Structural Analysis of the Polyomavirus Origin for DNA Replication

by

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ABSTRACT

Structural Analysis of the Origin for Polyomavirus DNA Replication

To define the minimal cis-acting sequences required for polyomavirus DNA replication, I subjected recombinant plasmids carrying subgenomic fragments of viral DNA to deletion mutagenesis. This was accomplished by mutagenesis with Bal 31 in vitro, cloning the mutant molecules in Escherichia coli, and analysis of their replicative capacity in permissive mouse cells producing large T antigen. These mouse cell lines, named MOP cells, were isolated by transformation of NIH 3T3 cells with a recombinant plasmid containing the polyomavirus early coding region fused to the SV40 early promoter. From these studies, I concluded that the polyomavirus origin for DNA replication comprises three distinct elements. Two of these elements, termed alpha (α) and beta (β), are functionally redundant and exert their effect in conjunction with a third element termed the core. To identify the functional sequences within each of these elements, I defined their early and late borders in greater detail. The results of my analysis revealed that these elements were composed of multiple sequence motifs.

I also attempted to elucidate the function of the α and β elements in DNA replication. Because the α and β elements contain enhancer and other transcriptional elements within their borders, I examined the effect of their position and orientation on origin function. Like the enhancer elements, the α and β elements function independent of their orientation relative to the core. By contrast to enhancers, the α and β elements could not activate DNA replication when placed away from the core. I propose a model for initiation of polyomavirus DNA replication which takes these observations and those of others into account.

RESUME

Structure de la région d'origine impliquée dans la réplication de l'ADN du virus de polyome.

Dans le but de déterminer les séquences agissant en cis absolument essentielles pour la réplication de l'ADN du virus de polyome, des plasmides recombinants comprenant des fragments subgénomiques d'ADN viral ont été soumis à des mutations par délétion. Ce processes de mutagénèse a été réalisé in vitro avec l'enzyme Bal 31, en clonant les molécules mutantes dans Escherichia coli et en analysant leur capacité de réplication dans des cellules permissives de souris produisant l'antigène large T. Ces lignées cellulaires, désignées cellules MOP, ont été isolées suite à la transformation de cellules NIH 3T3 par un plasmide recombinant comprenant les gènes précoces du virus de polyome sous le controle du promoteur précoce de SV40. Ces études permettent de conclure que l'origine de la réplication du virus de polyome comprend trois éléments distincts. Deux de ceux-ci, appelés alpha (α) et béta (β), sont redondants du point de vue fonctionnel et agissent en conjonction avec un troisième élément appelé core. Une étude des séquences limitrophes précoces et tardives de chacun de ces éléments a permis d'identifier leurs séquences fonctionnelles. Les résultats de cette analyse indiquent que ces éléments sont constitués par des motifs de séquences multiples. Le rôle fonctionnel des élément α et β dans la réplication de l'ADN viral a également été étudié. Du fait que ces éléments α et β comprenent un enhancer et d'autres éléments de transcription, l'effet de leur positin et de leur orientation sur l'origine de la réplication du virus a été examinée. De la même façon que l'enhancer, les éléments α et β fonctionnent quelle que soit leur orientation par rapport au core. Par contre, à la différence des enhancers, ces mêmes éléments α et β n'entrainent pas la replication de l'ADN viral lorsqu'ils sont éloignés du core. A partir de ces observations personnelles et des résultats obtenus par d'autres auteurs, un modèle du mécanisme à l'origine de la réplication de l'ADN du virus de polyome est proposé.

ACKNOWLEDGEMENTS

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. I isolated and characterized several large T antigen-producing mouse cell lines (MOP cells) that support the replication of polyomavirus recombinant plasmids.

2. I demonstrated that the polyomavirus origin of DNA replication was composed of multiple genetic elements.

3. I showed that two of these elements, which were named α and β, are functionally redundant and either of these when coupled to a third element, termed the core, was capable of forming a functional origin.

4. I defined the early and late borders of each replication element.

5. I showed that the α and β replication activators were composed of multiple sequence elements.

6. I demonstrated that the α and β elements could function independent of their orientation relative to the core but did not function when moved away from the core.

7. I showed that the α element could activate viral DNA replication from its native position.

CONTRIBUTIONS BY OTHERS

Chris Mueller constructed and sequenced the PB500 series. In addition, Chris Mueller sequenced the pdPd1300, pdPd1304, pdPd1326, pdPP1(B)R1, pdPd11-8, and pdPBHp mutants. The pdPd1300, pdPd1304, pdPd1326, and pdPd11-8 viral recombinants were constructed by Mike Mes-Masson. Betsy Pomerantz performed the T antigen-binding experiments with the MOP cell lines. All the other work described here was performed by William J. Muller.

LIST OF FIGURES

I. Organization and Structure of the Polyomavirus Genome

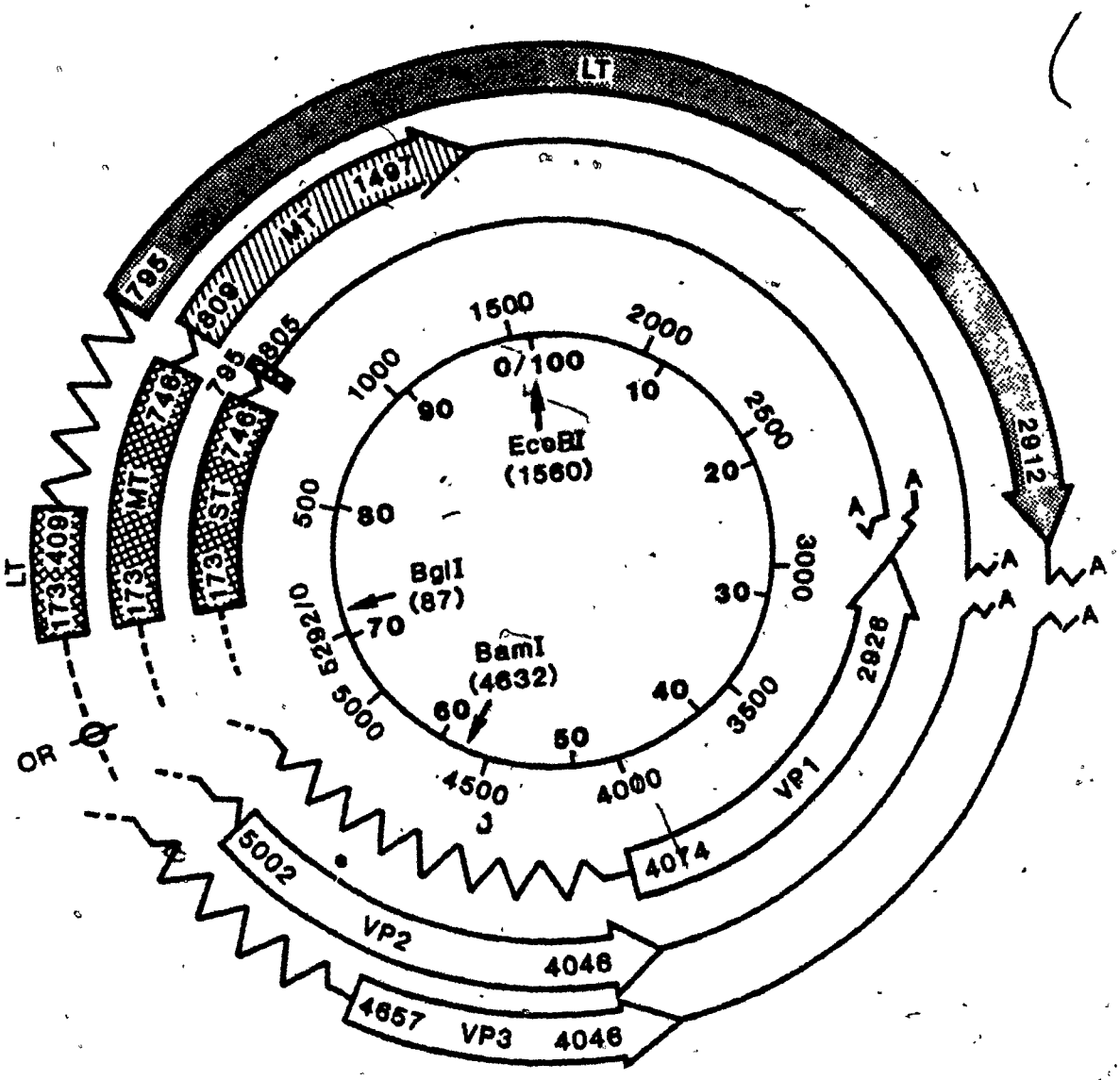
A. Introduction.

Polyomavirus (PyV) belongs to a class of DNA tumor viruses known as the papovaviruses. This group also includes simian virus 40 (SV40), the human papovaviruses (JC and BK) and the papillomaviruses among its members. Papovaviruses are characterized by a double-stranded circular DNA genome and a common virion architecture (Tooze, 1980). PyV was initially isolated by Ludwig Gross. Gross observed that a proportion of Ak mice injected with murine leukemia virus (MLV) extracts developed salivary (parotid) gland adenocarcinomas without showing overt signs of leukemia (Gross et al, 1953a, 1953b). Using differential filtration techniques, Gross was able to isolate a contaminating virus from the MLV stocks. Because of its unusual tumor spectrum, Gross referred to the contaminating virus as the parotid agent. Subsequently, Stewart et al (1958) showed that a variety of other neoplasms were induced when the parotid agent was injected into mice at high doses. For this reason, Stewart et al (1958) proposed that the parotid agent be called polyomavirus. The ability to inject mice with large amounts of virus became possible with the development of mouse embryo cell cultures that could support the replication of PyV. This heralded a new age in virology for it became possible to prepare sufficient amounts of virus to elucidate the structure and genetic organization of viral genome.

These studies revealed that the virions are icosahedral in symmetry and composed of three viral encoded proteins termed VP1, VP2 and VP3. VP1 is the major viral capsid protein whereas VP2 and VP3 proteins are present in the virion only in small quantities (Gibson, 1974; Herrick et al, 1975). Within the virion the circular double-stranded viral DNA is complexed with cellular histones. There are 21 to 26 nucleosomes per viral DNA molecule (Cremisi et al, 1976).

With the advent of DNA sequencing techniques (Maxam and Gilbert, 1977), it became possible to determine the precise nucleotide sequence of the viral DNA (Soeda et al, 1980;Deninger et al, 1980). These studies revealed that the A-2 strain of PyV is composed of 5292 base pairs (bp) (Soeda et al, 1980). The nucleotide numbering system proposed by Soeda et al (1980), which defines the Hpa II 3/5 restriction fragment junction as nucleotide position 1 and proceeds in a clockwise direction around the entire viral genome is used here (Fig. 1).

Figure 1: Physical map of the PyV genome. The nucleotide numbering scheme of Soeda et al (1980) is used and illustrated on the outer portion of the circle. The position of the viral BamHI, BglI and EcoRI restriction endonuclease sites are included for reference purposes. The approximate site at which DNA replication initiates is also included (or). The crosshatched region represents the reading frame of small T antigen (ST) and the N terminal portions of middle T (MT) and large (LT) antigens. The different reading frames of the C terminal portion of MT and LT proteins after the splice are denoted by the slashed and dotted regions respectively. The coding portion of the DNA thought to correspond to the proteins VP1, VP2 and VP3 are also illustrated.


LT
795
MT
1497
809
795
805
748
MT
748
ST
LT
173
409
173
173
0/100
10
20
30
40
50
60
70
80
90
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5292/0
EcoRI
(1560)
BglI
(87)
BamI
(4632)
2912
2928
OR
5002
VP2
4046
4657
VP3
4046
4074
VP1
A
A
A
A

The PyV genome is made up of two transcription units referred to as the early and late regions. The early region is expressed before the onset of viral DNA replication and is transcribed throughout the lytic cycle. It encodes the three tumor (T) antigens of the virus, large, middle and small T antigen. The late transcription unit is expressed principally at the onset and after viral DNA replication has started. It encodes the three viral structural proteins, VP1, VP2, and VP3. The early and late regions are transcribed by RNA polymerase II from opposite DNA strands (Kamen et al, 1974; Kamen and Shure, 1976; Beard et al, 1976; Kamen et al, 1976). The template DNA strands for early and late transcription are referred to as the E and L strands respectively. The sequences comprising the early and late regions and other features resident on the viral genome are illustrated in Figure 1.

B. The early coding region.

The major viral early mRNA starts are clustered between nucleotide positions 148 and 153 (Cowie et al, 1982; Kamen et al, 1982). Like most eucaryotic mRNAs, these viral mRNAs possess a cap structure at their 5' end (Cowie et al, 1982). The 3' end of the early mRNAs are co-terminal and are complementary to sequences downstream of an AAUAAA polyadenylation consensus sequence (Proudfoot and Brownlee, 1976; Soeda et al, 1980). A second polyadenylation site located between nucleotides 1476 and 1481 is infrequently used during the lytic cycle of the virus (Kamen et al, 1980). Following capping and polyadenylation, the primary RNA transcripts are differentially spliced to yield three early mRNAs encoding small, middle and large T antigens. Because these mRNAs use a common translation initiation codon (Soeda et al, 1978) they are translated in the same coding frame for the first 76 amino acids (Fig. 1). After the splice the reading frames for the three T antigen mRNAs change. The small T antigen mRNA remains in the same coding frame after the splice. Because a translational stop codon is situated close to the splice junction in this reading frame, small T mRNA encodes only 4 additional amino acids after the splice. However, the middle and large T antigen reading frames are altered by splicing. The middle T antigen mRNA is translated in a different coding frame than the large T antigen mRNA and encounters a translational stop codon at nucleotides 1498 to 1500 whereas translation termination of the large T mRNA does not occur until it reaches a stop codon between

nucleotides 2913 and 2914. Consequently, the carboxy termini of the three T antigens differ in size and amino acid composition. Nucleotide sequence analysis of the cloned cDNAs of the early mRNAs have confirmed the postulated splice donor and acceptor sites for the early mRNAs (Treisman et al, 1981a). Upon translation, the mature early mRNAs yield small, middle and large T antigen.

The PyV large T antigen is a nuclear phosphoprotein with a calculated molecular weight of 87,991 (Soeda et al, 1980; Deninger et al, 1980). Middle T antigen has a calculated weight of 49,710 daltons and is associated with the cytoplasmic membrane of the cell (Ito et al, 1977; Soeda et al, 1980) whereas the 22,866 dalton small T antigen is found in the cytoplasm of the host cell (Ito et al, 1977).

C. The late coding region.

Initiation of late transcription is highly heterogenous consisting of at least 15 distinct start sites (Cowie et al, 1981) . The RNA starts are distributed across a 94 bp segment of DNA situated between nucleotides 5168 and 5075 (Flavell et al, 1979; Flavell et al, 1980; Cowie et al, 1981). Like the early mRNAs, the late mRNAs are capped and polyadenylated (Kamen and Shure, 1976; Flavell et al, 1979). Late region transcripts are processed from precursor RNAs consisting of tandem repeats of the entire viral genome (Acheson et al, 1971; Birg et al, 1977; Acheson, 1978; Treisman and Kamen, 1981). Multiple copies of a 57 bp leader region are spliced from this precursor onto the body of the late mRNAs (Treisman, 1980). The result of these multiple splicing events is the generation of tandem repeats of the late leader region at the 5' ends of the mRNAs encoding the viral structural proteins. The late mRNAs are translated to yield the major capsid proteins, VP1, and the minor capsid proteins, VP2 and VP3 (Smith et al, 1976; Hunter and Gibson, 1978). VP1 of polyomavirus has a calculated molecular weight of 42,500, whereas VP2 and VP3 have calculated molecular weights of 36,400 and 22,800 repsectively (Soeda et al, 1980).

D. The noncoding region.

Between the early and late coding sequences resides a 465 bp segment of viral DNA that does not encode any known proteins. This region contains the cis-acting sequences required for transcription and replication of the

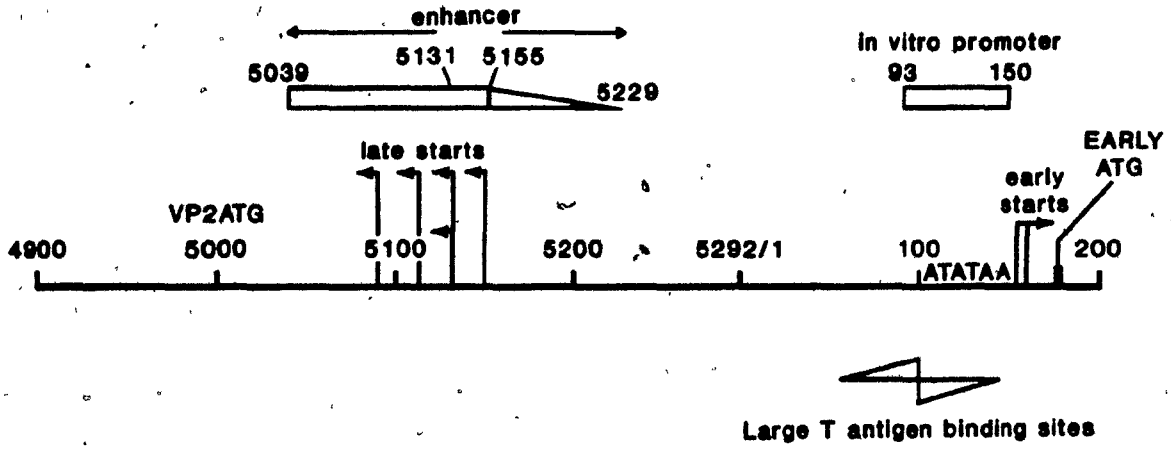
viral genome. These include the promoters for early and late gene transcription and regulation as well as the origin for DNA replication (Fig. 2).

The early promoter is made up of a number of distinct sequence elements. The first of these sequences is located 20 bp upstream of the early mRNA cap sites and is referred to as the Goldberg-Hogness box or "TATA" box (Goldberg, 1978). Although this sequence motif is a conserved feature of a variety of cellular and viral promoters, its deletion or mutation has little effect on viral gene expression in vivo (Bendig et al, 1979; Jat et al, 1982; Mueller et al, 1984). However, the RNA start sites are altered in such mutants suggesting that the "TATA" box is required to specify the precise initiation sites for transcription (Kamen et al, 1982).

Efficient PyV gene expression requires two additional upstream elements. These include a functionally redundant middle element and an enhancer (deVilliers et al , 1981; Tyndall et al, 1981; Jat et al, 1982; Mueller et al, 1984; Herbomel et al, 1984). The middle transcriptional element contains three repeats of the sequence 5'-CCACCC-3' within its borders (Mueller et al, 1984). In this respect it resembles the SV40 21 bp repeat element which contains 6 repeats of the related hexanucleotide sequence, 5'-CCGCCC-3', and is also required for efficient SV40 early and late transcription (Benoist and Chambon, 1981; Fromm and Berg, 1982).

The PyV enhancer has been mapped to a 190 bp region situated between nucleotides 5039 and 5229 (Mueller et al, 1984). Enhancers were operationally defined as transcriptional elements that can augment the expression of a linked gene in an orientation and position independent manner (Moreau et al, 1981; Banerji et al, 1981, Fromm and Berg, 1982). Although the functional sequences that constitute the PyV enhancer have not been defined, several sequence motifs have been identified within its borders that are conserved in other enhancers. These include 5'-GTGGTTT-3' and 5'-AGGAAGTGA-3'. The former has been observed within the enhancer regions of the SV40 early and immunoglobulin heavy chain genes (Weiher et al, 1983; Banerji et al, 1983) whereas the latter has been identified within the enhancer of the Ela transcription unit of human adenovirus type 5 (Hearing and Shenk, 1983). Deletion or mutation of these enhancer core sequences has been observed to have an adverse effect on gene expression (Hearing and Shenk, 1983; Weiher et al, 1983). Thus, these conserved core sequences are also functional components of enhancers.

Figure 2: Physical features in the PyV control region. The nucleotide numbering scheme of Soeda et al (1980) was used. The position of the early mRNA start sites, indicated by the arrows, were derived from Kamen et al (1982), whereas the position of late starts were derived from Cowie et al (1981). The early TATA box is shown near the early mRNA starts. The boxed region above this feature denotes the borders of the in vitro promoter. The high affinity large T antigen binding sites are shown below the line. The ATG for T antigen and the late VP2 translation are also included. The enhancer is represented by the boxed region above the solid numbered line. The early border of the enhancer is denoted by the slanted line. The boundaries of the enhancer and in vitro promoter were derived from Mueller et al (1984). The borders of the T antigen binding sites were derived from Pomerantz et al (1983a).


enhancer
5039
5131
5155
5229
in vitro promoter
93
150
late starts
EARLY
ATG
early
starts
VP2ATG
4900
5000
5100
5200
5292/1
100
ATATAA
200
Large T antigen binding sites

The PyV late promoter appears to be structurally different from the viral early promoter. Late mRNA start sites are heterogenous and are not preceeded by "TATA" boxes (Treisman, 1980; Cowie et al, 1981). Like the early promoter, the late promoter consists of a number of transcriptional elements (Kern et al, 1985; Featherstone, 1986). Interestingly, several of these sequence elements appear to be the same as those required for efficient early gene expression. These include parts of the viral enhancer and the middle transcriptional element (Kern et al, 1985; Featherstone, 1986). In DNA transfection experiments the late promoter is as strong as the early promoter even in the absence of viral DNA replication (Kern et al, 1985; Featherstone, 1986). Taken together, these results suggest that efficient transcription of both the early and late genes is dependent on common transcriptional elements.

II. The Polyomavirus Lytic Cycle

PyV can productively infect most murine cell lines in culture. Murine cells provide a permissive environment for the replication and expression of the viral genome. The early stages of the lytic cycle include the adsorption of the virus to the host cell (Bourgaux, 1964), penetration into the cell, transport to the nucleus and complete uncoating of virions (Mattern et al, 1966; MacKay et al, 1976). The viral early transcription unit is then expressed to yield small, middle and large T antigens through the action of cellular enzymes. When sufficient levels of the early gene products have accumulated, viral DNA replication begins. With the onset of DNA replication, the late region is efficiently expressed to yield the viral structural proteins. During the late phase of the lytic cycle, transcripts derived from the L strand become the dominant viral RNA species.

PyV large T antigen plays a key role in the regulation of viral gene expression. First, it represses its own transcription probably by binding to promoter proximal sites and thereby blocking transcription initiation (Cogen, 1978). Second, large T antigen is required for late gene transcription either by catalyzing viral DNA replication and thereby altering or increasing the template for late gene expression, or by transactivating late gene expression by a mechanism that does not require DNA replication.

The function of small and middle T antigens in the lytic cycle has not been clearly defined. PyV mutants which do not encode small and middle T antigens are restricted in their host range and are defective in transformation (hr-t mutants) (Benjamin, 1970; Staneloni et al, 1977; Benjamin, 1982; Garcea and Benjamin, 1983; Turler and Saloman, 1985). Although the hr-t mutants replicate efficiently in transformed or rapidly growing mouse cell lines, they grow poorly in established cell lines (Benjamin et al, 1970; Staneloni et al, 1977). The defect observed in the hr-t mutants can in part be accounted for by their inability to efficiently replicate their DNA in these cells. Nilsson and Magnusson (1983) observed that PyV hr-t mutants replicate poorly in mouse 3T6 cells. Interestingly, efficient viral DNA replication could be restored by providing PyV small and middle T antigen in trans (Nilsson and Magnusson, 1983). Consistent with these observations are the results of Templeton et al (1986). They found that an altered PyV genome encoding only small T antigen complements viral hr-t mutants for lytic growth in mouse 3T6 cells. Moreover, the amino terminus of middle T antigen can provide a similar complementing activity (Templeton et al, 1986). These results suggest that small T antigen functions in the lytic cycle by promoting viral DNA replication.

The final stages of the lytic cycle involve the assembly of viral chromatin into mature virion particles in the nucleus of the infected cell. After virion assembly, the cell lyses and mature virus particles are released into the extracellular space.

III. Transformation by Polyomavirus

PyV can induce tumors in newborn rodents and transforms a variety of cells in culture. The oncogenic potential of the virus resides in its early region which encodes the three tumor antigens (T antigens) (Hutchison et al, 1976; Ito et al, 1977; Silver et al, 1978; Smart and Ito, 1978). In addition to these gene products, viral mediated transformation is dependent on the host cell. Because mouse cells are permissive for PyV DNA replication, transformation of these cells by PyV is a rare event (Vogt and Dulbecco, 1960; Hellstrom et al, 1962; Todaro and Green, 1965). Consequently, most studies dealing with cellular transformation by the virus have required the use of semipermissive and nonpermissive established fibroblast cell lines.

The individual roles of the T antigens in transformation have not been completely elucidated. The PyV hr-t mutants are completely transformation defective (Benjamin, 1970; Staneloni et al, 1977). Because these mutants contain alterations in the primary structure of the small and middle T genes, either one or both these proteins may be required for cellular transformation (Feunten et al, 1976; Miller and Fried, 1976; Schaffhausen et al, 1978). Consistent with this view is the observation that recombinant plasmids containing the coding sequences for small and middle T antigens efficiently transform rat cells (Hassell et al, 1980; Novak et al, 1980). Recent studies have indicated that functional middle T antigen is absolutely required for efficient viral transformation (Mes and Hassell, 1982; Nilsson et al, 1983). Indeed, a cDNA clone encoding only middle T antigen is capable of efficiently transforming rat cells (Treisman et al, 1981b).

The roles large and small T antigens play in transformation are less well defined. PyV mutants encoding a temperature sensitive large T antigen transform rodent cell lines at much reduced frequencies when placed at the nonpermissive temperature (Fried, 1965; diMayorca et al, 1969; Eckhart, 1969; Della Valle et al, 1981). However, transformed cell lines established at the permissive temperature retain the transformed phenotype when shifted to the nonpermissive temperature (Fried, 1965; diMayorca et al, 1969; Eckhart, 1969; Sief and Cuzin, 1977; Fluck and Benjamin, 1979). These observations are consistent with the hypothesis that large T antigen is required to initiate but not to maintain the transformed state. Large T antigen may function in transforming infections to increase the dose of viral DNA per cell by inducing viral DNA replication (Hassell et al, 1980). Presumably, this would enhance the probability of stable integration of the viral DNA into the cellular DNA. It should be noted that large T antigen is not required during DNA mediated transfections to transform cells (Hassell et al, 1980; Novak et al, 1981; Della Valle et al, 1981). Conceivably, the use of relatively large quantities of DNA obviates the need for the viral large T protein.

Unlike established cell lines, transformation of primary cultures of rat embryo fibroblasts requires the concerted action of large and middle T antigen. Large T antigen is able to immortalize primary cells (Rassoulzadegan et al, 1983), but the activity of functional middle T and large T antigen is required to confer the full transformed phenotype (Rassoulzadegan et al, 1983).

The function of small T antigen in transformation of either primary or immortal cells is obscure. Cell lines that constitutively express small T antigen demonstrate anchorage independent growth and have an altered cytoskeletal morphology (Liang et al, 1984; D. Cook, personal communication). These observations suggest that cells expressing small T antigen exhibit certain features characteristic of transformed cells.

IV. Initiation of Polyomavirus DNA Replication

A. Introduction.

Historically, PyV has served as a model to study the mechanism of DNA replication in mammalian cells. The utility of the virus derives from its small genome and its almost complete reliance on cellular proteins for DNA replication. However, there exists at least one important difference between viral and cellular DNA replication. Unlike cellular DNA replication, the viral genome undergoes multiple rounds of DNA replication during a single cell cycle. This likely reflects a specific viral adaptation to circumvent cellular controls that limit chromosomal DNA replication to one round per cell cycle.

Examination of the replicative intermediates of PyV DNA revealed that replication initiates in a specific region and terminates 180° away (Crawford et al, 1973, 1974). Once initiated, replication proceeds by chain elongation in which one nascent DNA strand appears to grow continuously in the 5' to 3' direction copying a template of opposite polarity. The other strand is synthesized discontinuously also in the 5' to 3' direction from the retrograde template. DNA synthesis on the retrograde arms requires the de novo synthesis of short RNA primers 8 to 10 nucleotides in length which are subsequently removed to allow ligation of DNA fragments to form long nascent strands (Magnusson et al, 1973; Franke and Hunter, 1974). RNA primers also serve to initiate the first DNA chains at the origin of DNA replication (M. DePamphilis, personal communication). Recently, Buckler-White et al (1982) reported that while PyV DNA replication is bidirectional, examination of the replicative intermediates revealed that the two replication forks had travelled unequal distances from the origin. These authors propose that asynchronous intitiation of the two growing forks occurs and that unidirectional synthesis occurs preferentially in the direction of early transcription. A similar phenomenon has been described for the related papovavirus SV40 (Hay and DePamphilis, 1982).

Replication of PyV DNA is likely effected by cellular DNA polymerase α. DNA polymerase α is found associated with actively replicating PyV minichromosomes (Gourlie et al, 1981). In addition, studies with the closely related papovavirus, SV40, have shown that amphidocolin, a specific inhibitor of DNA polymerase α, will block all steps of SV40 DNA replication (Krokan et al, 1979). Therefore it is very likely that DNA polymerase α also replicates PyV DNA.

B. The role of permissive factors in the initiation of PyV DNA replication.

Biochemical and genetic analyses of PyV DNA replication have revealed that viral DNA replication requires at least three components. These include a permissive cellular environment, a viral gene product (large T antigen) and cis-acting viral sequences (ori).

PyV replicates in permissive mouse cells but poorly or not at all in cells derived from other species (Black et al, 1964). Restricted replication of PyV DNA is due to the absence of permissive cellular factors rather than to the presence of inhibitors in nonpermissive cells. This was demonstrated in two ways. First, stably integrated copies of the viral genome could be rescued from nonpermissive cell lines by fusion to permissive cells (Fogel and Sachs, 1969) and second, somatic cell hybrids containing chromosomes from both permissive and nonpermissive hosts support the replication of the virus (Basilico et al, 1970; Basilico and Wang, 1971). Interestingly, the extent of viral DNA replication observed in these cell hybrids correlated well with the number of chromosomes derived from the permissive host (Basilico et al, 1970). These results show that permissive factors are encoded by the genome of the permissive host and act in trans. Studies with synchronized cell populations have indicated that these factors may be expressed in the early S phase of the cell cycle (Pages et al, 1972; Loche, 1979). The nature and mechanism of action of these permissive cell factors is unknown. It is conceivable that these factors interact directly with the viral origin or with the large T antigen to elicit their effect.

C. The role of large T antigen in the initiation of viral DNA replication

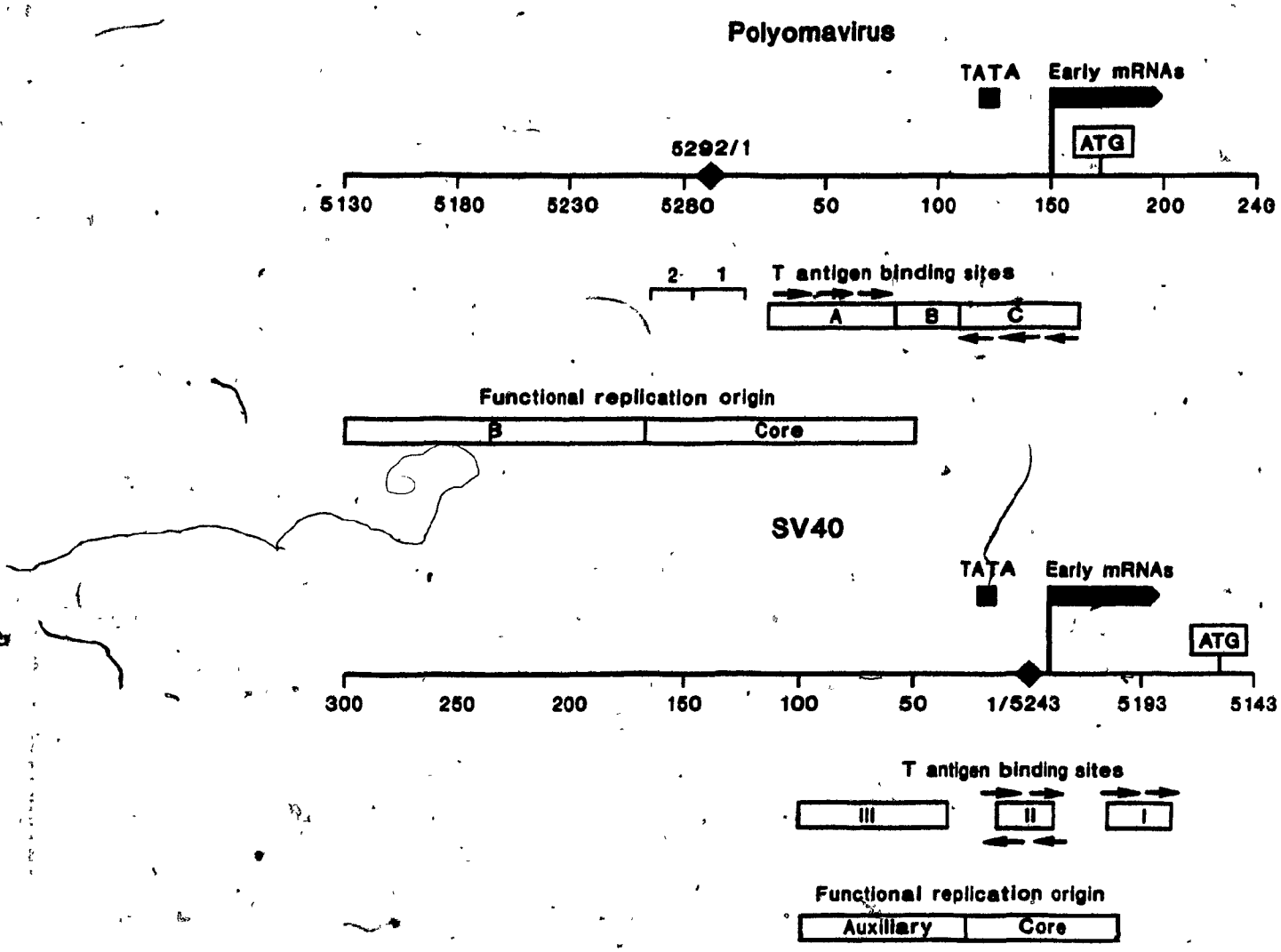
In addition to permissive factors, efficient viral DNA replication requires functional large T antigen. Mutants of PyV that encode a thermolabile large T antigen (Fried, 1965) fail to initiate new rounds of viral DNA replication at the restrictive temperature (Franke and Eckhart, 1973). Large T antigen is located in the nucleus of the host cell and is extensively phosphorylated (Schaffhausen et al, 1978). Although the predicted molecular weight of large T antigen is 87,991, direct measurements of its size by a number of different methods has led to higher estimates between 88,000 and 100,000 daltons (Tooze, 1980).

