CONSTRUCTION AND CHARACTERIZATION OF INSERTION AND DELETION MUTATIONS IN THE TRANSFORMING GENES OF HUMAN ADENOVIRUS TYPE 5

by

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Abstract

The process whereby a normal cell becomes malignant, termed transformation, can be initiated by a number of agents that have been implicated in carcinogenesis. Understanding the mechanism of oncogenic transformation is a necessary prelude to developing a rational approach to therapeutic and preventative treatment of this disease. This thesis examines the molecular mechanism of cell transformation caused by a member of a family of DNA tumor viruses, the human adenoviruses. The experimental approach taken in this work was to use recombinant DNA techniques to isolate the oncogenes encoded in the genome of adenovirus serotype 5 (Ad5). Defined mutations were constructed in these Ad5 oncogenes, and mutated plasmids were assayed for their transforming activity on primary rat and on primary hamster kidney cells in order to identify which Ad5 genes were necessary for primary cell transformation.

This study reports the construction and characterization of a library of recombinant bacterial plasmids, containing DNA restriction endonuclease fragments representing the entire Ad5 genome. This library of cloned viral DNA fragments has served as a useful source of reagents for both biological and biochemical studies on the molecular biology of adenovirus.

This study employs the prokaryotic transposable element Tn5 as an insertional mutagen for cloned Ad5 sequences. The results demonstrate the usefulness of bacterial transposable elements for gene mapping experiments with cloned eukaryotic genes. A number of insertion mutations located in the Ad5 oncogenes were constructed and were characterized by DNA sequence analysis as

a prelude to studies on their transforming activity. The results of studies on the specificity of target DNA sequences chosen for Tn5 insertion suggest that transposition is influenced by transcriptional activity in target DNA.

The results of morphological transformation experiments with primary rodent cells using plasmids containing the adenovirus oncogenes (early regions E1A and E1B), and with plasmids containing defined insertion and deletion mutations in these oncogenes, have demonstrated the following. First, viral genes located exclusively in the rightward transcribed DNA strand of E1A are essential for transformation. Secondly, the viral gene located in the promoter proximal region of early region E1B, encoding a Mr=21,000 product, is required for transformation. Third, the requirement for this E1B product could be replaced with serum supplements to cells transformed by only region E1A. Finally, it was demonstrated that not all primary cells require two cooperating oncogenes for cell transformation since insertion mutations located in E1A, which eliminate transformation on primary rat kidney cells, did not eliminate transformation on hamster kidney cells.

These results have helped to define the adenovirus genes encoding functions that are essential for cell transformation. Results presented also suggest possible roles for these viral functions in maintaining the transformed state. It will be of considerable interest to determine the biological properties of these gene products which allow them to bring about the process of cell transformation.

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Chapter I

Transformation by Human Adenovirus

1.1 Introduction

Cancer is a cellular disease characterized by abnormal proliferation, usually caused by a genetic alteration of a single cell. This transformed cell passes on the malignant phenotype to all daughter cells, and a progressive series of changes leads to the increased virulence of many advanced tumors. Although most types of differentiated cells can give rise to cancer cells and to a variety of different cancers, many types of cancers share common features suggesting a unifying molecular process (see Tooze, 1981). The introduction of cell culture techniques has provided an experimental approach for investigating the mechanism of carcinogenesis, a necessary prelude to the development of rational therapeutic and preventative treatment of this disease.

The event leading to the establishment of a cancer cell, transformation, may be initiated by mutagenic agents such as ionizing radiation, ultraviolet light, or various chemical carcinogens, resulting in the alteration of cellular gene expression. Alternatively, cell transformation may be caused by mutagenic events resulting from viral infection, or by transduction by certain viruses of genes whose expression in cells leads to the transformed phenotype (oncogenes). This thesis investigates the molecular mechanisms of cell transformation caused by one member of a family of DNA tumor viruses, the human adenoviruses.

1.2 Tumor Viruses

Viruses known to cause oncogenic transformation include both RNA and DNA tumor viruses. Significantly, the only RNA containing viruses that can transform cells are those which have a DNA stage in their replicative cycle, and with two possible exceptions (described below) transforming viruses integrate viral DNA sequences into the host cell genome during transformation. It is generally believed that for most of these viruses the continued expression of transduced viral oncogenes in these cells is required for maintenance of the transformed state. Examination of the transforming genes encoded by these viruses, and their associated tumor antigens, has been a central theme of tumor virus research during the past decade.

1.2.1 RNA Tumor Viruses and Cellular Oncogenes

The RNA tumor viruses (family retroviridae) are widely distributed amongst vertebrates and form a homogeneous group of single stranded RNA genome viruses that replicate by way of a DNA provirus intermediate integrated into cellular DNA (reviewed by Bishop, 1978). Infection with retroviruses leads to production of progeny particles without cell lysis, and can also lead to transformation of the host cell. It is probable that retroviruses play a significant role as a natural cause of cancer in animals and quite possibly in man.

Retroviruses include both acute (short latent period) and chronic (slow) transforming viruses. The prototype acute virus, Rous Sarcoma Virus (an avian retrovirus), induces sarcomas in 100% of infected birds within 2-4 weeks of infection (Bishop,1978) by transduction of a viral oncogene into cells. This oncogene, termed viral src (v-src), encodes a Mr=60,000 protein pp60^{src} with protein kinase activity (Collett and Erikson, 1978), and is required for the maintenance of

the transformed state (Kavai and Hanafusa, 1971). In fact, every acute-transforming retrovirus examined to date encode an oncogene, termed v-onc, derived from one of a family of cellular (c-onc) genes (see Bishop, 1981; Weinberg, 1982). Thus accute retroviruses have served to identify cellular oncogenes which can cause neoplastic transformation.

Two further strategies have unmasked cellular genes that are capable of cell transformation. First, analysis of the cellular location of proviruses in cells transformed by slow transforming retroviruses has shown that these viruses activate cellular oncogenes by promoter insertion (Hayward et al., 1981; Neele et al., 1981; Payne et al., 1981; Nusse and Varmus, 1982), and secondly, DNA from tumor cells has been shown to be able to transform rodent cells in DNA transfection experiments (Cooper, 1982; Weinberg, 1982). Taken together, these three lines of experimental evidence have identified a family of normal cellular genes that can cause neoplastic transformation. To date, the molecular events leading to activation of these c-onc genes (reviewed by Bishop, 1983), have been shown to be a result of (1) viral transduction, (2) promoter insertion, (3) transposition of controlling elements into c-onc loci, (4) translocation of c-onc to different chromosomal locations, (5) gene amplification, and (6) base pair mutation.

The normal function of the c-onc genes is not known, but the observation that they have been highly conserved during evolution (van Beveren et al., 1981; Hampe et al., 1982; Shibuya and Hanafusa, 1982) and that they may play a role in cellular differentiation (Bishop, 1983) suggests that they are intrinsic cellular functions whose abnormal expression leads to oncogenesis.

1.2.2 DNA Tumor Viruses

The DNA tumor viruses contain double stranded DNA as their genetic material, and include members from several distinct groups, including the large DNA viruses (Poxviruses and Herpesviruses) and the smaller DNA viruses (Hepadnaviruses, Papovaviruses, and Adenoviruses). The molecular biology of the DNA tumor viruses has been reviewed by Tooze (1981).

The Poxviruses, including myxomatosis virus and the Shope fibroma virus, cause cell proliferation during infection of their natural host, but generally do not produce a fatal disease. In contrast, Herpesviruses are associated with a number of diseases such as Marek's disease in chickens (T-lymphocyte transformation), Burkitt's lymphoma (Epstein Barr Virus), opportunistic infections in man (cytamegalovirus), and with cervical carcinoma (Herpes Simplex Virus). Studies on transformation of rodent cells in culture by HSV indicate that this class of herpesvirus may transform cells by mutation of cellular genes (reviewed by Galloway and McDougall, 1983), in contrast to the mechanism of transformation by acute retroviruses and by the small DNA tumor viruses.

The Papova viruses are a heterogeneous group of small viruses with a circular double stranded DNA genome, and include Papilloma (wart virus), murine Polyoma virus, and Simian virus SV40 from monkeys. Papilloma virus induces focal transformation, with viral DNA maintained in transformed cells as multiple episomal copies. DNA sequences of Bovine papilloma virus responsible for cell transformation and for maintenance of the episomal state have recently been distinguished (Nakabayashi et al., 1983), but transformation has not been closely studied until recently due to the absence of a convenient cell culture system for growth of this virus. Both Polyoma and SV40 induce tumors in newborn rodents and can transform semi or non-permissive cells efficiently in culture to neoplas-

tic growth. These viruses have received a great deal of attention from tumor virologists (reviewed in Tooze, 1981), and have been shown to transform cells by integration and expression of virally encoded functions.

The fifth class of DNA tumor viruses, Adenoviruses, are a conserved group of linear double stranded DNA viruses isolated from a variety of animal species, including 39 serotypes from man. Clinically they cause mild respiratory infections (one group is responsible for keratoconjunctivitis), but they gained notoriety as the first human viruses shown to have oncogenic properties when Trentin et al (1962) reported tumor induction after injection of newborn hamsters. Subsequently it was reported that human adenoviruses also transform cells in culture (reviewed in Tooze, 1981). This chapter will summarize our current understanding of the molecular biology of adenovirus transformation as a prelude to a description of my studies aimed at identifying the adenovirus encoded oncogenes.

1.3 Adenovirology

1.3.1 Classification

The adenovirus genus includes over 80 serotypes with 39 human isolates classified into six groups (Table 1.1) based on a variety of serological, biochemical and biological criteria. The group C human adenoviruses serotypes 2 and 5 (Ad2, Ad5) have been used as models for eukaryotic gene organization, gene expression, and cell transformation. The complete 36,000-bp nucleotide sequence of Ad2 and much of that of Ad5 have now been determined, and most if not all mRNA transcripts have been mapped to the genome. This wealth of information makes adenovirus a very convenient tool for examining the mechanism of cell transformation.

Table 1 -1

HUMAN ADENOVIRUS HOMOLOGY CLASSES

Class	Representative Serotypes	Hemag-glutina-tion Group *	% DNA Homology +	% G + C	Oncogenicity in Rodents	Target Tissue	Epidemiology
A	12, 18, 31	IV	a 48-69% b 8-20% c 50-80%	48%	high	gastrointestinal tract	cryptic gastrointestinal infection
B	3, 7, 11, 21	I	a 89-94% b 9-20% c 50-80%	51%	weak	pharynx lungs (upper & lower respiratory tract) hemorrhagic cystitis (lower urinary tract) conjunctivits (eye)	acute epidemic infection
C	1, 2, 5, 6	III	a 99-100% b 10-16% c 50-80%	58%	nil	pharynx (upper respiratory tract)	latent throat infection; cryptic gastrointestinal infection
D	8, 9, 19	II	a 94-99% b 4 -23%	58%	nil	keratoconjunctivitis (eye)	acute epidemic infection
E	4	III		58%	nil	upper respiratory tract	
F	EA				nil	gastrointestinal tract	enteritis-associated enteric infection

* I. Complete agglutination of monkey erythrocytes; II. Complete agglutination of rat ethryocytes; III. Partial agglutination of rat erthrocytes; IV. Minimal agglutination response.

+ a. Homology of members of same group; b. Homology of members of different groups; c. DNA sequence homology of members of different groups (0-4.5 map units and 15-17 map units).

Reprinted from Sambrook et al. (1981).

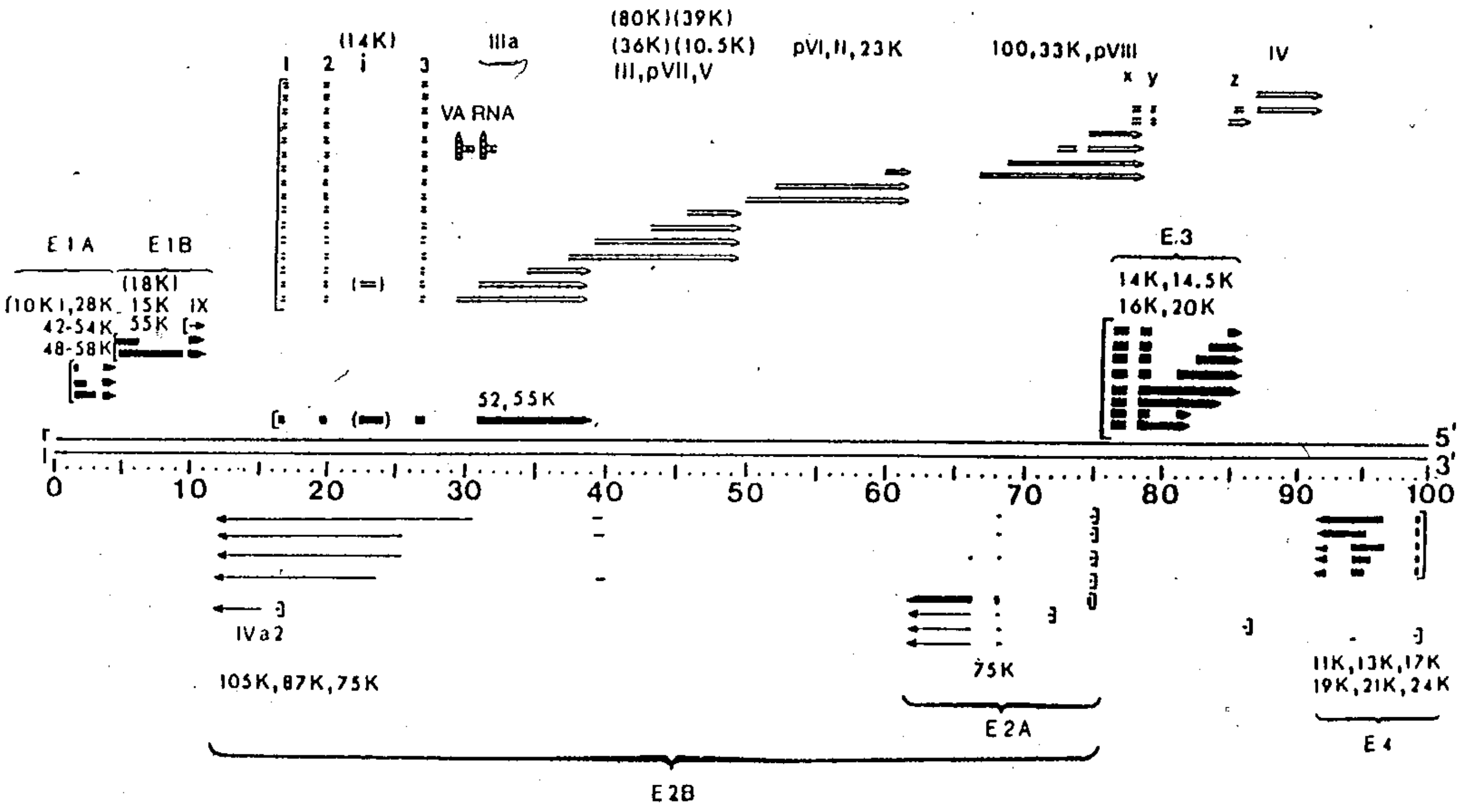
1.3.2 Genome structure and organization

The adenovirus genome ranges in size from 30-36,000-bp for different serotypes, and contains short (102 to 164-bp) inverted repeats at each molecular end depending on the serotype (Garon et al., 1972; Wolfson and Dressler, 1972). A short sequence of 6-bp at each molecular end (positions 9-14) is highly conserved between serotypes (Tolun et al., 1979; Shinagawa and Padmanabhan, 1980; Alestrom et al., 1982) and probably represents origin sequences for DNA replication (van Bergen et al., 1983). Covalently attached to each 5' molecular end is a Mr=55,000 protein (Robinson et al., 1973; Desiderio and Kelley, 1981) which was first implicated in priming DNA synthesis by Rekosh et al (1977), and has been shown to interact with a DNA polymerase encoded by the virus (Stillman et al., 1982) to initiate viral DNA replication (Lichy et al., 1982). The genome can be divided into left and right halves based on GC-content, with the left end including a packaging sequence (Hammarskjold and Weinberg, 1980) which preferentially directs this end into preformed, immature capsids (Sundquist et al., 1973; Edvardsson et al., 1976).

1.3.3 The lytic cycle

The lytic cycle of adenovirus has been reviewed by Tooze (1981) and by Pettersson and Akusjarvi (1983). Five early regions (E1A, E1B, E2, E3 and E4) expressed prior to the onset of DNA replication are located on both rightward (r) and leftward (l) transcribed strands (Figure 1), while a single major late transcriptional unit maps to the r-strand encoding predominantly virion structural components. Multiple mRNA species are generated from each of these primary transcripts by RNA splicing, a process common to all higher eukaryotes (Breathnach and Chambon, 1981) and first described in the adenovirus system (Chow et al., 1977; Kitchingman et al., 1977; Berget et al., 1977).

Figure 1. Transcription map of group C adenoviruses. Schematic representation of leftward (l) and rightward (r) transcribed adenovirus type 2 DNA strands. Genome is delineated in per cent map units (one map unit=355 base pairs). Arrowheads show the location of 3'-ends of the mRNAs and the promoter sites are indicated by brackets. Selected polypeptides which have been assigned to different coding regions are indicated. Thick lines: mRNAs expressed early after infection (includes early regions E1, E2, E3 and E4). Unfilled arrows: mRNAs expressed late (after onset of viral DNA replication). Thin lines: mRNAs expressed at intermediate and late times. Five separate cotermination families of late mRNA are present. The three segments which are spliced together to form the tripartite leader (1,2,3) are indicated, as well as the location of the i-leader. Two small viral associated RNAs, VA RNAI and VA RNAII map at position 30. From Pettersson and Akusjarvi (1983).


E1A
E1B
(18K)
(10K),28K
15K
IX
42-54K
55K
48-58K
1
2
(14K)
i
3
VA RNA
IIIa
(80K)(39K)
(36K)(10.5K)
III,pVII,V
pVI,II,23K
100,33K,pVIII
x
y
z
IV
E3
14K,14.5K
16K,20K
52,55K
5'
3'
0
10
20
30
40
50
60
70
80
90
100
IVa2
105K,87K,75K
75K
E2A
11K,13K,17K
19K,21K,24K
E4
E2B

Studies on lytic infection have been carried out predominantly in productively infected human cell lines (HeLa, KB) in culture. As early as 45 minutes after infection E1A transcripts are expressed, followed by all other early regions (Jones and Shenk, 1979b; Berk et al., 1979; Nevins, 1981). Two models have been proposed for the role of E1A in regulating early viral gene expression. An E1A product may act to inactivate a cellular repressor which otherwise inhibits transcription from the other early regions, since the requirement for E1A sequences can be relieved by addition of a protein synthesis inhibitor prior to infection (Nevins, 1981; Katze et al., 1983). Gaynor and Berk (1983) have also shown that in the absence of E1A, a time-dependent cis-acting modification of the viral DNA template is required prior to expression of other early region transcripts. Thus E1A products may also be required for a cis-acting effect.

Viral DNA replication commences 8 hours post infection, and requires three l-strand encoded early functions, the E2A Mr=72,000 DNA binding protein (Chow et al., 1979a; Lewis et al., 1976), the E2B Mr=87,000 precursor to the 55k terminal protein (Stillman et al., 1981; Alestrom et al., 1982; Gingeras et al., 1982; Smart and Stillman, 1982), and the putative E2B Mr=140,000 viral polymerase (Stillman et al., 1982; Lichy et al., 1982). Viral DNA replication proceeds by strand-displacement synthesis initiating at either end of the genome (Schilling et al., 1974; Lavelle et al., 1975; Tolun and Pettersson, 1975; Horwitz, 1976; Ariga and Shimojo, 1977) and synthesis is presumably primed by a dCTP residue covalently linked to the Mr=87,000 pre terminal protein (Rekosh et al., 1977; Pincus et al., 1981). The Mr=72,000 DNA binding protein (DBP) is multifunctional in adenovirus infection, being involved in initiation and elongation during DNA synthesis (van der Vliet and Sussenbach, 1975; van der Vliet et al., 1977; Horwitz, 1978), in regulation of early gene expression by decreasing the stability of early mRNA

(Babich and Nevins, 1981; Nevins and Winkler, 1980) including its own transcript (Carter and Blanton, 1978a; 1978b), and in viral host range specificity in a complex fashion involving processing of certain late viral mRNAs which, for human adenovirus are incorrectly spliced in monkey cells (Klessig and Chow, 1980). This block of human adenovirus replication in monkey cells can be relieved by mutations mapping in the DNA binding protein (Klessig and Grodzicker, 1979).

During productive infection by human adenovirus, host cell protein synthesis and DNA replication are both shut off before the onset of viral DNA replication (Ginsberg et al., 1967; Pina and Green, 1969). Host cell transcription is not inhibited however, and cellular RNA accumulates in the nucleus during infection (Price and Penman, 1972; McGuire et al., 1972) suggesting a preferential transport of viral mRNA to the cytoplasm.

After onset of DNA replication the pattern of viral transcription changes, with E2 transcripts initiating off a different promoter (Chow et al., 1979b) and transcripts that initiate off the major late promoter, which terminate after late region L1 (map position 40) early in infection (Lewis and Mathews, 1980; Shaw and Ziff, 1980; Akusjarvi and Persson, 1981), extending to include the entire r-strand late in infection (Fraser et al., 1979). These late long transcripts are processed to join a 5' tripartite leader sequence to one of five families of late mRNA transcripts, each with a common 3' terminus generated by endonucleolytic cleavage and polyadenylation within the primary transcript (Fraser et al., 1979; Shaw and Ziff, 1980). The tripartite leader encompases 203 nucleotides of 5' untranslated message including a sequence with complementarity to the 3' end of the 18s ribosomal RNA subunit, analagous to the Shine-Delgarno sequence in prokaryotes (Tooze, 1981). A fourth leader segment termed the "i" leader is incorporated into some mRNAs (Chow et al., 1979b; Akusjarvi and Persson, 1981).

This segment has an open translation reading frame following an ATG triplet, and could encode a hypothetical Mr=16,000 product (Gingeras et al., 1982; Virtanen et al., 1982).

The r-strand of the adenovirus genome also encodes two low molecular weight nontranslated RNAs, the virus associated or VA RNAs. Both are transcribed early in infection, and synthesis of VA RNAII declines relative to VA RNAI late in infection (Soderlund et al., 1976). Both are RNA polymerase III transcripts. It has been suggested that they play a role as adaptors in the RNA splicing reaction by binding to the 5' and 3' borders of exons creating a loop structure, facilitating a subsequent cleavage and ligation reaction (Mathews, 1980) as has been proposed for small nuclear ribonucleoproteins (snRNPs) for splicing of eukaryotic transcripts (Lerner et al., 1979; Rogers and Wall, 1980).

Assembly of virions commences at 12-15 hours post infection within the nucleus of infected cells. Virion maturation requires the proteolytic cleavage of several virion components including pVI, pVII, pVIII and the Mr=87,000 preterminal protein. An adenovirus mutant temperature sensitive for processing these precursors (Ad2ts1) maps in the late transcripts (Anderson et al., 1973; Bhatti and Weber, 1979).

1.3.4 Transformation by human adenovirus

Infection of cells either semi or nonpermissive for replication of human adenovirus, such as rodent cells, results in the transformation of a small fraction of infected cells. The requirement for a nonpermissive system presumably reflects the need to avoid expression of viral lytic functions, since permissive cells can be transformed by UV inactivated virus (Lewis et al., 1974), by replication defective viral mutants (Williams, 1973; Ginsberg et al., 1974; Williams et al., 1974), or by

DNA transfection using fragmented viral DNA (Graham et al., 1974b, 1977). Since transformation with wild type virus is limited to semi or nonpermissive cells, and represents an inefficient process requiring 10^4 to 10^6 infectious units per transformation event, and since adenovirus genes or gene products have not been detected in human tumors, it is unlikely that these viruses have any role in human neoplasia (Pettersson and Akusjarvi, 1983). However their value as an in vitro model for cell transformation cannot be overstated.

1.3.5 Identification of the transforming genes

Two lines of evidence indicate that viral early region E1 is both necessary and sufficient for transformation (reviewed by Tooze, 1981; Petterson and Akusjarvi, 1982; Graham, 1983). First, all transformed rodent cells retain at least the left end 14% of the viral genome (Gallimore et al., 1974; Sharp et al., 1974; Sambrook et al., 1974; Flint et al., 1976; Johansson et al., 1978), and secondly, more definitive studies on transformation with isolated DNA fragments using the calcium technique (Graham and van der Eb, 1973) have identified the left end 0-8% of the viral genome (early region E1) as the minimum DNA fragment capable of transforming in a DNA mediated assay (Graham et al., 1974a; 1974b; van der Eb et al., 1977; Shiroki et al., 1977; Mak et al., 1979). The organization of early region E1 is detailed in the following section.

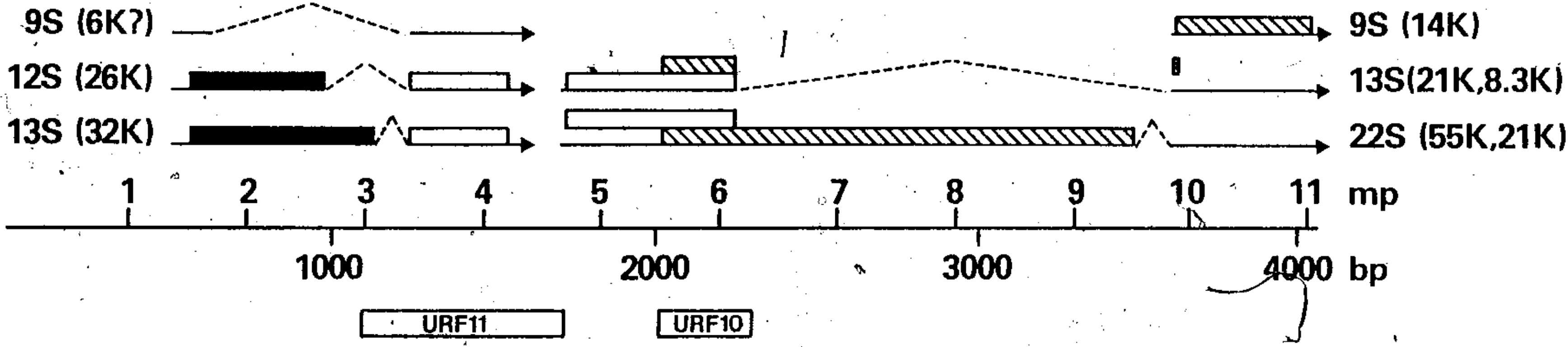
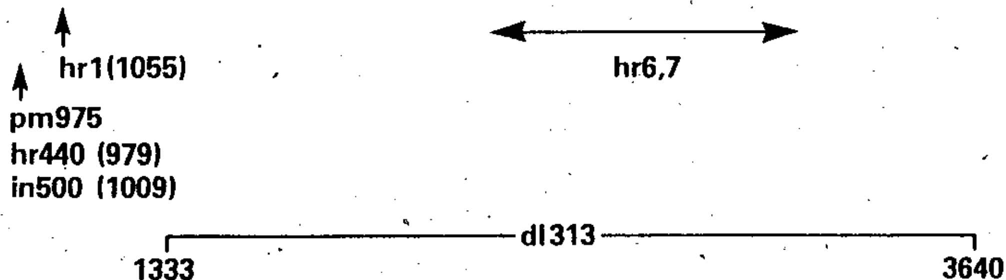
1.4 Early Region I

1.4.1 Organization of E1

The structure of early region I transcripts has been recently reviewed by Tooze (1981), Pettersson and Akusjarvi (1983), and Graham (1983). Figure 2 summarizes the genetic and physical map of E1 from Ad2 and the closely related Ad5 DNA. The transcription map in Figure 2 is a compilation of data obtained by S1 mapping experiments (Berk and Sharp, 1978), electron microscopy (Chow et al., 1979a; Kitchingman and Westphal, 1980), molecular cloning of cDNA (Perricaudet et al., 1979; 1980), and DNA sequencing studies (van Ormondt et al., 1980; Maat et al., 1979,1980; Gingeras et al., 1982).

E1 contains two independently promoted early RNA transcripts, E1A and E1B, transcribed rightwards from 1.4 and 4.8 map units respectively. As is the case with adenovirus transcripts in general, use of the coding information of this region is maximized by RNA splicing to generate multiple mRNAs for each transcript. E1A encodes 3 mRNAs (9s, 12s and 13s) with a common 5' cap site (position 499 from the left end), a common 3' poly(A) addition site (position 1632), and differing by the size of intervening sequence removed by splicing. Translation for each message begins in reading frame 2 at the first AUG, and for the 12s and 13s product continues in reading frame 1 after the splice junction, generating two proteins (289 and 243 amino acids long, Mr=32,000 and 26,000 respectively) differing only in the presence of 46 amino acids unique to the larger protein. The E1A 9s message is almost exclusively seen late, and translation terminates in reading frame 2 just downstream (3') of the splice junction generating a theoretical Mr=6100 protein (Virtanen and Pettersson, 1983).

Figure 2. Organization of early region E1. Genetic and physical map of the left end 11.5% (4100-bp) of early region E1 from group C adenoviruses. Top: r-strand transcripts; open translation reading frames are represented by open, closed, and hatched bars for reading frames 1,2, and 3 respectively. Unassigned reading frames URF10 and URF11 represent two large open reading frames on the l-strand starting from the first AUG initiator codon. Theoretical molecular weights for E1 proteins are shown in brackets. Bottom: map locations of several E1 mutants described in the text; horizontal bars indicate either the region in which mutations have been mapped (for host range mutants hr6 and hr7) or the extent of the defined deletion (for deletion mutants dl312, dl313, and dl1504). From Graham (1983).

E1A
E1B
9S (6K?)
12S (26K)
13S (32K)
9S (14K)
13S(21K,8.3K)
22S (55K,21K)
1 2 3 4 5 6 7 8 9 10 11 mp
1000 2000 3000 4000 bp
URF11
URF10
hr1(1055)
pm975
hr440 (979)
in500 (1009)
hr6,7
dl313
1333
3640
dl312
448
1349
453
560
dl1504

E1B has a single early transcript spliced to give 2 messages (22s and 13s) again with common 5' and 3' termini. Both messages encode a theoretical Mr=21,000 protein starting from the first AUG in reading frame 1. The 22s message also encodes a Mr=55,000 product in reading frame 3 starting from an internal AUG. Translation from this AUG in the 13s message would generate a Mr=8300 product terminating in reading frame 1 past the splice junction. This putative product probably has no function in viral lytic infection since an Ad5 mutant that does not produce the E1B-13s mRNA is wild-type for lytic infection (A.Berk, personal communication). The relative amounts of these two messages changes drastically during infection, with very little of the 13s mRNA present early in infection and abundant amounts of this mRNA present late in infection (Spector et al., 1978; Wilson et al., 1979; Wilson and Darnell, 1981). The second AUG on the 22s message is preferentially used for initiation of translation, whereas in the 13s message the first AUG is apparently chosen. The mechanisms governing the regulation of translation of these two messages are unclear at present. E1B also encodes an additional mRNA (Esche et al., 1980) slightly larger than the 13s message, but its exact structure has yet to be determined.

A third independent transcription block located at the 3' end of E1B encodes a 9s unspliced mRNA, which has a promoter lying within the intron common to the 13s and 22s E1B messages. This message shares the 3' poly(A) site with E1B transcripts, and encodes polypeptide IX (Alestrom et al., 1980), a virion structural component, which is predominantly a late product. The entire coding sequence for polypeptide IX is present in both 13s and 22s messages, although protein IX is apparently not made from either of these mRNAs.

