Shuber

MUTAGENESIS OF THE SECOND EXON OF THE AD5 E1A GENE

EFFECTS OF SITE DIRECTED MUTAGENESIS OF THE SECOND EXON OF THE ADENOVIRUS 5 E1A GENE ON TRANSCRIPTIONAL ACTIVATION

by

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ABSTRACT

The early region 1a oncogene of adenovirus 5 codes for proteins that can activate transcription of viral and cellular genes. This study describes the construction of three deletions and one point mutation that together span the entire coding region of the second exon of E1A. The exon-2 mutants were tested for their ability to activate transcription from the adenovirus early region 3 promoter (E3) in transient expression assays. Dl1116 (dl aa 205-221) did not affect transactivation of E3 in pKCAT-23. Sub1117 (dl exon-2 aa) and dl1115 (dl aa 188-204) were unable to activate transcription. Pm1131 (SER-219 to stop) had a reduced transactivating efficiency but was still able to stimulate transcription. These results define the 3' boundary of a transactivation domain on the E1A proteins as being between positions 188 and 204. Results obtained in our lab define the 5' boundary as being between 138-147 (Jelsma et al., 1988).

The mutants that could not transactivate were tested for their ability to block wildtype E1A transactivation of the E3 promoter in assays similar to those described by Glenn and Ricciardi (1987). Dl1115 and sub1117 appeared to block transactivation by WT E1A.

In transient expression assays, the fatty acid sodium butyrate was found to stimulate transcription of the CAT gene, when added to the medium of HeLa cells transfected with pKCAT-23. This suggests that sodium butyrate is transactivating the Ad 5 E3 promoter.

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LIST OF ABREVIATIONS

A	adenine
aa	amino acid
AC:CAM	acetylated chloramphenicol
Ad	adenovirus
ATP	adenine triphosphate
bp	base pair
C	cytosine
CAM	chloramphenicol
CAT	chloramphenicol acetyl transferase
ds	double stranded
dl	deletion mutant
G	guanine
mM	millimolar
NaB	sodium butyrate
NTP	nucleotide triphosphate
OD	optical density
pm	point mutant
R	residue
RF	recombinant form
rel	relative
rpm	revolutions per minute
ss	single stranded
T	thymine
ug	microgram
ul	microliter
WT	wild type

Part 1: INTRODUCTION

I. INTRODUCTION

(A). Discovery of adenoviruses

Adenoviruses were first isolated from human adenoidal tissue and identified as infectious virions in 1953 (Rowe et al.). These viruses were shown to cause the degeneration of cultured human adenoidal cells and of the cervical carcinoma cell line, HeLa. Furthermore, adenoviruses were shown to commonly cause upper respiratory tract infections in humans (Hilleman et al., 1954).

In 1962 it was shown that human adenovirus type 12 (Ad 12) is highly tumorigenic when injected intraperitonealy into newborn hamsters. This was the first human virus shown to have oncogenic properties (Trentin et al., 1962). Subsequent experimentation has shown that only a few of the many human adenovirus serotypes are highly tumorigenic and then only when injected into rodents. Adenoviruses have not been shown to be causative agents in human carcinogenesis.

(B). Classification

Since their initial discovery, numerous adenovirus serotypes have been isolated from mammalian and avian tissues. The genera of avian and mammalian adenoviruses belong to the family Adenoviridae and include all adenovirus serotypes isolated to date, including 41 human isolates. The human adenoviruses are divided into seven subgroups (A-G) and are further classified according to hemaglutination patterns

produced with rodent and simian red blood cells, DNA base composition (%[G+C]), and oncogenic potential.

Adenoviruses are used extensively in research as models to elucidate cellular mechanisms for the control of gene expression and the processes involved in the oncogenic transformation of normal cells. All human adenoviruses can transform cultured primary rodent cells. Tumorigenicity upon injection of virus directly into rodents varies from the highly oncogenic type A viruses (Ad 12, 18, 31) to the non-oncogenic viruses, including type C Ad 2 and Ad 5. Adenovirus type 2 and Ad 5 are highly homologous. Much of the research with adenoviruses has made use of these serotypes and it is assumed that properties ascribed to one apply to the other. Unless otherwise noted, references herein describe human adenovirus serotypes 2 and 5.

(C). Structure

Adenoviruses are non-enveloped, icosahedral, double stranded DNA viruses. The linear genome of approximately 36 kb contains inverted terminal repeats of about 100 base pairs and is capable of forming covalently closed circles (Ruben et al.,1983). The formation of these circles has been proposed as a mechanism of adenovirus replication and integration (Graham, 1984). The 5' ends of the genome have a 55K terminal polypeptide (TP) covalently attached to a terminal dCTP, by a phosphoserine linkage. This TP is involved in the priming of DNA replication. Furthermore, viral DNA with the TP attached is 1000 times more infectious than DNA stripped of this protein (Jones et al., 1978).

(D). Infectious cycle

The infectious cycle of adenoviruses lasts approximately 36 hours, beginning with attachment of the virion to the cell membrane and movement of the DNA into the nucleus. Transcription in the nucleus begins immediately and occurs in two phases. In the early phase the early regions (E1A, E1B, E2A, E2B, E3, and E4) which encode mostly non-structural polypeptides, and some of the major late promoter driven genes are transcribed. The late phase includes viral DNA synthesis and transcription of the late genes, encoding mostly structural or assembly associated proteins (Reviewed by Horwitz, 1985).

Viral RNA's are processed in the nucleus and translated in the cytoplasm. Indeed, the splicing and processing of mRNA was discovered in adenoviruses (Chow et al., 1977; Berget et al., 1977). Proteins synthesized in the cytoplasm move into the nucleus where assembly occurs. The linear adenovirus DNA forms a core histone-like complex with viral polypeptides and is enclosed in a regular icosahedral capsid composed of virally encoded hexon and penton polypeptide subunits. Glucosamine modified polypeptides project from the surface of the capsid. It is these polypeptide fibers that interact with the red blood cells used in the hemaglutination assay. Thousands of virions accumulate in the nucleus and are released lytically at the end of the cycle (Reviewed by Horwitz, 1985).

II. THE ADENOVIRUS EARLY REGIONS

(A). The E1 Region

Hybridization mapping of adenovirus early and late mRNA's has revealed that all the early regions and the major late promoter region are transcribed from separate promoters (Pettersonet al., 1976; Evans et al., 1977; Berk and Sharp, 1977). The transformation function of adenoviruses has been mapped to the left end of the viral genome, the E1 region (Figure 1). DNA sequencing and mRNA mapping has revealed that the E1 region can be subdivided into two independently promoted regions E1A and E1B that code for multiple mRNA species and translation products (Petterson et al., 1976; Berk and Sharp, 1977; Perricaudet et al., 1979).

(B). The E1A Region

The E1A region (1.3-4.5 map units) spans approximately 1700 bp at the left end of the viral genome (Hearing and Shenk, 1980; Perricaudet et al .,1979). The E1A gene gives rise to one primary transcript initiated at nucleotide 560, and variably spliced into two major (13S and 12S) and three minor (9S, 10S, 11S) mRNA's (Perricaudet et al., 1979; Stevens and Harlow, 1987; Ulfendahl et al., 1987). The two major mRNA's vary only in the size of the intron spliced out of the pre-mRNA (Figure 2). The 10S and 11S mRNA's have only recently been discovered, and the function of their protein products is unknown. These less abundant mRNA's are similar to the 12S and 13S mRNA's except for

Figure 1. The adenovirus genome and transcription units (from Broker, 1984)

Figure shows Ad-2 RNA transcripts and corresponding proteins.

THE ADENOVIRUS-2 CYTOPLASMIC RNA TRANSCRIPTS

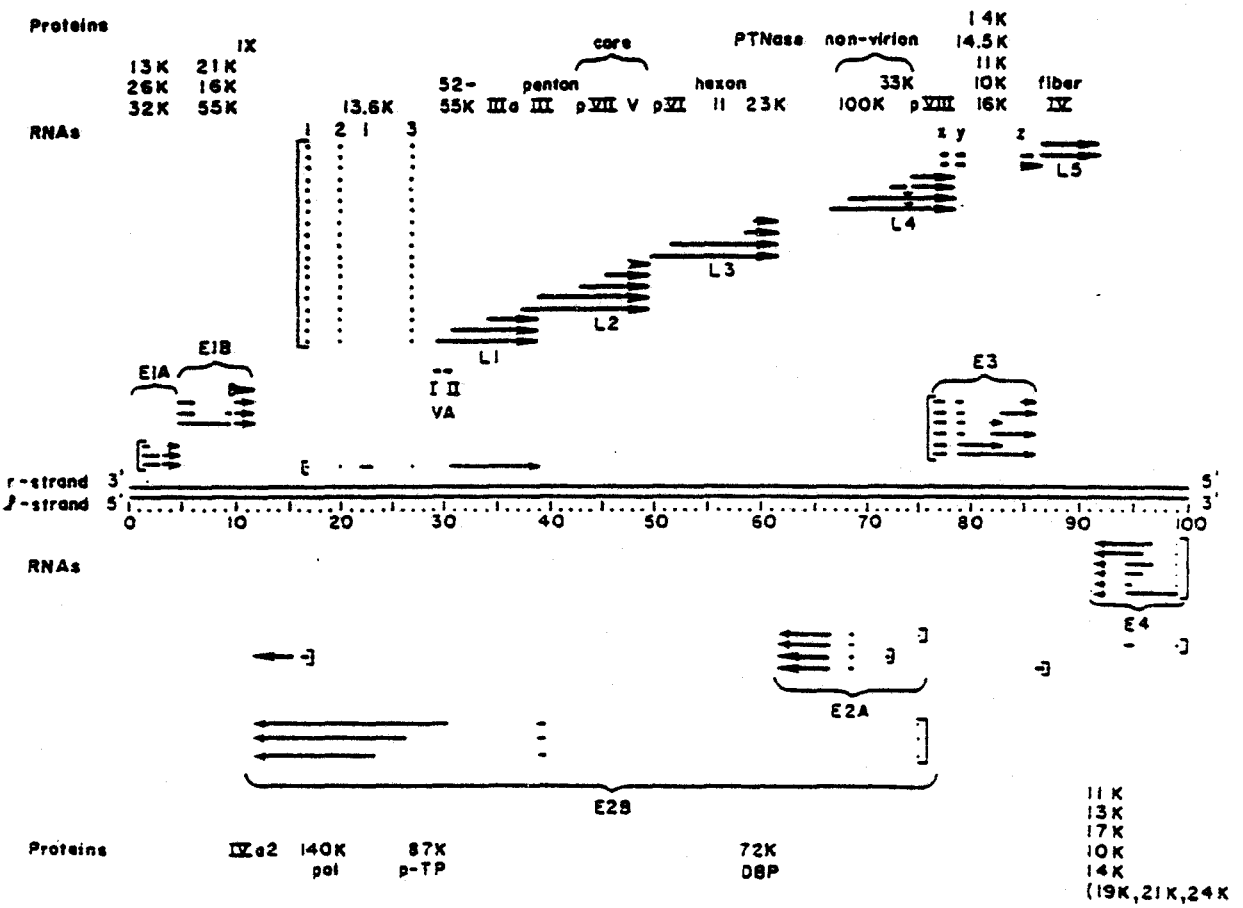
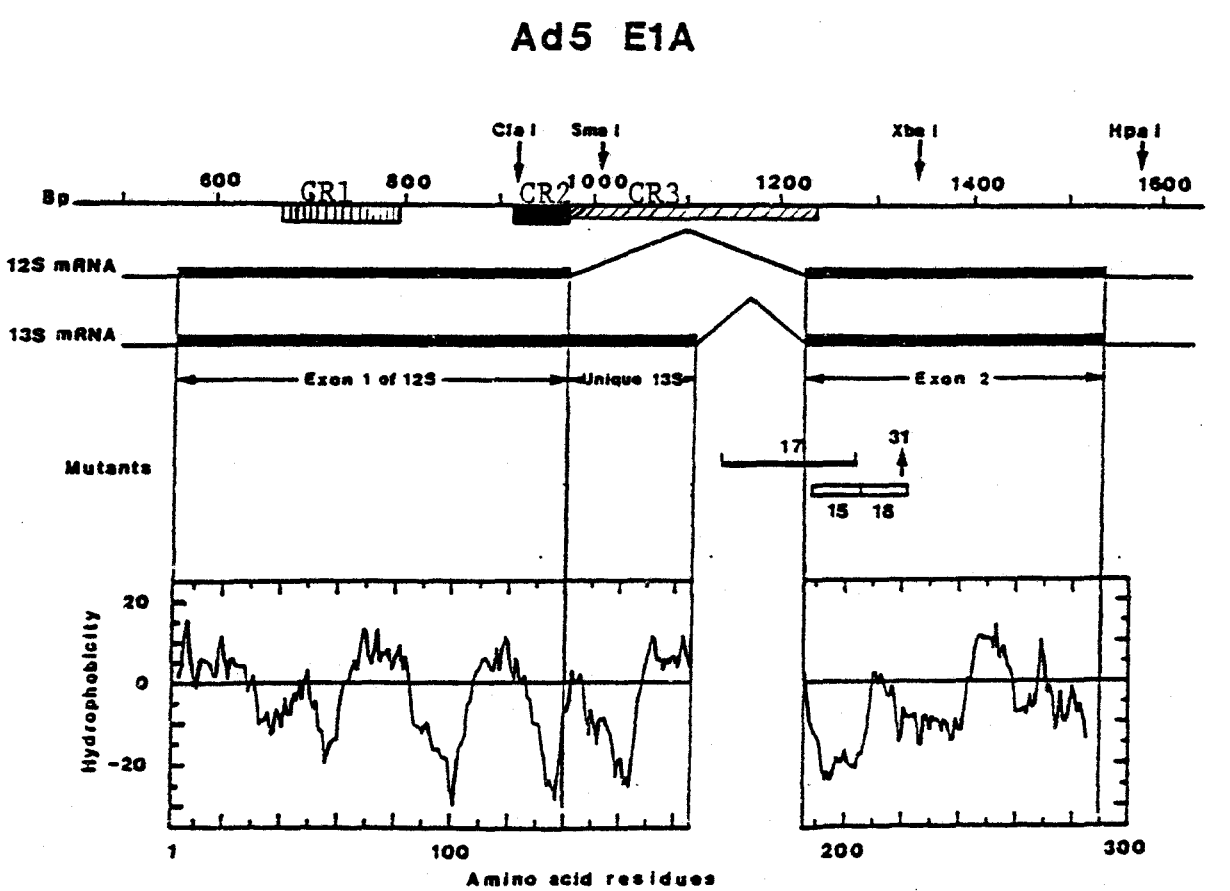


Figure 2. The Ad5 E1A region

(modified from Jelsma et al., 1988)

The Ad5 E1A coding region with 12S and 13S mRNA transcripts. Conserved regions (CR's) and mutations in exon-2 are indicated.

The hydropathy curve of the 289R polypeptide is shown.


Ad5 E1A
Cla I
Sma I
Xba I
Hpa I
Bp
600
800
1000
1200
1400
1600
CR1
CR2
CR3
12S mRNA
13S mRNA
Exon 1 of 12S
Unique 13S
Exon 2
Mutants
17
31
15
18
Hydrophobicity
20
0
-20
1
100
200
300
Amino acid residues

216 nucleotides removed by an additional splicing event. The 9S mRNA is made late in infection and the function, if any, of its protein product(s) is not known.

The 243R and 289R nuclear localized phosphoproteins encoded by the 12S and 13S mRNA's respectively share a common sequence with the exception of 46 amino acids unique to the 289R protein (Downey et al., 1984; Halbert and Raskas,1982; Smart et al.,1981; Perricaudet et al., 1979). Tryptic and chymotryptic peptide analysis of in vitro and in vivo translation products of the E1A 13S and 12S mRNA's revealed the presence of multiple products. Smart et al., (1981) identified 50K, 46K and 42K 13S mRNA products produced in Ad 2 infected HeLa cells, and 58K and 48K 13S mRNA products in cell free translation experiments. The 12S mRNA produced 46K, 42K and 38K polypeptides in vivo, and 54K and 42K proteins in vitro.

Similar in vitro experiments (Halbert and Raskas, 1982) revealed 53K, 47K, 41K and 35K E1A early translation products and a 28K late translation product. Up to eight variants of E1A proteins have been detected by immunoprecipitation and two dimensional gel electrophoresis; two major (52K, 48.5K) and two minor (37.5K, 29K) 289R variants , as well as two major (50K, 45K) and two minor (35K, 25K) 243R variants (Branton et al., 1985; Graham et al., 1984). The protein variants are probably due to the extent of phosphorylation of various serine residues. The major site of phosphorylation is serine-219 encoded by the second exon and common to all variants (Tsukamoto et al., 1986).

(C). The E1B Region

The E1B region (4.6-11.2 m.u.) begins immediately downstream of the E1A polyadenylation site and contains its own promoter (Berk and Sharp, 1977). E1B gives rise to up to five mRNA's including 22S, 13S, 14S and 9S messages. The 9S message is unspliced and codes for the structural protein IX (15K). The major mRNA's, 22S and 13S, are processed from a common transcript. The 22S message directs the synthesis of two polypeptides in two overlapping reading frames (Bos et al., 1981). The 19-21K and 53-58K tumor antigens are synthesized by initiation at alternative AUG triplets (Bos et al., 1981). The 13S mRNA encodes the same 19-21K protein as the 22S message as well as an 8.3K polypeptide. The two 14S mRNA's code for a 10K and a 17K polypeptide respectively (reviewed by Branton et al., 1985).

(D). The other early regions

The E2A region (67.9-61.5 m.u.) codes for a 72K single stranded DNA binding protein (DBP) essential for adenovirus replication and for transcriptional regulation of E4.

The E2B region (27.8-14.2 m.u.) codes for a 140K adenovirus DNA polymerase, and for the 80K precursor of the 55K terminal polypeptide.

The E3 region (76.6-86.3 m.u.) codes for several spliced mRNA's and for several proteins including a 19K glycoprotein that appears on the host cell surface. Adenovirus mutants lacking the E3 gene can

replicate at wild type levels in HeLa cells. Therefore, E3 is not essential for growth of virus in cell culture.

E4 (96.8-91.3 m.u.) codes several spliced mRNA species encoding several proteins, including an 11K nuclear matrix binding protein. Adenovirus mutants lacking a functional 11K polypeptide replicate at wild type levels on HeLa cells. Therefore, this protein does not appear to be essential for lytic infection in tissue culture (reviewed by Horwitz, 1985).

III. TRANSFORMATION

A. Adenoviruses transform mammalian cells

Adenoviruses are easily cultured in growth permissive human cells such as 293 cells -a human embryonal kidney cell line transformed by and expressing the E1 region (Graham et al., 1974; Graham et al., 1977; Aiello et al., 1979), KB cells -a human epidermoid carcinoma line, and HeLa cells, where they are able to complete the infectious cycle. Rodent cells such as baby rat kidney cells (BRK) and cloned rat embryo fibroblasts (CREF) are non-permissive for lytic infection with virus or when transfected with naked DNA. In non- permissive cells the viral DNA frequently integrates and may cause oncogenic transformation.

Fully transformed cells have certain distinguishing characteristics. They have reduced serum requirements and are sensitive to high concentrations of calcium ions. Transformed cells form foci of

rapidly growing cells of altered morphology. These cells exhibit anchorage independent growth and cause tumors when injected into rodents (Graham et al., 1984).

(B). Transformation function maps to E1 region

Soon after the discovery of the oncogenic properties of Ad12, it was shown that tumors induced and cells transformed by this serotype produce virus specific tumor antigens (Huebner et al., 1963). Furthermore, these antigens are specific for the transforming serotype, as they do not cross react with antibodies to other related serotypes (Huebner et al., 1964).

It was hypothesized that cells induced by Ad12 to a neoplastic state must contain viral DNA integrated within the host genome (Huebner et al., 1964). Fujinaga and Green (1966) showed that pulse labeled mRNA from Ad12 induced tumors and transformed cell lines contained a substantial proportion of virus specific mRNA's. Later, it was shown that Ad 2 is capable of transforming primary rat embryo cells and that these cells produce virus specific tumor antigens and mRNA's (Freeman et al., 1967). The use of restriction enzymes made possible the confirmation that cell lines transformed by Ad 2 contain stably integrated viral DNA, from the left 14 percent of the viral genome (Sharp et al., 1974; Gallimore et al., 1974; Flint, 1976). It was also shown that mRNA from Ad 2 transformed cells maps to the left 14 percent of the viral chromosome (Flint, 1975). Graham et al. (1975) showed that

primary rat cells could be transformed by a DNA fragment containing only the E1 region.

(C). E1 tumor antigens

Using antibodies several groups have shown that E1A, E1B, and sometimes E2A (72K DBP), and E4 (10.5K) polypeptides are expressed in transformed cells (Downey et al., 1983; Lassam et al., 1978; 1979; Levinson and Levine, 1977; Gilead et al., 1975), indicating that sometimes DNA sequences other than E1A and E1B integrate into the adenovirus genome. Rowe et al. (1984) used sera specific to the C-terminus of the E1A polypeptides to show that 12S and 13S mRNA products 52, 50, 48.5, and 45K can be detected at nearly equal levels in transformed hamster cell lines. C-terminal sera to E1B proteins were also used to detect 58K, 19K and a related 17K polypeptide in transformed cell lines. Similar E1A and E1B proteins have been identified in Ad 12 transformed cells (Jochemsen et al., 1982). E2, E3 and E4 polypeptides have been detected in adenovirus transformed cell lines. Although the proteins produced by these early regions are not necessary for transformation, they may have a role in the transformation phenotype (Graham et al., 1984).

The isolation of viral tumor antigens through the use of antigen specific antisera has made possible the assignment of certain transformation functions to specific E1 polypeptides. The E1A proteins are sufficient for immortalization of cells but require E1B proteins for a fully transformed phenotype. The cellular oncogene ras can substitute

for the E1B function(s) in E1A DNA mediated transformation assays of BRK cells. The human cellular oncogene Ha -ras can be used in place of the E1B gene to fully transform rodent cells (Ruley et al., 1983; Lillie et al., 1986; 1987; Schneider et al., 1987). E1A and ras plasmid mediated transformation of rodent cells has been used extensively for assaying the oncogenic properties of E1A mutants (Ruley, 1983; Lillie et al., 1986; 1987; Schneider et al., 1987). E1A-ras transformed cells do not have the same characteristics as E1A-E1B transformed cells (reviewed by Branton et al., 1985). It appears that transformation by E1A-E1B and E1A-ras occurs by a similar but not identical mechanism (Graham et al., 1984).

(D). Host-range mutants

E1 mutants defective for lytic infection of HeLa cells and for transformation of rodent cells have been isolated and assigned to one of two complementation groups, Host range I and II, (Harrison et al., 1977; Graham et al., 1978). These mutants cannot be propagated at wild type levels on normal host cells such as HeLa or KB. These and other E1 mutants can grow at wild type levels on 293 cells (Harrison et al., 1977; Ross et al., 1980). Group I host range mutants hr1, 2, 3, 4, 5 map to the unique region of the 289R E1A proteins. Group I transformed cells are defective for stimulation of DNA synthesis, express defective E1A antigens but normal E1B 58K and 19K proteins. Group I mutants can partially transform BRK cells at an efficiency greater than that of wild type (Graham et al., 1978; Ruben et al., 1982). These partly

transformed cells are not tumorigenic and do not exhibit many of the other characteristics of fully transformed cells (Rowe et al, 1984).

