

CHARACTERIZATION OF THE TRANSFORMING PROTEINS  
OF ADENOVIRUS TYPE 5  
SYNTHESIZED IN INFECTED HUMAN CELLS  
AND  
TRANSFORMED HAMSTER CELLS

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

© June, 1983

TRANSFORMING PROTEINS OF ADENOVIRUS

DOCTOR OF PHILOSOPHY (1983)

McMaster University  
Hamilton, Ontario

TITLE: Characterization of the Transforming Proteins of  
Adenovirus Type 5 Synthesized in Infected Human  
Cells and Transformed Hamster Cells

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NUMBER OF PAGES: xiv, 182.

## Abstract

It is known that human adenovirus type 5 gene products will cause the conversion of primary cells to a potentially oncogenic state. The transforming genes reside in a 4050 base pair (bp) region (early region 1; E1: 0-11.2 map units, mu) at the left end of the physical map of the linear duplex DNA genome (ca. 37,000 bp). Early region 1 is organized into two transcription units, E1A (0-4.5 mu) and E1B (4.5-11.2 mu), each of which produces multiple species of mRNAs and polypeptides. The results of studies on a library of baby hamster kidney cell lines transformed by adenoviral DNA fragments representing all (XhoI C:0-16 mu) or only a part (Hind III G:0-8 mu) of E1 established that the E1B-58K product was unnecessary for both maintenance of the transformed state and oncogenicity. It was further shown that although mutants defective for 58K expression are transformation deficient in virion mediated transformation assays, the DNA extracted from these mutant viruses possessed transforming activity comparable to wt in in vitro DNA transfection assays. The mutant transformed hamster cell lines which emerged from these experiments did not express the 58K product but exhibited a range of oncogenic potentials in newborn hamsters similar to wt transformed lines.

Hamster anti-tumor serum and a rabbit serum specific for a synthetic peptide corresponding to the carboxy terminus of the E1A proteins were used to identify E1A proteins (52K, 50K, 48.5K, 37.5K, 35K, 29K and 25K) and E1B proteins (19K and 58K) in infected and transformed cells. Interestingly, the few batches of anti-tumor sera positive for E1A products all recognized only a subset (52K, 48.5K, 37.5K) of the E1A proteins corresponding to the products of only the largest of the E1A mRNAs. These immunoreagents were used to study

the deposition of transforming proteins by cell fraction and indirect immunofluorescence techniques. The E1B-19K was resolved into two related cytoplasmic membrane-associated polypeptides (19K and 18.5K) neither of which were detected on the outer surface of the plasma membrane. The E1B-58K appeared to accumulate in the nucleus during the lytic cycle. E1A products localized in both nuclear and cytoplasmic compartments and, within the cytoplasm, these proteins showed a marked affinity for the cytoskeleton.

By examining the kinetics of early protein synthesis during lytic infection of KB cells by wild type (wt), it was shown that E1A, E1B, E2 (61.6 - 74.9  $\mu$ ) and E4 (91.4 - 99.1  $\mu$ ) proteins were expressed in a temporal relationship. Infections with the E1A host range (hr) mutant hr3 were characterized by severely depressed levels of early protein synthesis consistent with the proposed role of E1A gene products as activators of early transcription units. The normal pattern of expression was also perturbed by a defect in E1B as the E1B host range mutant hr6 showed generally delayed and reduced levels of early gene expression suggesting a role for E1B gene products in the efficient expression of early genes.

## ACKNOWLEDGEMENTS

I am indebted to so many for so much that it impossible for me to mention all of them individually let alone give them the credit they so richly deserve. The scientific council of the supervisory committee (my supervisor Frank Graham, and committee members Lud Prevec and James Smiley ) deserves special recognition as do the efforts of Philip Branton, Sui-Pok Yee, And Silvia Bacchetti.

In particular, I would like to thank Philip Branton for not hiring me and Frank Graham for not firing me. These two individuals have made this thesis possible, for without their wisdom and tolerance many of the things we have discovered would have remained unknown, and my resolve to complete this work would most surely have evaporated.

My studies were made profitable by the dedicated assistance of Chris Patterson and Judy Ng, whose efforts on my behalf I shall always remember. I was altogether very fortunate to be in the midst of people who could brighten the darkest days.

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## LIST OF ABBREVIATIONS

m.w.	molecular weight
l	liter
g	gram
cm	centimeter
mm	millimeter
°C	degrees celsius
K	$10^3$
min	minutes
hr(s)	hour(s)
Ci	curie(s)
mu	map units
Kb	kilobase(s)
M	molar
mM	millimolar
ml	milliliter
cpm	counts per minute
MOI	multiplicity of infection
$^{35}\text{S}$	sulfur-35
$^{32}\text{P}$	phosphorous-32
$\text{CaCl}_2$	calcium chloride
$\text{MgCl}_2$	magnesium chloride
$\text{NaCl}$	sodium chloride
$\text{ZnCl}_2$	zinc chloride
SDS	sodium dodecyl sulfate
EDTA	ethylenediamine tetracetic acid

ABBREVIATIONS (continued)

NP-40	nonidet P-40
PBS	phosphate buffered saline
Tris	tris(hydroxymehtyl)aminomethane
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
DNA	deoxyribonucleic acid
HS	horse serum
FBS	fetal bovine serum
MEM	minimal essential medium
BHK	baby hamster kidney
BRK	baby rat kidney
wt	wild type
p.i.	post infection
PAGE	polyacrylamide gel electrophoresis
2-D	2-dimensional
ELISA	enzyme-linked immunosorbant assay

"What do you know about this business?" the King said to Alice.

"Nothing," said Alice.

"Nothing whatever?" persisted the King.

"Nothing whatever," said Alice.

"That's very important," the King said, turning to the jury. They were just beginning to write this down on their slates, when the White Rabbit interrupted:

"Unimportant, your Majesty means of course," he said, in a very respectful tone, but frowning and making faces at him as he spoke.

"Unimportant, of course, I meant," the King hastily said and went on to himself in an undertone, "important - unimportant - unimportant - important - " as if he were trying which word sounded best.

Some of the jury wrote it down "important" and some "unimportant."

Alice's Adventures in Wonderland

LEWIS CARROLL

CHAPTER I

INTRODUCTION

## 1. Natural History of Adenoviruses

### 1.1 Discovery

For many years after the isolation of influenza virus in 1933 no new type of respiratory virus was found. During the winter and spring of 1952-53 Rowe et al. (1953), searching for the still unknown major causative agents of acute viral respiratory diseases, noted cytopathic changes characterized by progressive degeneration of epithelial cells in cultures of human adenoids. The agent responsible could be serially transmitted by filtered culture fluids to established lines of human cells and was called the adenoid degenerating (AD) agent. In the same year an agent responsible for a primary atypical pneumonia among military recruits at Fort Leonard Wood was shown to be related to AD by complement fixation and neutralization tests (Hilleman and Werner, 1954). The discovery of these prototype strains was followed in the next few years by the isolation of many viruses belonging to the same general group from humans and other mammals (Huebner et al., 1954; Hull et al., 1958; Kassenberg, 1959; Klein et al., 1959; Hartley and Rowe, 1960). The new agents were called "adenoviruses" to record the isolation of the original prototype strain from adenoid tissues (Enders et al., 1956).

### 1.2 Classification

The adenovirus group now has at least 80 members, categorized into six subgroups according to the natural host species (human, simian, bovine, canine, murine, avian) (Pereira et al., 1963). All of these viruses share the following properties: an icosahedral virion 600 A to 900 A in diameter composed of an inner core and an outer shell, which contains only DNA and protein and is resistant to inactivation by lipid solvents (i.e. has no envelope) and trypsin. All adenoviruses synthesize a soluble complement fixing antigen which cross-reacts with the antigens made by other members of

the group (Norby, 1971).

The adenoviruses isolated from humans have been divided into 31 distinct types on the basis of neutralization tests using antisera against identified prototype strains (Beladi, 1972). These serotypes have been gathered into groups according to different biologic and molecular properties. The four groups originally proposed by Rosen (1960), based on the differential haemagglutination properties of rat and monkey erythrocytes, have not been fully accepted because of the need to further classify the viruses into 10 subgroups (Hierholzer, 1973) and the difficulty in grouping adenovirus serotypes which either do not haemagglutinate or weakly haemagglutinate. Huebner (1967) and McAllister et al. (1969) grouped the adenovirus serotypes according to oncogenicity in newborn hamsters into four groups: A, highly oncogenic; B, weakly oncogenic; C and D, nononcogenic. The same four groups have been identified on the basis of genome homology (Green et al., 1979) molecular properties of structural polypeptides (Wadell, 1979) and early antigen reactivity (Gerna et al., 1982). A fifth group (group E) including only serotype 4 has been identified by molecular methods (Green et al., 1979) and a sixth group has been proposed (Wadell, 1979) to include enteric adenoviruses (Garg et al., 1979; Ritter et al., 1979). The correlations that have been discovered using different classification schemes to group the serotypes of human adenoviruses reinforce the validity of such endeavours and may reflect the evolutionary record of these viruses. It is interesting to note in this regard that despite many attempts, recombination between members of different subgroups has not been detected although members of any one subgroup have been observed to undergo this process in the laboratory (Flint, 1981).

### 1.3 Epidemiology

Although adenoviruses have been detected in several animal species there are no satisfactory animal models that mimic the infections of man. This problem has impeded progress in the study of adenovirus pathogenesis, and knowledge of human adenovirus infections has come primarily from clinical observations and experiments on volunteers (Davis et al., 1973). Most individuals are infected with one or more adenoviruses before the age of 15 and infections are either inapparent or characterized by self-limited respiratory illness followed by complete recovery and persistent type-specific immunity. Man is the only known reservoir for strains of adenovirus that infect humans and from a clinical point of view, these viruses may be divided into four groups. The first group (including serotypes 3,4,7, and 14) is associated with epidemic episodes of acute respiratory illnesses and antibodies to members of the group are rare in general human populations (Parker et al., 1961). The second category (most often involving serotype 8 and to a lesser extent serotype 7) characterized by the specific disease epidemic keratoconjunctivitis appears to be associated with trauma to the conjunctivitis produced with dust and dirt in shipyards and factories (Jawetz et al., 1958). The third group consisting of serotypes 1,2,5 and 6 has been isolated from 50% to 80% of tonsils and adenoids maintained in long-term cultures. These serotypes are responsible for most infections of young children and are apparently able to establish in lymphoid tissue latent or masked infections which persist for extended periods of time. As a result, a relatively high incidence of antibody to serotypes 1,2,5 and 6 exists in the general human population, but recurrent illnesses have not been shown to arise from these latent infections (Flint, 1981). The fourth group (serotypes 12,18 and 31) is regularly isolated from

feces of healthy individuals and incidence of antibodies to members of this group is relatively high. These serotypes do not appear to be associated with any specific disease and are rarely isolated from long-term cultures of adenoid or tonsil tissue (Davis et al., 1973; Flint, 1981).

Despite the large number and world wide distribution of adenoviruses their clinical importance appears to be limited to occasional epidemics of acute respiratory disease in military recruits and outbreaks of bronchiolitis among children. Infections are observed throughout the year but the greatest incidence and largest epidemics occur in the late fall and winter. Peculiarly, type 4 adenovirus is the common cause of acute respiratory disease in military recruits, but rarely produces infections in civilian populations. This epidemiological phenomenon is without parallel, and the underlying causative factors remain unknown.

## 2. Virion and Genome Structure

### 2.1 The outer shell

By far the greatest amount of information about the polypeptide composition and architecture of adenoviruses is derived from studies on human serotypes 2 and 5. These are particularly amenable to structural analysis as both the virions themselves and their structural constituents can be isolated in large quantities from infected cells. These viruses contain 87% protein and 13% DNA (Green and Pina, 1963). The outer shell is composed of 252 capsomers, 240 of which form the faces and edges of the 20 triangular facets of a regular icosahedron. The term hexon has been employed to denote that each of these is surrounded by six neighbors. The 12 vertex capsomers having only five neighbors have been termed pentons (Ginsberg et al., 1966). It was originally thought that the adenovirus particle was composed of only two kinds of proteins, hexons and pentons, in addition to the core. However,

using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, the polypeptide composition of purified virions displayed an unexpected complexity (Maizel et al., 1968a; Maizel, 1971) and estimates of the number of structural polypeptides involved still range from 9 (Maizel et al., 1968a) to 15 (Everitt et al., 1973; Weber et al., 1977). It appears well established that the virion contains at least nine unique polypeptides (II, III, IIIa, IV, V, VI, VII, VIII and IX) (Everitt et al., 1973; Everitt and Philipson, 1974) and several additional minor species which are present in only a few copies per virion or represent cleavage products which remain when certain capsid polypeptides mature during virion assembly.

Polypeptides II, III and IV with molecular weights of 120,000 (120K), 85K and 62K respectively, form the hexon, the penton base, and the fiber (Maizel et al., 1968b). The hexon purified from infected cells appears to have the shape of a conic triangular prism composed of three copies of polypeptide II (Horowitz et al., 1970; Cornick et al., 1973; Jornvall et al., 1974) whereas hexons obtained by dissociating virions are associated with two copies of polypeptide VI (m.w. 24K) (Everitt et al., 1973). Since polypeptide VI is not labeled when intact virions are iodinated by the lactoperoxidase method (Everitt et al., 1975) it has been suggested that this polypeptide is not exposed on the outer surface of the virion. Polypeptides VIII (m.w. 13K) and IX (m.w. 12.5K) are also hexon associated proteins but while IX appears to be located between facet hexons where it is thought to play some cementing role, the exact location and function of polypeptide VIII is unknown (Everitt et al., 1973, 1975).

The penton base, when purified to homogeneity, has an apparent molecular weight of about 246K as determined by neutron scattering techniques (Devaux et al., 1982) and contains only polypeptide III (Maizel et al., 1968b;

Anderson et al., 1973) suggesting a trimeric structure. This is an unexpected finding because of the pentagonal symmetry of the vertices of the icosahedral particle. The peripentonal hexons appear to be associated with polypeptide IIIa (m.w. 66K) which is present in approximately 60 copies per virion (Boudin et al., 1980; Devaux et al., 1982) implying five IIIa molecules per vertex. The role of polypeptide IIIa in cementing the penton base to peripentonal hexons remains conjectural, however temperature sensitive mutants of IIIa are defective in the assembly of virions and a role for IIIa in maintaining adenovirus capsid structure has thus been indicated (Devaux et al., 1982).

The fiber has a native molecular weight of 156K to 205K (Sundquist et al., 1973; Dorsett and Ginsberg, 1975; Devaux et al., 1982) and upon denaturation polypeptides of about 62K are released suggesting a trimeric structure. Evidence conflicts on whether the three polypeptide subunits are all polypeptide IV or whether two of the three chains are IV and one is different (Dorsett and Ginsberg, 1975; Pettersson et al., 1968; Herisse et al., 1981). The fiber, a highly asymmetric structure consisting of a rod with a terminal knob, forms a specific non-covalent association with the penton base and projects outwards when assembled into virions. Studies on adenovirus infection of cultured human cells suggest that the fiber recognizes specific receptors on the plasma membrane during the first stages of a productive infection (Levine and Ginsberg, 1967; Philipson et al., 1968; Sussenbach, 1967).

## 2.2 The inner core

Every infectious virion contains within it a DNA-protein core. This dense structure ( $1.42 \text{ g/cm}^3$ ) (Laver et al., 1968) contains 18-20% of the total protein of the virion and a single copy of the genome. Two

arginine-rich polypeptides - polypeptide V (m.w. 48.5K) and VIII (m.w. 18.5K). - are present in 180 and 1070 copies, respectively, per core (Everitt et al., 1973, 1975). A third polypeptide, present in only two copies, is associated with the molecular ends of the viral DNA (Robinson and Bellett, 1974; Rekosh et al., 1977).

The core structure is released from virions after treatment with heat, acetone, formamide, 10% pyridine or 5M urea (Philipson and Lindberg, 1974). Electron micrographs of cores prepared by a variety of techniques show particles that appear to be 660-670A in diameter and composed of 8-10 spherical subunits, 220A in diameter (Brown et al., 1975). Cores prepared by incubation of virions with 0.5% sarkosyl contain only polypeptide VII and the terminal protein-DNA complex suggesting that polypeptide V is less tightly bound to viral DNA than polypeptide VII (Brown et al., 1975). A nucleosome-like organization of DNA and protein has been suggested by studies showing that core-associated DNA has a limited susceptibility to staphylococcal nuclease treatment (Corden et al., 1976). Since initial cleavage fragments of 1800 nucleotides are approximately 5% the length of the genome, Corden et al. (1976) proposed a 20 subunit core with one subunit associated with each of the 20 faces of the icosahedral capsid. Alternatively, Brown et al. (1975) have suggested the core might consist of only 12 subunits, one per each vertex. The exact structure of the core and its relationship to the outer shell remain uncertain.

### 2.3 The viral genome

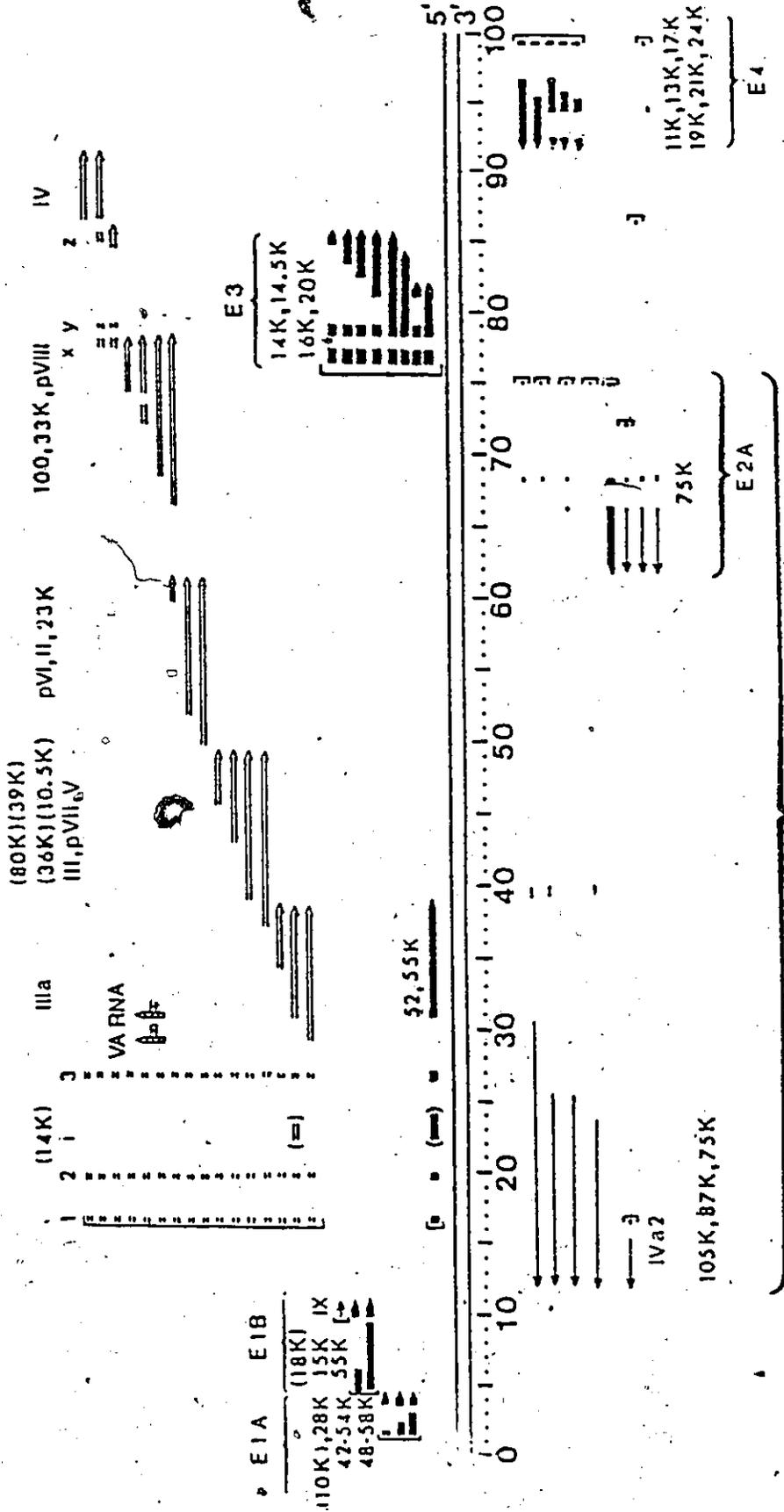
The adenoviral genome is a linear molecule of double-stranded DNA with no single-strand breaks (van der Eb and van Kesteren, 1966; Green et al., 1967; van der Eb et al., 1969). Adenovirus DNA is released from virions with the terminal protein covalently attached by treatment with 4M guanidium

hydrochloride. Pure viral DNA obtained after proteolytic enzyme digestion and phenol extraction sediments as 30.5S which corresponds to a molecular weight of  $21.8 \times 10^6$  daltons (Green et al., 1967; Doerfler and Kleinschmidt, 1970). The genome size varies only slightly (<5%) amongst the subgroups of human adenoviruses (Green et al., 1967). The most interesting topological feature of the genome is the presence of a short (100 nucleotides), inverted terminally repeated sequence (Garon et al., 1972, 1975; Wolfson and Dressler, 1972; Steenbergh et al., 1977; Arrand and Roberts, 1979). Single stranded adenovirus DNA forms circles with 'panhandle' double-stranded regions holding the ends together (Garon et al., 1975). Involvement of the genomic ends and the duplex panhandles in the replication of the linear viral DNA has been suggested (see below).

The complementary strands of the viral DNA separate in alkaline cesium chloride density gradients (Sussenbach et al., 1973). The heavy (h) and light (l) strands obtained correspond to the 'r' and 'l' strands, respectively, according to the widely used nomenclature of Sharp (1977) which refers to the strands by the respective rightward (r) or leftward (l) direction of the transcript. The approximate map co-ordinates of nuclear transcripts and messenger RNAs have been determined in several laboratories by hybridization of the RNAs to separated strands of DNA restriction fragments (Sharp et al., 1974; Pettersson et al., 1976; Flint, 1977; Craig et al., 1977; Smiley and Mak, 1978; Fraser and Ziff, 1978). The map was refined by electron microscopic examination of RNA-loops formed in double stranded DNA (Chow et al., 1977; Chow and Broker, 1978; Kitchingman and Westphal, 1980; Chow et al., 1980).

At present eight transcription units have been identified on the adenovirus genome (see Figure 1). Five transcription units (E1A, E1B, E2, E3

Figure 1. Transcription map of human adenovirus type 2. Arrowheads indicate the 3' ends of the mRNAs and the brackets show the position of promoter sites. Polypeptides which have been assigned to different regions are indicated above rightward reading messages and below leftward reading messages. Thick lines represent mRNAs which are expressed early after infection and unfilled arrows indicate late mRNAs. Thin lines designate mRNAs which are expressed at intermediate and late times after infection. Two small RNAs VA RNA I and VA RNA II, which map around position 30 are RNA polymerase III transcripts and do not encode proteins. (map modified from Chow et al., 1980).



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and E4) are expressed at early times (2-8 hours post-infection) before the onset of DNA replication, but one major late transcription unit is utilized for expression of almost all the viral structural components with the exception of polypeptides IX and IVa2 whose mRNAs are independently promoted and expressed at intermediate times post-infection (Pettersson and Mathews, 1977; Persson et al., 1979). A multiple of differently spliced mRNAs are generated from virtually all early and late transcription units by processing the primary transcripts in different ways. It has been possible, in some cases, to identify specific polypeptides as products of the different mRNAs.

There are few exceptions to the general pattern of mRNA formation. One involves the major late transcription unit which is promoted at map position 16.5 and generates an initial transcript which is 28,000 nucleotides long (Evens et al., 1977). Spliced late messages from this promoter share these short segments of 5' untranslated leader RNA with a combined length of 203 nucleotides and derived from sequences at map co-ordinates 16.5, 19.6 and 26.6 (Berget et al., 1977; Chow et al., 1977; Zain et al., 1979; Akusjarvi and Pettersson, 1979). The 3' ends of the mRNAs are made by an endonucleolytic cleavage of the initial transcript and addition of a poly A tail at one of five major poly A addition sites (Fraser et al., 1979; Nevins and Darnell, 1978). This allows the late mRNAs to be divided into five 3-co-terminal families, designated L1 to L5, which may be structurally polycistronic but are functionally monocistronic (see Figure 1). The term 'major late promoter' is in one respect misleading since it has been shown that this promoter is active at early times after infection but appears to synthesize only a subset of L1 RNA species (Fraser et al., 1979; Chow et al., 1980; Lewis and Mathews, 1980; Akusjarvi and Persson, 1981).

The positions and polarities of the sites complementary to early and

late mRNA are similar if not identical amongst the distantly related (in terms of DNA sequence homology) groups of human adenoviruses (Williams et al., 1975; Ortin et al., 1976; Smiley and Mak, 1978; Green et al., 1979). This conservation of physical organization suggests that genes with similar functions have most likely retained common map positions during the evolution of the adenovirus genome.

### 3. Lytic Infection by Adenoviruses

#### 3.1 Adsorption and uncoating

Adenovirions attach to cells through the interaction of their fibers with specific receptors (approximately 10,000 per cell) on the plasma membrane (Levine and Ginsberg, 1967; Lonberg-Holm and Philipson, 1969). This step is followed by either pinocytosis (Chardonnet and Dales, 1970a,b) or direct penetration of the membrane (Lonberg-Holm and Philipson, 1969; Morgan et al., 1969) with a concomitant loss of pentons and partial exposure of the genome (Sussenback, 1967; Philipson et al., 1968; Morgan et al., 1969). The pentonless virions are rapidly transported to the vicinity of the outer nuclear membrane by a poorly understood process involving microtubules (Dales and Chardonnet, 1973; Luftig and Weihing, 1975) and then appear to become lodged in the nucleopores (Chardonnet and Dales, 1970a,b; Dales and Chardonnet, 1973). The core is released into the nucleoplasm in an energy-dependent step leaving the outer shell behind at the nuclear membrane (Chardonnet and Dales, 1972). The final product of uncoating appears to be the naked DNA-terminal protein complex which becomes tightly associated with the nuclear matrix (Philipson et al., 1968; Youngusband and Maundrell, 1982). The uncoating process is completed in 0.5-2.0 hours depending upon the cell and does not appear to require de novo gene expression by the host since it can take place in the presence of inhibitors of protein and nucleic

acid synthesis (Lawrence and Ginsberg, 1967; Philipson, 1967).

### 3.2 Early gene expression

Not all eight transcription units are activated simultaneously during lytic infection by adenoviruses. Expression of viral genes follows a complex program of transcription activation and inactivation (Berk et al., 1979; Nevins et al., 1979; Ziff, 1980), promoter and splice site switching (Chow et al., 1979), and mRNA stabilization and translational control (Persson et al., 1981; Babich and Nevins, 1982). All the adenovirus genes are transcribed by the cellular RNA polymerase II with the sole exception of the VA-RNA genes (Price and Penman, 1972; Weinmann et al., 1974). Initially (ca. 2 hours p.i.), only the E1A region and the promoter proximal segment (L1) of the major late transcription unit are activated (Shaw and Ziff, 1980; Nevins and Wilson, 1981). Several lines of evidence suggest that the next step in lytic infection (ca. 3 hours p.i.) is the activation of the E1B, E2, E3 and E4 transcription units by E1A gene products (Berk et al., 1979; Jones and Shenk, 1979a; Persson et al., 1981; Solnick, 1981; Nevins, 1981). Although the precise mechanism by which E1A governs early gene expression has not been determined, it has been suggested that E1A product(s) inactivate a host repressor which would otherwise inhibit transcription (Nevins, 1981; Persson et al., 1981).

The kinetics of expression of RNA from each of the early transcription units is unique (Nevins et al., 1979; Shaw and Ziff, 1980), probably owing to the differential influence of multiple positive and negative feedback circuits. (These processes are discussed in detail in Chapter V.) By six hours p.i., just prior to DNA replication, promoters for the structural polypeptides IX and IVa2 are activated (Persson et al., 1978; Galos et al., 1979), but most early transcriptional activity subsides between six and eight

hours p.i. as the DNA template enters replication (Nevins et al., 1979; Shaw and Ziff, 1980).

### 3.3 Adenovirus DNA replication

The synthesis of viral DNA commences around six hours p.i. and reaches maximal levels six to ten hours later (Ginsberg et al., 1967; Pina and Green, 1969). Physical analysis and isotope incorporation studies of replicative intermediates from infected cells have established that initiation of viral DNA synthesis occurs with approximately equal frequency at both ends of the genome and proceeds by strand displacement down the entire length of the molecule (Sussenbach et al., 1973; van der Eb, 1973; Winnacker, 1975; Tolun and Pettersson, 1975; Flint et al., 1976b; Weingartner et al., 1976; Horowitz, 1976; Ariga and Shimojo, 1977; Lechner and Kelly, 1977). The discovery of the terminal protein (m.w. 55K) covalently attached to the 5' ends of the DNA (Robinson et al., 1973; Robinson et al., 1974; Rekosh et al., 1977) and the detailed knowledge of the inverted terminal repetition (Garon et al., 1972, 1975; Steenbergh et al., 1977; Arrand and Roberts, 1979) provided the basis for the current model of adenovirus replication. The key to this model is the proposal that a dCTP-terminal protein complex provides the 3' hydroxyl group necessary for priming of DNA polymerase at the terminus of the viral genome (Rekosh et al., 1977; Desidero and Kelly, 1980; Lichy et al., 1981; Enomoto et al., 1981). With this task accomplished, the rest of the replication scheme is just the elongation of the nascent chain and displacement of the parental chain. It has also been suggested that inverted repeats at the ends of displaced single strands may even self-anneal and form the panhandle structure to initiate complementary strand synthesis (Lechner and Kelly, 1977; Rekosh et al., 1977).

A missing piece of the adenovirus replication picture emerged recently

from studies using soluble cell extracts which can replicate exogenously added viral DNA (Arens et al., 1977; Kaplan et al., 1977; Horwitz, 1978; Challberg and Kelly, 1979). It was discovered that adenovirus encodes its own DNA polymerase (m.w. 140K) in E2B sequences between map co-ordinates 14.2 and 22.9, adjacent to the gene for the precursor to the terminal protein (m.w. 80K) between co-ordinates 23.5 and 28.9 (Lichy et al., 1982; Gingeras et al., 1982; Smart and Stillman, 1982; Stillman et al., 1982). Only the Ad DNA polymerase appears to be capable of initiating DNA replication in vitro and the preliminary data suggest that the first step is the Ad DNA polymerase-mediated linkage of dCMP to the precursor of the terminal protein (Lichy et al., 1982).

Host protein factors that stimulate the synthesis of the preterminal protein-dCMP complex have recently been described but a role for the E2A encoded single-stranded DNA binding protein (DBP) in initiation of DNA synthesis has not emerged (Lichy et al., 1982; Stillman et al., 1982; Nagata et al., 1982). Temperature-sensitive mutants of E2A are DNA negative and temperature shift experiments suggest that the DBP binds to single stranded tails during displacement synthesis and appears to be required only for elongation of nascent chains (van der Vliet and Sussenbach, 1975; van der Vliet et al., 1975; Horwitz, 1978; Klein et al., 1979).

Although the current understanding of adenovirus DNA replication seems relatively complete except for a few details, the final model could still involve circular genomic intermediates. Candidate structures have recently been isolated from infected cells (Ruben et al., 1983). Approximately 10% of the viral DNA in infected cells appears to be in the form of head-to-tail joined molecules some of which consist of covalently closed circles, but neither the mechanism which generates the circular molecules nor the

significance (if any) of head-to-tail joining in adenovirus replication have been determined.

