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Transformation by Polyomavirus:
(i) Dependence on Cell Cycle and
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Hong-Hwa Chen

has been accepted towards fulfillment
of the requirements for

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Transformation by Polyomavirus: (i) Dependence on Cell Cycle and (ii) Associated Interviral Recombination

By

Hong-Hwa Chen

A DISSERTATION

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ABSTRACT

Transformation by Polyomavirus: (i) Dependence on Cell Cycle and (ii) Associated Interviral Recombination

By

Hong-Hwa Chen

The cell cycle dependence of polyomavirus transformation was analyzed in infections of nonpermissive Fischer rat cells released from G0. A 20 to 50-fold difference in relative rate of transformation was found for cells infected in early G1 phase compared to cells infected in G2 phase. The transformation differential was reflected in an equally large differential in viral gene expression and was accounted only in part by a cell cycle dependence of viral adsorption (2–10 fold). My results suggest the existence of another timed step in early processing of the viral genome. Another major step in cell cycle dependence was observed, which does contribute to differences in transformation potential of cells infected at different phases of the cell cycle. Viral transcription (i.e. the early promoter) showed a strong cell cycle dependence with a large induction of viral transcripts (30–40 fold). Interestingly, viral gene expression was delayed until the G1 phase of the next cell cycle following infection.

Previous work has demonstrated the existence of high frequency of homologous interviral recombination coincidental with the integration of the viral genome in the

process of neoplastic transformation. I studied the role of large T-antigen in this process, using temperature sensitive mutants. My results do not support a role for large T-antigen, since high levels of recombination were still observed in conditions in which viral DNA synthesis was abolished. However, the experiments do not rule out the possibility that the dosage or the domain of large T-antigen required for recombination is different from that required for viral DNA replication.

In the course of the recombination studies, I observed a gradient of recombination frequency along the polyoma genome, with a 40-fold differential between the minimum in the enhancer region and the maximum between nucleotide 1245 and 1387.

To my parents

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Chapter 1

Literature Survey

1.1 Infection Cycle of Polyomavirus

1.1.1 Adsorption, penetration and decapsidation

Polyomavirus is a double stranded DNA tumor virus discovered in 1950. The early events of polyomavirus infection, from the time of viral attachment to the cellular receptor site to the time of viral DNA replication, can be broadly divided into two stages. The first stage involves those events related to the interaction of the viral particles with the host cell, i.e., attachment, penetration, and uncoating [33, 83, 143]. In the second stage, events related to the expression of the viral genetic information, i.e., transcription, translation, replication, and possibly derepression of certain host regulatory systems resulting in subsequent modification of cellular or viral constituents occur [222].

The course of polyomavirus infection can proceed in two distinct ways. One type of infection is productive in nature, which is described by the replication of virus in the cell nucleus. The virus then forms progeny virus particles, and subsequently kills the host cell [74, 167]. The other type of infection is nonproductive, in which the host

cells survives and acquires new properties of malignant cell [139, 168]. Whether a cell undergoes a productive or nonproductive infection largely depends on the species of cells being infected.

It is well known that the cellular receptors are important determinants of virus tropism and pathogenesis. However, the receptors of few viruses have been identified [229]. For examples, the receptor of poliovirus has been cloned and shown to be a member of the immunoglobulin superfamily [146], while the receptor of rhinovirus has been shown to be ICAM-1 [92, 188]. ICAM-1 is a member of the integrin family for the leukocyte [72]. In the case of SV40, it has been shown that the class I major histocompatibility proteins act as cell surface receptors [9].

The importance of sialic acid in the adsorption of polyomavirus to cells was first demonstrated by the abolition of viral mediated hemagglutination upon treatment of erythrocytes with neuraminidase [73]. Treatment of 3T6 cells with *Vibrio cholerae* neuraminidase to remove cell surface sialic acid prevents infection by polyomavirus. Susceptibility of 3T6 cells to infection can be fully restored by treating the cells with β-galactosideα2,3-sialytransferase and CMP-NeuAc which forms the sequence NeuAcα2,3Galβ1,3GalNAc common to oligosacharides of cell surface glycoproteins and glycolipids. These results suggest that the oligosarcharide sequence NeuAcα2, 3Galβ1, 3GalNAc serves as a specific cell surface receptor involved in both polyomavirus-mediated hemagglutination and polyomavirus infection of host cells [84].

The receptor of polyomavirus could be a complex formed by more than one cellular protein. The nonionic detergent octyl-D-glucopyranoside (OG) has been used to

stabilize peripheral cell surface proteins and lipids from a variety of isolated cell membranes as well as intact cells [129]. The extraction of mouse kidney cells in the presence of OG resulted in the isolation of polyomavirus receptor moieties with molecular weights of 95 kilodalton (Kd), 50 Kd, and 25 to 30 Kd [141]. The moieties were suggested to be subunits of a larger receptor complex, as seen in the adenovirus–receptor interaction which appears to involve more than one plasma membrane protein [99].