Two biochemical activities have been ascribed to large T antigen and linked to its role in the initiation of viral DNA replication. These include an ATPase activity and a specific DNA binding activity.

PyV large T antigen binds to multiple sites within the noncoding region. These include at least two high affinity binding sites, termed A and C (also known as sites I and II), located near the origin (Gaudray et al, 1981; Pomerantz et al, 1983a; Dilworth et al, 1984; Cowie and Kamen, 1984, 1986). PyV large T antigen also binds with lower affinity to a region between sites A and C (site B; Fig. 3) and to sites within the origin (sites 1 and 2; Fig. 3) (Cowie and Kamen, 1984, 1986).

Comparison of the sequences within the PyV large T antigen binding sites revealed the occurrence of a common sequence 5'-GAGGC-3' that is repeated within each binding site (Pomerantz et al, 1983a, 1984; Dilworth et al, 1984; Cowie and Kamen, 1984, 1986). Interestingly, the same sequence occurs within the large T antigen binding sties of other papovaviruses like SV40 (Fig. 3). The importance of the pentanucleotide repeat as a recognition sequence for large T antigen is supported by two lines of evidence. First, Pomerantz and Hassell (1984) showed that repetitions of a synthetic oligonucleotide containing this recognition sequence acted as a substrate for binding of PyV and SV40 large T antigens. Pomerantz and Hassell also showed that PyV large T antigen can bind specifically to the same sites in SV40 DNA bound by SV40 large T antigen and vice versa (see also Scheller and Prives, 1985). Second, Cowie and Kamen (1986) have shown that methylation of the guanine residues within this pentanucleotide repeat interferes with binding of large T antigen to its recognition site.

Figure 3: PyV and SV40 large T antigen binding sites relative to other control sequences. The regulatory regions of PyV and SV40 are shown. The nucleotide numbering scheme of Soeda et al (1980) was used for PyV and that of Buchman et al (1980) was used for SV40. The solid diamond represents the junction between the first and last nucleotide in the DNA sequence. The -TATA- consensus sequence and the 5' termini of abundant early mRNA are shown by the solid boxes (Tooze, 1980). The direction of early transcription is from left to right as shown by the arrow on the solid box representing the early mRNAs. The putative initiation codons for translation of early mRNAs are boxed. The large T antigen binding sites are illustrated by open boxes. The PyV low affinity T antigen binding sites are shown by the brackets. The boundaries of the PyV large T antigen binding sites were taken from Cowie and Kamen (1984) and those of SV40 were taken from DeLucia et al (1983). The arrows above and below the large T antigen binding sites refer to the -GAGGC- sequence, and their position above or below the box refer to their location on one or the other strand. The minimal DNA sequences which function as replication origins are also shown as open boxes. For each virus there are a minimum of two sequence domains comprising the functional origin. For PyV these are referred to as β and core, whereas for SV40 they are referred to as auxiliary sequences and core. The borders of these sequences are taken from Muller et al (1983a) for Py and from Myers and Tjian (1980), DiMaio and Nathans (1980) and Bergsma et al (1982) for SV40.


Polyomavirus
TATA
Early mRNAs
ATG
5292/1
5130
5180
5230
5280
50
100
150
200
240
2
1
T antigen binding sites
A
B
C
Functional replication origin
β
Core
SV40
TATA
Early mRNAs
ATG
300
250
200
150
100
50
1/5243
5193
5143
T antigen binding sites
III
II
I
Functional replication origin
Auxiliary
Core

There is little evidence with PyV to support the contention that large T antigen must bind to the origin to initiate viral DNA replication. However, analyses of mutants of SV40 strongly support this view. Mutants with base pair substitutions within SV40 large T antigen binding site II are defective for viral DNA replicaiton (Shortle and Nathans, 1979; DiMaio and Nathans, 1980). Revertants of these mutants, which are capable of productively replicating their DNA (Shortle et al, 1979), contain second site mutations within large T coding sequences that allow the altered protein to recognize the mutant origin (Margolskee and Nathans, 1984). Interestingly, these revertants contain mutations within the same domain of large T antigen as several replication defective mutants of SV40 (Gluzman and Aherns, 1982; Stringer, 1982; Manos and Gluzman, 1984; Kalderon and Smith, 1984; Pipas et al, 1985). The latter mutants encode large T antigens that are incapable of effectively binding to the viral origin (Prives et al, 1983; Clark et al, 1983; Manos and Gluzman, 1985; Paucha et al, 1986). From the analysis of these mutants, the SV40 large T antigen origin binding domain was mapped between amino acid residues 139 and 220 (Paucha et al, 1986). Interestingly, a region in PyV large T antigen exhibits considerable amino acid homology to the SV40 origin binding domain (Paucha et al, 1986). It is probable that this conserved feature of the two T antigen molecules allows them to bind to similar DNA sequences.

Although the physical interaction of PyV large T antigen with the origin is likely required for the initiation of viral DNA replication, it is not by itself sufficient for viral DNA replication. In fact, there exist mutants of PyV, which encode large T antigens that are capable of specifically binding to the viral origin, but are defective for the initiation of viral DNA replication (Hayday et al, 1983; Nilsson and Magnusson, 1984).

PyV large T antigen is capable of hydrolyzing ATP into ADP and Pi (Gaudray et al, 1980). It is not known whether this acitvity is required for the initiation of PyV DNA replication. However, studies with SV40 large T antigen have shown that its ATPase acitivity is closely associated with its replication function. In fact, SV40 large T mutants that are defective in ATPase activity are also incapable of initiating viral DNA replication (Clark et al, 1983; Manos and Gluzman, 1984; Manos and Gluzman, 1985). The origin binding and ATPase domains of SV40 large T antigen are

likely distinct because mutant large T antigen molecules defective in ATPase activity are capable of specifically binding the origin (Clark et al, 1983; Manos and Gluzman, 1985). Although the ATPase and origin binding activities of SV40 large T antigen are required for the initiation of viral DNA replication, replication defective large T antigens that possess both these activities have been reported (Manos and Gluzman, 1985). These observations suggest that these two activities are necessary but not sufficient for the initiation of viral DNA replication. The initiation of viral DNA replication may require other features of large T antigen that have not yet been discovered.

D. Cis-acting sequences required for the initiation of PyV DNA replication.

A third requirement for the initiation of PyV DNA replication is a functional origin. The limits of the PyV origin were initially inferred from the structural analyses of the DNA of viable deletion mutants (Bendig and Folk, 1979; Griffin and Maddock, 1979; Magnusson and Berg, 1979; Wells et al, 1979; Luthman et al, 1982) and evolutionary variants of the virus (Fried et al, 1975; Lund et al, 1977). These studies revealed that a region encompassing the noncoding sequences contains all the signals required for the replication of viral DNA but the entire noncoding segment is not required. These nonessential regions include a 100 bp stretch between the initiation codon for T antigen synthesis and the putative initiation site of viral DNA replication (Bendig and Folk, 1979; Griffin and Maddock, 1979; Magnusson and Berg, 1979; Wells et al, 1979) and a 134 bp region to the late side of the putative origin (Luthman et al, 1982).

More recently, recombinant DNA techniques and reverse genetics have been applied to map the PyV origin. The application of these methods to PyV showed that the origin is made up of at least two noncontiguous regions (Tyndall et al, 1981; Luthman et al, 1982; Muller et al, 1983a). One of these segments bears striking homology to the SV40 minimal origin (Soeda et al, 1978, 1979). This region, which I named the core (Muller et al, 1983a), contains several interesting sequence features. These include a stretch of 15 adenine:thymine residues (A:T) on its late border, a guanine:cytosine (G:C) rich palindrome comprising 34 bp in the centre of the core, and a stretch of about 30 bp near the early border that bears little resemblance to SV40 DNA and is devoid of any notable sequence

motifs. The palindrome contains several GAGGC motifs comprising large T antigen binding sites 1 and 2 (Fig. 3) (Cowie and Kamen, 1984, 1986). In addition to the core, sequences near the late region are also required to form a functional origin. This was shown by the analysis of the replication properties of viable mutants of PyV (Tyndall et al, 1981; Luthman et al, 1982) or viral recombinant plasmids (Muller et al, 1983a). This same region is also required for efficient early gene transcription and forms part of the enhancer (deVilliers and Schaffner, 1981; Jat et al, 1982; Mueller et al, 1984; Herbomel et al, 1984). Because these cis-acting replication sequences map to the same region as the viral enhancer, it was proposed that the same sets of sequences effect both transcription and DNA replication (Tyndall et al, 1981; Muller et al, 1983a).

V. Experimental Rationale

PyV is an ideal model system to study the initiation of DNA replication in mammalian cells. At the time these experiments were started little was known about the genetic organization of the PyV origin. For this reason, I decided to identify, by deletion mapping, the cis-acting sequences required for the initiation of PyV replication as a prelude to elucidating the mechanism of replication initiation. To facilitate the identification of the origin, I isolated several permissive mouse cell lines (MOP cells) that are capable of providing functional large T antigen and permissive factors in trans. In this way the replication properties of mutant viral recombinant could be attributed solely to cis-acting sequences because large T antigen and permissive factors are constitutively produced by the MOP cells.

To map the borders of the origin, a number of viral recombinant plasmids were constructed and assessed for their capacity to replicate in MOP cells. The advantage of this approach is that potentially cis-defective viral origins can be propagated in Escherichia coli and subsequently tested for their capacity to replicate in the MOP cell lines. To localize the origin to a small DNA segment, PyV recombinants containing portions of the viral genome were assessed for their capacity to replicate in MOP cells.

My analyses revealed that the viral origin comprises three sets of sequences. Two of these, termed alpha (α) and beta (β), are functionally redundant and work in conjunction with a third element termed the core. To

identify the active sequences within each element, I first defined the borders of the elements. This was accomplished by mutagenesis with Bal 31 nuclease *in vitro*, cloning of the mutant molecules, DNA sequencing and analysis of their replicative abilities in MOP cells. The results of these analyses have allowed me to define various sequences required for origin function.

I have also attempted to elucidate the mechanism of action of the α and β elements. First, I examined whether the α and β elements could function independent of their position or orientation relative to the core. The results of these experiments show that the α and β elements act independent of orientation but dependent on their position relative to the core region. Second, I introduced multiple point mutations within different regions of the α and β elements and measured the effect of these mutations on origin function. The results obtained show that α and β regions are composed of a mosaic of complementing sequence elements. I propose a model for the initiation of PyV DNA replication which takes into account my observations and those of others.

MATERIALS AND METHODS

A. Cell culture.

MOP and NIH 3T3 cell lines were grown on plastic dishes with Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) calf serum and antibiotics, and maintained at 37°C in a humidified CO_2 atmosphere. The Rat-1 cell line was treated in an identical manner except that they were grown in DMEM supplemented with 10% fetal calf serum. Cells were passaged by trypsinization after reaching confluence and were replated at approximately 10^4 cells per cm^2.

B. Preparation of DNA and its modification.

Recombinant plasmid DNAs were isolated from bacteria and purified by CsCl density centrifugation. Digestions of these DNA with restriction endonucleases were performed in accordance with the conditions specified by their manufacturers.

Reaction of the Klenow fragment of DNA polymerase I was performed by incubating 1 µg of DNA in a volume of 50 µl with 10 mM Tris-HCl pH 7.6, 1 mM dithiothreitol, the appropriate deoxynucleotide triphosphates (.1 to 1 mM), and 5 U of the Klenow fragment of *Escherichia coli* DNA polymerase I for 1 hour (h) at 15°C. The reaction was terminated by sequential phenol and chloroform-isoamyl alcohol (24:1, vol/vol) extraction, and the DNA was isolated by ethanol precipitation. Digestion of DNA with Bal 31 nuclease and ligation were carried out as described previously (Mes and Hassell, 1982).

C. Construction of recombinant plasmids.

The recombinant plasmids that served as substrates for mutagenesis or replication were derived from molecules whose construction has been described previously (Hassell *et al*, 1980; Mes and Hassell, 1982). I utilize the abbreviation pd (plasmid deleted) to denote those recombinant molecules whose plasmid sequences are derived from pML-2 DNA (Lusky and Botchan, 1981). Wherever possible I have tried to name the recombinant plasmids according to their composition. For example, pdPB1a refers to plasmid deleted (pd) polyomavirus (P), BamHI (B), isolate 1a. The clones pdPB1a, and pdPR1a were isolated by cloning the BamHI (pdPB1a) or EcoRI (pdPR1a) viral genomic fragments into the corresponding sites in pML-2.

The parent plasmid for many of the mutants described here was pPBR2 which is composed of the 2,220 base pair (bp) polyomavirus BamHI (nucleotide 4632)-to-EcoRI (nucleotide 1560) fragment and the large BamHI-to-EcoRI fragment of the plasmid pMK16.1 (Mes and Hassell, 1982). pPBR2 was modified by insertion of HindIII linker at the unique BglI within polyomavirus sequences (nucleotide 87) to create pPin7. This was accomplished by linearizing pPBR2 DNA with BglI, removing the 3 bp 3' extensions with the exonucleolytic activity with the Klenow fragment of E. coli DNA polymerase I, and ligating a synthetic HindIII linker to the blunted ends. After HindIII cleavage, ligation, and transformation of E. coli, the plasmid DNAs were screened, and one that contained a HindIII site in place of the BglI site was isolated and named pdPin7. This mutant DNA was sequenced across the site of insertion of the linker by the chemical method (Maxam and Gilbert, 1977). A 3 bp stretch of viral DNA (nucleotides 91, 92 and 93) has been removed and replaced with 10 bp of linker DNA. Both pPBR2 and pPin7 served as substrates for the isolation of viral DNA that were cloned in pML-2 DNA.

pdPBR2 was constructed by transfer of the polyomavirus sequences from pPBR2 to pML-2. This was accomplished by cleavage of both DNA species with BamHI and EcoRI and ligation of the viral species to the pML-2 vector sequences. The resulting recombinant plasmid, pdPBR2, contains the viral sequences from nucleotide 4632 to 1560 cloned between the BamHI and EcoRI sites of pML-2.

pdPBBg(H) was constructed by ligating the viral BamHI-to-HindIII fragment from pPin7 to pML-2 cut with these same enzymes. pdPBBg(H) contains the viral sequences from nucleotide 4632 to 90. pdPP1(B)Bg(H) was derived from pdPBBg(H) by cleavage with BamHI and extension of the 3'-recessed ends by incubation with the Klenow fragment of E. coli DNA polymerase I and the four deoxynucleotide triphosphates. The blunt-ended moleucles were then partially digested with PvuII to remove the viral sequences between nucleotides 4632 (the BamHI site) and nucleotides 5130 (the PvuII site). Circularization of the blunt ends by ligation with T4 ligase led to the isolation of pdPP1(B)Bg(H). This procedure resulted in the placement of a BamHI site at nucleotide 5131 of the viral insert. Sequencing of the viral-plasmid junction with pdPP1(B)Bg(H) confirmed that the viral sequences between nucleotides 5131 to 90 are contained within this recombinant at a BamHI-to-HindIII fragment. pdPP2(B)Bg(H) was

isolated in the same manner as pdPP1(B)Bg(H) except that it contains a BamHI site at the viral PvuII site located at nucleotide 5265. The pdPP2(B)Bg(H) recombinant plasmid contains the viral sequences between nucleotides 5265 and 90 within the BamHI-to-HindIII sites of pML-2. The recombinant plasmid, pdPP1(B)P2(X)Bg(H), was derived from pdPP1(B)Bg(H) by cleavage at the viral PvuII site (nucleotides 5265) and ligation of a XhoI synthetic linker to the blunt termini. After recircularization of the DNA with T4 ligase, transformation of E. coli and screening of the resulting colonies, one of the DNAs contained a unique XhoI site at the viral PvuII site (nucleotide 5265).

pdPP1(B)RI was constructed by ligation of the viral HindIII-to-EcoRI sequences from pPin7 (the sequences between the nucleotides 90 and 1560) between the HindIII and EcoRI of pdPP1(B)Bg(H) DNA. pdPP1(B)RI contains those polyomavirus sequences between nucleotides 5131 and 1560 and a 10 bp HindIII linker between nucleotides 90 and 94.

pdPBP1 was derived from pdPBR2 by digestion with BamHI and extension of the 3'-recessed ends after incubation with the Klenow fragment of E. coli DNA polymerase I and the four deoxynucleotide triphosphates. These molecules were then cleaved with PvuII to generate partial products, circularized with T4 ligase, and used to transform E. coli. After screening a number of colonies, we identified several that contained a recombinant plasmid from which viral sequences had been deleted between the BamHI site (nucleotides 4632) and the PvuII site at nucleotide position 5262. A BamHI site was created at the new junction between viral and plasmid sequences to yeild pdPBP1, which carries viral sequences between nucleotides 5265 (a BamHI site) and nucleotides 1560 (an EcoRI site) within pML-2 DNA.

The recombinant plasmid pdPBHp contains the viral BamHI site (nucleotide 4632)-to-HphI site (nucleotide 152) DNA fragment between the pML-2 BamHI and HindIII sites. The BamHI site was restored, but the HindIII and HphI sites were destroyed during the construction of pdPBHp. In short, pPBR2 DNA was digested with HphI, and the 3'-projections were removed with the Klenow fragment of E. coli DNA polymerase I. The DNA fragments were then reacted with BamHI, and the fragment containing the viral sequences enclosed by the BamHI (nucleotide 4632) and the HphI site (nucleotide 152) was isolated by agarose gel electrophoresis. This viral fragment was then ligated to pML-2 DNA that had been cleaved sequentially

with HindIII and BamHI. Before cleavage with BamHI, the vector was digested with HindIII and the 3'-recessed ends were extended by reaction with the Klenow fragment of DNA polymerase I and the four deoxynucleotide triphosphates. Ligation of vector and viral sequences to each other resulted in the formation of pdPBHp. The predicted sequences about the virus-plasmid joints were confirmed by sequencing them.

pdPBHp632 was derived form pdPBHp by partial digestion with PvuII followed by ligation to blunt ends with BamHI linkers. After transformation of E. coli, a colony contianing a plasmid with a BamHI linker at the PvuII site at nucleotide 5128 was isolated. This plasmid DNA was cleaved to completion with PvuII and a BglII linker was ligated to blunt ends. The resulting plasmid, pdPBHP632, contains BamHI sites located at nucleotides 4632 and 5128 (formerly a PvuII site), and a BglII site at nucleotide 5265.

The construction and final structure of pPSVE1 and pPH1-8 have been described elsewhere (Mes and Hassell, 1982; Featherstone et al, 1984). pPSVE1-B1a was constructed by ligating the carboxy-terminal coding sequences for large T antigen, the EcoRI-to-BamHI (nucleotides 1560 to 4632) DNA fragment from pdPB1a (a genomic clone of polyomavirus cloned as a BamHI fragment in pML-2 DNA), to EcoRI cleaved pPSVE1 DNA. pPSVE1 comprises pBR322 DNA and the SV40 early promoter (the SV40 HindIII C fragment) fused to the coding sequences for small and middle T antigen (polyomavirus nucleotides 154 to 1560). After ligation of the EcoRI-terminated ends with T4 ligase, the linear molecules were treated with the Klenow fragment of E. coli DNA polymerase I in the presence of the four deoxynucleotide triphosphates to fill in the single stranded ends and the molecules circularized with T4 ligase. The reaction mixture was then used to transform E. coli HB101 to ampicillin resistance, and individual colonies were isolated. Several transformed colonies were screened by restriction endonuclease analysis for the desired recombinant plasmid. After these colonies were found, one was chosen for further study. The structure of the plasmid DNA carried by these bacteria was confirmed by digestion with many restriction endonucleases.

D. Isolation of mutant recombinant plasmids.

The mutants that carry unidirectional deletions from the BamHI site (nucleotide 4632) in pdPBR2 were, with two exceptions, constructed by Bal

31 nuclease digestion of BamHI linearized pdPBR2 DNA. After reaction with the nuclease, a synthetic BamHI linker was ligated to the ends of the molecules, and they were then cleaved with BamHI and EcoRI. The BamHI and EcoRI terminated viral sequences were cloned within these same sites in pML-2 DNA. Each mutant DNA was sequenced by the chemical method to determine the end point of the deletion (Maxam and Gilbert, 1977). Two mutants in this series, pdPP1(B)Bg(H) and pdPBP1 (Fig. 9), were constructed as described previously.

pdPB503Bg(H) was derived by cleaving one of the mutants described above, pdPB503, with BglI. After incubation with the Klenow fragment of E. coli DNA polymerase I to remove 3 bp BglI 3' extension, a HindIII synthetic linker was ligated to the blunted ends. The DNA was then cleaved to completion with BamHI and HindIII and the BamHI-to-HindIII terminated viral sequences cloned into these same sites in pML-2. The resulting recombinant, pdPB503Bg(H), contains the viral sequences between 5039 and 90 within the BamHI and HindIII sites of pML-2.

The mutant DNAs that bear deletions within viral sequences from the EcoRI site in pdPBR2 DNA were constructed as described previously, with one exception. pdPBS1 was isolated after cleavage of pdPBR2 DNA with SstI and EcoRI. The SstI 3' projections and the EcoRI 3'-recessed ends were removed and extended with the Klenow fragment of DNA polymerase I in the presence of dATP, dGTP, and TTP. Intramolecular ligation across these ends resulted in the creation of an EcoRI site at the junction. pdPBS1 contains those viral sequences between nucleotides 4632 and 569 as a BamHI-to-EcoRI fragment within the large BamHI-to-EcoRI segment of pML-2 DNA.

Mutants with deletions internal to viral sequences were constructed as described below. pdPd11-8 was isolated after deletion of sequences about the unique BglI in pdPBR2 with Bal 31 nuclease (Hassell et al, 1982). The BamHI-EcoRI viral fragment bearing the deletion was subsequently cloned between the BamHI and EcoRI sites in pML-2 to yield pdPd11-8. Sequences between nucleotides 1 and 158 have been deleted from the viral insert.

pdPd1300 was derived from pdPBR2 by partial cleavage with PvuII followed by intramolecular ligation with T4 ligase. Among the bacteria transformed with this DNA were several that contained a recombinant plasmid with a deletion of the PvuII fragment between nucleotides 5130 and 5265. pdPd1304 and pdPd1326 were isolated after partial cleavage of pdPd1300 with PvuII, followed by digestion with Bal 31 nuclease. The eroded linear DNA

was then circularized with T4 ligase and used to transform E. coli. After screening a number of recombinant plasmids from independent colonies, I identified several with deletions about the PvuII site at the deletion junction (5130/5265) in pdPd1300 DNA. Sequencing across the deletions of two mutants revealed that pdPd1304 is deleted between nucleotides 5130 and 5277, whereas pdPd1326 is deleted between nucleotides 5126 and 5265.

The pdP500d1300 mutants (Fig. 11) were assembled by digesting pdPBBg(H) DNA (Fig. 10) to completion with BamHI and PvuII. The largest DNA fragment composed of pML-2 DNA and the viral sequences between nucleotides 5265 (a PvuII site) and 90 (a former BglI site, now a HindIII site), was purified by agarose gel electrophoresis and ligated to the gel-purified BamHI-to-PvuII fragments of viral DNA from the pdPB500 mutant series (Fig. 9).

The vector for assembly of the early and late alpha, beta and core deletions was constructed by cleavage of pML-2 DNA with BamHI and digestion of the linearized DNA with Bal 31 nuclease. After reaction with the nuclease, a synthetic XhoI linker was ligated to the ends of the molecules. DNA sequencing of deletion junction revealed that the plasmid sustained a deletion between nucleotides 465 and 325 in pBR322 sequences (Sutcliffe, 1979). The mutant bearing the XhoI site was then cleaved at its unique SalI restriction site and the 3'-recessed ends blunted with the Klenow fragment of E. coli. After ligation of BamHI synthetic linkers to the termini and digestion with BamHI, the molecule was recircularized with T4 DNA ligase. The resultant recombinant, pMLXB, contains a unique BamHI site (previously the SalI site) and a XhoI site at the deletion junction (fomerly the BamHI site).

Mutant recombinant plasmids that carry unidirectional deletions from the BamHI site (nucleotide 5131) in pdPP1(B)Bg(H) (ΔLB series; Fig. 16) were constructed by Bal 31 nuclease digestion of BamHI linearized pdPP1(B)Bg(H) DNA. After reaction with the nuclease, a synthetic XhoI linker was ligated to the ends of the molecules and the DNA was digested to completion with XhoI and HindIII. The XhoI and HindIII terminated viral sequences were then cloned between these same sites in pMLXB. The ΔLA mutants were constructed in a similar manner as the ΔLB deletion series except that these mutants bear deletions originating from the BamHI site (nucleotides 5039) in pdPB503d1300 DNA.

The ΔEB deletion series was derived by cleavage of pdPP1(B)Bg(H) with PvuII followed by digestion with Bal 31 nuclease. After ligation of synthetic XhoI linkers to the ends of the molecules, the DNA was then cleaved with BamHI and XhoI. The viral BamHI-to-XhoI fragments were then gel purified and ligated to the large BamHI-to-XhoI segment of pMLXB. Several colonies containing plasmids bearing viral inserts were isolated. The mutants ΔEB5240, ΔEB5223, ΔEB5173 and ΔEB5170 were derived by inserting the viral XhoI-to-HindIII (nucleotides 5265 to 90) fragment from pdPP1(B)P2(X)Bg(H) into the XhoI and HindIII sites of the aforementioned viral recombinants. Two other viral recombinants, ΔEB5202 and ΔEB5209, were isolated by directly cloning the mutated viral BamHI-to-XhoI fragments into the same sites in pdPP1(B)P2(X)Bg(H).

The ΔEA mutants were constructed in a similar manner as ΔEB5202 and ΔEB5209, except that these recombinants contain unidirectional deletions originating from the PvuII site (5130) of the plasmid, pdPB503d1300.

Mutants that carry undirectional deletions from the HindIII site (nucleotide 90) of pdPP1(B)Bg(H) were isolated by digestion of HindIII cleaved pdPP1(B)Bg(H) DNA with Bal 31 nuclease. After nuclease digestion, a synthetic XhoI linker was ligated to the ends of the molecule and the DNA was cleaved to completion with XhoI and BamHI. The BamHI-to-XhoI viral restriction fragments were then ligated into the BamHI and XhoI sites of pMLXB. Each mutant DNA was sequenced by the chemical method (Maxam and Gilbert, 1977) to determine the endpoint of the deletion.

E. Construction of the inversion and position mutants.

The inversion mutants, pdPBP1-5 and pdPBP5-1 (Fig. 25), were constructed by ligation of the viral BamHI (4632)-to-PvuII (5130) fragment of pdPBR2 to the BamHI-digested pdPBP1 DNA. After the BamHI cohesive ends of both species were joined, the molecules were reacted with the Klenow fragment of E. coli DNA polymerase I and the four deoxynucleotide triphosphates to fill in the remaining BamHI ends. The DNA was then circularized by ligation across the filled-in BamHI and PvuII ends and the DNA used to transform E. coli. After screening and characterizing the plasmid DNA from a number of colonies, I used two, pdPPB1-5 and pdPP5-1, for the experiments reported here. An identical strategy enabled me to clone the viral BamHI (4632)-to-PvuII (5130) fragment in both orientations at the BamHI site of pdPP1(B)Bg(H) (Fig. 10) to generate the recombinant plasmids pdPP1P1-5 and pdPP1P5-1 (Fig. 24).

The beta inversion mutants were constructed by ligating the small BamHI (5131)-to-BglII (5265) from pdPBHP-632 plasmid DNA to this same DNA cleaved with BamHI and BglII. After screening and characterizing the plasmid DNA from a number of colonies, two recombinants, pdPB632β+ and pdPB632β- bearing the BamHI (5131) to BglII (5265) fragment in opposite orientations were isolated.

The pdPB503Bg(H)α+ and pdPB503Bg(H)α- viral recombinants were isolated by ligating the BamHI to PvuII fragmen (5039 to 5130) isolated from pdPB503Bg(H) d1300 DNA to BamHI cleaved pdPP2(B)Bg(H) DNA. After the BamHI cohesive termini were joined, the molecules were reacted with the Klenow fragment of E. coli DNA polymerase I and the four deoxynucleotide triphosphates to fill in the remaining BamHI end. The DNA was then circularized by ligation across the blunt PvuII and BamHI ends and used to transform E. coli. These manipulations resulted in the placement of a BamHI site at the BamHI/PvuII junctions. After screening and characterizing the plasmid DNAs derived from a number of colonies, two recombinant plasmids containing the BamHI (5039)-to-PvuII (5130) in the + and - orientation next to the viral core sequences (nucleotides 5265 to 90) were isolated.

The late side position mutants (Fig. 27) were constructed in two steps. First, the XhoI-to-HindIII fragment (nucleotides 5265 to 90) from pdPP1(B)P2(X)Bg(H) was ligated to the large XhoI-to-HindIII segment of pMLXB DNA. The resultant recombinant plasmid DNA termed pdPP2(X)Bg(H), was then cleaved with BamHI and ligated to the BamHI (5039)-to-PvuII (5130) fragment from pdPB503d1300. After ligation across the cohesive BamHI termini, the remaining 3'-recessed BamHI end was back-filled with the Klenow fragment of E. coli DNA polymerase I in the presence of the four deoxynucleotide triphosphates. The DNA was recircularized with T4 ligase and used to transform E. coli. Several drug resistant colonies were screened by restriction endonuclease analysis for the desired recombinant plasmids. Two colonies containing plasmid DNAs bearing the BamHI-to-PvuII (5039-5130) in opposite orientations 185 bp away from the core (5265-90) were isolated. As a result of these manipulations, a BamHI site was generated at the BamHI and PvuII junctions. An identical strategy enabled me to clone the BamHI-to-PvuII fragments from pdPP1(B)Bg(H) (nucleotides 5131 to 5264) and pdPB503Bg(H) (nucleotides 5039-5265) in both orientations at the BamHI site of pdPP2(X)Bg(H).

The early side position mutants were constructed in a similar manner except that the vector pdPP2(X)Bg(H) was modified by introducing a BclI linker at the plasmids EcoRI site (Fig. 27). This was accomplished by cleaving pdPP2(X)Bg(H) DNA with EcoRI and back-filling the 5' extensions with the Klenow fragment of E. coli DNA polymerase I and the four deoxynucleotide triphosphates. After ligation of a synthetic BclI linker to the ends of the molecules, the DNA was used to transform the dam methylase deficient Gm 119 strain of E. coli. The resulting recombinant plasmid was cleaved with BclI and the viral BamHI fragments derived from the late side position mutants were ligated into this site. After transformation of the DH-1 strain of E. coli and screening the colonies, six viral recombinant plasmids containing these restriction fragments in both orientations at the BclI site were isolated.

F. Construction of the linker scanning and insertion mutants.

The mutant LS 5151/5166 (Fig. 21) was constructed in two steps. First, the BamHI (4632)-to-HindIII (5151) fragment from pdPd1801 (Mueller et al, 1984) was ligated to BamHI and XhoI digested ΔLB 5166 DNA. After ligation across the cohesive BamHI ends, the XhoI and HindIII ends were rendered blunt with the Klenow fragment of E. coli DNA polymerase I in the presence of the four deoxynucleotides and recircularized with T4 ligase. After transformation of E. coli with the ligation mixture a colony bearing the desired recombinant was isolated. In order to generate LS 5151/5166, this recombinant plasmid was cleaved partially with PvuII and digested to completion with BamHI. The large restriction fragment containing the viral sequences between nucleotides 5131 and 90 was gel purified and the BamHI ends filled in with Klenow fragment of E. coli DNA polymerase I. After ligation across the blunt PvuII and BamHI termini, the DNA was used to transform E. coli. Several colonies were screened and one containing the plasmid LS 5151/5166 was used for the experiments reported here.

The mutant LS 5173/5188 was constructed by ligating the BamHI (5131)-to-XhoI (5173) fragment from ΔEB5173 into the same sites in ΔLB5188 DNA. It should be noted that nucleotide sequence analysis of ΔEB5173 revealed that in addition to a XhoI linker it contained a 6 bp insert unrelated to the viral DNA sequence. Conceivably, this insert resulted from the ligation of a mutated XhoI linker to the ends of the molecule.

The LS 5202/5211 mutants was constructed by ligating the viral XhoI (5211)-to-HindIII (90) fragment from ΔLB5211 to XhoI and HindIII cleaved ΔEB5202 DNA. An identical procedure was used to generate the LS 5209/5218 mutant where the XhoI (5218)-to-HindII (90) fragment derived from ΔLB5218 was recombined with XhoI and HindIII cleaved ΔEB5209 DNA.

The LS 5240/5248 mutant was isolated by ligating the BamHI (5131)-to-XhoI (5240) fragment from ΔEB5240 to the same sites in ΔLB5248 DNA.

The mutant pdPB5075 LS 5151/5166 was constructed by ligating the BamHI (5131)-to-HindIII (90) fragment from LS 5151/5166 to PvuII and HindIII digested pdPB507Bg(H) DNA. The pdPB5075 Bg(H) vector contains viral sequences between nucleotides 5075 and 90 within the BamHI and HindIII sites of pML-2 and its construction is detailed elsewhere (Mueller et al, manuscript in preparation). After cohesive end ligation across the HindIII ends the 3' recessed BamHI end was rendered blunt as described previously. The DNA was recircularized across the blunt PvuII and BamHI termini with T4 ligase and used to transform E. coli. This procedure resulted in the generation of a BamHI site at nucleotide 5131 of the viral insert. A identical cloning strategy was used to clone the BamHI (5131)-HindIII (90) fragments from pdPP1(B)Bg(H) and LS 5173/5188 into the PvuII (5130) and HindIII (90) sites of pdPB5075Bg(H) to generate pdPB5075 wt and pdPB5075 LS 5173/5188.