Group II host range mutants map to the E1B region and affect the 58K protein specified by the 22S mRNA. In virus form Group II mutants cannot transform cells and do not produce normal 58K protein. They do stimulate DNA synthesis and produce the 19K polypeptide. Purified Group II DNA, however, can transform cells at wild type levels and these cells are tumorigenic (Rowe and Graham, 1983; Rowe et al., 1984). Therefore, the 58K polypeptide is not required for DNA mediated transformation (Rowe and Graham, 1983; Rowe et al., 1984) . Suprisingly, hr 50 -a group II mutant- does not synthesize 58K or 19K polypeptides, yet cells transfected with this mutant are fully transformed and can induce tumors in rodents (Rowe and Graham 1983). Certain Ad 2 and Ad 12 cyt mutants, which cause DNA degradation, are also defective for the 19K protein and can also induce tumors (Mak et al., 1984).

(E). Cold sensitive mutants

Cold sensitive (cs) host range group I mutants have provided more conclusive evidence that the products encoded by E1A are needed not only for the initiation of transformation, but also for the maintenance of the transformed phenotype (Ho et al., 1982; Babiss et al., 1983; Rowe et al., 1984). Hr1 deletes one nucleotide at 1055 (Figure 3A). The resulting reading frame shift causes a stop codon at nucleotide position 1144 (Ricciardi et al., 1981). Transformation of cloned rat

Figure 3.

(A). E1A coding sequence showing corresponding amino acid sequence.

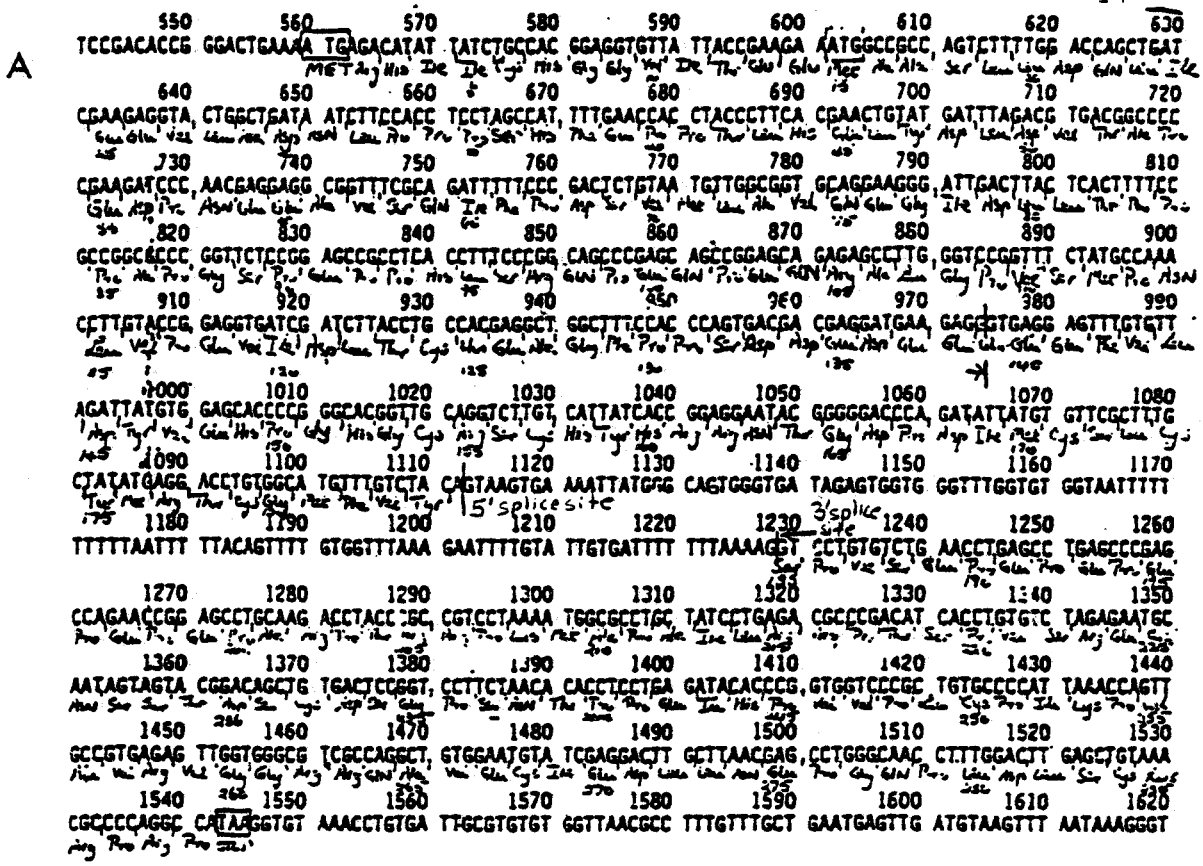
(modified from Ginsberg et al., 1984)

(B). Comparison of the E1A protein sequences of Ad5, Ad7, Ad12 and simian adenovirus type 7 (SA7).

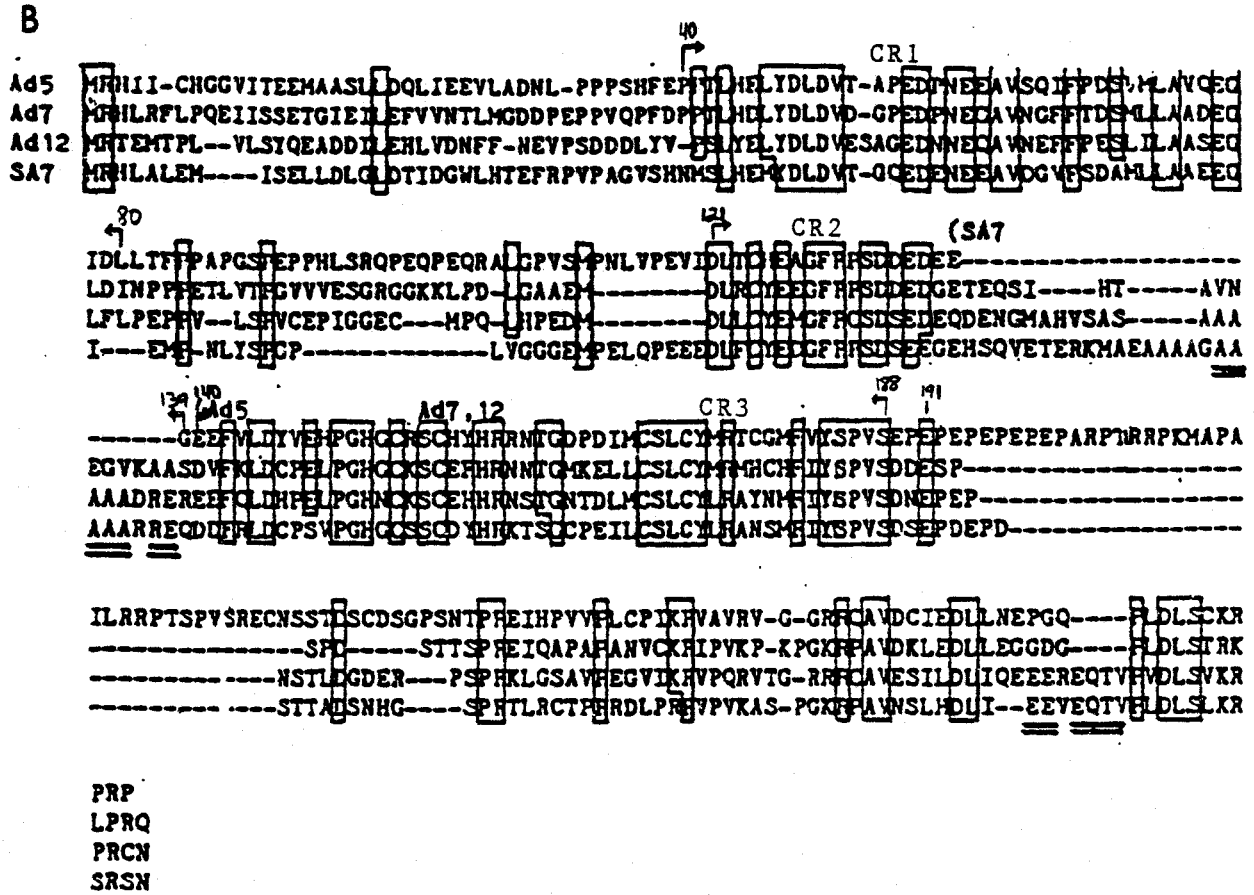
(modified from Kimmelman et al., 1985)

Boxed regions delineate regions of homology. Underlined residues show regions of homology between Ad12 and SA7. CR's of Ad5 indicated.

A



B



embryo fibroblasts (CREF) with hr1 occurs at efficiencies five fold higher than wild type, when cells are incubated at 37 C. When cells are transfected and incubated at 35.5 or 32.5, the cytopathic effect normally observed within five days and the formation of foci of transformed cells does not occur. However, if the cells are then shifted up to 37 C, the cytopathic effect occurs and surviving cells form foci of partially transformed cells. Amazingly, these cells can be shifted back and forth in temperature indefinitely and they will lose or regain the partly transformed phenotype (Babiss et al., 1983). These results indicate that an active E1A product is necessary for the maintenance of the transformed state.

Montel et al. (1984) have shown that mutant E1A viruses encoding only the 243R protein (dl1500) or only the 289R protein (pm975) can partially transform CREF cells. Both proteins, however, are needed for a fully transformed phenotype. The 243R proteins appear to be necessary for anchorage independent growth of transformed cells and for the propagation of virus in growth arrested human cells. Montel et al. (1984) postulated that the 243R function(s) which stimulates growth of virus in growth arrested cells may also function in the oncogenesis of rodent cells.

IV. E1A TRANSCRIPTIONAL ACTIVATION

(A). E1A activates transcription in trans

A gene product affected by the group I mutants is responsible for the transcription of E1B, E2, E3, and E4 regions (Berk et al., 1979). Jones and Shenk (1979) used dl312 , a deletion mutant lacking most of the E1A coding region, to also demonstrate that a product of the E1A gene is responsible for the transcriptional activation of the early genes. Furthermore, this defect could be provided in trans by 293 cells which produce the deleted E1A products.

Nevins et al., (1979) studied the time course of expression of mRNA from the early regions in Ad 2 infected HeLa cells. E1A was the first gene transcribed. E1A mRNA was first detected at 45 minutes post infection (p.i.), reaching a maximal level at three hours and was maintained thereafter for up to nine hours. Regions E3 and E4 were first transcribed at about one hour p.i., reached a maximal level at three hours p.i. and then declined. Region E1B began transcription at one hour p.i., reached a maximal rate at seven hours p.i. that was maintained for another nine hours. Region E2 was the last early region transcribed; transcription began at two hours p.i., reached a maximal rate at seven hours p.i. and then declined. It was noted that the decline of E2 and E4 mRNA transcription appeared to be a result of repression of these transcription units. This repression was inhibited when protein synthesis was inhibited. Similarly, the appearance of early gene products at different times has been reported (Rowe et al., 1984). It

was postulated that since the various early regions were transcribed at different times there must be different mechanisms of action of E1A transactivation (Nevins et al., 1979).

Mutations in the untranslated TATA region of the E1A gene reduce the level of E1A mRNA 5-10 fold. A similar decrease of E2 and E3 mRNA's is also detected. There is only a slight decrease in E4 and E1B transcription is not affected (Osborne et al., 1982). These results also indicate that the early genes are not dependent on E1A mediated transactivation to the same degree.

(B). 289R protein is the transactivator

Ricciardi et al. (1981) sequenced hr1 and showed that this mutation produces a truncated protein affecting only the 13S mRNA products. Since the 12S mRNA product is not affected and since hr1 is unable to transactivate the other early regions the transactivation function must be unique to the 13S mRNA product. Montel et al., (1984) created pm975, a point mutant expressing only the 13S mRNA. Pm975 prevents the formation of 12S mRNA but does not alter the 13S mRNA. This mutant was able to fully activate the transcription of the early adenovirus genes.

(C).Transcription in absence of E1A

Nevins (1981) showed that in the absence of an E1A product transcription from the other early genes does occur, but at less than eight percent of wild type levels. Nevins also showed that cells treated

with an inhibitor of protein synthesis and infected with dl312 had increased transcription from some of the early genes, notably E4. It was postulated that the inhibition of protein synthesis mimics the E1A function. Such a function could be to inactivate a cellular factor that inhibits transcription of the early genes, thereby allowing transcription to proceed.

Using similar protein inhibition experiments, Katze et al., (1981) showed that cells treated with protein synthesis inhibitors and infected with hr1 had abundant E3 and E4 mRNA production. It was also shown that the rates of transcription from all the early regions were equivalent. These workers concluded that E1A is responsible for accumulation of early mRNA's but not for transcription, and that inhibition of protein synthesis mimics the E1A function. However, it should be noted that since all the mRNA's were produced at the same rate and not differentially as in uninhibited wild type infection, it can be assumed that accumulation of mRNA due to protein synthesis inhibition probably proceeds by a different mechanism from E1A mediated transactivation.

Gaynor and Berk (1983) were unable to reproduce the "activation" of early genes by protein sythesis inhibition, as reported by Nevins (1981). They showed that the mechanism by which the early genes are transcribed in the absence of an E1A protein differs from the transactivating mechanism of E1A. Dl312 infected HeLa cells transcribe E3 mRNA late in infection due to a cis-acting mechanism. They proposed

that this mechanism may be a slow assembly of a transcriptional complex that is catalyzed in the presence of E1A protein.

Recently, Hearing and Shenk (1986) have shown that one of the three E1A enhancers is able to augment transcription of all the early regions on the adenovirus genome. These results may explain why dl312 and other mutants with intact non-coding regions have a basal level of early mRNA transcription. It would be of interest to see if a dl312 mutant with an additional deletion in the enhancer region can still produce early mRNA's late in infection, or in response to protein synthesis inhibition.

(D).Other E1A activities

Other adenovirus functions, possibly related to the mechanism of transformation and transactivation have been mapped to the E1A gene. E1A stimulates DNA synthesis in serum starved cells (Lillie et al., 1987), stimulates proliferation of cells by induction of growth factors (Quinlan et al., 1987), and induces undifferentiated F9 teratocarcinoma cells to undergo differentiation (Montano and Lane, 1987; Thangue and Rigby, 1987). The E1A product(s) are also antagonists to the anti-viral activity of interferon (Anderson and Fennie, 1987). Adenovirus infection has been shown to stimulate production of topoisomerase I in HeLa cells. It has been proposed that this induction may be due to E1A activation (Chow et al., 1985).

E1A has been shown to activate the transcription of endogenous cellular genes such as the human 70K heat shock protein (HSP) gene (Nevins, 1982; Kao and Nevins, 1983; Wu et al., 1986), the β-tubulin gene (Stein and Ziff, 1984), the dihydrofolate reductase gene (Yod et al.,1983), and transiently introduced cellular genes such as the rabbit and human β-globin genes (Green et al., 1983; Svensson and Akusjarvi,1984; Treisman et al., 1983).

E1A represses transcription from enhancers such as the SV40 (Velcich and Ziff, 1985), polyoma virus (Velcich et al., 1986) and the Ad 5 E2A enhancers (Zajchowski et al., 1985). The mouse class I MHC genes and human histone genes are also repressed, but it is not clear if this is due to suppression at the level of transcription, or to a suppression of DNA synthesis.

(E). **E1A acts indirectly on different promoter sequences**

The promoters of the early genes do not have any significant homology. The E2A gene has two promoters that do not contain the TATA boxes common to most promoter sequences including the other early gene promoters. The E2A promoters have different sequences but both respond to E1A-mediated transactivation (Zajchowski et al., 1985). Furthermore, E1A does not bind to any of the early gene promoters, or to any specific DNA sequences (Feldman et al., 1982). It therefore seems very unlikely that E1A transactivates genes by binding directly to upstream sequences.

Imperiale et al., (1983) showed that E1A products are able to activate the transcription of the E2 gene from the E2 promoter in a transient expression assay, or when an E2 plasmid was transfected into 293 cells. The pseudorabies immediate early gene product was also able to transactivate the E2 gene and at a higher efficiency. In fact, it has been demonstrated that the E1A transactivation deficiency in dl312 can be complemented by the herpesvirus immediate early gene product (Feldman et al., 1982). These results indicate that the mechanism of action of E1A may be a generalized trans-activation of genes that are available for transcription, and not a gene specific E1A induction.

(F). E1A and cellular factors are involved in transactivation

Glenn and Ricciardi (1987) reported that the transactivation defective point mutant hr5 can block the transactivation of adenovirus early promoters by wild type E1A. HeLa cells were cotransfected with salmon sperm DNA, a plasmid bearing an E3 promoter driven CAT gene, a wild type E1A plasmid, and increasing amounts of an hr5 plasmid. It was shown that acetylation of chloramphenicol was repressed by 50 percent with equimolar amounts of hr5 and wild type E1A plasmid. It was proposed that E1A interacts with a cellular protein(s) to activate promoters. Hr5 can still interact with this factor but can not transactivate.

Recent evidence also indicates that E1A acts with a cellular factor(s) to transactivate genes, and this or other factors can be similarly affected by other viral proteins, such as the HSV immediate

early protein. Reichel et al., (1987) identified an E1A inducible factor (E2F) present in HeLa cells (Kovesdi et al., 1986) and undifferentiated F9 cells that is involved with E2 promoter binding and activation. This factor or its affinity for the promoter is greatly increased upon infection by adenovirus. The activation or deactivation of a cellular protein has been proposed as a mechanism by which E1A induces differentiation in F9 cells (Montano and Lane, 1987). Since E1A does not bind to promoters, it seems very likely that cellular factors have a role in transactivation and probably in many of the other fuctions of E1A.

V. CONSERVED REGIONS

(A). Functional domains of E1A

Comparison of the amino acid sequences of human adenovirus serotypes 5, 7, 12 and simian adenovirus type 7 (SA7) has shown considerable conservation in three regions of the 289R protein, as well as a possible C-terminal rapid nuclear localization signal (Kimmelman et al, 1985),(see Figure 3B). Mutagenesis of the E1A gene has suggested that certain E1A functions may be closely associated with these regions. However, the limited number of in frame mutations in these regions has not made it possible to precisely define the boundaries of the various domains.

Conserved regions 1(aa 40-80) and 2 (aa 121-139) are common to the 243R and 289R proteins. Conserved region 3 (aa 140-188) is unique

to the 289R polypeptides (Moran and Mathews, 1987)(Figure 2). Mutations in CR1 and CR2 have been shown to affect the transformation function of E1A (Lillie et al.,1987; Schneider et al., 1987) as well as the ability of E1A to repress transcription from viral enhancers (Velcich et al., 1986). In fact, these two E1A functions are so tightly coupled that repression of cellular genes has been proposed as the mechanism by which E1A transforms cells (Lillie et al., 1986; 1987). Velcich et al. (1986) found that E1A represses transcription from the polyoma virus early and late promoter-enhancers. When the enhancer region was deleted, E1A was able to transactivate the early promoter but not the late promoter. Velcich et al. proposed that E1A affects transcriptional activation and repression by affecting the promoter or enhancer, respectively.

Lillie et al. (1986) proposed that E1A transforms rodent cells in cooperation with ras through a repression function. According to this model the 12S mRNA products appear to function as repressors and the 13S products function as transcriptional activators. In that study, mutants that affected E1A transformation also affected E1A repression of the SV40 enhancer.

Mutations in CR3 greatly reduce or completely eliminate the transactivation function of E1A (Jones and Shenk, 1979; Krippl et al., 1985; Lillie et al., 1986; Schneider et al., 1987). Therefore, the residues of the 289R polypeptide are alone responsible for the transactivation function of E1A. So far, mutations in the non-conserved regions have not been shown to affect repression, transformation or transactivation.

(B). E1A rapid nuclear localization signal

Mutant E1A proteins produced in E. coli and missing the C-terminal 67 amino acids, are slow to localize to the cell nucleus when microinjected into the cytoplasm of Vero cells (Krippl et al., 1985). Krippl et al. proposed that a rapid nuclear localization signal must be located at the C-terminus of E1A. The Ad 5 C-terminal residues (aa 279-289: PLDLSCKRPRP) share some homology with other adenovirus serotypes. The putative rapid nuclear localization signal (KRPR) shares homology with the nuclear localization signal of SV40 (KKKRK) and is identical to the polyomavirus internal nuclear localization signal (Krippl et al., 1985 and references therein). However, E1A mutants lacking only the terminal sequence have not been tested in a localization assay, so that the role of such a sequence has not been directly confirmed.

VI. Project aims and strategy

It has been demonstrated that the Ad5 E1A 13S mRNA product is responsible for the transcriptional activating function of E1A (Ricciardi et al., 1981; Montel et al., 1984). The unique region of the 289R protein appears to contain the transactivating domain, as point mutations in this region have been shown to eliminate this function (Ricciardi et al., 1981; Lillie et al.,1986). This region shares considerable homology with other human and simian adenovirus serotypes (Kimmelman et al., 1985; Figure 3B). The exon-1 encoded region common to both the 12S and 13S mRNA products may be needed for induction of cell proliferation, repression and establishment of cells in culture (Lillie et al.,1987; Quinlan et al., 1987).

Because of the large number of functions attributed to the E1A proteins, it would be valuable to determine the precise locations of domains responsible for the various activities, the ultimate aim being to gain an understanding of the processes involved in E1A mediated oncogenic transformation. Many of the mutants previously used to study E1A have been constructed by introducing random deletions or by removing restriction endonuclease fragments. This often created missence residues and prematurely terminated polypeptides. To help define the domains responsible for the various E1A functions, our lab has constructed a series of small in frame deletions and point mutations. Using the site directed mutagenesis technique of Zoller and Smith (1985), it has been

possible to make precise mutations that together span the entire coding region of the Ad5 E1A gene.

Much of the research on E1A has focused on the exon-1 encoded 12S mRNA region and on the region unique to the 13S mRNA. Because the residues encoded by the second exon are common to both the 243R and 289R proteins, and because the few mutation affecting only exon-2 have not been reported to significantly affect E1A functions, the role of the second exon encoded sequences has been overlooked.

To further define the role of exon-2 in transcriptional activation and transformation, three deletions and a point mutant encompassing the entire coding region of exon-2 have been created. Results presented here and partly in Jelsma et al. (1988) show that mutations in the 3' exon of Ad 5 E1A affect E1A-mediated transactivation but not transformation. Mutants made in this study can be used to further define E1A functions.

PART 2. MATERIALS AND METHODS

I. MATERIALS

A. Bacterial strains and DNA vectors

1. bacterial strains

LE-392(Pharmacia) -a dam+ *E. coli* strain

JM103 (Pharmacia) -a dam+.thi, lac pro, sup E, spcB, hsdR-,traD36, proAB, Z M13, *E. coli* strain

2. vectors

pHE-1 (a gift of F.L. Graham) contains the HpaI fragment of Ad5 (0-4%) and was derived from pXC1, a pBR322 vector carrying the Xho I C fragment of Ad5 (0-16%), inserted between the Sal I and Bam HI sites of pBR322. A Bam HI site at the left end non-coding region of the Ad5 sequence was created by Dr. Graham for this cloning procedure (McKinnon et al. 1982).

pLE-2 ,missing the pBR322 ClaI site, was derived from pCD2 (gift of F.L. Graham). pCD2 was originally derived from pXC1 and is missing the Ad5 SacI to SacI fragment (1770-5644).

pKCAT-23 (gift of M.B. Mathews) contains a chloramphenicol acetyl transferase (CAT) gene driven by the Ad5 E3 promoter, originally constructed by Weeks and Jones, 1983.

