CHARACTERIZATION OF PHOSPHORYLATION SITES ON THE E1A POLYPEPTIDES OF HUMAN ADENOVIRUS TYPE 5

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ABSTRACT

Human adenoviruses can induce tumors in rodents through the action of the products of two viral genes, termed E1A and E1B. E1A produces multiple transcripts as a result of differential splicing, however, only the products of the 13S and 12S mRNAs appear to play a role in oncogenicity and cell transformation. These mRNAs encode proteins of 289 and 243 residues that are identical except for the presence of a unique 46-amino internal sequence. Each of these species migrates in polyacrylamide gels as at least two electrophoretically separable forms which differ in apparent molecular mass by about 4 kDa. Previous studies had indicated that differential phosphorylation is largely responsible for this shift in gel migration. The objective of this thesis was to identify the phosphorylation events responsible for this mobility shift, and to determine the functional significance. These studies focussed on phosphorylation sites towards the amino terminus of E1A proteins. Mutants were generated by site-directed mutagenesis in which known phosphorylation sites at Ser89 and Ser96 were altered to alanine residues. Characterization of these and other mutants suggested that phosphorylation at Ser89 was largely responsible for the shift in gel migration of the E1A proteins, and may regulate phosphorylation at Ser96. Removal of the phosphorylation site at Ser89 was found to have no significant effect on the ability of E1A proteins either to transactivate E3 expression or to repress SV-40 enhancer activity, but it did reproducibly reduce E1A-mediated transforming activity by about three fold. These results suggested that phosphorylation at Ser89 may be of some regulatory significance. Ser89 as well as Ser219 were shown to be phosphorylated *in vitro* by the cell cycle protein $p34^{cdc2Hs}$ which had been purified by immunoprecipitation using specific antiserum. In addition, E1A proteins were

found to be most highly phosphorylated during mitosis, the period in the cell cycle of maximal $p34^{cdc2Hs}$ activity. These results suggested that $p34^{cdc2Hs}$ or a related protein kinase phosphorylates E1A proteins at these two sites *in vivo*.

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DEDICATION.

Without the love and understanding provided by my wife, Anne, completion of this thesis would not have been possible.

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LIST OF ABBREVIATIONS

A	adenosine
Acryl.	acrylamide
Ad5	Adenovirus Type 5
Ala	alanine
Amp.	ampicillin
APS	ammonium persulfate
Asp	aspartic acid
ATP	adenosine triphosphate
Bis	*N,N'* -methylene-bis-acrylamide
bp	base pair
BSA	bovine serum albumin
C	cytosine
CAT	Chloramphenicol acetyl transferase
°C	degrees Celsius
Chlor.	Chloramphenicol
cpe	cytopathic effect
CsCL	cesium chloride
ddDNA	double stranded DNA
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	each deoxynucleoside triphosphate
DTT	dithiothreitol
E1A	Early region 1A
E1B	Early region 1B
E2A(e)	Early region 2A
E3	Early region 3
EDTA	Ethylenediaminetetraacetic acid disodium salt
EGTA	Ethyleneglycol-bis-(ß-amino ethyl ether) *N,N,N',N'*-tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
FCS	fetal calf serum
F11	MEM+Earle's salts
ddH_2O	distilled "Super Q" water
G	guanosine
HS	horse serum
IP	immunoprecipitation

Joklik's	MEM (Joklik modified) (S-MEM)
l	liter
LB	Luria-Bertani
m	meter
m	mili-
μ	micro-
MEM	minimal essential medium
moi	multiplicity of infection
M	molar
MOPS	3-[N-Morpholino] propanesulfonic acid
n	nano-
N	nucleotide
NaOH	sodium hydroxide
NBCS	newborn calf serum
NEBL	New England Biolabs
NEN	New England Nuclear
OD_{nnn}	optical density at nnn wavelength
ON	overnight
PAGE	polyacrylamide gel electrophoresis
PBS^{2-}	phosphate buffered saline no Mg^{2+} and Ca^{2+}
PBS^{2+}	phosphate buffered saline with Mg^{2+} and Ca^{2+}
PEG	polyethylene glycol
pfu	plaque forming unit
PMSF	phenylmethlysulfonyl fluoride
PNK	T4 polynucleotide kinase
PPO	2,5-diphenyloxazole
Pro	proline
RF	replicative form
SDS	sodium dodecyl sulfate
Ser	serine
SSC	saline sodium citrate
ssDNA	single stranded DNA
T	thymine
TCA	trichloroacetic acid
TE	Tris. EDTA
TEMED	*N,N,N,N'* -tetramethylethylenediamide
Tet.	tetracycline
Tris.	tris(hydroxymethyl) aminomethane
UV	ultraviolet
wk.	week

CHAPTER I: INTRODUCTION

Adenoviruses were first discovered in 1953 as part of studies to identify viruses which could be linked to human disease (Rowe *et al.* 1953; Hillman and Werner, 1954). Continuing work has now lead to the identification of more than 41 human serotypes (*Table* **1.1**) (Horwitz, 1985).

Not all serotypes have been analyzed, however, the ones that have are comprised of a linear double stranded genome of approximately 36,000 bp. Viral genes are transcribed from both DNA strands (*Fig.* **1.1**). On the basis of DNA sequence homology, adenoviruses have been classified into five groups: A through E (Green *et al.*, 1979). They have also been grouped into three major classes based on their oncogenic potential in rodents: highly oncogenic, weakly oncogenic and non-oncogenic (Huebner, 1967; Bos and van der Eb, 1985) (*Table* **1.1**), and this classification agrees well with that based on DNA homology. In fact, all adenovirus serotypes can transform rodent cells in tissue culture, however, only adenoviruses from the highly oncogenic group A and weakly oncogenic group B viruses can cause tumors in rodents.

Table 1.1 **Classification of Adenoviruses.**

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

not classified: Ad 42

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

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

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

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

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

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

Taken from Brown (1990).

I. ADENOVIRUS REPLICATION.

Ia. VIRION STRUCTURE AND ADSORPTION.

Human adenoviruses are non-enveloped, double stranded DNA containing viruses with an icosohedral structure. The virions, which consist of an outer capsid, and an inner core structure of DNA and protein, are composed of twelve major virus encoded proteins designated VPII, VPIII, VPIIIa, VPIV-VPXII, and several minor polypeptides (*Fig.* 1.1) (Maizel *et al.*, 1968; Everitt *et al.*, 1973; Nermut, 1984).

The capsid is composed of two main structural units, hexons and pentons, which make up 252 subunits called capsomeres (Ginsberg, 1966). Hexons, of which there are 240 within the virion, are composed of three VPII (M_r=120 kDa) polypeptides which form a hexagonal structural bounded by hydrophobic and hydrophilic surfaces (Grütter and Franklin, 1974). The capsomeres at each end of the 12 vertices of the icosohedron are termed pentons because each is surrounded by five peripentonal hexons (Ginsberg, 1966). The penton is composed of a trimmer of VPIII (M_r=85 kDa) and a fibre composed of either two or three VPIV (M_r=62 kDa) polypeptides (Devaux *et al.*, 1982; Nermut, 1984). Other capsid proteins include the surface protein VPIX which is associated with the groups of 9 hexon structures (Everitt *et al.*, 1975; Boulanger *et al.*, 1979) and two major positively charged viral proteins V and VII, the are found associated with the DNA within the capsid (Everitt *et al.*, 1973; Nermut, 1984).

The initial stages of infection require the attachment of the adenovirion to the cell via the fibre of the virus capsid and a plasma membrane receptor (Londberg-Holm, 1969; Philipson, 1968; Svensson, 1981). The virus is internalized within 5 to 10 minutes after

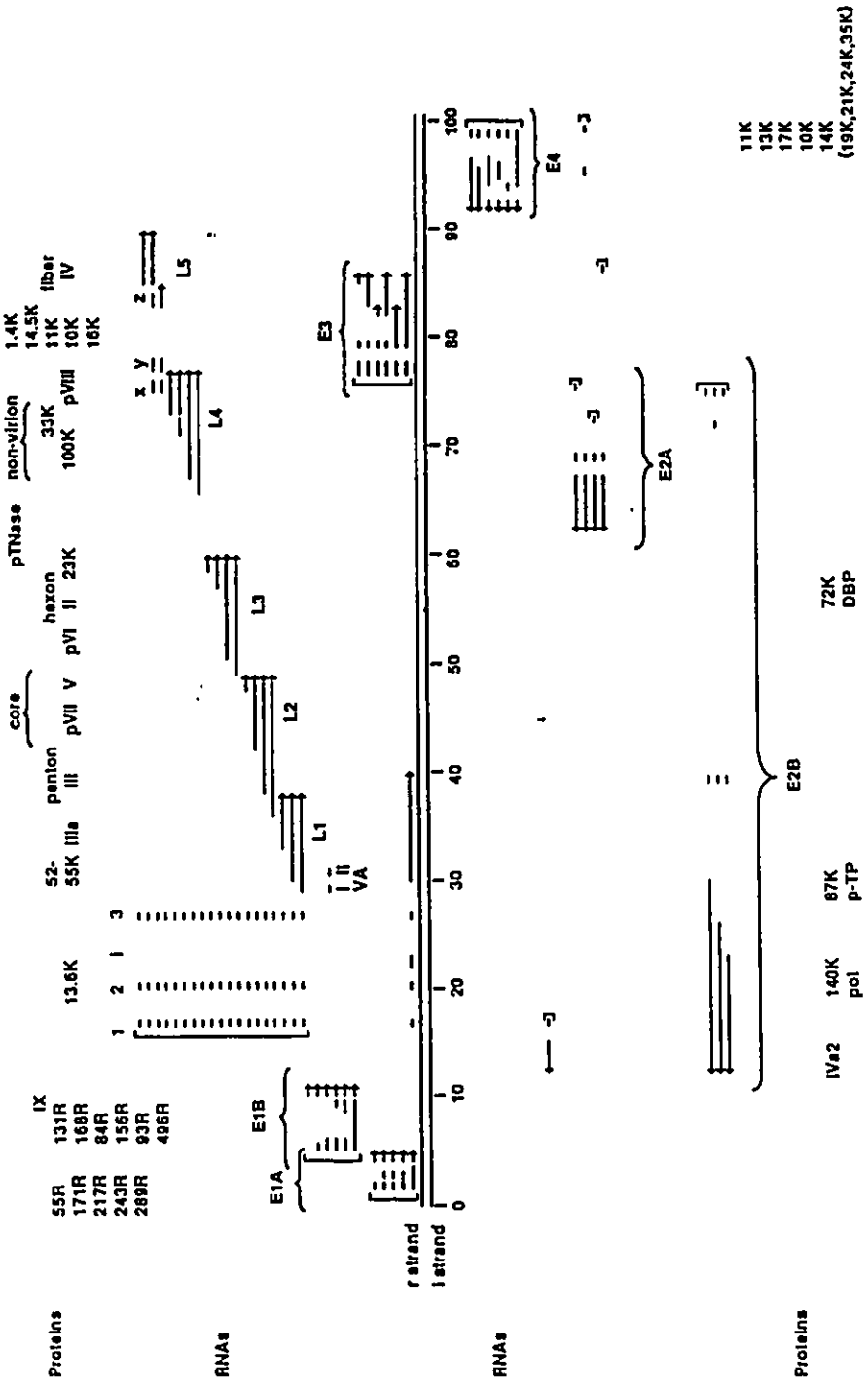
attachment by a mechanism that is not yet completely understood. Once internalized, the virion migrates to the nucleus and becomes associated with nuclear pores. During this process, at 10 to 45 minutes post absorption, the viral DNA becomes susceptible to DNAse as a consequence of destabilization of the virion which involves the loss of pentons (Sussenbach, 1967). Core proteins are then lost and viral DNA which is covalently linked to terminal proteins (see below) enters the nucleus (Younghusband and Madrel, 1982).

Ib. GENE TRANSCRIPTION EARLY DURING INFECTION.

Upon infection of cells by Adenovirus type 5 (Ad5) transcription is first detected from five regions, referred to as early (E) regions 1A, 1B, 2A, 3, and 4 (Berk and Sharp, 1977; see *Fig.* **1.1**). Some what later transcripts are produced from the major late promoter (MLP) (Lewis and Mathews, 1980; Miller *et al.*, 1980; Shaw and Ziff, 1980). Each early region gene possesses its own promoter and is expressed either from the upper strand (r-strand) (E1A, E1B, E3) or the lower strand (l-strand) (E2 and E4). Just before the onset of DNA replication four intermediate RNAs (pIX, Iva2, VAI, and VAII) are transcribed from their own promoters (Lewis *et al.*, 1977; Söderlund *et al.*, 1976; Alestrom *et al.*, 1980). Unlike all other adenovirus transcripts these VA RNAs are transcribed by RNA polymerase III (Weinman *et al.*, 1976). These low molecular weight transcripts do not encode any protein products, but are required for efficient viral translation late during infection (Thimmappaya *et al.*, 1982). The onset of DNA

Figure **1.1.** Transcription map of adenovirus genome. The linear genome of adenovirus type 5 is shown. Families of overlapping transcripts from each of the early regions, E1A, E1B, E2A/2B, E3, and E4, are depicted. The promoters are indicated by brackets at the 5' end of each set of mRNAs. The five blocks of late transcripts are shown (L1, L2, L3, L4, and L5) along with the tripartite leader (1,2,3) and the i leader sequences and the VA RNAs. The predicted translation products are depicted above the transcription map.

Figure 1.1 **Adenovirus type5 Transcription Map.**



Taken from Brown, (1990).

replication defines the switch from early to late gene expression which is accompanied by a 1000-fold increase in transcription from the major late promoter (Shaw and Ziff, 1980) and changes in RNA splicing (Fraser *et al.*, 1979; Chow *et al.*, 1979; Lewis and Mathews, 1980). Upon the onset of DNA replication, five late regions are transcribed from a common nuclear precursor RNA which is initiated from the major late promoter (MLP) at 16.5 map units (Ziff and Evans, 1978; Ziff and Fraser, 1978). Processing of the late mRNAs occurs through the use of one of five poly(A) addition sites and by differential splicing (Ziff and Fraser, 1978; Ziff and Evans, 1978; McGorgan and Raskas, 1978; Nevins and Darnell, 1978).

Ic. DNA REPLICATION.

Adenovirus DNA replication starts 8 to 10 hr postinfection and is maximal at 19 hr (Kelly, 1984). Replication of the linear viral genome is complicated by the need to ensure the integrity of the DNA termini (Graham *et al.*, 1989). Thus in order to preserve DNA ends, adenoviruses have developed several unique features of DNA replication. Replication proceeds by protein priming (Kelly, 1984) which is achieved by the linking of a dCMP nucleotide to the preterminal protein encoded within E2B. In fact the E2B region encodes two products used for Ad5 replication (see *Fig.* **1.1**), the 140 kDa viral DNA polymerase and the 80 kDa preterminal protein (pTP). pTP becomes covalently linked to dCMP which can then serve as a primer for DNA synthesis (Lichy *et al.*,1981; Pincus *et al.*, 1981; Challberg *et al.*, 1982; Tamanoi and Stillman, 1982). Replication of viral DNA utilizes a viral DNA polymerase and proceeds by strand displacement of the

parental strand (Ellens *et al.*, 1974; Schilling *et al.*, 1975; Lechner and Kelly, 1977). Viral DNA synthesis requires several accessory proteins provided by the host, and a DNA binding protein (72K or DBP) encoded by the virus (Challberg and Kelly, 1979; Rekosh *et al.*, 1977; Challberg *et al.*, 1980; Nagata *et al.*, 1983; Rosenfeld *et al.*, 1987; Wides *et al.*, 1987; Horwitz, 1978). The pTP is enzymatically cleaved by a virally encoded protease to yield a smaller form known as the terminal protein which is found at the 5' end of the mature viral genome (Challberg and Kelly, 1981; Stillman *et al.*, 1981). The displaced parental strand theoretically can be replicated via the formation of a panhandle structure formed by the annealing of the perfect inverted terminal repeats found at each end of the genome (Streenbergh *et al.*, 1977; Shinagawa and Padmanabhan, 1980). This replication intermediate has not been identified in infected cells, however, this structure is formed *in vitro* (Garon *et al.*, 1972). Initiation of DNA synthesis can then occur in the same fashion as described above.

Circularization of the displaced strand and a hypothesized "slippage mechanism" of the DNA polymerase at the ends of a circular genome provides a third mode of ensuring the integrity of the ends. Although this form of end repair has yet to be demonstrated directly, it has been proposed in order to explain how an adenovirus DNA with two defective ends can produce progeny with normal termini (Graham *et al.*, 1989).

II. TRANSFORMATION BY ADENOVIRUSES.

The discovery of the oncogenic potential of adenoviruses by Trentin *et al.* in 1962

led investigators to focus on adenoviruses as model systems to study oncogenesis. Although adenoviruses of the highly oncogenic group A might appear to be the best model, the fact that they replicate relatively poorly in tissue culture has generally inhibited their extensive study. The non-tumorigenic group C adenoviruses grow well *in vitro* and their ability to transform rodent cells in tissue culture is 10 to 50 times greater than group A viruses (Bos and van der Eb, 1985). These two important properties have moved these viruses, Ad2 and Ad5 in particular to the forefront of adenovirus research.

The discovery by Doerfler in 1968 that adenovirus-transformed cells contain integrated viral sequences provided the impetus to search for the viral genes responsible for the induction of the transformed phenotype. Gallimore *et al.*, (1974) analyzed adenovirus type 2 (Ad2)-transformed cell lines for viral DNA content and found sequences located at the left-most 14% of the viral genome were the only ones consistently integrated. This was one of the first reports suggesting that only a portion of the virus genome was required for maintenance of transformation.

Graham *et al.*, (1975) demonstrated using the newly developed calcium phosphate method (Graham and van der Eb, 1973) that the adenovirus type 5 (Ad5) *Hind* III-G fragment, which encompasses the left-most 8% of the viral genome, was sufficient to transform primary rodent cells. These data agreed with those from earlier studies using exonucleases which suggested that the transforming region was located at the left ends of the viral genome (Graham *et al.*, 1974). These results indicated that the transforming genes mapped to the E1 transcription unit.

IIa. ROLE OF E1A.

Early work by Houweling *et al.*, (1980) using the *Hpa* I-E fragment of Ad 5, which includes the left-most 4.5% of the viral genome, demonstrated that this viral DNA did not encode sufficient information to allow complete transformation of cells, but could immortalize cells in tissue culture. This was the first observation that transformation by adenovirus required the function of more than one gene product and that the immortalizing function mapped to the E1A region at the left most part of the viral genome. The E1A gene produces five mRNAs as a consequence of differential splicing (*Fig.* **1.2**). The two largest messages, which have sedimentation coefficients of 12S and 13S, are the major products and are produced immediately upon infection (Nevins *et al.*, 1979; Lewis and Mathews, 1980). The other messages, 9S, 10S, and 11S (*Fig.* **1.2**), are produced later in infection and are therefore referred to as delayed early messages. The products of these minor messages are dispensable for transformation (Stephens and Harlow 1987; Ulfendahl *et al.*, 1987).

The 13S message encodes a product of 289 residues (289R) which differs from the 243 residue product of the 12S message (243R) by an internal 46 amino acid region encoded by RNA that is spliced out of the latter (*Fig.* **1.2**). The products of each major E1A message (12S-243R and 13S-289R) migrate in SDS-polyacrylamide gels as two forms 50 and 45 kDa, and 52 and 48.5 kDa, respectively. These differences in migration have been shown to be the result of differential phosphorylation (this thesis; and Richter *et al.*, 1988; Dumont *et al.*, 1989; Smith *et al.*, 1989). In fact, these species share a precursor-product relationship in that the faster migrating E1A proteins are converted to

the slower migrating forms (Branton and Rowe, 1985). Studies using purified E1A protein produced in *E. coli* and microinjected into frog oocytes (Richter *et al.*, 1985) or introduced into mammalian cells by protoplast fusion (Ferguson *et al.*, 1985) have suggested that the slower migrating form (and as will be discussed later the hyperphosphorylated form) is the functionally more active E1A form.

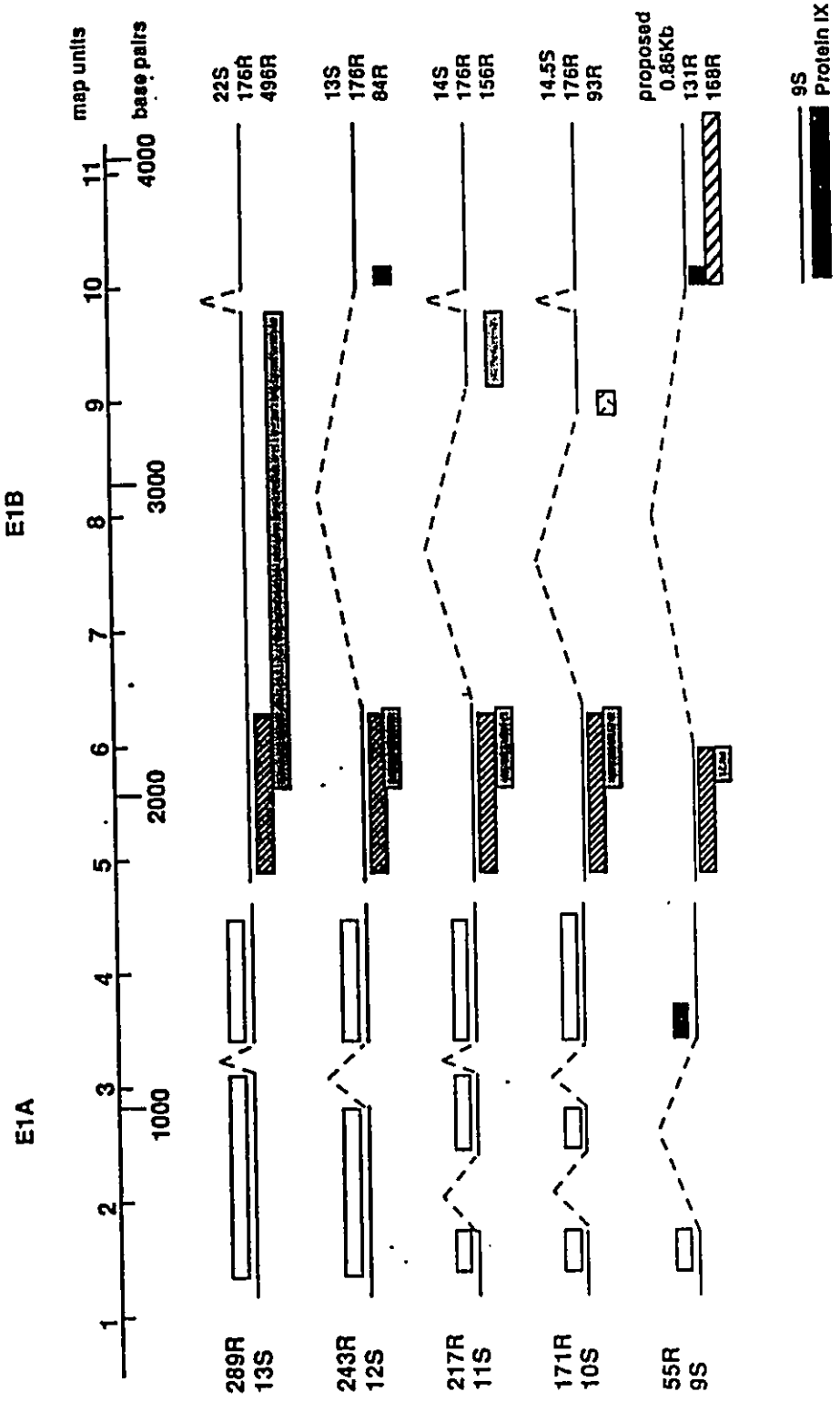
Experiments utilizing two dimensional thin layer chromatography (Tsukamoto *et al.*, 1986), or reverse phase high pressure liquid chromatography (Tremblay *et al.*, 1988; S. Whalen and P.E. Branton personal communication) of [^{32}P] labelled E1A polypeptides purified from infected cells have shown that E1A proteins are phosphorylated at a minimum of five sites. High resolution two dimensional electrophoresis of each of the major E1A forms has shown that each can be resolved into multiple species (Harlow *et al.*, 1985), suggesting that the E1A polypeptides are highly phosphorylated. Three of these sites are found in exon one at serines 89, 96, (Tremblay *et al.*, 1988) and 132 (S. Whalen and P.E. Branton personal communication) and the other sites in exon two, at serine 219 and on one or more serines between residues 227 and 237 (Tsukamoto *et al.*, 1986; Tremblay *et al.*, 1988).

IIb. ROLE OF E1B.

In addition to E1A, complete transformation of rodent cells requires the products of the E1B gene and that also produces multiple messages, including two major mRNAs of 22S and 13S (*Fig.* **1.2**). The 22S message encodes products of 176 residues (176R) and 496 residues (496R) which are translated from different reading frames. The 13S

Figure **1.2.** Structure of the E1 region of Ad5. The E1A and E1B RNAs are depicted together with a schematic of the proteins they encode. The C-terminal half of the 9S E1A product derives from a reading frame distinct from that of the 13S-10S RNAs. The 176R and 496R products from the E1B 22S RNA are derived from distinct reading frames. Except for the 156R product, all the C-terminal halves of the 176R- (131R) or 496R-related (84R, 93R, and 168R) products are derived from distinct reading frames.

Figure 1.2 E1 Transcription Unit



Taken from Brown, (1990).

message codes for 176R as well as a smaller 84R product which is related to the 496R species (Bos *et al.*, 1981; Perricaudet *et al.*, 1979; Virtanen *et al.*, 1985; Lewis and Anderson, 1987). At least two other E1B mRNAs are produced which yield 176R and other 496R-related products (*Fig.* **1.2**). The functions of the latter, especially with regard to transformation, remain unclear (Lewis and Anderson, 1987). Both of the major E1B products (176R or 496R) are individually able to cooperate with E1A to transform cells, however, the synthesis of both products results in increased numbers of transformed foci (White and Cipriani, 1990; McLorie *et al.*, 1991; Zhang *et al*, 1992), suggesting that this function of 176R and 496R transformation are additive.

The E1A polypeptides can also cooperate with either the product of an activated *H-ras* gene (Land *et al.*, 1983; Ruley, 1983), or polyoma middle T-antigen (Zerler *et al.*, 1986) to transform cells, suggesting that E1A supplies the immortalization function while these other oncogenes supply a second function necessary to produce the complete transformed state. The second function supplied by E1B is not equivalent to that supplied by *H-ras*, as transformants differ in colony morphology and other properties. For example, cells transformed by E1A plus E1B are epithelioid and will grow in medium which is low in calcium, whereas those transformed by E1A plus *H-ras* are fibroblastic and cannot be grown in medium low in calcium. Moreover, overexpression of *H-ras* alone produces foci at a low frequency while overexpression of E1B alone does not (E. White personal communication). These results suggest there are multiple ways to transform cells and that E1A supplies only the crucial immortalization step.

III. BIOCHEMICAL FUNCTIONS OF E1A PROTEINS.

IIIa. TRANSCRIPTIONAL TRANSACTIVATION.

Both major products from the E1A region are required for efficient growth of Ad5 in quiescent cells (Spindler *et al.*, 1985), however, the absolute requirement for the 12S 243R product can be bypassed in rapidly growing cells such as HeLa or KB cells (Montell *et al.*, 1982). It is thought that 243R plays a crucial role in activating entry of cells into S-phase. (Howe *et al.*, 1990) which in turn would ensure the presence of ample cellular products to aid in replicating the viral genome.

The 289R 13S product is essential for viral growth as it is responsible for activating *in trans* the transactivation of the other early viral genes. Mutational analysis has shown that the major transactivating activity maps to the 46 amino acid region unique to 289R (Berk *et al.*, 1979; Jones and Shenk, 1979; Jelsma *et al.* 1988; Fahnestock and Lewis, 1989) (*Fig.* **1.2**). In addition, 243R has been reported to transactivate some cellular genes such as proliferating nuclear antigen (PCNA), ß-tubulin, heat shock protein, and brain creatine kinase (CKB) (Jelsma *et al.* 1989; Zerler *et al.* 1987; Kaddurak-Daouk *et al.* 1990; Kao and Nevins, 1983; Nevins, 1982; Stein and Ziff, 1984).

The mechanism of E1A-mediated transactivating activity is still largely unknown. It is unlikely that E1A proteins function directly as transcription factors as, with one exception these polypeptides have not been shown to bind efficiently to DNA (Ferguson *et al.*, 1985). Chatterjee *et al.*, (1988) reported that E1A proteins were retained on a DNA column, however, binding occurred only after preincubation with cell extract,

suggesting that E1A proteins were retained on the column only after association with DNA-binding proteins. Evidence that E1A proteins function via protein/protein interactions is provided by two different kinds of experiments. A synthetic peptide corresponding to the unique region of E1A, (which did not bind to DNA) was capable of transactivating early Ad5 gene expression (Green *et al.*, 1988), thus indicating that sequences within the unique region were sufficient to activate E1A responsive genes in the absence of DNA-binding activity. A very different type of experiment utilizing E1A/Gal4 protein fusions and heterologous promoters containing Gal4 responsive elements demonstrated that the unique region of E1A could allow transactivation of the Gal4 reporter construct. These results suggested that the unique region of E1A transactivates promoters by recruiting transcription factors (Lillie and Green, 1989). In support of such a mechanism is the apparent lack of conserved upstream regulatory sequences in genes transactivated by E1A (Kingston *et al.*, 1984; Leff *et al.*, 1985). Such constructs from promoters recognized by other known transcription factors that have defined binding sites. Recruitment of multiple transcription factors may account for the ability of E1A proteins to transactivate multiple promoters because transactivation by E1A polypeptides would not be dependent on DNA sequence specificity, but rather on protein/protein interactions which would allow interaction with multiple factors and hence activation of genes from multiple promoters.

E1A proteins have also been shown to activate transcription from the E2 promoter as a result of E1A-dependent release of a transcription factor, termed E2F, from a high molecular weight inactive complex, a process dependent upon conserved regions 1 and

2 (see below) (Kovedsi *et al.*, 1986; Bagchi *et al.*, 1989; 1990; Yee *et al.*, 1989; Raychaudhuri *et al.*, 1990).

IIIb. E1A MEDIATED ENHANCER REPRESSION.

The E1A gene products have also been shown to repress transcription mediated by the simian virus 40 (SV-40), polyoma virus, immunoglobulin heavy chain, insulin and E1A enhancers (Borrelli *et al.*, 1984; Velcich and Ziff, 1985; Hen *et al.*, 1985; Stein and Ziff, 1987) and the principle product responsible for this effect seems to be 243R. Unlike transactivation by the unique region of 289R, no single region has been found to be responsible for repression (Velcich and Ziff, 1988; Jelsma *et al*, 1989). How E1A represses enhancers still remains unclear, although, repression is believed to occur through the recruitment of cellular enhancer binding proteins.

IV. FUNCTIONAL REGIONS WITHIN E1A PROTEINS

Comparison of E1A amino acid sequences from different adenoviruses has identified three highly conserved regions referred to as conserved regions 1, 2 and 3 (***Fig.* 1.3**; van Ormondt, 1980, Kimelman *et al.*, 1985; Moran and Mathews, 1988).

IVa. CONSERVED REGION 3.

Conserved region 3 (CR3) is comprised primarily of the 46-amino acid unique

region of 289R located from residue 141 to 187. Amino acids 154 to 174 contain a zinc binding finger and E1A from Ad5 has been shown to bind zinc which is believed to be important for E1A induced transcriptional activation (Culp *et al.*, 1988). As E1A proteins do not bind to DNA directly it is postulated either that the zinc finger stabilizes an interaction with a transcription factor or that once a complex is formed, the zinc finger is used by the complex to bind DNA (Culp *et al.*, 1988). The phenotype of the host-range mutant *hr*5 (Harrison *et al.*, 1977), which has a mutation immediately after the zinc binding domain (Glenn and Ricciardi, 1985), supports the notion that E1A interacts directly with a factor required for the initiation of transcription. This mutant is defective for transactivation, but unlike other host-range mutants when coinfected with *wt* Ad5 it interferes with *wt* viral growth, a phenomenon known as transdominance or squelching (Glenn and Ricciardi, 1987). The *hr*5 E1A transdominant effect is thought to occur through sequestering of limiting transcription factor(s) by binding them either irreversibly or with greater affinity than wild type E1A proteins (Glenn and Ricciardi, 1987). Mutations elsewhere in CR3 also greatly affect transactivation (Jelsma *et al.*, 1988; Jones and Shenk, 1979; Fahnestock and Lewis, 1989), suggesting that sequences flanking the zinc binding domain are also important.

IVb. CONSERVED REGION 2.

