CHARACTERIZATION OF HUMAN ADENOVIRUS TYPE 5 EARLY REGION 1 PROTEIN USING ANTI-PEPTIDE ANTIBODIES

by

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

October, 1985.

Characterization of Human Adenovirus type 5 Early Region 1 Proteins Using Anti-peptide Antibodies

DOCTOR OF PHILOSOPHY (1985)
(Medical Science)

MCMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Characterization of Human Adenovirus type 5 Early Region 1 Proteins Using Anti-peptide Antibodies

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NUMBER OF PAGES: 290

ABSTRACT

Human adenoviruses are known to transform rodent cells in culture and these cells are tumorgenic when injected into new born animals. It has been well established that the early region 1 (E1) of human adenovirus type 5 is necessary and sufficient for oncogenic transformation. The E1 region is comprised of two transcription units known as E1A (0 to 4.5% of the genome) and E1B (4.5 to 11.2%), each of which produces multiple species of mRNAs and polypeptides. E1A is also required to activate the transcription of other viral early regions. In the present study anti-peptide sera were used to identify and characterize these viral proteins.

Anti-peptide sera specific for the amino- and carboxy-termini of E1A were raised and these two sera precipitated an identical set of four major polypeptides of 52, 50, 48.5, and 45K and two minor species of 37.5 and 35K. Studies using E1A mutant viruses also revealed that 52, 48.5, and 37.5K polypeptides are derived from the 1.1 kb mRNA, and the 50, 45, and 35K species from the 0.9 kb mRNA of E1A. These sera were also used to identify polypeptides that are associated with E1 proteins. A set of five cellular

polypeptides consisting of >250K, 105K (doublet), 68K, and 65K species were found to co-precipitate with E1A proteins under various conditions and the nature of this association was investigated using the anti-peptide sera as well as an E1A-specific monoclonal activity.

Antisera against synthetic peptides corresponding to the both termini of E1B 58K were also raised and used to identfy 58K from wild-type and mutant-infected cells. It had previously been shown that protein kinase activity was associated with 58K. To ask if protein kinase activity was intrinsic to this viral protein several conventional methods were used to purify 58K and the results suggested that such activity may be intrinsic to this viral protein.

The anti-peptide sera were used to purify E1 proteins. A simple purification procedure using these sera and their corresponding synthetic peptides was developed and highly purified 58K and E1A proteins were obtained. Attempts were made to study protein kinase activity using these purified E1 proteins, however, the results were inconclusive and it was not possible to unequivocally determine if kinase activity was intrinsic to them.

ACKNOWLEDGEMENTS

It is difficult for me to adequately express my appreciation to my supervisor, Dr. Philip Branton, for his patience and guidance during the ups and downs of the study. Throughout these years he has not only taught me the excitment of science, but also provided encourgement in the tough time when nothing seemed to work. With his support I made this thesis a reality.

I would also like to thank the members of my supervisory committee Dr. James Smiley and Dr. Frank Graham for their advice throughout this work and thorough review of my thesis and the latter for also providing the various Ad5 mutants during these studies. My appreciation is also extended to Dr. Mark McDermott and Dr. N. Balachandran for their valuable suggestions on the immunological techniques.

The excellent technical help of Sylvia Cers, Joceline Otis and John Rudy are grateful appreciated. The helpful discussions of Michel Tremblay during the lunches, coffee breaks and ping-pong games, and as well the people in the lab, past and present, are greatly acknowledged. I would also like to thank Ernest Chan and his family for providing unlimited access to their Apple IIe and Applework for the preparation of this manuscript.

The work leading to this thesis would have never been completed without the encouragement and support of the persons to whom this thesis is dedicated.

This thesis is dedicated to

mom and dad

and

Wai-Sin

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

EDTA	ethylenediaminetetraacetic acid
dCTP	deoxycytosine triphosphate
dCMP	deoxycytosine monophosphate
M_R	molecular weight
DNA	deoxyribonucleic acid
mRNA	messenager ribonucleic acid
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
TCA	trichloroacetic acid
PPO	2,5-diphenyloxazole
DMSO	dimethylsulfoxide
SV40	simian virus 40
RSV	Rous sacroma virus
TEMED	N,N,N',N'-tetramethylethylenediamine
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ad5	adenovirus type 5
Ad2	adenovirus type 2
uCi	micro-Currie
ATP	adenosine triphosphate
ug	microgram
ml	millilitre
ul	microlitre
mM	millimolar
min	minute
vol	volume
Tris	Tris hydroxymethyl aminomethane
Tris-HCl	Tris-hydrochoric acid
m.u.	map unit

Chapter 1

Introduction

1.1 Human Adenoviruses

1.1.1. Classification of Human Adenoviruses

To date at least thirty-one recognized serotypes of human adenoviruses have been classified into several groups that share common properties (Flint, 1980a; Wadell et al, 1980). They have been classified into subgroups according to the base composition (G+C content) of their DNA (Pina and Green, 1965), the degree of their nucleic acid homology as measured by DNA:DNA and DNA:RNA hybridization (Lacy and Green 1964, 1965, 1967; Bartok et al 1874; Garon et al 1973), and also their ability to agglutinate erythrocytes of human, monkey, and rat in vitro (Rosen 1958, 1960; Kasel et al 1960, Zuschek 1961). They have also been gathered into four groups according to their ability to induce tumours in newborn hamsters. Serotypes in subgroup A which are highly oncogenic are able to induce tumours rapidly in the majority of inoculated animals. The weakly oncogenic subgroup B only has a limited capacity to induce tumours and members of subgroup C and D which are weakly oncogenic have a limited capacity to induce tumours (Huebner, 1967; McAllister et al., 1969). A fifth and sixth group

(groups E and F) have also been identified by molecular methods (Wadell, 1979; Gerna et al., 1982; Gary et al., 1982; Retter et al., 1979). These classification schemes are based on their biological properties and nucleic acid sequence homology and, surprisingly, there is a good correlation using the different schemes to group the serotypes.

Of all the human adenovirus serotypes, the closely related types 2 and 5, both members of subgroup C, are the best characterized. They share 98.8-99.4% DNA homology and are non-tumorgenic in newborn hamsters, but will transform cells in tissue culture. Unless otherwise stated, information presented in the remainder of this introduction pertains to these two serotypes.

1.1.2. Structure and Composition of Adenoviruses

The adenovirus virion is about 60-70 nm in diameter and is composed of an inner core and a highly characteristic icosahedral outer shell. The virus is nonenveloped and contains 87% protein and 13% DNA (Green and Pina,1963). The capsid is built up from 252 capsomers, comprising 240 hexons (termed hexon because each of these is surrounded by six neighbors) and 12

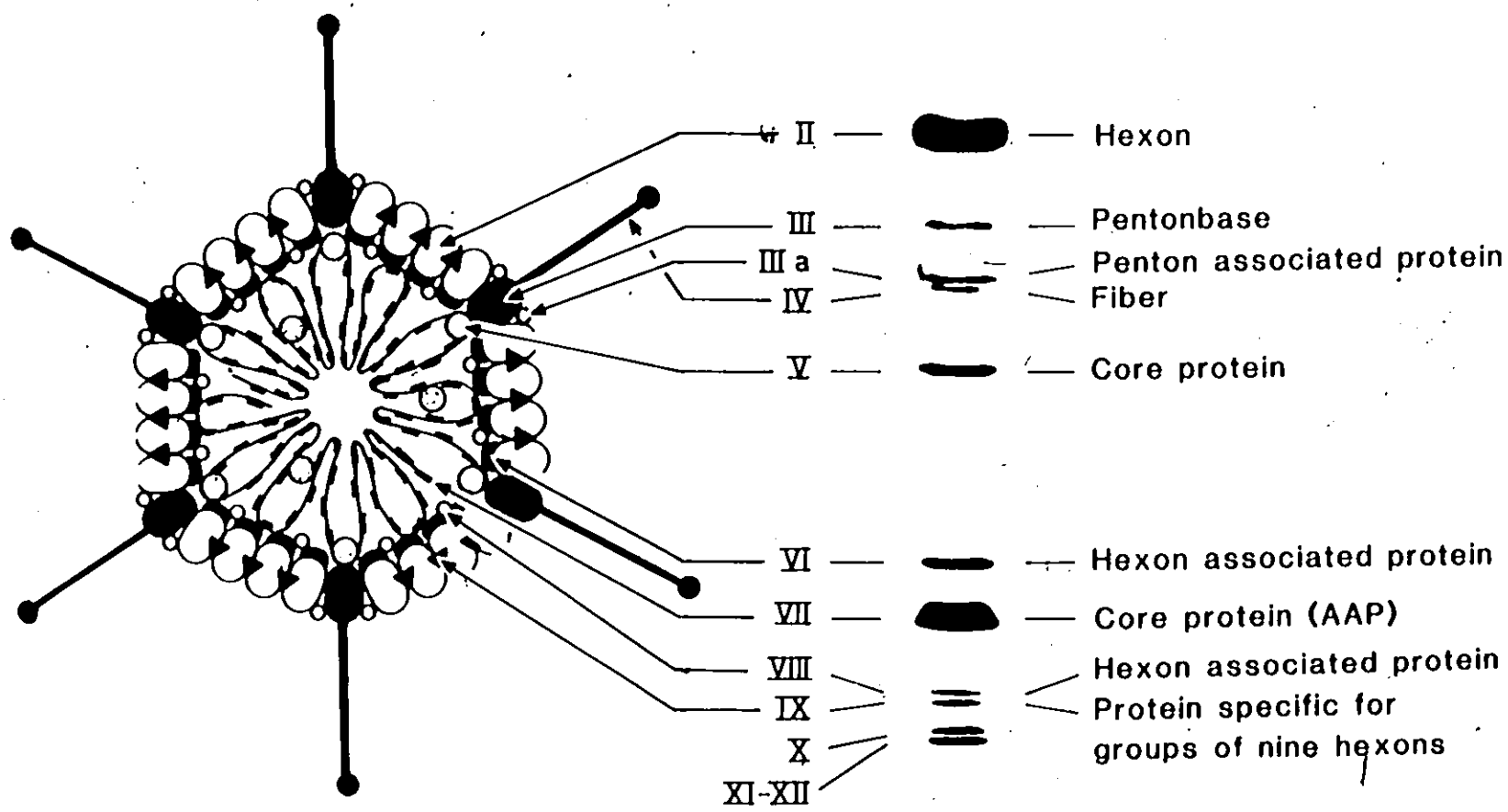
vertex capsomers at the vertices of the icosahedron each of which is surrounded by five peripentonal hexons and thus termed pentons. The pentons are composed of a base, which is anchored in the capsid, and an outward antenna-like projection termed the fibre. The core of the virion contains the viral genome, its associated nucleoproteins, and other polypeptides, some of which are involved in stabilizing the subunits of the capsid (reviewed by Ginsberg,1979; Philippison et al, 1975; Flint, 1980a). The viral core with a density of 1.42 g/cm^3 (Laver et al, 1968 mg) contains 18-20% of the total protein of the virion. These cores can be released by treatment of virions with heat, formamide, pyridine, and urea (Philippson and Lindberg, 1974).

The polypeptide composition of the adenovirus particle has been studied primarily by SDS-PAGE (see fig. 1). The results revealed an unexpected complexity (Maizel et al, 1968 and 1971) and up to 15 structural polypeptides have been described. It is now well established that virions contain at least nine unique polypeptides (II, III, IIIa, IV, V, VI, VII, VIII and IX) and several minor species which represent unique viral polypeptides present in only a few copies per

Figure 1

Virion Structure of Adenovirus.

(Adapted from Persson and Philipson, 1982).


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

virion, or cleavage products which remain when capsid polypeptides are cleaved during virion assembly.

Polypeptides II, III, and IV with molecular weights of 120,000, 85,000, and 62,000, respectively, are the major polypeptides of the capsid (Maizel et al, 1968b). Polypeptide II is the subunit of the hexon and three molecules constitute one hexon structure (Horowitz et al, 1970; Cornick et al, 1973; Jornvall et al, 1974). Polypeptide III is the subunit of the penton base which is probably comprised of five such subunits. Three molecules of polypeptide IV form the fibre protein. Polypeptide IX with a molecular weight of 12,500 is isolated with the groups of nine hexons and is thought to play some role in binding hexons together into ninemers (Everitt et al, 1973). It also appears to be partially exposed at the virion surface (Everitt et al., 1973; Maize; et al., 1968a,b). Polypeptide VIII (M_r=13,000) is found in association with individual hexons and appears to be located on the inner surface of the capsid. Polypeptide VI (M_r=24,000) is also localized internally, associated with hexons. Polypeptide IIIa (M_r=66,000) appears to be associated with peripentonal hexons close to the vertex regions of the virions. It can also be cross-linked to an internal

core polypeptide VII and may therefore extend into the interior of the virion (Everitt et al, 1973, 1975). Two arginine-rich polypeptides are associated with the cores, polypeptide V (M_r=48,500) and VII (M_r=18,500). They are present in 180 and 1100 copies in each particle (Everitt et al, 1973, 1975). A third polypeptide (M_r=55,000), present in only two copies, is covalently attached to the molecular ends of the viral DNA (Robinson and Bellett, 1974; Rekosh et al, 1977). The core-associated polypeptides probably mediate the packaging of adenovirus DNA within the virions, but the adenovirus DNA in the core particles appears not to be protected from nuclease attack in a way similar to cellular DNA packaged in nucleosomes (Tate and Philipson, 1979; Brown and Weber, 1980).

1.1.3. The Adenovirus Genome

The genome of adenovirus is an uninterrupted, linear molecule of double-stranded DNA (van der Eb and van Kesteren, 1966; Green et al, 1967; van der Eb, 1969). The genome size of adenovirus varies only slightly among the subgroups, from 20×10^6 daltons of the highly oncogenic serotypes 12, 18, and 31 to 23×10^6 daltons of the non-oncogenic serotypes 2 and 5 (Green

et al 1967). The strands of adenoviral DNA can be separated in alkaline cesium chloride equilibrum gradients (Sussenbach et al 1973), with heavy (h) and light (l) buoyant density strands corresponding to the 'r' and 'l' strands, respectively, according to an adopted nomenclature which names complementary strands according to the respective rightward (r) and leftward (l) direction of transcription (Sharp 1977) .

The viral DNA released from virions is covalently attached to a terminal protein at the 5' end. Adenovirus DNA molecules have two unique structural features: inverted terminal repetitions and palindromic sequences near the termini. The DNA molecules form single-stranded circles when denatured and renatured at low DNA concentrations due to the presence of complementary sequences at the end of each DNA strand (Garon et al, 1972; Wolfson and Dressler,1972). Using specific endonucleases to cleave DNA termini (Roberts et al 1974), and electron microscopy (Wu et al 1977) the Ad2 repetition has been estimated at 100-140 nucleotides. More recently direct sequencing of the ends of adenoviral DNA has confirmed this estimate, indicating 102 and 103 nucleotides for the repeats of Ad2 and Ad5, respectively (Steenbergh et al 1977;

Arrand and Roberts 1979). The second unusual structural feature of the adenovirus DNA molecules is the presence of palindromes. The exposed single-stranded DNA segment folds back on itself to form a hairpin structure, and this palindrome is located approximately 180 nucleotides from each terminus of adenoviral DNA (Padmanabhan and Green 1976). The hairpin structure was isolated by digestion with S1 nuclease and was shown by electrophoresis to be comprised of about 50 base-pairs. The functions of the inverted terminal repetition and palindrome remain largely unknown.

1.1.4. Functional Organization of the Viral Genome

The transcription of adenovirus RNA is conventionally divided into two stages, early and late, which are separated by the onset of viral DNA replication. Early viral RNA was shown to hybridize to four discrete and widely spaced regions of the viral genome designated E1 to E4 (Ortin et al 1976; Philipson et al 1974; Sharp et al 1974; Smiley and Mak 1978). Regions E1 and E3 are transcribed from the viral r strands, whereas regions E2 and E4 are transcribed from the viral l strands. Further studies have shown that region E1 can be subdivided into two separate

transcription units, termed E1A and E1B (Sehgal et al 1979; Wilson et al 1979). These transcription units are expressed at early times (2-8 hours post-infection) before the onset of DNA replication.

Eight transcription units have been identified on the adenovirus genome (see fig. 2). In addition to the five early transcription units there exist the major late transcription unit and two transcription units for mRNAs expressed at intermediate times of infection. The major late transcription unit is utilized for expression of almost all the virion polypeptides (Lewis et al 1980; Petterson and Mathews 1977) with the exception of polypeptides IX and IVa whose mRNAs are derived from two other transcription units and are promoted and expressed independently (Alestrom et al 1980; Wilson et al 1979; Chow et al 1977; Chow et al 1980). Both polypeptides IX and IVa are usually referred to as intermediate polypeptides because they accumulate before and independent of viral DNA replication (Chow et al 1980; Persson et al 1979).

Most transcription units produce multiple mRNA species as a result of splicing. Region E2 differs from the other transcription units by using alternative promoter sites for initiation of transcription (Chow et

Figure 2

Transcription map of Ad 2

(adapted from Sharp, 1984)

A
0
10
20
30
40
50
60
70
80
90
100
E Ia E Ib
E II
MLP
[VA]
52,55K
[IIIa]
[III]
[PVII]
[V]
[PVI]
[II]
72K
33K
100K
[P VIII]
[IX]
E III
E IV
140K
80K
E II (75)
E II (72)
(39)
(50)
(61.5)
(79)
(91.5)
l strand
r strand

al 1979). During the switch from early to late phase a promoter shift occurs so that the E2 mRNAs are preferentially transcribed from an alternative promoter. With the major late transcription unit, its promoter generates a primary transcript consisting of approximately 28,000 nucleotides (Evans et al 1977) that is then spliced in different fashions to generate the mRNAs for all the capsid proteins with the exception of polypeptide IX (Berget and Sharp 1979; Chow et al 1977; Lewis et al 1980; Pettersson and Mathews 1977). Spliced messages from the late promoter share three short segments of 5' untranslated leader RNA with a combined length of 203 nucleotides derived from sequences at map coordinates 16.5, 19.6 and 26.6 (Akusjarvi and Petterson 1979a; Akusjarvi and Petterson 1980b; Berget et al 1977; Chow et al 1977; Zain et al 1979). The 3' ends of the mRNAs are made by 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) and this allows the late mRNAs to be divided into five 3' coterminal families designated L1 to L5. The fact that many late adenovirus mRNAs share common 3' ends means that they are structurally polycistronic,

although in a functional sense they are monocistronic. Although this promoter is termed "major late promoter", it has been shown that it is also somewhat active at early times of infection (Akusjarvi et al 1981; Chow et al 1980; Fraser et al 1979; Kitchingman and Westphal 1980; Lewis and Mathews 1980).

1.1.5. Productive Infection by Adenoviruses

(a) Absorption and Uncoating

The viral infection starts with the absorption of the adenovirion to specific receptors on the plasma membrane of the host cells (Lonberg-Holm and Philipson, 1969; Levine and Ginsberg, 1967; Meager et al., 1977; Philipson et al., 1968). The virions are bound to the receptors through the fiber antigen (Dorsett and Ginsberg, 1975; Philipson et al., 1968; Sundquist et al., 1973) located at the 12 vertices of the virion (Valentine and Perira, 1965). Attempts have been made to isolate and characterize the virus receptor on KB cells and three polypeptides with apparent molecular weights of 78,000, 42,000, and 34,000 have been identified (Hennache and Boulange, 1977), of which one, the 42K species, is glycosylated (Svensson et al.,

1981). The binding of the virions leads to the redistribution of the viral receptors in the plasma membrane and subsequently results in a clustering of attached virions (Perrson et al., 1983; Pattersson and Russell, 1983). This reorganization mediates a destabilization of the virus particles and partial uncoating occurs at the outside of the plasma membrane leading to the loss of pentons and partial exposure of the genome (Lawerence and Ginsberg, 1967; Philipson, 1967; Sussenbach, 1967). Metabolic inhibitor studies suggest that metabolizing cells are required for uncoating (Svensson and Perrson, 1984). The modified virions are internalized within vesicles on route to the nucleus. Different mechanisms of internalization of the virions have been suggested, involving pinocytosis (Dales 1962; Chardonnet and Dales 1970a,b), direct penetration of the membrane (Lonberg-Holm and Philipson 1969; Morgan et al. 1969), or absorptive endocytosis (Svensson and Perrson, 1984) as described by Goldstein et al. (1979).

Within a few minutes of entering the cell, the pentonless particles move rapidly to the perinuclear cytoplasm by a poorly understood mechanism (Dales and Chardonnet, 1973; Luftig and Weihing, 1975) and appear

to become associated with pores in the nuclear envelope (Chardonnet and Dales, 1970a,b; Dales and Chardonnet, 1973). The remaining capsomers from the DNA-protein core are removed and the viral cores then enter into the nucleoplasm. Both uncoating at the nuclear membrane and entry into the nucleus seem to be mediated by an ATP-dependent activity (Chardonnet and Dales, 1972). The final stage of uncoating occurs to generate naked DNA-terminal protein complexes which become tightly associated with the nuclear matrix (Philipson et al. 1968; Younghusband and Maundrell 1982). The entire process is usually completed in about 2 hours (Philipson et al. 1968) and can occur in the presence of inhibitors of protein and nucleic acid synthesis, suggesting that de novo gene expression by the host cell is not required (Lawrence and Ginsberg 1967; Philipson 1967).

(b) Early Gene Expression

Not all of the eight transcription units are activated simultaneously during lytic infection by adenovirus. The production of adenovirus mRNAs during lytic infection is regulated by a complicated set of events involving both viral and cellular proteins. All

the adenovirus genes are transcribed by cellular RNA polymerase II except the VA RNA genes (Price and Penman, 1972; Weinmann et al. 1974). VA RNAs are low molecular weight viral RNAs that are synthesized by RNA polymerase III (Price and Penman, 1972; Soderland et al., 1976; Weinmann et al., 1974; Weinmann et al., 1976). The role of VA RNAs in viral replication is still largely unknown.

During the infectious cycle the five early transcription units are expressed with different kinetics (Nevins et al., 1979; Persson et al., 1981a). Region E1A is the first early gene to be expressed, and transcripts from this region can be detected as early as 45 min after infection. The maximum rate of transcription from the E1A promoter is thereafter maintained for at least 6 hours. Transcription from regions E3 and E4 begins around 1.5 hr post infection and reaches a maximum rate at about 3 hour post infection, followed by a decline. Region E2 is the last early transcription unit to be activated and the transcription rate peaks at 7 hours post infection then starts to decline (Nevins et al., 1979). The transcription rate from the E1B promoter appears to be

constant throughout the infection (Wilson and Darnell, 1981).

Expression of early regions of adenovirus is controlled at the level of transcription (Nevins 1981), at least in part by virus-encoded functions. Studies using host-range mutants (Berk et al., 1979) or deletion mutants (Jones and Shenk, 1979b) that have lesions in region E1A suggested that E1A products are necessary for efficient expression of other early regions. Cells infected with these mutants fail to accumulate cytoplasmic mRNA from early regions E1B, E2, E3, and E4. At low multiplicity of infection the growth of these mutants in HeLa cells is severely restricted. However, with 293 cells, which are Ad5-transformed human embryonic kidney cells that express RNAs and proteins from viral DNA encompassing the leftmost 11% of the viral genome (Graham et al., 1977; Aiello et al., 1979), both the host-range mutants and the deletion mutants grow as well as wild-type virus (Harrison et al. 1977; Jones and Shenk 1979a; Schrier et al., 1979; Persson et al., 1981b). Thus, the viral gene products from E1A are required for the expression of the other early regions.

Two mRNAs of 1.1 and 0.9kb in length are transcribed from E1A early after infection. These two mRNAs code for overlapping polypeptides (see below for more details). To differentiate functions of the overlapping E1A-specific gene products mutants with defects affecting either the 1.1 or 0.9k E1A mRNAs have been isolated and used to study their role for the expression of the other early regions. The results show that transcriptional control is mediated primarily by a product(s) of the 13S mRNA (Ricciardi et al., 1981; Montell et al., 1982). However, when recombinant plasmids that produce either the 13S or 12S gene products are cotransfected into HeLa cells with plasmids containing the viral EIIa or EIII transcription units, the individual products of the 13S and 12S E1A mRNAs both stimulate EIIa and EIII transcription (Leff et al.,1984). Two other Ad5 mutants, which produce either the 13S or 12S E1A mRNAs were constructed and used to characterize the function of the E1A mRNA gene products (Winberg and Shenk, 1984). The mutant that produces only 13S mRNA generated near wild-type yields in HeLa cells while the virus yields of the mutant that synthesizes only 12S mRNA were only 5-fold reduced. These results disagree with

the earlier findings and suggest that the products of both E1A early mRNAs are capable of stimulating the transcription of other early genes.

(c) Viral DNA Replication

The synthesis of viral DNA commences around six hours post infection and reaches maximal levels six to ten hours later (Ginsberg et al 1967; Pina and Green 1969). Ad5 DNA replication proceeds via a displacement mechanism. Replication may start at either end of the linear double-stranded genome and proceeds by a strand displacement mechanism until one of the parental strands is completely displaced (Lechner and Kelly 1977; Sussenbach et al., 1972; Sussenbach and Kuijk, 1977; 1978). Because the adenovirus DNA carries inverted terminal repetitions (Garon et al 1972; Wolfson and Dressler 1972), the liberated single strand presumably will form a circle which is maintained through base pairing between the two ends, thus generating a panhandle-like structure. The replication of the displaced single strand is then presumed to initiate in the panhandle structure. However, it has not been demonstrated directly whether the template for complementary strand synthesis is completely

single-stranded or via a panhandlelike structure (Daniell 1976). The finding that adenovirus genomes lacking part of the left inverted terminal repeat produce infectious progeny which contain two complete terminal repeats is consistent with the formation of a panhandle structure as an intermediate in adenovirus DNA replication (Stow 1982).

The development of an in vitro system that allows the replication of adenovirus DNA in soluble nuclear extracts has greatly increased our understanding of the DNA replication mechanism used by adenovirus (Arens et al 1977; Challberg and Kelly 1979; Enomoto et al 1981; Horwitz 1976; Ikeda et al 1981; Kaplan et al 1977; Kaplan et al 1979; Stillman 1981; Yamashita et al 1977). Extracts from infected cells can utilize exogenously added viral DNA as a template for replication. The availability of in vitro systems makes it possible to analyze the different steps in replication, to define the origin of replication, and furthermore to identify enzymes and other components that are necessary for adenovirus DNA replication. With this system it has been shown that the 80,000 dalton precursor for the terminal protein (pTP) can form a covalent complex with dCTP in the presence of

adenovirus DNA (Enomoto et al 1981; Lichy et al 1981; Tamanoi and Stillman 1982). The complex then binds to the origin sequence (Hay et al 1984) and serves as a primer for the elongation process. It has also been discovered that adenovirus encodes a viral DNA polymerase (M_r=140,000) in E2B between map coordinates 14.2 and 22.9 (Lichy et al 1982). The adenovirus DNA polymerase appears to be capable of initiating DNA replication in vitro by mediating linkage of dCMP to the precursor of the terminal protein (Lichy et al 1982; Stillman 1982). The exact role for the E2A encoded 72K single-stranded DNA binding protein (DPB) in DNA replication is not known. Temperature-sensitive mutants of E2A are DNA negative and temperature shift experiments suggest that the DBP is involved in both initiation and elongation (Horwitz 1978; van der Vliet and Sussenbach 1975; van der Vliet et al 1977). In addition to these three virally coded proteins two other cellular proteins, nuclear factors I and II with molecular weight of 47,000 and 39,000 respectively, are necessary, the former being required to stimulate pTP-dCMP initiation complex formation and the latter elongation reaction (Nagata et al 1982; Guggenheimer et al 1983; Nagata et al 1983; Guggenheimer et al 1984).

It is now generally believed that adenovirus DNA replication proceeds via displacement off linear DNA molecules. However, it has been shown that replication can initiate in vitro from termini embedded in plasmid DNA (Pearson et al,. 1983) and approximately 10% of the viral DNA in infected cells becomes joined as head-to-tail molecules due to the formation of covalently closed circles (Ruben et al., 1983). This covalently closed circular viral DNA was detected well before initiation of viral DNA synthesis. The Ad5 circles which had been cloned as plasmids were capable of generating infectious virus with an efficiency comparable with that of virion DNA following transfection into human cells (Graham, 1984b). These results suggested that these circles may play a role in viral replication.

(d) Transition from Early to Late Gene Expression

The major event governing the transition from early to late transcription appears to be the replication of viral DNA, because unreplicated DNA, when introduced into cells in the late phase, still fails to express late genes (Thomas and Mathews, 1980). The major late promoter at coordinate 16.5 controls

most of the transcriptional activity in the late phase and is responsible for the synthesis of functional mRNAs for most of the viral structural polypeptides. However, transcripts originating from the major late promoter have also been observed early after virus infection or in cells where viral DNA replication is blocked (Fraser et al., 1979; Chow et al., 1979; Kitchingnan and Westphal, 1980). The start site for transcription is the same at both early and late times of infection (Shaw and Ziff, 1980). The primary transcript, preterminated at early times and producing only two cytoplasmic RNA species from L1 (Chow et al., 1980; Akusjarvi and Perrsson, 1981), extends 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.

The switch from early to late phase also involves virus-induced changes in the splicing machinery (Akusjarvi and Perrson, 1981, Chow et al., 1980; Nevins and Wilson, 1981). The L1 nuclear RNA precursor is the same colinear RNA molecule at both early and late times. However, the early mRNA species has a quadripartite leader with an extra 440 nucleotide long leader segment derived from coordinates 22.0 to 23.2

(designated the i-leader) spliced into the message between the second and third late leader segments (Chow et al., 1980; Virtanen et al., 1982; Shaw and Ziff, 1980; Nevins and Wilson, 1982). The three E1A mRNAs, 13S, 12S and 9S, are generated by separate splicing events using the nuclear colinear transcripts as the only precursor RNA (Svensson et al., 1983). The 9S mRNA, which at early times is barely detectable, becomes the most prominent E1A mRNA late during the infectious cycle (Spector et al., 1978, Chow et al., 1979, Wilson and Darnell, 1981). It has been speculated that the change in abundance occurs as the result of virus-induced changes in the splicing enzymes (Svensson et al., 1983), however, it is also possible that the increased levels of 9S mRNA are due to an increased stability late in infection (Wilson and Darnell, 1981). In addition, it appears that early region 2 mRNAs are initiated at a different promoter site which is utilized exclusively at late times. During early infection, early region 2A makes use of a promoter at coordinate 75.2 which is also used by region 2B, but at late times region E2A RNA is synthesized from a different promoter at coordinate 72 (Chow et al., 1979).

The nature and mechanism of the changes in transcription termination sites, splicing patterns and promoter usuage which accompany the switch from early to late times remain largely unknown. However, it has been established that blockage of DNA replication either by DNA synthesis inhibitors or by DNA negative mutants prevents the formation of most late mRNAs (Berget et al., 1976; Carter and Ginsberg, 1976; Chow et al., 1979). When cells infected by ts125 that have been permitted to enter the late phase at a permissive temperature are shifted to a non-permissive temperature, late transcription continues for extended periods, even though viral DNA synthesis ceases rapidly (Carter and Ginsberg, 1976; Ginsberg et al., 1974; Thomas and Mathews, 1980). These results suggest that the onset of DNA replication is the critical factor, however, actively replicating adenoviral DNA molecules are not a prerequisite of transcription of late RNA.

(e) Assembly of Adenovirion

During the late phase of adenovirus productive infection, most of the viral polypeptides are rapidly released from polyribosomes and transported to the nucleus within three to six minutes (Horwitz et al.

1969; Velicer and Ginsberg 1970). During this short interval, monomeric structural polypeptides of the hexon, the penton base, and the fiber assemble into capsid units (Velicer and Ginsberg 1970), but the rates at which the different structural species become incorporated into mature virions varies considerably. Within 13 hours of infection the assembly of light intermediate particles begins (Sundquist et al., 1973), and these particles appear to be empty capsids and represent the first stage in particle assembly.

The cores or core precursor complexes are rapidly associated with viral DNA after their synthesis and the intermediate particles are formed by the insertion of the viral DNA and core-protein complexes into preformed capsids (Sundquist et al., 1973; Ishibashi and Maizel, 1974). Analysis of the viral DNA sequences associated with the intermediates 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). The loss of the polypeptide IVa from the intermediates signals the passage of these particles into the form of young virions (Ishibashi and Maizel, 1974).

In the final stage of the virus replication cycle young virions mature into infectious virions. Studies using a variety of temperature sensitive late mutants indicate that proteolytic cleavage of precursor polypeptides pVI, pVII, and pVIII are necessary for virion maturation (Leibowitz and Horwitz, 1975; Weber, 1976; Weber et al., 1977). Virions that are synthesized at the non-permissive temperature in cells infected by these temperature sensitive late mutants are not infectious (Weber et al., 1977).

Mature virions can be found in the nuclei of infected cells by 15 hours after infection (Green et al., 1970; Sundquist et al., 1973). Each infected cell can yield 4,000 to 10,000 progeny viral particles and the mature virions form large intranuclear eosinophilic inclusions (Boyer et al., Green and Daesch, 1961). The virus does not synthesize any enzymes which lyse cells and the release of virus particles appears to be due to cell death and lysis caused by attrition and may occur up to 48 hours after infection.

1.2. Transformation by Adenoviruses

A little over 20 years ago it was first reported that Ad12 induces tumors in newborn rats (Trentin et

al., 1962) and hamsters (Huebner et al., 1962). Subsequently, it became clear that while not all adenovirus species could induce tumors in animals, almost all human serotypes, including the non-oncogenic ones, could transform cells in culture (McAllister et al., 1969a,b). When an adenovirus infects a fully permissive human cell, the outcome is a productive infection ending in cell death. However, in a non-human cell, the virus is unable to complete the lytic cycle and few mature virions are formed. In some cases, the viral genome is integrated into cellular DNA leading to oncogenic transformation. The adenoviruses appear to preferentially transform cells that are nonpermissive for replication, and transformation is a very inefficient process, usually requiring 10^4 to 10^6 infectious units to give rise to one transformation event (reviewed by S.J. Flint, 1980b; Pettersson and Akusjarvi, 1983; van der Eb and Bernards, 1984; Graham, 1984).

Human adenoviruses can transform a variety of non-permissive rodent cells, including fibroblasts from hamster (Pope and Rowe, 1964), rat (Freeman et al., 1967), mouse (Younghusband et al., 1979; Starzinski-Powitz et al., 1982) and rabbit (Levinthal

and Peterson, 1965). Non-oncogenic Ad2 and Ad5 can replicate quite well in hamster cells (Takahashi et al., 1969; Williams, 1973) and transformation of these cells can be acheived by the use of virus that has been inactivated with ultraviolet light (Lewis et al., 1974), by the use of mutants that are defective for replication (Williams et al., 1974; Williams, 1973; Ginsberg et al., 1974), or by the use of sheared viral DNA (Graham et al., 1974b; Graham, 1977). Several adenovirus transformed human cells have also been isolated by DNA transfection including a human embryonic kidney cell line transformed with sheared Ad5 DNA known as 293 cells (Graham et al., 1973), and human retinoblast and human embryonic kidney cell lines transformed with a cloned DNA fragment of Ad12 (Byrd et al., 1982; Whittaker et al., 1984). It is now well established that adenovirus-transformed cells contain viral DNA, viral mRNA, and viral proteins.

1.2.1. Transforming Genes

It is well established that adenovirus DNA stably integrates into the host cell genome upon transformation (Flint, 1980b; Graham, 1984b). It is possible to analyse the viral genome present in

adenovirus-transformed cells in order to determine the viral sequences that are responsible for oncogenic transformation. These studies using a series of Ad2-transformed rat and hamster cell lines showed that the proportion of the viral genome retained in the cell lines varied considerably from one line to the other, however, all cell lines possessed the left-hand end of the viral DNA, some as little as 14% (Gallimore et al., 1974; Sharp et al., 1974a,b; Sambrook et al., 1975; Flint and Sharp, 1976). The left end of the viral genome corresponds to early region 1 (E1) which is one of five regions of the viral genome which are expressed prior DNA replication. These results therefore suggested that E1 is required for maintenance of transformation.

The finding of Graham and van der Eb (1973) that cells can be transformed by naked adenovirus DNA using the calcium phosphate technique provided direct proof that the transforming genes are located at the left end of the viral genome, since it is possible to introduce specific restriction enzyme fragments into cells and thus assay their transforming capacity. These studies showed that transformation can indeed be acheived by fragments containing only the left-hand end of Ad5 DNA.

In fact, not all of E1 is necessary for oncogenic transformation since as little as 8% of the viral genome is sufficient to induce a fully transformed phenotype (Graham et al., 1974a,b; van der Eb et al., 1977; Rowe et al., 1984). Subsequently, it was shown that partial or incomplete transformation can be obtained with the 4.5% Hpa I E fragment of Ad5 DNA (Houwelling et al., 1980; Dijkema et al., 1979). The transformation efficiency is considerably lower than with larger fragments, and the Hpa I E-transformed cells also show a difference in morphology compared to cells transformed by larger fragments. These cells are considered "immortalized" because of their unlimited life-span but not "fully transformed" as are those lines obtained using complete virus or larger DNA fragments (van der Eb et al., 1979). Taken together these results suggest that adenovirus transformation is a two-step process. DNA-mediated transformation using fragments containing E1A alone results in immortalization, whereas complete transformation can be acheived only by fragments containing E1A plus the 5' terminal part of E1B (Pettersson and Akusjarvi, 1983).

Recently it has been demonstrated that several viral and cellular transforming genes can on their own transform established cell lines, but fail to transform primary rat cells. The tumorigenic conversion of primary rodent fibroblasts requires two oncogenes, myc and ras (Land et al., 1983; Ruley, 1983). The mechanism may parallel the transformation by Ad5 which requires the action of two viral genes, E1A and E1B. From nucleotide and deduced amino acid sequence analyses it has been shown that the oncogenes myc, myb, and E1A are structurally related (Ralston and Bishop, 1983). E1A can substitute for myc and cooperate with ras, as well as polyoma middle-T, to induce oncogenic transformation of primary rat cells (Land et al., 1983; Ruley, 1983; Asselin et al., 1984). These results are consistent with the fact that viral transformation requires two different steps.

