FUNCTIONAL DOMAINS IN ADENOVIRUS 5 E1A

SEQUENCES IN ADENOVIRUS 5 E1A GENE THAT ARE REQUIRED FOR TRANSCRIPTIONAL ACTIVATION, ENHANCER REPRESSION, AND ONCOGENIC TRANSFORMATION

By

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ABSTRACT

The E1A gene of adenovirus 5 carries out a number of functions in infection and oncogenic transformation, including the transcriptional activation of viral and cellular genes, the repression of transcriptional enhancers, and cooperation with the adenovirus E1B gene or with the *ras* oncogene to transform primary cells. The purpose of this work was to investigate the mechanism of action of E1A, by determining the regions of the proteins that are required for these functions.

Deletion and point mutations were made in the region unique to the larger E1A mRNA, by exonuclease digestion and deletion loop mutagenesis respectively. These mutants and a series of mutants which delete sequences spanning the entire coding region, were examined for their effect on transcriptional activation, enhancer repression, and transformation.

The region which, when deleted, rendered E1A defective for transcriptional activation was found to be confined to the region unique to the 13S mRNA and the beginning of exon 2. Mutations in three regions, all within the 12S exon 1, affected repression activity. The first two, the N terminal region of the protein, and a region,

CR1, conserved between adenovirus serotypes, were essential for repression activity. The third region, at the end of exon 1 of the 12S mRNA, was probably only indirectly involved in repression.

Deletions in three regions of exon 1 resulted in a loss of the transforming function of E1A. The first two corresponded to the regions required for repression, suggesting that enhancer repression is a component of transformation. The third region, containing CR2, also conserved between adenovirus serotypes, is functionally distinct from the other two and appeared to affect the morphology of the transformants. These two functions did not operate efficiently when present on separate plasmids. The 13S unique region and exon 2 were not required for transformation but the loss of the transactivation function of E1A did result in an increased adhesiveness of the transformants.

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Above all I give thanks to my Heavenly Father, Who has given me the ability and desire to do this work, Who has guided and brought me this far, and Who will continue to keep us in His care.

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ABBREVIATIONS

A (e.g. A_{600})	absorbance
Ad 2, 5, 12	adenovirus types 2, 5, 12
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
bp	base pairs
BRK	baby rat kidney cells
CAT	chloramphenicol acetyltransferase
CR1, CR2, CR3	conserved regions 1, 2, 3
C, N Terminal	carboxy, amino terminal
Ci	curies
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
EC cells	embryonal carcinoma cells
EDTA	ethylenediamine tetraacetate
FBS	fetal bovine serum
g/L	grams per litre
Glu	Glutamic acid
Gly	Glycine
HeBs	Hepes buffered saline

hr	host range
Ile	Isoleucine
IPTG	Isopropylthio-beta-galactoside
K, kDa	kilodaltons
K_M	Michaelis-Menten constant
M	molar
m,u	milli (10^{-3}), micro (10^{-6})
Met	Methionine
mol	mole
mRNA	messenger ribonucleic acid
NCS	newborn calf serum
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
Phe	Phenylalanine
Pro	Proline
R	residue
RF	Replicative form
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	Serine
TE	Tris-EDTA
TLC	thin layer chromatography
U	units
Val	Valine
v/v	volume/volume
wt	wild type

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INTRODUCTION

A major concern in medical science today is the high incidence of cancer in society. In the last 150 years, there has been much success in reducing deaths due to infectious diseases by improving hygiene and public health in the 19th century and by vaccinations and antibiotics in the 20th century (Cairns, 1978 p. 8). As a result of this, life expectancy was increased greatly, so that by 1972, over 80% of all deaths in England were in people over 60 years of age. The two major causes of death are now both age-related, namely arterial disease, which includes strokes and heart attacks, and cancer. Combined, these accounted for over 70% of all deaths in the United States in 1968 (Cairns, 1978 p. 10). Since the proportion of old people in the population is increasing steadily, these two causes of death will become even more prominent in the future (Cairns, 1978 p. 13, 14).

While arterial disease directly involves only one, albeit important, part of the body, the circulatory system, this is not the case with cancer. Cancer can have an almost unlimited number of causes, and can arise anywhere in the body. Although it may be then that cancer is actually a class of diseases, all cancers have one underlying theme. Cancers generally arise from a single cell which has

undergone a mutation(s) to free it from the restraints on its growth (Cairns, 1978 pp. 17ff). The cancer cells divide uncontrollably, deprive the body of its nutrients, and eventually kill the host.

While most infectious diseases can be well controlled by proper hygiene and standard medical care, the treatment of cancer has not been as successful. To date, traditional chemotherapy is also relatively ineffective because its toxic effects are directed against all rapidly growing cells, not just cancer cells. As a result, it must be used in limited amounts. Surgery to remove the tumour and radiation to kill the cells of the tumour generally do not kill all of the cells, thus allowing the cancer to regenerate itself.

Clearly much research must be done to study the nature of cancer in order to be better able to understand and treat it.

The time between exposure to a carcinogen and the onset of cancer is usually of the order of decades (Cairns, 1978 p. 144), so it is difficult to study cause and effect relationships in cancer induction. In addition, because tumours generally are not detected until they are well established, it is impossible to study the initial stages of cancer <u>in vivo</u>. A rapid and reproducible model system

mimicking this process is needed to gain insight into carcinogenesis.

The discovery that a human virus, adenovirus serotype 12, could induce tumours following injection into newborn hamsters (Trentin et al., 1962), sparked interest in adenovirus as a possible cancer causing agent. The later realization that adenoviruses could morphologically transform rodent cells in culture (Freeman et al., 1967), and that these transformed cells closely resembled cultured cells taken from adenovirus-induced tumours, allowed the use of transformation by adenovirus as a convenient model system to study carcinogenesis in vitro.

Since the process of tumour induction by adenovirus is not well understood and must involve many factors, as a beginning it is simpler to study one component of tumourigenesis, namely transformation. Transformation is generally considered to be the in vitro analogue of tumourigenesis although there are factors involved in tumourigenesis that are not required for transformation. There are several features generally associated with transformed cells, discussed in Topp et al., (1981, p. 206, 209ff). Transformed cells, in contrast to untransformed cells, have a reduced requirement for serum growth factors, replicate indefinitely, are not subject to contact

inhibition, can grow in the absence of a solid support, and grow in a disorganized manner. Their normal cytoskeletal structure is disrupted so that the cells are small, rounded and with little cytoplasm. While many transformed cell types can induce tumours upon injection into suitable hosts, this is not a prerequisite for transformation.

Studies on the mechanism of action of transformation by adenovirus will provide valuable knowledge of the molecular events leading to both transformation and cancer in general.

GENERAL PROPERTIES OF ADENOVIRUSES

Adenoviruses are nonenveloped viruses with a linear double stranded DNA genome of approximately 35,000 bp, to which a terminal protein is attached at the 5' end of each strand. Adenoviruses have been isolated from many different species of mammals, birds, amphibians, and possibly even fish (reviewed in Ishibashi and Yasue, 1984), but the human adenoviruses are the most studied. There are at least 41 distinct human adenovirus serotypes which are associated with a number of different diseases. Adenovirus 5, the serotype used in the work described in this thesis, can cause upper respiratory tract infections (reviewed in Straus, 1984). Several methods exist for the subgrouping of

the different serotypes, but the one most relevant to the research described in this thesis is their ability to induce tumours upon injection into newborn rats (Green, 1970). By this criterion, adenoviruses are classified as highly oncogenic (type A), weakly oncogenic (type B), or nononcogenic (type C). Adenovirus type 12, mentioned earlier, belongs to type A, while adenovirus 5, used in this study is a type C adenovirus. The type C viruses Ad 2 and Ad 5 are the best characterized because they are cultured more readily than other more oncogenic adenoviruses.

The degree of oncogenicity of adenovirus appears to be related to the evasion of the host immune system by the cell transformed with the virus. For example, cells transformed by the nononcogenic type C adenovirus 2 can induce tumours in immunosuppressed rats (Gallimore, 1972) or thymectomized hamsters (Cook et al, 1979), but not in newborn rats or 21 day old hamsters.

The ability of adenoviruses to transform cells has been localized to the leftmost 8 to 10% of the adenovirus genome by two methods. Transfection studies with fragments of adenovirus DNA showed that the leftmost 7% of the viral genome was sufficient for transformation (Graham et al., 1974). Secondly, an examination of adenovirus sequences present in virus transformed cells has revealed that only

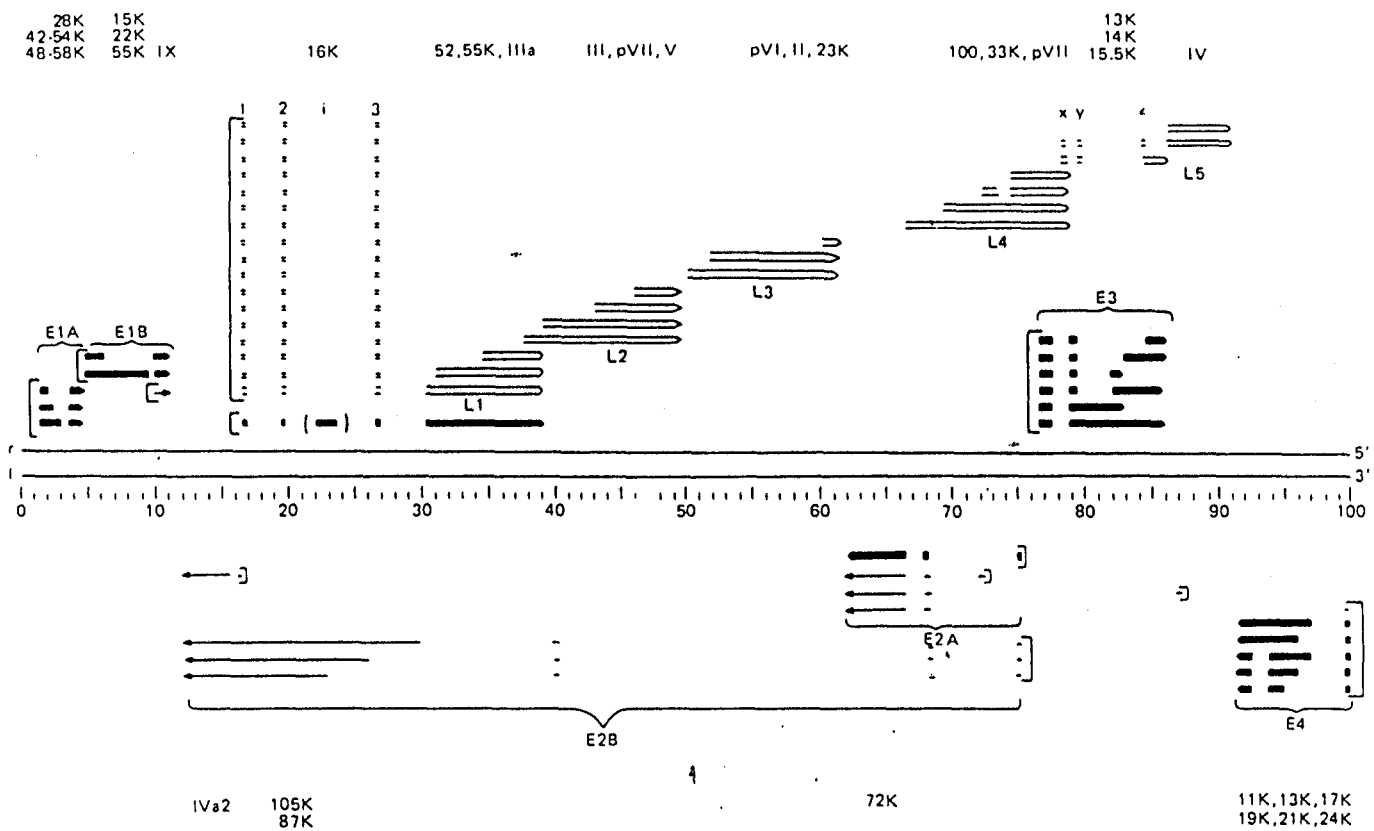
the leftmost 12-14% of the viral genome is consistently present (Gallimore et al., 1974). This region contains two transcription units, E1A and E1B (Wilson et al., 1979). While the entire region of E1B is present in virus transformed cells, only that which is contained in the leftmost 7% of the virus, i.e. the first half of E1B, is required for transformation with DNA fragments (Graham et al., 1974).

STRUCTURE OF ADENOVIRUS GENOME

The various adenoviruses that have been examined in sufficient detail have the same genomic organization. The adenovirus 5 (Ad 5) genome is approximately 36,000 bp in length and is expressed in two phases. Early genes are expressed before viral DNA replication and late genes are expressed afterwards. There are five early transcription units, E1A, E1B, E2, E3, and E4 (Figure 1). Since the work described in this thesis is an examination of the functions of E1A, it will be be described in detail. E1B is also involved in transformation by adenovirus so it will also be described in some detail. The other early regions are not directly relevant to this thesis, so their functions will be only briefly mentioned. The data described here were obtained from studies of Ad 5 or the nearly identical Ad 2.

Figure 1 Map of the adenovirus 2 genome. The adenovirus 2 and 5 genomes are highly homologous and have the same genetic organization. The direction of transcription of the early regions (1A, 1B, 2, 3, and 4) is indicated. Gaps in arrows indicate introns. More detailed maps of E1A and E1B are shown in Figures 2 and 3 respectively. The late transcription unit with its tripartite leader (1, 2, 3) is also indicated. Proteins are designated by their molecular weights in kilodaltons (K) or by Roman numerals (virion components). Taken from Sussenbach (1984).

Figure 1


28K 15K
42-54K 22K
48-58K 55K IX
16K
52,55K, IIIa
III, pVII, V
pVI, II, 23K
100,33K, pVII
13K
14K
15.5K
IV
1 2 i 3
x y
L5
L4
L3
L2
L1
E3
E1A E1B
5'
3'
0 10 20 30 40 50 60 70 80 90 100
E2A
E2B
E4
IVa2 105K
87K
75K
72K
11K,13K,17K
19K,21K,24K

None of the early gene regions encode only a single polypeptide, but through differential splicing they produce a variety of mRNA's and proteins.

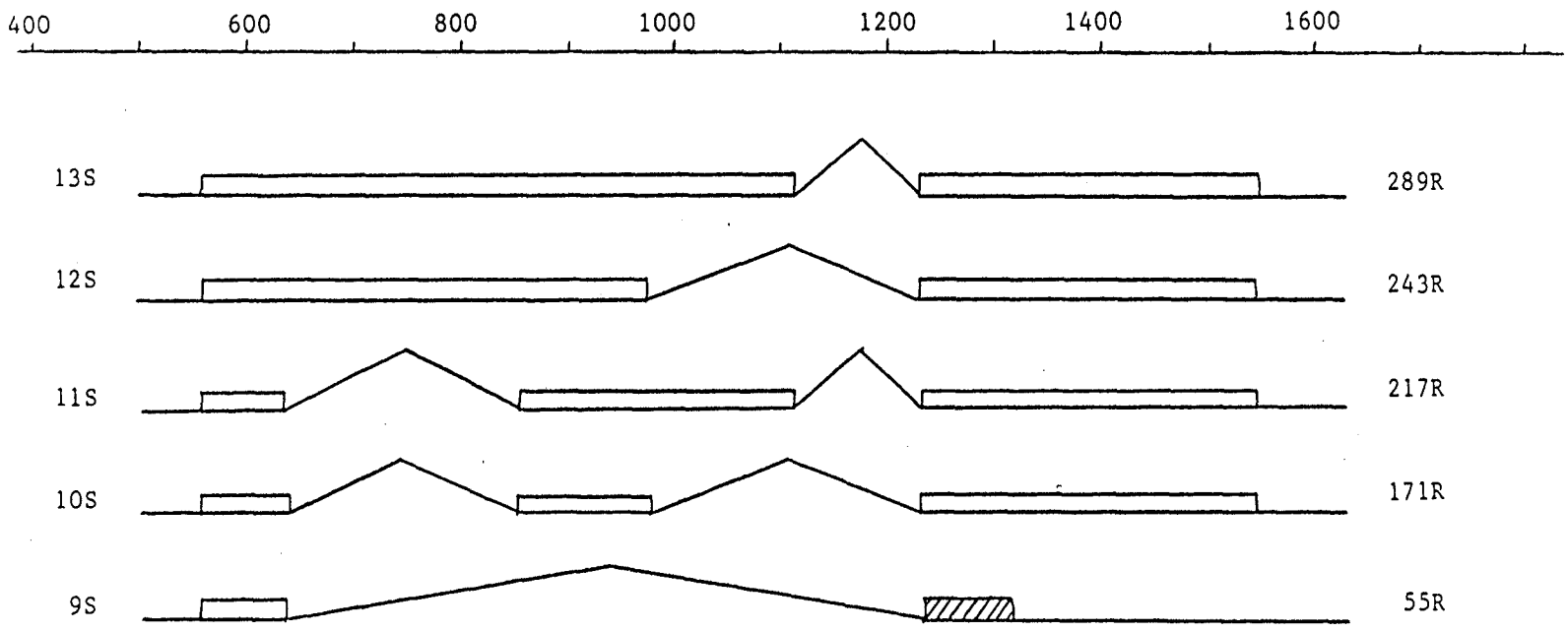
Most of the late transcription is from a single promoter, the major late promoter. The different mRNA's are formed from the primary transcript by the particular choice of the polyadenylation site and by the subsequent splicing pattern (Nevins and Darnell, 1978) (Figure 1). The late mRNA's code primarily for structural proteins of the virion.

a) E1A

E1A is located at the left end of the viral genome, at 1 to 4.5 map units (Figure 1). Five mRNA's are produced by differential splicing of E1A transcripts (Figure 2). The two largest of these, 13S and 12S, are the predominant messages early in infection and appear by 1 hour postinfection (p.i.), reaching maximum levels at 3-4 hours p.i. (Nevins et al., 1979). Late in infection the splicing pattern changes and the 9S mRNA is made in equal amounts to the 13S and 12S mRNA's (Spector et al., 1978). The 11S and 10S mRNA's are made in minor amounts and these appear only late in infection (Stephens and Harlow, 1987). The predicted products of the 13S and 12S mRNA's, of 289 amino acid residues (R) and 243R respectively, are identical

Figure 2 Transcripts and predicted products of Ad 5 E1A region. The lines indicate the mRNA's, the carets represent introns. The coding sequences are shown by the boxed regions. The cross-hatching of the coding region of exon 2 of the 9S mRNA indicates this sequence is read in a different reading frame. Except for the introns, all the other coding regions are identical. For further details refer to text.

Figure 2


400
600
800
1000
1200
1400
1600
13S
289R
12S
243R
11S
217R
10S
171R
9S
55R

except for the presence of 46 amino acids in the region unique to the 13S product. The 11S and 10S mRNA's have an additional splice near the beginning of the coding region which does not alter the reading frame. Thus the predicted products are similar to the 13S and 12S products and have molecular masses of 24K and 19K. The 9S mRNA is made by splicing out the sequences between the donor splice site of the first intron of the 10S and 11S mRNA's and the acceptor splice site of the second intron. Since the donor splice site is at the end of a codon (Glu 26) but the acceptor site is within the codon for the 13S and 12S mRNA's (Ser 185 and Gly 139 respectively), the splice of the 9S mRNA induces a frame shift. Thus exon 2 is read from a different frame in the 9S mRNA than the 13S and 12S messages, and the predicted product has a molecular mass of 6K, of which only the amino terminal portion is common to the other E1A proteins.

Although the predicted masses of the 13S and 12S products are 32K and 27K respectively, immunoprecipitates of E1A proteins reveals two major species for each mRNA with apparent molecular masses of 52 and 48.5 kDa (13S), and 50 and 45 kDa (12S). Numerous minor species have also been detected, and indeed Harlow et al. (1985) have resolved over 60 E1A species by high resolution two-dimensional gel electrophoresis. Although many of the less abundant species

may be breakdown products or products of the 11S and 10S mRNA's, much of the heterogeneity is due to the differences in phosphorylation on at least three, and possibly as many as seven sites on the protein (Tremblay et al., 1988). This phosphorylation may account for much of the difference between the predicted and apparent sizes of the E1A proteins as estimated on SDS-PAGE. Another factor that may also contribute to this anomalous migration in SDS-PAGE is the high proline content (16%) which may make the protein more rigid and unable to unfold completely in the presence of SDS.

As will be described in more detail later, the products of E1A are responsible for turning on the expression of the other early regions and for stimulating the host cell metabolism to maximize the production of progeny virus. These functions are carried out by the products of the 13S and 12S mRNA's, since these effects occur early in infection and the 11S, 10S and 9S mRNA's accumulate preferentially late in infection (Esche et al., 1980, Stephens and Harlow, 1987). The functions of the products of the latter mRNA's are not known.

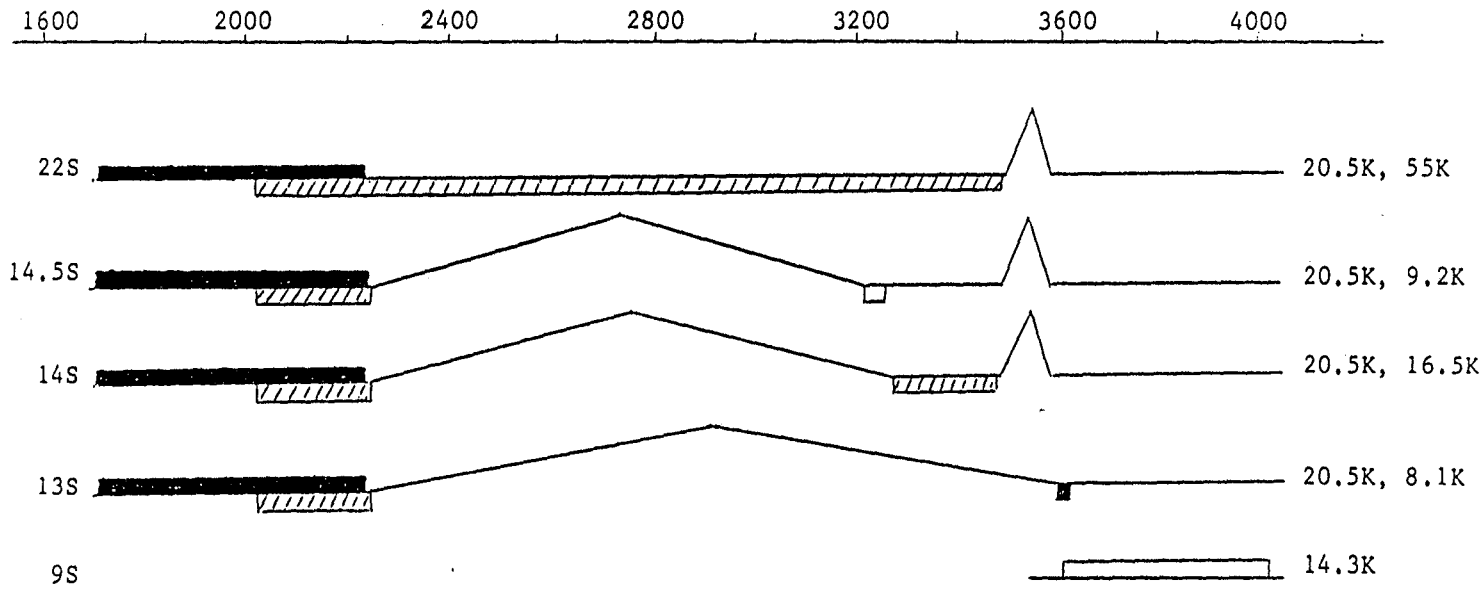
b) E1B

The E1B region is next to E1A, at approximately 4.5 to 11.5 map units (Figure 1). E1B messages first appear at 1.5 to 2 hours p.i., and reach maximum levels at 6 to 7 hour p.i. (Nevins et al., 1979). Early in infection two mRNA's, 22S and 13S, are produced by differential splicing. These mRNA's are made at similar levels early in infection, but late in infection the 13S mRNA becomes twentyfold more abundant (Spector et al., 1978). A third E1B mRNA, 9S, is transcribed late in infection from a separate promoter and codes for the structural polypeptide IX (Spector et al., 1978) which is not involved in the early functions of E1B. Two other minor messages, 14.5S and 14S, have also been identified (Virtanen and Petterson, 1985).

All four early mRNA's have two overlapping open reading frames (Figure 3). The first, coding for a 20.5 kDa protein, is present in the first exon of all four mRNA's. The size of the other open reading frame varies, depending on the mRNA. The molecular masses of the predicted products of this reading frame are 55 kDa, 9.2 kDa, 16.5 kDa, and 8.1 kDa for the 22S, 14.5S, 14S and 13S mRNA's respectively. All four proteins share the N terminal 78 residues in exon 1, but have different carboxy termini except for the

Figure 3 Transcripts and predicted products of the E1B region. The three reading frames are indicated by open, cross-hatched or filled boxes. The 9S mRNA coding for protein IX is produced from a separate promoter. Adapted from Virtanen and Petterson (1985).

Figure 3


1600
2000
2400
2800
3200
3600
4000
22S
20.5K, 55K
14.5S
20.5K, 9.2K
14S
20.5K, 16.5K
13S
20.5K, 8.1K
9S
14.3K

55kDa and 16.5kDa proteins which share the C terminal 107 residues.

The functions of the E1B proteins in infection and transformation are not completely understood. The 55 kDa protein is a predominately nuclear protein (Sarnow et al., 1982a) and appears to be involved in the shutoff of host protein synthesis in infection (Babiss and Ginsberg, 1984) and in the preferential transport or stabilization of viral mRNA's over cellular mRNA's (Pilder et al., 1986). In transformed cells the 55 kDa protein of adenovirus serotypes 2 and 5 (Ad2 and Ad5) but not Ad12, binds to the cellular tumour antigen p53 (Sarnow et al., 1982b), but the significance of this is unknown. Since the leftmost 7% of the viral genome is sufficient for transformation with viral DNA (Graham et al., 1974), an intact 55 kDa protein is not needed for this function. It is not clear however, whether the 20.5 kDa E1B protein alone functions in transformation or whether the N terminal portion of 55K is also required.

The 20.5K E1B protein which is associated with the membrane of infected and transformed cells (Persson et al., 1982) is involved in several functions in infection and transformation. Mutants of this protein induce the degradation of viral and cellular DNA (Pilder et al., 1984; Subramanian et al., 1984), display an enhanced cytopathic

effect (Takemori et al., 1984), form large plaques (Chinnadurai, 1983), and are defective for transformation of cell lines (Chinnadurai, 1983; Barker and Berk, 1987), although this protein may not be obligatory for transformation of primary cells (Bernards et al., 1986; Edbauer et al., 1988). 20.5K can also regulate the expression of E1A (White et al., 1988), but the purpose of this regulation is not clear. The functions of the minor E1B proteins are not known.

c) Other Regions

The products of E2 are involved mainly in the replication of the viral genome. These include an 80 kDa terminal protein, which acts as a primer for DNA replication (Challberg et al., 1982), a 140 kDa DNA polymerase (Stillman et al., 1982) and a 72 kDa DNA binding protein (van der Vliet and Levine, 1973). The 72kDa protein is also involved in regulation of the levels of other early mRNA's (Blanton and Carter, 1979).

Products of the E3 region appear to be involved in the evasion of the infected cell from immune surveillance. E19, an E3 protein, allows the cell to escape lysis by cytolytic T lymphocytes by associating with nascent class I MHC antigens and preventing them from being expressed on the

cell surface (Andersson et al., 1985). Another E3 protein, one of 14.7 kDa, protects the infected cell from lysis by tumour necrosis factor (TNF) (Gooding et al., 1988).

Little is known about the functions of the products of the E4 region. An E4 product can transactivate the E2 and E1B promoters in a manner distinct from that of E1A (Goding et al., 1985; Shiroki et al., 1984). E4 may also play a role in anchorage independance of adenovirus 12 transformed cells (Shiroki et al., 1984). In addition, a 25 kDa E4 protein is associated with the E1B 55 kDa protein during infection (Sarnow et al., 1984). Mutations of this protein result in a phenotype similar to mutants defective in 55K (Halbert et al., 1985) suggesting these two proteins function as a complex.

