STUDIES ON TUMOR ANTIGENS OF ADENOVIRUS TYPE 5

By

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ABSTRACT

The polypeptides specified by the transforming region of the Adenovirus 5 (Ad 5) genome have been studied by immunoprecipitating antigens (using the double antibody and protein A-Sepharose methods) from cells transformed by Ad 5 and from cells infected with Ad 5 wild type (wt) or host range mutants. Three different antisera were used: P antiserum specific for early viral products (Russel et al, 1967) and two hamster tumor antisera. With both the double antibody and protein A-Sepharose methods, all three antisera immunoprecipitated a 58,000 dalton polypeptide from wt-infected KB cells, while P antiserum precipitated additional polypeptides of molecular weight 72,000, 67,000 and 44,000. When the double antibody method was used, P antiserum and the hamster tumor antisera also immunoprecipitated a 10,500 dalton protein which was not observed with the protein A-Sepharose method. The Ad 5 hr mutants fall into two complementation groups designated I and II, both of which exhibit defective transformation activity (Harrison et al, 1977; Graham et al, 1979). In group I mutant infected cells, little 10,500 dalton protein was observed relative to that found with wt infected cells, whereas very little or no 58,000 dalton polypeptide was immunoprecipitated from cells infected with mutants from complementation group II. Since a 58,000 dalton antigen was also found in a number of Ad 5 transformed cell lines, but was not detected in the cells infected with transformation

defective group II mutants, it was concluded that the 58,000 dalton protein may be involved in the induction and/or maintenance of transformation.

Collett and Erikson (1978) have reported that the transformation gene product of Rous sarcoma virus is associated with protein kinase activity. Studies were undertaken to determine whether similar activity was also associated with the tumor antigens of Ad 5. The products immunoprecipitated from wt Ad 5 infected cells using tumor antisera were found to catalyse the transfer of ^{32}P from (γ-^{32}P) ATP into protein. Very little of this activity was observed with extracts from mock infected cells or when non-immune sera were used. The Ad 5 hr mutants were found to induce less protein kinase activity than wt virus. Evidence is presented suggesting that this activity is associated with early viral functions. Protein kinase activity was also detected in immunoprecipitates from Ad 5-transformed rat cells.

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ABBREVIATIONS

Ad 5	Adenovirus 5
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
HEK	human embryonic kidney
hr	host range
MES	(morpholino)ethanesulfonic acid
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
PBS	phosphate-buffered saline
PFU	plaque forming units
RNA	ribonucleic acid
RSV	Rous sarcoma virus
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid

THESIS-RELATED PUBLICATIONS

1. Lassam, N.J., Bayley, S.T. and Graham, F.L. (1978). Synthesis of DNA, late polypeptides and infectious virus by host-range mutants of Adenovirus 5 in nonpermissive cells. Virology 87: 463.

2. Lassam, N.J., Bayley, S.T., Graham, F.L. and Branton, P.E. (1979) Immunoprecipitation of protein kinase activity from Adenovirus 5-infected cells using antiserum directed against tumor antigens. Nature 277: 241.

3. Lassam, N.J., Bayley, S.T. and Graham, F.L. (1979). Transforming proteins of human Adenivirus type 5: Studies with infected and transformed cells. Cold Spring Harbour Symp. Quant. Biol. 44:477.

4. Branton, P.E., Lassam, N.J., Graham, F.L. and Bayley, S.T. (1979). T-antigen-related protein kinase activity in cells infected and transformed by human Adenoviruses 5 and 12. Cold Spring Harbour Symp. Quant. Biol. 44:487.

5. Lassam, N.J., Bayley, S.T. and Graham, F.L. (1979). Tumor antigens of Ad 5 in transformed cells and in cells infected with transformation defective host-range mutants. Cell 18:781.

6. Branton, P.E., Lassam, N.J., Downey, J.F., Siu-Pok, Y., Graham, F.L., Mak, S. and Bayley, S.T. Protein kinase activity immunoprecipitated from Adenovirus infected cells by sera from tumor-bearing hamsters. (in press).

INTRODUCTION

Adenovirus

The name Adenovirus (Enders et al, 1956) denotes a group of DNA viruses which have been isolated from a large variety of mammalian and avian species. Presently, more than 86 different adenovirus serotypes have been isolated, of which the majority show a close structural relationship (Ginsberg, 1979). In humans, most adenoviruses are found associated with respiratory infections, but some multiply in the intestinal tract (Fenner et al, 1974). Of the 33 human adenovirus serotypes presently known, the closely related types 2 and 5 are the best characterized. Unless otherwise stated, information presented in this introduction pertains to these two serotypes.

Structure and Composition of the Virion

The virions of adenoviruses have a highly characteristic icosahedral shape about 70 nm in diameter (reviewed by Philipson and Lindberg, 1974; Philipson et al, 1975). The virus lacks a lipid membrane and is composed of an inner DNA-protein core surrounded by a protein capsid. The capsid is composed of three major structural proteins arranged into 252 capsomeres, 240 of which have 6 neighbours and are called hexons, while the remaining 12 have 5 neighbours and are referred to as

pentons (Ginsberg et al, 1966). The virally coded capsid polypeptides can be removed by treating virions with acetone or formamide leaving a DNA-protein core (Valentine and Pereira, 1965). Of the three core proteins, two are virally coded arginine rich polypeptides (Russel et al, 1971; Laver, 1970; Lewis et al, 1975). The third core protein is found covalently associated with the 5' ends of the virion nucleic acid (Robinson and Bellet, 1974; Rekosh et al, 1977) and might be coded by a cellular gene (Green et al, 1979). The viral polypeptides and their most likely positions in the virion are shown in figure 1.

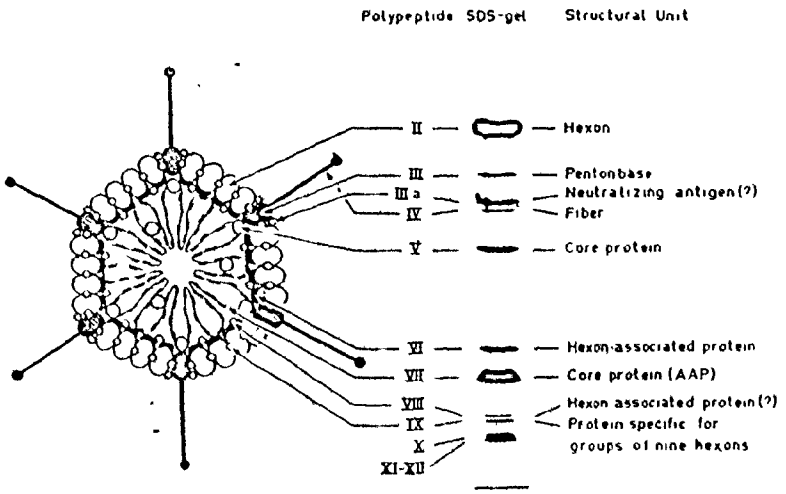


Figure 1. A model of the location of different proteins in the Ad 2 virion (from Philipson et al, 1975).

Adenovirus DNA is a linear duplex molecule with a molecular weight of about 23 x 10^6 daltons, sufficient to code for about 30 to 40 average sized proteins (Green et al, 1967; Philipson et al, 1975).

Digestion of the virion DNA with exonuclease III followed by annealing, does not generate circular molecules, indicating that viral DNA lacks terminal redundancies (Green et al, 1967). Instead, each strand of the viral DNA contains inverted terminal repetitions allowing denatured DNA strands to self-anneal into circular single-stranded molecules (Garon et al, 1972; Wolfson and Dressler, 1972). The length of the Ad 5 terminal repeat is 103 base pairs (Steenberg et al, 1977) while that of Ad 2 is 102 base pairs (Arrand and Roberts, cited by Winnacker, 1978). It is widely believed that these inverted terminal repetitions are involved in the replication of Adenovirus DNA but at present their actual function is unknown.

Virus-Cell Interactions

Adenovirus infection of a susceptible host leads to either of two cellular responses. If the host cell is permissive, infection proceeds through the viral replicative, or lytic, cycle resulting in cell death and release of progeny virus. During infection of non-permissive or semi-permissive hosts, some stage of viral replication is blocked and few, if any, new virions are released. Instead, a very small percentage of such infected cells may become virally transformed.

A. Productive infection

Lytic infection of human cells by adenovirus proceeds by an ordered expression of viral genes. Classically, two distinct phases

of gene expression have been described. The early phase is defined as including those events prior to viral DNA replication. During this phase, the viral genes required for cell transformation and viral DNA replication are expressed. The onset of the late phase begins with synthesis of viral DNA and structural polypeptides and proceeds until cell lysis.

During the early phase of infection, the virus absorbs to receptors on the plasma membrane of the host cell, enters into the cell and is rapidly uncoated (Philipson et al, 1968; Lonberg-Holm and Philipson, 1969). The DNA enters the nucleus and within 1 to 2 hours after infection virus-specified RNA is found in infected cells. Most viral mRNA is transcribed by the host RNA polymerase II (Price and Penman, 1972) and transported after post-transcriptional modifications to the cytoplasm. These modifications include splicing of the mRNA and capping of its 5' end, as well as polyadenylation of the 3' end of the messenger RNA (Berk and Sharp, 1978; Philipson et al, 1971; Lindberg and Persson, 1972; Hashimoto and Green, 1976; Gelinas and Roberts, 1977). Early mRNA sequences found in the cytoplasm are transcribed from both strands of the viral DNA and are complementary to about 30% of the viral genome (Petterson et al, 1976; Wold et al, 1977). Specific DNA fragments of the adenovirus genome produced by digestion with various restriction endonucleases have been used as probes for detailed transcription mapping (e.g. Petterson et al, 1976; Flint et al, 1975; Berk and Sharp, 1977; 1978; Galos et al, 1979). In addition, electronmicroscopy studies using the "R loop technique" have provided high resolution maps of the early viral genes (Chow et al, 1977). These investigations have defined the early

mRNA as mapping at five non-contiguous regions of the viral genome, referred to as early gene blocks (see figure 2).

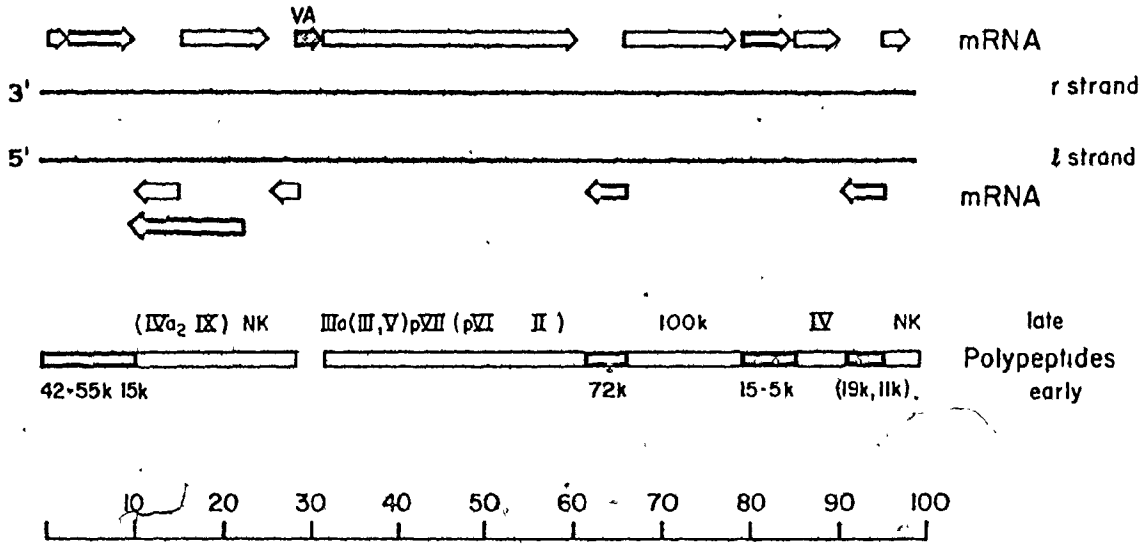


Figure 2. A map of the Ad 2 genome (from Flint, 1977). Solid lines indicate early viral gene products; open areas show late products.

Blocks 1 and 3 are located in the r strand between map units 1 to 11 and 76 to 86 respectively. The l strand early gene blocks 2 and 4 are found between map units 62 to 68 and 92 to 89 respectively. Galos et al (1979) have identified an additional l strand early gene sequence lying between 11 and 23 map units. The viral DNA strands have been designated as r or l to indicate the direction of transcription: the strand transcribed from left to right is called the r strand, whereas

its complement, the l strand, is transcribed from right to left.

The data presently available suggest that early after infection, two families of mRNA are transcribed from early region 1. One group of mRNA's is specified by the viral DNA sequences between 1.5 to 4.4 map units (region E1A) whereas the second family appears to be coded from the viral DNA sequences between 4.5 and 11.1 map units (region E1B) (van der Eb et al, 1979; Spector et al, 1978, 1979; Chow et al, 1977; Berk and Sharp, 1977, 1978). In good agreement with this arrangement of early region 1, Wilson et al (1979) have identified two independent early transcriptional promoters located on the viral DNA at about map positions 1.5 and 5.0. A third transcriptional promoter is apparently functional only at late times and specifies the late viral structural protein designated IX (Spector et al, 1978, 1979; Petterson and Mathews, 1977). The pattern of mRNA's specified by early regions 2, 3 and 4 appears to be less complex than that found with early region 1. Each of these early gene blocks contains a single transcriptional promoter and encodes a single family of mRNA's (Evans et al, 1977; Berk and Sharp, 1977, 1978; Chow et al, 1977).

A number of workers have detected Ad 2 or Ad 5 induced early proteins using SDS-polyacrylamide gel electrophoresis to compare in vivo labelled extracts of mock and infected cells (Harter et al, 1976; Saborio and Oberg, 1976; Neuwald et al, 1977; Chin and Maizel, 1976; Harter and Lewis, 1978). Since host protein synthesis is not shut off early in infection, a variety of methods have been used to facilitate detection of early proteins against the high background of host protein synthesis. Harter et al (1976), pretreated infected cells with cyclohexamide, while

Chinnadurai et al (1977) labelled cells in hypertonic medium. Both these treatments enhance the synthesis of early adenovirus proteins relative to host protein synthesis. Saborio and Oberg (1976) used antisera directed against Ad 5 infected cells to selectively immunoprecipitate early viral proteins from extracts of infected cells. In general, these studies show that a relatively large number of proteins are induced early after adenovirus infection. For example, Saborio and Oberg (1976) detected 14 additional polypeptides in extracts of Ad 2 infected HeLa cells which were not found in the mock infected extracts. While there is presently no precise consensus regarding the exact molecular weights, most of these studies report finding a major polypeptide of about 72,000 daltons, several proteins between 38,000 and 50,000 daltons, two proteins of approximately 25,000 and 21,000 daltons and a number of proteins between 10,000 and 19,000 daltons.

Early viral polypeptides have also been identified using cell-free protein synthesizing systems. Using RNA extracted from cells early after infection, Saborio and Oberg synthesized in vitro 15 early viral-induced polypeptides, ranging in molecular weight from 10,500 to 72,000. Lupker et al (1977), have translated viral coded proteins in vitro using as template early viral mRNA selected by hybridization to restriction endonuclease fragments of the Ad 5 genome. Lupker and co-workers found the leftmost 11% of the viral genome (early gene block 1) to code for a 14,000 dalton protein and four related polypeptides of molecular weight 38,000 to 51,000. RNA selected by a viral DNA fragment containing early gene block II (map units 62 to 68) coded for a 72,000 dalton polypeptide, whereas the rightmost 26% of the viral genome was found to encode

an early 12,000 dalton protein. In more detailed studies, Lewis et al (1976) and Harter and Lewis (1978) found early gene block I to code for a 15,000 dalton protein and several related proteins of 40,000 to 50,000 daltons, early gene block 2 to encode a 72,000 dalton protein, the viral DNA sequences between map units 76 and 86 (early region 3) to code for a 14,000 dalton protein and early region 4 (92 to 98 map units) to encode three proteins of molecular weight 11,000, 19,000 and 21,000. Using similar methods, Halbert et al (1979) concluded that region E1 encoded 6 polypeptides of molecular weight 53,000, 52,000, 47,000, 41,000, 35,000 and 15,000. The early translation map obtained by Lewis and co-workers is shown in figure 2.

Of the early virus-induced polypeptides described above, only the 72,000 dalton protein has been well characterized. This viral coded polypeptide is a phosphoprotein and unlike some other early proteins is synthesized in relatively large amounts both early and late after infection (Lewis et al, 1976; Levinson et al, 1977; Neuwald et al, 1977). In addition, the 72,000 dalton protein binds specifically to single-stranded DNA (van der Vliet and Levine, 1973; van der Vliet et al, 1975). The function of the 72,000 dalton protein has been investigated using an Ad 5 temperature-sensitive mutant; H5ts125, which contains a lesion in the structural gene coding for this polypeptide. At the non-permissive temperature, the 72,000 dalton protein synthesized in H5ts-125 infected cells fails to bind to viral DNA (van der Vliet et al, 1975). Temperature-shift experiments with H5ts125 suggest that the single-stranded DNA binding protein is required for initiation of viral DNA synthesis

(van der Vliet, 1977). The function of other early viral gene products is presently unknown.

During the late phase of productive infection, viral DNA synthesis begins and additional transcriptional units are expressed. Messenger RNA isolated late in infection contains sequences complementary to almost all of the viral genome (Tibbitts et al, 1974), including the five early gene blocks. However, transcription of some genes encoding early mRNA sequences is "shut off" at late times, suggesting that RNA's from these genes persist in the cell late after infection (Chinnaderai et al, 1976). The viral DNA sequences expressed as mRNA only late after infection are shown in figure 2. At least 22 virus-specific polypeptides have been detected in cells late after infection (Anderson et al, 1973). The viral proteins synthesized during this phase of infection are largely structural, destined for assembly in the nucleus into virion particles. Translation in vitro of mRNA selected by hybridization to viral DNA restriction enconuclease fragments has allowed direct mapping of most late proteins (Lewis et al, 1975) and is also shown in figure 2.

Adenovirus transformation

Human adenoviruses have been placed in four groups, A, B, C and D, according to their ability to induce tumors when injected into newborn rodents and serological activity (Green et al, 1970; McAllister et al, 1969). Viruses of group A are highly oncogenic whereas group B adenoviruses are weakly oncogenic; those of groups C and D are non-onco-

genic. Subgroup D viruses are distinguished by the fact that they fail to induce a tumor antigen common to all other groups (McAllister, 1969).

While only members of two adenovirus subgroups possess oncogenic potential, members of all human adenovirus subgroups can transform rodent cells *in vitro* (McBride and Weiner, 1964; McAllister *et al*, 1969, Green *et al*, 1970). In general, adenoviruses transform cells that are non-permissive (or semi-permissive) for viral replication. For example, the group C adenovirus 2 and 5 do not efficiently transform hamster cells which are permissive hosts for both these serotypes (Lewis *et al*, 1974; Williams, 1973). Similarly, adenovirus 12 (a member of the oncogenic group A adenoviruses) efficiently transforms hamster cells which are non-permissive for replication of Ad 12 (Strohl *et al*, 1967). It should be noted however, that cells permissive for adenoviruses can be transformed; for example, Graham *et al* (1974, 1977) transformed human embryonic kidney cells by transfection with DNA fragments obtained from Ad 5 and Williams *et al* (1973, 1974) has transformed hamster cells using temperature-sensitive mutants of Ad 5.

Several lines of evidence indicate that only a small portion of the viral genome is involved in the induction and maintenance of transformation. Using restriction endonuclease fragments of the Ad 5 genome to transform primary rat kidney cells, Graham *et al* (1974) found the DNA fragment comprising the leftmost 8.0% of the viral genome (Hin D III G fragment) sufficient to initiate and maintain transformation. Further, Gallimore *et al* (1974), and Flint *et al*, (1976) have characterized the viral DNA in a number of adenovirus 2 and 5 transformed rodent cell lines, finding that only the DNA sequences corresponding to the

leftmost 10 to 12% of the viral genome were common to all transformed cell lines examined. In agreement with these findings, Flint et al (1975, 1976) have examined the transcription of viral DNA in the same series of Ad 2 and Ad 5 transformed cell lines. Viral mRNA corresponding to the leftmost 12% of the viral genome was expressed in all transformed rodent cell lines analysed. These observations have been extended by Berk et al (1979) who found both Ad 5 infected and transformed cells to express the same family of mRNA's from region E1. Hence, of the five regions of the viral genome expressed early during lytic infection, only the leftmost region (early region 1) contains the DNA sequences specifying the viral transformation functions.

Identification of the viral polypeptides encoded by the transforming region of viral genome has been the subject of a number of studies. As mentioned above, Lupker et al (1978), Lewis et al (1976) and Harter and Lewis (1978) have shown by cell-free translation that the left 11% of the viral genome encodes several polypeptides of about 38,000 to 51,000 daltons and a smaller polypeptide of approximate molecular weight 15,000. Immunoprecipitation of labelled proteins has also been used to identify the proteins specified by early gene block 1. Gilead et al (1976) used antiserum produced against tumors of an Ad 2-transformed cell line (F17) to immunoprecipitate extracts of HeLa cells early after infection. Since the F17 transformed rat cell line contains only the leftmost 14% of the viral genome (Gallimore et al, 1974), the F17 antiserum should be specific for only those viral polypeptides encoded by the transforming region of the viral DNA. This antiserum was found to bind two early adenovirus-induced polypeptides of 53,000 and

15,000 daltons. In similar studies, Levinson and Levine (1977a, b) and Ross et al (1978) used antitumor sera generated against hamster tumors induced by Ad-transformed 14b cells to immunoprecipitate a 58,000 dalton protein from Ad 5 infected and transformed cells. The 14b hamster cell line expresses only the viral mRNA corresponding to the DNA sequences lying within the leftmost 11% of the viral genome (Flint et al, 1976). In more recent studies, Wold and Green (1979) used a variety of antisera to immunoprecipitate proteins from adenovirus 2-infected cells. Antisera directed against cell lines expressing all of early gene block 1 immunoprecipitated several polypeptides of molecular weight 53,000, 28,000, 18,000, 15,000, 14,500 and 12,000.

Analysis of polypeptides induced early after Adenovirus 5 infection.

Two central objectives underlie much of the research in the field of tumor virology. These goals are firstly, to identify the viral proteins involved in initiation and maintenance of transformation, and secondly, to determine the mechanisms by which such polypeptides induce transformation. The work presented in this thesis has been directed towards both these objectives.

A. Identification of Ad 5 transformation proteins.

To facilitate identification of the polypeptides specified by the transforming region of the Ad 5 genome, host range (hr) mutants of

Ad 5 were used. These mutants were isolated on the basis of their ability to replicate in the Ad 5-transformed human embryonic cell line 293 but not in HeLa or KB cells (Harrison et al, 1977). The 293 cell line, developed by Graham et al (1977), contains and expresses as mRNA the viral DNA sequences contained within early gene block 1 (Graham et al, 1977; Aiello et al, 1979). In addition, these transformed human embryonic kidney cells express adenovirus tumor antigen as detected by indirect immunofluorescence. (Graham et al, 1977, 1978). Since the 293 cells contain and express the leftmost region of the viral genome, it was expected that the host range mutants would be defective in those functions specified by the viral transformation genes.

The nine host range mutants isolated to date fall into two complementation groups, designated I and II, both distinct from complementation groups defined by the temperature sensitive mutants of Ad 5 (Harrison et al, 1977). Recombinational analysis indicates that the group I lesions map within the viral DNA sequences between 0 and 4.5 map units (region E1A) and that the group II mutants map in the region containing the viral DNA sequences between 6.1 and 9.0 map units (Frost and Williams, 1978; Galos and Williams, personal communication). Group I mutants are defective in viral DNA replication and fail to transform rat embryo brain cells but will abnormally transform baby rat kidney cells. In contrast, the group II mutants synthesize viral DNA but do not transform rat cells (Lassam et al, 1978; Graham et al, 1978). Interestingly, human embryonic kidney cells as well as 293 cells fully complement the group II defect (Harrison et al, 1977).

The problem of identifying adenovirus transformation proteins was approached by comparing those polypeptides synthesized early after infection with wild type Ad 5, which is transformation competent, to those induced by the transformation defective hr mutants. The demonstration of differences between the proteins induced early after infection with wild type and hr mutants would help to define those polypeptides involved in the viral transformation process. In addition, identification of such virally induced proteins in cells transformed by Ad 5 would provide further evidence for the association between specific viral polypeptides and the induction and maintenance of transformation.

Since the hr mutants were used throughout this study, adequate characterization of these mutants was a prerequisite. The synthesis of late viral proteins was examined in both hr mutant and wt infected cells to detect host range defects during the late phase of infection.

B. Function of the Ad 5 transformation proteins.

The mechanism by which adenovirus transformation proteins induce and maintain transformation is presently unknown, however, it is possible that these proteins function like the transformation proteins encoded by other tumor viruses. With Rous sarcoma virus transformation requires the expression of the src gene. As shown by Collett and Erikson (1978) the polypeptide specified by the src gene is associated with protein kinase activity. Protein kinases are enzymes found in all eukaryotic cells which catalyse the phosphorylation of some acceptor amino acids. This reaction

is of particular importance since phosphorylation regulates the activity of many mammalian enzymes (Greengard, 1978). A well known example is that of phosphorylase kinase. This enzyme, when itself activated by phosphorylation, catalyses the transfer of phosphate from ATP to phosphorylase B, an enzyme which catalyses the cleavage of glycogen to glucose 1 phosphate. In addition to enzymatic modification, the biological activity of a variety of other cellular proteins is also affected by phosphorylation. These polypeptides include histones and ribosomal and membrane proteins (Rubin and Rosen, 1975). Since many cellular activities are regulated by phosphorylation the finding that a viral transformation protein is associated with protein kinase activity is of significance. Viral transformation could result from changes in the pattern of cellular phosphorylation induced by virus associated protein kinase activity. The work presented here suggests that, like Rous sarcoma virus, products encoded by the transformation region of the Ad 5 genome may also be associated with protein kinase activity.

MATERIALS AND METHODS

I. Cells

A. Cell lines

The human KB cells used for propagation and assay of adenovirus 5 (Ad 5) were originally obtained from Dr. A. Rainbow. A number of Ad 5 transformed cells were also used in this study. Rat cell lines 637-C3 and 822-C2 were transformed by infection with wild type (wt) Ad 5 while 424-C1 was derived from baby rat kidney cells transfected with sheared Ad 5 DNA. The 424-C1 line has been shown by Visser et al (1979), to contain DNA corresponding to the entire viral genome whereas the 637-C3 line contains 25-30% of the leftmost end of the viral DNA (Martha Ruben, personal communication). The 512-C8, 889-C1 and 889-C3 rat cell lines were transformed with a viral restriction endonuclease fragment representing the left 8.0% of the Ad 5 genome (Graham et al, 1974). Rat cell lines 637-1 and 637-4 were transformed by Ad 5 group I host range mutants, hr 3 and hr 1 respectively (Graham et al, 1978). The 14b cell line was obtained by infecting hamster embryo cells at the restrictive temperature with the ts 14 mutant of Ad 5 (Williams et al, 1974). This hamster cell line contains 40% of the left end of the Ad 5 molecule and expresses only the mRNA corresponding to the sequences from the leftmost 10% of the viral genome (Flint et al, 1976). The transformed human 293 cell line was derived from human embryonic kidney cells

transfected with sheared Ad 5 DNA using the calcium technique (Graham et al, 1974, 1977). These cells contain and transcribe the left 10-12% of the Ad 5 genome (Graham et al, 1977; Aiello et al, 1979). The 14b cells were kindly provided by Dr. J.F. Williams; other Ad 5 transformed cell lines were obtained from Dr. F. Graham. Information on the Ad 5 transformed cell lines used in this study is summarized in Table 1.

B. Cell Culture

1.) KB and HeLa cells. Monolayer cultures of human KB and HeLa cells were grown in a modified minimal essential medium (αMEM, Flow Laboratories), supplemented with 5% horse serum or 10% fetal calf serum (GIBCO), 50 units/ml mycostatin (E.R. Squibb), 1.21 mg/ml penicillin G and 100 ug/ml streptomycin (GIBCO). Cell monolayers were subcultured by trypsinization: cells were dislodged from the glass substrate using 1 ml of 1 x trypsin-EDTA solution (GIBCO) and the resulting cell suspension was seeded with fresh media into sterile 32 oz screw-cap glass bottles. The cells were grown in an atmosphere of 5% CO_2 and 95% air at 37°C.

