CHARACTERIZATION OF CELLULAR PROTEINS FOUND IN ASSOCIATION WITH EARLY REGION 1A POLYPEPTIDES OF HUMAN ADENOVIRUS TYPE 5

by

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Abstract

Human adenoviruses are capable of oncogenic transformation of mammalian cells in culture and are a valuable model system for use in studying the molecular basis of cancer. The Early Region 1A (E1A) gene of the virus is involved in transformation, and its protein products have been previously reported to associate with cellular polypeptides termed p300, p107, p105, p68 and p65. The objective of the present investigation was to characterize these cellular proteins and to determine something of their function or biological significance with respect to E1a-mediated transformation.

It was found that p300, p68 and p65, as well as a number of other cellular proteins were able to bind with specificity to bacterially-produced E1a products covalently linked to Sepharose beads (E1a-Sepharose), suggesting that this would be suitable for affinity purification of E1a-binding proteins.

The binding sites for p300, p107 and p105 on the E1a polypeptides were mapped in a collaborative study with Dr. Stan Bayley's lab. A region at the amino terminus of E1a proteins was involved in binding p300, and p107 and p105 bound overlapping regions in CR2. CR1

played some role in binding all three proteins, but probably not as a primary binding site. E1a proteins which failed to bind either p300 or p105 were transformation defective, which indicated a role for both proteins in E1a-mediated transformation. E1a proteins which failed to bind both p300 and p105 were incapable of inducing DNA synthesis in quiescent cells, suggesting these proteins were cooperatively involved in suppressing DNA synthesis. p105 was confirmed to be the product of the retinoblastoma tumour suppressor gene, p105-Rb. Comparison of the peptide digestion products of p107 and p105-Rb and examination of the expression of p107 in retinoblastoma cells suggested that p107 and p105-Rb are from different, but related genes.

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This is for

W.A. and Myra,

for

Tanya

(the junior scientist),

and for

the Wolverines,

Bran, Morgan and Cass

all of whom were there.

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Ad5	adenovirus serotype 5
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
DAI	double-stranded RNA activated inhibitor (of protein synthesis)
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
dCMP	deoxycytosine monophosphate
EDTA	ethylenediaminetetraacetic acid
HPLC	high-performance liquid chromatography
kD	kiloDalton
mRNA	messenger ribonucleic acid
MOI	multiplicity of infection
ml	millilitre
mM	millimolar
mg	milligram
mCi	milliCurie
µg	microgram
µl	microlitre
nm	nanometre
PBS	phosphate-buffered saline
PFU	plaque-forming unit

PPO	2,5 diphenyloxazole
Tris	tris-hydroxymethyl aminomethane
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N', N'-tetramethylethylenediamine
wt	wild-type

Chapter 1

Introduction

Introduction

1.1 Adenoviruses: Etiology and Classification

1.1.1 Etiology

Human adenoviruses were first identified as the etiological agent of acute respiratory disease (ARD) in military recruits at Fort Bragg, North Carolina (U.S.Army Commission on Acute Respiratory Diseases, 1947). Acute respiratory disease was defined as one of a collection of severe respiratory ailments which were epidemic among new recruits, particularly in the late fall and early spring. Characterized by fever, sore throat, hoarseness, cough and drowsiness, ARD was seen as clinically distinct from the common cold in that the pharyngeal involvement was more pronounced, and the incubation period between infection and onset of symptoms was longer.

Subsequently, and in an independent investigation, Rowe et al. (1953) reported the isolation of an adenoid degeneration agent (A.D. agent) from cultured adenoid tissue obtained in operations on young children. A.D. agent was found to cause a characteristic degeneration pattern in a number of human epithelial cell cultures,

including adenoid, embryonic nose, pharynx and trachea, and HeLa cells, with an incubation period of four to eight days. Presumptive evidence for an immunological relationship between A.D. agent and the virus implicated in ARD was reported by Hilleman and Werner (1954) following the isolation of a viral agent, RI-67 from a patient with primary atypical pneumonia, during an outbreak of severe respiratory illness among recruits at Fort Leonard Wood, Missouri. Adenovirus serotypes 1-7 were isolated from one or the other of these sources (Jawetz et al., 1955).

Adenovirus serotype 8 was first isolated from the cornea of a seaman suffering from a condition known as "shipyard eye" (Jawetz et al., 1955), and was later determined to be the causative agent of this epidemic form of keratoconjunctivitis (Jawetz, 1959). Although the etiology of the shipyard eye agent did not resemble that of the other known adenoviruses, and it could not be neutralized by monotypic sera against serotypes 1 to 7, it did produce the characteristic cytopathic effect on HeLa cells and it reacted positively in complement fixation assays using anti-adenovirus antisera, thus clearly defining it as a member of this group (Jawetz et al., 1955).

A fourth clinical syndrome which has been associated with adenovirus infection involves acute gastroenteritis in young children. Because many adenovirus serotypes replicate efficiently in the intestine and may be excreted in the stool of both healthy and ill children, the relationship between adenovirus infection and gastrointestinal disease was not clear in early studies. It has more recently been reported, however, that the adenovirus serotypes which are enteric but which are not easily cultivable in tissue culture, are closely associated with gastroenteritis, and may account for approximately 15% of cases of viral gastroenteritis in hospitalized infants and children (Gary et al., 1979 and Retter et al., 1979). The fact that these serotypes are non-cultivable may have resulted in their being overlooked as potential pathogens in earlier studies.

There are now 41 known human serotypes of adenovirus, and the virus has been isolated from a number of other species, including cats, dogs, tree shrews, pigs, sheep, and some fowl (Flint, 1981a and Horwitz, 1990b). In animals and birds, infection tends to be associated with specific disease, but in humans, adenovirus associated disorders are often characterized by more general symptoms and involve only one third of

the known human serotypes (Horwitz, 1990b). Many serotypes, including serotypes 25 to 33, were either isolated from healthy individuals, or are not associated with any specific illness. Serotypes 40 and 41 have been implicated in gastroenteritis in children (Gary et al., 1979 and Retter et al., 1979), and serotypes 8, and more recently 19 and 37 are associated with the epidemic form of keratoconjunctivitis known as "shipyard eye" (Jawetz et al., 1955, Jawetz, 1959 and Horwitz, 1990b), but most of the remaining serotypes are involved in a variety of respiratory and pharyngeal conditions. These include acute febrile pharyngitis, with or without accompanying mild conjunctivitis, acute respiratory disease, pertussis-like syndrome, and pneumonia. Latency is often established, particularly in adenoid tissue, and virus has been recovered as late as two years after the initial infection (Horwitz, 1990b).

In 1962, using a novel approach to the search for human tumour viruses, Trentin et al. reported that adenovirus serotype 12 produced tumours when injected into newborn Syrian hamsters. The approach they used was based on the hypothesis that a potential human tumour virus might produce tumours only under some physiological conditions of the host which might arise some length of time after the manifestation of the acute disease

symptoms normally associated with the virus. Adenoviruses were seen as potentially interesting candidates because of their ability to cause acute illness in children, followed by latency, and because of their similarities to other animal tumour viruses, such as polyoma. Syrian hamsters were chosen as a test species because of their known susceptibility to cancer induction by animal tumour viruses or foreign tissue transplants. Of the nine adenovirus serotypes tested by Trentin et al., only Ad12 was capable of inducing tumours, which appeared one to three months later at the site of injection with all protocols used. It was suggested that, because 26% of the population carries neutralizing antibodies for Ad12, this virus could be etiologically related to cancer in man. Following the report of Trentin et al., it was reported that Ad18 was also highly oncogenic in hamsters (Huebner et al., 1962), and that serotypes 7 and 3 (Girardi et al., 1964, Larson et al., 1965 and Huebner et al. 1965) could produce tumours in hamsters as well, but only after a much longer incubation period (greater than 200 days). Even the serotypes which were not oncogenic when injected into animals could transform primary rodent cells in culture, so that the transformed cells were tumourigenic when injected into hamsters (Freeman et al., 1967 and Rowe et

al., 1984a). Adenoviruses were the first human viruses reported to be oncogenic in animals, and this finding generated a great deal of interest with regard to their involvement in human cancer. With the exception of one group's report on the detection of Ad12-related RNA sequences specifically in neurogenic tumours (Ibelgaufts et al., 1982), most attempts to detect adenovirus sequences in human tumours have failed (Green, 1970 and Green et al., 1979c). These results suggested that adenoviruses are not likely to be human cancer-causing agents (Green et al., 1979c and Horwitz, 1990b). Nevertheless these viruses, especially Ad2 and Ad5, have been well characterized at the molecular level, and have proven to be valuable model systems in which to study the molecular events involved in transformation of mammalian cells.

1.1.2 Classification

The family Adenoviridae is subdivided into two genera, Aviadenovirus, which includes the viruses that infect avian species, and Mastadenovirus, which includes the viruses which infect mammalian species (Norrby et al., 1976). The division of the family into two genera was made on the basis of an absence of any cross-reacting

structural proteins between avian and mammalian adenoviruses. Within the Mastadenovirus genus, most of the known serotypes are of human origin, but a number have also been isolated from simian species, and a few from equine, bovine, ovine, canine, murine, porcine and opossum species (Norrby et al., 1979 and Horwitz, 1990a). All serotypes within this genus show some cross-reactivity in complement-fixation assays, which are dependent on properties of the "group specific" antigen, or hexon capsid protein, which is highly conserved (Wadell et al., 1980). Complement-fixation analysis was originally the means of identifying new adenovirus isolates, and new serotypes were identified in neutralization assays with type-specific sera for established serotypes (Hilleman etal., 1954 and Jawetz et al., 1955). As the number of human adenovirus serotypes increased, however, it became expedient to develop a classification scheme based on a biological property of the virus which would facilitate identification of new isolates.

Rosen (1958) reported that adenoviruses had the ability to haemagglutinate red blood cells from a number of species of animals, or from humans, and that the pattern of blood cell types which could be agglutinated was characteristic of the serotype of virus. He

suggested three subclasses of human adenoviruses based on haemagglutination pattern which is largely dependent on properties of the fibre structural protein (Wadell et al., 1980 and Horwitz, 1990a). This classification scheme has been updated and revised by others including Zuschek, 1961 and Hierholzer, 1973, and now includes four subgroups. Subgroup 1 includes those serotypes which can produce complete agglutination of monkey erythrocytes, subgroup 2 gives complete agglutination of rat erythrocytes, subgroup 3, partial agglutination of rat erythrocytes, and subgroup 4 shows little or no agglutination of any red blood cell types assayed (Horwitz, 1990a).

Following reports of the transforming capabilities of a number of adenovirus serotypes (Trentin et al., 1962, Huebner et al., 1962, Girardi et al., 1964, Larson et al., 1965, Huebner et al., 1965 and Freeman et al. 1967), and analysis of the G + C content of the DNAs of the different serotypes (Lacy and Green, 1964, Lacy and Green, 1965 and Lacy and Green, 1967), Lacy and Green suggested that human adenoviruses be subclassified as one of three groups, based on the two properties of oncogenicity and G + C content. Highly oncogenic Group A viruses (serotypes 12, 18, 31) were very tumourigenic in animals and had a G + C content of 48-49%. Group B

viruses (serotypes 3, 7, 11, 14, 16 and 21) were weakly oncogenic in animals, producing fewer tumours and requiring a greater incubation period. These serotypes had a G + C content of 50-52%. Group C viruses, consisting of the remaining serotypes which had been described at that time, did not produce any tumours in test animals, but could transform cells in vitro, and had a somewhat higher G + C content of 55-61%. A later study by Piña and Green (1968), however, found that the highly oncogenic simian adenovirus SA7 had a significantly higher G + C content than that of the oncogenic human viruses, suggesting that the correlation between oncogenicity and G + C content was imperfect and that G + C content was probably not indicative of a particular biological property. That waiver aside, this classification scheme agreed to a large extent with that of the haemagglutination groups described above, with Group A serotypes possessing subgroup 4 agglutination characteristics, and Group B serotypes being of haemagglutination subgroup 1. Group C viruses were a mixture of subgroups 2 and 3 with respect to agglutination properties and this was later revised so that Group C currently refers to the haemagglutination subgroup 3 serotypes 1, 2, 5 and 6 and Group D to the subgroup 2 serotypes (Green, 1970).

The subdivision of human adenoviruses into these four groups (A-D) was consistent with DNA homology studies, which indicated a significantly higher degree of homology by DNA-DNA hybridization when comparing serotypes within a group (generally greater than 85% homologous, except within Group A, where it is 48-69%) to serotypes in two different groups (10-35%) (Lacy and Green, 1967, Garon et al., 1973 and Green et al., 1979a). In addition, the DNA homology studies indicated that Ad4 represented a unique serotype and it was suggested that it be classified as a member of a new group, Group E (Green et al., 1979a).

The validity of the Group A-D subdivisions, and the separate status for Ad4 as a member of Group E, was confirmed in studies comparing the numbers and molecular masses of the virion structural proteins by SDS-polyacrylamide gel electrophoresis, and by comparing restriction enzyme patterns from DNA digests of the viral genomes (Wadell, 1979 and Wadell et al., 1980). In addition, the more recently isolated enteric adenovirus serotypes 40 and 41 were characterized as a new subclass of serotypes, Group F, using these two criteria (Wadell et al., 1980 and Wadell et al., 1986). The above information on the classification of human adenoviruses is outlined in Table 1.

Table 1

Classification of human adenoviruses.

Sub-genus	Species	DNA			Apparent molecular weight of the major internal polypeptides			Hemagglutination pattern[3]	Oncogenicity in newborn hamsters
		Homology (%)[1]	G+C %	# of SmaI fragments[2]	V	VI	VII		
A	12,18 31	43-69 (8-20)	48	4-5	51-51.5K 46.5-48.5K[4]	25.5-26K	18K	IV	High (tumours in most animals in 4 months
B[5]	3,7,11,14, 16,21,34, 35	89-94 (9-20)	51	8-10	53.5-54.5K	24K	18K	I	Weak (tumours in few animals in 4-8 months
C	1,2,5,6	99-100 (10-16)	58	10-12	48.5K	24K	18.5K	III	nil
D[5]	8,9,10,13, 15,17,19, 20,22,23, 24,25,26, 27,28,29, 30,32,33, 36,37,38,39	94-99 (4-17)	58	14-16	50-50.5K[6]	23.2K	18.2K	II	nil
E	4	(4-23)	58	16-19	48K	24.5K	18K	III	nil
F	40	n.d.	n.d.	9	46K	25.5K	17.2K	IV	nil
G	41	n.d.	n.d.	11-12	48.5K	25.5K	17.7K	IV	nil

not classified: Ad 42

1 Per cent homology within the group and in brackets: homology with members of other groups

2 DNA fragments were analysized on 0.8-12% agarose gels. DNA fragment smaller than 400bp not resolved

3 I, complete agglutination of monkey erythrocytes ; II complete agglutination of monkey erythrocytes; III, partial agglutination of rat erythrocytes (fewer receptors); IV agglutination of rat erythrocytes only after addition of heterotypic antisera.

4 Polypeptide V of Ad 31 was a single band of 48K

5 Only DNA restriction and polypeptide analysis have been performed on Ad 32 - Ad 39

6 Polypeptides V and VI of Ad8 showed apparent molecular weights of 45K and 22K respectively. Polypeptide V of Ad30 showed an apparent molecular weight of 48.5K.

Modified from Wadell (1980)

In summary, the 41 known serotypes of human adenoviruses have been subclassified as Groups A-F according to a number of biological or molecular properties. These include oncogenicity, ability to agglutinate red blood cells from rats or monkeys, degree of homology by DNA-DNA hybridization, number and molecular masses of the structural polypeptides, and restriction digestion pattern of the viral DNA. It has been noted that these different methods of grouping adenovirus serotypes gave subgroups which were essentially in agreement with each other, even though the assays depend, to some extent, on properties of different regions across the entire genome of the virus (Flint, 1981a). This has been taken to indicate that the subgroups do represent distinct genetic entities which diverged shortly after the divergence of the adenovirus host species (Waddel et al., 1980 and Flint, 1981a), a theory which is supported by the observation that, although genetic recombination may occur between members of a subgroup, it has never been detected between members of different subgroups (Flint, 1981a).

1.2 Structural Characteristics of the Virion

Adenoviruses are non-enveloped, icosahedral viruses, with a single linear molecule of double-stranded DNA as their genome (Green and Piña, 1963, Horne et al. 1959 and van der Eb and van Kesteren, 1966). Most of the research which has gone into determining the structural characteristics of the virus has involved serotypes 2 or 5, which are very similar to each other. As these two serotypes are representative of adenoviruses as a group, both with respect to the structure of the virion and genomic organization (Horwitz, 1990a), the information outlined in the following two sections, although derived from Ad2 or Ad5 studies, is considered valid for adenoviruses in general.

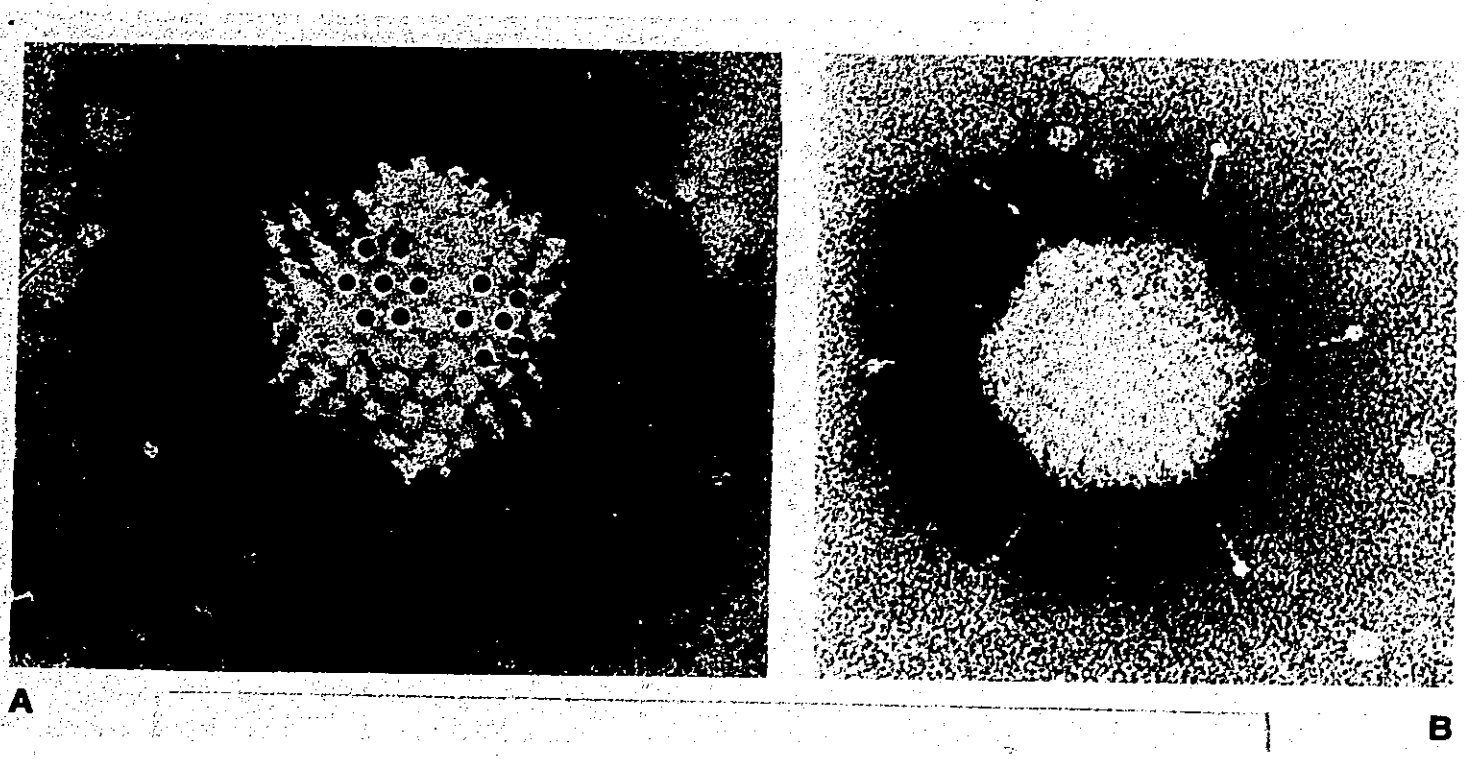
1.2.1 Virion Capsid

The capsid of the virus, which encloses the genomic material, has been shown by electron microscopy

to be icosahedral in shape, and approximately 70 nm in diameter. The capsid is made up of 252 distinct spherical protein subunits, termed capsomers, which are each approximately 7 nm in diameter (Horne et al., 1959). Each of the twenty facets of the icosahedron forms an equilateral triangle with six capsomers along each side (Figure 1a), and from each of the 12 vertices formed where the edges of any five facets converge, a proteinaceous fiber projects (Figure 1b). The fiber has a length of approximately 20 nm which differs slightly from serotype to serotype, a diameter of ~2 nm, and has a knob on the end distal to the capsid, of diameter 4 nm (Valentine and Pereira, 1965). Of the 252 capsomers which make up the capsid, 240 are situated so that each is surrounded by 6 others, and are therefore termed hexon capsomers, or hexons.

Figure 1

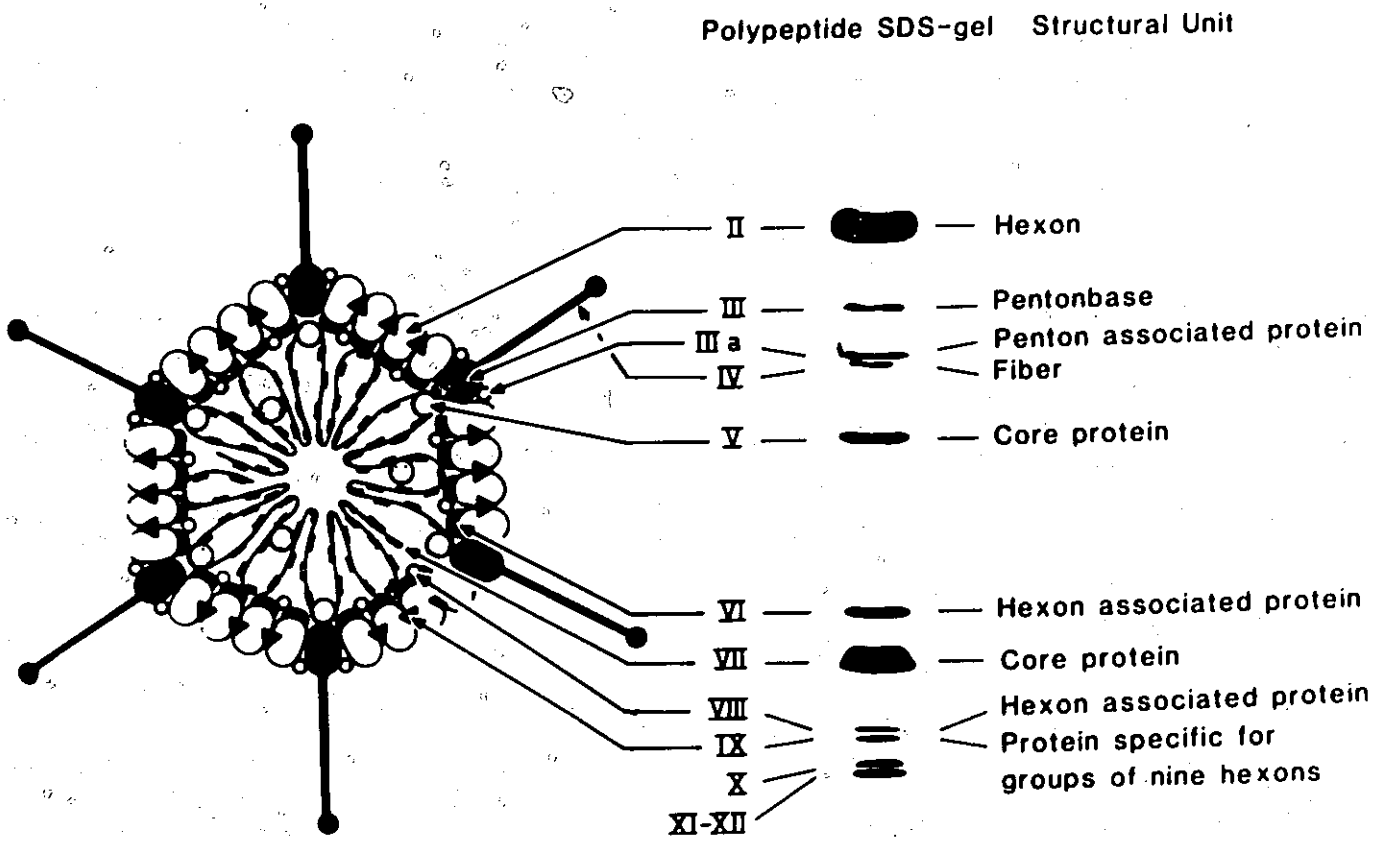
Electron micrographs of adenovirus serotype 5. **A:** The virion is an icosahedron. One of the 240 hexons surrounded by six identical hexons and one of the 12 pentons surrounded by 5 hexons are labeled. **B:** Six of the 12 fibers per virion are shown projecting from the vertex penton capsomers. Adapted from Horwitz, 1990a.


A
B

The 12 capsomers at the vertices are each surrounded by only 5 others, and are termed pentons. Each penton consists of a penton base plus its projecting fiber, and these two components can be isolated as separate entities from infected cells (Valentine and Pereira, 1965 and Ginsberg et al., 1966). Hexon, penton base and fiber are the three main soluble antigens found in infected cells, and all are produced in great excess (Flint, 1981a). Analysis of virion proteins by SDS-polyacrylamide gel electrophoresis has resulted in the identification of a number of other, less abundant virion components (Maizel et al., 1968a, Everitt et al., 1973, 1975). The relative gel mobilities of the virion proteins and a model of their arrangement in the virion capsid and core is illustrated in Figure 2 (from Horwitz, 1990a).

Figure 2

Schematic representation of an adenovirus. The mobilities and relative amounts of each protein after electrophoresis of the dissociated virus on a SDS-polyacrylamide gel are shown on the right. The roman numerals refer to a polypeptide designation described by Maizel et al. (1968). Adapted from Everitt et al., 1975.


Polypeptide SDS-gel
Structural Unit
II
Hexon
III
Pentonbase
IIIa
Penton associated protein
IV
Fiber
V
Core protein
VI
Hexon associated protein
VII
Core protein
VIII
IX
Hexon associated protein
Protein specific for
groups of nine hexons
X
XI-XII

1.2.2 Hexon

Hexon protein (polypeptide II) represents 51% of the protein present in the virion (Maizel, 1968b), and is the major group-specific antigen (Valentine and Pereira, 1965), although it may also possess some type-specific or subgroup (haemagglutinin) specific epitopes (Flint, 1981a). The hexon capsomer is elliptical in shape, with 11-12.5nm by 8-8.5nm dimensions by electron microscopy (Horne and Wildy, 1961). Based on the dimensions, the original estimate of molecular mass was ~210 kilodaltons (kD) (Valentine and Pereira, 1965), but analysis of purified hexon capsomers using methods such as sedimentation-diffusion, sedimentation-equilibrium, light scattering, and X-ray diffraction have given mass values of 313-380kD (Flint, 1981a). By SDS-polyacrylamide gel analysis, purified hexon capsomers contain only one protein component, of molecular mass 110-120kD, suggesting that each hexon capsomer consists of three non-covalently bound identical subunits (Maizel *et al.*, 1968a, Everitt *et al.*, 1973, 1975 and Furcinitti *et al.*, 1989).

1.2.3 Penton Base

The penton base (polypeptide III), which is a spherical capsomer of diameter 8 nm found only at the vertices of the adenovirus capsid, possesses both group and sub-group specific antigenic properties (Valentine and Pereira, 1965) and constitutes about 5% of the virion protein (Maizel *et al.*, 1968b). Purified penton base alone added to tissue culture cells may cause the cells to round up and detach from the plate, suggesting that this virion component is primarily responsible for the specific cytopathic effects seen in adenovirus infected cells (Pettersson and Högland, 1969). Penton base has also been reported to have a closely associated double-stranded or single-stranded endonuclease activity (Flint, 1981a), although more recently this activity has been seen to be dissociable from the penton base protein (Horwitz, 1990a), indicating that it is not intrinsic. The predicted molecular mass of the penton base capsomer, based on physical dimensions under the electron microscope is 210kD (Valentine and Pereira, 1965), and molecular mass determination by SDS-polyacrylamide gel was 70kD (Maizel *et al.*, 1968a), suggesting that each

penton base capsomer consists of more than one polypeptide.

1.2.4 Fiber

The fiber (polypeptide IV), which projects from the penton base at each of the 12 vertices of the capsid, constitutes 4% of the protein present in the virion (Maizel et al., 1968b) and has both subgroup and type specific properties associated with the tail and the distal knob respectively (Pettersson et al., 1968). It has been suggested that the knob functions in attachment of the virion to target cells (Valentine and Pereira, 1965). By electron microscopy the fiber dimensions have been shown to be 2nm by 20nm with the knob having a diameter of 4nm (Valentine and Pereira, 1965) and the molecular mass of the intact fiber by sedimentation estimated at 183kD (Dorsett and Ginsberg, 1975). By SDS-polyacrylamide gel electrophoresis the fiber protein has a molecular mass of about 60kD (Maizel et al., 1968a and Weber et al., 1989), and it has been shown to contain two residues of N-acetylglucosamine. The Ad2 fiber protein consists of 581 amino acids and is thought to form the intact fiber by dimerization (Philipson, 1983). Using a monoclonal antibody specific for an epitope near the

amino terminus of the fiber protein, Weber et al. (1989) demonstrated that the amino terminus of the protein was embedded in the penton base in the intact virion, with the remainder of the protein projecting outward to form the body of the fiber.

1.2.5 Minor Capsid Components

Other minor protein components of the capsid include polypeptide IIIa, which associates with the peripentonal hexons (Flint, 1981a), polypeptide IX which acts as a cement between hexon capsomers and binds them together in groups of nine (Everitt, 1973 and Furcinitti et al., 1989), and polypeptides VI and VIII, which are also hexon-associated and line the capsid interior (Horwitz, 1990a).

1.2.6 Virion Core

The capsid of the virus encloses the viral core, which consists of the viral genome and its associated proteins. The adenovirus genome is a single linear molecule of double stranded DNA which is approximately 35 kilobase pairs in length and has a molecular mass of 20-25 X 10^6 Daltons (Green and Piña, 1963, van der Eb and

van Kesteren, 1968 and Wu et al., 1977). The ends of the DNA molecule have inverted terminal repeat regions of approximately 100 nucleotides (Wolfson and Dressler, 1972), and the 5' end of each DNA strand has a 55kD viral protein covalently attached to it via its terminal dCMP residue (van der Eb et al., 1969, Sharp et al., 1976 and Rekosh, et al., 1977).

In the virion, adenovirus DNA is thought to be folded as a regular array of nucleosome-like subunits maintained through an association with the major core protein, polypeptide VII, which represents about 20% of the total virion protein (Maizel et al., 1968a, 1968b, Everitt et al., 1973, 1975 and Flint, 1981a). Two other DNA binding proteins, polypeptide V and protein μ, associate with the virion core as minor components which may help to condense and stabilize the core structure (Harpst et al., 1977, Vayda et al., 1983 and Lunt et al., 1988).

1.3 Organization of the Viral Genome

As described above, adenovirus serotypes 2 and 5 have a double-stranded linear genome. Shearing of the genomic DNA followed by analysis of each of the molecular halves has resulted in these being designated as the left

half or the right half of the genome based on the convention that the left half has a higher G + C content (60% G + C versus 53% for the right half of Ad2) (Green, 1970). Again by convention, nucleotides are numbered from the left end of the genome, and the two DNA strands are referred to as the r and l strands, according to whether transcription proceeds along them to the right or to the left (Sharp et al., 1975).

In early studies, using DNA-RNA hybridization techniques, Green et al. (1971) found that in productively infected cells, 80-100% of the adenoviral genome was transcribed, and that there were two waves of m-RNA production. Early messages, which were produced prior to DNA replication, hybridized to both the l and r strands, and late messages produced after DNA replication were transcribed mainly from the r strand. Sharp et al. (1975), again using DNA-RNA hybridization, but on restriction enzyme digested fragments of the viral genome, reported that early messages were produced from four discrete regions of the genome, two on the l strand and two on the r strand. Late messages, which coded for the structural proteins of the virion, were primarily produced from a single region on the r strand which lay between map units 30 and 95 on the genome (Sharp et al., 1975 and Chow et al., 1977b). The presence of

independent promoters for each of the early regions, termed early regions 1-4 (E1-E4), was demonstrated through ultra-violet mapping of the sites of initiation of the messages (Berk and Sharp, 1977).

By in vitro selection and translation of messages which hybridized to specific DNA fragments (Lewis et al., 1976), and by using electron microscopy to determine which regions of the genome selected messages hybridized to (Chow et al., 1977b), the number and size of the messages coming from both early and late regions was determined. This type of analysis demonstrated for the first time that messages of different sizes, and coding for different proteins, could come from the same gene, thus providing the first evidence for processing of nuclear RNA into spliced m-RNA products. It also indicated that there were two sites of initiation for RNA transcription within the E1 region, suggesting that it should be subdivided into Early region 1a (E1a) and E1b (Lewis et al., 1976).