M13mp11c1#5 an M13 phage vector containing the Bam HI to Xba I fragment from pHE-1 (Ad5 0-3.8%), inserted into the β-galactodidase gene, was used for all dideoxynucleotide sequencing and oligomer-directed mutagenesis reactions. M13mp11c1#5 was constructed by subcloning the Bam HI to Xba I fragment from the E1A plasmid pHE-1 into RF M13mp11 (gift of C. Evelegh).

B. Biochemicals and solutions purchased from suppliers

[14C] chloramphenicol (NEN)

[γ-32P] ATP (NEN)

[35S] ATP (NEN)

acetyl coenzyme A lithium salt (Pharmacia)

BIS (Bio-Rad)

N,N'- Methylene-bis-acrylamide

EDTA (BDH)

Ethylenediaminetetra-

acetic acid disodium salt

IPTG (BRL)

Isopropylthio-β-D-Galactoside

minimal essential medium (Gibco)

with L-glutamine

with ribonucleosides

with deoxyribonucleosides

with added sodium bicarbonate

new born calf serum (Gibco)

Pen-Strep 100X (Gibco)

10,000 u/ml penicillin (base)

10,000 mcg/ml streptomycin (base)

React-2 buffer 10X (BRL)

500mM Tris-HCl (pH 8.0)

100mM $MgCl_2$

500mM NaCl

React-3 buffer 10X (BRL)

500mM Tris-HCl (pH 8.0)

100mM $MgCl_2$

1000mM NaCl

React-4 buffer 10X (BRL)

200mM Tris-HCl (pH 7.4)

50mM $MgCl_2$

500mM KCl

sodium butyrate (Sigma)

TEMED (Bio-Rad)

N,N,N',N' -Tetramethylethylenediamine

Tris (Boehringer)

Tris (hydroxymethyl) aminomethan

Trypsin-EDTA 10X (Gibco)

X-gal (BRL)

5-Bromo-4-chloro-3indolyl-

D-galactoside

Xma I buffer 10X (Pharmacia)

250mM Tris-HCl (pH 7.8)

250mM KCl

100mM magnesium chloride

1000ug/ml bovine serum albumin

20mM β-mercaptoethanol

C. Solutions

alkaline SDS

0.2M NaOH

1% SDS

10X annealing buffer

0.2M Tris-HCL (pH 7.5)

0.1M $MgCl_2$

0.5M NaCl

0.01M DTT

chase solution (Pharmacia)

2mM dATP

2mM dCTP

2mM dGTP

2mM dTTP

10mM Tris-HCl (pH 7.5)

chloroform (24:1)

chlorofrom 24 parts

isoamyl alcohol 1 part

competence A solution

10mM NaCl

50mM $MnCl_2$

10mM sodium acetate (pH 5.6)

competence B solution

75mM CaCl

100mM $MnCl_2$

10mM sodium acetate (pH 5.6)

denaturing polyacrylamide gel

7 M urea

6% Bis-acrylamide (49:1)

0.08% ammonium persulfate

5 mM TEMED

Denhardt's solution 50X (500ml v/v water)

5g Ficoll

5g polyvinylpyrrolidone

5g bovine serum albumin

2.5mM dNTP's (Pharmacia)

2.5mM dATP

2.5mM dCTP

2.5mM dGTP

2.5mM dTTP

elution buffer

0.5 M ammonium sulfate

0.001 M EDTA

enzyme/dNTP mix

1ul solution B

4ul 2.5mM dNTP's

1ul 10mM ATP

1ul T4 DNA ligase (7u)

1ul DNA polymerase (Klenow) (7.5u)

2ul water

Ficol dye 10X

60% sucrose

0.02% (w/v) bromophenol blue

0.02% (w/v) xylene cyanol

0.025M EDTA

Hepes buffer 2X

16ug/ml NaCl

0.76ug/ml KCl

0.20ug/ml di-sodium hydrogen ortophosphate

10ug/ml Hepes

2ug/ml glucose

pH = 7.1 with NaOH

hybridization solution

6X SSC

10X Denhardt's solution

kinase buffer 10X

700mM Tris (pH 7.6)

100mM $MgCl_2$

50mM DTT

LB broth (1 L)

5g yeast extract

5g NaCl

10g tryptone

1g glucose

LB ampicillin agar plates

50ug/ml ampicillin

15g bacto-agar

low Tris buffer

10mM Tris-HCl (pH 7.5)

10mM NaCl

0.5mM EDTA

lysozyme solution (Sigma)

5mg/ml chicken egg white muramidase

in 50mM Tris (pH 7.5), 10mM EDTA

M9 supplemented agar plates

15 grams Bacto-agar per liter M9 medium

PBS

phosphate buffered saline

8.0g/l	NaCl
0.2g/l	KCl
1.15g/l	sodium hypophosphate
0.2g/l	di-potassium hydrogen orthophosphate

phenol:chloroform (1:1)

1 part phenol saturated in TE (pH 7.6)

1 part chloroform:isoamyl alcohol (24:1)

prehybridization solution

6X	SSC
10X	Denhardt's solution
1%	SDS

pL-1 buffer 10X

100mM Tris (pH 7.5)

100mM $MgCl_2$

50mM DTT

RF TE buffer

0.25M Tris (pH 8.0)

0.25M EDTA

20X SSC

3M NaCl

0.3M sodium citrate

0.01M EDTA

pH 7.2 with HCl

sonication buffer

0.25M Tris (pH 7.8)

0.05M EDTA

stop solution (Pharmacia)

0.3% (w/v) xylene cyanole

0.3% (w/v) bromophenol blue

in deionized formamide

sucrose buffer

25% sucrose

50mM Tris (pH 8.0)

10mM EDTA

TBE 1X

0.089 M Tris-HCl

0.089 M boric acid

0.002 M EDTA

TE

50mM Tris

10mM EDTA

pH adjusted with HCl

to 7.5

YT broth (1 L)

8g tryptone

5g yeast extract

5g NaCl

YT agar plates (1 L)

15g Bacto-agar

20ml per 100mm plate

METHODS

I. CONSTRUCTION OF dl AND pm MUTANTS

A. PREPARATION OF ssM13mp11 VECTOR DNA

1. Growth and maintenance of JM 103

Overnight cultures of JM103 were grown in sterile YT broth from single colonies at 37 C, with shaking, for 12 hours. Working stocks of JM103 were grown by inoculating 20 ml sterile YT broth with 100ul of an overnight JM103 culture, and incubating at 37C with shaking until the required optical density was reached. Cells to be made competent for the uptake of DNA were grown until OD550 reached 0.30. Cells used for making a lawn of JM103 (plating culture) were grown until OD550 reached 1.0. Optical densities were determined with a Spectronic 20 Spectrophotometer.

2. Growth of M13 phage and purification of single stranded DNA

An M13 phage vector containing the BamH I to XbaI fragment of pHE-1 was used for all dideoxynucleotide sequencing and oligomer directed mutagenesis reactions. Single stranded M13mp11 cl#5 DNA template was purified from M13 phage grown in E. coli strain JM103.

* underlined materials are included in Materials section.

JM103 stock was stored in 15% glycerol at -80 C, and was regularly streaked onto M9 supplemented agar plates and stored at 4 C for up to five weeks.

M13 phage were grown by inoculating 40 ml sterile YT broth with 500 ul JM103 at OD550=0.3 and 20 ul of lab stock M13mp11 cl#5 phage. The culture was grown at 37 C with shaking for seven hours. The bacteria were sedimented in an SS-34 rotor at 10,000 rpm for 10 min at 4 C. The supernatant containing the phage was transferred to new tubes and one ml was stored as stock at -20 C. Eight ml 2.5M NaCl/20% PEG 9000 were added to the remaining supernatant, vortexed and placed on ice for 15 min. The phage were sedimented by centrifugation at 10,000 rpm for 10 min at 4 C.

The phage pellet was resuspended in 750 ul TE (pH 7.5). ssDNA was extracted twice with phenol:chloroform, twice with chloroform and three times with ether. The aqueous phase was transferred to a new tube and the DNA was precipitated using the standard ethanol precipitation procedure.

(3). Standard ethanol precipitation procedure

DNA was precipitated by addition of two volumes 95 percent ethanol and one tenth volume 1M NaCl with immersion in liquid nitrogen for 30 sec. The DNA was sedimented by centrifugation for 10 min at 4 C in a Beckman Microfuge. The DNA pellet was resuspended in 100 ul water, ethanol precipitated and sedimented again. DNA was washed sequentially once with 70 percent ethanol and once with 95 percent ethanol, dried and resuspended in 50 to 100 ul water. The DNA was quantified by taking an

[γ-32P] end labeling of oligomer

1ul purified oligomer (0.5 pmol/ul)

5ul 10X kinase buffer

1ul T4 kinase (10u/ul)

1ul [32P] ATP (10 uCi/ul

42ul water

50ul total

ultraviolet light absorption scan from 210nm to 310nm in a Beckman DU7 Spectrophotometer. The OD260 was determined from the scan. One OD260 unit is the equivalent of 40ug ssDNA, 50ug dsDNA, or 20ug oligomer DNA per milliliter.

B. Preparation of oligonucleotides for sequencing and site directed mutagenesis

1. Synthesis and purification of oligomers

Four oligomers were prepared by the McMaster Institute for Molecular Biology and Biotechnology. The icosanucleotide SAM 95 was synthesized on a Biosearch SAM DNA synthesizer. The other icosanucleotide AB-53 and the two icosatrianucleotides AB-11 and AB-28 were prepared on an Applied Biosystems Model 380B DNA synthesizer. All the oligomers were synthesized using the phosphoramidite method, and purified by absorption to an SI polyanion column. Desalting was done on a Sephadex G-15 column. To visualize the purity of the oligomers, 0.5 pmol of the purified oligomers were 5' end labeled with [γ-32P] ATP and electrophoresed on a vertical 20 percent polyacrylamide gel with 1X TBE running buffer. The gel was autoradiographed with Kodak XK1 slow film for approximately 30 min.

2. Repurification of AB-11

Electrophoresis and autoradiography of [γ-32P] ATP labeled AB-11 revealed an unacceptable level of lower molecular weight DNA contamination. AB-11 was repurified by polyacrylamide gel

5' phosphorylation of oligomer

30ul AB-11 (50 pmol/ul)

10ul 10X kinase buffer

5ul 10mM ATP

5ul T4 kinase (10 u/ul)

50ul water

100ul total

electrophoresis. The oligomer was 5' phosphorylated with ATP and T4 polynucleotide kinase prior to electrophoresis, because certain compounds used in the purification procedure could inhibit subsequent phosphorylation. To visualize the icosatrianucleotide band, 0.5 pmol 5'[γ32P] ATP labeled AB-11 were added to the sample and the mixture electrophoresed on a vertical 20 percent polyacrylamide gel, with 1X TBE running buffer for 2 hr at 150v/20mamp, using an Eltech Designs VC300 power unit.

The slowest migrating band was cut out of the gel and chopped into very fine pieces. The gel pieces were incubated in 2 ml elution buffer at room temperature with shaking overnight. The eluate was recovered by centrifugation at 12,000 rpm for ten minutes at 4 C in an SS-34 rotor. The supernatant was transfered to a new tube and the gel pieces were back extracted with one ml elution buffer. The eluate was recovered by centrifugation. The combined eluates were filtered through siliconized glass wool to remove any remaining polyacrylamide. The DNA was recovered by ethanol precipitation and resuspended in 20 ul water.

C. Sanger chain termination dideoxynucleotide sequencing

1. Sequencing reaction

Pre-mixed sequencing reagents and protocol were purchased from Pharmacia. The oligomer primer was annealed to the template DNA and extended with the Klenow fragment of DNA polymerase I in a mixture of deoxy- and dideoxynucleotides, including [35S] ATP (NEN), which was used to label the chain terminated fragments. The [35S] ATP was chased

Annealing reaction

for sequencing

1ul pL-buffer

1ul oligomer DNA (0.5 pmol/ul)

1-2ul ss DNA template (0.7-1.5ug/ul)

7ul water

10ul total

incubated at 60 C for 5 min

cooled slowly to room temperature for 40 min

added 2.5ul 35 S ATP (0.1 uCi/ul) and 0.5ul DNA polymerase (Klenow) (7.5 u/ul)

aliquoted 2.5ul reaction mix to each x'/ddx and incubated at 50 C for 30 min

added 2ul chase solution to each reaction and incubated at 50 C for 30 min

added 3ul stop solution to each reaction and incubated at 100 C for 3 min and placed on ice

with 2 ul chase solution for thirty minutes, and the reaction was stopped with 3 ul stop solution. The DNA was denatured in a boiling water bath for three minutes and placed immediately on ice.

2. Electrophoresis

Three ul of each deoxy- and dideoxynucleotide reaction were loaded onto a vertical six percent denaturing polyacrylamide gel, and electrophoresed on an LKB Broma Electrophoresis Unit. Electrophoresis was at 3000v/120mamp for 75 minutes at 55C, for each loading. The gel plate was soaked in fresh 10% acetic acid for 30 min and baked at 80 C for two hours. The gel was autoradiographed with Kodak XK1 slow film for 24-48 hours.

D. Oligomer-directed mutagenesis

1. Preparation of mutagenic oligomer

(a). 5' phosphorylation of oligomer for mutagenesis reaction

All oligomers used for site-directed mutagenesis were first used as primers for sequencing reactions on ssM13mp11 c1#5 DNA template, to test that they bound specifically to one region and gave a clear sequence.

One hundred pmol oligomer was 5'phosphorylated with T4 polynucleotide kinase and ATP in 1X kinase buffer for 45 min at 37 C.

5' end labeling of oligomer

2ul oligomer (50 pmol/ul)

2ul 10X kinase buffer

5ul [32P] ATP (10 uCi/ul)

1ul T4 kinase (10 u/ul)

10ul water

20ul total

restriction enzyme digestion of RF DNA

24ul RF M13mp11 (0.1 ug/ul)

4ul React-2 buffer

1ul Bam HI (10 u/ul)

1ul Xba I (10 u/ul)

10ul water

40ul total

The kinase was heat inactivated at 65 C for 10 min, and the phosphorylated oligomer was stored at -20 C until used.

(b). 5' [γ-32P] ATP phosphorylation of oligomer for filter hybridization

One hundred pmol oligomer was 5' end labeled with [32P] ATP and T4 polynucleotide kinase for 45 min at 37 C. The kinase was heat inactivated at 65 C for 10 min and the labeled oligomer was stored at -20 C until used.

2. Preparation of RF M13mp11 (second primer)

RF M13mp11 was purchased from Pharmacia. One pmol was digested with Bam HI and Xba I for 2 hrs at 37 C. The DNA was extracted once with an equal volume phenol:chloroform and twice with chloroform. The organic phases were back extracted with water. Ten ug wheat germ tRNA (10ug/ul) were added as carrier and the DNA was precipitated as described previously. The pellet was air dried and resuspended in 3 ul water. The RF DNA was denatured in a boiling water bath for 10 min, placed on ice and used immediately.

3. Annealing reaction

The denatured RF M13mp11 primer and the mutagenic oligomer were annealed to the ss M13mp11cl#5 DNA template by incubating in 1X annealing buffer at 55 C for 5 min. The reaction was allowed to cool slowly at room temperature for 30 min.

Annealing reaction

3ul ss M13mp11 cl#5 (1 ug/ul)

1ul 10X annealing buffer

2ul 5' phosphorylated oligomer

3ul restricted RF primer

1ul water

10ul total

incubated at 55 C for 5 min and cooled slowly to room temperature for 40 min

Extension/ligation reaction

added 10 ul enzyme/dNTP mix to annealing reaction, and incubated at 15 C for 16 hours

4. Extension/ligation reaction

The annealed DNA was incubated with the Klenow fragment of DNA polymerase I, T4 ligase and dNTP's at 15 C overnight.

5. Transformation of JM103

(a). preparation of competent JM103 cells

JM103 cells were made competent to take in DNA by treatment with calcium chloride. One ml overnight culture of JM103 was added to 50 ml sterile YT broth and incubated at 37 C with shaking until OD550 = 0.3. Cells were harvested by centrifugation in an SS-34 rotor at 10,000 rpm for 5 min at 4 C. The supernatant was poured off and the cells resuspended in 10 ml cold competence A solution. The cells were incubated on ice for 30 min. The bacteria were harvested as described above and resuspended in 1 ml cold competence B solution. JM103 cells treated in this manner were stored on ice for no more than 24 hrs before being used.

(b). transformation reaction

Three ul of the mutagenesis reaction, mixed with 100 ul competent JM103 cells, were placed on ice for 30 min. The cells were heat shocked at 37 C for 15 min to take in the DNA.

(c). plaque formation

A series of 10 ml glass test tubes each containing 3 ml molten YT plating agar, 20 ul 100mM IPTG, 100ul 2% X-Gal and 100ul JM103 cells

(OD 550= 1.0) were prepared. Aliquots of 30, 20, 10, 5, and 1 ul transformed cells were added to each test tube respectively, mixed and poured onto prewarmed YT plates. Thirty ul non-transformed JM103 cells were plated as a control. The top agar was allowed to harden at room temperature and the plates were incubated upside down at 37 C overnight.

E. Screening for mutagenized M13mp11c1#5

Mutants were screened in a three step procedure:

1. Filter hybridization

(a). dot blot (Zoller and Smith, 1984)

Deletion mutants 1115, 1116, and point mutant 1131 were screened by dot blot hybridization in separate experiments. Thirty-six to seventy-two 10ml glass test tubes, each containing 2 ml YT broth and 100 ul JM103 (OD 550 =1.0), were prepared and inoculated with well isolated clear plaques picked from a lawn of JM103. The transfected cultures were incubated at 37 C with shaking for seven hours. One and one-half ml culture was transfered to sterile 1.5 ml Eppendorf tubes and centrifuged for 3 min in a Beckman Microfuge at room temperature. One ml supernatant was transfered to a new 1.5 ml Eppendorf tube, and the bacterial pellet with remaining supernatant was stored at -20 C. Two hundred ul 2.5M NaCl/20% PEG 9000 were added to each tube containing one ml supernatant, vortexed and placed on ice for 15 min. The phage were sedimented by centrifugation in a Beckman Microfuge for 10 min at 4 C. The supernatant was aspirated and the phage resuspended in 10 ul water. Two ul of

resuspended phage were spotted onto a nitrocellulose filter, allowed to air dry and baked at 80 C for 2 hrs. One or more spots of 1 ul purified ss M13mp11 cl#5 DNA (1-2 ug/ul) were used as negative controls.

(b). prehybridization and hybridization

The filter was prepared for hybridization by prehybridizing with 10 ml prehybridization solution in a sealed hybridization bag for one hour at 67 C with shaking. The prehybridization solution was removed and the filter was washed three times with 40 ml 6X SSC at room temperature. The nitrocellulose was hybridized in 50 ml hybridization solution with 20 ul 32P labeled mutagenic oligomer, at room temperature in a sealed bag with occasional shaking. The nitrocellulose was removed from the bag, and washed three times with 50 ml 6X SSC at room temperature, to remove background signal. Residual 6X SSC was removed by blotting the filter onto Whatman 3mm Chromatagraphy paper for 1 min. The filter was then placed on a 7 X 10 inch piece of 3mm paper, covered with a sheet of Saran Wrap and autoradiographed with Kodak XAR5 fast film. After a room temperature wash, the controls and almost all phage spots gave a strong signal. Starting 6 C below the temperature of dissociation (T_D = 4[G+C] + 2[A+T] degrees celcius), the filter was washed at successively higher temperatures with 20 ml pre-warmed 6X SSC for three minutes with shaking, until the signal from the control spots could no longer be detected with a hand held Geiger-Muller counter. The filter was then autoradiographed overnight with Kodak XAR5 fast film, as described above
.

(c). plaque lift hybridization

Sub1117 was initially screened by plaque lift hybridization. Plaques on a lawn of JM103 were prepared as described previously. One sterile nitrocellulose filter was placed on the lawn for 1 min and its position marked with pencil. The filter was peeled off and the plate stored at 4 C. The nitrocellulose was washed in 10 ml 6X SSC to remove any adhering bacteria, and was baked at 80 C for 1 hr. Prehybridization and hybridization were done as described above. The filter was washed at room temperature three times in 50 ml 6X SSC to remove any background signal, and autoradiographed overnight. Wild type plaques served as negative controls. Washings at successively higher temperatures in 20 ml 6X SSC were done as described above. At T_D + 5 C most of the signal due to wild type plaques could no longer be detected with a hand held Geiger-Muller counter. The filter was autoradiographed with Kodak XAR5 fast film overnight. Seven positive plaques were picked from the master plate and used to transfect two ml cultures of JM103 as previously described. One and one-half milliliters of culture was harvested in a Beckman Microfuge. The bacterial pellet, and supernatant were stored separately at -20 C. Phage was prepared as described previously and dot blotted onto nitrocellulose. The phage were probed with [γ-32P] ATP labeled AB-53 DNA. Only one plaque was positive for the mutation after this screening.

2. Restriction enzyme digestion and PAGE screening

(a). small scale extraction of RF M13 DNA

RF M13 DNA was extracted using a modification of the method for large scale plasmid isolation of Birnboim and Doly (1979). Cultures were prepared as described in preceeding sections. The frozen bacterial pellets were thawed at room temperature and centrifuged in a Beckman Microfuge for one minute at room temperature. Any remaining supernatant was removed and the cells were resuspended in 100 ul lysozyme solution and placed on ice for 30 min. Two hundred ul freshly prepared alkaline SDS were added to the lysates, mixed and incubated for 10 min at room temperature. One hundred and fifty ul 3M sodium acetate (pH 4.8) were then added and mixed. High molecular weight DNA and cell debris were precipitated by incubating on ice for 1 hr, and sedimented by centrifugation in an Eppendorf Centrifuge for 10 min at room temperature. The supernatants were transferred to new 1.5 ml Eppendorf tubes, and the RF DNA was precipitated with 1 ml cold 95 percent ethanol on ice for 10 min. The DNA was sedimented in a Beckman Microfuge for 10 min at 4 C. The pellet was resuspended in 100 ul water and ethanol precipitated twice. The DNA pellet was resuspended in 30 ul water.

Restriction enzyme digestion

10ul DNA

3ul React-4 buffer

1ul Sma I (10 u/ul)

1ul Xba I (10 u/ul)

15ul water

30ul total

incubated at 30 C for 2 hours

added 3ul 10X ficoll dye

and ran on a 6% polyacrylamide gel

(b). Restriction enzyme digests and PAGE screening

Ten ul of purified RF DNA were digested with Sma I and Xba I for 2 hrs at 30 C. Three ul 10X Ficoll dye were added to each reaction and the sample was loaded onto a vertical 6 percent polyacrylamide gel and run in 1X TBE buffer at 150v/20mamp, using an Eltech Designs VC300 power unit, for two to 3 hrs. The gel was stained in 500ml 1X TBE with 100 ul ethydium bromide (5mg/ml) for 60 sec. The gel was washed three times with 200 ml cold water and a photograph was taken with Polaroid type 57 Instant Sheet Film using short wave ultraviolet light illumination.

3. Sanger dideoxynuclectide sequencing of the mutation

One half ml JM103 (OD 550 =1.0) was added to 500 ml sterile YT broth and the culture was transfected with the 50 ul phage supernatant from a plaque that was positive for the mutation by hybridization and PAGE screening. Incubation was at 37 C, with shaking, for 7 hrs. The bacteria were sedimented by centrifugation in a JA-10 rotor in a Beckman Model J-21C centrifuge for 5 min at 6000 rpm. Forty ml of the phage supernatant were saved and used to prepare ss phage DNA. The remaining supernatant was discarded and the bacterial pellet stored at -20 C.

Single stranded DNA was prepared from 40 ml of supernatant containing M13 phage as described previously. The mutant was initially sequenced though the mutated region, by using primers that bound upstream of the mutation and later sequenced from the Sma I through the Xba I restriction sites.