The α internal deletions mutants were constructed by recombining mutants that carry unidirectional deletions on the late side of α with the ΔEA deletion series. The mutant pdPB5039 LS 5113/5109 was isolated by cohesive ligation of the BamHI (5039)-to-XhoI (5109) fragment from ΔEA 5109 to the BamHI site of ΔLAB 5113 DNA. Unlike the other late α mutants, ΔLAB 5113 has a BamHI site at its deletion endpoint. After ligation across the BamHI termini, the noncohesive ends were partially filled with the Klenow fragment of E. coli DNA polymerase I and dATP and dGTP and ligated across the 2 bp overhang. After transformation of E. coli, a colony containing the desired recombinant was isolated.

A similar approach was used to construct pdPB5039 LS 5108/5092. Briefly, another late α mutant containing a BamHI linker at its deletion endpoint, ΔLAB 5108, was cut with BamHI and ligated to BamHI (5039)-to-XhoI (5092) fragment from ΔEA5092. After ligation across the BamHI ends the remaining sites were blunted with Klenow fragment of DNA polymerase I and

the molecule recircularized and the DNA used to transform E. coli. After screening a number of colonies, one containing the desired plasmid, pdPB5039 LS 5109/5092, was isolated.

The mutant pdPB5039 LS 5102/5108 was constructed by ligating the BamHI (5039)-to-XhoI (5102) viral fragment from ΔEA 5102 to BamHI and XhoI cleaved ΔLA 5108. The structure of these viral recombinants was confirmed by DNA sequence analysis (Maxam and Gilbert, 1977).

G. DNA transfection.

CsCl gradient-purified supercoiled plasmid DNAs were transfected into cells by a modification of the DEAE-dextran transfection technique (McCutchan and Pagano, 1968; Sompayrac and Danna, 1981). A 60 mm dish containing 7.5×10^5 cells was washed twice with 5 ml of serum-free DMEM, and then incubated with 1 ml of DMEM supplemented with 250 μg of DEAE-dextran (molecular weight 500,000) per ml and 1-2 μg of plasmid DNA at 37°C for 4 h in a humidified CO_2 incubator. The cells were washed twice with serum-free DMEM and maintained in 5 ml of DMEM containing 10% calf serum.

In separate experiments I have varied the amount of DNA used in transfection between .02 and 2.0 μg per dish and found that the phenotype of the mutant plasmids does not change. I decided to routinely employ 1-2 μg of DNA per dish to insure I could detect the replication of a plasmid impeded in this capacity. A modification of the calcium phosphate technique (Wigler et al, 1978) was used to transfect Rat-1 cells with cesium chloride density gradient-purified supercoiled plasmid DNA. Transformed foci were scored 14 to 18 days post-transfection after the plates were fixed in 10% formalin-PBS (vol/vol) and stained with giemsa.

H. Isolation of MOP cell lines.

The mouse originless polymavirus (MOP) cell lines were established by transforming NIH 3T3 cells with pSVE1-Bla DNA. In brief, 10 separate cultures of sparsely seeded NIH 3T3 cell (1×10^5 to 2×10^5 cells per 100 mm diameter petri dish) were transfected with CsCl-purified supercoiled pPSVE1-Bla DNA (100 ng per petri dish) by a modification of the calcium phosphate technique (Wigler et al, 1978). The calcium-phosphate-containing medium was removed 5 hours after its initial application, and fresh DMEM containing serum was added. The medium was then changed every 3

days until foci of transformed cells appeared; 50 to 100 foci appeared after 2 weeks, and one focus was isolated from each dish and subsequently cloned (Hassell et al, 1980). A total of 10 independant colonies were established and characterized.

I. DNA replication assay.

At 48 to 72 hours post-transfection, low-molecular-weight DNA was isolated from cells by using the Hirt extraction procedure (Hirt, 1967). After the sedimentation of the high-molecular-weight DNA, 0.5 ml of the cleared lysate was diluted in 4.5 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and extracted once with buffer-saturated phenol and once with chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated by addition of 2.5 volumes of cold ethanol and storage overnight at -20°C. Precipitates were collected by centrifugation and suspended in 50 µl of TE buffer. A 15 µl portion of the DNA sample was sequentially digested with BamHI and DpnI restriction endonucleases.

The digested plasmid DNAs were subjected to electrophoresis through 1.0% (wt/vol) agarose gels, and the DNA fragments were transferred to nitrocellulose filters by the Southern (Southern, 1975) technique. The nitrocellulose filters were hybridized to ^{32}P-labeled, nick-translated pdPBR2 DNA (1×10^6 to 1×10^9 cpm/µg) by employing dextran sulphate (Wahl et al, 1979). After washing, the filters were dried and autoradiographed for 3 to 12 h with Kodak XAR-5 film and DuPont Lightning-Plus intensifying screens. I estimate that by 72 h post-transfection there are from 500 to 2,000 replicated plasmid molceules per cell. However, not all of these cells take up and replicate the recombinant plasmid DNA, and therefore the number of copies of replicated DNA per transfected cell is likely to be higher than this.

J. DNA and protein analysis.

The methods used to isolate low- and high-molecular-weight cellular DNA and to characterize that DNA after restriction endonuclease cleavage and Southern blotting-hybridization have been described previously (Pomerantz et al, 1983a).

Immunoprecipitation of the viral T antigens and their identification by fluorgraphy after electrophoresis through acrylamide-sodium dodecyl sulfate gels were also performed as described previously (Pomerantz et al, 1983b).

K. Binding of large T antigen to DNA.

The preparation of the reagents and the methods used to measure the binding of large T antigen to end-labeled DNA fragments have all be described previously (Pomerantz et al, 1983a). The substrates used in the binding reaction were the HinfI fragments of pPH1-8 DNA. This recombinant plasmid contains the origin-bearing HindIII-1 fragment of polyomavirus DNA cloned within the HindIII site of pBR322 (Sutcliffe, 1979). The HinfI-4 fragment of pPH1-8 (polyomavirus nucleotides 5073 to 385) contains all the large T antigen binding sites (Pomerantz et al, 1983a).

RESULTS

Chapter I: Isolation of Large T Antigen Producing Mouse Cell Lines Capable of Supporting Replication of Polyomavirus-Plasmid Recombinants

A. Transformation of NIH 3T3 cells with DNA containing a hybrid transcription unit.

Isolation of permissive mouse cells which express functional PyV large T antigen requires the use of a mutant viral DNA molecule with a defective origin. This is so because origin-containing DNAs are not stably maintained in the integrated state in the genome of a permissive cell producing functional large T antigen (Pellegrini et al, 1984). This problem can be avoided by using an origin-defective mutant DNA for transformation or by replacing the entire early promoter and origin region with a foreign promoter. I chose the latter approach because the exact borders of the PyV origin had not been defined when I began these experiments, thereby making it difficult to choose an appropriate site for mutagenesis. Moreover, the precise borders of important controlling elements of the early promoter also had not been defined, and therefore I had no way of knowing whether an origin-defective molecule would also prove to be debilitated in expression. Consequently, I chose to replace the entire upstream noncoding region of PyV with that of another strong promoter.

The SV40 early promoter has been well characterized (Benoist and Chambon, 1981; Fromm and Berg, 1982), and it has been shown that it functions nearly as well as the early PyV promoter to drive expression of downstream genes in rodent cells (Featherstone et al, 1984). The HindIII C fragment of SV40 contains all the viral regulatory elements, including the origin for DNA replication as well as the early and late promoters and the early start sites for transcription (Benoist and Chambon, 1981; Bergsma et al, 1982; Fromm and Berg, 1982). Therefore, I placed this SV40 DNA fragment in the appropriate orientation before the early region of PyV.

During lytic infection of mouse cells by PyV, expression of the T antigen is repressed at late times by the binding of large T antigen to promoter-proximal DNA sequences, thereby retarding transcription initiation (Cogen, 1978). The binding sites for PyV large T antigen all lie upstream of nucleotide position 154 in PyV DNA (Pomerantz et al, 1983a; Cowie et al,

1984). Moreover, the PyV principal mRNA 5' termini also map upstream of nucleotide position 154 (they are located between nucleotide positions 147 and 153) (Kamen et al, 1980; Treisman et al, 1981a). Because I wished to maximize expression of the PyV T antigens in permissive cells, I attempted to ensure against repression of early transcription. Therefore, I fused the SV40 early promoter to the PyV early region beginning at nucleotide 154 in PyV DNA. In this way I isolated a hybrid transcription unit, pPSVE1-B1a, whose coding sequences are expressed from a strong promoter and are independent of regulation (Fig. 4). I expected that pPSVE1-B1a would be transcribed in mouse cells to yield hybrid mRNA molecules whose 5' untranslated termini were derived from SV40 DNA but whose 3' sequences were derived from PyV DNA.

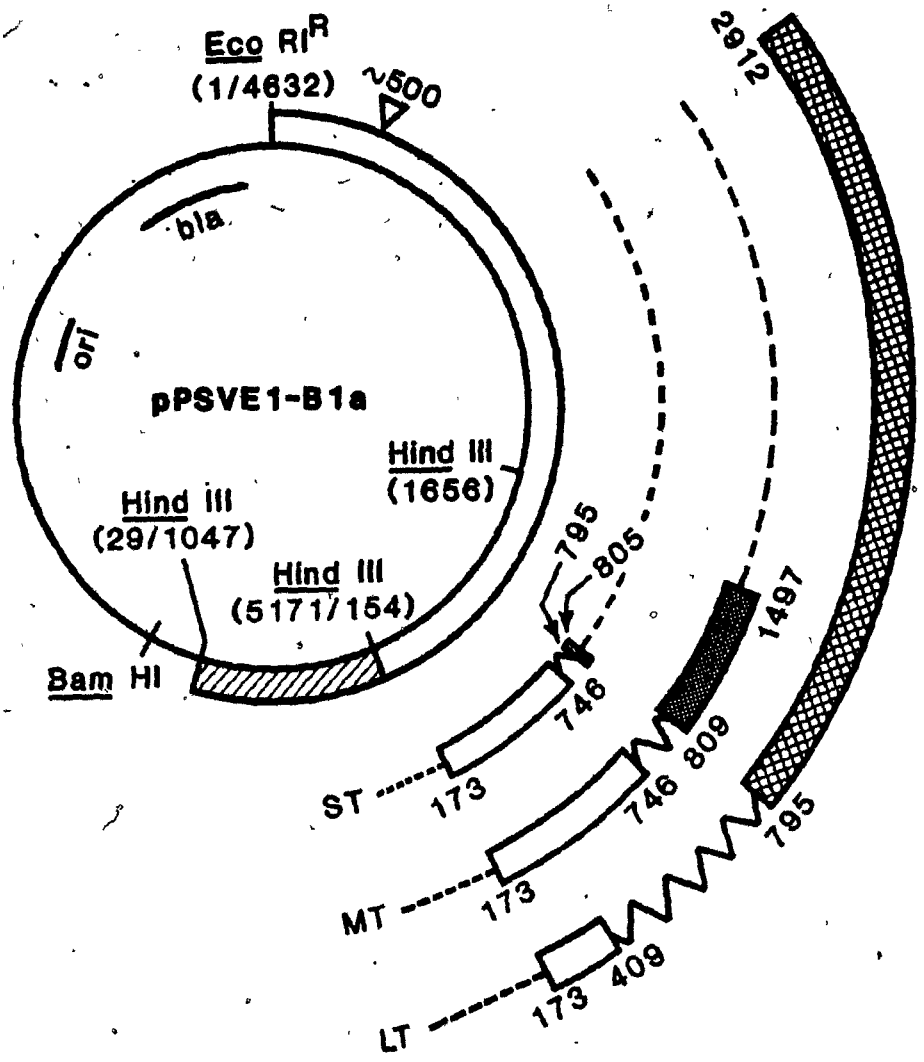
pSVE1-B1a DNA was used to transfect untransformed NIH 3T3 cells to the transformed phenotype as described in the Materials and Methods section. Transformed foci appeared in the cultures after 2 weeks. Ten independent foci were isolated from separate plates and recloned once before they were analyzed further. I named these lines MOP cells by analogy to COS cells (CV-1 originless SV40) (Gluzman, 1981).

B. Replication of origin-bearing plasmid DNA in MOP cell lines.

The MOP cell lines need only express middle T antigen to maintain the transformed cell phenotype (Hassell et al, 1980; Treisman et al, 1981b; Mes and Hassell, 1982). However, the coding sequences for middle T and large T antigen overlap across part of the viral genome (Fig. 4), and therefore I expected that a fair proportion of the lines would gratuitously produce large T antigen. To determine whether this was true and to ensure that active large T antigen was synthesized, I screened the 10 MOP lines for their capacity to support the replication of a recombinant plasmid bearing the PyV functional origin for DNA replication.

I chose the plasmid pdPR1a for this analysis (Muller et al, 1983b). pdPR1a comprises pML-2 DNA and the entire PyV genome. The viral DNA was inserted at the EcoRI site of pML-2 as an EcoRI genomic fragment. Because EcoRI cleaves PyV DNA in the middle of the early region (at nucleotide number 1560) (Fig. 4), this plasmid cannot encode large T antigen. Thus, pdPR1a will only replicate in permissive cells which produce active large T antigen in trans. To determine whether the pdPR1a plasmid could replicate in MOP cells, I transfected these cells with plasmid DNA,

Figure 4: Structure of pPSVE1-B1a DNA. pPSVE1-B1a DNA (ca. 9,430 base pairs [bp]) comprises pBR322 DNA (4,333 bp; thin line), the HindIII C fragment of SV40 DNA (1,119 bp; hatched box), and PyV DNA (ca, 3,978 bp; open box). The sequences in pBR322 DNA between its EcoRI (nucleotide 1) and HindIII (nucleotide 29) sites were substituted with the SV40-PyV hybrid transcription unit. The pBR322 EcoRI (nucleotide 1) and the PyV BamHI (nucleotide 4632) sites were destroyed at one joint, but the HindIII site was preserved at the other joint. The SV40 sequences are arranged such that its early promoter drives transcription of the PyV early region. The PyV sequences contain a deletion of about 500 bp (denoted by the triangle) encompassing the HindIII site at nucleotide 3918. The nucleotide schemes of Sutcliffe (1979), Buchman et al (1980) and Soeda et al (1980) were used for pBR322, SV40 and PyV DNA, respectively. The structures of the PyV early mRNAs (small [ST], middle [MT] and large [LT]) are illustrated outside the circular map of pPSVE1-B1a DNA. Transcription of the three coterminal mRNAs should originate in SV40 DNA and terminate in PyV DNA. The boxed areas of each mRNA represent translated sequences, and their different shadings denote different reading frames. The jagged lines connecting the mRNA coding regions represent sequences which are spliced from the nuclear precursors of the various mRNAs. The splice sites were taken from Treisman et al (1981a).


Eco RI^R
(1/4632)
~500
bla
ori
pPSVE1-B1a
Hind III
(1656)
Hind III
(29/1047)
Hind III
(5171/154)
Bam HI
2612
795
805
1497
746
ST
173
746
809
MT
173
795
LT
173
409

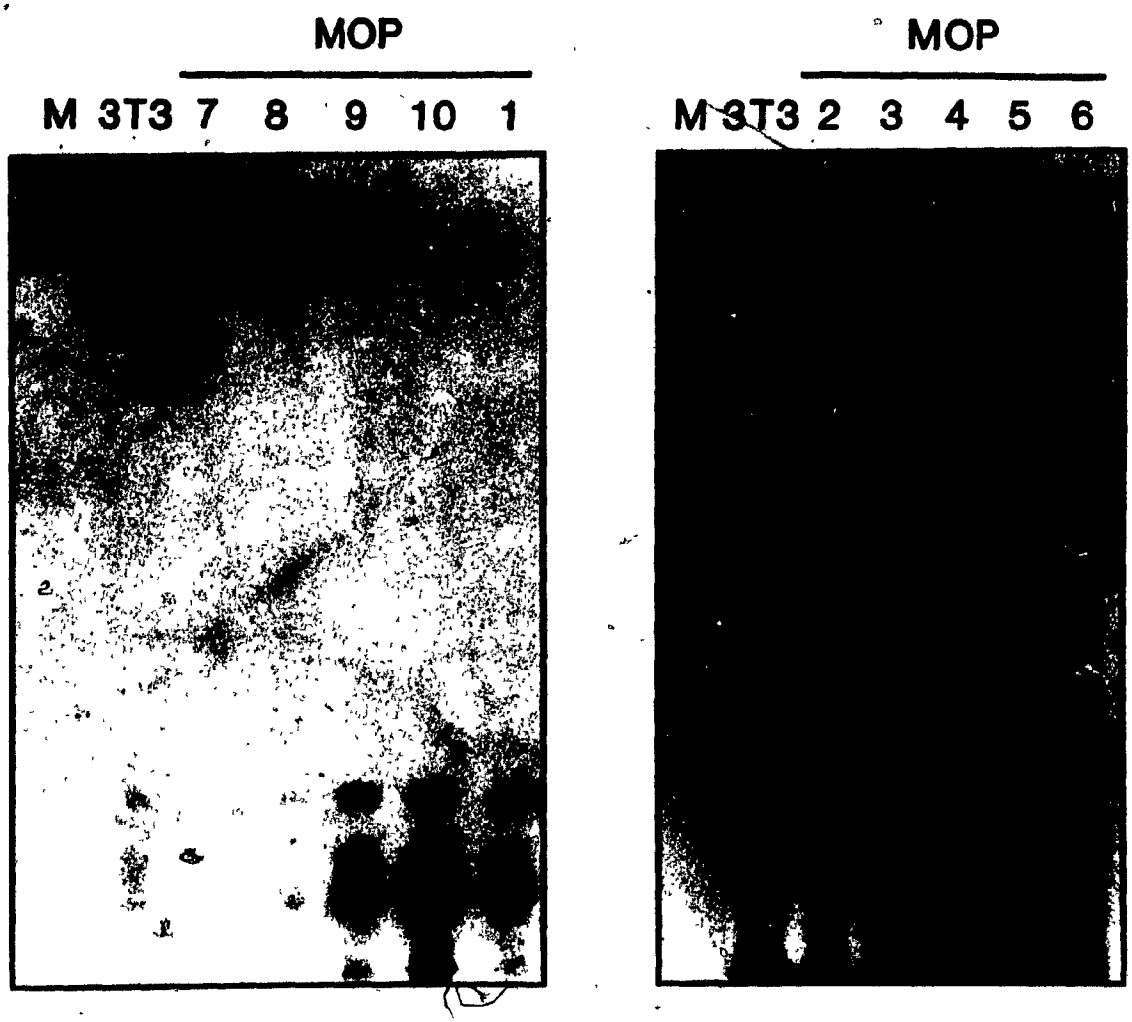
extracted the low-molecular-weight DNA 72 hours later, and assessed the state of methylation of the recombinant DNA as an indirect measure of replication after digestion with DpnI followed by Southern blotting-hybridization (Peden et al, 1980). Plasmid DNA that is propagated in Escherichia coli is methylated by the cell at adenine residues that lie within the sequence GATC. Such methylated sequences are recognized by DpnI (Lacks and Greenberg, 1977). However, DNA that is isolated from bacteria and subsequently replicated in mammalian cells is not methylated and therefore becomes resistant to cleavage by DpnI. Therefore, by digesting recombinant DNA that was recovered after transfection of mammalian cells with DpnI and SalI (the latter cleaves pdPR1a once), it was possible to distinguish the replicated DNA from the input DNA that did not replicate. The replicated DNA appears as a single species migrating with a linear marker of pdPR1a DNA, whereas the unreplicated DNA appears as numerous fragments of poorly separated, low-molecular-weight DNA at the bottom of the autoradiogram.

The outcome of screening 10 MOP cell lines for their capacity to support pdPR1a replication is illustrated in Fig. 5. Seven of the 10 lines supported pdPR1a replication to approximately the same extent (Fig. 5). As expected, the parental cell line (NIH 3T3) did not support pdPR1a replication because this line does not synthesize PyV large T antigen. These results show that many of the transformed MOP cell lines express active large T antigen. Because there were no large differences in the capacities of the various lines to complement PyV DNA replication, I randomly chose three cell lines, MOP-3, -6, and -8 for further characterization.

C. MOP cells with integrated pSVE1-B1a DNA.

To determine the physical state of pPSVE1-B1a DNA in the MOP-3, -6, and -8 cell lines I examined these cells for the presence of free plasmid DNA by preparing low-molecular-weight DNA from them and assaying this DNA for the presence of sequences homologous to pPSVE-B1a DNA by Southern blotting-hybridizations (Southern, 1975). I could not detect free plasmid DNA in these cells (Fig. 6A). Because I assayed the DNA from 3×10^6 cells and because I could readily detect 10^{-5} μg of marker DNA, I estimate that they are fewer than 0.3 molecules of free pSVE1-B1a DNA per cell.

Figure 5: Replication of pdPR1a DNA in the various MOP cell lines. A 1 μg sample of pdPR1a was transfected into ca. 5×10^5 cells growing on the surface of a plastic petri dish (6 cm diameter) by using DEAE-dextran as a facilitator of DNA uptake. Replication of the pdPR1a DNA was measured by isolating low-molecular-weight DNA and cleaving it with DpnI and SalI as described in the text. There are many sites of cleavage for DpnI, but only one SalI cleavage site in pdPR1a DNA. The replicated DNA is resistant to DpnI cleavage and appears as a band which comigrates with a linear marker of pdPR1a DNA (M). The marker lane (M) contained 10^{-5} μg of pdPR1a DNA. The input unreplicated DNA appears as a smear of low-molecular-weight fragments at the bottom of the autoradiogram.


MOP
M 3T3 7 8 9 10 1
MOP
M 3T3 2 3 4 5 6

Digestion of high molecular weight cellular DNA from MOP cells with restriction endonucleases which do not cleave pSVE1-B1a DNA, would establish whether this DNA is integrated, and the number of viral fragments detected after Southern blotting-hybridization would establish the number of separate insertions of transforming DNA (Botchan et al, 1976). BglII is an enzyme which does not cleave pSVE1-B1a DNA. Digestion of MOP-3, -6, and -8 cellular DNA with BglII followed by gel electrophoresis and Southern blotting-hybridization revealed the presence of integrated pPSVE1-B1a DNA in each of the cell lines (Fig. 6B). MOP-3 and MOP-6 cells contain two insertions, whereas MOP-8 cells contained a single insertion of transforming DNA.

To determine whether any of the three MOP cell lines contained multiple, tandemly reiterated pPSVE1-B1a genomes arranged in a head-to-tail fashion, I cleaved their cellular DNAs with BamHI, which cleaves pPSVE1-B1a DNA once. If a BamHI site was tandemly repeated, than I would expect to release linear pPSVE1-B1a DNA after cleavage of cellular DNA with BamHI. None of the three MOP cell lines yielded unit-length pPSVE1-B1a DNA after cleavage with BamHI (Fig. 6C). The cellular DNA of the MOP-8 line yielded two fragments after cleavage with BamHI, which is consistent with a single insertion of pSVE1-B1a in this cell line and the retention of the BamHI site within the integrated DNA. These results show that each of the MOP cell lines examined contained a small number of integrated pPSVE1-B1a genomes (from one to two) and that these insertions were not made up of long arrays of tandemly reiterated pSVE1-B1a DNA.

D. Viral T antigens expressed by MOP cell lines.

I examined the three prototype MOP cell lines for their capacity to express PyV T antigens. This was accomplished by immunoprecipitation of ^{35}S-labeled cell lysates with serum from tumor-bearing rats (Fig. 7). The three MOP cell lines contained large, middle and small T antigen although it was difficult to invariably demonstrate the presence of small T antigen in the MOP-6 and MOP-8 cell lines (Fig. 7). In separate independent experiments I could demonstrate no large differences in the amount of large, middle or small T antigen synthesized by the various cell lines (data not shown).

One biochemical activity associated with PyV large T antigen is its capacity to bind to specific viral sequences near the origin for DNA replication and the early promoter (Gaudray et al, 1981; Pomerantz et al,

Figure 6: Characterization of MOP cellular DNA. (A) Low-molecular-weight DNA was isolated from three MOP cell lines and probed for the presence of pPSVE1-B1a sequences by Southern blotting-hybridization as described in the text. The lane marked M in (A) contained a HindIII digest of 10^{-5} µg of pPSVE1-B1a DNA. Three fragments of ca. 6,809, 1,502 and 1,119 base pairs are displayed on the autoradiogram. (B) High-molecular-weight cellular DNA was isolated from three MOP cell lines and digested with BglII, which does not cleave the transforming pPSVE1-B1a DNA. The fragments complementary to pPSVE1-B1a DNA were detected by Southern blotting-hybridization and autoradiography as described in the text. The lane marked M contained a HindIII digest of 10^{-5} µg of pPSVE1-B1a DNA. (C) High-molecular-weight cellular DNA from three MOP lines was hydrolyzed with BamHI, which cleaves the transforming DNA, pPSVE1-B1a, once. The fragments bearing sequences homologous to pPSVE1-B1a DNA were detected as described previously. The lane labeled M contained 10^{-5} µg of BamHI-cleaved pPSVE1-B1a DNA. Arrows indicate the positions of the marker fragments.

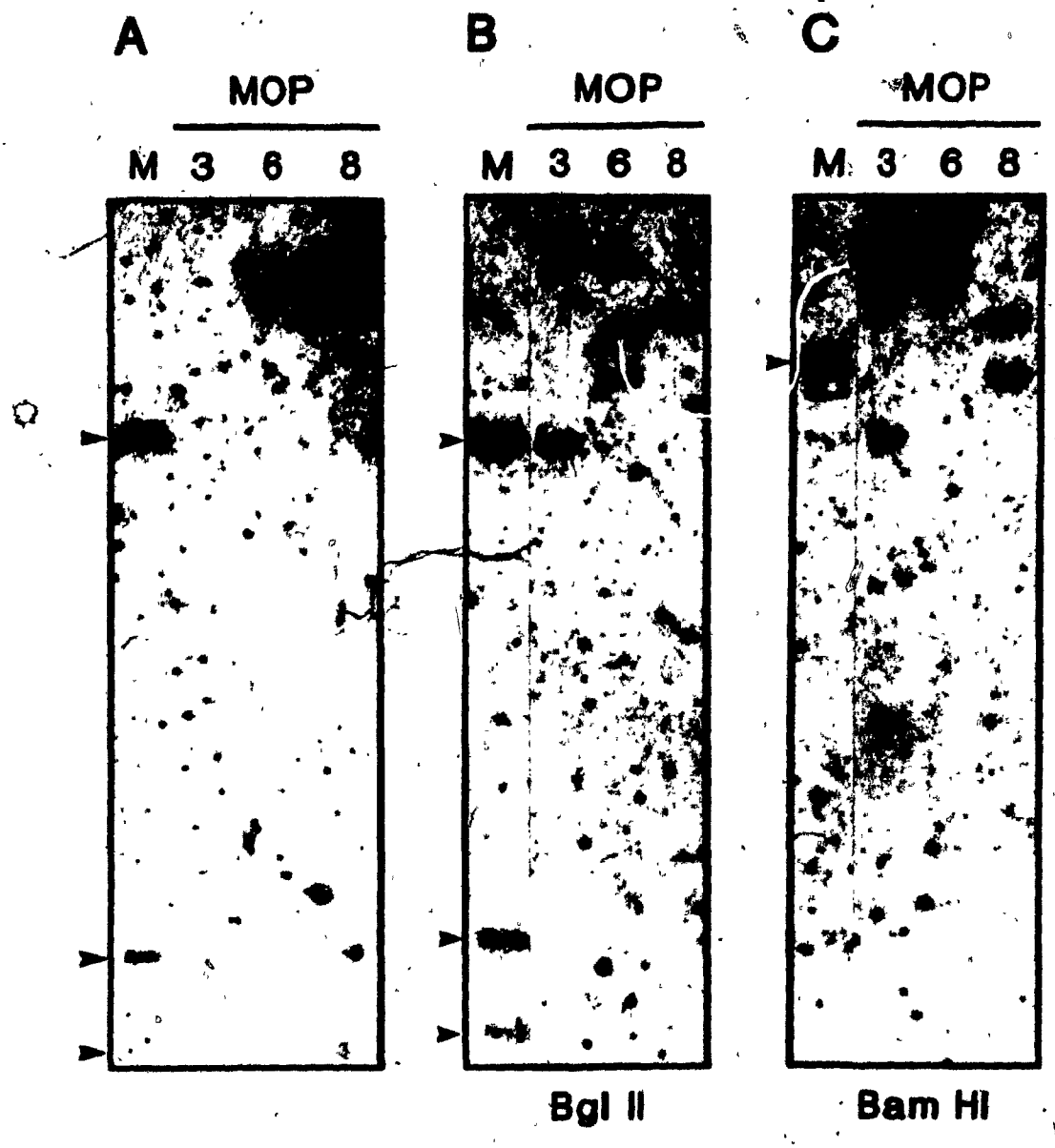
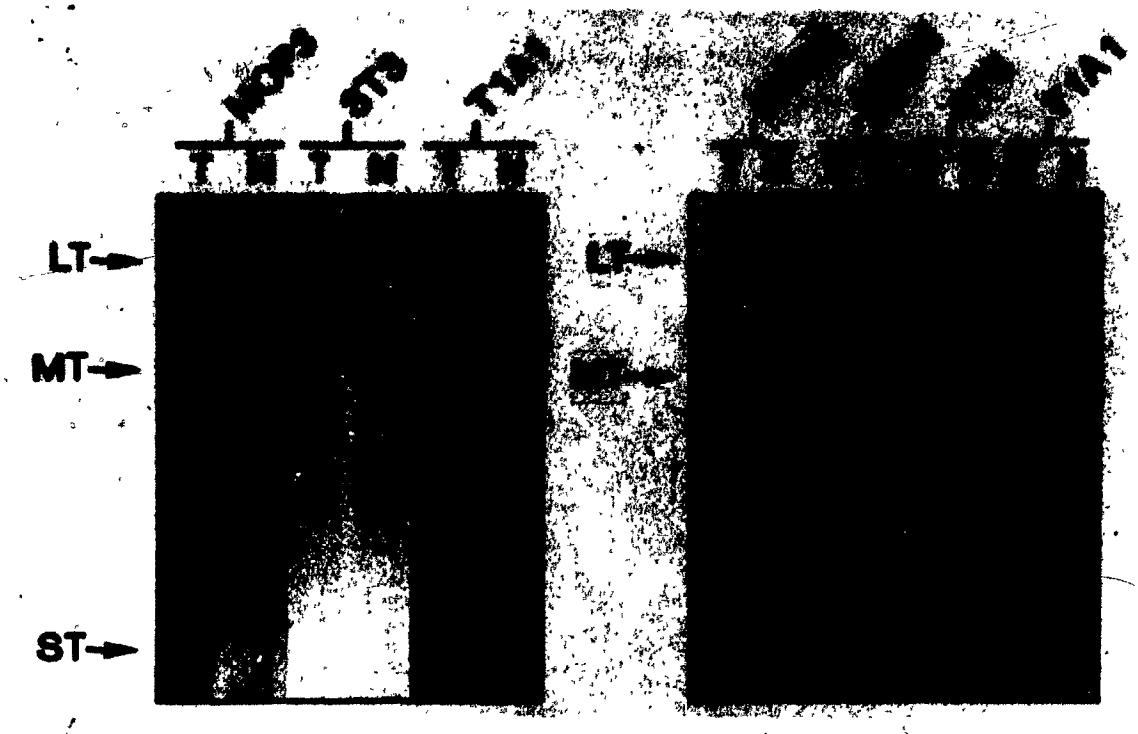
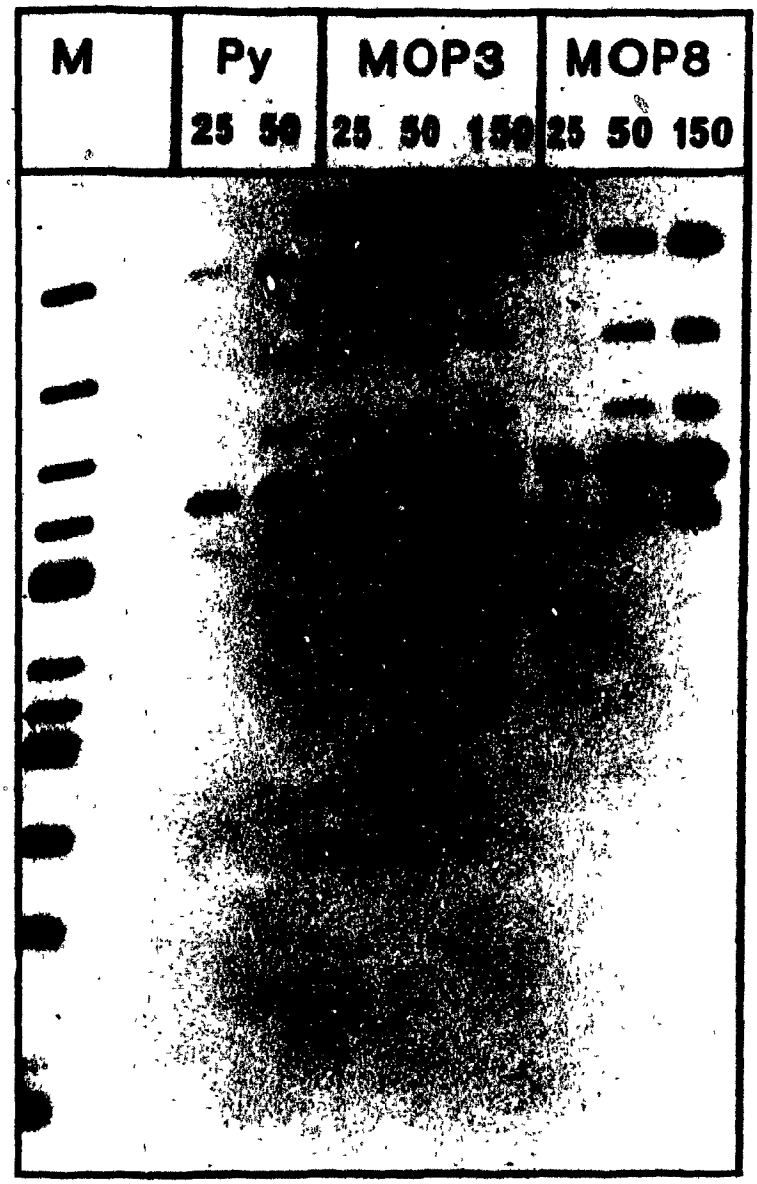

A
B
C
MOP
MOP
MOP
M 3 6 8
M 3 6 8
M 3 6 8
Bgl II
Bam HI

Figure 7: Immunoprecipitation of [^{35}S]methionine-labeled T antigens from the MOP cell lines. ^{35}S-labeled proteins were immunoprecipitated from three MOP cell lines, NIH 3T3 and the T1A1 rat cell line with antiserum from a Fisher rat which bore a tumor induced by a polyomavirus-transformed rat cell line (T). Serum from a normal Fisher rat was used as a control (N). The immunoprecipitated proteins were separated by electrophoresis through a 12.5% sodium dodecyl sulfate-polyacrylamide gel and autoradiographed as described in the text. Each panel represents a separate gel. LT, MT and ST refer to large, middle and small T antigen, respectively. Small T antigen was not visible in the autoradiogram of the gel represented by the rightmost panel. However, this protein was detected on longer exposure of the gel and in separate experiments (data not shown).