CR2 is located in exon 1 between residues 108 to 136 (*Fig.* **1.3**). Mutations in CR2 affect the ability of E1A to induce mitosis in quiescent rodent cells, to immortalize cells, to induce DNA synthesis, to induce PCNA, and to induce CKB (Murray *et al.*,

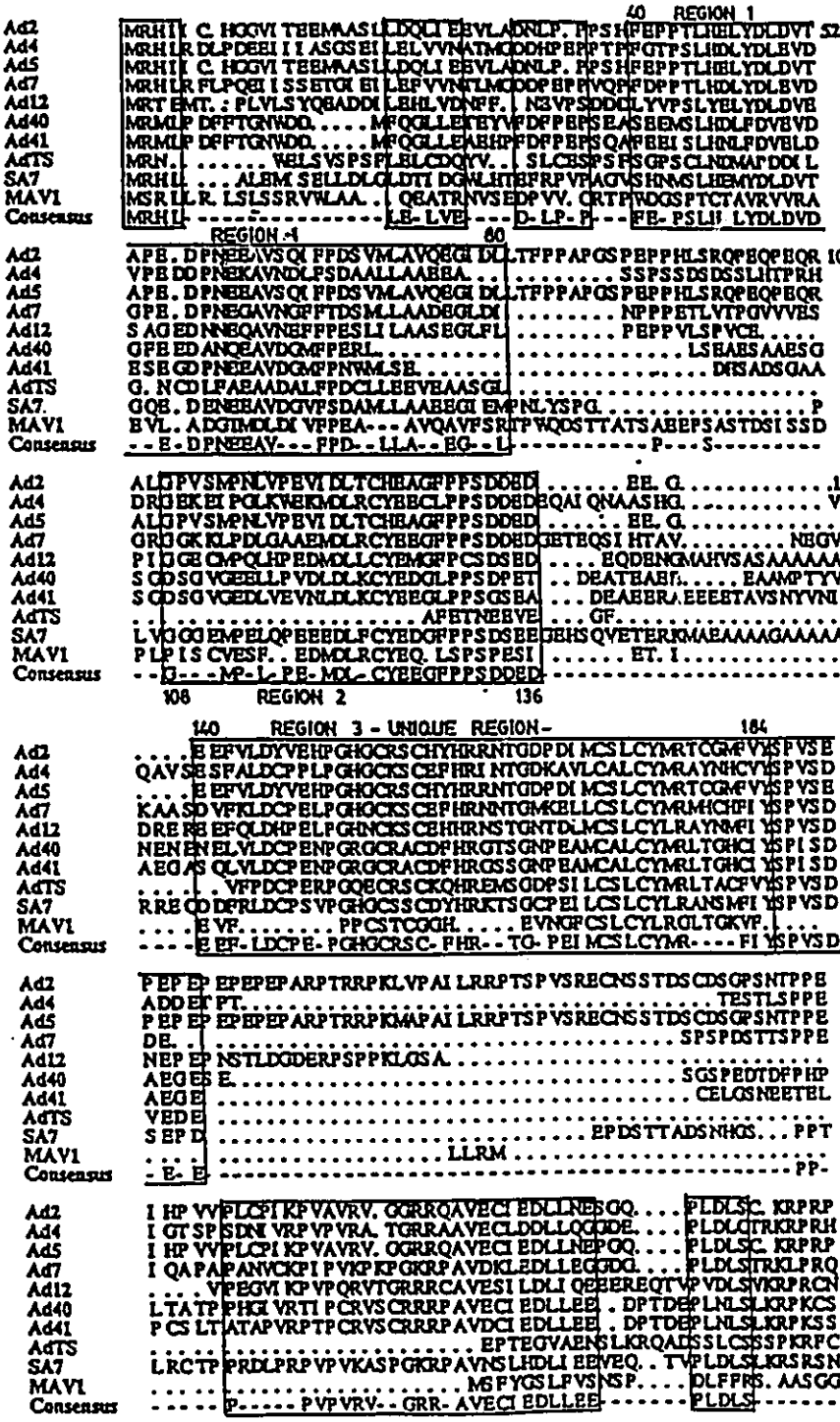
1982; Nevins, 1982; Kao and Nevins, 1983; Stein and Ziff, 1984; Zerler *et al.*, 1987; Jelsma *et al.*,1988, 1989; Fahnestock and Lewis, 1989; Howe *et al.*, 1990; Kaddurah-Daouk *et al.*, 1990). Mutations in CR2 also decrease the ability of E1A to repress transcription mediated by some enhancers and to transform primary rodent cells (Borrelli *et al.*, 1984; Hen *et al.*, 1984; Stein and Ziff, 1987; Velcich and Ziff, 1985; Velcich *et al.*, 1986). These results suggest that CR2 is involved in at least two E1A functions, enhancer repression and transformation. It seems apparent that these two biological effects could be induced through a common biochemical mechanism mediated by CR2. For some time it was believed that such was not the case as a mutant exists which is able to transform cells in cooperation with *ras*, but which fails to repress the transcriptional effects of a test enhancer (Velcich and Ziff, 1988). Now that it is apparent that different enhancers have different requirements for E1A regions CR1 and CR2, this question needs to be reexamined.

Vc. CONSERVED REGION 1.

The induction of DNA synthesis by E1A in quiescent rodent cells also involves CR1 which is located between residues 40 to 80 (*Fig.* **1.3**) (Howe *et al.*, 1990). In addition, mutations in this region adversely affect the ability of the E1A polypeptides to induce PCNA (Jelsma *et al.*, 1989) and CKB (Kaddurah-Daouk et al., 1990). The induction of PCNA is thought to be intimately linked with the induction of DNA synthesis (Bravo *et al.*, 1987; Prelich *et al.*, 1987). Increased amounts of CKB have been detected in numerous tumor cells (Gazdar *et al.*, 1981; Feld and White, 1977; Rubery *et*

Figure **1.3.** Alignment of E1A proteins from various species. Amino acid sequences were aligned by eye and gaps, depicted by periods, were introduced for maximum alignment. Regions of high sequence identity are boxed and where an amino acid is conserved in greater than 40% of the proteins it is depicted in the consensus sequence. Non conserved amino acids are depicted by dashes in the consensus sequence.

Figure 1.3 Alignment of E1A Proteins from Various Adenovirus Species.



Taken from Bautista, (1989).

al., 1982; Thompson *et al.*, 1980). CKB is involved in producing ATP in metabolically active cells (reviewed by Bessman, 1985) and thus its induction may be a requirement for efficient viral growth in quiescent cells. Mutations in CR1 also affect the ability of E1A proteins to repress enhancer driven transcription, to induce transformation, and to immortalize cells (Stein and Ziff, 1987; Velcich and Ziff, 1985; Velcich *et al.*, 1986; Jelsma *et al.*1988). Other studies have implicated sequences within and upstream to CR1 in efficient retention of E1A proteins in the nucleus (Richter *et al.*, 1985).

IVd. OTHER REGIONS.

The conserved stretch of basic amino acids at the carboxyl terminus of E1A proteins is required for rapid nuclear localization (Richarson *et al.*, 1986). A very similar type of sequence has been identified in other nuclear proteins (Kalderon *et al.*, 1984a,b; Kalderon and Smith, 1984), and introduction of this region into other polypeptides results in targeting to the nucleus (Kalderon *et al.*, 1984b; Colledge *et al.*, 1986).

A short region in the amino terminus is conserved between many human adenoviruses (*Fig.* **1.3**) and mutations within this sequence greatly reduce transformation and enhancer repression and adversely affect the ability of the E1A polypeptides to induce the production of an epithelial like growth factor in primary rat cells (Subramanian *et al.*, 1988; Quinlan *et al.*, 1988; Quinlan and Grodzicker, 1987). The induction of this growth factor is also affected by mutations in the less conserved region immediately to the right of CR3 (Quinlan *et al.*, 1988) (*Fig.* **1.3**). Moreover, a mutation in this region has been described which results in enhanced transforming activity (Subramanian *et al.*, 1989).

V. CELLULAR E1A BINDING-PROTEINS.

Using mild immunoprecipitation conditions, Yee *et al.* (1985) demonstrated that at least five cellular proteins specifically associate with E1A polypeptides. This result was corroborated by Harlow *et al.* (1986) who also identified several other E1A binding species. The most prominent of these proteins have apparent molecular weights of approximately 105 kDa, 107 kDa, and 300 Kda whereas the less prominent proteins migrate at 130kDa, 60kDa and 33kDa. Studies on the binding sites of the most prominent proteins on the E1A polypeptides using various E1A deletion mutants have shown that the 105 kDa and 107 kDa proteins bind primarily to overlapping regions within CR2. In addition, E1A sequences within CR1 contribute to the efficiency of binding to these proteins. The 300 kDa protein requires two discontinuous regions on the E1A proteins for binding, amino acids 4 to 25 and residues 48 to 60 within CR1 (Egan *et al.*, 1988; Whyte *et al.*, 1989). The binding of both the 300 kDa and the 107/105 kDa species by E1A products correlates with transformation (Egan *et al.*, 1988, Whyte *et al.*, 1989; Jelsma *et al.*, 1989), suggesting that the association with these proteins plays an important role in this function.

The cellular E1A binding proteins have also been found to interact with the oncogene products of other DNA tumor viruses. Simian virus-40 large tumor antigen (LT) has been shown to bind to both the 105 kDa (DeCaprio *et al.*, 1988) and the 107 kDa (Dyson *et al.*, 1989) proteins, and mutations in a region of LT which has homology

to CR2 of E1A abolishes both LT transforming activity and binding of these two proteins (Ewen *et al.*, 1989). Furthermore, this LT binding domain when substituted for CR2 within E1A can functionally replace E1A CR2, allowing this chimeric molecule to transform cells (Moran, 1988). Thus these tumor antigens may transform cells via similar mechanisms. In addition to SV40, the large T-antigens from BK virus-, SA12 virus-, polyoma virus and hamster lymphotropic virus (Dyson *et al.*, 1989) as well as the E7 gene product from the human papilloma viruses (HPV) -16 and -17 (Phelps *et al.*, 1988; Dyson *et al.*, 1989) all bind to the 105 kDa protein. These results suggest that these different DNA tumor viruses may transform by a common mechanism. Another common feature of transformation by DNA tumor viruses may involve interactions with a second cellular protein, p53. SV40 LT, HPV-16 and -17 E6 and the adenovirus type 5 E1B 496R proteins all bind to p53 (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al.*, 1982; Werness *et al.*, 1990). p53 usually is expressed at low levels in normal proliferating cells (Dippold *et al.*, 1981; Benchimol *et al.*, 1982; Thomas *et al.*, 1983; Rogel *et al.*, 1985), but in tumor cells it is often detected at much higher levels due either to increased mRNA stability, to increased expression levels, or both (Crawford *et al.*, 1981; Dippold *et al.*, 1981; Benchimol *et al.*, 1982; Rotter, 1983; Thomas *et al.*, 1983; Koefler *et al.*, 1986). Originally it was thought that p53 was encoded by a dominant cellular oncogene, however, it is now believed that p53 is a tumor suppressor and that p53 mutants can act in a dominant negative fashion and thus interfere with wild type p53 (Finlay *et al.*, 1988; Eliyahu *et al.*, 1988; Hinds *et al.*, 1989). SV40 LT, E6 and the 496R proteins may therefore mimic mutated forms of p53 and disrupt the cell cycle by binding

to and inactivating p53 (Finlay *et al.*, 1989; Werness *et al.*, 1990).

Identification of E1A associated proteins has been achieved either by cloning or by immunological approaches. p105 was shown to be the product of the *Rb*-1 tumour suppressor gene (Whyte *et al.*, 1988; Egan *et al.*, 1989). Deletion or other mutations affecting both alleles of *Rb*-1 are believed to induce retinoblastoma (reviewed by Hansen and Cavenee, 1988) and $p105^{Rb}$ is thought to control entry of cells into S-phase. The p107 protein has been cloned and shows limited homology to $p105^{Rb}$ (Ewen *et al.*, 1991). The 33kDa protein has been cloned and identified as *cdk2* a member of the *cdc2* family of kinases (Tsai *et al.*, 1991). p60 was shown to be cyclin A, a regulatory protein affecting cdc2 kinases (Giordano *et al.*, 1989; Pines and Hunter, 1990).

Recent studies have suggested a biochemical basis for cell cycle regulation by $p105^{Rb}$. $p105^{Rb}$ interacts with the cellular transcription factor E2F (Chellappan *et al.* 1991; Mudryi *et al.*1991; Bandara and LaThangue, 1991; Chittenden *et al.*, 1991; Bandara *et al.*, 1991) and a similar or related protein DRTF1 (Bandara and LaThangue, 1991). E2F is important for the transcription of cell cycle-regulated genes such as *c-myc*, and dihydrofolate reductase (DHFR) (Blake and Azizkhan, 1989). The interaction between E2F and $p105^{Rb}$ and the related p107 protein appears to control the transcriptional activity of E2F (Hiebert *et al.*,1992).

E2F was first described as a transcription factor required for E1A regulation of the adenovirus E2 promoter. Moreover, the heterodimeric complex of E2F with the 19K product from the E4 gene forms a very stable complex which further stimulates transcription from the E2 promoter (Hardy and Shenk, 1989; Hardy *et al.*, 1989; Huang

and Hearing, 1989; Neill *et al.*, 1990; Raychaudhuri *et al.*, 1990; Marton *et al.*, 1990). E2F is normally found in the cell in large inactive heteromeric complexes which prevent it from binding to the 19K E4 product (Bagchi *et al.*, 1990). E1A protein was shown to dissociate these complexes, thus making E2F available for heterodimeric association with the 19K E4 protein and increasing transcription from the E2 promoter (Bagchi *et al.*, 1990). Dissociation of E2F complexes by E1A products mapped to regions previously known to be important for oncogenesis (Mudryi *et al.*, 1991; Bandara *et al.*,1991; Deveto *et al.*, 1991; Cao *et al.*, 1992; Shirodkar *et al.*, 1992).

The regions of E1A polypeptides required for E2F dissociation were mapped to regions within CR1 and CR2 that are involved in binding of $p105^{Rb}$ and p107 (Raychaudhuri *et al.*, 1991). Both of these cellular proteins have been shown to be present in E2F complexes, and E1A products are believed to activate transcription by binding to $p105^{Rb}$ and p107, thus releasing and activating E2F (Raychaudhuri *et al.*, 1991).

E1A proteins may activate the cell cycle as a consequence of these interactions. E2F from synchronized NIH-3T3 cells is present in at least two types of complexes (Mudryi *et al.*, 1991). One accumulates during the G1 phase of the cell cycle and then disappears at the end of G1. A second distinct complex appears at the beginning of S-phase, accumulates during S-phase, and then disappears in G2 or mitosis. E2F is found associated with p107 as well as $p33^{cdk2}$ kinase (Deveto *et al.*, 1991; Cao *et al.*, 1992; Shirodkar *et al.*, 1992). In addition p107 is complexed to $p60^{cyclin\ A}$ (Ewen *et al.*, 1992; Faha *et al.*, 1992). Moreover the regulation of E2F activity may be more complicated because E2F has also been shown to be in a complex in G1 which contains p107 together

with cyclin E protein and $p33^{cdk2}$ (Lees *et al.*, 1992). How these interactions regulate the cell cycle is not immediately clear, however, transient expression assays with an E2F responsive reporter construct demonstrated that either $p105^{Rb}$ or p107 could block transcription from this promoter (Nevins, 1992)

It is believed that $p105^{Rb}$ is normally regulated by phosphorylation in a cell cycle dependent fashion. Whether this phosphorylation controls complex formation is not totally clear, however, only the hypophosphorylated $p105^{Rb}$ is found in the E2F complex (Chellappan *et al.*, 1991). High levels of phosphorylation are thought to inactivate $p105^{Rb}$ (Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Buchkovich *et al.*, 1989) which may release E2F, thus permitting progression through the cell cycle. A popular hypothesis is that association of $p105^{Rb}$ with oncogene products of DNA tumor viruses either sequesters and/or inactivates the function $p105^{Rb}$, and thus mimics its normal down-regulation by phosphorylation (DeCaprio *et al.*, 1989; Buchkovich *et al.*, 1989; Chen *et al.*, 1989; Furukawa *et al.*, 1990). Such interactions would release E2F and allow progression into S-phase.

VI. THE CELL CYCLE.

Cellular transformation can be viewed as the consequence of numerous insults to the cell that result in the deregulation of the cell cycle and unrestricted cellular proliferation. E1A proteins have been shown to induce quiescent cells to enter the cell cycle (Shimojo and Yamashita, 1968; Strohl, 1969; Younghusband, *et al.*, 1979; Howe *et al.*, 1990).

The cell cycle can be divided into five distinct phases: G_0, G_1, S, G_2, and M (***Fig.* 1.4**). Non-cycling cells in which very little macromolecular synthesis occurs are thought to be in an extended G_1-phase which is also referred to as G_0. G_1 is the preparatory phase before the onset of DNA synthesis (reviewed by Pardee, 1989) and it is believed that early during this phase the cellular decision of whether or not to enter S-phase is made (reviewed by Murray and Kirschner, 1989). This point has been defined in yeast as START and in mammalian cells as the R or restriction point (Nurse and Bissett, 1981; reviewed by Pardee, 1989). Once the cell enters S-phase it is committed to a new round of cellular division (reviewed by Laskey *et al.*, 1989). During this phase the chromosomes are replicated from multiple replication origins and although the time required for DNA synthesis varies among different cell types, it is generally constant for each cell type, suggesting that a well programmed set of events are involved in this process.

After the chromosomes have been replicated the cell enters G_2 during which time the cell prepares for mitosis (reviewed by Nurse, 1990). The exact biochemical events

occurring during this phase are not well understood. Mitosis can be divided into four discrete events: (i) prophase- condensation of chromosomes; (ii) prometaphase- positioning of the condensed chromosomes to form the "metaphase plate"; (iii) anaphase- separation of the chromosomes into equal parts and movement to opposite poles; and (iv) telophase- reformation of the nuclei and entry into interphase (reviewed by McIntosh and Koonce, 1989).

Much information about the cell cycle has come from the study of cell division cycle (*cdc*) mutants of yeast. Many *cdc* mutants have been isolated from both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* which have quite different cell cycles. *S. cerevisiae* divides by budding and its cell cycle has no true G_2 phase: S-phase and mitosis seem to be fused, and perhaps as a result of this the chromosomes do not condense and the nuclear membrane does not break down (reviewed by Nurse, 1985). In contrast, the cell cycle of *S. pombe* is comparable to that of mammalian cells and thus information gained with this organism may be relevant to the mammalian system (reviewed by Lee and Nurse, 1988). Analysis of *cdc* mutants has demonstrated that there are two critical control points in the cell cycle, START and the G_2/M transition point. Both of these points are controlled by the *cdc2* gene product .

VIa. GENETICS OF THE CELL CYCLE.

When temperature sensitive mutants (*ts*) of the *cdc2* gene (*cdc2ts*) of *S. pombe* are shifted to the non-permissive temperature they arrest at either of two points in the cell cycle, START or the G_2/M transition point, depending where within the cell cycle the

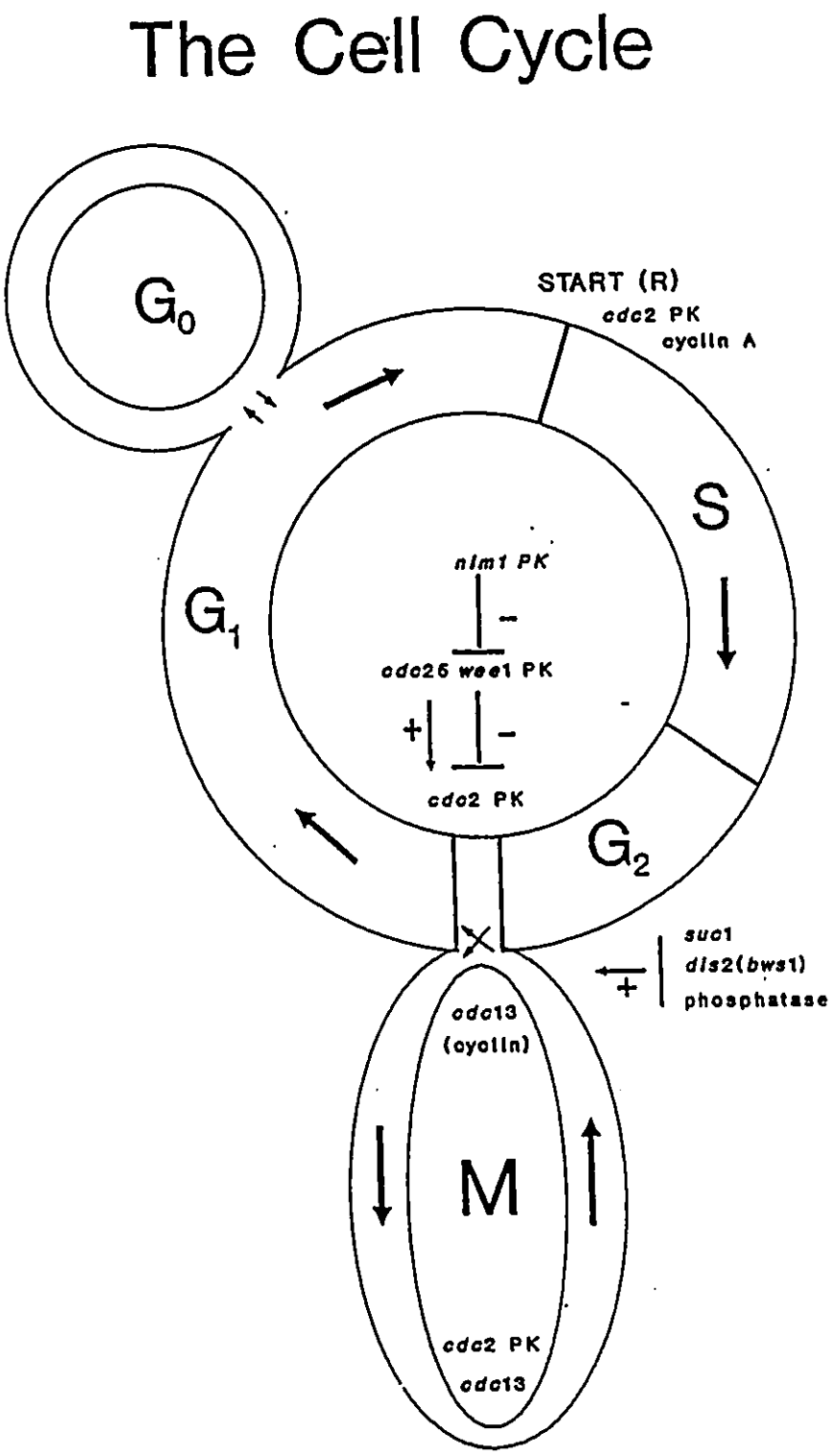
yeast cell was at the time of the temperature shift (*Fig.* **1.4**) (Simanis and Nurse, 1986). Little is known about the genes regulating *cdc2* activity at START, however, considerable information about genes regulating the onset of mitosis has been compiled.

cdc25ts mutants arrest in G_2 demonstrating that the product of this gene is clearly required for the onset of mitosis (Fantes, 1979; Russel and Nurse, 1986). Moreover, over-expression of the *cdc25* gene results in premature initiation of mitosis, thus suggesting that it is a rate-limiting inducer of mitosis (Russel and Nurse, 1986a). Many genes which are intimately involved in regulating mitosis have been identified as suppressors of *cdc* mutations. Suppressors of *cdc25ts* mutants map to two different genes, *wee1* and *cdc2*. The *wee1* mutation causes cells to enter the cell cycle prematurely (*i.e.* at a reduced cell size). The suppressors which map to the *cdc2* gene also produce the "wee" phenotype (Thuriaux *et al.*, 1978). On the basis of these and other experiments, Russel and Nurse (1989) have proposed that the *cdc25* and *wee1* gene products both play an important role in controlling mitosis by regulating the activity of the *cdc2* gene product in antagonistic fashions (*Fig.* **1.4**).

Yeast geneticists routinely use complementation experiments to clone genes and in such an experiment the *nim1* gene was identified and cloned serendipitously during an attempt to clone the *cdc25* gene. The predicted product from this gene is thought to be a serine/threonine kinase and when over-expressed can suppress *cdc25* mutations. This suppression is thought to be modulated through the post-translational down-regulation of *wee1* activity (*Fig.* **1.4**) (Russel and Nurse, 1986b). In a search for genes that were able to suppress a cold sensitive *cdc2* mutant which was defective only in its G_2 function a

Figure **1.4.** Schematic diagram of the cell cycle depicting the five phases of the cycle. Genes that alter this cycle in yeast are also depicted and their location in this diagram reflects the time point in the cell cycle that their gene products are thought to act.

Figure 1.4 Schematic Diagram of the Cell Cycle



gene was cloned that mapped to a previously identified *cdc* gene, *cdc13*. This suppression was allele specific, suggesting, and later confirmed by affinity chromatography, that the effect was due to a direct protein/protein interaction (Booher and Beach, 1987, Booher *et al.*, 1989).

suc1 is another gene isolated by complementation of a *cdc2* mutant. Only specific mutants of the *suc1* gene can suppress some *cdc2* mutants and this allele specific suppression suggested a direct interaction between these gene products (Hayles *et al.*, 1986a,b). Subsequently, using an immunoaffinity column directed against the *suc1* product, $p13^{suc1}$, the *cdc2* product $p34^{cdc2}$ was shown to be retained, confirming the direct protein/protein interaction between these species (Brizuela *et al.*, 1987). Null mutants of *suc1* arrest cells late in mitosis (Moreno *et al.*, 1989) while over-expression of *suc1* has an effect similar to *cdc2* mutants in that the onset of mitosis is delayed (Hindley *et al.*, 1987; Hayles *et al.*, 1986b). Thus the role of *suc1* in the cell cycle is unclear.

A gene affecting the completion of mitosis was isolated by two separate investigators using completely different criteria. Ohkura *et al.*(1989) isolated a cold sensitive mutant (*dis2*) which is defective in sister chromatid disjoining. The same gene was cloned by Booher and Beach (1989) in an attempt to clone *wee1* by complementation (*Fig.* 1.4).

VIb. BIOCHEMISTRY OF THE CELL CYCLE.

The cloning of many of the aforementioned *cdc* genes has facilitated the analysis of the biochemistry of the cell cycle. The predicted amino acid sequence of *cdc2* reveals

it has strong homology to serine/threonine kinases (Beach *et al.*, 1982). Supporting this prediction were results obtained using antipeptide serum directed against the carboxyl terminus of the *cdc2* product which specifically immunoprecipitates kinase activity (Simanis and Nurse, 1986). This serum also immunoprecipitates a protein of M_r =34 kDa, termed $p34^{cdc2}$ which has been shown to phosphorylate numerous substrates *in vitro*, such as histone H1 (Labbe *et al.*, 1988; Langan, 1978; Langan, 1989), casein (Simanis and Nurse, 1986), $p60^{c\text{-}src}$ (Shenoy *et al.*, 1989; Morgan *et al.*, 1989), RNA polymerase II (Cisek and Corden, 1989), SV40 LT (McVey *et al.*, 1989), elongation factor (Belle *et al.*, 1989), cyclin (Booher *et al.*, 1989; Lohka *et al.*, 1988), laminin A, B and C (Heald and McKeon, 1990; Peter *et al.*, 1990; Ward and Kirschner, 1990), p53 (Bischoff *et al.*, 1990) and *c-abl* (Kipreos and Wang, 1990). Whether these are true *in vivo* substrates of $p34^{cdc2}$ is not clear in all cases (reviewed by Moreno and Nurse, 1990).

The onset of mitosis is regulated by $p34^{cdc2}$ kinase activity which in turn is regulated by phosphorylation on tyrosine residue 15 (Y15). Y15 is located within the putative ATP binding site and it is believed that dephosphorylation of Y15 activates $p34^{cdc2}$ kinase activity. $p34^{cdc2}$ is also phosphorylated on serine and threonine residues and such phosphorylation also decreases as the cell enters mitosis, suggesting a regulatory role for these sites (Gould and Nurse, 1989).

The *cdc25* gene encodes a protein with a predicted M_r of 67 kDa, (Russel and Nurse, 1986a) and from sequence comparison may be the yeast homolog to the predicted product of the *string* gene in *Drosophila* (Edgar and O'Farrel *et al.*, 1989). Anti-serum raised against *cdc25* produced in *E. coli* immunoprecipitated a phosphoprotein with a M_r

of 80 kDa. This apparent larger size is probably due to posttranslational modifications such as phosphorylation. As one would predict for a rate limiting mitotic factor, the levels $p80^{cdc25}$ oscillate during the cell cycle, peaking at the G_2/M transition point (Moreno *et al.*, 1990). Expression of the *Drosophila string* gene also oscillates in the same fashion (Edgar and O'Farrel *et al.*, 1989), suggesting that the products of *cdc*25 and *string* perform the same functions in their respective organisms. It is now apparent that $p80^{cdc25}$ represents a novel phosphatase with activity against both phosphotyrosyl and phosphoseryl residues (Kumagai and Dunphy, 1991; Dunphy and Kumagai, 1992; Gautier *et al.*, 1992; reviewed in Millard and Russel, 1992).

The predicted product of the *wee1* gene has sequence homology to serine/threonine kinases. However, using anti-serum directed against the *wee1* product produced in bacteria it was determined using an *in vitro* kinase assay that *wee1* was able to phosphorylate serine and tyrosine residues (Featherstone and Russel, 1991; Parker *et al.*, 1991; Lundgren *et al.*, 1991; Russel and Nurse, 1991). This result may provide a possible mechanism for *wee1* to control *cdc2* function. In addition, over-expression of *wee1* results in entry of cells into the cell cycle at a later time (*i.e.* larger size), thereby suggesting that *wee1* codes for a dose-dependent negative regulator of the cell cycle (Russel and Nurse, 1987).

Yet another gene whose predicted product has homology to serine/threonine kinases is the *nim1* gene whose product has a predicted M_r of 50 kDa. The true biochemical activity of this protein remains unknown (Russel and Nurse, 1986b).

Two other gene products that are required for mitosis and are known to interact

directly with $p34^{cdc2}$ are the products from the *cdc13* and *suc1* genes. The predicted product of *cdc13* has homology to B-cyclins (Solomon *et al.*, 1988; Goebl and Byers, 1988; Moreno *et al.*, 1989; Hagan *et al.*, 1988; Booher and Beach, 1988) and two groups using antipeptide sera have identified proteins of M_r =56Kd (Moreno *et al.*, 1989) or 63Kd (Booher *et al.*, 1989) which are probably identical. The levels of $p56^{cdc13}$ oscillate during the cell cycle in the same fashion as cyclins (Evans *et al.*, 1983; Standart *et al.*, 1987), suggesting that $p56^{cdc13}$ is a cyclin. The importance of phosphorylation in regulating the cell cycle is also reflected in the fact that cyclins are usually phosphorylated just before they are degraded, suggesting phosphorylation may act as a signal which would initiate this process (Murray *et al.*, 1989; reviewed by Pines and Hunter, 1990). Very little is known about the biochemistry of the *suc1* product, apart from the fact $p13^{suc1}$ is found associated with $p34^{cdc2}$.

Dephosphorylation by protein phosphatases also plays a critical role in regulation of the cell cycle. As was alluded to earlier, $p34^{cdc2}$ is activated by dephosphorylation of Y15, a step required for the initiation of mitosis. Thus it was intriguing to find that the *dis2/bws1* gene which is required to exit mitosis encodes a product with homology to serine/threonine phosphatases and not tyrosyl phosphatases. These results suggest that dephosphorylation of serine/threonine residues is important for the completion of mitosis (Ohkura *et al.*, 1989; Booher and Beach, 1989). The substrate(s) for this phosphatase remains unknown.

VIc. MAMMALIAN HOMOLOGS.

Mammalian homologs have been found for all of the aforementioned genes with the exception of *wee1*, *dis2/bws1*, and *nim1*. A homolog to *dis2/bws1* in *Aspergillus nidulans* (*bimG*) has been reported (Doonan and Morris, 1989). Using an immunoaffinity column made with antibody directed against the $p13^{suc1}$ polypeptide from *S. pombe*, a human homolog was identified (Draetta *et al.*, 1987). This column also retained a number of other proteins, one of which was identified as the human $p34^{cdc2}$ (Draetta *et al.*, 1987). The presence of a *cdc2* homolog in human cells was also corroborated by other investigators (Lee and Nurse, 1987; reviewed by Norbury and Nurse, 1989).

The presence of homologs in such diverse species suggests a universality in the mechanism(s) of cell cycle control. In fact, corresponding proteins from *S. cerevisiae* (CDC28) and human (*Hscdc2*) are functionally interchangeable with the *cdc2* kinase of *S. pombe* (Lee and Nurse, 1987). Clearly protein phosphorylation plays a key role in controlling the cell cycle.

VII. PROTEIN PHOSPHORYLATION.

Many complex biological reactions within the cell are known to be regulated by phosphorylation including glycolysis and gluconeogenesis (reviewed by Pickett-Geis and Walsh, 1986), lipolysis (reviewed by Siess, 1989), contraction of vertebrate smooth muscle (reviewed by Cross, 1989), activation of receptors (reviewed by Ullrich and Schlessinger, 1990), activation of transcription factors (Montminy and Bilenzikjian, 1987;

Prywes *et al.*, 1988; Sorger *et al.*, 1987; Sorger and Pelham, 1988; Hoefler *et al.*, 1988), and progression of the cell cycle (reviewed by Nurse, 1990). Many of the protein kinases which catalyze these reactions are also regulated by phosphorylation. As summarized in Figure **1.5** it seems clear that many important biological signals are transmitted to the cell nucleus by a cascade of phosphorylation and dephosphorylation events (reviewed by Hunter, 1989).

VIIa. PROTEIN KINASES.

Protein kinases are classified according to whether they phosphorylate serine and threonine, or tyrosine residues. Serine/threonine kinases have been found throughout the cell, while with one exception, tyrosine kinases have not, been found in the nucleus. The product of the *c-abl* proto-oncogene ($p150^{c\text{-}abl}$) has been reported to be located in the nucleus as well as the cytoplasm (Wang and Baltimore, 1983). Many cell surface receptors for growth factors and other external stimuli are protein kinases, the vast majority belonging to the tyrosine kinase family (reviewed by Sibley *et al.* 1990). To date more than 30 tyrosine kinases and more than 50 serine/threonine kinases have been identified (Hunter, 1989) and with the advent of PCR technology this list continues to grow. Phosphotyrosine accounts for only 0.1% of the total acid stable phosphorylated residues in the cell, whereas phosphoserine and phosphothreonine account for almost 90% and 9%, respectively (Hunter and Sefton, 1980).

Figure **1.5.** Schematic of signal transduction pathway. The various pathways by which extracellular signals are transduced to the nucleus are depicted.

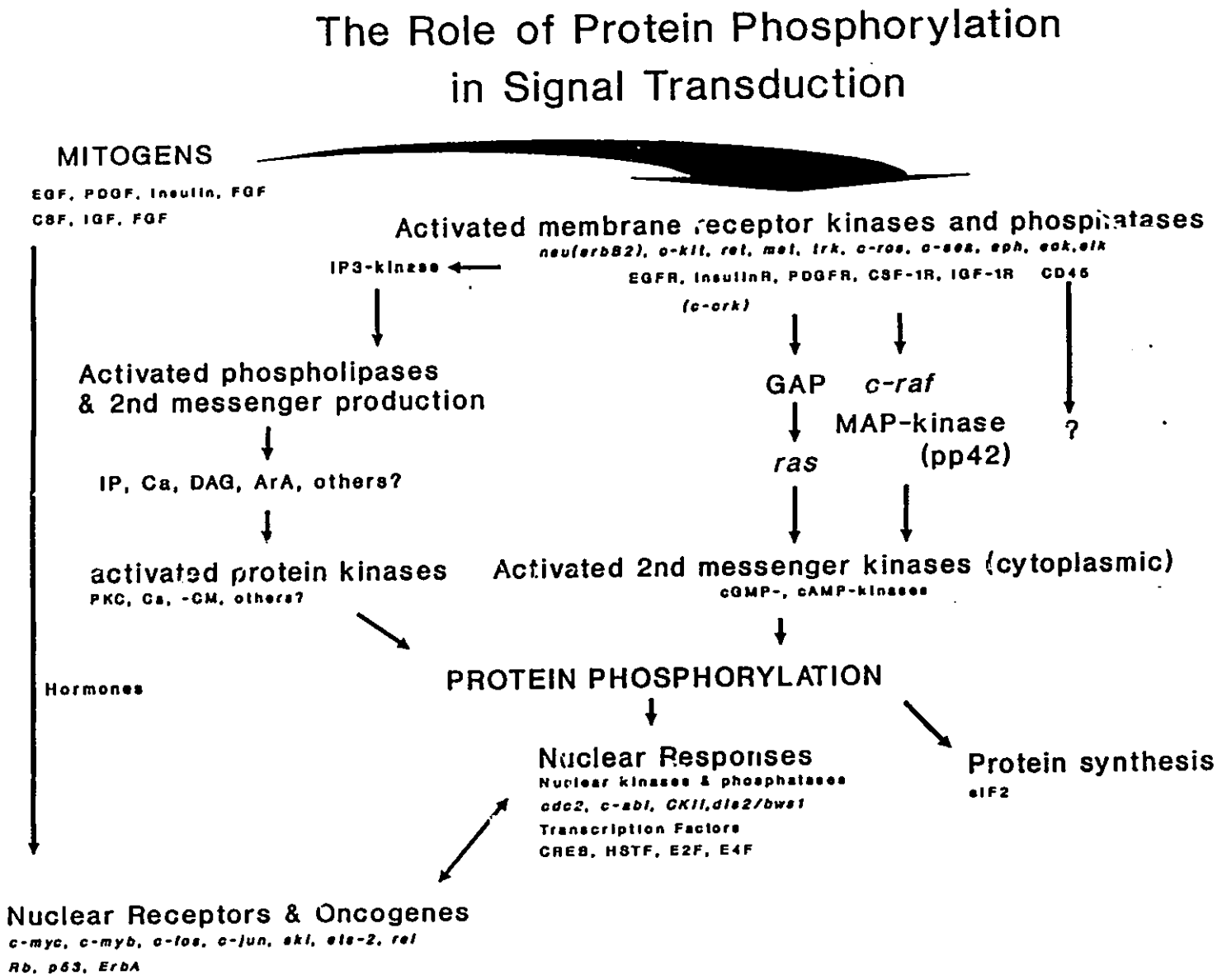


Figure 1.5 Schematic of Signal Transduction Pathway.