Although adenovirus E1A DNA can immortalize cells and induce partial transformation, it seems that region E1B has no transforming activity on its own. Ad5 group I host-range mutants, which contain defects in E1A but express E1B, are defective in transformation of primary rat kidney cells (Carlock and Jones, 1981; Solnick, 1981; Solnick and Anderson, 1982). When primary rat

kidney cells were transfected with a plasmid containing E1B under the control of an SV40 promoter to ensure high levels of E1B expression in the absence of E1A, no detectable transformants were found (van den Elsen et al., 1982). Experiments with the established rat cell line 3Y1 also failed to reveal any transforming activity of the SV40-E1B hybrid region, although E1B was fully expressed in the cells (Kimura et al., 1975; van den Elsen et al., 1983). These results suggest that the expression of E1B alone is not sufficient to cause cellular transformation.

1.2.2. Proteins encoded in E1

The adenovirus E1 region contains two transcription units, E1A and E1B, and each transcribes several messages (reviewed by Pettersson et al., 1984). E1A codes for three mRNAs of 1.1, 0.9, and 0.6kb in length (the 13S, 12S, and 9S mRNAs mentioned above). The 1.1 and 0.9kb mRNAs are expressed early during the lytic infection and in adenovirus-transformed cells. The 0.6kb mRNA is synthesized preferentially late in infection and it is not found in transformed cells and thus is probably not important for transformation.

Based on cDNA sequencing data the three E1A mRNAs of 1.1, 0.9, and 0.6kb should code for proteins of 289,

246, and 54 amino acids with predicted molecular weights of 32K, 26K, and 6.1K, respectively (Perricaudet et al., 1979; Virtanen and Pettersson, 1983). The sequencing data also indicate that these proteins should share a common amino terminal sequence. The 0.6kb mRNA product should be identical to the other E1A proteins for the initial 26 amino acids and due to its splicing pattern the polypeptide should be different from the other E1A products beyond the acceptor site (Virtanen and Pettersson,1983). Products derived from the 1.1 and 0.9kb mRNA should encode completely overlapping polypeptides, however, due to a larger internal splice in the 0.9kb mRNA, its product lacks an internal 46 amino acid stretch found in the product derived from the 1.1kb mRNA (Perricaudt et al., 1979).

Until the development of reagents produced in the course of experiments presented in this thesis, the study of E1A proteins had been hampered by the lack of tumor sera capable of immunoprecipitating them. These products are also made in small amounts during lytic infection and are present in even smaller amounts in transformed cells. In order to identify E1A products many studies used a variety of drugs to enhance the synthesis of E1A proteins in infected cells (Harter et al., 1976; Saborio and Oberg, 1976; Harter and Lewis,

1978; Brackmann et al., 1980; Gaynor et al., 1982). Several groups have also used in vitro translation of selected E1A mRNAs to detect the E1A products (Lewis et al., 1976; Harter and Lewis, 1978; Halbert et al., 1979; Esche et al., 1980; Spector et al., 1980; Lupker et al., 1981; Smart et al., 1981). These studies have suggested the existence of between two to six E1A polypeptides with molecular weights ranging between 35K to 50K. Studies using in vitro translation of selected mRNA also suggested potential products of 28K (Spector et al., 1980a,b; Esche et al., 1980; Halbert and Raskas, 1982), or 14K (van der Eb et al., 1979) for the 0.6kb E1A late mRNA. Both of these polypeptides have apparent sizes far exceeding the predicted molecular weight of 6.1K (Virtanen and Pettersson, 1983) However, these results are prone to artifacts that might accompany cell-free protein synthesis and metabolic inhibition.

Three mRNAs of 2.2, 1.0, and 0.5kb are derived from E1B. The 0.5kb mRNA produces the viral structural protein, polypeptide IX from its own promoter (Alestrom et al., 1980) and this protein which is not found in transformed cells probably plays no role in transformation. Sequencing data have indicated that the 2.2kb mRNA of Ad5 codes for a polypeptide of 176 amino acids (175 for Ad2) and, using a different start site

and reading frame, a polypeptide of 496 (495 for Ad2) amino acids (Bos et al, 1981; Gingeras et al., 1982). The predicted molecular weights of these two polypeptides are 21K and 55K, respectively. The 1.0kb mRNA should produce the same 21K protein as the 2.2kb mRNA (Bos et al., 1981; Gingeras et al., 1982).

The identification of proteins derived from E1B has been less difficult as compare to E1A proteins due to the fact that these proteins are generally made in larger amounts during lytic infection and in transformed cells, and tumor sera against these proteins are more readily available. Studies with Ad5 or Ad2 using in vitro translation of selected E1B mRNAs and immunoprecipitation using tumor sera have identified a protein with an apparent molecular weight in SDS-PAGE of 53-65K, corresponding to E1B-496R, and a protein of 15-21K, corresponding to E1B-176R (Harter and Lewis, 1978; Lassam et al., 1979a,b; Halbert et al., 1979; Schrier et al., 1979; Ross et al., 1980; Jochemson et al., 1980; 1981; Green et al., 1979, 1982; Halbert et al., 1982; Rowe et al., 1984). Using antibodies directed against synthetic peptides corresponding to specific amino acid sequences predicted from nucleotide sequencing data, these two proteins have been confirmed as the products of the 2.2kb and 1.0kb E1B mRNAs (Yee et al., 1983-this

thesis; Green et al., 1983; Anderson et al., 1984; Yee, 1984). These two proteins are often referred to as 58K and 19K for Ad5 (Graham, 1984). In addition to these major species, another protein with an apparent molecular weight of 20K has been identified from Ad2-infected and transformed cells (Green et al., 1979). This protein, which has been purified to homogeneity, is related to the E1B-58K (Green et al., 1982) and also translated in vitro from E1B-specific mRNA (Matsuo et al., 1982). Protein sequence studies further revealed that it shares completely overlapping polypeptides with E1B-58K. This protein is 155 amino acids in length and consists of the amino terminal 78 and carboxy terminal 77 residues of the E1B-58K (Anderson et al., 1984). This result is also confirmed by the fact that this protein is also precipitated by antibodies directed against synthetic peptides corresponding to the amino- and carboxy-termini of E1B-58K (Anderson et al., 1984; Luchner et al., 1984).

1.2.3. Function of E1

As described above, adenovirus early region 1 plays an important role in oncogenic transformation and region 1A also regulates the expression of other early regions and it has been suggested that the control

event occurs at the level of transcriptional initiation (Logan and Shenk, 1982; Weeks and Jones, 1983). The role of E1A in the regulation of expression of other early regions has been investigated by microinjection into cultured cells or frog oocytes of cloned viral DNA carrying E1A sequences. As a measure of E1A function a second plasmid has also been co-injected including sequences for the gene coding for the E2A Ad5 DNA binding protein (Rossini, 1983), or chimeric genes which were constructed with promoter sequences of other adenovirus early regions and the structural sequence of the bacterial enzyme chloramphemicol-3-o-actyltransferase (CAT) (Jones et al., 1983). These results confirmed that E1A is required to activate the transcription of other adenovirus early regions. More recently the E1A gene product itself has been synthesized in Escherichia coli, and using a Xenopus oocyte microinjection assay, it has been shown that the purified E. coli-produced E1A protein activates the transcription of other early regions and functions as efficiently as the E1A gene itself (Ferguson et al., 1984).

Several lines of evidence suggest that there is not an absolute requirement for E1A gene products to activate the transcription of other early viral regions. When cells are infected with E1A mutants at

low multiplicities, transcripts from other early regions can be detected but at a later time. By increasing the multiplicity of infection, this slow activation of early transcription can be enhanced (Nevins, 1981). These results suggest that E1A only acts to enhance or facilitate the transcription of other early regions. The E1A defect in these mutants can also be overcome by inhibiting protein synthesis. It has been suggested that E1A gene products function to eliminate a short-lived cellular factor that acts to repress early viral transcription (Nevins, 1981), or to block accumulation of early mRNAs (Katze et al., 1981;1983; Persson et al., 1981a,b). Treatment of cells before infection with protein synthesis inhibitors would thus reduce the concentration of this inhibitory protein. This model could also explain the observation obtained with high multiplicity infection of E1A mutants as enhanced expression of other early regions could be due to the presence of a large number of copies of early viral promoter regions in the cells that causes binding of all, or most, of the cellular repressor proteins.

More recently, Cross and Darnell (1983) have reinvestigated the cycloheximide stimulation of early transcription and found that expression of early viral genes, including E1A, is required before the addition

of cycloheximide can produce the stimulation. These results suggest that the mechanism of stimulation by cycloheximide is different from the mechanism of induction by E1A proteins. Furthermore, Gaynor and Berk (1983), using cells that were first infected with one E1A mutant, incubated until viral transcription was detectable, and then superinfected with a second E1A mutant with distinguishable transcripts from early region 3 (E3), found that transcription of E3 of the second virus was still delayed. These results are not compatible with the cellular repressor protein model since the first incoming virus ought to titrate out the host cell repressor proteins resulting in the immediate expression of all other early regions of the second virus. They proposed that E1A proteins induce the transcription of other early regions by catalyzing the assembly of the viral DNA into an active transcriptional complex.

It has been reported that certain cells, including the F9 mouse teratocarinoma line, express an E1A-like function. These cells, which also express the heat shock gene, a gene that can be induced by E1A (see below), are capable of expressing early adenovirus genes in the absence of E1A (Imperiale et al., 1984). There is also evidence demonstrating that immediate early proteins of herpesviruses, including pseudorabies

(Feldman et al., 1982; Imperiale et al., 1983), herpes simplex virus type 1 (Tremblay et al., 1985), and human cytomegalovirus (Tevethia and Spector, 1984), can induce adenovirus early transcription in the absence of E1A. These data suggest that the mechanism of transcriptional activation by E1A products and by herpesvirus immediate early proteins could be at least partially similar. Nevertheless, detailed knowledge of the mechanism of adenovirus early gene activation by E1A is still lacking and more experiments have to be done in order to understand this complex process.

Although gene activation by E1A is largely limited to early adenovirus transcription units, it has been found that E1A also activates the transcription of the mammalian heat-shock gene (and the synthesis of the 70K heat-shock protein) and of β-tubulin in adenovirus-infected HeLa cells (Nevins, 1982; Kao and Nevins, 1983; Imperiale et al., 1984; Stein and Ziff, 1984). In addition, infection with adenoviruses has also been found to induce alterations in the cell cycle and, using E1A mutants, it has been shown that this is a direct effect of E1A (Braithwaite et al., 1983). Furthermore, several experiments have shown that E1A products can activate the transcription of nonviral genes that are newly introduced into cells either by transfection or infection, even though the expression

of the endogenous copies of these genes is unaffected (Green et al., 1983; Gaynor et al., 1984; Svensson and Akusjarvi, 1984b). Differences in chromatin structure between the newly introduced nonviral genes and their endogenous counterparts probably plays a role in gene activation by E1A products. It is also interesting to note that transcription of an integrated copy of the adenovirus E3 gene is activated by E1A products (Curtois and Berk, 1984), suggesting that they possess a high degree of specificty for viral genes. All these observations tend to suggest that E1A is not only involved in regulating expression of other early viral genes, but also one of its roles is to activate the expression of certain cellular genes. An attractive hypothesis is that these E1A-induced alterations in cellular gene expression may lead to immortalization of primary cells (Graham, 1984) and an understanding of the biochemical functions of E1A may shed light on the mechanism of transformation.

In addition to enhancing transcription Borrelli et al (1984) recently showed that E1A also can cause a negative regulation of a viral enhancer. When HeLa cells are transfected with a plasmid containing a rabbit γ-globin gene linked to the enhancer of polyoma or SV40 virus, the enhancer stimulates transcription of the γ-globin gene, but transcription is repressed if

the cells are co-transfected with a plasmid that expresses E1A proteins. In competition experiments the negative effect of the E1A products can be relieved by co-transfecting increasing amounts of recombinants containing enhancer elements, including the E1A gene's own enhancer. These results suggested that the enhancer itself is probably the target for the negative regulation. Inhibition of the E1A enhancer activity by the E1A proteins also suggested that expression of E1A can be autoregulated during lytic infection.

The functions of E1B polypeptides are less clearly established. As described above, in addition to E1A, at least part of E1B is required to induce complete transformation in primary cells. The most important region is the 5' half of E1B, up to approximately 8.0 m.u. This segment contains the coding sequences for the 19K protein specified by the E1B 1.0kb mRNA, as well as a possible N-terminal region of a truncated 58K protein coded for by the 2.2kb mRNA. It seems likely therefore that 58K may not play an important role in maintaining the transformed cell phenotype. However, studies using E1B mutants that are defective in the synthesis of 58K suggest that it is required for initiation of transformation by virions (Graham et al., 1978; Rowe and Graham, 1983; Graham et al., 1983). Previous studies using Ad5 host range mutants have suggested

that E1B gene products do not function to replicate viral DNA but do affect the synthesis of late viral polypeptides (Lassam et al., 1978; Jones and Shenk, 1979). More recently, using deletion mutants that affect E1B 19K and 58K polypeptide species, Babiss and Ginsberg (1984) showed that both intact proteins were necessary for optimum viral replication but that they were not absolutely required for production of infectious virus and suggest that the 58K protein may also function to initiate the events leading to the shut off of host cell gene expression.

Following the original observation of Collett and Erikson (1978) that the transforming gene product, pp60$^{\underline{src}}$, of Rous sarcoma virus possess a protein kinase activity, a search for similar activity in adenovirus tumor antigens was carried out. A protein kinase activity was detected in immunoprecipitates from Ad5-infected and transformed cells containing the 58K antigen (Lassam et al., 1979a; Branton et al., 1979, 1981; Yee and Branton, 1983). The adenovirus-specific kinase phosphorylates 58K as well as exogenous substrates and phosphoserine and phosphothreonine residues (but no phosphotyrosine) are detected (Branton et al., 1981). A similar protein kinase has also been found in immunoprecipitates containing Ad12 tumor antigens (Raska et al., 1979; Branton et al., 1981).

However, it is still not clear whether the protein kinase activity is intrinsic or due to the binding of a cellular enzyme to E1B-58K.

Sarnow et al (1982) have shown that the E1B-58K present in adenovirus-transformed mouse cells is associated with a cellular 54K polypeptide. This protein is identical or closely related to a cellular protein p53 that is also complexed with the SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). These results suggested that the adenovirus 58K and SV40 large T antigen may share similar functions in transformation.

1.3. Antisynthetic peptide antibodies

Antibodies have proven to be powerful tools in the identification and characterization of viral proteins. However, many of these proteins are present in very small amounts and it is often difficult to obtain enough viral polypeptides to raise antisera. A different approach to circumvent the problem is to identify an antigenic determinant of the protein and to prepare a synthetic antigen that can mimic the conformation of the native protein. With the introduction of a simple and efficient peptide synthesis methodology (Merrifield, 1964) it is now

feasible to synthesize discrete segments of proteins. These synthetic antigens have been shown to elicit antibodies that can react against the native protein. Anderer (1963) prepared a carboxy-terminal hexapeptide of the tobacco mosiac virus (TMV) coat protein by tryptic cleavage and demonstrated that such a peptide coupled to bovine serum albumin (BSA) produces an antiserum which precipitates and neutralizes the virus. Moreover, synthetic fragments of the same peptide, when coupled to BSA as a carrier, also gave rise to antibodies that can inactivate the infectivity of the virus (Anderer and Schlumberger, 1965; 1966). A major antigenic determinant of the TMV coat protein has been localized in the internal region of the polypeptide (Stewart et al., 1966; Young et al., 1967). Subsequently, a decapeptide corresponding to this antigenic determinant was synthesized and antibodies against the synthetic peptide also reacted with the native protein (Fearney et al., 1971). Using the same approach Langbeheim and coworkers (1976) localized an antigenic determinant on the coat protein of bacteriophage MS-2 and synthesized a peptide corresponding to this determinant that gave rise to neutralizing antibodies against the virus. These results indicated that antibodies against small peptide

immunogens can also react with full-length proteins containing that peptide.

The antigenic sites or determinants of a protein can be localized by fragmentation of the protein by various enzymatic and chemical techniques and then these fragments can be analyzed to determine which ones retain the ability to interact with the antibodies (reviewed by Atassi, 1975; 1979; Crumpton, 1974). Once the antigenic region is identified, chemical analysis of the fragment can be used to determine the exact amino acid sequence involved in antibody binding. However, with the recent advances in molecular cloning and DNA sequencing techniques, it is now possible to obtain the nucleotide sequence of a viral gene and then its amino acid sequence can be easily deduced from the DNA sequencing data. It is then possible to synthesize small peptides with sequences corresponding to various portions of the protein. Since not all peptides elicit protein-reactive antibodies, a set of guidelines has been devised to select a region from the protein that is immunogenic when coupled to a carrier and that will give rise to antibodies that recognize the intact native protein in the absence of any experimental knowledge of the antigenic or three-dimensional structure of the protein (Shinnick et al., 1983; Walter and Doolittle, 1984; Lerner et al., 1981; Sutcliffe et

al., 1982). First, the peptide should contain sufficient numbers of polar or charged amino acids, since these peptides tend to be on the surface of the native protein (Hopp and Wood, 1981; Kyte and Doolittle, 1982). This is necessary for the region to be accessible to antibodies when in the native, full-length protein. Second, peptides should contain a minimum length of five amino acids since short peptides tend not to be immunogenic, and longer peptides appear to have a high probability of eliciting antibodies reactive with the native protein (Lerner et al., 1981). Third, peptides containing proline residues in addition to polar or charged residues because of the presence of secondary structure routinely elicit antibodies that react with the conformationally native protein. Fourth, peptides corresponding to the amino- or carboxy-termini have a better probability to produce antibodies active against the native protein as the termini are often exposed in many proteins.

The antisynthetic peptide antibodies are directed against predetermined sites on proteins and have proven to be a powerful tool to identify and characterize viral polypeptides (reviewed by Walter and Doolittle, 1984; Sutcliffe et al., 1983; Shinnick et al., 1983; Green et al., 1983a; Lerner, 1982; Lerner et al., 1981). Since these antibodies are raised against

synthetic peptides with amino acid sequences predicted from nucleotide sequencing data, they are particularly useful in identifying the protein product of an open reading frame and have been used to identify the SV40 large T antigen (Walter etal., 1980) and an envelope polypeptide in Moloney leukemia virus (Sutcliffe et al., 1980). A large body of information about antipeptide antibodies has now accumulated and this approach has been used to detect the putative products of the transforming genes of the Moloney sarcoma (Papkoff et al.,1982), simian sarcoma virus (Robbins et al., 1982), feline sarcoma virus (Sen et al., 1983), avian oncovirus MC29 (Patschinsky et al., 1984), Rous sarcoma virus (Tamura et al., 1983; Gentry et al., 1983; Nigg, et al., 1982a; 1982b; Wong and Goldberg, 1981), influenza virus hemagultinin (Green et al., 1982; Muller et al., 1982), hepatitis B surface antigens (Neurath et al., 1982; Prince et al., 1982; Bhatnagar et al., 1982; Dressman et al., 1982; Lerner et al., 1981; Hopp, 1981), polyoma middle T antigen (Schaffhausen et al., 1982; Walter et al., 1981), poliovirus genome protein (Semler et al., 1982; Baron and Baltimore, 1982a), VP1 protein of foot and mouth disease virus (Bittle et al., 1982), and herpes simplex virus-type 1 immediate early gene products 12 and 175 (Palfreyman et al., 1984).

The anti-synthetic peptide antibodies are not only useful for identifying viral proteins. Because of their predetermined specificity these reagents are also particularly useful in characterizing the processing of viral proteins and their biological functions. Antipeptide antibodies have been used to study the processing of polyprotein precursors in cells infected with poliovirus (Baron and Baltimore, 1982b), Moloney leukemia virus (Green et al., 1981), and to examine the cellular location of pp60^{src} (Nigg et al., 1982a,b), as well as the transforming proteins of adenoviruses (Yee et al., 1983-this thesis; Feldman and Nevins, 1983; Lucher et al., 1984). Peptide antisera are also powerful tools to study the correlation of structure with function. It has been used to inhibit the oncogene-coded tyrosine kinase activities of Rous sarcoma virus (Tamura et al., 1983; Gentry et al., 1983), feline sarcoma virus (Sen et al., 1983) and polyoma middle T antigen (Schaffhausen et al., 1982). Antipeptide sera can also provide a means for purifing the corresponding protein. Immunoaffinity columns prepared from antipeptide sera have been used to purify the middle T antigen of polyoma virus and the protein was eluted from the column by competition with excess synthetic peptide (Walter et al., 1982).

1.4. Proposal for this thesis

In view of the significance of the proteins coded for by the adenovirus transforming genes the present research project was initiated to identify and characterize these gene products and to study their biological functions, especially the E1B-58K associated protein kinase activity.

During the early phase of the project, information about the gene products derived from Ad5 E1A was limited and confusing. The then novel approach was taken to identify these gene products using antibodies against synthetic peptides with sequences corresponding to the predicted amino- and carboxy-termini of E1A. The viral polypeptides were identified by immunoprecipitation using these antibodies. The origin of the E1A multiple gene products has also been mapped using various E1A mutants. The anti-synthetic peptide antibodies also provided potentially powerful tools to purify these gene products and characterize their biological functions. In addition, antibodies against synthetic peptides corresponding to both termini of E1B-58K have also been raised. These antibodies were also used to identify this viral polypeptide from wild-type virus and host-range group II mutants. An attempt was also made to purify the E1 proteins and to

ask if protein kinase activity was intrinsic to them. E1B 58K was purified by several conventional methods and the results suggested that such activity may be intrinsic to this viral polypeptide. A simple purification procedure using anti-peptide sera and synthetic peptides was developed and this purifiction scheme was used to obtain highly purified 58K and E1A proteins. The E1 protein-associated protein kinase activity was also examined using these highly purified products, however, the data obtained were inconclusive and it was not possible to unequivocally determine if protein kinase activity was intrinsic to them. Further studies will be necessary to establish if Ad5 E1 proteins possesses intrinsic protein kinase activity.

CHAPTER 2

Materials and Methods

2.1. Cells and Viruses

Human KB cells were propagated as monlayers on 150mm plastic Petri dishes (Nunc) in alpha minimum essential medium (α-MEM) supplemented with 10% fetal calf serum. The 293 cell line was propagated as monlayers using Joklik's modified medium supplemented with 10% horse serum, as previously described (Graham et al., 1977). The Ad5-transformed hamster cell lines, including 14B and 983-2, were maintained as monolayers in α-MEM supplemented with 10% calf serum.

The wt strain of Ad5 and the host-range mutants hr 1, 2, 3, 4, 5, 6, 7, 50, and 51 have been described by Harrison et al. (1977) and Graham et al. (1978). The host-range mutants fall into two complementation groups: hr 1, 2, 3, 4, and 5 are in group I and possess defects in E1A; hr6, 7, 50, and 51 are in group II and possess defects in E1B. Other Ad5 mutants, including pm975 (Montell et al., 1982), dl313 (Colby and Shenk, 1981), and dl1504 (Osborne et al., 1982) were also employed in the present study. The wt, pm975, and dl1504 viruses were grown on either KB or 293 cells and other Ad5 mutants were propagated on 293 cells.

2.2. Infection and Radioactive Labelling of Cells

Virus stocks were diluted in 2 ml of either PBS or culture medium and confluent monolayers of KB cells in 150mm dishes were infected at 35 plaque-forming units per cell. After 45 to 60 min of absorption 20 ml of medium were added back to the cell cultures. Virus- or mock-infected cells were normally labeled from 7 to 11 h post-infection. The cell cultures were rinsed twice with warm PBS and each 150mm dish of cells was labeled with 100 uCi of [^{35}S]methionine in 4 ml of medium lacking methionine. For ^{32}P-labeled cells, cultures were washed twice with phosphate-free medium and each dish of cells labeled with 2 mCi of carrier-free ^{32}P-orthophosphate in 4 ml of medium lacking phosphate.

2.3. Preparation of Anti-peptide Sera

2.3.1. Conjugation of peptide to Bovine Serum Albumin

Synthetic peptides were purchased from Bachem (Torrance, California). Production was by sequential solid-phase synthesis (Stewart and Young,1969), and purity was greater than 90%. The synthetic peptides which contained a terminal tyrosine were conjugated to BSA with bis-diazotized benzidine as described by Bassiri et al (1979). Briefly, 50 mg of bovine serum

albumin (BSA) were dissolved at 0°C, along with a 40 molar excess of the peptide, in 10 ml of buffer containing 0.13M NaCl and 0.16M borate, pH9.0. The coupling was started by the addition of a 2 ml portion of bis-diazotized benzidine in 0.2M HCl and the pH was adjusted to 7.0 with 0.5M NaOH. The reaction was allowed to continue for 2 hours at 4°C with constant stirring. The conjugate was then dialyzed against 0.15M NaCl overnight and stored at -20°C until use. Peptides which did not contain a terminal tyrosine were coupled onto BSA using carbodiimide. Briefly, 25mg of BSA and a 25 times molar excess of peptide were dissolved in 10 ml of 0.15M NaCl and the pH was adjusted to 5.0. The coupling reaction was started by the addition of 225mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (ECDI) in 0.5 ml of H_2O and the reaction was carried out at room temperature for 30 min with constant mixing. The reaction mixture should have had a pH of 6.8 to 7.0 at the completion of the conjugation. The conjugate was then dialyzed against 0.15M NaCl for 24 hours, aliquoted and stored at -20°C until use.

2.3.2. Immunization of Rabbits and Preparation of Antisera

Antisera were prepared in 5 month-old male New Zealand white rabbits using 1 mg of the peptide-BSA

conjugate which had been emulsified in 1ml of complete Freund's adjuvant. The rabbits were injected at 10 different locations including intradermal, intramuscular, foot pads and subcutaneous sites. The rabbits were boosted 4 weeks later with 1 mg of conjugate emulsified in 1 ml of incomplete Freund's adjuvant. Blood was collected 7 to 10 days after each boost. Blood samples were allowed to clot at 37°C for 1 hour, and then they were incubated at 4°C overnight after which time the serum was collected by centrifugation and combined with PMSF at a final concentration of 5mM.

The antipeptide sera were further purified to remove impurities and most of the anti-BSA activity before use. BSA was added to a final concentration of 0.1 mg/ml and allowed to incubate at 4°C for 24 hours. The antibody-BSA aggregate was removed by centrifugation at 10,000xg for 10 min. A 5ml portion of the supernatant serum was then combined with 1 ml of PBS containing 5×10^7 unlabeled KB cells which had been disrupted by sonication. After 24 h at 4°C the insoluble particulate material was removed by centrifugation and the serum was stored at -20°C until use.

2.4. Preparation of cell extracts and immunoprecipitation

Cells were washed with PBS and removed by scraping with a rubber policeman into 10 ml of PBS. The cells were washed two times by resuspension in PBS and pelleted by centrifugation. The washed cell pellets were resuspended in RIPA buffer, which consisted of 50mM Tris(pH 7.2), 150mM NaCl, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100 and 100 KIU of aprotinin per ml, at a concentration of approximately 2×10^7 cells per ml. The lysate was vortexed vigorously and incubated on ice for 20 min. The insoluble material was removed by centrifugation at 12,500 x g for 10 min at 4°C. To each 1ml of cell extract was added an appropriate volume of antiserum and 250 ul of protein A-sepharose beads (Pharmacia Fine Chemicals) suspended (1:10,v/v) in RIPA buffer. The mixture was incubated at 4°C with constant mixing for 2 to 3 hours and then the sepharose beads with bound antibody were collected by centrifugation at 30 x g for 1 min and washed three times with 1 ml of RIPA buffer and twice with a ml of 100mM Tris (pH7.0) containing 200mM LiCl and 0.1% (v/v) 2-mercaptoethanol.

2.5. Protein kinase assay

Immunoprecipitates bound to sepharose beads were assayed for protein kinase activity in 50ul (final volume) of a solution containing 40mM 2-(N-morpholineethanesulfonic acid (MES) pH7.0, 40mM $MgCl_2$ and 75 ug of arginine-rich histone which had been boiled for 10 min just before used. The reaction was started by the addition of [γ-^{32}P]ATP to a final concentration of 7.5uM (specific activity, 250Ci/mmol) and the samples were incubated at 37°C for 2 min. The reaction was terminated by the addition of an equal volume of 2x sample buffer containing 0.2M Tris (pH6.8), 4% SDS, 2% 2-mercaptoethanol and 20% glycerol. The samples were boiled for 2 min and the sepharose beads were removed by low speed centrifugation. The supernatants were then analysed by SDS-PAGE.

2.6. One-dimensional Polyacrylamide Gel Electrophoresis

Radioactively labeled samples were analyzed by discontinous SDS-PAGE modified from Laemmli(1970), consisting of a 5% polyacrylamide stacking gel and a 12% polyacrylamide separating gel (unless otherwise mentioned). The ratio of acrylamide to N-N'-bis-methylene acrylamide was 30:0.8. In addition, the separating gel contained 0.375M Tris/HCl (pH8.8),

1% glycerol, 0.1% SDS. The stacking gel consisted of 5% acrylamide containing 0.125M Tris/HCl (pH6.8), 0.5% glycerol and 0.1% SDS. The gels were polymerized chemically by the addition of ammonium persulfate and TEMED to a final concentration of 0.05% (w/v) and 0.025% (v/v), respectively. The electrode buffer contained 0.025M Tris/HCl (pH8.3) and 0.192M glycine and 0.1% SDS. Electrophoresis was carried out at a contant voltage of 75 volts until the bromophenol blue marker reached the bottom of the gel (about 18 hours).

Following electrophoresis gels were either dried immediately or were first stained with Coomassie Blue and then destained with several changes of isopropanol-acetic acid. Some gels were treated by the fluorographic technique of Bonner and Laskey (1974) prior to drying to enhance the sensitivity of autoradiography. Briefly, gels were dehydrated by immersing them in about 20 volumes of DMSO for 30 min followed by a second 30 min immersion in fresh DMSO. The dehydrated gel was then soaked in 4 volumes of 22.5% (w/v) 2,5-diphenyloxazole (PPO) in dimethylsulfoxide (DMSO) for 3 hours and the gel was then immersed in water for at least one hour with several changes of fresh water prior drying to precipitate the PPO. Autoradiography was carried out using Kodak RP Royal X-Omat film.

2.7. Two-dimensional Gel Electrophoresis

For two-dimensional gel electrophoresis, isoelectric focusing was performed as described by O'Farrell (1975). Briefly, immunoprecipitates bound to sepharose beads were resuspended in lysis buffer containing 9.5M urea, 2% NP-40, 2% ampholines (1.6% pH range 4 to 6 and 0.4% pH range 3 to 10) and 5% 2-mercaptoethanol. Immunoprecipitates were incubated for at least 30 min at room temperature before loading. The gels were prerun without sample according to the following schedule: 200V for 15 min, followed by 300V for 30 min, and then 400V for another 30 min in order to form a linear pH gradient between pH6.5 and 4.1. The immunoprecipitates from approximately 10^7 cells were loaded onto the cylindrical gels and the gels were subjected to 400V for 16 hours and then 800V for 1 hour. The tube gels were then impregnated with sample buffer containing 0.625M Tris, pH6.8, 2% SDS, 1% 2-mercaptoethanol and 10% glycerol for 30 min at room temperature. Tube gels were either stored at -70°C or the second dimension was run immediately. The second dimension of electrophoresis was carried out using discontinuous SDS-PAGE as described above by placing the entire isoelectric focusing tube gel on the top of the stacking gel.

2.8. Cleveland Mapping

Proteins were analysed by partial hydrolysis with V-8 protease according to the method of Cleveland et al (1977). Bands containing the appropriate polypeptide were cut out of dried polyacrylamide gels using an autoradiogram as a template. The gel slices were cut into pieces, and the pieces were rehydrated with sample buffer containing 50mM Tris-HCl (pH6.8), 1% SDS, 10% glycerol 1% 2-mercaptoethanol and 0.001% bromophenol blue. The gel pieces were applied to a second 15% polyacrylamide gel with a 5% stacking gel in the presence of 50 ul of sample buffer containing 0, 1, 20, or 400 ug of Staphylococcal V-8 protease. The samples were electrophoresed at a constant voltage of 70 volts until the dye front reached the stacking gel-separating gel interface, at which time electrophoresis was stopped for 2 hours. Electrophoresis was then continued as usual and the gels were dried and processed for autoradiography as described above.

2.9. Immunofluorescence

HeLa cells were grown on glass cover slips to about 75% confluence and then infected with Ad5. At 12 hours postinfection Ad5- and mock-infected cells were washed three times with ice-cold PBS and were fixed

with methanol:acetone (1:1, v/v) at -20°C for 15 minutes. The cells were then again washed three times with ice-cold PBS. The cover slips were either air dried and stored at -20°C or used directly for immunofluorescence. For air dried cover slips, cells were rehydrated with PBS for at least 30 min at 37°C. Cover slips were incubated with either antipeptide serum or preimmune rabbit serum for 45 min at 37°C. After another three washes with PBS the cells were incubated with fluorescein isothiocyanate conjugated (FITC) goat anti-rabbit immunoglobulin G diluted 1:20 in PBS immediately before use. The fluorescent staining was visualized using an ultra-violet fluorescence microscope and fluorescence was recorded on Kodak Ektachrome 400 slide film.

2.10. Association of Ad E1A Polypeptides with Cellular Proteins

Cells were harvested by scraping with a rubber policeman into PBS. The cells were washed twice with PBS and then resuspended in lysis buffer containing 100 mM Tris-HCl (pH 7.0), 137 mM NaCl, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 1% (vol/vol) Nonidet P-40 and 100 KIU of aprotinin per ml at a concentration of 2×10^7 cells per ml. The cell lysate were briefly vortexed and then clarified by centrifugation at 12,000 x g for 10 min at

4°C. The supernatants were immunoprecipitated with 25 ul of serum and protein A-sepharose beads as described in Section 2.4. In some cases synthetic peptide was added to the supernatants and then mixed briefly before immunoprecipitation were carried out. The precipitates were washed three times with lysis buffer and then twice with 100 mM Tris-HCl (pH 7.0) containing 200 mM LiCl, 1 mM dithiothreitol and 100 KIU of aprotinin per ml.

2.11. Glycerol Gradient Centrifugation

Approximately $2x10^7$ 983-2 cells were labeled with [^{35}S]methionine for 12 hours and extracts were prepared in RIPA buffer as described before. The extract was layered on top of a 12ml linear gradient of 10 to 30% glycerol (v/v) in RIPA buffer and the gradient was centrifuged at 27,000 rpm in a Beckman SW40.1 rotor at 4°C for 16 hours. A mixture of proteins in RIPA buffer containing 50 ug each of ovalbumin (M_r=43,000), bovine serum albumin (M_r=67,000), aldolase (M_r=158,000), catalase (M_r=232,000), and ferritin (M_r=440,000) was analysed in a parallel gradient to provide molecular weight markers. Fractions of approximately 0.5ml were collected from the bottom of the tube and small aliquots were taken out from each fraction to quantify radioactivity directly using TCA precipitation. The

rest of the fraction was immunoprecipitated with 14B anti-tumour serum and analysed using SDS-PAGE. A parallel gradient containing extract from unlabeled 983-2 cells was also prepared and fractions were immunoprecipitated with 14B anti-tumor serum and analysed for protein kinase activity as described above. The amounts of 58K and protein kinase activity were determined by carrying out microdensitometer scans of autoradiograms of the 58K band and the histone H3 band, respectively.

2.12. Purification of viral polypeptide by ammonium sulfate precipitation

Ad5-transformed 983-2 hamster cells were labeled with [^{35}S]methionine for 12 hours and cells were harvested into PBS. The cells were washed and cell extracts were prepared at a concentration of about 2×10^7 cells per ml in RIPA buffer as described before. Sufficient amounts of 100% ammonium sulfate was added slowly with constant mixing to 2ml of cell extract to achieve the appropriate final concentration (w/v). The precipitation was allowed to carry on for 45min at 4^{o}C after which precipitates were collected by centrifugation at 12,500 x g for 10 min and the pellets were resolubilized in RIPA buffer and dialyzed against RIPA buffer overnight. The supernatant was also

collected and an appropriate amount of ammonium sulfate solution were added to achieve the next cut off concentration. The precipitates were processed as described above and the final supernatant was also dialyzed against RIPA buffer. ^{35}S-labeled samples were immunoprecipitated using 14B serum and the immunoprecipitates were analysed by SDS-PAGE. The unlabeled samples were also immunoprecipitated with 14B serum and protein kinase activity was examined as previously described.

2.13. Purification of 58K by DEAE-sephacel chromatography

Extracts from $4x10^7$ 983-2 cells labeled with [^{35}S]methionine were precipitated with ammonium sulfate as described above. The precipitates that were collected between 30 to 50% ammonium sulfate were solubilized in 10 mM Tris-HCl (pH8.0) containing 1 mM EDTA, 1mM 2-mercaptoethanol and 0.05% (v/v) Nonidet P40 (buffer A) and dialysed against buffer A. This material was loaded onto a DEAE-sephacel column (1.5 x 10.0 cm) equilibrated with buffer A. The column was then washed with 2 bed volumes of buffer A. Elution was carried out using an 80 ml gradient of 0 to 600 mM NaCl in buffer A and 1 ml fractions were collected. A small aliquot of each fraction was analysed directly for acid insoluble

radioactivity. Aliquots of some fractions were also immunoprecipitated using 14B serum and were either analysed directly by SDS-PAGE or were assayed for the presence of protein kinase activity.

2.14. Immunoaffinity Purification using Anti-synthetic Peptide Antibodies

Cell extracts were prepared and immunoprecipitations were carried out using anti-synthetic peptide antibodies as described above. The sepharose beads were sedimented by centrifugation at 2,500xg for 1 min at 4°C and washed five times with 1 ml of RIPA buffer. 500l of RIPA buffer containing 25ug of the corresponding peptide were added to the sepharose with constant shaking at 4°C for 3 hr to release the viral proteins. At the end of the incubation the sepharose was pelleted by centrifugation. The supernatant was collected and filtered through glasswool to remove any remaining sepharose beads. The supernatant containing the released material was either re-immunoprecipitated using another antiserum against a different epitope of the viral protein or it was mixed with an equal volume of double-strength sample buffer and analysed using SDS-PAGE. Residual bound material was also analysed by SDS-PAGE or assayed for protein kinase activity. For

controls, the procedure described above was carried out except that no synthetic peptide was added to release the viral proteins.

