and the 5'end of ppIX mRNA

A restriction fragment spanning nt 2938-3410 of Ad40 was 5'end labelled and used in S1 nuclease assay on cytoplasmic RNA obtained from Ad40 infected (I) and mock-infected (M) KBa+b cells at 42 hr p.i. Hybridization and S1 nuclease digestion were done at 55° and 42° respectively. The molecular weight marker (C) is a HpaII digest of pBR322, the size of the fragments (bp) is indicated. Arrows show the positions of protected fragments obtained.

end of E1B mRNA templates. No such products were observed most likely because long extended products are not made efficiently.

v) 3' ends of E1B, ppIX and IVa2 mRNAs

A common 3' end for E1B and ppIX transcripts was identified using a 565 nt 3'end labelled fragment (figure 9C) in S1 nuclease assay. This is a TaqI fragment where both strands are labelled. Figure 15 shows that two bands, of 268 and 298 nt, were obtained using this fragment; the 268 nt protected fragment corresponds to a 3' end around nt 3633, downstream of the polyadenylation signal at nt 3614-3619. This is the only poly A signal on the R strand in this region and thus maps the poly A site for E1B mRNAs and ppIX mRNA. There is a poly A signal on the other strand which, by analogy with Ad2, corresponds to the poly A signal of a IVa2 transcript. The 298 nt fragment maps the 3' end of the IVa2 transcript also around nt 3633, downstream of the sequence AAATAAATAAA (nt 3659-3649).

4.2.2.2. Analysis of cDNA by PCR amplification, cloning and sequencing

The mapping data described above indicated that the structure of Ad40 E1B late mRNA was analogous to the major transcripts found in other serotypes. The transcription map did thus not appear to offer any explanation as to the apparent lack of E1B 55K protein function. However, due to the lack of convenient restriction sites, some parts of the E1B region were not analysed in the experiments described above. In particular a site corresponding to the 1st splice acceptor site utilized in the 14S and 14.5S mRNAs of Ad2 and Ad12 (see figure 4) would not be identified by the restriction fragments used. In addition, the mapping data left some unanswered questions; the possibility of a splice acceptor site around nt 1480 raised by S1 analysis (figure 10) and the identity of 3-400 nt fragments produced by

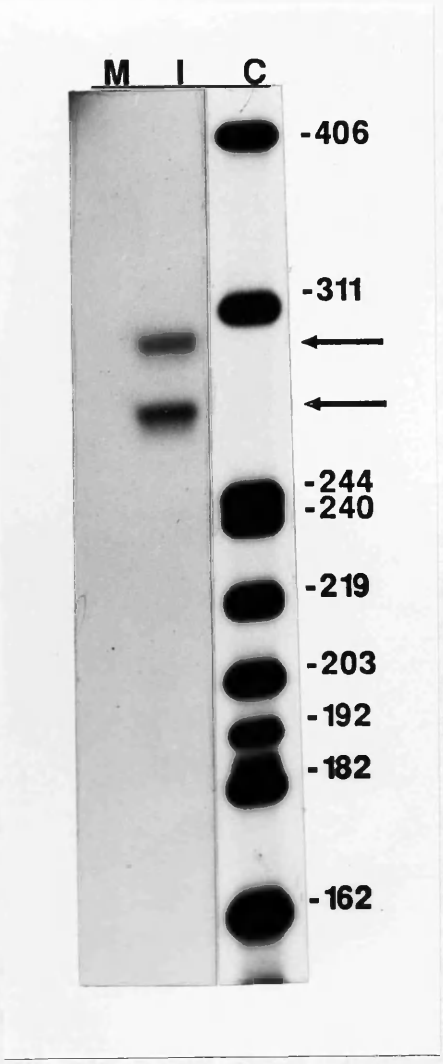


Figure 15: S1 nuclease analysis of the 3'ends of E1B, ppIX and IVa2 mRNAs

A restriction fragment spanning nt 3366-3690 of Ad40 was 5'end labelled and used in S1 nuclease assay on whole cell RNA obtained from Ad40 infected (I) and mock-infected (M) KBa+b cells at 42 hr p.i. Hybridization and S1 nuclease digestion were done at 55° and 42° respectively. The molecular weight marker (C) is a HpaII digest of pBR322, the size of the fragments (bp) is indicated. Arrows show the positions of protected fragments obtained.

extending primer VS1 (figure 11A) remained unsolved. Also, protected fragments seen in S1 analysis (see figure 13) and described in 4.2.2.1.iii, were not accounted for.

In order to extend the the analysis of E1B transcripts, RNA was subjected to cDNA-PCR mapping. The procedure is outlined in figure 16. After DNase treating a cytoplasmic RNA preparation in order to remove residual DNA which would interfere with a PCR reaction, cDNA was made using a single oligonucleotide primer. Prior to analysis, the cDNA was amplified using a pair of primers in a PCR reaction, making an amplified double stranded cDNA copy. PCR products were analysed on agarose gels and for further analysis amplified cDNA fragments were isolated, cloned and sequenced.

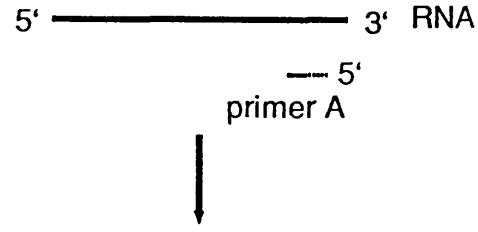
A cDNA-PCR assay using oligonucleotides RU4 and RU3 (see table 3 and figure 9B) was expected to amplify a 1457bp fragment from a cDNA copy of 22S mRNA. In addition these primers span a potential splice junction corresponding to the 1st splice junction in 14S and 14.5S mRNAs of other serotypes. Thus this experiment should determine if such transcripts are made in Ad40. The results are shown in figure 17A. When RNA from mock infected cells was used as a template no products were made (lane 5) whereas infected cell RNA gave a 334bp amplified product (lane 6). The absence of a 1457bp band corresponding to a 22S mRNA template can most likely be explained by incomplete reverse transcription of a long template since other evidence (S1 analysis and Northern blotting, see 4.2.2.3.) indicates the presence of 22S mRNA. A parallel PCR reaction using cloned viral DNA as template gave a 1457bp product (lane 7). For further analysis the 334bp cDNA fragment was isolated and cloned into the PstI site of pUC8 via PstI sites near the 5' end of each primer. The cloned fragment was sequenced (figure 17B), and the cDNA sequence found to correspond to nt 1719-1938 and 3061-3149 of the genomic DNA sequence (figure 17C). This confirms the splice donor site mapped to nt 1938 by S1 analysis, and reveals a hitherto undetected splice acceptor site at nt 3061 (TTAGGTTATATCCAG/C, nt 3046-3061). This corresponds to the splice acceptor used in the

Figure 16: cDNA-PCR procedure

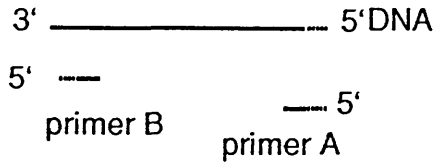
The flow chart outlines the procedure employed for the production of cDNA and amplification by the polymerase chain reaction followed by cloning and sequencing of the amplified product. See 3.18.2, 3.10. and 3.11. for details of methods.

DNase treated RNA

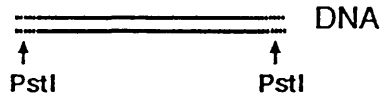
Reverse transcribed in PCR buffer using primer A



Taq polymerase and primer B added and cDNA template amplified by PCR



Double stranded cDNA product isolated from a low melt gel



primers wo cloning sites

primers with cloning sites

cDNA kinased ligated into SmaI site of M13

cDNA digested with PstI ligated into PstI site of pUC8

Sequenced

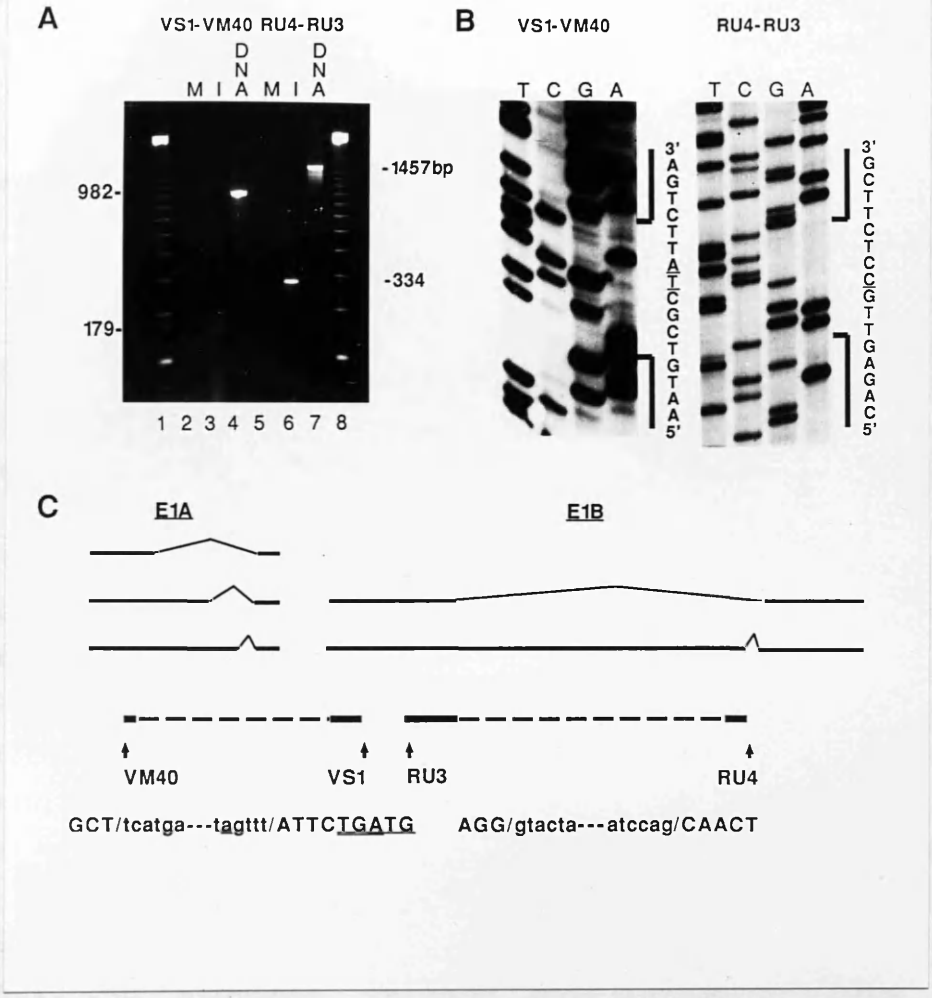
Figure 17: PCR amplification and sequence analysis of cDNA.

A) 5ug DNase 1 treated cytoplasmic RNA from mock infected (lanes 2 and 5) or Ad40 infected (lanes 3 and 6) KBa+b cells harvested at 42 hr p.i. was reverse transcribed and amplified in a PCR reaction as described (3.18.2.).[1] 10ng pNM82 (Ad40 E1) was amplified in parallel. One tenth of each PCR reaction was run on a 2% agarose gel. The size marker (lanes 1 and 8) is a 123bp DNA ladder (BRL). cDNA was made using oligonucleotide primers VS1 (lanes 2 and 3) and RU4 (lanes 5 and 6). Primer pairs VS1-VM40 (lanes 2-4) and RU4-RU3 (lanes 5-7) were used for PCR amplification (see table 3 for primer sequences and locations). Sizes of PCR products, in base pairs, are indicated on either side of the gel.

B) The 179bp VS1-VM40 cDNA fragment was cloned into M13 and sequenced using an M13 primer. The left panel shows the sequence around the E1A-E1B junction. The 334bp RU4-RU3 cDNA fragment was cloned into pUC8 and sequenced using RU4 as primer. The sequence at the splice junction is shown in the right panel.

C) E1A and predicted E1B mRNA structure. Locations of primers used in PCR analysis is indicated by arrows, and cDNA sequences are represented by bold solid lines and intron sequences by broken lines. The DNA sequence around the splice junction of each cDNA is shown with upper case for exon and lower case for intron sequences. The residues underlined in the VM40-VS1 sequence denote (from left to right) the E1B cap site a, the E1A ORF stop codon TGA, and the initiation codon ATG for the first E1B ORF.

[1] Conditions used for PCR reactions were as follows: Initial denaturation 94°C (7min) was followed by 30 cycles of 94°C denaturation (1min), 55°C annealing (1min) and 72°C elongation (3min).


A
VS1-VM40 RU4-RU3
DNA
M I A M I A
982-
179-
-1457bp
-334
1 2 3 4 5 6 7 8
B
VS1-VM40
RU4-RU3
T C G A
T C G A
3' AGTCTTATCGCTGTAA 5'
3' GCTTCTCCGTTGAGAC 5'
C
E1A
E1B
VM40
VS1
RU3
RU4
GCT/tcatga---tagttt/ATTCTGATG
AGG/gtacta---atccag/CAACT

Ad2 14S mRNA, creating an in frame splice in the 55K ORF in both serotypes. Thus a 14S mRNA is transcribed from the E1B region of Ad40. Oligonucleotides RU3 and RU4 include the translation initiation and termination codons respectively of the E1B large open reading frame. The RU3-RU4 cDNA clone (pVS1) therefore includes the whole coding region for a protein comprising the amino- and carboxy-termini of the predicted E1B 55K protein, the counterpart of the Ad2 E1B 15K protein (figure 4).

S1 analysis indicated a potential splice acceptor site around nt 1480, and extension of primer VS1 (located near the 5' end of E1B) produced fragments of approximately 330 and 420 nt in addition to the smaller products which defined the E1B 5' end. These results implied that some E1B transcripts have a 5' end further upstream, ie within the E1A region. A pair of oligonucleotides, VM40 and VS1 (see table 3 and figure 9B), spanning the region between nt 567-1548, were used in a cDNA-PCR reaction to look for E1A-E1B cotranscripts. The results are shown in figure 17A. A 179bp cDNA product was consistently amplified in this reaction using infected cell RNA as template, (lane 3) whereas using viral DNA as template a 982bp fragment was obtained (lane 4). No product was obtained from mock infected cell RNA template (lane 2). The 179bp cDNA product was isolated and in the absence of restriction sites in the primers, cloned into the SmaI site of M13 and sequenced (figure 17B). The sequence corresponded to nt 567-604 and 1408-1548 of the genomic DNA sequence (figure 17C). The 5' side of the junction (GCT/TCATGA, nt 602-610) bears no resemblance to a splice donor site and is located upstream of all the E1A splice sites mapped by van Loon *et al.*, (1987b). The 3' side of the junction maps between the E1B mRNA cap sites defined by primer extension analysis, and the sequence (TTTTAGTGTTAGTTT/A, nt 1393-1408) lacks a splice acceptor consensus. Five clones of the 179bp fragment obtained from two different RNA preparations gave an identical sequence and a fragment of this size was consistently obtained in numerous independent PCR reactions. This indicates that

this unconventional RNA junction is not an artefactual result of the PCR reaction but represents a real junction in E1A-E1B cotranscripts. Assuming that these transcripts share a 5' end with E1A mRNAs they contain the first 40 codons of the E1A ORF linked to E1B. No new ORFs are created by the junction and the E1A ORF terminates with one codon added after the junction. The nature of these cotranscripts was further investigated by Northern blotting (see 4.2.2.3.).

A cDNA-PCR reaction was used to analyse the splice junction in the 22S mRNA. This was done in order to clarify if the potential splice donor site indicated by S1 analysis around nt 3088 (figure 13; see 4.2.2.1.iii) as well as the predicted site at nt 3151 was utilized in the 22S mRNA. Oligonucleotides VS4 and VS6 (table 3; figure 9B), spanning nt 3000-3287 amplified a single spliced product (not shown). The size of this fragment (217bp) was consistent with the use of a splice donor at nt 3151 and a splice acceptor at nt 3222. This indicated that the potential splice donor sites mapped by S1 analysis around nt 3088 (figure 13) are not used in the 22S mRNA. Primer VS6 lies upstream of the splice acceptor site used in the 14S mRNA and thus this PCR product is not amplified from a 14S template. Moreover, the 14S cDNA (described above) was amplified using primer RU4 demonstrating that this mRNA species is not spliced at nt 3088 either. The formal possibility remains that an mRNA species exists which uses the splice acceptor at nt 3061, a splice donor around nt 3088 and the splice acceptor site at nt 3222, which would account for the 147 and 151nt bands detected by S1 analysis in figure 13. A more likely explanation is that those bands are artefactual and the site at nt 3151 is the only splice donor site in this region (see figure 9A).

In order to distinguish between a 14S mRNA structure, identified above, and a possible 13S mRNA structure analogous to the Ad2 E1B 13S mRNA, several attempts were made to amplify cDNA using primers from the first and the last E1B exons. A seemingly ideal primer pair was VS4-RU3

(see figure 9B). However, using VS4 in primer extension analysis resulted in extended products from mock infected as well as infected cells (Hela as well as KBa+b), and these products were amplified using RU3-VS4 in a PCR reaction, even under stringent conditions. From the primer extension data it was clear that these products were much more abundant than products extended from E1B mRNA template (similar in abundance to products from ppIX mRNA template, not shown). This would explain why they appeared to be preferentially amplified over E1B cDNA template in a PCR reaction. Consequently no E1B specific products were identified by this method. Using RU3-VS5 as a primer pair in this reaction did not give reproducible results. Thus this approach did not clarify if the Ad40 E1B region is transcribed into a 13S mRNA as in Ad2 or if such a transcript is absent as is the case in Ad12.

4.2.2.3. Northern blot analysis of E1 transcripts

From the primer extension analysis it was clear that the E1B cap site is used, while evidence from the cDNA analysis showed that some E1B transcripts are linked to a fragment from the 5' end of E1A. As an approach to determine which types of E1B transcripts have an E1A 5' end and to further resolve the structure of E1 transcripts, total RNA harvested at 42 hr p.i. was separated on a methyl mercury gel. This gel system was chosen in order to get a better resolution of mRNA species than that obtained on the formaldehyde gels used previously (figure 8). RNA was blotted onto a nylon membrane and blots probed with selected restriction fragments from the E1 region. Based on the cDNA sequence data, one E1A probe was selected that would not detect the predicted E1A-E1B cotranscripts and another one chosen that would hybridize to the E1A sequences in such transcripts. In addition the E1B probe that was used was a fragment near the 3' end of E1B that should recognize all E1B transcripts and ppIX mRNA but not IVa2 mRNA.

When the RNA was probed with the E1A fragment nt 709-1211

(figure 18, lane 2) two bands were observed, corresponding to E1A 12S and 13S transcripts. However, when the adjacent fragment nt 1-709, comprising the 5' end of E1A, was used (figure 18, lane 1) two additional bands were seen. Those bands comigrated with the 22S and 14S bands detected by the E1B probe, nt 2857-3410 (figure 18, lane 3). This shows that the E1A-E1B cotranscripts identified by cDNA analysis exist as two types, having 22S and 14S E1B structure. The band migrating below the 14S cotranscript in lane 3 is likely to be the E1B 14S mRNA which would differ from its cotranscript counterpart by 180nt and should thus be separated in this part of the gel (the E1A 12S and 13S differ by 84nt). On the other hand the 22S cotranscript would not be separated from its E1B counterpart on this type of gel. The intensity of the 14S cotranscript bands in lane 1 and 3 demonstrates that the specific activity of the probes is similar. The increased intensity of the E1B 22S signal as compared to the corresponding E1A signal thus indicates that the E1B band is heterogeneous, containing 22S type transcripts from both the E1A and the E1B promoter. The E1B probe also gave a strong 9S band, corresponding to the predicted mRNA encoding structural polypeptide IX. A potential E1B 13S mRNA is predicted to be 90nt smaller than the 14S mRNA. The absence of a band between the 14S and ppIX mRNAs (lane 3) thus implies that a 13S mRNA is not produced from the Ad40 E1B region.

4.2.2.4. Summary

The Ad40 E1B transcription map was determined from RNA produced at late times in infected KBa+b cells. The results are shown in figure 9A. E1B transcripts corresponding to Ad2 14S 22S and 9S mRNA were identified but no 13S mRNA equivalent was detected, a pattern similar to that seen in the Ad12 transcription map. The coding potential for E1B 19K, 55K and 15K proteins and for polypeptide IX is retained in the Ad40 transcripts. In addition novel E1A-E1B cotranscript counterparts of the 14S and 22S mRNAs were detected.

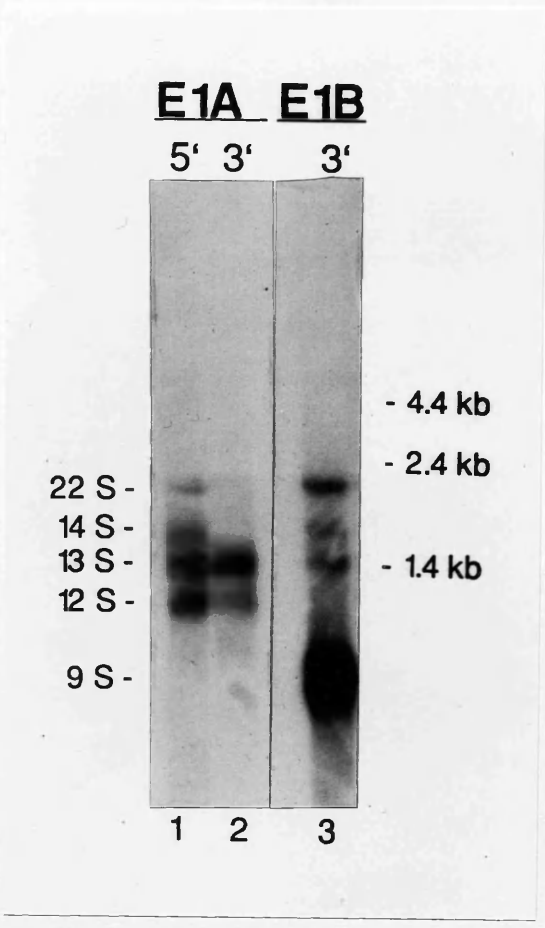


Figure 18: Northern blot of E1 mRNA

Total RNA from Ad40 infected KBa+b cells harvested at 42 hr p.i. was separated on a 1% agarose/methyl mercury gel and analysed on a Northern blot. The blot was probed with E1A (lanes 1 and 2) and E1B (lane 3) DNA restriction fragments and exposed overnight. The E1A 5' probe (lane 1) is a PstI fragment of pNM82 (nt 1-709), the E1A 3' probe (lane 2) is a PstI-AccI fragment of pNM82 (nt709-1211), and the E1B 3' probe is an EcoRI-PstI fragment of pNM86 (nt 2857-3410). Numbers on the right are the sizes of the RNA marker (9.5-kb RNA ladder, BRL). Numbers on the left are S values as referred to in the text, but do not signify specific S values for these mRNA species (note that E1B 14S comigrates with E1A 13S).

These are predicted to be 5' coterminal with E1A transcripts and contain the first 40 codons of the E1A first exon linked to a site 4-5 nt downstream of the E1B cap site, retaining all the coding potential of the E1B mRNAs. No new open reading frames are created by the junction, and the E1A ORF terminates with one codon added after the junction. The E1A-E1B junction is unusual in that it does not conform to splice consensus sequences and thus may not be generated by a conventional splicing mechanism.

4.2.3. A timecourse of E1 transcription and DNA replication in Ad40 infected Kba+b cells and Hela cells

The mapping of E1B mRNA led to the unexpected observation of E1A-E1B cotranscripts. Although these transcripts do not appear to be formed by conventional splicing mechanism they may be the product of a primary transcript extending from the E1 cap site to the E1B poly A site. Such a transcript would be made by a read through of the E1B promoter, and if it was made early in infection it could interfere with transcription from the E1B promoter.

The experiments described below were performed to address several questions. Firstly, to see if E1A-E1B cotranscripts could be detected at early times in infection, before transcripts from the E1B promoter are detectable; Secondly, to look at a timecourse of DNA replication in parallel with a timecourse of transcription in order to see if E1B transcripts can be detected before the onset of DNA replication; Thirdly to compare the pattern of transcription and replication in the permissive KBa+b cells with that seen in Hela cells, which are only semi-permissive (see 4.4.).

KBa+b cells and Hela cells on 50mm plates were infected with 100µl of a 1/10 dilution of Ad40 p9 and harvested at various times post infection. After incubation at 37°C two plates of each cell type were pooled and harvested at each timepoint. Cytoplasmic RNA was extracted and DNA was recovered from the nuclei by Hirt extraction. Subsequently the RNA was run on three 1% agarose/5mM methyl mercury gels (1/4 harvest of a 50mm plate/lane) and blotted onto Hybond-N membranes. Blots were probed with three different DNA fragments from the E1 region, selected to distinguish between E1A and E1B mRNAs and cotranscripts, labelled by the random priming method. Hirt DNA (1/4 of a 50mm plate) was applied to Hybond-N on a slot blot and probed with a nick translated Ad40 E1 plasmid (pNM82).

The results are shown in figure 19. Panel A shows a timecourse of Ad40 infected Hela cell and KBa+b cell RNA probed with an E1A fragment (nt 709-1211). This probe does not overlap E1A sequences found in the E1A-E1B cotranscripts

Figure 19: A timecourse of E1 transcription and DNA replication in Hela cells and KBa+b cells

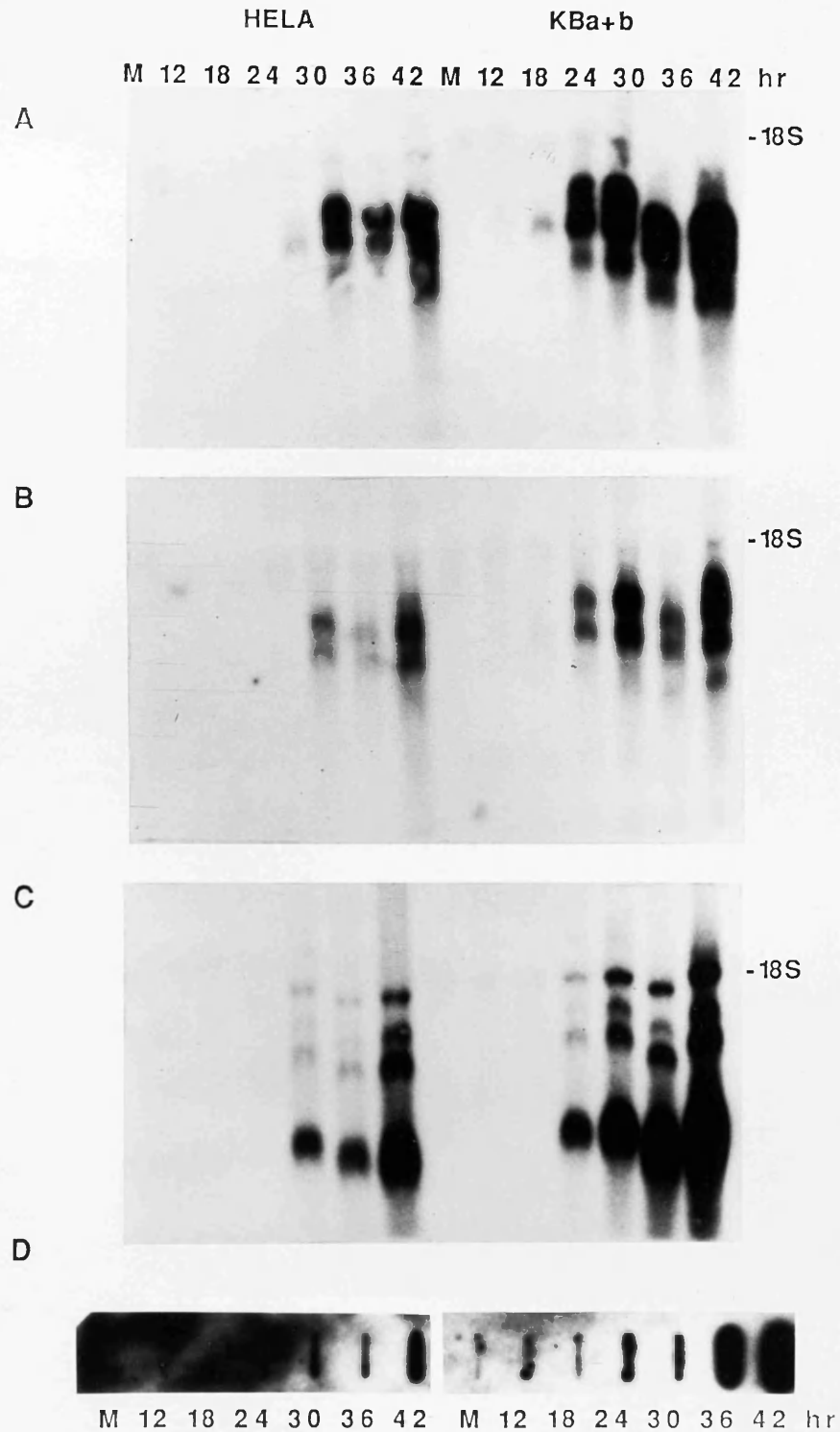
Cytoplasmic RNA was harvested from mock infected and Ad40 infected Hela cells and KBa+b cells at the times indicated post infection. DNA was harvested from the nuclei in parallel by Hirt extraction. Aliquots of RNA were separated on three 1% agarose/5mM methyl mercury gels (1/4 harvest of a 50mm plate/lane) and blotted onto nylon membranes. Hirt DNA (1/4 harvest of a 50mm plate) was applied to a nylon membrane on a slot blot.