LT
MT
ST

1983a; Cowie and Kamen, 1984). To determine whether the large T antigen produced by the MOP cell lines could bind to PyV DNA, an indirect assay described by McKay (1981) was used. Terminally labeled HinfI fragments of pPH1-8 DNA were incubated with crude nuclear extracts from lytically infected 3T6 cells, MOP-3 cells and MOP-8 cells, and the fragments bearing bound large T antigen were immunoprecipitated with antitumor serum and Staphylococcus aureus bacteria. The immunoprecipitated DNAs were separated by agarose gel electrophoresis and visualized by autoradiography (Fig. 8). The large T antigen from each cell line specifically bound to the HinfI-4 fragment of pPH1-8 DNA, which carries all of the T antigen binding sites (Pomerantz et al, 1983a). The amount of nuclear extract required to immunoprecipitate an equivalent amount of HinfI-4 fragment was nearly the same for all three extracts. These results show that all three MOP cell lines analyzed contained large T antigen as well as middle T and small T antigen and that two of these lines (MOP-3 and MOP-8) contained large T antigen molecules capable of binding to the same viral sequences as those recognized by the large T antigen synthesized in lytically infected 3T6 cells.

Figure 8: Immunoprecipitation of ^{32}P end-labeled HinfI fragments of pPH1-8 DNA after reaction with nuclear extract from various cell lines. Nuclear extracts were prepared from polyomavirus-infected 3T6 cells (Py), MOP-3 cells and MOP-8 cells and were incubated with the end-labeled fragments of pPH1-8 DNA. Several volumes of nuclear extract, 25, 50 and 150 µl, were used. The lane marked M contained a portion of the original substrate (pPH1-8 cleaved with HinfI) used in the binding reactions. The fourth-largest, 604 base pair fragment contained the binding sites for large T antigen.


M
Py
25 50
MOP3
25 50 150
MOP8
25 50 150

Chapter II: Structural Requirements for the Function of the Polyomavirus Origin for DNA Replication

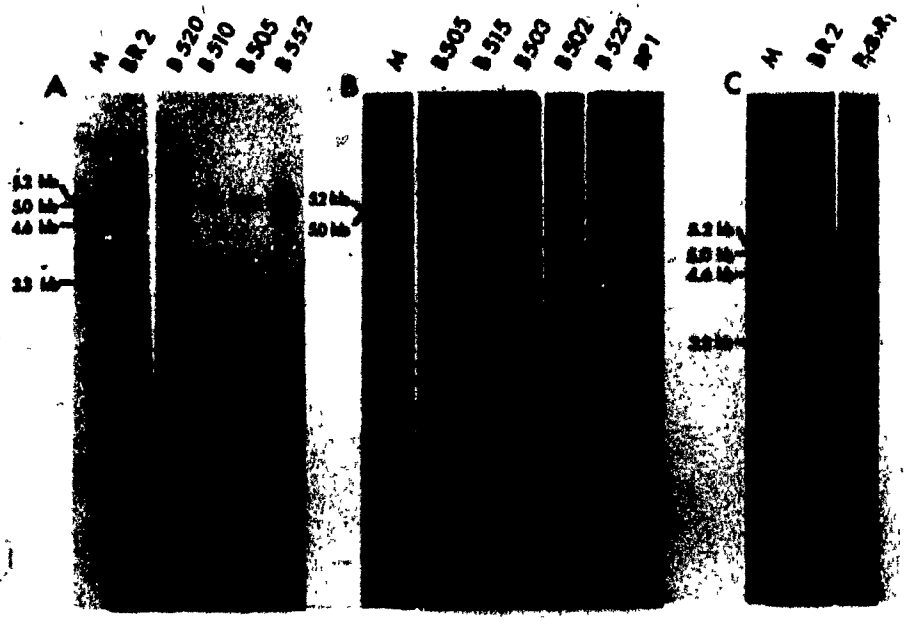
A. Limits of the PyV sequences required in cis for DNA replication.

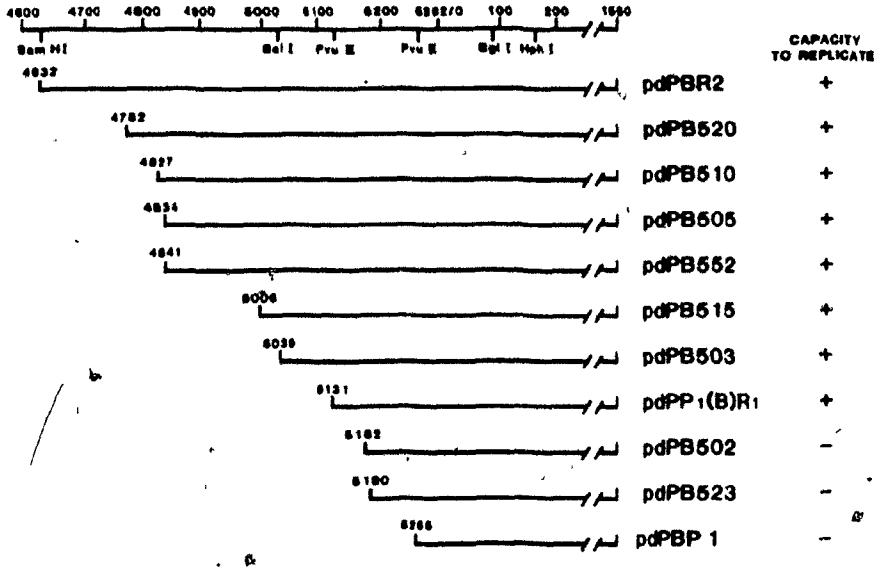
My initial objective in isolating permissive cells capable of complementing the replication of mutant PyV was to use such cells to map the borders of the PyV functional origin for DNA replication (Muller et al, 1983a). To define the borders of the PyV origin for DNA replication (ori), I constructed a number of PyV DNA-plasmid recombinants and measured their replicational capacity in MOP-8 cells. I employed pML-2 as a vector in these experiments because, like Lusky and Botchan (1981), I observed that sequences within pBR322 DNA inhibited the replication of PyV-pBR322 DNA molecules in MOP-8 cells (data not shown). These "poison" sequences are not present in pML-2 DNA (Lusky and Botchan, 1981). The replicative capacity of the PyV recombinant plasmids was assessed at 72 hours post-transfection using the DpnI assay.

Application of this method to measure the replication of the various PyV-pML-2 recombinant molecules in MOP-8 cells revealed that all of the cis-acting sequences required for replication resided within the small BamHI (nucleotide 4632)-to-EcoRI (nucleotide 1560) fragment of PyV DNA (pdPBR2; Fig. 9A). I employ the nucleotide numbering system proposed by Soeda et al (1980) and report the boundaries of the DNA fragments from the clockwise direction around the circular PyV genome. This 2,220 bp fragment includes the region from which DNA replication initiates in vivo (Crawford et al, 1973).

To more precisely delineate the boundaries of the PyV ori, I constructed a series of mutants that contain progressive deletions within viral DNA sequences originating from either the BamHI site (nucleotide 4632) or the EcoRI site (nucleotide 1560) in pdPBR2 DNA and measured their capacity to replicate in MOP-8 cells. The structure and replication competence of the various DNAs are shown in Fig. 9. PyV recombinant plasmids possessing deletion endpoints up to nucleotide position 5131 were found to replicate within the MOP-8 cell line at levels comparable to those of the parent plasmid pdPBR2. However, further deletions extending to nucleotides 5182, 5190 and 5265 rendered these recombinant plasmids replication defective (Fig. 9). On the basis of these results, the late border of the PyV origin can be positioned between nucleotides 5131 and 5182.

Figure 9: Deletion analysis of late boundary of the PyV ori. (A, B and C) A 2 μg sample of PyV recombinant plasmid DNA was transfected into MOP-8 cells, and replication was assayed 72 hours post-transfection as described in the text. After simultaneous digestion with BamHI and DpnI, the plasmid DNAs were subjected to electrophoresis through a 1% agarose gel and transferred to nitrocellulose filters. Replicating recombinant plasmid DNA can be visualized as a DpnI-resistant band comigrating with the linearized parental plasmid after hybridization and autoradiography. The smear that appears at the bottom of the autoradiogram represents the closely spaced fragments of DpnI-cleaved recombinant plasmid DNA that has not replicated. Because large quantities of DNA were used to transfect the cells, only a small fraction of the total input DNA is replicated in these MOP-8 cells. The autoradiograms shown in each panel are taken from the same film, which was clipped to allow comparisons to be made between adjacent lanes. The lanes labeled M contain DNA fragments of known sequence. The size of each fragment is shown adjacent to its position in the autoradiogram. (D) Schematic illustration of the structures of deletion mutants relative to known restriction endonuclease cleavage sites on the poloymavirus genome. The nucleotide numbering is according to Soeda et al (1980). Because no attempt was made to quantitate the precise level of DNA replication, the replicational phenotypes are designated as either + or -.


A
M
BR2
B520
B510
B505
B552
B
M
B505
B515
B503
B502
B523
BP1
C
M
BR2
PdBR1
52 kb
50 kb
46 kb
33 kb


D
4600
4700
4800
4900
5000
5100
5200
5282/0
100
200
1550
Bam HI
Bal I
Pvu II
Pvu II
Bal I
Hph I
CAPACITY TO REPLICATE
4632
pdPBR2
+
4752
pdPB520
+
4827
pdPB510
+
4834
pdPB505
+
4841
pdPB552
+
5006
pdPB515
+
5039
pdPB503
+
5131
pdPP1(B)R1
+
5162
pdPB502
–
5180
pdPB523
–
5266
pdPBP 1
–

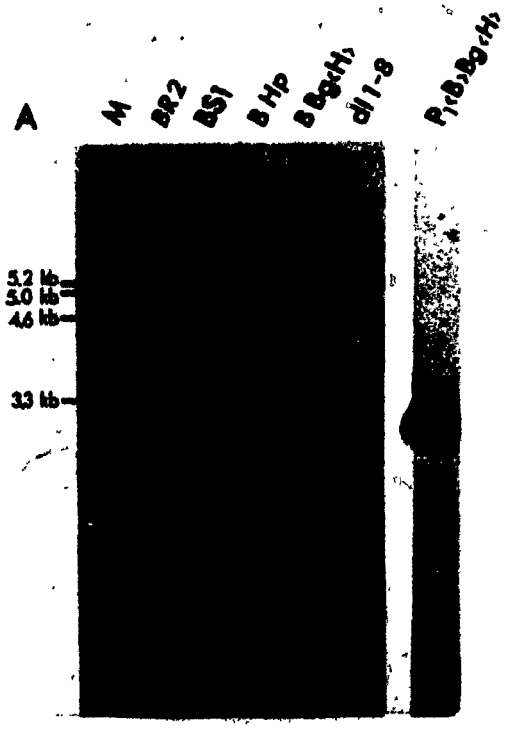
To establish the early boundary of ori, deletions were created from the EcoRI site toward the BamHI site through viral sequences in pdPBR2 DNA. The structure and replicative capacity of these recombinant plasmids are illustrated in Fig. 10. All recombinant plasmids, including one (pdPBBg[H]) with a 1,470 bp deletion from the EcoRI site, were capable of autonomous replication in MOP-8 cells (Fig. 10). These data demonstrate that the early border of ori is located to the left of nucleotide position 90 in PyV DNA.

To position this boundary more precisely, I employed a derivative of pdPBR2, termed pdPd11-8, which bears a deletion between nucleotides 1 and 148 (Hassell et al, 1982). Measurement of the capacity of this mutant DNA to replicate revealed that it was defective (Fig. 10). This observation and previous results permits me to define the early boundary of ori between nucleotides 1 and 90. On the basis of these measurements I predict that one boundary of ori is between nucleotides 5131 and 5182 and that the other is between nucleotide positions 1 and 90. This prediction was tested by cloning a 251-bp fragment of PyV DNA between the PvuII site at nucleotide 5131 and the BglI site at nucleotide 90, as a BamHI-HindIII fragment within pML-2 DNA, and measuring its replication potential in MOP-8 cells. The result (Fig. 10) demonstrates that this viral recombinant plasmid, pdPP1(B)Bg(H), is capable of autonomous DNA replication in MOP-8 cells. Therefore, the 251-bp DNA segment situated between nucleotides 5131 and 90 contains all the necessary genetic information required in cis for PyV DNA replication.

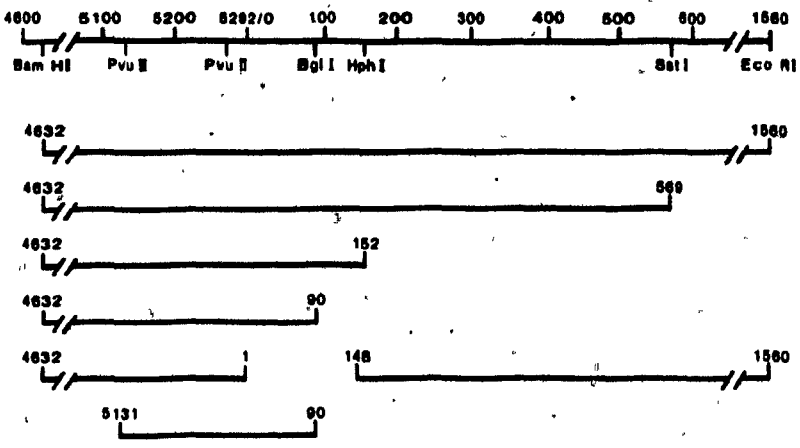
B. PyV ori comprises multiple genetic elements.

To determine whether the viral ori is a single contiguous element or is composed of multiple sequence motifs, I constructed deletions within ori and then measured the replicational capacity of the derived mutant plasmids in MOP-8 cells. To carry out these experiments I planned to introduce deletions about the PvuII site at nucleotide position 5265 in pdPBR2 DNA. For a variety of reasons these initial experiments led to the isolation of deletion mutants which lacked all the sequences between nucleotides 5130 and 5265 (sites of cleavage for PvuII) as well as sequences that flanked these positions. As a control, I tested these recombinants for their capacity to replicate and was surprised to discover that two of the aforementioned DNAs were fully capable of autonomous DNA replication in

Figure 10: Deletion analysis of the early boundary of the PyV ori. (A) The replicative capacities of the PyV recombinant plasmids were determined as described for Fig. 9. (B) The physical maps and replication phenotypes of the viral deletions mutants are illustrated as described for Fig. 9.



B



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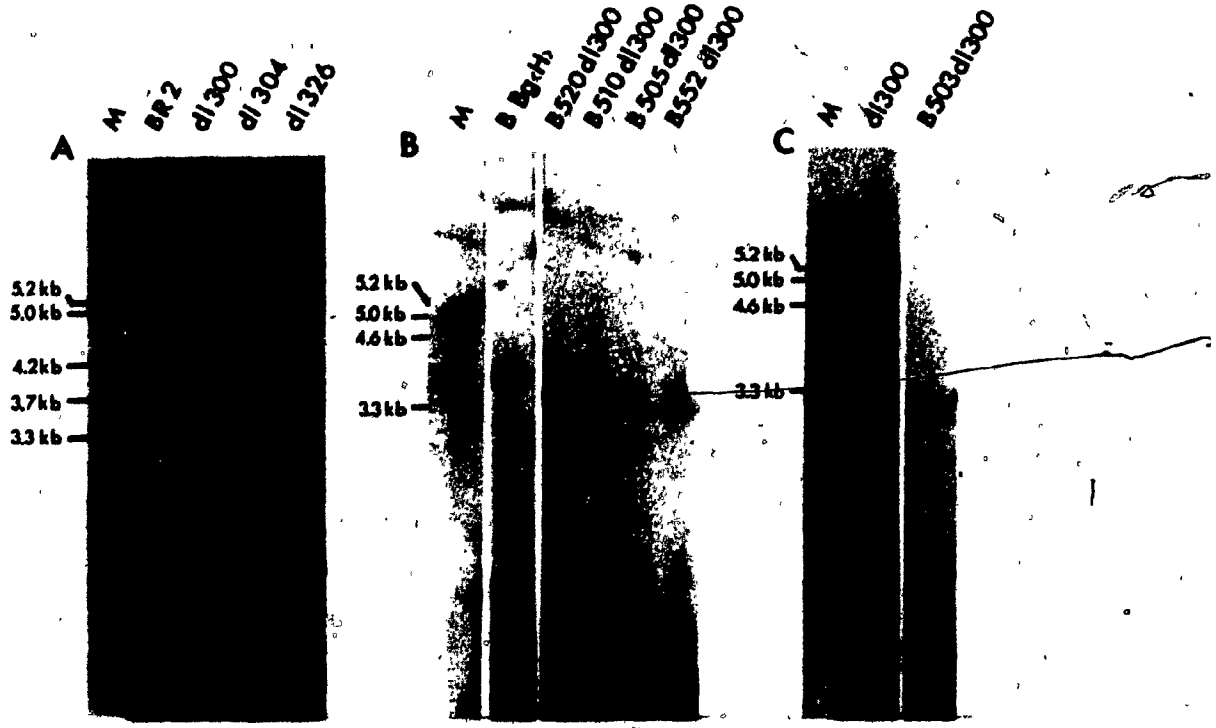
MOP-8 cells (Fig. 11: pdPd1300 and pdPd1326). These two DNAs lack sequences that span the region between nucleotides 5126 and 5265 (Fig. 11). The sequences missing in these recombinants had previously been identified as forming part of ori (Fig. 10). These conflicting observations could be reconicled if I assume that sequences elsewhere within the viral genome (the BamHI-EcoRI fragment) could functionally substitute for those deleted in pdPd1300 and pdPd1326.

To pursue this hypothesis I attempted to identify these sequences by deleting viral DNA from pdPd1300. Deletion of viral sequences between nucleotide positions 90 (a BglI site) and 1560 (A EcoRI site) from pdPd1300 did not render the plasmid defective for replication (data not shown). This observation suggested that the functionally redundant sequences were located between nucleotides 4632 and 5126 in pdPd1300 DNA (Fig. 11A, D). To examine this possibility I constructed a set of deleted derivatives of pdPd1300 that bear lesions which originate at the BamHI site (nucleotide 4632) and terminate at various positions within viral DNA (Fig. 11). These deleted recombinants were constructed by cloning the BamHI-to-PvuII fragments from the B500 series of mutants (Fig. 9) within pdPBBg(H) DNA, which had been cleaved to completion with BamHI and PvuII (Fig. 10). The resulting recombinants were then tested for their capacity to replicate within MOP-8 cells. The results are presented in Fig. 11B and C and summarized in Fig. 11D. They show that viral sequences between nucleotides 5039 and 5130 can functionally substitute for those located between nucleotides 5131 and 5265.

I have named these cis-acting sequences the α and β elements respectively. The α element and β element together are incapable of forming a functional origin (Fig. 10; pdPd11-8). Yet another sequence motif located between 5265 and 90 is required to form ori. This conclusion is supported by the isolation of a mutant, pdPd1304 which carries a deletion of 12 bp between nucleotides 5265 and 5277, which is replication defective (Fig. 11A, D). On the basis of these deletion studies I postulate that the PyV ori comprises at least three genetic elements (Fig. 12). These elements include two upstream functionally equivalent regions mapping on either side of the PvuII site at nucleotide 5130, termed the α and β elements. In addition, an area of the viral genome containing one high-affinity large T antigen binding site, a 34-bp palindrome, and a tract of eight adenine:thymine (A:T) residues designated the core region is

Figure 11: Internal deletion analysis of the PyV ori. (A, B and C). The replicative capacities of the viral deletion mutants were assessed as described for Fig. 1. The recombinant plasmids pdPBR2, pdPBBg(H), and pdPd1300 were included in (A), (B) and (C), respectively, for comparative purposes. (D) The structures of the deletion mutants and their associated replicational properties are illustrated as described for Fig. 9.


A
M
BR2
dl300
dl304
dl326
5.2kb
5.0kb
4.2kb
3.7kb
3.3kb
B
M
B Bg+Hb
B520dl300
B510dl300
B505dl300
B552 dl300
5.2kb
5.0kb
4.6kb
3.3kb
C
M
dl300
B503dl300
5.2kb
5.0kb
4.6kb
3.3kb

D

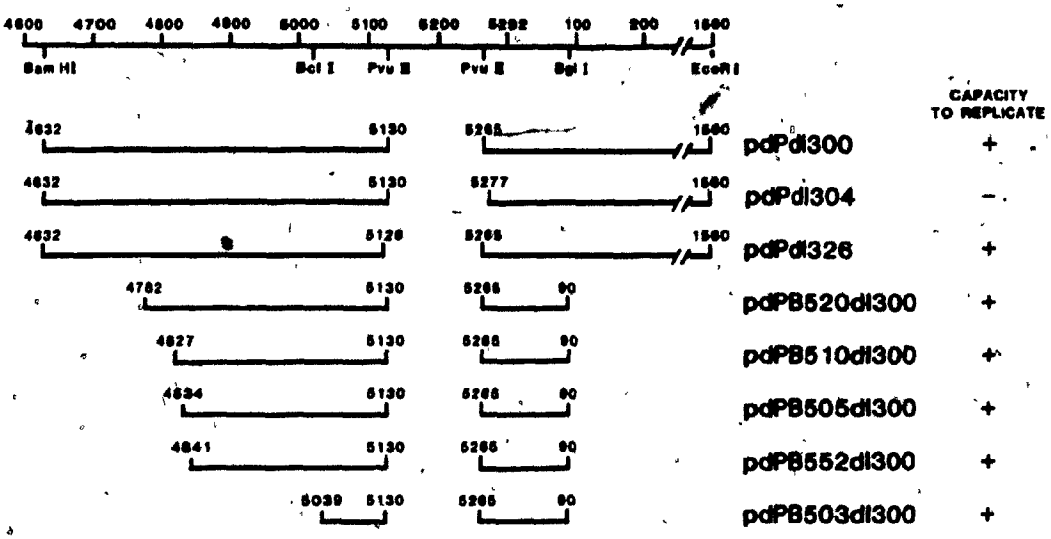

4600
4700
4800
4900
5000
5100
5200
5282
100
200
1580
Bam HI
Bcl I
Pvu II
Pvu II
Bgl I
EcoR I
4632
5130
5265
1580
pdPdl300
+
4632
5130
5277
1580
pdPdl304
−
4632
5128
5265
1580
pdPdl326
+
4782
5130
5265
90
pdPB520dl300
+
4827
5130
5265
90
pdPB510dl300
+
4834
5130
5265
90
pdPB505dl300
+
4841
5130
5265
90
pdPB552dl300
+
5039
5130
5265
90
pdPB503dl300
+
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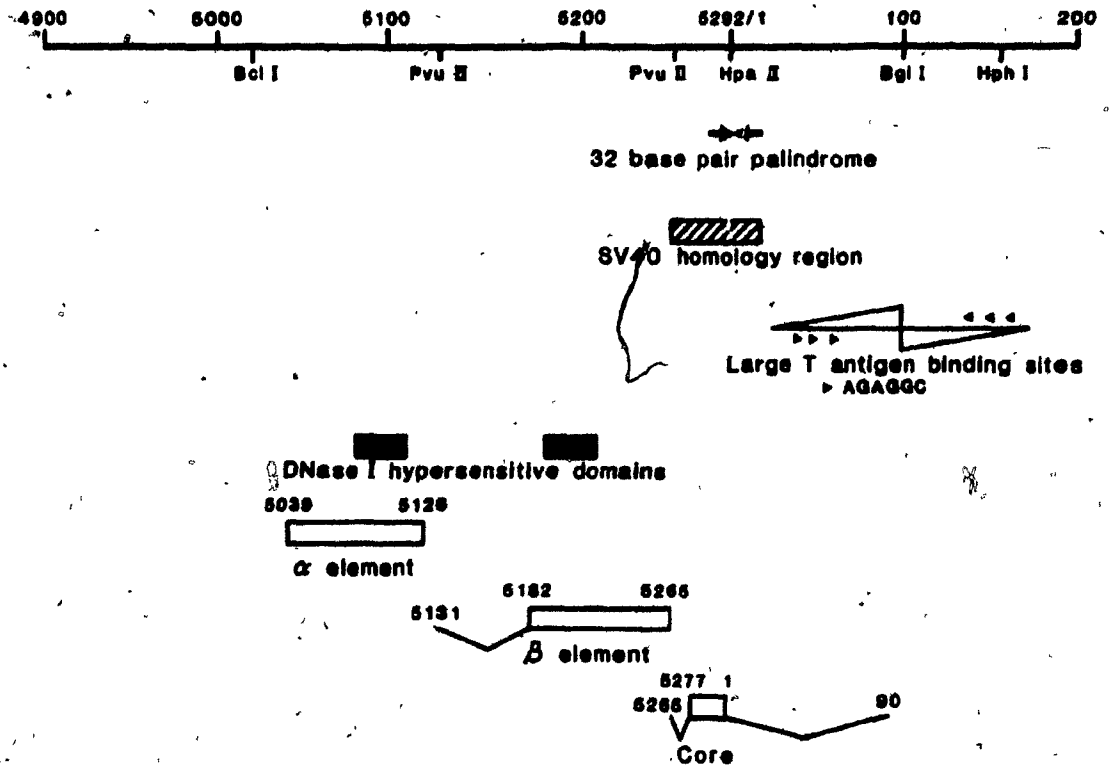
Figure 12: Physical features near the PyV ori. The viral large T antigen binding sites are positioned according to Pomerantz et al (1983a). Repeats of the nucleotide sequence -AGAGGC- are designated by arrowheads. The 32 bp region of dyad symmetry is indicated by bold arrows. Domains of DNase I hypersensitivity are shown according to Herbomel et al (1981). The various replicational elements are shown at the bottom of the figure. The borders of the elements are denoted by a V-shaped line, where they are known.


4900
5000
5100
5200
5262/1
100
200
Bcl I
Pvu II
Pvu II
Hpa II
Bgl I
Hph I
32 base pair palindrome
SV40 homology region
Large T antigen binding sites
AGAGGC
DNase I hypersensitive domains
5039
5126
α element
5182
5266
5131
β element
5277
1
5266
90
Core

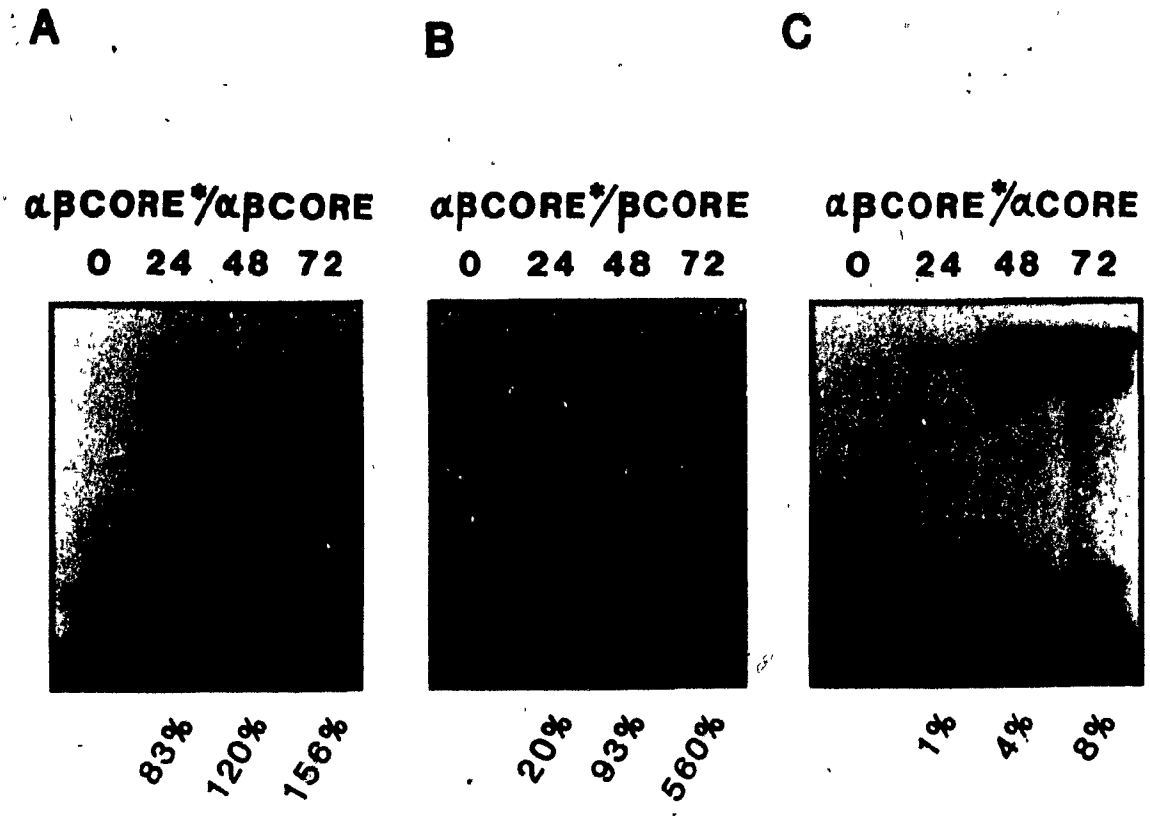
required in cis for viral DNA replication. Furthermore, neither the core region alone nor the upstream α and β elements, alone or together, is capable of autonomous DNA replication in MOP-8 cells (data not shown). This latter observation suggests that a replicationally active recombinant plasmid must maintain in cis the coordinate presence of at least one of the upstream elements (α or β) and an intact core region to remain active in replication.

C. Replicative capacity of the PyV minimal ori regions.

To quantitate the replication efficiencies of viral recombinant plasmids containing the different PyV origin configurations (α-core, β-core, αβ-core) I measured the replicative capacity of these plasmids in MOP-8 cells at 0, 24, 48 and 72 hours post-transfection. The αβ-core recombinant plasmid (pdPB503Bg[H]) is composed of the viral sequences located between nucleotide 5039 (a BamHI site) and 90 (a HindIII site) cloned within the BamHI and HindIII sites of pML-2. The other two origin configurations, α-core (pdPB503d13000; Fig. 11C, D) and β-core (pdPP1[B]Bg[H]; Fig. 10A, B) have been described previously. Because I wished to determine the relative replication efficiencies of the origin bearing plasmids I included a competitor plasmid in these transfections. I chose to use a recombinant plasmid containing all three replication elements, αβ-core* for this purpose (pdPBBg[H]; Fig. 10B). The latter can be distinguished from the other origin containing plasmids after gel-electrophoresis because it is larger. It contains additional viral sequences between nucleotides 4632 and 5039 as well as those between nucleotides 5039 and 90. To insure competition between the two DNA species, 500 ng of each was used to transfect 5 x 10^5 MOP-8 cells per plate. The replication capacity of each origin-bearing viral recombinant was measured by densitometric scanning of the autoradiographs and normalized to that of αβ-core*.

The results are illustrated in Fig. 13. Comparison of the replication properties of the various viral recombinant plasmids indicated that peak DNA replication occured at 48 hours post-transfection with all three replication origins. Replication of the viral recombinants increased slightly or leveled off at 72 hours post-transfection.

Figure 13: Relative replication efficiencies of the PyV minimal ori regions. A 500 ng sample of the αβ-core* viral recombinant plasmid and each of the other origin containing viral recombinants were cotransfected into MOP-8 cells and their replication assessed at 0, 24, 48 and 72 hours (top of autoradiogram) post-transfection as described previously (Fig. 9). The extent of replication of the origin containing DNAs relative to the αβ-core* plasmid was calculated as described in the text and is illustrated at the bottom of the autoradiogram. The replication capacity of the αβ-core, β-core and α-core recombinants (lower bands) are shown in panels A, B and C respectively.


A
B
C
αβCORE*/αβCORE
0 24 48 72
83%
120%
156%
αβCORE*/βCORE
0 24 48 72
20%
93%
560%
αβCORE*/αCORE
0 24 48 72
1%
4%
8%

As expected the αβ-core* replicated to approximately the same levels as the αβ-core* plasmid at all three time points examined (Fig. 13A). Therefore, the additional viral DNA sequences present in the αβ-core* (nucleotides 4632 to 5039) do not enhance or retard plasmid replication in MOP-8 cells.

Examination of the replicative capacity of the β-core plasmid (pdPP1[B]Bg[H]) revealed that this viral recombinant replicated to 5-fold lower levels that the αβ-core* control plasmid at 24 hours post-transfection. Surprisingly, the β-core plasmid had replicated to the same or greater extent than the αβ-core* plasmid by 72 hours post-transfection (Fig. 13B).

