MUTATION ANALYSIS

OF

AD 5 E1A PROTEIN

DELETION MUTATION ANALYSIS

OF

THE REGION UNIQUE TO THE 289 RESIDUE PROTEIN

FROM THE

E1A REGION OF ADENOVIRUS TYPE 5

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science.

McMaster University,

Feb., 1988

MASTER OF SCIENCE (1988)
(biology)

MCMASTER UNIVERSITY
Hamilton. Ontario

TITLE: Deletion mutation analysis of the region unique to the 289 residue protein from the E1A region of adenovirus type 5

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NUMBER OF PAGES: X, 143

ABSTRACT

The early gene region, E1A, of Adenovirus is responsible for two mRNAs that appear to be, along with their protein products, necessary for oncogenic transformation. The two proteins differ only in that the larger 289R protein has an extra internal sequence of 46 amino acids. This single difference must account for the functional differences between the two proteins. One function associated with this unique sequence is transactivation, the ability to transcriptionally activate the other early viral genes.

In this thesis the construction and analysis of three in-frame deletion mutants are described. These three deletions, along with a fourth previously made, span the entire unique region.

All three mutants had lost their transactivation ability, suggesting that the entire domain is necessary for transactivation. Transformation assays with these mutants also suggest that this function blocks transformation. Thus, the unique domain must encode another as yet unidentified function necessary for full transformation. Further evidence for another function in the unique domain comes from the differently reduced abilities of the mutants to grow on HeLa cells. Each mutant has

differentially affected some function that is also necessary for lytic infection.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Stan Bayley for his encouragement and advice while I was working in his lab. I am indebted to Carole Evelegh and Tony Jelsma for their help in the lab. Special thanks to my parents for their constant support and encouragement.

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ABBREVIATIONS

Ad5	Adenovirus serotype 5
BRK	baby rat kidney cells
DTT	dithiothreitol
E1A	Adenovirus early gene region 1A
EDTA	ethylenediaminetetracetic acid
FCS	fetal calf serum (Gibco)
HBS	HEPES buffered saline
IPTG	isopropylthiogalactose
MEM	minimal essential medium (Gibco)
NCS	newborn calf serum (Gibco)
PBS	phosphate buffered saline
PEG	polyethylene glycol
SSC	sodium chloride-sodium citrate solution
TBE	tris-boric acid-EDTA buffer
Xgal	5-bromo-4-chloro-3-indolyl-B-D-galatopyranoside

INTRODUCTION

Adenovirus was first isolated from humans by Rowe and his colleages in 1953 from cultured adenoidal tissue. A year later Hilleman and Warner (1954) isolated a virus from army recruits suffering from an influenza-like illness, which was later shown to be a member of the same adenovirus family (Huebner et. al., 1954).

Since then, the adenovirus family has been shown to have a broad species host range including bovine, canine, avian, and simian, as well as human. In humans alone, over forty different serotypes have been isolated. They are responsible for a number of clinical effects, principally acute respiratory and ocular illnesses (Straus, 1984).

Although early research was concerned with the clinical effects of Adenovirus, ongoing research has now switched to its use as a tool in the study of oncogenic transformation and eukaryotic gene regulation. The switch in interest occurred as the result of a significant discovery by Trentin and his associates (1962). They reported that adenovirus serotype 12 was able to induce tumours in newborn hamsters at the site of injection. This was the first human virus shown to be oncogenic. Their observations were quickly confirmed and extended to other serotypes and other rodents (Huebner et. al., 1962; 1965; Yabe et.

al., 1962; 1963; 1964; Girardie et. al., 1964; Rabson et. al., 1964; Pereira et. al., 1965). The obvious implication was that these oncogenic serotypes could also be responsible for some human cancers, but various studies have been unable to link any of the serotypes with any human cancers (Green et. al., 1979; McKay et. al., 1979; Wold et. al., 1979). Adenovirus is still useful as a research tool since the mechanism of tumour induction is presumably similar in all mammals.

Serotypes vary in their ability to induce tumours in rodents. A few such as Ad12 are highly oncogenic and able to induce tumours within three months in most of the treated animals. Others are only weakly oncogenic, having a lower efficiency of transformation and requiring a longer incubation time. Still others are unable to produce any tumours (Trentin et. al., 1962; Huebner et. al., 1962; 1965; Girardie et. al., 1964).

The adenovirus family has been subdivided into five groups based partially on their oncogenic ability, but also on other characteristics such as the guanine-cytosine content of their DNA (Pina and Green, 1965), and their DNA sequence homology (Green et. al., 1979b). These groups are as follows: A (Ad12, 18 and 31) highly oncogenic, B (Ad3, 7, 11, 14, 16, and 21) weakly oncogenic, and C (Ad1, 2, 5, and 6), D (Ad8-10, 13, 15, 17, 19, 20, 22-30), and E (Ad4) all non-oncogenic (Green et al., 1979b).

Of all these serotypes the best characterized are Ad2 and Ad5, which share over ninety-nine percent sequence homology and have had over seventy percent of their genomes sequenced (Green et al., 1979b; Sussenbach 1984). Although they are nononcogenic serotypes, they are able to transform cultured rat embryo cells. The transformed cells are immortal and are morphologically indistinguishable from Ad12 transformed cells (Freeman et al., 1967; Gilden et al., 1968). These transformed cells can in turn produce tumours in rats if the rats are first immunosuppressed (Freeman et al., 1967; Gallimore 1972). Thus, the study of Ad5 can provide useful information on the mechanism of transformation.

In addition, Ad5 can be used as a tool to study eukaryotic gene regulation. Ad5 is a DNA virus which uses the human host cell's transcriptional and translational machinery to replicate itself. The viral genes are transcribed, and the mRNAs translated by the host's machinery in a specific temporal pattern suggesting that the activity is precisely regulated. In order to regulate its own gene activity, the virus must be able to control the host cell's machinery. This indicates that there is interaction between the viral and human systems of gene regulation, and that the two systems must, therefore, be very similiar. Since Ad5 has a smaller and simpler genome, it is obviously easier to study. Anything learned from the virus can then be applied to the more complicated human system. This has already led to such important

discoveries as messenger RNA splicing (Berget et. al., 1977; Klessig, 1977).

Lytic Infection: Transcription and Translation of the E1A and E1B Gene Regions

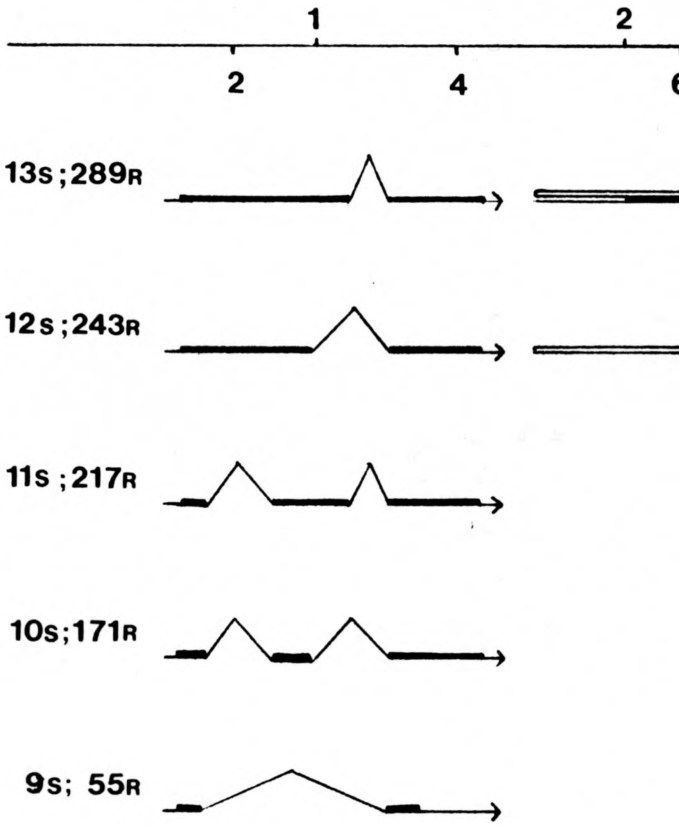
The virus life cycle can be roughly divided into early and late phases separated by the onset of DNA replication. There are six gene regions active in the early phase, each with their own promoter: E1A, E1B, E2A, E2B, E3, and E4. In the late phase, transcription is initiated at the major late promoter to produce a long primary transcript which is processed by a complicated splicing pattern to produce the late phase mRNAs. The major late promoter is also active to a limited extent early in infection (Berk and Sharp, 1978; Chow et al, 1979a;b). The two regions, E1A and E1B (Figure 1), are the ones of most interest since, as will be shown later, they are both necessary for full oncogenic transformation. In addition, the E1A region plays an important role in the activation and repression of the other early gene regions.

E1A is the first early gene region to be transcribed in an infection. Transcription is first detectable forty-five minutes after infection of cultured HeLa cells and reaches a maximum rate two to three hours later. Transcription from E1B does not begin until sixty to ninety minutes after infection. The rate of

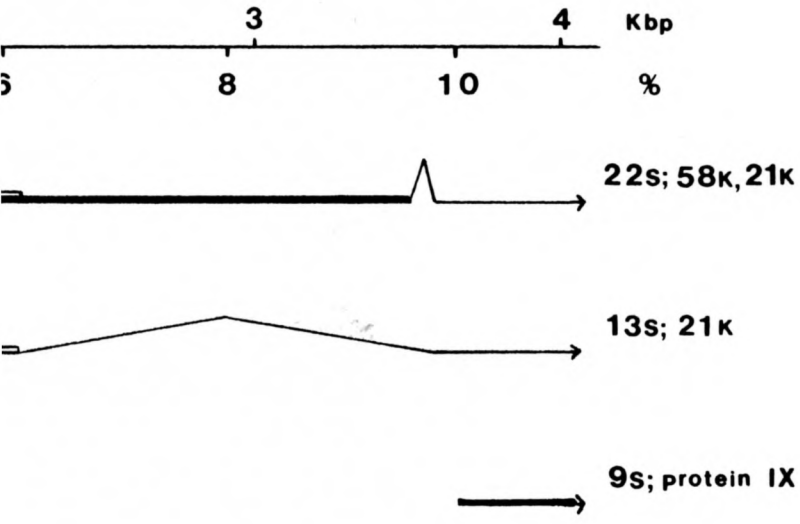
Figure 1:

Map of the early gene regions E1A and E1B.

The organization of the Ad5 E1A and E1B gene regions is illustrated. Detailed descriptions of the mRNAs and their polypeptide products are given in the text.



E1A



E1B

transcription from E1B increases steadily to a maximum at approximately seven hours post infection which is then maintained into the late phase (Nevins et al., 1979).

Early region 1A is transcribed from the extreme left part of the viral r strand between 1.3 and 4.6% of the genome. From it are produced five mRNAs with approximate sedimentation coefficients of 13S, 12S, 11S, 10S, and 9S, the 11S and 10S only recently discovered. All five mRNAs are processed by differential splicing from a common primary transcript and have identical 5' and 3' termini. The 13S, 12S and 9S differ only in the size of their single intron (Berk and Sharp, 1978; Chow et al., 1979a,b; Perricaudet et al., 1979; Kitchingman and Westphal, 1980; Virtanen and Pettersson, 1983), while the two recently discovered mRNAs differ in that they each have a second splice (Stephens and Harlow, 1987; Ulfendahl et al., 1987).

It should be noted that although the early region E1A is the first to be transcribed, it is mainly the 13S and 12S that are produced at this stage. The 9S mRNA, although present in small quantities early in infection, is preferentially produced in the late phase (Spector et al., 1978; Chow et al., 1979). The 11S and 10S appear to be synthesized only in the late phase and only in small quantities (Stephens and Harlow, 1987; Ulfendahl et al., 1987).

With the sequences of complementary DNA copies of the mRNAs now known, it is possible to determine the exact splice sites of the mRNAs, and to predict the exact sequence of their protein products (Figure 1). The 13S, 12S, and 9S RNAs use the same splice acceptor site at nucleotide 1229, but differ in their donor splice sites, using nucleotides 1112, 974, and 636 respectively (Perricaudet, 1979; Virtanen and Pettersson 1983; Roberts et al., 1985). The 11S has one splice identical to the 1112/1229 splice of the 13S mRNA, while the 10S has one identical to the 12S 974/1229 splice. Both mRNA's then have an additional splice removing the nucleotides between 637 and 854 (Stephens and Harlow, 1987; Ulfendahl et al., 1987).

All the mRNA's are translated in the same reading frame except for the 9S which switches to a different frame in its second exon due to the structure of its splice junction (Perriceaudet et al., 1979; Virtanen and Petterson, 1983; Stephens and Harlow, 1987; Ulfendahl et al., 1987). As a result the 9S should produce a fifty-five amino acid (55R) polypeptide, of which only the amino terminal twenty six are in common with the other proteins. This also means that the 289 amino acid (289R) protein coded for by the 13S and the 243 amino acid (243R) protein encoded by the 12S differ only in the presence of a unique internal sequence of forty-six amino acids in the larger protein. The 11S codes for a 217 amino acid (217R) protein identical to the 289R

except for missing amino acids 27 to 98, while the 171 amino acid (171R) protein produced from the 10S is identical to the 243R except that it also lacks amino acids 27 to 98.

Early attempts to examine these E1A proteins directly were hampered by their relatively low concentrations in infected cells, and poor avidity for antisera. More effective antiserum was raised against a synthetic peptide corresponding to the predicted carboxy terminus common to all but the 55R polypeptide (Yee at al., 1983). This antiserum was used successfully to precipitate E1A proteins produced early in infection, and Yee et al were able to identify four major proteins with molecular masses of 52, 50, 48.5, and 45kD, and two minor proteins with masses 37.5 and 35kD. Later experiments using mutant viruses suggested that the 13S mRNA is responsible for the 52 and 48.5kD, while the 50 and 45kD are produced from the 12S mRNA (Rowe et al., 1983). The 37.5kD and 35kD species may be the products of the 11S and 10S mRNAs (respectively) discovered by Harlow et al (1985).

Harlow et al., (1985) created a variety of monoclonal antibodies, and used these to precipitate E1A proteins at various times in after infection. The immunoprecipitated proteins were run on 2-dimensional electrophoretic gels to reveal at least sixty different protein species. Some of these species were shown to be products of neither the 13S or 12S mRNAs, and this led the authors to the discovery of the 11S and 10S mRNAs. The 11S and

10S protein products have molecular masses of 35 and 30kD respectively and are detectable only late in infection (Stephens and Harlow, 1987; Ulfendahl et al., 1987).

The only mRNA whose product is still uncertain is the 9S. It has been shown to have a 28kD translation product in vitro (Esche et al. 1980; Spector et al., 1980), but no in vivo protein products have been detected.

There are two interesting features of the E1A proteins. One, they all have much higher apparent molecular masses, as determined by SDS gels, than predicted from the sequences. And, two, there appears to be a large number of species. One explanation for the former is that the proteins have an unusually high proline content. Proline is actually an imino acid and probably alters the polypeptides' tertiary structure so that their movement through the gel is retarded to give anomalously high molecular weights.

Another explanation for the anomalously high molecular weights, and one that could also account for the variety of species, is that the proteins undergo post-translational modifications. Since all the early proteins have the same N-terminal sequences (Downey et al.,1984), and C-terminal sequences (Yee et al., 1983), it is unlikely that the proteins are cleaved. The major modification is probably phosphorylation (Yee et al., 1983).

Some of the many protein species that Harlow found may represent only partially phosphorylated precurser proteins. Others may simply be artifacts such as breakdown products (Harlow et al., 1985). Still others may be the products of mRNAs, such as the 11S and 10S, which are present in only minute quantities. It is not known if these mRNAs are due to normal or aberrant splicing patterns. The functional significance of the variety of protein species and phosphorylation is not yet known.

In a lytic infection, the E1B gene region is expressed after E1A and at about the same time as E3 and E4 (Nevins et al., 1979). It has two promoters, one at 4.6% and the other at 9.7%. The latter promoter is responsible for an unspliced mRNA preferentially transcribed late in infection (Alestrum et al., 1980). It codes for polypeptide IX which appears to be a structural protein involved in packaging of the genome (Alestrum et al., 1980; Pettersson, 1984; Ghosh-Choudhury et al., 1987). The promoter at 4.6% is the one active early in infection. Two mRNAs, 2.2kb and 1.0kb long with sedimentation coefficients of 22S and 13S respectively, are transcribed from it. They, like the early E1A mRNAs, have identical 5' and 3' termini and differ only in the size of their single intron (Perricaudet et al., 1980; Bos et al., 1981).

Based on sequencing data (Perricaudet et al., 1980; Bos et al., 1981), a 21kD polypeptide could be translated from both the

13S and the 22S mRNAs. A 58kD protein could also be translated from the 22S by using an internal AUG site and a different reading frame. The two proteins would have no amino acids in common (Bos et al., 1981). (See figure 1). These two predicted protein species have been found (Halbert et al., 1979; van der Eb, 1979). Recently three other E1B protein species have also been found. All three proteins are encoded by mRNAs which are produced by cryptic splicing of the 22S mRNA (Lewis and Anderson, 1987).

Transformation

Depending on the virus serotype, a host cell can be permissive, semi-permissive, or non-permissive for lytic infection. In semi- or non-permissive infections, the virus replication cycle is either partially or completely blocked. As a result there are few progeny virus, and a small percentage of the cells become transformed. In transformation these cells undergo a number of changes. For instance, they are immortal and can form cell lines. In addition, they often have different growth properties, being able to grow to high cell density, and without anchorage. They also usually have altered morphologies (Flint, 1981; Graham, 1984).

These transformants have been shown to have stably integrated adenoviral DNA into their genome. Studies with a number of independently derived cell lines indicated that usually

only a portion of the viral genome was integrated, and that although the amounts varied, the left-hand 12 to 14% was always preserved (Sharp et al., 1974; Gallimore et al, 1974; Flint et al., 1976; Johansson et al, 1977). The stable integration of only the left 12-14% suggested that the E1A and E1B early gene regions were involved in transformation.

By transfecting primary rat cells with viral DNA fragments, Graham et al (1974a,b) were able to show that the left-hand 7% of the genome containing the entire E1A and part of the E1B gene region was both necessary and sufficient to fully transform cells. Cells could be transformed with a smaller fragment containing only the E1A region, but the frequency was much lower, and the cells, although immortal, did not exhibit all the morphological changes (Graham et al., 1974b; van der Eb, 1979; Houweling et al., 1980).

To show that transformation by the virus was not a hit-and-run process, assays were performed with cold sensitive transformation mutants (Ho et al., 1982; Babiss et al., 1984). Cells were infected with the mutants and incubated at the permissive termperature. Just before the foci were expected to become large enough to be visible, the incubation temperature was dropped to 32°C. Foci never developed. Those cells transformed at the permissive temperature were unable to continue to grow at the non-permissive temperature. Since these were E1A mutants,

they demonstrated that continued activity of the E1A region was necessary to maintain transformation.

It should be noted that in virion mediated transformation the entire E1A and E1B regions appeared to be needed, while in DNA mediated transformation the whole E1B region was no longer necessary. In the latter situation, DNA fragments lacking the 3' end of the E1B gene were able to transform cells even though only the 20kD protein and a truncated 58kD protein could be produced. It was later suggested that the 58kD E1B protein was only required for virion mediated but not DNA mediated transformation (Graham et al., 1978; van der Eb et al., 1979; Rowe and Graham, 1983). It is likely that the 58kD E1B protein is required by the virion for initiation of transformation, but that its role is not critical and can be bypassed in DNA transfection (Rowe and Graham, 1983).

In an attempt to separate the roles of E1A and E1B in transformation, these regions were inserted into separate plasmids and the plasmids' abilities to transform were compared (van den Elsen et al., 1982). Together the plasmids gave complete and normal transformation. When E1A and E1B plasmids from different serotypes were cotransfected, the oncogenecity of the transformed cell lines correlated with the oncogenicity of the serotype of the E1B and not with that of the E1A.

On its own, E1A was able to transform to a limited extent, but as in the previously mentioned experiments, the cells were

immortal but lacked the normal morphology of fully transformed cells. E1B, on the other hand, was unable to transform at all (van den Elsen et al., 1982). This negative result could have been due to a low level of E1B proteins since, as will be discussed later, E1B transcription is promoted by E1A. To ensure transcription the SV40 promoter-enhancer was inserted into the E1B plasmid, but still E1B was unable to transform. Even when the E1B plasmid was transfected into an established cell line rather than into primary cells, the E1B was unable to transform (van den Elsen et al., 1983).