After binding to its receptors, the virus particles penetrate into the cells. Penetration of virus particles across the cell membrane was observed to occur in two forms. In the first form, virus particles containing the viral genome can enter the cytoplasm in monopinocytotic vesicles. Thus, the virus particle is first tightly surrounded by the cell membrane, engulfed by the cell membrane which then pinches off and forms a monopinocytotic vesicle which specifically migrates to the nuclear membrane. Virus particles are now in the nucleus devoid of a membrane, and are rapidly uncoated. In the second form of penetration, virus particles without DNA are observed to enter the cytoplasm in large membrane–enclosed phagocytotic vesicles [137]. The results from the direct electron microscopy visualization of the virus suggest that the virus uncoats between the nuclear membranes [143], whereas biochemical fraction studies [32] show that the virus reaches the nucleus intact, implying that uncoating occurs within the nucleus. Recently, it has been suggested by Kasamatsu and her colleagues (1991) that SV40 enters its host cell through the nuclear pore and then decapsidates in the nucleus [235].

1.1.2 Transcription, replication and translation

In the productive infection of polyomavirus in mouse cells, virus coded early proteins, large T-antigen, middle T-antigen, and small T-antigen , start to be expressed by 10 to 12 hours after the uptake of virus into cells. Synthesis of a variety of cellular enzymes is induced, beginning 12 to 15 hours after infection, to carry out both cellular and viral DNA replication. After that, late viral mRNA is then made in large quantities, and viral capsid proteins are synthesized. Infectious viral progeny begins to be observed 20 to 25 hours after infection and assembly of the viral capsids with the virions continues until 60 to 70 hours.

Transcription

Polyoma virus specific RNA in the cytoplasm of permissive cells, is observed immediately after decapsidation of virus particles. Before viral DNA replication, the cytoplasm of infected cells contains a single viral early RNA sedimenting at 19S [222, 225, 226]. The early RNA is then alternatively spliced into 3 messages encoding for the early viral proteins large T-antigen, middle T-antigen and small T-antigen. Viral cytoplasmic RNA is polyadenylated [166, 224].

After the initiation of viral DNA replication, two relatively abundant species of cytoplasmic viral RNA are present. The more abundant sediments at 16 S, which is the late gene product while the less abundant sediments at 19 S [38, 208, 225]. The late SV40-specific 16 S and 19 S RNA molecules share common nucleotides sequences [225]. The 19 S RNA is the precursor of 16 S RNA, and it can be alternatively spliced into 3 messages encoding for viral capsid proteins VP1, VP2 and VP3 [4]. The late viral

RNA transcripts are far more abundant than the early viral RNA transcripts in permissive cells.

The 5' ends of the early and late strand transcripts are proximal to the point on the genome where bidirectional viral DNA replication initiates [110, 111, 114, 169]. The sequence between the early and late transcription start sites is the enhancer region and is about 380 base pairs. The enhancer region of polyomavirus can facilitate viral transcription as well as viral DNA synthesis.

There is more than one transcription initiation site for polyoma early transcripts. During the late phase of productive infections, there are late–early transcripts synthesized from upstream transcription start sites and the RNA is not capped [55]. The 5' proximal region of the late–early transcripts encodes an additional protein of 23 amino acids [37]. The down stream initiation site is used during the early phase of infection, leading to the synthesis of large T–antigen. The binding of large T–antigen to the origin derepresses the transcription from down stream initiation sites as well as induces the replication of DNA [37, 87, 95]. The onset of DNA replication then activates transcription from upstream sites.

The early promoter of polyomavirus is autoregulated by large T–antigen [115, 159, 201] via the direct interaction of large T–antigen with viral DNA [203, 205]. Among the three large T–antigen binding sites (I, II, and III), the autoregulation of large T–antigen involves the interaction of large T–antigen with binding sites I and II [160].

Transcription of polyoma DNA during the late phase of productive infections in mouse cells gives rise to "giant" RNA molecules. The giant RNA molecules contain up

to 3–4 times the size of the viral genome [2, 25, 131, 163], and no detectable nonviral sequences [1]. A small proportion of these hybrid molecules contain single-stranded branches or deletion loops in characteristic positions, indicating that RNA splicing may occur on these high molecular weight nuclear transcripts [1].

Hyde–DeRuyscher and CarMichael (1990) showed that the multi-genome length transcripts can be spliced leader–to–leader, and produce a high level of polyomavirus late RNA containing multiple leaders [105]. Cytoplasmic RNA from polyomavirus–infected cells contains between 1 and 12 tandem leader units at the 5' ends of all three late mRNA types for VP1, VP2, and VP3. It is possible that before viral DNA replication, transcription is efficient and allows the production of half–genome length primary transcripts. However, these transcripts are inefficiently spliced, and most are degraded in the nucleus. The transcript with multiple leaders is a prerequisite for the efficient accumulation of polyomavirus late mRNA.