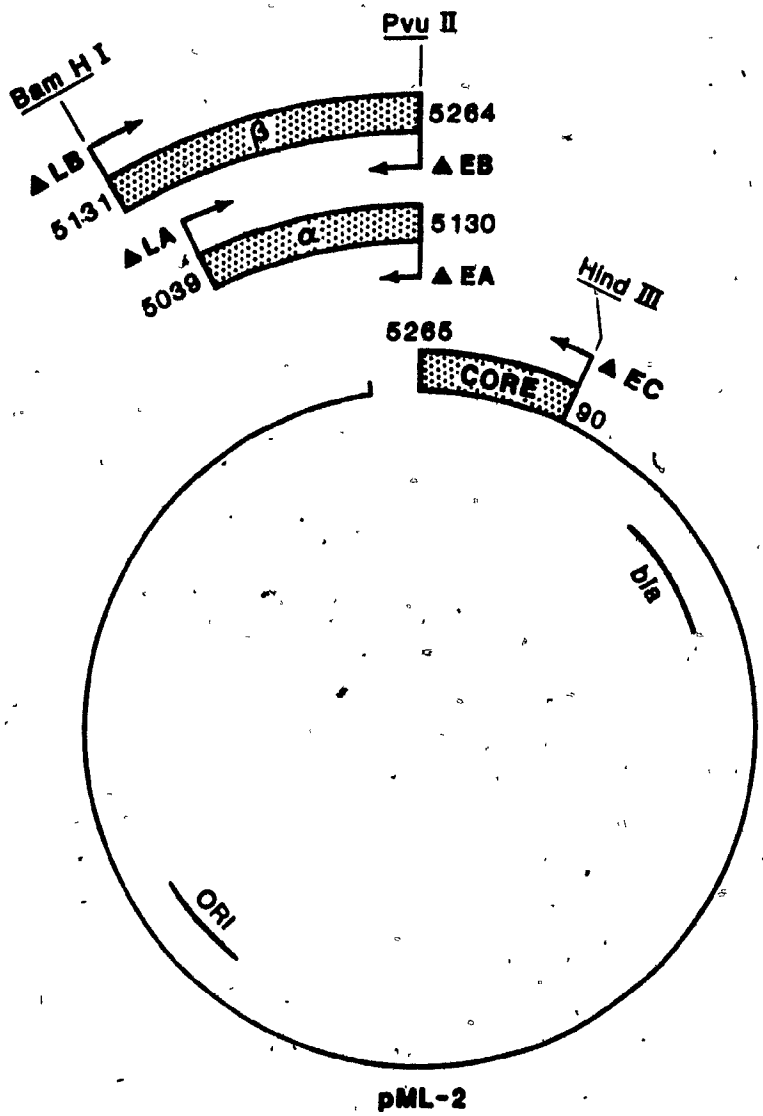
These results show that the β-core replication origin is at an initial disadvantage with respect to αβ-core* origin for DNA replication. However, as the β-core continues to replicate, it eventually outcompetes the αβ-core* recombinant plasmid (Fig. 13B).

Measurement of the replicative capacity of the α-core viral recombinant plasmid showed that it replicated poorly in competition with the control plasmid at all time points examined (Fig. 13C). The relative replication efficiency of this plasmid varied from 1 to 8% the level of the αβ-core* recombinant plasmid. Thus, these results indicate that sequences residing within the β-element (absent in the α-core viral recombinant) confer a competitive advantage in viral DNA replication.

D. Fine mapping the borders of the replication elements.

To aid the identification of the functional sequences within each replication element, I mapped their borders in greater detail. This was accomplished by cloning the α and β elements separately near the late border of the core to yield plasmids containing either the α-core (pdPB503Bg[H]d1300) or the β-core (pdPP1[B]Bg[H]) configurations of the functional origin (Fig. 14). To facilitate the isolation of deletion mutants, unique restriction endonuclease sites were introduced at the late and early borders of each element. In no case did the presence of these linkers affect the replicative capacity of the viral recombinants. The origin containing plasmids were resected at these restriction endonuclease sites and subjected to limited digestion with Bal 31 nuclease. To ensure the recovery of unidirectional deletion mutants, the α and β elements were recloned adjacent to the late core border after mutagenesis. In this way,

Figure 14: Fine mapping of the borders of the replication activators. The parental plasmids used for this deletion analysis were comprised of either of the β-core (pdPP1[B]Bg[H]; Fig. 9) or α-core (pdPB503d1300; Fig. 10) origin configuration cloned the BamHI to HindIII sites of pML-2. The nucleotide numbering scheme of Soeda et al (1980) is used for PyV. The arrows indicate the site and directions of deletions generated. The ΔLB and ΔLA deletion series were made by cleaving the α-core and β-core viral origins at the BamHI site and subjecting them to Bal 31 nuclease digestion. The ΔEB and ΔEA mutants were constructed by resecting the α-core and β-core recombinants at the PvuII and digesting them with Bal 31 nuclease whereas the ΔEC mutants were generated by cleavage and nuclease treatment of HindIII cleaved β-core DNA. The pML-2 plasmid origin for DNA replication and ampicillin resistance marker are included for reference purposes.


Pvu II
Bam H I
5264
β
▲LB
▲EB
5131
▲LA
5130
α
▲EA
5039
Hind III
5265
CORE
▲EC
90
bla
ORI
pML-2

5 series of recombinant plasmids bearing deletions through the late and early borders of the α element (ΔLA and ΔEA), the β element (ΔLB and ΔEB) and the early border of the core region (ΔEC) were generated (Fig. 14).

To measure the replicational capacity of these deletion mutants, 1 μg of plasmid DNA was transfected into MOP-8 cells and the extent of plasmid DNA replication assessed at 48 hours post-transfection by cleavage of the isolated low-molecular-weight DNA with DpnI and an appropriate one-cut restriction endonuclease (see Materials and Methods).

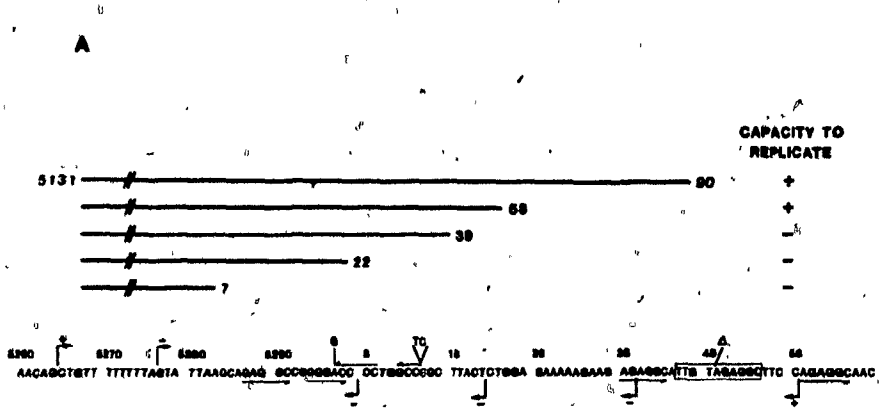
E. The borders of the core.

Previous deletion mapping of the core region established that its early border was situated between nucleotides 1 and 90 and that its late border was located between nucleotides 5265 and 5277 (Chapter 2, sections A, B). Although the late border of the core was defined to a relatively small DNA stretch, its early border was less well defined. For this reason I decided to map more precisely the early border of the core region. This was accomplished by determining the replication capacity of recombinant plasmids containing unidirectional deletions originating from nucleotide position 90. Because these mutant viral recombinant plasmids possess a functional β element (nucleotides 5131 to 5265), the replicational phenotypes exhibited by these mutants can be ascribed solely to the deletion of core sequences. As shown in Fig. 15, mutants possessing viral DNA sequences up to nucleotide 58 were capable of autonomous DNA replication in MOP cells. However, deletion of viral DNA to nucleotides 39, 22, or 7 resulted in the production of replication defective plasmids. From these results I conclude that the early border of the viral core region is between nucleotides 58 and 39. Because the strain of PyV used in these studies contains a 10 bp deletion relative to the A-2 strain (nucleotides 44 to 55), the early boundary of the core is actually located within an 8 bp stretch of viral DNA (Fig. 15A).

F. The borders of the β element.

To identify the minimal DNA sequences required for β element function, I measured the replication of the late and early β deletion mutants (ΔLB and ΔEB) in MOP-8 cells. The structures and replication phenotypes of these mutants are illustrated in Fig. 16. Deletions of viral DNA through the late side of the β element up to nucleotide 5172 did not affect

Figure 15: The early core boundary. (A) Schematic illustration of the structures of the mutants relative to the PyV nucleotide sequence. The nucleotide numbering scheme is according to Soeda et al (1980). The strain of PyV used in this research, which I will refer to as A-1, differs from the A-2 in DNA sequence at several positions. The A at nucleotide position 5 is replaced by a G in the A-1 strain and the A-1 strain contains an insertion of 2 bp (TC) between nucleotides 14 and 15. The A-1 strain contains a 10 bp deletion relative to the A-2 strain between nucleotides 44 and 55 (shown by the box region with the Δ above it). The solid arrows represent the large T antigen recognition-binding pentanucleotide sequence motif (5'-GAGGC-3') whereas dotted arrows refer to the related large T antigen binding sequence (5'-GGGGC-3') described by DeLucia et al (1983). The replication phenotypes of mutants are illustrated by the arrows above (late mutants) or below (early mutants) the sequence. Because no attempt was made to quantitate the precise level of DNA replication, the replication phenotypes are designated as either + or -. (B) A 1μg sample of the PyV recombinant plasmid was transfected into MOP-8 cells, and its replication assayed at 48 hours post-transfection as described previously (Fig. 9).


A
CAPACITY TO REPLICATE
5131
90
68
39
22
7
+
+
–
–
–
TC
Δ


B
ΔEC Deletions
pWP1 (Wild-type)
ΔEC68
ΔEC39
ΔEC22
ΔEC7

replication of the resulting plasmids (Fig. 16A). However, mutants bearing deletions extending to nucleotides 5182, 5225 and 5248 did not replicate in MOP-8 cells. Even after prolonged exposure of the blot, no replicated DNA was detected. These results define the late border of β between nucleotides 5172 and 5182. Although the mutant defining the late β border (ΔLB 5172) is replication competent, I have consistently observed that it replicates at 5 to 10% the level of the wild type β-core replication origin. Because the ΔLB 5147 mutant replicates close to wild type levels, this result suggests that viral DNA between nucleotides 5147 and 5172 may contribute to β function.

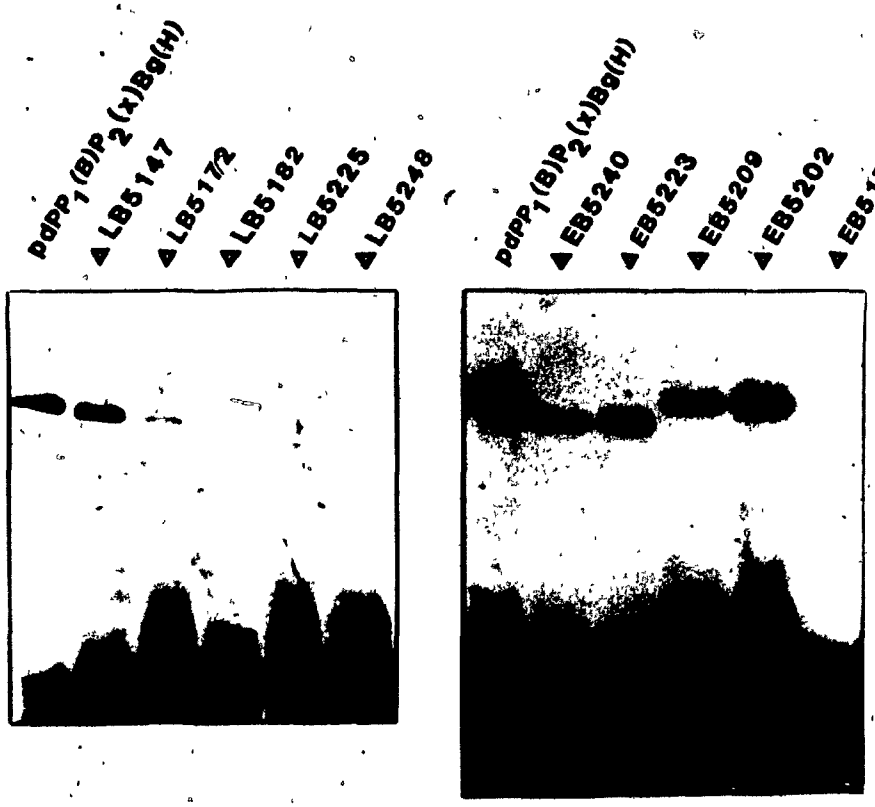
PyV recombinant plasmids bearing deletions on the early side of the β element up to nucleotide position 5202 were capable of autonomous DNA replication in MOP-8 cells (Fig. 16B). However, deletion of viral sequences to nucleotide 5170 rendered the mutant DNA (ΔEB 5170) replication defective. These observations demonstrate that the early border of the β element is located between nucleotides 5202 and 5170.

Taken together, these results indicate that the β element is composed of at least two sets of sequences. The first of these regions is located between nucleotides 5147 and 5172. Although this region is not absolutely essential for β element function, deletion of these sequences impairs viral replication. A second region positioned between nucleotides 5172 and 5202 is absolutely required for β element activity. Several interesting sequence features are located within this 33-bp stretch of viral DNA. These include a 9 bp guanine:cytosine (G:C)-rich inverted repeat (nucleotides 5170 to 5187) and a 9 bp tract homologous to the SV40 enhancer core consensus sequence (Weiher et al, 1983; Fig. 16C).

G. The borders of the α element.

To map the borders of the α element, the replicative capacity of comparable deletion mutants (ΔLA and ΔEA) were determined. The results of this analysis are shown in Fig. 17. Measurement of the replicational capacity of the ΔLA mutants revealed that deletion of viral DNA up to nucleotide position 5097 resulted in the production of replication competent molecules. However, deletion of 26 bp beyond this endpoint resulted in a replication-defective viral recombinant. These results indicate that the late boundary of the α element is situated between nucleotides 5097 and 5120.

Figure 16: Sequences required for the function of β. (A, B) The replicative capacities of the PyV recombinant plasmids were determined as described in Fig. 15. The recombinant plasmid pdPP1(B)P2(X)Bg(H) was included for comparative purposes. The difference in electrophoretic mobility for the mutants ΔEB5240 and ΔEB5223 relative to the control plasmid is due to deletion of plasmid sequences in these constructs (see Materials and Methods). (C) Schematic illustration of the structures of the ΔLB and ΔEB deletion mutants: The nucleotide sequence of A-1 strain contains several differences relative to the PyV A-2 strain. These include an insertion of a T between nucleotides 5171 and 5172, an insertion of an A between nucleotides 5185 and 5186, and an insertion of an A between nucleotides 5209 and 5210. The boxed region refers to the sequence homologous to the SV40 enhancer core sequence (Weiher et al, 1983). The solid arrows represent the 9 bp G;C rich inverted repeat within β. The arrows above the sequence refer to mutants containing deletions on the late side of β whereas the arrows below represent mutants bearing deletions on the early side of β. The + and - refer to the replication phenotypes of the deletion mutants. The structure and replication phenotypes of the additional mutants not shown in panel A and B are also included.


A.
Δ LB Deletions
B.
Δ EB Deletions
pdPP1(B)P2(x)Bg(H)
ΔLB5147
ΔLB5172
ΔLB5182
ΔLB5225
ΔLB5248
pdPP1(B)P2(x)Bg(H)
ΔEB5240
ΔEB5223
ΔEB5209
ΔEB5202
ΔEB5170

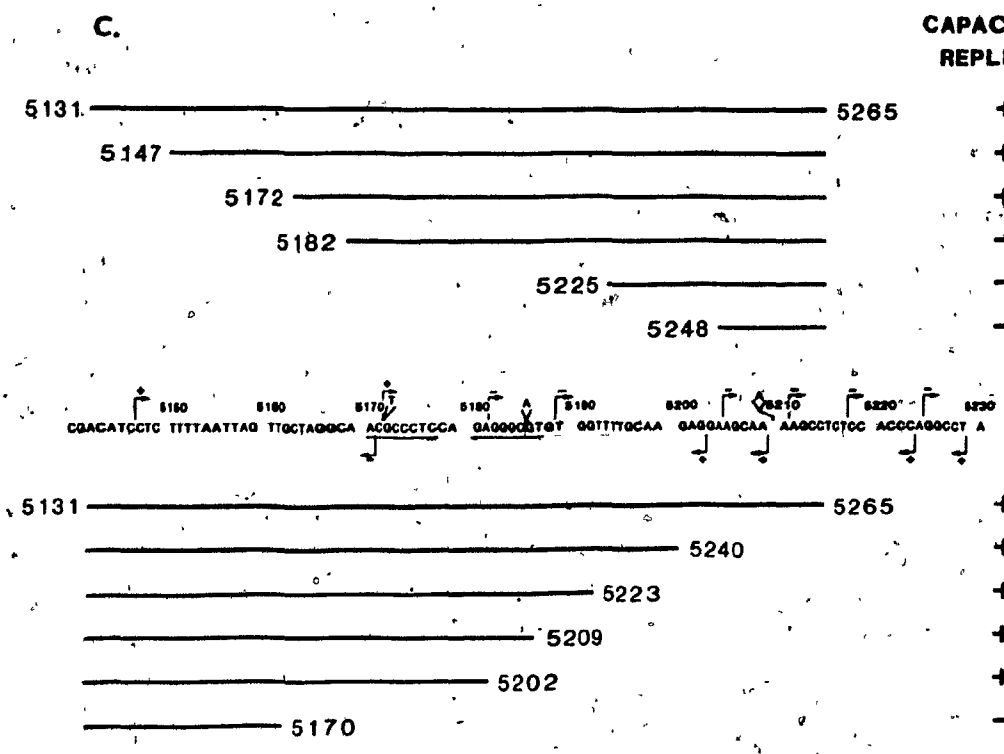
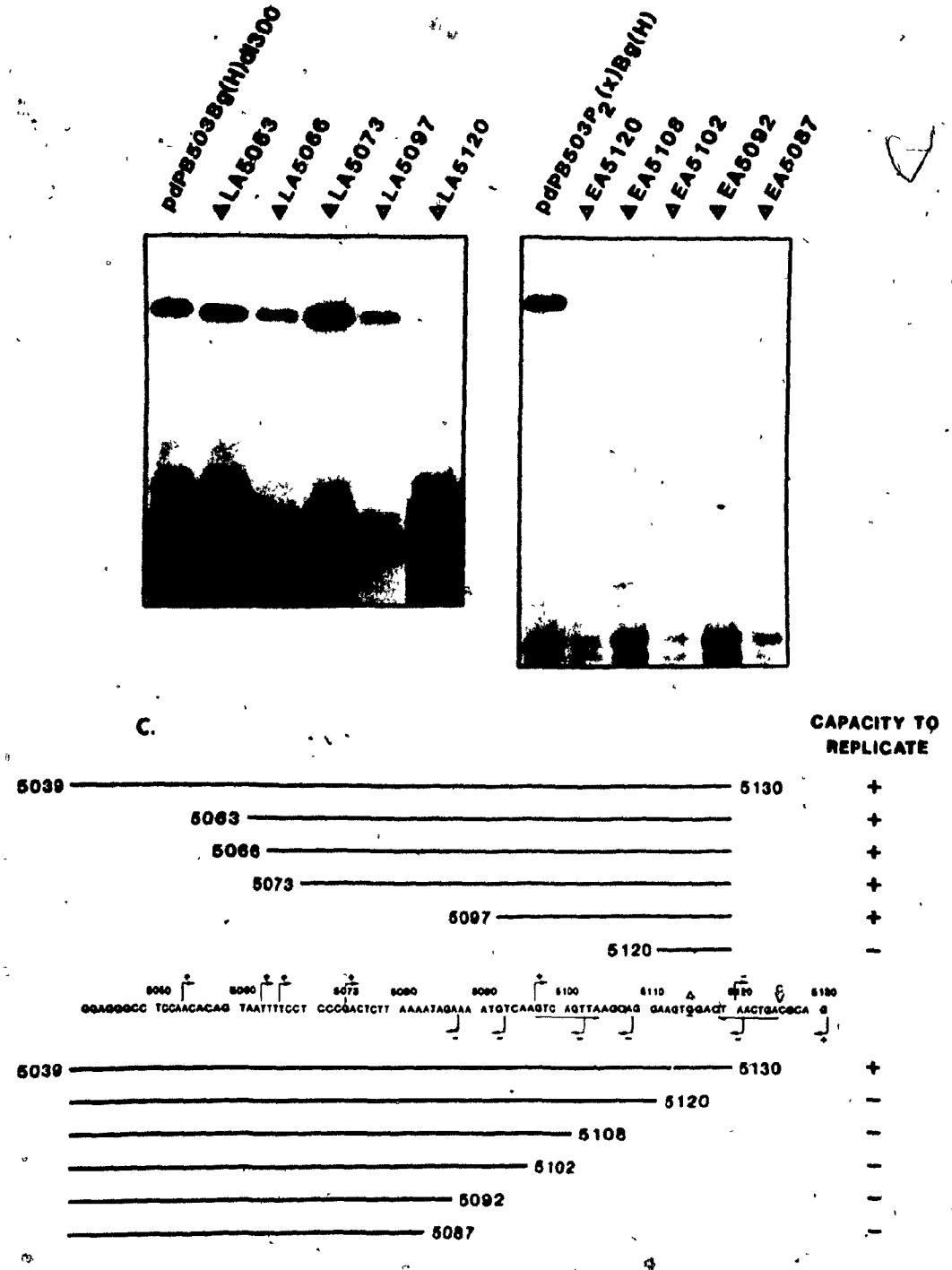

C.
CAPACITY TO REPLICATE
5131
5265
+
5147
+
5172
+
5182
−
5225
−
5248
−
5150
5160
5170
5180
5190
5200
5210
5220
5230
CGACATCCTC TTTTAATTAG TTGCTAGGCA ACGCCCTCGA GAGGGCTGT GGTTTTGCAA GAGGAAGCAA AAGCCTCTCC ACCCAGGCCT A
5131
5265
+
5240
+
5223
+
5209
+
5202
+
5170
−

Figure 17: Sequences required for the function of α. (A, B) The replicative capacities of the PyV mutants were determined as described in Fig. 15. The recombinant plasmids pdPB503Bg(H) d1300 and pdPB503P2(X)Bg(H) were included as controls. (C) Schematic illustration of the structures of the ΔLA and ΔEA deletion mutants relative to the PyV nucleotide sequence. The A-1 strain differs from the A-2 strain in several positions in the viral sequence. These include a deletion of a G at nucleotide 5115 (shown by triangle above sequence) and an insertion of a C between nucleotide 5125 and 5126. The boxed region represents the 10 bp region homologous to the adenovirus E1a transcriptional enhancer (Hearing and Shenk, 1983). The solid arrows underlining the sequence refer to the 8 bp inverted repeat sequence motif. The arrows above the sequence refer to mutants bearing deletions on the late side of α whereas those below represent mutants containing deletions on the early side of α. The structure and replication phenotypes of additional mutants not shown in panels A or B are also illustrated.


A. ΔLA Deletions
B. ΔEA Deletions
pdPB503Bg(H)dB00
ΔLA5063
ΔLA5066
ΔLA5073
ΔLA5097
ΔLA5120
pdPB503P2(x)Bg(H)
ΔEA5120
ΔEA5108
ΔEA5102
ΔEA5092
ΔEA5087
C.
CAPACITY TO REPLICATE
5039
5130
5063
5066
5073
5097
5120
5060
5070
5080
5090
5100
5110
5120
5130
GGAGGGCC TCCAACACAG TAATTTTCCT CCCGACTCTT AAAATAGAAA ATGTCAAGTC AGTTAAGCAG GAAGTGGACT AACTGACGCA G
5039
5130
5120
5108
5102
5092
5087
+
+
+
+
+
+
−
+
−
−
−
−
−

To determine the early border of the α element, mutant recombinant plasmids containing unidirectional deletions starting from nucleotide 5130 were assayed for their capacity to replicate in MOP-8 cells. Deletions of 10 bp or more from this endpoint rendered the viral recombinant plasmids replication defective (Fig. 17B).

To ensure that the replication defect observed in the ΔEA mutants was not due to an inhibitory effect of the XhoI linker on DNA replication, I tested the effect of inserting an XhoI linker between the intact α element and the core region. As shown in Figure 17B, the resulting recombinant (pdPB503P2[X]Bg[H]) plasmid replicated well in MOP-8 cells. On the basis of these results it appears that the replication defect observed in the mutants is due to deletion of critical cis-acting sequences located between nucleotides 5130 and 5120. I found previously that unidirectional deletions on the early side of the α element, up to nucleotide 5126, had no effect on DNA replication (Muller et al, 1983a). Therefore, the early border of the α element is located between nucleotides 5126 and 5120.

The 30 bp fragment containing the minimal α element (nucleotides 5097 to 5126) contains several interesting sequence features (Fig. 17C). These include a 10 bp sequence homologous to the adenovirus 5 E1a enhancer core sequence and an 8 bp inverted repeat.

In summary, my results suggest that the arrangement of the sequences within the α and β element is remarkably similar. Both elements contain homologies to enhancer core sequences and both possess distinctive inverted repeats. Interestingly, the inverted repeats in α and β are related in their primary sequence. A consensus sequence 5'-A/C,ACTG,A/C,C-3' can be drawn for these repeat structures. The coincidence of these replication elements with conserved enhancer core sequences suggest that components of the viral enhancer are required in cis for the initiation of viral DNA replication.

H. Relative replicative capacity of PyV mutant origins.

I previously noted that some of the deletion mutants replicated at a reduced capacity by comparison to their wild type parent (Chapter 2, section F). To determine whether this was true for other mutants as well I measured their replication in competition with a control plasmid, referred to previously as αβ-core* (Chapter 2, section C). The αβ-core* plasmid used in these experiments contains all three replication elements.

In this way I was able to assess the contribution to replication of sequences that flank the minimal core, β and α elements. The total amount of DNA used in these experiments was above saturation (500 ng of each DNA species) to insure their competition for replication factors in MOP cells. I also assessed the capacity of each of the mutant DNAs to replicate independently in MOP cells. In order to maintain the total amount of transfected DNA at 1 μg, 500 ng of each mutant DNA template was cotransfected with an equal amount of pML-2 DNA. The replication capacity of these recombinants was assessed by densitometric scanning of the autoradiographs and normalized to the appropriate PyV origin configuration.

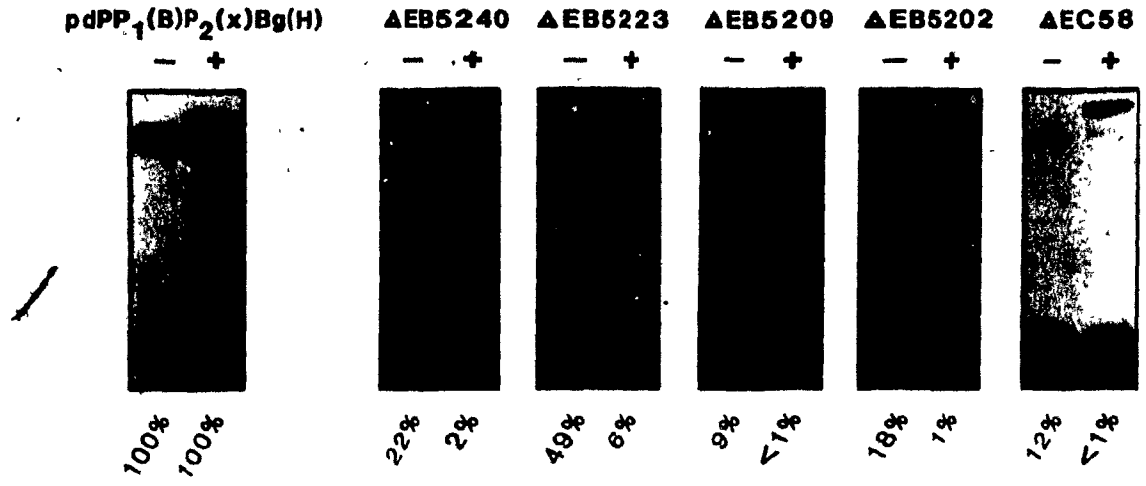
For the competition analysis the ratio of the replication capacity of the wild type origin configuration (β-core or α-core) to that of the αβ-core* origin is taken to be 100%. The resulting ratio of replication capacity of either the mutant β-core or the mutant α-core origins to the competitor plasmid are compared against these wild type values.

Measurement of the capacity of the ΔEB mutants to replicate independently (cotransfected with pML-2) revealed that there was no clear correlation between the size of deletion and the replication phenotype exhibited by the mutant (Fig. 18). Rather the replication efficiencies of these mutants fluctuated between 9% and 49% of the wild type β-core recombinant (pdPP1[B]P2[X]Bg[H]). It should be noted that the wild type recombinant used in these experiments contains an XhoI linker between the β and core elements.

Cotransfection of these same ΔEB mutants with the αβ-core* plasmid exaggerated the difference between replication of the mutant DNAs by comparison to their parent (pdPPP1[B]P_2[X]Bg[H]). As observed previously, the β-core parent plasmid replicated to approximately twice the level of αβ-core* plasmid (Fig. 13B). Mutants that replicated well independently competed poorly with the αβ-core* plasmid (i.e. ΔEB 5223; 6%), whereas mutants that replicated poorly when cotransfected with pML-2 DNA replicated at barely detectable levels in the presence of the competitor plasmid (i.e. ΔEB 5209; >1%).

Because deletion of viral DNA on the early side of the β element element also alters the spatial relation of the β element relative to the core, it is unclear whether the replication phenotypes observed with the ΔEB deletion mutants is due to deletion of essential viral sequences or to

Figure 18: The relative replication efficiencies of the ΔEB and ΔEC mutants. A 500 ng sample of mutant DNA was cotransfected with either equal amounts of pML-2 DNA (lanes with -) or αβ-core* DNA (lanes with +) into MOP-8 cells. Their replication capacity was assayed 48 hours later as described previously (Fig. 9). The replication capacities of the mutant DNA were determined as described in the text and are shown at the bottom of the panels. The pdPP1(B)P2(X)Bg(H) recombinant plasmid was used as a control for these experiments. All the panels were clipped from a single autoradiogram.


pdPP1(B)P2(x)Bg(H)
ΔEB5240
ΔEB5223
ΔEB5209
ΔEB5202
ΔEC58
– +
100% 100%
22% 2%
49% 6%
9% <1%
18% 1%
12% <1%

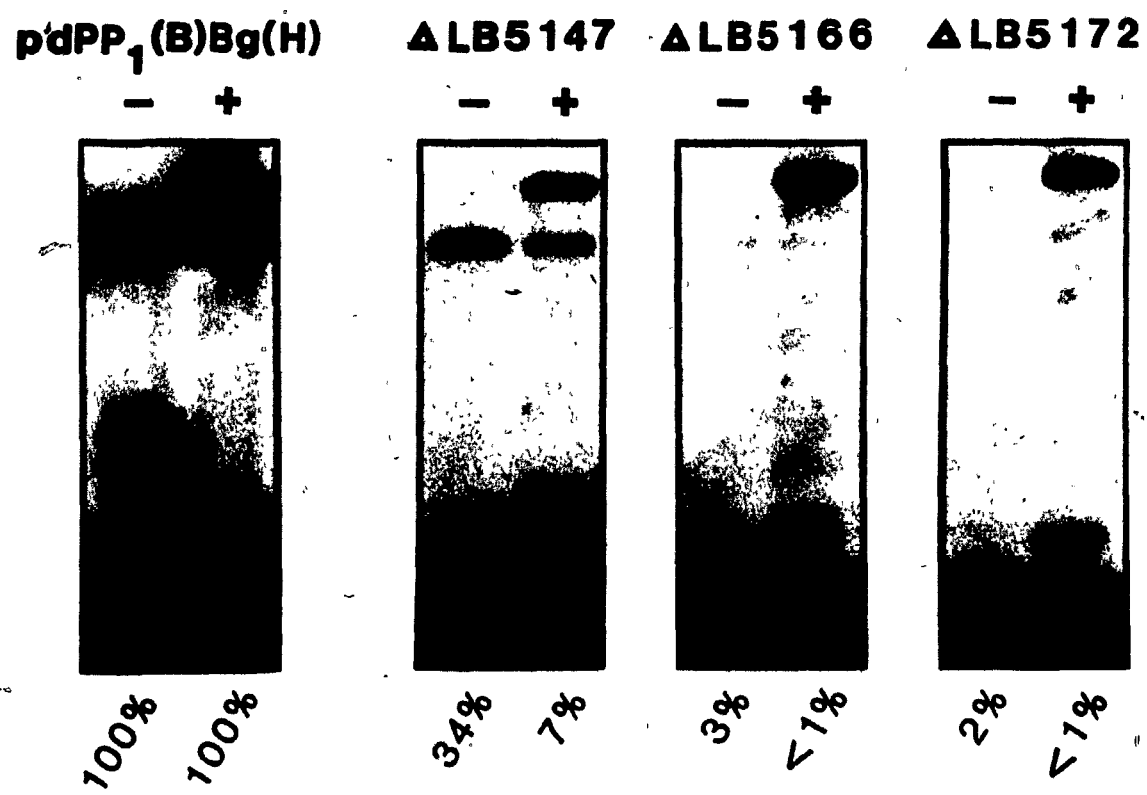
the repositioning of the β element relative to the core. Evidence that the latter phenomenon is occuring is suggested by the observation that certain mutants bearing large deletions replicated better than others containing smaller deletions (ΔEB 5223 > ΔEB 5240; ΔEB 5202 > ΔEB 5209).

Deletion analysis of the core had previously established that its early border was situated near nucleotide 58 (ΔEC 58; Fig. 15). To determine whether this mutant demonstrated an impaired replication phenotype, I measured its replication capacity alone and in competition with the αβ-core* viral recombinant plasmid (Fig. 18). The results showed that the ΔEC 58 mutant replicated at 10-fold reduced levels compared to the wild type β-core plasmid. As observed with other impaired replication origins, replication of this mutant was barely detectable when cotransfected with αβ-core*. Interestingly, the ΔEC 58 deletion impinges upon large T antigen binding site A. These results suggest that the presence of large T antigen binding site A may confer a cis-acting advantage in viral DNA replication.

Measurement of the capacity of the late β mutants (ΔLB) to replicate independently indicated that ΔLB 5147 replicated to 3-fold lower levels than did the wild type control plasmid (pdPP1[B]Bg[H]; Fig. 19). Because I have routinely observed 2-fold variations in the replication capacity of identical mutants, it is not clear whether this difference is significant. Extension of the deletion to nucleotides 5166 or 5172 resulted in a futher 10 to 15-fold drop in replication efficiency of the mutant DNAs. These results are consistent with previous observations (Fig. 16) and indicate that viral sequences adjacent to the late border of the β element (nucleotides 5172 to 5182) contribute to its activity. Furthermore, the observation that ΔLB 5166 replicated poorly whereas ΔLB 5147 replicated well suggests that there are important sequences between nucleotides 5147 and 5166 that affect the activity of the β element.