The evidence so far suggests that the E1B 19kD protein is necessary for complete morphological transformation. The E1B 58kD, on the other hand, appears to be involved only in the very early stages of virion-mediated transformation and can be by-passed with DNA transfection. Of course, the N-terminal portion of the E1B 58kD could still be important in transformation since the DNA fragments used in transfection were capable of producing a truncated protein. E1A is responsible for the initial immortalization, and is also involved in the morphological transformation.

Role of Individual E1A proteins in Transformation

The E1A gene region is responsible for five overlapping mRNAs and their numerous species of polypeptides. Up to this time

the only E1A proteins found in transformed cells have been those from the 13S and 12S mRNAs. Little is known about the possible roles of the other mRNAs' proteins in transformation. Recent assays using mutants producing only the 217R, 171R, or 55R protein have suggested that none is capable of transformation in the absence of the 289R or 243R proteins (Moran et al., 1986a; Stephens and Harlow, 1987; Ulfendahl et al., 1987). On the other hand, another group (Roberts et al., 1985) did demonstrate that cells transfected with a plasmid containing a complementary DNA copy of the 9S transcript were slightly altered in morphology, although not as dramatically as those with the 13S or 12S cDNAs. It is possible that the products of the 11S and 10S can function only in conjunction with the 289R or 243R proteins, or in some manner that can't be observed in tissue culture. One argument against the 11S, 10S and 9S and their proteins having any role in transformation in that they are preferentially produced late in infection. Since transformation is an early function, it is therefore likely that only the 289R and 243R protein species play a significant role.

Three mutants have proved to be invaluable in attempting to separate the roles of the 13S and 12S mRNAs and their products. The first is Ad5 hr1 (Harrison et al., 1977), which has a single base deletion within the unique region of the 13S mRNA. It produces a normal 243R and only a truncated 289R (Riccardi et al.,

1981). The second, Ad2 d11500, was mutated at the 13S donor splice site so that only a 12S mRNA is produced (Montell et al., 1984). And the third mutant, pm975, was constructed with a single base altering the 12S donor splice signal so that it could only produce a 13S mRNA (Montell et al., 1982). All three mutants have been used in transformation assays, and they reveal some striking differences in the activities of their polypeptide products.

Ad5 hr1 has been shown to be unable to transform rat embryo or embryo brain cells, and to induce only an abnormal transformation in rat kidney cells, although at a higher efficiency of focus formation than wild type Ad5 (Graham et al., 1978). For instance, this transformation was cold sensitive and at the lower temperature (32°C) only the wild type Ad5 virus was still able to transform (Ho et al., 1982; Babiss et al., 1983). In addition only polyclonal cell lines could be developed (Graham et al., 1978; Ruben et al., 1982), and the cells did not exhibit the fully transformed phenotype. They were fibroblastic rather than epithelial, were defective for anchorage independant growth, and were significantly less tumourigenic (Ruben et al., 1982).

As will be explained later E1A transcriptionally activates the E1B gene region, but as the Ad5 hr1 transformed cell lines were producing E1B proteins, these abnormalities were not due simply to a lack of the E1B proteins (Ruben et al., 1982). Since hr1 can produce a truncated 289R protein, its effect could not be

ruled out. Dl1500, though, does not produce any 13S mRNA. Experiments with dl1500 (Montell et al., 1984) indicate that the 12S is responsible for this cold sensitive abnormal transformation.

The mutant pm975 lacks the 12S mRNA and therefore its proteins. Transformation by pm 975 is not cold sensitive, but it does have a lower frequency of focus formation than the wild type virus. Pm975 transformants also appear to be defective for anchorage independant growth. Again E1B proteins were being produced (Montell et al., 1984).

In a slightly different approach complementary DNA copies of the mRNAs were inserted into retroviral vectors and then into a mouse cell line. Those cells with either the 13S or 12S cDNA grew with diminished adherence to solid support. The cells with the 13S were also cuboidal, while those with the 12S were elongated and had altered contact inhibition so that cells were overlapping each other (Robert et al, 1985).

This last experiment illustrated that the 289R and 243R proteins are responsible for different morphological and growth alterations. And since none of the three mutants were able to duplicate the wild type transforming ability, it appears that the protein products from both mRNAs are required and have different and specific activities. What these activities are and how they relate to the lytic cycle functions is still undetermined.

E1A Functions

Since the roles of the E1A gene region in lytic infection and transformation are not well understood, some researchers have taken the approach of identifying as many activities of the E1A proteins as possible and then trying to determine how they could function in lytic infection and transformation.

1. Nuclear Localization

Immunofluorescence techniques have detected E1A proteins in diffuse areas in the cytoplasm and in discrete patches in the nucleus (Yee et al., 1983). In other experiments the purified proteins were microinjected into the cytoplasm of oocytes (Krippl et al., 1985; Ferguson et al, 1985). Both the 289R and 243R proteins moved into the nucleus within thirty minutes. Deletions introduced into these proteins now show that a short five amino acid sequence at the carboxy terminus is responsible for the rapid nuclear localization (Krippl et al., 1985; Lyons et al., 1987). Both the 217R and 171R proteins, but not the 55R protein, should also have this ability.

2. Transactivation

In a lytic infection E1A is the first early gene region to be transcribed, preceding the others by at least thirty minutes (Nevins et al., 1979). Early evidence for E1A's importance came

from work with E1A mutants such as d1312, which lacks the entire E1A gene region, and hr group 1 (Harrison et al., 1977; Berk et al., 1979; Jones and Shenk, 1979). These mutants could not grow in Hela cells, the usual human host cell, only in 293 cells, a human cell line in which E1A and E1B are constitutively expressed (Graham et al., 1977). Analysis of early mRNAs produced by these mutants suggested that the lack of E1A prevented transcription from the other early regions (Berk et al., 1979; Jones and Shenk 1979; Nevins 1981). The E1A region is not required absolutely. If the infection was given more time, or the cells were infected with an excess of virus particles, transcription levels did increase (Nevins, 1981) and low yields of progeny virus could be obtained (Shenk et al., 1979). Early experiments with protein synthesis inhibitors led Nevins (et al.,1979; 1981) to suggest that there was a shortlived cellular factor which inhibits viral transcription except from E1A. E1A proteins, once produced, could then block the cellular factor to allow transcription from the other genes. Later experiments (Gaynor and Berk, 1983) did not support this hypothesis, and there is evidence that E1A is actually a transcriptional activator. That is, the E1A proteins turn on other genes, either directly or, more likely, through a cellular intermediate (Weeks and Jones 1983; Jones et al., 1983; Ferguson et al., 1984).

To demonstrate E1A's ability to transcriptionally activate the other early genes, Weeks and Jones (1983) devised a transient expression assay based on the CAT assay (Gorman et al., 1982). In this assay, a recombinant plasmid was created which contained the chloramphenicol acetyltransferase gene (CAT) attached to one of the early viral promoters such as E2A, E3 or E4. This plasmid was then transfected into a mammalian cell line where it was the only source of the enzyme. The amount of enzyme was indirectly measured by the ability of a cell extract to acetylate radioactively labeled chloramphenicol. If a second plasmid containing the E1A gene was cotransfected, the amount of the CAT enzyme activity would increase eight to ten fold.

Assays with the individual mRNAs and their products have shown that the 243R and 171R proteins do not seem to have any transactivation ability (Montell et al., 1982; Svensson and Akusjarvi, 1984; Moran et al., 1986; Ulfendahl et al., Stephens and Harlow, 1987), while the 55R may have slight but erratic activity (Svensson and Akusjarvi, 1984; Moran et al., 1986). The 217R does have some activity, although not as great as the 289R, which appears to induce wild type levels of activation (Montell et al., 1982; Ferguson et al., 1984; Svensson and Akusjarvi, 1984; Moran et al., 1986; Ulfendahl et al., 1987).

The transactivation ability of the 289R protein also appears to extend to some non-viral genes, suggesting at least one

possible role E1A could have in transformation. For instance, the Beta tubulin and 70kD heat shock genes endogenous in Hela cells were found to be specifically activated by adenovirus infection (Stein and Ziff, 1984; Kao and Nevins, 1983; Wu et al., 1986). Also, the 70kD protein was transactivated only by those mutant viruses producing an unaltered 289R protein (Wu et al., 1986), confirming that it was still this protein that was responsible.

E1A was not enhancing overall transcription (Stein and Ziff, 1984), and a number of genes such as Beta globin and preproinsulin were not activated under the same conditions as above (Gaynor et al., 1984, Stein and Ziff, 1984). If the same genes were inserted into a plasmid or virus and then cotransfected with E1A, they were successfully transactivated (Treisman et al., 1983; Gaynor et al., 1984; Stein and Ziff, 1984; Svensson and Akusjarvi, 1984). It can be inferred from the two opposing results that exogenous plasmid or viral genes are open for transactivation while endogenous cellular genes are protected by some general transcriptional blocking mechanism involving perhaps nucleosomes or methylation patterns.

3. Enhancer Repression

Evidence suggests that the E1A proteins have another transcriptional regulation function, that of enhancer repression. In an elegant series of experiments Borelli et al., (1984),

clearly showed that E1A proteins were able to inhibit the transcriptional activation of enhancers. The authors had performed a number of cotransfection experiments using a combination of different plasmids, and then measured the amounts of mRNAs transcribed from the plasmid target genes. From their results they concluded that in the absence of an enhancer in the target gene, E1A proteins stimulate transcription, but that if the enhancer is present, the E1A proteins will abolish its activity. Also, both the 289R and 243R proteins appeared to have enhancer repression activity, although this is disputed by some recent experiments (Lillie et al., 1986) which suggest only the 243R is capable of repression. It could be that in the 289R the repression function is overridden by the transactivation function.

In addition, Borelli et al., (1984) found that the repression effect could be titrated out by adding increasing amounts of a third plasmid containing the enhancer element alone. Repression must therefore act at the enhancer sequence and shouldn't interfere with transcription activation at the promoter. Their work has been confirmed by other independent groups (Treisman etal., 1983; Svensson and Akusjarvi, 1984; Velcich and Ziff, 1985; Hen et al., 1985; Velcich et al., 1986; Stein and Ziff, 1987).

Since transactivation is unique to the 289R, while enhancer repression is either common to both or specific to the 243R; and

since repression acts at the enhancer element, while transactivation appears to act at the promoter, probably at the TATA box itself (Wu et al., 1987), it is probable that these are two distinct and independent functions (Borelli et al., 1984).

The dual ability of E1A to transactivate and repress independently allows for a complex regulation of viral and host cellular genes through a delicate balance between the two opposing activities. In fact, it appears that E1A is able to autoregulate itself through this same dual control. It has its own enhancer element which allows initial transcription early in the lytic cycle, and is stimulated by its own proteins (Smith et al., 1985). At the same time its proteins are also able to block its own enhancer effect (Borelli et al., 1984).

4. Requirement for Lytic Infection in Growth Arrested Cells

Ad5 hr1 and Ad2 dl1500 are both host range mutants in that they replicate with at least a thousand fold less efficiency in HeLa cells compared to 293 cells (Graham et al., 1977; Montell et al., 1984). Mutant viruses producing only the 171kD or 217kD proteins also appeared to have severely impaired growth, although the latter was not quite as drastically affected (Stephens and Harlow, 1987; Ulfendahl et al., 1987). Only pm975 appears to grow equally well in HeLa and 293 cells (Montell et al., 1982). The results suggest that only the 289R is required for normal lytic

infection, and do not indicate what possible functions the 243R may have.

Experiments altering tissue culture conditions suggest that the 243R is necessary for replication in growth arrested cells (Montell et al., 1984; Spinder et al., 1985). When cells are infected with Ad5 virus while growing in medium containing very low serum concentrations (0.2%), the rate of infection and yield of virus is decreased. Under these conditions the growth of the mutant virus dl1500 was no more reduced than wild type, but that of the pm975 virus was significantly more impaired. In natural infections, the normal target cells are end differentiated respiratory epithelial cells and in these cells the 243R protein would be required for efficient replication. The 243R protein's actual function in this instance is not yet known but it is possibly related to its ability to induce DNA synthesis.

5. Induction of DNA synthesis and Mitosis

The early work of Shimojo and Yamashita (1968) suggested that Adenovirus 5 infection of non- or semi-permissive cells stimulated cellular DNA synthesis. E1A was later implicated when it was shown that only E1A mutant viruses were unable to induce synthesis (Braithwaite et al., 1983). To ensure that induction was an actual function of the E1A region and not an indirect effect of the infection, complementary DNA plasmids of the 13S,

12S, and 9S mRNAs were microinjected into quiescent cells (Stabel et al., 1985). Both the 13S and 12S plasmids were able to stimulate DNA synthesis. Even the direct microinjection of their polypeptide products was sufficient to induce cellular DNA replication (Kaczmarek et al., 1986).

The results of another group are not in complete agreement with Stabel et al (1985). Spindler and her associates (1985) were trying to determine why pm 975's growth on quiescent cells was so reduced (see previous section). They found that the levels of early and late proteins were the same in both infection assays; the only difference was that pm975 was unable to induce wild type levels of viral or cellular DNA synthesis. Dl1500 was also unable to induce wild type levels of viral DNA synthesis but this can be attributed to the indirect effect of a lack of early proteins (no transactivation function). Cellular DNA synthesis, on the other hand, was successfully induced by dl1500. Other groups have agreed with these results (Quinlan and Grodzicker, 1987; Quinlan et al., 1987; Zerler et al., 1987). Thus, the 243R protein is able to induce DNA synthesis, but it is not yet known if the 289R also shares this ability.

Induction of mitosis is another function in which the relative roles of the 289R and 243R proteins are unclear. Ad5 hr1, which can produce only a truncated 289R, was shown to be unable to induce mitosis (Braithwaite et al., 1983). Other groups

(Quinlan and Grodzicker, 1987; Quinlan et al., 1987; Zerler et al., 1987) have been able to induce proliferation with mutants producing only the 243R protein.

6. Differentiation of Cells

Montano and Lane (1987) have recently shown that E1A proteins, both the 289R and 243R, are able to induce differentiation of F9 teratocarcinoma cells. These stem cells, when transfected with plasmids producing either the 289R or 243R, underwent extensive morphological alterations specific to differentiation. In the process a stem cell-specific protein was lost and differentiation-specific proteins began appearing. F9 stem cell differentiation can be activated by a range of signals, and it is possible that this is an incidental ability of the E1A proteins and has no functional significance.

7. Thesis Experiments

The E1A region is necessary for both normal lytic infection and for oncogenic transformation. Although this region in responsible for five distinct messenger RNA's, only the two early ones, the 13s and 12s, have been shown to play a role in both processes. It is probable that their roles in the transformation process are related to their normal functions in a lytic infection. Although their proteins may have some activities in

common such as enhancer repression, and induction of DNA synthesis, they also have some that are distinct. For example, the 243R is responsible for replication in growth arrested cells, while the 289R is able to transcriptionally activate other genes. In addition, both polypeptides are required for complete transformation. These differences in function, by necessity, must be related to the unique sequence of forty-six amino acids in the 289R polypeptide since in every other aspect they are identical. This unique region probably adds a functional domain to the 289R, but could also alter the structure of the protein as a whole, and could also disrupt or alter a functional domain as it exists in the 243R protein.

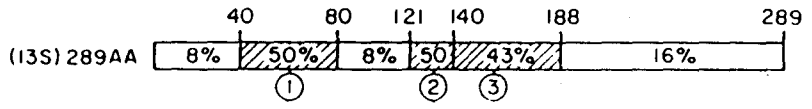
The concept of functional domains within the proteins is suggested by the pattern of conserved sequences in different serotypes. A comparison of amino acid sequences for a number of adenovirus serotypes (Kimelman et al., 1985) has revealed that there are three distinct regions of conserved sequences separated by relatively unconserved sequences (see figure 2). One conserved domain is basically the unique sequence of the 289R protein. Another is a stretch of forty-one amino acids within exon one, and common to both polypeptides. And the last region is the last twenty amino acids of exon 1. In adenovirus types 2 and 5, this later region is joined directly to the conserved unique region of

Figure 2:

Sequence of the 289R protein indicating conserved regions.

The amino acid sequence of the 289R Ad2 E1A protein is given in the single letter code. The Ad2 and Ad5 serotypes are 99% homologous. Below is a representation of the conserved and non-conserved regions of the protein found in a survey of serotypes 5, 7 and 12. The numbers 1, 2 and 3 identify the three conserved regions. Respectively 50, 50 and 43% of the amino acids are identical between the three serotypes. (Reprinted from Moran and Mathews, 1987).

	M	R	H	I	I	C	H	G	G	V	I	T	E	E	M	A	A	S	L	L	20
	D	Q	L	I	E	E	V	L	A	D	N	L	P	P	P	S	H	F	E	P	40
1	P	T	L	H	E	L	Y	D	L	D	V	T	A	P	E	D	P	N	E	E	60
1	A	V	S	Q	I	F	P	D	S	V	M	L	A	V	Q	E	G	I	D	L	80
	F	T	F	P	P	A	P	G	S	P	E	P	P	H	L	S	R	Q	P	E	100
	Q	P	E	Q	R	A	L	G	P	V	S	M	P	N	L	V	P	E	V	I	120
2	D	L	T	C	H	E	A	G	F	P	P	S	D	D	E	D	E	E	G	E	140
	E	F	V	L	D	Y	V	E	H	P	G	H	G	C	R	S	C	H	Y	H	160
3	R	R	N	T	G	D	P	D	I	M	C	S	L	C	Y	M	R	T	C	G	180
	M	F	V	Y	S	P	V	S	E	P	E	P	E	P	E	P	E	P	E	P	200
	A	R	P	T	R	R	P	K	L	V	P	A	I	L	R	R	P	T	S	P	220
	V	S	R	E	C	N	S	S	T	D	S	C	D	S	G	P	S	N	T	P	240
	P	E	I	H	P	V	V	P	L	C	P	I	K	P	V	A	V	R	V	G	260
	G	R	R	Q	A	V	D	C	I	E	D	L	L	N	E	S	G	Q	P	L	280
	D	L	S	C	K	R	P	R	P												289



the 289R polypeptide, but in other serotypes there are intervening sequences of 15 - 25 residues. This implies that the unique region constitutes a separate domain from the other conserved sequence.

Recently researchers have begun to define functional domains including the unique region using specific site directed mutants (Lillie et al., 1986; Moran et al., 1986). By analyzing the altered activities of these mutants it would then be possible to determine which sequences are essential for which functions, and relate these to their transforming abilities.

I have been particularly interested in the significance of the unique region for the reasons already mentioned. For this thesis, I have constructed three deletion mutants that, together with a fourth previously made, encompass the entire unique region. I have assayed these mutants for their transactivation ability, their transformation ability, and their ability to replicate in Hela cells versus 293 cells.

MATERIALS AND METHODS

Unless otherwise described in solutions, all solutions were made as specified in Maniatis et al (1982).

Cell lines

293 cells were provided by Dr. F.L. Graham (McMaster University). The cells were maintained in Joklik-modified MEM with 10% horse serum (Gibco), unless otherwise stated. The 293 cell line was created by transforming a human embryonic kidney cell with sheared Ad5 DNA (Graham et al, 1977).

HeLa cells were supplied by Dr. F. Graham (McMaster University). They were maintained in Alpha-MEM with 10% newborn calf serum.

Virus

Ad5 dl309 virus was obtained from Dr. F.L. Graham. This mutant has a single Xba I site at map position 3.8 (Jones and Shenk, 1979) and grows like the wild type virus.

Plasmids

The plasmid pLE2 (Fig. 8) (provided by A. Jelsma) contains the entire E1A gene region. It is the same as plasmid pCD2 (obtained from Dr. F.L. Graham) except that the Cla I site at position 23 in the pBR322 sequence had been removed by cleaving with Cla I, treating with DNA polymerase (Klenow fragment) and religating. The plasmid pCD2 was derived from plasmid pXC-I which contains the Xho I C fragment of Ad5. It was constructed by adding a Bam HI linker to the left hand end of the virus, digesting the virus with Xho I, and inserting the Bam HI - Xho I fragment between the Bam HI and Sal I sites of plasmid pBR322 (McKinnon et al, 1982). In plasmid pCD2 the Ad5 sequence between the Sac I sites at bases 1770 and 5644 was deleted, removing most of the E1B gene region.

The plasmid pKCAT 23 was a gift from M. B. Mathews (Cold Spring Harbor Labs), and originally came from Dr. N. Jones (Perdue university, Indiana). This plasmid contains the bacterial gene chloramphenicol acetyl transferase (CAT), driven by the E3 promoter of Ad5 (Weeks and Jones, 1983).

The plasmind pEJras 1 was obtained from I. Mak (McMaster University) and originally came from R. Weinburg (Massachusetts Insitute of Technology, Mass.). This plasmid contains the oncogene Harvey ras 1.