### 3.4 Late gene expression

A major shift in adenovirus gene expression, known as the early to late switch, is closely coupled to the onset of DNA replication and dramatically increases the abundance of 'late transcription unit' mRNAs. The primary transcript, preterminated at early times and producing only two cytoplasmic RNA species from L1 (Chow et al., 1980; Akusjarvi and Persson, 1981), extends from co-ordinate 16.5 to the right end of the genome at late times and is spliced into about 20 different mRNAs allowing expression of genes coded in L2-L5. Accompanying the increase in activity of RNA polymerases II (and III) at late times is a change in the splicing machinery (Akusjarvi and Persson, 1981; Chow et al., 1980; Nevins and Wilson, 1981). The L1 nuclear precursor is the same molecule at early and late times, however, at early times an extra 440 nucleotide leader segment (designated the i-leader) derived from co-ordinates 22.0-23.2 is spliced into the message between the second and third late leader segments (see Figure 1) (Chow et al., 1980; Virtanen et al., 1982; Shaw and Ziff, 1980; Nevins and Wilson, 1981). Expression of early genes has also been detected during the late phase however profound shifts occur in the splice site preferences during the late phase, particularly for early region 1 messages (see Chapter VI) (Flint and Sharp, 1976; Spector et al., 1978; Chow et al., 1980). In addition, it appears that early region 2 mRNAs are initiated at a different promoter site which is utilized exclusively at late times (Chow et al., 1979).

The changes in promoter usage, transcript termination sites and splicing patterns which accompany the early to late switch almost surely involve viral specific factors and although the nature of these factors remains unknown,

candidate components have been studied. The most interesting of these are the virus associated (VA) RNAs, VA-RNA<sub>I</sub> (156 nucleotides; map co-ordinates 29.0 to 29.5) and VA-RNA<sub>II</sub> (157 nucleotides; map co-ordinates 29.7 to 30.2) (Gingeras et al., 1982). Both VA-RNAs can be detected early during infection, however, transcription of VA-RNA<sub>II</sub> accelerates after the onset of DNA replication becoming the most prevalent RNA species at late times (Soderlund et al., 1976). Both VA-RNAs are transcribed by RNA polymerase III (Price and Penman, 1972; Soderlund et al., 1976; Weinmann et al., 1974) utilizing promoter sequences which lie within the structural gene (Fowlkes and Shenk, 1980). VA-RNAs bind to unfractionated late viral RNA and VA-RNA<sub>I</sub> binds to cloned cDNA of late fiber mRNA but not to cloned genomic fragments (Mathews, 1980). Early mRNA and uninfected cell mRNA bind less well. These results have suggested that VA-RNA may be hybridizing across splice junctions, and possibly involved in bridging nascent transcripts during the splicing reaction (Mathews, 1980).

Although the factors mediating the early to late shift are not known, it has been established that blockage of DNA replication whether by inhibitors of DNA synthesis or by DNA minus conditionally lethal mutants prevents the formation of most late mRNA (Berget et al., 1976; Carter and Ginsberg, 1976; Chow et al., 1979). Temperature shift experiments with the E2A mutant ts125 have revealed that the onset of DNA replication is the critical factor since late mRNA synthesis continues after a shift to the non-permissive temperature that causes DNA replication to cease during the late phase (Ginsberg et al., 1974; Carter and Ginsberg, 1976).

Maximal rates of late viral protein synthesis occur around 15 hours p.i. (Russell and Skehel, 1972; Walter and Maizel, 1974). However, some polypeptides (including II and IV) continue to be made in large amounts for

an additional 10-15 hours while the synthesis of others (III, V and IX) declines. The relative rate of translation of a specific message possessing the tripartite leader (including messages encoding polypeptides II, 100K, III, IIIa, IV, V, pVI, PVII and pVIII among others) is directly proportional to the abundance of that mRNA species in the cytoplasm (Lawrence and Jackson, 1982). Thus the chief mechanism for controlling levels of individual late gene products appears to be at the level of mRNA processing.

### 3.5 Assembly of adenovirions

Most of the viral polypeptides made during the late phase are rapidly released from polyribosomes and transported to the nucleus in three to six minutes (Horwitz et al., 1969; Velicer and Ginsberg, 1970). Nuclear monomeric structural polypeptides assemble to form the multimeric constituents of the capsid (i.e., hexon, fiber, penton base) (Velicer and Ginsberg, 1970).

The process by which these constituents are put together has not been as well characterized as other aspects of adenoviral biology. Core proteins appear to associate with virions within 15 minutes of their synthesis which contrasts with the several hours required before hexons appear in virions (Horwitz et al., 1969). These studies suggested that cores or core precursor complexes are inserted into preformed capsids. Pulse-chase experiments confirm that the 'empty' capsids bear a precursor product relationship with complete virions (Sundquist et al., 1973; Ishibashi and Maizel, 1974). Analysis of the viral DNA sequences associated with particles of densities which are intermediate between empty capsids and complete virions show enrichment of sequences homologous to the left end in a pattern suggesting that viral DNA sequences enter preformed capsids by the left-end first (Daniell, 1976; Tibbits, 1977; Daniell et al., 1978).

Studies with a variety of temperature sensitive assembly mutants show that several viral gene products facilitate assembly and are responsible for the proteolytic cleavages of pVI, pVII and pVIII (Leibowitz and Horwitz, 1975; Weber, 1976; Weber et al., 1977). Virion maturation is dependent upon proteolytic cleavage as conditionally-lethal mutants defective in this post-translational processing of viral polypeptides assemble precursor-containing virions which are non-infectious (Weber et al., 1977).

The infection process culminates with the production of 4,000-10,000 progeny viral particles per cell, which form into large intranuclear eosinophilic inclusions (Boyer et al, 1959; Green and Daesch, 1961). Progeny tend to remain in the nucleus and less than 1% of the total virus output is free in the culture fluid when the maximum viral titer is attained (Boyer et al., 1959). When these observations are considered in conjunction with the data suggesting that only 10-15% of the viral DNA and proteins made become incorporated into virions (Davis et al., 1973), the process of adenovirus assembly and dissemination seems remarkably inefficient compared to the well-ordered sequence of events which usurp a cell's synthetic machinery and direct the manufacture of huge stockpiles of virion components.

#### 4. Interaction of Adenovirus with Heterologous Hosts

##### 4.1 Semi and non-permissive infections and transformation

The study of semi-permissive and non-permissive infection had originally attracted the attention of oncologists seeking experimental systems with which to study cellular transformation and tumorigenesis. It was proposed that viruses ordinarily causing other diseases in man might, under the proper conditions, initiate tumors. While testing a large number of viruses Trentin et al. (1962) discovered that serotype 12 adenovirus induced formation of undifferentiated sarcomas when inoculated into newborn hamsters.

Subsequently, it was determined that while not all human serotypes could induce tumors in newborn rodents, adenoviruses from species as divergent as man and chicken were capable of transforming cultured cells to a potentially oncogenic state (Trentin et al., 1962; Sarma et al., 1965; Darbyshire, 1966; Huebner, 1967; Green, 1970).

This phenomenon appears to depend in part on the species and tissue origin of the cells (McBride and Weiner, 1964; Kusano and Yamane, 1967; Casto, 1973; Gallimore, 1974), the length of time they have spent in culture (Casto, 1973), and the multiplicity at which they are infected. For particular serotypes of adenovirus, infection may be permissive, semi-permissive, or non-permissive, depending upon the species, and at present there is no a priori means of determining the consequences of infection of different cells with different serotypes of adenovirus. For example, adenovirus serotypes 2 and 5 replicate quite well in hamster cells although the yield of infectious virus is reduced (Takahashi et al. 1969; Williams, 1973). Infected cells succumb to viral replication producing 100-1000 plaque-forming units (pfu)/cell and build-up of late proteins toxic to the cell. Transformation of hamster cells by serotypes 2 and 5 requires the use of viruses disabled by UV-irradiation (Lewis et al., 1974) or the presence of a conditionally lethal mutation (Williams, 1973). In contrast, serotype 12 infection of hamster cells is completely abortive as no viral DNA replication or late gene expression takes place (Doerfler, 1968, 1969; zur Hausen and Sobol, 1969; Doerfler and Lundholm, 1970; Mak, 1975). Apparently, the pattern of early gene expression in Ad12 infected hamster cells is very similar to that of a normal lytic infection (Ortin et al., 1976). Some of the incoming viral DNA becomes integrated into the cellular chromosomes (which also become rearranged) (zur Hausen, 1968; Doerfler,

1969, 1970) and some becomes degraded (zur Hausen and Sobol, 1969). Since the frequency of transformation is very low (1 focus forming unit/ $10^4$  to  $10^6$  pfu) only a rare interaction between virus and host is expected to lead to the establishment of adenovirus transformants.

#### 4.2 Integration of the viral genome into the host chromosomes

All viral transformants regardless of serotype of virus or species and origin of cell contain integrated viral DNA sequences and express viral proteins. The number of integration sites is usually low but frequently the viral and abutting cellular DNA sequences have become amplified (Sutter et al., 1978; Ibelgafts et al., 1980; Stabel et al., 1981; Green et al., 1981; Visser et al., 1981). The patterns of integration of some 70 different Ad12 or Ad2 transformed cell lines show no site specificity by Southern blotting analyses (Sutter et al., 1978; Sambrook et al., 1979; Ibelgafts et al., 1980; Stabel et al., 1980; Vardimon and Doerfler, 1981; Kuhlmann and Doerfler, 1982), but recent nucleotide sequencing of the junction between viral and cellular DNAs has revealed multiple patch homologies between adjacent viral and cellular sequences (Deuring et al., 1981; Gahlmann et al., 1982). Interestingly, Ad12 DNA is often integrated co-linearly and nearly intact into cellular DNA, while inserted Ad2 DNA is usually segmented. Special circumstances (either mutant viruses or unusual cell types) seem to be necessary before co-linear integration of viral DNA from Ad2 (or the closely related Ad5) can be observed (Dorsch-Hasler et al., 1980; Ruben et al., 1982; Fisher et al., 1982). For both Ad12 and Ad2, recombination of viral DNA with cellular DNA seems to entail losses of nucleotides from the ends of the viral genome (Deuring et al., 1981; Gahlmann et al., 1982; Visser et al., 1982; Stabel and Doerfler, 1982) possibly explaining the failure to rescue infectious virus from any adenovirus transformed cell studied to date.

The frequent retention of both the left and right end of the viral genome by transformed cells and the occasional isolation of cell-lines which have integrated these regions as a head-to-tail joint (Sambrook et al., 1979; Stabel et al., 1980; Visser et al., 1981, 1982) are consistent with the observation that shortly after infection of primary rodent cells adenovirus DNA molecules can be detected in the form of covalently closed circles (Rubin et al., 1983). It is not clear whether circular molecules represent an obligate or alternative intermediate in integration or have any role at all in this process.

#### 4.3 Transforming genes and gene products

The study of the viral genes and gene products necessary and sufficient for transformation has occupied much of the adenovirus literature during the last decade (for reviews see Tooze, 1981; Flint, 1982; Graham, 1983) and is the subject examined in this thesis. Each of the following chapters deals with related work on different aspects of the characterization of the transforming gene products. Chapters II and III report the establishment and characterization of a library of adenovirus type 5 DNA fragment transformed hamster cell lines. In Chapter II the transforming genes of early region I and the current state of knowledge concerning the involvement of these genes in the process of oncogenic transformation are described. The original work presented involves the analysis of DNA fragment transformed cell lines for expression of viral information, oncogenicity, and the specificity of antisera raised against tumors. The study suggests that one E1 protein, the 58K, is not required for in vitro transformation with DNA fragments even though complementation group II host range mutants defective in this product had been previously shown to be unable to transform cells in vivo (Graham et al., 1978).

In addition, no role for the 58K protein was indicated in determining the oncogenicity of transformed hamster cells since the cell lines which did or did not express 58K appeared to have the same broad range of oncogenic potentials in newborn hamsters. The apparent contradiction between in vivo and in vitro transformation studies concerning the role of the 58K protein was resolved by the experiments described in Chapter III. It was shown that DNA extracted from the virions of Group II mutants is phenotypically wt in in vitro transformation assays suggesting that virion mediated and DNA fragment mediated transformation follow separate routes in achieving the same end.

During the investigations outlined above a large bank of anti-tumor sera was collected. Using these sera to detect viral tumor antigens, the properties of the transforming proteins synthesized during the normal lytic infection were analysed. In Chapter IV, the intracellular localization was examined by cell fractionation and immunofluorescence techniques. Early region 1A products and the ELB-58K were recovered from both cytoplasmic and nucleoplasmic compartments. The ELB-19K antigen was found to be almost completely cytoplasmic and associated exclusively with membranes. In Chapter V, the effects of mutations in the transforming genes on the kinetics of protein synthesis from the regions expressed early after lytic infection were investigated. These studies identified and characterized an ELB function apparently necessary for the efficient expression of viral genes at early times. Finally, an analysis of the in vivo forms of ELA gene products is presented in Chapter VI. For these studies, anti-tumor sera and an antiserum specific for the carboxy terminus of the ELA proteins were employed. Using mutants with lesions in the ELA gene, the formation of multiple species of protein from individual ELA messages was shown to

require peptide sequences in the body of the polypeptide and was not due to either proteolytic degradation, premature termination or the use of potential internal initiation sites for translation. Prior to this study these proteins were only detected following in vitro translation or after treatment of cells with drugs to enhance accumulation of E1A messages.

CHAPTER II

Establishment and Characterization of Hamster Cell Lines  
Transformed by Restriction Endonuclease Fragments  
of  
Adenovirus Type 5

## INTRODUCTION

Human adenovirus infection of cultured rodent fibroblasts has been shown to induce a morphological transformation to a potentially oncogenic state (Pope and Rowe, 1964; McBride and Weiner, 1964; Freeman et al., 1967; Williams, 1973). Although the majority of transformed cell lines established after Ad2 or Ad5 infection appear to stably integrate only a portion of the viral DNA into the cellular genome, the cells invariably retain and express the left-most approximately 14% of the viral genome (Gallimore et al., 1974; Flint et al., 1976). Studies on transformation by DNA transfection have established that only sequences of early region 1 [E1, 0-11.2% map units (mu) of the viral genome) are required for transformation of rodent cells (Graham et al., 1974; Shiroki et al., 1977; van der Eb et al., 1977; Mak et al., 1979). The mRNAs complementary to this region define two distinct non-overlapping transcription units: E1A, 1.5-4.4 mu (Berk and Sharp, 1978; Chow et al., 1979) and E1B, 4.5-11.2 mu. The three E1A mRNA species of 1.1, 0.9 and 0.6 Kilobases (Kb) direct the synthesis of seven proteins detectable by a combination of in vivo and in vitro techniques. On two-dimensional polyacrylamide gels the in vivo translation products of the 1.1 and 0.9 Kb mRNAs form a cluster containing at least six species which range in molecular weight from 58K to 42K (Halbert et al., 1979; Esche et al., 1980; Smart et al., 1981; Rowe et al., 1983); the product of the 0.6 Kb mRNA is a 28K protein which has only been observed in vitro (Spector et al., 1980). The E1B 1.0 and 2.2 Kb mRNAs both encode a 19K major tumor antigen, but the 2.2 Kb message also directs the synthesis of a 58K major tumor antigen by utilizing an internal translation initiation site for a different reading

frame which partially overlaps the coding sequences for the 19K antigen (Bos et al., 1981). It is not clear whether all or only part of E1 is responsible for the induction of oncogenic transformation. Primary baby rat kidney cells can be transformed by fragments of Ad5 DNA as small as the Hind III G fragment (0-8%) and such transformants are morphologically similar to cells transformed by larger DNA fragments or by virus (Graham et al., 1974; Schrier et al., 1979). Thus viral sequences with transforming activity appear to include only the 5' half of the E1B transcription unit. Transformation by DNA fragments containing even less of the E1B coding sequences (Kpn I-H, 0-5.9 mu) and HpaI E, 0-4.5 mu) has been reported but required less stringent assay conditions for transforming activity than has been used for larger DNA fragments and virions (Schrier et al., 1979; Houwelling et al., 1980; Shiroki et al., 1979). In addition, the resulting transformed lines were morphologically different from cells transformed by Hind III G or larger fragments (more fibroblastic, slower growing, lower saturation densities). Thus, the regions of the Ad5 genome required for establishment of "fully" transformed cells may extend to a point between approximately 6 and 8 mu from the left-end of the Ad5 genome.

An important question related to viral DNA sequences involved in transformation is whether cells transformed by DNA fragments comprising less than the entire E1 region are capable of inducing tumors in appropriate animals. Ad2 and Ad5 virus transformed hamster cells induce tumors when injected into newborn hamsters (Williams, 1973; Lewis et al., 1974; Graham et al., 1974); whereas rat cells transformed by these viruses only induce tumors when injected into young immunosuppressed rats (Gallimore, 1972) or nude mice (Gallimore et al., 1977). In contrast Ad12 transformed rodent cells induce tumors both in syngeneic animals and in nude mice (Mak et al., 1979;

Gallimore et al., 1977, 1980; Jochemsen et al., 1982). Although the reasons for the differences in tumorigenicity of virus-transformed rodent cells in their respective species remains unknown, it has been reported that neither BRK cells transformed by Ad12 Hind III G (0-7.2 mu) fragment nor cells transformed by Hind III G (0-8.0 mu) of Ad5 are capable of inducing tumors in nude mice van der Eb et al., 1979; Jochemsen et al., 1982). In addition, characterization of BRK cells transformed by plasmid DNA containing Ad5/Ad12 hybrid early region 1 has suggested that the differences in oncogenic potential in nude mice between cells transformed by 'highly oncogenic' (Ad12) and 'nononcogenic' serotypes (Ad5) are due to differences in the expression of E1B (Bernards et al., 1982).

These results raise a second and related question concerning which viral proteins are made in cells containing varying extents of Ad DNA and what effects these proteins have on tumorigenicity. The results of a number of studies on expression of viral RNA in transformed rat and hamster cell lines have suggested that the splicing patterns of E1 messages in transformed rodent cells are very similar to those found in infected human cells (Lewis and Mathews, 1981; Wilson et al., 1978; Green et al., 1981; Esche and Siegmann, 1982; Matsuo et al., 1982). A dozen or more major and minor proteins have been detected by immunoprecipitation of extracts from in vivo labeled transformed cells with anti-tumor sera (Schrier et al., 1979; Cassam et al., 1978; Matsuo et al., 1982; Ruben et al., 1982; Johannson et al., 1978; Levinson and Levine, 1977). Of these, only the E1B 58K and 19K antigens have been identified unequivocally in BRK cells transformed by DNA fragments of varying length (Schrier et al., 1979) and by comparative tryptic peptide mapping (Levinson and Levine, 1977; Matsuo et al., 1982).

In this chapter I describe the transformation of primary Syrian hamster

kidney cells with Hind III digested Ad5 DNA, purified Xho I C fragment (0-16 mu) or purified Hind III G fragment (0-8 mu). A number of transformed lines were established and characterized for content of viral DNA and proteins and ability to induce tumors following injection into newborn hamsters.

## MATERIALS AND METHODS

### Cells and virus

KB cells were cultured as monolayers on 150 mm diameter dishes (Lux) in alpha-MEM supplemented with 10% horse serum. For preparation of suspension cultures the cells were seeded in Joklik's modified medium supplemented with 10% horse serum at a density between  $5 \times 10^4$  and  $1 \times 10^5$ /ml and grown up to a density of  $3 \times 10^5$ /ml in Bellco spinner culture flasks. For transformation assays primary baby hamster kidney cells prepared from kidneys of one week old Syrian hamsters were cultured as monolayers in alpha-MEM supplemented with 10% fetal bovine serum. The 14b hamster cell line has been previously described (Williams, 1973) as has the human cell line 293 (Graham et al., 1977).

The Wt strain of Ad5 used in this study has been described previously (Harrison et al., 1977). Titers of Wt Ad5 were determined by plaque assay on monolayers of 293 cells; the particle/pfu ratio of virus preparations was in the range of 50 to 100.

### Isolation of DNA fragments

Viral DNA digested with restriction endonucleases Hind III or Xho I was electrophoresed on 1% agarose slab gels and individual fragments collected on Whatmann paper strips as described by Girvitz et al. (1980).

### Transformation assays

Primary baby hamster kidney (BHK) cells in 60mm petri dishes were

transformed with DNA using the calcium technique (Graham and van der Eb, 1973a,b) with modifications (Graham et al., 1980). Specifically, restriction endonuclease fragments of viral DNA were mixed with high molecular weight carrier DNA (rat embryo DNA) at 10 ug/ml in 250 mM CaCl<sub>2</sub>. The DNA in CaCl<sub>2</sub> was then added slowly to 2x concentrate Hepes buffered saline (HeBS, ref. Graham and van der Eb, 1973a) with bubbling (Wigler et al., 1979). After 30 min the DNA-calcium phosphate co-precipitate was added to BHK monolayers at 0.5 ml suspension per culture dish containing 5 ml medium. After 20 hrs incubation at 37°C the medium was changed (MEM plus 10% fetal bovine serum) and the cells were incubated a further two days at which time the medium was changed to selective medium (Joklik's modified MEM plus 5% horse serum) as previously described (Graham and van der Eb, 1973b). Negative controls (cultures treated with carrier DNA and untreated cultures) gave no colonies.

#### Analysis of viral DNA content in transformed cells

The methods used for purification, digestion, and Southern blotting of cell DNA are as described by Ruben et al. (1983). DNA probes, consisting of the Hind III G fragment of viral DNA purified from agarose gels, or other viral DNA fragments cloned into plasmid vectors, were labeled in vitro by nick translation and hybridized to cell DNA.

#### Tumorigenicity

Confluent monolayers of transformed cells were trypsinized and suspended in phosphate buffered saline (PBS) with 2% HS. After counting, the cells were centrifuged and resuspended in PBS without serum at a concentration of  $5 \times 10^7$ /ml; newborn Syrian hamsters were inoculated subcutaneously with 0.1 ml of cell suspension.

#### Labeling of infected and transformed cells

Suspension cultures of KB cells were concentrated 10 times and infected

with 50 pfu/cell of virus. After 30 min of absorption at 37°C the concentration of cells was made 1x with the spent culture medium which had been kept at 37°C. At 7 hours p.i., cells were centrifuged and resuspended in one-tenth volume methionine-free medium containing 50 uCi [<sup>35</sup>S]methionine/10<sup>7</sup> cells. After two hours period, the cells were washed once with PBS and collected by centrifugation. To detect the presence of Ad5 early antigens in transformed cells, monolayers of 150 cm Petri dishes were labeled for six hours with 100 uCi of <sup>35</sup>S-methionine in 5 ml of methionine-free medium per dish. For <sup>32</sup>P labeling, monolayers were incubated for 4 hours with 1 mCi of <sup>32</sup>P-orthophosphate in 4 ml of phosphate-free medium per dish.

#### Immunoprecipitation and SDS-polyacrylamide gel analysis

The protein A-Sepharose immunoprecipitation technique (Schaffhausen et al., 1978) with modifications (Lassam et al., 1979) has been described previously. Briefly, the labeled cells were washed once with PBS and lysed in 1 ml of precipitation buffer (20 mM Tris pH 7.5, 1.0% Triton X100, 1.0% sodium deoxycholate, 150 mM NaCl, 0.2% SDS) per 10<sup>7</sup> cells. After 20 min at 4°C the lysed cells were sonicated (Biosonik III, setting 30) and centrifuged at 5000g for 10 min. Usually 5 to 20 ul of antiserum and 30 ul of equilibrated settled beads were added per ml of supernatant. After 6 hours of constant mixing at 4°C the samples were recovered by low speed centrifugation, washed three times with equal volumes of washing buffer (50 mM Tris pH 7.5, 250 mM LiCl, and 0.1% 2-mercaptoethanol), suspended in 50 ul of electrophoresis sample buffer (0.625M Tris pH 6.8, 0.2% SDS, 0.19 2-mercaptoethanol, 10% glycerol and 0.02% bromphenol blue) and analysed by the discontinuous gel electrophoresis techniques (Laemmli, 1970).

For two-dimensional gel electrophoresis, the isoelectric focusing tube

gels were as described by O'Farrell (1975). Briefly, immunoprecipitated extracts of infected or mock-infected cells were resuspended in sample preparation buffer (9.5M urea, 2.0% NP.40, 2% ampholines [1.6% pH range 4 to 6, 0.4% pH range 3 to 10] and 5.0% 2-mercaptoethanol). Usually, the immunoprecipitate from  $10^7$  cells was loaded into the isoelectric focusing cylindrical gel in 35 ul of sample buffer. The gels were run as described (O'Farrell, 1975), and formed a linear pH gradient between pH 6.5 at the top and 4.1 at the bottom. The second dimension SDS-slab gels were of 12% polyacrylamide (30:0.8 acrylamide to bis-acrylamide ratio) 12 cm in length and run at a constant 100V for 4 hours. Fixed and dried gel slabs were exposed for autoradiography on Kodak XAR-5 x-ray film.

## RESULTS

### I. Transformation of primary baby hamster kidney cells

Primary BHK cells prepared from kidneys of one week old Syrian hamsters were transformed by Ad5 DNA fragments using the calcium technique (Graham and van der Eb, 1973) with minor modifications (Graham et al., 1980). Three different DNA preparations were used to transform: a limit Hind III digest of Ad5 DNA, unfractionated; the Hind III G fragment (left 8%) purified by agarose gel electrophoresis; and similarly purified Xho I C fragment (left 16%). As transformed colonies appeared and reached a suitable size (after 4-5 weeks), cell lines were established by isolating clones or by subculturing cells from dishes in which a single colony was visible. In two cases (lines 945-C1 and 954-C4) the dishes were kept after clonal isolation and later passaged to establish lines 945-4 and 954-21, respectively. Thus 945-C1 and 945-4, and 954-C4 and 954-21 are not independently isolated and, as will be discussed later, appear to be two sets of identical twins.

Because BHK cells are not particularly suited for quantitative assays and because the purpose of these experiments was to establish a number of lines for further study, a determination of the specific activity of different DNA fragments was not attempted. However, the number of colonies appearing in unstained cultures after four to five weeks suggested that the efficiency of transformation was similar (i.e. within 2-3-fold) for all three DNA preparations. Quantitative assays carried out previously on baby rat kidney cells have indicated that Hind III G fragments transform rat kidney cells with the same efficiency as larger DNA fragments (Graham et al., 1974; van der Eb et al., 1977).

In total, 14 Ad5 transformed BHK cell lines were established: 3 lines (945-C1=945-4; 954-C4=954-21 and 954-2) transformed by a total Hind III digest of Ad5 DNA; 5 lines (954-1, 954-C1, 954-C5, 954-5 and 954-6) transformed by purified Hind III G fragment; and 6 lines (972-1, 972-2, 972-3, 983-2, 983-3, 983-4) transformed by purified Xho I C fragment (see Table I).

The DNA content of these lines has been analysed by Southern blotting of transformed cell DNA cleaved with Hind III and hybridized to probes consisting of the Hind III fragments of Ad5 DNA. As shown in Table I, all the transformed cell lines contained sequences hybridizing with Hind III G (0-8) but only the cell lines established after transformation with the Hind III digest of viral DNA or with the Xho I C (0-15.5 mu) fragment contained sequences hybridizing to the Hind III E (8-17.0 mu) fragment. With few exceptions the number of integration sites of viral DNA into the host genome was low (Table I), and lines 945-C1 and 945-4 displayed identical integration patterns, as did lines 954-C4 and 954-21. Since each pair of lines was derived from the same petri dish, it appeared that the cell lines

Table I  
Tumorigenicity of Ad 5 Transformed Baby Hamster Kidney Cells

Line	Transformed by	Contains sequences hybridizing with	# of inserts of EI sequences	Tumorigenicity <sup>a</sup>		
				# of Experiments	Average Latent Period (days)	Cumulative Fraction positive
945.C1	Hind III digest	G + E	1	3	25	24/24
945-4	Hind III digest	G + E	1	NT <sup>c</sup>		
954-21	Hind III digest	G + E	4	4	60	3/31
954.C4	Hind III digest	G + E	4	NT		
954-2	Hind III digest	G + E	1	1	16	9/9
954.C1	Hind III G	G	1	5	48	7/33
954.C5	Hind III G	G	3	NT		
954-1	Hind III G	G	1	4	25	24/31
954-5	Hind III G	G	2	2	50	6/14
954-6	Hind III G	G	6	2	40	4/16
972-1	Xho I C	G + E	4	2	30	9/13
972-2	Xho I C	G + E	2	NT		
972-3	Xho I C	G + E	4	2	100	1/13
983-2	Xho I C	G + E	2	3	25	16/19
983-3	Xho I C	G + E	4	1	25	1/8
983-4	Xho I C	NT <sup>c</sup>		3	35	5/20
268.C3	Sheared DNA	NT		1	100	1/9

<sup>a</sup>5 x 10<sup>6</sup> cells, sc into newborn Syrian hamsters

<sup>b</sup>represent pairs of identical cell lines

<sup>c</sup>Not tested

obtained from the dishes kept after clonal isolation were in fact of the same lineage as the clonally derived cells.

Tumorigenicity of hamster cells transformed by Ad5 DNA fragments

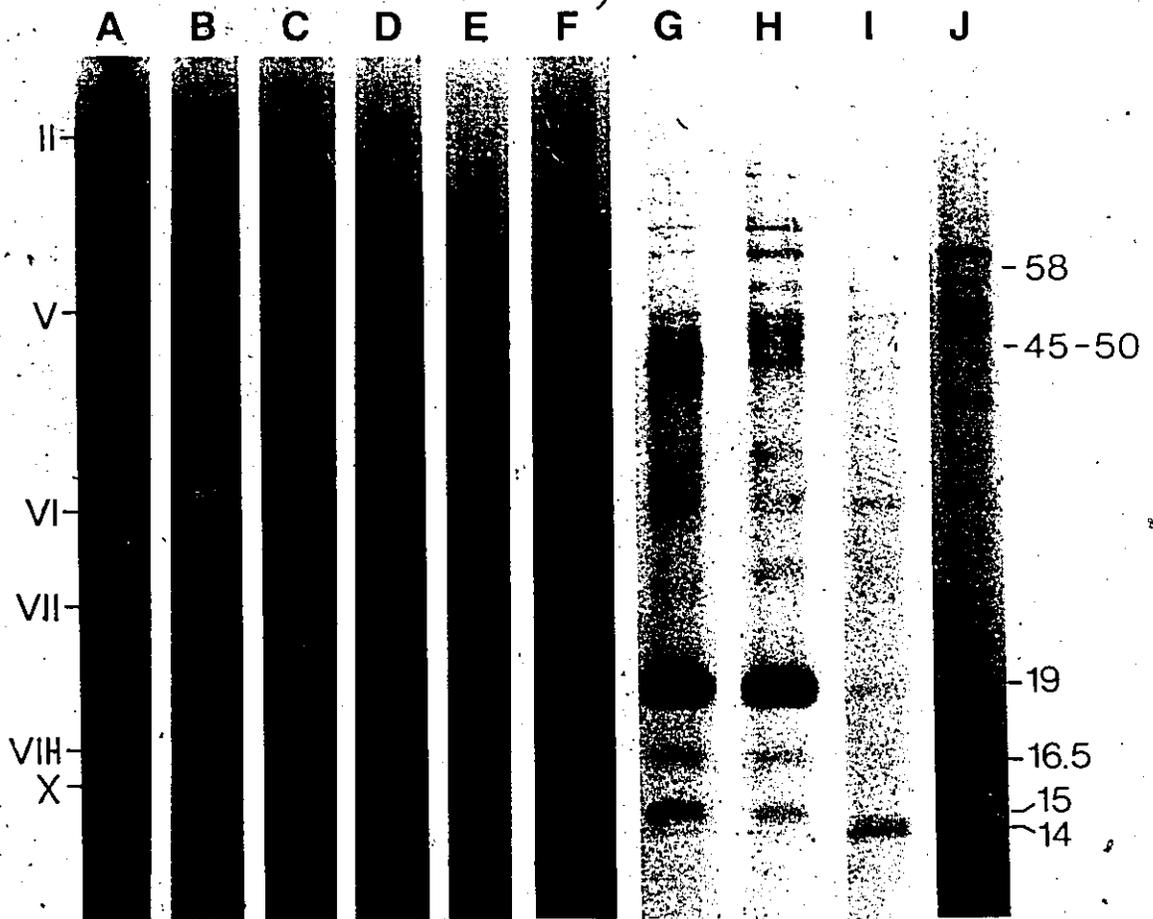
Characterization of the oncogenic potential of cell lines established after transformation of primary baby rat kidney cells with the Hind III G fragment of Ad12 (0-7.2 mu) has led to conflicting conclusions concerning the E1 gene products responsible for oncogenic transformation. Shiroki et al. (1978) reported that the Hind III G fragment transformed lines induced tumors in rats when injected either intraperitoneally or subcutaneously, while Jochemsen et al. (1982) found that similarly transformed rat cells were non-oncogenic in nude mice. Since G fragment transformed lines contain less than the entire E1 region, and therefore must synthesize a subset of E1 viral functions, it is of some importance to establish whether or not such cells are oncogenic. To address this question I have examined several Ad5 Hind III G (0-7.8 mu) and Xho I C (0-15.5 mu) transformed hamster cell lines for their tumorigenicity in hamsters as well as lines transformed by a total Hind III digest. Tumorigenicity assays were carried out by injecting  $5 \times 10^6$  cells subcutaneously into newborn or one day old hamsters. The degree of tumorigenicity observed for different cell lines varied widely (Table I), and the results failed to show any clear correlation between the fragment used to transform and the tumorigenicity of the resulting cell line. It was clear, however, that cells transformed by and containing only the Ad5 Hind III G fragment were capable of inducing tumors following injection into newborn hamsters. Some G fragment transformed lines (e.g. 954-1) appeared to be as tumorigenic as some Xho I C fragment transformed lines (e.g. 983-2) and perhaps more tumorigenic than other Xho I C transformed lines (e.g. 972-3).