Further research, using additional techniques such as S1 nuclease mapping and DNA sequencing, provided a greater understanding of how the adenovirus genome was organized, where nuclear transcripts were initiated and terminated, and the locations and number of splice junctions present in the processed transcripts. Figure 3

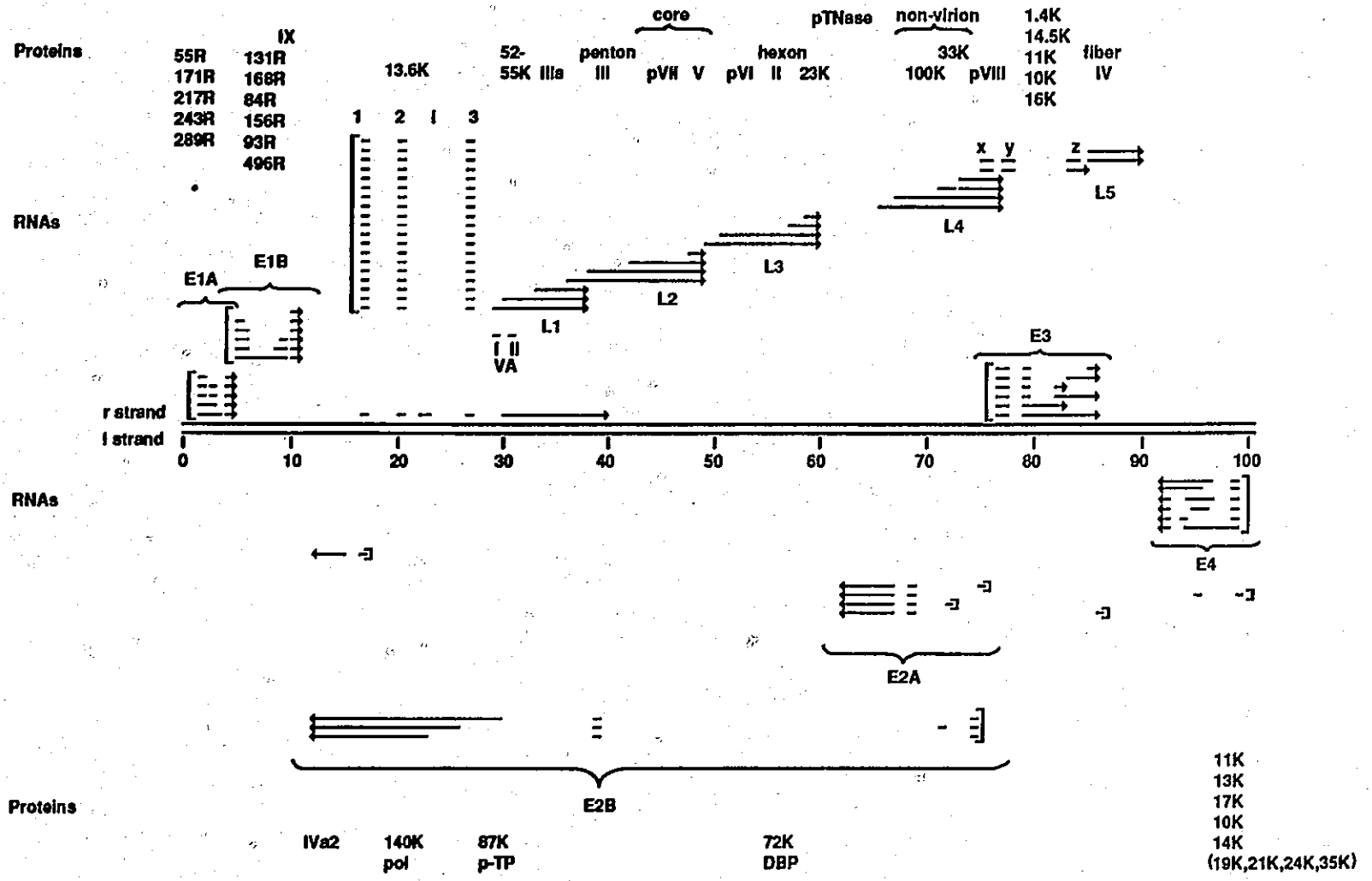
represents a schematic map of the Ad2 genome, indicating the location of early and late genes, their messages, and the protein products, where known. A brief description of the gene products follows, and a more detailed analysis of the known functions of the proteins, particularly the E1 proteins, in lytic infection and in transformation, is provided later.

1.3.1 Region E1a

Early Region 1, at the left end of the genome, encompasses two independently promoted genes transcribed from the r strand; E1a, which is located between 1.3 and 4.5 map units, and E1b, which is found between 4.6 and 11.2 map units (Horwitz, 1990a). The E1a region produces

Figure 3

Transcription and translation map of adenovirus type 2. Adapted from Horwitz, 1990a.


Proteins
IX
55R
171R
217R
243R
289R
131R
168R
84R
156R
93R
496R
13.6K
core
pTNase
non-virion
1.4K
14.5K
11K
10K
16K
52-
55K IIIa
penton
III
pVII V
hexon
pVI II 23K
33K
100K pVIII
fiber
IV
RNAs
1 2 I 3
x y
z
L5
L4
L3
L2
L1
E1A
E1B
I II
VA
E3
r strand
l strand
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A
E2B
Proteins
IVa2
140K
pol
87K
p-TP
72K
DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

a single nuclear transcript, which is spliced to produce five messages. These messages are referred to by their sedimentation values as 13S, 12S, 11S, 10S and 9S, and are translated from the same initiation codon to produce proteins which all share the same amino terminus (Perricaudet et al., 1979, Green et al., 1979b, Virtanen and Pettersson, 1983a, Stephens and Harlow, 1987 and Ulfendahl et al., 1987). The four largest messages, of which the 13S and 12S predominate, differ with respect to the number and size of their internal splices, but share the same reading frame across each message so that the proteins produced are highly related throughout the length of the polypeptide (Perricaudet et al., 1979, van Ormondt et al., 1980 and Smart et al., 1981). These four messages produce a family of acidic nuclear phosphoproteins which migrate on SDS-polyacrylamide gels as proteins with molecular masses of 52kD, 48.5kD (from the 13S message), 50kD and 45kD (from the 12S message) (Green et al., 1979b, Rowe et al., 1983b and Yee et al., 1983) and 35kD and 30kD from the 11S and 10S messages respectively (Stephens and Harlow, 1987 and Ulfendahl et al., 1987). The 9S message splices into a different reading frame from that of the other four messages, and has been predicted from its sequence to produce a protein of molecular mass 6.1kD which has the same amino terminal

27 amino acids as the products of the other messages but has an entirely different sequence following residue 28 (Virtanen and Pettersson, 1983a). A 28kD protein has been reported to be the product of *in vitro* translation of the 9S message (Spector *et al.*, 1980 and Esche *et al.*, 1980), but the relationship between this protein and the predicted *in vivo* 9S product is unclear (Smart *et al.*, 1981 and Virtanen and Pettersson, 1983a).

1.3.2 Region E1b

The E1b region produces two major messages. One, of length 2.2kb predominates early in infection and the other, of length 1.05kb accumulates later in infected cells (Perricaudet *et al.*, 1980, Bos *et al.*, 1981 and Glenn and Ricciardi, 1988). Two minor messages, of length 1.26kb and 1.31kb are also produced (Virtanen and Pettersson, 1983b, Anderson *et al.*, 1984 and Lewis and Anderson, 1987). All four of these messages, when translated from the first initiation codon, produce a 176 residue protein, which migrates as a 19kD doublet in SDS-polyacrylamide gels (Rowe *et al.*, 1983a, McGlade *et al.*, 1987 and McGlade *et al.*, 1989). All four messages may also be translated from the second initiation codon and in a different reading frame to produce, due to

differential splicing, a family of proteins which are related to each other but not to the 19kD protein. In Ad5 these are a 496 residue (58kD) protein from the 2.2kb message, a 156 residue protein (25kD doublet) from the 1.26kb message, a 93 residue protein (about 20kD) from the 1.31kb message and an 84 residue (20kD) protein from the 1.05kb message. These four related proteins share 79 common amino terminal residues, and the 1.26kb message splices back into the same reading frame as the 2.2kb message so that the 156 residue protein and the 58kD protein share 78 common carboxy terminal residues. The 1.31kb and 1.05kb messages are spliced into different reading frames which gives each of the resulting proteins a unique carboxy terminus (Bos et al., 1981, Green et al., 1982, Matsuo et al., 1982, Lewis and Anderson, 1987 and Brown et al. (submitted)).

1.3.3 Region E2

Region E2 is transcribed from the l strand of the genome from map unit positions 67.9 to 61.5 (E2a) and 29 to 14.2 (E2b) (Horwitz, 1990a). The transcripts from E2a and E2b are produced coordinately from the same promoter, and share leader sequences, but utilize different polyadenylation sites (Berk and Sharp, 1978). E2a

transcripts are found at levels about 100 fold greater than those of E2b, possibly due to preferential use of the E2a polyadenylation site (Stillman et al., 1981). Early in infection this region is transcribed from a promoter found near map position 68, and late in infection transcription is promoted from further upstream, as far as 87 or 99 map units (Chow et al., 1980). The E2a transcripts code for the 72kD DNA binding protein which is essential for viral replication (Lewis et al., 1976), and the E2b transcripts code for both the 105kD viral DNA polymerase (Alestrom et a., 1982 and Stillman et al., 1982) and the 80kD precursor to the 55kD terminal protein (Stillman et al., 1981 and Smart and Stillman, 1892), both of which are also required for viral replication.

1.3.4 Region E3

The E3 region, transcribed from the r strand of the genome, lies between map unit positions 76.6 and 86.2 (Horwitz, 1990a). It produces at least nine messages, all of which share the same 5' untranslated region and all of which, through the use of alternative splice sites and one of two possible polyadenylation sites, have different open reading frames (Carlin et al., 1989 and

Ginsberg et al., 1989). This region is not essential for viral growth in tissue culture cells, but appears to produce products which function to benefit the virus in some way during infection of the normal host. Four unique proteins have been identified in vivo from this region, and three of these have been at least partially characterized with respect to function. These include a 19kD glycoprotein (Chow et al., 1980, Burgert and Kvist, 1987 and Ginsberg et al., 1989), a 14.7kD protein (Gooding et al., 1988 and Ginsberg et al., 1989), a 11.6kD protein (Ginsberg et al., 1989) and a 10.4kD protein (Carlin et al., 1989 and Ginsberg et al., 1989).

1.3.5 Region E4

Early region 4 is transcribed from the l strand of the genome between map units 99.3 and 91.3, and produces at least nine messages which have the same initiation and polyadenylation sites but differ with respect to internal splicing (Chow et al., 1980). At least five proteins have been detected in vivo fom this region. These have molecular masses of 14kD (Downey et al., 1983), 19kD and 21kD (Harter and Lewis, 1978), and 17kD and 34kD (Cutt et al., 1987).

1.3.6 Late Region

Following DNA replication, messages are produced primarily from the late region, which is transcribed off of the r strand of the genome (Sharp et al., 1975). This region, which codes for most of the virion structural proteins, produces a single nuclear transcript late in infection which extends from map position 16.4 to 99 (Berget and Sharp, 1979 and Shaw and Ziff, 1980). The twenty or more messages which come from this transcript share a common tripartite leader segment (Chow et al., 1977a and Klessig, 1977) but terminate at one of five polyadenylation sites to give five "families" of messages (L1-L5) (Chow et al., 1980), which are spliced in different ways to produce a large number of open reading frames (Lewis et al., 1977). Messages from the L1 region appear early in infection, indicating that the major late promoter is active early as well as late (Shaw and Ziff, 1980). At early times, however, the primary transcript from the major late promoter terminates within the L2 region (Akusjärvi and Persson, 1981). The L1 message which predominates early in infection is that which codes for two related proteins of molecular masses 52kD and 55kD (Chow et al., 1980). More than half of these

messages include an extra leader sequence, the i sequence (Chow et al., 1980), which interferes with normal translation, possibly by introducing its own open reading frame upstream of the one normally utilized (Akusjärvi and Persson, 1981). Later in infection, the L1 transcript is preferentially spliced to produce the message for structural protein IIIa (Chow et al., 1980 and Akusjärvi and Persson, 1981). The L2-L5 mRNA families begin to be produced at the onset of viral DNA replication, and their production is dependent on this event (Shaw and Ziff, 1980). The L5 messages may also have leader sequences x, y and z in addition to the tripartite leader (Zain et al., 1979).

Two virion structural proteins, protein IX and protein IVa_2, are transcribed at intermediate times during infection from genes which are promoted independently of the major late promoter. Protein IX, which is a hexon-associated protein, is coded for on the r strand of the genome, between map units 9.4 and 11.1 (Pettersson and Mathews, 1977). Protein IVa_2 is a maturation protein translated from a message transcribed from the l strand of the genome, between map positions 18.1 and 11.3 (Persson et al., 1979).

1.3.7 VA RNAs

Two small RNA species which do not code for proteins are transcribed by the host Pol III RNA polymerase from the r strand of the genome at about map position 30 (Weinmann et al., 1976 and Horwitz, 1990a). These VA (virus-associated) RNAs possess considerable secondary structure (Weinmann et al., 1976), and it has been suggested that they may function in promoting the splicing of some viral messages (Horwitz, 1990a) or in positively regulating translation of viral mRNAs (Svensson and Akusjärvi, 1984). With the exception of these VA RNAs, all other adenovirus genes are transcribed by the host Pol II polymerase (Price and Penman, 1972).

1.4 Productive Infection

Human Adenovirus serotype 5 normally infects the epithelial cells of the upper respiratory tract (Horwitz, 1990b), and so the lytic infectious cycle of the virus has mainly been studied in tissue culture cells of epithelial origin, such as KB or HeLa cells. The infectious cycle may be divided into a number of stages, beginning with adsorption of the virus to the target

cell, and penetration of the plasma membrane, followed by uncoating of the virion and transport of the viral DNA to the cell nucleus. In the nucleus, early transcription takes place, followed by viral DNA replication and late transcription, resulting in the accumulation of virion structural proteins. Assembly and release of the mature virions completes the lytic infectious cycle.

1.4.1 Adsorption, Penetration and Uncoating

Ad5 virions bind to the external surface of target cells through a highly specific and non-energy requiring interaction between the virion fiber protein, and a host cell receptor (Lonberg-Holm and Philipson, 1969). This adsorption can be inhibited by the addition of purified fiber protein to the cells prior to virus challenge, but is unaffected by the addition of other virion structural components (Levine and Ginsberg, 1967). Using fiber protein alone, or fiber protein attached to penton base, or whole virions as affinity substrates, a number of groups have identified three cell surface glycoproteins which constitute major adenovirus receptor components. These have mobilities on SDS-PAGE gels corresponding to molecular masses of 70kD, 40kD and 34kD (Meager et al., 1976, Hennache and Boulanger, 1977 and

Svensson et al., 1981). There are estimated to be 10^5 to 10^6 receptors per cell, but the maximum number of virions which can attach to one cell is in the range of 10^4 (Philipson et al., 1968), suggesting multivalent binding by each virion (Patterson and Russel, 1983). Following initial receptor binding the adsorbed virus particles are redistributed over the cell surface into clusters or "caps" (Persson et al., 1983 and Patterson and Russel, 1983). This is an energy requiring process which Patterson and Russel (1983) have suggested is mediated by the cytoskeleton, and which appears to play an important role in efficient viral penetration. Analysis of infected cells by electron microscopy has indicated that the virus may penetrate the plasma membrane of the cell directly (Morgan et al., 1969), or may be endocytosed in coated or uncoated vesicles (Chardonnet and Dales, 1970a and Patterson and Russel, 1983). The half-life of this process is 15 minutes (Lonberg-Holm and Philipson, 1969), and it involves loss of some of the virion capsid, possibly the fiber and penton base components, leaving the internalized virion with a core which is slightly sensitive to DNase (Sussenbach, 1967 and Svensson and Persson, 1984). The internalized virion is transported rapidly to the nucleus by a mechanism which is thought to involve an association with microtubules (Chardonnet and

Dales, 1972 and Dales and Chardonnet, 1973). At the nuclear membrane, further uncoating of the virion is accomplished, and the core is released. This may require a nuclear pore-associated ATPase activity (Chardonnet and Dales, 1972 and Dales and Chardonnet, 1973). The viral DNA enters the nucleus via the nuclear pores (Chardonnet and Dales, 1970a, 1972). The entire process of adsorption, penetration and nuclear uptake of the viral genome is complete 60 to 90 minute after infection at 37°C (Chardonnet and Dales, 1970a,b).

1.4.2 Early Transcription

Early transcripts are defined as those which appear before the beginning of DNA synthesis, and which can be detected in the presence of inhibitors of DNA synthesis. These come primarily from the four early regions E1-E4, although low levels of transcription from some late regions have been detected. Messages from region L1 and a region between map units 17 and 21.5 can be detected early in infection, but at levels which are extremely reduced compared to those expressed after DNA replication (Lewis and Mathews, 1980 and Nevins and Wilson, 1981). Similarly, transcripts from two independently promoted regions coding for structural

proteins IVa2 and polypeptide IX begin to appear at intermediate times, just prior to DNA synthesis, but increase dramatically at later times (Chow et al., 1980 and Horwitz, 1990a). During early infection, viral RNA represents 0.1% or less of the total cell RNA, but viral messages constitute 5-18% of the cytoplasmic mRNA (Flint and Broker, 1981). Of the five early regions, E1a is transcribed first, followed temporally by E2, E3, E4 and E1b (Glenn and Ricciardi, 1988). The early phase of viral transcription ends with the onset of DNA replication at about 8 hours post infection in KB or HeLa cells, although transcripts from early regions may still be produced at late times (Chow et al., 1980 and Flint and Broker, 1981).

1.4.3 Early Proteins: Function in Lytic Infection

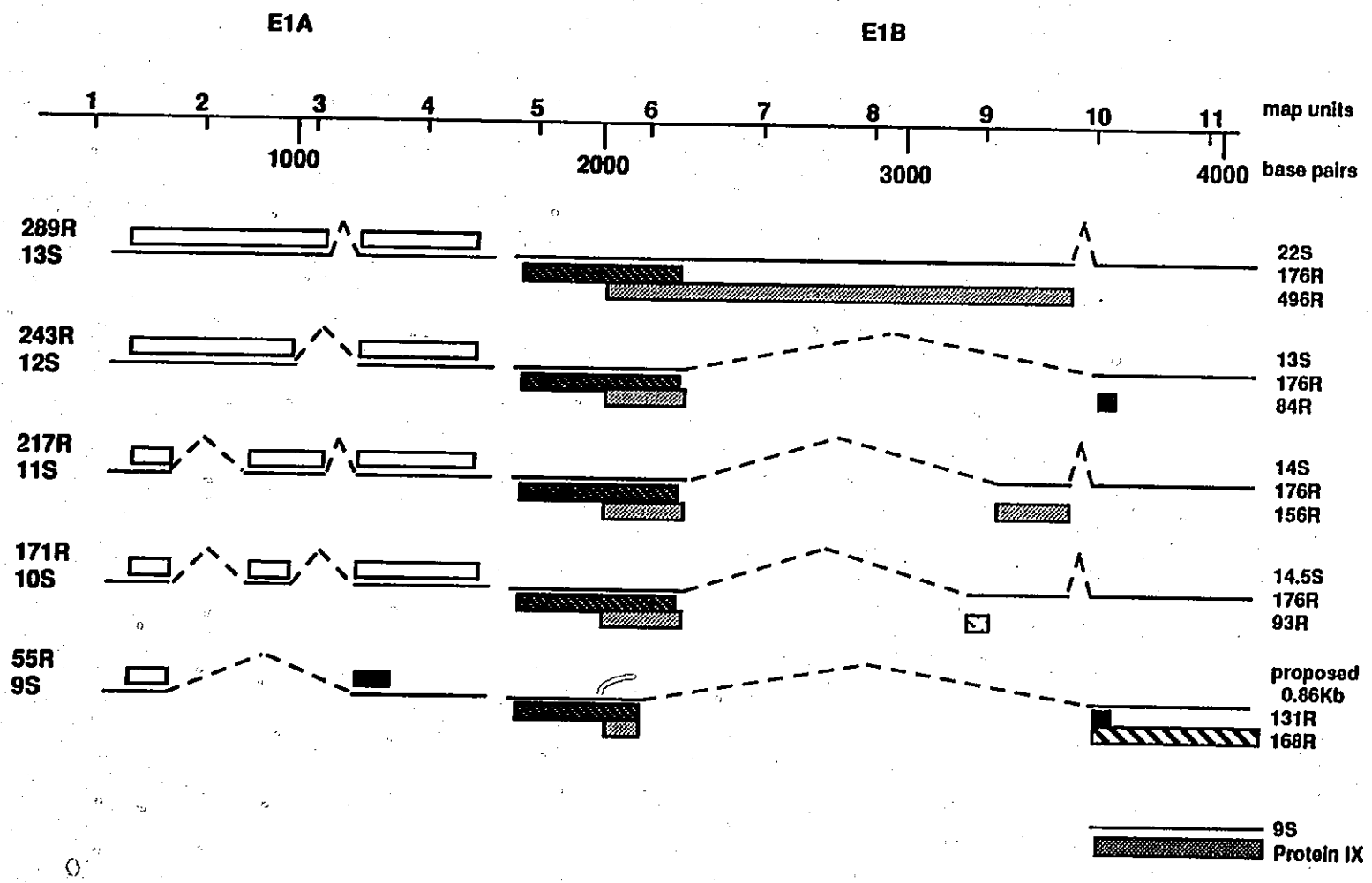
E1a Proteins

The E1a proteins are the first of the viral early gene products to appear in infected cells, and can be detected within two hours of infection (Rowe et al., 1984b). The protein species which predominate early in infection are translated from the 13S and 12S messages (Chow et al., 1980 and Glenn and Ricciardi, 1988). The products of the 11S and 10S messages are minor species

which are produced primarily late in infection (Stephens and Harlow, 1987 and Ulfendahl et al., 1987). As previously described, these four messages share common 5' and 3' ends and are translated in the same reading frame, but differ with respect to the size and number of their internal splices (Figure 4). They produce a highly related, heterogeneous group of phosphoproteins which migrate as a series of bands on one dimensional SDS-PAGE gels with mobilities corresponding to molecular masses of 52kD, 48.5kD (from the 13S message), 50kD and 45kD (from the 12S message) (Green et al., 1979b, Rowe et al., 1983b and Yee et al., 1983) and 35kD and 30kD from the 11S and 10S messages respectively (Stephens and Harlow, 1987 and Ulfendahl et al., 1987). On two-dimensional SDS-PAGE gels, this heterogeneity is even more pronounced, and it has been suggested that E1a products can be resolved into as many as 60 polypeptide species (Harlow et al., 1985). Studies on the post-translational modifications of these proteins indicate that most, if not all of this heterogeneity is due to differential phosphorylation (Yee et al., 1983, Tsukamoto et al., 1986b, Tremblay et al., 1988, Richter et al., 1988 and Tremblay et al., 1989). The molecular mass shift of about 4kD from the faster to the slower migrating species of each of the 12S and 13S products is brought about primarily by a single

Figure 4

Transcription and translation map of early region 1 of adenovirus type 5. (With permission of P.E. Branton)


E1A
E1B
1
2
3
4
5
6
7
8
9
10
11
map units
1000
2000
3000
4000
base pairs
289R
13S
22S
176R
496R
243R
12S
13S
176R
84R
217R
11S
14S
176R
156R
171R
10S
14.5S
176R
93R
55R
9S
proposed
0.86Kb
131R
168R
9S
Protein IX

phosphorylation event at serine residue 89 (Dumont et al., 1989 and Smith et al., 1989), although this shift in mobility may also be contributed to by a phosphorylation event near the carboxy terminus of the protein (Richter et al., 1988).

E1a proteins have a very short half-life of about 40 minutes (Branton and Rowe, 1985) and following translation, they are rapidly localized to the nucleus by the nuclear transport system in the cell which recognizes a pentapeptide nuclear localization signal at the carboxy terminus of the E1a polypeptides (Krippl et al., 1984, Ferguson et al., 1985, Lyons et al., 1987 and Yamasaki et al., 1989). In cell fractionation studies, E1a proteins have been found in both nuclear and cytoplasmic fractions (Rowe et al., 1983, Feldman and Nevins, 1983, Branton et al., 1984 and Chatterjee and Flint, 1986), but using indirect immunofluorescence, it has been shown that they are primarily localized in the nucleus (Feldman and Nevins, 1983, Yee et al., 1983 and Schmitt et al., 1987). This nuclear association may be fragile and easily disrupted by cell fractionation techniques (Schmitt et al., 1987). A portion of the 13S gene product has been found to associate with the nuclear matrix (Feldman and Nevins, 1983, Chatterjee and Flint, 1986 and Schmitt et al., 1987).

One of the primary functions of E1a proteins in lytic infection is to activate transcription from the other four early regions (Jones and Shenk, 1979a and Berk et al., 1979). The E1a promoter itself, which is active in the absence of viral gene products, can be up-regulated by the E1a proteins (Berk et al., 1979 and Nevins, 1981). In the absence of E1a proteins, only very low levels of other early transcripts are detectable and viral replication is severely restricted (Katze et al., 1981 and Nevins, 1981). The products of both the 12S and 13S messages act as transactivators early in infection, but the 13S products are much more efficient (Winberg and Shenk, 1984, Ferguson et al., 1985, Moran et al., 1986, Ferguson et al., 1986 and Fahnestock and Lewis, 1989). E1a proteins do not bind DNA directly, but are thought to act by mediating the effect of transcription factors which bind to the promoter regions of the other early genes (Kovesdi et al., 1986, Wu et al., 1987, Fahnestock and Lewis, 1989 and Pei and Burk, 1989). There is no single common promoter region or transcription factor through which E1a proteins exert their effect on target genes, although some factors such as ATF and E2F are involved in the transactivation of more than one of the viral promoters (Jones et al., 1988). Generally, transcriptional activation by E1a proteins can be

effected in the absence of new cell protein synthesis, and it has been suggested that E1a proteins activate transcription factors which are already present in the cell rather than induce synthesis of new factors (Kovesdi et al, 1986, Jalinot et al., 1987, Reichel et al., 1988, Pei et al., 1989 and Bagchi et al., 1989). This activation may involve a post-translational modification, such as phosphorylation, to alter the DNA binding capabilities of the factor (Reichel et al., 1988, Sassone-Corsi, 1988, Yamamoto et al., 1988, Raychaudhuri et al., 1989 and Bagchi et al., 1989), or it could involve bringing appropriate factors together to form an active complex (Jalinot et al., 1987).

The E1a 13S gene product, which is a more potent transactivator than the 12S product, has a unique internal sequence of 46 amino acids. This region, which by itself has been shown to be sufficient to transactivate other viral genes (Lillie et al., 1987, Green et al., 1988 and Lillie and Green, 1989), consists of an activation domain and a recognition domain separated by a metal-binding domain which has a zinc-finger motif (Culp et al., 1988, Lillie and Green, 1989 and Fahnestock and Lewis, 1989). Mutations in any one of these three domains severely reduces the ability of 13S products to transactivate (Glenn and Ricciardi, 1987,

Jelsma et al., 1988, Green et al., 1988, Culp et al., 1988 and Fahnestock and Lewis, 1989). It has been suggested that this 46 amino acid region acts to bring 13S proteins to the promoter of a target gene through an interaction between the recognition domain and a transcription factor bound to the promoter. In this model the activation domain, once it has been positioned at the promoter, functions to enhance transcription (Lillie and Green, 1989). The zinc finger, a motif found in a number of transcription factors (Mitchell and Tijan, 1989), may contact the DNA in such a way so as to promote the formation or increase the stability of the transcription complex on the DNA (Culp et al., 1988).

In addition to transcriptional activation of the other early genes, E1a proteins repress enhancer-driven transcription from their own promoter (Borrelli et al., 1984, Smith et al., 1986). It has been suggested that this function may become active as E1a proteins accumulate in early infection, and may allow them to down-regulate their own synthesis (Borrelli et al., 1984, Smith et al., 1986). Both the 13S and 12S gene products have been reported to repress enhancer driven transcription (Smith et al., 1986), but the 12S product appears to function more effectively in this capacity (Lillie et al., 1986 and Dery et al., 1987). The 12S

product has also been reported to repress transcription from the other early promoters (Guilfoyle et al., 1985 and Dery et al., 1987), and again, it has been suggested that this may function to reduce transcription from the other early promoters after E1a proteins have accumulated, and sometime following their early transactivation of these same promoters (Dery et al., 1987).

A third function of E1a proteins in lytic infection involves the stimulation of DNA synthesis in quiescent cells. Both the 12S and 13S products have been reported to be able to induce DNA synthesis in quiescent, non-transformed human cells (Spindler et al., 1985 and Kaczmarek et al., 1986) and in primary rat epithelial cells (Nakajima et al., 1987, Zerler et al., 1987, Quinlan and Grodzicker, 1987, Moran and Zerler, 1988 and Smith and Ziff, 1988). In G_0-arrested human WI-38 lung fibroblasts, expression of the 12S product was found to be required for maximal viral replication (Spindler et al., 1985). In the natural course of infection, the target cells for the virus are terminally differentiated epithelial cells of the upper respiratory tract, which are quiescent (Horwitz, 1990b). It has been suggested that the ability to induce cellular DNA synthesis in these cells is of benefit to the virus because it results

in more efficient viral DNA replication (Spindler et al., 1985 and Quinlan and Grodzicker, 1987).

E1a proteins also function to protect infected cells against the anti-viral action of interferons. Interferons are a class of proteins which can be produced by virus-infected cells, and which induce a cascade of events in neighbouring cells, designed to protect them against infection (Lengyel, 1982). The abrogation of this protection by E1a proteins enables the virus to replicate in interferon-treated cells, and as such is beneficial to the virus in lytic infection. E1a proteins have been reported to repress transcription of genes induced by interferon (Reich et al., 1988), and to directly or indirectly inhibit specific interferon-induced activities which would normally impair the replication of an interferon-sensitive virus such as vesicular stomatitis virus (Anderson and Fennie, 1987). E1a proteins also induce polymerase III transcription during infection (Yoshinaga et al., 1986), resulting in transactivation of the adenovirus VA RNA genes, at least one of which antagonizes the interferon-induced eIF2-α kinase (Kitajewski et al., 1986 and O'Malley et al., 1986).

E1b Proteins

The products of both of the open reading frames of this region (Figure 4) are required for productive infection in KB cells, as mutants affecting either the 19kD protein or the 58kD protein are host range (Harrison et al., 1977), and defective in viral DNA replication (Babiss and Ginsberg, 1984). The 19kD protein, which has been shown by indirect immunofluorescence to be primarily associated with the nuclear membrane and perinuclear region (Rowe et al., 1983 and McGlade et al., 1987) is modified both by fatty acid acylation (Grand et al., 1985 and McGlade et al., 1987) and by phosphorylation at a single serine residue (McGlade et al., 1989). Infection of cells with viruses carrying mutations in the 19kD coding region results in a cytocidal effect which gives a large plaque phenotype, and the rapid degradation of both cellular and viral DNA (Chinnadadurai, 1983, Mak and Mak, 1983, Subramanian et al., 1984, Takimori et al., 1984 and White et al., 1984). The 19kD protein has been reported to be involved in transactivation of the other early genes (Herrmann et al., 1987), and the relief of E1a-mediated enhancer repression (Yoshida et al., 1987), but these functions appear to depend on the type of assay used (White and Stillman, 1987 and Herbst et al., 1988), and may be an indirect result of the ability of the 19kD

protein to protect DNA from degradation (Herrmann and Mathews, 1989).

The E1b 58kD protein is detected by indirect immunofluorescence mainly in the perinuclear region of infected cells (Rowe et al., 1983a and Yee et al., 1983). It is a phosphoprotein which has a protein kinase activity associated with it when it is isolated from cell extracts by immunoprecipitation (Branton et al., 1981 and Yee and Branton, 1983). The 58kD protein forms a complex with the E4 25kD protein (Sarnow et al., 1984 and Cutt et al., 1987), and functions in facilitating the transport of late viral messages, as well as inhibiting the accumulation of cellular mRNAs, thus contributing to the shut-off of host protein synthesis during lytic infection (Babiss and Ginsberg, 1984, Cutt et al., 1987, Samulski and Shenk, 1988 and Leppard and Shenk, 1989). The functions of the smaller 58kD-related proteins have not for the most part been well characterized, but the 156 residue protein may be required for viral replication (Brown and Branton, unpublished).

E2 Proteins

E2a messages code for a 72kD DNA binding protein which has a number of functions in the lytic cycle. This is a nuclear phosphoprotein which is found in vivo both

as a full length 72kD polypeptide and a 48kD cleavage product (Sugawara et al., 1977 and Axelrod, 1978). The cleavage product, which lacks the amino terminal 120 amino acids of the protein, can be isolated in vitro by cleavage of the full length polypeptide with chymotrypsin. The 72kD protein has functional domains at both its carboxy and amino termini. The amino terminal 120 amino acids contains most, if not all of the phosphorylation sites (Klein et al., 1979), and has been reported to function in the regulation of late gene expression, and to contribute to the host range of the virus (Klessig and Grodzicker, 1979 and Brough et al., 1985). This region may also be involved in autoregulation of the 72kD gene, by enhancing its own expression, and phosphorylation at a number of sites appears to be critical for this function (Morin et al., 1989). The amino terminus of the protein is capable of binding ATP and may have a protein kinase activity associated with it (Branton et al., 1985b). The carboxy terminal domain functions in binding the 72kD protein to both single-stranded DNA and RNA (Klein et al., 1979 and Cleghon and Klessig, 1986), an interaction which is required for viral DNA replication (Van der Vliet and Sussenbach, 1975). This region is also involved in the down-regulation of viral early gene expression, both by

blocking transcriptional initiation specifically from the E4 promoter (Nevins and Winkler, 1980 and Handa et al., 1983), and by destabilizing early viral mRNAs (Carter and Blanton, 1978 and Lazaridis et al., 1988). Later in infection, the carboxy terminal domain mediates the role of the 72kD protein in assembly of new virion particles (Nicolas et al., 1983).

E2b messages code for the 80kD precursor of the 55kD terminal protein which binds to the 5' termini of the viral genome (Rekosh et al., 1977), and for the 140kD viral DNA polymerase (Friefeld et al., 1983). Both of these proteins, in addition to the 72kD DNA-binding protein, are required for viral replication (Lichy et al., 1983 and Friefeld et al., 1983), and their functions in replication are discussed below.

E3 Proteins

The E3 region of the genome, which is not essential for virus propagation in tissue culture, produces at least four proteins, three of which have been found to function in the modulation of the host's response to viral infection in vivo. The 19kD glycoprotein produced from this region complexes non-covalently in infected cells with major histocompatability antigens, and prevents their terminal

glycosylation and expression at the cell surface (Burgert and Kvist, 1987). This is thought to permit infected cells to evade destruction by the host's cytotoxic T lymphocytes, which recognize viral antigens present on the surface of infected cells, only in the presence of major histocompatability antigens (Burgert and Kvist, 1987, Tanaka and Tevethia, 1988 and Ginsberg et al., 1989). Adenovirus infected cells carrying viruses which lack the E3 region are also susceptible to lysis by tumour necrosis factor, a protein hormone which is secreted by some cells of the immune system in response to inflammation or viral infection. A 14.7kD E3 protein functions to protect infected cells from this cytolytic effect (Gooding et al., 1988). A third E3 protein, of molecular mass 10.4kD, has an amino acid sequence similar to the cytoplasmic portion of the epidermal growth factor receptor. This protein induces internalization and degradation of the receptor in infected cells, possibly through the formation of hetero-oligomers. It has been suggested that this may function to constitutively induce the mitogenic effect normally associated with the internalization of the receptor in response to epidermal growth factor, resulting in increased proliferation of infected cells, and resultant stimulation of viral DNA synthesis. Alternatively the internalization of the

receptor in the absence of ligand may abrogate EGF-induced cellular responses leading to inflammation and recruitment of the host's immune system (Carlin et al., 1989 and Ginsberg et al., 1989). The function of the fourth E3 protein, of molecular mass 11.6kD, has not been determined (Ginsberg et al., 1989).