Bacteriophage M13

The Bam HI-Xba I fragment of pLE2 containing the Ad5 sequence was inserted into the appropriate sites of the cloning region of the M13 mp11 phage supplied by Pharmacia.

Bacteria

The M13 phage was cultured in Escherichia coli strain JM103, while the plasmids were grown in E. coli strain LE392. Both strains were supplied by Pharmacia.

Enzyme digests

Enzymes were bought from either BRL or Pharmacia and used as recommended.

Phenol extraction and ethanol precipitation of DNA

To extract DNA from a solution containing protein an equal volume of phenol (saturated with 10mM Tris pH 8.0) was added and mixed in. The two phases were separated by centrifugation and the aqueous DNA solution was pipetted out. This extraction was repeated with phenol:chloroform (1:1) and then ether. Each step of the extraction was repeated, if necessary, to remove all the protein.

The DNA was then precipitated by adding one-tenth volume 1M NaCl, 2 to 2.5 volumes 95% ethanol and standing at -70°C. The precipitated DNA was pelleted by spinning at 12,000rpm and the ethanol was aspirated off.

Preparation of M13 single strand DNA

Single strand M13 DNA was prepared according to Pharmacia's suggested protocol. 40ml of YT broth was inoculated with 200ul from an overnight culture of JM103. The culture was then started from either an individual plaque or with 20ul from a small previously made phage stock. The culture was grown at 37°C with agitation for 6 to 7 hrs.

The culture was spun at 8,000rpm for 10 minutes and the supernatant was poured into a second tube containing 8ml of 2.5M NaCl:20% PEG. The solution was mixed and kept on ice for 30 minutes. It was then spun at 10,000rpm at 4°C for 30 minutes. The phage pellet was resuspended in 500ul of 10mM Tris:1mM EDTA (pH 7.6) and phenol extracted and ethanol precipitated.

Oligonucleotide synthesis

The oligonucleotides were synthesized by the phosphotriester method on either a Bioresearch SAM 1 or an Applied Biosystems Model 380B DNA Synthesizer. They were purified by

absorption to an S I polyanion column and elution with a gradient phosphate buffer. The DNA was then desalted on a Sephadex G15 column.

Production of mutants

The E1A mutants were created using oligonucleotide directed mutagenesis as developed by Zoller and Smith (1982, 1984). The template for mutagenesis was an M13 phage with the Bam HI - Xba I fragment from pLE2 inserted into the cloning region. This fragment contained the Ad5 E1A sequence to be mutated (see Figure 1). Two primers were annealed to this template. One was the original M13 mp11 cut with Bam HI and Xba I, and the other was the phosporylated mutagenic oligonucleotide (Figure 5). 20pmoles of the oligonucleotide, 1pmol of the second primer and 1pmol of the template were combined in a 10ul volume of annealing buffer and heated to 55°C for five minutes. After gradual cooling to room temperature, an equal volume of extension/ligation reaction mix was added. The reaction was allowed to proceed overnight at approximately 15°C to fill in the gaps in the heteroduplex.

The following day fresh competent JM103 cells were prepared as follows. A colony was grown to OD_{550}=0.2-0.3 in 75ml of YT broth and then spun down at 6,000rpm for five minutes. The cells were resuspended in 15ml of competent A solution and kept on ice for twenty minutes. The cells were resedimented and

resuspended in 1.5ml of competent B solution. The cells were used within one hour.

In the transformation 3ul of the DNA solution was added to 100ul of the competent cell suspension and left on ice for thirty minutes. These cells were then warmed at 37°C for 15 minutes. Half of the cells were added to 200ul of an overnight JM103 culture and then mixed with 3ml of molten (44°C) plating agar containing 20ul of 100mM IPTG and 100ul of 2% w/v Xgal and poured onto YT plates. After incubation overnight at 37°C, plaques were visible.

Screening for mutants

The M13 clones were screened for the mutants using the dot blot hybridization technique as outlined by Zoller and Smith (1982).

A number of well separated clear plaques were picked and grown with 15ul from an overnight culture of E. coli JM103 in 2ml YT at 37°C for five to six hours. 500ul of the culture were saved for stock. The remainder was centrifuged to pellet the cells and the phage precipitated from the supernatant at room temperature for 15 minutes with 200ul of 2.5M NaCl:20% PEG. A five minute spin at 14,000rpm brought down the phage and they were resuspended in 5ul of water. Each M13 clone was then spotted onto a nitrocellulose filter which was baked at 70°C for 2 hours. The

nitrocellulose was sealed into a bag with 5mls prehybridization buffer for a 1 hour incubation at 67°C. The filter was rinsed with 50ml 6X SSC at room temperature for 1 minute before hybridization.

The mutagenic oligonucleotide was used as the probe in the hybridization. 20pmoles were labelled with approximately 50uCi of ^{32}P-ATP using T_4 Kinase. The unincorporated ATP was removed from the probe by chromatography using Whatman DE 52. About 250ul of DE 52 equilibrated in 10mM Tris : 1mM EDTA (pH 8) was packed into the tip of a 1ml disposable pipette blocked with siliconized glass wool. 200ul of the TE solution was added to the kinased oligonucleotide and this was applied to the column. The column was then washed with TE five times. The free ATP was eluted with 5 1ml washes with 0.3M NaCl in TE, and then the oligonucleotide was eluted with three 1ml washes with 1M NaCl in TE solution.

For hybridization the labeled probe was combined with 4ml of the hybridization buffer and sealed into the bag with the filter for 1 hour incubation at room temperature. The filter was washed 3 times in 6X SSC at room temperature for 10 minutes and exposed to film for 1 hour. The wash was repeated at a higher temperature and the filter exposed to film. The approximate T_m of the probe was calculated by adding 2°C for every A or T nucleotide and 4°C for every G or C in the oligonucleotide (Wallace et al, 1979). The final wash was done at this temperature. Any clones

that still had the probe bound were sequenced to confirm that they had the appropriate deletion.

Sequencing by dideoxynucleotide method

Sequencing was performed according to the protocol suggested by Pharmacia and their premixed deoxy-dideoxy solutions were used. Single stranded DNA preparations were made from several clones that the filter hybidization screen suggested had been mutated. 0.5pmoles of the appropriate sequencing primer was combined with 1 to 2ug of each of these single stranded DNA preparations in PL-1 buffer and heated to 60°C for 10 minutes. The DNA solution was then allowed to cool slowly to 37°C to anneal the primer to the template before adding 3 units of DNA pol 1 (Klenow fragment) and 20uCi of ^{35}S-dATP.

For the sequencing reactions 2.5ul of the annealed DNA was aliquoted into 4 tubes containing each of the dNTP/ddNTP mixes. The reactions were allowed to go for 30 minutes at 37°C (or 50°C if there were problems with secondary structure interfering with the sequencing). 2ul of chase solution was added to each tube and the reactions continued for a further 30 minutes. The reactions were stopped by the addition of 3ul of stop solution and the samples were placed in a boiling water bath for 5 minutes. 2ul was then loaded onto a sequencing gel. After the electophoresis, the gel was dried and exposed to film.

Preparation of double strand M13 DNA

DNA was prepared according to New England Nuclear Laboratory's protocol. 40ul of the M13 stock were added to 500ml YT to which 300ul of an overnight JM103 culture had also been added. The phage was grown for approximately 7 hours at 37°C with agitation. The bacteria were sedimented for 5 min. at 6,000rpm and resuspended in 2ml of sucrose buffer with 10mg lysozyme. After 5 min. incubation on ice, 2ml of T:E (5:1) and 250ul of RNAse were added. After another 5 min. on ice, 2.5ml of buffered triton solution were added and this was left for a further 10 min. The samples were spun at 12,000rpm for 20 min. at 4°C.

The DNA in the supernatant was run on a CsCl gradient for purification. The volume of the supernatant was brought up to 8.5ml with T:E (5:1). 8gm of CsCl was dissolved into the solution before 320ul of ethidium bromide (5mg/ml) was added. This was centrifuged at 40,000rpm for 40 hours. The DNA band was extracted with a syringe through the side of the tube and the ethidium bromide removed with isoamyl alcohol extraction. The CsCl was removed by dialyzing in a low tris buffer; 3 times with 2l for 1hr., and once overnight with 4l. The DNA was then ethanol precipitated.

Rescue of deletion mutants into pLE2

2pmoles of the pLE2 plasmid was cut with Sma I, phenol extracted and then ethanol precipitated. The cut pLE2 was combined with 10pm of RF M13 and both were digested with Xba I and Bam HI. The DNA was again phenol extracted and ethanol precipitated. The DNA was resuspended in a small volume of the recommended buffer solution for ligation with T_4 DNA ligase overnight at 14°C.

Competent cells were prepared for the transformation as outlined in Maniatis et al (1982). A small 3ml overnight culture of strain LE392 was diluted 1 in 100 in 50ml of Luria Broth and was grown to a density of OD_{660}=0.5. The cells were sedimented at 5,000rpm for 5 min., resuspended in 15ml sterile 75mM $CaCl_2$:5mM Tris (7.6), and left on ice for 20 min. The cells were again sedimented and resuspended in 2ml of the same solution.

The ligated DNA was added within 1 hour to the competent cells. The DNA was incubated with the cells on ice for 1 hour, before the cells were heat shocked at 42°C for 2 min. 8ml of warm Luria Broth was added and the cells were incubated for a further 30 min at 37°C. 200ul of this was added to Luria Broth plates with 100ug/ml ampicillin and incubated overnight at 37°C.

Screening of colonies for rescue into plasmid

Colonies were picked and grown for 8 hours in 2ml Luria Broth supplemented with ampicillin (100ug/ml). While 500ul of the bacteria were saved, the rest was pelleted at 12,000rpm for one minute. The DNA was harvested using a modified Birnboim technique (Maniatis et al, 1982).

The cells were resuspended in 100ul lysozyme solution and then 200ul of alkaline SDS was added. After a 10 min. incubation on ice, 150ul of 3M NaAc (pH 4.8) was added. The cell suspension was incubated for a further 15 min. on ice before the debris and cellular DNA was spun down at 12,000rpm for 5 min. The DNA was precipitated from the supernatant with 2 volumes of 95% ethanol without the addition of more salt. The plasmid DNA was resuspended in 20ul of water, and 5ul was digested with either Hae II or Xba I and Sma I. The digests were analysed on 6% polyacrylamide gels with the appropriate markers.

Large scale plasmid preparations

The first large scale preparations were made according to Maniatis et al (1982). A 10ml overnight culture was used to inoculate a 1l culture (50ug/ml ampicillin). When the OD_{660} reached 0.5-0.7, chloramphenicol was added to a final concentration of 180ug/ml. The culture was incubated 16-20 hours.

The cells were spun down for 5 min. at 6,000rpm. and resuspended in 10ml lysozyme solution. After 5 min., 10ml of alkaline SDS was mixed in and the lysed cells put on ice for 10 min. 15ml of 3M KAc (pH 4.8) was added and the solution was left on ice for a further 10 min. The debris was spun down at 10,000rpm for 15 min., 0.6 volumes of isopropanol was added and after 15 min. at room temperature, the nucleic acids were spun down at 10,000rpm for 15 min.

For CsCl gradient centrifugation, the DNA was resuspended in 9.2ml of 0.1X SSC in which 10.8g of CsCl had already been dissolved. 600ul of ethidium bromide (5mg/ml) was added and the gradient centrifuged for 36 to 48 hrs. at 36,000rpm. The DNA band was extracted and the ethidium bromide removed by isoamyl alcohol extraction. The CsCl was removed by dialysis in 1l 0.1X SSC at 4°C for 45min. 3 times, then in 1l 0.01M Tris: 0.001M EDTA (8.0) at 4°C a further 3 times. The DNA was ethanol precipitated.

In later preparations a CsCl gradient was not used. Instead the DNA was purified according to the method of Pelham (1982). After the isopropanol precipitation, the nucleic acids were resuspended in 1ml 10mM Tris: 1mM EDTA (pH 7.4). The RNA was precipitated by adding 2ml 5M LiCl and cooling on ice for 30 min. The RNA was pelleted at 12,000rpm for 15 min. Two volumes of 95% ethanol was added to the supernatant to precipitate the DNA, and the solution was respun for another 15 min. The DNA was

resuspended in 400ul 10mM Tris (pH 7.5): 15mM NaCl and RNAse added to a final concentration of 40ug/ml. After a 30 min. incubation at 37°C the DNA was precipitated with an equal volume of 13% PEG : 1.6M NaCl on ice for 30 min. A centrifugation at 12,000rpm for 10 min. pelleted the DNA. Again the DNA was resuspended, this time in 10mM Tris: 1mM EDTA (pH 7.4), phenol extracted and ethanol precipitated.

Transfection of HeLa cells for CAT assay

100mm dishes of 80% confluent HeLa cells were used for the CAT assay. DNA was transfected into the cells using the calcium phosphate-DNA coprecipitation method as developed by van der Eb and Graham (1980). Sonicated salmon sperm DNA was added to HBS buffer to a final concentration of 40ug/ml. 500ul was aliquoted into separate tubes for each dish. The appropriate amounts of pKCAT 23 and pLE2 or mutant plasmid were then added to each tube. 50ul of 2.5M $CaCl_2$ was added and a precipitate usually formed immediately. After 20 min. the precipitate was generaly flocular and was added dropwise to the dishes.

5 to 6 hours after the transfection the cells were osmotically shocked with glycerol as suggested by Weeks and Jones (1984). The medium in the dishes was replaced with 2ml 20% glycerol in HBS. Within 1 minute the glycerol was aspirated off,

and the cells rinsed with HBS. Fresh medium was added and the cells were incubated for 38 to 45 hours after the transfection.

CAT assay

The CAT assay was performed according to the method of Gorman et al (1982).

The transfected dishes of HeLa cells were rinsed with PBS and 1ml of 40mM Tris (pH 7.4): 1mM EDTA: 15mM NaCl was added. The dishes were left at room temperature for 5 min. before scraping the cells off. The cells were transferred to a 15ml falcon tube and sedimented gently at 2,000rpm for 5 min. They were resuspended in 100ul of 0.25M Tris (pH 7.8) and were sonicated at 0-4°C. Usually 20 1s bursts of 50W with a needle probe were required to break open the cells. The cells were checked under the microscope to confirm that >80% of the cells were broken. The debris was sedimented at 3,000rpm for 5 min.

The entire supernatant was assayed immediately for CAT activity as it was found to drop with freezing. To the supernatant from each dish, Acetyl CoA was added to a final concentration of 1mM. 0.2uCi of ^{14}C chloramphenicol was also added. The reaction was allowed to proceed for 1 hr. at 37°C. 800ul of ethyl acetate was added to stop the reaction and extract the chloramphenicol. The solution was vortexed for 30s and spun in an Eppendorf microfuge for 1 min. The ethyl acetate was

extracted and dried in a Speed Vac. The choramphenicol was resuspended in 20ul of ethyl acetate and spotted onto silica gel thin layer chromatograph plates. The ascending buffer used for the chromatography was chloroform :methanol (95:5). After 45 min. the plates were removed, dried, and exposed to XAR-2 Kodak film for 18 to 22 hrs. The radioactive spots were scrapped from the chromatograph plates, suspended in omniflour and counted in a scintillation counter.

Preparation of primary rat kidney cells

Primary baby rat kidney cells were prepared as suggested by van der Eb and Graham (1980). Kidneys were dissected from rats between 1 and 6 days old and cleaned of enveloping membranes and blood vessels. The kidneys were cut into small pieces with scissors. The cells were then digested in a solution of 0.1% trypsin in PBS for 15 min. at room temperature with agitation. The supernatant was added to 10ml cold FCS, while 30ml more trypsin was added to the remaining debris for further digestion. This was repeated once or twice more to free as many cells as possible.

The cells were sedimented at 3,000rpm for 5 min. and resuspended in 100ml Alpha-modified MEM supplemented with 10% FCS. After incubation for 15 min. at 37°C, the cells were filtered through cheesecloth and more medium was added to bring the volume

up to the required level. In general, this worked out to 50ml for every pair of 6 day old kidneys. 5ml of the cell suspension was aliquoted into each 60mm dish. The cells were allowed to settle overnight and the following day the dishes were rinsed once with PBS and fresh Alpha-modified MEM supplemented with 10% FCS was added.

BRK transformations

The assay for transformation was performed as outlined by Ruley (1983). The dishes of BRK cells were usually 60-80% confluent 2 days after plating. The transfection was carried out as described by Wigler et al (1979). In the first step a fresh sterile solution of 1mM Tris :0.1mM EDTA (pH 7.9) was made. To this was added $CaCl_2$ from a 2.5M stock to a final concentration of 250mM, and carrier DNA. The amount of carrier DNA used, either rat kidney or Hela cell, was based on the quality of precipitate produced, and therefore varied from assay to assay. Tests were done with increasing amounts until a fine but distinct precipitate was seen.

1.25ml of the Tris-$CaCl_2$-carrier DNA solution was aliquoted into separate tubes for each group of five replicate dishes. At this point the pEJras and plasmid DNAs were added, either 625ng, 1250ng, or 2500ng of each. In the final step each of the solutions was added gradually to a 1.25ml solution of 2X

concentrated HBS while air was bubbling through the latter. The precipitate formed within a few seconds but was allowed to sit for a further 20 min. 0.5ml was then added dropwise to each of the dishes.

16 to 20 hours after the transfection, fresh Alpha-modified MEM with 10% FCS was added. 3 days later the medium was replaced with Eagle's MEM, 5% FCS and 0.12ug/ml Fungizone (Gibco), and the medium was changed every 3 to 4 days thereafter. Whenever fungus appeared that dish was immediately removed and fixed. Foci were counted every two days after their initial appearance until 36 to 41 days post-transfection when the experiment was finished.

Fixing and staining of cells

Cells were fixed by removing the medium and rinsing the cells with PBS. Carnoy's: PBS (1:1) was added and left on the cells for 10 min. Then straight Carnoy's was added and the dishes left for 30 min. The dishes were then dried and stained with Giemsa according to the manufacturer's specifications.

Establishment of cell lines

Cell lines were grown from individual foci using the method of van der Eb and Graham (1980). Foci were picked early in

the transformation assay while still small, and from separate dishes. Small glass cylinders were stuck to the dish with silicon grease to isolate a specific focus. The focus was then treated with 0.05% trypsin and lifted from the dish. The cells were transferred to a 30mm dish with 2ml medium, and then to larger dishes as necessary.

Growth on soft agar

This assay was based on the protocol used by Macpherson and Montagnier (1964). 3.5ml of 1% bactoagar was combined with an equal volume of 2X concentrated Eagle's MEM supplemented with 10% FCS and plated onto a 60mm dish as a base layer. Between 1,000 to 5,000 cells were used to seed the dishes and their volume was brought up to 1ml with the same medium. The cells were added to 1ml of 0.5% bactoagar and pipetted onto the base layer. The same number of cells were also added to 60mm dishes with 1X Eagle's MEM and 5% FCS. The cultures were incubated for a week.

Rescue of deletion mutants into virus

The deletion mutants were rescued into virus using the technique developed by Stow (1981). Purified d1309 DNA was cut with Cla I and Xba I. The RF M13 or plasmid DNAs were also cut with Xba I. The DNAs were then combined, and immediately phenol

extracted and ethanol precipitated. This cut DNA was resuspended and ligated with T_4 DNA ligase overnight at 14°C.

2pmoles of viral DNA and a 20 pmoles of plasmid or RF M13 DNA was used for every dish to be transfected. The DNA was transfected into 293 cells which had been grown to confluency on 60mm dishes. The cells in the dishes were grown in Alpha-MEM rather than Joklik-modified MEM as the quality of precipitate was better in the former medium. The DNA was transfected as previously described for the CAT assay transfection. The cells were also osmotically shocked with glycerol as with the CAT assay. After the glycerol treatment the cells were given an overlayer consisting of supplemented 2X concentrated Eagle's MEM mixed 1:1 with sterile 1.6% agarose. Plaques appeared within one week when the rescue was successful.

Screening rescued virus for mutations

Plaques were picked by piercing with a pasteur pipette through the overlayer. The agarose plug was added to 1ml PBS solution with 20% glycerol. 200ul was then used to infect a 60mm dish of 293 cells. The medium was aspirated and the virus adsorbed for 1 hour at 37°C. Fresh medium was added and the cells were left until 80-90% of the dish was showing cytopathic effect. This usually took between 36 and 48 hours.

The DNA was extracted using a modified Hirt extraction (Hirt, 1967). The medium was removed and 500ul of lysing buffer with 800ug/ml of pronase was added to the cells. After 2 hours digestion at 37°C the pronased cells were scraped into eppendorf tubes, phenol extracted and ethanol precipitated. The DNA was resuspended in 20ul of water and half was digested with Sma I and Xba I. The digests were analyzed on 6% polyacrylamide gels.