VIIai. Receptor Kinases.

Receptor kinases are composed of three major domains: (i)- an extracellular ligand binding domain; (ii)- a hydrophobic transmembrane domain; (iii)- and a cytoplasmic protein kinase domain (reviewed by Ullrich and Schlessinger, 1990). In order to be activated, receptors must oligomerize, a process which is stimulated either by ligand binding by a single receptor, which induces conformational changes resulting in oligomerization, or by binding of two receptors to a bivalent ligand (Greenfield *et al.*, 1989; Seifert *et al.*, 1989; Helden *et al.*, 1989; Hammacher *et al.*, 1989). This oligomerization results in autophosphorylation or transphosphorylation on tyrosine residues and enhances phosphorylation of serine/threonine residues by associated regulatory molecules. These events are important for activation of receptor kinase activity which in turn is required for both the mitogen response, in the case of growth factors, and proper receptor recycling (Felder *et al.*, 1990).

The receptors for PDGF and EGF have been found in large complexes that are comprised of regulatory factors and substrates termed "Signal Transfer Particles" (STP) (Margolis *et al.*, 1989; 1990; Williams *et al.*, 1990). This observation suggests that at least the initial components of the signal transduction pathway are physically associated with the primary transducing molecule, thus ensuring a rapid and specific response to the external signal.

VIIIai. Cytoplasmic Kinases.

The majority of cytoplasmic kinases identified to date are of the serine/threonine

kinase class. The exceptions are the p150$^{c\text{-}abl}$, and the pp60$^{c\text{-}src}$ family of tyrosine kinases (reviewed by Hunter, 1989). pp60$^{c\text{-}src}$ is found in the cytoplasm but due to N-terminal myristoylation, it associates with membranes (Buss and Sefton, 1985; Schultz and Oroszlan, 1984; Schultz *et al.*, 1984). This membrane association is critical for transformation because mutants of the viral pp60$^{c\text{-}src}$ which lack the myristoylation site are transformation defective (Kamps *et al.*, 1985; Buss *et al.*, 1986). The kinase activity of pp60$^{c\text{-}src}$ is regulated by phosphorylation. Kinase activity is down-regulated by phosphorylation on tyrosine 527 and activated by phosphorylation on tyrosine 416 (reviewed by Cooper *et al.*, 1989). Tyrosine residue 416 is a site of autophosphorylation, while tyrosine 527 is phosphorylated by a novel class of tyrosine kinase (Nada *et al.*, 1992). In addition, pp60$^{c\text{-}src}$ is phosphorylated on serine and threonine residues, however, mutations in the major phosphorylation site (serine 17), which is phosphorylated by cyclic-AMP dependent protein kinase A, and another site (serine 12) phosphorylated by calcium-phospholipid-dependent protein kinase C have thus far been found to have no apparent biological effect (Hirota *et al.*, 1988; Yaciuk *et al.*, 1989). However, mutations in serine 17 cause an increase in the phosphate incorporated in serine 12, suggesting that phosphorylation at serine 17 down-regulates the phosphorylation at serine 12 (Yaciuk *et al.*, 1989). pp60$^{c\text{-}src}$ is also phosphorylated on serine residues 34, 72 and on threonine 46 during mitosis (Shenoy *et al.*, 1989) and during this portion of the cell cycle pp60$^{c\text{-}src}$ has increased kinase activity (Shenoy *et al.*, 1989; Morgan *et al.*, 1989).

The cell cycle dependent phosphorylation of another tyrosine kinase, p150$^{c\text{-}abl}$

which is found predominately in the nucleus (Kipreos and Wang, 1990), further supports the notion that tyrosine phosphorylation plays an integral part in regulating the cell cycle. The product of the activated viral *c-abl* counterpart, $p160^{gag\text{-}v\text{-}abl}$ is no longer found in the nucleus, but is found predominantly in the cytoplasm due to deletion of the normal amino terminus and fusion to viral myristoylated *gag* sequences. The amino terminus of $p150^{c\text{-}abl}$ contains sequences needed for nuclear targeting, and thus transformation by *v-abl* may be due to a change in the cellular localization of its product. As previously mentioned, phosphorylation of Y15 of $p34^{cdc2}$ regulates entry into mitosis. It has been proposed that $p34^{cdc2}$ may be a substrate of $p150^{c\text{-}abl}$ and that reduced $p34^{cdc2}$ tyrosine phosphorylation as consequence of decreased nuclear $p150^{c\text{-}abl}$ may result in a more active $p34^{cdc2}$, thus enhancing cellular proliferation (Van Etten *et al.*, 1989). However, the tyrosine kinase activity of $p150^{c\text{-}abl}$ is required for transformation (reviewed by Rosenburg and Witte, 1988), suggesting that like $pp60^{c\text{-}src}$, both cellular localization and kinase activity are requirements.

A possible link between receptor kinases and cytoplasmic kinases was suggested by reports that the activity of the serine/threonine kinases encoded by the proto-oncogene *c-raf* and the microtubule-associated protein kinase (MAP-kinase) are regulated by tyrosine phosphorylation (Morrison *et al.*, 1989; Rossomando *et al.*, 1989). The *c-raf* protein kinase is believed to be phosphorylated by the PDGF receptor (Morrison *et al.*, 1989), while MAP-kinase is phosphorylated upon mitogen stimulation (Rossomando *et al.*, 1989). The *c-raf* substrates are unknown, however, MAP-kinase has been shown *in vitro* to phosphorylate microtubule-associated protein 2 as well as ribosomal protein S6

kinase II (Sturgill *et al.*, 1988). These observations support the concept of a kinase/kinase cascade involved in signal transduction.

The degradation of membrane lipids offers another mechanism through which the cell transmits external stimuli (reviewed by Rozengurt, 1986). In fact, some growth factors and tumor promoters are capable of activating this turnover. A key player in this turnover is the 3' phosphatidylinositol (PI-3) kinase (*Fig.* **1.5**) which is phosphorylated on tyrosine residues upon stimulation with PGDF (Kaplan *et al.*, 1990). The membrane degradation products diacylglycerol (DAG), arachidonic acid (ArA), and inositol 1,4,5-triphosphate (IP3) (*Fig.* **1.5**), are responsible for activating protein kinase C, producing type E prostaglandins which stimulate adenylate cyclase activity, and mobilizing calcium (reviewed by Rozengurt, 1986). All of these end products produce agents known to stimulate protein kinases, thus further supporting the role of protein phosphorylation and kinase/kinase cascades in signal transduction.

Another protein kinase activity known to increase as a result of mitogen treatment is casein kinase II (CKII) (Sommercorn *et al.*, 1987; Klarlund and Czech, 1988; Ackerman and Osheroff, 1989; Carroll and Marshak, 1989) which is present in all cell compartments (Hathaway and Traugh, 1982), and acts at serine/threonine residues followed by a stretch of acidic residues.

VIIb. PHOSPHORYLATION WITHIN THE NUCLEUS.

CKII has been shown to phosphorylate some nuclear oncoproteins which include the E7 protein from several serotypes of HPV (Barbosa *et al.*, 1990),

$p64^{c\text{-}myc}$ (Lüscher *et al.*, 1989) and $p75^{myb}$ (Lüscher *et al.*, 1990). The role of these phosphorylation events remain unclear, however, the phosphorylation of the HPV E7 proteins is thought to be an important step in oncogenesis (Barbosa *et al.*, 1990). In addition, the phosphorylation of $p64^{myc}$ is thought to be important for its localization to the nucleus (Lüscher *et al.*, 1989). The role of $p75^{myb}$ phosphorylation has one important ramification as the CKII sites are found on a region which is deleted in the activated *v-myb*, and phosphorylation of this region *in vitro* inhibits DNA binding (Lüscher *et al.*, 1990). Experiments using *myb* antisense oligonucleotides demonstrated an inhibition of mitogen stimulation, suggesting that $p75^{c\text{-}myb}$ plays an important role in cellular proliferation (Gewirtz *et al.*, 1989). Taken together these results suggest that a protein kinase cascade involving $p75^{c\text{-}myb}$ as the final substrate may be involved in mediating cellular responses to some mitogens.

The *c-fos* and *c-jun* proto-oncogene products comprise the transcription factor AP-1 which is involved in the immediate response to mitogens (reviewed by Eisenman, 1989). These proteins associate as homo- and heterodimers (reviewed by Ziff, 1990) and both are extensively phosphorylated (Barber and Verma, 1987; Cohen and Curran, 1988; Nakabeppu *et al.*, 1988). The role phosphorylation plays in regulating transcription activation or dimer association is unclear, however, studies with the CREB/ATF transcription factor may provide some clues. Phosphorylation of CREB by protein kinase C induces it to dimerize and to bind to DNA, resulting in an increased rate of transcription (Yamamoto *et al.*, 1988). Other transcription factors that are phosphorylated upon activation include the yeast heat shock factor (HSTF) (Sorger and Pelham, 1988),

E2F and E4F (Raychaudhuri *et al.*, 1990). Thus phosphorylation may represent a general post-transcriptional mechanism by which transcription factors are regulated in very rapid response to external stimuli.

As previously described, $p105^{Rb}$ and p53 polypeptides are both nuclear phosphoproteins which are thought to down-regulate cellular proliferation. As discussed above, phosphorylation is believed to regulate $p105^{Rb}$ activity and thus the cell cycle. $p105^{Rb}$ is believed to be capable of binding DNA (Lee *et al.*, 1989) although it has no clear sequence specificity. It now appears that $p105^{Rb}$ regulates transcription by complexing and inactivating the transcription factor E2F (Bagchi *et al.*, 1990). Phosphorylation probably affects this process. The role phosphorylation plays in controlling p53 function is not clear, however, it has been shown that both the cyclin A/$p34^{cdc2}$ and cyclin B/$p34^{cdc2}$ kinase complexes phosphorylate p53 on serine 315 (Bischoff *et al.*, 1990).

VIII. CONCLUDING REMARKS.

It is clear that both phosphorylation and the formation of protein complexes play key roles in regulating cellular proliferation. Thus further information about these events will undoubtedly lead to a better understanding of the mechanism of oncogenesis.

IX. OBJECTIVES OF THIS STUDY

The present studies were initiated to determine the role that phosphorylation plays in regulating adenovirus E1A function. As described above, E1A proteins are phosphorylated at multiple sites, however, the biological functions of these modifications remain unclear. In the present studies, site-directed mutagenesis was used to convert the previously mapped serine phosphorylation sites in the first exon to alanine residues. These studies demonstrated that the large decrease in gel mobility in SDS-PAGE was due to phosphorylation at serine 89. This mutation also resulted in a decreased efficiency in the ability of E1A products to cooperate with either E1B or *ras* to transform primary rodent cells. Subsequent *in vitro* and *in vivo* experiments also demonstrated that the cell cycle protein kinase $p34^{cdc2Hs}$ phosphorylates E1A at serine residues 89 and 219.

CHAPTER II: MATERIALS AND METHODS

I. BACTERIA AND BACTERIOPHAGE CULTURE.

Ia. *E. coli* GROWTH.

Cultures of *E. coli* were grown at 37°C under standard conditions in LB broth plus the appropriate antibiotic for plasmid selection, if required.

Ib. GROWTH AND ISOLATION OF M13.

Bacteriophage M13 was propagated on exponentially growing *E. coli* cells under standard conditions (Zoller and Smith, 1984).

Table 2-1. **Bacteria and Bacteriophage Strains.**

Name	Genotype
MV1190	Δ(*lac-pro* AB), *thi*, *sup*E,Δ(*sr1-rec*A)306::Tn10 [F': *tra*D36, *pro*AB,*lac*I^{q}ZΔM15]7
CJ236	*dut*, *ung*, *thi*, *rel* A; pCJ105
XL1-Blue	*end*A1, *hsd*R17($r_K^-m_K^+$), *sup*E44, *thi*-1, lambda $^-$, *rec*A1, *gyr*A96, *rel*A1, Δ(*lac*), [F',*pro*AB,*lac*^{q}ZΔM15, Tn10]
LE392	F$^-$*hsd*R17($r_K^-m_K^+$), *sup*E44, *sup*F58, *lac*Y1 or Δ(*lac*IZY)6 *gal*K2 *met*B1 *trp*R55 lamba$^-$.
M13mp11Ad5	Containing Ad 5 sequences from Nucleotide 22-1340. (Jelsma *et al.*, 1988; McKinnon *et al.*, 1982.)

II. CELL AND VIRUS CULTURE.

IIa. TISSUE CULTURE.

Human KB and HeLa cells were cultured on 100mm plastic dishes in α-MEM supplemented with 10% FCS. Human Hep2 cells were cultured in Dulbecco's complete medium supplemented with 10% NBCS. The Ad5 transformed 293 cells (Graham, *et al.* 1977) were cultured in F11 medium supplemented with 10% NBCS. All of these cells were cultured under standard conditions.

Baby rat kidney cells were prepared from fresh kidneys obtained from 4-6 day old Wistar rats. The connective tissue was removed and the kidneys diced with scissors to a homogeneous soup. This soup was trypsinized twice with 20ml of 2X trypsin for 20 min at room temperature (RT) in a 100ml Gibco bottle with spinning. Then the cells were decanted away from the undigested material into 20ml of ice cold FCS. After trypsinization of the tissue the cell preparations were combined and resuspended in 100ml of α-MEM+10% FCS and placed at 37°C for 30 min. The coagulate which formed during this incubation was filtered through sterile cheese cloth and the cell suspension brought to the appropriate volume with α-MEM+10% FCS. Normally about ten 60 mm dishes of cells could be obtained from each pair of kidneys. The medium was changed after a day and the cells were used in transformation assays (see below).

IIb. VIRUS GROWTH.

Wildtype Ad5 used was the strain described by Harrison *et al.*, (1977). It was grown on monolayers of either 293 or Hep2 cells plated in plastic 150mm dishes under standard conditions.

IIc. VIRUS TITER.

All viruses were titered on monolayers of 293 cells plated on 60mm dishes using well established protocols.

IId. METABOLIC LABELING.

IIdi [^{35}S] Methionine.

Infected or mock infected cells were labeled for 2 hr with 100μ*Ci* of [^{35}S]methionine (Amersham Corp.; 1300 *Ci*/mmol, or Trans ^{35}S-label; ICN Radiochemicals; 1,159 *Ci*/mmol) in 2ml of 199 medium lacking methionine.

IIdii. [^{32}P]Phosphate.

Cells were labeled for 4 hr with 2.5 m*Ci* of [^{32}P]orthophosphate (NEN; 3000 *Ci*/mmol) in phosphate free 199 medium supplemented with only 1% L-glutamine.

IIe. PREPARATION OF CELL EXTRACTS.

After the labeling period cells were washed twice in ice cold PBS^{2-} and removed from the dish by scrapping with a rubber-policeman. The cells were pelleted by centrifugation in a chilled (4°C) table-top centrifuge at 2000 rpm for 10 min and then

lysed in 1ml of RIPA^{++++} (50mM Tris (Ph 7.2), 150mM NaCl, 1% Triton X100, 1% Na deoxycholate, 0.1% SDS, 100 KIU/ml of aprotinin, 500µM $NaVO_4$, 50mM NaF, 1mM EDTA, and 1mM ß-glycerophosphate) per plate of cells for all purposes except the study of E1A-associated proteins. For this latter purpose RIPA^{-} (50mM Tris (Ph 7.2), 150mM NaCL, 1% Triton X100, 100 KIU/ml aprotinin) was used to lyse the cells. The cell pellet was solubilized by pipeting up and down once or twice after which the cell extract was chilled on ice for 10 min. Cell debris and nuclei were removed by centrifugation at 4°C using a Sorval SS34 rotor spun at 18000 rpm for 1 hr at 4°C. The supernatant was used immediately or occasionally frozen at -20°C.

IIf. ANTISERA AND IMMUNOPRECIPITATION.

Antibodies to the E1A, E1B, and E2A gene products were used in these studies. Three types of antibodies were used to immunoprecipitate the E1A polypeptides: (i) a rabbit anti-peptide serum (E1A-C1) raised against a peptide containing the predicted amino acid sequence of the carboxyl terminus (Yee *et al.*, 1983); (ii) a rat monoclonal (R28) antibody raised against the 13S E1A protein made in and purified from *E. coli* (Tsukamoto *et al.*, 1986); and (iii) a mouse monoclonal antibody (M73) that recognizes an epitope mapping to the second exon of E1A polypeptides (Harlow *et al.*,1985). The R28 antibody was obtained from culture supernatant or from the generation of ascites fluid in nude mice. The M73 antibody was purchased from Oncogene Science.

Antibodies which reacted with the products from the E1B region were generated using synthetic peptides deduced from the putative amino acid sequence obtained from

their mRNAS (McGlade *et al.*, 1987; D. Takayesu and P.E. Branton unpublished results). The E1B 19K serum was directed against the predicted carboxyl terminus of the protein (McGlade *et al.*, 1987). The E1B 58K antiserum was made to the predicted amino terminus of 58K and thus it immunoprecipitated all of the 58K-related products (D. Takayesu and P.E. Branton unpublished results) The 72KD protein encoded by the E2A gene was immunoprecipitated using H2-67 mouse monoclonal antibody (Branton *et al.*, 1985).

E1A proteins were immunoprecipitated according to a standard protocol. Immunoprecipitation reactions which required preincubation with peptide against which the antibody was raised were incubated with the antibody for 1 hr with mixing prior to the addition of cell extract. Once the precipitates were washed they were resuspended into 40µl of 1X sample buffer (0.125M Tris base, Ph 6.8., containing 2% 2-mercaptoethanol, 2% SDS, and 20% (vol/vol) glycerol).

IIg. GENERATION OF ASCITES IN NUDE MICE.

Nude mice were used to generate ascites tumors with R28 hybridoma cells because no suitable rat host was available. Nude mice obtained from the mouse colony at Guelph University were injected intraperitoneally with 0.5ml of pristane (Sigma) followed a week later by injection with $1x10^7$ hybridoma cells. Ascites fluid was apparent 2 to 3 weeks after injection after which time it was tapped (usually twice) and clarified by centrifugation prior to storage at -20°C in 20µl aliquotes.

IIh. INDIRECT IMMUNOFLUORESCENCE.

HeLa cells plated onto coverslips at a density of 6 X10^4 cells/well in a 24 well tissue culture dish (Nunc) a day before use were infected at different moi's. Cells were washed twice with PBS^{2+} and then fixed with 5% formalin in PBS^{2-} at RT for 20 min after which they were washed twice for 5 min with PBS^{2-} and three times with immunofluorescence dilution buffer (3% BSA, 0.05% (vol/vol) Tween-20 (Sigma), and 0.02% (wt/vol) NaAzide (BDH)). The fixed cells were permeabilized by immersing them in a 1% (vol/vol) solution of NP-40 (Sigma) in PBS^{2-} for 20 min at RT. The cells were washed twice after this step in dilution buffer. A few drops of M73 antibody at 20µg/ml in dilution buffer was added to the coverslips which were then placed at 37°C for 60 min. The coverslips were washed 5 times with dilution buffer for 5 min and after the final wash a few drops of biotinalated goat anti-mouse antibody (BRL) at 6µg/ml in dilution buffer were added and incubated at 37°C for 45 min. This second antibody was removed and the coverslips washed 5 times for 5 min with dilution buffer. To identify the immunocomplexes, a streptavidin-fluorescein isothiocyanate conjugate (SA-FITC) diluted 1:12000 in dilution buffer was added to the coverslips and incubated at 37°C for 30 min in the dark. The coverslips were washed 3 times with PBS^{2-} and once with ddH_2O. The coverslips were mounted with Aquamount. The immunocomplexes were examined using an immunofluorescence microscope set to the standard FITC filter set (blue excitation) and photographed with Kodak 400ASA film.

III. ELECTROPHORESIS AND CHROMATOGRAPHY.

IIIa. SDS-PAGE AND PROTEIN MANIPULATIONS.

The discontinuous gel system described by Rowe *et al.*, (1983) was used to resolve proteins.

IIIai. Fluorography.

To decrease the exposure time with [^{35}S]-methionine-labeled proteins the gels were treated twice with DMSO for 1 hr and then immersed in a 10% PPO/DMSO solution for at least 3 hr following which they were rehydrated with water and washed several times before drying under vacuum.

IIIaii. Protein extraction.

Preparatory gels containing [^{32}P]-labeled E1A proteins were either dried without fixing or exposed as wet gels to Kodak XAR-5 film for 3-20 hr, as warranted. The protein bands of interest were excised and the gel slices were rehydrated with ddH_2O and the paper backings removed. The gel pieces were crushed with a teflon plunger and 10ml of elution buffer (50 mM Ammonium bicarbonate, pH8, containing 0.1% (wt/vol) SDS, and 0.5% (vol/vol) 2-mercaptoethanol) was added. The slurry was placed in a boiling water bath for 10 min after which it was mixed end-over-end at 37°C overnight. Gel pieces were sedimented by centrifugation at 5000 rpm for 10 min in a table top clinical centrifuge and the supernatant was combined with an additional 5ml of elution buffer. These tubes were again boiled for 10 min, and elution was carried out for 2 to 3 hr after which the acrylamide was removed as described above. The supernatants were pooled and filtered through siliconized glass wool to remove residual acrylamide. The filtrate

was placed on ice and 200µg of human globin (Sigma) was added as carrier and the solutions were made to 20% TCA and left on ice for 3 to 4 hr. The precipitate was collected by centrifuging the samples at 10000 rpm in a Sorval SS34 rotor for 1 hr at 4°C.

The protein pellet was washed with either 95% EtOH or acetone chilled to -20°C, it was air dried, and then combined with 200µl of fresh performic acid (9ml of 98% formic acid and 1ml of hydrogen peroxide), as described by Hirs (1967) . This mixture was mixed well and allowed to sit at RT for 1 hr before use. The protein solution was frozen in liquid N_2 and the frozen solution was overlayed with ddh_2O to act as plug to limit the loss of proteins during lyophilization. The dried protein pellets were resuspended twice in 100µl ddH_2O and dried in a speedvac. The extracted protein was now ready for further manipulations.

IIIaiii. Trypsin digests.

Oxidized gel-purified proteins were resuspended into 200µl of 50 mM ammonium bicarbonate, (pH8), containing 50µg of TPCK-trypsin (Worthington) and the tubes were incubated at 37°C for 4 hr after which time another 50µg of trypsin was added for an additional 4 hr incubation. The digested protein was lyophilized ON and used for further analyses.

IIIaiv. *In situ* trypsin digests.

Tryptic peptides were also generated by excising E1A proteins from gels and washing the gel piece twice in gel fix lacking glycerol (20% methanol and 10% acetic acid (vol/vol)) and twice with ddH_2O. The gel pieces were mashed with a teflon plunger

and resuspended in 300µl of 50 mM ammonium bicarbonate (pH8) containing 100µg of TPCK-treated trypsin (Worthington) and the tubes mixed end-over-end at 37°C ON. The acrylamide was sedimented by centrifugation and the supernatant removed. To further extract the peptides an additional 300µl of ddH_2O was added to the acrylamide, it was vortexed and again centrifuged. The two supernatants were pooled and, depending on the size of the final volume dried either by speedvac (small volumes) or lyophilizer. The dried protein pellet was oxidized and lyophilized as described above.

IIIb. TWO DIMENSIONAL THIN LAYER CHROMATOGRAPHY.

All peptide analysis by TLC was carried out at Ph 8.9. For [^{32}P]-labeled proteins 2000-4000 cpm were spotted onto the cellulose plate (Polygram CEL 300) and the spot was overlayed with 0.5µl of tracking dye (0.1% Orange G, and 0.1% Xylene Cyanol in water). Using a refrigerated TLC apparatus, electrophoresis was carried out at 1400V until the orange dye reached the edge of the wick (approximately 45 min). The dried TLC was then subjected to ascending chromatography using N-butanol-pyridine-formic acid-water (75-50-15-60; vol/vol/vol/vol). The TLC was removed dried in a fume hood and exposed to film. [^{35}S] labeled proteins were treated exactly in the same fashion except that 4000 to 6000 cpm were spotted when possible and after the chromatographic step plates were treated with the fluorographic agent Enhance (NEN) and exposed to film.

IIIc. PHOSPHOAMINO ACID ANALYSIS.

Gel purified protein was hydrolysed with 6N Hcl at 110°C for 1 hr. The hydrolysate was frozen in liquid N_2 and overlayed with ddh_2O and frozen again after which the sample was lyophilized. The dried hydrolysate was twice resuspended in 50µl of ddH_2O and dried in a speedvac and then resuspended into 20µl of ddH_2O. A portion of this material containing about 2000 cpm was mixed with 5µg of cold phosphoserine, phosphothreonine, and phosphotyrosine markers (Sigma) and spotted onto a cellulose thin layer plate (TLP) (Polygram CEL300). This spot was overlayed with 0.5µl of TLC tracking dye and the phosphoamino acid analysis was performed in two fashions: (i) Two dimensional- The TLP was electrophoresed in the first dimension at pH1.9 (2.6% formic acid, and 8.2% acetic acid (vol/vol) in ddH_2O), at 1000V, until the orange dye reached the wick. It was then dried, rotated 90° and electrophoresed in the second dimension at 1000V using pH3.5 buffer (0.5% pyridine, 5% acetic acid,(vol/vol) and 1mM EDTA in ddH_2O) (ii) One dimensional-The procedure was similar to that described above except that only the pH3.5 dimension was used. In both procedures the positions of the cold phosphoamino acids were detected by staining with ninhydrin and those of labeled material by autoradiography.

IIId. HPLC.

Tryptic peptides were separated by HPLC using procedures adapted from Tremblay *et al.*(1989). Peptides were dissolved into 500µl of 20% formic acid in ddH_2O and the mixture was filtered through a 0.22µm filter. Separation was achieved at RT on

a Waters dual pump HPLC system with a 600E controller using a 4.6 X 250 mm Ultrasphere ODS (C18) reverse phase column which had been prequilibrated with buffer A (5% formic acid in ddH_2O). Peptides were eluted from the column by a linear 1 to 63% gradient of buffer B (5% formic acid in ethanol) for 95 min at a flow rate of 1ml/min. Detection of radio-peptides was achieved with an on-line isotope detector (Berthoid, LB507A).

IIIe. PAGE.

IIIei. Gel preparation.

DNA fragments were resolved in either 4% or 5% acrylamide gels electrophoresed in TBE (89mM Tris, (pH8.3), containing 89mM boric acid, and 25mM EDTA) according to standard protocols.

IIIeii. DNA extraction.

DNA fragments ranging from 3000bp to 100bp were separated by PAGE. After staining the gel with EtBr in complete darkness, the DNA fragment of interest was detected using a UV lamp and excised. The gel piece was mashed with a teflon plunger and the DNA was eluted by shaking ON at 37°C with 0.5 to 1.0ml of DNA elution buffer (500mM ammonium acetate, 10 mM Mg acetate, 1mM EDTA, and 1% SDS) the following day the acrylamide was removed by centrifugation and it was extracted using an additional 0.5ml of elution buffer. The supernatants were pooled and filtered through siliconized glass wool. The DNA was precipitated with one-tenth volume of 2.5M ammonium acetate, pH8, and 2.5 volumes of EtOH precooled to -20°C. The DNA was

pelleted by centrifugation at 12000rpm in a microcentrifuge at 4°C and the DNA pellet was washed at RT with 70% EtOH, 95% EtOH, and then dried under vacuum in a speedvac.

IIIf. AGAROSE GELS.

Agarose gels were cast according to well established protocols and ran submerged using TBE buffer.

IIIg. DNA SEQUENCING.

IIIgi. Gel preparation.

Sequencing acrylamide (40%, 19:1) was deionized by adding 1g of AG 501-X8 Ion Exchange Resin (BioRad) /100 ml and stirred at room temperature (RT) for 1 hour. The resin was removed by filtration. Sequencing gels were cast and electrophoresed according to standard lab protocols.

IIIgii. Sequencing reactions.

All sequencing reactions utilizing ssDNA as a template were carried out according to the protocol supplied with the T7-DNA polymerase sequencing kit purchased from Pharmacia. Sequencing of dsDNA involved additional standard steps to denature the template. Unless stated otherwise all reactions were done with [α-^{35}S]ATP (Amersham; 1000-1500 *Ci*/mmol) as the isotope.

IV. RECOMBINANT DNA.

IVa. PLASMID ISOLATION.

IVai. Small Scale Isolation of Episomal DNA.

Small scale isolation of plasmid DNA was done by alkali lysis following well established lab protocols (Maniatis *et al.*, 1989).

IVaii. Large Scale Preparation of Episomal DNA.

Large scale plasmid and M13 RF isolation was done according to established lab protocols (Maniatus *et al.*, 1989). Further purification was accomplished by ultracentrifugation through a CsCl density gradient according to established protocols (Maniatus *et al.*, 1989).

Table 2.2 **Plasmids used in these studies.**

Plasmid	Special Characteristics	Source
PLE2	Genomic E1A gene	Dr. S. Bayley
pXC38	Adenovirus N22 to 5788	Dr. F. Graham
pJM17	Complete viral genome+pBX1	Dr. F. Graham
pJF12	12S cDNA of E1A	Dr. N. Jones
pE1*ras*	activated H-*ras* gene	Dr. S. Bayley
pKCAT23	E3 promoter driving CAT	Dr. S. Bayley

IVb. TRANSFECTION OF MAMMALIAN CELLS.

IVbi. Transient Assay.

The day before the DNA transfection was to be done, HeLa cells were seeded at a cell density of 1 X 10^6 cells/60mm dish. The medium was changed and cells were

transfected 4 hr later. During this time DNA cocktails were made as follows:

Volumes for one 60mm dish.

Plasmid DNA	volume for desired concentration.
carrier DNA	volume to bring total DNA to 15µg
2.5M $CaCl_2$	25µl
sterile ddH_2O	to a final volume of 250µl

This mixture was mixed well and added drop-wise to 0.25ml of 2X HEPES buffered saline (1.6g of NaCl, 0.074g KCl, 0.02 Na_2HPO_4, and 1.0 g of HEPES made to 100 ml with ddH_2O and adjusted to pH 7.1) and mixed. The precipitate was allowed to form for 1 hr after which the DNA cocktail was added drop wise to cells which were incubated ON then given fresh medium.

IVbii. Rescue of Mutations into Virions.

The night before use, 293 cells were seeded at 1 x 10^6 cells/60mm dish and the following morning the medium was changed and cells were transfected 4 to 5 hr later. The procedure used was taken from McCrory *et al.*,1988. Briefly, 5µg of E1 or E1A plasmid DNA was cotransfected with 5µg of pJM17 plasmid DNA (*Table* **2.2**). The following morning the medium was removed and 10ml of overlay medium (F11 medium supplemented with 5% HS, 1% L-glutamine, and 1% penicillin/streptomycin (vol/vol) containing 0.7% (wt/vol) agarose) was added. Plaques were visible 7 to 10 days post-transfection. The plaques were picked and expanded on monolayers of 293 cells plated on 60mm dishes. Upon attaining 90-95% cytopathic effect (cpe), viral DNA was extracted and analyzed by restriction digestion (see section **IVe. Extraction of Viral DNA**).

IVbiii. Transformation Assays.

Primary baby rat kidney cells were used in all DNA-mediated transformation

assays. The culture medium on cells prepared the day before was changed and they were transfected 4 to 5 hr later. DNA cocktails were prepared as described for transient assays except that the DNA/$CaCl_2$ mixture was added drop-wise into 2X HEPES buffered saline which had a stream of air bubbling through it to ensure efficient mixing. The precipitate was allowed to form for 1 hr and it was added to the cells and left ON. The following morning the medium was changed and cells were grown for 2 days after which medium was replaced with selection medium. For E1A and E1B cooperation assays selection was carried out in Joklik's medium supplemented with 5% HS, and for E1A and *ras* cooperation assays selection was in α-MEM supplemented with 5% FCS. The medium was changed every 3 days and foci were usually detected by the second to third week post-transfection. Foci were identified by removing the medium and fixing the cells in cell fixative (10% acetic acid and 50% methanol in ddH_2O) for 30 min and then the dishes were washed once with water and allowed to completely dry. Foci were stained with a 2% Geimsa (Fisher) stain for 30 min after which the cells were washed with water, dried and the foci counted.

IVbiv. CAT ASSAYS.

CAT assays were done as described by Nordeen *et al.* (1987). Briefly, HeLa cells were cotransfected with the reporter construct and either a *wt* E1A expressing plasmid or E1A mutants (*Table* **2.2**). Cells were harvested 40 to 48 hr post-transfection in ice-cold wash buffer (40mM Tris-HCl (pH 7.4), containing 150mM NaCl, and 1mM EDTA) and sedimented for 3 seconds in a microcentrifuge at 4°C. The cell pellet was resuspended in freeze thaw buffer (250mM Tris-HCl (pH 7.8), containing 0.005% (wt/vol) PMSF), and

disrupted by 3 consecutive freezings in liquid N_2 and thawings at 37°C. The cell debris was removed by centrifugation and the cell extract was placed at 60°C for 10 min as described by Crabb and Dixon, (1987). The precipitated protein was removed by centrifugation and the extract was either frozen at -70°C or used immediately. Preliminary experiments demonstrated that the protein content present in equal volumes of cell extract from separate plates of cells did not vary significantly and thus protein determinations were not done for every assay.

IVc. EXTRACTION OF VIRAL DNA.

Monolayers of 293 cells plated on 60mm dishes were infected with individual plaque isolates and upon attaining 95% cpe the cells were removed by gentle pipeting and then collected by centrifugation at 1000 rpm for 5 min at RT in a bench-top clinical centrifuge. The cell pellets were resuspended into 500µl of viral extraction buffer (10mM Tris (pH 7.8), containing 5mM EDTA, and 0.5% (wt/vol) SDS) containing 50µg/ml of proteinase K (BRL). The mixtures were incubated at 37°C for 30 min and phenol extracted once. The DNA was ethanol precipitated and sedimented by centrifugation at 4°C in a microcentrifuge. The DNA pellet was washed once with 70% and 95% EtOH, then dried in a speedvac and resuspended into 50µl of TE, (pH8).