2.15. Measurement of Incorporation of Radioactivity by TCA Precipitation

From each sample two small aliquots of reaction mixture were taken and combined with 200 µl of 0.1% BSA solution. Five ml of ice-cold 10% TCA were added and the mixture was allowed to incubate at 4°C for at least 1 hour. The precipitates were collected by centrifugation for 10 min at 1000xg at 4°C and pellets were redissolved in 0.2 ml of 1.0 M NaOH. The samples were reprecipitated by the addition of 5 ml of ice-cold TCA and the final precipitates were collected by filtration through glass fibre filters. The filters were washed three times with 10 ml of TCA and twice with 10 ml ice-cold 95% ethanol. The filters were then allowed to air-dry and the amount of radioactivity present was determined by liquid scintillation counting.

2.16. Estimates of protein abundance

Ad5-infected cells were labeled with [^{35}S]methionine at 7 to 11 h post-infection. Cell extracts equivalent to 1x10^{6} cells were

immunoprecipitated using E1A-C1, 14B, and non-immune serum. Excess serum (approximately 100ul) was used in order to precipitate all of the E1 proteins. To ensure the complete removal of E1 proteins residual cell extracts after the first immunoprecipitation were subjected to at least two more rounds of precipitation using the same serum. The precipitates were analysed by SDS-PAGE. Autoradiography showed that no E1 proteins were detected after the first precipitation (data not shown). Bands containing E1A proteins and E1B 58K were excised from the dried gel and solubilized in 1ml of NCS at 60°C for 1 hr. The radioactivity present in these proteins was determined by liquid scintillation counting. The radioactivity present in the corresponding regions of gels containing samples prepared with non-immune serum were also determined to provide the background activity. The amounts of E1A proteins and E1B 58K were expressed as a percentage of the total radioactivity present in the cell extracts used for immunoprecipitation. The calculation assumed that the rates of synthesis and degradation of the E1 proteins were similar to those of total cell protein.

CHAPTER 3

Analysis of Ad5 E1 proteins using anti-peptide antisera

3.1 Analysis of E1 Polypeptides Using Antisera to Synthetic Peptides Corresponding to the Carboxy Termini

3.1.1. Preparation of Antisera Specific for the Carboxy Termini of Ad5 E1 Proteins

The nucleotide sequence of Ad5 region E1 has been analysed using both viral DNA and cDNA clones derived from E1 mRNAs (Bos et al., 1981; Maat et al., 1980; Maat and van Ormondt, 1979; Perricaudet et al., 1979; van Ormondt et al., 1980) and thus the amino acid sequence of E1 gene products can be deduced from these data. Using these predicted sequences it should be possible to produce synthetic peptides which correspond to various portions of E1 proteins and then use them to generate antisera with activity against these viral products. Synthetic peptides were therefore prepared which corresponded to the carboxy termini of the E1B-58K protein and of the E1A 1.1 and 0.9 kb mRNA gene products. Fig. 3 shows the predicted carboxy-terminal residues of E1B-58K and E1A proteins and the synthetic peptide sequences. For the 58K protein, the peptide was designated as 58-C and consisted of the six carboxy terminal amino acids of 58K, with a tyrosine residue added at the amino end to serve as a means of coupling the peptide to the BSA carrier using bis-diazotized benzidine. For the E1A proteins, the peptide was

Figure 3

The predicated carboxy-terminal sequences and synthetic peptides of Ad5 58K and E1A proteins. The carboxy-termini of the Ad5 E1B 58K species and of the Ad5 E1A gene products were deduced from viral DNA and mRNA sequencing data (Bos et al., 1981; Perricaudet et al., 1979; van Ormondt et al., 1980). The synthetic peptides corresponding to the carboxy termini were synthesized by a sequential solid-phase system (Stewart and Young, 1969).

Carboxy Terminal Sequences and Synthetic Peptides

E1B-58K NH_2.....-GLY-SER-SER-ASP-GLU-ASP-THR-ASPCOOH

58-C PEPTIDE NH_2TYR-SER-ASP-GLU-ASP-THR-ASPCOOH

E1A NH_2.....-LEU-SER-CYS-LYS-ARG-PRO-ARG-PROCOOH

E1A-C PEPTIDE NH_2TYR-GLY-LYS-ARG-PRO-ARG-PROCOOH

designated as E1A-C and consisted of the five carboxy terminal amino acids of the products of the 1.1 and 0.9kb mRNAs. The sixth residue of the protein sequence, a cysteine, was replaced with a glycine both to serve as a spacer and to prevent the formation of intermolecular disulfide bridges and thus the generation of peptide dimers. Again an amino terminal tyrosine residue was added for coupling purposes. These peptides were then coupled to BSA and the peptide-BSA conjugates were used to immunize rabbits as described in Materials and Methods.

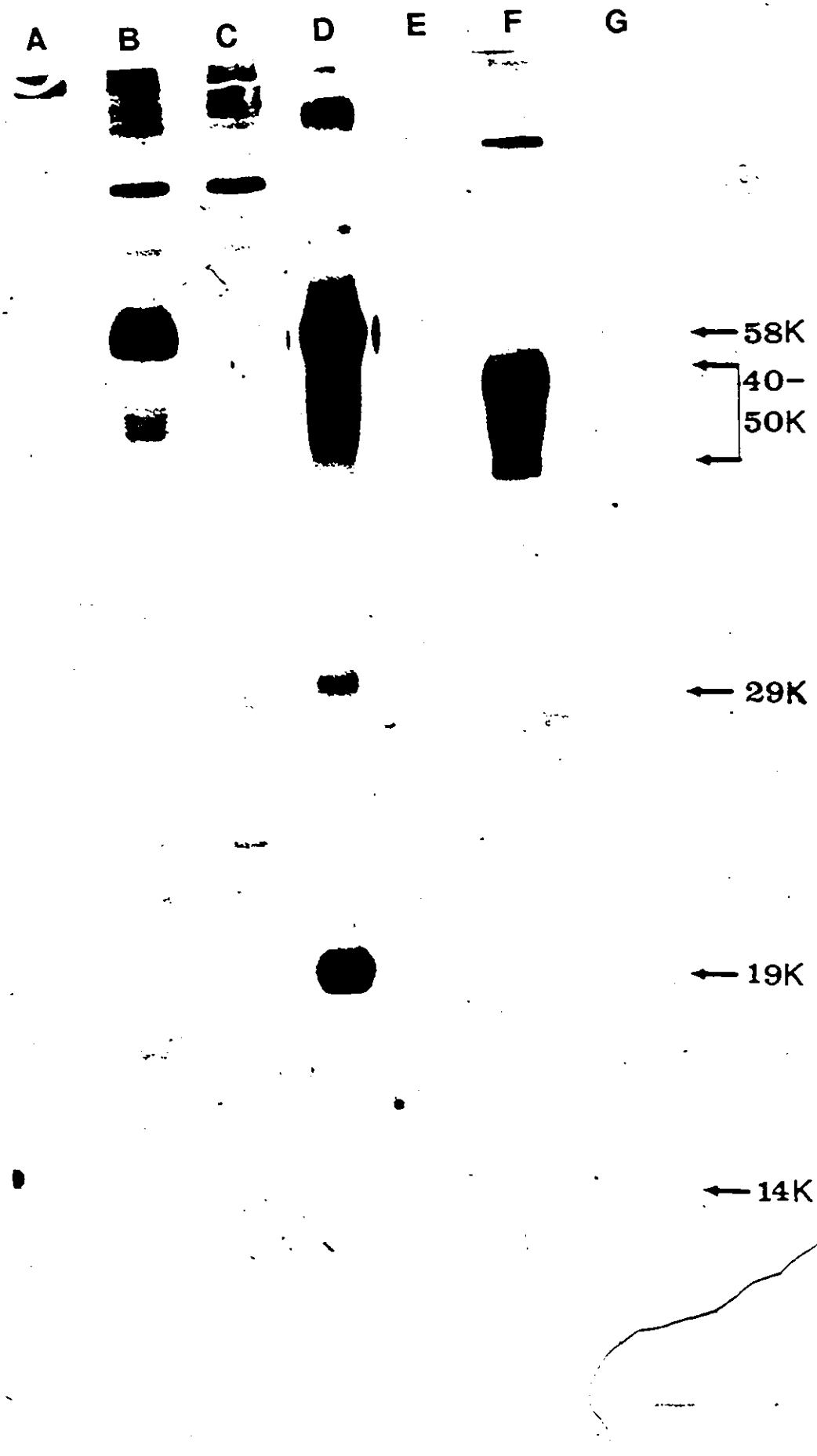
3.1.2. Immunoprecipitation of Ad5 Polypeptides with Antisera Raised Against the Synthetic Peptides Corresponding to the Carboxy Termini of E1 Proteins

Sera obtained from rabbits immunized with the synthetic peptides were tested for their ability to immunoprecipitate Ad5 polypeptides. Extracts from Ad5- and mock-infected cells labeled with [^{35}S]methionine from 7 to 11 h postinfection were precipitated either with combined hamster antitumour serum (cf. Rowe et al., 1983b) or with antisera raised against the peptides E1A-C and 58-C (sera designated E1A-C1 and 58-C1, respectively). The antitumor serum precipitated polypeptides from Ad5-infected cells with apparent

molecular weights of 58K, 29K, and 19K and a collection of proteins at about 40K to 50K (fig. 4, lane D). Previous studies (Lassam et al., 1979a,b, Rowe et al., 1984) using various host range mutants and cells transformed by specific fragments of Ad5 DNA, have suggested that the 58K and 19K polypeptides are coded for by the E1B region and that the 40K to 50K polypeptides are coded for by E1A. The origin of the 29K polypeptide is not known for certain but it probably represents the Ad5 E4 25K protein which has been reported to be associated with 58K under certain conditions (Sarnow et al., 1984) and which has been identified in immunoprecipitates prepared using certain Ad5 anti-tumour sera (Downey et al., 1983). None of these polypeptides was precipitated from mock-infected cells (fig. 4, lane E) or from Ad5-infected cells using hamster nonimmune serum (data not shown). The rabbit 58-C1 antipeptide serum precipitated almost exclusively a 58K polypeptide (fig. 4, lane B). Little material was precipitated by normal rabbit preimmune serum (fig. 4, lane A). It is unclear why 58-C1 (and the 58K-specific amino terminal anti-peptide serum, 58-N1, discussed below) failed to co-precipitate the E4 25K protein. However, the conditions of immunoprecipitation used in the present study were somewhat different than those employed by Sarnow et al. (1984) and in addition, this

Figure 4

Immunoprecipitation of Ad5 E1 polypeptides by antitumour and C-terminal antipeptide sera. Ad5- and mock-infected cells were incubated with [^{35}S]methionine from 7 to 11h postinfection, and whole cell extracts were immunoprecipitated with normal rabbit preimmune serum, combined hamster antitumour serum, or antipeptide serum 58-Cl or E1A-Cl. Lanes: A, preimmune rabbit serum with Ad5-infected cells; B and C, 58-Cl serum with Ad5-infected and control cells, respectively; D and E, combined antitumour serum with Ad 5-infected and control cells, respectively; F and G, E1A-Cl serum with Ad5-infected and control cells, respectively.

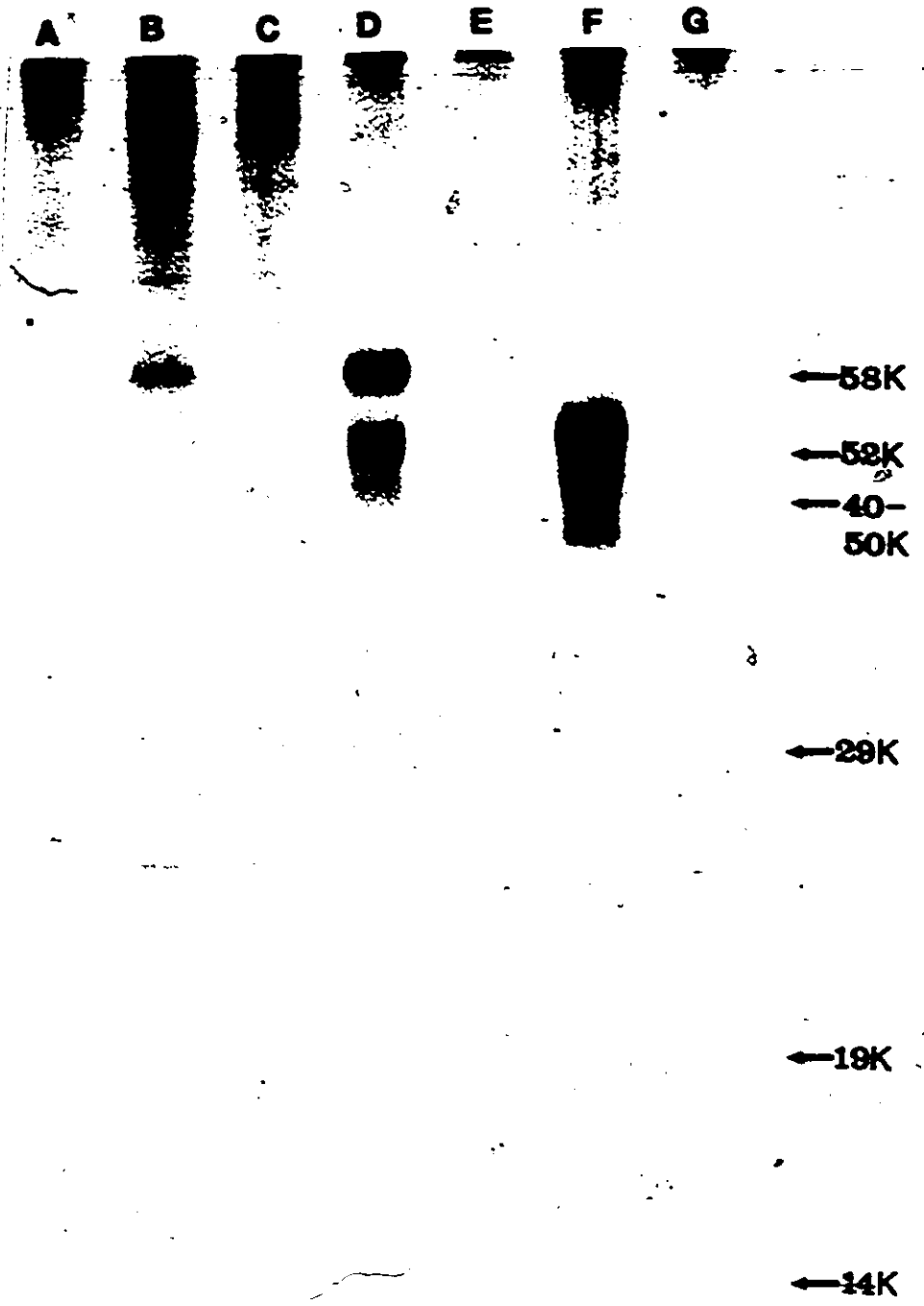

A
B
C
D
E
F
G
58K
40-
50K
29K
19K
14K

species has not always evident with all sera that recognize 58K (Sarnow et al., 1982; Anderson et al., 1984; Green et al., 1983; 1984; Lucher et al., 1984). The rabbit E1A-C1 antipeptide serum (fig.4, lane F) precipitated a collection of polypeptides from Ad5-infected cells with apparent molecular weights ranging from about 35K to 50K which had migration properties similar to those of the E1A 40K to 50K gene products seen with antitumor serum. The E1A-C1 serum precipitated little from mock-infected cells (fig. 4, lane G).

To study the specificity of the antipeptide sera further, the same sera were used to immunoprecipitate extracts from ^{32}P-labeled cells as it was already known that the 58K and E1A gene products were phosphoproteins (Ross et al., 1978; Malette et al., 1983; Gaynor et al., 1982). The antitumor serum (fig. 5, lane D) and the 58-C1 antipeptide serum (fig. 5, lane B) both precipitated a ^{32}P-labeled 58K species from infected cells. No such phosphoprotein was detected using preimmune serum (fig. 5, lane A) or by immunoprecipitation of extracts from mock-infected cells with antitumour or antipeptide sera (fig. 5, lane C and E). The antitumor serum also precipitated two other phosphoproteins from infected cells of about 40K to 50K. The E1A-C1 serum also precipitated a group of

Figure 5

Immunoprecipitation of ^{32}P-labeled Ad5 E1 polypeptides by antitumour and C-terminal antipeptide sera. Ad5- and mock-infected cells were incubated with $^{32}P_i$ from 7 to 11h postinfection, and whole cell extracts were immunoprecipitated with normal rabbit preimmune serum, combined hamster antitumour serum, or antipeptide serum 58-Cl or E1A-Cl. Lanes: A, preimmune rabbit serum with Ad5-infected cells; B and C, 58-Cl serum with Ad5-infected and control cells, respectively; D and E, combined antitumour serum with Ad 5-infected and control cells, respectively; F and G, E1A-Cl serum with Ad5-infected and control cells, respectively.


A
B
C
D
E
F
G
58K
52K
40-
50K
29K
19K
14K

phosphoproteins from infected cells ranging from about 40K to 50K (fig. 5, lane F). No labeled polypeptides were detected by immunoprecipitation of extracts from uninfected cells with E1A-C1 serum (fig. 5, lane G). To obtain better resolution of these polypeptide species in a one-dimensional gel, ^{32}P-labeled polypeptides precipitated by antitumor serum and by E1A-C1 serum were again separated on a 12% gel under slightly different conditions. Electrophoresis was carried out as described in Materials and Methods but continued for a further 60 min after the bromophenol blue dye marker had run out of the gel. As shown in fig. 6, the E1A-C1 serum clearly precipitated four major phosphoproteins of 52K, 50K, 48.5K, and 45K and two minor species of 37.5K and 35K (lane C1). The hamster antitumor serum recognized only the 52K, 48.5K, and 37.5K species, as well as the E1B 58K protein (lane PS). The ^{32}P-labeled immunoprecipitate obtained using E1A-C1 serum was further analysed by two-dimensional gel electrophoresis. As shown in fig. 7 the viral polypeptides precipitated by E1A-C1 serum were resolved into a cluster of four major and two minor phosphoproteins with pIs ranging between about 5.0 to 4.5. It should be noted that on some gels, each of these species could be partially resolved into a family of subspecies. These results demonstrated that sera

Figure 6

Comparsion of phosphoproteins precipitated by combined hamster antitumour and E1A-Cl sera. ^{32}P-labeled Ad5-infected cells were immunoprecipitated with combined antitumour serum (PS) or E1A-Cl serum (Cl). The samples were analysed on a 12% SDS-polyacrylamide gel as described in Materials and Methods. The gel was electrophoresed for 60 min further after the dye front had reached the bottom of the gel.

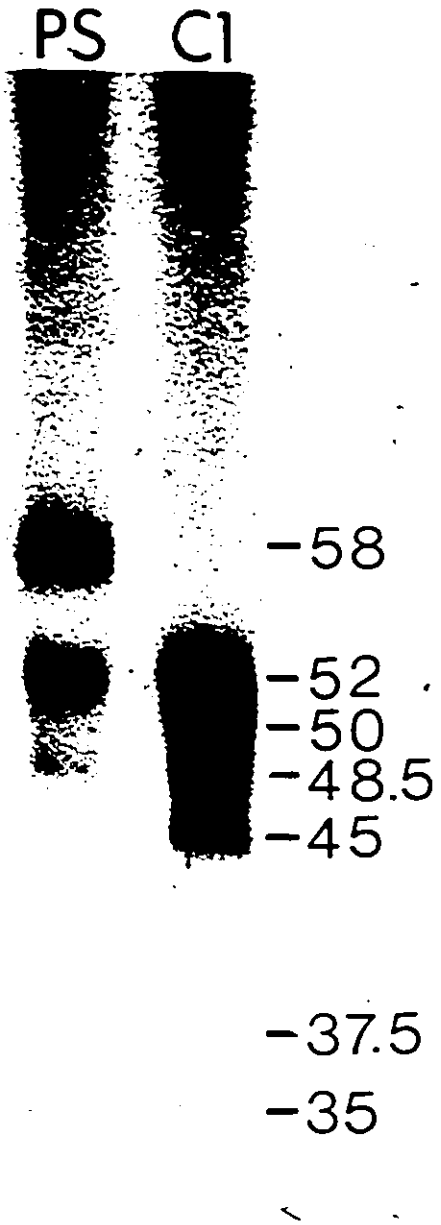
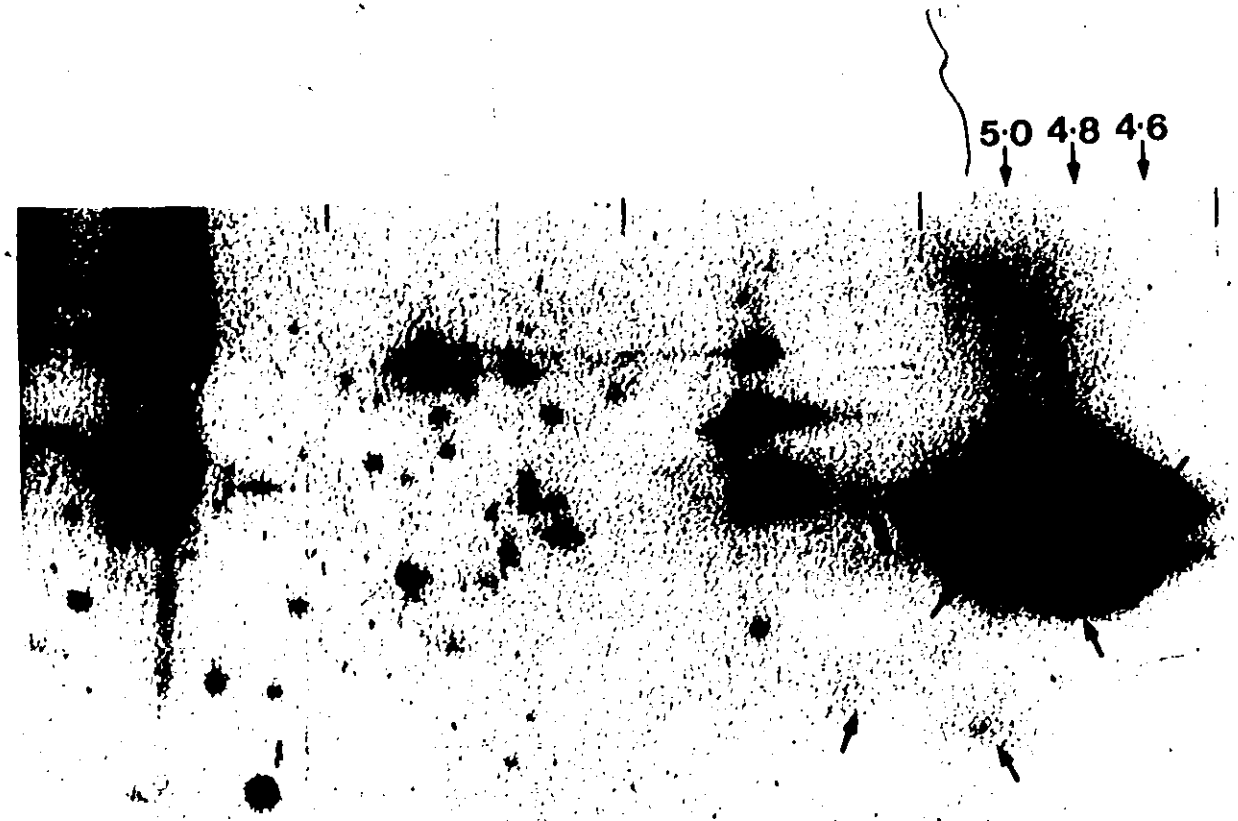

PS
C1
-58
-52
-50
-48.5
-45
-37.5
-35

Figure 7

Two-dimensional gel electrophoresis of Ad5 E1A proteins precipitated by E1A-C1 serum. Ad5-infected cells were labeled with [^{35}S]methionine from 7 to 11 h postinfection and immunoprecipitated with E1A-C1 serum. Immunoprecipitated proteins were separated in an isoelectric focusing gel (pH 4-6) and then in a 12% SDS-polyacrylamide gel as described in Materials and Methods. The arrows indicate the pH of the isoelectric focusing gel, as measured as described in Materials and Methods.



E1A-C1

raised against synthetic peptides with sequences corresponding to carboxy termini of E1 proteins are active against these viral proteins and there are apparently four major and two minor polypeptide species derived from E1A of Ad5, each of which may consist of a family of subspecies.

3.1.3. Specificity of antipeptide sera

Another way of demonstrating that these viral proteins were specifically immunoprecipitated by antipeptide sera was to precipitate extracts in the presence of various concentrations of the corresponding synthetic peptides. The synthetic peptides and viral proteins should compete for the antipeptide antibodies and thus with increasing amounts of synthetic peptide the precipitation of proteins with antigenic sites specified by these sera should be increasingly blocked. As shown in fig. 8, neither the 58C (lane A-D) nor E1A-C (lane I-L) peptides had any significant effect on the precipitation of the 58K and the 40K to 50K polypeptides by the hamster antitumor serum. These data suggest that antibodies against the epitopes defined by these peptides are present only in low amounts in the antitumour sera. In addition the 58C peptide had no effect on precipitation of the 35K to 52K E1A complex precipitated by E1A-C1 antipeptide serum (fig. 9, lane

Figure 8

Effect of synthetic peptides on precipitation of viral proteins by antitumour and C-terminal antipeptide sera. ^{35}S- or ^{32}P-labeled Ad5-infected cells were precipitated with either antitumour or C-terminal antipeptide serum in the presence of various amounts of synthetic peptides, as follows: ^{35}S-labeled cells with antitumour serum and 0 ug (lane A), 0.1 ug (lane B), 1.0 ug (lane C), and 10 ug (lane D), of 58-C peptide; ^{35}S-labeled cells with 58-C1 serum and 0 ug (lane E), 0.1 ug (lane F), 1.0 ug (lane G), and 10 ug (lane H) of 58-C peptide; and ^{32}P-labeled cells with antitumour serum (lane I-L) or E1A-C1 serum (lane M-P) and the same respective amounts of E1A-C peptide as described for 58-C.

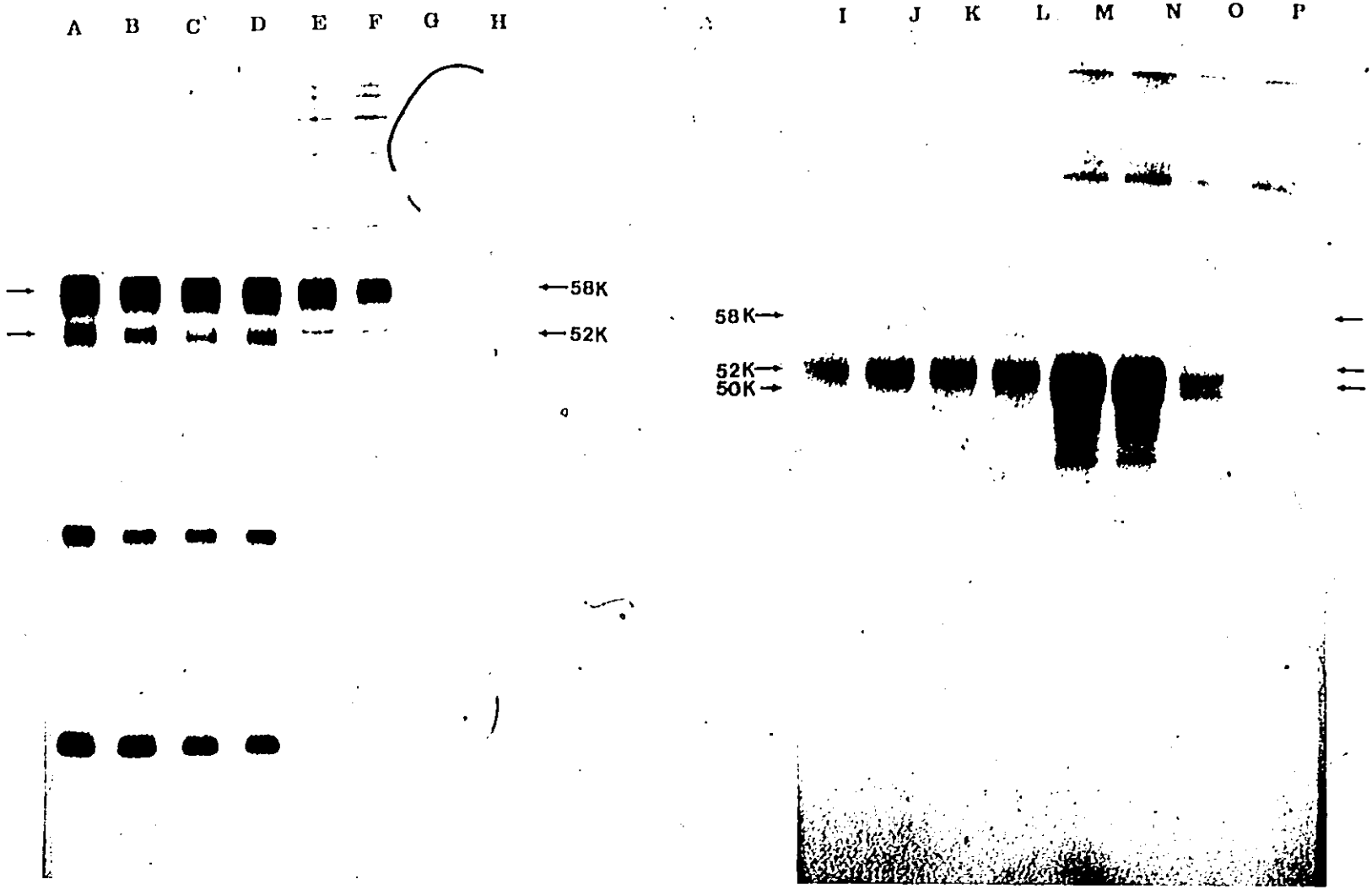
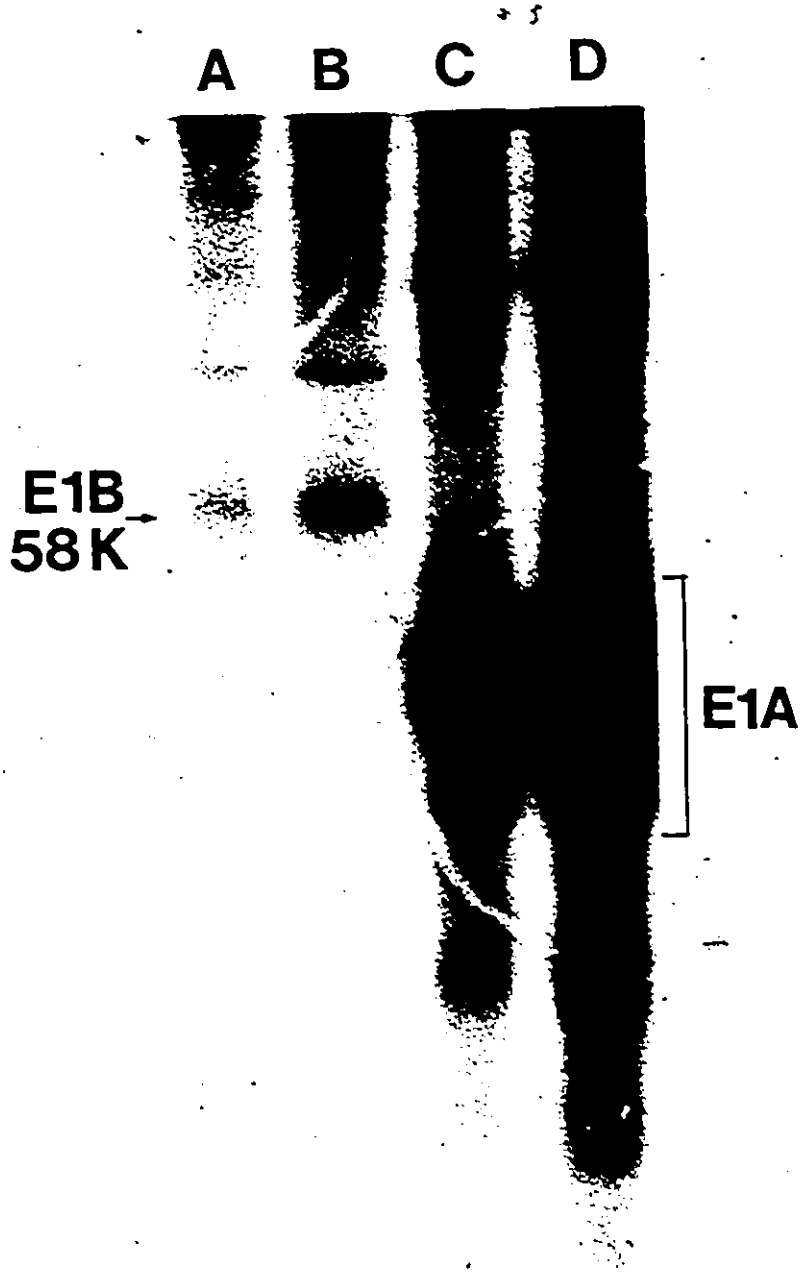

A B C D E F G H
58K
52K
I J K L M N O P
58K
52K
50K

Figure 9

Effect of heterologous peptide on precipitation of viral proteins by C-terminal antipeptide sera. ^{35}S-labeled Ad5-infected cell extracts were immunoprecipitated with either 58-C1 or E1A-C1 sera in the presence or absence of heterologous synthetic peptide, as follow: ^{35}S-labeled cells with 58-C1 serum and 0 ug (lane A) and 25 ug (lane B) of E1A-C peptide; and ^{35}S-labeled cells with E1A-C1 serum and 0 ug (lane C) and 25 ug (lane D) of 58-C peptide.


A
B
C
D
E1B
58K
E1A

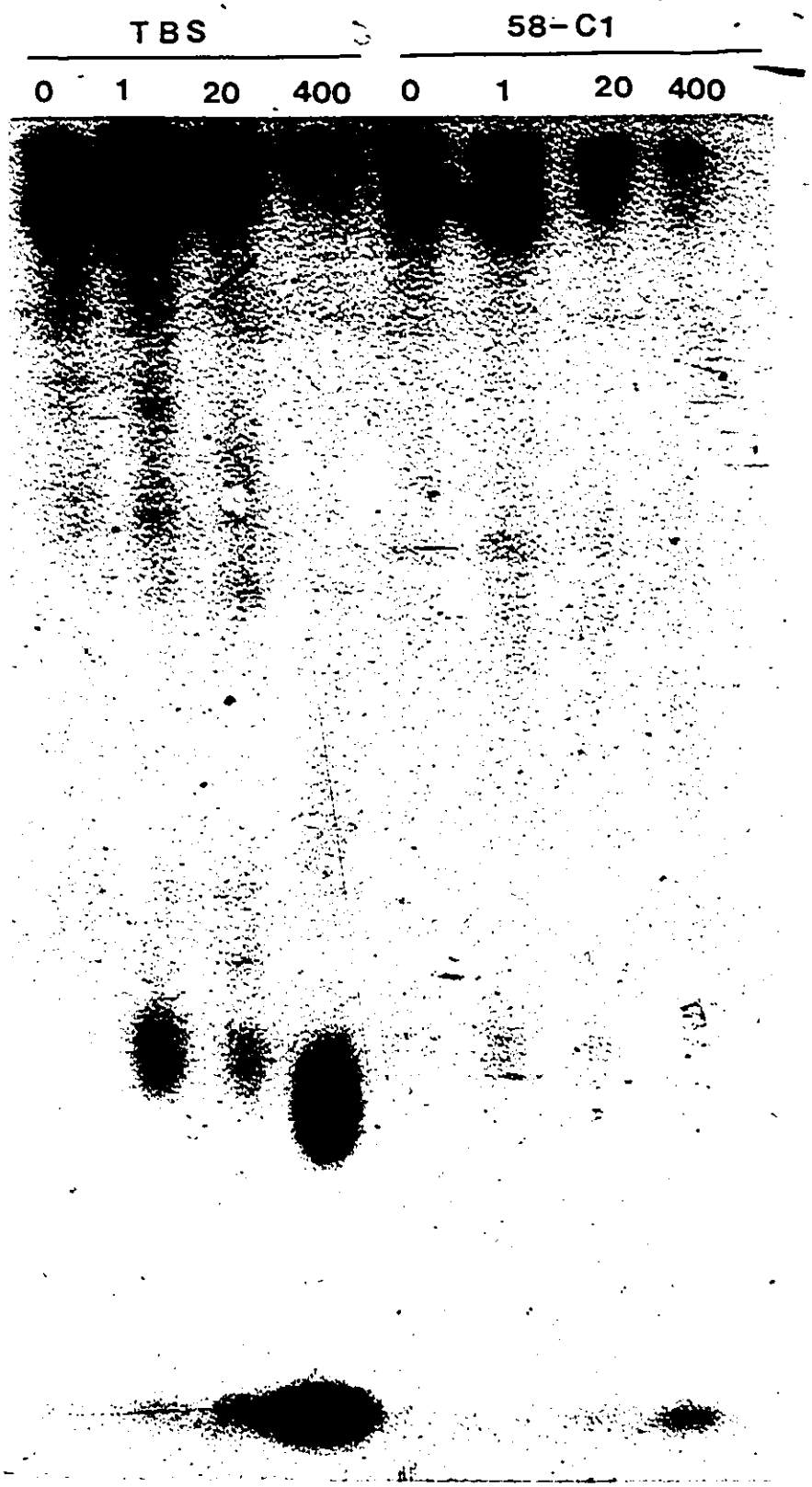
D), nor did the E1A-C peptide affect 58K precipitation by 58-C1 antipeptide serum (fig. 9, lane B). However, increasing concentrations of the 58-C peptide blocked the precipitation of the 58K protein by 58-C1 serum (fig. 8, lane E-H), and the E1A-C peptide blocked the precipitation of the 35K to 52K complex by E1A-C1 serum (fig. 8, lane M-P). In addition to these major viral species, several other polypeptides were detected in the immunoprecipitates, often at low levels. The presence of most of these was unaffected by the addition of peptides and thus they probably represented non-specific contaminants of the precipitation. However, the level of some of the minor species was reduced by the presence of the peptides. Thus these proteins could either contain the antigenic site specified by the peptide or be specifically bound to the Ad5 proteins containing these sites (see below, Chapter 4).

