A GENETIC ANALYSIS OF CELLULAR TRANSFORMATION BY POLYOMAVIRUS

by

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I dedicate this thesis to my father, the late John Mes,

and to my mother, Margeret Mes van Dinther - who believed.

ABSTRACT

Ph.D. Anne-Marie Mes-Masson Microbiology

A genetic analysis of cellular transformation by polyomavirus

To determine whether small and/or middle T antigen of polyomavirus are required for transformation, mutants of recombinant plasmids which bear the viral oncogene(s) were constructed and tested for their capacity to transform Rat-1 cells in culture. Insertion and deletion mutations in sequences encoding small and middle T antigens rendered the DNA incapable of transforming Rat-1 cells as measured by the focus assay. Similar mutations in sequences which encode middle but not small T antigen generally abolished the transforming activity of the DNA. However, two mutants (pPdl1-4 and pPdl2-7) retained the capacity to transform cells. From these studies, I concluded that small T antigen alone is insufficient to cause transformation and that middle T antigen is required for transformation either by itself or in combination with small T antigen.

Because cellular transformation mediated by polyomavirus requires the action of middle T antigen, an attempt was made to define the domains of the viral protein important for its transforming activity. One of the domains of middle T antigen thought to be important for its function is tyrosine 315, the major site of tyrosine phosphorylation *in vitro*, and the stretch of acidic amino acids preceding this residue. Characterization and analysis of three mutant recombinant plasmids (pPdl1-4, pPdl2-7, and pdPbs25) proved that the major site of tyrosine phosphorylation in middle T antigen and the acidic amino acids preceding it are not essential for its transforming activity.

A second domain of middle T antigen thought to be important for its transforming activity is a cluster of hydrophobic amino acids found at the carboxy-terminus (C-terminus) of the protein. Three mutant recombinant plasmids (pPdl1, pPdl15, and pPdl90), which lack either all or some of these hydrophobic amino acids, are totally defective in the transformation assay, leading to the conclusion that these sequences are absolutely required to maintain middle T antigen transforming activity. However, a single mutant (pPdl6) which retains activity in the transformation assay, despite a small alteration of the amino acid composition of the C-terminus of middle T antigen, suggests that the protein can sustain some alterations at its C-terminus without a loss of transforming activity.

RESUME

Ph.D. Anne-Marie Mes-Masson Microbiologie

Analyse génétique de la transformation cellulaire par le virus de polyome.

Afin de déterminer si l'antigène moyen et/ou petit T du virus de polyome est nécessaire pour la transformation, des mutants de plasmides contenant le ou les oncogène viraux ont été construite et testés en ce qui concerne leur capacité de transformation des cellules Rat-1 in vitro. Des mutations causant des insertions ou des délétions dans les séquences qui codent pour les antigènes petit et moyen T, ont entrainé l'incapacité de l'ADN de transformer les cellules Rat-1. Des mutations similaires dans les séquences qui codent pour l'antigène moyen T, mais non pas pour l'antigène petit T, ont eu pour effet d'abolir l'activité de transformation de l'ADN. Par contre, deux mutants (pPdl1-4 et pPdl2-7) ont conservé cette capacité de transformation des cellules. Il en ressort que l'antigène petit T seul n'est pas suffisant pour causer la transformation, tandis que l'antigène moyen T est nécessaire pour assurer la transformation, soit seul, soit en association avec l'antigène petit T.

Du fait que la transformation cellulaire produit par le virus de polyome requiert la participation de l'antigène moyen T, on a essayé de déterminer les régions de la protéine virale qui sont essentielles pour la transformation. Une de ces régions considérées comme importantes pour cette fonction serait le site de phosphorylation in vitro de la tyrosine 315 ainsi que la zone occupée par les amino acides dicarboxyliques qui précèdent cet acide aminé. En fait l'analyse et la caractérisation de trois mutants (pPdl1-4, pPdl2-7 et pPbs25) ont montré que le principal site de phosphorylation de la tyrosine

dans l'antigène moyen T, de même que les acides aminées dicarboxyliques qui la précèdent, ne sont pas essentiels pour l'activité de transformation.

Une deuxième région de l'antigène moyen T considérée comme importante pour son activité de transformation correspondant à la zone d'acides aminés hydrophobes que l'on trouve dans la partie carboxy-terminale de la protéine virale a été également considérée comme un deuxième foyer possible de l'activité de transformation. Trois mutants (pPdl1, pPdl15 et pPdl90), privés en totalité ou en partie des acides hydrophobes se sont avérés totalement défectueux en ce qui concerne la transformation cellulaire. Par conséquent, il semble que ces séquences hydrophobes sont nécessaires pour la fonction de transformation de l'antigène moyen T. Seul un mutant (pPdl6) a conservé son activité de transformation, malgré une légère modification des acides aminées se trouvent dans la partie carboxy-terminale de l'antigène moyen T. Ces résultats indiquent que l'antigène moyen T peut subir quelques modifications dans son extrémité carboxy-terminale sans perdre son activité de transformation.

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Finally, I wish to thank my supervisor, John Hassell, who served as a tremendous source of assistance, advice and motivation. You're right John, I'll always remember you (with fondness).

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. Mutant recombinant plasmids bearing insertions (pPin67) or deletions (pPdl1-8, pPdl1-10, pPdl2-2, pPdl2-3, pPdl2-5, pPdl2-12, pPdl1, and pPdl15) in middle T antigen coding sequences, but which retain the coding sequences of small T antigen, as well as the transcriptional regulatory sequences for small T antigen mRNA synthesis, are completely defective in the transformation assay. Therefore, small T antigen of polyomavirus alone is insufficient to cause transformation of Rat-1 cells in culture.

2. Insertion and deletion mutations in sequences which encode middle but not small T antigen generally abolish the transforming activity of the DNA. Therefore, middle T antigen of polyomavirus is absolutely required for cellular transformation.

3. Mutant recombinant plasmids bearing deletions which either effectively abolish the hydrophobic domain (pPdl1 and pPdl15) or alter the amino acid composition (pPdl90) of the hydrophobic tail of middle T antigen are completely defective in the transformation assay. Thus, the hydrophobic amino acids at the carboxy-terminus of middle T antigen are required for the transforming activity of this protein.

4. A mutant recombinant plasmid (pPdl6) contains a deletion which alters the sequences at the carboxy-terminus of middle T antigen such that there is a significant reduction in the net positive charge at the carboxy-terminus of the novel middle T antigen species at physiological pH. Despite this difference in charge, pPdl6 DNA is still active in the transformation assay indicating that there is some flexibility in the amino acid sequence at the

carboxy-terminus of middle T antigen.

5. A mutant (pdPbs25) containing a single base substitution exchanges tyrosine 315, the major site of in vitro tyrosine phosphorylation in middle T antigen, for a serine residue. This mutant continues to transform Rat-1 cells in culture. Therefore, the major site of tyrosine phosphorylation is not essential for transformation by polyomavirus.

6. Two mutant recombinant plasmids, pPdl1-4 and pPdl2-7, containing deletions in sequences encoding middle but not small T antigen, retain the capacity to transform Rat-1 cells at high frequencies. The middle T antigen encoded by pPdl1-4 lacks part of the acidic string of amino acids preceding tyrosine 315 whereas the middle T antigen encoded by pPdl2-7 lacks the entire acidic amino acid stretch as well as both the major (tyrosine 315) and the alternate (tyrosine 322) sites of tyrosine phosphorylation in vitro. These results prove that the major and alternate sites of tyrosine phosphorylation in middle T antigen, as well as the acidic amino acids which precede it, are not essential for its transforming activity.

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LITERATURE REVIEW

LITERATURE REVIEW

I. STRUCTURE OF POLYOMAVIRUS

A) Introduction

Polyomavirus is a member of the Papovavirus family. The virus was originally described by Ludwig Gross in 1953 (Gross, 1953a, 1953b). Gross accidently stumbled onto the virus while attempting to isolate a murine leukemia virus from leukemic Ak mice extracts. Although the majority of mice developed leukemia when injected with filtered extracts, a small percentage of the mice developed salivary gland (parotid) adenocarcinomas, but remained free of leukemia. Gross was able to demonstrate that this activity was due to a contaminating virus which he called the parotid agent. It was later discovered that while parotid tumors are the most common consequence of inoculation by the parotid agent, a variety of other tumors including medullary adrenal tumors, epithelial thymic tumors, mammary gland carcinomas, renal carcinomas, liver hemangiomas, and subcutaneous fibrosarcomas also arise (Stewart et al., 1958). Because the parotid agent was able to transform so many different cell types, the name "polyoma virus" was proposed (Stewart et al., 1958).

Polyomavirus has been found to be endemic for a large fraction of both wild (Rowe, 1961) and laboratory-bred mice (Yabe et al., 1961). A major breakthrough in understanding the structure and the replication of polyomavirus came after the development of an in vitro culture system which supported the replication of the virus. The first culture systems made use of mouse embryo cells as a permissive environment for the virus (Stewart

et al., 1958). The availability of cultures of permissive cells made it possible to grow relatively large amounts of polyomavirus in sufficient quantity for chemical analysis. These studies revealed that the viral particle consists of a covalently closed, double-stranded DNA molecule (Dulbecco and Vogt, 1963; Weil and Vinograd, 1963) which associates with host histones to form the viral chromatin (Frearson and Crawford, 1972; Schaffhausen and Murakami, 1972; Murakami and Schaffhausen, 1974; Fey and Hirt, 1975) Early attempts to calculate the molecular weight (M.W.) of the DNA molecule suggested that the polyomavirus DNA molecule had a M.W. of $3.5X10^6$ (Crawford, 1963, 1964; Weil et al., 1967). This estimate corresponds well with the absolute molecular weights which was calculated once the nucleotide sequence of the viral DNA was determined (Friedmann et al., 1978a, 1978b, 1979; Soeda et al., 1978, 1979, 1980; Deninger et al., 1980). The sequence analysis revealed that polyomavirus DNA is comprised of 5292 base pairs (b.p.). Nucleotide numbers given in this text will be according to the numbering system of Soeda et al., (1979, 1980). The viral chromatin is surrounded by an icosahedral capsid consisting of 72 capsomers. These capsomers are composed of the major capsid protein VP1 and two minor capsid proteins VP2 and VP3.

Because it has only recently been possible to sequence the entire polyomavirus genome, a physical map, consisting of a series of reference points whose distances from each other were known, was used to define a physical map of reference points in polyomavirus DNA. The most widely used method to map viral genomes involves the use of restriction endonuclease cleavage sites. The polyomavirus genomes was arbitrarily divided into 100 map units (m.u.), with the unique EcoR1 site defined as m.u. 0/100, and with m.u. increasing in a clockwise direction (Griffin et al., 1974).

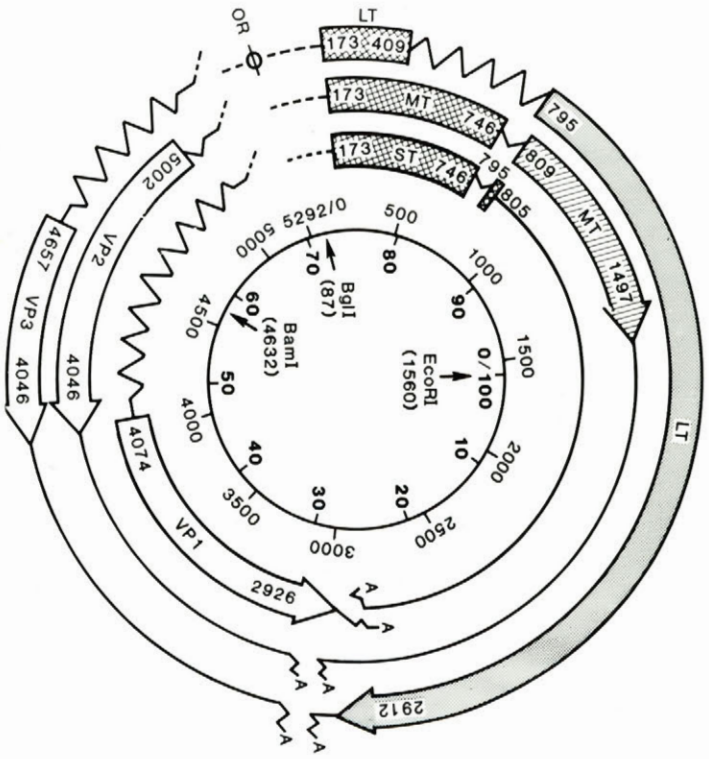
Using a number of different restriction endonucleases, a detailed cleavage map of the A-2 strain of polyomavirus (Fried et al., 1974) was constructed (see Fried and Griffin, 1977).

Two temporal classes of stable viral mRNAs are present in the cytoplasm of cells infected with polyomavirus; early mRNAs, which by definition are synthesized before the onset of viral DNA replication, and late mRNAs, which are produced in significant quantity only after viral DNA replication begins. These mRNAs are transcribed from different strands of the DNA known as the E strand for early RNA and the L strand for late RNA which define the 5' to 3' orientation of the viral DNA strands (Summers, 1975; Kamen et al., 1974, 1976). Because of the temporal nature of viral gene expression, the polyomavirus genome has been divided into an early and a late region. Between these two regions lies the early and late promoters of transcription as well as the viral origin of DNA replication (Figure 1). The salient features of the coding and non-coding regions of polyomavirus, which will be dealt with in greater detail further in the text, are shown in Figure 1.

B) The Early Coding Region

The early coding region of polyomavirus is situated approximately between m.u. 73 and 26 (clockwise) or nucleotides 173 and 2912 (Figure 1). The early region codes for three early proteins collectively known as tumor antigens (T antigens). They are the large T, middle T, and small T antigen (Ito et al., 1977a, 1977b, 1977c; Hunter et al., 1978; Hutchinson et al., 1978; Schaffhausen et al., 1978; Benjamin et al., 1979; Soeda et al., 1979). These T antigens are translated from three differentially spliced mRNAs which sediment at about

Figure 1: Physical map of the polyomavirus genome. The numbers on the inner portion of the circle represent map units, with the unique EcoR1 site serving as the 0/100 reference point. The numbers displayed on the outer portion of the circle represent nucleotides numbered according to Soeda et al., (1979, 1980). The relative position and the nucleotide numbers are given for the cleavage sites of the three restriction endonucleases Bgl1 (87), EcoR1 (1560) and BamH1 (4632). The figure shows the coding sequences for the three T antigens. Note that the N-terminal portion of the three T antigens (before the proximal splice) as well as the distal portion of small T antigen are in the same reading frame. The C-terminal portion of middle and large T antigen (after the distal splice) are in the two remaining reading frames and are different from each other. The coding portion of the DNA thought to correspond to the proteins VP1, VP2 and VP3 are also shown. In addition, the approximate location of the site at which DNA replication initiates (or) is indicated.


OR
LT
173
409
173
MT
746
173
ST
746
795
809
MT
1497
795
805
LT
2912
5002
VP2
4537
VP3
4046
4046
4074
VP1
2926
A
A
A
A
A
5292/0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
10
20
30
40
50
60
70
80
90
0/100
BglI
(87)
BamI
(4632)
EcoRI
(1560)

20S (Kamen and Shure, 1976; Bacheler, 1977). The three mRNA species share a common 5' end near 73.3 m.u. and 3' end at 25.8 m.u. Fine structure mapping places the major starts for the 5' end of early mRNA at nucleotides 148 and 152 (Kamen et al., 1982). The polyadenylation signal, AAUAAA, common to eukaryotic mRNAs (Proudfoot and Brownlee, 1976), is located at the end of the early region between nucleotides 2915 and 2920. There is an additional polyadenylation signal at nucleotides 1476-1482 which is infrequently used to generate mRNAs which can code for either middle or small T antigen, but not for large T antigen (Kamen et al., 1980). In vitro translation of RNA fractionated by size showed that the mRNA for large T antigen is smaller than the mRNA for small and middle T antigen (Hunter et al., 1978). Originally the introns for the early mRNAs were determined by S1 mapping and comparison to consensus splicing sequences (Kamen et al., 1980). They were conclusively determined when cDNAs of each early messenger was cloned and sequenced (Treisman et al., 1981; Tyndall et al., 1981; Rassoulzadegan et al., 1982). These studies indicated that the introns extended from nucleotides 409-809, 795-805, and 795-809 for large, middle, and small T antigen respectively.

The net effect of transcription, splicing, and translation is that the T antigen proteins share common amino acids at their amino-termini (N-termini) but contain varying lengths of unique sequences at their carboxy-termini (C-termini). All three proteins begin with a single initiation codon between nucleotides 173 and 175 (Soeda et al., 1978; Friedman et al., 1978b) and initially share the same coding frame (Smart and Ito, 1978; Hutchinson et al., 1978). Large T antigen shares the same reading frame as middle and small T antigen until nucleotide 409 or amino acid 79. The large T antigen intron causes a shift in the reading frame downstream of

the splice so that the large T antigen is encoded in an open reading frame until a termination codon is reached at nucleotides 2913-2915. This gives rise to a protein which is 785 amino acids long and has a calculated M.W. of 87,991. While all three T antigens share a 79 amino acid N-terminal sequence, middle and small T antigens share an additional 112 amino acids in a region that is within the intron for large T antigen. The intron for small T antigen results in four amino acids being encoded downstream of the splice in the same reading frame as the N-terminus of the protein. This results in a protein which is 195 amino acids long and has a calculated M.W. of 22,785. The intron for middle T antigen results in the addition of an extra 230 amino acids which are acquired in a reading frame distinct from those used for the C-terminal portion of both small and large T antigen. Thus middle T antigen contains 421 amino acids and has a calculated M.W. of 48,506.

C) The Late Coding Region

The late region of polyomavirus is situated approximately between m.u. 65 and 26 (counterclockwise) or nucleotides 5002 to 2926 (Figure 1). The late region is actively transcribed after DNA replication has been initiated and codes for the three viral capsid proteins VP1, VP2, and VP3. Late viral mRNAs species are polyadenylated, and probably make use of the polyadenylation signal AAUAAA between nucleotides 2913-2918 (Soeda et al., 1980). Late mRNA sediments at 19S and 16S. S1 nuclease mapping studies revealed that the 19S mRNA corresponds to most of the late region while the 16S mRNA represents sequences in the distal half of the late region (Kamen and Shure, 1976). Both the 19S and 16S mRNA species are spliced. The 16S mRNA codes for the major capsid proteins while two different 19S mRNA species code for the

minor capsid proteins VP2 and VP3 (Smith et al., 1976; Hunter and Gibson, 1978).

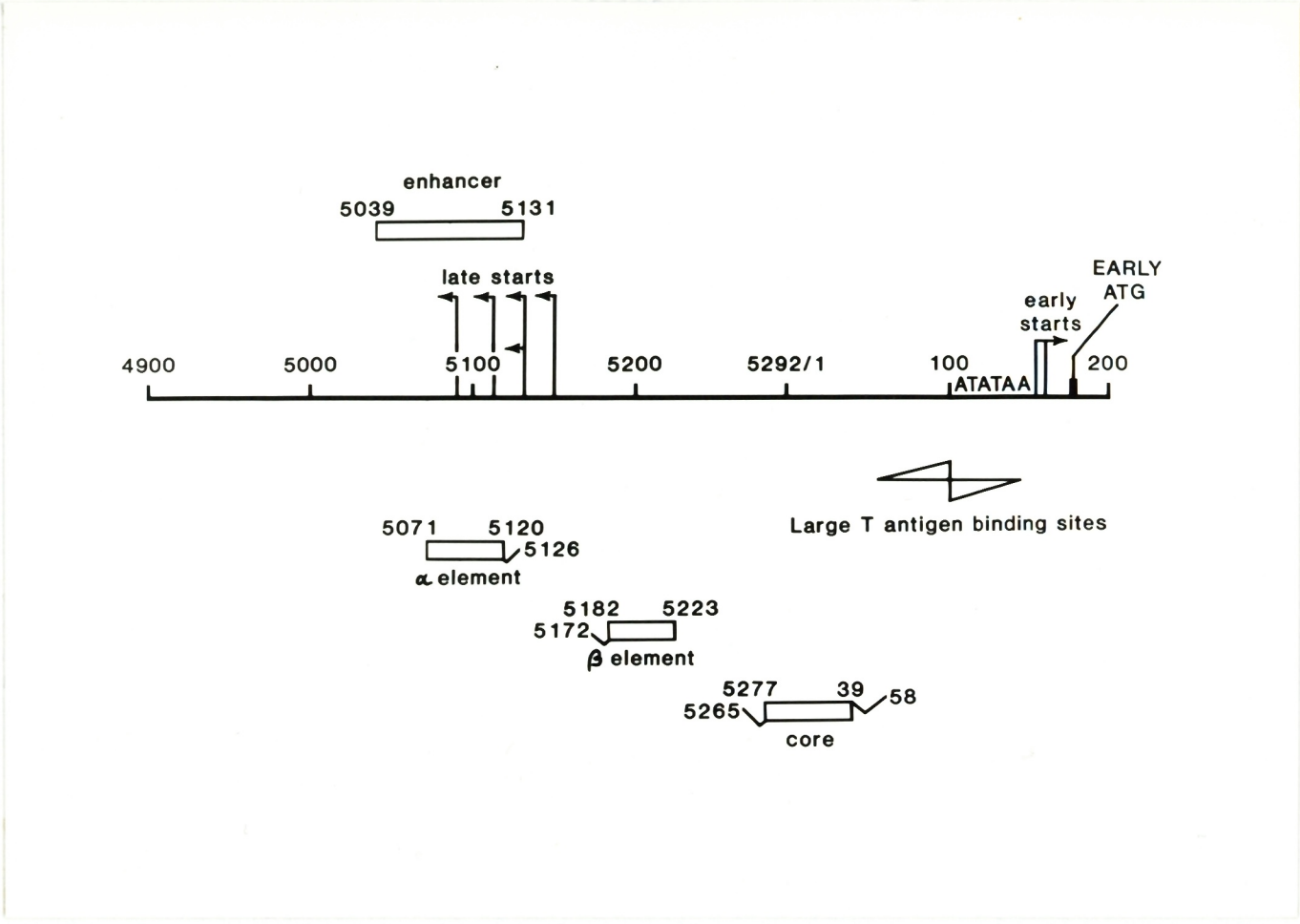
VP1 of polyomavirus appears to be composed of 383 amino acids, has a M.W. of 42,500, and is thought to be encoded by sequences between nucleotides 4074 and 2926 (Soeda et al., 1980). VP2 and VP3, which are unrelated to VP1 (Gibson, 1974; Herrick et al., 1975), have a calculated M.W. of 36,400 and 22,800 respectively. VP2 is thought to be encoded by sequences from 5002 to 4046 while VP3 arises from sequences between 4657 and 4046 (Soeda et al., 1980).

D) The Non-Coding Region

The sequences between m.u. 65 and 73 (clockwise) or nucleotides 5002 to 173 are not known to code for any viral proteins. However, this region of the viral DNA contains the transcriptional promoters for both early and late mRNA synthesis as well as the viral origin for replication. Figure 2 is a schematic representation of the important features of the non-coding region.

The early promoter is active throughout the lytic cycle, as well as in transformed cells. The functional elements of the early promoter resembles many other viral and cellular promoters which have been described. These include a Hogness-Goldberg 'TATA' consensus sequence (Goldberg, 1979) found in the DNA preceeding the principal early mRNA cap sites. This sequence appears to determine the position at which RNA transcription begins, and while its presence is required in vitro (Jat et al., 1982a, 1982b; Mueller et al., 1984), it does not appear to be required in vivo (Tyndall et al., 1981; Hassell et al., 1982; Jat et al., 1982b; Mueller et al., 1984). The major cap sites

Figure 2: Physical features in the non-coding region of polyomavirus DNA. The relative position of the large T antigen binding sites and the various replicational elements are shown at the bottom of the figure. At the top of the figure are shown the 5' termini of late and early mRNAs, the viral enhancer region, the early TATA box (ATATAA), and the ATG which likely serves as the initiation codon for the three T antigens.


enhancer
5039
5131
late starts
EARLY
ATG
early
starts
4900
5000
5100
5200
5292/1
100
ATATAA
200
Large T antigen binding sites
5071
5120
5126
α element
5182
5223
5172
β element
5277
39
58
5265
core

are somewhat heterogeneous, but at early times they are all positioned approximately 31 b.p. from the beginning of the 'TATA' box (Kamen et al., 1982). A more heterogeneous array of cap sites for early mRNA is found late in infection (Kamen et al., 1982). The early promoter is under the influence of a viral enhancer sequence. The enhancer is defined as a sequence which can increase the expression of promoters over considerable distances and in an orientation independent manner (deVilliers and Schaffner, 1981; Tyndall et al., 1981; Hassell et al., 1982; Jat et al., 1982b; Mueller et al., 1984). At the present time, the smallest portion of polyomavirus sequences which have been shown to contain enhancer function lies between nucleotides 5039-5131 (Mueller et al., 1984).

The position of the late promoter is not completely known. The late transcription unit functions during infection predominantly after the onset of viral DNA replication and is not normally expressed in transformed cell lines. Transcripts from the late region use one of at least 15 different cap sites (Cowie et al., 1981) and gives rise to mRNA species containing variable numbers of the 5' non-coding leader sequence (Treisman, 1980). These head-to-tail, tandemly repeated leaders are probably generated by RNA splicing of giant tandem transcripts of the entire circular viral genome which are the nuclear precursors of the late mRNAs (Acheson et al., 1971; Birg et al., 1977; Acheson, 1978; Treisman and Kamen, 1981). The late promoter does not contain the usual 'TATA' sequence although a related sequence TAATTAAAA is found between nucleotides 5158-5150 (Jat et al., 1982a) which lies within the heterogeneous late mRNA cap sites (Cowie et al., 1981). It is not known whether the enhancer of early transcription also functions to enhance transcription from the late promoter.

The initiation of viral DNA replication begins near 70 m.u. or nucleotide 5292/0 (Crawford, 1973; Crawford et al., 1974). The cis-acting sequences which comprise the polyomavirus origin of replication have been primarily defined through the use of viable deletion mutants (Bendig and Folk, 1979; Griffin and Maddock, 1979; Magnusson and Berg, 1979; Wells et al., 1979) or by using viral/plasmid recombinants (Tyndall et al., 1981; Muller et al., 1983a). These results indicate that the viral origin is comprised of several genetic elements. Muller et al., (1983a) found that the viral origin was composed of two functionally redundant sequences termed alpha and beta which act in concert with a third region called the core region. The exact role these elements play in viral replication is presently being studied.

II. THE LYTIC CYCLE OF POLYOMAVIRUS

Cells infected by polyomavirus may exhibit various responses. Depending on the cell-type, cells may support the replication of the virus and subsequently the viral lytic cycle (permissive cells). However, a small percentage of these cells may undergo abortive transformation while an even smaller number can become stably transformed. Cells in which only a fraction of the population support viral replication (semipermissive cells), or cells not supporting viral replication (nonpermissive cells), abortive transformation is the common response, and stable transformation can take place. In this section, the lytic cycle of polyomavirus in permissive cells will be briefly introduced, whereas polyomavirus-directed transformation is covered in the following section.

The first steps in infection are adsorption, penetration and uncoating

of the virus. Upon infection, the virus particles adsorb to cell monolayers in 1-2 hours. Pinocytotic vesicles harboring the virus appear in the cytoplasm shortly after infection (Mattern et al., 1966) and are thought to act in transporting the virus to the nucleus. Intact virions are detected in the nucleus of infected cells as early as 30 minutes after viral infection (Mackay and Consigli, 1976). Although intact virions can be found in the nucleus suggesting that uncoating can occur in the nucleus, Frost and Bourgaux, (1975) have found evidence that uncoating may begin in the cytoplasm. The viral DNA in the nucleus can now undergo the subsequent steps in the viral lytic cycle.

The polyomavirus genome is temporally regulated and during the early phase the three non-structural T antigens are made. The early phase is operationally defined as beginning with the appearance of viral RNA in the nucleus and terminating with the start of viral DNA synthesis. Early mRNAs represent only 0.01-0.02% of the total cytoplasmic RNA when radioactively labelled for a long period during the early phase (Acheson and Mieville, 1978). Transcription of early mRNAs is dependent on the early promoter which is negatively regulated by one of its own end products, the large T antigen (Cogen, 1978; Kamen et al., 1980). Large T antigen probably affects early transcription by binding to specific sites in the viral DNA (Gaudray et al., 1981; Pomerantz et al., 1983; Dilworth et al., 1984) with the net affect of reducing the rate of synthesis of all early mRNAs.

Viral DNA replication can occur as early as 12-15 hours after infection of permissive cells and marks the beginning of the late phase of productive infection. Viral DNA replication is semiconservative (Hirt, 1969) and bidirectional (Crawford et al., 1973, 1974; Griffin et al.,

1974). The initiation, but not the elongation, of each round of viral DNA replication requires functionally active large T antigen as shown by experiments with temperature-sensitive mutants of polyomavirus affecting large T antigen (Francke and Eckhart, 1973). DNA replication terminates where the two replication forks meet and is not dependent on specific termination signals (Griffin and Fried, 1975).

The bulk of late mRNA is synthesized after DNA replication begins. Late viral mRNA represents 0.13% of total cytoplasmic RNA at late times (Bacheler, 1977). This amount of late mRNA is approximately 5,000 fold greater than that at early times. This is consistent with the notion that late mRNAs are needed in large quantities because they code for the viral capsid proteins VP1, VP2, and VP3, which are themselves needed in large quantities to form the viral capsid. The high overall rate of viral RNA synthesis during the late phase is probably due to an increase in viral template DNA (Jat et al., 1982a). The role, if any, of large T antigen in the activation of the late transcription unit has not yet been assessed for polyomavirus.

Although little is known about the mechanism of virus assembly, it appears that empty viral capsids are formed initially, and they subsequently interact with viral DNA-histone complexes to form intact virions. There appears to be no specific mechanism for release of progeny polyomavirus from the infected cell. Indeed, many viral particles remain cell-associated even after cell death has occurred. New infectious virus can appear as early as 20-25 hours post-infection, and can continue to appear up to 60-70 hours post-infection, the time at which cell death usually occurs.

III. TRANSFORMATION BY POLYOMAVIRUS

A) Introduction

Infection of permissive cells by polyomavirus usually results in productive infection but at a low frequency either abortive or stable transformation can occur. In semipermissive and nonpermissive cells, abortive and stable transformation are the common responses. Abortive transformation results in the infected cells temporarily assuming a transformed phenotype (Stoker, 1968), while stable transformation permanently alters the cellular phenotype. The duration of abortive transformation coincides with the duration of synthesis, in the infected cells, of virally coded proteins (Stoker and Dulbecco, 1969). Early evidence indicated that in stably transformed cells, polyomavirus DNA is covalently linked to cellular DNA sequences (Shani et al., 1972; Folk, 1973; Manor et al., 1973). Therefore, the difference between stable and abortive transformation may depend on the integration of the viral genomes into the host chromosome as well as the continued expression of the viral genes required for transformation. Although transformed cells acquire a number of new characteristics, the most striking is the loss of growth control. Cell division is tightly controlled in normal cells. Transformed cells continue to multiply under conditions which prevent the multiplication of normal cells. Under normal conditions, polyomavirus is not oncogenic in its natural host. Polyomavirus behaves as a tumor virus when it is injected into newborn mice (Gross, 1953a, 1953b; Stewart and Eddy, 1958), hamsters (Eddy et al., 1958), rats (Eddy et al., 1959b), or rabbits (Eddy et al., 1959a), and in each case can give rise to a wide variety of tumors. In adult animals the virus can also induce tumors when injected at high concentrations, and these tumors usually develop at the site of injection

(Defendi, 1960).

Interest in polyomavirus stems mainly from its ability to induce neoplastic transformation of cultured cells, which provides a model system for the study of the cellular events that lead to the development of primary tumors in animals. Cells freshly explanted from an animal are known as primary cells. These cells generally grow poorly in vitro, and after a number of generations they usually enter crisis and die. Dawe and Law, (1959) showed that primary fibroblasts could be given extended life in culture after infection with polyomavirus. It was later shown that these cells had acquired an increased rate of cell growth and the ability to form tumors when reintroduced into susceptible hosts (Vogt and Dulbecco, 1960). A breakthrough in the study of in vitro transformation by polyomavirus came with the development of clonal cell lines susceptible to transformation by the virus (MacPherson and Stoker, 1962; Stocker, 1962; Stoker and Abel, 1963). Although the majority of primary cells enter crisis and die in culture, rare survivors can be isolated. These survivors can be maintained indefinitely in culture and provide a genetically homogeneous population of cells. Although these cell lines are "immortalized", they are not considered to be transformed. Studies using cell lines have allowed for the characterization of novel properties displayed by transformed cells.

B) Properties of transformed cells

Many of the phenotypic changes unique to transformed cells affect the rate and pattern of cell growth (Table 1). In the case of untransformed cell lines, cell-to-cell contact results in a density-dependent growth inhibition (Holley and Kiernan, 1968). Polyomavirus transformed cells can grow to a much

Table 1. Properties of cells transformed by polyomavirus.[a]

Growth:

1. High or indefinite saturation density.
2. Different, usually reduced, serum requirement.
3. Growth in agar or Methocel suspension-anchorage independence.
4. Tumor formation upon injection into susceptible animals.
5. Not susceptible to contact inhibition of movement.
6. Growth in a less-oriented manner.
7. Growth on monolayers of normal cells.