E4 Proteins

The E4 region of the genome codes for functions which are important in viral DNA replication, accumulation of late messages and proteins, and viral growth (Halbert et al., 1985, Hemstrom et al., 1988 and Bridge and Kettner, 1989), but for the most part, specific functions have not been attributed to the five proteins which have been detected in vivo from this region. The 25kD protein (34kD) detected by Sarnow et al. (1984) and Cutt et al. (1987) forms a complex with the E1b 58kD protein which is functionally involved in host cell shutoff and late viral polypeptide synthesis (Cutt et al., 1987). A second E4 protein is involved in activation of the E2F transcription factor which is required for maximal transcriptional activation of the E2 promoter (Reichel et al., 1989 and Hardy et al., 1989).

1.4.4 DNA Replication

Replication of the double-stranded linear genome can be initiated at either end, and proceeds semi-conservatively by continuous elongation, resulting in the displacement of one non-replicated parental strand (Lechner and Kelly, 1977 and reviewed in Horwitz, 1990a). This strand is probably replicated in the same way following the annealing of the complementary sequences at either end to form a panhandle structure which resembles the ends of the viral genome, and which acts as an origin of replication (Lechner and Kelly, 1977, Lichy *et al.*, 1983 and Lippé and Graham, 1989).

Studies with temperature-sensitive mutants *in vivo*, and purified viral proteins *in vitro* have demonstrated that the three viral proteins coded for by the E2 region are required for viral DNA replication (Van der Vliet and Sussenbach 1975, Horwitz, 1978, Ikeda *et al.*, 1981, Stillman *et al.*, 1982 and Lichy *et al.*, 1983). The reaction is primed by the 80kD precursor to the 55kD terminal protein (Rekosh *et al.*, 1977). This protein complexes in a 1:1 ratio with the virus-encoded DNA polymerase, binds to the terminus of the adenovirus genome, and forms a covalent attachment through a serine

residue to the 5' hydroxyl of a dCMP nucleotide (Desiderio and Kelly, 1981 and Lichy et al., 1982). The polymerase then utilizes the free 3' hydroxyl end of the nucleotide to initiate the polymerization reaction, and synthesizes the daughter strand continuously in a 5' to 3' direction to the end of the genome (Stillman et al., 1982). The 72kD DNA binding protein, also coded for by the virus, is required for chain elongation and may play a role in unwinding the DNA at the end of the genome to facilitate the initiation of the replication reaction (Lichy et al., 1983 and Stuiver and van der Vliet, 1990).

A number of factors coded for by the host cell are either required for, or contribute to the efficiency of the replication process (Lichy et al., 1983 and Horwitz, 1990a). Nuclear factor I, which has also been isolated as a transcription factor termed CTF (Jones et al., 1987), binds near the origin between nucleotides 17 and 48 and is necessary for initiation and elongation of the nascent strand (Nagata et al., 1982 and Leegwater et al., 1985). Nuclear factor II, which is a type I topoisomerase, is required later in the elongation process, presumably to reduce torsional stress (Nagata et al., 1983). Nuclear factor III also binds near the origin, and recognizes a specific sequence between nucleotides 36 and 54 (Pruijn et al., 1986, 1988). This

factor, which enhances activity of the origin, has also been recognized as a transcription factor (Pruijn et al., 1987 and O'Neill and Kelly, 1988). Topoisomerase II, which is not required for adenovirus replication in vitro, may be necessary in vivo to mediate the separation of daughter strands which are closely linked to the nuclear matrix (Schaack et al., 1990).

1.4.5 Host Cell Shutoff

In lytic infection of KB or HeLa cells, inhibition of host cell macromolecule synthesis begins to be seen at about the same time as viral DNA replication, and follows the early stages of infection (Bello and Ginsberg, 1967 and Ginsberg et al., 1967). Host protein synthesis is almost completely shut off by 16 hours post-infection as a result of preferential transport of virus-specific messages from the nucleus to the cytoplasm, although host messages are still transcribed and processed correctly (Beltz and Flint, 1979). There is no rapid breakdown of pre-existing cytoplasmic host messages (Horwitz, 1990a), but initiation of translation of these messages may be inhibited by the virus-induced increase in double-stranded RNA activated inhibitor (DAI) protein kinase activity, which results in phosphorylation and

inactivation of the α-subunit of the translation initiation factor eIF-2 (O'Malley et al., 1989). Host cell DNA synthesis is gradually reduced, starting at about 6 hours following infection of randomly growing KB cells (Ginsberg et al., 1967). Using logarithmically growing HeLa cells which were cell-cycle synchronized, Hodge and Scharff (1969) found that the initiation of a new round of host cell DNA synthesis was inhibited if viral DNA replication began when the cells were in G_1, and cell DNA synthesis was severely reduced but not completely inhibited if viral DNA was replicated during S phase. It was suggested that host replicons which were initiated early in S phase could complete their round of replication, but those which would normally replicate later in S phase were prevented from initiating. The shutoff of host protein synthesis may also contribute to a reduction in the ability of the cell to replicate DNA, if proteins normally involved in the replication process were in short supply (Horwitz, 1990a).

1.4.6 Late Transcription

Following viral DNA replication, late transcripts are preferentially produced from the replicated templates (Sharp et al., 1975), and reach levels 10 fold in excess

of those detected during early infection (Horwitz, 1990a). Some, such as the independently promoted transcripts for protein IX and polypeptide IVa2, and transcripts from the L1 region downstream of the major late promoter, are detectable in lower levels earlier in infection, but are greatly amplified following DNA replication (Chow et al., 1980). The majority of late transcripts, however, are promoted by the major late promoter and require in cis the prior replication of the template they are transcribed from (Thomas and Mathews, 1980). The controls for this early to late switch are not well understood, but it has been suggested that it may involve displacement of components with which the unreplicated genome is associated, or alterations in the structure of the DNA itself (Thomas and Mathews, 1980). There is recent evidence that the promoter for protein IX is inactive early in infection due to its position within the E1b coding region, which results in its occlusion due to active transcription across this region (Vales and Darnell, 1989). The authors suggest that replication of the DNA template is required to clear the promoter, thus allowing the required transcription factors to interact with it.

Most of the late transcripts code for virion structural proteins or their precursors. These

polypeptides are being synthesized at a maximal rate by 20 hours post-infection (Horwitz, 1990a) and are transported rapidly to the nuclei, where new virion assembly is initiated (Flint and Broker, 1981).

1.4.7 Assembly and Release of New Virions

Hexon polypeptides trimerize into hexon capsomers within a few minutes of their release from polyribosomes (Horwitz et al., 1969) in a process mediated by the 100kD scaffolding protein (Cepko and Sharp, 1983). These capsomers then self-assemble into empty shells in the infected cell nucleus and develop through "light intermediate capsid", "heavy intermediate capsid" and "young virion" stages before becoming mature virion particles (Flint and Broker, 1981). A number of minor capsid components, such as polypeptides VI, VIII, and IIIa associate with the hexon shell to form a light intermediate capsid structure (D'Halluin et al., 1978). Viral DNA enters the capsid beginning at its left end, directed by the packaging signal which is located between nucleotides 290 and 390 at the left end of the genome (Hearing et al., 1987). The light intermediate capsid may be isolated from infected nucleii in association with a portion of the left end of the viral genome, which is

thought to result from shearing of the full-length genome during isolation (Horwitz, 1990a). The chromosome enters the assembling structure covalently attached to the 80kD terminal protein, but in the absence of any of the virion core proteins with which it associates in the mature virion (Horwitz 1990a). Incomplete capsids containing full length DNA constitute the "heavy intermediate" class of structures (Flint and Broker, 1981). Assembly of the penton capsomers from penton base and fiber proteins proceeds more slowly than assembly of the hexon capsomers (Horwitz et al., 1969), but these are found in the capsid by the young virion stage (Horwitz 1990a), as are protein IX (Ghosh-Choudhury et al., 1987), precursors to the other minor capsid components, and the virion core proteins (Horwitz, 1990a). The final maturation involves cleavage of precursor capsid components by a 23kD protein from the L3 region (Bhatti and Weber, 1979) to give the mature products, and a general compacting of the entire capsid to give a structure which is impermeable to nucleases (Horwitz, 1990a).

The virus lytic cycle is complete within 32 to 36 hours (Horwitz, 1990a), and results in 4,000-10,000 viable progeny virus particles per cell (Green and Daesch, 1961). Adenoviruses do not specifically code for functions which lyse the host cell (Flint and Broker,

1981). The cell dies through attrition, and virus particles remain cell-associated for some time after cell death (Horwitz, 1990a).

1.5 Transformation by Human Adenoviruses

Human adenoviruses are classified into three subgroups according to their ability to cause tumours in baby rats and hamsters (Lacy and Green, 1967). Subgroup A viruses, which include serotypes 12 and 18, are highly oncogenic and produce tumours with a high incidence at the site of injection within three months (Trentin et al., 1962 and Huebner et al., 1962). Subgroup B viruses such as Ad7 also induce tumours in injected animals, but are only weakly oncogenic, producing tumours in a small proportion of animals (7-21%) only after a long latency (200-400 days) (Girardi et al., 1964 and Larson et al., 1965). Tumour cells from hamsters injected with viruses from either Subgroup A or B can be successfully passaged through adult hamsters, and new tumours appear within three weeks (Trentin et al., 1962 and Larson et al., 1965). Subgroup C viruses, which include serotypes 1, 2 and 5, are not oncogenic and do not produce tumours in injected animals, even after long periods of time (Trentin et al., 1962, Huebner et al., 1962 and Lewis et

al., 1974). Although not tumourigenic in rodents, Subgroup C viruses can transform primary hamster or rat cells in vitro (Freeman et al., 1967 and McAllister et al., 1969a), and these cells may be tumourigenic when injected back into suckling hamsters or immunosuppressed juvenile rats (McAllister et al., 1969b, Lewis et al., 1974 and Williams et al., 1974). Most, if not all adenovirus serotypes are capable of transforming mammalian cells in culture (Branton et al., 1985a and references therein), and transformation of both primary and differentiated cells of a number of mammalian species, including human, have been reported, using either virus or DNA-mediated procedures (Levinthal and Peterson, 1965, Reed, 1967, Graham and van der Eb, 1973, Graham et al., 1977, Byrd et al., 1982, Whittaker et al., 1984 and Woodworth and Ison, 1987). Rat or hamster cells transformed in vitro by non-oncogenic serotypes have characteristics similar to cells transformed by oncogenic serotypes (Freeman et al., 1967), and closely resemble cells explanted from virus-induced tumours (McAllister et al., 1969a, Branton et al., 1985). Transformed cells are capable of indefinate growth in vitro, are not contact-inhibited at high densities, and are able to grow in soft agar, and in medium with low levels of calcium (0.1 mM) (Freeman et al., 1967 and McAllister et al., 1969b).

Early studies showed that adenovirus-transformed cells did not produce infectious virus, but expressed serotype-specific mRNA and produced viral antigens that could be detected by indirect immunofluorescence or in complement-fixation assays (Huebner et al., 1962, Girardi, 1964, Larson et al., 1965, Reed, 1967, Freeman et al., 1967, McAllister et al., 1969a,b and Lewis et al., 1974). This led investigators to search for specific adenovirus tumour antigens, and the oncogenes that coded for them.

1.5.1 Isolation of Transforming Genes

A number of lines of evidence led to the identification of Early Region 1 (E1) as the region coding for transforming functions. Firstly, examination of adenovirus transformed rodent cells indicated that only a portion of the viral DNA became stably integrated into the host genome, and that although the actual amount of integrated material varied between cell lines, the leftmost approximately 14% of the virus genome was always present (Gallimore et al., 1974, Flint, 1981b and Green et al., 1981). Secondly, DNA-RNA hybridization analysis provided evidence that the viral messages produced in transformed cells were also transcribed from a small fraction of the viral genome during lytic infection, and

that they came only from early genes (Green, 1970, Graham et al., 1977, Aiello et al., 1979 and Green et al., 1981). Thirdly, there was the demonstration by Graham et al. (1974a,b) that small fragments of Ad5 DNA from the left end of the genome were sufficient to transform rat, hamster or human cells, with the suggestion that the transforming region was of a size likely to contain two contiguous genes mapping to an area between 1% and 6% on the viral genome. Cells transformed by sheared viral DNA or restriction fragments containing the leftmost 8% of the genome were tumourigenic and did not appear to differ morphologically from cells which had been transformed by the intact virus, indicating that genes outside of the E1 region did not contribute to the transformed phenotype (Graham et al., 1974a,b, van der Eb et al., 1977, Rowe and Graham, 1983 and Rowe et al., 1984a).

1.5.2 E1a and E1b: Roles in Transformation

In early studies, the involvement of products from both of these genes in transformation was suggested by the presence of messages and proteins specific for both E1a and E1b in transformed cells (Gilead et al., 1976, Graham et al., 1977, van der Eb et al., 1977, Schrier et al., 1979, Lassam et al., 1979, Green et al.,

1981 and Lewis and Mathews, 1981). Viruses with mutations in either gene were found to be potentially defective for transformation (Graham et al., 1978, Jones and Shenk, 1979b, Carlock and Jones, 1981 and Solnick and Anderson, 1982). Neither gene on its own, if transfected into primary rodent cells, could induce the fully transformed phenotype seen if both E1a and E1b genes were present (van den Elsen et al., 1982), indicating that both contributed to the transformation process.

The products of E1a alone are capable of partially transforming, or immortalizing primary rodent cells (Dijkema et al., 1979, Houweling et al., 1980 and Graham et al., 1984). These cells can be passaged indefinitely in culture, but are more fibroblastic in morphology than fully transformed cells, and are generally not tumourigenic (Graham et al., 1984 and Branton et al., 1985). The efficiency of immortalization by E1a depends at least in part on levels of expression of E1a products (Senear and Lewis, 1986) and the culture conditions used to select for immortal cells (Graham et al., 1984). The products of both the 12S and 13S E1a messages are present in transformed cells (Rowe et al., 1984a and Branton and Rowe, 1985), and products of both contribute to the transformed phenotype (Montell et al., 1984, Haley et al., 1984, Hurwitz and Chinnadurai, 1985,

and Kuppuswamy and Chinnadurai, 1988). E1a proteins, in addition to being able to transform cells in conjunction with E1b, can cooperate with polyoma virus middle-T antigen or with the Ha-ras oncogene to transform primary rodent cells (Ruley, 1983, Land et al., 1983 and Branton et al., 1985).

E1b gene products, in the absence of E1a products have no detectable transforming activity in either primary or established cells (Solnick and Anderson, 1982, van den Elsen et al., 1982, 1983 and Ruley, 1983). In DNA-mediated transformation assays, and in cooperation with E1a, only approximately the left half of the E1b gene, coding for the 19kD protein and the amino terminus of the 58kD protein is required to produce fully transformed cells (Graham et al., 1974a,b, van der Eb and Houweling, 1977, Rowe and Graham, 1983 and Rowe et al., 1984). With viral-mediated transformation, however, the entire E1 region is required and it has been suggested that the carboxy terminal half of the 58kD protein is not necessary for transformation but it may be involved in mediating the integration of virion DNA into the host chromosome (Rowe and Graham, 1983). There is recent evidence that E1a products in conjunction with either the 19kD protein or the 58kD protein are able to transform cells, but that transformation is more efficient in the

presence of both E1b products, suggesting that they may function via independent pathways in transformation (Edbauer et al., 1988, White and Cipriani, 1990, McLorie et al., 1990 and Zhang et al., 1990).

The biological mechanisms by which E1a and E1b proteins transform cells are not well understood, but the development of anti-peptide and monoclonal antibody probes for these proteins, as well as site-directed mutagenesis of the E1 genes, has facilitated the approach to understanding their functions (Rowe et al., 1983b, Yee and Branton, 1985a, Harlow et al., 1985, Egan et al., 1988, Jelsma et al., 1988, 1989, McLorie et al., 1990 and Brown and Branton, unpublished).

1.6 Mechanisms of Transformation

It is generally accepted that transformed cells have developed an abnormal proliferative capacity as a result of genetic alteration. This alteration may be the result of spontaneous mutation, or mutation induced in the cell genome by an external agent such as a chemical carcinogen or ultra violet light. It may also result from the introduction into the cell of oncogenes from an external source, such as a virus. As a greater understanding of the molecular processes which regulate

normal growth and development is acheived, so are the mechanisms by which these processes can be disrupted, leading to oncogenic transformation. Cellular genes that play critical roles in maintaining normal growth processes may be eliminated in transformed cells, or altered so that the proteins they produce function abnormally. Two such classes of genes normally present in cells include proto-oncogenes, whose products may become actively oncogenic following mutation, and anti-oncogenes, or tumour suppressor genes, whose products are eliminated or inactivated in the transformed cell (reviewed by Bishop, 1983, Hunter, 1984, Varmus, 1985, Hunter, 1987, Weinberg, 1988, and Sager, 1989).

The mechanisms by which oncogenic viruses alter normal cell processes to produce a transformed phenotype, have provided a great deal of insight into how those normal processes work, and how they are sometimes disrupted in naturally occuring cancers. The study of transforming retroviruses, in particular, was instrumental in the discovery of the proto-oncogene class of cellular genes. Many retroviruses carry in their genome an activated oncogene which is homologous to one of the cellular proto-oncogenes. Comparison of the activated and normal versions of these genes, both by sequence analysis and biochemical function has provided

information on how they may become activated in human malignancies and what the functional properties of the activated form are which lead to neoplastic transformation (reviewed by Bishop, 1983, 1985, Hunter, 1984, and Varmus, 1988).

The mechanisms by which DNA tumour viruses such as adenoviruses and the papova viruses transform cells are less well understood. The transforming genes have been identified but these are unlike those of the transforming RNA viruses, in that they do not have extensive sequence homology with known cellular proto-oncogenes (reviewed by Green, 1985 and Bishop, 1985). Some homology at the level of secondary structure exists between E1a proteins and the myc family of oncogenes (Ralston and Bishop, 1983 and Branton et al., 1985a), although the functional significance of this is not clear. There has been no detectable correlation between an enzymatic activity intrinsic to the oncoproteins of the DNA tumour viruses, and their ability to transform cells. One approach to understanding the mechanism of action of these proteins has been to search for cellular components with which they interact. Early reports indicated that a number of cellular proteins could be detected in association with the T-antigens of polyoma and SV40 viruses in infected and transformed cells (Lane

and Crawford, 1979, Courtneidge and Smith, 1983, Grussenmeyer et al, 1985, Murphy et al., 1986 and Courtneidge and Heber, 1987). One of the proteins, termed p53, which specifically complexed with SV40 large T-antigen, could also be detected in association with the Ad5 E1b 58kD protein (Sarnow et al., 1982). This suggested that binding to p53 might represent a common mechanism by which oncoproteins from different DNA tumour viruses were involved in transformation, and stimulated interest in the characterization of other cellular proteins found in association with the products of DNA tumour virus oncogenes.

1.7 Cellular Proteins Associated with E1a Products

Yee and Branton (1985b) were the first to report an association between Ad5 E1a proteins and a number of cellular polypeptides. These cellular proteins could be immunoprecipitated in conjunction with E1a 12S and 13S products, from infected KB cells or transformed human 293 cells. Their relative molecular masses as determined by reducing SDS-PAGE analysis were 65kD, 68kD, 105kD, 107kD and 300kD, with the 105kD, 107kD and 300kD species being the most readily detectable. These proteins were shown to be specifically complexed with E1a polypeptides by

virtue of the fact that they could be detected in immune complexes using antisera directed against different epitopes on the E1a proteins. They were detected in similar quantities in infected and uninfected cells, as determined by mixing experiments, indicating that they are not induced by viral infection. The 300kD (p300), 107kD (p107) and 105kD (p105) proteins were shown to be phosphoproteins, and the other species were not phosphorylated. These results were later confirmed and extended by Harlow et al. (1986), who reported that the major detectable cellular species (p300, p107, and p105) associated with only a small fraction of the E1a proteins present in transformed cells, and appeared to form individual complexes with the E1a proteins, as analysed by separation on sucrose gradients. Both groups reported a number of minor proteins whose presence in the immune complexes was either inconsistent or difficult to detect due to their low levels, and these were not characterized.

1.8 Thesis Proposal

The research described in this thesis was carried out in order to further characterize the cellular

proteins found in association with Ad5 E1a polypeptides, and, if possible, to determine something of their biological significance with respect to E1a-mediated transformation.

The ability of bacterially-produced E1a to form complexes with the cellular proteins was examined to determine its suitability as an affinity substrate for purification. Attempts to raise monoclonal antisera to gel-purified E1a binding proteins by *in vivo* or *in vitro* immunization of Balb/C mice were unsuccessful. A number of attempts to directly identify the proteins as already known cellular gene products were also unsuccessful. The biological significance of the proteins was investigated in a collaborative study with Dr. Stan Bayley's lab in which the regions on the E1a proteins involved in binding the cellular polypeptides were identified. Binding of E1a proteins to both p300 and p105 was shown to be essential for transformation. Binding of E1a proteins to either p300 or p105 or both correlated with the ability of E1a proteins to induce DNA synthesis in quiescent cells. The role of p107 in transformation was not clear, but it did not appear to play a role in induction of DNA synthesis. Following reports by others that the product of the retinoblastoma tumour suppressor gene could be found in association with E1a proteins and SV40 large T

antigen, p105 was confirmed as the Rb gene product, and it was determined that most of this protein complexed with E1a products in infected cells. Binding of p300, p105 and p107 to E1a proteins in a number of human tumour cell lines was investigated. P300 was present in all cell lines investigated but it was found that p107 was expressed at barely detectable levels in retinoblastoma lines with defects in p105-Rb. Cleveland peptide analysis of these two proteins indicated a possible structural relationship between them, but further studies will be necessary to determine the nature and extent of this relationship.

Chapter 2

Materials and Methods

Materials and Methods

2.1 Viruses

The wild-type strain of human adenovirus serotype 5 used in these studies has been described previously (Harrison et al., 1977 and Graham et al., 1978), as has the host-range mutant hr5 (loc. cit.), which contains a point mutation altering residue 139 in the 13S E1a gene product and residue 185 in the 12S product. The mutants dl1504 (Osborne et al. 1982) and dl313 (Colby and Shenk 1981) lack 14 amino acids at the amino terminus and 70 amino acids at the carboxy terminus of the E1a proteins, respectively. The mutant dl520 contains a deletion in E1a which removes the 5' splice donor for the 13S message and thus produces only the 12S message (Haley et al., 1984). The mutants Ad89-A and Ad96-A (Tremblay et al., 1989) carry point mutations in the first exon of the E1a products and produce E1a proteins with serine residues 89 and 96 respectively altered to alanine residues. The dl1101 to dl1115 series of deletion mutants lacks short stretches of amino acids encompassing most of the region amino to residue 204 of the 13S product, and residue 158 of the 12S product. This series of mutants, and dl1141, dl1142, and dl1143 were created in Dr. Stan Bayley's lab, and their development and phenotype have been previously

described (Jelsma et al. 1988, 1989). Viruses carrying these E1a mutations either alone or in combinations of two mutations were rescued into dl520 background to produce viruses expressing mutated 12S products only (Howe et al., 1990). All virus stocks were propagated on monolayers of 293 cells.

2.2 Cell lines

Human KB cells, an established cell line derived from nasopharangeal carcinoma tissue, were cultured as monolayers on 100mm plastic tissue culture dishes (Nunc) in alpha minimal essential medium (α-MEM) with 10% foetal calf serum. The 293 cell line, derived from human embryonic kidney cells transformed by the leftmost end of the adenovirus 5 genome (Graham et al. 1977), which constitutively express E1a and E1b gene products, were cultured as monolayers on 150mm tissue culture dishes in Joklik's modified medium supplemented with 10% horse serum. The retinoblastoma lines were grown as non- or semi- adherent cell masses and were propagated on 100mm plates in α-MEM supplemented with 15% foetal calf serum. These lines were all derived from primary retinoblastoma tumours and lines Y79, WERI, Rb537, Rb522, Rb544, Rb383, Rb414 and Rb430 have been partially characterized (Friend

et al., 1986, Dryja et al., 1986, Lee et al., 1988, Goddard et al., 1988 and Zhu et al., 1989). The established human retinal cell line HER3.10, transformed by sequences at the left end of Ad12 (Byrd et al. 1982), was propagated as a monolayer on 100mm plates in α-MEM supplemented with 10% foetal calf serum. Most of the retinal lines used in this study were provided by Dr. Brenda Gallie (The Hospital for Sick Children, Toronto). Small cell lung carcinoma (SCLC) cell lines RG-1, H-209 and H-69, were derived from primary tumours, with lines H-69 and H-209 having been previously described (Harbour et al., 1988). These were grown as non-adherent cell masses in RPMI medium plus 15% foetal calf serum in 100mm plates. Non-small cell carcinoma lines A549, A427 and Calu-1, also derived from lung carcinomas, were cultured as adherent or semi-adherent monolayers on tissue culture plates in α-MEM supplemented with 10% foetal calf serum. Line A549 has been previously described (Yokota et al., 1988). All lung cell lines were acquired from Dr. Ted Bradley (l'Institut du Cancer de Montréal).

2.3 Antisera

Anti-peptide antisera E1a-N1 and E1a-C1 are directed against the amino and carboxy termini,

respectively, of Ad5 E1a proteins and have been previously described (Yee and Branton 1985 and Yee et al. 1983). The mouse monoclonal antibody M73 (Harlow et al. 1985) was made against a TrpE-E1a fusion protein purified from bacteria (Spindler et al. 1984), and it recognizes an epitope within the region coded for by the second exon of the E1a proteins (Stephens and Harlow, 1987). The rat monoclonal antisera R7 and R28 were made to the same bacterially-produced protein as the M73 antibody (Tsukamoto et al., 1986a), but they react with an epitope within a region bounded by amino acid residues 26 and 70 of the E1a polypeptide (this thesis). The mouse monoclonal antibody H-219 recognizes the Ad5 72K DNA binding protein and has been previously described (Branton et al. 1985). The anti-E1b 58K monoclonal antiserum used was purchased from Oncogene Science and it reacts with an epitope near the amino terminus of the 58K protein (C. Egan and P.E. Branton, unpublished results).

2.4 Purification of bacterially-produced E1a proteins

Bacterially-produced E1a 13S or 12S gene products were purified from Escherichia coli (cell line AR120) containing either the expression vector pAS1-E1A410 (for expression of 13S product) or pAS1-E1A412 (for production

of 12S product). These cells and the E1a protein purification procedure were kindly provided by Dr. Martin Rosenberg (Smith, Kline and French Labs, New Jersey), and the construction of the vectors is described elsewhere (Rosenberg et al. 1983 and Ferguson et al. 1984). E1a proteins were purified from these cells by a procedure modified from that of Krippl et al. (1984). Briefly, bacteria were cultured overnight in Yeast Tryptone (YT) Broth (10g tryptone, 5g yeast extract, 10g NaCl in total volume 1 litre), and an aliquot of the overnight culture was diluted 1 in 100 in YT plus 50ug/ml Ampicillin. This culture was grown at 37°C to an optical density of 650nm (OD_{650}) of 0.4 -0.6, and then E1a protein production was induced by the addition of nalidixic acid (Calbiochem) in 1M NaOH to a final concentration of 60ug/ml. The bacterial culture was allowed to continue incubating with aeration at 37°C for 7 hours, and the cells pelleted by centrifugation for 20 minutes at 12,500g. This procedure yielded approximately 1.2g of bacterial cells per 250 ml of bacterial culture, and the cell pellets were either stored overnight at -70°C, or were resuspended immediately in lysis buffer to begin protein purification.

To extract E1a proteins, cells were resuspended in Lysis Buffer (50mM Tris pH7.5, 1.0mM

ethylenediaminetetraacetic acid (EDTA), 1.0mM dithiothreitol (DTT), 5.0% glycerol) at a volume of 60ml/20g cells, and lysozyme was added to a concentration of 10mg lysozyme/20g cells. The suspension was stirred for 30 minutes at 4°C, and phenyl-methyl-sulfonyl-fluoride (PMSF) was added to 1.0mM final concentration, using a PMSF stock solution of 100mM in ethanol. The cells were sonicated for 6 minutes and then pelleted by centrifugation for 20 minutes at 12,500g at 4°C. This resulted in the release of some of the E1a protein into the supernatant, but as the insoluble portion remaining in the pellet represented a purer starting material, E1a proteins were preferentially purified from the pellet fraction, and the supernatant discarded. The pellet was resuspended in Lysis Buffer plus PMSF, sonicated 1 minute and centrifuged at 12,500g for 20 minutes at 4°C, then the pellet was resuspended, sonicated briefly, and centrifuged again. E1a protein remaining in the pellet in insoluble form was then extracted by resuspension in 20 ml of Extraction Buffer (50mM Tris pH8.0, 1.0mM EDTA, 5.0mM DTT and 6.0M Urea) per 20g of starting material, brief sonication, and centrifugation at 12,500g for 20 minutes at 4°C. The supernatant fraction was loaded onto a DEAE Trisacryl column which had been equilibrated in Column Buffer (50mM Tris pH 8.0, 1.0mM EDTA, 1.0Mm DTT

50mM NaCl and 2.0M Urea), using 20ml of resin per 20g starting material. Proteins were eluted from the column using a 50mM to 500mM NaCl gradient in a volume equal to ten times the column resin volume. Fractions of eluate of approximately 1 ml volume were collected and assayed for protein content using a commercially prepared Bio-Rad protein assay dye reagent and determination of absorbance at 595nm. Fractions were pooled into larger volumes representing protein peaks which eluted from the column at discrete NaCl concentrations, and portions of these pooled peaks were run on sodium dodecyl sulfate (SDS) polyacrylamide gels, transferred to nitrocellulose, and probed by western blot to determine which peaks contained E1a protein. Samples containing E1a protein were dialysed against E1a Buffer (50mM Tris pH 7.5, 1.0mM EDTA, 1.0Mm DTT, 20mM NaCl) and again assayed for protein concentration using the Bio-Rad reagent and absorbance at 595nm. Percent purity of the E1a proteins was estimated by running a fraction of the sample on an SDS polyacrylamide gel and staining with Coomassie Brilliant Blue dye. All purified E1a protein samples used appeared to be of at least 80% or greater purity. Aliquots of purified protein were frozen at -20°C for further use.

2.5 Conjugation of bacterially-produced E1a proteins to cyanogen bromide-activated Sepharose

Bacterially-produced E1a proteins in E1a Buffer, purified as described above, were lyophilized, and the pellet resuspended at a concentration of 5 mg/ml in Coupling Buffer (0.1M $NaHCO_3$, pH8.3 plus 0.5M NaCl). Cyanogen bromide-activated Sepharose beads (Pharmacia) were swelled for 15 minutes in 1mM HCl to give a final volume equivalent to half of the volume of protein sample which was to be coupled (1g of dry Sepharose gives approximately 3.5ml of swelled beads). The swelled Sepharose beads were then washed three times with 1.0mM HCl, followed by one wash with Coupling Buffer before addition of the E1a proteins in Coupling Buffer. The protein and beads mixture was allowed to couple overnight at 4°C or two hours at room temperature on an end-over-end mixer. The beads with protein coupled to them were then pelleted by centrifugation, the supernatant removed, and remaining reactive groups on the beads were blocked with Blocking Buffer (1.5M Tris, pH8.0) using a 2:1 ratio of buffer to beads and mixing on an end-over-end rotator either overnight at 4°C or for two hours at room temperature. The conjugated beads were then pelleted and

washed with Coupling Buffer followed by Acetate Buffer (0.1M Acetate, pH4.0 plus 0.5M NaCl) three times for a total of six washes to remove any non-covalently adhering protein residues, washed a final time in coupling buffer, and resuspended in E1a Buffer at a 9:1 volume of buffer to volume of bead ratio. E1a-Sepharose beads were stored at 4°C.

2.6 Infection and radiolabeling of cells

KB cells, and non-small cell lung carcinoma cells were infected with wild-type or mutant virus at a multiplicity of infection of 35 plaque forming units (pfu) per cell in 1 ml of spent medium per 100mm plate. Retinoblastoma and small cell lung carcinoma lines were infected with wild type virus at approximately 100 pfu/cell in 1 ml of spent medium per 100 mm plate. The virus was allowed to adsorb to the cells for 1-2 hours, after which the 1 ml adsorption volume was diluted out to approximately 10 mls per 100mm plate with spent medium, and the infection was allowed to continue for a further 5-7 hours. The cells were then radiolabeled for a period of 2-4 hours by removing the spent medium, washing the cells once with 199 Medium minus methionine (Gibco), and incubating in fresh 199 Medium minus methionine

containing 50uCi/ml ^{35}S methionine (Amersham Corporation; specific activity 1,300Ci/mM) in 2ml per 100mm plate prior to harvesting. For orthophosphate labeling, cells were washed in 199 Medium minus phosphate and incubated for four hours in 199 Medium minus phosphate containing 1.25mCi/ml of ^{32}P-orthophosphate (New England Nuclear) in 4ml per 100mm plate.

2.7 Preparation of cell extracts and immunoprecipitation

After infection and/or labeling, cells of lines which grew as adherent monolayers were harvested by aspirating the spent medium, scraping the plates with a rubber scraper, resuspending the cells in cold (4°C) Phosphate-Buffered Saline (PBS) and transferring them to a 12ml polypropylene Falcon centrifuge tube. Cells of lines which grew as non-adherent masses were transferred to Falcon tubes and the cells pelleted prior to removal of the spent medium. Cells were then washed a number of times with cold PBS, pelleted and lysed by vortexing in 1ml per 100mm plate of Ripa$^{=}$ (50mM Tris pH7.2, 150mM NaCl, 1% Triton-X 100, 100 KIU Aprotinin (Sigma)), or modified Schaffhausen Buffer (200mM Tris pH7.0, 137mM NaCl, 1.0mM $CaCl_2$, 0.4mM $MgCl_2$, 10% glycerol, 1% NP-40).

Cell lysates were clarified by centrifugation at 12,500g for 20 minutes at 4°C.

For immunoprecipitations, 0.5ml aliquots of clarified cell extract were transferred to Eppendorf tubes, 250µl of a suspension of Protein-A Sepharose beads were added (suspension prepared by pre-swelling the beads in the appropriate lysis buffer, washing, and resuspending the bead pellet in a nine-fold volume of buffer), and an appropriate volume of antibody. This mixture was allowed to react for a period of time varying from three hours to overnight on an end-over-end mixer at 4°C. The Protein-A Sepharose beads were pelleted, the supernatant was removed, and the beads were washed either three times with Ripa$^{=}$ followed by two times with Ripa$^{\equiv}$ (50mM Tris pH7.2, 150mM NaCl, 100 KIU Aprotinin), or three times with modified Schaffhausen Buffer followed by two times with LiCl Wash Buffer (100mM Tris pH7.0, 200mM LiCl, 50mM DTT, 1.0mM $CaCl_2$, 0.4mM $MgCl_2$). The pelleted beads were resuspended in 40µl of Laemmli Sample Buffer (50mM Tris pH6.8, 1% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 5 minutes.