3.1.4. Comparison of viral proteins precipitated by antitumor and antipeptide sera by Cleveland peptide mapping

To further prove that the proteins precipitated by the antipeptide sera were in fact the same virus-specific polypeptides recognized by antitumor serum, the ^{32}P-labeled viral proteins were analysed by

Figure 10

Partial hydrolysis of the 58K polypeptide precipitated by antitumour or 58-Cl serum with Staphyloccal V-8 protease. ^{32}P-labeled Ad5-infected cells were immunoprecipitated with antitumour serum or 58-Cl serum, and the labeled 58K protein precipitated by each was removed from an SDS-polyacrylamide gel and rerun in the presence of 0, 1, 20, or 400 ug of Staphyloccal V-8 protease per ml as described in Materials and Methods. TBS, Combined hamster antitumour serum (tumour bearing serum).


TBS
58-C1
0
1
20
400
0
1
20
400

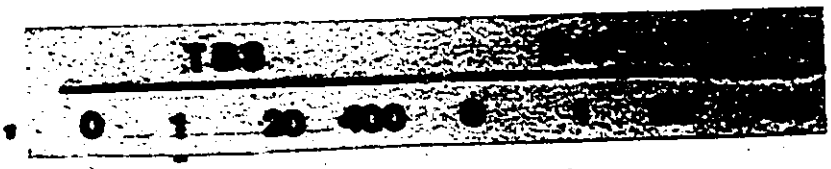
Cleveland partial peptide mapping (cf. Cleveland et al., 1977). The 58K polypeptide species precipitated by both the antitumor and 58-Cl sera were hydrolysed with increasing concentrations of Staphylococcal V-8 protease. As shown in fig. 10, the 58K protein precipitated by both sera produced identical peptide patterns characterized by a single major phosphopeptide. Similarly, hydrolysis of the 52K protein precipitated by the antitumuor and the E1A-Cl sera produced identical patterns of multiple phosphopeptides (fig. 11). Thus, the antipeptide sera precipitated the same virus-specific polypeptides recognized by the hamster antitumor serum. All four major phosphoproteins precipitated by E1A-Cl serum were related as revealed by their similar tryptic phosphopeptide maps (Yee et al., 1983; Tremblay and Branton, personal communication). This was expected if all were E1A products.

3.1.5. Localization of Ad5 proteins by immunofluorescence

The antipeptide sera were used to localize the viral proteins in Ad5-infected cells by indirect fluorescent antibody staining. Ad5- and mock-infected cells growing on coverslips were fixed at 10 h postinfection and then incubated with either 58-Cl or

Figure 11

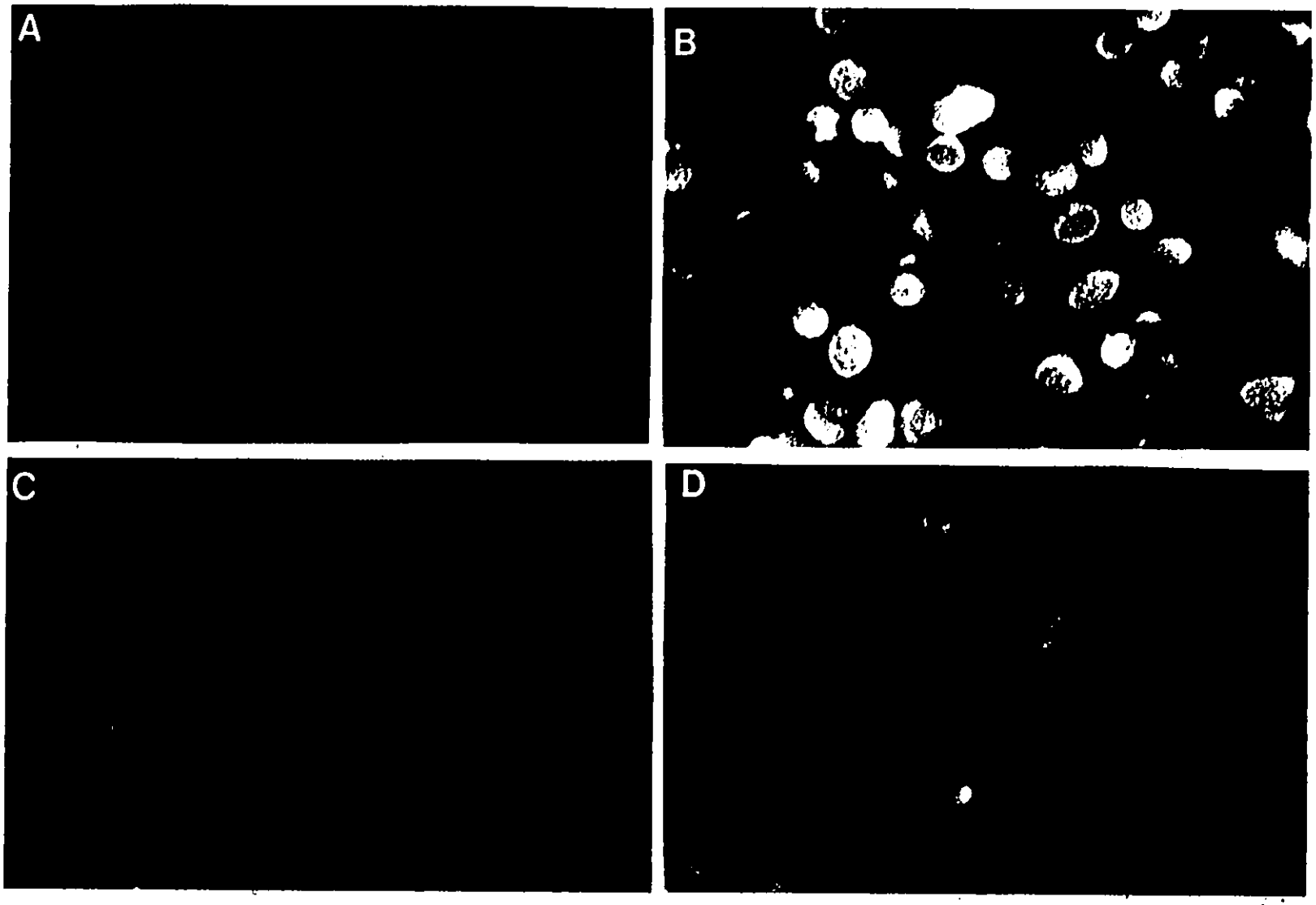
Partial hydrolysis of the 52K polypeptide precipitated by antitumour or E1A-C1 serum with Staphyloccal V-8 protease. ^{32}P-labeled Ad5-infected cells were immunoprecipitated with antitumour serum or E1A-C1 serum, and the labeled E1A 52K protein precipitated by each was removed from an SDS-polyacrylamide gel and rerum in the presence of 0, 1, 20, or 400 ug of Staphyloccal V-8 protease per ml as described in Materials and Methods. TBS, Combined hamster antitumour serum (tumour bearing serum).


TDS
0 1 20 100

E1A-C1 sera. The rabbit antibodies were stained by fluorescein isothiocynante conjugated goat anti-rabbit immunoglobulin antibody and visualized using an ultraviolet fluorescence microscope. Neither of these sera stained mock-infected KB cells to any significant degree (fig. 12, A and C). The 58-C1 serum stained Ad5-infected cells in both the nucleus and cytoplasm (Fig. 12, B). The cytoplasmic fluorescence was concentrated in the perinuclear region, and the remaining cytoplasm was only weakly stained. These data suggested that at early times after infection the major portion of E1B-58K protein is located in or surrounding the nucleus. The E1A-C1 serum also stained both the nuleus and the cytoplasm (fig. 12, D). The nuclear staining was largely in concentrated speckled areas, whereas the cytoplasmic staining was weaker and diffuse. These data suggest that the majority of E1A proteins are located and may function within the nucleus.

Figure 12

Fluorescent-antibody staining with C-terminal antipeptide sera. Ad5-infected (B and D) and mock-infected (A and C) cells were fixed and stained at 12 h postinfection, using the indirect fluorescent-antibody staining technique with the antipeptide sera 58-C1 (A and B) and E1A-C1 (C and D).


A
B
C
D

3.2. Analysis of E1 Polypeptdie Using Antisera to Synthetic Peptides Corresponding to the Amino Termini

3.2.1. Preparation of Antisera Against the Amino-termini of E1 polypeptides

Antisera against synthetic peptides could cross-react with cellular proteins that harbour regions with identical or similar amino acid sequences. In addition, such sera may not recognize mutant viral proteins in which the mutation has altered or abolished the predetermined antigenic site. To avoid these problems a second set of antipeptide sera against the amino termini of E1 proteins was raised. Fig. 13 shows the amino-terminal residues and synthetic peptide sequences. The synthetic peptide corresponding to the amino terminus of the E1B 58K protein was designated as 58-N and consisted of the six amino terminal amino acids with a tyrosine residue added at the carboxy end to serve as a linker for coupling the peptide to BSA. For the E1A gene products, the peptide (designated E1A-N) consisted of seven amino acids corresponding to the amino terminus of E1A polypeptides with the fifth residue, a cysteine, being substituted for by alpha-amino butyric acid. It has been shown that, antigenically, alpha-amino butyric acid is a good replacement for cysteine (Hopp, 1983) and this

Figure 13

The predicted amino-terminal sequences and synthetic peptides of 58K and E1A proteins. The amino-termini of the Ad5 E1B 58K species and of the Ad5 E1A gene products were deduced from viral DNA and mRNA sequencing data (Bos et al., 1981; Perricaudet et al., 1979; van Ormondt et al., 1980). The symbol ABA stands for alpha-amino butyric acid which was substituted for cys^6 in the E1A-N1 peptide. The synthetic peptides corresponding to the amino termini were synthesized by a sequential solid-phase system (Stewart and Young, 1969).

Amino Terminal Sequences and Synthetic Peptides

E1B 58K NH_2Met-Glu-Arg-Arg-Asn-Pro-Ser-Gly.....COOH

58-N NH_2Met-Glu-Arg-Arg-Asn-Pro-TyrCOOH

E1A NH_2Met-Arg-His-Ile-Ile-Cys-His-Gly-Gly.....COOH

E1A-N NH_2Met-Arg-His-Ile-Ile-αABA-His-GlyCOOH

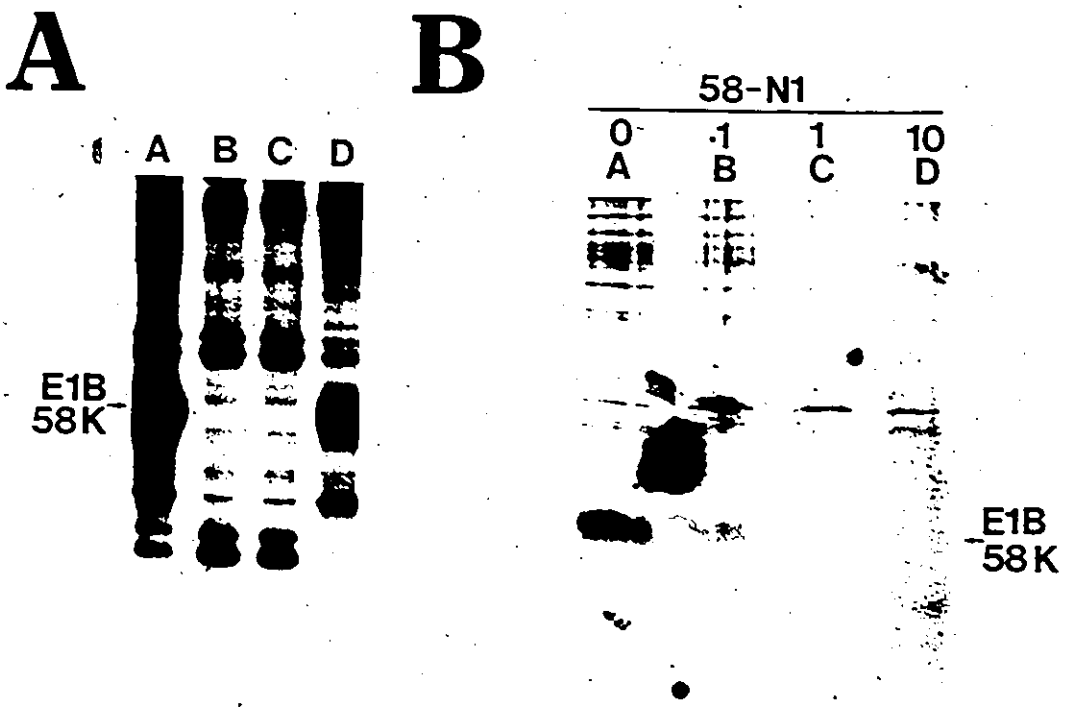
substitution was carried out to prevent the formation of interpeptide disulfide bridges. The peptides were coupled to BSA, E1A-N by treatment with carbodiimide to crosslink the carboxy terminus directly, and 58-N by bis-diazotized benzidine to couple via the tyrosine residue as before. The peptide-BSA conjugates were then used to immunize rabbits as described in Materials and Methods.

3.2.2. Immunoprecipitation of Ad5 polypeptides with antiserum raised against the amino termini synthetic peptide

The antisera raised against the synthetic peptides 58-N and E1A-N, designated 58-N1 and E1A-N1, respectively, were tested for their ability to precipitate viral polypeptides from Ad5-infected cells. The carboxy terminal specific rabbit antipeptide sera, 58-C1 and E1A-C1, which were described previously, were used as a positive control in these studies. Extracts from Ad5- and mock-infected cells labeled with [^{35}S]methionine from 7 to 11 hour postinfection were precipitated with either 58-N1 or 58-C1 sera. As shown in fig. 14A, lane A, 58-C1 precipitated a 58K polypeptide and 58-N1 also precipitated a polypeptide which co-migrated with the E1B 58K protein (fig. 14A, lane D). Little material was precipitated by 58-N1 from

Figure 14

Immunoprecipitation of 58K and effect of synthetic peptide on precipitation by 58-N1 serum. ^{35}S-labeled mock- or Ad5-infected cells were immunoprecipitated with preimmune rabbit serum, or antipeptide serum 58-N1 or 58-C1. Fig. 13A: Ad5-infected cell extracts precipitated with 58-C1 serum (lane A), mock-infected cell extracts precipitated with 58-N1 serum (lane B), Ad5-infected cell extracts precipitated with preimmune rabbit serum (lane C) and 58-N1 serum (lane D). Fig. 14B: Ad5-infected cell extracts precipitated with 58-N1 serum in the presence of 0 (lane A), 0.1 (lane B), 1 (lane C), and 10 ug (lane D) of 58-N peptide.

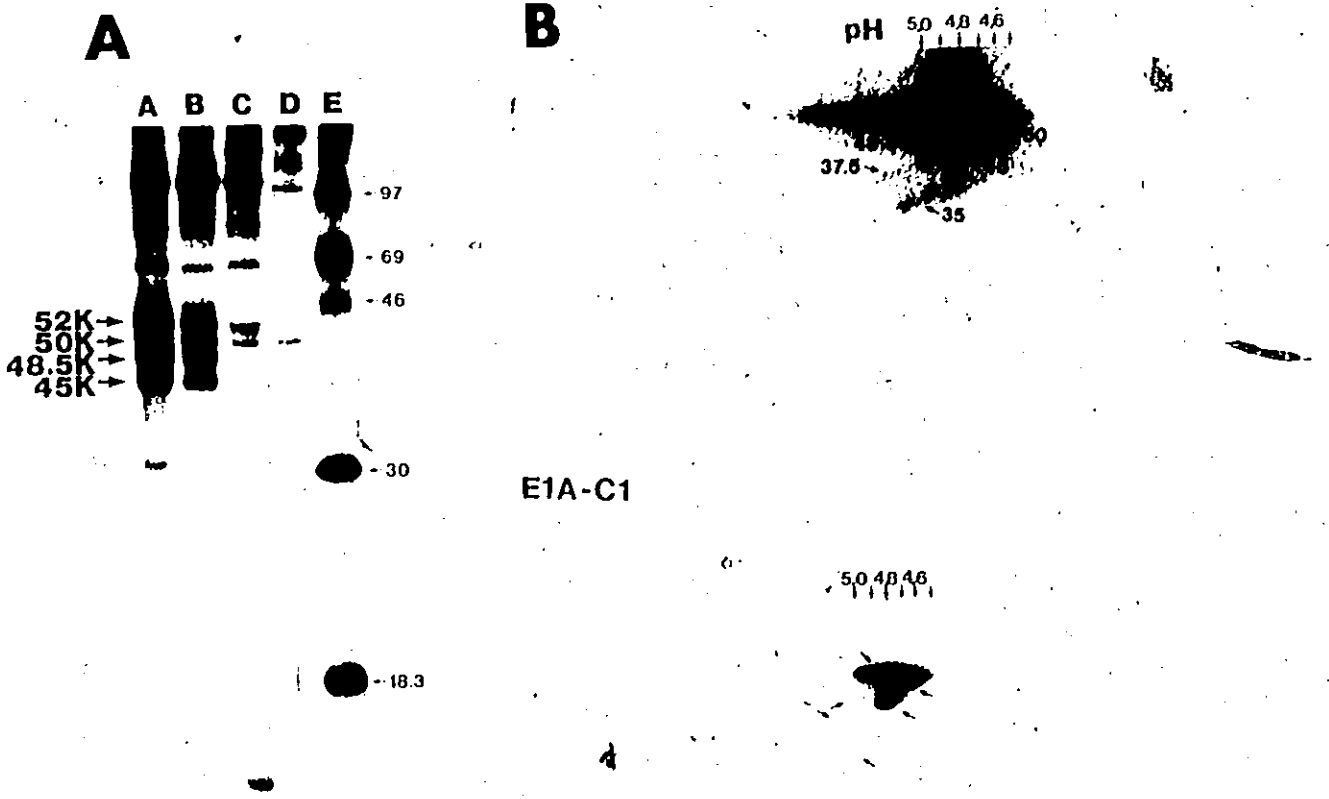

A
B
A
B
C
D
E1B
58K
58-N1
0
A
1
B
1
C
10
D
E1B
58K

mock-infected cells (fig. 14A, lane B). Addition of increasing amounts of 58-N peptide blocked the precipitation of the 58K phosphoprotein (fig. 14B). These results suggested that 58-N1 serum was also active against E1B 58K proteins.

To examine the specificity of the E1A-N1 serum, Ad5-infected or mock-infected cells were labeled with either ^{32}P-orthophosphate or [^{35}S]methionine from 7 to 11h postinfection and cell extracts were immunoprecipitated with either E1A-N1 or E1A-C1 sera. Fig 15 shows the results obtained when ^{35}S-labeled samples were analysed on a one-dimensional gel. The antiserum E1A-N1 precipitated a total of six polypeptides from Ad5-infected cells. These polypeptides consist of the four major species with apparent molecular weights of 52K, 50K, 48.5K, 45K and, two minor species of 37.5K and 35K (lane B) which had migration properties identical to those of the E1A polypeptides precipitated by E1A-C1 serum (lane A). None of these polypeptide species were detected using nonimmune rabbit serum (lane C) or extracts from mock-infected cells treated with E1A-N1 serum (lane D). Addition of increasing amounts of E1A-N peptide blocked the precipitation of the E1A species (see below, fig. 19). These data suggested that the E1A-N1 serum was active against the gene products derived from the Ad5

Figure 15

Analysis on one- and two-dimension gels of E1A proteins immunoprecipitated by E1A-N1 and E1A-C1 antipeptide sera. ^{35}S-labeled mock- or Ad5-infected cells were immunoprecipitated with preimmune rabbit serum, or antipeptide serum E1A-N1 or E1A-C1. Precipitates were analysed by one-dimensional SDS-PAGE using 12% polyacrylamide gel or two-dimensional gels, as described in Materials and Methods. Fig. 15A: ^{35}S-labeled samples analysed by one-dimensional SDS-PAGE. Ad5-infected cells precipitated with E1A-C1 serum (lane A), or E1A-N1 serum (lane B), or pre-immune rabbit serum (lane C). Mock-infected cells precipitated with E1A-N1 serum (lane D) and ^{14}C-labeled molecular weight markers (lane E). Figure 15B: ^{32}P-labeled samples from Ad5-infected cells precipitated with E1A-N1 or E1A-C1 serum and analysed by two-dimensional gel electrophoresis. Numbers along the top of the autoradiograms indicated approximate pH of isoelectric focusing gel.


A
A B C D E
- 97
- 69
- 46
52K
50K
48.5K
45K
- 30
- 18.3
B
pH 5.0 4.8 4.6
37.5
35
E1A-C1
5.0 4.8 4.6
E1A-N1

E1A 1.1 and 0.9kb mRNAs. Analysis of equal amounts of immunoprecipitates prepared using the same volumes of E1A-N1 serum was about one-half that of E1A-C1 (data not shown). This difference persisted even when rabbits were boosted further with peptide (data not shown).

To further characterize these E1A products, ^{32}P-labeled infected cell extracts were also immunoprecipitated with E1A-N1 serum and then analysed by two-dimensional gel electrophoresis. Fig. 15B shows that the immunoprecipitate obtained using E1A-N1 serum precipitated the same set of polypeptides as E1A-C1 serum, i.e. four major (52, 50, 48.5, and 45K) and two minor (37.5 and 35K) acidic phosphoproteins. These data indicated that the multiple products of the E1A 1.1 and 0.9kb mRNA, including the minor species, are not generated by proteolytic cleavage at either terminus and suggested that all are full length proteins that are phosphorylated.

3.3.1. Analysis of E1A polypeptides synthesized by Ad5 Mutants mapping in E1A

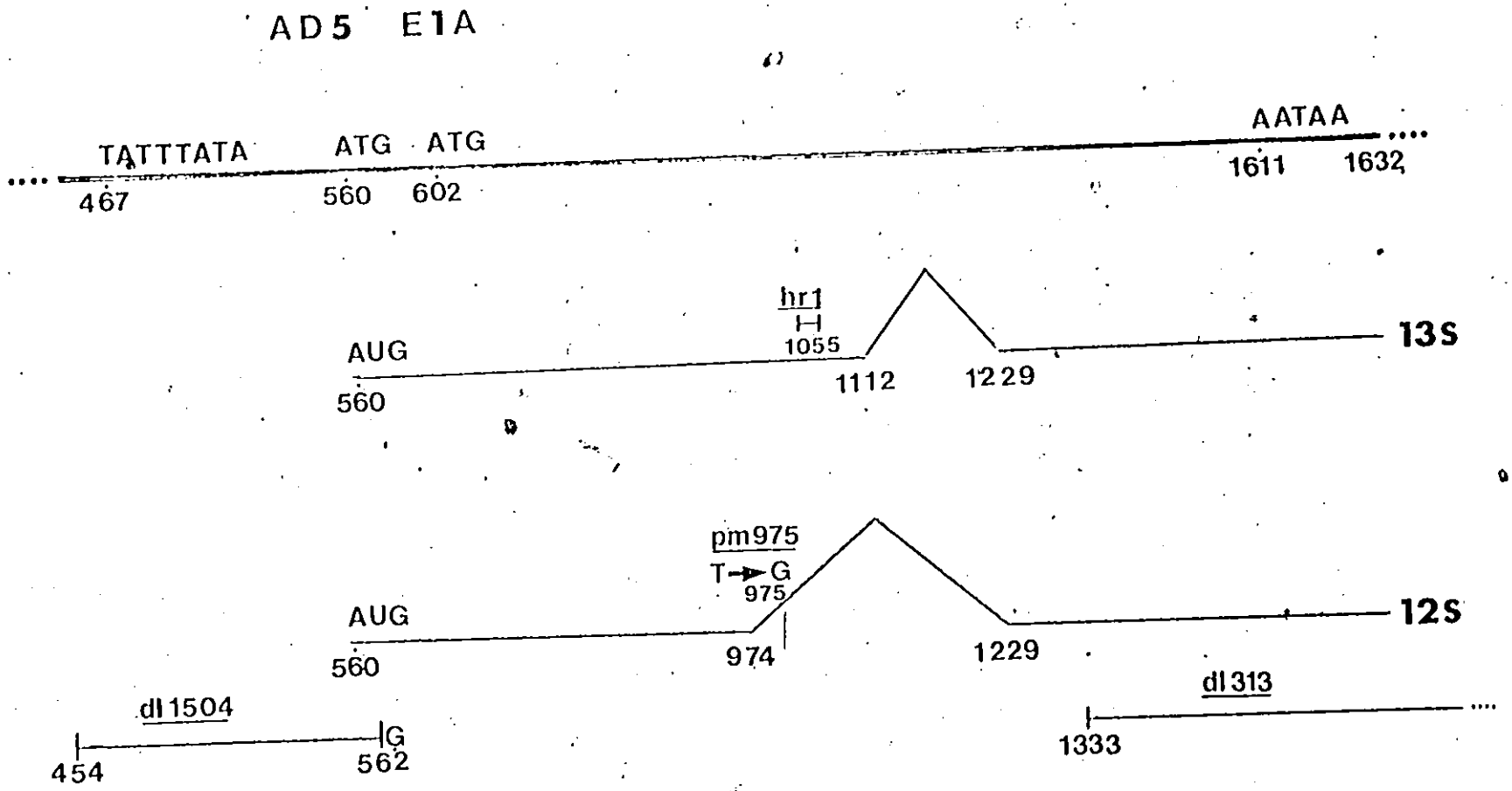
As described in the Introduction, two mRNAs of 1.1 and 0.9 kb are transcribed from E1A early during the lytic infection. Both E1A-N1 and E1A-C1 sera precipitated an identical family of polypeptides consisting of of four major and two minor species

derived from these two mRNAs. According to cDNA sequencing data, products derived both of these mRNAs encode completely overlapping polypeptides. To confirm the origin of E1A polypeptide species, the E1A products synthesized by two E1A mutants, pm975 and hr1, were analysed.

The point mutant, pm975, contains a transversion of a T to G that eliminates the 5' splice site of the E1A 0.9kb mRNA, while leaving the reading frame of the 1.1kb mRNA unaffected. As a result of the mutation only the 1.1kb mRNA is produced (Montell et al., 1982). The Ad5 host range mutant hr1 (Harrison et al., 1977) contains a single base deletion at position 1005 causing a frame shift that produces a nonsense triplet (TGA) 11 codons downstream (Ricciardi et al., 1981). Thus this mutation should generate a truncated polypeptide from the 1.1kb mRNA but should not affect the product of the 0.9kb mRNA. The positions of these two mutations relative to the E1A mRNAs have been indicated in Fig. 16. Cells infected with either pm975 or hr1 were labeled with [^{32}P]orthophosphate from 7 to 11 h post-infection and cell extracts were precipitated with E1A-N1 serum and analysed by two-dimensional gel electrophoresis. Fig.17 shows that with pm975, which synthesizes only the products of the 1.1 kb mRNA, the major phosphoproteins, 52K, 48.5K migrating at a pI of

Figure 16

Map locations of Ad5 E1A mutants. The spliced mRNA species derived from the r-strand of E1A are shown and the relative locations of mutations present in various Ad5 E1A mutants have been noted in the figure (see Chapter 3 for details).


AD5 E1A
TATTTATA
ATG
ATG
AATAA
467
560
602
1611
1632
hr1
1055
AUG
560
1112
1229
13S
pm975
T→G
975
AUG
560
974
1229
12S
dl1504
454
G
562
dl313
1333

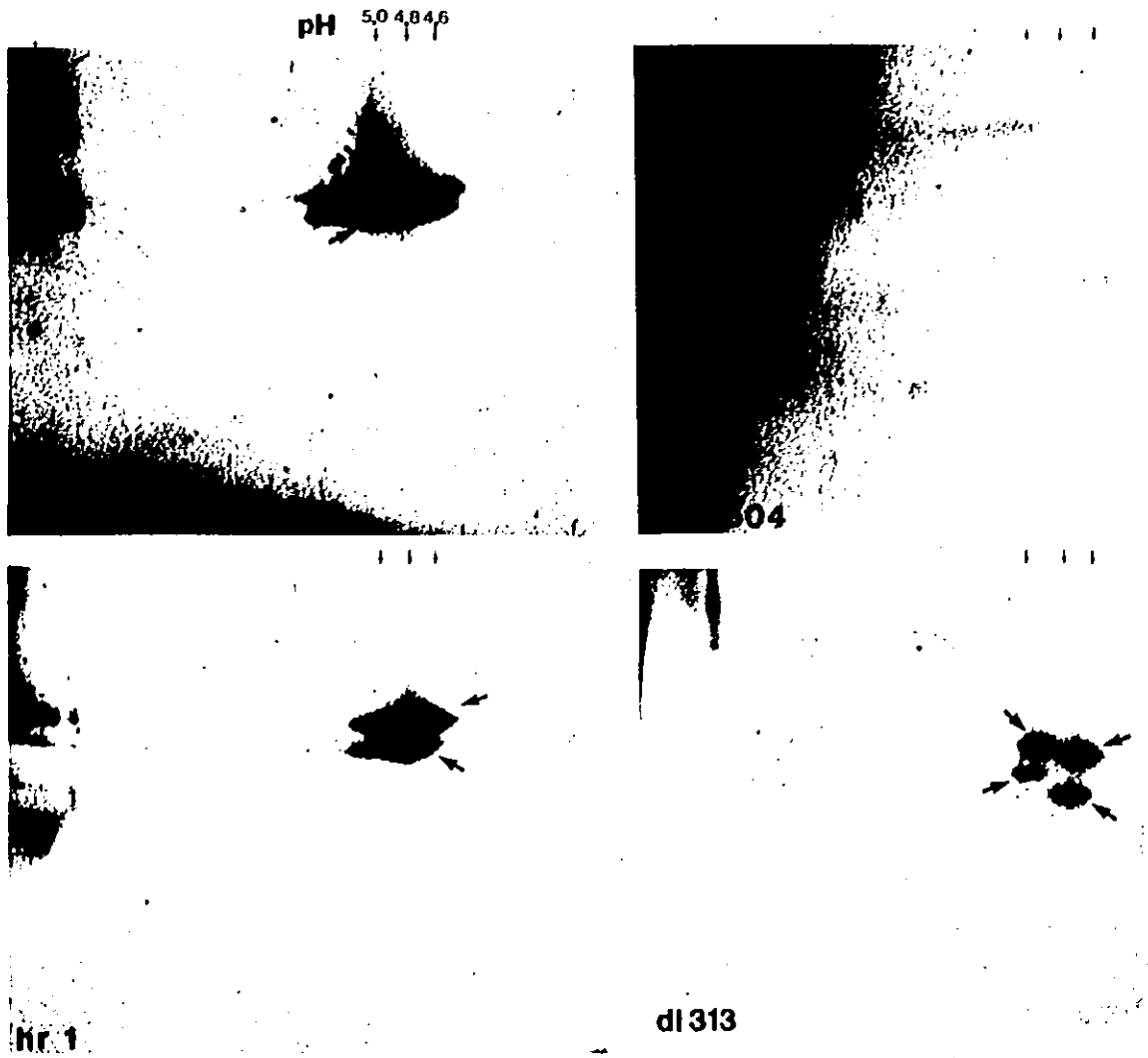
about 4.9, and the minor 37.5K species were detected. Conversely, with hr1, only the 50K and 45K major products migrating at a pI of about 4.7 and the minor 35K species originating from the 0.9 kb mRNA were observed. These results were identical to those obtained previously in a collaborative study not presented in this thesis using E1A-C1 serum (Rowe et al., 1983). In both these and other experiments employing E1A-N1 serum and cells labeled with [^{35}S]methionine (data not shown), the truncated 1.1 kb mRNA product predicted for hr1 (Ricciardi et al., 1981) was not detected. This product must either be extremely short-lived or possess an altered configuration making the N-terminal antigenic site unavailable for reaction with the antibody. These results clearly demonstrated that the two major 52K and 48.5K species and the minor 37.5K species are derived from the 1.1 kb mRNA, and the 50K and 45K major species and the minor 35K species are produced by the 0.9 kb mRNA.

It is now clear that more than one polypeptide species is produced by each of the E1A mRNAs and it is still not known what is the source of these multiple species. Since both E1A-N1 and E1A-C1 sera precipitate identical multiple polypeptide species, the multiple products of the E1A 0.9 and 1.1 kb mRNA are probably not generated by proteolytic cleavage at either

terminus. To further characterize these species, the abberant E1A proteins encoded by Ad5 deletion mutants mapping in E1A were examined. The mutant dl1504 (Osborne et al., 1982) contains a deletion near the 5'-cap site of E1A mRNA, including the AU of the first in-phase AUG initiation codon (see fig. 16). The E1A proteins are initiated at the next AUG site which is 42 nucleotides downstream in the E1A protein coding region (Downey et al., 1984). Another mutant dl313 carries a deletion at the 3' end of E1A (nucleotides 1334 to 3639) that fuses its coding sequence with that of protein IX at the right end of region E1B (see fig.16). This deletion eliminates 70 amino acids from the carboxy termini of E1A proteins and results in the addition of 15 amino acids encoded by an unused reading frame within the protein IX coding sequence, terminating in an in-phase stop codon (Colby and Shenk, 1981). Cells infected with either dl1504 or dl313 were labeled with [^{32}P]orthophosphate and cell extracts were precipitated with E1A-N1 serum and analysed by two-dimensional gel electrophoresis. Fig. 17 shows that with dl1504 no E1A products were detected with E1A-N1 serum as expected since the deletion removed 15 amino acids from the amino terminus of the E1A proteins. Previous collaborative studies (Rowe et al., 1983) demonstrated that four major E1A proteins were detected

Figure 17

Two-dimensional gel electrophoresis of E1A proteins synthesized in mutant-infected cells and immunoprecipitated by E1A-N1 serum. Cells were infected with various E1A mutants and were labeled with ^{32}P-orthophosphate from 7 to 11 h postinfection. Cell extracts were immunoprecipitated with E1A-N1 serum and analysed by two-dimensional gel electrophoresis. Numbers along the tops of the panels represent the pH at the positions indicated. Arrows in the figure indicate the positions of the 52 and 48.5K products of the 1.1 kb mRNA (→) and the 50 and 45K products of the 0.9 kb mRNA (←).



Hr. 1

dl 313

using E1A-C1 serum and they migrated on SDS-PAGE faster than the E1A proteins derived from wt-infected cells. With d1313, a cluster of four polypeptides with apparent molecular weights of about 35K to 45K were observed. These proteins had a slightly lower pI and a faster migration rate in SDS-PAGE than the E1A proteins derived from wt-infected cells. As expected, previous collaborative studies indicated that E1A-C1 serum did not recognize the E1A proteins encoded by d1313-infected clells since the mutation eliminates the carboxy terminal antigenic site recognized by this antipeptide serum (Rowe et al., 1983). These data suggested that post-translational modifications in the C-terminus are not responsible for the generation of the E1A multiple species. Previous results had suggested that this region might be involved in generating the multiple species (Esche et al., 1980). These data also confirmed that multiple protein species derived from the E1A 1.1 and 0.9 kb mRNAs did not result from proteolytic degradation from either termini of the E1A proteins, and furthermore, suggested that the amino terminal 15 and carboxy terminal 70 amino acids were not involved. In summary, as tabulated in Table 1, the patterns of immunoprecipitation of E1A proteins by E1A-N1 and E1A-C1 sera were entirely

Table 1: Immunoprecipitation of Ad5 E1A Proteins coded for by wt and mutant virus by E1A-N1 and E1A-C1 sera

		1.1 kb mRNA product		0.9 kb mRNA product	
Mutant	Serum:	E1A-C1	E1A-N1	E1A-C1	E1A-N1
wt		+	+	+	+
pm975		+	+	-	-
hr1		-	-[a]	+	+
dl1504		+[b]	-	+[b]	-
dl313		-	+[b]	-	+[b]

*The ability of E1A-N1 and E1A-C1 to precipitate Ad5 E1A proteins from wt- and mutant-infected cells was determined by experiments described in fig. 16 and by studies published previously by Rowe et al. (1983a).

[a] no truncated polypeptide detected

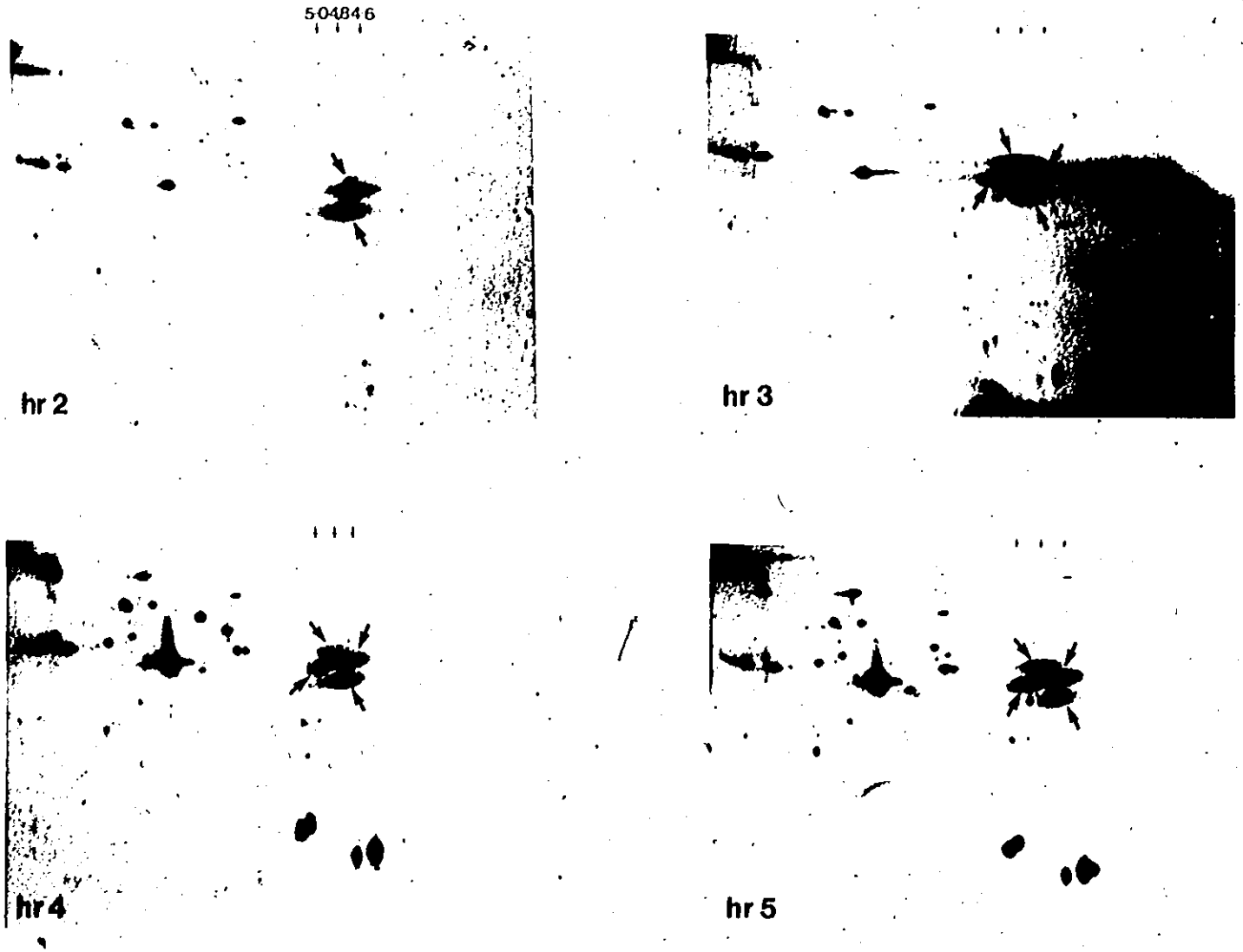
[b] truncated forms of the protein observed

compatible with those predicted from the proposed structures of wt and mutant E1A products.