F. Rescue of the mutation into plasmid

1. Large scale purification of RF M13 DNA

After confirmation of the mutation by sequencing, RF DNA was prepared from the stored bacterial pellet. The bacteria were thawed at room temperature and resuspended in 2 ml sucrose buffer. The cells were incubated with 1 ml lysozyme solution on ice for 5 min. Two ml RF TE buffer were added and the lysate was incubated on ice with 250 ul RNase (10mg/ml) for 5 min. Two and one-half ml Triton-X 100 were added and the cells incubated on ice for 10 min. High molecular weight DNA and cellular debris were sedimented by centrifugation in an SS-34 rotor at 12,000 rpm for 20 min at 4 C. The supernatant was transferred to a 10 ml graduated cylinder, to which 8 g cesium chloride were added and the volume brought up to 10 ml with RF TE buffer. The sample was transferred to a 16 X 76 mm Beckman Quick-Seal centrifuge tube and 320 ul ethidium bromide (5mg/ml) were added. The tube was heat sealed and centrifuged at 40,000 rpm for 20 hrs at 14.9 C, in a Beckman L8-70 ultracentrifuge.

The lowest band containing the RF M13 DNA was collected with a syringe, and extracted three times with water saturated isoamyl alcohol to remove the ethidium bromide. The DNA solution was dialyzed against several changes of low-Tris buffer, and ethanol precipitated. The DNA was resuspended in 100 ul water and ethanol precipitated again. The pellet was washed sequentially once with 1 ml 70 percent ethanol and once with 1 ml 95 percent ethanol. The air dried pellet was resuspended in 100 ul water, and the DNA was quantitated as described previously.

The DNA was digested with Sma I and Xba I to check for proper restriction fragment patterns.

2. Large scale purification of plasmid DNA

(a). plasmid purification

Two methods were used in the purification of plasmid DNA.

(1). CsCl density centrifugation

(Birmboim and Doly, 1979)

To 500 ml sterile LB broth, 25 mg ampicillin and 10 ul stock LE-392 cells carrying pLE-2 or pHE-1 were added. Plasmid DNA was amplified by the addition of chloramphenicol (180ug/ml) after the OD600 had reached 0.6. The cells were then incubated at 37 C overnight with shaking. The bacteria were harvested by centrifugation in a JA-10 rotor at 6000 rpm for 5 min at 4 C. The supernatant was discarded and the pellet resuspended in 10 ml lysozyme solution. The cells were incubated on ice for 30 min. Twenty ml alkaline SDS were added to the lysate and incubated for 10 min on ice. Fifteen ml 3M sodium acetate (pH 4.8) were added and the sample was incubated on ice for 1 hr.

High molecular weight DNA and cellular debris were sedimented by centrifugation in an SS-34 rotor at 12,000 rpm for 15 min. The supernatant was transfered to a new tube and incubated with two volumes 95 percent ethanol on ice for 10 min. Plasmid DNA was recovered by centrifugation as described above and the pellet was dissolved in 9 ml

0.1X SSC. Eleven grams cesium chloride were added, and the solution transferred to 16 X 76 mm Beckman Quick-Seal centrifuge tubes. Three hundred and fifty ul ethidium bromide were added and the tube was heat sealed. The plasmid DNA was banded in a Beckman Ultracentrifuge at 40,000 rpm for 24 hours at 14.9 C. The plasmid band was removed with a syringe, and the ethidium bromide was extracted with water saturated isoamyl alcohol three times. The plasmid DNA solution was dialyzed against several changes of low Tris buffer, ethanol precipitated twice and washed sequentially with 1 ml 70 percent ethanol and 1 ml 95 percent ethanol. The pellet was dried under vacuum, and resuspended in 100 ul water. The DNA was digested with SmaI and XbaI or with Hae II to check for proper restriction fragment patterns.

(2). Lithium chloride procedure

Five hundred ml cultures of LE-392 cells carrying pHE-1 or pLE-2 were grown in LB with ampicillin. Plasmid DNA was amplified with chloramphenicol and the cells were harvested as described above. The bacterial pellet was resuspended in 10 ml lysozyme solution and incubated at room temperature for 30 min. Ten ml alkaline SDS were added to the lysate, mixed gently and placed on ice for 10 min. Seven and one-half ml 3M potassium acetate were added, vortexed and the lysate incubated on ice for 10 min. High molecular weight DNA and bacterial debris were sedimented by centrifugation in an SS-34 rotor at 12,000 rpm for at least 40 min at 4 C. The supernatant was transferred to a new tube and 16 ml isopropanol were added. RNA and plasmid DNA were precipitated by incubating at room temperature for 15 min. The

precipitate was sedimented by centrifugation in an SS-34 rotor at 12,000 rpm for 10 min at 21 C. The pellet was washed with 2 ml cold 95 percent ethanol, allowed to air dry, and resuspended in 2 ml TE buffer (10:1). RNA was precipitated by the addition of 2 ml 5M lithium chloride and incubation on ice for 15 min. The RNA was sedimented by centrifugation in an SS-34 rotor at 12,000 rpm for 15 min at 4 C. The supernatant was transferred to a new tube. Plasmid DNA was precipitated with 4 ml cold 95 percent ethanol on ice for 15 min. The DNA was sedimented by centrifugation as described above. The pellet was resuspended in 500ul TE and ethanol precipitated and resuspended in 500 ul RNase buffer. Remaining RNA was digested with 10 ul RNase (10mg/ml), at 37 C for 1 hr.

The DNA was resuspended in 500 ul 2.5M NaCl/20 percent PEG 9000, and incubated on ice for 30 min. Plasmid DNA was sedimented by centrifugation in a Beckman Microfuge for 15 min at 4C. The pellet was resuspended in 400 ul water, and was phenol:chloroform extracted once, chloroform extracted three times and ethanol precipitated Plasmid DNA was precipitated twice in ethanol, 1.1M ammonium acetate, by incubation at -20 C for 30 min. The DNA pellet was washed sequentially once with 1 ml 70 percent ethanol and once with 1 ml 95 percent ethanol. The DNA was air dried and resuspended in 50-100 ul water. The DNA was digested with Hae II or with Sma I and Xba I to check for proper restriction fragment patterns.

3. subcloning of Xma I/Xba I fragment

(a). restriction and ligation reactions

Three ug RF mutant M13 DNA and 0.5 ug pHE-1 or pLE-2 were incubated with 2 units Xma I for 6 hrs at 37 C in 1X Xma I buffer (Pharmacia). The restricted DNA was then incubated with 5 units Xba I in 50mM NaCl for 2 hrs at 37 C. The DNA solution was extracted once with phenol:chloroform, and twice with chloroform. Organic phases were back extracted with water. One ul wheat germ tRNA (10ug/ul) was added and the DNA was ethanol precipitated. The DNA was resuspended in 40 ul ligation buffer and ligated with 7.3 units T4 DNA ligase at room temperature for 4 hrs.

(b). transformation of LE-392

LE-392 cells were made competent to take in plasmid DNA by treatment with calcium chloride. One-half ml of an overnight culture of LE-392 was used to inoculate 40 ml sterile LB broth and incubated at 37 C until OD 600= 0.5. The cells were harvested by centrifugation in an SS-34 rotor at 10,000 rpm for 10 min at 4 C. The pellet was resuspended in 10 ml 75mM calcium chloride, 5mM Tris (pH 7.6) and placed on ice for 30 min. The cells were harvested as described above and resuspended in 2 ml 75mM calcium chloride: 5mM Tris (pH 7.6). LE-392 cells treated with calcium chloride were stored on ice for no more than 24 hrs before being used.

One hundred ul of suspended, competent LE-392 cells and 20 ul of ligation reaction were mixed and placed on ice for 30 min. The cells were heat shocked at 42 C for 2 min to take in the DNA. One ml warm LB was added to the cells and vortexed. The cells were incubated at 37 C for 30 min. Aliquots of 100, 200, and 300 ul transformed cells were spread onto LB amp plates and incubated at 37 C overnight.

4. Screening for recombinant plasmids

(a). small scale growth and isolation of plasmid DNA

Ten to twenty well separated, single colonies were picked and grown in two ml LB with ampicillin (20ug/ml) overnight at 37 C with shaking. One and one-half ml cultures were harvested by centrifugation in a Beckman Microfuge for 3 min at room temperature. The remainder of the cultures were stored at 4 C. Plasmid DNA was extracted by the modified Birnboim procedure as described previously.

(b). polyacrylamide gel electrophoresis (PAGE)

Ten ul extracted plasmid DNA were digested with Sma I and Xba I in React-4 buffer, at 30 C for 2 hrs, as described previously. Three ul 10X Ficoll dye were added and the sample was electrophoresed on a 6 percent polyacrylamide gel as previously described. Recombinant plasmids were screened for a decrease in size of the Sma I/Xba I fragment from the wild type length (332 bp). Point mutant 1131 was

rescued into dl1115-pLE-2, and recombinant plasmids were screened for an increase in fragment size from 282bp to wild type length.

(c). growth and large scale purification of recombinant plasmids

Five hundred ml cultures of LE-392 carrying the deletion or point mutations in pHE-1 or pLE-2 were grown and harvested as previously described. Plasmid DNA was purified using the lithium chloride procedure as described previously. The DNA was restricted with Hae II or with Sma I and Xba I and electrophoresed on a vertical 6 percent polyacrylamide gel to check for proper restriction fragment patterns as described previously.

II. ASSAY OF MUTATIONS FOR TRANSACTIVATION FUNCTION

A. Transient expression (CAT) assays

1. growth and maintanance of HeLa cells

HeLa cells were a gift of Mary Schneider (McMaster University). The cells were grown in minimal essential medium (α-MEM; Gibco), supplemented with 10% new born calf serum (Gibco), and 1% penicillin-streptomicin (Gibco). The medium from one confluent 160mm dish was aspirated and the cells were washed with 5 ml PBS. The cells were treated with 3 ml 1X trypsin (Gibco) for 10 min, and were dislodged from the plate by agitation. The cells were diluted into 50 ml supplemented medium, and plated onto two 160mm tissue culture dishes.

The dishes were incubated at 37 C in a 5 percent carbon dioxide incubator for 24 hrs until confluent. The cells were then diluted into four 160mm dishes and incubated at 37 C for 24 hrs as described above. Two dishes were used to prepare 10-12 100mm dishes of log phase HeLa cells for transfection. The cells were diluted into 100-120 ml supplemented medium and 10ml were plated onto 100mm tissue culture dishes. The dishes were incubated at 37 C overnight and were transfected the next day. Cells prepared for transfection in this manner were between 60 and 80 percent confluent at the time of transfection.

2. transfection with plasmid DNA

A working stock of 2X Hepes buffer (0.5ml/sample), sonicated salmon sperm (20 ug/dish), and pKCAT-23 (5ug/dish) was prepared and 0.51 ml aliquoted into 15 ml disposable polypropylene tubes. Appropriate amounts of plasmid DNA were added to each tube and vortexed. Fifty ul cold 2.5M calcium chloride were added to each tube and vortexed. The samples were precipitated at room temperature for 30 min. The precipitates were added dropwise onto each 100mm dish of HeLa cells respectively and left undisturbed for 5 min in the tissue culture hood before being moved to the incubator. After 6 hrs the medium was removed by aspiration and 2 ml 20% glycerol in 1X Hepes buffer were added to each dish, and aspirated 80 seconds later. Four ml 1X Hepes buffer were added to each dish to wash off any remaining glycerol and removed by aspiration. Ten ml supplemented medium were then added to each dish. The plates were incubated at 37 C, for 40-48 hours.

3. Assay for CAT enzyme

After 40 hrs the medium was removed by aspiration, and the cells were washed with 10 ml PBS. One ml 0.04M Tris (pH 7.6), 0.01M NaCl was added to each dish and the cells harvested by scraping. The resuspended cells were transferred to 15 ml disposable polypropylene tubes and centrifuged in a Damon IEC PR-J centrifuge, at 3000 rpm for 30 sec at 4 C. The supernatant was removed and the cells were resuspended in 100 ul 0.25M Tris. The cells were lysed by sonicating in an ice-water bath using a Bronwill Biosonik III sonicator, at a setting of 30 with 50-80 one second bursts. Cell debris was sedimented by centrifugation in an IEC PR-J centrifuge at 3000 rpm for 5 min at 4 C. The supernatant was transferred to a 1.5 ml Eppendorf tube and incubated with 2 ul [14C]-chloramphenicol (0.2uCi/ul), and 4 ul 20mM acetyl Coenzyme A at 37 C for 30-60 min. The chloramphenicol was extracted with 700 ul ethyl acetate. The ethyl acetate was evaporated by centrifugation in a Speed-Vac centrifuge for 30 min under vacuum. The chloramphenicol was resuspended in 20 ul ethyl acetate and spotted onto pre-coated silica gel thin layer chromatography plates (Analtech). Acetylated and non-acetylated forms of [14C]-chloramphenicol were separated by ascending chromatography in a closed glass chamber, with a solvent of chloroform:methanol (95:5) for 30 min. The plates were air dried and autoradiographed with Kodak XK1 or XAR5 film. The positions of the chloramphenicol spots were determined by superimposing the TLC plate over the autoradiogram. The spots were scrapped off with a scalpel. Radioactivities were determined by counting the samples in a cocktail of

Omnifluor/toluene (5g/l), in a Beckman Scintillation counter for one minute.

B. Sodium butyrate transient transactivation assays

CAT assays with sodium butyrate were performed as described for the transient transactivation assays. Sonicated salmon sperm DNA (20ug) and pKCAT-23 (5ug) were transfected into HeLa cells. A stock solution of 1M Na-butyrate was freshly prepared and an appropriate amount was added to the medium of each 100mm dish of HeLa cells, after glycerol shock.

C. Transactivation blocking assays

An assay similar to that described by Glenn and Ricciardi (1987) was performed using dl1115, sub1117, and IN819:pLE-2 as the competing plasmids. A stock solution of 2X Hepes buffer (0.5ml/sample), sonicated salmon sperm DNA (20ug/dish), pKCAT-23 (5ug/dish), and pLE-2 (1ug/dish) was prepared and 0.51 ml was aliquoted to each 15 ml polypropylene tube. Increasing concentrations of the mutant E1A plasmid were added to each tube. The samples were precipitated and transfected onto 100mm dishes of HeLa cells as described above. Cells were harvested at 40 hours and a cellular extract was prepared and assayed for CAT enzyme activity as described previously.

PART 3: RESULTS

I. Strategy

The Ad5 E1A polypeptides are multifunctional proteins with at least three functional domains that appear to act independently of each other. To gain a better understanding of the various properties of E1A and to determine a more precise location of the various domains on the E1A proteins, our lab has constructed in frame deletions and point mutations spanning all of the E1A coding region. These mutants were constructed in an M13 Ad5 E1A vector using the oligomer directed mutagenesis technique of Zoller and Smith (1984). Mutations rescued into plasmids and adenoviruses can be used to study E1A mediated events including repression, transformation and transactivation.

This thesis describes the construction of three deletions and one point mutant that together span all of the E1A exon-2 encoded residues. These mutants have been used to study E1A transactivation of the Ad5 E3 promoter.

The Ad5 E1A mutants were originally designed so that they could be rescued into dl309, an Ad5 virus that has only one Xba I restriction site, using the technique of Stowe (1981). This procedure makes use of the single Xba I restriction site in dl309 and M13mp11c1#5 for transfering the mutated fragment from the vector to the virus. This technique is useful only for mutations upstream of the Xba I site at nucleotide 1339. To remove sequences downstream of this site pm1131 was constructed. Pm1131 was made with an icosamer spanning G-1331 but changing it to a C (Table 1). This changes the triplet TCA to TGA in the

Table 1: Oligomers and mutants

mutant designation	Ad5 E1A DNA sequence altered	Ad5 E1A protein sequence altered	oligomer designation	oligomer length	oligomer sequence
dl1115	1237-1287 (51bp deleted)	188-204 (17 residues deleted)	AB11	icosatriamer	5' AAGGTCCTGTGCGCCGTCCTAAA 3'
dl1116	1288-1338 (51bp deleted)	205-221 (17 residues deleted)	AB28	icosatriamer	5' CAAGACCTACCTCTAGAGTCGAC 3'
pm1131	C-1331 to G (TCA to TGA)	stop codon effectively deletes aa 219-289	SAM95	icosamer	5' GACGCCCGACATGACCTGTG 3'
sub1117	AB-53 insertion and 1142-1287 (146bp deleted) C-1331 to G	missense lysine-186 stop codon at 187 effectively deletes E1A aa 186-289 non-sense	AB53	icosamer	5' GGTGATCGACCTTACCTGCC 3'
pm922	T-922 to C	silent mutation			

Table describes mutants and oligomers used to construct them.

coding sequence and replaces serine-219 with a termination in the protein sequence (See Figure 3A). Dl1115 (dl aa 188-204) and dl1116 (dl aa 205-221) were designed to remove small stretches at the 5' end of the exon-2 encoded residues. These mutants delete seventeen amino acids each, in regions of the protein that are hydrophilic and hydrophobic respectively. Sub1117 removes the 3' splice site in exon-2 and effectively deletes all of the exon-2 encoded amino acids (Table 1).

II. Mutagenesis

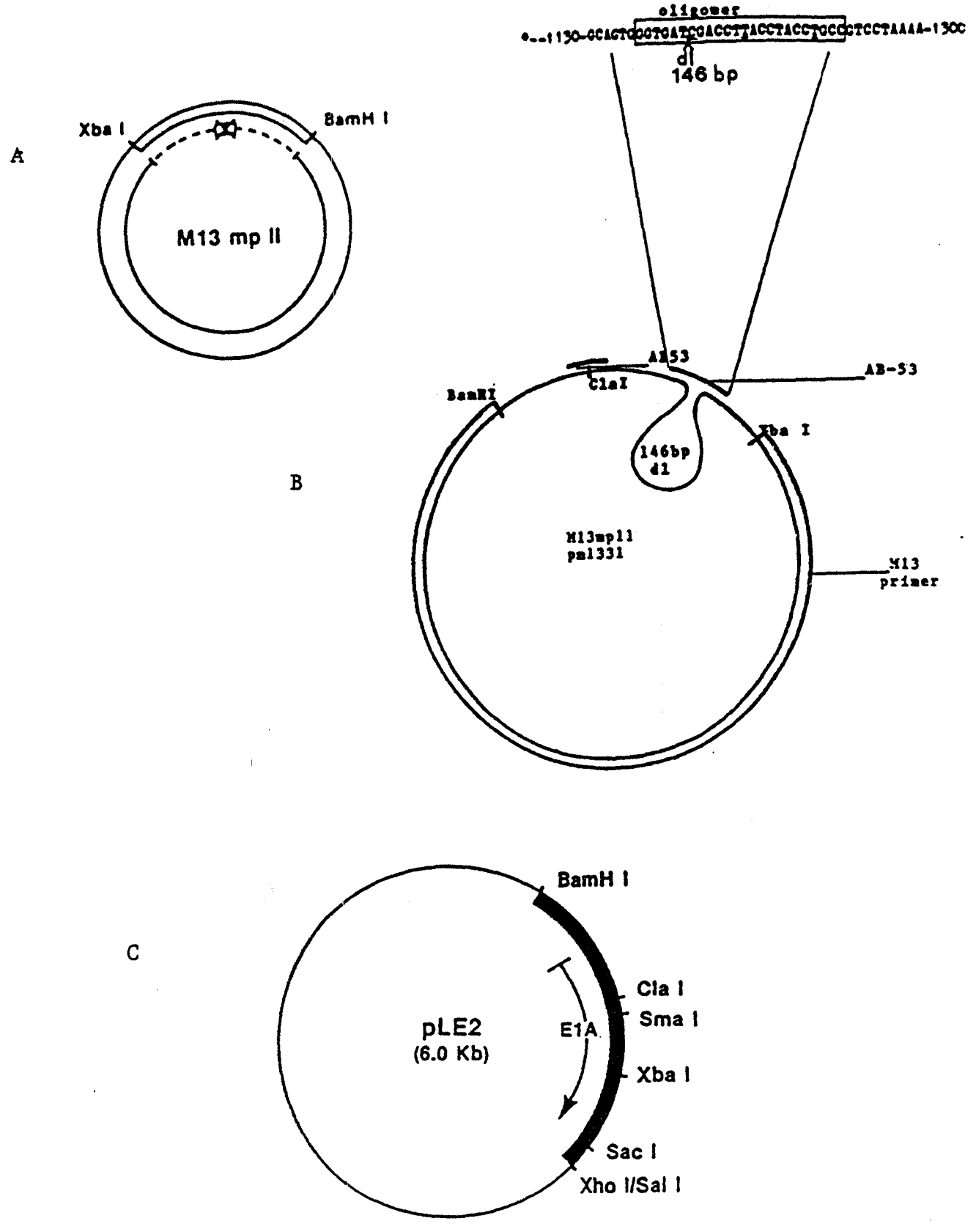
Mutants were constructed using the two primer method of Zoller and Smith (1984). Synthetic oligomers designed for deletions were made such that 12 or 11 nucleotides of an icosatriamer flanked the region of the sequence to be deleted. Single stranded M13mp11cl#5 DNA, an M13 vector containing the BamHI to XbaI fragment of pHE-1 (Ad5 0-3.8%), was mutated with oligomers designed to delete or make single base changes in the coding region of the second exon of E1A. When the oligomer is hybridized to the single stranded M13mp11cl#5 template, it causes the sequence to be deleted to loop out (Figure 4A). The second primer was denatured RF M13 DNA cut with BamH I and Xba I and hybridized to the M13 sequence in M13 mp11 cl#5. The gaps were filled in with the Klenow fragment of DNA polymerase I and ligated with T4 DNA ligase. The resulting products consisting of wild type and mutated sequences were used to transfect E. coli JM103, which was then plated onto YT agar plates, with X-Gal and IPTG.

Figure 4.

A. Standard site-directed mutagenesis of M13mp11 c1#5.
(from Jelsma et al., 1988)

B. A-53 mutagenesis of m13mp11pm1131 which created sub1117.

C. pLE-2 restriction map (modified from Jelsma et al., 1988)



* Nucleotide sequence around AB-53 insert (boxed). Underlined bases are inserts or mismatches relative to the wild type sequence.

Hybridization screening

Mutants were initially screened by dot blot or plaque lift hybridization, using the mutagenic oligomer as a probe. For dot blot hybridization, M13 phage was prepared by infecting 2 ml cultures of JM103 with clear, well separated, individual plaques and incubating at 37 C, for 8 hrs. Phage was prepared from the supernatant. The bacterial pellet containing RF M13 DNA was saved for further screening. The phage were spotted onto a nitrocellulose filter and were screened with the [γ-32P] ATP labeled oligomer (Figure 5). The frequency of mutated clones isolated varied from 1 to 5 percent (data not shown). Plaque lift hybridization (Figure 6) increased the frequency of mutant plaques isolated to 14 percent (data not shown), because of the larger number of plaques that could be screened. In this procedure a nitrocellulose filter was placed directly on top of the agar plate containing plaques from the mutagenesis reaction. The filter was removed, baked and probed with the end labeled mutagenic oligomer.

Figure 5. Dot blot hybridization autoradiograms: 78 plaques were picked from a lawn of JM103 cells transfected with single stranded M13mp11cl#5 DNA mutagenized with AB-28. Phage were prepared and blotted onto a nitrocellulose filter. The filter was hybridized with [γ-32P] ATP-labeled AB-28 DNA as described.
(A) Autoradiography after one hour revealed that after a room temperature wash in 6X SSC the probe hybridized to the control spots of ss M13mp11 cl#5 DNA ,denoted by C, as well as most of the phage spots.
(B) Autoradiography for 14 hours revealed that after a wash at 68 C (T_D-2) in 6X SSC the probe washed off the control spots and all but two of the plaque spots, both of which were found to be mutants.