IVd. SOUTHERN ANALYSIS OF VIRAL DNA.

IVdi. Transfer.

Viral DNA was prepared as described above and digested with the appropriate

restriction enzymes. The digests were electrophoresized ON in a 2% agarose gel and DNA fragments visualized by EtBr staining. The gel was soaked in denaturation solution (0.5M NaOH, and 1.5M NaCL) for 1 hr at RT and then neutralized for 1 hr in 1M Tris (pH7.5) containing 1.5M NaCl. The transfer was done with 20X SSC by capillary action as described in Maniatis *et al.*, (1989).

IVdii. Hybridization.

Prehybridized of the nitrocellulose filters was in freshly prepared 6X SSPE (20X= 200mM NaH_2PO_4, (pH 7.7) containing 3M NaCL and 20mM EDTA) containing 0.5% (wt/vol) SDS, and 5X Denhardt's (50X= 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA wt/vol) which was done at the T_m of the oligonucleotides. The T_m for an oligomer of 14 to 20 nucleotides in length was defined as [2° for each A or T plus 4° for each G or C] - 5°C.

IVe. SITE DIRECTED MUTAGENESIS.

Table 2.3. **Oligonucleotides Used in These Studies.**

Oligonucleotide	Sequence	Use
AB147	TTTCGCAGATCTTTCCCGACT	Silent *Bgl* II site
AB216	GGCGCCCGGTGCTCCGGAGCC	Ser89 to Ala
AB217	GCCTCACCTTGCGCGGCAGCCCG	Ser96 to Ala
AB431	GGCGCCCGGTTCTGCGGAGCCGC	Pro90 to Ala
AB432	GGCGCCCGGTGACCCGGAGCCGC	Ser89 to Asp
AB485	GTGGCAGATAATATGTCTCATTTT	E1A primer
AB560	ACAGGCCAGTAACGCCAGACTTCTC	ß-globin primer

IVei. Phosphorylation of Oligonucleotide.

All oligonucleotides used for mutagenesis (described in *Table* **2.3**) were phosphorylated as described in Zoller and Smith, (1984).

IVeii. Mutagenesis.

Mutagenesis was done using the *in vitro* mutagenesis kit purchased from BioRad. This kit is based on the procedure described by Zoller and Smith (1984), as modified by Kunkel (1985). The procedure is outlined in Figure **3.1** and was carried out in the following manner: (i)- M13mp11Ad5 was propagated on CJ236 cells (*dut⁻ ung⁻*) using a very low moi so as to minimize the contribution of non-passaged phage to the population; (ii)- To test for the presence of sufficiently methylated DNA the progeny virus was titered on both *wt* [MV1190 or XL1-Blue (*dut⁺ung⁺*)] and CJ236 (*dut⁻ung⁻*) bacteria. If the titer difference on *wt* and CJ236 was not greater than 1 X 10^4 pfu the M13 was not used; (iii)- M13 from a 15ml culture was precipitated with 2ml of 20% PEG 8000 containing 2.5M NaCL for 30 min at RT. The phage was sedimented by centrifugation at 13000 rpm for 15 min in a microcentrifuge at RT. The phage pellet was phenol extracted three times and ethanol precipitated and sedimented by centrifugation using standard lab protocols. The DNA pellet was washed once each with 70% and 95% EtOH and dried in a speedvac. The DNA was resuspended in TE (pH8); (iv)- The phosphorylated oligonucleotides were annealed to the M13 DNA as described in the BioRad manual; (v)- synthesis of the complementary strand using T4 DNA polymerase was done according to the provided protocol. The success of this procedure was monitored by running the products on an 1% agarose gel; (vi)- The synthesis mixture was diluted and used to

transform *wt E. coli*. Plaques were then picked and expanded as 1.5ml preps and screened directly by sequencing (see *Figure* **3.1**).

Large scale RF preparations were done on doublely plaque purified clones as described in section IVai. Which were subsequently subcloned into plasmid using *BstX* I or with *Sst* II plus *Xba* I. The presence of these mutations were verified in all subsequent cloning steps by digestion with diagnostic restriction enzymes.

V. PRIMER EXTENSION ANALYSIS.

Va. INFECTION OF HEP2 CELLS.

Hep2 cells were used in these experiments because it has been reported by Lillie and Green (1986) that they allow for a more E1A-independent expression of the ß-globin gene on the in 340-2/ß-globin $^{enh(+)}$ virus.

The 12S E1A expressing viruses used in these studies were titered a minimum of 3 times. Hep2 cells seeded at 1 X 10^6/60mm dish the night before were coinfected with E1A expressing viruses at an moi of 10, while a moi of 100 was used for the ß-globin expressing virus. Virus was allowed to absorb for 1 hr after which it was removed and fresh medium containing 40µg/ml of AraC was added. The cells were refed the following day with medium containing AraC and harvested 48 hr post-infection.

Vb. ISOLATION OF TOTAL RNA.

The isolation of RNA was accomplished using RNAzol (CINNA/BIOTECX Lab. Int.) according to the protocol provided by the manufacturer with some added

modifications. Cells were washed twice with PBS^{-2} and were scraped from the dishes with a rubber policeman. These cells were sedimented by a brief spin in a microcentrifuge at 4°C and the cell pellet was solubilized in 500µl of RNAzol by pipeting and combined with 50µl of chloroform and left on ice for 15 min. The suspension was centrifuged for 15 min at 4°C after which the upper aqueous phase was removed and extracted two more times with phenol/chloroform/isoamyl alcohol (25:24:1; vol/vol/vol). The RNA was precipitated with an equal volume of isopropanol and sedimented by centrifugation in a microcentrifuge at 4°C. The pellet was resuspended in 0.3M DEPC-treated Na acetate (pH 5.2) and reprecipitated with 2.5X volume of 95% EtOH, sedimented by centrifugation in a microcentrifuge at 4°C, and then washed once each with 70% and 95% EtOH. The RNA pellet was resuspended in DEPC-treated 0.3M Na acetate (pH 5.2). To help dissolve the pellet the tube was heated to 60°C for 10 min. The concentration of RNA was determined by its absorption at OD_{260}.

Vc. END-LABELING OLIGONUCLEOTIDES.

The procedure for end labeling oligonucleotides with [τ^{32}P]ATP was identical to that described in the mutagenesis section except that the cold ATP was replaced with 50 *µCi* (3000 *Ci*/mmol, NEN) of radioactive ATP. The unincorporated [τ^{32}P]ATP was removed by size exclusion chromatography using mini-columns containing Sephadex G-50 equilibrated with TE (pH8).

Vd. PRIMER EXTENSION.

Primer extension reactions were done following the procedure described by Jones *et al.*, (1985). Briefly: (i)- 20µg of RNA was EtOH precipitated, washed with 70% and 95% EtOH and then dried in a speedvac with no heat for 1 hr; (ii)- The RNA pellet was resuspended in TE (pH 7.9) containing 200,000 cpm (Cerenkov units) labeled oligonucleotide.; (iii)- To this 2µl of TE (pH 7.9) containing 1.25M KCl was added the RNA and DNA were allowed to hybridize at 60°C (temperature used for both E1A and ß-globin oligonucleotides, table 2.3) for 1 hr, and allowed to cool slowly to RT; (iv)- 25µl of reverse transcription buffer (20mM Tris-HCL (pH8.7), 10mM $MgCl_2$, 5mM DTT, 0.33mM of each dNTP, and 10µg/ml of actinomycin D) containing 10 units of AMV reverse transcriptase (Life Sciences) was added and the reaction mixtures were incubated at 37°C for 1 hr; (v)- 300 µl of 95% EtOH were added and the precipitated extension products were sediment by centrifugation in a microcentrifuge at 4°C. The pellet was washed with 70% and 95% EtOH and dried in a speedvac.; (vi)- the extension products were resuspended in 4µl of ddH_2O and 4µl of sequencing STOP buffer (deionized formamide solution containing 10mM EDTA, 0.3% (w/v) xylene cyanol and 0.3% (w/v) bromophenol blue); (vii)- 4µl of this solution was loaded onto a 8% sequencing gel.

VI. CELL CYCLE EXPERIMENTS.

VIa. CELL CYCLE BLOCK.

HeLa cells growing on 100mm plastic dishes were subcultured the day before use such that they were 75 to 85% confluent for the next day. Cells were either infected with

Ad5 or they were mock infected and after 1 hr either α-MEM medium alone (untreated), or α-MEM containing 10mM hydroxyurea (G_1/S block), or α-MEM containing 40ng/ml of nocodazole (Mitotic block) were added.

Metabolic labeling of cells was as previously described. The untreated and hydroxyurea-blocked cells were harvested at 48 hr post-infection, as described previously. The cells blocked in mitosis were harvested by mitotic shake-off. This was done by either gently tapping the side of the dish or by a gentle wash with medium, and the cells that floated off were collected.

The mitotic index was determined by swelling a small sample of cells in 0.5ml of 1% Na citrate for 10 min at RT and then 0.5ml of Carnoy (Methanol-acetic acid, 3:1; vol/vol) was added to fix the cells, after which 1ml of 1% Na citrate was added. The cells were sedimented by centrifugation at 1000rpm for 10min in a table top clinical centrifuge at RT and the supernatant was removed and the cells resuspended in a few drops of 1% Na citrate. A drop of this suspension was added to a slide, allowed to dry completely and the chromosomes were stained using 2% Orcein in 50% acetic acid. The mitotic indices were 30-40%, 1%, and 85-95%, for untreated, hydroxyurea, and nocodazole treated cells, respectively.

VIb. *In vitro* KINASE ASSAY.

289R-E1A produced in *E. coli* was purified as previously described (Ferguson *et al.*, 1984). Histone H1 was purchased from Sigma and both proteins were used at a concentration of 5 mg/ml. $p34^{cdc2Hs}$ was purified from cells by immunoprecipitation with

an anti-peptide serum raised against the predicted carboxyl terminus (Simanis, and Nurse, 1986). Cells were lysed in cdc2 lysis buffer (20mM Tris (pH 7.4), containing 5mM EDTA, 100mM NaCl, 1% (vol/vol) Triton X-100, 1mM PMSF, 5mM NaPyrophosphate, 1mM NaOrthovanadate, 1mM NaF, 1mM EGTA, 1mM ß-glycerophosphate, and 5µg/ml of Leupeptin, and Pepstatin) and the lysates were cleared by centrifugation at 18000 rpm in a Sorval SS34 rotor for 1 hr at 4°C. Immunoprecipitates were prepared as previously described and then washed sequentially with lysis buffer, lysis buffer containing 2M NaCl, lysis buffer and finally with HB buffer (25mM MOPS (pH 7.2), containing 15 mM EGTA, 15 mM p-nitrophenyl phosphate, 60mM ß-glycerophosphate, 15mM $MgCl_2$, 1mM DTT, 0.1mM Na Orthovanadate, 1% (vol/vol) Triton X-100, 5µg/ml of Leupeptin and Pepstatin). The Sepharose beads containing the immunoprecipitates were resuspended in 10µl of HB buffer and combined with 5µg of either histone H1 or E1A along with 1mM ATP containing 50 µCi of [τ^{32}P]ATP. Reaction mixtures were incubated at 37°C for 10 min and stopped by addition of an equal volume of double strength SDS-PAGE sample buffer. Equal aliquots were analyzed by SDS-PAGE using gels containing 9% polyacrylamide.

CHAPTER III: ANALYSIS OF THE ROLE OF PHOSPHORYLATION SITES IN THE AMINO TERMINAL PORTION OF Ad5 E1A PROTEINS.

Previous studies had indicated that serine residues 89 and 96 of Ad5 E1A proteins are sites of phosphorylation. In an attempt to study the role of these sites a series of mutants was constructed by oligonucleotide-directed mutagenesis in which serine residues 89 and 96 were converted to alanines.

I. PREPARATION OF MUTANTS WITH DEFECTS AFFECTING PHOSPHORYLATION AT Ser89 AND 96.

Ia. OLIGONUCLEOTIDE MUTAGENESIS.

To facilitate the identification of mutagenized DNA in all subsequent cloning steps throughout these experiments bacteriophage M13Ad5 (*Table* **2-2**) which contains adenovirus type 5 nucleotide sequences 22 to 1339 was subjected to oligonucleotide-directed mutagenesis using oligonucleotide AB147 (*Table* **2-3**). This oligonucleotide introduced a diagnostic *Bgl* II restriction site while preserving the *wt* coding sequences. This mutant (mp11-BX-754) was used as a template for all subsequent mutagenesis.

A schematic representation of the steps used to generate and identify all of

Figure **3-1.** Schematic diagram of steps involved in generating and identifying mutants. (A) EtBr stained 1% agarose gel from synthesis reactions of mutageneses primed with AB216 (lane C), AB217 (lane D), and AB216+AB217 (lane E). The single stranded (lane B) and double stranded (lane A) forms of mp11-BX-754 were run as markers. (B) Sequence of the region mutated by AB147 (pm754), AB216 (Ser89 to Ala, N824 a T to G) and AB217 (Ser96 to Ala, N845 a T and N847 a C both changed to G)

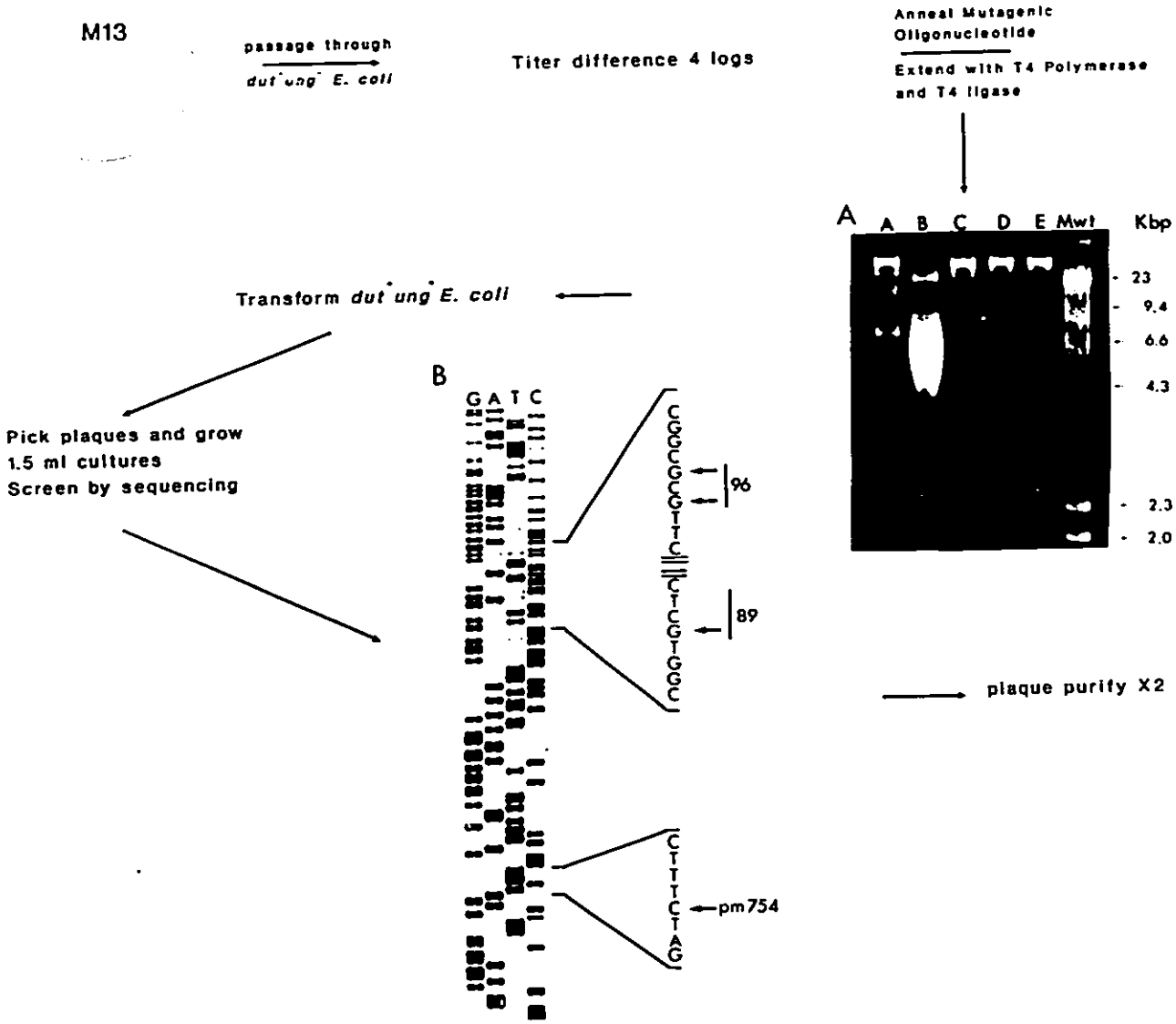
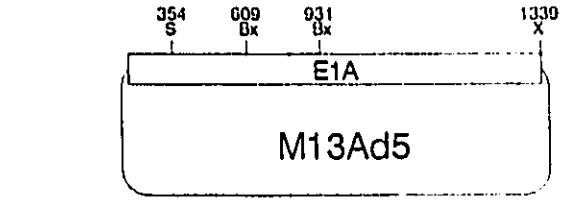
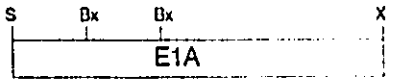

M13
passage through
dut⁻ung⁻ E. coli
Titer difference 4 logs
Anneal Mutagenic Oligonucleotide
Extend with T4 Polymerase and T4 ligase
A
A B C D E Mwt
Kbp
23
9.4
6.6
4.3
2.3
2.0
Transform dut⁻ung⁻ E. coli
Pick plaques and grow
1.5 ml cultures
Screen by sequencing
B
G A T C
96
89
pm754
plaque purify X2

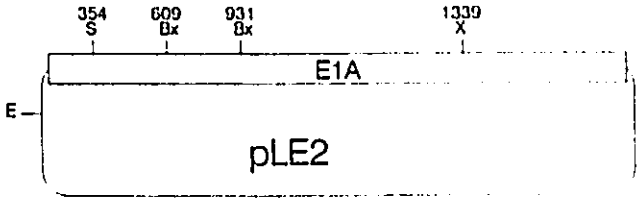
Figure **3-2.** Schematic diagram illustrating the procedure of rescuing mutations into the various E1A and E1 vectors.



Digest with *Sst* II
Xba I and gel Purfiy



Ligate into pLE2



Digest with *BstX* I and
Gel Purify Fragment
Ligate into pJF12
(12S E1A)

Digest with *EcoR*
Xba I and gel Purfiy
Fragment
Ligate into pXC38
(E1)

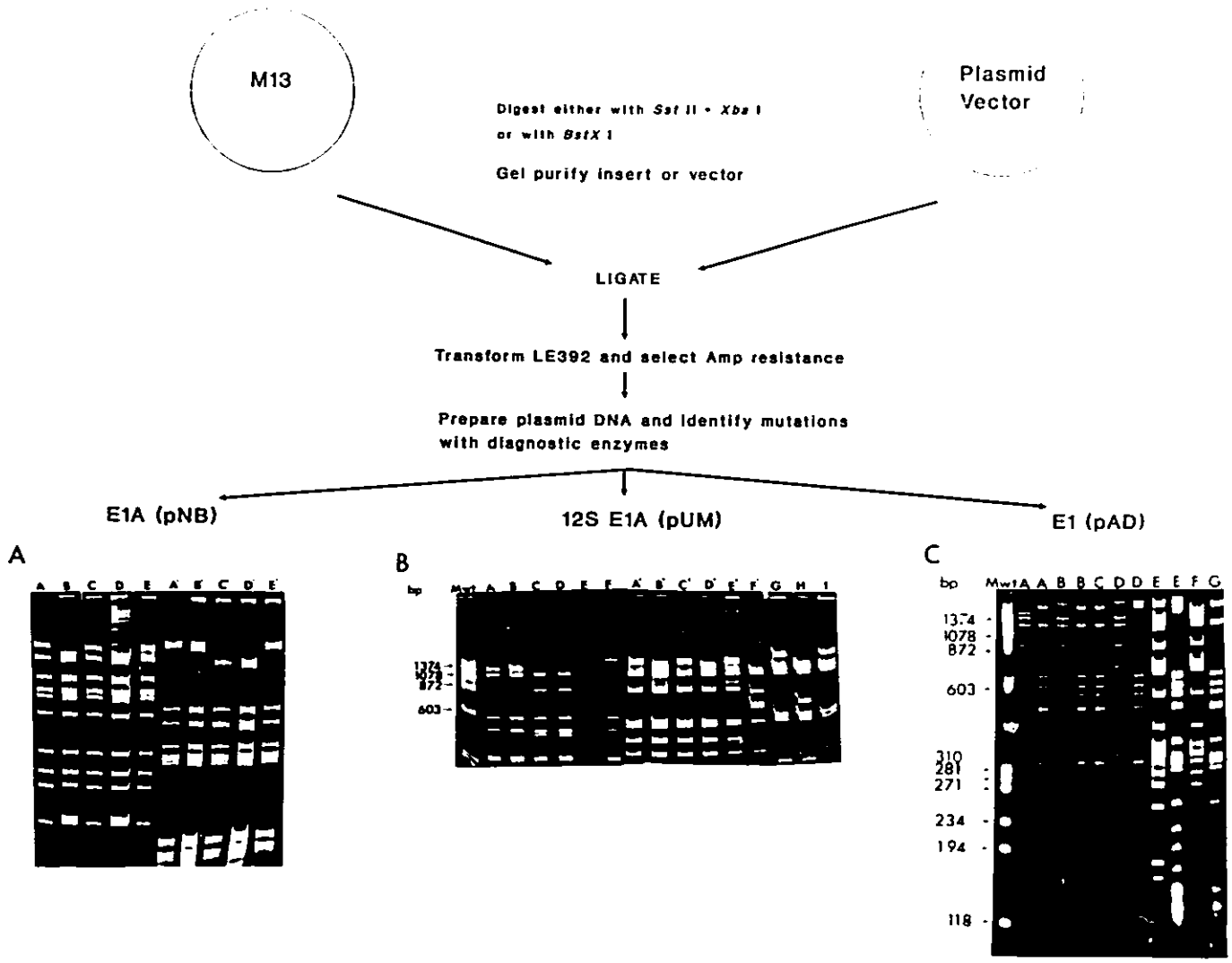
Bx: *BstX* I
E : *EcoR* I
S : *Sst* I
X : *Xba* I

the mutants produced by this type of mutagenesis is presented in Figure **3-1**. To determine the success of elongation of the complementary strand *in vitro*, a small portion of the synthesis reactions were electrophoresed in 1% agarose gels and visualized with UV after EtBr staining (*Fig.* **3-1A**). Lanes C-E show the products of the mutagenesis reactions using oligonucleotides AB216 (Ser89-Ala), AB217 (Ser96-Ala), and AB216+AB217 (*Table* **2-3**), respectively. The shift from the ssDNA form (as in *Fig.* **3-1A**, lane B) to the circular RF form (*Fig.* **3-1A**, lane A) was verified with all mutagenesis reactions and the success of the mutagenesis was dependent on this conversion. In fact synthesis reactions that did not result in such a conversion produced high backgrounds of non-mutated *wt* plaques.

The DNA products of successful synthesis reactions were transformed into a *dut*$^+$*ung*$^+$ E. coli (MV1190 or XL-1 Blue) and resulting plaques were picked the following day and screened directly by DNA sequencing. Figure **3-1B** displays the DNA sequence of an M13 clone isolated from the AB216+AB217 (Ser89 and 96 to Ala) mutagenesis, demonstrating the T to C transition (pm754) and the nucleotide changes in the codons for Ser89 (T to G at N824) and Ser96 (T at 845 and C at N847 both changed to G) which both result in conversions to Ala codons. In most instances the mutagenesis frequency was over 80%. Mutants were plaque purified twice and all nucleic sequences destined to be rescued into plasmids for further study were sequenced.

In order to transfer the mutations into plasmids, large scale preparations of RF DNA were cleaved with *Sst* II (N354) and *Xba* I (N1339) (*Fig* **3-2**), after which

Figure **3-3.** Schematic diagram of method of rescuing mutations in the various vectors and their identification. After sequencing the DNA destined to be rescued into plasmid the M13 RF DNAs containing the different mutations were digested with *Sst* I (N354) and *Xba* I (N1339) and fragments were gel purified and cloned into a similarly digested plasmid vector as described in *Figure* **3-2.** The presence of the mutations were identified with diagnostic enzymes.
(A) E1A plasmids PNB1 (lanes A and A'), pNB89A (lanes B and B'), pNB96A (lanes C and C'), pNB89A96A (lanes D and D'), and pLE2 (lanes E and E') were digested with either *Bsp1286* I (lanes A-E, respectively), or *Tha* I (lanes A' to E' respectively).
(B) 12S E1A plasmids pUM1 (lanes A and A'), pUM89A (lanes B and B') , pUM96A (lanes C and C'), pUM89A96A (lanes D and D'), pJF12 (lanes E and E'), and pLE2 (genomic E1A) (lanes F and F') were digested with either *Tha* I (lanes A to F, respectively) or *Bsp1286* I (lanes A' to F', respectively). Further to this plasmids pUM1 (lane G), pUM89A (lane H), and pJF12 (lane I) were digested with *HgiA* I.
(C) E1 expression vectors were also analyzed by digestion with diagnostic enzymes. pAD89A96A (lanes A and A'), pAD90A96A (lanes B and B'), pAD89D96A (lane C), pXC38 (lanes D and D'), pAD1 (lanes E and E') were digested with either *Bsp1286* I (lanes A, B, D, and E, respectively), or *BstU* I (isoschizomer=*Tha* I)(lanes A', B', C, D', and E', respectively). The single mutants pAD89A (lane F) and pAD96A (lane G) were also digested with *Bsp1286* I and *BstU* I, respectively. All digests were resolved in 5% polyacrylamide gels and DNA was visualized by UV after staining with EtBr.


M13
Plasmid Vector
Digest either with Sst II + Xba I
or with BstX I
Gel purify insert or vector
LIGATE
Transform LE392 and select Amp resistance
Prepare plasmid DNA and identify mutations with diagnostic enzymes
E1A (pNB)
12S E1A (pUM)
E1 (pAD)
A
B
C
bp
1374
1078
872
603
310
281
271
234
194
118

the fragment containing the mutated codon was gel purified and ligated into the appropriately digested E1A (pLE2), or E1 (pXC38) vectors. Subsequent to this the 12S E1A vectors were constructed from *BstX* I (N609, N931) digests of E1A plasmids and gel purified fragments were ligated into *BstX* I digested pJF12 (*Fig.* **3-2**). To ascertain whether the rescue of the mutated codon was successful the presence of the mutated DNA was initially identified by the presence of the new *Bgl* II restriction site (data not shown). Moreover, the presence of mutations was determined more conclusively by digestion with the appropriate diagnostic restriction enzymes for which sites had been engineered into the mutagenic oligonucleotides. Oligonucleotide AB216 which changed Ser89 to Ala was designed such that it introduced a *Bsp1286* I site. Similarly changing Ser96 to Ala with AB217 introduced a new *Tha* I (isoschizomer=*BstU* I) site, and changing Ser89 to Asp with AB432 produced a new *BstE* II site. No diagnostic restriction site could be designed when changing Pro90 to Ala, thus AB431 was used to revert the Ala89 mutation in the Ala89Ala96 double mutant to produce Ala90Ala96 (*Table* **2-3**) and initial identification of the Pro90 to Ala change was for the absence of the novel *Bsp1286* I site characteristic of the presence of Ala89 mutation.

Figure **3-3A**, shows a representative example typical of many digests done to determine the presence of the mutations in the E1A plasmids resolved on a 5% polyacrylamide gel. Although no molecular weight markers were electrophoresed in the gel presented in panel A, size estimates were calculated from similar gels (data not shown). Lanes A through E (*Fig.* **3-3A**) show the fragments which were resolved after plasmids were digested with the enzyme *Bsp 1286* I, which were diagnostic for the

presence of an Ala codon at residue 89. Lanes A (pNB1), C (pNB96A), and E (pLE2) did not contain this codon change and thus produce identical restriction fragment profiles characteristic of unaltered E1A sequences. On the other hand, lanes B (pNB89A) and D (pNB89A96A) did contain the Ala89 codon resulting in the generation of the diagnostic *Bsp 1286* I site, and in fact the presence of an additional restriction site was clearly evident. The largest band detected in plasmids containing an unaltered Ser89 codon migrating with an approximate size of 1.2 kb (lanes A, C, and E) was cleaved in Ala89 containing plasmids (lane B and D) to produce a faster migrating fragment of approximately 1.0 kb and an additional fragment migrating at approximately 0.18 kb thus demonstrating the presence of the Ser89 to Ala89 mutation. These results demonstrate the successful transfer of the Ala89 mutation into these plasmids.

A similar analysis for the presence of the Ala96 codon was performed on these plasmids and the presence of a novel *Tha* I restriction site which is diagnostic for the Ser96 to Ala change was determined (*Fig.* **3-3A**, lanes A'-E'). Plasmids pNB1 (lane A'), pNB89A (lane B'), and pLE2 (lane E') did not contain this alteration and thus produced identical restriction fragment profiles. However, pNB96A (lane C') and pNB89A96A (lane D') had identical profiles which were different from the others. The largest DNA fragment produced from plasmids containing the Ser96 codon (lanes A', B', and E') migrated at approximately 1.4 kb, whereas with plasmids encoding Ala96 (lanes C' and D') this fragment was cleaved to produce a faster migrating fragment of approximately 1.0 kb and an additional smaller fragment of 0.4 kb which was not resolved away from other fragments in the gel. This fragment was evident when digests were resolved under

different gel conditions (data not shown). These results demonstrated that the mutations which converted Ser96 to Ala had been successfully rescued into pNB96A and pNB89A96A.

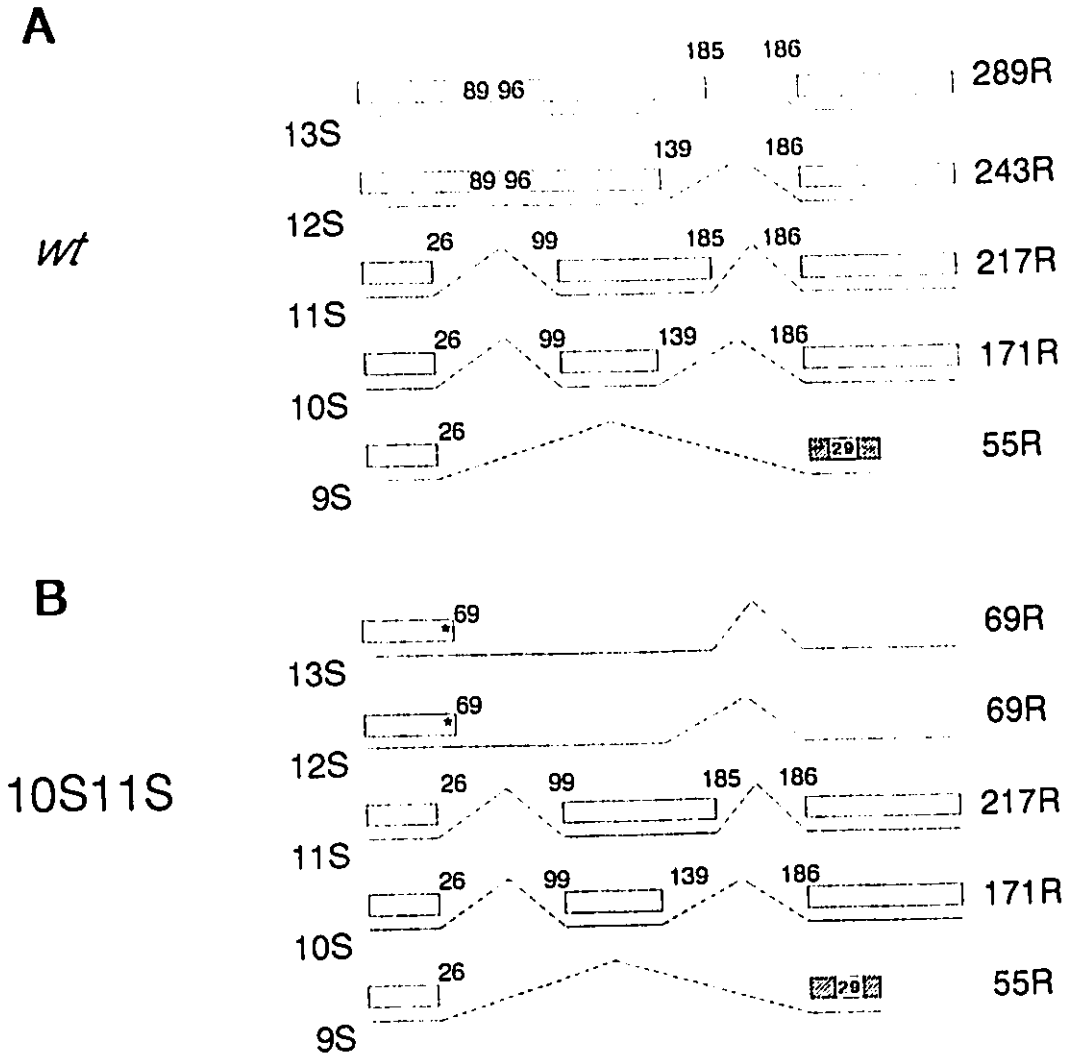
All the mutations were subcloned into the 12S E1A vector (pJF12) by digestion with *BstX* I (N609, N931) and ligation into pJF12 using the E1A vectors as a source of DNA (*Fig.* **3-2**). The presence of these mutations was verified in the same fashion as described for Figure **3-3A**. Figure **3-3B** (lanes A to E) demonstrates the restriction fragment profiles of the 12S E1A plasmids digested with *Tha* I when resolved in a 5% polyacrylamide gel. Plasmids pUM1 (lane A), pUM89A (lane B), and pJF12 (lane E) did not contain the Ser96 to Ala codon change and thus produced identical restriction patterns. However, plasmids pUM96A (lane C) and pUM89A96A (lane D) contained this change which was clearly evident by the different restriction profiles. The largest fragment produced from *Tha* I digests of the Ser96 containing plasmids (pUM1, pUM89A, and pJF12; lanes A, B, and E respectively) migrated at approximately 1.3 kb and was cleaved in Ala96 containing plasmids (pUM96A and pUM89A96A; lanes C and D respectively) to give rise to a faster migrating fragment of 0.87 kb and a new fragment of approximately 0.47 kb.