Host range mutations in region E1A generated by chemical mutagenesis have produced mutants that are phenotypically deficient in the formation of viral DNA and late protein synthesis (Dassam et al., 1978). Recently, the mutations in hr3, 4, and 5 have been localized and identified by DNA sequencing (Glenn and Ricciardi, 1985). In hr3 and 4, single base pair substitutions affecting only the 1.1 kb mRNA products were detected. Methionine at position 176 was replaced by lysine, and leucine at position 173 was replaced by phenylalanine in hr3 and 4, respectively. In hr5, due to the splicing patterns of the two mRNAs, a single base pair alteration produced missense mutations affecting both the 1.1 and 0.9 kb mRNA products. Serine at position 185 of the 1.1 kb mRNA products was replaced by asparagine, while aspartic acid replaced glycine at position 139 in the 0.9 kb mRNA products. Analysis of the E1A proteins produced in cells infected with these mutants, including hr2, 3, 4, and 5, might provide clues concerning the generation of the E1A multiple species. Thus cells infected with these mutants were labeled with [^{35}S]methionine from 7 to 11 h postinfection and extracts were immunoprecipitated with E1A-N1 serum. As shown in fig. 18, E1A-N1

Figure 18

Analysis on two-dimensional gels of E1A proteins from host-range group I mutants immunoprecipitated by E1A-N1 serum. Cells were infected with various host-range group I mutants and were labeled [^{35}S]methionine from 7 to 11 h postinfection. Cell extracts were immunoprecipitated with E1A-N1 serum and analysed by two-dimensional gel electrophoresis. Numbers along the tops of the panels represent the pH at the positions indicated.


504846
hr 2
hr 3
hr 4
hr 5

precipitated a cluster of four major polypeptides from cells infected with hr3, 4, and 5, with migration properties identical to those precipitated from wt-infected cells (see fig. 15). A different pattern of E1A proteins was detected from hr2-infected cells. Only two major polypeptide species were precipitated using E1A-N1 serum, with migration properties identical to those seen with hr1-infected cells (see fig. 17). Identical results were also observed in previous collaborative studies not included in this thesis using E1A-C1 serum (Rowe et al., 1983). Thus, these results demonstrated that E1A proteins synthesized by hr3, 4, and 5 were indistinguishable from those synthesized by wild-type virus. The identical phenotypes of hr1 and 2 suggested that the defects of hr2 most likely involved a premature termination of the 1.1 kb mRNA similar to hr1. While hr1 and 2 are defective for the synthesis of the 1.1 kb gene products, all of these mutants are capable of synthesizing multiple polypeptide species derived from the individual E1A early messages.

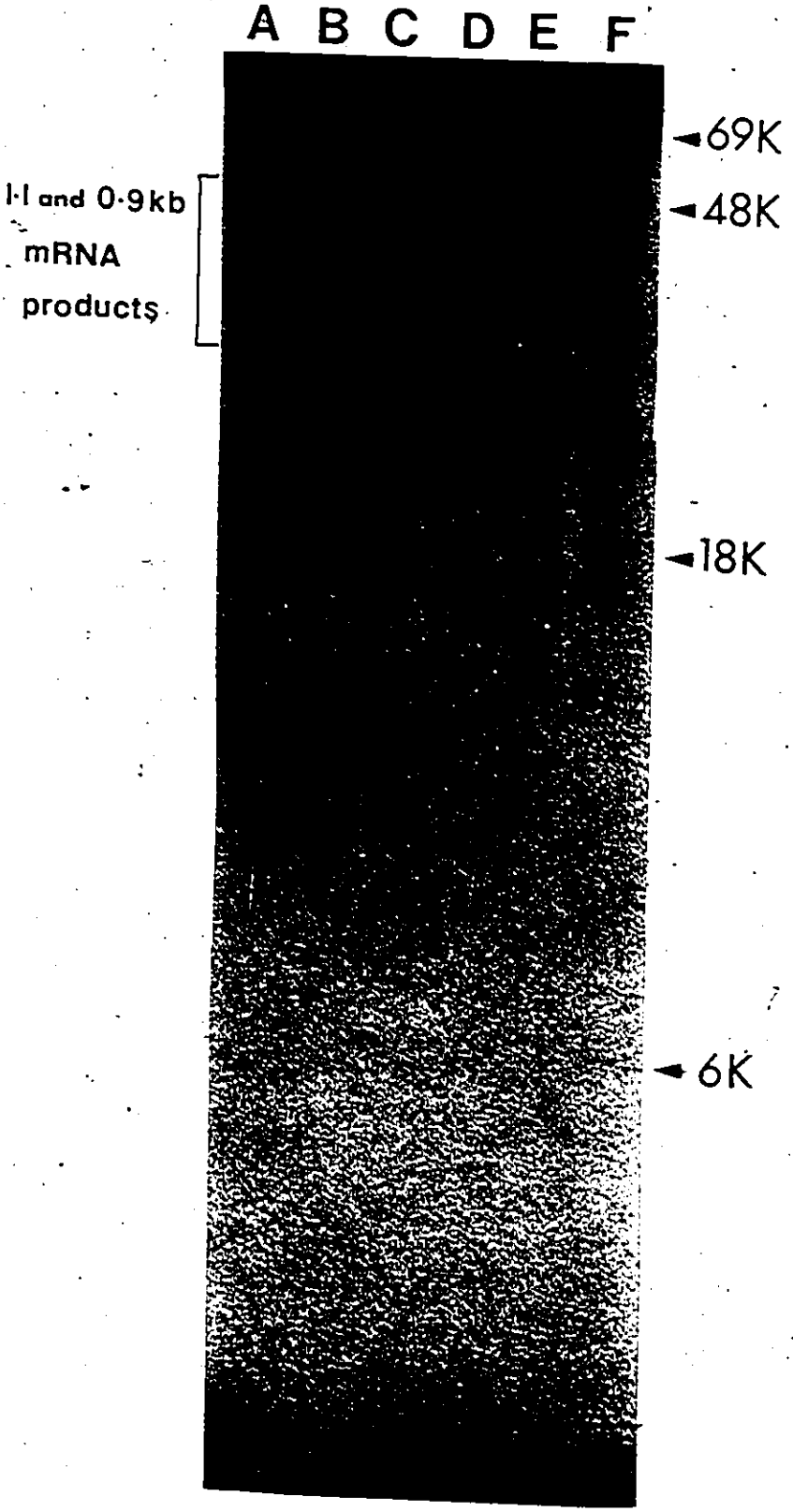
3.3.2. Failure to detect products from the 0.6kb E1A mRNA using E1A-N1 serum

As mentioned above in the Introduction (Chapter 1), a third mRNA of 0.6kb is also transcribed from E1A, predominantly late after the onset of viral DNA

replication (Spector et al., 1978; Green et al., 1981; Lewis and Mathews, 1981). DNA sequencing data have predicted that this mRNA codes for a polypeptide with an amino terminus identical to the polypeptides derived from 1.1 and 0.9kb mRNAs, but with a different carboxy terminus as a result of the use of a different reading frame after the 3' RNA splice acceptor site (Virtanen and Pettersson, 1983). Thus the antipeptide serum E1A-N1, which was raised against the amino terminus of E1A, should recognize the product derived from this mRNA. The antipeptide serum E1A-C1, which recognizes the carboxy termini of the products of the 1.1 and 0.9kb mRNAs should not react against the 0.6kb mRNA protein product. In order to detect this product Ad5- and mock-infected cells were labeled with [^{35}S]methionine from 20 to 24 h postinfection and cell extracts were prepared and immunoprecipitated with E1A-N1 and E1A-C1 sera. To ensure that any species observed was truly recognized by the E1A-N1 serum, increasing amounts of the synthetic peptide E1A-N were added to the cell extracts. The results, shown in fig. 19, indicated that the products from the 1.1 and 0.9kb mRNAs were still synthesized at high levels even at these late times after infection. The late structural hexon polypeptide, which is synthesized in large quantities at this time during the infection

Figure 19

Analysis of E1A proteins synthesized late during infection. Ad5- and mock-infected cells were labeled with [^{35}S]methionine from 20 to 24 h postinfection and cell extracts were immunoprecipitated with either E1A-N1 or E1A-C1 serum, in some cases in the presence of increasing amounts of E1A-N peptide, and then analysed on a 15% polyacrylamide gel. Molecular weight markers are shown on the right. A: mock-infected cells and E1A-N1 serum. B: Ad5-infected cells and E1A-C1 serum, C-F: Ad5-infected cells and E1A-N1 serum in the presence of 0 (C), 0.1 (D), 1.0 (E) and 10 (F) ug of E1A-N peptide.


A B C D E F
69K
48K
18K
6K
1·1 and 0·9kb
mRNA
products

(Philipson, 1983), was also non-specifically precipitated with all of the sera. Increasing concentrations of E1A-N peptide efficiently blocked the precipitation by E1A-N1 serum of products derived from 1.1 and 0.9 kb mRNAs. No polypeptide(s) was detected that was recognized by E1A-N1 serum but not by E1A-C1 serum. This was particularly true in the range of 6K to 28K which should contain polypeptides of a size comparable to that predicted for the 0.6kb mRNA (Virtanen and Pettersson, 1983) or to those suggested by previous *in vitro* and *in vivo* experiments (Spector et al., 1980a,b; Esche et al., 1980; van der Eb et al., 1980; Winberg and Shenk, 1984). Thus no *in vivo* product of the 0.6 kb mRNA was detected using E1A-N1 serum. Reasons for the failure to detect a product for the 0.6 kb mRNA are discussed in Chapter IV.

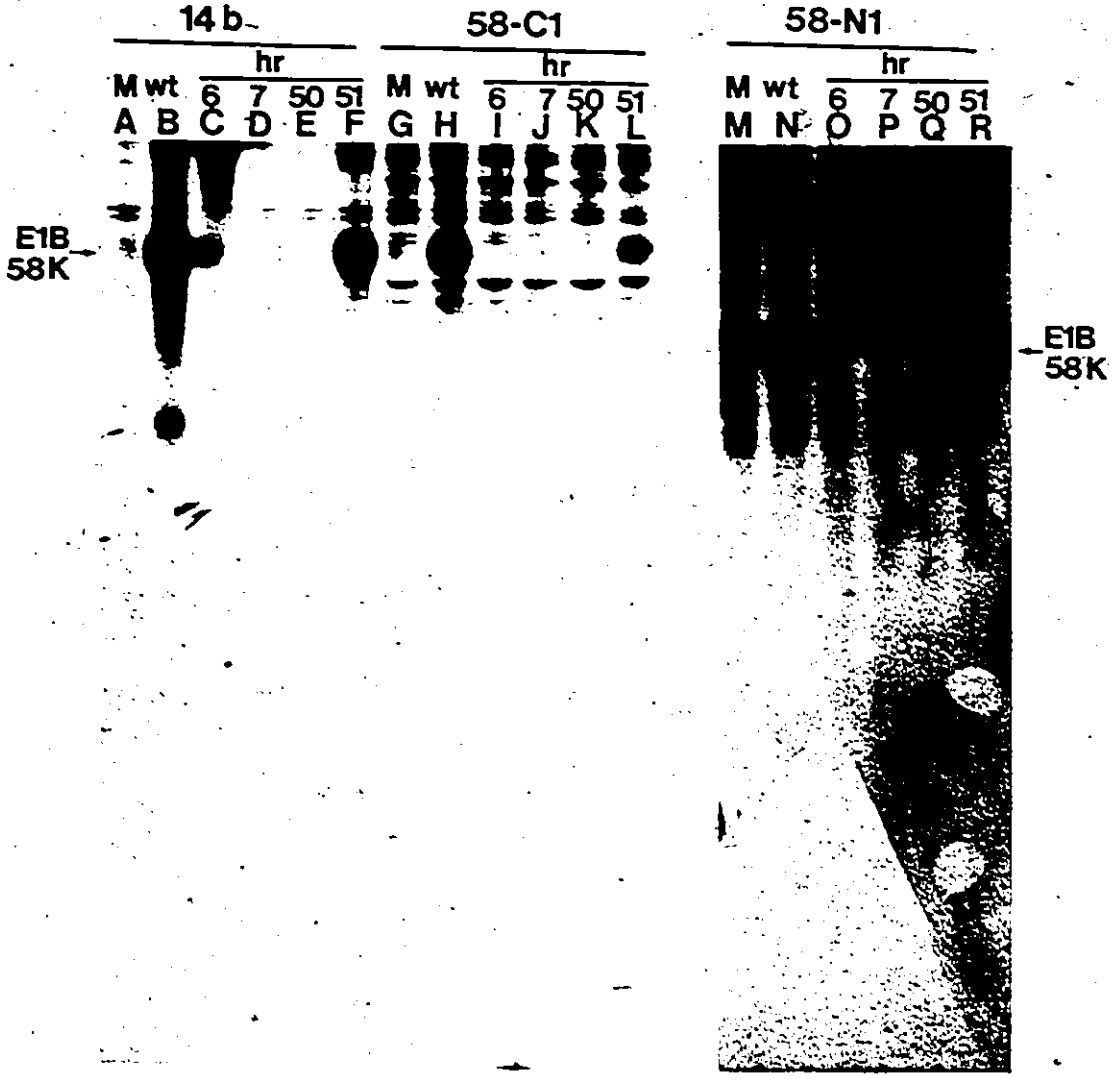
3.3.3. Immunoprecipitation of E1B 58K Synthesized in Cells Infected with Wild-type or Group II Host-range Mutants of Ad5

Ad5 group II *hr* mutants are defective in early region 1 and have been mapped to the sequences of the E1B 22S message which codes for 58K (Galos et al., 1980). The *hr* mutations were generated by chemical mutagenesis and probably represent single base changes (Harrison et al., 1978). Thus the mutant protein

products could contain either single amino acid changes or, in the case of the formation of an aberrant stop codon, a truncated version of the 58K polypeptide. The anti-synthetic peptide sera 58-N1 and 58-C1, and the hamster antitumor serum 14B, were used to immunoprecipitate E1B 58K (or 58K-related viral polypeptides) synthesized by the host range group II mutants. Cells were infected with wild-type or mutant viruses, including hr6, 7, 50, 51, they were labeled with [^{35}S]methionine at 12 to 16 h postinfection and cell extracts were immunoprecipitated with 58-N1, 58-C1, or 14B sera and the precipitates were examined by SDS-PAGE. As shown in fig. 20, the anti-synthetic peptide serum 58-N1 precipitated the E1B 58K from wt-infected cells (lane N). No E1B 58K or other viral polypeptides were detected from cells infected with hr6, 7, and 50 (lanes O to Q). However, a viral polypeptide which co-migrated with 58K was precipitated from hr51-infected cells (lane R). Similar results were also observed using another anti-synthetic peptide serum, 58-C1 (fig. 20, lanes H to L) and 14B hamster antitumor serum (fig. 20, lanes B to F). The viral polypeptide detected in hr51-infected cells was recognized by all three sera and, although it appeared to be produced at somewhat reduced levels relative to wt, it co-migrated with 58K from wt-infected cells,

Figure 20

Analysis of 58K-related viral proteins from host-range group II mutants by antitumour and antipeptide sera. Mock-infected cells and cells infected with wt- or hr group II mutants were labeled with [^{35}S]methionine from 12 to 16 h postinfection and cell extracts were immunoprecipitated with either 14B, 58-C1, or 58-N1 sera and then analysed on either a 15% (lane A to L) or 12% (lane M to R) polyacrylamide gel. Lane A to F: 14B serum with mock-, wt, hr6, 7, 50, and 51, respectively; lane G to L: 58-C1 serum with mock-, wt, hr6, 7, 50, and 51, respectively; and lane M to R: 58-N1 serum with mock-, wt, hr6, 7, 50, and 51, respectively.


14 b
58-C1
58-N1
hr
M wt 6 7 50 51
A B C D E F
G H I J K L
M N O P Q R
E1B
58K
E1B
58K

suggesting it was related to 58K. Similar results were also obtained in previous studies using 14B serum (Lassam et al., 1979a, b). These results suggested that all hr group II mutants except hr51 are defective in the synthesis of E1B 58K.

The studies described in this Chapter indicated that antipeptide sera could be produced to both the N and C termini of E1A proteins and 58K and that they were highly specific for these viral products. These sera therefore represent powerful tools for the further characterization of the structural and functional properties of Ad5 E1 proteins in infected and transformed cells.

Chapter 4

Association of Ad5 E1A Polypeptides with Cellular Proteins in Ad5-infected cells

4. Association of Ad5 E1A Polypeptides with Cellular Proteins in Ad5-infected Cells

It is now known that E1A polypeptides play an important role in gene expression as well as in oncogenic transformation. However, little is known about the mechanisms involved in either. As one approach to learning more about the mechanism of action of these viral products, the antipeptide sera were used to attempt to identify cellular and viral proteins that interact with E1A products. It has been shown that other viral polypeptides appear to be present in functional complexes with cellular proteins including both Ad5 E1B 58K protein and SV 40 large T antigen with cellular p53 (Sarnow et al., 1982, Lane and Harlow, 1982), RSV $pp60^{src}$ with 50K and 90K cellular proteins (Brugge et al., 1981), and polyoma middle T antigen with the cellular c-src protein (Courtneige and Smith, 1983). Thus it is possible that E1A proteins also do not act alone but they too form functional complexes with cellular proteins. To demonstrate such an association, immunoprecipitations were carried out using E1A-C1 and E1A-N1 sera and precipitates were analysed for the presence of polypeptides other than the E1A species. In order to identify cellular proteins that were specifically associated with E1A products

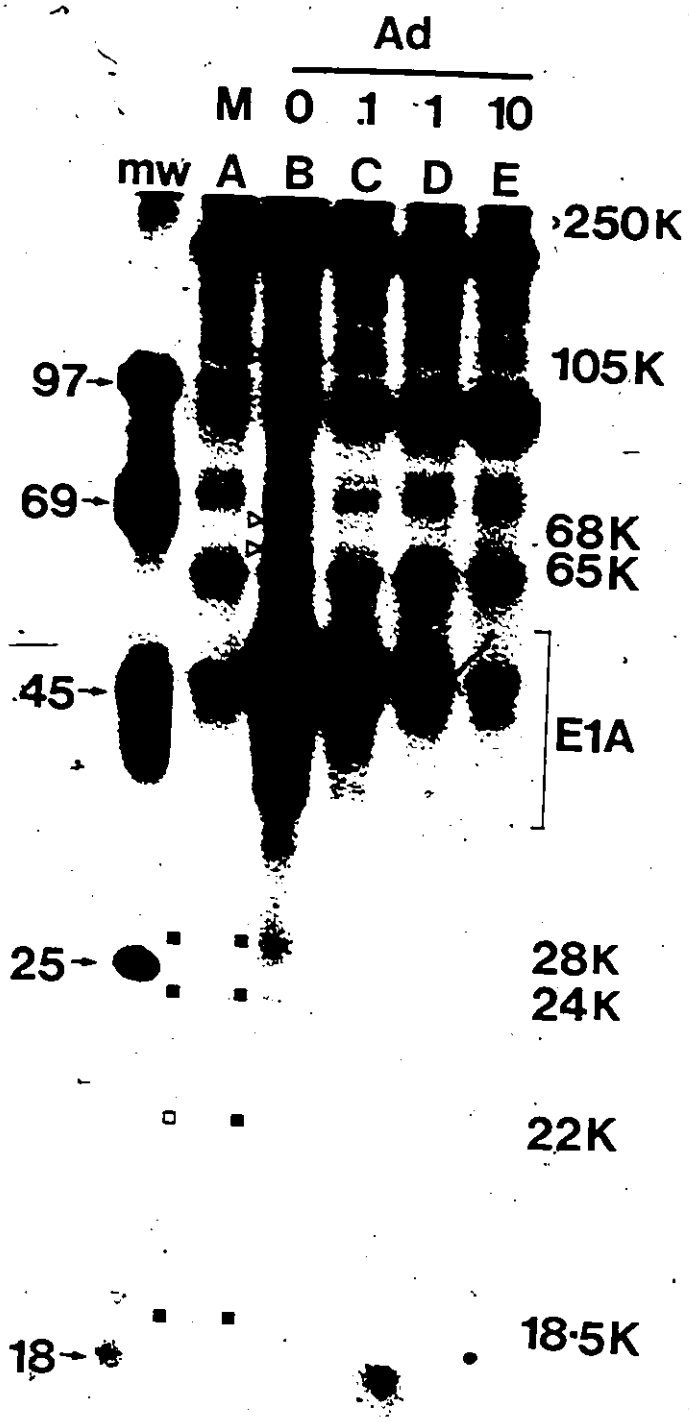
immunoprecipitations were carried out in the presence of increasing amounts of the corresponding synthetic peptides. As discussed above, the presence of synthetic peptides should reduce the amount of E1A polypeptides precipitated and, in addition, should proportionally decrease quantities of any cellular proteins present in precipitates because of their association with E1A polypeptides. While some cellular proteins could possibly cross-react with an epitope defined by one of the E1A-specific synthetic peptides, it was considered very unlikely that the same cellular proteins could possess antigen sites recognized by both E1A-N1 and E1A-C1 sera. Thus proteins which were present in immunoprecipitates prepared from infected cells using both E1A antipeptide sera and absent in precipitates from mock-infected cells were deemed to be good candidates. A lysing buffer containing a physiological salt concentration, Ca and Mg ions, and only a non-ionic detergent was used to prepare cell extracts. These milder conditions were designed so as to better preserve protein complexes as protein-protein interactions might be easily distrupted by the ionic detergents present in RIPA buffer.

In order to examine these conditions of immunoprecipitation Ad5- and mock-infected cells were labeled with [^{35}S]methionine 7 to 11 h postinfection

and cell extracts were prepared and precipitated with E1A-C1 serum containing increasing amount of E1A-C peptide. As shown in fig. 21, E1A polypeptides were precipitated by E1A-C1 serum as a series of major species of 40-50K (lane B) and addition of synthetic peptide E1A-C efficiently blocked the precipitation of these E1A species (lane B-E). The immunoprecipitation was relatively free of contaminating protein species. Several polypeptides were also detected but addition of peptide did not reduce their levels and therefore they were probably present nonspecifically (see below and fig. 22). However, the precipitation of some additional proteins appeared to be reduced by the presence of E1A-C peptide, including species of about 18.5K, 22K, 24K, 28K, 65K, 68K, 105K, and one in excess of 250K (>250K) which, under the the present gel conditions barely entered the separating gel. The >250K species will be discussed further below (see fig. 26). Small amounts of 18.5, 24 and 28K species were also present in precipitates from mock-infected cells (lane A). These species became more evident upon longer exposures of this gel (data not shown). Thus these protein species may have represented host cell polypeptides that contained antigenic sites recognized by the E1A-C1 serum. A protein of approximately 110K was also detected in both Ad5- and mock-infected cells and it

Figure 21

SDS-PAGE analysis of polypeptides coprecipitated with E1A proteins using E1A-C1 serum. Ad5- and mock-infected cells were labeled with [^{35}S]methionine from 7 to 11 h postinfection and extracts were prepared and immunoprecipitated with E1A-C1 serum, in some cases in the presence of increasing amounts of C-terminal peptide, as described in Materials and Methods. Precipitates were then analysed on a 9% gel. mw: ^{14}C-labeled molecular weight markers. A: mock-infected cells. B-E: Ad5-infected cells precipitated in the presence of 0 (B), 0.1 (C), 1.0 (D), or 10 (E) ug of C-terminal synthetic peptide.


Ad
M 0 .1 1 10
mw A B C D E
250K
97
105K
69
68K
65K
45
E1A
25
28K
24K
22K
18
18·5K

was not affected by the addition of E1A-C peptide. This species clearly migrated slightly slower than the 105K polypeptide seen in extracts from infected cells. To demonstrate that the amounts of the 105K, 68K and 65K species present were proportional to the amount of E1A proteins precipitated, the quantities of these proteins species and the 48.5K and 45K E1A products were estimated by microdensitometer scans of the autoradiogram. The amounts of four other contaminating proteins were also measured. The quantities of all of these proteins were plotted against the amounts of E1A-C peptide present in the immunoprecipitation mixture. As shown in fig. 22, the levels of the four contaminating species remained more or less constant in the presence of various amounts of E1A-C peptide. The amounts of 105K, 68K and 65K decreased reasonably proportionately with the reduction of the 45K E1A species. These results suggested that the 105K, 68K and 65K species (and possibly 22K) either cross-reacted with the C-terminus of E1A proteins or were associated with these viral proteins.

To further distinguish between these two possibilities, a similar competition experiment was carried out using both E1A-C1 and E1A-N1 sera. As shown in Fig. 23, in addition to E1A proteins a set of polypeptides including 24K, 28K, 65K, 68K and 105K was

Figure 22

Analysis of the levels of polypeptides coprecipitated with E1A proteins using E1A-C1 serum. Fig. 22A: Ad5-infected cells were labeled with [^{35}S]methionine from 7 to 11 h postinfection and extracts were immunoprecipitated with E1A-C1 serum in the presence of various amounts of E1A-C peptide. The number above each lane indicates the amount of peptide in ug added to the cell extracts. Fig. 22B: The levels of Ad5 E1A 45K, the coprecipitated 105K, 68K, 65K proteins, and four arbitrary cellular bands (A, B, C, and D as indicated in fig. 22A) were quantitated by scanning the autoradiogram in fig. 22A using a microdensitometer. With 105K a contaminating band was present which migrated only slightly slower then this protein and it was not possible to separate these species in the microdensitometer scans. Thus in this case the amount of 105K was estimated by subtracting the value obtained for the sample from mock-infected cells. The levels of proteins detected are plotted against the amount of E1A-C peptide present in the cell extracts.

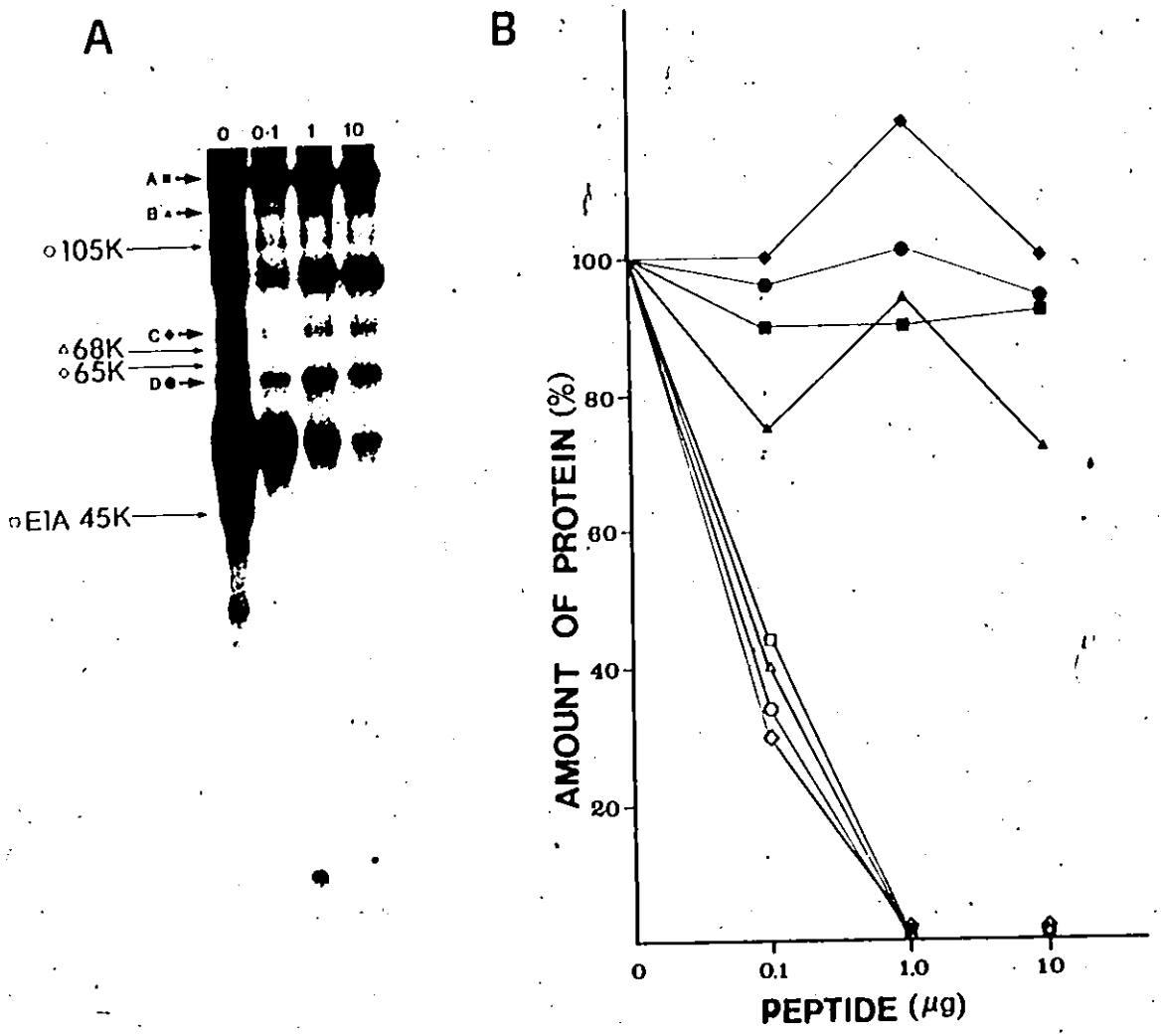
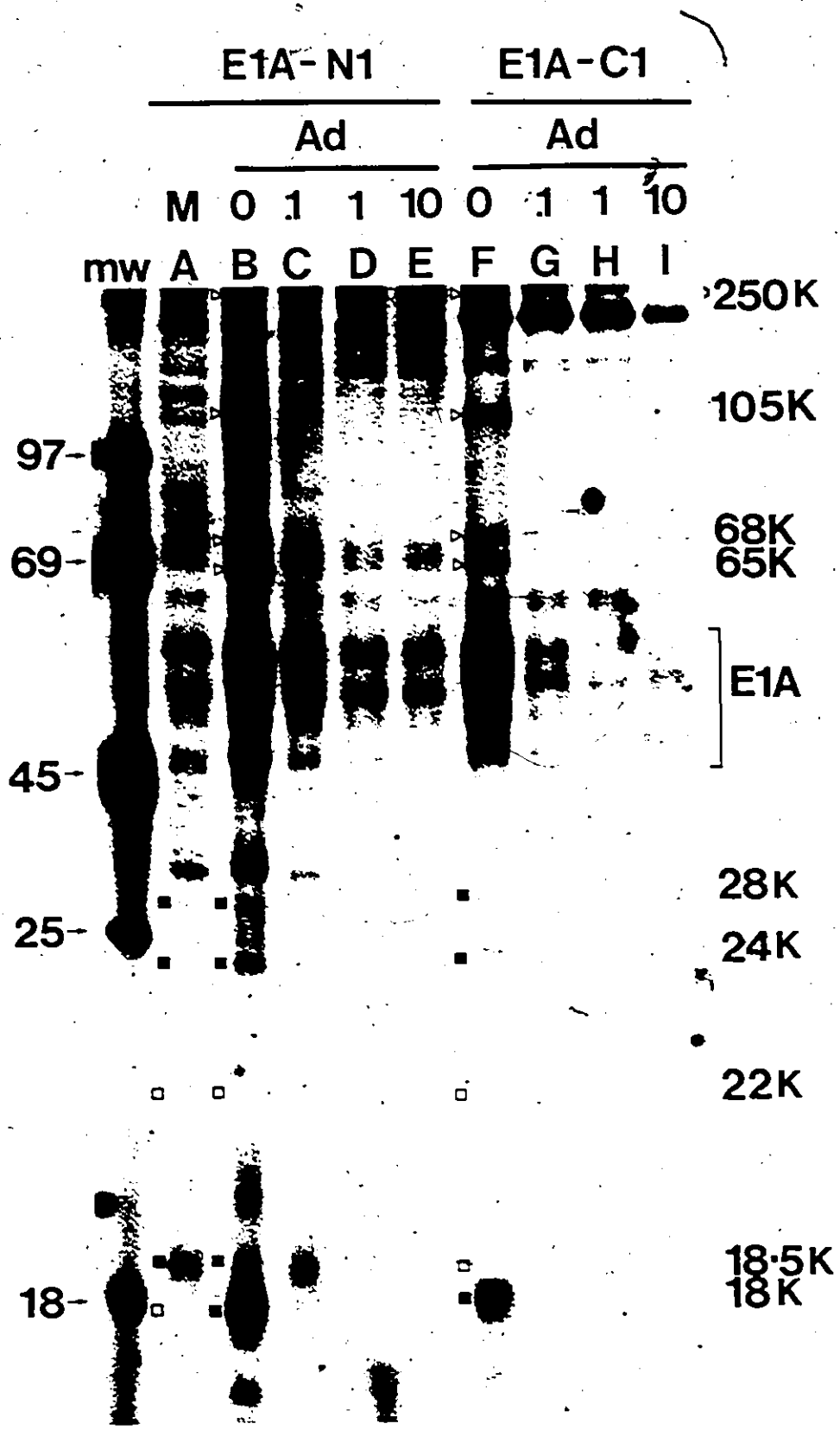

A
0
0·1
1
10
A
B
105K
68K
C
65K
D
E1A 45K
B
100
80
60
40
20
AMOUNT OF PROTEIN (%)
0
0.1
1.0
10
PEPTIDE (μg)

Figure 23

SDS-PAGE analysis of polypeptides coprecipitated with E1A proteins using E1A-C1 and E1A-N1 sera. Ad5- and mock-infected cells were labeled with [^{35}S]methionine from 7 to 11 h postinfection and extracts were prepared and immunoprecipitated with E1A-C1 or E1A-N1 sera, in some cases in the presence of increasing amounts of homologous synthetic peptide, as described in Materials and Methods. Precipitates were then analysed on a 9% gel. mw: ^{14}C-labeled molecular weight markers. A: mock-infected cells precipitated with E1A-N1 serum. B-E: Ad5-infected cells precipitated with E1A-N1 serum in the presence of 0 (B), 0.1 (C), 1.0 (D) or 10 (E) ug of N-terminal synthetic peptide. F-I: Ad5-infected cells precipitated with E1A-C1 serum in the presence of 0 (F), 0.1 (G), 1.0 (F) or 10 (I) ug of C-terminal peptide.


E1A-N1
E1A-C1
Ad
Ad
M 0 .1 1 10 0 .1 1 10
mw A B C D E F G H I
250K
105K
97-
68K
69-
65K
E1A
45-
28K
25-
24K
22K
18.5K
18-
18K

detected using E1A-C1 serum and again the precipitation of these proteins was reduced by the addition of E1A-C peptide. In this experiment no 18.5K or 22K species were observed but a new protein of about 18K was detected. The amino terminal-specific E1A-N1 serum precipitated the multiple E1A species and several other proteins including the 18.5K, 24K, 28K, 65K, 68K, and 105K species. The levels of both E1A products and these protein species were reduced by the addition of the N-terminal peptide (lane B-E). However, as with E1A-C1 serum, 18.5, 24, and 28K were also seen in extracts from mock-infected cells (lane A) and thus were probably present nonspecifically. Labeled material at the top of the gel consistent with the presence of >250K was observed in this experiment, but in further studies in which conditions were such that this protein was clearly resolved (see below, fig. 25), E1A-N1 serum was not found to consistently precipitate this polypeptide. It is not certain, at this point, if >250K is an associated protein. The 18K species was also present with E1A-N1 serum and was competed out by the addition of peptide. Since the detection of both 18K and 18.5K was inconsistent over the course of several experiments, these proteins will be disregarded for the present and not mentioned further in the present studies. It is possible that one of these two species

could correspond to the 19 and 18.5K proteins coded for by E1B of Ad5 (Rowe et al., 1983b). However, these species do not precisely comigrate with the E1B proteins and, in addition, anti-peptide sera specific for the N- and C-termini of these viral products do not co-precipitate E1A proteins (McGlade and Branton, unpublished results). Table 2 summarizes all of the data obtained to this point and suggests that the 65K, 68K, 105K, and perhaps the >250K proteins are associated with E1A proteins.