Viral clones that did have deletions were plaque purified to ensure that the virus stock contained only the mutant virus. The virus was serially diluted with PBS up to 10^6fold. These serial dilutions were used to infect as before, except that an agarose overlayer (see virus rescue) was added. After incubation for 1 week plaques were visible. These were picked and screened as described. The new clones were plaque purified once more before viral stocks were grown.

Host range assays of virus

Virus stocks were grown by infecting 8 or 9 dishes of 293 cells with 500ul each of the supernatant saved from the second plaque purification. Within 24 hours 80-100% of the dishes were showing cytopathic effect and were harvested. The cells were centrifuged gently and then resuspended in about 4ml of PBS with 20% glycerol. The virus was released from the cells by rapid freeze-thawing four times.

These virus stocks were titred on both 293 and HeLa cells. Cells were seeded onto 60mm dishes and grown to confluency. The virus was serially diluted with Eagle's MEM, mixing well at each step. The medium was aspirated from the dishes and 200ul from each dilution was added to the separate dishes. The virus was adsorbed for 60 min. before an agarose overlayer was added. The dishes were then incubated for at least 7 days. To see the plaques more clearly on the HeLa dishes, neutral red dye was added to a concentration of 1.5% in fresh overlay solution and 5ml was added to each dish. The number of plaques was generally counted between 6 and 8 days after the transfection. Titres were repeated at least 3 times on each cell type.

SOLUTIONS

Unless listed here, all solutions were made as specified in Maniatis et al (1982).

Annealing buffer (10X)
0.2M Tris (pH 7.5)
0.1M $MgCl_2$
0.5M NaCl
0.01M DTT

Extension/ligation reaction mix
20mM Tris (pH 7.5)
10mM $MgCl_2$
10mM DTT
1mM ATP
1mM dGTP, dATP, dTTP, dCTP
3 units T_4 DNA ligase
3 units E. coli pol 1 (large fragment)

Competence A
10mM NaCl
50mM $MnCl_2$
10mM CH_3COONa (pH 5.6)

Competence B
75mM $CaCl_2$
100mM $MnCl_2$
10mM CH_3COONa (pH 5.6)

Prehybidization buffer
3ml 20X SSC
2ml 50X Denhardts
0.1ml 20% w/v SDS
4.9ml H_2O

Hybridization buffer
3ml 20X SSC
2ml 50X Denhardts
5ml H_2O

PL-1 sequencing buffer
100mM Tris (pH 7.5)
100mM $MgCl_2$
50mM DTT

Sequencing gel
6% acrylamide
42% urea
1X TBE

T:E (5:1)
50mM Tris (8.0)
10mM EDTA

Sucrose buffer
25% w/v sucrose in T:E (5:1)

Buffered triton
2% v/v triton X-100 in T:E (5:1)

Low Tris buffer
10mM Tris (pH 7.5)
10mM NaCl
0.5mM EDTA

HBS (pH 7.1)
2.0g NaCl
0.095g KCl
0.025g Na_2HPO_4
1.25g HEPES
to 250ml with water

Carnoy's fixative
3 volumes ethanol
1 volume acetic acid

Supplemented 2X concentrated Eagle's MEM
4ml 5% yeast extract
10ml horse serum
2ml Fungizone (Gibco)
2ml Pen-strep (Gibco)
78ml 2X concentrated Eagle's MEM
(4ml 1M MgCl for HeLa cell overlay)

Lysing buffer
10mM Tris (pH 7.4)
10mM EDTA
0.4% w/v SDS

Pronase
The pronase was dissolved in 10mM Tris (pH 7.5) to a final concentration of 5mg/ml. It was incubated for 15 min. at 56°C and then for 60 min. at 37°C.

RESULTS

Production of Mutants

The unique region of the 13S mRNA contains 138 nucleotides. In order to examine the entire region, and possibly identify functional domains within it, a group of four in-frame deletions were planned which together would encompass the entire sequence (figure 3)

One of these deletions, d1[illegible]0, was made previously. It extends from nucleotides 976 to [illegible]038, and as it has deleted the 12S acceptor splice site, should only produce the 13S mRNA. Three more deletions were then planned to collectively delete the remaining sequences. The first, d1[illegible]2, removes bases 1040 to [illegible]063 inclusive, and thus deletes the amino acids [illegible]61 to [illegible]68. The second, d1[illegible]3, extends from nucleotide 1064 to [illegible]090 inclusive and deletes the next nine amino acids. The third, d1[illegible]4, removes bases 109[illegible] to [illegible] and the last seven amino acids of the unique region.

The third deletion, d1[illegible]4, alters the second base before the 13S acceptor splice site from a cytosine to a guanine. A study comparing splice site consensus sequences found that guanine was as frequent at this site as cytosine (Mount, [illegible]982). For this reason, d1[illegible]4 was not expected to unduly affect splicing.

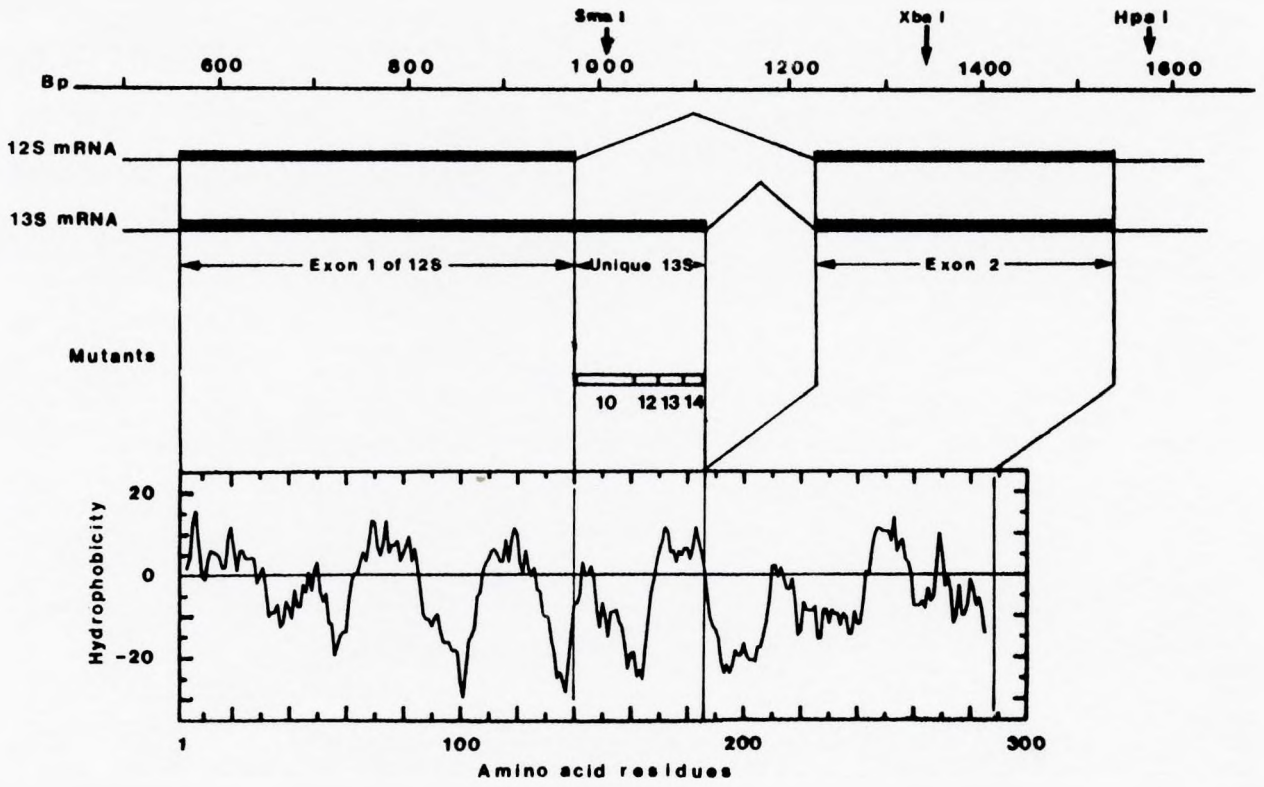
Based on hydropathy curves (figure 3), the unique region

Figure 3:

Location of deletions

This map shows the two early E1A mRNAs and the relative positions of the deletions within the unique region of the larger mRNA. The hydropathy curve shown below indicates the relative hydrophobicity of the deleted amino acid sequences. Amino acid sequences below the x-axis are hydrophillic while those above are hydrophobic. (Adapted from Jelsma et al, 1988).

Ad5 E1A


Sma I
Xba I
Hpa I
Bp
600
800
1000
1200
1400
1600
12S mRNA
13S mRNA
Exon 1 of 12S
Unique 13S
Exon 2
Mutants
10
12
13
14
Hydrophobicity
20
0
-20
1
100
200
300
Amino acid residues

can be subdivided into two sections of roughly equal length. The amino terminal half is primarily hydrophilic and is almost completely deleted by dl1110; The remaining portion is deleted in dl1112. The carboxy half of the unique region is hydrophobic. The two mutants dl1113 and dl1114, together delete this hydrophobic sequence.

The three deletion mutants were constructed using oligonucleotide directed mutagenesis, a technique developed by Zoller and Smith (1982, 1984). An M13 mp11 vector containing the left hand Ad5 sequence up to the Xba I site was used as a template since it included the 13S unique region (see figure 3 for Ad sequences). Twenty-three base oligonucleotides were used as primers for the mutagenesis (Fig. 4). These oligomers were constructed to correspond with the appropriate Adenovirus sequences. Eleven bases were matched with one side of the intended deletion and twelve with the other side so that the oligonucleotide would hybridize stably to the template.

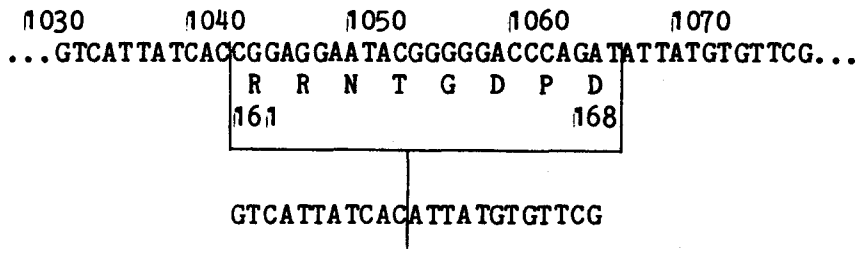
To check that the mutagenic oligomers would hybridize specifically to the intended sites, they were used as primers in Sanger dideoxy nucleotide sequencing reactions with the template already described (Zoller and Smith, 1982; 1984). By reading the sequences produced, it was possible to determine if the oligomers had hybridized to the correct site and only to the correct site. The oligomer for dl1113 was the only one that gave an ambiguous

Figure 4:

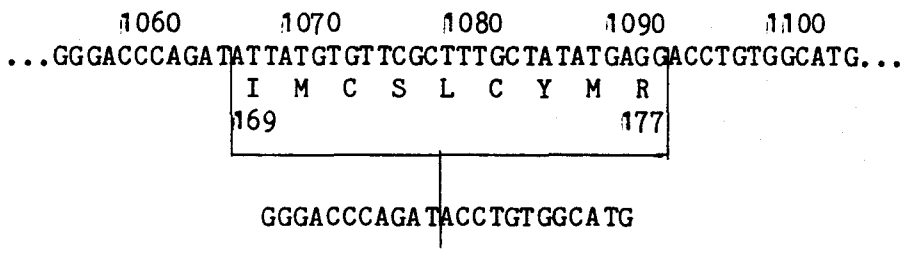
Sequence of the mutagenic oligonucleotides.

Twenty-three base oligonucleotides were used to make all three deletions. This figure illustrates the oligonucleotides used, and the exact bases and amino acids deleted from the Ad5 sequence.

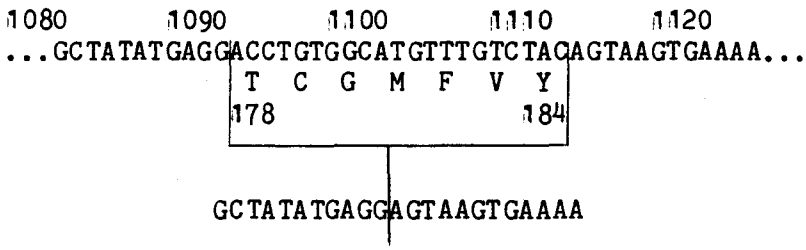
d11112



d11113



d11114



sequence, each band in the sequence appearing to be a doublet. It was suspected that this was due to the presence of incomplete oligomers. For every size band in the sequence produced with the full length primer, there would also be one a base shorter produced with the incomplete oligomers. Despite this, the oligomer was used successfully for mutagenesis.

For mutagenesis (Fig. 5), the mutagenic primer was annealed along with a second primer to the M13 template. The second primer was the original M13 phage cut at the BamH I and Xba I sites. The heteroduplex thus produced only needed its gaps filled in using DNA polymerase I (large fragment) and T4 DNA ligase. This construct had one strand that was wild type and one that contained the deletion. When the M13 was transfected into Escherichia coli both strands replicated to produce clones that contained either the wild type or the deletion mutant sequence.

Screening Mutants

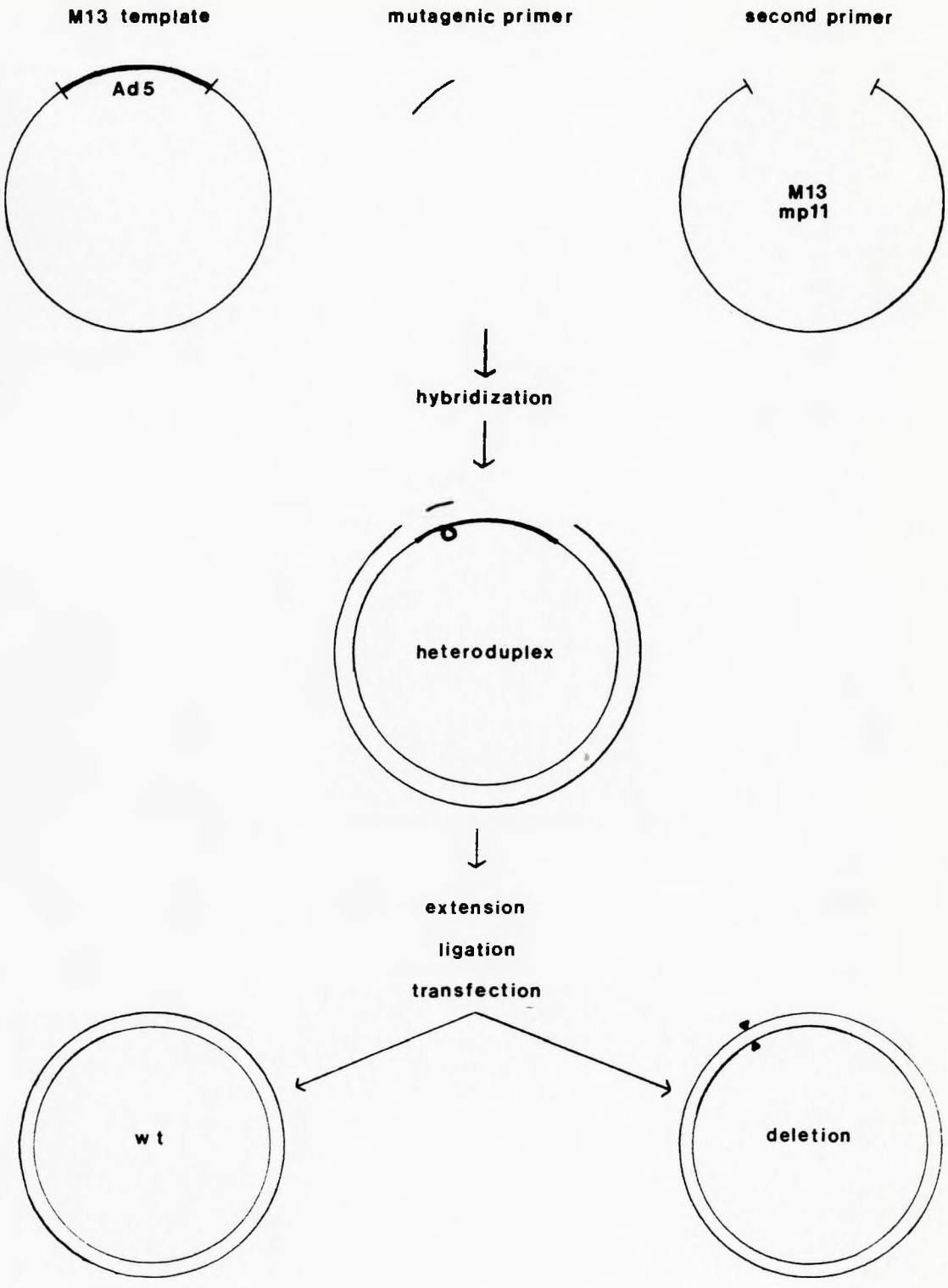
Since the clear plaques could contain clones with either the wild type sequence or the deletion mutant sequences it was necessary to screen them.

The dot blot hybridization technique (Zoller and Smith, 1982; 1984) was used. Single stranded phage were precipitated from cultures grown from distinct plaques and blotted onto nitrocellulose. The original M13 template was also blotted to

Figure 5:

Mutagenesis reaction.

In mutagenesis, the M13 template, mutagenic primer, and second primer were annealed together to produce a gapped heteroduplex. The only mismatch was at the site of the deletion where a loop was created in the M13 template. The gaps in the heteroduplex were filled in, and the constucted M13 phage was transfected into E. coli. The plaques produced contained phage which were either wild type or deletion mutant.


M13 template
mutagenic primer
second primer
Ad5
M13
mp11
hybridization
heteroduplex
extension
ligation
transfection
wt
deletion

provide a control for non-specific hybridization. The mutagenic oligomer endlabelled with ^{32}P was then hybridized to the phage on the filter.

After a room temperature wash, an autoradiograph showed that the probe had hybridized non-specifically to all the clones (Fig. 6). Successively hotter washes were used until the probe was removed from the original M13 template and any clones that had the wild type E1A sequences. The final wash was done at T_m, to ensure that the probe remained bound only to those clones containing the deletion. To confirm that these clones had the intended deletions they were then sequenced by the Sanger dideoxy nucleotide technique (Fig. 7). The entire Ad5 fragment in M13 was sequenced, using specifically synthesized oligonucleotide primers, to ensure that no other alterations had been created.

Rescue of Mutated Ad5 E1A Sequences into Plasmid

Deletions were rescued into the pLE2 plasmid, which contains a wild type copy of the entire E1A gene (Fig. 8). The plasmid was digested with the restriction enzymes Sma I, Xba I and Bam HI. The XbaI and BamHI cut out the wild type Ad sequence, while the SmaI cut into the sequence to reduce the frequency of religation. Each M13 mutant was also digested with Xba I and Bam HI, and then ligated with the plasmid. The recombinant was transfected into E. coli.

Figure 6:

Selection of mutant phage by filter hybridization.

All three mutants were screened using filter hybridization. The mutant dl1113 is shown here as an example. After mutagenesis, phage from distinct plaques were blotted onto nitrocellulose. They were probed with the mutagenic oligomer endlabelled with ^{32}P. The C indicates the original phage used as a control for non-specific binding of the oligomer. A shows an autoradiograph of the dot blot after a room temperature wash. At this temperature the oligomer is bound to all the clones. B is an autoradiograph of the same blot after a 74°C wash. At this temperature the probe has been removed from the control and the unmutated clones. The probe remains bound to eight clones.

A

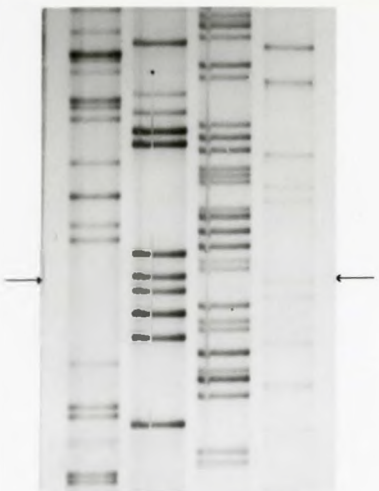
B

Figure 7:

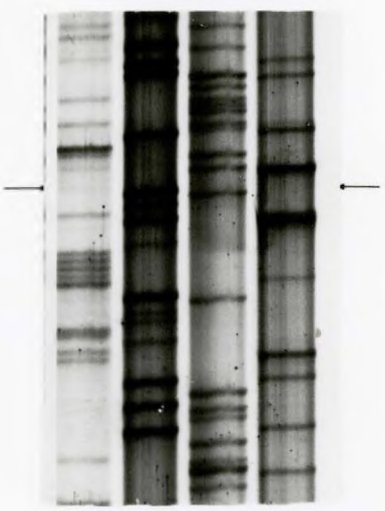
Sequences of deletion mutants.