The presence of the Ser89 to Ala89 codon change was also determined in these plasmids by digestion with *Bsp 1286* I (Figure **3-3B** lanes A' to E'). Plasmids pUM1 (lane A'), pUM96A (lane C'), and pJF12 (lane E') did not contain the codon change which produces the Ser89 to Ala89 alteration and thus had identical restriction profiles. Moreover, plasmids pUM89A and pUM89A96A produced identical patterns which were

different from the former plasmids. The largest fragment produced from Ser89 containing plasmids (pUM1, pUM96A, and pJF12; lanes A', C', and E' respectively) migrating at approximately 1.3 kb was cleaved to produce a faster migrating form of approximately 1.2 kb and a novel fragment of 0.18 kb which had been run off the bottom of this gel, but was evident in gels run with higher acrylamide concentrations (data not shown). In order to demonstrate more conclusively whether or not the Ala89 mutation was indeed present in plasmids pUM1 (lane G), pUM89A (lane H), and pJF12 (lane I), DNA from these plasmids was digested with *HgiA* I whose recognition sequence overlapped that of *Bsp 1286* I, but was less degenerate, thus producing a different restriction profile. Plasmid pUM1 (lane G) and pJF12 (lane I) produced identical restriction profiles, while pUM89A produced a profile which was characteristic of the presence of an additional restriction site. The largest fragment of approximately 1.9 kb produced from pUM1 and pJF12 (lanes G and I; respectively) was cleaved in DNA from pUM89A thus producing a faster migrating band of approximately 1.2 kb and a novel fragment of approximately 0.65 kb demonstrating conclusively the presence of the Ala89 mutation in the 12S plasmid. A similar analysis was performed on pUM89A96A with identical results (data not shown). Included in this gel was a digestion of pLE2 (genomic E1A) with *Tha* I and *Bsp 1286* I (lanes F and F', respectively) as a comparison.

Figure 3-3C represents a similar restriction analysis performed with the E1 plasmid constructs (pAD series). The *Bsp1286* I restriction pattern of plasmids with a *wt* Ser codon at position 89 are represented by plasmids pAD90A96A (lane B), pXC38 (lane D), and pAD1 (lane E). Lanes A (pAD89A96A) and F (pAD89A) represented

Figure **3-4.** Detection of the mutation within pAD89A96A. Schematic of Ad5 *wt* (A) E1A and 10S11S mutant (B) mRNA isoforms and their predicted translation products. (C) dsDNA sequence of pADS89A96A (10S11S mutant) demonstrating the deletion of the T nucleotide. (D) The predicted translation product from the 12 and 13S messages from the 10S11S virus.


A
wt
185
186
89 96
289R
13S
139
186
89 96
243R
12S
26
99
185
186
217R
11S
26
99
139
186
171R
10S
26
29
55R
9S
B
10S11S
69
69R
13S
69
69R
12S
26
99
185
186
217R
11S
26
99
139
186
171R
10S
26
29
55R
9S
C
A T G C A T C
C
C
C
T
T
T
C
T
A
G
T
D
Gln64 Ile Phe Pro Asp Ser Val70
wt
CAG ATC TTT CCC GAC TCT GTA
Gln64 Ile Phe Pro Thr Leu60 End
10S11S
CAG ATC TTC CCG ACT CTG TAA
T
Translation Product
Translation Product (different reading frame)
Processed mRNA
Stop codon

two digests of plasmids which contain a Ser89 to Ala mutation that introduced a diagnostic *Bsp1286* I restriction site. The slowest migrating restriction fragment (approximately 1.7 kb) found in plasmids encoding Ser89 (lanes B, D, and E) was cleaved in those encoding Ala 89 containing plasmids to produce a faster migrating fragment of 1.5 kb and a novel fragment of approximately 0.18 kb. Due to its size, the presence of the 0.18 kb fragment could only be clearly detected upon overloading the gel with DNA, as shown in lane F (pAD89A).

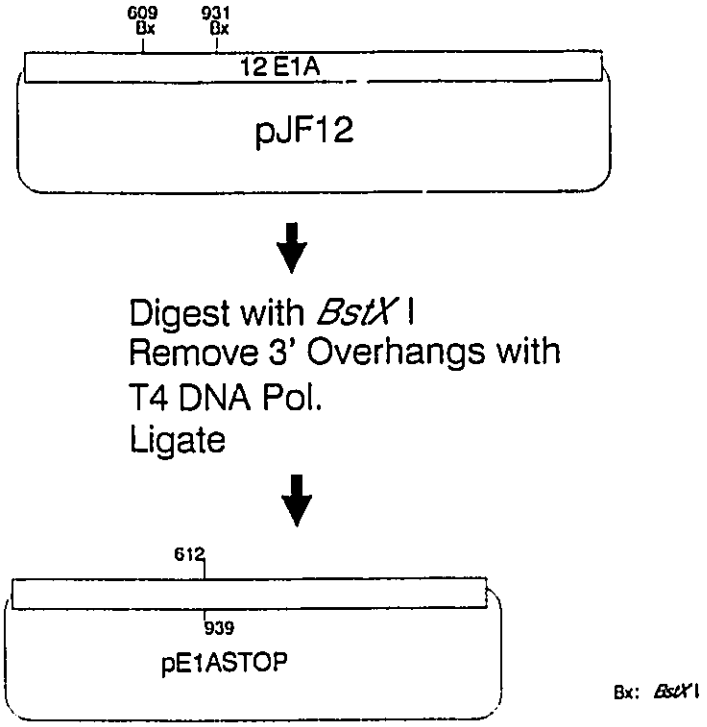
The presence of the Ser96 to Ala codon change was also analyzed in an similar fashion. Plasmids encoding Ser96 would not contain the diagnostic *BstU* I site (isoschizomer of *Tha* I) and thus produced the characteristic restriction pattern seen for plasmids pXC38 (lane D'), and pAD1 (lane E'). Moreover, plasmids that encoding Ala96 would have an additional restriction site which was clearly evident in lanes A' (pAD89A96A), B' (pAD90A96A), C (pAD89D96A), and G (pAD96A), where the slowest migrating fragment of approximately 1.7 kb found in plasmids encoding Ser96 (lanes D' and E') was cleaved to produce a faster migrating 1.2 kb fragment and a new smaller 0.5 kb fragment which under different gel conditions could be resolved away from the other DNA fragments in the gel (data not shown). Taken together these results and others (data not shown) demonstrated that the engineered codon changes had been rescued into the various plasmids.

Ib. 10S11S MUTANT.

During the generation of the pAD89A96A mutant, a deletion of a single T at

Figure **3-5.** (A) Schematic diagram depicting the construction of the deletion mutant, pE1ASTOP.
(B) The predicted translational product from all mRNAs (13, 12, 11, 10, 9S) produced from the E1ASTOP mutant.

A



B

612

GCC AGC TGG CTT TCC ACC CAG TGA

939

A S[18] W L S I N STOP

nucleotide 755 was detected by dsDNA sequencing (*Fig.* **3-4C**). This deletion produced a frame-shift resulting in the addition of two non-related amino acids and premature termination of translation after amino acid 69 (*Fig.* **3-4B**, and **D**). Since this mutation was within the intron of the E1A 10S and 11S messages (*Fig.* **3-4A** and **B**), the products of these mRNAs should not be affected, whereas the 243R and 289R products would be truncated at amino acid residue number 69 (*Fig.* **3-4B** and **D**). This mutation was also cloned into all vectors described previously and utilized to analyze the biological role of these smaller E1A products.

Ic. E1ASTOP MUTANT.

In order to generate a plasmid and a virus that would produce mRNA transcripts but no significant translational products, a deletion mutant was constructed that introduced a frameshift within the E1A coding sequences resulting in premature termination of translation (*Fig.* **3-5**). It was hoped that this mutant could act as a control for effects that could be attributed both to E1A protein function and to effects of E1A mRNAs which could sequester essential posttranscriptional or translation factors in E1A enhancer repression assays.

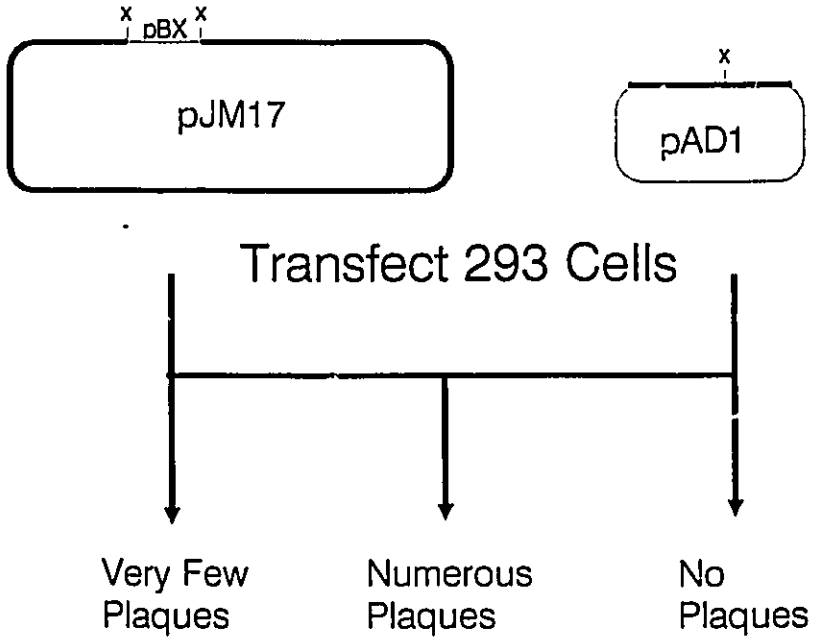
This mutant, was constructed by self ligation of *BstX* I digested pJF12 DNA after the removal of the 3' protruding ends by digestion with T4 DNA polymerase (*Fig.* **3-5A**). This construction resulted in the generation of a small deletion (N613-938) which also caused a frameshift resulting in premature termination of translation after Ser18 (*Fig.* **3-5B**). The presence of this deletion was distinguished by restriction digests and sequencing

(data not shown), this mutant was designated pSTOP and the virus derived from this plasmid was called E1ASTOP.

Id. RESCUE INTO VIRIONS.

Rescue of mutations into virions was accomplished according to the method of McCrory *et al.* (1988) which is based on the fact that intact circular forms of Ad5 are produced during infection of rodent cells and, if a plasmid origin of replication is provided, these complete Ad5 genomes can be rescued from infected cells and propagated as plasmids in *E. coli*. pJM17 is an Ad5 recombinant that contains a pBR322 (pBX) plasmid derivative cloned into the *Xba* I site in the E1A gene (*Fig.* **3-6**) that is too large to allow packaging of the virus. Cotransfection of cells with DNA from pJM17 and E1 plasmids will therefore only yield viable viruses which have been produced by homologous recombination. There are two principal modes by which plaques can arise using this approach, either by deletion of pJM17 sequences or by homologous recombination with the co-introduced E1 plasmids. Both of these products are easily determined by restriction analysis of the resulting plaques. Plaques from cotransfections of pJM17 and the different E1 vectors were picked and expanded on monolayers of 293 cells. Recombinant viruses were first identified by the detection of a novel *Bgl* II site (data not shown). These viruses were then plaque purified twice on 293 cells and DNA prepared and digested with additional enzymes to ensure that the virus contained no gross abnormalities. Figure **3-7** is an example of many digests done with all the recombinants and in this figure the 12S virus UM89A (lanes A, A', and A''), the genomic double

Figure **3-6.** Schematic of the method used to rescue mutations into virions by co-transfection of pJM17 with the different E1 plasmids.



Nucleotide Sequences.

pBR ——

Ad5 ——

Restriction Site

X: *Xba* I

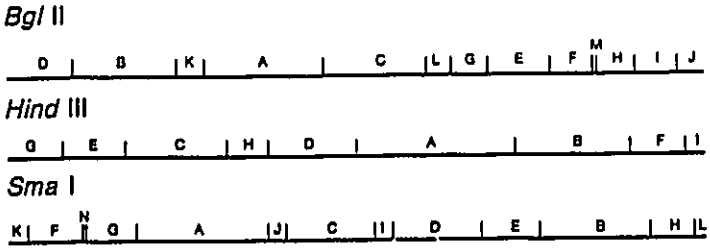
mutant AD90A96A (lanes B, B', and B''), and dl309 (lanes C, C' and C'') which is the virus from which pJM17 was constructed, were digested with several enzymes. Digestion of Ad5 DNA with *Bgl* II produced 13 fragments which have been designated A through M according to their size, A being the largest and M the smallest. The *Bgl* II D-fragment found at the left most end of the genome (*Fig.* **3-7A**) contained the E1A gene and migrated with an apparent size of 3.5 kb (*Fig.* **3-7B**, lane C). This fragment was cleaved in DNA prepared from all recombinant viruses made for these studies. An example of this effect has been illustrated with DNA made from cells infected with AD90A96A (lane B) and UM89A (lane A). In the case of AD90A96A, the *Bgl* II D-fragment was cleaved to produce a faster migrating band with an approximate size of 2.8 kb, while the new fragment from UM89A (lane A) migrated at approximately 2.3 kb. The smaller fragment was due to the deletion of intron sequences in the 12S cDNA virus. In addition, the appearance of a novel 0.75 kb fragment could be detected in these digests, however, this fragment was not always apparent due to its size and the presence of cellular RNA (*Fig.* **3-7B**, lane B, and data not shown).

Digestion of these DNAs with *Hind* III produced 9 fragments of which the *Hind* III G-fragment (*Fig.* **3-7A**), migrating at approximately 3 kb, contained the E1A gene. The restriction patterns for all recombinant viruses were identical to dl309 (lanes B', C' and data not shown), except for the 12S cDNA viruses which produced a smaller *Hind* III G-fragment migrating at approximately 2.5 kb (lane A') due to the deletion of the intron to generate the 12S cDNA. Similarly, digestion of all recombinant viruses with *Sma* I produced restriction fragment profiles identical to those produced from DNA

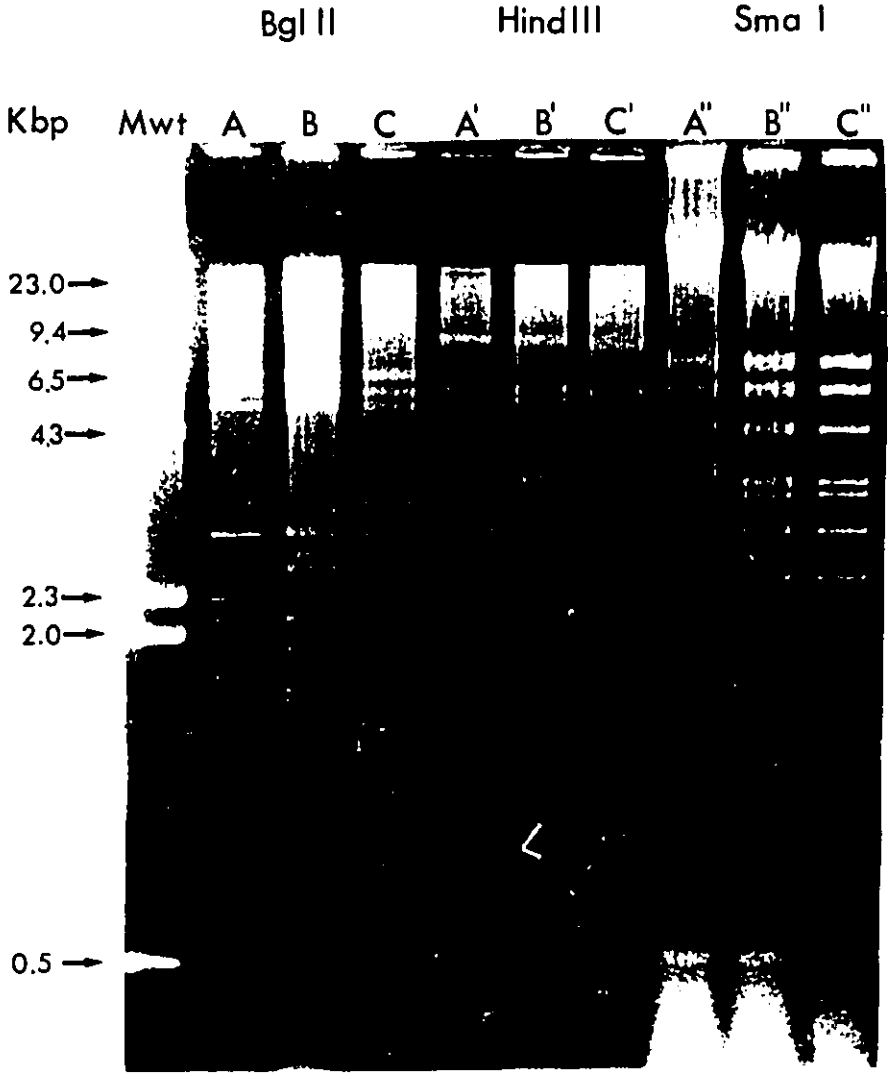
obtained from dl309 infected cells, except for the 12S recombinant viruses. The intron deleted in these viruses as a consequence of RNA processing also deleted a *Sma* I site, hence the *Sma* I K and F fragments (*Fig.* **3-7A**) which migrate at approximately 3.3 and 0.9 kb, respectively (*Fig.* **3-7B**, lanes B'' and C'') were fused (lane A'') and produced a fragment of approximately 3.7 kb. A fragment smaller than the sums of the K and L fragments was produced due to the deletion of the intron in the 12S cDNA virus. To further ensure that the mutations had been rescued into virus, viral DNA was digested with restriction enzymes that were diagnostic for the presence of the altered codons and the restriction fragments were resolved in a 2% agarose gel. Viral DNAs were either visualized directly by EtBr staining or by Southern analysis with a [^{32}P]-labelled oligonucleotide (AB147). Viral DNAs extracted from cells infected with AD89A96A and dl309 (*Fig.* **3-8**, lanes A and C, respectively) and their corresponding E1 plasmids pAD89A96A and pXC38 (*Fig.* **3-8**, lanes B and D, respectively) were digested with *BstU* I (isoschizomer of *Tha* I) which was the restriction enzyme site engineered to be diagnostic of the Ser96 to Ala codon change. The fragments were resolved in a 2% agarose gel and visualized by EtBr staining and UV illumination. Digestion of dl309 (lane C) and pXC38 (lane D) DNA with *BstU* I produced numerous small fragments, however, one of the larger BstU I fragments of 1561 bp (N414 to N1975) which co-migrated with other viral DNA fragments (lanes A and C) contained the Ser96 codon. This fragment was cleaved in DNA prepared from AD89A96A infected cells to produce a faster migrating fragment of approximately 1100 bp which was also present in similarly digested pAD89A96A (*Fig.* **3-8**, lanes A and B (arrow)). AD89A96A was initially

Figure 3-7. Restriction digests of extracted viral DNAs. (A) The Ad5 restriction map for the different restriction enzymes used. (B) Total DNA from UM89A (lanes A, A', and A''), AD90A96A (lanes B, B', and B''), and *dl*309 (lanes C, C', and C'') infected cells were digested with *Bgl* II (lanes A,B,and C), *Hind* III (lanes A',B', and C'), and with *Sma* I (lanes A'', B'', and C'').

A


Bgl II
D B K A C L G E F M H I J
Hind III
G E C H D A B F I
Sma I
K F N G A J C I D E B H L

B


Bgl II
Hind III
Sma I
Kbp
Mwt
A
B
C
A'
B'
C'
A''
B''
C''
23.0
9.4
6.5
4.3
2.3
2.0
0.5

identified by the presence of the diagnostic *Bgl* II site, thus the presence of a diagnostic *Bsp1286* I site (Ala89 mutation) was not verified because the *Bgl* II site was only a short distance upstream of the Ala96 codon change and it was thought that the likelihood of a double recombination event occurring between these mutations resulting in the loss of the Ala89 mutation would be extremely low.

The presence of the Ser96 and Ser89 codon changes to Ala in the single mutants was tested by digestion with *BstU* I and *Bsp1286* I (*Fig.* **3-10**, lanes A to C, and A' to C', respectively). Viral DNA digested with *BstU* I, the restriction enzyme which was diagnostic for the presence of the Ser96 to Ala codon change, produced numerous small fragments. Using a radiolabelled oligonucleotide (AB147) to probe a membrane to which these digestion fragments had been transferred, revealed a single band that migrated as a 1561 bp fragment in DNA obtained from AD89A and dl309 infected cells (lanes B and C, respectively), whereas a fragment of 340 bp was detected in DNA obtained from AD96A (lane A) infected cells. These digests proceeded to completion as verified by EtBr staining and UV illumination (data not shown). Thus the data were consistent with the presence of a new restriction site and demonstrated that the Ser96 to Ala96 codon change was present in this virus. Digestion of these viruses with *Bsp1286* I, the restriction enzyme diagnostic for the Ser89 to Ala89 codon change, produced a single band, as detected by radiolabelled AB147 in DNA purified from AD96A and dl309 infected cells (lanes A' and C', respectively), which migrated at 1001 bp, whereas the fragment detected from DNA extracted from AD89A infected cells migrated at 754 bp (lane B'). These results were consistent with the presence of a new restriction site

Figure **3-8.** Resolution of viral DNA in 2% agarose gel. AD89A96A and *dl*309 DNA was extracted from infected cells and digested with *BstU* I (lanes A and C, respectively). Plasmid DNAs were also digested and run along side the viral DNA (lanes B and D respectively). Digestion of these DNA's with *BstU* I demonstrated that only AD89A96A and pAD89A96A produced faster migrating fragments (arrow).

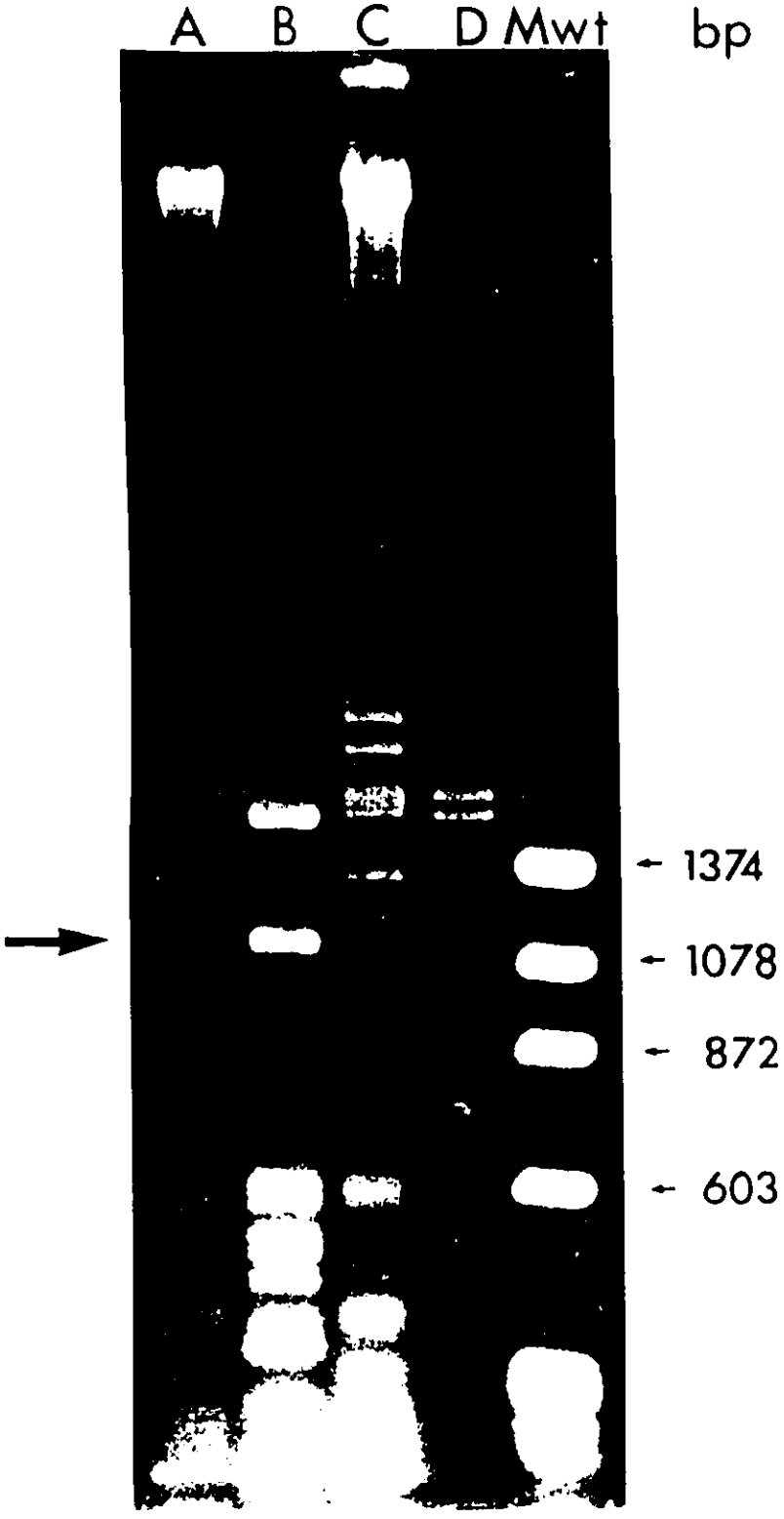
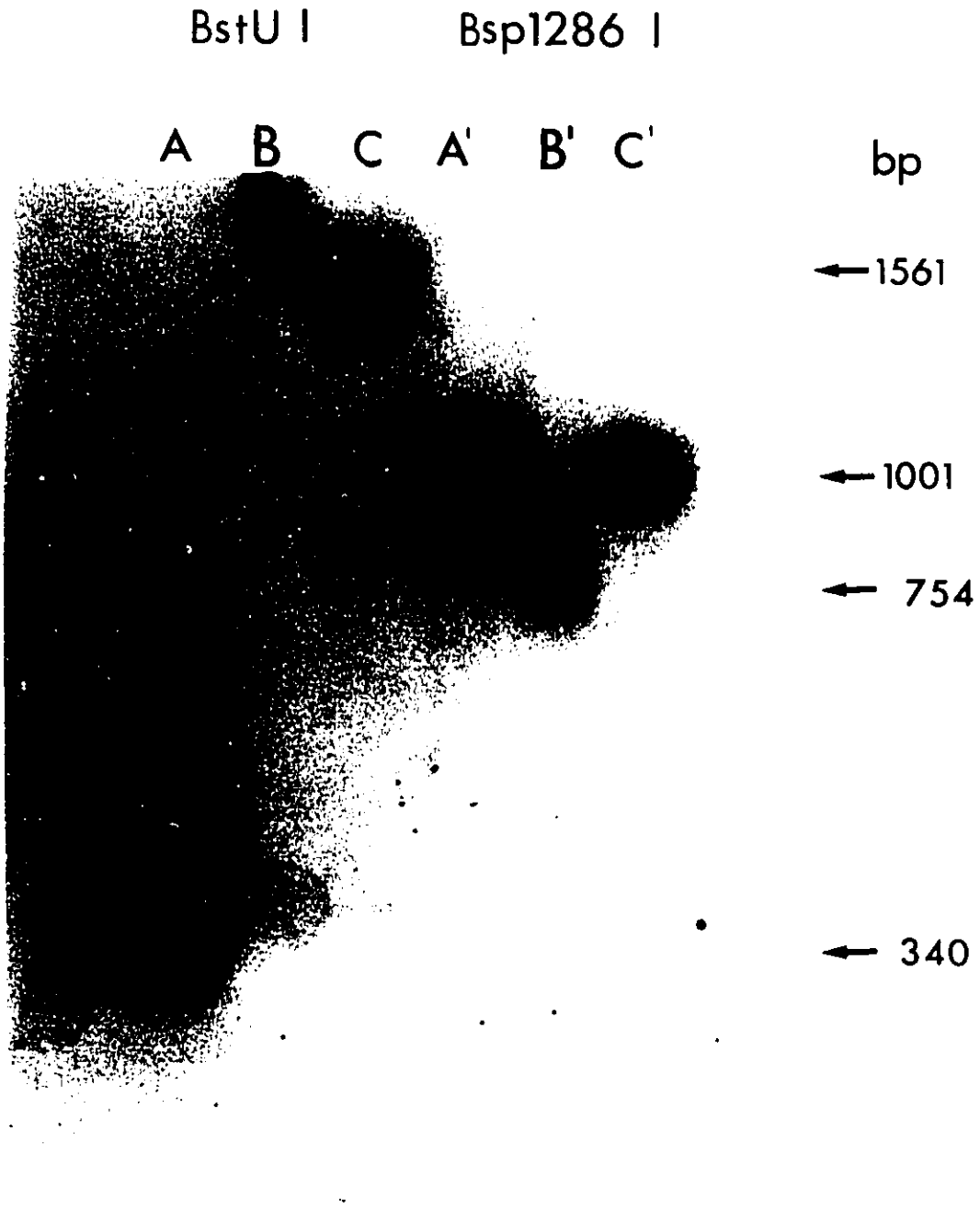

A
B
C
D
Mwt
bp
1374
1078
872
603

Figure **3-9.** Southern analysis of DNA extracted from infected cells. DNA from AD96A, AD89A, and *dl*309 infected cells was digested with either *BstU* I (lanes A,B, C, respectively) or *Bsp1286* I (lanes A',B', and C' respectively) resolved on a 2 % agarose gel, transferred to nitrocellulose and probed with [^{32}P]-labelled AB147.


BstU I
Bsp1286 I
A
B
C
A'
B'
C'
bp
1561
1001
754
340

diagnostic for the presence of the Ala89 codon. Such analyses were performed for all viruses used in the following studies in order to confirm the presence of all desired mutations.

II. ANALYSES OF E1A PROTEINS FROM MUTANT AND WT VIRUSES.

To determine the effects of mutations at the Ser89 and 96 sites on the overall pattern of E1A protein phosphorylation, a number of biochemical studies were carried out using both mutant and *wt* Ad5 viruses.

IIa. PHOSPHOAMINO ACID ANALYSIS OF E1A PROTEINS LABELLED *IN VIVO*.

To identify the amino acid(s) which are phosphorylated in the E1A polypeptides E1A proteins labelled *in vivo* with [^{32}P]-orthophosphate were immunoprecipitated and purified by SDS-PAGE and then subjected to acid hydrolysis. Phosphoamino acid content was determined by 2-dimensional thin layer electrophoresis (TLE). Figure **3-10** confirmed previous observations (Tsukamoto *et al.*, 1986, Richter *et al.*, 1988) that E1A proteins contain phosphoserine and possibly a very small amount of phosphothreonine. In contrast, Tremblay *et al.* (1988) found exclusively phosphoserine. The method of isolation and processing of E1A polypeptides in both this study and in the Tremblay *et al.* (1988) study were very similar, however, the polyacrylamide concentrations of the preparative gels used to isolate the E1A products were different. Tremblay *et al.* (1988)

utilized 12% polyacrylamide gels and this study used 10% gels. The discrepancy in phosphothreonine content could have resulted from the inclusion of contaminating cellular phosphoproteins which co-migrated with the E1A proteins and which may have been excluded by the use of a higher gel concentration. These studies also utilized multiple phosphatase inhibitors which had not been previously used, thus if the phosphorylated threonine residue(s) was very sensitive to dephosphorylation these inhibitors may have prevented the action of phosphatases.

IIc. ANALYSIS OF TRYPTIC PEPTIDES BY REVERSE PHASE HPLC.

E1A tryptic peptides were analyzed by reverse phase HPLC (RP-HPLC) under conditions similar to those described previously (Tremblay *et al.*, 1988), and for E1A proteins produced by *wt* Ad5 (*Fig.* **3-11A**), very similar results were obtained. However, recent work (S. Whalen, unpublished data) utilizing different deletion mutants has shown that the identities of some of the peptides were incorrect. The peptide previously identified as P3 was believed to be composed of amino acids 224 to 258 (*Table* **3-1**, peptide T12) exclusively, and thought to elute from RP-HPLC as a heterogeneous peak from 58 to 94 minutes. It is now apparent that P3 actually consists of multiple peptides (S. Whalen, unpublished results), including a portion of the large T2 peptide (*Table* **3-1**, amino acids 3 to 97) which was originally thought to be totally retained on the column (Tremblay, *et al.*,1988).

[^{32}P]-labelled E1A proteins produced from *wt*, AD89A, AD96A, AD89A96A, AD89D96A, and AD90A96A infected cells were purified from SDS-PAGE and separated

Figure **3-10.** Phosphoamino acid analysis of [^{32}P] labelled E1A proteins. The positions of the non-radioactive phosphoamino acid markers identified by ninhydrin staining have been indicated.