Recently, a series of mouse monoclonal antibodies directed against E1A proteins has been developed by E. Harlow at the Cold Spring Harbor Laboratory (Harlow et al., 1985). One of these antibodies, M73, is specific for an epitope present somewhere in the carboxy-half of E1A proteins (Harlow, personal communication). This antibody does not react with either the N or C-termini as the addition of 25 ug of either synthetic peptide to an immunoprecipitation mixture failed to reduce the amount of E1A proteins precipitated. This antibody was also used to study the association of cellular proteins with E1A species. Ad5- and mock-infected cells were labeled with [^{35}S]methionine from 7 to 11 h postinfection and cell extracts were prepared and precipitated with either E1A-C1 serum or M73 monoclonal antibody. Fig. 24 shows that again, the E1A multiple

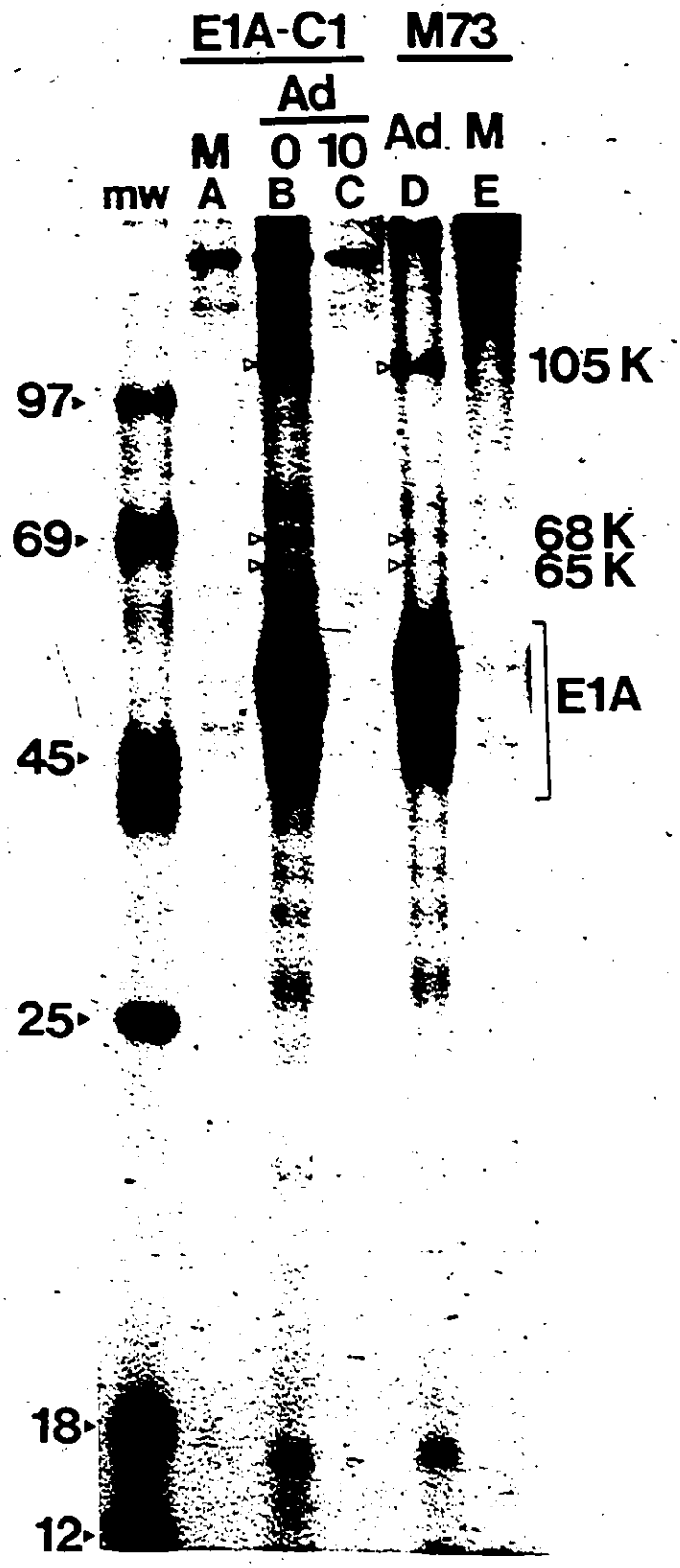
Table 2: Analysis of Proteins Associated with Ad5 E1A Products*

Protein	Present with E1A-C1 serum	Present with E1A-N1 serum	Detected in mock-infected cells	Comments
>250K	+	+/-	-	E1A-associated?
105K (doublet)	+	+	-	E1A-associated
68K	+	+	-	E1A-associated
65K	+	+	-	E1A-associated
28K	+	+	+	non-specific
24K	+	+	+	non-specific
22K	+/-	-	-	inconsistent
18.5K	+	+	+	non-specific
18K	+/-	+	-	inconsistent

* The data summarized in this table were derived from experiments presented in Fig. 21-23 and from other similar studies (data not shown).

Figure 24

SDS-PAGE analysis of polypeptides coprecipitated with E1A-C1 serum and mouse monoclonal antibody M73. Ad5- and mock-infected cells were labeled with [^{35}S]methionine from 7 to 11 h postinfection and extracts were prepared and immunoprecipitated with E1A-C1 serum or monoclonal antibody M73, in some cases in the presence of increasing amounts of C-terminal peptide, as described in Materials and Methods. Precipitates were then analysed on a 9% gel. mw: ^{14}C-labeled molecular weight markers. A: mock-infected cells precipitated with E1A-C1 serum. B-C: Ad5-infected cells precipitated with E1A-C1 serum in the presence of 0 (B) or 10 (C) ug of C-terminal peptide. D: Ad5-infected cells precipitated with M73. E: mock-infected cells precipitated with M73.


E1A-C1
M73
Ad
M
0
10
Ad.
M
mw
A
B
C
D
E
97
69
45
25
18
12
105 K
68 K
65 K
E1A

species and 65, 68, and 105K were clearly present in E1A-C1 precipitate from infected cells (lane B) and their presence was reduced by the addition of 10ug of E1A-C peptide (lane C). M73 also precipitated E1A proteins and 65, 68, and 105K species from infected cells (lane D) and none of these proteins were detected in mock-infected cells (lane F). The >250K species was also detected using M73 monoclonal antibody under gel conditions that clearly resolved this species (data not shown). These results indicated that M73, which possesses a different specificity for E1A proteins than E1A-C1 and E1A-N1 sera, also precipitated the same 65, 68, 105, and >250K species along with E1A products, thus further substantiating the fact that these proteins may be associated with E1A proteins.

It should be pointed out that the particular conditions of immunoprecipitation and gel electrophoresis used were essential for the consistent detection of these species. Failure to allow electrophoresis to proceed according to the prescribed protocol often resulted in the failure to separate 65 and 68K and in the incomplete separation of these species, and 105K, from cellular polypeptides detected in extracts from mock-infected cells. More importantly, the presence of Ca^{++} and Mg^{++} affected the ability of anti-E1A sera to precipitate three of the proteins.

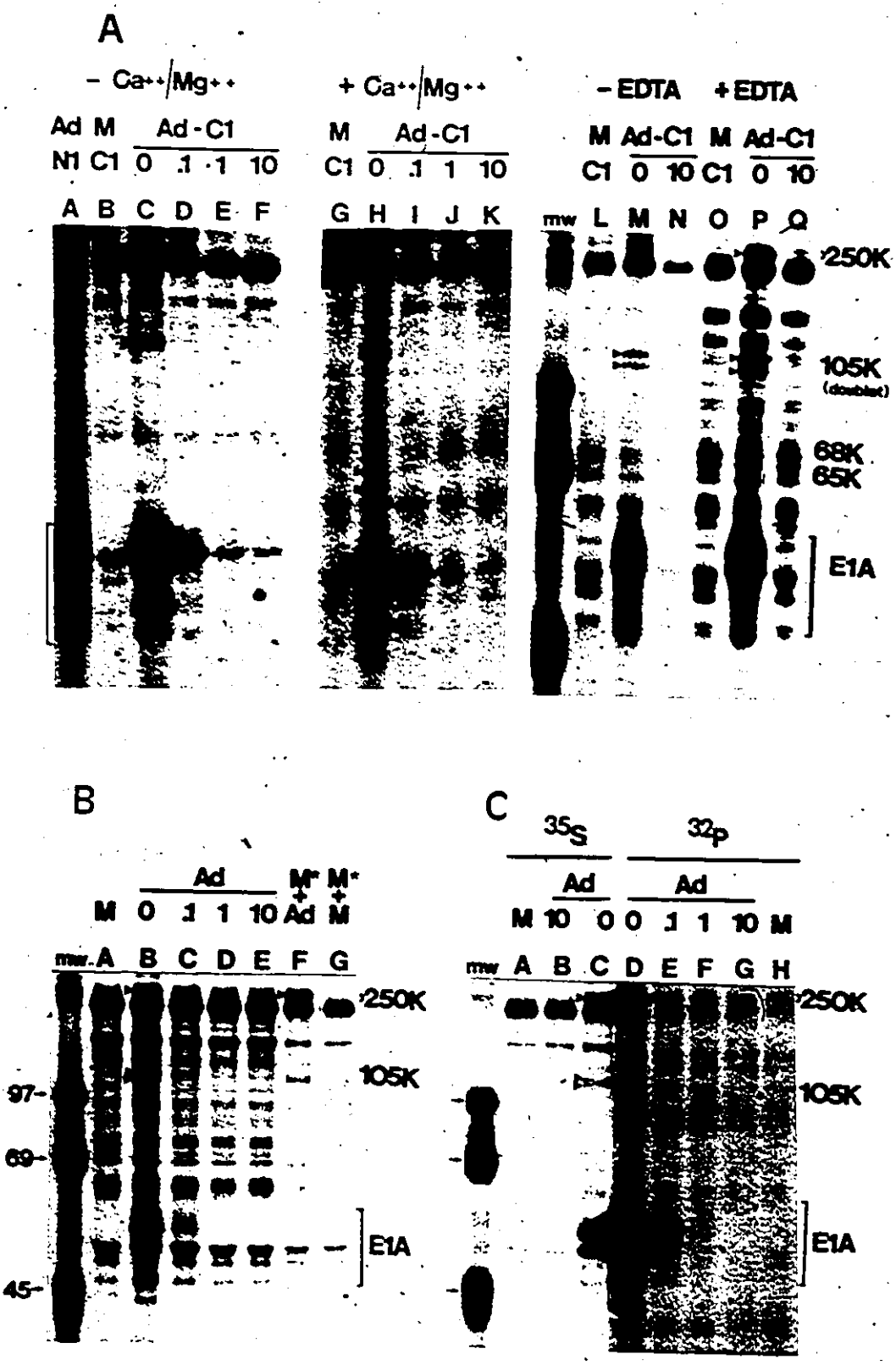
Cell extracts were prepared and immunoprecipitated in lysis buffer either containing or lacking Ca^{++} and Mg^{++} and the precipitates were analyzed on a gel that was electrophoresed longer to allow the resolution of the >250K species. Fig. 25A shows that this protein was detected in extracts from infected cells prepared in lysis buffer lacking divalent cations using E1A-C1 serum (lane C), it was reduced in quantity upon addition of E1A-C peptide (lanes D-F), and it was not detected in precipitates from mock-infected cells (lane B). Identical results were also obtained using M73 monoclonal antibody (data not shown). As discussed above, E1A-N1 failed to precipitate >250K under these conditions (lane A). However, in experiments carried out using lysis buffer containing divalent cations, little >250K was evident with extracts from either mock- or Ad5-infected cells (lanes H-K). Furthermore, upon the addition of EDTA to lysis buffer containing these divalent cations, >250K was again detectable (compare lanes P and M). Conversely, detection of 68K and 65K was inconsistent with either anti-peptide serum unless Ca^{++} and Mg^{++} were present (lane H and C; and other data not shown). The 105K species was found under all conditions employed in these experiments, but as is evident in Fig. 25 (lane C and P), 105K actually is comprised of two polypeptides that are separable if

Figure 25

Analysis of the association of the >250K protein with E1A polypeptides. Fig. 25A Effect of divalent cations. Ad5- or mock-infected cells were labeled with [^{35}S]methionine from 7 to 11 h postinfection and cell extracts prepared in lysis buffer either containing or lacking Ca^{++} and Mg^{++} were immunoprecipitated with antipeptide serum and analysed on a 9% polyacrylamide gel. The samples were run for at least an additional 90 min after the dye front had reached the bottom of the gel. Lanes A to F: lysis buffer lacking divalent cations. Lane A: infected cells precipitated with E1A-N1 serum. Lane B: mock-infected cells with E1A-C1 serum. Lanes C-F: infected cells with E1A-C1 serum in the presence of 0 (C), 0.1 (D), 1.0 (E) or 10 (F) ug of C-terminal peptide. Lane G-K: lysis buffer containing divalent cations. Lane G: mock-infected cells precipitated with E1A-C1 serum. Lane H-K; infected cells precipitated with E1A-C1 serum in the presence of 0 (H), 0.1 (I), 1.0 (J) or 10 (K) ug of C-terminal peptide. mw: molecular weight markers. Lane L to Q: lysis buffer and EDTA. Lysis buffer alone with E1A-C1 serum and mock-infected cells (L) and Ad5-infected cells in the presence of 0 (M) or 10 (N) ug of

C-terminal peptide. Lysis buffer plus 2.5 mM EDTA with E1A-C1 serum and mock-infected cells (O) and Ad5-infected cells in the presence of 0 (P) or 10 (Q) ug of C-terminal peptide. Fig. 25B In vitro association of >250K with E1A proteins. Ad5- and mock-infected cells were labeled, precipitated with E1A-C1 serum using lysis buffer lacking divalent cations, and analysed on gels as described in Fig. 25A. These profiles were compared to similar precipitates prepared by mixing extracts from mock-infected cells which had been labeled for 4 h with unlabeled extracts from either infected or uninfected cells as described in Fig. 26. mw: molecular weight markers. A. labeled mock-infected cells. B-E: labeled infected cells precipitated in the presence of 0 (B), 0.1 (C), 1.0 (D) or 10 (E) ug of C-terminal peptide. Lanes F to G: labeled mock-infected cells mixed with either unlabeled infected cell extract (F) or with unlabeled mock-infected cell extract (G). Fig. 25C Phosphorylation of >250K. Ad5- and mock-infected cells were labeled either [^{35}S]methionine or ^{32}P-orthophosphate as described in Fig. 27 and cell extracts were prepared in lysis buffer lacking divalent cations, precipitated in with E1A-C1 serum and analysed

on gels as in Fig. 25A. mw: molecular weight markers. Lanes A-C: ^{35}S-labeled samples. A: mock-infected cells. B-C: infected cells precipitated in the presence of 0 (B) or 10 (C) ug of C-terminal peptide. Lanes D-H: ^{32}P-labeled samples. D: mock-infected cells. E-H: Ad5-infected cells precipitated in the presence of 0 (E), 0.1 (F), 1.0 (G) or 10 (H) ug of C-terminal peptide.


A
– Ca++/Mg++
+ Ca++/Mg++
– EDTA
+ EDTA
Ad M Ad-C1
N1 C1 0 .1 1 10
M Ad-C1
C1 0 .1 1 10
M Ad-C1 M Ad-C1
C1 0 10 C1 0 10
A B C D E F
G H I J K
mw L M N O P Q
250K
105K
(doublet)
68K
65K
E1A
B
Ad
M* + Ad
M* + M
M 0 .1 1 10
mw A B C D E F G
250K
105K
97-
69-
45-
E1A
C
35S
32P
Ad
Ad
M 10 0 0 .1 1 10 M
mw A B C D E F G H
250K
105K
E1A

electrophoresis is carried out for a long enough period. Similar results were also obtained using the monoclonal antibody M73 (data not shown). These data suggested that 65K, 68K and >250K may be more tenuously associated with E1A proteins, and that divalent cations may be involved in the formatiom of such a complex.

The data presented so far do not eliminate the possibility that these proteins represent high molecular weight aggregates of E1A polypeptides which persisted during SDS-PAGE. In order to determine whether or not 65, 68, 100 and >250K are cellular proteins, and furthermore, to establish if association with E1A polypeptides could be demonstrated in vitro, the following experiment was performed. Cell extracts prepared from mock-infected cells which were labeled for four hours with [^{35}S]methionine were incubated with cell extracts from unlabeled mock- or Ad5-infected cells. Infected cells labeled from 7 to 11 h post-infection were also included as a postive control. The mixtures were then immunoprecipitated with E1A-C1 serum and analysed by SDS-PAGE. As shown in fig. 26, the 105K, 68k, and 65K protein species were again identified by a peptide competition experiment (lane B to C). These proteins were not detected when labeled mock-infected cell extracts were incubated with those from unlabeled mock-infected cells (lane F). However,

Figure 26

Complexing of cellular proteins with E1A polypeptides *in vitro*. Mock- and Ad5-infected cells were labeled with [^{35}S]methionine from 7 to 11 h postinfection and extracts were immunoprecipitated with E1A-C1 serum, in one case in the presence of C-terminal peptide. In addition, mock-infected cells were labeled with [^{35}S]methionine for 4 h and then extracts prepared in lysis buffer were mixed with equal amounts of similar extracts from unlabeled infected or uninfected cultures, in one case in the presence of C-terminal peptide. After 5 min at 0°C these mixtures were then immunoprecipitated with E1A-C1 serum for 2 h as described in Materials and Methods. The pattern of labeled proteins was analysed on a 9% gel. mw: molecular weight markers. A: ^{35}S-labeled mock-infected cells. B-C: ^{35}S-labeled Ad5-infected cells precipitated in the presence of 0 (B) or 10 (C) ug of C-terminal peptide. D-F: extracts from ^{35}S-labeled mock-infected cells incubated *in vitro* with those from unlabeled Ad5-infected cells in the presence of either 0 (D) or 10 (E) ug of C-terminal peptide or with those from uninfected cells (F).


M
Ad
M
Ad
M
M
0
10
Ad
10
M
mw
A
B
C
D
E
F
105K
97
69
68K
65K
45
E1A
45
18

when these labeled mock-infected cell extracts were incubated with extracts from unlabeled Ad5-infected cells (which contained unlabeled E1A proteins) the precipitate clearly contained the 105K, 68K and 65K polypeptides species lane D). In a similar experiment in which the immunoprecipitation was carried out using E1A-C1 serum in the presence of 10 ug of E1A-C synthetic peptide, none of these polypeptides species was present (lane E). These data indicated that the 65K, 68K and 100K polypeptides are cellular proteins and that they could form a complex with E1A polypeptides in vitro.

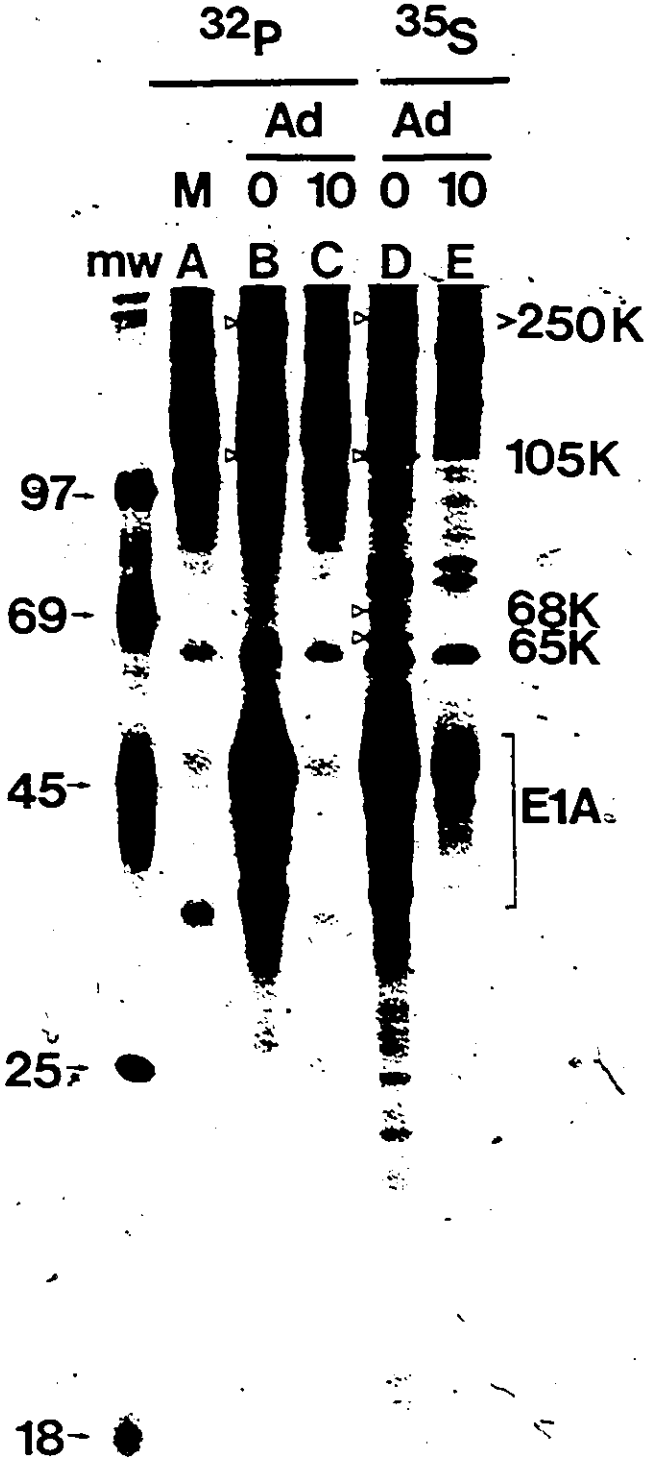
In order to assess the association of >250K with E1A proteins, a mixing experiment was carried out using cell extracts prepared in lysis buffer lacking divalent cations and immunoprecipitates were analysed on a gel that was electrophoresed longer in order to resolve this high molecular weight species. Fig. 25B shows that no >250K species was detected from labeled mock-infected cells (lane A). It was present in the precipitate from labeled infected cells (lane B) and addition of E1A-C peptide efficiently blocked the precipitation of this species (lanes C-E). Furthermore, this polypeptide was precipitated upon mixing extracts from labeled mock-infected cells with those from unlabeled infected cells (lane F) but not with

unlabeled mock-infected cells (lane G). Similar results were also obtained using the monoclonal antibody M73 (data not shown). Thus even though this species did not consistently co-precipitate with E1A proteins using E1A-N1 serum, nevertheless it appeared that it was capable of associating with these viral polypeptides.

To further characterize these cellular proteins, Ad5-infected cells were labeled with ^{32}p-orthophosphate or [^{35}S]methionine and cell extracts were immunoprecipitated with E1A-C1 serum in the presence of increasing amounts of E1A-C peptide. As shown in Fig. 27 E1A polypeptides were precipitated using E1A-C1 and a ^{32}p-labeled species which appeared to comigrate with ^{35}S-labeled 105K species was present in these precipitates. The precipitation of the E1A polypeptides and the ^{32}p-labeled 100K species was blocked by the addition of E1A-C peptide. In other studies in which the two 105K closely-migrating species were separated, both were shown to be labeled with ^{32}p (data not shown). No ^{32}p-labeled species were found that corresponded to 68K or 65K. Similar experiments were carried out in which lysing buffer lacking divalent cations was used and in which gel conditions allowed the >250K species to be resolved. Clearly this protein was also labeled with ^{32}p (fig. 25C). Thus the 105K and >250K polypeptides appeared to be phosphoproteins.

Figure 27

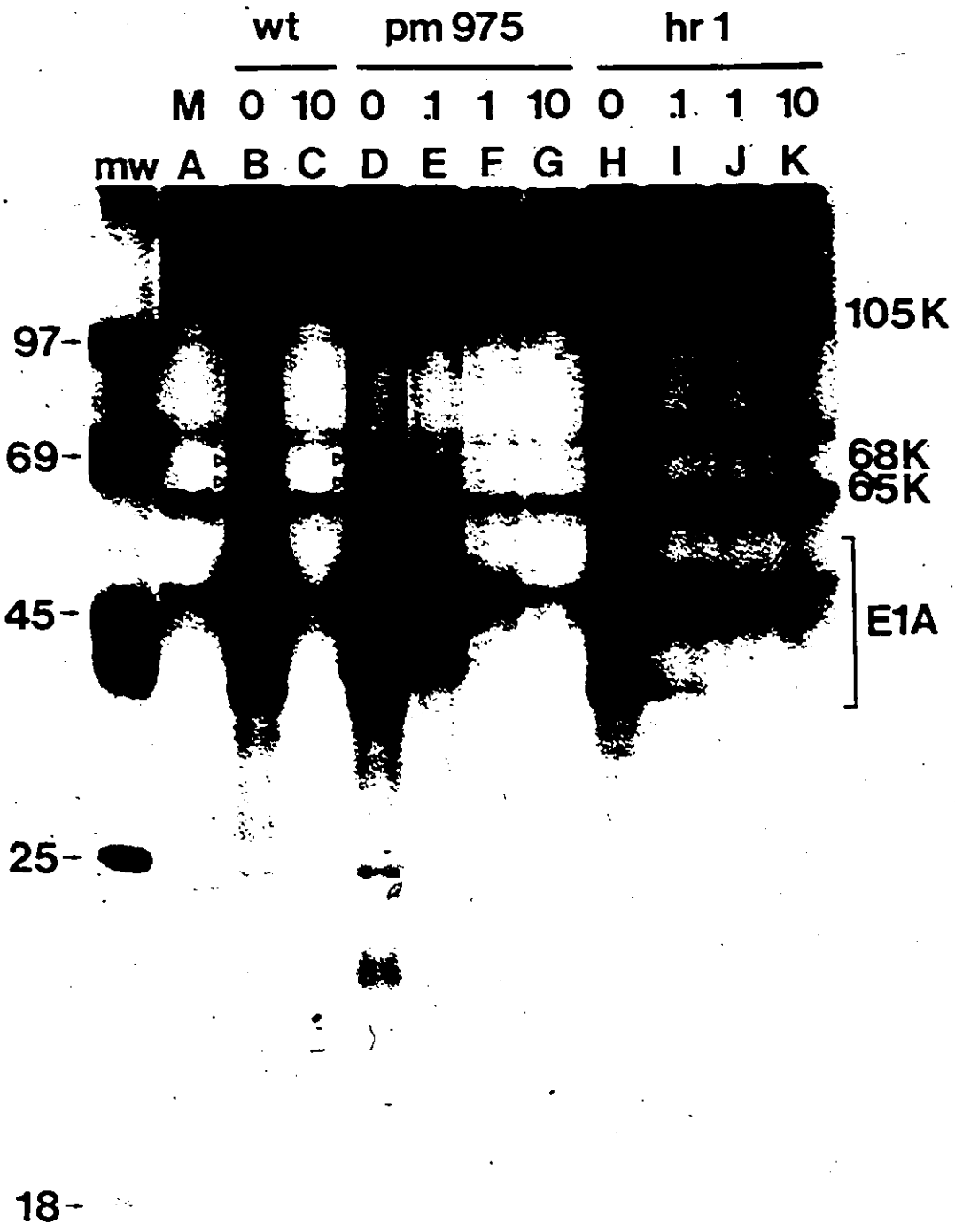
Phosphorylation of E1A-associated proteins. Ad5- and mock-infected cells were labeled with either $^{32}P_i$ or [^{35}S]methionine from 7 to 11 h postinfection and cell extracts were immunoprecipitated with E1A-C1 serum, in some cases in the presence of increasing amounts of C-terminal peptide. mw: molecular weight markers. A-C: ^{32}P-labeled mock- (A) or Ad5-infected cells in the presence of 0 (B) or 10 (C) ug of C-terminal peptide. D-E: ^{35}S-labeled Ad5-infected cells precipitated in the presence of 0 (D) or 10 (E) ug of C-terminal peptide.


32P
35S
Ad
Ad
M
0
10
0
10
mw
A
B
C
D
E
>250K
105K
97-
69-
68K
65K
45-
E1A
25-
18-

The E1A-C1 and E1A-N1 sera recognize both the 1.1 and 0.9kb E1A mRNA products, and thus it was important to determine if these various cellular proteins are associated with proteins coded for by both mRNAs. In order to examine this question, cells were infected with pm975 or hr1 which, as described above in Chapter 3, produce only the 1.1 or 0.9kb mRNAs, respectively. In addition, wt Ad5- and mock-infected cells were also prepared and all cultures were labeled with [^{35}S]methionine and cell extracts were precipitated with E1A-C1 serum in the presence of increasing amounts of E1A-C peptide. Fig. 28 shows that the 105K species was present at high levels in precipitates from cells infected with both pm975 (lanes D-G) and hr1 (lanes H-K). Separate analysis indicated that both species present in the 105K doublet were found using either mutant (data not shown). In a similar experiment run under the appropriate conditions, >250K was also found using either mutant (data not shown). However, the 68K and 65K species were present in precipitates from cells infected with pm975 (lanes D-G), but were barely detectable with hr1 (lanes H-K). These data indicated that 105K and >250K appeared to be associated with the products of both of the E1A early mRNAs, whereas 68K and 65K appeared to be more prominantly associated with those of the 1.1kb mRNA.

Figure 28

Analysis of immunoprecipitates from pm975- and hr1-infected cells. Mock-infected cells and cells infected with wt, pm975 or hr1 Ad5 were labeled with [^{35}S]methionine from 7 to 11 h postinfecton and extracts were precipitated with E1A-C1 serum, in some cases in the presence of C-terminal peptide. mw: molecular weight markers. A: mock-infected cells. B-C: wt-infected cells precipitated in the presence of 0 (B) or 10 (C) ug of C-terminal peptide. D-G: pm975-infected cells with 0 (D), 0.1 (E), 1.0 (F) or 10 (G) ug of C-terminal peptide. H-K: hr-infected cells with 0 (H), 0.1 (I), 1.0 (J) or 10 (K) ug og C-terminal peptide.


wt
pm 975
hr 1
M 0 10 0 .1 1 10 0 .1 1 10
mw A B C D E F G H I J K
97-
69-
45-
25-
18-
105K
68K
65K
E1A

Little is known about the identity or function of these E1A-associated cellular proteins. However, the data presented in this Chapter demonstrating their existence may lead to a more detailed understanding of how E1A products interact with the cellular machinery, and thus to insights into the mechanism of E1A action in infected and transformed cells.

Chapter 5

Analysis of 58K and its Associated Protein Kinase Activity Using Conventional Methods of Protein Purification

It has been shown that protein kinase activity is present in immunoprecipitates from Ad5-infected cells containing either the E1A gene products (Branton et al., 1984; Rowe and Branton, unpublished results) or E1B 58K (Lassam et al., 1979; Branton et al., 1980; 1981). It was still not clear whether or not these activities arise from cellular kinases bound to the E1 proteins or if they were due to activities that are intrinsic to the viral gene products. To further investigate these possibilities attempts were made to separate Ad5 58K and its associated kinase activity by both conventional methods.

5.1. Ammonium Sulfate Fractionation of Ad5 E1B 58K

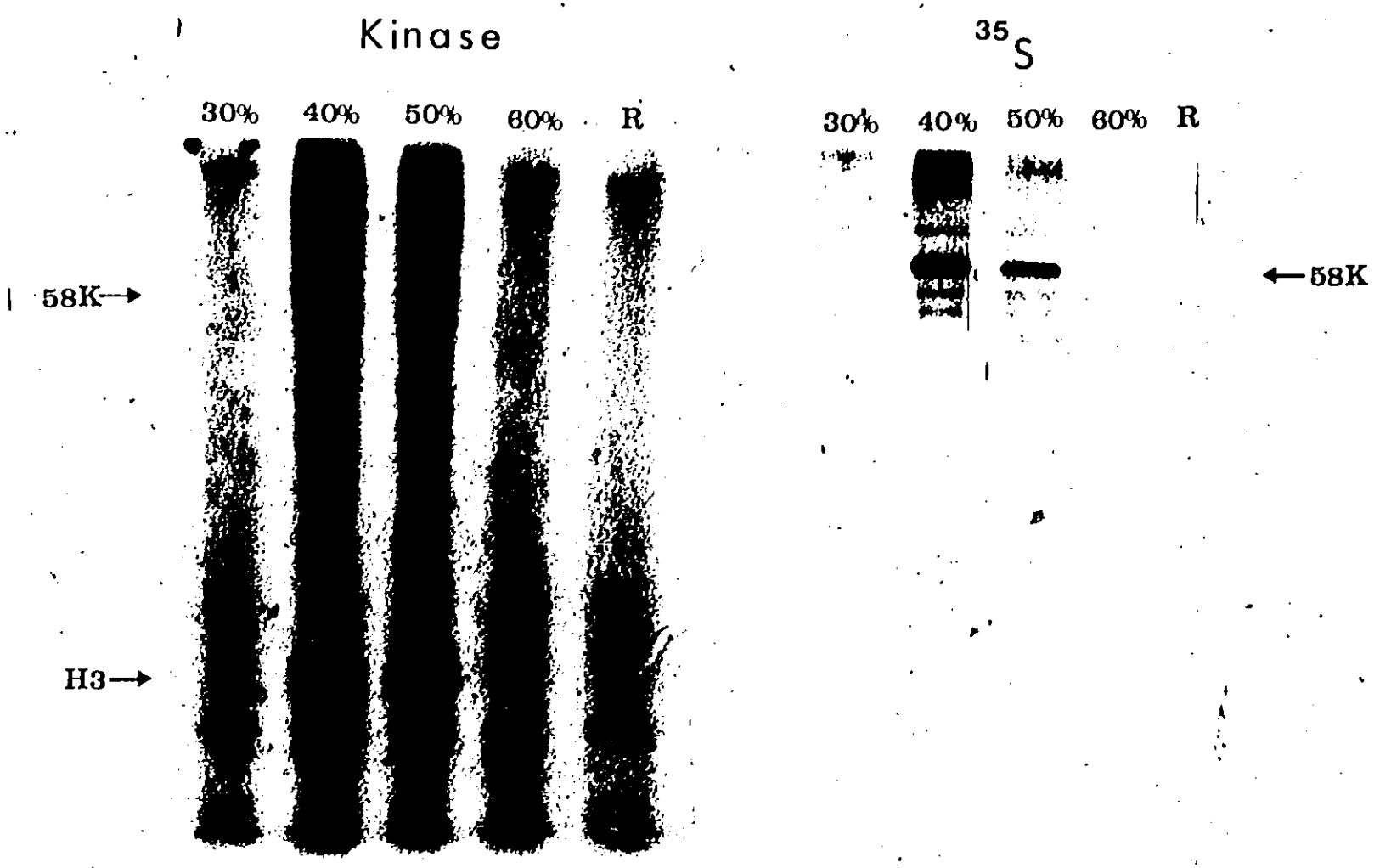
Ad5 E1B-58K was partially purified from an Ad5-transformed cell line, 983-2. This cell line was isolated from cultures of baby hamster kidney cells which had been treated with the XhoI C fragment (0 to 16%) of Ad5 DNA (Rowe and Graham, 1983; Rowe et al., 1984). This cell line was chosen for the present study because it can be grown easily in culture in large quantities and it synthesizes 58K constitutively and without the need of viral infection.

Extracts were prepared from [^{35}S]methionine-labeled or unlabeled 983-2 cells using RIPA buffer, and proteins were precipitated by ammonium

sulfate using 30%, 40%, 50%, and 60% cuts, as described in Materials and Methods. The precipitates were resolubilized, dialyzed, and then immunoprecipitated with 14B hamster antitumour serum. The labeled immunoprecipitates were examined directly by SDS-PAGE for the presence of 58K. Protein kinase activity was detected by incubating the unlabeled immunoprecipitates with [γ-^{32}P]ATP and exogenous histone substrate, as described in Materials and Methods. The results shown in fig. 29 indicated that Ad5 E1B-58K was primarily precipitated by 40% and 50% ammonium sulfate and that little (less than 5%) was present in the 30% and 60% precipitates or in the residual supernatant. The total cellular protein recovered from the pooled 40% and 50% ammonium sulfate fractions represented 50% of the starting material, as estimated by the incorporation of [^{35}S]methionine. Thus close to a two fold purification of 58K was achieved using ammonium sulfate precipitation. The pattern of the protein kinase activity, shown in fig. 29, indicated that activity was detected mainly in the 40% and 50% ammonium sulfate precitates, and in general levels paralleled the amount of 58K present. Thus using this approach, 58K and its associated kinase activity co-purified.

Figure 29

Purification of 58K by ammonium sulphate precipitation. Ad5-transformed 983-2 hamster cells were labeled for 4 h with [^{35}S]methionine and cell extracts were combined with sufficient 100% (w/v) ammonium sulfate to give the require concentrations. The precipitates were collected and resolubilized and dialysed against RIPA buffer. The ^{32}S-labeled samples were immunoprecipitated with 14B antitumour serum and precipitates were analysed by SDS-PAGE. The unlabeled samples were immunoprecipitated with 14B serum and the precipitates were assayed for kinase activity as described in Materials and Methods. The pattern of phosphorylation was determined by SDS-PAGE. The percentage (w/v) ammonium sulfate including residual material remaining (R) has been noted, as have the positions in the gel of 58K and histone H3.