Surface:

1. Increased agglutinability of plant lectins.
2. Changes in composition of glycoproteins and glycolipids.
3. Tight junctions missing.
4. Fetal antigens revealed.
5. Virus-specific transplantation antigen.
6. Different staining properties.
7. Increased rate of transport of nutrients.
8. Increased secretion of proteases or activators.

Intracellular:

1. Disruption of the cytoskeleton.
2. Changed amounts of cyclic nucleotides.

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[a] Adapted from DNA Tumor Viruses, J. Tooze (ed.).

higher saturation density (Todaro et al., 1963), and appear to be less contact inhibited (Stoker and MacPherson, 1961) than untransformed cells. Transformed cells continue to grow once a cell monolayer is reached and actually pile up upon each other to form dense foci of cells which can easily be visualized by staining (Temin and Rubin, 1958). Normal cells tend to grow in a regularly oriented manner, while transformed cells tend to be randomly oriented. Even individually transformed cells appear different from their normal counterpart; they are more bipolar and tend to be rounded or stellate in shape (Stoker, 1964; Weisburg, 1964). These changes may be due in part to the breakdown of the actin cable architecture which has been noted in many transformed cells (Pollack et al., 1975; Verderame et al., 1980). By contrast with normal cells, many transformants display a decreased serum requirement for growth (Jainchill and Todaro, 1970) and many lines will grow efficiently in semisolid medium (MacPherson and Montagnier, 1964).

In addition to changes in growth patterns, transformed cells also display a number of cell surface and biochemical changes (Table 1). At the cell surface a new antigen preventing transplantation of tumor cells into isogeneic adult mice, termed the tumor-specific transplantation antigen (TSTA), has been detected (Habel, 1961; Sjogren, 1961). The structure of the plasma membrane is also changed as measured by an increased agglutinability with plant lectins (Burger, 1969). There also appears to be an increased amount of surface fibronectin in transformed cells (Chen et al., 1976). At the biochemical level, it has been noted that transformed cells display an increased transport of various metabolites (Cunningham and Pardee, 1969; Foster and Pardee; 1969) and transformed cells can secrete large amounts of plasminogen activator into the medium (Ossowski et al.,

1973; Pollack et al., 1976).

Although many of the properties described above can be associated with neoplastic transformation, many transformed cell lines display only a subset of these properties. Cells transformed by simian virus 40 (SV40), a papovavirus which is closely related to polyomavirus, were isolated without a selection procedure. A number of these transformed cell lines display only some of the properties of transformed cells, indicating that none of these new phenotypes is necessarily acquired in parallel (Risser and Pollack, 1976). The observed variation in the number of transformed cell properties has led to the description of transformants as either "minimally" or "fully" transformed. Not all transformants are capable of causing tumors when injected into susceptible animals. The ability to grow in an anchorage independent manner, such as growth in agar or in methylcellulose, appears to be the property that most parallels tumorigenicity (Freedman and Shin, 1974; Shin et al., 1975) although there are many exceptions (Kahn et al., 1980).

Most quantitative assays for transformation are carried out in semipermissive or nonpermissive cells because this obviates the problem of killing transformants by productive viral infection. A variety of assays have been used to assess transformation and all of these assays depend in one way or another on an increase in the cell's nutritional autonomy following transformation. The ability of cells to grow in soft agar or methylcellulose, to form dense foci, or to grow in low serum are the most widely used properties for quantitating and isolating viral transformants.

C) Viral Genes Involved in Transformation of Established Cell Lines by Polyomavirus

There is a wealth of evidence which links transformation by polyomavirus to the early region of the viral genome. Abortively transformed cells express viral T antigens, and the reversion of these cells to the "normal" phenotype occurs concomitantly with the disappearance of viral T antigens (Stoker and Dulbecco, 1969), indicating that there is a link between continued expression of the early genes of the virus and the maintenance of the transformed phenotype. Studies of cell lines transformed by polyomavirus indicate that these cells invariably contain cytoplasmic mRNA from the early region of the viral genome while RNA corresponding to the late region is rarely found (Kamen et al., 1974, 1980; Beard et al., 1976; Grady et al., 1977; Bacheler, 1977; Lania et al., 1980b). In addition, the gene products of the early region, but not the late region, could be found in transformed cells (Ito et al., 1977a; Hutchinson et al., 1978; Schaffhausen et al., 1978).

More evidence for the involvement of the early region of polyomavirus in transformation came with the isolation of two classes of viral mutants having altered transformation properties and whose lesions mapped to the early region of the virus. They are the host range, transformation-defective mutant class (hr-t) which are unable to transform cells in vitro or to induce tumors in rodents (Benjamin, 1970; Staneloni et al., 1977), and the temperature-sensitive mutant class (ts-a), which transform cells at a lower frequency at the nonpermissive than at the permissive temperature (Fried, 1965; diMayorca et al., 1969; Eckhart, 1969). The hr-t and the ts-a functions have been shown to complement each other in transformation and tumorigenesis (Eckhart, 1977; Fluck and Benjamin, 1979; Fluck et al., 1977). The gene products which are altered in ts-a and hr-t mutants

have been identified; the middle and small T antigen are altered by hr-t mutations (Silver et al., 1978; Schaffhausen et al., 1978), and large T antigen is altered in ts-a mutations (Paulin and Cuzin, 1975). Marker rescue experiments have mapped the ts-a lesion to the distal portion of the early region (Miller and Fried, 1976; Feunteun et al., 1976; Thomas et al., 1981). Four ts-a mutations have been sequenced and the results indicate that one mutant carries a lesion in the extreme C-terminus of large T antigen while the other three are in the region 500 to 600 amino acids from the N-terminus of large T antigen (Deininger et al., 1976; Thomas et al., 1981). Marker rescue experiments have localized the hr-t mutations to the proximal portion of the early region (Feunteun et al., 1976). Most hr-t mutants appear to be deletions which are positioned in the sequences common to middle and small T antigen (Staneloni et al., 1977; Feunteun et al., 1976; Hattori et al., 1979). Three hr-t mutants show a similar alteration; each contains a G to A transition at nucleotide 706 along with the insertion of the three bases TAA (Carmichael and Benjamin, 1980). The result is that the amino acid aspartic acid is lost and two new amino acids, isoleucine and asparagine, are inserted into both small and middle T antigen coding sequences.

Although the evidence indicates that the early region is required for transformation, it does not appear that the entire early region is required. Several investigators failed to find mRNA sequences complementary to the distal portion of the polyomavirus early region in some transformed cells (Kamen et al., 1974; Bacheler, 1977; Grady et al., 1977). Moreover, many transformed cell lines do not appear to contain large T antigen (Hutchinson et al., 1978; Benjamin et al., 1979; Israel et al., 1979b; Lania et al., 1980a,1980b) while other

transformed cell lines contain only fragments of the protein (Lania et al., 1980a, 1980b; Novak et al., 1980; Ito and Spurr, 1980; Hassell et al., 1980). In fact, interruption of the early region sequences in the distal portion of large T antigen appeared to increase the tumorigenicity of polyomavirus DNA and suggested that intact large T antigen was not required for oncogenesis (Israel et al., 1979, 1980; Moore et al., 1980). Using isolated or cloned subgenomic fragments of the polyomavirus genome it was shown directly that portions of the viral genome comprising the entire coding sequences for middle and small T antigen, but only a portion of large T antigen coding sequences, are sufficient for transformation (Bastin et al., 1980; Chowdhury et al., 1980; Hassell et al., 1980; Novak et al., 1980). This result was further suggested by the fact that cells transformed by ts-a mutants at the permissive temperature often retain the transformed phenotype when transferred to the nonpermissive temperature (Fluck and Benjamin, 1979; Eckhart, 1969; diMayorca et al., 1969; Fried, 1965; Seif and Cuzin, 1977). In fewer instances the opposite result has been observed (Kimura, 1975) and the outcome may depend on the conditions used to select the transformants (Rassoulzadegan et al., 1978, 1980).

Because we know that temperature sensitive mutants of large T antigen transform cells at a lower frequency at the nonpermissive temperature than the permissive temperature, what then is the role of large T antigen in transformation? Della Valle and co-workers (1981) compared both the transforming ability, and the pattern of integration, of both wild-type (wt) and ts-a DNA. They found that not only is the ts-a DNA 10-25 fold less transforming at the nonpermissive temperature, but also that the frequency of tandem integration was higher in cells transformed at the permissive, versus

the nonpermissive, temperature. These results suggest that the large T antigen may play a role in transformation by facilitating integration of the viral genome into the cellular chromosome. Thus, it appears that large T antigen is not required to initiate transformation, but it may, under certain circumstances, facilitate its establishment. This conclusion is strengthened by the observation that while ts-a mutants are defective in stable transformation, they are normal with respect to abortive transformation (Stocker and Dulbecco, 1969; Fluck and Benjamin, 1979). These results suggest that large T antigen acts in "fixing" the transformed phenotype, perhaps by facilitating integration of the viral genome into cellular sequences. Recently, the transformation frequency of replication-competent and replication-defective recombinant plasmids containing polyomavirus sequences coding for large T antigen were compared (Muller et al., 1983b). The authors found that recombinant plasmids capable of replicating in transfected cells transformed these cells at frequencies approximately sixfold greater then their replication-defective counterparts. These results indicate a relationship between the replicating capacity of a recombinant plasmid and its transforming potential.

The hr-t function appears to be absolutely required for transformation. However, hr-t mutants carry lesions in both the middle and small T antigen coding sequences. In order to determine whether middle and/or small T antigen was required for transformation, a number of different laboratories have isolated mutants in the coding region common to the large and middle T antigens (Griffin and Maddock, 1979; Magnusson and Berg, 1979; Bendig et al., 1980; Novak and Griffin, 1981; Mes and Hassell, 1982; Templeton and Eckhart, 1982; Carmichael et al., 1982, 1984; Ding et al., 1982; Nilsson et al., 1983; Oostra et al., 1983; Templeton

et al., 1984). Different mutations have been found to affect transformation to differing extents, but the general conclusion is that middle T antigen is absolutely required for transformation and that small T antigen, either alone or in combination with large T antigen, is unable to cause transformation. However, none of these studies could assess whether middle T antigen acted alone or in concert with small T antigen to bring about transformation. This question was resolved by Treisman et al., (1981). They found that an altered polyoma virus genome expressing only middle T antigen was capable of transforming cultured rat cells. Therefore, it is now clear that middle T antigen of polyomavirus is sufficient to maintain the transformed state in established cell lines.

D) Viral Genes Involved in Transformation of Primary Cells and in Tumorigenesis.

Although it is clear that middle T antigen alone is sufficient to cause transformation of established cell lines, a completely different result is obtained if primary cells are used. Using recombinant polyomavirus genomes each encoding only one of the T antigens, it was possible to identify independent functions for large and middle T antigen in the acquisition of the transformed phenotype (Rassoulzadegan et al., 1982, 1983). Middle T antigen by itself does not transform primary cells. Transformation by middle T antigen requires the presence of at least 40% of the N-terminal portion of large T antigen. Because primary cells in which large T antigen is expressed continue to grow in culture indefinitely, it has been suggested that large T antigen confers on cells an unlimited growth potential in culture, termed an "immortalizing" function (Rassoulzadegan et al.,

1982). Large T antigen also appears to reduce the serum concentration needed for growth of large T antigen immortalized cell lines. In addition it was found that in the highly serum-dependent rat line FR3T3 (Rassoulzadegan et al., 1981) middle T antigen alone could confer a transformed phenotype, but the cells did not remain transformed when shifted to low serum growth condition (Rassoulzadegan et al., 1982, 1983). Thus, it has been suggested that large T antigen exerts its effect by modulating the cells responsiveness to cellular growth factors (Rassoulzadegan et al., 1982).

Rat-1 cells, which have been transformed by an altered polyomavirus genome expressing only middle T antigen, can form tumors when introduced into nude mice or syngeneic Fisher rats (Asselin et al., 1983). However, the middle T antigen alone is not sufficient to form tumors in newborn Fisher rats (Asselin et al., 1983). The inability to form tumors can be complemented with either small T antigen or large T antigen. Small T antigen is not implicated in immortalization functions (Rassoulzadegan et al., 1983) suggesting that it complements middle T antigen in tumorigenesis in a manner different then large T antigen does. Recently it has been reported that middle T antigen alone is sufficient to cause tumors in newborn hamsters, although it is far more tumorigenic in the presence of small T antigen (Asselin et al., 1984). This result provides strong evidence that the middle T antigen protein is primarily responsible for tumor induction by polyomavirus.

IV. Middle T Antigen of Polyomavirus

A) Introduction

The predicted amino acid sequence of middle T antigen (Figure 3) can be deduced from the DNA sequence of the virus (Friedmann et al., 1978a, 1978b, 1979; Soeda et al., 1978, 1979, 1980; Deininger et al., 1980) and the known structure of the viral mRNAs (Kamen et al., 1980; Treisman et al., 1981). The protein is 421 amino acids long, and the 191 N-terminal amino acids are shared with small T antigen. Although middle T antigen has a predicted M.W. of 48,506, two species of middle T antigen with apparent M.W. of 56,000 (56K) and 58K have been detected in lytically-infected and transformed cells. The difference between the two middle T antigen species is due to post-translational phosphorylation at different sites in the C-termial region upstream from amino acid 300 (Schaffhausen and Benjamin, 1981a, 1981b). Both forms of the protein are predominantly phosphorylated in vivo at serine residues, and to a much lesser extent at threonine and tyrosine residues (Schaffhausen and Benjamin, 1981a, 1981b; Segawa and Ito, 1982). It is thought that the minor, 58K species is a post-translational modification product of the 56K form because the in vitro translation product of middle T antigen mRNA has a M.W. of 56K (Hunter et al., 1978). However, in many instances only a single broad band can be seen in immunoprecipitates of middle T antigen, which probably represents the two unresolved species.

A number of distinguishing features are present in the amino acid sequence of middle T antigen and are underlined in Figure 3. Two cysteine clusters (cys-x-cys-x-x-cys) which are in the region common to small and middle T antigen (residues 120-125 and 148-153) are reminiscent of similar cysteine clusters seen in the alpha and beta subunits of such hormones as TSH, LH, and FSH (Friedman et al., 1978a). In the unique amino acids of middle T antigen there is also a proline rich sequence (residues 336-341)

Figure 3: The predicted amino acid sequence of middle T antigen. Underlined are two cysteine clusters (residues 120-125 and 148-153) matching similar cysteine clusters found in the hormones LH, TSH, and FSH, the major site of tyrosine phosphorylation (tyrosine 315) as well as the stretch of six glutamic acid residues which precede it (residues 309-314), the proline rich sequence (residues 336-344) which is also found in human chorionic gonadotropin, and the stretch of 22 hydrophobic amino acid residues (394-415) at the C-terminus of the protein. The asterisk (*) indicate the positions of the basic amino acids which flank the hydrophobic stretch. Adapted from Schaffhausen, (1982).

MET-ASP-ARG-VAL-LEU-SER-ARG-ALA-ASP-LYS-GLU-ARG-LEU-LEU-GLU-[15]

LEU-LEU-LYS-LEU-PRO-ARG-GLN-LEU-TRP-GLY-ASP-PHE-GLY-ARG-MET-[30]

GLN-GLN-ALA-TYR-LYS-GLN-GLN-SER-LEU-LEU-LEU-HIS-PRO-ASP-LYS-[45]

GLY-GLY-SER-HIS-ALA-LEU-MET-GLN-GLU-LEU-ASN-SER-LEU-TRP-GLY-[60]

THR-PHE-LYS-THR-GLU-VAL-TYR-ASN-LEU-ARG-MET-ASN-LEU-GLY-GLY-[75]

THR-GLY-PHE-GLN-VAL-ARG-ARG-LEU-HIS-ALA-ASP-GLY-TRP-ASN-LEU-[90]

SER-THR-LYS-ASP-THR-PHE-GLY-ASP-ARG-TYR-TYR-GLN-ARG-PHE-CYS-[105]

ARG-MET-PRO-LEU-THR-CYS-LEU-VAL-ASN-VAL-LYS-TYR-SER-SER-CYS-[120]

SER-CYS-ILE-LEU-CYS-LEU-LEU-ARG-LYS-GLN-HIS-ARG-GLU-LEU-LYS-[135]

ASP-LYS-CYS-ASP-ALA-ARG-CYS-LEU-VAL-LEU-GLY-GLU-CYS-PHE-CYS-[150]

LEU-GLU-CYS-TYR-MET-GLN-TRP-PHE-GLY-THR-PRO-THR-ARG-ASP-VAL-[165]

LEU-ASN-LEU-TYR-ALA-ASP-PHE-ILE-ALA-SER-MET-PRO-ILE-ASP-TRP-[180]

LEU-ASP-LEU-ASP-VAL-HIS-SER-VAL-TYR-ASN-PRO-LYS-ARG-ARG-SER-[195]

GLU-GLU-LEU-ARG-ARG-ALA-ALA-THR-VAL-HIS-TYR-THR-MET-THR-THR-[210]

GLY-HIS-SER-ALA-MET-GLU-ALA-SER-THR-SER-GLN-GLY-ASN-GLY-MET-[225]

ILE-SER-SER-GLU-SER-GLY-THR-PRO-ALA-THR-SER-ARG-ARG-LEU-ARG-[240]

LEU-PRO-SER-LEU-LEU-SER-ASN-PRO-THR-TYR-SER-VAL-MET-ARG-SER-[255]

HIS-SER-TYR-PRO-PRO-THR-ARG-VAL-LEU-GLN-GLN-ILE-HIS-PRO-HIS-[270]

ILE-LEU-LEU-GLU-GLU-ASP-GLU-ILE-LEU-VAL-LEU-LEU-SER-PRO-MET-[285]

THR-ALA-TYR-PRO-ARG-THR-PRO-PRO-GLU-LEU-LEU-TYR-PRO-GLU-SER-[300]

ASP-GLN-ASP-GLN-LEU-GLU-PRO-LEU-GLU-GLU-GLU-GLU-GLU-GLU-TYR-[315]

MET-PRO-MET-GLU-ASP-LEU-TYR-LEU-ASP-ILE-LEU-PRO-GLY-GLU-GLN-[330]

VAL-PRO-GLN-LEU-ILE-PRO-PRO-PRO-ILE-ILE-PRO-ARG-ALA-GLY-LEU-[345]

SER-PRO-TRP-GLU-GLY-LEU-ILE-LEU-ARG-ASP-LEU-GLN-ARG-ALA-HIS-[360]

PHE-ASP-PRO-ILE-LEU-ASP-ALA-SER-GLN-ARG-MET-ARG-ALA-THR-HIS-[375]

ARG-ALA-ALA-LEU-ARG-ALA-HIS-SER-MET-GLN-ARG-HIS-LEU-ARG-ARG-[390]

* * * *

LEU-GLY-ARG-THR-LEU-LEU-LEU-VAL-THR-PHE-LEU-ALA-ALA-LEU-LEU-[405]

*

GLY-ILE-CYS-LEU-MET-LEU-PHE-ILE-LEU-ILE-LYS-ARG-SER-ARG-HIS-PHE[421]

* * * *

which is also found in the beta subunit of human chorionic gonadotropin (Benjamin et al., 1980). It is not known whether the sequences similar to those found in peptide hormones are significant in the function of middle and/or small T antigen. A portion of middle T antigen is highly acidic, with a striking string of six consecutive glutamic acid residues between amino acids 309-314, followed by a tyrosine residue at amino acid 315. Also noteworthy is a stretch of 22 hydrophobic amino acids (394-415), flanked on both sides by short clusters of basic amino acids, which is found at the C-terminus of middle T antigen. These last two features of middle T antigen play a role in the function of the protein. Tyrosine 315 and the surrounding acidic amino acids play a role in the capacity of middle T antigen to become phosphorylated at tyrosine residues in vitro (Eckhart et al., 1979; Schaffhausen and Benjamin, 1979; Smith et al., 1979, 1980) and the hydrophobic stretch including the flanking basic amino acids plays a role in the association of middle T antigen with the plasma membrane (Ito et al., 1977a, 1977b, 1977c; Silver et al., 1978; Ito, 1979; Schaffhausen et al., 1982b).

B) In Vitro Kinase Activity Associated with Middle T Antigen

The study of protein phosphorylation in relation to tumor virology was stimulated by the finding of Collett and Erickson, (1978) that the src gene product of Rous sarcoma virus is a protein kinase. The virally coded phosphoprotein $pp60^{src}$ was able to phosphorylate the heavy chain of immunoglobulin in immunoprecipitates and this activity had the unusual specificity of phosphorylating tyrosine residues (Hunter and Sefton, 1980). A number of other retroviral oncogenes have now been shown to be associated

with a tyrosine kinase activity, but this is by no means a universal property of retroviral oncogenes (reviewed by Bishop, 1983). Because protein kinases play a fundamental role in the control of many aspects of cellular metabolism (see Greegard, 1978; Hunter et al., 1981) it is possible that one of the mechanisms by which tumor viruses transform cells involves the phosphorylation of specific host cell substrates. Indeed, high levels of cellular proteins phosphorylated at tyrosine residues are seen in cells transformed by Rous sarcoma virus (Sefton et al., 1980). Although protein cellular targets of viral tyrosine kinases have been identified (Sefton et al., 1981; Cooper et al., 1983), it is unclear whether these proteins have an important biological effect in transformation. More recently it has been suggested that $pp60^{src}$ may activate pathways leading to the malignant phenotype through the generation of second messengers and not by the covalent modification of various proteins. The new work shows that $pp60^{src}$ can phosphorylate phosphatidylinositol, thereby increasing the formation of polyphosphoinisitide, which acts as a second messenger for several hormones, neurotransmitters, and growth factors (Sugimoto et al., 1984; discussed in Marx, 1984). Therefore, it would seem that tyrosine kinase activity may play an important role in transformation by certain retroviruses.

The finding that $pp60^{src}$ could phosphorylate substrates in vitro spurred researchers to see if a similar activity was associated with the transforming protein of polyomavirus. Indeed, it was found that middle T antigen was associated with a protein kinase activity which phosphorylates middle T antigen itself (Eckhart et al., 1979; Schaffhausen and Benjamin, 1979; Smith et al., 1979, 1980) and that this reaction was specific for tyrosine (Smith et al., 1979a).

However, unlike cells transformed by Rous sarcoma virus, polyomavirus transformed cells do not display higher than normal levels of tyrosine phosphoproteins (Sefton et al., 1980). While both the 56K and the 58K middle T antigen species are phosphorylated, the 58K species has a much higher specific activity (Schaffhausen and Benjamin, 1981a). The evidence that middle T antigen itself is the substrate which becomes phosphorylated in vitro is twofold. First, the mobility of the ^{32}P-labelled band is appropriately different in viral strains and deletion mutants whose middle T antigen migrates differently from wt (Schaffhausen and Benjamin, 1979, 1981a, 1981b; Smith et al., 1979, 1980). Secondly, the partial proteolytic peptide maps of in vitro, ^{32}P-labelled protein after digestion with chymotrypsin or Staphylococcus aureus V8 protease yields a pattern which matches the pattern obtained with ^{35}S-labelled middle T antigen. The principal site of middle T antigen phosphorylation in vitro is tyrosine 315 (Schaffhausen and Benjamin, 1981b), and the binding of an antibody which is specific to tyrosine 315 and its surrounding amino acids inhibits in vitro kinase activity (Schaffhausen et al., 1982a). This tyrosine residue is preceded by a cluster of six glutamic acid residues reminiscent of the amino acid sequence surrounding sites of tyrosine phosphorylation in the transforming proteins of several retroviruses (Patchinski et al., 1982; reviewed in Bishop, 1983). In addition, tyrosine 322 may also become phosphorylated in vitro (Oostra et al., 1983; B. Schaffhausen, personal communication).

The in vitro kinase activity is clearly a property associated with middle T antigen. ts-a mutants retain kinase activity at the nonpermissive temperature (Schaffhausen and Benjamin, 1979) and cells lacking large T antigen continue to display the in vitro kinase activity

(Eckhart et al., 1979; Schaffhausen and Benjamin, 1979). By contrast, all hr-t mutants are completely defective in the kinase reaction (Eckhart et al., 1979; Schaffhausen and Benjamin, 1979; Smith et al., 1979, 1980). Many mutations affecting middle T antigen coding sequences, but not small T antigen coding sequences, are crippled or defective in the kinase assay (Smith et al., 1979, 1980; Griffin et al., 1980; Ito et al., 1980; Carmichael et al., 1982, 1984; Nilsson et al., 1983; Mes-Masson et al., 1984a). Finally, cells transformed by altered forms of polyomavirus which can only code for middle T antigen are active in the in vitro kinase assay (Treisman et al., 1981).

Presently it is still unclear whether the kinase activity seen in immunocomplexes is an intrinsic activity of middle T antigen itself or whether middle T is associated with a cellular tyrosine kinase which provides the in vitro activity. hr-t mutants (see Schaffhausen and Benjamin, 1981b) and transformation defective N-terminal deletion mutants of middle T antigen (Templeton and Eckhart, 1984) are totally inactive in the in vitro kinase reaction suggesting that middle T is catalytically active. However, middle T antigen fails to become labelled with ATP affinity reagents 8-azido-ATP and 2,3,-dialdehyde ATP which have been used to label the catalytic subunit of protein kinases and ATP-utilizing enzymes (Schaffhausen et al., 1982b). Monomers of middle T antigen, purified by immunoaffinity chromatography, are not active in the kinase assay (Walter et al., 1982). In addition, middle T antigen expressed in Escherichia coli does not have attendant kinase activity (B. Schaffhausen, personal communication) in contrast to the src viral protein, which retains kinase activity when expressed in bacteria (Gilmer and Erickson, 1982). Recent evidence indicates that polyomavirus middle T antigen

forms a complex with the product of the c-src cellular gene, and it has been suggested that the tyrosine kinase activity measured *in vitro* in immunoprecipitates of middle T antigen is the activity of the associated c-src protein (Courtneidge and Smith, 1983, 1984).

Whether the *in vitro* tyrosine phosphorylation is important to the transforming activity of polyomavirus is a matter of some debate. A number of mutants have been isolated which provide strong genetic evidence linking kinase activities to transforming ability (Griffin *et al.*, 1980; Ito *et al.*, 1980; Ding *et al.*, 1982; Nilsson *et al.*, 1983; Carmichael *et al.*, 1982, 1984; Templeton and Eckhart, 1984). By contrast, mutants which retain kinase activity but are defective in transformation (Nilsson *et al.*, 1983; Templeton *et al.*, 1984) and mutants lacking kinase activity but retaining transforming activity (Mes-Masson *et al.*, 1984) have recently been described. These later results strongly question the link between transformation and *in vitro* kinase activity. *In vivo* labeling experiments have found little (Segawa and Ito, 1982) or no (Schaffhausen and Benjamin, 1981a) phosphate associated with tyrosine residues of middle T antigen. This may reflect that phosphorylation of middle T antigen *in vitro* is but a marker of activity, rather then itself being of direct biological importance.

C) Membrane Association of Middle T Antigen

Middle T antigen has been shown to be associated with the membrane fraction of infected or transformed cells (Ito *et al.*, 1977a; Ito, 1979; Silver *et al.*, 1978), although approximately 50% is also found

in the soluble fraction (Schaffhausen and Benjamin, 1981b). Several lines of evidence indicate that middle T antigen is predominantly associated with the plasma membrane of the cell (Ito et al., 1977a; Schaffhausen et al., 1982b). Pulse-chase experiments have shown that the entry of middle T antigen into the membrane is a slow process (Ito et al., 1977a). There is a body of evidence which suggests that middle T antigen is oriented towards the inside of the cell. The protein kinase activity associated with middle T antigen cannot be detected extracellularly, but is associated with inside out vesicles (Schaffhausen and Benjamin, 1981b). Attempts to label middle T antigen by iodination with lactoperoxidase, or to cleave middle T antigen with proteases, using intact cells, has proved unsuccessful (Schaffhausen et al., 1982b).

There is a cluster of hydrophobic amino acids at the carboxy terminus of middle T antigen extending from amino acid residues 394-415, flanked on either side by basic amino acids. In fact, this hydrophobic stretch is followed by 4 basic amino acids among the terminal 6 amino acids. This arrangement of amino acids is typical of membrane associated proteins (Natheson et al., 1981; Tomita and Marchesi, 1975; Porter et al., 1979; Rose et al., 1980), and it has been hypothesized that this arrangement of amino acids is sufficient to allow the spontaneous insertion of proteins into membranes (Engelman and Steitz, 1981). It is likely, but by no means proven, that the hydrophobic C-terminus of middle T antigen spans the membrane in which it is located. The hydrophobic region appears to be important for membrane association, because a polyomavirus mutant lacking the last 37 amino acids of middle T antigen synthesizes a deleted protein found exclusively in the cytosol (Carmichael et al., 1982). The hydrophobic tail is important for the transforming activity of

middle T antigen because a number of mutants having alterations in this region show a concomitant loss of transforming activity (Novak and Griffin, 1981; Mes and Hassell, 1982; Carmichael et al., 1982; Templeton and Eckhart, 1982; Templeton et al., 1984; Mes-Masson et al., 1984b). Prematurely truncated forms of middle T antigen have also been found to be defective in middle T antigen protein kinase activity (Carmichael et al., 1982; Templeton and Eckhart, 1982). Middle T antigen species which do not associate with the plasma membrane may never acquire kinase activity because they do not associate with the c-src gene product (Courtneidge and Smith, 1983, 1984) whose location is also the plasma membrane (Courtneidge et al., 1980). Templeton et al., (1984) have contructed a recombinant gene encoding a polyomavirus middle T antigen whose C-terminus is derived from the hydrophobic C-terminal amino acids from vesicular stomatitis virus glycoprotein G. This fusion protein was associated with cell membranes and possessed tyrosine protein kinase activity, but was unable to bring about cellular transformation. Taken together, the biochemical and genetic analysis indicate that membrane association is essential for transformation, and this association must be precise for middle T antigen to cause transformation.

MATERIALS AND METHODS

MATERIALS AND METHODS

MUTAGENESIS PROTOCOL FOR INSERTION AND DELETION MUTATIONS

A) Basic Strategy

The mutagenesis protocol used was similar to the method developed to construct SV40 mutants (Shortle et al., 1979). The basic strategy involved linearizing recombinant plasmid molecules by cleavage of the DNA with restriction endonucleases. The plasmid molecules contained, among other sequences, a bacterial origin of replication, a gene coding for antibiotic drug resistance, and sequences corresponding to a portion of the polyomavirus genome. The free ends of linear molecules were then modified by treatment with various enzymes which either deleted or added sequences at the ends of the molecule. The deletion or addition of sequences at the ends of the linear molecules led to the loss of the restriction endonuclease site which was originally used to linearize the plasmid. Plasmids were then recircularized, transfected into bacteria and drug resistant colonies were isolated. Plasmid DNA was purified from individual bacterial colonies and was tested for the loss of the restriction endonuclease site. Recombinant plasmids having lost the restriction endonuclease site were tested for biological activity in mammalian cells.

B) Isolation of Permuted Linear DNA Molecules

Two parental plasmids carrying fragments of polyomavirus DNA were used

as substrates for *in vitro* mutagenesis. One of these plasmids, pPH1-8 (Figure 4A), contains the *Hind*111-1 fragment of polyomavirus DNA (45 m.u. to 1.4 m.u. or nucleotides 3918 to 1656 clockwise) inserted at the *Hind*111 site of the plasmid pBR322 (Bolivar *et al.*, 1977; Sutcliffe, 1979). The second plasmid, pPBR2 (Figure 4B) contains the *Bam*H1/*Eco*R1 fragment of polyomavirus DNA (58.5 m.u. to 100/0 m.u. or nucleotides 4632 to 1560 clockwise) within the *Bam*H1/*Eco*R1 double-digest product of the plasmid pMK16.1 (Kahn *et al.*, 1979; Gluzman *et al.*, 1980). Both of these recombinant plasmids contain the polyomavirus sequences which code for middle and small T antigen and the N-terminal 40% of large T antigen. A number of restriction endonuclease sites within the coding sequences for the viral T antigens were mutagenized. They included the *Sst*1 sites (81.3 and 96.5 m.u. or nucleotides 569 and 1373), the *Ava*1 sites (82.9 and 89.7 m.u. or nucleotides 657 and 1016), the *Pvu*11 site (92.4 m.u. or nucleotide 1144), the *Pst*1 site (79.7 m.u. or nucleotide 484), and the *Eco*R1 site (0/100 m.u. or nucleotide 1560). Of these restriction endonucleases only *Pst*1 and *Eco*R1 cleave the recombinant plasmid, pPBR2, once. *Ava*1, *Pvu*11, and *Sst*1 all cleave either pPH1-8 or pPBR2 DNA more than once.

To use the mutagenesis protocol, full length linear molecules were required. In order to generate these linear molecules with multicut enzymes, one ug of recombinant plasmid DNA was cleaved with the restriction endonuclease in the presence of 20-40 ug/ml of ethidium bromide (Parker *et al.*, 1977) under conditions specified by the manufacturer for 1 hour (hr) at 37^{o}C. Under these conditions, most restriction endonucleases will either make a single-stranded break or a double-stranded cut at only one of a number of sites, to generate permuted linear or circular molecules.