2.8 Separation of proteins by SDS polyacrylamide gel electrophoresis

Discontinuous sodium-dodecyl-sulfate polyacrylamide gel electrophoresis in one dimension was carried out by a procedure adapted from Laemmli (1970). Proteins were separated using vertical slab gels, with a 5% acrylamide stacking gel (125mM Tris pH6.8, 0.1% SDS, 0.05% glycerol, 0.0625% ammonium persulfate, 0.075% N,N,N',N'-tetremethylethylenediamene (TEMED), using a 30:1.0 stock solution of acrylamide : bis-acrylamide) and an 8% separating gel (375mM Tris pH8.8, 0.1% SDS, 0.1% glycerol, 0.05% ammonium persulfate, 0.06% TEMED). Solutions were degassed extensively prior to addition of activating agents, and allowed to polymerize for a minimum of 1 hour before loading. Gels were run overnight in Electrode Buffer (0.025M Tris pH8.3, 0.192M Glycine and 0.1% SDS) at constant current which was pre-adjusted to allow stacking to be carried out at 40 to 60 volts, and electrophoresis was terminated when the Bromophenol Blue tracking dye reached the bottom of the gel. Prior to fluorography, gels were soaked in dimethylsulfoxide (DMSO) twice for 0.5 hours each time, followed by 3 hours in a 22.5% w/v solution of 2,5-diphenyloxazole (PPO) in DMSO. Gels were then rehydrated

for 1 hour in water, dried on Watman chromatography paper, and exposed to Kodak RP X-Omat film at -70°C (Bonner and Laskey 1974).

2.9 Cleveland peptide mapping

The partial digestion products of proteins were compared by hydrolysis with Staphylococcus aureus V-8 Protease (Bohringer-Mannheim), using the method described by Cleveland et al. (1977). Briefly, protein samples were run on 8% SDS polyacrylamide gels, the gels were dried and exposed to film. The developed fluorogram was used to locate bands representing the proteins of interest and gel pieces containing the appropriate polypeptides were excised. The gel pieces were re-swelled in Electrode Buffer, the paper backing was removed, and the intact pieces were loaded into the wells of a 15% SDS gel (5% stacking gel). For V-8 analysis the wells were then loaded with 50µl of Laemmli Sample Buffer containing 0.1, 1.0 or 10.0µg Staphyloccus V-8 Protease. Electrophoresis was started at 50V, and the current was shut off for 1 hour when the dye front reached the stacking gel/separating gel junction. After turning the current back on, the gel was run on constant current until the dye front reached the bottom of the gel.

Enhancement and fluorography were carried out as described above.

2.10 Western blotting

Immunoprecipitated samples were prepared and run on 8% SDS gels as described above. Following electrophoresis, proteins were transferred to nitrocellulose using a Bio-Rad Electroblotting apparatus. The transfer was carried out overnight at 200mA at 4°C in Transfer Buffer (200mM glycine, 25mM Tris, 20% methanol). The nitrocellulose membranes were then washed at room temperature 5 minutes in phosphate-buffered saline, and blocked by washing 30 minutes in Tris- Saline Buffer (TS) (10mM Tris pH7.5, 150mM NaCl) containing 5% w/v Carnation Powdered Skim Milk, followed by two 5 minute washes with TS. Blots were probed with an appropriate first antibody, diluted 1/1000 in TS plus 5% milk for 60 minutes, then washed twice with 10 minute washes in TS, and probed with the appropriate second antibody (either ^{125}I conjugated goat anti-rabbit IgG or ^{125}I conjugated goat anti-mouse IgG (ICN)) 1.0μCi/ml in TS plus 5% milk, for 60 minutes. Blots were then washed twice for 10 minutes per wash in TS, dried and exposed to film at room temperature.

Chapter 3

Binding of Cellular Polypeptides to E1a proteins produced in *Escherichia coli*

Binding of Cellular Polypeptides to E1a proteins produced in *Escherichia coli*

3.1. Association of cellular proteins with soluble E1a polypeptides produced in bacteria

It was previously shown by Yee and Branton (1985b), that Ad5 E1a polypeptides associated in infected KB cells with at least five cellular proteins termed p300, p107, p105, p68 and p65. The ability of these cellular proteins to associate with bacterially produced E1a gene products was investigated in order to characterize the specificity of binding and to potentially provide a method of purifying them for further study. E1a proteins were prepared from bacteria expressing E1a products, as described in Chapter 2.

In the first experiment, the ability to bind cellular proteins was examined using both purified E1a products synthesized in *E. coli* and those from Ad5 infected human KB cells. For these studies cell extracts were prepared in either Ripa$^{=}$ or modified Schaffhausen Buffer, which contains divalent cations. Both buffers were employed because in previous studies it had been shown that in some cases divalent cations significantly affected the

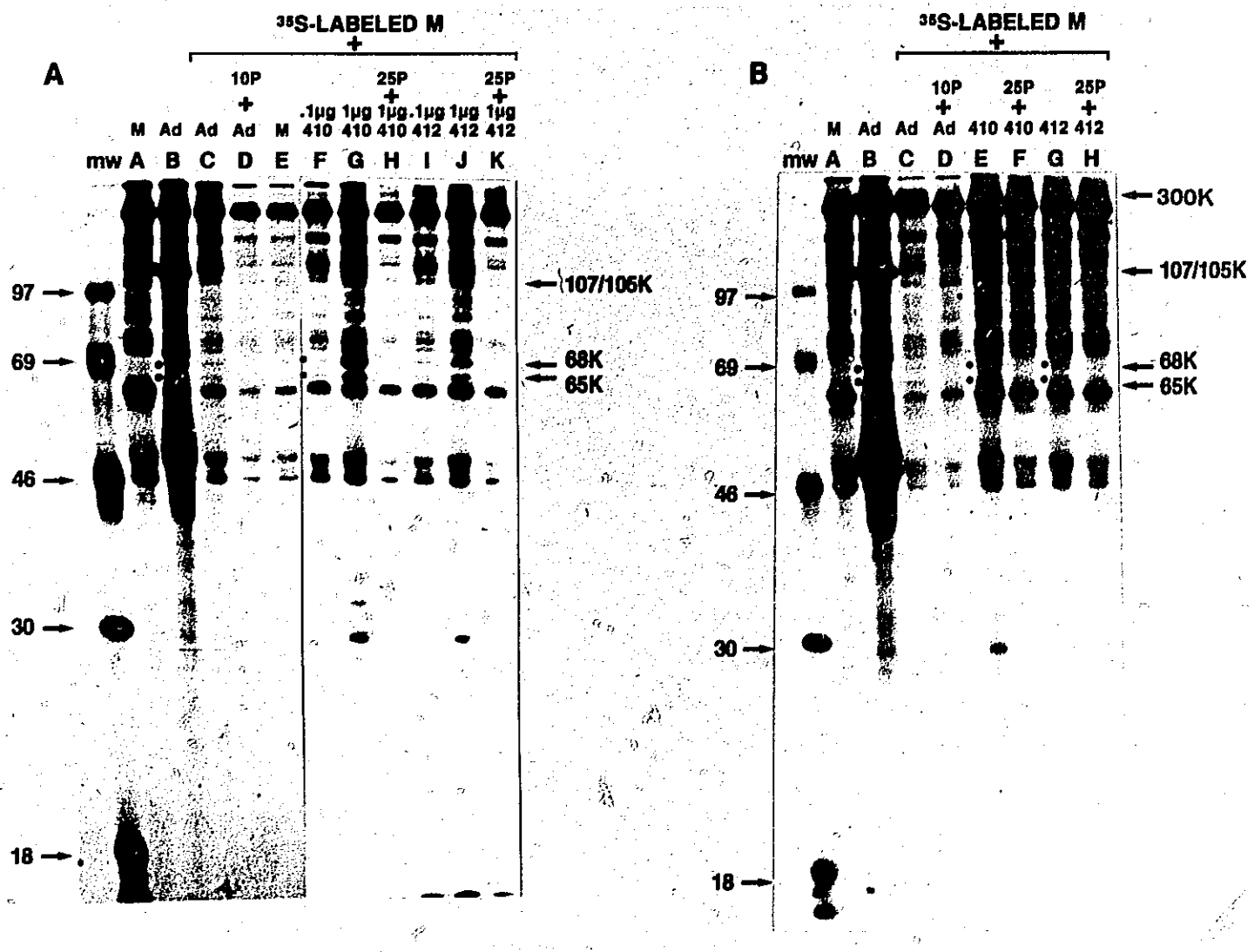
binding of the cellular proteins to E1a products (Yee and Branton, 1985). Figure 5A shows that, using modified Schaffhausen Buffer which contains Ca^{++} and Mg^{++} and the anti-peptide serum E1a-C1 which is specific for the carboxy terminus of E1a proteins (Yee et al., 1983), E1a polypeptides as well as the p107, p105, p68 and p65 cellular proteins were immunoprecipitated from extracts of Ad5 infected cells labeled with ^{35}S-methionine (lane B), but not from those of labeled mock-infected cells (lane A). In a similar experiment using $Ripa^{=}$ which lacks divalent cations, these species as well as the p300 cellular protein were seen (Figure 5B, lane B). P105 and p107 were poorly resolved in these gels, but in other experiments (see below) they were clearly separable as two closely migrating proteins. Figure 5 also shows, as demonstrated previously (Yee and Branton, 1985), that mixing of extracts from labeled uninfected cells with those from unlabeled infected cells resulted in the coprecipitation of these labeled cell proteins along with unlabeled E1a products using E1a-C1 anti-peptide serum (lane C, Figures 5A and 5B). Precipitation of all species was eliminated by the addition of an excess of the carboxy terminal peptide (E1a-C) to which the antiserum was produced (lane D, Figures 5A and 5B).

In order to investigate further the nature of the association, a similar mixing experiment was carried out, except that E1a proteins synthesized in E. coli transformed by plasmids pAS1-E1A410 (12S) or pAS1-E1A412 (13S) (Ferguson et al., 1984, 1985), were used in place of those from infected cells. These proteins are identical in sequence to authentic E1a polypeptides except that they lack the arginine residue at position 2 at the amino terminus. Previous studies had shown that these products are efficiently immunoprecipitated by the E1a-C1 serum (B. Ferguson and P. Branton, unpublished results). Extracts from ^{35}S-methionine labeled uninfected cells were mixed with varying amounts of the purified E. coli-expressed E1a proteins either in the presence or absence of excess of E1a-C peptide and the mixtures were precipitated with E1a-C1 serum. In the presence of divalent cations (using modified Schaffhausen Buffer) small amounts of p65 and p68 were precipitated using 0.1μg of the 13S mRNA product (Figure 5A, lane F), and substantially more was detected using 1.0μg of this protein (Figure 5A, lane G). Precipitation of p68 and p65 proteins was completely blocked by the addition of the E1a-C peptide (Figure 5A, lane H). Similar results were also obtained in the absence of divalent cations but the amounts of p68 and p65 were reduced, as usually

occurs under these conditions (Figure 5B, lanes E and F). Using the E1a 12S mRNA product (Figure 5A, lanes I to K and Figure 5B, lanes G to H) comparable results were obtained, except that the amount of binding of p68 and p65 was somewhat less than that seen with the 13S mRNA product. This observation was consistent with the previous results of Yee and Branton (1985), indicating that p68 and p65 associated somewhat more strongly with the virally-produced E1a 13S gene product. Only a very small amount of binding of material that comigrated with p107 and p105 was observed with either of the E. coli-expressed E1A proteins (Figure 5A, lanes F to K, and Figure 5B, lanes E to H). In the case of p300, binding was poor even in the absence of divalent cations (Figure 5B, lanes E to H). This result was surprising as p300, p107 and p105 generally represented the major species detected in association with virally-produced E1a products. These results indicated that while p65 and p68 bound efficiently to bacterially-produced E1a gene products, p300, p107 and p105 did not.

Figure 5

Association of cellular proteins with Ad5 E1a products from Ad5-infected human cells or synthesized in bacteria from cloned c-DNAs. Ad5 infected and uninfected KB cells were labeled with ^{35}S-methionine from 8 to 10 hours post-infection. These cells, and unlabeled cultures were harvested either in the presence (modified Schaffhausen buffer, Figure 5a) or absence (Ripa$^{=}$, Figure 5b) of divalent cations. In some cases labeled extracts were immunoprecipitated with 25μl of the E1a C-terminal anti-peptide serum, E1a-C1 either in the presence or absence of E1a-C peptide. In others, extracts from labeled uninfected cells were mixed with those from unlabeled infected or mock-infected cells and the mixtures were immunoprecipitated with E1a-C1 serum as described above. Finally, extracts from labeled uninfected cells were mixed with 0.1 or 1.0μg of bacterially-produced E1a 12S or 13S product, and these mixtures were precipitated with E1a-C1 serum in the presence or absence of peptide. Samples were analysed by SDS-PAGE using 9% polyacrylamide gels. E1a proteins from infected cells are present in lane B of panels **A** and **B** and are seen as a diffuse group of bands near the middle of the gel. **A:** Extracts prepared in modified Schaffhausen buffer containing divalent cations. mw, ^{14}C-labeled molecular weight markers (New England Nuclear); lane A, mock-infected cells; B, Ad5-infected cells; C, D, labeled uninfected cells (^{35}S-labeled M) mixed with unlabeled infected cells (Ad) in the absence (C) or the presence (D) of 10μg of E1a-C1 peptide (10P); E, labeled uninfected cell extract mixed with unlabeled mock-infected cell extract immunoprecipitated as for lane C, as a negative control; F, G, and H, labeled uninfected cells mixed with 0.1 (F) or 1.0 (G) μg of purified <u>E. coli</u>-expressed E1a 13S product (410) and 25μg of E1a-C peptide (H); I, J, K, as in F to H except using the E1a 12S product (412). **B:** Extracts prepared in Ripa$^{=}$ lacking divalent cations. mw, ^{14}C-labeled molecular weight markers; lanes A-D, same as lanes A-D in **A**; lanes E-F, same as lanes G-H in **A**; lanes G-H, same as lanes J-K in **A**.


A
35S-LABELED M
+
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M Ad Ad Ad M .1μg 1μg .1μg 1μg 1μg
10P + Ad
25P + 410
25P + 412
410 410 410 412 412 412
97
69
46
30
18
107/105K
68K
65K
B
35S-LABELED M
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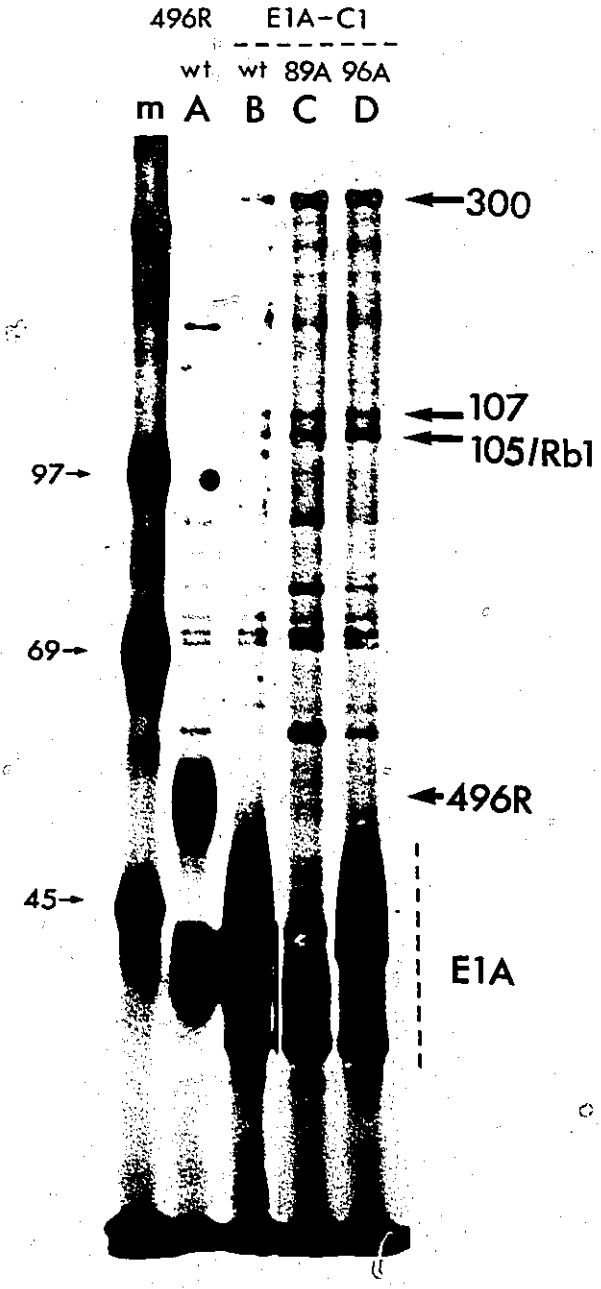
mw A B C D E F G H
M Ad Ad Ad 410 410 412 412
10P + Ad
25P + 410
25P + 412
97
69
46
30
18
300K
107/105K
68K
65K

3.2 Effect of phosphorylation sites in exon 1 on binding

Virus-encoded E1a proteins produced in KB cells are potentially phosphorylated at a number of sites, two of which (serine residues 89 and 96), are in the first exon of the E1a proteins (Tremblay et al., 1988), near regions which play a role in the binding of p300, p107 and p105 (Egan et al., 1988 and see below). E1a proteins produced in bacteria should not be phosphorylated, and it was thought that the reduced binding of the cellular polypeptides might have been due to the lack of phosphorylation at serine 89 or 96. To investigate this possibility, KB cells were infected with either wt Ad5, or with one of the mutant viruses, Ad89-A or Ad96-A, in which the serine residues at positions 89 and 96 respectively were changed to alanines (Tremblay et al., 1989). E1a proteins were immunoprecipitated under mild conditions using the anti-E1a monoclonal antibody M73, and the immunoprecipitates were analysed by SDS-PAGE. As shown in Figure 6, the association of p300, p107 and p105 with E1a products was just as efficient with Ad89-A or Ad96-A (lanes C and D) as with E1a from the wt virus (lane B). Immunoprecipitation of the E1b 58kD protein was used as a negative control as this protein does not

Figure 6

Binding of cellular proteins to mutants lacking phosphorylation sites at Ser-89 and Ser-96. KB cells were infected with wt Ad5 or with the mutants AD89-A and AD96-A which contain alanine residues in place of serines 89 or 96. m: ^{14}C-labeled molecular weight markers (indicated at left of the figure); A-B: wt Ad5 precipitated with monoclonal antibody to the E1b 58kD protein (A) or M73 serum (B); C-D: AD89-A (C) or AD96-A (D) precipitated with M73 serum. The positions of the 300, 107 and 105 kDa proteins, 496R and E1A products are indicated at the right.


496R
E1A-C1
wt
wt
89A
96A
m
A
B
C
D
300
107
105/Rb1
97
69
496R
45
E1A

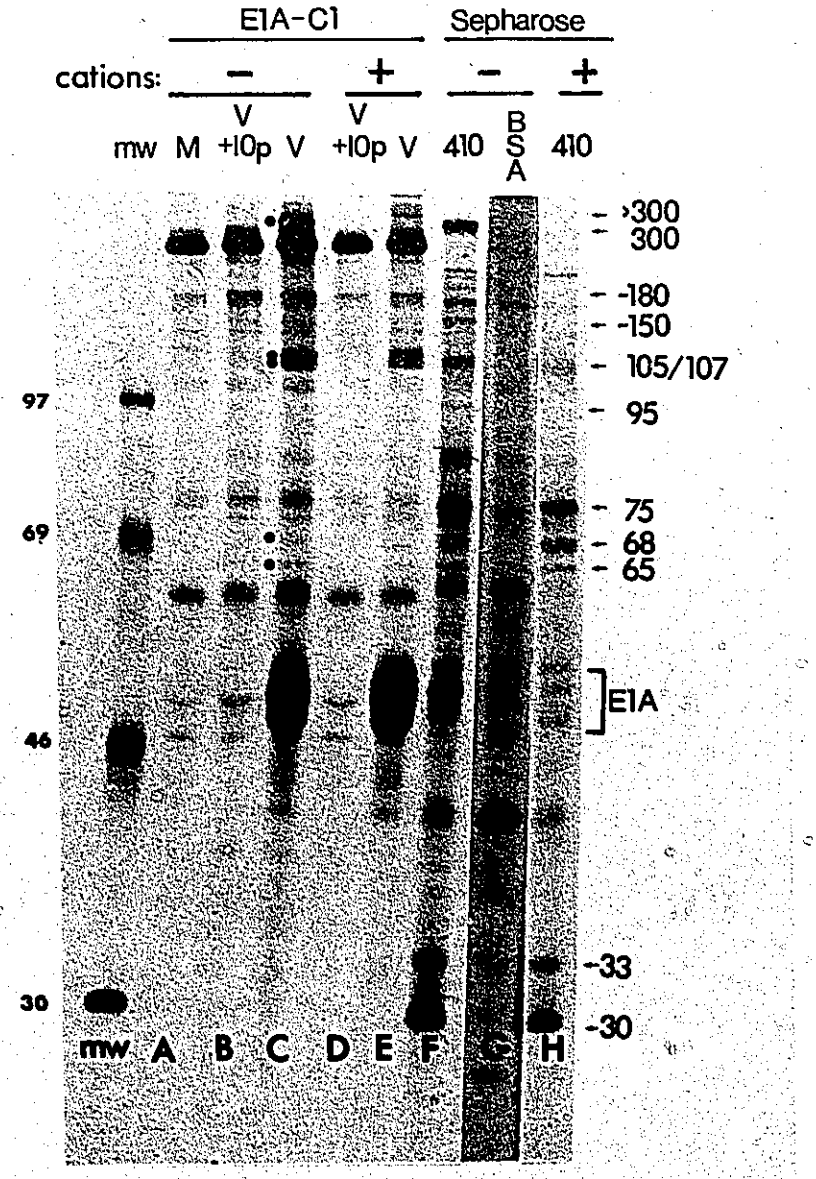
associate with the E1a-binding proteins, and thus there was no binding detected (lane A).

3.3 Association of cellular proteins with bacterially-produced E1a products conjugated to Sepharose beads

Binding of cellular proteins was also examined using the E. coli-produced 13S mRNA product coupled by cyanogen bromide to Sepharose beads (hereafter termed E1a-Sepharose). As a control, bovine serum albumen (BSA)-Sepharose conjugates were also examined. Again, in a standard experiment using labeled uninfected or Ad5 infected KB cells, p107 and p105 were evident (Figure 7, lanes C and E). p300 was seen in the absence (lane C), but not the presence (lane E) of divalent cations. In addition, p68 and p65 were seen at low levels (lane C). In all cases, addition of an excess of E1a-C peptide blocked precipitation of all species (lanes B and D), and none of the cellular proteins were precipitated from mock-infected cells (lane A). When E1a-Sepharose was mixed with extracts from labeled uninfected cells prepared in the absence of divalent cations (Figure 7, lane F), several cellular proteins were detected in association with the beads. Among these were some that were also present when BSA-Sepharose was used (lane G).

Figure 7

Binding of cellular proteins to E. coli-produced E1a protein linked to Sepharose beads. The E. coli-produced E1a 13S mRNA product (410), and as a control BSA, were linked to cyanogen-bromide-activated Sepharose beads as described in Methods and Materials. These beads were mixed for 3 hours at 4°C with extracts from ^{35}S-methionine-labeled uninfected KB cells which were prepared using modified Schaffhausen lysis buffer containing divalent cations or Ripa$^{=}$ lysis buffer, without divalent cations. The beads were then washed and analysed by SDS-PAGE as for immunoprecipitations. In addition a standard experiment using labeled Ad5- or mock-infected cells precipitated with E1a-C1 serum was carried out. mw, ^{14}C-labeled molecular weight marker; lanes A-C, extracts in buffer containing divalent cations and precipitated with E1a-C1 serum; A, mock-infected cells; B, Ad5-infected cells with 10μg of E1a-C peptide; C, Ad5-infected cells; D-E, extracts in lysis buffer lacking divalent cations and precipitated with E1a-C1 serum; D, infected cells and C-terminal peptide; E, infected cells; F, E1a-Sepharose with extracts from uninfected cells prepared in the absence of divalent cations; G, BSA-Sepharose with labeled extracts prepared in the absence of divalent cations; H, E1a-Sepharose with labeled extracts prepared in the presence of divalent cations. The molecular masses (X10^{-3}) of proteins which bound to E1a-Sepharose and not to BSA-Sepharose are noted at the right.


E1A-C1
Sepharose
cations:
−
+
−
+
V
V
mw M +10p V +10p V 410 BSA 410
>300
300
180
150
105/107
95
75
68
65
E1A
33
30
97
69
46
30
mw A B C D E F G H

In addition to these proteins, which presumably bound non-specifically, there were several other prominent species that appeared to be E1a specific. Two of these proteins co-migrated with p68 and p65, and a third with p300. This latter protein was not detected in the presence of divalent cations (lane H), and it was thus considered likely that it was the p300 previously described. In addition, a protein was present which migrated in the gel in a position between p107 and p105, but it was not clear whether this protein was related to one or the other or both. In a number of experiments this band was always seen to migrate in the same position relative to the p107 and p105 bands, and was of the same relative intensity. The fact that it was found to be phosphorylated (data not shown), and p107 and p105 are also phosphoproteins (Yee and Branton, 1985b) suggested that it could be related to one or the other. These results suggested that at least p300, p68 and p65 are capable of binding to E1a-Sepharose. In addition to these species, several other proteins showed affinity for the E1a-Sepharose, but not BSA-Sepharose, including major species of 75, 33 and 30kD, a 150kD protein and more minor species of 95, 180 and >300kD. Thus these polypeptides may also represent E1a-binding proteins. The >300kD protein, in particular, was reproducibly found

in anti-E1a immunoprecipitation reactions, and has been detected with antisera directed against different epitopes on the E1a proteins, suggesting that it is associated with E1a products (see below and data not shown). Polypeptides of about 33 and 30kD were observed in a previous study (Yee and Branton, 1985), but they were seen inconsistently. Upon prolonged exposure of fluorographs, low amounts of these proteins, as well as the 75kD species, were also found with BSA-Sepharose, and so the status of their specificity is unclear. The 30kD species comigrates with histone H3 (data not shown), but identity between the two proteins has not been established. It has been reported that the E1a proteins of Ad12 can associate with histones (Grand and Gallimore, 1984). The 30kD species did not comigrate with the RNA polymerase II-associated protein RAP30, and so was considered not to have identity with this polypeptide (data not shown).

Chapter 4

Mapping the Binding Sites of the Cellular Proteins on the E1a Polypeptides and Correlation of Binding with Biological Activity

Mapping the binding sites of the cellular proteins on the E1a polypeptides and correlation of binding with biological activity

The two major proteins coded for by the E1a region have lengths of 289 residues (the 13S product) and 243 residues (the 12S product), and are identical except for the presence of an additional 46 internal amino acids in the larger species (Perricaudet et al., 1979). Structurally, the 289 residue protein can be divided into three regions: residues 1-139, which are common to both the 289 and 243 residue proteins (exon 1); residues 140-185 which are unique to the 289 residue protein (the unique region); and residues 186-289 which are again common to both proteins (exon 2) (see also Figure 16). E1a proteins are involved in a number of functions, including transactivation of viral and cellular genes (Berk et al., 1979, Jones and Shenk, 1979a, 1979b, Nevins, 1981, 1982, Kao and Nevins, 1983, and Stein and Ziff, 1984) transcriptional repression (Borrelli et al., 1984, Hen et al., 1985, Velcich and Ziff, 1985 and Hen et al., 1986), induction of DNA synthesis and mitosis in quiescent cells (Spindler et al., 1985, Nakajima et al., 1987 and Quinlan et al., 1987), immortalization, and, in

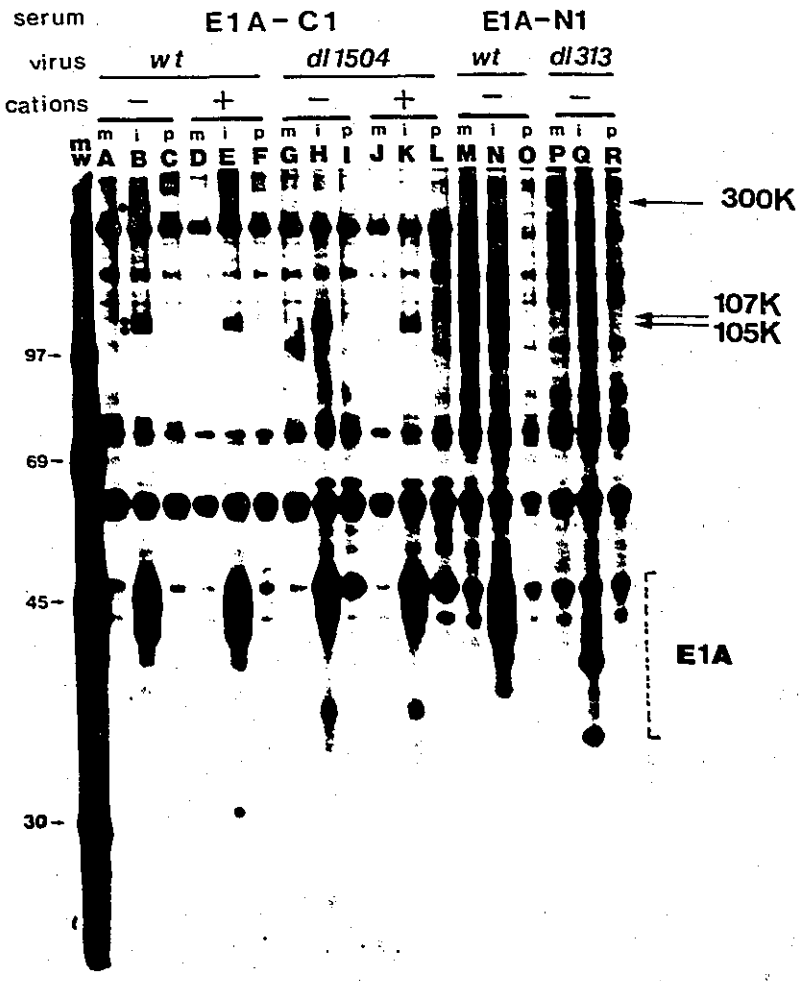
cooperation with E1b proteins, oncogenic transformation (Branton et al., 1985a, and references therein). At the time this research was initiated, others were beginning to find that specific regions or domains of the E1a proteins were involved in specific functions (Jochemsen et al., 1984, Lillie et al., 1986, Moran et al., 1986b and Zerler et al., 1986). As nothing was known at the time about the biological functions of the cellular E1a-binding proteins, a collaborative study with Dr. Stan Bayley was undertaken, in which E1a deletion mutants were examined for their ability to bind to the cellular proteins, and a correlation between binding and biological function was looked for. It was thought that this approach might provide some insight into the E1a-mediated function(s) with which the cellular proteins were involved, and indicate what the normal biological roles of the proteins were in uninfected cells. Most of the E1a deletion mutants used in this study were made and characterized with respect to biological function in Dr. Bayley's lab by Tony Jelsma, Nina Cunniff, Carole Evelegh, John Howe and Joe Mymryk, and the details of their production and characterization have been published (Jelsma et al., 1988, 1989 and Howe et al., 1990).

4.1 Role of the E1a amino and carboxy termini

To examine the importance of the amino and carboxy termini of E1a products in the binding of cellular proteins, human KB cells were infected with wt Ad5 or with mutant dl1504 or dl313 which lack sequences encoding the first 14 residues at the amino terminus (Osborne et al., 1982) or the last 70 amino acids at the carboxy terminus (Colby and Shenk, 1981), respectively. Ad5-infected and mock-infected cultures were labeled with ^{35}S-methionine and cell extracts prepared in either the presence or absence of divalent cations were immunoprecipitated with anti-peptide antiserum E1a-C1 or E1a-N1 which are specific for the carboxy or amino termini respectively of both the E1a 13S and 12S products (Yee et al., 1983, and Yee and Branton, 1985a). In some cases the peptides to which these antisera were generated were also added in the mixture. Immunoprecipitates were analyzed by SDS-PAGE on 8% polyacrylamide gels and migration patterns were determined by fluorography. Figure 8, lane B, shows that with extracts from wt-infected cells, E1a-C1 serum precipitated p300, p105 and p107 in addition to E1a products. As shown previously (Yee and Branton, 1985b), p300 was absent when extracts

Figure 8

E1a-associated proteins in cells infected with the mutants dl1504 and dl313. Human KB cells infected with wt Ad5 or with mutant dl1504 or dl313 were labeled with ^{35}S-methionine and cell extracts prepared either in the presence or absence of divalent cations were immunoprecipitated using either E1a-C1 or E1a-N1 anti-peptide serum, in some cases in the presence of the appropriate synthetic peptides. Lanes: mw, ^{14}C-labeled molecular weight markers (DuPont NEN Research Products); m, mock-infected cells; i, Ad5 infected cells; p, precipitations carried out in the presence of 10μg of appropriate peptide. Extracts were prepared in the presence (+) or absence (-) of divalent cations. To the right of the gel are shown the positions of p300, p107 and p105; to the left are shown the molecular masses of the ^{14}C-labeled markers (÷ 10^3).


serum
E1A-C1
E1A-N1
virus
wt
dl1504
wt
dl313
cations
−
+
−
+
−
−
m i p m i p m i p m i p m i p m i p
mw A B C D E F G H I J K L M N O P Q R
300K
107K
105K
97-
69-
45-
30-
E1A

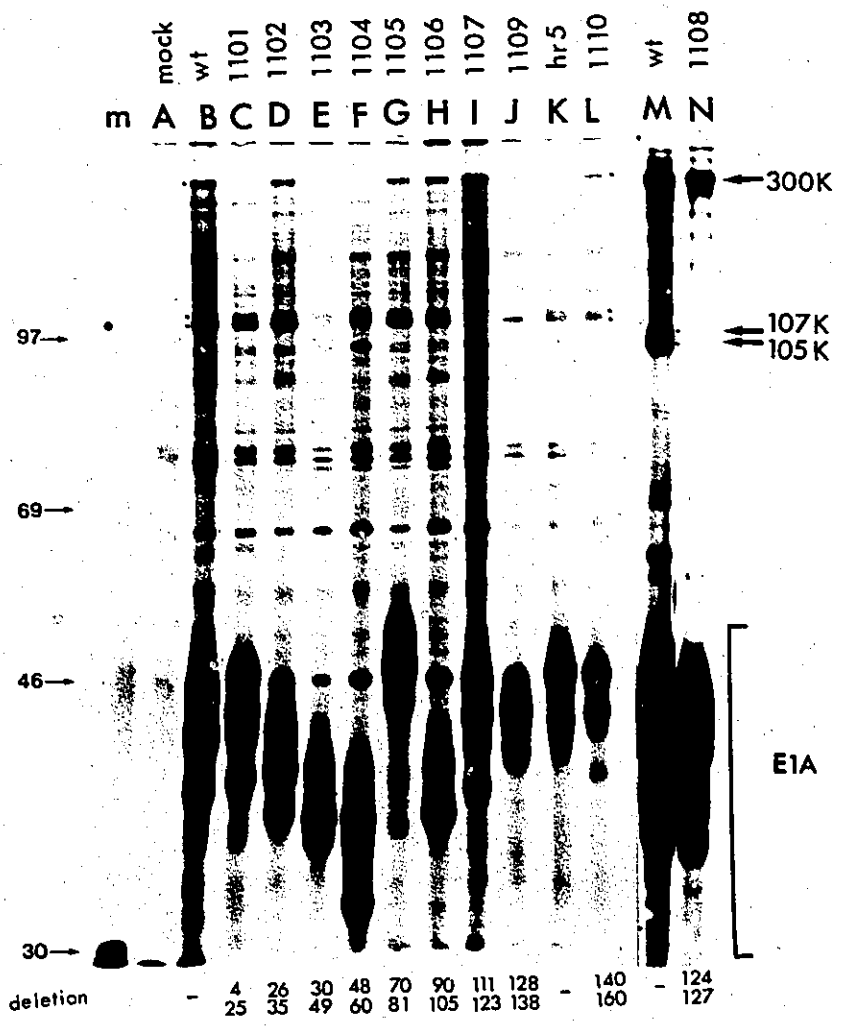
were precipitated in the presence of divalent cations (lane E). The three cellular proteins were not precipitated from mock-infected cells (lanes A, D, G and J), and addition of E1a-C peptide blocked their precipitation (lanes C, F, I and L). With E1a-C1 serum, and extracts from cells infected with mutant dl1504, p107 and p105 were detected, but p300 was not (lanes H and K). With extracts from wt or dl313-infected cells precipitated with E1a-N1 serum p107 and p105 were present but p300 was not (lanes N and Q). These results were compatible with previous data that indicated that E1a protein complexes containing p300 were not precipitated by serum prepared against the amino terminus (Yee and Branton, 1985b). These data suggested that the amino terminus of E1a products may be involved in binding p300 but that neither this region or the last 70 residues at the carboxy terminus plays a significant role for p107 and p105.