Specificity of anti-tumor sera induced by Ad5 DNA fragment transformed hamster cells

As one approach to determining which viral products were expressed in the fragment transformed cells, serum was collected from tumor bearing animals and used to immunoprecipitate proteins from extracts of virus infected cells labeled with  $^{35}\text{S}$ -methionine for two hours at seven hours post-infection (pi). Analysis of the precipitated proteins by SDS-polyacrylamide gel electrophoresis resulted in patterns such as those shown in Figure 1. From these experiments it appeared that different cell lines induced immune responses against different proteins. The Hind III digest transformed line, 945-C1 (Fig. 1I) for example, induced a response primarily against a 14K antigen while other lines, e.g. 954-6 (Fig. 1G), produced a response mainly against a 19K protein, and still other lines, e.g. 983-2 (Fig. 1C), 972-2 (Fig. 1D), and T4b (Fig. 1B), induced a response against a 58K protein. Because the sera used in most of these immunoprecipitations were obtained by pooling serum from several animals I suggest that their specificity was largely characteristic of the cell line injected. The characteristic specificities of sera obtained following injection of a number of lines are summarized in Table 2. For every line shown, at least two separate serum pools from two independent litters were tested. One serum batch obtained following injection of 983-2 cells was particularly active against a 52-48.5K protein (see Fig. 1) but this same specificity was not obtained from successive injections of 983-2 cells. With the exception of this single 983-2 directed serum batch the specificity of serum collected following independent injections of a given cell line was reproducible.

In total, using all the sera prepared in this study, proteins of 58K,

Figure 1. Specificities of various hamster anti-tumor sera for Ad5 early antigens in infected KB cells. Suspension cultures of KB cells were infected at an m.o.i. of 50 pfu/cell and labeled with  $^{35}\text{S}$ -methionine at 7 hours p.i. for a period of 2 hours. Samples of immunoprecipitated proteins (see Materials and Methods) were electrophoresed on 15% polyacrylamide slab gels. A) Marker Virus with structural proteins indicated at left. Anti-tumor sera against the following cell lines were used for immunoprecipitation; B) 14b, C) 983-2, D) 972-2, E) 954-1, F) 954-5, G) 954-6, H) 954-C4, I) 945-C1, J) normal hamster serum. Molecular weights are indicated at right.



52-48.5K, 19K, 16.5K, 15K and 14K could be immunoprecipitated from infected cell extracts. Using antisera directed against synthetic peptides corresponding to the predicted carboxy termini of the E1 proteins, we have identified the 58K antigen as a product of the E1B-2.2 Kb message and the 52-48K band as the product of the E1A-1.1 Kb message (Yee et al., 1983; Chapter VI ). Based on the results of high multiplicity infections with the E1 deletion mutants dl312 and dl313 (Downey et al., 1982) and the analysis of the transformed cells reported here, the 19K antigen has been mapped to E1B sequences between 4.5 and 8.0 mu. The N-terminal amino acid sequence of the 14K antigen has identified this protein as a product of early region 4 (91.4-99.1) at the right end of the genome (Downey et al., 1982). The map positions of the 16.5K and 15K minor antigens have not as yet been determined.

I found that injection of lines transformed by the Xho I C fragment as well as of the virus transformed line 14b, resulted in anti-tumor sera directed predominantly against the 58K protein with little activity against smaller polypeptides (Table II). The serum from hamsters bearing tumors induced by Hind III G fragment transformed cells contained antibodies predominantly against the E1B-19K with some batches of sera having a minor specificity for the E1A-52K-48K (Fig. 1, Table II). One exception was the 954-5 line, which although transformed by the Hind III G fragment induced a strong response against the E1B-58K antigen as well as the 19K antigen (Fig. 1). The two independent lines, 945-C1 and 954-C4, transformed by Hind III digested viral DNA, induced markedly different antigenic specificities in sera from tumor-bearing animals (Fig. 1). Several batches of 945-C1 serum were almost monospecific for the E4-14K antigen and showed a very low avidity for the E1A-52K-48K and the E1B-19K. In contrast the specificity of 954-C4

Table II

## Specificity of Hamster Anti-Tumor Sera

Line	Transformed by	58	48- 52	19	14
945.C1	Hind III digest		+	+	++
945-4	Hind III digest			+	++
954.C4	Hind III digest		+	++	
954.C1	Hind III G			+	
954-1	Hind III G	+	+	++	
954-5	Hind III G	++		++	
954-6	Hind III G		+	++	
972-1	Xho I C	++			
972-3	Xho I C	++			
983-2	Xho I C	++	+		
983-3	Xho I C	++			
983-4	Xho I C	++			
14b	Virus	++			

<sup>a</sup> ++ indicates major avidity

anti-tumor sera was more like that of a typical Hind III G fragment transformed cell line.

#### Immunoprecipitation of Ad5 early proteins from transformed cells

Anti-tumor sera with different specificities were pooled to prepare a combined serum capable of efficiently immunoprecipitating all the proteins listed in Table II. This combined anti-tumor serum was then used to identify the tumor antigens present<sup>a</sup> in the various transformed cell lines. Figure 2 shows the electrophoretic patterns of immunoprecipitates of tumor antigens from representative transformed lines. A summary of the polypeptides which could be detected reproducibly in the transformed cells is presented in Table III. Of the proteins found to co-migrate with viral polypeptides from infected cells (and not found in untransformed or uninfected cells or in immunoprecipitations with normal hamster serum) the 58K, when present, was usually the most prominent protein. The anti-tumor serum raised in hamsters by transformed cells expressing the 58K (Xho I C transformants) appeared to be specific for this antigen and showed little or no activity against other tumor antigens. A 58K was also detected in lines 954-C1 and 954-21 (Hind III digest transformed cells) consistent with the presence of Hind III E sequences in these cells but also suggesting that the transforming DNA had either religated prior to or during integration or contained small amounts of incompletely digested molecules. The amount of 58K detected in these cells was, however, much less than that found in cells transformed by Xho I C fragments (Fig. 2) and this protein did not form the major antigenic target of anti-tumor sera raised against 954-C4 cells (Table II).

The E1B-58K antigen was not detected in the 945-C1 cells even though Southern blot analysis revealed the presence of sequences hybridizing to Hind III E, nor was the protein found in any of the cell lines transformed by

Table III  
Antigens Detected in Ad5 Transformed Cells

Line	Transformed by	58K	48-a 52K	19K	14K
945.C1	Hind III Digest	-	+	+	+
954.C4	Hind III Digest	+		+	+
954.21	Hind III Digest	+		+	+
954-1	Hind III G	-	+	+	-
954.5	Hind III G	-	+	+	-
954.6	Hind III G	-	+	+	-
983.2	Xho I C	+	+	+	-
983-4	Xho I C	+		+	-
972-2	Xho I C	+	+	+	-
972.3	Xho I C	+	+	+	-
268-C3	Sheared DNA	+		+	-
637.C3	Virus	+		+	-
293	Sheared DNA	+	+	+	-
BHK	-	-		-	-

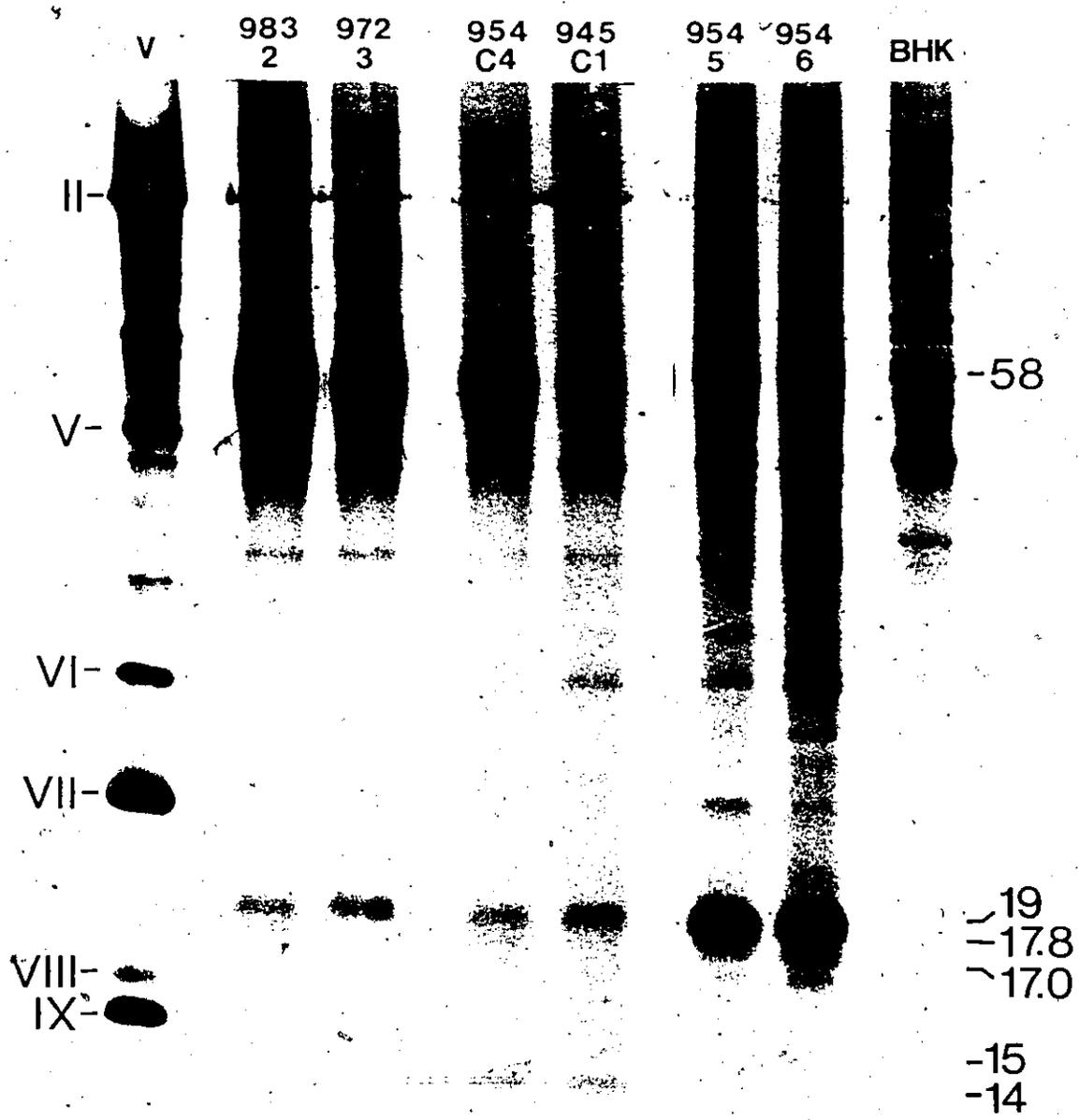
<sup>a</sup> Detected by E1A-C1 serum in <sup>32</sup>P-labeled cells. All cells tested to date are positive.

purified Hind III G fragment (Fig. 2, Table III). However, two of these lines, 954-1 and 954-5, raised anti-tumor antibodies capable of recognizing 58K and thus might synthesize either a truncated form of 58K or a chimeric polypeptide composed in part of amino acids specified by cellular sequences. Although this seems likely, the putative 58K-like products of these cell lines have not as yet been detected by immunoprecipitation.

The E1B-19K antigen was detected in all the cell lines examined but only cells which did not express the high levels of 58K found in Xho I C transformants raised an anti-tumor serum having a high avidity for this protein. As seen in Fig. 2, lines 954-5 and 954-6 appear to contain elevated levels of 19K; successive experiments however, do not support the conclusion that lines transformed by the G fragment express increased amounts of 19K. We have recently shown that high resolution 15% polyacrylamide gel analysis of immunoprecipitates from infected and transformed cells permits separation of 19K and 18.5K species which were not clearly resolved on gels of lower acrylamide concentration ( Chapter IV ). Pulse-chase analysis has suggested that the two major closely related species of 18.5K and 19K may share a precursor-product relationship with minor species of 17.8K and 17K, a possibility which is consistent with the observation that only anti-tumor sera which react with the 18.5K-19K major band, were capable of detecting 17.8K and 17K. Thus the minor species 17.8K and 17K detected in transformed cells (Fig. 2) may be related to the 19K; moreover, their presence in Hind III G fragment transformed cells does indicate that, if they are indeed viral products, they must be encoded by sequences to the left of 8.0 mu.

Another minor protein detected in some transformed cell lines co-migrated with the 14K from infected cells. I have shown that the presence of this product in transformed cells correlates with the presence of

Figure 2. Detection of Ad5 early antigens in transformed hamster cells. Monolayers of transformed hamster cells were labeled as described in Materials and Methods and extracts were immunoprecipitated with a polyvalent serum (a serum with specificity for all the early region I antigens made by pooling sera with different specificities). 983-2, 972-3, Xho I C fragment transformed hamster cell lines; 954-C4, 954-C1, Hind III G digest transformed cell lines; 954-5, 954-6, Hind III G fragment transformed cell lines. V, virus marker; BHK, untransformed secondary baby hamster kidney cells.



viral DNA sequences corresponding to the Hind III F fragment (89.1-97.1 mu) and amino acid sequencing has proved that the 14K is in fact encoded by early region E4 at the right end of the viral genome (Downey et al., 1983). While both the independent Hind III digest transformed lines analysed in this study contain E4 sequences and express the 14K, only the 945-C1 cell line (and its twin 945-4) induced anti-tumor sera which recognized this protein. Thus, although there is some similarity (compare Tables II and III) between the spectrum of proteins found in, and the specificity of antisera against, transformed cells, it is clear that neither the presence nor absence of a particular protein is necessarily reflected in the immune response obtained following injection into newborn hamsters.

#### Characterization of E1A polypeptides in transformed cells

In the preceding analysis of transformed cell polypeptides using immunoprecipitation with anti-tumor sera, I was unable to detect proteins in the range of 45-52K which correspond to E1A products. Although the long incubation periods used for labeling the transformed cells with <sup>35</sup>S-methionine were not well suited for detecting proteins which may have been rapidly turning over, the most serious problem has been the low avidity of the anti-tumor sera for the E1A polypeptides. In a separate publication (Yee et al., 1983) I have described the preparation of rabbit antiserum (E1A-C1) against a synthetic peptide (gly-lys-arg-pro-arg-pro) corresponding in sequence to the carboxyl terminus of the E1A proteins as predicted from the structure of the E1A mRNAs of Ad5 (Perricaudet et al., 1979). Using this antiserum to immunoprecipitate wt and mutant infected cell extracts I identified four major (52K, 50K, 48.5K, 45K) and four minor (37.5K, 35K, 29K, 25K) phosphoproteins (Fig. 4a) of which the 52K, 48.5K and 37.5K were shown to be products of the E1A-1.1 Kb mRNA and the 50K, 45K and 35K were products

of the E1A-0.9 Kb mRNA. There were no major differences in the migration patterns of these phosphoproteins when they were labeled with either  $^{35}\text{S}$ -methionine or  $^{32}\text{P}$ -orthophosphate, however, the  $^{32}\text{P}$ -labeled proteins tended to be more readily and clearly detected on one- and two-dimensional gels. When anti-tumor sera reacting with the E1A products were retested using  $^{32}\text{P}$ -labeled infected cell extracts and for comparison, E1A-C1 anti-peptide serum, it was discovered that only the 52K and 48.5K products of the 1.1 Kb mRNA were immunoprecipitated by anti-tumor sera (Fig. 3). The number of different cell lines which induced this narrow range of serum specificity included cells transformed by virus, Xho I C or Hind III G fragments, and Hind III digests of viral DNA. None of the hamster anti-tumor sera tested so far has shown any avidity for the E1A-0.9 Kb mRNA products. (E1A 0.6 Kb mRNA has not been observed in either hamster rat or human Ad-transformed cell lines (Green et al., 1981; Lewis and Mathews, 1981). Thus, it seems possible that the antigenic sites recognized by hamster anti-tumor sera lie within or are determined by the sequence of 46 amino acids which are unique to the products of the 1.1 Kb mRNA.

Since the unique segment of 46 amino acids in the 1.1 Kb mRNA products might alter the conformation (and thus the antigenicity) of these proteins, the immune response of tumor-bearing hamsters could be limited to producing antibodies to them if 0.9 Kb mRNA products were not made in transformed cells (even though translatable 0.9 Kb mRNA has been detected in transformed hamster cells, Green et al., 1981). To determine whether 0.9 Kb mRNA products were synthesized in transformed cells, extracts of  $^{32}\text{P}$ -labeled transformed cells were immunoprecipitated with E1A-C1 serum and then analysed by two-dimensional (2-D) polyacrylamide gel electrophoresis (Fig. 4). The 2-D profile of the immunoprecipitate of  $^{32}\text{P}$ -labeled wt infected Kb cell and

Figure 3. Specificity of various hamster anti-tumor sera for Ad5 early region 1A antigens in infected KB cells. Monolayers of KB cells were infected at an m.o.i. of 50 pfu/cell and labeled with  $^{32}\text{P}$ -orthophosphate from 7 to 9 hours p.i. Immunoprecipitates were prepared as described in Materials and Methods using anti-tumor sera which displayed some specificity for E1A products and a rabbit antiserum (E1A-C1) specific for the carboxy terminal peptide of E1A proteins. A) Normal hamster serum, B) 14b, C) 983-2, D) 972-1, E) 945-C1, F) 954-C4, G-I) different batches of 954-1, J) 954-5, K) 954-6, L) E1A-C1.

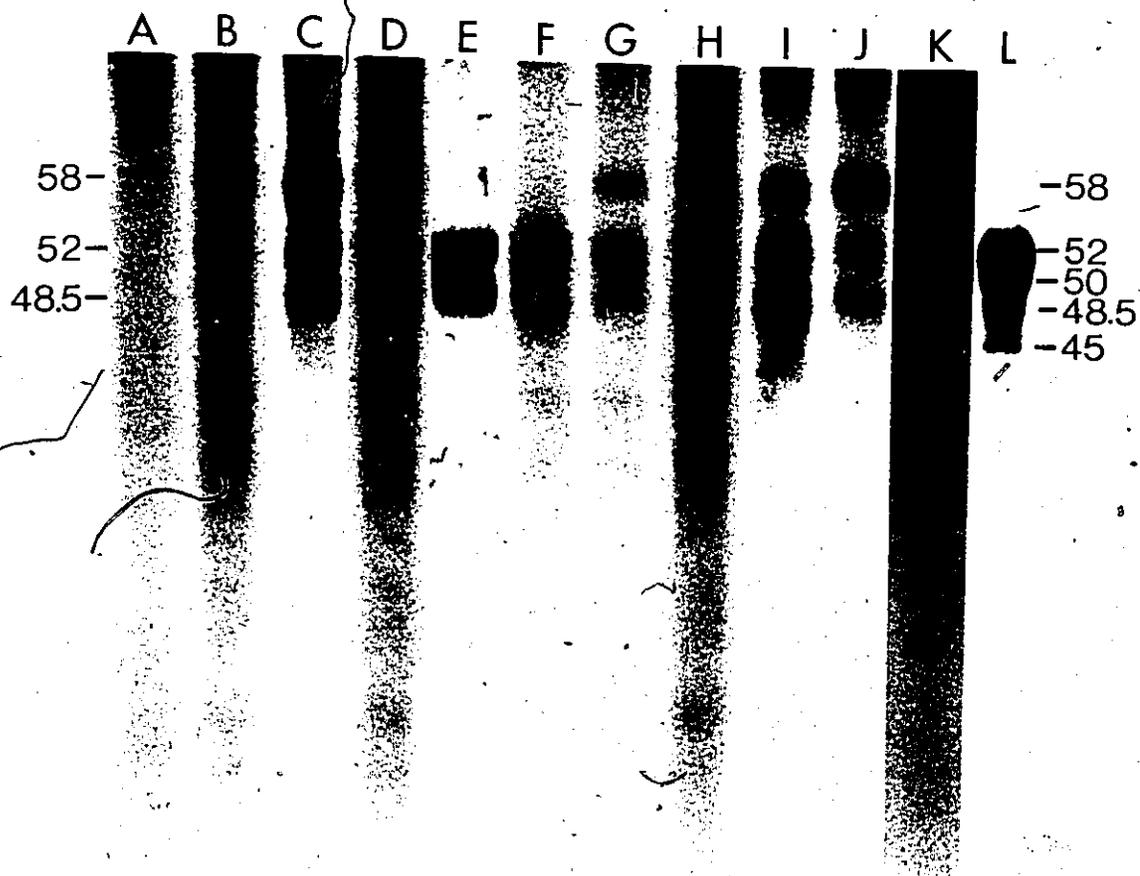


Figure 4. Two-dimensional gel separation of  $^{32}\text{P}$ -labeled E1A proteins immunoprecipitated from the 954-5 cell line with E1A-C1 serum. A)  $^{32}\text{P}$ -labeled wt-infected cells immunoprecipitated with E1A serum. B) Schematic representation of proteins detected in (A). Black spots indicate 1.1 Kb mRNA products; white spots, 0.9 Kb mRNA products. Numbers represent apparent molecular weights. C) and D) Two exposures of  $^{32}\text{P}$ -labeled E1A products from 954-5 cells.

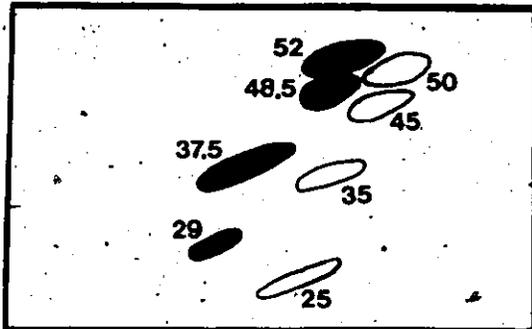
**A**



**C**



**B**



**D**



the corresponding schematic representation (Fig. 4a and 4b) illustrate for comparison the four major and four minor species and their mRNA assignments. Fig. 4c and d show different exposures of the 2-D gel containing immunoprecipitated E1A products from an extract of 954-5 cells. The pattern, which is typical of the other transformed hamster cell lines I have examined thus far, indicated that much less of the 48.5K and 45K major species was present relative to the 52K and 50K but clearly showed that the transformed cells contained both 1.1Kb mRNA products and 0.9Kb mRNA products in roughly equal proportions. The 2-D electrophoretic properties of the  $^{32}\text{P}$ -labeled E1A proteins of two lines 983-2 and 954-1 were further examined by co-electrophoresis with  $^{35}\text{S}$ -methionine labeled E1A proteins from infected KB cells. As shown in Fig. 5 the  $^{32}\text{P}$ -labeled E1A proteins from the 983-2 and 954-1 cell lines co-migrated with  $^{35}\text{S}$ -labeled E1A products made in infected cells from both 1.1 Kb and 0.9 Kb mRNAs. These results suggest that E1A products made in the hamster cells transformed by various viral DNA fragments are very similar if not identical to the products made in KB cells early after Ad5 infection. Thus the failure of anti-tumor sera to recognize 0.9 Kb mRNA products cannot be due to the absence of these products from transformed cells.

#### DISCUSSION

This chapter describes the establishment and characterization of a library of baby hamster kidney cell lines transformed by Ad5 DNA fragments comprising all (Xho I C; 0-16 mu) or only a part (Hind III G; 0-7.8 mu) of region E1 (0-11.2 mu). Transformation of hamster cells by smaller fragments (i.e. HpaI E 0-4.5 mu) has not been observed (data not shown) even under the less stringent selection conditions which have been used to isolate such transformants from cultures of primary

Figure 5. Two-dimensional gel comparison of  $^{32}\text{PO}_4$ -labeled E1A proteins from transformed cells with  $^{35}\text{S}$ -labeled E1A proteins from Ad5-infected KB cells. Aliquots of  $^{35}\text{S}$ - or  $^{32}\text{P}$ -labeled samples immunoprecipitated with E1A-C1 serum were mixed prior to analysis by two-dimensional gel electrophoresis. The dried gels exposed a double overlay of x-ray films for 8 days such that the upper film detected only the higher energy emission of the  $^{32}\text{P}$  decay while the film next to the gel was exposed by  $^{35}\text{S}$ - and  $^{32}\text{P}$ -decay. The gel was re-exposed for 18 days after 8 half-lives of  $^{32}\text{P}$  permitting detection of the  $^{35}\text{S}$ -decay in the relative absence of a  $^{32}\text{P}$  signal. For each of the cell lines examined the infected cell proteins are shown on the left, the transformed cell proteins on the right, and the combined signal is presented in the center.

**983-2**



**954-1**



baby rat kidney cells (Houwelling et al., 1980; Graham, unpublished). The Hpa I-E transformed rat cells differed from the cells transformed by larger fragments in a number of their properties (morphology, growth rate, saturation density) and have been referred to as 'partially' or 'incompletely' transformed cells. Thus, in addition to E1A specified proteins, products of E1B coded between 4.5 and 7.8 mu appear to be required for the establishment of 'fully' transformed rodent cells. The cells transformed by the Xho I C fragment, like cells transformed by larger fragments or by virus, express E1A proteins (52K, 50K, 48.5K, 45K, 37.5K, 35K, 29K, 25K) and E1B proteins (19K and 58K). Cells transformed by the Hind III G appeared to express all of these proteins with the exception of the 58K protein (Table III). Previous studies using immunoprecipitation have demonstrated that the E1B-58K antigen was not detected in rat cell lines transformed by the Ad5 Hind III G fragment (Schrier et al., 1979) in agreement with these results. Thus, it seems likely that Ad DNA fragment transformation of rat and hamster cells leads to expression of a similar if not identical spectrum of viral antigens.

The specificity of antisera from tumor bearing hamsters exhibited several interesting features. Firstly, the E1B-19K protein was weakly antigenic in cell lines which expressed the E1B-58K protein, but for the cell lines which did not express 58K protein the major antigenic specificity was most often for the 19K protein. Only two Hind III G fragment transformed lines, 954-1 and 954-5, induced an immune response to both the 58K and 19K (Table II). Since these cells contain sequences coding only for the amino portion of the 58K protein, it seems possible that they synthesize an as yet unidentified altered form of this protein, most likely a truncated or hybrid species. It is not clear why anti-954-5 sera always appears to have a

stronger avidity for 58K than does anti-954-1 sera, however this difference raises the possibility that other (and perhaps all) Hind III G fragment transformed cells express some form of 58K antigen. In addition, the efficient expression of the intact full length 58K protein may be needed for this product to dominate the immune response.

Another interesting characteristic of the immune response concerned the specificity of anti-tumor sera for E1A products. Most batches of antiserum, regardless of the cell lines used to induce the tumor, showed little avidity for E1A products, and the few examples of positive antisera were able to immunoprecipitate <sup>35</sup>S-labeled E1A proteins that resolved only as a diffuse blur in the region of 48-52K on most polyacrylamide gels (Fig. 1). The development of an antiserum (E1A-C1) directed against a synthetic peptide corresponding to the carboxy terminus of the E1A proteins allowed the identification of E1A 1.1 Kb mRNA products (52K, 48.5K, 37.5K) and 0.9 Kb mRNA products (50K, 45K, 35K) in infected cell extracts (Yee et al., 1983; Chapter VI). By using gels with better resolution in the range of 40-50K it was found that E1A-positive anti-tumor sera recognized only the products of the 1.1 Kb mRNA (Fig. 4). These results suggested that the major antigenic determinant of the E1A products lies within or is determined by the sequence of 46 amino acids unique to the products of the 1.1 Kb mRNA. This conclusion was strengthened by the analysis of the E1A proteins made in transformed cells using E1A-C1 to immunoprecipitate labeled proteins. All the major and minor forms of E1A products identified in infected cell extracts were also detectable in the transformed cells. The distribution of radioactivity amongst these products strongly favored accumulation in the 52K and 50K major products of the 1.1 Kb and 0.9 Kb messages, respectively, but the significance of this observation will require further understanding of

the functional roles of each of the species derived from the E1A messages. Nevertheless, the observation that 1.1 Kb and 0.9 Kb mRNA products are present in equivalent amounts in transformed cells does support the conclusion that the antigenicity of E1A products is strongly influenced by the stretch of 46 amino acids in the middle of the 1.1 Kb mRNA products.

Characterization of the hamster cell lines for their tumorigenicity in newborn hamsters suggested that no distinction could be drawn between cells transformed by Xho I C and Hind III G fragments with respect to oncogenic potential. Cell lines established with either fragment showed a broad range of oncogenicity, but every line tested was able to induce tumors. Previously, Jochemsen et al (1982) have suggested that the oncogenic potential of primary baby rat kidney cells transformed by Ad12 DNA fragments, analogous to the Xho I and Hind III fragments used in this study, correlated with the presence of the Ad12 E1B-60K (related to the Ad5-58K, Bos et al., 1982). Recently, Raska and Gallimore (1982) reported that this pattern of tumorigenicity for Ad12 fragment transformed cells bears an inverse relationship to the susceptibility of the cell lines to killing by syngeneic natural killer (NK) cells. Although it has been tempting to suggest some role for the Ad12 E1B-60K antigen in the resistance of transformed cells to NK activity, no comparable role may be suggested for the Ad5 58K. The differences in oncogenic potential between Ad5 and Ad12 transformed cells may be due to differences in the cell-animal systems being used in different laboratories, but it has been suggested that the differences in tumor-inducing capabilities of Ad-transformed hamster cells in hamsters is also a reflection of susceptibility to host cell-mediated immune destruction (Lewis and Cook, 1982). Our results utilizing tumor induction assays in newborn hamsters do not indicate any role for the Ad5-58K in these

tumor-rejection processes. It may be useful to characterize the tumorigenicity of Ad12-DNA fragment transformed hamster cells in hamsters to determine the contribution of E1B antigens in tumorigenicity.

Determining the role of E1 proteins in the tumorigenicity of Ad-transformed cells must include consideration of the potential effects of truncated or chimeric products (eg. Hind III G' fragment transformed cell lines) and the additional minor antigens (eg. 16.5K and 15K). Furthermore, recent evidence has suggested that a leftward reading 20S mRNA may specify a polypeptide from open reading frames URF-10 (5.5-6.3 mu) on the 1-strand of early region 1 (Katze et al., 1982; Gingeras et al., 1982). The contribution of this or other 1-strand products towards oncogenic transformation remains to be explored.