The three deletion mutants were sequenced in the M13 phage using Sanger dideoxy technique. The arrows indicate the boundaries of each deletion.

dl 1112



dl 1113



dl 1114

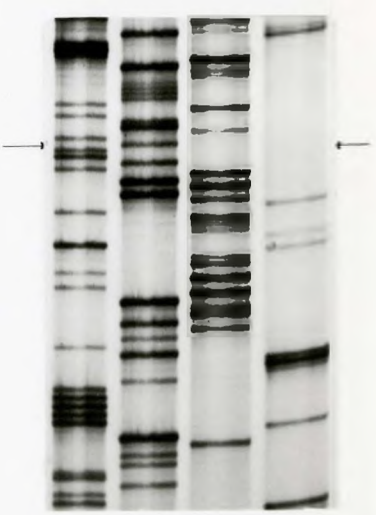
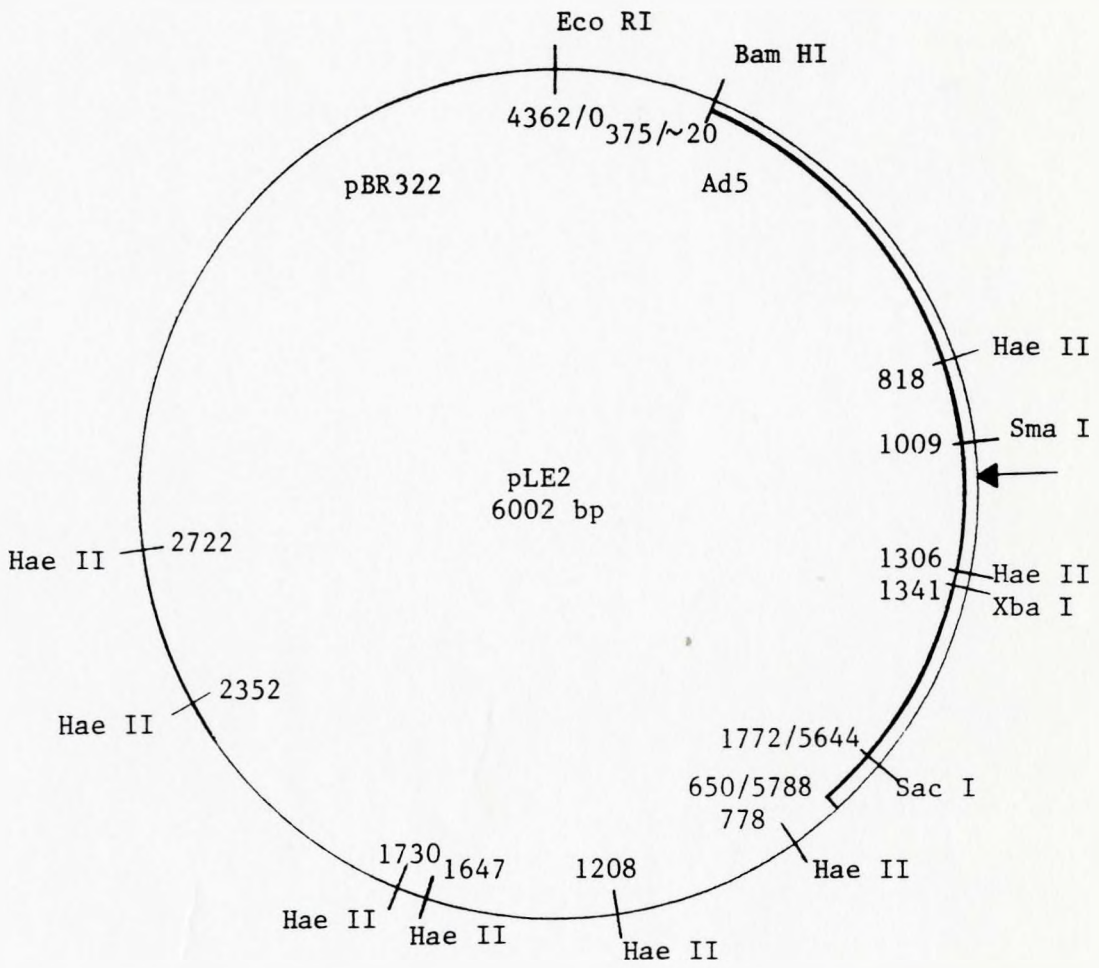


Figure 8:

Restriction map of the plasmid pLE2.

Relevant restriction enzyme sites are shown on this map of pLE2. The double line represents the inserted Ad5 E1A sequences. Refer to text in Materials and Methods for construction of plasmid. The approximate location of the deletions is given by the arrow.


Eco RI
Bam HI
4362/0
375/~20
pBR322
Ad5
Hae II
818
Sma I
1009
pLE2
6002 bp
1306
Hae II
1341
Xba I
Hae II
2722
2352
Hae II
1772/5644
650/5788
Sac I
778
Hae II
1730
1647
1208
Hae II
Hae II
Hae II

Clones were screened by digesting with Sma I and Xba I and analysing on a 6% polyacrylamide gel. The two enzymes cut into the Ad5 sequences to produce a fragment of 332 nucleotides with wild type, and a recognisably smaller one with the deletion mutants (Fig. 9). That is, the fragments were 24, 27, and 21 nucleotides shorter with dl1112, dl1113 and dl1114 respectively.

Transactivation

One of the specific functions of the 289R protein is to transcriptionally activate the other early Ad genes. The three deletions were tested for their effect on this ability using the CAT assay (Weeks and Jones, 1983). A recombinant pBR322 plasmid, pKCAT23, which contains the chloramphenicol acetyl transferase gene (CAT) linked to the E3 promoter of Adenovirus type 5 was used. The plasmid was transfected into HeLa cells using the calcium-phosphate co-precipitation technique (Weeks and Jones, 1983). After thirty-six to forty-five hours, the cells were harvested and assayed for CAT enzyme activity.

The CAT enzyme acetylates ^{14}C-chloramphenicol which can be separated from its unacetylated form by thin layer chromatography. By autoradiography, the radioactive spots can be identified, scraped and counted. From the counts the approximate percent acetylation is calculated and therefore the relative levels of CAT activity can be determined.

Figure 9:

Restriction digest patterns of mutants rescued into the pLE2 plasmid.

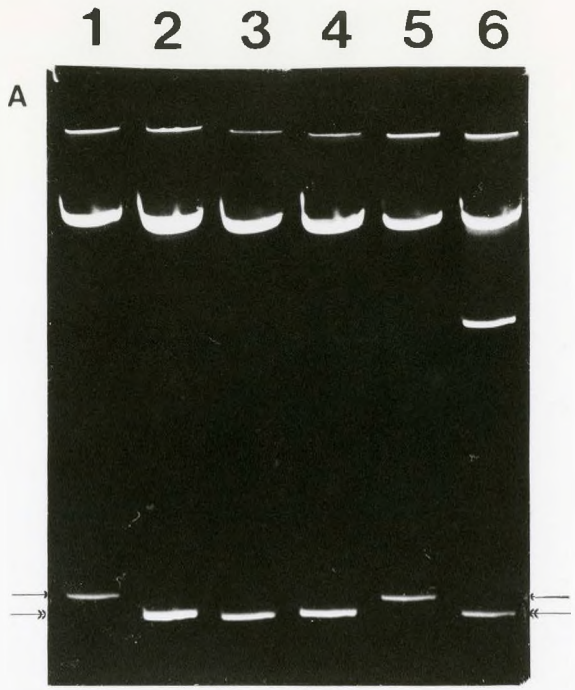
To screen for deletion mutants, plasmid DNA was cut with restriction enzymes and analysed on 6% polyacrylamide gels.

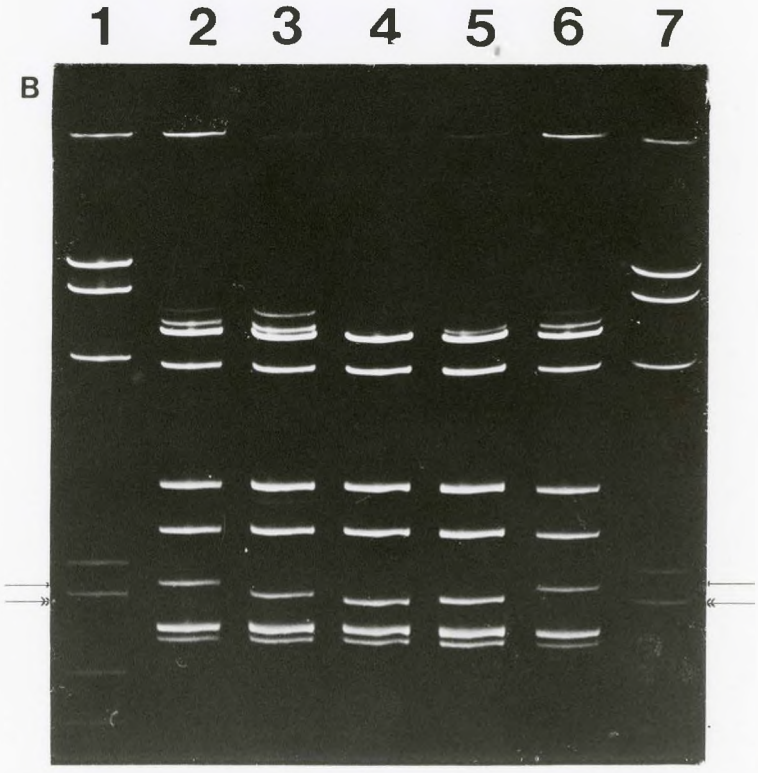
A. The enzymes, Xba I and Sma I, were used to create a fragment 332 nucleotides long in the wild type pLE2 (single arrow). This fragment is identifiably smaller in the three mutants (double arrow).

Lane: 1. pLE2
2. pdlΔΔΔ4
3. pdlΔΔΔ3
4. pdlΔΔ2
5. pLE2
6. M13 dlΔΔΔ3

B. The enzyme Hae II was used since it gave a number of fragments. The only one which was altered is 488 nucleotides long in wild type (single arrow). The double arrow indicates the deletion length fragments.

Lane: 1. M13 dlΔΔΔ4
2. pLE2
3. pdlΔΔΔ4
4. pdlΔΔΔ3
5. pdlΔΔ2
6. pLE2
7. M13 dlΔΔΔ4


1 2 3 4 5 6
A


1 2 3 4 5 6 7
B

Since the CAT enzyme is not normally present in HeLa cells the pKCAT23 plasmid is the only source. In the assays, one 100mm dish was always transfected with the pKCAT23 plasmid alone. The level of acetylation from this dish was consistently low (less than 1%). When a functional E1A plasmid was cotransfected with the pKCAT23 plasmid, the E1A proteins were able to transactivate the E3 promoter, and a significant increase in CAT activity of at least twenty-fold was seen.

In their assays, Weeks and Jones (1983) used 20 ug of pKCAT23 and 40 ug of pLE2 per transfection, and then only a portion of each cell extract. In the present CAT assays, sufficient levels of acetylation were achieved with smaller amounts of plasmid by using the entire cell extract (see figure 10 and table 1). Autoradiographs of two sample CAT assays are shown in Figures 10 and 11, and their quantitative results given in Tables 1 and 2. These CAT assays had been repeated at least three times for each mutant. In these repeats between 1 and 20ug of plasmid DNA were used. In each case, none of the deletion mutants had been able to stimulate transcription of the CAT gene. The levels of acetylation were equivalent to the dishes transfected with the pKCAT23 plasmid alone. All three deletions had successfully destroyed the transactivation function.

Figure 10:

Autoradiograph of CAT assay with plasmids pLE2 and pdl1113 at two concentrations.

The amounts and type of plasmid transfected into each dish were:

Lane 1. 5ug pKCAT 23

2. " , 1ug pdl1113

3. " , 10ug pdl1113

4. " , 1ug pLE2

5. " , 10ug pLE2

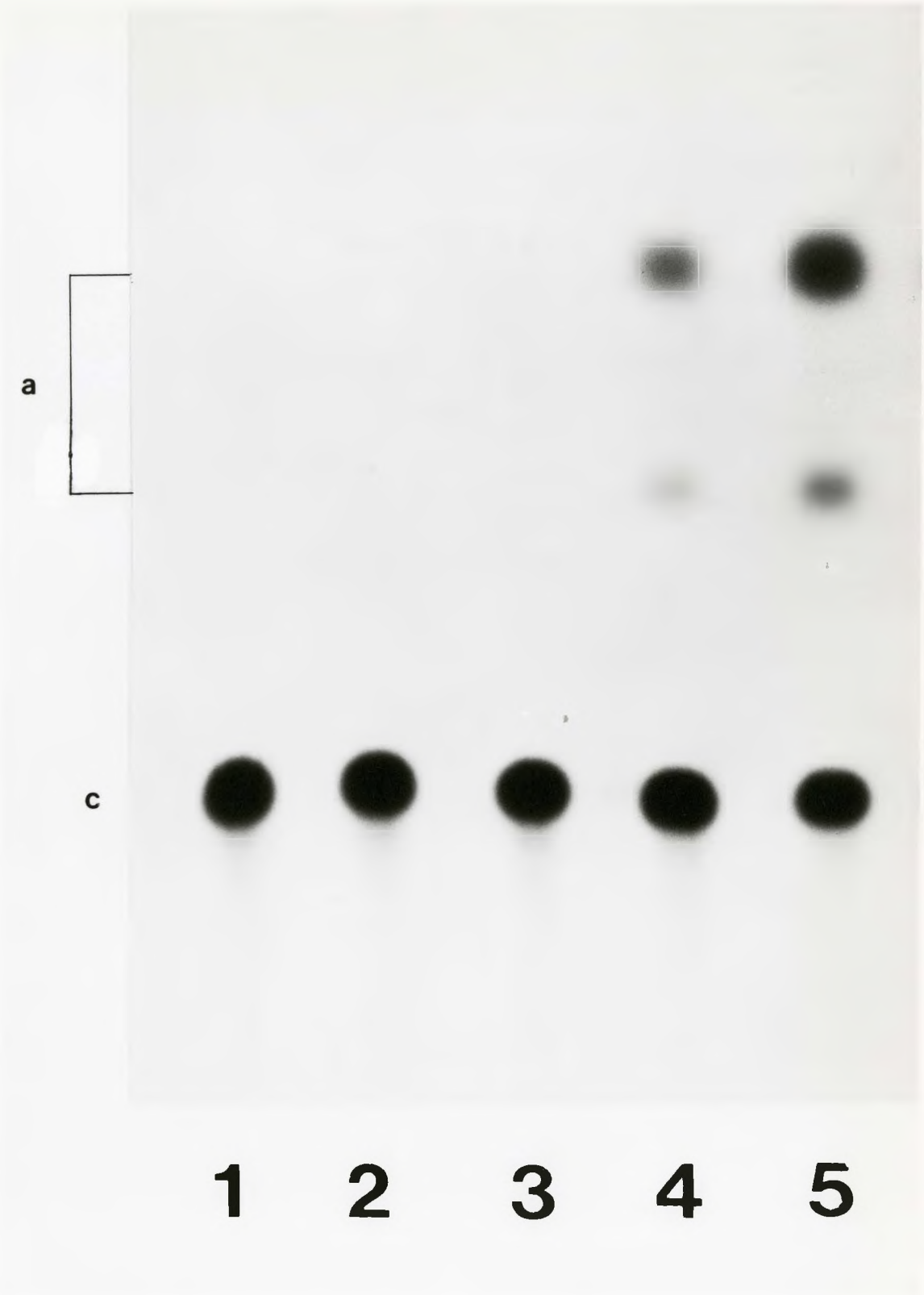

a
c
1
2
3
4
5

Table 1 Quantitative results from Figure 10

Lane	Plasmids Transfected	% Chloramphenicol Acetylated	Increase Over pKCAT Alone
1	pKCAT alone	0.2	---
	pKCAT + pd1111113		
2	(1 ug)	0.3	1.5 fold
3	(10 ug)	0.3	1.5 fold
	pKCAT + pLE2		
4	(1 ug)	11.7	58.5 fold
5	(10 ug)	35.7	178.5 fold

Quantitative results were obtained by scraping the spots from the thin layer chromatography plates corresponding to the unacetylated and acetylated chloramphenicol and counting the radioactivity . From the counts, the percentage of acetylated chloramphenicol and the relative increases in CAT activity were determined.

Figure 11:

Autoradiograph of CAT assay with plasmids pLE2, pdl1112, pdl1113 and pdl1114.

Each lane of this and the following autoradiographs represents the acetylation activity of the cell extract from a single dish of HeLa cells. The c indicates the unacetylated chloramphenicol, while the a indicates the two acetylated forms. The plasmids and amounts transfected were:

Lane: 1. 20ug pKCAT 23

2. " , 20ug pLE2

3. " "

4. " , 20ug pdl1112

5. " , 20ug pdl1113

6. " , 20ug pdl1114

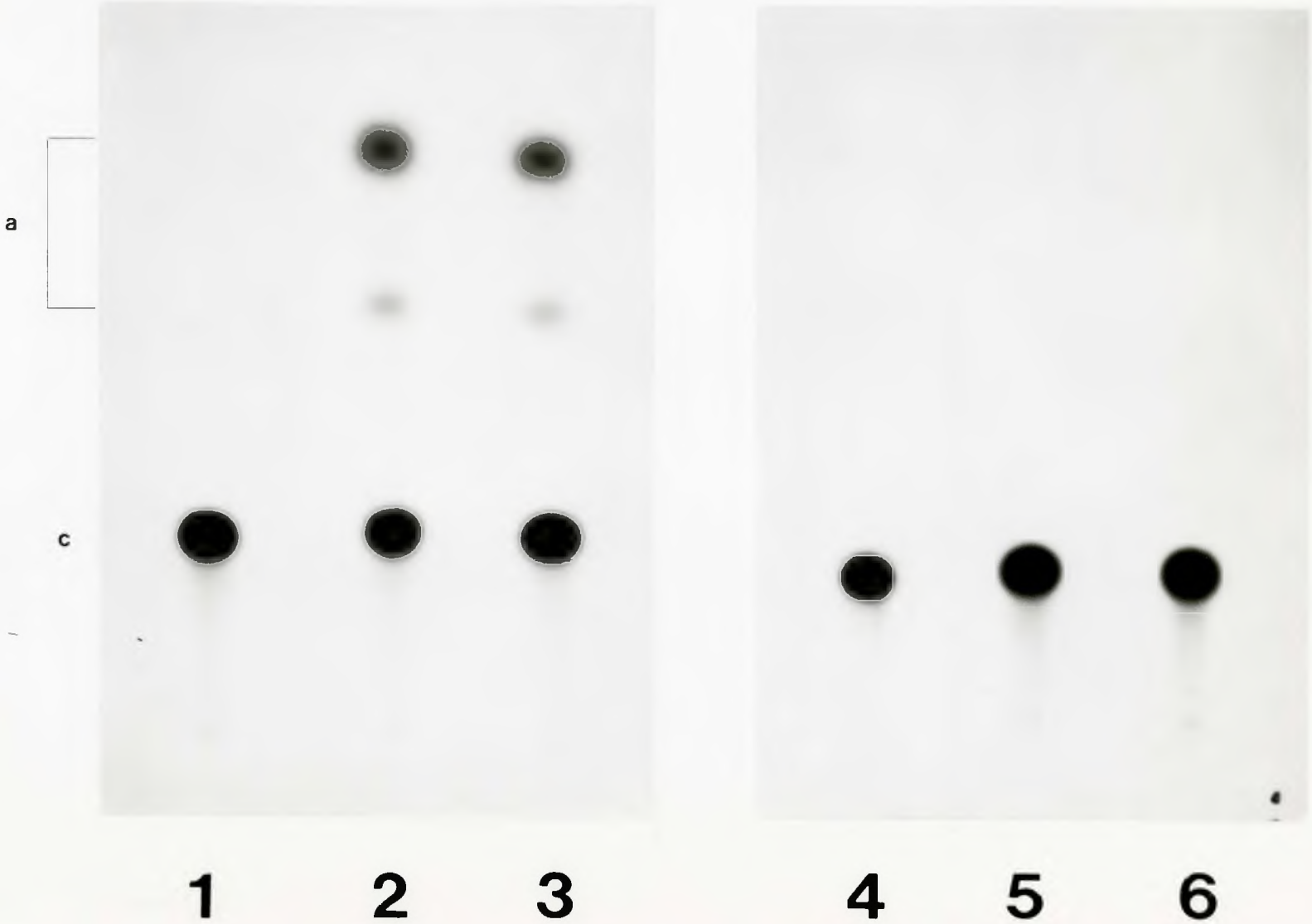

a
c
1
2
3
4
5
6

Table 2 Quantitative results from Figure 1

Lane	Plasmids Transfected	% Chloramphenicol Acetylated	Increase Over pKCAT Alone
1	pKCAT alone	0.4	---
2	pKCAT + pLE2	20.0	50 fold
3	pKCAT + pLE2	12.2	30 fold
4	pKCAT + pd1111112	0.3	none
5	pKCAT + pd1111113	0.2	none
6	pKCAT + pd1111114	0.4	none

Transactivation inhibition

Recent experiments by Glenn and Ricciardi (1987) suggested that not only could a mutant E1A have lost its transactivation ability, but it could also block the activity of wild type E1A. In order to determine whether the three deletion mutants could interfere with transactivation, the CAT assays were repeated as follows. Each dish of HeLa cells was cotransfected with 5ug of pKCAT 23, 1ug of pLE2, and either 0, 1.0, 2.5, or 10.0ug of one of the mutant plasmids.