4.2 Studies with other deletion mutants and hr5

Cells infected with wt Ad5, with hr5, or with one of a series of deletion mutants were labeled with ^{35}S-methionine, and extracts were precipitated with the E1a-specific monoclonal antibody M73 and analyzed by SDS-

Figure 9

E1a-associated proteins in cells infected with various deletion mutants and hr5. Cells were infected with wt or mutant Ad5 or they were mock-infected. The Ad5 deletion mutants dl1101 to dl1107 (1101 to 1107), dl1109 (1109), dl1110 (1110) and dl1108 (1108) all possess in frame deletions (see Figure 16 and Table 2 for details of the deleted sequences) and their production has been described previously (Jelsma et al., 1988). These mutants were all propagated in cell line 293 (Graham et al., 1977). Cells were labeled with ^{35}S-Methionine and extracts prepared in Ripa$^{=}$ lysis buffer were immunoprecipitated using M73 anti-E1a monoclonal antibody directed against an epitope in the second exon of the E1a polypeptides (Harlow et al., 1985). Lanes: m, molecular weight markers; A, mock-infected cells; B, wt Ad5 infected cells; C through L, mutant-infected cells (as indicated); M and N, wt Ad5 (M) and dl1108 (N) from a separate experiment. The residues missing in the deletion mutants are indicated at the bottom of the figure. Positions of p300, p107 and p105 are shown at the right of the gel; to the left are shown molecular masses of the ^{14}C-labeled markers.


m
A mock
B wt
C 1101
D 1102
E 1103
F 1104
G 1105
H 1106
I 1107
J 1109
K hr5
L 1110
M wt
N 1108
97
69
46
30
300K
107K
105K
E1A
deletion
- 4 25 26 35 30 49 48 60 70 81 90 105 111 123 128 138 - 140 160 - 124 127

PAGE. Mutant hr5 contains a point mutation at nucleotide 1229 which alters Gly-139 in the 12S product to Asp and alters Ser-185 in the 13S product to Asn (Glenn and Ricciardi, 1985). The other mutants contain in-frame deletions and yield E1a products lacking various regions (Jelsma et al., 1988, 1989, Howe et al., 1990; see Figure 16, Tables 2 and 3, and the legend to Figure 9 for complete details). All of these mutants produced reasonable amounts of E1a proteins, although migration rates were anomalous and increased or even decreased in a fashion unrelated to the size of the deletion (Figure 9). An analysis (not shown) indicated that removal of acidic residues and to a lesser extent proline was the principal cause of increased gel mobility of mutant E1a proteins. These results suggested that high contents of these two types of amino acids cause E1a proteins to migrate much slower than would be expected from their molecular masses.

Figure 9 shows that p300, p107 and p105 coprecipitated with E1a products from cells infected with wt Ad5 and hr5 but were reduced or absent with some of the deletion mutants. To quantify the amount of binding in the experiment shown, appropriate exposures of the gel in Figure 9 were scanned by microdensitomer. The amounts of p300, p107 and p105 were normalized to the total

amount of E1a present relative to the values obtained with wt Ad5 (Table 2). The numbers obtained from scanning this gel represented a reasonable approximation of the amount of binding of the cellular proteins seen with these mutants in a number of experiments (Table 2). p300 was undetectable with dl1101 and dl1104, greatly reduced with dl1103, and somewhat reduced with dl1105. These results, together with that obtained with dl1504 showed that binding of p300 was affected by deletions within two regions of exon 1, namely between residues 1 and 25 and between amino acids 36 and at least 60 (Figure 16). The involvement of the first 14 to 25 residues at the amino terminus supported observations made previously (Yee and Branton, 1985b) and in the present study (Figure 8) which indicated that the E1a-N1 anti-peptide serum failed to recognize E1a protein complexes containing p300. This failure may have resulted from the binding of p300 to this region, although it could also have been due to a conformational change induced by binding at another site. p105 was undetectable with dl1107 and somewhat reduced with dl1103 and dl1104, indicating that the region between residues 111 and 123 (and perhaps the region between residues 36 and 60) plays a role in binding this protein. The increased binding with dl1102 was not seen in other experiments in which levels similar

Table 2

Binding to wt background mutants and correlation with transformation.

virus	residues deleted	binding to p300[a] (%wt[b])	binding to p107 (%wt)	binding to p105 (%wt)	transformation with ras[c]
wt	0	++ (100)	++ (100)	++ (100)	+
dl1101	4-25	- (0)	++ (60)	++ (120)	-
dl1102	26-35	++ (160)	++ (150)	++ (180)	+
dl1103	36-49	+/- (10)	+ (40)	+ (50)	-
dl1104	48-60	- (0)	++ (90)	+ (50)	-
dl1105	70-81	+ (50)	++ (90)	++ (120)	+
dl1106	90-105	++ (130)	++ (120)	++ (170)	+
dl1107	111-123	++ (140)	+ (50)	- (0)	-
dl1106[d]	124-127	++ (100)	- (0)	- (0)	-
dl1109	128-138	++ (130)	+ (80)	++ (130)	-
dl1110	140-160	++ (160)	++ (110)	++ (100)	+
dl1111	161-168	++	++	++	+
dl1113	169-177	++	++	++	+
dl1114	178-184	++	++	++	+
hr5		++	++	++	+
dl1504	1-14	-	++	++	+[e]
dl313	220-289	++	++	++	+
dl1141	61-69	+	++	++	nt
dl1142	82-92	++	++	++	nt
dl1143	38-60	-	++	+	nt

a amount of binding obtained by visual examination of relative intensities of the protein bands seen in a number of experiments.

- ++ binding similar that seen with wt E1a
- + binding reduced relative to that seen with wt E1a, but still readily detectable
- +/- binding not detected or extremely reduced

b Values in parentheses are expressed as a percentage of binding relative to that detected with wt E1a proteins and were obtained following microdensitometer scan of the gel shown in Figure 9 and normalization of the intensity of the associated protein bands to the total amount of E1a present.

c Transformation assays were carried out with BRK cells with plasmid pLE2 containing mutated E1a regions and an activated ras gene, as described previously (Lillie et al., 1986 and Schneider et al., 1987). The specific transformation data has been published (Jelsma et al., 1989)

- + transformation
- - no transformation

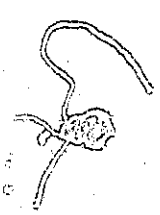
d Values were obtained in a separate experiment.

e Data of Osborne et al. (1982), by a different transformation assay involving virions.

to wt Ad5 were observed (Figure 12 and data not shown). With p107, binding was somewhat reduced with mutants dl1101, dl1103, dl1107 and dl1109. It was considered unlikely that residues 1 to 25 were involved in binding this protein, as reduced binding was not seen in other experiments with mutant dl1101 (see Figures below and data not shown), and was never seen with dl1504 (Figure 8) which lacks residues 1 to 14. Less binding was seen reproducibly in several experiments with dl1103 and dl1109, suggesting that residues 36 to 47 and 128 to 138 could be of some importance.

Because none of the previous mutants completely eliminated binding of p107, mutant dl1108 was constructed in which residues between those removed by mutants dl1107 and dl1109, namely 124 to 127, were deleted. With this mutant, binding of p300 was similar to that seen with wt Ad5 (Figure 9, lane M) but neither p107 nor p105 were detected (lane N). This indicated that the region necessary for binding of p105 could be extended to residue 127, and that residues 124-127 were required to bind to p107. Deletions in the unique region (dl1110, dl1112, dl1113 and dl1114) or in the second exon between residues 188 and 204 (dl1115) did not affect binding of any of the proteins (Table 2 and data not shown). Further mutational analysis in exon 1 showed that the

loss of residues 61 to 69 (dl1141) resulted in somewhat reduced binding of p300 (Figure 14, lane D and Table 2), but loss of residues 82 to 92 (dl1142) had no effect on the binding of any of the cellular proteins (Figure 14, lane F and Table 2). Deletion of residues 38 to 60 (dl1143) resulted in loss of binding of p300, and reduced binding of p105 (Table 2 and data not shown) again suggesting that residues within CR1 affected binding of these two proteins. With the exception of one short region in exon 1 between residues 106 and 110 and one region in exon 2 between amino acids 205 and 219, deletions across the entire E1a polypeptide were examined for their effect on binding the cellular proteins, and the results are summarized in Table 2 and Figure 16. In brief, a region at the amino terminus of E1a proteins, between residues 1 and 25 was involved in binding p300; a region within CR2 between residues 111 and 127 was involved in binding p105; and residues 124 to 127 were involved in binding p107. Deletions in CR1 between residues 36 and 60 resulted in loss of binding of p300, and reduced binding of both p107 and p105.

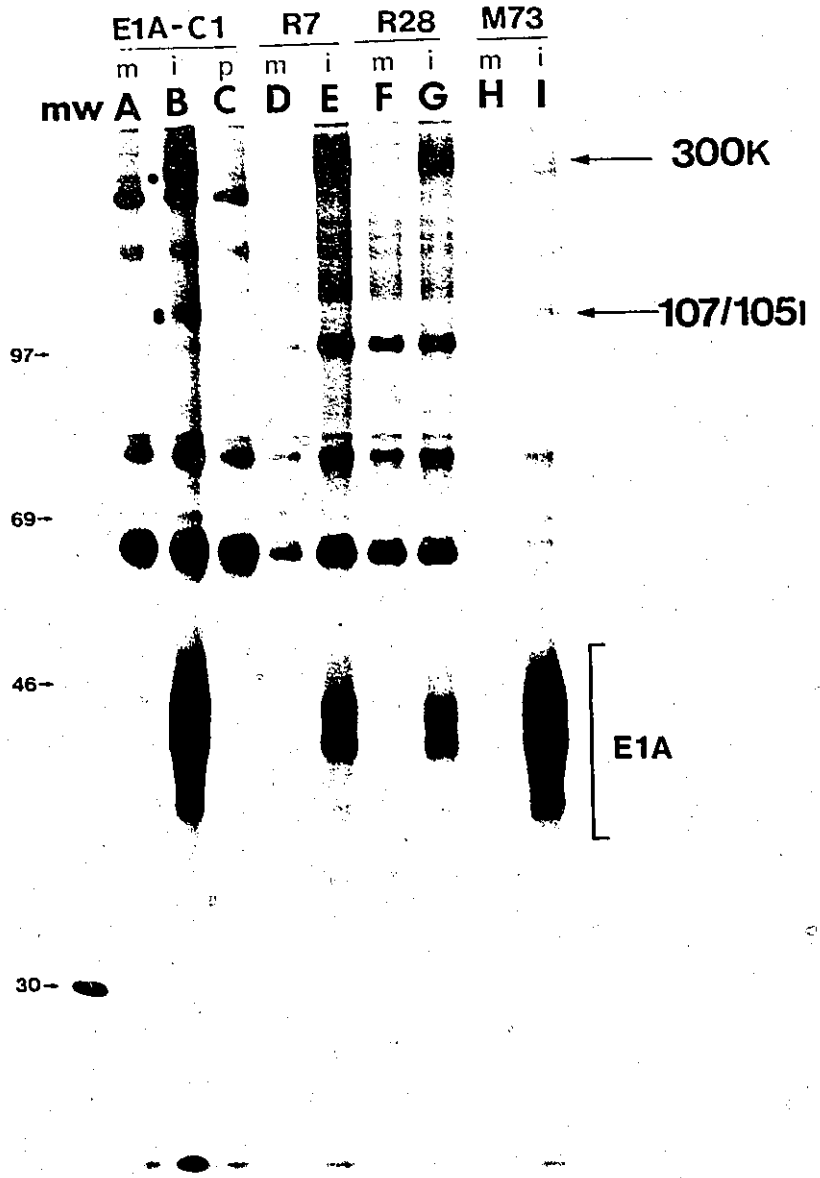


4.3 Role of region between residues 36 and 60

Small deletions between residues 36 and 60 appeared to affect the binding of all three cellular proteins to some extent, but it was not clear whether this region represented an actual binding site for p300, p107 or p105, or whether these sequences contributed conformationally to binding at another site. In order to further investigate the role of this region, KB cells were infected and cell extracts immunoprecipitated with the E1a-specific rat monoclonal antibodies R7 and R28, which had been previously reported to recognize an immunodominant epitope lying somewhere between residues 23 and 120 (Tsukamoto et al., 1986a). In immunoprecipitation reactions, both R7 and R28 recognized E1a proteins complexed with p300, but neither recognized E1a proteins bound to p107 or p105 (Figure 10, lanes E

Figure 10

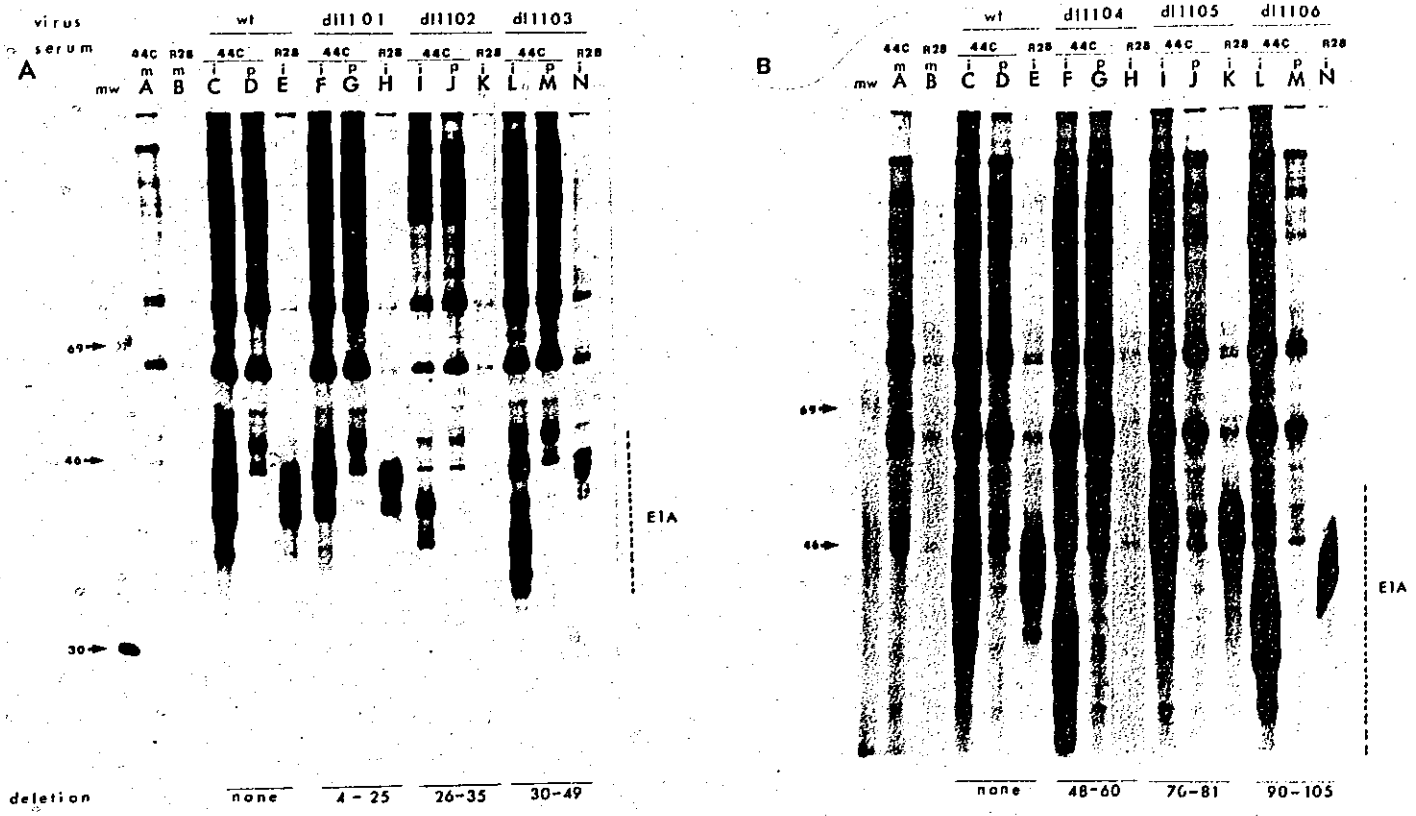
Immunoprecipitation of E1a-associated proteins using various E1a-specific antisera. Cells were infected with wt Ad5, or they were mock-infected, and after labeling with ^{35}S-methionine, the cells were lysed in Ripa$^{=}$ lysis buffer and the extracts immunoprecipitated with either the anti-peptide serum E1a-C1, the rat monoclonal antibodies R7 or R28, or the mouse monoclonal antibody M73. Symbols are as described in the legend to Figure 8.


E1A-C1
R7
R28
M73
m i p m i m i m i
mw A B C D E F G H I
300K
107/105l
97-
69-
46-
E1A
30-

and G), although E1a gene products in association with all three cellular proteins could be precipitated from the same cell extracts with the M73 monoclonal which recognizes an epitope in the second exon of E1a proteins (lane I), or with the E1a-C1 anti-peptide antiserum (lane B, with peptide competition shown in lane C). None of the E1a-binding proteins were precipitated by any of these antisera using extracts from uninfected cells (lanes A, D, F or H). In order to more closely delineate the amino acid residues which constituted the R7/R28 epitope, KB cells were infected with wt Ad5 or one of the dl1101 series of deletion mutants, and the extracts were immunoprecipitated with either the E1a-C1 anti-peptide serum or the R28 monoclonal. As shown in Figure 11, the R28 antibody failed to immunoprecipitate E1a proteins from three of the deletion mutants, dl1102, dl1103 and dl1104 (Figure 11A, lanes K and N and Figure 11B, lane H) although E1a proteins from these same cell extracts could be immunoprecipitated by the E1a-C1 anti-peptide antiserum (Figure 11A, lanes I and L and Figure 11B, lane F, with peptide competitions in the succeeding lanes). The R28 monoclonal recognized E1a proteins from cells infected with wt virus, or with dl1101, dl1105 or dl1106 (Figure 11A, lane H and Figure 11B, lanes K and N). These data indicated that the epitope for R28 (and for R7

Figure 11

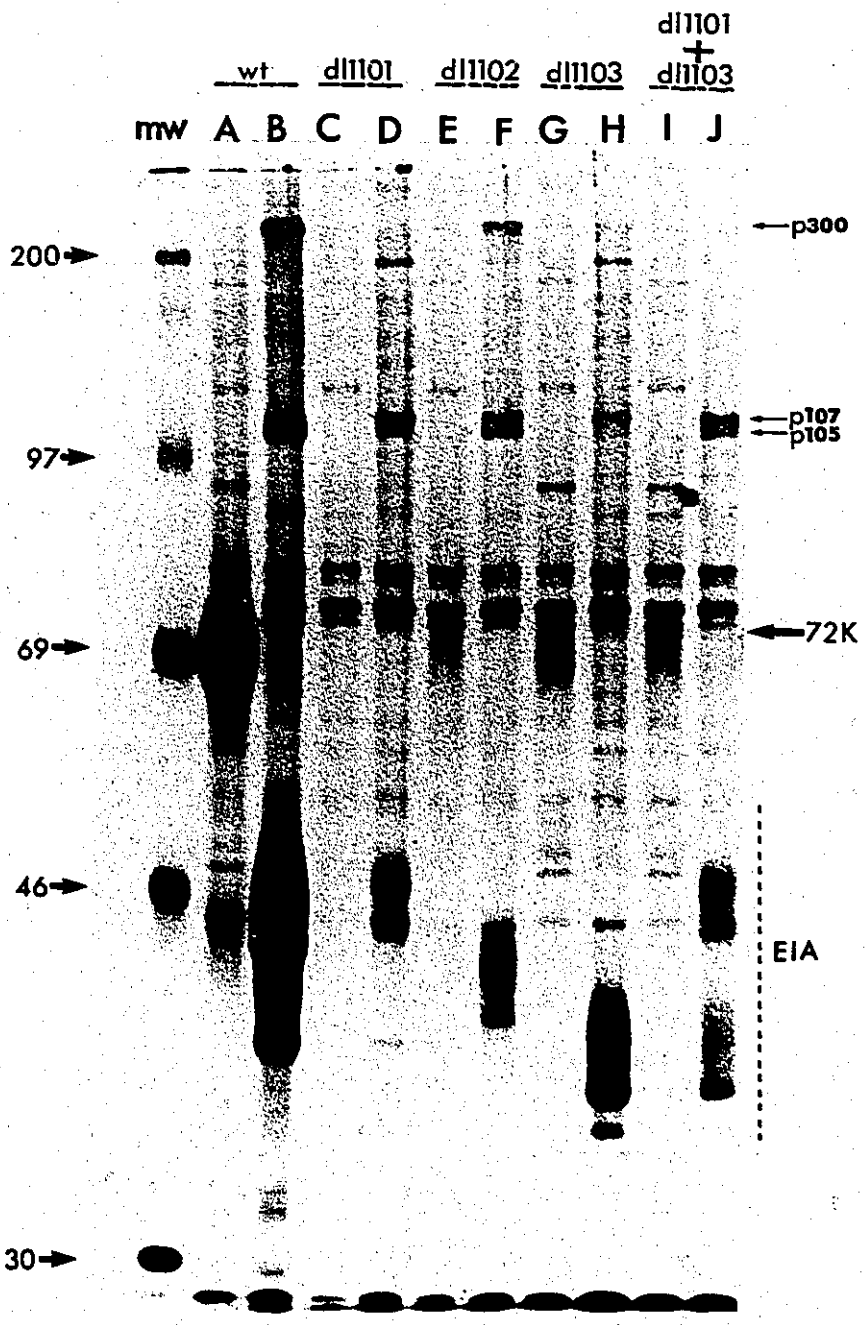
Determination of the binding site of the R28 monoclonal antibody. Cells were infected with the various deletion mutants or wt Ad5, or were mock-infected, and after labeling, cell extracts were immunoprecipitated using either E1a-C1 serum or the R28 rat monoclonal antibody. A: mw, molecular weight markers; A, B, extracts from mock-infected cells immunoprecipitated with either the E1a-C1 serum (A) or the R28 serum (B); C to N, extracts from cells infected with wt Ad5 (C to E) or cells infected with the E1a deletion mutants dl1101 (F to H), dl1102 (I to K) or dl1103 (L to N) immunoprecipitated with E1a-C1 serum in the absence (C, F, I and L) or the presence (D, G, J and M) of E1a-C peptide, or immunoprecipitated with R28 serum (E, H, K and M). B: Symbols are as for A but extracts were immunoprecipitated from cells infected with wt Ad5 (lanes C to E) or one of the E1a deletion mutants dl1104 (lanes F to H), dl1105 (lanes I to K) or dl1106 (lanes L to N), or from mock-infected cells (lanes A and B).


A
virus
serum
wt
dl1101
dl1102
dl1103
44C
R28
mw
A
B
C
D
E
F
G
H
I
J
K
L
M
N
69
46
30
E1A
deletion
none
4 - 25
26-35
30-49
B
wt
dl1104
dl1105
dl1106
48-60
70-81
90-105

which reacts with the same or an overlapping epitope (Tsukamoto et al., 1986a) required the entire region between amino acid residues 26 and 60, suggesting that this region has a degree of secondary structure which was disrupted by the deletions in dl1102, dl1103 and dl1104. The inability of these antibodies to recognize E1a proteins bound to p107 or p105 suggested that p107 and p105 interact in some way with the region between residues 36 and 60, and that this binding either blocks or alters the epitope recognized by R28. The fact that p300 can be immunoprecipitated in association with E1a proteins by the R7 or R28 antisera suggested that the region between residues 36 and 60 is not a primary binding site for this protein. This, in turn, implies that the role of this region may be to confer a degree of secondary structure near the amino terminus of E1a proteins which allows p300 to bind at the amino terminus. This suggestion is consistent with the result shown in Figure 12, demonstrating that two of the regions important in binding p300, between residues 4 and 25, and 30 and 49, do not complement each other for binding p300 in trans but must be present on the same E1a molecule in cis. For this experiment cells were infected with wt Ad5, or dl1101, or dl1103 or were co-infected with both

Figure 12

Double infection with dl1101 and dl1103 does not complement for p300 binding in trans. Extracts from ^{35}S-methionine-labeled KB cells infected with wt Ad5 (lanes A and B) or one of the E1a deletion mutants dl1101, (lanes C and D) dl1102 (lanes E and F) or dl1103 (lanes G and H), or infected with both dl1101 and dl1103 (lanes I and J) were immunoprecipitated using the hamster monoclonal antibody H219, directed against the E2a 72kD protein (lanes A, C, E, G, I) or the anti-E1a mouse monoclonal antibody M73 (lanes B, D, F, H, J). mw, ^{14}C-labeled molecular weight markers.


dl1101
+
wt
dl1101
dl1102
dl1103
dl1103
mw A B C D E F G H I J
200
97
69
46
30
p300
p107
p105
72K
EIA

dl1101 and dl1103 to provide the two regions in trans, and extracts were immunoprecipitated with the M73 anti-E1a monoclonal antibody (Figure 12, lanes B, D, F, H, J) or the H-219 hamster monoclonal against the E2a 72kD DNA binding protein (lanes A, C, E, G, I) as a negative control. The amount of p300 precipitated in conjunction with E1a proteins from extracts of cells infected with both dl1101 and dl1103 (lane J) was essentially the same as the amount precipitated from cells infected with either dl1101 (lane D) or dl1103 (lane H), and was clearly less than the amount precipitated with E1a from cells infected with wt Ad5 (lane B) or the mutant dl1102 (lane F) which binds p300 at wt levels. The ability of these mutants to transform primary baby rat kidney cells in conjunction with activated ras, and their ability to bind the cellular proteins has been summarized in Table 2 (see also Egan et al., 1988 and Jelsma et al., 1989). Mutants producing E1a products which failed to bind to p300, p105, or both p107 and p105 were found to be transformation deficient.

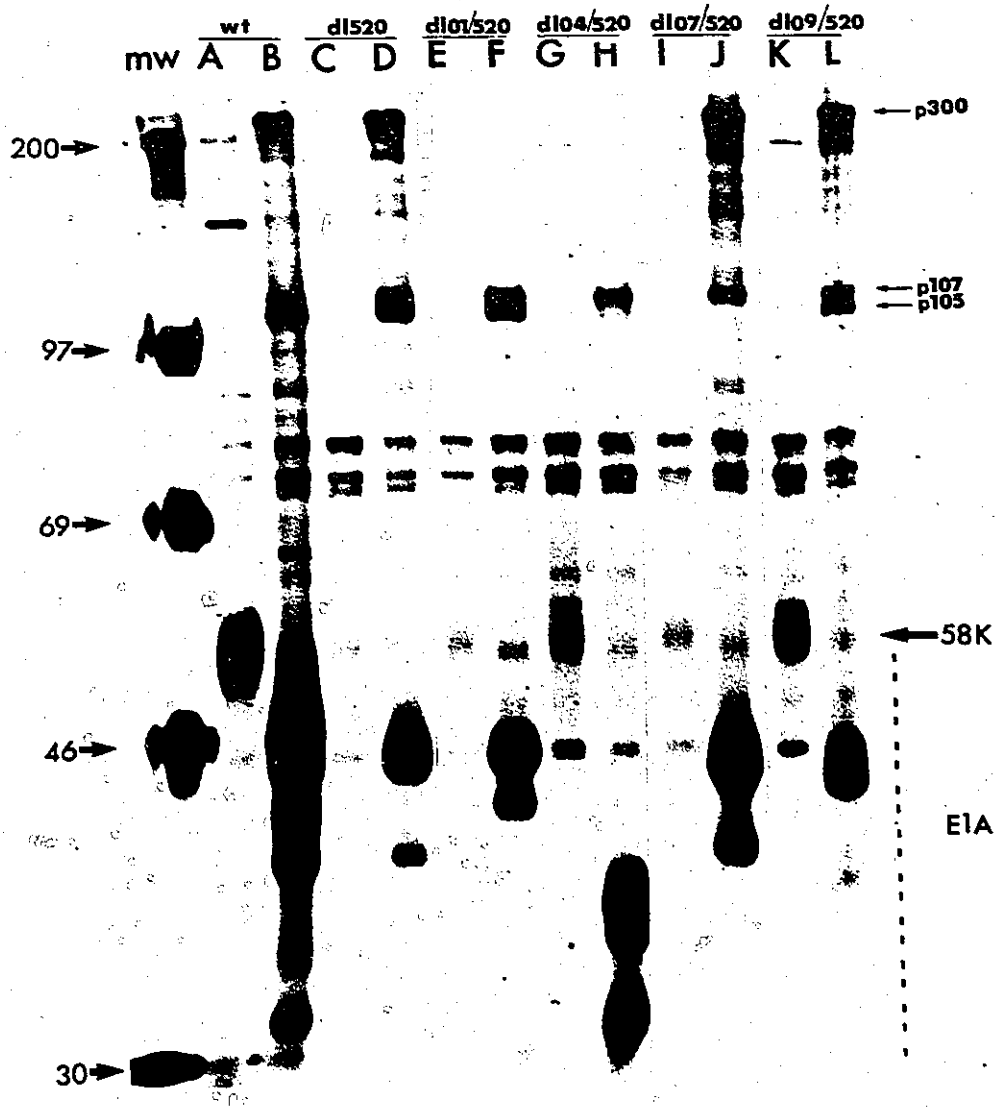
4.4 Studies with single and double mutants in a 12S background

In order to characterise the ability of E1a deletion mutants to induce DNA synthesis in quiescent cells, viruses which produced E1a gene products from the dl1101 series of mutants rescued into a 12S background were developed (ie. dl1101/520, dl1102/520 etc.) (Howe et al., 1990). The DNA synthesis assays required that the 12S products alone be tested, as the 13S products were found to be cytolytic over the extended period of time required to assay this function (Howe et al., 1990). The E1a products of these 12S deletion mutants were examined for their ability to associate with the cellular proteins. Previous results obtained using the E1a mutants dl520 and dl313, which produce only 12S or 13S products, respectively, had indicated that p300, p105 and p107 bound as efficiently to the 12S E1a proteins as they did to the 13S proteins (Yee and Branton, 1985b). These results were consistent with the mapping described above, indicating that all three cellular proteins bound to regions in exon 1 and were unaffected by mutations in the unique region. Thus, it was expected that these cellular proteins would bind in a comparable fashion to exon 1 deletion mutants expressed in 12S background (ie.

dl1101/520, dl1102/520 etc.). To test this, KB cells were infected with either wt virus, dl520, or one of the dl1101/520 series of E1a mutants, and the extracts were immunoprecipitated with M73 monoclonal antibody or, as a negative control, a mouse monoclonal against the E1b 58kD protein. Representative immunoprecipitations are shown in Figure 13 and the data are summarized in Table 3. Essentially the same results were seen with the 12S series of mutants as with those expressed in a wt background. E1a products from the mutants dl1101/520 and dl1104/520 failed to bind p300 (lanes F and H) and dl1103/520 and dl1105/520 products associated poorly with p300 (data not shown). Products from dl1107/520 failed to bind p105 (lane J) and E1a proteins from dl1108/520 did not associate with either p105 or p107 (data not shown). Again, the mutants dl1103/520 and dl1104/520 produced E1a proteins which associated with reduced amounts of p107 and/or p105 (lane H and data not shown). The dl1109/520 mutant bound reduced amounts of p107 relative to the amounts of p300 and E1a proteins in the immunoprecipitate (lane L and data not shown). The reduced amount of p105 in the immunoprecipitate in lane L was not seen in other experiments. In order to test directly whether a mutant which showed reduced binding in the wt background showed the same degree of reduced

Figure 13

E1a-associated proteins in cells infected with deletion mutants expressed in 12S background. Extracts from ^{35}S-methionine-labeled KB cells infected with wt Ad5 (lanes A and B), the dl520 mutant which expresses only the E1a 12S products (Haley et al., 1984) (lanes C and D), or with one of the 12S background deletion mutants dl1101/520 (lanes E and F), dl1104/520 (lanes G and H), dl1107/520 (lanes I and J), or dl1109/520 (lanes K and L), were immunoprecipitated using an anti-E1b 58kD mouse monoclonal antibody (lanes A, C, E, G, I and K) or the M73 monoclonal antibody specific for E1a proteins (lanes B, D, F, H, J and L). The production of these 12S background mutants has been described elsewhere (Howe et al., 1990). mw, ^{14}C-labeled molecular weight markers.