It has been suggested that the cellular factors required for polyadenylation and termination in polyomavirus–infected mouse cells are present in limited amounts. Less than 20 % of the giant RNA molecules are polyadenylated. Efficient polyadenylation and termination of late polyomavirus transcripts are the results of these limiting factors.

The termination site of polyoma late transcription is weak so that run through transcription occurs. To test this, the 94 nucleotides of the rabbit β–globin polyadenylation signal was inserted upstream of the late strand polyadenylation signal. Results showed that efficient termination of late transcription by polymerase II can occur and result in a 1.4 to 2.5 fold increase in polyadenylated virus RNA [125].

Replication

DNA replication of polyomavirus requires the interaction of large T-antigen with sequence elements within the virus origin. The origin region contains two primary elements: a core element and auxiliary components. The core component is required for viral DNA replication under all conditions. The auxiliary components, containing promoter and enhancer elements, are involved in transcription as well as viral DNA replication. The core component per se is sufficient to constitute a functional replication origin, but the presence of auxiliary domains increases its activity 5 to 100 fold [66, 100, 128, 132].

The polyomavirus origin core can initiate replication only in the presence of large T-antigen and the DNA polymerase α-primase complex from permissive cells. Large T-antigen can initiate DNA replication by binding to its recognition sequence within the origin and then unwinding the DNA in its vicinity via its helicase activity [63, 70, 187]. The unwinding of double stranded DNA at the origin core may permit DNA polymerase α-primed-DNA synthesis within the origin core exclusively on the template strand at one of several possible initiation sites [98, 78, 197]. Transition sites on each strand of the origin where RNA-primed initiation events stop and continuous DNA synthesis begins occur in the origin core. Subsequent initiation of RNA primed nascent DNA chains (Okazaki fragments) occurs only on the retrograde arm of the replication forks [98].

An enhancer element is required for polyomavirus origin-dependent replication in developing two-cell mouse embryos, in mouse embryonic and differentiated cells, and

in the animal [41, 162, 199, 212, 232]. The enhancer function is normally provided either by the α or β element, corresponding to enhancer domains A and B, respectively. It has been shown that the non-polyomavirus enhancer can substitute for α and β. *Cis*–acting mutations in these sequences can allow polyoma core to initiate replication in mouse cell types that are normally nonpermissive.

Normally, wild type polyomavirus can replicate only in differentiated cells. There are polyomavirus mutants that can replicate in mouse embryonic carcinoma cells. These mutants have mutations in the enhancer B domain of wild type polyomavirus DNA resulting in sequence rearranged merits [5]. For example, fPyF9 can replicate in undifferentiated F9 cells. fPyF9 has three exogenous sequences inserted into the B domain [8]. The sequences are homologous to each other, and the consensus sequence of the inserts, GCATTCCATTGTTGTCAAAAG is termed box DNA. The box DNA can decrease activity of the SV40 promoter and enhancer in undifferentiated F9 cells. Thus, this sequence appears to be a negative regulatory sequence specific to undifferentiated cells.

The enhancer element determines cell type specificity for the activation of the polyoma origin–core. It has been suggested that species–specific permissive factors do not interact with the auxiliary domains but rather with either the origin–core or large T–antigen or both to effect DNA replication [21]. The permissivity is most likely caused by large T–antigen induced modification of cellular proteins required to replicate the polyomavirus origin. A possible target for the large T–antigen induced modifications is DNA polymerase α–DNA primase [200].

Enhancer and promoter elements can function in either orientation, but they must

be in close proximity to the AT-rich side of the polyoma origin–core [46, 128, 106]. The AT-rich region nearby which is not protected by large T–antigen in footprinting studies, is nevertheless essential for replication [190]. A role for the AT-rich sequences is to open the duplex at the replication origin. Thus, the polyomavirus origin–core components are affected by transcriptional elements [68], since RNA transcript can assist the melting of the origin. The transcriptional activation of the origin has also been observed in prokaryotic systems [12]. This mechanism for altering the topological state of the origin probably accounts for the transcriptional activation first observed in the initiation of phage lambda [86].

In *in vitro* systems, the main components from the host cell necessary to replicate the double stranded circular genome of polyomavirus are polymeraseα–DNA primase, polymerase δ, proliferating cell nuclear antigen (PCNA), single-strand binding protein (SSB) which is a helix-destabilizing protein and an important polymerase accessory protein (RF–C) [210]. DNA polymerase δ synthesizes DNA on the leading (continuous) strand, whereas DNA polymerase α makes the DNA on the lagging (discontinuous) strand [210]. ATP is required for both binding of large T–antigen to the DNA and for separating the double strands by the helicase activity of large T–antigen. ATP is hydrolyzed in a DNA-dependent manner for the latter case, and the large T–antigen molecules split in half and move outward in both directions from its initial site on the DNA [3].

1.2 Neoplastic Transformation by Polyomavirus

In nonpermissive cells, such as rat and hamster cells, infection of polyomavirus is nonproductive and neoplastic transformation of the host cells is the major event.