E1A FUNCTIONS

E1A proteins carry out a number of different functions in infection and transformation. E1A can activate transcription of both viral and cellular genes. These genes are expressed at a low level in the absence of E1A, but E1A proteins greatly stimulate transcription. E1A can also negatively regulate gene expression by repressing the effect of transcriptional enhancer sequences. As mentioned earlier, E1A and E1B can cooperate to transform primary

rodent cells. Some other effects of E1A, which may be involved in transformation are the ability of E1A to induce cellular DNA synthesis, to induce progression through the cell cycle, and to stimulate the proliferation of quiescent cells (Zerler et al., 1987). The primary E1A functions, namely transcriptional activation, enhancer repression and oncogenic transformation, are described in detail below.

a) Transactivation

The best characterized E1A function is its ability to activate transcription of other genes in trans (transactivation).

E1A is the first viral gene expressed in lytic infection and the E1A products turn on the other viral early regions by activating transcription from these genes (Jones and Shenk, 1979; Berk et al., 1979). E1A also transactivates a number of cellular genes (Liu et al., 1985), including topoisomerase I (Chow and Pearson, 1985), several heat shock genes (Wu et al., 1986; Simon et al., 1987), beta-tubulin (Stein and Ziff, 1984), the oncogenes c-fos and c-myc (Sassone-Corsi and Borrelli, 1987), thymidylate synthase, and proliferating cell nuclear antigen (PCNA) (Zerler er al., 1987). E1A also stimulates the expression of class III genes, including the VA genes of

adenovirus (Hoeffler and Roeder, 1985) and exogenous tRNA genes (Gaynor et al., 1985). It is not clear, however, whether the activation of all of these genes is like that of the viral early genes, or whether some genes are activated by a different mechanism, possibly by an indirect effect of E1A. For example, PCNA is efficiently induced by the 12S product of E1A (Zerler et al., 1987), whereas transactivation of the viral early regions is a function of the 13S product only (Montell et al., 1984).

Two approaches have been used to study the mechanism of action of E1A in transactivation. On the one hand, studies such as that described in this thesis focus on the E1A protein itself to determine which regions of the protein are directly involved in the transactivation function.

The approach of others has been to focus on the targets of E1A transactivation, the genes that are activated by E1A, to determine the sequences required and the mechanism of this activation. Although the most obvious mode of action would be for E1A proteins to bind to specific target sequences to activate transcription, numerous attempts have not demonstrated a DNA-binding activity of E1A (discussed in Branton et al., 1985). Thus E1A likely activates genes indirectly, presumably by interacting with

cellular transcription factors and modulating their activity.

Several such factors and the sequences they interact with have been identified (reviewed in Jones et al., 1988) and some are discussed below. These targets of E1A action are not mutually exclusive and any gene may contain different elements or multiple copies of a single target sequence, or both.

"TATA" elements containing the TATAA sequence are targets for E1A transactivation. Some examples of these are the "TATA" boxes of E1B (Wu et al., 1987), the 70kDa heat shock gene (hsp70), c-fos, histone H1 (Simon et al., 1988) and Beta-globin (Green et al., 1983). "TATA" elements not containing the TATAA sequence are not acted on by E1A. These include the SV40 early, E2A (Simon et al., 1988) and E4 (Gilardi and Perricaudet, 1986) genes. (The E2A and E4 genes are transactivated by different mechanisms). These results suggest that not all "TATA" boxes are alike in terms of their function, and that there may be a specific TATAA binding factor which is activated by E1A.

Another target for E1A transactivation contains the sequence (T/G)(T/A)CGTCA. It is bound by a 43 kDa cellular transcription factor designated activating transcription factor (ATF) (Lee et al., 1987). ATF binding sites have

been identified in the upstream sequences of E4 (Lee and Green, 1987), E2, E3 (Lee et al., 1987), E1A (SivaRaman et al., 1986) as well as c-fos, hsp70 and others (Lee et al., 1987).

Since the activation of ATF by E1A is not accompanied by an increase in binding (SivaRaman and Thimmappaya, 1987), presumably the activated ATF has the same affinity for its target sequence, but functions more efficiently.

The ATF binding sequence is also present in the cAMP response element of the somatostatin gene, and the phosphorylation of the 43K protein (possibly ATF) that binds this element was increased 3-4 fold upon stimulation of this gene by cAMP (Montminy and Bilezikjian, 1987). This suggests that E1A transactivation may mimic the cellular system of cyclic AMP-induced gene activation.

Another factor, EIIF, is a 54K protein (Nevins et al., 1988), which is involved in transactivation of E2A and E1A. EIIF binds to the sequence TTTCGCGG, which is present in two copies in both E1A and E2 (SivaRaman and Thimmappaya, 1987). In contrast to what was observed with the activation of ATF, the binding of EIIF is increased severalfold in the presence of E1A. Since transactivation can occur in the absence of protein synthesis (Nevins et al., 1988; Green et

al., 1988), it does not involve an increase in the amount of EIIF, but E1A modifies EIIF to increase its binding activity. Since undifferentiated F9 teratocarcinoma cells also contain this binding activity, and lose it upon differentiation (Reichel et al., 1987), it appears that this activation of EIIF also occurs in undifferentiated cells and is reproduced by the action of E1A.

For the stimulation of RNA polymerase III-transcribed genes, E1A products either directly or indirectly modify transcription factor IIIC (TFIIIC), probably by phosphorylation. This modification of TFIIIC, which can also be induced by serum stimulation, makes TFIIIC more active in transcription by increasing its affinity for its target sequence (Hoeffler et al., 1988).

There are a number of other factors which bind to sequences upstream of E1A-inducible genes, as discussed in Jones et al. (1988), but although they are important for expression of these genes, their role in the induction by E1A is not clear. Once they have been isolated and characterized, the action of E1A on these factors can be examined more directly.

Other transactivating proteins are also able to activate E1A-responsive genes. These include the HTLV II X gene product (Chen et al., 1985), the immediate early

proteins of pseudorabies (Imperiale et al., 1983) and cytomegalovirus (Tevethia and Spector, 1984), and the HPV16 E7 gene product (Phelps et al., 1988). Undifferentiated F9 teratocarcinoma cells also contain an activity that can complement the E1A$^-$ mutant dl 312 in transactivation, as shown by the activation of EIIF in these cells (Reichel et al., 1987; Imperiale et al., 1984); this activity is lost upon differentiation. In addition, preimplantation stage mouse embryos also contain this E1A-like activity which disappears around the time of implantation (Suemori et al., 1988). Thus E1A and the other viral transactivating proteins may imitate a normal cellular protein which functions in undifferentiated cells.

b) Repression

In addition to being able to activate genes, the E1A products can negatively modulate gene activity by repressing the expression of enhancer-driven genes to the levels that would be obtained without the enhancer. Some enhancers which can be repressed by E1A are the SV40, polyoma and adenovirus E1A enhancers (Borrelli et al., 1984, Velcich and Ziff, 1985, Smith et al., 1985, Velcich et al., 1986), the immunoglobin heavy chain enhancer in lymphoid cells (Hen et al., 1985) and the insulin gene enhancer in Beta cells

(Stein and Ziff, 1987). Although the mechanism of enhancer repression is not known, it can be titrated out by excess copies of the enhancer sequence (Borrelli et al., 1984). Thus E1A may activate an endogenous enhancer repressing protein.

A similar enhancer repression activity has been demonstrated in undifferentiated embryonal carcinoma (EC) cells, and is lost upon differentiation (Gorman et al., 1985). Like the E1A-mediated repression activity, it can be titrated out by excess copies of the enhancer sequence. As appears to be the case with transactivation, the enhancer repression function of E1A may mimic a similar function which is present in undifferentiated cells. The loss of this endogenous repression activity upon cell differentiation implies that repression is somehow involved in the maintenance of the undifferentiated state.

c) Transformation

The fact that E1A mimics activities in undifferentiated cells in transactivation and enhancer repression suggests that one of the roles of E1A in adenovirus infection is to return the cell to an undifferentiated state. In this state the cell might be metabolically more active and produce higher yields of

progeny virus. This E1A-induced "undifferentiated" state may be related to the role of E1A in oncogenic transformation. These same functions may be involved in the ability of E1A to suppress the differentiation of myoblasts in culture (Webster et al., 1988).

Upon infection of nonpermissive rodent cells by adenovirus, the infection process is aborted, and progeny virus is not made. The expression of the E1A and E1B products however, can lead to oncogenic transformation at a low frequency.

As described earlier, the E1A and E1B genes of adenovirus are both necessary and sufficient for transformation by adenovirus. Cells can be immortalized by E1A alone, but their morphology resembles that of untransformed cells (Houweling et al., 1980; Ruley, 1983) and E1B is needed for the expression of the fully transformed phenotype. This cooperation of two transforming genes in transformation is also seen with a number of other oncogenes, which can be divided into two general classes. One group of genes functions in immortalization and includes E1A (Houweling et al., 1980, Ruley, 1983), v-myc, polyomavirus large T (Land et al., 1983), and p53 (Jenkins et al., 1984). The second class of genes is involved in morphological transformation and includes ras, polyomavirus

mid T (Land et al., 1983, Ruley, 1983), and E1B. The assignment of E1B to the latter group is complicated by the fact that E1B requires E1A for its expression, but by virtue of its cooperation with E1A, it can be assigned to this group. Although it is not clear whether the products of all the genes in each group function in the same manner, it is intriguing that the products of the immortalization genes are localized in the nucleus (Lyons et al., 1987, Donner et al., 1982, Richardson et al., 1986, Dippold et al., 1981), while each of the transformation group genes produces proteins that are membrane-associated (Ito et al., 1977; White et al., 1984; Willingham et al., 1980). This hints at a similar mode of action for the members of each complementation group.

The exact function(s) of E1A in transformation is not clear. E1A does bring about the establishment or immortalization of cells (Houweling et al., 1980, Ruley et al., 1983), but E1A also plays a role in transformation since established cell lines are not morphologically transformed by ras alone (Franza et al., 1986). High level expression of E1A can elicit alterations in cell morphology (Senear and Lewis, 1986; Roberts et al., 1985; van den Elsen et al., 1985) which may contribute to the process of transformation but it is not known to what extent these

alterations in morphology overlap those generally attributed to E1B or *ras*.

The ability of E1A to stimulate cellular DNA synthesis, induce mitosis, and stimulate the proliferation of quiescent cells (Zerler et al., 1987) may also be components of the actions of E1A in transformation.

BASIS OF EXPERIMENTAL WORK IN THIS THESIS

Tumourigenesis is a multistep process and therefore is difficult to study as a whole. To begin to understand the molecular events that are associated with tumourigenesis, a rapid and easily controlled model system is needed. Transformation by adenovirus is a relatively convenient *in vitro* model system in which at least two components of tumourigenesis, namely cellular immortalization, and the change in cell morphology can be examined. The work described in this thesis is an examination of the role of E1A in transformation. E1A carries out a number of functions in viral infection and transformation. Three E1A functions were investigated in this work, namely transcriptional activation, enhancer repression and the ability to cooperate with the *ras* oncogene to transform cells. The regions of the E1A protein that are involved in these functions were examined with a

series of site-specific mutations spanning the entire E1A coding region. By determining the regions of E1A that were required for each of these activities, the relationships between these functions were investigated. In particular, these experiments examined the involvement of transcriptional activation and enhancer repression in the transformation function of E1A as a means of elucidating some of the molecular events comprising transformation and by extrapolation, tumourigenesis.

MATERIALS AND METHODS

ENZYMES

Enzymes were purchased from BRL, Pharmacia, and Boehringer-Mannheim, and were used according to the suppliers' directions.

PLASMIDS USED

pHE1 (Figure 4), obtained from Dr. F. L. Graham, McMaster University, is a derivative of pXCI (McKinnon et al., 1982) which contains the adenovirus 5 XhoI C fragment (bp 1-5788) inserted into the Bam HI and SalI sites of pBR322. A BamHI linker was added to the terminus of the adenovirus DNA to allow it to be ligated to the BamHI cohesive end of pBR322. XhoI and SalI have compatible cohesive ends. Upon sequencing of the terminal adenovirus sequences in pXC1, it was found that the terminal 21 bp were not present and were replaced by 35 bp of apparently random DNA (McKinnon, 1984). pHE1 was obtained from pXC1 by removing the sequences between the Hpal site in the Ad5 sequence (bp 1574) and the PvuII site in the pBR322 sequence (Figure 4) (McKinnon, 1984). pHE1 contains the entire coding sequence of E1A, but lacks the polyadenylation signal.

pCD2, also obtained from Dr. F. L. Graham, was generated from pXC1 by deleting the sequences between the two SstI sites at bp 1772 and 5644 of the Ad5 sequence. It contains the entire E1A sequence.

pLE2 (Figure 4) was generated from pCD2 by removing the ClaI site at position 24 of the pBR322 sequence by cutting with ClaI, treating with Klenow fragment and religating. The only other ClaI site, that at bp 920 in the Ad5 sequence, contains the dam methylation sequence GATC. Since this preparation of pCD2 had been grown in dam^+ bacteria (LE392) this sequence was resistant to cleavage by ClaI during this cloning procedure.

pKCAT23, obtained from Dr. M. B. Mathews, Cold Spring Harbour Laboratories, contains the chloramphenicol acetyltransferase gene driven by the E1A sensitive adenovirus 5 early region 3 promoter (Weeks & Jones 1983).

pSV_2CAT, obtained from Dr. F. L. Graham, (originally from Dr. B. H. Howard, National Cancer Institute, Bethesda, Maryland) contains the chloramphenicol acetyltransferase gene driven by the SV40 promoter/enhancer (Gorman et al., 1982).

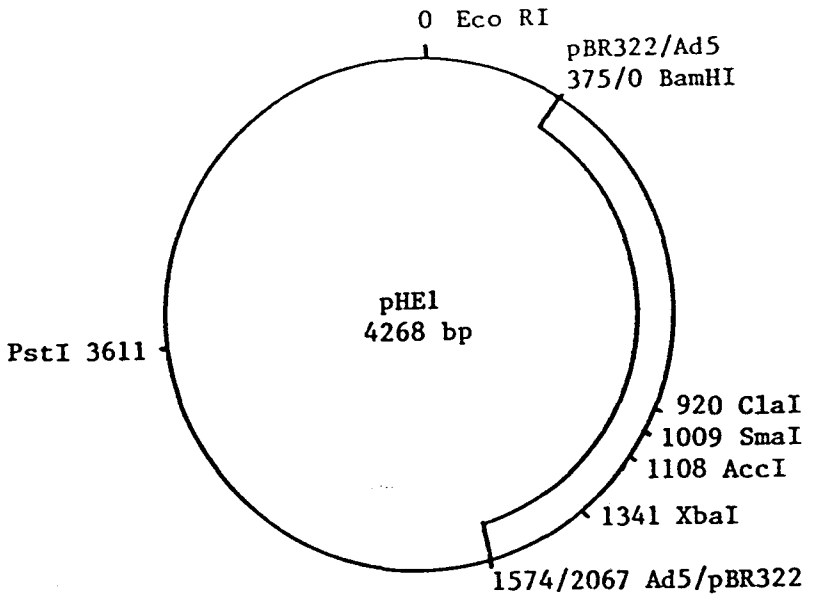
pMBCAT8, obtained from D. Bautista, is a derivative of pSV_2CAT, which also expresses the chloramphenicol

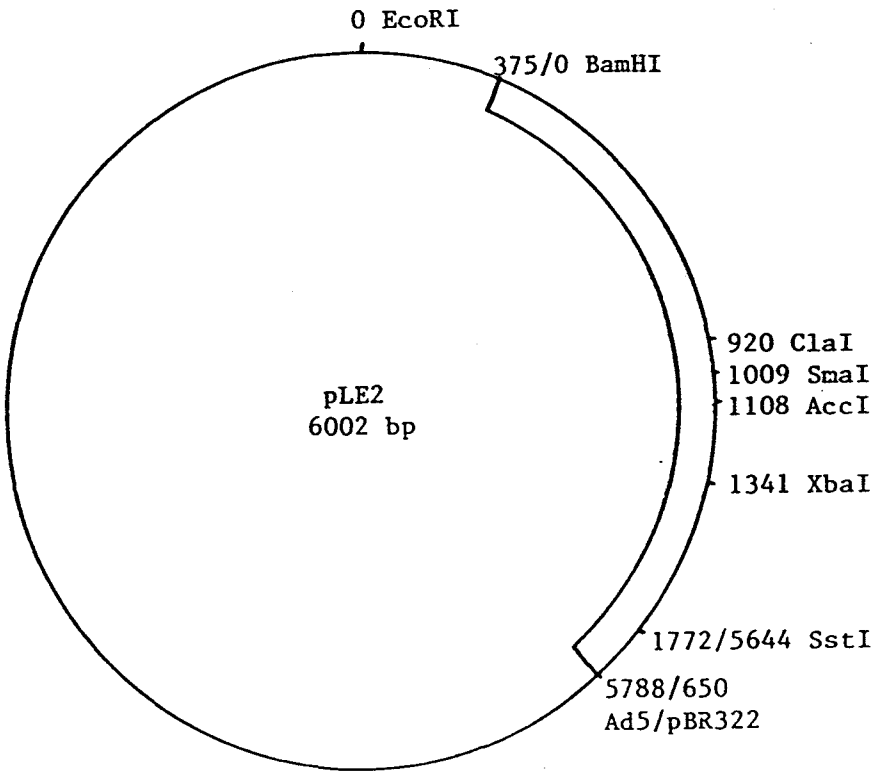
Figure 4 Restriction map of E1A plasmids used in this work. The origins of the plasmids are described in Materials and Methods. The recognition sites for the commonly used enzymes are indicated.

Double line = adenovirus sequence

Single line = pBR322 sequence

Figure 4


0 Eco RI
pBR322/Ad5
375/0 BamHI
pHE1
4268 bp
PstI 3611
920 ClaI
1009 SmaI
1108 AccI
1341 XbaI
1574/2067 Ad5/pBR322


0 EcoRI
375/0 BamHI
pLE2
6002 bp
920 ClaI
1009 SmaI
1108 AccI
1341 XbaI
1772/5644 SstI
5788/650
Ad5/pBR322

acetyltransferase gene under the control of the SV40 promoter/enhancer sequences.

pEJ-ras, obtained from Dr. S. Mak, is a derivative of pBR322, and contains the activated Ha-ras oncogene (Land et al., 1983).

OTHER MUTANTS USED IN THESE STUDIES

A number of other mutants were produced by others in the laboratory (Jelsma et al., 1988). These were small in-frame deletions and point mutations which essentially spanned the entire E1A gene. The mutations are listed in Table 1 and their positions are shown in Figure 10. These mutants were made using the oligonucleotide-directed mutagenesis technique (Zoller and Smith, 1984). Where possible, these mutations were chosen to remove regions of hydrophobicity or hydrophilicity to delete internal or external sequences of the protein. These mutations cloned into pLE2 were used for the experiments described in this thesis.

The mutants dl 1101 to dl 1109 are small deletions spanning the 12S exon 1, while dl 1119 deletes essentially all of 12S exon 1. Dl 1112 to dl 1114, along with dl 1110 which will be described later, together delete the 13S unique region. Dl 1115, dl 1116, and pm 1131 which is a

point mutation that introduces a stop codon in place of ser 219, together delete exon 2 except for residues 186 and 187 at the splice acceptor site. Sub 1117 was produced fortuitously during the production of another mutant, and substitutes 11 bases for the sequences between bp 1142 and bp 1288. The net effect of this is the removal of the acceptor splice site of E1A. The predicted product of this mutant includes 12S exon 1, the 13S unique region including Ser 185, and a lysine residue encoded by the intron.

The dl 520 mutation was also used in these studies. It deletes nucleotides 1107 to 1117 surrounding the 13S splice donor site, thus allowing only a 12S message to be made (Haley et al, 1984). This mutation was used in plasmid form by inserting the XmaI to XbaI (bp 1009 to 1341) fragment of dl 520 containing the mutation, into pLE2.

The dl 520 mutation was also cloned into pLE2 containing deletions in exon 1. The resulting recombinants make only the 12S products with mutations in exon 1. In a similar manner, the dl 1112 mutation was cloned into pLE2 plasmids containing the dl 1101 to dl 1109 deletions, with the exception of dl 1108. These recombinants produced both 12S and 13S products, but the 13S products were mutated by the dl 1112 mutation.

E. COLI STRAINS

LE392, obtained from Dr. F. L. Graham, was used in general for cloning and propagation of the various plasmids.

K58, (ung^-) obtained from Dr. A. E. Smith, National Cancer Institute for Medical Research, Mill Hill, London, U.K., was used during the deletion loop mutagenesis technique.

GM48, obtained from Dr. S. Mak, McMaster University is a dam^- strain that was used to propagate plasmids that could be cut at the ClaI site in E1A.

LARGE SCALE PLASMID PREPARATIONS

For large scale plasmid preparations, the alkaline lysis procedure described in Birnboim and Doly (1979) was used with some modifications.

In general, preparations were made using 500 mL cultures containing 250 mL Luria Broth, 225 mL TB and 25 mL 0.17M KH_2PO_4, 0.72M K_2HPO_4 and 50 ug/mL ampicillin (Sigma or Ayerst). When the A_{600} of the cultures reached 0.6 - 0.9, chloramphenicol (Boehringer-Mannheim) was added to a concentration of 180 ug/mL and the cultures were incubated at 37^{o}C overnight with shaking.

The cells were pelletted at 8,000 rpm for 15 minutes at 4°C in a Beckman JA-10 rotor. The cells were resuspended in 10 mL lysozyme solution (5 mg/mL lysozyme, 50mM glucose, 25mM Tris HCl pH 8.0, 10mM EDTA) and placed on ice for 10 minutes. To this cell suspension was added 20 mL alkaline SDS (0.2M NaOH, 1% SDS) with swirling, followed by a further 10 minute incubation on ice. 15 mL of high salt (3M Na Acetate, pH 4.8) was added, the bottle was shaken to mix the contents thoroughly, and the mixture was left on ice for a further 10 minutes. The bulk of the cell debris was removed by filtering through cheesecloth, and the rest was removed by centrifuging the remaining solution at 12,000 rpm in a Sorval SS34 rotor for 15 minutes at 4°C. 26 mL of isopropanol was added to the supernatant. After 20 minutes at room temperature, the solution was centrifuged as before at room temperature and the supernatant was discarded. The pellet was rinsed once with 70% ethanol and the tube was inverted for several minutes to drain the liquid from the pellet. The pellet was resuspended in 2 mL TE pH 7.4, and 2 mL 5M LiCl was added; this was placed on ice for 30 minutes. The mixture was centrifuged as before at 4°C. The supernatant was collected and to it 10 mL 95% ethanol was added, with a 30 minute incubation at -20°C. The mixture was centrifuged again as before at 4°C and the DNA pellet

was resuspended in 500 uL 0.1M Tris HCl pH 7.4, 15mM NaCl. 5 uL 10 mg/mL RNAse A (Sigma) (prepared as described in Maniatis et al., 1982), was added and the tube was incubated at 37°C for 15 minutes. 500 uL of 13% polyethylene glycol (BDH), 1.6M NaCl was added and the tube was kept on ice for 30 minutes. It was spun in a Beckman Microfuge at 4°C for 10 minutes, and the supernatant was discarded. The pellet was resuspended in 500 uL TE pH 7.4. The sample was thoroughly extracted using a 1:1 phenol/chloroform solution, 3-6 times (phenol saturated with 0.1M Tris HCl, pH 8.0, chloroform was a 24:1 chloroform : isoamyl alcohol (IAA) solution), chloroform/IAA extraction once, and ether several times. The solution was made to 0.1M NaCl, and 2 1/2 volumes 95% ethanol were added to precipitate the DNA. The sample was then redissolved in about 500 uL H_2O. The purity of the DNA preparation was checked by determining the absorbance at 260 and 280 nm. An A_{260}/A_{280} ratio of 1.8 indicates pure DNA. The plasmid concentration and absence of cellular DNA was confirmed by comparing the restriction digestion pattern with a known standard.

SMALL SCALE PLASMID PREPARATIONS

Small scale plasmid preparations were made in a similar manner to the large scale preparations. Bacteria

from 2.5 mL cultures grown overnight in Luria Broth with 50 ug/mL ampicillin were pelleted in the Microfuge. The treatment of the cells with lysozyme, alkaline SDS, and high salt was the same as in the large scale procedure, except 100 uL, 200 uL, and 150 uL of the respective solutions were added. After the high salt step, cell debris was pelletted in the Microfuge for 5 minutes at $4^{o}C$. The supernatant was added to 1 mL ice cold 95% ethanol and the mixture was centrifuged as before. The pellet was resuspended in 200 uL TE (10mM Tris pH 7.4, 1mM EDTA) and 200 uL 5M LiCl was added. After a 30 minute incubation on ice, the mixture was centrifuged as before, the supernatant was added to 1 mL 95% ethanol, and the precipitated DNA was pelletted by centrifuging as before. The treatment with LiCl was not done in earlier plasmid preparations, but was added later because it removed much of the RNA and residual cellular debris. The DNA was twice resuspended in 100 uL 0.1M Tris HCl pH 7.4, 15mM NaCl, adding 200 uL 95% ethanol and centrifuging as before. The final pellet was resuspended in 10-20 uL H_2O. For restriction digestions, generally half the sample was used per reaction.

Luria Broth

5 g/L yeast extract (Difco)
5 g/L NaCl
10 g/L tryptone (Difco)
1 g/L glucose
Sterilized by autoclaving

TB (BRL Focus, Spring 1987)

0.4% v/v glycerol
12 g/L tryptone
24 g/L yeast extract
Sterilized by autoclaving

PREPARATION OF COMPETENT E. COLI CELLS FOR PLASMID TRANSFORMATION

50 mL bacterial cultures were grown in Luria broth with agitation until the A_{550} reached approximately 0.5. The cells were pelletted at 8000 rpm in the Sorval SS34 rotor for 5 minutes at 4°C and were then resuspended in 15 mL sterile competence solution (75mM $CaCl_2$, 5mM Tris HCl, pH 7.4) at 4°C for 20 minutes. The cells were pelletted as before, resuspended in 2 mL competence solution, and kept at 4°C overnight before transforming. For convenience, in later experiments 100 uL aliquots of fresh competent cells

were frozen at -70°C until used, even though this gave a lower efficiency of transformation.

TRANSFORMATION OF COMPETENT E. COLI CELLS

In earlier experiments the transformation procedure was as follows. 100 uL of competence solution containing 1-5 ug of recombinant plasmid was added to 2 mL of the competent E. coli cell suspension. This was kept on ice for 30-60 minutes with occasional shaking. The cells were heat shocked at 42°C for 2 minutes, then quickly cooled by placing on ice for a few seconds. Four volumes of warm Luria broth were added and the cells were incubated at 37°C for 15-30 minutes. 10^{0} and 10^{-1} dilutions of the cell suspension were plated on Luria agar (Difco) plates containing 50 ug/mL ampicillin. Colonies appeared after overnight incubation at 37°C.

In later experiments, transformations were carried out using the 100 uL aliquots of competent cells frozen in 15% glycerol at -70°C in 15 mL polypropylene tubes (Falcon). 5-10 uL of competence solution containing 0.1 ug of recombinant plasmid was used to transform the cells, using the procedure described above.