A) A Northern blot probed with an E1A restriction fragment (nt 709-1211) which does not overlap the E1A-E1B cotranscripts, labelled by the random priming method. The blot was exposed for 7 days.

B) A Northern blot probed with an E1A restriction fragment (nt 1-709) overlapping the E1A-E1B cotranscripts, labelled by the random priming method. The blot was exposed for 7 days.

C) A Northern blot probed with an E1B restriction fragment (nt 2857-3410) which overlaps all E1B mRNAs but not IVa2. The fragment was labelled by the random priming method and the blot exposed for 3 days. A longer exposure did not reveal E1B mRNA at earlier timepoints than seen here.

D) A slot blot of Hirt DNA was probed with a nick translated plasmid from the Ad40 E1 region (pNM82) and exposed for 5 days. On the original autoradiograph the levels seen in the timecourse on Hela cells up to 24 hr are similar to that seen in mock infected KBa+b cells.


HELA
KBa+b
M 12 18 24 30 36 42 M 12 18 24 30 36 42 hr
A
-18S
B
-18S
C
-18S
D
M 12 18 24 30 36 42 M 12 18 24 30 36 42 hr

mapped previously. Panel B is a parallel blot probed with an adjacent E1A fragment (nt 1-709) which should identify the E1A-E1B cotranscripts as well as E1A transcripts. The blot in panel C was probed with an E1B fragment (nt 2857-3410) which detects all E1B mRNAs as well as the E1A-E1B cotranscripts. A parallel timecourse of nuclear DNA in both cell types is shown in panel D. Panel C shows that the E1A-E1B cotranscripts appear at the same time as E1B mRNAs and are not seen at early times in infection. Therefore they are not likely to be the cause of a lack of early E1B transcription by promoter occlusion, although the possibility remains that they are made early but in undetectable amounts or that they are unstable. In KBa+b cells the 12S E1A mRNA is first seen at 18 hr p.i. and all other E1 mRNAs, including E1A-E1B cotranscripts, appear between 18 and 24 hr p.i. The first increase in viral DNA levels in KBa+b cell nuclei is also seen at 24 hr p.i. In Hela cells E1 mRNAs become detectable even later and DNA replication is more delayed, E1A 12S and 13S mRNAs are seen at 24 hr p.i. while E1B mRNAs and E1A-E1B cotranscripts appear between 24 and 30 hr p.i. A slight increase in DNA levels is seen at 30 hr p.i. with a further increase between 36 and 42 hr p.i. Thus the pattern of transcription and replication appears to be similar in KBa+b cells and Hela cells. E1A mRNA appears first and six hours later the E1B 22S, 14S and ppIX transcripts as well as the E1A-E1B cotranscripts are detectable along with a slight increase in DNA levels. Over the following six hours RNA levels increase but change in DNA levels is not detectable. After a further six hours DNA levels have risen and increase rapidly after that in KBa+b cells. This procedure begins later in Hela cells than in KBa+b cells and increase in DNA replication is less dramatic at least up to 42 hr p.i.

These experiments are not conclusive as to whether E1B mRNA is made before the onset of DNA replication. Mautner *et al.*, (1990) found that they could not detect E1B mRNA until 30 hr p.i, after the onset of DNA replication at 18-24 hr p.i. in KBa+b cells and they did not see accumulation of

RNA in the presence of a replicative block up to 42 hr p.i. Furthermore, they did not detect any E1B mRNA up to 42 hr p.i. in Hela cells but saw an increase in DNA levels at 30 hr p.i. Differences in extraction methods and sensitivity of probes may at least partly explain this discrepancy. Moreover, their experiment used a different virus stock, thus the multiplicity of infection may differ between the experiments. A higher multiplicity of infection in the experiment described here would account for an increase in transcription. The onset of DNA replication is detected at the same time in both experiments but they detected E1B mRNA at later times than seen in this experiment. However, the results described here are in agreement with an RNA time-course described in section 4.2.1., thus supporting this data.

The onset of DNA replication and E1B transcription around the same time may suggest that one is dependent on the other. It has been shown previously that the E1B 55K protein is required for DNA replication in Ad12 (Shiroki *et al.*, 1986) but not in Ad5 (Babiss and Ginsberg, 1984). In Ad40 E1B mRNA does not accumulate when DNA replication is blocked (Mautner *et al.*, 1990). The reason for this could be that in Ad40, DNA replication is inefficient in the absence of the E1B 55K protein and that the E1B region is only transcribed on newly replicated DNA. This would be consistent with the small increase in DNA level seen 24-30 hr p.i. followed by the detection of E1B RNA and later increased DNA replication. It would also explain the finding that E1B mRNA does not accumulate under a replicative block. An alternative explanation could be that E1B transcription is inefficient and therefore not detectable until after DNA replication when an increase in template results in a detectable transcription level.

The Ad40 E1 transcription pattern is remarkably different from that seen in an Ad5 infection on Hela cells at a moderate multiplicity of infection (Glenn and Ricciardi, 1988). Ad5 E1A transcripts were detectable at 2 hr p.i., E1B 22S at 3.5 hr p.i. and low levels of E1B 13S were seen

early with an increase after 8 hr p.i. In addition the ppIX 9S mRNA was detectable after 8 hr p.i. with a rapid increase at 10-12 hr p.i., and a IVa2 mRNA was seen at 9.5 hr p.i. The onset of DNA replication was not determined in this study. Not only is the whole transcription process severely delayed in Ad40 as compared to Ad5 but also the time lag between early and late transcription appears absent, since E1B transcripts are not seen before the ppIX mRNA. It is however notable that the time lag between the onset of E1A and E1B transcription is seen, suggesting that Ad40 E1B transcription may be activated in the presence of E1A and that the delay is due to a delay in transcription of E1A. Although this may partly explain the late onset of E1B transcription it can not be the only reason since providing E1A by coinfecting with an E1B mutant virus or using a cell line expressing E1A does not result in complementation of Ad40 growth.

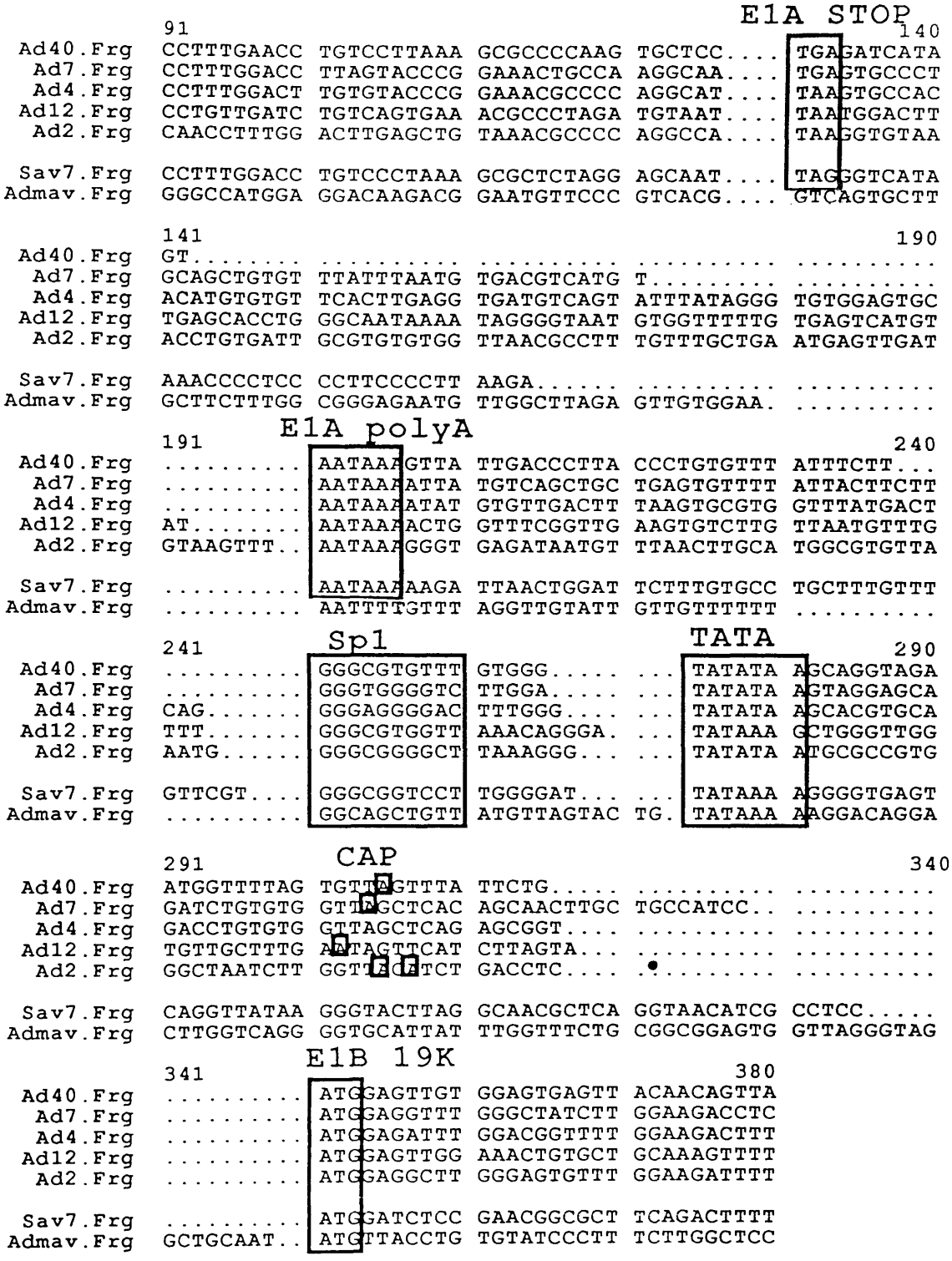
4.3. The Ad40 E1B promoter

E1B transcripts were not detectable before the onset of DNA replication in KBa+b cells and Hela cells (see above). This implies that the E1B promoter may be inactive at early times in infection. The E1B promoter region is discussed in detail in section 1.3.2. The Ad2 E1B promoter is a simple promoter, consisting of a TATA box and an Sp1 binding site (GC box) (Wu *et al.*, 1987). Only mutations in the TATA box interfere with E1A transactivation, suggesting that E1A activation of E1B is mediated through the TATA box. Parks *et al.*, (1988) identified four protein binding sites in the upstream region of the Ad5 E1B promoter (see figure 3) and suggested that they might be involved in stimulation of transcription in the absence of a functional GC box. Figure 20 shows a comparison between the E1B promoter sequences of Ad40 and other adenovirus serotypes of different subgroups. The sequences have been lined up with respect to the E1A termination codon, E1A poly-adenylation signal, Sp1 binding site, TATA box and the initiation codon for the 19K ORF. The TATA box sequence of Ad40 is identical to that of Ad2, 4 and 7 and is in a similar position relative to the cap site. The consensus sequence for an Sp1 binding site is 5'(G/T)GGGCGG(G/A)$_2$(C/T) (Kadonaga *et al.*, 1986). The Ad2 sequence fits this consensus perfectly, but all the other sequences vary slightly. The Ad40 sequence differs from Ad12 by one base in the flanking sequence so that both sequences have two bases different from the consensus. Of the upstream elements identified by Parks et al.,(1988), only one, site IV (nt 224-231 in figure 20), is apparent in the Ad40 E1B promoter. The sequence, TGTGTTTA (-59 to -52 relative to the cap site) has a one base difference from the TGTGGTTA (-137 to -125) in Ad5 E1B and similar sequences are present in Ad4, Ad7 and Ad12.

In general the Ad40 E1B promoter appears similar to E1B promoters of other serotypes. It has a potential Sp1 binding site, shown to be important for E1B transcription in Ad2, and it has a TATA box identical to that of Ad2, where

Figure 20: E1B upstream sequences

A comparison of E1B promoter sequences of Ad40 (subgroup F), Ad7 (subgroup B), Ad4 (subgroup E), Ad12 (subgroup A), Ad2 (subgroup C), simian adenovirus 7 (Sav7) and mouse adenovirus 1 (Admav). The sequences were lined up with respect to the E1A stop codon, E1A polyA signal, Sp1 binding site, TATA box and E1B 19K start codon. The known E1B cap sites are indicated. The numbering system is arbitrary and does not refer to the genomic sequences. The figure was prepared by Dr V. Mautner. DNA sequences were obtained from the EMBL databank.



E1B UPSTREAM SEQUENCES

the TATA box has been shown to be important for E1A trans-activation of E1B. So the differences in E1B gene expression between Ad40 and other serotypes do not reflect obvious differences in sequences of important promoter elements. However, there might be more subtle differences between promoters which are not apparent from simple sequence comparisons. As an approach to studying the Ad40 E1B promoter the expression of a reporter gene under the control of this promoter was determined in the presence or absence of plasmids expressing the E1A genes of Ad40 or Ad5.

4.3.1. Construction of CAT reporter plasmids

In order to assess the activity of the Ad40 E1B promoter, plasmid constructs were made with the chloramphenicol acetyl transferase (CAT) gene under the control of the Ad40 E1B promoter. The plasmid pE1B-CAT was used as a basis for these constructs (Dery et al., 1987). This plasmid consists of the Ad5 E1B promoter (nt 1336-1702) inserted between the HindIII-XhoI sites of pBA-CAT, upstream of a promoterless CAT gene (figure 21B). It was shown to have a low activity when transfected into Hela cells but cotransfection with an Ad5 E1A containing plasmid resulted in a 2-3 fold increase in CAT activity.

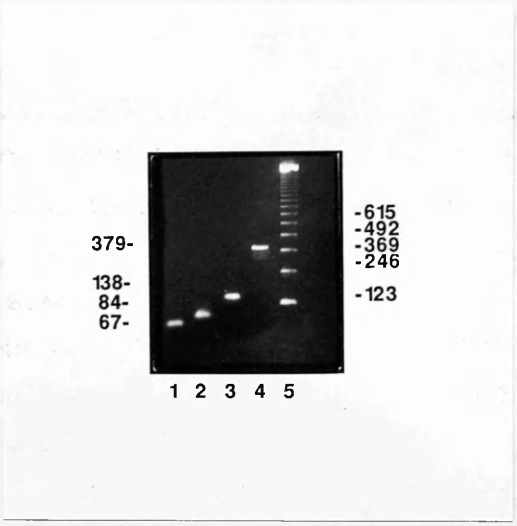
Four DNA constructs containing Ad40 E1B promoter sequences were made by PCR. The downstream and upstream primers contained HindIII and XhoI restriction sites respectively to facilitate cloning of the constructs into an expression vector. Primer VS13 was used as a downstream primer for all the constructs. It includes the E1B cap site (-8 to +9, see figure 20) and has a HindIII restriction site near the 5' end. Four upstream primers (VS9-VS12), all including a 5' XhoI site, were used in order to make constructs with different lengths of upstream promoter sequences (see figure 21B). The primers were designed so that the shortest construct (9/13) would include only the TATA box, the second one (10/13) extends just beyond the Sp1 binding site, the third fragment (11/13) extends beyond the

Figure 21: Construction of CAT expression vectors

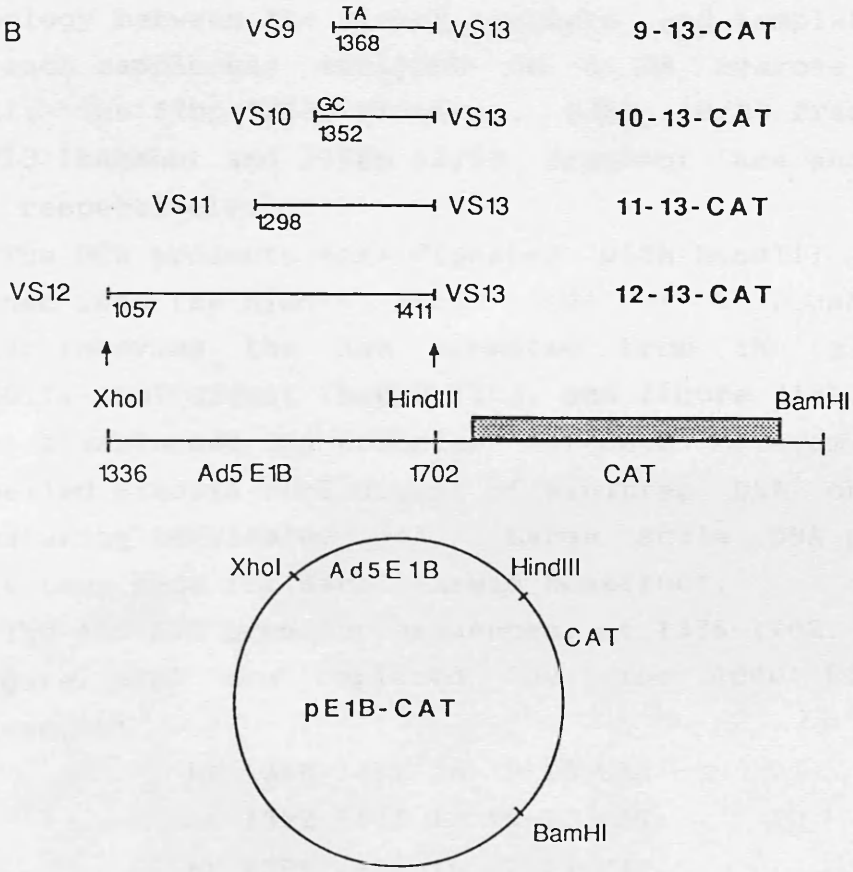
A) PCR products containing E1B promoter sequences were analysed on a 2% agarose gel prior to restriction digestion and cloning (see text for details). The size of each fragment (bp) is indicated on the left; numbers on the right refer to the size marker. Lane 1: a 67bp product amplified using primers VS9 and VS13; lane 2: an 84bp fragment amplified using primers VS10 and VS13; lane 3: a 138bp fragment amplified using primers VS11 and VS13; lane 4: a 379bp fragment amplified using primers VS12 and VS13; lane 5: a 123bp ladder was used as a size marker. Primers VS9-VS12 contain a XhoI restriction site 5' to Ad40 sequences. Primer VS13 contains a HindIII restriction site 5' to Ad40 sequences.

B) The plasmid pE1B-CAT containing the CAT gene in a HindIII-BamHI fragment, under the control of the Ad5 E1B promoter, in a XhoI-HindIII fragment, was used as a basis for the construction of CAT expression vectors containing the Ad40 E1B promoters. The Ad5 E1B promoter was removed from the plasmid by XhoI-HindIII digestion and the backbone isolated from a NuSieve agarose gel. The PCR products seen in lanes 1-4 in A were digested with HindIII and XhoI and ligated into the pE1B-CAT backbone, creating the plasmids 9-13-CAT, 10-13-CAT, 11-13-CAT and 12-13-CAT respectively. Numbers in the diagram refer to the Ad40 genomic sequence (Ad5 sequence for pE1B-CAT). TA and GC indicates the position of the TATA box and Sp1 binding site.

A


379-
138-
84-
67-
-615
-492
-369
-246
-123
1 2 3 4 5

B


VS9
TA
1368
VS13
9-13-CAT
VS10
GC
1352
VS13
10-13-CAT
VS11
1298
VS13
11-13-CAT
VS12
1057
1411
VS13
12-13-CAT
XhoI
HindIII
BamHI
1336
Ad5 E1B
1702
CAT
XhoI
Ad5E1B
HindIII
CAT
pE1B-CAT
BamHI

E1A poly A signal and includes site IV and the longest fragment (12/13) is comparable in length with the Ad5 promoter in pE1B-CAT (see below) and extends into the E1A intron. Primer sequences are shown in table 3 (section 2.6.).

PCR reactions were set up as described (see 3.18) using 10ng pNM82, an Ad40 E1-containing plasmid, as a template. Amplification was performed as follows; One cycle of denaturation at 94°C/5 min, annealing at 35°C/0.5 min and extension at 72°C/0.5 min followed by 30 cycles of denaturation at 94°C/0.2 min, annealing at 55°C/0.2 min and extension at 72°C/0.2 min. Initial annealing of primer at a low temperature in the first round of amplification was essential since the T_M of VS13 is very low in the absence of homology between the linker sequence and template. A tenth of each sample was analysed on a 2% agarose gel (figure 21A). The 67bp 9/13 fragment, 84bp 10/13 fragment, 138bp 11/13 fragment and 379bp 12/13 fragment are shown in lanes 1-4 respectively.

The PCR products were digested with HindIII and XhoI and cloned into the HindIII-XhoI sites of the plasmid pE1B-CAT after removing the Ad5 promoter from the plasmid by a HindIII-XhoI digest (see 3.11.3. and figure 21B). DH1 cells were transformed and colonies screened by examining 3' end labelled HindIII-XhoI digest of miniprep DNA on a 12% non-denaturing acrylamide gel. Large scale DNA preparations were then made for each plasmid construct.

The Ad5 E1B promoter sequences, nt 1336-1702, in pE1B-CAT (figure 21B) are replaced by the Ad40 E1B promoter sequences:

nt 1368-1411 in 9-13-CAT
nt 1352-1411 in 10-13-CAT
nt 1298-1411 in 11-13-CAT
nt 1057-1411 in 12-13-CAT

In order to confirm that promoter sequences in the clones do not differ from the viral sequence, plasmids 10-13-CAT and 11-13-CAT were sequenced in both directions using the primers that were used to create the inserts (VS10/VS13 and

VS11/VS13 respectively), and 12-13-CAT was sequenced using primers VS11 and VS13. All Ad40 sequences were identical to the published sequence. However, in 12-13-CAT the junction between the inserted fragment and the HindIII site of the plasmid was created by a blunt end ligation between the full length primer sequence (not HindIII digested) and the backbone. This resulted in a duplication of the HindIII site, effectively giving a 12bp insert between the Ad40 cap site and the initiation codon of the CAT gene. Since this insert is located in a leader sequence 14 bp downstream of the cap site and 43 bp upstream of the AUG, it is unlikely that it will affect either transcription or translation of the CAT gene.

4.3.2. Transient transfection assays

The Ad40 and Ad5 E1B CAT reporter plasmids were transfected into WS Hela cells with or without plasmids expressing E1A, in order to investigate the activity of the E1B promoter constructs and their response to E1A transactivation. The ability of an unrelated 'universal' transactivator, the 140K immediate early protein of varicella zoster virus (VZV) (see Everett, 1987) to affect CAT gene expression from 12-13-CAT and E1B-CAT was also tested. The E1A expression vectors used were pNM80, which encodes Ad40 E1A, pLE1A which expresses Ad5 E1A and MT13S which contains the E1A 13S cDNA sequence under the control of the mouse metallothionein-1 promoter.

Cell plates transfected with 4µg of CAT plasmid and 4µg of inducer plasmid were harvested after 48 hr incubation at 37°C. Total DNA was made up to 8µg with pUC8 in the absence of an inducer plasmid. Sonicated cell extracts (1/3 of a 50mm plate) were incubated 1 hr/37°C with ^{14}C Chloramphenicol in the presence of butyryl CoA, subsequently extracted with TMPD/Xylenes and CAT enzyme activity i.e. the yield of butyrylated chloramphenicol, determined by scintillation counting of the organic phase.

The results are shown in table 5, expressed as counts per

Table 5. CAT Activity[a]

Plasmid/ Exp. no.	1	2	3	4	5	mean
Backgr.	334	456	343	331	308	354
pE1B	425	512	518	384	430	454
+pNM80	736	582	691		479	622
+pLE1A	1502	729	1503	624	778	1027
+MT13S		1347		964	1012	1108
+p140				2854	3669	3261
12-13	364	476	340	383	396	392
+pNM80	419	458	385	399	405	413
+pLE1A	382	450	388	358	454	406
+MT13S		394		342	412	383
+p140				518	563	541
11-13	348	522	337	337		386
+pNM80	268	500	318	369		364
+pLE1A	341	446	350	394		383
+MT13S		453		309		381
10-13	387	494	368	349		400
+pNM80	429	497	360	342		407
+pLE1A	382	495	391	377		411
+MT13S		489		367		428
9-13	480	558	416	374		457
+pNM80	427	508	358	359		413
+pLE1A	454	478	329	393		413
+MT13S		500		368		434

[a] activity is expressed as cpm

minute. The results for every experiment are also presented in table 6 and figure 22A, where the CAT activity in the presence of an activator is expressed as a ratio of the activity of each promoter construct on its own. The mean values and standard error of the mean are also shown. Figure 22A is based on the mean values in table 6 and shows the effect cotransfecting with inducer plasmids has on CAT activity, which is taken to be indicative of promoter activity.

The activity of each vector in the presence of different activators was compared to expression from the vector on its own by applying students t-test on the paired data from each experiment. The plasmid E1B-CAT which has the CAT gene under the control of the Ad5 E1B promoter, shows a 1.3 fold increase in activity in the presence of Ad40 E1A ($p<10\%$). In the presence of Ad5 E1A a 2.3 fold increase is seen ($p<5\%$) and a 2.5 fold increase in the presence of Ad5 E1A 13S ($p<2\%$). On the other hand none of the Ad40 E1B CAT vectors show a significant increase in activity in the presence of E1A of either Ad40 or Ad5 ($p>10\%$). 9-13-CAT, which includes promoter sequences up to -35, is less active in the presence of pNM80 ($p<5\%$) than on its own. In order to test the effect of a strong promiscuous transactivator on the Ad40 E1B promoter, 12-13-CAT and pE1B-CAT were cotransfected with p140, a plasmid expressing the VZV 140K immediate early protein. A 1.4 fold increase in CAT activity from 12-13-CAT was seen in the presence of 140K and an 8 fold increase from pE1B-CAT. This indicates that while the Ad40 E1B promoter can respond to this strong trans-inducing factor which activates the Ad5 E1B promoter, the effect is less marked. The VZV 140K protein is a homologue of the HSV1 $V_{MW}175$ immediate early protein (McGeoch *et al.*, 1986) and the pseudorabies virus IE protein (Cheung *et al.*, 1989). These immediate early herpes virus proteins, particularly the VZV 140K and the PrV IE proteins, are strong trans-activators capable of activating several cellular and viral promoters (for review see Everett 1987). Wu and Berk (1988a) have shown that the TATA box region is required for

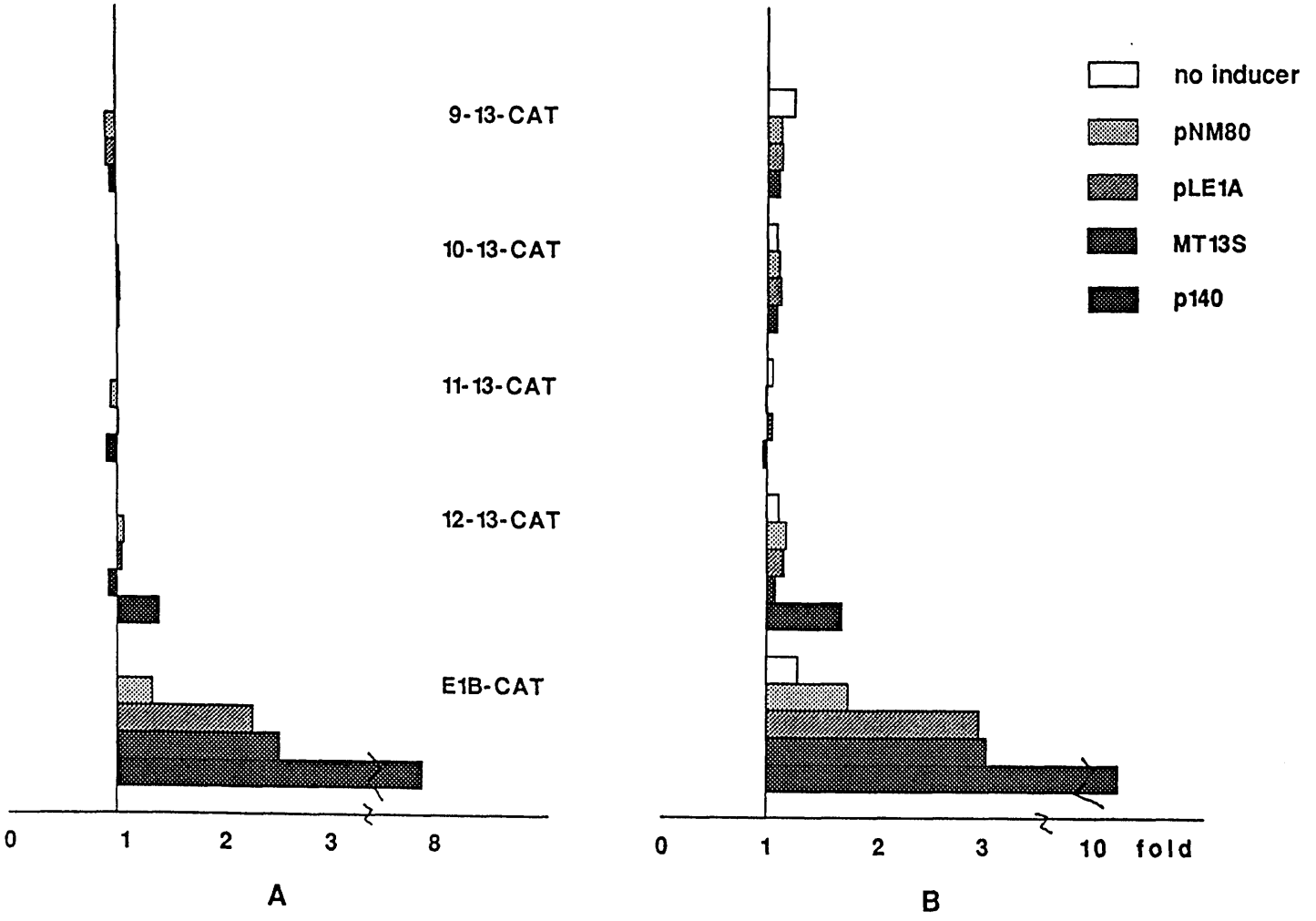
Table 6. Activation of CAT expression vectors[a]

Plasmid/ Exp. no.	1	2	3	4	5	mean	SEM
pE1B	1.0	1.0	1.0	1.0	1.0	1.0	
+pNM80	1.73	1.14	1.33		1.11	1.33	0.143
+pLE1A	3.53	1.42	2.90	1.63	1.81	2.26	0.408
+MT13S		2.63		2.51	2.35	2.50	0.081
+p140				7.43	8.53	7.98	0.550
12-13	1.0	1.0	1.0	1.0	1.0	1.0	
+pNM80	1.15	0.96	1.13	1.04	1.02	1.06	0.035
+pLE1A	1.05	0.95	1.14	0.93	1.15	1.04	0.046
+MT13S		0.83		0.89	1.04	0.92	0.062
+p140				1.35	1.42	1.39	0.035
11-13	1.0	1.0	1.0	1.0		1.0	
+pNM80	0.77	0.96	0.94	1.09		0.94	0.066
+pLE1A	0.98	0.85	1.04	1.17		1.01	0.066
+MT13S		0.87		0.92		0.90	0.025
10-13	1.0	1.0	1.0	1.0		1.0	
+pNM80	1.11	1.01	0.98	0.98		1.02	0.031
+pLE1A	0.99	1.00	1.06	1.08		1.03	0.022
+MT13S		0.99		1.05		1.02	0.030
9-13	1.0	1.0	1.0	1.0		1.0	
+pNM80	0.89	0.91	0.86	0.96		0.91	0.021
+pLE1A	0.95	0.86	0.79	1.05		0.91	0.056
+MT13S		0.90		0.98		0.94	0.040

[a] for each experiment, the activity of a vector in the presence of an activator is expressed as a ratio of the activity of that vector in the absence of an activator

Figure 22: E1B promoter activity

Plasmids containing the CAT gene under the control of the Ad40 E1B promoter (9-13-CAT; 10-13-CAT; 11-13-CAT; 12-13-CAT) or the Ad5 E1B promoter (pE1B-CAT) were transfected into Hela cells in the absence of an activator or cotransfected with an activator plasmid: pNM80 (Ad40 E1A); pLE1A (Ad5 E1A); MT13S (Ad5 E1A 13S); p140 (VZV 140K immediate early protein). Cells were harvested after 48 hr and cell extracts were assayed for CAT activity. After a phase extraction of acylated chloramphenicol the activity was determined by scintillation counting. The activity of each promoter construct in the presence of an activator is expressed as a ratio of the activity in the absence of an inducer in table 6. Panel A represents the mean values based on 2-5 experiments (table 6). A background level of counts was obtained from extracts without a CAT expression vector as a result of <0.5% of the free chloramphenicol being extracted into the organic phase. In table 7 the activity of uninduced and induced extracts is expressed as a ratio of this background for each experiment. Panel B represents the mean values of this ratio (same experiments as before, see table 7).