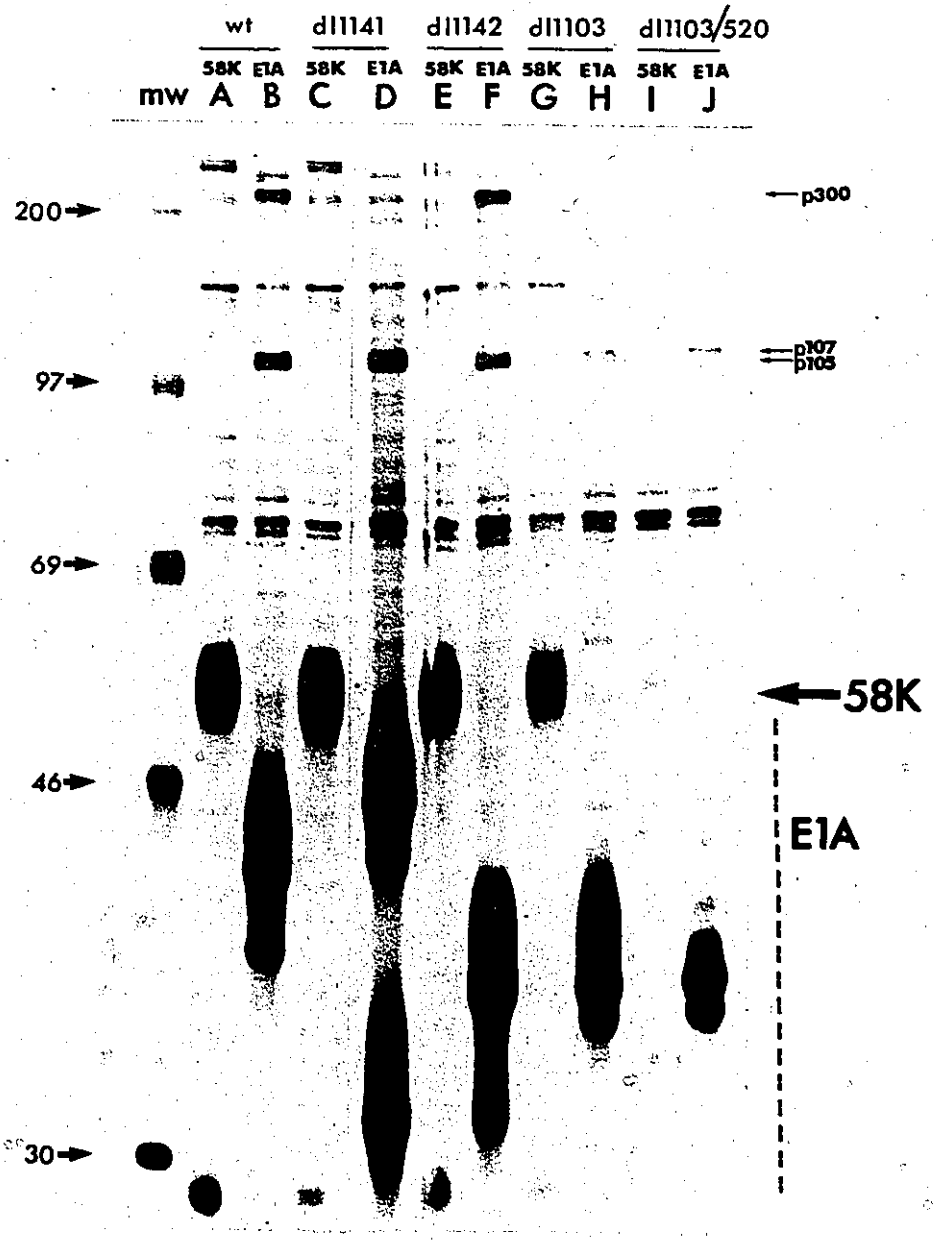

wt
dl520
dl01/520
dl04/520
dl07/520
dl09/520
mw A B C D E F G H I J K L
200
97
69
46
30
p300
p107
p105
58K
E1A

binding in the 12S background, KB cells were infected with wt Ad5 or with dl1103 or dl1103/520, both of which produce E1a products which bind only reduced amounts of p300 and p105. Extracts from infected cells were immunoprecipitated with M73 or the anti-58kD monoclonal as a negative control, and the results, shown in Figure 14 indicated that the amounts of p300 and p105 bound to E1a products from dl1103 or dl1103/520 (lanes H and J) were the same, but clearly much reduced relative to wt (lane B), even though similar amounts of E1a proteins were precipitated in all reactions. In this experiment, the ability of the E1a-binding proteins to bind to E1a polypeptides missing residues 61 to 69 (dl1141) (Figure 14, lane D) or residues 82 to 92 (dl1142) (lane F) was also investigated. Neither of these deletions appeared to affect binding of p107 or p105, but loss of residues 61 to 69 resulted in reduced association with p300 (lane D).

A series of E1a mutants carrying these exon 1 mutants alone or in various combinations in a 12S background was produced by John Howe and Joe Myrmyk in Dr. Stan Bayley's lab and characterised with respect to their ability to induce DNA synthesis in quiescent rat cells (Howe et al., 1990). Again, in order to determine the relationship, if any, between the ability of the

Figure 14

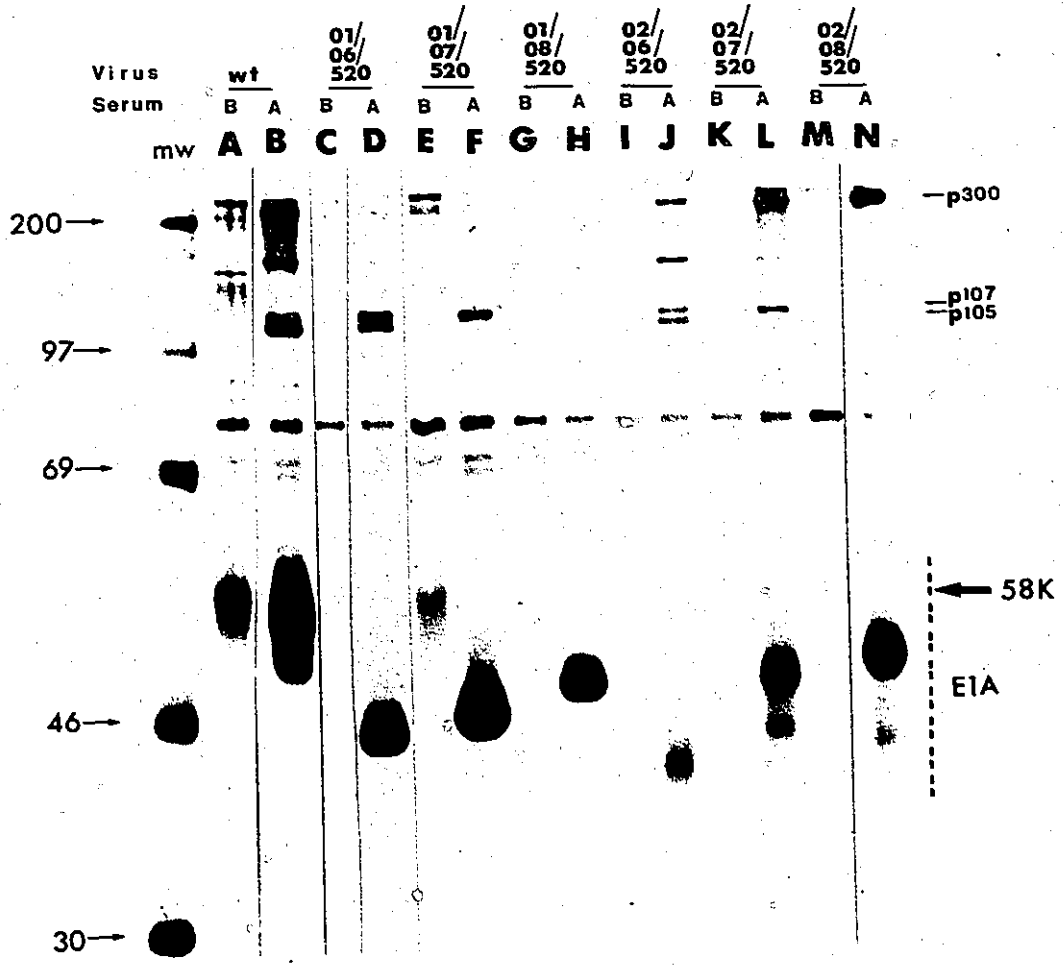
E1a-associated proteins in cells infected with the deletion mutants dl1141, dl1142, dl1103, and dl1103/520. Extracts were made from ^{35}S-methionine-labeled KB cells infected with wt Ad5 (lanes A and B), or with one of the exon 1 deletion mutants dl1141 (lanes C and D), dl1142 (lanes E and F) or dl1103 (lanes G and H), or with the mutant dl1103/520 producing only a 12S product containing the dl1103 exon 1 mutation (lanes I and J), and were immunoprecipitated with an anti-E1b 58kD monoclonal antibody (lanes A, C, E, G, I), or with the M73 monoclonal antibody directed against E1a proteins (lanes B, D, F, H, J). mw, ^{14}C-labeled molecular weight marker. The unusual migration pattern of the E1a polypeptides from the mutant dl1141 may result from the creation of a new DNA sequence identical to the splice acceptor site of the 12S and 13S products, across the junction of the deleted region. If this splice acceptor site were to be used in conjunction with the upstream splice donor for the 10S and 11S messages, it could result in the production of an additional heterogeneous group of proteins missing additional amino acid residues between residues 27 and 60, which would be expected to migrate on an SDS polyacrylamide gel in a manner similar to the group of smaller E1a products seen in Lane D.


wt
dl1141
dl1142
dl1103
dl1103/520
58K E1A 58K E1A 58K E1A 58K E1A 58K E1A
mw A B C D E F G H I J
200
97
69
46
30
p300
p107
p105
58K
E1A

mutated E1a products carrying combined deletions to associate with p300, p107 and p105, KB cells were infected with wt virus, or with one of the deletion mutants and extracts were immunoprecipitated with M73 monoclonal antibody or with the anti-58kD monoclonal. Representative results are depicted in Figure 15 and results for all mutants examined are summarized in Table 3. The results were consistent with those previously shown: ie. viruses containing the dl1101 mutation at the amino terminus in combination with the deletions in dl1106, dl1107, or dl1108 (lanes D, F and H), produced E1a proteins which failed to bind p300. p300 associated somewhat better with E1a proteins from dl1103/08/520 and dl1104/08/520 than with those from dl1103/520 and dl1104/520 (Tables 2 and 3 and data not shown). If the region deleted in dl1104 and dl1103 has a conformational effect on a p300 binding site at the amino terminus, then it may be possible for the additional deletion of residues in dl1108 to alter the conformation in some other way so as to provide a slightly different effect on binding of p300. E1a proteins produced by viruses having the dl1107 mutation in combination with any other deletion failed to bind p105 (ie. lanes F and L), and those from viruses with the dl1108 mutation combined with another deletion failed to bind to both p107 or p105 (ie.

Figure 15

E1a-associated proteins in cells infected with mutants carrying double deletions in exon 1 of the E1a 12S products. Extracts were made from ^{35}S-methionine-labeled KB cells infected with wt Ad5 (lanes A and B), or with one of the exon 1 double deletion mutants in 12S background, dl1101/1106/520 (referred to as dl01/06/520) (lanes C and D), dl01/07/520 (lanes E and F), dl01/08/520 (lanes G and H), dl02/06/520 (lanes I and J), dl02/07/520 (lanes K and L) or dl02/08/520 (lanes M and N), and were immunoprecipitated with an anti-E1b 58kD monoclonal antibody (lanes A, C, E, G, I, K and M), or with the M73 monoclonal antibody directed against E1a proteins (lanes B, D, F, H, J, L and N). mw, ^{14}C-labeled molecular weight marker.


Virus
Serum
wt
01/06/520
01/07/520
01/08/520
02/06/520
02/07/520
02/08/520
B A B A B A B A B A B A B A
mw
A B C D E F G H I J K L M N
200
97
69
46
30
p300
p107
p105
58K
E1A

lanes H and N). E1a proteins which bound to either p300 or p105 could induce cellular DNA synthesis but those failing to bind to both cellular proteins were deficient at induction of DNA synthesis (Table 3 and Howe et al., 1990). The ability to induce DNA synthesis did not appear to be affected by the binding or failure to bind to p107.

The binding site data is summarized in Figure 16, which shows a schematic representation of the E1a polypeptide, indicating the locations of the conserved regions, the deletion mutants and antisera used in this study, and the regions on the E1a polypeptide involved in binding p300, p107 and p105.

Table 3

Binding to 12S background single and double mutants and correlation with induction of DNA synthesis.

virus	binding to p300	binding to p107	binding to p105	induction of DNA synthesis
dl520	++	++	++	++
dl312	-	-	-	-
dl1101/520	-	++	++	++
dl1102/520	++	++	++	++
dl1103/520	+/-	+	+	+
dl1104/520	-	++	+	+
dl1105/520	-	++	++	++
dl1106/520	++	++	++	++
dl1107/520	++	+	-	++
dl1108/520	++	-	-	++
dl1109/520	++	+	++	++
dl01/07/520	-	++	-	-
dl01/08/520	-	-	-	-
dl43/08/520	-	-	-	-
dl03/08/520	+	-	-	+
dl04/08/520	+	-	-	+
dl1143/520	-	++	+	+
dl01/06/520	-	++	++	++
dl02/06/520	++	++	++	++
dl02/07/520	++	++	-	++
dl02/08/520	++	-	-	++
dl05/07/520	++	++	-	++

The amount of binding of the cellular proteins was obtained by visual examination of the relative intensities of the protein bands seen in a number of experiments and is expressed as

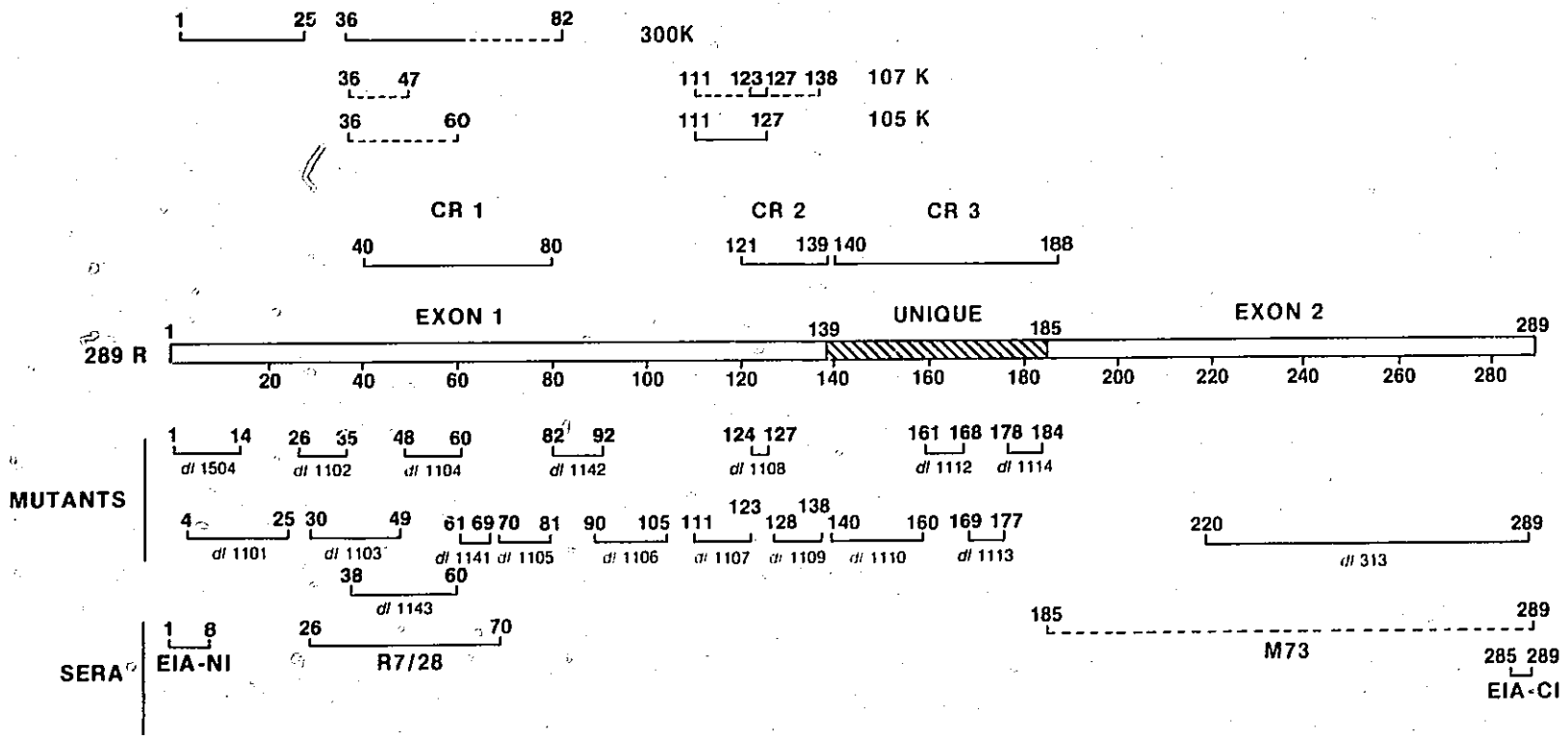
- ++ binding similar that seen with wt E1a
- + binding reduced relative to that seen with wt E1a, but still readily detectable
- +/- binding not detected or extremely reduced

DNA synthesis data for these mutants has been published (Howe et al., 1990). Briefly, DNA synthesis was measured in baby rat kidney cells which were plated on 60 mm dishes, and infected with virus at a m.o.i. of 10, three days after plating. At the time of infection, or 24 or 48 hours post-infection, the cells were labeled over a 24 hour period with 50μCi of ^{3}H-thymidine in fresh medium. The cells were harvested and lysed in 0.3M NaOH, DNA was precipitated from the lysate with trichloroacetic acid and collected on Watman glass fibre filters, which were counted in a scintillation counter. Ability to induce DNA synthesis is expressed as

- ++ induction at a level comparable to that of dl520 (wt E1a 12S product)
- + induction at a level reduced relative to dl520 induction, but greater than that seen in the absence of E1a products (ie. with dl312)
- - poor induction, comparable to that seen with dl312 (no E1a products)

Figure 16

Ad5 E1a 289R (13S) product, mutants, antisera, conserved regions and binding sites. The 289R protein is shown with its three structural regions: that encoded by exon 1 of the 12S mRNA (residues 1 to 138), the region unique to the 289R product of the 13S mRNA and the region encoded by exon 2 of both E1a mRNAs (residues 186 to 289 of the 289R protein). Shown below are the regions believed to be recognized by various E1a-specific sera and the locations of deletions in the various Ad5 mutants. Shown above are the positions of conserved regions 1, 2 and 3 (CR1, CR2 and CR3 respectively) and the locations of the regions which play a role in the binding of p300, p107 and p105, as interpereted from data obtained in the present report. ————, Sequences essential for binding; -------, sequences having some effect on binding.


1 25 36 82
300K
36 47
111 123 127 138
107 K
36 60
111 127
105 K
CR 1
CR 2
CR 3
40 80
121 139 140 188
EXON 1
UNIQUE
EXON 2
289 R
1
139
185
289
20 40 60 80 100 120 140 160 180 200 220 240 260 280
MUTANTS
1 14
dl 1504
26 35
dl 1102
48 60
dl 1104
82 92
dl 1142
124 127
dl 1108
161 168
dl 1112
178 184
dl 1114
4 25
dl 1101
30 49
dl 1103
61 69
dl 1141
70 81
dl 1105
90 105
dl 1106
111 123
dl 1107
128 138
dl 1109
140 160
dl 1110
169 177
dl 1113
38 60
dl 1143
220 289
dl 313
185 289
M73
285 289
EIA-CI
SERA
1 8
EIA-NI
26 70
R7/28

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Chapter 5.

Identification of p105 as Rb, product of the retinoblastoma tumour suppressor gene

Identification of p105 as Rb, product of the retinoblastoma tumour suppressor gene

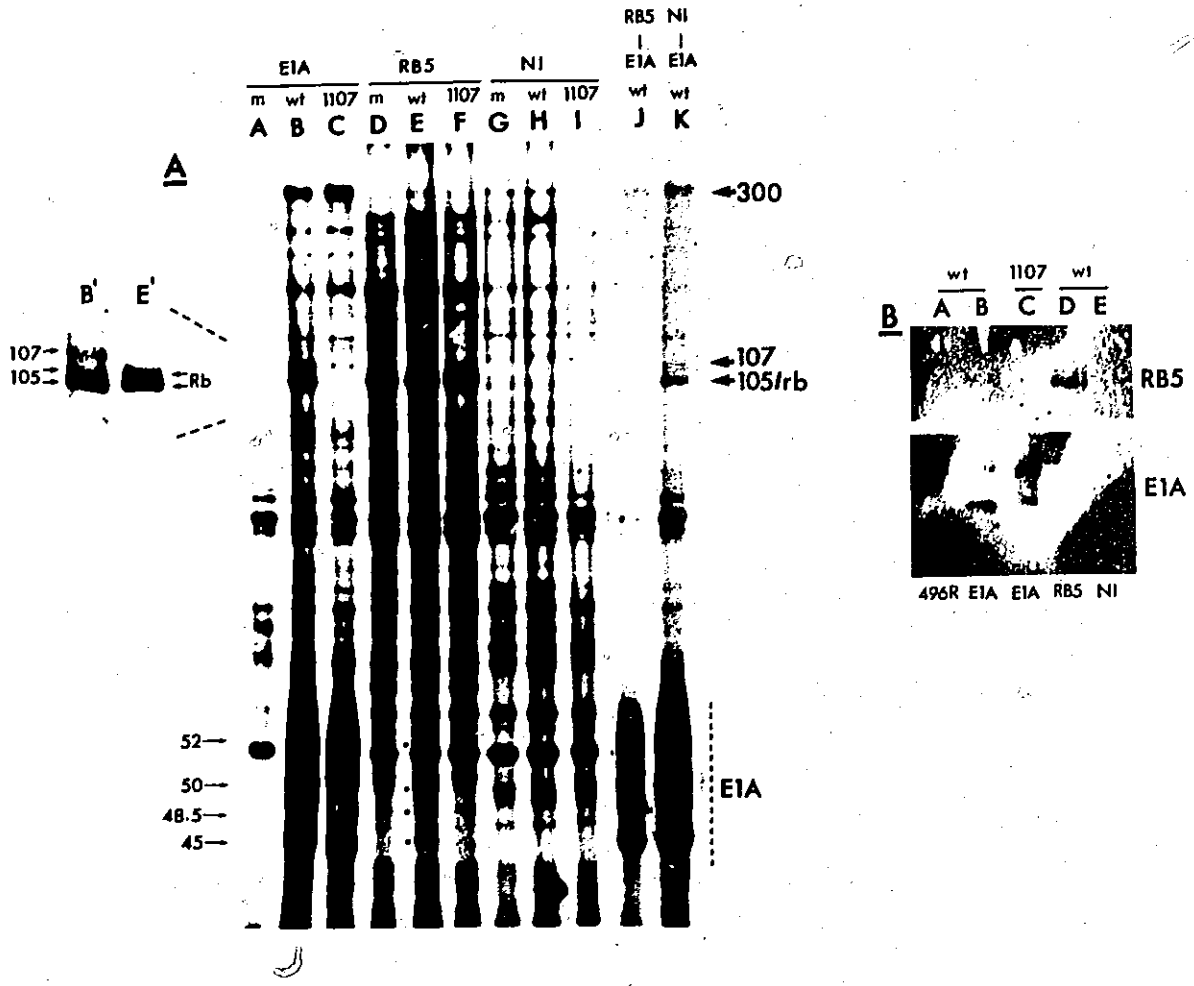
Little was known of the identity or function of cellular proteins found in association with E1a gene products prior to the report of Whyte et al. (1988a) identifying the 105kD E1a-binding protein observed by Harlow and coworkers as the product of the Rb1 recessive oncogene. This gene is absent or altered in retinoblastomas and other human cancers and encodes a nuclear phosphoprotein associated with DNA binding activity (Friend et al., 1986, 1987, Fung et al., 1987, and Lee et al., 1987a, 1987b). The Rb1 gene product was also reported to bind to the large T antigen of SV40 (DeCaprio et al., 1988). These data suggested that the effective removal of the Rb protein into complexes with DNA tumour virus oncogene products could be of some importance in transformation by these viruses. The following experiments were undertaken in order to confirm that the 105kD E1a-binding protein first reported by Yee and Branton (1985) was the product of the Rb1 gene, and to investigate the amount of Rb protein which complexed to E1a products in infected and transformed cells.

5.1 The E1a-binding protein p105 is the product of the Rb1 gene

To investigate the relationship between the E1a-binding protein p105 and the product of the Rb1 gene, extracts from Ad5-infected or uninfected KB cells labeled with ^{35}S-methionine were immunoprecipitated with an E1a-specific monoclonal antibody (M73) or with the rabbit anti-peptide serum RB#5 which recognizes the Rb protein (Whyte et al. 1988a). Figure 17A shows that E1a-specific serum precipitated the 52 and 48.5 kDa forms of the 13S product as well as the 50 and 45 kDa 12S species (lane B). The three cellular E1a-binding proteins, p300, p107 and p105 were also detected in precipitates from Ad5-infected cells (lane B) but not from uninfected cultures (lane A). As previously demonstrated (Egan et al., 1988), with the mutant dl1107, which lacks residues 111 to 123, p300 was present in amounts comparable to those found with wt Ad5, p107 was somewhat reduced, and p105 was totally absent (lane C). These results confirmed the requirement for the sequence between residues 111 and 123 within CR2 for binding of p105. With extracts from mock-, wt Ad5-, and dl1107-infected cells (lanes D, E and F, respectively), the RB#5

Figure 17

Immunoprecipitation of E1A proteins and the Rb1 gene product. A: Extracts from cells infected with wt Ad5 or dl1107, or mock-infected cells, were immunoprecipitated with the E1A-specific mouse monoclonal antibody M73, with RB#5 anti-peptide serum against the product of the Rb1 gene, or with nonimmune rabbit serum. m, mock-infected cells; wt, wt Ad5-infected cells; 1107, dl1107-infected cells. Lanes A to C: M73 serum. Lanes D to F: RB#5 serum. Lanes G to I: nonimmune rabbit serum. Lane J: this extract was precipitated twice with RB#5 serum and then with M73 serum. Lane K: this extract was precipitated twice with nonimmune rabbit serum and then with M73 serum. The inset (lanes B' and E') shows a shorter exposure of fluorographs similar to those presented in lanes B and E. The positions of the 300, 107 and 105 kDa E1A-binding proteins and the E1A products are shown to the right. The positions of the individual 52, 50, 48.5 and 45 kDa E1A proteins are shown at the left. B: Immunoblotting of E1A products and the Rb protein. Extracts from wt Ad5-infected cells precipitated with monoclonal antibody to E1b 58kD protein (lane A), M73 serum (lane B), RB#5 serum (lane D), or nonimmune rabbit serum (lane E), or those from dl1107-infected cells precipitated with M73 serum (lane C) were separated by SDS-PAGE and the proteins were transferred to nitrocellulose paper which was divided in two. The top half was treated with RB#5 serum and the bottom half with E1A-C1 anti-peptide serum, as described in Materials and Methods.

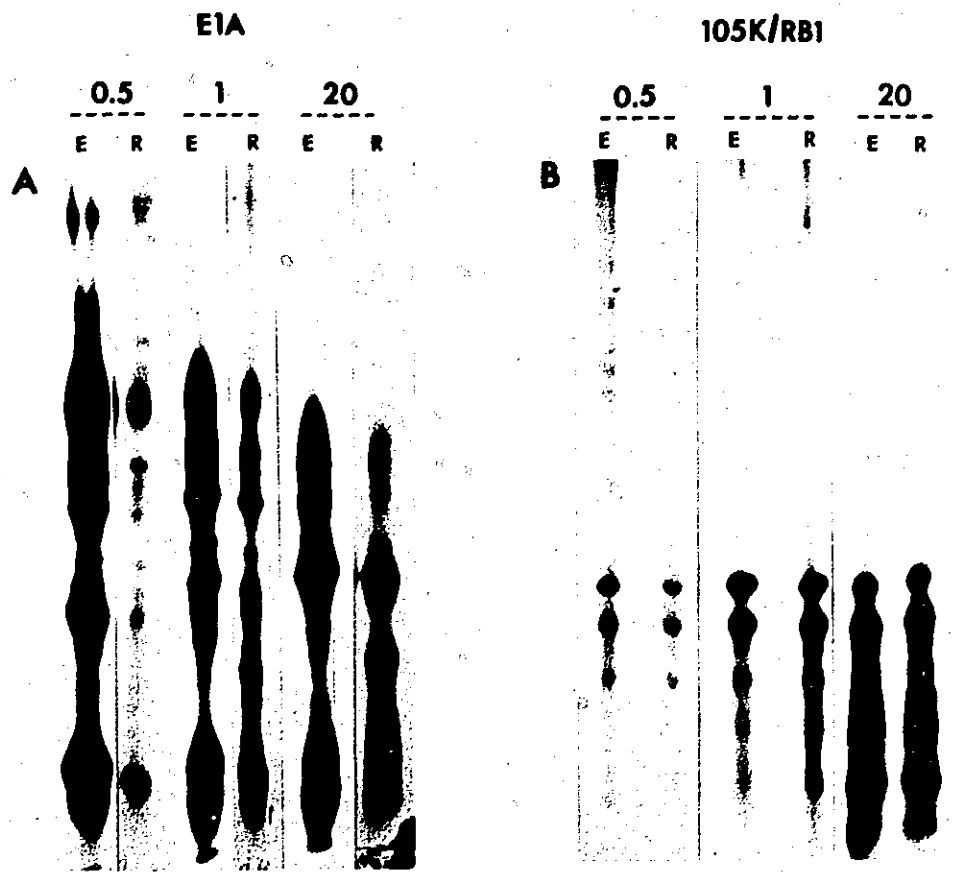

E1A
RB5
NI
RB5 NI
I I
E1A E1A
m wt 1107 m wt 1107 m wt 1107 wt wt
A B C D E F G H I J K
A
B' E'
107
105
Rb
300
107
105/rb
52
50
48.5
45
E1A
B
wt 1107 wt
A B C D E
RB5
E1A
496R E1A E1A RB5 NI

antibody precipitated comparable amounts of a protein that comigrated with p105. It was noted that with appropriate exposures of the gel (see the inset to Figure 17A), both RB#5 serum (lane E) and E1a-specific antibodies (lane B) actually precipitated two p105 polypeptides, a major species and a minor slower-migrating polypeptide. These species were not precipitated using normal rabbit serum (lanes G, H and I). It was apparent that low levels of the 52, 50, 48.5 and 45kDa E1a proteins were present in the RB#5 precipitates from wt Ad5-infected cells (lane E) but not in those from mock- or dl1107-infected cultures (lanes D and F). The identity of these E1a species was confirmed by comparing the S. aureus V-8 protease peptide patterns of the 52 kDa proteins precipitated by E1a-specific antibody and RB#5 serum (Figure 18A). Using 0.5, 1.0 or 20.0 μg of the V-8 protease, E1a proteins precipitated with anti-E1a antiserum (lanes labeled E) yielded digestion patterns that were identical to those obtained from the proteins precipitated by RB#5 serum (lanes labeled R). The similarity of peptide patterns suggested that the Rb protein is associated with E1a products in infected cells.

To further investigate the relationship between the Rb protein and p105, cell extracts that had been

Figure 18

Analysis of E1A products, the p105 E1A-binding protein and the product of the Rb1 gene by partial proteolysis with S. aureus V-8 protease. Extracts from Ad5-infected cells were immunoprecipitated using either M73 or RB#5 serum and the proteins were separated by SDS-PAGE. Areas of the gel containing either the 52 kDa E1A protein or the p105-Rb protein were excised and treated with 0.5, 1 or 20 μg of S. aureus V-8 protease as described by Cleveland et al. (1977). A: E1A 52 kDa protein precipitated by M73 (E) or RB#5 (R) serum. B: p105 precipitated by M73 (E) or RB#5 (R) serum.


E1A
105K/RB1
0.5
1
20
E
R
A
B

precipitated with either RB#5 or normal rabbit antibody (ie. lanes E and H) were reprecipitated with E1a-specific serum. Figure 17A (lane K) shows that after precipitation with normal rabbit serum, E1a-specific antibody still precipitated large amounts of E1a products as well as p300, p107 and p105. However, following treatment with RB#5 (lane J), E1a-specific serum precipitated p300, p107 and E1a products, but no detectable p105. These data strongly suggested that the E1a-binding protein p105 is the Rb1 gene product.

To examine the relationship between these proteins further, extracts from cells infected with either wt Ad5 or dl1107 were immunoprecipitated with E1a-specific antibody, RB#5, or normal rabbit serum, or with a rat monoclonal antibody against the Ad5 E1b 58kD protein. Proteins were separated by SDS-PAGE and then transferred to nitrocellulose paper which was divided in half. Each half was then immunoblotted, the top portion of the gel containing p105 with RB#5 antibody, and the bottom half containing E1a proteins with E1a-C1 serum, and both were treated with ^{125}I-labeled goat anti-rabbit IgG antibody. With E1a-C1 serum (Figure 17B, bottom), the 52 and 50kDa major E1a products were detected with precipitates from wt Ad5- and dl1107-infected cells prepared using E1a-C1 serum (lanes B and C) but not with

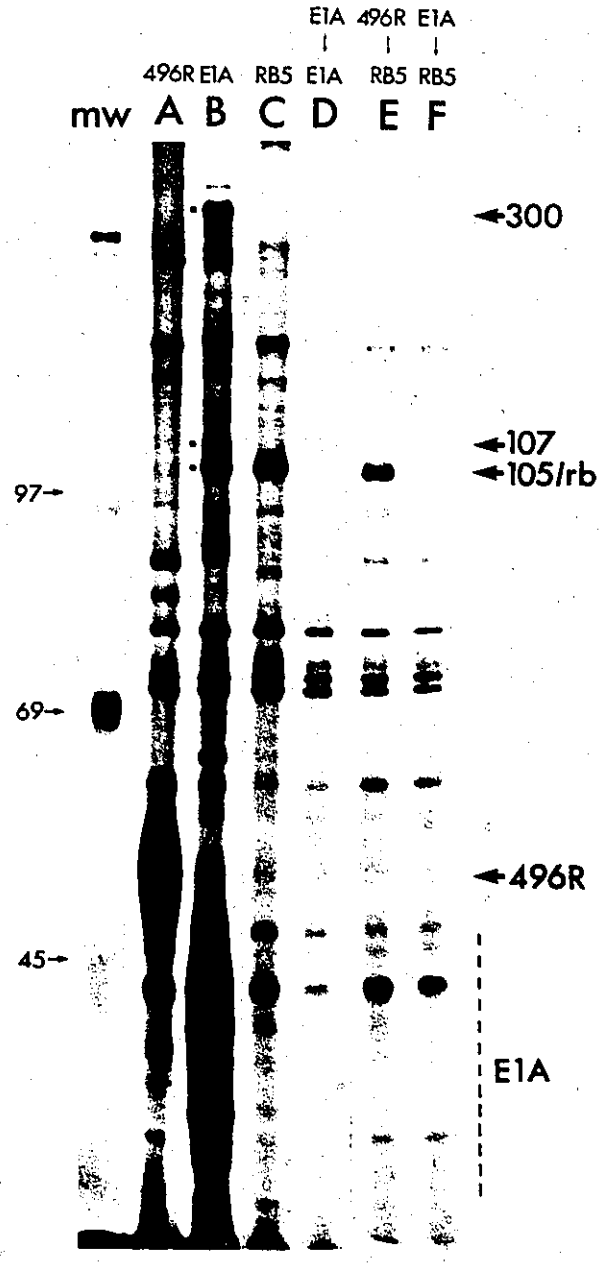
the other samples. The RB#5 serum (Figure 17B, top) detected the Rb1 product precipitated from wt Ad5-infected cells using RB#5 serum (lane D) and also p105 precipitated using E1a-C1 (lane B). No Rb protein was detected in precipitates obtained with the E1b 58kD-specific serum (lane A), normal rabbit serum (lane E) or with extracts from cells infected with dl1107 (lane C). These results again suggested that the E1a-binding protein p105 is the product of the Rb1 gene.
To demonstrate the relationship between p105 and the Rb protein directly, each polypeptide was excised from a polyacrylamide gel and treated with S. aureus V-8 protease. Figure 18B shows that the V-8 peptides generated from the two proteins were identical, thus proving that the p105 is the product of the Rb1 gene.