It was expected that the dish transfected with only the pkCAT 23 and pLE2 plasmids would give the highest percent acetylation, and that as increasing amounts of the mutant E1A plasmid were added the level of acetylation would drop. In order to demonstrate this, the levels of acetylation were calculated as a percentage of the activity from the dish transfected with just the pkCAT 23 and pLE2 plasmids. The level of acetylation from the dish transfected with only pkCAT was subtracted from all the other acetylation levels to remove the background.

This assay was repeated three times with the mutants pd1111 2 and pd1111 4, and six times with pd1111 3. Figure 12 is given as an example autoradiograph of one assay. There was considerable variability in the levels of relative CAT activity between dishes within an experiment and between matched dishes in separate experiments. As a result no consistent patterns of

Figure 12:

Autoradiograph showing effect of adding increasing amounts of pdl1112 on wildtype pLE2 induced CAT activity.

The same techniques were used for this CAT assay as for the previous ones. All three deletion mutants were assayed a number of times. This autoradiograph is shown as an example. The plasmids and amounts transfected into each dish were:

Lane 1. 5ug pKCAT 23

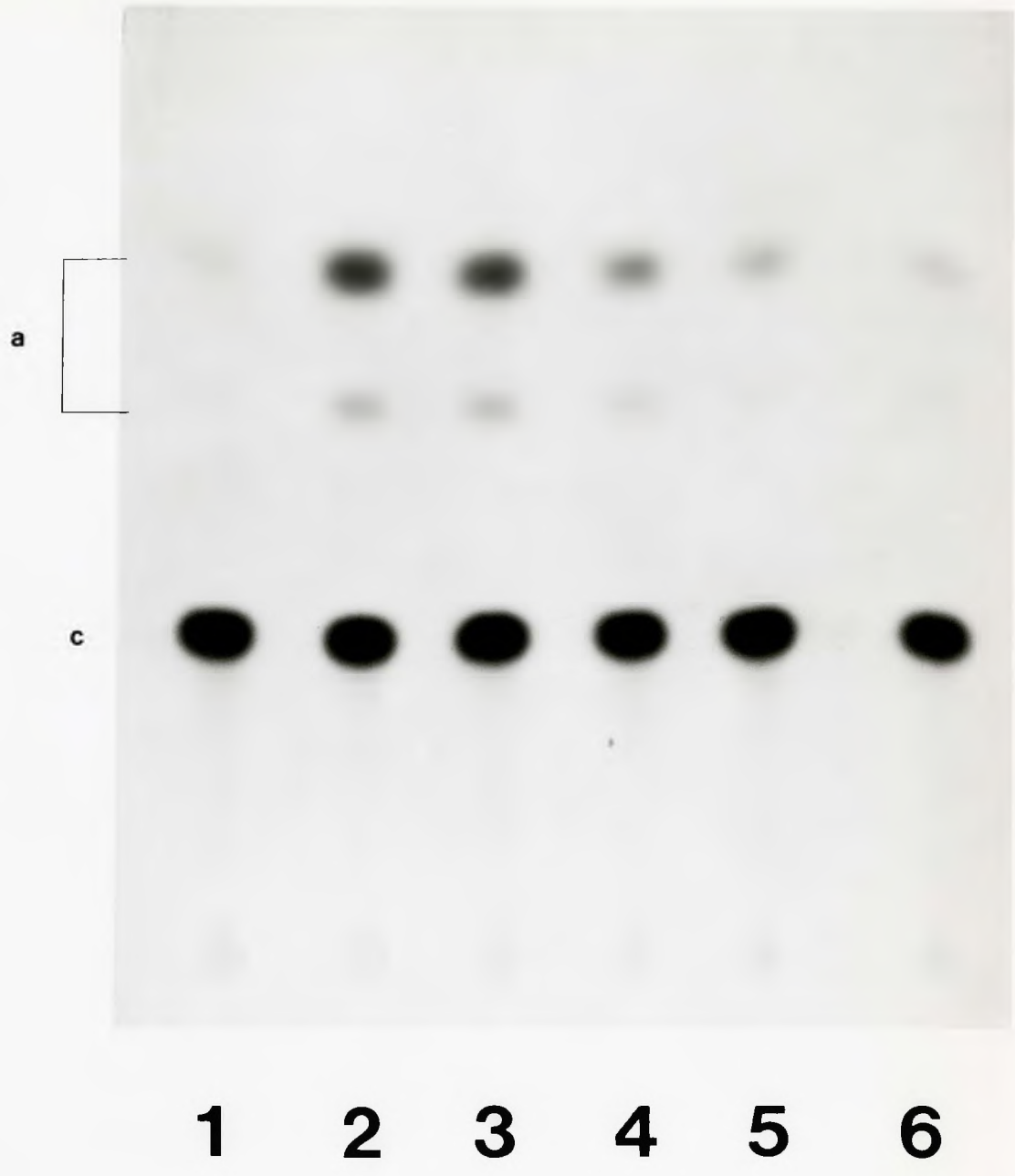
2. " 1ug pLE2

3. " " 1.0u pdl1112

4. " " 2.5ug pdl1112

5. " " 5.0ug pdl1112

6. " " 10.0ug pdl1112


a
c
1
2
3
4
5
6

decreasing CAT activity could be shown, although each mutant did have a definite effect.

At equimolar concentrations of mutant and wild type E1A, the mutant pd1∏∏∏2 (See Figure ∏2 and Table 3) showed the strongest effect of the three mutants, reducing CAT activity to between 43 and 78% of wild type levels. Then, as the amount of pd1∏∏∏2 increased to ten times that of the wild type, the activity continued to decrease to between ∏4 and 47% of the wild type levels.

The mutant pd1∏∏∏3 had the most inconsistent effect on CAT activity (see Table 4). At equimolar concentrations of the wild type and mutant plasmids the relative level of CAT activity decreased in only two of the experiments. In the other four, the levels of acetylation either remained the same or actually increased. In two experiments the level of acetylation more than doubled. On its own, pd1∏∏∏3 has no transactivation ability. When the amount of pd1∏∏∏3 plasmid was increased to ten times that of the wild type plasmid the level of acetylation did drop between 70 and 95%.

At equimolar concentrations of pd1∏∏∏4 and pLE2, the pd1∏∏∏4 had only a small effect on CAT activity (see Table 5). In two experiments the levels were essentially unchanged, while in another the activity dropped by 35%. As the concentration of pd1∏∏∏4 was increased the levels of acetylation decreased. When

Table 3 Quantitative results of assays showing effects of adding increasing amounts of pdl1012 on pLE2 induced CAT activity.

Exp. no.	% chloramphenicol acetylated					
	pkCAT alone	pkCAT + pLE2	Concentration of pdl1012 plasmid (ug) 1.0	2.5	5.0	10.0
1	5.4	53	26	---	---	28
2	0.1	1.8	1.1	0.9	0.6	0.5
3	0.3	7.3 8.5	6.2	1.9	1.9	1.4
	activity as % of wild type E1A plasmid alone					
1		100	43	---	---	47
2		100	56	44	28	22
3		100	78	21	21	14

Table 4 Quantitative results of assays showing effects of adding increasing amounts of pd1[illegible]3 on pLE2 induced CAT activity.

Exp. no.	% chloramphenicol acetylated					
	pkCAT alone	pkCAT + pLE2	Concentration of pd1[illegible]3 plasmid (ug)			
			[illegible].0	2.5	5.0	[illegible]0.0
[illegible]	0.7	[illegible]1	26	9.[illegible]	7.8	2.7
2	[illegible].2	[illegible]8	2.9	---	2.9	5.3
3	0.2	[illegible]2	26	4.7	[illegible]1	2.2
4	0.6	25	49 40	5.7 5.7	[illegible]8 [illegible]0	[illegible].8 [illegible].7
5	0.[illegible]	[illegible].8	[illegible].9	3.6	0.4	0.4
6	0.3	7.3 8.5	3.6	[illegible].1	2.9	2.6
	activity as % of wildtype E1A plasmid alone					
[illegible]		[illegible]00	240	8[illegible]	68	[illegible]9
2		[illegible]00	[illegible]0	----	[illegible]0	27
3		[illegible]00	220	39	90	[illegible]7
4		[illegible]00	[illegible]80	2[illegible]	54	4.7
5		[illegible]00	[illegible]06	206	28	[illegible]8
6		[illegible]00	43	[illegible]1	34	30

Table 5 Quantitative results of CAT assays showing the effect of increasing amounts of pdl1114 on pLE2 induced CAT activity.

Exp. no.	% chloramphenicol acetylated					
	pkCAT alone	pkCAT + pLE2	Concentration of pdl1114 plasmid (ug) 1.0	2.5	5.0	10.0
1	0.7	11	12	2.0	1.0	1.0
2	0.1	1.8	1.2	0.6	0.3	0.1
3	2.2	20	18	12	4.4	3.5
	activity as % of wild type E1A plasmid alone					
1		100	110	13	2.9	2.9
2		100	65	29	12	0
3		100	89	56	12	7.2

2.5ug of the plasmid was added the levels of acetylation ranged between 56 and 13% only. With 10ug of the plasmid,acetylation levels declined between 93 and 100%.

Transformation with Ad5 E1A and the Ha ras 1 oncogene

Since the 289R protein is required for oncogenic transformation, it was important to determine how the deletions affected E1A's ability to transform. Ruley (1983) developed a transformation assay involving the co-transfection of an E1A plasmid and the pEJras plasmid into primary baby rat kidney cells. The T24 Harvey ras 1 oncogene is unable to transform cells alone, but can complement E1A to produce full transformation at a high efficiency (Ruley, 1983; Franza et al, 1986).

Tables 6 - 9 summarize the results of four separate transformation experiments. In each of the experiments there were a number of control dishes as well as the dishes transfected with the pEJras and either the pLE2 or mutant plasmids. Generally two or three dishes were untransfected, while another four or five were transfected with carrier DNA alone, and ten to fifteen with varying concentrations of the pEJras plasmid alone. Of all the control dishes, only one of the dishes transfected with the pEJras plasmid developed foci (Table 9). This was approximately five weeks after the transfection.

Table 6:

Transformation of BRK cells by pLE2, pdl1112, and pdl1113 with pEJras.

Two day old BRK cells were prepared and plated onto 60mm dishes. They were transfected three days later when they were 75% confluent. Foci began appearing eleven days later, and rapidly spread throughout the dishes. As each dish filled with foci it was stained. After forty-nine days the remaining dishes were stained and final counts were made.

Table 6

Plasmid(s) Transfected	# dishes with foci			ave. # foci per dish with foci		
	Days Post-Transfection					
(ng)	11	16	49	11	16	49
Carrier	0/4	0/4	0/4	0	0	0
pEJras						
125	0/5	0/5	0/5	0	0	0
250	0/5	0/5	0/5	0	0	0
500	0/4	0/4	0/4	0	0	0
pEJras + pLE2						
125	0/5	0/5	0/5	0	0	0
250	1/5	1/5	2/5	1.0	9.0	*
500	1/5	2/5	3/5	3.0	4.0	*
pEJras + pdl1112						
125	0/5	1/5	4/5	0	1.0	*
250	2/5	4/5	5/5	1.0	4.5	*
500	3/5	5/5	5/5	1.3	5.4	*
pEJras + pdl1113						
125	0/5	1/5	3/5	0	1.0	*
250	1/5	4/5	5/5	1.0	1.5	*
500	3/5	4/5	5/5	1.0	1.8	*

* numerous

Table 7:

Transformation of BRK cells by pLE2, pdl1112 and pdl1114 with pEJras.

Six day old BRK cells were plated onto 60mm dishes and were 80% confluent when they were transfected with the plasmids two days later. As the foci spread over each dish it was stained. All the remaining dishes were stained by the forty-first day, and final counts were made.

Table 7

Plasmid(s) Transfected	# dishes with foci			ave. # foci per dish with foci		
	Days Post-Transfection					
(ng)	16	28	41	16	28	41
Carrier	0/5	0/5	0/5	0	0	0
pEJras						
125	0/5	0/5	0/5	0	0	0
250	0/5	0/5	0/5	0	0	0
500	0/5	0/5	0/5	0	0	0
pEJras + pLE2						
125	0/5	0/5	1/5	0	0	10.0
250	0/5	0/5	3/5	0	0	20.3
500	0/5	2/5	4/5	0	1.5	13.3
pEJras + pd11112						
125	5/5	5/5	5/5	2.6	*	*
250	4/4	4/4	4/4	7.5	*	*
500	3/5	5/5	5/5	2.0	*	*
pEJras + pd11114						
125	5/5	5/5	5/5	3.4	*	*
250	5/5	5/5	5/5	6.4	*	*
500	5/5	5/5	5/5	8.0	*	*

* numerous

Table 8:

Transformation of BRK cells by pLE2, pdl1113 and pdl1114 with pEJras.

Kidney cells were taken from one day old rats. These cells reached 60% confluency two days after plating and were transfected. Foci began appearing within two weeks and each dish was stained as it filled with foci. Final counts were made thirty-six days after transfection.

Table 8

Plasmid(s) Transfected (ng)	# dishes with foci			ave. # foci per dish with foci		
	Days Post-Transfection					
	14	24	36	14	24	36
Carrier	0/2	0/2	0/2	0	0	0
pEJras						
250	0/5	0/5	0/5	0	0	0
500	0/5	0/5	0/5	0	0	0
pEJras + pLE2						
125	0/5	0/5	1/5	0	0	2.0
250	1/5	1/5	1/4	1.0	2.0	*
500	0/4	2/4	3/4	0	7.5	*
pEJras + pdl1113						
125	1/5	3/5	3/5	2.0	*	*
250	2/5	3/5	3/5	1.0	*	*
500	3/5	4/5	4/5	1.0	*	*
pEJras + pdl1114						
125	3/5	3/5	5/5	3.3	*	*
250	3/5	3/5	3/5	1.0	*	*
500	4/5	4/5	4/5	1.3	*	*

* numerous

Table 9:

Transformation of BRK cells by pLE2, pdl1111112, pdl1111113 and pdl1111114 with pEJras.

Kidneys were removed from five day old rats and the cells plated onto 60mm dishes. The dishes were transfected two days later when they had reached 60% confluency. Dishes were stained throughout the experiment as before and final counts were performed forty days after transfection.

Table 9

Plasmid(s) Transfected	# dishes with foci			ave. # foci per dish with foci		
	Days Post-Transfection					
(ng)	12	17	40	12	17	40
Carrier	0/5	0/5	0/5	0	0	0
pEJras						
250	0/5	0/5	1/5	0	0	4.0
500	0/5	0/5	0/5	0	0	0
pEJras + pLE2						
125	1/5	3/5	4/5	1.0	3.7	*
250	4/5	4/5	4/5	1.0	1.5	*
500	0/5	1/5	2/5	0	2.0	*
pEJras + pdl1112						
125	3/3	3/3	3/3	1.7	*	*
250	3/4	4/4	4/4	1.3	9.0	*
500	3/5	5/5	5/5	1.3	11.8	*
pEJras + pdl1113						
125	4/5	5/5	5/5	1.0	11.2	*
250	5/5	5/5	5/5	1.8	13.8	*
500	4/5	5/5	5/5	2.5	*	*
pEJras + pdl1114						
125	3/5	5/5	5/5	2.0	7.4	*
250	5/5	5/5	5/5	1.6	7.0	*
500	2/5	4/5	5/5	1.5	5.8	*

* numerous

Each of the mutants were assayed in three of the four experiments. Equal amounts of both the E1A and pEJras plasmids were co-transfected in increasing concentrations of 125, 250, and 500 ng per dish. Five 60 mm dishes were used for each of the three plasmid concentrations.

The numbers of foci are recorded at three time points. The first count indicates when foci first began to appear, the second when the numbers began to escalate, and the third was at the end of the experiment after the dishes had been stained. The transformation efficiency was calculated in two ways, namely from the number of dishes with foci, and from the average number of foci on those dishes with foci.

In Table 6, the deletion mutants d11112 and d11113 are compared to the wild type E1A. Foci were only just visible eleven days after transfection. They first appeared on the dishes transfected with both the mutant and wild type plasmids at the two higher concentrations. By the sixteenth day almost all of the dishes transfected with 250 ng and 500 ng of either the d11112 or d11113 plasmid had foci. Foci had also begun appearing on the dishes with the lowest concentration of mutant plasmid.

By the end of the experiment all of the dishes transfected with the mutants except for a few at the lowest concentration had foci. There was no real difference between the two mutants. On the other hand, by the end of the experiment, only half of the

dishes transfected with the higher concentrations of the pLE2 plasmid had foci, and those with the lowest never developed foci.

In terms of numbers of foci, each dish usually had between one and five foci at 14 days. As the foci grew larger, the cells at the centre tended to lift off and move around the dish so that it would become filled with foci. These large foci counts no longer accurately represented the transformation frequency and are symbolized simply with a star.

In Table 7, the transformation efficiency of the plasmid d1∆∆∆2 is compared to that of pd1∆∆∆4, and both are compared to pLE2. In this experiment there was a large gap between the appearance of foci on the wild type E1A and the mutant E1A transfected dishes.

With wild type, foci appeared twenty-eight days post-transfection only on the dishes with the highest plasmid concentration. Over the next two weeks, foci also began appearing on the other dishes, and by the end of the experiment even one of the dishes with the lowest plasmid concentration had foci. Foci appeared much earlier with pd1∆∆∆2 and pd1∆∆∆4. Both mutants behaved similarly, and almost all their dishes had foci by the sixteenth day. These foci had, in fact, already begun to lift and spread. By the twenty-eighth day the dishes were filled with foci. The wild type transfected dishes, on the other hand, had only just begun to spread in the last week of the experiment.

Table 8 summarized the results of an assay comparing the mutants plasmids pdlΠΠΠ3 and pdlΠΠΠ4, and wild type. Fourteen days after transfection only one focus had appeared on the pLE2 plasmid transfected dishes. At the same time six dishes of the pdlΠΠΠ3, and ten of the pdlΠΠΠ4 plasmid transfected dishes had foci.

Within ten days the number of pdlΠΠΠ3 plasmid transfected dishes with foci had increased to ten out of fifteen, and these foci had spread throughout their respective dishes. The number of pdlΠΠΠ4 transfected dishes with foci increased more gradually, and on the thirty-sixth day of the experiment four-fifths of the dishes were covered with foci. In comparison, the number of wild type pLE2 plasmid transfected dishes with foci only increased slightly, and by the end of the experiment only five of thirteen dishes had foci. The foci on these dishes had also grown to cover the entire dish except in one instance. Since the foci on the one dish only appeared a few days before the dishes were stained they did not have a chance to spread.

The last table (Table 9), shows all three mutants and wild type in one combined assay. It was in this assay that one dish transfected only with the pEJras plasmid developed foci in the last week of the experiment. In this assay the differences between wild type and the three mutants are still distinct although not as obvious as in the first two experiments. Foci

were visible on nearly all the mutant transfected dishes on the twelfth day of the experiment. Within five days the remaining dishes had foci, and the counts had begun to increase. Foci were also visible on the wild type transfected dishes by the twelfth day, but only on a third of the dishes. By the end of the experiment the number of dishes with foci had doubled and these foci had spread throughout each dish.