Suspension cultures of KB cells required for virus purification and mRNA isolation were maintained at 37°C in suspension medium (Joklik's modified suspension medium, GIBCO) supplemented with 5% horse serum. The cell density of the cultures was kept between 2.5 and 5 x 10^5 cells per ml by regular dilution with prewarmed suspension medium. The cells were kept in suspension with a magnetic stirring bar.

2). Adenovirus Transformed Cells. Adenovirus transformed cells were maintained as monolayers on 150 mm plastic petri dishes (LUX). Cells were grown in suspension medium supplemented with 10% fetal calf serum (GIBCO). Transformed rodent cells were subcultured by trypsinization. The adenovirus transformed human cell line 293 was subcultured using an isotonic buffer containing EDTA (0.5 mM EDTA, 136 mM NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM glucose).

3). Primary Cultures. Primary cultures of baby rat kidney cells were obtained from 7 day old Wistar rats. The rats were anaesthetized using ether, killed and their kidneys removed. The kidneys were then minced using scissors in a small volume of phosphate buffered saline (PBS; 136 mM NaCl, 2.5 mM KCl, 8mM Na_2HPO_4, 1.5 mM KH_2PO_4). The suspension of kidney fragments was diluted with additional prewarmed PBS and placed in a small capped vessel containing a magnetic stirring bar. Trypsin-EDTA was added and the suspension agitated for 20 minutes at 37°C. Undigested tissue fragments were then allowed to settle briefly and the supernatant containing disaggregated cells was decanted into sterile tubes. The cells were collected by centrifugation in an IEC centrifuge for 10 minutes at 1,000 x g. The resulting cell pellet was resuspended in suspension medium supplemented with 10% fetal calf serum and antibiotics and inoculated into 150 mm petri dishes. Secondary cultures were obtained by treatment with trypsin-EDTA. Both primary and secondary cultures were maintained in suspension medium supplemented as described above. Human embryonic kidney cells, kindly supplied by Dr. F. Graham, were maintained like the secondary baby rat kidney cells.

II. Virus

A. Virus Strains

The wild type strain of Adenovirus 5 (Ad 5) and the Ad 5 host range mutants used in these studies were provided by Dr. F. Graham. The isolation and properties of the Ad 5 host range (hr) mutants have been described in the introduction. Information on the Ad 5 hr mutants is also summarized in Table 2.

B. Viral Infection

1). Suspension Cultures. Suspension cultures were used for the preparation of Ad 5 wild type virus. KB cells growing in suspension (about 3 litres) were collected by a 10 minute centrifugation at 500 x g in an IEC centrifuge at 20°C. One half of the suspension medium was saved to be used later as conditioned medium. The cell pellet was resuspended in fresh Joklik's MEM at a concentration of 1 - 2 x 10^7 cells per ml, and the cells were infected by addition of purified adenovirus at a multiplicity of infection (MOI) of 5 plaque forming units (PFU) per cell. The infected cultures were then agitated at 37°C for 90 minutes. After absorption the cells were diluted to their original volume, using 50% conditioned medium and 50% fresh Joklik's MEM supplemented with 5% horse serum and the 37°C incubation continued.

2). Monolayer Cultures. In most experiments about 1 - 2 x 10^7 KB cells grown to confluency in 32 oz glass bottles were used. The monolayer

growth medium was removed and the cells were washed once with fresh Joklik's MEM. The cell monolayer was then inoculated with 0.5 ml of virus appropriately diluted in Joklik's MEM or PBS. Thirty minutes after infection, 30 ml of αMEM supplemented with 5% horse serum was added per bottle.

C. Purification of Wild Type Virus

Wild type adenovirus 5 was purified by the method of Green and Pina (1963). Adenovirus 5-infected suspension cultures of KB cells were harvested 48 hours post-infection by centrifugation in an IEC centrifuge at 500 x g for 10 minutes. The resulting supernatants were decanted and the cell pellet suspended in 50 ml of 100 mM Tris buffer (pH 8.1). The suspension was then frozen at -70°C until convenient to continue the preparation. After thawing in a 37°C water bath, the suspension was sonicated (Bisonic III, setting 30) in 7.0 ml aliquots for three 10 second bursts. An equal volume of Freon 113 (Matheson) was then added and this material was homogenized for 1.0 minute in a Sorval omnimixer (setting 7.5). The homogenate was then centrifuged in an IEC centrifuge for 2 minutes at 1,000 x g. The virus containing supernatant was removed and placed in a cold 250 ml graduated cylinder and the remaining Freon phase re-extracted twice with 20 ml of 100 mM Tris (pH 8.1) as described above. The supernatants were pooled, poured into a buret and slowly dripped into Beckman 1" x 3-1/2" ultracentrifuge tubes containing 7.0 ml of CsCl Tris buffer 1 (100 mM Tris, pH 8.1, 1.43 gm/ml CsCl). This material was then centrifuged in a Beckman type

SW-27 rotor at 50,000 x g for 90 minutes. After centrifugation, the supernatant was removed by aspiration, leaving the opalescent virus band on top of the CsCl cushion. This viral material was collected using a pipette, adjusted to a density of 1.34 gm/ml using crystalline CsCl and centrifuged in a Beckman type 65 rotor at 70,000 x g for 20 hours. After centrifugation the virus band was collected, resuspended in CsCl-Tris buffer 2 (100 mM Tris, pH 8.1, 1.34 gm/ml CsCl) and again centrifuged for 20 hours at 70,000 x g. The virus band was collected and the concentration of virus was estimated by measuring the absorbance at 260 nm. An absorbance of 1.0 at 260 nm is approximately 3.5×10^{11} particles per ml (Mak, 1971). The purified virus was diluted to about 10^{10} pfu/ml with an appropriate volume of Tris-buffered saline (136 mM NaCl, 5 mM KCl, 1.5 mM Na_2HPO_4, 12 mM glucose, 50 mM Tris, pH 7.5, 30% glycerol) and stored at -70°C. About 20 Ad 5 virus particles is equivalent to 1 pfu (I. Mak, personal communication).

III. Radiolabelling of Cells

At the appropriate time post infection, the growth medium was removed from the infected KB or HeLa monolayers (about $1 - 2 \times 10^7$ cells) and the cultures were washed twice with PBS. One ml of prewarmed methionine-free medium containing 30 uCi of ^{35}S-methionine (Amersham, specific activity 350 - 1,400 Cimmol^{-1}) was added to each bottle and the cells were incubated at 37°C. Every 30 minutes the bottles were gently rocked to evenly distribute the labelled medium. After the required labelling period, the cells were scraped from the glass surface with a rubber policeman, suspended in 10 ml of PBS, and centrifuged in an IEC centrifuge at

1,000 x g for 10 minutes. Samples were then either prepared for immunoprecipitation or for subsequent analysis by polyacrylamide gel electrophoresis. In the latter case, the cell pellet was suspended in 100 µl of sterile water, sonicated, and 100 µl of TSM buffer (100 mM Tris, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$) containing 50 ug/ml of DNase was added. After incubating for 30 minutes at 37°C the samples were solubilized by addition of 200 µl of 2 x electrophoresis sample buffer (1.25 M Tris, pH 6.8, 20% glycerol, 0.002% bromphenol blue, 4% 2-mercaptoethanol, 4% SDS).

IV. Immunoprecipitation

To detect ^{35}S-methionine labelled antigens synthesized *in vivo*, three different antisera were employed. Two antitumor sera were obtained from hamsters bearing tumors induced by either 14b or 297-C43 cells. The 14b cell line has been described above. The 297-C43 line was derived by transformation of hamster kidney cells with sheared Ad 5 DNA (Graham *et al*, 1974) and contains the viral DNA sequences from 0 to 23 and 32 to 52 map units (Visser *et al*, 1979). The antitumor sera obtained by pooling sera collected from 10 to 20 hamsters were kindly provided by Dr. F. Graham. The P antiserum, kindly supplied by Dr. W. Russell, was prepared in rabbits against Ad 5 infected RK 13 rabbit cells and is directed against early Ad 5 proteins (Russell *et al*, 1967). In addition, non-immune hamster and rabbit sera obtained from normal animals were used as controls.

Extracts from about 1 - 2 x 10^7 infected KB cells or about 10^8 Ad 5 transformed cells were immunoprecipitated using the above sera. Two immunoprecipitation methods were employed:

1) Double antibody technique: labelled cells were washed twice by centrifugation with PBS and to the final cell pellet one ml of ice cold precipitation buffer (20 mM Tris pH 7.5, 1.0% NP40 1.0% sodium deoxycholate, 150 mM NaCl, 0.2% SDS) was added. The samples were then stored at -70°C until convenient to continue the preparation. After thawing in a 37°C water bath, the suspension was frozen and thawed twice, sonicated three times for 15 seconds (Biosonik III, setting 30) at 4°C and then centrifuged at 26,000 x g for 20 minutes in a Sorvall centrifuge at 5°C. The supernatant, used as the antigen source, was placed in a 1.5 ml Brinkman centrifuge tube and incubated with one of the antisera described above for 12 hours at 4°C. An IgG directed against the first IgG (goat anti-rabbit or rabbit anti-hamster from Cappel Laboratories, Cochranville, Pa.) was added at equivalence and incubated for a further 12 hours at 4°C. (Equivalence was crudely determined by reacting a constant amount of the first antibody with varying concentrations of second antibody. The proportion of second antibody to first antibody which gave rise to the largest visible immunoprecipitate was taken as equivalence). The precipitate was sedimented at 2,000 x g in an IEC centrifuge for 20 minutes at 4°C and washed twice with cold precipitation buffer. The antigen-antibody pellet was then dissolved in 30 µl of 0.1 M NaOH, precipitated with 1.0 ml of cold 10% trichloroacetic acid, washed with ethanol and solubilized in 2 x electrophoresis sample buffer.

2) Protein A-Sepharose technique: using a method similar to that of Schaffhausen et al, (1978) labelled cells were washed twice with cold PBS, rinsed with cold buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10% glycerol (v/v) and then lysed in the same buffer containing 1% NP40 (v/v) (NP40 lysing buffer). After in-

cubating the cells at 4°C for 20 minutes, nuclei were removed by centrifugation at 10,000 x g for 20 minutes at 4°C in a Sorvall centrifuge. The supernatant was then centrifuged again at 10,000 x g for 20 minutes. The resultant supernatant was placed in a 1.5 ml Brinkman centrifuge tube and combined with 10 µl of appropriate antiserum and 250 µl of equilibrated protein A-Sepharose CL-4B (Pharmacia) diluted 1 to 10 with NP40 lysing buffer. After incubating for between 2 and 6 hours in the cold with constant mixing, the Sepharose beads with bound antibody were pelleted by centrifugation at 30 x g for 1.0 minute at 4°C in an IEC centrifuge, washed 3 times with ice cold 100 mM Tris (pH 8.0) containing 200 mM LiCl and 0.1% 2-mercaptoethanol, and finally suspended in 100 µl of 2 x electrophoresis sample buffer.

In some experiments using the protein A-Sepharose technique, nuclei were also analysed. Following fractionation of the nuclei and cytoplasm, 200 µl of ice cold double antibody lysis buffer was added to the nuclear pellet. The samples were then frozen, thawed, sonicated and finally centrifuged at 26,000 x g for 20 minutes in a Sorvall centrifuge at 4°C. The supernatant was removed, made up to 1.0 ml with NP40 lysing buffer, combined with antibody and protein A-Sepharose and incubated and washed as described above.

V. Protein Kinase Assay

A. Immunoprecipitation for Protein Kinase Assay

In general, about 1 - 2 x 10^7 KB cells or 10^8 adenovirus transformed cells were used for each protein kinase assay. Adenovirus 5

infected KB cells were harvested 9 or 12 hours post infection. Immunoprecipitates were obtained with normal hamster or 14b antiserum using the protein A-Sepharose technique. The protocol used was identical to that described above for the detection of labelled viral antigens from infected cells except for the following modifications. The pH of the NP40 lysis and wash buffer was changed from 8 to 7 since the pH optimum for the protein kinase activity was found to be 7.0 (see Results). In addition, after the final LiCl wash, the beads were washed twice with cold 1mM Tris (pH 7.0) containing 150 mM NaCl, and processed as described in the following section.

B. Assay for Protein Kinase Activity

In most experiments, the washed protein A-Sepharose beads with bound immune complex were assayed for protein kinase activity in a final volume of 50 µl containing 40 mM MES and 50 mM $MgCl_2$. Usually 75 ug of arginine-rich histone (Sigma) which had been boiled for 10 minutes just prior to use was also present. In some experiments cyclic AMP or cyclic GMP was also added at various concentrations. Prior to incubation, all components used in the assay were held on ice. The reaction mixture was preincubated for 2 minutes at 30°C, (γ-^{32}P) ATP (New England Nuclear) and unlabelled ATP was added to a final concentration of 7.5 µM and a specific activity of 250 Ci/nmole, and the samples were incubated at 30°C for from 30 seconds to 10 minutes. The reaction was terminated by the addition of an equal volume of 2 x electrophoresis sample buffer and the samples were boiled for several minutes. Aliquots of the boiled samples (usually 10 µl) were removed for measurement of radioactivity and for SDS-PAGE.

C. Measurement of ^{32}P Incorporation in Immunoprecipitates

The reactions were centrifuged for 5 minutes at 1,000 x g to pellet the protein A-Sepharose beads. Two 10 µl aliquots of reaction supernatant were removed from each sample and combined with 400 µg of bovine serum albumin and 5 ml of ice-cold 5% trichloroacetic acid (TCA). After 1 hour on ice, samples were centrifuged for 10 minutes at 400 x g at 4°C. The pellets were dissolved in 0.2 ml of 1.0 N NaOH and combined with 5 ml 5% TCA. The precipitate was collected by centrifugation, solubilized in NaOH and subsequently precipitated with TCA. The final precipitates were collected by filtration through glass fibre filters and the filters washed with 20 ml of 5% TCA and 10 ml of 95% ethanol. After air-drying the filters, the amount of radioactivity present was determined by liquid scintillation counting. Counts from duplicate samples generally differed by less than 12%. Phosphate incorporation in fmoles (10^{-15} moles) was calculated from the amount of radioactivity present.

VI. SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the discontinuous buffer system described by Laemmli (1970). The slab gel apparatus used is similar in design to that shown by Studier (1973). Gels were formed as slabs 1.0 mm thick and 150 or 180 mm long. The running gel contained either 15 or 18% acrylamide, the stacking gel 4.5% acrylamide and the ratio of acrylamide to bisacrylamide was 30:0.19. Samples were prepared for electrophoresis by boiling in 2 x sample buffer. Electrophoresis was generally for 16 hours at 70 volts.

After electrophoresis the slab gels were stained, destained and in most experiments using ^{35}S-methionine a fluorographic technique was employed to increase the sensitivity of autoradiography (Bonner and Laskey, 1974). Kodak X-ray film (PR/R-2, X-Omat) was used for autoradiography.

In some experiments the radioactivity present in specific protein bands in the gel was quantified by excising the band from the gel and placing it in a scintillation vial containing 0.3 ml of H_2O_2. After incubation overnight at 37°C, 10 ml of Omnifluor (New England Nuclear) was added to the digested gel fragment, and the radioactivity present determined by liquid scintillation counting. A second method made use of the Joyce Lobel MKIII CS double beam recording microdensitometer. The appropriate bands in the gel autoradiograph were scanned and the relative radioactivity estimated from the peak areas.

TABLE 1

Summary of Cell Lines Used in This Study

CELL LINE	ANIMAL CELL TYPE	TRANSFORMED BY	VIRAL DNA CONTENT (MAP UNITS)	REFERENCE
637C3	Rat	WT Ad 5 virus	0-25	M. Ruben, personal communication
822C2	Rat	WT Ad 5 virus	unknown	
424C1	Rat	Sheared WT Ad 5 DNA	0-100	Visser et al, 1979
512C8	Rat	HIND III G fragment of WT Ad 5 DNA	0-8	Graham et al, 1974
889C1	Rat	"	"	"
889C3	Rat	"	"	"
637-1	Rat	Ad 5 host range mutant hr 3	unknown	Graham et al, 1978
637-4	Rat	Ad 5 host range mutant hr 1	0-100	M. Ruben, personal communication
14b	Hamster	H5 ts 14 virus	0-40	Williams et al, 1974; Flint et al, 1976
297-C43	Hamster	Sheared WT Ad 5 DNA	0-23, 35-52	Graham et al, 1974; Visser et al, 1979
293	Human	Sheared WT Ad 5 DNA	0-12	Graham et al, 1974, 1977; Aiello et al, 1979

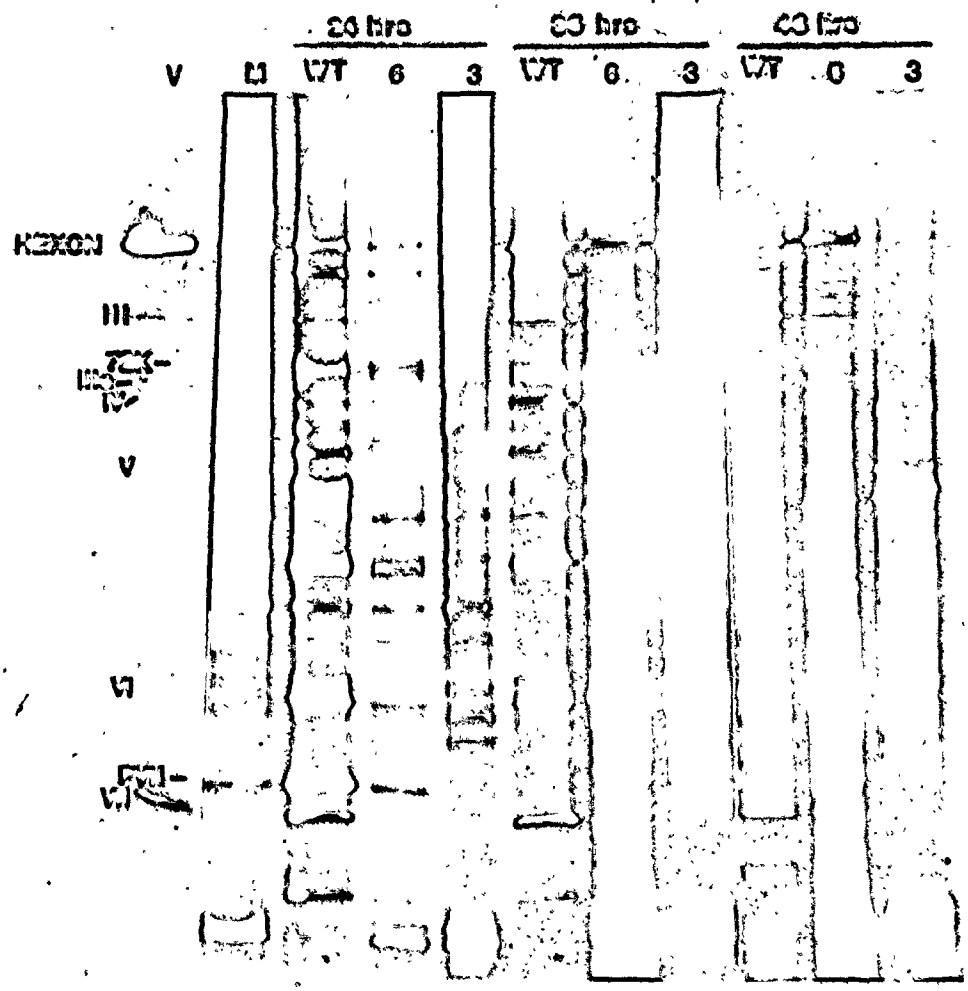
RESULTS

I. Late Viral Protein Synthesis: Virus-specific polypeptides synthesized in HeLa cells late after infection with wild type and host range mutant adenovirus.

To gain an understanding of the viral gene functions affected by the host range (hr) lesions the synthesis of late viral structural polypeptides was first investigated. HeLa cells were infected with adenovirus 5 (Ad 5) wild type (wt), group I mutant hr 3 or group II mutant hr 6 at a multiplicity of 5 plaque forming units (PFU) per cell, and labelled for 1 hour with ^{35}S-methionine at 24, 36 and 48 hours post infection. Cellular extracts were then prepared and analysed on SDS-polyacrylamide slab gels (Figure 3). In extracts from wt infected cells, a number of late viral polypeptides are observed, eg. hexon, III, V, VI, and VII, which clearly co-migrate with the polypeptides from purified ^{35}S-methionine labelled Ad 5 virus particles. With the group II mutant hr 6, some late viral products are seen, though in reduced amounts relative to wild type. In group I mutant hr 3 infected cells, synthesis of late virus specified polypeptides appears largely blocked since even 48 hours post infection no viral structural proteins can be discerned.

Synthesis of two of the late viral proteins seen in figure 3 was quantified (see figure 4). The bands corresponding to hexon and to protein VII and its precursor pVII were excised from the gel, and their radioactivities determined as described in the Materials and Methods. In extracts from wt infected cells, production of hexon declined 36 hours after

Figure 3. Autoradiograph of gel electrophoresis patterns of ^{35}S-methionine-labelled extracts from infected HeLa cells. Confluent cultures of HeLa cells were infected at an moi of 5 pfu/cell with wt or hr mutant adenovirus and labelled for 1 hour with ^{35}S-methionine at 24, 36 and 48 hours post infection. After labelling the cells were harvested and prepared for electrophoresis as described in materials and methods. M, extract of mock-infected cells; WT, extracts of wt-infected HeLa cells; hr 6, extracts of hr 6-infected cells; and hr 3, extracts of hr 3-infected cells. In this and subsequent figures, V represents a ^{35}S methionine labelled Ad 5 virus marker.


24 hrs
48 hrs
72 hrs
V
WT
6
3
WT
6
3
WT
6
3
EXON
III
IV
V
VI
VII

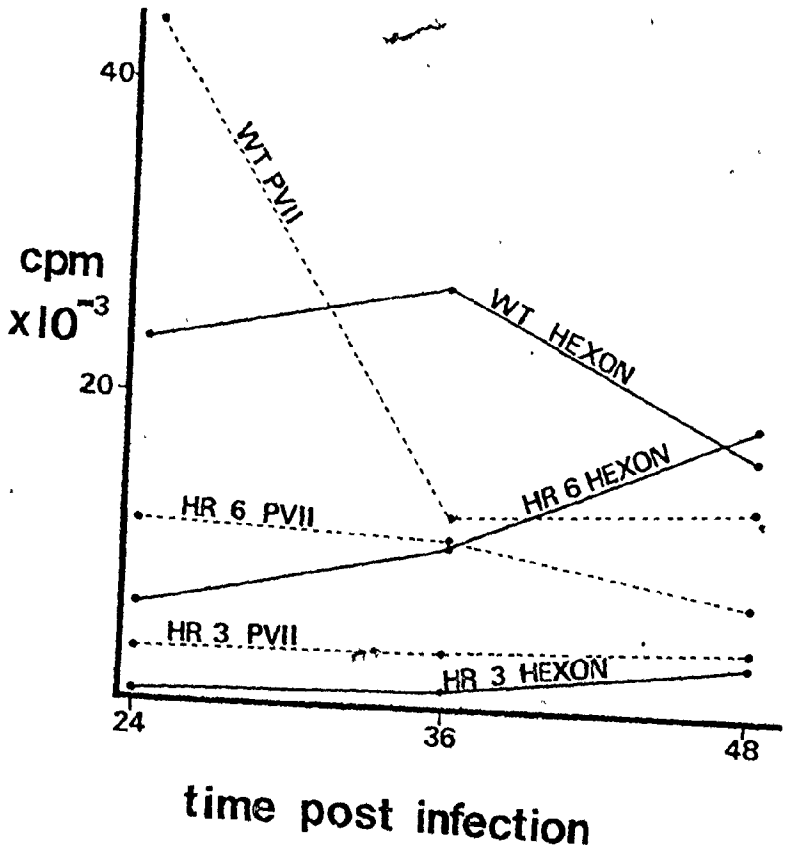


Figure 4. Radioactive incorporation into polypeptides VII and pVII and hexon. The amounts of radioactive label in these polypeptides were determined by cutting the appropriate bands from the SDS-polyacrylamide gel shown in figure 1 and solubilizing them in hydrogen peroxide. Aquasol (New England Nuclear) was then added to each sample and the radioactivity was measured by liquid scintillation counting. The total incorporation into VII and pVII together is shown as pVII.

infection, whereas incorporation into PVII + VII declined abruptly after 24 hours. With hr 6, synthesis of hexon continued to increase while production of PVII + VII declined slightly during the course of the experiment. In addition, synthesis of PVII + VII in hr 6 infected HeLa cells was significantly less than that seen in wt. The group I mutant hr 3 induced no significant synthesis of these polypeptides.

II. Early Protein Synthesis

A. Immunoprecipitation of proteins from adenovirus 5 wild type and host range mutant infected KB cells using the double antibody technique.

Since the hr lesions map within early region I, it was possible that in infecting non-permissive cells they might be defective in the production of certain early polypeptides. Preliminary studies indicated that early after infection few viral-specific polypeptides could be observed against the background of host protein synthesis. To detect early viral proteins relatively free of contaminating cellular proteins immunoprecipitation was used. In initial experiments, immunoprecipitation was performed using the double antibody method with three different antisera. P antisera, a gift from Dr. W.C. Russell, was generated in rabbits and is directed against early Ad 5 proteins. Two tumor antisera directed against the Ad 5 transformed hamster cell lines 14b and C43 were also used. The 14b antiserum is of particular interest, since as described above, this antiserum should be specific for proteins encoded by the transforming region of the Ad 5 genome. Results obtained with 14b and C43 antisera were always identical and in later experiments only 14b antiserum was used.

Details of the double antibody immunoprecipitation method have been

described in the Materials and Methods. Briefly, KB cells were infected with wt or hr mutant adenovirus at a moi of 35 pfu/cell and labelled with ^{35}S-methionine 7 to 9 hours after infection. A soluble cellular extract was prepared, incubated with the appropriate antiserum, and the resulting complex precipitated by the addition of a second antibody directed against the first. The labelled immunoprecipitate was then analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the radioactive proteins identified by autoradiography.