These full length nicked or cleaved species were resolved on agarose gels containing 5 ug/ml of ethidium bromide. The desired species were eluted from the agarose gel by electrophoresis (Yang et al., 1979) as follows: the band corresponding to full length nicked circles or linears was cut from the gel and placed in a dialysis bag containing 500 ul of 5 ug/ml bovine serum albumin (BSA) in electrophoresis buffer. Current was passed through the bag until the DNA was eluted from the gel slice, at which point the current was reversed for 20 seconds (sec). The solution was extracted with chloroform:isoamyl alcohol (24:1), the salt concentration was adjusted to 0.1 M NaCl, and the DNA precipitated with two volumes of ethanol. The permuted circles and linears were then ready for mutagenesis.

C) Modification of Free Ends

A number of different protocols were used to modify the recombinant plasmid DNA. Small deletions were created by using nuclease S1, which degrades single-stranded but not double-stranded DNA (Vogt, 1973). Nuclease S1 was used to remove the single-stranded tails left by the staggered cut made by some restriction endonucleases to produce blunt ends. A modification of a previously described procedure was used to remove the single-stranded ends (Shenk, 1977). One ug of permuted linears was resuspended in 90 ul water and 10 ul of 10 X nuclease S1 buffer (2 M NaCl, 0.5 M sodium acetate pH 4.5, 10 mM $ZnSO_4$, 5% (vol/vol) glycerol) was added. One unit of nuclease S1 was added and the reaction was allowed to proceed at $37^{o}C$ for 30 minutes (min). The reaction was stopped with one ul of 0.5 M ethylenediaminetetraacetic acid (EDTA).

Larger deletions were generated through the use of the enzyme nuclease

Bal 31. This enzyme possesses a single-stranded specific endonuclease activity similar to nuclease S1 as well as a processive exonuclease activity for double-stranded DNA (Gray et al., 1975). Nuclease Bal 31 was used for both permuted circles and linears in order to generate deletions of varying sizes. Although a number of different reaction conditions were used initially, the following was the most successful (Maniatis et al., 1982). One ug of permuted circles or linears were resuspended in BSA (500 ug/ml) with an equal volume of freshly prepared 2 X Bal 31 buffer (24 mM $CaCl_2$, 24 mM $MgCl_2$, 400 mM NaCl, 40 mM Tris-HCl pH 8.0, 2 mM EDTA), the Bal 31 enzyme was added (0.2 unit/ug of DNA) and incubated at 15°C for one or two min. The reaction was stopped by succesive phenol, phenol:chloroform (1:1), and chloroform:isoamyl alcohol (24:1) extractions.

Insertion mutations were generated through the use of RNA-dependent DNA polymerase, better known as reverse transcriptase. This enzyme has a 5' to 3' polymerase activity as well as a processive 5' and 3' ribonuclease activity (Verma, 1977). Reverse transcriptase was used to back fill the 5' single-stranded projections left by certain restriction endonucleases. The procedure used was according to Arrand et al., (1974). One ug of permuted linear DNA was resuspended in 85 ul water, 10 ul 10 X reverse transcriptase buffer (100 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 500 mM KCl), 4 ul of 0.1 M deoxyribonucleotides (dNTP) and 5 units of reverse transcriptase. The reaction was allowed to proceed for 2 hr at 37°C and the reaction was stopped by extraction with chloroform:isoamyl alcohol (24:1).

Whatever the protocol used to mutagenize the free ends of linear molecules, the resulting linear recombinant plasmid molecules possessed blunt ends. These blunt ends were joined to form circular molecules through the use

of T4 DNA ligase. DNA was resuspended in nine parts water and one part 10 X ligation buffer (0.66 M Tris-HCl pH 7.6, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP) to a final DNA concentration of 100-150 ug/ml. One unit of T4 ligase was added per ug of DNA and the reaction was incubated at 15°C for 15 hr. Aliquots of ligated DNA were used to transform competent Escherichia coli.

D) Transformation of E. coli

Recircularized recombinant plasmids were transformed into either X1776 (Curtiss et al., 1977), HB101 (Maniatis et al., 1982) or DH1 (Hanahan, 1983), all of which are strains of E. coli. In order to obtain stable drug resistant transformants, the calcium chloride technique was used with varying efficiency for all E. coli strains (Mandel and Higa, 1970). The basic procedure was as follows. One ml of an overnight bacterial culture was used to inoculate 100 ml of L-broth (10 gm/l tryptone, 5 gm/l yeast extract, 85 mM NaCl) and was grown at 37°C with vigorous shaking to a final density of 5 X 10^7 cells/ml ($O.D._{550}$ = 0.5). The culture was chilled on ice for 10 min and subsequently centrifuged at 4,000Xg for 5 min at 4°C. Cells were resuspended in one half the original culture volume of ice cold 50 mM $CaCl_2$ and 10 mM Tris-HCl pH 8.0. The cell suspension was placed on ice for 15 min and was again centrifuged at 4,000Xg for 5 min. Cells were resuspended in one fifteenth of the original volume in ice cold 50 mM $CaCl_2$ and 10 mM Tris-HCl pH 8.0. Cells were stored on ice and used with maximum efficiency 12-24 hr later. DNA in ligation buffer was added to a 200 ul aliquot of $CaCl_2$ shocked bacteria and kept on ice for 30 min, then transferred to 42°C for 2 min. L-broth (800 ul) without

antibiotics was added and the cells were incubated without shaking for 1 hr at 37°C. At this stage an appropriate quantity of cells (10-200 ul) were plated onto 1.5% (wt/vol) agar plates containing selective media consisting of L-broth supplemented with either ampicillin (100 ug/ml) or kanamycin (50 ug/ml). The plates were incubated at 37°C for 12-16 hr after which drug resistant colonies appeared. This transformation protocol routinely yielded 5 X 10^5 transformants per ug of intact plasmid DNA. Higher transformation frequencies were obtained when more elaborate protocols were used (Hanahan and Meselson, 1980; Hanahan, 1983).

E) Plasmid DNA Purification and Identification of Mutated Recombinant Plasmids

Individual drug resistant bacterial colonies were isolated and small scale isolation of plasmid DNA was performed. Although many procedures were used successfully, one of the most rapid and reliable has been described by Klein et al. (1980). Briefly, 10 ml of bacterial cultures were grown in a 50 ml conical tube with vigorous shaking for a period of 12-15 hr at 37°C. The cultures were subsequently centrifuged at 4,000Xg for 5 min. The bacterial pellet was resuspended in 0.5 ml of 50 mM Tris-HCl pH 8.0 and the suspension transferred to an Eppendorf tube and 1 mg/ml of lysozyme was added, and allowed to incubate at room temperature for 15 min. At this point 0.5 ml of buffered phenol was added, the phases were mixed gently by inverting the tube a number of times, and spun in a microfuge for 15 min at room temperature at 10,000 rpm. The aqueous phase was removed and extracted with phenol:chloroform (1:1) followed by a chloroform:isoamyl alcohol (24:1) extraction. The salt concentration was adjusted to 0.1 M NaCl and the DNA was

precipitated with 2 volumes of ethanol at -70°C for 30 min. The mixture was spun in a microfuge for 5 min at room temperature at 10,000 rpm and the DNA pellet was resuspended in 100 ul TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) containing 20 ug/ml ribonuclease A. To monitor for the loss of a restriction endonuclease site, 20 ul of plasmid DNA was cut with the appropriate enzyme, followed by agarose gel electrophoresis. The DNA bands were visualized under ultraviolet light after staining the gel with ethidium bromide (approximatelly 0.5 ug/ml).

Recombinant plasmids which had lost the appropriate restriction endonuclease cleavage site were characterized further. For this purpose, relatively large quantities of recombinant plasmid DNA was required. From a 1 ml saturation culture of bacteria, 100 ml of L-broth were inoculated and were left shaking vigorously at 37°C for 15 hr. The culture was subsequently centrifuged at 4,000Xg for 15 min, washed once in 50 ml TE, and centrifuged a second time at 4,000Xg for 15 min. The bacterial pellet was resuspended in 1.5 ml ice cold 25% (wt/vol) sucrose in 0.05 M Tris-HCl pH 8.0. To this was added 0.5 ml of freshly prepared lysozyme solution (10mg/ml in 25 mM Tris-HCl pH 8.0) and 0.5 ml of 0.5 M EDTA pH 8.5. The whole mixture was swirled gently on ice for 5 min. At this point 2.5 ml of ice cold Triton solution (1ml of 10% (vol/vol) Triton X-100, 12.5 ml of 0.5 M EDTA pH 8.5, 5 ml of 1M Tris-HCl pH 8.0, and 80 ml H_2O) was added and the mixture was swirled gently on ice, for 10 min. The lysed bacteria were transferred to a Type 65 ultracentrifuge tube and spun at 30,000 rpm for 1 hr. The supernatant was then removed and the volume was measured. An equal weight (wt/vol) of cesium chloride (CsCl) was added assuming that the density of the supernatant was 1 mg/ml and ethidium bromide was added to a final concentration of 0.2 mg/ml. The CsCl solution was transferred to a tube suitable for centrifugation in a

Type 65 rotor, and was centrifuged at 40,000 rpm at 20°C for 48 hr. The lower plasmid band consisting of closed circular plasmid DNA was collected through a hypodermic needle inserted into the side of the ultracentrifuge tube. Ethidium bromide was removed by repeated extractions with isopropanol equilibrated with CsCl saturated water. The CsCl was removed by dialysis against several changes of TE at 4°C. The DNA was then extracted once with phenol and twice with chloroform:isoamyl alcohol (24:1). Traces of organic solvent were removed by repeated dialysis against TE at 4°C. The concentration of the DNA was determined spectroscopically by determining the optical density of the final solution at 260 nm. This preparation of DNA was then used for restriction endonuclease analysis, DNA sequence analysis, and in transformation assays with mammalian cells .

F) Sequence Analysis of Mutations

The appropriate region of the mutated recombinant plasmids were sequenced using the technique of Maxam and Gilbert, (1977). The DNA was opened using restriction endonuclease digestion. The free ends were then labelled either by the kinase exchange reaction (Berkner and Folk, 1977) or by using the Klenow fragment of E. coli DNA polymerase 1 (Jacobson et al., 1974) to back fill the recessed 3' ends of DNA. The kinase exchange reaction was performed on DNA which had been cleaved by a restriction endonuclease, extracted with chloroform:isoamyl alcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in 25 ul water, 5 ul 10 X kinase exchange buffer (500 mM Imidazole-HCl pH 6.0, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA), 3 ul 5 mM ADP, 10 ul [γ-^{32}P]ATP (100 uCi: 10.0 mCi/ml, 3,000 Ci/mmol) and 5 units of T4 polynucleotide

kinase. The mixture was incubated for 30 min at 37°C. The reaction was terminated by heat inactivating the enzyme at 65°C for 5 min. After adding 150 ul of 2 M ammonium acetate and 2 ug transfer RNA (tRNA) the labelled DNA was precipitated with 2 volumes of ethanol.

End labelling of the DNA with the Klenow fragment of E. coli DNA polymerase 1 was more rapid and more efficient. The reaction was carried out immediately after digesting the DNA with a restriction enzyme. There was no need to remove or heat-inactivate the restriction endonuclease and there was no need to change buffers. After restriction endonuclease digestion, the appropriate ^{32}P-labelled deoxyribonucleotide (10 uCi: 3,000 Ci/mmol, 10.0 mCi/ml) was added to the reaction mixture along with 5 units of the Klenow fragment of E. coli DNA polymerase 1. The reaction was allowed to proceed for 1 hr at 15°C. The reaction was stopped by heat inactivating the enzyme at 65°C for 5 min.

For the sequencing reaction to work, only one strand of the DNA must be labelled. Because both of the end labelling techniques described above label both ends of the DNA, a subsequent step which removes the label from one end of the DNA must be performed. This can be done either by cutting with a second restriction endonuclease or by separating the two DNA strands. The first protocol relies on cleaving the DNA with a second restriction endonuclease whose site of cleavage is past the site to be sequenced. DNA labelled by the kinase exchange reaction was resuspended after ethanol precipitation in the appropriate digestion buffer and cleaved with a restriction endonuclease. When DNA was labelled using the Klenow fragment of E. coli DNA polymerase 1 the second restriction endonuclease was added to the reaction mix directly. The labelled DNA fragments were then separated by electrophoresis on either agarose or polyacrylamide gels. The

procedure for eluting DNA from agarose has been described previously.

To elute DNA from a polyacrylamide gel the apex of a 1,000 ul pipette tip was sealed with heat and the tip was plugged tightly with siliconized glass wool. The acrylamide gel slice containing the desired fragment of DNA was then introduced into the plugged and sealed pipette tip. The gel slice was crushed with a glass rod and 0.6 ml of gel elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 10 ug/ml tRNA) was added. The top of the tip was then sealed with parafilm and heated at 37^{o}C for 10 hr. The parafilm was removed and the sealed point of the tip was cut off. The liquid was forced through the glass wool and an extra 200 ul of gel elution buffer was used to wash the tip. The salt concentration was adjusted to 0.1 M NaCl and the DNA was precipitated in ethanol.

The second procedure used to ensure that only one free end of DNA was labelled involved DNA strand separation. The DNA, with ^{32}P-label at both ends, was resuspended in 40 ul of 30% (vol/vol) dimethylsulfoxide (DMSO), 1 mM EDTA, 0.05% (wt/vol) xylene cyanol, and 0.05% (wt/vol) bromophenol blue. This mixture was heated at 90^{o}C for 2 min, quick chilled on ice, and loaded onto a strand separation gel (5% (wt/vol) acrylamide, 0.1% (wt/vol) methylene bis-acrylamide, 50 mM Tris-borate pH 8.3, 1 mM EDTA). After electrophoresis, the appropriate portion of the gel was isolated and the DNA was eluted as described above.

The purified DNA which was prepared for sequencing was resuspended in 40 ul of distilled water and used in five separate reactions. For convenience, each reaction will be described separately.

G reaction:

In an Eppendorf tube 5 ul of end labelled DNA, 1 ul sonicated calf thymus (CT) DNA (1 mg/ml in water) and 200 ul of G buffer (50 mM sodium cacodylate

pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA) were mixed. The mixture was chilled on ice, then 1 ul of dimethylsulfate (DMS) was added and the reaction was allowed to proceed for 12 min at 20°C. The reaction was stopped with 50 ul of G stop buffer (1.5 M sodium acetate, pH 7.0, 1 M mercaptoethanol, 100 ug/ml tRNA) and 750 ul ethanol.

G + A reaction:

To 10 ul of end labelled DNA and 1 ul of CT DNA (1 mg/ml) was added an additional 10 ul of water. This mixture was chilled on ice in an Eppendorf tube. To this was added 50 ul of formic acid and the reaction was allowed to proceed for 10 min at 20°C. The reaction was terminated by the addition of 200 ul of HZ stop (0.3 M sodium acetate pH 7.0, 0.1 mM EDTA, 25 ug/ml tRNA) and 750 ul ethanol.

T + C reaction:

In an Eppendorf tube 10 ul of end labelled DNA, 1 ul of CT DNA (1 mg/ml) and 10 ul of water were chilled on ice. To this was added 30 ul of 95% hydrazine, and the reaction was allowed to proceed for 15 min at 20°C. The reaction was stopped with the addition of 200 ul of HZ stop buffer and 750 ul ethanol.

C reaction:

The C reaction was identical to the T + C reaction except that the starting mixture consists of 5 ul of end labelled DNA, 1 ul of CT DNA (1 mg/ml), and 15 ul of 5 M NaCl.

A > C reaction:

An Eppendorf tube containing 5 ul of end labelled DNA, 1 ul CT DNA (1 mg/ml), and 100 ul of A > C buffer (1.2 N sodium hydroxide, 1 mM EDTA) was prepared. This mixture was heated to 90°C for 15 min and the reaction was terminated with the addition of 150 ul of 1 N acetic acid, 5 ul tRNA (1 mg/ml), and 750 ul ethanol.

At this point in the protocol, all the reactions were treated similarly. The DNA in ethanol and stop buffer was frozen at -70°C for 5 min and then centrifuged at room temperature. The DNA pellets were resuspended in 200 ul of 0.3 M ammonium acetate and mixed vigorously. Once again 750 ul of ethanol was added, the mixture was frozen and centrifuged. The DNA pellets were vacuum dried for 5 min and subsequently resuspended in 100 ul of 1 M piperidine. All the reactions were then placed at 90°C for 30 min, after which they were frozen and lyophilized for approximately 3 hr. The DNA was successively resuspended in 50, 20, and 10 ul of distilled water and frozen and lyophilized. The DNA was finally resuspended in 10 ul 80% (vol/vol) deionized formamide, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 0.1% (wt/vol) xylene cyanol, 0.1% (wt/vol) bromophenol blue. The samples were heated at 90°C for 3 min and quickly chilled on ice. The samples were then loaded and electrophoresed through sequencing gels.

Both 10% and 20% sequencing gels were commonly used in this work. To obtain a 20% sequencing gel, 25 ml of 40% (wt/vol) acrylamide, 2% (wt/vol) methylene bis-acrylamide, 25 gm ultra-pure urea, 5 ml 10 X TBE (0.89 M Tris-base, 0.89 M boric acid, 0.025 M EDTA) were mixed and the final volume adjusted to 50 ml with water. This solution was degased, and 500 ul of 10% (wt/vol) ammonium persulfate (AP) amd 30 ul of Temed were added. The solution was mixed and rapidly poured into a preformed sequencing gel mold. The same procedure was used for 10% acrylamide gels except that 12.5 ml of 40% (wt/vol) acrylamide, 2% (wt/vol) methylene bis-acrylamide was used to prepare the gel. After electrophoresis, the sequencing gels were wrapped in Saran wrap, exposed to X-ray film in cassettes containing Dupont lightning plus intensifying screens, and the cassettes were placed at -70°C for varying lengths of time. The autoradiographs were developed using an X-omat machine

and the sequence was read directly from the autoradiograms.

PROTOCOL FOR OLIGONUCLEOTIDE DIRECTED MUTAGENESIS

A) Basic Strategy

The method of oligonucleotide mutagenesis stems from the observations that DNA duplexes containing mismatches can be stabilized under certain condition (Gillam et al., 1975; Gillam and Smith, 1979) and that the Klenow fragment of E. coli DNA polymerase 1 is able to extend oligonucleotide primers hybridized to single-stranded DNA templates (Hutchinson et al., 1978; Razin et al., 1978). The basic principle involves hybridizing a short (9-15 nucleotides) single-stranded oligonucleotide to a single-stranded circular template. The oligonucleotide is completely complementary to a region of the template except for a mismatch that directs the mutation. The enzymatic activity of DNA polymerase is then used to extend the 3' end of the oligonucleotide to the primer until a complete circular double-stranded molecule is formed. The molecule can then be closed by ligation of the newly synthesized strand with T4 DNA ligase. Upon transformation of E. coli cells with the in vitro synthesized closed circular DNA (CC-DNA), a population of mutant and wt molecules are obtained. Mutant molecules are distinguished from wt by one of a number of screening procedures. The protocols used were kindly provided by Mark Zoller and Michael Smith (Department of Biochemistry, University of British Columbia).

B) Sequence Determination of the Mutagenic Oligonucleotide

Tyrosine 315 of polyomavirus middle T antigen is the main site of tyrosine phosphorylation *in vitro*. In order to test the importance of this amino acid in both the kinase assay and the transformation assay, oligonucleotide mutagenesis was employed to convert the codon for tyrosine 315 into a codon for a serine residue. For this purpose the oligonucleotide, 5' OH-GAGGAGTCCATGCCA-OH 3', was purchased from ChemGenes Corporation. The oligonucleotide matches the sequence for middle T antigen except that there is an A-C transversion at nucleotide position seven in the oligonucleotide. The oligonucleotide was designed so that the mismatch was located near the middle of the molecule. Placement of the mismatch in the middle yields the greatest binding differential between a perfectly matched duplex and a mismatched duplex, and prevents the correction of the mismatch by the 3' to 5' exonuclease activity of the Klenow fragment of *E. coli* DNA polymerase 1 (Gillam and Smith, 1979).

In order to verify the sequence of the oligonucleotide, the end labelled oligonucleotide was sequenced by a modified Maxam and Gilbert, (1977) procedure. Because the oligonucleotide contains a 5' OH, it was phosphorylated using the kinase reaction. An Eppendorf tube containing 7 ul of [γ-^{32}P]ATP (70 uCi: 10.0 mCi/ml, 3,000 Ci/mmol), 1 ul 10 X kinase buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT), 1 ul oligonucleotide (100 ng), and 1 ul T4 polynucleotide kinase was prepared and allowed to incubate at 37^{o}C. After 30 min 1 ul of 10 X kinase mix, 1 ul of 10 mM ATP, 7 ul of H_2O, and 1 ul of T4 kinase were added and the reaction was allowed to proceed for an additional 30 min at 37^{o}C. The reaction was stopped by heat inactivating the enzyme for 5 min at 65^{o}C. The labelled oligonucleotide was run on a 5% acrylamide gel and purified as described

previously. The DNA was resuspended in 32 ul of distilled water. Four separate sequencing reactions were performed. These reactions resemble those previously described and only the differences are emphasized here.

G reaction:

The reaction was performed in an Eppendorf tube containing 5 ul of end labelled DNA, 1 ul CT DNA (1 mg/ml), 300 ul of G buffer, and 2 ul of DMS. The reaction was allowed to proceed for 15 min at 37°C.

G + A reaction:

The reaction consisted of 10 ul of end labelled DNA, 1 ul CT DNA (1 mg/ml), 10 ul H_2O, and 3 ul formic acid mixed in an Eppendorf tube which was allowed to react for 20 min at 37°C.

T + C reaction:

An Eppendorf tube containing 10 ul of end labelled DNA, 1 ul CT DNA (1 mg/ml), 15 ul H_2O, and 30 ul hydrazine was incubated for 15 min at 55°C.

C reaction:

The C reaction was identical to the T + C reaction except that only 5 ul of end labelled DNA was used, and instead of 15 ul of H_2O the reaction contained 20 ul of 5 M NaCl.

After the reactions were completed, 5 ul of tRNA (1 mg/ml) was added and the samples were processed as described previously. The results of the sequencing gel confirmed that the oligonucleotide was the correct sequence (GAGGAGTCCATGCCA).

C) Oligonucleotide Directed Mutagenesis

A preliminary *in vitro* test was performed in order to insure

that the primer could function under the reaction conditions used. M13mp8BRcMT was constructed by Don Cook in our laboratory by inserting the BamH1/EcoR1 fragment (58.5 to 100/0 m.u. or nucleotides 4632 to 1560 clockwise) of an altered polyomavirus genome encoding only middle T antigen (Treisman et al., 1981) into the BamH1/EcoR1 fragment of the double stranded form of the bacteriophage cloning vehicle M13mp8 (Messing and Vieira, 1982). In the annealing step of the reaction, 50 ng of end labelled oligonucleotide DNA was added to M13mp8BRcMT DNA (1 ug/ml). To this was added 1 ul solution A (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl, 10 mM DTT) and the volume was adjusted to 10 ul with water. This mixture was placed in a water bath at 55°C for 5 min and then allowed to cool to 23°C for 5 min. To this mixture 4 ul of a 2.5 mM stock of all four deoxynucleotides (dNTP) and 1 unit of the Klenow fragment of E. coli DNA polymerase 1 was added and incubated for 5 min at 23°C. The reaction was terminated by heating at 65°C for 10 min, 5 units of the restriction enzyme EcoR1 were added, and the digestion was allowed to proceed for 1 hr at 37°C. After an equal volume of formamide/dye solution (80% (vol/vol) formamide, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 0.1% (wt/vol) xylene cyanol, 0.1% (wt/vol) bromophenol blue) had been added, the sample was boiled for 3 min and electrophoresed through a 5% sequencing gel. If the priming initiated by the oligonucleotide was specific, a 389 b.p. fragment, corresponding to the 5' end of the oligonucleotide at 1171 to the EcoR1 site at 1560, should be visible upon autoradiography of the gel. Indeed, such a fragment was found and it was the predominant labelled species on the gel, indicating that priming by the oligonucleotide was specific.

In the mutagenesis protocol, the annealing reaction was identical to the annealing reaction described above. In order to extend and ligate the

annealed oligonucleotide, 1 ul of solution B (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT), 4 ul dNTP (10 mM), 1 ul ATP (10mM), 2 units ligase and 2.5 units of the Klenow fragment of E. coli DNA polymerase 1 were added and the entire mixture was incubated for 15 hr at 15°C. The sample was then electrophoresed through a 1% (wt/vol) agarose gel containing 5 ug/ml ethidium bromide. The band corresponding to CC-DNA was eluted as described previously. The CC-DNA was resuspended in 1 X ligase mix (0.066 M Tris-HCl pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP) and transfected into $CaCl_2$ treated E. coli JM103 by a procedure which was essentially identical to the one described for plasmid DNA. However, at the end of the transfection protocol, JM101 cells were mixed with 2.5 ml of molten YT top agarose (8 g/l tryptone, 5 g/l yeast extract, 85 mM NaCl, 6 g/l agarose) and poured onto a YT plate (8 g/l tryptone, 5 g/l yeast extract, 85 mM NaCl, 15 g/l agar). The top agarose was allowed to harden at room temperature for 15 min. The plates were then incubated at 37°C for 12 hr, at which time clear plaques could be seen.

D) Screening and Isolation of Mutated Phage

In order to identify phage bearing the mutation specified by the oligonucleotide, two consecutive procedures were used. The first protocol relies on the fact that the oligonucleotide will anneal more stringently to a mutant phage, which matches the oligonucleotide perfectly, than with a wt phage which bears a single mismatch. In this protocol 5 ml of JM103 cells which had been growing in 2 X YT media (16 g/l tryptone, 10 g/l yeast extract, 85 mM NaCl) for 2 hr at 37°C in a shaking incubator, were diluted to 45 ml in 2 X YT media. One ml of these cultures were distributed

to 45 culture tubes. Phage plaques were cored using Pasteur pipettes and the agarose plug was blown into the culture tube. The tubes were then incubated for 5 hr at 37°C with vigorous shaking. The contents of each tube was transferred to an Eppendorf tube and spun for 5 min. The phage-containing supernatant was decanted into a fresh Eppendorf tube. The supernatant containing the phage was spotted directly onto a nitrocellulose filter using the "dot blot" apparatus marketed by Bethesda Research Laboratories. To individual wells, 20 ul of phage supernatant were added, and washed twice with 100 ul of 10 mM Tris-HCl pH 8.0. The filter was air dried and then baked at 80°C for 2 hr in a vacuum oven. The filter was prehybridized with 6 X SSC (20 X SSC = 3 M NaCl, 0.3 M sodium citrate), 10 X Denhardt's (0.2% (wt/vol) BSA, 0.2% (wt/vol) polyvinyl pyrollidone, 0.2% (wt/vol) Ficoll), and 0.2% (wt/vol) SDS for 1 hr at 67°C. After an hour the filter was rinsed in 50 ml of 6 X SSC for 1 min at room temperature. The filter was then exposed to a 4 ml solution of 6 X SSC, 10 X Denhardt's containing 2 ng of end labelled oligonucleotide, and incubated at 23°C for 1 hr. The filter was then washed three times in 50 ml 6 X SSC for 10 min. The dried filter was then autoradiographed. Thereafter, the filter was re-washed three times at 47°C for 10 min and again the filter was autoradiographed. When the two autoradiograms were compared, only three spots continued to hybridize well at 47°C and the phage represented by these spots were chosen for further study.

In order to confirm that the three phage populations chosen contained mutant phage, a second protocol was used to screen these phage. This method takes advantage of the fact that the mutation caused by the oligonucleotide creates a new Hinf1 restriction endonuclease site. Double stranded phage DNA was prepared by the small scale plasmid DNA isolation technique described

earlier. Restriction digest analysis revealed that two of the three plaques chosen contained phage whose DNA bore a new Hinf1 site at nucleotide 1178. One of the mutant phages, M13mp8BRcMT25 was chosen for further study. The polyomavirus BamH1/EcoR1 fragment of the mutant phage was recloned into the large BamH1/EcoR1 fragment of the bacterial plasmid pML2 (Lusky and Botchan, 1981) and this recombinant plasmid will be referred to as pdPbs25. For comparative purposes, the BamH1/EcoR1 fragment of the altered polyomavirus DNA expressing only middle T antigen was also cloned in the large BamH1/EcoR1 fragment of pML2. The exact nature of the mutation suffered by pdPbs 25 was confirmed by sequence analysis (Maxam and Gilbert, 1977).

CELL CULTURE AND TRANSFORMATION

A) Cell Culture

All cell lines were propagated in Dulbecco's modification of Eagles medium (DMEM) supplemented with gentamicin (50 ug/ml) and fungizone (2.5 ug/ml). The media for growth of Rat-1 cells, a subclone of Fischer rat F2408 cells (Freeman et al., 1973), was supplemented with 10% fetal bovine serum (FBS) whereas the media of transformed cell lines was supplemented with 5% FBS. All cell lines were maintained at 37°C in a humidified CO_2 incubator.

Transformed cell lines were established from transfections of Rat-1 cells which yielded foci. The plates containing the foci to be cloned were washed with 10 ml of PBS (8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4). Independent foci from separate plastic dishes were

gently scraped off the plastic dish with a Pasteur pipette and placed in a microtiter well containing trypsin (650 ug/ml) in versene (8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.1 g/l $CaCl_2$, 0.0592 g/l $MgSO_4$, 0.2 g/l EDTA) and allowed to incubate at 37°C for 10 min. The cells were then transferred to a 35 mm dish containing medium. A second round of cloning was performed after diluting cells until well separated individual colonies could be isolated. These individual colonies were recloned as described above.

B) Transformation Assay

A modification of the calcium phosphate technique (Wigler et al., 1978) was used to transfect Rat-1 cells with CsCl density gradient purified supercoiled plasmid DNA. Rat-1 cells were plated on 100 mm plastic dishes at a cell density of 3.5×10^5 cells/plate, and were incubated for 15 hr in DMEM supplemented with 10% FBS. The medium was then changed and 4 hr later the DNA calcium phosphate precipitate was added. The precipitate was prepared as follows. One drop of 2 M $CaCl_2$ was added to 3.375 ml of H_2O. To this was added, drop by drop, 1 ml of water containing DNA (200 ug total, consisting of the desired amount of recombinant plasmid DNA with the balance being made up of CT DNA). Additional 2 M $CaCl_2$ was added drop by drop until the final volume of 2 M $CaCl_2$ added was 630 ul. The entire solution was vortexed. This solution was gently bubbled into a tube containing 5 ml of 2 X HBS pH 7.1 (50 mM Hepes, 280 mM NaCl) and 0.1 ml of 0.07 M Na_2HPO_4. The resulting precipitate was allowed to form for 30-45 min at room temperature. One ml of the precipitate was added per plate by directly pipetting it onto the medium. The precipitate remained on the

plates for 4-5 hr after which the cells were washed in 10 ml of PBS, and new medium was added. The medium was changed the next day, and routinely every three days after, until the plates were ready to be stained. In order to visualize and quantitate the foci, cells were fixed to the plate using 10 ml of 10% (vol/vol) formaline in PBS for 10 min. The plates were then covered with a 1/25 dilution of Giemsa stain in PBS. The cells were stained for 24 hr, then the plates were rinsed with water, allowed to dry, and the foci were counted.

C) Growth in Agar and Tumorigenicity of Transformed Cell Lines

To test the capacity of cells to grow without a solid support, 10^5 cells were suspended in 0.33% (wt/vol) agarose in DMEM containing 5% FBS and plated over a layer of 0.60% (wt/vol) agarose in the same medium. After one week, the plates were scored for the presence of colonies.

To test the tumorigenicity of the various transformed cell lines, 10^6 cells were injected subcutaneously into three week old Fischer rats. After 21 days, rats were examined for the presence of solid tumors at the site of injection, and the number of animals with tumors scored.

D) Identification of Viral Sequences in Transformed Cells

In order to identify viral sequences in transformed cells, cellular DNA was isolated as follows. Transformed cell lines were grown to confluence on 100 mm plates. The plates were then washed in 10 ml of cold PBS, the cells were scraped off the plate, and resuspended in 1 ml of PBS/plate. Cells were centrifuged at 2,000Xg for 5 min and the cellular pellet was resuspended in 1

ml of TE, 0.5% (wt/vol) SDS per plate. One mg of pronase per one ml of cell suspension was added and the mixture was allowed to incubate for 8 hr at 37°C. The DNA was then extracted once with buffered phenol, and twice with chloroform:isoamyl alcohol (24:1). Residual organic solvent was removed by dialyzing the cellular DNA against successive changes of TE at 4°C. A total of 5-10 ug of this cellular DNA was cut with a 10 fold excess of an appropriate restriction endonuclease, and the DNA fragments were separated on an agarose gel.

In order to visualize only the viral sequences, the DNA was transferred to a membrane filter and immobilized by a modification of the method developed by Southern, (1975). The relative positions of the DNA fragments in the gel are preserved during their transfer to the filter. The DNA attached to the filter was then hybridized to viral ^{32}P-labelled DNA, and autoradiography was used to locate the position of any bands complementary to the radioactive probe. The specific protocol used was one supplied by the manufacturer's of Biodyne nylon membranes. Briefly, after electrophoresis, the gel was placed in 150 ml of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 45 min, then it was rinsed in water and placed in 150 ml of neutralizing solution (3 M sodium acetate pH 5.5) for 45 min. A reservoir, containing 20 X SSC, was spanned with a glass plate. A sheet of filter paper, saturated with 20 X SSC, was place over the glass plate, and the two ends of the paper were place in the reservoir. Excess gel was trimmed off, and the gel was then placed on the paper/glass support, over which was placed a Biodyne membrane, two layers of filter paper, and a stack of paper towels, all of which were cut to the dimensions of the gel, and finally on top of this was placed a second glass plate and a one kg weight. The DNA from the gel was allowed to transfer to the membrane for at least 15 hr. At this point the membrane was

removed from the gel surface, air dried, and baked at 80°C for 1 hr in a vacuum oven.