Between the three mutants there did not seem to be any significant differences in either first appearance of foci, numbers of dishes with foci, or numbers of foci. In general, the three mutants behaved similarly to each other and distinctly different from the wild type. Their foci appeared sooner than wild type. At every plasmid concentration and at every time point, more of the mutant transfected dishes had foci than the wild type transfected dishes. By the end of every experiment, most if not all of the mutant transfected dishes had foci. In comparison, only between one-third and two-thirds of the wild type transfected dishes had foci. The mutant foci grew faster and spread more rapidly than wild type. Within three weeks the mutant foci were overgrown, while wild type foci took closer to four weeks or even longer to overgrow their dishes.

Morphology of Transformed Cells

In the transformation assays, the foci produced by pLE2-wt, dl1012, dl1013, and dl1014 all appeared essentially the same. That is, the cells were generally epithelial rather than fibroblastic. The cells grew in multiple layers, and would often lift off in large clumps and move around the dish.

Foci were picked and grown up as cell lines. The foci were taken from separate dishes to ensure that the lines were from independently transformed cells. In this way, five distinct lines were derived from each mutant, and two from wild type. Cells from all the lines tended to lift off easily so that it was difficult to grow them to a reasonable cell density.

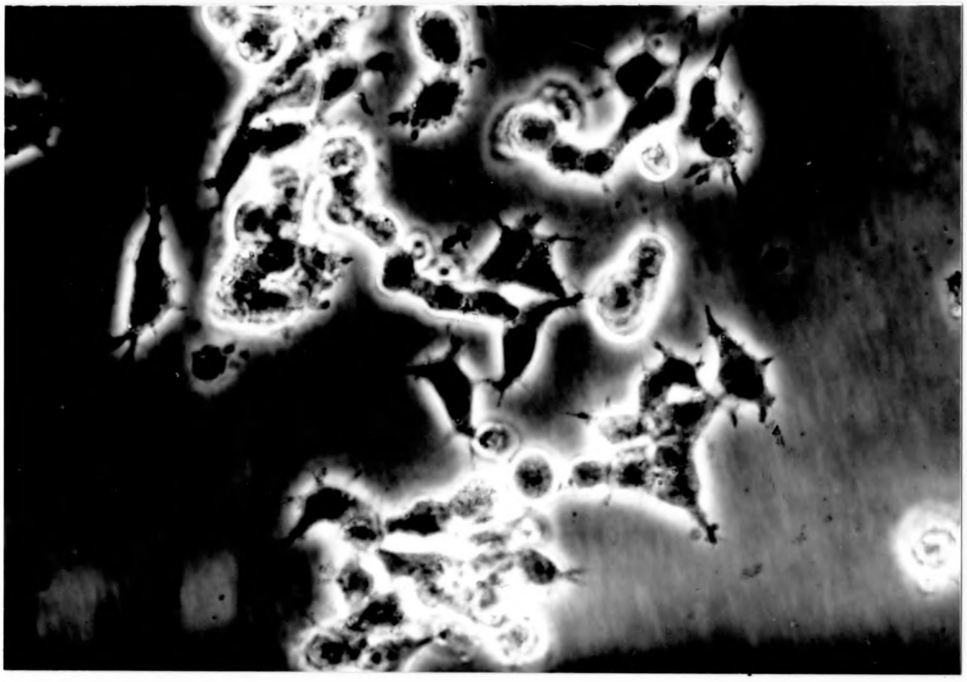
Once the cells were being grown as lines, differences in morphology and growth became apparent. As Figure 13 illustrates, the wild type transformants were less fibroblastic than the original untransformed cells and most of the cells had become almost spherical.

The cells transformed by the mutant plasmids, although indistinguishable from each other were distinctly different from the wild type transformants (Figures 13 - 16): these cells were less epithlial and more fibroblastic, they grew in irregular arrays, and they tended to overlap each other considerably.

Figure 13:

Wild type E1A transformed BRK cells.

Live BRK cells from two separate lines transformed by the plasmids pLE2 and pEJras were photographed under phase contrast.



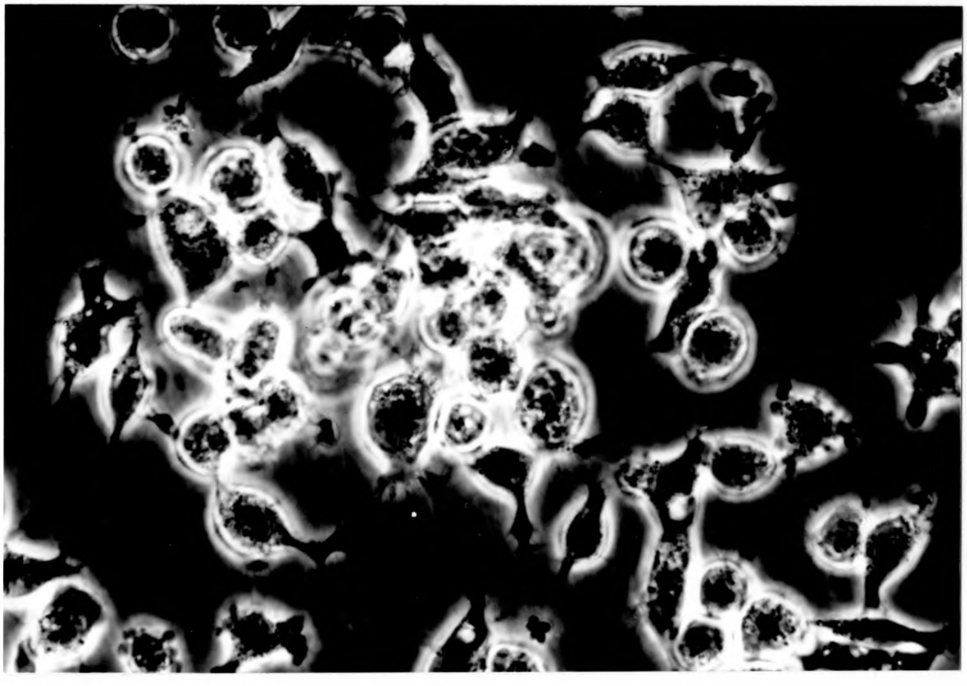
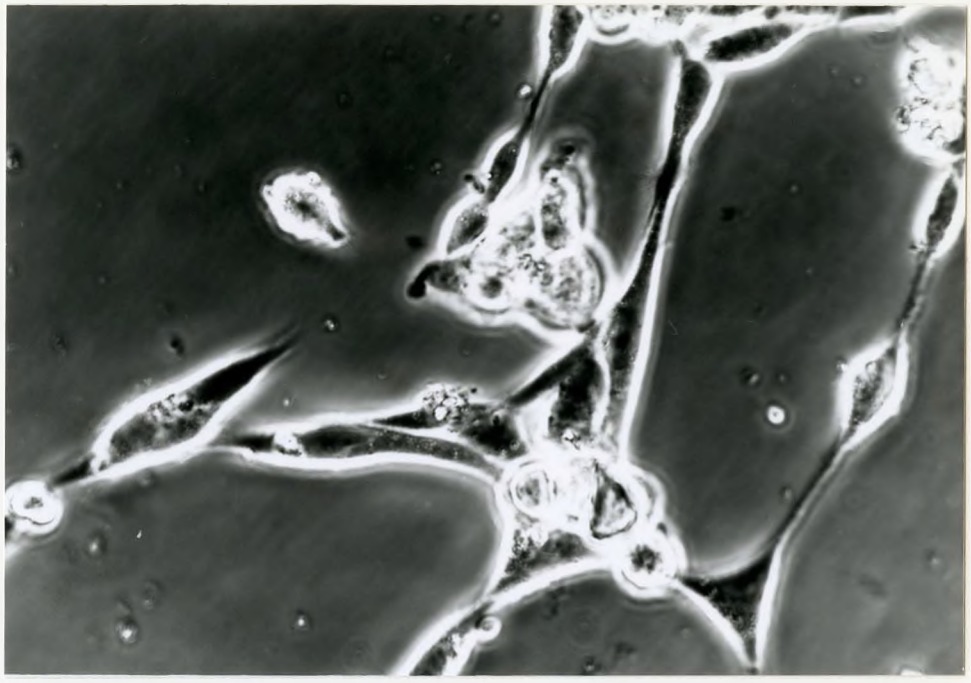


Figure 14:

Dl1112 E1A transformed BRK cells.

Two distinct lines of BRK cells transformed by the plasmids pdl1112 and pEJras were photographed live under phase contrast.



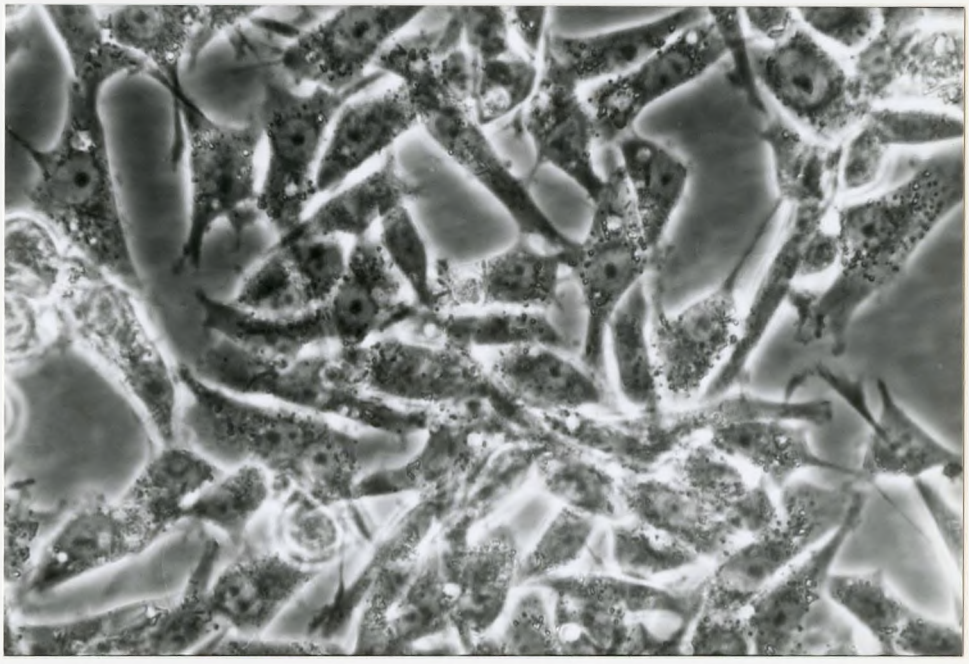
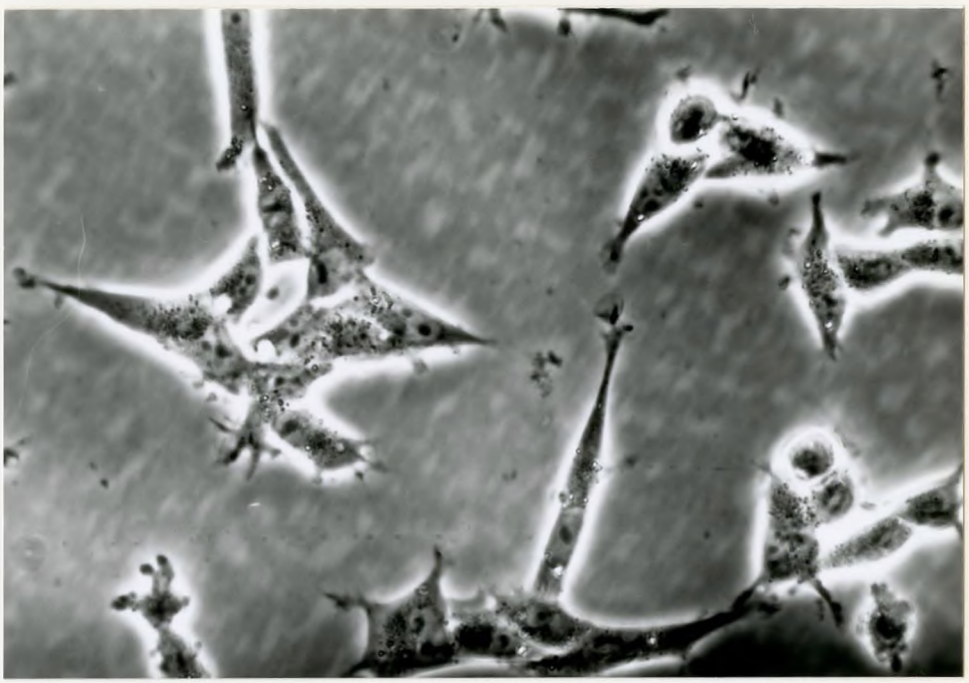


Figure 15:

Dl1113 E1A transformed BRK cells.

These live BRK cells photographed under phase contrast were from two different lines transformed by the plasmids pdl1113 and pEJras.



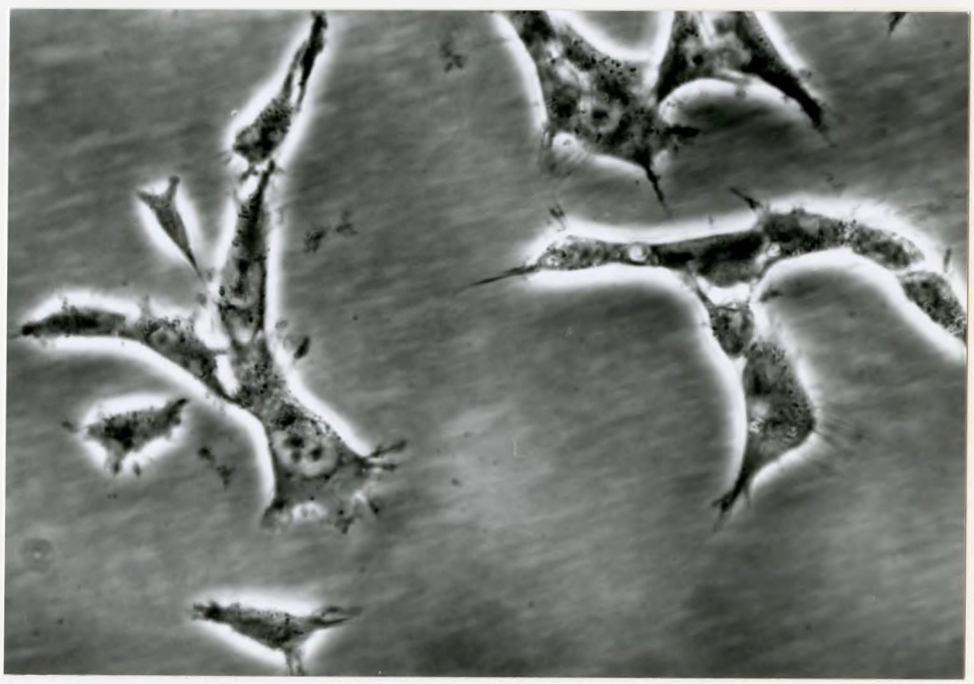
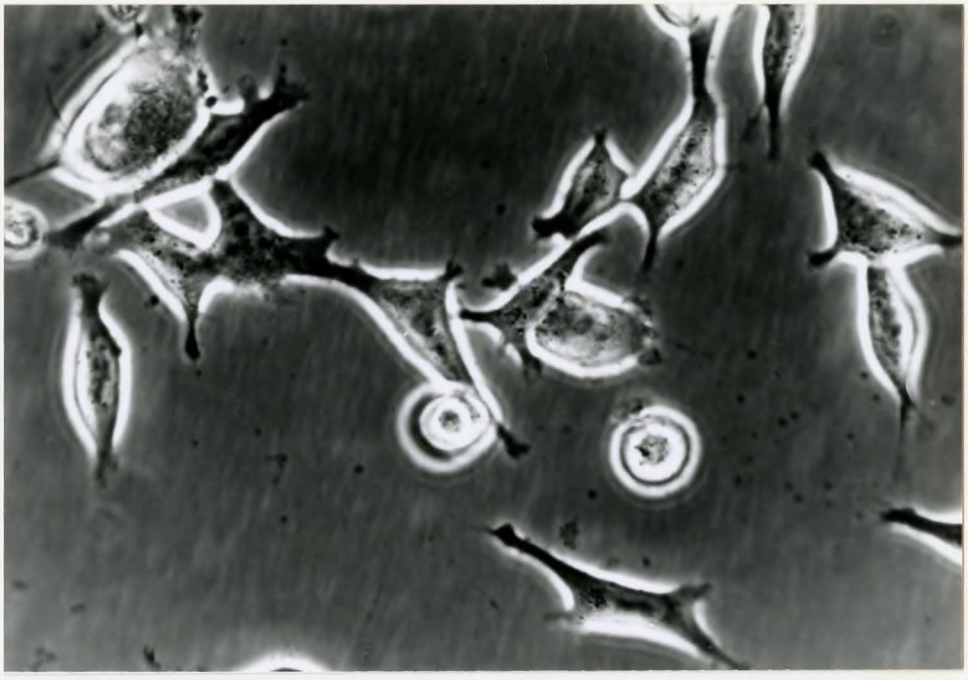
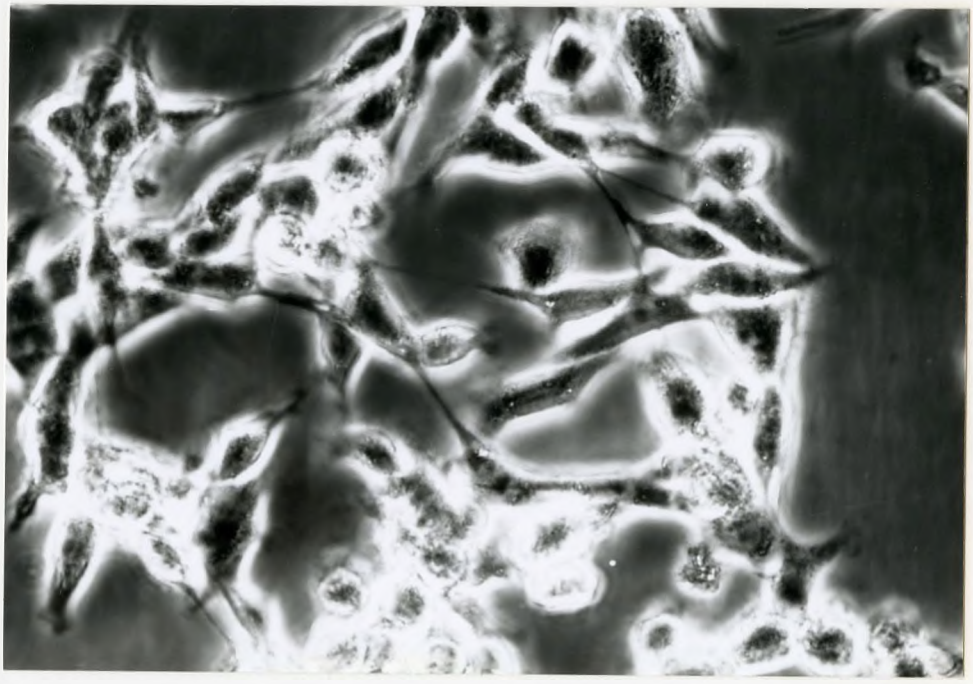


Figure 16:

Dl1114 E1A transformed BRK cells.

Photographed under phase contrast these live BRK cells were from two separate lines of pdl1114 and pEJras transformed cells.





Growth on Soft Agar

One feature of fully transformed cells is an ability to grow without solid support. Each of the mutant and wild type cell lines were tested for their ability to grow on soft agar. Approximately one to five thousand cells were mixed in medium containing 0.25% bacto-agar, and then plated on more medium containing 0.5% bacto-agar. At the same time an equal number of cells were plated on 60mm dishes without the agar. Within a week small colonies were visible within the agar.

The dl1112, dl1113, and wild type transformed cell lines all grew equally well on the soft agar. Only two of the dl1114 cell lines grew on the soft agar at first, although they were all able to grow on the dishes with solid support. When the number of seeded cells was increased a hundred fold the other three dl1114 cell lines were able to grow on soft agar.

The colony sizes varied, but there was as much difference between lines from the same mutant as between lines from different mutants, or between the mutants and wild type. Wild type and mutant colonies differed in that while all the mutant colonies were tightly packed and essentially spherical, the wild type colonies were more dispersed. The cells around the edges of the colonies were only loosely packed, and some appeared completely unattached.

Transformation by E1A alone

The transformation assays were repeated, in order to compare the mutants with each other and with wild type, without the Harvey ras1 oncogene. Although each mutant was assayed at least four times, in only one experiment were there significant numbers of foci. The results of this experiment are shown in Table 10.

In this experiment there were a number of colonies of cells with unaltered morphology (counts given in parentheses). Although most of the kidney cells had died within two weeks, these colonies were still growing at the end of the experiment twenty-seven days later. Attempts to grow cell lines from these colonies were unsuccessful. The cells stayed as monolayers with no overlapping. As a result they were difficult to see and accurate counts were only obtained after staining.