CHAPTER III

Transformation of Rodent Cells by DNA  
Extracted from  
Transformation-defective Adenovirus Mutants

Early region I (EI) of adenovirus 5 (Ad5), located within the left 12% of the genome, contains the viral genes necessary and sufficient for transformation of cultured rodent cells (Gallimore et al., 1974; Graham et al., 1974). Host range (hr) mutations in this region have been isolated (Harrison et al., 1978) by screening mutants for differential plaquing efficiency on HeLa and 293, a transformed human embryonic kidney cell line which contains the left 12.5% of the Ad5 genome and expresses viral mRNA from EI (Aiello et al., 1979; Graham et al., 1977). These hr mutants define two complementation groups (I and II) (Harrison et al., 1978) which map in the non-overlapping transcriptional units E1A (1.5 - 4.5) and E1B (4.5 - 11.0) respectively (Berk and Sharp, 1978; Bos et al., 1982; Frost and Williams, 1978; Galos et al., 1980). The Group I complementation class of Ad5 hr mutants are phenotypically deficient in viral DNA and late protein synthesis (Lassam et al., 1978). The mutations appear to affect one or more gene products which stimulate synthesis of viral mRNA from other early regions, including E1B, and thus this region may not be directly involved in DNA replication or control of late gene expression (Berk et al., 1979; Persson et al., 1981). Group I mutants are defective in the transformation of most rodent cells but will induce a semi-abortive or abnormal transformation of primary rat kidney cells (Graham et al., 1978; Ruben et al., 1982). In contrast the hr Group II mutants are not defective in viral DNA synthesis or late gene expression (Lassam et al., 1978), but are defective in the synthesis of the 58K major tumor antigen as determined by immunoprecipitation and polyacrylamide gel electrophoresis (Lassam et al., 1979), and our unpublished results). The hr mutations, most of which were generated by chemical mutagenesis (Harrison et al., 1978) and probably represent single base changes, have been mapped to the sequences of the E1B 22S message (Galos et al.,

1980) which are spliced out of the E1B 13S mRNA. The 13S and 22S messages both direct the synthesis of the 19K major tumor, but the 22S message also encodes the 58K antigen by utilizing an internal translation initiation site for a different reading frame which partially overlaps the coding sequences for the 19K antigen (Bos et al., 1982; Halbert et al., 1979; Perricaudet et al., 1980; Ross et al., 1980; Schrier et al., 1979; van Ormondt et al., 1980). For Fig 7, the one mutant which has been studied by S1 nuclease gel analysis, the pattern of early cytoplasmic RNA expressed from region E1B in non-permissive cells is indistinguishable from that of wild-type (wt) (Berk et al., 1979). Thus, the reason for the failure of Group II mutants to synthesize detectable 58K protein is not immediately clear. It remains possible that during Group II mutant infections a form of the 58K protein or a truncated version of this antigen is synthesized which does not react with the battery of sera we have used to detect it. Another possibility is that this antigen is rapidly degraded in Group II mutant infected non-permissive cells. In any case, defective 58K synthesis together with the failure of the Group II mutants to transform any of the cell types tested so far (Graham et al., 1978) suggest a role for the 58K antigen in adenovirus induced transformation.

In contrast to these results with the group II mutant virions, studies on the biological activity of purified viral DNA fragments have shown that only the left hand 8% (HindIII G fragment) of the Ad5 genome is needed to transform rat kidney cells (Graham et al., 1974). These fragment transformed cells do not synthesize an immunoprecipitable 58K antigen (Schrier et al., 1979 and our unpublished results) but are otherwise similar in growth and morphology to cells transformed by larger fragments or by wt virus. In

particular Hind III G<sup>v</sup> fragment transformed hamster cells, like hamster cells transformed by larger DNA fragments, are capable of inducing tumors following injection into newborn hamsters ( Chapter II ). These results would suggest that the sequences which code for the carboxyl portion of the 58K antigen are not necessary for transformation in vitro in apparent contradiction to the conclusions derived from studies with Group II hr mutants.

To resolve this apparent paradox we have postulated that initiation of transformation by viral DNA proceeds through a mechanism slightly different from that operating during virion-mediated transformation (Rowe et al., 1982). Evidently the latter process requires the 58K function, whereas DNA mediated transformation is independent of 58K. An obvious prediction of this model is that DNA extracted from Group II mutant virions should be as efficient in transformation as DNA derived from wt virions. The results shown in Fig. 1 indicate that this is indeed the case. Following transfection of baby rat kidney cells with DNA from wt or Group II mutants hr 6 or hr 50 the frequency of transformation increases in direct proportion to the amount of DNA applied to the cells and DNA from both hr 6 and hr 50 can transform rat cells with the same efficiency as wt DNA. Thus the block to transformation by Group II mutant virions disappears when transformation is assayed with purified DNA. Group II mutant DNA was also capable of converting primary baby hamster kidney cells to morphologically fully transformed cells, a finding which made it possible to study the oncogenicity of Group II mutant transformed hamster cells in newborn hamsters. Table 1 shows the tumorigenicity of six lines measured in terms of the fraction of positive animals and the time elapsed between injection and appearance of palpable

Figure 1. Transformation of primary baby rat kidney (BRK) cells by Ad5 DNA purified from wt, hr 6, and hr 50 virions. BRK cells in 60 mm petri dishes were transformed with DNA using the calcium technique (Graham and van der Eb, 1973a,b) with modifications (Graham et al., 1980). Specifically, viral DNA which had been digested with restriction endonuclease XhoI to eliminate infectivity was mixed with high molecular weight carrier DNA (rat embryo DNA) at 10 µg/ml in 250 mM CaCl<sub>2</sub>. The DNA in CaCl<sub>2</sub> was then added slowly to 2x concentrate Hapes buffered saline (HeB9, ref Graham and van der Eb, 1973a) with bubbling (Wigler et al., 1979). After 30 min. the DNA-calcium phosphate co-precipitate was added to BRK cell cultures 0.5 ml suspension per culture dish containing 5 ml medium. After 20 hrs. incubation at 37°C the medium was changed (α MEM plus 10% fetal bovine serum) and the cells were incubated a further two days at which time the medium was changed to selective medium (Jokliks modified MEM plus 5% horse serum) as described previously (Graham and van der Eb, 1973b). Negative controls (cultures treated with carrier DNA and untreated cultures) gave no colonies. The results are expressed as colonies per dish after 12 days incubation in selective medium.

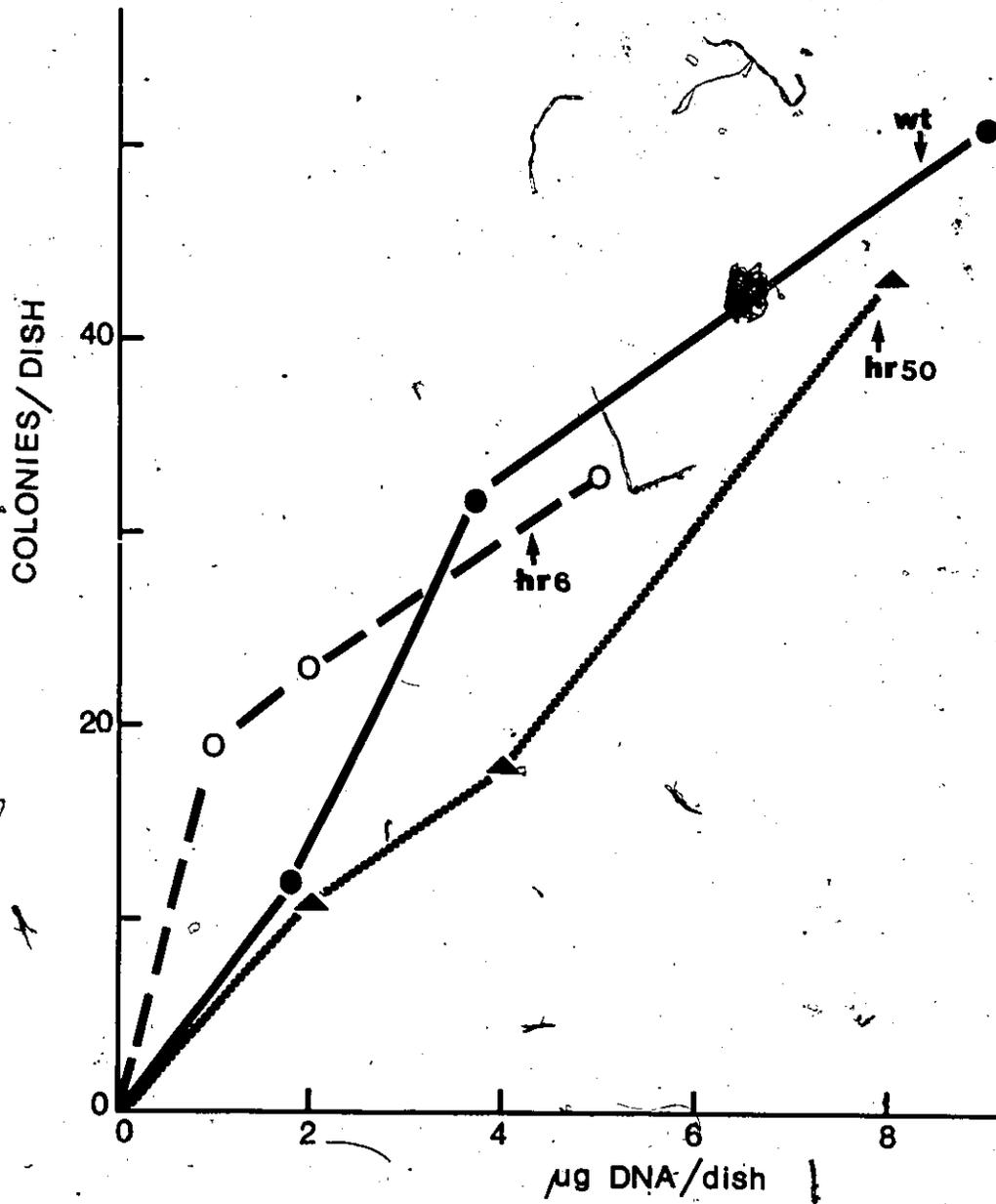


Table 1

Tumorigenicity of hr transformed hamster cells

Cell line	Transformed by:	Tumorigenicity <sup>a</sup>	
		Fraction positive	Latent period
1019-C1	hr 6 DNA	1/19	100 days
1019-1	hf 6 DNA	4/20	25 days
1019-2	hr 6 DNA	3/10	110 days
1019-1T	(derived from tumor induced by line 1019-1)	7/11	16 days
1019-C3	hr 50 DNA	4/24	90 days
1019-3	hr 50 DNA	16/20	16 days

- Compare to wt transformed cells

14b	Ad5 ts 14, virus <sup>b</sup>	20/20	12 days
972-1	Ad5, XhoI C DNA	2/9	40 days
983-2	Ad5, XhoI C DNA	9/12	27 days
268-C3	Ad5, sheared DNA	0/13	-
983-4	Ad5, XhoI C DNA	9/12	27 days
297-	Ad5, sheared DNA	13/22	25 days

<sup>a</sup>  $5 \times 10^6$  cells injected s.c. into newborn hamsters.

<sup>b</sup> Ref. Williams, 1973.

tumors. Following injection of some lines only a few of the animals produced tumors and then only after long latent periods. One cell line, 1019-3 and another line, 1019-1T derived from a 1019-1 tumor appeared to be relatively tumorigenic as most of the animals injected bore tumors in 3 weeks or less, though the tumors were smaller and grew more slowly than the tumors of animals injected with wt transformed cells. Thus, although the tumorigenicity of some Group II mutant transformed cells was low, all lines tested were capable of inducing tumors and since not all wt transformed lines are tumorigenic (cf 268-C3) Group II mutant transformed cells may possess the same range of oncogenic potential as wt transformed hamster cell lines. Though it is difficult to draw firm conclusions from in vivo studies involving a limited number of animals, these results suggest a role for the 58K coding sequences chiefly in initiation of transformation by viruses (but not by viral DNA). Thereafter expression of 58K may not play any major role in determining the transformed phenotype and in particular may not be needed for tumorigenicity of cells transformed in vitro by DNA.

Characterization of serum from tumor-bearing hamsters by immunoprecipitation of Ad5 antigens from S<sup>35</sup>-labelled KB cells early after wt infection (Table 2) revealed that none of the sera raised against hr mutant transformed cells contained antibodies to the 58K antigen. Most of the sera reacted with the 19K E1<sub>0</sub> protein or the 14K E4 protein (Downey et al., 1983) and antisera from 1019-3 injected hamsters appeared to be specific for the 44K E1A protein. The possibility that sera from Group II mutant tumor-bearing hamsters contain antibodies to a 58K-like antigen but do not react with the wt-58K seems remote. When these sera were used to immunoprecipitate early antigens from hr 6-infected cells no novel antigen was observed upon

Table 2

## Specificity of hamster antitumor sera

Cell line	Transformed by:	Early Antigen				
		58	44	29	19	14 <sup>a</sup>
1019-1	hr 6				++	+
1019-1T	hr 6		+		++	+
1019-71	hr 6					
1019-2	hr 6					++
1019-3	hr 50		++			
1019-C3	hr 50		++			
983-2	wt	++		+		

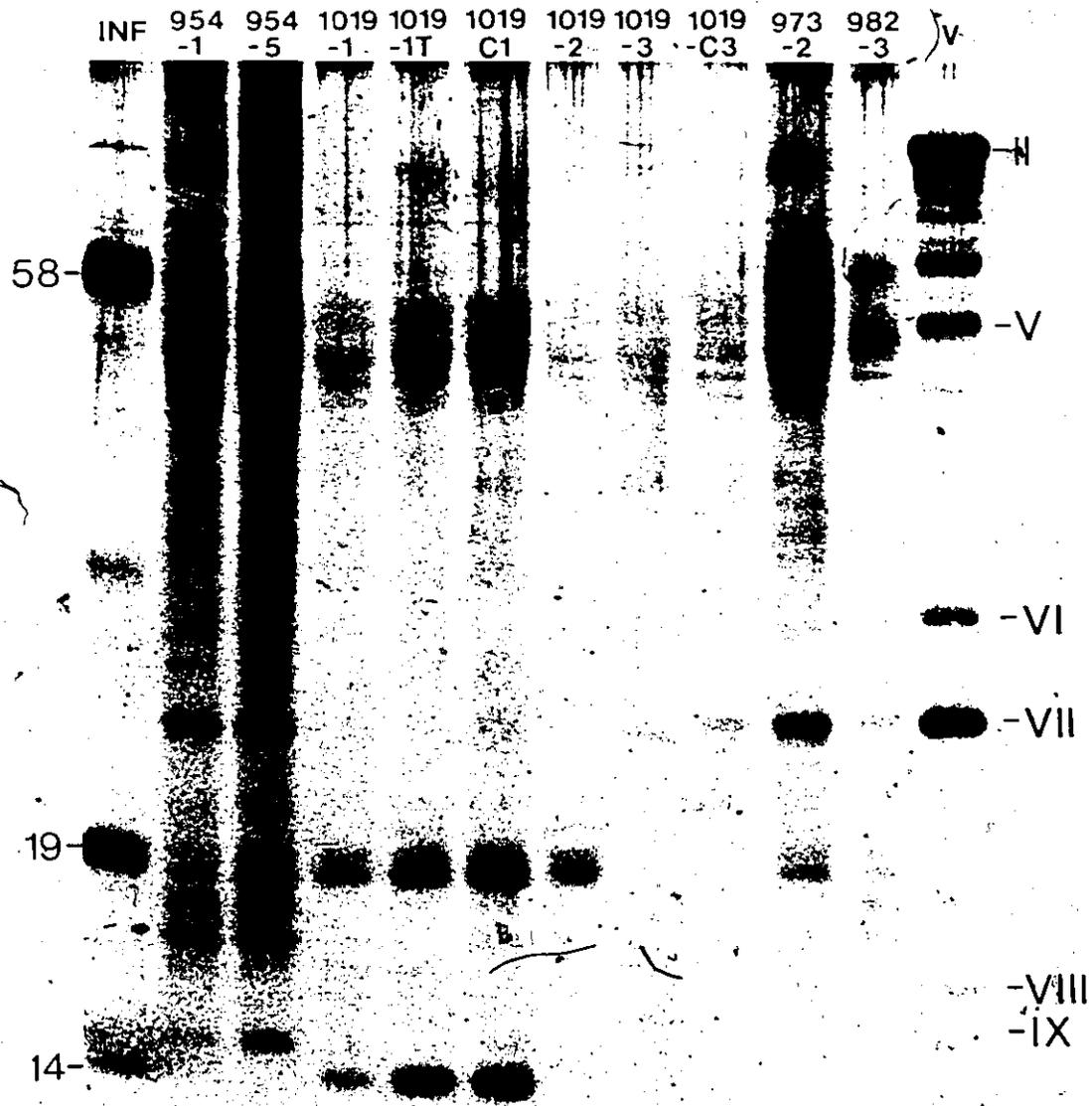
<sup>a</sup> An E4 encoded protein (8).

Ad5 early antigens present in  $10^7$  KB cells labelled with  $50 \mu\text{G S}^{35}$  methionine from 8-10 hours post-infection were detected with 30  $\mu\text{l}$  of anti-tumor serum using the Sepharose-protein A immunoabsorption technique (Schaffhausen et al., 1978). Major avidity is indicated by ++ symbol.

polyacrylamide gel electrophoresis and autoradiography (data not shown) suggesting that at least this mutant does not induce the synthesis of a stable truncated form of 58K.

The absence of a 58K antigen from Group II mutant infected cells (Lassam et al., 1979) and the absence of antibodies to the 58K antigen in Group II mutant transformed cell anti-tumor sera suggests that the phenotype of the Group II mutants in infected cells (lack of 58K protein) was likely expressed in the transformed hamster cells. To test this, and to ensure that the cells had indeed been transformed by Group II mutants and not by wt revertants, six mutant transformed lines were assayed for the presence of tumor antigens by immunoprecipitation with anti-tumor serum capable of reacting with 58K and other E1 antigens. The autoradiogram in fig. 2 shows that cells transformed by hr DNA fragments or by fragments of wt DNA which exclude the 3' part of E1B (954-1, 945-C1) fail to synthesize the E1B 58K antigen which is readily detectable in cells transformed by wt DNA fragments containing all of E1 (983-2, 972-3). Hr 6 transformed hamster cell lines expressed the E1B-19K antigen which has been detected in all cell lines transformed by wt-virus or DNA fragments and infected cells. (Fig. 2 and our unpublished results). On high resolution polyacrylamide gels the 19K band has been shown to be composed of two proteins which share a common amino acid sequence ( Chapter IV ). Somewhat surprisingly the hr 50 transformed cell lines 1019-3 and 1019-C3 produced no detectable quantities of either the 58K or 19K antigen, consistent with the specificity of tumor sera obtained from hamsters injected with these transformed cells. The present study does not rule out the possibility that E1B antigens are made and then rapidly degraded in the Group II mutant transformed cells.

Figure 2. Autoradiograph of a 14% polyacrylamide gel (Laemmli, 1970) showing the separation of Ad5 early viral antigens from infected and transformed cells. To detect antigens early during lytic infection approx.  $10^7$  spinner grown KB cells were infected with 50 pfu/cell Ad5 and labeled with 100  $\mu$ C  $S^{35}$ -methionine at 10-12 hr post infection. For transformed cells approximately  $10^8$  cells in Petri dishes were labeled for 8 hr with 150  $\mu$  Ci of  $S^{35}$ -methionine in 3 ml of methionine deficient Eagles medium. The immunoprecipitation procedure using the protein A-sepharose technique (Schaffhausen et al., 1979) with modifications has been described (Lassom et al., 1979). A number of tumor antisera were selected and pooled for these experiments to provide an immunoreagent which would react with all the viral early antigens of region 4. The molecular weights of the early antigens detected in the infected cells (INF) appear on the left and structural proteins of the virion (MV) are indicated by Roman numerals on the right. 954-1 and 954-5 are both hamster cell lines transformed by the Hind III G (0-7.8%) fragment of Ad5 DNA, 1019-1 1019-C1 and 1019-2 are hamster lines transformed with purified hr 6 DNA, and 1019-IT is a line derived from a 1019-1 induced tumor. Lines 1019-3 and 1019-C3 were transformed by hr 50 DNA. The lines 972-3 and 983-2 were transformed with the XhoI C (0-15%) fragment of wt DNA.



Although the 58K has never been detected in hr 6 or hr 50 infected KB cells even with very short labeling times, both these mutants induce the synthesis of an immunoprecipitable 19K in non-permissive infections. (Lassam et al., 1979, and our unpublished results). Thus hr 50 transformed cell lines 1019-3 and 1019-C3 exhibited the unique phenotype of being fully transformed and relatively tumorigenic in the absence of any detectable E1B antigens.

These results are not in agreement with the findings of Jockensen et al. (Jockensen et al., 1982) which show that Ad12 Hind III G (0-7.2%) fragment transformed rat cells were unable to induce tumors in nude mice. It is possible that the different conclusions are due to inherent differences in the oncogenicity testing systems employed in the two studies. Bernards et al. (4) also examined the oncogenicity in nude mice of rat cells transformed by Ad5/Ad12 hybrid E1 plasmids and have suggested that the oncogenicity of Ad transformed cells is determined by products specified by region E1B. Although a role for the E1B proteins in determining the degree of tumorigenicity cannot be ruled out, these studies indicate that the 58K E1B antigen is not absolutely required for oncogenic transformation and suggest that even cells lacking the 19K protein can be oncogenic.

There are superficial similarities between the results I have obtained here and biochemical and genetic studies of polyoma DNA transformation. Recent evidence suggests that polyoma DNA fragments encoding only the small and middle T antigens are sufficient for transformation in vitro (Hassell et al., 1980; Novak et al., 1980) and for tumorigenicity (Moore et al., 1980) although it has been established that tsA mutants of polyoma virus which affect only large T are greatly impaired in their ability to transform rat

and hamster cells at non permissive temperatures (Di Mayorca et al., 1969; Eckert, 1975; Fried, 1965). Large T antigen appears to promote integration of viral DNA in tandem arrays, a mode of insertion which increases the efficiency of transformation (Della Valle et al., 1981). The formation of tandem head to tail integration is suggested to occur through aberrant replication intermediates present in infected cells (Della Valle et al., 1981) and since large T is required to initiate DNA replication (Eckert, 1975), mutants of large T which fail to synthesize DNA would ultimately be transformation deficient. This particular model does not appear to be applicable to adenovirus 58K involvement in cell transformation since the 58K does not appear to be required for viral DNA replication. Nevertheless, a role for the 58K antigen in the initial step of integration cannot be ruled out and indeed such a role seems likely. The Group II mutant hr 6 supports adeno-associated virus DNA replication but is unable to rescue the integrated form of the AAV viral genome in human Detroit 6 cells latently infected with AAV (Ostrove and Berns, 1980). This finding suggests that the mechanisms by which viral information is integrated into or excised from the cellular genome during adenovirus infection may be influenced by viral gene products from early region 1B, a model which is consistent with the results presented here.

CHAPTER IV

Intracellular Localization  
of  
Adenovirus Type 5 Tumor Antigens  
in Productively Infected Cells

## INTRODUCTION

The early phase of lytic infection with adenoviruses involves a complex co-ordinated expression of at least five early regions of the viral genome prior to viral DNA replication (Flint, 1977; Galos et al., 1979). Of greatest interest have been the products of early region 1 (E1:1.5-11.2 map units) which contains the viral genes necessary and sufficient for transformation of cultured rodent cells (Gallimore et al., 1974; Graham et al., 1974). The six mRNAs complementary to this region define two distinct non-overlapping transcription units: E1A: 1.5-4.4 map units and E1B, 4.5-11.2 map units, plus a third transcription unit starting within E1B (Berk and Sharp, 1978; Chow et al., 1979). The three E1A mRNA species of 1.1, 0.9 and 0.6 Kilobases (Kb) direct the synthesis of seven proteins detectable by a combination of in vivo and in vitro techniques. The in vivo translation of products of the 1.1 and 0.9 Kb mRNAs form a cluster of at least six species on 2-dimensional polyacrylamide gels which range in molecular weight from 58K to 42K (Halbert et al., 1979; Esche et al., 1980; Smart et al., 1981) and the product of the 0.6 Kb message is a 28K protein which has only been observed in vitro (Spector et al., 1980). The E1B 1.0 and 2.2 Kb mRNAs both direct the synthesis of the 19K major tumor antigen, but the 2.2 Kb message also encodes the 58K major tumor antigen by utilizing an internal translation initiation site for a different reading frame which partially overlaps the coding sequences for the 19K antigen (Bos et al., 1981).

Further understanding of the role of E1A coded antigens in lytic infection and in the initiation and maintenance of the transformed state may be gained by an analysis of the intracellular sites at which these products

act. In an earlier study, Lenk et al. (1980) reported that several proteins expressed early after adenovirus infection may be associated with the cytoskeleton but did not show that these proteins were in fact viral tumor antigens and drew no distinction between viral-coded and viral-induced proteins. Harter and Lewis (1978) fractionated what were later shown to be E1A proteins (Smart et al., 1981) into cytoplasmic and nucleoplasmic compartments but their studies required the use of drug enhancement of early protein synthesis and consequently the localizations may not have been an accurate reflection of normal infections. More recently, the E1B-15K protein of Ad2 has been found to be associated with the membrane fraction of lytically infected cells (Persson et al., 1982), an important finding since studies on Ad5 transformation with fragments of the viral genome have suggested that the E1B-19K protein (identical to the 15K protein of Ad2) may be responsible for some of the properties of transformed cells (Houwelling et al., 1980; Schrier et al., 1979). In the present study I examine the intracellular localization of the Ad5-E1 tumor antigens and an E4 protein during the early stages of productive infection.

## MATERIALS AND METHODS

### Cells and Virus

The KB cells and Ad5 transformed cells used in this study are as described in the Materials and Methods section of Chapter II.

### Infection and Labeling of Cells

Confluent monolayers of KB cells in 150 mm culture dishes were infected with 35 plaque forming units of Ad5 wt per cell in 2 ml of phosphate buffered saline (PBS). After 30 minutes of adsorption at 37°C, the cultures received 20 ml of alpha-MEM supplemented with 5% serum. At the appropriate time post-infection, the cells were rinsed once with PBS, and labeled with 100 uCi of <sup>35</sup>S-methionine in 4 ml of methionine-free medium. After the labeling period (usually 60 min), the cells were washed once with PBS and fractionated as described below.

### Immunoprecipitation and Polyacrylamide Gel Electrophoresis

These techniques were performed as described in the Materials and Methods section of Chapter II.

### Cell Fractionation

To prepare whole cell extracts labeled cells were washed once with PBS

and lysed in 1 ml of radioimmunoprecipitation buffer (RIPA: 50 mM Tris pH 7.5, 1.0% Triton X100, 1.0% sodium deoxycholate, 150 mM NaCl, 0.2% SDS) per  $10^7$  cells. After 20 min at  $4^{\circ}\text{C}$ , the cell lysate was sonicated (Biosonic III setting 30, 30 sec) and centrifuged at  $10,000\times g$  for 10 min.

Cytoplasmic and nuclear fractions were prepared by resuspending  $10^7$  cells in 1 ml of 100 mM Tris pH 7.1, 0.25M sucrose, 25 mM NaCl, 5 mM MgCl, and then adding Nonidet P40 and sodium deoxycholate to final concentrations of 1% w/v. After incubating for 20 min at  $4^{\circ}\text{C}$  the cells were disrupted by 10 passes in a tight pestle Dounce homogenizer. Nuclei were removed by centrifugation at  $10,000\times g$  for 10 min, and the supernatant subsequently referred to as cytoplasmic extract was collected. The nuclei were resuspended in 50 mM Tris pH 7.1 ( $1\text{ ml}/10^7$ ), incubated for 20 min on ice, and disrupted by brief sonication (Biosonic III, setting 30). After centrifugation at  $10,000\times g$  for 10 min the nucleoplasm supernatant was removed and a one-tenth volume of 10x RIPA was added. The chromatin pellet was resuspended in 1 ml of RIPA buffer per  $10^7$  cell equivalents and sonicated for 3 min. Insoluble material was removed by centrifugation at  $10,000\times g$  for 10 min.

The membrane fraction was prepared by a modification of the procedure previously described by Warren (1974). Briefly,  $10^7$  cells were suspended in 1 ml of a hypotonic buffer (10 mM Tris pH 7.1, 0.5 mM  $\text{ZnCl}_2$ ) and incubated for 20 min at  $4^{\circ}\text{C}$ . Cells were disrupted by Dounce homogenization (10-20 passes) and centrifuged at  $3000\times g$  for 10 min. To the crude membrane supernatant, an appropriate volume of 5M NaCl was added to give a final concentration of 1M NaCl. After incubating for 4 hours at  $0^{\circ}\text{C}$  the membrane preparation was centrifuged at  $100,000\times g$  at  $4^{\circ}\text{C}$  for one hour. The membrane pellet was resus-

ended in 1 ml of RIPA buffer and sonicated for 3 min. The cytosol supernatant was dialyzed for 16 hours against RIPA buffer prior to immunoprecipitation.

Cytoskeletal fractions were prepared according to the method of Gupta et al. (1982). Cell monolayers were rinsed twice with PBS and lysed by addition of 1 ml of a microtubule stabilizing buffer (50% glycerol, 5% dimethyl sulfoxide, 0.5 mM GTP, 0.5 mM GTP, 0.5 mM MgCl<sub>2</sub>, 0.5 mM ethylene glycol bis (-aminoethylether)-N,N,N',N-tetraacetic acid, 0.1% Triton X100, 1 mM phenylmethylsulfonyl fluoride and 10 mM potassium phosphate buffer, pH 7.0) per 10<sup>7</sup> cells. After a 5 min incubation at room temperature the cells were harvested into centrifuge tubes by scraping, vortexed vigorously, and centrifuged at 100,000xg for 1 hour at 25°C. The microtubule rich pellet was resuspended in a microtubule-dissociation buffer (10 mM Tris pH 6.8, 0.1% Triton X100) at 4°C. Microtubule-associated proteins, released by the cold extraction, were recovered in the supernatant of a second centrifugation at 100,000xg for 1 hour at 4°C.

## RESULTS

Polypeptides Immunoprecipitated from Cytoplasmic and Nuclear Extracts of Infected Cells

Whole cell, cytoplasmic, nucleoplasmic, membrane, cytoskeletal, and cytosolic fractions were routinely prepared as described in Materials and Methods. Analyses of the protein yield for each fraction by these procedures (Table I) indicated that recovery of protein from Ad5 infected cells was the same as from mock-infected cells and within the range obtained by other workers (Fleischer and Kervina, 1974). The SDS-PAGE analysis of cellular proteins recovered from the cytoskeletal fractions showed enrichment of cytoplasmic proteins that are components of the intracellular network of filaments, particularly actin, tubulin, and alpha-actinin (data not shown). These constituents were also recovered in the cytosolic fraction because the purification procedure used to prepare cytosolic extracts does not provide for the preservation of cytoskeletal filaments.