The results of cotransfection experiments involving the αβ-core* competitor plasmid and the late β mutants are shown in Fig. 19 (lanes with +). As observed previously (Chapter 2, section C), the β-core wild type plasmid (pdPP1[B]Bg[H]) replicated to approximately 2-fold higher levels than the αβ-core* plasmid. By contrast, ΔLB 5147 mutant replicated to approximately 15-fold reduced levels in competition with the αβ-core* plasmid (Fig. 19). Only very weak replication (<1%) could be observed with ΔLB 5166 and ΔLB 5172 in competition with the αβ-core* plasmid. These

Figure 19: The relative replication efficiencies of the ΔLB mutants. The replicative capacities of the ΔLB recombinant plasmids were determined as described for Fig. 18. The recombinant plasmid pdPP1(B)Bg(H) was included as a control.


p'dPP1(B)Bg(H)
ΔLB5147
ΔLB5166
ΔLB5172
- + - + - + - +
100% 100% 34% 7% 3% <1% 2% <1%

results show that compared to the wild type β-core viral recombinant ΔLB 5147 replicates poorly in competition (7% of the levels of wild type β core). Moreover, deletion of viral DNA sequences to nucleotide number 5166 abolishes the capacity of mutants to replicate in competition with the αβ-core* plasmid. Thus, the results of the competition experiments suggest that in addition to the sequences between 5147 and 5166, a second segment of viral DNA between nucleotides 5131 and 5147 contributes to β element activity.

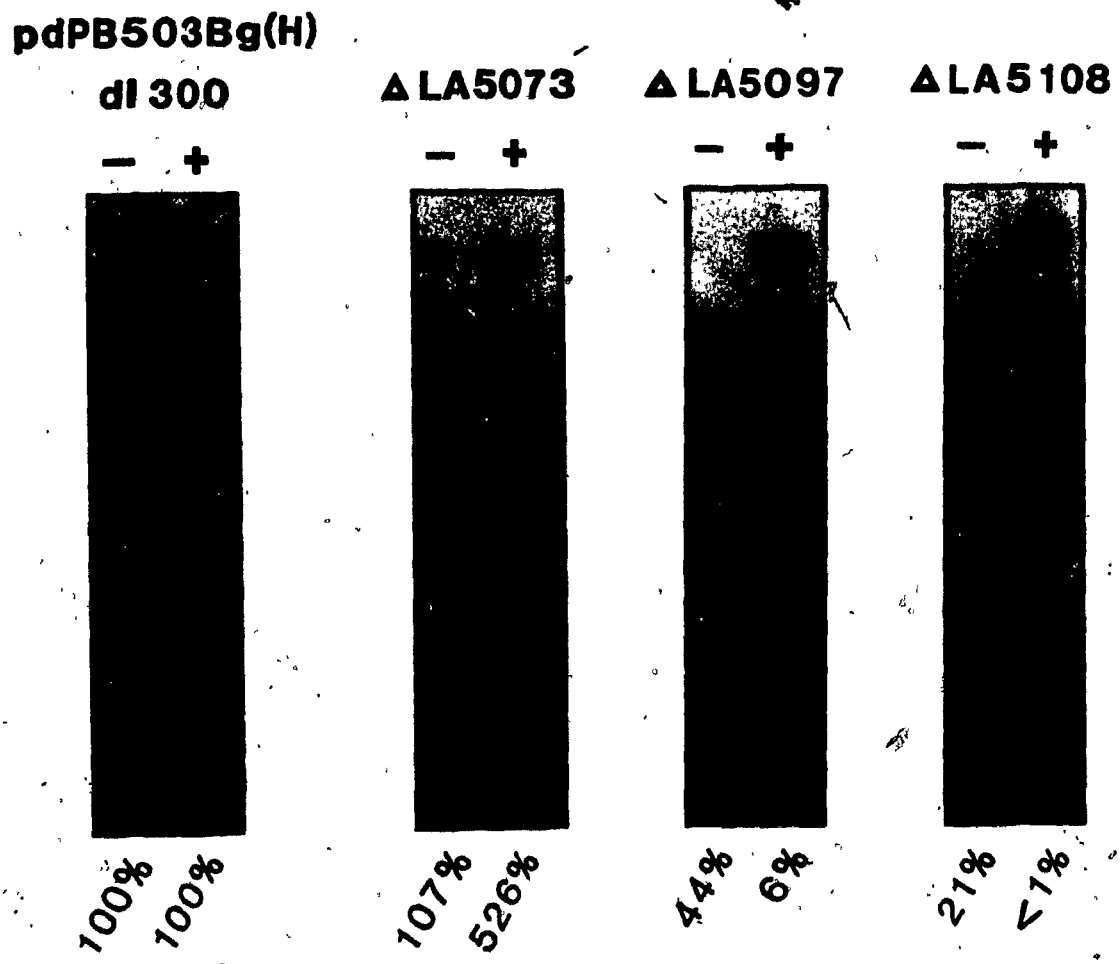
The capacity of mutants bearing deletions on late side of α to replicate in MOP cells alone or in competition with the αβ-core* is shown in Fig. 20. Measurement of the capacity of these mutants to replicate alone revealed that deletion to nucleotide numbers 5073, 5097 and 5108 resulted in a gradual decline in replication competence (107%, 44% and 21% respectively) relative to the α-core plasmid recombinant (pdPB503d1300). These and previous results (Fig. 11) indicate that the late border of the α element is located between nucleotides 5108 and 5126.

As observed previously, the α-core plasmid (pdPB503d1300) replicated poorly when cotransfected with the αβ-core* competitor plasmid (Fig. 20). Surprisingly, ΔLA 5073 replicated to 5-fold higher levels in competition with the αβ-core* plasmid than did the wild type α-core. These results suggest that sequences situated between nucleotides 5039 and 5073 may have a negative effect on viral DNA replication. The replication defects exhibited by the ΔLA 5097 and ΔLA 5108 mutants were more pronounced in competition with the αβ-core* plasmid. The ΔLA 5097 mutant replicated in competition to 6% the level of the wild type α-core plasmid whereas the ΔLA 5108 mutant replicated to barely detectable levels. These results indicate the viral sequences located between 5073 and 5108 constitute an important functional domain of the α element.

I. <u>β comprises multiple sequence elements</u>.

The results of my deletion analyses predict that the minimal sequences required for β function reside between nucleotides 5172 and 5202. However, I could not readily test this prediction for no convenient restriction sites are near these endpoints to allow for the cloning of β. Consequently, I attempted another approach whose application was built into the design of the early and late β mutants. This method, called linker scanning mutagenesis, allows for the substitution of wild type DNA by

Figure 20: The relative replication efficiencies of the ΔLA mutants. The replicative capacities of the ΔLA mutants were determined as described for Figure 18. The recombinant plasmid pdPB503d1300 was included as a control.


pdPB503Bg(H)
dl 300
ΔLA5073
ΔLA5097
ΔLA5108
− +
− +
− +
− +
100%
100%
107%
526%
44%
6%
21%
<1%

linker DNA, thereby causing mutations of 8, 10 or 12 bp at a time, depending upon the length of the linker (McKnight and Kingsbury, 1982). The advantage of this approach is that the spatial arrangement of the β sequences relative to the core remains unaltered. Consequently, the replication phenotypes of these mutants is solely the result of alteration of the primary structure of the DNA. To accomplish this, sets of appropriately spaced late and early β mutants were recombined in vitro using the unique XhoI restriction site at the borders of the deletion mutants. In this manner, clustered point mutations were introduced into β sequences at the linker junction. Because my library of early and late deletion mutants was not perfect, it was not always possible to recombine deletion mutants at their XhoI-marked termini to exactly substitute wild type sequences with XhoI linker sequences. As a result, some of the linker scanning mutants contain 1 bp deletions or substitutions (Fig. 21). The linker scanning mutants were then assessed for their capacity to replicate autonomously (co-transfected with pML-2 DNA) or in competition with a recombinant plasmid containing all three replication elements (αβ-core*).

The structure and replication capacities of these mutants are illustrated in Figures 21 and 22 respectively. The control for these experiments was the wild type β-core replication origin configuration (pdPP1[B]Bg[H]; Fig. 10). Consistent with previous results (Fig. 13), the wild type β-core plasmid replicated to approximately 5-fold higher levels than did the αβ-core* viral recombinant. As described previously (Fig. 18) the ratio of the replication capacity of the linker scanner mutants to the competitor plasmid (αβ-core*) are normalized against this value.

Substitution of an 8 bp XhoI linker for 7 bp between nucleotides 5240 and 5248 (nucleotides 5241 through 5247 are missing) resulted in a net insertion of 1 bp (Fig. 21). Measurement of the capacity of this mutant, LS 5240/5248, to replicate independently (-) or in competition with αβ-core* (+) revealed that it replicated close to control β-core levels (93% and 73% respectively; Fig. 22B). This observation indicates that the viral DNA present between these two endpoints is dispensible for β element function. Previous results indicated that although the sequences between nucleotides 5240 and 5265 are nonessential for β function, deletion of these sequences had a inhibitory effect on viral DNA replication (ΔEB5240; Fig. 18). In light of the wild type replication properties of LS 5240/5248, the

Figure 21: Structure of the β-linker scanning mutants. The top sequence represents the wild type nucleotide sequence in β. The nucleotides above this sequence refer to the changes present in the A-1 strain as noted previously (Fig. 16). The boxed regions in the sequence refer to the immunoglobulin heavy chain enhancer homology (left box) or the SV40 enhancer homology (right box). The inverted arrows denote inverted repeats present within this sequence. The boxed sequences below the wild type sequence refer to the sequences altered in the various mutants. The squares represent deletion of a base pair whereas the insertion of a base pair is denoted by the inverted V.

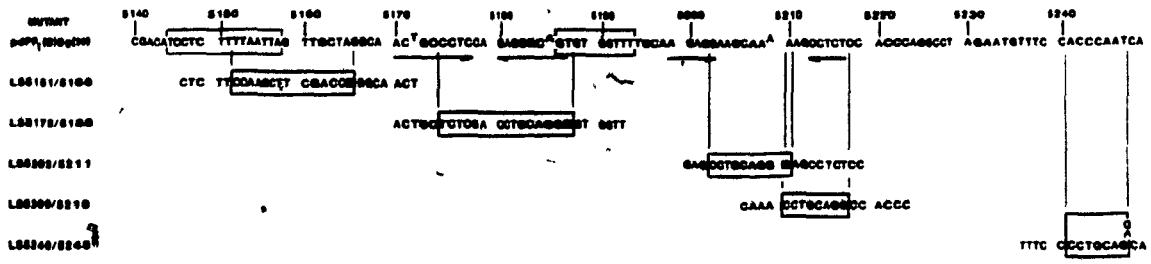
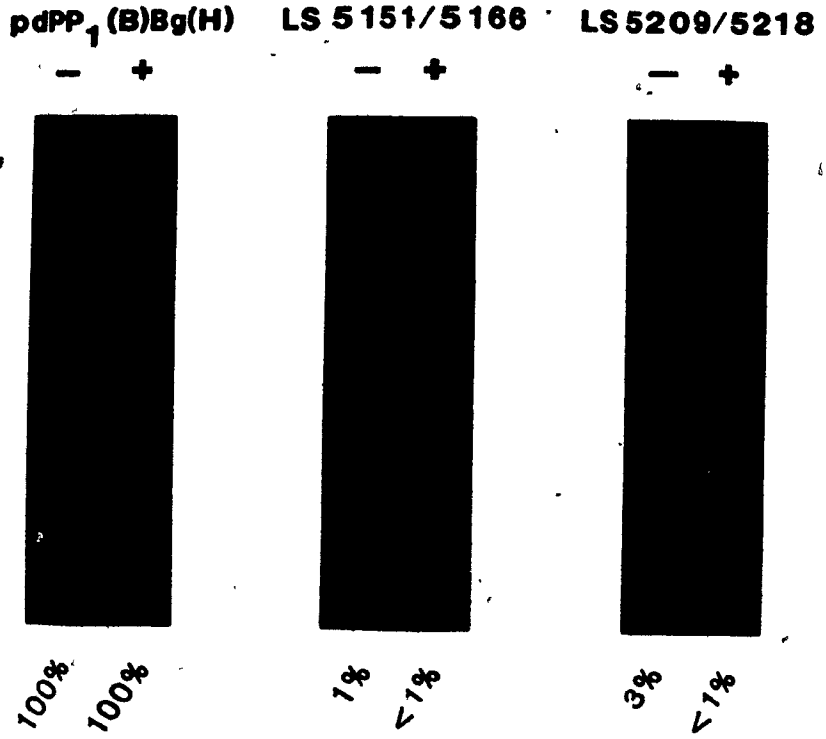
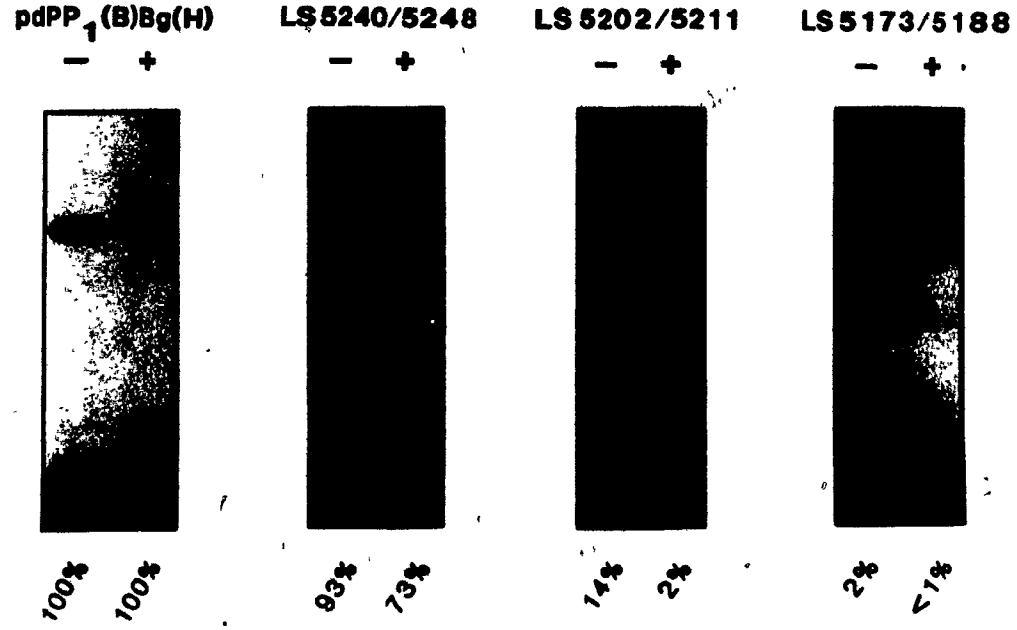


Figure 22: Replication capacities of β linker scanning mutants. (A, B) The replication capacities of the mutants were calculated as described in Fig. 18. The autoradiograms shown in each panel are taken from the same film, which was clipped to allow comparisons to be made between adjacent lanes. The recombinant plasmid pdPP1(B)Bg(H) was included for comparative purposes.

A


pdPP1(B)Bg(H)
LS 5151/5166
LS 5209/5218
− +
− +
− +
100%
100%
1%
<1%
3%
<1%

B


pdPP1(B)Bg(H)
LS 5240/5248
LS 5202/5211
LS 5173/5188
− +
− +
− +
− +
100%
100%
93%
73%
14%
2%
2%
<1%

replication defect exhibited by ΔEB5240 can be attributed to either alteration of the spatial alignment between β and core or to deletion of viral DNA sequences between nucleotides 5248 and 5265.

The LS 5209/5218 mutant contains an 8 bp XhoI linker between nucleotides 5209 and 5218 (nucleotides 5210 to 5217 are missing) and as a result does not contain any insertions or deletions relative to the wild type β-core recombinant (Fig. 21). Measurements of the capacity of this viral recombinant to replicate independently in MOP cells revealed that it replicated to only 1% the level of the wild type β-core origin. Only very faint replication could be detected when this mutant was cotransfected into MOP-8 cells with the competitor plasmid (αβ-core*). The LS 5209/5218 mutant defines a cis-acting sequence required for β element activity.

The mutant LS 5202/5211 contains an 8 bp XhoI linker between nucleotides 5202 and 5211 (nucleotides 5203 and 5210 are missing) (Fig. 21) and was generated by recombining the late β mutant ΔLB 5211 with the early β mutant ΔEB 5202 (see Materials and Methods). Because the strain of PyV used in these experiments contains an insertion of 1 bp between nucleotide 5209 and 5210 (Fig. 21), the mutant LS 5202/5211 actually contains a net deletion of 1 bp relative to the wild type β-core origin (pdPP1[B]Bg[H]). Measurement of the capacity of this mutant to replicate independently (cotransfected with pML-2 DNA) revealed that it replicated to approximately 6-fold reduced levels relative to the β-core origin. When the LS 5202/5211 was assessed for its capacity to replicate in competition (+) with the αβ-core* viral recombinants, it replicated to only 6% the level observed with the wild type β-core plasmid. These results show that the alteration of the viral DNA sequences between nucleotide 5202 and 5211 has a moderate inhibitory effect on β element function.

The sequences altered in the LS 5209/5218 and LS 5202/5211 mutants are located outside the mapped early border of β (between nucleotides 5202 and 5170). Interestingly, the early β mutants from which these LS mutants were derived (ΔEB5202 and ΔEB5209) also exhibited impaired replication phenotypes (Fig. 18). The results of the linker scanning analyses suggest that the sequences deleted in these early β mutants are required for efficient β function.

The LS 5151/5166 and LS 5173/5188 mutants contain a net 1 bp deletion relative to the wild type β-core plasmid (Fig. 21). One of the mutations

maps outside the borders of β (LS 5151/5166) whereas the other (LS 5173/5188) maps within β. Both mutants replicated at 1% the levels of the control in the absence of a competitor, and their replication was barely detectable in a competitive situation (Fig. 22). These results strongly suggest that the viral sequences altered in these recombinant plasmids are essential for efficient β element function.

The replication properties of the LS 5151/5166 and LS 5173/5188 mutants are consistent with results obtained with the late β mutants (Fig. 16). This analysis revealed that the viral sequences between nucleotides 5147 and 5166, and 5172 and 5182 were required for efficient β function. The LS 5151/5166 and LS 5173/5188 mutants map within these critical β sequences.

This experimental approach confirmed that essential β sequences reside between nucleotides 5172 and 5202, and also showed that sequences between nucleotides 5151 and 5166, at the late β border and between 5209 and 5218 at its early border are also important for β function.

There are several interesting sequence features present within these three areas. Located within the viral DNA sequences between nucleotides 5151 to 5166 is an extensive region of homology to the immunoglobulin enhancer (Banerji et al, 1983; Fig. 21). The sequences between nucleotides 5173 and 5188 overlaps with a 9 bp G:C rich inverted and a homologue to the SV40 enhancer core consensus sequence (Weiher et al, 1983). The LS 5209/5218 linker scanning mutant disrupts a 5 bp inverted repeat structure. Interestingly, the LS 5202/5211 mutant which mutates only 1 bp of this inverted repeat replicated in MOP cells to intermediate levels.

J. α comprises multiple sequence elements.

The location of the α element was defined by deletion mutagenesis between nucleotides 5108 and 5126. However, viral DNA to the late side of this region between nucleotides 5073 and 5108 augmented viral DNA replication up to 5-fold (Fig. 20). To confirm the functional significance of sequences within this region, linker scanning and internal deletions mutants were constructed and assessed for their capacity to replicate alone or in competition with αβ-core*.

The structures and replicative capacities of these mutants are shown in Figures 23 and 24. The recombinant plasmid pdPB503d1300 (α-core) served as a wild type control for these experiments. As observed previously (Fig.

Figure 23: Structure of the α linker scanner and insertion mutants. The top sequence represents the wild type nucleotide sequence in α. The nucleotides above this sequence refer to the changes present in the A-1 strain as noted previously (Fig. 17). The boxed region in the sequence refer to E1a enhancer core homology. The inverted arrows denote the 8 bp inverted repeat within this region. The boxed sequences below the wild type sequence refer to sequences altered in the various mutants. The squares represent deletion of a base pair.

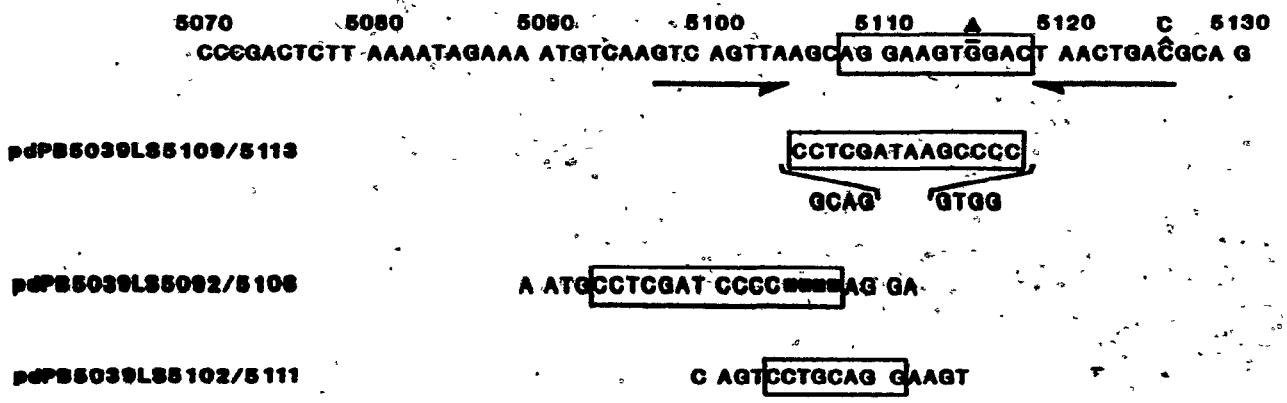
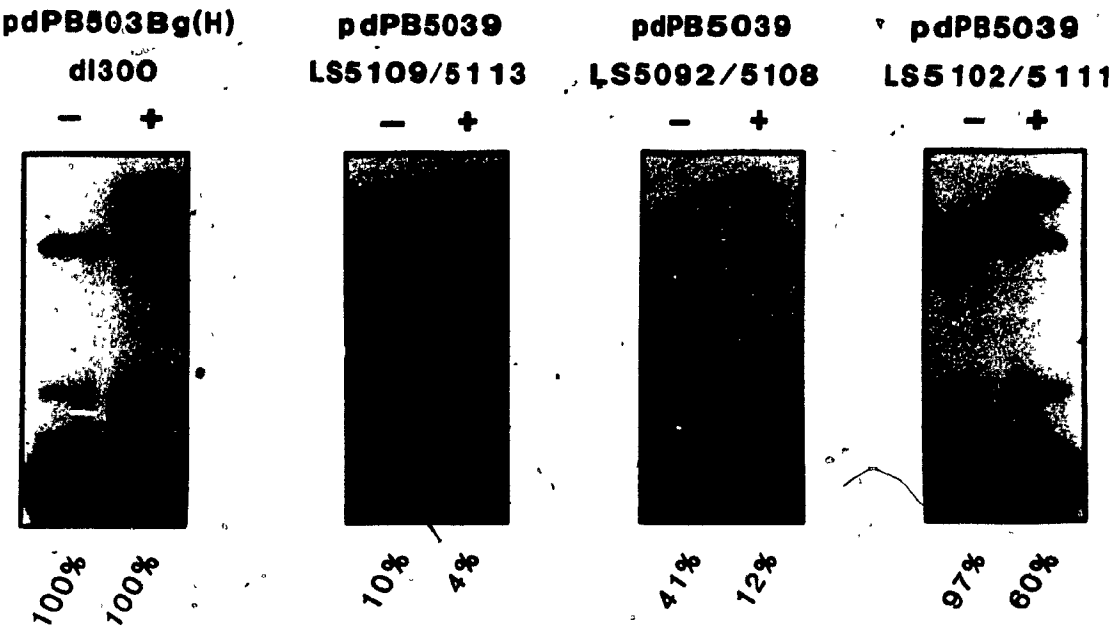

5070
5080
5090
5100
5110
5120
C
5130
CCCGACTCTT AAAATAGAAA ATGTCAAGTC AGTTAAGCAG GAAGTGGACT AACTGACGCA G
pdPB5039LS5109/5113
CCTCGATAAGCCCC
GCAG
GTGG
pdPB5039LS5092/5108
A ATGCCTCGAT CCGC AG GA
pdPB5039LS5102/5111
C AGTCCTGCAG GAAGT

Figure 24: Replication capacities of α insertion and linker scanner mutants. The replication capacities of the mutants were calculated as described in Fig. 18. The pdPB503d1300 recombinant was included as a control.


pdPB503Bg(H)
dl300
pdPB5039
LS5109/5113
pdPB5039
LS5092/5108
pdPB5039
LS5102/5111
− +
− +
− +
− +
100%
100%
10%
4%
41%
12%
97%
60%

13), the α-core recombinant plasmid replicated poorly in competition with the αβ-core* plasmid (approximately 10-fold lower levels). The replicative capacities of mutant α-core origins in competition were normalized against this ratio.

A mutant was isolated, pdPB5039 LS 5109/5113*, that contains a deletion of 3 viral bp (nucleotides 5110 to 5112 are missing) and an insertion of 14 bp of linker DNA (Fig. 23). Although the sequences mutated in this viral recombinant plasmid reside within the borders of the minimal α element, this viral recombinant plasmid was capable of autonomous in MOP-8 cells albeit at reduced levels relative to the wild type α-core viral recombinant (Fig. 24). In the presence of the competitor plasmid, this mutant replicated to reduced but detectable levels (Fig. 24). Surprisingly, deletion of nucleotides 5039 through 5109 from this viral recombinant plasmid led to the generation of a replication defective molecule (data not shown). This result implies that sequences located between nucleotides 5039 and 5109 can partially compensate for deletion of critical α sequences between nucleotides 5109 and 5113. Interestingly, deletion of viral DNA sequences on the late side of pdP5039 LS 5109/5113 to nucleotide 5075 did not affect the replication of viral recombinant (data not shown). This observation indicates that these compensatory sequences are located between nucleotides 5075 and 5109.

The pdPB5039 LS 5108/5092 mutant contains a deletion of 15 bp (nucleotides 5107 to 5091 are missing) and an insertion of 12 bp of linker DNA (Fig. 23) resulting in a net deletion of 3 bp relative to its parent, pdPB503d1300 (the wild type α-core plasmid). It replicated to approximately 40% the level of pdPB503d1300 without a competitor plasmid. A 4-fold drop in replication relative to the wild type α-core plasmid was observed when this mutant DNA was assessed for its capacity to replicate in competition with αβ-core* (Fig. 24). These results suggest that viral sequences located between nucleotides 5092 to 5108 confer a competitive advantage in DNA replication.

The mutant pdPB5039 LS 5111/5102 contains an 8 bp deletion in viral sequences (nucleotide 5110 to 5103 are missing) substituted with an 8 bp XhoI linker (Fig. 23). By contrast to other mutant α-core origins, this mutant replicated alone or in competition to levels close to the wild type α-core viral recombinant plasmid. Because the XhoI linker restores viral DNA sequences between nucleotides 5111 and 5108, only the viral DNA

sequences between nucleotides 5102 and 5105 are altered in this mutant. This observation indicates that sequences between 5102 and 5105 are dispensible for α function.

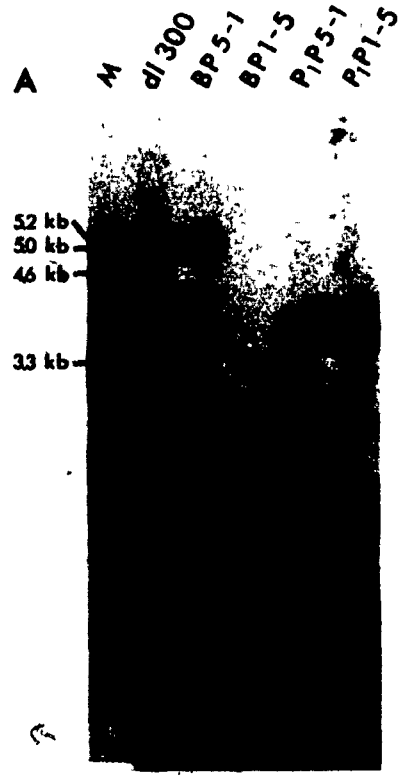
The results of the mutational analyses within α suggests that it, like β, comprises multiple sequence elements. The first of these elements is situated within the borders of the minimal α element (nucleotides 5108 to 5126) whereas the other is located adjacent to the late border of α (nucleotides 5075 to 5102). Although only the 5108 to 5126 element can independently activate core replication, its activity is increased by the presence of the 5075 to 5102 region. In addition to the functional similarities between the α and β elements, the results of this genetic analysis suggest that they also share a similar structural organization.

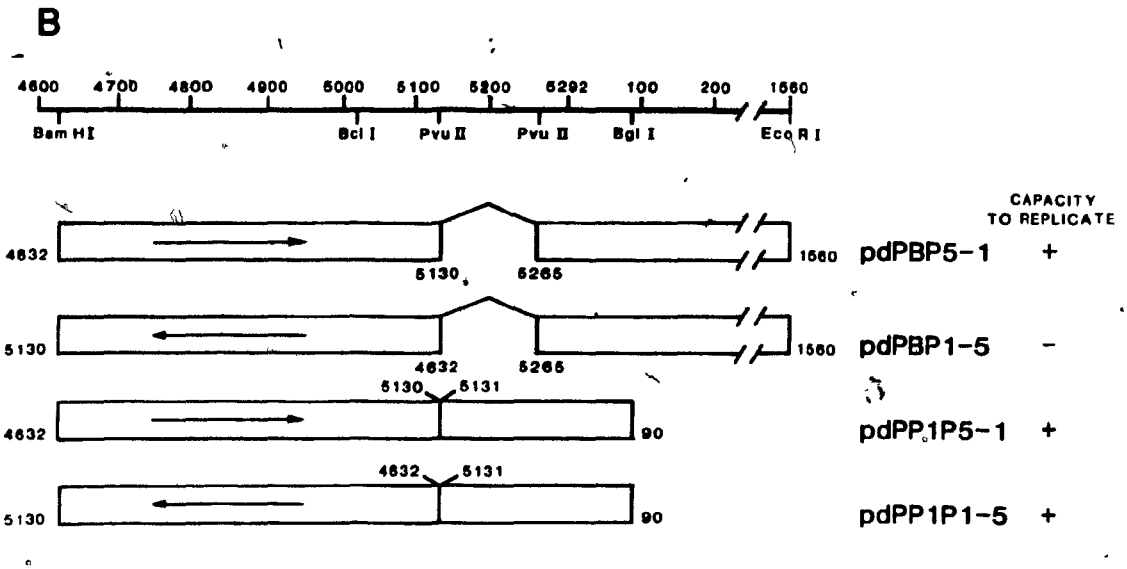
K. Spatial requirements for the activation of PyV DNA replication.

In their natural position, the α and β elements are physically removed from the PyV core. Moreover, the α and β elements map within the borders of the PyV enhancer. Because enhancers function irrespective of their orientation or position to augment gene expression, I was curious to learn whether α and β could also function independent of orientation and position to activate DNA replication.

I first examined whether these elements could activate core replication independent of orientation. To determine whether the α element could function in an orientation independent manner, a 498 bp restriction fragment (nucleotides 4632 to 5130) encompassing the α element was inserted in both orientations at the BamHI site of the viral recombinant pdPBP1, which possesses only the PyV core region (Fig. 25). One of the resulting recombinants, pdPBP5-1, which contains the α element in its native orientation relative to the core region, replicated at levels indistinguishable from those of the pdPd1300 viral recombinant plasmid (α-core; Fig. 11). This occurred despite the insertion of 4 bp at the junction between the α and core regions. However, inversion of the 498 bp fragment in front of the core, as occurs in pdPBP1-5, did not restore replication competence to the pdPBP1 recombinant plasmid (Fig. 25). It is noteworthy that the inversion mutant pdPBP1-5 not only alters the orientation of α relative to the core region, but also effectively places 400 bp between the α element and core (Fig. 25).

Figure 25: The α replicational element demonstrates an orientation or position dependence. (A) A 2 μg sample of DNA was transfected into MOP-8 cells and the replicational capacities of the mutant DNA assayed at 72 hours post-transfection as described previously (Fig. 9). The recombinant plasmid pdPd1300 is included for comparative purposes. (B) The structures of the PyV recombinant plasmids and their associated replicational phenotype are illustrated as described for Fig. 9.


A
M
dl 300
BP5-1
BP1-5
P1P5-1
P1P1-5
52 kb
50 kb
46 kb
33 kb


B
4600
4700
4800
4900
5000
5100
5200
5292
100
200
1560
Bam HI
Bcl I
Pvu II
Pvu II
Bgl I
Eco R I
CAPACITY TO REPLICATE
4632
5130
5265
1560
pdPBP5-1
+
5130
4632
5265
1560
pdPBP1-5
-
5130
5131
4632
90
pdPP1P5-1
+
4632
5131
5130
90
pdPP1P1-5
+

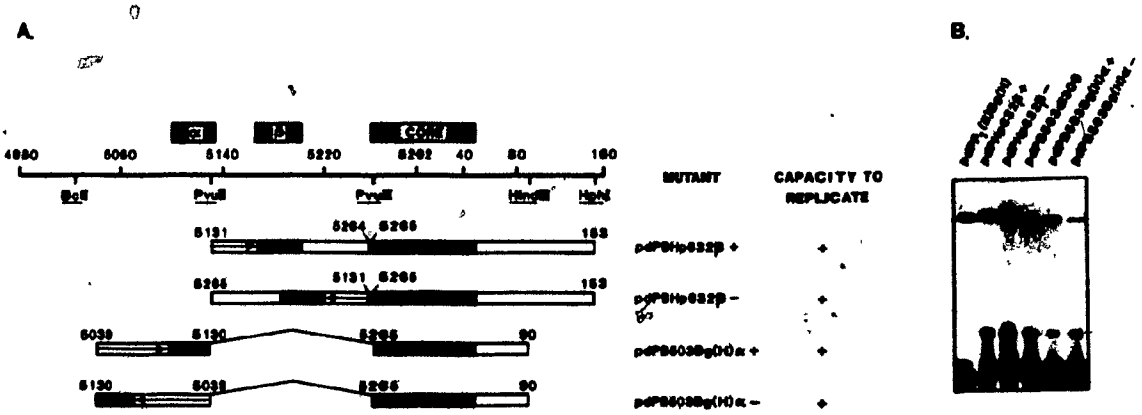
To ensure that the phenotype of the α inversion mutant was not due to the inadvertant generation of sequences inhibitory to DNA replication, the same 498 bp restriction fragment bearing α was inserted at the BamHI site of a replication competent plasmid, pdPP1(B)Bg(H), possessing both the β and core regions. The replicative capacities of the resulting recombinants, pdPP1P5-1 and pdPP1P1-5, were unaltered regardless of the orientation of the usptream α element (Fig. 25). It is therefore unlikely that the inversion of the α element in front of the core leads to the chance creation of an inhibitory sequence detrimental to DNA replication. Instead, it seems more likely that either the orientation or the spacing (or both) of the α and core regions relative to each other is critical to create a functional origin.