Two large open reading frames with ATG initiation codons are located on the l-strand complementary to E1, designated unassigned reading frames

(URF10, URF11). They could encode polypeptides of theoretical Mr=14,000 and 23,000 respectively. No mRNAs have been mapped to this region, although Katz et al (1982) have translated in vitro an Mr=11,000 product from a 20s message selected by hybridization to the l-strand of E1, which may represent a product from URF10. Given the predisposition of adenovirus to generate multiple mRNA species from a given coding region, it is unwarranted to assume that any product from URF10 or URF11 would have the theoretical size of any open reading frames predicted from the DNA sequence. Thus any prediction of the coding potential of this region requires mRNA mapping data in order to be more definitive.

In summary, the left end 12% of the genome containing the adenovirus oncogene(s) is comprised of several transcriptional units and generates multiple RNA products. The assignment to these transcripts of viral proteins, seen in lytic infection and as tumor antigens, is discussed in the next section.

1.4.2 Proteins from E1

Viral proteins encoded in E1 have been identified using a variety of techniques (reviewed by Graham, 1983). These include immunoprecipitation of viral antigens from both infected and transformed cells, using antisera from tumor bearing animals and sera raised against synthetic polypeptides, and by in vitro translation of hybridization selected messenger RNA. E1A products include two families of highly acidic proteins mapping to the 12s and 13s messages (Smart et al., 1981; Rowe et al., 1983b). One family, with Mr values of 52k, 48.5k and 37.5k, map to the 13s E1A message, while the second group with values of 50k, 45k and 35k map to the 12s message. At present it is not clear why each E1A message encodes multiple products, nor are the post translational modifications responsible for this

heterogeneity understood. No in vivo product has yet been mapped to the 9s E1A message expressed late in infection (Virtanen and Pettersson, 1983).

E1B products include a Mr=58,000 protein assigned to the 22s message and a Mr=19,000 protein assigned to both 22s and 19s messages (see Graham, 1983). Additional proteins assigned but not mapped to E1B include a product larger than the 13s 19k protein which is related to 58k (Green et al., 1982) and a Mr=14,000 product from the l-strand of E1B (Katz et al., 1982) possibly encoded by URF10.

1.4.3 Genetics of adenovirus transformation

Mutations affecting adenovirus transformation map in four complementation groups (reviewed by Graham, 1983) located in early regions E1 and E2. Mutations in E2A such as H5ts125 affect the Mr=72,000 DBP (Ensinger and Ginsberg, 1972, van der Vliet et al., 1975) and have been found to enhance transformation (Ginsberg et al., 1974; Logan et al., 1981; Fisher et al., 1982), an effect which may be due to the increased stability of E1 messages in the absence of DBP described in **Section 1.3.3.**

Mutations in the N-group (E2B) such as H5ts36 affect the viral encoded polymerase (Stillmann et al., 1982) and are defective for initiation of transformation (Williams et al., 1974). It is not clear why a mutation altering the polymerase gene affects transformation, since viral DNA replication is not required for transformation (see Graham, 1983). It is possible that the polymerase, which interacts with the Mr=87,000 precursor to the Mr=55,000 terminal protein prior to DNA replication (Enomoto et al., 1981; Lichy et al., 1982) forms a complex which interacts with the ends of viral DNA and facilitates integration of the viral genome during transformation (Graham, 1983).

Clearly the most important transformation defective mutants isolated to date are those mapping in E1 (summarized in Figure 2). Harrison et al. (1977) isolated a series of host range (hr) mutants falling into two complementation groups, hr group I and group II. These mutants are able to replicate on an Ad5 transformed human embryonic kidney cell line, 293 cells (Graham et al., 1977) which contain and express E1 (Aiello et al., 1979), but replicate poorly on HeLa or KB cells (Harrison et al., 1977) and are defective for transformation in primary rodent cells (Graham et al., 1978). Group I mutants map in E1A (Frost and Williams, 1978), and one mutant hr1 has been located by partial DNA sequencing and was shown to contain a single base pair deletion at map position 1055 (Ricciardi et al., 1981). This deletion results in a frame shift which prematurely terminates translation of the E1A 13s message, but does not affect translation of the other E1A mRNAs. Group I mutants fail to synthesize either viral DNA or late proteins in HeLa cells (Lassam et al., 1979a; Ross et al., 1980). Group II hr mutants map in E1B sequences between 6.1 and 8.5 map units (Galos et al., 1980) corresponding to the intervening sequence of the E1B-13s message. In HeLa cells, group II mutants synthesize both viral DNA and late viral proteins, but are defective in the synthesis of the E1B-22s product Mr=58,000 (Lassam et al., 1979a; Ross et al., 1980).

Jones and Shenk (1978) also used 293 cells to isolate transformation defective hr deletion mutants mapping in E1, the most extensively characterized of which are dl312 with a deletion in E1A from 448-bp to 1349-bp (Shenk et al., 1979), dl311 with a deletion in E1A from 1282 to 1339-bp, and dl313 from 1333 to 3640-bp deleting part of both E1A and E1B (Colby and Shenk, 1981) as shown in Figure 2. Ad5dl312 complements hr group II mutants, and like hr group I is DNA negative and defective in expression of other early regions in HeLa cells (Jones

and Shenk, 1979a, 1979b; Galos et al., 1980). Surprisingly, both hr1 and dl312 can complement dl313 (Jones and Shenk, 1979a), although the deletion in dl312 overlaps that in dl313 in the coding sequences for the carboxy terminal region of E1A, suggesting that this domain of E1A products is not essential for viral replication in HeLa cells. Unlike group II mutants, dl313 does not synthesize viral DNA in HeLa cells (Jones and Shenk, 1979a) suggesting that a function mapping between 4.5 and 6.1 map units is required for DNA replication.

Finally, a number of very interesting mutants (shown in Figure 2) mapping in E1A have been recently constructed by use of more sophisticated and very powerful techniques. Solnick (1981) has constructed a host range mutant hr440 containing mutations near the 5' splice site of the E1A 12s message, which affects production of the 12s mRNA and introduces an amber mutation in the E1A-13s coding sequence. This mutant is transformation negative on rat cells, and defective in E2 and E3 but not E1B or E4 mRNA expression (Solnick and Anderson, 1982). Carlock and Jones (1981) constructed an octanucleotide insertion mutant (in500) at position 1009 in E1A which, like hr1, introduces a frame shift in translation of the E1A 13s message resulting in a truncated product but does not affect the E1A 12s product. This virus, like hr1, is defective for DNA replication in nonpermissive cells and is also transformation defective (Carlock and Jones, 1981). Although expression of E2 and E4 is reduced in in500 infected cells, E1B and E3 expression are not impaired. Montell et al. (1982) have used the technique of synthetic oligodeoxynucleotide mutagenesis (Zoller and Smith, 1982) to construct a virus pm975 with a single base transversion at position 975 in E1A. This mutation does not affect the E1A-13s mRNA (due to the degeneracy of the genetic code) but eliminates the 12s mRNA 5' splice sequence. Hence pm975 does not produce an E1A-12s mRNA during infection, but is phenotypically wild type

for replication in HeLa cells, suggesting that the important E1A function for viral replication is the E1A 13s message product(s) and that the 12s product(s) play only a minor role if any in lytic infection.

Taken together, the results of studies on lytic infection with E1A mutants suggest that the 13s E1A transcript alone is required for expression of other Ad5 early genes. Mutations affecting this function alter both viral DNA synthesis and production of late viral proteins. Furthermore, results with group II hr mutants and dl313 suggest that the E1B 55k product is essential for lytic infection but not required for either DNA replication or late gene expression, while 21k may be required for DNA replication.

The most extensively studied hr mutants for transformation are the group I and II hr viruses. Group I hr mutants induce a semi-abortive abnormal transformation of baby rat kidney cells (Graham et al., 1978). This transforming phenotype is not due to an inability of hr1 mutants to express E1B, since these semi-transformed cells express normal levels of E1B proteins (Lassam et al., 1979a, 1979b; Ruben et al., 1982) but fail to express a fully transformed phenotype (Graham et al., 1978; Ruben et al., 1982). Thus an E1A function missing in group I hr mutants is required for expression of the complete transformed phenotype.

Group II mutants map in a region of E1B that is not essential for transformation by isolated viral DNA. Consistent with this map location, isolated DNA from group II hr mutants can transform rat cells (Rowe and Graham, 1983a), suggesting that the E1B 22s product Mr=58,000 is required for initiation of transformation by virions but dispensable for transformation by isolated DNA fragments.

In summary, functions encoded in both E1A and E1B are required for transformation. An E1A product (possibly the 13s E1A function, required for expression of the other early genes) is required for maintenance of the transformed phenotype. An E1B function mapping to the region encoding the Mr=58,000 product is required for initiation of transformation by virus, but not by purified viral DNA. These results help to localize the E1 encoded adenovirus oncogenes, but in light of the coding potential of this region they do not define the functions involved. Although the E1A 12s mRNA product is not required for viral gene expression, it has not been entirely ruled out as a candidate for transforming activity. Also, the role of URF11 in transformation has not been defined. To date no viral mutants have been characterized that map in the region of E1B upstream (5') of the Mr=58,000 coding region. Hence the role of either the Mr=19,000 or URF10 products in lytic infection or in transformation has yet to be elucidated. These questions must be addressed before we can unambiguously define the adenovirus oncogenes.

1.5 Purpose of Investigation

Identification of the adenovirus encoded DNA sequences responsible for transformation is a necessary prelude to understanding the mechanism of cell transformation. The aim of this work was to identify which regions of E1 were necessary for DNA-mediated morphological transformation of primary cells.

The approach taken has been to construct a recombinant plasmid containing the E1 functions involved in transformation (McKinnon et al., 1982). Insertion mutations were constructed in this plasmid by transposition mutagenesis, using the prokaryote transposable element Tn5, and a number of E1-insertion mutants were mapped by DNA sequence analysis (McKinnon et al., 1983) and

characterized for their transforming activity. The results suggest (1) that E1A sequences are required for transformation of primary rat cells, but are not absolutely essential for transformation of primary hamster cells, (2) that E1B sequences encoding the Mr=19,000 protein are essential for transformation of both BHK and BRK cells, and (3) that the requirement for 19k can be relieved by serum supplements to transformed cells.

Chapter II

Materials and Methods

2.1 Chemicals and Radiochemicals

The ^{32}P labelled nucleotides used in this work were γ-^{32}P-ATP (specific activity >3,000 Ci per mmole) and α-^{32}P-dCTP (specific activity 800 Ci per mmole), from Amersham. Levels of radioactivity were determined in solution by Cerenkov counting (Elrick and Parker, 1968) in a Beckman LS6900 liquid scintillation counter. All materials used were standard laboratory chemicals and reagents. Solutions were prepared using double distilled water.

2.2 Bacterial Cell Culture Techniques

All recombinant DNA experiments were carried out under level B biological containment conditions as specified by the Medical Research Council of Canada guidelines (1980). This work describes construction of a number of recombinant plasmids containing DNA from human adenovirus type 5 inserted into derivatives of pBR322 (Bolivar et al., 1977), a plasmid derived from a resistance factor R1 isolated from the wild in London from Salmonella paratyphi B (Datta and Kontomichalou, 1965).

2.2.1 Bacterial Strains and Plasmids

The bacterial strains used during the course of this work and their genotypes are listed in Table 2.1. E.coli LE392, obtained from J.R.Smiley, was used as a host for all recombinant plasmids isolated. E.coli strain C600 pBR322, obtained from C.P. Stanners, was the source for this plasmid DNA.

2.2.2 Propagation and Maintenance of Bacterial Strains

Bacterial culture was performed in aluminum hoods which were flooded with UV light when not in use to maintain aseptic conditions. Glassware as well as culture medium was sterilized by autoclaving, and culture dishes for plating bacteria were purchased from Fischer Scientific Co. E.coli strain LE392 was grown in Luria-Bertani (LB) broth with shaking in either a Controlled Environment Incubator floor shaker (New Brunswick Scientific Co. Inc., Edison N.J.) for large cultures, or an Eberbach Corp. Shakerbath (cat.no.6250) for overnight cultures less than 50 ml. For selection of antibiotic resistance, medium was supplemented with 40 μg per ml kanamycin (Kan) sulphate (Sigma), 40 μg per ml ampicillin (Amp, Ayerst Laboratories), and/or 40 μg per ml tetracycline (Tet,Sigma). Stock antibiotic solutions (20 mg per ml in ddH_2O) were sterilized by filtration (Nalgene milipore filters) and stored in 2.0 ml aliquots at -20°C. To obtain a clonal isolate of a bacterial population, overnight liquid cultures were streaked using a flame sterilized wire loop on agar plates containing the appropriate antibiotics. E.coli strain JF1106 was grown in LB containing 0.2% maltose, or on plates containing broth with 15g (for plates) or 7g (for top agar) Bacto-agar.

Bacteria were stored for short periods (several weeks) on agar plates inverted at 4°C. For long term storage (several years), fresh overnight (o/n) cultures were diluted with an equal volume of sterile 40% glycerol and 2 ml aliquots were frozen and maintained at -70°C. Viable bacteria were recovered by thawing these cultures and transferring a loop of bacteria to broth, and the stock returned to the freezer.

Table 2.1: Bacterial Strains

Bacterial Strains Used in this work

Strain	Genotype	Reference
C600	F^-,thy-1,thr-1,leuB6,lacY1, tonA21,supE44,	Maniatis et al., 1982
LE392	F^-,hsdR514($r^-m_k^+$),supE44,supF58 lacY1/ (lacIZY)6,galK2,galT22, metB1, trpR55,	Maniatis et al., 1982
JF1106	F^-,$r_k^+m_k^+$,sup6,	J.Friesen, pers.comm.
KY895	F^-,$r_k^+m_k^+$, su^+,	J.Friesen, pers.comm.

2.2.3 Transformation of E.coli LE392

Transformation of LE392 was essentially carried out according to the method of Goodman and MacDonald (1977). Fresh o/n cultures in LB were diluted 1:50 in sterile medium without antibiotics and grown to log phase (generally to OD660=0.7, usually 2.5 hrs at 37°C), then collected by centrifugation (4,000 rpm, 5 min, 4°C) in 50 ml Corning tubes. After decanting the supernatant fluid, the bacteria were resuspended and maintained on ice for 20 min in 25 ml of 75 mM $CaCl_2$, 5 mM Tris.HCl (pH 7.6), collected by centrifugation as above and resuspended in 2 ml Tris-$CaCl_2$ plus plasmid DNA. Bacteria were incubated on ice

for 60 min with intermittent shaking, heat shocked (42°C, 2 min), then diluted with 8 ml warm Luria broth. For selection of Kan^r it was necessary to incubate the bacteria at 37°C for at least 30 min at this step before adding the antibiotics in order to allow time for transformed bacteria to express this phenotype. Serial dilutions of these bacteria (generally 10^{-1}, 10^{-2}, and 10^{-3} dilutions) were plated on Luria plates (0.2 ml per plate) containing the appropriate antibiotics to select for bacteria which have acquired the transfected plasmid. Transforming efficiency was generally $5\text{-}10 \times 10^5$ colonies per µg of plasmid DNA.

2.2.4 Propagation and Maintenance of Bacteriophage

E.coli strain JF1106 obtained from J.Friesen was used for propagation of the lambda phage derivative λ467 (λ kan hopper, λb221 rex::Tn5 cI857 Oam29 Pam80), also from J.Friesen, which was constructed by N.Kleckner (personal communication) from a λ rex::Tn5 phage isolated by Berg et al (1975).

The bacterophage λ kan hopper was titered on the suppressor tRNA strain JF1106 by mixing 200 µl of serial diluted phage stock with 200 ul of a fresh o/n culture after making the broth 10 mM $MgSO_4$. This mixture was incubated at 37°C for 30 min for preadsorption, then infected cells were mixed with 5 ml 56°C H-top agar and poured over two plates of H-agar (2.5 ml each). On plates in which the cell monolayer was totally lysed after o/n incubation (37°C), top agar was scraped into 50 ml Corning tubes, 10 drops of chloroform were added, and the tubes were vigorously shaken then centrifuged (5,000 rpm, 10 min, 4°C) and the supernatant fluid was stored at 4°C as a fresh phage stock. The titer of phage stocks usually decreased by a magnitude of one log unit per month when stored at 4°C.

2.2.5 Tn5 Transposition into LE392 plasmid DNA

The protocol for Tn5 transposition is shown in Figure 10. Overnight cultures of LE392 containing target plasmid DNA (pXC1 or pHE1) grown in LB plus ampicillin and 0.4% maltose were infected at an moi of >10 with λ467. Preparation of a phage stock is described in **Section 2.2.4** above. After preadsorbtion (20 min, room temperature) infected cells were collected by centrifugation, resuspended in 250 ml LB plus Amp, and incubated at 37°C to allow cell division and promote transposition. After 2 hrs cells were harvested (4,000 rpm, 10 min, 4°C) and resuspended in 5 ml LB plus Amp, then plated (0.5 ml per plate) on Luria agar plates with Amp and kanamycin (Kan) and incubated at 37°C o/n. Amp^r Kan^r bacteria were harvested by scraping the plates with a silicon spatula and grown in 1.3 l LB plus Amp and Kan. When the A660 of this culture reached 0.8 chloramphenicol was added (50 μg per ml) and the culture incubated o/n. Plasmid DNA was extracted using the procedure of Birnboim and Doly (1979) described in **Section 2.4.1**, and banded on CsCl-EtBr density gradients. This DNA was then used to transform <u>E.coli</u> LE392 (Amp^s Kan^s) cells as described in **Section 2.2.3.** Transformed cells in 2 ml Tris-$CaCl_2$ buffer were diluted to 10 ml with LB plus Amp and incubated at 37°C for 30 min, then plated on Luria agar plates with Amp and Kan. Isolated colonies were screened for the presence and location of Tn5 sequences in plasmid DNA using the Birnboim procedure (**Section 2.4.1**).

2.3 Mammalian Cell Culture Techniques

2.3.1 Cells and viruses

In addition to cell lines established during the course of this work (Table 5.6) two permanent cell lines were used in these studies, the cervical carcinoma line HeLa and the 293 line of human embryonic kidney cells transformed by human adenovirus type 5 (Graham et al., 1977) which contains and expresses viral early region 1 (Aiello et al., 1979).

The wild-type strain of Adenovirus type 5 used in this study is from the Glasgow stock described by Harrison et al (1977). The nondefective deletion mutant virus dl309 (Jones and Shenk, 1978), selected as an XbaI endonuclease-resistant varient of the wild-type Ad5 stock H5wt300 from H.Ginsberg, was obtained from T.Shenk. Mutant viruses pm975 (Montell et al., 1982) and dl1504 (Osborne et al., 1982) were obtained from A.Berk. Virus stocks were prepared as freeze thawed suspensions of infected cells and were stored at -70°C in PBS plus 20% glycerol. For viral DNA, virus was purified and stored as described by Green and Pina (1964). Titers of virus were determined by plaque assay on monolayers of 293 cells.

2.3.2 Propagation and maintenance of cell lines

All cell culture work was performed in Laminar flow hoods (Containment Control Inc., Lansdale Penn. 19446) under sterile conditions and following level B containment conditions (MRC Guidelines, 1980). Cells were grown as monolayers at 37°C in 150 mm Lux dishes (Lux Scientific Corp., Newbury Park, California), or in Corning flasks. Growth medium was either α-Minimal Essential Medium (α-MEM, Stanners et al., 1971) or Joklik's modified essential medium supplemented with 1.2 mg/ml L-glutamine, penicillin-streptomycin (Gibco Laboratories, Grand

Island, N.Y.), fungizone (Squibb Canada Inc, Montreal), and either 5% horse or 10% fetal bovine serum (Gibco). Culture medium was changed every 2-3 days, and at confluency cells were passaged by aspirating spent medium, washing the monolayer twice with prewarmed Gibco trypsin-EDTA diluted 10-fold in PBS^{-}, and incubating at 37°C with 2 ml diluted trypsin per 150 mm Lux plate until cells could be detached from the plate by gentle tapping on the side of the plate. For 293 cells described above, cells were detached using saline EDTA. Detached cells were diluted in fresh medium and distributed to new plates (generally 1:4 to 1:10 dilution, depending on the growth rate of the cells; 293 cells were split 1:2). For storage of cell lines, trypsinized cultures were centrifuged (3500 rpm, 10 min, 4°C) in sterile Corning tubes, the trypsin removed by aspiration, and the cells were resuspended in 1 ml per Lux dish of complete medium plus 8% DMSO (Fisher). Cells were then frozen in 1.5 ml Nunc minivials (Inter Med) by suspension over a tank of liquid nitrogen for 4 hrs, then stored in racks in liquid nitrogen. Such cells generally are viable after several years of storage provided proper precautions are taken to maintain storage conditions. For retrieval of a cell line, minivials were rapidly thawed by immersion in 37°C water and immediately plated in prewarmed medium. After 4 hrs to allow viable cells to attach, the medium (containing nonviable cells and DMSO from the storage vial) was replaced by fresh medium and cells were maintained as described above.

2.3.3 DNA Purification

A. Mammalian cell DNA purification

Carrier DNA used for morphological transformation assays was isolated from primary baby hamster kidney cells or from hamster embryo cells. To extract DNA from these cells, monolayers were rinsed once with PBS⁻ and digested with 500 µg/ml pronase (Calbiochem) in .01 M Tris (pH 7.4), 10 mM EDTA and .4% SDS. Pronase stock at 5 mg/ml in TE-buffer was pretreated at 56°C for 10 min and 37°C for 60 min to inactivate any contaminating DNase and RNase activity, and stored at -20°C. Lysed cells were gently scraped into 50 ml Corning tubes with a sterile silicon spatula (to avoid drying out of the plates during digestion) and incubated at 37°C for 4-12 hrs, then the pronase treated DNA was gently extracted once with saturated phenol. After centrifugation (4000 rpm, 10 min) the aqueous phase was extracted once with 24:1 chloroform:isoamyl alcohol, dialyzed extensively against .1x SSC, and finally dialyzed o/n against TE-buffer for transformed cell DNA or against HeBS pH7.1 (Graham and van der Eb, 1973) plus .1 mM EDTA for carrier DNA. Purified cellular DNA was stored at 4°C. These preparations consist of aproximately 33% DNA and 66% RNA, and yields of DNA range around 200 µg DNA per 150 mm dish of confluent cells. For transformation assays it was not necessary to remove RNA from carrier DNA preparations (Graham et al., 1980).

B. Viral DNA purification

For purification of viral DNA, infected cell monolayers were rinsed once in PBS⁻ at 40 hrs post infection then scraped into 50 ml Corning tubes, pelleted, and resuspended in .1% deoxycholate. Lysed cells were incubated for 2 hrs at 37°C with 1 µg/ml DNase, sonicated for 60 sec (until the solution became clear), then to each 3.2 ml of lysate was added 1.8 ml of 1.98 g/ml CsCl (saturated at room

temperature) and virus particles were banded at 35,000 rpm (4°C) o/n. Bands were collected by puncture of the tube with a 21 gauge needle using high vacuum grease (Dow Corning) to prevent leakage, pooled, and rebanded in CsCl as described above. The final banded virus was dialysed extensively against 50 mM Tris HCl (pH 8.1) then treated with pronase-SDS, phenol, and chloroform as described above for preparation of cellular DNA, and precipitated with 2 volumes of ethanol at -20°C. The ethanol pellet was resuspended in a minium volume of .3 M NaHAc, reprecipitated, the pellet washed with 96% ethanol, thoroughly dried, then resuspended in an appropriate volume of TE-buffer.

2.3.4 Primary cell culture

Primary cultures of baby rat (BRK) and baby hamster (BHK) kidney cells were used for the morphological transformation assay described in this study. A detailed description of the establishment of monolayer cultures is described by van der Eb and Graham (1980). Litters of 6-7 day old Hooded Lister rats (Woodlyn, Guelph Ont.) or LVG hamsters (Charles River Breeding Laboratories, Inc.) were shipped with their maternal parent. Kidneys were removed asceptically from pups immediately after cervical dislocation. Kidneys are most accesable after removal of the abdominal skin and severing the spine with scissors just posterior to the rib cage. Kidneys were removed with forceps, washed in PBS^{--}, and freed from enveloping membrane and traces of urinary duct and blood vessels by extensive peeling using sterile forceps. Cleaned kidneys were transferred to a suitable sterile vessle and exhaustively minced with scissors, then trypsinized in a .5% trypsin solution in PBS^{--}. After 15-20 min of stirring at 37°C the suspension was pipetted up and down fairly vigorously and suspended cells were removed, by decanting, and pipetted into a 4°C solution of 50% PBS^{++} 50% FCS to inactivate

the trypsin. The remaining tissue clumps were further digested with up to 3 additional incubations with trypsin until only a collagenous matrix remained. Cells were collected, after pooling the trypsin digests in PBS^{++}/FCS, by centrifugation (2500 rpm, 10 min, 4°C) and the cell pellet was suspended in 50 ml alpha-MEM plus 10% FCS. This suspension was incubated at 37°C for 30 min to allow aggregation of noncellular fibrous material then filtered through 3 layers of sterilized cheesecloth into an appropriate volume of α-MEM plus antibiotics and 10% FCS. Routinely, cells were suspended in 30 ml medium for each pair of kidneys used and distributed into 6 plastic 60 mm petri dishes. After incubation at 37°C for 12-18 hrs the culture medium was replaced with fresh α-MEM plus 10% FCS. Two days after seeding the monolayers usually reached a stage of 70-80% confluency and were ready for transfection with DNA (see below). Best results (highest number of transformed foci) were obtained with subconfluent (70-80%) monolayers, which generally reached confluency during the period of exposure to the DNA.

2.3.5 DNA Transfection

Transformation assays (DNA transfection experiments) of subconfluent monolayers of primary kidney cells with viral DNA and cloned DNA fragments were carried out using the calcium technique (Graham and van der Eb, 1973) using carrier DNA isolated from primary BHK cells as described in **Section 2.3.3.** Precipitates were prepared by mixing an appropriate volume of plasmid DNA to give the desired final concentration (from 2 to 20 µg/ml, 0.5 ml/dish) with carrier DNA (final concentration approx. 5 µg/ml; Graham et al., 1980) and dissolved by gentle mixing in TE-buffer. This solution of plasmid and carrier DNA was slowly mixed with 1/10 volume of sterile 2.5 M $CaCl_2$. Once the $CaCl_2$ was added, this solution

was added dropwise to an equal volume of 2xHeBS while introducing a steady stream of bubbles to the HeBS solution, to produce a visible precipitate of somewhat coarse and flakey morphology (see Figure 8 of Graham et al., 1980). Best transformation results were obtained with a coarse morphology precipitate which was optimal when formed in the presence of approximately 5 μg/ml final concentration of high molecular weight carrier DNA (Graham et al., 1980). It is not clear whether the effect of precipitate morphology is to optimize the sedimentation of DNA onto the cell monolayer when added to the culture medium, or if there is some peculiarity about the form of this specific precipitate which enhances its uptake by the cells. After addition of DNA-$CaCl_2$ to the 2xHeBS the precipitated DNA was aliquoted to recipient dishes, and cells were incubated for 4-20 hrs. Best results were obtained if the medium was changed between 12-16 hrs post transfection using nonselective (α-MEM, 10% FCS) conditions. The cells grew to a confluent monolayer by 24 hrs post transfection under these conditions, and incubation in selective medium (Joklik's MEM, 5%HS) was initiated at day 4. For BRK cells under these conditions the primary cells began to detach from the plates by day 8 and transformed epithelial colonies were visible by eye shortly thereafter. Dishes were routinely processed by day 16 by discarding the spent medium and staining with a solution of 1% crystal violet (Harleco, No192, Philadelphia) in 30% formaldehyde, and foci of transformed cells were counted by eye. For BHK cells the cell monolayer was more persistent and transformed foci could generally be recognized using an inverted microscope as epithelial cells growing on a monolayer of fibroblasts after 2 weeks. The untransformed cells generally detached by day 20. These dishes were generally scored for transformed foci by staining as described above for BRK cells. For both the primary BHK and BRK cells used in these experiments the background of morphologically transformed cells in untreated and in control dishes was zero.

2.3.6 Establishment of Cell Lines

Cell lines were established from colonies of morphologically transformed cells by trypsinization of either isolated colonies (using sterile aluminum cloning cylinders) or trypsinization of whole dishes containing from 1 to several colonies, and passage of cells in flasks with continued selection in Joklik's MEM supplemented with 5% horse serum.

2.4 Biochemical and Recombinant DNA Techniques

2.4.1 Plasmid DNA Purification

A. Analytical plasmid DNA preparations

For screening recombinant clones the rapid alkaline extraction procedure of Birnboim and Doly (1979) was used for all plasmid DNA preparations. After transformation of LE392, colonies were picked from agar plates with a sterilized wire loop and used to innoculate 10 ml LB cultures. Overnight cultures were centrifuged, resuspended in 1 ml of broth (after removing 9 ml of broth from supernatant), and transferred to 1.5 ml eppendorf centrifuge tubes. All remaining steps were performed in these tubes. Bacteria were centrifuged (15 sec in Eppendorf bench centrifuge), resuspended in 100 µl of 50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl (pH 8.0) plus freshly added 5 mg per ml lysozyme (Sigma), and incubated for 30 min. Spheroplasts were then lysed and cellular protein plus high molecular weight nucleic acids were denatured by addition of 200 µl freshly prepared 0.2 N NaOH, 1% SDS. The tubes were inverted several times, then the denatured macromolecules were precipitated as a clot by addition of 150 µl 3 M NaHAc (pH 4.8). After a 60 min incubation the tubes were centrifuged (5 min, Eppendorf) and the supernate fluid transferred by decanting to a fresh tube con-

taining 1 ml cold 96% ethanol, and the plasmid DNA (which remains intact after alkali denaturation due to its small, covalently closed circular structure) precipitated at -70°C. DNA was recovered by centrifugation and routinely resuspended in 100 µl of 50 mM Tris (pH8.0) 100 mM NaHAc, dissolved, and reprecipated with 2 volumes ethanol. The final ethanol pellet was washed once with 1 ml 96% ethanol, dried either in a Speed-vac (Savant Instruments, Inc.) or by overnight incubation at 37°C, then resuspended in 50 µl TE (pH 7.5). Usually 5 µl of this DNA was sufficient for analysis by gel electrophoresis after digestion with appropriate restriction endonucleases.

B. Preparative scale plasmid DNA preparations

For large scale DNA purifications 1 liter cultures were harvested after overnight growth in the presence of 170 µg per ml chloramphenicol (Boehringer Mannheim; stock solution of 35 mg per ml in ethanol stored at 4°C and added to cultures at A660=0.4). Extraction of plasmid DNA was again according to Birnboim and Doly (1979) with the following modifications: bacteria were collected by centrifugation (5,000 rpm, 10 min, 4°C) and resuspended in 8 ml lysozyme buffer, lysed with 16 ml alkaline-SDS, and finally protein and high molecular weight nucleic acids were denatured with 12 ml of 3 M NaHAc. After removing the precipitate by centrifugation, the supernatant fluid was ethanol precipitated. The final DNA pellet was dried thoroughly, dissolved in 8 ml of 10 mM Tris.HCl (pH 8.0) 1 mM EDTA, then 1 g per ml CsCl was added and dissolved. This was overlayed in a Beckman nitrocellulose tube with 0.8 ml of 10 mg per ml EtBr, the tubes were capped, mixed by inversion, and centrifuged (35,000 rpm, 48 hrs, 15°C) in a Beckman type 50 Ti fixed angle rotor. Covalently closed circular plasmid DNA (the lower band visible by UVfluorescence) was collected from the gradient in a

3 cc syringe by puncture with a 21 gauge needle. The EtBr was extracted with three changes of CsCl-saturated isoamyl alcohol, and the DNA was extensively dialysed first against 0.1x SSC then overnight against 10 mM Tris.HCl (pH 7.5) 1 mM EDTA. Yields varied from 0.5 to 1.0 mg plasmid DNA per liter culture.