The membrane was prehybridized in 4 ml of hybridization solution (5 X Denhardt's, 5 X SSC, 50 mM sodium phosphate pH 6.5, 0.1% (wt/vol) SDS, 250 ug/ml CT DNA, 50% (vol/vol) formamide) per 100 cm^2 of membrane in a sealed plastic bag placed at 42°C for 1 hr. Fresh hybridization solution (2 ml per 100 cm^2 of membrane) containing the nick-translated radioactive probe (Kelly et al., 1970) was boiled for 10 min and was used to replace the prehybridization fluid. The labelled probe was prepared by incubating 5 ul of 10 X NT buffer (0.5 M Tris-HCl pH 7.2, 0.1 M $MgSO_4$, 1 mM DTT, 500 ug/ml BSA), 2.5 ul of each radioactively labelled dNTP (25 uCi, 3,000 Ci/mmol, 10 mCi/ml), 1 ul DNase (10^{-4} dilution of a 1 mg/ml stock), 0.2 ug of DNA, and water to a final volume of 50 ul. Five units of E. coli DNA polymerase 1 (holoenzyme) were added and the reaction was incubated at 15°C for 1 hr, at which point the reaction was terminated with the addition of 2 ul of 0.5 M EDTA. The nick-translated DNA was separated from unincorporated dNTPs by passage through a Sephadex G-50 column. The specific activity of the probe was usually > 10^8 cpm/ug of DNA. The probe was allowed to hybridize to DNA attached to the Biodyne membrane for 15 hr at 42°C after which the filter was washed three times for 5 min at room temperature, and twice for 30 min at 50°C, in 0.1 X SSC, 0.1% (wt/vol) SDS. The membrane was then air dried and autoradiographed.

ANALYSIS OF POLYOMAVIRUS T ANTIGENS

A) Immunoprecipitation of Viral Tumor Antigens

Immunoprecipitation of the viral tumor antigens of Rat-1 cells transformed by polyomavirus, or transformed by recombinant plasmids bearing mutations in the polyomavirus sequences, was performed as described by Ito (1979). Cells were plated on 60 mm plastic dishes at a density of 5 X 10^4 cells/cm^2 in DMEM containing 5 % FBS. After 24 hr, the media was removed, and the monolayers were washed twice with 5 ml of PBS. Cells were then incubated for 1 hr at 37°C in methionine-free DMEM, and the monolayers were subsequently washed twice with PBS. This was followed by a 4 hr incubation in methionine-free DMEM (1 ml) containing 300 uCi of [^{35}S]methionine (1,000 Ci/mmol). Labelling of cellular proteins was terminated by removing the medium and washing the cell monolayers twice with 5 ml of ice cold TBS (8 g/l NaCl, 0.38 g/l KCl, 1 g/l Na_2HPO_4, 1 g/l dextrose, 3 g/l Tris-base).

Cells were lysed directly on the plate with 0.8 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% (vol/vol) Nonidet P40 (NP40)) for 10 min on ice. The supernatant was then transferred to an Eppendorf tube and spun for 10 min in a microfuge. The supernatant was removed and placed in a fresh Eppendorf tube, and 30 ul of normal rat serum (NRS) from brown Norwegian (BN) rats was added. The mixture was left on ice overnight. The next day 120 ul of 20% formalin-fixed S. aureus (Kessler, 1976) was added and incubated for 30 min on ice. The mixture was spun in a microfuge for 2 min and the supernatant was equally divided into two Eppendorf tubes. To one tube 15 ul of anti-tumor antisera (anti-T) was added, while the second tube received 15 ul of NRS. In all immunoprecipitations, ascites fluid from BN rats, which had been injected with PyB4a cells (Silver et al., 1978), was used as a source of anti-T serum. The antibody-antigen complexes were allowed to form overnight in ice. Once again 120 ul of 20%

formalin-fixed S. aureus was added and incubated for 30 min on ice. The mixture was centrifuged for 20 sec and the pellet was washed three times with 1 ml of NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.5). The pellet was finally resuspended in 50 ul of 7 M urea in 0.08 M Tris-HCl pH 8.0 and incubated at room temperature for 30 min. The mixture was spun in a microfuge for 2 min and the supernatant was removed and diluted in 450 ul NET. The immunocomplexes were allowed to reform overnight. The next day 120 ul of 20% formalin-fixed S. aureus was added and allowed to react with the antibody-antigen complexes for 30 min on ice after which the mixture was centrifuged. The pellet was washed twice with NET and finally resuspended in 50 ul of sample buffer (2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.08 M Tris-HCl pH 6.8, 0.2% (wt/vol) bromophenol blue, 5% (wt/vol) mercaptoethanol).

The immunoprecipitated samples were boiled for 10 min and then run on a 1.5 mm thick SDS-polyacrylamide gel composed of a short stacking gel (1.67 ml 30% acrylamide/0.8% methylene bis-acrylamide (wt/vol), 1.25 ml 1 M Tris-HCl pH 6.8, 50 ul 20% (wt/vol) SDS, 7 ml H_2O, 50 ul 10% (wt/vol) AP, 10 ul Temed) and a 10% polyacrylamide running gel (10 ml 30% acrylamide/0.8% methylene bis-acrylamide (wt/vol), 11.2 ml 1 M Tris-HCl pH 8.8, 150 ul 20% (wt/vol) SDS, 8.7 ml H_2O, 100 ul 10% (wt/vol) AP, 25 ul Temed). In some cases the concentration of the running gel was increased to 12.5%. Samples were electrophoresed at 100V in glycine running buffer (0.025 M Tris-base, 0.192 glycine, 0.1% (wt/vol) SDS) until the bromophenol blue dye reached the bottom of the gel. Gels were stained for 12 hr (260 ml H_2O, 202 ml methanol, 26.5 ml glacial acetic acid, 0.0625 gm Coomassie blue), destained for 1 hr (260 ml H_2O, 202 ml methanol, 26.5 ml glacial acetic acid) and fluorographed with Enhance according to the manufacturer's specifications.

The gels were then dried down and the ^{35}S-labelled proteins were visualized by autoradiography.

B) In Vitro Kinase Assay

To measure the capacity of middle T antigen to become phosphorylated in vitro, a procedure described by Benjamin and Schaffhausen (1981b) was used. Briefly, transformed cells were grown to 60% confluence on 100 mm plastic dishes in DMEM supplemented with 5% FBS, the cells were washed three times with PBS and rinsed in washing buffer (0.135 M NaCl, 0.02 M Tris-HCl pH 9.0, 0.001 M $MgCl_2$, 0.001 M $CaCl_2$). Cells were lysed and the T antigens extracted for 20 min at 4°C using 1.1 ml of washing buffer supplemented with 1% (vol/vol) NP40. The extracts were cleared by centrifugation and allowed to react with either NRS or anti-T serum in the presence of 80 ul of 50% formalin-fixed S. aureus for 30 min at 4°C. The immunoprecipitates were washed three times with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris-HCl pH 6.8, and finally with distilled water. These washed immunoprecipitates were then resuspended in 0.4 ml of 0.02 M Tris-HCl pH 7.5, 0.005 M $MgCl_2$, containing 5 uCi of [γ-^{32}P]ATP. The reaction was allowed to proceed for 15 min at 22°C, after which the immunoprecipitates were collected and washed as described above. The final pellet was resuspended in sample buffer, and the proteins released from the immunocomplexes after boiling for 10 min were electrophoresed through SDS-polyacrylamide gels as described in the previous section. The ^{32}P-labelled proteins were visualized by autoradiography.

C) Partial Proteolytic Mapping of Middle T Antigen

The technique of partial proteolysis has been described previously (Schaffhausen et al., 1978; Schaffhausen and Benjamin, 1979, 1981a, 1981b) and I wish to express my gratitude to Brian Schaffhausen (Department of Biochemistry, Tuft's University) for the assistance he provided in this portion of the work.

Immunoreactive proteins were collected after the in vitro kinase assay as described previously. The proteins were eluted in sample buffer and separated on cylindrical gels. The bottom of the cylindrical tube (15 cm X 2.5 mm) was plugged with 2 cm of 1% (wt/vol) agarose. The 10% SDS-polyacrylamide running gel was poured using a length of flexible tubing long enough to reach the bottom of the cylinder. The 1.5 cm 3% SDS-polyacrylamide spacer gel was poured in a similar fashion. Electrophoresis was carried out in a vertical gel apparatus until the bromophenol blue reached the bottom of the gel using 50 to 80 volts of constant voltage. After electrophoresis, the glass tube was gently broken with a hammer and the gel was then rinsed in water to remove glass fragments.

Two cylindrical gels were compared by placing the gels head to head on top of a 12.5% SDS-polyacrylamide gel. The electrophoresis apparatus was then filled with running buffer and two ml of digestion solution (0.0125 M Tris-HCl pH 6.8, 0.01 M EDTA, 0.0075% (wt/vol) bromophenol blue, 20% (vol/vol) glycerol containing 50 ug/ml BSA, 30 ug/ml S. aureus V8 protease) were layered on top. Electrophoresis was carried out at 50V for approximately 16 hr until the bromophenol blue reached the bottom of the gel. The labelled products of S. aureus V8 protease digestion were visualized by autoradiography.

RESULTS

RESULTS

CHAPTER 1: THE ROLE OF SMALL AND MIDDLE T ANTIGEN IN CELLULAR TRANSFORMATION

I. In Vitro Mutagenesis, Isolation, and Characterization of Mutants

A) Introduction

Two parental plasmids carrying fragments of polyomavirus DNA were used as substrates for *in vitro* mutagenesis. One of these plasmids, pPH1-8 (Figure 4A, 4B) contains the Hind111-1 fragment of polyomavirus DNA (nucleotides 3918-1656) inserted at the Hind111 site of the bacterial plasmid pBR322. The construction and transforming activity of this plasmid have been described previously (Hassell *et al.*, 1980). The second recombinant plasmid, pPBR2 (Figure 5A, 5B), which was specifically constructed for this work, contained the BamH1/EcoR1 fragment of polyomavirus DNA (nucleotides 4632-1560) within the large BamH1/EcoR1 double digestion product of the plasmid pMK16.1. Both of these recombinant plasmids contain the sequences which code for the polyomavirus small and middle T antigens (Figure 4B, 5B), and both have been shown by the focus assay to cause transformation of Rat-1 cells in culture at approximately the same frequency.

The first objective of this research was to determine whether small and/or middle T antigen was required for transformation. To this end, a recombinant plasmid DNA bearing the coding sequences of small and middle T antigen was mutated. Because the coding sequences of the proximal portion of small and middle T antigens overlap, only two different classes of mutants

Figure 4A: Map of restriction endonuclease cleavage sites in pPH1-8 DNA. pPH1-8 contains the Hind111-1 (45 tp 1.4 m.u.) fragment of polyomavirus DNA (thick line) inserted at the Hind111 site of the plasmid pBR322 (thin line).

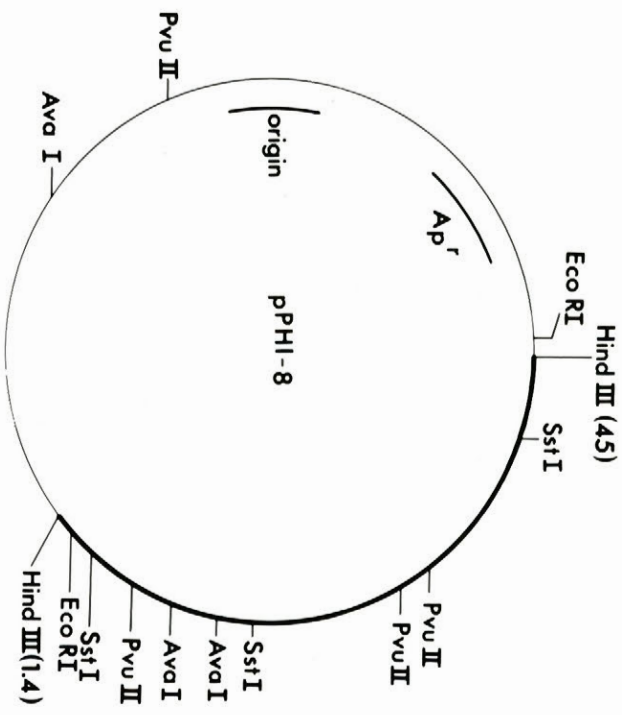

Hind III (45)
Eco RI
Sst I
Ap r
pPH1-8
origin
Pvu II
Ava I
Pvu II
Pvu II
Sst I
Ava I
Ava I
Pvu II
Sst I
Eco RI
Hind III(1.4)

Figure 4B: Physical map of pPH1-8 DNA. pPH1-8 contains the Hind111-1 (nucleotides 3918-1656) fragment of polyomavirus DNA (thick line), inserted into the Hind111 site of the plasmid pBR322 (thin line). The figure also shows the coding sequences for large, middle, and small T antigen which are present in pPH1-8 DNA. Note that the N-terminal portion of the three T antigens (before the proximal splice) as well as the distal portion of small T antigen are in the same reading frame. The C-terminal portion of middle and large T antigen (after the distal splice) are in the two remaining reading frames and are different from each other.

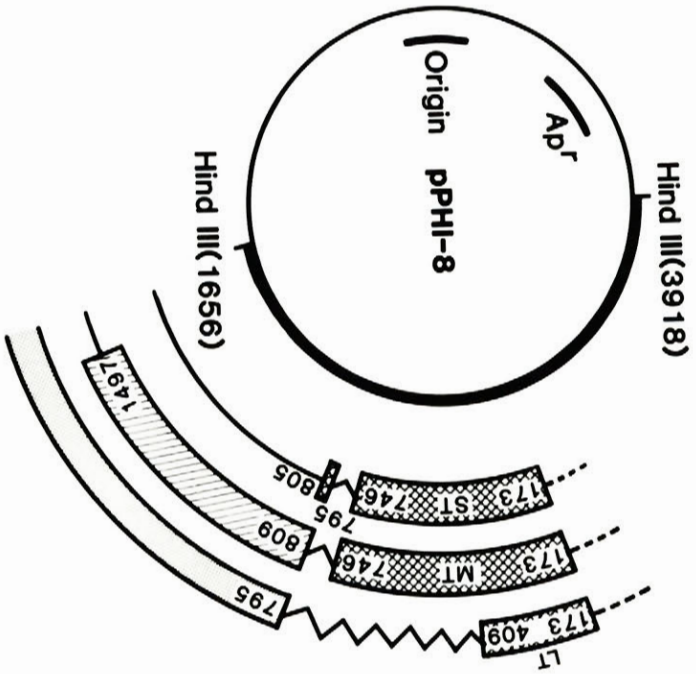

Hind III(3918)
Ap^r
Origin
pPHI-8
Hind III(1656)
1497
795
809
805
795
173
ST
746
173
MT
746
173
409
LT

Figure 5A: Map of restriction endonuclease cleavage sites in pPBR2 DNA. pPBR2 is composed of the BamH1/EcoR1 (58.5 to 100/0 m.u.) fragment of polyomavirus DNA (thick line) inserted in the BamH1/EcoR1 digestion product of the plasmid pMK16.1 (thin line).

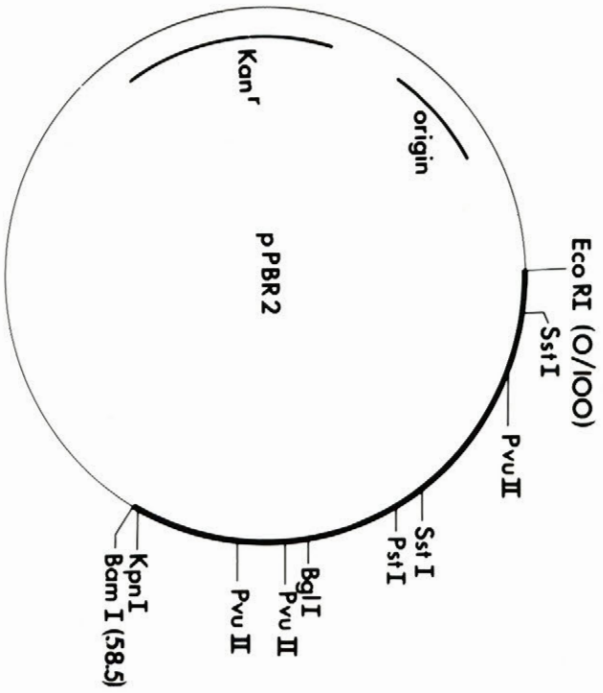
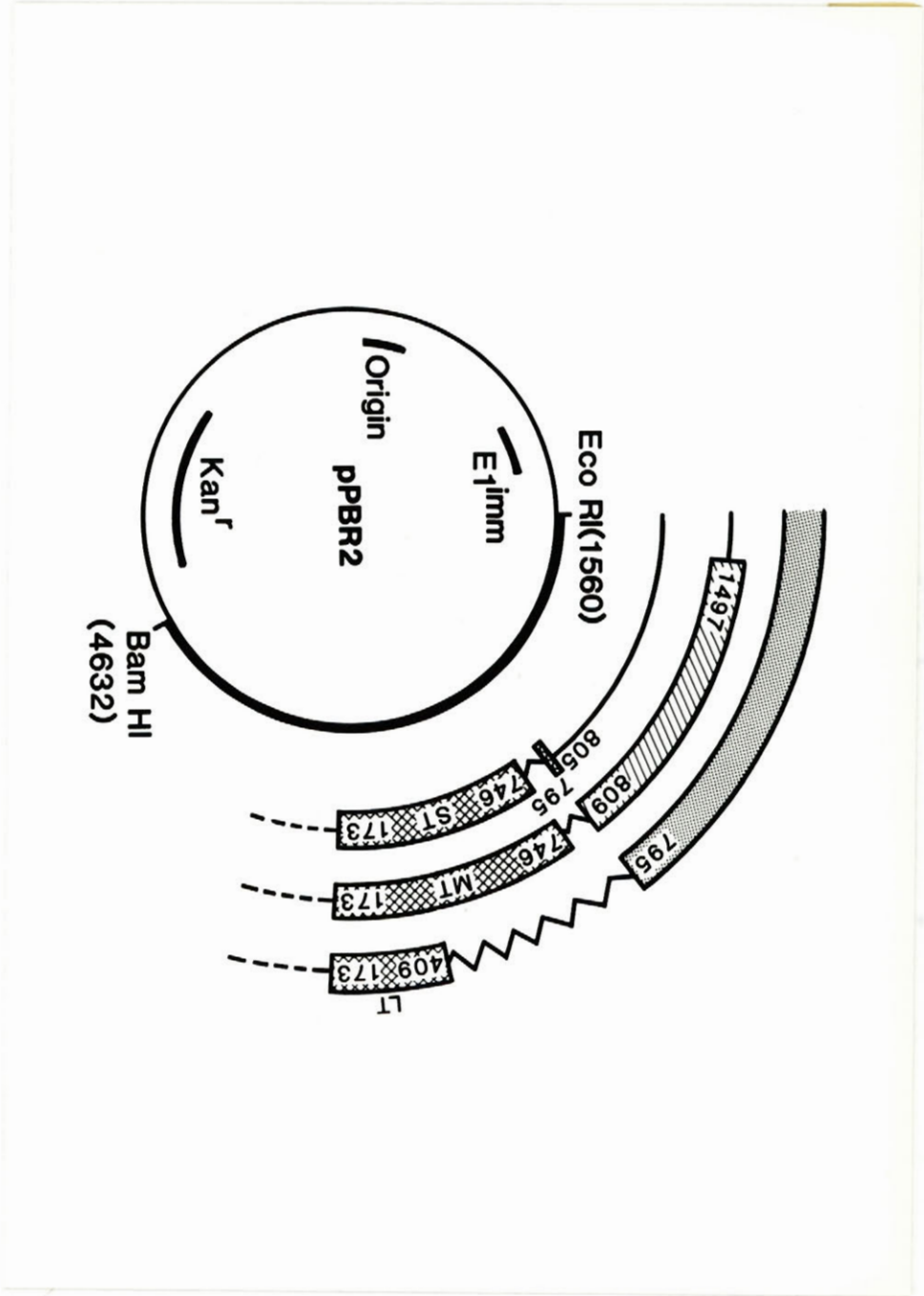

Eco RI (O/IOO)
Sst I
Pvu II
Sst I
Pst I
Bgl I
Pvu II
Pvu II
Kpn I
Bam I (58.5)
pPBR2
origin
Kan r

Figure 5B: Physical map of pPBR2 DNA. pPBR2 contains the BamH1/EcoR1 (nucleotides 4632-1560) fragment of polyomavirus DNA (thick line) inserted in the BamH1/EcoR1 digestion product of the plasmid pMK16.1 (thin line). The figure shows the coding sequences for large, middle, and small T antigen which are present in pPBR2. Note that the N-terminal portion of the three T antigens (before the proximal splice) as well as the distal portion of small T antigen are in the same reading frame. The C-terminal portion of middle and large T antigen (after the distal splice) are in the two remaining reading frames and are different from each other.


Origin
pPBR2
E1imm
Kanr
Eco RI(1560)
Bam HI
(4632)
1497
809
805
795
746 ST 173
746 MT 173
409 173
LT

could be obtained; one class having altered coding sequences for both small and middle T antigens, and a second class carrying lesions in the coding sequences for middle T antigen alone. To create deletions or insertions within sequences known to encode polyomavirus small or middle T antigen, one or the other of the parental plasmid DNAs described above was digested singly with one of several restriction endonucleases under conditions designed to favor linearization of the plasmid DNA. The ends of the linear DNA were then modified enzymatically, and the resulting mutated DNAs were cyclized with T4 ligase. Individual mutated plasmid DNAs were then cloned by transformation of E. coli and monitored for the loss of the appropriate restriction endonuclease site (Figure 6). In this way, mutations were introduced within coding sequences around nucleotide 484 (Pst1), 569 (Sst1), 657 (Ava1), 1016 (Ava1), 1144 (Pvu11), and 1373 (Sst1).

B) Mutations at the Pst1 site

The restriction endonuclease Pst1 cleaves the DNA of the recombinant plasmid pPBR2 only once (Figure 5A). The Pst1 site resides in the coding sequences which code for both small and middle T antigen (Figure 5B). Four deletion mutants were isolated which lacked the Pst1 site at nucleotide 484. These were isolated after Pst1 digestion of pPBR2 DNA and treatment with the Bal 31 nuclease (Table 2A). The size of the deletions were estimated by cleaving the mutant DNAs with Pvu11 and comparing the size of the fragments after gel electrophoresis to the fragments obtained when parental plasmid DNA was digested with Pvu11 (Figure 7d). It was determined that two of the mutants, pPd13 and pPd111, contained small deletions (less than 15 b.p.) whereas the other two mutant DNAs, pPd14 and pPd110, contained progressively larger deletions. The deletion in pPd14 was estimated to be 60

Figure 6: Analysis of mutated, recombinant plasmids by restriction endonuclease digestion and agarose gel electrophoresis. Plasmid DNA was monitored for the loss of a restriction endonuclease cleavage site. (a) In the first lane, arrows point to the four products obtained when the parental plasmid pPH1-8 was cleaved with Pvu11. DNAs lacking the site at 92.1 m.u. lost fragments 2 and 3 and gained a new fragment which migrated close to, or with, fragment 1. The bottom portion of the gel was derived from a different exposure in order to clearly show the smallest fragments. (b) Arrows indicate the three fragments obtained when pPH1-8 was cleaved with Ava1. DNAs lacking the Ava1 site at 82.9 m.u. (pPin2) lost fragment 3, retained fragment 2, and had a slower-migrating fragment 1. When the Ava1 site at 89.7 m.u. was mutated, fragment 3 was lost, fragment 1 was retained, and here fragment 2 migrated more slowly. (c) pPBR2 is cleaved twice by Sst1, and arrows indicate the position of the digestion products (left panel). When the Sst1 site at 81.3 m.u. (pPd19) or the Sst1 site at 96.5 m.u. (pPd11 and pPd115) was lost, digestion of these mutant DNAs with Sst1 produced full-length linears. When pPH1-8 was digested with Sst1, three fragments were obtained (right-most panel). pPd117 had lost the Sst1 site at 81.3 m.u. When digested with Sst1, pPd117 DNA yielded two fragments, one that comigrated with fragment 1 of the pPH1-8 digest and one that migrated more slowly then fragment 2 and at the same position as a partial digestion product of pPH1-8.

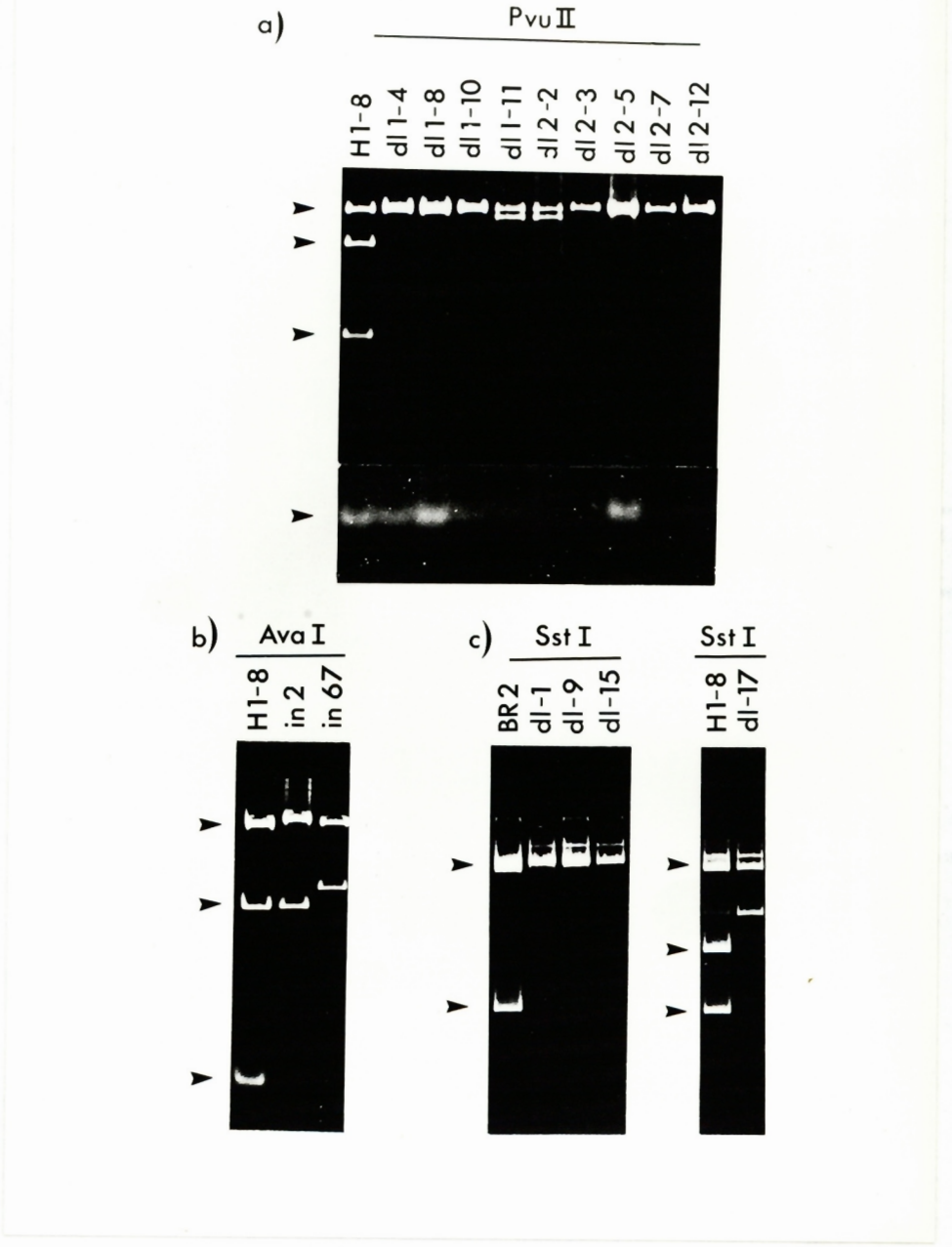

a)
PvuII
H1-8
dl 1-4
dl 1-8
dl 1-10
dl 1-11
dl 2-2
dl 2-3
dl 2-5
dl 2-7
dl 2-12
b)
AvaI
H1-8
in 2
in 67
c)
SstI
BR2
dl-1
dl-9
dl-15
SstI
H1-8
dl-17

Figure 7: Estimation of the size of deletions or insertions by restriction endonuclease digestion and gel electrophoresis. Mutant DNAs were cleaved with various restriction endonucleases, and the products were separated through 1.4% agarose gels along with molecular weight markers in order to determine the approximate size of the lesions. Molecular sizes are indicated above each gel. The upper portion of each gel is a longer exposure of the negative of the gel; this was done to clearly display all fragments.

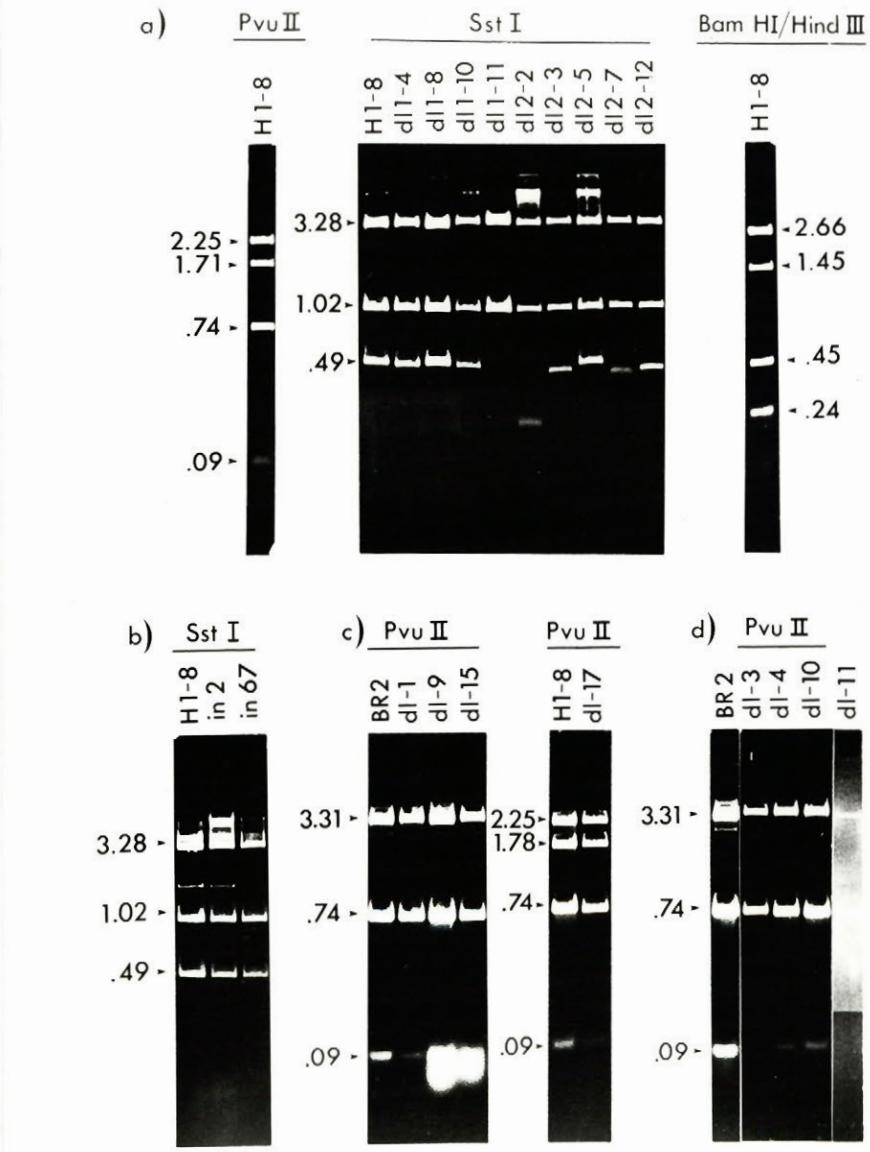

a)
Pvu II
Sst I
Bam HI/Hind III
H1-8
dl1-4
dl1-8
dl1-10
dl1-11
dl2-2
dl2-3
dl2-5
dl2-7
dl2-12
2.25
1.71
.74
.09
3.28
1.02
.49
2.66
1.45
.45
.24
b)
Sst I
H1-8
in 2
in 67
3.28
1.02
.49
c)
Pvu II
BR2
dl-1
dl-9
dl-15
3.31
.74
.09
Pvu II
H1-8
dl-17
2.25
1.78
.74
.09
d)
Pvu II
BR2
dl-3
dl-4
dl-10
dl-11
3.31
.74
.09

Table 2A. Characterization of DNAs with mutations at the PstI site.

DNA source	Parent plasmid	Mode of mutagenesis	Site mutagenized (m.u.)	Site mutagenized (nucleotide)	T antigen affected[a]
pPd13	pPBR2	Bal 31 nuclease	79.7	484	sT and mT
pPd14	pPBR2	Bal 31 nuclease	79.7	484	sT and mT
pPd110	pPBR2	Bal 31 nuclease	79.7	484	sT and mT
pPd111	pPBR2	Bal 31 nuclease	79.7	484	sT and mT

[a] sT, small T antigen; mT, middle T antigen.