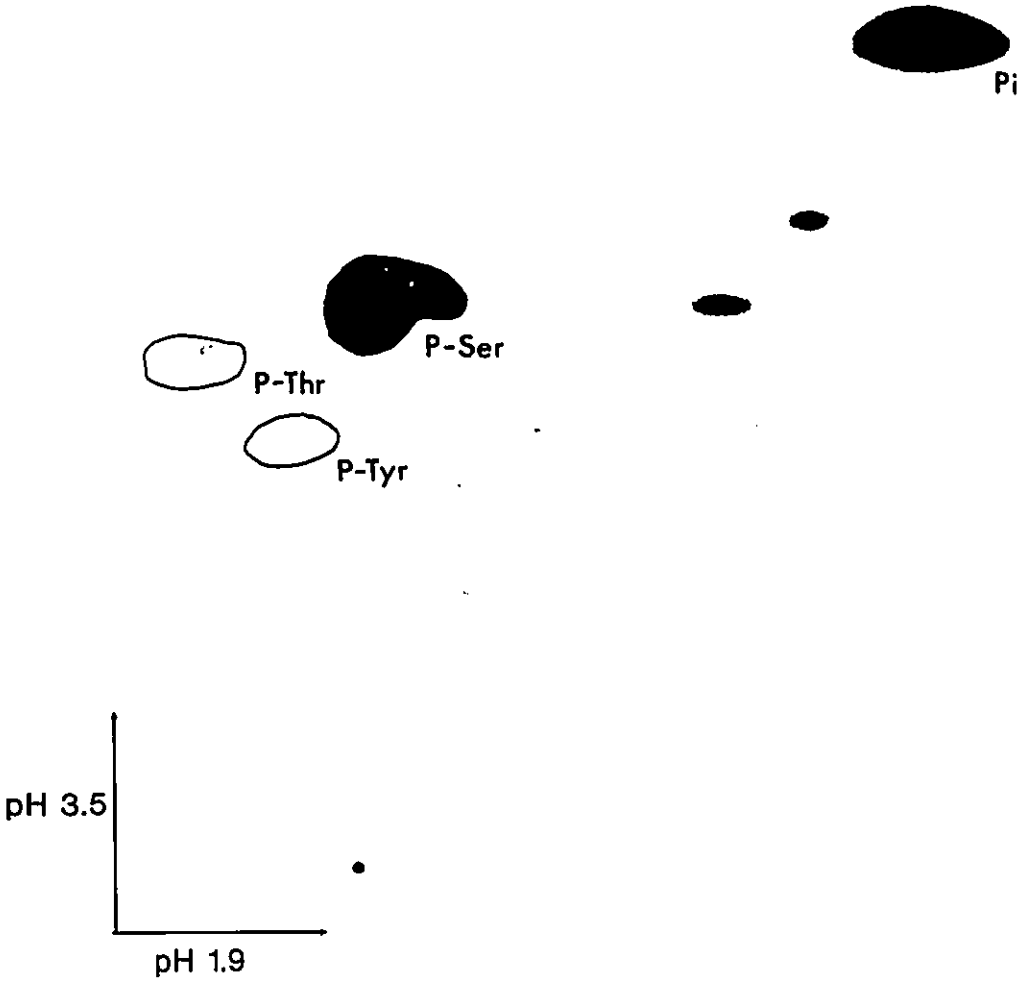
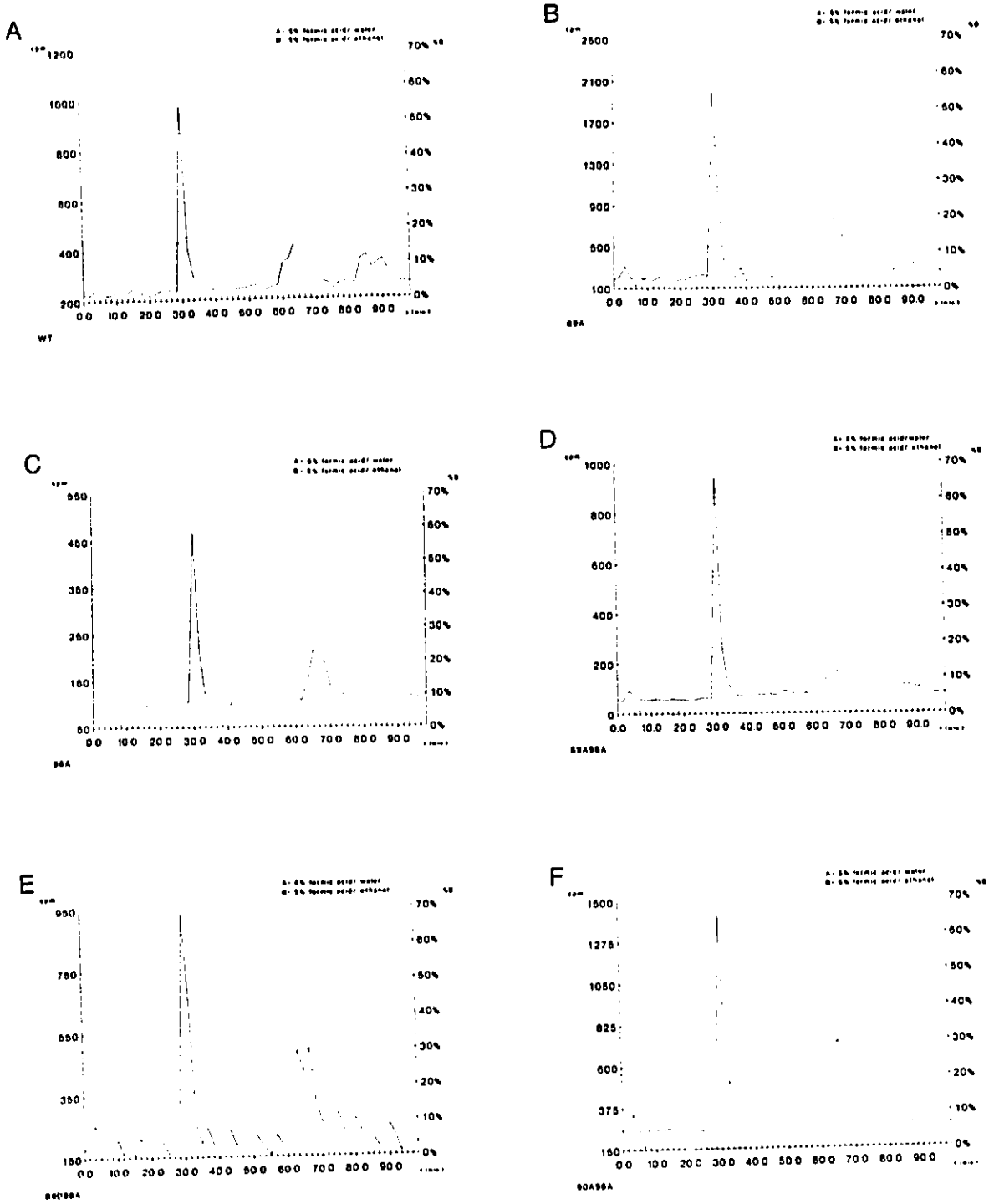

Pi
P-Ser
P-Thr
P-Tyr
pH 3.5
pH 1.9

Table **3-1.** Summary of Tryptic Peptides from E1A Proteins

Peptide Number	Amino Acid Residues
T1	1-2
T2	3-97
T3	98-105
T4A*	106-155
T4B*	106-139/186-205
T5*	156-161
T6*	162
T7*	163-177
T8*	178-205
T9	206-208
T10	209-215
T11	216-223
T12	224-258
T13	259-262
T14	263
T15	264-285
T16	286-289

*Unique to the 12S product and *unique to the 13S product due to splicing.

Figure **3-11.** RP-HPLC profiles of E1A proteins purified from AD1 (A), AD89A (B), AD96A (C), AD89A96A (D), AD89D96A (E) , and AD90A96A (F) infected KB cells labelled with [^{32}P]-orthophosphate, gel purified and digested with trypsin.


A
B
C
D
E
F

by RP-HPLC. The resulting phosphopeptides from all samples eluted in patterns similar to that obtained for *wt* Ad5 (*Fig.* **3-11B-F**) except that the material eluting at 82 to 94 min was either reduced or absent. The elution profiles for the single mutants (89A and 96A) and double mutants were very similar. The identical patterns found for both single and double mutants suggested that perhaps only one site was phosphorylated, however this interpretation could not explain the identical profiles obtained with the individual single mutants (AD89A and AD96A). The results could be explained if T2 eluted from the column only when it is phosphorylated at both sites, however as will be seen later, this explanation can not be reconciled with the *in vitro* phosphorylation experiments. Thus, the basis of this result remains unclear, but may be due to other posttranslational modifications of the E1A proteins that are yet to be discovered.

IId. ANALYSIS OF MUTANTS BY TLC.

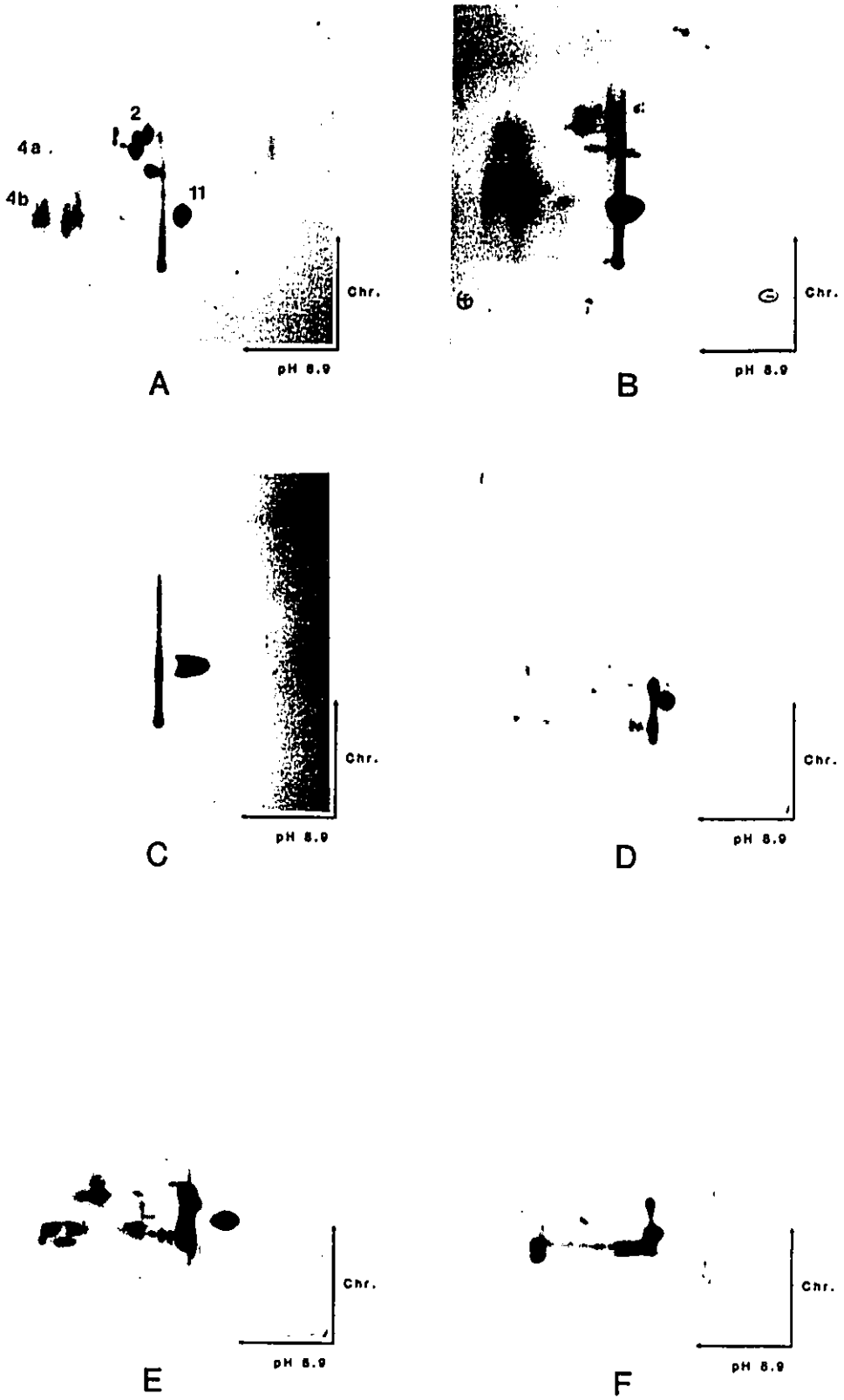
The analysis of tryptic peptides by RP-HPLC is a powerful technique, however, it suffers from the fact that peptides may bind irreversibly to the column matrix. The analysis of peptides by TLC allows detection of all the peptides produced from a particular digest. Thus the combined use of RP-HPLC and TLC should provide a more complete picture.

To identify the peptide(s) containing phosphorylation sites involved in production of the slower migrating species, peptide maps produced from the slower migrating 50 kDa and faster migrating E1A species were compared to the 45 kDa faster migrating species.

(*Fig.* **3-12A, B**). All of the species within a cluster of spots found directly above the origin appeared to originate from T2, for the reasons described below. The two species which contained the most label within this cluster from the *wt* E1A proteins (*Fig.* **3-12A**, arrows) were absent from proteins isolated from the faster migrating 45 kDa proteins (*Fig.* **3-12B**), suggesting that phosphorylation on this residue(s) produced the decrease in gel mobility. On the whole there was a reasonable amount of reproducibility in the E1A phosphopeptide patterns from experiment to experiment. Some minor variations were noted, for example in peptide T11 which had previously shown to contain the major phosphorylation site at Ser219 (Tsukamoto *et al.*, 1988). With the E1A sample (*Fig.* **3-12A**), this species migrated further to the right of the origin than with the 45K sample. This difference probably arose because of two factors: differences in the removal of SDS from the protein samples may have affected the electrophoretic mobility of the peptides; and secondly, overloading this protein sample may have caused a trailing effect due to precipitation during both the electrophoretic and chromatographic phases.

To determine whether the cluster of species above the origin did in fact originate from T2, tryptic phosphopeptides of E1A proteins from various mutants and from the products of the 11S and 10S mRNAs were resolved on TLC. The 37.5 and 35 kDa species encoded by the 11S and 10S E1A mRNAs lack much of T2 (residues 26 to 99) as a result of splicing. Figure **3-12C** shows that analysis of phosphopeptides produced from a mixture of these proteins yielded primarily only peptide T11 and that all of the species within the cluster of peptides above the origin were absent. In addition, Figure **3-12, D-F** shows that peptides from various deletion mutants within the T2 coding sequence

Figure **3-12.** Analysis of tryptic phosphopeptides by 2D TLC. [^{32}P]-orthophosphate labelled E1A proteins from A- *wt* 52 kDA, B- *wt* 45 kDa, C- *wt* 37.5+35 kDa, D- *dl*1104 (amino acids 48-60 deleted), E- *dl*1105 (amino acids 70-81 deleted), and F- *dl*1143 (amino acids 30-60 deleted) were gel purified, digested with trypsin and resolved by TLC. Position of known tryptic peptides (*Table* **3-1**) are indicated on the TLC made from E1A proteins isolated from *wt* Ad5 infected cells (A). The phosphopeptides absent in the 45K species are identified on the TLC made from the 50 kDa protein by arrows.

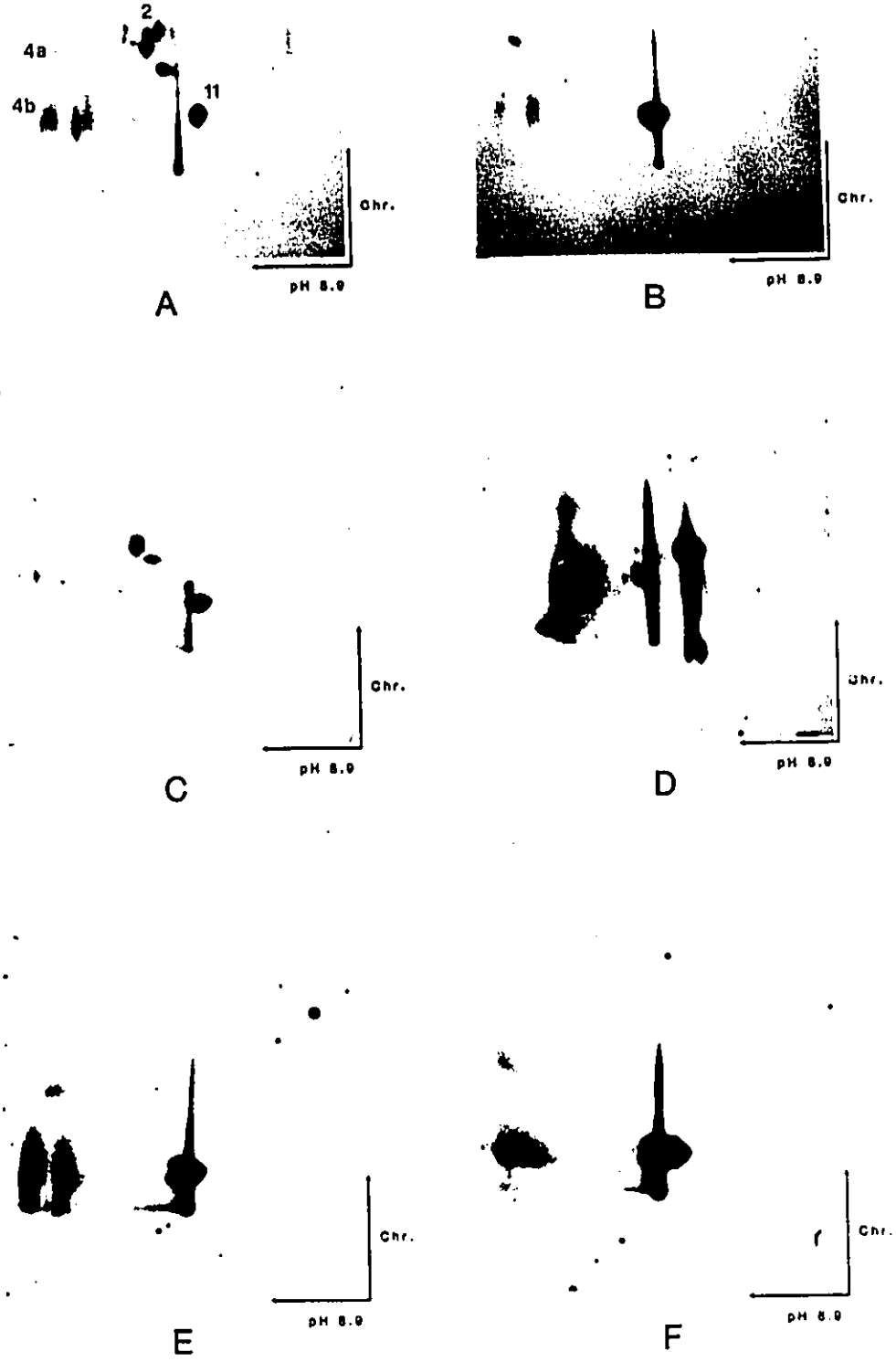

4a
2
1
4b
11
Chr.
pH 8.9
A
Chr.
pH 8.9
B
Chr.
pH 8.9
C
Chr.
pH 8.9
D
Chr.
pH 8.9
E
Chr.
pH 8.9
F

affected the migration of the entire cluster of species. These included *dl*1104 (***Fig.* 3-12D**, amino acids 48-60 deleted), *dl*1105 (***Fig.* 3-12E**, amino acids 70-81 deleted), and *dl*1143 (***Fig.* 3-12F**, amino acids 30-60 deleted). These data again suggested that the entire cluster of spots directly above the origin must have originated from T2. As discussed below, these multiple species could result from the existence of additional phosphorylation sites within T2, from the presence of phospho-isomers, or from the formation of partial trypsin digestion products.

To study the phosphorylation of Ser89 and Ser96 more carefully, phosphopeptides produced from *wt* Ad5 and from mutants affecting these residues were examined. E1A phosphopeptides from mutant AD89A (***Fig.* 3-13B**) produced a pattern on TLC that was very similar to that of *wt* Ad5 (***Fig.* 3-13A**) except that the entire T2 cluster was missing. Peptides T11 and T4a and b were present, but all species migrating above the origin were absent. This result was very surprising as our group had previously obtained evidence to suggest that Ser96 was phosphorylated (Tremblay *et al.*, 1989). The data could be explained by any of four possibilities: (i)- Ser96 is not phosphorylated (ii)- phosphorylation of Ser89 is required for phosphorylation of Ser96; (iii)- the mutation affecting Ser89 altered the mobility of these peptides, or (iv)- the mutation altered protein conformation such that phosphorylation at other site(s) was inhibited.

The TLC pattern obtained from the AD96A mutant (***Fig.* 3-13C**) tended to argue against the first and third possibilities because the number of spots found in the T2 cluster above the origin with this mutant was reduced, as would be expected from the elimination of the Ser96 site, but no apparent altered mobility was observed. Thus the data suggested

Figure **3-13.** Analysis of tryptic phosphopeptides of A- AD1, B- AD89A, C- AD96A, D- AD89A96A, E- AD89D96A, and F- AD90A96A by 2D-TLC.


2
4a
4b
11
Chr.
pH 8.9
A
Chr.
pH 8.9
B
Chr.
pH 8.9
C
Chr.
pH 8.9
D
Chr.
pH 8.9
E
Chr.
pH 8.9
F

that Ser96 is indeed phosphorylated, although at a low level and may depend on phosphorylation of Ser89.

The tryptic phosphopeptide patterns from E1A proteins containing double mutations affecting Ser89, Ser96 or Pro90 were also examined. The profile obtained with AD89A96A (*Fig.* **3-13D**) was poor, but nevertheless indicated the absence of the entire T2 cluster. AD89D96A (*Fig.* **3-13E**), in which Ser89 had been altered to an acidic Asp residue, also showed an absence of the T2 cluster. Finally, removal of Pro90 also led to the elimination of all T2 spots (AD90A96A- see *Fig.* **3-13F**), suggesting that this residue is necessary for the phosphorylation of Ser89. The reason for this effect will be discussed below.

III. CHARACTERIZATION OF MUTANTS.

IIIa. TRANSFORMATION.

The E1A mutants described above were used to determine the role of E1A protein phosphorylation in the transformation of primary baby rat kidney (BRK) cells in cooperation with either E1B or *H-ras*. BRK cells were either transfected with DNA from plasmids which encoded the entire E1 (E1A + E1B) region (pAD series) or they were cotransfected with DNA from plasmids expressing E1A (pNB series) and *H-ras* (pEJras), and the numbers of transformed foci were determined.

Table **3-2A** shows a summary of results obtained from five separate experiments and Table **3-2B** shows the statistical analysis of these data involving a Student's t-test of

differences in transforming efficiency (vs *wt*) at the 5% confidence limit. The absolute number of foci varied somewhat from experiment to experiment, but some clear trends were apparent. Mutation of Ser96 to Ala alone (pAD96A) had no significant effect on transformation in 4 of 5 experiments, suggesting that phosphorylation at this site is of little importance in this function. In all experiments using both E1A+E1B and E1A+*ras* the change from Ser89 to Ala (pAD89A) significantly reduced transformation efficiency. These data suggested that phosphorylation at Ser89 had some role in the regulation of E1A transforming activity. This idea was strengthened by additional data obtained with the double mutant pAD89A96A which in all cases except experiment 1 yielded transformants at efficiencies significantly less than *wt* (about 2-to-8 fold). In all cases tested, mutant pAD90A96A, which was shown above to prevent phosphorylation at Ser89, also transformed at a significantly reduced rate. Two possibilities existed to explain these results. First, they may have suggested that phosphorylation at Ser89 is functionally important for E1A-mediated transformation. It was also possible that the effect was caused by changes in protein conformation induced by alteration at amino acids 89 and 90 and that phosphorylation at these residues was of little importance. These possibilities could only be distinguished through the introduction of other mutations near to Ser89, but that do not alter its phosphorylation. Nevertheless, the simplest explanation remains that phosphorylation at Ser89 plays some functional role. The data obtained with a mutant containing Asp89 (pD89D96A) were also of interest. In some experiments (number 1 and the *ras* study) this mutant transformed at efficiencies not significantly different from *wt*, whereas in others (numbers 2-4) the efficiency was diminished. Thus it was not clear if

Table **3-2A.** Cooperation of E1A products with E1B and *H-ras*. BRK cells were transfected with either E1 encoding plasmids or co-transfected with E1A and *H-ras* encoding plasmids. The results are expressed as averages with the standard deviation (see *Table* **3-1B** for statistical analyses).

Cooperation with E1B

	Experiment			
PLASMID	**1**	**2**	**3**	**4**
pAD1	52.4 (±12.2)	9.5 (±3)	39 (±7)	7.8 (±13)
pAD89A	9.4 (±1.1)	0.5 (±0.8)	18.8 (±2)	1.7 (±2.7)
pAD96A	44 (±6.2)	4.2 (±3.3)	31.8 (±7.4)	2.7 (±5.5)
pAD89A96A	36.8 (±11)	3.4 (±3.3)	8 (±1.4)	1.2 (±2.6)
pAD89D96A	75.2 (±16)	4.3 (±2.5)	14.8 (±4.1)	2.5 (±4.7)
pAD90A96A	15 (±5.1)	4.5 (±2.4)	5.7 (±1.5)	-
10S11S	0 (±0)	0 (±0)	0 (±0)	-

Cooperation with *H-ras*

PLASMID	
pNB1	0.67 (±0.6)
pEJ*ras*	0.5 (±1)
pNB1+pEJ*ras*	4.7 (±1.4)
pNB89A+pEJ*ras*	2.8 (±0.4)
pNB96A+pEJ*ras*	4.5 (±2.7)
pNB89D96A+pEJ*ras*	5.3 (±9.1)
pNB89A96A+pEJ*ras*	2.3 (±0.7)

Table **3-2B.** Statistical analyses of transformation assays testing the ability of E1A proteins to cooperate with E1B or *H-ras*. Statistical analysis were using a two-sided student t-test to test the hypothesis $\mu_1=\mu_2$. One assumption made is that variances are unknown but are equal and the degrees of freedom = n_1+n_2-2.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{Sp\sqrt{1/n_1+1/n_2}}$$

PLASMID	N	$\varepsilon(x)^2$	εx_i	$\bar{X}$	Sp	t	Hypothesis* ($\mu_1=\mu_2$)
		Experiment 1					
pAD1	5	14326	262	52.4	-	-	
pAD89A	5	447	47	9.4	8.67	7.83	R
pAD96A	5	9870	220	44	9.92	1.34	A
pAD89A96A	5	7240	184	36.8	11.54	2.14	A
pAD89D96A	5	29330	376	75.2	14.37	2.5	A
pAD89D96A[v]	5	5446	162	32.4	9.96	3.17	A
pAD90A96A	5	1231	75	15	9.38	6.31	R
		Experiment 2					
pAD1	5	595	57	9.5			
pAD89A	5	5	3	0.5	2.39	6.53	R
pAD96A	5	157	25	4.17	3.26	2.83	A
pAD89A96A	5	24	10	2.0	2.53	4.9	R
pAD89A96A[v]	4	10	4	1.0	2.72	3.12	R
pAD89D96A	5	110	20	4.0	3.05	4.6	R
pAD90A96	6	151	27	4.5	2.88	3.01	R
		Experiment 3					
pAD1	4	6248	156	39			
pAD89A	4	1419	75	18.75	5.42	5.27	R
pAD96A	4	4195	127	31.75	7.38	1.39	A
pAD89A96A	4	262	32	8.0	5.32	8.24	R
pAD89D96A	4	921	59	14.75	5.98	5.73	R
pAD90A96A	3	321	27	9.0	6.96	5.65	R
		Experiment 4					
pAD1	6	433	47	7.8			
pAD89A	6	30	10	1.7	2.8	3.78	R
pAD96A	6	70	16	2.7	3.03	2.9	R
pAD89A96A	6	21	7	1.17	2.79	4.12	R
pAD89D96A	6	61	15	2.5	2.97	3.09	R
		ras Experiment					
pNB1	6	140	28	4.7			
pNB89A	5	40	14	2.8	1.06	2.96	R
pNB96A	6	159	27	4.5	2.16	0.16	A
pNB89A96A	6	36	14	2.3	1.13	3.69	R
pNB89D96A	6	216	32	5.3	2.34	0.45	A

([v] Different DNA preparations; * A=accept, R=reject)

replacement of Ser89 phosphorylation site with a negatively charged amino acid was able to restore *wt* transforming activity.

The 10S and 11S E1A products were also tested and found to be completely defective in transforming activity (*Table* **3-2A**). These results were not surprising because the novel intron in these mRNAs contains the coding sequence for CR1 which is known to be required for transformation.

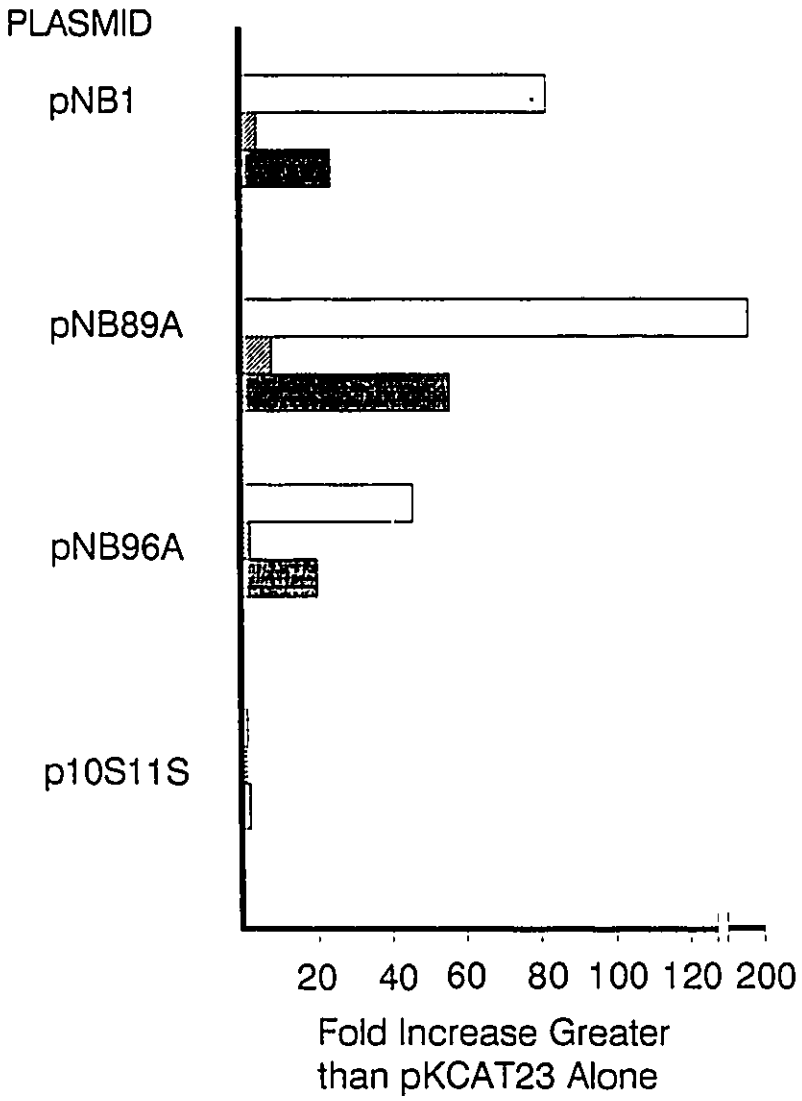
One concern in this type of assay was that the expression of E1B is known to be dependent on the E1A transactivation function. Thus, mutants that failed to transactivate the E1B gene to sufficient levels could appear transformation negative. The data obtained using E1A plasmids cotransfected with a *H-ras* encoding plasmid circumvented this problem as the latter is expressed in an E1A-independent fashion. As described above, the results obtained in these experiments, (*Table* **3-1A** and **B**), followed the same general trend as the E1A + E1B assays

IIIb. TRANSACTIVATION.

The abilities of the different mutant E1A products to transactivate early viral promoters were tested in two different fashions: (i)- transactivation of an E3CAT reporter construct in transient assays; (ii)- production of early viral products *in vivo* following infection of KB cells.

All mutants tested were capable of transactivating the E3 promoter, although the transient assay employed produced absolute values that varied somewhat from experiment to experiment, the relative differences in transactivation for various mutants were consistent. Figure **3-14** illustrates the results from three separate experiments. In

Figure **3-14.** Transient assay to test the ability of different E1A products to transactivate the E3 promoter. HeLa cells were cotransfected with pKCAT23 alone or with pNB1, pNB89A, pNB96A, and pNB10S11S and CAT levels were determined 48 hr later. The figure shows a graphic representation of three separate experiments (indicated by open, cross-hatched and stipled bars). The basal level of CAT activity from cells transfected with pKCAT23 alone was taken as equaling 1.


PLASMID
pNB1
pNB89A
pNB96A
p10S11S
20
40
60
80
100
120
200
Fold Increase Greater
than pKCAT23 Alone

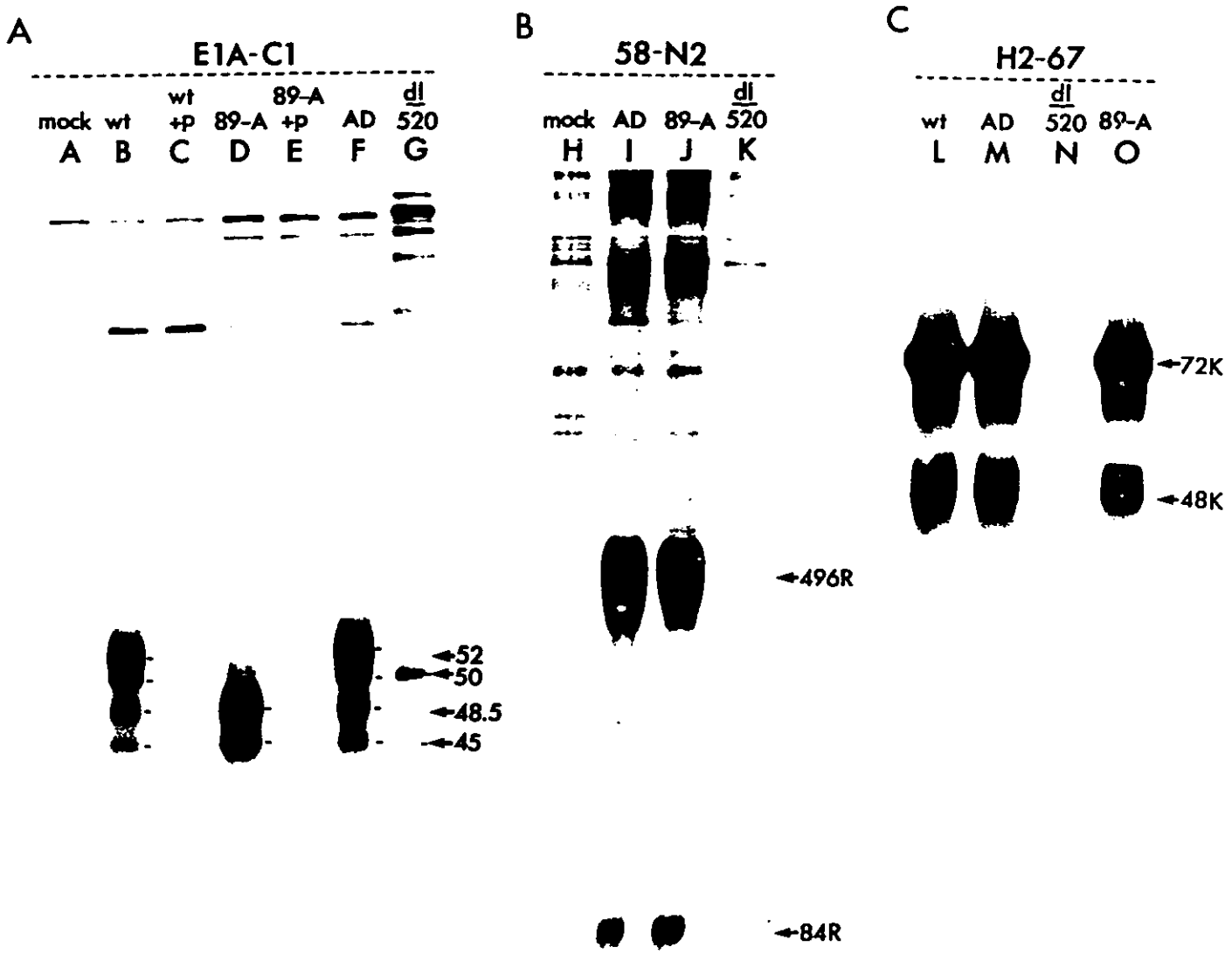
all cases CAT activity was stimulated about the same amount by *wt* E1A (pNB1) and pNB96A which contained Ala96. p10S11S which encodes only the products of the 10S and 11S E1A mRNAs had little or no transactivating activity with the E3 promoter. The reason for this observation was unclear as the 11S product should contain the CR3 region known to function in gene activation. It is possible that these products were highly unstable and thus unable to function in transactivation. Of some interest was the observation that in all three experiments with pNB89A, in which Ser89 had been altered to Ala, transactivation was enhanced by about 2-fold relative to *wt*. While the inherent variability of this type of assay and the low number of experiments performed made it difficult to draw firm conclusions, these data suggested that phosphorylation at Ser89 may negatively affect the ability of E1A products to activate E3 transcription.