5.2 Proportion of the Rb1 product bound to E1a proteins in infected and transformed cells

To determine what proportion of p105-Rb in infected cells could be found in association with E1a products, cell extracts were precipitated either with E1a-specific M73 monoclonal antiserum (Figure 19, lane B) or with monoclonal antibody against E1b-58kD which precipitates large amounts of this E1b polypeptide but

Figure 19

Proportion of p105-Rb protein bound to E1A products. Extracts from Ad5-infected cells were precipitated with monoclonal antibody against E1b 58kD protein (496R) (lane A), M73 serum (lane B) or RB#5 serum (lane C). Lanes D and F, extracts precipitated twice with M73 serum and then reprecipitated with either M73 serum (lane D) or RB#5 (lane F). Lane E, extracts precipitated twice with monoclonal antibody to 58kD and then reprecipitated with RB#5. The positions of p300 and p107, p105-Rb, 58kD, and E1A products are shown at the right. The positions of ^{14}C-labeled molecular weight markers (Amersham Corp.) are shown at the left (lane mw).


E1A 496R E1A
496R E1A RB5 E1A RB5 RB5
mw A B C D E F
300
107
105/rb
97
69
496R
45
E1A

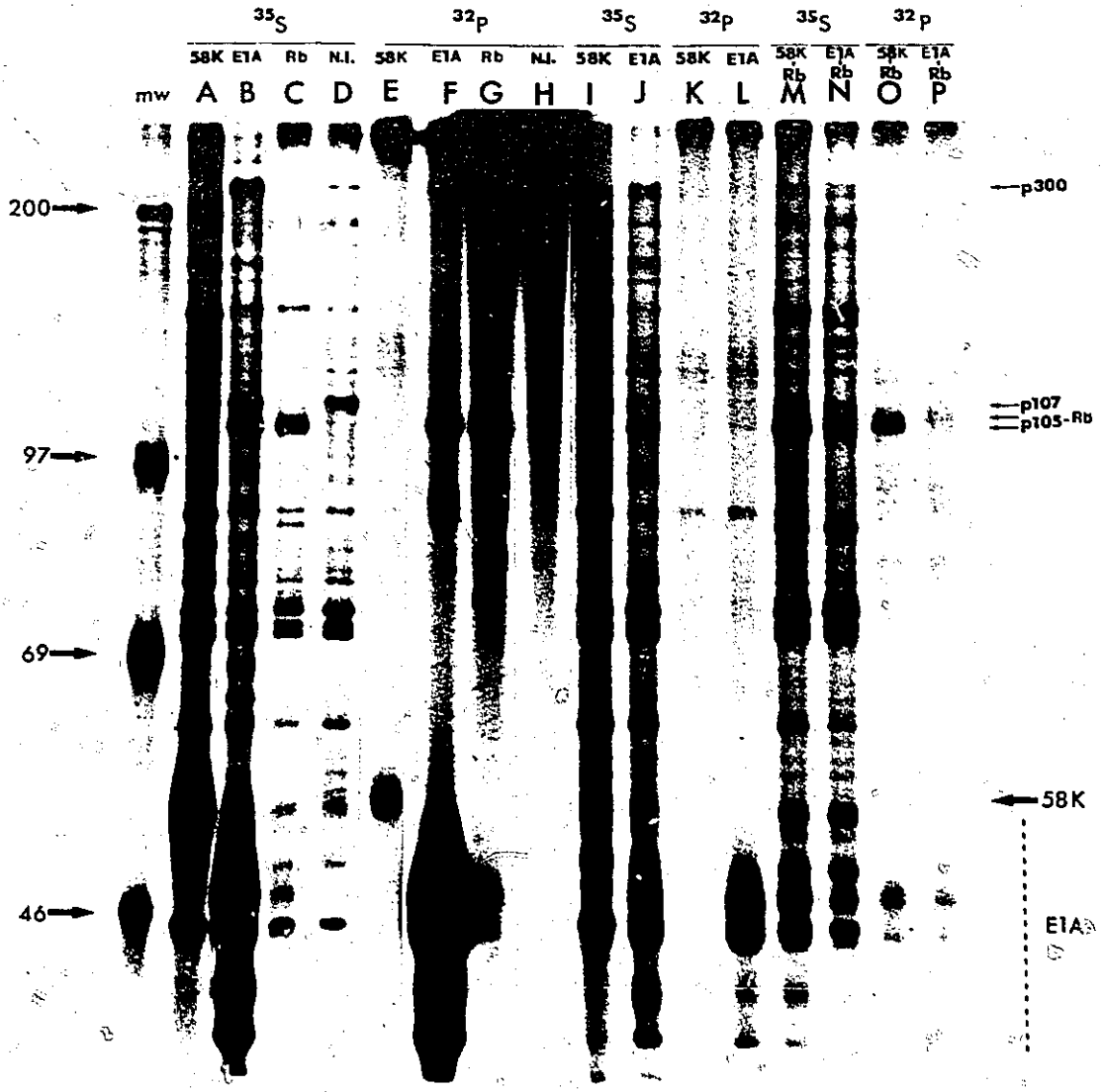
not the E1a-binding proteins (Figure 19, lane A). The amount of E1a-specific antibody used in this experiment was sufficient to remove all of the E1a proteins present in the extract, as shown by their absence in a subsequent reaction with M73 serum (Figure 19, lane D). These extracts were then reprecipitated with RB#5 serum. Figure 19 (lane F) shows that only a small proportion of p105-Rb protein failed to associate with E1a products in infected cells. Much of this material appeared to be the minor slower migrating species recognized by RB#5 antibody. The levels of p105-Rb protein in lanes E and F were estimated by microdensitometer scans of appropriate exposures of this fluorograph and revealed that only about 25% or less failed to associate with E1a products. Thus most of the p105-Rb protein in infected cells is associated with E1a products. A similar proportion of p105-Rb was found in association with E1a products in Ad5 transformed 293 cells (data not shown).

It had been previously shown by Yee and Branton (1985b) that p105 associated with E1a polypeptides is a phosphoprotein. To determine whether there was a difference in the degree of phosphorylation of p105-Rb bound or not bound to E1a proteins, infected KB cells were labeled with ^{35}S-methionine or ^{32}P-orthophosphate and the extracts were immunoprecipitated with monoclonal

antibodies against the E1b 58kD protein or normal rabbit serum as negative controls, or with M73 anti-E1a monoclonal antibody or RB#5 anti-peptide rabbit serum. As in the previous experiment, excess anti-E1a or 58kD antibody was used and the extracts were reprecipitated with the same E1a or 58kD antibody to ensure removal of all E1a or 58kD protein before being reprecipitated with anti-Rb antiserum. The results are shown in Figure 20. With extracts from cells labeled with ^{35}S-methionine (lanes A and B) or ^{32}P-orthophosphate (lanes E and F), p300, p107 and p105-Rb were found in association with E1a proteins (lanes B and F) but not with 58kD proteins (lanes A and E), confirming that all three associate with E1a polypeptides as phosphoproteins. Rb protein which was immunoprecipitated by RB#5 antiserum from lysates labeled with either ^{35}S or ^{32}P (lanes C and G) appeared as a doublet band with the slower migrating species appearing to be more heavily phosphorylated (lane G). In lanes C and G the four major E1a species could be detected in association with the immunoprecipitated p105-Rb proteins, indicating that p105-Rb binds to phosphorylated forms of E1a proteins from both the 12S and 13S messages. In immunoprecipitations of ^{32}P-labeled extracts, using normal rabbit serum, no p105-Rb or associated E1a proteins were detected (lane H). To ensure removal of

Figure 20

Phosphorylation of p105-Rb protein bound to E1A products. Lanes A to D, extracts from ^{35}S-methionine labeled Ad5-infected cells were precipitated with monoclonal antibody against E1b 58kD protein (lane A), M73 serum (lane B), RB#5 serum (lane C) or non-immune serum (lane D). Lanes E to H were immunoprecipitated as for lanes A to D except that the extracts were prepared from ^{32}P-orthophosphate labeled cells. Following immunoprecipitation of 58kD or E1a proteins, the cell extracts from the reactions shown in lanes A, B, E and F were re-immunoprecipitated for 58kD (lanes I and K from extracts A and E respectively) or E1a proteins (lanes J and L from extracts B and F respectively) to ensure that these viral proteins were cleared from the extracts. These same four cell extracts were then precipitated a third time, using RB#5 antiserum and the precipitates run in lanes M to P, with lanes M and O representing precipitates which were precleared of 58kD protein (extracts A and E) and lanes N and P representing extracts that were precleared of E1a proteins (and E1a-Rb complexes) in the reactions shown in lanes B and F. mw, molecular weight markers.


35S
32P
35S
32P
35S
32P
58K
E1A
Rb
N.I.
58K
E1A
Rb
N.I.
58K
E1A
58K
E1A
58K Rb
E1A Rb
58K Rb
E1A Rb
mw
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
200
97
69
46
p300
p107
p105-Rb
58K
E1A

all E1a and 58kD proteins from the immunoprecipitation reactions shown in lanes A, B, E, and F, these lysates were reprecipitated against the same proteins, using an equal amount of antiserum, and the reprecipitations were run in lanes I to L, respectively. These same four lysates were then immunoprecipitated a third time using an excess of RB#5 antiserum, and the immunoprecipitates run in lanes M to P respectively. In lane M p105-Rb protein present in cell extracts which had been pre-cleared of the 58kD is illustrated. As the 58kD protein does not associate with p105-Rb, the amount of p105-Rb appeared to be the same as that in lane C, which was expected, and the protein appeared as the characteristic doublet, with the faster-migrating species being the most evident in an ^{35}S-labeled extract. Lane N depicts the same type of immunoprecipitate, but the extracts had been pre-cleared of all the E1a proteins together with whatever p105-Rb had been bound to them. The p105-Rb protein which was left is significantly reduced in amount, in comparison with lane M, and appeared to consist of equal amounts of the slower and faster migrating species, suggesting that it was primarily the faster migrating form of p105-Rb which binds to E1a proteins, consistent with the results shown in Figure 19. The immunoprecipitates in lanes O and P duplicated those

in lanes M and N, except that they were from ^{32}P-orthophosphate labeled cell extracts. In lane O, as in lane G, the ^{32}P-labeled p105-Rb band appeared to consist of two species, of about the same intensity. As the slower migrating form appears to represent a minor protein species when ^{35}S-methionine labeled cell extracts are examined, this may suggest that the slower migrating form of the protein is the more heavily phosphorylated. Others have demonstrated that increased phosphorylation of Rb protein accompanies a shift in gel mobility, from a faster to a slower migrating form in monkey cells (Ludlow et al., 1989) and in HeLa cells (Buchkovich et al., 1989). When the cell extracts were pre-cleared of E1a proteins (lane P), the faster-migrating form of p105-Rb again appeared to be preferentially removed, suggesting that E1a proteins complex more readily with the less phosphorylated form of p105-Rb.

Chapter 6

Detection of E1a-associated proteins in human tumour cells and potential relatedness of p107 and p105-Rb

Detection of E1a-associated proteins in human tumour cells and potential relatedness of p107 and p105-Rb

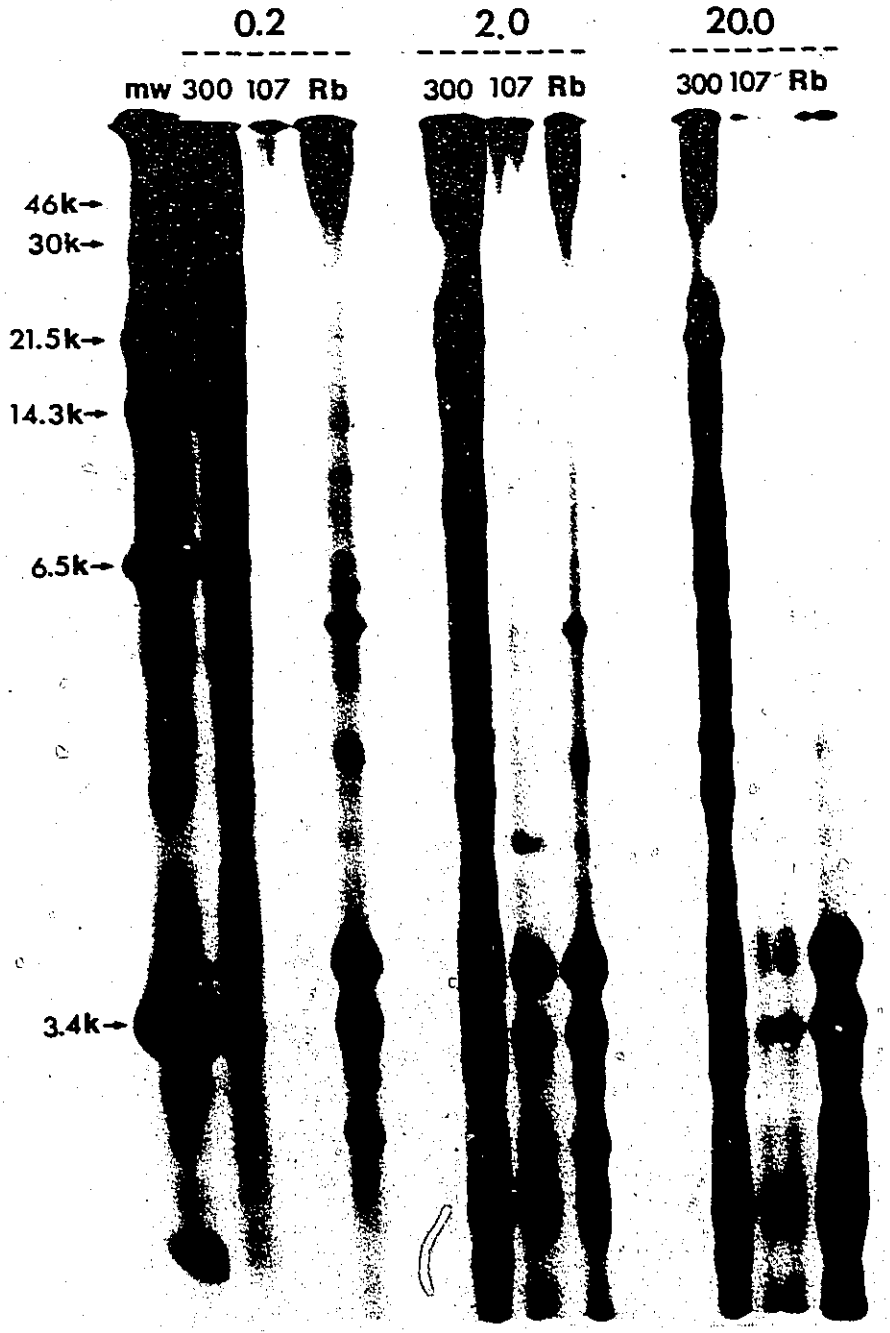
6.1 Cleveland peptide comparison of p107 and p105-Rb

Studies on the binding of the cellular E1a-associated proteins to E1a deletion mutants indicated that p107 and p105-Rb both bind to E1a proteins in conserved region 2 (CR2), and require overlapping, but not identical amino acid sequences for their association (Egan et al., 1988 and see Chapter 4). This similarity in binding specificity, combined with the failure to detect p107 in retinoblastoma cells lacking both alleles of the Rb gene (Egan et al., 1989 and see below), suggested an investigation into the degree of identity between these proteins by comparison of their partial V-8 protease degradation products. KB cells were infected with wt Ad5, labeled with ^{35}S-methionine from 8 to 10 hours post-infection and the lysates were precipitated using the M73 mouse monoclonal antibody specific for E1a products or as a negative control the monoclonal antibody specific for the E1b 58kD protein which does not

associate with the E1a binding proteins. The immunoprecipitates were separated by SDS-PAGE on 6% gels, and the bands corresponding to p107 and p105-Rb were located by autoradiography and excised. Both the gel from which the bands were taken and the excised bands themselves were re-exposed to film to verify that the gel pieces to be digested contained only p107 or p105-Rb respectively. The gel pieces were inserted into the wells of a 15% gel and digested with increasing amounts of Staphylococcus V-8 protease. Proteolysis of p300 served as a means of comparing the p107 and p105-Rb digestion products with those of an unrelated protein. An alternative approach to isolating p107 and p105-Rb proteins for analysis was to immunoprecipitate p107 in association with E1a products from KB cells infected with the Ad5 mutant dl1107, whose E1a products are missing residues 111-123 and are able to bind p107 but not p105-Rb (Egan et al., 1988). For these preparative gels, p105-Rb was obtained in immunoprecipitations from uninfected cells using the mouse monoclonal antibody Rb-02 (Pharmingen), and again, bands excised from the gel and the gel itself were re-exposed a second time to visualize what the gel pieces contained prior to digestion. Results similar to those shown in Figure 21 were obtained using both preparative methods.

Figure 21

Cleveland peptide comparison of p107 and p105-Rb. Gel pieces containing p105-Rb, p107 or p300 were excised from 6% preparative gels, loaded into the wells of a 15% polyacrylamide gel, and overlaid with sample buffer containing 0.2μg, 2.0μg or 20.0μg of S. aureus V-8 protease, as indicated at the top of the figure. Lanes labeled 300 indicate digestion products of p300; lanes labeled 107 are digestion products of p107 and lanes labeled RB are digestion products of p105-Rb. mw, molecular weight marker.


0.2
2.0
20.0
mw 300 107 Rb
300 107 Rb
300 107 Rb
46k
30k
21.5k
14.3k
6.5k
3.4k

The digestion pattern of p300 (lanes labeled 300) is different from those of the other two proteins, but p107 (lanes labeled 107) and p105-Rb (lanes labeled Rb) appeared to be similar but not identical at the level of primary sequence, as they shared several common peptides. In order to examine the possibility that p107 and p105-Rb might be products of alternatively spliced mRNAs of the same gene a number of other retinoblastoma lines with defects in the Rb gene were examined to look for expression of p107.

6.2 Detection of E1a-binding proteins in retinoblastoma cell lines

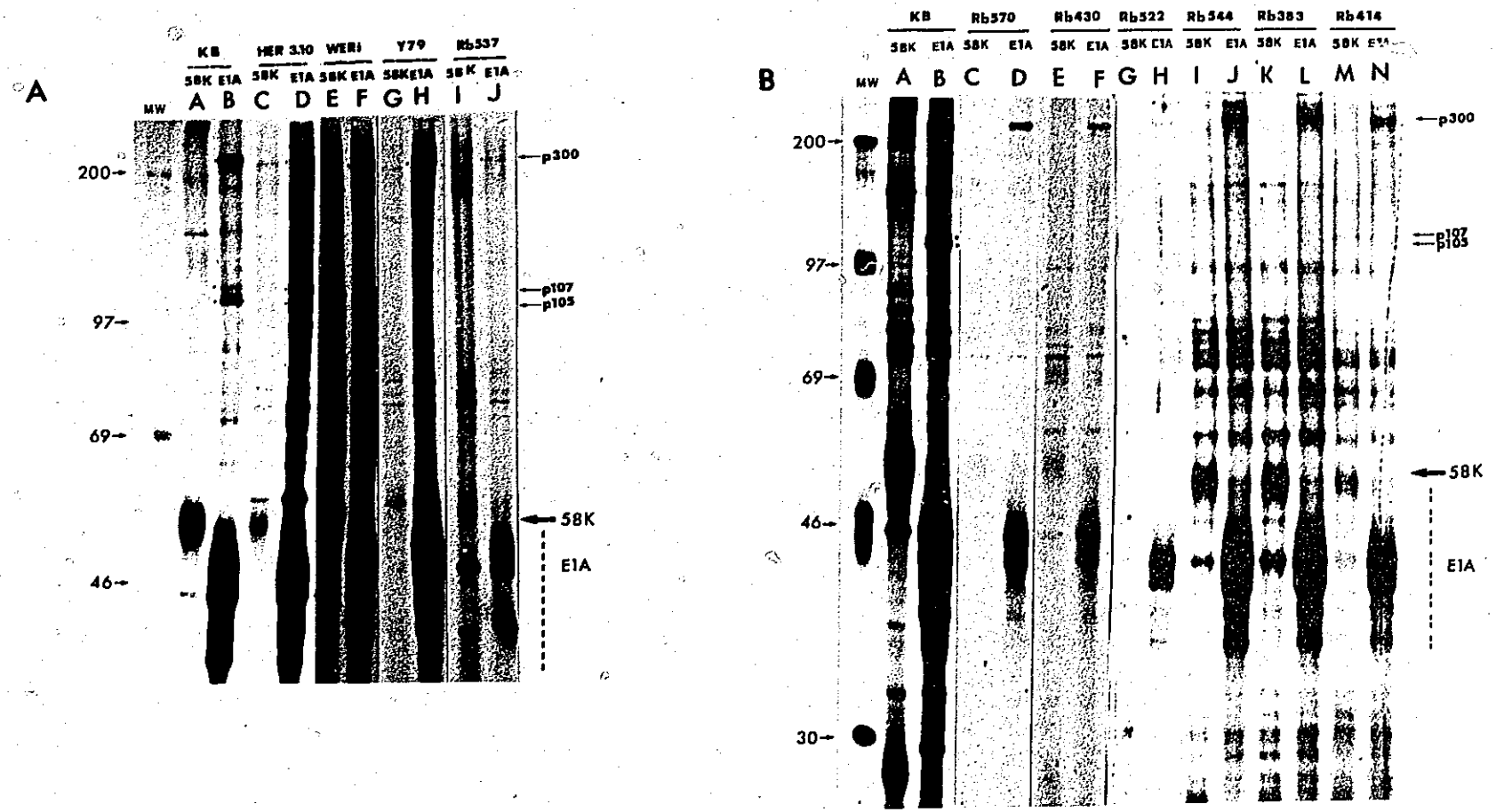
To determine whether p107 could be found complexed to E1a products in retinoblastoma cells, cells from a number of retinoblastoma lines, and one non-retinoblastoma retinal line were infected with Ad5, using a multiplicity of infection of 100pfu/cell for the retinoblastoma lines and 35 pfu/cell for the non-retinoblastoma lines and the E1a-associated protein complexes were immunoprecipitated from cell extracts using the M73 antibody. The immunoprecipitates were analysed by SDS-PAGE on 8% gels. These cells were infected at a higher multiplicity of infection than that

normally used to infect KB cells in order to ensure the production of reasonable amounts of E1a polypeptides. Infected cells were labeled with ^{35}S-methionine from 8 to 10 hours post-infection, as it had been previously determined that a two hour labelling period allowed for optimal detection of all the proteins of interest (data not shown). Immunoprecipitations using the monoclonal antibody directed against the E1b 58kD protein were used as a negative control for each cell line. Cell lysates from infected KB cells were immunoprecipitated as positive controls.

The results are shown in Figure 22. Binding of p107 to E1a proteins was undetectable in any of the 9 retinoblastoma lines tested (Figure 22a, lanes E-J and 22b, lanes C-N), but binding of p300 was seen in all lines (lanes D, F, H, J, L and N). p105-Rb was also absent from the immune complexes in all cases, but this was expected as the Rb gene has been predicted to be absent or mutated in retinoblastoma cells. Although the protein products have not been directly examined, the Rb1 gene has been characterized as deleted or altered in WERI, Y79, Rb537, Rb570, Rb383 and Rb414 lines, (Lee et al., 1985, Goddard et al., 1988 and Zhu et al., 1989). In all retinoblastoma cell lines examined, sufficient amounts of E1a proteins were produced to be able to

Figure 22

Detection of E1a-associated proteins in retinoblastoma cell lines. Extracts from KB cells, Ad12-transformed retinal cells or retinoblastoma cells infected with wt Ad5 and labeled with ^{35}S-methionine from 8 to 10 hours post-infection were immunoprecipitated with an anti-58kD monoclonal antibody or with the M73 mouse monoclonal specific for E1a proteins. **A:** Extracts from KB cells (lanes A, B), HER3.10 retinal cells (Ad12-transformed) (lanes C, D), or one of the retinoblastoma cell lines WERI (lanes E, F), Y79 (lanes G, H) or Rb-537 (lanes I, J) immunoprecipitated for 58kD proteins (lanes A, C, E, G, I) or E1a proteins (lanes B, D, F, H, J). **B:** Extracts from KB cells (lanes A, B), or from the retinoblastoma lines Rb-570 (lanes C, D), Rb-430 (lanes E, F), Rb-522 (lanes G, H), Rb-544 (lanes I, J), Rb-383 (lanes K, L) and Rb-414 (lanes M, N), immunoprecipitated for 58kD proteins (lanes A, C, E, G, I, K, M) or E1a proteins (lanes B, D, F, H, J, L, N).


A
MW
KB
58K E1A
A B
HER 3.10
58K E1A
C D
WERI
58K E1A
E F
Y79
58K E1A
G H
Rb537
58K E1A
I J
200
97
69
46
p300
p107
p105
58K
E1A
B
MW
KB
58K E1A
A B
Rb370
58K E1A
C D
Rb430
58K E1A
E F
Rb522
58K E1A
G H
Rb544
58K E1A
I J
Rb383
58K E1A
K L
Rb414
58K E1A
M N
200
97
69
46
30
p300
p107
p105
58K
E1A

clearly visualize the cellular p300 protein associated with them. p107 in infected or transformed cells and in the immortalized HER3.10 retinal cells (Figure 22a, lane B and D and Figure 22b, lane B) is seen as a band of about the same magnitude of intensity as p300. The absence of this band in the lanes containing immunoprecipitates from retinoblastoma lines suggested that the protein was either absent in these cells, or expressed at extremely reduced levels, or that it was expressed in a form which was unable to complex with E1a proteins.

To attempt to distinguish between these three possibilities, WERI or Y79 cells were labeled for longer periods of time using a five-fold greater amount of ^{35}S-methionine than was usually used, lysed and precipitated as described above. The data indicated that very small amounts of p107 could, in fact, be detected (Figure 23 lane D'). It was noted that the intensity of the band representing p107 in WERI cells was extremely reduced relative to the intensity of the band in the KB cell immunoprecipitation reactions, but the intensities of the bands representing p300 in both cell lines was essentially the same (Figure 23, lanes B and D). Cleveland peptide analysis of the p107 band from KB cells and the comigrating species seen in WERI cells was used

Figure 23

Detection of low amounts of p107 in WERI cells. KB cells or WERI retinoblastoma cells were infected with wt Ad5 and labeled from 7 to 10 hours post-infection with ^{35}S-methionine (1.0 mCi per 100mm dish). Extracts from these cells were immunoprecipitated with a mouse monoclonal antibody specific for E1b 58kD proteins (lanes A, C) or with the M73 mouse monoclonal specific for E1a proteins (lanes B, D). Lanes A, B, immunoprecipitates from KB cells; Lanes C, D, immunoprecipitates from WERI cells; lanes C' and D' are from a longer film exposure of the material in lanes C and D.

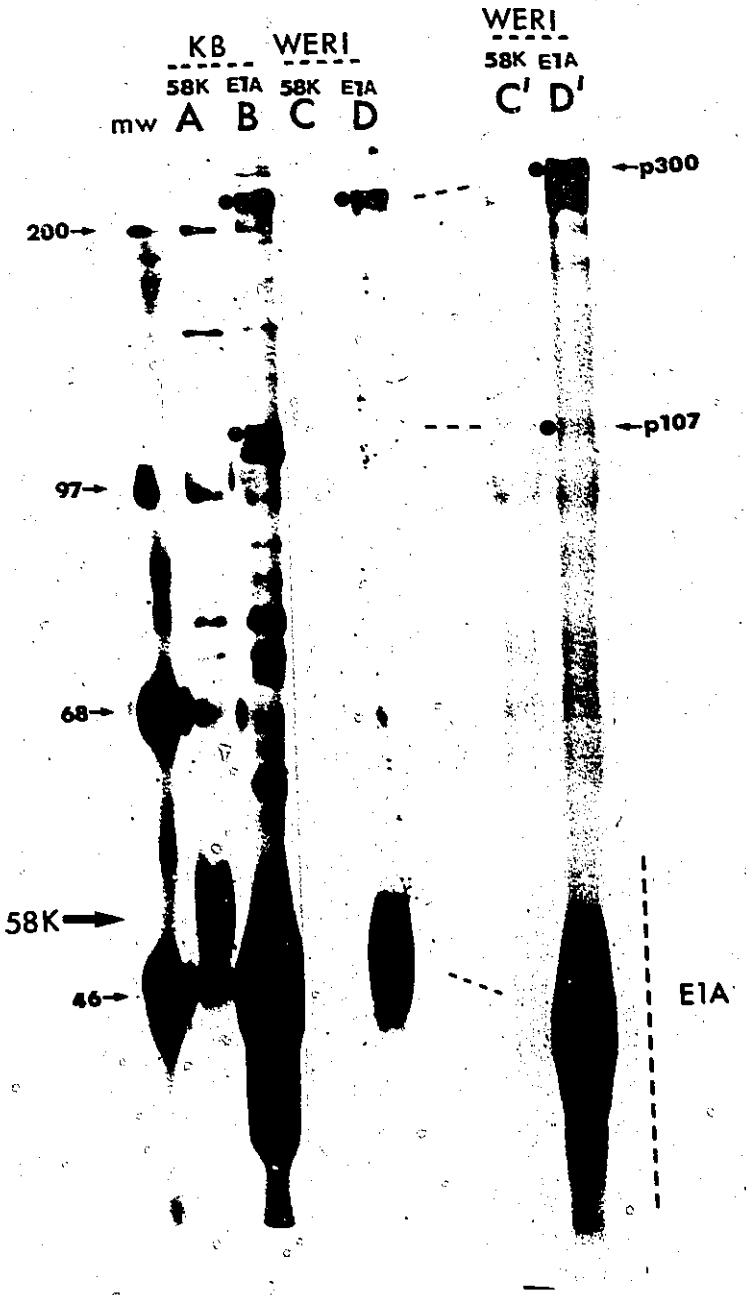
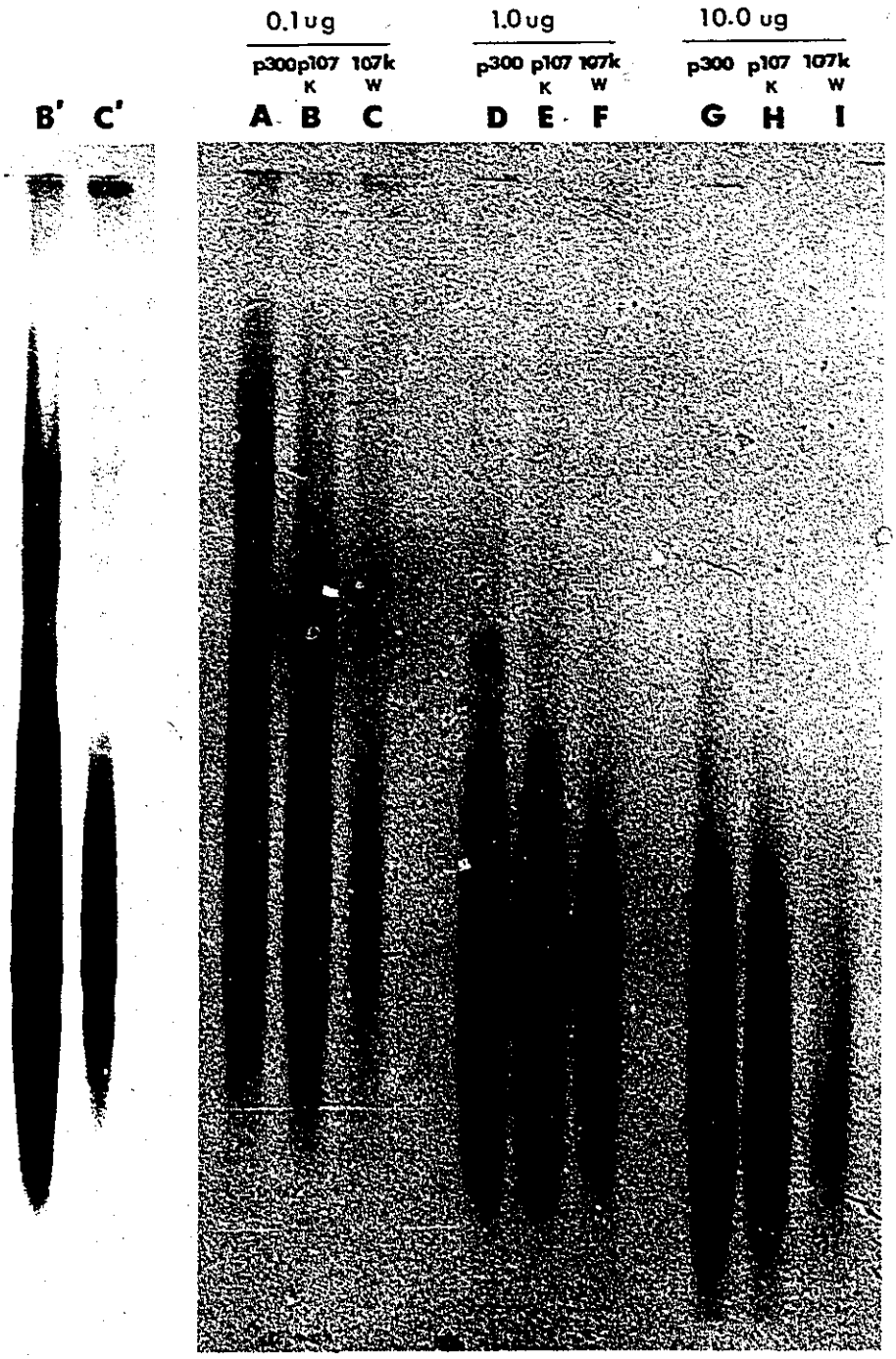

KB
WERI
WERI
58K E1A 58K E1A
58K E1A
mw A B C D
C' D'
200
97
68
58K
46
p300
p107
E1A

Figure 24

V-8 peptide comparison of p107 from KB cells and the comigrating 107kD protein from WERI cells. Gel pieces containing p300 or p107 from KB cells or the 107kD protein from WERI retinoblastoma cells were excised from preparative gels, loaded into the wells of a 15% polyacrylamide gel and overlaid with sample buffer containing 0.1μg (lanes A, B and C), 1.0μg (lanes D, E, anf F) or 10.0μg (lanes G, H, and I) of S. aureus V-8 protease. Lanes A, D and G, digestion products of p300; lanes B, E and H, digestion products of p107 and lanes C, F and I, digestion products of the 107kD protein from WERI cells. Lanes B' and C' are from a longer film exposure of the material in lanes B and C. mw, molecular weight marker.