9-13-CAT
10-13-CAT
11-13-CAT
12-13-CAT
E1B-CAT
0
1
2
3
8
A
no inducer
pNM80
pLE1A
MT13S
p140
0
1
2
3
10
fold
B

transactivation of the Ad2 E1B promoter by the PrV IE protein as well as E1A. It is likely that the 140K protein, like the PrV IE protein and E1A, exerts its effect on the E1B promoter through the TATA box and thus the weak response of the Ad40 E1B promoter to the 140K protein is further indication that this promoter is less susceptible to activation through the TATA box than its Ad5 counterpart.

As a control in these experiments an extract from non-transfected cells was assayed for CAT activity. This gave a background level resulting from <0.5% of the free chloramphenicol being extracted into the organic phase. In table 7 and figure 22B the data is presented as the mean of the ratio of measured enzyme activity against this background. This is to give a better indication of the basal level of expression from each promoter construct. All the constructs show CAT activity slightly above background, although only 9-13-CAT and 10-13-CAT ($p<5\%$) and E1B-CAT ($p<2\%$) differ significantly from the background.

These results indicate that while transcription from the Ad5 E1B promoter is activated in the presence of either Ad40 or Ad5 E1A, the Ad40 E1B promoter is not affected to a detectable level. Due to the low basal activity of the E1B promoters seen in these experiments the results do not give conclusive answers as to whether the activity of the Ad5 and Ad40 E1B promoters in the absence of an inducer differ significantly. Others have found that expression from the Ad2 E1B promoter is inefficient in the absence of E1A transactivation in *in vitro* assays (Wu *et al.*, 1987). Early transcription of Ad2 E1B *in vivo* is dependent on E1A activation. The lack of activation of the Ad40 E1B promoter by E1A, as suggested by these results, most likely explains the finding that E1B mRNA is not detectable at early times in infection. This could in turn be the basis for the growth restriction of Ad40 in tissue culture since E1B 55K mutant viruses of other serotypes are restricted for growth in tissue culture (see 1.3.5.). In particular, Ad12 mutant viruses defective for the E1B 55K protein function are severely impaired for viral DNA synthesis (Shiroki *et al.*,

Table 7. CAT activity above background[a]

Plasmid/ Exp. no.	1	2	3	4	5	mean	SEM
Backgr.	1.0	1.0	1.0	1.0	1.0		
pE1B	1.27	1.12	1.51	1.16	1.40	1.29	0.073
+pNM80	2.20	1.28	2.01		1.56	1.76	0.209
+pLE1A	4.50	1.60	4.38	1.89	2.53	2.98	0.615
+MT13S		2.95		2.91	3.29	3.05	0.121
+p140				8.62	11.91	10.27	1.645
12-13	1.09	1.04	0.99	1.16	1.29	1.11	0.052
+pNM80	1.25	1.00	1.12	1.21	1.31	1.18	0.054
+pLE1A	1.14	0.99	1.13	1.08	1.47	1.16	0.081
+MT13S		0.86		1.03	1.34	1.08	0.141
+p140				1.56	1.83	1.70	0.135
11-13	1.04	1.14	0.98	1.02		1.05	0.034
+pNM80	0.80	1.10	0.93	1.11		0.99	0.074
+pLE1A	1.02	0.98	1.02	1.19		1.05	0.047
+MT13S		0.99		0.93		0.96	0.030
10-13	1.16	1.08	1.07	1.05		1.09	0.024
+pNM80	1.28	1.09	1.05	1.03		1.11	0.057
+pLE1A	1.14	1.09	1.14	1.14		1.13	0.013
+MT13S		1.07		1.11		1.09	0.020
9-13	1.44	1.22	1.21	1.13		1.25	0.066
+pNM80	1.28	1.11	1.04	1.08		1.13	0.053
+pLE1A	1.36	1.05	0.96	1.19		1.14	0.087
+MT13S		1.10		1.11		1.11	0.005

[a] for each experiment, the activity is expressed as a ratio of the background level in non-transfected cell extracts

1986; Breiding *et al.*, 1988; Mak and Mak, 1990).

4.4. Ad40 growth in continuous cell lines

The growth restriction of Ad40 in tissue culture poses a practical problem for *in vitro* studies of the virus. This problem has been partially overcome by growing the virus on the permissive KBa+b cells. However, these cells express Ad2 E1 gene products, which may affect Ad40 gene expression and DNA replication and are thus not an ideal system in which to study Ad40. A better model system would be a cell line which can support efficient Ad40 growth, but which does not express viral gene products. A high number of viral particles in stools from Ad40 infected children (Gary *et al.*, 1979; Retter *et al.*, 1979; Takiff and Straus, 1982) indicates that the virus replicates efficiently *in vivo*. Ad40 is known to infect cells in the small intestine but it is not clear which cell type or types are infected. It is most likely that the growth restriction of the virus in tissue culture in some way reflects the tissue specificity of the virus and thus the ideal tissue culture system would be cells with properties similar to the natural host cells of the virus. As an attempt to identify such a cell line several cell types were tested for their ability to support replication of Ad40.

Figure 23 shows the results from one such experiment where four different cell lines, EJ, HT29, pancreatic carcinoma and Tera-2 C13 cells, were infected and compared to a parallel infection of KBa+b cells and Hela cells. Monolayers of each cell type on 50mm plates were infected with 100µl of a 1/10 dilution of Ad40 p9. Following incubation at 37°C for 72 hr virus was harvested and virion DNA prepared. The DNA was analysed by restriction enzyme digestion (not shown) and by slot blot analysis. Concentration of virion DNA was compared by densitometry. Ad40 replication on Hela cells is around 17% of that seen on KBa+b cells and replication on all the cell lines tested is negligible. Thus EJ, HT29, pancreatic carcinoma and Tera-2 C13 cells are all nonpermissive for Ad40.

Figure 24 shows slot blot analysis of Ad40 DNA from

Figure 23: Ad40 virion production in different cell lines

KBa+b, Hela, EJ, HT29, pancreatic carcinoma (PC) and Tera-2 C13 cells were mock infected or infected with 100µl of 1:10 diluted Ad40 p9 stock. Virus was harvested at 72 hr p.i., the harvest of two 50mm plates pooled and virion-packaged DNA extracted. Dilutions (1/40, 1/120 and 1/360) were applied to a nylon filter on a slot blot and probed with a mixture of Ad40 E1A and E1B containing plasmids (pNM80 and pNM81). The top panel shows extracts from Ad40 infected cells, the lower panel shows mock infected cell extracts. The bottom row shows plasmid control (5, 1.0 and 0.2ng unlabelled probe).

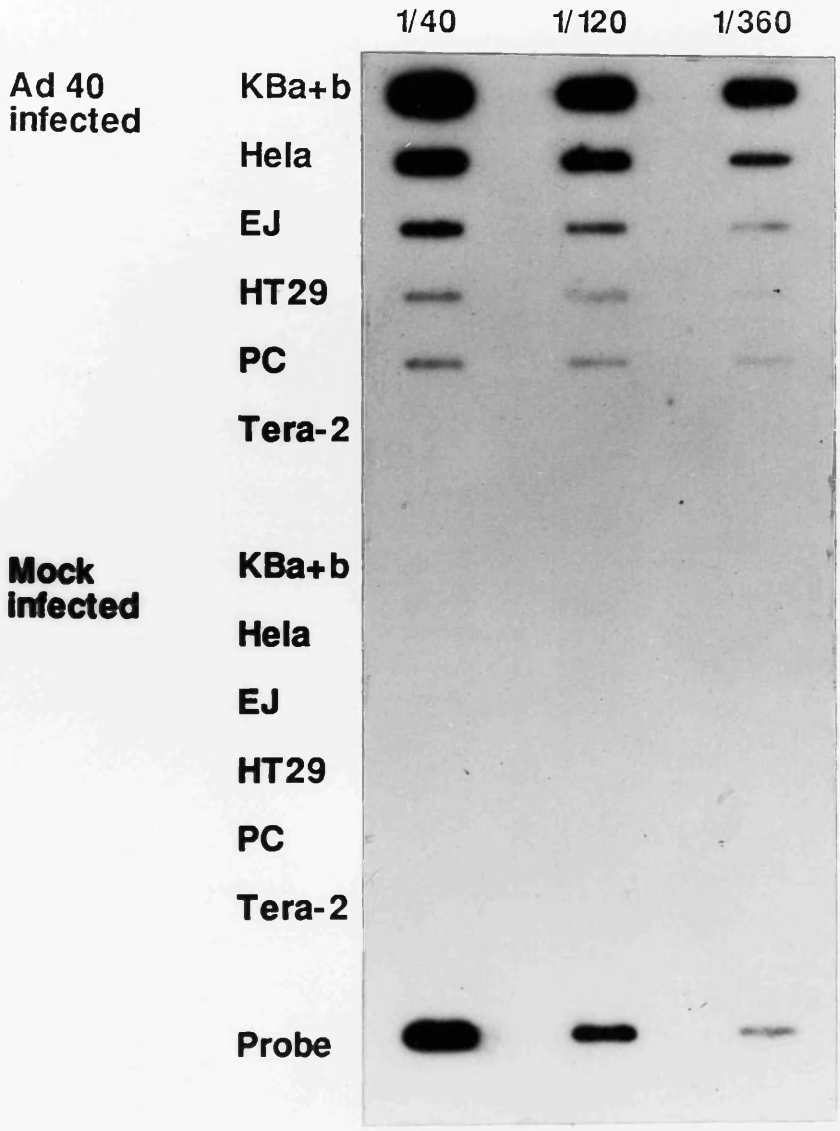
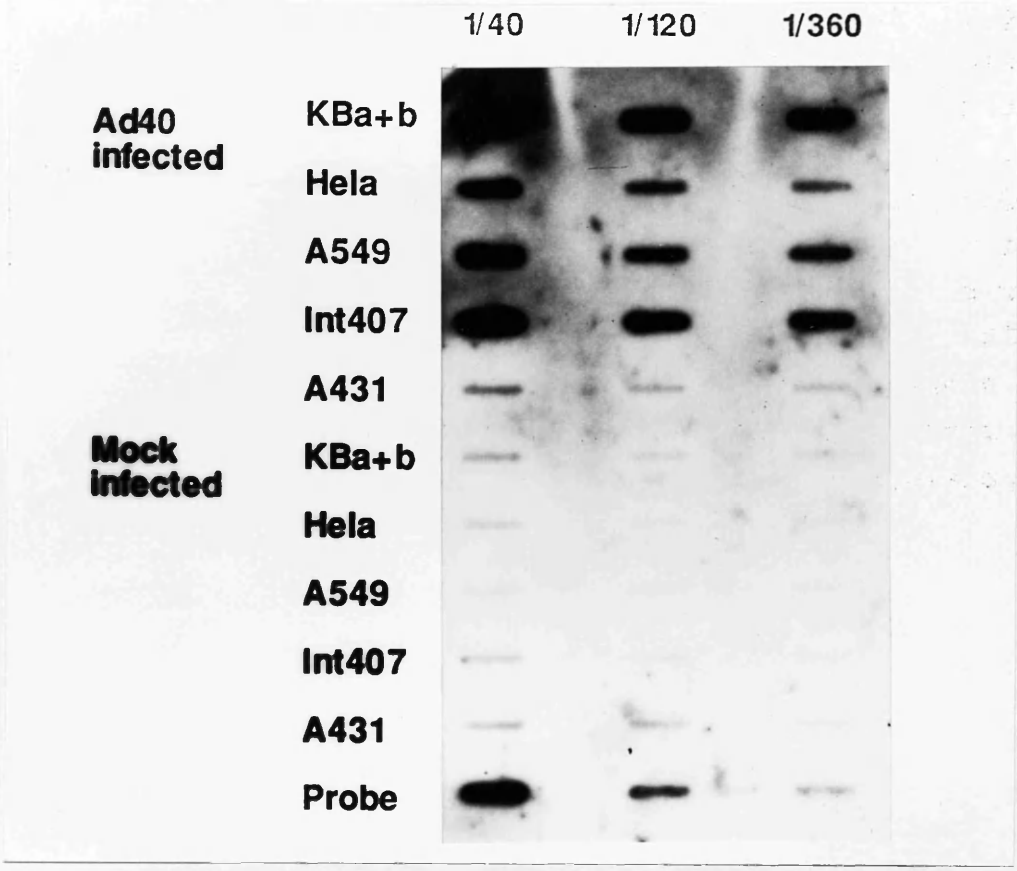

1/40
1/120
1/360
Ad 40 infected
KBa+b
Hela
EJ
HT29
PC
Tera-2
Mock infected
KBa+b
Hela
EJ
HT29
PC
Tera-2
Probe

Figure 24: Ad40 virion production in different cell lines

KBa+b, Hela, A549, Int407 and A431 cells were mock infected or infected with 100µl of 1:10 diluted Ad40 p9 stock. Virus was harvested at 72 hr p.i., the harvest of two 50mm plates pooled and virion-packaged DNA extracted. Dilutions (1/40, 1/120 and 1/360) were applied to a nylon filter on a slot blot and probed with a mixture of Ad40 E1A and E1B containing plasmids (pNM80 and pNM81). The top panel shows extracts from Ad40 infected cells, the lower panel shows mock infected cell extracts. The bottom row shows plasmid control (5, 1.0 and 0.2ng unlabelled probe).


1/40
1/120
1/360
Ad40 infected
KBa+b
Hela
A549
Int407
A431
Mock infected
KBa+b
Hela
A549
Int407
A431
Probe

three additional cell lines, A549, Int407 and A431, infected in parallel with Hela cells and KBa+b cells as described above. Virus replication on Hela cells was around 17% of that seen on KBa+b cells as before and virion DNA was not detectable in A431 cells. However, both A549 cells and Int407 show increase in DNA levels as compared to Hela cells. The DNA yield is 35% and 70% of that seen on KBa+b cells, for A549 and Int407 respectively. This was reflected in the cytopathic effect seen on the cells at 72 hr p.i., where a marginal cpe was seen on Hela cells with a more marked effect on A549 and Int407 cells and a full cpe on KBa+b cells. It is possible that with a longer incubation time higher yields of DNA could have been obtained. However, these preliminary experiments indicate that Ad40 can grow in tissue culture in the absence of any complementing adenovirus functions. This is not an "on and off" effect since there is a range of permissivity from Hela cells to Int407. This could be due to increasing amounts of some limiting factor or factors in these cells, possibly a factor essential for early transcription or at least for efficient early transcription from the E1B promoter. An alternative explanation could be that the need for an E1B 55K function varies between these cells and possibly the more permissive cells provide a function that can replace the E1B 55K protein to some extent. Replication of Ad40 in Int407 cells may be related to the intestinal origin of this cell line. Further investigation of the differences in Ad40 gene expression and replication between Hela cells and the more permissive Int407 cells may give some further insight into the molecular basis of the growth restriction of Ad40 in tissue culture and possibly how that reflects the tissue specificity of the virus.

5. DISCUSSION

The work described in this thesis was aimed at understanding the molecular basis for the growth restriction of Ad40 in tissue culture. The involvement of the E1B region in the growth restriction is discussed in section 5.1. In section 5.2. the E1B transcription map is discussed as well as the significance of the E1A-E1B cotranscripts. Section 5.3. considers the basis for the lack of early E1B protein functions in Ad40 infected cells and how this may reflect the tissue specificity of the virus. Future work towards understanding the growth properties of Ad40 is discussed in section 5.4.

5.1. Involvement of the E1B region in the growth restriction of Ad40.

The E1 region was first implicated in the growth restriction of the enteric adenoviruses when it was shown that these viruses grow on 293 cells, HEK cells transformed with the Ad5 E1 region, and not in primary HEK cells (Takiff *et al.*, 1981). A specific involvement of the E1B region in the growth restriction of Ad40 was demonstrated when Ad40 was shown to grow equally well on KBb and KBa+b cells but the growth was as restricted on KBa cells as on KB cells lacking any adenovirus sequences (Mautner *et al.*, 1989). Complementation of Ad40 by an Ad5 E1A (dl312) but not E1B (dl313) mutant virus further shows that supplying the E1A region *in trans* in the absence of E1B does not help Ad40 replication while supplying the E1B region *in trans* in the absence of E1A results in complementation of Ad40 growth (section 4.1.). The involvement of other adenovirus gene products in the complementation of Ad40 by Ad5 and Ad12 viruses can not be ruled out. However, it should be noted that none of the viruses used in the coinfection assays supported Ad40 growth to levels as high as seen in a single infection on KBa+b cells, which is comparable to that seen on KBb cells (Mautner *et al.*, 1989). This may reflect a

difference between cell types since KB cells are slightly more permissive than Hela cells (Mautner *et al.*, 1989). It also shows that providing the E1B region *in trans* in the absence of any other viral gene product is sufficient to give complementation of Ad40 as high as in the presence of any additional viral gene products. The finding that Ad40 can complement dl312 growth further suggests that Ad40 expresses an E1A gene product capable of transactivating adenovirus early genes. This is consistent with reports from van Loon *et al.*, (1987a) and Ishino *et al.*, (1988) which show that in transient transfection assays the Ad40 E1A region is capable of activating transcription from Ad2 and Ad5 early promoters, albeit with lower efficiency than the Ad5 or Ad12 E1A gene products. The ability of Ad40 to complement an Ad12 E1A mutant virus could not be tested since the laboratory stock of the mutant hr700 grew as well as wild type on Hela cells (figure 7). This mutant was originally described as being severely defective for growth on Hela cells (Breiding *et al.*, 1988), suggesting that the virus stock used in the experiment described here may contain a revertant virus or be contaminated with wild type virus.

Complementation of Ad40 by E1A but not E1B mutant viruses indicates that the growth defect of Ad40 may be related to a lack of E1B 55K protein function. However, Ad40 appears to provide some helper function for the Ad2 and Ad12 E1B 55K mutants dl1520, in602 and dl620 (figures 6 and 7). None of these mutants express detectable amounts of 55K protein (Barker and Berk, 1987; Byrd *et al.*, 1988). It is possible that Ad40 expresses low levels of the 55K protein or alternatively a 55K protein which is functionally less active in Hela cells than its Ad2 or Ad12 counterparts, which could account for some complementation of viruses devoid of any 55K protein. However, no E1B 55K product has been detected in Ad40 infected cells, either by immunoprecipitation using antipeptide antisera against the N- and C-terminal domains of the Ad40 55K protein, or by Western blotting (Mautner *et al.*, 1990), suggesting that if the 55K

protein is made, the quantity is below the level of detection. Since the antisera do not precipitate *in vitro* translated 55K protein the quality of the sera is not known and the possibility that the 55K protein is expressed can not be excluded. It is equally possible that an increase in the levels of some other viral products in the presence of Ad40 may account for the increased replication of the 55K mutant viruses in double infections. The Ad12 mutants in602 and dl620 express very low levels of E1 proteins at a low multiplicity of infection (Byrd *et al.*, 1988). Moreover, the replication of the Ad5 55K mutant dl1520 on Hela cells is multiplicity dependent (Barker and Berk, 1987) suggesting that increased expression of gene products other than the E1B 55K protein may partially overcome the growth restriction. Tiemessen and Kidd (1988) have shown that Ad41 growth on non-permissive cells is complemented in a coinfection with Ad2. Complementation is more effective when the cells are preinfected with Ad2. However, these experiments did not address the question which gene products are involved in the complementation, although it is likely to be E1 gene products since Ad41 grows well on 293 cells.

5.2. The E1B transcription map

The organization of open reading frames in the E1 region of human adenoviruses is very well conserved. Where transcription maps have been determined they are similar, with E1A and E1B regions as separate transcription units. The E1B transcription map reported here for adenovirus 40 shows some important differences from the well characterized Ad2, namely the relative abundance of a 14S mRNA and the presence of E1A-E1B cotranscripts.

5.2.1. Comparison to other serotypes

The major mRNAs from the Ad40 E1B promoter correspond in structure to the Ad2 22S and 14S transcripts, but no 13S mRNA equivalent is found (figure 9). In contrast, in Ad2

the 22S and 13S mRNAs are abundant whereas the 14S, as well as a 14.5S mRNA are minor species (see figure 4). However, in Ad12, although a 13S mRNA has been reported based on S1 analysis (Sawada and Fujinaga, 1980; Bos *et al.*, 1981), by cDNA cloning Virtanen *et al.*, (1982) detected two related 14S but no 13S type mRNA. This is not inconsistent with the S1 data since the splice sites used in a potential 13S mRNA are also used in a 14S mRNA, and suggests that Ad12, like Ad40, does not have a 13S mRNA. The amino acid sequences of the Ad40 E1B proteins are more closely related to Ad12 than to Ad2 (Ishino *et al.*, 1988), and the same appears to be true for the E1B splicing patterns. The effect of this difference in mRNA structure on gene expression is not clear.

The Ad40 E1B transcripts retain the coding potential for the major 19K and 55K E1B polypeptides. The 19K protein encoded by the Ad2 13S mRNA is also encoded by the 14S and 22S mRNAs. However, the counterpart of the 82R polypeptide expressed from the 55K ORF of the Ad2 13S mRNA (figure 4) is likely to be absent from Ad40 as well as Ad12. The function of this protein is unknown. The 14S mRNA also encodes a protein from the 55K ORF, comprising the amino and carboxy termini of the E1B 55K protein. This 155R (15K) polypeptide has not been assigned a function in Ad2 so the significance of the relatively high abundance of the 14S transcript is not clear. In Ad2 the 15K protein cannot substitute for the 55K protein in RNA transport or host shut off during infection (Babiss and Ginsberg, 1984; Babiss *et al.*, 1985; Pilder *et al.*, 1986). The Ad40 15K protein has been immunoprecipitated from infected cell extracts harvested at late times post infection (A. Bailey and N. Mackay, personal communication). The antiserum used was raised against a polypeptide expressed in bacteria from the 15K cDNA clone (see 4.2.2.2.). Although this antiserum immunoprecipitates *in vitro* translated 55K as well as 15K E1B proteins, only the 15K protein has been identified in infected cell extracts.

The Ad40 E1B transcription map was determined from RNA

harvested at late times in infection. This was necessary since E1B transcripts are not detectable at early times in infection. Transcription maps of the E1B regions of other adenovirus serotypes have mostly been determined using RNA from cells infected at a high multiplicity and early mRNA allowed to accumulate by using cytosine arabinoside (ara-C) block of DNA replication (Berk and Sharp, 1977b; 1978; Yoshida and Fujinaga, 1980; Kitchingman and Westphal, 1980; Dijkema *et al.*, 1982; Virtanen *et al.*, 1982; Virtanen and Pettersson, 1985; Hashimoto *et al.*, 1984). This approach was not possible in the case of Ad40 since even in the presence of an ara-C block early E1B transcripts fail to accumulate to detectable levels (Mautner *et al.*, 1990). Moreover, although the infectious titre of the Ad40 virus stock was not determined in the absence of a suitable titration method, virus particle counts indicate that the virus concentration used for infections is approximately 10^3 particles /cell. In spite of this high virus input, a low yield of Ad40 E1B RNA was obtained. The yield of E1B RNA from an Ad40 infection is at least 10-fold less than from an Ad5 infection at a modest multiplicity (Mautner *et al.*, 1990). It has previously been reported that while the yield of Ad40 virions was 3-10-fold less than that obtained from an infection with other adenovirus serotypes the yield of infectious virus was 100-1000-fold lower (Brown, 1985). This suggests that a high proportion of Ad40 particles are defective and may explain the low RNA yield if only a subset of cells are productively infected, or if the defective particles interfere with the infection. Ad41 virus stocks have been reported to contain a large proportion of defective particles lacking the core protein V (Pieniazek, D. *et al.*, 1990). Similar studies have not been performed with Ad40.

Attempts to select messenger RNA using oligo dT resulted in loss of material and were thus not successful. Consequently it has not been formally proven that the mapped transcripts are poly-adenylated mRNAs. By analogy with other serotypes no E1B mRNA species are predicted to be made

at early times in infection that are not seen at late times. It is therefore likely that the transcripts described here represent all major E1B mRNA species. However, the possibility of minor RNA species cannot be excluded.

5.2.2. E1A-E1B cotranscripts

A novel type of E1A-E1B cotranscript was detected in Ad40 infected cells, consisting of a short fragment from the 5' end of E1A linked to the E1B region 4-5 nt downstream of the major cap site. Both the 22S and the 14S type spliced products exist as E1A-E1B cotranscripts as well as being transcribed from the E1B promoter. The size of RNA seen on Northern blot is compatible with the cotranscripts being 5' coterminal with the E1A transcripts, starting at the E1A cap site mapped previously at nt 418 (van Loon *et al.*, 1987b), although the 5' ends have not been analysed in detail. No new open reading frames are created by the E1A-E1B junction but if the cotranscripts start at the E1A cap site they include the first 40 codons of the E1A open reading frame, followed by one amino acid and a termination codon overlapping the E1B 19K ORF. It is not clear whether these transcripts could be utilized in the cell to make E1B proteins; most capped eucaryotic mRNAs appear to be translated by a scanning mechanism (Kozak, 1981) and the first AUG a scanning ribosomal subunit would encounter on an E1A-E1B cotranscript is the E1A start codon. With further AUGs downstream, preceding the E1B region, it seems unlikely that translation will be initiated from the E1B start signals.

The sequences around the E1A-E1B junction in the cotranscripts do not conform to splice consensus sequences, suggesting that they may not be formed by a conventional splicing mechanism. The sequence around the 5' end of the junction is CGC/T/TCATGA, and the 3' sequence is TTTTAGTGTTAGTT/T/A (the T residue at the junction could originate from the 5' or the 3' end as indicated; see figure 17C), compared to the consensus sequences (C/A)AG/GT(A/G)AGT

and $(T/C)_{11}N(C/T)\underline{AG}/G$ (Mount, 1982). The cotranscript sequence was obtained consistently from several cloned fragments amplified from different RNA preparations. Moreover, Northern blotting experiments confirmed the presence of E1A-E1B cotranscripts, and the detection of these transcripts by a restriction fragment from the 5' end of E1A and not by the 3' adjacent fragment (see figure 18) is consistent with the position of the junction as mapped by cDNA sequencing. However, the location of the junction in the genomic sequence, and in particular the absence of a GT dinucleotide at the 5' end of the predicted intron, and an AG dinucleotide at the 3' end was somewhat unexpected.