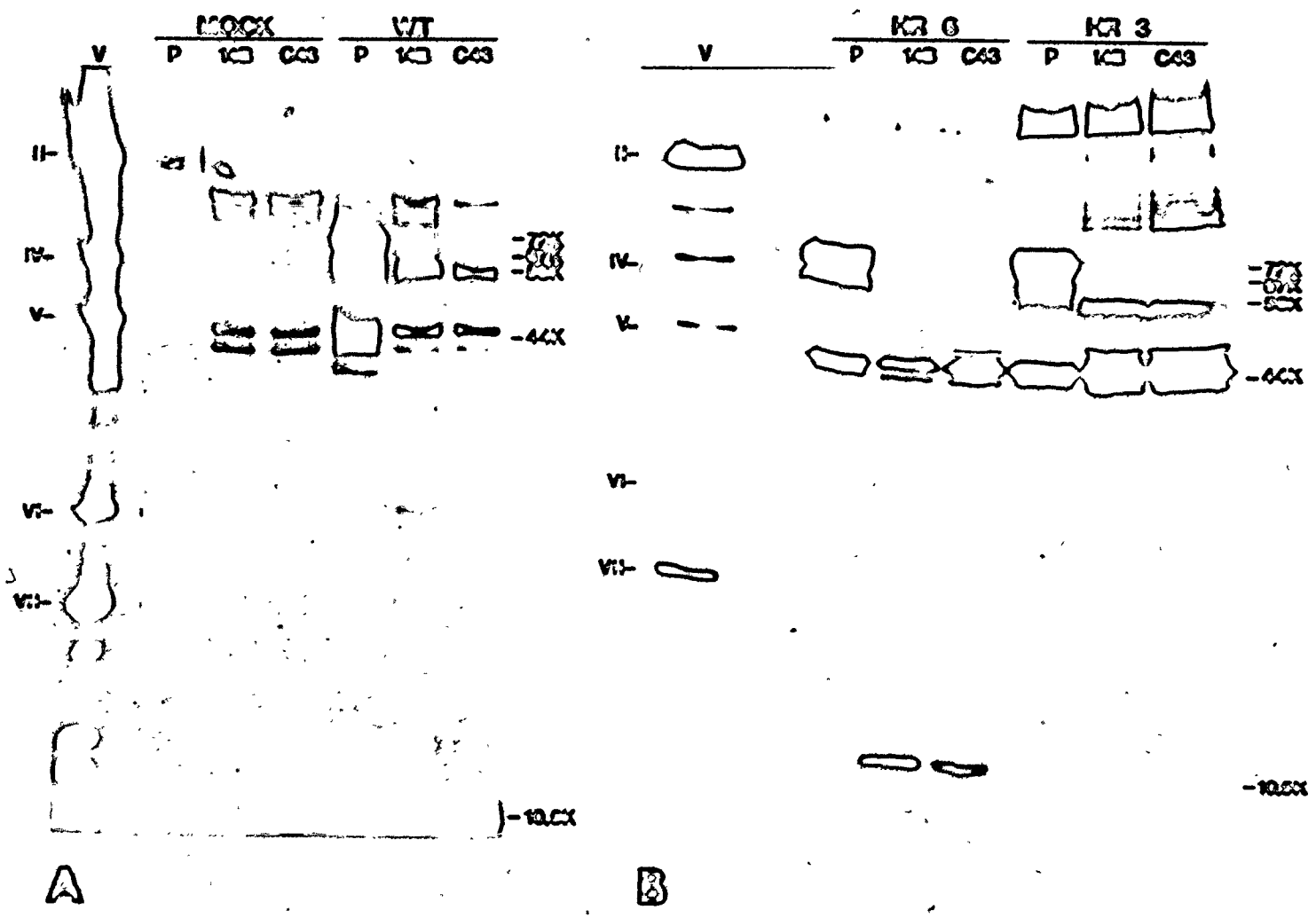
Figure 5 shows an autoradiograph of ^{35}S-methionine labelled antigens from mock and wt Ad 5 infected KB cells immunoprecipitated with P, 14b or C43 antiserum. A number of polypeptides are nonspecifically immunoprecipitated by all antisera from mock infected cells, but additional proteins with distinct mobilities are seen as major bands in extracts from productively infected cells. With the wt infected extracts P antiserum binds predominantly a protein of 72,000 daltons and smaller amounts of 67,000 and 44,000 dalton polypeptides. Two other adenovirus-specific antigens of 58,000 and 10,500 daltons were also immunoprecipitated. Detection of these last two proteins with P antiserum was variable (see figure 5b) and appeared dependent on the batch of serum used. The 14b and C43 antisera both immunoprecipitated the 58,000 and 10,500 dalton proteins from infected cell extracts.

Radiolabelled polypeptides immunoprecipitated from cells infected with the group I mutant hr 3 or group II mutant hr 6 are shown in figure 5b. With both mutants, synthesis of the 72,000, 67,000 and 44,000 dalton polypeptides is similar to that seen with wt. However, the 14b and C43 antisera which precipitate the 58,000 and 10,500 dalton proteins from wt infected cells, bound only the 10,500 dalton protein from extracts of hr

Figure 5. Immunoprecipitation of ^{35}S-methionine labelled polypeptides from KB cells infected with Ad 5 wt or hr mutants by the double antibody method. KB cells were infected at an moi of 35 pfu/cell and labelled with ^{35}S-methionine from 7 to 9 hours post infection. Extracts were prepared and immunoprecipitated by the double antibody technique as described in materials and methods. For each cell extract, three different immunoglobulins were used in the first step of immunoprecipitation as indicated, namely P = rabbit P antiserum; 14b = hamster 14b tumor antiserum; C43 = hamster C43 tumor antiserum. Material from approximately 2 x 10^6 cells was loaded into each of the wells.

Figure 5a: Mock infected, or infected with wt Ad 5;

Figure 5b: Infected with group II mutant hr 6 or group I mutant hr 3.

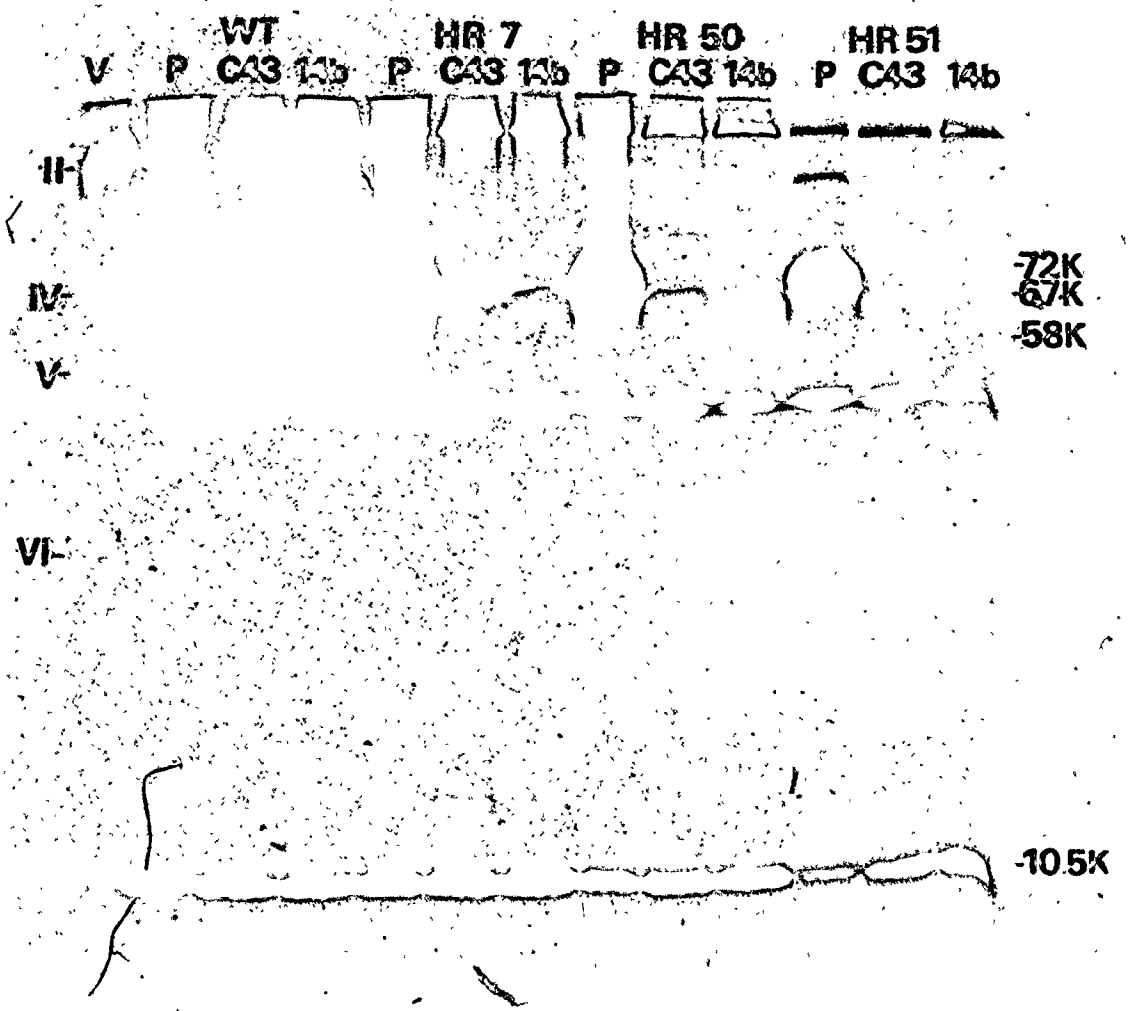

MOCK
WT
V
P
Kir
Cx43
II-
IV-
V-
VI-
VII-
-44Cx
-10.5Cx
A
Kir 6
Kir 3
B

6 infected KB cells. In contrast, the 58,000 dalton polypeptide was immunoprecipitated from group I mutant infected cell extracts but little 10,500 dalton protein was detected relative to that seen with hr 6 or wt.

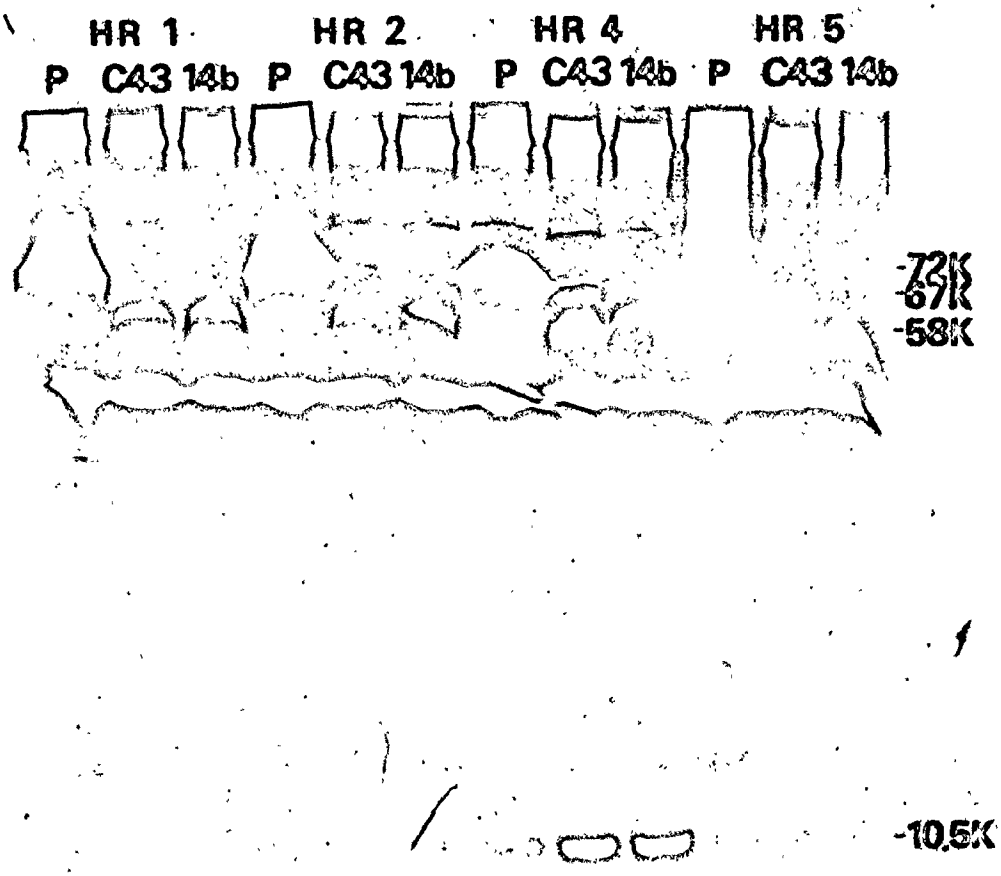
Figure 6 shows the results of analysis of proteins from cells infected with additional hr mutants. Figure 6a compares immunoreactive proteins induced by wt and three group II mutants. Two of these mutants, hr 7 and hr 50, failed to induce any immunoreactive 58,000 dalton polypeptide. Infection with hr 51 did result in the production of the 58,000 dalton protein but in amounts greatly reduced relative to that seen in wt infected extracts. The amount of 10,500 dalton protein seen immunoprecipitated from group II mutant infected extracts is similar to that found after wt Ad 5 infection. Figure 6b shows results obtained with four different group I mutants. All produce an immunoreactive 58,000 dalton protein and three of the mutants show the 10,500 dalton protein at levels much lower than those observed with wt or the group II mutants. More of the 10,500 dalton protein was precipitated from hr 4 infected cell extracts than from the other group I mutants. Much of the 10,500 dalton protein produced in hr 4 infected cells may be due to leakiness, since J. Williams (personal communication) has found this mutant to be less defective than other host range mutants of this complementation group.

In several experiments the amount of immunoprecipitable 10,500 dalton protein in wild type and group I mutant hr 3 infected cell extracts was quantified by microdensitometer scanning. The amount of 10,500 dalton protein observed after hr 3 infection was found to be consistently less than 15% that of wild type.

Figure 6. Autoradiograph of gel electrophoresis patterns of ^{35}S-methionine labelled antigens immunoprecipitated using the double antibody method from KB cells infected with wt, group I or group II mutants. Figure 6a shows results obtained using wt, hr 7, hr 50 and hr 51. Figure 6b shows results obtained using hr 1, 2, 4 and 5. Experimental details and symbols are the same as in figure 5.



A


HR 1
HR 2
HR 4
HR 5
P C43 14b P C43 14b P C43 14b P C43 14b
72K
67K
58K
10.5K

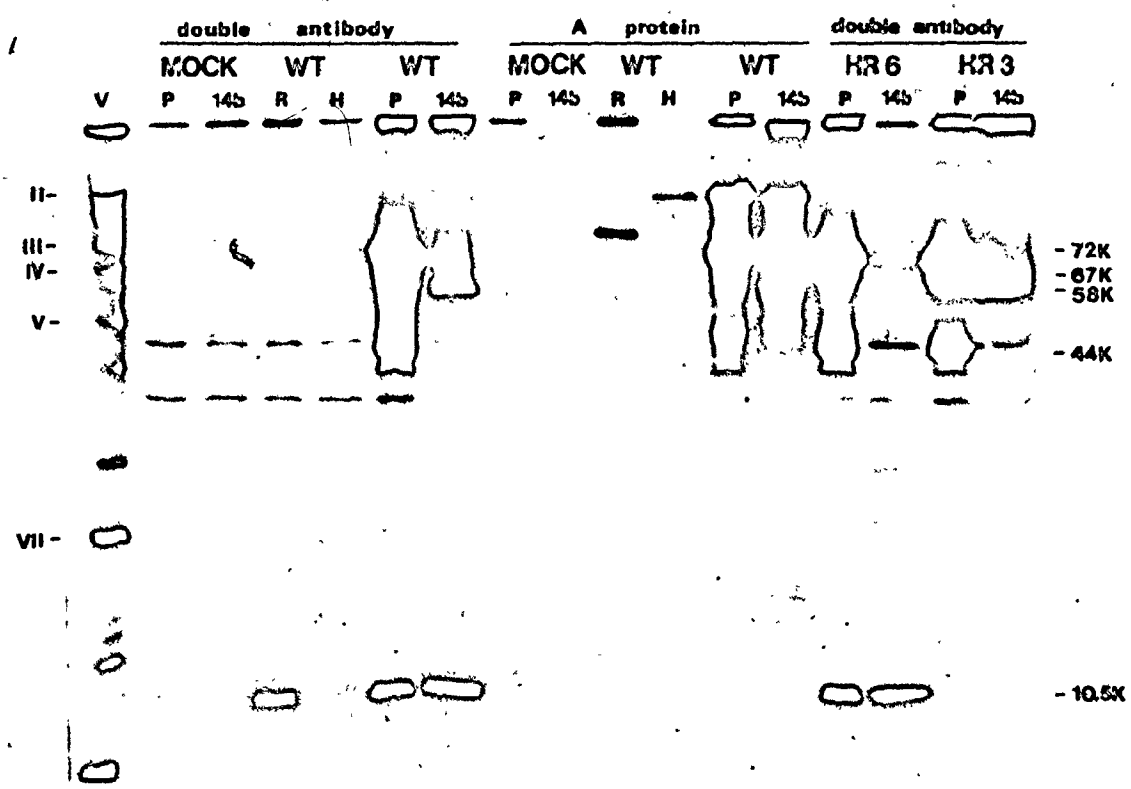
B

B. Immunoprecipitation of polypeptides from wild type and host range mutant infected KB cells using the protein A-Sepharose method.

A second widely used method for immunoprecipitating labelled antigens is the protein A-Sepharose method. To confirm the results obtained with the double antibody method the experiments described above were repeated and additional studies carried out using this second technique. In these experiments KB cells were infected and labelled with ^{35}S-methionine as described above for the double antibody technique. The cells were then harvested, and fractionated into cytoplasmic and nuclear material. In most experiments the cytoplasmic fraction was used as the antigen source and incubated with the appropriate antiserum. In place of the second antibody, protein A-Sepharose beads were used to bind the antigen-antibody complex. After washing the beads, the precipitates were analysed on polyacrylamide gels as before.

Figure 7 compares results obtained using either the double antibody or the protein A-Sepharose technique. With one significant exception, the results obtained with the two procedures were very similar. In both cases, P antisera bound the 72,000, 67,000 and 44,000 dalton polypeptides and the 14b antiserum bound the 58,000 dalton protein. However, only with the double antibody technique was the 10,500 dalton polypeptide immunoprecipitated by either serum. Failure of the protein A-Sepharose method to immunoprecipitate the 10,500 dalton protein was not a consequence of the extract used for immunoprecipitation since this polypeptide was not precipitated with this method from either cytoplasmic or nuclear extracts (see figure 14). The inability of the protein A-Sepharose method to immuno-

Figure 7. Immunoprecipitation of labelled polypeptides from infected KB cells using either the double antibody or protein A-Sepharose technique. KB cells were mock infected (MOCK), or infected with Ad 5 wt (WT), group II mutant hr 6 (HR 6), or group I mutant hr 3 (HR 3). The cells were infected at an moi of 35 pfu/cell and labelled with ^{35}S-methionine from 7 to 9 hours post infection. Double antibody and protein A-Sepharose immunoprecipitations were as described in materials and methods. R and H refer to immunoprecipitations using normal rabbit or normal hamster serum, respectively. Other symbols are the same as in figure 5.


double antibody
A protein
double antibody
MOCK
WT
WT
MOCK
WT
WT
KR6
KR3
V
P
14b
R
H
P
14b
P
14b
R
H
P
14b
P
14b
P
14b
II-
III-
IV-
V-
VII-
-72K
-67K
-58K
-44K
-10.5K

precipitate the 10,500 dalton polypeptide is the major difference between this technique and the double antibody method.

Since the 10,500 dalton protein was not immunoprecipitated by the protein A-Sepharose method, it is possible that this protein was immunoprecipitated non-specifically using the double antibody method. This is also suggested by the fact that, as shown in figure 7, the 10,500 dalton protein was immunoprecipitated to a small extent by non-immune hamster serum but to a greater extent by non-immune rabbit serum. Furthermore, different batches of non-immune rabbit or hamster sera immunoprecipitated varying amounts of this polypeptide, although in amounts less than when immune sera was used (data not shown). In an effort to determine whether the 10,500 dalton protein was immunoprecipitated non-specifically, immunoprecipitates were prepared using the double antibody method and washed with buffer containing concentrations of NaCl from 150 mM (the amount normally present in the wash buffer) to 1.0M. The effect of a high salt buffer should be that of preferentially removing non-specifically bound material. Results from an experiment of this kind using P, non-immune rabbit and 14b sera are shown in figure 8. It is apparent that even at the highest salt concentration used, no selective reduction of the 10,500 dalton protein relative to the other Ad 5 induced polypeptides such as thed 58,000 and 72,000 dalton polypeptides was observed. Hence this data is inconclusive and does not indicate whether or not the 10,500 dalton protein is specifically bound by antibodies in the 14b antiserum.

Figures 9 and 10 show labelled antigens immunoprecipitated using the protein A-Sepharose method from cells infected with hr mutants. As before, group II mutants failed to induce the synthesis of an immunoreactive 58,000 dalton protein (hr 6, figure 9 and hr 50, figure

Figure 8. Immunoprecipitation of labelled proteins from wt Ad 5 infected KB cells using the double antibody method. Experimental details are as described in figure 5 except that the labelled immunoprecipitates were washed with buffer containing increasingly higher salt concentrations: A, 0.125 M NaCl; B, 0.25 M NaCl; C, 0.5 M NaCl; D, 1.0 M NaCl. The other symbols are the same as in figure 7.

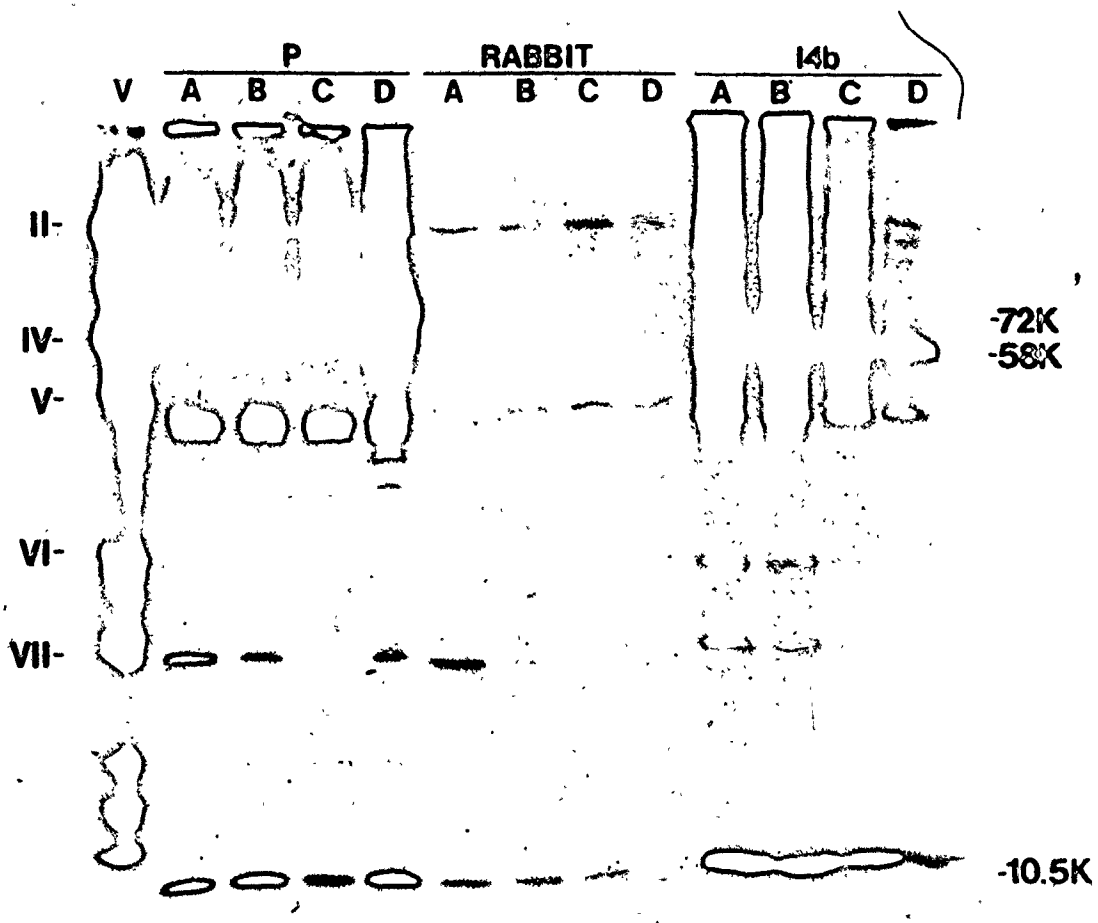

P
RABBIT
14b
V A B C D A B C D A B C D
II-
IV-
V-
VI-
VII-
-72K
-58K
-10.5K

Figure 9. Immunoprecipitation of labelled polypeptides from wt, hr 3 and hr 6 infected KB cells using the protein A-Sepharose technique. The experimental details and symbols used are the same as in figure 7.

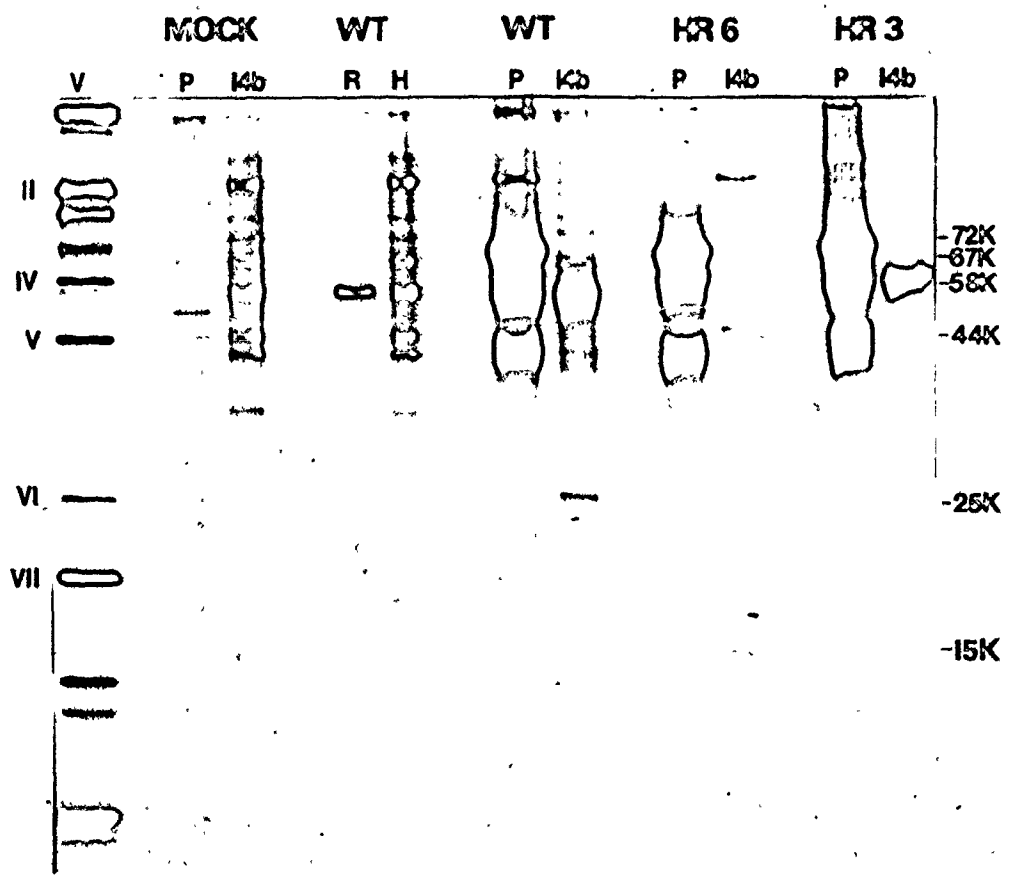
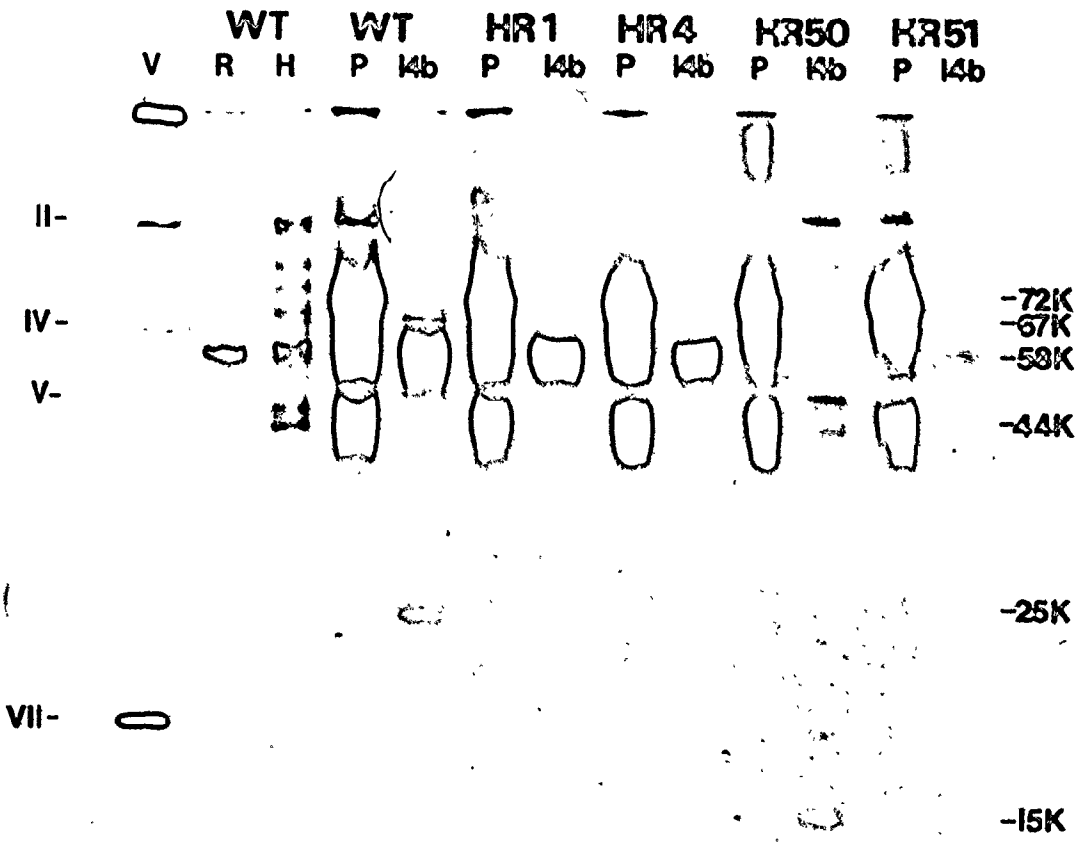

MOCK
WT
WT
HR 6
HR 3
V
P
Kb
R
H
P
Kb
P
Kb
P
Kb
II
IV
V
VI
VII
-72K
-67K
-58K
-44K
-25K
-15K

Figure 10. Immunoprecipitation of labelled polypeptides from wt or hr mutant infected KB cells using the protein A-Sepharose technique. Hr 1 and hr 4, KB cells infected with group I mutants; hr 50 and hr 51, KB cells infected with group II mutants. The experimental details and symbols used are the same as in figure 7.