Localization of viral proteins during the early phase of adenovirus lytic infection requires techniques which will allow the detection of viral products within the considerable background of host cellular proteins. In vivo labeling with  $^{35}\text{S}$ -methionine followed by immunoprecipitation with anti-tumor sera has been used by a number of groups to isolate viral gene products from infected and transformed cells (Persson et al., 1981; Green et al., 1979b; Johansson et al., 1978; Ross et al., 1980a). In other studies I have found that sera derived from tumor-bearing hamsters have specificities which in general are characteristic of the cell line used to induce tumors

TABLE I: Protein Content in Cell Fractionation

Cell Fraction	% of Whole Cell Protein	
	Ad5-Infected	Mock-Infected
Cytoplasm	42.0	49.8
Nucleoplasm	26.5	25.8
Chromatin	11.2	9.2
Cytoskeleton	14.7	14.8
Cytosol	15.2	16.2
Membranes	5.9	6.6

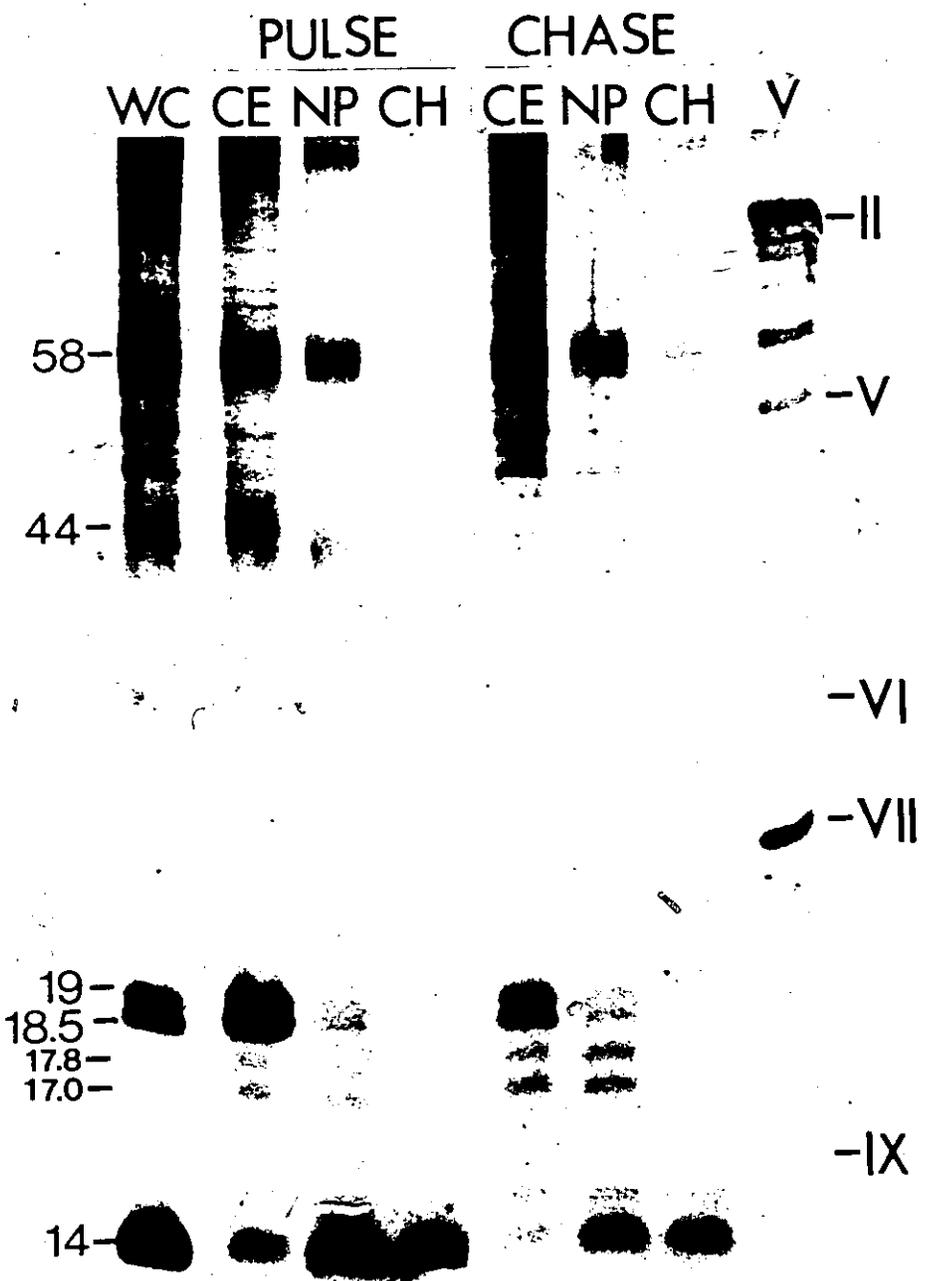
and to some extent reflect the expression of viral proteins in the transformed cells ( Chapter II ). Fig. 1 shows that tumor serum made in response to 954-C4 cells was directed principally against the E1B-19K antigen, sera from animals bearing tumors induced by 1019-3 cells which have been found not to express the E1B antigens (Chapter III ) were always limited to a response against the 44K antigen. The 945-C1 serum was almost monospecific for the E4-14K, though a low avidity for the E1A-44K and E1B-19K antigens was also observed. Antisera derived from hamsters with tumors induced by 983-3 were primarily active against the E1B-58K antigen but some batches also appeared to react weakly with a number of other antigens. A polyvalent antiserum made by pooling these anti-tumor sera was used to detect labeled viral proteins in the various fractions prepared from infected cells.

KB cells were infected with Ad5 and pulse labeled for one hour at 7 hours p.i. After the labeling period, some of the cells were immediately fractionated into cytoplasmic and nucleoplasmic preparations, and the other portion of the cells was washed once with PBS and incubated for an additional four hours in medium containing excess cold methionine to allow the labeled viral products to undergo any potential post-translational modifications that might influence their final intracellular deposition. In immunoprecipitates of whole cell extracts, five major immunoprecipitable proteins of 58K, 44K, 19K, 18.5K and 14K were reproducibly detected on polyacrylamide gels (Fig. 2). Of these, the 14K (a major early protein encoded in early region 4; Downey et al., 1983) was detected principally in the nuclear fractions isolated from pulse-labeled cells (Fig. 2). This observation is consistent with previous reports of a major early protein of similar molecular weight

Figure 1. Autoradiogram of polyacrylamide gel separation of antigens immunoprecipitated from infected KB cells by various anti-tumor sera. Whole cell extracts prepared from infected cells labeled with <sup>35</sup>S-methionine from 7 to 9 hours p.i. were immunoprecipitated with antisera raised in hamsters bearing tumours induced with the following transformed cell lines; A) 983-3, B) 1019-3, C) 954-6, D) 945-C1. Immunoprecipitation of infected cell extracts with normal hamster serum is presented in lane E.



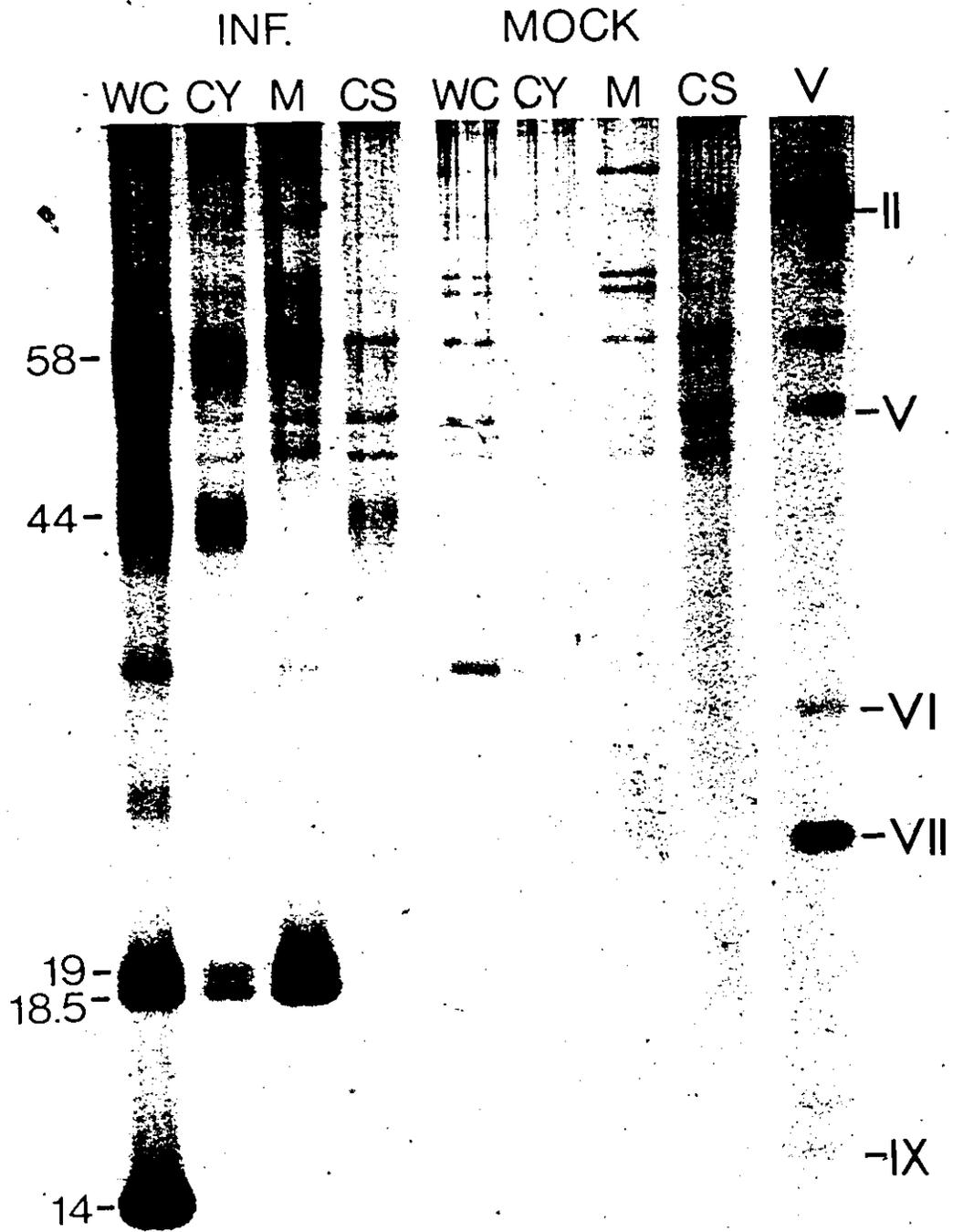
Figure 2. Autoradiogram of a 15% polyacrylamide gel analysis of immunoprecipitates from a pulse-chase cell fractionation experiment. Ad5 infected KB cells were pulse-labeled for one hour at 7 hours p.i. Half of the labeled cells were then incubated in a chase medium containing excess unlabeled methionine for four hours. Fractions were prepared as described in Materials and Methods and symbols are as follows: (V) virus marker, (WC) whole cell, (CE) cytoplasmic extract, (NP) nucleoplasm, (CH) chromatin.



being enriched in cell fractions containing the nuclear matrix (Chin and Maizel, 1977; Lenk et al., 1980). This was the only antigen examined in this study which showed a significant association with a fraction composed almost exclusively of histone proteins. Although a greater than 2-fold reduction in the intensity of the 14K band was observed following the chase period, the relative proportions of 14K in each fraction did not change significantly.

In polyacrylamide gels of immunoprecipitates from infected cell extracts the diffuse band which migrates in the region of 44K is thought to be composed of a number of closely related products encoded in region E1A (Harter and Lewis, 1978; Ross et al., 1980; Smart et al., 1981). These products were detected in nearly equal amounts in the cytoplasmic and nucleoplasmic extracts, but products partitioning in cytoplasmic extracts showed a qualitatively different migration pattern from those found in nucleoplasmic fractions (Fig. 2). The significance of this is not clear and the composition of the E1A products detected in the cytoplasm and nucleus of infected cells is currently under investigation. In cells chased for 4 hours following labeling, the E1A products were absent from both nuclear and cytoplasmic fractions (Fig. 2) in agreement with my previous studies which showed these products to be extremely labile (Rowe et al., submitted). Within the cytoplasm the 44K band was detected in both the cytosol and the cytoskeletal fractions but was not recovered in significant quantities from the membrane preparation (Fig. 3). It appeared to migrate slightly faster than the band from whole cell extracts but as with the 44K band observed in nucleoplasm preparations it was not possible to determine whether all or only a subset of the related proteins comprising this band were present in these fractions.

Figure 3. Autoradiogram of 15% polyacrylamide gel analysis of the labeled proteins detected in immunoprecipitates of infected and mock-infected whole cell (WC), cytosol (CY), membrane (M) and cytoskeletal (CS) extracts made at 8 hours p.i. Aliquots of the immunoprecipitated samples derived from an equivalent number of cells were analysed.



The EIB 58K antigen was present in approximately equal amounts in the cytoplasmic and nucleoplasmic fractions of pulse-labeled cells, but only a small amount was associated with chromatin (Fig. 2). During the chase period there was a slight accumulation of 58K antigen in the chromatin fraction.

Very little of the 58K protein was recovered in any of the cytoplasmic subfractions, a small amount being detected in the cytosol, and little or none in either membrane or cytoskeletal fractions of infected cells (Fig. 3). Since the preparation of cytoplasmic subfractions yields contaminated 'nuclear' pellets and most of the cytoplasmic 58K appears to be associated with these nuclei, it suggests that most of the 58K antigen synthesized in a one hour pulse was localized in and around the nucleus of infected cells.

Two antigens, 18.5K and 19K, present in nearly equal amounts in whole cell extracts were both detected almost exclusively associated with cytoplasmic extracts (Fig. 2). Only a small amount of either of these products was recovered from the nucleoplasm and neither product was associated with chromatin. In pulse-chase experiments both the 18.5K and 19K were reduced more than 2-fold in intensity after the chase period and two slightly faster migrating bands, designated 17K and 17.8K, barely detectable in one hour pulses, increased in intensity. These bands were of approximately equal intensity, but unlike the 18.5-19K doublet were associated with the nucleoplasm as well as the cytoplasmic extract. In addition, about 75% of the label incorporated into the 18.5 and 19K during the one hour pulse could be accounted for when the 17K and 17.8K bands were

included in the estimate of label remaining after the chase period (data not shown). Taken together these results and the observation that only anti-tumor sera which reacted with the 18.5 and 19K were capable of immunoprecipitating the 17.0K and 17.8K (data not shown) suggested that perhaps a precursor-product relationship might exist amongst these products.

The most striking observation was that almost all of the 18.5 and 19K antigens were recovered from the membrane fraction (Fig. 3). Only a comparatively small amount of each of these proteins was present in the cytosol and no significant association with the cytoskeleton was observed. In a recent study, Persson et al. (1982) reported the purification of the Ad2 E1B-15K protein (equivalent to the 19K protein of Ad5) from membrane preparations similar to the ones discussed above. However, these workers observed only one band in polyacrylamide gel analyses of the purified membrane preparations whereas we have identified at least two bands of equal intensity (Fig. 3). A possible relationship between the 18.5 and 19K proteins which co-purified in membrane preparations was therefore explored further.

#### Synthesis and Processing of the E1B-19K Antigen

Many workers have reported the ability of anti-tumor sera to immunoprecipitate a 19K-E1 protein from adenovirus infected and transformed cells (Gilead et al., 1976; Green et al., 1979a; Persson et al., 1982; Ross et al., 1980a; Schrier et al., 1979). By using 15% SDS-polyacrylamide gels which provide greater resolution of low molecular weight species, we have found that it was possible to resolve two separate bands in the region of 19K

which were not observed on gels of lower acrylamide concentration. Neither of these products was immunoprecipitated from uninfected cells and neither product was observed in immunoprecipitates with non-immune serum (Fig. 3 and unpublished observations). Since both species were observed in all the transformed cell lines we have examined (data not shown) including cell lines transformed by purified Ad5 Hind III G fragment (0-8%), it is likely that both polypeptides are encoded to the left of 8.0 map units.

To further examine the relationship between the 18.5K and 19K proteins extracts of Ad5 infected KB cells pulse-labeled for two hours from 6 to 8 hours p.i. were immunoprecipitated and separated by preparative scale gel electrophoresis. The 18.5K band was cut from the dried gel using the autoradiogram as a template. After the gel was re-exposed to ensure the complete isolation of 18.5K from 19K, the second band was cut out and both proteins were subjected to partial proteolysis peptide mapping. In the autoradiogram shown in Fig. 4, all partial peptides detected in the 18.5K digest were also present in the 19K digest suggesting that these proteins shared common amino acid sequences. The isolated 19K protein appeared to be contaminated by some of the 18.5K and all attempts to obtain 19K free of any contaminating 18.5K have failed even though the purified 18.5K product was readily obtainable by these procedures. A possible explanation for this might be that 19K is converted to the 18.5K species during or after purification from gels.

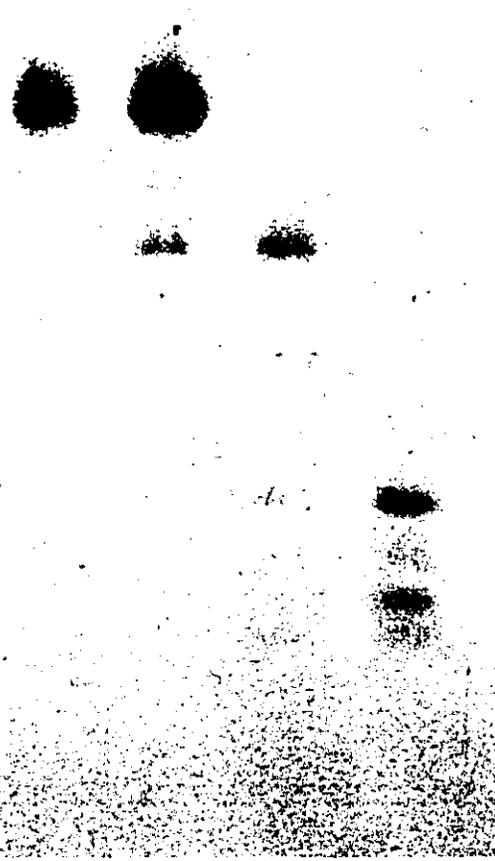
#### Immunofluorescence Studies of Infected KB Cells

The preceding biochemical analysis of viral proteins synthesized during

Figure 4. Partial proteolysis peptide mapping of the 18.5K and 19K proteins. Immunoprecipitates were run on preparative scale 15% polyacrylamide gels and the bands cut out using the autoradiogram as a template. Partial proteolysis with V-8 protease was carried out according to the method of Cleveland et al. (1977). Numbers above each well indicate the concentration of V-8 protease in ug/ml applied to each well.

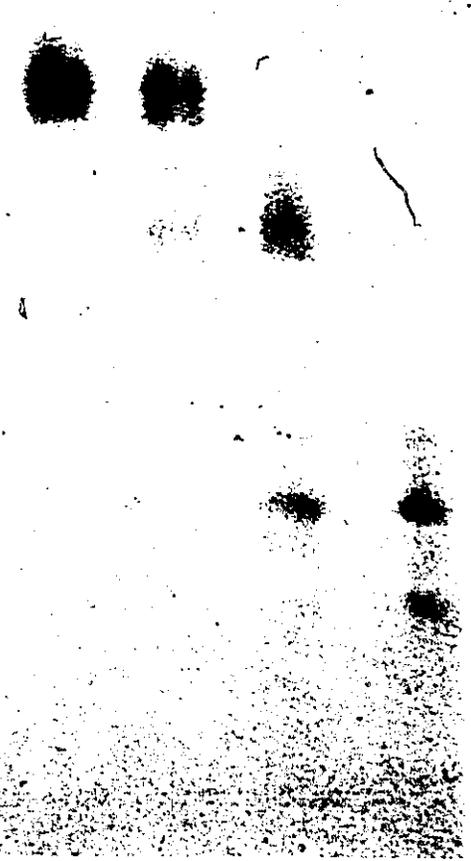
18.5

0 0.75 15 300



19

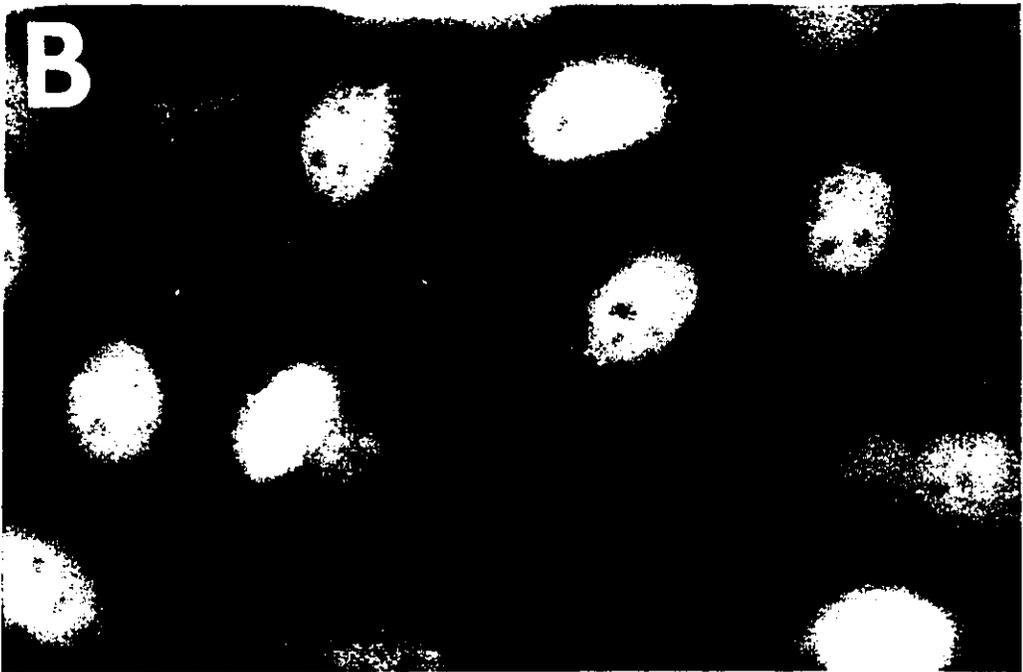
0 0.75 15 300



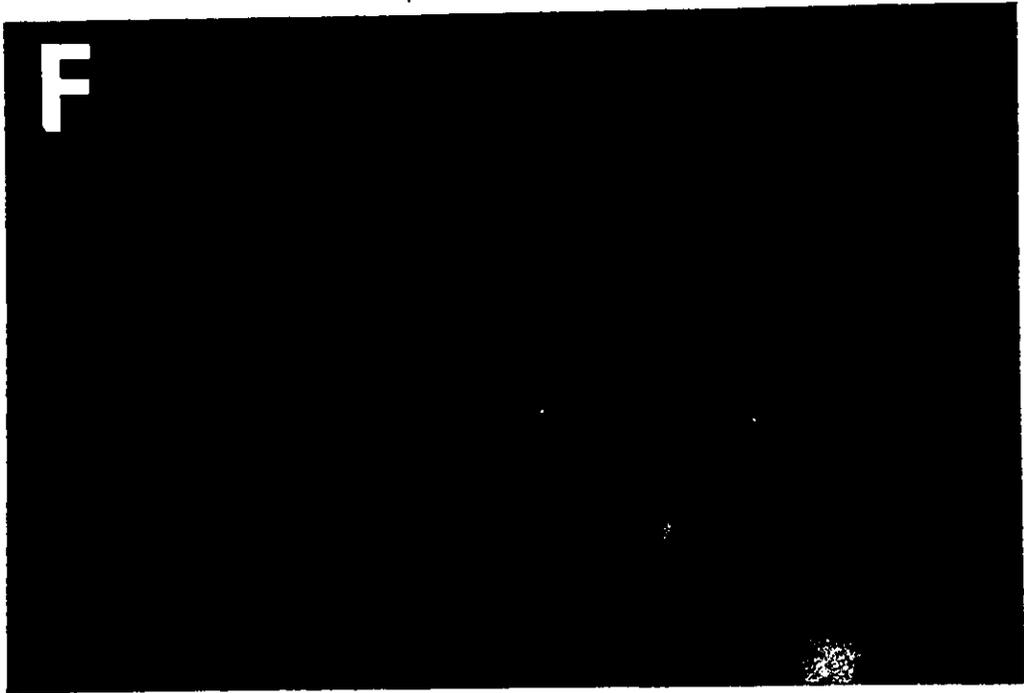
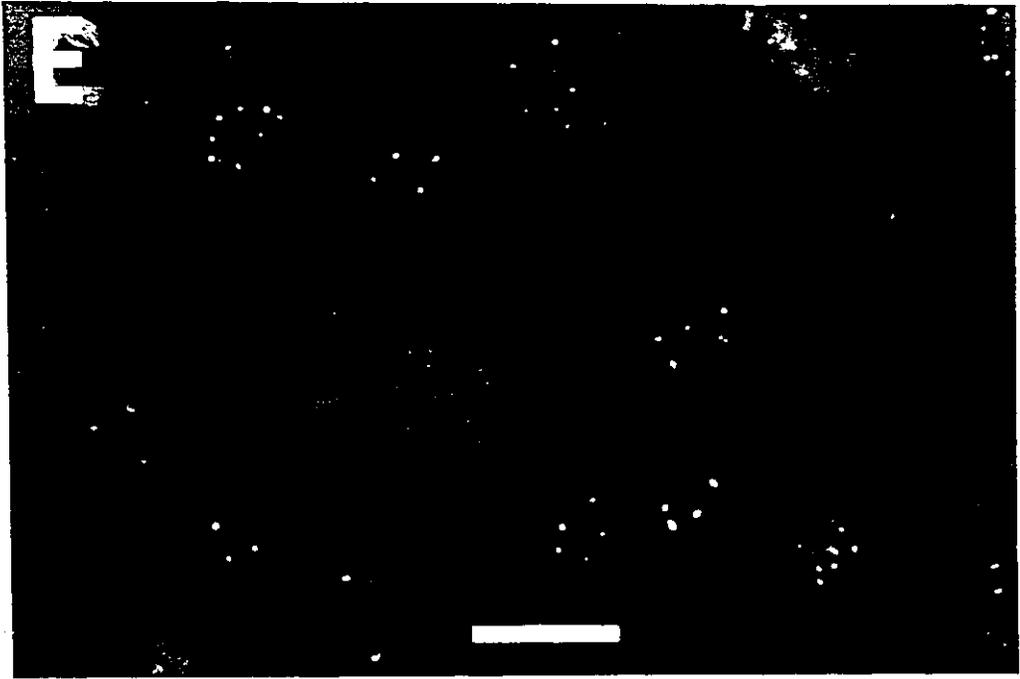
the early phase of adenovirus lytic infection requires the pooling of extracts from large numbers of cells to allow detection of the viral products synthesized at low levels. Direct visualization of tumor antigens, presumably encoded in E1, has been reported for both infected and transformed cells by immunofluorescence using anti-tumor sera (van der Eb et al., 1977; Lassam et al., 1979; Ross et al., 1980b; Vasconcelos-Costa and Ribeiro, 1982). However, the range of immune sera tested in those studies was limited and the antigens being detected by immunofluorescence were not well characterized.

Using the well-characterized and relatively monospecific antisera shown in Fig. 1, the immunofluorescent staining patterns of mock- and virus-infected HeLa cells were examined. None of the anti-tumor sera showed any immunofluorescent staining of mock-infected cells that was significantly different from the immunofluorescence seen using normal hamster serum on mock-infected cells (data not shown). Similarly, normal hamster serum did not show immunofluorescent staining of infected cells (Fig. 5F). The 983-3 anti-tumor serum, with a major avidity for the 58K protein, displayed two different immunofluorescent staining patterns depending upon the time p.i. at which the cells were fixed. Cells fixed between 4 and 8 hours p.i. showed mainly cytoplasmic perinuclear staining with a very few cells showing nuclear fluorescence (Fig. 5A). The number of cells showing nuclear fluorescence increased after 8 hours p.i. until by 12 hours p.i. most cells were displaying a strong nuclear fluorescence (Fig. 5B). A correlation may be drawn between the shift in fluorescence pattern observed with 983-3 anti-tumor serum at different times p.i. and the apparent increase in the nuclear association of 58K antigen detected by pulse-chase cell fractionation

Figure 5. Indirect immunofluorescent staining of T antigens in HeLa cells infected with Ad5. Monolayers were infected with 25 pfu per cell, fixed with methanol:acetone (1:1) at indicated times p.i. and stained with the sera described in Figure 9. Infected cells fixed at (A) 6 hours p.i. or (B) 12 hours p.i. stained with 983-3 antiserum; (C) infected cells fixed at 6 hours p.i. stained with 1019-3 antiserum. Infected cells fixed at 12 hours p.i. stained with (D) 954-C4, (E) 945-C1, or (F) normal hamster serum. Exposure time was 60 seconds. The bar in (E) represents 10  $\mu$ m.







experiments (Fig. 1), assuming that the immunofluorescence observed is in fact due to the detection of the 58K protein. This is likely since in other studies I have shown that this antiserum does not show immunofluorescent staining of transformed cells that do not possess the 58K antigen but which express other E1 antigens (Chapter III).

The staining of infected HeLa cells with the 1019-3 antiserum was low in intensity but showed a faint perinuclear fluorescence and a speckling associated with the nucleus at six hours p.i. (Fig. 5C). In some experiments it was possible to detect exclusively perinuclear staining as early as 2 hours p.i., and interestingly, some cells displayed only perinuclear fluorescence even at late times p.i. The assumption that the immunofluorescence is due to the 44K antigen is consistent with the results of these cell fraction studies which showed this protein was recovered from nuclear and cytoplasmic preparations.

Infected HeLa cells stained with the 954-C4 anti-tumor serum generally displayed a diffuse cytoplasmic fluorescence, however some cells showed a strikingly brilliant solid whole cell fluorescence. Fig. 5D shows three of these unusual cells, but such specimens were rarely observed this close together as less than 1% of the infected population displayed this remarkable feature. This pattern of fluorescence was observed in infected and not uninfected cells and only with antisera possessing a major avidity for the 19K antigen. It is not clear what causes a small minority of the population of infected cells to appear to contain 10-100x more antigen than the cells that surround them. The cytoplasmic immunofluorescence with 954-C4 antiserum correlated well with the cytoplasmic and membrane association of the 19K

antigen (Figs. 2 and 4) but attempts to visualize the 19K antigen on the surface of living cells or cells fixed in formaldehyde and impermeable to antibodies have been unsuccessful. Experiments involving lactoperoxidase-catalysed cell-surface iodination or proteolytic digestion of intact cells have also failed to demonstrate the presence of 19K antigen on the surface of infected cells (unpublished observations). If the 19K antigen is indeed located on the outer surface of the cells, it must be incorporated into structures which protect it from detection by these various techniques.

Immunofluorescence with the 945-C1 antiserum showed a nuclear speckling pattern which could be detected within six hours p.i. (Fig. 5E). This observation is consistent with the major avidity of the antiserum for the E4-14K antigen which was also shown to be a nuclear antigen by cell fractionation studies (Fig. 1; Chin and Maizel, 1976; Sarnow et al, 1982). Cells stained with 945-C1 (14K) antiserum displayed a fluorescence similar in pattern to that seen with 1019-3 (44K) antiserum but contained fewer, and more intense, spots in the nuclei.

## DISCUSSION

Recent work has suggested the products of early region 1 are directly involved in transcriptional and translational control of mRNA from the early regions of the viral genome (Berk et al., 1979; Persson et al., 1981). By determining the intracellular localization of these products in infected cells I have attempted to define more clearly the sites and structures that might be involved in the proposed regulatory functions. A number of workers have identified E1A translation products in vivo and in vitro as a class of closely related proteins migrating in a cluster from 35K to 52K on polyacrylamide gels (Harter and Lewis, 1978; Green et al., 1979; Esche et al., 1980; Smart et al., 1981). On the gel system I have used for these studies, which accentuates the separation of low molecular weight species in the range of 10K to 20K, the E1A products resolve only as a diffuse blur in the region of 44K (Fig. 2). Association of 44K with the nucleoplasm was indicated by the cell fractionation experiments but the speckled immunofluorescence in nuclei of infected cells obtained using anti-tumor sera (Fig. 9C) suggested that the 44K products might be further localized in a number of discrete regions within the nucleus. A more detailed analysis of the structures which appear to concentrate E1A products within the nucleus might provide important clues on the role of the E1A gene products in the stimulation of early viral expression. The cytoskeletal adherence of the E1A-44K proteins (Fig. 2) was another finding which in view of the cytoskeletal association of adenovirus messages in infected KB cells (van Venrooij et al., 1981) could indicate a second site where E1A products are involved in the regulation of gene expression.

Recent work with mutants affecting another adenovirus tumor antigen, the E1B-58K protein, has suggested that this protein may act at the post-transcriptional level to modulate the translation of adenoviral mRNA (Ross et al., 1980b; Chapter V ). This protein was found to be associated with both the cytoplasmic and nuclear fractions in approximately equal amounts but pulse-chase and immunofluorescence studies suggested an increase in the nuclear association occurred late in infection (Fig. 1 and Fig. 5). The high levels of 58K in the nucleus at late times parallels the reported restriction of host mRNA transcripts to the nucleus late in infection (Beltz and Flint, 1979) and analyses of the effects of 58K mutants on the levels of host cytoplasmic messages may help to determine whether 58K plays any role in this process.

In contrast to the partitioning of the E1A-44K and E1B-58K antigens in a number of cytoplasmic and nuclear subfractions, the E1B-19K antigen was found to be almost exclusively associated with the membrane fraction of infected cells (Fig. 5). Recently, Persson et al. (1982) reported the purification of the Ad2 E1B-15K protein (equivalent to the 19K of Ad5) from the membrane fraction of infected HeLa cells. Using 15% polyacrylamide gels I have resolved the Ad5-19K antigen into two related membrane-associated proteins designated 18.5K and 19K (Figs. 5 and 7) and have shown that both products were made in a variety of Ad5-transformed human, hamster and rat cell lines. Persson et al. (1982) reported that the Ad2-15K protein was not labeled by tritiated sugars added to the medium of infected cultures and I have been unable to detect any incorporation of phosphate into the 18.5-19K doublet in cells labeled for four hours with  $^{32}\text{P}$ -orthophosphate (data not shown). In long chase experiments the 18.5-19K doublet lost label while 17K and 17.8K

species increased in intensity on autoradiograms (Fig. 1). Although it was not possible to recover sufficient radioactivity from these proteins for peptide mapping studies, it seemed possible that these bands were degradation products of the 18.5-19K doublet. This conclusion is consistent with the observation that these products were only immunoprecipitable by antisera possessing a major specificity for the 18.5-19K antigens (data not shown).