There are several ways of explaining how the anomalous junction may be formed. Firstly, the virus is passaged on a complementing cell line which provides Ad2 E1A and E1B functions, so an E1A deficient virus could grow on these cells. Thus the virus stock, or a variant within it may have a deletion in the E1 region corresponding to the junction in the cDNA. In order to test that possibility virion DNA was amplified using the same primers that were used for the cDNA amplification. A product corresponding to the genomic DNA was obtained with no detectable smaller fragments, indicating that such a deletion is absent from the virus stock. Secondly, the DNA sequence of the virus stock might differ from the published sequence. The cDNA sequenced was identical to the published sequence but intron sequences might be different. To conform to the GT-AG rule there would have to be a point mutation T→G at the splice donor site and a deletion of 2-3 T residues at the splice acceptor site. A way to address this possibility would be to amplify viral DNA in a PCR reaction using primers flanking either end of the junction, and sequence without cloning, looking for any ambiguity in the sequence at the junction. In a preliminary experiment, covering the 3' end of the junction, the sequence appeared uniform and identical to the published sequence, thus rendering this possibility unlikely.

A third possibility is that this E1A-E1B cotranscript is

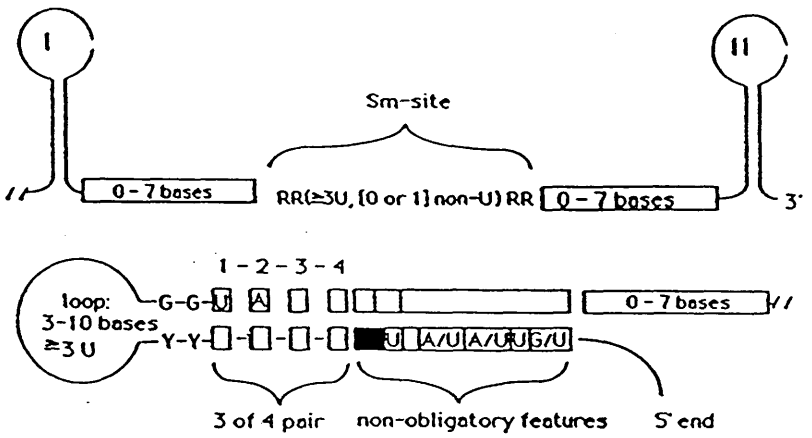
formed by a novel type of RNA processing which does not rely on sequences necessary for conventional splicing. It is not likely that the junction is formed by a conventional *cis*-splicing mechanism. The selection of a 5' splice site involves a direct base pairing between the U1 small nuclear RNA (snRNA) and sequences around the splice site (Lerner *et al.*, 1980; Rogers and Wall, 1980; Zhuang and Weiner, 1986). Such an interaction would not take place between the U1 sequence ^{3}'GUC CAUUCAU^{5}' and the 5' sequence around the junction in the cotranscript, ^{5}'CGC↓U↓UCAUGA^{3}' (the arrows denote the position of the splice junction). A different splicing mechanism, termed *trans*-splicing, has been observed in the mammalian parasite *Trypanosoma brucei* and related species and in the nematode *Caenorhabditis elegans* (see Blumenthal and Thomas, 1988; Laird, 1989 for review). This involves the transfer of a 5' fragment from a spliced leader transcript (SL RNA) to a precursor mRNA with a 3' splice acceptor site. In nematodes the reaction takes place in the same nuclear compartment as *cis*-splicing and proceeds through Y-branched intermediates, analogous to the lariats formed in *cis*-splicing (Murphy *et al.*, 1986; Sutton and Boothroyd, 1986). In nematodes *cis*-splicing occurs as well as *trans*-splicing and small nuclear ribonucleoprotein particles (snRNPs), corresponding to those essential for *cis*-splicing in other organisms (U1, U2, U4/U6, and U5) have been identified (Thomas *et al.*, 1988). Like snRNPs from other animals these can be immunoprecipitated with antibodies to the Sm antigen (Thomas *et al.*, 1988), which is common to all these snRNPs (Bringmann and Luhrmann, 1986). In trypanosomes *cis*-splicing has not been identified and a homologue for U1 has not been detected although the other snRNPs have been identified. However, the spliced leader sequences in trypanosomes and nematodes contain a consensus sequence for an Sm binding site (A[U_{3-6}]G; Liautard *et al.*, 1982; Mattaj and De Robertis, 1985; Riedel *et al.*, 1987) and can be immunoprecipitated by anti-Sm antibodies (Bruzik *et al.*, 1988; Thomas and Conrad, 1988; van Doren and Hirsh, 1988). These authors have suggested that an SL-snRNP

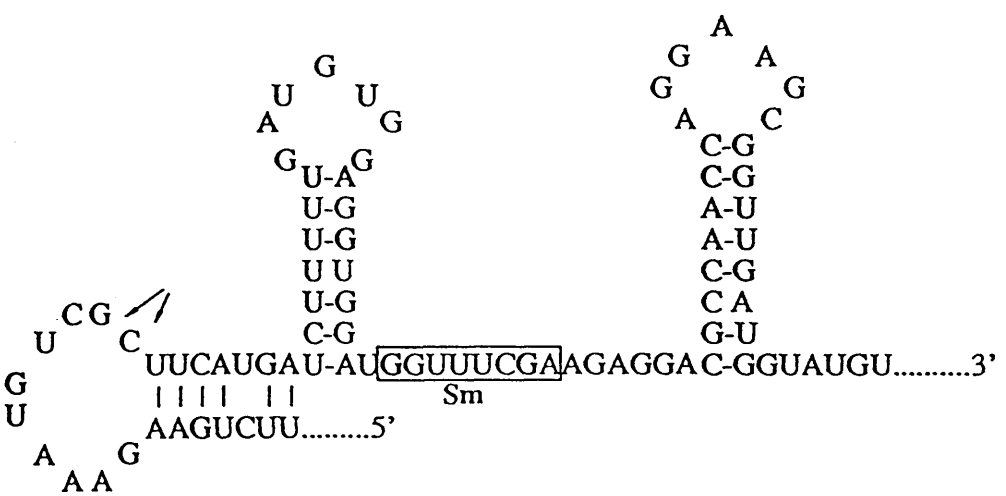
particle may have a role similar to that of U1 in rendering the 5' splice site susceptible to attack in a splicing reaction. Recently Dandekar and Sibbald (1990) used several known *trans*-splicing structures as a basis for a computer search for putative new *trans*-splicing sites. They predict that *trans*-splicing may occur in a wide variety of organisms including the vertebrates. Their model predicts three stem-loop structures and an Sm binding site (RR(≥3U[0-1 non U]RR) in a single stranded region near the 5' splice site. The model is shown in figure 25, top panel. A similar structure could be predicted around the 5' splice site in the E1A-E1B cotranscripts, where the predicted Sm binding site is GGUUUCGA (figure 25, lower panel). Some of the obligatory features in the stem-loop structure are not consistent with the model, in particular the GG dinucleotide at the splice junction is absent in the Ad40 sequence. However, it would be of interest to see if this site can indeed bind Sm, which would raise the possibility of a mechanism related to *trans*-splicing having a role in the processing of some Ad40 transcripts. The requirements for the 3' splice site in *trans*-splicing have not been analysed as extensively. However, it is unlikely that a transcript from the E1B promoter could act as a 3' splice acceptor since the 3' site of the junction in the E1A-E1B cotranscript is only 5 nt downstream of the E1B cap site. Therefore a splicing reaction in which the 5' end of an E1A transcript acts as an snRNA would most likely take place on a transcript overriding the E1A poly A signal, extending from the E1A cap site to the E1B poly A site.

Adenovirus E1A-E1B cotranscripts have been detected previously at low abundance in Ad2 and Ad12 infected and transformed cells (Büttner *et al.*, 1976; Berk and Sharp, 1978; Kitchingman and Westphal, 1980; Chinnadurai *et al.*, 1976; Sawada and Fujinaga, 1980; Saito *et al.*, 1983; van den Elsen *et al.*, 1983). The Ad12 cotranscripts are reported to have 5' ends and splice sites identical to the major E1A mRNAs and to extend to the 3' end of E1B, with a splice corresponding to the splice in E1B 22S mRNA. However, cDNA

Figure 25: The predicted secondary structures for the 5' splice sites involved in *trans*-splicing and for the 5' splice site in the E1A-E1B cotranscripts.

The top panel shows a predicted RNA structure around 5' splice sites involved in *trans*-splicing (Dandekar and Sibbald, 1990). The model is based on RNA sequences in six known *trans*-splicing structures. The lower panel shows a potential secondary structure for the RNA sequences around the 5' splice site in the E1A-E1B cotranscripts. A consensus sequence for a binding site for the Sm antigen is indicated. The arrows denote the splice junction in the E1A-E1B cotranscripts. (The top panel was reproduced from Dandekar and Sibbald, 1990).


I
II
Sm-site
0 - 7 bases
RR(≥3U, [0 or 1] non-U) RR
0 - 7 bases
3'
1 - 2 - 3 - 4
loop:
3-10 bases
≥3 U
G-G-U
A
Y-Y-
U
A/U
A/U
U
G/U
0 - 7 bases
3 of 4 pair
non-obligatory features
5' end


UUCAUGAU-AUGGUUUCGAAGAGGAC-GGUAUGU.....3'
AAGUCUU.....5'
Sm

sequences have not been determined. Berk and Sharp (1978) describe an Ad2 transcript consisting of fewer than 50 bp from the 5' end of E1A, linked to a site near the 5' end of E1B, resulting in a 22S type E1A-E1B cotranscript similar to the one described here, but the precise structure was not analysed, so it is not clear if it is made by conventional splicing.

Hashimoto *et al.*, (1984) report *in vitro* translation of Ad2 E1A proteins from E1B selected transcripts, indicating a different structure for E1A-E1B cotranscripts. They suggested that a splice acceptor consensus sequence downstream of the E1B cap site (CTCCTCCCAG/G, nt 1809-1819) may play a role in such transcripts. A similar sequence can be observed downstream of the Ad40 E1B cap site (CTTCTGCCAG/A, nt 1472-1482). The use of this potential splice acceptor would account for the 170 nt protected fragment seen in figure 10. However, no further evidence was obtained for the use of this splice site. A cotranscript with the structure predicted by Hashimoto *et al.*, (1984) including this splice site would have been expected to be detected by the cDNA-PCR amplification across the E1A-E1B junction (see figure 17). This was not seen and the only cDNA fragment obtained in this reaction corresponded to the E1A-E1B cotranscript described above. This suggests that this alternative structure of an E1A-E1B cotranscript is absent in Ad40 and indicates that the 170 nt fragment obtained by the S1 analysis (figure 10) may be artefactual (protection of a transcript from the other strand cannot be excluded). It is possible that the shortest template in the PCR reaction was preferentially amplified and a longer template, corresponding to the structure predicted by Hashimoto *et al.*, (1984) was therefore not detected. However, Northern blotting experiments (see figure 18) support the conclusion that the cotranscripts described here are the only E1A-E1B cotranscripts present in detectable amounts.

The presence of minor RNA species not detected by the methods used here for the transcription mapping cannot be ruled out. A possible way of extending the analysis would

be to prepare and screen a cDNA library from Ad40 infected cell RNA. This was not done here since the main purpose of the transcription mapping was to determine if the E1B transcripts retained the coding potential for the major E1B polypeptides predicted by analogy with other adenoviruses.

5.3. What is the basis for the lack of E1B early protein functions?

Although Ad40 E1B transcripts are made encoding the major E1B proteins they are not seen before the first increase in viral DNA in KBa+b cells or Hela cells (figure 19). Furthermore, E1B transcripts do not accumulate when DNA replication is blocked by cytosine arabinoside (Mautner *et al.*, 1990). This indicates that this conventionally early transcription unit (Berk and Sharp, 1977a; Evans *et al.*, 1977) behaves like a late gene in Ad40. However, since the first increase in E1B RNA and viral DNA is seen at the same timepoint (figure 19) it is not possible to determine if an increase in either E1B transcription or DNA replication occurs first, perhaps triggering off an immediate increase in the other. Although the E1B 55K protein is not essential for DNA replication in Ad5 (Babiss and Ginsberg, 1984), it is required for replication of Ad12 DNA in infected cells (Shiroki *et al.*, 1986). This protein may be equally important for the replication of Ad40 DNA since replication begins earlier and is more efficient in KBa+b cells, where the 55K protein is provided *in trans*, than in Hela cells. However, a sudden increase in Ad40 E1B expression in the presence of Ad2 E1B gene products is unlikely to have a great effect on DNA replication. Inefficient transcription from the E1B promoter would account for the late detection of E1B mRNA. The increase in E1B transcription seen around the onset of DNA replication may reflect transcription from an increasing number of templates. Alternatively, the promoter may be more active on newly replicated DNA; such activation has been suggested for the ppIX promoter (Venkatesh and Chinnadurai, 1987). Both explanations are

consistent with the finding that E1B mRNA does not accumulate under a replicative block.

Transcription from the E1A region of Ad40 as well as the E1B region is severely delayed compared to Ad5. Ad40 E1A and E1B transcripts are first detected at 24 and 30 hr p.i. respectively in Hela cells and 18 and 24 hr p.i. respectively in KBa+b cells (figure 19) whereas in an Ad5 infection of Hela cells their counterparts are seen at 2 and 3.5 hr p.i. respectively (Glenn and Ricciardi, 1988). However, the lack of E1A gene products cannot explain the late appearance of E1B transcripts since Ad2 E1A proteins are expressed in KBa+b cells. Ishino *et al.*, (1988) have previously shown that the Ad40 E1A promoter is weaker that its Ad12 counterpart, and the effect is cell type dependent. This may at least partly explain the late detection of E1A mRNA as seen in figure 19. Mautner *et al.*, (1990) have shown that E1 transcripts do not accumulate in the nuclei; however, the possibility that E1 transcripts are made but are unstable cannot be excluded.

The most likely explanation for the lack of early E1B gene products in an Ad40 infection is that transcription from the E1B promoter is inefficient or absent at early times in infection. Early transcription of the E1B region like other early regions is dependent on transactivation by an E1A gene product. E1A activation is mediated through the E1B TATA box, while the Sp1 binding site is essential for efficient transcription from the E1B promoter but does not affect E1A transactivation (Wu *et al.*, 1987; Parks *et al.*, 1988). The lack of response to E1A transactivation by the Ad40 E1B promoter (table 6; figure 22A) can not however be attributed to a lack of a functional TATA box. The Ad40 E1B TATA box is identical to that of Ad5, which does respond to E1A activation, and also to TATA boxes of several other serotypes (see figure 20). Moreover, the Ad40 E1B promoter is capable of responding to another transactivator, the VZV 140K protein, which is thought to act through the TATA box, although the activation is weak compared to the Ad5 E1B promoter (figure 22A). It is not clear if the GC box in the

Ad40 E1B promoter is a functional Sp1 binding site. It is possible that weak binding of Sp1 to this site results in a low basal activity of the promoter. Mutations in the Sp1 binding site in the Ad2 E1B promoter result in a 5-fold decrease in activity (Wu *et al.*, 1987). These authors used S1 analysis to detect transcription from mutated E1B promoters. The CAT assays described in section 4.3.2. show an induction of expression from the Ad5 E1B promoter by Ad5 E1A similar to that seen previously (Dery *et al.*, 1987). However, if the basal activity of the Ad40 E1B promoter is much lower than its Ad5 counterpart, 2-3-fold activation by E1A might not be detected in this system. Because no activity of the Ad40 E1B promoter above background could be detected it is not possible to determine by this method if the lack of transcription is due to a weak Sp1 binding site, the absence of E1A transactivation or some other factor. A substitution of one or both promoter elements, including flanking sequences, by its Ad5 counterpart might distinguish between these possibilities. This could also give an indication if any negative regulatory elements are present in the Ad40 E1B region.

The lack of early E1B gene expression in tissue culture raises the question what happens in an *in vivo* Ad40 infection. The growth restriction of Ad40 in tissue culture does not reflect a defect in virus growth *in vivo* since a large number of virus particles can be isolated from infected children (Gary *et al.*, 1979; Retter *et al.*, 1979; Takiff and Straus, 1982). Efficient virus growth *in vivo* suggests that either the E1B region is expressed at early times in infection in the host cells, or E1B early gene products are nonessential in these cells. The cell type that supports Ad40 growth in the small intestine has not been identified. Early E1B transcription in enteric cells could suggest the presence of specific transcription factors essential for E1B transcription in the enteric adenoviruses but not other adenovirus serotypes. Alternatively, negative regulatory factors, interacting specifically with the enteric adenoviruses and present in most tissue culture cell

lines may be absent in those cells. If E1B early products are nonessential in the natural host cells, that would suggest that late mRNAs are efficiently transported to the cytoplasm in the absence of the E1B 55K/E4 34K protein complex. A549 cells (human lung carcinoma cells) are more permissive for Ad40 than Hela cells (figure 24). These cells are also more permissive for an E4 34K mutant virus (dl355) and host cell shut off, a function of the 55K/34K complex, is less affected in these cells than in Hela cells (Halbert *et al.*, 1985). This suggests that the need for the 55K/34K complex is partly overcome in these cells and is cell type dependent. Moreover, Ad12 E1B mutant viruses are less restricted for growth on A549 cells than on Hela cells (Breiding *et al.*, 1988). An indication that the 55K protein may not be essential at early times in infection in all cell types comes from recent studies using the intestinal cell line Int407. These cells are nearly as permissive for Ad40 growth as KBa+b cells (figure 24). However, E1B transcripts are not detectable at early times in infection (R. Ullah, personal communication), suggesting that virus replication is not dependent on E1B early gene expression. It is possible that these cells are more related to the natural host cells of Ad40 than either Hela cells or KB cells. However, even in Int407 cells Ad40 replication is inefficient compared to Ad5 growth in tissue culture, thus indicating that these cells may not entirely reflect the conditions of virus growth *in vivo*. It is of importance to identify the natural host cells of the virus in order to study the host cell functions important for the propagation of Ad40.

5.4. Future work

Detailed studies on Ad40 first became feasible when the virus was shown to propagate in KBa+b cells (Mautner *et al.*, 1989) thus providing a tissue culture system in which to grow the virus. The work presented here and by others in the last few years provides the basis for several

interesting lines of studies.

The identification of the E1A-E1B cotranscripts opens several questions: (i) how are they made? (ii) are they important for virus growth? (iii) are they unique to Ad40 or can similar structures be found in other serotypes? An approach to study the mechanism by which the cotranscripts are made was discussed in section 5.2.2. Binding of the Sm antigen near the 5' splice site would open the possibility that a mechanism related to *trans*-splicing is involved in this procedure. Mutational analysis, in order to study the importance of the cotranscripts for the virus, is difficult until more is known about which sequences are important for the creation of the junction. It is possible that these cotranscripts are not unique to Ad40. A report of a transcript with a similar structure in Ad2 (Berk and Sharp, 1978) suggests that they may be found in other serotypes as well, although in this case the sequence around the junction was not determined. It would be of interest to use PCR-cDNA analysis to look for E1A-E1B cotranscripts in Ad2 and other adenovirus serotypes.

If Ad40 E1B proteins are not essential in the natural host cells of the virus, they may not be functionally equivalent to their counterparts in other serotypes. This can be tested by removing the E1B coding regions from an Ad5 virus and substituting the Ad40 E1B coding regions. It can then be seen if the Ad40 E1B proteins, expressed from the Ad5 E1B promoter can compensate for the lack of Ad5 E1B proteins. This type of experiment is currently being performed (A. Bailey, personal communication).

The lack of expression from the Ad40 E1B promoter can be investigated in several ways. A comparison of protein binding to the Ad40 and Ad5 E1B promoters might reveal important differences which could account for the different activities of the promoters. Moreover, it would be of interest to see if there is a difference in protein binding to the Ad40 E1B promoter using cell extracts from early and late times in infection. By substituting individual promoter elements by their Ad5 counterparts the importance of the

Ad40 Sp1 binding site and TATA box can be individually assessed.

In order to fully understand the growth requirements of Ad40 a system which supports virus growth to levels similar to that seen for other adenovirus serotypes must be found. It is likely that the virus is largely dependent on cell functions unique to its natural host cells, making it very cell specific. It is not clear if Ad40 replicates in cells other than in the small intestine in an *in vivo* infection. It may be well adapted to one particular cell type, and thus have a very restricted host range. The host cell functions involved can only be studied in a permissive cell system.

REFERENCES

ABMAYR, S.M., WORKMANN, J.L. and ROEDER, R.G. (1988). The pseudorabies immediate early protein stimulates *in vitro* transcription by facilitating TFIID:promoter interactions. *Genes Dev.* 2, 542-553.

AKUSJÄRVI, G., ALESTRÖM, P., PETTERSSON, M., LAGER, M., JÖRNVALL, H. and PETTERSSON, U. (1984). The gene for the adenovirus 2 hexon polypeptide. *J. Biol. Chem.* **259**, 13976-13979.

AKUSJÄRVI, G. and PETTERSSON, U. (1979). Sequence analysis of adenovirus DNA. IV. The genomic sequences encoding the common tripartite leader of late adenovirus messenger RNA. *J. Mol. Biol.* 134, 143-158.

AKUSJÄRVI, G. AND WADELL, G. (1990). Genetic map of adenovirus. In Genetic Maps, S.J. O'Brien (Ed). Cold Spring Harbor Laboratory Press.

ALBERT, M.J. (1986). Enteric adenoviruses. *Arch. Virol.* **88**, 1-17.

ALESTRÖM, P., AKUSJÄRVI, G., PERRICAUDET, M., MATHEWS, M.B., KLESSIG, D.F. and PETTERSSON, U. (1980). The gene for polypeptide IX of adenovirus type 2 and its unspliced messenger RNA. *Cell* 19, 671-681.

ALLARD, A. and WADELL, G. (1988). Physical organisation of the enteric adenovirus type 41 early region 1A. *Virology* **164**, 220-229.

ANDERSON, C.W. (1990). The proteinase polypeptide of adenovirus serotype 2 virions. *Virology* **177**, 259-272.

ANDERSON, C.W., SCHMITT, R.C., SMART, J.E. and LEWIS, J.B. (1984). Early region 1B of adenovirus 2 encodes two coterminal proteins of 495 and 155 amino acid residues. *J. Virol.* 50, 387-396.

ANDERSON, K.P. and KLESSIG, D.F. (1984). Altered mRNA splicing in monkey cells abortively infected with human adenovirus may be responsible for inefficient synthesis of the virion fiber polypeptide. *Proc. Natl. Acad. Sci.* **81**, 4023-4027.

ANGEL, P., ALLEGRETTO, E.A., OKINO, S.T., HATTORI, K., BOYLE, W.J., HUNTER, T. and KARIN, M. (1988). Oncogene *jun* encodes a sequence-specific *trans*-activator similar to AP-1. *Nature* 332, 166-171.

ANGEL, P., IMAGAWA, M., CHIU, P., STEIN, B., IMBRA, R.J., RAHMSDORF, H.J., JONAT, C., HERRLICH, P. and KARIN, M. (1987). Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* 49, 729-739.

AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SMITH, J.A., SEIDMAN, J.G. and STRUHL, K. (1987). Current Protocols in Molecular Biology. Greene Publishing Associates, and John Wiley & Sons Inc USA.

BABICH, A. and NEVINS, J.R. (1981). The stability of early adenovirus mRNA is controlled by the viral 72kd DNA-binding protein. *Cell* 26, 371-379.

BABISS, L.E. (1989). The cellular transcription factor E2F requires viral E1A and E4 gene products for increased DNA binding activity and functions to stimulate adenovirus E2A gene expression. *J. Virol.* 63, 2709-2717.

BABISS, L.E., FISHER, P.B. and GINSBERG, H.S. (1984). Effect on transformation of mutations in the early region 1b-encoded 21- and 55-kilodalton proteins of adenovirus type 5. *J. Virol.* 52, 389-395.

BABISS, L.E. and GINSBERG, H.S. (1984). Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. *J. Virol.* 50, 202-212.

BABISS, L.E., GINSBERG, H.S. and DARNELL, J.E. (1985). Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. *Mol. Cell. Biol.* 5, 2552-2558.

BABISS, L.E., YOUNG, C.S.H., FISHER, P.B. and GINSBERG, H.S. (1983). Expression of adenovirus E1A and E1B gene products and the *Escherichia coli* XGPRT gene in KB cells. *J. Virol.* 46, 454-465.

BAGCHI, S., RAYCHAUDHURI, P. and NEVINS, J.R. (1989). Phosphorylation-dependent activation of the adenovirus inducible E2F transcription factor in a cell-free system. *Proc. Natl. Acad. Sci.* 86, 4352-4356.

BAICHWAL, V.R. and TJIAN, R. (1990). Control of *c-Jun* activity by interaction of a cell specific inhibitor with regulatory domain δ: differences between *v-* and *c-jun*. *Cell* 63, 815-825.

BAKER, C.C. and ZIFF, E.B. (1981). Promoters and heterogeneous 5' termini of the messenger RNAs of adenovirus serotype 2. *J. Mol. Biol.* 149, 189-221.

BALL, A.O., BEARD, C.W., REDICK, S.D. and SPINDLER, K.R. (1989). Genome organization of mouse adenovirus type 1 early region 1: A novel transcription map. *Virology* 170, 523-536.

BALL, A.O., WILLIAMS, M.E. and SPINDLER, K.R. (1988). Identification of mouse adenovirus type 1 early region 1: DNA sequence and a conserved transactivating function. *J. Virol.* 62, 3947-3957.

BANKIER, A.T., WESTON, K.M. and BARRELL, B.G. (1987). Random cloning and sequencing by the M13/dideoxynucleotide chain termination method. *Meth. in Enzymol* **155**, 51-93.

BARKER, D.D. and BERK, A.J. (1987). Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology* **156**, 107-121.

BERGER, S.L. and FOLK, W.R. (1985). Differential activation of RNA polymerase III-transcribed genes by the polyomavirus enhancer and the adenovirus E1A gene products. *Nucl. Acids Res.* **13**, 1413-1428.

BERGET, S.M., MOORE, C. and SHARP, P. (1977). Spliced segments of the 5' terminus of adenovirus 2 late mRNA. *Proc. Natl. Acad. Sci.* **74**, 3171-3175.

BERK, A.J. (1986a). Functions of adenovirus E1A. *Cancer Surveys* **5**, 367-387.

BERK, A.J. (1986b). Adenovirus promoters and E1A transactivation. *Ann. Rev. Genet.* **20**, 45-79.

BERK, A.J. and SHARP, P.A. (1977a). Ultraviolet mapping of the adenovirus 2 early promoters. *Cell* **12**, 45-55.

BERK, A.J. and SHARP, P.A. (1977b). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**, 721-732.

BERK, A.J. and SHARP, P.A. (1978). Structure of the adenovirus 2 early mRNAs. *Cell* **14**, 695-711.

BERNARDS, R., DE LEEUW, M.G.W., HOUWELING, A. and VAN DER EB, A.J. (1986). Role of the adenovirus early region 1B tumor antigens in transformation and lytic infection. *Virology* **150**, 126-139.

BEUTLER, B. and CERAMI, A. (1989). The biology of cachectin-/TNF - A primary mediator of the host response. *Ann. Rev. Immunol.***7**, 625-655.

BHATTI, A.R. and WEBER, J. (1979). Protease of adenovirus type 2. *J. Biol. Chem.* **254**, 12265-12268.

BLUMENTHAL, T. and THOMAS, J. (1988). *Cis* and *trans* mRNA splicing in *C. elegans*. *Trends Genet.* **4**, 305-308.

BOHMANN, D., BOS, T.J., ADMON, A., NISHIMURA, T., VOGT, P.K. and TJIAN, R. (1987). Human proto-oncogen *c-Jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP1. *Science* **238**, 1386-1392.

BOHMAN, D. and TJIAN, R. (1989). Biochemical analysis of transcriptional activation by *Jun*: differential activity of *c-* and *v-Jun*. *Cell* **59**, 709-717.

BORELLI, E., HEN, R. and CHAMBON, P. (1984). Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature* **312**, 608-612.

BOS, J.L., POLDER, L.J., BERNARDS, R., SCHRIER, P.I., VAN DEN ELSEN, P.J., VAN DER EB, A.J. and VAN ORMONDT, H. (1981). The 2.2kb E1B mRNA of human ad12 and ad5 codes for two tumor antigens starting at different AUG triplets. *Cell* **27**, 121-131.

BOS, J.L. and TEN WOLDE-KRAAMWINKEL, H.C. (1983). The E1b promoter of Ad12 in mouse L tk$^-$ cells is activated by adenovirus region E1A. *EMBO J.* **2**, 73-76.

BOS, T.J., BOHMAN, D., TSUCHIE, H., TJIAN, R. and VOGT, P.K. (1988). *v-Jun* encodes a nuclear protein with enhancer binding properties of AP-1. *Cell* **52**, 705-712.