2.4.2 Nuclease Digestions

A. Restriction endonucleases

All restriction endonuclease digestions were performed at 37°C for at least 2 hrs (routinely o/n) in Eppendorf vials. Reactions contained 50 mM KCl, 10 mM NaCl, 10 mM Tris (pH 7.5) and 1 mM EDTA with up to 1 µg per µl DNA and 0.1 to 1 units enzyme per ug DNA. When necessary to inactivate enzymes after digestion, reactions were terminated by heating at 56°C for 15 min.

B. RNase digestions

Pancreatic RNase A (Sigma) was dissolved at a concentration of 2 mg per ml in 10 mM Tris.HCl (pH 7.5) and 15 mM NaCl, heated to 100°C for 2 min to inactivate contaminating DNase, and cooled to room temperature. Stock solutions were stored in 10 ml aliquots at -20°C. Digestions were carried out at 37°C for 30 min with 1 µg per ml enzyme and samples were then phenol extracted after digestion.

2.4.3 Gel Electrophoresis

The equipment used in this work, including vertical and horizontal gel boxes and high voltage power regulators, were obtained from Eltech (Hamilton). Glass plates were purchased from Lloyds Glass, Hamilton. Electrophoresis buffer (E-buffer) was made up as a 50X (Tris-acetate) or 10X (Tris-borate) stocks (Table 2.3).

A. Agarose gel electrophoresis

Electrophoresis of DNA fragments through agarose (Miles Laboratories) gels was at 1 volt per cm (horizontal gels) or up to 4 volts per cm (vertical gels). After casting the gel between glass plates mounted in the vertical apparatus, the well former was removed and loose agarose was removed from the wells by aspiration. The sample to be electrophoresed was applied into the wells (submerged in 1X E-buffer) after addition of 10% v/v "stopper" (20% glycerol, 2% SDS, 0.5% bromophenol blue). The mobility of the dye front was used as an indicator for monitoring the electrophoresis. Table 2.2 gives a summary of the mobilities of various DNA fragments relative to the tracking dye on various gel types (from Maniatis et al., 1982). After electrophoresis, gels were removed from their support and stained in a solution of 1X E-buffer containing 0.5 µg per ml ethidium bromide (Sigma) for 20 min, then photographed with a mounted Polaroid land camera with Polaroid type 59 film illuminated with UV light using a Toshiba monochromatic (red) filter.

B. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis buffer was either Tris-acetate (analytical gels) or Tris-borate (sequencing gels). Acrylamide stocks were filtered through Whatman No.1 filter-paper and stored at 4°C. For sequencing gels, stock solutions were deionized by stirring with 1 gram per 100 ml stock of Biorad AG501-X8 Mixed Bed Resin before filtering. Ammonium persulfate (BDH) stock solution (10% in ddH_2O) was prepared fresh weekly and stored at 4°C. Acrylamide solutions were prepared for polymerization between sealed glass plates according to the recipes in Table 2.3.

Table 2.2: Electrophoretic Mobility of Marker Dyes

Gel type	XC	BB
1% agarose	2500-bp	400-bp
3% PAGE	700-bp	110-bp
5% PAGE	230-bp	55-bp
8% PAGE	160-bp	45-bp
5% PAGE, 7M urea	130-n	35-n
6% PAGE, 7M urea	106-n	26-n
8% PAGE, 7M urea	72-n	19-n
10% PAGE, 7M urea	55-n	14-n
12% PAGE, 7M urea	42-n	12-n
20% PAGE, 7M urea	29-n	10-n

Size of DNA fragment (n=nucleotides; bp=base pairs) with which dyes comigrate.
XC: xylene cyanol
BB: bromophenol blue

(i) Analytical acrylamide gels:

Glass plates 20x30x.15cm were mounted on a vertical apparatus (Eltech, Hamilton) using plexiglass spacers to separate the plates, and the bottom was sealed with agarose to suport the acrylamide during polymerization. When the apparatus was assembled, polymerization of the acrylamide solution catalysed by ammonium persulfate was initiated by addition of N,N,N',N'-tetramethylethylene diamine (TEMED, Sigma) and the acrylamide solution was immediately poured into the apparatus. The well former was clamped in place and

Table 2.3: Composition of Gel Mixtures

Stocks:	10xTBE[a]	50xTAE[b]	40% acrylamide[c]
Tris base	108 g	242 g	38 g acrylamide
boric acid	55 g	-	2 g bis-acryl.
glacial acetic acid	-	57.1ml	
.05M EDTA	40 ml	100 ml	
	1000 ml	1000 ml	100 ml

Gel mixtures:	5%	6%	8%	20%	25%
10xTBE	5 ml	5 ml	5 ml	5 ml	5 ml
urea	21 g	21 g	21 g	21 g	21 g
40% acrylamide	6.25ml	7.5ml	10 ml	25 ml	31.25ml
10% ammonium persulfate	100 ul	100ul	100ul	100ul	100 ul
TEMED	35 ul	35ul	35ul	35ul	35ul
	50 ml	50 ml	50 ml	50 ml	50 ml

a) 10x Tris-borate EDTA

b) 50x Tris-acetate EDTA

c) acrylamide stocks were deionized with 1 g Biorad AG501X8 mixed-bed resin (with stirring, room temp for 1 hr.), filtered (Whatman No.1), and stored at 4°C.

the gel was left to sit at room temperature for 30 min to polymerize. After polymerization, the gel was ready for use after removal of the well former and aspiration of loose bits of acrylamide in the wells. Buffer was added to each tank and samples were applied to the gel in loading buffer (DNA samples plus 1/10 volume of 20% glycerol, 2% SDS, and .05% bromophenol blue). Electrophoresis was at 2 to 3 volts per cm until the dye marker had migrated the required length (Table 2.2) and the bands were visualized for photography by UV fluorescence after EtBr staining as described for agarose gels.

(ii) Sequencing gels:

Sequencing gels were cast between 25x50 cm glass plates separated by 0.3 mm Mylar spacers along both vertical edges. To facilitate pouring, the inside face of one plate was coated with 1 ml of 5% dichloro dimethylsilane using Kim Wipes to spread the solution over the plates prior to assembly of the gel cast. Plates were sealed along the bottom and sides using 3M electrical tape. Polyacrylamide gel solutions were prepared as shown in Table 2.3 and poured into the cast after addition of TEMED. The well former was placed in the top of the gel, and after polymerization this was removed and the wells washed out with E-buffer. The tape was then removed from the plates and the backside plate was coated with a thin layer of Wakefield thermal compound (Electrosonic, Toronto) and an aluminum plate 25x40x.5 mm was placed over the back plate. The apparatus was then mounted on a vertical gel apparatus and connected to an Eltech VIP3000 power supply. The aluminum plate was used for even heat transfer in order to prevent retardation of samples loaded in the outside lanes caused by differential heat exchange along the surface of the outer glass plate. This aluminum plate was mounted such that it was not in contact with the E-buffer in the bot-

tom (anode) tank. Electrophoresis was at 15 mA (1500-2200 volts) until the dye fronts migrated to their appropriate positions (Table 2.2). **Section 2.4.8** describes the preparation of samples for sequencing gels. After electrophoresis the glass plates were removed from the apparatus, separated (the gel remains on the uncoated plate), and the gel was transferred to an exposed (dispensable) X-ray film and wrapped in Saran Wrap for autoradiography.

2.4.4 Autoradiography

DNA sequencing gels were exposed to 35x43 cm Kodak XRP-1 X-ray film at -70°C in film cassettes (supplied by A.Rainbow). For purification of labelled DNA fragments on preparative gels, the gel was wrapped in Saran Wrap and exposed to an 8x10 inch sheet of XRP-1 film in a Kodak X-ray exposure holder for 5-30 min. Exposed films were developed in a Kodak Rapid Process (RPX) developer (Health Science Center, McMaster U).

2.4.5 Purification of DNA Fragments

DNA restriction endonuclease fragments were isolated after electrophoresis on agarose or acrylamide gels using the technique of Maxam and Gilbert (1980). Bands were visualized by UV flourescence, the gel containing the band was excised with a scalpel, and the DNA was electroeluted into .5 ml of E-buffer inside a small dialysis membrane bag sealed by tying knots at both ends. The elution of DNA from the gel slice to the wall of the dialysis bag was monitered by UV flourescence, and after electrophoresis in a horizontal apparatus (150 volts, 2-4 hrs) the DNA was recovered by ethanol precipitation.

DNA fragments end labelled with ^{32}P for DNA sequencing (**Section .2.4.7**) were isolated from acrylamide gels after location of the bands by autoradiography. The gel was lined up with the autoradiogram (the position of the

wells was marked on the film for orientation) and the labelled DNA located with a Geiger-Mueller counter. Gel slices containing the DNA were exhaustively minced in a 1.5 ml Eppendorf tube using a plunger from a 1 cc syringe, and eluted into 500 µl of buffer (.5 M ammonium acetate, .01 M magnesium acetate, 1 mM EDTA, .1% SDS) at 37°C for 12-18 hrs. After incubation, the acrylamide was centrifuged in an Eppendorf bench centrifuge for 10 min, the aqueous phase collected, the acrylamide washed with 200 µl elution buffer and again centrifuged, then the supernatant fluids pooled, filtered by gravity through glass wool, precipitated twice from 66% ethanol and the ethanol pellet finally dried in a Speed-vac. This pellet was ready for chemical degradations as described in **Section 2.4.8.**

2.4.6 T4 DNA Ligase conditions

Ligations were performed at 14°C overnight in 10 mM $MgCl_2$, 20 mM dithiothreitol, 50 M Tris HCl (pH 7.5) and 1 mM ATP with 0.1 units of T4 DNA ligase (Bethesda Research Laboratories) per µg DNA.

2.4.7 T4 Polynucleotide kinase conditions

DNA was end labelled using [^{32}P]-ATP and T4 polynucleotide kinase (BRL) as described by Maxam and Gilbert (1980). After kinasing, labelled DNA was separated from ATP by centrifuging through Sephadex G50 as described by Maniatis et al (1982).

2.4.8 Maxam-Gilbert DNA Sequencing

The strategy for sequencing DNA fragments is outlined in the legends of appropriate figures. End labelled DNA was chemically cleaved using the base-specific chemical modification reactions exactly as described by Maxam and Gilbert (1980), except that in place of the pyridinium formate reaction (G+A) the purine

specific reaction described by Maniatis et al (1982) was used. Briefly, 50 µl of 98% formic acid (Baker Chemical Co.) was added to 10 µl ddH_2O and 10 µl of end labelled DNA. This reaction (15 min, 20°C) was stopped as described for the hydrazine reactions and the cleavage products from the sequencing reactions were resolved on denaturing acrylamide gels (**Section 2.4.3**) as described (Maxam and Gilbert, 1980).

Chapter III

Construction of Recombinant Plasmids Containing Ad 5 DNA

3.1 Introduction

As an approach to studying the transforming genes of Ad5 we chose to construct recombinant bacterial plasmids containing Ad5 DNA restriction fragments. Cloning the left end sequences would provide a convenient approach to studying these genes since (1) specific fragments of the Ad5 genome could be obtained biochemically pure, (2) large amounts of a unique subgenomic DNA fragment could be purified from E.coli by isolating recombinant plasmid DNA, and (3) cloned Ad5 DNA sequences could be mutagenized in vivo or in vitro and the effects of these sequence alterations could be examined by cloning and purifying plasmids containing the altered structure and examining their biological activity (or the loss thereof) in eukaryotic cells.

The cloning vector pBR322 (Bolivar et al., 1977), depicted in Figure 3A, was used for construction of all recombinant plasmids described in this work. This plasmid has a mumber of advantages for recombinant DNA work involving isolated eukaryotic genes such as the adenovirus genome. (1) It is a small plasmid (4.3 kilobase pairs) and can accomodate reasonably large DNA inserts. This work describes inserts of up to 20 kbp. (2) It is a high copy number plasmid and under stringent replication conditions there can be from 1,000 to 3,000 copies per cell (Lewin, 1977). (3) It encodes two drug resistance determinants, ampicilin resistance (Amp^r) and tetracycline resistance (Tet^r), facilitating selection of E.coli containing recombinant molecules on the basis of drug resis-

tance phenotypes. And finally, (4) pBR322 has numerous unique restriction endonuclease cleavage sites within the Amp^r and Tet^r genes, allowing a wide choice of sites for insertion of foreign DNA.

This chapter describes the construction of chimeric plasmids containing segments of Ad5 DNA inserted into pBR322. This section also contains a detailed structural analysis of several plasmids which were used in studies to be described in later chapters. Recombinant plasmids have been named according to the Ad5 DNA restriction endonuclease fragment they contain, such as plasmids containing the XhoI-C fragment (pXC1) and the HindIII-G fragment (pHG1). Diagramatically, plasmid DNA is represented either as a circle or schematically linearized at a restriction site unique to pBR322 (vector) DNA. The vector sequences are represented as a solid line while inserted viral DNA is represented as a boxed region, and appropriate endonuclease restriction sites are marked for orientation.

3.2 Results and Discussion

3.2.1 Cloning the left end

The cloning of the BamHI-A and BamHI-B fragments of Ad5 DNA was carried out by Dr.F.L. Graham. The approach taken is illustrated in Figure 3A. To prepare viral DNA for blunt end ligation to BamHI linker DNA it was necessary to treat Ad5 DNA with limited exonuclease III digestion followed by S1 nuclease digestion. This procedure eliminates the terminal protein or peptide which is covalently attached to the 5' end of viral molecules (Rekosh et al., 1977; Desiderio and Kelly, 1981) but it should not affect the transforming activity provided digestion is limited to less than about 300 bp (Graham et al., 1974a). Viral DNA, ligated to

BamHI linker molecules, was digested with BamHI and ligated to BamHI digested pBR322 as described in the legend to Figure 3. This DNA was then used to transform E.coli LE392, and Amp^r Tet^s clones were isolated and screened using the technique of Birnboim and Doly (1979) for rapid analysis of plasmid DNA. Using this approach, 9 recombinant plasmids containing the left 21 kbp BamHI-A fragment (including pFG25, Figure 3A) and one plasmid, pFG23 (Figure 3A) containing the right 15 kbp BamHI-B fragment of Ad5 DNA were isolated (McKinnon et al., 1982).

3.2.2 Construction of pXC1

The extent of exo III digestion in the 9 plasmids containing the BamHI-A fragment was determined by sizing the left terminal Ad5 XbaI-E band by agarose gel electrophoresis, and comparing it to the corresponding band from wild-type viral DNA. Plasmid DNA isolated from these 9 clones was digested with BamHI and XbaI and electrophoresed on a 2% agarose gel shown in Figure 3B. Digestion of these plasmids generates 4 fragments including the XbaI-B fragment and the leftmost 3.8% XbaI-E fragment. This latter band was largest for the plasmid pFG25 shown in lane 9 of Figure 3B, and this plasmid was chosen for further work. The precise extent of exoIII digestion in pFG25 will be discussed below.

The orientation of the BamHI-A fragment in pFG25 was determined by analysis of the HindIII digestion products shown in Figure 3B (lane 10). In addition to the HindIII-C,D,E, and H fragments, two junction fragments representing pBR322 sequences attached to viral fragments G and A are observed. The sizes of these hybrid fragments agree with the structure of pFG25 as shown in Figure 3A with E1A sequences proximal to the EcoRI site in pBR322 sequences. A similar analysis of pFG23 revealed that the BamHI-B viral fragment was oriented as

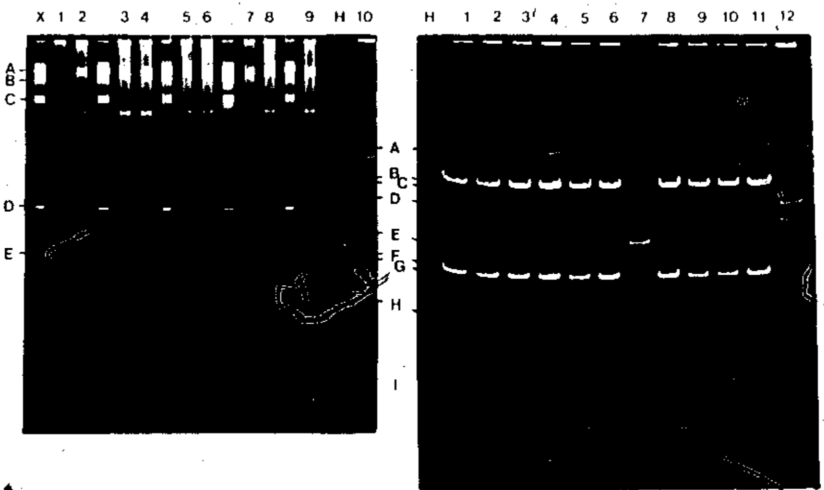
Figure 3. Molecular cloning of Adenovirus type 5 DNA.

A. Cloning protocol. The conditions for all nuclease digestions are described in **Section 2.4.2.** Ad5 DNA was first treated with exonuclease III and then S1 as described by Graham et al (1974a), phenol-extracted, ligated to synthetic decanucleotide BamHI "linker" fragments, then digested with BamHI. This DNA was ligated to pBR322 DNA which had been digested with BamHI then alkaline phosphatase to prevent recircularization. Ligated DNA was used to transform E.coli. LE392, and Amp^r Tet^s colonies containing Ad5 DNA sequences were identified by colony hybridization using [^{32}P] Ad5 DNA as a probe. Plasmid DNA from positive colonies was screened by HindIII digestion and agarose gel electrophoresis for the presence of Ad5 inserts. Initially, one plasmid (pFG23) was identified. In a subsequent experiment the BamHI-A fragment from Ad5 DNA was isolated by gel electrophoresis after attachment of BamHI linkers and digestion with BamHI. This DNA was cloned in pBR322 as above, and nine recombinants containing the BAmHI-A fragment were obtained.

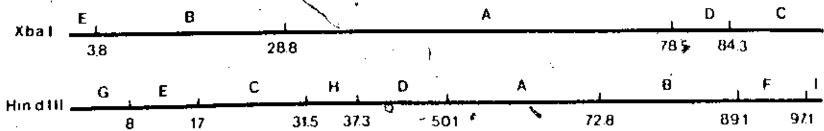
B. Plasmid DNA from the nine BamHI-A containing clones was digested with XbaI plus BamHI and electrophoresed on a 1.2% vertical agarose gel (lanes 1-9). The orientation of the A-fragment in clone 9 (pFG25) was determined by HindIII digestion (lane 10). Markers: (X) Ad5 XbaI digestion products; (H) Ad5 HindIII digestion. The restriction map of Ad5 for XbaI and HindIII is shown in the cartoon below.

C. Construction of pXC1. DNA from clone 9 (pFG25) was digested with XhoI plus SalI and the entire reaction volume was treated with T4 DNA ligase. After transformation of LE392 with this DNA, Amp^r Tet^s colonies were isolated and plasmid DNA was analysed from eleven clones by digestion with HindIII and gel electrophoresis (lanes 1-11). Lane 12:pFG23 DNA digested with HindIII (see text). H: Ad5 HindIII digestion (marker).

B



C



depicted in Figure 3A, with the right end E4 sequences located distal to the EcoRI site of pBR322. The location of the synthetic BamHI linker molecules ligated to the ends of exoIII treated cloned viral DNA is shown by an asterisk in Figure 3A.

The plasmid pXC1 containing only Ad5 E1 sequences and little else was constructed as shown in Figure 3A. Digestion of pFG25 with SalI (3 sites in Ad5 sequences and one site in pBR322 DNA) and XhoI (three sites in Ad5, one of which is shown) gives a 9.9 kbp fragment containing β-lactamase (Amp^r) and origin sequences of pBR322 with 4 bp cohesive ends (SalI and XhoI generate the same "sticky-ends" after digestion of DNA). The reaction was treated with T4 DNA ligase, then the DNA was used to transform LE392. Amp^r Tet^s colonies were isolated and plasmid DNA analyzed by digestion with HindIII as shown in Figure 3C in order to identify clones carrying pXC1. 90% of the clones screened (10/11) contained the construct pXC1 which gives two fragments of 6850 and 3150-bp after digestion with HindIII as shown in Figure 3C. The eleventh clone, shown in lane 7 of Figure 3C, was not further characterized.

The plasmid pXC1 contains the entire Ad5 E1 sequences oriented clockwise in pBR322 from position 375 (BamHI site) to 650 (SalI site) in pBR322 sequences. As will be described in Chapter V, pXC1 is capable of transforming primary rodent cells in a morphological transformation assay. The following section describes the construction of a number of plasmids derived from pXC1 which are used in Chapter V to examine the oncogenes encoded in pXC1.

3.2.3 Plasmids derived from pXC1

A. pHG1, pCD1, pHE1

The strategy used to construct 3 plasmids derived from pXC1 is detailed in the legend to Figure 4. The plasmid pHG1, containing the left 8% viral HindIII-G fragment (Figure 4A) was constructed by first isolating this BamHI-HindIII fragment from pXC1 DNA then inserting it into the compatible sites in pBR322. Vector DNA was treated with alkaline phosphatase in order to prevent religation of pBR322. This procedure reduced the transforming efficiency of ligated DNA ($2x10^3$ Amp^r colonies per µg input pBR322), but resulted in a high proportion of colonies containing chimeric plasmids (28/30 or 93% of colonies were Amp^r Tet^s). All colonies analysed (5/5) contained the plasmid pHG1 with the Ad5 HindIII-G fragment correctly inserted into pBR322 as illistrated in Figure 4A.

The plasmid pCD1 shown in Figure 4A deletes most of the E1B sequences of Ad5 and is one of 3 derivatives constructed by Dr.Graham. SacI recognizes 3 sites in pXC1, generating viral SacI fragments E (0-5%), G (5-10.3%), and F (10.3-16.5%). Only the two leftmost Ad5 SacI sites are illustrated in Figure 4A. The plasmid pXC1 was partially digested with SacI, treated with T4 DNA ligase, then used to transform LE392. Amp^r Tet^s colonies were then screened for the presence of plasmids which had deleted the SacI-G fragment (pCD1). In addition, the plasmids pCD3 (missing the SacI-F fragment) and pCD2 (missing both G and F fragments) were isolated. Plasmids pCD1 and pCD3 were analysed to ensure that the SacI-F and -G fragments, respectively, had not been inverted during the construction (data of Dr.F.Graham).

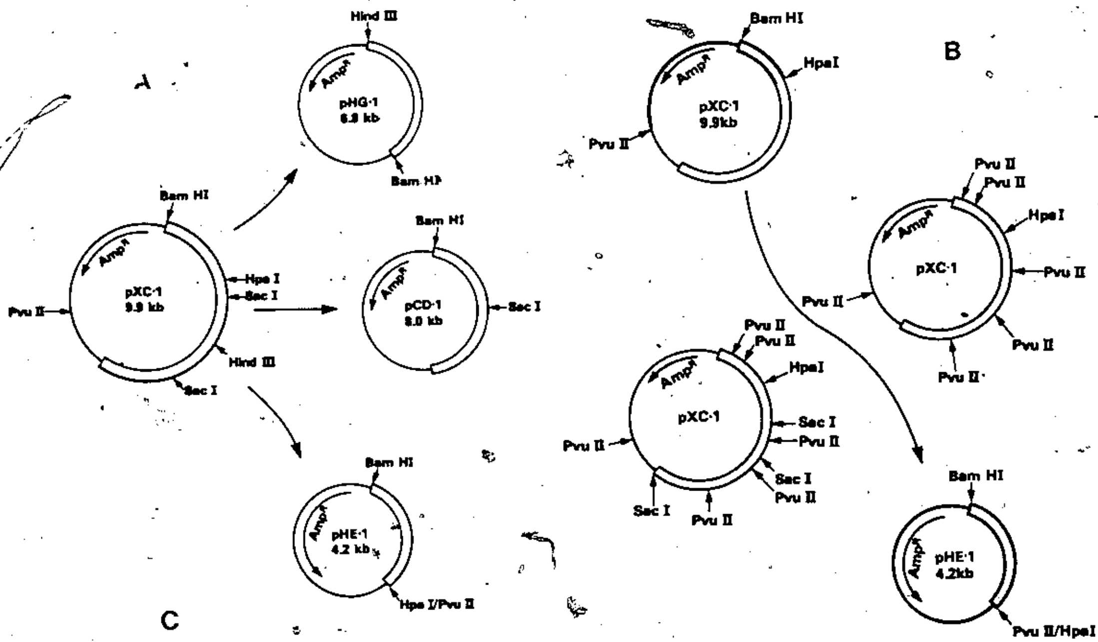
Construction of pHE1, containing only E1A sequences, is detailed in Figure 4B. Since the origin of replication of pBR322 is located between the Amp^r

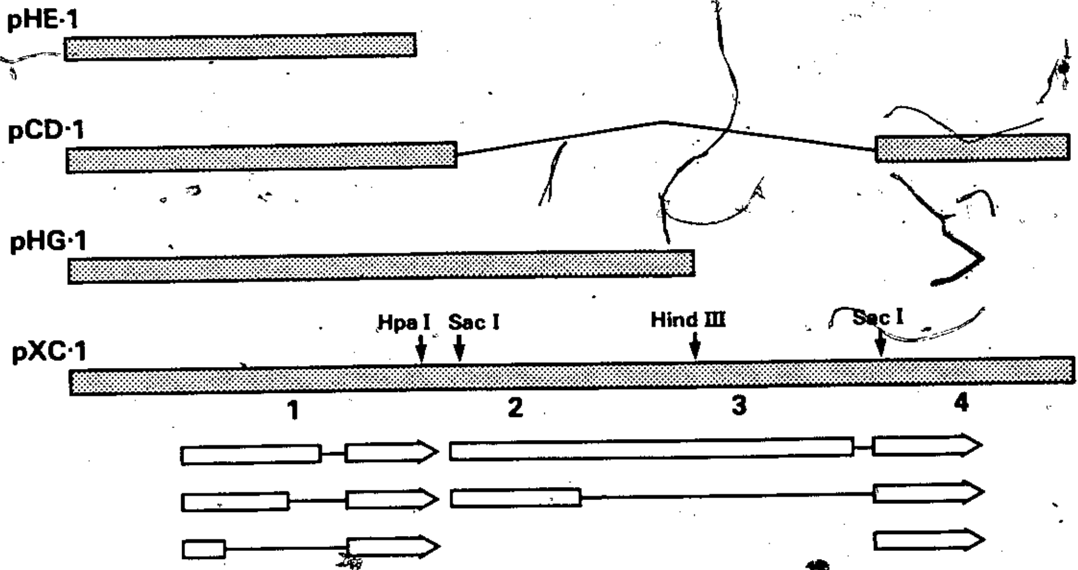
Figure 4 Construction of plasmids pHG1, pCD1 and pHE1.

A. The plasmid pHG1 was constructed by isolating the 2.8-kbp HindIII fragment from pXC1 after agarose gel electrophoresis of digested DNA. Recovered fragment DNA was ligated to pBR322 DNA which had been digested with BamHI, treated with alkaline phosphatase (as described in the legend to Figure 3), then digested with HindIII. Ligated DNA was used to transform E.coliLE392 and Amp^r colonies were tested for Tet^s. All Amp^r Tet^s colonies screened contained the plasmid pHG1. The plasmid pCD1 was constructed by digestion of pXC1 with SacI then ligating this DNA and transforming LE392. Amp^r Tet^s colonies were screened for clones containing the construct pCD1.

B. Construction of pHE1. After digestion of pXC1 with HpaI and SacI (total digestion) and with PvuII (partial digestion), this DNA was ligated with T4 DNA ligase and used to transform LE392. Amp^r Tet^s colonies were screened for a 4.2-kbp plasmid containing the 171-bp PvuII fragment.

C. Schematic representation of Ad5 DNA present in plasmids pXC1 (0-16% XhoI-C fragment), pHG1 (0-8% HindIII-G fragment), pHE1 (0-4.5% HpaI-E fragment) and pCD1 (0-5% plus 10-16% SacI-E and -F fragments). A transcription map of E1 mRNAs is included for orientation. Numbers indicate kilobase pairs from the extreme left end of the viral genome.


A
B
Hind III
pHG·1
6.8 kb
Bam HI
Amp^R
pXC·1
9.9 kb
Pvu II
Hpa I
Sac I
pCD·1
8.0 kb
pHE·1
4.2 kb
Hpa I/Pvu II
9.9kb
HpaI
pXC·1
4.2kb
Pvu II/HpaI


C
pHE·1
pCD·1
pHG·1
pXC·1
Hpa I
Sac I
Hind III
Sac I
1
2
3
4

gene (β-lactamase) and the PvuII site shown in the top construct of Figure 4B, the region of pXC1 between the HpaI site (1574 bp on the Ad5 map) and this PvuII site could be deleted without altering the viability of this plasmid in E.coli. Since HpaI (recognition sequence 5'-GTT'AAC-3') and PvuII (recognition sequence 5'-CAG'CTG-3') both produce flush 5' and 3' termini after digestion, these ends can be ligated to produce the fusion sequence 5'-GTTCTG-3' which would subsequently not be recognized by either enzyme. The difficulty in constructing this plasmid was the presence of 2 sites for PvuII within E1A as shown in the second construct in Figure 4B. It was thus necessary to digest pXC1 partially with PvuII, anticipating that amongst the digestion products would be molecules that had been cleaved at the PvuII site in pBR322 DNA but had not been cut at either of the 2 sites in E1A sequences. After partial digestion with PvuII (room temperature, 1 hr) this enzyme was heat inactivated (65°C, 15 min) then this DNA was digested to completion with HpaI and SacI. SacI cleaves at 3 sites in pXC1 (see the third construct in Figure 4B) and produces termini that are incompatible for ligation to blunt end termini, thus reducing the background of ligatable molecules which contained E1B PvuII fragments. Ligated DNA was used to transform LE392 and Amp^r colonies were screened by PvuII digestion of plasmid DNA. Out of 19 colonies screened, 2 were found to contain the plasmid pHE1.

The adenovirus DNA present in pXC1, pHG1, and pCD1 is shown in Figure 4C relative to the transcription map of early region E1. These 4 plasmids have been characterized extensively (**Chapter V**) for their transforming activity, and a detailed analysis of their structure is presented in **Section 3.2.6**.

B. pXC386, pHC1

The construction of a plasmid containing only E1B sequences is shown in Figure 5. First, pXC1 DNA was linearized by digestion with HpaI, then ligated in the presence of BamHI linker DNA (decanucleotide 5'-pCCGGATCCGG-3'). After transformation of E.coli LE392 and selection for Amp^r bacteria, colonies were screened for clones carrying a 10-kbp plasmid which gave 2 fragments (8.4 and 1.6-kbp) on digestion with BamHI. The resulting plasmid pXC386 was then digested with BamHI, treated with T4 DNA ligase, then DNA was used to transform LE392 and Amp^r colonies were screened for a plasmid which had lost the 1.6-kbp BamHI fragment (pHC1).

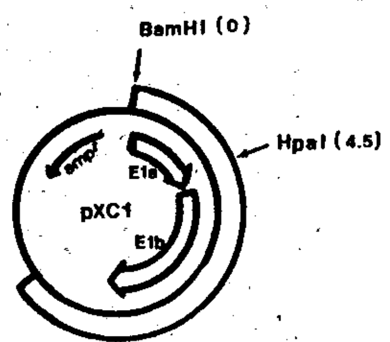
C. pVU16, pHI4

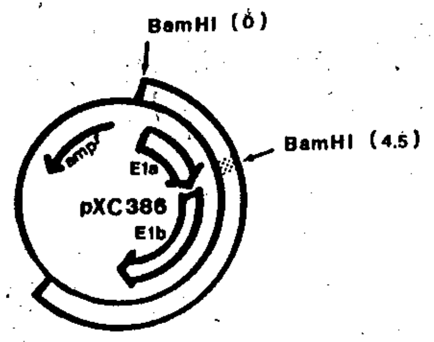
Two additional plasmids, containing the extreme left terminal PvuII fragment (pVU16) and the extreme right terminal HindIII-I fragment (pHI4), were constructed as follows. To construct pVU16 the plasmid pXC1 was digested to completion with PvuII (see Figure 4B) then treated with T4 DNA ligase, and transformed E.coli were screened for colonies containing a 3.1-kbp plasmid with a 454-bp BamHI-PvuII insert. To construct pHI4, the plasmid pFG23 shown in Figure 3A was digested with HindIII and ligated, and transformed E.coli were screened for colonies containing a 5.4-kbp plasmid with the HindIII-I fragment of Ad5 (97-100%) inserted between BamHI and HindIII sites in pBR322.