Because the inversion of a large DNA fragment (498 bp) bearing α changed not only the orientation, but also the position of α relative to the core, it was not clear which parameter affected replication of the inverted α-core construct adversely. For this reason, I cloned smaller restriction fragments containing the α and β elements next to the core.

To establish whether the α element could also function in an orientation independent manner, a 90 bp restriction fragment (nucleotides 5039 to 5130) containing the asymmetrically located α element was cloned in both orientations next to the core. The resulting recombinant plasmids, pdPB503Bg(H)α+ and pdPB503Bg(H)α-, both replicated in MOP-8 cells but to differing extents (Fig. 26). The mutant plasmid containing the α element in the inverted orientation replicated poorly (10%) by comparison to both the pdPB503d1300 and pdPB503Bg(H)α+ plasmids. It is noteworthy that inversion of the 90 bp fragment containing α effectively places 60 bp between the α element and the core. From these results it is apparent that the α element can function independent of its orientation when placed next to the core.

To determine whether the β element could also function independent of its orientation, the PvuII-4 fragment (nucleotides 5131 to 5265) bearing β was cloned in both orientations adjacent to the core (Fig. 26A). One of the viral recombinant plasmid pdPBHp632β+, contains the 134 bp PvuII-4 restriction fragment in its native orientation relative to the core. Despite the insertion of 8 bp of linker DNA between the β sequences and the core, this viral recombinant replicated at levels indistinguishable from the control plasmid (pdPP1[B]Bg[H]). Inversion of this 134 bp fragment

Figure 26: The α and β elements function independent of orientation relative to the core. (A) The structure and activity of the PyV origin containing inversions of β (pdPBHp632β-) and α (pdPB503Bg[H]α-) replication activators. The arrow within each segment refers to the orientation of the insert relative to the core. The dotted regions within each segment denotes the minimal functional core, whereas the hatched boxes refers to the minimal functional α and β elements. The filled-in box represents the 8 bp BglII linker between the β and core regions in the pdPHp632β+ and - mutants. (B) A 1 μg sample of DNA was transfected into MOP-8 cells and the replicational capacities of the mutant DNA assayed at 48 hours post-transfection as described previously (Fig. 9). The recombinant plasmids pdPP1(B)Bg(H) and pdPB503d1300 were included for comparative purposes.


A.
B.
4980
5060
5140
5220
5292
40
80
160
BclI
PvuII
PvuII
HindIII
HpaI
MUTANT
CAPACITY TO REPLICATE
5131
5264
5266
163
90
+
−

adjacent to the core resulted in a recombinant plasmid (pdPBHp632β-) that replicated to approximately 3-fold higher levels than the wild type β-core plasmid (Fig. 26). Interestingly, the inversion of the PvuII-4 fragments brings the β element closer to the core. These results indicate that the β element activates DNA replication independent of its orientation.

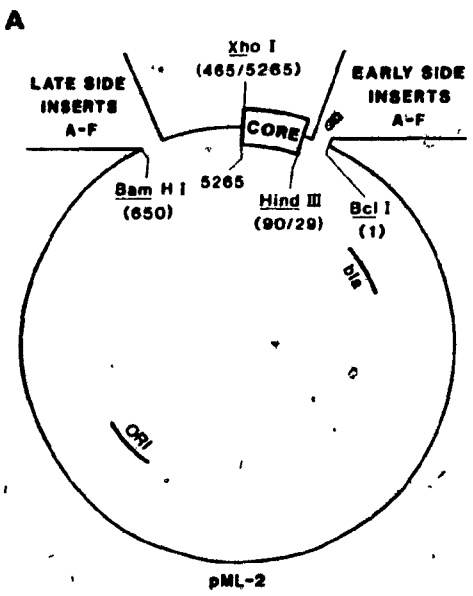
The results obtained by altering the orientation of α and β relative to the core show that both of these elements activate DNA replication irrespective of orientation. Interestingly, bringing the β element closer or moving the α element farther away from the core resulted in up and down effects respectively on replication. These results and those described earlier (Fig. 25) strongly hinted that each of the elements acted in a position dependent way.

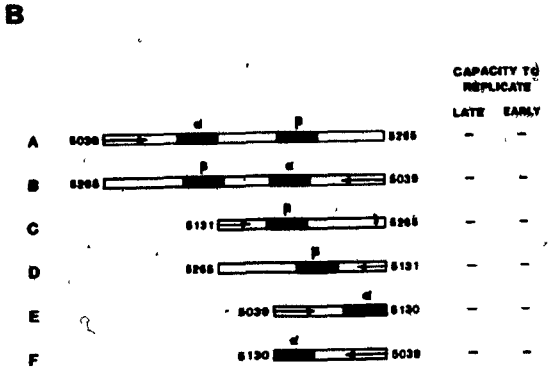
To directly test whether the α and β elements could function independent of position, restriction fragments containing the individual α and β elements, or a DNA fragment containing both elements, were cloned in either orientation at a site in pML-2 DNA positioned 185 bp from the late border of the core, or at a site 50 bp removed from its early border (Fig. 27, B). The result of examining replicative capacity of these constructs are shown in Fig. 27C and D. They demonstrate that the auxiliary replication elements could no longer activate DNA replication when moved away from the core. On the basis of these results, I conclude that the α and β elements activate DNA replication dependent on their position relative to the core. Replication of the various recombinant plasmids only occurs when α and/or β are located near the late border of the core.

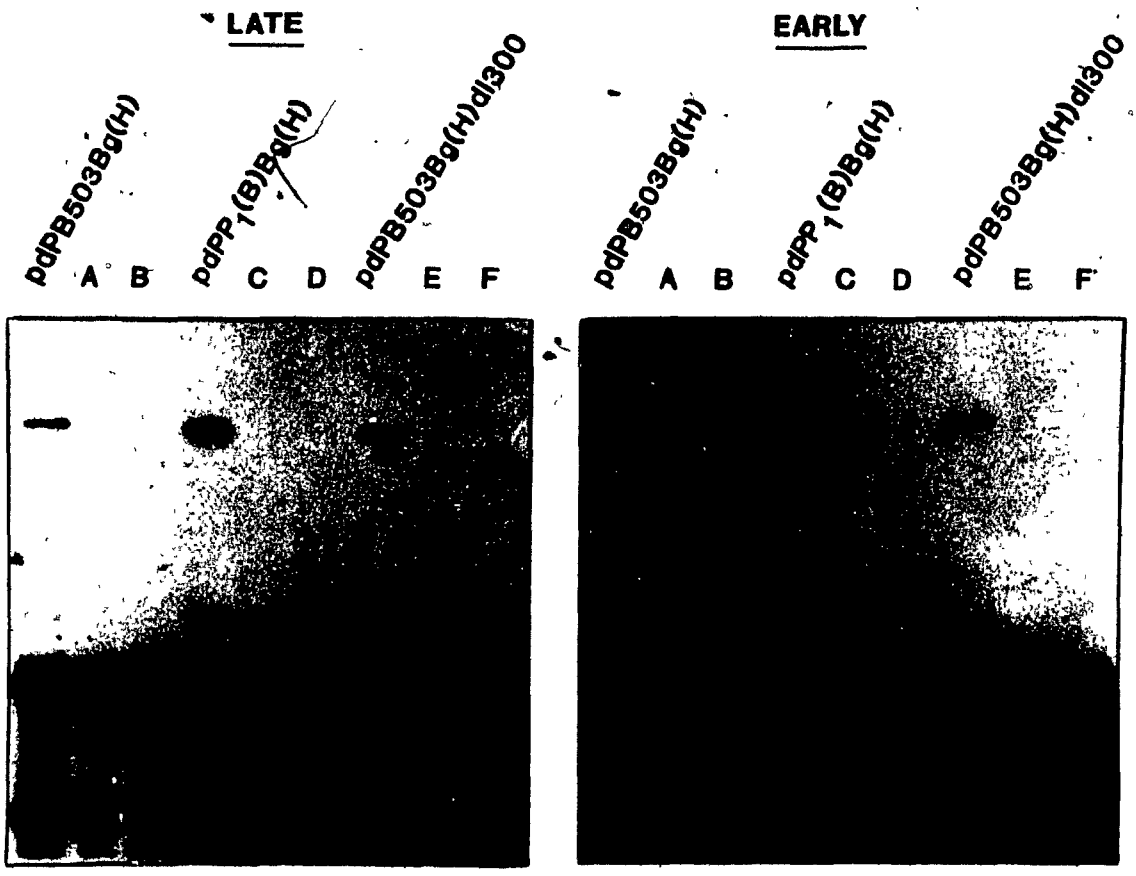
L. The α element activates replication from its native position.

In its native position the α element is more than 134 bp upstream of the core region. I was therefore curious to learn whether the α element functions to activate core DNA replication in its normal sequence context. To test this I cloned a restriction fragment containing the α element (nucleotide 5075 to 5130) next to 2 mutant viral recombinant plasmids bearing mutant β elements (LS 5151/5166 and LS 5173/5188; Fig. 21) next to the core. It should be noted that all these recombinants, including the control plasmid (pdPB5075Bg[H]), contain a 4 bp insertion between the α and β elements. These recombinant plasmids were named pdPB5075 LS 5151/5166 and pdPB5075 LS 5173/5188. These plasmids contain multiple point mutations in their β elements that severely impair their capacity to function (Fig.

Figure 27: The structure and replicational activity of PyV position mutants. (A) The structure of the vector used to clone and assess the replicational activity of recombinant plasmids containing the replication activators positioned at a distance from the core. The DNA segments shown in panel B were cloned in the vector at unique BamHI site (late side inserts) or BclI (early side inserts) in both orientations to yield six recombinant plasmids. (B) Composition and orientation of the various DNA fragments that were cloned in vector shown in panel A. The arrow within each segment refers to the orientation of the insert relative to the core. Arrows pointing to right are meant to depict the activator in the same orientation as it naturally occurs in PyV DNA relative to the core. (C, D) A 1 μg sample of recombinant plasmid DNA was transfected into MOP-8 cells and the extent of replication assayed at 48 hours post-transfection as described previously (Fig. 9). The results obtained with the late side position mutants are illustrated in panel C, whereas those with the early side mutants are shown in panel D. The letters above the lane refer to the inserts described in B. The recombinant plasmids pdPB503Bg(H) (αβ-core), pdPP1(B)Bg(H) (β-core) and the pdPB503Bg(H) d1300 (α-core) recombinants were included for comparative purposes.


A
Xho I
(465/5265)
LATE SIDE
INSERTS
A-F
EARLY SIDE
INSERTS
A-F
CORE
Bam H I
(650)
5265
Hind III
(90/29)
Bcl I
(1)
bla
ori
pML-2


B
CAPACITY TO REPLICATE
LATE
EARLY
A
B
C
D
E
F

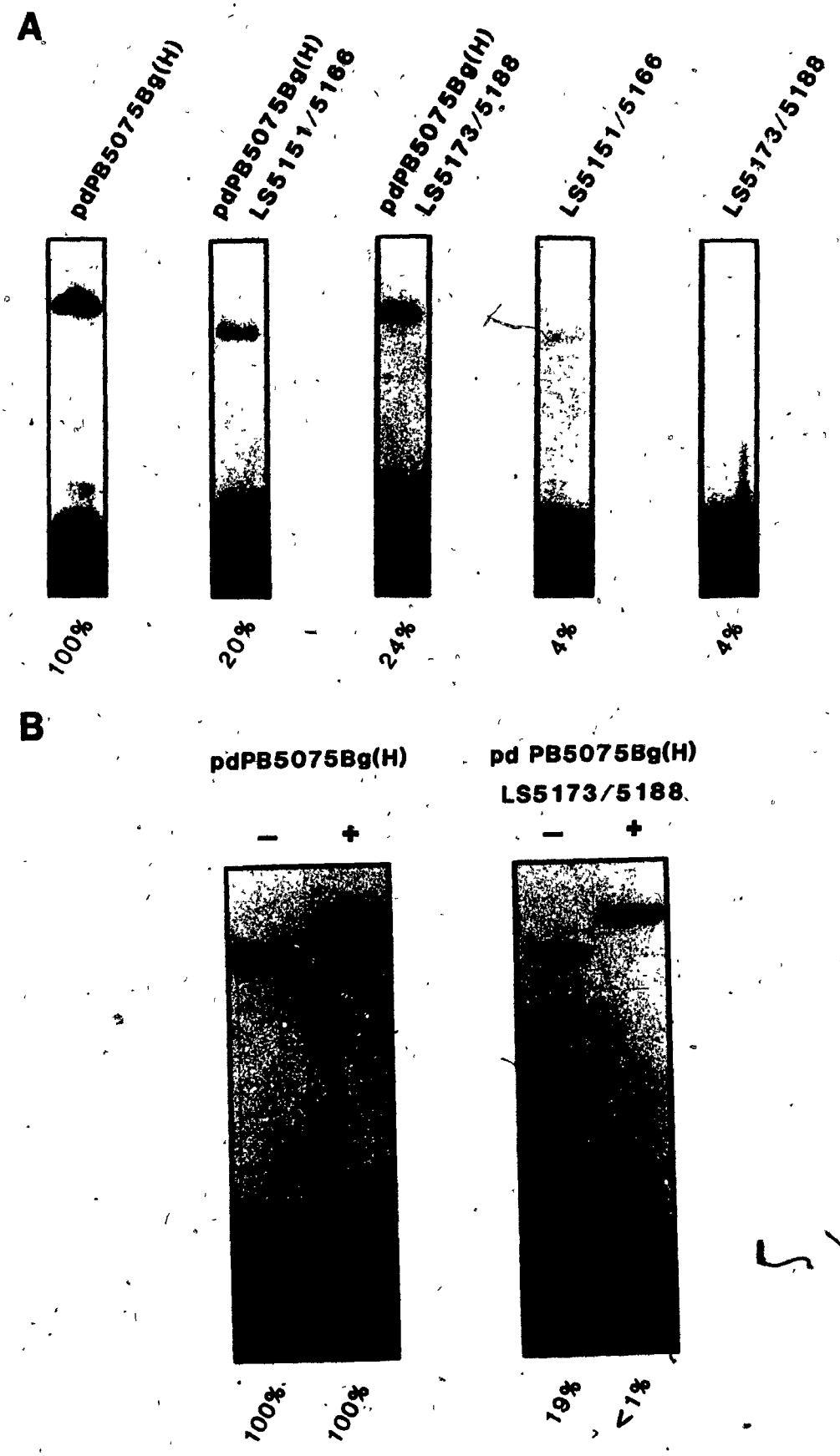

C
LATE
pdPB503Bg(H)
pdPP1(B)Bg(H)
pdPB503Bg(H)dl300
A B C D E F
D
EARLY
pdPB503Bg(H)
pdPP1(B)Bg(H)
pdPB503Bg(H)dl300
A B C D E F

22). Therefore, the replicational activity exhibited by the pdPB5075 LS 5151/5166 and pdPB5075 LS 5173/5188 viral if it occurred, could be attributed largely to the activity of the adjacent α element. Because the α element is now in essentially its native position in these mutants, replication of these mutant DNAs would suggest that α could function from its normal site.

The replicational properties of these recombinants are shown in Fig. 28. Compared to the control plasmid, pdPB5075 Bg(H), the pdPB5075 LS 5151/5166 and pdPB5075 LS 5173/5188 mutant DNAs replicated to reduced levels (20-25% of wild type; Fig. 28). However, compared to the parental LS 5151/5166 and LS 5173/5188 plasmids, these viral recombinant plasmids replicated to 5-fold higher levels.

To examine the phenotype of these mutants more rigorously, I tested the capacity of one of the mutants (pdPB5075 LS 5173/5188) to replicate alone and in competition with the αβ-core* origin as described previously (Chapter 2, section C). The results of this analysis is shown in Figure 28B. As observed previously, pdPB5075Bg(H) LS 5173/5188 replicated alone to about 20% the control level (pdPB5075Bg[H]). By contrast, this recombinant plasmid replicated in competition with the αβ-core* plasmid to barely detectable levels. Taken together, these results suggest that α is capable of activating core DNA replication from its native position. However, in the context of a mutated β element, the α element failed to compete effectively with the αβ-core* viral recombinant. Conceivably, this defect is the result of competition for limiting replication factors between the mutant and wild type templates.

Figure 28: The α element functions from its native position. (A) The replication capacities of the mutants were determine as described in Fig. 15 and are illustrated at the bottom of the autoradiogram. (B) The replicative capacities of the mutants were calculated as described in Fig. 18.


A
pdPB5075Bg(H)
pdPB5075Bg(H) LS5151/5166
pdPB5075Bg(H) LS5173/5188
LS5151/5166
LS5173/5188
100%
20%
24%
4%
4%
B
pdPB5075Bg(H)
pd PB5075Bg(H) LS5173/5188
–
+
–
+
100%
100%
19%
<1%

DISCUSSION

Chapter I: Isolation of Large T Antigen-Producing Mouse Cell Lines Capable of Supporting the Replication of Polyomavirus-Plasmid Recombinants

I constructed a recombinant plasmid, pPSVE1-B1a, containing a hybrid transcription unit comprising the SV40 early promoter fused to the T antigen coding sequences of PyV. pPSVE1-B1a DNA was used to transform NIH 3T3 cells, and the resulting transformed cells were screened for the presence of active large T antigen. The majority (70%) of the transformed MOP cell lines proved capable of supporting the replication of recombinant plasmids containing the polyomavirus origin for DNA replication. These results prove that permissive mouse cells readily tolerate active large T antigen provided that excision of the integrated viral DNA is blocked by removal or mutation of the polyomavirus origin for DNA replication.

I analyzed the state and structure of pPSVE1-B1a DNA in three MOP cell lines and found no evidence of free pPSVE1-B1a DNA in any of them. Instead, each MOP cell line contained integrated pPSVE1-B1a DNA. The MOP-3 and MOP-6 lines contained two independent insertions of transforming DNA, whereas the MOP-8 cell line contained a single insertion. None of the lines contained complete head-to-tail tandemly arranged copies of pPSVE1-B1a DNA. The fact that free unintegrated transforming DNA was not detected in these cells suggests that PyV large T antigen cannot act at the SV40 origin for DNA replication to trigger excision in mouse cells.

Examination of the MOP-3, -6 and -8 cell lines for the physical presence of PyV T antigens showed that each line synthesized large, middle and small T antigen. These proteins were the same size as those synthesized in the transformed rat T1A1 cell line. It has been shown previously that the T1A1 T antigens are the same size as those synthesized in lytically infected mouse cells (Ito and Spurr, 1979). It is very likely that the middle T antigen of the MOP cell lines is functional because each line is transformed, and this phenotype is strongly correlated with middle T antigen activity (Hassell et al, 1980; Mes and Hassell, 1982; Treisman et al, 1981b). I do not know whether the small T antigen present in the MOP lines is active or not. However, the large T antigens were functional for DNA replication, and they were capable of specifically binding to the large T antigen binding sites near the PyV early promoter and origin for DNA replication.

I compared the extent to which the three MOP cell lines support the replication of origin-bearing plasmids and found no large differences among them. I estimate that each cell in the population yields from 500 to 2,000 replicated plasmid molecules by 72 hours post-transfection (Muller et al, 1983a). Because less than 10% of the cell population was effectively transfected by this procedure, the yield of replicated DNA per transfected cell is at least 10 times higher than this.

I have also compared the yield of replicated plasmid DNA obtained from MOP-8 cells with that obtained from COS cells. These experiments were conducted by transfection of MOP-8 cells with pML-2 containing the minimal PyV origin for DNA replication (pdPP1[B]Bg[B]; Muller et al, 1983a) and by transfection of COS-1 cells with pML-2 containing the HindIII C fragment of SV40. Both cell lines yielded the same number of replicated plasmid DNA molecules 72 hours after transfection (data not shown).

An important application of transformed permissive cells is their use to complement the replication of early mutants of PyV, or early-replacement viral vectors. Unfortunately, the MOP cell lines which I characterized in greatest detail did not yield high titers of mutant viruses (data not shown). I believe that this was due to the nature of the cell line (NIH 3T3) rather than to the efficiency of complementation by large T antigen. Although 3T3 cells are permissive for PyV replication, the yield of infectious virus produced by them is at least 1,000-fold reduced compared to infected 3T6 or baby mouse kidney cells (data not shown). To correct this deficiency, I have recently isolated another suite of transformed permissive 3T6 cells by using a slightly different transformation strategy. Among those lines are several which encode a temperature-sensitive large T antigen. I anticipate that these 3T6 MOP cell lines will support the efficient replication of early PyV mutants and early-replacement vectors.

Two other transformed mouse cell lines have been described which support the replication of PyV-plasmid recombinants (Tyndall et al, 1981). These lines were derived by transformation of C127 mouse cells with an origin-defective PyV genome. Because a detailed characterization of these cell lines has not been published I cannot compare them to the MOP cell lines described here. Moreover, I also do not know whether C127 mouse cells permit the efficient replication of PyV, and therefore I do not know whether their transformed derivatives allow for the production of defective polyomaviruses.

Despite this shortcoming, the MOP cell lines have already proved useful to map the borders of the PyV origin for DNA replication (Muller et al, 1983a), and they have other applications as well. For example, they can be used as a ready source of large T antigen for DNA binding studies or as a source of middle and small T antigen. The amount of active large T antigen I obtained from the MOP-6 and MOP-8 cell lines was nearly 30% that obtained from lytically infected 3T6 cells. The MOP cell lines can also be used to rescue integrated PyV DNA sequences from transformed cells. Finally, by cloning foreign genes in pML-2 plasmids bearing the PyV origin, it should be possible to study their expression in mouse cells. Therefore, MOP cells provide an alternative to COS cells when it is important to measure gene activity in a murine cell environment.

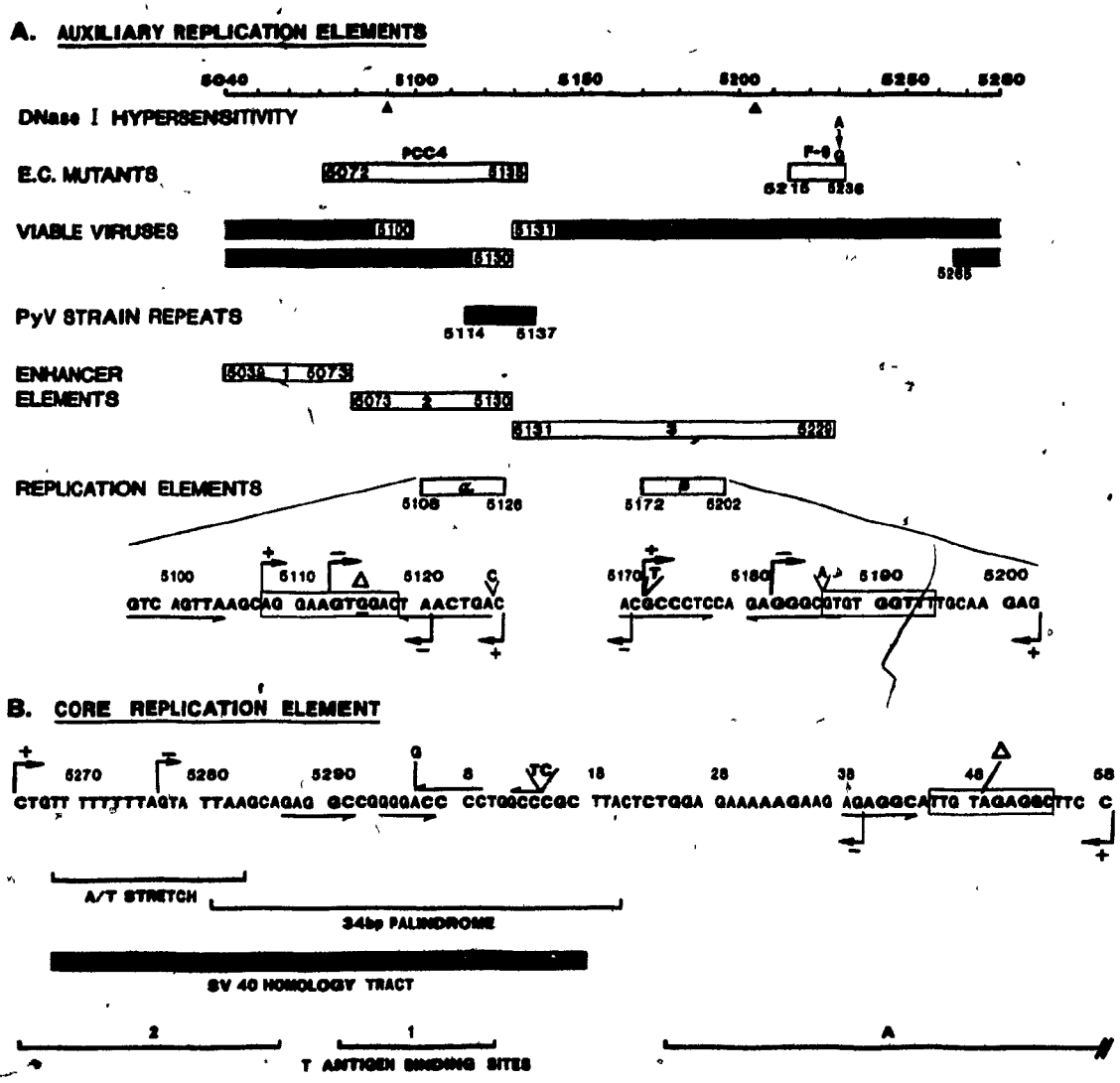
Chapter II: Structural Requirements for the Function of the Polyomavirus Origin for DNA Replication

A. PyV origin comprises multiple genetic elements.

I used the MOP-8 cell line to identify the *cis*-acting sequences required for PyV DNA replication. I constructed a series of deletion mutants, sequenced the deletion junctions and then measured their capacity to replicate in MOP-8 cells. The results revealed that the PyV origin for DNA replication (*ori*) comprises several genetic elements. These include two functionally equivalent but unique sets of viral sequences, designated alpha (α) and beta (β), that are situated near a third element termed the core. The simultaneous presence of either α or β and an intact core region is required to form a functional origin. All three elements reside within a noncoding stretch of the viral genome no more than 242 bp in length between nucleotides 5108 and 58. The results of my deletion analysis indicate that these elements are noncontiguous. The sequences that comprise *ori* and other features resident in this area of the viral genome are summarized in Fig. 29.

The multi-element organization of the origin is supported by the analysis of viable virus mutants of PyV. Luthman *et al* (1982) have described the construction of a viable deletion mutant of PyV (dl1024) from which all but 8 bp of the 134 bp PuvII-4 fragment have been deleted. Because this viable variant lacks the β element, their results together with mine suggest that two noncontiguous regions of viral DNA, namely α and core, are capable of forming a functional origin. A second viable mutant of PyV containing a deletion between nucleotides 5100 and 5131 removes the minimal α element (Tyndall *et al*, 1981). It is likely that in this mutant the β-core origin configuration functions to allow initiation of viral DNA replication. Interestingly, the same group have isolated other unidirectional and bidirectional viral mutants about the PvuII site located at nucleotide number 5130. Many of the bidirectional mutants fail to replicate, whereas the unidirectional mutants that are deleted toward either the early or late transcription units do replicate (Tyndall *et al*, 1981). These results are readily explained by the model I have presented. The bidirectional mutants were replication defective because sequences comprising both α and β were deleted. By contrast, the unidirectional deletion mutant DNA bear lesion that remove either α or β and are therefore replication competent.

Figure 29: Schematic of the features within the PyV origin for DNA replication. (A) The top line represents noncoding PyV DNA according to the scheme proposed by Soeda et al (1980). The open triangles below the line depict sites in viral chromatin that are hypersensitive to DNase I cleavage (Herbomel et al, 1981). The borders of DNA that are commonly duplicated in PyV mutants selected for their capacity to replicate in PCC4 or F9 embryonal carcinoma (EC) cells are shown by the open boxes. The stippled boxes represent those sequences that are present within the genomes of two viable deletion mutants of PyV. The hatched box shows a region in PyV DNA that is commonly duplicated in various wild type strains of PyV (Ruley and Fried, 1983). The positions of the three DNA segments that are thought to be enhancer elements are shown as three numbered open boxes. The α and β replication elements are shown together with their sequences. The arrows that underline the sequences in α and β represent the inverted repeats, whereas the boxed sequenced represent enhancer core sequences. The arrows above and below the sequence represent deletion endpoints on the late or early side of each element. The + and - designates whether that endpoint replicates (+) or not (-) in MOP-8 cells. The DNA sequence changes present in this strain have been previously noted. (B) The arrows above the sequence represent deletion endpoints on the late side of the core, whereas those below the sequence represent deletions on the early side of core. The solid arrows underlining the sequence represent the large T antigen recognition sequence 5'-GAGGC-3'. The broken arrows represent the related pentanucleotide 5'-GGGGC-3' described by DeLucia et al (1983). The lines below the sequence denote the A/T rich and 34 bp palindrome regions. The stippled box represents the PyV region homologous to the SV40 ori. The T antigen binding sites are illustrated by the lines beneath the sequence and their borders were derived from Cowie and Kamen (1984). The DNA sequence changes within this region have been previously noted.


A. AUXILIARY REPLICATION ELEMENTS
5040
5100
5150
5200
5250
5290
DNase I HYPERSENSITIVITY
E.C. MUTANTS
PCC4
F-9
5215
5236
VIABLE VIRUSES
5100
5131
5130
5265
PyV STRAIN REPEATS
5114
5137
ENHANCER ELEMENTS
5038
5075
5130
5131
5229
REPLICATION ELEMENTS
α
5106
5126
β
5172
5202
5100
5110
5120
GTC AGTTAAGCAG GAAGTGGACT AACTGAC
5170
5180
5190
5200
ACGCCCTCCA GAGGGCGTGT GGTTTTGCAA GAG
B. CORE REPLICATION ELEMENT
5270
5280
5290
8
TC
18
28
38
48
58
CTGTT TTTTTTAGTA TTAAGCAGAG GCCGGGGACC CCTGGCCCGC TTACTCTGGA GAAAAAGAAG AGAGGCATTG TAGAGGCTTC C
A/T STRETCH
34bp PALINDROME
SV 40 HOMOLOGY TRACT
2
1
A
T ANTIGEN BINDING SITES

Although the α and β elements can functionally substitute for each other in viral DNA replication they do not do so with equal efficiency. In competition with a viral recombinant plasmid that contains all three elements (αβ-core*), the β-core origin configuration replicated best (Fig. 13). By contrast, a recombinant plasmid bearing the α-core origin replicated poorly in competition with the αβ-core* recombinant (at approximately 10-fold lower levels). The impaired replicational activity of the α-core recombinant plasmid could be due to either the inherent activity of this element relative to β, or to a suboptimal alignment of the α sequences relative to the core in the α-core plasmid. Interestingly, the β-core recombinant plasmid replicated to higher levels than the αβ-core* competitor plasmid at later time intervals (72 hours post-transfection). It is possible that sequences within α have an inhibitory effect on β function. Further experiments are required to precisely elucidate the nature of this effect.

Recently, Veldman et al (1985) reported that recombinant plasmids containing essentially the β-core origin configuration replicated to ten to twenty-fold reduced levels compared to the complete origin (αβ-core). I therefore measured the replication efficiency of β-core viral recombinant plasmid (pdPP1[B]Bg[H]) in competition with another recombinant plasmid which contains all three replication element (αβ-core*). The results indicated that the pdPP1(B)Bg(H) viral recombinant replicated 2 to 5-fold higher levels than the αβ-core* origin at 48 h post-transfection. The difference between these results and those of Veldman et al (1985) may be due to differences in the amounts of large T antigen present in the MOP-8 and COP-5 cell lines or to different assay conditions used to assess the replicative capacities of these viral recombinants. Alternatively, the levels of a limiting cellular factor required for β function may differ in these cell lines and this parameter may affect the replication phenotypes of the viral mutants. Whatever the explanation, there is little doubt that the β element can independently activate core DNA replication.

B. Sequences required for core function.

The core region is composed of no more than 77 bp (Fig. 29B). Its late border resides between nucleotides 5265 and 5277, whereas its early border is located between nucleotides 39 and 58. Located within this span are several unusual sequence features. These include a 8 bp stretch of

adenine:thymine (A:T) residues, and a guanine:cytosoine (G:C)-rich 34 bp palindrome (between nucleotides 5281 and 20), which includes the low-affinity large T antigen binding sites 1 and 2 (Cowie et al, 1984, 1986).

Removal of the entire tract of eight consecutive A:T-bp, as occurs in pdPd1304 (Fig. 11) or four of the eight A:T residues (Luthman et al, 1982) renders the DNA replication defective. These observations suggest that the A:T tract is an essential component of the core. Interestingly, this sequence is conserved in the DNA of three other papovaviruses, SV40, JC and BK virus, and it is known to be a functional component of the SV40 origin for DNA replication (Bergsma et al, 1982; Fromm and Berg, 1982).

There is also evidence to support the contention that the PyV 34 bp palindrome is required for origin function. Point mutations or deletions within the center of the 34 bp region have been observed to render the viral DNA replication defective (Luthman et al, 1982; Triezenberg and Folk, 1984; Luthman et al, 1984). However, base pair substitutions located near the ends of the dyad symmetry have been reported to have little or no effect on viral DNA replication (Triezenberg and Folk, 1984).