BRANDT, C.D., KIM, H.W., RODRIGUEZ, W.J., AROBIO, J.O., JEFFRIES, B.C., STALLINGS, E.P., LEWIS, C., MILES, A.J., GARDNER, M.K. and PARROTT, R.H. (1985). Adenoviruses and paediatric gastroenteritis. *J. Infect. Dis.* **151**, 437-443.

BRANDT, C.D., KIM, H.W., YOLKEN, R.H., KAPIKIAN, A.Z., ARROBIO, J.O. RODRIGUEZ, W.J., WYATT, R.G., CHANOCK, R.M. and PARROTT, R.H. (1979). Comparative epidemiology of two rotavirus serotypes and other viral agents associated with pediatric gastroenteritis. *Am. J. Epidemiol.* **110**, 243-254.

BREIDING, D.E., EDBAUER, C.A., TONG, J.Y., BYRD, P., GRAND, R.J.A., GALLIMORE, P.H. and WILLIAMS, J. (1988). Isolation and characterization of adenovirus type 12 E1 host-range mutants defective for growth in nontransformed human cells. *Virology* **164**, 390-402.

BRIDGE, E. and KETNER, G. (1989). Redundant control of adenovirus late gene expression by early region 4. *J. Virol.* **63**, 631-638.

BRIDGE, E. and KETNER, G. (1990). Interaction of adenoviral E4 and E1B products in late gene expression. *Virology* **174**, 345-353.

BRIGATI, D.J., MYERSON, D., LEARY, J.J., SPALHOLZ, B., TRAVIS, S.Z., FONG, C.K.Y., HSIUNG, C.D. and WARD, D.C. (1983). Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labelled hybridization probes. *Virology* **126**, 32-50.

BRINCKMANN, U., DARAI, G. and FLÜGEL, R.M. (1983). Tupaia (tree shrew) adenovirus DNA: sequence of the left-hand fragment corresponding to the transforming early region of human adenoviruses. *EMBO J.* **2**, 2185-2188.

BRINGMANN, P. and LUHRMANN, R. (1986). Purification of the

individual snRNPs U1, U2, U5 and U4/U6 from Hela cells and characterization of their protein constituents. *EMBO J.* 5, 3509-3516.

BROCKMANN, D., TRIES, B. and ESCHE, H. (1990). Isolation and characterization of novel adenovirus type 12 E1A mRNAs by cDNA PCR technique. *Virology* 179, 585-590.

BROWN, D.T., WESTPHAL, M., BURLINGHAM, B.T., WINTERHOFF, U. and DOERFLER, W. (1975). Structure and composition of the adenovirus type 2 core. *J. Virol.* 16, 366-387.

BROWN, M., (1985). Selection of nonfastidious adenovirus species in 293 cells inoculated with stool specimens containing adenovirus 40. *J. Clin. Microbiol.* 22, 205-209.

BROWN, M., PETRIC, M. and MIDDLETON, P.J. (1984). Diagnosis of fastidious enteric adenoviruses 40 and 41 in stool specimens. *J. Clin. Microbiol.* 20, 334-338.

BRUDER, J.T. and HEARING, P. (1989). Nuclear factor EF-1A binds to the adenovirus E1A core enhancer element and to other transcriptional control regions. *Mol. Cell. Biol.* 9, 5143-5153.

BRUZIK, J.P., VAN DOREN, K., HIRSH, D. and STEITZ, J.A. (1988). *Trans* splicing involves a novel form of small nuclear ribonucleoprotein particles. *Nature* 335, 559-562.

BYRD, P.J., CHIA, W., RIGBY, P.W.J. and GALLIMORE, P.H. (1982). Cloning of DNA fragments from the left end of the adenovirus type 12 genome: Transformation by cloned early region 1. *J. Gen. Virol.* 60, 279-293.

BYRD, P.J., GRAND, R.J.A., BREIDING, D., WILLIAMS, J.F. and GALLIMORE, P.H. (1988). Host range mutants of adenovirus type 12 E1 defective for lytic infection, transformation and oncogenicity. *Virology* 163, 155-165.

CARLIN, C.R., TOLLEFSON, A.E., BRADY, H.A., HOFFMAN, B.L. and WOLD, W.S.M. (1989). Epidermal growth factor receptor is downregulated by a 10,400 MW protein encoded by the E3 region of adenovirus. *Cell* 57, 135-144.

CARTER, T.H. and BLANTON, R.A. (1978a). Possible role of the 72,000-Dalton DNA-binding protein in regulation of adenovirus type 5 early gene expression. *J. Virol.* 25, 664-674.

CARTER, T.H. and BLANTON, R.A. (1978b). Autoregulation of adenovirus type 5 early gene expression. II. Effect of temperature-sensitive early mutations on virus RNA accumulation. *J. Virol.* 28, 450-456.

CEPKO, C.L. and SHARP, P.A. (1982). Assembly of adenovirus major capsid protein is mediated by a non-virion

protein. *Cell* 31, 407-415.

CEPKO, C.L. and SHARP, P.A. (1983). Analysis of Ad5 hexon and 100K *ts* mutants using conformation-specific monoclonal antibodies. *Virology* 129, 137-154.

CHALLBERG, M.D., DESIDERIO, S.V. and KELLY, T.J., JR. (1980). Adenovirus DNA replication *in vitro*: Characterization of a protein covalently linked to nascent DNA strands. *Proc. Natl. Acad. Sci.* 77, 5105-5109.

CHALLBERG, M.D. and KELLY, T.J. (1989). Animal virus DNA replication. *Ann. Rev. Biochem.* 58, 671-717.

CHALLBERG, M.D. and RAWLINS, D.R. (1984). Template requirement for the initiation of adenovirus DNA replication. *Proc. Natl. Acad. Sci.* 81, 100-104.

CHALLBERG, S.S. and KETNER, G. (1981). Deletion mutants of adenovirus 2: Isolation and initial characterization of virus carrying mutations near the right end of the viral genome. *Virology* 114, 196-209.

CHANG, L.-S. and SHENK, T. (1990). The adenovirus DNA-binding protein stimulates the rate of transcription directed by adenovirus and adeno-associated virus promoters. *J. Virol.* 64, 2103-2109.

CHATTERJEE, P.K., VAYDA, M.E. and FLINT, S.J. (1986). Identification of proteins and protein domains that contact DNA within adenovirus nucleoprotein cores by ultraviolet light crosslinking of oligonucleotides ^{32}P-labelled *in vivo*. *J. Mol. Biol.* 188, 23-37.

CHEUNG, A.K. (1989). DNA nucleotide sequence analysis of the immediate early gene of pseudorabies virus. *Nucl. Acids. Res.* 17, 4637-4646.

CHIBA, S., NAKATA, S., NAKAMURA, I., TANIGUCHI, K., URASAWA, S., FUJINAGA, K. and NAKAO, T. (1983). Outbreak of infantile gastroenteritis due to type 40 adenovirus. *Lancet* 2, 954-957.

CHINNADURAI, G. (1983). Adenovirus 2 *lp'* locus codes for a 19kd tumor antigen that plays an essential role in cell transformation. *Cell* 33, 759-766.

CHIU, R., ANGEL, P. and KARIN, M. (1989). *Jun-B* differs in its biological properties from, and is a negative regulator of, *c-Jun*. *Cell* 59, 979-986.

CHOMCZYNSKI, P. and SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.

CHOW, L.T., BROKER, T.R. and LEWIS, J.B. (1979). Complex splicing patterns of RNAs from the early regions of

adenovirus 2. *J. Mol. Biol.* 134, 265-303.

CHOW, L., GELINAS, R., BROKER, T. and ROBERTS, R. (1977). An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNAs. *Cell* 1, 1-8.

CLADRAS, C., BHAT, B. and WOLD, W.S.M. (1985). Mapping the 5' ends, 3' ends, and splice sites of mRNAs from the early E3 transcription unit of adenovirus 5.

CLADRAS, C. and WOLD, W.S.M. (1985). DNA sequence of the early E3 transcription unit of adenovirus 5. *Virology* **140**, 28-43.

COHEN, S.N., CHANG, A.C.Y. and HSU, L. (1973). Non-chromosomal antibiotic resistance in bacteria: Genetic transformation of *E.coli* by R-factor DNA. *Proc. Natl. Acad. Sci.* 69, 2110-2114.

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

CORDEN, J., ENGELKING, M. and PEARSON, G.D. (1976). Chromatin-like organization of the adenovirus chromosome. *Proc. Natl. Acad. Sci.* 73, 401-404.

CORTES, P., BUCKBINDER, L., LEZA, M.A., RAK, N., HEARING, P., MERINO, A. and REINBERG, D. (1988). EIVF, a factor required for transcription of the adenovirus E4 promoter, binds to an element involved in E1A-dependent activation and cAMP induction. *Genes Dev.* 2, 975-990.

COUREY, A.J., HOLTZMAN, D.A., JACKSON, S.P. and TJIAN, R. (1989). Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. *Cell* 59, 827-836.

COUREY, A.J. and TJIAN, R. (1988). Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* 55, 887-898.

CRUZ, J.R., CACERES, P., CANO, F., FLORES, J., BARTLETT, A. and TORUN, B. (1990). Adenovirus types 40 and 41 and rotaviruses associated with diarrhea in children from Guatemala. *J. Clin. Microbiol.* 28, 1780-1784.

CUTT, J.S., WEBSTER, L.C., FRIEDMAN, D.J., SMITH, C.L., HUANG, W.-J., WU, F.Y.-H., ROSENBERG, M. and RICCIARDI, R.P. (1988). The 289-amino acid E1a protein of adenovirus binds zinc in a region that is important for transactivation. *Proc. Natl. Acad. Sci.* 85, 6450-6454.

CUTT, J.R., SHENK, T. and HEARING, P. (1987). Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. *J. Virol.* 61, 543-552.

DALES, S. (1978). Penetration of animal viruses into cells. In "Transport of macromolecules in cellular systems". Ed. Silverstein, S.C.,. Dahlem Konferenzen, Berlin.

DANDEKAR, T. and SIBBALD, P.R. (1990). *Trans*-splicing of pre-mRNA is predicted to occur in a wide range of organisms including vertebrates. *Nucl. Acids Res.* **18**, 4719-4725.

DANIELL, E. (1976). Genome structure of incomplete particles of adenovirus. *J. Virol.* **19**, 685-708.

DE CAPRIO, J.A., LUDLOW, J.W., FIGGE, J., SHEW, J.-Y., HUANG, C.-M., LEE, W.-H., MARSILIO, E., PAUCHA, E. and LIVINGSTON, D.M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**, 275-283.

DE JONG, J.C., WIGAND, R., KIDD, A.H., WADELL, G., KAPSENBERG, J.G., MUZERIE, A.G., WERMENBOL, A.G. and FIRTZLAFF, R.-G. (1983). Candidate adenoviruses 40 and 41: fastidious adenoviruses from human infant stool. *J. Med. Virol.* **11**, 215-231.

DE VRIES, E., VAN DRIEL, W., TROMP, M., VAN BOOM, J. and VAN DER VLIET, P.C. (1985). Adenovirus DNA replication *in vitro*: site-directed mutagenesis of the nuclear factor I binding site of the Ad2 origin. *Nucl. Acids Res.* **13**, 4935-4952.

DEFER, C., BELIN, M.-T., CAILLET-BOUDIN, M-L. and BOULANGER, P. (1990). Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. *J. Virol.* **64**, 3661-3673.

DERY, C.V., HERRMANN, C.H. and MATHEWS, M.B. (1987). Response of individual adenovirus promoters to the products of the E1A gene. *Oncogene* **2**, 15-23.

DESIDEIRO, S.V. and KELLY JR, T.J., (1981). Structure of the linkage between adenovirus DNA and 55K terminal protein. *J. Mol. Biol.* **145**, 319-337.

DEVAUX, C., ADRIAN, M., BERTHET-COLOMINAS, C., CUSACK, S. and JACROT, B. (1990). Structure of adenovirus fibre. I. Analysis of crystals of fibre from adenovirus serotypes 2 and 5 by electron microscopy and X-ray crystallography. *J. Mol. Biol.* 215, 567-588.

DEVAUX, C., BERTHET-COLOMINAS, C., TIMMINS, P.A., BOULANGER, P. and JACROT, B. (1984). Chrystal packing and stoichiometry of the fibre protein of adenovirus type 2. *J. Mol. Biol.* **174**, 729-737.

DEVAUX, C., ZULAUF, M., BOULANGER, P. and JACROT, B. (1982). Molecular weight of adenovirus serotype 2 capsomers: a new characterization. *J. Mol. Biol.* **156**, 927-939.

D'HALLUIN, J.C., ALLART, C., COUSIN, C., BOULANGER, P.A. and MARTIN, G. (1979). Adenovirus early function required for the protection of viral and cellular DNA. *J. Virol.* **32**, 61-71.

DIJKEMA, R., DEKKER, B.M.M. and VAN ORMONDT, H. (1982). Gene organization of the transforming region of adenovirus type 7 DNA. *Gene* **18**, 143-156.

DIJKEMA, R., DEKKER, B.M.M., VAN ORMONDT, H., DE WAARD, A., MAAT, J. and BOYER, H.W. (1980). Gene organization of the transforming region of weakly oncogenic adenovirus type 7: The E1a region. *Gene* **12**, 287-299.

DOERFLER, W. (1986) (ed.). Adenovirus DNA. Martinus Nijhoff, Boston.

DOWNEY, J.F., ROWE, D.T., BACCHETTI, S., GRAHAM, F.L. and BAYLEY, S.T. (1983). Mapping of a 14,000-dalton antigen to early region 4 of the human adenovirus 5 genome. *J. Virol.* **45**, 514-523.

DUERKSEN-HUGHES, P., WOLD, W.S.M. and GOODING, L.R. (1989). Adenovirus E1A renders infected cells sensitive to cytolysis by tumor necrosis factor. *J. Immunol.* **143**, 4193-4200.

DYNAN, W.S. and TJIAN, R. (1983). The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* **35**, 79-87.

DYSON, N., BUCHKOVICH, K., WHYTE, P. and HARLOW, E. (1989). The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and JC virus. *Cell* **58**, 249-255.

EDBAUER, C., LAMBERTI, C., TONG, J. and WILLIAMS, J. (1988). Adenovirus type 12 E1B 19 kilodalton protein is not required for oncogenic transformation in rats. *J. Virol.* **62**, 3265-3273.

EGAN, C., BAYLEY, S.T. and BRANTON, P.E. (1989). Binding of the RB1 protein to E1A products is required for adenovirus transformation. *Oncogene* **4**, 383-388.

EGAN, C., JELSMA, T.N., HOWE, J.A., BAYLEY, S.T., FERGUSON, B. and BRANTON, P.E. (1988). Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. *Mol. Cell. Biol.* **8**, 3955-3959.

ESTES, M.K., PALMER, E.L. and OBIJESKI, J.F. (1983). Rotaviruses: a review. *Curr. Top. Microbiol. Immunol.* **105**, 123-184.

EVANS, R.M., FRASER, N., ZIFF, E., WEBER, J., WILSON, M. and DARNELL, J.E. (1977). The initiation sites for RNA transcription in Ad2 DNA. *Cell* **12**, 733-739.

EVERETT, R.D. (1987). The regulation of transcription of viral and cellular genes by Herpes virus immediate early gene products. *Anticancer Research* 7, 589-604.

EVERITT, E., LUTTER, L. and PHILIPSON, L. (1975). Structural proteins of adenoviruses. XII. Location and neighbour relationship among proteins of adenovirion type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. *Virology* 67, 197-208.

EVERITT, E., SUNDQUIST, B., PETTERSSON, U. and PHILIPSON, L. (1973). Structural proteins of adenoviruses. X. Isolation and topography of low molecular weight antigens from the virion of adenovirus type 2. *Virology* 52, 130-147.

EZOE, H., LAI FATT, R.B. and MAK, S. (1981). Degredation of intracellular DNA in KB cells infected with *cyt* mutants of human adenovirus type 12. *J. Virol.* 40, 20-27.

FEINBERG, A.P. and VOGELSTEIN, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132, 6-13.

FLEWETT, T.H., BRYDEN, A.S., DAVIES, H.A. and MORRIS, C.A. (1975). Epidemic viral enteritis in a long-stay childrens ward. *Lancet* 1, 4-5.

FLÜGEL, R.M., BANNERT, H., SUHAI, S. and DARAI, G. (1985). The nucleotide sequence of the early region of the Tupaia adenovirus DNA corresponding to the oncogenic region E1b of human adenovirus 7. *Gene* 34, 73-80.

FREYER, G.A., KATOH, Y. and ROBERTS, R.J. (1984). Characterization of the major mRNAs from adenovirus 2 early region 4 by cDNA cloning and sequencing. *Nucl. Acids Res.* 12, 3503-3519.

FUHRMAN, S.A. ENGELKE, D.R. and GEIDUSCHEK, E.P. (1984). HeLa cells RNA polymerase III transcription factors. *J. Biol. Chem.* 259, 1934-1943.

FUKUI, Y., SAITO, I., SHIROKI, K. and SHIMOJO, H. (1984). Isolation of transformation-defective, replication-non-defective early region 1B mutants of adenovirus type 12. *J. Virol.* 49, 154-161.

FURCINITTI, P.S., VAN OOSTRUM, J. and BURNETT, R.M. (1989). Adenovirus polypeptide IX revealed as capsid cement by difference images from electron microscopy and crystallography. *EMBO J.* 18, 3563-3570.

GARCIA, J.A., WU, F. and GAYNOR, R.B. (1987). Upstream regulatory regions required to stabilize binding to the TATA sequence on an adenovirus early promoter. *Nucl. Acids Res.* 15, 8367-8385.

GARON, C.F., BERRY, K.W. and ROSE, J.A. (1972). A unique form of terminal redundancy in adenovirus DNA molecules. *Proc. Natl. Acad. Sci.* **69**, 2391-2395.

GARY, G.W. JR., HIERHOLZER, J.C. and BLACK, R.E. (1979). Characterization of noncultivable adenoviruses associated with diarrhoea in infants: a new subgroup of human adenoviruses. *J. Clin Microbiol.* **10**, 96-103.

GAYNOR, R.B., FELDMAN, L.T. and BERK, A.J. (1985). Transcription of class III genes activated by viral immediate early proteins. *Science* **230**, 447-450.

GELINAS, R.E. and ROBERTS, R.J. (1977). One predominant 5'-undecanucleotide in adenovirus 2 late messenger RNAs. *Cell* **11**, 533-544.

GENTZ, R., RAUSCHER, F.J.,III, ABATE, C. and CURRAN, T. (1989). Parallel association of *Fos* and *Jun* leucine zippers juxtaposes DNA binding domain. *Science* **243**, 1695-1699.

GHOSH-CHOUDHURY, G., HAJ-AHMAD, Y. and GRAHAM, F.L. (1987). Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. *EMBO J.* **6**, 1733-1739.

GILARDI, P. and PERRICAUDET, M. (1984). The E4 transcriptional unit of Ad2: far upstream sequences are required for its trnsactivation by E1A. *Nucl. Acids Res.* **12**, 7877-7888.

GILARDI, P. and PERRICAUDET, M. (1986). The E4 promoter of adenovirus type 2 contains an E1a-dependent *cis*-acting element. *Nucl. Acids Res.* **14**, 9035-9049.

GINGERAS, T.R., SCIAKY, D., GELINAS, R.E., BING-DONG, J., YEW, C.E., KELLY, M.M., BULLOCK, P.A., PARSONS, B.L., O'NEILL, K.E. and ROBERTS, R.J. (1982). Nucleotide sequences from the adenovirus-2 genome. *J. Biol. Chem.* **257**, 13,475-13,491.

GINSBERG, H.S. (1984) (Ed.). The viruses: The adenoviruses. Plenum Press, New York.

GINSBERG, H.S., PEREIRA, H.G., VALENTINE, R.C. and WILCOX, W.C. (1966). A proposed terminology for the adenovirus antigens and morphological subunits. *Virology* **28**, 782-783.

GLENN, G.M. and RICCIARDI, R.P. (1988). Detailed kinetics of adenovirus type 5 steady state transcripts during early infection. *Virus Research* **9**, 73-91.

GONZALES, G.A. and MONTMINY, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675-680.

GONZALES, G.A., YAMAMOTO, K.K., FISHER, W.H., KARR, D., MENZEL, P., BIGGS, W. III, VALE, W.W. and MONTMINY, M.R. (1989). A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature* **337**, 749-752.

GOODING, L.R., ELMORE, L.W., TOLLEFSON, A.E., BRADY, H.A. and WOLD, W.S.M. (1988). A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. *Cell* **53**, 341-346.

GOODING, L.R. and WOLD, W.S.M. (1990). Molecular mechanism by which adenoviruses counteract anti-viral immune defenses. *CRC Rev. Immunol.* **10**, 53-71.

GOUGH, J.A. and MURRAY, N.E. (1983). Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J. Mol. Biol.* **166**, 1-19.

GRÄBLE, M. and HEARING, P. (1990). Adenovirus type 5 packaging domain is composed of a repeated element that is functionally redundant. *J. Virol.* **64**, 2047-2056.

GRAHAM, F.L., SMILEY, J., RUSSEL, W.C. and NAIRN, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**, 59-72.

GRAND, R.J.A. (1987). The structure and functions of the adenovirus early region 1 proteins. *Biochem. J.* **241**, 25-38.

GRANT, A.G., DUKE, D. and HERMON-TAYLOR, J. (1979). Establishment and characterization of primary human pancreatic carcinoma in continuous cell culture and in nude mice. *Br. J. Cancer* **39**, 143-151.

GRASS, D.S., READ, D., LEWIS, E.D. and MANLEY, J.L. (1987). Cell- and promoter-specific activation of transcription by DNA replication. *Genes Dev.* **1**, 1065-1074.

GREEN, M., BRACKMANN, K.H., CARTAS, M.A. and MATSUO, T. (1982). Identification and purification of a protein encoded by the human adenovirus type 2 transforming region. *J. Virol.* **42**, 30-41.

GREEN, N.M., WRIGLEY, N.G., RUSSEL, W.C., MARTIN, S.R. and McLACHLAN, A.D. (1983). Evidence for a repeating cross-beta sheet structure in the adenovirus fibre. *EMBO J.* **2**, 1357-1365.

GRÜTTER, M. and FRANKLIN, R.M. (1974). Studies of the molecular weight of the adenovirus type 2 hexon and its subunit. *J. Mol. Biol.***89**, 163-178.

GUGGENHEIMER, R.A., STILLMANN, B.W., NAGATA, K., TAMANOI, F. and HURWITZ, J. (1984). DNA sequences required for the *in vitro* replication of adenovirus DNA. *Proc. Natl. Acad. Sci.* **81**, 3069-3073.

HAI, T., LIU, F., ALLERGRETTO, E.A., KARIN, M. and GREEN, M.R. (1988). A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1. *Genes Dev.* 2, 1216-1226.

HAI, T., LIU, F., COULOS, W.J. and GREEN, M.R. (1989). Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* 3, 2083-2090.

HALBERT, D.N., CUTT, J.R. and SHENK, T. (1985). Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J. Virol.* 56, 250-257.

HALBERT, D.N., SPECTOR, D.J. and RASKAS, H.J. (1979). *In vitro* translation products specified by the transforming region of adenovirus type 2. *J. Virol.* 31, 621-629.

HANAHAN, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166, 557-580.

HANAKA, S., NISHIGAKI, T., SHARP, P.A. and HANDA, H. (1987). Regulation of *in vitro* and *in vivo* transcription of early-region IV of adenovirus type 5 by multiple *cis*-acting elements. *Mol. Cell. Biol.* 7, 2578-2587.

HANDA, H., KINGSTON, R.E. and SHARP, P.A. (1983). Inhibition of adenovirus early region IV transcription *in vitro* by a purified DNA binding protein. *Nature* 302, 545-547.

HANSEN, M.F. and CAVENEE, W.K. (1988). Tumor suppressors: rescessive mutations that lead to cancer. *Cell* 53, 172-173.

HARDY, S., ENGEL, D.A. and SHENK, T. (1989). An adenovirus early region 4 gene product is required for induction of the infection specific form of cellular E2F activity. *Genes Dev.* 3, 1062-1074.

HARDY, S. and SHENK, T. (1988). Adenoviral control regions activated by E1a and cAMP response element bind to the same factor. *Proc. Natl. Acad. Sci.* 85, 4171-4175.

HARLOW, E., FRANZA, B., JR. and SCHLEY, C. (1985). Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. *J. Virol.* 55, 533-546.

HARLOW, E., WHYTE, P., FRANZA, B.R., JR., and SCHLEY, C. (1986). Association of adenovirus early-region 1A proteins with cellular polypeptides. *Mol. Cell. Biol.* 6, 1579-1589.

HARRIS, M.P.G. and HAY, R.T. (1988). DNA sequences required for the initiation of adenovirus type 4 DNA replication

in vitro. *J. Mol. Biol.* 201, 57-67.

HARRISON, T., GRAHAM, F. and WILLIAMS, J. (1977). Host range mutants of adenovirus type 5 defective for growth in HeLa cells. *Virology* 77, 319-329.

HASHIMOTO, S., SYMINGTON, J. and MATSUO, T. (1984). Cell-free translation of adenovirus 2 E1A- and E1B-specific mRNAs and evidence that E1A related polypeptides are produced from E1A-E1B overlapping mRNA. *J. Biol. Chem.* 259, 7016-7023.

HASSON, T.B., SOLOWAY, P.D., ORNELLES, D.A., DOERFLER, W. and SHENK, T. (1989). Adenovirus L1-52 and 55 kilodalton proteins are required for assembly of virions. *J. Virol.* 63, 3612-3621.

HAY, R.T., STOW, N.D. and McDOUGALL, I.M. (1984). Replication of adenovirus mini-chromosomes. *J. Mol. Biol.* 175, 493-510.

HAYES, B.W., TELLING, G.C., MYAT, M.M., WILLIAMS, J.F. and FLINT, S.J. (1990). The adenovirus L4 100-kilodalton protein is necessary for efficient translation of viral late mRNA species. *J. Virol.* 64, 2732-2742.

HEARING, P., SAMULSKI, R.J., WISHART, W.L. and SHENK, T. (1987). Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J. Virol.* 61, 2555-2558.

HEARING, P. and SHENK, T. (1983). The adenovirus type 5 E1A transcriptional control region contains a duplicated enhancer element. *Cell* 33, 695-703.

HEARING, P. and SHENK, T. (1986). The adenovirus type 5 enhancer contains two functionally distinct domains. One is specific for E1A and the other modulates transcription of all early units in cis. *Cell* 45, 229-236.

HEMSTRÖM, C., NORDQVIST, K., PETTERSSON, U. and VIRTANEN, A. (1988). Gene product of region E4 of adenovirus type 5 modulates accumulation of certain viral polypeptides. *J. Virol.* 62, 3258-3264.

HEN, R., BORELLI, E. and CHAMBON, P. (1985). Repression of the immunoglobulin heavy chain enhancer by the adenovirus-2 E1a products. *Science* 230, 1391-1394.

HEN, R., BORELLI, E., FROMENTAL, C., SASSON-CORSI, R. and CHAMBON, P. (1986). A mutated polyoma virus enhancer which is active in undifferentiated embryonal carcinoma cells is not repressed by adenovirus-2 E1A products. *Nature* 321, 249-251.

HERBST, R.S., HERMO, H. JR., FISHER, P.B. and BABISS, L.E. (1988). Regulation of adenovirus and cellular gene expression and of cellular transformation by the E1B-

encoded 175-aminoacid protein. *J. Virol.* 62, 4634-4643.

HERISSE, J., COURTOIS, G. and GALIBERT, F. (1980). Nucleotide sequence of the EcoRI D fragment of adenovirus 2 genome. *Nucl. Acids Res.* 8, 2173-2192.

HERISSE, J. and GALIBERT, F. (1981). Nucleotide sequence of the EcoRI E fragment of adenovirus 2 genome. *Nucl. Acids Res.* 9, 1229-1240.

HERRMANN, C.H., DERY, C.V. and MATHEWS, M.B. (1987). Transactivation og host and viral gene by the adenovirus E1B 19K tumor antigen. *Oncogene* 2, 25-35.

HERRMANN, C.H. and MATHEWS, M.B. (1989). The adenovirus E1B 19-kilodalton protein stimulates gene expression by increasing DNA levels. *Mol. Cell. Biol.* 9, 5412-5423.

HIERHOLZER, J.C., WIGAND, R. and DE JONG, J.C. (1988). Evaluation of human adenoviruses 38, 39, 40 and 41 as new serotypes. *Intervirol.* 29, 1-10.

HILLEMAN, M.R. and WERNER, J.R. (1954). Recovery of a new agent from patients with acute respiratory illness. *Proc. Soc. Exp. Biol. Med.* 85, 183-188.

HIRAI, S.-I., RYSECK, R.-P., MECHTA, F., BRAVO, R. and YANIV, M. (1989). Characterization of *junD:* a new member of the *jun* proto-oncogene family. *EMBO J.* 8, 1433-1439.

HIRT, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26, 365-369.

HOEFFLER, J.P., MEYER, T.E., YUN, Y., JAMESON, J.-L. and HABENER, J.F. (1988a). Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. *Science* 242, 1430-1433.

HOEFFLER, W.K., KOVELMAN, R. and ROEDER, R.G. (1988b). Activation of transcription factor IIIC by the adenovirus E1A protein. *Cell* 53, 907-920.