WT
WT
HR1
HR4
KR50
KR51
V
R
H
P
14b
P
14b
P
14b
P
14b
P
14b
II-
IV-
V-
VII-
-72K
-67K
-58K
-44K
-25K
-15K

10) or produced it at very low levels (hr 51, figure 10). The major immunoreactive proteins detected in wild type infected cells, namely the 72,000, 67,000 58,000 and 44,000 dalton proteins were also observed in cells infected with group I mutants. When extracts of wild type infected cells were immunoprecipitated with non-immune hamster serum, no 58,000 dalton protein was observed but some of this protein was detected with non-immune rabbit serum.

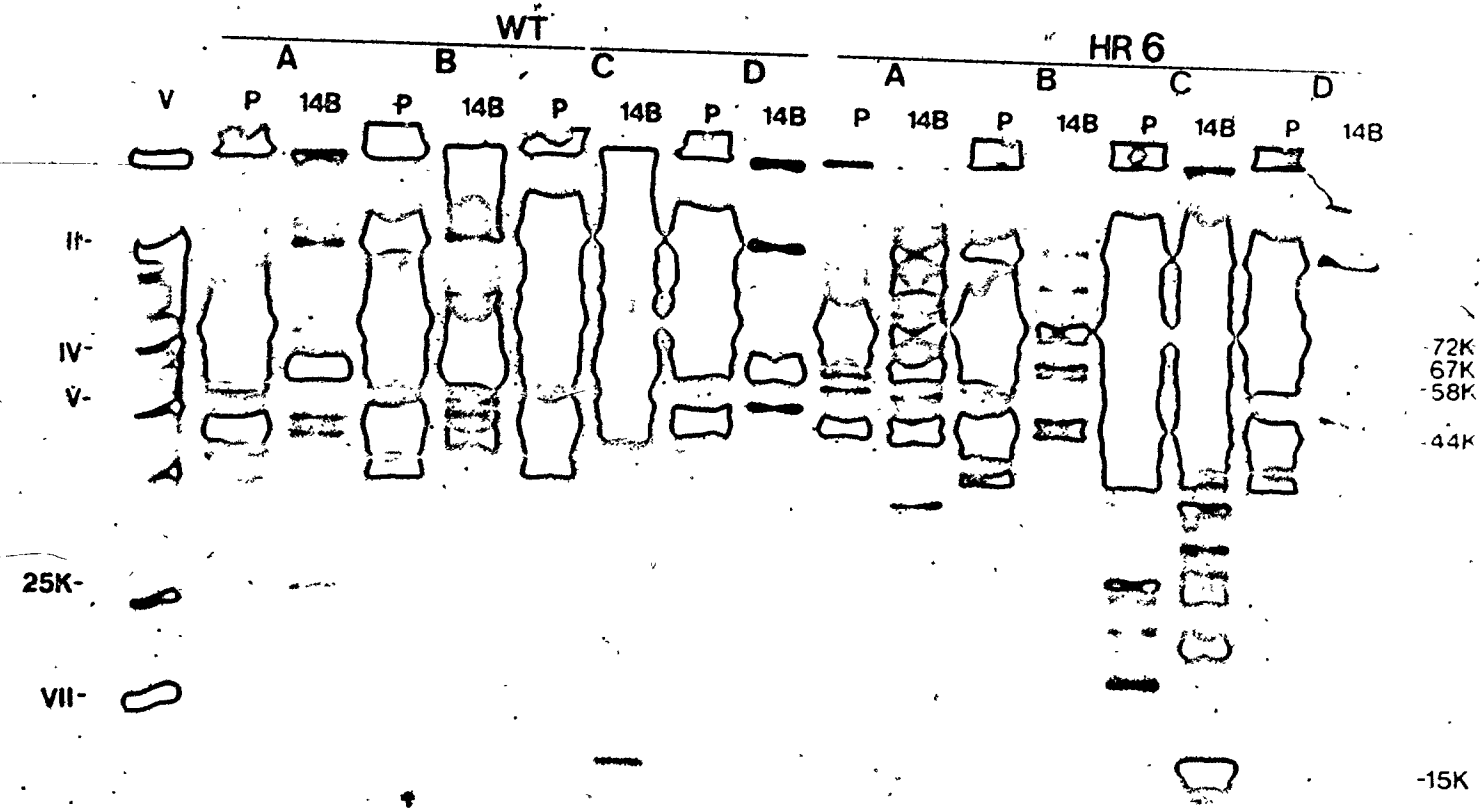
Synthesis of the 58,000 dalton polypeptide in group I hr 3 mutant and wild type infected cell extracts was quantified by microdensitometry in a number of experiments. With the protein A-Sepharose method the amount of this antigen immunoprecipitated from hr 3 infected cell extracts was about 20 to 65% that of wild type. Slightly higher values were obtained when the double antibody technique was used.

In addition to the major immunoreactive polypeptides discussed above, two minor virus-specific polypeptides were frequently detected in immunoprecipitates using the protein A-Sepharose technique. Firstly, a polypeptide of molecular weight 25,000 daltons was often observed in wild type infected extracts immunoprecipitated with 14b antiserum (visible in figures 9, 10, 15, 20 and 21). This polypeptide was not detected in immunoprecipitates from group I or group II host range mutant infected KB cells nor was it bound by any non-immune serum. Secondly, a 15,000 dalton protein was often detected in immunoprecipitates from group II mutant infected extracts obtained using 14b antiserum. This protein, which frequently migrated as a doublet, is seen in figures 9, 10, 11 and 15. With prolonged exposures some 15,000 dalton protein was also occasionally detected in extracts of wt-infected cells though in amounts reduced relative to that seen with group II mutant infections.

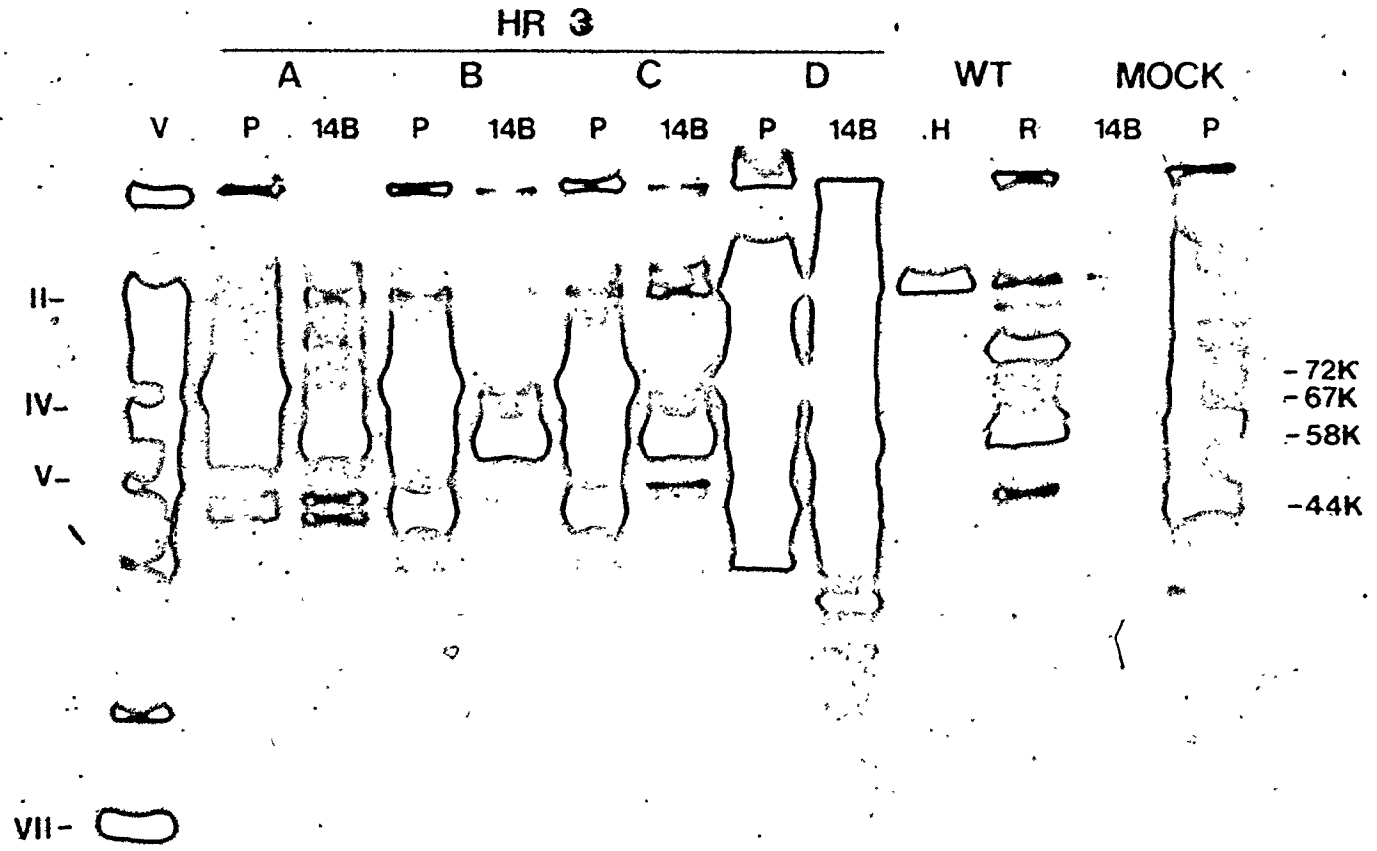
C. Time of appearance of the adenovirus induced polypeptides immunoprecipitated by P and 14b antisera.

To determine when the immunoreactive polypeptides described in the previous sections were synthesized, KB cells infected with Ad 5 wild type, hr 3 or hr 6 were labelled with ^{35}S-methionine 3-8, 8-13, 13-18 or 18-22 hours post infection. Cytoplasmic extracts were prepared after the labelling period and immunoprecipitated with P or 14b antiserum by the protein A-Sepharose method. Figure 11 shows the immunoprecipitated polypeptides separated by SDS-polyacrylamide gel electrophoresis. The major Ad 5 specific antigens of molecular weight 72,000, 61,000 and 44,000 were first detected 3-8 hours post infection and were also synthesized late (18-22 hours) after infection. The 58,000 dalton protein, while not immunoprecipitated from hr 6 infected cell extracts, was synthesized in wild type and hr 3 infected cells during all the labelling periods used. The minor 15,000 dalton protein is clearly seen in extracts from group II mutant hr 6 infected cells, but its synthesis appears retarded relative to the major immunoprecipitated polypeptides, with maximal production occuring 13-18 hours after infection. A small amount of this protein was also immunoprecipitated from wild type infected cell extracts. The 25,000 dalton polypeptide was detected only from wild type infected cells and its synthesis declined after the first labelling period. Non-immune hamster serum did not react with any virally induced proteins, but a small amount of the 58,000 dalton protein was immunoprecipitated with non-immune rabbit serum from wild type infected cells labelled 8-13 hours after infection. None of these proteins were detected in mock infected cells immunoprecipitated with P or 14b antiserum.

Figure 11. Time of appearance of labelled polypeptides immunoprecipitated from wt, hr 3 and hr 6 infected KB cells using the protein A-Sepharose method with P or 14b antiserum. Infected cells were labelled with ^{35}S-methionine between 3-8 (A), 8-13 (B), 13-18 (C) or 18-22 (D) hours after infection. Figure 11a shows immunoprecipitates from wt and group II mutant hr 6 infected cell extracts. Figure 11b shows immunoprecipitates from group I mutant hr 3 infected cell extracts. The wt infected cell extracts immunoprecipitated with non-immune sera were labelled with ^{35}S-methionine from 8-13 hours post infection. The symbols used are the same as in figure 7.



A


HR 3
A
B
C
D
WT
MOCK
V
P
14B
P
14B
P
14B
P
14B
H
R
14B
P
II-
IV-
V-
VII-
-72K
-67K
-58K
-44K

B

These data suggest that the major viral specific proteins detected by immunoprecipitation are early proteins since they were observed by 3-8 hours after infection, a time period which precedes most viral DNA replication (Green et al, 1970). In agreement with these results, the major immunoreactive proteins described here were also detected in infected KB cells treated with cytosine arabinoside to prevent viral DNA synthesis (see figure 15).

Synthesis of the 72,000 and 58,000 dalton proteins shown in figure 11 was analysed by microdensitometry. Figure 12 compares the amount of ^{35}S-labelled 72,000 dalton protein immunoprecipitated from extracts of wt, hr 3 and hr 6 infected cells as a function of time post infection. With wild type, synthesis of the 72,000 dalton protein rises abruptly between 3-8 and 8-13 hours post infection. After 13-18 hours, its production declines and by 18-22 hours incorporation of ^{35}S-methionine into the 72,000 dalton protein is reduced to the levels observed at the earliest time interval. Synthesis of the 72,000 dalton antigen in cell extracts infected with group II mutant, hr 6 parallels that seen with wild type extracts but appears to be overproduced relative to wild type. Early after infection, the group I mutant hr 3 induced less 72,000 dalton protein than either wild type or hr 6 and only late in infection does the synthesis of the 72,000 dalton protein rise to the levels observed with wild type. Synthesis of the 58,000 dalton protein in the wild type and hr 3 infected cell extracts is shown in figure 13. With wild type infection, synthesis of this protein was maximal 8-13 hours post infection and declined thereafter. In hr 3 infected cells, less of the 58,000 dalton protein was synthesized and maximal production was delayed, occuring 13-18 hours after infection.

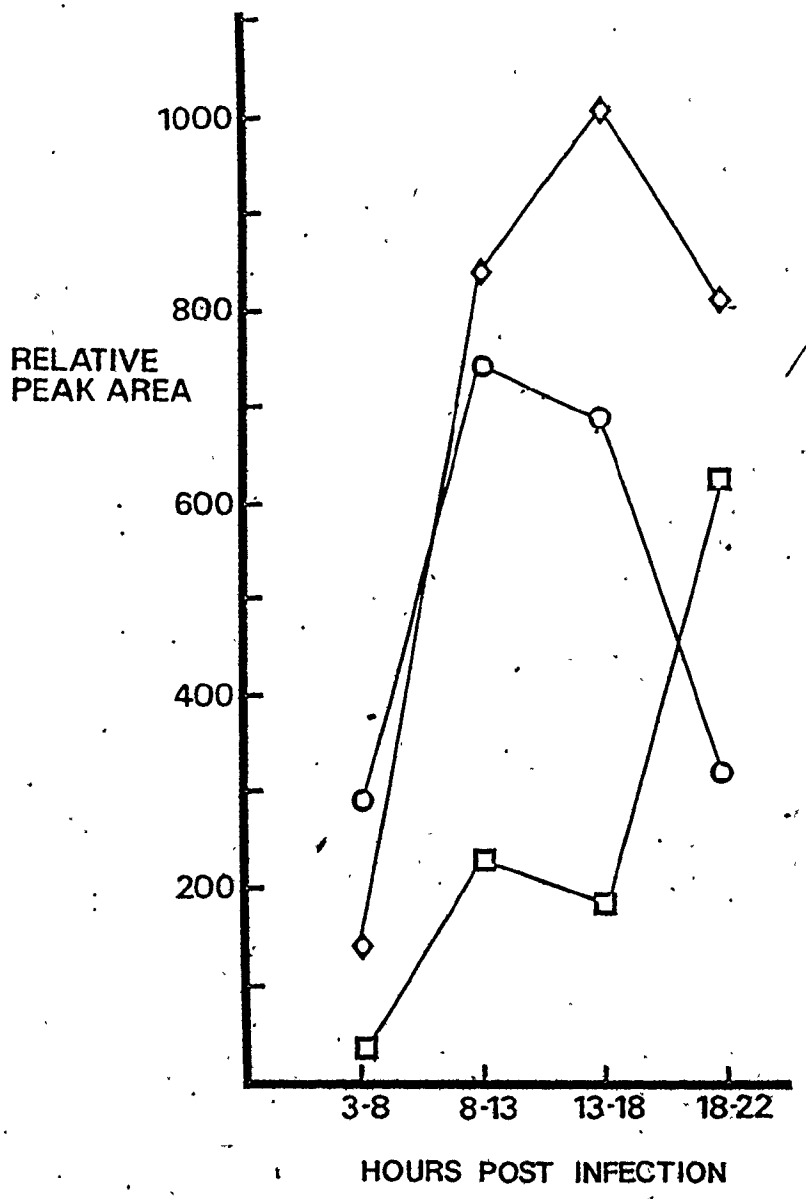


Figure 12. Time of appearance of the 72,000 dalton antigen in wt, hr 3 and hr 6 infected cells. The relative areas under densitometer scans of appropriate bands in the autoradiographs in figure 11 were used as a measure of the incorporation of ^{35}S-methionine into the 72,000 dalton antigen. WT (○—○); hr 3 (□—□); hr 6 (◇—◇).

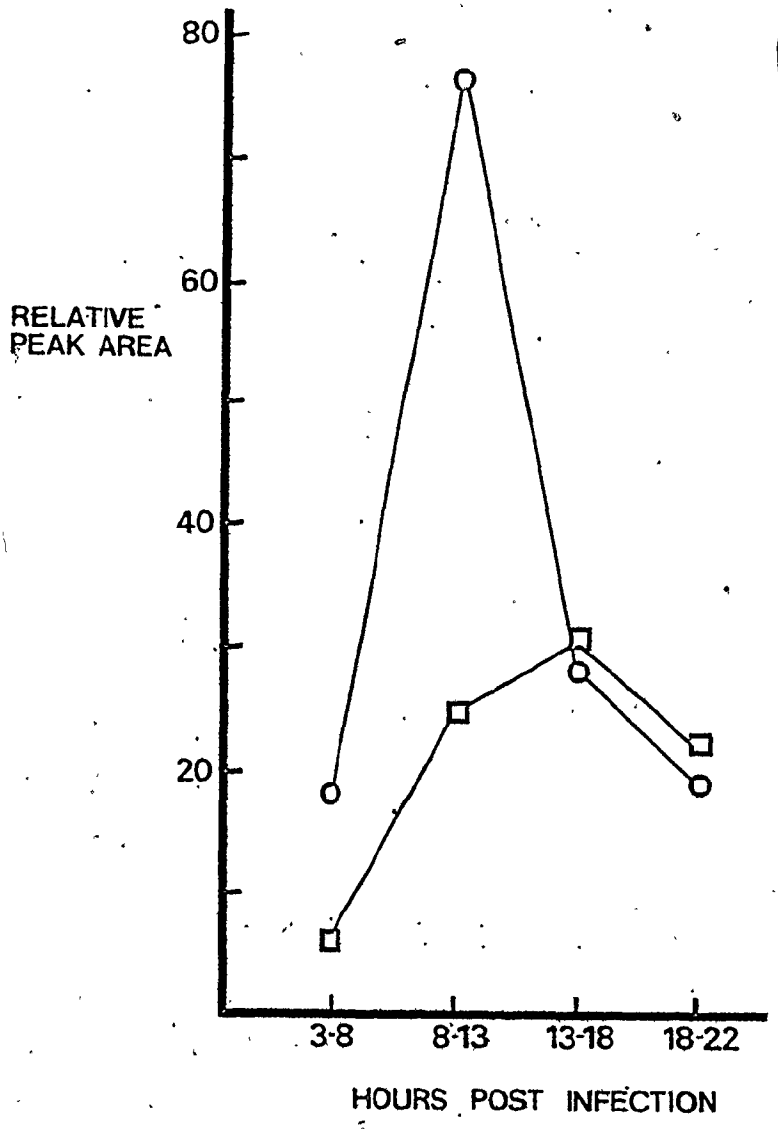


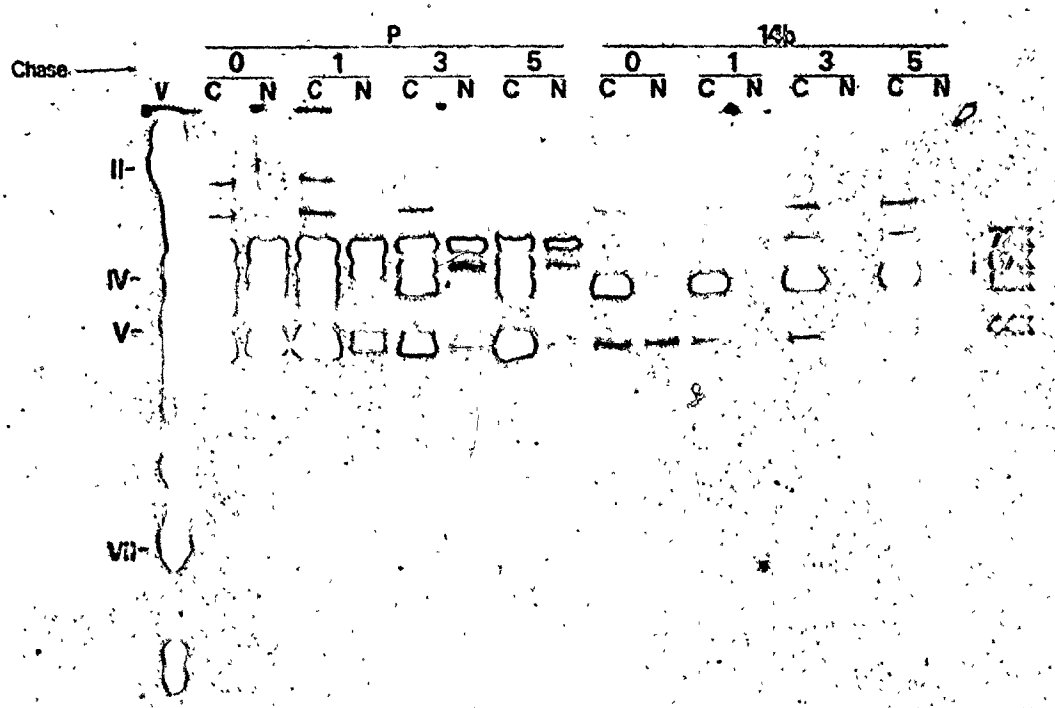
Figure 13. Time of appearance of the 58,000 dalton antigen in wt and hr 3 infected cells. The relative areas under densitometer scans of appropriate bands in the autoradiographs in figure 11 were used as a measure of the incorporation of ^{35}S-methionine into the 58,000 dalton antigen. WT (O—O); hr 3 (□—□).

D. Immunoprecipitation of Adenovirus 5 infected KB cell extracts pulse labelled with ^{35}S-methionine and chased with unlabelled methionine.

Pulse-chase experiments were designed and executed in collaboration with D. Rowe. These studies were initiated in an effort to answer two questions: do the major viral antigens, specifically the 58,000 dalton polypeptide, arise from proteolytic cleavage; and, what is the cellular location of the early viral antigens?

Suspension cultures of KB cells were infected at an moi of 50 pfu/cell. The cells were labelled with ^{35}S-methionine between 8 and 9 hours post infection and the label was then chased by adding medium containing excess unlabelled methionine and incubation was continued for a further 1, 3 or 5 hours. Finally, the radiolabelled cultures were fractioned into nuclear and cytoplasmic extracts both of which were immunoprecipitated with P or 14b antiserum. Figure 14 shows an autoradiograph of the labelled polypeptides immunoprecipitated by the protein A-Sepharose method and separated on an SDS-polyacrylamide gel. In immunoprecipitates obtained with 14b serum, only the major 58,000 dalton polypeptide was observed. This protein appears to be localized predominantly in the cytoplasm since only trace amounts of it were seen in immunoprecipitates obtained from the nuclear extracts. The electrophoretic mobility of the 58,000 dalton antigen did not appear to vary even after a 5 hour chase, so that if the 58,000 dalton protein undergoes post-translational modification, these modifications probably occur within the first 1-2 hours after translation. The P antiserum bound all of the four major viral antigens. As with 14b antiserum, the 58,000 dalton protein is not seen in any of the nuclear extracts.

Figure 14. Immunoprecipitation of labelled proteins from wt Ad 5 infected KB cells using the protein A-Sepharose method. KB cells were infected at an moi of 50 pfu/cell, labelled with ^{35}S-methionine from 8 to 9 hours after infection and the label was chased by adding excess unlabelled methionine for 0, 1, 3 or 5 hours. Cytoplasmic and nuclear extracts were prepared and immunoprecipitated using P or 14b antiserum as described in the materials and methods. 0, 1, 3 and 5 refer to the length (in hours) of the chase time. Other symbols are as described in figure 7.

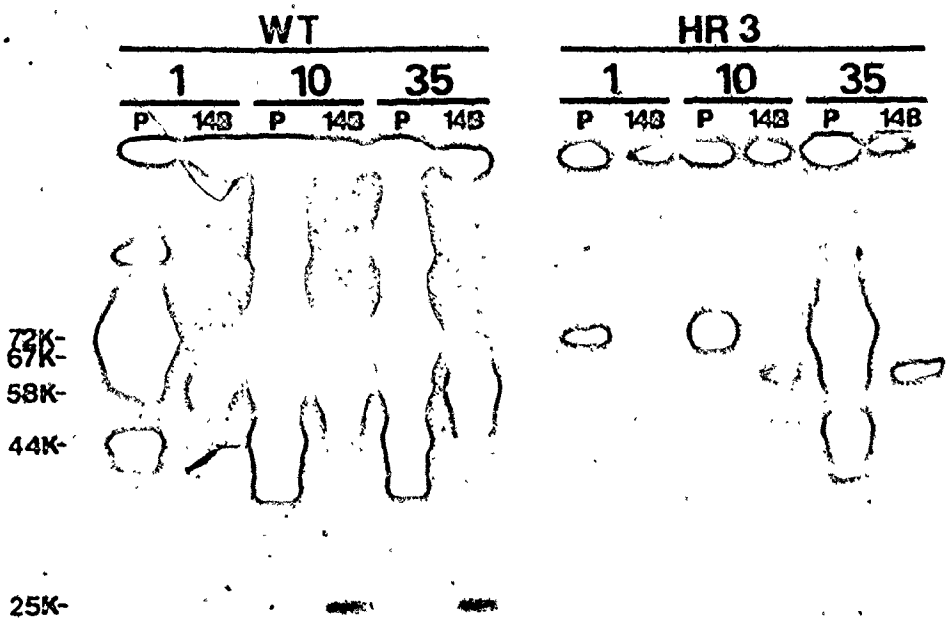

P
14b
Chase
0
1
3
5
V C N C N C N C N C N C N C N C N
II-
IV-
V-
VII-

The 72,000 and 67,000 dalton proteins are found in both nuclear and cytoplasmic extracts, whereas the 44,000 dalton protein was found predominantly in the cytoplasm.