MUTAGENESIS

a) Deletion Mutagenesis

Deletions were made in the SmaI site in pHE1 for use in deletion loop mutagenesis. The digestion conditions required to produce a deletion of 30 to 50 bp were determined as part of an undergraduate thesis project. 5 ug pHE1 was digested with 9 U SmaI in a 30 uL reaction volume for one hour, then was phenol extracted and ethanol precipitated. The ends were digested with 13.4 units (U) of Exonuclease III for 2 1/4 minutes at 30^{o}C, in a 50 uL reaction volume. The reaction was stopped by addition of an equal volume of phenol/chloroform. After phenol extraction and ethanol precipitation the DNA was treated with S1 nuclease at 37^{o}C in a 40 uL reaction volume, using 17 U of S1 and an additional 17 U after 15 minutes, to remove the single stranded tails and create blunt ends. 0.8 ug of the DNA was removed, digested with HaeII, and run on a 6% polyacrylamide gel to check the extent of the ExoIII/S1 digestion. After phenol extraction and ethanol precipitation, the remaining DNA was ligated overnight using 7.5 units of T4 DNA ligase at room temperature. One of the deletion mutants obtained, d1 1111, was used to create point mutants by deletion loop mutagenesis.

b) Deletion Loop Mutagenesis

5 ug each of wt pHE1 and pHE1/d1 1111 were digested with excess EcoRI and XbaI respectively, combined and ethanol precipitated. The plasmids were resuspended in 23 uL H_2O, then 4.8 uL 1M NaOH was added and the solution was left at room temperature for 10 minutes. The following was added sequentially: 280 uL H_2O, 40 uL 0.5 M Tris HCl pH 8.0, and 48 uL 100 mM HCl. The DNA was immediately placed at 63°C for two hours, then cooled slowly overnight.

The DNA was ethanol precipitated and resuspended in 50 uL H_2O. To this was added 150 uL sodium bisulphite solution, 1.33 uL 1M DTT, and 100 uL paraffin oil. The tube was wrapped in foil and incubated at 37°C for 4 hours. The Sodium Bisulphite Solution was made as follows:

331.4 mg $Na_2S_2O_5$
148.8 mg Na_2SO_3
1 mL H_2O

Dithiothreitol (DTT) was substituted for hydroquinone, which was used by Kalderon et al. (1982) as the antioxidant in this procedure.

After the mutagenesis reaction, the sodium bisulphite was removed from the DNA by dialysis. The

initial three dialyses were carried out at 4°C to minimize the activity of the sodium bisulphite still present in the DNA solution.

To remove the sodium bisulphite, the DNA was dialysed extensively in the following buffers (Shortle and Nathans, 1978).

- 2x 500 mL 5mM potassium phosphate pH 6.8, 0.5mM DTT, for 2 hours each at 4°C
- 500 mL 5mM potassium phosphate pH 6.8, overnight at 4°C
- 500 mL 0.2mM Tris HCl pH 9.2, 50mM NaCl, 2mM EDTA, for 16 hours at 37°C
- 500 mL 2mM Tris HCl pH 8.0, 2mM NaCl, 0.2mM EDTA, for 24 hours at 4°C

The DNA was then ethanol precipitated and used to transform competent K58 E. Coli cells.

SEQUENCING OF DELETION AND MISSENSE MUTATIONS

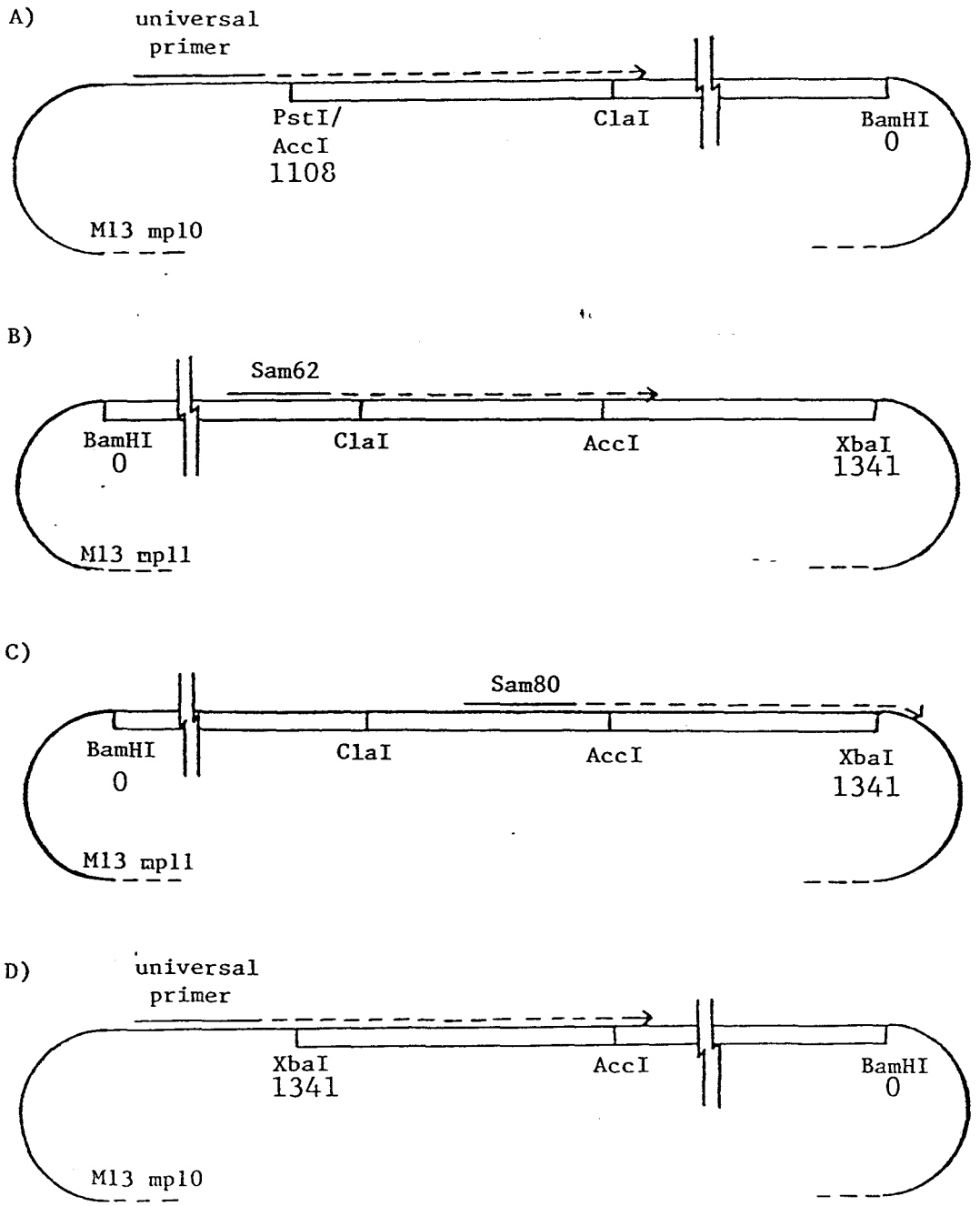
a) M13 Recombinants Used for Sequencing

Fragments of DNA containing the mutations were cloned into M13 for dideoxy-nucleotide sequencing.

The M13 recombinant used to sequence the deletion and missense mutations is shown in Figure 5A. The mutant pHE1 was cut with AccI, treated with the Klenow fragment of

Figure 5 Recombinant M13 clones used for sequencing deletion mutants dl 6 and dl 1111, and missense mutants made by deletion loop mutagenesis. For convenience, only the relevant part of the recombinant is shown. Construction of each clone is described in Materials and Methods. The boxed regions contain the insert from the mutant pHE1. The binding site for the sequencing primers (universal primer, Sam 62 and Sam 80) are indicated. The direction and extent of the extension of this primer by Klenow fragment during sequencing is indicated by a broken arrow.

Figure 5


A)
universal primer
PstI/ AccI 1108
ClaI
BamHI 0
M13 mp10
B)
Sam62
BamHI 0
ClaI
AccI
XbaI 1341
M13 mp11
C)
Sam80
BamHI 0
ClaI
AccI
XbaI 1341
M13 mp11
D)
universal primer
XbaI 1341
AccI
BamHI 0
M13 mp10

DNA polymerase I to make blunt ends, and cut with BamHI. The BamHI to AccI fragment (bp 0 to 1108) was ligated to M13 mp10 which had been similarly treated with PstI, Klenow fragment, and BamHI. The appropriate recombinants were sequenced from the AccI site to the ClaI site (bp 1108 to bp 920) with the universal primer.

To confirm the sequence of the missense mutants, the complementary strand was cloned into M13 mp11 and sequenced. The BamHI to XbaI fragment (bp 0-1341) was cloned into the BamHI and XbaI sites of M13 mp11. The sequence from the ClaI site to the AccI site was obtained using the synthetic oligonucleotide Sam 62 as a primer (Figure 5B). Sam 62, which was originally made to generate dl 1106, described elsewhere, contained sequences corresponding to bp 816 to 826 and bp 875 to 886.

This same recombinant was used to sequence further from the AccI site to the XbaI site with the oligonucleotide Sam 80 as primer (Figure 5C). Sam 80, which was originally made to generate dl 1112, contained sequences corresponding to bp 1029 to 1039 and bp 1064 to 1075.

The opposite strand was also sequenced from the XbaI site to the AccI site with the recombinant shown in Figure 5D. The BamHI to XbaI fragment of E1A was inserted

into the corresponding sites of M13 mp10. The universal primer was used to obtain the sequence.

b) Use of M13 for Sequencing

Recipes for the solutions are at the end of each section. M13 mp10 and mp11 were purchased from Pharmacia.

i) Preparation and Transformation of Competent E. Coli With M13 DNA

E. coli cells of strain JM103 (Pharmacia) were grown on Minimal Media Agar to maintain the F episome. Colonies from these plates were grown in 50 mL of YT medium until the A_{550} reached 0.2-0.3. The cells were pelletted in a Sorval SS34 rotor at 6,000 rpm for 5 minutes at 4°C. The cell pellet was resuspended in 10 mL of sterile Competence Solution A (10mM NaCl, 50mM $MnCl_2$, 10mM Na Acetate, pH 5.6) and kept on ice for 20 minutes. The cells were pelletted as before and resuspended in 1 mL sterile Competence Solution B (75mM $CaCl_2$, 100mM $MnCl_2$, 10mM NaAcetate, pH 5.6). The cells were used immediately for transformation.

20 uL of the ligation reaction was added to 150 uL of competent JM103 cells. This was incubated on ice for 30 minutes with occasional mixing. The cells were then

incubated at 37°C for exactly 2 minutes, chilled on ice for a few seconds, and plated out.

30 uL and 140 uL of the transfected JM103 cells were added to two 5 mL sterile plastic tubes containing 200 uL plating culture (JM103 cells grown to late log phase of growth, A_{550} about 1). 3 mL of plating agar at 45°C was added to each tube. The contents of the tubes were mixed thoroughly, and were immediately poured onto YT agar plates. The plates were incubated at 37°C overnight. Recombinant M13 clones could be identified as colourless plaques on a lawn of cells. Nonrecombinant M13 gave blue-green plaques.

Minimal Media Agar

12 g/L Na_2HPO_4

6 g/L KH_2PO_4

1 g/L NaCl

2 g/L NH_4Cl

Sterilized by autoclaving

30 g/L agar (Difco) sterilized by autoclaving

The above solutions were combined 1:1 after autoclaving and the following solutions were added:

1 mL/L 1M $MgSO_4$

1 mL/L 0.1M $CaCl_2$

1 mL/L 1M Thiamine HCl

5 mL/L 40% glucose

All sterilized by filtration

YT Medium

8 g/L Tryptone (Difco)

5 g/L yeast extract (Difco)

5 g/L NaCl

Adjusted to pH 7.2-7.4 with NaOH

Sterilized by autoclaving

YT Agar

1.5% agar in YT

Plating Agar

0.6% agar in YT medium

containing per 3 mL agar:

0.1 mL 2% Xgal (IBI) in dimethylformamide

0.02 mL 100 mM IPTG (BRL)

The latter components were added to the agar at $45^{o}C$ immediately before use.

c) Isolation of M13 DNA

To screen the M13 phage for the proper recombinant, small colourless plaques were picked using a sterile pasteur pipette and were placed in 5 mL YT containing 5 uL plating culture. These cultures were incubated for 8 hours at $37^{o}C$ with agitation.

i) Preparation of Double-Stranded (RF) DNA

Cells from 3 mL of the culture were pelletted in the Microfuge for 30 seconds at room temperature. 1.5 mL of the supernatant (containing the phage) was saved and stored at $-20^{o}C$. The cell pellet was treated using the procedure described earlier for the small scale plasmid preparation to obtain the RF form of the M13 DNA; the LiCl step was not

used. The M13 RF DNA was analysed by restriction digestion to identify the proper recombinant.

ii) Preparation of Single-Stranded M13 DNA

To prepare single stranded M13 DNA to use as a template for sequencing, 20 uL of the stored phage supernatant (see section i: Preparation of RF DNA) was added to 40 mL of YT medium containing 20 uL plating culture. This was incubated for 8 hours at 37^{o}C with agitation. The culture was centrifuged in a Sorval SS34 rotor at 8,000 rpm for 15 minutes at 4^{o}C. The supernatant was transferred to another tube and to it was added 8 mL of 2.5M NaCl, 20% polyethylene glycol 8000. This was kept on ice for 30 minutes, after which the phage were pelletted by centrifuging as above. The supernatant was poured off and the tube was left inverted for a few minutes to drain the liquid from the pellet. The phage pellet was resuspended in 1 mL TE pH 7.5 and phenol extracted extensively. The DNA was precipitated using 1/10 volume 3M NaAc pH 6.0 and 2.5 volumes of cold 95% ethanol. The DNA was resuspended in 100 uL TE pH 7.5. The concentration of DNA was determined by the A_{260} reading.

DIDEOXYNUCLEOTIDE SEQUENCING

The procedure used for the dideoxy sequencing reactions was that described in the ^{35}S sequencing manual by Pharmacia, with only one change. The reactions were carried out at 37°C or 50°C instead of room temperature. The reagents were all obtained from Pharmacia with the exception of the oligonucleotide primers: the universal primer was obtained from New England Biolabs, while Sam 62 and Sam 80 were synthesized by the phosphotriester method using a Bioresearch Saml Synthesizer. The Sam oligonucleotides were originally used to generate the deletion mutants dl 1106 and dl 1112.

Single stranded template, 1.5 ug, and 2.5 ng of primer were combined with 10 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, and 5 mM DTT to a final volume of 10 uL, incubated at 60°C for 10 minutes and then allowed to cool slowly to room temperature over a period of about 30 minutes. Two units Klenow fragment, and 20 uCi ^{35}S-ATP, (NEN) 500 Ci/mmol, were added to a total volume of 13 uL.

This solution (2.5 uL) was added to each of four tubes containing 2 uL of the A,C,G, and T deoxy/dideoxynucleotide mixes defined at the end of this section. These reactions were incubated at 37°C or 50°C for

30 minutes. One uL of chase solution (2mM each of dATP, dCTP, dGTP and dTTP, 10mM Tris HCl pH 7.5) was added and the reactions were continued for an additional 30 minutes. They were then stopped by the addition of 3 uL Stop solution (0.3% xylene cyanol (Eastman Kodak), 0.3% bromophenol blue (BDH), in deionized formamide (BDH)). The solutions were incubated in a boiling water bath for 3 minutes, immediately placed on ice and then loaded on the sequencing gel.

The reaction mixtures were run on a linear 6% polyacrylamide sequencing gel (19:1 acrylamide : bisacrylamide (Bio-Rad), 7M urea (Bio-Rad)) in 1x TBE using an LKB sequencing gel apparatus, as described in the LKB instruction manual. The gel, bonded to the glass plate, was washed with 10% acetic acid and dried at $80^{o}C$ overnight prior to exposing it to film.

1x TBE

12.11 g/L Tris HCl

5.1 g/L Boric Acid

0.37 g/L EDTA

Deoxy/dideoxynucleotide mixes (Taken from Pharmacia ^{35}S sequencing manual)

Component	A mix	C mix	G mix	T mix
ddATP	13 uM	----	----	----
ddCTP	----	25 uM	----	----
ddGTP	----	----	45 uM	----
ddTTP	----	----	----	330 uM
dATP	----	----	----	----
dCTP	62.5 uM	4.1 uM	82 uM	82 uM
dGTP	62.5 uM	82 uM	4.1 uM	82 uM
dTTP	62.5 uM	82 uM	82 uM	4.1 uM
$MgCl_2$	10 mM	10 mM	10 mM	10 mM
Tris HCl pH 7.5	10 mM	10 mM	10 mM	10 mM
Dithiothreitol	1 mM	1 mM	1 mM	1 mM

CAT ASSAYS

In the CAT assays, HeLa cells were transfected with the appropriate plasmids, and the extracts of these cells were assayed for CAT activity. HeLa cells were supplied by Dr. F. L. Graham. They were cultured in Alpha-MEM, 10% newborn calf serum and 1% penicillin-streptomycin (all obtained from Gibco).

(a) Transactivation

The transactivation CAT assays were carried out basically as described in Weeks & Jones (1983).
The mixture used to transfect the HeLa cells contained:

0.5 mL 2x HeBS pH 7.1 (defined at end of section)

0.5 mL H_2O

10 uL 2 mg/mL sonicated salmon sperm DNA (Sigma)

5 ug pKCAT 23

10 ug pLE2 (wild type or mutant)

The mixture was vortexed to mix thoroughly. Fifty microlitres 2.5M $CaCl_2$ was added, and the mixture was vortexed again for 5 seconds, and left standing at room temperature for 20 minutes. The precipitate was very fine and gave a cloudiness to the solution. The solution was added to a 100 mm dish of HeLa cells (about 70% confluency).

Four to eight hours later, the cells were glycerol shocked by removing the medium, and adding 2 mL of 20% glycerol in 1x HeBS. After 90 seconds, the excess glycerol solution was removed. At 2 minutes, 4 mL 1x HeBS was added to wash the cells. The solution was removed and 10 mL fresh medium was added.

Forty-eight hours after adding the DNA precipitate, the cells were harvested. The medium was removed from the dishes and replaced by 1 mL 40mM Tris HCl pH 7.4, 1mM EDTA, 0.15M NaCl. Five minutes later the cells were scraped off with a rubber policeman and pelletted in the Microfuge for 15 seconds. The supernatant was removed by aspiration and the cells were resuspended in 100 uL 0.25M Tris-HCl pH 7.8. The cells were transferred to clear polystyrene tubes and sonicated in an ice water bath with 15 x 1 second bursts. The cell lysate was transferred to centrifuge tubes, and centrifuged for 5 minutes at $4^{o}C$ to pellet the debris. The supernatant was transferred to another centrifuge tube to assay the CAT activity.

The reaction mixture consisted of:

4 uL 20mM Acetyl CoA (Pharmacia)
2 uL ^{14}C-chloramphenicol
(50 mCi/mmol, 0.1 uCi/uL)
100 uL extract

The reaction was carried out at $37^{o}C$ for one hour.

The ^{14}C-chloramphenicol was extracted into 900 uL ethyl acetate and dried in the Speedvac for 30 minutes. The sample was resuspended in 20 uL ethyl acetate and spotted on

Silica Gel G thin layer chromatography (TLC) (Analtech) plates. The products were separated by ascending chromatography in chloroform:methanol (95:5) and after drying the TLC plates were exposed to Kodak XAR film overnight. The CAT activity was quantified by scraping the radioactive spots on the TLC plates into Omnifluor Scintillation fluid (NEW England Nuclear) and measuring the radioactivities by scintillation counting.

HeBS Solutions (from Graham and Bacchetti, 1983)

1x HeBS	2x HeBS	
8 g/L	16 g/L	NaCl
0.38 g/L	0.76 g/L	KCl
0.1 g/L	0.2 g/L	Na_2HPO_4
5 g/L	10 g/L	Hepes (Gibco)
1 g/L	2 g/L	glucose

brought to pH 7.1 and sterilized by filtration

(b) Enhancer Repression

The transfection mixture contained the following:

0.5 mL 2x HeBS pH 7.1

0.5 mL H_2O

10 uL sonicated salmon sperm DNA

5 ug pSV_2CAT or pRSVCAT

2-4 ug E1A plasmid or pUC18

The transfection was carried out as described in the previous section. The harvesting procedure and the CAT assay were modified slightly for the enhancer repression assays to obtain more controlled conditions. After the cells were scraped from the dishes and pelletted, they were resuspended in 150 uL 0.25M Tris-HCl pH 7.8, 5mM EDTA. After sonicating as described above, the cells were checked by microscope to ensure that they were completely lysed. The cell lysate was then incubated at $60^{o}C$ for 10 minutes. The lysate was spun in the microfuge at $4^{o}C$ for 5 minutes to pellet debris, and the supernatant was assayed for CAT activity.

The reaction mixture consisted of:

24 uL 0.25M Tris-HCl pH 7.8,
5mM EDTA
4 uL 20mM Acetyl CoA
2 uL ^{14}C-chloramphenicol
20 uL extract

The reaction was carried out at 37°C for 20 minutes. The extraction, chromatography, and quantitation were the same as for the transactivation assay.

TRANSFORMATION ASSAYS

a) Preparation and Transfection of BRK Cells

All media used contained 1% penicillin-streptomycin (Gibco), and 1% fungizone (Squibb). All tissue culture dishes were obtained from Gibco.

Cells were prepared according to van der Eb and Graham (1980) with minor modifications.

Baby rat kidney cells were obtained from 2 or 5 day old Wistar rats (Charles River). Fewer cells were obtained from 2 day than 5 day old rats, but they consistently gave more foci of transformed cells with a shorter lag time.

Two day old rats were killed by decapitation, and 5 day old rats by breaking their necks. The kidneys were prepared and minced as described by van der Eb and Graham,

(1980). The minced kidneys were then incubated at room temperature for 15 minutes in 35 mL 1 g/L Trypsin, 0.4 g/L EDTA (Gibco) in sterile PBS^{--} (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4) with stirring. The tissue clumps were allowed to settle, and the supernatant containing the freed cells was removed and placed in 10 mL ice-cold fetal bovine serum (FBS) or newborn calf serum (NCS) (Gibco) to stop the trypsin reaction. Fresh trypsin solution was added to the tissue clumps and the incubation was repeated twice using 20 and 25 minute incubation times.

The remainder of the preparation of the cells was carried out as described by van der Eb and Graham, (1980). The cells were plated in Alpha-MEM (Gibco) plus 10% FBS. In general, 2 day old rats gave 8-9, while 5 day old rats gave 13-14 60 mm dishes per pair of kidneys.

The dishes were placed in a $37^{o}C$ CO_2 incubator overnight. They were rocked gently for approximately 15 minutes after plating to allow the cells to settle evenly throughout the dish. The following morning the cultures were washed to remove debris and unattached cells by aspirating the medium, rinsing the cells with PBS^{--} and adding fresh Alpha-MEM plus 10% FBS. Cells were transfected on the second day after plating, when the dishes were 60-70% confluent.

The preparation of the carrier DNA and transfection were carried out as described by Graham and Bacchetti (1983) with minor modifications. The DNA from ten 150 mm dishes of HeLa cells was dissolved in 10 mL TE for use as carrier DNA. The amount of carrier HeLa cell DNA needed to produce a suitable precipitate was determined empirically to obtain the optimum consistency of precipitate. The concentration of TE used to dissolve the DNA was increased tenfold from that described in Graham and Bacchetti (1983), to 10mM Tris pH 8.1, 1mM EDTA. In general, 7-15 uL of carrier DNA per dish was used.

The precipitate was left on the cells for approximately 16 hours, after which the medium was removed and replaced with fresh Alpha-MEM plus 10% FBS. Thereafter medium was replaced twice weekly with MEM (Gibco), plus 5% FBS.

Starting one week after transfection, the dishes were examined every other day for the appearance of foci. The dishes were stained at four to five weeks after transfection. The number of foci in a dish often increased rapidly, probably by the production of secondary foci. To minimize this, dishes were stained earlier if they contained ten or more foci.

To stain the cells, the medium was removed, and the cells were washed with PBS--. They were fixed by treating with Carnoy's fixative (95% ethanol:glacial acetic acid 3:1):PBS-- (1:1) for 10 minutes and Carnoy's for 30 minutes. The Carnoy's was removed and the dishes were air dried. The cells were stained with Giemsa stain (4% v/v Giemsa stain (Fisher) in 4.1mM Na_2HPO_4, 2.6mM H_2PO_4) for 30 minutes. The dishes were rinsed with water to remove the stain and allowed to dry.

b) Transformation Using Multiwell Trays

The preparation of the BRK cells was as described above. The cells were plated into 24-well trays, 400 uL per well, 16mm diameter, using a repeater pipet. The transfections were carried out as described above, using 40 uL of precipitate-containing solution per well.

The medium changes were carried out as described above, with 400 uL medium per well, using a repeater pipet.

c) Transformation Using Agar Overlay

The preparation and transfection of BRK cells, and the initial medium changes were done as with the normal dishes. At 8 or 9 days post-transfection, or earlier if foci appeared, the medium was removed and replaced with 8 mL

medium containing 0.35% agar: 2x MEM plus 10% FBS at 45°C was mixed 1:1 with melted 0.7% agar in water at 45°C and added to the dishes. The dishes were kept at room temperature for about 15 minutes until the agar hardened, and then incubated at 37°C. After one week, 2 mL of additional overlay was added twice weekly until 4-5 weeks post transfection.

d) Transformations at 33°C

The cells were prepared and transfected as described for the normal dishes. The precipitates were left on the cells overnight at 37°C. After the medium change the following morning, the cells were incubated at 33°C for the duration of the experiment.

RESULTS AND DISCUSSION

MUTAGENESIS

To identify regions in E1A that are involved in transactivation, enhancer repression, and transformation, site-directed mutations were made in E1A, and the effect of these mutations on different E1A functions was examined. At the time these studies were initiated, little was known about important regions in E1A. The 13S unique region was chosen first for mutagenesis because it comprised the difference between the 13S and 12S products and because mutations affecting only the unique region (eg. hr1) are defective for virus growth and transformation (Harrison et al., 1977; Ricciardi et al., 1981). The contribution of a particular region in a protein to a specific E1A function can best be studied using missense mutations since they cause a minimum perturbation to the tertiary structure of the protein. However, in this case, although the unique region was known to be important for E1A function, the particular residues that were important for this were not known. For this reason a shotgun mutagenesis technique was needed to examine a number of residues at a time. Deletion loop mutagenesis was well suited for this as it allowed the

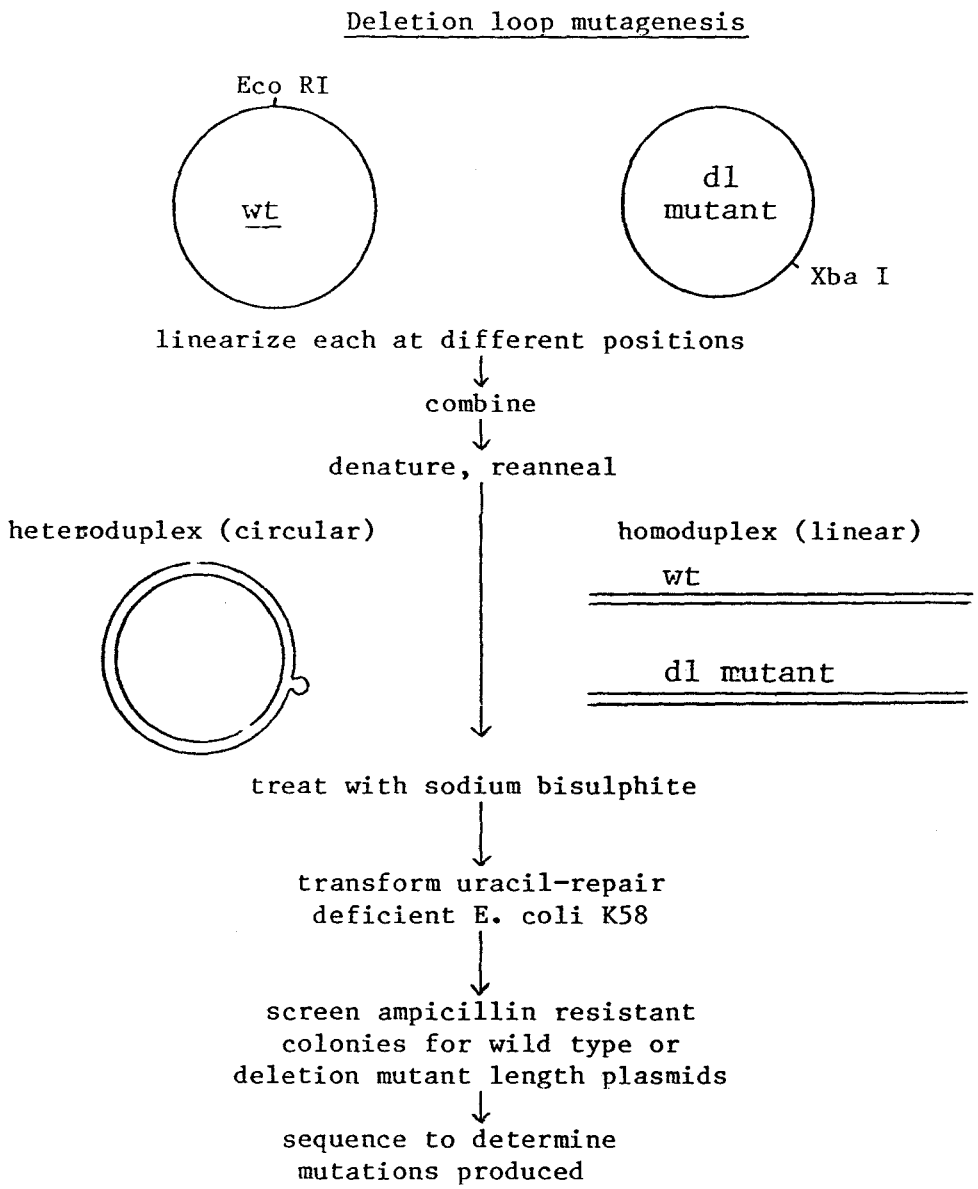
production of a large number of missense mutations collectively mutating most of the residues within a defined region. The sensitivity of this region to mutation could also be examined using mutants with more than one mutation.