HOEFFLER, W.K. and ROEDER, R.G. (1985). Enhancement of RNA polymerase III transcription by the E1A gene product of adenovirus. *Cell* 41, 955-963.

HORNE, R.W., BRENNER, S., WATERSON, A.P. and WILDY, P. (1959). The icosahedral form of an adenovirus. *J. Mol. Biol.* 1, 84-86.

HORWITZ, M.S. (1990). Adenoviruses. In Virology. Ed. B.N. Fields, D.M. Knipe *et al.*, Raven Press Ltd. New York.

HOWE, J.A., MYMRYK, J.S., EGAN, C., BRANTON, P.E. and BAYLEY, S.T. (1990). Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. *Proc. Natl. Acad. Sci.* 87, 5883-5887.

HUANG, M. and HEARING, P. (1989a). The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. *Genes Dev.* 3, 1399-1410.

HUANG, M. and HEARING, P. (1989b). Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. *J. Virol.* 63, 2605-2615.

IMPERIALE, M.J., FELDMAN, L.T. and NEVINS, J.R. (1983). Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in *trans* and by a *cis*-acting adenovirus enhancer element. *Cell* 35, 127-136.

IMPERIALE, M.J., HART, R.P. and NEVINS, J.R. (1985). An enhancer-like element in the adenovirus E2 promoter contains sequences essential for uninduced and E1A-induced transcription. *Proc. Natl. Acad. Sci.* 82, 381-385.

IMPERIALE, M.J., KAO, H.-T., FELDMAN, L.T., NEVINS, J.R. and STRICKLAND, S. (1984). Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. *Mol. Cell. Biol.* 4, 867-874.

ISHINO, M., OHASHI, Y., EMOTO, T., SAWADA, Y. and FUJINAGA, K. (1988). Characterization of adenovirus type 40 E1 region. *Virology* 165, 95-102.

ISHINO, M., SAWADA, Y., YAEGASHI, T., DEMURA, M. and FUJINAGA, K. (1987). Nucleotide sequence of the adenovirus type 40 inverted terminal repeat: close relation to that of adenovirus type 5. *Virology* 156, 414-416.

JACOBSON, P.A., JOHANSSON, M.E. and WADELL, G. (1979). Identification of an enteric adenovirus by immunoelectroosmophoresis (IEOP) technique. *J. Med. Virol.* 3, 307-312.

JALINOT, P., WINTZERITH, M., GAIRE, M., HAUSS, C., EGLY, J.M. and KEDINGER, C. (1988). Purification and functional characterization of a cellular transcription factor that binds to an enhancer element within the adenovirus early EIIa promoter. *Proc. Natl. Acad. Sci.* 85, 2484-2488.

JANSEN-DURR, P., BOEUF, H. and KEDINGER, C. (1988). Replication-induced stimulation of the major late promoter of adenovirus is correlated to the binding of a factor to sequences in the first intron. *Nucl. Acids Res.* 16, 3771-3786.

JANSEN-DURR, P., BOEUF, H. and KEDINGER, C. (1989a). Cooperative binding of two E2F molecules to an E1A responsive promoter is triggered by the adenovirus E1A, but

not by a cellular E1A-like activity. *EMBO J.* 8, 3365-3370.

JANSEN-DURR, P., MONDESERT, G., and KEDINGER, C. (1989b). Replication-dependent activation of the adenovirus major late promoter is mediated by the increased binding of a transcription factor to sequences in the first intron. *J. Virol.* 63, 5124-5132.

JANSEN-DURR, P., WINTZERITH, M., REIMUND, B., HAUSS, C. and KEDINGER, C. (1990). Two distinct cellular proteins interact with the E1A-responsive element of an adenovirus early promoter. *J. Virol.* 64, 2384-2387.

JELSMA, T.N., HOWE, J.A., EVELEGH, C.M., CUNNIFF, N.F., SKIADOPOULOS, M.H., FLOROFF, M.R., DENMAN, J.E. and BAYLEY, S.T. (1988). Use of deletion and point mutants spanning the coding region of the adenovirus 5 E1A gene to define a domain that is essential for transcriptional activation. *Virology* 163, 494-502.

JELSMA, T.N., HOWE, J.A., MYMRYK, J.S., EVELEGH, C.M., CUNNIFF, N.F. and BAYLEY, S.T. (1989). Sequences in E1A proteins of human adenovirus 5 required for cell transformation, repression of a transcriptional enhancer, and induction of proliferating cell nuclear antigen. *Virology* 171, 120-130.

JOHANSSON, M.E., UHNOO, I., KIDD, A.H., MADELEY, C.R. and WADELL, G. (1980). Direct identification of enteric adenovirus, a candidate new serotype associated with infantile gastroenteritis. *J. Clin. Microbiol.* 12, 95-100.

JOHANSSON, M.E., UHNOO, I., SVENSSON, L., PETTERSSON, C.A. and WADELL, G. (1985). Enzyme-linked immunosorbent assay for detection of enteric adenovirus 41. *J. Med Virol.* 17, 19-27.

JONES, K.A., KADONAGA, J.T., ROSENFELD, P.J., KELLY, T.J. and TJIAN, R. (1987). A cellular DNA binding protein that activates eucaryotic transcription and DNA replication. *Cell* 48, 79-89.

JONES, N.C., RIGBY, P.W.J. and ZIFF, E.B. (1988). Trans-acting protein factors and the regulation of eucaryotic transcription: lessons from studies on DNA tumor viruses. *Genes Dev.* 2, 267-281.

JONES, N. and SHENK, T. (1979a). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* 17, 683-689.

JONES, N. and SHENK, T. (1979b). An adenovirus type 5 early gene function regulates expression of other early viral genes. *Proc. Natl. Acad. Sci.* 76, 3665-3669.

KADDURAH-DAOUK, R., LILLIE, J.W., DAOUK, G.H., GREEN, M.R.,

KINGSTON, R. and SCHIMMEL, P. (1990). Induction of a cellular enzyme for energy metabolism by transforming domains of adenovirus E1a. *Mol. Cell. Biol.* **10**, 1476-1483.

KADONAGA, J.T., CARNER, K.R., MASIARZ, F.R. and TJIAN, R. (1987). Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* 51, 1079-1090.

KADONAGA, J.T., COUREY, A.J., LADIKA, J. and TJIAN, R. (1988). Distinct regions of Sp1 modulate DNA binding and transcriptional activation. *Science*, **242**, 1566-1570.

KADONAGA, J.T., JONES, K.A. and TJIAN, R. (1986). Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem. Sci.* 11, 20-23.

KAO, C.C., YEW, P.R. and BERK, A.J. (1990). Domains required for *in vitro* association between the cellular p53 and the adenovirus 2 E1B 55K proteins. *Virology* 179, 806-814.

KATZE, M.G., DeCORATO, D., SAFER, B., GALABRU, J. and HOVANESSIAN, A.G. (1987). Adenovirus VAI RNA complexes with the 68,000 Mr protein kinase to regulate its autophosphorylation and activity. *EMBO J.* 6, 689-697.

KAWASAKI, E.S. and WANG, A.M. (1989). Detection of gene expression. In PCR Technology: Principles and applications for DNA amplification. Ed. H.A. Erlich. Stockton Press.

KENNY, M.K. and HURWITZ, J. (1988). Initiation of adenovirus DNA replication. II. Structural requirements using synthetic oligonucleotide adenovirus templates. *J. Biol. Chem.* 263, 9809-9817.

KIDD, A.H. and ERASMUS, M.J. (1989). Sequence characterization of the adenovirus 40 fibre gene. *Virology* **172**, 134-144.

KIDD, A.H. ERASMUS, M.J. and TIEMESSEN, C.T. (1990). Fibre sequence heterogeneity in subgroup F adenoviruses. *Virology* 179, 139-150.

KIDD, A.H. and MADELEY, C.R. (1981). *In vitro* growth of some fastidious adenoviruses from stool specimens. *J. Clin. Pathol.* 34, 213-216.

KIDD, A.H., ROSENBLATT, A., BESSELAAR, T.G., ERASMUS, M.J., TIEMESSEN, C.T., BERKOWITZ, F.E. and SCHOUB, B.D. (1986). Characterization of rotaviruses and subgroup F adenoviruses from acute summer gastroenteritis in South Afrika. *J. Med. Virol.* 18, 159-168.

KIMELMAN, D., MILLER, J.S., PORTER, J.D. and ROBERTS, B.E. (1985). E1A regions of the human adenoviruses and of the

highly oncogenic simian adenovirus 7 are closely related. *J. Virol.* 53, 399-409.

KIMURA, T., SAWADA, Y., SHINAWAWA, M., SHIMIZU, Y., SHIROKI, K., SHIMOJO, H., SUGISAKI, H., TAKANAMI, M., UEMIZU, Y. and FUJINAGA, K. (1981). Nucleotide sequence of the transforming early region E1b of adenovirus type 12 DNA; structure and gene organization, and comparison with those of adenovirus type 5 DNA. *Nucl. Acid Res.* 9, 6571-6589.

KINLOCH, R., MACKAY, N. and MAUTNER, V. (1984). Adenovirus hexon. Sequence comparison of subgroup C serotypes 2 and 5. *J. Biol. Chem.* 259, 6431-6436.

KITCHINGMAN, G.R. and WESTPHAL, H. (1980). The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. *J. Mol. Biol.* 137, 23-48.

KLEIN, G. (1988). The approaching era of the tumor suppressor genes. *Science* 238, 1539-1545.

KLESSIG, D.F. (1977). Isolation of a variant of human adenovirus serotype 2 that multiplies efficiently of monkey cells. *J. Virol.* 21, 1243-1246.

KLESSIG, D.F. and CHOW, L.T. (1980). Incomplete splicing and deficient accumulation of the fiber messenger RNA in monkey cells infected by human adenovirus type 2. *J. Mol. Biol.* 139, 221-242.

KLIEWER, S., GARCIA, J., PEARSON, L., SOULTANAKIS, E., DASGUPTA, A. and GAYNOR, R.B. (1989). Multiple transcriptional regulatory domains in the human immunodeficiency virus type 1 long terminal repeat are involved in basal and E1A/E1B induced promoter activity. *J. Virol.* 63, 4616-4625.

KLIMKAIT, T. and DOERFLER, W. (1985). Adenovirus types 2 and 5 functions elicit replication and late expression of adenovirus type 12 DNA in hamster cells. *J. Virol.* 55, 466-474.

KLIMKAIT, T. and DOERFLER, W. (1987). E1B functions of type C adenoviruses play a role in the complementation of blocked adenovirus type 12 DNA replication and late gene transcription. *Virology* 161, 109-120.

KORNUC, M., KLIEWER, S., GARCIA, J., HARRICH, D., LI, C. and GAYNOR, R. (1990). Adenovirus early region 3 promoter regulation by E1A/E1B is independent of alterations in DNA binding and gene activation of CREB/ATF and AP1. *J. Virol.* 64, 2004-2013.

KOTLOFF, K.L., LOSONSKY, G.A., MORRIS, J.G. JR., WASSERMAN, S.S., SINGH-NAZ, N. and LEVINE, M.M. (1989). Enteric adenovirus infection and childhood diarrhea: an epidemiologic study in three clinical settings. *Pediatrics*

84, 219-225.

KOVESDI, I., REICHEL, R. and NEVINS, J.R. (1987). Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. *Proc. Natl. Acad. Sci.* **84**, 2180-2184.

KOZAK, M. (1981). Mechanism of mRNA recognition by eucaryotic ribosomes during initiation of protein synthesis. *Curr. Top. Microbiol. Immunol.* 93, 81-123.

KUPPUSWAMY, M.N. and CHINNADURAI, G. (1987). Relationship between the transforming and transcriptional regulatory functions of adenovirus 2 E1a oncogene. *Virology* **159**, 31-38.

LAI FATT, R.B. and MAK, S. (1982). Mapping of an adenovirus function involved in the inhibition of DNA degradation. *J. Virol.* **42**, 969-977.

LAIRD, P.W. (1989). *Trans* splicing in trypanosomes - archaism or adaption. *Trends Genet.* 5, 204-208.

LALLY, C., DÖRPER, T., GRÖGER,W., ANTOINE, G. and WINNACKER, E.-L. (1984). A size analysis of the adenovirus replicon. *EMBO J.* 3,333-337.

LANDSCHULZ, W.H., JOHNSON, P.F. and McKNIGHT, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240**, 1759-1764.

LARSEN, P.L. and TIBBETTS, C. (1987). Adenovirus E1A gene autorepression: Revertants of an E1A promoter mutations encode altered E1A proteins. *Proc. Natl. Acad. Sci.* **84**, 8185-8189.

LASSAM, N.J., BAYLEY, S.T. and GRAHAM, F.L. (1978). Synthesis of DNA, late polypeptides, and infectious virus by host range mutants of adenovirus 5 in nonpermissive cells. *Virology* 87, 463-467.

LASSAR, A.B., MARTIN, P.L. and ROEDER, R.G. (1983). Transcription of class III genes: formation of preinitiation complex. *Science* 222, 740-748.

LAVER, W.G., YOUNGHUSBAND, H.B. and WRIGLEY, N.G. (1971). Purification and properties of chick-embryo-lethal orphan virus (an avian adenovirus). *Virology* **45**, 598-614.

LEE, K.A.W., FINK, J.S., GOODMAN, R.H. and GREEN, M.R. (1989). Distinguishable promoter elements are involved in transcriptional activation by E1A and cyclic AMP. *Mol. Cell. Biol.* 9, 4390-4397.

LEE, K.A.W. and GREEN, M.R. (1987). A cellular transcription factor E4F1 interacts with an E1a-inducible

enhancer and mediates constitutive enhancer function *in vitro*. *EMBO J.* 6, 1345-1353.

LEE, K.A.W., HAI, T.-S., SIVARAMAN, L., THIMMAPPAYA, B., HURST, H.C., JONES, N.C. and GREEN, M.R. (1987a). A cellular protein activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. *Proc. Natl. Acad. Sci.* 84, 8355-8359.

LEE, W., HASLINGER, A., KARIN, M. and TJIAN, R. (1987b). Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature* 325, 368-372.

LEE, W., MITCHELL, P. and TJIAN, R. (1987c). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* 49, 741-752.

LEITE, J.P.G., NIEL, C. and D'HALLUIN, J.C. (1986). Expression of the chloramphenicol acetyl transferase gene in human cells under the control of early adenovirus subgroup C promoters: effect of E1A gene products from other subgroups on gene expression. *Gene* 41, 207-215.

LEITE, J.P., PEREIRA, H.G., AZEREDO, R.S. and SCHATZMAYR, H.G. (1985). Adenoviruses in faeces of children with acute gastroenteritis in Rio de Janeiro, Brazil. *J. Med. Virol.* 15, 203-209.

LEONG, K., BRUNET, L. and BERK, A.J. (1988). Factors responsible for the higher transcriptional activity of extracts of adenovirus-infected cells fractionate with the TATA box transcription factor. *Mol. Cell. Biol.* 8, 1765-1774.

LEPPARD, K.N. and SHENK, T. (1989). The adenovirus E1B 55kd protein influences mRNA transport via an intranuclear effect on RNA metabolism. *EMBO* 8, 2329-2336.

LERNER, M.R., BOYLE, J.A., MOUNT, S.M., WOLIN, S.L. and STEITZ, J.A. (1980). Are snRNPs involved in splicing? *Nature* 283, 220-224.

LEVINE, A.J. (1990). The p53 protein and its interactions with the oncogene product of the small DNA tumor viruses. *Virology* 177, 419-426.

LEVINE, A.J. and GINSBERG, H.S. (1967). Mechanism by which fibre antigen inhibits multiplication of type 5 adenovirus. *J. Virol.* 1, 747-757.

LEVINE, M. and MANLEY, J.L. (1989). Transcriptional repression of eucaryotic promoters. *Cell* 59, 405-408.

LEWIN, B. (1990). Commitment and activation at PolII promoters: a tail of protein-protein interactions. *Cell* 61, 1161-1164.

LEWIS, E.D. and MANLEY, J.L. (1985). Control of adenovirus late promoter expression in two human cell lines. *Mol. Cell. Biol.* **5**, 2433-2442.

LEWIS, J.B. and ANDERSON, C.W. (1987). Identification of adenovirus type 2 early region 1B proteins that share the same amino terminus as do the 495R and 155R proteins. *J. Virol.* **61**, 3879-3888.

LEWIS, J.B. and MATHEWS, M.B. (1980). Control of adenovirus 2 early gene expression: a class of immediate early products. *Cell* **21**, 303-313.

LEZA, M.A. and HEARING, P. (1988). Cellular transcription factor binds to adenovirus early region promoters and to a cyclic AMP response element. *J. Virol.* **62**, 3003-3013.

LEZA, M.A. and HEARING, P. (1989). Independent cyclic AMP and E1A induction of adenovirus early region 4 expression. *J. Virol.* **63**, 3057-3064.

LIAUTARD, J.-P., SRI-WIDADA, J., BRUNEL, C. and JEANTEUR, P. (1982). Structural organization of ribonucleo-proteins containing small nuclear RNAs from Hela cells. *J. Mol. Biol.* **162**, 623-643.

LILLIE, J.W. and GREEN, M.R. (1989). Transcription activation by the adenovirus E1A protein. *Nature* **338**, 39-44.

LILLIE, J.W., GREEN, M. and GREEN, M.R. (1986). An adenovirus E1a protein region required for transformation and transcriptional repression. *Cell* **46**, 1043-1051.

LILLIE, J.W., LOEWENSTEIN, P.M., GREEN, M.R. and GREEN, M. (1987). Functional domains of adenovirus type 5 E1A proteins. *Cell* **50**, 1091-1100.

LIN, Y.S. and GREEN, M.R. (1988). Interaction of a common transcription factor, ATF, with regulatory elements in both E1a- and cyclic AMP-inducible promoters. *Proc. Natl. Acad. Sci.* **85**, 3396-3400.

LIU, F. and GREEN, M.R. (1990). A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1A protein. *Cell* **61**, 1217-1224.

LOEKEN, M.R. and BRADY, J. (1989). The adenovirus EIIA enhancer. Analysis of regulatory sequences and changes in binding activity of ATF and EIIF following adenovirus infection. *J. Biol. Chem.* **264**, 6572-6579.

LUCHER, L.A., BRACKMANN, K.H., SYMINGTON, J.S. and GREEN, M. (1984). Antibody directed to a synthetic peptide encoding the NH2-terminal 16 amino acids of the adenovirus type 2 E1B-53K tumor antigen recognizes the E1B-20K tumor antigen. *Virology* **132**, 217-221.

LUDLOW, J.W., DE CAPRIO, J.A., HUANG, C.-M., LEE, W.-H., PAUCHA, E., and LIVINGSTON, D.M. (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell* **56**, 57-65.

MAK, S. and MAK, I. (1986). Oncogenic transformation by human adenoviruses and their DNA. In: Adenovirus DNA. Ed. Doerfler, W. Boston, Martinus Nijhoff.

MAK, I. and MAK, S. (1990). Separate regions of an adenovirus E1B protein critical for different biological functions. *Virology* **176**, 553-562.

MALETTE, P., YEE, S.-P. and BRANTON, P.E. (1983). Studies on the phosphorylation of the 58,000 dalton early region 1B protein of human adenovirus type 5. *J. Gen. Virol.* **64**, 1069-1078.

MANOHAR, C.F., KRATOCHVIL, J. and THIMMAPAYA, B. (1990). The adenovirus EII early promoter has multiple E1A-sensitive elements, two of which funtion cooperatively in basal and virus-induced transcription. *J. Virol.* **64**, 2457-2466.

MARINUS, M.G. (1973). Location of DNA methylation genes on the *E. coli* K12 genetic map. *Mol. Gen. Genet.* **127**, 47-55.

MATSUI, T., MURAYAMA, M. and MITA, T. (1986). Adenovirus 2 peptide IX gene is expressed only on replicated molecules. *Mol. Cell. Biol.* 6, 4149-4154.

MATTAJ, I.W. and DE ROBERTIS, E.M. (1985). Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. *Cell* 40, 111-118.

MAUTNER, V., MACKAY, N. and MORRIS, K. (1990). Enteric adenovirus type 40: expression of E1B mRNA and proteins in permissive and non-permissive cells. *Virology* **179**, 129-138.

MAUTNER, V., MACKAY, N. and STEINTHORSDOTTIR, V. (1989). Complementation of enteric adenovirus type 40 for lytic growth in tissue culture by E1B 55K function of adenovirus types 5 and 12. *Virology* **171**, 619-622.

McGEOCH, D.J., DOLAN, A., DONALD, S. and BRAUER, D.H.K. (1986). Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. *Nucl. Ac. Res.* 14, 1727-1764.

McGLADE, C.J., TREMBLAY, M.L. and BRANTON, P.E. (1989). Mapping of a phosphorylation site in the 176R (19KDa) early region 1B protein of human adenovirus type 5. *Virology* **168**, 119-127.

McGLADE, C.J., TREMBLAY, M.L., YEE, S.-P., ROSS, R. and BRANTON, P.E. (1987). Acylation of the 176R (19 kilodalton) early region 1B protein of human adenovirus type 5. *J. Virol.* **61**, 327-3234.

MELLITS, K.H., KOSTURA, M. and MATHEWS, M.B. (1990). Interaction of adenovirus VA RNA1 with the protein kinase DAI: nonequivalence of binding and function. *Cell* **61**, 843-852.

MESSING, J. and VIEIRA, J. (1982). A new pair of M13 vectors for selecting either strand of double digest restriction fragments. *Gene* **19**, 269-276.

MILLER, J.S., RICCIARDI, R.P., ROBERTS, B.E., PETERSON, B.M. and MATHEWS, M.B. (1980). Arrangement of messenger RNAs and protein coding sequences in the major late transcription unit of adenovirus 2. *J. Mol. Biol.* **142**, 455-488.

MITCHISON, H.M., GRAND, R.J.A., BYRD, P.J., JOHNSON, G.D., PARTON, A. and GALLIMORE, P.H. (1990). The expression of the adenovirus 12 early region 1B 19K protein using a recombinant simian virus 40 system. *J. Gen. Virol.* **71**, 1713-1722.

MOFFETT, R.B. and WEBB, T.E. (1983). Characterization of a messenger RNA transport protein. *Biochem. Biophys. Acta* **740**, 231-242.

MONTELL, C., COURTOIS, G., ENG, C. and BERK, A. (1984a). Complete transformation by adenovirus 2 requires both E1A proteins. *Cell* **36**, 951-961.

MONTELL, C., FISHER, E.F., CARUTHERS, M.H. and BERK, A.J. (1982). Resolving the functions of overlapping viral genes by site-specific mutagenesis of a mRNA splice site. *Nature* **295**, 380-384.

MONTELL, C., FISHER, E.F., CARUTHERS, M.H. and BERK, A.J. (1984b). Control of adenovirus E1B mRNA synthesis by a shift in the activities of RNA splice sites. *Mol. Cell. Biol.* **4**, 966-972.

MORAN, E., GRODZICKER, T., ROBERTS, R.J., MATHEWS, M.B. and ZERLER, B. (1986a). Lytic and transforming functions of individual products of the adenovirus E1A gene. *J. Virol.* **57**, 765-775.

MORAN, E. and MATHEWS, M.B. (1987). Multiple functional domains in the adenovirus E1A gene. *Cell* **48**, 177-178.

MORAN, E., ZERLER, B., HARRISON, T.M. and MATHEWS, M.B. (1986b). Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. *Mol. Cell. Biol.* **6**, 3470-3480.

MORIN, N., DELSERT, C. and KLESSIG, D.F. (1989). Mutations that affect phosphorylation of the adenovirus DNA-binding protein alter its ability to enhance its own synthesis. *J. Virol.* **63**, 5228-5237.

MOUNT, S.M. (1982). A catalogue of splice junction sequences. *Nucl. Acids Res.* **10**, 459.

MOYNE, G., PICHARD, E. and BERNHARD, W. (1978). Localization of simian adenovirus 7 (SA 7) transcription and replication in lytic infection. An ultracytochemical and autoradiographical study. *J. Gen. Virol.* **40**, 77-92.

MUCHARDT, C., LI, C., KORNUC, M. and GAYNOR, R. (1990). CREB regulation of cellular cyclic AMP-responsive and adenovirus early promoters. *J. Virol.* **64**, 4296-4305.

MULLIS, K.B. and FALOONA, F.A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. *Meth. Enzymol.* **155**, 335-350.

MULLIS, K., FALOONA, F., SCHARF, S., SAIKI, R., HORN, G. and ERLICH, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* **51**, 263-273.

MURPHY, W.J., WATKINS, K.P. and AGABIAN, N. (1986). Identification of a novel Y branch structure as an intermediate in trypanosome mRNA processing: evidence for *trans* splicing. *Cell* **47**, 517-525.

NAGATA, K., GUGGENHEIMER, R.A. and HURWITZ, J. (1983). Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. *Proc. Natl. Acad. Sci.* **80**, 6177-6181.

NEILL, S.D., HEMSTRÖM, C., VIRTANEN, A. and NEVINS, J.R. (1990). An adenovirus E4 gene product transactivates E2 transcription and stimulates stable E2F binding through a direct association with E2F. *Proc. Natl. Acad. Sci.* **87**, 2008-2012.

NERMUT, M.V. (1979). Structural elements in adenovirus cores. Evidence for a core shell and linear structures in 'relaxed cores'. *Arch. Virol.* **62**, 101-116.

NERMUT, M.V. (1980). The architecture of adenoviruses: Recent views and problems: Brief review. *Arch. Virol.* **64**, 175-196.

NEVINS, J.R. (1981). Mechanism of activation of early viral transcription by the adenovirus E1A gene product. *Cell* **26**, 213-220.

NEVINS, J.R. (1982). Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. *Cell* **29**, 913-919.

NEVINS, J.R. (1989). Mechanisms of viral-mediated trans-activation of transcription. *Adv. Vir. Res.* **37**, 35-83.

NEVINS, J.R., GINSBERG, H.S., BLANCHARD, J.-M., WILSON, M.C. and DARNELL, J.E., JR. (1979). Regulation of the primary expression of the early adenovirus transcription units. *J. Virol.* 32, 727-733.

NEVINS, J.R. and WILSON, M.J. (1981). Regulation of adenovirus-2 gene expression at the level of the transcriptional termination and RNA processing. *Nature* **290**, 113-118.

NEVINS, J.R. and WINKLER, J.J. (1980). Regulation of early adenovirus transcription: a protein product of early region 2 specifically represses region 4 transcription. *Proc. Natl. Acad. Sci.* 77, 1893-1897.

NEWCOMBE, W.W., BORING, J.W. and BROWN, J.C. (1984). Ion etching of human adenovirus 2: structure of the core. *J. Virol.* **51**, 52-56.

NICOLAS, J.C., SARNOW, P., GIRARD, M. and LEVINE, A.J. (1983). Host range, temperature-conditional mutants in the adenovirus DNA-binding protein are defective in the assembly of infectious virus. *Virology* **126**, 228-239.

NORRBY, E. (1968). Biological significance of structural adenovirus components. *Curr. Top. Microbiol. Immunol.* **43**, 1-43.

NORRBY, E. (1969). The relationship between the soluble antigens and the virion of adenovirus type 3. IV. Immunological complexity of soluble components. *Virology* **37**, 565-576.

NORRBY, E. and WADELL, G. (1969). Immunological relationship between hexons of certain human adenoviruses. *J. Virol.* 4, 663-670.

O'MALLEY, R.P., DUNCAN, R.F., HERSHEY, J.B.W. and MATHEWS, M.B. (1989). Modification of protein synthesis initiation factors and the shut-off of the host protein synthesis in adenovirus-infected cells. *Virology* **168**, 112-118.

OOSTEROM-DRAGON, E.A. and ANDERSON, C.W. (1983). Polypeptide structure and encoding location of the serotype 2 adenovirus late, non structural 33K protein. *J. Virol.* **45**, 251-263.

OOSTEROM-DRAGON, E.A. and GINSBERG, H.S. (1981). Characterization of two temperature-sensitive mutants of type 5 adenovirus with mutations in the 100,000-dalton protein gene. *J. Virol.* **40**, 491-500.

ORNELLES, D.A. and SHENK, T. (1991). Localization of the adenovirus early region 1B 55-kilodalton protein during

lytic infection: association with nuclear viral inclusions requires the erly region 4 34-kilodalton protein. *J. Virol.* 65, 424-439.

OSBORNE, T.F., ARVIDSON, D.N., TYAU, E.S., DUNSWORTH-BROWNE, M. and BERK, A.J. (1984). Transcription control region within the protein-coding portion of adenovirus E1A genes. *Mol. Cell. Biol.* 4, 1293-1305.

PARKS, C.L., BANERJEE, S. and SPECTOR, D.J. (1988). Organization of the transcriptional control region of the E1B gene of adenovirus type 5. *J. Virol.* 62, 54-67.

PARKS, C.L. and SPECTOR, D.J. (1990). *cis*-Dominant defect in activation of adenovirus type 5 E1B early RNA synthesis. *J. Virol.* 64, 2780-2787.

PASTAN, I., SETH, P., FITZGERALD, D. and WILLINGHAM, M. (1986). Adenovirus entry into cells: Some new observations on an old problem. In "Consepts in viral pathogenesis II" (A.L. Notkins and M.B.A. Oldstone eds.). 141-146 Springer-Verlag, New York.