1.2.1 Definition of transformed cells

Polyomavirus was first observed to transform cultured cells by Dawe and Law in 1959 [62]. Ordinarily cells grow to a limited extent in culture then enter crisis and die. A proportion of polyoma-infected nonpermissive cells are found to grow at an increased rate and acquire a transformed phenotype [214].

The differences between transformed and normal cells involve changes in the regulation of cell growth as well as in structural morphology [19]. The growth of normal cells is restricted by cell density, availability of growth factors, and the need for anchorage. However, transformed cells can escape the above three limits to various extents. Transformed cells can be selected by these properties: loss of the contact inhibition which gives rise to foci of cells in the monolayer, and loss of anchorage-dependence gives rise to the growth in soft agar [20, 138].

An important feature that distinguishes normal cells from transformed cells is the ability of the cells to regulate the entry into and out of the cell cycle in response to factors such as population density and serum [15]. In the growth of normal cells, cells will cease their growth due to cell-cell contact inhibition when they reach 100 % confluency. The density regulation of growth can thus limit the maximal number of cells growing in a particular size of culture plate. Transformed cells, on the other

hand, fail to show cell-cell contact inhibition when they reach 100 % confluency. Multilayered growth of transformed cells can be observed even when the cells are still subconfluent.

There are several mitogenic factors present in serum to support cell growth, such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin, and insulin-like growth factor. PDGF can induce fibroblasts into the competent state, which can be progress to S phase in the presence of plasma [189]. Serum also contains components that promote attachment and spreading of cells on a solid substratum and that are needed for anchorage-dependent growth. Other components such as nonpeptide hormones, vitamins, Ca^{2+} and H^{+} ions can affect the density of cell growth.

Addition of serum or growth factors to normal resting cell cultures can induce cells to a competent state and further to DNA synthesis and mitosis. However, transformed cells lose serum-dependent growth and grow to high density with low serum.

1.2.2 Abortive and stable transformation

When nonpermissive cells (hamster or rat) are exposed to high dosage of polyomavirus, a large proportion of the cells develop a transformed phenotype, divide several times in the agar and then stop dividing. This phenomenon is referred to as abortive transformation [191, 192]. Only a small proportion of infected cells retain the transformed characters indefinitely and this occurs only after stable integration of the viral genome into host DNA. This is referred as stable transformation. Lines of stably transformed hamster cells occur at a maximum rate of about 5 % after

polyoma infection. In Fischer rat cells, the transformation rate is less than 1 %.

Stoker showed that infection of baby hamster kidney (BHK21) cells with tsa mutant at the non–permissive temperature could initiate a temporary change in BHK21 cells, but stable transformation did not occur [191, 192]. The defect of large T-antigen in the tsa mutant resulted in the failure of stable integration of the viral genome, so that stable transformation could not occur. These results with the tsa mutants suggested that the defect of large T–antigen is not in the expression of the transformed phenotype but rather in the events leading to stable perpetuation of the viral genome.

1.2.3 Initiation and maintenance of transformation

There are at least two steps in process of transforming a normal cell to a transformed cell. The first step is initiation and the second step is maintenance. Large T–antigen is required for the initiation of transformation in nonpermissive cells. Fried (1965) showed in hamster cells infected by a tsa mutant of polyomavirus that once the effect of one temperature on transformation is produced, the reversion of transformation cannot be obtained by shifting the infected cells to the other temperature [85]. For example, the cells transformed at $31.5^{o}C$ retained their transformed state upon cultivation at $38.5^{o}C$. Thus large T–antigen is important in the initiation of the transformed state, but not for the maintenance of transformation state.

Middle T–antigen is required for the maintenance of the transformed state of the cells [81]. Both F111 and normal rat kidney cells were coinfected with hr–t mutants and ts-a mutants of polyoma virus. The majority of clones selected from F111 cells expressed both middle T–antigen and small T–antigen, whereas the expression of the

large T-antigen was only detected in some of the clones. This result suggested that middle T-antigen, rather than large T-antigen, is required for the maintenance of the transformed state of the cells.

1.2.4 The role of large T-antigen in polyoma integration /transformation

In cells transformed by polyomavirus in tissue culture system, one finds viral genomes stably integrate into host chromosomes [170]. Thus the integration of the polyoma genome is correlated to transformation. The integrated DNA exists as head-to-tail tandem repeats of unit-length polyoma DNA attached to cellular DNA [24].

Large T-antigen is important for the integration pattern of head-to-tail tandem repeats of viral DNA. Della Valle et al. (1981) showed that in the absence of a functional large T-antigen, transformants contained multiple nontandem insertions of viral DNA segments shorter than the infecting polyoma molecule [65]. The efficiency of transformation was about 20 fold lower in the absence of large T-antigen.

The integration sites of the polyomavirus DNA in different transformed cell lines are different [107, 124]. Sequence rearrangements or alterations occur immediately adjacent to the viral insert, possibly as a consequence of the integration of viral DNA [97].