Immunofluorescence studies with antisera almost monospecific for the 18.5-19K proteins revealed a small number of infected cells that showed a strikingly brilliant solid fluorescence greatly exceeding the normal range of fluorescence intensities expected from studies with other adenovirus antigen-antibody combinations. At present it is not clear what causes a small minority of the population of HeLa cells to express prodigious amounts of stainable antigen but the frequency of occurrence of these cells (approximately 0.1-1.0%) coupled with the fact that the moi was 35 pfu<sup>cell</sup> suggests that it is a property of the cells and not the infecting Ad5 virus. Since the E1B-19K product(s) appear to be responsible for many of the morphological properties characteristic of transformed cells (Houwelling et al., 1980, Schrier et al., 1979), expression of copious amounts of 19K by rare infected cells might be an important determinant in the initiation of adenovirus transformation of rodent cells. Interestingly, cellular heterogeneity in cloned populations of BHK-21 cells has been suggested to be the underlying basis for the semi-permissivity of this cell line to polyoma virus infection and may play a role in determining the frequency of polyoma virus transformation (Folk et al., 1981).

One of the major early viral proteins detected in vivo is the E4-14K antigen. It is a nuclear protein, apparently associated with the nuclear

matrix (Fig. 2; Chin and Maizel, 1977; Lenk et al., 1980; Sarnow et al, 1982) and was the only antigen analysed in this study which showed significant association with a nuclear subfraction composed almost entirely of histones (Fig. 1). In cells stained with 14K specific antiserum a speckled nuclear fluorescence similar to the fluorescence with anti-44K sera was observed. Determination of a role for this protein in lytic infection awaits the isolation of mutants with defects in the E4 coding region. Initial studies with deletion mutants which lack in addition to other regions the coding sequence for the 14K suggest that these viruses are defective in the expression of certain late viral gene products (Challberg and Ketner, 1981). A role for 14K in the expression of late genes is in doubt since a frame shift-deletion mutant in 14K coding sequences, dl 341, has no effect on the one step growth curve of the virus (Sarnow et al, 1982).

The E1 coded proteins characterized in these studies play an important role in the control of viral and cellular gene expression. The preliminary intracellular localization of these proteins establishes a basis from which more detailed investigations on the role of the adenovirus tumor antigens in lytic infection and transformation may be conducted.

CHAPTER V

The Kinetics of Synthesis of Early Viral Proteins  
in KB Cells Infected with  
Wild-Type and Transformation-Defective Host-Range Mutants  
of Human Adenovirus Type 5

## INTRODUCTION

Expression of the adenovirus genome in productively infected cells is temporally divided into two distinct phases, early and late, corresponding to events occurring before or after the onset of viral DNA replication. During the early phase only a portion of the genome, consisting of five discrete regions and involving both strands of the viral DNA, is expressed (Flint, 1977; Galos et al., 1979). Early nuclear RNA is transcribed from the 'r' strand of early region 1 (E1:1.5 to 11.2 map units, m.u.), from the 'l' strand of early region 2 (E2A:61.6 to 74.9 and E2B:11.2 to 23.5), from the 'r' strand of early region 3 (E3:76.8 to 86.0), and from the 'l' strand of early region 4 (E4:91.4 to 99.1) (Berk and Sharp, 1978; Chow et al., 1979, Gingeras et al., 1982, Stillman et al., 1981). Small amounts of RNA from the 'r' strand of the region 16.5 to 40.0 (L1) representing a portion of the sequences expressed from the major late promoter operating after DNA replication, have also been detected at early times (Lewis and Mathews, 1980; Shaw and Ziff 1980). Several lines of evidence have implicated viral early gene products as regulators of gene expression. S1 nuclease gel analysis of steady state levels of viral RNA in cells infected with host range (hr) mutant hr1 (which maps in E1A; Galos et al., 1980) has implicated one or more E1A products as a positive effector in the synthesis of stable nuclear RNA from E1B, E2, E3, and E4 regions (Berk et al., 1979). The 13S and 12S mRNAs complementary to this region are processed from alternative 5' splice sites (5'SS) to a common 3' splice site (3'SS) of the same initial transcript and are translated into polypeptides detected both in vitro and in vivo which share common amino and carboxyl peptides but differ in the length of the internal amino acid sequences (Esche et al., 1980; Smart et al., 1981;

Perricaudet et al., 1979; Halbert et al., 1979). The 9S mRNA has recently been shown to be processed at a unique 3' SS as well as a unique 5' SS and thus has only amino terminal peptides in common with 12S and 13S RNAs (A. Vertanen, personal communication). Hr1 carries a single base deletion in sequences which are unique to the 13S mRNA and synthesizes a truncated 13S product but a normal 12S and 9S product. (Ricciardi et al., 1981). Recent studies on the kinetics of RNA synthesis in E1A mutant infected cells show that early transcription is repressed in dl 312 [a deletion mutant expressing no E1A proteins (Jones and Shenk, 1981)] or in hr1 infections (Nivens, 1981) and that to a degree this repression can be overcome by protein synthesis inhibitors. To date the studies with mutants of E1A and drug treated cells have identified a requirement for an E1A gene product as an activator of early gene expression and raised the possibility of further involvement of E1A products in stabilization and translation of early mRNA (Berk et al., 1979; Nevins et al., 1979; Jones and Shenk, 1980; Persson et al. 1981 a,b; Katze et al., 1981; Nevins, 1981; Solnick, 1981; Solnick and Anderson, 1982; Shaw and Ziff, 1982;

Studies of early transcription with the Ad5 E2A mutant, ts 125, which produces a temperature sensitive DNA binding protein (DBP) have shown that this product specifically represses transcription of region E4 (Nevins and Winkler, 1980). In addition, the cytoplasmic stabilities of at least E1 mRNAs have been shown to be three to five times greater in ts125 infected cells than in wt-infected cells at the non-permissive temperature implying a role for the DBP not only as a repressor of E4 but also as a destabilizer of early adenovirus RNA (Babich and Nevins, 1981).

The potential involvement of E1B in the regulation of early gene expression has also been explored but the results have not revealed as clear

a role for E1B products as has been found for E1A and E2A. E1B encodes 13S and 22S mRNAs spliced from alternative 5'SS to a common 3'SS and an independently promoted unspliced 9S mRNA. 13S and 22S both direct the synthesis of the 19K major tumor antigen but the 22S also directs the synthesis of the 58K major tumor antigen by utilizing an internal translation initiation site for a different reading frame which partially overlaps the coding sequence for the 19K antigen (Bos et al., 1981). The 9S mRNA contains a single open reading frame encoding the virion structural polypeptide IX (Alestrom et al., 1980). The steady state levels of early cytoplasmic mRNA synthesized in HeLa cells by the E1B mutant hr7 are not significantly different from wt as determined by S-1 nuclease gel analysis (Berk and Sharp, 1979). Comparison of levels of DBP in wt and hr7 infected cells pulse-labeled for four hrs at 12 hrs p.i. lead Ross et al., (1980) to the conclusion that this product was overproduced in hr7 infections and suggested that region E1B may encode a negative regulator of adenovirus early gene expression. Lassam et al. (1978) reported that similar levels of DBP were synthesized by wt and hr6 (an E1B mutant phenotypically identical to hr7; Harrison et al., 1977) infected cells in one hour pulses at 24 hr p.i. but the level of late gene expression in hr6 infected cells was reduced compared to wt. Both hr6 and hr7 exhibit a multiplicity dependent leakiness for virus production but synthesize no 58K tumor antigen detectable by immunoprecipitation and polyacrylamide gel electrophoresis (Lassam et al., 1979; Ross et al., 1980) even at very high (100 pfu/cell) multiplicities.

Most of the studies described above have examined either steady state or kinetic levels of RNA synthesis or limited pulse-labeling analysis of protein synthesis in wt and mutant infected cells. Only Neuwald et al. (1977) have provided data on the time course of early protein synthesis in infected KB

cells. Although the coding regions of the early proteins examined (72K, 19K, and 11K) were not precisely identified, it is now clear that the 72K was the E2-DBP (Ginsberg et al., 1977; Levine et al., 1974), the 19K was the E3 membrane glycoprotein (Persson et al., 1979, 1980) and the 11K was an E4 nuclear protein (Chin and Maizel, 1977; Downey et al., 1983; Sarnow et al., 1982). Each protein showed unique kinetics of expression which was in rough agreement with the subsequently determined transcriptional activity of the corresponding early regions (Nevins et al., 1979). In the present study I examine the effect of mutations in E1A and E1B upon the expression of E1, E2, and E4 at the level of protein synthesis in infected KB cells. I show that expression of E1B as well as E1A is involved in the regulation of early gene expression at early times post-infection.

## MATERIALS AND METHODS

### Cells

The cultivation of KB cells for these studies has been described in the Materials and Methods section of Chapter II.

### Infection and labelling of Cells

This procedure was performed as described in the Materials and Methods section of Chapter II.

### Antiserum

The anti-tumor sera prepared against hamster cell lines for these studies are described in Chapter II.

The techniques for production and characterization of mouse hybridomas

secreting monoclonal antibodies to E2A antigens were previously described (Killington et al., 1981). Briefly,  $5 \times 10^6$  disrupted cells harvested 8 hours after infection with Ad5 were injected in complete Freund's adjuvant intraperitoneally (IP) and intramuscularly into BALB/c mice. Two subsequent IP boosts of virus infected cells without adjuvant at 2 week intervals were given and spleens were fused 3 days after the second boost. Positive poly-clones were detected by ELISA, cloned, and injected intraperitoneally into pristane-primed BALB/c mice. The resulting ascitic fluid containing monoclonal antibodies was used in this study.

#### Immunoprecipitation and polyacrylamide gel electrophoresis

These techniques were performed as described in the Materials and Methods section of Chapter II. Relative incorporations of radioisotope into protein bands were measured by scanning autoradiograms with a Joyce-Lobel double-beam recording densitometer.

RESULTSImmunoprecipitation of early viral proteins with anti-tumor serum

Analysis of viral proteins synthesized during the early phase of adenovirus lytic infection requires techniques which will allow detection of viral products within the considerable background of cellular protein synthesis. In vivo-labeling with  $^{35}\text{S}$ -methionine followed by immunoprecipitation with anti-tumor sera has been used by a number of groups to isolate viral gene products from infected and transformed cells (Green et al., 1979; Johansson et al., 1978; Ross et al., 1980; Lassam et al., 1978; Schrier et al., 1979). This procedure requires that appropriate sera be available in order to precipitate and analyse an array of viral proteins. Figure 1 shows that different anti-tumor sera used to immunoprecipitate viral antigens labeled from 7-9 hours p.i. varied greatly in avidity for any particular protein. Sera derived from hamsters bearing tumors seemed to have specificities which in general reflected the expression of viral proteins in the transformed cells used to induce the tumor (Chapter II). Figure 1 shows that tumor serum made in response to 954-C cells was directed principally against the 19K antigen. The immune response to 983-2 cells was

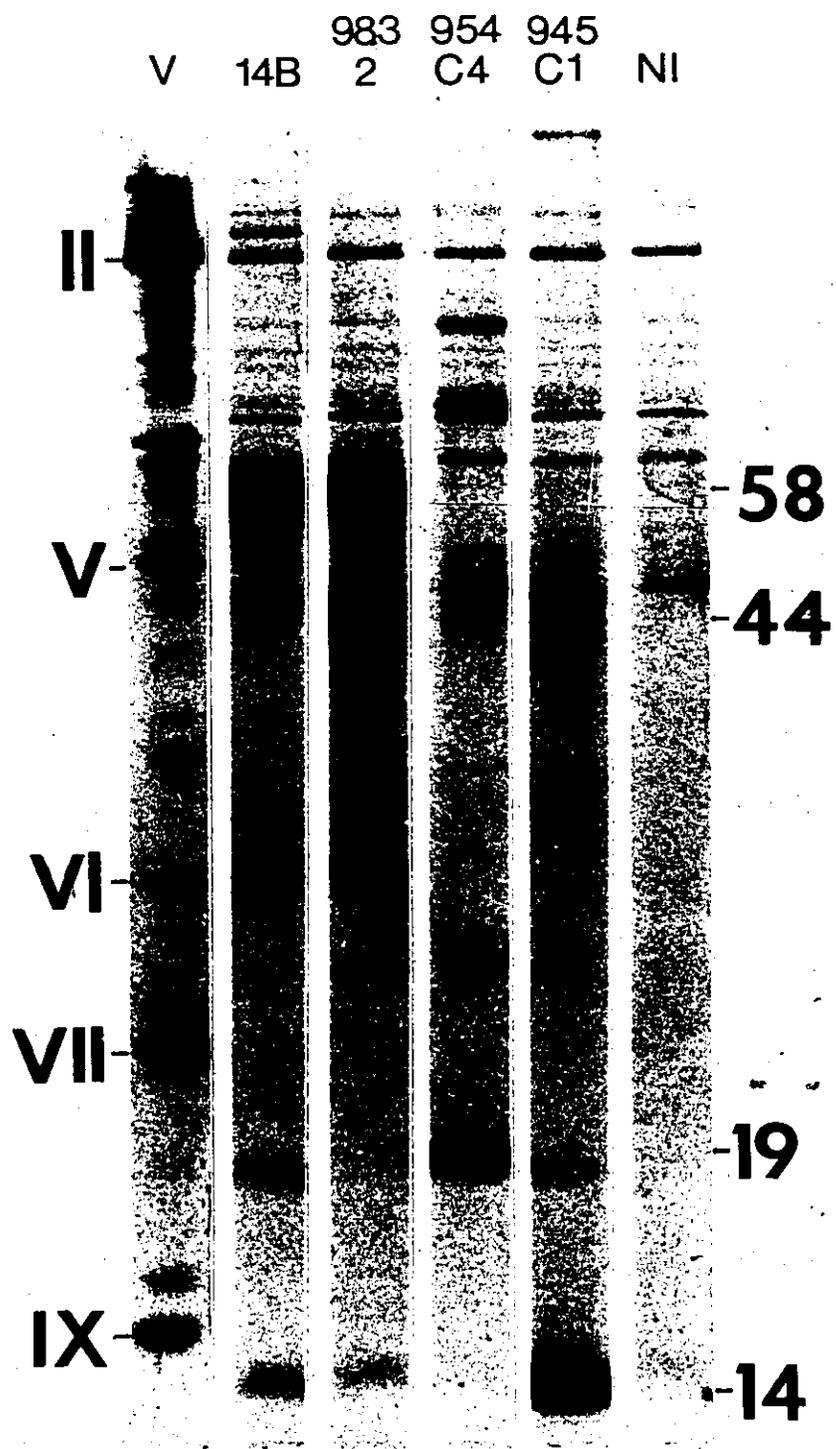
against the E1B-58K antigen, and a complex of several proteins migrating as a diffuse band around 44K. The 945-C1 serum was almost monospecific for the 14K, though a low avidity for the 44K and 19K antigens was also observed, and finally, 14b tumor serum was primarily active against the 58K antigen but also appeared to react weakly with a number of other antigens.

The characterization of a variety of transformed cell lines for viral DNA and protein content and analysis of tumor antigen synthesis in cells infected with various deletion mutants has permitted the tentative identification of the regions of the viral genome that encode the various viral proteins seen in Fig. 2 (Downey et al., 1983; Chapters II and IV). In those studies the 44K complex was assigned to E1A, the 58K and 19K products to E1B, and the 14K protein to E4 assignments which were consistent with the reports of other workers (Bos et al., 1981; Ross et al., 1980; Smart et al., 1981; Sarnow et al., 1982). By pooling the sera whose specificities are shown in Fig. 1, a polyvalent serum was made which was capable of immunoprecipitating the E1A 44K complex, the E1B 58K and 19K products and the E4-14K protein. With this pooled serum it was possible to examine the time course of expression of these early transcription units at the level of translation in Ad5 infected cells.

#### Kinetics of Early Protein Synthesis during wt Infection

In a time course experiment KB cells grown in suspension and infected at 50 pfu/cell were pulse-labeled for one hour with  $^{35}\text{S}$ -methionine every hour until 12 hrs p.i. The synthesis of late polypeptides was detected in whole cell extracts beginning at 8 hours p.i. Prior to 8 hours p.i. the pattern of protein synthesis in infected cells was indistinguishable from that of uninfected controls with the exception of the appearance of detectable levels of the 72K protein (see below). Aliquots of the infected cell extracts were

Figure 1. Autoradiogram of polyacrylamide gel separated antigens immunoprecipitated from Ad5 infected cells. Extract prepared from infected cells labeled with  $^{35}\text{S}$ -methionine from 7 to 9 hours p.i. were immunoprecipitated with various anti-tumor sera as described in the Materials and Methods. From left to right: V, purified virus marker; baby hamster kidney cell lines used to induce tumors were; 14B, transformed by a ts Ad5 mutant (Williams, 1973); 983-2, transformed by Xho I C fragment (0-16%); 954-C4 and 945-C1, transformed by Hind III digest of Ad5 DNA. Roman numerals refer to various structural components, numbers refer to M.W. in daltons as determined by SDS-PAGE.

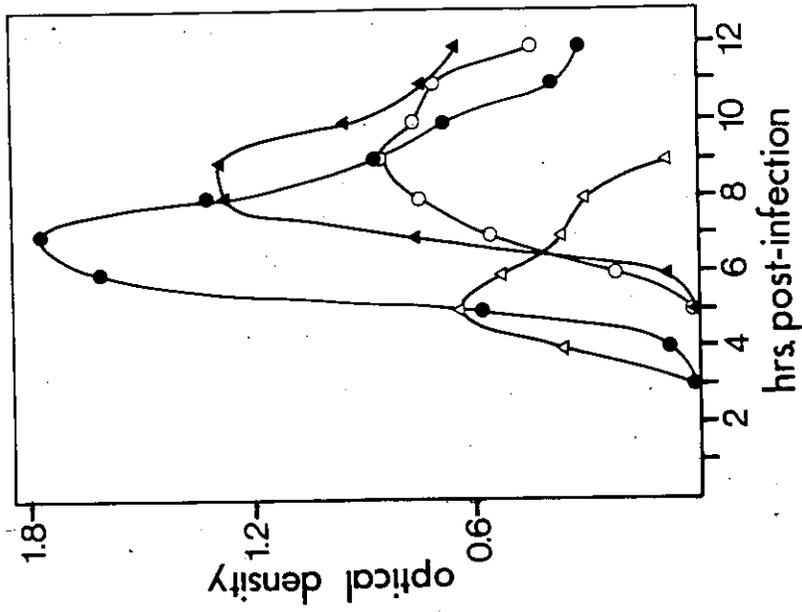
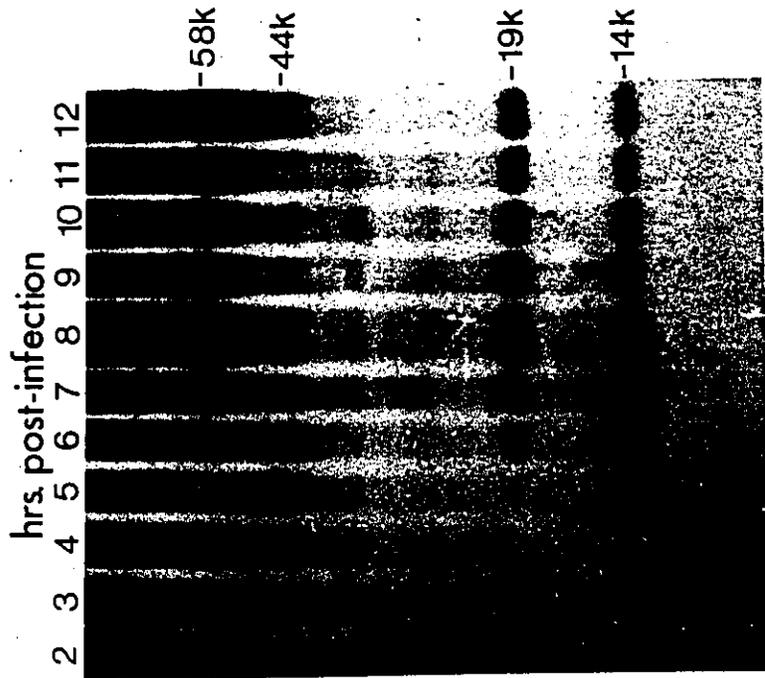


immunoprecipitated with the polyvalent tumor antiserum described above and the antigens recovered were separated on a polyacrylamide gel (Figure 2). It was possible to detect synthesis of E1A products within three hours of addition of virus to the cells and these proteins migrated as a diffuse band in the region of 44K as has been previously observed (Harter and Lewis, 1978; Ross et al., 1980a; Persson et al., 1981). Maximal rates of synthesis occurred by five hours p.i. and then declined until by at 10 hours p.i. the band was no longer detectable. Since E1A is the first to be transcribed (Nevins et al., 1979) the rapid appearance of E1A translation products was expected, but the decrease in protein synthesis after five hours p.i. contrasts with the continuous transcription and accumulation of cytoplasmic message throughout infection (Nevins et al., 1979; Shaw and Ziff, 1980; Spector et al., 1978).

The kinetics of expression of the early region 1B 19K protein was very similar to that of the E1B-58K protein. These products were first detected at approximately six hours p.i. and continued to be made in readily detectable quantities through 12 hours p.i. For both proteins, the rate of synthesis was maximal at approximately eight hours p.i. and then expression declined to a lower level which was maintained well into the late phase. Maximal rates of E1B RNA synthesis have been reported to occur around seven hours p.i. (Nevins et al., 1979) but the rate of accumulation of E1B 13S mRNA increases 50-fold relative to the accumulation of E1B 22S message. Infected cells labeled with <sup>35</sup>S-methionine for one hour at 24 hours p.i. show synthesis of 19K but not 58K (data not shown) even though the 22S message has been shown to persist in minute quantities at late times (Spector et al., 1978).

The E4-14K protein first appeared at four hours p.i. and reached a maximal rate of synthesis between six and seven hours p.i. and declined

Figure 2. Kinetics of early protein synthesis in adenovirus infected cells. Cells were labeled with  $^{35}\text{S}$ -methionine for one hour every hour from two to twelve hours p.i. Immunoprecipiate of whole cell extracts were analyzed on SDS-PAGE and densitometer tracings of the autoradiograms were made and plotted. Symbols used: 44K;  $\Delta$  , 19K;  $\circ$  , 58K;  $\blacktriangle$  , 14K  $\bullet$ .



sharply thereafter. This pattern is virtually identical to that reported by Neuwald et al. (1977) and both our results and those of Neuwald et al. (1977) conform to the pattern of transcription that has been reported for region E4 (Nevins et al., 1979). Thus, the rates of translation of E1B and E4 proteins observed in this study are in accordance with the available data on the rates of RNA transcription and accumulation and protein synthesis from these regions. However, the E1A translation pattern, which showed a cut-off after about seven hours p.i., was in contrast with the reported E1A transcription and mRNA accumulation that has been found to occur at much later times (Nevins et al., 1979; Shaw and Ziff, 1980; Spector et al., 1978).

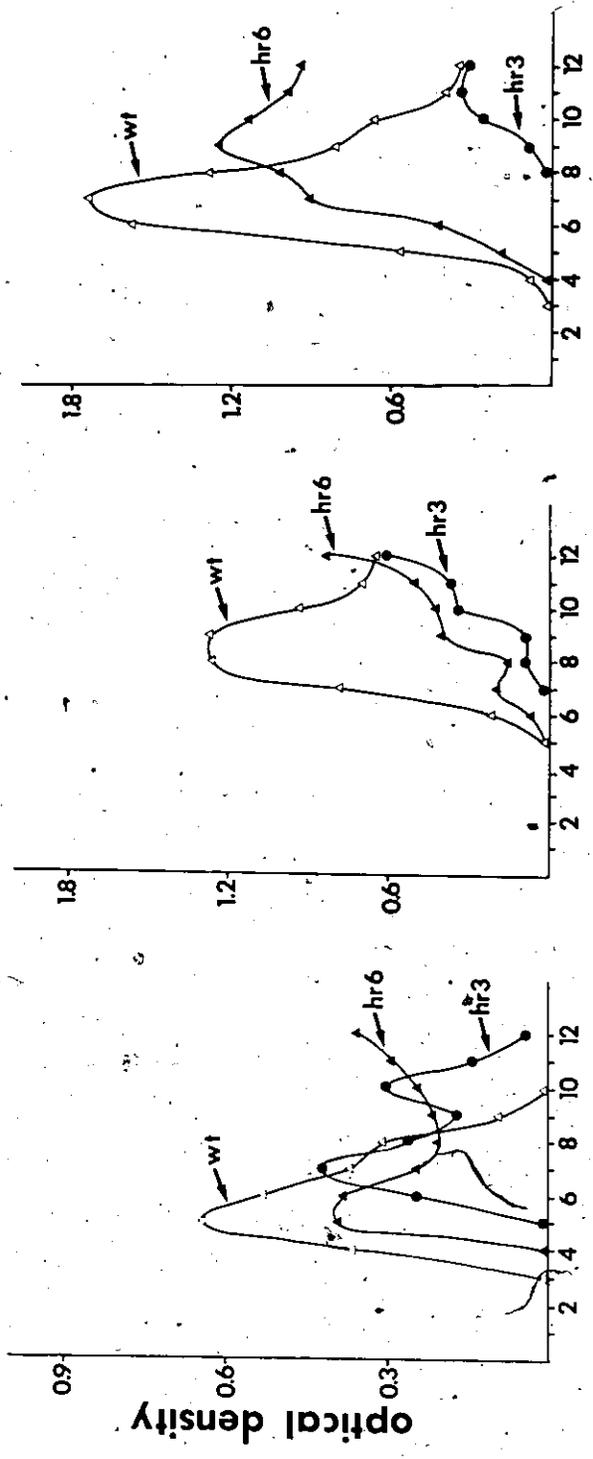
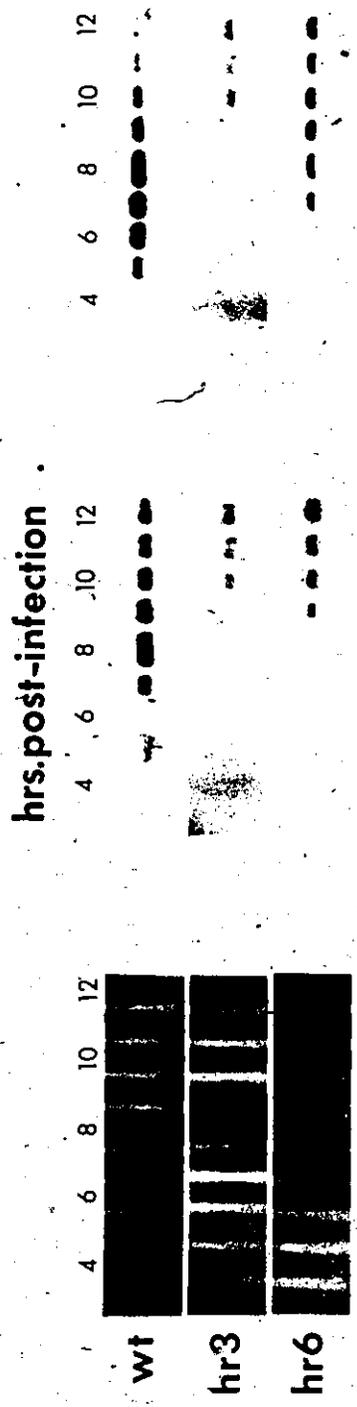
#### Effect of Host-Range Mutations on Early Protein Synthesis

Investigators of viral RNA synthesis utilizing deletion and host-range mutants and translational inhibitors have identified a role for E1A products in the stimulation of early Ad5 transcription (Berk et al., 1979; Nevins, 1980; Shaw and Ziff, 1982). To examine this control of expression as manifested at the translational level, the rate of translation of tumor antigens from regions E1A, E1B and E4 was analysed and compared to KB cells infected with wt virus or the E1A mutant hr3 (Fig. 3). Expression of E1A proteins in hr3 infected cells was characterized by a longer lag period relative to wt kinetics and two separate peaks of E1A protein synthesis appeared at seven and ten hours p.i. before a cessation of translational activity nearly 12 hours p.i. (Figure 3A). The normal early peak of expression of E1B-19K and E4-14K was absent from hr3 infections, but at late times translation of both proteins appeared to have recovered to levels comparable to wt infected cells (Figure 3B and C). Synthesis of the E1B-58K antigen was not detected in immunoprecipitates of hr3 infected cells during the course of this experiment. It was occasionally possible to detect

Figure 3. Kinetics of protein synthesis in hr mutant infected KB cells. Infected cells were pulse-labeled with  $^{35}\text{S}$ -methionine as described in legend of Fig. 2. Portion of the autoradiogram containing the viral antigen analyzed is shown above the plot of the densitometer scans. A, 44K; B, 19K; C, 14K. Symbols used:  $\triangle$ , Wt;  $\blacktriangle$ , hr6;  $\bullet$ , hr3.



**A** **B** **C**



synthesis of reduced amounts of 58K in hr3 infections particularly at higher m.o.i. where the leakiness of this host range mutant was also characterized by the ability to synthesize increased amounts of viral DNA (Rowe and Graham, 1981). These results are consistent with the proposed role of E1A gene products in the control of early viral gene expression.

To determine whether a product of early region 1B was involved in the regulation of early gene expression a similar comparison of translational activity was performed using the E1B mutant hr6. This mutant, though it shows a multiplicity dependent leakiness for virus production, synthesizes no immunoprecipitable 58K antigen even at high moi (Lassam et al., 1979; Ross et al., 1980). Synthesis of the E1A-44K in hr6 infected cells peaked at five hours p.i., similar to wt, but reached a lower maximal rate, then after decreasing to a minimum level of expression at eight hours p.i. increased again over the next four hours and showed no indication of abatement by 12 hours p.i. in contrast to wt which had shut off E1A translation by 10 hours p.i. (Figure 3A). The translation of the E1B-19K increased slowly over the eight hour period from four to 12 hours p.i. but showed no early peak of expression characteristic of wt infection. The pattern of E4-14K synthesis was also perturbed by the hr6 defect which caused a lower maximal rate of expression and shifted the peak to a later time after infection (Figure 3C). While it is difficult at present to suggest a single mechanism by which all these regulatory effects occur, these results suggest that E1B may encode some regulatory function which modulates expression of early viral proteins at early times p.i.

#### Kinetics of E1A-72K synthesis in Wt and hr Mutant Infections

The 72K-DBP encoded by the E2A region is an abundant early phosphoprotein (van der Vliet and Levine, 1973; Levinson et al., 1977;

Axelrod, 1978) with a complex role in adenovirus gene expression (Nevins and Winkler, 1980; Babich and Nevins, 1981; Klessig and Grodzicker, 1979; Nicolas et al., 1982) and DNA replication (van der Vleit et al., 1975; Nagata et al., 1982). Although the kinetics of synthesis of this protein may be measured directly by polyacrylamide gel electrophoresis of pulse-labeled infected cell extracts (Neuwald et al., 1977; Axelrod, 1978), most workers enhance detection of E2A products by immunoprecipitation with polyvalent or monospecific antisera derived from a variety of sources (Ross et al., 1980b; van der Vleit et al., 1977; Nicolas et al., 1982; Asselbergs et al., 1983). For this study we have used a murine monoclonal IgG antibody, H2-19 (see Materials and Methods) which specifically immunoprecipitates the major forms of the DBP (Axelrod, 1978; Jeng et al., 1977) as well as some of the minor related species (Figure 4; Asselbergs et al., 1983). Since the subset of E2A polypeptide species immunoprecipitated by H2-19 appear to be the same products detected by the rabbit antiserum prepared against purified DBP, it is possible that the site recognized by the monoclonal antibody is within the sequences corresponding to amino acids 170 to 240 which bears the antigenic determinants recognized by rabbit antiserum (Asselberg et al., 1983). The monoclonal antibody, which was obtained in high titers from murine ascites tumors, was used to immunoprecipitate E2A proteins from extracts of cells infected with wt, hr3 or hr6 and pulse-labeled for one hour at various times p.i. (Figure 5). Synthesis of E2A proteins (72K, 67K, 50K and 48K) in wt infected cells was detected three hours p.i., was maximal by six hours p.i., and declined gradually after eight hours p.i. The kinetics of E2A translation in cells infected with hr3 (E1A mutant) showed no early peak of expression but the rate of synthesis increased slowly to wt levels by 12 hours p.i., a pattern very similar to the kinetics of synthesis of E1B-19K

Figure 4. Specificity of H2-19 monoclonal antibody. Polyacrylamide gel analysis of Ad5 proteins labeled from 7 to 9 hours p.i. with  $^{35}\text{S}$ -methionine and immunoprecipitated with H2-19 antibody or anti-tumor serum. A, virus marker; B, mock-infected cells immunoprecipitated with H2-19; Ad5 infected cells immunoprecipitated with C, H2-19 and D, anti-tumor serum.