Table 2B. Characterization of DNAs with mutations at the PstI site.

DNA source	Boundaries of deletions (nucleotides)	Number of base pairs deleted	Predicted amino acid composition of proteins produced from mRNAs having small and middle T antigen donor and acceptor splice sites
pPd13	485-491	7	105 sT[a] + 4 n.c.(2)[b]
pPd14	468-490	23	98 sT + 9 n.c.(3)[c]
pPd110	468-514	47	98 sT + 1 n.c.(2)
pPd111	485-489	5	104 sT + 9 n.c.(3)

[a] sT, amino acids corresponding to the N-terminal portion of small T antigen before the deletion.

[b] n.c.(2), amino acids from non-coding sequences corresponding to the reading frame used for the C-terminal portion of middle T antigen.

[c] n.c. (3), amino acids from non-coding sequences corresponding to the reading frame used for the C-terminal portion of large T antigen.

b.p., whereas the mutant pPd110 contained a larger deletion estimated to be 90 b.p.

In order to unequivocally determine the size and extent of the mutations, Maxam and Gilbert sequencing was performed on each mutant recombinant plasmid. The DNAs were individually labelled by the kinase exchange reaction at the Aval site at nucleotide 657. The products of the sequencing reactions were run on 10% denaturing polyacrylamide gels for 1.5 X the length of time it took the xylene cyanol to migrate to the bottom of the sequencing gel at constant voltage. The results are summarized in Table 2B. The predicted amino acid composition of the proteins produced from mRNAs having small and middle T antigen donor and acceptor splice sites could be determined for all mutant recombinant plasmids. In the case of mutants at the Pstl site only a single altered T antigen would be produced if the deletion caused a change in the reading frame such that a stop codon was introduced before the proximal splice site for both small and middle T antigen mRNA. Indeed, this was found to be the case for all four plasmids bearing mutations at the Pstl site and the amino acid composition of these altered T antigens is summarized in Table 2B.

C) Mutations at the Aval sites

The DNA of the parental plasmid pPH1-8 contains three Aval sites (Figure 4A). One site is in pBR322 sequences while the two other sites are in polyomavirus sequences. The sites in polyomavirus DNA occurred at nucleotide 657, in the coding sequences for both small and middle T antigens, and at nucleotide 1016, in the coding sequences for middle T antigen (Figure 4B). Insertions were created at these sites by back-filling the 5' projections left by Aval with reverse transcriptase (Table 3A). Two mutant DNAs,

Table 3A. Characterization of DNAs with mutations at the AvaI sites.

DNA source	Parent plasmid	Mode of mutagenesis	Site mutagenized (m.u.)	Site mutagenized (nucleotide)	T antigen affected[a]
pPin2	pPH1-8	reverse transcriptase	82.9	657	sT and mT
pPin67	pPH1-8	reverse transcriptase	89.7	1016	mT

[a] sT, small T antigen; mT, middle T antigen.

Table 3B. Characterization of DNAs with mutations at the AvaI sites.

DNA source	Boundaries of insertions	Number of base pairs inserted	Predicted amino acid composition of proteins produced from mRNAs having small and middle T antigen donor and acceptor slice sites
pPin2	657-658	4 (CCGA)	163 sT[a] + 2 N[b] + 25 n.c.(2)[c]
pPin67	1016-1017	4 (CCGA)	wild type small T antigen 262 mT[d] + 304 LT[e] + 4 p[f]

a
sT, amino acids corresponding to the N-terminal portion of small T antigen before the insertion.

b
N, novel amino acids coded for by the inserted nucleotides.

c
n.c.(2), amino acids from non-coding sequences corresponding to the reading frame used for the C-terminal portion of middle T antigen.

d
mT, amino acids corresponding to the N-terminal portion of middle T antigen before the insertion.

e
LT, amino acids corresponding to those normally found in large T antigen.

f
p, amino acids derived from pBR322 sequences at the polyomavirus/plasmid junction in the recombinant plasmid.

pPin2 and pPin67, which lacked the Ava1 site at nucleotide 657 and 1016 respectively (Figure 6b), were isolated and characterized. When pPin2 and pPin67 DNA were cut with Sst1, fragments which comigrated with those of the parental plasmid pPH1-8 were found (Figure 7b), suggesting that the insertions were small.

In order to determine that the 5' projections left by Ava1 digestion were completely back-filled, and that no additional lesions were suffered during the mutagenesis protocol, the DNA of both pPin2 and pPin67 was sequenced. The DNA of pPin2 was sequenced from the Ban1 site at nucleotide 594 which was radioactively labelled using the Klenow fragment of E. coli DNA polymerase 1. pPin67 DNA was sequenced from the Pvu11 site at nucleotide 1144 which had been labelled by the kinase exchange reaction. The products of the sequencing reactions were electrophoresed through a 10% sequencing gel until the bromophenol blue reached the bottom of the gel in the case of pPin2, or until the xylene cyanol reached the bottom of the gel in the case of pPin67. Both mutants contained the 4 b.p. insertion, CCGA, (Table 3B) which altered the reading frame of the sequences past the insertion. The plasmid pPin2 was mutated in sequences common to small and middle T antigen, and presumably coded for a single altered T antigen species (Table 3B). By contrast, the lesion in pPin67 DNA was in sequences unique to middle T antigen, and as such coded for a wt small T antigen and an altered form of middle T antigen (Table 3B).

D) Mutations at the Sst1 Sites

The recombinant plasmid pPBR2 contains two sites of cleavage for Sst1 (Figure 5A). One of these sites is at nucleotide 569, within the coding sequences for small and middle T antigen; the other maps within coding

sequences for the distal portion of middle T antigen at nucleotide 1373 (Figure 5B). Small deletions were created at these locations by digesting permuted linear pPBR2 DNA molecules generated by Sst1 cleavage with the nuclease S1 (Table 4A). Sst1 left a 4 b.p. 3' projection which served as the substrate for S1 nuclease. Three mutants were isolated which lacked an Sst1 site in polyomavirus sequences (Figure 6c). The mutant pPd19 was deleted at the Sst1 site at nucleotide 569, while both pPd11 and pPd115 bore deletions at the Sst1 site at nucleotide 1373. In each case the deletions were small (approximately 10 b.p.) because cleavage of the mutant DNAs with the restriction endonuclease Pvu11 yielded fragments which comigrated with those of pPBR2 DNA (Figure 7c). In addition to these three mutants, one mutant was isolated by treating Sst1 partially digested pPH1-8 DNA, which has three Sst1 sites in polyomavirus sequences (Figure 4A), with Bal 31 nuclease (Table 4A). This mutant, pPd117, was resistant to Sst1 cleavage at nucleotide position 569 (Figure 6c), thus positioning the mutation in the sequences common to small and middle T antigen (Figure 4B). Digestion with Pvu11 (Figure 7c) revealed that the deletion was small (approximately 15 b.p.).

The DNAs of all four recombinant plasmids with mutations at polyomavirus Sst1 sites were sequenced. pPd19 and pPd117 were sequenced from the Ava1 site at nucleotide 657 while pPd11 and pPd115 were sequenced from the Hinf1 site at nucleotide 1335. The free ends of the molecules to be sequenced were in all cases radioactively labelled using the kinase exchange reaction. The products of the sequencing reactions were electrophoresed on 10% denaturing acrylamide gels until the bromophenol blue reached the bottom of the gel in the case of pPd11 and pPd115, or until the xylene cyanol reached the bottom of the gel in the case of pPd19 and pPd117. Both pPd19 and pPd117 contained deletions which could cause the production of only a single

Table 4A. Characterization of DNAs with mutations at the SstI sites.

DNA source	Parent plasmid	Mode of mutagenesis	Site mutagenized (m.u.)	Site mutagenized (nucleotide)	T antigen affected[a]
pPd11	pPBR2	S1 nuclease	96.5	1373	mT
pPd19	pPBR2	S1 nuclease	81.3	569	sT and mT
pPd115	pPBR2	S1 nuclease	96.5	1373	mT
pPd117	pPH1-8	Bal 31 nuclease	81.3	569	sT and mT

[a] sT, small T antigen; mT, middle T antigen.

Table 4B. Characterization of DNAs with mutations at the SstI sites.

DNA source	Bounderies of deletions (nucleotides)	Number of base pairs deleted	Predicted amino acid composition of proteins produced from mRNAs having small and middle T antigen donor and acceptor splice sites
pPd11	1373-1383	11	wild type small T antigen 379 mT[a] + 59 LT[b] + 14 p[c]
pPd19	570-576	7	133 sT[d] + 3 n.c.(2)[e]
pPd115	1373-1379	7	wild type small T antigen 379 mT + 6 n.c.(1)[f]
pPd117	565-578	14	130 sT + 3 n.c.(3)[g]

a
mT, amino acids corresponding to the N-terminal portion of middle T antigen before the deletion.

b
LT, amino acids corresponding to those normally found in large T antigen.

c
p, amino acids derived from pBR322 sequences at the polyomavirus/plasmid junction in the recombinant plasmid.

d
sT, amino acids corresponding to the N-terminal portion of small T antigen before the deletion.

e
n.c.(2), amino acids from non-coding sequences corresponding to the reading frame used for the C-terminal portion of middle T antigen.

f
n.c.(1), amino acids from non-coding sequences corresponding to the reading frame used for the N-terminal portion of small, middle and large T antigen.

g
n.c.(3), amino acids from non-coding sequences corresponding to the reading frame used for the C-terminal portion of large T antigen.

altered and truncated T antigen (Table 4B). By contrast, both pPd11 and pPd115 retained sequences capable of encoding a wt small T antigen and an altered form of middle T antigen (Table 4B).

E) Mutations at the Pvu11 site

The restriction endonuclease Pvu11 cleaves pPH1-8 DNA at four sites (Figure 4A); one site is located within pBR322 sequences, two sites are located within non-coding sequences in polyomavirus DNA (nucleotides 5128 and 5262), and one site (nucleotide 1144) is located within sequences which encode middle but not small T antigen (Figure 4B). Deletions of various sizes were created about the site at nucleotide 1144 by digesting populations of Pvu11 cleaved permuted circular molecules of pPH1-8 DNA with Bal 31 nuclease (Table 5A). Bal 31 nuclease was capable of cleaving molecules with a single-stranded scission and subsequently progressively deleting the open linear ends. Nine individual mutated DNAs were obtained which had lost the Pvu11 site at nucleotide 1144 (Figure 6a). The size of the deletions were estimated after digestion of mutant recombinant plasmids with Sst1 comparing, the size of the fragments obtained with those generated after cleavage of the parental plasmid with Sst1 (Figure 7a). Three of the mutants (pPd11-4, pPd11-8 and pPd12-5) contained small deletions (less then 45 b.p.), four recombinants (pPd11-10, pPd12-3, pPd12-7 and pPd12-12) contained medium sized deletions (less then 120 b.p.), and two mutated recombinants (pPd11-11 and pPd12-2) sustained large deletions (greater then 350 b.p.).

The exact boundary of each deletion was determined by sequence analysis. All the DNAs were labelled at the Ava1 site at nucleotide 1016 except for the plasmid pPd12-2. The plasmid DNA of pPd12-2 had lost the Ava1 site at

Table 5A. Characterization of DNAs with mutations at the PvuII site.

DNA source	Parent plasmid	Mode of mutagenesis	Site mutagenized (m.u.)	Site mutagenized (nucleotide)	T antigen affected[a]
pPd11-4	pPH1-8	Bal 31 nuclease	92.1	1144	mT
pPd11-8	pPH1-8	Bal 31 nuclease	92.1	1144	mT
pPd11-10	pPH1-8	Bal 31 nuclease	92.1	1144	mT
pPd11-11	pPH1-8	Bal 31 nuclease	92.1	1144	mT
pPd12-2	pPH1-8	Bal 31 nuclease	92.1	1144	sT and mT
pPd12-3	pPH1-8	Bal 31 nuclease	92.1	1144	mT
pPd12-5	pPH1-8	Bal 31 nuclease	92.1	1144	mT
pPd12-7	pPH1-8	Bal 31 nuclease	92.1	1144	mT
pPd12-12	pPH1-8	Bal 31 nuclease	92.1	1144	mT

[a] sT, small T antigen; mT, middle T antigen.

Table 5B. Characterization of DNAs with mutations at the PvuII site.

DNA source	Boundaries of deletions (nucleotides)	Number of base pairs deleted	Predicted amino acid compostion of proteins produced from mRNAs having small and middle T antigen donor and acceptor splice sites
pPd11-4	1144-1164	21	303 N-mT[a] + 111 C-mT[b]
pPd11-8	1147-1150	4	304 N-mT + 39 n.c.(1)[c]
pPd11-10	1137-1162	26	300 N-mT + 1 N[d] + 165 LT[e] + 4 p[f]
pPd11-11	1147-1592	446	304 N-mT + 26 n.c.(1)
pPd12-2	804-1258	455	194 sT[g] + 80 n.c.(1)
pPd12-3	1110-1186	77	292 N-mT + 157 LT + 4 p
pPd12-5	1147-1157	11	304 N-mT + 167 LT + 4 p
pPd12-7	1087-1203	117	282 N-mT + 98 C-mT
pPd12-12	1106-1190	86	290 N-mt + 1 N + 155 LT + 4 p

a
N-mT, amino acids corresponding to the N-terminal portion of middle T antigen before the deletion.

b
C-mT, amino acids corresponding to the C-terminal portion of middle T antigen after the deletion.

c
n.c.(1), amino acids from non-coding sequences corresponding to the reading frame used for the N-terminal portion of small, middle and large T antigen.

d
N, novel amino acids formed at the junction of the deletion.

e
LT, amino acids corresponding to those normally found in large T antigen.

f
p, amino acids derived from pBR322 sequences at the polyomavirus/plasmid junction in the recombinant plasmid.

g
sT, amino acids corresponding to the N-terminal portion of small T antigen before the deletion.

nucleotide 1016. However, the rearrangement of sequences in the deletion fortuitously recreated a new AvaI site at the junction of the deletion and this site was used to determine the sequence at both sides of the deletion. The free ends of the molecules were labelled by the kinase exchange reaction. The products of the sequencing reactions were generally run on 10% sequencing gels until the xylene cyanol reached the bottom of the gel except for pPdl2-7 DNA. In this case the gel was electrophoresed until the bromophenol blue reached the bottom of the gel. The sequencing reactions of pPdl2-2 DNA were run on a 20% sequencing gel until the bromophenol blue had migrated 1/3 the length of the gel. The results of the sequence analysis are summarized in Table 5B. Eight of the mutated recombinant molecules are capable of encoding a wt small T antigen. Only pPdl2-2 contained a deletion which removed the termination codon for small T antigen, and therefore was incapable of encoding wt small T antigen. Seven of the mutated recombinant plasmids (pPdl1-8, pPdl1-10, pPdl1-11, pPdl2-3, pPdl2-5 and pPdl2-12) contained deletions which altered the reading frame for middle T antigen. Out-of-frame deletions in this area of the genome would result in the synthesis of hybrid proteins containing middle T antigen coding sequences up to the 5' boundary of the deletion, whereas the remainder of the protein would be derived from sequences which normally do not code for proteins (the small T antigen reading frame) or from sequences encoding large T antigen (Table 5B). Two mutated plasmid DNAs (pPdl1-4 and pPdl2-7) contained in frame deletions of middle T antigen (Table 5B).

II. Transforming Activity of Mutant, Recombinant Plasmid DNAs

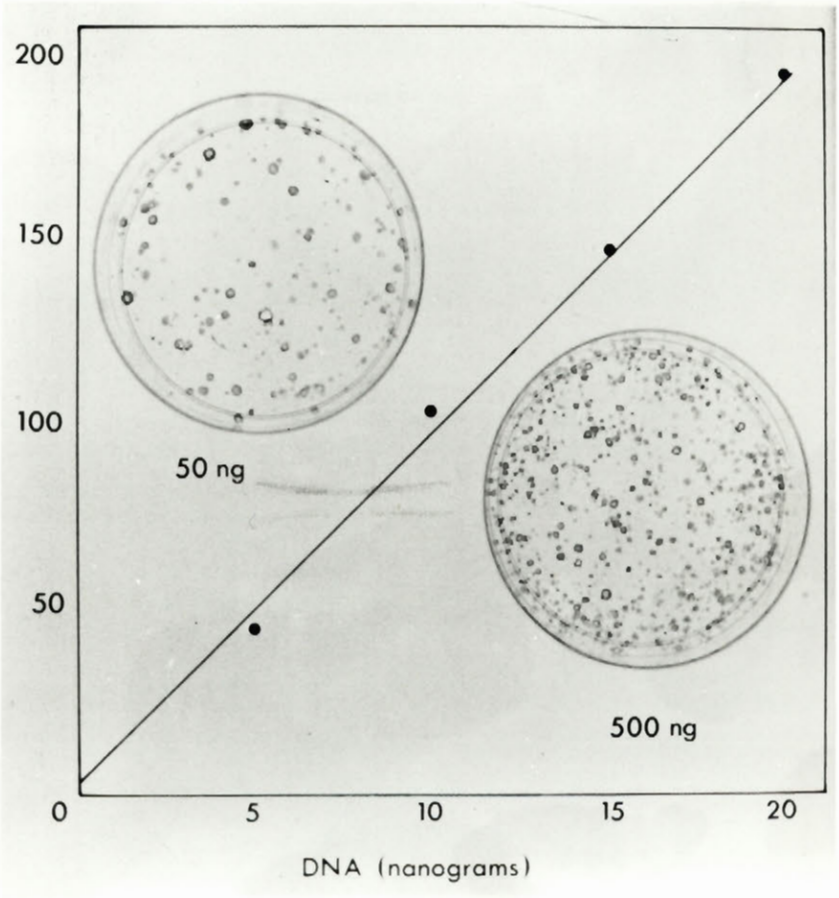
A) The Transformation Assay

The transformation assay was optimized by transfecting Rat-1 cells at a low cell density (3.5 X 10^5 cells/100 mm plate) with small quantities of DNA (5-100 ng/100 mm plate). Under these conditions the transformation assay was linear and directly proportional to DNA concentration between 5 and 20 ng (Figure 8). Within this range of DNA concentrations, the specific transforming activity of the DNA remained constant. Foci were visible and could be readily scored 10 to 14 days post-transfection (Figure 8). As the DNA concentration was increased beyond 50 ng/3.5 X 10^5 cells, the total number of foci increased until a plateau was reached at 500 to 1,000 ng of DNA/3.5 X 10^5 cells. However, within this range of DNA concentrations the specific transforming activity of the DNA continuously declined and finally reached a level of about 500-1,000 foci/ug of DNA/3.5 X 10^5 cells inoculated. Within an experiment, the precision of the transformation assay was very good. However, the specific transforming activity of wt or mutant DNA could vary over a threefold range from one experiment to the next. For this reason, comparisons between mutant and wt DNA were made in a single experiment over a range of DNA concentrations.

In the original screening of the mutant recombinant plasmids, each DNA sample was tested in triplicate at 32.5°C and 38.5°C on at least two separate occasions by transfecting with 1,000 ng of supercoiled, recombinant plasmid DNA per dish (3.5 X 10^5 cells). Foci were scored 2 weeks post-transfection for positive samples, whereas negative samples were incubated up to 6 weeks before the cells were fixed and stained. The transforming activity of positive samples was measured against wt DNA over a range of DNA concentrations and are discussed in a later section (see Table 9).

B) Transforming Activity

Figure 8: Transfection with recombinant plasmid DNA. The graph shows the linear relationship between the amount of transforming DNA and the number of foci obtained per dish. Also shown are plates whose foci have been stained to demonstrate their appearance. The number below the plates indicate the amount of DNA used to transfect the cells on that plate.


200
150
100
50
0
5
10
15
20
50 ng
500 ng
DNA (nanograms)

The transforming activity of mutant, recombinant plasmid DNAs was determined and the results are summarized in Table 6. Mutations which affected the coding sequences for small and middle T antigens, regardless of their location (nucleotides 484, 567, 657) or nature (insertion or deletions), completely abolished the transforming activity of the DNA. The mutant pPdl2-2, which contained a large deletion at the Pvu11 site at nucleotide 1144, was completely unable to encode small T antigen because the acceptor splice site for small T antigen mRNA had been removed. This recombinant plasmid, whose deletion also affected the coding sequences for middle T antigen, was completely inactive in the transformation assay.

The majority (7/9) of the DNAs which carried mutations affecting the coding sequences of middle T antigen but not small T antigen were also incapable of transforming Rat-1 cells. These include mutations around nucleotide 1016, 1144 and 1373. By contrast, the two mutants pPdl1-4 and pPdl2-7, both of which contained deletions around the Pvu11 site at nucleotide 1144, transformed Rat-1 cells. By measuring the transforming activity of these DNAs over a range of DNA concentrations and comparing them with pPH1-8 DNA (data presented elsewhere, see Table 9), I determined that pPdl1-4 DNA transformed cells at close to wt frequencies whereas pPdl2-7 DNA was slightly impaired in its ability to transform cells.

Table 6. Transforming activity of various DNAs with mutations in sequences encoding polyomavirus T antigens.

DNA source	T antigen affected[a]	Avg. no. of foci/ug of DNA[b]
pPd13	sT + mT	<1.0
pPd14	sT + mT	<1.0
pPd110	sT + mT	<1.0
pPd111	sT + mT	<1.0
pPd19	sT + mT	<1.0
pPd117	sT + mT	<1.0
pPd12-2	sT + mT	<1.0
pPin2	sT + mT	<1.0
pPin67	mT	<1.0
pPd11-4	mT	~3,000
pPd11-8	mT	<1.0
pPd11-10	mT	<1.0
pPd11-11	mT	<1.0
pPd12-3	mT	<1.0
pPd12-5	mT	<1.0
pPd12-7	mT	~300-900
pPd12-12	mT	<1.0
pPH1-8	-	~3,000-6,000
pPBR2	-	~3,000-6,000

a
sT, small T antigen; mT, middle T antigen.

b
3.5×10^5 cells on 100 mm plastic dishes were transfected with 1 ug of DNA/plate and the foci counted 14 post-transfection. The results are the average number of foci/plate from three plates per experiment. The range of values represent the extremes from two separate experiments.

CHAPTER 2: THE ROLE OF THE CARBOXY-TERMINAL DOMAIN OF MIDDLE T ANTIGEN IN TRANSFORMATION

I. Sequences at the Carboxy Terminus of Middle T Antigen are Required for Transformation

A) Introduction

The recombinant mutant plasmid pPdl15 contained a 7 b.p. deletion starting at nucleotide 1373. The net affect of this out-of-frame deletion would be the production of a hybrid protein containing middle T antigen coding sequences up to amino acid 379, whereas the remaining 6 amino acids of the protein (ILE-GLN-CYS-ASN-ALA-THR) would be derived from sequences which normally do not code for proteins (the small T antigen reading frame). The hybrid protein should lack the last 42 amino acids of middle T antigen. pPdl15 DNA was completely inactive in the transformation assay. These results suggest that the C-terminus of middle T antigen plays a significant role in the function of the protein. The deletion in pPdl15 removed, among other sequences, the cluster of hydrophobic amino acids extending from amino acid residues 394-415, as well as the flanking basic amino acids. In order to assess the role of these hydrophobic and basic amino acids in the transforming function of middle T antigen, mutant recombinant plasmids bearing smaller deletions in the sequences which code for the C-terminus of middle T antigen were characterized.

B) Mutations at the EcoR1 site

The recombinant plasmid pPBR2 contains a single cleavage site for EcoR1 (Figure 5A) which is downstream of the termination codon for middle T antigen (Figure 5B). Two mutants, pPd16 and pPd190, containing deletions extending into middle T antigen coding sequences, were produced by Bal 31 nuclease cleavage of EcoR1 linearized pPBR2 (these mutants were isolated by William Muller). Although both mutants contained small deletions in middle T antigen coding sequences, only one of the mutants, pPd16, was capable of transforming cells in culture. By contrast, pPd190 was completely inactive in the transformation assay.

In order to assess the difference between these two mutants, the DNA of both recombinant plasmids was sequenced. Both DNAs were sequenced from the Aval1 site at nucleotide 1412, which had been radioactively labelled using the kinase exchange reaction. The Aval1 fragment containing the deletion was isolated and the two DNA strands were separated by gel electrophoresis. The products of the sequencing reactions were then run on 10% sequencing gels until the bromophenol blue reached the bottom of the gel. Sequence analysis revealed that pPd16 DNA contained a 126 b.p. deletion which encompassed both polyomavirus sequences as well as plasmid sequences coding for the Colicin E1 immunity gene product. The deletion began in polyomavirus sequences at b.p. 1489, removed all subsequent polyomavirus sequences up to the EcoR1 site at b.p. 1560, and in addition removed 54 b.p. from the 3' end of the Colicin E1 gene. This deletion removed the DNA which coded for the last three amino acids and the translational stop codon at the C-terminus of middle T antigen. By juxtaposing the remaining polyomavirus and plasmid sequences it was possible to predict the amino acid sequence for a fusion middle T antigen protein. This protein contained three new amino acids (ASP-LEU-ILE) before encountering a translational stop codon within plasmid DNA (Figure 9).

Figure 9: A partial amino acid sequence of the C-terminus of polyomavirus middle T antigen. The long and short arrows indicate the amino acids deleted in fusion middle T antigens encoded by pPd190 and pPd16 DNA respectively. Also shown are the new amino acids (bold face type) coded for by either pPd16 or pPd190 DNA, before a translational stop codon is encountered.

WILD TYPE
NH2 ..... ALA-HIS-SER-MET-GLN-ARG-HIS-LEU-ARG-ARG-LEU-GLY-ARG
THR-LEU-LEU-LEU-VAL-THR-PHE-LEU-ALA-ALA-LEU-LEU-GLY-ILE-CYS
LEU-MET-LEU-PHE-ILE-LEU-ILE-LYS-ARG-SER-ARG-HIS-PHE-COOH
pPdl 6NH2 ..... ASP-LEU-ILE-COOH
pPdl 90NH2 ..... GLN-MET-GLY-LEU-LEU-ALA-PRO-ASN-LYS-VAL-ASP-TYR-ILE-ALA-COOH

pPdl90 contained a 197 b.p. deletion. In polyomavirus DNA, 99 b.p. were missing starting with b.p. 1462, thereby removing sequences which coded for the last 12 amino acids of middle T antigen. In addition, 98 b.p. corresponding to the 3' end of the Colicin gene were deleted. The resulting DNA sequence could encode a fusion middle T antigen protein in which the last 12 amino acids of authentic middle T antigen were replaced by 14 novel amino acids (GLN-MET-GLY-LEU-LEU-GLY-PRO-ASN-LYS-VAL-ASP-TYR-ILE-ALA) encoded by plasmid sequences before encountering a translational stop codon (Figure 9).

II. Characterization of Cell Lines Transfected with pPdl6 DNA

A) The Transforming Activity of pPdl6 DNA

While pPdl6 DNA was unable to encode wt middle T antigen, the plasmid DNA retained the capacity to transform Rat-1 cells. In order to determine the specific transforming activity of the mutant DNA, pPdl6 and pPBR2 DNA were compared in two separate experiments over a range of DNA concentration (10 and 50 ng of DNA/3.5 X 10^5 cells). The results are summarized in Table 7. pPdl6 DNA was capable of transforming Rat-1 cells at approximately 50-70% of wt frequencies. Cell lines were established after transfection with pPdl6 DNA and two cell lines, dl6:2A and dl6:4B, were extensively characterized. These cells were shown to be transformed by a number of criteria including the capacity to form foci, the capacity to form colonies in soft agar, and the capacity to form tumors when injected into young rats (Table 8).

B) Viral Sequences in Cells Transformed by pPdl6 DNA

The integrated polyomavirus sequences found in cells transformed by

Table 7. Titration of the transforming activity of pPd16 DNA.

DNA source	Amount of DNA/plate	No. of foci per plate	Av. no. of foci per ug of DNA
Experiment A			
pPBR2	10 ng	55, 62, 64	6,000 (100%)
pPd16	10 ng	39, 49, 41	4,300 (71%)
pPBR2	50 ng	150, 1321 147	2,860 (100%)
pPd16	50 ng	82, 78, 77	1,580 (55%)
Experiment B			
pPBR2	10 ng	53, 49, 56	5,260 (100%)
pPd16	10 ng	26, 23, 107	2,600 (49%)
pPBR2	50 ng	112, 123, 107	2,280 (100%)
pPd16	50 ng	79, 71, 82	1,550 (68%)

Table 8. Properties conferred on Rat-1 cells by the novel T antigen produced in cells transformed by pPd16 DNA.

Cell line	Growth in agar[a]	Tumorigenicity[b]
Rat-1	-	0/6
BR2(9)[c]	+	3/4
DL6:2B	+	4/4
DL6:4B	+	4/4

a
All lines are scored + if $\geq$60% of cells formed colonies $\geq$16 cells in size when suspended in a semi-solid medium of 0.33% agarose and allowed to grow for 1 week.

b
10^6 cells were injected subcutaneously into 3 week old Fisher rats and visible tumors were scored 21 days later.

c
A clone of Rat-1 cells transformed by pPBR2 served as the positive control. This cell line synthesized wild type middle and small T antigen (data not presented).

pPdl6 were probed by Southern blot analysis. Cellular DNA isolated from both dl6:2A and dl6:4B was hydrolyzed with Bgl11, a restriction endonuclease which does not cleave pPdl6 DNA (a no-cut enzyme). By using a no-cut enzyme the number of plasmid insertions in cellular DNA could be determined. Both dl6:2A and dl6:4B cellular DNA contained only a single insertion of pPdl6 DNA (Figure 10). To ensure that the integrated sequences represented pPdl6 DNA, and not contaminating wt DNA, cellular DNA from dl6:2A and dl6:4B were doubly-digested with either BstE11/Sst1 or Pst1/EcoR1. In all cases diagnostic fragments revealed that the integrated sequences in dl6:2A and dl6:4B cell lines were derived from pPdl6 DNA and not wt DNA (Figure 10).

C) T Antigen Analysis

The T antigens produced in the cell lines dl6:2A and dl6:4B were identified by immunoprecipitation of ^{35}S-labelled proteins (Figure 11). Polypeptides which comigrated with authentic middle and small T antigen were identified in both dl6:2A and dl6:4B cell lines. However, immunoprecipitates of cell line dl6:2A consistently revealed low levels of middle and small T antigen. The fusion proteins encoded by pPdl6 DNA removed three amino acids from middle T antigen and these were replaced by three new amino acids encoded by plasmid sequences (Figure 9). Therefore, the fusion middle T antigen not surpisingly comigrated with authentic middle T antigen.

Middle T antigen was also detected in dl6:2A and dl6:4B cells after performing in vitro kinase assays. A reactive species, which was indistinguishable from wt middle T antigen, was identified (Figure 12). Although the dl6:2A cell line appeared to make very little middle T antigen (Figure 11), extracts from these cells were as reactive in the kinase assay as extracts from cell lines with normal amounts of middle T antigen.

Figure 10: Detection of DNA fragments which contain polyomavirus or pMK16.1 DNA by Southern blot analysis followed by autoradiography. (A) Markers of pPBR2 (lane M1) and pPd16 (lane M2) DNA (100 pg per well each) were double digested with BstE11 and Sst1. BstE11 cleaves both pPBR2 and pPd16 only once, in the E1 immunity gene (see Figure 5B). The left-most top arrow indicates the position of the BstE11/Sst1 fragment, extending from polyomavirus sequences past the EcoR1 site and into plasmid sequences, in the parent plasmid pPBR2. The top double-headed arrow indicates this same fragment in pPd16 DNA, which migrates more quickly during electrophoresis because of the deletion in pPd16 DNA. The bottom arrow in both cases indicates the position of the Sst1 fragment derived from polyomavirus sequences (see Figure 5A). The cellular DNA (10 ug per well) of two cell lines transformed by pPd16, DL6:2A (lane A) and DL6:4B (lane B) was also double-digested with BstE11 and Sst1. As expected, both cell lines contained the small (bottom arrow) Sst1 fragment derived from polyomavirus sequences which code for middle T antigen. One cell line (DL6:2A, top double-headed arrow) contained a fragment which comigrated with the BstE11/Sst1 fragment of pPd16 DNA. No fragment corresponding to the BstE11/Sst1 double-digest product of either pPBR2 or pPd16 DNA was found in DL6:4B cellular DNA. This probably indicates the loss of the plasmid BstE11 site during integration. (B) Markers of pPBR2 DNA (10 pg per well) represent either uncut DNA (lane M1) or EcoR1/Pst1

double digested DNA (lane M2). DL6:2A (lane A) and DL6:4B (lane B) cellular DNA (10 ug per well) were also doubly-digested with EcoR1/Pst1. The arrow indicates the position of the EcoR1/Pst1 fragment extending from polyomavirus sequences (which code for middle T antigen) to the EcoR1 site which links polyomavirus and plasmid sequences (see Figure 5A). This band is not found in either lane A or B even on autoradiograms which have been exposed for long periods. Because the Pst1 site is probably present in the integrated sequences, this result indicates that the EcoR1 site is absent from the integrated DNA, providing further evidence that the integrated sequences correspond to pPd16 DNA. (C) Markers of pPBR2 DNA (100 pg per well) represent either uncut (lane M1) or linear (lane M2) DNA. DL6:2A (lane A) and DL6:4B (lane B) cellular DNA (10 ug per well) were cleaved with Bgl11, a no-cut restriction endonuclease for pPd16 DNA. The arrows indicate the unique insertion of polyomavirus DNA in both DL6:2A and DL6:4B cellular DNA. The autoradiograms of the markers in panels A and C represent a lower exposure then the adjacent cellular DNA.