In addition to integrated viral genomes, there are 20 to 50 copies of free viral DNA per cell [156, 238]. Most of these molecules exist in the supercoiled form in the nuclei of the transformed cells. Basilico and his colleagues suggested that the origin of these

free viral DNA was due to the high rate of excision and amplification of integrated viral genomes [50]. The presence of homologous regions in the integrated viral sequences is required for viral amplification and excision [50]. The excision events occur via intramolecular homologous recombination which is promoted directly or indirectly by the large T-antigen [16, 18, 29, 30, 31, 50, 148, 169].

1.2.5 The role of middle T-antigen in the maintenance of transformation phenotype

Middle T-antigen is the transforming protein of polyomavirus, and is located on the cytoplasmic membrane. Expression of middle T-antigen leads to profound changes in the cells and causes cell transformation. For example, the transformed state of cells can be induced by the expression of middle T-antigen cDNA in normal rat fibroblasts [209]. Different levels of transformation can be observed by using the dexamethasone-inducible promoter of the mouse mammary tumor virus LTR. At low levels of expression, rat fibroblast cells showed loss of actin cables and decreased adhesion. At a higher level of induction, the cells are able to form foci; and at the maximal expression, the cells are able to grow in the soft agar [157].

Middle T-antigen is one of the important elements in the cascade of signal transduction events required to induce cell transformation. Middle T-antigen exerts its function by binding to the Src tyrosine protein kinase [54], and phosphatidylinositol 3-kinase [112, 230]. The formation of complexes between middle T-antigen and Src can lead to a 10 to 50-fold increase of Src kinase activity [28, 53]. Before middle

T-antigen forms complexes with Src, phosphorylation of middle T-antigen by serine kinases at two to three sites is required [172]. Then the middle T-antigen-Src complex binds to a phosphatidylinositol kinase [112, 230].

Middle T-antigen possesses associated protein kinase activity of $pp60^{c-src}$ and $p62^{c-yes}$, which are in the *src*-family of protein kinases [119]. The amino-terminal portion of middle T-antigen between residues 78 and 191 have been shown to associate with $pp60^{c-src}$. Recently, another member of the *src* protein kinase, $p60^{fyn}$ was shown to associate with in murine polyomavirus-transformed rat cells [104]. Cook and Hassell (1990) showed that the amino terminus of polyomavirus middle T-antigen is required for transformation [51]. The first four amino acids of constitute part of a domain required for activation of the $pp60^{c-src}$ tyrosyl kinase activity and for consequent cellular transformation.

Middle T-antigen also activates cellular gene expression by enhancing transcription via the cellular transcription factor PEA1 [221], which is similar to the mouse Jun protein (AP1) [7, 127, 155]. PEA1 binds to polyoma enhancer A [155], and overlaps in its target specifically with TPA-inducible genes. Middle T-antigen, activated *ras*, *v-src*, and *raf* all stimulate PEA1-mediated transcription [221].

1.3 Cell Cycle Regulation of Gene Expression

1.3.1 Cell cycle

A functional cell cycle is divided into G1, S, G2, and M phases. The G1 phase of the cell cycle is a period during which cells prepare for S phase. The S phase is marked

by beginning of DNA, histone, and some enzyme synthesis. G1 was originally defined as a time interval, a gap between the readily observed events of mitosis and DNA synthesis.

The G1 events can begin during the previous cycle, at the same time as other events such as DNA synthesis or preparation for mitosis. Thus the observed G1 interval between M and S phase depends on how much progress has been made in the previous cycle [76].

Cells *in vivo*, for example hepatocytes and neurons, remain healthy for very long periods in the nonproliferating or quiescent state called G0. Cells in culture can also be arrested in G0 by contact inhibition or serum starvation. G0 arrested cells have an unduplicated DNA content, smaller size because their protein and RNA molecules are degraded and are not rapidly synthesized. Synthesis of macromolecules are about one-third as rapid in G0 as in proliferating cells [15, 58, 67].

G1 has been divided into subphases [164, 189, 202], depending on the effects of the limiting growth factors, nutrients, or inhibitors, as measured by time to reach S phase after the block in G0 is removed. These subphases are referred to as competence, entry, progression and assembly [153].

Treatment of cells blocked in G0 with PDGF or plasma (which lacks PDGF) alone cannot stimulate normal BALB/c 3T3 cells to enter S. If first treated with PDGF and then with plasma, the cells can progress to S phase, but not vice versa. The PDGF-treated cells are then competent to progress to S phase [15, 58, 67, 164, 189, 202]. For competent BALB/c 3T3 cells, it takes about 12 hours to reach S phase, the same amount of time it takes cells in G0 phase.

The addition of plasma, which provides factors such as EGF and insulin, for competent cells, can lead these cells to progress to S phase [130, 236]. Competent cells incubated with plasma in a medium without essential amino acids reach a point called V. It requires 6 hours for cells at V to reach S phase after the essential amino acids are provided. This length of time is very similar to the duration of G1 phase for the cycling cells.