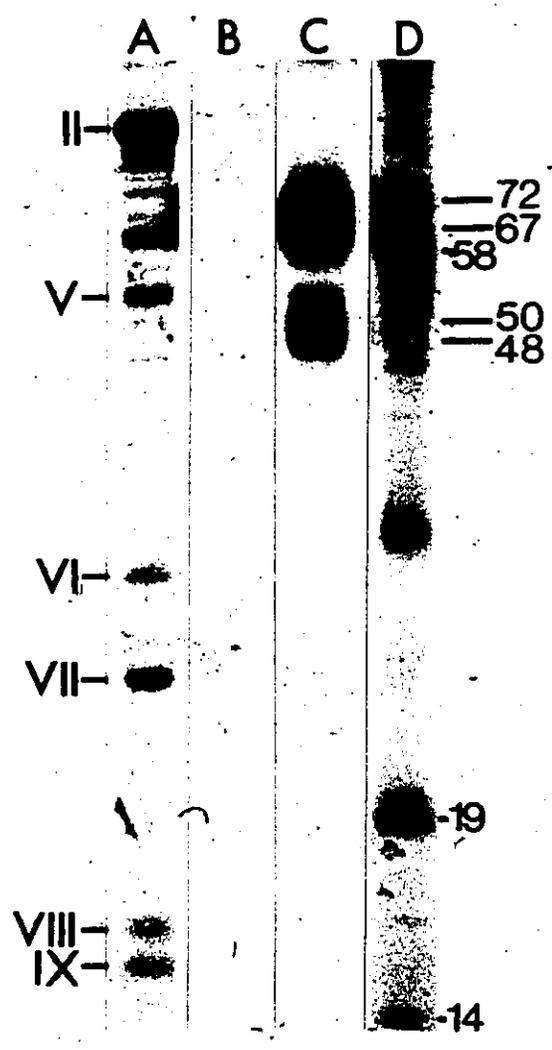
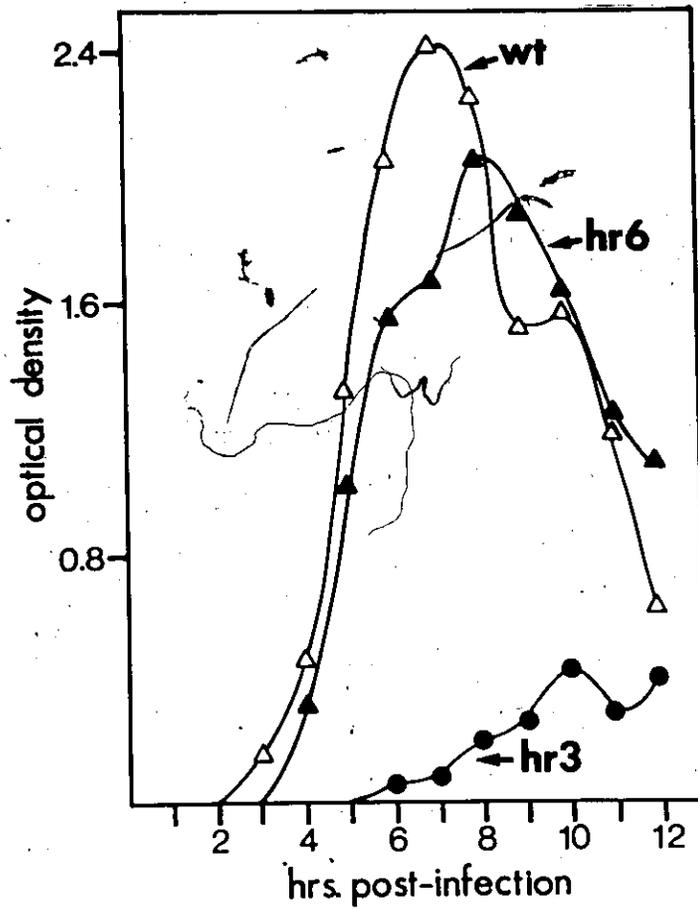
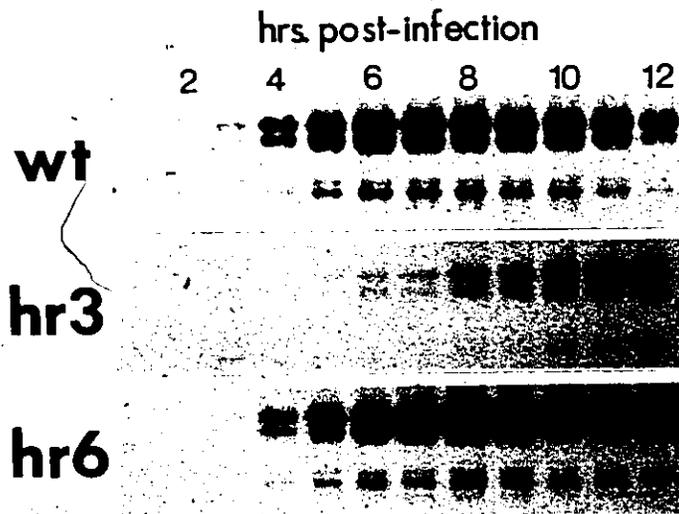


Figure 5. Kinetics of E2A protein synthesis in hr mutant infected KB cells. Infected cells were pulse-labeled as described in legend of Fig. 2 and immunoprecipitated with H2-19 monoclonal antibody. The portion of the autoradiogram containing the E2A proteins is shown above the plot of the densitometer scans.

Wt;  $\Delta$  hr6;  $\blacktriangle$  hr3  $\bullet$ .



and E4-14K in hr3 infected cells (Figure 3B and C). Synthesis of E2A proteins in hr6 infected cells was not severely affected and followed a pattern similar to that seen for E4-14K expression. Previously it had been suggested that the E2A DBP was overproduced in hr7 (an E1B mutant of the same complementation group as hr6) infected cells assayed by a 4 hour pulse-label from 12 to 16 hours p.i. (Ross et al., 1980b). Our results suggest that the rate of E2A protein synthesis in hr6 infected cells does not exceed normal levels until late times when E2A expression was curtailed in wt infected cells.

#### DISCUSSION.

Examining the kinetics of early protein synthesis during adenovirus infection permits the observation of the ultimate effects of the complex viral regulatory system on the expression of early genes. The time of appearance of proteins from early region 1A, 1B, 2 and 4 was unique in each case, as was the occurrence and duration of the maximal rate of expression (Figure 2). The reduction of E1A protein synthesis during the course of wt infection is qualitatively similar to the reduction which was seen in protein synthesis from other early regions, but temporally distinct, reaching very low levels at the same time as peak expression of E1B and E2. This effect on E1A gene expression is likely to be mediated at the level of translation as E1A 13S and 12S RNA have been reported to accumulate in the cytoplasm at a constant rate throughout infection (Wilson and Darnell, 1981; Spector et al., 1978).

The kinetic studies with the E1A mutant hr3 revealed a delay in protein synthesis and decreased levels of translation of E1B, E2 and E4. While these

results are in general agreement with the proposed role of E1A functions for the regulation of expression of other early regions at the level of transcription (Berk et al., 1979; Ricciardi et al., 1981; Nevins, 1980; Jones and Shenk, 1979), E1A protein synthesis in hr3 infected cells peaked twice within 12 hours which contrasted with a single peak for wt infected cells (Figure 3A) and may indicate a role for E1A functions in the control of E1A expression. It is not clear from this study whether E1A autoregulation acts at the transcriptional or post-transcriptional level or whether this effect is indirectly caused by reduced amounts of other early gene products.

During lytic infection the E1B hr6 defect was characterized by deviations from normal early gene expression for all products examined. In general, the mutant appeared to induce delayed and reduced maximal levels of protein synthesis from E2 and E4 and no early phase of E1B-19K expression (Figures 3 and 5). As with the hr3 mutant, the kinetics of E1A protein synthesis in hr6 infected cells were more complex than wt, indicating a failure of the normal regulation of E1A expression but leaving open the question of how this defect is mediated. Hr6 infections of non-permissive cells are also characterized by reduced levels of late gene expression (Lassam et al., 1978), yields of infectious virus which are reduced by as little as one order of magnitude (Rowe and Graham, 1981) and marked multiplicity dependent leakiness. All of these effects appear to be due to the failure of hr6 to synthesize a 58K E1B protein suggesting that this product is not required for execution of the viral genetic program but may be necessary for the efficient expression of viral genes at both early and late times p.i. Cell fractionation and immunofluorescence studies of the 58K have suggested that this protein is associated with the nucleus at late times ( Chapter VI ). This high level of late nuclear association

parallels the reported restriction of host mRNA transcripts to the nucleus late in infection (Beltz and Flint, 1979). A role for the 58K protein in this process would explain most of the phenotypic effects of hr6 and other E1B mutants.

CHAPTER VI

Characterization of Human Adenovirus Type 5  
Early Region 1A Polypeptides Using Anti-Tumor Sera  
and  
an Antiserum Specific for the Carboxy Terminus

## INTRODUCTION

The early phase of lytic infection with adenovirus (before viral DNA replication) involves a complex coordinated expression of at least five regions of the viral genome (Flint, 1977; Galos et al, 1979). Early RNA is transcribed from the 'r' strand of early region 1 (E1, 1.5 to 11.2 map units), the 'l' strand of early region 2a (E2a, 61.6 to 74.9) and 2b (E2b, 11.2 to 31.5), from the 'r' strand of early region 3 (E3, 76.8 to 86.0) and from the 'l' strand of early region 4 (E4, 92.4 to 99.1) (Berk and Sharp, 1978; Chow et al., 1979; Stillman et al., 1981). Small amounts of RNA from the 'r' strand of the region 16.5 to 40.0 (L1) representing a portion of the sequences expressed from the major late promoter operating after DNA replication, have also been detected at early times (Lewis and Mathews, 1980). Recent work has suggested that the products of early region 1 (E1) are involved in transcriptional and translational control of early RNA expression during lytic infection (Berk et al., 1979; Nevins, 1981; Jones and Shenk, 1979a; Persson et al., 1981).

The six mRNAs complementary to E1 define two distinct non-overlapping transcription units: E1a, 1.5 - 4.4 and E1B, 4.5 - 11.2 map units, plus a third transcription unit starting within E1B. The three E1A mRNA species of 1.1, 0.9 and 0.6 Kilobases (Kb) are thought to be processed from alternative 5' splice sites (5'SS) to a common 3' splice site (3'SS) of the same initial transcript (Berk and Sharp, 1978; Chow et al., 1979). From data on nucleotide sequencing of genomic DNA and cloned cDNA (Perricaudet et al., 1979; van Ormondt et al., 1980), the mRNA, the 1.1 Kb and 0.9 Kb messages code for two proteins of 289 and 243 amino acids in length which share common amino and carboxy terminal peptides but differ in that 46 residues of the

larger polypeptide (amino acids 140 to 185) are absent from the smaller product. In vitro translation of Ad2 early viral mRNA selected by hybridization to viral DNA fragments representing only the sequences of E1A has permitted the identification of four major products of 53K, 47K, 41K and 35K (Halbert et al., 1979; Harter and Lewis, 1978). Cell-free translation of size fractionated E1A mRNA suggests that the 53K and 41K are encoded by the 1.1 Kb mRNA and the 47K and 35K are the products of the 0.9 Kb mRNA (Esche et al., 1980). Various values for the molecular weights of the four major in vitro E1A products have been reported by a number of groups (Halbert et al., 1979; Green et al., 1979; Esche et al., 1980; van der Eb et al., 1980) and it has been suggested that the differences are primarily due to the gel systems and molecular weight markers used in the different laboratories (van Ormondt et al., 1980). All the estimates of molecular weight are however much higher than the 32K and 26K products predicted from the sequences of the 1.1 Kb and 0.9 Kb mRNA species, respectively (Perricaudet et al., 1979). The product of the E1A 0.6 Kb message has only been detected in vitro. Van der Eb et al. (1980) reported a 14K product which is approximately the size predicted from the sequence (van Ormondt et al., 1980) whereas in another study, Spector et al. (1980) reported that the cell-free translation product of the Ad2.0.6 Kb mRNA was a 28K protein.

Harter and Lewis (1978) identified six in vivo translation products in infected cells pretreated with cycloheximide before labeling with <sup>35</sup>S-methionine. All these products were also detected by in vitro translation of E1A mRNAs and the in vitro and in vivo products resolved on two-dimensional gels with isoelectric points of ca. 5.9 - 6.0. Analysis of tryptic peptides by amino acid sequencing showed that the three products focusing ca. 6.0 were encoded by the 1.1 Kb message and the other three

products focusing ca. 5.9 were encoded by the 0.9 Kb message (Smart et al., 1981). Other workers have identified only four in vivo and in vitro products (Green et al., 1979) or as few as two products after in vitro translation (Ricciardi et al., 1981). It is not clear what factors are important in the synthesis from the same mRNA molecules of the various numbers of products that have been reported by the different groups.

The effects of mutations within E1A on the polypeptides synthesized from the 1.1 Kb and 0.9 Kb mRNA have been studied by in vitro translation. Transformation-defective complementation group 1 host range mutants (Graham et al., 1978; Harrison et al., 1978) displayed two phenotypes with respect to the synthesis of E1A polypeptides. Hr 1 and hr2 failed to synthesize the longer of two E1A products detected by in vitro translation reactions programmed with mRNA from wt or hr3, 4 or 5 (Ricciardi et al., 1981). The hr1 mutant was further shown to contain a single base deletion at position 1055 creating a frame shift that introduces a termination signal 11 codons downstream. This mutation is spliced out of the 0.9 Kb mRNA and thus affects only the products of the 1.1 Kb message. A novel 28K polypeptide detected by Ricciardi et al. (1981) in hr1 mRNA programmed translations was assumed to represent the truncated product predicted from the introduction of the nonsense codon by the hr1 mutation. In vitro translation studies of Ad5 hr deletion mutants constructed around the XbaI cleavage site at map position 4.0 (Jones and Shenk, 1979b) have identified faster migrating products on polyacrylamide gels with molecular weights that are consistent with the size and location of the DNA deletions (Esche et al., 1980).

Most of the E1A polypeptides described above were the products of in vitro translation reactions or were synthesized in cells which had been pretreated with drugs to enhance E1A mRNA accumulation and therefore are

potentially prone to artifacts that might accompany cell-free synthesis or metabolic inhibition. The present study makes use of anti-tumor sera and an antiserum prepared in rabbits against the synthetic peptide (tyr-gly-lys-arg-pro-arg-pro) corresponding to the terminus of the E1A proteins (Yee et al., submitted) to analyse the synthesis of E1A products in vivo in the absence of any manipulation or interference that might affect these important regulatory proteins.

## MATERIALS AND METHODS

Cells

The cell lines used for this study are those described in Chapters II and III.

Viruses

The wt strain of Ad5 and the hr mutants hr1, 2, 3, 4 and 5 used in this study have been described previously (Harrison et al., 1977; Graham et al., 1978). The Ad5 deletion mutant dl313 (provided by T. Shenk) carries a deletion of 2307 base pairs between nucleotide positions 1334 and 3639 within region E1 (Colby and Shenk, 1981). This mutant displays a host-range phenotype, and was grown on monolayers of 293 cells. The Ad5 E1A deletion mutant dl1504 (Osborne et al., 1982) and the E1A point mutant pm975 (Montell et al., 1982) were kindly provided by A. Berk and replicate on HeLa cells as efficiently as wild-type. Titers of wt and E1A mutants were determined by plaque assay on monolayers of 293 and HeLa cells, and for mutants with host-range phenotype ratios of titers on 293 versus HeLa were typically in the range of  $10^2$  to  $10^3$ .

Infection and labeling

This procedure was performed as described in the Materials and Methods section of Chapter II.

Immunoprecipitation and polyacrylamide gel electrophoresis

These techniques were performed as described in the Materials and Methods section of Chapter II.

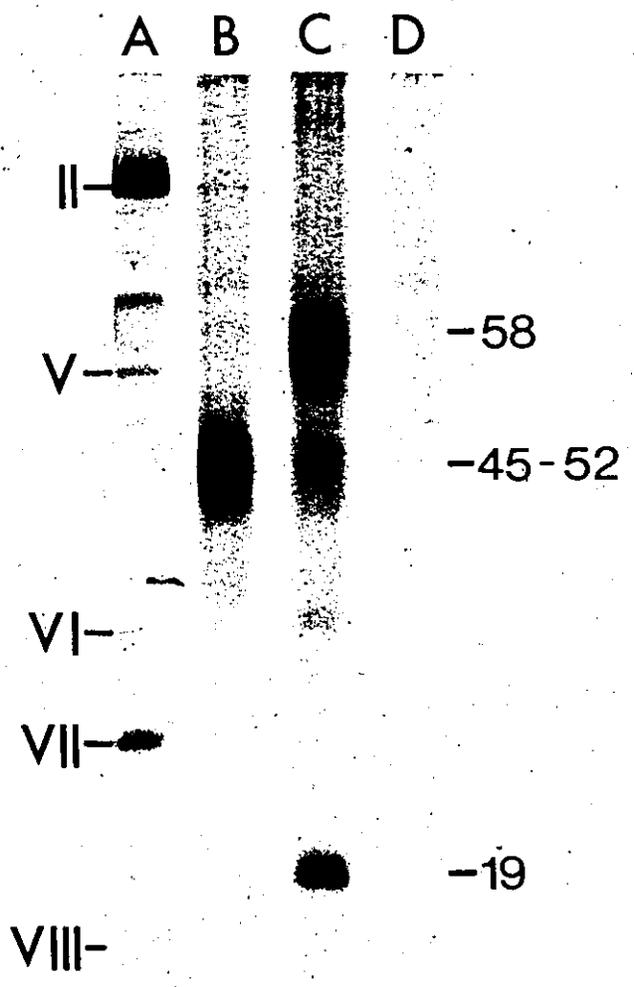
## RESULTS

Immunoprecipitation of E1A polypeptides with anti-tumor serum

Analysis of E1A polypeptide products in lytically infected cells requires techniques which will allow detection of viral proteins within the considerable background of cellular protein synthesis. In vivo labeling with  $^{35}\text{S}$ -methionine followed by immunoprecipitation with anti-tumor serum has been used by a number of groups in attempts to detect E1A proteins in infected and transformed cells (Green et al., 1979; Lassam et al., 1979; Röss et al., 1980; Schrier et al., 1979). The proteins observed have been poorly characterized compared with the mapping studies of the in vitro synthesized polypeptides (Esche et al., 1980; Smart et al., 1981). In part, the difficulty in obtaining sufficient quantities of E1A polypeptides from infected cells has been the low levels of E1A protein synthesis, but the problem has also been that the few anti-tumor sera which react with the E1A products do so with only very low avidity.

I have described a polyvalent antiserum made by pooling anti-tumor sera with differing major antigenic specificities which is capable of immunoprecipitating the proteins of early region 1 (Chapter II). Recent studies with cell lines transformed by DNA fragments of Group II hr mutants have shown that hr 50 transformed hamster cells exhibited the unique phenotype of being fully transformed and relatively tumorigenic in the absence of detectable E1B antigens (Chapter III). As shown in Fig. 1 hamster anti-tumor sera raised against the hr50 transformed cell line 1019.3 appeared to respond only to the E1A-45K to 52K proteins. This avid and specific antiserum used alone or combined with other anti-tumor sera greatly improved the sensitivity of immunoprecipitation assays for E1A antigens. In an initial step toward a

Figure 1. Autoradiogram of a 14% polyacrylamide gel analysis of adenovirus proteins. Infected KB cells were pulse-labeled with  $^{35}\text{S}$ -methionine for 2 hours from 7 to 9 hr post-infection and immunoprecipitated with anti-tumor serum. A) marker virus, B) infected cell- and C) mock-infected cell extracts immunoprecipitated with anti-1019-3 tumor serum, D) infected cell extract immunoprecipitated with a polyvalent combined anti-tumor serum.



characterization of the in vivo synthesized E1A products, infected and mock infected cells were labeled for 2 hours with  $^{35}\text{S}$ -methionine or  $^{32}\text{P}$ -orthophosphate at 6 hours p.i. and immunoprecipitated with a combined anti-tumor serum which included the anti-1019.3. Fig. 2 shows that the combined anti-tumor serum was capable of immunoprecipitating E1A proteins in the molecular weight range of 52K to 45K, and the E1B-58K and 19K products. In particular, the E1A products migrated in two broad bands of approximately 52K and 48K and both species were labeled with  $^{32}\text{P}$ -orthophosphate. While I found no major difference in the migration patterns of these phosphoproteins when they were labeled with either  $^{35}\text{S}$ -methionine or  $^{32}\text{P}$ -orthophosphate, for reasons which are not clear the  $^{32}\text{P}$ -labeled proteins always tended to be better resolved in one dimensional gel electrophoresis. Of the 58K and 19K E1B antigens detected by immunoprecipitation of  $^{35}\text{S}$ -methionine labeled infected cells, only the 58K product was labeled by  $^{32}\text{P}$ -orthophosphate (Fig. 2).

Two-dimensional resolution of E1A polypeptides immunoprecipitated by anti-tumor serum

The analysis of the translation products of E1A messages in vitro has permitted the identification on two dimensional gel systems of as many as six closely migrating species which have been difficult to resolve by one-dimensional gel electrophoresis (Harter and Lewis, 1978; Smart et al., 1981). To characterize further the E1A products from metabolically labeled infected cells, immunoprecipitates were analysed by two-dimensional gel electrophoresis. As shown in Fig. 3, I found that isoelectric focusing gels with a pH range of 4 to 6 resolved two major species with a pI ca. 4.8 in infected cells which were not present in mock-infected cell extracts.

Figure 2. Autoradiogram of a 14% polyacrylamide gel analysis of immunoprecipitates from Ad5-infected KB cells. Infected and mock-infected KB cells were pulse-labeled with  $^{35}\text{S}$ -methionine or  $^{32}\text{P}$ -orthophosphate for 2 hours from 7 to 9 hours p.i. and the labeled polypeptides were immunoprecipitated with the polyvalent anti-tumor serum.

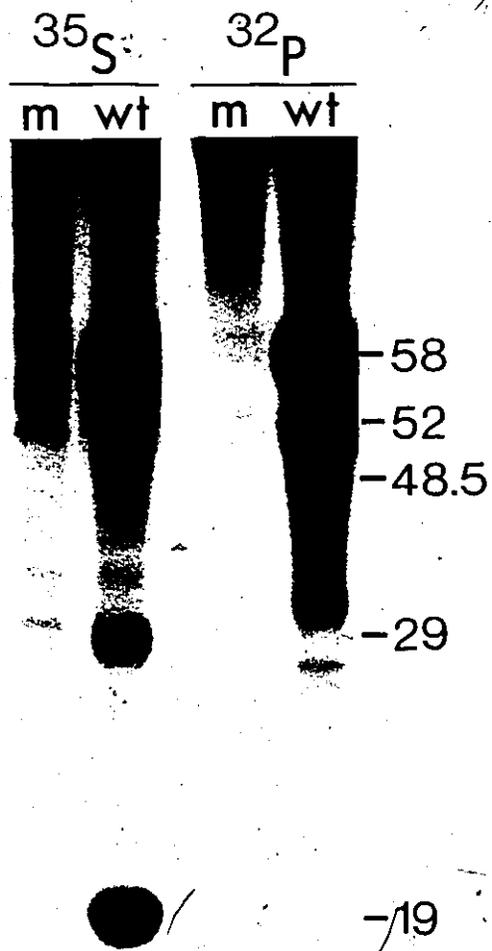


Figure 3. Autoradiogram of two dimensional gel electrophoresis of infected and mock-infected cells. Cells were labeled with  $^{35}\text{S}$ -methionine for 2 hours from 7 to 9 hours p.i. and immunoprecipitated with the polyvalent anti-tumor serum. Immunoprecipitated proteins were separated in pH 4-6 isoelectric focusing gels (horizontal dimension) and then in SDS-12% polyacrylamide slab gels (vertical dimension) as described in Materials and Methods. The basic side (-) of the isoelectric focusing dimension is on the left. The arrow indicates a pI of approximately 4.8.

MOCK



INFECTED



Several minor spots were also observed which trailed most often towards the acidic end of the isoelectric focusing gels but trailing could also be observed towards the basic end (Fig. 3). To study the relationship of the polypeptides detected by two-dimensional electrophoresis, immunoprecipitates of cells infected with wt, hr1 and pm975 were compared. Nucleotide sequencing of hr1 DNA has revealed a single base deletion at position 1055 causing a frame shift termination affecting only the translation of the 1.1 Kb mRNA (Riccardi et al., 1981). The point mutant of pm975, a T to G transversion, eliminates the 5' splice site of the E1A 0.9 Kb mRNA while leaving the reading frame of the 1.1 Kb mRNA unaffected. Infection of cells with this mutant leads to expression of only the 1.1 Kb mRNA but no detectable synthesis of the 0.9 Kb mRNA (Montell et al., 1982). As shown in Figure 4c, infections with the hr1 mutant produced neither the 52K nor 48.5K polypeptides detected in wt infected cells (Fig. 4b), whereas both these products were detected in pm975 (Fig. 4d) infected cells. These results suggested that the 52K and 48.5K protein detected by immunoprecipitation with anti-tumor serum were both products of the 1.1 Kb message. In addition, it appeared that only the products of the 1.1 Kb mRNA were being detected in immunoprecipitates of wt-infected cells using anti-tumor serum.

#### Identification of E1A products in immunoprecipitates with anti-peptide serum

In a recent publication Yee et al. (1983) described a preparation of rabbit antiserum against a synthetic peptide corresponding to the carboxyl terminus of the E1A proteins (gly-lys-arg-pro-arg-pro) as predicted from the structure of the E1A-mRNA of Ad5 (Perricaudet et al., 1979). This anti-peptide serum (E1A-C1) reacted with the E1A-52K and 48.5K proteins detected by anti-tumor serum (Fig. 5a) but also immunoprecipitated a

Figure 4. Two-dimensional gel separation of  $^{35}\text{S}$ -methionine labeled proteins synthesized in wt and mutant infected cells. Cells were labeled for 2 hours from 6 to 8 hours p.i. and immunoprecipitated with anti-tumor serum A) mock, B) pm975, C) hr1, D) Wt. Only the portion of the gel which contains the E1A proteins is shown. The arrow in each autoradiogram represents the isoelectric position of a host cell contaminant protein.

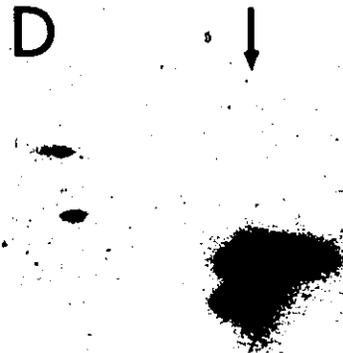
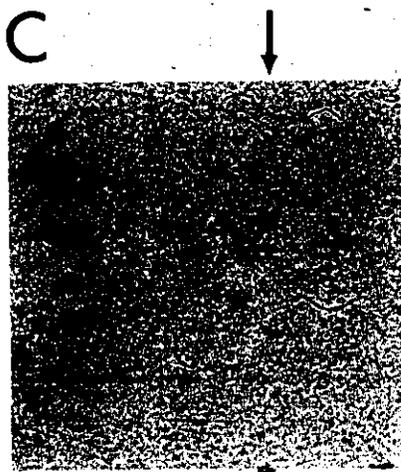
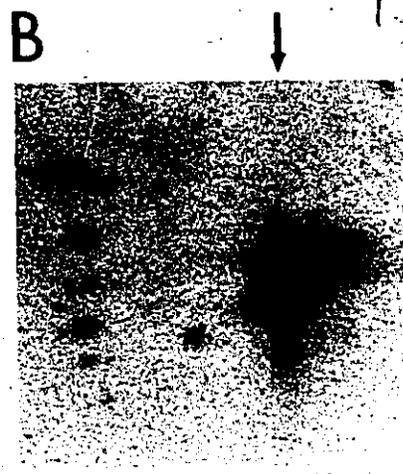
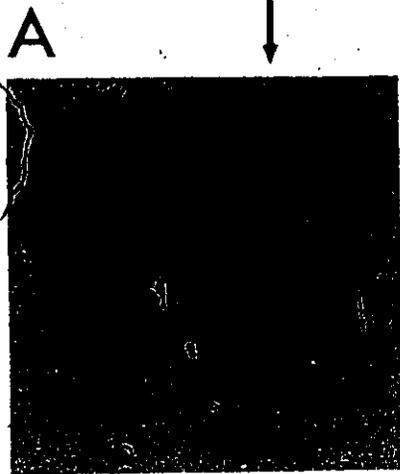


Figure 5. A) 10% polyacrylamide gel comparison of the E1A polypeptides immunoprecipitated from infected cells with E1A-C1 serum or polyvalent anti-tumor serum and separated on 10% polyacrylamide gel. Ad5 infected KB cells were pulse-labeled with  $^{32}\text{P}$ -orthophosphate for two hours from 6 to 8 hours p.i. B) Two-dimensional separation of the E1A polypeptides immunoprecipitated from  $^{32}\text{P}$ -labeled infected cells with the E1A-C1 rabbit anti-peptide serum. Numbers along the top of the autoradiogram represent approximate pH at the position indicated. The pH measurements were made on consecutive fractions of an isoelectric focusing gel poured and run in a parallel with the gel shown in the figure.

PS C1



-58

-52

-50

-48.5

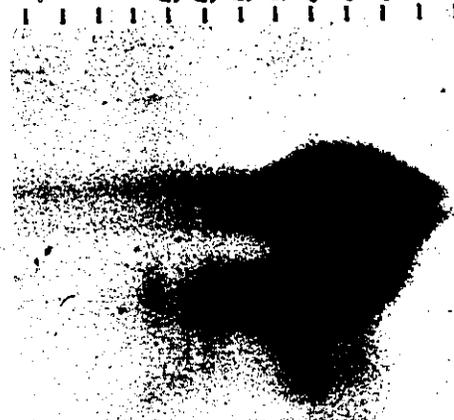
-45

-37.5

-35

**A**

-5.33  
-5.22  
-5.14  
-5.00  
-4.88  
-4.75  
-4.63  
-4.55



-52  
-50  
-48.5  
-45  
-37.5  
-35

**B**

50K, 45K, 37.5K and 35K from infected KB cells labeled from six to eight hours p.i. with  $^{32}\text{P}$ -orthophosphate. Two-dimensional gel electrophoresis revealed that the 50K and 45K had a slightly lower pI than the 52K and 48.5K (Fig. 5b) but all six proteins resolved as a cluster on the acidic side of the two-dimensional gel. The 37.5K and 35K were always recovered in reduced amounts compared to the four slower migrating products and were more readily visualized in autoradiograms of  $^{32}\text{P}$ -labeled than  $^{35}\text{S}$ -labeled samples.

The origin of the products detected in vivo with the anti-peptide serum was investigated by immunoprecipitation of extracts prepared from  $^{35}\text{S}$ -labeled KB cells infected with hr1 or pm975. Immunoprecipitates of  $^{35}\text{S}$ -labeled KB cells fortuitously contained minute quantities of contaminating cellular proteins which could be used as positional markers. The results (Fig. 6) clearly indicated that, of the four major species detected in wt infected cells with E1A-C1 serum, only the more basic 52K and 48.5K were present in pm975 infected cells. The reduced amounts of these products compared to wt may reflect the failure of mutants defective in the synthesis of E1A-1.1 Kb mRNA products to induce high levels of mRNA from region E1A (Gaynor et al., 1982). Hr1 infected cells appeared to synthesize only the 50K and 45K species. Analysis of  $^{32}\text{P}$ -labeled samples showed that the 37.5K product was present only in pm975 infected cells and the 35K product was present only in hr1 infected cells (data not shown). Taken together these results indicated that the 52K, 48.5K and 37.5K were products of the 1.1 Kb mRNA and the 50K, 45K and 35K were the products of the 0.9 Kb mRNA.