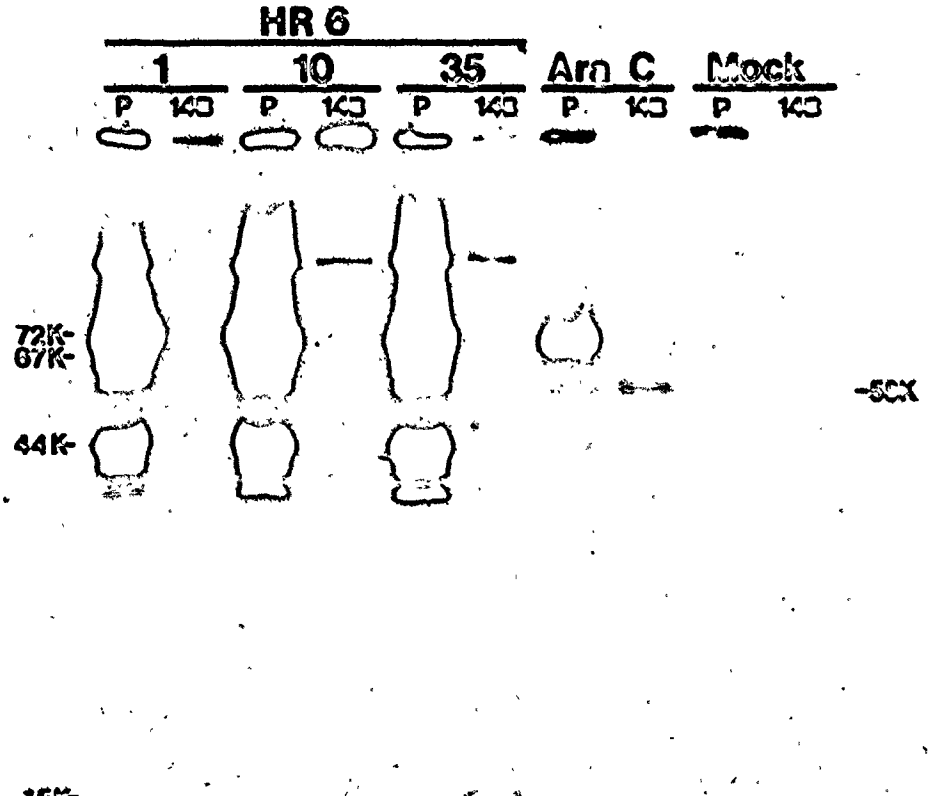
E. The effect of multiplicity of infection on the production of early viral specific antigens.

In the immunoprecipitation experiments described above, a relatively high moi of 35 pfu/cell was used. At this moi there is a tendency for the hr mutants, and particularly those of group II, to leak through the block resulting from the hr lesion and proceed through the lytic cycle to produce complete progeny virus (F. Graham, personal communication). Hence, some of the antigens detected at high moi might be absent if low moi's were used. To investigate the effect of moi's on the synthesis of early immunoreactive proteins, KB cells were infected with Ad 5 wild type, hr 6 or hr 3 at moi's of 1, 10 and 35 pfu/cell, labelled with ^{35}S-methionine from 7 to 9 hours after infection and immunoprecipitated with P or 14b antiserum.

Figure 15 shows an autoradiograph of the labelled polypeptides separated by electrophoresis. Figures 16 and 17 show incorporation of ^{35}S-methionine into the 72,000 and 58,000 dalton proteins as determined by microdensitometry. With wild type infected extracts, the four major virus-specific antigens (i.e. the 72,000, 67,000, 58,000 and 44,000 dalton proteins) were detected at all moi's used. Synthesis of the 72,000 dalton protein was much greater at a moi of 10 pfu/cell than at 1 pfu/cell, but higher input multiplicity resulted in no further increase. In contrast, more of the 58,000 dalton protein was synthesized as the moi was increased.



A



B

Figure 15. Immunoprecipitation of labelled proteins from KB cells infected at moi's of 1, 10 or 35 pfu/cell with Ad 5 wt, hr 3 or hr 6. 1, 10 and 35 refer to the multiplicity of infection used. The remaining symbols and experimental details are the same as in figure 7. Figure 15a shows immunoprecipitates obtained from wt and hr 6 infected cell extracts. Figure 15b shows immunoprecipitates from hr 3 infected cell extracts. Also shown in figure 15b are immunoprecipitates obtained from hr 3 infected cells treated with cytosine arabinoside (Ara C) at a concentration of 25 µg/ml to block DNA synthesis. The treated cultures were rinsed in Ara C containing medium prior to infection and maintained throughout the experiment in medium containing Ara C. Labelling and immunoprecipitation was performed as described in figure 7.

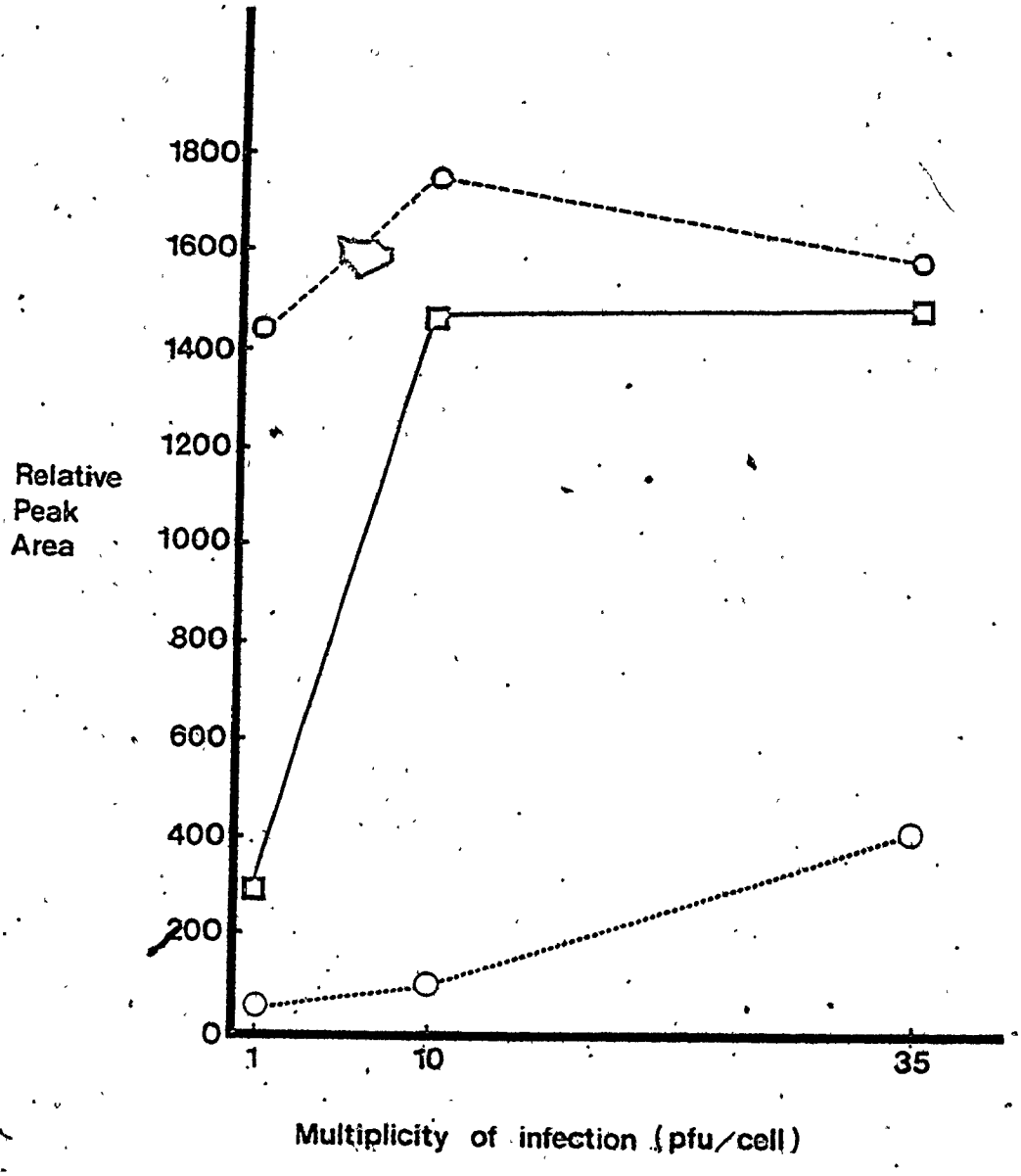


Figure 16. Relative synthesis of the 72,000 dalton protein in KB cells infected at moi's of 1, 10 and 35 pfu/cell with Ad 5 wt, hr 6 and hr 3. The relative areas under densitometer scans of appropriate bands in the autoradiographs in figure 15 were used as a measure of the incorporation of ^{35}S-methionine into the 72,000 dalton antigen. WT, (□–□); hr 6, (O--O); hr 3, (O···O).

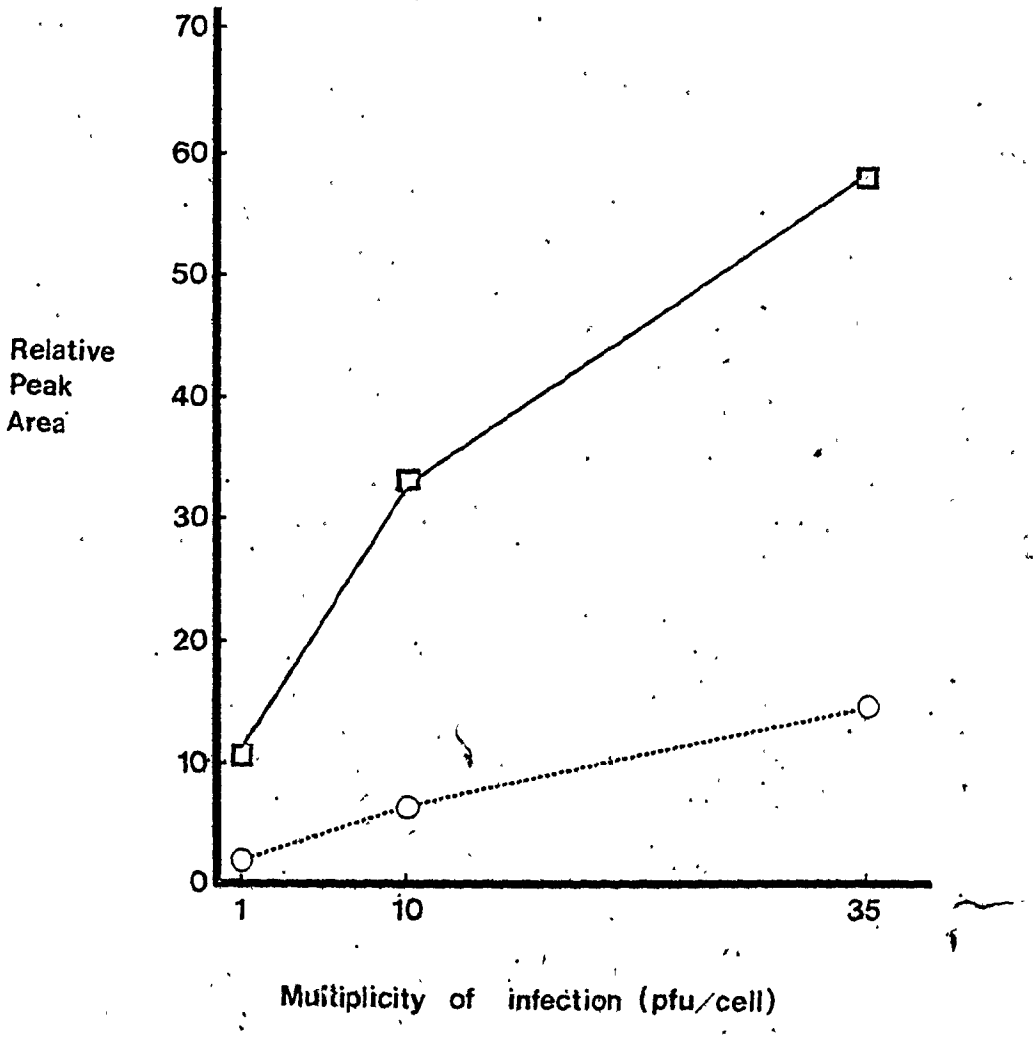


Figure 17. Relative synthesis of the 58,000 dalton protein in KB cells infected at moi's of 1, 10 and 35 pfu/cell with Ad 5 wt and hr 3. The relative areas under densitometer scans of appropriate bands in the autoradiographs in figure 15 were used as a measure of the incorporation of ^{35}S-methionine into the 58,000 dalton antigen. WT (□—□); hr 3, (○····○).

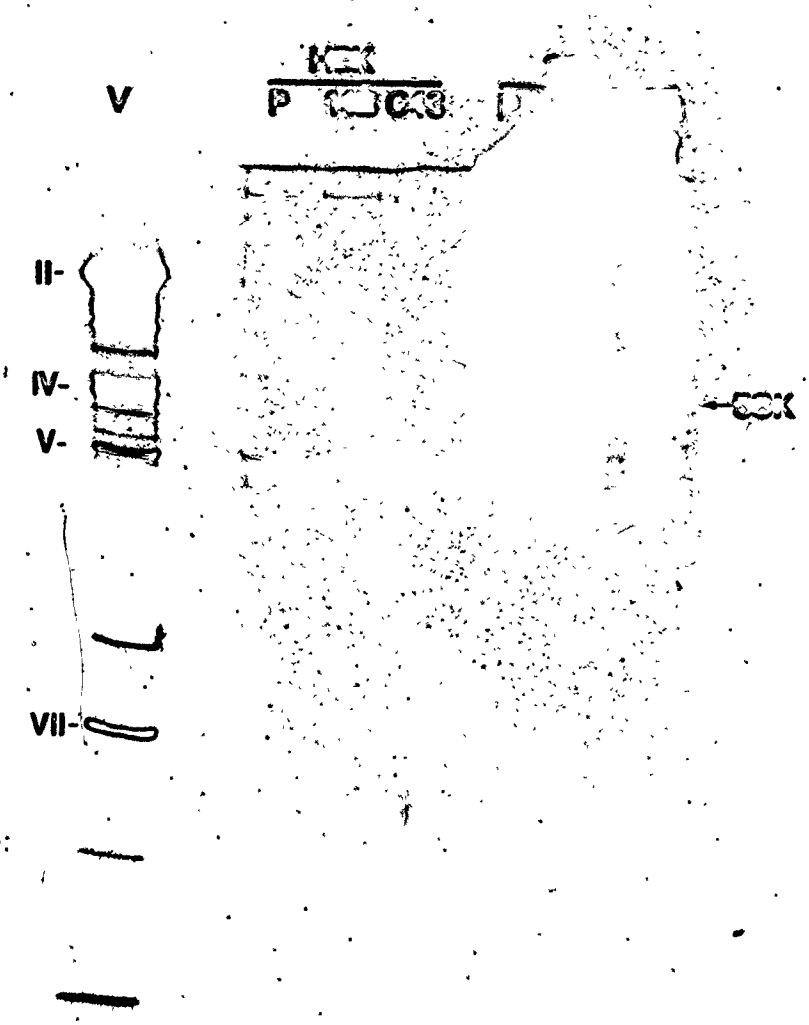
In hr 6 infected cells, more 72,000 dalton antigen was produced than after wild type infection. In addition, the amount of 72,000 dalton protein in immunoprecipitates from hr 6 infected cells was approximately the same at all moi's tested. The group I mutant hr 3 induced the synthesis of the 72,000, 67,000, 58,000 and 44,000 dalton proteins at all three moi's used, although production was reduced relative to wild type. More of both the 58,000 and 72,000 dalton proteins were produced as the moi was increased.

Also shown in figure 15 are the results of an experiment in which Ara C was used to prevent DNA synthesis. The fact that the viral antigens of molecular weight 72,000 and 58,000 were immunoprecipitated from the Ara C treated cultures indicates that these proteins are synthesized during the early phase of lytic infection.

F. Polypeptides immunoprecipitated from Ad 5 transformed cells.

Since the host range mutants were selected on the basis of their ability to replicate efficiently in the Ad 5 transformed human cell line (293 cells) and not in HeLa or KB cells, it was of interest to determine what polypeptides could be immunoprecipitated from these Ad 5 transformed cells. Secondary cultures of normal human embryonic kidney (HEK) and 293 cells were labelled for 6 hours with ^{35}S-methionine and immunoprecipitated with P, 14b or C43 antiserum by the double antibody technique. Figure 18 shows an autoradiograph of labelled polypeptides immunoprecipitated from HEK and 293 cells. Both C43 and 14b antisera, and to a lesser extent, P antiserum, immunoprecipitated a protein migrating as a diffuse band of molecular weight 58,000 from 293 cell extracts. This polypeptide

Figure 18. Immunoprecipitation of labelled polypeptides from human embryonic kidney (HEK) and Ad 5 transformed human embryonic kidney cells (293) using the double antibody method. Cells were labelled and immunoprecipitated as described in materials and methods. Symbols are as described in figure 5.


V
P
II-
IV-
V-
VII-

was not detected in HEK cells, nor was any other protein immunoprecipitated exclusively from the 293 cells.

Figure 19 shows the labelled polypeptides immunoprecipitated from a number of additional Ad 5 transformed cell lines using the double antibody method and 14b antiserum. Information on these rat cell lines is summarized in Table 1. An additional experiment comparing immunoprecipitates obtained from HEK and 293 cell extracts is also shown. Results obtained from extracts of mock and wild type infected KB cells and from normal baby rat kidney cells are included as controls.

Immunoprecipitates from all four Ad 5 transformed rat cell lines exhibit a 58,000 dalton protein which comigrates with a polypeptide of identical molecular weight found in 293 cells and in wild type Ad 5 infected KB cells. This protein was not detected in the untransformed baby rat kidney or human embryonic kidney cells. The 14b antiserum also immunoprecipitated a number of other polypeptides from transformed cells. Of these, only the polypeptides of molecular weight 15,000 and 11,000 were consistently detected in different transformed cells and therefore may be virus-specific. However, these proteins were not immunoprecipitated from Ad 5-transformed cells when the protein A-Sepharose method was used (see below). Hence, like the 10,500 dalton protein found in infected cell extracts, the significance of these low molecular weight polypeptides is unclear.

Labelled polypeptides were also immunoprecipitated from Ad 5 transformed cell lines using the protein A-Sepharose technique. Figure 20 shows immunoprecipitates obtained from the four adenovirus transformed rat cell lines described immediately above and the 14b hamster cell line.

Figure 19. Immunoprecipitation of labelled polypeptides from Ad 5 infected and transformed cells using the double antibody technique. M, mock infected KB cells; WT, KB cells infected with wt Ad 5; HEK, human embryonic kidney cells; 293, Ad 5 transformed HEK; BRK, baby rat kidney cells; Ad 5 transformed rat cells, 424-C1, 822-C2, 512-C8, and 637-C3. All immunoprecipitations were performed using 14b antiserum.

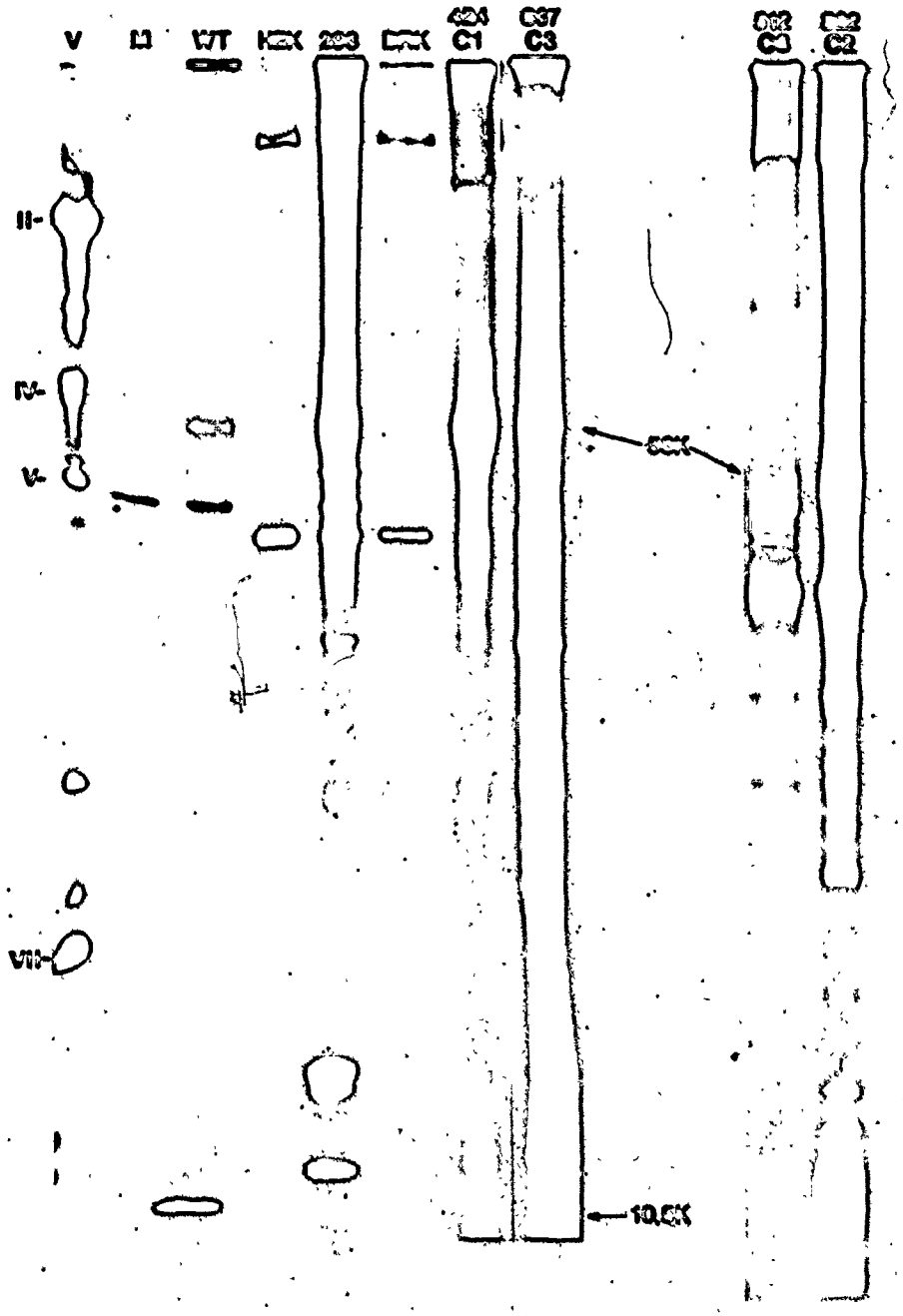
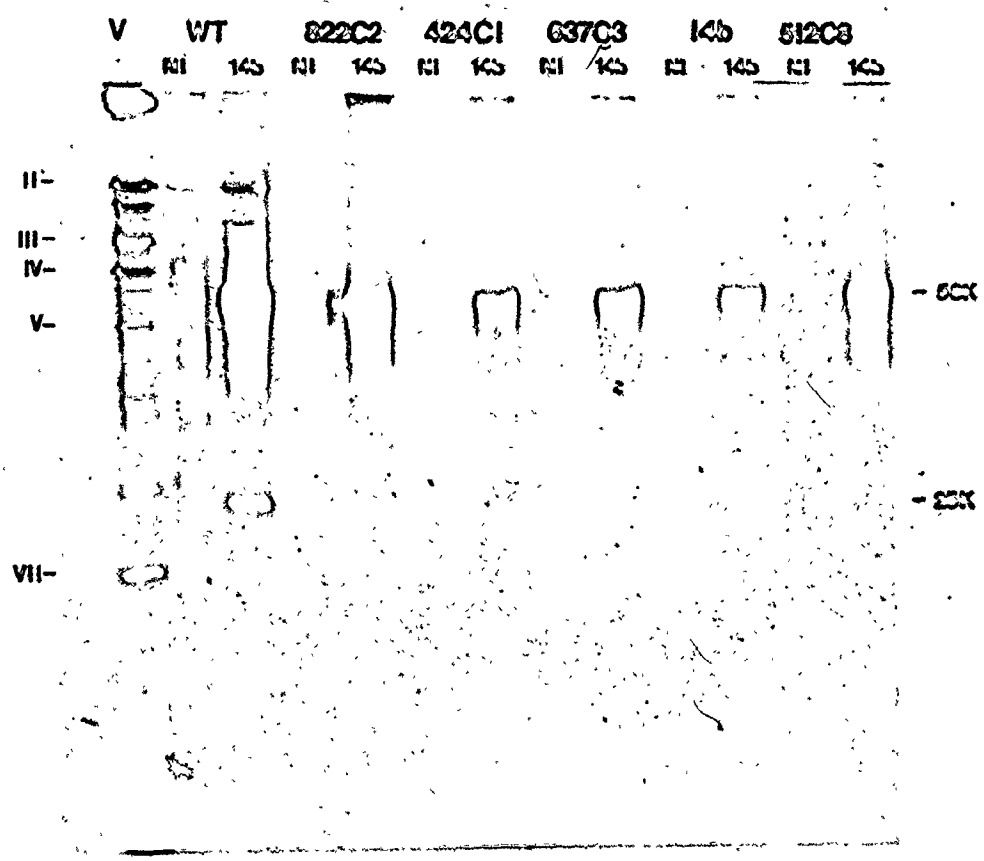

V
WT
C1
C3
C3
C2
II-
VII-
10.CK

Figure 20. Immunoprecipitation of labelled polypeptides from infected and Ad 5 transformed cells using the protein A-Sepharose technique. WT, KB cells infected with wt Ad 5; Ad 5 transformed rat cells, 822-C2, 424-C1, 637-C3 and 512-C8; 14b, Ad 5 transformed hamster cells. Cell extracts were immunoprecipitated with both non-immune hamster sera (NI) and 14b antiserum (14b).

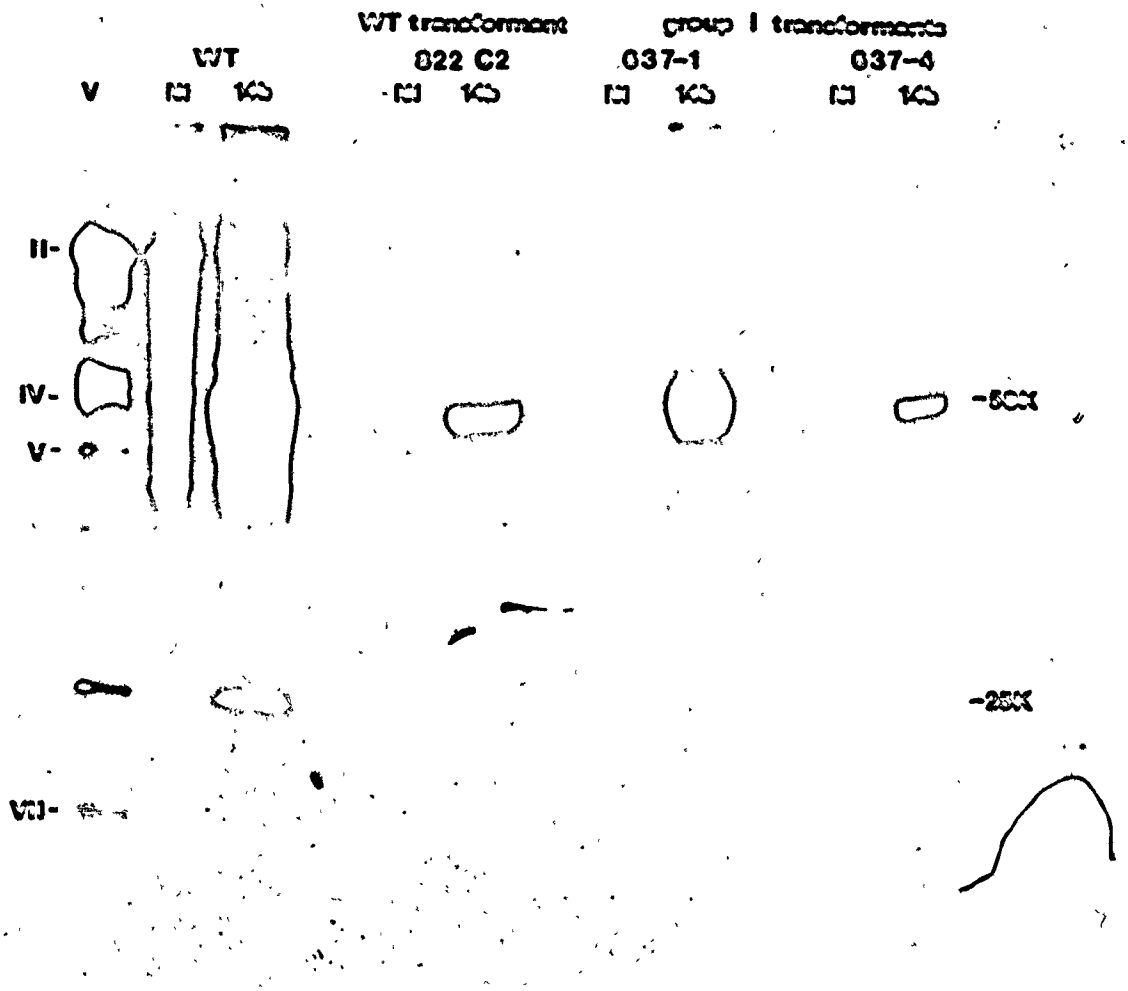

V
WT
822C2
424C1
637C3
14b
512C8
NI
14S
II–
III–
IV–
V–
VII–
– 6CX
– 2CX

Cytoplasmic extracts prepared from these cell lines were immunoprecipitated with either non-immune hamster sera or 14b antiserum. A 58,000 dalton polypeptide is clearly seen in immunoprecipitates from all the Ad 5 transformed rat cell lines and from the Ad 5 transformed hamster cell line, 14b. This protein is indistinguishable from that detected in wild type Ad 5 infected KB cells and is not detected in the cytoplasmic extracts immunoprecipitated with non-immune hamster sera. No other polypeptides specific to the 14b immunoprecipitates were detected. In addition, nuclear extracts were also routinely prepared from Ad 5 transformed cells and immunoprecipitated with 14b antiserum using the protein A-Sepharose method. In some experiments the 58,000 dalton antigen was observed in these extracts (data not shown).