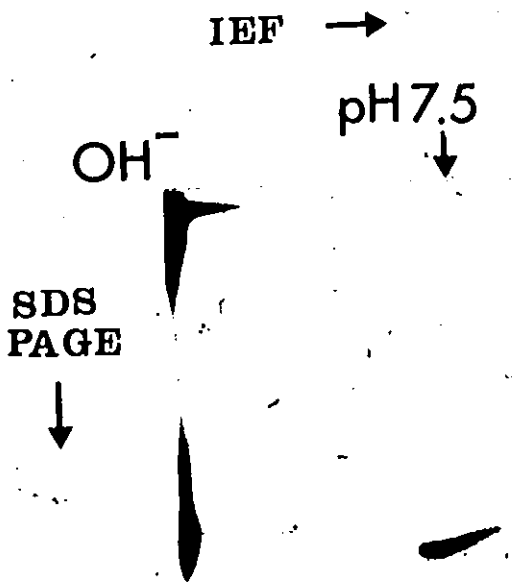

Kinase
30%
40%
50%
60%
R
58K
H3
35S
30%
40%
50%
60%
R
58K

5.2. Fractionation of Ad5 E1B 58K using Ion-exchange Chromatography

In a further effort to fractionate 58K and its associated kinase activity, the material precipitated by 40% and 50% ammonium sulfate was pooled together and was further fractionated by DEAE-sephacel chromatography. In order to determine the proper pH for the buffer used in the DEAE chromatography the pI of 58K was determined by two-dimensional gel electrophoresis. An immunoprecipitate obtained from Ad5-infected cells using 14B antiserum was analyzed and, as shown in fig. 30, 58K separated as a somewhat heterogeneous species with a pI of about 7.5. This heterogeneity could have been caused by variations in the degree of phosphorylation of 58K. A tris buffer of pH 8.0 was used for the anion exchange chromatography. Elution of 58K was carried out using a gradient of 0 to 600 mM NaCl. Portions of each fraction were analysed directly for acid-insoluble radioactivity. Aliquots of some fractions were immunoprecipitated using 14B serum and analysed by SDS-PAGE to determine the presence of 58K and the amount of 58K was estimated by microdensitometer scans of autoradiograms. Other fractions were also immunoprecipitated by 14B serum and incubated with [γ-^{32}P]ATP and histone and protein

Figure 30

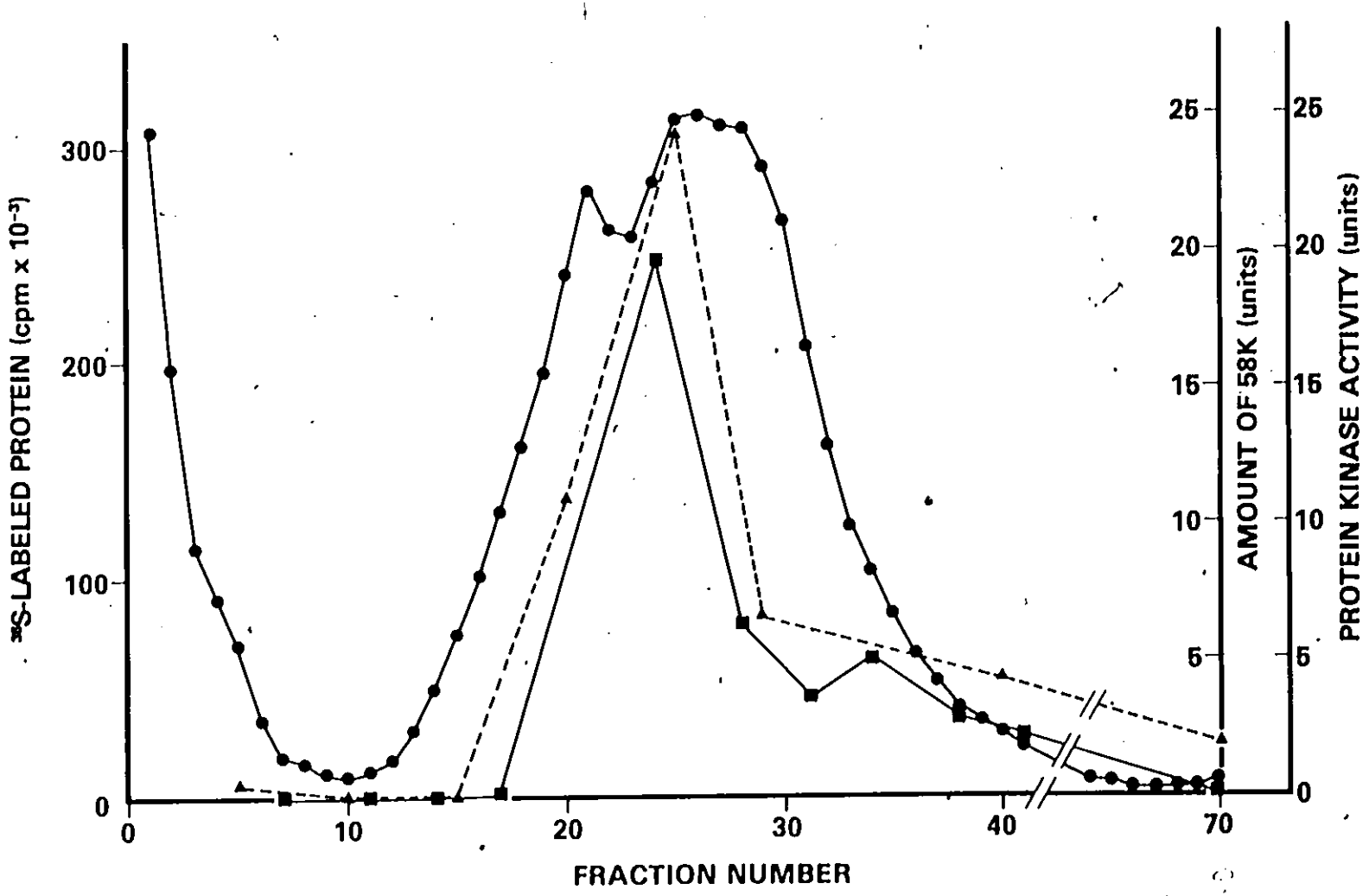
Two-dimensional gel electrophoresis of immunoprecipitates containing 58K. Ad5-infected cells were labeled with [^{35}S]methionine from 12 to 16 h postinfection and cell extracts were immunoprecipitated with 14B antiserum. The precipitates were analysed by two-dimensional gel electrophoresis.


IEF
pH7.5
OH⁻
H⁺
SDS
PAGE

kinase activity was estimated by incorporation of acid-insoluble radioactivity. Fig. 31 shows that total cellular proteins eluted as a broad peak. The 58K eluted in a slightly narrower peak and the majority of 58K eluted between 175 to 200 mM NaCl (fractions 20 to 27). The amount of 58K represented about 0.014% of the starting material after ammonium sulfate precipitation and about 0.083% of the peak fractions (fraction 20 to 27). Thus DEAE-sephacel chromatography yielded about a 6-fold purification and the combined ammonium sulfate precipitation and DEAE-sephacel purification was 12 fold. No attempt was made in these studies to measure the absolute recovery of 58K from the chromatography procedure. Assays for protein kinase activity also indicated a peak in the same fractions (20-27) that contained 58K. The ratio of kinase activity to the amount of 58K in these fractions as measured by microdensitometer scans of autoradiograms was 0.844 which was almost identical to the ratio of 0.859 measured in the starting material following ammonium sulfate precipitation. These data showed therefore that even after a further 6-fold purification of 58K, little change in the specific activity of the associated kinase was observed.

Figure 31

Analysis of Ad5 E1B 58K and protein kinase activity by DEAE-sephacel chromatography. Extracts from [^{35}S]methionine labeled 983-2 cells were precipitated with ammonium sulfate and the proteins precipitated by between 40% and 50% were solubilized and then chromatographed on a DEAE sephacel column as described in Materials and Methods. Fractions were then assayed for total radioactivity (●———●), for 58K precipitated by 14B serum (■———■), and for protein kinase activity in precipitates obtained using 14B serum (▲-----▲). The total radioactivity is given as ^{35}S counts per min. The amount of 58K and protein kinase activity is given in arbitrary units obtained from microdensitometer scans of autoradiograms.

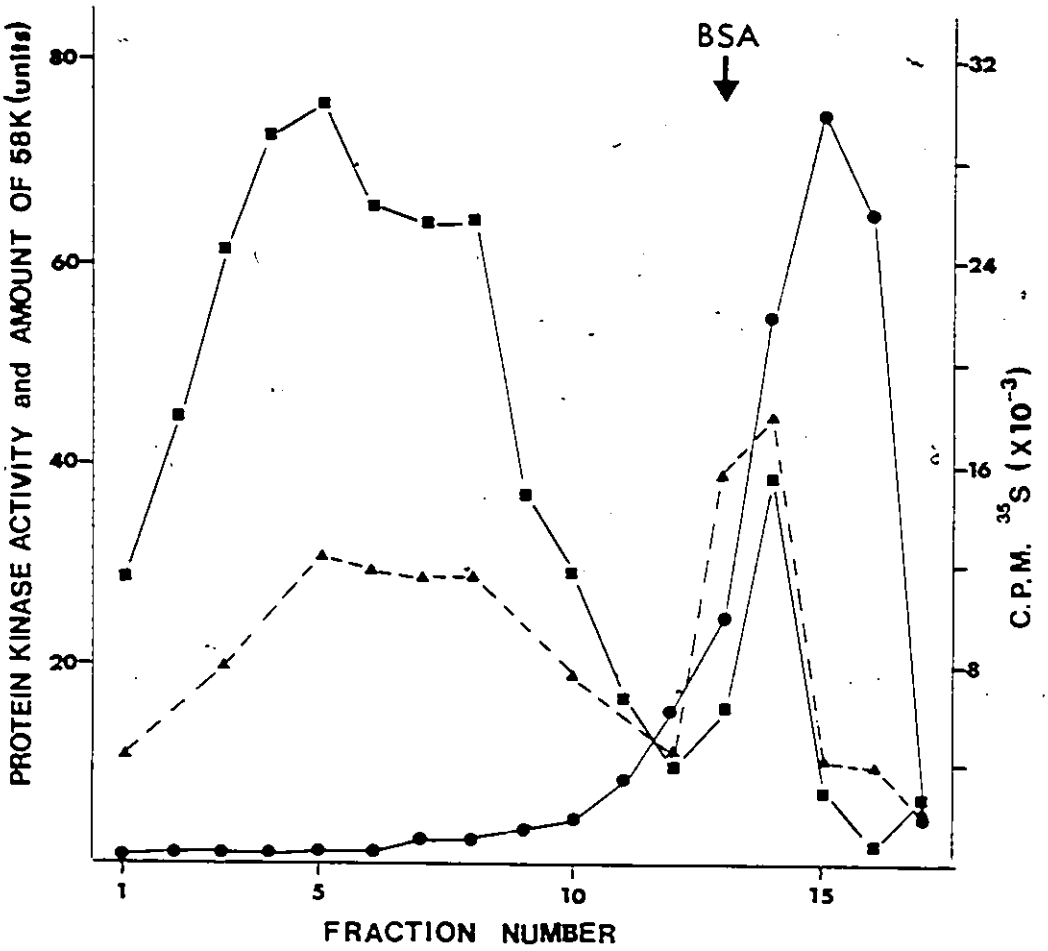

^{35}S-LABELED PROTEIN (cpm x 10^{-3})
300
200
100
0
0
10
20
30
40
70
FRACTION NUMBER
25
20
15
10
5
0
AMOUNT OF 58K (units)
PROTEIN KINASE ACTIVITY (units)

5.3. Glycerol Gradient Centrifugation

Another means to analyze E1B-58K and its associated protein kinase activity is via size fractionation. Extracts from [^{35}S]methionine-labeled 983-2 cells were separated on glycerol gradients. Fractions were analyzed for total radioactivity and they were also immunoprecipitated with 14B serum in order to detect 58K and to measure protein kinase activity. The results shown in fig. 32 indicated that the majority of the cellular protein remained at the top of the gradient as low molecular weight components. E1B-58K separated into two peaks, a narrow one in the position of monomeric 58K, as judged by sedimentation of molecular weight markers in a parallel gradient, and a second large peak in the position of high molecular weight complexes. Protein kinase activity co-sedimented with both 58K peaks. The monomeric 58K contained about 30% of the total kinase activity but only about 8% of the 58K, whereas the complex contained about 65% of the kinase activity and 90% of the 58K. Glycerol gradient sedimentation resulted in about a threefold purification of monomeric 58K and a 15- to 20-fold purification of the 58K complex. These data again showed that protein kinase activity co-purified with 58K and that the specific activity was highest in a

Figure 32

Analysis of Ad5 E1B 58K and protein kinase activity by glycerol gradient centrifugation. Extracts from [^{35}S]methionine labeled 983-2 cells were analysed on glycerol gradients and fractions were assayed for 58K and protein kinase activity as described in Materials and Methods. (●-----●) total ^{35}S label. (■-----■) 58K precipitated by 14B serum. (▲-----▲) protein kinase activity present in immunoprecipitats obtained using 14B serum. The direction of sedimentation was from right to left.


BSA
PROTEIN KINASE ACTIVITY and AMOUNT OF 58K (units)
C.P.M. ^{35}S (X 10^{-3})
FRACTION NUMBER
80
60
40
20
32
24
16
8
1
5
10
15

species that sedimented in the position of monomeric uncomplexed 58K.

Thus using these conventional methods of protein purification there was no indication that 58K was separable from its associated protein kinase activity, that the activity was either intrinsic to or tightly associated with 58K.

Chapter 6

Purification of Ad5 E1 Proteins by Immunoaffinity Using Anti-peptide Sera

The methods of fractionation of 58K discussed in Chapter 5 failed to yield highly-purified preparations of this viral antigen. For this reason it was difficult to make meaningful conclusions about the association of kinase activity with 58K. The need for such purified E1 proteins for this and potentially other purposes inspired attempts to find other methods of purification of Ad5 E1 antigens. The anti-peptide sera presented a potentially powerful tool to purify these viral antigens because of their high degree of specificity towards them. Conventional methods of immunoaffinity chromatography employ chaotropic agents or low pH to uncouple antigen-antibody complexes. However, such harsh conditions might be expected to inactivate the biological activities of the purified proteins. The anti-peptide antisera offer an alternative as addition of synthetic peptide to antigen-anti-peptide antibody complexes might be expected to uncouple the bound protein antigen. Thus with this procedure the proteins would be subjected to mild conditions, thus reducing the possibility of denaturation (or inactivation). This method also could provide a rapid and efficient purification process. It has been reported that a 2,500 fold purification in a single step was obtained with

polyoma middle T antigen using this approach (Walter et al., 1982).

6.1. Releasing of E1 Proteins from Immunoprecipitates using Corresponding Synthetic peptide

The anti-synthetic peptide antibodies described above were used to purify the E1 proteins. The purification protocol is outlined in fig. 33. The Ad5 E1 proteins were first collected by immunoprecipitation using the appropriate anti-peptide sera and protein A-sepharose. The bound E1 proteins were then eluted by incubating the sepharose with buffer containing an excess (25ug) of the corresponding synthetic peptide. Controls were carried out by incubating the sepharose with buffer only.

To investigate the release of E1A proteins from immunoprecipitates, Ad5-infected cells were labeled with [^{32}P]orthophosphate from 7 to 11 hr post-infection and E1A proteins were precipitated using E1A-C1 serum. The immunoprecipitate was then incubated for 1 h with RIPA buffer either lacking or containing 25ug of E1A-C peptide. The material eluted from the immunoprecipitate was analysed by SDS-PAGE. Fig. 34 shows that the material released in the presence of peptide (lane D) consisted primarily of E1A proteins, and most of the other proteins remained bound to the immunoprecipitate

Figure 33

Purification scheme of Ad5 E1B proteins using antipeptide sera.

Purification of Ad5 E1 Proteins using Anti-peptide Antibodies

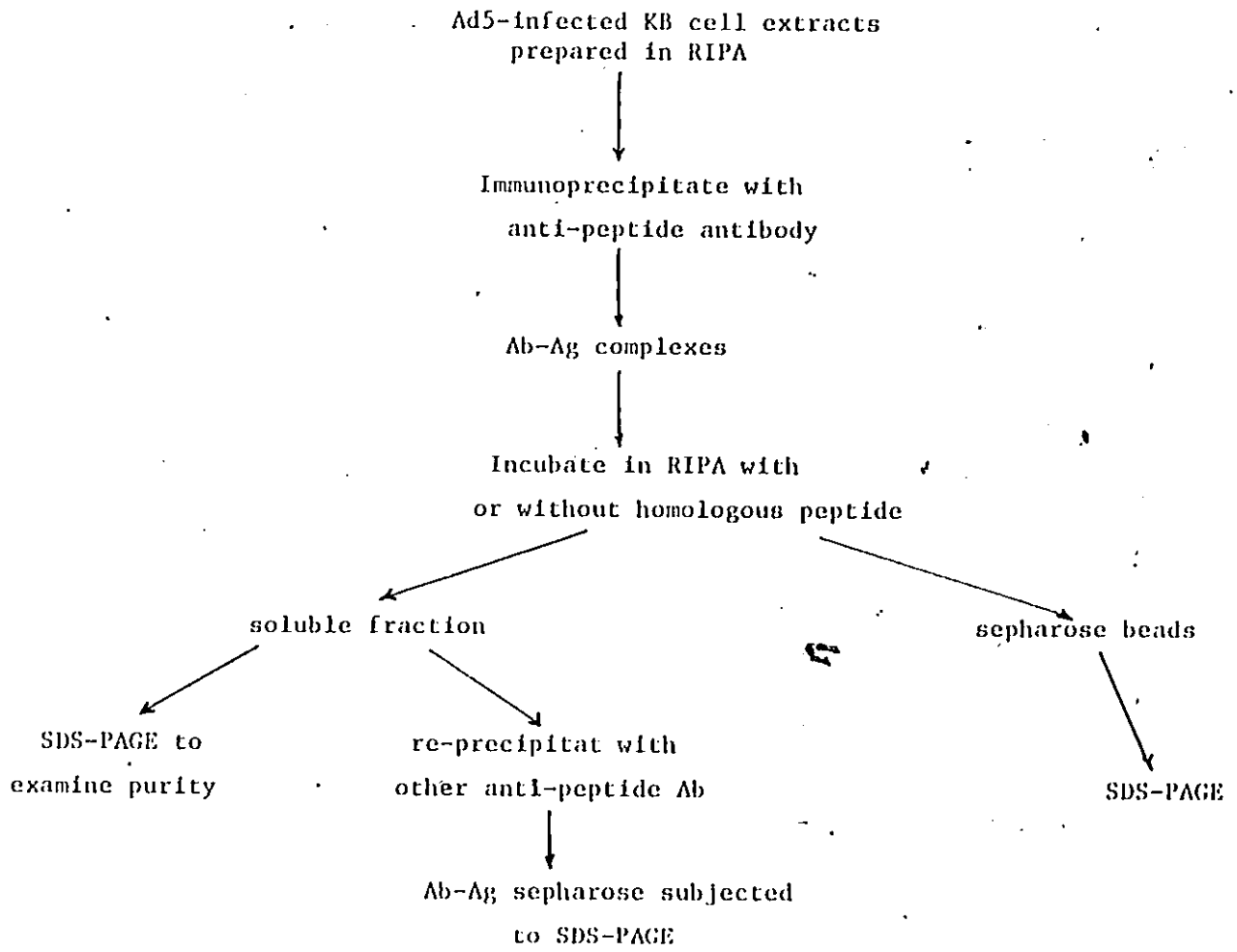


Figure 34

Displacement of E1A proteins from E1A-C1 immunoprecipitates by E1A-C peptide. ^{32}P-labeled Ad5-infected cell extracts were precipitated with E1A-C1 and the precipitates were then incubated in RIPA buffer either lacking (-) or containing (+) 25ug of E1A-C peptide for 1 or 3 h. The material eluted from (E) and remaining bound to (R) the immunoprecipitates after the incubation were analysed on a 12% polyacrylamide gel. A to D: 1 h incubation. Material remaining bound to (lane A) and eluted from (lane B) the precipitates in buffer lacking E1A-C peptide and, with buffer containing 25ug of E1A-C peptide, material remaining bound (lane C) and eluted from (lane D) the precipitates; E to H: # h incubation. Lanes as described for A to D.

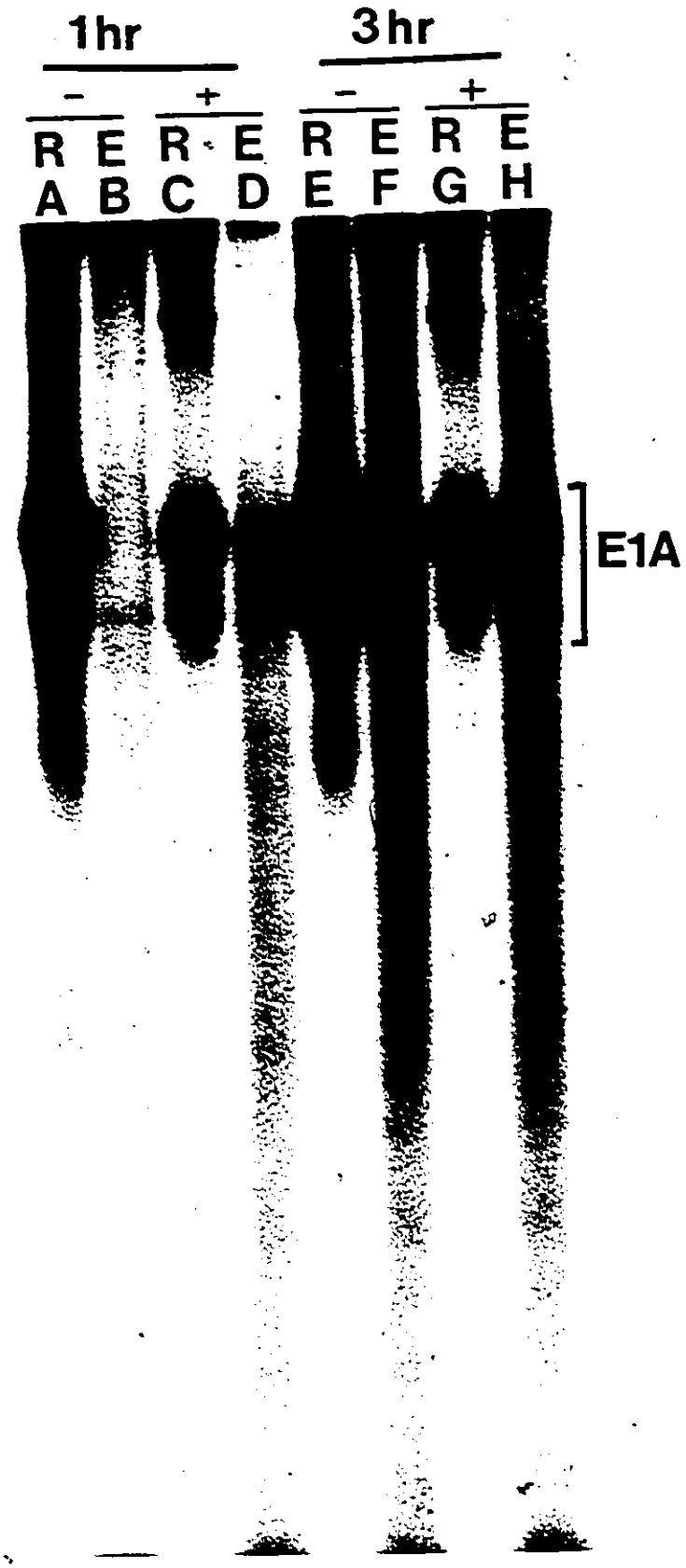

1hr
3hr
-
+
-
+
R E R E R E R E
A B C D E F G H
E1A

Figure 35

Displacement of 58K from 58-Cl immunoprecipitates by 58-C peptide. ^{32}P-labeled Ad5-infected cell extracts were precipitated with 58-Cl and the precipitates were then incubated in RIPA buffer either lacking (-) or containing (+) 25ug of 58-C peptide for 1 or 3 h. The material eluted from (E) and remaining bound to (R) the immunoprecipitates after the incubation were analysed on a 12% polyacrylamide gel. A to D: 1 h incubation. Material remaining bound to (lane A) and eluted from (lane B) the precipitates in buffer lacking 58-C peptide and, with buffer containing 25ug of 58-C peptide, material remaining bound (lane C) and eluted from (lane D) the precipitates; E to H: 3 h incubation. Lanes as described for A to D.


1 hr
3 hr
–
+
–
+
R E R E R E R E
A B C D E F G H
E1B
58K

(lane C). Although the amount of peptide present was more than sufficient to bind all of the E1A-C1 antibodies, as shown by the competition experiment in fig. 8, only about 8% of the E1A protein was released. In a separate preparation the E1A-C peptide was incubated with a similar immunoprecipitate for 3 h and about 43% of the E1A protein was released (lane H). However, incubation beyond 3 h did not result in any further release of E1A species (data not shown). Clearly release of E1A proteins was due to the presence of the synthetic peptide E1A-C as incubation of the immunoprecipitates with RIPA buffer alone indicated that most of the E1A protein remained bound to the antibody-antigen complexes (lanes A and E) and none was detected in the supernatant even after a 3 h incubation (lane F).

The release of E1 proteins from E1A-N1, 58-C1, and 58-N1 antibody-antigen complexes by the corresponding peptides was also examined in a similar series of experiments. With E1B-58K and 58-C1 serum about 5% and 12% of the 58K protein was released from the precipitate after 1 and 3 h of incubation, respectively, with 58-C peptide (fig. 35, lane D and H). However, not all of the synthetic peptides were as effective. Fig 36 and 37 shows that neither E1A proteins nor 58K were released from the E1A-N1 and

Figure 36

Displacement of E1A proteins from E1A-N1 immunoprecipitates by E1A-N peptide. ^{32}P-labeled Ad5-infected cell extracts were precipitated with E1A-N1 and the precipitates were then incubated in RIPA buffer either lacking (-) or containing (+) 25ug of E1A-N peptide for 1 or 3 h. The material eluted from (E) and remaining bound to (R) the immunoprecipitates after the incubation were analysed on a 12% polyacrylamide gel. A to D: 1 h incubation. Material remaining bound to (lane A) and eluted from (lane B) the precipitates in buffer lacking E1A-N peptide and, with buffer containing 25ug of E1A-N peptide, material remaining bound (lane C) and eluted from (lane D) the precipitates; E to H: 3 h incubation. Lanes as described for A to D.

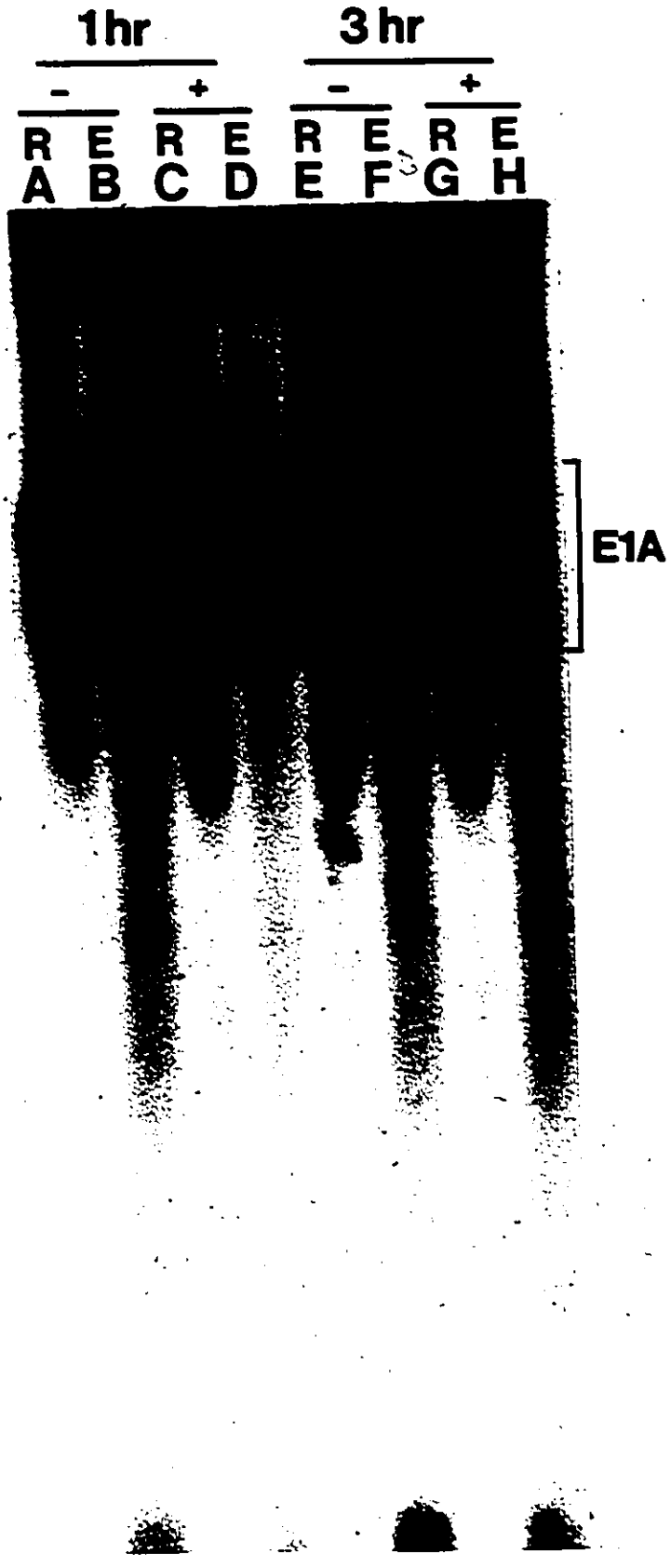

1hr
3hr
-
+
-
+
R E R E R E R E
A B C D E F G H
E1A

Figure 37

Displacement of 58K from 58-N1 immunoprecipitates by 58-N peptide. ^{32}P-labeled Ad5-infected cell extracts were precipitated with 58-N1 and the precipitates were then incubated in RIPA buffer either lacking (-) or containing (+) 25ug of 58-N peptide for 1 or 3 h. The material eluted from (E) and remaining bound to (R) the immunoprecipitates after the incubation were analysed on a 12% polyacrylamide gel. A to D: 1 h incubation. Material remaining bound to (lane A) and eluted from (lane B) the precipitates in buffer lacking 58-N peptide and, with buffer containing 25ug of 58-N peptide, material remaining bound (lane C) and eluted from (lane D) the precipitates; E to H: 3 h incubation. Lanes as described for A to D.

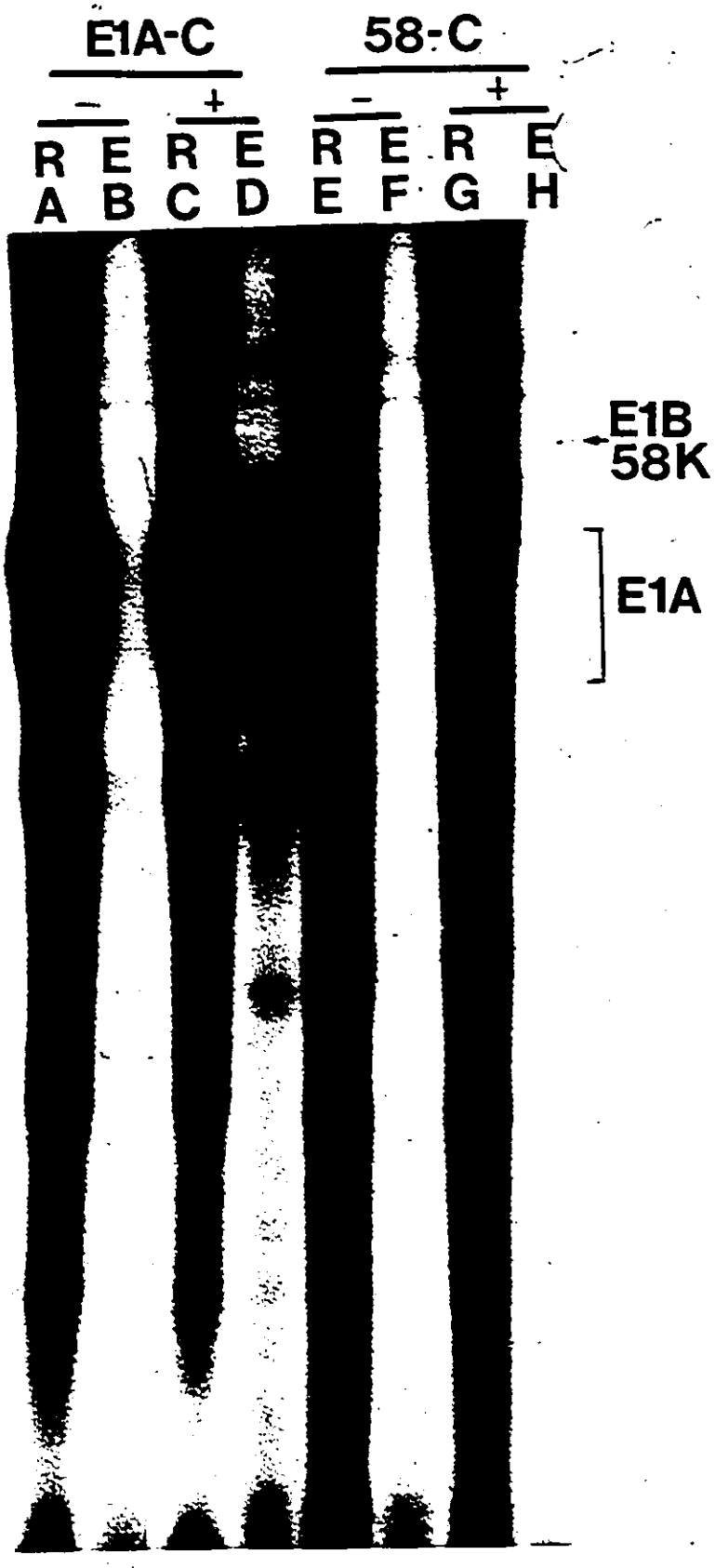

1 hr
3 hr
−
+
−
+
R E R E R E R E
A B C D E F G H
E1B
58K

58-N1 immunoprecipitates by incubating with their homologous peptides. These results indicated that under the present conditions not all synthetic peptides were capable of eluting antigens from their corresponding antibody-antigen complexes and that even with those that did, the efficiencies varied considerably.

The requirement of ionic detergents in the elution of E1 proteins from immune complexes by synthetic peptides was examined. Ad5-infected cells were labeled with [^{32}P]orthophosphate from 7 to 11 h postinfection and cell extracts were immunoprecipitated using either E1A-C1 or 58-C1 serum. The precipitate was then incubated for 3 h with 25 ug of the corresponding peptide in RIPA buffer which lacked both SDS and deoxycholate. The materials remaining bound or eluting from the precipitates were analysed using SDS-PAGE and the autoradiograph was purposely over-exposed in order to detect any minute amount of eluted materials. Fig. 38 shows that less than 5% of the E1A proteins were released from the precipitate (lane D) and very little, if any, 58K was eluted under these conditions (lane H). Similar results were also obtained when the elution was carried out in PBS (data not shown). These results suggested that ionic detergents such as SDS and deoxycholate acid are required to enhance peptide release of E1 proteins.

Figure 38

Effect of ionic detergents in the displacement of E1 proteins from immunoprecipitates. ^{32}P-labeled Ad5-infected cell extracts were precipitated with either E1A-C1 serum or 58-C1 serum. The immunoprecipitates were then incubated for 3 h with or without 25ug of the corresponding peptide in RIPA buffer lacking both SDS and deoxycholate. The material remaining bound or eluting from the precipitates were analysed using SDS-PAGE. A to D: E1A-C1 precipitates. Material remained bound (lane A) and eluted from (lane B) the precipitates in the absence of E1A-C peptide; with buffer containing E1A-C peptide material remained bound (lane C) and eluted from (lane D) the precipitates. E to F: 58-C1 precipitats. With buffer lacking 58-C peptide material bound (lane E) and eluted from (lane F) the precipitates and in the presence of 58-C peptide material bound (lane G) and eluted from (lane H) the precipitates.

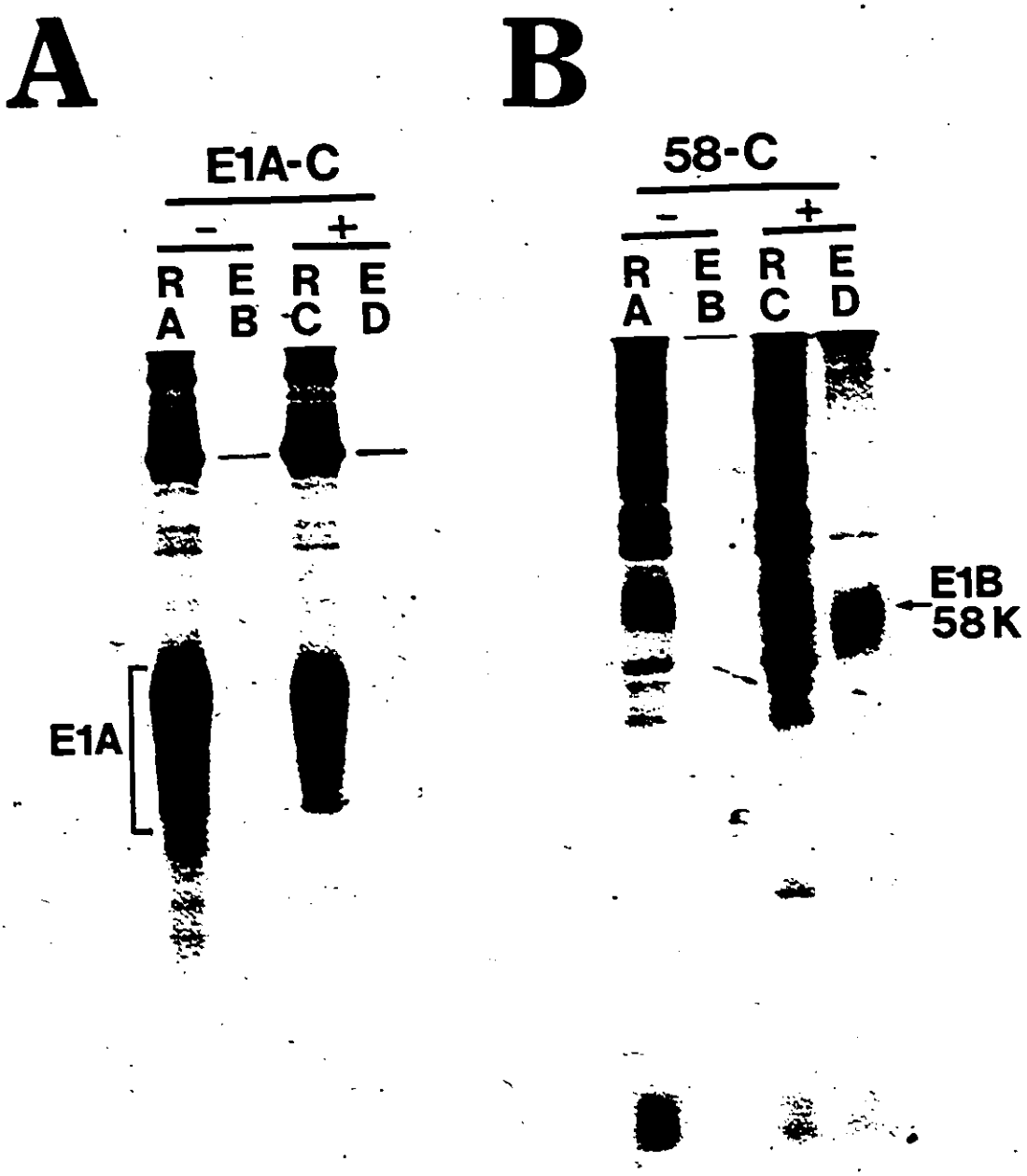

E1A-C
58-C
–
+
–
+
R E R E R E R E
A B C D E F G H
E1B
58K
E1A

6.2. Purification of Ad5 E1 Proteins using Immunoaffinity Chromatography

Ad5 E1 proteins were purified by immunoaffinity using the anti-peptide sera and estimates of the degree of purity of each were made. Ad5-infected cells were labeled with [^{35}S]methionine from 7 to 11 h post-infection and cell extracts were prepared using RIPA buffer as described before. E1 proteins were first precipitated using either anti-peptide serum, E1A-C1 or 58-C1, under conditions of antigen excess where approximately 25% of total E1A proteins and 20% of total 58K from infected cells were bound to the antibody. The conditions were selected in these preliminary studies in order to economize on the amount of peptide needed for the next step of the purification. Viral proteins were then released from the antibody-antigen complexes by incubating with homologous peptide in RIPA buffer for 3 hours at 4^{o}C. The material eluted from and also remaining bound to the immunoprecipitates was analysed by SDS-PAGE. As shown in fig. 39, in the absence of homologous peptide, E1A proteins (fig. 39A, lane B) or 58K (fig. 39B, lane B) failed to be eluted and all of these E1 proteins and many other cellular proteins remained bound to the sepharose (fig. 39A, lane A and fig. 39B, lane A).