To test further the ability of these E1A polypeptides to transactivate early Ad5 genes, the levels of the E1B-176R, -496R and -84R proteins and the E2a-72K DNA binding protein were determined in extracts from infected cells labelled with [^{32}P]-orthophosphate or [^{35}S]methionine by immunoprecipitation using specific antisera. All mutants produced high levels of the E2a-72K DNA binding protein (*Fig.* **3-15**, and **3-16C**). They also produced high levels of E1B proteins (*Fig.* **3-16B**, and data not shown). Although this type of analysis did not allow for accurate quantitative comparisons, it did illustrate that the levels of E1B and E2a protein produced upon infection at an equal multiplicity of infection (35 pfu/cell) with the various mutant viruses was not drastically different. The levels of these proteins produced by AD10S11S will be described in a later section. Thus in total these results suggested that phosphorylation at residues 89 and 96 did not seem to have a major effect on the ability of E1A to transactivate the E1B, E2a,

Figure **3-15.** Immunoprecipitation of the E2A-72K DNA binding protein from AD1 (lane A), AD89A (lane B), AD96A (lane C), AD89A96A (lane D), AD89D96A (lane E), and AD90A96A (lane F) infected cells labelled with [^{32}P]-orthophosphate.


A B C D E F

Figure **3-16.** Immunoprecipitation of E1A proteins, E1B products, and the E2A-72K DNA binding protein from cell extracts made from [^{35}S]methionine labelled cells infected with *wt* or mutant viruses. (A) E1A proteins from *wt* Ad5 (lane B), AD89A (lane C), AD1 (lane D), and *dl*520 (lane E) were immunoprecipitated with antipeptide serum E1A-C1, in some cases with 10μg of peptide (lanes C, and E). (B) These extracts were then reimmunoprecipitated with 58-N2 rabbit antipeptide serum, and then again with H2-67 mouse monoclonal antibody against the Ad5 E2A-72K DNA-binding protein (lanes L to O).


A
E1A-C1
mock
wt
wt +p
89-A
89-A +p
AD
dl 520
A
B
C
D
E
F
G
52
50
48.5
45
B
58-N2
mock
AD
89-A
dl 520
H
I
J
K
496R
84R
C
H2-67
wt
AD
dl 520
89-A
L
M
N
O
72K
48K

and E3 promoter.

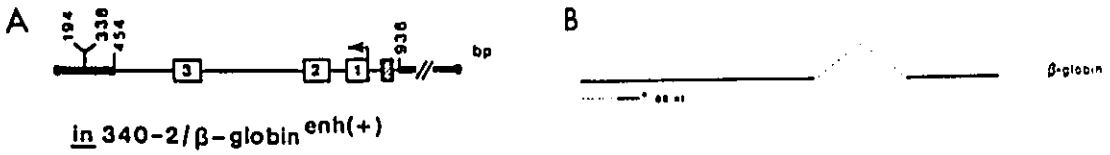
IIIc. ENHANCER REPRESSION.

In order to test the ability of the mutant E1A polypeptides to repress enhancer mediated transcription, the mutations were rescued into 12S cDNA vectors (UM series) which were then used to produce mutant virions. The 12S viruses were coinfected with the in 340-2/ß-globin $^{enh(+)}$ virus (Lillie and Green, 1986) in which a portion of the E1A gene had been deleted and replaced by the human ß-globin gene driven by the SV40 enhancer (*Fig.* **3-17A** and **B**). This virus had been used previously to detect the effects of E1A products on SV40 enhancer activity (Lillie and Green, 1986).

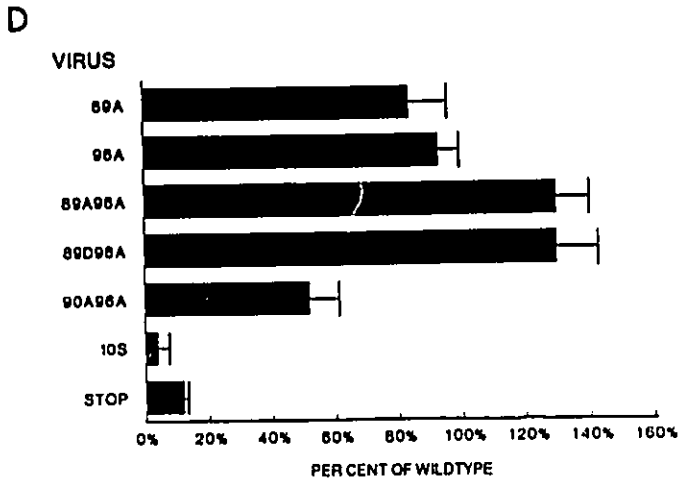
In order to detect the levels of ß-globin transcripts, an oligonucleotide was synthesized that was complementary to the sequences from +61 to +86 upstream of the CAP site of ß-globin mRNA (*Table* **2-3**, AB560). Primer extensions with this oligonucleotide should produce an extension product of 86 nucleotides (*Fig.* **3-17B**).

Hep-2 cells were coinfected with the mutants and the ß-globin virus and total RNA was analyzed for both the levels of ß-globin and E1A mRNAs. Figure **3-17C** shows results from a representative experiment using this type of analysis. Primer extensions performed on RNA extracted from cells infected with only the ß-globin virus produced a single extension product of 86 nucleotides (*Fig.* **3-17C**, lane H). In cells coinfected with this virus and *wt* 12S virus, the ß-globin signal was not detected (*Fig.* **3-17C**, lane I), thus demonstrating repression of the SV40 enhancer. All the amino acid substitution mutants (Ala89, Ala96, Ala89Ala96, Asp89Ala96, Ala90Ala96) tested were

Figure **3-17.** Repression of the SV40 enhancer by E1A proteins. (A) Diagram of ß-globin reporter virus. Thick solid lines represent Ad5 sequences, thin lines are ß-globin intron sequences while open boxes are exon sequences, and the crosshatched box represents the SV40 enhancer sequences. (B) Schematic representation of a portion of the ß-globin mRNA produced; the position to which the oligonucleotide hybridizes, and the length of the predicted extension product. (C) Representative experiment of a coinfection of Hep-2 cells with the ß-globin virus and UM10S (lane A), UM89A (lane B), UM96A (lane C), UM89A96A (lane D), UM89D96A (lane E), 90A96A (lane F), E1ASTOP (lane G), ß-globin only (lane H), and UM1 (lane I). (D) Graphic representation of four different experiments levels are given as a percent of *wt* activity.


A
194
338
454
3
2
1
936
bp
B
β-globin
in 340-2/β-globin enh(+)


C
A B C D E F G H I


D
VIRUS
89A
96A
89A96A
89D96A
90A96A
10S
STOP
0%
20%
40%
60%
80%
100%
120%
140%
160%
PER CENT OF WILDTYPE

capable of effectively repressing the SV40 enhancer, with three exceptions. UM90A96A, in which both Pro90 and Ser96 were converted to Ala residues, was not quite as efficient as the others (*Fig.* **3-17C**, lane F). Also the STOP mutant, which produces truncated E1A products due to an in frame STOP codon after amino acid residue 18 (see *Fig.* **3-5**), as anticipated, was completely defective for repression (*Fig.* **3-17C**, lane G), as was the 10S virus which yields only the 10S E1A mRNA products (*Fig.* **3-17C**, lane A). Similar results were found consistently in four different experiments which have been summarized in Figure **3-17D**. Differences in E1A mRNA levels with the different mutants was never more than 2 fold (data not shown). Thus phosphorylation at Ser89 and 96 did not appear to affect E1A mediated enhancer repression.

IIId. GEL MIGRATION OF E1A PRODUCTS.

In order to determine the effect of phosphorylation of Ser89 on the mobility of E1A products in SDS-PAGE, KB cells were infected with AD89A, E1A proteins were labelled with [^{35}S]methionine and immunoprecipitated with the antipeptide serum E1A-C1, and analyzed by SDS-PAGE. Figure **3-16A** shows that both *wt* viruses (lanes B & F) produced the 52, 50, 48.5, and 45 kDa forms of E1A products, whereas dl520, a deletion mutant that removes the 13S splice donor site, produced only the 12S mRNA products (50 and 45 kDa proteins; lane G). E1A proteins from AD89A infected cells migrated predominantly as the faster migrating forms, 48.5 and 45 kDa (lane D), however, some material was detected which migrated slightly slower than the 48.5 kDa species. Precipitation of these products was blocked by the addition of E1A-C peptide (lanes C

Figure **3-18.** Migration of E1A polypeptides from the different mutants through 9% SDS-PAGE. KB cells were either mock infected (lane A) or infected with AD1 (lane B), AD89A (lane C), AD96A (lane D), AD89A96A (lane E), 89D96A (lane F), and AD90A96A (lane G) at an moi of 35 labelled with [^{32}P]-orthophosphate and immunoprecipitated with M73 antibody.

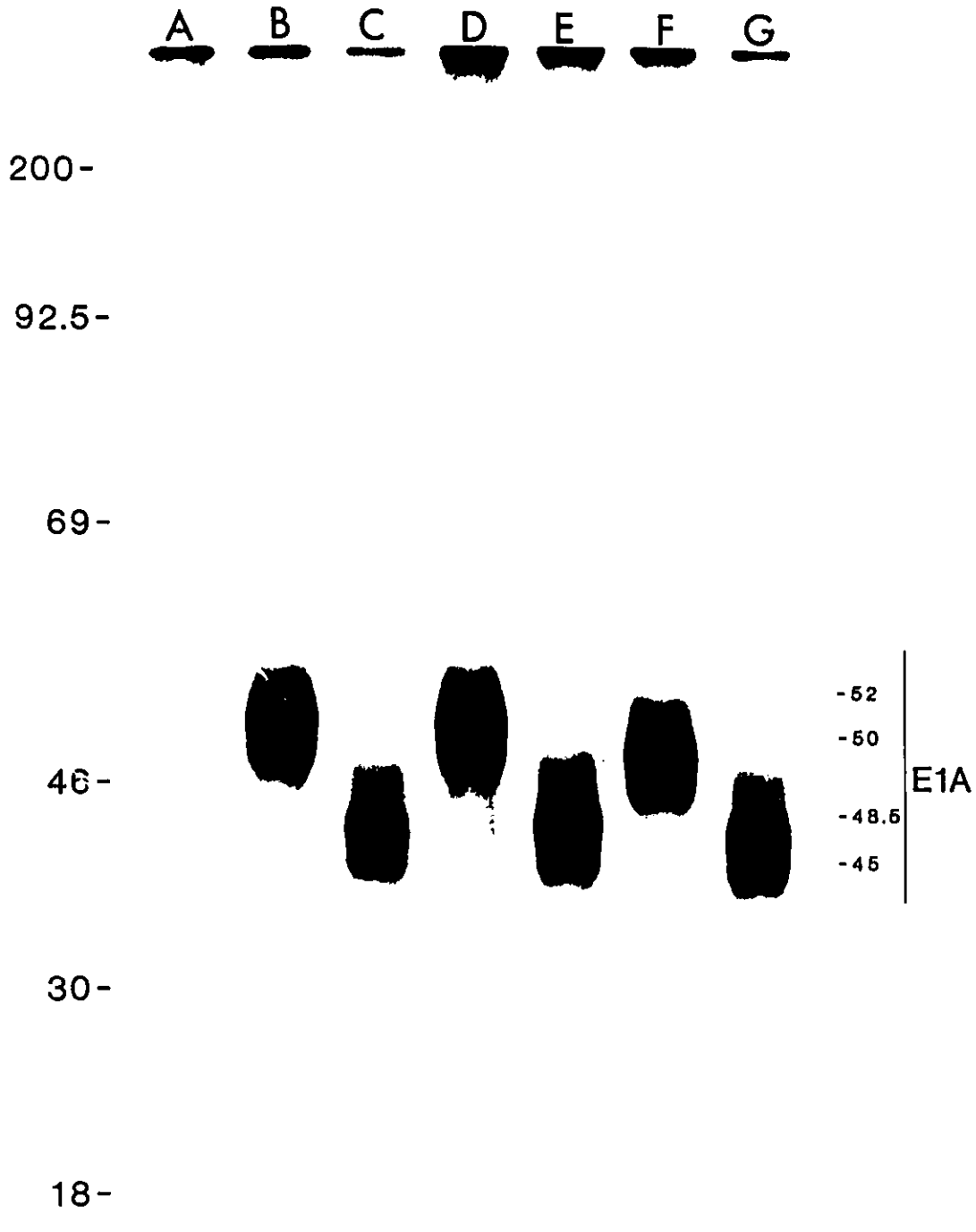
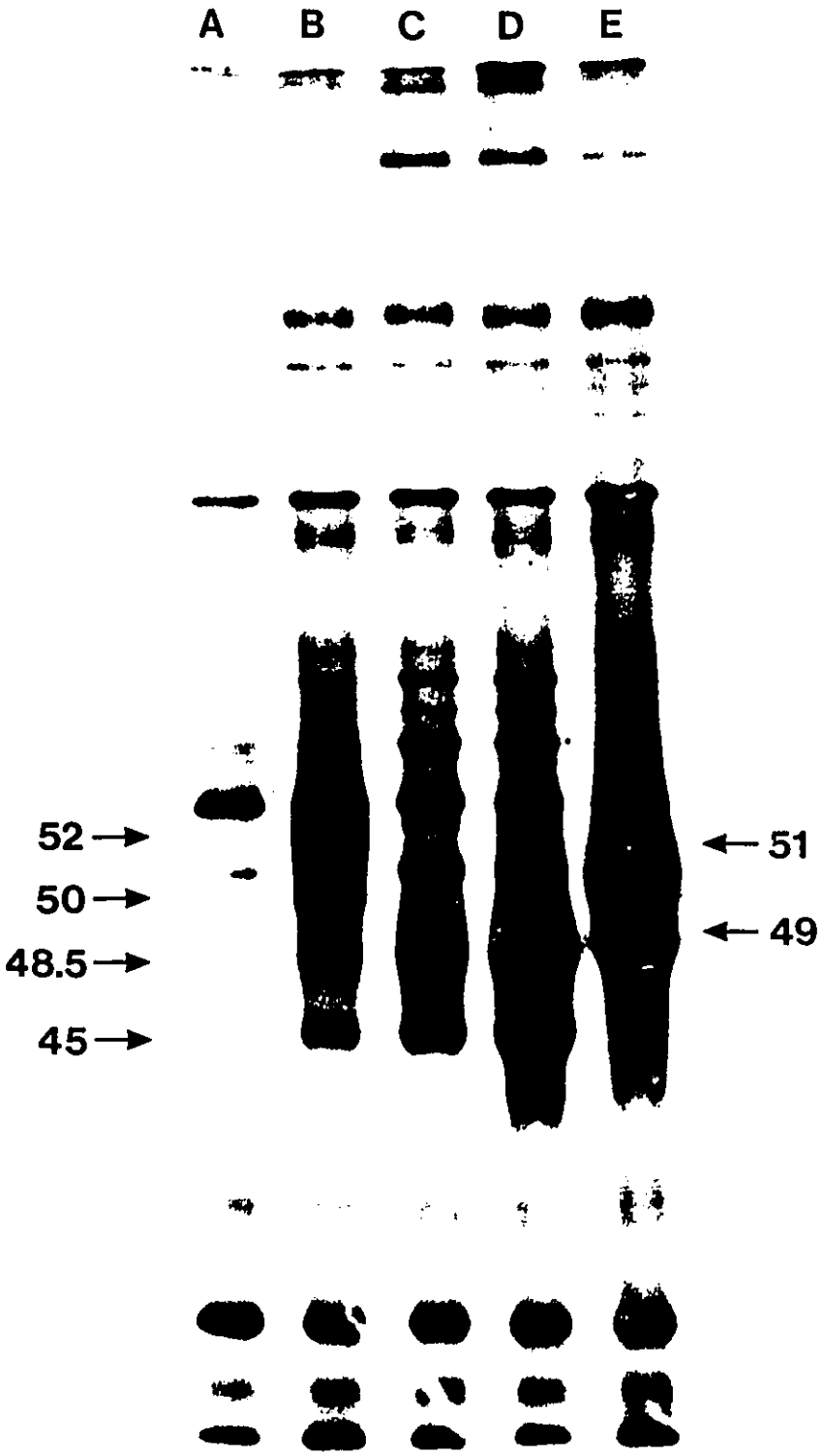

A
B
C
D
E
F
G
200-
92.5-
69-
46-
30-
18-
-52
-50
-48.5
-45
E1A

Figure **3-19.** Migration of E1A polypeptides from the different mutants through 9% SDS-PAGE. A- KB cells were either mock infected or infected with B- AD1, C- AD89A, D- AD89A96A, or E- 89D96A at an moi of 35 labelled with [^{35}S]methionine and immunoprecipitated with M73 antibody. Position of 13S (52 and 48.5 kDa) and 12S (50 and 45 kDa) E1A products are shown by arrows on the left, and the position of the 13S (51 kDa) and 12S (49 kDa) AD89D96A E1A products by arrows on the right.


A
B
C
D
E
52
50
48.5
45
51
49

and E), thus demonstrating the specificity of the antiserum. These data suggested that phosphorylation at Ser89 is largely responsible for the shift in gel migration. The small amount of slower migrating species may have been due to phosphorylation at another site(s) at serine residues 227, 228, 231, 234, and 237 which occurs at very low levels in infected KB cells (S. Whalen, unpublished results).

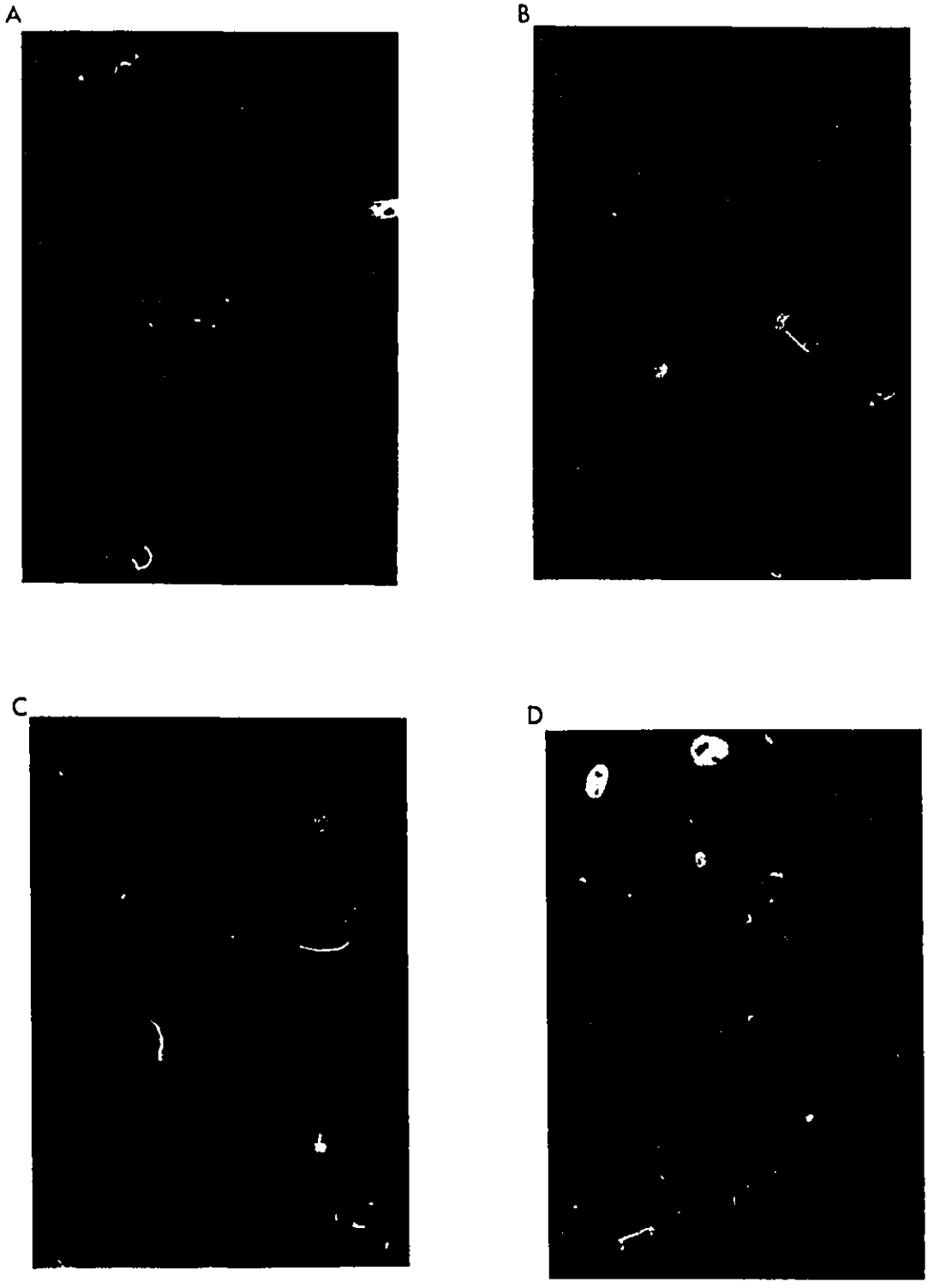
A similar study was carried out to examine the E1A proteins produced by other mutants affecting phosphorylation at Ser89 and Ser96. The parental *wt* AD, and AD96A, produced all four 52, 50, 48.5 and 45 kDa forms (*Fig.* **3-18**, lanes B & D). Thus conversion of Ser96 to Ala had no effect on gel mobility. Replacement of both Ser89 and Ser96 with Ala (AD89A96A; *Fig.* **3-18**, lane E) resulted in E1A proteins that migrated in a fashion similar to AD89A for which only the 48.5 and 45 kDa forms were apparent (*Fig.* **3-18**, lane C). Mutant AD90A96A, which was shown previously to block phosphorylation at Ser89 and other sites in peptide T2, yielded E1A products like those from AD89A infected cells, that is, only the 48.5 and 45 kDa products. Phosphorylation at Ser89 would add two additional negative charges to the molecule at physiological pH. To determine whether a negative charge at this residue was sufficient to induce a conformational change and result in an increased gel mobility, the AD89D96A mutant was tested. Figure **3-18** (lane F) demonstrates that these E1A proteins migrated slower than those of AD89A96A, however, the decrease in mobility was not as great as seen with the *wt* 52 and 50 kDa products. Thus the negative charge introduced by an Asp residue only partially compensated for the lack of phosphorylation at Ser89. Another interesting observation was that unlike the E1A polypeptides produced by *wt* Ad5, which

contain both slower and faster migrating forms which reflect the presence of a phosphate at serine 89, all E1A proteins from 89D96A should have a uniform negative charge at residue 89 and thus all should have the same gel mobility. This effect was observed in Figure **3-18** (lane F) and **3-19** (lane B). The 13S and 12S E1A products produced from cells infected with AD89D96A migrated predominantly as single forms with M_r of 51 and 49 kDa, respectively, while those from *wt* migrated with relative molecular masses of 52 and 48.5 kDa for the 13S products and 50 and 45 kDa for the 12S products. Thus, in total, these data indicated that phosphorylation at Ser89 is largely responsible for the decrease in gel mobility of the E1A 13S and 12S products, and that introduction of an acidic amino acid (Asp) at this position can partially induce the change in gel migration.

IIIe. INTRACELLULAR LOCALIZATION OF E1A PROTEINS.

In order to determine whether mutations in phosphorylation sites of E1A products affected intracellular localization, infected cells grown on coverslips were fixed and E1A products were examined by indirect immunofluorescence. The multiplicity of infection used in these studies was chosen such that not all cells would be infected, thus providing an internal control for the specificity of the antibody. The E1A proteins produced by AD1, AD89A, AD96A, and AD89A96A (*Fig.* **3-20 A**,**B**,**C**,and **D**, respectively) were all found to be located within the nucleus with a similar staining pattern. Thus, conversion of Ser 89 and 96 to Ala did not affect nuclear localization.

Figure **3-20.** Intracellular localization of E1A proteins. HeLa cells were infected with AD1 (panel A), AD89A (panel B), AD96A (panel C), and AD89A96A (panel D), and the localization of E1A polypeptides within the cell was determined by indirect immunofluorescence using M73 as the primary antibody.


A
B
C
D

IIIf. CHARACTERIZATION OF 10S11S VIRUS.

At the time that this work was initiated there were few studies that had been done to characterize the properties of the 10S and 11S E1A products. Although the 10S11S virus produced E1A proteins that lacked both Ser89 and Ser96, it was thought that perhaps studies on this virus may provide some insight into the roles of these E1A proteins which lack the functionally important CR1 region. Studies that first identified the 10S and 11S E1A transcripts (Ulfendahl *et al.*, 1987; Stephens *et al.*, 1987) and work from our group (M.L. Tremblay, unpublished data) demonstrated that the 10S and 11S mRNAs produced E1A polypeptides which migrated in SDS-PAGE with a M_r of 35 kDa and 37.5 kDa, respectively. In order to determine whether the 10S11S virus produced these products, E1A proteins were immunoprecipitated with E1A specific M73 serum from cell extracts from infected KB cells labelled with [^{32}P]-orthophosphate. Figure **3-21** (lane B) shows that only the 37.5 and 35 kDa forms of E1A proteins were observed with AD10S11S, thus confirming the absence of the slower migrating species encoded by the 13S and 12S mRNAs.

To determine whether these E1A products could transactivate E1B and E2A transcription, extracts from KB cells infected with the 10S11S virus and labelled with [^{35}S]methionine were immunoprecipitated with antibody directed against the 496R (58K) E1B and the 72K E2A gene products. The 10S11S virus was able to synthesize both products at reasonably high levels (*Fig.* **3-22**, lanes A and B). To compare the levels of synthesis of these products relative to *wt* Ad5, a parallel experiment with Ad5 *wt* was carried out using an equal multiplicity of infection (35 pfu/cell) and an equal amount of cell extract was immunoprecipitated in the presence of excess antibody in an attempt to

detect all the proteins of interest in the extract. Although the autoradiography in Figure **3-22** was overexposed, it was clear that the 10S11S virus produced somewhat lower amounts of these products than *wt* (lanes C and D). As previously described in Figure **3-14**, this mutant was very deficient in transactivation of the E3 promoter, and the present data suggested that even though the 11S product contains CR3 which is largely responsible for transactivation, it appeared to produce somewhat lower amounts of E1B and E2a products than *wt*. The plaquing efficiency of this virus on HeLa cells relative to 293 cells was more than $4X10^4$-fold lower (data not shown), again suggesting a defect for transactivation of early viral genes. In fact, viruses containing no E1A coding sequences have been found to plaque at efficiencies close to this level. Similar results have been described for a 10S11S virus produced by site directed mutagenesis (Ulfendahl *et al.*, 1987). To determine if this defect was due to reduced levels of E1A transcripts, KB cells were infected with either *wt* or 10S11S virus and total cytoplasmic RNA was harvested at either 6 hr or 12 hr postinfection and subjected to primer extension analysis using a primer specific for E1A transcripts (*Table* 2-3, and *Fig.* **3-23A**). E1A mRNA levels produced by AD10S11S at 6 hr postinfection were considerably lower than those of *wt* Ad5 and AD1 (*Fig.* **3-23B**, lanes A, B, and C respectively). However, the levels at 12 hr postinfection were about equal (*Fig.* **3-23B**, lanes A',B',and C', respectively), suggesting that the defect was not due to a low level of E1A transcription, but to an inherent decreased ability of the 10S and 11S products to compensate for 12S and 13S products.

Figure **3-21.** Immunoprecipitation of the E1A proteins produced from *wt* (lane A) and AD10S11S (lane B) infected KB cells labelled with [^{32}P]-orthophosphate.

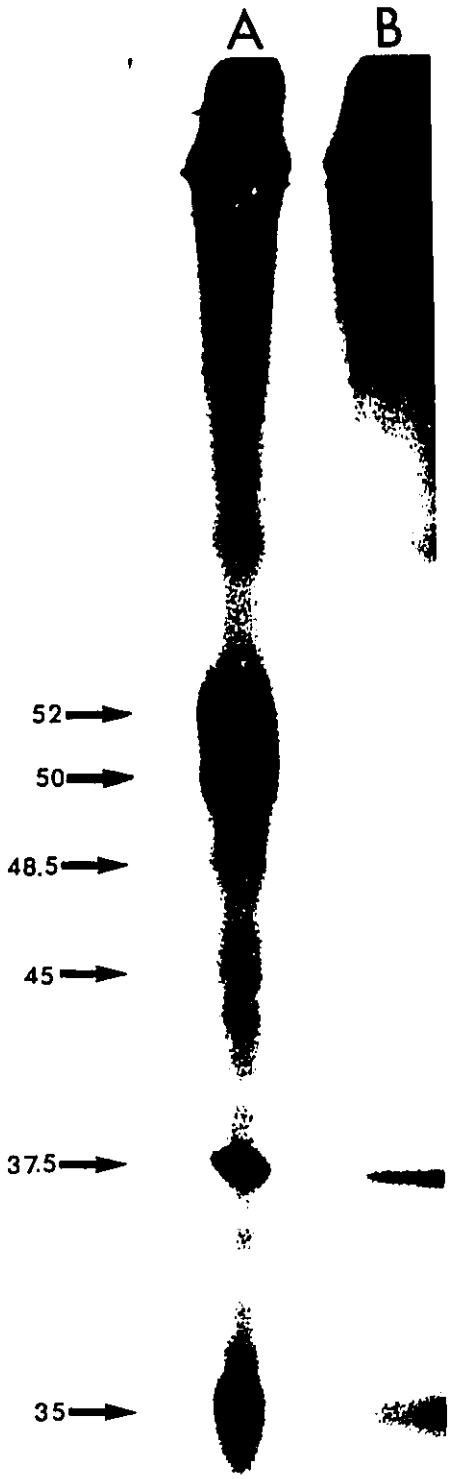

A
B
52
50
48.5
45
37.5
35

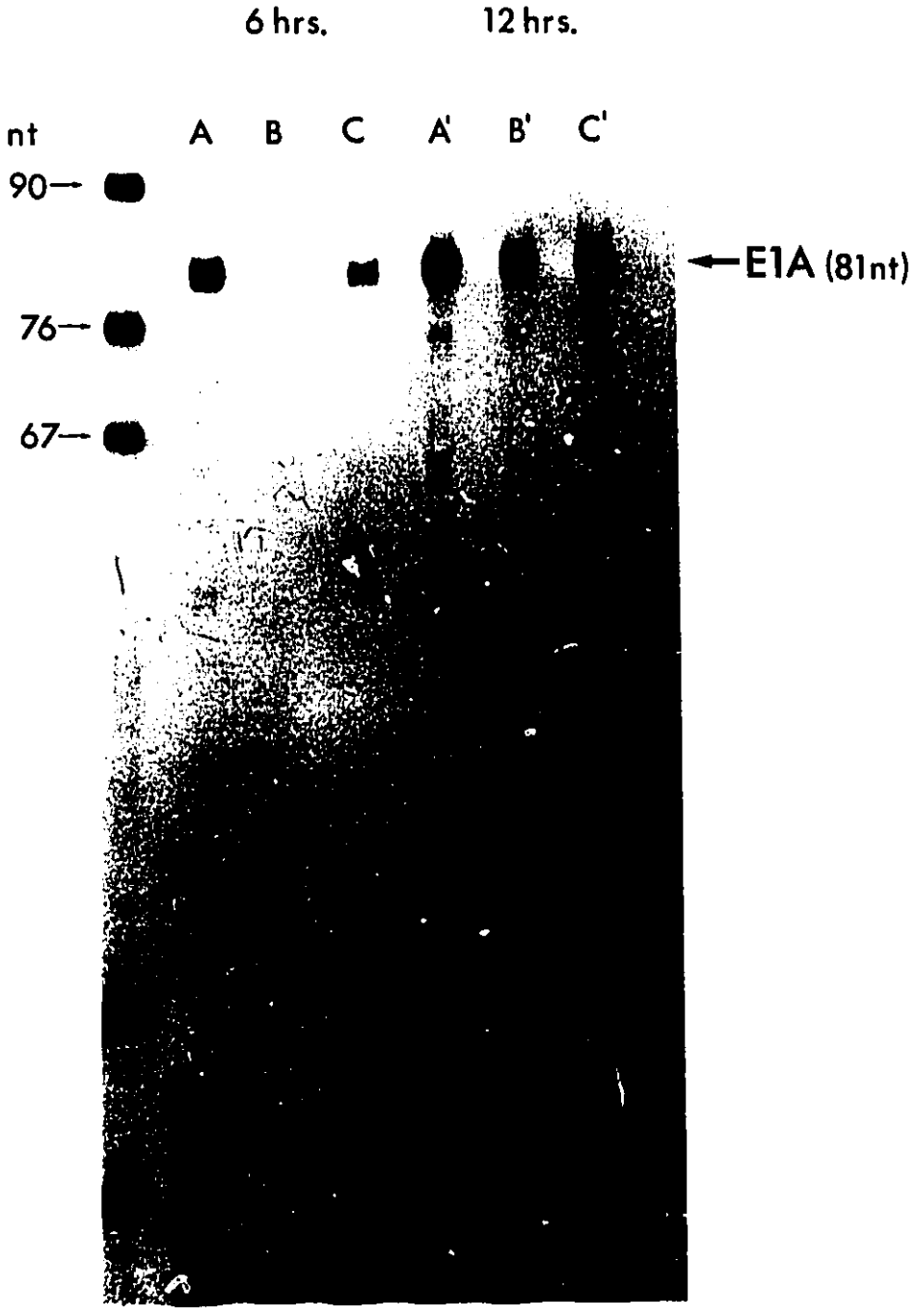
Figure **3-22.** Immunoprecipitation of E2A-72K DNA-binding protein and E1B-58K from 10S11S and *wt* infected KB cells. Lanes A and B are immunoprecipitates prepared from 10S11S infected cells immunoprecipitated with the 58K antipeptide serum (lane A) and the 72K monoclonal (lane B). Lanes C and D are similar immunoprecipitations prepared from *wt* Ad5 infected cells (lanes C and D, 58K and 72K immunoprecipitations, respectively). Infected cells were metabolically labelled with [^{35}S]methionine.


A
B
C
D

Figure **3-23.** E1A mRNA levels from *wt*, 10S11S, and AD1 infected KB cells. (A) Schematic of E1A mRNAs produced and the expected extension product size. (B) E1A mRNA levels measured at 6 and 12 hr post-infection from *wt* (lanes A,A)', 10S11S (lanes B,B'), and AD1(*wt*) (lanes C,C') infected cells.

A

B



CHAPTER IV: IDENTIFICATION OF A PROTEIN KINASE INVOLVED IN THE PHOSPHORYLATION OF AD5 E1A PROTEINS.

Increasing information has now begun to link specific families of protein kinases with regulatory roles in cell growth and progression through the cell cycle. The identification of potential consensus sequences at Ser89 and Ser219 in E1A proteins which were characteristic of the sites phosphorylated by the $p34^{cdc2}$ family of protein kinases, lead to a series of studies to determine if in fact this enzyme was responsible for E1A protein phosphorylation at these sites.

Ia. IN VITRO PHOSPHORYLATION OF E1A BY $p34^{cdc2}$.