Extracts of group I mutant transformed cells were also prepared to determine whether any viral specific polypeptides could be immunoprecipitated from these cells. Figure 21 compares polypeptides from an Ad 5 wild type transformant (822-C2) to those from two cell lines, 637-1 and 637-4, transformed by group I mutants hr 3 and hr 1, respectively. The 58,000 dalton protein is clearly seen in both mutant transformed cell lines.

The above data shows that a polypeptide of 58,000 daltons could be detected in all Ad 5 transformed cell lines tested. However, it must be pointed out that in two of these cell lines, 512-C8 and 293, this protein was not detected consistently. With 293 cells, this protein was observed in 3 out of 10 experiments and in the case of the 512-C8 cell line only 3 out of more than 20 trials were positive. The 512-C8 rat cell line was transformed with a viral DNA fragment representing the left 8.0% of the Ad

Figure 21. Immunoprecipitation of labelled polypeptides from infected cells and from cells transformed by wt or hr mutant virus using the protein A-Sepharose technique. KB cells infected with wt Ad 5 (WT); Rat cells transformed with wt Ad 5 (822-C2) or with group I mutants hr 3 (637-1) or hr 1 (637-4). Cytoplasmic extracts were immunoprecipitated with both non-immune hamster (NI) and 14b antiserum (14b).


WT transformant
group I transformants
WT
822 C2
037-1
037-4
V
-
+
-
+
-
+
II-
IV-
V-
-500x
-250x
VII-

genome (Graham et al, 1974). Three other rat lines transformed by the same DNA fragment have each been tested 2 or 3 times with negative results (data not shown). At present, the reason for the variability in these results is unknown since in all other cell lines examined the 58,000 dalton protein was reproduceably observed. It should be noted that D. Rowe (personal communication) using 14b antiserum, has reproduceably immuno precipitated a polypeptide of identical molecular weight from the 293 cells.

III. Protein Kinase Activity Associated with Adenovirus 5 Tumor Antigens

A. Immunoprecipitation of protein kinase activity associated with Adenovirus 5 tumor antigens from productively infected KB cells.

The results presented in previous sections were directed towards identifying the polypeptides involved in the Ad 5 transformation process. Until recently, very little was known concerning the mechanism of action of such proteins. However, Collett and Erikson in 1978 showed that the transformation-specific gene product (src) of Rous sarcoma virus (RSV) is associated with protein kinase activity. Using antisera directed against RSV-coded polypeptides, it was found that phosphorylation of the antibody occured when immunoprecipitates containing the src gene product were incubated with (γ-^{32}P) ATP. The studies of Collett and Erikson prompted me to determine whether a similar activity was associated with the transforming proteins of Adenovirus 5.

To assay for such protein kinase activity the following method was used. KB cells infected with wt or hr\ Ad 5 were harvested 9 hours post

infection and a cytoplasmic extract was prepared and incubated with 14b antisera. Mock infected cultures and infected cell extracts treated with non-immune hamster serum were included as controls. Immune complexes isolated using protein A-Sepharose method were incubated with (γ-^{32}P) ATP as described in Materials and Methods and the resulting ^{32}P-labelled products were analyzed by SDS-polyacrylamide gel electrophoresis. An autoradiograph obtained from such an experiment is shown in figure 22. With wild type adenovirus 5 infected cell extracts and 14b antiserum, a major ^{32}P-labelled polypeptide was observed which comigrated with the heavy chain of the antibody. Several minor labelled bands of higher molecular weight were also observed but no incorporation into the light chain of the antibody was detected. Little incorporation was observed using extracts from wild type infected cells immunoprecipitated with non-immune hamster serum or with extracts from mock infected cells immunoprecipitated with 14b antiserum. With the host range mutants hr 6 and hr 50 of group II and hr 1 and hr 3 of group I incorporation of ^{32}P into the heavy chain of the antibody was less than that observed with wild type Adenovirus 5. Different batches of non-immune hamster sera showed similar low levels of activity (data not shown). These results demonstrate that 14b antiserum will immunoprecipitate protein kinase activity from extracts of Ad 5 infected cells.

To determine if an exogenous protein would act as a substrate for this Ad 5-associated protein kinase activity, arginine-rich histones were added to the immunoprecipitates prior to addition of (γ-^{32}P) ATP. Figure 23 shows that arginine-rich histones, particularly histone H3, were

Figure 22. *In vitro* phosphorylation of immunoprecipitates from Ad 5 and mock infected KB cells. Confluent KB cells were infected with Ad 5 *wt*, group II mutants *hr* 6 and *hr* 50 and group I mutants *hr* 1 and *hr* 3. Cytoplasmic extracts were prepared 9 hours after infection and treated with 14b or non-immune hamster serum. Immunoprecipitates were incubated for 10 minutes with (γ ^{32}P) ATP as described in materials and methods and analysed by SDS-PAGE. Mock infected cells treated with 14b (a) or non-immune (b) serum. Wild type Ad 5 infected cells treated with 14b (c) or non-immune (d) serum. Cells infected with Ad 5 group II host range mutants *hr* 6 (e) and *hr* 50 (f) treated with 14b antiserum. Cells infected with Ad 5 group I host range mutants *hr* 1 (g) and *hr* 3 (h) treated with 14b antiserum. Coomassie blue stained gel of wild type infected cells treated with 14b antiserum (i).

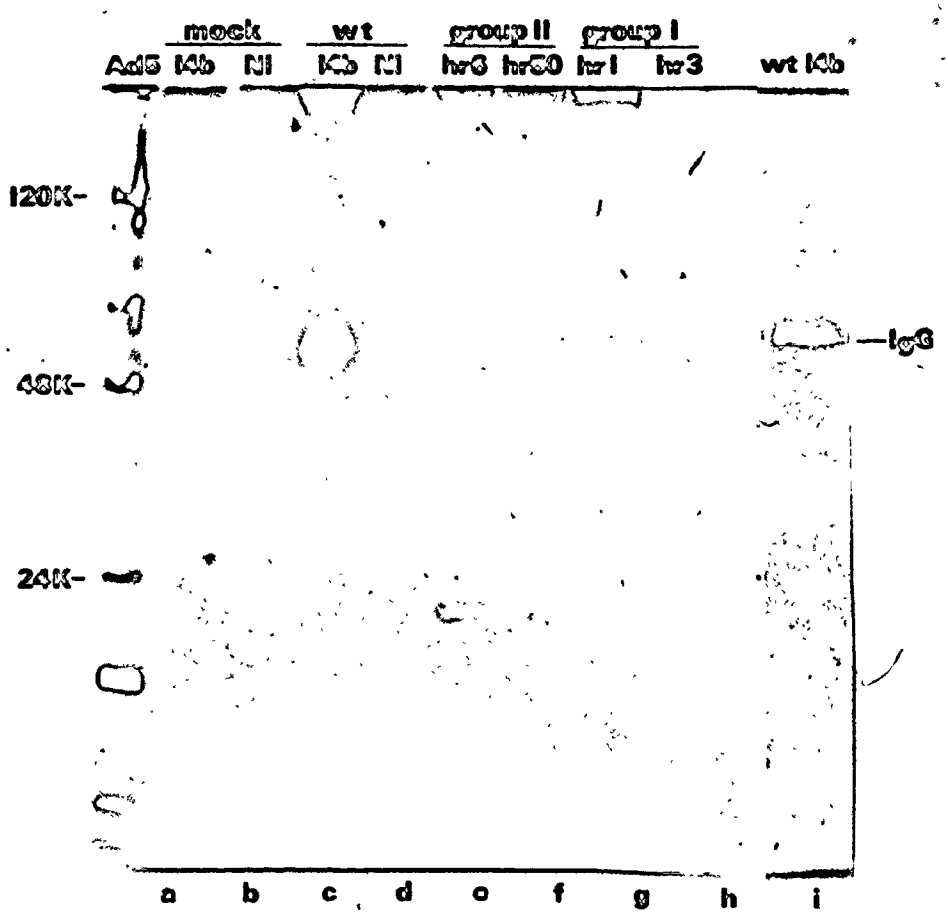
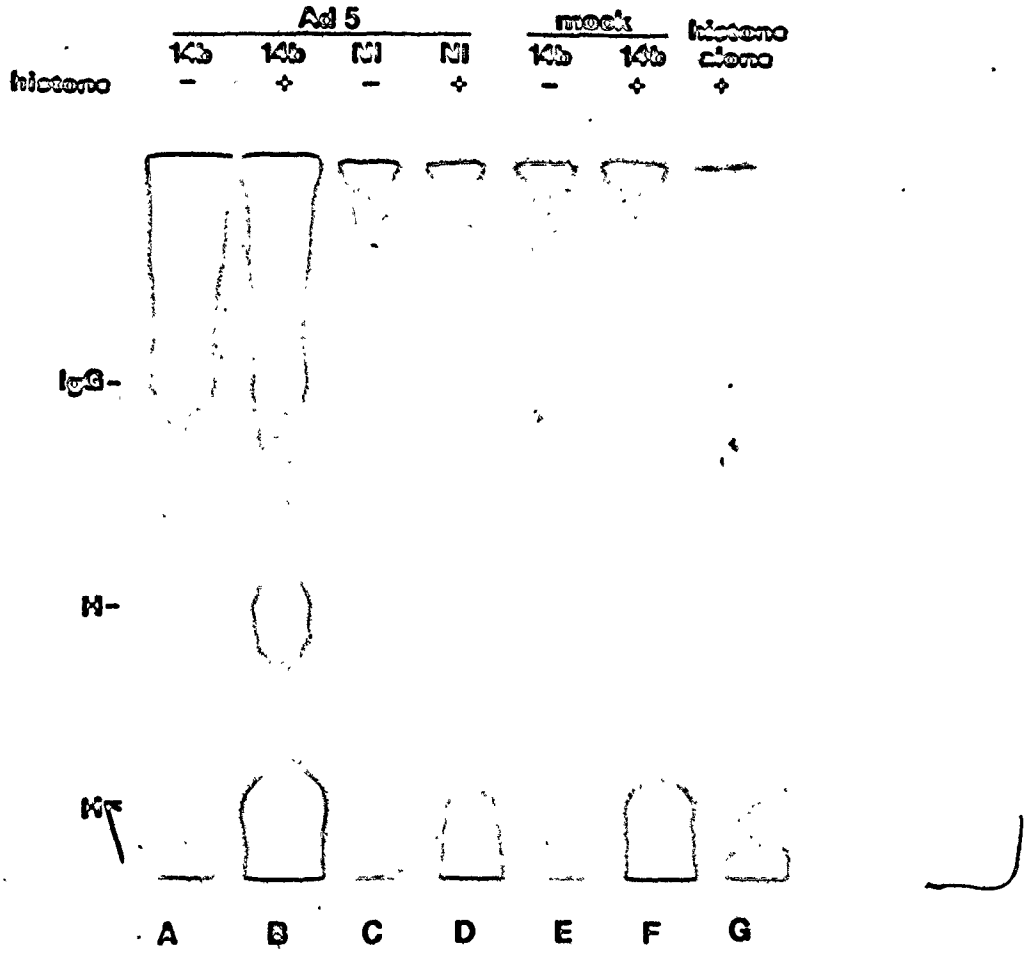

mock
wt
group II
group I
Ad5
Kb
NI
Kb
NI
hr3
hr80
hr1
hr3
wt Kb
120K-
48K-
24K-
IgG
a
b
c
d
e
f
g
h
i

Figure 23. *In vitro* phosphorylation of immunoprecipitates from Ad 5 and mock infected KB cells. Ad 5 infected or mock infected KB cells were harvested at 12 hours post infection and cytoplasmic extracts were prepared and treated with 14b or non-immune serum. Immunoprecipitates were incubated for 10 minutes with ATP (γ ^{32}P) as described in materials and methods, in the presence or absence of 75 µg of boiled arginine-rich histone and analyzed by SDS-PAGE. Ad 5 infected extracts with 14b antiserum, incubated in the absence (A) or presence (B) of histone. Ad 5 infected extracts with non-immune serum, incubated in the absence (C) or presence (D) of histone. Mock-infected extracts with 14b serum, incubated in the absence (E) and presence (F) of histone. Histone alone with no cell extract or antiserum (G).

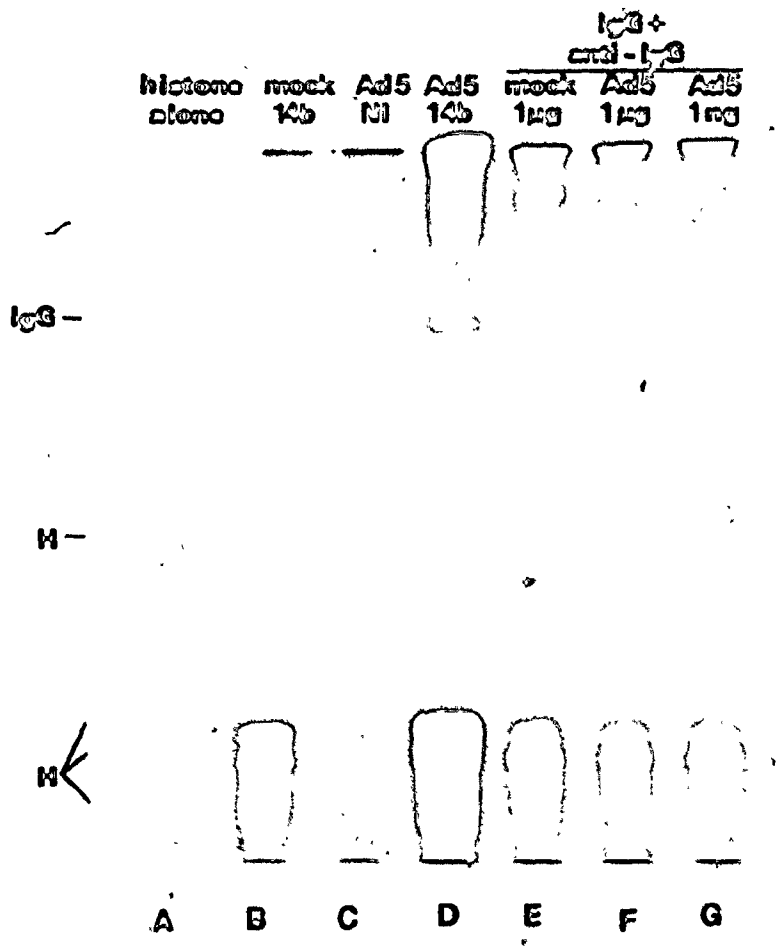

Ad 5
mock
hisomo
14h
14h
NI
NI
14h
14h
hisomo clono
hisomo
−
+
−
+
−
+
+
IgG-
M-
K-
A
B
C
D
E
F
G

phosphorylated by extracts from Ad 5-infected cells immunoprecipitated with 14b antiserum. Incorporation of ^{32}P into the heavy chain of the antibody occurred to a similar extent in the presence or absence of histone. Considerably less histone phosphorylation was observed in the case of immunoprecipitates from mock infected cells or from infected cell extracts incubated with non-immune hamster sera. In addition, preferential phosphorylation of histone H3 was not observed in these controls. When the histone preparation alone was incubated with (γ-^{32}P) ATP, little incorporation was observed. Since addition of histone provided a convenient and sensitive measure of the protein kinase activity it was routinely used in subsequent experiments.

B. Immunoprecipitation of protein kinase activity using 14b and heterologous antisera.

The phosphotransferase activity found in immunoprecipitates from Ad 5 infected cell extracts using 14b antiserum could be the result of host protein kinases nonspecifically trapped in the antigen-antibody complex. To examine this possibility, 1 μg or 1 ng of purified human IgG was reacted with sheep anti-human IgG antiserum in the presence of cytoplasmic extracts from mock and wild type infected KB cells. These immune complexes, and others from mock and Ad 5 infected cell extracts treated

Figure 24. *In vitro* phosphorylation in the presence of heterologous immunoprecipitates. To cell extracts prepared from mock and Ad 5 infected cells harvested 12 hours post infection, were added either 1 ng or 1 µg of human IgG followed by sheep anti-human IgG serum. The immunoprecipitates were bound to protein A-Sepharose beads and assayed for kinase activity in the presence of boiled histone. Histone alone (A); mock infected with 14b serum (B); Ad 5 infected with 14b (C) or non-immune (D) serum. Mock infected with 1 µg human IgG, and anti-IgG serum (E). Ad 5 infected with 1 µg (F) or 1 ng (G) human IgG and anti-IgG serum.


IgG+
anti-IgG
histone
clone
mock
14b
Ad5
Nil
Ad5
14b
mock
1µg
Ad5
1µg
Ad5
1ng
IgG –
H –
H
A
B
C
D
E
F
G

with non-immune hamster or 14b serum, were isolated, incubated with (γ ^{32}P) ATP and analyzed by gel electrophoresis. Figure 24 shows that when human IgG and sheep antihuman IgG antiserum were used, little ^{32}P incorporation was observed relative to that seen with immunoprecipitates from infected KB cell extracts treated with 14b antiserum. This low level of phosphorylation activity was observed using either 1 µg or 1 ng of human IgG. In addition, following immunoprecipitation of human IgG, no phosphorylation of the heavy chain of the antibody was observed and the small amount of ^{32}P incorporation seen was evenly distributed among all histone types. The Ad 5 tumor antigen related protein kinase phosphorylated the heavy chain of the antibody and again demonstrated a preference for histone H3. Similar results were obtained in parallel experiments using bovine serum albumen (BSA) and rabbit anti-BSA (data not shown). Taken together, these data suggest, but do not prove, that the Ad 5 related protein kinase activity results from a virally induced enzyme and not from non-specific entrapment of a host protein kinase.

C. Characterization of the Adenovirus 5 tumor antigen-related protein kinase.

A number of properties of the Ad 5 tumor antigen-related protein kinase were characterized. Protein kinase assays were carried out as described in the legends to figures 25 to 31, using histones as a substrate and the amount of ^{32}P incorporated into TCA insoluble material was determined as described in the materials and methods. Samples were also analyzed by SDS-polyacrylamide gel electrophoresis to confirm results

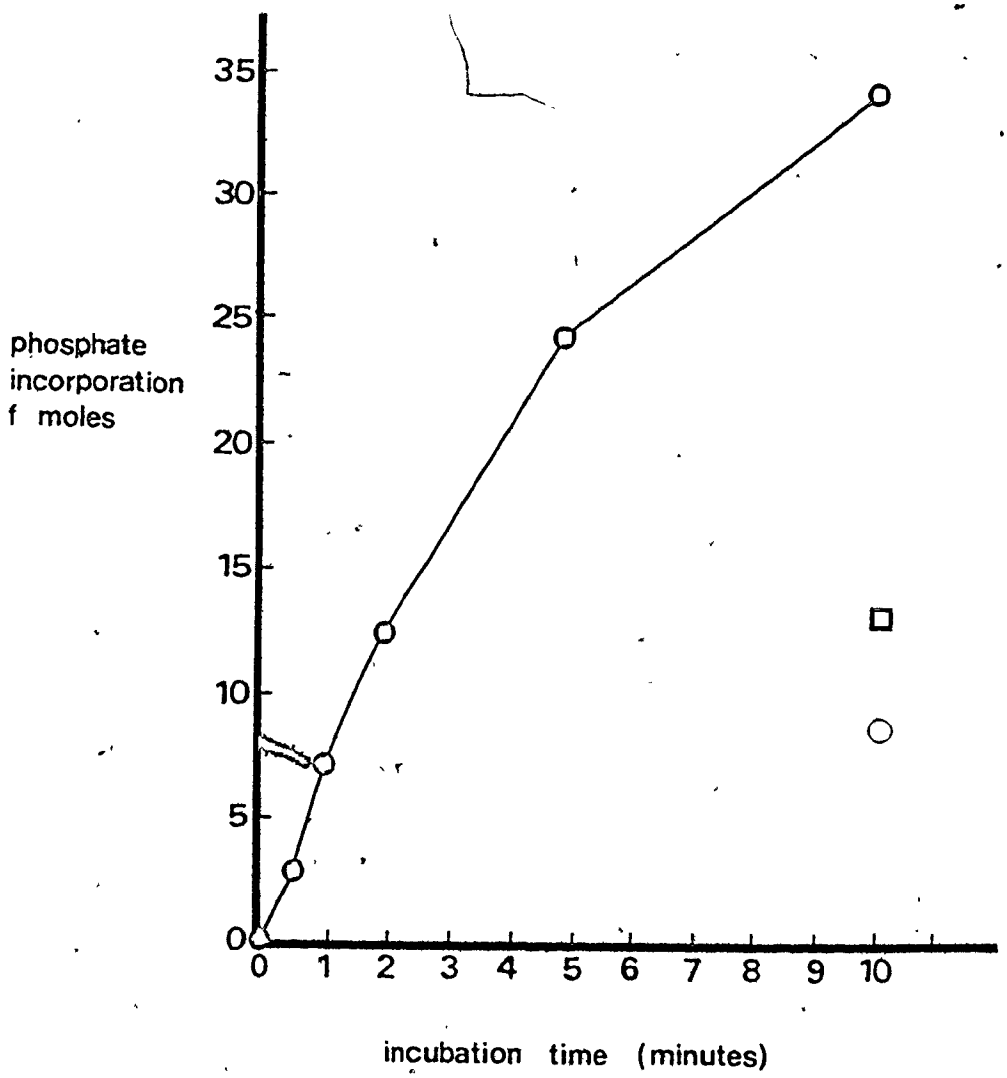


Figure 25. Rate of in vitro phosphorylation in immunoprecipitates from KB cells infected with wt Ad 5. Immunoprecipitates were prepared using 14b antiserum and incubated for the periods indicated with (γ-^{32}P) ATP and histone. The amount of ^{32}P incorporation into TCA precipitable material was determined as described in materials and methods. The number of f moles of phosphate incorporated was calculated from the radioactivity present. Ad 5 infected cell extracts prepared 12 hours post infection and immunoprecipitated with 14b antiserum, (O—O); mock infected cells and 14b antiserum (□); histone incubated alone with (γ-^{32}P) ATP in the absence of immunoprecipitate (0).

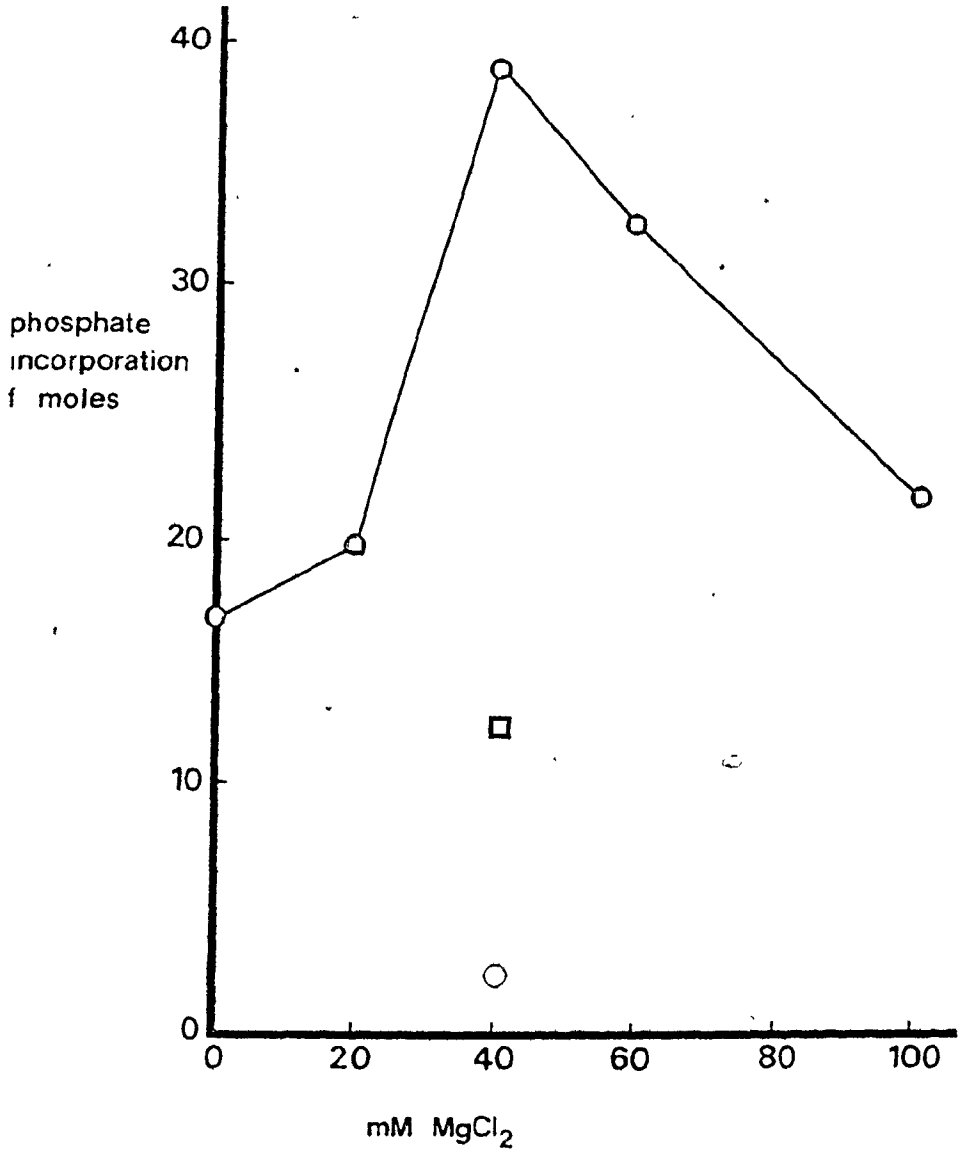


Figure 26. Effect of Mg^{2+} concentration on the immunoreactive protein kinase activity. Immunoprecipitates prepared using 14b antiserum and extracts of wt Ad 5 infected KB cells harvested 12 hours post infection were incubated for 1 minute with (γ-^{32}P) ATP and different concentrations of $MgCl_2$. The number of fmoles of phosphate incorporated was calculated as described in figure 25. The amount of phosphate incorporation obtained using histone alone has been subtracted. Ad 5 infected cell extracts immunoprecipitated with 14b antiserum (O—O); mock infected cells and 14b antiserum (□); Ad 5 infected cell extracts immunoprecipitated with non-immune hamster serum (0).