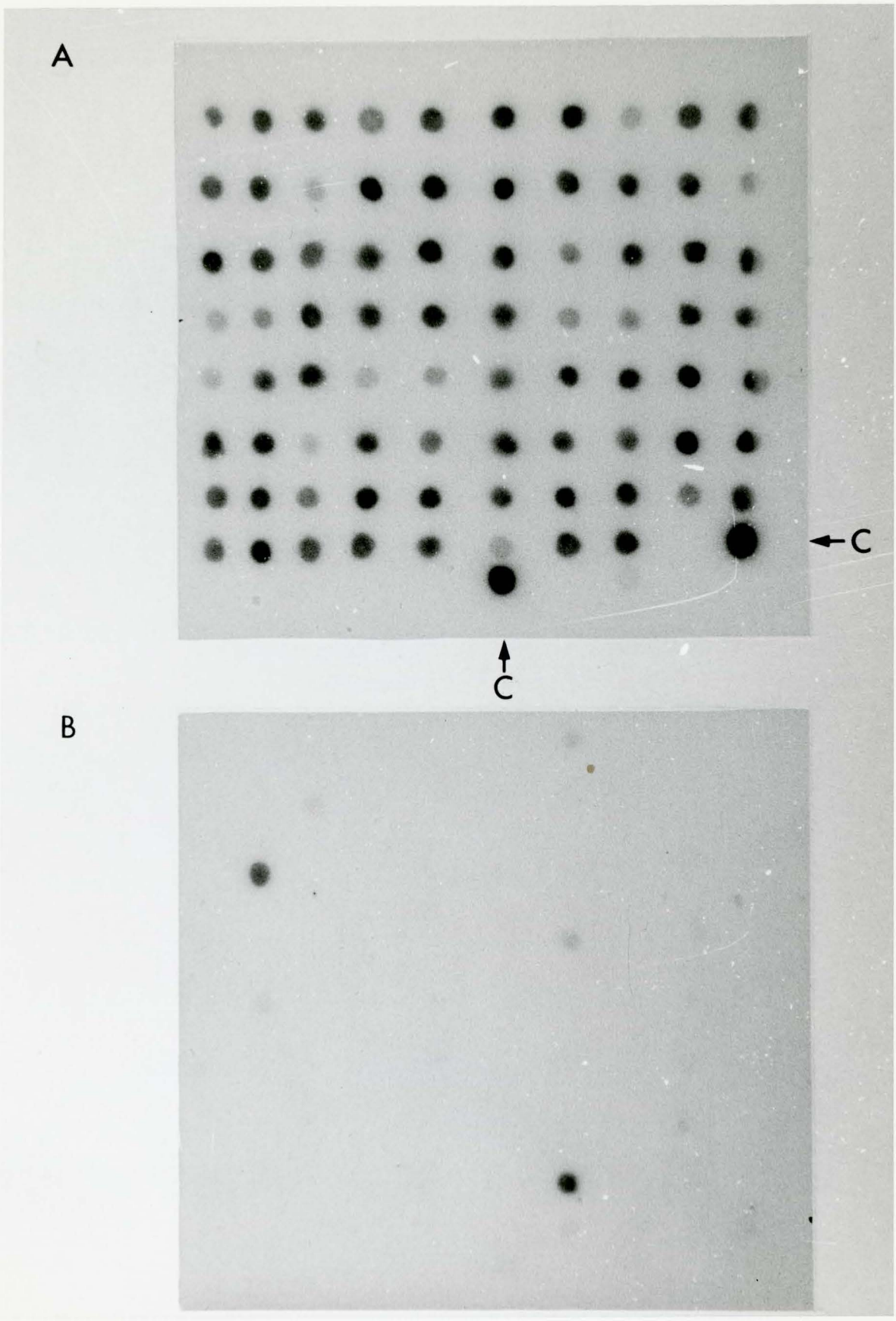
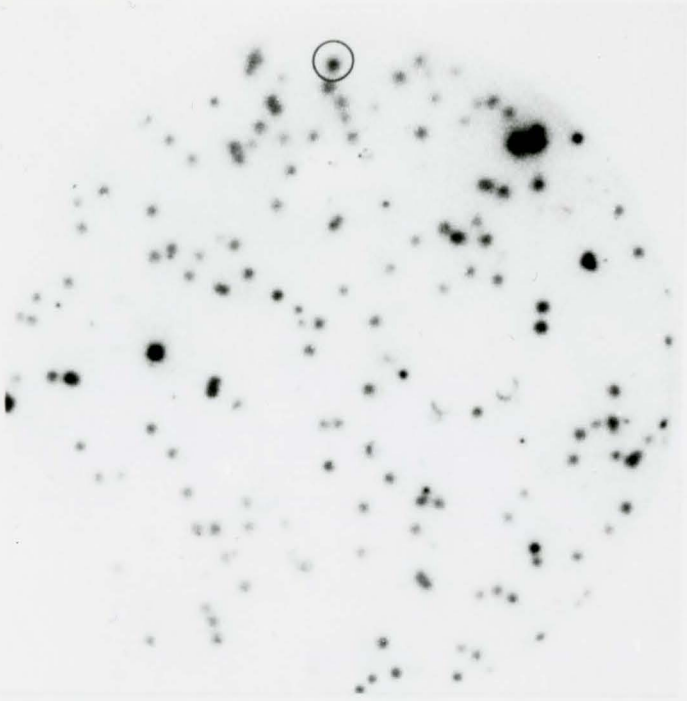

A
C
C
B

Figure 6. Plaque lift hybridization autoradiogram: JM103 cells were transfected with single stranded M13mp11 cl#5 DNA mutagenized with AB-53and spread onto a YT agar plate, which was then incubated at 37 C for 12 hours. A nitrocellulose filter was placed on top of the agar, removed and hybridized with [γ-32P] ATP-labeled AB-53 DNA as described.

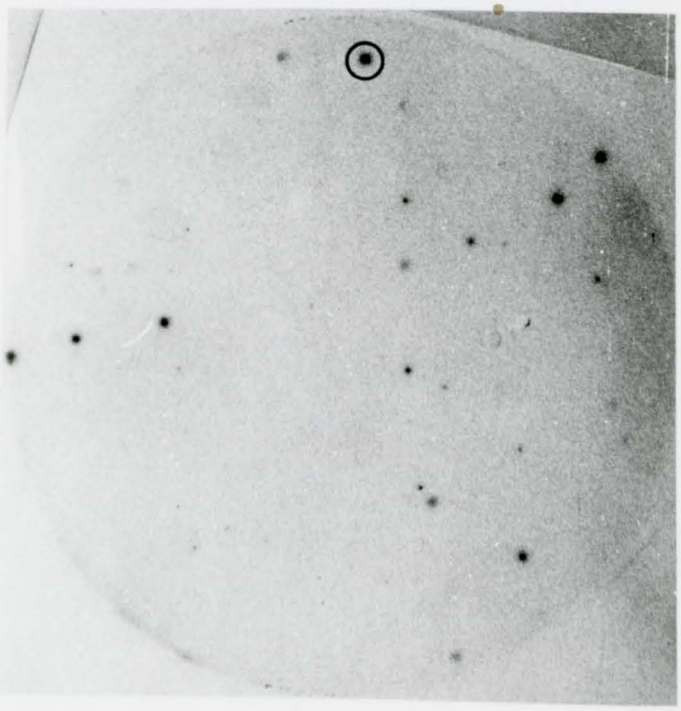
(A) Autoradiography for 12 hours revealed that after a room temperature wash in 6X SSC the oligomer hybridized to many of the plaques.

(B) The filter was washed at succesively higher temperatures in 6X SSC until most of the signal due to oligomer hybridization with wild type plaques could no longer be detected with a hand held Geiger-Muller counter. Autoradiography for 12 hours revealed that after a 60 C wash in SSC, the label bound to only a few plaques. The encircled plaque was picked and later shown to contain a large deletion as well as the intended point mutation.

A



B



PAGE screening

Mutant clones isolated by hybridization screening were then screened by restriction enzyme digestion and polyacrylamide gel electrophoresis (PAGE) (data not shown). RF DNA was prepared from the stored bacterial pellet and was digested with Sma I and Xba I, and run alongside Sma I/Xba I restricted wild type RF M13mp11 cl#5 DNA on a 6 percent polyacrylamide gel. The dl1115 and dl1116 Sma I/Xba I fragment ran faster than the wild type fragment, consistant with the predicted size of 282bp. The Sma I/Xba I fragment of sub1117 also ran faster than the wild type fragment, consistant with the predicted size of 191bp. The restriction fragments patterns of pm1131 are identical to wild type.

Dideoxynucleotide sequencing

Single stranded DNA was prepared from clones containing a mutation as judged by hybridization and PAGE screening, and was sequenced with a primer that bound upstream of the mutated region, usually an oligomer used to make an upstream mutation. All mutations were initially sequenced through the mutated region by the Sanger dideoxynucleotide sequencing method (Figure 7). Later, the entire region between the Sma I and Xba I restriction sites was sequenced in all the mutants studied, to ensure that no other nucleotides had been altered. Except for the predicted mutated sequences, the region between SmaI and Xba I was found to be wild type in all the mutants studied. M13mp11:pm1131 was rescued into dl1115-pLE-2 (see below), and the Bam HI to Xba I fragment of pm1131:pLE-2 was then subcloned into M13mp10. The

Figure 7. Autoradiograms of sequencing gels:
Mutants in single stranded M13 were sequenced with the Sanger dideoxynucleotide technique using 35S-ATP as the radioactive label, and were run on denaturing polyacrylamide gels, as described. Guanine (G), adenine(A), thymine(T) and cytosine(C) lanes are indicated.

(A)ss M13 mp11 sub1117 DNA was sequenced with SAM-61*. The arrow indicates residue C-1142, the first base to deviate from the wild type sequence.

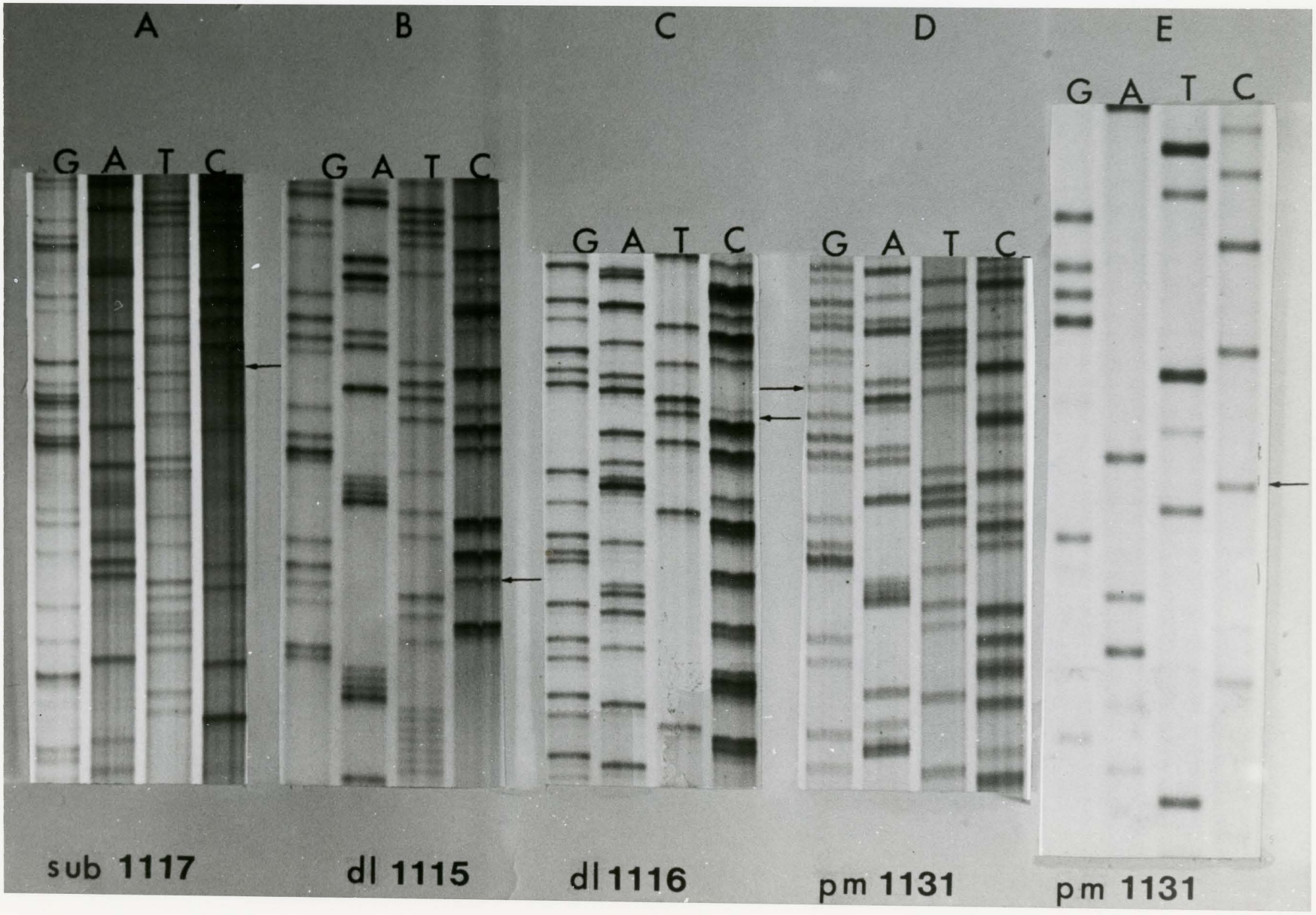
(B) ss M13mp11 dl1115 DNA was sequenced with SAM 80*. The arrow indicates residue C1288, the first base following the 1237 to 1287 deletion and is preceded by residue G-1236.

(C) ss M13 mp11 dl1116 DNA was sequenced with SAM 80. The arrow indicates residue T-1339 the first base following the 1288 to 1338 deletion and is preceded by residue C-1287.

(D) ss M13mp11 pm1131 DNA was sequenced with SAM 80. The arrow indicates the C to G -1331 transition.

(E) ss M13mp10 pm1131 DNA , the antisense strand of pm1131, was sequenced with a primer that binds immediately upstream of the M13 Xba I site in M13 (Universal primer, Pharmacia). The arrow indicates the G to C-1331 transition.

* SAM61 primes transcription from nucleotide 814.
SAM80 primes transcription from nucleotide 1063.


A
B
C
D
E
G A T C
G A T C
G A T C
G A T C
G A T C
sub 1117
dl 1115
dl 1116
pm 1131
pm 1131

antisense strand in M13mp10 was sequenced to confirm the single base change observed by sequencing in M13mp11. The G to C transition on the antisense strand is shown in Figure 7E.

Sub1117 arose fortuitously in an attempt to remove the Cla I site at nucleotide 922 (see Figure 4) in M13mp11:pm1131, using AB-53. The lack of a Cla I site in pm1131, as judged by restriction with ClaI and polyacrylamide gel electrophoresis, would be used as a marker for future work with this mutant. Sequencing of a mutant clone isolated by plaque lift hybridization confirmed that the T-922 to C transition had occured. Subsequent restriction enzyme digestion and polyacrylamide gel electrophoresis of this clone in M13 revealed that there was a large deletion somewhere between the Sma I and Xba I restriction sites. The mutant was sequenced downstream of the Sma I site to determine if the deletion was a useful one. Sequencing revealed that in addition to the T-922 to C transition, AB-53 deleted 146 bases between 1141 and 1289, a region that includes the 3' splice site and the splicing branch point of E1A. It appears that AB-53 shares considerable homology with the sequences flanking this deleted region, and except for three mismatches it is a perfect oligomer for creating the resulting deletion (Figure 4B). The formation of this mutant stressed the importance of fully sequencing the M13 fragment to be subcloned into the E1A plasmids. Even though it arose accidentally, this mutation was in itself useful as the removal of the 3' splice site and splicing branch point insures that the mRNA cannot splice. Translation from such an mRNA species should result in a 186 residue polypeptide comprised of E1A residues 1-185

followed by a missense lysine-186. Translation is stopped by a termination codon at position 187.

[This unexpected mutagenesis was not an isolated incident. Wild type M13mp11cl#5 template was also mutated with AB-53. The single AB-53 directed point mutant created on M13mp11cl#5, isolated by dot blot hybridization, was later used by Dan Dumont (McMaster University) as template for site directed mutagenesis. Subsequent sequencing revealed that AB-53 had inserted near nucleotide 1325 in the E1A gene and altered the sequence in that region (Dan Dumont, personal communication)].

III. Rescue of mutations into E1A plasmids

Large scale preparations of RF M13 were made by the cesium chloride method (Methods). The Bam HI to Xba I fragments of M13mp11:dl1115 and dl1116 where originally rescued into pHE-1, a pBR322 plasmid that contains the entire E1A coding sequence (0-1574), but lacks the E1A polyadenylation signal sequence. Both vectors were cut with Bam HI and Xba I and then ligated with T4 DNA ligase. The resulting products were transfected into E. coli strain LE-392 and were plated onto LB agar plates with ampicillin. Ampicillin resistant colonies were picked and grown in 2 ml LB broth with ampicillin. DNA was prepared using the rapid plasmid isolation technique described by Maniatis et al. (1982), and was screened for a decrease in the Sma I to Xba I fragment size by restriction with these enzymes and electrophoresis on a 6 percent polyacrylamide gel. Later, the Xma I(Sma I) to Xba I fragment of M13mp11:dl1115, dl1116 and sub1117 was subcloned into pLE-2, a pBR322

plasmid containing the entire E1A coding region (0-1772), including the polyadenylation signal (Figure 3C), by cutting the M13 vector and wild type pLE-2 with Xma I and Xba I and then ligating. The resulting products were transfected into LE-392 and plated onto LB agar plates with ampicillin. Ampicillin resistant colonies were picked and grown in 2 ml LB broth with ampicillin, and plasmid DNA was purified. Recombinant plasmids were screened for a decrease in the Sma I to Xba I fragment by restriction with these enzymes and electrophoresis on a 6 percent polyacrylamide gel. Dl1115 and dl1116 both delete 51 bases from the wild type Ad5 E1A sequence. Dl1116 deletes the Hae II restriction site at 1304. Therefore, these mutants can be distinguished by digestion with Hae II (Figure 8 lanes a,b). The SmaI/XbaI fragment of sub1117:pLE-2 migrates at 191bp. The Xma I(Sma I) to Xba I fragment of M13mp11:pm1311 was rescued into dl1115:pLE-2. Recombinant plasmids were screened for an increase in the Sma I to Xba I fragment size from 282bp to wild type length of 332bp. The restriction fragment patterns of wild type pLE-2 and pm1131:pLE-2 are identical (Figure 8 lanes d,e and j,k).

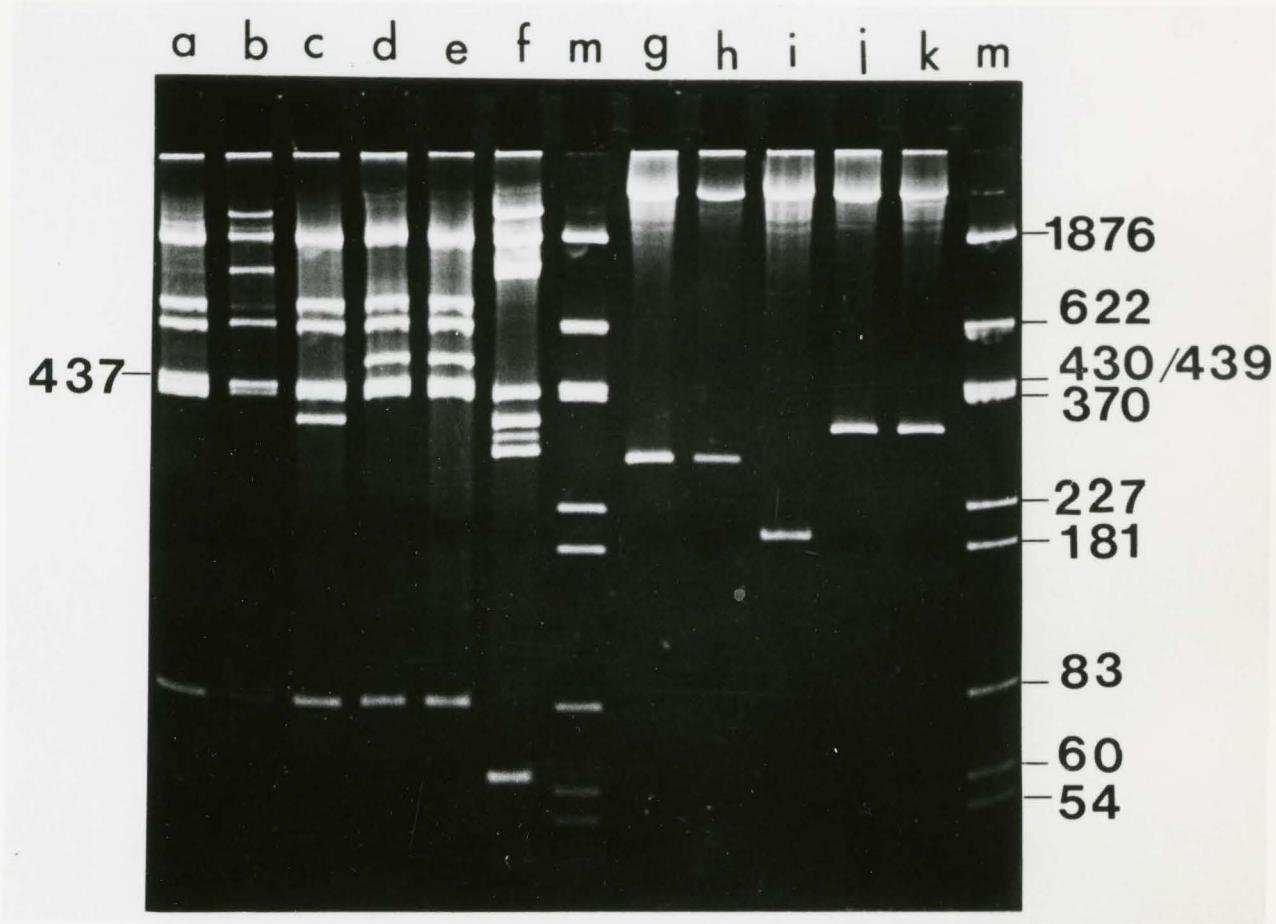
Figure 8 Restriction enzyme analysis of pLE-2 mutants.

(a-f) DNA was digested for two hours with Hae II as described;

(a) dl1115-pLE-2 , the 437bp Hae II fragment includes a 51 bp deletion from the wild type (488bp); (b) dl1116:pLE-2 is missing the Hae II site at 1304 in E1A, so that a larger fragment of 1049bp is produced;
(c) sub1117:pLE-2 E1A Hae II fragment migrates at 352bp; (d) pm1131:pLE-2 has a restriction fragment pattern identical to wild type pLE-2 (lane e);
(f) pKCAT-23; (m) pBR322 cut with Hae II, fragment sizes are indicated on the right.