On average two thirds of the wild type transfected dishes had colonies, with an average of two per dish. A slightly higher proportion of the pd1111112 plasmid transfected dishes had colonies, and the average number of 2.7 per dish was also slightly higher. All the dishes transfected with the pd1111114 plasmid had colonies and the average number of colonies per dish was 4.6. Foci were visible on both the pd1111112 and pd1111114 plasmid transfected dishes twenty-three days into the experiment. The d1111112 foci grew more rapidly than the d1111114 foci, but more of the pd1111114 plasmid transfected dishes had foci than the pd1111112 plasmid transfected

Table 10:

Transformation of BRK cells by pLE2, pdlΔΔΔ2 and pdlΔΔΔ3 without pEJras.

In this assay kidney cells were taken from six day old rats. The dishes were 80% confluent when the cells were transfected two days after plating. The experiment was ended forty-one days later when all the dishes were fixed and stained. The numbers in parantheses refer to colonies of cells that were still growing after the untransformed kidney cells had died, but the colonies did not have the phenotype characteristic of fully transformed foci.

Table 10

Plasmid(s) Transfected	# dishes with foci		ave. # foci per dish with foci	
	Days Post-Transfection			
(ng)	23	41	23	41
Carrier	0/5 (0/5)	0/5 (0/5)	0 (0)	0 (0)
pLE2				
125	0/5 (0/5)	0/5 (3/5)	0 (0)	0 (2.0)
250	0/5 (1/5)	0/5 (2/5)	0 (1.0)	0 (1.5)
500	0/5 (1/5)	1/5 (5/5)	0 (1.0)	1 (2.2)
pdl1112				
125	1/5 (2/5)	1/5 (4/5)	1.0 (1.0)	1 (3.0)
250	1/5 (2/5)	1/5 (3/4)	2.0 (1.0)	* (1.7)
500	0/4 (1/4)	1/4 (3/4)	0 (2.0)	* (3.3)
pdl1114				
125	1/5 (0/5)	2/5 (5/5)	1.0 (0)	1.5 (5.8)
250	0/5 (0/5)	1/4 (4/4)	0 (0)	1.0 (4.8)
500	2/5 (0/5)	2/5 (5/5)	1.0 (0)	2.5 (3.4)

* numerous

dishes. In comparison only one wild type E1A focus appeared by the end of the experiment.

Rescue of mutated E1A regions into Virus

For further studies on E1A proteins, virus induced transformation, and lytic infection, the deletion mutants needed to be rescued into virus. The procedure used was that developed by Stow (1981). Ad5 d1309 is an essentially wild type virus which has lost all its XbaI sites except for one in E1A. This virus was cut in the E1A region with ClaI, and then both the virus and the deletion mutant M13 phage were cut with XbaI and ligated together.

The ligation mixture was transfected into 293 cells using the calcium-phosphate coprecipitation technique (Graham et al., 1977). 293 cells were used since they produce E1A proteins and would therefore permit growth of the mutant viruses even if their E1A proteins were non functional.

Plaques appeared within one week when the transfection was successful. The plaques were picked and used to infect 60 mm dishes of 293 cells. The viral DNA was extracted, digested with SmaI and XbaI and analysed on a 6% polyacrylamide gel to screen for the deletions. The fragment of interest was only 332 nucleotides long and its altered mobility readily showed if the

deletion was present (Figure 17). Positive clones were then plaque purified and checked twice before viral stocks were grown.

Viral Growth on HeLa cells

Up to this point the mutant viruses had been grown on 293 cells which were able to complement any defects in the E1A gene region. To determine how successfully these mutants were able to grow depending solely on their own E1A proteins, the mutant viruses were titred on both 293 and HeLa cells.

Each virus was serially diluted, and a range of these dilutions were used to infect 60 mm dishes of either 293 or HeLa cells. Over the following eight days the dishes were checked for the appearance of plaques. A minimum of two dishes were infected per dilution, and only those dishes possessing approximately twenty to one hundred plaques were included in the titre. Titres were repeated on at least three separate occasions with each cell type. The results are given in Table 11.

Of the mutants, Ad5 dl1112 was found to be least able to grow on HeLa cells. The titre on 293 cells indicated that there were 9.0×10^{10} viral plaque forming units per ml, but on Hela cells only 2.5×10^{8}. This was a 360 fold reduction in growth. In addition, while the plaques could be counted on the 293 cells within five or six days, the HeLa cells often had to be maintained for eight days for plaques large enough to count.

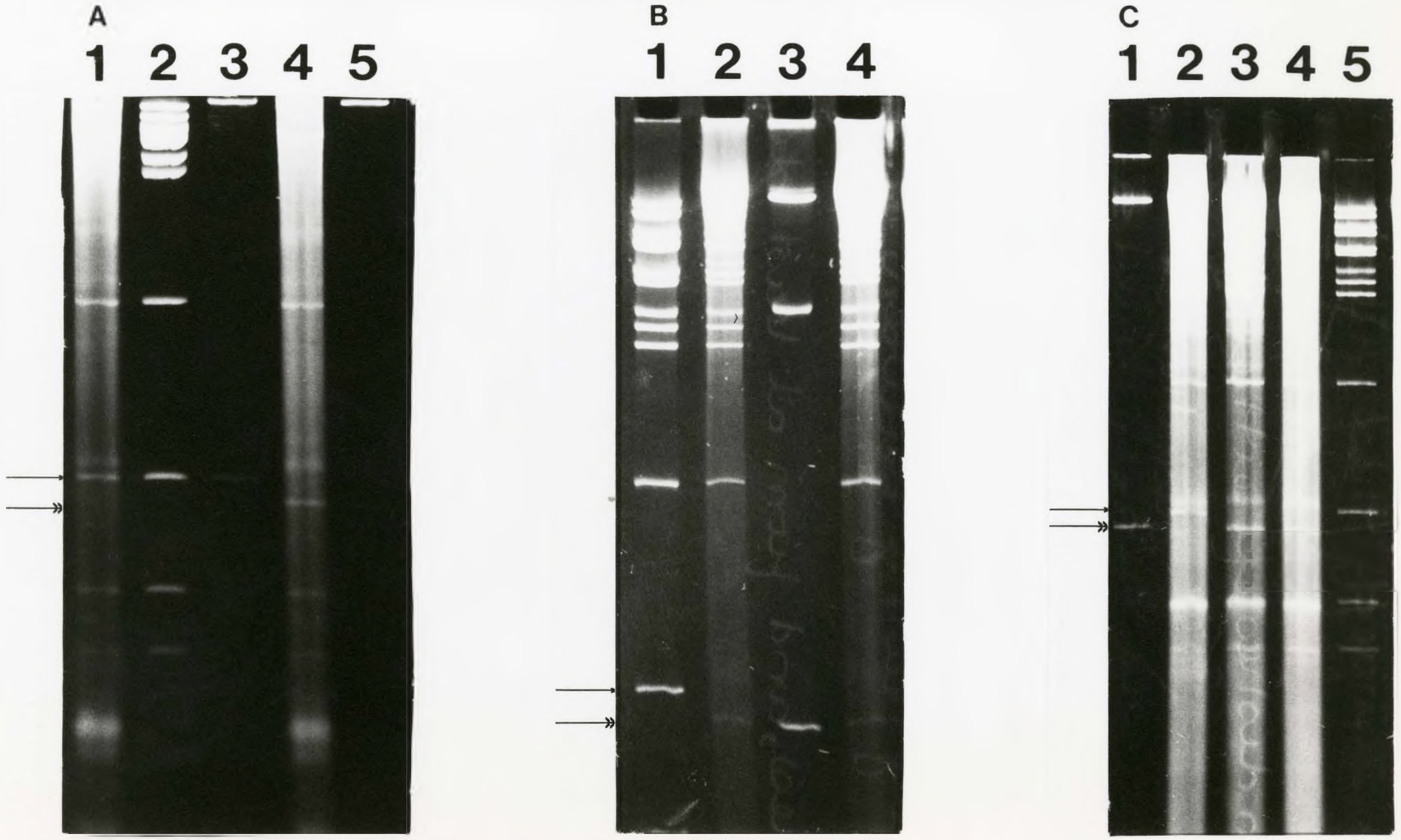

A
1 2 3 4 5
B
1 2 3 4
C
1 2 3 4 5

Table 11:

Virus titres on 293 and HeLa cells.

Virus stocks were serially diluted, and a range of dilutions used to infect 60mm dishes of either HeLa or 293 cells. At least two dishes were infected with each dilution. Plaques were usually counted six to eight days after the infection. Counts were taken from dishes generally having between twenty and hundred plaques.

Table 11

Virus	titre exp. no.	titre (pfu/ml) 293	HeLa	Ratio 293/HeLas
dl1112	1	4.1×10^{10}	---	
	2	1.2×10^{11}	---	
	3	1.1×10^{11}	2.8×10^{8}	
	4	---	3.2×10^{8}	
	5	---	1.4×10^{8}	
	ave.	9.0×10^{10}	2.5×10^{8}	360
dl1113	1	3.0×10^{9}	---	
	2	3.2×10^{9}	---	
	3	3.2×10^{9}	2.2×10^{7}	
	4	---	1.4×10^{7}	
	5	---	1.5×10^{7}	
	ave.	3.1×10^{9}	1.7×10^{7}	180
dl1114 (stock 1)	1	1.7×10^{10}	---	
	2	2.2×10^{10}	---	
	3	1.8×10^{10}	---	
	4	---	1.6×10^{9}	
	5	---	1.4×10^{9}	
	ave.	1.9×10^{10}	1.5×10^{9}	13
(stock 2)	1	6.8×10^{9}	2.0×10^{8}	
	2	3.0×10^{9}	4.2×10^{8}	
	3	2.6×10^{9}	2.5×10^{8}	
	ave.	4.1×10^{9}	2.9×10^{8}	14

Ad5 dl∏∏3 virus also indicated reduced growth on HeLa cells, although not as great as that of dl∏∏2. The plaque count was 180 fold reduced on HeLa cells. The plaques on HeLa cells usually appeared later than on 293 cells, and were usually countable on the seventh day rather than the fifth or sixth. The Ad5 dl∏∏3 plaques on HeLa cells were also the most diffuse and difficult to see of all the mutants.

The Ad5 dl∏∏4 virus was the best able of the three mutants to grow on HeLa cells. Plaques were visible on these cells at the same time as on 293 cells, and were very clear and distinct. The first stock of virus made showed a slight decrease in titre of approximately thirteen-fold. A second fresh stock of Ad5 dl∏∏4 was prepared and titred on both cell types. Again, a slight difference was noted between the two titres. The titre on HeLa cells was fourteen fold lower than the titre on 293 cells.

DISCUSSION

The purpose in studying deletion mutants spanning the unique region of the E1A 289R protein was three-fold. One part was to examine the transactivation domain to see if it extended throughout the unique region. The second was to determine if this transactivation ability was required for transformation. This was an important point because it has been suggested that transformation may be caused in some cases by an aberrant expression of endogenous cellular genes (Weinberg, 1985). The third part was to determine if any functions other than transactivation could be associated with the unique region.

The mutant dl1112 had lost 8 amino acids, residues 161 to 168, located in the centre of the unique region. Mutant dl1113 had the next 9 amino residues from 169 to 177 inclusive deleted, while dl1114 had lost the carboxy 7 amino acids. As was clearly shown all three deletions resulted in a total loss of transactivation activity. A fourth deletion mutant, dl1110, made in our lab deletes the entire amino half of the unique region. It has also lost all transactivation ability (Jelsma at al, 1988).

With deletion mutants it could be argued that they had caused alterations in the tertiary folding of a protein and were not necessarily located within the functional domain. But recent

work by our lab (Jelsma at al, 1988) and others (Glenn and Ricciardi, 1985; Lillie et al, 1986; 1987; Schneider et al, 1987) with point mutants which extend from one end to the other of this domain provide strong evidence that the entire unique region and the first amino acids of the second exon are involved in transactivation. In fact, Lillie et al (1987) have just produced a 49R synthetic peptide corresponding to this domain that is able to transactivate.

Not much is known about how transactivation actually works. It is generally supposed that the E1A proteins interact with some cellular factor that in turn binds to DNA and stimulates transcription. It is known that E1A proteins bind to some cellular proteins although they have not been identified yet (Egan et al, 1987; Harlow et al, 1986). It has also been indicated that stimulation of transcription by the E1A proteins requires protein synthesis (Richter et al, 1987). That is, it has been shown that protein synthesis inhibitors will also block E1A induced transactivation.

On the other hand, the evidence concerning transcription factor binding to E1A inducible genes is controversial. Some have found that binding patterns have been altered by the presence of E1A proteins (Kovesdi et al, 1986), while others have not (Devaux et al, 1987). Devaux et al (1987) suggests that E1A proteins may alter the functionality of bound factors rather than increasing

binding. If in fact E1A proteins interact with some cellular factors then there should be a specific binding domain in the E1A protein.

In the cotransfection CAT assays the results were inconsistent. Despite this, they do suggest that all three mutants were able to inhibit wild type E1A induced CAT activity with varying degrees of success. The mutant pdl1113 gave the most erratic results. At equimolar amounts of the wild type and mutant plasmids the acetylation levels were actually enhanced in some assays and decreased in others. Only when the mutant plasmid was present in excess was there a clear decrease in acetylation levels.

The assay results with mutants pdl1112 and pdl1114 were more consistent. At equimolar concentrations, pdl1112 reduced acetylation levels at least 22%, while pdl1114 reduced levels between 0 and 25%. As the amount of mutant plasmids increased the levels of acetylation continued to drop. Pdl1114, at a 10-fold excess was able to completely block wild type E1A induced acetylation.

One argument against the significance of these results is that the cell system may have simply been overloaded with the excess plasmids. For example, the excess number of mutant plasmids could have outcompeted the wild type for transcription and translation factors to produce the E1A proteins. This is not

likely. The results from a straightforward CAT assay using varying amounts of plasmid demonstrate that 10ug results in higher acetylation than 1ug (see figure 10 and table 1). Therefore, 1 1ug of plasmid is probably not overloading the cells.

Another argument is that although the transactivation function has been lost the repression function has not been affected. The decreased acetylation could simply be due to repression by these mutant proteins. The three mutants had somewhat different abilities to block acetylation. Since they are all equally unable to transactivate and should be equally able to repress, this explanation may not be sufficient. Further work needs to be done to determine if repression plays a role in the inhibition, and to get more consistent results. It would also be interesting to find out if d11113 can actually enhance wild type induced acetylation at equimolar concentrations.

Although the variable results make it difficult to draw any strong conclusions, it is still fairly clear that these mutants do have some effect on E1A induced transactivation. If E1A proteins act through a cellular intermediate, it is possible that the mutant proteins are competitively binding to this factor, but the complex is unable to transactivate. These mutants could prove to be useful in later work to learn more about this unknown cellular factor, their sites of interaction, and the actual process of transactivation.

To look at the transformation ability of these deletion mutants they were co-transfected into primary BRK cells as plasmids with another plasmid carrying the Harvey ras 1 oncogene. The oncogene was used for several reasons. One, E1A alone is able to transform cells with only a very low efficiency (Graham et al, 1974b; van der Eb, 1979). And two, unlike the E1B gene, ras gene expression is independent of E1A so that the assay did not depend on the E1A proteins' abilities to transactivate. And finally, since the ras oncogene has no independent transformation ability but is able to complement E1A, this provided a sensitive assay for E1A's transformation ability (Ruley, 1985; Franza et al, 1986; these results).

All three mutants were able to transform primary baby rat kidney cells with the ras oncogene at a higher frequency than the wild type E1A. The mutant induced foci appeared sooner after the transfection, were present in higher numbers, and spread throughout the dishes faster than the wild type E1A induced foci.

Since all three deletion mutants had a higher efficiency of focus formation than wild type E1A, it suggested that the transactivation function was not only not necessary for transformation but may have actually interfered with the process. A number of other labs have been able to dissociate transactivation and transformation.

For instance, Lillie et al (1986) found that two transformation defective mutants that had single base changes in exon 1 had not lost their transactivation function, while Moran et al (1986) also produced point mutants that were transformation defective but still able to transactivate. In addition, they showed that two point mutants with changes in the 289R unique region were unable to transactivate, but were still able to transform.

Recently Schneider et al (1987) presented a number of mutants that were transformation defective without being transactivation defective and others that were the reverse. Thus, the evidence indicates that transformation does not require the 289R transactivation function and that the 289R protein must play another role in transformation.

In fact, these papers noted a correlation between the ability to repress enhancer activity and transform cells (Lillie et al, 1986; 1987; Schneider et al, 1987). None of the papers were able to present a mutant that could transform cells without also being able to repress enhancer activity. Those mutants in which transformation ability had been affected had also reduced ability to repress. The latter correlation was not as clear because two mutants that had completely lost their transformation ability still had some repression activity (Schneider et al, 1987).

The argument in favour of repression playing a role in transformation is a plausible one since cell growth can be and probably is controlled as much by proliferation inhibitors as by stimulators. If the genes for these inhibitors have E1A sensitive enhancers, their transcription could be artificially depressed and cell growth would no longer be under control (Schnieder et al, 1987).

Although enhancer repression rather than transactivation appears to be necessary for transformation, transactivation should not be ignored. As pointed out already, the three mutants dl1112, dl1113 and dl1114 were all able to transform primary baby rat kidney cells with greater efficiency compared to the wild type E1A. These results were similiar to those obtained in transformation assays with the E1A 243R protein.

In these early assays the ability of the two E1A mRNAs and their protein products to transform were assayed separately. Viruses such as pm975 and dl1500, that could only produce one or the other of the E1A mRNAs, were used. The assays presented here used E1A plasmids cotransfected with the ras oncogene. Thus, it should be pointed out that the experimental conditions are not completely comparable. While the 289R proteins alone transformed with decreased efficiency, the 243R had a higher efficiency (Graham et al, 1978; Montell et al, 1984).

Transformation induced by 243R, although at a higher efficiency, was aberrant and there are distinct differences between its transformed cells and those transformed by dl1114, dl1115 and dl1116. 243R transformed cells were fibroblastic not epithelial, and were defective for anchorage independent growth. In addition only polyclonal cell lines could be developed (Graham et al, 1978; Ruben et al, 1982). A final point was that transformation by the 243R protein was cold sensitive so that at 32°C no foci could develop (Ho et al, 1982; Babiss et al, 1983).

Neither dl1114, dl1115 nor dl1116 were assayed for their ability to transform at 32°C, although it would be interesting to determine if any share this feature with the 243R protein. Three other differences were observed. One, clonal cell lines were readily grown from single foci with all three mutants. Two, the cells although less rounded than the wild type E1A transformants were not epithelial. And three, only the dl1116 cell lines were defective for growth on soft agar.

Thus, the three mutants did not behave identically to the 243R protein in transformation. Their behaviour suggests that even when the 289R protein has lost its transactivation function it still behaves distinctly differently from the 243R. In addition, the dl1116 mutant has behaved differently from the other two mutants in that it is the only one defective for anchorage independent growth.

Another assay that demonstrates some differences between the three mutants is their ability to grow on HeLa cells compared to 293 cells. It should be remembered that 293 cells are able to complement defects in the E1A proteins whereas HeLa cells have no such ability. Unlike host range group 1 mutants that had shown severely reduced growth on HeLa cells (Harrison et al, 1977), these deletion mutants had only somewhat reduced titres. That is, their titres were reduced between thirteen-fold for dl1114 and 360-fold for dl1112, while the titres for the host range mutants dropped about a thousand-fold. That all these mutants, deletion and host range, display different growth abilities despite their mutations being in the same unique region and all equally negative for transactivation suggests that they have variably affected some other property of this domain.

Both the 243R and the 289R proteins are necessary for full and complete transformation (Graham et al, 1974a; b). From this, it is obvious that the 289R must have some property that is distinct from the 243R. From the evidence presented here it appears that this property is definitely not transactivation. Since enhancer repression is either a common ability or specific to the 243R (Borelli et al, 1984; Lillie et al, 1986), this property is not repression either. As has been mentioned previously, the only difference between the two proteins is the existence of an extra internal sequence of 46 amino acids in the

289R protein. This sequence must play a role, other than transactivation, in transformation.

The three deletion mutants all exhibit some subtle but distinct functional differences between each other which is consistent with the concept that the unique region must determine another function in addition to transactivation. It could be either a direct one involving an as yet unidentified function or an indirect one involving the join between the two exons and a possibly altered tertiary configuration. The next steps could be to identify this function and determine what its role is in transformation. The three deletion mutants would be useful in the process because they have all apparently affected the unknown function slightly differently.

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