Figure 4-1A shows that the amino acid sequences surrounding serine residues 89 and 219 resemble the consensus sequence for sites phosphorylated by the $p34^{cdc2}$ family of protein kinases (Cisek and Corden, 1989; Heald and McKeon, 1990; Hill *et al.*, 1990; Kipreos and Wang, 1990; McVey *et al.*, 1989; Morgan *et al.*, 1989; Peter *et al.*, 1990; Shenoy *et al.*, 1989; Ward and Kirschner, 1990; reviewed in Moreno and Nurse, 1990). To determine if these or other sites are phosphorylated by $p34^{cdc2}$, E1A-289R protein synthesized in and purified from *E. coli* (Ferguson *et al.*, 1984) or purified histone H1 (Sigma) were incubated with $p34^{cdc2}$ which had been purified by immunoprecipitation using a $p34^{cdc2}$-specific anti-peptide serum (Simanis and Nurse, 1986). Cell extracts were prepared from either unsynchronized HeLa cells or those blocked either in mitosis or

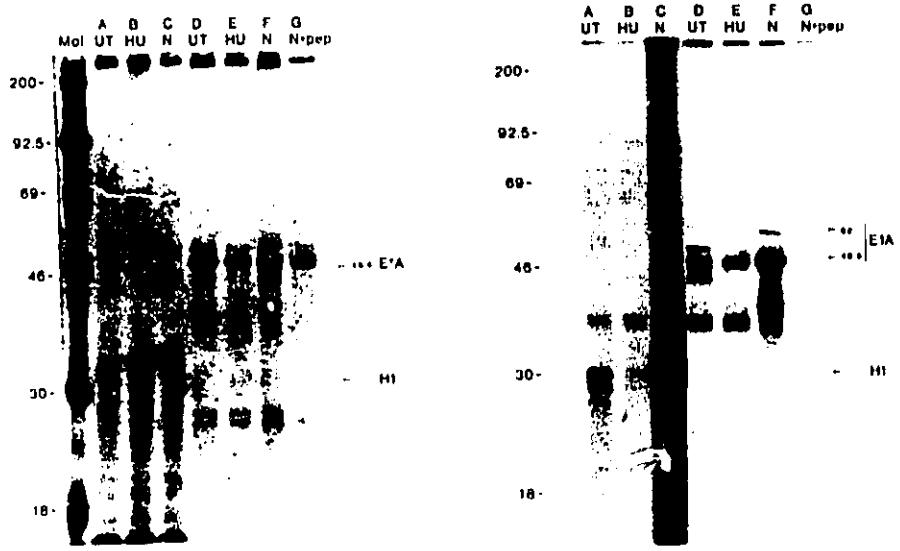
G1/S using nocodazole (Zieve *et al.*, 1980) or hydroxyurea (Bacchetti and Whitmore,1969), respectively. As shown in Figures **4-1B** and **C** (lanes A-C), and as found previously (Moreno and Nurse, 1990), $p34^{cdc2}$ kinase activity measured using histone H1 as substrate was seen to be high during mitosis and considerably lower in cells in G1/S. Figures **4-1B** and **C** (lanes D-F) show that while E1A-289R was phosphorylated in all cases, the specific activity was considerably higher using $p34^{cdc2}$ purified from mitotic cells. Incorporation of ^{32}P was largely inhibited when the immunoprecipitate was prepared in the presence of the peptide to which the serum was raised (*Fig.* **4-1B** and **C**, lane G). These data indicated that phosphorylation was carried by the kinase to which the antibodies had been raised. Although this competition strongly suggested that the kinase activity observed was due to $p34^{cdc2Hs}$, the possibility existed that other closely related kinases could also have been recognized by the $p34^{cdc2}$ specific anti-serum. Phosphoamino acid analysis of labelled histone and E1A-289R indicated that whereas $p34^{cdc2}$ phosphorylated the former on serine and threonine residues at approximately equal levels, phosphorylation of E1A proteins was almost exclusively (over 95%) on serine residues (*Fig.* **4-1D**). The much higher specific activity detected for histone relative to E1A may have reflected either a greater affinity of the enzyme for histone over E1A, or just the fact that histone H1 contains numerous *cdc*2 sites whereas E1A contains just two such sites.

Of some interest was the generation of a slower-migrating E1A species following incubation with $p34^{cdc2}$ purified from mitotic (*Fig.* **4-1B**, lane F) and unsynchronized

Figure **4-1.** *In vitro* phosphorylation of E1A by $p34^{cdc2}$. (A) Table of proteins and the sites known to be phosphorylated by $p34^{cdc2}$. (B) Comassie stained gel of *in vitro* kinase assay. (C) Autoradiogram of the gel presented in panel B. *In vitro* kinase assays were done with $p34^{cdc2}$ purified from cell extracts from either untreated cells (UT), lanes A and D, or cells treated with hydroxyurea (HU), lanes B and E, or nocodazole (N), lanes C and F. Immunoprecipitation of cell extracted made from nocodazole treated cell with 25µl of anti-peptide serum preincubated with 10µg of peptide to which the serum was raised against (lane G). (D) Gel purified kinase products were subjected to acid hydrolyse and analyzed for phospho-amino acid content. The position of cold markers detected by ninhydrin staining are indicated.

cdc2 PHOSPHORYLATION SITES

SUBSTRATE	SEQUENCE PHOSPHORYLATED
Histone H1	Arg/Lys Ser/Thr Pro X Arg
Lamin B	Pro Leu Ser Pro Thr Arg
pp60$^{c\text{-}src}$	Gln Thr** Pro Asn Lys Arg Thr** Pro Ser Arg Thr Ser** Pro Ser Arg
Murine RNA Pol II	(Thr Ser Pro Ser Tyr)$_n$
SV40 Large T	Ser Thr*** Pro Pro Arg
Consensus	Thr/ser Pro Polar Basic
E1A	Gly Ser** Pro Glu Pro Pro His Thr Ser*** Pro Val Ser Arg
c-abl	Asp Thr*** Pro Glu Leu Val Ser*** Pro Leu Leu
Rb-1	Many sites that fit consensus

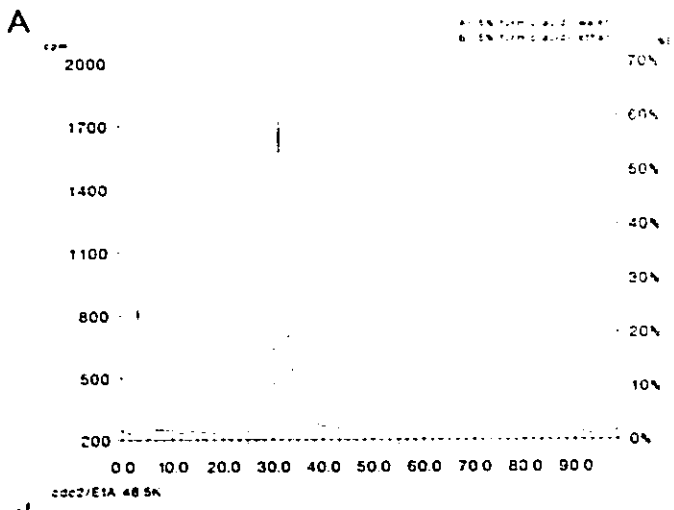


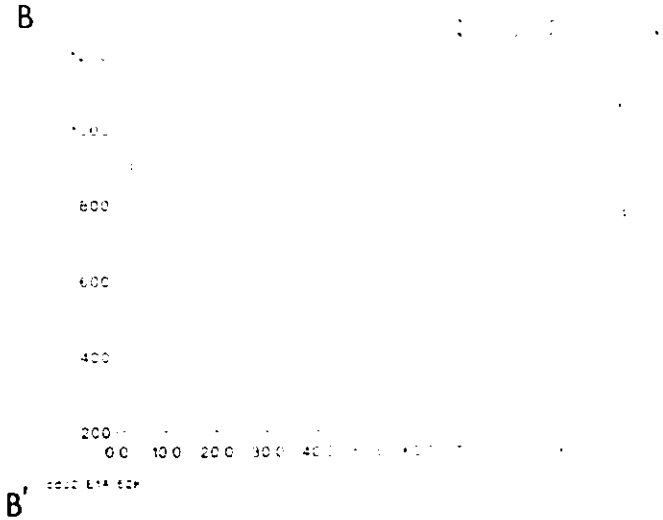
(lane D) cells, but not hydroxyurea-treated cultures (lane E). The E1A-289R protein purified from *E. coli* migrated on SDS-PAGE as a single species at a nominal molecular mass of 48,500 (see *Fig.* **4-1A** lanes D-G and Chen *et al.*, 1989). The data presented earlier in this thesis and other studies indicated that the slower migrating 52 kDa E1A species is produced through phosphorylation (Richter *et al.*, 1988; Tremblay *et al.*, 1988; Dumont *et al.*, 1989; Smith *et al.*, 1989), and that phosphorylation at serine-89 is largely responsible (this thesis; Dumont *et al.*, 1989; Smith *et al.*, 1989). These data suggested that $p34^{cdc2}$ phosphorylates serine residues in E1A-289R *in vitro* and that one of these sites is probably serine-89.

A band migrating at approximately 35K was also apparent in assays using either E1A or histone as subtrates (see *Fig.* **4-1C**). This species may have resulted from the autophosphorylation of $p34^{cdc2}$.

To identify the sites on E1A-289R phosphorylated by $p34^{cdc2}$, both the faster- and slower-migrating E1A proteins labelled with $p34^{cdc2}$ purified from mitotic cells were digested with trypsin and analyzed by RP-HPLC and 2D-TLC. While E1A products are phosphorylated *in vivo* at several sites, the faster-migrating E1A protein labelled *in vitro* contained only one ^{32}P-labelled phosphopeptide (*Fig.* **4-2A'**). This species was peptide T11 comprised of residues 216-223 (*Table* **3 1**) and containing the major phosphorylation site at serine-219 (Tremblay *et al.*, 1989; Tsukamoto *et al.*, 1986). The slower-migrating E1A protein yielded at least three labelled species (*Fig.* **4-2B'**), one of which was again peptide T11. The other two species were apparent in digests of E1A products labelled *in vivo* (*Fig.* **4-4A**; **3-12** and **3-13**) and eluted as a heterogenous peak from 85 to 90 min

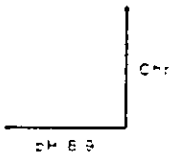
Figure **4-2.** Tryptic digests of E1A 48.5K (A and A') and E1A 52K (B and B') phosphorylated *in vitro* and resolved either by HPLC or 2D-TLC.


A
2000
1700
1400
1100
800
500
200
70%
60%
50%
40%
30%
20%
10%
0%
0.0 10.0 20.0 30.0 40.0 50.0 60.0 70.0 80.0 90.0


B
800
600
400
200
0.0 10.0 20.0

A'

B'


CH

(S. Whalen, unpublished data). For reasons described previously, both major species appeared to correspond to peptide T2 (*Table* **3-1**). The fact that two species were present may have resulted from incomplete trypsin digestion. Tryptic peptide T2 should be produced by cleavage at Arg 97, however, the existence of proline just two residues downstream may reduce the efficiency of this cleavage. Thus these species may correspond to a T2-T3 partial product and the T2 peptide alone. These results indicated that incubation *in vitro* of purified E1A protein with $p34^{cdc2}$ resulted in phosphorylation of serine residues 219 and 89.

IVb. IN VIVO PHOSPHORYLATION OF E1A BY $p34^{cdc2}$.

To determine the pattern of phosphorylation of E1A proteins *in vivo* at different stages of the cell cycle, Ad5-infected cells which had been blocked either in G1/S or mitosis or left unsynchronized were labelled with either [^{32}P]-orthophosphate or [^{35}S]methionine and E1A proteins were immunoprecipitated and analyzed by SDS-PAGE. Figure **4-3** shows that although approximately equal amounts of E1A products were synthesized during the labelling period (*Fig.* **4-3**, lanes A'-C'), phosphorylation of E1A was considerably higher in mitotic cells which contain the highest levels of $p34^{cdc2}$ kinase activity (*Fig.* **4-3**, lanes A-C). TLC analyses of E1A tryptic peptides from samples shown in Figure **4-3** (lanes Band C) were performed. It should be noted that even though peptides were generated from E1A proteins derived from equal amounts of cell extract, and such samples were processed in parallel, it was difficult to produce such preparations in a strictly quantitative fashion. Thus a rigorous comparison of peptide patterns was not

possible. However, Figure **4-4** indicated that peptide T11 containing Ser219, peptide T2 containing Ser89, and two other species which have been found to correspond to peptides T4a and T4b containing Ser132 (Whalen *et al.*, unpublished results), were all phosphorylated at seemingly higher levels in mitotic cells (*Fig.* **4-4B**) than in G1/S (*Fig.* **4-4A**) cells, although clearly some phosphorylation of most of these peptides was apparent in the latter. The potential significance of these data and the problems in their interpretation will be discussed below.

Figure **4-3.** Cell cycle dependent phosphorylation of E1A *in vivo*. HeLa cells were infected with *wt* Ad5 were either untreated ((UT),lanes A and A') or treated with hydroxyurea ((HU),lanes B and B') or nocodazole ((N), lanes C and C') and labelled with either [^{32}P]-orthophosphate (lanes A to C) or [^{35}S]methionine (lanes A' to C').

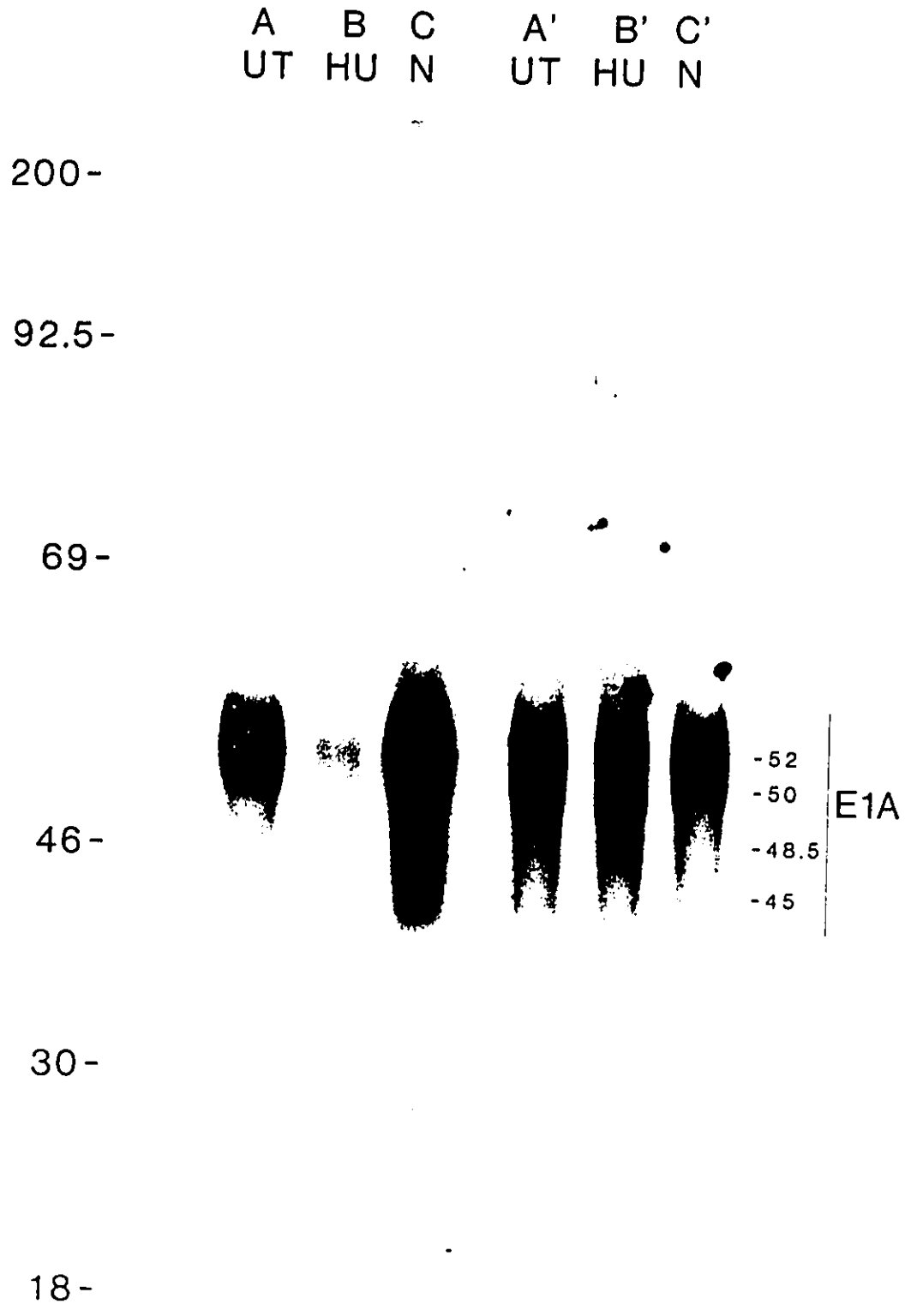
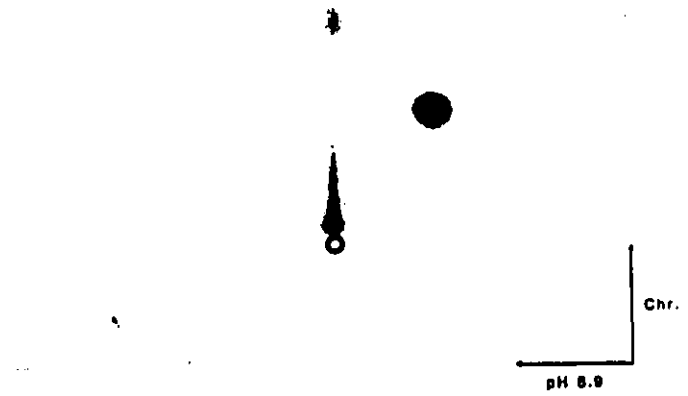

A
UT
B
HU
C
N
A'
UT
B'
HU
C'
N
200-
92.5-
69-
46-
30-
18-
-52
-50
-48.5
-45
E1A

Figure **4-4.** 2D-TLC profiles of tryptic peptides produced from E1A proteins phosphorylated at different points in the cell cycle, (A) G_1/S (hydroxyurea block) and (B) Mitosis (nocodazole block).



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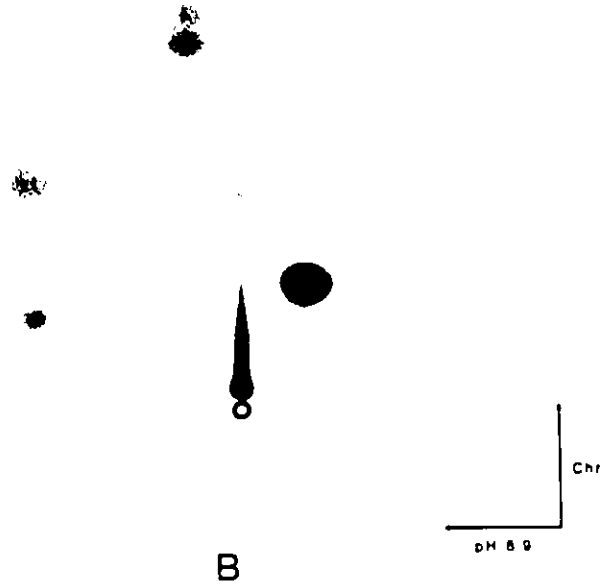

B

CHAPTER V: DISCUSSION

I. E1A PHOSPHORYLATION.

For many proteins, phosphorylation represents a critical mechanism for the regulation of biological activity. E1A proteins appear to be multifunctional and multiphosphorylated, and thus the possibility exists that phosphorylation at one or more sites could play an important regulatory role. The objective of the present work was to further localize the E1A phosphorylation sites and through the generation of mutants at these sites to test the functional role of each.

Previous studies had indicated that Ad5 E1A proteins are phosphorylated at Ser89 and 219 , and suggested that Ser96 as well as one or more Ser residues between amino acids 227 to 237 (Tremblay *et al.*, 1988; 1989). In addition studies carried out very recently have shown that Ser132 is also phosphorylated (Whalen and Branton, in preparation). When the present studies commenced, nothing was known about the protein kinases involved in such phosphorylation nor the biological consequences of such events.

The present studies have indicated that phosphorylation at Ser89 is responsible for the major shift in gel mobility of the E1A 289R and 243R products. Several caveats must be considered in making this conclusion. First, it is possible that the substitution of Ala for Ser89, regardless of its phosphorylation, could induce a conformational change in E1A proteins leading to altered gel mobility. However, the increased gel mobility of E1A proteins from the Ala90Ala96 mutant and the partial restoration of this shift by the

Asp89Ala96 mutant argues that phosphorylation, *i.e.* the presence of a negative charge at amino acid 89 does in fact induce this alteration in gel mobility. Second, the shift in gel mobility might be the direct result of another unidentified posttranslational modification which is dependent upon phosphorylation at Ser89. Nevertheless, the elimination of the more slowly migrating forms of E1A proteins by phosphatase treatment (Lyons *et al.*, 1987; Richter *et al.*, 1988; Tremblay *et al.*, 1989) strongly suggested that phosphorylation at Ser89 is directly involved. Thirdly, tryptic peptide analysis of single and double mutants suggested that phosphorylation of Ser89 seemed to be required for the phosphorylation of Ser96 and perhaps additional sites on the T2 peptide. Thus the increase in gel mobility of E1A proteins may be the due to phosphorylation at multiple sites, and these phosphorylation events may all be dependent upon phosphorylation at Ser89. Whether phosphorylation at Ser89 creates part of the recognition sequence for the protein kinase that phosphorylates Ser96 or induces structural changes in the E1A proteins such that Ser96, and perhaps other sites, become accessible to protein kinases is not clear.

The analysis of tryptic peptides on 2D-TLC is complicated by the fact that multiple species for the T2 peptide were consistently detected. Multiple species representing a single peptide have been described in the case of a tryptic peptide prepared from $p130^{gag\text{-}fps}$ proteins (Weinmaster *et al.*, 1984). The authors of this study proposed several possibilities to account for this phenomenon, and apart from partial proteolysis of the protein they suggested that circularization of N-terminal glutamine resulted in the production of the different isoforms of the peptide. The E1A T2 peptide, however, does not contain N-terminal glutamine, and thus this possibility may be excluded. Multiple

species can be attributed either to incomplete digestion by trypsin which would produce a combination of T2, T1-T2, T2-T3, or T1-T2-T3 peptides which would all contain phosphate. The short T1 peptide may not provide a good site for cleavage at the N-terminus as it represents only two amino acids at the N-terminus. In addition, a proline residue is located just two amino acids from the T2-T3 cleavage site which may reduce the efficiency of cleavage. In addition to such problems of trypsinization, it is also possible that another type of post-translational modification of the T2 peptide exists which could also alter the mobility of the T2 peptide. It is also possible that T2 is phosphorylated at other sites that have thus far escaped detection. These sites would also contribute to the pattern of multiple species. Phosphorylation of Ser89 could regulate phosphorylation at other sites by causing a change in the subcellular localization of E1A proteins, thus permitting exposure to a different array of kinases. In fact, it has been shown in frog oocytes that the generation of the slower migrating E1A forms (*i.e.* phosphorylation of Ser89) is required for the retention of E1A proteins in the nucleus (Richter *et al.*, 1985). These data may suggest that upon transport into the nucleus, E1A proteins are phosphorylated at Ser89 by $p34^{cdc1s}$, thus increasing nuclear retention and susceptibility to phosphorylation by other nuclear protein kinases. However, this hypothesis is hard to reconcile with the following: i) as determined by indirect immunofluorence, E1A proteins from *wt* and mutant Ad5 viruses were found in similar distributions within the nucleus ; ii) only a fraction of E1A proteins are phosphorylated on Ser89; iii) a precursor-product relationship exists between the faster and slower migrating species which differ by phosphorylation at Ser89 (Branton and Rowe, 1985).

In summary it appears that phosphorylation at Ser89 is independent of phosphorylation at the carboxyl terminus and perhaps at the newly identified Ser132 site (S.Whalen, unpublished data), but that it somehow regulates phosphorylation at Ser96 and other sites in T2.

Another group using deletion mutants yielding E1A products lacking regions containing the Ser89 and Ser96 sites and the site(s) between Ser227 and Ser237, reported that phosphorylation in both regions could independently induce a mobility shift (Richter *et al.*, 1988). In these studies and others (S. Whalen, unpublished data), phosphorylation toward the carboxyl terminus has been proposed to have some effect on gel mobility, but not to be sufficient to induce the major shift in gel mobility. The experiments in the present report differed from those of Richter *et al.* (1988) in that while the latter used E1A proteins produced *in vitro* either in a coupled transcription-translation system or after microinjection of E1A mRNA into frog oocytes, the present studies used proteins produced *in vivo* in cells lytically infected with Ad5. In addition, E1A products containing deletions are known to have aberrant, and to some extent unpredictable, gel mobilities (Egan *et al.*, 1988), and it may therefore be inappropriate to compare the effects of phosphorylation of such molecules with those induced in full-length proteins. This discrepancy may also be explained by recent findings which indicated that in cells containing high levels of cAMP, such as HeLa and KB cells, E1A proteins appear to be hypo-phosphorylated in the carboxyl terminal sites, whereas in cells with low cAMP levels (e.g. S49 cells) these sites are hyper-phosphorylated (Kleinberger *et al.*, 1990; S. Whalen and P.E. Branton, unpublished results). Therefore cAMP appears to block

phosphorylation of these carboxyl terminal sites, and/or to induce their dephosphorylation. Thus decreased levels of cAMP in frog oocytes or in *vitro* translation mixtures could lead to a hyper-phosphorylation of the carboxyl terminal region, producing what would seem to be an alteration in gel mobility equivalent to the alteration in gel mobility seen from infected HeLa or KB cells.

The present results could suggest that phosphorylation at Ser89 induces conformational changes in the E1A proteins such that electrophoretic mobility is altered. There are many precedents for such an effect, including RNA polymerase II (Bartholomew *et al.*, 1986; Cadena and Dahmuus, 1987), the anti-oncogene product from the Rb-1 gene, $p105^{Rb}$, (Buchkovich *et al.*, 1989; DeCaprio *et al.*, 1989; Chen *et al.*, 1989), $p55^{c\text{-}fos}$ (Barber and Verma, 1987), nucleoplasmin (Cotten *et al.*, 1986) and many others. In some of these cases phosphorylation is thought to be either a functionally activating or inactivating event. The results presented in this report suggested that phosphorylation of Ser89 may be an activating event. Replacement of Ser89 with Asp was found to partially mimic the presence of a phospho-serine residue in that some shift in gel mobility was observed. However, this substitution did not result in any phosphorylation of the T2 peptide, suggesting that phosphorylation of Ser89 and not simply the presence of a negative charge is required for phosphorylation at Ser96 (or other sites in T2). In addition, the presence of a Pro residue immediately following Ser89 is required for its phosphorylation, suggesting that Ser-Pro is an important part of the recognition sequence for the protein kinase that acts on Ser89.

Previous studies had suggested that Ser96 is phosphorylated, but it was not

possible to be certain from results obtained by phosphoamino acid sequencing (Tremblay *et al.*, 1988, 1989). Comparison of E1A phosphopeptide maps obtained using *wt* or the Ala96 mutant revealed the absence of two T2 species, indicating clearly that Ser96 is phosphorylated. Analysis of this mutant revealed that phosphorylation at Ser96 had little effect on gel mobility and biological function of E1A products. Thus the role of this phosphorylation event remains unknown.

E1A proteins produced from mutants containing Ala89, Ala89Ala96, or Ala90Ala96 were all less efficient at cooperating with either E1B or *Ha-ras* in cell transformation, suggesting that the hyperphosphorylated form of E1A proteins is the most active in this type of assay. This phenotype could be partially rescued by replacing Ala89 with Asp. At physiological pH an Asp residue would supply a single negative charge whereas two would be produced by a phosphate group. Thus two negative charges may be functionally more effective than one. The partial restoration of the shift in gel mobility in this mutant also supports this idea.

In contrast to these studies Smith *et al.*, (1989), have suggested that phosphorylation at Ser89 affects the ability of the E1a polypeptides to repress the SV-40 enhancer. The discrepancy between this observation and the results presented here may be due to the type of assay used. Whereas the present studies utilized infection of Hep-2 cells with recombinant viruses, which leads to a much greater efficiency of DNA uptake, Smith *et al.* (1989) utilized a HeLa cell-based transient transfection assay. All of their mutants with the exception of one (Ser96 to Gly), produced similar results suggesting that they were observing secondary effects due to either transfection differences, or squelching

of enhancer-factors away from the SV-40 enhancer. Another possible interpretation is that HeLa cells provide a cellular environment which makes E1A proteins more sensitive to perturbations in structure than do Hep-2 cells. The nature of these discrepancies can only be resolved after repeating the assays with each mutant under identical conditions.

The Ser89 mutant produced *wt* levels of transcriptional activation from E1B, E2a, and E3 promoters. These data agreed in principle with those of Richter *et al.*, (1988) and Smith *et al.*, (1989). However, considering recent results regarding the effects of E1A on E2F activation and complex formation (Chellappan *et al.*, 1991; Bagchi *et al.*, 1991; Chittenden *et al.*, 1991) these assays should probably be carried out again using a system that measures the activation and/or complex formation of the transcription factor E2F more specifically.

The 10S and 11S E1A mRNAS are produced later during productive infections. Their function during infection is not known, however, they are dispensable both for growth in tissue culture and for cell transformation (Ulfendahl *et al.* 1987; Stephens and Harlow, 1987). The studies in this report demonstrated that even though the 11S product contains all of the putative transactivating domain it is only partially functional. In fact it was unable to stimulate viral growth to any significant extent. These results were surprising considering the levels of E1A, E1B, and E2a products detected after infection with the 10S11S virus. These data were in complete agreement with those of Ulfendahl *et al.* (1987) and Stephens and Harlow (1987), suggesting that perhaps there is a threshold of transcriptional activation that must be attained during infection and that the 11S product can not attain this level early enough during infection. A more attractive

hypothesis is that regions outside the transactivating domain are required to interact with certain components of the transcriptional machinery and that these regions are deleted in the 11S product. Thus this E1A product can only interact with a limited number of cellular factors, resulting in poor growth of the virus. In fact, experiments which utilized chimeric E1A molecules suggested that E1A activates transcription through the association with cellular transcription factors (Lillie and Green, 1989; Martin *et al.*, 1990). It now seems clear that E1A activates transcription through at least two mechanisms, one which involves the unique CR3 region (which is present in the 11S product), but also via a second which involves E2F and the CR2 region of E1A proteins which is absent in the 11S product. The inability of the 10S11S virus to grow well on HeLa cells demonstrates that there must be another region required for viral transactivation that is indispensable for viral growth which is deleted in the 10S and 11S products.

It now seems apparent that substrates of $p34^{cdc2Hs}$ include products of both viral (McVey *et al.*, 1989; Morgan *et al.*, 1989; Shenoy *et al.*, 1989) and cellular (Hill *et al.*, 1990) oncogenes. Phosphorylation by $p34^{cdc2Hs}$ appears to significantly affect DNA binding activity of the large T antigen of simian virus 40 (McVey *et al.*, 1989) and to have a small effect on protein tyrosine kinase activity of $pp60^{c\text{-}src}$ (Morgan *et al.*, 1989; Shenoy *et al.*, 1989). With Ad5 E1A proteins, $p34^{cdc2Hs}$ appeared to phosphorylate two sites which both resembled substrate consensus sequences (*Fig.* **3-24A**). The functional significance of phosphorylation at these sites is unknown. Although it is the major E1A phosphorylation site, alteration of Ser219 has thus far not been found to affect any aspect of viral replication or E1A-mediated transformation (Tsukamoto *et al.*, 1988; Smith *et al.*,

1989). As described above, however, phosphorylation of Ser89 by $p34^{cdc2Hs}$ does seem to have some regulatory role.

In conclusion, these studies demonstrated that phosphorylation of Ser89 induces a major shift in mobility of E1A products on SDS-PAGE, due presumably to conformational changes resulting from this event. This phosphorylation seems to increase the ability of E1A products to cooperate with either Ad5 E1B or activated *c-Ha-ras* in transforming primary rat cells. Transforming activity was reduced two to three-fold in mutants containing alanine residues in place of serine-89 and even though this reduction in activity was modest, it was nonetheless entirely reproducible and statistically significant.

It appears that all of the phosphorylation sites on E1A proteins have been identified. It remains to be seen if modifications of multiple sites yield a more dramatic phenotype than that observed for the Ser89 site.

II. SPECULATION ON THE ROLE OF E1A PHOSPHORYLATION.

At the conclusion of these studies the question still remains, "Why does complex differential phosphorylation of E1A proteins occur if it plays no major role in regulating biological function?". The answer may lie in the assays used to evaluate E1A function which at best, are very crude. As we learn more about transcriptional activation the assays used to evaluate E1A function and the role of phosphorylation will be further refined.

E1A proteins are phosphorylated at residues found in regions which are thought to be dispensable for transcriptional transactivation, cell transformation, and enhancer repression (Tremblay *et al.*, 1988, 1989). However, it now appears that other regions of the molecule may be important for transactivation (reviewed in Braithwaite *et al.*, 1991). Also, in this study transactivation assays were performed in either KB or HeLa cells, the same cells used to analyze E1A phosphorylation. Thus the pattern of E1A phosphorylation relative to the biological assay system was known. In contrast, transformation assays were done in rodent cells, thus begging the question, "Are E1A proteins phosphorylated to the same extent in rodent cells?". The phosphorylation pattern of E1A polypeptides can vary in different cell types (S. Whalen and P.E. Branton, unpublished results), suggesting that the choice of cell for the assay could influence the outcome. Until a study is completed to determine the phosphorylation pattern in rodent cells, the role of phosphorylation in E1A-mediated transformation will remain uncertain.

E1A transforming activity appears to result largely from the formation of complexes with a number of cellular proteins, including p105/*Rb-1*, the product of the *Rb-1* tumor suppressor gene (Whyte *et al.*, 1988; Egan *et al.*, 1989). Previous studies indicated that unphosphorylated E1A products synthesized in *E. coli* bind some of these cellular proteins less efficiently than those made *in vivo* in infected cells (Egan *et al.*, 1987). Thus phosphorylation at Ser89 could affect transformation by altering binding efficiency. In addition, one of these cellular polypeptides is p60-cyclin A (Pines and Hunter, 1990). Thus E1A proteins not only serve as substrates for $p34^{cdc2Hs}$ but also interact with one of its regulatory subunits. Furthermore, it has been proposed that

p105/*Rb-1* acts in the cell cycle to regulate entry into S-phase and that phosphorylation, probably by a member of the $p34^{cdc2}$ family may control this activity (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989). By acting as substrates for $p34^{cdc2Hs}$, E1A proteins could be involved in the creation of functional complexes which alter or regulate $p34^{cdc2Hs}$ kinase activity, or in the enhancement of interactions between $p34^{cdc2Hs}$ and regulatory subunits or substrates. Preliminary studies with the Ala89 mutant indicated a small decrease in the binding of p105/*Rb-1*, p107 or p300 (D. Barbeau, unpublished results). However, more extensive quantitative studies will have to be carried out to determine if phosphorylation at Ser89 does indeed have any affect on the formation of these complexes.

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