(g-k) DNA was digested for two hours with SmaI and XbaI;

(g) dl1115 282 bp SmaI/XbaI fragment contains a 51 base deletion from the wild type length (332bp);
(h) dl1116:pLE-2 SmaI/XbaI fragment migrates at 282bp;
(i) sub1117:pLE-2 SmaI/XbaI fragment at 191bp;
(j) pm1131:pLE-2 has a restriction fragment pattern identicle to wild type pLE-2 (lane k).


a b c d e f m g h i j k m
437
1876
622
430/439
370
227
181
83
60
54

IV. E1A transient expression assay

(a). Transactivation of the Ad5 E3 promoter

Preliminary CAT assays were done with plasmid DNA preparations banded on cesium chloride gradients (Methods). Later, it was found that even after RNAse treatment the banded DNA contained nucleic acid contaminants. However, CAT assay results with these preparations (data not shown) did not differ from assays utilizing more purified DNA .

Plasmid DNA used in subsequent assays was prepared by the new lithium chloride procedure (Methods). This method utilizes steps designed to remove contaminating nucleic acids. DNA was quantitated by UV absorption scanning from 210nm to 310nm. The absorbance at 260nm was used to calculate the plasmid DNA concentration. Relative concentrations were confirmed by restriction enzyme digestion and electrophoresis on 6 percent polyacrylamide gels, by comparing relative intensities of bands after ethidium bromide staining. DNA markers of known concentration were run alongside the new DNA preparations.

Dl1115, dl1116, sub1117 and pm1131 were assayed for their ability to stimulate transcription of pKCAT-23, in plasmid based transient expression assays. Semi-confluent 100mm dishes of HeLa cells were transfected with a DNA co-precipitate of E1A plasmid (10ug), pKCAT-23 (5ug), and sonicated salmon sperm DNA (20ug). A cell extract was prepared at forty hours post-transfection and an assay performed to measure the activity of CAT enzyme synthesized. All of the extract was incubated in the presence of 1mM acetyl CoA and [14C]-chloramphenicol

(0.2 uCi). Acetylated and unacetylated forms of [14C]-chloramphenicol were separated by ascending chromatography. The chromatography plates were autoradiographed and acetylation was determined by scintillation counting.

Initially, dl1115 and dl1116 were assayed in pHE-1 for transactivation of pKCAT-23. Dl1116:pHE-1 was found to stimulate transcription as well as wild type pLE-2. Dl1115:pHE-1 was unable to transactivate pKCAT-23 (data not shown).

Dl1115, dl1116, sub1117 and pm1131 in a pLE-2 background, were assayed for transactivation of pKCAT-23 (Figure 9A). Dl1115 (dl aa 188-204) and sub1117 (dl aa 186-289) were unable to stimulate transcription significantly above the basal level of pKCAT-23 (5ug). Dl1116:pLE-2 (dl aa 205-221) stimulated transcription as well as wild type pLE-2; that is about 25 fold over pKCAT-23 alone (Table 2).

Results with pm1131 were variable. In one experiment pm1131 stimulated transcription as well as wild type (Table 2, exp. a). However, with other plasmid preparations values ranged between 8 and 26 percent of wild type (Table 2, b-e). It is not clear why pm1131 gave such variable results, but since the wild type transactivation levels in experiment (a) (Table 2) were not reproducible only experiments b-e were used to obtain an average transactivating efficiency. Other truncated E1A mutants have been similarly assayed for transactivation of the E3 promoter (Dody Bautista, McMaster University). Cell extracts prepared at various times after transfection of HeLa cells with these mutants, and

Table 2: Transactivation efficiency of E1A exon-2 mutants relative to wild type E1A

E1A mutant	a	b	c	d	e	mean
sub1117	0.01	0.04	0.06	0.04	0.04	0.04
dl1115	0.04	0.02	0.05	0.03	0.02	0.03
dl1116	0.90	0.84	1.01	1.04	1.04	0.97
pm1131	1.14	0.20	0.26	0.18 0.15 0.17 0.18	0.08	0.17*
pKCAT-23 (5ug)	0.01	0.09	0.07	0.004	0.01	0.04
WT pLE-2 (10ug) (% acetylation)	21%	81%	78%	83%	40%	---

Transient expression assays were conducted as described in Methods, with 10ug mutant plasmid DNA.

Relative transactivation efficiencies of mutant E1A plasmid and pKCAT-23, as compared to WT E1A (1.00), are shown. Percent conversion of CAM to AC:CAM for wild type pLE-2 is indicated.

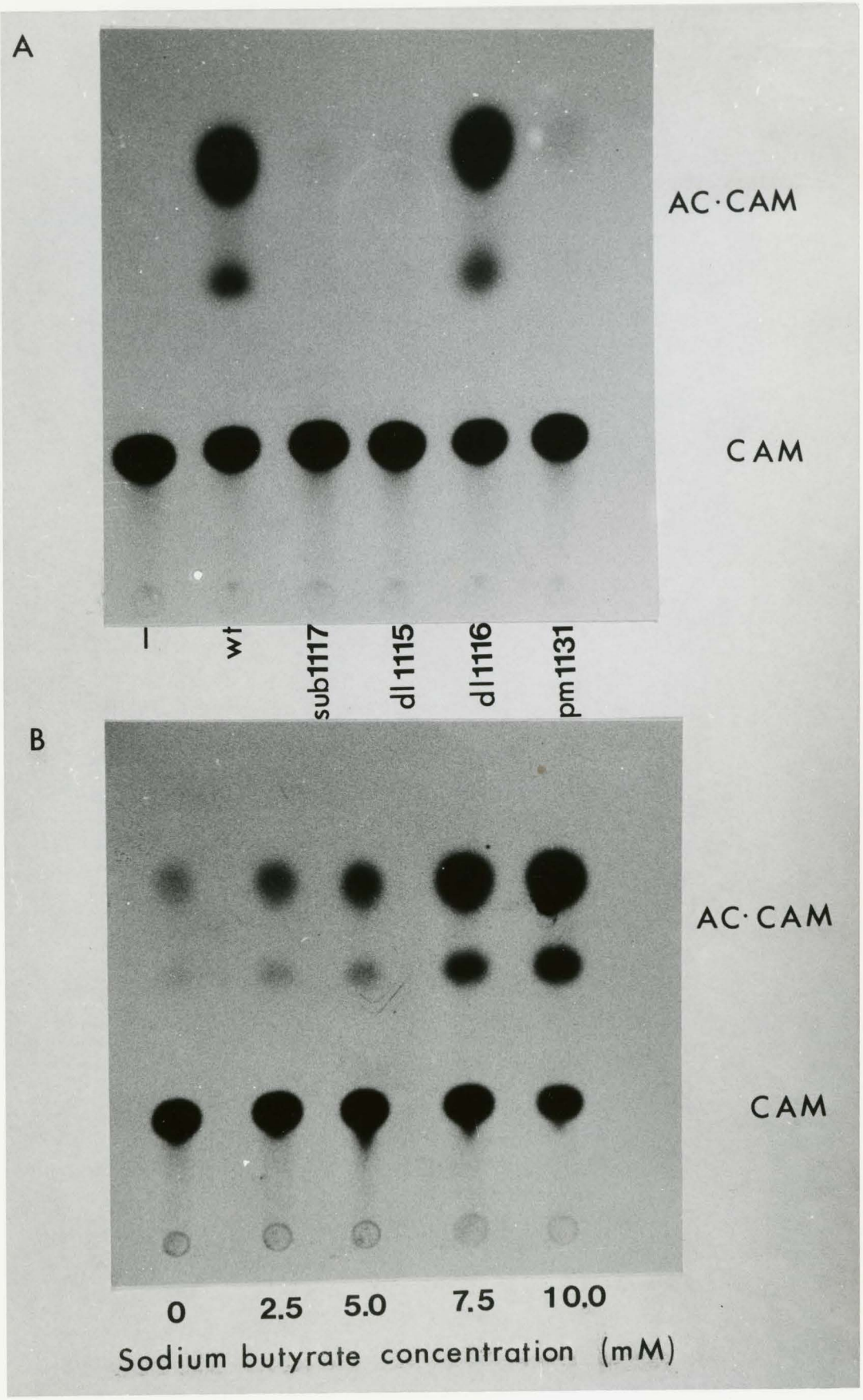
$$\text{relative efficiency} = \frac{\%\text{ acetylation by mutant E1A}}{\%\text{ acetylation by wt E1A}} \times 100$$

*Mean does not include exp. (a).

Figure 9 CAT assay autoradiograms.

(A) 100mm dishes of HeLa cells were transfected with sonicated salmon sperm DNA (20ug), pkCAT-23 (5ug) and wild type(WT) or mutant pLE-2 (10ug) as indicated. Cell extracts were prepared and incubated with acetyl CoA and [14C] chlorampenicol for 30 min. Unacetylated (CAM) and acetylated forms of chloramphenicol (AC:CAM) separated by chromatography on silica gel plates and autoradiographed are indicated.

(B) 100mm dishes of HeLa cells were transfected with sonicated salmon sperm (20ug) and pKCAT-23 (5ug) as described. After glycerol shock, 1M sodium butyrate (NaB) was added to the medium to the indicated final concentrations. The cells were incubated for 40 hours, cell extracts were prepared incubated in the presence of acetyl CoA and [14C]-chloramphenicol for 30 minutes as described. Unacetylated (CAM) and acetylated forms of chloramphenicol (AC:CAM) were separated by chromatography on silica gel plates and autoradiographed.


A
AC·CAM
CAM
–
wt
sub1117
dl 1115
dl 1116
pm1131
B
AC·CAM
CAM
0
2.5
5.0
7.5
10.0
Sodium butyrate concentration (mM)

assayed for CAT activity also yielded variable results (Dody Bautista, personal communication). Extracts prepared at early times post transfection had low percent acetylation of chloramphenicol, while extracts prepared later gave the equivalent of wild type acetylation.

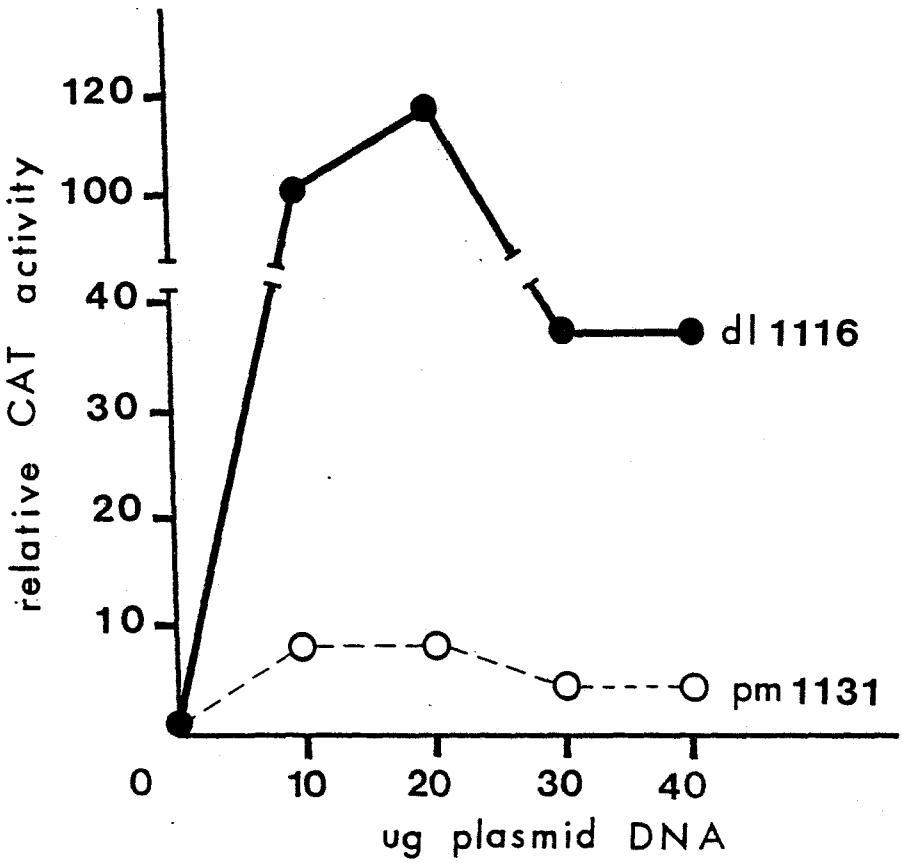
It should be emphasized that the CAT assay is by no means a precise tool for measuring absolute transactivation efficiency, but rather is useful in discerning mutants that can or cannot transactivate. However, in the case of pm1131 it can be concluded with some certainty that this mutant can activate transcription but not at the high level of wild type E1A. Dl1116 did not affect transactivation, therefore residues 205-221 do not appear critical for the transactivation fuction of E1A in these transient expression assays. Deletion of residues 186-289 in sub1117 resulted in a complete loss of the E1A transactivation function, suggesting that exon-2 encoded residues are involved in transacativation. Dl1115 further defines the critical exon-2 encoded amino acids as being between residues 187-205. These residues are necessary for E1A mediated transactivation in these transient expression assays.

(b) Effect of increasing the amount of transfecting mutant pLE-2 DNA on transactivation

Increasing amounts of mutant pLE-2 plasmids were assayed for transient transactivation of pKCAT-23, in an assay similar to the CAT assay previously described. Ten, 20, 30, and 40ug of dl1116:pLE-2 and pm1131:pLE-2 were assayed for transactivation of pKCAT-23 (5ug), with sonicated salmon sperm DNA (20ug) (Figure 10). Conversion of CAM reached maximal levels at 20 ug dl1116:pLE-2 and declined at 30ug and 40ug. Increasing pm1131:pLE-2 plasmid amounts over 20ug showed a similar effect as that with dl1116:pLE-2, indicating that maximal stimulation of transactivation occurs with 10-20 ug.

Figure 10 Effect of increasing the amount of mutant plasmid DNA on transactivation.

100mm dishes of HeLa cell monolayers were transfected with sonicated salmon sperm DNA (20ug), pKCAT-23 (5ug), and increasing amounts of dl1116 or pm1131 DNA. Cell extracts were prepared and assayed for CAT activity as described. Points on graph indicate relative activity with respect to WT pLE-2 (100) and were part of one experiment (exp. e ;Table 2) .


relative CAT activity
120
100
40
30
20
10
0
10
20
30
40
ug plasmid DNA
dl 1116
pm 1131

V. Sodium Butyrate stimulates transcription of the E3 promoter driven CAT gene in pKCAT-23

Use of sodium butyrate in transient expression (CAT) assays began as an attempt to increase the number of HeLa cells available for harvesting after the 40 hour incubation period. Sodium butyrate (5mM) has been shown to stop the growth of HeLa cells (Ginsberg et al., 1973). This in effect would reduce the number of cells that came off the plate before the end of the incubation period.

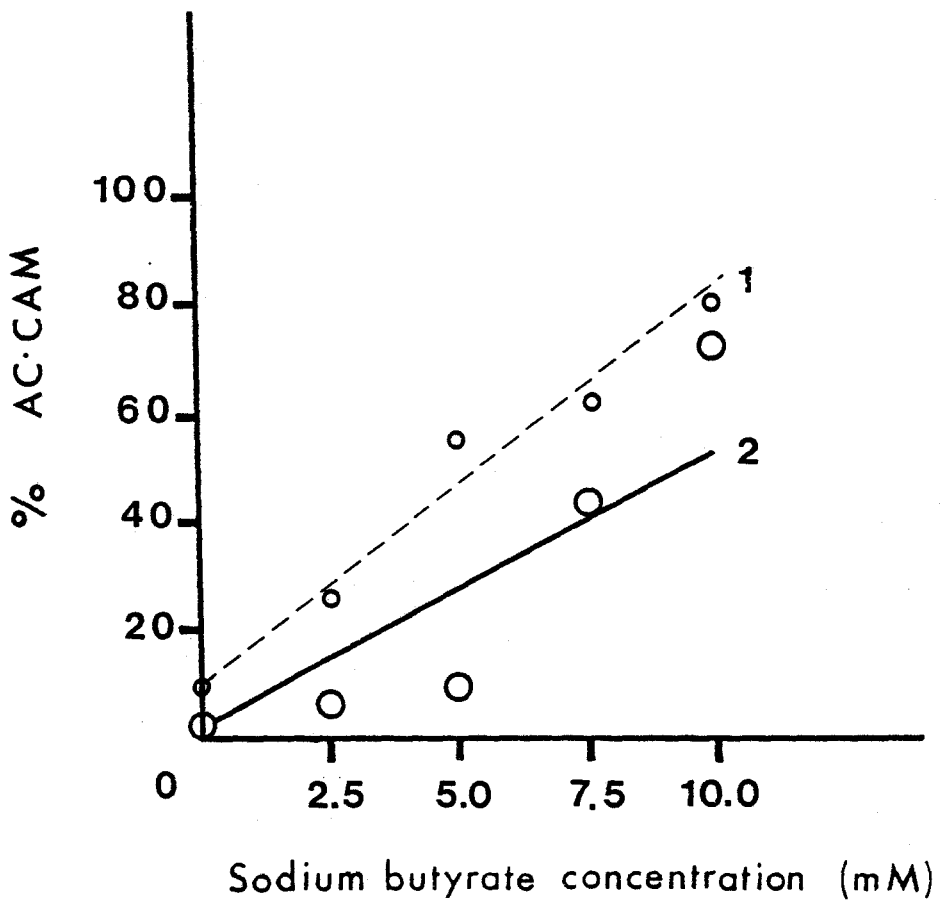
In transient expression assays with pLE-2, it was found that although the overall conversion of CAM increased in the presence of sodium butyrate, background levels with pKCAT-23 alone were also increased. This effect did not seem to be simply due to an increase in the number of cells harvested, as the number of NaB treated cells was not appreciably different from untreated cells.

Addition of sodium butyrate (NaB) to the medium of HeLa cells transfected with sonicated salmon sperm DNA (20ug) and pKCAT-23 (5ug), after glycerol shock, induced transcription of the CAT gene (Figure 9B). Stimulation of transcription was measured indirectly by assaying the amount of CAM acetylated by the CAT enzyme, as previously described. Conversion of CAM increased in proportion to increasing NaB concentrations. It is not clear from these experiments if the dose responce curve is linear, however these results indicate that sodium butyrate induces transcription from the adenovirus E3 promoter.

Figure 11 Sodium butyrate stimulates transcription of E3 promoter driven CAT gene.

100mm dishes of HeLa cell monolayers were transfected with 20ug salmon sperm DNA and 5ug pKCAT-23 and were incubated in the presence of increasing concentrations of sodium butyrate. Cell extracts were prepared as described and assayed for CAT activity. Graphs indicate percent conversion of CAM to AC:CAM from two experiments using different preparations of pKCAT-23 DNA.

Autoradiogram of exp. 2 is shown if Figure 9B.


% AC·CAM
100
80
60
40
20
0
2.5
5.0
7.5
10.0
1
2
Sodium butyrate concentration (mM)

VI. Transactivation blocking assays

Dl1115 and sub1117 were assayed for their ability to block transactivation of the E3 promoter by wild type E1A, in a plasmid based transient expression assay similar to that described by Glenn and Ricciardi (1987) (see p.21).

(a). dl1115:pLE-2 blocks E1A transactivation

HeLa cell monolayers were cotransfected with sonicated salmon sperm DNA (20ug), pKCAT-23 (5ug), wild type pLE-2 (1ug) and increasing amounts of dl1115:pLE-2 up to a 20 fold molar excess of mutant E1A (Figures 12A, 13, 14, Table 3). At low concentrations of dl1115, results were variable. In two out of five experiments (Table 3 b and e) there was an apparent increase in acetylation. It is not clear why this occured, but it possible that the transfection efficiency in those dishes was higher than in the control dishes. Alternatively, it may be that at low concentrations the mutant dl1115 protein cannot compete with wild type E1A. The mean of five experiments indicates that a 50 percent reduction in acetylation of CAM was obtained by cotransfecting with a five fold molar excess of dl1115:pLE-2. Complete inhibition of transactivation was obtained with 20ug dl1115:pLE-2. The addition of 20ug wild type pLE-2 in place of dl1115-pLE-2 did not reduce transactivation of the E3 promoter indicating that reduction of acetylation was not simply due to a plasmid concentration effect on transfection efficiency. These experiments suggest that dl1115 is sequestering a cellular protein that is involved in transactivation.

Figure 12 Autoradiograms of transactivation blocking assays with (A) dl1115 and (B) sub1117

100mm dishes of HeLa cells were transfected with 20ug sonicated salmon sperm DNA, and 5ug pKCAT-23; lanes b-g contained 1ug wild type pLE-2; (c) contained an additional 20ug pLE-2; lanes d-g contained increasing amounts of dl1115:pLE-2 or sub1117 as follows: (d) 1ug; (e) 5ug; (f) 10ug; (g) 20ug.

Cell extracts were incubated in the presence of acetyl CoA and [14C]-chloramphenicol for 30 min as described. Acetylated (AC:CAM) and unacetylated forms of chloramphenicol (CAM) were separated by chromatography on silica gel glass plates and autoradiographed.

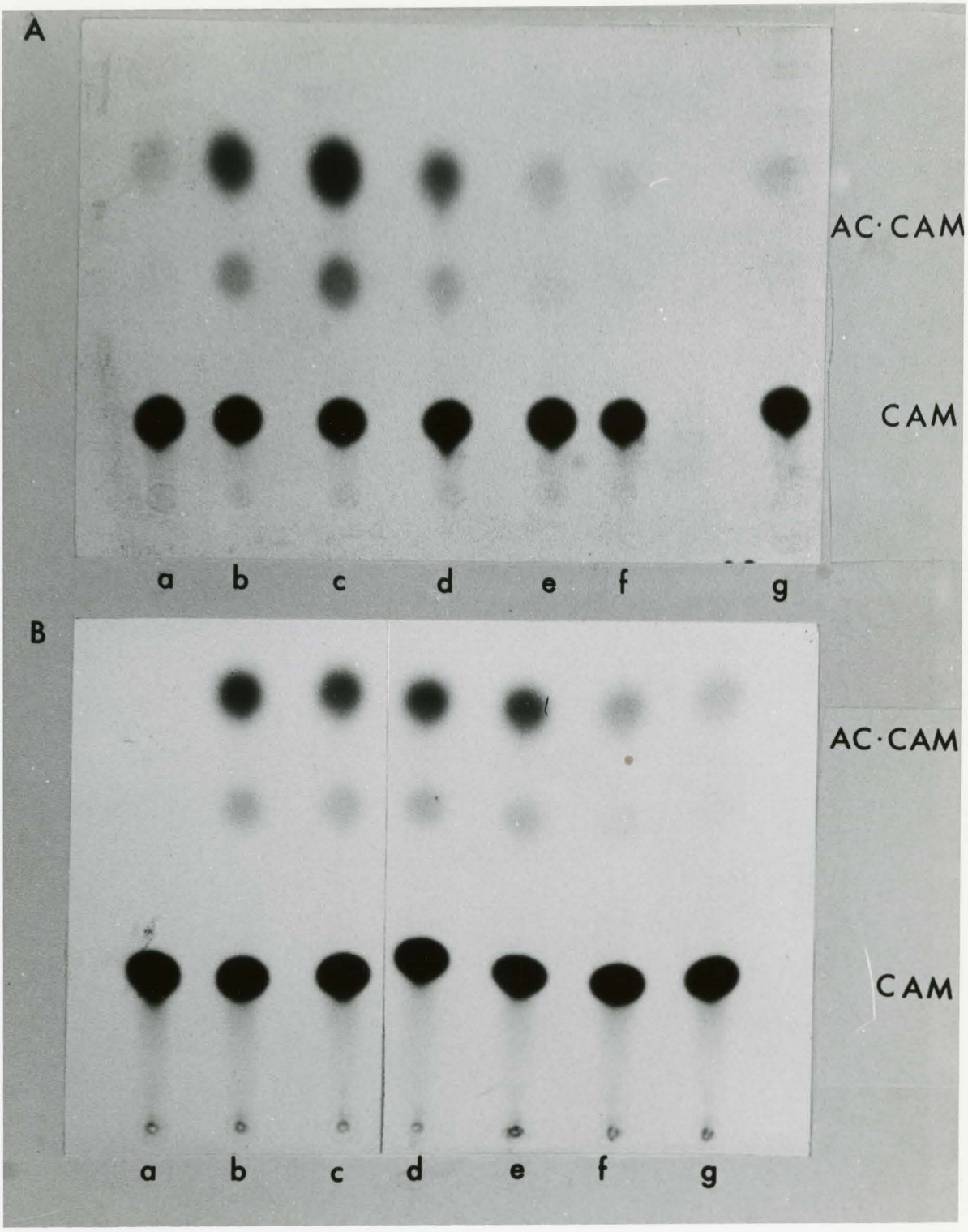

A
AC·CAM
CAM
a b c d e f g
B
AC·CAM
CAM
a b c d e f g

Table 3: Relative activity with dl1115 in transactivation blocking assays

ug mutant DNA	a	b	c	d	e	f*	g**	h**	mean (a-e)
1.0	----	115	72	61	182	48	60	98	107
5.0	99	47	26	7	71	7	117	83	38
10.0	41	18	34	0	6	0	41	18	14
20.0	19@	3	6	0	0	--	17	0	2
21 ug WT pLE-2	----	54	98	240	82	240	315	22	118
(% acetylation) 5ug pKCAT-23	2.2%	1.5%	0.5%	1.7%	0.3%	1.7%	0.8%	2.5%	----
(% acetylation) 1ug WT pLE-2	85.2%	53.3%	33%	16.8%	12.8%	16.8%	11.5%	90.5%	----

Experiments (a-f) were performed as described in Methods and correspond to Figure 13. Relative activities with respect to WT pLE-2 (1ug) are indicated. Percent acetylation with pKCAT-23 (5ug) and with WT pLE-2 (1ug) are indicated.

$$\text{relative activity} = \frac{\%\ \text{AC:CAM with mutant} - \%\ \text{AC:CAM of pKCAT-23}}{\%\ \text{AC:CAM WITH WT pLE-2} - \%\ \text{AC:CAM of pKCAT-23}} \times 100$$

@ 16ug dl1115 were used. This figure is therefore not used in calculating the mean

* expt. (f) dl1113 was used as the co-transfecting plasmid

** expts. (g-h) were performed as described in Methods with the exception that 0.5ug pLE-2 were used throughout the experiment in place of 1ug pLE-2 (Corresponding graphs are shown in Figure 15.