The basis of deletion loop mutagenesis (Kalderon et al., 1982) is to hybridize one strand of the wild type plasmid to the complementary strand of a deletion plasmid. This results in a heteroduplex with a single stranded loop on the wild type strand corresponding to the deletion on the other strand. The heteroduplexes are then treated with sodium bisulphite which converts cytosine to uracil. Since sodium bisulphite acts only on single stranded DNA, only the deletion loop is susceptible to the mutagen. The mutagenized DNA is transfected into E. coli, and resulting colonies are screened for those plasmids which contain the point mutations. The procedure is outlined in Figure 6.

The deletion loop mutagenesis technique involved first generating a suitably sized deletion in this region using the nucleases ExoIII and S1. The intended size of the deletion was 30 to 50 base pairs, which could provide a large target area for mutagenesis, but is small enough to allow the isolation of mutants with relatively few mutations. In this way the importance of particular residues could be demonstrated.

Figure 6 Outline of deletion loop mutagenesis (Kalderon et al., 1982). The starting plasmids used were wt pHE1 and pHE1/dl 1111. Details of the procedure are provided in the text.

Figure 6


Deletion loop mutagenesis
Eco RI
wt
dl
mutant
Xba I
linearize each at different positions
combine
denature, reanneal
heteroduplex (circular)
homoduplex (linear)
wt
dl mutant
treat with sodium bisulphite
transform uracil-repair
deficient E. coli K58
screen ampicillin resistant
colonies for wild type or
deletion mutant length plasmids
sequence to determine
mutations produced

(a) Creation of Deletion

Deletions were made in the 13S unique region in plasmid pHE1, a derivative of pBR322 which contains the leftmost 3.8% of the Ad5 genome, up to the HpaI site at position 1574 (Figure 4). pHE1 was first digested with SmaI to linearize the plasmid. Excess enzyme was used to minimize the amount of uncut pHE1. These would survive the mutagenesis procedure since they would not have free ends and therefore would be resistant to the exonucleases ExoIII and SI. The next step in this procedure was the digestion of the 3' ends of the plasmid DNA by ExoIII to remove 15 to 25 bases from each end (30-50 bases in total). The conditions used, ie. amount of enzyme, temperature and digestion time required, to produce the desired deletion size were determined empirically. To remove the single stranded tails that remained after ExoIII digestion, the DNA was treated with S1 nuclease. The blunt ends were joined together by T4 DNA ligase.

To eliminate plasmid molecules that may have escaped digestion during the first SmaI treatment, DNA in the ligation reaction was again treated with SmaI. While those plasmids which contained the deletion had lost the SmaI site and were unaffected, plasmid which did not contain a deletion would be linearized by SmaI. Since linear DNA

transforms at a low efficiency relative to circular DNA, these linear molecules would not interfere in the screening for deletion plasmids. Competent E. coli were transformed with the DNA, and ten colonies were grown for plasmid DNA isolation. To determine the approximate size of the deletion the samples were digested with HaeII, which gives a 488 bp fragment containing this region, and were run on a polyacrylamide gel. A semilog plot of distance migrated vs. fragment size was used to estimate the sizes of the deletions. Three clones, dl 1110, dl 1111, and dl 6, which appeared to have deletions of about 30-50 base pairs, were chosen for further study.

In order to determine the exact sizes of these deletions, the region around the deletion was sequenced for the three mutants. The method used was the Sanger dideoxy sequencing technique, with M13. Dl 1110 was sequenced by Judy Denman. For each mutant, two separate clones were sequenced to ensure the accuracy of the sequencing data. The sizes of the deletions in dl 1110, dl 1111, and dl 6 were 63 bp, 43 bp, and 38 bp respectively. The deletion in dl 1110 began at the third base of codon 139, and ended at the second base of codon 160. This in effect changed codon 139 from GGT to GGC. Because of the degeneracy in the genetic code however, both code for glycine, so the net

effect of the deletion was the removal of amino acid residues 140-160. Since this was an in-frame deletion, dl 1110 was used for further study along with the mutants made by oligonucleotide-directed mutagenesis. The deletions in dl 1111 and dl 6 (bp 996 - 1038 and 1001 to 1038 respectively) were not multiples of three bases, so in both cases the deletions caused a frameshift in the sequences downstream of the deletion.

It is notable that the 3' boundary of all three deletions was at the same position (bp 1038) while the 5' boundary varied widely. The DNA sequence is exceptionally G-C rich at the 3' boundary (CCGGAGG) and the stronger base pairing perhaps stalled the progress of the ExoIII through this sequence.

Although dl 1110 deletes sequences in the 13S unique region only, both 13S and 12S mRNA's are affected. Dl 1110 removes the consensus donor splicing sequence for the 12S mRNA and thus no 12S mRNA will be made. If an intact 12S mRNA product is needed for a particular E1A function, then dl 1110 should be defective in that function. The 13S product lacks almost half of the unique region, and any function requiring an intact 13S product will also be defective in dl 1110.

(b) Deletion Loop Mutagenesis

To generate the heteroduplexes for deletion loop mutagenesis, wild type pHE1 and pHE1/dl 1111 were digested with EcoRI and XbaI respectively, and ethanol precipitated. Both enzymes cut only once in different parts of pHE1 and are not in the immediate vicinity of the region to be mutated. The cut plasmids were combined in water and NaOH was added to denature the plasmids. HCl and Tris HCl pH 8.0 were added to renature the DNA. For proper reannealing the sample was immediately placed in a $63^{o}C$ water bath, and allowed to cool slowly overnight. Upon reannealing, two types of products were formed: homoduplexes, where both strands were from either the wild type or deletion plasmid, or heteroduplexes which had one strand from each plasmid. The homoduplexes were linear since both strands had been cut at the same place. On the other hand, the heteroduplexes were circular, with nicks at the EcoRI and XbaI sites on the wild type and deletion strands respectively. The wild type strand contained the loop opposite the dl 1111 deletion.

The conditions used for the sodium bisulphite mutagenesis reaction were the same as those used by Kalderon et al., (1982) and were sufficient to give a high frequency of mutants, yet not so high as to saturate the region with

mutations. After the mutagenesis reaction the sodium bisulphite was removed from the DNA by dialysis.

The DNA was then used to transform competent K58 E. coli cells, a strain which is deficient in uracil-N-glucosidase and will not select against DNA strands containing uracil residues, ie. the mutated DNA. The homoduplexes were not removed from the transfection mixture because being linear, they had a low efficiency of transformation, and did not interfere in the process of screening for mutants (Kalderon et al., 1982).

The colonies of bacteria transformed by the mutagenized plasmids contained plasmids of deletion length, wt length, or perhaps both. To determine whether the colonies contained wt length or deletion length plasmids, plasmid DNA was digested with HaeII and run on a 6% polyacrylamide gel. Wt length plasmids gave a 488 bp HaeII fragment, while deletion length plasmids gave a 445 bp band. The wt length plasmids were those which contained the mutations induced by the sodium bisulphite treatment. Out of 62 colonies analyzed, 15 contained plasmids of wt length only. These were colony purified and stored for future analysis.

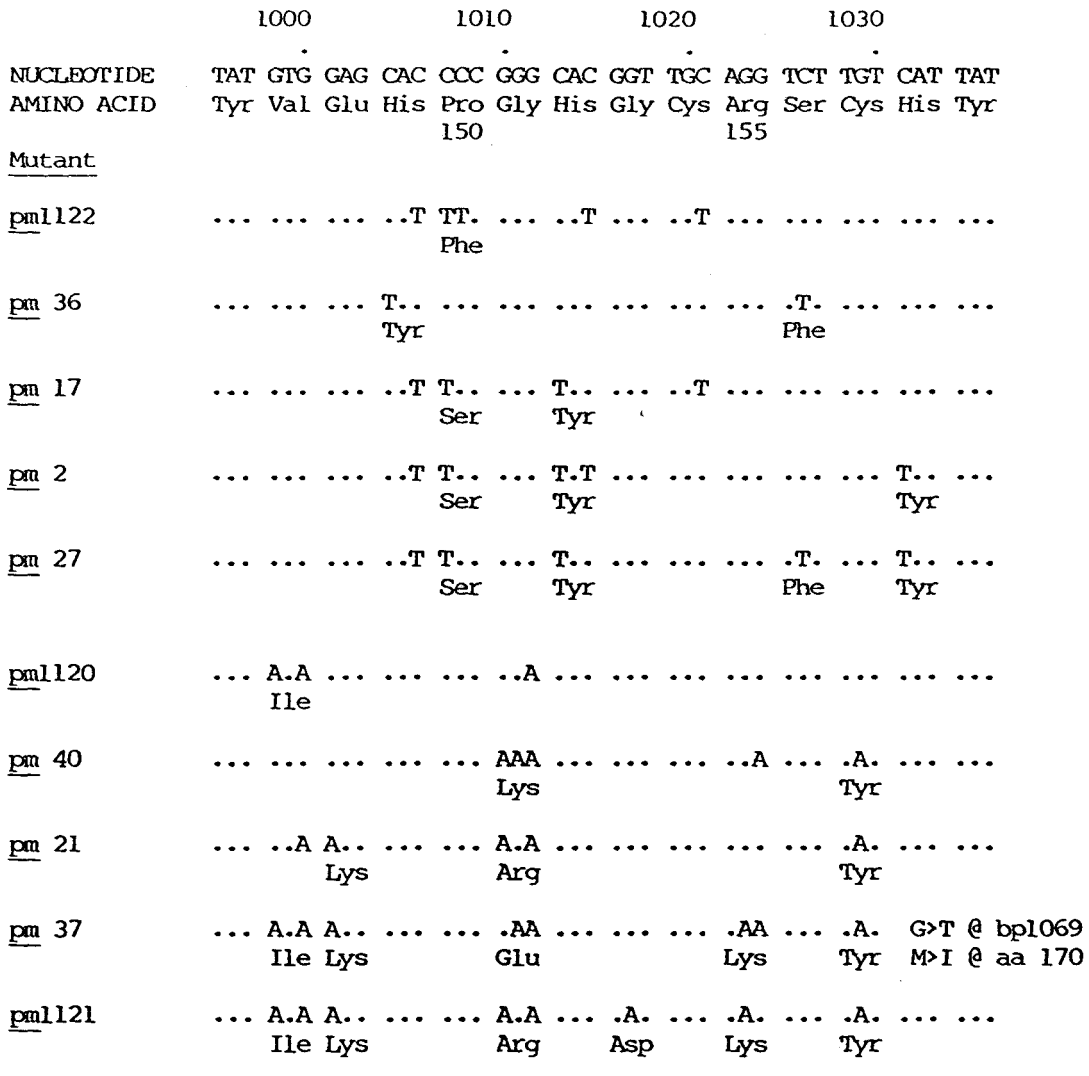
It was not clear whether colonies containing plasmids of both lengths were actually mixed populations of bacteria

containing wt length and those with deletion length plasmids. Colony purification of such colonies would have shown whether this was the case. However, since enough clones were available that had wt length plasmid only, nothing further was done with those that had both size plasmids.

Ten different mutants were sequenced initially in the region around the mutations. The sequencing of this region was done in two sections, from ClaI to AccI (bp 920 to 1108), and from AccI to XbaI (bp 1108 to 1341). To be certain of the mutations in this region, both strands of the mutants were sequenced. Their sequences are shown in Figure 7. The sequencing data for three mutants that were examined further is shown in Figure 8. Two types of changes occurred, C to T, or G to A, depending on which strand of the wt plasmid was present in the heteroduplex. In total, 19 of the 24 susceptible nucleotides were mutated, resulting in changes in 11 out of the 13 susceptible amino acid residues. Of the 8 nucleotides that were not mutated, or mutated only once, seven participate in a hypothetical hairpin loop structure comprised of nucleotides 1003 to 1021 (Figure 9). In this loop these bases are in a double stranded-like context and thus would not have been as accessible to the bisulphite. Nucleotides in this loop that

Figure 7 Mutants generated by deletion loop mutagenesis. All mutations are in the 13S unique region in the vicinity of the SmaI site. The nucleotide and amino acid changes are indicated. The wild type sequence of this region is shown above the mutants. Mutants with C to T changes are above; mutants with G to A mutations are below. Pm 37 also has a point mutation outside the loop, at bp 1069 (amino acid residue 170).

Figure 7



	1000				1010				1020				1030					
NUCLEOTIDE	TAT	GTG	GAG	CAC	CCC	GGG	CAC	GGT	TGC	AGG	TCT	TGT	CAT	TAT				
AMINO ACID	Tyr	Val	Glu	His	Pro	Gly	His	Gly	Cys	Arg	Ser	Cys	His	Tyr				
					150					155								
Mutant																		
pml122	...	...	...	..T	TT.	...	..T	...	..T	...	...	...	...	...				
					Phe													
pm 36	...	...	...	T..	...	...	...	...	...	...	.T.	...	...	...				
				Tyr							Phe							
pm 17	...	...	...	..T	T..	...	T..	...	..T	...	...	...	...	...				
					Ser		Tyr											
pm 2	...	...	...	..T	T..	...	T.T	...	...	...	...	...	T..	...				
					Ser		Tyr						Tyr					
pm 27	...	...	...	..T	T..	...	T..	...	...	...	.T.	...	T..	...				
					Ser		Tyr				Phe		Tyr					
pml120	...	A.A	...	...	...	..A	...	...	...	...	...	...	...	...				
		Ile																
pm 40	...	...	...	...	...	AAA	...	...	...	..A	...	.A.	...	...				
						Lys						Tyr						
pm 21	...	..A	A..	...	...	A.A	...	...	...	...	...	.A.	...	...				
			Lys			Arg						Tyr						
pm 37	...	A.A	A..	...	...	.AA	...	...	...	.AA	...	.A.	G>T @ bp1069					
		Ile	Lys			Glu				Lys		Tyr	M>I @ aa 170					
pml121	...	A.A	A..	...	...	A.A	...	.A.	...	.A.	...	.A.	...	...				
		Ile	Lys			Arg		Asp		Lys		Tyr						

Figure 8 Mutations in pm 1120, pm 1121, and pm 1122. The region containing the mutations in pm 1120 (A), pm 1121 (B), and pm 1122 (C) (bp 995-1036) is shown. The autoradiograph is oriented to match the printed sequence. The mutated nucleotides are underlined.

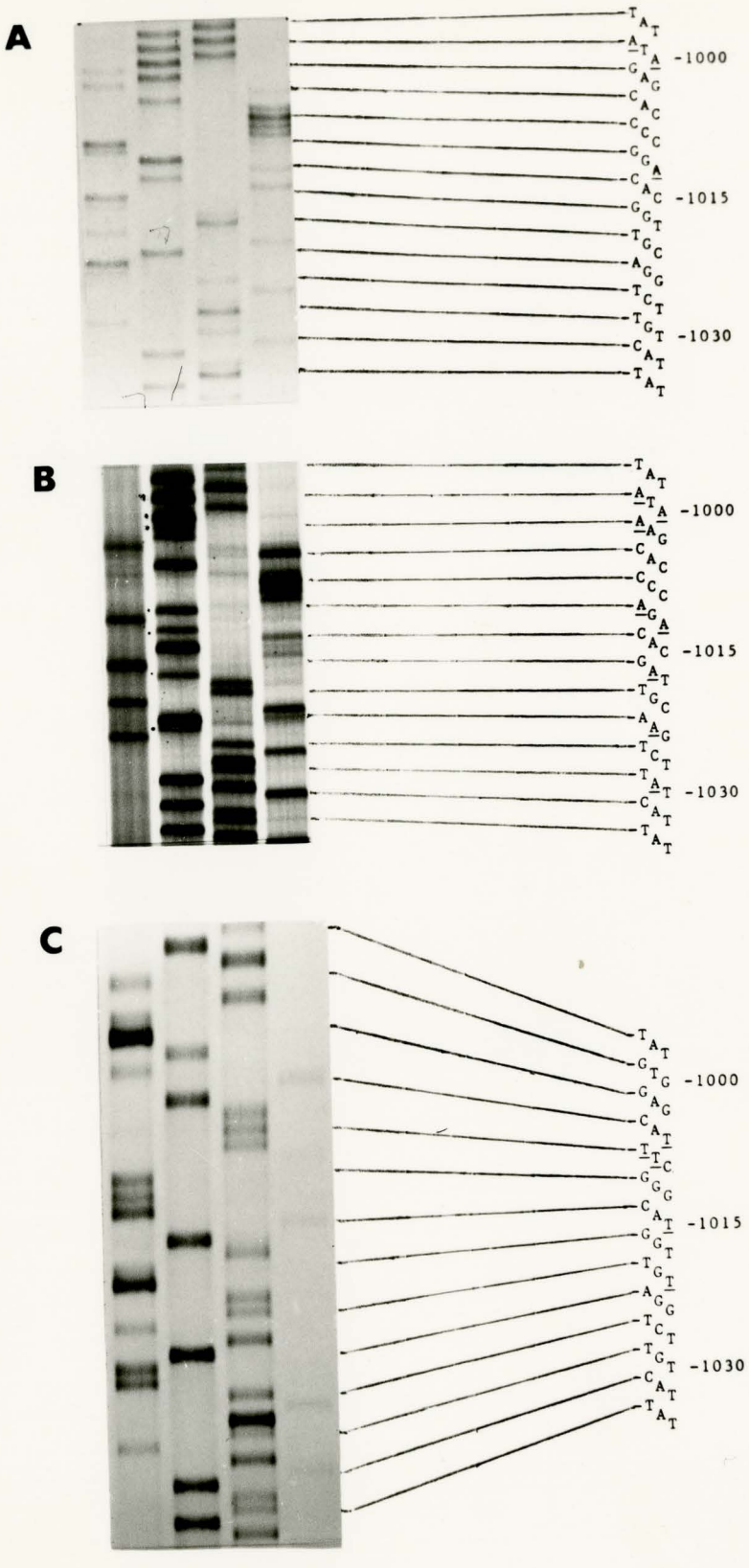
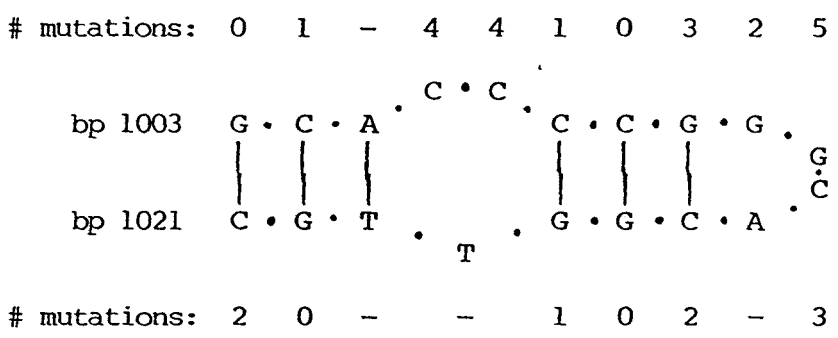

A
B
C
-1000
-1015
-1030

Figure 9 Hypothetical secondary structure of part of the deletion loop during deletion loop mutagenesis, from nucleotide 1003 to 1021. Above and below each nucleotide is the number of times it was mutated in the mutants shown in Figure 8. A's and T's were not susceptible to the mutagen.

Figure 9


mutations: 0 1 - 4 4 1 0 3 2 5
C • C
bp 1003 G • C • A C • C • G • G
G
C
bp 1021 C • G • T G • G • C • A
T
mutations: 2 0 - - 1 0 2 - 3

are not predicted to be base-paired were mutated more frequently. Because of their proximity to the end of the loop, G 1010 and C 1015 may not actually have been base paired and thus may have been accessible to the bisulphite. Although this loop is hypothetical, it does explain the different sensitivities of the various bases to the mutagen.

Pm 37 had an additional G to T transversion at base pair 1069 (Met 170 to Ile)(Figure 7). Since sodium bisulphite induces only C to U(T) transitions (Hayatsu, 1976), this mutation was not caused by the bisulphite, but was a random mutation that probably occurred spontaneously during the growth and manipulation of the plasmids.

The number of resulting amino acid changes in the mutants ranged from one to six. Five of these mutants were chosen for further study and were fully sequenced from the ClaI to the XbaI site. Pm 1120 contained a single conservative change, Val 147 to Ile, and pm 1122 contained a single nonconservative change, Pro 150 to Phe. These mutants were used to study the sensitivity of this region to single mutations. Pm 21, pm 27, and pm 1121 which had 3, 4, and 6 amino acid changes respectively, were also included to examine the effect of more numerous changes. Since preliminary results indicated that pm 1122 and pm 1121 with 1 and 6 changes respectively had the same phenotype in

transactivation and transformation (described in the following sections), these studies concentrated only on the mutants pm 1120, pm 1121 and pm 1122. It was not anticipated that the other mutants would have different properties than pm 1121 and pm 1122.

To avoid the possibility that mutations had occurred elsewhere on the plasmid besides in the deletion loop, a fully sequenced restriction fragment containing the mutated region was cloned into a background of a wild type E1A plasmid. The smallest convenient restriction fragment containing the mutations was the ClaI-XbaI fragment, from bp 920 to bp 1341.

The ClaI to XbaI fragment of the mutant plasmid was rescued into plasmid pLE2. pLE2 is similar to pHE1 except the entire E1A sequence is present, up to position 1772, as well as more of the pBR322 sequence (Figure 2). Determination of the proper recombinant involved screening for the loss of the SmaI site in the mutated fragment, since all these mutants had mutations in the SmaI site, and a HaeII digestion pattern that was characteristic of pLE2.

OTHER MUTANTS USED IN THESE STUDIES

To examine the role of regions in the rest of E1A in E1A functions, a series of mutants with mutations throughout E1A (Table 1 and Figure 1Ø) was used. These mutants were made using the synthetic oligonucleotide-directed mutagenesis technique (Zoller and Smith, 1984). Dl 11Ø1-dl 11Ø9, created by John Howe, Michael Floroff, Judy Denman, and Carole Evelegh, are small in-frame deletions which together delete most of 12S exon 1. Where possible, the deletions were chosen to remove regions of hydrophilicity or hydrophobicity, which may be exterior or interior domains of the protein, respectively. The entire 13S unique region was deleted by the small in-frame deletions, dl 1112 - dl 1114, made by Nina Cunniff, small in-frame deletions, along with dl 111Ø. Dl 1115 and dl 1116, generated by Mario Skiadopoulos, are in-frame deletions at the 5' end of exon 2 and delete a hydrophilic and hydrophobic region respectively. Pm 1131 (by M. Skiadopoulos) is a nonsense mutation at codon 219 which in effect deletes the C terminal 71 residues of the E1A proteins. Sub 1117 (by M. Skiadopoulos) is missing the acceptor splice site of E1A and thus is predicted to produce a readthrough product consisting of 13S exon 1, plus a

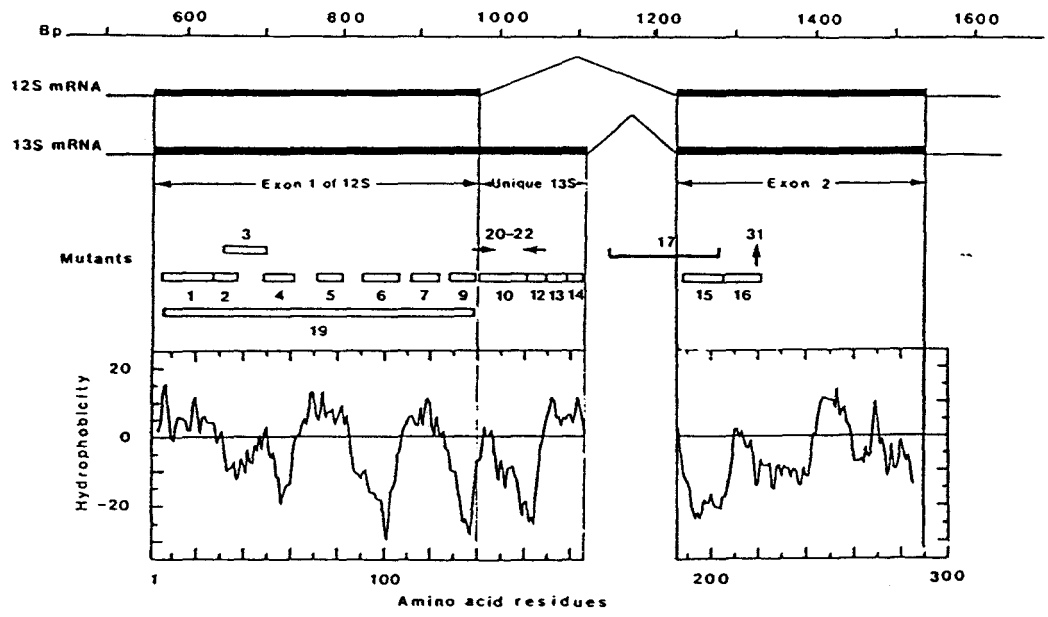
Table 1 Sequences deleted by E1A mutants. All the mutants except dl 1110 were made by oligonucleotide-directed mutagenesis. The particular nucleotides and amino acids deleted by each mutation are indicated. The number of nucleotides and amino acids deleted are in parentheses. In the case of pm 1131 and sub 1117, the codons themselves have not been deleted, but they are not translated because of the mutation. The amino acid residue numbers refer to the 13S product.

Table 1

Mutant	bp deleted	aa deleted
dl 1101	569- 634 (66)	4- 25 (22)
dl 1102	635- 664 (30)	26- 35 (10)
dl 1103	647- 706 (60)	30- 49 (20)
dl 1104	701- 739 (39)	48- 60 (13)
dl 1105	767- 802 (36)	70- 81 (12)
dl 1106	827- 874 (48)	90-105 (16)
dl 1107	890- 928 (39)	111-123 (13)
dl 1108	929- 940 (12)	124-127 (4)
dl 1109	941- 973 (33)	128-138 (11)
dl 1119	569- 973 (405)	4-138 (135)
dl 1110	976-1038 (63)	140-160 (21)
dl 1112	1040-1063 (24)	161-168 (8)
dl 1113	1064-1090 (27)	169-177 (9)
dl 1114	1091-1111 (21)	178-184 (7)
dl 1115	1237-1287 (51)	188-204 (17)
dl 1116	1288-1338 (51)	205-221 (17)
pm 1131	C->G@1331	219-289 (71)
sub 1117	1142-1288 (146)	186-289 (104)

Figure 10 Mutants used in this work deleting parts of the E1A protein. The 13S and 12S mRNAs and their coding regions are indicated. The regions deleted by each of the mutants are shown in blocks. For simplicity, only the last digit(s) of the mutant numbers are shown (e.g. 1 = dl 1101, etc.). Pm 1131 (31) has a missense point mutation at the position indicated (arrow). Pm 1120 to pm 1122 (20-22) have point mutations between the arrows in the 13S unique region. Below is a hydropathy plot of the 13S product, showing hydrophobic regions (positive) and hydrophilic regions (negative). For further details on the mutants, refer to Table 1 and Figure 7.