#### E1A proteins synthesized by Ad5 hr group I mutants.

The preceding analysis using defined mutants hr1 and pm975 revealed that at least three in vivo products could be assigned to each of the 0.9 Kb and

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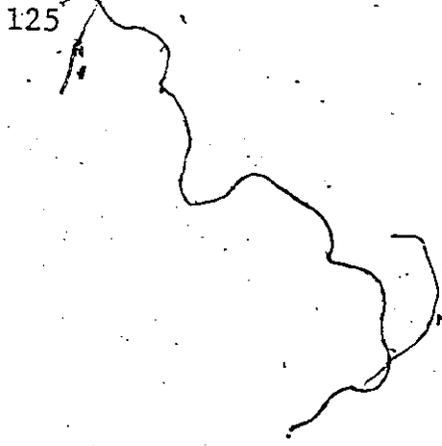
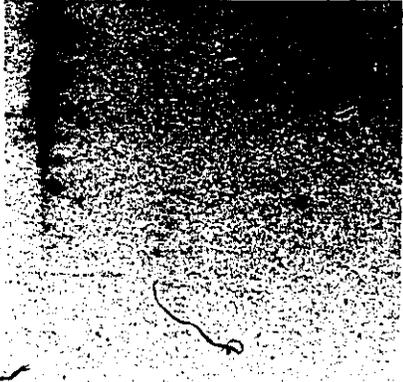
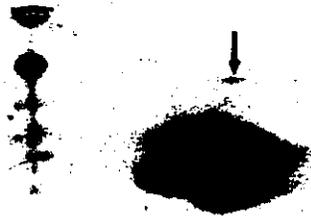


Figure 6. Two dimensional gel separation of  $^{35}\text{S}$ -methionine labeled proteins synthesized in wt and mutant infected cells. Cells were labeled for 1 hours from 6 to 8 hours p.i. and immunoprecipitated with the E1A-C1 antiserum, A) mock, B) wt, C) hr1, D) pm975. The arrow in each autoradiogram indicates the isoelectric position of the same host cell contaminant protein in each sample.

A



B



C



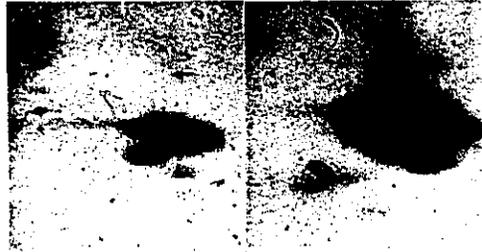
D



1.1 Kb E1A mRNAs. Characterization of the products synthesized in KB cells infected with additional hr mutants of complementation group I might provide clues concerning the nature of the presumably different proteins encoded by the same message. Extracts of  $^{35}\text{S}$ -methionine or  $^{32}\text{P}$ -orthophosphate labeled cells infected with wt or hr mutants 2-5 were immunoprecipitated with the E1A-C1 antiserum. As shown in the profile of Fig. 7,  $^{32}\text{P}$ -labeled E1A proteins immunoprecipitated from wt infected cells was identical to the profile of  $^{35}\text{S}$ -labeled proteins. The reduced amounts of the 45K protein was not entirely unexpected since we have noted over the course of these studies that both the 45K and 48.5K were often recovered with reduced intensities from infected KB cells compared to the 50K and 52K. An interpretation of this finding awaits a more complete characterization of the function of these proteins in lytic infection. Infections with hr3,4 and 5 produced two-dimensional electrophoretic profiles of immunoprecipitable E1A proteins similar to wt for both  $^{35}\text{S}$ - and  $^{32}\text{P}$ -labeled infected cells (Fig. 7). Only hr2 produced a different pattern of spots with both  $^{35}\text{S}$  and  $^{32}\text{P}$  profiles showing only two major species. The identity of these products was investigated by mixing equal aliquots of hr2 with the defined mutants hr1 and pm975 prior to two-dimensional gel electrophoresis. As shown in Fig. 8 mixing hr2 with pm975 produced a wt-like profile of four major species identical to that produced by mixing hr1 and pm975. In addition, mixing hr1 and hr2 immunoprecipitates indicated that the two products synthesized in hr2 infected cells could not be electrophoretically distinguished from the 0.9 Kb mRNA products synthesized in hr1 infected cells. The truncated products predicted from the sequence of hr1 were not detected by immunoprecipitation with anti-tumor serum and no equivalent hr2 product was detected with either anti-peptide or anti-tumor serum (data not shown). The identical phenotype

Figure 7. Two dimensional gel separation of  $^{35}\text{S}$ -methionine or  $^{32}\text{P}$ -orthophosphate labeled E1A proteins from wt and hr mutant infected cells. Cells were infected with 10 pfu/cell mutant or wt virus, labeled for 2 hours from 7 to 9 hours p.i., and immunoprecipitated with E1A-C1 antiserum. For each virus,  $^{35}\text{S}$ -labeled proteins are presented on the left,  $^{32}\text{P}$ -labeled proteins on the right.

**wt**



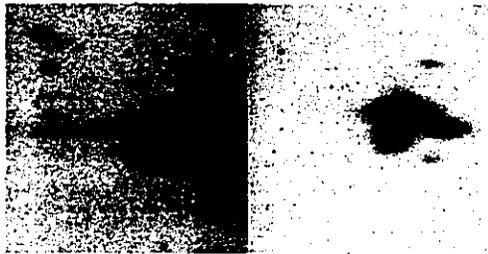
**hr2**



**hr3**



**hr4**



**hr5**

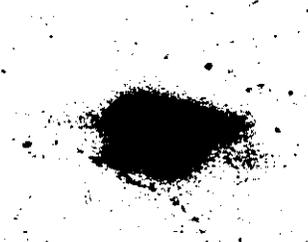


Figure 8. Two dimension gel separation of  $^{32}\text{P}$ -labeled E1A proteins from cells infected with mutants hr1, hr2 or pm975. Cells were infected with 10 pfu/cell, labeled from 7-9 hours p.i. and immunoprecipitated with E1A-C1 antiserum. For autoradiograms of combined samples, equal aliquots of the immunoprecipitated extracts for each mutant were mixed prior to gel analysis.

pm975



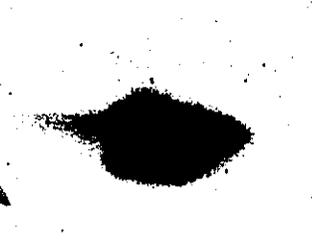
pm975+hr2



hr2



pm975+hr1



hr1



hr1+hr2



of these two mutants indicates that the defect of hr2 most likely involves a premature termination of the 1.1 Kb mRNA translation similar to hr1. These results suggest that although some of the host range mutants (hr1 and hr2) were defective for the synthesis of the 1.1 Kb mRNA products, all the mutants were capable of synthesizing the multiple forms of the E1A products derived from the individual messages (Fig. 9).

#### Effects of deletion mutants within E1A on the synthesis of E1A proteins

The in vivo translation products of each of the 1.1 Kb and 0.9 Kb mRNAs have been resolved into several electrophoretically distinct forms (Fig. 5). It is not known what factors influence the synthesis of E1A products possessing different properties. In an effort to determine what amino acid sequences might be important in generating the various forms of the E1A translation products, two mutants, dl1504 and dl313, containing deletions affecting E1A coding sequences were examined. dl1504 carries a 106bp deletion and is missing 44 bps upstream and 62 bps downstream from the normal 5' cap site of E1A mRNA including the AU of the first in phase AUG (Osborne et al., 1982). The mutant induces reduced levels of early mRNAs (including E1A) in infected HeLa cells but replicates with essentially wt kinetics to produce relatively high yields of virus. dl313 is a 2307 bp deletion between nucleotide 1334 and 3639 that fuses E1A coding sequences to the coding sequences of the protein IX at the right end of region E1b. This deletion results in the elimination of 70 amino acids from the carboxyl terminal of E1A proteins and the addition of 15 novel amino acids encoded by an unused reading frame within the protein IX coding sequences before a fortuitous in phase stop codon is reached.

The <sup>32</sup>P-labeled E1A proteins synthesized in dl1504 infected KB cells

were immunoprecipitated with anti-tumor or anti-peptide serum and analysed by one- and two-dimensional gel electrophoresis (Fig. 9). All four major E1A proteins from dl1504 infected cells showed a significantly faster migration rate compared to the four major species present in wt-infected cells. Identification of the products of the 1.1 Kb message by immunoprecipitation with anti-tumor serum revealed that the order of migration of the multiple forms of 1.1 Kb and 0.9 Kb mRNA products was not affected by the deletion mutation (Fig. 9a). These results suggest that the E1A mRNA of dl1504, which lacks the first AUG initiator codon at position 560, might be initiating protein synthesis at novel internal sites on the message, possibly at the next in phase AUG at position 602. Two-dimensional gel electrophoresis (Fig. 9b and c) revealed that the mutant proteins retained the generally acidic properties characteristic of the wt proteins, but the mutant products ca43.5K and 40K corresponding to the 48.5K and 45K of wt appeared to be more basic than the wt parental forms, which always focused very close to the 52K and 50K species.

Analysis of the E1A proteins synthesized in dl313-infected KB cells was only possible using anti-tumor serum since the mutation eliminates the carboxyl terminal antigenic site recognized by anti-peptide serum. The two species which were detected in dl313 infected cells with anti-tumor serum (Fig. 10) migrated slightly faster than the corresponding 52K and 48.5K species of wt. No E1b-58K tumor antigen was detected in the mutant infected cells because the deletion of dl313 includes the 58K coding sequences (2019-3506). Two-dimensional gel electrophoresis of proteins immunoprecipitated from wt or dl313 infected cells revealed that the products of the 1.1 Kb mRNA of wt and the corresponding message of dl313 had very similar isoelectric focusing properties (Fig. 10b and d). Neither of the

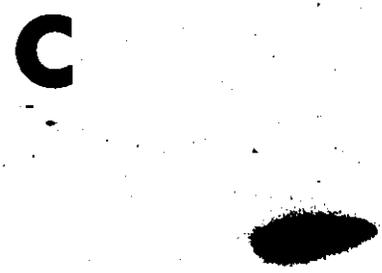
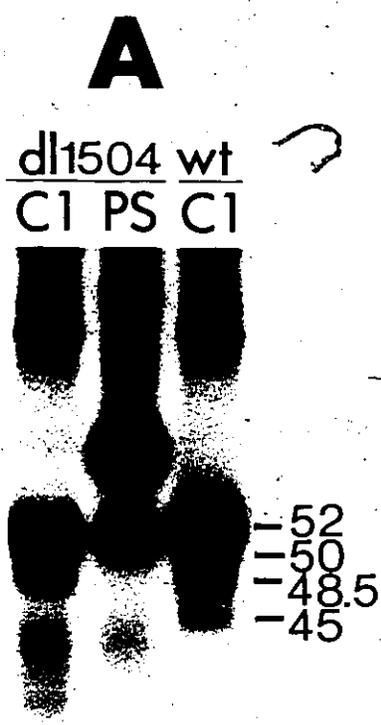
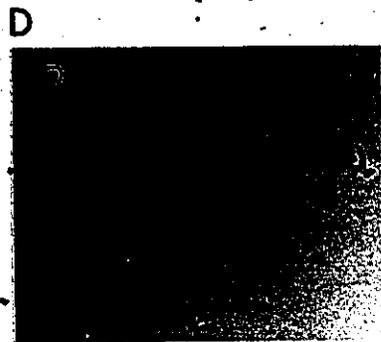
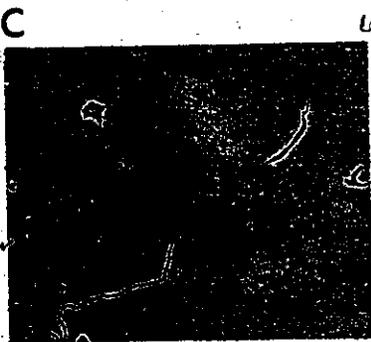
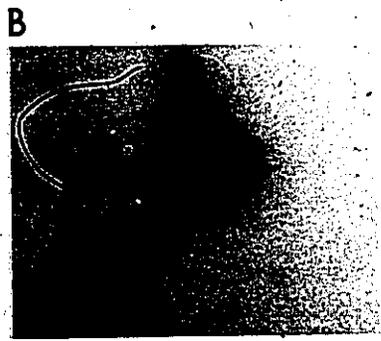
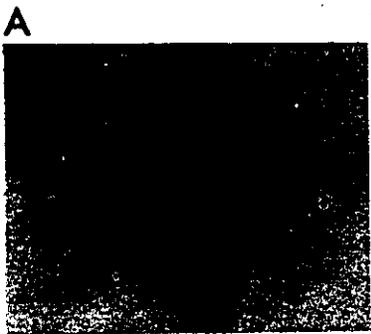


Figure 9. One-dimensional and two-dimensional gel separation of  $^{32}\text{P}$ -labeled E1A proteins from cells infected with wt or dl1504. Cells were labeled for 2 hours from 6 to 8 hours p.i. and immunoprecipitated with either E1A-C1 antiserum or polyvalent anti-tumor serum (PS). One-dimensional gel analysis is presented in (A) and two-dimensional resolution of E1A proteins from dl1504 infected cells immunoprecipitated with B) E1A-C1 antiserum or C) PS antiserum is shown on the right.

Figure 10. One-dimensional and two-dimensional gel separation of  $^{32}\text{P}$ -labeled E1A proteins from cells infected with wt or dl313. Cells were labeled for 2 hours from 6 to 8 hours p.i. One-dimensional gel analysis presented on the left: mock (M), dl313 and wt infected cells were immunoprecipitated with polyvalent anti-tumor serum. Two-dimensional gel analysis is presented on the right. A and B: wt infected cells immunoprecipitated with either E1A-C1 or PS antiserum, respectively. C and D: dl313 infected cells immunoprecipitated with either E1A-C1 or PS antiserum respectively.



products detected by anti-tumor serum in dl313 infected cells were immunoprecipitated by the E1A-C1 serum (Fig. 10c) which was capable of detecting all the 0.9 Kb and 1.1 Kb mRNA products in wt infected cell extracts (Fig. 10a). These results suggest that the 70 amino acids removed from the carboxyl terminal of dl313 E1A products were not directly involved in the generation of the multiple forms of E1A proteins derived from a single mRNA species.

## DISCUSSION

A number of recent reports have described the products of in vitro translation of E1A mRNA or of proteins made in drug-treated cells (Harter and Lewis, 1978; Halbert et al., 1979; Esche et al., 1980; Smart et al., 1981; Ricciardi et al., 1981) but none have examined these short-lived regulatory proteins as they are synthesized in infected cells in the absence of any treatment with metabolic inhibitors. Although these techniques include the possibility of artifactual products, such manipulations have been necessary to enhance the synthesis of E1A mRNAs which are normally made at only very low levels in infected cells. During the course of these studies on adenovirus tumorigenicity, I have found that anti-tumor sera generally react very weakly or not at all with the E1A proteins but several batches of antiserum raised in response to tumors induced in newborn hamsters with 1019-3 cells (transformed by hr50 DNA) showed a much stronger avidity for the same E1A antigens (Fig. 1). Using this antiserum I identified two major species of <sup>35</sup>S-labeled E1A proteins, 52K and 48.5K, which were also labeled by <sup>32</sup>P-orthophosphate (Fig. 2). Subsequent studies with a rabbit antiserum directed against a synthetic E1A-carboxyl terminal peptide revealed that these products represented a subset of the four major E1A phosphoproteins 52K, 50K, 48.5K and 45K (Fig. 5a). On two-dimensional gels these proteins focused as a cluster with a pI ca. 4.8 (52K and 48.5K ca. pI 4.9; 50K and 45K ca. pI 4.6). In addition, two minor species of 37.5K and 35K were often observed (Fig. 5b) in both <sup>35</sup>S- and <sup>32</sup>P-labeled samples.

The relationships of the major and minor species were studied by infection of KB cells with mutants pm975 (which synthesizes no 0.9 Kb mRNA) and hr1 (which synthesizes a truncated 1.1 Kb mRNA product) and permitted the

identification of the 52K, 48.5K and 37.5K species as products of the 1.1 Kb mRNA and the 50K, 45K and 35K species as products of the 0.9 Kb mRNA (Fig. 6). The pattern suggested by the results is that each E1A mRNA produces two major and one minor species, and it is possible that these products may differ by a post-translational modification of the polypeptide chain. In many instances, the 48.5K and 45K major products were recovered in greatly reduced amounts compared to the corresponding slower migrating species 52K and 50K. It is interesting to note in this regard that the numerous reports concerning E1A translation products in vitro or from drug treated cells vary with respect to both the number and the isoelectric points of the proteins observed (Halbert et al., 1979; Esche et al., 1980; Smart et al., 1981; Ricciardi et al., 1981; Gaynor et al., 1982). The reasons underlying these differences may eventually be understood in view of the many forms of E1A proteins which I have observed in vivo. The experiments also revealed that, in infected cell extracts containing all the E1A proteins, anti-1019-3 tumor serum was capable of recognizing only the products of the 1.1 Kb mRNA (Figs. 4 and 10). Anti-tumor sera directed against other transformed hamster cell lines which showed some specificity for E1A proteins also showed this narrow range of specificity (data not shown). Thus, it seems possible that the antigenic sites recognized by anti-tumor sera lie within or are determined by the sequence of 46 amino acids which are unique to the products of the 1.1 Kb mRNA.

A first step in examining the nature of the different forms of the E1A products was the characterization of the E1A products synthesized in KB cells infected with complementation group I host range mutants. Hr1 and hr2 were defective only for the synthesis of the 1.1 Kb mRNA products and the profiles of the hr3,4 and 5 could not be distinguished from wt. Therefore, none of

the host-range mutations appears to affect the production of multiple forms of E1A products from the individual mRNAs except in the case of hr1 and hr2 where all forms of 1.1 Kb mRNA products appear to have been affected. It is not clear from these results whether complete translation products are necessary to the formation of the multiple forms of E1A products since the truncated proteins expected in hr1 and hr2 infected cells from in vitro translation studies were not detected by immunoprecipitation. Thus it remains possible that multiple forms of these truncated products were present in the mutant infected cells. With these reservations, the results with these two mutants and pm975 do indicate that the products of either the 1.1 Kb mRNA or the 0.9 Kb mRNA alone were sufficient for the formation of multiple species of proteins from E1A message.

It seems likely that the different forms of the E1A translation products are due to modifications of the nascent polypeptides, possibly related to the phosphorylation of specific sites within the amino acid chain. Preliminary studies on the tryptic phosphopeptides of 52K, 48.5K, 50K and 45K suggest that some phosphorylation differences do exist (Yee et al., 1983). Studies with two mutants, d11504 and d1313, containing deletions affecting E1A coding sequences suggested that neither the amino terminal 15 amino acids nor the carboxyl terminal 70 amino acids were directly involved in the generation of the multiple forms of the E1A products (Fig. 9 and 10). The results of studies with these mutants and the E1A-C1 serum also suggest that neither proteolytic degradation, premature termination, nor use of potential internal translation initiation sites are implicated as mechanisms for producing any of the in vivo species of E1A proteins. Thus it seems that amino acid sequences in the body of the protein, excluding the 46 amino acids unique to the 1.1 Kb products, harbour the sites that are important for the

expression of the properties of the E1A products revealed by two-dimensional gel electrophoresis.

In a recent publication Yee et al. (1983) have reportedly obtained no evidence for the synthesis of a product from the E1A 0.6 Kb mRNA which accumulates late in infection (Spector et al., 1978). Since the anti-tumor sera do not recognize the products of the 0.9 Kb mRNA, this result might be expected in experiments employing immunoprecipitation with anti-tumor serum as the means of detecting 0.6 Kb mRNA products. However, no polypeptide possessing either the size or kinetics that might correspond to a 0.6 Kb mRNA product has been observed by immunoprecipitation with the E1A-C1 serum. Although a number of explanations might account for these observations, recent evidence suggests that the 3' splice site of the 0.6 Kb message is not identical to the 3' SS of the 1.1 Kb and 0.9K mRNAs (Dijkema et al., 1982; A. Vertanen, personal communication). The different splicing arrangement permits the use of a novel reading frame for the carboxy portion of the 0.6 Kb mRNA product thus terminating this protein with a unique amino acid sequence. Therefore, neither the anti-tumor sera nor the E1A-C1 serum appear to recognize the product of the 0.6 Kb mRNA. Anti-peptide sera with specificity for other regions of the E1A polypeptides are currently being developed which should encompass antigenic sites of the 0.6 Kb mRNA product(s).

CHAPTER VII

## CONCLUDING REMARKS

A role for adenovirus genes in the conversion of primary rodent cells to a potentially oncogenic state has been implied by studies on the viral DNA sequences retained and expressed in virus transformed cells (Gallimore et al., 1974; Lewis and Mathews, 1981; Green et al., 1981) and by studies of transformation by DNA transfection (Graham et al., 1974; Shiroki et al., 1977; Mak et al., 1979; van der Eb et al., 1979; Chapter II). The viral sequences with transforming activity appear to include all of the E1A transcription unit (0-4.5 m) and only the 5' half (4.5-8.0 m) of the E1B transcription unit. Of the two rightward transcribed Ad5-E1B early gene products (19K and 58K) the protein which appears to be dispensible in the establishment of morphologically transformed cells is the 58K (Schriber et al., 1979; Chapter II). Cells transformed by the Hind III G fragment (0-8.0 mu) of Ad5 did not appear to express 58K but were morphologically indistinguishable from transformed cells which contained and expressed all of E1 including the 58K. Recent studies with baby rat kidney cells transformed by the Hind III G fragment of either Ad5 or Ad12 showed that unlike cells expressing the E1B 58K (60K for Ad12, Bos et al., 1981) neither of these types of transformed rodent cells were tumorigenic in nude mice (van der Eb et al., 1979; Jochemsen et al., 1982). In contrast the Ad5 Hind III G fragment transformed hamster cells described in Chapter II possessed the same range of oncogenic potential in newborn hamsters as the cell lines which contained and expressed all of E1. These differences may in part be due to differences in the cell-animal systems being used in the different laboratories and sharply point out

the danger of drawing general conclusions concerning oncogenicity from investigations on a single animal model system.

It is also difficult to firmly identify the amount of ElB information required for cell transformation since this seems to depend to some extent on the conditions of the transformation assay. As shown in Chapter III transformation by virions requires expression of the intact ElB transcription unit whereas only part of ElB is necessary for transformation by DNA transfection. In addition transformation by DNA fragments containing ElA alone has been achieved but required less stringent assay conditions than has been employed in transformations with larger fragments and virions (Houweling et al., 1980). Such transformants are morphologically different from cells transformed by larger DNA fragments or virions suggesting that expression of ElB is required for establishment of 'fully' transformed cells. Apparently fully transformed and tumorigenic cell lines (1019-3 and 1019-C3) in which synthesis of neither the ElB-19K nor 58K can be detected were established by DNA transfection with DNA extracted from virions of the ElB mutant hr50 (Chapter III). This mutant expresses 19K following infection of HeLa or KB cells (Ross et al., 1980; our unpublished observations) - and recently several more rat and hamster cell lines have been established with hr 50 DNA transfection and preliminary data suggests that these lines express a normal ElB 19K product. It is possible that the lines 1019-3 and 1019-C3 are examples of transformed cells in which expression of ElB genes has been shut off (no ElB RNA has been detected in these cell lines; L. DeTina, personal communication). Thus, as with the requirement for 58K in virion-mediated transformation, expression of 19K may only play a transitory initial role in the

establishment of transformed cells. Recently cold-sensitive ElB host range mutants of Ad5 have been isolated but thus far neither the precise site of the lesion nor the contribution of the defective product towards maintenance of the transformed state have been determined (Ho et al., 1982). Similar mutants with lesions in ElA have provided the first genetic evidence that expression of viral proteins is required to maintain the transformed phenotype.

Determining the role of El proteins in transformation and oncogenicity must include consideration of the potential effects of truncated or chimeric products. The characterization of antisera from animals bearing tumors induced by Hind III G fragment transformed cells showed that some lines induced a response against the ElE-58K protein even though this product could not possibly be present in these cells (Chapter II). Since the cells contain the sequences coding for the amino half of this protein it seems likely that they synthesize a truncated or altered form of this product. Anti-tumor sera also detect additional minor antigens (16.5K and 15K) which have not as yet been mapped to the viral genome. Recent evidence has suggested a leftward reading 20S m RNA may specify products from open reading frames (URF-10 and URF-11) on the 1-strand early region I' (Katze et al., 1982; Gingeras et al., 1982). While the effects of these and other products remain unknown, the available evidence suggests that neither the ElB 19K nor 58K may be absolutely required for tumorigenicity or maintenance of transformation (Chapters II and III). The results of these studies and those previously reported (Graham et al., 1974; Shiroki et al., 1977; van der Eb et al., 1979; Jochemsen et al., 1982) suggest that tumorigenicity is a property largely determined by the expression of

cellular rather than viral genes. Nevertheless, some evidence does indicate that expression of E1B products in transformed cells may be responsible for the marked differences in oncogenicity which have been used to group the various serotypes of human adenoviruses (Jochemsen et al., 1982; Bernardis et al., 1982).

One approach to learning more about transforming gene products has been to study their synthesis and deposition in cells infected with wt virus or with mutants bearing lesions in the transforming region. The intracellular localizations described in Chapter IV utilize the ability of anti-tumor sera to specifically recognize and remove the products of E1A and E1B from solubilized extracts of infected cells. Multiple forms of the E1B-19K were discovered and found to be associated with membrane preparations but not found exposed on the outer surface of the infected cell. None of the forms of E1B-19K appear to be glycosylated (Persson et al., 1982) or phosphorylated (Chapter VI, Fig. 2). Pulse chase analysis has suggested that the 18.5-19K doublet band is the precursor to the 17.8 and 17K proteins (which might represent degradation products). It is of some interest to note that the association of virus coded transformation related proteins with internal cytoplasmic membranes has been reported for a number of other viruses including Rous sarcoma virus (Willingham et al., 1979; Rohrschnieder, 1979), Harvey murine sarcoma virus (Willingham et al., 1980) and polyoma (Ito, 1979; Griffin and Dilworth, 1983). The functional significance of these correlations remains unclear.

In contrast to the specific localization of the E1B-19K product, the E1B-58K was found in both the nucleus and cytoplasm but could not be localized to any specific structures. The 58K showed a greater affinity

for the nucleus at late times p.i. Since studies with ElB mutants (Ross et al., 1980a and Chapter V) have suggested some role for this product in regulating gene expression, it is interesting to note that this elevated nuclear association at late times parallels the reported restriction of host messages to the nucleus late in infection (Beltz and Flint, 1978). An analysis of the effects of 58K mutants on the levels of host cytoplasmic messages may be helpful in determining if 58K is involved in this process.

The study of ElA protein localization using anti-tumor sera has achieved only limited success. Firstly, the sera used reacted only weakly with ElA products although evidence for nuclear, cytoplasmic and particularly cytoskeletal association was obtained (Chapter IV). Then, after these studies were completed, it was discovered that the specificity of anti-tumor sera for ElA proteins was limited to the products of the 13S message (Chapters II and VI). Recently the subcellular distribution of ElA products was studied using ElA-C1 anti-peptide serum (Yee et al., 1983; my unpublished observations). These proteins appear to be located principally in the cytoplasm with the 52K and 50K major products associated with cytoskeletal preparations and the 48.5K and 45K major products enriched in membranes. Analyses of ElA proteins in mutant infected cells and examination of ElA protein associations with other subcellular components is currently in progress to help determine the exact meaning of the surprising properties of the closely related ElA gene products.

For some time it has been clear that El genes are involved in the regulation of early viral gene expression. The experiments described in Chapter V examined the roles of ElA and ElB in gene regulation by

comparing the kinetics of synthesis of tumor antigens in cells infected with wt or host range mutants (with lesions in E1A or E1B). In general, E1A defects have previously been characterized as causing an abrogation of early gene expression (Berk et al., 1979; Jones and Shenk, 1979a,b; Nevins, 1981; Persson et al., 1981; Shaw and Ziff, 1982; Katze et al., 1982) and although there is not complete agreement on the precise mechanism the consensus appears to be that the normal E1A products are required to activate early gene expression. The kinetics of protein synthesis for regions E1A, E1B, E2 and E4 in hr3 (E1A mutant) infected cells are consistent with the proposed role of E1A gene products in the regulation of gene expression (Chapter V). While the kinetic experiments I have done do not indicate a mechanism for E1A regulatory effects, direct interaction with viral templates (although not ruled out), seems unlikely considering the cytoplasmic localization of the E1A proteins discussed above. Indeed, other workers have also concluded that E1A products exert their transcriptional effects indirectly by inactivating cellular factors which would otherwise somehow block viral early transcription (Nevins, 1981; Katze et al., 1982).

The kinetics of early translation in cells infected with the E1B mutant hr 6 were generally characterized by delayed and reduced levels of protein synthesis (Chapter VI). The defect of hr6 does not appear to block any particular step in the lytic cycle since the mutant executes a complete program of gene expression including viral DNA replication; late protein synthesis and capsid assembly (Lassam et al., 1979; Chapter VI). At both early and late times, however, it appears that the efficient expression of viral genes is impaired. Further analyses of the accumulation and translation of mRNA in hr6 infected cells will be

required before the mechanism of this inhibition is understood. It seems clear from these studies that E1B products are involved in regulating gene expression and that the principle defective product is the 58K major tumor antigen.

An important advance in the study of adenovirus tumor antigens has been the development of antisera specific for chemically synthesized oligopeptides corresponding to short regions of the E1 proteins as determined from the nucleotide sequence (Yee *et al.*, 1983). Previous studies on E1A protein have required the use of *in vitro* translation of selected messages or drug-treated cells because these products are normally made at only very low levels in infected and transformed cells (Harter and Lewis, 1978; Halbert *et al.*, 1979; Esche *et al.*, 1980; Smart *et al.*, 1981; Ricciardi *et al.*, 1981). Such manipulations are prone to artifacts and indeed various values for the number, molecular weight and pI of E1A proteins have been obtained. The studies described in Chapter VI represent the first detailed examination of the E1A proteins made by cells early after lytic infection. Using an antiserum specific for the carboxy terminus of the E1A proteins and a variety of E1A mutants it has been possible to identify at least eight related products which appear to be made from the E1A 12S and 13S mRNAs. The multiple products made from each message are almost certainly the result of post-translational modifications, possibly involving phosphorylation. Multiple products are synthesized in cells infected with E1A deletion mutants dl1504 and dl313 indicating that neither the amino terminal 15 amino acids nor the carboxy terminal 70 amino-acids are involved in the generation of the multiple species. Determining the type and location of the modification(s) responsible for generating multiple species and how

These changes correlate with the intracellular deposition of the E1A products will be necessary steps in elucidating the mode of action of these transforming genes.



Publications Arising from Thesis-related Work

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- 2) Rowe, D.T. and Graham, F.L. (1983). Transformation of rodent cells by DNA extracted from transformation-defective adenovirus mutants. *J. Virol.* 46: 1039-1044.
- 3) Rowe, D.T., Yee, S.P., Otis, J., Graham, F.L. and Branton, P.E. (1983). Characterization of human adenovirus type 5 early region 1A polypeptides using anti-tumor serum and an antiserum specific for the carboxy terminus. *Virology* 127: 253-271.
- 4) Rowe, D.T., Ruben, M., Bacchetti, S. and Graham, F.L. (1982). Identification of an adenovirus function required for initiation of cell transformation possibly at the level of DNA integration. *Proc. 4th Tech. Transfer Workshop*, ed. M. Pearson and N. Steinberg, Raven Press.
- 5) Rowe, D.T., Graham, F.L. and Branton, P.E. (1983). Intracellular localization of adenovirus type 5 tumor antigens in productively infected cells. *Virology*, in press.
- 6) Rowe, D.T., Branton, P.E., Yee, S.P., Bacchetti, S. and Graham, F.L. (1983). Establishment and characterization of hamster cell lines transformed by restriction endonuclease fragments of adenovirus type 5. *J. Virology*, accepted for publication.
- 7) Rowe, D.T., Branton, P.E. and Graham, F.L. (1983). The kinetics of synthesis of early viral proteins in KB cells infected with wild-type and transformation-defective host-range mutants of human adenovirus type 5. *J. gen. Virol.*, accepted for publication.

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