3.2.4 Structure of the junction between pBR322 and E1 sequences

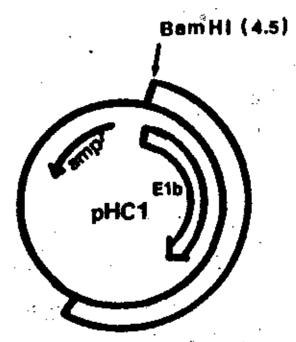
Analysis of the PvuII fragments from viral and plasmid DNA is shown in Figure 6. Digestion of wild type Ad5 DNA with PvuII gives 22 bands resolvable on acrylamide (Figure 6A) and on agarose (Figure 6B) gels. DNA sequence analysis of the left end of Ad5 viral DNA (Tooze, 1981) predicts that the left terminal fragment

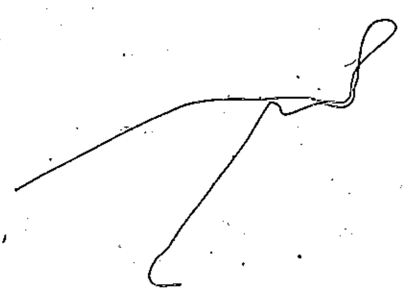
Figure 5. Construction of pXC386 and pHC1. pXC1 DNA was digested with HpaI, ligated in the presence of BamHI decanucleotide fragments, then used to transform E.coli LE392. Of 36 Amp^r colonies screened, one contained a plasmid (pXC386) with two BamHI cleavage sites. The remainder contained pXC1. The E1B plasmid pHC1 (HpaI–C fragment) was constructed from pXC386 by BamHI digestion followed by ligation with T4 DNA ligase. This DNA was used to transform LE392 to Amp^r, and all clones screened (4) contained an 8.4-kbp plasmid with a single BamHI site. Center: sequence of Ad5 DNA at the 3' end (C-terminus) of E1A, showing the translation stop codon at position 1543 and the poly(A) addition signal UUAUUU at position 1611. Shaded sequence represents the decanucleotide insertion (BamHI linker DNA) present at position 1574 in pXC386.


BamHI (0)
HpaI (4.5)
amp
E1a
pXC1
E1b


BamHI (0)
BamHI (4.5)
amp
E1a
pXC386
E1b

BamHI (4.5)
linker)
C terminus; Ela proteins
1550
1570
1610
1620
1630
poly(A) Ela RNAs
CGCCCCAGGCCATAAGGTGT....GTGGTTCCGGATCCGGAAC...TTAATAAAGGGTGAGATAATGTTT


BamHI (4.5)
amp
E1b
pHC1

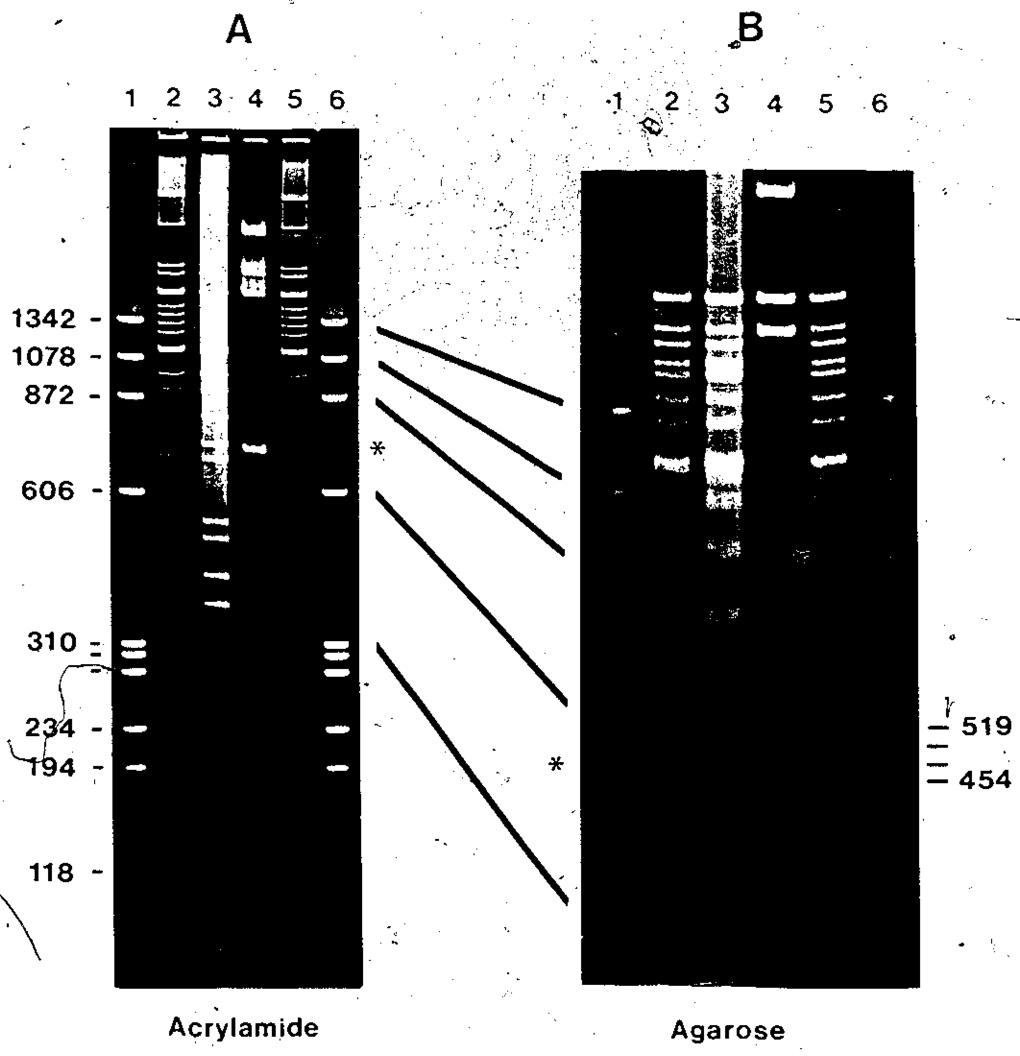


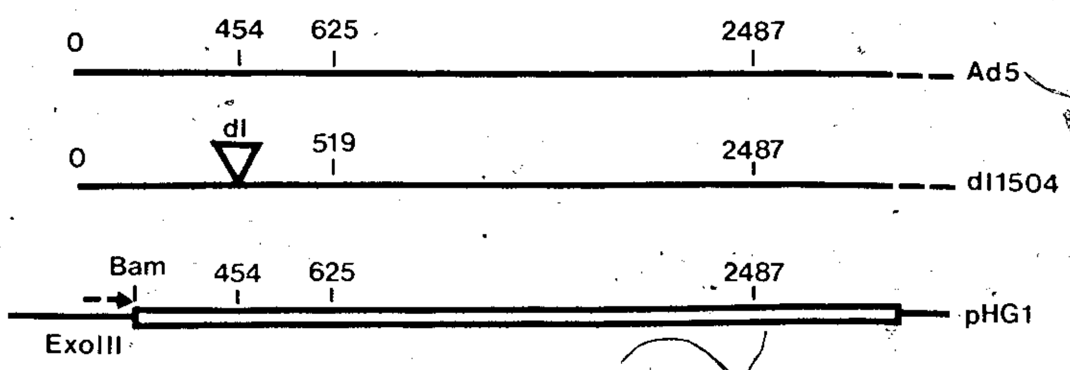
should be 454-bp, with a penultimate 171-bp fragment. The 454-bp fragment is identified in Figure 6B as the lower band that is absent from the deletion mutant virus dl1504 (lane 3) and present in wild type DNA (lanes 2,5). dl1504 has a 106-bp deletion spanning the leftmost PvuII restriction site (Osborne et al., 1982) and is missing both the 454 and 171-bp bands while generating a novel 519-bp fusion PvuII band.

While these bands migrate according to their molecular weight on agarose gels (Figure 6B), they migrate anomalously on acrylamide gels (Figure 6A). The 519-bp fusion band from dl1504 (lane 3) migrates slower than the 606-bp marker band in acrylamide, in contrast to its position relative to this marker band in agarose gels. The 454-bp band from wild type viral DNA comigrates with a 690-bp PvuII fragment which shows increased intensity (lanes 2 and 5). Thus in both wt and mutant virus the leftmost PvuII fragment migrates through acrylamide as though it were approximately 200-bp larger than either the predicted size or the size observed on gels. There are 3 possible explanations for this: (1) a strain difference between the virus sequenced (van Ormondt et al., 1980) and the two virus isolates shown here; (2) effects of residual peptide sequences covalently attached to the 5' ends of viral DNA after proteinase digestion (Rekosh et al., 1977) altering the mobility of these bands in acrylamide but not agarose gels; and (3) secondary structure of the DNA unique to this restriction fragment altering its mobility in acrylamide.

The first possibility can be ruled out since the leftmost PvuII fragments from both wt virus and dl1504 migrate as predicted (from the DNA sequence) on agarose gels (Figure 6B). The second possibility can be ruled out by examining the mobility of the 454-bp fragment from cloned plasmid DNA, as shown in Figure 6 for pHG1. Digestion of pHG1 with BamHI and PvuII gives 5

Figure 6. Structure of cloned left end sequences. Viral DNA (Ad5, dl1504) and plasmid DNA (pHG1) was digested with either PvuII or BamHI plus PvuII and electrophoresed on (A) 5% acrylamide and (B) 2% agarose gels. Lanes 1,6: marker DNA (λ DNA digested with HindIII); lanes 2,5: Ad5 DNA digested with PvuII; lane 3: dl1504 digested with PvuII; lane 4: pHG1 digested with PvuII plus BamHI. Numbers on the left refer to size of marker DNA fragments in base pairs. Numbers on the right refer to the size of the left terminal PvuII fragments from Ad5 DNA and from dl1504 DNA as depicted in the cartoon below. The location of PvuII restriction sites in the left end of Ad5 and dl1504 DNA, and in pHG1, is shown in the cartoon. Numbers indicate distance (base pairs) from the left end.


A
B
1 2 3 4 5 6
1342
1078
872
606
310
234
194
118
519
454
Acrylamide
Agarose


0
454
625
2487
Ad5
0
dl
519
2487
dl1504
Bam
454
625
2487
pHG1
ExoIII

fragments including one band that migrates as approximately 700-bp on acrylamide (Figure 6A, asterisk) and as 475-bp on agarose gels (Figure 6B). Since terminal peptide sequences are not present in the cloned fragment from pHG1, the altered mobility of this band in acrylamide must be due to secondary structure. It should be possible to demonstrate this directly by electrophoresing this DNA on denataturing acrylamide gels, although this was not attempted.

The size of the leftmost cloned PvuII fragment in pHG1 (475-bp, Figure 6B) is surprisingly large since exoIII digestion should have reduced its size relative to the 454-bp band seen in wt viral DNA. The mobility of this band suggested that it was 20-30-bp larger than the wt viral fragment. As will be discussed below this moblilty shift is due to a cloning artifact which resulted in the insertion of a 35-bp sequence of unknown origin between the BamHI linker and the end of the viral DNA after combined exoIII-S1 digestion. This sequence is present in pXC1 and in all plasmids derived from pXC1, and is presumably present in pFG25. Other plasmids containing the BamHI-A fragment were not examined in detail for the presence of this insert, although it is probably unique to pFG25 since this plasmid was selected as described above based on the increased size of the leftmost XbaI-E band relative to those from the other 8 BamHI-A clones (Figure 3B).

3.2.5 DNA sequence analysis

The extent of exonuclease digestion during construction of pXC1 (as shown in Figure 3) was determined by DNA sequence analysis as shown in Figure 7 (panels A-C). DNA sequencing was carried out according to Maxam and Gilbert (1980) using the strategy as shown for pXC386 in the upper cartoon of Figure 7 and as outlined in detail in the legend to Figure 7. The approach was to isolate a DNA

fragment uniquely end labelled with ^{32}P at the left end BamHI site, perform the chemical degradations described in **Section 2.4.8**, and analyse the cleavage products on a 20% 7M urea sequencing gel. The results for sequencing the left end BamHI linker region of pXC386 are shown in panels B and C of Figure 7. Identical results were obtained with a mixture of comparable end labelled DNA fragments from 3 derivatives of pXC1 described in **Chapter IV** (pXC2, pXC15 and pXC16) as shown in panel A of Figure 7. The DNA sequence is interpreted adjacent to each tract in the autoradiogram, and is summarized in the upper cartoon of Figure 7. Several points can be made from these results. First, the BamHI linker fragment 5'-CCGGATCCGG-3' (underlined sequence) is correctly inserted since the sequenced portion (starting from the 5' penultimate G) is as expected. It should be noted that this analysis does not formally rule out the possibility that multiple BamHI linker molecules were ligated next to the end of the exoIII-S1 treated viral termini, since the sequence analysis shown in Figure 7 commences at the rightmost BamHI site at the left end-pBR322 junction. Such a possibility seems remote, however, since the viral DNA with BamHI linkers attached was digested with BamHI prior to insertion into pBR322 (see legend to Figure 3) and this would remove concatameric linkers at the ends.

The second observation from the left end sequence analysis in Figure 7 is that exonuclease III treatment followed by S1 digestion resulted in the loss of 21 nucleotide pairs from the leftmost termini of the viral fragment that was cloned into pFG25. All derivatives of this plasmid (including pXC2, pXC15, pXC16, and pXC386 shown in Figure 8) have lost the first 21-bp of Ad5 DNA in the left terminal repeat and show a 1:1 correspondence from position 22 rightwards with the published sequence for Ad5 DNA (Tooze, 1981). A similar DNA sequence analysis of the right end BamHI linker site in pFG23 (Figure 3A) demon-

Figure 7. DNA sequence analysis of cloned Ad5 DNA. The sequencing strategy for pXC386 is depicted in the upper cartoon. BamHI digested DNA was treated with alkaline phosphatase, then the 1.6-kbp left-end fragment was isolated by gel electrophoresis. DNA was electroeluted into a dialysis bag, ethanol precipitated, then end-labelled with [^{32}P]-ATP and T4 polynucleotide kinase. Labelled DNA was separated from ATP by Sephadex G25 chromatography, ethanol precipitated, digested with SmaI, then electrophoresed on a 5% acrylamide gel. The two end-labelled fragments were located by autoradiography and the acrylamide slice containing ^{32}P DNA was minced and incubated o/n in buffer (**Section 2.4.5**). Finally the DNA was ethanol precipitated from the buffer, dissolved in ddH_2O, and subjected to base specific chemical cleavages (G, A>G, T>C, C) as described by Maxam and Gilbert (1980) except that formic acid was used for the purine cleavage reaction (**Section 2.4.8**). Cleavage products were electrophoresed on sequencing gels as described in **Section 2.4.4**.

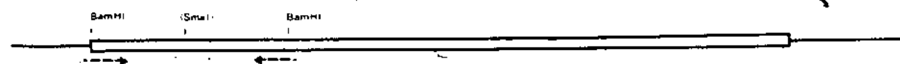
A. 20% acrylamide gel; DNA end labelled at the single left end (0-map units) BamHI site from three plasmids derived from pXC1 (pXC2, pXC15, pXC16) was mixed, cleaved, then run in two sets such that nucleotides 1-22 from the end label (right set) and 8-50 (left set) were visualized.

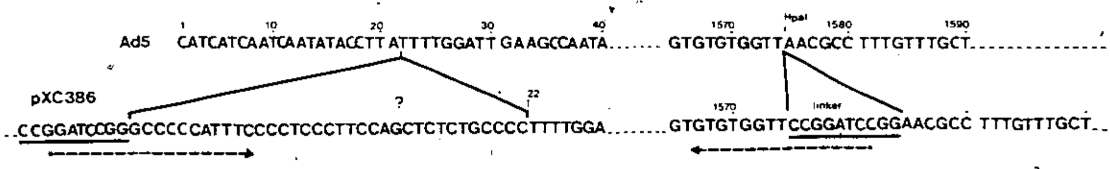
B. 20% gel with DNA end labelled at the 0-map unit BamHI site of pXC386, comparable to the right set in panel A.

C. 20% gel with DNA end labelled at the 0-map unit BamHI site of pXC386, comparable to the left set in panel A.

D,E. 20% gel with DNA end labelled at the 4.5-map unit BamHI site of pXC386.

The top cartoon shows the sequence of Ad5 DNA (1-40 and 1565-1590-bp) and the sequence of the junction between pBR322 and Ad5 DNA in pXC1 derivatives (from panels A,B and C) as well as the sequence accross the BamHI site at 1574-bp in pXC386 (panel D). Arrows indicate the direction of sequencing (bottom of gel is closest to the end label). Underlined sequences represent the decanucleotide linker inserts. (?): sequence of unknown origin (see text).





A B C D E

strated that 24-bp were removed by combined exoIII-S1 treatment during construction of this plasmid (data not shown).

Finally, for all derivatives of pXC1 sequenced to date, a 35-bp sequence of unknown origin is inserted between the BamHI linker and the end of exoIII digestion at position 22. This sequence, shown in Figure 7, bears no homology to either linker DNA or to the end of Ad5 DNA. Since it was apparently ligated into position, after exoIII digestion, in the presence of BamHI linker DNA, it could conceivably be a contaminant sequence present in linker DNA or it may represent the ligation of nucleotides derived from the exoIII digestion of viral DNA (reaction products 5'-pN-3') to each other in random order, although the distribution of bases in this sequence (66% GC) does not reflect the distribution of bases in the first 20 bp of viral DNA (20% GC). The presence of this extra 35-bp at the left end gives a net increase of 21-bp (counting from the BamHI cleavage site) in cloned left end sequences, and explains the size of the leftmost PvuII fragment as seen on agarose gels (Figure 6B, lanes 2 and 4). The presence of these extra sequences presumably has no effect on the biological activity of these plasmids, and have been considered as vector sequences for the purpose of this thesis.

Figure 7 also shows a sequence analysis of the BamHI linker decanucleotide introduced at position 1575 (HpaI site) in pXC386. Panels D and E of Figure 7 show that the linker was inserted as predicted, and that sequences upstream (leftward) of this linker site agree with the published sequence as shown in the upper cartoon in Figure 7. A similar analysis of the rightward boundary of this linker site was also carried out (data not shown). This analysis again does not rule out the possibility of multiple linker inserts. However, this is improbable since digestion with HinfI produces a fragment spanning this linker site in pXC386 with a mobility on acrylamide gels consistent with the anticipated size (data not

shown). Therefore pXC386 contains the decanucleotide BamHI linker molecule properly inserted at the HpaI site in pXC1.

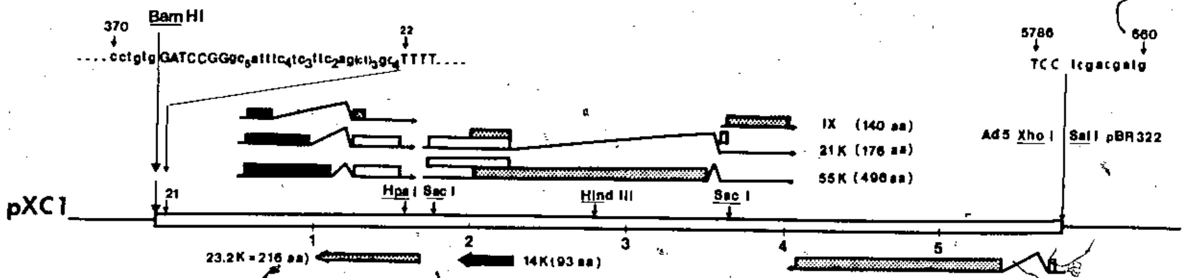
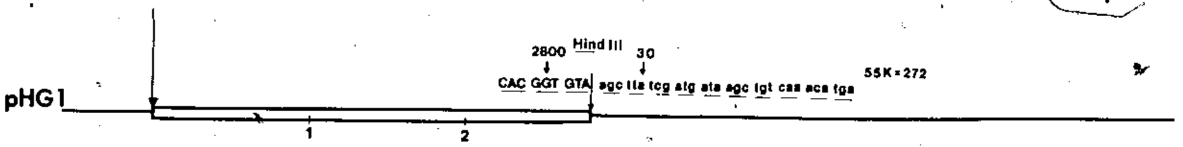
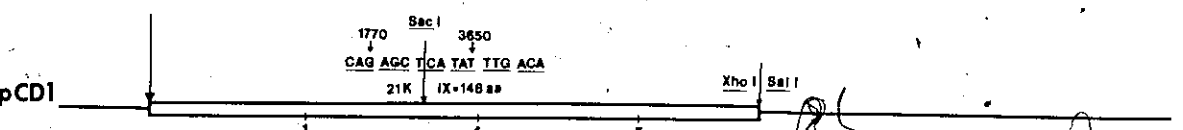
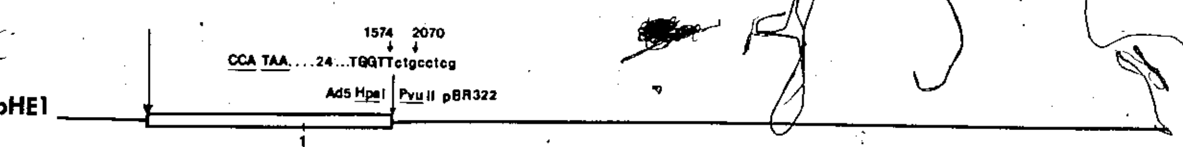
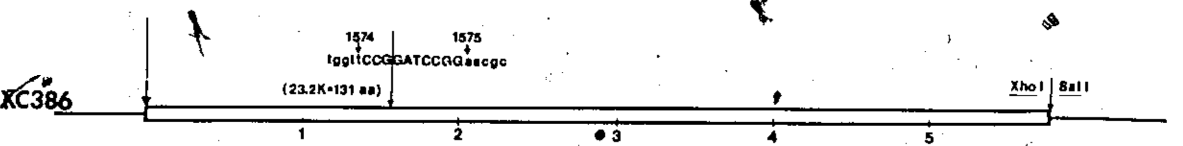
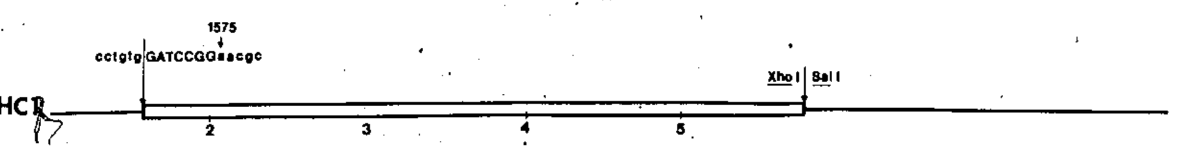
3.2.6 Summary of E1 plasmids

Figure 8 shows a schematic representation of six recombinant plasmids whose construction was detailed above. The structure of the Ad5 genes present in these plasmids will be described as a prelude to studies on the transforming activity of these plasmids examined in **Chapter V**. For orientation purposes the transcriptional map of E1 described in the **Introduction** (see Figure 2) is shown in the top of Figure 8.

The plasmid pXC1 contains all of E1 in addition to the structural component of the gene encoding IVa2. The left boundary of Ad5 and pBR322 DNA is the site of the BamHI linker molecule followed by a 35-bp sequence of unknown origin (discussed above) then Ad5 sequences commencing at position 22 in the left terminal repeat. The right boundary between Ad5 and pBR322 DNA is a ligation between XhoI and SalI cohesive ends producing a sequence that is recognized by neither enzyme. As far as has been determined by restriction endonuclease analysis and by DNA sequence analysis (see **Chapter IV**), the XhoI-C fragment of Ad5 DNA present in pXC1 is structurally identical to that of the published DNA sequence for this region of Ad5 DNA (Tooze, 1981).

The plasmid pHG1 contains Ad5 DNA from nucleotide position 22 to position 2804-bp (the left 8% HindIII-G fragment). All of Ad5 E1A sequences are present in pHG1, as well as the structural coding sequences for the E1B 21k protein and the URF10 product. In addition, the N-terminal sequences of 55k are present, but fusion of Ad5 and pBR322 sequences results in the introduction of 9 new codons preceding a premature termination of 55k which would result in a

Figure 8. Structure of E1 plasmids. Representation of plasmids containing Ad5 DNA (boxed) and vector (single line) sequences. Plasmids are schematically linearized in the vector DNA, and are aligned with the BamHI linker site at 0-map units in pXC1. Top cartoon: pXC1 with a transcription map of E1A and E1B as described in the legend to Figure 2. Junction sequences between pBR322 and Ad5 DNA sequences are presented in lower case, while viral DNA sequences and linker DNA are in upper case letters. Translation codons are indicated by underlined triplets, and where theoretical products are extended by translation of vector sequences these sequences are indicated. Hypothetical products include a 272 aa truncated protein from pHG1 and a 148 aa extended polypeptide IX from pCD1. Nucleotide positions indicated (small arrows) represent the map positions from pBR322 DNA and from Ad5 viral DNA.


370
Bam HI
22
5786
660
TCC tcgacgatg
IX (140 aa)
21K (176 aa)
55K (496 aa)
Ad5 Xho I
Sal I pBR322
pXC1
21
Hpa I
Sac I
Hind III
Sac I
1
2
3
4
5
23.2K = 216 aa)
14K (93 aa)

2800
Hind III
30
CAC GGT GTA agc tta tcg atg ata agc tgt caa aca tga
55K = 272
pHG1
1
2

Sac I
1770
3650
CAG AGC TCA TAT TTG ACA
21K
IX = 148 aa
Xho I
Sal I
pCD1
1
4
5

1574
2070
CCA TAA....24...TGGTTctgcctcg
Ad5 HpaI
PvuII pBR322
pHE1
1

1574
1575
tggttCCGGATCCGGaacgc
(23.2K = 131 aa)
Xho I
Sal I
pXC386
1
2
3
4
5

1575
cctgtg GATCCGGaacgc
Xho I
Sal I
pHC1
2
3
4
5

truncated product of theoretical molecular weight of 30k (272 aa). Note that this plasmid construct lacks Ad5 sequences for termination of the E1B transcript and for polyadenylation, in addition to having lost the 3' splice acceptor site for the 13s E1B mRNA. Since a mRNA product for the l-strand URF10 product has not been mapped it is not possible to predict the structural alterations of this product at present.

The plasmids pCD1 and pHE1 represent two E1A containing constructs. pCD1 deletes most of E1B sequences leaving the 5' and 3' transcription signals intact. By fusing SacI sites at 1774 and 3645-bp, the first 22 codons of 21k in E1B are fused in phase to the 128 C-terminal codons of protein IX producing a theoretical 14k fusion product. On the other hand pHE1 deletes all of E1B sequences as well as IVa2. The HpaI site (1574-bp) is 5' to the transcriptional stop and polyadenylation signals for E1A transcripts, but lies downstream from the stop codon used for E1A translation products. Thus, like the situation for the 21k E1B product in pHG1, all the translational information for E1A products is present in pHE1 but transcriptional termination signals are deleted.

The plasmid pXC386 contains a 10-bp BamHI linker DNA insert at the HpaI site in E1A. As for pHE1, alteration of this site should not affect expression of E1A products. However, it does cause a frameshift of the putative product of URF11 resulting in a missense polypeptide terminating at position 1332 in the l-strand. This is the only alteration of E1 products in this plasmid, since E1A, E1B, and URF10 coding sequences are unaffected by this insertion.

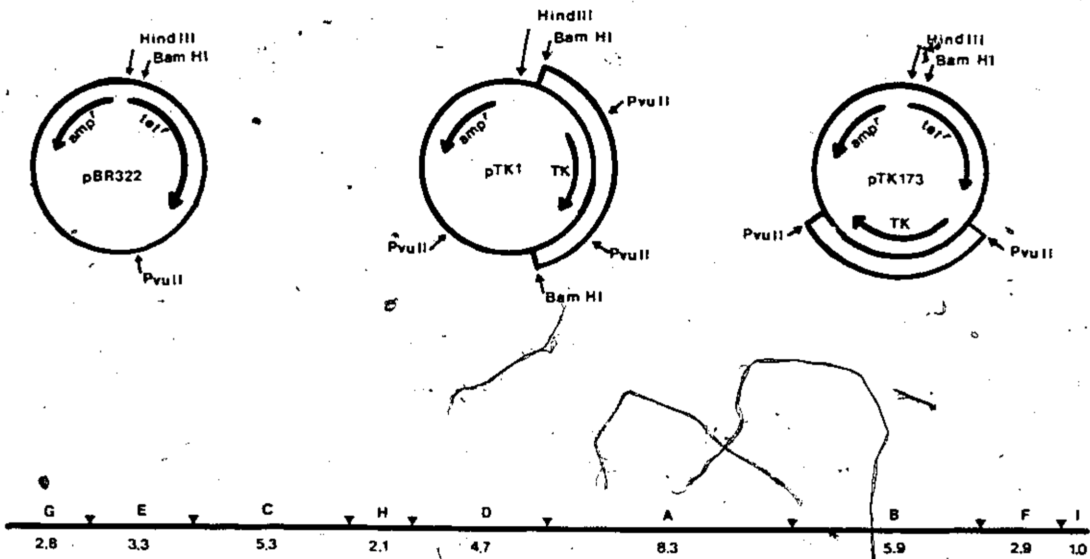
The plasmid pHC1 deletes all of E1A sequences between the BamHI linker at position 22 and the HpaI site at 1577-bp. This plasmid contains the entire E1B region, as well as sequences for IVa2.

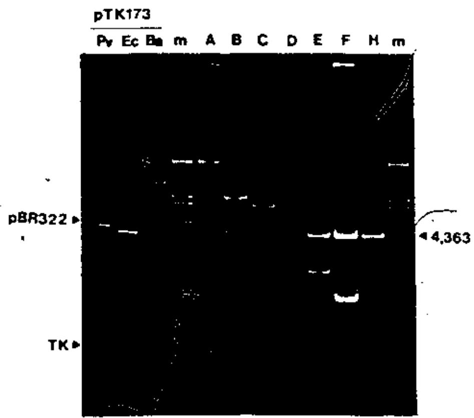
3.2.7 Construction of an Ad5 gene bank

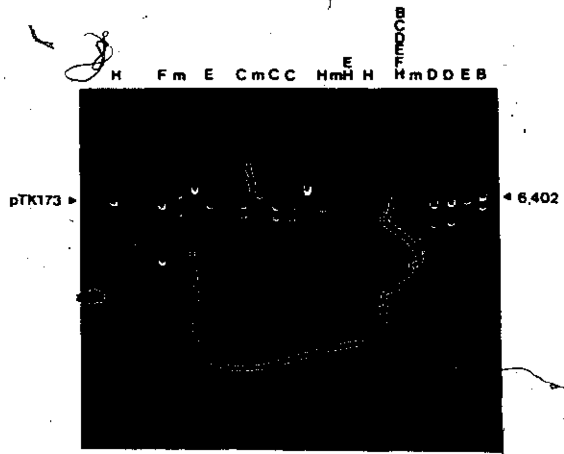
In addition to plasmids containing Ad5 E1 sequences described above, several recombinant plasmids were constructed containing internal Ad5 HindIII fragments. The vector used for construction of these plasmids, pTK173, is shown in Figure 9. pTK173 is a 6.5 kbp recombinant plasmid, constructed by inserting a 2 kbp PvuII fragment containing the HSV-1 (strain KOS) thymidine kinase gene (TK) from the plasmid pTK1 (Graham et al., 1980), into the PvuII site of pBR322 (unpublished results). This vector, analogous to a plasmid pAG0 constructed by Colbere-Garapin et al. (1979), has all the structural features that render its parental plasmid a useful cloning vector. In addition it contains a selectable eukaryotic marker (TK) which allows it to be used to introduce cloned DNA into mammalian cells by cotransfection.