After 3T3 cells have reached V point, the only growth factor required to progress to S phase is insulin–like growth factor–1 (IGF–1) [130, 236]. Rapid synthesis is required during this middle part of G1, and enzymes required for DNA synthesis are made during progression. In the assembly subphase, movement of enzymes into the nucleus and organization of these enzymes into a complex to catalyze DNA synthesis may require considerable time.

Induction of cellular genes after external stimulation

Competent cells produce immediate–early mRNA including *c-fos*, which appears in a few minutes after treatment with mitogenic factors and *c-myc*, which appears several hours later. Some of these mRNA turn over rapidly; their levels peak for only a short time. The presence of these mRNA are observed even when synthesis of new protein is inhibited, suggesting that transcriptions of early mRNA do not require production of new proteins.

Progression through the cell cycle is regulated primarily during the G1 phase [154]. Synthesis of new mRNA is required for progress through G1. Inhibitors and mutations affecting G1 are effective in blocking proliferation. For examples, α–amanitin, which

is an inhibitor of RNA polymerase II, blocks cells in G1 [216] , and a cell line that is arrested in G1 at the non-permissive temperature is defective in RNA polymerase II Protein synthesis in early G1, possibly directed by newly transcribed messages, is also required for cell growth [154, 177]. It has been estimated in mouse fibroblast cells by subtractive or differential hybridization that 3 % of the mRNA species in exponentially growing cells are absent in non-growing cells [231]. Most molecules are made continuously to serve house-keeping functions.

A number of normal cellular genes have been postulated to have roles in the control of cell proliferation. Included among these are those characterized as proto-oncogenes, those whose expression is cell cycle dependent, and those whose protein products are components of the cytoskeletal framework. Cellular proto-oncogenes encode proteins with three major sites of action: the cell surface membrane, cytoplasm, and nucleus [26, 223]. The nuclear acting group of proto-oncogenes includes *c-fos, c-myc, c-myb, c-erbA*, p53, and *c-jun* etc. [215]. Because of their nuclear location, it has been suggested that products of these genes function as transcriptional regulators [117]. The *c-erbA* gene codes for a thyroid hormone receptor [171, 227]. The *c-Myc* protein acts in conjunction with MAX protein [27], and functions as a transcriptional activator. *c-jun* codes the transcription factor AP-1, and binds to a DNA sequence in the upstream promoter regions of many genes. *c-fos* codes for a trans-activator which does not operate via direct binding to DNA but by interacting with *c-jun*/AP-1 [47]. The wild type p53 is a tumor suppressor gene, and regulates the cell growth. If both alleles of p53 are mutated, p53 becomes an oncogene resulting in loss of the control of cell growth [75, 80, 101]. The *c-ras* gene can be activated

in mid-G1. The Ras protein is a guanosine triphosphate (GTP)-binding G protein, and it is membrane localized.

The early gene responses can also be induced by viral infection. When quiescent cells are infected by polyomavirus, cellular DNA synthesis and cell division are induced to allow viral replication. Zullo et al. showed that infection of quiescent BALB/c 3T3 cells by polyomavirus resulted in the biphasic accumulation of RNA from the early response genes *c-fos, c-myc*, and JE [239]. They found that the first peak was due to the interaction of VP1, the major virus capsid protein, with its receptors. The second peak was due to the expression of polyomavirus early messages. Studies with virus mutants indicated that large T-antigen alone was not sufficient to induce the second peak. Middle T-antigen was dispensable, and small T-antigen either alone or together with large T-antigen, may be the regulator for the second wave of induction [90]. Furthermore, Glenn and Eckhart (1990) showed that infection of both BALB/c 3T3 and NIH 3T3 cells with polyomavirus lead to the expression of early-response genes,*c-fos, c-myc*, and *c-jun*, biphasically. Large T-antigen was not sufficient, and middle T-antigen was dispensable for the induction of the early-response genes [90].

In mid-G1, several enzymes activities increase. These enzymes includes transin (which is a protease), ornithine decarboxylase (which catalyzes polyamine synthesis), hydroxymethylglutaryl coenzyme A reductase for isoprenoid synthesis, and a 68 Kd nuclear protein that is a RNA helicase. Both p53 and p68 proteins are increased [61, 102, 113].

In late G1 phase, several gene products involved indirectly in DNA synthesis are increased. These include thymidine kinase [52, 181], dihydrofolate reductase [79],

thymidylate synthetase [11], DNA polymerase α, and proliferating cell nuclear antigen (PCNA). Induction of DNA polymerase α activity during the S phase of the cell cycle [14, 48] suggests that expression of this gene is closely coupled to the onset of DNA replication.

At the end of the G1 phase, the migration of the enzymes, which are produced on ribosomes in the cytoplasm, to the nucleus to catalyze DNA synthesis is observed. Enzymes in the nucleus then form a multienzyme complex called replitase [153]. The replitase includes enzymes for DNA replication, such as DNA polymerase α [42, 217], and enzymes that catalyze precursor synthesis, such as ribonucleotide reductase, dihydrofolate reductase, and thymidine kinase (TK) [69, 94, 96, 120, 149, 158, 211, 213]. After G1 phase, DNA synthesis and histone synthesis occur, and the cell cycle is completed by mitosis and cytokinesis.