Figure 10


Bp
600
800
1000
1200
1400
1600
12S mRNA
13S mRNA
Exon 1 of 12S
Unique 13S
Exon 2
Mutants
3
20-22
17
31
1 2 4 5 6 7 9 10 12 13 14
15 16
19
Hydrophobicity
20
0
-20
1
100
200
300
Amino acid residues

missense lysine encoded in the intron (Jelsma et al., 1988). In total the mutants available in the lab delete the entire E1A gene with the exception of a few small gaps, most notably in 12S exon 1 between dl 1104 and dl 1105 (9 amino acids), and between dl 1105 and dl 1106 (8 amino acids).

The original intent was to use these mutants to identify regions in E1A that were required for different E1A functions. Once these regions were identified, missense mutations could be made within the region of these deletions, by deletion loop mutagenesis or oligonucleotide-directed mutagenesis to identify important residues in these regions. However, during the course of these studies other groups identified similar regions using deletion and point mutants which corroborated our data and made much of this further mutagenesis unnecessary.

Another mutant, dl 520, was used to produce only the 12S product. Dl 520 has a 11 bp deletion of the 13S donor splice site and thus no 13S mRNA can be made (Haley et al., 1984).

TRANSACTIVATION

A transient expression CAT assay was used to study the transactivation function of E1A. The test plasmid pKCAT23 contains the E1A sensitive Ad5 E3 promoter coupled to the gene for chloramphenicol acetyl transferase (CAT). Expression of the CAT enzyme coded by pKCAT23 can thus be stimulated by the transactivation function of E1A (Weeks and Jones, 1983). In these experiments HeLa cells were cotransfected with pKCAT23 and the wild type or mutant E1A plasmid pLE2, or, in one instance, with pMBCAT8 alone. Forty eight hours post transfection the cells were harvested and the extracts were assayed for CAT activity by measuring the ability of the extracts to acetylate ^{14}C-chloramphenicol.

(a) Optimization of Transfection Procedure

In order to obtain the highest transfection efficiency, the effect on the yield of CAT activity of altering two variables in the transfection procedure, was examined in a pilot experiment. These were the length of time the precipitate was on the cells, and the inclusion of a glycerol treatment of the cells after incubation with the precipitate. Because the reason for the glycerol treatment was to facilitate uptake of the DNA, this had to be done at

the end of the incubation with the DNA precipitate, so the time of glycerol treatment was dependent on the time of incubation with the precipitate.

When the Hela cells were transfected the precipitates were left on the cells for 4, 8, or 16 hours. For those dishes that were to be treated with glycerol, the medium and precipitate were removed after these times, and a 20% glycerol solution was added for 2 minutes. The glycerol was then removed, the dishes were rinsed and fresh medium was added. For those dishes that were not treated with the glycerol shock, the medium and precipitate were removed and replaced with fresh medium. Forty eight hours post transfection the cells were harvested and assayed for CAT activity. The results are shown in Table 2.

At both the 4 and 8 hour time points the glycerol treatment gave an approximately 40-fold increase in CAT activity. Without the glycerol shock, near background levels of CAT activity were obtained. When the precipitate was left on the cells for 16 hours the effect of the glycerol shock was less pronounced. A longer incubation time can conceivably have two effects on the cells. It may allow more cells to take up the DNA, but the longer incubation time may also reduce the viability of the cells taking up the DNA. The $CaCl_2$ or another component of the

Table 2 Effect of glycerol shock and of differing exposure of cells to precipitate on CAT activity of cell extracts. Amounts of plasmid DNA used were 20 ug pKCAT23, 40 ug pLE2, and 20 ug pMBCAT8. The length of exposure in hours of cells to precipitate (time), presence or absence of glycerol shock (glycerol), percentage of chloramphenicol acetylated (% acet.), and fold increase in CAT activity due to glycerol treatment (fold increase) are indicated.

Table 2

Plasmid	Time	Glycerol	% Acet.	Fold Increase
pKCAT23	4	-	0.8%	-
+ pLE2		+	36%	45
pKCAT23	8	-	0.8%	-
+ pLE2		+	31%	39
pKCAT23	16	-	0.6%	-
+ pLE2		+	5.3%	8.8
pMBCAT8	4	-	1.6%	-
		+	86%	54
pMBCAT8	8	-	3.0%	-
		+	84%	28
pMBCAT8	16	-	0.4%	-
		+	2.6%	6.5
mock	-	-	0.2%	-

precipitate mixture may kill the cells directly, or may weaken them so that they could not survive a further insult such as the glycerol treatment. Although this experiment did not distinguish between these two possibilities, it was evident that at the 16 hour incubation with the precipitate, the toxic effects of the precipitate outweighed any possible increase in transfection efficiency.

The CAT activity obtained with the test plasmid pMBCAT8, which expresses the CAT enzyme constitutively under the control of the SV40 enhancer/promoter sequences, was also greatly stimulated by the glycerol shock treatment, and also to the greatest extent at the 4 and 8 hour time points (Table 2).

On the basis of this experiment, the protocol for transfection included a 4 to 8 hour incubation of the precipitate on the cells (generally 6 hours), and a glycerol shock.

(b) Exon 1 Mutants

Mutants dl 1101 - dl 1107 and dl 1109 with small deletions in 12S exon 1 were tested in this assay to examine the role of 12S exon 1 in the transactivation function of E1A. The results are shown in Table 3.

Table 3 Effect of exon 1 mutations on transactivation of pKCAT23 by E1A. HeLa cells were transfected with the amounts indicated of the exon 1 mutant pLE2 plasmid (E1A) and of pKCAT23 (CAT). The per cent of chloramphenicol acetylated by the cell extracts is presented for the mutant pLE2 (mutant) as well as the controls for each experiment, wt pLE2 (wt) and pKCAT23 alone (CAT). The ratio of the mutant to wt activities is also indicated.

Comments: 1) Mutant value was an average of duplicate dishes.

2) Wt value was an average of duplicate dishes.

Table 3

ug E1A	ug CAT	% Acetylation mutant	% Acetylation wt	CAT	mutant / wt	Comments
dl 1101						
10	5	70	73	9	1.0	
dl 1102						
10	5	74	63	4	1.2	
10	5	59	73	9	0.8	
dl 1103						
10	5	74	63	4	1.2	
10	5	84	73	9	1.2	
dl 1104						
20	20	75	64	1	1.2	1, 2
10	5	76	73	9	1.0	
10	5	34	20	3	1.7	
dl 1105						
20	20	62	64	1	1.0	1, 2
10	5	64	73	9	0.9	
10	5	26	20	3	1.3	
dl 1106						
20	20	52	71	6	0.7	1
20	20	40	35	0	1.1	1
10	5	68	73	9	0.9	
dl 1107						
10	5	68	63	4	1.1	
10	5	66	73	9	0.9	
dl 1109						
20	20	59	71	6	0.8	1
20	20	49	35	0	1.4	1
10	5	50	73	9	0.7	
dl 1119						
10	5	5	63	4	0.1	
10	5	2	30	5	0.1	

The degree of transcriptional activation by E1A can be expressed in two ways in these experiments, by the absolute increase in acetylated chloramphenicol and by the relative increase in activity compared to that obtained with pKCAT23 alone. The absolute increase in CAT activity depends largely on the efficiency of transfection. The relative increase in CAT activity (mutant/CAT) depends both on the absolute increase in CAT activity and the activity obtained with pKCAT23 alone. The wide range of activity with pKCAT23 alone, may have reflected the presence of an endogenous transactivator, the levels of which may vary depending on the state of the cells. Imperiale et al. (1984) have postulated that such an E1A-like activity exists in HeLa cells.

A wide variability was seen in the yield of CAT activity between experiments. For example, wt pLE2 gave CAT activities ranging from 20% to 73% acetylation.

For the CAT reaction to be an accurate reflection of the amount of CAT enzyme present and hence the amount of transactivation that occurred, the amount of chloramphenicol acetylated must be directly proportional to the amount of CAT enzyme present. This requires that the concentration of the substrates for the reaction, acetyl CoA and chloramphenicol, which were 800uM and 40uM respectively, be

well above their K_M's for the enzyme, which were 50uM and 17uM respectively (Shaw, 1975). Excessive depletion of the chloramphenicol would decrease the rate of reaction and would lessen the difference in percent acetylation between two extracts of different activity. As shown in Table 3 the reactions frequently gave over 60% acetylation, bringing the final concentration of chloramphenicol to a point less than the K_M for the reaction. As a result, quantitative comparisons of small differences in CAT activity between different samples could not be accurately determined.

An examination of Table 3 shows that there was a wide variability in the CAT activity seen between experiments. This was most probably due to variations in the efficiency of transfection and/or harvesting from experiment to experiment. In spite of this, a comparison of wt and mutant within an experiment showed that the exon 1 mutants dl 1101 to dl 1107 and dl 1109 stimulated CAT activity to approximately the same extent as wt pLE2. The last column in Table 3, showing the ratio of the CAT activities of the mutant to wt, demonstrates that regardless of the overall transfection efficiency of the experiment or the amount of plasmid DNA used, wt and mutant plasmids had similar activities. The data clearly show that the sequences removed in dl 1101 to dl 1107 and dl 1109 are not

not required for transactivation by E1A. Some additional results for most of the mutants were obtained by John Howe in our laboratory corroborating this conclusion (cf Jelsma et al., 1988).

These results suggested that exon 1 was not involved in transactivation and that any deletion in exon 1 would not affect transactivation. To test this, another mutant, dl 1119, which deletes almost the entire exon 1, residues 4 to 138, was also examined for its ability to transactivate. As shown in Table 3, dl 1119 was unable to transactivate. The significance of this result will be discussed later, in defining the transactivation domain of E1A.

(c) Unique Region Mutants

To study the effect of mutations in the unique region on transactivation by E1A, deletion and missense mutations spanning this region were also tested in the CAT assay. The results are shown in Table 4. To ensure that the lack of any transactivating activity was not due to faulty plasmid preparations, each mutant was tested using different amounts of DNA, and with different plasmid preparations.

Pm 1120 - pm 1122 are missense mutants in the region of the SmaI site and have one, six and one amino acid

Table 4 Effect of 13S unique region mutations on transactivation of pKCAT23 by E1A. The headings are the same as in Table 3.

Table 4

ug E1A	ug CAT	% Acetylation mutant	% Acetylation wt	CAT	mutant / wt	Comments
pm 1120						
20	20	1	22	0	0.04	1
1	5	6	66	2	0.09	
10	5	14	76	2	0.18	
10	5	25	63	4	0.40	
pm 1121						
20	20	2	11	1	0.18	
20	20	2	11	1	0.18	
10	5	1	23	0	0.04	
10	5	2	30	5	0.07	
pm 1122						
20	20	0	22	0	0.00	1
20	20	1	64	1	0.02	1, 2
1	5	2	85	2	0.02	
4	5	1	84	2	0.01	
10	5	1	81	2	0.01	
10	5	0	23	0	0.00	
dl 1110						
20	20	1	64	1	0.02	1, 2
10	5	0	23	0	0.00	
dl 1112						
10	5	0	23	0	0.00	
dl 1113						
10	5	1	23	0	0.04	
dl 1114						
10	5	0	23	0	0.00	

changes respectively (Figure 8). All three were impaired in their ability to transactivate. Pm 1120 retained a partial transactivating activity, giving as high as 25% acetylation, or 40% of the wt activity. Pm 1121 and pm 1122 on the other hand, were completely defective in transactivation and did not stimulate CAT activity to any extent above background levels, yielding no more acetylation. Similarly, dl 1110, which deletes the region containing the pm 1120 - pm 1122 mutations, was also unable to transactivate (Table 4). The reason that pm 1120 was only partially defective may have been due to the fact that the Val 147 to Ile change was a conservative mutation (valine and isoleucine differ only by a methyl group), or that the mutation was in a region of the transactivation domain which was not as critical for function.

Dl 1112-1114, three deletion mutants made by Nina Cunniff in our laboratory, together delete residues 161-184, the remainder of the unique region not deleted by dl 1110. These mutants were shown to be defective in transactivation (Cunniff, 1988; Jelsma et al., 1988a). I obtained one result for each mutant that confirmed this (Table 4).

The exon 2 mutants dl 1115, dl 1116, pm 1131, and sub 1117 were made by Mario Skiadopolous in our laboratory. Dl 1115 and dl 1116 lack residues 188 to 204 and 205 to 221

respectively, while sub 1117 and pm 1131 delete the C terminal 107 and 71 residues respectively. When these mutants were examined for their transactivation ability sub 1117 and dl 1115 were unable to transactivate, while dl 1116 gave wild type levels of CAT activity. Pm 1131 was variable, with levels ranging from near background to wild type levels (Skiadopoulos, 1988; Jelsma et al., 1988).

(d) Assignment of a Transactivation Domain

A diagram of the mutants used in this work is shown in Figure 5. With the exception of dl 1119, which will be discussed later, deletions in 12S exon 1 did not affect transactivation, indicating that the transactivation domain did not include 12S exon 1. All the mutations in the unique region except pm 1120, and dl 1115 in the N terminal portion of exon 2 were totally inactive in transactivation, suggesting that this region contained the transactivation domain. This domain begins between residues 139 (dl 1109) and 147 (pm 1120) and continues to a point between residues 188 and 204 (the sequence deleted in dl 1115). Results obtained with dl 1116 showed that residues 205 to 221 were not required for transactivation. Since pm 1131 was partially defective in transactivation, this suggested that either some part of the C terminal 71 residues of E1A was

also part of the transactivation domain, or that the removal of this region adversely affected either the conformation or the stability of the domain itself. However, Schneider et al. (1987) showed that a similar mutant deleting the C terminal 67 residues is wild type for transactivation. This suggests that the C terminal region of the protein does not contain part of the transactivation domain but pm 1131 is defective in transactivation because the large deletion caused the transactivation domain to be unstable.

The reason dl 1119 failed to transactivate may have been because it lacks a portion of the transactivation domain that was not removed in the other exon 1 mutants, or, as was postulated for pm 1131, the large deletion may have caused the remainder of the protein to be in an inactive conformation or rapidly degraded. Consistent with the last possibility is the fact that E1A proteins could not be seen in immunoprecipitates of cells infected with the dl 1119 virus whereas the other exon 1 mutants gave easily detectable levels of E1A proteins (C. Egan, personal communication, Egan et al., 1988).

The unique region and the N terminal portion of exon 2, residues 140 to 188, is highly conserved between different adenovirus serotypes (Kimelman et al., 1985; Moran and Matthews, 1987). Since these studies were initiated, a

synthetic peptide corresponding to this conserved region was shown to be able to transactivate upon microinjection into HeLa cells (Lillie et al., 1987), showing that this indeed was the transactivation domain. This suggested that the reason dl 1119 and pm 1131 were defective in transactivation was not because they lacked a part of the transactivation domain, but instead these mutations affected the stability of the protein or the conformation of the transactivation domain. These effects on stability and conformation could also apply to the synthetic peptide of Lillie et al., (1987), but the large quantity injected, 1000 - 2000 molecules per cell (Green et al., 1988), would ensure that enough remained in the correct conformation for transactivation to occur. Alternatively, the peptide of Lillie et al. (1987) may have acted inefficiently, but enough of the peptide was present to give a signal in their assay. A similar efficiency of the dl 1119 product would not be detected in the CAT assay described here because of the lower levels of E1A protein present in cells transfected with dl 1119.

Although in this assay the activation of the E3 promoter only was examined, these results can probably be extended to the activation of the other adenovirus early promoters. Schneider et al. (1987) obtained identical

results in transactivation studies using the E2 and E3 promoters. However, this may not be the mode of activation of all cellular genes induced by E1A. For example, the unique region is dispensable for the induction of PCNA (Zerler et al., 1987; Jelsma et al., 1989).

These results indicate that the transactivation domain is comprised of a discrete region in the unique region and N terminal portion of exon 2 and, notwithstanding the results seen with dl 1119 and pm 1131, is relatively insensitive to mutations elsewhere in the protein.

(e) Inhibition of wt Transactivation by Mutant E1A Proteins

The E1A protein produced by the transactivation negative mutant hr 5 was reported to inhibit wild type E1A in transactivation when plasmids containing each were cotransfected into HeLa cells (Glenn and Ricciardi, 1987). This inhibition was at least 50% at equimolar amounts of wt and hr 5 E1A, and was essentially complete at a fivefold molar excess of the mutant gene. In contrast, two other point mutations in the 13S unique region, hr 3 and hr 4, did not significantly inhibit wt E1A. Glenn and Ricciardi (1987) suggested that the hr 5 protein was sequestering the cellular transcription factors that E1A normally binds to in transactivation, and removing them from the available pool

of these factors. This would suggest a two component process in transactivation, namely binding to the putative transcription factor, and activation of this protein (Glenn and Ricciardi, 1987). Hr 5 has a G-A substitution at bp 1113, at the splice acceptor site in exon 2, which changes Ser 185 to Asn in the 13S product and Gly 139 to Asp in the 12S product.

To test whether this is a more general phenomenom, or is confined to hr 5, Nina Cunniff in our lab tested dl 1112, dl 1113, and dl 1114 for this inhibitory activity. CAT assays were carried out using pKCAT23, pLE2 and varying amounts of the mutant E1A plasmids. The presence of the mutant plasmid in general resulted in a reduction of CAT activity, but often this occurred only with at least a 2.5-fold excess of the mutant E1A plasmid over the wt E1A (Cunniff, 1988). There were several obstacles to our interpreting these results as showing an inhibition of transactivation by these mutants. Firstly, the amount of DNA transfected in each sample was not kept constant by using a nonfunctional plasmid such as pBR322 or pUC18. This may have resulted in a difference in transfection efficiency between dishes. Secondly, the addition of the mutant E1A plasmids increased the competition for the various factors needed for transcription and translation of the E1A

products, since the mutant E1A products were expressed as well as wild type E1A. This may have given a nonspecific inhibition of the expression of wild type E1A. Thirdly, although the mutants were defective for transactivation, they were still able to repress enhancer sequences (see Introduction and following chapter). Since the E1A enhancer is also repressed by E1A (Smith et al., 1985), the addition of the mutant plasmids could have changed the balance between transactivation and repression of the E1A genes in favour of repression, and thus also have given a nonspecific inhibition of transactivation.

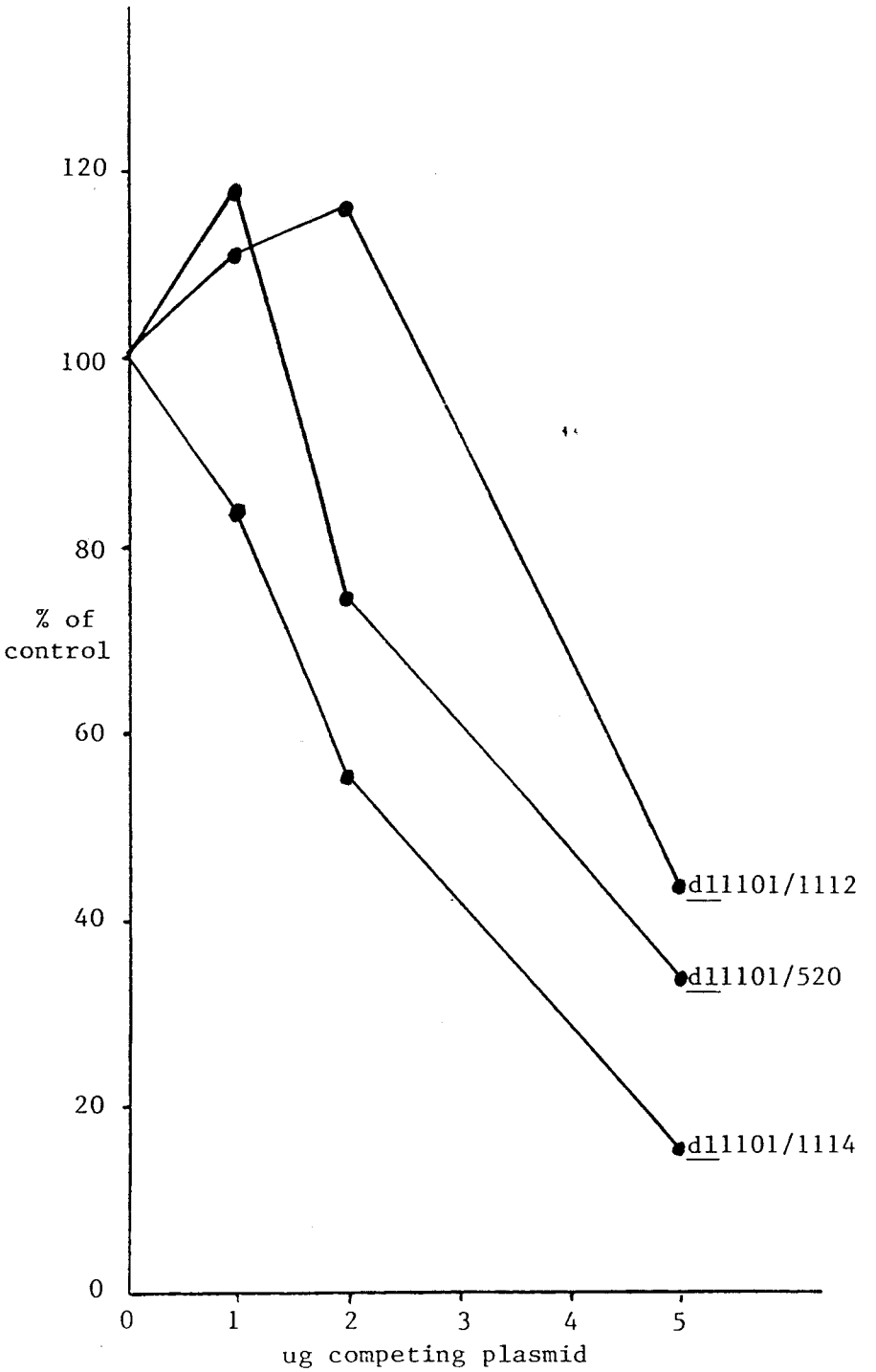
The experimental design was therefore modified as follows. Firstly, pUC18 was added to the transfection mixture where necessary, to maintain a constant amount of DNA in all samples. Secondly, to control for the number of copies of the E1A gene product, different amounts of dl 520 were tested in parallel with the mutants. Since this mutant does not make the 13S product, almost none of the transactivation domain was present. Any decrease in CAT activity seen with this mutant would not have been due to the type of inhibition described for hr 5 (Glenn and Ricciardi, 1987) but would have been due to a nonspecific competition for factors required for transcription and translation of the E1A products. Thirdly, to avoid the

effect of the increase in the enhancer repression activity, the d1 1101 mutation was cloned into all the E1A plasmids used. This mutation, deleting residues 4-25, did not affect the ability of E1A to transactivate, but, as will be described in the following section, rendered the E1A protein unable to repress enhancer sequences.

HeLa cells were transfected with 5 ug pKCAT23, 1 ug d1 1101, used as the "wild type" transactivator, 0, 1, 2, or 5 ug of d1 1101/d1 1112, d1 1101/d1 1114 or d1 1101/d1 520, and pUC18 to bring the total amount of plasmid DNA transfected to 11 ug. The results are graphed in Figure 11. Although at a 5:1 ratio both d1 1101/1112 and d1 1101/1114 caused a large reduction in CAT activity, this was similar to the reduction in CAT activity caused by d1 1101/520. Even though the amount of transfecting DNA was kept constant and there was no interference by an increase in enhancer repression, the competition for transcription and translation factors by the mutant E1A genes caused a large reduction in the amount of transactivating E1A protein present. D1 1101/1112 did not show any hr 5-like inhibitory activity as it gave even less of a reduction than d1 1101/520. D1 1101/1114 gave a greater reduction in CAT activity than d1 1101/520, but if the difference in activities obtained between d1 1101/1112 and d1 1101/520 can

Figure 11 Effect of addition of dl 1101/1112, dl 1101/1114, or dl 1101/520 double mutants on transactivation of pKCAT23 by dl 1101. Cells were transfected with 5 ug pKCAT23, 1 ug pLE2 dl 1101, and 1, 2, or 5 ug of the plasmids indicated (competing plasmid). The CAT activity of the extracts is expressed as the percent of the activity obtained with 1 ug pLE2 dl 1101 alone.

Figure 11


120
100
80
% of
control
60
40
20
0
0
1
2
3
4
5
ug competing plasmid
dl1101/1112
dl1101/520
dl1101/1114

be used as an indication of the inherent variability of the assay, the difference between dl 1101/1114 and dl 1101/520 is not significant. In any case the magnitude of this difference is small relative to the reduction in CAT activity seen with dl 1101/520.

These results indicate that based on this assay, dl 1112 does not have the ability to inhibit wt E1A in transactivation as described for hr 5 (Glenn and Ricciardi, 1987). If such an ability exists for dl 1114, it is very minor relative to hr 5 and cannot be conclusively shown by this type of assay.

ENHANCER REPRESSION

Another E1A function that was examined was the repression of enhancer sequences. A transient expression CAT assay was also used in these experiments to test the ability of the E1A mutants to repress the SV40 enhancer. The test plasmid used, pSV_2CAT, contains the gene for chloramphenicol acetyltransferase (CAT), driven by the SV40 enhancer/promoter sequences (Gorman and Howard, 1982). Full repression by E1A reduces the expression of the gene to that which would be present without the enhancer (Borrelli et al., 1984). In the present experiments pSV_2CAT was cotransfected with E1A plasmid into HeLa cells. Forty-eight hours after transfection, the cells were harvested and assayed for CAT activity. Enhancer repression was measured by the reduction in CAT activity from that obtained with pSV_2CAT and a nonrepressing carrier plasmid, pUC18.

(a) Optimization of Harvesting Procedure

During the experiments on enhancer repression, it soon became apparent that due to the different experimental design from the transactivation CAT assays, the experiments were inherently more difficult to control. First of all,

the transactivation assays involved an E1A-induced increase in CAT activity from a low level, while the enhancer repression experiments involved a decrease from high levels of CAT activity. Because of this, the variability in these assays made it difficult to accurately measure mutants with low repression activity. Secondly, while the transactivation experiments generally showed as much as a tenfold stimulation in activity, the enhancer repression experiments showed only a four to fivefold repression, making it more difficult to detect an inefficient repression activity. Thirdly, in the transactivation CAT assays, the kinetics of the CAT reaction were not ideal, since the final concentration of chloramphenicol was often lower than the K_M for the reaction. For these experiments, the amount of acetylation needed to be kept low to avoid depletion of substrate, yet high enough to yield a measurable signal. Since, as shown in the transactivation experiments, there was a wide variation in CAT activity obtained between experiments, a more efficient harvesting procedure that yielded stable extracts was needed.

Two activities are present in cell extracts that reduce the final yield of acetylated chloramphenicol. Cell extracts contain an activity which hydrolyses acetyl CoA, one of the substrates of the reaction, and they contain

proteases which break down the CAT enzyme (Crabb and Dixon, 1987). Two changes were therefore made in the harvesting protocol to minimize these activities. Included in the sonication buffer was 5mM EDTA, to minimize the acetyl CoA hydrolyzing activity of the extract, and, to inactivate the protease activity, immediately after the sonication step each sample was incubated at $60^{o}C$ for 10 minutes. The CAT enzyme itself is resistant to this heat treatment (Crabb and Dixon, 1987). The activity of extracts prepared in this way was higher and more reproducible than that obtained in the transactivation experiments. Whereas in the transactivation CAT assays the whole extract was needed, a small fraction of the total extract gave sufficient CAT activity for these experiments. These additions to the procedure also made the CAT activity in the extracts more stable. The extracts could be stored at $4^{o}C$ for at least three days with no measurable loss of activity. The advantage of this increased stability was that the reactions could be repeated with different amounts of extract and incubation times if the amount of acetylation was too high or too low.

Because of the variability of this assay, each mutant was tested a number of times and with at least two or three different plasmid preparations to ensure the reliability of the data. Since this involved a considerable

amount of work, the repression assays of the exon 1 mutants were shared between John Howe and myself. The results to be discussed were done by myself; John did a similar number of experiments with similar results.