0.1 ug
1.0 ug
10.0 ug
p300
p107
K
107k
W
A
B
C
D
E
F
G
H
I
B'
C'

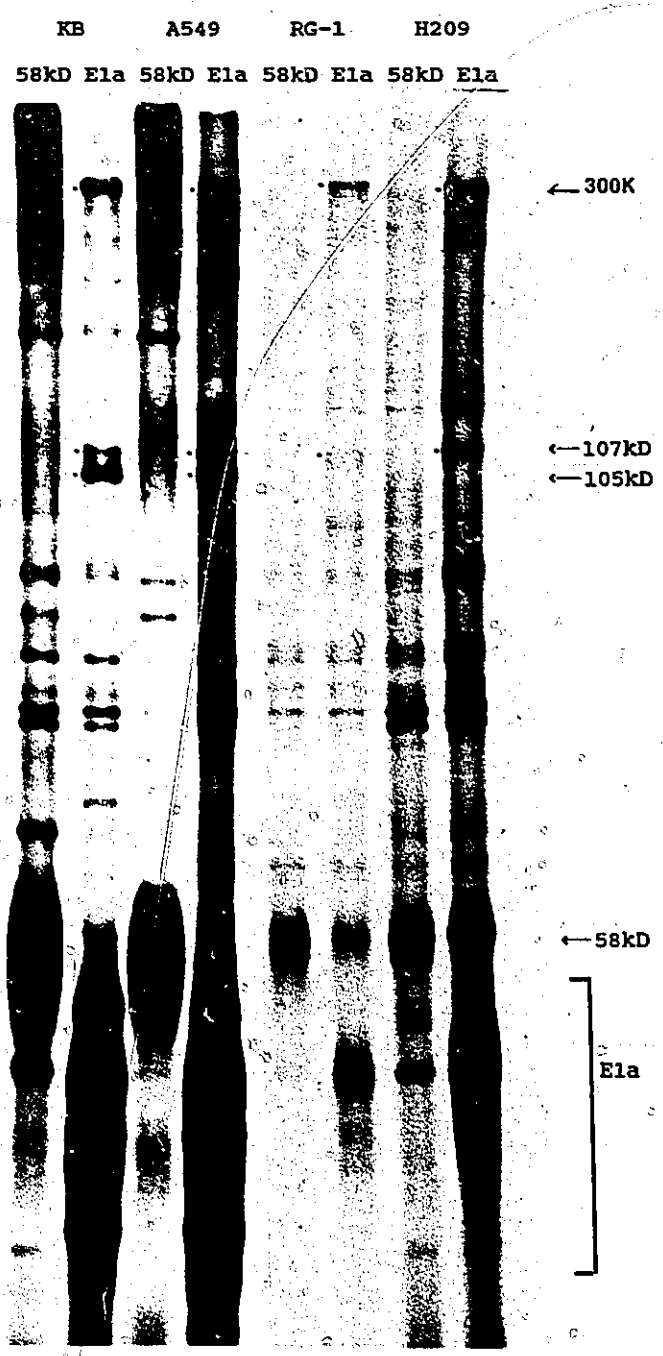
to show that these were identical proteins (Figure 24).Whether the reduced amounts of p107 seen were due to only a low amount of p107 being present in retinoblastoma cells, or whether a loss of Rb gene function results in severely reduced ability of p107 to bind to E1a was not determined. The detection of p107in even low amounts in WERI cells, which lack both Rb alleles, clearly indicated that p107 could not be a product of the Rb gene.

6.3 Detection of E1a-associated proteins in lung cell carcinoma lines

To further characterize the expression and/or ability of p107 to bind to E1a products in cells with defects in the Rb gene, cells from a number of lines of small-cell or non-small cell carcinoma of the lung were investigated. Small-cell carcinoma of the lung has been characterized as one of the cancers with which defects of the Rb gene have been associated. Lysates of these cells were prepared and immunoprecipitated as described for retinoblastoma cell lines. The immunoprecipitates were separated on 8% SDS PAGE gels, and results were as illustrated in Figure 25. Infected KB cell extracts immunoprecipitated for 58K (lane A) or E1a proteins (lane B) were again used to demonstrate that p300, p107 and

Figure 25

Detection of E1a-associated proteins in Small Cell Lung Carcinoma (SCLC) lines having defects in Rb. Extracts from ^{35}S-methionine-labeled KB cells infected with wt Ad5, or infected lung carcinoma cells were immunoprecipitated with anti-58kD mouse monoclonal (lanes labeled 58kD) or M73 anti-E1a mouse monoclonal (lanes labeled E1a). Numbering from the left side of the gel, lanes 1, 2, KB cell extracts; lanes 3, 4, extracts from A549 non-small-cell carcinoma line; lanes 5, 6, extracts from RG-1 SCLC line; lanes 7, 8, extracts from H209 SCLC line.


KB
A549
RG-1
H209
58kD E1a 58kD E1a 58kD E1a 58kD E1a
←300K
←107kD
←105kD
←58kD
E1a

p105 were all detectable in this experiment (lane B). Infected A549 cells, from a (non-small cell) adenocarcinoma of the lung, have been characterized as having normal Rb mRNA and protein expression (Yakota et al., 1988). p300, p107, and p105-Rb proteins from these cells were all detected in association with E1a products (Figure 25, lane 4). RG-1 and H-209 (lanes 5, 6 and 7, 8) are both lines developed from small-cell carcinoma of the lung (SCLC). P105-Rb was not detected in association with E1a proteins in either of these lines, consistent with evidence that the Rb protein and/or message is not expressed in a majority of SCLC lines (Harbour et al., 1988 and Yakota et al., 1988). Interestingly, and unlike the situation in retinoblastoma cells, p107 was detected at normal levels bound to E1a gene products, as was p300 (Figure 25, lanes 6 and 8). The reduced intensity of the p300 and p107 bands in the immunoprecipitations from RG-1 cells was likely due to the small amount of E1a present relative to the other cell lines. Even with reduced amounts of E1a proteins it was noted that the intensities of the p300 and p107 bands were of essentially comparable magnitudes, consistent with the relative intensities seen in the other lines. Two other non-small-cell carcinoma lines (A427 and Calu-1) and one other small-cell carcinoma line (H-69) yielded similar

results (Table 4 and data not shown). A427 and Calu-1 both produced p300, p107, and p105-Rb proteins which could be immunoprecipitated in association with E1a gene products. H-69, which produces only trace amounts of Rb mRNA (Harbour et al., 1988), exhibited no detectable binding of p105-Rb to E1a, but normal amounts of p300 and p107 were seen. These results are summarized in Table 4.

Table 4

Expression of E1a-associated proteins in human tumour lines.

	E1a	p300	p107	p105-Rb
Retinoblastoma				
WERI	+	+	reduced/-	-
Y79	+	+	-	-
Rb537	+	+	-	-
Rb522	+	+	-	-
Rb544	+	+	-	-
Rb383	+	+	-	-
Rb414	+	+	-	-
Rb430	+	+	-	-
Retinal Cells of non-retinoblastoma origin				
Her3.10 (Ad12 immortalized)	+	+	+	+
Small Cell Lung Carcinoma				
RG-1	+	+	+	-
H-209	+	+	+	-
H-69	+	+	+	-
Non-Small Cell Lung Carcinoma				
A549	+	+	+	+
A427	+	+	+	+
Calu-1	+	+	+	+

Chapter 7

Discussion

Discussion

The results presented in this thesis describe the further characterization of cellular proteins which are found in association with Ad5 E1a polypeptides in infected and transformed cells, and which were originally described by Yee and Branton (1985b). In the present studies the cellular proteins, termed p300, p107, p105-Rb, p68 and p65, were initially characterized with respect to their ability to bind to E1a gene products synthesized in E. coli, as such products could be of use in affinity purification (Egan et al., 1987). Using soluble bacterially-produced 12S or 13S products mixed with labeled cell extracts, it was found that two of the cellular proteins, p68 and p65 bound very efficiently, but p300, p107 and p105 bound very poorly or not at all. p68 and p65 bound somewhat more efficiently to the 13S products than to the 12S products, consistent with a previous observation of Yee and Branton (1985b) using E1a protein complexes from cells infected with mutant Ad5 virions.

The failure of the bacterially-produced E1a products to associate with p300, p107 and p105 could have resulted from differences in the degree or type of post-

translational modifications generated in prokaryotic and eukaryotic cells. In mammalian cells E1a proteins are phosphorylated at a number of sites (Tsukamoto et al., 1986, Tremblay et al., 1988 and Dumont et al., 1989), and this is the only known modification of these proteins (Harter and Lewis, 1978, Yee et al., 1983 and Yee and Branton, 1985a). Phosphorylation at one of these sites, serine 89, causes a characteristic change in the migration pattern of E1a proteins on SDS-polyacrylamide gels (Dumont et al., 1989), and this change in migration is not seen with bacterially-produced E1a products (Richter et al., 1985), indicating that phosphorylation at this site does not occur. As serine 89 and a second phosphorylation site at serine 96 (Dumont et al., 1989) lie near to the region involved in binding to p107 and p105 (see below and Egan et al., 1988, and elsewhere, Moran, 1988 and Whyte et al., 1989), E1a mutants containing Ala-89 or Ala-96 were examined for their ability to associate with p107 and p105 in infected cells. It was found that E1a proteins produced by both mutants were able to associate with p300, p107 and p105 at levels essentially the same as wt E1a products. Other studies using a mutant altered at both Ser-89 and Ser-96 yielded similar results (Dumont and Branton, unpublished results) These data suggested that the inability of

bacterially-produced E1a products to bind to these three cellular proteins was not due to their lack of phosphorylation at either of these sites, but did not rule out the possibility that other phosphorylation sites or other differences in post-translational modification could play a role in affecting the association.

The bacterially-produced E1a polypeptides used in the present study also differed from E1a proteins produced by wt virus in that they lack arginine residue 2 (Ferguson et al., 1984). As E1a deletion mutants with alterations at the amino terminus generally fail to bind to p300 (Egan et al., 1988 and Whyte et al., 1989), and E1a point mutants altered at Arg-2 also fail to bind p300 (Whyte et al., 1989) this could also represent a possible reason for the failure of the bacterially-produced E1a products to associate efficiently with p300. For p107 and p105, it was observed that their ability to associate in vitro with virally-produced E1a proteins in mixing experiments was reduced when compared to in vivo experiments and so the inefficient binding to the bacterially-produced E1a proteins may have resulted in part from the in vitro conditions of the experiment. A further possibility was that one or more of the cellular species required the presence of some viral product other than E1a proteins in order to bind to them efficiently.

To test this possibility, bacterial E1a products were mixed with cell extracts from uninfected cells or cells infected with dl312 virus (deleted in E1a but producing all other viral products). No difference in the association between E1a proteins and the cellular polypeptides could be detected in the presence of other viral products (data not shown).

To further test the possibility of utilizing the bacterially-produced E1a products as an affinity substrate for purification of the cellular proteins, the bacterially-produced 13S products were covalently attached to cyanogen bromide-activated Sepharose, and this E1a-Sepharose was examined for its affinity for the cellular proteins. p68 and p65 again bound efficiently to this substrate, suggesting that either soluble bacterially-produced E1a proteins or E1a-Sepharose could be used to further purify these two proteins either for antibody production or for microsequencing. Interestingly, p300, which did not associate efficiently with the free bacterial E1a proteins, could bind quite well to E1a-Sepharose. One possible explanation for the difference in binding of this protein to uncoupled versus coupled bacterially-produced E1a polypeptides could be that the process of attachment to cyanogen bromide-activated Sepharose, which involves coupling through free

amino groups, favours the formation or presentation of an appropriate binding site at or near the amino terminus of the E1a proteins.

A number of other proteins were detected in association with E1a-Sepharose, but not with BSA-Sepharose, suggesting that they too may be E1a-binding proteins. These included proteins with apparent mobilities which indicated molecular masses of 30, 33, 75, 95, 106, 150, 180 and >300 kD. Polypeptides of about 33 and 30kD were observed in a previous study (Yee and Branton, 1985b), but they were seen inconsistently. In this present study, upon prolonged exposure of the fluorographs, low amounts of these proteins, as well as of the 75kD species were also found with BSA-Sepharose, and so the status of their specificity is unclear. The 30kD species comigrated with histone H3, which may be of interest as Grand and Gallimore (1984) have found that E1a proteins of Ad12 can associate with histone. The biological importance of this property remains to be established.

The 106kD protein was consistently found to migrate at a position midway between that of p107 and p105. The 106kD protein was found to be a phosphoprotein. This species was not characterized

further with regard to its relationship with p107 and p105.

The >300kD species was detected in association with soluble bacterially-produced E1a products as well as E1a-Sepharose. This same protein, or one migrating in a similar position, was consistently found in association with E1a proteins precipitated from infected cell extracts using E1a-C1 or M73 antibodies which recognize different epitopes on the E1a polypeptides. Thus this protein may represent an E1a-binding protein which has not yet been reported or characterized.

The first indication that any of these cellular proteins were functionally involved in E1a-mediated transformation came from studies done in collaboration with Dr. Stan Bayley's lab, in which the regions on the E1a polypeptides with which the cellular proteins interacted were mapped and the ability of E1a deletion mutants to bind the cellular proteins was correlated with biological activity (Egan et al., 1988). E1a proteins contain three regions which are highly conserved among different adenovirus serotypes (Kimelman et al., 1985). Two of these regions are involved in E1a-mediated transformation and are present in both the 12S and 13S products. These two regions are found in exon 1 between residues 40 and 80 (conserved region 1; CR1) and residues

121 and 140 (CR2). CR3 consists mostly of the 13S unique region and maps on the 13S product between residues 140 and 188 (Moran and Mathews, 1987). It has been suggested that these conserved regions represent functional domains, with CR3 being both necessary and, in some cases sufficient (Green et al., 1988) for transactivation of other viral early promoters (Moran et al., 1986a, 1986b, Lillie et al., 1987, Moran and Mathews, 1987 and Jelsma et al., 1988). CR1 and CR2 are involved in a number of transformation-related functions, such as the induction of DNA synthesis and mitosis in quiescent cells, induction of synthesis of proliferating cell nuclear antigen, and induction of epithelial cell growth factor (Moran et al., 1986b, Zerler et al., 1987, Moran and Mathews, 1987, Subramanian et al., 1988, Smith and Ziff, 1988 and Howe et al., 1990). It was thought that some information on possible biological functions of the E1a-associated proteins could be obtained from a knowledge of which E1a regions they interacted with.

Using a series of small in frame deletion mutants which spanned almost the entire E1a coding sequence, the regions with which the cellular proteins interacted were determined. It was felt that deletions which eliminated or reduced binding of one or more of the cellular proteins must exist in regions involved in the

association with cellular polypeptides either by exerting conformational effects on a binding site, or by binding directly to the cellular protein. For p300, two regions of E1a products were found to be involved in binding and these were located at the amino terminus, within the region between residues 1 and 25, and in CR1 between residues 36 and 49. Deletions between residues 50 and 82 resulted in reduced binding. It was not clear if these two regions combined to form a single binding site or if one of these regions is necessary only for the formation of an appropriate protein structure at the other site. The result showing that the epitope for the R28 rat monoclonal anti-E1a antibody required the entire region between residues 26 and 60 may indicate that there is some degree of tertiary structure within this region. The R28 antibody recognized E1a proteins bound to p300, but the anti-peptide antiserum E1a-N1, directed against the amino terminus of E1a proteins failed to immunoprecipitate E1a proteins in association with p300. These results may suggest that the binding site for p300 is at the amino terminus of E1a polypeptides, and that this binding hinders antibody recognition of the amino terminal epitope, but not the R28 epitope.

For p107 and p105, only a single putative binding site was apparent, between residues 124 and 127 and

residues 111 to 127, respectively. It seemed likely that other regions of the E1a protein within CR1 which, when deleted resulted in only partial reduction in binding, may play a role in the tertiary structure of the primary binding site, or may stabilize binding at the primary site through weaker protein:protein interactions. Results which were essentially in agreement with these, and which further showed that E1a residues 86 to 120 were not required for p105 binding were later reported by Moran (1988) and Whyte et al. (1989) in an independent study on E1a-binding proteins which this group was investigating (Harlow et al., 1986).

The fact that the E1a-binding proteins appeared to interact with exon 1 of E1a proteins was of great interest, as this region had been linked in a number of studies to immortalization and oncogenic transformation (Moran et al., 1986b, Lillie et al., 1986, 1987, Moran and Mathews, 1987, Subramanian et al., 1988 and Smith and Ziff, 1988). For transformation in association with an activated ras gene, both CR1 and CR2 are required. The deletion mutants described in the present study were used in plasmid form to map the regions in exon 1 necessary for transformation in cooperation with ras. The results showed that, in addition to CR1 and CR2 a region near the amino terminus was also essential (Jelsma et al., 1989). Others had previously demonstrated the importance of the amino terminus, but had not shown that this region was

functionally separable from CR1 (Whyte et al., 1988b), although other amino-terminal transformation-negative mutants have been reported since (Subramanian et al., 1988 and Smith and Ziff, 1988). There was an excellent correlation between the failure to bind one or more of the E1a-associated proteins and loss of transforming activity (Table 1). The only exceptions were dl1109 and dl1504. The former transformed poorly, even though the mutant E1a product appeared to bind the E1a-associated species at levels near to that of wild type Ad5. The latter, which failed to bind p300 has been shown by others, using whole virus, to be capable of transformation (Osborne et al., 1982), although it was not tested under the present assay conditions. These results suggested that at least one of the reasons why CR1, CR2 and the amino terminus are necessary for transformation could be the requirement to form functional complexes with all three E1a-associated proteins. The results further suggested that the major role of CR1 was to provide some degree of secondary structure in exon 1 which was required for binding of p300 at the amino terminus and which stabilized the binding of p107 and p105 to residues in CR2.

Little was known of the identity or biological function of these cellular polypeptides prior to the

accounts of Whyte et al. (1988a) and DeCaprio et al. (1988) who reported that the product of the Rb1 retinoblastoma tumour suppressor gene could be detected in association with E1a proteins and with large T antigen of SV40, respectively. This gene is absent or altered in retinoblastomas (Friend et al., 1986, 1987, Fung et al., 1987 and Lee et al., 1987a) as well as in some other tumour types including osteosarcomas (Toguchida et al., 1988, 1989), small-cell lung carcinomas (Harbour et al., 1988 and Yakoda et al., 1988), breast carcinomas (T'ang et al., 1988, Lee et al., 1988b and Varley et al., 1989), and other soft tissue sarcomas (Friend et al., 1987 and Reissmann et al., 1989). Because it is the loss of function of the gene that is associated with malignancy, Rb1 has been termed a tumour suppressor gene, or anti-oncogene, the putative function of which is to block cell proliferation. The Rb1 gene product was originally described by Lee et al. (1987b) as a nuclear phosphoprotein which, when immunoprecipitated from human cells and analysed on SDS-polyacrylamide gels, migrated as a doublet band of molecular mass 110kD. The protein was reported to have some DNA binding activity, and analysis of the protein sequence showed that it had putative metal-binding domains and proline rich sequences similar to those of some transcription factors (Lee et

al., 1987a). The protein was found to be expressed in a number of cell types, and the gene appeared to be highly conserved (Lee et al., 1987a, 1987b).

The results presented in this study were compatible with those of Whyte et al. (1988a) and demonstrated that the E1a-binding protein p105 is the product of the retinoblastoma tumour suppressor gene. The identity of these two proteins was demonstrated in a number of ways. Rb protein immunoprecipitated using anti-Rb anti-peptide serum comigrated on gels with p105 precipitated in association with E1a proteins, and both p105 and Rb from ^{35}S-methionine labeled cell extracts ran as doublets, with the slower migrating species appearing as the minor band. Preclearing infected cell extracts with anti-Rb serum prior to immunoprecipitating the E1a proteins resulted in loss of p105-E1a complexes in the immunoprecipitates, although E1a proteins complexed to p300 and p107 could still be detected. E1a proteins from both 12S and 13S messages could be found in association with Rb proteins in anti-Rb immunoprecipitation reactions, as shown by Cleveland peptide digestion of the E1a products. Western blot analysis demonstrated that anti-Rb antiserum could directly recognize p105. Finally, Cleveland peptide analysis of p105 and Rb indicated that the peptide products from the two proteins

were identical, clearly showing that p105 was the product of the retinoblastoma tumour suppressor gene, hereafter termed p105-Rb. By preclearing cell extracts of all E1a proteins (and thus of all p105-Rb protein complexed to E1a polypeptides) and then reprecipitating the same extracts for p105-Rb, it was shown that only a very small amount of p105-Rb existed in uncomplexed form (Egan et al., 1989). This result was consistent with the model presented by Whyte et al. (1988a) which suggested that transformation by E1a proteins results from binding to, and inactivating p105-Rb, thus mimicking the inactivation or loss of this gene product in naturally occurring tumours. The model predicts that E1a proteins must bind to most of the p105-Rb present in the cell, as the results discussed above confirmed, and the model also predicts that binding to p105-Rb is required for transformation. This requirement had already been demonstrated (see above), but it had also been shown that binding to p105-Rb was not sufficient for E1a-mediated transformation. Binding to at least one other cellular protein, p300, was also necessary for transformation. E1a proteins which failed to bind to either p105-Rb or p300 were transformation defective (Egan et al., 1988 and Whyte et al., 1989). The E7 proteins of a number of human papilloma viruses also bind to p105-Rb, suggesting

that the inactivation of p105-Rb through interaction with a tumour antigen may represent a common mechanism of transformation for different DNA tumour viruses (Münger et al., 1989 and Dyson et al., 1989a).

In immunoprecipitation reactions from cells labeled with ^{32}P-orthophosphate, it was demonstrated that both the slower and faster migrating species of p105-Rb which co-precipitate with E1a products were phosphorylated, and these species appeared to be of the same intensity. In immunoprecipitations from ^{35}S-labeled cells however, the slower migrating species is seen as a much less intense band, suggesting that it is a minor protein species. These results together may indicate that the slower migrating, minor species is more heavily phosphorylated. It has been shown by Ludlow et al. (1989) that SV40 large T antigen binds only to under- or non-phosphorylated forms of p105-Rb, suggesting there may be some difference in the specificity of binding of large T compared to E1a proteins. Recently it has been shown that p105-Rb has characteristics of a cell cycle regulatory element and is phosphorylated in a cell cycle-dependent manner (Chen et al., 1989, DeCaprio et al., 1989 and Buchkovich et al., 1989). The protein exists in a form which is not detectably phosphorylated during G_1, and first becomes phosphorylated at G_1/S. It then

undergoes further phosphorylation events in G_2 and early M, following which it is dephosphorylated, so that when the cells re-enter G_1, all the p105-Rb present is unphosphorylated. It has been suggested that the non-phosphorylated form of the protein is the active form, and that the phosphorylation event at G_1/S inactivates the protein, thus allowing DNA synthesis to proceed (Chen et al., 1989, DeCaprio et al., 1989 and Buchkovich et al., 1989). T antigen associates with p105-Rb in a cell cycle-dependent manner, complexing with only with the non-phosphorylated form present during G_1 and so presumably only inactivating the active form (Ludlow et al., 1990). The results discussed above suggest that E1a proteins may show less discrimination in their binding to p105-Rb, and their binding may not show any cell cycle dependency, but this has yet to be shown directly. The ability to inactivate a suppressor of DNA synthesis is thought to be of benefit to viruses such as adenoviruses which normally infect quiescent epithelial cells, as the induction of DNA synthesis in these cells would likely promote viral DNA replication as well.

Further evidence for the ability of p105-Rb to act as a suppressor of DNA synthesis was obtained in a study in which E1a mutants coding for 12S products with single or double deletions in exon 1 were examined to

determine whether they could induce DNA synthesis in quiescent rat cells (Howe et al., 1990). These mutants were also used to infect KB cells, and the ability of the E1a products to bind to p300, p107 and p105-Rb was examined. The results (Table 2) again showed a correlation between binding of the cellular proteins and the induction of DNA synthesis. E1a mutants which bound either p300 or p105-Rb or both, at levels similar to the wt control (dl520) could induce DNA synthesis at levels comparable to wt. Mutants which failed to bind to both p300 and p105-Rb (or which bound extremely low amounts of these proteins), did not induce DNA synthesis at levels greater than the E1a-minus virus, dl312 which was used as a negative control. Mutants which bound reduced levels of either p300 or p105-Rb or both were capable of induction of DNA synthesis to levels greater than that of dl312 but not as great as that induced by dl520. There was no correlation between the binding of p107 and induction of DNA synthesis. One of the mutants, dl1104/08/520 was capable of inducing DNA synthesis at a level greater than would have been expected, given that the E1a products from this mutant failed to bind p105-Rb and bound extremely reduced amounts of p300. It is possible that the products of this mutant interact in some novel way with an as yet unidentified cellular

factor which is involved in the induction of DNA synthesis. Another possibility is that the reduced binding of the E1a proteins to p300 which was seen in KB cells was not as reduced in the rat cells in which the DNA synthesis assays were carried out. The results suggested that p300 as well as p105-Rb acts to suppress DNA synthesis and that the two proteins act in a cooperative manner. If one or both of these proteins is inactivated through an interaction with E1a products, DNA synthesis is induced. It is only in cells carrying E1a proteins which bind neither p300 nor p107, that the quiescent state of the cells is maintained. The fact that only one of the proteins must be inactivated to allow DNA synthesis to proceed, but both must be inactivated to allow the cell to become transformed, suggests that one or both of p300 and p105-Rb have functions other than the suppression of DNA synthesis. Others have shown that the regions on E1a with which these two proteins interact may be distinguished on a functional basis with respect to the induction of DNA synthesis and other functions such as focus formation, induction of an epithelial cell growth factor and induction of mitosis in cells in which DNA synthesis has already come about (Moran and Mathews, 1987, Subramanian et al., 1988 and Smith and Ziff, 1988). Further studies

will be required to fully delineate the functions of these two cellular proteins.

The research described above suggested that both p300 and p105-Rb are involved in the suppression of DNA synthesis, and that both must be inactivated for cells to become transformed, indicating that binding of at least these two proteins by E1a products has some biological significance. The biological significance of the association of E1a proteins with p107 was not made clear in these studies, although p107 did not appear to play any role in the suppression of DNA synthesis. A role for p107 in E1a-mediated transformation was not apparent as all E1a mutants which were characterized as failing to complex to p107 also failed to bind p105-Rb. Such mutants were found to be transformation defective, but so were mutants which failed to bind to p105-Rb only. E1a mutants producing proteins which do not complex with p107, but do associate with both p105-Rb and p300 have not been described.

Cleveland peptide analysis indicated that there was some degree of homology between p107 and p105-Rb, but the digestion patterns although similar, were not identical indicating, in agreement with Ewen et al. (1989) and Dyson et al. (1989b), that p107 and p105-Rb are not identical proteins. The extent of homology

between these two proteins could not be determined by this type of analysis, but further information on the degree of homology and the identity of common peptides could possibly be obtained following separation of tryptic peptides from both proteins by high performance liquid chromatography.

Retinoblastoma cell lines were infected with wt Ad5 in order to characterize the binding of the E1a-associated proteins in these lines and it was noted that p107 was not detectable in any of the 9 retinoblastoma lines tested, although E1a proteins were produced during infection of these cells, and a third E1a-binding protein p300, was readily detectable in all cases (Table 3). p107 could be detected in retinal cells of non-retinoblastoma origin, indicating that it is normally produced in this cell type. With the radiolabeling procedure used in these experiments, p300 and p107 appeared in control lanes on the fluorograms as bands of about the same intensity, and so it was expected that p107 would be detected in the retinoblastoma cells under these labeling conditions. Failure to detect p107 suggested that either p107 was not present in retinoblastoma cells, or that it was present but at extremely reduced and therefore undetectable levels, or that it was produced at normal levels but was unable to

complex to E1a proteins in the absence of functional p105-Rb. It had previously been shown that binding of p105-Rb to E1a proteins was not required for binding of p107 (Egan et al., 1988), and so the third possibility suggested rather that functional p105-Rb was necessary to modify or alter p107 in some way before p107 could interact with E1a proteins. When WERI-RB1 retinoblastoma cells were labeled for an extended period of time using increased amounts of ^{35}S-methionine, it was found that a very small amount of p107 could be detected. This indicated in agreement with reports by Ewen et al. (1989) and Dyson et al. (1989) that p107 was expressed in these cells, and that it could not therefore be an alternatively spliced product of the Rb1 gene, both alleles of which are missing in WERI-RB1 cells. Clearly however, the protein was either expressed at extremely reduced levels in WERI-RB1 cells or only a small fraction of the p107 present in these cells could bind to E1a. When small cell lung carcinoma cells which lacked a functional Rb1 gene product were examined however, it was found that normal amounts of p107 were found in association with E1a proteins, suggesting that there was no requirement for p105-Rb to modify or act on p107 as a prerequisite to formation of a complex with E1a products. This is consistent with the hypothesis that it is the

level of expression of p107 and not its ability to associate with E1a proteins which is reduced in retinoblastoma cells, although in the absence of a probe for p107 mRNA, no definitive conclusions with respect to levels of expression could be made.

These results may suggest a possible relationship between the product of the retinoblastoma tumour suppressor gene, p105-Rb, and p107, with which it has been shown to share a number of characteristics. Both of these proteins bind to adenovirus E1a proteins and to SV40 large T antigen, and for both proteins large T binds only the non- or under-phosphorylated form (Ewen et al., 1989), whereas E1a proteins associate with phosphorylated and non-phosphorylated species (Yee and Branton, 1985b, this thesis and Buchkovich et al., 1989). Both p107 and p105-Rb bind to E1a proteins in overlapping regions (Egan et al., 1988 and Whyte et al., 1989). Mutations that remove amino acid residues 111-123 on E1a polypeptides eliminate p105-Rb binding, and mutations that remove residues 124-127 abolish binding of both p105-Rb and p107. Mutations in CR1 in the first exon of E1a proteins result in reduced binding of p107 and p105-Rb, possibly due to an effect on protein conformation. Both p107 and p105-Rb interact with a region of SV40 large T between residues 105 and 114 which is highly homologous to the

120-127 region of E1a proteins (Moran, 1988 and Ewen et al., 1989). They are both ubiquitous proteins, which have been detected in a number of tissue types and in a number of mammalian species (Yee and Branton, 1985b, DeCaprio et al., 1989, Ewen et al., 1989 and Dyson et al., 1989). They are of similar molecular mass, and appear to be expressed in similar quantities. The results discussed suggest that p107 and p105-Rb may be products of related genes.

In conclusion, it has been shown that an association between E1a proteins and at least two of the cellular proteins, p300 and p105-Rb is involved in E1a-mediated transformation. Both of these proteins were also found to be involved in the regulation of DNA synthesis in quiescent cells, and appeared to act in a cooperative manner to suppress DNA synthesis. These results suggested that p300 may be the product of a tumour suppressor gene with a function similar to p105-Rb. The ability of p300 to bind to E1a-Sepharose could make E1a-Sepharose suitable for affinity purification of p300 in order to develop a probe for the p300 gene, possibly by microsequencing the protein. Cloning the gene for p300 would clearly be of interest. The role of p107 in transformation was not determined in these studies, as E1a mutants which could bind p105-Rb but not p107 have

not been reported, but this protein did not appear to function as a suppressor of DNA synthesis. There seemed to be some degree of homology by Cleveland peptide analysis between p107 and p105-Rb but further comparison of the digestion products of these two proteins, possibly by complete tryptic digestion and separation of the peptides by high performance liquid chromatography, will be required to determine the specific relationship. Such an analysis could result in the identification of p107 peptides having enough sequence identity to known regions of p105-Rb that they could be used to develop probes for the p107 gene. The results described in this study suggested that p107 may be from a gene related to p105-Rb, and so cloning of the p107 gene is clearly of interest and could help to elucidate the biological significance of the association between E1a proteins and p107.

Appendix 1. Amino acid sequence of the E1a 12S and 13S gene products. The region unique to the 13S product is shown as a shaded area.

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                                                  10                  20
Met Arg His Ile Ile Cys His Gly Gly Val Ile Thr GLU GLU Met Ala Ala Ser Leu Leu

                                                  30                  40
ASP Gln Leu Ile GLU GLU Val Leu Ala ASP Asn Leu Pro Pro Pro Ser His Phe GLU Pro

                                                  50                  60
Pro Thr Leu His GLU Leu Tyr ASP Leu ASP Val Thr Ala Pro GLU ASP Pro Asn GLU GLU

                                                  70                  80
Ala Val Ser Gln Ile Phe Pro ASP Ser Val Met Leu Ala Val Gln GLU Gly Ile ASP Leu

                                                  90                 100
Leu Thr Phe Pro Pro Ala Pro Gly Ser Pro GLU Pro Pro His Leu Ser Arg Gln Pro GLU

                                                 110                 120
Gln Pro GLU Gln Arg Ala Leu Gly Pro Val Ser Met Pro Asn Leu Val Pro GLU Val Ile

                                                 130                 140
ASP Leu Thr Cys His GLU Ala Gly Phe Pro Pro Ser ASP ASP GLU ASP GLU GLU Gly GLU

                                                 150                 160
GLU Phe Val Leu ASP Tyr Val GLU His Pro Gly His Gly Cys Arg Ser Cys His Tyr His

                                                 170                 180
Arg Arg Asn Thr Gly ASP Pro ASP Ile Met Cys Ser Leu Cys Tyr Met Arg Thr Cys Gly

                                                 190                 200
Met Phe Val Tyr Ser Pro Val Ser GLU Pro GLU Pro GLU Pro GLU Pro GLU Pro GLU Pro

                                                 210                 220
Ala Arg Pro Thr Arg Arg Pro Lys Met Ala Pro Ala Ile Leu Arg Arg Pro Thr Ser Pro

                                                 230                 240
Val Ser Arg GLU Cys Asn Ser Ser Thr ASP Ser Cys ASP Ser Gly Pro Ser Asn Thr Pro

                                                 250                 260
Pro GLU Ile His Pro Val Val Pro Leu Cys Pro Ile Lys Pro Val Ala Val Arg Val Gly

                                                 270                 280
Gly Arg Arg Gln Ala Val GLU Cys Ile GLU ASP Leu Leu Asn GLU Pro Gly Gln Pro Leu

ASP Leu Ser Cys Lys Arg Pro Arg Pro
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