PEI, R. and BERK, A.J. (1989). Multiple transcription factor binding sites mediate adenovirus E1A transactivation. *J. Virol.* 63, 3499-3506.

PERRICAUDET, M. AKUSJÄRVI, G., VIRTANEN, A. and PETTERSSON, U. (1979). Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. *Nature* 281, 694-696.

PERRICAUDET, M., LE MOULLEC, J.M. and PETTERSSON, U. (1980a). Predicted structure of two adenovirus tumor antigens. *Proc. Natl. Acad. Sci.* 77, 3778-3782.

PERSSON, H., MATHISEN, B., PHILIPSON, L. and PETTERSSON, U. (1979). A maturation protein in adenovirus morphogenesis. *Virology* 93, 198-208.

PERSSON, H., ÖBERG, B. and PHILIPSON, L. (1978). Purification and characterization of an early protein (E14K) from adenovirus type 2-infected cells. *J. Virol.* 28, 119-139.

PETTERSSON, U. (1984). Structural and nonstructural adenovirus proteins. In: The viruses: The adenoviruses. Ed. H.S. Ginsberg, Plenum Press, New York.

PETTERSSON, U. and HOGLUND, S. (1969). Structural proteins and adenovirus type 2 penton antigen. *Virology* 39, 90-106.

PETTERSSON, U., PHILIPSON, L. and HOGLUND, S. (1968). Structural proteins of adenoviruses. II. Purification and characterization of adenovirus type 2 fiber antigen. *Virology* 35, 204-215.

PHILIPSON, L. (1967). Attachment and eclipse of adenovirus. *J. Virol.* 1, 868-875.

PHILIPSON, L. (1984). Structure and assembly of adenoviruses. In: The viruses: The adenoviruses. Ed. H.S. Ginsberg, Plenum Press, New York.

PHILIPSON, L., LONBERG-HOLM, K. and PETTERSSON, U. (1968). Virus-receptor interaction in an adenovirus system. *J. Virol.* 2, 1064-1075.

PIENIAZEK, D., PIENIAZEK, N.J., MACEJAK, D., COWARD, J., RAYFIELD, M. and LUFTIG, R.B. (1990a). Differential growth of human enteric adenovirus 41 (TAK) in continuous cell lines. *Virology* 174, 239-249.

PIENIAZEK, D., PIENIAZEK, N.J., MACEJAK, D. and LUFTIG, R.B. (1990b). Enteric adenovirus 41 (Tak) requires low serum for growth in human primary cells. *Virology* 178, 72-80.

PIENIAZEK, N.J., SLEMENDA, S.B., PIENIAZEK, D., VELARDE, J. JR. and LUFTIG, R.B. (1989a). Sequence of the human enteric adenovirus type 41 Tak fibre protein gene. *Nucl. Acids Res.* 17, 9474.

PIENIAZEK, N.J., SLEMENDA, S.B., PIENIAZEK, D., VELARDE, J., JR. and LUFTIG, R.B. (1990). Human enteric adenovirus type 41 (Tak) contains a second fibre protein gene. *Nucl. Acids Res.* 18, 1901.

PIENIEZEK, N.J., VELARDE, J.JR., PIENIAZEK, D. and LUFTIG, R.B. (1989b). Nucleotide sequence of human enteric adenovirus type 41 hexon-associated protein VIII precursor (pVIII) including the early region E3 promoter. *Nucl. Acids Res.* 17, 5398.

PILDER, S., LOGAN, J. and SHENK, T. (1984). Deletion of the gene encoding the adenovirus 5 early region 1B 21,000 molecular weight polypeptide leads to degradation of viral and host cell DNA. *J. Virol.* 52, 664-671.

PILDER, S., MOORE, M., LOGAN, J. and SHENK, T. (1986). The adenovirus E1B 55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol. Cell. Biol.* 6, 470-476.

PUGH, B.F. and TJIAN, R. (1990). Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* 61, 1187-1197.

RAMOS-ALVAREZ, M. and SABIN, A.B. (1958). Enteropathogenic viruses and bacteria - role in summer diarrheal diseases of infancy and early childhood. *JAMA* 167, 147-156.

RAUSCHER, F.J.,III, VOULALAS, P.J., FRANZA, B.R., JR., and CURRAN, T. (1988). *Fos* and *Jun* bind cooperatively to the AP-1 site: reconstitution *in vitro*. *Genes Dev.* 2, 1687-1699.

RAWLE, F.C., TOLLEFSON, A.E., WOLD, W.S.M. and GOODING, L.R. (1989). Mouse anti-adenovirus cytotoxic T lymphocytes. Inhibition of lysis by E3 gp19K but not E3 14.7K. *J. Immunol.* **143**, 2031-2037.

RAWLINS, D.R., ROSENFELD, P.J., WIDES, R.J., CHALLBERG, M.D. and KELLY JR., T.J. (1984). Structure and function of the adenovirus origin of replication. *Cell* **37**, 309-319.

RAYCHAUDHURI, P., BAGCHI, S., NEILL, S.D. and NEVINS, J.R. (1990). Activation of the E2F transcription factor in adenovirus-infected cells involves E1A-dependent stimulation of DNA-binding activity and induction of cooperative binding mediated by an E4 gene product. *J. Virol.* **64**, 2702-2710.

RAYCHAUDHURI, P., BAGCHI, S. and NEVINS, J.R. (1989). DNA-binding activity of the adenovirus-induced E4F transcription factor is regulated by phosphorylation. *Genes Dev.* **3**, 620-627.

RAYCHAUDHURI, P., ROONEY, R. and NEVINS, J.R. (1987). Identification of an E1A-inducible cellular factor that interacts with regulatory sequences within the adenovirus E4 promoter. *EMBO J.* **6**, 4073-4081.

REICHEL, R., KOVESDI, I. and NEVINS, J.R. (1988). Activation of a pre-existing cellular factor as a basis for adenovirus E1A-mediated transcriptional control. *Proc. Natl. Acad. Sci.* **85**, 387-390.

REICHEL, R., NEILL, S.D., KOVESDI, I., SIMON, M.C., RAYCHAUDHURI, R. and NEVINS, J.R. (1989). The adenovirus E4 gene, in addition to the E1A gene, is important for transactivation of E2 transcription and for E2F activation. *J. Virol.* **63**, 3643-3650.

REKOSH, D.M.K., RUSSELL, W.C. and BELLET, A.J.D. (1977). Identification of a protein linked to the ends of adenovirus DNA. *Cell* **11**, 283-295.

RETTER, M., MIDDLETON, P.J., TAM, J.S. and PETRIC, M. (1979). Enteric adenoviruses: detection, replication and significance. *J. Clin. Microbiol.* **10**, 574-578.

RICCIARDI, R.P., JONES, R.L., CEPKO, C.L., SHARP, P.A. and ROBERTS, B.E. (1981). Expression of early adenovirus genes requires a viral encoded acidic polypeptide. *Proc. Natl. Acad. Sci.* **78**, 6121-6125.

RIEDEL, N., WOLIN, S. and GUTHRIE, C. (1987). A subset of yeast snRNAs contains functional binding sites for the highly conserved Sm antigen. *Science* **235**, 328-331.

RIGBY, P.W.J., DIECKMANN, M., RHODES, C. and BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA

polymerase I. *J. Mol. Biol.* 113, 237-251.

RIJNDERS, A.W.M., VAN BERGEN, B.G.M., VAN DER VLIET, P.C. and SUSSENBACH, J.S. (1983). Specific binding of the adenovirus terminal protein precursor-DNA polymerase complex to the origin of DNA replication. *Nucl. Acids Res.* 24, 8777-8789.

ROBERTS, M.M., WHITE, J.L., GRÜTTER, M.G. and BURNETT, R.M. (1986). Three-dimentional structure of the adenovirus major coat protein hexon. *Science* 232, 1148-1151.

ROBERTS, R.J., AKUSJÄRVI, G., ALESTRÖM, P., GELINAS, R.E., GINGERAS, T.R., SCIAKI, D. and PETTERSSON, U. (1986). A consensus sequence for the adenovirus-2 genome. In Doerfler, W. (ed) *Adenovirus DNA*, 1-51, Martinus Nijhoff.

ROBINSON, A.J., YOUNGHUSBAND, H.B. and BELLET, A.J.D. (1973). A circular DNA-protein complex from adenoviruses. *Virology* 56, 54-69.

ROCHETTE-EGLY, C., FROMENTAL, C. and CHAMBON, P. (1990). General repression of enhansons activity by the adenovirus-2 E1A proteins. *Genes Dev.* 4, 137-150.

ROESLER, W.J., VANDENBARK, G.R. and HANSON, R.W. (1988). Cyclic AMP and the induction of eucaryotic gene transcription. *J. Biol. Chem.* 236, 9063-9066.

ROGERS, J. and WALL, R. (1980). A mechanism for RNA splicing. *Proc. Natl. Acad. Sci.* 77, 1877-1879.

ROONEY, R.J., RAYCHAUDHURI, P. and NEVINS, J.R. (1990). E4F and ATF, two transcription factors that recognize the same site, can be distinguished both physically and functionally: a role for E4F in E1A *trans* activation. *Mol. Cell. Biol.* 10, 5138-5149.

ROSS, S.R., LEVINE, A.J., GALOS, R.S., WILLIAMS, J. and SHENK, T. (1980). Early viral proteins in Hela cells infected with adenovirus type 5 host range mutants. *Virology* 103, 475-492.

ROSSINI, M. (1983). The role of adenovirus early region 1A in the regulation of early regions 2A and 1B expression. *Virology* 131, 49-58.

ROWE, D.T. and GRAHAM, F.L. (1983). Transformations of rodent cells by DNA extracted from transformation-defective adenovirus mutants. *J. Virol.* 46, 1039-1044.

ROWE, W.P., HUEBNER, A.J. GILMORE, L.K., PARROTT, R.N. and WAND, T.G. (1953). Isolation of a cytopathic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. Soc. Exp. Biol. Med.* 84, 570-573.

RUIGROK, R.W.H., BARGE, A., ALBIGES-RIZO, C. and DAYAN, S.

(1990). Structure of adenovirus fibre. II. Morphology of single fibres. *J. Mol. Biol.* 215, 589-596.

RUSSELL, W.C. and PRECIOUS, B. (1982). Nucleic-acid binding properties of adenovirus structural polypeptides. *J. Gen. Virol.* 63, 69-79.

SAIKI, R.K. SCHARF, S., FALOONA, F., MULLIS, K.B., HORN, G.T. ERLICH, H.A. and ARNHEIM, N. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350-1354.

SAITO, I., SATO, J.-I., HANDA, H., SHIROKI, K. and SHIMOJO, H. (1981). Mapping of RNAs transcribed from adenovirus type 12 early and VA regions. *Virology* 114, 379-398.

SAITO, I., SHIROKI, K. and SHIMOJO, H. (1983). mRNA species and proteins of adenovirus type 12 transforming regions: Identification of proteins translated from multiple coding stretches in 2.2 kb region 1B mRNA *in vitro* and *in vivo*. *Virology* 127, 272-289.

SALAS, M. (1983). A new mechanism for the initiation of replication of ϕ29 and adenovirus DNA: priming by the terminal protein. *Curr. Top. Microbiol. Immunol.* 109, 89-106.

SANDLER, A.B. and KETNER, G. (1989). Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. *J. Virol.* 63, 624-630.

SANGER, F., NICKLEN, S. and COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74, 5463-5467.

SARNOW, P., HEARING, P., ANDERSON, C.W., HALBERT, D.N., SHENK, T. and LEVINE, A.J. (1984). Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. *J. Virol.* 49, 692-700.

SARNOW, P., HEARING, P., ANDERSON, C.W., REICH, N. and LEVINE, A.J. (1982a). Identification and characterization of an immunologically conserved adenovirus early region 11,000 Mr protein and its association with the nuclear matrix.

SARNOW, P., HO, Y.S., WILLIAMS, J. and LEVINE, A.J. (1982b). Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54kd cellular protein in transformed cells. *Cell* 28, 387-394.

SASSONE-CORSI, P., RANSONE, L.J., LAMPH, W.W. and VERMA, I.M. (1988). Direct interaction between *fos* and *jun* nuclear oncoproteins: role of the 'leucine zipper' domain. *Nature* 336, 692-695.

SAWADA, Y. and FUJINAGA, K. (1980). Mapping of adenovirus 12 mRNAs transcribed from the transforming region. *J. Virol.* 36, 639-651.

SCHMIDT, M.C., ZHOU, Q. and BERK, A.J. (1989). Sp1 activates transcription without enhancing DNA-binding activity of the TATA box factor. *Mol. Cell. Biol.* 9, 3299-3307.

SCHMITT, R.C., FAHNESTOCK, M.L. and LEWIS, J.B. (1987). Differential nuclear localization of the major adenovirus type 2 E1a proteins. *J. Virol.* 61, 247-255.

SCHNEIDER, J.F., FISHER, F., GODING, C.R. and JONES, N.C. (1987). Mutational analysis of the adenovirus E1A gene: the role of transcriptional regulation in transformation. *EMBO J.* 6, 2053-2060.

SCHUGHART, K., BAUSE, E. and ESCHE, H. (1985). Structure and expression of adenovirus type 12 E1B 58K protein in infected and transformed cells: Studies using antibodies directed against a synthetic peptide. *Virus Res.* 3, 41-56.

SCHÜTTE, J., VIALLET, J., NAU, M., SEGAL, S., FEDORKO, J. and MINNA, J. (1989). *jun-B* inhibits and *c-fos* stimulates the transforming and *trans*-activating activities of *c-jun*. *Cell* 59, 987-997.

SEED, B. and SHEEN J.-Y. (1988). A simple phase-extraxtion assay for chloramphenicol acyltransferase activity. *Gene* 67, 271-277.

SHAW, A. and ZIFF, E.B. (1980). Transcripts from the adenovirus major late promoter yield a single early family of 3' coterminal mRNAs and five late families. *Cell* 22, 905-916.

SHIROKI, K., KATO, H. and KAWAI, S. (1990). Tandemly repeated hexamer sequences sequences within the beta interferon promoter can function as an inducible regulatory element in activation by the adenovirus E1B 19-kilodalton protein. *J. Virol.* 64, 3063-3068.

SHIROKI, K., OHSHIMA, K., FUKUI, Y. and ARIGA, H. (1986). The adenovirus type 12 early-region 1B 58,000-Mr gene product is required for viral DNA synthesis and for initiation of cell transformation. *J. Virol.* 57, 792-801.

SHU, L., PETTIT, S.C. and ENGLER, J.A. (1988). The precise structure and coding of mRNAs from early region 2B of human adenovirus serotype 2. *Virology* 165, 348-356.

SIMON, M.C., FISCH, T.M., BENECKE, B.J., NEVINS, J.R. and HEINTZ, N. (1988). Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for E1A regulation. *Cell* 52, 723-729.

SIMON, M.C., KITCHENER, K., KAO, H.-T., HICKEY, E., WEBER, L., VOELLMY, R., HEINTZ, N. and NEVINS, J.R. (1987). Selective induction of human heat shock gene transcription by the adenovirus E1A gene products, including the 12S E1A product. *Mol. Cell. Biol.* 7, 2884-2890.

SLEMENDA, S.B., PIENIAZEK, N.J., VELARDE, J.JR., PIENIAZEK, D. and LUFTIG, R.B. (1990). Nucleotide sequence of the region coding for 100K and 33K proteins of human enteric adenovirus type 41 (Tak). *Nucl. Acids Res.* **18**, 3069.

SMART, J.E. and STILLMAN, B.W. (1982). Adenovirus terminal protein precursor: Partial amino acid sequence and the site of covalent linkage to virus DNA. *J. Biol. Chem.* **257**, 13499-13506.

SMILEY, J.K., YOUNG, M.A. and FLINT, S.J. (1990). Intranuclear location of the adenovirus type 5 E1B 55-kilodalton protein. *J. Virol.* 64, 4558-4564.

SMITH, D.H. and ZIFF, E.B. (1988). The amino-terminal region of the adenovirus serotype 5 E1a protein performs two separate functions when expressed in primary baby rat kidney cells. *Mol. Cell. Biol.* **8**, 3882-3890.

SPECTOR, D.J., McGROGAN, M. and RASKAS, H.J. (1978). Regulation of the appearance of cytoplasmic RNAs from region 1 of the adenovirus 2 genome. *J. Mol. Biol.* **126**, 395-414.

SPINDLER, K.R., ENG, C.Y. and BERK, A.J. (1985). An adenovirus early region 1A protein is required for maximal viral DNA replication in growth-arrested human cells. *J. Virol.* 53, 742-750.

SPINDLER, K.R., ROSSER, D.S.E. and BERK, A.J. (1984). Analysis of adenovirus transforming proteins from early region 1A and 1B with antisera to inducible fusion antigens produced in *E. Coli* cells. *J. Virol.* **49**, 132-141.

STEIN, R.W., CORRIGAN, M., YACIUK, P. WHELAN, J. and MORAN, E. (1990). Analysis of E1A-mediated growth regulation functions: Binding of the 300-Kilodalton cellular product correlates with E1A enhancer repression function and DNA synthesis-inducing activity. *J. Virol.* **64**, 4421-4427.

STEIN, R.W. and WHELAN, J. (1989). Insulin gene enhancer activity is inhibited by adenovirus 5 E1a gene products. *Mol. Cell. Biol.* 9, 4531-4534.

STEIN, R.W. and ZIFF, E.B. (1987). Repression of insulin gene expression by adenovirus type 5 E1a proteins. *Mol. Cell. Biol.* 7, 1164-1170.

STEPHENS, C. and HARLOW, E. (1987). Differential splicing

yields novel adenovirus 5 E1A mRNAs that encode 30kd and 35kd proteins. *EMBO J.* 6, 2027-2035.

STILLMAN, B.W., LEWIS, J.B., CHOW, L.T., MATHEWS, M.B. and SMART, J.E. (1981). Identification of the gene and mRNA for the adenovirus terminal protein precursor. *Cell* 23, 497-508.

STILLMAN, B.W., TAMANOI, F. and MATHEWS, M.B. (1982). Purification of an adenovirus-coded DNA polymerase that is required for initiation of DNA replication. *Cell* 31, 613-623.

STOW, N.D. (1982). The infectivity of adenovirus genomes lacking DNA sequences from their left hand termini. *Nucl. Acids Res.* 10, 5105-5119.

SUBRAMANIAM, A., THIRUNAVUKKARASU, M. and RAJAMANICKAM, C. (1990). Role of cytosol in the stimulation of RNA transport *in vitro* during cardiac hypertrophy in rats. *Biochem. J.* 267, 133-140.

SUBRAMANIAN, T., KUPPUSWAMY, M., GYSBERS, J., MAK, S. and CHINNADURAI, G. (1984a). 19kDa tumor antigen coded by early region E1B of adenovirus 2 is required for efficient synthesis and for protection of viral DNA. *J. Biol. Chem.* 259, 11777-11783.

SUBRAMANIAN, T., KUPPUSWAMY, M., MAK, S. and CHINNADURAI, G. (1984b). Adenovirus *cyt*$^{+}$ locus, which controls cell transformation and tumorigenicity, is an allele of the *lp*$^{+}$ locus, which codes for a 19-kilodalton tumor antigen. *J. Virol.* 52, 336-343.

SUTTON, R.E. and BOOTHROYD, J.C. (1986). Evidence for *trans* splicing in trypanosomes. *Cell* 47, 527-535.

SVENSSON, U. (1985). Role of vesicles during Ad2 internalization into HELA cells. *J.Virol.* 55, 442-449.

SVENSSON, U. and PERSSON, R. (1984). Entry of adenovirus 2 into Hela cells. *J. Virol.* 51, 687-694.

SVENSSON, U., PERSSON, R. and EVERITT, E. (1981). Virus-receptor interaction in the adenovirus system. I. Identfication of virion attachment proteins of the Hela cell plasma membrane. *J. Virol.* 38, 70-81.

TAKEMORI, N., CLADARAS, C., BHAT, B., CONLEY, A.J. and WOLD, W.S.M. (1984). *cyt* gene of adenovirus types 2 and 5 is an oncogene for transforming function in early region E1B and encodes the E1B 19,000-molecular-weight polypeptide. *J. Virol.* 52, 793-805.

TAKEMORI, N., RIGGS, J.L. and ALDRICH, C. (1968). Genetic studies with tumorigenic adenoviruses. I. Isolation of cytocidal (*cyt*) mutants of adenovirus type 12. *Virology* 36, 575-586.

TAKIFF, H.E., REINHOLD, W., GARON, C.F. and STRAUS, S.E. (1984). Cloning and physical mapping of enteric adenoviruses (candidate types 40 and 41). *J. Virol.* **51**, 131-136.

TAKIFF, H.E. and STRAUS, S.E. (1982). Early replicative block prevents the efficient growth of fastidious diarrhea-associated adenoviruses in cell culture. *J. Med. Virol.* 9, 93-100.

TAKIFF, H.E., STRAUS, S.E. and GARON, C.F. (1981). Propagation and *in vitro* studies of previously non-cultivable enteral adenovirus in 293 cells. *Lancet* ii, 832-834.

TAMANOI, F. and STILLMAN, B.W. (1983). Initiation of adenovirus DNA replication *in vitro* requires a specific DNA sequence. *Proc. Natl. Acad. Sci.* **80**, 6446-6450.

THIMMAPPAYA, B., WEINBERG, C., SCHNEIDER, R.J. and SHENK, T. (1982). Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. *Cell* 31, 543-551.

THOMAS, G.P. and MATHEWS, M.B. (1980). DNA replication and the early to late transition in adenovirus infection. *Cell* **22**, 523-533.

THOMAS, J.D., CONRAD, R.C. and BLUMENTHAL, T. (1988). The *C. elegans trans*-spliced leader RNA is bound to Sm and has a trimethylguanosine cap. *Cell* **54**, 533-539.

THOMPSON, S., STERN, P.L., WEBB, M., WALSH, F.S., ENGSTROM, W., EVANS, E.P., SHI, W.K., HOPKINS, B. and GRAHAM, C.F. (1984). Cloned human teratoma cells differentiate into neuron-like cells and other cell types in retinoic acid. *J. Cell Sci.* 72, 37-64.

TIBBETTS, C., LARSEN, P.L. and JONES, S.N. (1986). Autoregulation of adenovirus E1A gene expression. *J. Virol.* **57**, 1055-1064.

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

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

TIEMESSEN, C.T., WEGERHOFF, F.O., ERASMUS, M.J. and KIDD, A.H. (1989). Infection by enteric adenoviruses, rotaviruses, and other agents in a rural African environment. *J. Med. Virol.* **28**, 176-182.

TIGGES, M.A. and RASKAS, H.J. (1984). Splice junctions in adenovirus-2 early region 4: multiple splice sites

produce 18 to 24 RNAs. *J. Virol.* 50, 106-117.

TOKUNAGA, O., YAEGASHI, T., LOWE, J., DOBBS, L. and PADMANABHAN, R. (1986). Sequence analysis in the E1 region of adenovirus type 4 DNA. *Virology* 155, 418-433.

TOLLEFSON, A.E., KRAJCSI, P., PURSLEY, M.H., GOODING, L.R. and WOLD, W.S.M. (1990b). A 14,500 MW protein is coded by region E3 of group C human adenoviruses. *Virology* 175, 19-29.

TOLLEFSON, A.E., KRAJCSI, P., YEI, S., CARLIN, C.R. and WOLD, W.S.M. (1990a). A 10,400-molecular-weight membrane protein is coded by region E3 of adenoviruses. *J. Virol.* 64, 794-801.

TOLLEFSON, A.E. and WOLD, W.S.M. (1988). Identification and gene mapping of a 14,700-molecular-weight protein encoded by region E3 of group C adenoviruses. *J. Virol.* 62, 33-39.

TOOGOOD, C.I.A. and HAY, R.T. (1988). DNA sequence of the adenovirus type 41 hexon gene and predicted structure of the protein. *J. Gen. Virol.* 69, 2291-2301.

TOOGOOD, C.I., MURALI, R., BURNETT, R.M. and HAY, R.T. (1989). The adenovirus type 40 hexon: sequence, predicted structure and relationship to other adenovirus hexons. *J. Gen. Virol.* 70, 3203-3214.

TSUKAMOTO, A.S., PONTICELLI, A., BERK, A.J. and GAYNOR, R.B. (1986). Genetic mapping of a major site of phosphorylation in adenovirus type 2 E1A proteins. *J. Virol.* 59, 14-22.

TULLIS, R.H., FETHEROLF, P. and O'HARA, B. (1989). Synthesis considerations for PCR primers and probes. *Amplifications: a forum for PCR users* 3, 17-19.

TURNER, R. and TJIAN, R. (1989). Leucine repeats and a novel DNA binding domain mediate the formation of functional *cFos-cJun* heterodimers. *Science* 243, 1689-1694.

UHNOO, I., OLDING-STENKVIST, E. and KREUGER, A. (1986). Clinical features of acute gastroenteritis associated with rotavirus, enteric adenovirus, and bacteria. *Arch. Dis. Child.* 61, 732-738.

UHNOO, I., WADELL, G., SVENSSON, L. and JOHANSSON, M. (1983). Two new serotypes of enteric adenovirus causing infantile diarrhoea. *Dev. Biol. Standard* 53, 311-318.

UHNOO, J., WADELL, G., SVENSSON, L. and JOHANSSON, M.E. (1984). Importance of enteric adenoviruses 40 and 41 in acute gastroenteritis in infants and young children. *J. Clin. Microbiol.* 20, 365-372.

ULFENDAHL, P.J., LINDER, S., KREIVI, J.-P., NORDQVIST, K., SVENSSON, C., HULTBERG, H. and AKUSJÄRVI, G. (1987). A novel adenovirus-2 E1A mRNA encoding a protein with transcription activation properties. *EMBO J.* **6**, 2037-2044.

VALES, L.D. and DARNELL, J.E., JR. (1989). Promoter occlusion prevents transcription of adenovirus polypeptide IX mRNA until after DNA replication. *Genes Dev.* **3**, 49-59.

VAN BERGEN, B.G.M., VAN DER LEY, P.A., VAN DRIEL, W., VAN MANSFELD, A.D.M. and VAN DER VLIET, P.C. (1983). Replication of origin containing adenovirus DNA fragments that do not carry the terminal protein. *Nucl. Acids Res.* **11**, 1975-1989.

VAN DAM, H., OFFRINGA, R., SMITS, A.M.M., BOS, J.L., JONES, N.C. and VAN DER EB, A.J. (1989). The repression of the growth factor-inducible genes, JE, *c-myc* and stromelysin by adenovirus E1A is mediated by conserved region 1. *Oncogene* **4**, 1207-1212.

VAN DEN ELSEN, P., KLEIN, B., DEKKER, B., VAN ORMONDT, H. and VAN DER EB, A. (1983). Analysis of virus-specific mRNAs present in cells transformed with restriction fragments of adenovirus type 5 DNA. *J. gen. Virol.* **64**, 1079-1090.

VAN DEN HEUVEL, S.J.L., VAN LAAR, T., KAST, W.M., MELIEF, C.J.M., ZANTEMA, A. and VAN DER EB, A.J. (1990). Association between the cellular p53 and the adenovirus 5 E1B-55Kd proteins reduces the oncogenicity of Ad-transformed cells. *EMBO J.* **9**, 2621-2629.

VAN DER VLIET, P.C. and SUSSENBACH, J.S. (1975). An adenovirus type 5 gene function required for initiation of viral DNA replication. *Virology* **67**, 415-426.

VAN DOREN, K. and HIRSH, D. (1988). *Trans*-spliced leader RNA exists as small nuclear ribonucleoprotein particles in *caenorhabditis elegans*. *Nature* 335, 556-559.

VAN LOON, A.E., MAAS, R., VAESSEN, R.T.M.J., REEMST, A.M.C.B., SUSSENBACH, J.S. and ROZIJN, T.H. (1985a). Cell transformation by the left terminal regions of the adenovirus 40 and 41 genomes. *Virology* **147**, 227-230.

VAN LOON, A.E., GILARDI, P., PERRICAUDET, M., ROZIJN, T.H. and SUSSENBACH, J.S. (1987a). Transcriptional activation by the E1A regions of adenovirus types 40 and 41. *Virology* **160**, 305-307.

VAN LOON, A.E., LIGTENBERG, M., REEMST, A.M.C.B., SUSSENBACH, J.S. and ROZIJN, T.H. (1987b). Structure and organization of the left-terminal DNA regions of fastidious adenovirus types 40 and 41. *Gene* **58**, 109-126.

VAN LOON, A.E., ROZIJN, T.H., DE JONG, J.C. and SUSSENBACH, J.S. (1985b). Physicochemical properties of the DNAs of the fastidious adenovirus species 40 and 41. *Virology* **140**, 197-200.

VAN OOSTRUM, J. and BURNETT, R.M. (1985). Molecular composition of the adenovirus type 2 virion. *J. Virol.* **56**, 439-448.

VAN ORMONDT, H. and HESPER, B. (1983). Comparison of the nucleotide sequence of early region EIB DNA of human adenovirus types 12, 7 and 5 (subgroups A, B and C). *Gene* **21**, 217-266.

VAN ORMONDT, H., MAAT, J. and DIJKEMA, R. (1980). Comparison of nucleotide sequence of the early E1a regions for subgroups A, B and C of human adenoviruses. *Gene* **12**, 63-76.