(b) 12S Exon 1 Mutants

In initial repression experiments, no repression was observed with the wt E1A plasmid pLE2. On the other hand, dl 1112 and dl 520 gave a three to fivefold reduction in CAT activity. Dl 1112 contains a deletion in the unique region, and dl 520 lacks the donor splice site for the 13S mRNA and thus produces only the 12S product (Haley et al., 1984). Both mutants are defective for transactivation.

Although the extent of repression seen in these experiments was generally three to fivefold, the actual level of repression may be much higher. In the plasmid transfection assays used here, a small proportion of cells that would receive the pSV_2CAT plasmid only would substantially reduce the apparent repression activity. For example, if the actual degree of repression were tenfold, and if 10% of the cells receiving pSV_2CAT did not receive the E1A plasmid, the apparent level of repression would be just over fivefold. Although the extent of such an occurrence is not known in these experiments, the nature of

this type of assay makes it difficult to detect a high degree of repression. Nevertheless the levels of repression that were observed gave a clear indication of the effects of the mutations on repression of the SV40 enhancer.

The results with dl 1112 and dl 520 suggested that the transactivation function of the wt pLE2 was masking the repression activity. To avoid this, experiments were done using the exon 1 mutants into which the dl 520 mutation had been cloned. Because of the dl 520 mutation, these exon 1/dl 520 double mutants were transactivation defective. These plasmids contained the XmaI to XbaI fragment (bp 1009 to 1341) from dl 520, containing the mutation, cloned into each exon 1 mutant.

The results of the repression assays are shown in Table 5. The last column is an average of all the experiments for each mutant. One hundred percent repression was defined as the reduction in CAT activity obtained by dl 520 in each experiment and the reduction obtained by the mutants was related to this value. A negative repression value indicated that the mutant caused an increase (usually slight) in CAT activity. While this may have been due to a transactivating activity of the 12S mRNA product, the increase was less than the variability of the assay as shown by the standard deviation for each mutant. Dl 1101/520,

Table 5 Effect of exon 1 deletions on repression of pSV_2CAT by pLE2/d1 520. Average results for each mutant are shown in the right column. The average value of the relative repression was determined by comparing the average of the activity of the mutant relative to pUC18 to the average of pUC18. pUC18 refers to samples containing pUC18 in place of the E1A plasmid.

relative repressing activity =

$$\frac{\text{activity with pUC18 - activity with mutant}}{\text{activity with pUC18 - activity with d1 520}}$$

Where relative repressing activity < 0, mutant and pSV_2CAT gave higher activity than pUC18 and pSV_2CAT.

Average relative repressing activity for d1 1109 was not determined because of the wide variation in results.

Each mutant also contained the d1 520 deletion.

Table 5

% Acetylation
Activity relative to pUC18
Repressing activity Relative to *dl* 520

1	2	3	4	5	6	7	8	9	10	Ave.	±SD
pUC18											
12	69	88	23	97	31	23	12	39	64		
dl 520											
2	26	28	4	20	10	8	4	12	14		
0.2	0.4	0.3	0.2	0.2	0.3	0.3	0.3	0.3	0.2	0.27	±0.07
1	1	1	1	1	1	1	1	1	1	1	
dl 1101											
–	76	97	–	95	–	37	13	34	–		
–	1.1	1.1	–	1.0	–	1.6	1.1	0.9	–	1.13	±0.24
–	<0	<0	–	0.0	–	<0	<0	0.2	–	<0	
dl 1102											
–	47	44	9	69	–	–	–	–	–		
–	0.7	0.5	0.4	0.7	–	–	–	–	–	0.58	±0.15
–	0.5	0.7	0.7	0.4	–	–	–	–	–	0.6	
dl 1103											
–	84	82	–	95	–	–	–	–	–		
–	1.2	0.9	–	1.0	–	–	–	–	–	1.03	±0.15
–	<0	0.1	–	0.0	–	–	–	–	–	<0	
dl 1104											
13	70	87	40	96	–	–	16	31	–		
1.1	1.0	1.0	1.7	1.0	–	–	1.3	0.8	–	1.13	±0.29
<0	<0	0.0	<0	0.0	–	–	<0	0.3	–	<0	
dl 1105											
5	43	43	–	38	–	–	–	–	24		
0.4	0.6	0.5	–	0.4	–	–	–	–	0.4	0.46	±0.09
0.7	0.6	0.8	–	0.8	–	–	–	–	0.8	0.7	
dl 1106											
–	–	–	–	48	–	12	–	–	17		
–	–	–	–	0.5	–	0.5	–	–	0.3	0.43	±0.12
–	–	–	–	0.6	–	0.7	–	–	0.9	0.7	
dl 1107											
4	27	37	–	–	–	13	–	–	21		
0.3	0.4	0.4	–	–	–	0.6	–	–	0.3	0.40	±0.12
0.8	1.0	0.8	–	–	–	0.7	–	–	0.9	0.8	
dl 1108											
–	–	–	–	–	–	11	–	–	12		
–	–	–	–	–	–	0.5	–	–	0.2	0.35	±0.21
–	–	–	–	–	–	0.8	–	–	1.0	0.9	
dl 1109											
–	–	–	–	–	29	23	13	46	36		
–	–	–	–	–	0.9	1.0	1.1	1.2	0.6	0.96	±0.23
–	–	–	–	–	0.1	<0	<0	<0	0.6	–	

dl 1103/520 and dl 1104/520 gave no reduction in CAT activity. Dl 1109/520 usually did not repress, but on one occasion in my experiments and occasionally in John's it had substantial repression activity, but less than wt dl 520. Dl 1102/520 and dl 1105/520 were about two thirds as effective as dl 520, and dl 1106/520, dl 1107/520, and dl 1108/520 gave reductions in CAT activity approaching that of dl 520.

These data indicated that deletions in three regions of 12S exon 1 severely impaired the ability of E1A to repress the SV40 enhancer. These were the deletion of residues 4 to 25 (dl 1101), 36 to 60 (dl 1103 and dl 1104, excluding residues deleted by dl 1102), and 128 to 138 (dl 1109). Deletions between these regions did not abolish repression, although activity was sometimes diminished.

c) Assignment of Repression Domain

There are two regions in E1A which are highly conserved between different adenovirus serotypes, besides the one discussed earlier which is involved in transactivation. These are residues 40 to 80, designated conserved region 1 (CR1), and residues 121 to 139, CR2 (Moran and Mathews, 1987). The repression defective mutants dl 1103 and dl 1104 delete parts of CR1, while dl 1109

deletes part of CR2, implying that these regions are involved in repression. However, dl 1105 deletes part of CR1 and dl 1107 and dl 1108 delete parts of CR2 but are still able to repress. Conversely, dl 1101 does not delete any part of CR1 or CR2 yet is unable to repress.

The region deleted in dl 1101 may constitute a separate domain from that deleted in dl 1103 and dl 1104, or, by tertiary folding of the protein, the two regions may be part of one domain. In this case the region deleted by dl 1102 would be a nonessential linker region between the two. Since the sequence deleted by dl 1101 is not conserved between different adenovirus species, the structure, rather than the sequence in this region may be important for function. For example, this region may form a core structure for the correct folding of CR1, which may be the actual repression domain.

Conflicting results have emerged from other labs examining enhancer repression which have made it difficult to assign functional domains for repression. For example, Velcich and Ziff (1988) suggested that the structural integrity of the whole protein was needed to maintain function, since several deletions throughout E1A reduced repression activity. Our data, and that of other labs, however, have assigned functional domains for repression,

but not all the data agree. Our results indicate that the N terminal region of E1A is required for repression. This is supported by preliminary evidence that dl 1504 which lacks the N terminal 15 residues (Osborne et al., 1982; Downey et al., 1985) is also defective for repression (J. Howe, personal communication). In contrast, Velcich and Ziff (1988) report that deletion of residues 1 to 14 did not greatly affect repression. A substitution mutant of Subramanian et al., (1988) at residue 19 was also wt for repression. These results suggest that the entire N terminal region is not required for repression, but parts of it are, and perhaps the different mutations had different effects on the folding of this region.

Our data suggest that the entire CR1 is not needed for repression, since dl 1105 which removes the C terminal 11 residues of CR1 retains substantial activity. In contrast, two point mutations in the region deleted by dl 1105, at residues 72 and 73, abolish repression activity (Lillie et al., 1987). The reason for this discrepancy is not clear.

Our results also show that at least part of CR2, residues 121 to 127, is dispensable for enhancer repression. This is in direct conflict with results obtained from other groups who showed that deletion of residues 121-125

(Schneider et al., 1987) or mutation of residue 126 (Lillie et al., 1986) severely impaired repression. The reason for these discrepancies is not clear, but, as with the N terminal region, CR2 may not be directly involved in repression, but may affect the folding of the domain for repression. Different mutants may have different effects on the interaction between CR2 and the repression domain. Similarly, dl 1109/520 is at least partially defective in repression, but preliminary results indicate that dl 1109/1112 is not (J. Howe, personal communication). Thus the presence of the unique region may negate the effect of the dl 1109 deletion on repression. Together these results suggest that CR2 is probably not directly involved in enhancer repression.

The unique region itself is not required for repression since dl 520, which produces only the 12S product, represses as efficiently as dl 1112, which produces both 13S and 12S products. Exon 2 is also not required since sub 1117 which lacks exon 2 is capable of repressing (Jelsma et al., 1988b). This is in direct conflict with data of Velcich and Ziff (1988) who have a mutant deleting residues 185 to 216, which is defective for repression. The reason for this discrepancy is not clear. Thus the enhancer

repression function maps within 12S exon 1, to the N terminal region, CR1, and possibly part of CR2.

As mentioned earlier, in our initial experiments the transactivation function of E1A masked the effects of repression. This was also seen by Lillie et al., (1986) in a different assay where the SV40 enhancer-driven gene was cloned into adenovirus, and where transcription was measured directly by primer extension. However, in still other assays of plasmids containing the SV40 or polyoma enhancer, where transcription was measured by Northern analysis (Velcich and Ziff, 1988) or S1 nuclease analysis (Borrelli et al., 1984; Schneider et al., 1987) there was no such effect of transactivation. The reason for these discrepancies is not clear and may be a function of the different assay methods used. Since transactivation and repression occur at the same range of E1A concentrations (Borrelli et al., 1984), the amount of E1A present in the different assays is not a factor.

Regardless of these discrepancies, however, the assays used in this work circumvented the interfering effect of transactivation and identified the N terminal region and CR1 as being required for repression, and the C terminal portion of CR2 as having at least an indirect effect on repression.

TRANSFORMATION

The assay chosen to study the effect of the mutations on the role of E1A in transformation was the transformation of primary baby rat kidney (BRK) cells by plasmids containing E1A and *ras* (Ruley, 1983). In using *ras* instead of E1B as the second transforming oncogene, the interactions between E1A and E1B were avoided. These interactions include the requirement of transactivation by E1A for E1B expression (transactivation defective E1A mutants can be examined in this assay since *ras* is expressed constitutively), the repression of E1A expression by E1B (White et al., 1988) and the inhibition by E1B of the enhancer repression function of E1A (Yoshida et al., 1987). However, the E1A and *ras* genes may have functions in common which are required for transformation, and removal of this function by an E1A mutation would not be apparent in this assay. For this reason, the results obtained do not necessarily apply to transformation by E1A and E1B.

Even though transformation by E1A and *ras* is not part of a normal infectious process, these studies are nevertheless worthwhile. First, a reproducible system of transformation lends itself to systematic research into the molecular events leading to the cancerous state. Second,

since E1A can bring about these effects, they presumably also play some role in the life cycle of the virus. Thus the study of the mode of action of the various E1A functions in transformation is also relevant to the role of E1A in viral infection.

In these transformation experiments, 0.25 ug each of the wild type or mutant E1A plasmids and a plasmid containing the EJ-ras oncogene (Land et al., 1983) were transfected into primary baby rat kidney (BRK) cells from 2 or 6 day old Wistar rats by the $CaPO_4$ coprecipitation technique. The appearance of transformed foci was monitored throughout the experiment. The dishes were generally stained at 4-5 weeks post-transfection.

At the beginning of this work, it was suggested that the difference in the transforming abilities of the wild type and mutant E1A plasmids may be concentration dependent. To determine whether this was the case, the early experiments used three different amounts of the E1A and ras plasmids, 0.125, 0.25, and 0.5 ug per dish. Over the fourfold range of plasmid concentration, differences in amounts of DNA did not make much difference in the numbers of foci obtained, so subsequent experiments used only 0.25 ug of each plasmid.

(a) Unique Region and Exon 2 Mutants

i) Normal Medium

The results of the transformation assays with the transactivation defective missense and deletion mutants in the 13S unique region are shown in Table 6 and are summarized in Table 7. Likewise the data for the exon 2 mutants are shown in Tables 8 and 9. For comparison, data for wild type in the same experiments are also presented. In these experiments cells from one focus frequently lifted off and established secondary foci elsewhere in the dish. Because these secondary foci distorted the focus count, the data are expressed as the number of dishes with foci out of the total, total number of foci, and average number of foci per dish. On occasion the secondary foci spread throughout a dish and made accurate counting impossible. Such dishes, which were not included when calculating the average number of foci per dish, are shown in the tables with an asterisk. Thus it was impossible to determine how many original foci there were in these dishes. However, these dishes needed to be included in the experimental data. Therefore, in order to determine the average number of foci over all the experiments, these dishes were counted as having had the average number of foci for that experiment. Since these dishes were part of the

Table 6 Effect of unique region mutations on transformation of primary baby rat kidney (BRK) cells. BRK cells were transfected with wt or mutant pLE2 and ras plasmids as described in Materials and Methods. The data are expressed as the number of dishes containing foci out of the total, the total number of foci, and average number of foci per dish. Dishes that were filled with foci are indicated with an asterisk (*), and were not included in the calculation of average number of foci per dish.

Table 6

Number of Dishes with Foci
Total Number of Foci
Average Number of Foci/Dish

Expt.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mutant															
wt pLE2	9/13	9/15	14/14	20/20	13/15	10/10	8/12	7/7	4/8	4/4	4/4	6/7	6/6	6/6	3/5
	44	23	39	153	110+1*	88	68	44	19	24	18	21	53	21	10
	3.4	1.5	2.8	7.6	7.9	8.8	5.7	6.3	2.4	6.0	4.5	3.0	8.8	3.5	2.0
pm 1120	–	–	14/15	–	15/15	–	–	–	–	–	3/4	–	–	–	–
	–	–	102	–	128+2*	–	–	–	–	–	10	–	–	–	–
	–	–	6.8	–	9.8	–	–	–	–	–	2.5	–	–	–	–
pm 1121	–	–	15/15	–	15/15	–	–	–	6/8	–	4/4	–	–	–	–
	–	–	179+1*	–	140+1*	–	–	–	128	–	16	–	–	–	–
	–	–	12.8	–	11.0	–	–	–	16.0	–	4.0	–	–	–	–
pm 1122	–	15/15	–	15/15	–	10/10	–	–	7/8	–	4/4	–	–	–	–
	–	340	–	165	–	122	–	–	121+1*	–	35	–	–	–	–
	–	22.7	–	11.0	–	12.0	–	–	17.3	–	8.8	–	–	–	–
dl 1110	14/15	–	–	–	–	–	–	–	8/8	–	4/4	–	–	–	–
	24+7*	–	–	–	–	–	–	–	193	–	15	–	–	–	–
	3.0	–	–	–	–	–	–	–	24.1	–	3.8	–	–	–	–
dl 1112	–	–	–	–	–	–	–	4/4	–	4/4	4/4	–	–	–	–
	–	–	–	–	–	–	–	189	–	110	35	–	–	–	–
	–	–	–	–	–	–	–	47.2	–	27.5	8.8	–	–	–	–
dl 1113	–	–	–	–	–	–	–	8/8	–	–	4/4	–	–	–	–
	–	–	–	–	–	–	–	164	–	–	31	–	–	–	–
	–	–	–	–	–	–	–	20.5	–	–	7.8	–	–	–	–
dl 1114	–	–	–	–	–	10/10	10/10	4/4	–	–	4/4	–	–	–	–
	–	–	–	–	–	158	206	89	–	–	40	–	–	–	–
	–	–	–	–	–	15.8	20.6	22.2	–	–	10.0	–	–	–	–
dl 520	–	–	–	–	–	–	–	–	8/8	–	4/4	8/8	6/6	6/6	5/5
	–	–	–	–	–	–	–	–	206	–	53	154	211	86	42
	–	–	–	–	–	–	–	–	25.8	–	13.2	19.3	35.2	14.3	8.4

Table 7 Summary of transformation data with unique region mutants. The data from Table 6 are summarized for each mutant and are expressed as in Table 6. The number of foci for the dishes marked with an asterisk (*) was set at the average number of foci obtained in the experiment in which it occurred. For comparison, the data for the wt pLE2 in the same experiments is also presented.

Table 7

	No. of Dishes with Foci Total No. of Foci Average No. of Foci/Dish Mutant	wild type	No. of Expts.
pm 1120	32/34 240+2* 7.5	31/33 167+1* 5.1	3
pm 1121	40/42 463+2* 11.1	35/41 186+1* 4.6	4
pm 1122	51/52 783+1* 15.1	47/57 301 5.3	5
dl 1110	26/27 232+7* 8.9	17/25 81 3.2	3
dl 1112	12/12 334 27.8	15/15 86 5.7	3
dl 1113	12/12 195 16.3	11/11 62 5.6	2
dl 1114	28/28 493 17.6	29/33 218 6.6	4
dl 520	37/37 752 20.3	29/36 142 3.9	6

Table 8 Effect of exon 2 mutations on transformation of BRK cells. BRK cells were transfected with wt or mutant pLE2 and ras plasmids as described in Materials and Methods. The data are expressed in Table 6.

Table 8

Number of Dishes with Foci
Total Number of Foci
Average Number of Foci/Dish

Experiment:	1	2	3	4	5	6	7
Mutant							
wt pLE2	13/15	14/15	13/15	10/10	8/12	4/8	4/4
	21+2*	39	110+1*	88	68	19	18
	1.6	2.6	7.9	8.8	5.7	2.4	4.5
sub 1117	–	15/15	15/15	–	12/12	–	–
	–	60	103+2*	–	182	–	–
	–	4.0	7.9	–	15.2	–	–
dl 1115	12/14	15/15	–	–	12/12	8/8	4/4
	28+1*	244	–	–	221	163	76
	2.2	16.3	–	–	18.4	20.4	19.0
dl 1116	15/15	14/14	–	–	10/12	–	4/4
	37+2*	30	–	–	128	–	29
	2.8	2.1	–	–	10.7	–	7.3
pm 1131	–	15/15	15/15	10/10	10/10	–	4/4
	–	216	68+6*	82	141	–	16
	–	14.4	7.6	8.2	14.1	–	4.0

Table 9 Summary of transformation data with exon 2 mutants. The data from Table 10 are summarized for each mutant and are expressed as in Table 6. The number of foci for the dishes marked with an asterisk (*) was set at the average number of foci obtained in the experiment in which it occurred. For comparison, the data for wt pLE2 in the same experiment is also presented.

Table 9

No. of Dishes with Foci
Total No. of Foci
Average No. of Foci/Dish

	Mutant	wild type	No. of Expts.
sub 1117	42/42 345+2* 8.6	35/42 217+1* 5.4	3
dl 1115	51/53 732+1* 13.8	43/54 165+2* 3.1	5
dl 1116	43/45 224+2* 5.1	39/46 146+2* 3.2	4
pm 1131	54/54 523+6* 10.5	49/56 323+1* 5.9	5

experiment, they needed to be included in the determination of overall transforming efficiency, and the average for that experiment is the best estimate of the actual number of foci the dish contained. To minimize the occurrence of dishes with too many foci to count, dishes were stained when they had ten or more foci.

Although this may have biased the focus count in favour of those samples with a lower focus forming efficiency, it was not feasible to end the entire experiment when one dish became filled with foci because many dishes did not yet show any foci. Because of these difficulties in obtaining accurate counts of primary foci, these data cannot be used quantitatively to detect subtle differences in transforming efficiency.

The results in Tables 6 and 7 show that all the mutants in the 13S unique region produced as many and usually more foci than wt E1A, suggesting that this region is not required for transformation. Except for pm 1120, all the mutants were completely defective in transactivation and gave more foci than wt, suggesting that transactivation is perhaps inhibitory to transformation. Pm 1120 has a partial transactivating activity and did not give many more foci than wt.

Of the exon 2 mutants (Table 8), dl 1115, like the unique region mutants, gave on average more foci than wild type. Dl 1116 did not consistently give more foci than wt. Pm 1131 and sub 1117 both gave variable results, where at times they gave similar numbers of foci to wt while at other times they gave many more foci. Although the reason for this variability is not clear, it is evident that these two mutations did not delete sequences that are required for transformation.

Because of the production of secondary foci and the variability of the assay, the difference in transforming efficiency between wt and mutants could not be determined precisely. To examine further the extent of this difference, two approaches were used to prevent the formation of secondary foci and thereby obtain more accurate data.

(ii) Multiwell Trays

In the first approach cells were cultured in multiwell trays which had wells 15 mm in diameter. In effect this divided each 60 mm dish into 16 compartments, so transformed cells could not spread throughout a dish, as they did in the normal experiments, but were confined to one well. The results for some of the unique region mutants and

dl 1115 are shown in Table 10. As it was impossible to determine if a well had had one or more primary foci, the data in these experiments were tabulated as number of wells with foci out of the total number of wells. There was a wide variation in the number of wells with foci seen with the different mutants. Since the medium changes of the multiwell trays were done using a repeater pipet, this more vigourous treatment of the cells probably washed many foci from the dishes. In these experiments all the mutants gave considerable numbers of wells with foci, while wt gave almost none. Wt foci were formed in normal dishes transfected in parallel, but evidently the foci were lost from the multiwell dishes. The fact that wt but not mutant foci were washed away indicates that the wt foci were less adhesive than the mutant foci.

Although these experiments with multiwell trays did show that there was a difference in adhesion between the wt and mutant transformants, they did not give accurate estimates of the transforming efficiency of the wt or mutant E1A plasmids.

(iii) Agar Overlay

In the second approach used to assess more accurately the transforming efficiency of the unique region

Table 10 Transformation of BRK cells in multiwell trays. BRK cells cultured in multiwell trays with wells 15 mm in diameter were transfected with the mutant pLE2 plasmids indicated and with the ras plasmid, as described in Materials and Methods. The data are expressed as the number of wells containing foci, out of the total number of wells. For comparison, the data obtained with wt pLE2 is also presented.

Table 10

	No. of Wells with Foci	
	Mutant	Wild Type
pm 1121	9/48	1/44
pm 1122	27/43	1/44
dl 1110	4/41	1/44
dl 1112	29/34	0/39
dl 1114	13/36	0/39
dl 520	15/47	1/44
dl 1115	24/40	1/44

mutants and dl 1115, the cells were cultured under medium containing 0.35% agar. This agar overlay was intended to prevent the free diffusion of transformed cells which would establish secondary foci, and to avoid the washing away of foci, since the medium was not changed in these experiments. The low agar concentration was intended to allow the free diffusion of nutrients to the cells, and minimize any possible physical restraint on the growth cells.

The cells were prepared and transfected as described for the normal dishes. The cells were cultured in normal medium for one week post-transfection. Since the fixation of transformation occurs during the initial round of host cell DNA synthesis after adenovirus infection (Casto, 1973) and presumably after DNA-mediated transfection as well, the actual transformation event occurred under the same conditions as in the experiments using medium without agar. This week of culturing in normal medium also allowed most of the untransformed cells to die and lift off before focus formation was apparent. At this stage the transformants had not had time to form large foci so the number of cells lifting off to form secondary foci was minimal. At one week post-transfection the medium was replaced with the agar-containing medium. This overlay was maintained on the

cells for one week, after which an additional overlay was added twice weekly.

The results in Table 11 show firstly, that fewer foci were obtained with the mutants under the agar overlay than without the overlay, suggesting that many of the foci obtained with these mutants in normal growth medium were secondary foci.

Since wt transformants also produced secondary foci in normal medium (Table 6), it was expected that with the agar overlay fewer foci would be seen. Since the number of foci produced by wt E1A under agar was similar to that in normal medium, the reduction in secondary foci was balanced by an increased retention of wt foci under agar. This indicated that wt foci were being retained under the agar overlay, that would have been lost in experiments with normal growth medium. The decreased adhesiveness of wt relative to mutant transformants, described in the previous section, suggests that in experiments using normal growth medium, some of the wt foci lifted off or were washed away during medium changes. The possibility cannot be ruled out however, that the presence of the agar could have had a differential effect on the growth of the wt versus the mutant transformed cells.

Table 11 Effect of agar overlay on transformation of BRK cells with unique region mutants and dl 1115. BRK cells were transfected with wt or mutant pLE2 and ras plasmids and overlaid with agar as described in Materials and Methods. The data are expressed as in Table 6. The values in parentheses refer to the average number of foci per dish in normal growth medium in the same experiments.

Table 11

No. of Dishes with Foci
Total No. of Foci
Average No. of Foci/Dish

	Mutant	wild type	No. of Expts.
wt pLE2	-	34/38	8
	-	231	
	-	6.1 (4.4)	
pm 1120	4/4	3/4	1
	6	7	
	1.5 (2.5)	1.8 (4.5)	
pm 1121	7/8	6/7	2
	28	33	
	3.5 (12.0)	4.7 (3.1)	
pm 1122	8/8	6/7	2
	30	33	
	3.8 (17.2)	4.7 (3.1)	
dl 1110	8/8	6/7	2
	38	33	
	4.8 (20.8)	4.7 (3.1)	
dl 1112	19/19	17/18	4
	154	83	
	8.1 (27.8)	4.6 (5.7)	
dl 1113	4/4	3/4	1
	11	7	
	2.8 (7.8)	1.8 (4.5)	
dl 1114	4/4	3/4	1
	17	7	
	4.3 (16.1)	1.8 (5.6)	
dl 520	31/32	28/32	6
	448	193	
	14.0 (18.7)	6.0 (3.8)	
dl 1115	8/8	6/7	2
	45	33	
	5.6 (19.9)	4.7 (3.1)	

Thus the agar overlay gave a more accurate estimate of transforming efficiency because it not only prevented the production of secondary foci, but it also retained the less adhesive primary foci. Under these conditions, wt E1A, the unique region mutants, and dl 1115 all gave similar numbers of foci. This indicated that wt and the mutants transformed with approximately the same efficiency and that the higher numbers of foci obtained with the mutants in normal growth medium was due to a difference in the nature of the mutant transformed cells. Although under the agar overlay, dl 1112 and dl 520 did give significantly more foci than wt as judged by the Student's "t" test (one tailed, $P<0.025$), it is difficult to attach a biological significance to a twofold variation in transforming efficiency.

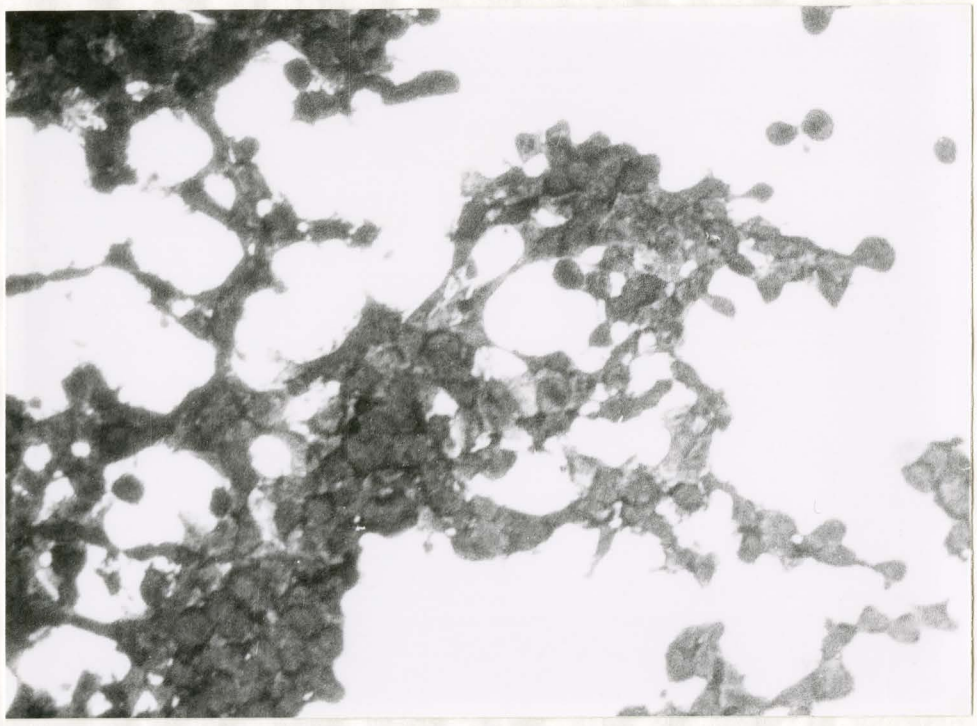
Since most of the unique region and exon 2 mutants are unable to transactivate, but transform as well as wt, transactivation is not required for transformation in this assay. However the transactivation function does alter the character of the transformed cells, to make them less adhesive. The exact nature of this difference is not known, but it is tempting to speculate that a cellular gene which is transactivated by E1A reduces the ability of the cell to adhere to the dish.