The results above were obtained from proliferative cells stimulated from serum-deprived, metabolically blocked, or spatially restricted quiescent cultures. These methods perturb normal cell growth by artificially bringing cells into a noncycling or G0 phase from which, up on stimulation, cells must re-enter the cell cycle. The G0 phase is usually not present in the exponentially growing cells. For example, Wahl et al. (1988) have studied the expression of human DNA polymerase α in both quiescent cells stimulated to proliferate as well as in actively growing cells separated into progressive phases by counterflow centrifugal elutriation. Results showed that the regulation of human DNA polymerase α was positively correlated with cellular transformation and activation of proliferation. However, results using exponentially growing cells separated into different phases by elutriation showed that DNA poly-

merase α was constitutively expressed throughout the cell cycle, with only a moderate elevation prior to the S phase and a slight decrease late in the G2 phase [217]. These results suggested that what is observed in the serum-released cells may not occur in the exponential growing cells.

1.3.2 Effect of cell cycle on polyomavirus infection

It has been shown that the process of viral infection is a function of the physiological state of the host cell. The infection of polyomavirus can be initiated during the cell cycle while the integration of viral DNA into the host cell DNA occurs during the S phase.

Integration

Eremenko and Volpe (1984) showed that the integration of the viral genome into host cell DNA was not observed until the next S phase. These cells were synchronized by the double thymidine block, infected by SV40 occurred middle G2 phase in the mouse 3T3 cells. In the other experiment, cells were synchronized by mitotic shake off, where cells were infected in early G1 phase, the integration of the viral DNA was seen in late S phase of the same cell cycle. These results suggested that the integration of SV40 viral DNA occurred preferentially during S phase, and that the G1 phase was necessary for the viral integration [77].

Transformation

The neoplastic transformation induced by polyomavirus in nonpermissive cells is very low [193]. It has been shown that the physiological state of the cells can affect its competence for neoplastic transformation. Basilico and Marin (1966) showed that the susceptibility of baby hamster kidney cells (BHK21) to transformation by polyomavirus varied in different stages of the mitotic cycle [17]. They found that the transformation rate was about 2 fold higher in cells infected in G2 cells than in G1. The 2–fold difference observed was suggested to be directly related to the 2–fold increase of DNA content in G2 cells. The duplicated cellular DNA thus offers twice as many targets for viral integration which can then lead to 2–fold higher of transformation frequency.

In mouse 3Y1 cells infected by SV40, Tamura (1983) found that the highest transformation frequency was observed when the growth of resting 3Y1 cells was stimulated by sparse replating after virus inoculation, and the lowest frequency was in resting stage [198]. A 8 to 30–fold increase in transformation frequency was observed in resting cells infected with SV40 compared to infection of growing cells. In his experiment, similar results were also observed after infection of 3Y1 cells with polyomavirus. The reduction of transformation in proliferating cells infected with SV40 was also observed in BALB/c 3T3 mouse cells [142].

Viral DNA replication

The relation between replication of SV40 DNA and the various periods of the host–cell cycle was investigated in synchronized CV1 cells by Pages et al. (1973). CV1 cells

synchronized by a double thymidine block were infected with SV40 at the beginning of S, middle of S or in G2 phase. Infection with SV40 was also performed on cells obtained in early G1 through mitotic shake off. Their results showed that as long as cells were infected in G1 phase (either early, middle or late G1), the viral progeny DNA molecules were detected during the S phase of same cell cycle. However, if infection took place once the cells had entered the S phase, no progeny DNA molecules were detected until the S phase of the next cell cycle [152]. The explanation for these results were that the infected cells has to pass through a critical stage situated near the end of G1 or the very beginning of S in order to gain competence for the eventual initiation of viral DNA synthesis.

Adsorption

The influence of the various phases of the cell cycle on adsorption of SV40 was studied by measuring the binding of tritiated thymidine–labeled virus to cells in various phases. The results showed that a 2 to 3–fold increase of viral adsorption was obtained in G1–infected cells compared to cells infected at G2 or M phase [152].

1.4 Recombination

Mammalian cells readily integrate foreign DNA into their chromosomes. In the integration of the polyoma viral genome, there is little or no nucleotide sequence homology at the joint between viral DNA and chromosomal DNA. The absence of homology indicates that polyoma integration is nonhomologous recombination.

Homologous recombination are present in the polyomavirus such as in gene amplifications [50, 16, 195], excision [29, 30, 31], and interviral recombination [93]. Hacker and Fluck (1989) showed that high levels of interviral recombination were obtained among the integrated viral genomes in transformed Fischer rat (F111) cells [93].

When duplicated regions of the polyoma genomes are present, homologous sequences can recombine and produce complete viral molecules that can be excised and circularized as free DNA [31, 50]. The newly replicated molecules can recombine with homologous sequences of the parental strands. This type of recombination can lead to an increase in the copy number of the integrated viral genomes [50, 195].