The internal Ad5 HindIII fragments were cloned into the unique HindIII site of pBR322 (Figure 9B) and into the HindIII site of pTK173 (Figure 9C) by ligating a mixture of HindIII digested Ad5 DNA plus vector DNA, transforming E.coli LE392 with ligated DNA, then isolating and screening Amp^r Tet^s colonies for clones carrying the various Ad5 DNA inserts. Each internal HindIII fragment from Ad5 has been isolated in both of these vectors, and in addition multiple inserts have been isolated in the vector pTK173 (Figure 9C). The orientation of these inserts was not determined. The establishment of such a library of TK-Ad5 plasmids should allow the establishment of cell lines containing specific viral DNA fragments by transformation with any one of the plasmids and selection for TK. In principle such cell lines would be useful for isolation of adenovirus host range mutants, analogous to the group I and II hr mutants isolated on 293 cells discussed in Chapter I. This has not been pursued in this work.

Figure 9. Construction of an Ad5 gene bank. Top: structure of pBR322, pTK1 and pTK173. Center: HindIII restriction map of Ad5 DNA. Bottom: recombinant clones containing Ad5 HindIII fragments inserted into (left) pBR322 and into (right) pTK173 vectors. DNA was digested with HindIII and electrophoresed on 1.2% agarose gels, and lanes are marked by the viral HindIII fragment present in each clone. Marker (m): Ad5 HindIII digestion products. Positions of linearized pBR322 DNA (4,363-bp) and pTK173 (6,402-bp) are indicated on their respective gels. The left gel includes three lanes with pTK173 DNA digested with BamHI (Ba), EcoRI (Ec), and with PvuII (Pv) showing the 2-kbp HSV-TK DNA fragment present in pTK173.


HindIII
Bam HI
amp^r
tet^r
pBR322
PvuII
pTK1
TK
Bam HI
PvuII
pTK173
G E C H D A B F I
2.8 3.3 5.3 2.1 4.7 8.3 5.9 2.9 1.0


pTK173
Pv Ec Ba m A B C D E F H m
pBR322
TK
4,363


pTK173
6,402

In summary, the plasmids shown in Figure 8 and Figure 9, as well as the plasmids pHI4 and pVU16 described in Section 3.2.3, represent a complete gene bank of individually cloned Ad5 DNA fragments. The availability of these cloned viral fragments as reagents for both biological and biochemical studies has greatly facilitated many of the research projects initiated in our laboratory during the past three years.

Chapter IV

Tn5 Mutagenesis of Early Region I

4.1 Introduction

A variety of approaches have recently been used for in vitro mutagenesis of cloned genes. For example, plasmid DNA can be linearized in vitro using a unique site restriction endonuclease and deletion mutants can be generated by treatment with either Bal 31 exonuclease (Bryant and Parsons, 1982) or with exonuclease III followed by S1 nuclease (Sakonju et al., 1980), or conversely insertion mutants can be constructed by ligation of synthetic DNA linker molecules to this linearized DNA (McKnight and Kingsbury, 1982). Mutated sequences can be cloned as plasmid DNA in E.coli after transformation with ligated DNA, and the altered sequences characterized by DNA sequence analysis. Even more precise point mutations can be generated in vitro by directed sodium bisulfate mutagenesis (Shortle and Nathans, 1978) or the more elegant technique of synthetic oligodeoxynucleotide generated mutagenesis (Zoller and Smith, 1982).

These techniques represent powerful tools for analysing the functional domains of cloned genes which have been characterized by restriction endonuclease analysis or (preferably) by DNA sequence analysis. They are however of limited value in gene mapping experiments designed to identify a genetic locus within a genome. Recent studies on transposable prokaryotic elements (transposons) suggest that these elements may be useful as mutagens for in vivo genetic engineering (Kleckner et al., 1977). In particular, insertional mutagenesis could be a useful approach to identify genetic loci. Transposable elements are particularly

adaptable for gene mapping since (1) they insert at many sites in bacterial DNA and thus circumvent the limitations of the restriction endonuclease-dependent mutagenesis approaches described above, (2) interrupted genes suffer complete loss of function, (3) the point of insertion can be accurately mapped by physical analysis, and (4) many transposons carry drug-resistance determinants providing a positive selection for mutagenesis in vivo. The properties of bacterial transposable elements have been recently reviewed by Calos and Miller (1980) and Kleckner (1981). As discussed in Chapter I, E1 encodes at least 7 potential gene products that may be involved in transformation. I have used the transposon Tn5 for insertional-mutagenesis of pXC1, as an approach to identifying which of these genes is involved in transformation by this plasmid and in order to construct mutants which should be valuable in further studies on E1 genes. This element has all of the advantages listed above for insertion mutagenesis (see Berg and Berg, 1983 for a recent review of Tn5).

Tn5 is a composite element comprised of inverted repeats of the 1534-bp transposable sequences IS50 (Berg et al., 1982a) surrounding a central 2400-bp unique segment encoding the kanamycin resistance determinant (Jorgensen et al., 1979; Beck et al., 1982) as shown in Figure 11. The Kan^r determinant is also a dominant selectable marker in eukaryotic cells ($G418^r$, Colbere-Garapin et al., 1981, Southern and Berg, 1982). The central unique region of Tn5 also encodes a streptomycin resistance determinant expressed in Rhizobium meliloti (Putnoky et al., 1983). The rightmost repeat, IS50R, encodes both the transposase protein (Rothstein et al., 1980) and a smaller repressor protein (Johnson et al., 1982; Isberg et al., 1982; Lowe and Berg, 1983) translated from the same reading frame. The leftmost repeat, IS50L, is identical to IS50R except for a single base pair nonhomology which results both in premature termi-

nation of transcription of repressor and transposase and creation of an IS50L encoded promoter proximal to the neomycin resistance gene (Rothstein and Reznikoff, 1981).

The evidence to date suggests that Tn5 transposition is conservative and results in the loss of donor DNA sequnces (Berg et al., 1982b; Berg, 1983). This is in contrast to the semi-conservative (replicative) models of transposition (Shapiro, 1979; Arthur and Sherratt, 1979) proposed for transposition of elements such as Tn3, which encode a site specific recombination system capable of resolving cointegrate structures formed as a consequence of relicative transposition (Kostriken et al., 1981; Reed, 1981; Kitts et al., 1982).

This chapter describes the construction and mapping of derivatives of pXC1 containing the kanamycin resistance element Tn5. A library of mutants with Tn5 mapping within E1 sequences was characterized by DNA sequence analysis as a prelude to studies on the transforming activity of these mutants presented in **Chapter V**. These studies represent the first report of genetic mapping experiments involving in vivo mutagenesis of cloned eukaryotic viral genes using a prokaryotic transposable element, and illustrate the use of transposons for mapping the coding boundaries of cloned eukaryotic genes.

4.2 Results and Discussion

4.2.1 Mutagenesis of pXC1

A. "Lambda kan hopper"

Tn5 insertion events in pXC1 DNA were obtained using the protocol shown in Figure 10 using the λ::Tn5 transducing phage λ467 (λ kan hopper). This phage is unable to integrate upon infection because the phage attachment site is deleted, and it cannot replicate because of two amber mutations (Oam 29 and Pam80) which affect functions essential for viral replication (Lewin, 1977). These amber mutations restrict growth of this phage to cells carrying the suppressor tRNA sup6 (J.Friesen, personal communication). As shown in Table 4.1, λ kan hopper can replicate on LE392 (supE, supF), but only if the phage stock used for the infection is grown on JF1106 (sup6). Presumably supE (glycine insertor) and/or supF (tyrosine insertor) can suppress the O and P amber mutations, albeit less efficiently than sup6 (leucine insertor) since plaques on LE392 cells are much smaller than those on JF1106.

B. Transposition

After infection of LE392pXC1 with λ kan hopper as shown in Figure 10, approximately 10^5 Amp^r Kan^r colonies were obtained as estimated by plating a serial dilution of infected cells on agar with Amp and Kan. Assuming that Kan^r colonies represent transposition events from λ kan hopper DNA to E.coli or plasmid DNA it is possible to estimate the number of pXC::Tn5 isolates in this population. The E.coli chromosome is 4.6×10^6 base pairs, of which approximately 50% encodes indispensable functions (Bukhari, 1976). Therefore the "available" E.coli DNA for transposition is 2.3×10^6-bp. The available DNA in pXC1 is 9.2×10^3-bp,

Table 4.1: Lytic Growth of Lambda-467

titered on	sup	phage grown in LE392	phage grown in JF1106
KY895	sup⁻	0	0
LE392	E,F	0	$2x10^6$
JF1106	sup6	$1x10^7$	$3x10^6$

Titers of phage stock prepared from either LE392 or from JF1106 cells and titered on the listed strains. sup: suppressor genotype of strain (see Table 2.1)

assuming β-lactamase and the origin of replication are essential sequences for cell survival in the presence of ampicillin, and assuming one copy of pXC1 per cell (a minimun estimate). Therefore the proportion of transposition events leading to pXC::Tn5 insertions is $4x10^{-3}$, or 1 in 250 events. Thus this population of Amp^r Kan^r cells should contain a minimum of 400 independent pXC::Tn5 insertion events.

In order to isolate plasmid insertion events from chromosomal insertions, plasmid DNA was purified from these cells and used to transform Amp^s Kan^s-LE392 cells to Amp^r Kan^r (Figure 10). Since kanamycin is an inhibitor of

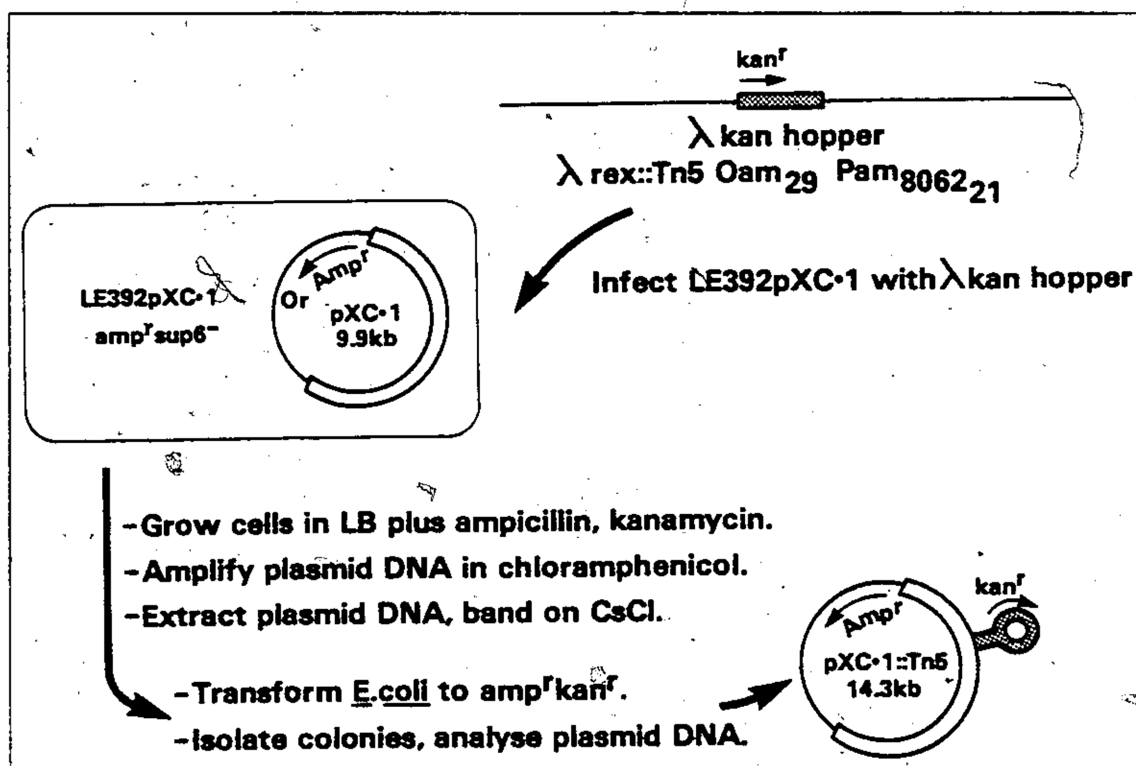


Figure 10: Tn5 transposition with Lambda-467

E.coliLE392 pXC1 (Amp^r) cells were infected at an moi of 0.1 pfu per cell in 10mM $MgSO_4$ for 20 min at room temperature with the lambda derivative λ kan hopper. Cells were pelleted to remove unadsorbed phage, resuspended in 500 ml broth containing ampicillin, and incubated at 37°C with shaking for 3 h (A660<.8) at which time they were collected by centrifugation, plated on agar containing both ampicillin and kanamycin, then incubated overnight. Amp^r Kan^r cells were scraped off the plates and resuspended in 1.31 Luria broth with ampicillin and kanamycin, grown to A660=.08, then chloramphenicol was added (50 µg/ml) and incubation was continued o/n. Plasmid DNA was extracted (**Section 2.4.1**), banded on CsCl-EtBr density gradients, recovered from the gradient, dialysed, and finally used to transform LE392 cells to Amp^r Kan^r. Individual clones were screened for the presence of Tn5 sequences in pXC1 DNA by endonuclease digestion of plasmid DNA and gel electrophoresis (see text).

protein synthesis it was necessary to preincubate transformed cells in LB containing only Amp (no Kan) for 30 min to allow cells to express the Tn5 encoded neo^r function (Table 4.2). Directly plating transformed cells on agar containing Amp and Kan resulted in 20-fold less Amp^r Kan^r bacteria, consistent with the hypothesis that expression of the Tn5 encoded neo^r gene is required to release the block in protein synthesis caused by kanamycin.

Table 4.2: Transformation of LE392 with pXC::Tn5 DNA

selection	colonies/ug DNA
Amp^r	$2x10^5$
Amp^rKan^r (a)	250
Amp^rKan^r (b)	10

LE392 cells transformed with pXC::Tn5 DNA were plated on Luria agar plates containing either amp or amp + kan. Amp + kan: cells plated either with (a) or without (b) 30 min preincubation at 37°C in LB+ amp to allow expression of Tn5 encoded neo.

4.2.2 Mapping Tn5 in pXC1 DNA

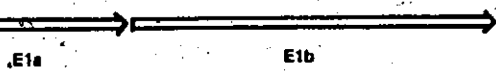
Plasmid DNA from individual Amp^r Kan^r colonies was analysed by digestion with BamHI plus PvuII and electrophoresis on 2% agarose gels. As shown in Figure 11, these enzymes cut pXC1 at 7 sites including the single PuvII site in pBR322 sequences. The lower cartoon in Figure 11 schematically represents an insertion event with Tn5 transposed into pXC1 at a site in E1B sequences. PvuII cuts Tn5 at 4 sites including twice in the inverted repeats, and BamHI cuts once in the unique region of Tn5 DNA. Analysis of 144 Amp^r Kan^r colonies revealed 57 clones with single insertions of Tn5 into pXC1 DNA as indicated by the absence of a single band corresponding to a pXC1 fragment and the presence of the predicted Tn5 fragments including two bands corresponding to the junction of pXC1 and Tn5 sequences. The remaining [illegible]es screened gave ambiguous results and were not analysed further.

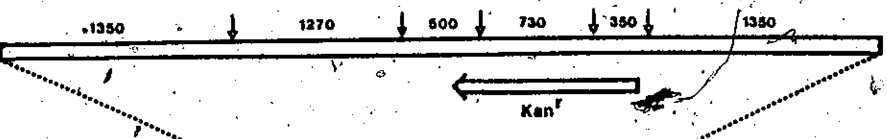
The distribution of integration sites observed in these 57 pXC::Tn5 plasmids is summarized in Figure 12. Since the 2670-bp fragment containing β-lactamase and the origin is essential for replication in LB plus Amp, a low frequency of Tn5 insertions into this region was expected. Indeed, only 2 events mapped to the 2670-bp fragment, both presumably located in nonessential pBR322 sequences. Ad5 DNA, on the other hand, should be nonessential for plasmid replication and consequently a uniform distribution of insertion events was expected within this region of pXC1. Instead, as shown in Figure 12, there was an overrepresentation of Tn5 insertions in the 1482-bp band and fewer events were observed in the 1862-bp band than would be expected assuming Tn5 transposition occurs at random.

Since PvuII cleaves Tn5 once within each inverted repeat (Figure 11), it is not possible to distinguish the right and left junction fragments in pXC::Tn5

Figure 11. Mapping Tn5 in pXC1 DNA. Cartoon representation of pXC1 DNA (top) and of pXC::Tn5 DNA (bottom) schematically linearized at the single PvuII site in pBR322 sequences (thin line). Ad5 DNA is shown as a hatched box, while Tn5 is represented as an open box. Open arrows under constructs represent transcription units for pBR322 (Amp^r), for Tn5 (Kan^r), and for Ad5 (E1A, E1B). Small arrows represent PvuII (open) and BamHI (closed) restriction sites, and sizes of expected fragments are shown in base-pairs above respective fragments. Bottom construct shows Tn5 inserted into the 1482-bp Ad5 PvuII fragment, with the 1350-bp PvuII fragments at the ends of Tn5 covalently attached (dotted lines) to Ad5 sequences.

pXC·1
2670
625
1862
1482
1046
2300
Origin
Amp^r
E1a
E1b




IS50R
IS50L
1350
1270
500
730
350
1350
Kan^r

pXC::Tn5
2670
625
1862
1046
2300

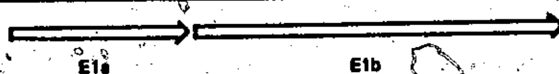
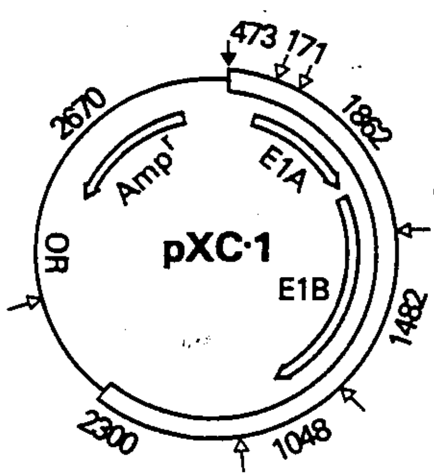

Amp^r
E1a
E1b

Figure 12. Distribution of Tn5 insertions in pXC::Tn5 plasmids. Plasmid DNA was digested with PvuII plus BamHI and electrophoresed on 1.2% agarose gels. pXC::Tn5 plasmids were identified by the presence of the predicted bands as shown in Figure 11, including two junction fragments. Assignment of Tn5 to within one pXC1 fragment was based on the absence of a single pXC1 band. Expected distribution is based on random integration of Tn5 into pXC1 DNA assuming that the 2670-bp fragment was unavailable for insertion as described in the text. Number of insertions mapped=57.

Location of Tn5 Insertions in pXC·1



Fragment size (b.p.)	No. Insertion Events expected[1]	observed[2]
2670		2
2300	17	18
1862	14	2
1482	11	23
1048	8	12
473	5	0
171	1	0

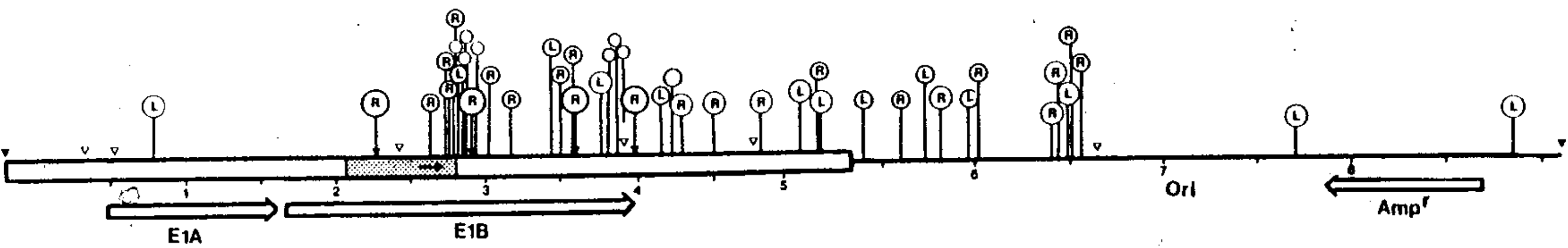
1. Assuming random integration of Tn5 into pXC·1.
2 Number of insertions mapped = 57.

plasmids by analysis using this enzyme. Thus the above analysis serves only to locate Tn5 insertions within one of the seven BamHI plus PvuII fragments of pXC1, and does not precisely map the position of Tn5 in these plasmids. Therefore the location, and the orientation, of Tn5 in a number of pXC::Tn5 plasmids was more accurately mapped using the endonucleases HindIII and SmaI (Figure 13). After digestion with each of these enzymes, plasmid DNA was electrophoresed on 1.2% agarose gels (not shown) and the mobility of the junction fragments was determined. The location of the 5.7-kbp Tn5 insert is represented for each mutant in Figure 13 as a vertical bar, with the orientation of Tn5 in the individual plasmid isolate encircled above. Those mutants which were not analysed for orientation are depicted by small circles in Figure 13.

The results of this mapping study illustrate that a preferred region for Tn5 insertion maps between 2500-bp and 4500-bp in the Ad5 DNA insert in pXC1. This preferred target region is immediately downstream from a fortuitous prokaryotic promoter sequence in Ad5 E1B (J.Waye and F.Graham, personal communication). The minimum DNA fragment containing promoter activity for an E1B-lacZ fusion gene in plasmid pFG66 (constructed by F.Graham) was defined as the shaded region of pXC1 in Figure 13 (McKinnon et al., submitted). The implication of these findings for the mechanism of Tn5 transposition will be discussed in **Section 4.3.**

Several transposition events in Ad5 E1 sequences contained in pXC1 were selected for further study based on their distribution throughout E1. Five plasmids with Tn5 mapping in E1B, and a single E1A mutant, were more closely mapped using the restriction endonucleases shown in Figure 14. The upper cartoon in Figure 14 shows the approximate location of Tn5 in these mutants relative to the transcription map of early region E1. Mapping by this type of analysis is pre-

Figure 13. <u>Position and orientation of Tn5 in pXC::Tn5 plasmids.</u> Representation of pXC1 linearized at the unique BamHI site (0-map units in Ad5 DNA sequences), showing positions of E1A, E1B, Amp^r, and origin of plasmid DNA replication sequences. The location of 44 Tn5 insertions represented by circles above pXC1 DNA was determined by digestion of plasmid DNA with HindIII and digestion with BamHI plus PvuII and electrophoretic analysis of the size of the junction fragments. The orientation of Tn5 was determined by SmaI digestion for those inserts shown: L, IS50L maps closest to the left end of E1; R, IS50R maps closest to the left end. Inserts that were not mapped for orientation are shown by small open circles. Mutants which have been mapped by DNA sequencing are shown by large circles, and positioned with closed arrows. Closed triangles: BamHI site; open triangles: PuvII sites. Hatched region: minimum Ad5 DNA fragment known to contain a fortuitous promoter sequence for an E1B-lacZ fusion gene (see text); small arrow shows possible location of 5' end of E1B transcript in E.coli. (J.Waye, personal communication).


E1A
E1B
Ori
Amp^r
1
2
3
4
5
6
7
8

cise to within about 200 bp due to the error involved in sizing DNA fragments larger than 1-2-kbp after acrylamide gel electrophoresis. A more exact determination of the Tn5 locations by direct DNA sequence analysis will be presented below.

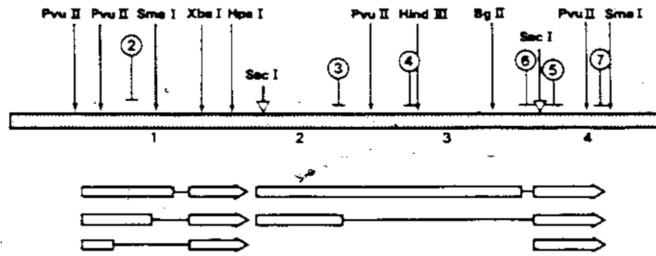
4.2.3 Mutagenesis of pHE1

As discussed in **Chapter I**, mutations in E1A are of particular interest since this region is involved in both regulation of viral gene expression and in cell transformation. Unfortunately, the distribution of Tn5 insertions in pXC::Tn5 isolates was biased against insertions in E1A. Therefore Tn5 transposition was repeated using the plasmid pHE1 as a target for insertion. Since this plasmid contains only E1A sequences and the region of pBR322 DNA essential for replication in selective medium, any Amp^r Kan^r colonies should represent E1A insertions. Using the protocol for E1A mutagenesis shown in Figure 15A, 5 insertion mutants were isolated mapping between EcoRI and XbaI. These mutants were more precisely mapped using the restriction endonucleases shown in Figure 15B, and the locations of these E1A mutants relative to the transcription map of E1 is shown in Figure 15C.

4.2.4 Reconstruction of E1B in pHE::Tn5 plasmids

In order to assay the transforming activity of these E1A mutants (see **Chapter V**) it was necessary to reintroduce E1B sequences into the pHE::Tn5 plasmids. The protocol for E1A insertional mutagenesis shown in Figure 15A was designed such that it would be possible to rescue these mutants into pXC1 using the restriction endonucleases EcoRI and XbaI. These two enzymes cut once each in pHE1 and do not cut Tn5. Thus pHE::Tn5 DNA was digested with EcoRI and XbaI, mixed with a 10-fold molar excess of pXC1 DNA digested with the same enzymes, and ligat-

Figure 14. Location of E1 insertions. Plasmid DNA was digested with the indicated enzymes and electrophoresed on a 5% acrylamide gel. Lanes (1): pXC1; lanes (2)-(7): pXC2-pXC7. Upper cartoon shows the transcription map of E1 (as described in the legend to Figure 2) and shows the approximate location (horizontal bars) of Tn5 in six pXC::Tn5 mutants (represented by circles numbered 2-7).


Pvu II
Pvu II
Sma I
Xba I
Hpa I
Sac I
Pvu II
Hind III
Bgl II
Sac I
Pvu II
Sma I
1
2
3
4

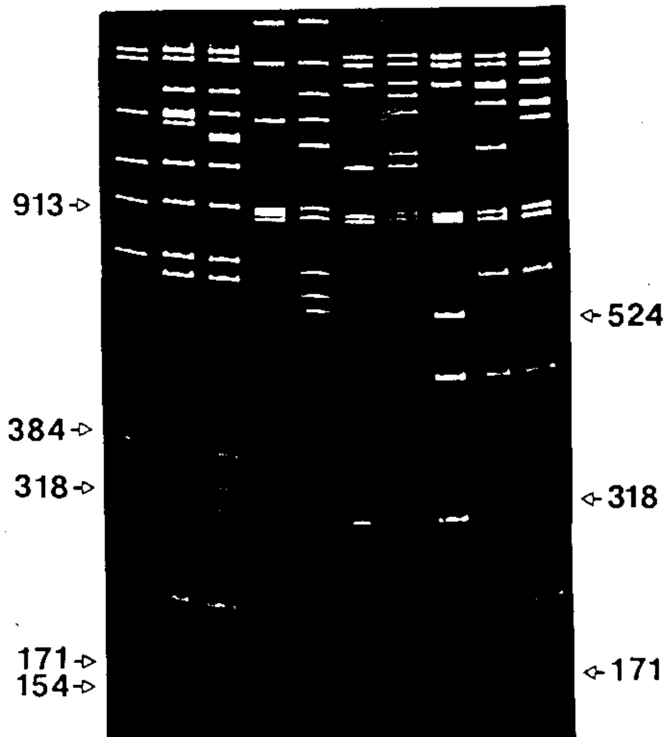
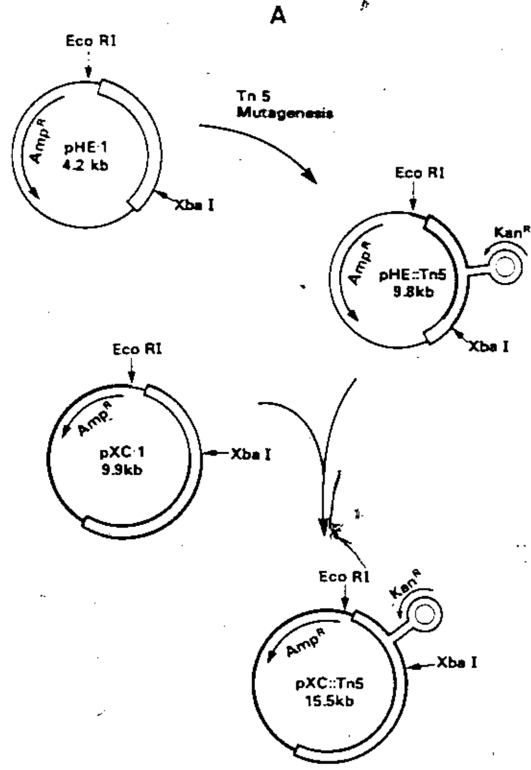
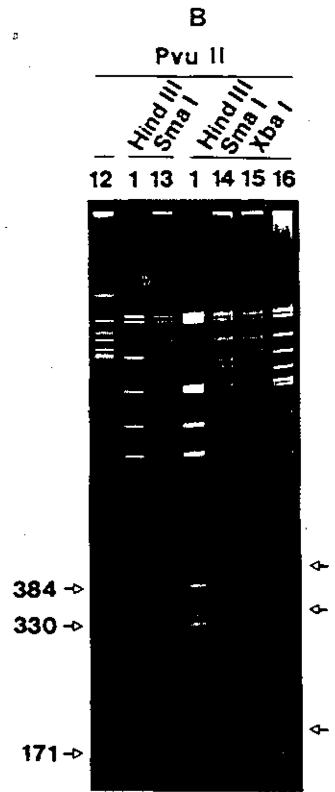

Pvu II
Hind III
Sma I
Hpa I
Hind
Hind III
Bgl II
1 2 7 1 3 1 4 1 5 6
913
384
318
171
154
524
318
171

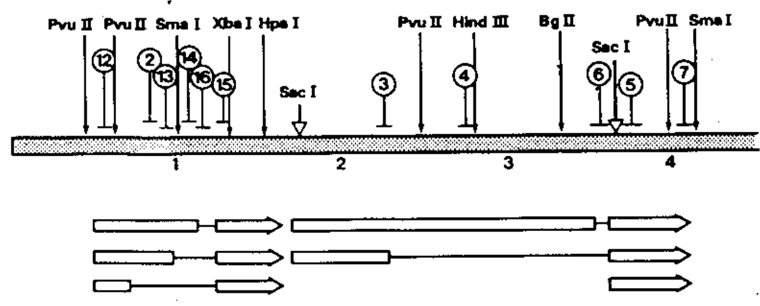
Figure 15. Tn5 mutagenesis of pHE1.

A. Protocol for mutagenesis of pHE1 and rescue of E1A insertion mutants into pXC1. E.coli. LE392 cells were infected with λ467 and Amp^r Kan^r cells were isolated as described in the legend to Figure 10. Plasmid DNA extracted from these cells was used to transform LE392, and individual Amp^r Kan^r colonies were screened for the presence of Tn5 as described in the legend to Figure 12. Five clones (pHE12-pHE16) containing Tn5 in E1A were chosen, and E1B sequences were reconstructed in these plasmids by digestion with EcoRI plus XbaI, mixing these individual digests with a 5-fold molar excess of similarly digested pXC1 DNA, ligating these five samples, then transforming LE392 to Amp^r Kan^r using ligated DNA. Colonies were screened for pXC::Tn5 plasmids derived from these 5 initial pHE::Tn5 isolates by digestion of plasmid DNA with HindIII and electrophoresis on a 1.2% agarose gel. Of 4 colonies screened for each mutant, 2-3 clones represented pXC::Tn5 derivatives in each case.