Figure 39

Purification of E1A proteins and 58K using C-terminal antipeptide sera. ^{35}S-labeled Ad5-infected cell extracts were precipitated with either E1A-C1 serum or 58-C1 serum. The immunoprecipitates were then incubated for 3 h with or without 25ug of the corresponding peptide in RIPA buffer. Fig. 39A: Purification of E1A proteins using E1A-C1 serum. Material remaining bound (lane A) and eluted from (lane B) the precipitates in the absence of E1A-C peptide; with buffer containing E1A-C peptide material remaining bound (lane C) and eluted from (lane D) the precipitates. Fig. 39B: Purification of 58K using 58-C1 serum. With RIPA buffer lacking 58-C peptide material bound to (lane A) and eluted from (lane B) the precipitates and in the presence of 58-C peptide material bound to (lane C) and eluted from (lane D) the precipitates.


A
E1A-C
–
+
R A
E B
R C
E D
E1A
B
58-C
–
+
R A
E B
R C
E D
E1B 58K

However, as shown above, in the presence of homologous peptide the eluted material consisted primarily of E1A proteins (fig. 39A, lane D) and 58K (fig. 39B, lane D). About 9% of E1A species and 56% of 58K protein was released from the precipitates by incubating with homologous peptide and most of the other proteins remained bound to the immunoprecipitate (fig. 39A, lane C and fig. 39B, lane C). E1A species and 58K protein represented 37.6% and 52.5%, respectively, of the total proteins in the eluted material, as estimated by the levels of incorporation of [^{35}S]methionine. The fractions of E1A protein and 58K in the starting material were 0.005% and 0.016% of total infected cell proteins. Thus a 7,500- and 3,300-fold purification of E1A species and 58K, respectively, were achieved in a single step. Since only 25% of total E1A proteins and 20% of total 58K were precipitated initially, the recoveries of E1A proteins and 58K in this experiment were 9.4 and 10.5%, respectively.

Two species of about 25,000 and 24,500 daltons were also present in the purified 58K preparation (fig. 39B, lane D). This doublet was only released from the precipitate in the presence of 58-C peptide and it was not found in the control (fig. 39B, lane B). These species were not been observed in previous immunoprecipitations using 58-C1 serum (fig. 4, lane B)

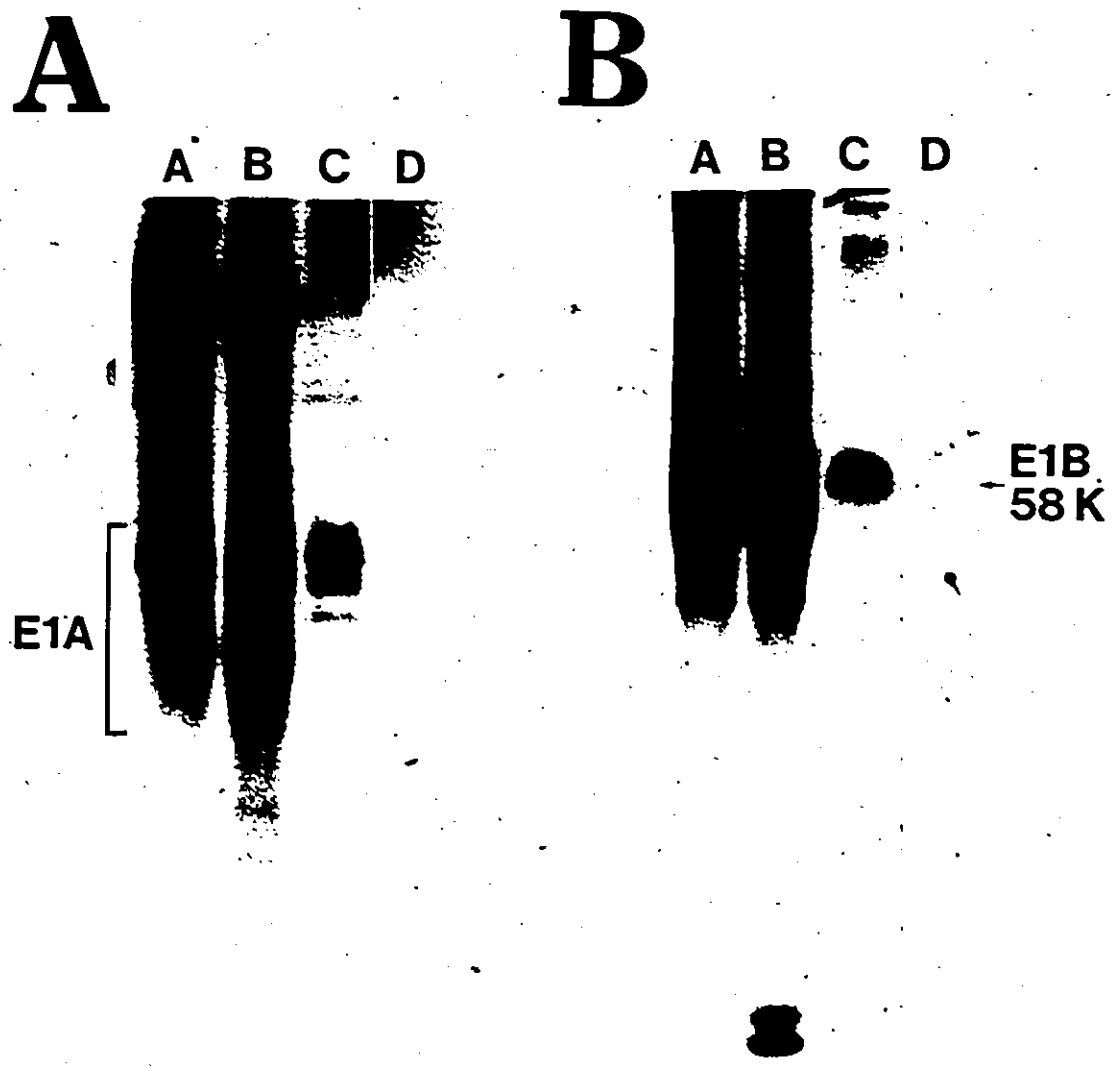
and it is possible that they represented either degradation products of 58K formed during the purification process or Ad5 E4 25K protein which has been found to co-precipitate with 58K under certain conditions (Sarnow et al., 1984).

Although E1A species and 58K protein were not significantly released from the E1A-N1 and 58-N1 precipitates by incubation with homologous peptides, it was still possible to further purify the E1 proteins using these sera. A separate purification was carried out using E1A-C1 and 58-C1 sera as described above and the eluted E1A proteins and 58K protein were then re-precipitated using E1A-N1 and 58-N1 sera, respectively. For controls, experiments were carried out in the same fashion except that no peptide was added to the first immunoprecipitates.

Fig. 40A shows that in the presence of E1A-C peptide, E1A proteins were eluted from E1A-C1 immunoprecipitates and that E1A-N1 serum efficiently immunoprecipitated them (lane C). The residual supernatant after the precipitation using E1A-N1 serum was also analysed by SDS-PAGE and no E1A species were detected (data not shown). Approximately 27.9% of the E1A protein was recovered by this two-step method, as estimated by the level of incorporation of radioactivity. Since approximately 25% of the total E1A

Figure 40

Two-step purification of E1A proteins and 58K using antipeptide sera. ^{35}S-labeled Ad5-infected cell extracts were precipitated with either E1A-C1 serum or 58-C1 serum. The immunoprecipitates were then incubated for 3 h with or without 25ug of the corresponding peptide in RIPA buffer. The eluted material was then re-precipitated with the corresponding amino terminal serum. Fig. 40A: Purification of E1A proteins. Material remaining bound to E1A-C1 precipitates after incubation with RIPA buffer containing (lane A) or lacking (lane B) E1A-C peptide; re-precipitation of material eluted from RIPA buffer containing (lane C) or lacking (lane D) E1A-C peptide using E1A-N1 serum. Fig. 40B: Purification of 58K. Material remaining bound to 58-C1 precipitates after incubation with RIPA buffer containing (lane A) or lacking (lane B) 58-C peptide; re-precipitation of material eluted from RIPA buffer, containg (lane C) or lacking (lane D) 58-C peptide using E1A-N1 serum.


A
A B C D
E1A
B
A B C D
E1B
58 K

proteins were initially precipitated by E1A-C1 serum, the recovery of E1A proteins represented 6.9% of the starting material. The purified E1A protein represented 76.3% of the total protein present in the E1A-N1 immunoprecipitate, as compared to 0.005% in the starting material. Therefore a purification of approximately 15,000-fold was achieved in these two steps. In control experiments, E1A-C1 precipitate was incubated with RIPA buffer only and no E1A species were detected in either the E1A-N1 immunoprecipitate (lane D) or in the residual supernatant after the immunoprecipitation (data not shown). The material which remained bound to the E1A-C1 precipitates after incubation with or without E1A-C peptide was also analysed. In the absence of E1A-C peptide, a large amount of the E1A protein remained bound to the E1A-C1 precipitate (lane B) as compared to the sample that was eluted with E1A-C peptide (lane A). These results confirmed that E1A species was released from E1A-C1 precipitate by incubation with E1A-C peptide and all the eluted E1A species were re-precipitated by E1A-N1 serum.

Similar experiments were also carried out to purify 58K protein using both 58-C1 and 58-N1 sera. The 58-C1 immunoprecipitate was incubated with 58-C peptide and the eluted material was re-precipitated using 58-N1

serum. Fig 40B shows that 58K protein was detected in the eluted material using 58-N1 serum (lane C). Approximately 15.3% of the bound 58K protein was eluted from the 58-C1 precipitate and re-precipitated using 58-N1 serum. Since 20% of the total 58K was initially precipitated by 58-C1 serum, thus only 3.1% of 58K in the starting material were recovered. The residual supernatant after the precipitation using 58-N1 serum was also analysed by SDS-PAGE. Only a small quantitiy of 58K was detected and less than 5% of the eluted 58K protein remained in this residual supernatant (data not shown). It was possible that this portion of 58K protein was denatured during the purification process and was not recognized by 58-N1 serum. However, a more likely explanation was that insufficient quantities 58-N1 serum were added to precipitate all of the eluted 58K protein. The purified 58K represented 69.7% of the protein present in the 58-N1 immunoprecipitate as estimated by the incorporation of [^{35}S]methionine. Since 58K made up 0.016% of total protein in the starting material, a 4,350-fold purification was obtained in the two-step fractionation. In a control experiment 58-C1 precipitate was incubated with RIPA buffer only and no 58K protein was detected in the eluted material using 58-N1 serum (lane D) or in the residual supernatant after the immunoprecipitation

(data not shown). The material bound to 58-C1 precipitates after incubation with or without 58-C peptide was also analysed. In the absence of 58-C peptide a greater amount of 58K remained bound to 58-C1 precipitate (lane B) than with samples that were incubated with 58-C peptide (lane A). These results again confirmed that 58K protein was released from the 58-C1 precipitate by incubation with 58-C peptide and 58-N1 serum was capable of re-precipitating the eluted 58K protein, thus providing a highly purified sample in two-steps. These E1 proteins can be eluted off the precipitates using other conventional methods and should provide highly purified material for raising monoclonal antibodies or for other biochemical studies.

One immediate use for the highly purified E1A and 58K preparations would have been to determine if they possessed protein kinase activity. Such attempts were made, however, as discussed below in Chapter 7, the data suggested that the level of kinase activity did not corrleate with the amount of viral protein present, however, the results were sufficiently ambiguous that no definite conclusion could be drawn. Thus more detailed studies will be required to unequivocally answer this question.

Chapter 7

Discussion

In the present study antisera against synthetic peptides homologous to the termini of Ad5 E1A proteins were used to characterize these products. The two sera, E1A-N1 and E1A-C1, were raised against peptides corresponding to the first eight amino acids at the amino terminus and last five at the carboxy terminus of the proposed sequences of E1A gene products. The sequences for the synthetic peptides were chosen based on guidelines described in the Introduction. The synthetic peptides were hydrophilic thus increasing the probability that they contained antigenic sites recognizable on the complete protein (Hopp and Wood, 1981). These sera precipitated identical patterns of multiple polypeptide species from Ad5-infected cells as determined by one- and two-dimensional gel electrophoresis. The latter also revealed that the E1A gene products are acidic proteins with pI's ranging between 5.0 to 4.5. Four major species with apparent molecular weight of 52K, 50K, 48.5K, and 45K, and two minor species of 37.5K and 35K were detected, although the precise size varied somewhat depending on the exact conditions of electrophoresis and the maximum apparent molecular weight observed have been adopted and used throughout. These data therefore confirmed the validity of the proposed sequence and reading frame of the E1A region mRNA (Pericaudet et al., 1979; van Ormondt et

al., 1980). However, the hamster antitumor serum precipitated three of the six E1A gene products including the 52K, 48.5K, and 37.5K species. Partial hydrolysis of the 52K species precipitated by E1A-C1 and the hamster antitumour serum using V-8 protease, demonstrated that both recognized the same 52K viral polypeptides.

The origin of these multiple E1A polypeptides was studied using the mutants pm975, which only synthesizes polypeptides derived from the 0.9kb mRNA, and hr1, which synthesizes normal 0.9 kb mRNA gene products and is predicted to produce a truncated polypeptide derived from the 1.1kb E1A mRNA. These mutants permitted the identification of the 52K, 48.5K, and 37.5K species as products of the 1.1kb mRNA and 50K, 45K, and 35K species as products of the 0.9kb mRNA. These results, and previous collaborative studies (Rowe et al., 1983a), showed that the hamster antitumor serum only recognizes the products of the 1.1kb mRNA. It is not clear why this is the case. One possibility is that tumor serum is specific for the 46 amino acids that are unique to the 1.1kb mRNA products. It is also possible that the presence of this additional internal sequence drastically alters the structure of polypeptides encoded by the 1.1kb mRNA, such that they become much more antigenic. The nature of the E1A gene products

from different group I host-range mutants were also examined using E1A-N1. The results showed that hr1 and hr2 were defective for the synthesis of the 1.1kb mRNA products whereas the two-dimensional electrophoretic profiles of hr3, 4, and 5 were indistinguishable from that of wild-type virus. Thus none of the host-range mutations appears to affect the synthesis of multiple species from each of the E1A messages.

Several other groups have also made attempts to identify the E1A gene products. These studies primarily employed in vitro translation of selected E1A mRNA and analysis of viral proteins by two-dimensional gel electrophoresis. The cells were pretreated with cycloheximide to augment the amount of E1A mRNA and gene products. These studies identified a cluster of about six acidic proteins derived from E1A (Harter and Lewis, 1978; Smart et al., 1981). These species were shown to be related, as determined by tryptic peptide mapping, and amino acid sequencing of the tryptic peptides revealed that they were the products of the 1.1 and 0.9kb E1A mRNAs (Smart et al., 1981). Other groups using both in vivo and in vitro approaches have detected only four acidic proteins (Green et al., 1979; Brackmann et al., 1980), or as few as two products (Riccardi et al., 1981; Gaynor et al., 1982). These studies were potentially prone to artifacts due to the

cell-free translation or, in some cases, the use of drugs as metabolic inhibitors. The present study is of particular interest because the results were obtained by short-term labeling of cells *in vivo* which should avoid such problems.

It is now clear that each E1A mRNA produces two major and at least one minor species. However, what causes the multiple polypeptide species from the individual E1A mRNA is still largely unknown. Using *in vitro* translation of size-fractionated E1A mRNAs, Esche et al. (1980) had suggested that modifications responsible for the generation of the multiple forms of E1A proteins were located in the carboxy terminal 70 amino acids which are absent in the products produced by d1313. However, the results in the present study clearly demonstrated that this mutant does produce multiple species which are slightly smaller and more acidic than those made by wild-type virus (Fig. 17). Studies with mutant d11504, which deletes the first 15 amino acids at the amino terminus, demonstrated that this mutant also produces multiple species (Rowe et al., 1983a; Downey et al., 1984). These results thus suggested that neither the amino terminal 15 amino acids or the carboxy terminal 70 residues are directly involved in the generation of multiple species. The fact that all the E1A multiple species were recognized

by both E1A-N1 and E1A-C1 also indicated that these products represent full-length molecules and neither proteolytic degradation, premature termination, nor use of internal translation initiation sites are responsible for producing the E1A multiple species. It seems likely that they are generated by some forms of post-translational modification. All of the E1A multiple forms are phosphorylated and preliminary studies suggested that there are differences in the sites of phosphorylation among various species (Yee et al., 1983; Tremblay and Branton, personal communication). Thus it is possible that differential phosphorylation could generate the multiple species. While it might be expected that phosphorylation differences would primarily affect only the isoelectric point of a protein, it has been reported that phosphorylation of canine phospholambin induces a 2,000 dalton shift in mobility on SDS-PAGE (Wegner and Jones, 1984) and that of p140^{fps} an apparent 5,000 dalton change (T. Pawson, personal communication).

According to nucleotide sequencing data, the N-terminus of the E1A 0.6kb late mRNA product should be identical to other E1A gene products (Virtanen and Petterson, 1983). Thus E1A-N1 serum should have be capable of immunoprecipitating this viral polypeptide. However, despite of a variety of approaches no

potential product was detected using this serum. The failure to detect this protein could be explained in a number of ways. (1) It is possible that the amino acid sequence deduced from the 0.6kb mRNA is incorrect. (2) The product could be extremely short-lived, although it was still not possible to detect such a protein even using short labeling periods of 15 min. (3) There may be no protein produced in vivo from this late E1A mRNA. (4) The product could be particularly insoluble in RIPA buffer. (5) It is possible that the 0.6kb mRNA product constitutes only a small portion of the total E1A gene products and thus may go undetected because of competition for binding to the antiserum by the large excess of 1.1 and 0.9 kb mRNA products. However, immunoprecipitations have been carried out first using E1A-C1 to remove most, if not all, of the 1.1kb and 0.9kb mRNA products, and then followed by precipitation with E1A-N1 serum in an attempt to identify the 0.6kb mRNA product. However, even under these conditions no such product was detected (data not shown). (6) Finally, unlike the other E1A proteins, the product of the 0.6kb mRNA could be folded in such a way that the amino terminus is inaccessible to the antibody. There is precedent for this possibility as anti-peptide serum specific for the common amino terminal peptide of SV40 large T and small T antigens immunoprecipitates the

large T antigens but not the small SV40 antigen (Walter et al., 1981). In addition to the failure to detect the products of the 0.6kb mRNA, the anti-synthetic peptide serum E1A-N1 should also have been able to precipitate a truncated form of the 1.1 kb mRNA product predicted for hr1. However, no such product was detected and some of these explanations could apply in this case as well.

The antipeptide sera were also used to look for proteins that may be physically associated with E1A products. Five cellular proteins of 65K, 68K, 105K (doublet) and >250K were likely candidates. Of the five, four (65, 68 and the 105K doublet) co-precipitated with E1A proteins using M73, a monoclonal antibody specific against the carboxy-half of E1A proteins, as well as both antipeptide sera, and their precipitation was efficiently blocked by the addition of homologous synthetic peptides. These results suggested that they were not present because of non-specific precipitation or cross-reactivity with epitopes present in E1A proteins. The fifth cellular protein, >250K, was consistently detected with E1A-C1 serum and M73 monoclonal antibody, but not with E1A-N1 serum. The possibility that this protein was associated with E1A products was strengthened by results from in vitro mixing experiments. It is possible therefore that the failure to detect >250K with E1A-N1 serum could

reflect an involvement (either directly or indirectly) of the amino terminal region of E1A proteins in the association with >250K.

The mixing experiment using unlabeled Ad5-infected cell extracts and labeled mock-infected cell extracts also clearly showed that these polypeptides are cellular proteins. The fact that they are capable of associating with E1A proteins *in vitro* also suggested that such an association may take place *in vivo* as well. However, it is nevertheless possible that all of these observations could be explained by the formation of protein complexes in the extracts after lysis of infected cells. This argument could be somewhat clarified if it were possible to obtain antisera against these cellular proteins and demonstrate by immunofluorescence they are present in similar intracellular locations as the E1A products.

Similar experiments were also carried out using Ad5-infected Hep 2 and HeLa cells and Ad5-transformed 293 cells and results identical to those present in this thesis were obtained (data not shown). An array of E1A-associated proteins has also been detected in infected cells using a series of mouse monoclonal antibodies specific for Ad5 E1A polypeptides (Harlow and Schley, unpublished results) and it is likely that

some or all of these species are identical to those described in this thesis.

Other viral transforming proteins are also found to associate with cellular proteins. The RSV transforming protein pp60^{src} is associated with two cellular proteins of 90K and 50K (Brugge, et al., 1981), polyoma middle T antigen with cellular c-src protein (Courtneige and Smith, 1983, 1983), both the Ad5 E1B-58K protein and SV40 large T antigen with p53 (Sarnow, et al., 1982; Linzer and Levine, 1979; Lane and Crawford, I979; Chang et al., 1979; Kress et al., 1979; Smith et al., 1979), and Ad5 E1B 58K with E4 25K in Ad5-infected cells (Sarnow et al., 1984). However, little is known about the functional significance of these complexes. It is known that E1A plays a role both in transformation and transcriptional activation, but the mechanism of action is still unclear. It has been proposed that E1A proteins may interact with the transcription complex (Logan and Shenk, 1982) and thus it would be important to determine if these cellular proteins are part of this complex. Of some interest in this regard was the study using Ad5 E1A mutants which demonstrated that the 105K doublet and >250K were complexed with the products of both 1.1 and 0.9kb E1A mRNAs but that the 68K and 65K species were less evidently associated with 0.9 kb mRNA products. It has

been found that transcriptional enhancement by E1A is primarily induced by the 1.1kb mRNA products and these results, taken together, may suggest that these cellular proteins could be involved in gene activation whereas 105K and >250K could be associated with another function of the E1A proteins. It is possible that such protein-protein interactions could modify, stablize or regulate the biological activity of the protein species or of E1A products. It has been suggested that normal cells contain an E1A-like activity (Imperiale et al., 1984) and it is possible that these cellular proteins may play an important role in normal cell activities as well.

Several questions about the significance and relationships between the E1A products and the cellular proteins remain to be answered. In the present study it was not possible to determine if all of the E1A proteins are associated with cellular proteins. It was also not determined if these cellular proteins are associated with the E1A proteins as one complex. Studies using sedimentation analysis could potentially answer these questions. The cellular location of the complexes would also be valuable in order to suggest possible functions for these structures. The identity of these cellular proteins is not known and antisera

against them will be invaluable in their identification and characterization.

The 58-C1 and 58-N1 sera which were raised against peptides corresponding to the carboxy and amino termini of the large 58K E1B gene product, respectively, also precipitated the 58K viral polypeptide. This polypeptide was specifically recognized by the antipeptide sera as addition of homologous synthetic peptides significantly blocked its precipitation. Partial hydrolysis with V-8 protease also revealed that this viral polypeptide is identical to the E1B 58K species recognized by antitumour serum. It is now known that the E1B 58K is acetylated at the amino terminus (Anderson et al., 1984). Although the 58-N synthetic peptide was nonacetylated, antibody prepared against this peptide was still active against the 58K polypeptide species, suggesting that the acetylated amino terminus of 58K does not significantly alter its antigenicity. Since the synthetic peptide sequences were obtained from an amino acid sequence deduced from nucleotide sequencing data, the results in the present study further confirmed the validity of the proposed sequences of the E1B DNA and mRNAs and the reading frame of the mRNA (Bos et al., 1981; Maat et al., 1980; Maat and van Ormondt, 1979).

Another viral protein with an apparent molecular weight of 20K, which is related to E1B 58K, has been identified in Ad2-infected and transformed cells (Green et al., 1979, 1982; Matsuo et al., 1982). This protein is also acetylated and its termini consist of the amino terminal 78 and carboxy terminal 77 residues of the E1B-58K protein (Anderson et al., 1984). These data suggest that antisera prepared against either the amino- or carboxyl-terminus of E1B-58K should precipitate the 20K viral protein. Antiserum raised against an acetylated amino terminal nonapeptide (Anderson et al., 1984) corresponding to the E1B-58K protein was shown to be capable of precipitating both the E1B-58K and 20K polypeptides. Lucher and coworkers (1984) also prepared two synthetic peptides designated as peptides 6 and 2. Peptide 6 consists of 16 amino acids corresponding to the amino terminus of E1B 58K from amino acid 2 to 17, and peptide 2 contains the 8 amino acid sequence at the carboxy end of peptide 6 (i.e. amino acids 10 to 17). Antibody directed against peptide 6 immunoprecipitated both E1B 58K and 20K species, however, antiserum raised against peptide 2 was active only against the E1B-58K protein. These results, taken together, suggested that the nine amino terminal residues of E1B 58K harbour a specific antigenic site found in the E1B-20K polypeptide.

However, the 58-N1 antipeptide serum used in the present study failed to precipitate the E1B 20K polypeptide. This failure can be explained in a number of ways. The synthetic peptide 58-N is nonactylated, as well as much shorter than the other two peptides that were successfully used in raising antisera against the 20K polypeptide. These differences may reduce the avidity of this serum against the E1B-20K species. It has also been suggested that this viral polypeptide is present in only small amounts in infected cells and that a high,multiplicity of infection and treatment with metabolic inhibitors are required to augment its synthesis. However, even under these conditions, 58-N1 serum still failed to precipitate E1B-20K. According to the predicted protein sequences, antisera against the carboxy terminus of E1B-58K should also precipitate both the E1B-58K and 20K proteins. Although the antisera raised against synthetic peptides corresponding to the carboxy terminus of E1B 58K prepared by Green and coworkers (1983) and 58-C1 used in the present study were capable of precipitating the E1B 58K protein, none of them were active against the 20K polypeptide. It is not known why this is so. One possible explanation is that the proposed amino acid sequence of E1B-20K is incorrect at the carboxy terminus. It is also possible that the protein

conformations in the carboxy terminal half of the 58K and 20K species are different.

The 58-N1 and 58-C1 antipeptide sera were also used to analyse the E1B-58K protein synthesized by group II host-range mutants. These mutants have been mapped to sequences of the E1B 2.2kb message (Galos et al., 1980) and they are defective in the production of E1B 58K protein (Lassam et al., 1979a, b). Although these mutants fail to induce transformation in most rodent cells (Graham et al., 1978), DNA extracted from mutants virions can transform both rat and hamster cells in vitro (Rowe and Graham, 1983). These results suggested that the 58K protein is required for initiation but not for maintenance of transformation. The hr mutations were generated by chemical mutagenesis (Harrison et al., 1978) and probably represent single base changes which could result either in single amino acid differences or, in the case of the generation of an abnormal termination codon, in truncated forms of the protein. Thus it remains possible that the amino terminal region of a 58K-related polypeptide could play a role in transformation. The 58K-specific antipeptide sera (in particular 58-N1) were used to search for aberrent 58K-related polypeptides generated from group II mutants. The results obtained using 58-N1 and 58-C1 antipeptide sera and 14B antitumour serum indicated

that no E1B 58K protein or related products were detected in cells infected with hr6, 7, and 50. In no case was a truncated form of 58K observed using 58-N1 or the other two sera. A viral polypeptide which co-migrated with 58K was precipitated in hr51-infected cells using all three sera. These data confirmed previous findings that except for hr51 all of these mutants are defective in the synthesis of the 58K protein (Lassam et al., 1979a, b). The exact nature of the mutations and the reasons for the failure of hr6, 7, and 50 to synthesize any detectable 58K-related protein remain unclear. The pattern of early cytoplasmic RNA derived from E1B of hr7 has been studied by S1 nuclease analysis and it was found to be indistinguishable from that of wt virus (Berk et al., 1979). Thus it is still possible that aberrent forms of the 58K protein are synthesized in cells infected with the mutant viruses but that they are extremely short-lived or, for reasons similar to those discussed above for hr1, that they are not recognized by the 58-N1 serum.

Because the antipeptide sera described in this thesis are highly specific for E1A or E1B 58K polypeptides, they have been extremely useful in studying the cellular location of these viral proteins by indirect immunofluorescence. The results showed

that at early times after lytic infection, the E1B-58K protein, as detected by 58-C1, was found predominantly in the perinuclear region of the cytoplasm and to some extent within the nucleus. The E1A proteins, as detected by E1A-C1 and E1A-N1 sera were found in discrete patches within the nucleus and in diffuse areas of the cytoplasm. Using mutants pm975 and hr1 it has been found that the 1.1 kb mRNA products are primarily located within the nucleus, whereas the 0.9 kb mRNA products are detected in diffuse area in the cytoplasm (data not shown; R. Ross and Branton, unpublished results). However, in the present study it was not possible to determine the location of each of the individual E1A polypeptide species. With the appropriate monoclonal antibodies which react with individual E1A protein species it may at some point in the future be possible to do these types experiments. Results obtained from cell fractionation studies (Rowe et al., 1983b) were in general agreement with the present immunofluorescence data. Further cell fractionation studies using E1A-C1 serum showed that nuclei contained predominantly only the 48.5 and 45K E1A polypeptides, whereas all four major species were detected in the cytoplasm (Branton et al., 1984). This type of experiment is hard to interpret because it is difficult to role out the possibility that large

amounts of E1A proteins were lost from nuclei during cell fractionation. Using antiserum against the E1A 1.1 kb mRNA products, Feldman and Nevins (1983a) also demonstrated that these E1A proteins are located predominantly in the nucleus. Thus, both the 58K protein and the E1A proteins may function, at least in part, in or near the nucleus. Whether the presence of protein in the cytoplasm represents newly-synthesized species in transit to the nucleus, or whether it indicates that the proteins may be partially located and function in the cytoplasm is not clear. However, recent results showed that when purified E1A polypeptides produced in E. coli expressing a cloned cDNA copy of the 1.1 kb mRNA are microinjected into the cytoplasm of mammalian cells, E1A protein is rapidly taken up and localized in the nucleus (Ferguson et al., 1984; Krippl et al., 1984). The presence of E1A proteins in the nucleus is consistent with the fact that these proteins apparently play an important role in the regulation of gene expression.

The antipeptide sera were also employed to purify E1 proteins using immunoaffinity chromatography. Since the antisera were raised against synthetic peptides they should possess a higher affinity towards the corresponding peptides than to the complete E1 proteins. Thus it was possible, at least in some cases,

to elute the antigens from the antibody-antigen complexes by incubation with homologous peptide in isotonic solution at neutral pH. This approach presents several advantages over more traditional purification regimes. Since no chaotropic agents or extreme pH are involved, the protein recovered should generally retain biological activity. The release of antigens should be specific for the protein because the sequence present in the synthetic peptide. Thus most of the contaminants present in the immunoprecipitates should remain bound to the column resulting in a substantial purification over a short period of time in a single step. In the present study over a 3,000-fold purification of 58K protein was obtained in about 4 to 5 hours. This technique can be used sequentially. For example a protein purified through the use of one immunoaffinity absorbant can be further purified by a second immunoaffinity column using antibodies directed against a different region of the same protein. This approach will eliminate crossreacting proteins that are present after the first purification. Since this procedure is fast and simple, it is useful for the purification of labile proteins, especially in a situation where multiple samples, such as wild type and mutant proteins, are being purified and analysed at the same time. Unlike conventional methods, this procedure

allows the purification of proteins in a small scale. In the present study as little as 10^6 cells were used for the purification of E1 proteins. For large amounts of protein it should be possible to employ immunoaffinity columns of large capacity. It is difficult generally to purify proteins that are present at low concentrations using conventional methods.

There are also several drawbacks of this purification procedure. The recovery of E1 proteins was relatively low. However, the present study was carried out to develop a purification procedure for the viral proteins and experiments were carried out to economize on peptide use. For more efficient recovery or large scale of purifications excess antibody should be used to precipitate all of the viral proteins from infected or transformed cells. Large amounts of synthetic peptide would then be needed to release the viral proteins from the antibody, however, for routine use the peptide could be recovered by a variety of methods and then reused. Under these conditions the recovery of viral proteins should be equal to the amount of viral proteins released from the antigen-antibody complexes. However, not all synthetic peptides were capable of eluting the E1 proteins from their corresponding antibody-antigen complexes. For example the synthetic peptides E1A-N and 58-N corresponding to the

amino-termini of E1A products and 58K failed to elute the E1 proteins. Nevertheless, the E1A-N and 58-N1 sera were useful in a two-step purification in the present study and over a 15,000- and 4,000-fold purification of E1A proteins and 58K protein were obtained, respectively. These E1 proteins could then potentially be eluted off the antibody-antigen complexes using conventional methods such as chaotropic agents or low pH for further study. Ionic detergents were generally required for the elution of antigen. It is possible that the presence of these detergents could cause a mild denaturating effect on the antibody-antigen complexes such that the antibody binding site is more acessible to the synthetic peptide. It has been reported that SV 40 large T antigen bound to Sepharose by antibodies directed against a carboxy-terminal peptide can be eluted in the absence of detergents. Thus these results suggested that the conditions for release of an antigen bound to an antipeptide antibody vary with the characteristics of both the antibody and its homologous peptide (Walter et al., 1982).

Not all of the E1 protein bound to the antibody-antigen complexes was eluted by incubation with homologous peptide and amounts varying between 10 to 50% of the E1 proteins were released. The different efficiencies in releasing the proteins could be due to

the different affinities of antibodies from different batches of sera. To achieve a more consistent and efficient elution of the antigen, antipeptide antibody could be purified according to its affinity to the synthetic peptide. This can be done by preparing a synthetic peptide-Sepharose column and allowing the corresponding antipeptide antibody to bind to the column. The antibody can then be eluted stepwise from the column by agents with increasing stringency such as increasing salt concentration or decreasing pH. Thus a population of antibody molecules with a homogeneous affinity against the peptide could be isolated and tested for its suitability in protein purification. Theoretically one would want an antibody with high affinity to the peptide and a somewhat lower affinity to the complete protein.

It has been shown that protein kinase activity is present in immunoprecipitates from Ad5-infected cells containing E1A proteins (Branton et al., 1984; Rowe and Branton, personal communication) and E1B 58K protein (Lassam et al., 1979; Branton et al., 1981). It is still not clear whether the kinase activity is intrinsic to the E1 proteins or to a cellular enzyme bound to the immunoprecipitates. These issues could be resolved through the production of highly purified preparations of E1 proteins, the use of E1 protein

produced by in vitro translation of E1-specific mRNA, or the expression of E1 proteins from Ad5 E1 genes cloned into bacteria. In the present study E1 proteins were purified and the kinase activities were examined. Various conventional methods have been employed to purify the 58K protein. While none of them led to a high degree of purification, nevertheless in all cases protein kinase activity remained associated with the 58K protein. It is still not clear whether the kinase activity is intrinsic to 58K because of the low degree of purity in these preparations. An observation of considerable interest is that when extracts from Ad5-transformed 983-2 cell were analysed on glycerol gradients, most of the 58K protein was present in a complex rather than as monomeric 58K molecules. At present it is not known if this complex is comprised of multimers of 58K protein, of 58K molecules bound to other proteins, or both. Sarnow and co-workers (1982) reported that Ad5 E1B 58K protein appears to be associated with the p53 cellular non-viral T antigen in Ad5-transformed mouse cells, but little else is known concerning these complexes. Of considerable importance was the observation that the majority of the kinase activity from these glycerol gradient fractions precipitated by 14B antitumour serum was from the 58K protein monomer region. These results suggest that the

kinase activity may be intrinsic to the monomeric 58K protein or some other protein sedimenting in glycerol gradients in the same position. In addition, the activities present in the positions of 58K monomers and complexes could be due to two different protein kinases which co-purify with 58K.

As described in this thesis E1A proteins and E1B-58K were purified using the antipeptide sera in a two-step purification. Attempts were made to monitor protein kinase activity throughout the entire process. The viral polypeptides were eluted from C-terminal antibody precipitates using appropriate synthetic peptides and then reprecipitated with N-terminal antisera. A state of 15,000- and 4,350-fold purification was obtained for E1A proteins and 58K, respectively. In control experiments, purifications were carried out without using any synthetic peptides to elute the viral polypeptides. All the E1A proteins and 58K remained bound to the precipitates prepared using C-terminal antibody and none of these viral polypeptides were detected by immunoprecipitating this material with N-terminal antibody (fig. 39). A similar low level of kinase activity was detected both in the purified E1A proteins and 58K samples as well as in the corresponding controls (data not shown). This low level probably represented background activity due to a

contaminating kinase(s) from the histone substrate (Branton et al., 1981). Thus it seems that little kinase activity could be specifically ascribed to the E1A proteins or 58K. As described in Chapter 6, the elution of viral proteins from immunoprecipitates using synthetic peptides was rather inefficient and a large portion of viral proteins remained bound to the immunoprecipitates (fig. 39 and 40). The kinase activity was also monitored in these samples. A high level of kinase activity was detected as compared to the purified samples. However, a similar high level of kinase was detected in precipitates to which no synthetic peptide had been added (data not shown). Thus it was not possible to demonstrate a direct correlation between the level of kinase activity and the amount of E1 proteins present. Furthermore, a number of the contaminating cellular proteins were also heavily phosphorylated in all of these samples. Thus it is still not clear whether the kinase activity is intrinsic to the viral polypeptides or if it resulted from cellular enzymes trapped in the precipitates. It is not certain if employing the present approach alone will be able to differentiate these possibilities. As E1A proteins appear to bind specifically to several host cell proteins, it is extremely difficult to purify E1 products to homogeneity. Furthermore, the background

level of kinase activity present in the histone substrate also interferes with the interpretation of the data. Finally, it would be ideal to assay the kinase activity in E1 products that are free of the antibody. An attempt has been made to assay the kinase activity from the material eluted from the first precipitates using synthetic peptides. However, no kinase activity was detected (data not shown) and it is possible that inactivation by the ionic detergents present in the supernatant was responsible. Other methods such as the expression of E1 proteins from E1 genes cloned into bacteria might be more useful in resolving this question. Malette et al. (1983) demonstrated that the patterns of 58K phosphopeptides obtained in vivo and in vitro are different and thus suggested that the 58K-associated kinase detected in vitro does not mimic the phosphorylation of 58K in vivo. Furthermore, both E1A proteins and 58K protein failed to bind ATP using a photoaffinity probe (Branton et al., 1984). These data suggested that the E1 proteins are not ATP-binding proteins. Thus these results, taken together, tend to argue that kinase activity is not intrinsic to the E1 proteins.

In summary, the studies presented in this thesis were the first to employ antisera to synthetic peptides in the analysis of adenovirus proteins, and were among

the earliest in general using this technique. They have provided immunological tools that have proved and should continue to prove extremely useful in the study of the structure and functions of human adenovirus type 5.

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