Figure 13. Graphs of dl1115 transactivation blocking assays.

Graphs represent data presented in Table 3 (a-f).

expt. (f) was done with dl1113 as the competing plasmid.

_____ WT pLE-2

---------mutant

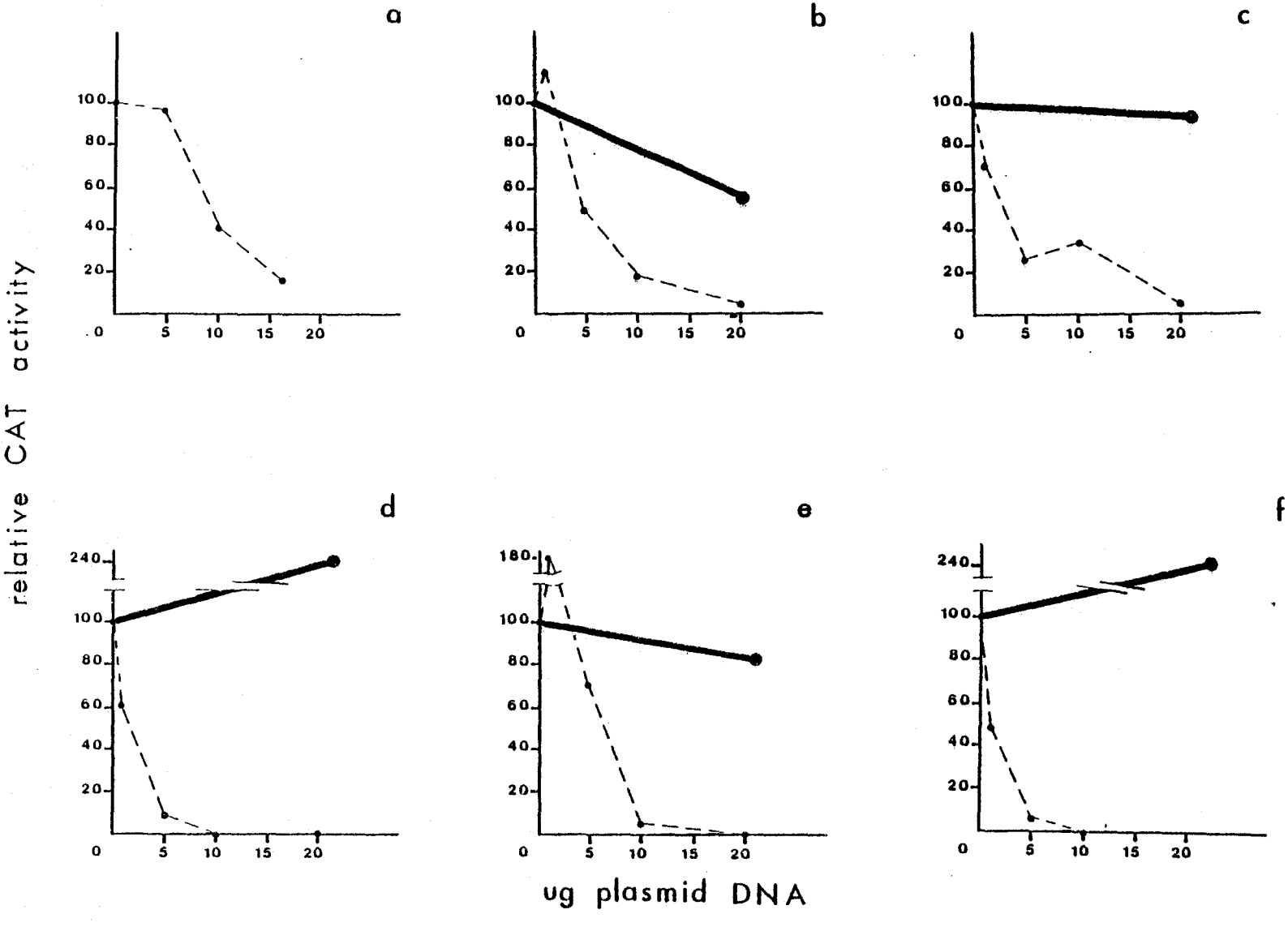

a
b
c
d
e
f
relative CAT activity
ug plasmid DNA
0
5
10
15
20
40
60
80
100
180
240

Figure 14. Graph of mean dl1115 transactivation blocking activity.

Graph represent mean of data presented in Table 3 (a-e).

_____ WT pLE-2

---------- dl1115

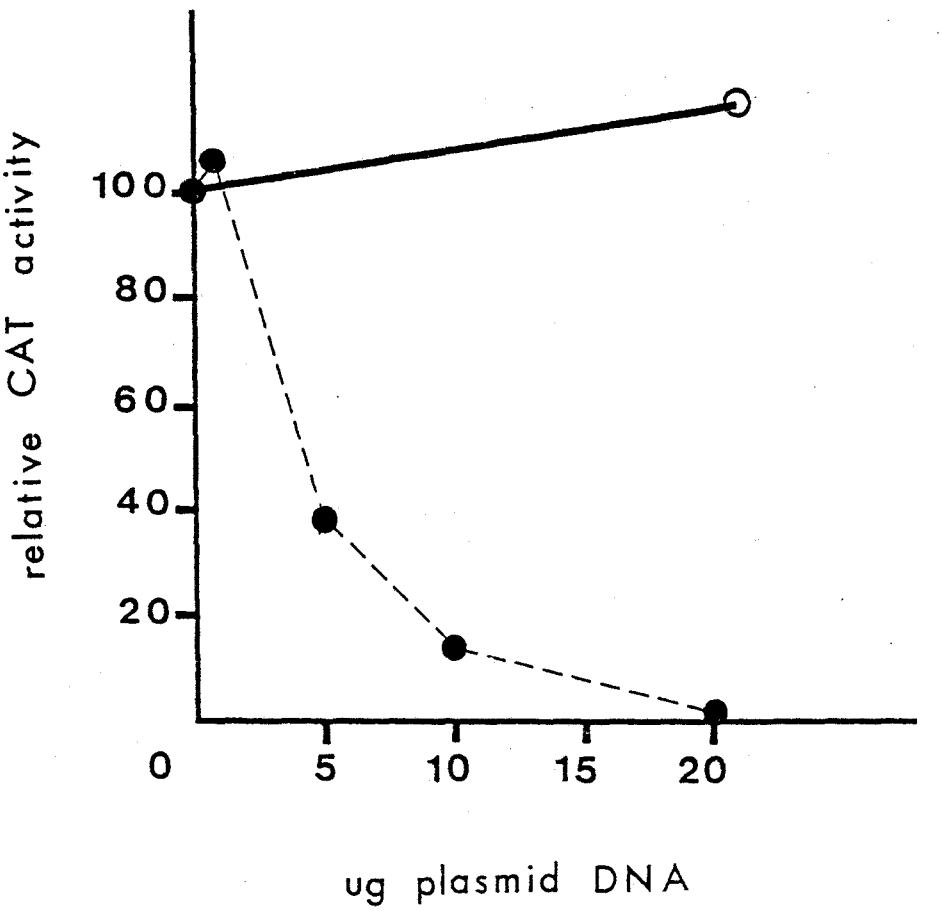
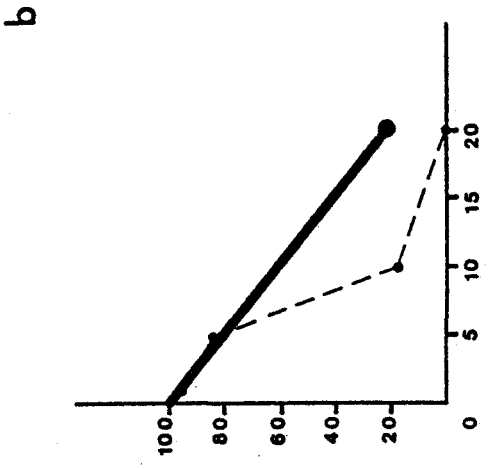

relative CAT activity
100
80
60
40
20
0
5
10
15
20
ug plasmid DNA

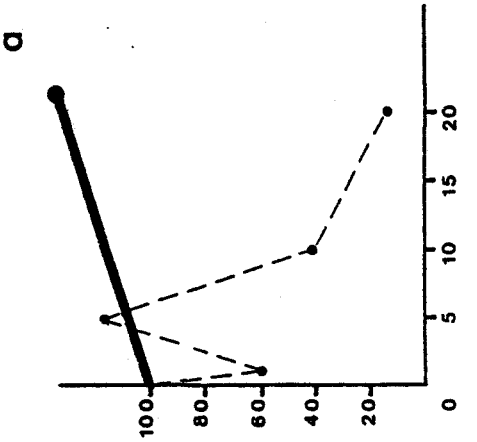
Figure 15. Graphs of dl1115 transactivation blocking assays. (modified, see discussion p.102)

Graphs represent data presented in Table 3 (g-h)

_____WT pLE-2

---------- dl1115


b
100
80
60
40
20
0
5
10
15
20


a
100
80
60
40
20
0
5
10
15
20

(b). Sub1117:pLE-2 weakly blocks wild type E1A transactivation

Sub1117:pLE-2 was assayed for its ability to block wild type pLE-2 transactivation of the E3 promoter in pKCAT-23, in assays similar to those described above. Increasing amounts of sub1117:pLE-2 were assayed as described (Table 4). Again, at low concentrations of mutant plasmid, there was an apparent increase in acetylation in one out of two experiments. The mean of the two experiments indicates that at least a 10 fold molar excess of sub1117 was necessary to produce a 50 percent reduction in pKCAT-23 transactivation (Figures 12B, 16, 17). Over 70 percent inhibition was obtained with 20ug sub1117:pLE-2. The addition of 20ug wild type pLE-2 in place of sub1117:pLE-2 DNA did not inhibit transactivation. Therefore, the amount of cotransfecting plasmid used in these assays was not a factor in the reduction of transactivation efficiencies. These results suggest that sub1117 may be sequestering a cellular protein that, along with E1A, is involved in transactivating the E3 promoter.

Table 4: Sub1117 transactivation blocking assays

ug mutant DNA	a	b	mean
1.0	----	92	92
5.0	134	81	108
10.0	83	24	54
20.0	42	15	29
21ug WT pLE-2	98	82	90
5ug pKCAT-23 (% acetylation)	1.5%	0.2%	-----
1ug WT pLE-2 (% acetylation)	53.3%	12.8%	-----

Assays were performed as described in Methods.
Relative activities with increasing concentrations of mutant plasmid DNA are shown.
Percent acetylation with pKCAT-23(5ug) alone and with pLE-2 (1ug) are indicated.
See Table 3 for calculation of relative activity.
Corresponding graphical representation of data is presented in Figure 16 and 17.

Figure 16. Graphs of sub1117 transactivation blocking assays.

Graphs represent data presented in Table 4.

_____ WT pLE-2

---------- sub1117

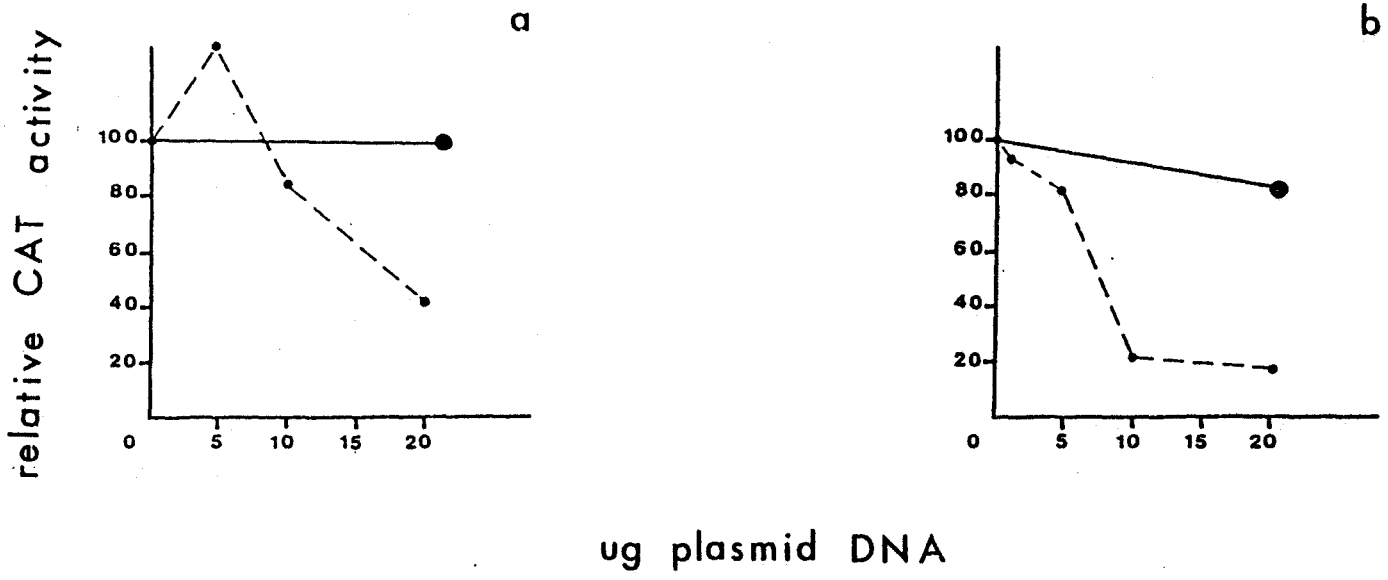
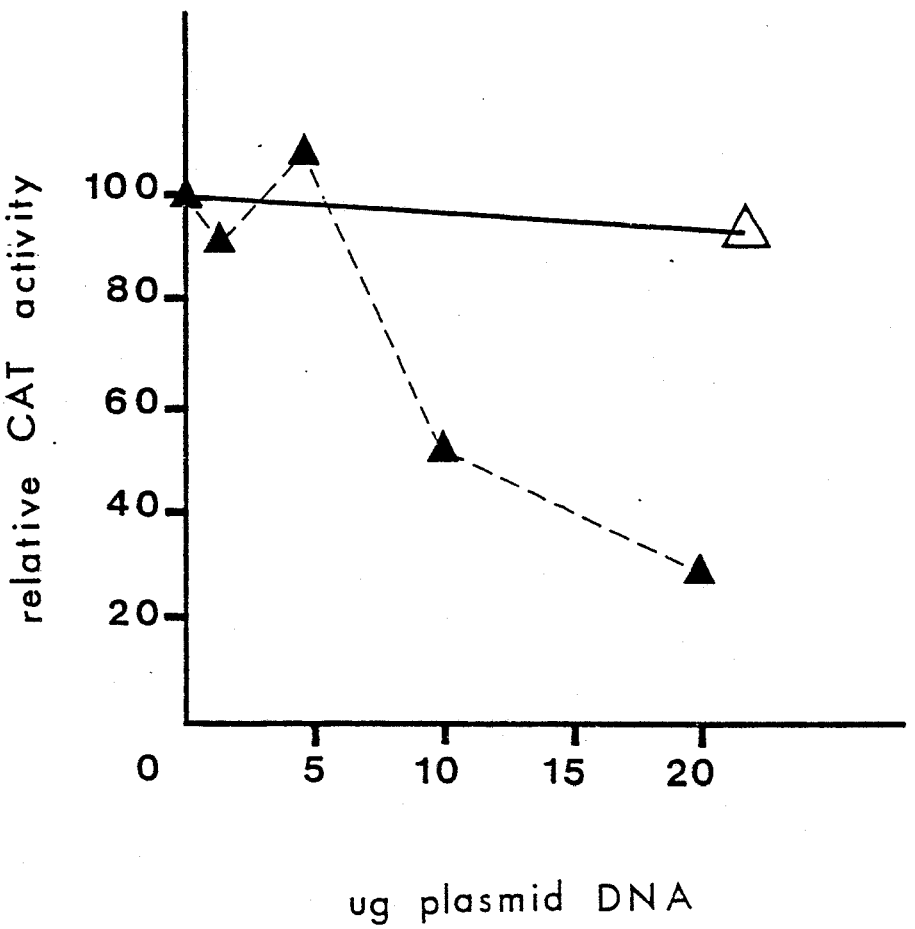

a
b
relative CAT activity
100
80
60
40
20
0
5
10
15
20
ug plasmid DNA

Figure 17. Graph of mean sub1117 transactivation blocking activity. Graph represents mean of data presented in Table 4 and Fig. 16.

_____ WT pLE-2

---------- sub1117


relative CAT activity
100
80
60
40
20
0
5
10
15
20
ug plasmid DNA

(d). IN819 does not block E1A mediated transactivation

To test whether the transactivation blocking properties of dl1115 and sub1117 were due to plasmid sequences other than those in the mutated regions or to a plasmid concentration effect, an E1A insertion mutant that codes for a severely truncated polypeptide was used in transactivation blocking assays. IN819 was originally created by Dody Bautista (McMaster University) by linker insertion mutagenesis. The 39 bases inserted at Ad5 E1A nucleotide position 819 code for two missense residues followed by a termination codon at residue 89. The upstream enhancer-promoter region in this mutant is fully intact. Tony Jelsma (McMaster University) subcloned this mutant into pLE-2. In a transient expression assay, IN819:pLE-2 (1ug) was unable to transactivate the E3 promoter in pKCAT-23 (5ug) (data not shown).

In transactivation blocking experiments, results with this mutant were variable as the percent acetylation of pLE-2 (1ug) with IN818 was higher than with pLE-2 (1ug) alone. The addition of more wild type pLE-2 (20ug) gave similar increases in acetylation. This may have been due to a variability in transfection efficiencies in the dishes. In three transactivation blocking assays up to a 20 fold molar excess of pIN819 was insufficient to block wild type E1A transactivation of the E3 promoter (Figures 18, 19 and Table 5). These results suggest that transactivation blocking properties of dl1115 and sub1117, especially at higher plasmid concentrations, are not due to an effect on transfection efficiencies.

Figure 18 Autoradiogram of transactivation blocking assay with IN818. 100mm dishes of HeLa cells were transfected with 20ug sonicated salmon sperm DNA, 5ug pKCAT-23. Lanes a-d,f,g contained 1ug wild type pLE-2, lane g contained an additional 20ug pLE-2. Lanes a-d contained increasing amounts of IN818 as follows:
(a) 1ug; (b) 5ug ; (c) 10ug ; (d) 20 ug;
Cell extracts were incubated in the presence of acetyl CoA and [14C]-chloramphenicol for 30 min as described. Acetylated and unacetylated forms of chloramphenicol were separated by chromatography on silica gel plates and autoradiographed as described.