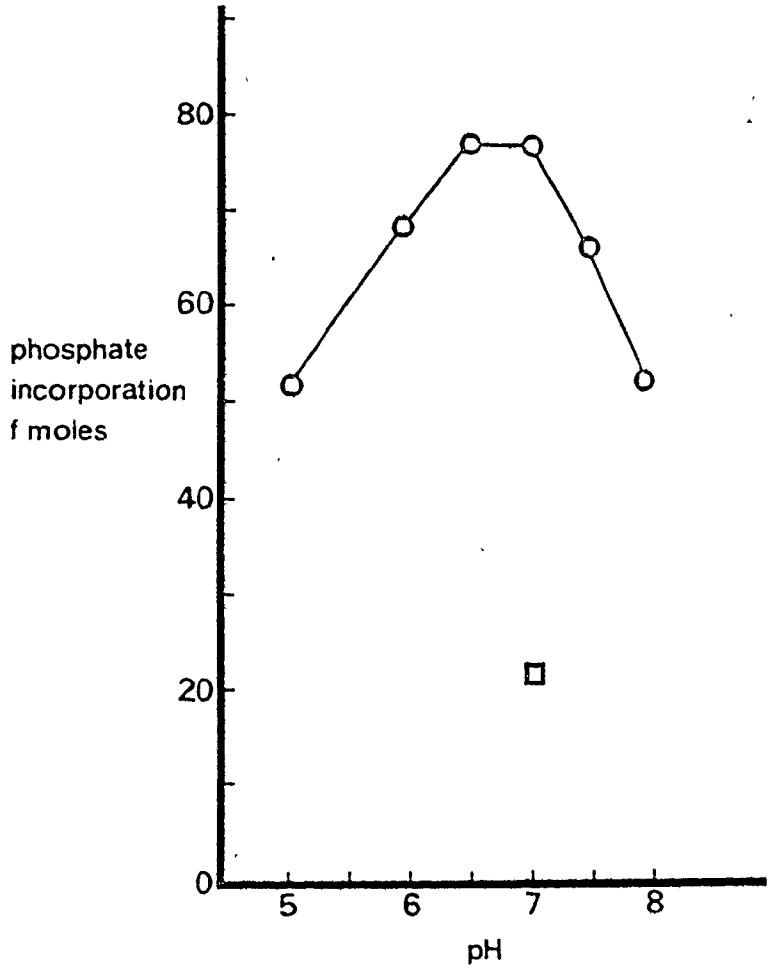


Figure 27. Effect of pH on the immunoreactive protein kinase activity. Immunoprecipitates from wt Ad 5 infected KB cells prepared using 14b antiserum were incubated for 1 min with (γ-^{32}P) ATP at pH 5, 6, 6.5, 7, 7.5 and 8. The number of fmoles of phosphate incorporated was calculated as described in figure 25. The amount of phosphate incorporation obtained using histone alone has been subtracted. Ad 5 infected cell extracts prepared 12 hours post infection and immunoprecipitated with 14b antiserum (O—O); mock infected cells immunoprecipitated with 14b antiserum (□).

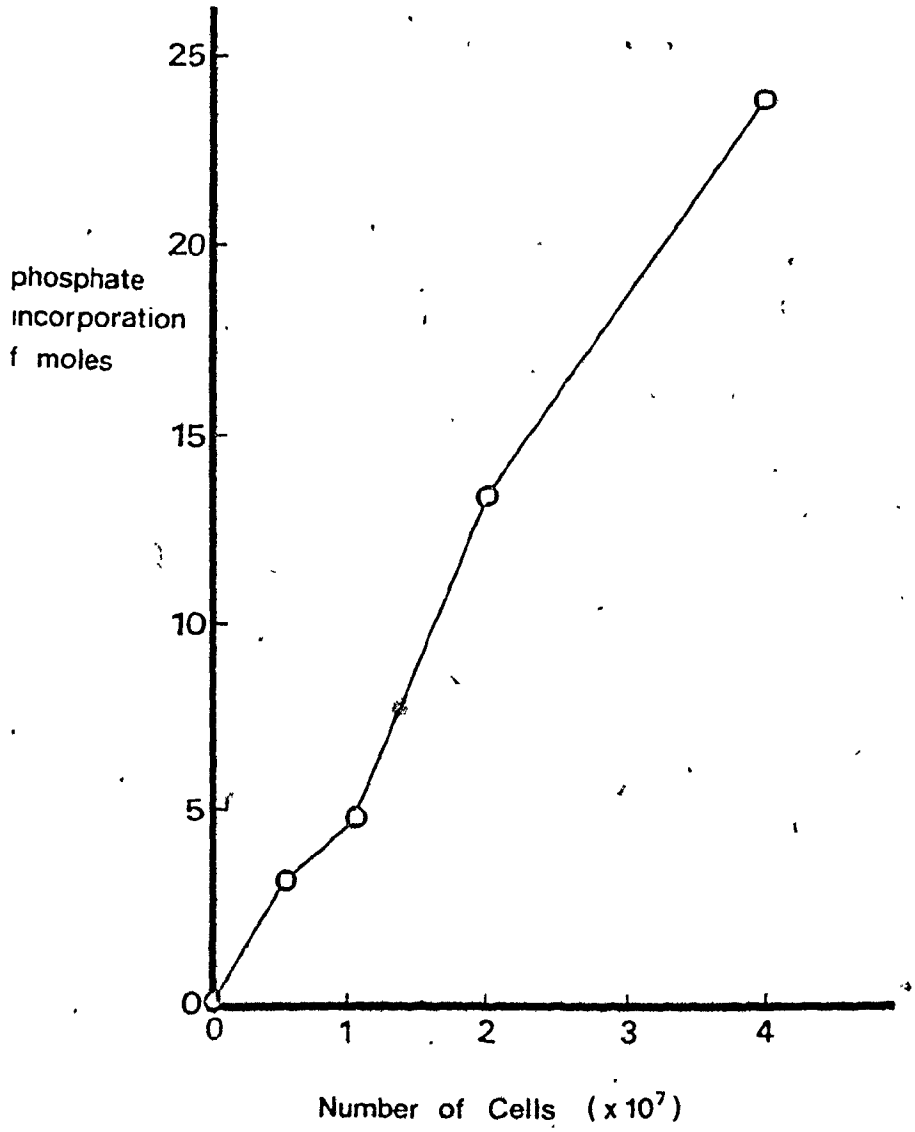


Figure 28. Effect of amount of immunoprecipitate on the immunoreactive protein kinase activity. Immunoprecipitates were prepared using 14b antiserum and extracts from 1 x 10^8 Ad 5 infected cells harvested 12 hours post infection. The immunoprecipitates were combined and then divided into aliquots representing material from 0.5, 1, 2 and 4 x 10^7 cells. The aliquots were then incubated for 1 min with (γ-^{32}P) ATP. The number of fmoles was determined as described in figure 25. The amount of phosphate incorporation obtained using histone alone has been subtracted.

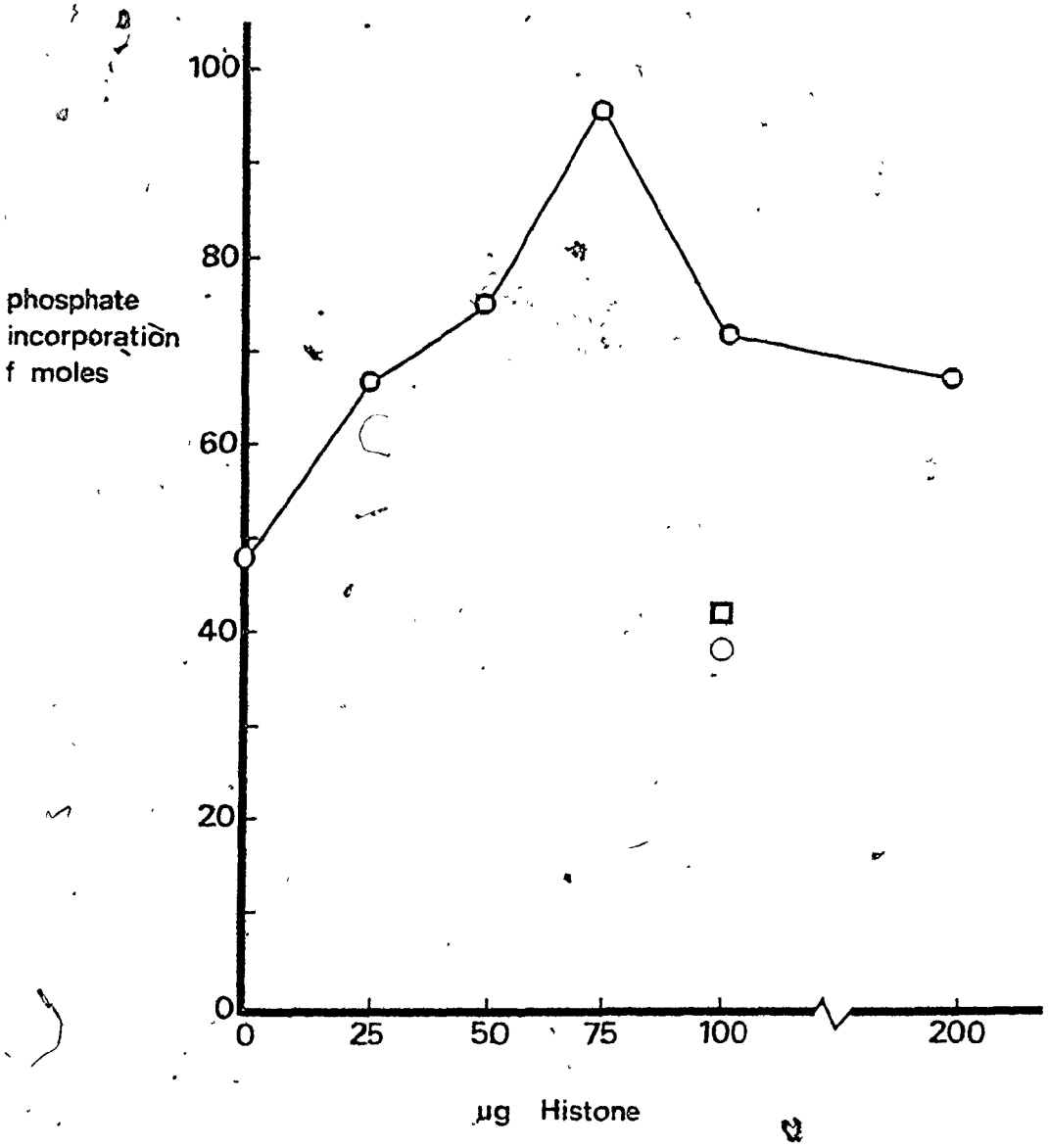


Figure 29. The effect of histone concentration on the immunoreactive protein kinase activity. Immunoprecipitates prepared using 14b antiserum and extracts of wt Ad 5 infected KB cells harvested 12 hours post infection were incubated for 1 min with (γ-^{32}P) ATP and different concentrations of histone. The number of fmoles of phosphate incorporated was calculated as described in figure 25. The amount of phosphate incorporation obtained using histone alone has been subtracted. Ad 5 infected cell extracts immunoprecipitated with 14b antiserum (O–O); mock infected cells and 14b antiserum (□); Ad 5 infected cells and non-immune hamster serum (O).

obtained using TCA precipitation (data not shown) and the resulting autoradiographs corresponded well with the direct measurements of ^{32}P incorporation.

The rate of the protein kinase reaction was monitored by incubating immunoprecipitates with (γ ^{32}P) ATP at 30°C for varying periods of time. As shown in figure 25, incorporation of ^{32}P was linear for 2 minutes and declined thereafter. All subsequent experiments involving characterization of the protein kinase activity were performed with an incubation time of 2 minutes or less (i.e. when the reaction rate was linear).

The effect of magnesium ion concentration on the immunoprecipitable protein kinase activity is shown in figure 26. Maximal incorporation of ^{32}P was observed at a concentration of 40 mM $MgCl_2$.

The effect of pH on the rate of phosphorylation is shown in figure 27. The pH optimum was relatively broad with maximal activity occurring between pH 7.0 - 7.5. Similar values were obtained when Tris buffer was substituted for MES buffer (data not shown).

Figure 28 shows phosphotransferase activity using varying amounts of immunoprecipitate. Incorporation of ^{32}P was directly proportional to the amount of immunoprecipitate present in the reaction.

As shown in figure 29, incorporation of ^{32}P into added histone was maximal with 75 µg of histone. Higher quantities of histone added to the reaction resulted in reduced activity.

To determine if immunoprecipitable protein kinase activity was dependent upon cyclic nucleotides, immunoprecipitates were incubated with (γ ^{32}P) ATP and histone in the presence of varying concentrations of cyclic AMP or cyclic GMP. As a control, purified cyclic AMP-dependent

beef heart protein kinase was assayed under similar conditions. Figure 30 shows that in the presence of 10^{-6} or 10^{-5} M cyclic AMP, the beef heart protein kinase was stimulated more than 3-fold. With immunoprecipitates from Ad 5 infected extracts, little stimulation was observed with either cyclic nucleotide. The results presented here show the highest level of stimulation observed in a number of experiments, so that it appears unlikely the immunoprecipitable protein kinase is cyclic nucleotide dependent. The small amount of stimulation observed may be due to the presence of low levels of contaminating host enzymes in the immunoprecipitate.

Figure 31 shows the effect of antibody dilution. The 14b antiserum was diluted in water and immunoprecipitations were carried out using a constant amount of infected cytoplasmic extract. No significant change in ^{32}P incorporation was seen with the antiserum diluted as much as 1:4, but at higher dilutions protein kinase activity decreased. Hence, with undiluted antiserum, immunoprecipitation of protein kinase activity was occurring under conditions of antibody excess.

D. Time of appearance of the tumor antigen related protein kinase activity during productive infection.

To determine when during the productive cycle of Ad 5 infection protein kinase activity appeared, cytoplasmic extracts from Ad 5 infected KB cells were prepared at various times after infection, immunoprecipitated with 14b antiserum and incubated with (γ ^{32}P) ATP for 1 minute. As seen in figure 32, the immunoreactive protein kinase activity was first detected between 6-8 hours post infection. The reaction rate increased

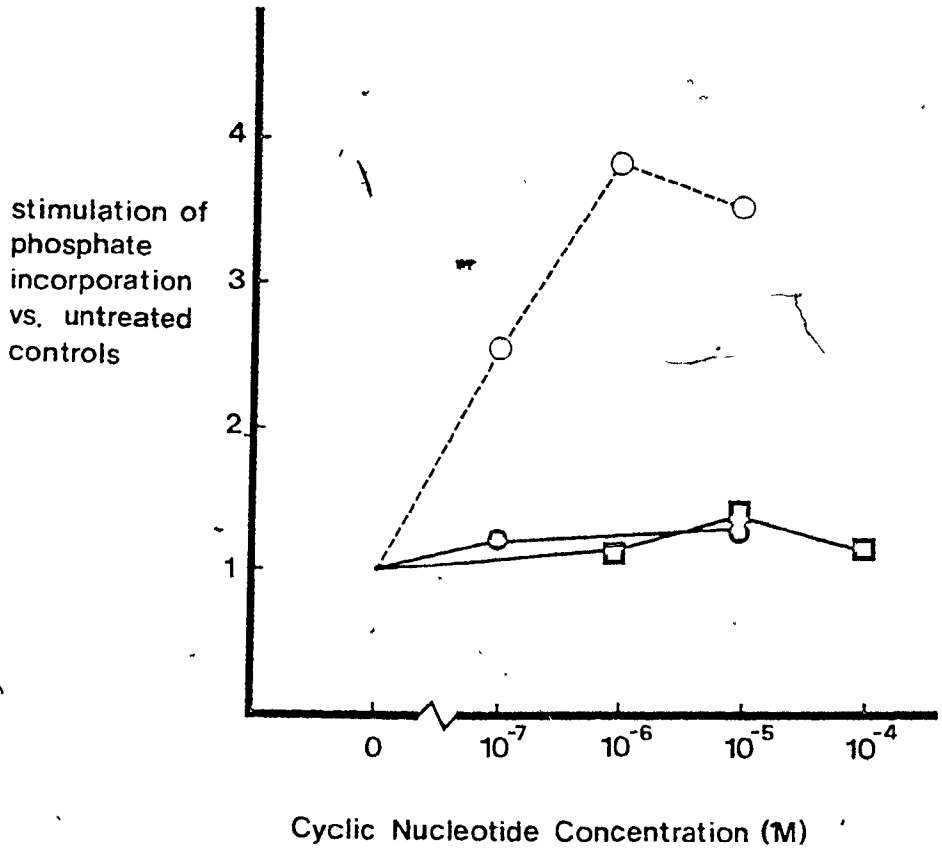


Figure 30. The effect of cyclic nucleotides on the immunoreactive protein kinase activity. Immunoprecipitates prepared using 14b antiserum and extracts of wt Ad. 5 infected KB cells harvested 12 hours post infection were incubated for 2 min with (γ-^{32}P) ATP in the absence or presence of varying concentrations of cyclic AMP (O—O) or cyclic GMP (□—□). Purified cyclic AMP-dependent beef heart protein kinase assayed in the same fashion in the absence or presence of cyclic AMP (O--O). The data has been presented as the ratio of ^{32}P incorporated in the presence vs. the absence of cyclic nucleotides. The amount of ^{32}P incorporation obtained using histone alone has been subtracted.

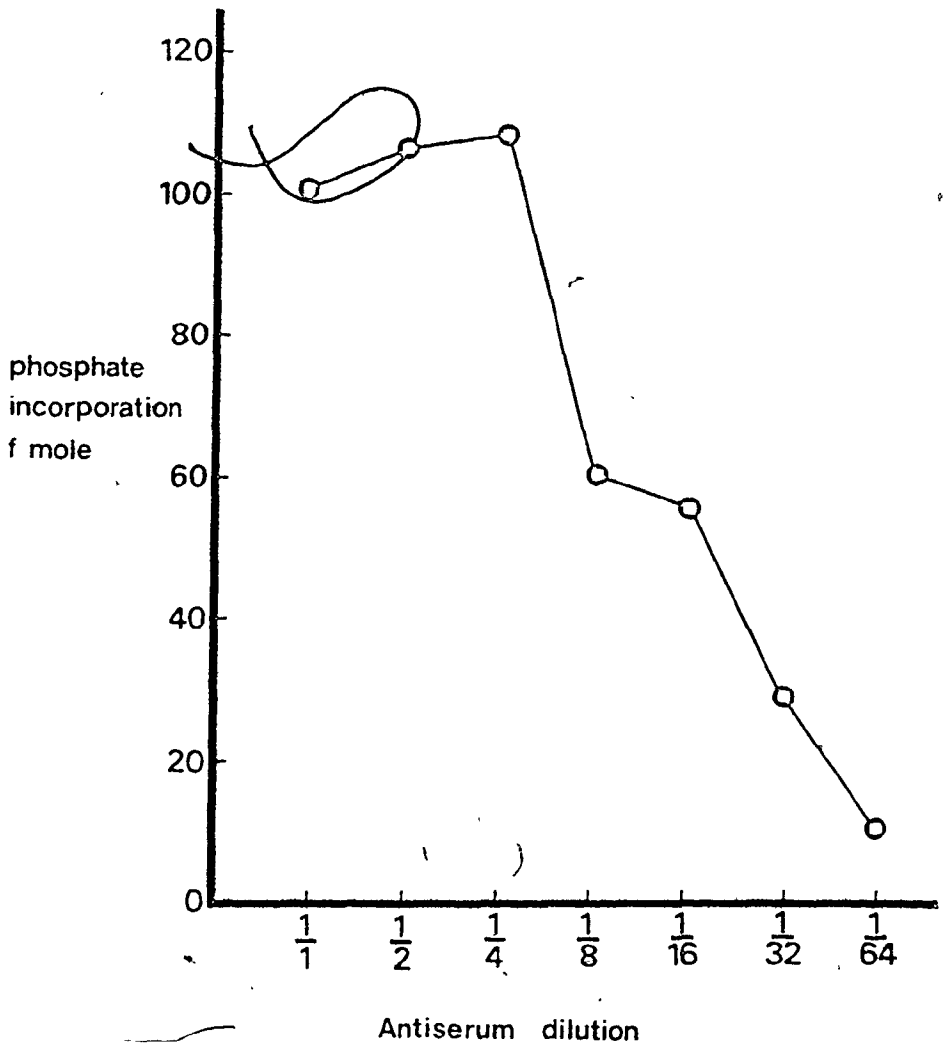


Figure 31. Effect of antiserum concentration on the immunoreactive protein kinase activity. The 14β antiserum was diluted in water as shown and immunoprecipitates were carried out using a constant amount of cell extract from Ad 5 infected KB cells harvested 12 hours post infection. Incubation with (γ-^{32}P) ATP was for 1 min. The number of fmoles of phosphate incorporated was calculated as described in figure 25. The amount of phosphate incorporation obtained using histone alone has been subtracted.

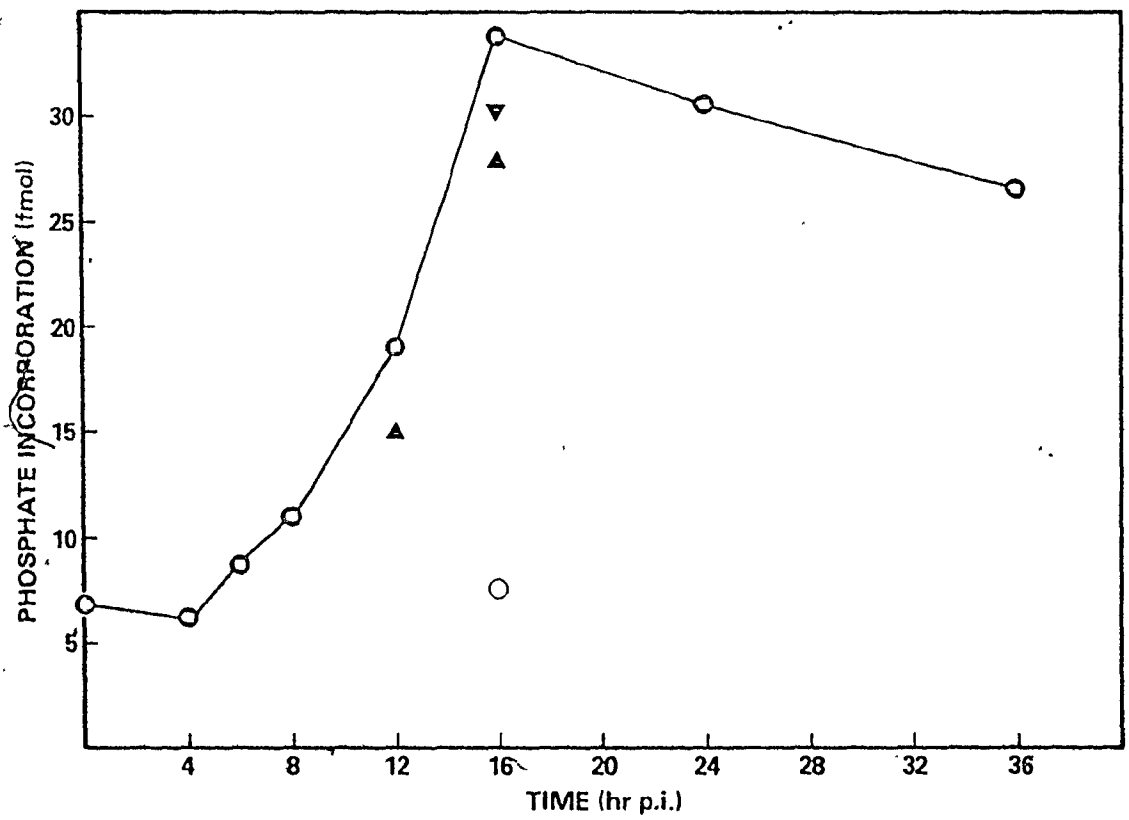


Figure 32. Appearance of Ad 5 tumor antigen-related protein kinase activity during the course of infection. Protein kinase activity was assayed in immunoprecipitates obtained from cells at various times after infection, using 14b antiserum. The number of fmoles of phosphate incorporated was calculated as described in figure 25.. Evidence (provided by Dr. F. Graham) showing the absence of viral DNA synthesis in the Ara C treated cultures was obtained by analytical ultracentrifugation. Some cultures were treated with cytosine arabinoside (Ara C) (5 or 20 µg/ml). (O—O) Ad 5 infected untreated cells. Ad 5 infected cells treated with Ara C at 5 (▼) or 20 (▲) µg/ml. (O) Ad 5 infected cells immunoprecipitated with non-immune serum.

up to 16 hours after infection and thereafter decreased. Figure 32 also shows results obtained using infected cells treated with cytosine arabinoside (Ara C) to block viral DNA replication. The protein kinase activity immunoprecipitated from such cells was comparable to that found with the untreated cultures. These results indicate that the immunoreactive protein kinase activity is associated with an early viral function.

E. Rate of phosphorylation in immunoprecipitates prepared from cells infected with wild type Adenovirus 5 or Adenovirus 5 host range mutants.

The data presented in figure 23 shows that Ad 5 host range mutants of both complementation groups are defective in the induction of immunoprecipitable protein kinase activity. To extend these observations, the rates of phosphorylation in immunoprecipitates from group I mutant hr 3 and group II mutant hr 6 infected cell extracts were compared to extracts from cells infected with wild type Ad 5. As shown in figure 33, with immunoprecipitates from wild type Ad 5 infected cell extracts, the rate of reaction was constant for about 2 minutes and then decreased. With extracts from hr 3 infected cells, the initial rate of reaction was lower than with preparations from wild type infected cells and after the 10 minute incubation the total amount of ^{32}P incorporation was less than with wild type. Immunoprecipitates from hr 6 infected cells exhibited a very low rate of ^{32}P incorporation. Results similar to these were obtained when

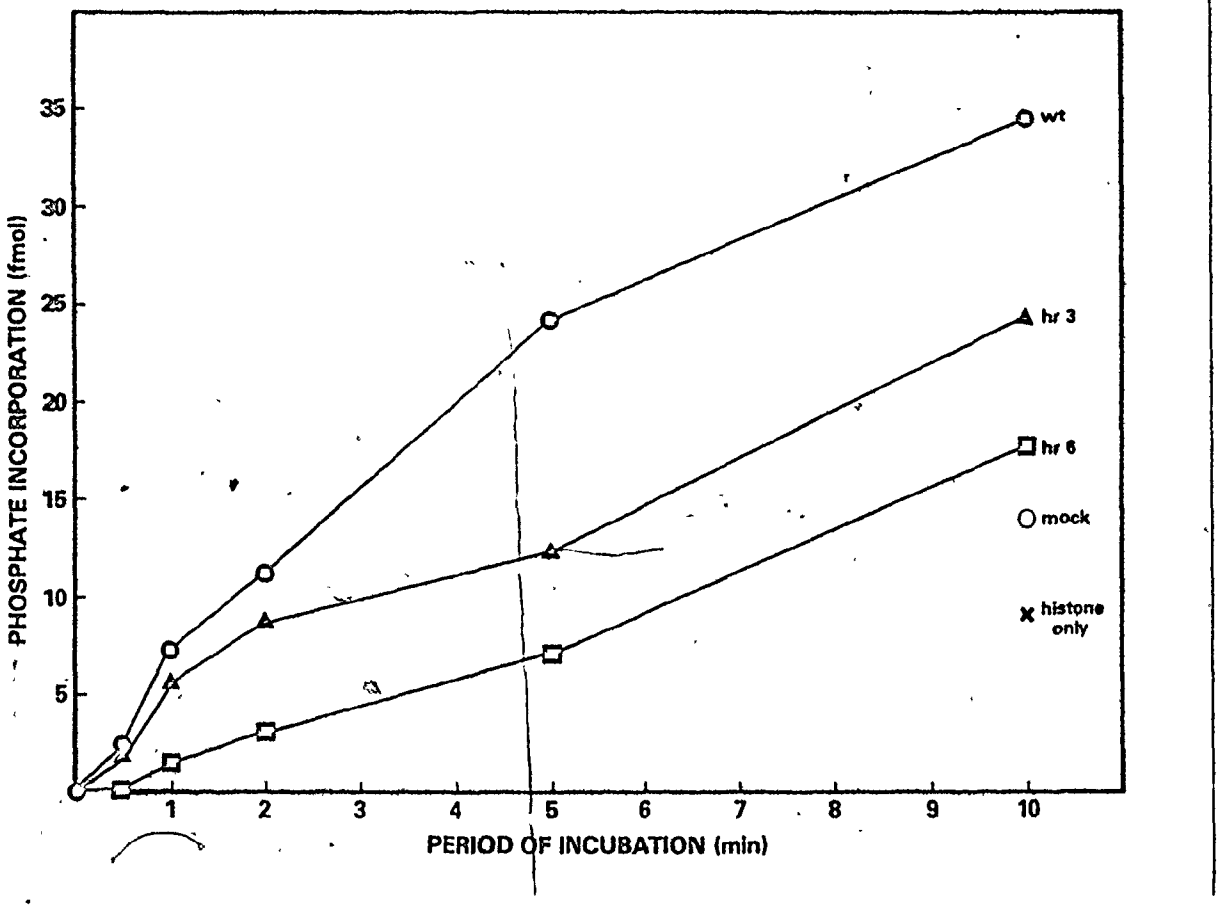


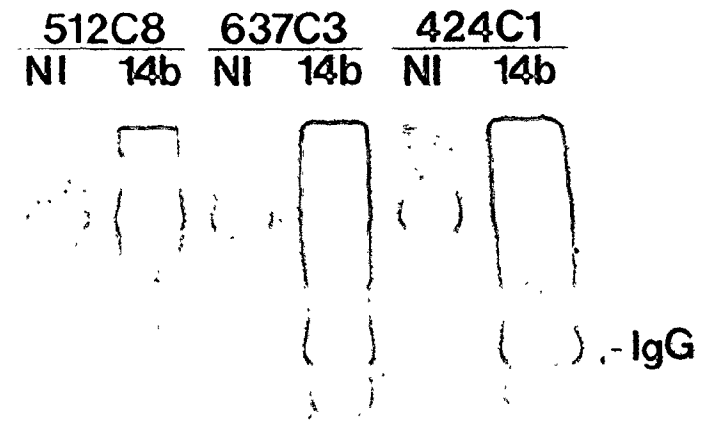
Figure 33. *In vitro* phosphorylation by immunoprecipitates from cells infected by wild type or host range mutants of Ad 5. KB cells were infected with Ad 5 *wt*, group I host range mutant *hr* 3 or group II host range mutant *hr* 6 and harvested 12 hours post infection. Immunoprecipitates prepared using 14b antiserum were incubated for different periods of time with ATP (γ-^{32}P) and histone. The number of fmoles incorporated was calculated as described in figure 25. Wild type Ad 5 (O—O); *hr* 3 (Δ—Δ); *hr* 6 (□—□); mock infected (O); histone alone (X).

immunoprecipitates were incubated with (γ ^{32}P) ATP in the absence of added histone (data not shown). This data indicates that the hr mutants of both complementation groups are deficient in inducing the Ad 5 related protein kinase, group II more so than group I.