VANHAESEBROECK, B., TIMMERS, H.T., PRONK, G.J., VAN ROY, F., VAN DER EB, A.J. and FIERS, W. (1990). Modulation of cellular susceptibility to the cytotoxic/cytostatic action of tumor necrosis factor by adenovirus E1 gene expression is cell type dependent. *Virology* **176**, 362-368.

VARGA, M.J., BERGMAN, T. and EVERITT, E. (1990). Antibodies with specificities against a dispase-produced 15-kilo-dalton hexon fragment neutralize adenovirus type 2 infectivity. *J. Virol.* **64**, 4217-4225.

VAYDA, M.E., ROGERS, A.E. and FLINT, S.J. (1983). The structure of nucleoprotein cores released from adeno-virions. *Nucl. Acids Res.* **11**, 441-460.

VELCICH, A. and ZIFF, E. (1985). Adenovirus E1a proteins repress transcription from the SV40 early promoter. *Cell* **40**, 705-716.

VELCICH, A. and ZIFF, E. (1988). Adenovirus E1a *ras* coope-ration activity is separate from its positive and negative transcription regulatory functions. *Mol. Cell. Biol.* **8**, 2177-2183.

VENKATESH, L.K. and CHINNADURAI, G. (1987). Activation of the adenovirus 2 protein IX promoter by DNA replication in a transient expression assay. *Nucl. Acids Res.* **15**, 2235-2250.

VIEIRA, J. and MESSING, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259-268.

VIRTANEN, A., GILARDI, P., NASLUND, A., LEMOULLEC, J.M., PETTERSSON, U. and PERRICAUDET, M. (1984). mRNAs from human adenovirus 2 early region 4. *J. Virol.* **55**, 822-831.

VIRTANEN, A. and PETTERSSON, U. (1983). The molecular structure of the 9S mRNA from early region 1A of adenovirus serotype 2. *J. Mol. Biol.* **165**, 496-499.

VIRTANEN, A. and PETTERSSON, U. (1985). Organization of early region 1B of human adenovirus type 2: Identification of four differentially spliced mRNAs. *J. Virol.* **54**, 383-391.

VIRTANEN, A., PETTERSSON, U., LE MOULLEC, J.M., TIOLLAIS, P. and PERRICAUDET, M. (1982). Different mRNAs from the transforming region of highly oncogenic and nononcogenic human adenoviruses. *Nature* **295**, 705-707.

VOELKERDING, K. and KLESSIG, D.F. (1986). Identification of two nuclear subclasses of the adenovirus type 5-encoded DNA-binding protein. *J. Virol.* **60**, 353-362.

VOS, H.L., VAN DER LEE, F.M., REEMST, A.M.C.B., VAN LOON, A.E. and SUSSENBACH, J.S. (1988). The genes encoding the DNA binding protein and the 23K protease of adenovirus types 40 and 41. *Virology* **163**, 1-10.

WADA, T., WATANABE, H., USUDA, Y. and HANDA, H. (1991). Different biological activities of the hetero- and homodimers formed by the 47- and 43-kilodalton proteins of transcription factor ATF/E4TF3. *J. Virol.* **65**, 557-564.

WADELL, G., ALLARD, A., JOHANSSON, M., SVENSSON, L. and UHNOO, I. (1987). Enteric adenoviruses. *Ciba Foundation Symposium* **128**, 63-91.

WADELL, G. and NORRBY, E. (1969). Immunological and other biological characteristics of pentons of human adenoviruses. *J. Virol.* **4**, 671-680.

WANG, E.W., SCOTT, M.O. and RICCIARDI, R.P. (1988). An adenovirus mRNA which encodes a 14,700-Mr protein that maps to the last open reading frame of region E3 is expressed during infection. *J. Virol.* **62**, 1456-1459.

WEBER, J.M., DERY, C.V., MIRZA, M.A. and HORVATH, J. (1985). Adenovirus DNA synthesis is coupled to virus assembly. *Virology* **140**, 351-359.

WEINBERG, D.H. and KETNER, G. (1986). Adenoviral early region 4 is required for efficient viral DNA replication and for late gene expression. *J. Virol.* **57**, 833-838.

WEINMANN, R., BRENDLER, T.G., RASKAS, H.J. and ROEDER, R.G. (1976). Low molecular weight viral RNA transcribed by RNA polymerase III during adenovirus 2 infection. *Cell* **7**, 557-566.

WHITE, D.O. and FENNER, F. (1986) (eds.). Medical Virology 3rd. ed. Academic Press, London.

WHITE, E., BLOSE, S.H. and STILLMAN, B.W. (1984a). Nuclear envelope localization of an adenovirus tumor antigen maintains the integrity of cellular DNA. *Mol. Cell. Biol.* **4**, 2865-2875.

WHITE, E. and CIPRIANI, R. (1989). Specific disruption of intermediate filaments and the nuclear lamina by the 19kDa product of the adenovirus E1B oncogene. *Proc. Natl. Acad. Sci.* **86**, 9886-9890.

WHITE, E. and CIPRIANI, R. (1990). Role of adenovirus E1B proteins in transformation: Altered organization of intermediate filaments in transformed cells that express the 19-kilodalton protein. *Mol. Cell. Biol.* **10**, 120-130.

WHITE, E., DENTON, A. and STILLMAN, B. (1988a). Role of the adenovirus E1B 19,000-dalton tumor antigen in regulating early gene expression. *J. Virol.* **62**, 3445-3454.

WHITE, E., FAHA, B. and STILLMAN, B. (1986). Regulation of adenovirus gene expression in human WI38 cells by an E1B-encoded tumor antigen. *Mol. Cell. Biol.* **6**, 3763-3773.

WHITE, E., GRODZICKER, T. and STILLMAN, B.W. (1984b). Mutations in the gene encoding the adenovirus early region 1B 19,000 molecular weight tumour antigen cause the degradation of chromosomal DNA. *J. Virol.* **52**, 410-419.

WHITE, E., SPECTOR, D. and WELCH, W. (1988b). Differential distribution of the adenovirus E1A proteins and colocalization of E1A with the 70-kilodalton cellular heat shock protein in infected cells. *J. Virol.* **62**, 4153-4166.

WHITE, E. and STILLMAN, B. (1987). Expression of adenovirus E1B mutant phenotypes is dependent on the host cell and on synthesis of E1A proteins. *J. Virol.* **61**, 426-435.

WHITELAW, A., DAVIES, H. and PARRY, J. (1977). Electron microscopy of fatal adenovirus gastroenteritis. *Lancet* **1**, 361.

WHYTE, P., BUCHKOVICH, K.J., HOROWITZ, J.M., FRIEND, S.H., RAYBUCK, M., WEINBERG, R.A. and HARLOW, E. (1988a). Association between an oncogene and an antioncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334**, 124-129.

WHYTE, P., RULEY, H.E. and HARLOW, E. (1988b). Two regions of the adenovirus early region 1A proteins are required for transformation. *J. Virol.* **62**, 257-265.

WHYTE, P., WILLIAMSON, N.M. and HARLOW, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. *Cell* **56**, 67-75.

WILLCOX, N. and MAUTNER, V. (1976). Antigenic determinants of adenovirus capsids. II. Homogeneity of hexons and accessibility of their determinants in the virion. *J. Immunol.* **116**, 25-29.

WILLIAMS, J.F. (1970). Enhancement of adenovirus plaque formation on Hela cells by magnesium chloride. *J. Gen. Virol.* **9**, 251-255.

WILLIAMS, J.F. (1986). Adenovirus genetics. In: Adenovirus DNA. Ed. W. Doerfler, Martinus Nijhoff, Boston.

WILLIAMS, J., KARGER, B.D., HO, Y.S., CASTIGLIA, C.L., MANN, T. and FLINT, S.J. (1986). The adenovirus E1B 495R protein plays a role in regulating the transport and stability of the viral late messages. *Cancer Cells* **4**, 275-284.

WILSON, M.C. and DARNELL, J.E., JR. (1981). Control of messenger RNA concentration by differential cytoplasmic half-life. *J. Mol. Biol.* **148**, 231-251.

WILSON-RAWLS, J., SAHA, S.K., KRAJCSI, P., TOLLEFSON, A.E., GOODING, L.R. and WOLD, W.S.M. (1990). A 6700MW membrane protein is encoded by region E3 of adenovirus type 2. *Virology* **178**, 204-212.

WITT, D.J. and BOUSQUET, E.B. (1988). Comparison of enteric adenovirus infection in various human cell lines. *J. Virol. Meth.* **20**, 295-308.

WOHLFART, C. (1988). Neutralization of adenoviruses: kinetics, stoichometry and mechanisms. *J. Virol.* **62**, 2321-2328.

WOLD, W.S.M., CLADARAS, C., MAGIE, S.C. and YACOUB, N. (1984). Mapping a new gene that encodes an 11,600-molecular-weight protein in the E3 transcription unit of adenovirus 2. *J. Virol.* **52**, 307-313.

WOLD, W.S.M. and GOODING, L.R. (1989). Adenovirus region E3 proteins that prevent cytolysis by cytotoxic T cells and tumor necrosis factor. *Mol. Biol. Med.* **6**, 433-452.

WOLFSON, J. and DRESSLER, D. (1972). Adenovirus 2 DNA contains an inverted terminal repetition. *Proc. Natl. Acad. Sci.* **69**, 3054-3057.

WOLGEMUTH, D.J. and HSU, M.-T. (1981). Visualization of nascent RNA transcripts and simultaneous transcription and replication in viral nucleoprotein complexes from adenovirus 2-infected Hela cells. *J. Mol. Biol.* **147**, 247-268.

WONG, M.-L. and HSU, M.-T. (1989). Linear adenovirus DNA is organized into supercoiled domains in virus particles. *Nucl. Acids Res.* **17**, 3535-3550.

WU, L. and BERK, A.J. (1988a). Transcriptional activation by the pseudorabies virus immediate early protein requires the TATA box element in the adenovirus 2 E1B promoter. *Virology* **167**, 318-322.

WU, L. and BERK, A.J. (1988b). Constraints on spacing between transcription factor binding sites in a simple adenovirus promoter. *Genes Dev.* **2**, 403-411.

WU, L., ROSSER, D.S.E., SCHMIDT, M.C. and BERK, A. (1987). A TATA box implicated in E1A transcriptional activation of a simple adenovirus 2 promoter. *Nature* **326**, 512-515.

YANISCH-PERRON, C., VIEIRA, J. and MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

YEE, A.S., RAYCHAUDHURI, P., JAKOI, L. and NEVINS, J.R. (1989). The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. *Mol. Cell. Biol.* **9**, 578-585.

YEE, S.-P. and BRANTON, P.E. (1985). Deletion of cellular proteins associated with human adenovirus type 5 early region E1A polypeptides. *Virology* **147**, 142-153.

YEW, P.R., KAO, C.C. and BERK, A.J. (1990). Dissection of functional domains in the adenovirus 2 early 1B 55K polypeptide by suppressor-linker insertional mutagenesis. *Virology* **179**, 795-805.

YOLKEN, R.H., LAWRENCE, F., LEISTER, F., TAKIFF, H.E. and STRAUS, S.E. (1982). Gastroenteritis associated with enteric type adenovirus in hospitalized infants. *J. Pediatr.* **101**, 21-26.

YOSHIDA, K. and FUJINAGA, K. (1980). Unique species of mRNA from adenovirus type 7 early region 1 in cells transformed by adenovirus type 7 DNA fragment. *J. Virol.* **36**, 337-352.

YOSHIDA, K., NARITA, M. and FUJINAGA, K. (1989). Binding sites of Hela cell nuclear proteins on the upstream region of adenovirus type 5 E1A gene. *Nucl. Acids Res.* **17**, 10015-10034.

YOSHINAGA, S., DEAN, M., HAN, M. and BERK, A.J. (1986). Adenovirus stimulation of transcription by RNA polymerase III: evidence for an E1A dependent increase in transcription factor IIIC concentration. *EMBO J.* **5**, 343-354.

ZAIN, B.S. and ROBERTS, R.J. (1978). Characterization and sequence analysis of a recombination site in the hybrid virus Ad2+ND1. *J. Mol. Biol.* **120**, 13-31.

ZAJCHOWSKI, D.A., BOEUF, H. and KEDINGER, C. (1985). The

adenovirus-2 early EIIa transcription unit possesses two overlapping promoters with different sequence requirements for E1a-dependent stimulation. *EMBO J.* **4**, 1293-1300.

ZANTEMA, A., SCHRIER, P.I., DAVIS-OLIVIER, A., VAN LAAR, T., VAESSEN, R.T.M.J. and VAN DER EB, A.J. (1985). Adenovirus serotype determines association and localization of the large E1B tumor antigen with cellular tumor antigen p53 in transformed cells. *Mol. Cell. Biol.* **5**, 3084-3091.

ZERIAL, M., TOSCHI, L., RYSECK, R.-P., SCHUERMANN, M., MÜLLER, R. and BRAVO, R. (1989). The product of a novel growth factor activated gene, *fosB*, interacts with *JUN* proteins enhancing their DNA binding activity. *EMBO J.* **8**, 805-813.

ZERLER, B., MORAN, B., MARUYAMA, K., MOOMAW, J., GRODZICKER, T. and EARL RULEY, H. (1986). Adenovirus E1A coding sequences that enable ras and pmt oncogenes to transform cultured primary cells. *Mol. Cell. Biol.* **6**, 887-899.

ZERLER, B., ROBERTS, R.J., MATHEWS, M.B. and MORAN, E. (1987). Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. *Mol. Cell. Biol.* **7**, 821-829.

ZHUANG, Y. and WEINER, A.M. (1986). A compensatory base change in U1 snRNA suppresses a 5' splice site mutation. *Cell* **46**, 827-835.

ZIFF, E. and EVANS, R. (1978). Coincidence of the promoter and capped 5' terminus of RNA from the adenovirus 2 major late transcription unit. *Cell* **15**, 1463-1475.

AD40 E1 SEQUENCE

```
CATCATCAATAATATACCTTAAAACTGGAAACGAGCCAATATGATAATGAGGGAGGAGGGACTAGGGGTGGTGTA    75

AGGTGACGTAGAGGCGGGCGGGGTGGGAAAGGGTGGAGGCGGATGACGTGTGGGGTCGGAGGACGGGCGCGGTGC   150

GGCGGAAGTGACGGAAAATCTGGTGTATTGGGCGGGTTTTTGTAACTTTTGGCCATTTTGGCGCGAAAACTGAGT   225

AATGAGGACGTGGGACGAACTTTGGACTTTTGTGTTTATGGAGGAAAAACTGCTGATTATTACTGAACTTTGGCC   300

CATGACGAACCGGTTTTTCTACGTGGCAGTGCCACGAGACGGCTCAAAGTCCTAATTTTTTATTGTGTGCTCAGC   375

CCGTTTGAGGGTATTTAAACACAGCCAGAACATCAAGAGGCCACTCTTGAGTGCGAGCGAGTAGAGTTTTCTCCT   450

                                        M   R   M   L   P   D   F   F   T   G    10
CCATTGCTGTTGGCGCTTTTGACATAGCCACCAAG ATG AGA ATG CTG CCG GAT TTT TTT ACC GGG   515

 N   W   D   D   M   F   Q   G   L   L   E   T   E   Y   V   F   D   F   P    29
AAC TGG GAT GAC ATG TTC CAG GGG TTG CTG GAG ACT GAA TAT GTG TTT GAT TTC CCT   572

 E   P   S   E   A   S   E   E   M   S   L   H   D   L   F   D   V   E   V    48
GAA CCT TCT GAG GCT TCT GAA GAA ATG TCG CTT CAT GAT CTT TTT GAT GTG GAG GTG   629

 D   G   F   E   E   D   A   N   Q   E   A   V   D   G   M   F   P   E   R    67
GAT GGT TTC GAA GAG GAC GCC AAC CAG GAA GCG GTT GAT GGT ATG TTT CCC GAG AGG   686

 L   L   S   E   A   E   S   A   A   E   S   G   S   G   D   S   G   V   G    86
TTG CTG TCC GAG GCT GAG AGC GCT GCA GAG AGC GGT TCG GGT GAT TCT GGG GTT GGC   743

 E   E   L   L   P   V   D   L   D   L   K   C   Y   E   D   G   L   P   P   105
GAA GAG TTG TTG CCG GTT GAT CTG GAT TTG AAA TGC TAT GAA GAC GGT TTG CCT CCT   800

 S   D   P   E   T   D   E   A   T   E   A   E   E   E   A   A   M   P   T   124
AGC GAT CCT GAA ACT GAT GAG GCT ACA GAG GCG GAA GAA GAG GCG GCT ATG CCG ACT   857

 Y   V   N   E   N   E   N   E   L   V   L   D   C   P   E   N   P   G   R   143
TAT GTG AAT GAA AAT GAA AAT GAG CTG GTG CTG GAC TGT CCA GAG AAC CCT GGG CGA   914

 G   C   R   A   C   D   F   H   R   G   T   S   G   N   P   E   A   M   C   162
GGT TGT CGG GCT TGT GAT TTC CAT CGG GGC ACT AGT GGC AAT CCT GAA GCT ATG TGT   971

 A   L   C   Y   M   R   L   T   G   H   C   I   Y   S                       176
GCT TTG TGT TAT ATG CGT TTA ACT GGA CAC TGT ATC TAC AGT AAGTAAAAAAGTTTTTATT 1032

                                                        P   I   S   D         4
TGTTTGGTGGTGTTGGTTAATATGAACAAGAGTTAACGACTTTTTGTTATTTTAGGT CCA ATT TCA GAT   1101

 A   E   G   E   S   E   S   G   S   P   E   D   T   D   F   P   H   P   L    23
GCG GAA GGG GAG TCT GAG TCG GGG TCG CCT GAG GAC ACT GAT TTT CCC CAC CCT TTA 1158

 T   A   T   P   P   H   G   I   V   R   T   I   P   C   R   V   S   C   R    42
ACC GCC ACG CCG CCA CAT GGA ATT GTG AGA ACC ATC CCG TGC AGA GTT TCT TGT AGA 1215

 R   R   P   A   V   E   C   I   E   D   L   L   E   E   D   P   T   D   E    61
CGA CGC CCA GCT GTT GAG TGC ATA GAA GAT TTA CTT GAG GAA GAT CCA ACA GAT GAA 1272

 P   L   N   L   S   L   K   R   P   K   C   S                               73
CCT TTG AAC CTG TCC TTA AAG CGC CCC AAG TGC TCC TGAGATCATAGTAATAAAGTTATTGAC 1335

CCTTACCCTGTGTTTATTTCTTGGGCGTGTTTGTGGGTATATAAGCAGGTAGAATGGTTTTAGTGTTAGTTTATT 1410

     M   E   L   W   S   E   L   Q   S   Y   Q   N   L   R   R   L   L   E    18
CTG ATG GAG TTG TGG AGT GAG TTA CAA AGT TAT CAG AAC CTC CGA CGC TTG CTG GAG 1467
```

```
 L   A   S   A   R   T   S   S   C   W   R   I   L   F   G   S   T   L   T     37
TTG GCT TCT GCC AGA ACT TCC AGC TGT TGG AGA ATC CTT TTT GGC TCA ACT TTA ACT 1524

 N   V   I   Y   R   A   K   E   E   Y   S   S   R   F   A   D   L   L   S     56
AAT GTA ATC TAT AGA GCT AAG GAG GAG TAC TCT TCG CGG TTT GCT GAC CTT TTG TCG 1581

 H   N   P   G   I   F   A   S   L   N   L   G   H   H   S   F   F   Q   E     75
CAT AAC CCT GGA ATT TTT GCT TCT TTG AAT TTG GGG CAT CAC TCA TTT TTT CAA GAA 1638

 I   V   I   R   N   L   D   F   S   S   P   G   R   T   V   S   G   L   A     94
ATT GTG ATC AGA AAT TTA GAT TTT TCT TCT CCT GGC CGT ACG GTT TCT GGG CTT GCT 1695

                                 M   E   R   P   N   S   S   V   A   G   L   Y   S   G   L     15
 F   I   C   F   I   L   D   Q   W   S   A   Q   T   H   L   S   Q   G   Y   T   L   D   Y    117
TTT ATT TGT TTT ATA TTG GAT CAATGGAGCGCCCAAACTCATCTGTCGCAGGGTTATACTCTGGATTA 1763

 H   G   N   G   S   V   E   N   L   A   T   E   E   E   G   L   R   L   L   A   G   A   A   S   A     40
   M   A   M   A   L   W   R   T   L   L   R   R   K   R   V   L   G   C   L   P   A   Q   R   P   H    142
CATGGCAATGGCTCTGTGGAGAACCTTGCTACGGAGGAAGAGGGTCTTAGGTTGCTTGCCGGCGCAGCGTCCGCA 1838

 R   F   G   S   S   A   G   R   G   G   G   G   G   E   P   E   G   R   P   G   P   F   N   G   I     65
   G   L   D   P   V   Q   E   E   E   E   E   E   E   N   L   R   A   G   L   D   P   S   T   E   L    167
CGGTTTGGATCCAGTGCAGGAAGAGGAGGAGGAGGAGGAGAACCTGAGGGCCGGCCTGGACCCTTCAACGGAATT 1913

 V   T   E   P   D   P   E   E   G   T   S   S   G   Q   R   G   G   I   N     84
 _                                                                             167
GTAACT GAG CCT GAT CCC GAA GAG GGT ACT AGC AGT GGG CAA AGG GGG GGC ATT AAT  1970

 G   Q   R   G   T   K   R   K   M   E   N   E   G   E   D   F   L   K   E    103
GGG CAA AGG GGG ACA AAG AGA AAG ATG GAA AAC GAG GGG GAG GAC TTT TTA AAG GAG 2027

 L   T   L   S   L   M   S   R   R   H   H   E   S   V   W   W   A   D   L    122
TTA ACC TTG AGT TTA ATG TCT CGT CGC CAT CAT GAG TCT GTT TGG TGG GCT GAT TTG 2084

 E   D   E   F   K   N   G   E   M   N   L   L   Y   K   Y   T   F   E   Q    141
GAA GAT GAG TTT AAA AAC GGT GAA ATG AAT TTG TTA TAC AAG TAT ACA TTT GAA CAG 2141

 L   K   T   H   W   L   E   A   W   E   D   F   E   L   A   L   N   T   F    160
CTG AAG ACA CAT TGG CTG GAG GCT TGG GAG GAT TTT GAG TTA GCT CTG AAC ACT TTT 2198

 A   K   V   A   L   R   P   D   T   I   Y   T   I   K   K   T   V   N   I    179
GCC AAA GTG GCT CTT CGC CCG GAC ACT ATT TAT ACC ATT AAG AAG ACT GTT AAT ATA 2255

 R   K   C   A   Y   V   L   G   N   G   A   V   V   R   F   Q   T   C   D    198
CGT AAA TGT GCC TAT GTG CTG GGG AAT GGA GCT GTG GTG CGG TTT CAA ACA TGT GAC 2312

 R   V   A   F   N   C   A   M   Q   S   L   G   P   G   L   I   G   M   S    217
CGT GTA GCC TTT AAC TGC GCA ATG CAG AGC TTG GGC CCT GGG CTT ATT GGC ATG AGT 2369

 G   V   T   F   M   N   V   R   F   V   V   E   G   F   N   G   T   V   F    236
GGG GTA ACT TTT ATG AAT GTG AGA TTT GTA GTG GAG GGA TTT AAT GGC ACA GTG TTT 2426

 A   S   T   T   Q   L   T   L   H   G   V   F   F   Q   N   C   S   G   I    255
GCT TCT ACC ACT CAA TTA ACC TTG CAT GGT GTG TTT TTT CAA AAT TGC AGC GGT ATC 2483

 C   V   D   S   W   G   R   V   S   A   R   G   C   T   F   V   A   C   W    274
TGC GTG GAT TCC TGG GGT AGG GTG TCT GCC AGA GGG TGT ACG TTT GTT GCA TGT TGG 2540

 K   G   V   V   G   R   N   K   S   Q   M   S   V   K   K   C   V   F   E    293
AAA GGG GTG GTG GGG CGA AAC AAA AGT CAA ATG TCT GTA AAG AAG TGT GTG TTT GAA 2597

 R   C   I   M   A   M   V   V   E   G   Q   A   R   I   R   H   N   A   G    312
CGT TGC ATT ATG GCC ATG GTG GTA GAA GGT CAG GCG CGG ATT CGC CAT AAT GCG GGC 2654
```

```
 S   D   N   V   C   F   L   L   L   K   G   T   A   S   V   K   H   N   M   331
TCT GAT AAT GTG TGT TTT TTA CTG CTA AAG GGA ACT GCC AGT GTA AAG CAT AAC ATG 2711

 I   C   G   G   G   H   S   Q   L   L   T   C   A   D   G   N   C   Q   A   350
ATT TGT GGC GGT GGT CAC TCT CAG CTG CTA ACC TGT GCA GAT GGA AAC TGT CAG GCT 2768

 L   R   V   F   H   V   V   S   H   P   R   R   P   W   P   V   F   E   H   369
CTG AGA GTG TTT CAC GTA GTA TCT CAT CCC CGC CGC CCC TGG CCT GTT TTT GAG CAC 2825

 N   M   L   M   R   C   T   V   H   L   G   A   R   R   G   M   F   S   P   388
AAC ATG CTT ATG CGC TGT ACT GTG CAT TTG GGA GCT CGT CGT GGC ATG TTT TCT CCA 2882

 Y   Q   S   N   F   C   H   T   K   V   L   M   E   T   D   A   F   S   R   407
TAC CAG AGT AAC TTT TGC CAC ACT AAA GTT TTA ATG GAA ACT GAT GCT TTT TCT CGG 2939

 V   W   W   N   G   V   F   D   L   T   M   E   L   F   K   V   V   R   Y   426
GTA TGG TGG AAC GGG GTA TTT GAT TTA ACC ATG GAG CTA TTT AAA GTG GTG AGG TAT 2996

 D   E   S   K   V   R   C   R   P   C   E   C   G   A   N   H   I   R   L   445
GAT GAG TCA AAG GTT CGT TGT CGC CCC TGT GAG TGT GGA GCT AAT CAT ATT AGG TTA 3053

 Y   P   A   T   L   N   V   T   E   Q   L   R   T   D   H   Q   M   M   S   464
TAT CCA GCA ACT CTG AAC GTG ACC GAG CAG CTG CGT ACG GAC CAC CAG ATG ATG TCG 3110

 C   L   R   T   D   Y   E   S   S   D   E   D   -                           476
TGT CTG CGT ACT GAC TAC GAA TCC AGC GAT GAG GAT TAA GGGTAAGGGGCGGAGCCTATTAC 3172

                                                 M   S   G   F   T   E   G     7
AGGTATAAAGGTTGGGGTAGAGTAAAAAAAAGGGAAGTTACAAA ATG AGT GGC TTC ACG GAA GGA   3237

 N   A   V   S   F   E   G   G   V   F   S   P   Y   L   T   T   R   L   P   26
AAC GCT GTG AGT TTT GAG GGT GGG GTG TTT AGC CCA TAT CTG ACA ACC CGT CTT CCC 3294

 S   W   A   G   V   R   Q   N   V   V   G   S   N   V   D   G   R   P   V   45
TCT TGG GCA GGA GTG CGT CAG AAT GTG GTG GGG TCC AAC GTT GAT GGT CGT CCT GTC 3351

 A   P   A   N   S   T   T   L   T   Y   A   T   I   G   S   S   V   D   T   64
GCC CCT GCC AAC TCG ACA ACC CTT ACC TAC GCC ACT ATT GGA TCG TCG GTG GAT ACC 3408

 A   A   A   A   A   A   S   A   A   A   S   T   A   R   G   M   A   A   D   83
GCT GCA GCT GCT GCC GCG TCT GCT GCT GCC TCT ACT GCT CGT GGC ATG GCA GCA GAT 3465

 F   G   L   Y   N   H   L   A   A   S   R   L   R   E   E   D   A   L   S   102
TTT GGA CTG TAC AAT CAT CTG GCC GCG TCT CGG TTA AGA GAA GAA GAT GCC CTG TCC 3522

 V   V   L   T   R   L   E   E   L   S   Q   Q   L   Q   D   M   S   A   K   121
GTG GTG TTG ACC CGC TTG GAG GAG CTG TCT CAG CAG TTG CAA GAT ATG TCT GCC AAA 3579

 M   A   L   L   N   P   P   A   N   T   S   -                               132
ATG GCT CTG CTT AAC CCT CCC GCT AAT ACT TCT TAA TAAAGACACAATTGGTTGGAAAAGTCA 3642

AAAGTGTTTATTTATTTCTTTTGCGGTAGGCCCTAGACCACCTGTCGCGGTCGTTTAAAACTTTATGGATGTTTT 3717

CCAAGACCCGGTACAGGTGGGCTTGGATGTTCAAATACATAGGCATTAGGCCGTCCCTGGGATGCAGGTAGGACC 3792

ACTGGAGGGCGTCATGCTCTGGGGTGGTGTTGTAAATAATCCAATCGTAGCAGGGTTTTTGAGCATGAAACTGGA 3867

AGATGTCCTTAAGGAGGAGGCGAATGGCCAGAGGTAGCCCCTTGGTCAAGGTATTAACAAATCGAT       3933
```