An examination of the foci obtained with the wt and mutants revealed a number of different morphologies of transformed cells. An example of representative foci of wt and dl 520 is shown in Figure 12. The other unique region mutant transformants had similar morphologies to dl 520. While the shapes of the cells are not radically different, the dl 520 transformed cells appear to be more spindly than the wt transformants. These processes on the dl 520 transformants probably anchor the cell to the surface of the dish and may be the cause of the increased adhesion of the dl 520 and other unique region mutant transformants.

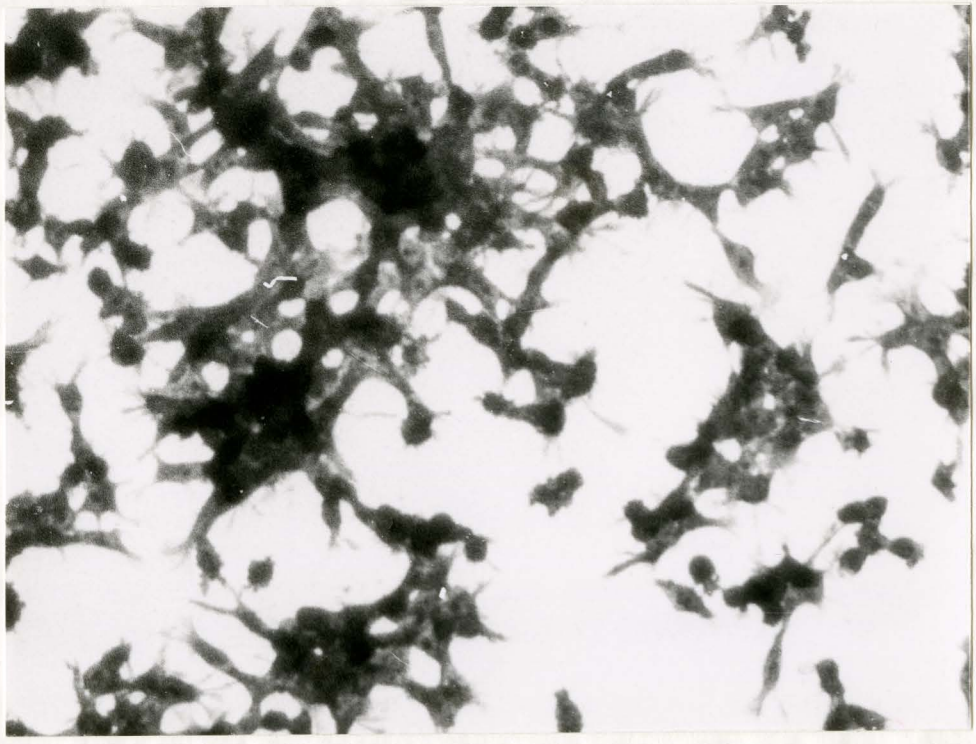
Others have also shown that the 13S unique region is not essential for transformation of BRK cells by E1A and ras (Schneider et al., 1987; Zerler et al., 1986). In both these reports similar or slightly lower numbers of foci were obtained with the unique region mutants relative to wt, and the increased numbers of secondary foci were not observed. The reason for this discrepancy with the data shown here is not clear but may be due to some subtlety in the particular protocol or materials used. Another report stated that two unique region mutants in 13S plasmid form gave few and abnormally small foci in relation to wt 13S (Lillie et al., 1986). These same mutants were also defective for the transformation with ras of rat embryo fibroblasts (Lillie et

Figure 12 Foci of wt E1A and dl 520 transformed BRK cells. Primary BRK cells were transfected with wt pLE2 (A) or pLE2 dl 520 (B) and the ras plasmid and cultured as described in Materials and Methods. The transformed foci were fixed and stained with Giemsa. Magnification is 300x.

A



B



al., 1986). In a different type of assay examining morphological transformation of an established rat cell line, REF 52, by E1A and ras, plasmids producing only the 12S or 13S products transformed equally well and only slightly less efficiently than wt E1A (Velcich and Ziff, 1988).

In the case of transformation by E1A and E1B, in both viral and plasmid form, the transactivation function of E1A is also not required for focus formation, but the morphology of cells transformed by transactivation defective mutants is fibroblastic as compared to the epithelial morphology of wt transformed cells. This is seen both with transformation by virus (Montell et al., 1984; Winberg and Shenk, 1984; Haley et al., 1984, Lillie et al., 1986) and by E1A and E1B plasmids (Haley et al., 1984). These mutant transformants may also have a limited lifespan (Winberg and Shenk, 1984).

In summary, the transactivation function of E1A is not required for focus formation for transformation by E1A and ras or by E1A and E1B. However, it does contribute to the phenotype of the transformed cell to make it less adhesive. This effect is much more apparent when the cooperating oncogene used is E1B than when ras is used. Perhaps the function of ras partially overlaps that of

transactivation by E1A, thereby masking the difference between *wt* and transactivation defective transformants.

(b) 12S Exon 1 Mutants

The results of the transformation experiments using the exon 1 mutants are shown in Table 12 and are summarized in Table 13. As before, the data are expressed as the fraction of dishes with foci, the total number of foci, and the average number of foci per dish. Dishes which had too many foci to count are indicated with an asterisk (*) and were generally not included in the calculation of the average number of foci per dish. However, in instances where the average number of foci per dish was less than 1, (e.g. *dl* 1103 and *dl* 1104), these dishes were included in the calculation and were counted as having one focus. This arbitrary assignment is valid because it is obvious that these dishes had at least one focus, yet by the Poisson distribution it is highly improbable (P = 0.01) that these dishes contained more than one focus.

As shown in Table 12, *dl* 1101, *dl* 1103, *dl* 1104, *dl* 1107, *dl* 1108 and *dl* 1109 were all severely defective in transformation, giving only the occasional focus. *Dl* 1105 and *dl* 1102 gave slightly fewer foci than *wt*, and *dl* 1106 gave slightly more foci than *wt*.

Table 12 Effect of exon 1 mutations on transformation of BRK cells. BRK cells were transfected with wt or mutant pLE2 and ras plasmids as described in Materials and Methods. The data are expressed as in Table 6. When the average number of foci per dish was less than one, dishes filled with foci (*) were counted as having one focus.

Table 12

Number of Dishes with Foci
Total Number of Foci
Average Number of Foci/Dish

Expt.	1	2	3	4	5	6	7	8	9	10	11	12	13
Mutant													
wt pLE2													
	4/15	9/13	6/15	13/15	14/14	13/15	4/4	6/7	6/6	6/6	3/5	7/8	5/8
	6	44	18+2*	21+2*	39	110+1*	24	21	53	21	10	23	12
	0.4	3.4	1.4	1.6	2.8	7.9	6.0	3.0	8.8	3.5	2.0	2.8	1.5
dl 1101													
	–	–	0/15	1/15	–	–	0/4	–	–	0/6	0/6	0/8	2/7
	–	–	0	1	–	–	0	–	–	0	0	0	2
	–	–	0	0.1	–	–	0	–	–	0	0	0	0.3
dl 1102													
	–	–	6/15	13/15	–	14/15	4/4	–	–	–	–	–	–
	–	–	18	34	–	52	18	–	–	–	–	–	–
	–	–	1.2	2.3	–	3.5	4.5	–	–	–	–	–	–
dl 1103													
	–	–	1/15	1/15	–	–	0/4	–	–	–	–	–	–
	–	–	1*	1*	–	–	0	–	–	–	–	–	–
	–	–	0.1	0.1	–	–	0	–	–	–	–	–	–
dl 1104													
	1/13	2/15	–	–	2/15	–	1/4	–	–	–	–	–	–
	1	2+1*	–	–	3	–	1	–	–	–	–	–	–
	0.1	0.2	–	–	0.2	–	0.2	–	–	–	–	–	–
dl 1105													
	0/15	8/15	–	–	12/15	12/15	2/4	–	–	–	–	–	–
	0	10	–	–	27	38+3*	9	–	–	–	–	–	–
	0	0.7	–	–	1.8	3.2	2.2	–	–	–	–	–	–
dl 1106													
	–	14/15	–	–	15/15	–	4/4	–	–	–	–	–	–
	–	75	–	–	94	–	36	–	–	–	–	–	–
	–	5.0	–	–	6.3	–	9.0	–	–	–	–	–	–
dl 1107													
	–	4/14	0/15	–	2/15	–	0/4	–	–	0/6	0/6	–	–
	–	9	0	–	2	–	0	–	–	0	0	–	–
	–	0.6	0	–	0.1	–	0	–	–	0	0	–	–
dl 1108													
	–	–	–	–	–	–	–	0/8	0/6	0/6	0/6	0/8	0/8
	–	–	–	–	–	–	–	0	0	0	0	0	0
	–	–	–	–	–	–	–	0	0	0	0	0	0
dl 1109													
	–	6/15	0/15	–	3/14	–	1/3	–	–	–	–	1/8	0/8
	–	9	0	–	3	–	1	–	–	–	–	1	0
	–	0.6	0	–	0.2	–	0.3	–	–	–	–	0.1	0

Table 13 Summary of transformation data with exon 1 mutants. The data from Table 11 are summarized for each mutant and are expressed as in Table 6. The number of foci for the dishes marked with an asterisk (*) was set at the average number of foci obtained in the experiment in which it occurred. For comparison, the data for wt pLE2 in the same experiment is also presented.

Table 13

Number of Dishes with Foci
Total Number of Foci
Average Number of Foci/Dish

Mutant	Mutant	wild type	No. of Expts
dl1101	3/61 3 0.05	44/61 129+4* 2.2	7
dl1102	37/49 122 2.5	36/49 173+5* 3.8	4
dl1103	2/34 2* 0.06	23/34 63+4* 2.0	3
dl1104	6/47 7+1* 0.2	31/46 113 2.5	4
dl1105	34/64 84+3* 1.5	44/61 223+1* 3.8	5
dl1106	33/34 205 6.0	27/31 107 3.5	3
dl1107	6/60 11 0.2	42/57 156+2* 2.8	6
dl1108	0/42 0 0	33/40 140 3.5	6
dl1109	11/63 14 0.2	45/62 160+2* 2.6	6

These results defined three regions in 12S exon 1, which when deleted, severely impaired transforming activity. These were the N terminal region, residues 4 to 25; residues 36 to 60 containing part of CR1; and residues 111 to 138 containing CR2.

The sequence of the N terminal region of E1A is not conserved between different adenovirus serotypes, but is required for transformation. As was shown in the chapter on repression, this region is also required for enhancer repression, and by tertiary folding may be part of the functional domain for repression and transformation, which includes CR1.

CR1 is directly involved in transformation since dl 1103 and dl 1104, which delete parts of CR1, are defective in transformation. The entire CR1 is not essential for transformation since dl 1105 which deletes the C terminal 11 residues of CR1 could still transform. However, the assignment of the boundaries of the conserved regions is arbitrary, and especially in the case of the C terminus of CR1, the end of sequence conservation is not abrupt. Of the 12 amino acids that dl 1105 deletes, only 4 are strictly conserved in Ad5, Ad7, Ad12, and SA7 (Kimelman et al., 1985). The presence of conserved regions is useful

as a guide to possible functional domains, but these domains must still be defined experimentally.

One of the cellular E1A-associated proteins, that with a molecular mass of 300K DNA, binds to the N terminal and CR1 regions of E1A, and dl 1101, dl 1103, and dl 1104 are defective for this binding (Egan et al., 1988). Thus 300K binding and enhancer repression both appear to be required for transformation.

While the difference was not large, dl 1106 gave significantly more foci than wt in these assays as judged by the Student's "t" test ($P < 0.05$). However, it is difficult to attach any biological significance to such a small difference in transforming activity.

The pattern of transforming ability for the mutants dl 1101 to dl 1106 parallelled their repression activity. Mutants that were completely defective for enhancer repression also did not transform. This suggests that enhancer repression is a part of the transformation process induced by E1A. One can envisage an enhancer-driven cellular gene whose function it is to regulate the growth of the cell, for example by preventing entry into the cell cycle. Repression of this gene could lead to uncontrolled growth of the cell. However, slight reductions in the level of repression were not reflected by similar changes in the

transforming activity (cf dl 1105 and dl 1106). Perhaps another function of this region is responsible for the difference in the number of foci obtained between dl 1105 and dl 1106. Dl 1115 and sub 1117 are also reduced in repression activity (Jelsma et al., 1989) but transform as well as wt. Thus even a reduced repression activity appears to be sufficient for transformation, implying that the target gene in transformation is sensitive even to small amounts of repression.

This requirement of repression for transformation was not seen by Velcich and Ziff (1988) who had two mutants, one in the N terminal region, and one in exon 2, that were defective for repression, yet could transform. These discrepancies may have been due to the difference in the assays for repression and transformation, or perhaps the two functions can be separated, but were not by the mutants used in this thesis. If the latter possibility is the case, then the link between repression and transformation, as shown in this thesis, is coincidental, and another as yet unknown function mapping to the same region is required for transformation.

The origin of the few foci that were seen with dl 1101, dl 1103 and dl 1104 is not clear. Since ras alone or mock transfected dishes did not give foci in these

experiments (Table 13), it is not likely that these were spontaneous transformants. A photograph of one of the foci obtained with dl 1104 is shown in Figure 13A. The cells appeared to be slightly larger than the wt cells, (Figure 12A) but were rounded like the wt transformants, and not spread out like untransformed cells, so there was a morphological transformation. In a few instances the cells from these foci filled the dish with secondary foci e.g. dl 1103 and dl 1104. Perhaps dl 1101, dl 1103, and dl 1104 retained a small amount of transforming activity which was enough to produce the occasional transformant. Alternatively, the foci that arose were from cells that had had a mutation which in itself was not sufficient to fully transform the cell, but was sufficient to complement the defect in these mutants and thus produce a fully transformed cell. This hypothesis makes the assumption that E1A carries out more than one function in transformation, only one of which is defective in these mutants. One cannot determine what had occurred to generate these foci.

The third region in exon 1 that was required for transformation corresponded to conserved region 2. The three mutants that removed parts of CR2, namely dl 1107, dl 1108, and dl 1109, were all severely defective in transformation. Since dl 1107 and dl 1108 had wt repression

activity, repression alone is not sufficient for transformation and another function which is inactivated by the dl 1107 and dl 1108 deletions is also required. Since dl 1109 deletes a large part of CR2, it is probably also defective in this function although the possibility that it transforms poorly solely because of a defect in enhancer repression cannot be ruled out.

The cellular E1A-associated proteins 107 kDa and 105 kDa in size (107K and 105K) appear to bind to a region in CR2. Dl 1107 has reduced binding to 107K and does not bind 105K, while dl 1108 does not bind either of these proteins (Egan et al., 1988). This suggests that either or both these proteins may be involved in transformation, and may carry out the function postulated for this region. 105K is a likely candidate for this as it has been shown to be the product of the recessive anti-oncogene Rb1 (Whyte et al., 1988; Egan et al., 1989). In contrast, dl 1109 does bind these two proteins, albeit at reduced levels (Egan et al., 1988), yet does not transform. Perhaps binding to these proteins alone is not sufficient but there must also be a modulation of their activity, for which dl 1109 is defective. Alternatively, if the function of this binding is to titrate out a factor that suppresses transformation, even a slightly reduced efficiency of binding may be

insufficient for transformation. It is also possible that binding of either of these proteins is irrelevant to transformation but the demonstrated relationship between loss of Rb1 and oncogenesis (Friend et al., 1986) makes this unlikely.

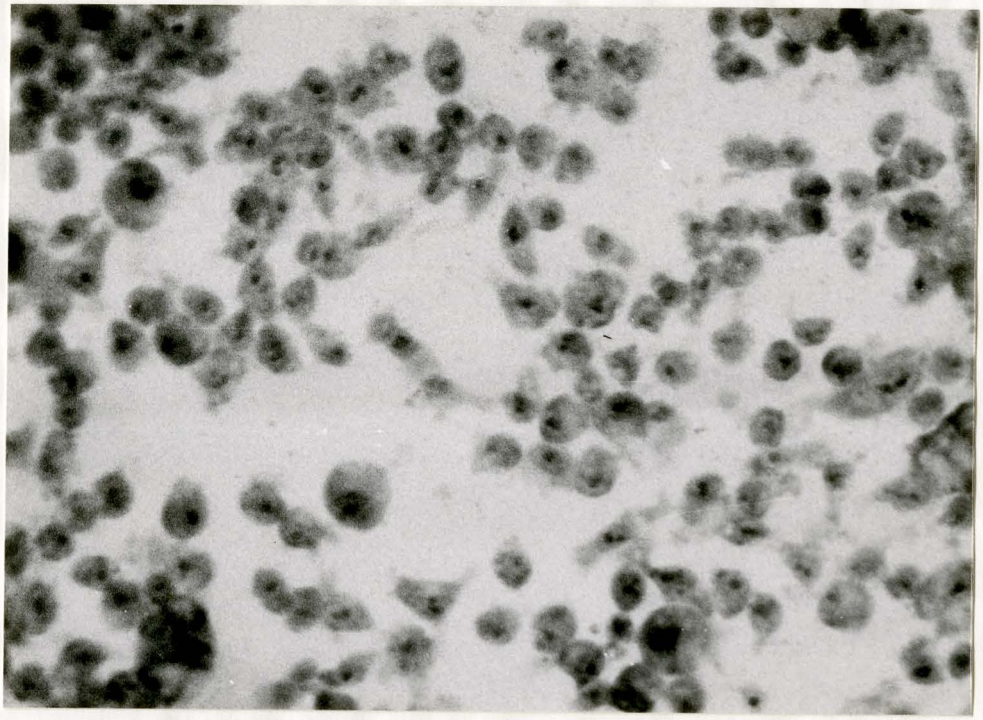
Cells from the few foci that were observed with dl 1107 and dl 1109 looked different from wt transformants and resembled untransformed cells. One sample focus induced by dl 1109 is shown in Figure 13B. The cells were much more spread out than the wt transformants and were difficult to see under the microscope. These cells could not be grown into lines and thus were not true transformants.

The nature of the function of CR2 in transformation is not apparent, but it apparently affects the morphology of the cells, to make them more rounded. Moran and Zerler (1988) suggested that it was involved in the cessation of cellular DNA synthesis, because cells infected with a mutant deleting CR2 tended towards polyploidy.

To ensure that the reason few foci were seen with the transformation defective mutants was not because they were washed away, transformation experiments were carried out using the agar overlay. The results are shown in Table 14. Dl 1101, dl 1104, dl 1107, dl 1108 and dl 109 were as transformation defective under the agar overlay as

Figure 13 Foci of dl 1104 and dl 1109 transformed BRK cells. Primary BRK cells were transfected with pLE2 dl 1104 (A) or pLE2 dl 1109 (B) and the ras plasmid and cultured as described in Materials and Methods. The transformed foci were fixed and stained with Giemsa. Magnification is 300x.

A



B

Table 14 Transformation of BRK cells with exon 1 mutants under agar overlay. BRK cells were transfected with wt or mutant pLE2 and ras plasmids and overlaid with agar as described in Materials and Methods. The data are expressed as in Table 6.

Table 14

Number of Dishes with Foci
Total Number of Foci
Average Number of Foci/Dish

Expt. 1	2	3	4	5
Mutant				
wt pLE2				
4/4	8/8	6/6	6/6	2/5
18	38	95	23	4
4.5	4.8	15.8	3.8	0.8
dl1101				
0/3	–	–	0/6	0/6
0	–	–	0	0
0	–	–	0	0
dl1102				
4/4	–	–	–	–
15	–	–	–	–
3.8	–	–	–	–
dl1103				
1/4	–	–	–	–
6	–	–	–	–
1.5	–	–	–	–
dl1104				
0/4	–	–	–	–
0	–	–	–	–
0.0	–	–	–	–
dl1105				
0/4	–	–	–	–
0	–	–	–	–
0	–	–	–	–
dl1106				
4/4	–	–	–	–
18	–	–	–	–
4.5	–	–	–	–
dl1107				
0/4	–	–	0/5	0/6
0	–	–	0	0
0	–	–	0	0
dl1108				
–	0/8	0/6	0/6	0/6
–	0	0	0	0
–	0	0	0	0
dl1109				
2/4	–	–	–	–
2	–	–	–	–
0.5	–	–	–	–

they were in normal growth medium. Dl 1103 gave foci, but fewer than wt and they were all in one dish. These may have been due to the formation of some secondary foci before the overlay was added or they may have formed in spite of the overlay. Dl 1105 gave no foci under agar although in normal growth medium it did give foci. However, the sample size, four dishes, was too small to demonstrate conclusively whether cells transformed by this mutant were truly defective for growth under agar.

In general, the results of these experiments were not inconsistent with the results of the experiments carried out under normal growth conditions and showed that, even under the agar overlay, the transformation defective mutants did not form foci.

Thus 12S exon 1 has three regions important for transformation. The first two, the N terminal region and part of CR1, are involved in enhancer repression, and binding to the 300 kDa cellular protein. The third region, containing CR2, is most likely not required for enhancer repression, but is involved in a separate function required for transformation.

Since these experiments were carried out using saturating amounts of plasmid DNA, minor differences in transforming efficiency may not have been detected for

mutants which had a different dose-response curve. For example, with nonsaturating amounts of DNA, the differences in transforming efficiency between wt and dl 1102 or dl 1105 may have been greater. Still, these experiments defined distinct regions that are absolutely required for transformation and therefore may be functional domains for transformation.

The results of this work are consistent with data from other labs which showed that exon 1 contains distinct transforming regions separated by nonessential regions (Zerler et al., 1987; Whyte et al., 1988). While earlier studies had implied that CR1 and CR2 alone were required for transformation (Moran and Matthews, 1987; Lillie et al., 1987) the functional domains needed to be defined experimentally. Subsequently, others have shown the importance of the N terminal region (Whyte et al., 1988; Subramanian et al., 1988) using mutations at residues 2 and 19. The extent of the important sequences in this region needs to be further defined, however, with other mutants.

While it is generally accepted that CR1 is required for transformation (Schneider et al., 1987; Velcich and Ziff, 1988; Lillie et al., 1987), there are conflicting results regarding the extent of this required region. For example, Lillie et al. (1987), in assaying the

transformation of cloned rat embryo fibroblasts (CREF), an immortal cell line, found that point mutations at residues 72 and 73 drastically reduced transforming ability. In contrast, dl 1105 which deletes residues 70 to 81, has only a slight reduction in transforming efficiency. However, it is difficult to compare data obtained by different assay systems.

CR2 also is important for transformation, but different regions appear to have different sensitivities to mutation. Whyte et al. (1988) have reported that the deletion of residues 121 to 127 abolished focus formation while deletion of residues 128 to 139 merely reduced the efficiency of transformation. Similarly, a mutation of residue 125 reduced transforming activity by only 35% (Schneider et al., 1987), but the mutation of residue 126 almost eliminated transformation entirely (Lillie et al., 1986). This is in contrast to the data shown in this work, where all three CR2 mutants were essentially unable to transform.

(c) Complementation Between Exon 1 Mutants

To examine the possibility that the two functional domains in exon 1 could operate on separate proteins, complementation experiments were carried out using

combinations of mutants, one defective in each function. BRK cells were transfected with 1/4 ug of each of two exon 1 mutants and of ras. Complementation was examined by comparing the numbers of foci obtained with the two mutants combined to the total number of foci obtained with each mutant separately. The results are summarized in Table 15.

Two forms of the exon 1 mutants were tested in this assay, those with and without the d1 520 mutation. The presence of the d1 520 mutation appeared to increase the number of foci observed, with the result that more foci were obtained with the mutants alone, but did not affect the interpretation of the results. An agar overlay was also included on half of the dishes in two experiments; this made no difference to the results except that fewer foci were seen under agar on dishes transfected with d1 520 than in normal growth medium. Both with and without the d1 520 mutation, the combination of d1 1101 and d1 1107, d1 1108, or d1 1109 gave more foci than each mutant gave separately, but the numbers of foci did not approach wt levels. Because there are few data points,however, the statistical significance of this difference can not be properly estimated. The combination of d1 1108 and d1 1109 did not give any foci in two experiments (data not shown) showing firstly that the increased number of foci obtained with

Table 15 Summary of complementation transformation experiments. BRK cells were transfected with ras and with two transformation defective mutants separately or combined. The data are expressed as in Table 6. For comparison the wt data from the same experiments is also shown.

Table 15

d11101	d11107	d11101 + d11107	wt	No. of Expts.
0/24	0/23	6/22	17/22	2
0	0	8	58	
0	0	0.36	2.64	
d11101	d11108	d11101 + d11108	wt	
2/39	0/40	14/47	29/38	4
2	0	26	93	
0.05	0	0.55	2.45	
d11101	d11109	d11101 + d11109	wt	
2/15	1/16	10/24	12/16	2
2	1	16	35	
0.13	0.06	0.67	2.19	
d11101/520	d11107/520	d11101/520+d11107/520	d1520	
2/24	5/24	12/21	22/22	2
2	11	29	197	
0.08	0.46	1.38	8.95	
d11101/520	d11108/520	d11101/520+d11108/520	d1520	
2/24	6/23	8/22	22/22	2
2	13	17	197	
0.08	0.57	0.77	8.95	
d11101/1112	d11109/1112	d11101/1112+d11109/1112	d1520	
1/8	0/8	2/6	8/8	1
3	0	4	137	
0.38	0	0.67	17.13	

other combinations was not a nonspecific effect e.g. of adding double the amount of DNA, and secondly, that dl 1108 and dl 1109 are defective in the same function and cannot combine to produce foci.

In these assays the possibility existed that recombination occurred between the two deletions to produce a wild type E1A gene which in itself could produce foci. To control for this, I created another mutant, PvuII$^-$, which lacks the sequences between the PvuII sites at nucleotides 454 and 625 in E1A (-45 to +126 relative to the transcription initiation site). This mutant lacks the first two E1A methionine codons at bp 560 and 602 (amino acid residues 1 and 15), and the next ATG, at bp 698, is in a different reading frame. The only in-frame exon 1 initiation codon, at bp 770 (residue 71), has a sequence context that is rarely seen with initiation codons, and is predicted to be an inefficient start site for translation (Kozak, 1983). Thus PvuII$^-$ probably does not make any functional E1A proteins.

Since the end of the PvuII$^-$ deletion (bp 625) is near the end of the dl 1101 deletion (bp 634), it should recombine as efficiently as dl 1101 with dl 1108 or dl 1109, to produce a wild-type E1A. Thus PvuII$^-$ could be used to determine the recombination frequency in these assays. As

shown in Table 16, no foci were seen when PvuII$^-$ was cotransfected in combination with dl 1108. PvuII$^-$ and dl 1109 gave some foci, but these did not resemble wt foci, but looked like those seen with dl 1109 alone (cf Table 12, expt 5; Figure 13B) and thus were probably not due to recombination. These results indicate that recombination to produce a wt E1A probably did not contribute to focus formation in these experiments.