F. Detection of the Ad 5 tumor antigen related protein kinase in Ad 5 transformed rat cells.

It was of interest to determine if the immunoreactive protein kinase activity seen with productively infected cells could also be detected in Ad 5 transformed cells. The three Ad 5 transformed cell lines which were examined, 637-C3, 424-C1 and 512-C8, have been described above. Extracts from approximately 10^8 cells were immunoprecipitated with either non-immune hamster serum or 14b antiserum and incubated for 10 minutes with (γ ^{32}P) ATP and histone. Figure 34 shows an autoradiograph of the labelled products separated on the gel. With extracts from two transformed cell lines, 637-C3 and 424-C1, more protein kinase activity was observed using 14b antiserum than with non-immune serum. The pattern of phosphorylation in these 14b immunoprecipitates is similar to that seen when extracts of lytically infected cells were used. In the case of the 512-C8 cell line, which contains only the leftmost 8% of the viral genome, little or no activity was observed.

Figure 34. *In vitro* phosphorylation in immunoprecipitates from Ad 5 transformed cells using non-immune hamster serum (NI) or 14b antiserum (14b). Immunoprecipitates were incubated for 10 minutes with (γ-^{32}P) ATP in the presence of histone as described in materials and methods and analysed by SDS-PAGE.


512C8
NI
14b
637C3
NI
14b
424C1
NI
14b
IgG


H

DISCUSSION

As pointed out earlier, a number of studies have established that no more than the leftmost 11% of the Adenovirus genome is required for cell transformation. This region encompasses early region 1, one of 5 gene blocks expressed early in productive infection. The work presented here has focused on the polypeptides encoded by the E1 region of the viral genome. To assist in the study of these polypeptides, Ad 5 host range mutants have been used. These mutants are of particular interest because they contain lesions mapping within this leftmost early region of the viral genome and are altered in their ability to transform (Frost and Williams, 1978; Galos and Williams, personal communication; Graham et al, 1978).

The discussion of the work described here falls into three sections. The first section deals with the early viral proteins synthesized in wt infected cells. The second and third sections are concerned with the defects expressed by the hr mutants and the characterization of protein kinase activity found associated with the early Ad 5 polypeptides.

I. Early Viral Antigens in Adenovirus 5 Infected Cells

A. Virus-induced 58,000 dalton antigen.

The results presented here show the presence of a 58,000 dalton protein in Ad 5-infected KB cells. This antigen was detected in immunoprecipitates from wild type Ad 5 infected and transformed cells using

three different antisera. The 58,000 dalton protein was not detected in mock infected cell extracts treated with immune sera or in immunoprecipitates obtained from infected extracts using non-immune hamster serum and only very small amounts of this protein were found when non-immune rabbit serum was used.. These results indicate that the 58,000 dalton antigen is both virally induced and specifically immunoprecipitated by immune sera.

Three lines of evidence presented here indicate that the 58,000 dalton protein is induced 'early' in productive infection. The 58,000 dalton antigen was first detected between 3 and 8 hours after infection. This time interval precedes most viral DNA synthesis (Green et al, 1970) and hence the expression of late viral genes. Secondly, the kinetics of synthesis of the 58,000 dalton protein during lytic infection was similar to that observed for the 72,000 dalton antigen immunoprecipitated with P antiserum. As discussed below, the 72,000 dalton protein probably corresponds to the single-stranded DNA binding protein which is known to be a viral coded early protein (Lewis et al, 1976). Finally, the 58,000 dalton protein was detected in immunoprecipitates obtained from infected cells treated with cytosine arabinoside to prevent replication of DNA.

Since the adenovirus single-stranded DNA binding protein may form proteolytic breakdown products (Rosenwirth et al, 1976), it is important to know whether the 58,000 dalton antigen is simply a degradation product. This possibility appears unlikely in view of the following evidence. The 14b antiserum which binds the 58,000 dalton antigen does not react with the 72,000 dalton protein. Consistent with this result is the fact that

the 14b cell line does not contain the adenovirus DNA sequences which specify the early 72,000 dalton protein (Lewis et al, 1976; Flint et al, 1976). In addition, peptide mapping studies by J. Downey and D. Rowe (personal communication) indicate that the 58,000 dalton antigen does not share any peptides with the 72,000 dalton protein.

Several groups have immunoprecipitated adenovirus antigens from cells early after infection. However, some disagreement exists regarding the molecular weight of the major tumor antigen reported here as being 58,000 daltons. Using 14b antiserum, Levinson and Levine (1977a, b) and Ross et al (1978), have also immunoprecipitated a 58,000 dalton polypeptide from KB cells early after Ad 5 infection. While it is likely that this 58,000 dalton protein corresponds to the antigen of identical molecular weight reported here, direct confirmation would require proteolytic mapping studies. Van der Eb et al (1979), have detected a 65,000 dalton protein from Ad 5 infected cells using several anti-tumor antisera which is identical to the 58,000 dalton antigen identified by Levinson and Levine (1977a, b; S. Ross and P. Schrier, unpublished results; cited by van der Eb et al, 1979). Gilead et al (1976) and Wold and Green (1979), have immunoprecipitated a 53,000 dalton antigen from Ad 2-infected cells. This Ad 2-induced antigen was found to be closely related to a slightly larger antigen immunoprecipitated from Ad 5-infected cell extracts (M. Green, personal communication) which may correspond to the 58,000 dalton antigen described here. These reported differences in molecular weight estimation may be the result of variation in electrophoresis method.

A central question regarding the 58,000 dalton protein is whether this antigen is coded by viral or host sequences. The following evidence based on work reported here and from several different laboratories, suggests that this antigen is encoded by the region of the viral genome responsible for transformation and, more specifically, by the viral DNA sequences located between map units 4.5 and 11 (region E1B). 1.) As previously discussed, the 14b cell line expresses only the viral mRNA transcribed from the leftmost 11% of the Ad 5 genome (Flint et al, 1976). Antiserum raised against tumors of 14b cells specifically bound the 58,000 dalton protein, suggesting that the 58,000 dalton protein is encoded by the viral DNA sequences corresponding to the leftmost 11% of the viral DNA molecule. Consistent with this finding, the 58,000 dalton antigen was detected in extracts from 14b cells. 2.) The 58,000 dalton protein was also detected in the Ad 5-transformed human cell line designated as 293. This observation has been confirmed by D. Rowe (personal communication) who has immunoprecipitated a protein of identical molecular weight from this human cell line. Since the 293 cells have been shown to contain and transcribe only the Ad 5 early gene block I (Graham et al, 1977; Aiello et al, 1979), this data also suggests that the 58,000 dalton protein is encoded by the viral DNA sequences within the leftmost 11% of the Ad 5 genome. 3.) Ross and Levine (personal communciation) have found that dl 313, an Ad 5 deletion mutant lacking the viral DNA sequences from map units 3.5 to 10.5 (Jones and Shenk, 1979), is defective in the synthesis of the 58,000 dalton protein. 4.) Wold and Green (1979) have obtained rat antisera specific for a number of Ad 2-transformed cell lines. Antiserum directed against the F17 rat cell line, which contains and expresses

only the leftmost 11% of the Ad 2 genome (Gallimore et al, 1974; Flint et al, 1975) was found to immunoprecipitate predominantly two polypeptides of molecular weight 53,000 and 15,000 daltons from KB cells early after Ad 2-infection. When antiserum directed against a rat cell line transformed by a DNA fragment comprising the left 8% of the Ad 5 genome was used only the 15,000 dalton antigen was immunoprecipitated. This data suggests that at least some of the sequences coding for the Ad 2 53,000 dalton protein may be located to the right of map unit 8.0. 5.) Van der Eb et al (1979) have selected early Ad 5 viral mRNA by hybridization to specific DNA fragments from the viral transformation region. When messenger RNA specified by the viral DNA between map units 4.5 and 11.0 was translated in vitro, a 65,000 dalton protein was detected. Little or none of this polypeptide was produced when the mRNA template was selected by the DNA sequences contained within the leftmost 4.5% of the viral genome. In good agreement with these results, van der Eb and coworkers were able to immunoprecipitate the 65,000 dalton protein only from transformed cells containing a minimum of the leftmost 11% of the viral genome; cell lines transformed by the leftmost 8% of the Ad 5 DNA induced no detectable 65,000 dalton protein. These same workers have also determined the nucleotide sequences of the leftmost 11.8% of the Ad 5 genome. The only open reading frame for protein synthesis in region E1B begins at about map unit 4.5 and extends to map unit 10. The viral DNA sequences within this reading frame could code for a protein having a molecular weight of 65,000. As previously mentioned, the 65,000 dalton protein found by van der Eb et al (1979), is identical to the Ad 5-induced 58,000 dalton antigen detected by Levinson and Levine (1977a, b). 6.) Finally, the Ad 5

group II host range mutants which are defective in the induction of the 58,000 dalton antigen, map within the viral DNA sequences between 6.1 and 9.0 map units (J. Williams, personal communication).

Taken together, these results provide strong evidence that the 58,000 dalton protein is encoded by the viral DNA sequences between map units 4.5 and 11.0. It is therefore difficult to explain how a 58,000 dalton protein was immunoprecipitated from cells transformed by a viral DNA fragment comprising the leftmost 8.0% of the Ad 5 DNA (512-C8 cells). However, it must be noted that this protein was detected in extracts from 512-C8 cells in only 3 out of more than 20 trials and was not observed in immunoprecipitates from two other cell lines transformed by the same viral DNA fragment. It is possible that detection of a 58,000 dalton protein in 512-C8 cells resulted from inadvertant viral infection of the 512-C8 cells or contamination of the 512-C8 cell line with other virally transformed cells.

To summarize the results discussed thus far, the evidence presented here and elsewhere suggests that the major Ad 5 tumor antigen of approximate molecular weight 58,000 may be classified as a polypeptide induced during the early phase of viral infection. This antigen does not appear to be a proteolytic degradation product of the early 72,000 dalton protein (described below) and is probably encoded by the viral DNA sequences lying between map units 4.5 and 11.0.

B. Other Virus-induced Antigens

P antiserum immunoprecipitated predominantly the 72,000 dalton polypeptide. This antigen is induced during the early phase of viral infection since it was first detected between 3 and 8 hours post infection and synthesized in infected cells treated with cytosine arabinoside to prevent viral DNA replication. The 72,000 dalton protein detected here is presumably identical to the well-characterized single-stranded DNA binding protein of 72,000 daltons identified by van der Vliet and Levine (1973) and van der Vliet et al (1975). Both these polypeptides are major early viral proteins which are also synthesized late in infection and neither protein is related to the 58,000 dalton antigen (Levinson and Levine, 19771, b; Rosenwirth et al, 1978; Harter et al, 1976; Neuwald et al, 1977; D. Rowe and J. Downey, personal communication). Studies with Ad 5 temperature-sensitive mutants which induce a thermolabile DNA binding protein suggest that this 72,000 dalton polypeptide is required for the initiation of viral DNA replication (van der Vliet et al, 1977). Lewis et al (1976) have mapped the sequences encoding this polypeptide to the viral DNA between map units 58.5 and 70.7 (early gene region 2). In agreement with this mapping data tumor antisera raised against the 14b and C43 cells, which lack viral DNA sequences corresponding to early region 2, did not react with the 72,000 dalton protein.

In addition to the 72,000 dalton antigen, P antiserum also immunoprecipitated two other virus-specific polypeptides of molecular weights 67,000 and 44,000. These antigens were not found in immunoprecipitates obtained with tumor antisera. In a previous study, Saborio and Oberg

(1976) detected proteins of similar molecular weight in Ad 2-infected cell extracts immunoprecipitated with P antiserum. Tryptic fingerprint analysis of the Ad 5 44,000 and 72,000 dalton proteins indicates that the 44,000 dalton polypeptide may be a proteolytic breakdown product of the 72,000 dalton protein (Rosenwirth et al, 1976). In addition, the 67,000 dalton protein shares methionine containing peptides in common with the 72,000 dalton protein (D. Rowe and J. Downey, personal communication). Hence, both the 67,000 and 44,000 dalton proteins may represent proteolytic breakdown products of the 72,000 dalton antigen.

With the double antibody technique all three antisera immunoprecipitated a 10,500 dalton protein from lytically infected KB cells. This polypeptide was reproduceably detected in wild type infected cell extracts but not in mock infected extracts, indicating that it is virally induced. However, when non-immune hamster or rabbit serum was employed with the double antibody method some 10,500 dalton protein was immunoprecipitated from infected cell extracts. In addition, with the protein A-Sepharose method no 10,500 dalton was detected from either cytoplasmic or nuclear extracts. Although these data suggest that the 10,500 dalton polypeptide may have been immunoprecipitated non-specifically, no selective removal of this protein was observed when immunoprecipitates containing this protein were washed with buffer containing high salt concentrations. Hence it is presently not clear whether the 14b and C43 antisera contain antibodies specifically directed against the 10,500 dalton protein. In earlier work, Saborio and Oberg (1976) reported that a 10,500 dalton antigen could be synthesized in vitro using as a template mRNA extracted early after Ad 2 infection. Ross et al, (1978) have immunopre-

cipitated a 10,000 dalton protein from Ad 5-infected KB cell extracts using a number of anti-tumor antisera including C43 and 14b. Harter and Lewis (1978) and Chin and Maizel (1977) have both detected an early viral specific polypeptide of molecular weight 11,000 to 11,500 which is localized primarily in the cell nucleus. The gene coding for an early 11,000 dalton protein has been mapped by Lewis et al (1976) to the viral DNA sequences between 91.5 and 96.8 map units. The relationship of these polypeptides to the 10,500 dalton protein described in this thesis is presently unknown.

Two minor virus-specific antigens of molecular weights 25,000 and 15,000 were frequently detected with 14b antiserum using the protein A-Sepharose method. The early 25,000 dalton protein did not appear to be induced by host range mutants of either complementation group nor was this polypeptide found in immunoprecipitates using any non-immune sera. Peptide mapping studies by J. Downey (personal communication) and the pulse-chase experiments described here, suggest that the 25,000 dalton antigen is not generated from the 58,000 dalton protein. Recently, two groups have identified the viral gene encoding an early viral protein of similar or identical molecular weight. Spector et al (1979) have isolated three early Ad 2 mRNA species transcribed from the viral DNA sequences between map units 1 and 4.5. One of these mRNA's was found to direct the synthesis of an early 28,000 dalton protein. In a similar study, van der Eb et al (1979) tentatively mapped the gene coding for an early Ad 5 25,000 dalton protein to the leftmost 4.5% of the viral genome. It is not presently known whether the 25,000 dalton protein described here is related or identical to the polypeptides of similar molecular weight reported by these authors. A second minor antigen of 15,000 daltons was

occasionally detected in immunoprecipitates from wild type infected cells. More of this polypeptide appeared to be synthesized by group II host range mutants than by wild type Ad 5 or group I mutants. Identification of an early Ad 2 or Ad 5 polypeptide of similar molecular weight has been reported in several studies (cf. Wold and Green, 1979; Gilead et al, 1976; van der Eb et al, 1979). The sequences coding for an early Ad 2 or Ad 5 15,000-dalton protein have been mapped to the region of the viral genome between 4.5 and 11.0 map units (Lewis et al, 1976; Harter and Lewis, 1978; Spector et al, 1979).

II. The Defect in Adenovirus 5 Host Range Mutants

A. Group II Mutants

The group II lesion is contained within the viral DNA sequences between map units 6.1 and 9.0 (Gallos and Williams, personal communication). Since this region of the viral genome is expressed early after infection (Flint et al, 1977; Berk and Sharp, 1977) it is somewhat surprising that the defect expressed by the group II mutants does not significantly affect late viral functions (see table 2). The group II mutant hr 6 synthesized wild type levels of viral DNA in non-permissive cells even when low multiplicities of infection were used (Lassam et al, 1978). At similar input multiplicities (5 pfu/cell) this mutant also induced the synthesis of late viral structural polypeptides.

The group II host range mutants were found to be defective in

TABLE 2

SUMMARY OF THE DEFECTS EXPRESSED BY THE ADENOVIRUS 5 HOST RANGE MUTANTS

Virus	Viral DNA[a] Replication	Late Protein Synthesis	Transformation[b] Activity	Induction of Early Viral Specific Proteins 72,000	67,000	58,000	44,000	10,500
Ad 5 wt	+	+	+	+	+	+	+	+
Ad 5 hr Group II Mutant hr 6	+	+	-	+	+	-	+	+
Ad 5 hr Group I Mutant hr 3	-	-	c.	+	+	+[d.]	+	-[e.]

a. Lassam et al, 1978
b. Harrison et al, 1977.
c. Graham et al, 1977. The Ad 5 group I mutants exhibit defective transformation activity in that they will not transform primary rat embryo brain cells or rat embryo fibroblasts, but will "abortively" transform rat embryo kidney cells.
d. The amount of immunoprecipitated 58,000 dalton protein found after group I mutant infection was between 20-65% that observed after wt infection.
e. Very little 10,500 dalton protein (less than 15% that seen with wild type) was detected in immunoprecipitates obtained from extracts of group I mutant infected cells.

the production of an immunoprecipitable 58,000 dalton protein, an observation which has since been confirmed by Ross and Levine (personal communication). At present, it is not clear if these mutants fail to induce the synthesis of any 58,000 dalton polypeptide, or whether they produce an altered, non-immunoreactive protein. It would be surprising if all four mutants of group II made a 58,000 dalton protein which did not react with antibody, and it seems more probable that these mutants fail to induce the synthesis of this protein.

With the exception of the 58,000 dalton antigen, the group II mutants induced the synthesis of all other major early viral antigens found in wt infected KB cells (see table 2). Since these transformation-negative mutants are capable of synthesizing DNA and late viral proteins, it may be tentatively concluded that the 58,000 dalton protein is involved in initiation and/or maintenance of transformation. The conclusion that this polypeptide plays a role in transformation is in agreement with the fact that the 58,000 dalton antigen was detected in a number of Ad 5-transformed cell lines.

B. Group I Mutants

At low multiplicities of infection, the group I mutants appear unable to express major late viral functions. Using a multiplicity of infection of 5 pfu/cell no late virus structural proteins were detected in extracts of hr 3 infected HeLa cells. Furthermore, Lassam et al (1978) observed no detectable viral DNA synthesis in cells infected with hr 3 at the same moi. These results suggest that the group I lesion is expressed early after infection and that this lesion effec-

tively blocks the expression of late viral functions.

At present, the relationship between the defects expressed by group I mutants and the production of early virus-specific antigens in cells infected with these mutants is unclear (see table 2). The group I mutants induced the synthesis of the 72,000, 67,000 and 58,000 dalton antigens but not the minor viral antigens of molecular weights 25,000 and 15,000. When the double antibody technique was used, significantly less 10,500 dalton protein was immunoprecipitated from group I infected cell extracts than from cells infected with wild type or the group II mutants. However, as discussed above, the origin of this polypeptide is not clear since it may have been immunoprecipitated non-specifically. Hence, the connection between the reduction in synthesis of the 10,500 dalton protein and the group I defect is obscure. Recently, Ross and Levine (personal communication) have reported that the group I mutant hr 1 induced little or no synthesis of an immunoreactive 10,000 dalton protein. This 10,000 dalton antigen could be identical to the 10,500 dalton protein described here.

The finding that the group I mutants induced the synthesis of the 72,000 dalton protein is in conflict with results reported by Berk et al (1979). In their study, Berk and co-workers analysed the expression of early viral mRNA in HeLa cells after infection with host range mutants of both complementation groups. Briefly, the group II mutants were found to induce the same early virus-specific mRNA's detected after wild type infection. However, after group I mutant infection, only the RNA transcribed from the viral DNA sequences between map units 1.5 and 4.5 was expressed at wild type levels. (The amount of mRNA corresponding to

early region 2 which encodes the 72,000 dalton protein (Lewis et al, 1976) was found to be 300 fold less in group I mutant infected cells than when wild type virus was used.) Lewis (personal communication) has also examined early viral mRNA synthesis in host range mutant infected cells and, in marked contrast to Berk et al (1979), found no substantial reduction in expression of early viral mRNA from early region 2 in group I mutant infected cells.

These discrepancies could be attributed to leakiness of the host range lesions. At high multiplicities of infection there is a tendency for the host range mutants to leak through the block resulting from the host range lesion and proceed through the productive cycle (F. Graham and J.F. Williams, personal communication). Hence, synthesis of the 72,000 dalton protein in group I mutant infected cells could be a consequence of the relatively high input multiplicity (35 pfu/cell) routinely used in the immunoprecipitation studies. However, this possibility appears unlikely since this antigen was detected in hr 3 infected cells even when a multiplicity of infection of 1 pfu/cell was used. At present, the reasons behind the conflicting data outlined above is obscure.

III. Protein Kinase Activity Associated with Transformation Antigens

The data discussed above was principally concerned with identification of the early Ad 5 proteins involved in adenovirus transformation. The possible function(s) of these proteins was also investigated in this study. Some insight into the function of viral transformation proteins has been provided by Collett and Erikson (1978) who reported that the src

gene product of Rous sarcoma virus was associated with protein kinase activity. These results were later confirmed and extended by Levinson et al (1978), who detected protein kinase activity in immunoprecipitates obtained using antibody specifically directed against the src protein. Furthermore, the src gene product synthesized in vitro using segments of the RSV genome containing the viral transformation gene also exhibits protein kinase activity (Erikson et al, 1978; Sefton et al, 1979). Since the pioneering studies of Collett and Erikson (1978), protein kinase activity has been found associated with a transformation-related protein of another RNA tumor virus, murine sarcoma virus (Sen and Todaro, 1979); with a transformation antigen of the DNA tumor virus polyoma virus (Smith et al, 1979; Eckhardt et al, 1979; Schafthausen and Benjamen, 1979) and a transformation gene product of the related simian virus 40 (Griffin et al, 1979), although some controversy exists about the latter results (Tjian and Robbins, 1979).

The studies presented here provided the first evidence that phosphotransferase activity, similar to that found with the RNA tumor virus RSV, was also associated with the transformation related polypeptides of a DNA tumor virus. When wild type Ad 5 infected cell extracts were immunoprecipitated with 14b antiserum and incubated with (γ-^{32}P) ATP, phosphorylation of the heavy chain of the antibody occured. Only a small amount of phosphotransferase activity was observed with immunoprecipitates from mock infected cells or wild type infected cells immunoprecipitated with non-immune hamster sera. The immunoprecipitable protein kinase activity was also found in two lines of Ad 5 transformed rat cells. These results demonstrate that the Ad 5 protein kinase activity was

dependent upon and associated with the presence of polypeptides immunoprecipitated by 14b antiserum. Very recently, Raska et al (1979) and P. Branton, S. Mak and S. Bayley (personal communication) have found the transformation antigens of a different adenovirus serotype, Ad 12, to be associated with protein kinase activity.

It could be argued that the protein kinase activity detected in Ad 5 infected cells resulted from contaminating cellular protein kinases trapped within the antigen-antibody complex. However, in view of the results obtained using unrelated antibody, this possibility appears unlikely. When human IgG and rabbit anti-human IgG were immunoprecipitated in the presence of wild type infected cell extracts only a low level of phosphotransferase activity was observed. Further, little protein kinase activity was detected in a similar experiment using bovine serum albumen (BSA) and rabbit anti-BSA. In these experiments, the amount of antigen-antibody complex was probably far in excess of that obtained using infected cell extracts and 14b antiserum. Although not conclusive, these results suggest that only low levels of protein kinase are trapped non-specifically in immunoprecipitates. Hence it is unlikely that the Ad 5 associated protein kinase activity resulted from entrapment.

The phosphotransferase activity immunoprecipitated from Ad 5 infected cell extracts first appeared between 6 and 8 hours after infection, with maximal activity observed using cell extracts prepared 16 hours post infection. When cytosine arabinoside was used to prevent viral DNA synthesis, the Ad 5 related protein kinase activity was only slightly less than that found with the untreated cultures. These data

indicate that the immunoreactive protein kinase activity is associated with an early Ad 5 function. This conclusion is also supported by the fact that the phosphotransferase activity was immunoprecipitated with 14b antiserum. As mentioned above, this antiserum should contain antibodies directed against only those viral polypeptides encoded by the transforming region of the Ad 5 genome.

The Ad 5 host range mutants of both complementation groups were found to be defective in inducing the immunoprecipitable protein kinase activity. With immunoprecipitates from group II mutant hr 6 infected cells, the rate of phosphotransferase activity observed was only slightly above that detected with control mock infected extracts. The activity obtained using group I mutant hr 3 infected cell immunoprecipitates was also reduced relative to that found with wild type but was nevertheless considerably greater than that seen with extracts from hr 6 infected cells.

The observed protein kinase activity could be due to at least three formal possibilities: a) a virus-induced cell protein kinase specifically bound by the 14b antiserum; b) a cellular enzyme tightly associated with one or more proteins bound to the 14b antiserum; c) a virus coded phosphotransferase enzyme. As discussed immediately below, the present data tends to support the latter two possibilities.

a) The Ad 5 protein kinase activity was detected in infected human KB cells and in transformed rat cells using 14b antiserum obtained from hamsters. Hence, if the phosphotransferase activity was due to a virus induced cell enzyme, then the relevent protein kinase in human, rat and hamster cells would necessarily be antigenically very similar. Furthermore,

P. Branton (personal communication) has found that the Ad 5 immunoreactive protein kinase activity is not immunoprecipitated from tumor antisera directed against Ad 12 transformed cells. Similarly, the 14b antiserum does not immunoprecipitate the Ad 12 induced protein kinase. Hence, if a virally induced host enzyme was responsible for the protein kinase activity, each adenovirus serotype must induce antigenically distinct host-coded protein kinases. Together these results suggest that the Ad 5 protein kinase is not due to a host enzyme specifically induced by viral infection. However, this possibility cannot be excluded in view of studies with RSV indicating that the endogenous sarc protein kinases from a variety of species all cross-react immunologically (Sefton, personal communication).

b) The phosphotransferase activity could also be due to a cellular protein kinase tightly associated with one or more viral transformation proteins. At present, neither the data presented here nor that published in any of the tumor virus systems, rules out this possibility. In fact, Tjian and Robbins (1979) have suggested that the SV-40 induced protein kinase is actually a cellular enzyme which may be separated from SV-40 tumor antigens. However, in a similar study by Griffin et al (1979), highly purified SV-40 tumor antigens were found to retain phosphotransferase activity.

c) Finally, the protein kinase activity could be due to a virally coded protein. Should this prove to be the case, the candidate polypeptide(s) must be among those viral specified antigens immunoprecipitated by 14b antiserum. With the available data, it is not possible to speculate as to which of these proteins could be a protein kinase.

The data presented here and the work of others mentioned above, suggests that the transforming region of many tumor virus genomes codes for a polypeptide associated with protein kinase activity. This suggests that viral transformation might proceed by a common mechanism involving phosphorylation of cellular proteins. Participation of a protein kinase in the events leading to viral transformation is a particularly attractive idea, since protein phosphorylation is known to be involved in the regulation of a wide variety of cellular activities (Greengard, 1978; Rubin and Rosen, 1975). At present, the target(s) of these viral associated protein kinases is unknown.

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