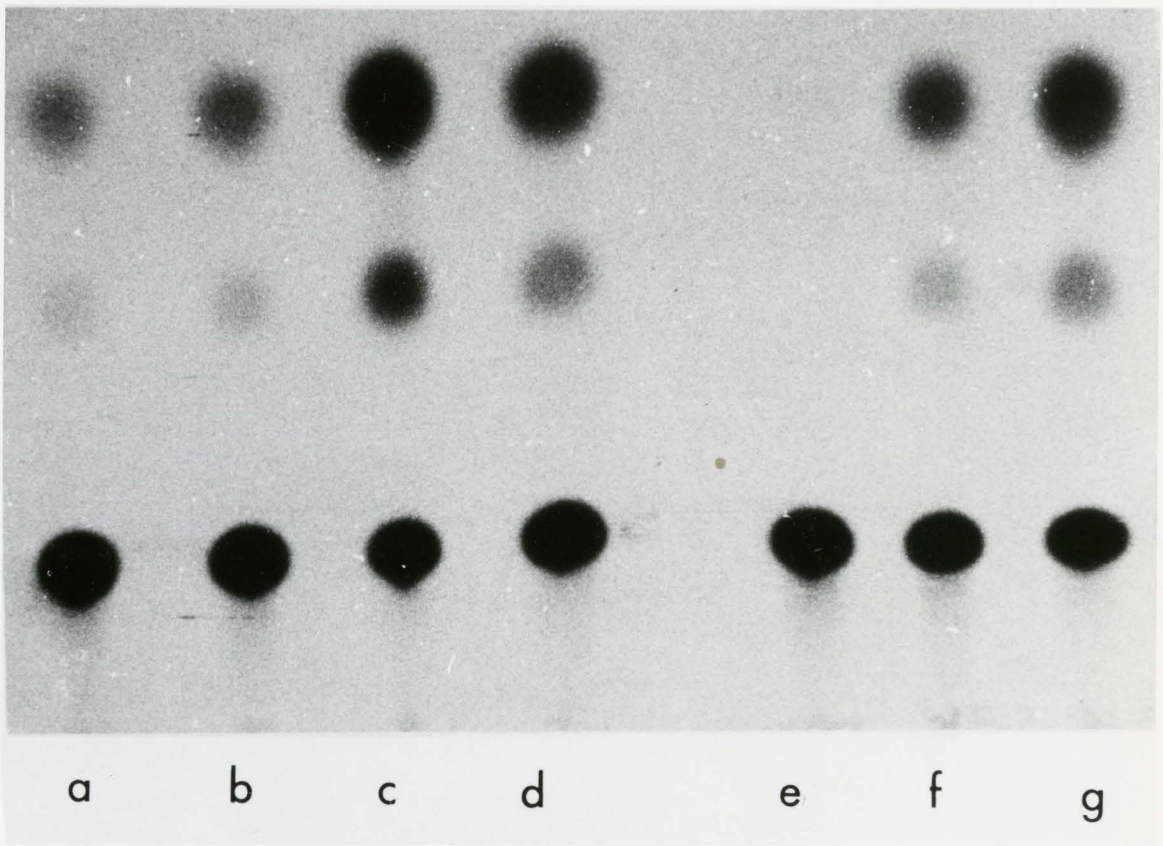

a
b
c
d
e
f
g

Table 5: IN818 transactivation blocking assays

ug mutant DNA	a	b	c
1.0	18	51	103
5.0	---	75	77
10.0	418	415	108
20.0	302	213	51
21ug WT pLE-2	367	213	69
5ug pKCAT-23 (%acetylation)	0.6%	0.4%	0.3%
1ug WT pLE-2 (%acetylation)	14%	12%	43%

Assays were performed as described in Methods.
Relative activities with increasing concentrations of mutant plasmid DNA are shown.
Percent acetylation with pKCAT-23(5ug) alone and with pLE-2 (1ug) are indicated.
See Table 3 for calculation of relative activity

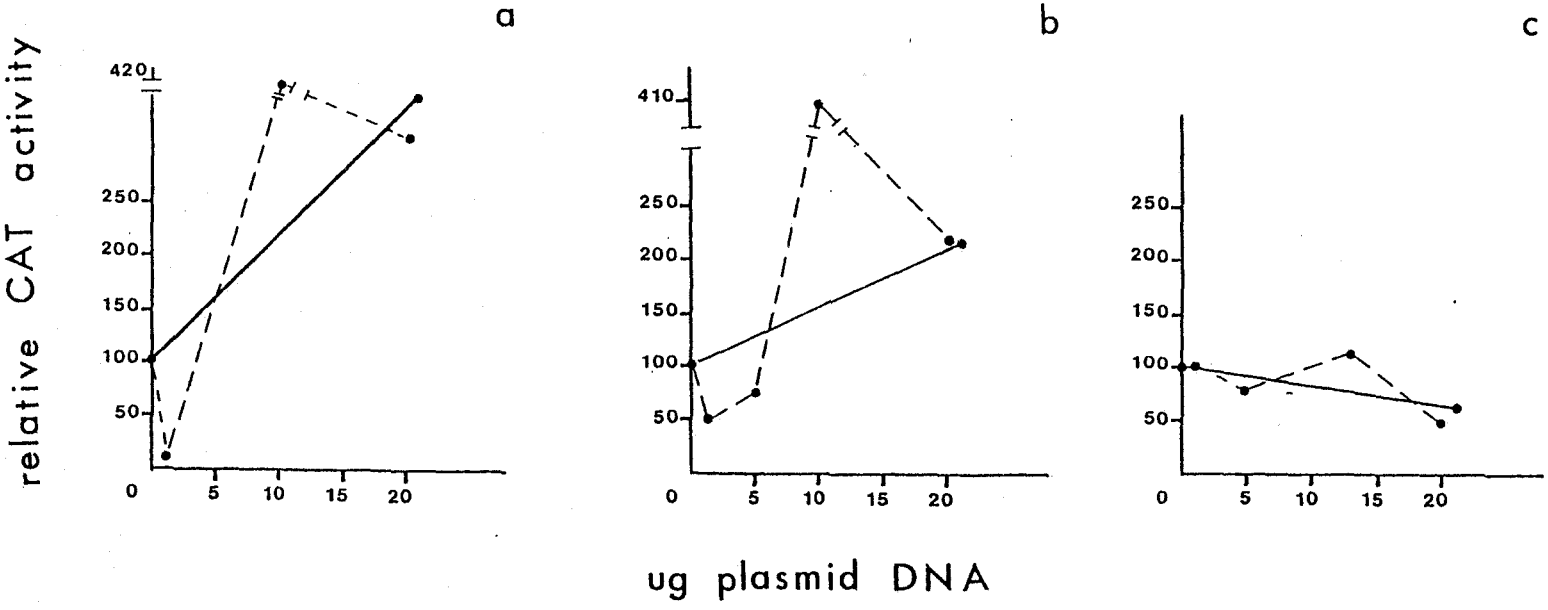
Corresponding graphical representation of data is presented in Figure 19.

Figure 19. Graphs of IN818 transactivation blocking assays.

Graphs represent data presented in Table 5 (a-c).

_____ WT pLE-2

---------- pIN818


a
b
c
relative CAT activity
420
410
250
200
150
100
50
0
5
10
15
20
ug plasmid DNA

PART 4: DISCUSSION

I. Transactivation

Our lab has constructed a series of deletions and point mutations spanning the entire coding region of the Ad5 E1A gene. Small in frame deletions are an efficient way to survey a gene the size of E1A. Areas of the protein that seem interesting can then be altered with point mutations. In fact, deletion mutants can be used to make point mutations in the area that is deleted on wild type plasmid, using the technique of Kalderon et al., (1982). This technique has already been used in our lab to create point mutations in the 13S unique region (Jelsma et al., 1988).

To study the role of amino acids 188-289 in exon-2 of E1A, two in frame deletion mutants and one point mutation were constructed. M13mp11 cl#5, an M13 vector carrying the Bam HI to XbaI fragment of pHE-1 was mutated with oligomers, using the site directed mutagenesis technique of Zoller and Smith (1984). Dl1115 (dl aa 188-204) and dl1116 (dl aa 205-221) were designed to remove small regions from the 5' end of the second exon of E1A. Pm1131 creates a stop codon in place of the codon specifying serine-219 and effectively deletes amino acids 219-289. Sub1117, which arose fortuitously, removes the 3' splice site of E1A and effectively deletes residues 186-289. The mutants created in this study can be used to study the role of the residues encoded by the second exon, in E1A mediated events.

The exon-2 mutants were assayed for their ability to stimulate transcription from the adenovirus 5 early region 3 promoter in

pKCAT-23. In plasmid based transient expression assays, HeLa cell monolayers were cotransfected with salmon sperm DNA, pKCAT-23 and E1A plasmid DNA coprecipitates. Assays were performed to indirectly measure the ability of mutant E1A to stimulate transcription of the CAT gene.

Dl1115 was shown to eliminate the transactivation function of E1A, as determined by the transient expression assays. Dl1115 affects residues 188-204 in both the 12S and 13S mRNA products and is the 3'-most mutant shown to eliminate the transactivation function of E1A. Sub1117 also eliminates the transactivation function. Deletion of residues 205-221 (dl1116) had no effect on transcriptional activation as determined with the CAT assay. Pm1131 effectively deletes residues 219-289 in both the 12S and 13S mRNA products by introducing a stop codon in place of the codon specifying serine-219. Transient expression assays with this mutant show that it can transactivate the E3 promoter, but at less than wild type efficiency. In eight out of nine experiments, pm1131 was shown to have only 17% of the transactivating potential of wild type E1A (Table 2). In one CAT assay this mutant was wild type for transactivation, but this result was not reproducible. Pm1131 is unusual in that this mutant decreases but does not completely eliminate the transactivation function of E1A. Furthermore, the region deleted is far from CR3.

Dl1115 and dl1116 remove hydrophilic and hydrophobic regions in the E1A proteins respectively. In making alterations in a protein sequence, it is recognized that a given function may be lost due to a deletion of an active site, or to an alteration in the conformation of

the protein. However, small deletions have been made in several regions of the E1A coding region without any effect on transcriptional activation or transformation (Jelsma et al., 1988; this thesis). This would indicate that the E1A proteins can withstand small alterations in their structure. The results obtained with the CAT assays indicate that some of the residues between positions 187 and 205 are absolutely critical for the transactivation function of E1A, in such an assay. CAT assay results with other mutants constructed and assayed in our lab, along with the results presented in this thesis, define the transactivation domain of E1A as starting between residues 138-147 and ending between 188-204.

It can be predicted from the sequence comparisons of Kimmelman et al. (1985) that the 3' boundary of the Ad5 E1A transactivating domain lies very close to glutamine-191, as that residue is common to human and simian adenovirus serotypes (Figure 3B). Kimmelman et al. (1985) has shown that the E1A proteins of several human adenovirus serotypes and a simian adenovirus share several regions of homology. The Ad5 13S unique region residues share 13 out of 21 residues (from 171 to 191) with other human and simian adenovirus serotypes (Figure 3B).

Lillie et al. (1987) synthesized a 49 residue polypeptide containing the amino acids between residues 139 and 189 of the 13S mRNA product. This peptide was microinjected, along with a plasmid carrying the E2A gene, into HeLa cells. Fifty five percent of the cells produced the E2A polypeptide, as detected by indirect immunofluorescence.

Coinjection of the E2A gene and a plasmid containing the wild type E1A gene was equally efficient in stimulating E2A protein (DBP) production.

In a complementation assay, HeLa cells were microinjected with the 49R peptide and then infected with dl312. Thirty-eight percent of the cells produced the 72K DBP as determined by indirect immunofluorescence. Peptides from conserved region one or two where unable to complement the E1A defect in dl312 (Lillie et al., 1987).

It is clear from these experiments that the 49R peptide has transcriptional activating properties. These results suggest that the transactivation domain starts at position 140 and ends at residue 188 in the E1A protein. However, since the exact amount of 49R peptide used to activate the E2 gene is not stated, and because purified E1A protein was not used in control experiments, it is not possible to make a comparison of the efficiency of the 49R peptide transactivation with respect to wild type E1A transactivation. Point mutations at the 5' and 3' ends of the known transactivation domain would help to further define its boundaries.

Results in our lab have shown that small stretches of the E1A coding region upstream and downstream of the transactivation domain can be deleted without eliminating the transactivation function (Jelsma et al., 1988). It can therefore be predicted that all the residues flanking the transactivation domain can be removed without affecting the transactivation function. As reported in Jelsma et al. (1988), deletion of residues 4-140 and 219-289 results in a loss of the transactivation

function. The discrepancy between these results and those of Lillie et al. may be due to differences in the amount of polypeptide assayed.

The results obtained with pm1131 contradict results obtained by Schneider et al. (1987) with a similar mutant GCE-R. GCE-R, which replaces residues 223-289 with a serine, was shown to be wild type for transactivation. Several possibilities can account for the discrepancy between the results obtained with pm1131 and GCE-R. Pm1131 and GCE-R differ by at least five C-terminal amino acids (219-223), however three of the five residues have been shown to be dispensable for transactivation, as they are deleted in dl1116 (205-221) without any effect.

If deletion of the C-terminal rapid nuclear localization signal in pm1131 results in a decrease in transactivation efficiency, then perhaps the C-terminal residues in GCE-R act as a substitute rapid nuclear localization signal. Richter et al. (1985) reported that residues 23-120 contain a slow nuclear localization signal. C-terminal mutants, such as pm1131, with this region still intact can migrate to the nucleus, but at a slower rate.

It is not clear if the E1A coding region in GCE-R ends in a stop codon or if translation can proceed into pBR322 specified message. If the latter is the case, then any additional non-E1A specified amino acids may be providing a function deleted in pm1131. Alternatively, it may be that the protein conformation of pm1131 is such that the

transactivating domain is modified to a small extent resulting in a reduction of transactivation.

Because the number of residues deleted in pm1131 is fairly large, it is difficult to draw a conclusion as to which missing residues are responsible for the decrease in transactivation efficiency. A series of smaller deletions including one removing only the rapid nuclear localization signal and a construct of pm1131 with the rapid nuclear localization signal at its C-terminus would be useful in determining why this mutant is transactivation defective.

Krippl et al (1985) reported that mutant E1A proteins lacking the C-terminal end of E1A were slow to localize to the nucleus when microinjected into the cytoplasm of Vero cells. Jones and Shenk (1979) reported the effect of deleting the C-terminal end of the E1A polypeptides in adenovirus infections. D1311 (dl 1280-1339) alters the reading frame and had up to a 1000 fold reduction in plaque forming units when grown on HeLa cells. Hearing and Shenk (1985) reported that this and other mutants with altered reading frames are defective for growth on HeLa cells and have at least a five-fold reduction in cytoplasmic mRNA as compared to wild type adenovirus infection. D1310 (dl 1324-1350), an in frame deletion mutant, is wild type for growth on HeLa cells and produces wild type levels of early mRNA's. Hearing and Shenk suggested that premature termination of translation in truncated mutants may cause the mRNA's to get degraded. This may also explain why pm1131 has reduced transactivating properties. Rescue of this mutant

into adenovirus and analysis of mRNA and protein production from virus infected cells may yield more conclusive results.

II. **Transactivation blocking**

Glenn and Ricciardi (1987) found that hr5, but not hr3 or hr4, can block wild type E1A transactivation of the E3 and E4 promoters in transient expression assays. These workers postulated that the mutant hr5 protein sequesters cellular proteins that normally interact with wild type E1A to activate transcription. These cellular proteins can interact with hr5 and therefore become unavailable to the wild type E1A.

Dl1115 and sub1117 were tested in similar transient expression assays for their ability to block wild type E1A transactivation of the E3 promoter in pKCAT-23. When compared to hr5, dl1115 and sub1117 were less efficient at blocking transactivation. At low concentrations of mutant cotransfecting plasmid, there was significant variation. At low concentrations of mutant competitor DNA, there was an apparent increase in activity. It is not clear why this happened but the apparent increase in acetylation may have been due to a variation in the transfection efficiencies of the HeLa cells. At the highest concentration of dl1115 (20-fold molar excess) transactivation was completely blocked. The standard deviation at that concentration for five experiments was less than five. Significantly, equivalent amounts of wild type E1A or IN819:pLE-2 (dl aa 87-289), which codes for a severely truncated E1A

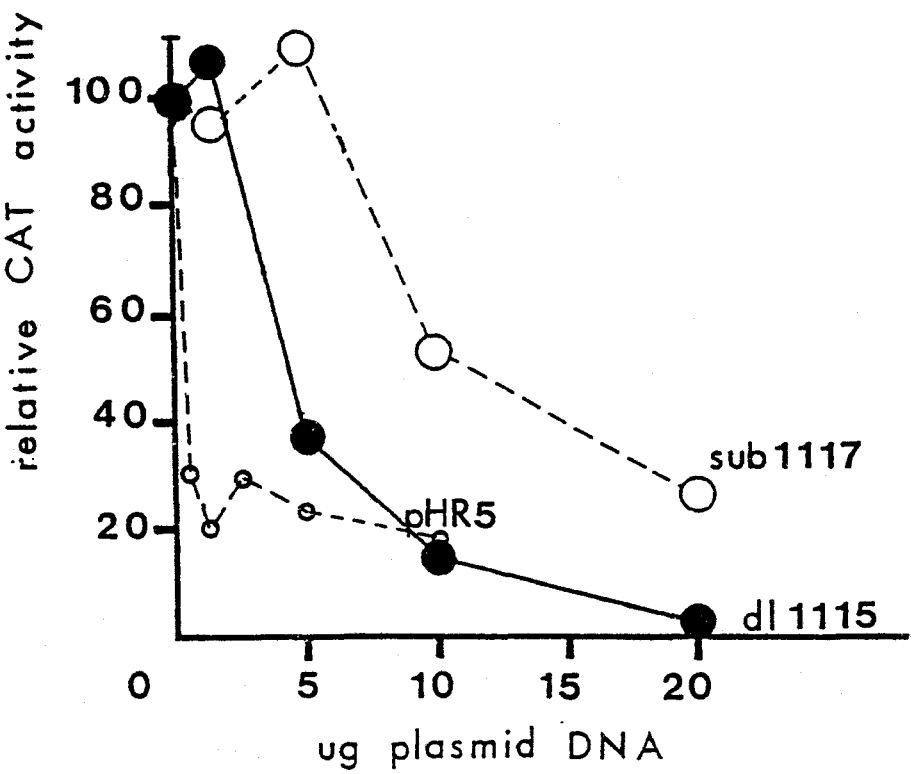
protein, were unable to reduce conversion of CAM in these assays (Figure 19).

The condition of the HeLa cell monolayer and the quality of the DNA coprecipitate appeared to affect the transfection efficiency. When acetylation of CAM is high transactivation blocking cannot be readily detected. In an attempt to overcome this problem, the amount of transactivating wild type pLE-2 was decreased from 1ug to 0.5ug. Results obtained in these assays did not differ significantly from previous assays (Figure 15), as the overall percent acetylation of CAM was still high. Perhaps a further reduction of the wild type pLE-2 concentration would give more reproducible results. Rando et al. (1986) experienced similar difficulties in transactivation blocking experiments utilizing HPV expression vectors. At low concentrations of competing plasmid, there was an apparent increase in activity.

As determined by calculating the plasmid concentration required to produce 50 percent inhibition (Figure 20), dl1115 was five fold less efficient and sub1117 was at least ten fold less efficient than hr5 in blocking E1A mediated transactivation. In one assay dl1113:pLE-2 (dl aa 169-177, created by N. Cunniff) appeared to block transactivation as well as hr5 (Figure 13, f).

The fact that E1A acts on non-homologous promoter-enhancer sequences, but does not actually bind to promoters (Feldman et al., 1982) also points to the possibility of a cellular co-factor involved in E1A mediated transactivation. It has been known for some time that the

Figure 20. Relative transactivation blocking activities of hr5, dl1115, sub1117.


relative CAT activity
100
80
60
40
20
0
5
10
15
20
ug plasmid DNA
sub 1117
pHR5
dl 1115

E1A transactivation deficiency in dl312 can be complemented by other viral tumor antigens such as the pseudorabies and HSV antigens (Feldman et al., 1982; Person et al., 1985). Imperiale et al. (1984) found that a factor in F9 murine embryonal stem cells could also complement this deficiency in dl312. Thangue and Rigby (1987) showed that these cells contain a cellular factor that is analogous to E1A and may be involved in cellular differentiation. E1A has been shown to induce differentiation in undifferentated F9 cells (Montano and Lane, 1987). It seems unlikely that E1A acts on specific genes but rather transactivates and represses sets of genes by interacting with cellular proteins.

Two cellular factors, EPF and E2F, have been identified in HeLa cells (SivaRaman et al., 1986; Yee et al., 1987). These proteins bind to separate sites on the E2 promoter and are induced by E1A. Cellular proteins that form complexes with E1A may have a role in transformation and have been detected with E1A antibodies (Egan et al., 1987; Harlow et al., 1986).

The mechanism of action of E1A may be affected by the differential affinity of the 243R and 289R proteins for various cellular proteins. The resulting E1A-cellular protein complex or perhaps a cellular protein modified by E1A may in turn have varying affinities for promoters or enhancers. In some cases this results in an activation or repression of transcription. Such effects may also provide a mechanism for transformation of cells.

III. Sodium butyrate stimulates transcription from the E3 promoter

HeLa cells were transfected with pKCAT-23 (5ug) and sonicated salmon sperm DNA (20ug) and were incubated in the presence of increasing concentrations of NaB. Enzyme activity was assayed as described previously. Acetylation of chloramphenicol was directly proportional to the concentration of NaB in the medium (Figure 9B, and 11). These results indicate that sodium butyrate activates transcription from the E3 promoter.

Sodium butyrate increases transcription of plasmid constructs containing the SV40 promoter (Gorman et al., 1983). Plasmid constructs without the enhancer had a slight stimulation in transcription in the presence of NaB, but the presence of an enhancer and NaB gave the highest stimulation. The magnitude of this effect varied depending on the cell line used.

The effects on cells incubated in the presence of NaB are numerous. NaB inhibits the action of certain hormones (Martel et al., 1983), enhances the activity of some enzymes and enhances interferon activity in cells incubated in its presence. NaB induces transcription of the rat metallothionein-I gene (Birren and Herschman, 1986), the human β and γ-globin genes in transient expression assays (Partington et al., 1984) and has been shown to induce erythroid cell differentiation (Leder and Leder, 1975). Sodium butyrate also reversibly blocks cell division and DNA synthesis (Daniell, 1980). Sendai virus and vesicular stomatitis virus replication is inhibited by sodium butyrate

(Ginsberg et al., 1973), but HeLa cells incubated in its presence can succesfully support lytic adenovirus 5 infection (Daniell, 1980).

NaB action on gene transcription has been attributed to hyperacetylation of histone proteins. This is thought to relax the chromatin stucture and allow protein complexes to initiate transcription. However, the evidence for such a model is not complete and cannot explain all of the NaB induced effects.

Transformed cell lines incubated with NaB lose some of their transformation phenotype characteristics. The cells regain the transformed morphology when the sodium butyrate is removed. This fact coupled with the fact that NaB stimulates transcription makes it tempting to speculate on a more direct role for this compound in transcriptional activation. Is NaB interacting with a cellular protein involved with transcription, in a manner similar to E1A? Some of the effects of sodium butyrate on cells seem to be analogous to the effects of E1A, yet others seem to be the opposite of E1A effects.

If NaB modifies cellular proteins so that they can transactivate or if it enhances their binding to promoters, it may be a useful tool in the study of activation of transcription in trans. The effects of sodium butyrate on cellular and viral proteins involved with transactivation can be studied. If HeLa cells incubated in the presence of sodium butyrate could support dl312 replication, then it can be argued that NaB may be able to stimulate transcription from viral promoters in a manner analogous to E1A. Further studies on the mechanism of action of NaB transactivation of viral promoters may yield valuable information.

IV. Transformation

It has been demonstrated that mutations in the unique region of the 13S mRNA destroy the transactivating function of the E1A 289R proteins (Ricciardi et al., 1981; Lillie et al., 1986; Schneider et al., 1987; Jelsma et al., 1988). It has also been shown that 13S unique region mutant adenoviruses transform CREF and BRK cells at a greater efficiency than the wild type viruses (Graham et al., 1978; Montell et al., 1984). Although only the 13S mRNA product is required for transactivation, both the 12S and 13S mRNA products are needed for full transformation of rat cells in adenovirus infections. (Montell et al., 1984).

Point mutants in conserved regions two and three affect repression and transactivation, respectively (Lillie et al., 1986; 1987). Schneider et al. (1987) studied the relationship between transformation, repression and transactivation. Deletion and substitution mutants were assayed for transactivation and transformation functions. Mutations that affected transformation mapped to CR1 and CR2 also affected the ability of E1A to repress the SV40 enhancer. The authors concluded that the E1A transformation function is not linked to the transactivation function, and that the transformation and repression functions were closely associated.

Exon-2 mutants constructed in this study were tested for their ability to transform baby rat kidney cells using the technique of Ruley (1983). Sixty millimeter dishes of baby rat kidney cell were

cotransfected with a plasmid containing the EJ ras oncogene and mutant E1A plasmid. Dl1115 in pHE-1 was able to transform BRK cells at a higher efficiency than wild type E1A in one assay (data not shown). All the exon-2 mutants tested, in pLE-2, transformed BRK cells in cooperation with EJ-ras (unpublished observations, T. Jelsma and C. Evelegh). Dl1116 (205-222) transformed at an efficiency comparable to wild type E1A. Dl1115, sub1117 and pm1131 appeared to transform at an efficiency greater than wild type E1A, but results were not conclusive. Exon-2 mutants have previously been shown to transform BRK cells in cooperation with Ha-ras. Mutants pMX (dl aa 186-289) and GCE-R (dl aa 223-289) of Schneider et al. (1987) transformed BRK cells at least at wild type levels.

Further transformation experiments with dl1115 and pm1131 including assays for cold sensitivity, anchorage independent growth and ability to transform cells in cooperation with E1B in adenovirus will further define the nature of these mutations.

Finally, the positive transformation results obtained with the transactivation defective exon-2 mutants in pLE-2 confirm that the plasmid constructs used in this study can produce active E1A proteins.

PART 5: REFERENCES

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