B. Plasmid DNA from pXC::Tn5 isolates was digested with the indicated enzymes and electrophoresed on a 5% acrylamide gel. Lanes (1): pXC1; lanes (12)-(16): pXC12-pXC16. Lower cartoon summarizes the location of the 6 mutants described in Figure 14 as well as the location of the mutants derived from pHE::Tn5 plasmids as described in A above.


A
Eco RI
pHE·1
4.2 kb
Xba I
Tn 5
Mutagenesis
pHE::Tn5
9.8kb
Kan^R
pXC·1
9.9kb
pXC::Tn5
15.5kb


B
Pvu II
Hind III
Sma I
Xba I
12 1 13 1 14 15 16
384
330
171


Pvu II
Sma I
Xba I
Hpa I
Sac I
Hind III
Bg II
Sma I
1
2
3
4

ed with T4 DNA ligase as described in the legend to Figure 15. After transformation of LE392, Amp^r Kan^r colonies were screened for clones carrying pXC::Tn5 plasmids.

Figure 15C summarizes the location of eleven pXC::Tn5 plasmids that are further characterized in this thesis. Six E1A mutants were used in transformation experiments (**Chapter V**), including five mutants obtained by pHE1 mutagenesis (mutants 12-16) and one obtained by pXC1 mutagenesis (mutant 2). In addition, five E1B mutants obtained by pXC1 mutagenesis (mutants 3-7) were selected for transformation experiments based on their location in different positions in E1B. These eleven pXC::Tn5 plasmids represent a library of mutants with Tn5 located throughout early region E1.

4.2.5 DNA sequence analysis

The above restriction endonuclease analysis can only map the location of Tn5 in these mutants to within 150-200-bp in Ad5 sequences, and represents a first approximation based on sizing of DNA fragments on acrylamide gels. Since the organization of E1 coding sequences is complex (Figure 2), it was necessary to precisely map the position of Tn5 in these mutants in order to unambiguously interpret the results of transformation assays (**Chapter V**). Therefore the exact location of Tn5 in a number of pXC::Tn5 mutants depicted in Figure 15 was precisely mapped by DNA sequence analysis using the protocol of Maxam and Gilbert (1980) as described in **Section 4.2.8.**

The strategy used to sequence the insertion site of two of these mutants, pHE15 and pHE16, is shown in the upper cartoon of Figure 16. DNA fragments spanning both the left and the right junction between Ad5 and Tn5 DNA were isolated from both of these plasmids, and after end labelling and

Figure 16. DNA sequence analysis of Tn5 insertion sites: pHE15 and pHE16. The strategy for sequencing two E1A insertion mutants, pHE15 and pHE16, is shown in the upper cartoon. For each mutant the right junction site between Tn5 and Ad5 DNA was sequenced by digesting 500 μg of DNA with XbaI, treating with alkaline phosphatase then γ-[^{32}P] ATP and T4 polynucleotide kinase as described in the legend to Figure 7. ^{32}P-labelled DNA was then digested with BamHI, electrophoresed on a 5% acrylamide gel, and the recovered end labelled DNA was chemically cleaved as described in the legend to Figure 7. Cleavage products were electrophoresed on an 8% sequencing gel for pHE16 (panel B) and on a 20% gel for pHE15 (panel D). The Left junction for these two mutants was sequenced by digesting plasmid DNA with BamHI plus XhoI, isolating the 2-kbp left end-Tn5 junction fragment by agarose gel electrophoresis, and treating this DNA with HinfI then alkaline phosphatase as described above. The HinfI junction fragment was purified from acrylamide gels, end labelled with ^{32}P, cleaved with HhaI, and finally electrophoresed on a 5% acrylamide gel in order to obtain DNA uniquely end labelled at the HinfI site proximal to the junction site. After recovery of end labelled fragments they were chemically cleaved then electrophoresed on a 20% sequencing gel, shown in panel A for pHE16 and in panel C for pHE15.

Top. The DNA sequence of Ad5 DNA in E1A between 1250-bp and 1330-bp (lower sequence), showing the 9-bp duplicated "target" sequence (underlined) for Tn5 insertion in mutants pHE16 and pHE15. The sequence above illustrates the structure of the left and right junction for each of these mutants, showing the 9-bp target sequence and 6-bp of the left and right termini of Tn5. Shaded regions represent Tn5 sequences.

Hinf I   Xba I

GAACCGGAG CTGACT......AGTCAG GAACCGGAG
CTTGGCCTC GACTGA*......TCAGTC CTTGGCCTC
1272

ATCCTGAGA CTGACT......AGTCAG ATCCTGAGA
TAGGACTCT GACTGA*......TCAGTC TAGGACTCT
1320

16   15

1260   1280   1300   1330

----TGAGCCCGAG CCAGAACCGG AGCCTGCAAG ACCTACCCGC CGTCCTAAAA TGGCGCCTGC TATCCTGAGA CGCCCGACAT----
----ACTCGGGCTC GGTCTTGGCC TCGGACGTTC TGGATGGGCG GCAGGATTTT ACCGCGGACG ATAGGACTCT GCGGGCTGTA----

A   B   C   D

G G G C T C G G T C T T G G C C T C
1272
G A C T

G G A C G A T A G G A C T C T
1320
G A C T

T C A G T C T A G G A C T C T G C G G G C T G T A G T G

chemical cleavage as described in the legend to Figure 16 the products were resolved on sequencing gels as shown in Figure 16. Using this sequencing strategy only one DNA strand for each of these mutants was sequenced. However, the observed sequences are consistent with the corresponding regions of the published sequence of Ad5 (Tooze, 1981) and the ends of Tn5 (Auerswald et al., 1981), and can be interpreted unambiguously. The sequence analysis shown in Figure 16 reveals that for both mutant's Tn5 transposition generated a 9-bp direct repeat of plasmid DNA sequences at the site of insertion, consistent with published observations on Tn5 transposition (Schaller, 1980).

The two mutants pHE15 and pHE16 analysed in Figure 16 represent unique isolates with Tn5 mapping within 50-bp of each other just downstream of the 3' splice acceptor site common to all 3 E1A mRNAs (see Figure 19). Although the insertion sites in pXC15 and pXC16 (derived from pHE::Tn5 mutants as shown in Figure 15A) were not sequenced, it can be assumed that there has been no rearrangement of Tn5 in these derivatives since a restriction analysis of these plasmids shown in Figure 15B gives the expected fragments.

The location of Tn5 in the 5 remaining mutants sequenced (pHE14, pXC3, pXC4, pXC6 and pXC7) was determined as shown in Figure 17. The strategy for sequencing the insertion site in these mutants is described in the legend to Figure 17, and depicted in the cartoon in this figure. For all mutants sequenced, Tn5 maps within 200-bp of the predicted location from the restriction enzyme analysis presented in Figures 14 and 15, and no discrepancies were detected for the corresponding regions of the published sequence of Ad5 or Tn5. For the mutants analysed in Figure 17, only one junction (either left or right) was sequenced, and it is assumed that the unsequenced junction for each of these mutants would also contain the 9-bp duplication of target sequences known to be

generated by Tn5 transposition (Schaller,1978) and directly demonstrated for pHE15 and pHE16 (Figure 16). Four of the E1 mutants that are characterized in **Chapter V** (pHE12, pHE13, pXC2 and pXC5) were not mapped by DNA sequencing.

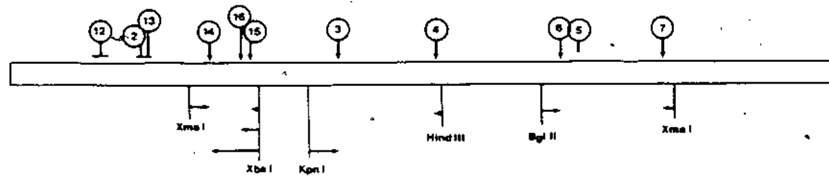
The Ad5 DNA sequences at and surrounding the target site for Tn5 insertion in the 7 mutants sequenced is shown in Figure 18. This figure represents the 9-bp target site duplicated during transposition in bold face, and shows Ad5 sequences for 40-bp bracketing the insertion site. Analysis of these target DNA sequences revealed no sequence specificity for Tn5 insertion, as will be discussed further in **Section 4.3.1.** A summary of the location of Tn5 in the E1 mutants with respect to viral transcripts is presented below.

4.2.6 Summary of Tn5 mutants mapping in E1

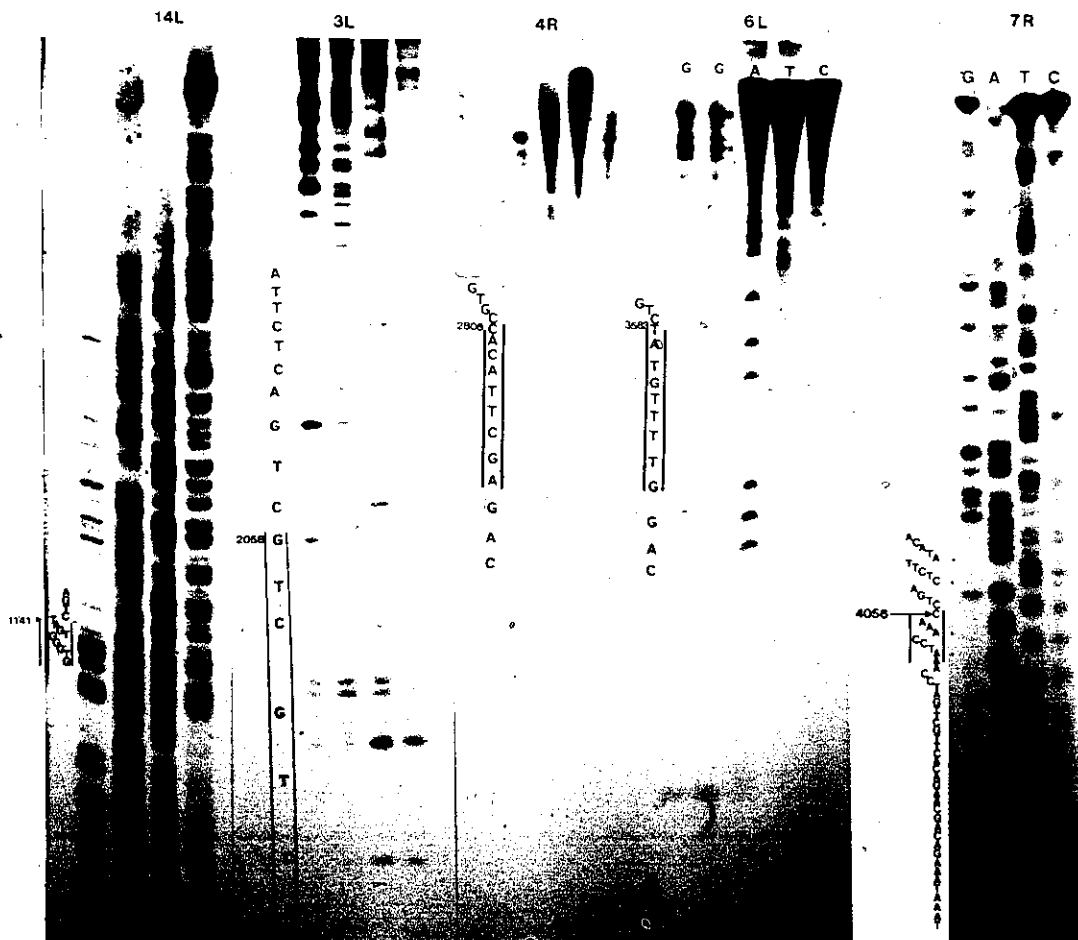
Figure 19 summarizes the location of the 11 pXC::Tn5 mutants characterized for transforming activity in **Chapter V.** Four of these mutants (pXC12, pXC14, pXC6, and pXC7) are located in nontranslated regions of E1. The plasmid pXC12 mapped by restriction analysis to the region of the E1A promoter, although sequencing would be required to rule out the possibility that it actually maps within the amino-terminal region of these products. Two mutants, pXC14 and pXC6, are located within intervening sequences spliced out of messages from E1A and E1B, respectively. The fourth mutant, pXC7, is located between the poly(A) addition sequence AAUAAA and the wild type poly(A) addition site at the 3' end of E1B transcripts.

For the mutants mapping within E1 protein coding sequences (pXC2, pXC13, pXC15, pXC16, pXC3 and pXC4), insertion of Tn5 introduces a stop codon mapping in the ends of Tn5 downstream from the site of insertion. For mutants pXC15 and pXC16, this results in the truncation of the E1A 12s and 13s

Figure 17. DNA sequence analysis of pHE14, pXC3, pXC4, pXC6 and pXC7. The strategy used to sequence one junction site only for each of pHE14, pXC3, pXC4, pXC6 and pXC7 was to isolate DNA uniquely end labelled at the restriction site indicated for each mutant in the top cartoon, using the approaches outlined in the legend to Figure 16 for pHE15 and pHE16. End labelled DNA was then chemically cleaved as described in the legend to Figure 7 and electrophoresed on 6% sequencing gels (14L, 7R) or on 20% sequencing gels (3L, 4R, 6L) as described. The 9-bp target site is marked for each mutant by vertical bars on the sequencing tracts, and the point of insertion in Ad5 DNA is indicted. Extra bands in panel 3L are cleavage products from ATP, since this mutant was sequenced from an end labelled nucleotide within the 9-bp repeat. L and R refer to the junction of each mutant which was sequenced. The top cartoon illustrates which of the 11-E1 mutants shown in Figure 15 have been mapped by restriction analysis (horizontal bar attached to circle) and mapped by DNA sequence analysis (arrow attached to circle).



12
2
13
14
16
15
3
4
6
5
7
Xma I
Xba I
Kpn I
Hind III
Bgl II
Xma I



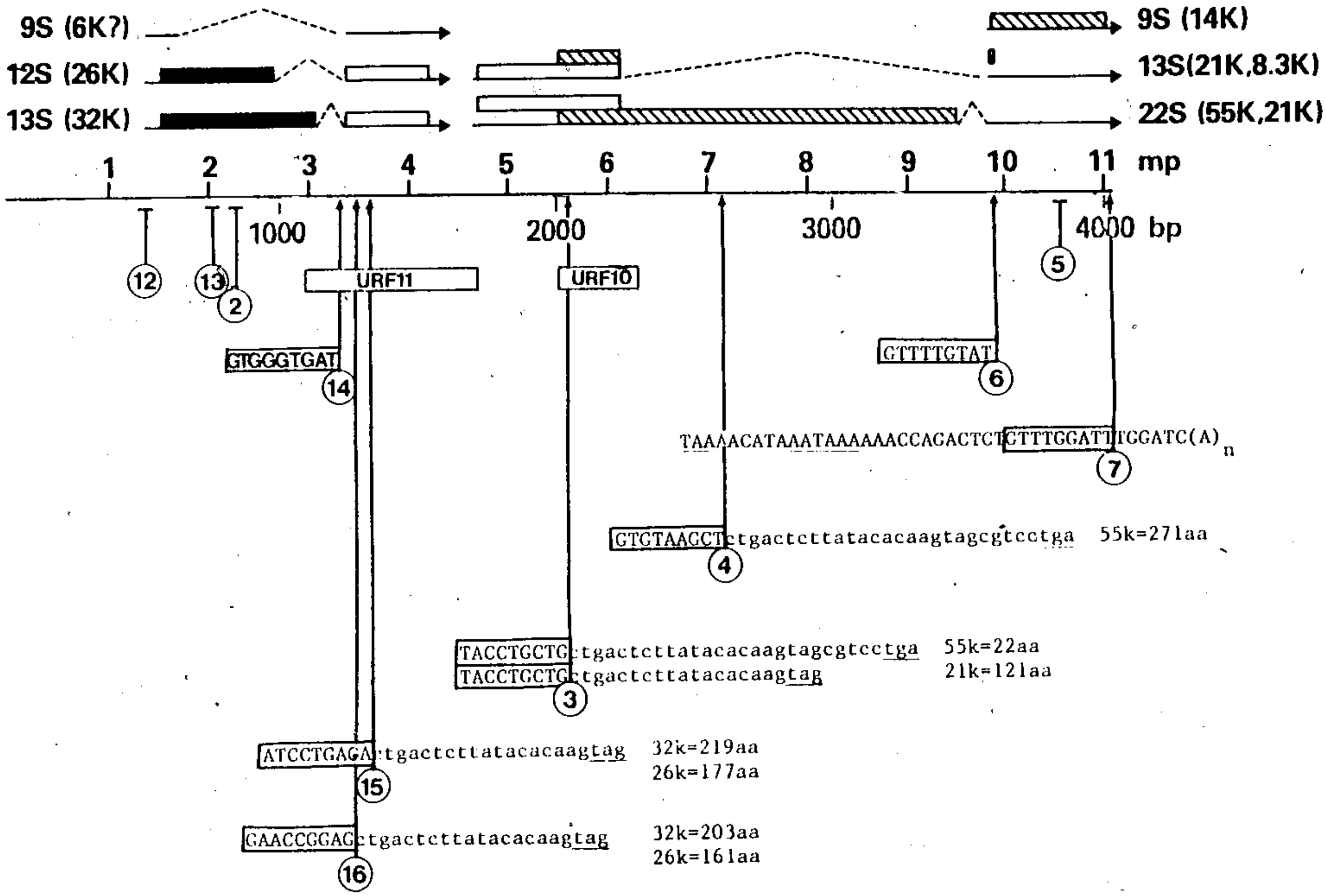
14L
3L
4R
6L
7R
G G A T C
G A T C
1141
2068
2806
3583
4056

Mutant	Sequence
pXC3	AAACCCATCTGAGCGGGGGG**TACCTGCTG**GATTTTCTGGCCATGCATCT
pXC4	TACCAACCTTATCCTACACG**GTGTAAGCT**TCTATGGGTTTAACAATACC
pXC6	TAAGGTGGGGGTCTTATGTA**GTTTTGTAT**CTGTTTTGCAGCAGCCGCCG
pXC7	TAAATAAAAAACCAGACTCT**GTTTGGATT**TGGATCAAGCAAGTGTCTTG
pHE14	GTAAGTGAAAATTATGGGCA**GTGGGTGAT**AGAGTGGTGGGTTTGGTGTG
pHE15	GTCCTAAAATGGCGCCTGCT**ATCCTGAGA**CGCCCGACATCACCTGTGTC
pHE16	CTGAGCCTGAGCCCGAGCCA**GAACCGGAG**CCTGCAAGACCTACCCGCCG

<u>Figure 18</u>: Ad5-DNA sequences at the site of Tn5 insertion.

The sequences of the 9-bp duplicated "target" sequence are shown in bold face for each mutant sequenced, with surrounding Ad5 sequences shown in small script. Five mutants were sequenced at one junction site only (Figure 17), while two mutants (pHE15 and pHE16) were sequenced at both left and right junction sites (Figure 16). For all insertion sites sequenced, only one DNA strand was sequenced.

Figure 19 Summary of eleven pXC::Tn5 mutant plasmids. Location of Tn5 mutant relative to the E1 transcription units. Top: transcription map of E1 (see legend to Figure 2). Bottom: mutants are identified by the numbers in circles and are positioned by horizontal bars (for those mapped by restriction enzyme analysis) and by arrows (for those mutants mapped by DNA sequence analysis). For mutants that have been sequenced, the 9-bp duplicated Ad5 target site sequence is shown in upper case (boxed), and for those which map within coding sequences the juxtaposed Tn5 sequences (lower case) are included up to the first in phase nonsense codon (underlined). The predicted sizes of truncated polypeptides are shown next to the size of the theoretical wild type product for each termination mutant. Mutant pXC7 maps 21-bp downstream from the AAUAAA polyadenylation signal in E1B and 16-bp upstream from the wild type poly(A) addition site. Mutants pXC14 and pXC6 map within intervening sequences for the E1A-13s and the E1B-22s mRNAs, respectively.


E1A
E1B
9S (6K?)
12S (26K)
13S (32K)
9S (14K)
13S(21K,8.3K)
22S (55K,21K)
1
2
3
4
5
6
7
8
9
10
11
mp
1000
2000
3000
4000 bp
URF11
URF10
GTGGGTGAT
GTTTTGTAT
TAAAACATAAATAAAAAACCAGACTCTGTTTGGATTTGGATC(A)n
GTGTAAGCTctgactcttatacacaagtagcgtcctga 55k=271aa
TACCTGCTGctgactcttatacacaagtagcgtcctga 55k=22aa
TACCTGCTGctgactcttatacacaagtag 21k=121aa
ATCCTGAGActgactcttatacacaagtag 32k=219aa
26k=177aa
GAACCGGACctgactcttatacacaagtag 32k=203aa
26k=161aa

products by 54 and 69 codons, respectively. In the case of pXC3, Tn5 inserted downstream of the AUG for the 55k protein initiation and therefore would affect 55k, 21k and URF10 translation products. For the 21k protein, an amber mutation decreases the size of the predicted product from 176 aa to 121 amino acids, while the 55k protein would be severly truncated (22 amino acids). For pXC4, which maps within the 13s intron in E1B, 55k would be the only product predicted to be truncated, from 496 to 271 amino acids. The exact position of Tn5 was not determined for the two E1A mutants pXC2 and pXC13.

It is possible to predict the structures of the carboxy termini of each altered E1 polypeptide in these mutants based on the DNA sequence of the ends of Tn5. Thus for the two E1A products altered in pXC15 and pXC16 as well as for the E1b 21k product altered in pXC3, the truncated product should have a common carboxy-terminal sequence X-leu-thr-leu-ilu-his-lys-COOH. For both pXC3 and pXC4, the carboxy terminal sequences of the truncated 55k product would be X-ala-asp -ser-tyr-thr -gln-val-ala -ser-COOH.

4.2.7 Plasmids derived from Tn5 insertion mutants

One objective of pXC1 mutagenesis was to rescue mutant plasmid sequences into full length viral DNA. However, the packaging constraints for Ad5 virions have not been rigorously defined and it was not known if pXC::Tn5 mutants could be directly rescued into virus. Studies on adeno-SV40 hybrid viruses (reviewed in Tooze, 1981) suggest an upper limit of about 38-kb for virion DNA. Addition of Tn5 (5.7-kb) to wt viral DNA (36-kbp) would amount to a 16% increase in genome size which probably exceeds the packaging constraints of Ad5 virions. It was anticipated that pXC::Tn5 plasmids would have to be altered in order to rescue these mutations into virus. This section describes (A) the construction of

pXC::Tn5 deletion-insertion plasmids designed for use in defining the packaging constraints of Ad5 virions, and (B) construction of pHE::Tn5 derivatives containing the HSV-TK gene.

A. Insertion-deletion mutants

A series of insertion-deletion mutants were constructed starting from the pXC::Tn5 plasmids shown in Figure 14. These derivative plasmids, shown in Figure 20, were constructed by removal of BglII restriction fragments from the parental plasmid as described in the legend to Figure 20. The resulting plasmids have net size increases relative to pXC1 ranging from -1000-bp to +3450-bp, and should serve useful for investigations on the packaging constraints of Ad5 virions. Since these plasmids have deleted various lengths of Ad5 DNA in addition to the insertion of Tn5 sequences, the nature of the E1 sequence alterations is complex. Hence the effect of these substitutions on viral gene expression would be difficult to interpret in light of the structure of E1 in resulting mutant virus. At the present time none of these plasmids have been rescued into viral DNA sequences.

B. Palindromic plasmids

Because Tn5 contains a unique region encoding neo^r bordered by 1.5-kbp inverted repeat sequences, in vitro deletion of the center of Tn5, using restriction enzymes such as HpaI, XhoI, or BglII, would produce a large palindrome on subsequent ligation. This approach to reducing the size of inserts in pXC::Tn5 plasmids would have two advantages. First, the size of the insert could be defined by choice of endonuclease used to cleave plasmid DNA and a range of insertion sizes could conceivably be generated from any one mutant. Secondly, these derivatives would maintain a point insertion mutation in the tailored constructs, in contrast to the more complex substitution mutants described above. However, several

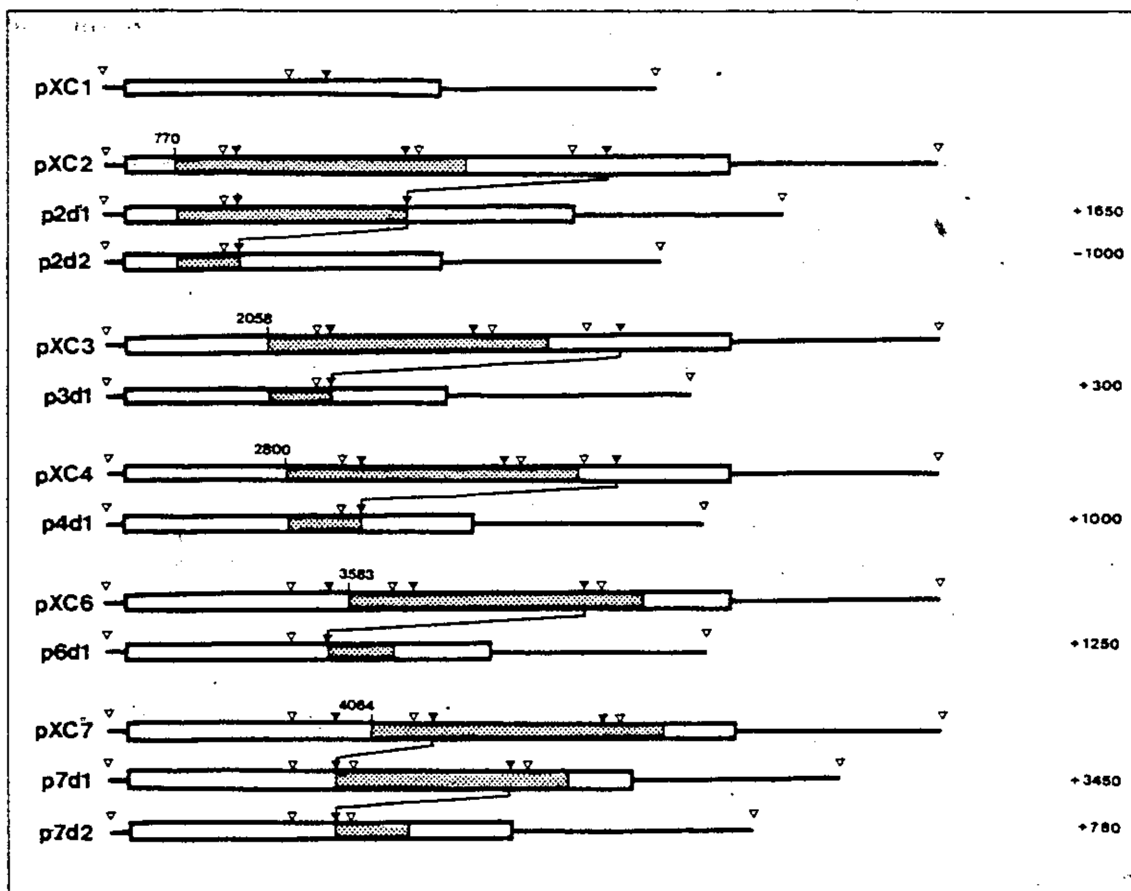


Figure 20: Insertion-deletion mutants derived from pXC::Tn5 plasmids.

Representation of plasmids derived from pXC1 by digestion with BglII followed by ligation with T4 DNA ligase then cloning in LE392. Single lines represent pBR322 sequences; open box: Ad5 sequences; shaded box: Tn5 sequences. Open triangles represent HindIII sites; closed triangles represent BglII sites. Numbers above each pXC::Tn5 mutant represent the location of Tn5 in Ad5 sequences. Numbers to the right of each mutant represent the net size increase of derivative plasmids relative to pXC1. Arrows represent the extent of the deletion from the parent plasmid.

attempts at cloning such palindromes in LE392 were unsucessful, consistent with recent reports showing that large palindromes are nonviable in E.coli (Lilley, 1981; Collins, 1980; Collins et al., 1982). The use of plasmids containing Tn5 as cloning vehicles based on the lethality of palindromic sequences in plasmids has recently been described (Hagan and Warren, 1982).

C. Substitution mutants: pHE142, pHE144

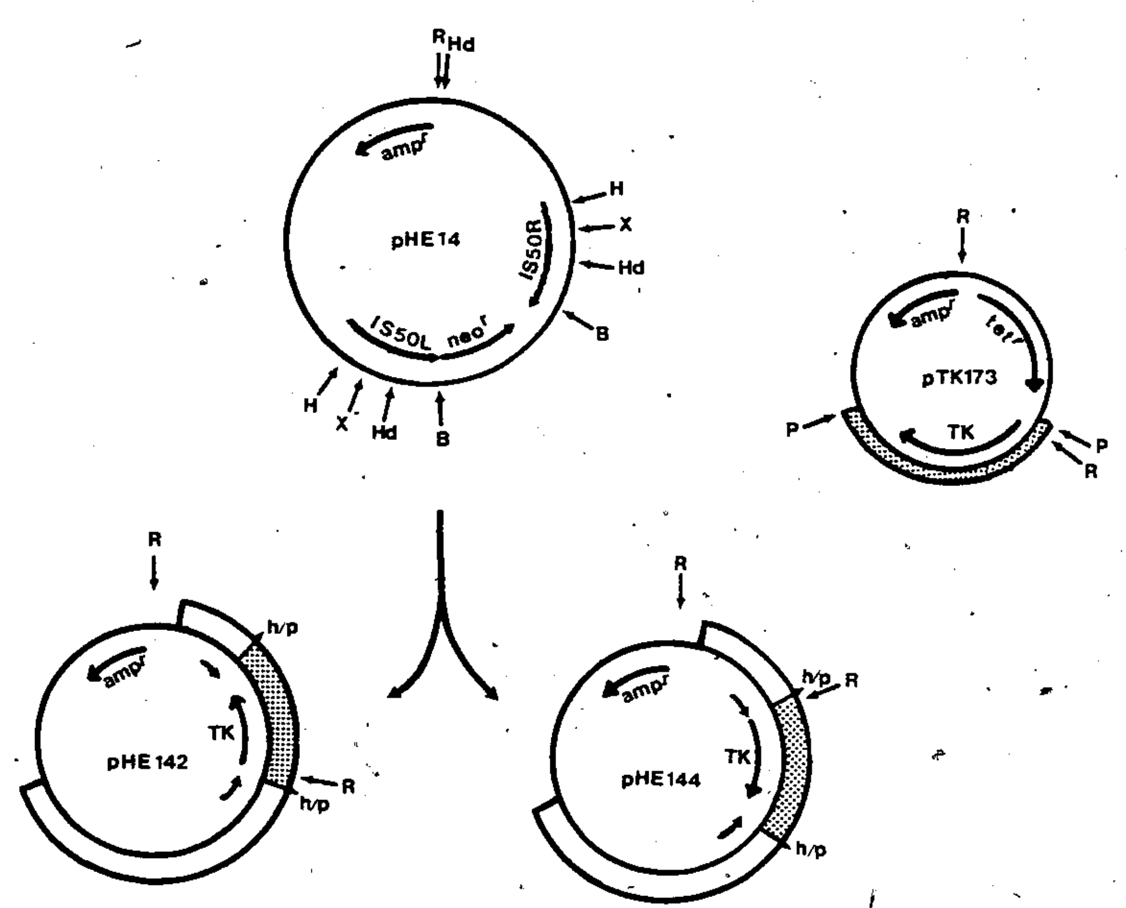
Another approach used to construct derivatives of Tn5 mutants with "packageable" inserts is shown in Figure 21. In this strategy, the insert size of pHE14 was reduced by replacing most of Tn5 sequences with the HSV-1 TK gene. The 2-kbp HSV-TK PuvII fragment from pTK173 (shown in Figure 9) was isolated by agarose gel electrophoresis and ligated to HpaI digested pHE14 DNA as described in the legend to Figure 21. Transformation of LE392 gave Amp^r colonies at an efficiency of $1x10^3$ colonies/ug input vector DNA, 85% of which (69/80 colonies screened) were Kan^s, and 75% (9/12) of Amp^r Kan^s colonies contained the TK gene in either orientation as shown in Figure 21.