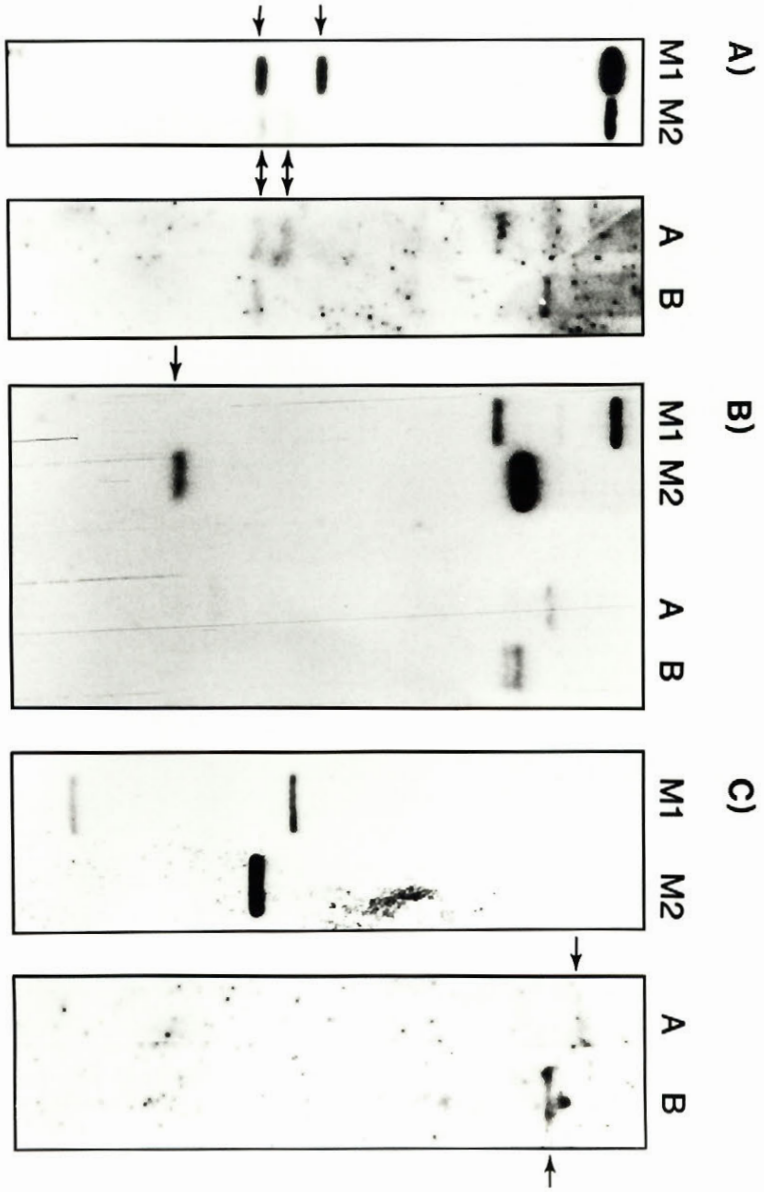

A)
M1 M2
A B
B)
M1 M2
A B
C)
M1 M2
A B

Figure 11: Autoradiogram of a 10.0% SDS-polyacrylamide gel of proteins from control cells (Rat-1), or wt (T1A1) and mutant (DL6:2A and DL6:4B) transformed cells, labelled with [^{35}S]-methionine and immunoprecipitated with either normal (N) or antitumor (T) antiserum. The location of middle T (mT) and small T (sT) antigens is shown. The right hand panel is a longer exposure of proteins immunoprecipitated in the DL6:2A cell line.

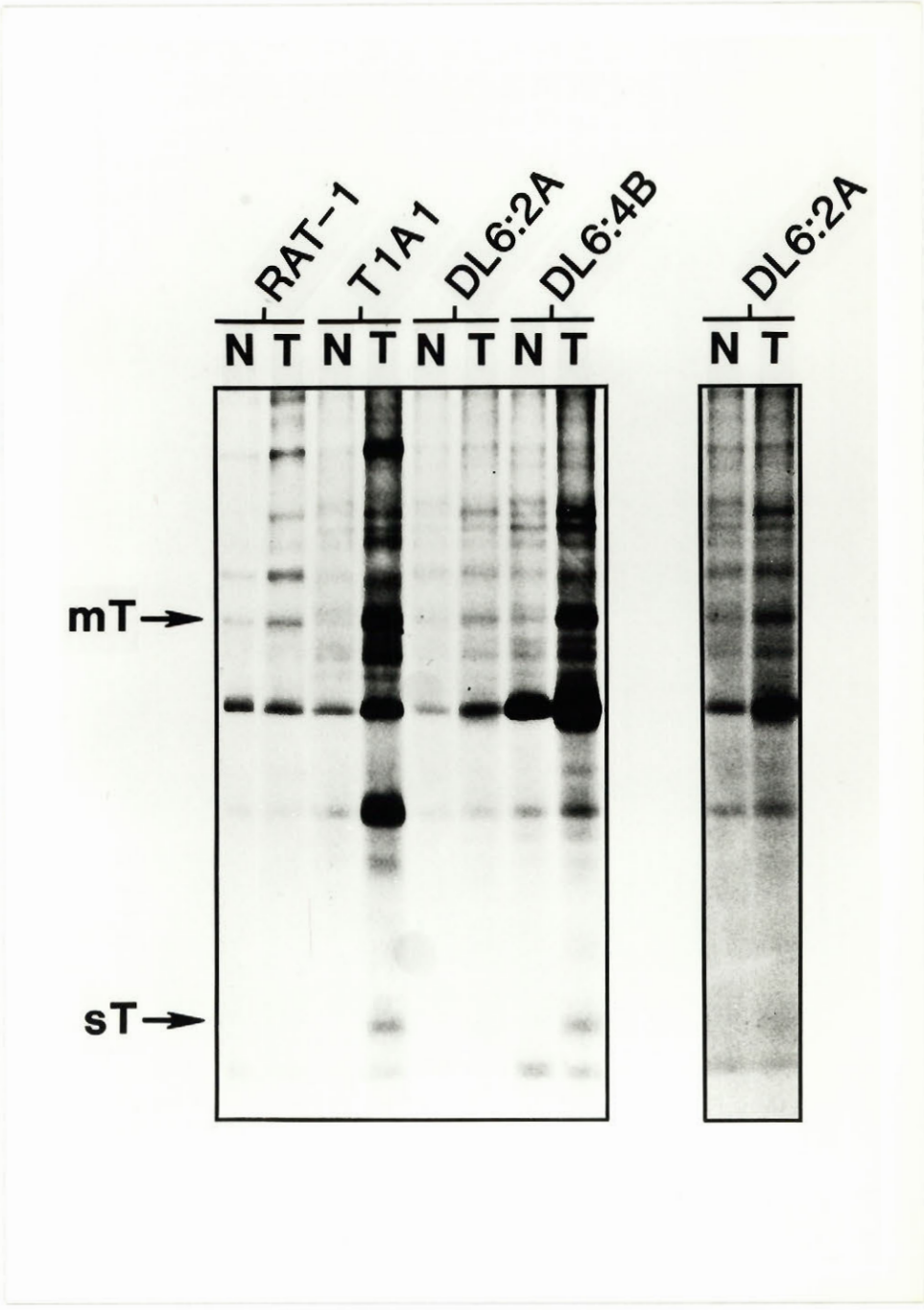

RAT-1
T1A1
DL6:2A
DL6:4B
DL6:2A
N T N T N T N T
N T
mT
sT

Figure 12: Autoradiogram of a 10.0% SDS-polyacrylamide gel showing *in vitro* tyrosine phosphorylation of middle T antigen (mT) in wt (T1A1), and mutant (DL6:2A and DL6:4B) transformed cell lines. No ^{32}P-labelled proteins are seen in the Rat-1 control.


Rat-1
T1A1
DL 6:2A
DL 6:4B
mT

CHAPTER 3: THE ROLE OF THE MAJOR SITE OF IN VITRO TYROSINE PHOSPHORYLATION OF MIDDLE T ANTIGEN IN TRANSFORMATION

I. Deleted Recombinant Plasmids

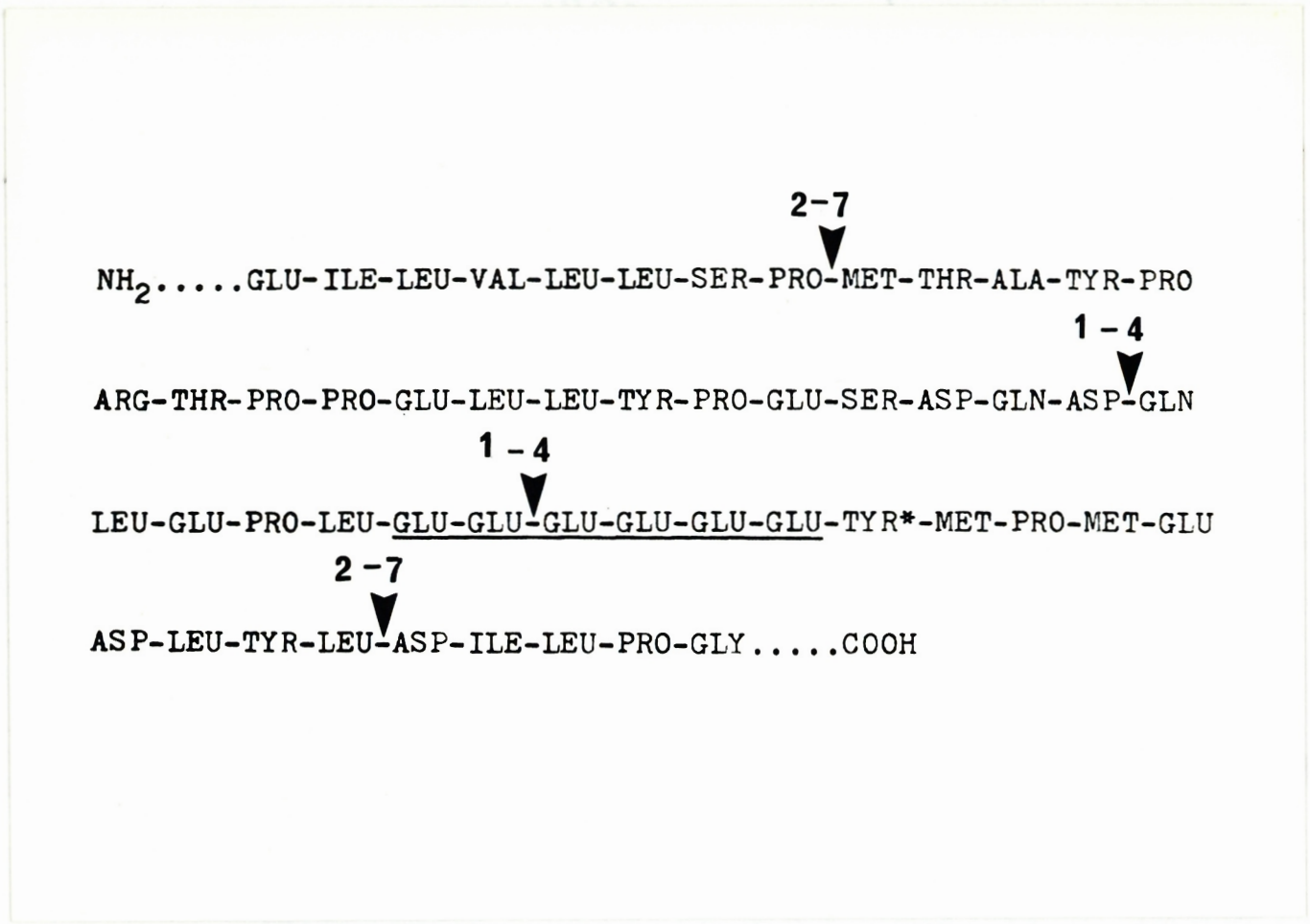
A) Introduction

A total of nine recombinant plasmids bearing deletions in and around the Pvu11 site at nucleotide 1144 were recovered after Bal 31 mutagenesis of Pvu11 permuted circles of pPH1-8 DNA. Of these nine recombinants, only two, pPd11-4 and pPd12-7, retained the capacity to transform Rat-1 cells (Table 6). Both pPd11-4 and pPd12-7 DNA sustained in phase deletions of polyomavirus middle T antigen coding sequences. pPd11-4 DNA was deleted of 21 b.p. by comparison to wt DNA. The middle T antigen encoded by this DNA should lack amino acids 304-310 inclusively (Figure 13). These amino acids include the first two of six glutamic acid residues which precede the major site of *in vitro* phosphorylation, tyrosine 315. pPd12-7 DNA contained a 117 b.p. deletion. The middle T antigen encoded by pPd12-7 DNA should be deleted of amino acids 285-323 inclusively (Figure 13). This large deletion should result in the loss of 39 amino acids from middle T antigen including all six glutamic acid residues as well as tyrosine 315 and tyrosine 322. Both pPd11-4 and pPd12-7 were characterized further as these mutants define sequences in middle T antigen which were dispensable for tranformation.

B) The Transforming Activity of pPd11-4 and pPd12-7 DNA

Both pPd11-4 and pPd12-7 DNA were compared in the transformation assay

Figure 13: A partial amino acid sequence the polyomavirus middle T antigen extending from amino acids 277 to 323. Shown by the vertical arrows are the amino acids predicted to be deleted from the middle T antigens encoded by the mutant recombinant plasmids pPdl1-4 and pPdl2-7. Tyrosine 315 is indicated by the asterisk.


2-7
NH2.....GLU-ILE-LEU-VAL-LEU-LEU-SER-PRO-MET-THR-ALA-TYR-PRO
1-4
ARG-THR-PRO-PRO-GLU-LEU-LEU-TYR-PRO-GLU-SER-ASP-GLN-ASP-GLN
1-4
LEU-GLU-PRO-LEU-GLU-GLU-GLU-GLU-GLU-GLU-TYR*-MET-PRO-MET-GLU
2-7
ASP-LEU-TYR-LEU-ASP-ILE-LEU-PRO-GLY.....COOH

to the DNA of the parental plasmid pPH1-8, at different DNA concentrations (10 and 50 ng/3.5 X 10^5 cells), in order to carefully determine the specific transforming activities of these DNAs. The results from two different experiments are summarized in Table 9. pPdl1-4 DNA transformed cells at a frequency which was comparable to wt pPH1-8 DNA (65-75% of wt) whereas pPdl2-7 DNA transformed Rat-1 cells at an average frequency between 20-35% of wt DNA.

To determine whether cells transformed by these mutant DNAs exhibited the full complement of transformed cell characteristics, Rat-1 cell lines transformed by either pPdl1-4 DNA (dl1-4:1A and dl1-4:3A) or pPdl2-7 DNA (dl2-7:5B and dl2-7:6A) were isolated. All four cell lines grew to an indefinite saturation density (data not shown) and were capable of forming colonies in semi-solid medium to the same extent (Table 10). Moreover, all four experimental cell lines induced tumors when injected subcutaneously into three week old Fisher rats, and did so at frequencies and latencies comparable to Rat-1 cells transformed with a wt control plasmid DNA (Table 10). Therefore, the phenotype of cells transformed by pPdl1-4 DNA, pPdl2-7 DNA, or wt DNA were not significantly different when compared by these assays.

C) Viral Sequences in Cells Transformed by pPdl1-4 or pPdl2-7 DNA

To determine the number of sites at which plasmid DNA had been integrated into the cellular sequence of the dl1-4:1A, dl1-4:3A, dl2-7:5B and dl2-7:6A cell lines, cellular DNA from all four cell lines was hydrolyzed using the no-cut enzyme Bgl11. The results indicated that each of these independent cell lines contained only a single insertion of the transforming plasmid DNA (Figure 14).

Table 9. Titration of the transforming activity of pPd11-4 and pPd12-7 DNA.

DNA source	Amount of DNA/plate	No. of foci per plate	Av. no. of foci per ug of DNA
Experiment A			
pPH1-8	10 ng	25, 23, 22	2,300 (100%)
pPd11-4	10 ng	16, 17, 15	1,600 (69%)
pPd12-7	10 ng	8, 9, 8	850 (36%)
pPH1-8	50 ng	68, 63, 64	1,300 (100%)
pPd11-4	50 ng	64, 54, 57	1,160 (89%)
pPd12-7	50 ng	36, 30, 30	640 (49%)
Experiment B			
pPH1-8	10 ng	36, 40, 42	3,900 (100%)
pPd11-4	10 ng	25, 27, 23	2,500 (64%)
pPd12-7	10 ng	6, 6, 9	700 (18%)
pPH1-8	50 ng	95, 105, 97	1,980 (100%)
pPd11-4	50 ng	75, 69, 81	1,500 (76%)
pPd12-7	50 ng	29, 18, 17	430 (21%)

Table 10. Properties conferred on Rat-1 cells by novel middle T antigens encoded by pPd11-4 and pPd12-7 DNA.

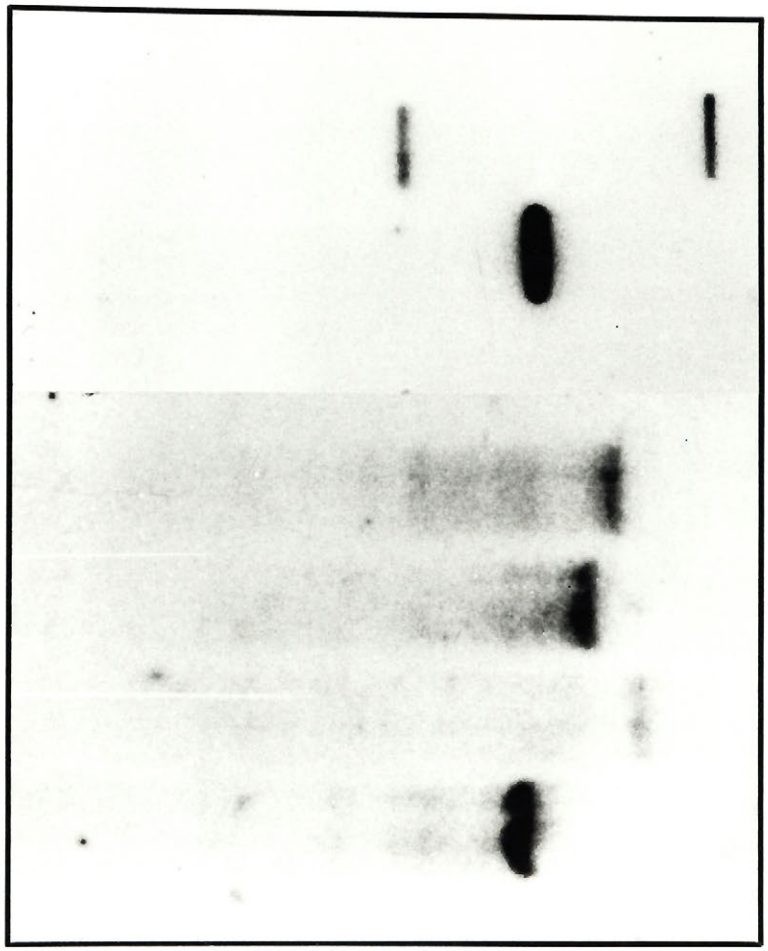
Cell line	Growth in agar[a]	Tumorigenicity[b]
Rat-1	-	0/6
BR2(9)[c]	+	3/4
DL1-4:1A	+	3/4
DL1-4:3A	+	4/4
DL2-7:5B	+	3/4
DL2-7:6A	+	3/4

[a] Cell lines were scored + if ≥60% of cells formed colonies ≥16 cell in size when suspended in a semi-solid medium of 0.33% agarose and allowed to grow for 1 week.

[b] 10^6 cells were injected subcutaneously into 3 week old Fisher rats and visible tumors were scored 21 days later.

[c] A clone of Rat-1 cells transformed by pPBR2 DNA served as the positive control. This cell line synthesized wild type middle and small T antigens (data not presented).

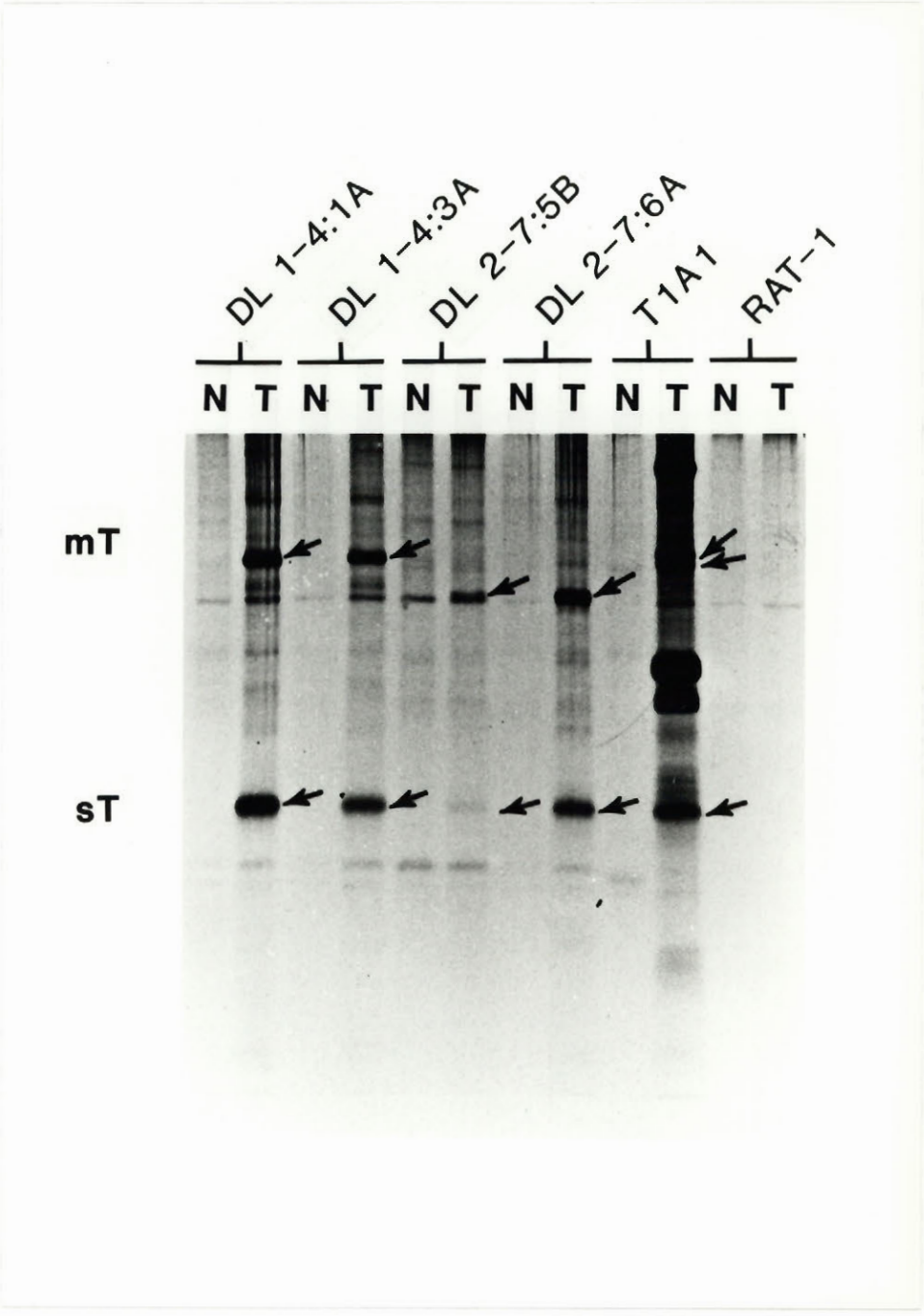
Figure 14: Detection of DNA fragments which contain polyomavirus or pBR322 DNA by Southern blot analysis followed by autoradiography. Markers (100 pg per well) represent either uncut (lane M1) or linear (lane M2) pPH1-8 DNA. Cellular DNA, from the transformed cell lines DL1-4:1A (lane A), DL1-4:3A (lane B), DL2-7:5B (lane C) and DL2-7:6A (lane D), was digested with Bgl11, which is a no-cut enzyme for pPH1-8 DNA. The presence of a single band in lanes A, B, C, and D indicates that each cell line harbors a single insertion of recombinant plasmid DNA. The autoradiogram of the markers is a lower exposure than the adjacent cellular DNA.


M1 M2 A B C D

D) T Antigen Analysis

The T antigens within cell lines transformed with the pPdl1-4 and pPdl2-7 mutant DNAs were immunoprecipitated using antitumor serum and compared to the T antigens synthesized by cell transformed with a wt polyomavirus genome (T1A1). The middle T antigen expressed by cells transformed with pPdl1-4 DNA (dl1-4:1A and dl1-4:3A) was slightly smaller than the wt middle T antigen synthesized by the T1A1 cell line (Figure 15). The relative molecular weight of the pPdl1-4 encoded middle T antigen was estimated to be 55K and that of the wt middle T antigen was estimated to be 56K. The measured molecular weight of the truncated pPdl1-4 encoded middle T antigen was that expected of a 7 amino acid deletion. The small T antigens expressed by these same cell lines were the same size as the wt small T antigen synthesized by the T1A1 cell line (Figure 15). The middle T antigens expressed by cells transformed with pPdl2-7 DNA (dl2-7:5B and dl2-7:6A) were much smaller then that encoded by the T1A1 cell line, and the measured molecular weight of the Pdl2-7 encoded middle T antigen (47K) was much smaller than expected of a 39 amino acid deletion (Figure 15). The latter observation is consistent with reports of other investigators who have previously shown that the removal of acidic amino acids from middle T antigen has a much greater than expected effect on the mobility of the protein in SDS-polyacrylamide gels (Griffin and Maddock, 1979; Ding et al., 1982). As expected, the small T antigen encoded by the pPdl2-7 genome in the two cell lines examined were the same size as that encoded by the wt genome in the T1A1 cell line (Figure 15). The amount of middle T antigen synthesized by the various transformed cell lines were not significantly different. Only the dl2-7:5B cell line yielded consistently lower amounts of middle and small

Figure 15: Autoradiogram of a 12.5% SDS-polyacrylamide gel showing the location of ^{35}S-labelled middle (mT) and small (sT) T antigens immunoprecipitated with antitumor serum (T) from wt (T1A1) and mutant (DL1-4:1A, DL1-4:3A, DL2-7:5B, DL2-7:6A) transformed cell lines. No T antigens are observed in Rat-1 cells immunoprecipitated with T serum. Tracks to the left of each T lane show similar immunoprecipitation results using normal antiserum (N).


DL 1-4:1A
DL 1-4:3A
DL 2-7:5B
DL 2-7:6A
T1A1
RAT-1
N T N T N T N T N T N T
mT
sT

T antigen. Although two forms of middle T antigen were observed in lysates prepared from the T1A1 cell line, two closely migrating forms of middle T antigen were not found in any of the cell lines transformed with mutant DNA.

The altered middle T antigens encoded by pPdl1-4 and pPdl2-7 DNA were tested for their ability to act as subtrates for the middle T antigen associated tyrosine kinase. A 56K molecular weight protein was phosphorylated in immunoprecipitates prepared from extracts of the T1A1 cell line (Figure 16) whereas no proteins were labelled when extracts from untransformed Rat-1 cells were reacted under identical conditions. Reaction of extracts from the various cells lines with normal rat serum did not lead to the phosphorylation of any proteins (data not shown). The mutant middle T antigens encoded by pPdl1-4 (55K) and pPdl2-7 (47K) were labelled in the in vitro kinase assay (Figure 16). However, the pPdl2-7 encoded middle T antigen was phosphorylated to a much lesser extent than either the wt middle T antigen or the mutant pPdl1-4 encoded protein (Figure 16). Incubation of SDS-polyacrylamide gels containing the ^{32}P phosphate-labelled proteins in alkali (1.0 M NaOH, 1 hr at 55^{o}C) did not significantly reduce the intensity of the labelled 56K (T1A1), 55K (dl1-4:1A and dl1-4:3A), and 47K (dl2-7:5B and dl2-7:6A) molecular weight T antigens (data not shown). By contrast, ^{32}P phosphate was completely removed from the less intensely labelled proteins shown in Figure 16.

To confirm that the 47K protein labelled in the in vitro kinase assay was a mutant form of middle T antigen encoded by pPdl2-7 DNA, and to map the site(s) of tyrosine phosphorylation of this 47K protein, the in vitro phosphorylated 47K protein and the 56K middle T antigen encoded by the T1A1 cell line were compared by partial proteolysis. Partial digestion of the 47K middle T antigen by S. aureus V8 protease yielded three peptides (Figure 17). These three peptides had the same mobility as three

Figure 16: Autoradiogram of a 10.0% SDS-polyacrylamide gel showing proteins which become phosphorylated *in vitro* in the presence of [γ-^{32}P]ATP in T antigen immunoprecipitates of polyomavirus wt (T1A1) and mutant (DL1-4:1A, DL1-4:3A, DL2-7:5B, DL2-7:6A) transformed cell lines. The position of the 56K middle T antigen in T1A1, the 55K truncated middle T antigen in DL1-4:1A and DL1-4:3A, and the 47K middle T antigen in DL2-7:5B and DL2-7:6A cell lines is indicated. No ^{32}P-labelled proteins were observed in immunoprecipitates of the Rat-1 cell line.