Located on either strand of the 34 bp palindrome are 2 repeats of the sequence, 5'-GAGGC-3'. This pentanucleotide repeat is the recognition-binding sequence for PyV and SV40 large T antigens (Tegtmyer et al, 1983; DeLucia et al, 1983; Pomerantz and Hassell, 1984). PyV large T antigen specifically contacts the guanine residues within these repeats (Cowie and Kamen, 1986). Interestingly, mutation of certain of these guanine residues located about the center of the palindrome abolishes viral DNA replication (Triezenberg and Folk, 1984). These results suggest that the large T antigen binding sites in this region of the core are required for the initiation of viral DNA synthesis.

A 27 bp palindrome that is related in sequence to that of the PyV core is also found with the origin-core of SV40. Moreover, genetic analyses have demonstrated that the 27 bp palindrome is an essential component of the SV40 origin (Shortle and Nathans, 1979; Gluzman et al, 1979). Interestingly, the SV40 27 bp palindrome also contains repeats of the GAGGC pentanucleotide that serve as T antigen binding sites, and these are arranged in a similar manner to those in the PyV core (Tjian, 1978; DeLucia et al, 1983; Tegtmyer et al, 1983; Jones et al, 1984).

Located adjacent to the PyV 34 bp palindrome near its early border are several high affinity large T antigen binding sites (sites A and C)

(Gaudray et al, 1981; Pomerantz et al, 1983a; Cowie and Kamen, 1984, 1986). Surprisingly, the replication competent mutant (ΔEC58; Fig. 15) that defines the early core boundary lacks DNA sequences that contain binding site A (Pomerantz et al, 1983a; Cowie and Kamen, 1984). Although I have not tested the ability of this mutant to bind large T antigen, these results suggest that the integrity of this large T antigen binding site is dispensible for the initiation of viral DNA replication. Conceivably, the lower affinity T antigen binding sites located within the 34 bp palindrome are the functionally important sites required for the initiation of viral DNA replication.

Quantitation of the replication capacity of the ΔEC58 mutant revealed that it replicated at 10-fold reduced levels relative to wild type β-core plasmid. Furthermore, when the ΔEC58 mutant was cotransfected with αβ-core* recombinant containing all three replication elements, it replicated at barely detectable levels. These results strongly suggest that large T antigen binding site A is not necessary but does contribute to PyV core function. Consistent with this view is the observation that lesions within large T antigen binding site A result in an impaired replication phenotype (Katinka and Yaniv, 1983). Moreover, it is probably not coincidental that all viable deletion mutants of PyV isolated to date retain sequences that include large T antigen binding site A (Bendig and Folk, 1979; Magnusson and Berg, 1979; Wells et al, 1980; Luthman et al, 1982).

The minimal SV40 origin also includes sequences that efficiently bind SV40 large T antigen (site II) (Tjian, 1978; Myers and Tjian, 1980; DiMaio and Nathans, 1982). SV40 large T antigen binding site II is located within the borders of the 27 bp palindrome. Interestingly, DeLucia et al (1986) observed that the SV40 core, containing site II, replicated to 10-fold higher levels when linked to the adjacent high affinity T antigen binding site I near its early border. In this respect, SV40 large T antigen binding site I functionally resembles PyV site A. These observations suggest that the T antigen binding sites located in the viral palindromes comprise the functionally important sequences for the initiation of PyV and SV40 DNA replication and that the origin proximal T antigen binding sites (site A in PyV and site I in SV40) facilitate replication. It is tempting to speculate that this effect occurs through protein:protein interaction between T antigen molecules bound to sites I or A with T antigen bound to

the palindrome sites. These interactions may stabilize binding of T antigen. Alternatively, T antigen bound at site I or site A may stabilize or facilitate the binding of other replication factor(s) to the core regions.

In summary, these observations suggest that an A:T rich stretch, a G:C-rich region of dyad symmetry, and large T antigen binding sites are features that are not only conserved among the papovaviruses origin cores but are also functionally important for the initiation of viral DNA replication.

C. Sequences required for β function.

Cis-acting viral sequences in addition to the core are needed to form a functional PyV origin. One such element that can, together with the core region, form a functional ori has been termed β. The minimal β element is located within the PvuII-4 fragment and is no larger than 33 bp. One of its borders maps between nucleotides 5172 and 5182, and the other is positioned between nucleotides 5202 and 5170 (Fig. 29). In addition to these sequences, others that flank the β element also contribute to its activity. These include the sequences between nucleotides 5131 and 5172, on its late border and those between nucleotides 5202 and 5218 on its early border.

The importance of the region between nucleotide 5131 and 5172 in β function was revealed by the analysis of late β deletion mutants (Fig. 16, 19). Deletion of the late sequences flanking β resulted in 20-fold drop in replication capacity of the mutant DNA (ΔLB5166, ΔLB5172) when they were assayed alone, whereas the replication of these mutants was barely detectable in competition with the αβ-core* viral recombinant. The replication phenotype of the ΔLB5147 mutant suggests that the late border of this flanking region is close to nucleotide 5147. This mutant was clearly capable of efficient replication in MOP cells, but it replicated poorly when placed in competition with the αβ-core* viral recombinant.

The analysis of the replicational properties of linker scanning mutants within the region flanking the late β border confirms that sequences to the late side of β are required for β function. The mutant LS 5151/5166, in which a linker disrupts this region, replicated at only 1% the level of its parent. Because this mutation leaves other sequence features in β intact, the replication defect observed in this mutant can be

ascribed solely to the alteration of sequences between nucleotides 5151 and 5166. These observations strongly suggest that sequences that neighbor the late border of β (5131-5172) influence the activity of β.

There are several interesting sequence features present in this region. First, a segment of this region between nucleotides 5150 and 5166 is A:T rich (10/11 of base pairs) and second this region contains a segment homologous to sequences within the immunoglobulin heavy chain enhancer (Banerji et al, 1983). Mutants that delete (i.e. ΔLB5166) or alter this region (i.e. LS 5151/5166) replicate at impaired levels. Interestingly, the ΔLB 5147 mutant, which replicated poorly in competition with the αβ-core* origin, impinges on the first two base pairs of the immunoglobulin enhancer homology. This result suggests that the immunoglobin enhancer homologue near the late border of β (nucleotides 5145 to 5158) contributes to β function.

Both early and late border deletion analyses defined the minimal β element between nucleotides 5172 and 5202. This DNA segment contains a 9 bp, G:C rich, inverted repeat and a 9 bp sequence homologous to the SV40 enhancer core described by Wieher et al (1983) (Fig. 29). The importance of the 9 bp inverted repeats in β function was assessed by their mutation. Deletion of the inverted repeat near the late border as occurs in ΔLB5182, abolished β function. This mutant retained the SV40 enhancer core and part of the inverted repeat at the early β border. These results indicate that the inverted repeat sequence is essential for β activity and that the SV40 enhancer core sequence cannot by itself activate viral DNA replication.

Interestingly, the linker scanner mutant, LS 5173/5188, whose mutation disrupts both the inverted repeats and the SV40 enhancer homology, replicated, albeit poorly. This result further supports the notion that the SV40 core homology and inverted repeats are important functional units of the β element and in addition, highlights the importance of flanking sequences.

Viral DNA sequences located near the early border of the β element also affect β function. Viral recombinant plasmids bearing deletions on the early side of β replicate from two- (ΔEB5223) to ten-fold (ΔEB5209) lower levels than did the wild type β-core origin (Fig. 16). Because these deletions also alter the spatial arrangement of the β sequences relative to the core, it is not clear whether the impaired replication phenotypes were

due to removal of critical β sequences or to the alteration of the position of the β element relative to the core. However, the replicational properties of the linker scanning mutants, LS 5202/5211 and LS 5209/5218, strongly suggest that the debilitated replication phenotypes exhibited by at least two of the early β mutants (ΔEB5209 and ΔEB5202) was due in part to the alteration of the primary DNA sequence. The LS 5202/5211 mutant replicated its DNA to 14% the level observed with the wild type β-core viral recombinant. By contrast, the LS 5209/5218 viral recombinant replicated at 3% the levels observed with the control β-core origin. The replication defect observed with both these mutants was readily apparent in competition with the αβ-core* plasmid. Because the spatial relationship between β and core is unaltered in these mutants, these results indicate that the viral sequences mutated (nucleotides 5202 to 5218) in these two recombinants contribute to β function.

Within this region of viral DNA is a 5 bp inverted repeat consisting of the sequence 5'-AGGAG-3' spaced by 10 bp from its complement (Fig. 21). Interestingly, the more severely impaired mutant (LS 5209/5218) contains a linker which disrupts this 5 bp inverted repeat whereas the LS 5202/5211 mutant, which replicates to intermediate levels, contains a linker between the 5 bp inverted repeats. Perhaps the LS 5202/5211 mutant replicates better than the LS 5209/5218 mutant because it more nearly preserves the integrity of the 5 bp inverted repeat.

Although mutation of the sequences on the early side of β can have a profound effect on DNA replication, there is evidence to suggest that these sequences are dispensible for virus viability. Indeed, PyV variants containing deletions on the early side of β from nucleotide 5265 to nucleotides 5211 or to 5214 are viable (Tyndall et al, 1981; N. Acheson, personal communication). The difference between these observations and mine may be explained by the fact that these viruses also possess α and the presence of this element compensates for the deletion of β sequences. Indeed, one of these viable mutants possesses a duplicated α element (N. Acheson, personal communication).

Sequences located between nucleotides 5240 and 5248 are clearly dispensible for β function since a mutation (LS 5240/5248) altering these sequences replicates at wild type levels. It is interesting to note that a direct repeat of the sequence 5'-TCCACCCA-3' (nucleotides 5217 to 5225 and nucleotides 5238 to 5245) is contained within this region. This sequence is present within the distal enhancer of bovine papillomavirus (Lusky et

al, 1983). Because the LS 5240/5248 mutant replicates as well as wild type, the integrity of this sequence cannot be necessary for β element function.

Piette et al (1985) recently reported that sequences residing within the β element are protected from nuclease digestion by cellular factors. One of these protected regions is located near the late border of β within the sequences homologous to the immunoglobulin enhancer (nucleotides 5145 to 5160). A second protected region was found that contains the 9 bp inverted repeat. Interestingly, Piette et al (1985) observed that deletion of the late side region including the immunoglobulin enhancer homologue (nucleotides 5145 to 5158) impaired the binding of cellular factors to the central, G:C rich, 9 bp inverted repeat. These observations suggest that these factors may recognize viral DNA sequences and bind in a cooperative manner.

It is striking that these two protected regions overlap to sequences that comprise functional components of β. The impaired replicational phenotypes of viral recombinants that contain mutations within the immunoglobulin enhancer homology in β could be ascribed to their reduced capacity to bind cellular factors. Conceivably, factors bound to this region faciliate the binding of factors to the minimal β element (nucleotides 5172 to 5202). A similar phenomenon may also account for the capacity of sequences on the early side of β to augment β function. Indeed, Piette et al (1985) have observed nuclease hypersensitive sites that are suggestive of the binding of a factor in this region. It is tempting to suggest that factors bound on either side of the minimal β stabilize the binding of factors to β through protein:protein interactions.

The β element resides within an area of the viral genome that is tandemly duplicated and mutated by base pair substitutions in variants of PyV, which unlike wild type virus, are capable of productively infecting F9 embryonal carcinoma cells (Sekiwa and Levine, 1981; Vasseur et al, 1982). The observation that coinfection of F-9 embryonal carcinoma cells with wild type and mutant strains of PyV yields only mutant virus progeny, despite the presence of complementing large T antigen (in the infected cells), indicates that the sequence modifications in these mutants likely allow them to overcome a cis-acting defect in viral DNA replication present in wild type DNA (Fujimura and Linney, 1982). One class of F9 PyV mutants contain multiple base pair substitutions within the 9 bp inverted repeat present in β (Vasseur et al, 1982). A second class of variants contain a

single base pair transition at nucleotide 5230 (A→G; Fig. 29) and tandem duplications of sequences surrounding and including this base pair substitution (Fujimura et al, 1981; Sekiwa and Levine, 1981). Interestingly, these alterations are positioned outside the mapped borders of the minimal β element. These mutations may promote the binding of limiting factor(s) within EC cells to the β element or to its adjacent sequences. In this regard, it is interesting to note that the A to G transition at nucleotide 5230 results in the generation of a sequence homologous to SV40 enhancer core motif (Wieher et al, 1983).

D. Sequences required for the function of α

Another genetic element designated α, when coupled to the core, can also form a functional origin. I have defined the limits of this element to a 19 bp region situated between nucleotides 5108 and 5126 (Fig. 20). Within the borders of the α element is a 10 bp region homologous to the adenovirus 5 E1a enhancer core sequence (Hearing and Shenk, 1983) and an 8 bp inverted repeat. The results of my deletion analysis indicate that the early side repeat (nucleotide 5119 to 5125) is absolutely required for α function because its deletion results in a replication defect (ΔEA5120; Fig. 17). The adenovirus E1a homology box also appears to be a functional component of the α element because a deletion impinging on this element to nucleotide 5113 rendered the DNA replication defective (data not shown). These results demonstrate that the minimal α element is composed of an adenovirus 5 E1a enhancer core sequence and the early side 8 bp inverted repeat.

In addition to the minimal α element, I have mapped another 36 bp segment between nucleotides 5075 to 5109 which augments its activity. The importance of this region for α function was revealed by two lines of evidence. First, late α mutants bearing deletions of this region (ΔLA 5097, ΔLA5108; Fig. 20) replicated at reduced levels. The impaired replication phenotypes exhibited by these mutants was particularly pronounced when they were tested in competition with the αβ-core* origin configuration. Second, replication of a defective recombinant which contains a deletion impinging on the late border of α (nucleotide 5113), could be restored by cloning the sequences between nucleotides 5039 and 5109 adjacent to it at its late border (pdPB5039 LS 5113/5109; Fig. 23, 24). I have recently reduced the length of this functionally redundant

sequence to 24 bp between nucleotides 5075 and 5109 (data not shown). Interestingly, this region is A:T rich (20/28 bp) and contains a sequence identical to the 8 bp inverted repeat found in α.

The integrity of the structure that could be formed by the 8 bp inverted repeat flanking the Ela enhancer core sequence is not required for α function, because the mutant LS 5102/5111, which replicates at wild type levels, alters 2 bp within this repeat. Moreover, deletion of the late side inverted repeat does not abolish replication of α-core. It is conceivable that distinct cellular factors recognize and bind to both the early side repeat and the Ela homology. Binding of factors to the minimal α element may be facilitated by the binding of factors to sequences upstream of the late border of α (nucleotides 5075 to 5109). This may account for the capacity of these sequences to augment α function.

Recently, Veldman et al (1985) reported that a recombinant plasmid containing the viral sequences between nucleotides 5108 to 5130 next to core replicated poorly. Surprisingly, wild type levels of viral DNA replication could be obtained by simply duplicating the sequences between nucleotide 5108 and 5130 (the minimal α element is situated between nucleotide 5108 and 5126) (Veldman et al, 1985). In this regard, it is interesting to note that many strains of PyV contain duplications of the minimal α element (Fig. 29; Ruley and Fried, 1983). Perhaps the duplication of the minimal α element stabilizes or facilitates the binding of factors to this region.

Curiously, a region to the late side of α (nucleotides 5039 to 5073), has a cis-inhibitory effect on DNA replication. This was demonstrated by the observation that a mutant lacking these sequences (ΔLA5073) replicated in competition experiments with αβ-core* to five-fold higher levels than a comparable plasmid containing these sequences (pdPB503d1300; Fig. 20). Because only one deletion mutant defines this effect, this result must be interpreted cautiously. Nonetheless, it is interesting to note that when this region is linked to a hybrid transcription unit composed of the PyV enhancer and the chloramphenicol acetyl transferase gene (CAT gene), it represses CAT expression in mouse embryos cultured in vitro (M. DePamphilis, personal communication).

PyV productively grows in most murine cells, but it cannot replicate in the undifferentiated PCC4 embryonal carcinoma cells. However, variants of PyV can be isolated that replicate efficiently in these cells (Katinka et al, 1980; Vasseur et al, 1982). These variants possess common sequence

rearrangements that generally result in the duplication of viral sequences between nucleotides 5072 and 5137 (the borders of the minimal α element are situated between nucleotides 5108 to 5126) and deletion of those sequences between nucleotides 5185 and 5215 (the borders of β are between nucleotides 5172 and 5202; Katinka et al, 1980). It is likely that the PCC4 variants lack β and contain duplications of the α element. Conceivably, these alterations allow limiting cellular factors present in the PCC4 cell line to recognize the α element.

E. Comparison of the structure of α and β.

The arrangement of the functional sequences within the α and β auxiliary elements is surprisingly similar. Both elements appear to be composed of a number of distinct sequence motifs. The late border of each element contains an A:T rich region which augments its activity. In addition, both elements contain enhancer core homologies and distinctive inverted repeats. The α element contains a 10 bp homologue to sequences repeated in the enhancer region of E1a transcription unit of adenovirus 5 (Hearing and Shenk, 1983), whereas β contains a homologue of the SV40 enhancer core sequence (Weiher et al, 1983). Interestingly, the 9 bp inverted repeat in β and the 8 bp inverted repeat in α are related in sequence. A 7 bp consensus sequence, 5'-A/C,ACTG,A/C,C-3', can be derived for the inverted repeats. It is possible that these inverted repeats are recognized by a common cellular factor.

Recently, Wirak et al (1985) found that PyV origin configurations containing the β element (αβ-core, β-core) could replicate in a T antigen-dependent manner within mouse embryos cultured in vitro. Surprisingly, under the same conditions, the α-core configuration of origin did not replicate in early mouse embryos. One possible explanation of these observations is that the factors in early mouse embryos that interact with α are missing or inactive. Alternatively, mouse embryos may contain a repressor that inactivates α function. Interestingly, the α-core constructs used by Wirak et al (1985) contained the putative repressor binding sequence (5039 to 5073) alluded to earlier.

F. The α and β elements overlap with viral transcriptional enhancer

The α and β elements are located within the borders of the PyV enhancer. Recently, it has been demonstrated that PyV enhancer comprises three distinct domains termed elements 1, 2 and 3 (Mueller et al, manuscript in preparation; Fig. 29). Individual elements are incapable of

acting as an enhancer but pairs of these elements can function to augment gene expression nearly as well as all 3 elements. The minimal α element is located within the borders of enhancer element 2 (nucleotides 5075 to 5130). Deletion of the inverted repeat located at the late side of the adenovirus core (nucleotides 5097 to 5104) has little effect on element 2 function. By contrast, deletion of the inverse of this sequence (nucleotides 5120 to 5126) abolishes enhancer element function. These mutations affect α in a similar way, and imply that the same sequences in α and element 2 mediate replication and transcription enhancement. Similarly, reiteration of the sequences that make up the minimal α element impart enhancer function to the DNA and increase its capacity to activate DNA replication (Veldman et al, 1985). These observations strongly suggest that the sequences within this region function to both enhance gene expression and to activate DNA replication.

The β replication element is located within the borders of enhancer element 3. This region of viral DNA has been reported to possess weak enhancer activity in mouse fibroblast cells (Herbomel et al, 1984; Mueller et al, manuscript in preparation). Because the mutants I have generated that define the β element have not been tested yet for enhancer activity it is not known whether the same sequences implicated in β function are also involved in enhancer element 3 function in mouse fibroblasts. Mutants of PyV that replicate in F9 embryonal carcinoma cells contain sequence alterations on the early side of β that allow this region to function as an independent enhancer in mouse embryo carcinoma cells (Linney and Donnerly, 1983; Herbomel et al, 1984). Moreover, it has recently been found that the wild type β element functions as an enhancer in early mouse embryos cultured in vitro (M. DePamphilis, personal communication). These results suggest that the sequences resident within β possess intrinsic enhancer activity that can be fully expressed in certain cellular environments.

The PyV enhancer contains a third region (element 1) that can functionally substitute for deletion of either element 2 or 3. However, enhancer element 1 is not capable of activating PyV DNA replication. This element is also completely incapable of independently enhancing gene expression. These observations suggest that element 1 is intrinsically weak, perhaps because it is unable to serve as an independent binding site for trans-acting factors. In this regard it is noteworthy that element 1 contains a sequence that is a poor match to the SV40 enhancer core and

another sequence that is also a poor match to the conserved inverted repeat motif that is present in enhancer elements 2 (α) and 3(β).

G. Spatial requirements for the function of the α and β elements

The coincidence of the replication and enhancer elements suggest that the same sequences effect viral DNA replication and transcription. Like enhancer elements the α and β elements function independent of their orientation relative to the core. Consistent with these findings, Veldman et al (1985) reported that a tandem duplication of the sequences comprising α (nucleotides 5108 to 5130) could activate viral DNA replication irrespective of its orientation relative to the core region.

However, replication activation by the α and β elements did not occur when they were moved away from the core (Fig. 27). The replication defect observed in these spacing mutants was likely not due to the inadvertant creation of inhibitory sequences that retarded DNA replication because these elements were cloned at two distinct sites located on either side of the core. The replication properties of several α inversion mutants are also consistent with the view that the replication elements cannot activate viral DNA from a distance. These α-core origins replicated poorly or not at all depending on the distance between the inverted α element and the core. Inversion of a large 498 bp fragment containing α adjacent to the core resulted in a replication defect (Fig. 25), whereas inversion of smaller restriction fragment containing the same α sequences next to the core resulted in the generation of replication capable but enfeebled molecules (Fig. 26). Therefore, the replication defect observed in the 498 bp α inversion mutant was likely due to a position effect (more that 400 bp separate α from the core in this construct) rather than an orientation effect. Taken together, these observations suggest that the auxiliary replication elements (α and β) and the core must be in close proximity in order to form a functional origin.

By contrast to these results, de Villiers et al (1984) recently reported that the α and β elements could activate viral DNA replication when placed more than 800 bp away from the core. It is noteworthy that the viral recombinant plasmids used in this study encode functional large T antigen. Because the replication elements also possess enhancer activity it is conceivable that the weak replication observed was due to enhanced levels of expression of large T antigen and this influenced core DNA

replication. Indeed, it is known that PyV large T antigen is limiting in the COP cells which were used in these experiments (Katinka and Yaniv, 1983). If this explanation is correct, then enhanced levels of active large T antigen may compensate for α and β.

Although the α element cannot activate viral DNA replication when moved approximately 200 bp away from the core there is evidence to suggest that it can function, albeit poorly, from its normal position in the viral genome, where it is situated more than 134 bp from the core. This was demonstrated by the observation that viral recombinant plasmids containing the α element next to a mutated β-core origin (LS 5151/5166 and LS 5173/5188) replicated to five-fold higher levels than the mutant β-core origins lacking α (Fig. 28A).

Interestingly, the early side position mutants place α closer to core than it is in its native position and yet these mutants are replication defective (Fig. 27). These results suggest that α can only effect its function from the late side of the core. The α element may have to be aligned with sequences located at the late side of core (i.e. A:T rich stretch). Alternatively, α may effect its function through sequences present within the mutated β element. It may be that protein(s) bound to α stabilize the binding of factors to the mutated β element through protein-protein interaction. Whatever the mechanism, it is clear that α can activate viral DNA replication from its normal position in the viral genome.

H. Comparison of the PyV and SV40 origins for DNA replication.

The PyV origin structurally resembles the origin for SV40 DNA replication. In SV40 DNA the functional origin is composed of a core segment and an adjacent region consisting of two perfect and one imperfect G:C rich 21 bp long direct repeats that stimulate DNA replication (Bergsma et al, 1982; Fromm and Berg, 1982). By contrast to the PyV core, the SV40 core, when linked to plasmid sequences, is capable of efficiently replicating in COS cells (Bergsma et al, 1982). The difference between the replication capacity of the SV40 and PyV cores may be due to the different affinities by which the viral large T antigens bind to their respective origins. It is known that SV40 large T antigen binds to its origin independent of auxiliary sequences (Jones et al, 1984; DeLucia et al, 1986). By contrast, PyV large T antigen binds very poorly to sites 1 and 2

within the core (Cowie and Kamen, 1984). Perhaps in PyV, efficient T antigen binding to the core requires the presence of factors bound to the α and β elements. These protein-protein interactions may stabilize large T antigen binding. Alternatively, some other feature of viral cores accounts for this difference in replication properties of the two core origins.

The SV40 auxiliary region containing the 21 bp repeats has an enhancing effect on replication efficiency that is dependent on the number of copies of the 21 bp repeats (Bergsma et al, 1981; Fromm and Berg, 1982). Recently, DeLucia et al (1986) reported that the 21 bp and 72 bp repeats can functionally substitute for each other in enhancing SV40 core replication. In this respect, they resemble the PyV α and β elements. Interestingly, when the SV40 auxiliary elements are moved away from the core there is corresponding decrease in the levels of viral DNA replication (Innis et al, 1984). These observations suggest that the mechanism by which these auxiliary elements activate core DNA replication is similar in both PyV and SV40.

Surprisingly, either the SV40 21 bp or 72 bp repeats activate PyV core DNA replication (deVilliers et al, 1982, 1984; Campbell and Villareal, 1985; Bennett and Hassell, manuscript in preparation). Indeed, it has been demonstrated that the immunoglobulin heavy chain enhancer activates PyV core DNA replication in a tissue specific manner (deVilliers et al, 1984). These elements share little sequence homology, yet they apparently function in the same manner. Within cells, these various elements are relatively free of nucleosomes and hypersensitive to DNAase I digestion (Fig. 29) (Sargosti et al, 1980; Herbomel et al, 1981). There is evidence to suggest that these elements serve as recognition sites for cellular proteins. Dynan and Tjian (1983) demonstrated that the SV40 promoter specific transcription factor, Sp1, binds to 21 bp repeats, and other cellular factors are known to specifically bind the SV40 72 bp repeats (Wildeman et al, 1984; Sassone- Corsi et al, 1985), the polyomavirus β element (Piette et al, 1985) and the immunoglobulin heavy chain enhancer (Ephrussi et al, 1985). The only common feature shared among these various elements is that they act as components of the promoters in each system.

I. A model for PyV DNA replication

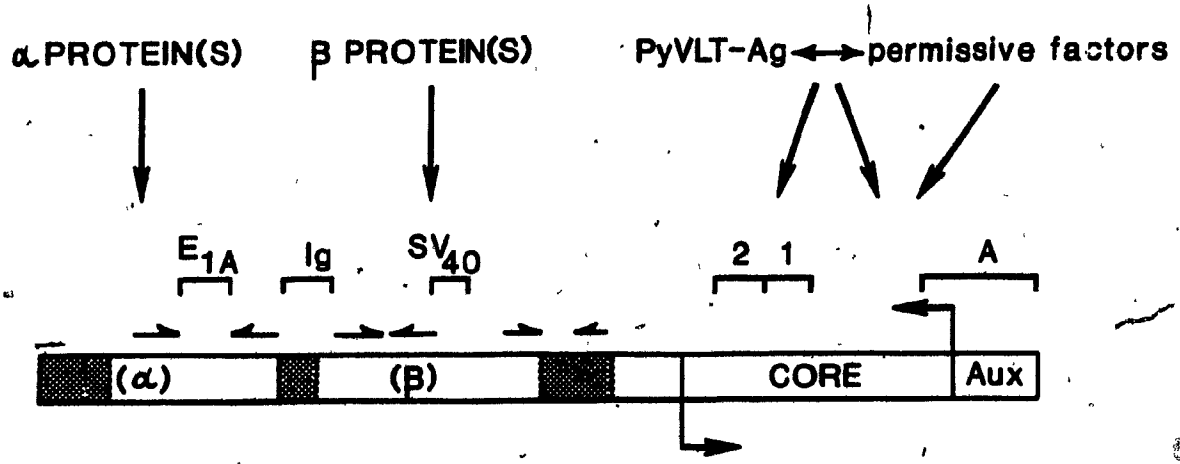
I have attempted to devise a model for the initiation of PyV DNA replication which takes into account the apparent dual role of the α and β

elements in viral transcription and DNA replication. The observation that enhancers potentiate transcription but not replication from a distance suggests to me that while the initial reactions leading to these processes might be the same, subsequent pathways are likely to be different. I propose that transcriptional factors bound upstream of the A:T rich stretch facilitate entry of replication proteins on the late side of the core region. These replication factors in turn interact with PyV large T bound to the core region. Permissive factors may also be associated with the viral large T antigen or exert their effect by directly binding the core region (Fig. 30). Local binding transcription factors near the late side of the core may lead directly to separation of DNA strands near a 15 bp A:T rich region situated at the late border of the core. The A:T rich stretch is an essential component of the PyV core as well as the SV40 origin for DNA replication (Luthman et al, 1982; Muller et al, 1983). Alternatively, RNA polymerase may recognize this complex of proteins and facilitate the denaturation of this region. This single stranded region may then be recognized by DNA polymerase α-primase complex and DNA synthesis initiated. Consistent with this model is observation that primers for PyV leading and lagging strand DNA synthesis are initiated on either side, but not within, the core (M. DePamphilis, personal communication). Therefore, unlike SV40, no primers are initiated within the genetically defined PyV origin core (M. DePamphilis, personal communication). This may be due to the presence of a PyV LT complex bound to both strands of DNA helix that blocks the synthesis of primers within this region.

This model predicts that the proteins bound to the auxiliary replication elements must be near the A:T rich region to exert their effect. This is consistent with my data that alterations of the spatial relationship between these elements can have a profound effect on viral DNA replication.

The protein:protein interactions occuring across the enhancer/core boundary may not be as critical for enhancer function. It may be that the protein-DNA complex comprising the enhancer element serves as entry site for RNA polymerase II (Moreau et al, 1981). Once on the DNA template, RNA polymerase may then move along until it encounters transcription factor-DNA complexes which align it for initiation. This would explain why enhancers activate gene expression from a considerable distance.

Figure 30: A model for the initiation of PyV DNA replication. Solid lines within the boxes represent the boundaries of the PyV replication activators. The arrows parallel to the boxed regions denote inverted repeat structures present within each element. The arrows perpendicular to the boxed regions represent the origin of bidirectional DNA synthesis. The brackets on top of boxes refer to either enhancer core homologies (E.A., Ig and SV40) or PyV large T antigen binding sites (1, 2 and A). The Aux region represents the sequence on the early side of core which contributes to core function but is not absolutely required. The dotted regions within the α and β elements refer to viral sequences that contribute to the activity of each replication activator.


α PROTEIN(S)
β PROTEIN(S)
PyVLT-Ag
permissive factors
E1A
Ig
SV40
2 1
A
(α)
(β)
CORE
Aux

The transcriptional activation model I have proposed for the initiation of PyV DNA replication is applicable to SV40. In SV40 transcriptional elements (the 21 bp or 72 bp repeats), located to the late side of the core, enhance viral DNA replication in *cis* (Bergsma *et al*, 1982; Fromm and Berg, 1982; DeLucia *et al*, 1986) and, like PyV, these elements appear to demonstrate a spatial dependence relative to the core (Innis *et al*, 1984). Recently, Takahashi *et al* (1986) reported that the 21 bp repeats and the early TATA box within the SV40 core requires a precise sterospecific alingment in order to effect efficient transcription. These results suggest that protein:protein interactions occur across the core. Interestingly, point mutations within the SV40 early TATA box have a *cis* inhibitory effect on viral DNA replication (Wasylyk *et al*, 1983; DeLucia *et al*, 1986). It is conceivable that the factor that binds to TATA box in the viral core region interacts with factors (i.e. Sp1) bound to the 21 bp repeats. This protein:protein interaction may be critical for both viral transcription and DNA replication. It is interesting to note that a restriction fragment spanning the 21 bp repeats and the SV40 early TATA will compete efficiently for limiting factor(s) required for SV40 DNA replication *in vitro* (Yamaguchi and DePamphilis, 1986). These results too suggest that components of the transcriptional machinery are also involved in the initiation of SV40 DNA replication.

Activation of SV40 DNA replication does not require transcription since α amanitin, which is an inhibitor of RNA polymerase II, does not interfere with SV40 DNA replication *in vitro* (Li and Kelly, 1984). Nontheless, this observation does not rule out the role for RNA polymerase II in the initiation of viral DNA replication. In the model I propose, RNA polymerase II is required to form a complex in the core that causes DNA strand separation. Transcription per se is not a requirement for activation of DNA replication.

Transcription activation has been implicated as a key event in the initiation of DNA replication at the *Escherichia coli* and λ origins of DNA replication (Furth and Wickner, 1983). Like the papovaviruses, transcriptional activation in these systems must occur near the origin (Furth and Wickner, 1983). However, the mechanism by which local transcription activates these *ori* regions is not known.

The multi-element organization of the PyV and SV40 origins is not unique to the papovavirus group. The adenovirus 2 origin comprises two

functionally distinct segments. The presence of one of these segments is sufficient to initiate viral DNA replication (Tapanoi and Stillman, 1983; Rawlins et al, 1984). A second adjacent region enhances viral DNA replication (Rawlins et al, 1984). Interestingly, a cellular factor, known as NF1, binds to this sequence (Nagata et al, 1983) and binding of this factor is known to be required for efficient adenovirus DNA synthesis. Although the role of NF1 in adenoviral replication is unclear, it is tempting to speculate that like the factors that bind to the PyV and SV40 auxiliary elements, it also plays a role in transcription. In this regard it is interesting to note that a 21 bp sequence identical in sequence to the SV40 21 bp repeats has been reported to reside within the inverted repeat termini of simian adenovirus 7 DNA (Tolun et al, 1979). Although it is not known whether these adenoviral sequences are elements of the adenovirus 7 functional origin their location near the sites of viral DNA replication is suggestive of a role in this process.

Recently, the origin of Epstein Barr virus (ori-P) has been shown to be also composed of two cis-acting components (Reisman et al, 1985). Interestingly, one of these components appears to be a transcriptional enhancer element (Reisman et al, 1985). In addition to the viral origins, the yeast chromosomal ARS elements which are thought to be cellular origins for DNA replication are also composed of multiple sequence elements (Kearsey, 1984). Taken together, these observations suggest that a multi-element organization may be a common feature of viral and by analogy, cellular replication origins.

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