The plasmids pHE142 and pHE144 represent interesting derivatives of pHE14, since they contain a selectable eukaryotic gene (TK) and their net insertion size (approximately 2.4-kbp) is within the packaging constraints of Ad5 virions. Derivatives of these plasmids have recently been rescued into Ad5 viral DNA (Yousef Haj-Ahmad, unpublished results).

4.3 Discussion

The results presented above demonstrate that Tn5 can be used to generate insertion mutants of cloned eukaryotic DNA. A number of interesting insertion mutants mapping in Ad5 E1 sequences were constructed and characterized, and

Figure 21. Construction of pHE142, pHE144. The 2-kbp HSV-TK gene was isolated from pTK173 DNA by agarose gel electrophoresis (**Section 2.4.5**) and ligated to HpaI digested pHE14 DNA. After transformation of E.coli LE392 with ligated DNA, Amp^r colonies were screened by digestion of plasmid DNA with EcoRI for clones containing the TK gene inserted clockwise (pHE144) and counterclockwise (pHE142) in pHE14.



R
Hd
amp^r
pHE14
H
X
Hd
IS50R
B
IS50L
neo^r
H
X
Hd
B
R
amp^r
tet^r
pTK173
P
TK
P
R
R
h/p
amp^r
TK
pHE142
R
h/p
R
h/p
R
amp^r
TK
pHE144
h/p

the biological activity of these pXC::Tn5 insertion plasmids is examined in **Chapter V.** The above results have also contributed to the understanding of the process of Tn5 transposition in E.coli, and the following sections discuss these results in terms of the mechanism and specificity of Tn5 transposition.

4.3.1 Target site specificity for Tn5 insertion

The DNA sequences shown in Figure 18 represent the largest library of Tn5 insertion sites analysed to date. Several conclusions can be drawn from this data regarding the DNA sequence specificity of Tn5 insertion.

It is evident from Figure 18 that there is no consensus target sequence either at or surrounding the site of Tn5 insertion. This is in contrast to the symmetrical 6-bp sequence which is the target site determining Tn10 insertion specificity (Halling and Kleckner, 1982). It is also evident from Figure 18 that the region surrounding the site of Tn5 inserton is not particularly A+T rich (49% A+T), as found at the site of insertion for Tn3, Tn9 and IS1 (Tu and Cohen, 1980; Galas et al., 1980; Meyer et al., 1980). In fact, one region of pXC1 (the intron of the 13s mRNA from E1A) is 85% A+T (Tooze, 1981) yet none of the 57 pXC::Tn5 insertions mapped in this region. Thus A+T rich sequences are not favoured sites of Tn5 transposition.

Several groups have reported limited sequence homologies between the molecular ends of insertion sequences, such as Tn9 and Tn3, and their target sites of insertion (Galas et al., 1980; Tu and Cohen, 1980; Saedler et al., 1981). Bossi and Ciampi (1982) examined the sequences at the site of 3 Tn5 insertions in the histidine operon of Salmonella, and reported a weak correspondence (7/12 matches) between sequences near the insertion sites and a region of Tn5 from 15 to 26-bp from the molecular ends. A computer search for homologies between the

ends of Tn5 and insertion sites presented in Figure 18 was performed using the homology search program (developed by Ray Wu) with help from J.R.Smiley. Setting the minimum homology limit at 40% (demanding at least 4/10 matches to score homology), the program searched for matches between the sequences presented in Figure 18 and the first 50-bp at the ends of Tn5. No region of Tn5 examined showed consistent homologies with target sequences, and no striking matches were found. In fact, at the level of homology searched there were as many matches made between the ends of Tn5 and randomly chosen pBR322 sequences as found when comparing Tn5 with the target sites shown in Figure 18. Thus these results argue against any role of target sequence homologies for Tn5 insertion specificity.

Berg et al (1983) have similarly analysed the specificity of Tn5 insertion into the Tet^r gene of pBR322. Of 75 insertions examined, 47 mapped to five "hotspots" located in the first 300-bp of the 1250-bp Tet^r gene. DNA sequence analysis of these 5 target sites revealed no sequence specificity, consistent with the results presented above. Their analysis revealed that a GC base pair occupies position one and position nine in the duplicated 9-bp target sequence of each hotspot examined, suggesting a GC cutting preference during Tn5 transposition as found at the insertion sites of Tn9 (Galas et al., 1980). However, from the results presented in Figure 18 it is clear that a GC cutting preference is not an absolute requirement for Tn5 insertion, since an AT base pair occurs at least once in positions 1 and 9 in most sites analysed in Figure 18, and one site (pXC15) has an AT base pair at both ends of the 9-bp repeat. Thus it can be concluded that while a GC base pair at positions 1 and 9 may help define preferred targets for Tn5 transposition this is by no means a prerequisite for Tn5 insertion.

Taken together, the above observations argue against any clear DNA sequence specificity for Tn5 insertion. From the results presented in Figure 13, however, it appears that some mechanism is operative in directing Tn5 insertions to preferred regions. Several other groups have reported nonrandom insertion of Tn5 (Shaw and Berg, 1979; Berg et al., 1980; Miller et al., 1980). Since this observation cannot be explained on the basis of DNA sequences directing Tn5 to the site of insertion, some other mechanism must be invoked to account for preferential insertion. As will be argued in the following sections, it is possible that Tn5 transpositon is stimulated by transcription of target DNA sequences, and that a fortuitous promoter in Ad5 E1B sequences is responsible for the distribution of insertions seen in Figure 13.

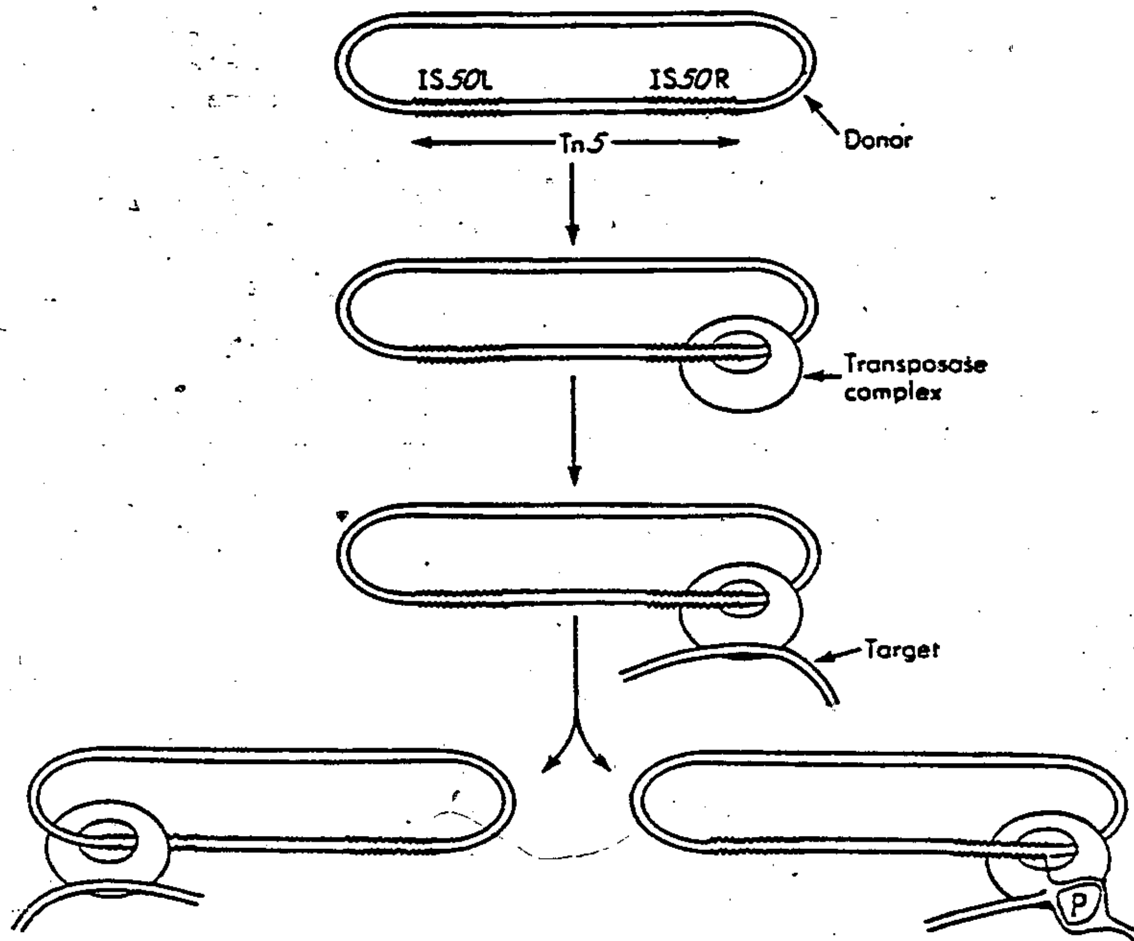
4.3.2 Mechanism of Tn5 transposition

Tn5 shows nonrandom orientation when inserting downstream from active promoters. Berg et al (1982b; 1983) found that IS50R mapped closest to the promoter in 19 of 23 independent insertions into the Amp^r gene of pBR322, whereas in the Tet^r gene both orientations were equally frequent. Because Amp^r is transcribed more strongly than Tet^r in pBR322 (Stuber and Bujard, 1981) this observation suggests that transcription of the target molecule may play a role in controlling transposition. In support of this, when the very strong M13 gene II promoter is placed upstream from the Tet^r gene of pBR322, IS50R preferentially maps closest to this promoter after Tn5 transposition (Berg et al., 1982b). Thus transcriptional activity affects the process of Tn5 transposition.

In addition, the IS50R encoded transposase of Tn5 (Rothstein et al., 1980) acts only in cis (Berg et al., 1982b), suggesting that newly translated transposase protein envelopes the DNA as it is being made. These observations led

Berg to propose a "donut" model for Tn5 transposition (see diagram), in which nascent transposase protein wraps around adjacent DNA immediately following coupled transcription and translation. Interaction between transposase adjacent to the ends of IS50R and the target DNA, possibly facilitated by transient denaturation at the site of transcription on the target DNA molecule; results in the preferential insertion of IS50R closest to the promoter. After initiating insertion of Tn5 at the right end, transposase then feeds through Tn5 DNA until in comes to the end of IS50L and completes the process of Tn5 transposition (Berg et al., 1982b).

An analysis of 35 pXC::Tn5 plasmids using the endonuclease SmaI and HindIII (Figure 13) showed that Tn5 was nonrandomly oriented in these plasmids. In the 500-bp region of preferred insertion in E1B sequences, 7 out of 8 inserts (88%) were in the rightward orientation (IS50R closest to the left end of Ad5). For inserts mapping in the 2670-bp PvuII band of pXC1, in contrast, the orientation of Tn5 was less polarized with 9/15 insertions (60%) in the rightward orientation (Figure 13). Thus the region of pXC1 which has an overrepresentation of Tn5 insertions also displays a preferential orientation, with IS50R mapping closest to the E1A region in pXC1. These observations support the concept that Tn5 insertions show nonrandom orientation (Berg et al., 1982b), and suggest that a promoter located close to 2500-bp in Ad5 E1B sequences oriented rightward (clockwise in pXC1) was actively directing Tn5 transposition into this preferred region of pXC1.



The donut model for transposase action. Transposase is postulated to bind to and wrap around the DNA molecule containing IS50R, during or following coupled transcription and translation. It is able to move along the IS50R containing molecule, and thus act on IS50L. Transposase can not act on IS50 elements present on separate DNA molecules. The site on transposase used for binding to target DNA, which is not specific for any particular DNA sequence, is postulated to lie on an outer surface of the protein. A possible role for RNA polymerase (designated P) or transcription in the target DNA is presented.
From Berg et al., Stadler Symposium, Vol.14 (1982)

4.3.3 Preferred Tn5 insertion downstream from a promoter

Since the target for Tn5 mutagenesis in this work is from a eukaryotic source, it was anticipated that this DNA would be transcriptionally silent in E.coli. However, independent evidence demonstrated the presence of a strong fortuitous prokaryotic promoter within E1B sequences in pXC1. Dr.Graham has constructed a plasmid, pFG66, containing the promoterless lacZ gene from plasmid pLG400 (Guarente et al., 1980) fused to the HindIII site in E1B. This plasmid contains Ad5 DNA (22-2804-bp) immediately 5' to the lacZ coding region, with the lacZ coding region in phase with the single open reading frame in this region of Ad5. Surprisingly, pFG66 expressed high levels of β-galactasidase activity in E.coli (80% of induced levels of the wild-type lac operon, J.Waye personal communication), suggesting that adenoviral sequences were able to promote transcription in E.coli.

This promoter activity was mapped within Ad5 sequences of pFG66. A derivative of this plasmid lacking Ad5 DNA between the KpnI site (2052-bp) and the HindIII site (2804-bp) was constructed by J.Waye. This derivative has no detectable β-galactasidase activity, mapping the promoter to this region of the Ad5 sequences (the shaded region in Figure 13). Analysis of the DNA sequence in this region of pXC1 revealed an area with striking agreement with the consensus E.coli RNA polymerase promoter sequence proposed by Scherer et al (1978), with a predicted 5' end of transcribed RNA mapping at 2698-bp in pXC1 and with a potential ribosome binding site downstream from this site (J.Waye, personal communication). The existence of a fortuitous promoter sequence upstream from the preferred region of Tn5 insertion in pXC1, and the nonrandom orientation of Tn5 in the region immediately downstream from this promoter, supports the suggestion that transcription may stimulate Tn5 transposition (Berg et al., 1982b). It

is conceivable that transcription transiently opens up the DNA helix facilitating transposition, analogous to the role of host chromosomal replication forks during transposition by bacteriophage Mu (Fitts and Taylor, 1980) or of transient melting at A+T rich regions postulated as important for Tn3 transposition (Tu and Cohen, 1980).

Sasakawa et al (1982) reported that transcription impinging on the ends of IS50 elements inhibits further transposition. The transposition frequency of IS50L is 8-fold lower than that of IS50R, presumably because transcription of the neo^r gene initiating within IS50L interferes with the action of the transposase on the end of IS50L (Sasakawa et al., 1982). Thus, Tn5 apears to have evolved a mechanism of transcriptional regulation of transposition. Transcription in a target DNA molecule serves to both stimulate and direct the orientation of Tn5 insertion. In addition once Tn5 is inserted downstream from an active promoter any further transposition is repressed, a process which may serve to decrease the frequency of IS50 and Tn5 induced insertional mutations in these cells.

4.4 Summary

A number of observations have been described in this chapter, and they can be summarized as follows:

1. First, it is clear from the above that Tn5 can be used as an insertional mutagen for cloned eukaryotic genes. The ability to manipulate the size of the insertions in such plasmids also makes this a useful system for genetic engineering of cloned DNA, as shown by the construction of a variety of insertion-deletion plasmids from pXC::Tn5 mutants (Figure 20).
2. Tn5 transposition is apparently nonrandom with respect to the target DNA molecule, and seems to preferentially insert downstream from an active transcriptional promoter sequence.

3. The orientation of Tn5 insertion downstream from active promoters is nonrandom, with IS50R mapping closest to the promoter region in most cases. This may indicate that the cis-acting transposase protein seeks out transiently denatured regions to initiate transposition as first suggested by Berg et al (1982b).

Chapter V

Cell Transformation

5.1 Introduction

As outlined in **Chapter I**, one of the major objectives of this work was to identify the adenovirus genes encoded in early region E1 responsible for cell transformation. This chapter describes the morphological transformation of primary rodent cells using the plasmids described in **Chapters III and IV.** As discussed by van der Eb and Graham (1980), the transforming activity of adenovirus DNA and DNA fragments can be assayed on a variety of cell types. For most transformation experiments, rat and hamster cells have been used because of their high transforming efficiency and low background of spontaneous transformants.

It is important to recognize that interpretation of results of transformation assays depends on the cell system used. For example, different results are seen for transformation of primary cells than for transformation of immortalized cells (Land et al., 1983; Ruley, 1983). In addition, it is important to consider growth conditions used for selection of transformants, since different media (high vrs low calcium concentrations) select for partial (Houweling et al., 1980) or for full morphological transformants (van der Eb et al., 1977). The transformation assays described here use primary rodent (baby rat and baby hamster kidney) cells, selecting for morphological transformants in medium containing low calcium ions (Joklik's MEM) supplemented with 5% horse serum. Under these conditions untransformed fibroblasts are nonviable, and only morphologically trans-

formed cells can survive. These cells are phenotypically similar to cells explanted from viral induced tumors (reviewed by Graham, 1983), and it can be assumed that this assay is a reasonable in vitro analogue to in vivo oncogenicity by human adenovirus'.

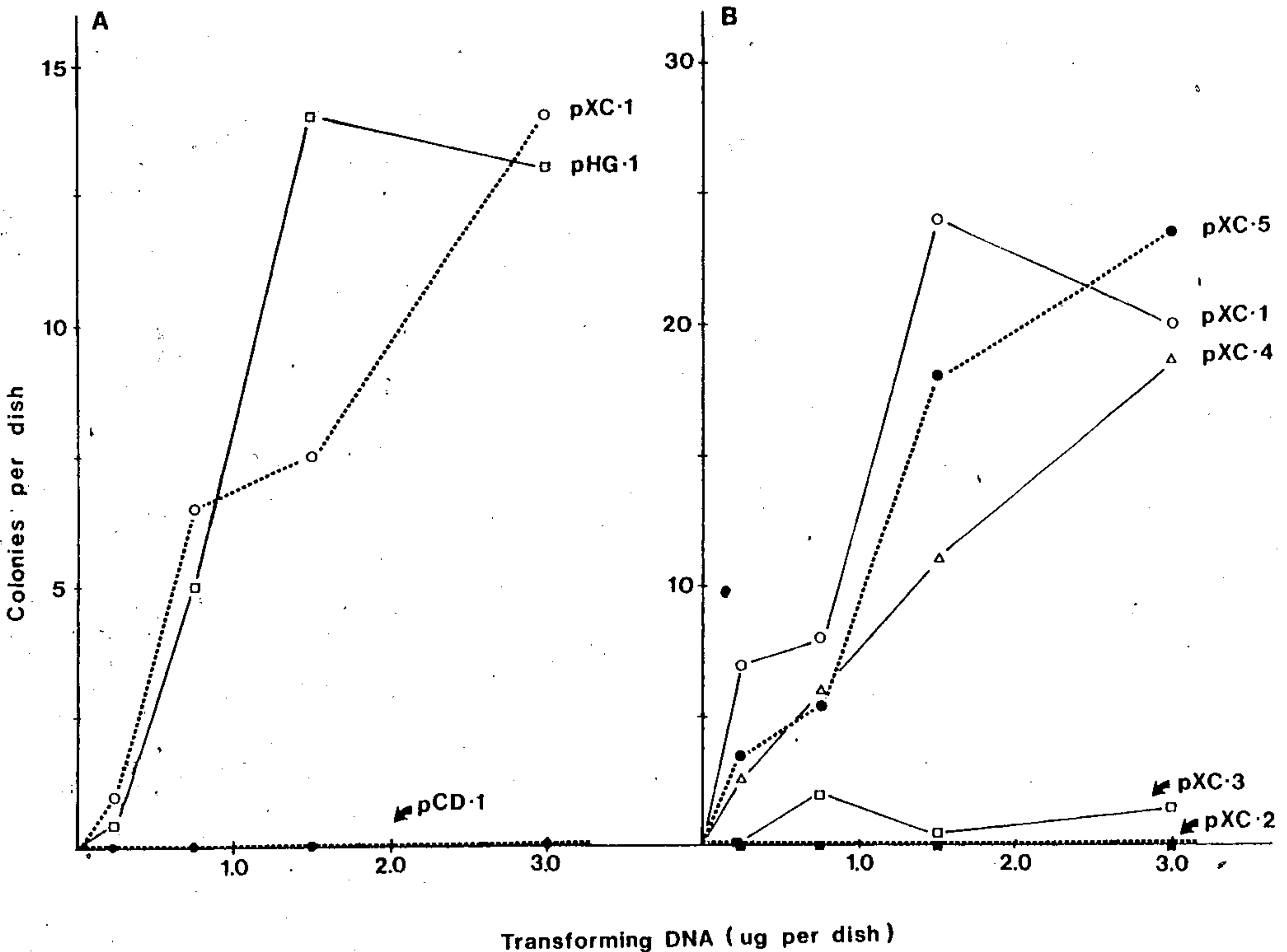
5.2 Results

5.2.1 BRK Transformation; dose response

As discussed in **Section 3.2.2**, the plasmid pXC1 is capable of transforming primary baby rat kidney (BRK) cells. Examples of transformed cell foci can be seen in Figure 23. In this assay transformed cells expand as visible colonies of small epithelioid cells, and the background of non-transformed fibroblastic cells lift off the dishes after 7-10 days. Transformed foci were usually counted by eye, and the epithelioid morphology of transformed cells was confirmed by microscope examination of dishes stained with crystal violet after 15 days (**Section 2.3.5**).

The dose-response for transformation of BRK cells with pXC1 is shown in Figure 22A. The curve is linear over the range of plasmid DNA concentrations tested, and continues linear up to 10 μg per dish (not shown) with a specific activity of approximately 5 colonies per μg plasmid. This represents one transformant per 10^6 cells per μg plasmid DNA (10^{11} molecules of pXC1). The absence of a plateau effect over this concentration range indicates that this assay is DNA limited. Presumably at higher DNA concentrations a saturation level would be observed representing the number of "competent" cells that are able to take up DNA, as has been seen in TK transformation experiments (Wigler et al., 1979). All transformation experiments reported in this work were done with covalently closed circular plasmid DNA. No attempt was made to quantify the effect of lin-

Figure 22. BRK transformation: dose response. Primary baby rat kidney cells (BRK) were established in monolayer culture as described in **Section 2.3.4.** When approximately 80% confluent (ca.48 hrs), plasmid DNA was added using the calcium technique as described in **Section 2.3.5,** and the DNA coprecipitate was left on the cells for 20 hrs during which time the monolayer became 100% confluent. Cells were exposed to selective medium (Joklik's MEM plus 5% horse serum) two days later, and the medium was changed every 3 days. Dishes were stained and counted for foci on day 20. A: Ad5 plasmids pXC1, pHG1, pCD1; B: pXC::Tn5 mutants pXC2, pXC3, pXC4, pXC5.


A
B
Colonies per dish
15
10
5
30
20
10
pXC·1
pHG·1
pCD·1
pXC·5
pXC·1
pXC·4
pXC·3
pXC·2
1.0
2.0
3.0
1.0
2.0
3.0
Transforming DNA (ug per dish)

ear vrs circular plasmid on transforming efficiency, since previous studies on TK transformation of mouse L cells indicated that circular plasmid DNA transformed cells as efficiently as did linear plasmid DNA (Graham et al., 1980).

5.2.2 Transformation with pXC::Tn5 plasmids

Transformation of BRK cells with several representative Tn5 insertion mutants is shown in Figure 22B, and the results of a number of experiments are summarized in Table 5.1. Again the dose response is linear and the values in Table 5.1 represent data compiled from a number of dishes assayed at from 1 to 20 µg plasmid DNA per dish for each experiment. The plasmid pXC1 transformed in these experiments with an efficiency of 5 to 15 colonies per µg plasmid DNA. The variability between experiments is due to a number of factors, including the density and growth rate of the primary cells at the time that the DNA calcium coprecipitate was added, and the morphology of the precipitate. Untreated control dishes do not give rise to foci, and spontaneous morphological transformants of either BRK or BHK cells were never observed under the selective conditions used in this assay. The results of transformation experiments using E1 containing plasmids and the Tn5 insertion mutant plasmids shown in Figure 19 can be summarized as follows:

1. All E1A insertion mutant plasmids are transformation negative on BRK cells (Figure 22 and Table 5.1). In addition, the E1B plasmid pHC1 which deletes E1A sequences is also transformation defective (Table 5.2). These results indicate that E1A is needed for DNA mediated transformation. However, these results cannot distinguish between a direct requirement for an E1A encoded oncogene required for the maintenance of cell transformation, or an indirect requirement for the E1A encoded activator of

Table 5.1: BRK Transformation: pXC::Tn5 Plasmids

(colonies per ug plasmid DNA)

plasmid	experiment 1	2	3	4	5	6	$\bar{X} \pm$ SEM
pXC1	5.2	7.6	14.9	5.9	11.4	6.6	8.6 ±1.54
pXC12	-	-	-	0	0	0	0
pXC13	-	-	-	0	0	0	0
pXC2	0	0	0	0	0	0	0
pXC14	-	-	-	0	0	0	0
pXC15	-	-	-	0	0	0	0
pXC16	-	-	-	0	0	0	0
pXC3	0.7	0.2	0.8	.05	0.8	0	.43 ±.15
pXC4	0.6	2.7	10.4	1.3	6.9	-	4.4 ±1.9
pXC6	5.1	2.3	8.6	2.7	3.9	-	4.5 ±1.11
pXC7	7.8	4.4	-	9.3	5.1	-	6.7 ±1.15
pCD1	0	0	-	-	0	0	0
control	0	0	0	0	0	0	0

Values represent the average from 4 (experiments 2,5,6), 6 (expt.1) and 8 (expt.3,4) dishes assayed at from 1 to 10 ug plasmid DNA per dish. Values are normalized to the size of pXC1 (10-kbp).

E1B gene expression as first proposed by Berk et al (1979). This will be dealt with further in the Discussion.

2. Since pXC2, pXC12 and pXC13 are transformation negative on BRK cells, and they map downstream of the l-strand 23.2k URF11 product, it is unlikely that this product is the sole E1A function involved in transformation. This conclusion was substantiated by transformation with pXC386 as shown in Table 5.2. This plasmid has a decanucleotide insertion which alters the reading frame of URF11 but does not affect the R-strand transcripts (discussed in Chapter III), and transformed with efficiencies comparable to pXC1. Thus, the essential products for transformation of BRK cells probably are encoded by R-strand transcripts of E1A.

3. The presence of Tn5 in the promoter-proximal region of E1B (pXC3) drastically reduces the transforming activity of this DNA (Figure 22B, Table 5.1), indicating that this region is required for transformation. The absolute requirement for E1A sequences mapping between 5% and 8% is further shown using the plasmid pCD1 which deletes all of this region of E1B and is transformation negative on BRK cells (Figure 22A, Table 5.1). Since pHG1 (0-8%) can transform, this data demonstrates that the region between 5% and 8% encoding the Mr=21,000 tumor antigen is essential for transformation.

4. In contrast, the presence of Tn5 sequences mapping in the promoter-distal region of E1B, as in pXC4, pXC5, pXC6 and pXC7, did not affect the transforming activity of these plasmids (Figure 22, Table 5.1). These results indicate that viral sequences to the right of 8% are not required for DNA mediated transformation. This is substantiated by transformation with pHG1 which contains only 0-8% sequences and transforms with

an efficiency equivalent to pXC1 (Figure 22A), and confirms previous data using DNA fragments isolated from viral DNA (Graham et al., 1974a, 1974b; van der Eb et al., 1977).

Table 5.2: BRK Transformation: E1 Plasmids

plasmid	Ad5	plates[a]	ug DNA	colonies	efficiency[b]
pXC1	E1	3	6.9	46	6.7
pXC386	E1	10	38.5	95	2.5
pHG1	E1A,21k	3	10.5	40	4.7
pCD1	E1A	6	63.0	0	0
pHE1	E1A	3	45.0	0	0
pHC1	E1B	7	75.0	0	0

[a] number of 60 mm plates assayed
[b] colonies per ug plasmid DNA

5.2.3 BRK Transformaton: Selection Regimens

The results of transformation assays presented above indicate that viral products from both E1A and E1B are essential for morphological transformation of BRK cells. In these experiments transformants were selected in low calcium medium (Joklik's MEM) supplemented with 5% horse serum. Houweling et al (1980) have

reported the partial transformation (immortalization) of BRK cells with E1A alone when transformants were selected in high calcium medium supplemented with 10% fetal bovine serum. It was therefore of interest to determine whether the high calcium medium or the high serum supplement (10% FCS) was required to achieve transformation with E1A alone.

Figure 23 shows the results of a transformation assay with BRK cells selected with either Joklik's or alpha MEM supplemented with 5% horse or 10% fetal bovine serum. As can be seen in the control dishes in Figure 23, exposure of BRK cells to stringent selection conditions (Joklik's MEM plus 5% HS) eliminates the cell monolayer within 2 weeks. In contrast, incubation with alpha MEM plus 10% fetal calf serum results in the persistence of primary BRK cells. It should be emphasized that morphological transformants are not observed in control dishes under any of the selective conditions used.

The results presented in Figure 23 and summarized in Table 5.3 again demonstrate that, when selective medium contains 5% horse serum, the minimum transforming fragment of Ad5 DNA was approximately 0-8% (pHG1). Interestingly, no appreciable difference was observed in transformation efficiency with pXC1 when selection medium was either alpha or Joklik's MEM (normal and low calcium medium, respectively) with 5% horse serum (Table 5.3).

In marked contrast, however, when transformants were selected in medium supplemented with 10% fetal bovine serum rather than 5% horse serum, it was found that plasmids containing only E1A were able to morphologically transform primary BRK cells (Figure 23 and Table 5.3). The efficiency of transformation with E1A plasmids was slightly higher with alpha MEM than with Joklik's MEM, and was reduced approximately 10-fold relative to transformation with pXC1 (Table 5.3). E1A transformed colonies resemble transformed cells

Figure 23. <u>BRK transformation: selection conditions.</u> Primary BRK cells established as described in the legend to Figure 23 were transfected with plasmids containing either E1 (pXC1,pHG1) or containing E1A (pCD1). Cells were exposed to the DNA coprecipitates for 20 hrs, then nonselective medium (alpha MEM plus 10% fetal calf serum, FCS) was added. After 2 days the cells were refed with the indicated medium (alpha or Joklilk's MEM) supplemented with either 5% horse serum (HS) or 10% FCS, and refed twice weekly. Dishes were stained after 15 days.