In order for true complementation to have occurred, the foci seen with the combination of mutants must be identical to foci induced by wt E1A, or dl 520 in the experiments involving the dl 520 double mutants. This was generally not the case, however. Almost all resembled foci induced by dl 1107, dl 1108, or dl 1109 alone. Representative foci obtained with dl 1101 and dl 1108, and with dl 1101 and dl 1109, are shown in Figure 14. These cells clearly do not have the morphology of wt transformants (Figure 12A) but resemble untransformed cells. Thus there was no true complementation in these instances. Only in one experiment did there appear to be complementation to produce foci that resembled wt transformants. However, on the basis of this single experiment one cannot come to a conclusion whether complementation occurred or not.

Table 16 Test for recombination in complementation transformation experiments using PvuII$^-$ mutant. BRK cells were transfected with ras and with PvuII$^-$ and dl 1108 or dl 1109 separately or combined. The data are expressed as in Table 6. For comparison, the wt data from the same experiments is also shown.

Table 16

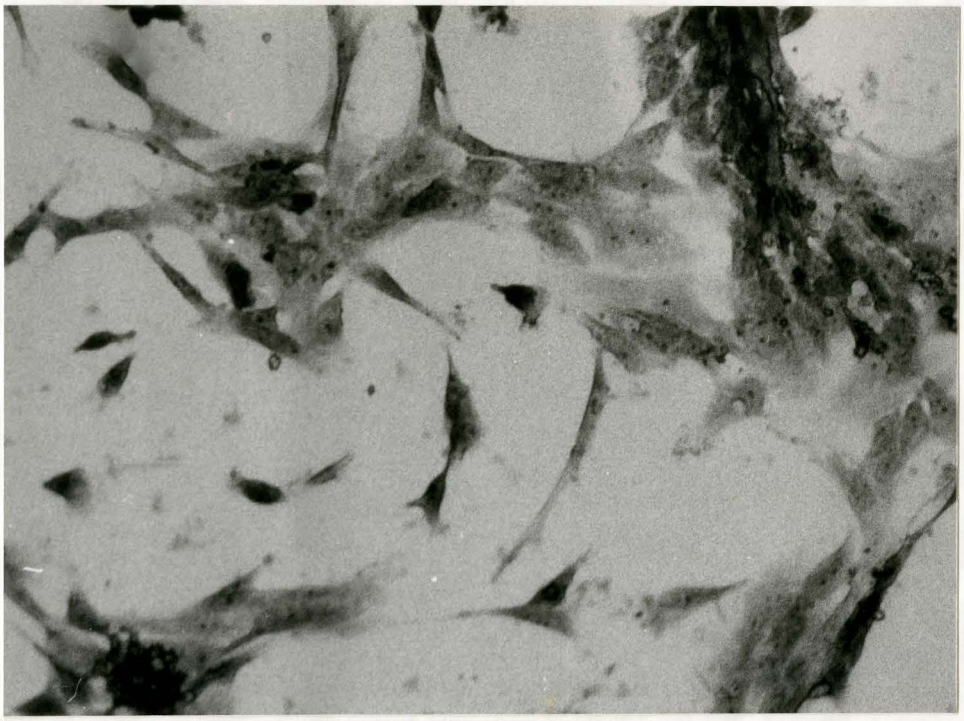
PvuII-	dl 1108	PvuII- + dl 1108	wt	No. of Expts.
0/16	0/16	0/24	12/16	2
0	0	0	35	
0	0	0	2.19	

PvuII-	dl 1109	PvuII- + dl 1109	wt	No. of Expts.
0/16	1/16	4/24	12/16	2
0	1	5	35	
0	0.06	0.21	2.19	

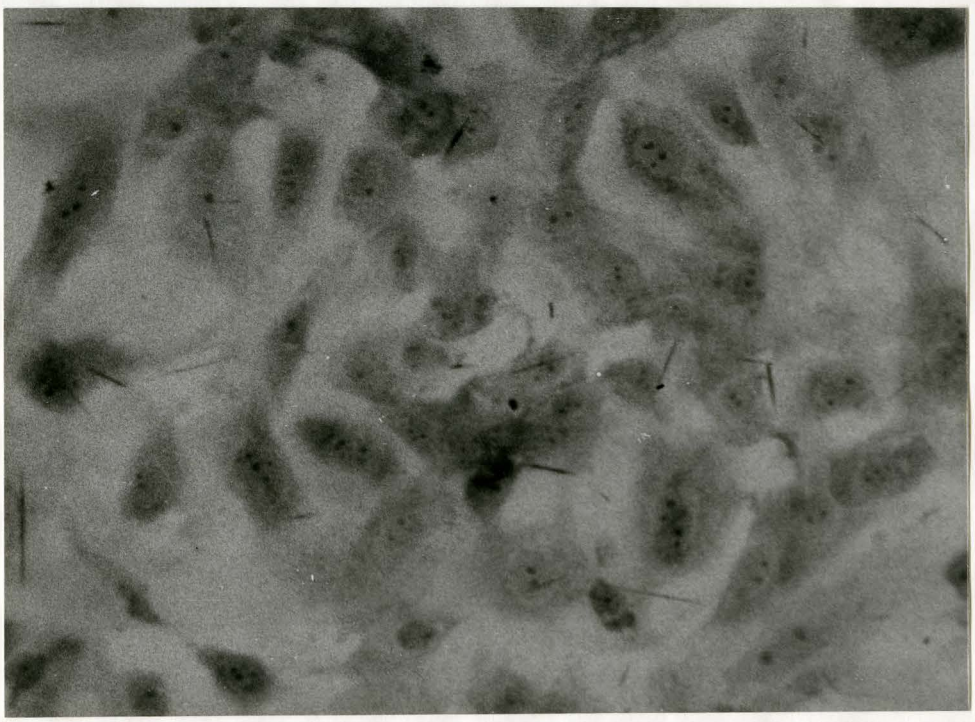
PvuII-/ 1112	dl 1109/ 1112	PvuII-/1112 + dl 1109/1112	dl 1112	No. of Expts.
0/8	0/8	0/7	8/8	1
0	0	0	137	
0	0	0	17.1	

Figure 14 Foci of BRK cells transfected by dl 1101 and dl 1108 or dl 1109. BRK cells were transfected with ras and with a combination of dl 1101 and dl 1108 plasmids (A) or with dl 1101 and dl 1109 plasmids (B) as described in Materials and Methods. Magnification is 300x.

A



B



Nevertheless, these experiments did reveal that although exon 1 codes for two distinct functions in transformation, these functions cannot operate efficiently on separate plasmids. It is unlikely that the reason for this inefficiency is the difficulty of transfecting both plasmids into the same cell, because in a cotransfection, large amounts of the transfected DNA are incorporated in the host cell genome (Perucho et al., 1980) and the presence of one plasmid would not preclude the entry of the other, since the two are identical except for the deletions.

The lack of complementation does not necessarily mean that the regions of exon 1 are involved in a single function. These functions are distinct as shown by their role in enhancer repression, but may need to operate together on the same molecule. For example, the cellular proteins which bind to E1A and which may mediate E1A functions may operate only when they are all present in the protein complex.

Alternatively, there could be a third E1A function in transformation which requires both transforming regions. If this were so, then both mutants would be defective in this function and would not complement each other.

Although true complementation was not consistently seen, the presence of dl 1101 did appear to enhance the

ability of dl 1107, dl 1108 or dl 1109 to produce foci. If, as postulated above, there are three exon 1 functions in transformation, then two of the functions would be accounted for in these experiments, resulting in more foci than if only one function were present. Without further study of these foci, e.g. an examination of the expression of both mutant E1A proteins in these cells, little more can be said about the nature of a possible contribution of dl 1101 to focus formation by dl 1107, dl 1108, or dl 1109.

Moran and Zerler (1988) have carried out similar complementation experiments using two mutants with large deletions in exon 1. These mutants were unable to transform alone, but in combination gave foci at 1/8 to 1/4 the efficiency of wt. These transformants were not a result of recombination between the two mutants, and they resembled wt transformants in their ability to grow into cell lines. Moran and Zerler suggested that the two domains in exon 1 are able to complement in transformation on separate molecules, although less efficiently than wt.

The difficulty in interpreting the results in these complementation assays lies in determining whether complete transformation by the complementating mutants has occurred. Transformation is a multistep process, and the progressive accumulation of mutations during clonal expansion of a

partially transformed cell could lead to full transformation. These cells would be indistinguishable from wt transformants. For example, it appeared as though the intact CR2 in dl 1101 enhanced the growth of cells containing an intact N terminal region and CR1 (Table 15). Although these cells were not fully transformed, extended growth of these cells may in time have yielded fully transformed cells. This may also have occurred in the experiments of Moran and Zerler.

Without a better understanding of the molecular events leading to transformation, the significance of the increased numbers of foci obtained and the lack of full complementation which were seen in this work cannot be fully appreciated.

CONCLUSIONS

Research on E1A has advanced to the point where a large number of E1A functions have been identified. The work described in this thesis has focussed on the three best studied of these, namely transactivation, enhancer repression, and transformation, and has identified regions which are required for these functions.

The techniques used to do this were site-directed mutagenesis of the entire E1A gene with small deletions and point mutations, and analysis of the effects of these mutations on these three E1A functions. The domains identified in this work correspond well with data obtained from other groups, with a few exceptions. One possible explanation for conflicting data obtained when using site-directed mutagenesis to map functional domains is the effects of different mutations in the same region of the protein. The ideal mutation completely inactivates the region or residue being mutated, but does not affect the rest of the protein. However, occasionally the mutated sequence may functionally substitute for the wt sequence. This can occur with a conservative missense mutation, but is also possible with deletion mutants. One example of this may be the E1A mutant pSVXL105 of Velcich and Ziff (1988),

which lacks residues 38 to 44 and transforms almost as well as wt. Since CR1 is defined as residues 40 to 80, the results with pSVXL105 suggest that the N terminal 5 residues of CR1 are dispensable for transformation. However, since pSVXL105 lacks residues 38 to 44, the N terminal 5 residues are in effect replaced by residues 33 to 37. A comparison of the two sequences reveals that 3 of the 5 residues are identical between the two sequences and a fourth, Thr 42 is replaced by a similar amino acid, Ser, in the next position:

	40				44
wt E1A	Pro	Pro	Thr	Leu	His
pSVXL105	Pro	Pro	Pro	Ser	His
	33				37

Thus while the data obtained with this mutant may suggest that the N terminal 5 residues are dispensable for transformation, it may be that residues 33 to 37 functionally substitute the missing part of CR1.

Another possible effect of either a deletion or a missense mutation is that an important functional domain remote from the site of the mutation (in terms of primary structure) is affected by the alteration in the tertiary structure of the protein. This may have occurred for example with dl 1109/520 which was defective for repression even though it probably did not delete part of the functional domain for enhancer repression.

It is conceivable that the protein products of some mutants are degraded too rapidly to have any activity, as may have occurred with dl 1119. However, all the mutants except dl 1119 could perform at least one of the functions examined, indicating that the mutant proteins were acting in these cases. In addition, immunoprecipitations of ^{35}S labelled E1A proteins from dl 1101-dl 1109 in virus form showed approximately equivalent amounts of wt and mutant E1A proteins (Egan et al., 1988). Thus these mutations probably had little effect on the stability of the mutant proteins.

In spite of these isolated cases of the side effects of mutation, it is possible to use mutational analysis in mapping functional domains in E1A. All three E1A functions analyzed in this work were mapped to distinct regions of the protein and were relatively insensitive to mutations outside their respective functional domains.

The mutations in exon 1 were chosen where possible to delete regions of relative hydrophilicity or hydrophobicity. This was done to attempt to delete intact domains of the protein and induce minimal perturbation of the rest of the protein. While this is a valid rationale for the design of deletion mutants, in retrospect it can be seen that the hydropathy profile had little to do with the pattern of functional domains. For example, of the three hydrophilic regions of exon 1, deleted by dl 1104, dl 1106,

and dl 1109, only two were functionally important, and of two regions which pass from a hydrophobic domain to a hydrophilic domain, dl 1102 and dl 1108, only the latter was important. There was also little correlation between the hydropathy profile and the presence of the conserved region. Dl 1107 and dl 1115, which delete regions of hydrophobicity and hydrophilicity respectively, delete only the extreme edges of CR2 and CR3 respectively.

This work has revealed a number of features of the action of E1A. Firstly, it is clear that the functions of E1A do not result from a single action, but from several E1A activities, which are carried out by different regions in E1A. Thus E1A appears to be comprised of discrete functional domains joined together in one protein. This organizational strategy of the virus using one protein, rather than a group of proteins, one for each function, is advantageous for the virus. Considering the packaging constraints of the virion, one multifunctional gene is much more economical than a set of genes. The coordinate control of these functions is also easily achieved if they are present in the same protein. Minor differences in optimum levels of expression can also be attained by differential splicing. For example, the 12S product may exist to dilute

the transactivation function while maintaining the levels of the other E1A functions.

Secondly, the multistep nature of oncogenic transformation is also apparent in the actions of E1A in transformation. At least two distinct functions appear to be provided by E1A in addition to that supplied by the ras gene used in these experiments. This work suggested that enhancer repression was required, but not sufficient for transformation, since mutants defective in enhancer repression also did not transform. A second function of E1A, which has not been identified, but which has been mapped to conserved region 2 in E1A, is also required. Transactivation, on the other hand, was not required, although it did reduce the adhesiveness of the transformed cells as shown by the increased production of secondary foci by transactivation defective mutants, and by the increased retention of wt transformants under the agar overlay.

The indication by the data shown here that enhancer repression is a component of transformation suggests that there are genes that maintain the untransformed state. These genes may be involved in bringing about the morphology of untransformed cells, they may repress cell growth and replication until the cells are specifically stimulated to

do so, and these genes may also program the replicative capacity of cells.

Alternatively, the enhancer repression function of E1A may regulate the expression of a cellular gene which in turn represses the expression of genes which bring about the transformed phenotype. These genes may induce the cell to grow and divide or they may express morphological characteristics of untransformed cells. These two possible types of cellular targets of enhancer repression in transformation are not mutually exclusive, and may both be involved in transformation by E1A.

Although enhancer repression has not been reported for other transforming genes, the same control pathways may be affected in other transformation systems. For example, since E1A, polyomavirus large T and v-myc can all cooperate with ras to transform cells, they may modulate the expression of the same genes in transformation. This may, however, be done at different control points in these pathways.

It is now clear that E1A carries out many functions in the infected cell. E1A not only transactivates the expression of viral genes to set the infection process in motion, but it also acts directly on the cell to stimulate its metabolic activities in order for it to be better able

to produce progeny virus. It does this in a number of ways to induce the quiescent cell to reenter the cell cycle. E1A stimulates DNA synthesis, and the production of PCNA and thymidylate synthase (Zerler et al., 1987), and it induces the production of a growth factor (Quinlan et al., 1987). As the cell enters S phase, the enzymes that are required for DNA synthesis are produced, but are pirated by the virus for the synthesis of viral DNA. By its action both on viral and cellular genes E1A stimulates the process of progeny virus production.

Once we have come to a better understanding of the control of the target genes by the transforming functions of E1A, it may be possible to determine whether these same control pathways are altered in tumours induced by a variety of different agents. There are a large number of cellular oncogenes which, when activated by one means or another, are involved in tumourigenesis. This indicates that there are many control points in the regulation of cell growth where a transforming gene can act. In time this complex control system may be well enough understood that attempts can be made to manipulate the growth of cells at these control points as a treatment against cancer.

FUTURE DIRECTIONS

Although much is now known about the role of E1A in infection and transformation, much more remains to be elucidated. Several areas of research are available which can contribute much to the understanding of the mechanism of action of E1A. Firstly, the results described in this thesis can be directly extended by using these and other available mutants to identify regions required for other E1A functions than those examined here. Some of this work is already in progress in this laboratory, such as the study of the regions required for the stimulation of DNA synthesis and the induction of proliferating cell nuclear antigen (PCNA). Other E1A activities that can be examined with these mutants are the induction of an epithelial cell growth factor (Quinlan et al., 1987), and the inhibition of differentiation of myoblasts (Webster et al., 1988). The activity profiles of the E1A mutants in these functions will demonstrate which regions in E1A are involved in these functions, and how these regions act to bring about transformation. For example, since the inhibition of differentiation of myoblasts by E1A is concomitant with the repression of certain muscle-specific genes (Webster et al.,

1988), the ability of enhancer repression defective mutants to inhibit this differentiation can indicate whether enhancer repression is necessary and/or sufficient for this effect.

The understanding of the mechanism of action of transactivation, repression and transformation can also be extended by examining the molecular mechanism of action of E1A in these functions. Much of this work has already been done in transactivation, to identify E1A-responsive DNA sequences, isolate cellular factors binding to these sequences, and probe the action of E1A on these factors (reviewed in Jones et al., 1988). Other transactivating genes, e.g. ICP4, herpes IE proteins, etc., can be examined in a similar manner to determine if they use similar mechanisms of action as E1A.

The mechanism of action of enhancer repression can be studied in the same way. The susceptibility of different enhancer motifs can be determined and cellular factors binding to these motifs can be identified to elucidate the action of E1A on these factors.

Since transformation is the end result of multiple effects, further studies on transformation would do best to focus on individual components of the transformation process. For example, the role of enhancer repression in

transformation can be studied by identifying and examining cellular genes which are repressed by E1A.

The contribution of CR2 to transformation can also be explored to determine the molecular mechanism of action of this domain. This region appears to be involved in the cessation of DNA synthesis (Moran and Zerler, 1988) and/or the induction of mitosis (Zerler et al., 1987). The effects of E1A on the expression of genes involved in progression through the cell cycle at these points may give an indication of the role of CR2 in transformation. Although these genes are not yet known, the CR2 mutants available can help in the identification of such genes.

The action of E1A in transformation can also be compared to that of other oncogenes by exchanging possible functional domains with other oncogenes. This has been done between SV40 and E1A where CR2 was replaced by the analogous sequence of SV40 to produce a functional E1A/SV40 hybrid protein (Moran, 1988). Comparison of the hydropathy profiles of E1A and v-myc reveals a similar profile from residue 7 to 86 (112 to 181 in v-myc) and from residue 115 to 167 (213 to 265 in v-myc) (Branton et al., 1985). Replacement of one of these regions in E1A by the corresponding region of v-myc may also yield a functional E1A/v-myc hybrid protein. Other oncogenes can also be

examined for the possibility of substituting similar regions.

Finally, it would be most useful to produce large amounts of E1A with the baculovirus vector system in order to obtain the crystal structure of the protein. Such a crystal structure would do much to explain the structure-function relationship of E1A, and will help in the design of better mutants which will have a more specific effect on a particular domain and avoid the perturbation of other regions of the protein. However, because of the degree of heterogeneity in E1A at the level of phosphorylation and because of the instability of the protein itself, it will be difficult to obtain the pure preparation of one form of E1A which is required for crystal formation. Phosphatase treatment may remove all the phosphates and give a more homogeneous product, but this in turn could alter the structure of the protein so that what would then be seen would not be the fully functional E1A structure. Large amounts of E1A proteins produced by the baculovirus vector system can be used for <u>in vitro</u> studies on the mechanism of action of various E1A functions, in particular its action on the cellular factors which mediate transactivation and enhancer repression by E1A.

SUMMARY

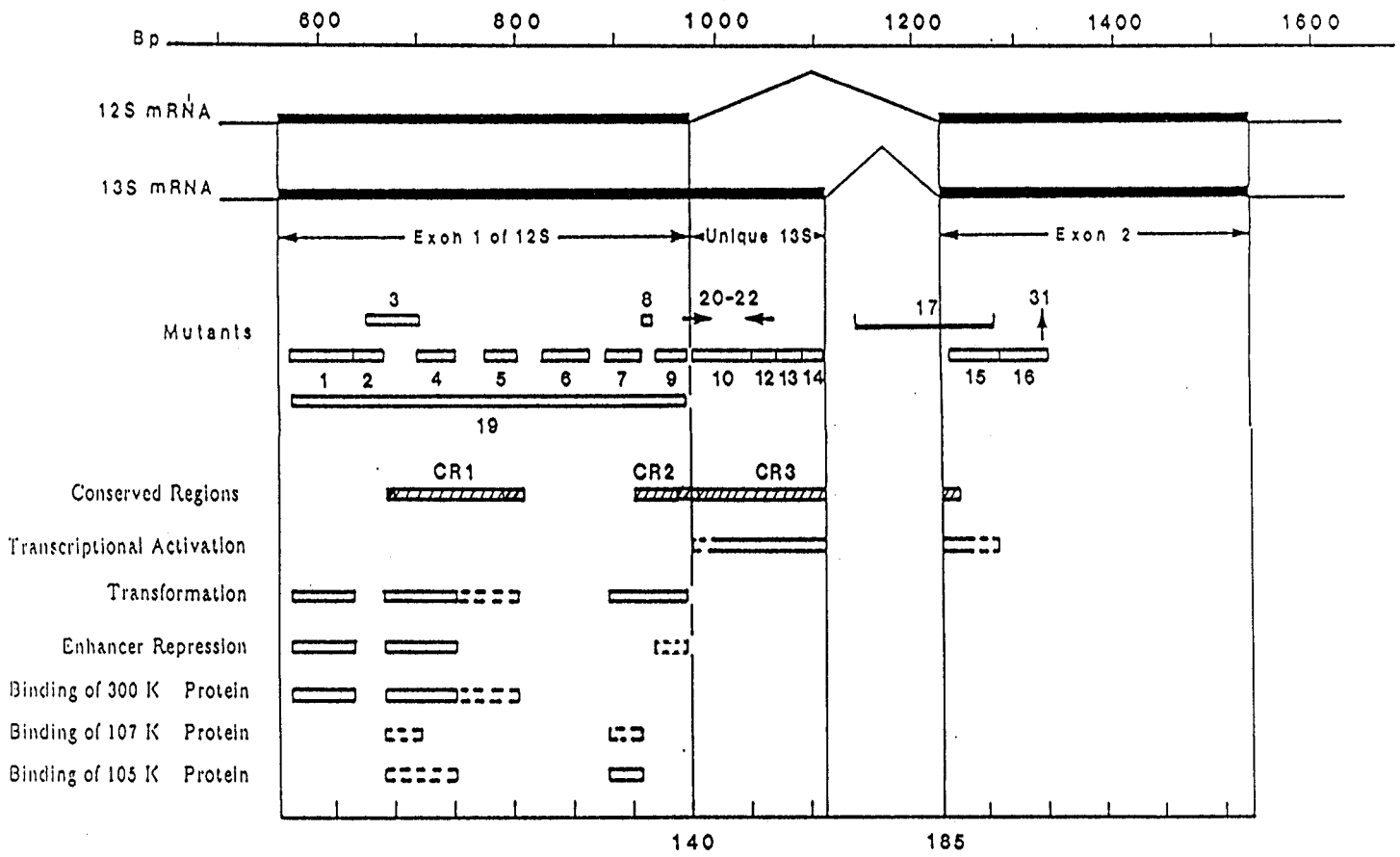
This thesis describes the study of the mechanism of action of the products of the adenovirus E1A gene. The intent of this work was to define regions of the protein that are required for transcriptional activation, enhancer repression, and transformation, by making and examining site-specific mutations throughout E1A.

The first part of the project involved site-directed mutagenesis of the 13S unique region. To do this, deletions were made in the 13S unique region at the SmaI site with exonucleases ExoIII and S1. One of the mutants obtained by this method contained an in-frame deletion of approximately the first half of the unique region and was used for further study. Another mutant with a smaller deletion was used in the deletion loop mutagenesis technique to generate a series of missense mutants centered around the SmaI site in the unique region.

Three of these mutants and a series of other mutants which in combination deleted essentially the entire E1A region, were examined for three E1A functions, namely transactivation, enhancer repression and transformation. A summary of the results is diagrammed in Figure 15.

Figure 15 Regions in E1A required for transcriptional activation, transformation, enhancer repression, and binding to cellular proteins. The E1A mRNA's, products and mutants shown as in Figure 5. The regions conserved between adenovirus serotypes, CR1, CR2, and CR3 are also indicated. The regions containing sequences that are required for the different E1A functions are boxed. Sequences outside each box are not directly required for these functions. Deletion of regions boxed in broken lines gives a reduced activity of that function.

Figure 15


Bp
600
800
1000
1200
1400
1600
12S mRNA
13S mRNA
Exon 1 of 12S
Unique 13S
Exon 2
Mutants
3
8
20-22
17
31
1
2
4
5
6
7
9
10
12
13
14
15
16
19
CR1
CR2
CR3
Conserved Regions
Transcriptional Activation
Transformation
Enhancer Repression
Binding of 300 K Protein
Binding of 107 K Protein
Binding of 105 K Protein
140
185

The transactivation experiments revealed that the 13S unique region and the N terminal portion of exon 2 were required for transactivation (Figure 15). These data, combined with data from other laboratories indicated that the functional domain for transactivation is Conserved Region 3 of E1A. This region is remarkably compact (49 amino acids), and is relatively independent of the rest of the protein since it is insensitive to deletions immediately adjacent to this domain, and since a synthetic peptide corresponding to this domain is also functional. However, the remainder of the protein does play a role in maintaining the stability of this domain.

The enhancer repression function of E1A is not related to transactivation as the two activities map to separate regions of the protein. Deletions in three regions of exon 1 reduce the ability of E1A to repress the SV40 enhancer (Figure 15). Two of these, the N terminal region and a region within CR1, are probably parts of the same functional domain, for several reasons. They are close to each other, separated only by a short joining loop which is not required for repression, they both are required for enhancer repression, and are both required for the binding of E1A to a 300 kDa cellular protein (Egan et al., 1988a).

This suggests that the 300K mediates enhancer repression, which in turn is a component of transformation.

The third region in exon 1, which when deleted reduces repression, includes CR2, but is probably not directly involved in enhancer repression, for several reasons. Firstly, preliminary evidence indicated that the deletion of two other parts of CR2 did not affect repression, while all three deletions abolished transforming activity. Secondly, dl 1109 retained a partial repressing activity, suggesting that there was no absolute requirement for these sequences in repression. Finally, when the 13S unique region was present, dl 1109 was less defective in repression, (J. Howe, personal communication) suggesting that the dl 1109 mutation affected repression only when the immediately adjacent unique region was also deleted. Thus, while there may be an interaction between CR1 and CR2 such that some mutations in CR2 affect the function of CR1, the region of dl 1109 does not seem to be directly involved in enhancer repression.

The unique region was not required for repression since dl 520, which deletes the entire unique region, repressed efficiently. Other work by John Howe has shown that exon 2 is also not required for repression (Jelsma et al., 1988b).

Three regions of exon 1 are required for transformation (Figure 15). The first two, the N terminal region and the region within CR1, are also required for enhancer repression and binding of the 300kDa cellular protein, suggesting that these two activities are involved in transformation. The third region required for transformation, including CR2, is not directly involved in enhancer repression and binding to 300K, indicating that these two functions may be necessary, but are not sufficient for transformation. Two mutants in this region, dl 1107 and dl 1108, are defective in binding to a 107kDa and a 105kDa cellular protein (Egan et al., 1988), the latter of which is the product of the antioncogene Rb1 (Whyte et al., 1988; Egan et al., 1989). However, the third CR2 mutant, dl 1109, does bind these proteins, but at reduced levels. If these three mutants are all defective in the same function, it may be either that the binding to these cellular proteins is irrelevant to that function, or that complete binding is required, or that binding alone is not sufficient, but this domain of E1A must activate or inactivate these proteins in some manner to bring about transformation. The function of this domain is not clear, but it does affect the morphology of the cells since the few transformants that were obtained or given by CR2 mutants resembled untransformed cells.

transformants that were obtained or given by CR2 mutants resembled untransformed cells.

Although the N terminal/CR1 and CR2 regions do carry out distinct functions, they cannot transform when present on separate E1A proteins. The combination of two mutants, one defective in each region, produced more foci than either alone, but the cells did not resemble wt transformants.

The unique region and exon 2 were not required for transformation. Loss of the transactivation function by some of these mutants did increase the adhesiveness of the transformants however, resulting in the production of more secondary foci.

The work described in this thesis is a systematic analysis of E1A to identify the regions of the protein that are responsible for the major E1A functions, and provides valuable insight into the mechanism of action of the control of eukaryotic gene expression and of oncogenic transformation.

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