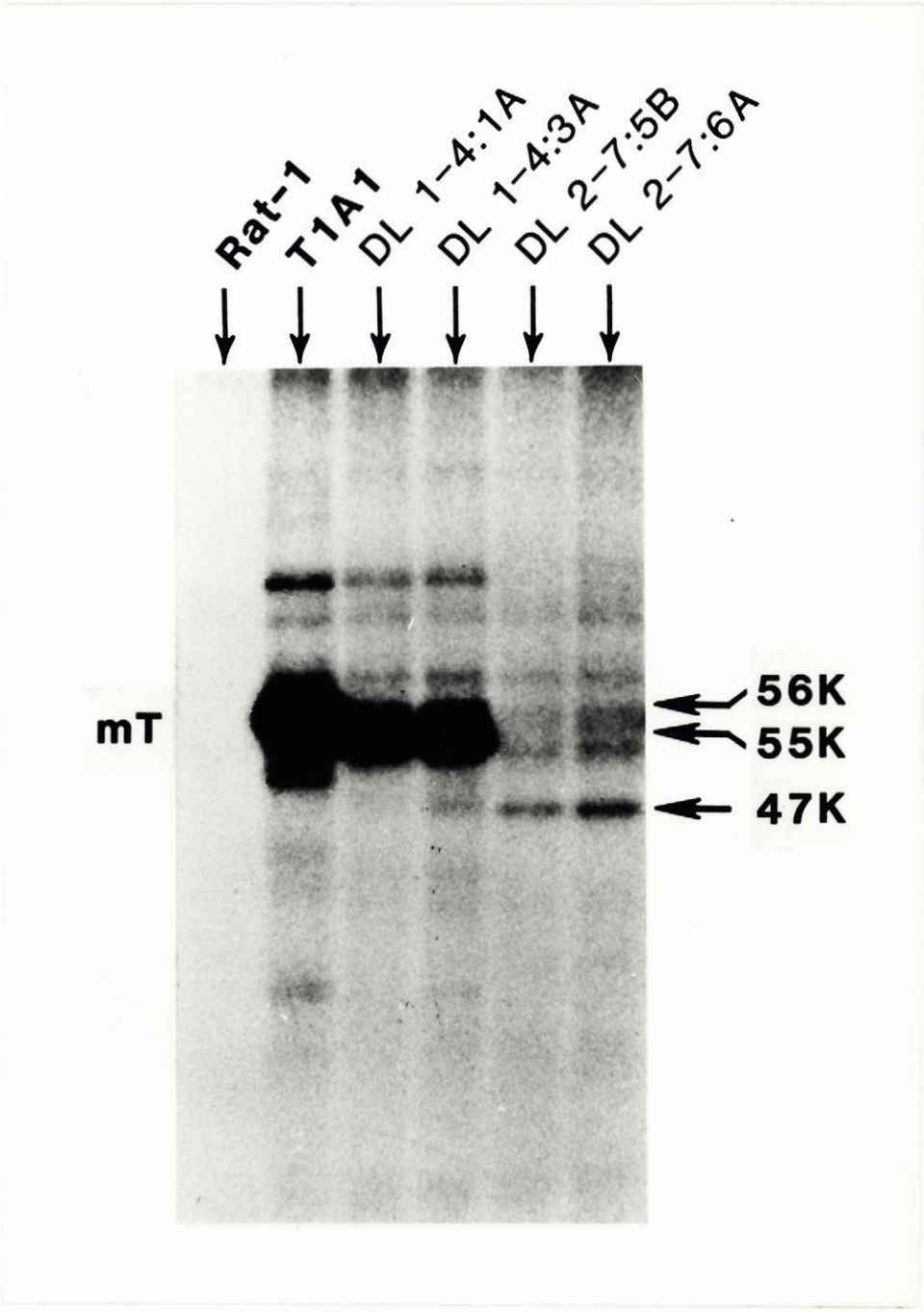
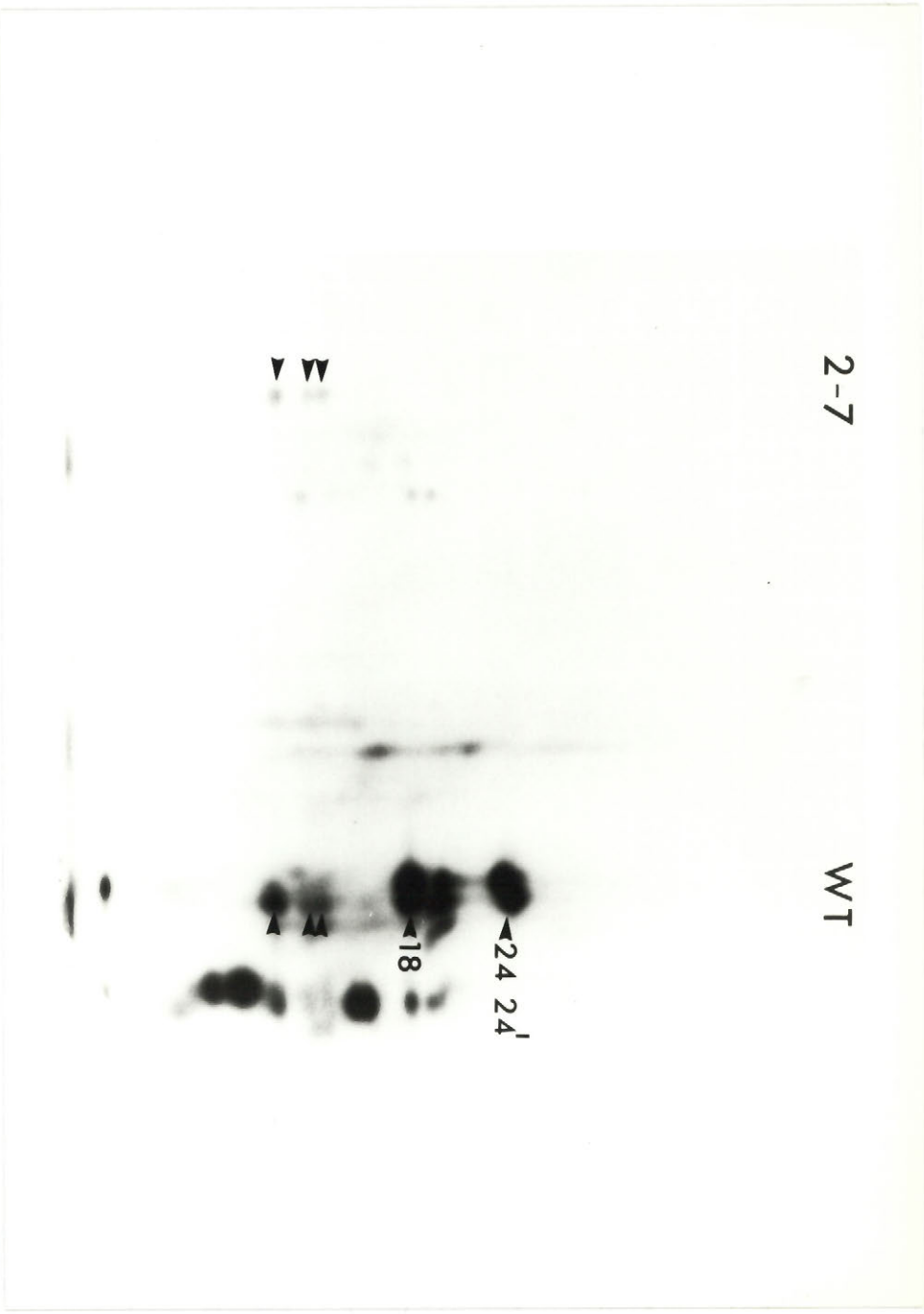

Rat-1
T1A1
DL 1-4:1A
DL 1-4:3A
DL 2-7:5B
DL 2-7:6A
mT
56K
55K
47K

Figure 17: Partial S. aureus V8 protease digestion of wt T1A1 (right) and pPdl2-7 (left) middle T antigens labelled in vitro with [γ-^{32}P]ATP. The major C-terminal peptides (24, 24', 18K) of wt middle T antigen are indicated. As well, the arrows indicate the three peptides obtained by cleavage of pPdl2-7 middle T antigen (left) and the corresponding minor spots observed in the wt pattern (right). The labelled spots to the right and left of the major spots in the wt lane are the partial V8 protease products of other proteins which become labelled with ^{32}P-phosphate in immunoprecipitates of T1A1 cell lysates (see Figure 16).


2-7
WT
18
24
24'

minor spots observed in the wt pattern, but they were distinct from the major carboxy terminal peptides of the wt middle T antigen (24K, 24'K and 18K) which contained the major tyrosine acceptor site (residue 315).

II. Oligonucleotide-Directed Mutagenesis of Recombinant Plasmids

A) Characterization of the Base Substitution in Recombinant Plasmids

The single stranded cloning vector M13mp8 carrying the BamH1/EcoR1 fragment of an altered polyomavirus DNA which could encode only the middle T antigen (nucleotides 4632-1560) was used as a substrate for oligonucleotide directed mutagenesis. To determine the role of the major site of *in vitro* tyrosine phosphorylation, the amino acid tyrosine 315 was converted to a serine residue using the oligonucleotide GAGGAGTCCATGCCA. This oligonucleotide matches the sequences in and around tyrosine 315 except that the triplet codon for tyrosine (TAC) was changed for the triplet codon for serine (TCC). The polyomavirus BamH1/EcoR1 fragment from a recombinant bacteriophage harboring the oligonucleotide-directed alteration was recloned in the large BamH1/EcoR1 fragment of the bacterial plasmid pML2. This mutant, pdPbs25, was sequenced by the method of Maxam and Gilbert in order to ensure that the DNA specified the correct base substitution at nucleotide 1178. The DNA of pdPbs25 was sequenced from the Xho11 site at nucleotide 1191 which was labelled using the large fragment of DNA polymerase. The products of the sequencing reactions were run on a 20% sequencing gel until the bromophenol blue reached the bottom of the gel. The autoradiogram of the gel is shown in Figure 18. The sequence clearly indicated that the mutant pdPbs25 was identical to wt except that there was a substitution of cytosine for adenine

Figure 18: Autoradiogram of end labelled pPbs25 DNA subjected to chemical degradation and polyacrylamide (20%) gel electrophoresis. The vertical line indicates the bases which correspond to the sequences of the oligonucleotide used as a mutagenizing agent. The asterisk indicates the site of the A-C transversion in the mutant DNA.

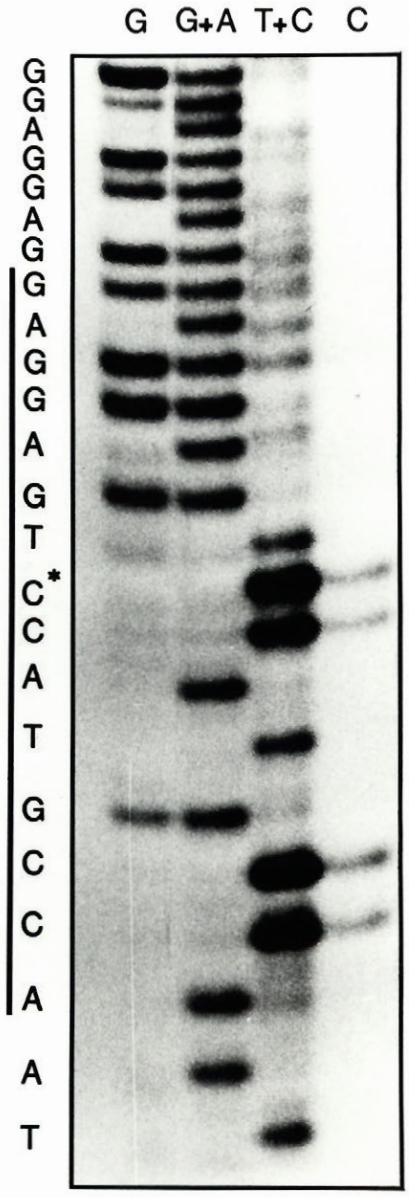

G G+A T+C C
G
G
A
G
G
A
G
G
A
G
G
G
A
A
G
T
C*
C
A
T
G
C
C
A
A
T

Figure 19: Oligonucleotide-directed mutagenesis substituting cytosine for adenine at nucleotide 1178. A partial nucleotide and amino acid sequence is shown for both the wt and the mutant. The asterisk indicates the position of the A-C transversion as well as the position of the tyrosine to serine substitution.

WILD TYPE MIDDLE T ANTIGEN

(1170) G GAG GAG T*AC ATG CCA A (1186) : nucleotide sequence

(313) GLU GLU T*YR MET PRO (317) : amino acid sequence

MUTANT MIDDLE T ANTIGEN

(1170) G GAG GAG T*CC ATG CCA A (1186) : nucleotide sequence

(313) GLU GLU S*ER MET PRO (317) : amino acid sequence

at nucleotide 1178 resulting in the conversion of tyrosine at residue 315 to a serine residue (Figure 19).

B) The Transforming Activity of pdPbs25 DNA

The transforming activity of pdPbs25 DNA was compared to pdPBR1 (the BamH1/EcoR1 fragment of wt polyomavirus inserted into the large BamH1/EcoR1 fragment of the plasmid pML2) and to pdPcMTBR1 (the BamH1/EcoR1 fragment of an altered polyomavirus genome expressing only middle T antigen inserted into the BamH1/EcoR1 fragment of pML2) over a range of DNA concentrations (10, 50 and 100 ng/3.5 X 10^5 cells) in two separate experiments. The results are summarized in Table 11. The plasmid pdPcMTBR1 transformed Rat-1 cells at approximately the same frequency (70-95%) as the wt plasmid pdPBR1. When pdPbs25 was compared to the wt plasmid pdPBR1, the mutant DNA transformed cells at 15-45% the frequency of the wt plasmid. Moreover, the foci first appeared at day 12 post-transfection on plates transfected with either pdPBR1 or pdPcMTBR1 DNA, whereas foci were only visible 17 days post-transfection on plates transfected with pdPbs25 DNA. pdPbs25 DNA also transformed cells at 15-45% of wt frequencies when plates were incubated at 32^{o}C, but the foci appeared much later (27 days) at this temperature.

Transformed cell lines were isolated after transfection of Rat-1 cells with pdPBR1 DNA (BR1:1C and BR1:2A), pdPcMTBR1 DNA (cMTBR1:1A and cMTBR1:2A) and pdPbs25 DNA (BS25:1B, BS25:2B and BS25:3A). In all cases, these cell lines appeared to be transformed by a number of criteria including the capacity to form foci, the capacity to grow to high saturation densities (data not shown), and the capacity to grow in soft agar (Table 12).

Table 11. Titration of the transforming activity of pdPbs25 DNA.

DNA source	Amount of DNA/plate	No. of foci per plate	Av. no. of foci per ug of DNA
Experiment A			
pdPBR1	10 ng	22, 33, 29	2,800 (100%)
pdPcMTBR1	10 ng	25, 34, 23	2,700 (96%)
pdPbs25	10 ng	3, 6, 4	400 (14%)
pdPBR1	50 ng	116, 93, 80	1,920 (100%)
pdPcMTBR1	50 ng	116, 71, 56	1,620 (84%)
pdPbs25	50 ng	39, 41, 49	860 (44%)
pdPBR1	100 ng	126, 131, 183	1,460 (100%)
pdPcMTBR1	100 ng	141, 116, 138	1,310 (89%)
pdPbs25	100 ng	45, 56, 59	530 (36%)
Experiment B			
pdPBR1	10 ng	46, 20, 25	3,000 (100%)
pdPcMTBR1	10 ng	40, 25, 24	2,900 (96%)
pdPbs25	10 ng	9, 3, 9	700 (23%)
pdPBR1	50 ng	130, 61, 94	1,900 (100%)
pdPcMTBR1	50 ng	55, 64, 77	1,300 (68%)
pdPbs25	50 ng	49, 45, 43	900 (47%)

Table 12. Properties conferred on Rat-1 cells by the novel middle T antigen encoded by pdPbs25 DNA.

Cell line	Growth in agar[a]
Rat-1	-
BR1:1C	+
BR1:2A	+
cMTBR1:1A	+
cMTBR1:2A	+
BS25:1B	+
BS25:2B	+
BS25:3A	+

[a] Cell lines were scored + if $\geq$60% of cells formed colonies of $\geq$16 cells in size when suspended in a semi-solid medium of 0.33% agarose and allowed to grow for 1 week.

C) T Antigen Analysis

The altered middle T antigen produced in cells transformed by pdPbs25 DNA was tested for its capacity to act as a substrate in the *in vitro* kinase assay. A 56K species, which likely represents middle T antigen, was phosphorylated in immunoprecipitates prepared from extracts of cells transformed by either pdPBR1 (BR1:1C, BR1:2A) or pdPcMTBR1 DNA (cMTBR1:1A, cMTBR1:2A) whereas no protein(s) with a similar mobility was found to be labelled when extracts from untransformed Rat-1 cells were reacted under identical condition (Figure 20A). In the extracts of cells transformed by pdPbs25 DNA (BS25:1B, BS25:2B, BS25:3A) a protein which migrates close to wt middle T antigen became phosphorylated in the *in vitro* kinase assay and presumably represents the mutant middle T antigen encoded by pdPbs25 DNA (Figure 20A). The novel middle T antigen, which is expressed by cells transformed by pdPbs25 DNA, appeared to migrate slightly more rapidly in a 10.0% SDS-polyacrylamide gel than wt middle T antigen. The reason for this different migration pattern is not well understood because the only alteration known to have occurred in pdPbs25 DNA resulted in a simple amino acid substitution, and this should not alter the mobility of the protein. Restriction endonuclease analyses of pdPbs25 DNA did not reveal any deletions. However, pdPbs25 DNA may have sustained a small deletion which has gone undetected. A similar mutant, Py1178-T, which contains a phenylalanine substitution for tyrosine, codes for a middle T antigen species which is indistinguishable in size from wt middle T antigen (Carmichael *et al.*, 1984). This does not exclude the possibility that the base substitution encoded by pdPbs25 DNA in some way alters its post-translational modification and this may affect its mobility during electrophoresis.

Figure 20A: Autoradiogram of a 10.0% SDS-polyacrylamide gel showing proteins which become phosphorylated *in vitro* in the presence of [γ-^{32}P]ATP in T antigen immunoprecipitates of polyomavirus wt (BR1:1C, BR1:2A, cMTBR1:1A, cMTBR1:2A) and mutant (BS25:1B, BS25:2B, BS25:3A) transformed cell lines. The position of the middle T antigen (mT) species phosphorylated in both wt and mutant cell lines is indicated. No ^{32}P-labelled proteins were observed at the position corresponding to middle T antigen in immunoprecipitates of the Rat-1 cell line.

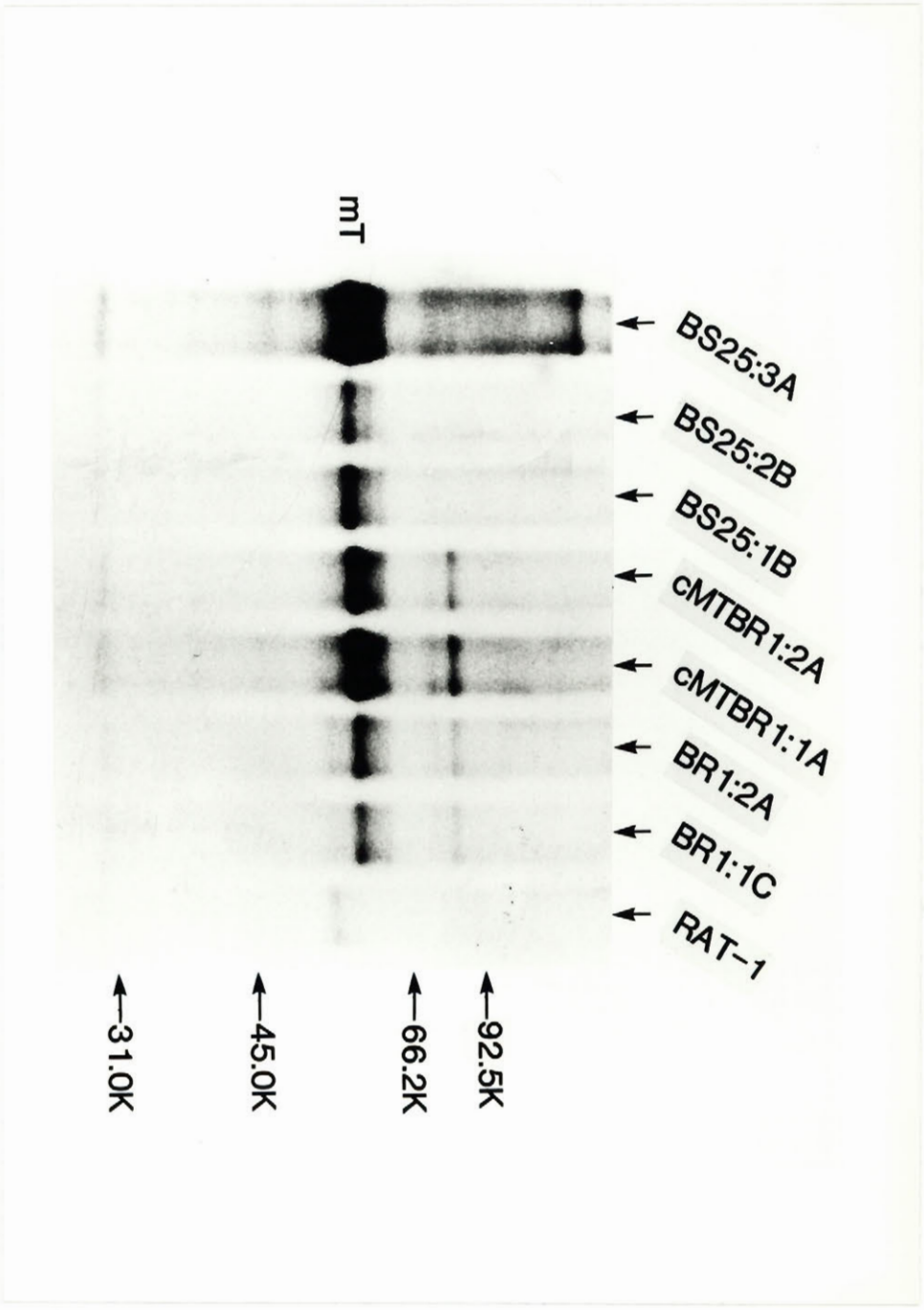
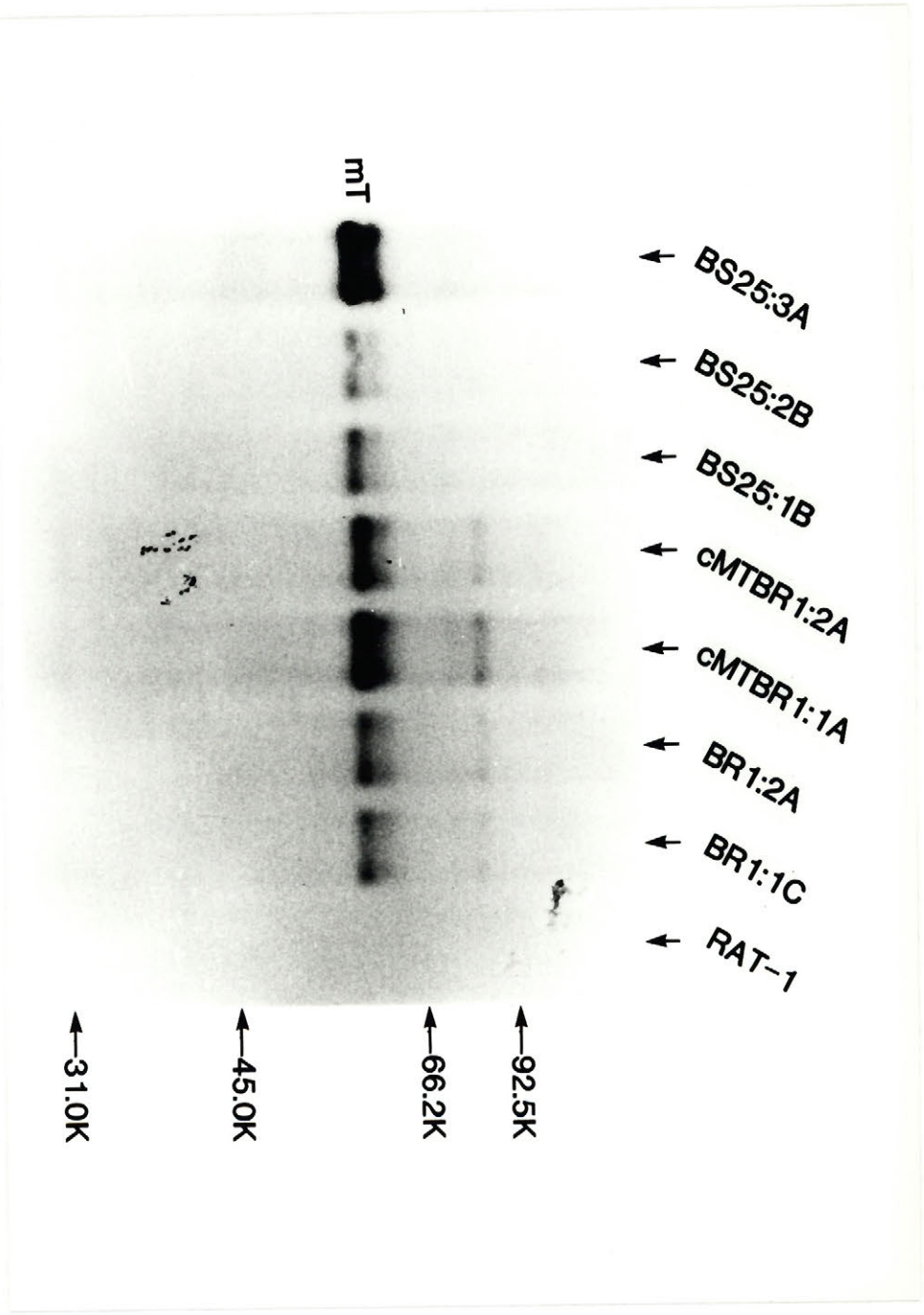

mT
BS25:3A
BS25:2B
BS25:1B
cMTBR1:2A
cMTBR1:1A
BR1:2A
BR1:1C
RAT-1
92.5K
66.2K
45.0K
31.0K

Figure 20B: Autoradiogram of a 10.0% SDS-polyacrylamide gel identical to the one shown in Figure 20A except that the gel was soaked in alkali (1 N NaOH, 1 hr, 55^{o}C) before the gel was dried down. The position of the middle T antigen (mT) species phosphorylated in both wt and mutant cell lines is indicated. Treatment in alkali does not significantly reduce the radioactivity in either the wt or mutant cell lines indicating that the labelled phosphate is probably associated with tyrosine residues.


mT
BS25:3A
BS25:2B
BS25:1B
cMTBR1:2A
cMTBR1:1A
BR1:2A
BR1:1C
RAT-1
92.5K
66.2K
45.0K
31.0K

The ^{32}P-labelled middle T antigen encoded by pdPbs25 is probably phosphorylated either exclusively or predominantly at tyrosine residues in the *in vitro* kinase assay because incubation of SDS-polyacrylamide gels containing the ^{32}P phosphate-labelled proteins in alkali (1.0 M NaOH, 1 hr at 55^{o}C) did not significantly reduce the intensity of the labelled middle T antigen (Figure 20B). By contrast, ^{32}P phosphate was almost completely removed from the less intensely labelled proteins shown in Figure 20A. This result suggests that the serine residues at position 315 is not the major site of *in vitro* phosphorylation, but rather tyrosine 322 is acting as the acceptor site for phosphate.

DISCUSSION

DISCUSSION

CHAPTER 1: THE ROLE OF SMALL AND MIDDLE T ANTIGEN IN CELLULAR TRANSFORMATION

To identify the polyomavirus tumor antigen(s) responsible for causing transformation of cells in culture, mutations were created within a cloned fragment of polyomavirus DNA known to carry the transforming gene(s). This was accomplished by *in vitro* mutagenesis of recombinant plasmid DNA coupled with the recovery of mutants in *E. coli*. These mutants were then tested for their capacity to cause transformation of normal rat cells in culture. Because propagation of a recombinant plasmid in *E. coli* is independent of the foreign DNA it carries, totally defective cis- or trans-acting mutations can be readily obtained.

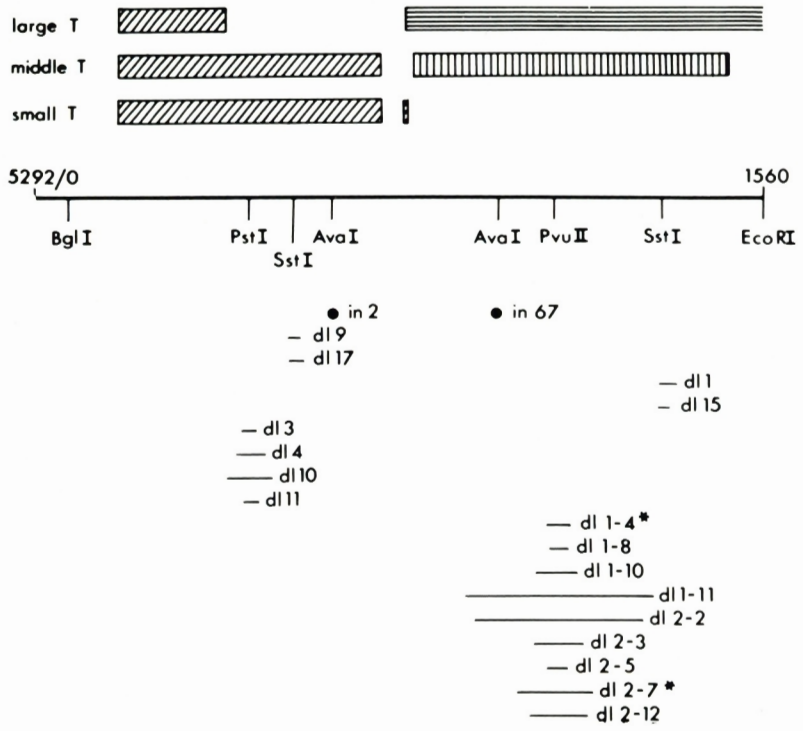
The mutagenesis protocol involved the enzymatic modification of the free ends of linear molecules. Circular recombinant molecules were linearized using a one-cut restriction endonuclease or alternatively, a multicut restriction endonuclease in the presence of ethidium bromide. The advantage of using a one-cut restriction endonuclease is obvious; all the mutations are located in the desired area in the recombinant plasmid. However, in the case of a multicut enzyme, the frequency of mutations at the desired location is proportional to the frequency at which this site is located at the ends of permuted linears. Although one might expect a multicut enzyme to cleave all sites equally, digestion of permuted linears with a second enzyme revealed that certain fragments were more abundant than others (data not shown), indicating that some restriction sites were preferentially cleaved by the enzyme and thus were more frequently represented at the open ends of permuted linears. This preferential site-dependent cleavage by restriction

endonucleases has been observed by others (Thomas and Davis, 1975; Goldstein et al., 1975; Rubin and Modrich, 1978), and was primarily due to the nature of nucleotide sequences adjacent to the site of cleavage (Armstrong and Bauer, 1982). The frequency with which mutants were recovered at a given site reflects the frequency with which this site was present at the open ends of permuted linears. For instance, while mutants were easily obtained at the Pvu11 site at nucleotide 1144, the preferred cleavage site for the enzyme Pvu11, mutants were difficult to obtain at the Ava1 site at nucleotide 1016, the least preferred site of cleavage by the restriction endonuclease Ava1. Although this presents a constraint in the in vitro mutagenesis protocol, it can be easily overcome by screening a large sample of drug resistant colonies after transfection of mutated recombinant plasmids into bacteria.

Mutant recombinant plasmids isolated after the mutagenesis protocol were first characterized by restriction endonuclease digestion followed by gel electrophoresis. Subsequently, the precise boundaries of the deletions or insertions were determined by sequence analysis. Comparison of these two methods reveals that while restriction endonucleases are useful in determining the location of the lesion, they are somewhat less useful when estimating the size of deletions or insertions. For example, while the deletion in the mutant pPdl1-4 was estimated, by restriction endonuclease digestion and gel electrophoresis, to be 45 b.p., sequence analysis revealed the deletion to be only 21 b.p. in length. A similar pattern was seen with the other mutants characterized, thus restriction endonuclease analysis proved to consistently overestimate the size of the deletion. Although restriction endonuclease analysis did not reveal the correct size of the deletions, it did however correctly order the plasmids according to the relative sizes of their deletions.

A total of 19 mutants of recombinant plasmid pPH1-8 or pPBR2 were constructed and characterized and the location of these mutations is summarized in Figure 21. Eight of these mutant recombinant plasmids contained lesions affecting the coding sequences of both small and middle T antigens. Four recombinant plasmids (pPdl3, pPdl4, pPdl10, and pPdl11) with mutations at the Pst1 site at nucleotide 484 were recovered. Interestingly, two of these mutants, pPdl3 and pPdl11, carried unidirectional deletions after mutagenesis of Pst1 permuted linears with Bal 31 nuclease, while the two other mutants, pPdl4 and pPdl10, carried bidirectional, but not symmetrical, deletions. The deletion in all four mutant recombinant plasmids caused a shift in the reading frame in sequences common to small and middle T antigen. All of these out-of-frame deletions would lead to the synthesis of a single truncated protein because in each case a termination codon is encountered before the proximal splice junction for small and middle T antigen. Two mutant recombinant plasmids (pPdl19 and pPdl17) were recovered with deletions at the Sst1 site at nucleotide 569. pPdl17 is a non-symmetrical bidirectional mutant generated by Bal 31 digestion of Sst1 permuted linears of pPH1-8 DNA. pPdl9, on the other hand, was obtained after digestion of Sst1 permuted linears of pPBR2 with the enzyme S1 nuclease. S1 nuclease usually displays a specificity for single stranded DNA (Vogt, 1973) and should have removed only the single stranded projections between nucleotides 570-573 left by Sst1 hydrolysis. However, sequence analysis revealed that the deletion in pPdl9 extended from nucleotide 570-576. This "nibbling" phenomenon of S1 nuclease has been previously observed (Shenk, 1977) and is thought to occur because the open ends of linear molecules tend to "breathe", thus temporarily creating regions of single stranded DNA. Both pPdl9 and pPdl17 carry out-of-frame deletions, and both would encode a truncated protein because in both cases a stop codon is encountered before

Figure 21: Schematic summary of mutations affecting the coding sequences of polyomavirus T antigens. The figure shows the coding sequences for large, middle, and small T antigens. Note that the N-terminal portion of the three T antigens (before the proximal splice) as well as the distal portion of small T antigen are in the same reading frame. The distal portion of middle and large T antigens (after the distal splice) are in the two remaining reading frames and are different from each other. Shown as well is the approximate position and size of the lesion generated in the T antigen coding sequences. Symbols: ●, insertion; -, deletion. The asterisk indicates lesions which do not abolish the transforming capacity of the DNA.


large T
middle T
small T
5292/0
1560
BglI
PstI
SstI
AvaI
AvaI
PvuII
SstI
EcoRI
● in 2
– dl 9
– dl 17
● in 67
– dl 1
– dl 15
– dl 3
— dl 4
—— dl 10
– dl 11
— dl 1-4*
— dl 1-8
—— dl 1-10
dl 1-11
dl 2-2
—— dl 2-3
— dl 2-5
——— dl 2-7*
——— dl 2-12

the proximal splice for both small and middle T antigen. A single mutant recombinant plasmid (pPin2) bearing an insertion at the Aval site at nucleotide 657 was characterized. This mutant was generated by treating Aval permuted linears of pPH1-8 with the enzyme reverse transcriptase. This enzyme has a 5' to 3' polymerase activity which is capable of back-filling the 5' projections left by Aval hydrolysis. If the polymerase activity acts to completion, a four b.p. insertion of the nucleotide CCGA, which corresponds to a duplication of the single stranded projection left by Aval, should be present. Sequence analysis of pPin2 DNA revealed that it does indeed carry the expected four b.p. insertion. This would lead to the synthesis of a single truncated protein because the out-of-frame insertion introduces a stop codon before the proximal splice for small and middle T antigen. Surprisingly, one of the deletion mutants recovered after Bal 31 nuclease digestion of Pvull permuted circles, pPdl2-2, also affected the coding sequences of both small and middle T antigen. Although the Pvull site, which was used to generate mutations, lies within sequences which are unique to middle T antigen at nucleotide 1144, the deletion in pPdl2-2 was so large that it removes the distal splice for middle T antigen and the stop codon for small T antigen. This deletion results in the synthesis of a single altered protein which consists of the first 194 amino acids of small T antigen in addition to 80 novel amino acids encoded by sequences in the same reading frame as the N-terminal portion of small, middle and large T antigen.

When all eight mutant recombinant plasmids bearing mutations in sequences encoding both small and middle T antigen of polyomavirus were tested in the transformation assay, all eight were found to be transformation defective. These results are analogous to previous results which were obtained using viral mutants. The recombinant plasmid mutants which carry

mutations affecting the primary structure of both small and middle T antigens resemble in phenotype the hr-t viral mutants (transformation defective) which also carry mutations in the sequences common to small and middle T antigen (Benjamin, 1970; Staneloni et al., 1977). Apart from confirming the necessity for either small or middle T antigen in the transformation process, these results also indicate that truncated forms of large T antigen are not sufficient to cause transformation of Rat-1 cells. This conclusion was reached because the mutations at the Pst1 site (484), the Sst1 site (569) and the Ava1 site (657) all map within a region known to be absent in large T antigen mRNA. Thus, a truncated form of large T antigen which may be synthesized from recombinant plasmids is, by itself, insufficient to cause transformation and this is in agreement with results indicating that complete large T antigen by itself is incapable of conferring the transformed phenotype to untransformed cells (Schlegel and Benjamin, 1978; Lania et al., 1979).