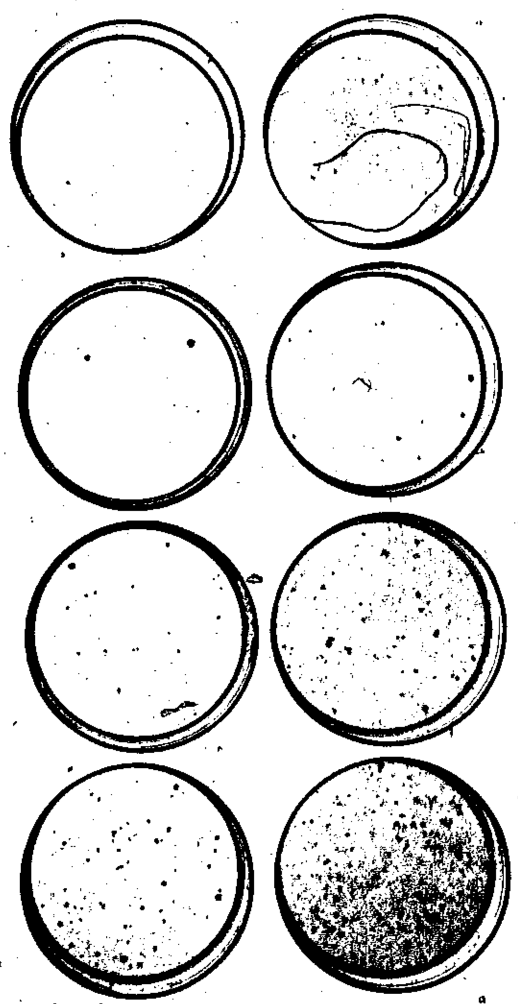
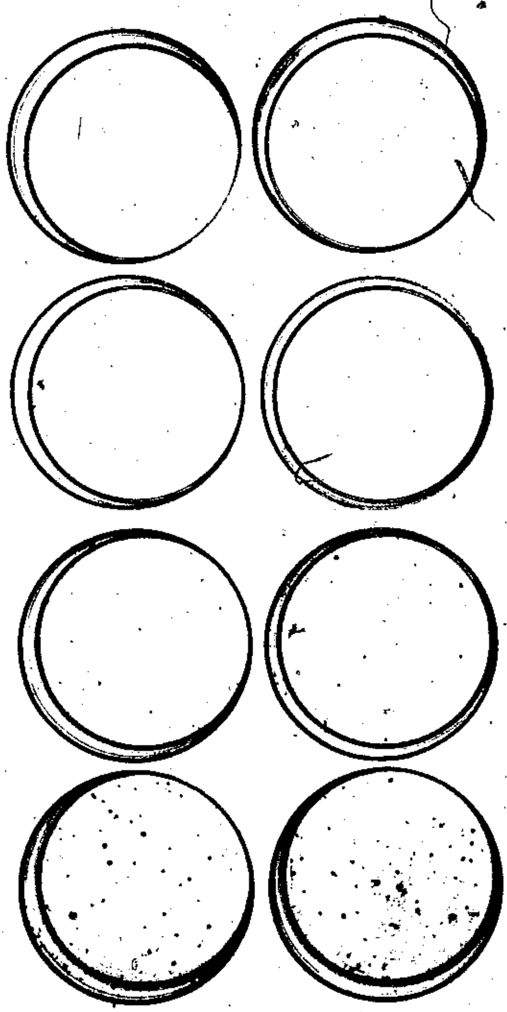


5 % Horse Serum
10 % Fetal Calf Serum
Joklik's
Alpha MEM
Joklik's
Alpha MEM
Control
E1A (0-5%) pCD1
E1A,21k (0-8%) pHG1
E1A,1B (0-16%) pXC1

Table 5.3: Transformation of BRK cells: Selection Criteria

(Colonies per dish)[b]

Selection		Plasmid DNA		
Medium	Serum[a]	control	E1A	E1A+E1B
Joklik's MEM	5% HS	0,0,0,0	0,0,0,0	77,67
alpha MEM	5% HS	0,0,0,0	0,0,0,0	115,67
Joklik's MEM	10% FCS	0,0,0,0	1,3,2,1	82,44
alpha MEM	10% FCS	0,0,0,0	13,4,5,8	70,74

a) serum supplement either 5% horse (HS) or 10% fetal bovine serum to indicated medium.

b) number of transformed foci per 60 mm dish.

from pXC1 treated dishes in both cell morphology (epitheloid) and colony shape (dense, round colonies). These results suggest that a factor present in fetal bovine serum but not in horse serum can replace the requirement for E1B sequences for morphological transformation of BRK cells. The implications of these findings are discussed below.

5.2.4 BHK transformation

A. Tn5 insertion mutants

Transformation with pXC::Tn5 mutants was also investigated in primary baby hamster kidney cells as shown in Table 5.4. The initial objective of this work was to establish a library of transformed cell lines containing these plasmids in order to study (1) the integration of plasmid DNA, (2) the expression of E1 mRNA and proteins in these cells, and (3) the tumorgenicity of cells transformed with different plasmids. The results presented below examine the efficiency of transformation of BHK cells with pXC::Tn5 plasmids.

Table 5.4 summarizes the results of three experiments studying BHK transformation with pXC::Tn5 plasmids. Selective medium for these experiments was Joklik's MEM supplemented with 5% horse serum. As with BRK cells, pXC1 gave approximately 5 transformants per μg plasmid DNA. Transformants are observed as foci growing on a monolayer of untransformed primary cells (which detach in selective medium after a period of approximately 3 weeks). Colonies were generally counted by microscopic examination without staining dishes, and were maintained in culture in order to establish cell lines. In cases where no colonies could be detected the plates were stained and scored for foci routinely by day 21 post transfection. The results in Table 5.4 can be summarized as follows:

1. Tn5 mutants mapping in E1A are capable of transforming BHK cells. Plasmids pXC12, pXC13, pXC15 and pXC16 all showed reduced but non-zero transforming activity. In contrast, pXC14 transformed BHK cells at a slightly enhanced level compared to pXC1.
2. As with BRK cell transformation, the promoter-distal region of E1B was not required for DNA transformation of BHK cells since pXC4, pXC5, pXC6 and pXC7 transformed with efficiencies comparable to pXC1.

Table 5.4: BHK Transformation: pXC::Tn5 Plasmids

(Colonies per ug plasmid DNA)[a]

plasmid	experiment 1	2	3	$\overline{X} \pm$ SEM
pXC1	4.0	6.7	-	5.4 ± 1.20
pXC12	-	3.4	0.9	2.2 ± 1.20
pXC13	-	0	0.2	0.1 ± .09
pXC2	0	-	-	0
pXC14	-	19.1	5.0	12 ± 7.07
pXC15	-	0.9	0.5	0.7 ± .21
pXC16	-	1.3	0.2	.75 ± .57
pXC3	0	0	-	0
pXC4	3.3	2.5	3.0	2.9 ± .23
pXC6	7.5	12.4	-	10 ± 2.40
pXC7	10.2	9.4	-	9.8 ± .04
pCD1	0	-	-	0
control	0	0	0	0

a) values normalized to the size of pXC1 (10-kbp)

3. The insertion mutant pXC3 on the other hand was transformation defective in BHK cells as was the E1B deletion plasmid pCD1, indicating again that the promoter-proximal E1B sequences are essential for DNA mediated transformation.

Table 5.5 summarizes the results of transformation of both BRK and BHK cells with these plasmids. The values for transformation efficiency (colonies per μg plasmid DNA) were averaged for the data presented in Tables 5.1 and 5.4 and are expressed as a fraction of the efficiency of pXC1 DNA in these same experiments. These values can be taken as an estimate of the efficiency of transformation by mutant plasmids relative to wild type plasmid DNA, and serve to highlight the results presented above:

1. The E1A mutants used in these studies were transformation negative on BRK cells. In contrast, most E1A mutants had approximately 10-fold reduced transforming activity on BHK cells, but were not absolutely deficient for BHK transformation.
2. pXC14, with Tn5 inserted within the intron of the E1A 13s mRNA had approximately two-fold enhanced transforming efficiency on BHK cells.
3. pXC3, with Tn5 inserted in the Mr 21,000 E1B coding sequences, was defective for BHK transformation and had drastically reduced transforming activity on BRK cells.
4. The promoter-distal E1B mutants pXC4, pXC5, pXC6 and pXC7 had transforming efficiencies comparable to pXC1.

Table 5.5: Transformation of Rodent cells with pXC::Tn5 Plasmids.

Efficiency (colonies per ug)[a]

plasmid	BRK	BHK
pXC1	1.0	1.0
pXC12	0	.34 ±.17
pXC13	0	.02 ±.02
pXC2	0	-
pXC14	0	1.9 ±.96
pXC15	0	.11 ±.02
pXC16	0	.12 ±.08
pXC3	.05 ±.02	0
pXC4	0.4 ±.11	0.6 ±.13
pXC6	0.5 ±.12	1.9 ±.01
pXC7	1.0 ±.30	2.0 ±.58
control	0	0

a) values represent the average of 6 (BRK) and 3 (BHK) experiments expressed relative to the efficiency of pXC1 as described in the text, and are normalized to the size of pXC1 (10 kbp).

B. Cell lines

A number of hamster cell lines were established during the course of this work, as listed in Table 5.6. In order to expedite the process of establishing this library of transformed lines, dishes containing transformed foci were trypsinized without cloning individual colonies, and then passaged in flasks until aliquots from these cultures could be frozen away in liquid nitrogen. Cell lines are named after the plasmid used to transform the initial dish, such as the pXC14 transformed hamster lines HXC14a and HXC14b.

Table 5.6: Ad5 Transformed BHK Cell Lines

line	plasmid	expt.	colonies[a]
HXC12a	pXC12	346	3
HXC14a	pXC14	373	7
HXC14b	pXC14	373	
HXC6a	pXC6	294	1[b]
HXC6b	pXC6	346	3
HXC6c	pXC6	346	14
HXC6d	pXC6	373	8
HXC7a	pXC7	346	7
HXC7c	pXC7	346	5

[a] number of colonies in original 60 mm dish
[b] clonal cell line

Chapter VI

Discussion

Adenovirus early region I is both necessary and sufficient for DNA mediated oncogenic transformation. Identifying and characterizing the functions of the adenovirus oncogenes encoded in E1 is paramount to our understanding of the mechanisms of cell transformation. The above transformation results have helped to define the adenovirus encoded oncogenes, and I will summarize these results in the context of recent literature, with reference to the E1A (**Section 6.1**) and the E1B (**Section 6.2**) encoded functions as shown in Figure 2.

6.1 Transformation functions encoded in E1A

As discussed in **Chapter I**, E1A is required for expression of all other viral early regions (Jones and Shenk, 1979b; Berk et al., 1979) including two regions that are required for initiation of transformation by virus, region E1B (group II-hr mutants, Graham et al., 1978) and region E2B (N-group mutants, Williams et al., 1974). Berk et al (1979) first proposed that the requirement for an E1A function in transformation may only be to activate expression of these other early regions. However, results from studies with group I hr viruses, reviewed in **Chapter I**, suggest that an E1A function is also required for expression of the full transformed phenotype (Graham et al., 1978; Ruben et al., 1982). Thus E1A encodes at least two separate functions involved in cell transformation.

6.1.1 R-strand transcripts are required for transformation

The transformation results with E1A mutants presented in **Section 5.2.2** demonstrate that E1A is necessary for transformation of primary BRK cells, as has been previously demonstrated with the hrI mutant viruses (Graham et al., 1978), with the deletion mutant virus dl312 (Jones and Shenk, 1979a), and more recently with E1A cold sensitive (cs) mutants (Ho et al., 1982). E1A encodes three possible gene products, including the r-strand 12s and 13s transcripts and the putative l-strand URF11 gene product (Figure 2). Three lines of evidence indicate that URF11 is not required for transformation.

First, there has been no report to date of either a mRNA transcript or a protein product from URF11, either in lytically infected cells or in transformed cells. Secondly, the frame shift deletion mutant Ad5dl311 (Jones and Shenk, 1978) lacks nucleotides 1280-1340-bp in E1A, affecting both the C-terminal exon of r-strand transcripts and URF11 on the l-strand, yet transforms like wild-type virus (Jones and Shenk, 1979b). Finally, the plasmid pXC386, with a decanucleotide insert affecting only the URF11 reading frame of E1A (Figure 9) transforms with wild-type levels (Table 5.2). Thus this l-strand coding sequence is not required for cell transformation, and as yet no function has been assigned to this region during the lytic cycle.

6.1.2 Role of the 12s and 13s E1A transcripts

The r-strand of E1A encodes two products, 12s and 13s mRNAs, translated in the same reading frame to generate related products differing only by the presence of 46 amino acids unique to the larger (13s) product (Figure 2). Post-translational modification of these two products gives rise to two families of discrete, related products from these mRNAs (Smart et al., 1981; Rowe et al., 1983b). The differ-

ences between these two families of protein products may have functional significance, since only the 13s products are detected with antitumor antiserum even though both 12s and 13s products are present in transformed cells (Rowe et al., 1983b).

The roles of the 12s and 13s products in lytic infection and transformation have been elucidated by construction of mutants that are defective in the synthesis of one or the other product. Montell et al (1982) constructed a virus pm975 defective in splicing of the E1A-12s mRNA but wild-type for production of the E1A-13s mRNA (described in **Chapter I**). This virus is wild-type for growth on HeLa cells (Montell et al., 1982) but defective for lytic infection of growth arrested human cells (Montell et al., 1984). Thus the E1A-13s product alone is sufficient to initiate lytic infection under normal cell culture conditions, but the 12s product is also required for infection of quiescent cells.

Montell et al (1984) have characterized the transforming activity of pm975 as well as a second mutant, dl1500 (wild-type for the E1A-12s mRNA but defective in splicing of the 13s mRNA). Their results suggest that both 12s and 13s products are required to produce the fully transformed phenotype, independent of the role of E1A products in activating E1B gene expression. They also report that transformation with dl1500 (expressing only the E1A-12s mRNA) is cold sensitive (Montell et al., 1984) as has been found with the E1A-13s mutant hr1 (Ho et al., 1982; Babiss et al., 1983). Thus E1A encodes two functions required for transformation, the 13s mRNA product required for expression of other early regions and for full transformation, and the 12s mRNA product (cold-sensitive) required for complete transformation.

6.1.3 Supertransforming mutants

Results from several groups suggest that mutations affecting the E1A-13s mRNA product actually increase the efficiency of transformation by adenovirus. It is difficult to assess the role of the 13s product in transformation however, since mutations affecting this function necessarily alter the expression of other viral early genes required for the initiation of transformation by virus. For example, the E1A-13s mutant in500 described in **Chapter I** (see Figure 24) expresses normal levels of E1B mRNA but expression of E2 is severely reduced and thus the transformation defective phenotype of in500 (Carlock and Jones, 1981) may be due to lack of expression of the E2B polymerase required for initiation of transformation. The mutant hr440 described in **Chapter I** is also transformation negative on BRK cells, both as virus and viral DNA (Solnick and Anderson, 1982). The defective transforming phenotype of this virus is therefore not due to the lack of expression of E2 by hr440, since E2 is not required in a DNA mediated transformation assay. Since hr440 has mutations altering E1A gene products (Figure 24) yet is able to express normal levels of E1B, this transformation defect supports previous conclusions that both E1A and E1B encode separate functions required for complete transformation (Solnick and Anderson, 1982).

Solnick and Anderson (1982) constructed a better defined mutant (dlA) by inserting a 330-bp DNA fragment (SmaI to XbaI) from hr440 in the comparable sites in E1A from wild type (dl309) DNA. This 330-bp segment contains one missense codon to the left of the 13s 5' splice site (Figure 24), and the resulting mutant has slightly reduced infectivity on HeLa cells. However, the alteration does not affect transformation. In fact, the transforming efficiency with dlA is proportional to input moi (Solnick and Anderson, 1982), whereas an inverse correlation is found when wild type virus is used. This suggests that the partial mutant

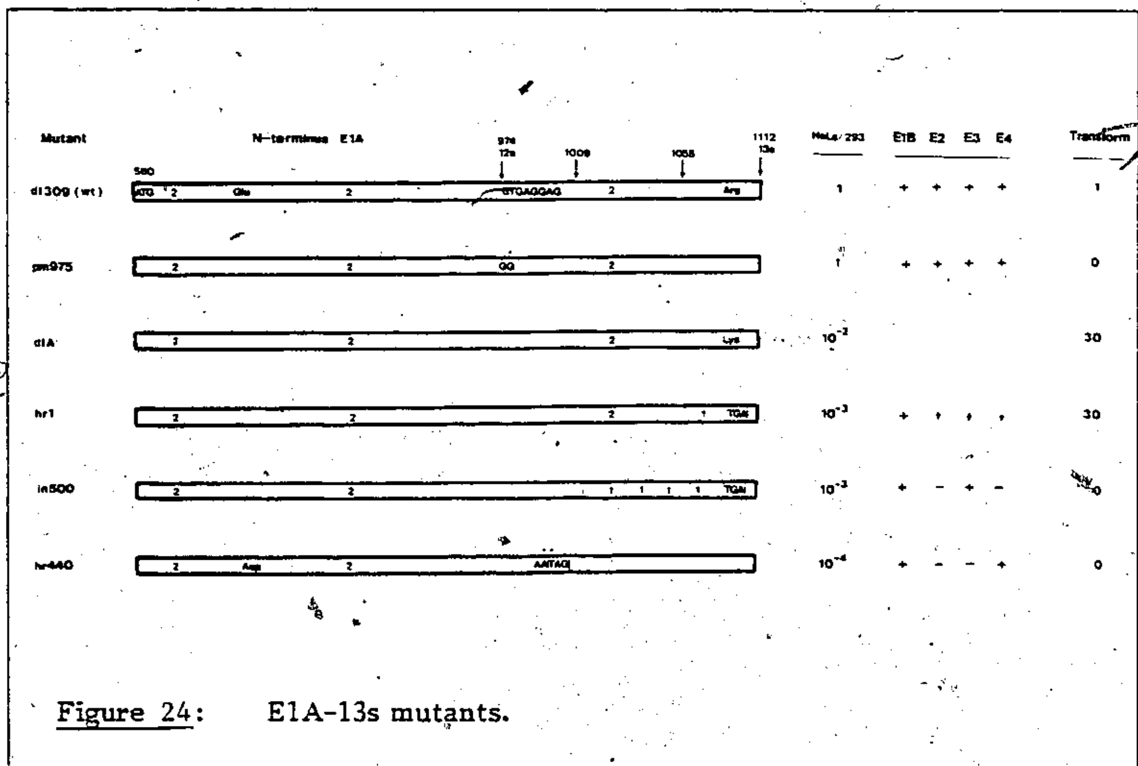


Figure 24: E1A-13s mutants.

Left: representation of the N-terminal exon of E1A. Top structure shows wild type (dl309) mRNAs, including the initiation codon (560-bp), the sequence at the 5'-splice site for 12s mRNA (974-bp) and the 13s mRNA (1112-bp), and the wild-type sequences that are altered in various mutants depicted below. Numbers within the boxes represent translation reading frames. Right: phenotype of mutants including virus titers on HeLa and 293-cells, levels of early region gene expression in HeLa cells (wild type=[+], nondetected=[-], decreased=[↓]), and transforming activity of viruses relative to wild type (see text).

phenotype of dlA may reduce the cytopathic effects of this virus, allowing a higher transforming efficiency in BRK cells. This may also explain why mutants hr1 (Graham et al., 1978; Babiss et al., 1983) and dl1500 (Montell et al., 1984) transform BRK cells with increased frequencies compared to wild-type virus.

Since hr1 has at least one sequence alteration affecting the 13s product (Figure 24) in the region removed from the 12s product (Ricciardi et al., 1981), it also may have reduced cytotoxicity in BRK cells allowing a greater number of transformants to survive (Ruben et al., 1982). The observation that hr1 transformed BRK cells are abnormal in that they are difficult to establish as transformed cell lines (Graham et al., 1978) suggests that the mutations in hr1 has altered an E1A function involved in immortalization of primary cells. The observation that dl1500 transformed cells are defective in anchorage independent growth (Montell et al., 1984) further suggests that the E1A-13s product is required for the fully transformed phenotype. However, the above observations suggest that mutations affecting 13s reduce viral cytotoxicity and thereby increase the frequency of transformation. Thus the E1A-13s protein appears to be multifunctional in transformation.

6.1.4 E1A Function: immortalization

Houweling et al (1980) have shown that the E1A DNA fragment alone can induce a partial transformation, or immortalization, of BRK cells. A similar incomplete transformation with the E1B deletion virus dl313 has been reported, both on the rat 3Y1 embryo cell line (Shiroki et al., 1981) and on primary BRK cells at high multiplicities of infection (Mak and Mak, 1982). Also, several viral and cellular transforming genes, which can transform immortal cell lines such as NIH 3T3 cells but not primary cells, have been shown to complement E1A in transforma-

tion of primary BRK cells (Ruley, 1983; Land et al., 1983). This suggests that E1A alters primary cells such that they can be fully transformed by oncogenes from a second complementation group. These observations are consistent with the conclusion that E1A and other complementing oncogenes induce an immortalization of primary cells.

Other oncogenes which fall into the E1A complementation group, including Polyoma large-T antigen and c-myc (Ruley,1983), encode proteins localized in infected and transformed cell nuclei (Donner et al., 1982; Abrams et al., 1982; Ito et al., 1977; Feldman et al., 1983) as is E1A (Rowe et al., 1983c; Feldman and Nevins, 1983). These observations are consistent with the hypothesis that immortalization is a consequence of E1A activation of cellular gene expression. Cellular genes activated by transformation may include functions shown to be induced during adenovirus infection of both permissive and nonpermissive cells, such as cellular thymidine kinase and DNA synthesis, heat shock proteins, abnormal mitoses, and chromosomal aberrations (Cheetham and Bellett, 1982; Bellet et al., 1982a,b; Nevins, 1982; Braithwaite et al., 1983). E1A of Ad12 (but not Ad5) has also recently been shown to switch off expression of class I major histocompatibility antigens in transformed rat cells (Schrier et al., 1983). This phenomenon may account for the higher tumorigenicity of group A adenoviruses (Table 1.1), since by switching off this locus these transformed cells may be able to avoid T-cell immunity in the host (Bernards et al., 1983).

Thus it is possible that one class of viral oncogenes, represented by E1A, can regulate cellular gene expression and thereby cause cell immortalization. It is likely that the function of E1A in immortalization is intrinsic to its role in regulating viral and cellular gene expression during lytic infection by adenovirus.

6.1.5 Functional domain of E1A proteins

Several lines of evidence indicate that the functional domain of E1A proteins is located in the N-terminal exon of both the 12s and 13s products. As discussed above, the mutant dl311 lacks the C-terminal exon of E1A due to a frame shift deletion at 1280-bp, yet is able to transform cells (Jones and Shenk, 1979a). In addition, both dl311 and the larger deletion mutant dl313, which lacks most of the C-terminal exon of E1A as well as E1B sequences (Figure 2), express RNA from all other early regions (Jones and Shenk, 1979b). Finally, both Shiroki et al (1981) and Mak and Mak (1982) have shown that dl313 retains the E1A immortalization function. Therefore the C-terminal exon of E1A is not required for either transformation or gene expression. The function of this coding region in E1A is not clear at present.

6.1.6 Nonequivalence of BHK and BRK cell transformation

The results presented in **Section 5.2.4** suggest that BHK cells may not have an absolute requirement for a functional E1A oncogene for transformation. In parallel experiments the DNA precipitates from each of these E1A mutants was able to transform BHK cells, albeit at approximately 10-fold reduced levels compared to pXC1 (Table 5.5). These results raise the issue of the nature of primary BHK cells. It is possible that these cells are partially transformed by the process of tissue culture, and therefore are similar to immortal cell lines such as NIH 3T3 cells which can be transformed by Polyoma middle-T and by c-ras, two oncogenes which fall into the same complemention group as E1B (Ruley, 1983; Land et al., 1983).

The observation that pXC14 transforms BHK cells with efficiencies comparable to pXC1, whereas it is unable to transform BRK cells (Table 5.5), also

points to a difference in these two cell types. Since Tn5 is located in the E1A-13s intron in pXC14, it is possible that BHK cells are able to remove Tn5 sequences from E1A transcripts by mRNA processing, while BRK cells are unable to properly splice such a hybrid transcript. Although no attempt has been made to examine RNA processing in pXC14 transformed cells in this work, this is an intriguing observation that deserves further examination.

6.2 Functions encoded in E1B

6.2.1 55k: initiation (integration function?)

The transformation results presented in Table 5.5 demonstrate that functions encoded in the promoter distal region of E1B are not required for transformation, confirming previous observations using isolated viral DNA fragments (Graham et al., 1974a, 1974b; van der Eb et al., 1977). However, group II mutant hr viruses (Harrison et al., 1977) are transformation negative on BRK cells (Graham et al., 1978), and their only known defect maps to this carboxy-terminal region of E1B (Galos et al., 1980) affecting the synthesis of the E1B 22s mRNA product 55k, seen on SDS-PAGE as 58k (Lassam et al., 1979a; Ross et al., 1980). This contradiction (transformation with HindIII-G DNA but not with hrII virions) may be more apparent than real, however, since hrII viral DNA is able to transform BRK cells (Rowe and Graham, 1983).

The apparent contradiction may actually reveal a difference in transformation assays. For example, transformation with virions could induce cytopathic effects in infected cells, an effect which may be avoided in DNA transfection experiments due to shearing of the viral DNA. A second explanation may be that during DNA transfection experiments more copies of viral DNA are

delivered to competent cells (input of $3x10^{10}$ copies per μg viral DNA) than are delivered to infected cells in a typical virion transformation experiment (usual moi<1, representing 25-50 particles per cell or $<10^8$ copies of viral DNA per dish).

A third possibility is that this observation reflects a different mechanism of stable integration of viral DNA into the transformed cell chromosome. During DNA transfection experiments the DNA integrates after formation of high molecular weight "peckalosomes", or concatameric structures involving both transforming and carrier DNA (Wigler et al., 1979). This may be in contrast to an active "integration" function, defective in hrII virus (ie 55k), that might be required for integration during an adenovirus infection (Graham et al., 1983), as discussed in **Chapter I.**

To date there is no direct evidence that adenovirus has an integrative or proviral stage of infection in tissue culture. However, the mechanism of persistent infections in man with this virus is not understood, and an integrative phase is an intriguing possibility.

6.2.2 21k: maintenance of transformation

The results of transformation experiments presented in **Section 5.2.2** demonstrate that the N-terminal region of E1B is essential for transformation. There are three possible genes in this region of E1B, the 13s product (21k), the N-terminal portion of the 22s mRNA product (55k), and the putative l-strand encoded URF10 (see Figure 19). Support for the argument that the 21k product is required for transformation comes from the observations of Matsuo et al (1982) that this protein is present in all Ad5 transformed cell lines.

Since the insertion in pXC3 is located 13 codons past the initiation AUG of 55k, creating 9 missense codons (from Tn5 sequences) before a nonsense

triplet, the 55k gene product would be severly truncated in this mutant if made at all. In both the 21k and the URF11 reading frames, the mutation in pXC3 interrupts C-terminal sequences, and thus if translated could conceivably produce a truncated product with residual activity. This could explain why pXC3 has reduced but nonzero transforming activity, while pHE1 and pCD1 are completely transformation negative.

Recently, Chinnadurai (1983) localized the mutations in two Ad2 large plaque (lp) mutants, lp3 and lp5. Both have hydroxylamine induced base substitutions, affecting the E1B-21k protein (lp3) and affecting the E1B-21k, 55k, and URF10 (lp5), and both mutants are defective for transformation of the 3Y1 rat embryo cell line (Chinnadurai, 1983). Interestingly, the mutation in lp3 causes only a conservative substitution (valine for alanine) at position 1718 immediatly adjacent to the N-terminus of 21k. DNA sequence analysis of this region of Ad5 (Bos et al., 1981) and Ad2 (Gingeras et al., 1982) reveal that while the C-terminal portion of 21k shows amino acid substitutions, the N-terminal region is highly conserved. These observations support the conclusion that the N-terminus of 21k is an important functional domain involved in transformation.

6.2.3 Expression of hybrid genes in transformed cells

If as argued above the E1B-21k protein is required for cell transformation, then this gene must be expressed as a hybrid mRNA transcript in cells transformed with the HindIII-G DNA fragment since this DNA fragment does not contain the viral processing signals for the 3' splice acceptor site or the poly(A) addition involved in RNA maturation (Figure 8). It is probable that the integrated DNA in transformed cells uses cellular 3' processing signals for expression of this protein. This has been recently demonstrated for three cell lines transformed by the

HindIII-G fragment (Klessig et al., 1982). Presumably transformation assays select for cells in which viral maintenance genes have stably integrated into a cellular chromosome such that the viral information can be expressed.

An extension of this argument is that cells transformed by pXC::Tn5 plasmids may express hybrid transcripts, allowing the expression of the viral encoded gene products. Although insertion of Tn5 into these plasmids introduces nonsense codons immediately downstream from the site of insertion, it is not clear what other transcriptional, processing, or translational signals may be present in this bacterial DNA sequence. Thus it is likewise not clear what effect these insertions will have on transcription or processing of viral genes in transformed cells. Since pXC3 transforms BRK cells, although at a very much reduced level compared to pXC1 (Table 5.4), it is possible that these rare transformants represent events in which the plasmid DNA integrated upstream from cellular RNA processing signals allowing expression of E1B sequences.

6.2.4 E1B relieves serum dependency of primary cells

The results presented in **Section 5.2.3** suggest that one E1B function during cell transformation is to relieve a serum dependency of the untransformed cell. As shown in Table 5.4 and Figure 23, morphological transformants were obtained after transformation with E1A plasmids only when selection medium was supplemented with 10% fetal bovine serum. The E1A transformed cells shown in Figure 23 appear morphologically transformed, but when these cells are expanded as cell lines they are more fibroblastic than cells transformed by all of E1 (FLG, personal communication). Therefore it is also possible that the E1B function is also responsible for morphological alteration of the transformed cell. Rassoulzadegan et al (1982) have reported that the polyoma large T tumor anti-

gen lacks intrinsic oncogenic potential but can relieve the serum dependency of both normal and transformed cells. Thus it appears that viral transformed cells express a viral encoded function which is a component present in serum and is required for cell growth.

It is possible that this function present in sera is a mitogenic growth factor, analogous to Platelet Derived Growth Factor (PDGF). Evidence for this first came from the observation by Scher et al (1978) that the loss of serum dependency or the requirement for PDGF is a necessary, although insufficient, step in the malignant transformation of fibroblasts. PDGF is a peptide hormone involved in regulating the mitotic cycle of connective tissue cells (see Stiles, 1983), and has recently been shown to be actively transcribed in some human sarcomas (Eva et al., 1982). Evidence that PDGF may be directly involved in malignant transformation is provided by recent reports (Waterfield et al., 1983; Doolittle et al., 1983) that the oncogene transduced by the simian sarcoma virus (v-sis) is the structural gene for PDGF.

Thus it appears that a second class of oncogenes active in malignant cells, and transduced by oncogenic viruses, are involved in regulating the mitotic cycle. It may be that the DNA tumor viruses such as Polyoma and adenovirus encode their own polypeptide regulating the cell cycle, which may increase mitotic activity in infected cells and thereby increase the cellular capacity to replicate virus. The presence of these genes in the transformed cell therefore would increase the replicative capacity of these cells. one hallmark of a malignancy.

6.3 Summary

Adenovirus encodes several functions involved in oncogenic transformation, including functions required for both initiation and maintenance of the transformed state. The E1A-13s product is required for viral gene expression (Berk et al., 1979; Ricciardi et al., 1981; Montell et al., 1982). This gene product activates expression of two other early genes, the E1B-22s product (58k) and the E2B N-group product (DNA polymerase) that are possibly involved in integration of viral DNA into the host cell chromosome during transformation. However, only the left end 8% (early regions E1A, E1B) are required for maintenance of the transformed state, and this region is sufficient for transformation in a DNA-mediated assay. The objective of this study was to identify those regions of E1 necessary for DNA-mediated transformation. The approach taken was to construct recombinant plasmids containing all or portions of E1 (**Chapter III**), and E1 plasmids containing defined mutations using the prokaryotic transposable element Tn5 (**Chapter IV**), and to characterize the transforming activity of recombinant plasmids on primary rodent cells (**Chapter V**).

The results presented in this study demonstrate the usefulness of prokaryotic transposable elements as insertional mutatagens for cloned eukaryotic genes. The transposon Tn5 was used to define the coding regions of E1 oncogenes, and this technique could conceivably be applied to gene mapping for any system which has a biological assay. The ability to define the site of insertion of Tn5 by DNA sequence analysis permits the interpretation of the phenotype of these mutants.

Results presented in **Chapter IV** have demonstrated several interesting phenomenon associated with Tn5 transposition. It was found that Tn5 preferably inserts downstream from a prokaryotic promoter sequence fortuitously located in

adenovirus type 5 E1B sequences. In addition, the orientation of Tn5 downstream from this promoter sequence was found to be nonrandom, with the end of Tn5 encoding the cis-acting transposase protein (IS50R) located nearest the promoter. These results support the "donut" model of Tn5 transposition proposed by Berg (1982b), and suggest that transposase seeks out transiently denatured regions (transcriptionaly active regions) on target DNA molecules to initiate transposition.

The morphological transformation results presented in Chapter V have demonstrated (1) the requirement for r-strand E1A transcripts in transformation, (2) the requirement for E1B sequences and the replacement of this requirement with serum supplements, and (3) the nonidentity of primary BHK and BRK cell transformation assays. These results have contributed to defining the adenovirus genes required for cell transformation. What remains to be determined are the biological properties of these gene products which allow them to bring about the immortality and unlimited proliferative capacity of transformed cells.

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