The remaining 11 mutants carry mutations affecting the coding sequences of middle T antigen but not small T antigen. A single insertion mutation (pPin67), which had lost the Ava1 site at nucleotide 1016, was recovered after treatment of Ava1 permuted linears of pPH1-8 with reverse transcriptase. Sequence analysis of this mutant revealed that the polymerase activity of reverse transcriptase worked to completion as witnessed by the four b.p. insertion of the nucleotides CCGA. This out-of-frame insertion would lead to the synthesis of an altered form of middle T antigen consisting of authentic amino acid sequences of middle T antigen up to the point of the insertion, followed by a long stretch of foreign protein at its C-terminus. A total of eight mutants, whose lesions affected only the sequences for middle T antigen, were recovered after Bal 31 nuclease digestion of Pvu11 permuted circles. The enzyme Pvu11 hydrolyzes polyomavirus DNA at

nucleotide 1144, leaving a blunt-ended molecule. Of the eight Pvu11 mutants containing lesions affecting only middle T antigen coding sequences, three of them (pPd11-8, pPd11-11 and pPd12-5) contained unidirectional deletions after Bal 31 mutagenesis. The remaining five mutants contained bidirectional deletions, although the deletion was rarely symmetrical. For example, pPd11-4 contained a 21 b.p. deletion, with 3 nucleotides removed from the 5' side of the Pvu11 cleavage site and 18 nucleotides removed from the 3' side of the Pvu11 cleavage site. One mutant DNA, pPd11-11, contained a large deletion which extended past the termination codon for middle T antigen. This DNA could presumably encode a protein containing 330 amino acids, of which 304 amino acids are from the N-terminal portion of authentic middle T antigen and 26 amino acids are foreign amino acids. Seven of the mutants around the Pvu11 site at nucleotide 1144 contain deletions which fall exclusively within the coding sequences for middle T antigen. Of these seven, five (pPd11-8, pPd11-10, pPd12-3, pPd12-5, and pPd12-12) contain deletions altering the reading frame for the C-terminal portion of middle T antigen. All of these out-of-frame deletions would lead to the synthesis of altered forms of middle T antigen which would be composed of the N-terminal amino acids of authentic middle T antigen up to the beginning of the deletion, while the C-terminus of the protein would carry varying lengths of foreign amino acids depending on the size and position of the deletion. Two of the recombinant plasmids bearing deletions around the Pvu11 site at nucleotide 1144, pPd11-4 and pPd12-7, did not alter the reading frame of middle T antigen after the deletion. Both of these DNAs should encode middle T antigen species which lack some internal amino acids. Finally, two deletion mutants, pPd11 and pPd115, which had lost the Sst1 site at nucleotide 1373, were recovered after digestion of Sst1 permuted linears of pPBR2 with the S1 nuclease. Sequence analysis revealed that, in both cases,

treatment with S1 nuclease removed the single stranded projections left after Sst1 hydrolysis, as well as removing a small number of nucleotides of double-stranded DNA from both sides of the restriction endonuclease site at nucleotide 1173. Both pPdl1 and pPdl15 carry out-of-frame deletions which could lead to the synthesis of a protein containing 379 amino acids from the N-terminal portion of middle T antigen followed by a short stretch of foreign amino acids in the case of pPdl15, or a long stretch of foreign amino acids in the case of pPdl1, at the carboxy end of the protein.

Of the 11 mutants, whose lesions affected the coding sequences of middle T antigen, 10 retained the coding sequnces for small T antigen as well as all the regulatory sequences thought to be required for the expression of small T antigen. Only one, pPdl1-11, retained the coding sequences for small T antigen but possibly removed some of the regulatory sequences, including the mRNA polyadenylation signal, thought to be important for mRNA processing. The 11 mutants carrying mutations affecting the coding sequences of middle T antigen, but not the coding sequences of small T antigen, were tested in the transformation assay. The majority, 9 out of 11, were found to be completely transformation defective. Two mutants, pPdl1-4 and pPdl2-7, retained the capacity to transform Rat-1 cells. pPdl1-4 DNA transformed Rat-1 cells at the same frequency as the wt parental plasmid, pPH1-8, whereas pPdl2-7 DNA transformed Rat-1 cells at an average frequency of 20% of wt DNA. Viable viral mutants with lesions affecting the coding sequences of middle but not small T antigen have been described (Griffin and Maddock, 1979; Magnusson and Berg, 1979; Bendig et al., 1980; Templeton and Eckhart, 1982; Carmichael et al., 1982, 1984; Ding et al., 1982; Nilsson et al., 1983). Of these viral mutants, MOP1033 (Templeton and Eckhart, 1982), dl22 (Ding et al., 1982), dl23 (Griffin and Maddock, 1979) and Py1387-T (Carmichael et al., 1982), all appear to be

totally defective in the transformation assay, a result analogous to the results with the mutant recombinant plasmids pPin67, pPdl1-8, pPdl1-10, pPdl2-3, pPdl2-5, pPdl2-12, pPdl1 and pPdl15. The viable viral mutants dl2208 (Nilsson et al., 1983), dl1013 and 1015 (Magnusson and Berg, 1979), Py1178-T (Carmichael et al., 1984) resemble in their phenotype the mutant recombinant plasmid pPdl2-7 (reduced capacity to transform cells in culture). Finally, some viable viral mutants, such as dl17 (Ding et al., 1982), dl8 (Griffin and Maddock, 1979), dl45 (Bendig et al., 1980), dl2209 and dl2210 (Nilsson et al., 1983) retain essentially wt transforming activity, much like the mutant recombinant plasmid pPdl1-4.

Two major conclusions can be derived from these results. First, small T antigen alone is insufficient to establish the transformed state. Rat-1 cells transfected with recombinant plasmids which carry the coding sequences for small T antigen and all the regulatory sequences thought to be required for its expression failed to become transformed as measured by the focus assay. Secondly, middle T antigen appears to be essential for cellular transformation. Most mutations in middle T antigen coding sequences either abolished or reduced the transforming activity of the DNA. This latter conclusion is strengthened by the finding that an altered polyomavirus genome capable of encoding only middle T antigen retains the capacity to transform cells in culture (Treisman et al., 1981). The mutant recombinant plasmids and the viable viral mutants which retain activity in the transformation assay in all likelihood delete amino acids which are dispensable for the functioning of middle T antigen in cellular transformation.

CHAPTER 2: THE ROLE OF THE CARBOXY TERMINAL DOMAIN OF MIDDLE T ANTIGEN IN TRANSFORMATION

In order to determine which of the polyomavirus tumor antigens plays a role in cellular transformation, *in vitro* mutagenesis was performed on recombinant plasmids harboring polyomavirus DNA known to carry the transforming gene(s) of the virus. These studies revealed that middle T antigen is absolutely required for cellular transformation. In addition, sequence analysis of two of the mutants strongly indicated that the amino acids at the C-terminus of middle T antigen are required for the protein to be active in transformation. Both pPdl1 and pPdl15 encode proteins which should lack the last 42 amino acids of middle T antigen, and both are completely inactive in the transformation assay, despite sharing considerable homology with wt middle T antigen. However, the mutation in pPdl1 repositions the nucleotide sequences in the recombinant plasmid such that while the first 379 amino acids correspond to those normally found in middle T antigen, the C-terminus of the protein would be composed of a long stretch of foreign amino acids. The presence of these foreign amino acids, rather then the lack of sequences normally found at the C-terminus of middle T antigen, might conceivably be responsible for the transformation negative phenotype. This possibility is less likely when considering the amino acid sequence of the protein encoded by pPdl15 DNA. The protein produced from mRNAs having middle T antigen donor and acceptor splice sites would have 379 amino acids corresponding to authentic middle T antigen, which would be followed by 6 novel amino acids derived from non-coding sequences in the reading frame corresponding to the reading frame used for the N-terminal portion of small, middle and large T antigen. These six novel amino acids

(ILE-GLN-CYS-ASN-ALA-THR) are either uncharged-polar or non-polar amino acids and by virtue of their small number and their nature, are unlikely to have a profound effect on the conformation of the protein. Therefore, in the case of pPdl15, the loss of transforming activity is likely due to the loss of amino acids normally found at the C-terminus of middle T antigen.

The last 42 amino acids, which are deleted in pPdl1 and pdl15, include a cluster of hydrophobic amino acids extending from amino acid residue 394-415, as well as the basic amino acids which flank this hydrophobic region. This arrangement of amino acids is typical of membrane associated proteins (Tomita and Marchesi, 1979; Porter et al., 1979; Rose et al., 1980; Natheson et al., 1981) and because middle T antigen has been shown to associate with the membrane fraction of infected or transformed cells (Ito et al., 1977a; Ito, 1979; Silver et al., 1978) it seems likely that the amino acids at the C-terminus of middle T antigen play a role in anchoring the protein in membranes. Therefore, it is plausible that the lesions present in pPdl1 and pPdl15 prevent the insertion of altered middle T antigen species encoded by these DNAs into the plasma membrane of cells, where it is predominantly located (Ito et al., 1977a; Schaffhausen et al., 1982a). The fact that pPdl1 and pPdl15 are transformation defective would then suggest that middle T antigen must associate with membranes in order to bring about cellular transformation. The results with pPdl1 and pPdl15 are in agreement with results found with the viable viral mutant Py1387-T (Carmichael et al., 1982) and with the mutant recombinant plasmid p12 (Novak and Griffin, 1981). p12 contains a deletion which removes the last 15 amino acids of authentic middle T antigen and adds two novel amino acids at the C-terminus of the protein (Novak and Griffin, 1981). Py1387-T contains a single base change which introduces a stop codon

in middle T antigen coding sequences at nucleotide 1387, producing a truncated middle T antigen lacking the last 37 amino acids at the C-terminus of middle T antigen (Carmichael et al., 1982). The latter protein closely resembles the protein encoded by pPd115 DNA. The virus Py1387-T and the plasmid p12 are unable to transform cells in culture. In addition, the deleted protein produced by Py1387-T is exclusively found in the cytosol demonstrating directly that the protein can no longer associate with membranes (Carmichael et al., 1982). Truncated middle T antigen, made in cells infected with Py1387-T, is defective in both tyrosine protein kinase activity as well as in vivo phosphorylation of serine or threonine residues at the 58K sites of middle T antigen (Carmichael et al., 1982). The authors suggest that middle T antigen associated kinase activity is regulated and/or activated by cellular kinase(s) localized in the membrane. The recent finding that middle T antigen associates with the product of the c-src gene substantiates this contention and has led to the proposal that this binding may play a part in transformation (Courtneidge and Smith, 1983, 1984). If binding of middle T antigen to the product of the c-src gene is truly a requirement for the transformation process then it is conceivable that the defect in pPd115, p12 (Novak and Griffin, 1982), and Py1387-T (Carmichael et al., 1982) is related to their inability to insert into the plasma membrane and thereby interact with c-src, whose normal location is also the plasma membrane (Courtneidge et al., 1980). However, although membrane association and concomitant in vitro tyrosine kinase activity appear to be important in transformation, recent evidence indicates that these two properties may not be sufficient to bring about cellular transformation. Templeton et al., (1984) constructed a fusion gene product consisting of the N-terminal 379 amino acids of

polyomavirus middle T antigen, followed by the C-terminal 60 amino acids of the vesicular stomatis virus glycoprotein G. This hybrid gene contains the coding region for the C-terminal hydrophobic membrane-spanning domain of the G protein which substitutes for the C-terminal hydrophobic domain of middle T antigen. Although the hybrid protein, produced in COS cells under the control of the late SV40 promotor, associated with cell membranes and displayed a tyrosine protein kinase activity *in vitro*, the plasmid encoding the hybrid protein failed to transform mouse and rat cells in culture. However, the authors found that the fusion protein and middle T antigen, under the control of the late SV40 promoter, appeared to be associated with the endoplasmic reticulum, which contradicts previous results localizing the middle T antigen to the plasma membrane (Ito *et al.*, 1977a; Schaffhausen *et al.*, 1982a). Thus, the loss of transforming activity may be due to an inappropriate localization of the hybrid middle T antigen. In addition, the authors admit that the transformation defective phenotype of the mutant may be attributable to the fact that the fusion protein is not expressed, under the control of the SV40 late promoter, at sufficiently high levels in mouse and rat cells to cause transformation (Templeton *et al.*, 1984).

In order to further delineate the minimal sequences required at the C-terminus of middle T antigen for transformation, two deletion mutants carrying small alterations at the C-terminus of middle T antigen were characterized. The mutant recombinant plasmid pPd190 should encode a protein which contains the first 409 amino acid from the N-terminus of middle T antigen, at which point the deletion caused the removal of the last 12 amino acids of authentic middle T antigen (6 hydrophobic and 4 basic amino acids) and their replacement with 14 new amino acids (7 hydrophobic and 1 acidic

amino acid) encoded by plasmid sequences. In addition, the deletion in pPd190 DNA also removed the internal polyadenylation signal AAUAAA (Proudfoot and Brownlee, 1974; Fitzgerald and Shenk, 1981). pPd190 DNA was completely inactive in the transformation assay. The complete loss of transforming activity with pPd190 DNA is probably due to the amino acids changes this DNA encodes, and is unlikely to be due entirely to the loss of the internal polyadenylation signal since a separate mutant recombinant plasmid, p46 (Novak and Griffin, 1981), retains some, albeit low, transforming activity, despite the loss of the polyadenylation signal. The lesion in p46 DNA results in the synthesis of a protein with 78 new amino acids replacing the last 12 amino acids of authentic middle T antigen (Novak and Griffin, 1981). While both pPd190 and p46 cause the removal of the last 12 amino acids of middle T antigen, only p46 retains partial activity in the transformation assay. This would suggest that the difference between these two mutants resides in the nature of the novel amino acids found at the C-terminus of the proteins encoded by these DNAs. In this case, while the loss of amino acids at the C-terminus probably plays a role in determining the phenotype of these mutants, the nature of the new amino acids added to the C-terminus may also account for their impaired capacity to transform cells. An effort was not made to isolate normal cells which harbored pPd190 DNA; thus, it was not possible to determine whether the altered midde T antigen produced by pPd190 DNA could associate with the plasma membrane.

The basic amino acids, which normally flank hydrophobic domains in proteins, have been hypothesized to interact with the phosphate moiety of membrane phospholipids and may function to prevent the C-terminus of the protein from entering the hydrophobic lipid bilayer (Natheson et al., 1981). A recombinant plasmid, pPd16, bearing a deletion affecting only the

basic amino acids, but not the hydrophobic stretch, found at the C-terminus of middle T antigen was characterized. The deletion suffered by pPdl6 results in the synthesis of a protein which contains the N-terminal 419 amino acids of middle T antigen, followed by three novel amino acids encoded by plasmids sequences before a stop codon is encountered. While middle T antigen has four basic amino acids at its C-terminus, the fusion middle T antigen of pdl6 has two basic and one acidic amino acid. Interestingly, pPdl6 DNA retained nearly wt activity in the transformation assay. This is a surprising result, because a difference in charge at the C-terminus of middle T antigen appears to have little effect on transformation. These results imply that despite a significant reduction in net positive charge at the C-terminus of the protein at physiological pH, the fusion middle T antigen protein of pPdl6 could insert efficiently into membranes, as evidenced indirectly by both its capacity to transform Rat-1 cells at the same frequency as wt DNA, as well as its reactivity in the kinase assay. pPdl6 is unlike previously described mutants which affect the 3' coding sequences of middle T antigen. Novak and Griffin, (1981) found that the removal of the termination codon for middle T antigen, either alone or together with a few additional amino acids, drastically decreased or abolished the transforming efficiency of the DNA. In particular, the mutant p42 encodes the entire amino acid sequence of middle T antigen with the addition at the C-terminus of 8 novel amino acids. Despite the fact that the entire hydrophobic region as well as the flanking basic amino acids are present in the fusion protein, p42 transforms at 4% of the wt frequency (Novak and Griffin, 1981). This low transforming activity is not due to the loss of regulatory sequences in the 3' non-coding sequences which follow the stop codon of middle T antigen because the loss of these same sequences in pPdl6 had no apparent effect on the transforming activity of the

DNA. Thus, the difference between their data and the results obtained with pPdl6 is probably due to the nature of additional amino acids derived from plasmid sequences, although it is not at all clear what the nature of this difference is. In summary, the C-terminus of middle T antigen is essential for transformation, and both the deletion of amino acids, as well as the addition of novel amino acids, at the C-terminus of middle T antigen, can significantly affect its transforming activity. The results with pPdl90 and pPdl6 suggests that at the hydrophobic terminal domain of middle T antigen, it is the composition rather than the amino acid sequence *per se*, which is essential to maintain its transforming activity.

CHAPTER 3: THE ROLE OF THE MAJOR SITE OF IN VITRO TYROSINE PHOSPHORYLATION OF MIDDLE T ANTIGEN IN TRANSFORMATION

Using molecularly-cloned polyomavirus DNA and *in vitro* mutagenesis techniques, a series of deletion mutants whose lesions affected the coding sequences of middle T antigen were isolated. These mutations were centered in and around the *Pvu*II site at nucleotide 1144 in polyomavirus DNA. Of the seven deleted recombinant plasmids characterized whose lesions were entirely within only middle T antigen coding sequences, five (pPdl1-8, pPdl1-10, pPdl2-3, pPdl2-5, and pPdl2-12) were completely incapable of transforming Rat-1 cells. These five mutants all contained deletions which altered the reading frame for the C-terminal portion of middle T antigen whose importance in transformation was discussed in the previous chapter.

Two deleted recombinant plasmids, pPdl1-4 and pPdl2-7, retained the capacity to transform Rat-1 cells. pPdl1-4 DNA transformed Rat-1 cells at approximately wt frequencies whereas pPdl2-7 DNA transformed cells at 20-35% the frequency of wt DNA. Cell lines established after transformation of Rat-1 cells by either pdl1-4 or pPdl2-7 DNA were indistinguishable from wt transformants as judged by a number of criteria, including the capacity to form tumors in animals. These two deletion mutants are of particular interest as they define portions of middle T antigen which are not essential for transformation of cells in culture.

Polyomavirus middle T antigen has been shown to be, or to be associated with, a tyrosine kinase (Eckhart *et al.*, 1979; Schaffhausen *et al.*, 1979; Smith *et al.*, 1979). Recent evidence indicates that polyomavirus middle T antigen forms a complex with the product of the c-src cellular gene, and it has been suggested that the tyrosine kinase activity

measured in vitro in immunoprecipitates of middle T antigen is the activity of the associated $pp60^{c\text{-}src}$ protein (Courtneidge and Smith, 1983, 1984). Whatever the nature of the tyrosine kinase, middle T antigen appears to be a substrate for this activity in vitro and it has been shown that the major site of phosphorylation in middle T antigen is tyrosine 315 (Schaffhausen and Benjamin, 1981). In addition, recent evidence indicates that tyrosine 322 may also become phosphorylated in vitro (Oostra et al., 1983; B.Schaffhausen, manuscript in preparation).

While transformation is considered to be an all or none phenomenon, the fact that certain mutants retain only partial activity in the transformation assay would appear to contradict this assumption. If transformation is truly an all or none phenomenon then an explanation is required to explain how mutants like pPd12-7 retain only 20-35% activity in the transformation assay. One possible explanation is the amount of middle T antigen required in transformation, i.e. that a certain threshold level of middle T antigen is required within the cell in order to bring about cellular transformation. In the case of an altered middle T antigen, it may either be crippled in its intrinsic transforming activity or alternatively it may be more susceptible to proteolytic degradation. Therefore, a higher threshold of transforming protein would be required to bring about cellular transformation. If this were the case, transformation could only occur in cells where the deleted middle T antigen was produced in higher then normal quantities. These higher protein levels could be attained if cells transformed by poorly transforming mutant DNAs contained multiple copies of the recombinant plasmid DNA. However, Southern blot analysis of cells transformed by pPd12-7 DNA revealed that the cellular DNA harbors only a single copy of the recombinant plasmid. Nonetheless, this does not rule out the possibility that a single copy of the

viral sequences is expressed at high levels. Immunoprecipitates of cells transformed by pPd12-7 DNA do not appear to contain greater then normal quantities of middle T antigen. However, under the labelling conditions used, higher levels of protein synthesis would not have been found if the protein turnover was great. The role, if any, that the quantity of middle T antigen within a given cell plays in transformation is still uncertain and requires further study.

The discovery that middle T antigen associates with the product of the c-src gene (Courtneidge and Smith, 1983, 1984) suggests an alternate explanation for the low transforming activity of certain mutants. It is possible that the levels of c-src, and not the level of middle T antigen, may determine the outcome of the transformation process. If mutant middle T antigens associate poorly with the c-src product, and if this association is required in the transformation process, then cells with high levels of c-src may allow the middle T antigen/c-src complex to form more readily. To date there is no evidence to substantiate this proposal, although certain predictions could be made if this proposal was correct. For instance, if high levels of c-src facilitate transformation, especially of low transforming mutant DNAs, then one might predict that cell lines which constitutively produce high levels of c-src would be transformed at higher frequencies by mutant DNAs than cell lines containing low levels of c-src. In addition, if the level of the c-src product does play a role in transformation, then cells originally transformed by a mutant DNA presumably contain high levels of c-src. Some revertants, which no longer display the transformed phenotype due to the loss of viral sequences, should also presumably contain uniformly high levels of c-src and should subsequently become re-transformed with the mutant DNA at frequencies comparable to wt DNA. It has yet to be determined

whether either hypothesis presented above serves to explain why some mutant DNAs transform cells at a reduced frequency when compared to wt DNA.

Early studies with retroviruses suggested that the sites of *in vitro* tyrosine phosphorylation were preceded by one or more acidic amino acid residues (Patschinsky *et al.*, 1982). Tyrosine 315, the major site of *in vitro* tyrosine phosphorylation (Schaffhausen and Benjamin, 1981), is located in a highly acidic portion of middle T antigen. In particular, it is preceded by a stretch of six glutamic acid residues (amino acids 309-314). A number of polyomavirus mutants lacking either a part or all of the DNA encoding this region of middle T antigen have been isolated. The polyomavirus mutant dl17 contains a deletion which causes the removal of amino acids 300-310, thereby deleting the first two residues from the stretch of six glutamic acid residues (Ding *et al.*, 1982). dl2210 contains a large deletion from middle T antigen coding sequences which results in the removal of amino acids 296-312 from middle T antigen. This amino acid tract includes the first four of six glutamic acid residues which precede tyrosine 315 (Nilsson *et al.*, 1983). Both dl17 and dl2210 transform cells at close to wt frequencies. These results have led to the suggestion that all the acidic amino acids need not precede tyrosine 315 in order to preserve the transforming activity of middle T antigen. Our data with pPdl1-4 confirm these results. pPdl1-4 middle T antigen lacks amino acids 304-310 thus removing the first two glutamic acid residues. Moreover, pPdl1-4 DNA transforms Rat-1 cells at wt frequencies. The deletion in pPdl1-4 DNA repositions sequences such that the milieu of tyrosine 315 is still generally acidic. However, these new acidic amino acids do not seem to be required for middle T antigen function because the deletion in dl2210 removes them with no apparent loss in transforming activity. The dispensibility of these acidic

amino acids is also demonstrated by mutant recombinant plasmids which retain wt transforming activity even when as many as four of the glutamic acid residues are replaced by leucine residues (Oostra et al., 1983).

Three polyomavirus deletion mutants have been isolated whose middle T antigens lack the entire glutamic acid residue stretch, as well as tyrosine 315. They include dl23 (Griffin and Maddock, 1979), dl22 (Ding et al., 1982), and dl2208 (Nilsson et al., 1983); the middle T antigens of these mutants lack amino acids 302-335, 287-319, and 298-316 respectively. All three mutants are incapable of transforming cells in culture. These mutations appear to define a region in polyomavirus middle T antigen which is absolutely required for transformation. However, our results with pPdl2-7 contradict this conclusion. pPdl2-7 middle T antigen is deleted of amino acids 285-323. This deletion includes all six glutamic acid residues as well as tyrosine 315 and tyrosine 322. However, pPdl2-7 DNA transforms Rat-1 cells at 20-35% the frequency of wt DNA. These results indicate that amino acids 285-323 are not absolutely required for transformation by middle T antigen. It is difficult to reconcile the difference between our observations and previous results, especially in view of the fact that all the sequences deleted from dl22 DNA and dl2208 DNA are smaller subsets of those deleted from pPdl2-7 DNA. One possible explanation of these results is that the deletion suffered in the middle T antigen encoded by pPdl2-7 allows the protein to retain structural features important for its transforming activity. By contrast, proteins encoded by other mutants (dl23, dl2208, and dl22), may not assume a conformation compatible with the normal functions of middle T antigen. The structure of middle T antigen likely affects its stability as well as its capacity to interact with cellular proteins such as the c-src product, and perhaps other substrates. However, recent evidence

suggests that this interpretation may be too simplistic. Courtneidge and Smith, (1984) have shown that the middle T antigen species produced by two non- or weakly-transforming mutants, dl23 and dl2208, become weakly phosphorylated in the in vitro kinase assay but that these same middle T antigens species bind c-src and are able to efficiently phosphorylate the IgG heavy chain of TBR serum (Courtneidge and Smith, 1984). These results suggest that while binding to c-src may be one of the steps involved in the transformation process, it is by no means sufficient to cause transformation. Whether the middle T antigen produced by pPdl2-7 is capable of binding c-src, and whether pPdl2-7 middle T antigen can phosphorylate exogenous substrates such as the IgG heavy chain of TBR serum, has not been assessed. It still remains unclear why the smaller deletion mutants dl23 and dl2208 are inactive in the transformation assay while the large deletion mutant pPdl2-7 retains transforming activity, although the block does not appear to be at the level of simply binding to the c-src protein, because dl2208 and dl23 are still capable of forming a complex with c-src.

In order to directly assess the role of the major site of in vitro tyrosine phosphorylation in transformation, tyrosine 315 was substituted by a serine residue. This was accomplished by oligonucleotide-directed mutagenesis. The resulting mutant recombinant plasmid, pdPbs25, retained activity in the transformation assay. pdPbs25 DNA transformed Rat-1 cells at approximately the same frequency as the mutant pPdl2-7, i.e. 20-35% of wt frequencies. However, unlike transfections with pPdl2-7 DNA, transformed foci appeared five days later, after transfections with pdPbs25 DNA when compared with wt DNA. Cloned populations of transformed cells isolated, after transfection with pdPbs25 DNA, were morphologically indistinguishable from cells transformed with the wt DNA and were able to

grow well in soft agar. In addition, the middle T antigen encoded by pdPbs25 DNA was active in the *in vitro* kinase assay and the majority of the label appeared to be associated with tyrosine residues because incubation of gels containing the ^{32}P phosphate-labelled protein in alkali did not significantly reduce the intensity of the labelled middle T antigen species. Presumably, the pdPbs25 middle T antigen species is phosphorylated at tyrosine 322, an alternate site of tyrosine phosphorylation in middle T antigen (Oostra *et al.*, 1983; B. Schaffhausen, manuscript in preparation). However, this result needs to be confirmed by partial proteolysis mapping with cyanogen bromide. Because cyanogen bromide mapping allows the separation of middle T antigen residue 315 from residue 322, it should also be possible to determine whether serine, occupying the site which is normally the major site of *in vitro* phosphorylation, becomes phosphorylated.

These results are in agreement with those obtained with a recombinant plasmid mutant, pAS131, whose middle T antigen contains a substitution of phenylalanine for tyrosine 315 (Oostra *et al.*, 1983). pAS131 DNA appears to retain wt transforming activity in the transformation assay, although these assays were done at high DNA concentrations which could mask relatively small differences (less then 10-fold) in transforming activity. Like pdPbs25 DNA, foci appear more slowly after transfection with pAS131 DNA than the foci on control plates. In addition, cells transformed by pAS131 DNA are indistinguishable from wt transformants by a number of criteria, including the ability to grow in soft agar as well as forming tumors in animals (Oostra *et al.*, 1983). The pAS131 middle T antigen species retains activity in the *in vitro* kinase assay, and tyrosine 322 was shown to be the major site of tyrosine phosphorylation (Oostra *et*

al., 1983). The results with pdPbs25 and pAS131 are in sharp contrast with the results reported for the viable viral mutant Py1178-T, which contains a substitution of phenylalanine for tyrosine 315 (Carmichael et al., 1984). Py1178-T displays a drastically reduced transforming activity and the middle T antigen encoded by this mutant virus displays a reduced capacity (20% by comparison to the wt protein) to become phosphorylated at tyrosine residues in vitro (Carmichael et al., 1984). However, Carmichael et al., (1984) measured the kinase activity of middle T antigen in extracts prepared from virally-infected baby mouse kidney cells, and these authors used rat F-111 cells to determine the transforming activity of the mutant virus. By contrast, pdPbs25 and pAS131 (Oostra et al., 1983) were tested in the transformation assay using Rat-1 cells, and the kinase activity associated with middle T antigen was tested in the resulting transformed cells. Because of the close similarities of all three mutants it seems likely that the choice of cells (F-111, Rat-1, or baby mouse kidney cells) influences the outcome of the experiments. This could be because the various cell types may produce differing amounts of the c-src product and it is possible that the concentration of this protein is crucial to effect transformation, to allow association with middle T antigen, and consequently to permit phosphorylation of middle T antigen. This question could be resolved by testing the transforming activity of pdPbs25 and pAS131 DNA in F-111 cells.

Whether phosphorylation of middle T antigen at tyrosine residues is sufficient or even necessary for its transforming activity is open to debate. There are mutants of polyomavirus affecting middle T antigen which fail to transform cells, but their middle T antigen are efficiently phosphorylated in vitro (Magnusson et al., 1981; Schaffhausen and Benjamin,

1981; Templeton et al., 1984). Therefore, the capacity of middle T antigen to become phosphorylated in vitro is not sufficient for transforming activity. Whether the capacity of middle T antigen to become phosphorylated in vitro is required for its transforming activity is a more contentious issue. All of the middle T antigens encoded by mutants with lesions (deletions or base substitutions) in middle T antigen coding sequences, which are relatively active in the transformation assay, are efficiently phosphorylated in vitro. Conversely, many mutants with lesions (deletions or base substitutions) in middle T antigen sequences encode proteins which are poorly transforming and which are poor substrates for the in vitro kinase activity. pPd12-7 is the only mutant which transforms cells at high efficiency (20-35% the frequency of wt DNA) yet encodes a middle T antigen which is a poor substrate for the associated tyrosine kinase. The 47K middle T antigen encoded by pPd12-7 lacks the acidic amino acid stretch, tyrosine 315, and tyrosine 322. Nonetheless, the 47K middle T antigen is phosphorylated in vitro, albeit poorly, at tyrosine residues. It appears likely that the weakly phosphorylated 47K species seen in the kinase assay is in fact middle T antigen, although mapping studies have not ruled out the possibility that the 47K species is a cellular protein which becomes phosphorylated at tyrosine residues in immunoprecipitates of the polyomavirus T antigens. If the truncated 47K protein is indeed middle T antigen, then it must be phosphorylated at a tyrosine residue(s) other than tyrosine 315 or tyrosine 322. The V8 protease partial digests of the 47K protein revealed that the site of tyrosine phosphorylation is in the N-terminal region of middle T antigen. There are 11 tyrosine residues between the N-terminus in middle T antigen and tyrosine 315. However, which of these 11 tyrosine serve(s) as sites of phosphorylation

in the 47K middle T antigen is unknown. None of these tyrosines are surrounded by amino acids commonly found N-terminal to sites of tyrosine phosphorylation in viral $p60^{src}$ and other $p60^{src}$-related transforming proteins (Patschinsky et al., 1981; Bishop, 1983). The consensus phosphorylation sequence includes a basic amino acid seven residues to the N-terminal side of tyrosine and one or more acidic residues between this basic amino acid and tyrosine. Only one, but not both, of these features precede the major sites of tyrosine phosphorylation in middle T antigen (315 and 322) thereby making it difficult to choose among the remaining putative, weak sites of tyrosine phosphorylation in the 47K middle T antigen.

In summary, although it is tempting to dismiss the phosphorylation of tyrosine in middle T antigen as an in vitro artifact especially because elimination of the major sites of phosphorylation do not invariably result in a loss of transforming activity, there are other data worthy of consideration before reaching such a conclusion. First, there is evidence that a small fraction of middle T antigen is phosphorylated at tyrosine in vivo (Segawa and Ito, 1982). Second, the weak tyrosine phosphorylation site in the 47K middle T antigen is also a minor site of phosphorylation in the wt middle T antigen. One could argue that the phosphorylation of this site is relevant to the transforming activity of middle T antigen, whereas phosphorylation of tyrosine 315 and/or 322 is not. The physiological significance of the tyrosine phosphorylation of middle T antigen will only be proven when all sites of tyrosine phosphorylation are removed from the protein by mutation. Nonetheless, the results with pPdl2-7 and pdPbs25 demonstrate unequivocally that the major site of tyrosine phosphorylation in polyomavirus middle T antigen is not required for cellular transformation.

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