1.4.1 Mechanisms of recombination

Nonhomologous recombination

The nonhomologous recombination is a two–step process: Free ends of double strand DNA are generated in the first step and joined in the second. DNA ends arise from errors of DNA metabolism, and are subsequently eliminated by sticking them together. The end joining is the general defense mechanism in mammalian cells for dealing with broken chromosomes.

Homologous recombination

Two working models have been proposed to explain the results of homologous recombination of DNA introduced into mammalian cells. One is the double-strand break repair (DSBR) model [10, 34, 35, 82, 108, 121, 185, 186], and the other is the

single–strand annealing (SSA) model [6, 36, 43, 133, 134, 178, 218]. Both models are based on the observation that double–strand breaks in DNA at certain locations can stimulate recombination.

In the DSBR model [196], recombination is initiated by a double-strand break in one of the DNA molecules (the recipient) that is enlarged to a double strand gap. That gap is then repaired by using the second molecule (the donor) that shares homology with the region flanking the double-strand break in the first molecule. In this model, only the recipient molecule needs to contain a double-strand cut in the homologous region. In contrast, both substrates must be linearized to generate recombinants by the SSA model. According to SSA model, the DNA ends act as entry sites for a strand-specific exonuclease. Degradation of DNA by such a nuclease generates complementary single-stranded DNA for pairing [133, 134]. Alternatively, the single–stranded DNA can be generated by helicase unwinding of the linearized DNA duplex without extensive exonuclease degradation. The essential feature of the SSA model is that double–strand breaks made close to the region of homology shared by each parental DNA molecule are required to initiate recombination.

Although both the DSBR and SSA models emphasize the importance of generating single-strand DNA for pairing steps in the reactions, the outcomes of recombination predicted by the two models are very different. In the SSA model [43, 178, 218], recombination is nonconservative and produces crossover products exclusively. The flanking sequences of the recombinant DNA produced by SSA model are usually rearranged. However, in the DSBR model, only about 50 % of the recombinant DNA is associated with crossover of the flanking markers if there is no bias in the resolution

step of the reaction.

1.4.2 Enzymology for recombination

RecA and auxiliary proteins

In prokaryotic systems, two types of homologous recombination are observed. One type of recombination utilizes the RecA protein, does not require extensive DNA synthesis, and is found in phage, bacterial, and fungal systems. The other type is RecA-independent and replication-dependent,such as in phage lambda, T7 and T4 [56].

The RecA protein is an ATP-dependent, single-strand DNA binding protein [150]. It is also, a protease and is required in the regulation of its own synthesis [233]. The protease activity of RecA can cleave the lexA repressor [161], which regularly binds to the RecA promoter, and allows the expression of RecA.

The principal function of the RecA protein in recombination is to catalyze strand invasion [71]. RecA is able to promote the annealing of a single strand to a recipient molecule of double helix and form a D-loop by hydrolyzing ATP in the process [182, 144]. After binding to the single-strand DNA, RecA is able to partially denature the recipient duplex DNA molecule to accept the invading single strand DNA [59]. Then a reciprocal exchange of DNA strands between the two double helixes can then occur. That is, as a strand from a donor double helix invades a recipient double helix, the displaced strand in the recipient molecule can invade the first double helix [228]. This is the same as the initial step in recombination in the Holiday model [103].

Although the RecA protein can catalyze a variety of DNA strand-exchange reactions, three other *E. coli* proteins play roles in conjunction with the RecA protein [71]. These proteins are *E. coli* single-strand DNA binding protein (SSB), topoisomerases, and the RecBC enzymes. The *E. coli* SSB protein has an even greater affinity for single-strand DNA than does the RecA protein [184]. When the SSB protein is added to the various reactions with RecA, it dramatically improves the efficient use of RecA and ATP, so that less RecA protein and less ATP are required to generate an equivalent level of strand exchange [145, 183]. When a topoisomerase is added to a basic reaction mixture containing RecA, ATP, single-strand circles, and supercoiled duplex rings, the single-stranded circles become more stably interwrapped with the duplex ring at a region of homology [60].

The RecBC enzyme is a multisubunit protein product of the RecB and RecC proteins in *E. coli* [207]. The RecBC enzyme was first identified as a potent exonuclease as well as an endonuclease driven by the hydrolysis of ATP [13, 151, 91]. RecBC can initiate genetic recombination by traveling internally down a DNA double helix and creating a region of local denaturation. In the denatured region, several hundred base pairs of positive and negative strand DNA are held apart so that their hydrogen bonding surfaces are exposed. In this reaction the RecBC works with the SSB protein, whose role is to stabilize the separated strands. This is the DNA molecule that will initiate recombination by serving as the donor. One of its exposed single-stranded regions will attack a second DNA molecules [71].

Topoisomerase

The DNA topoisomerases are involved in nearly all biological transaction of DNA. These include the relaxation of negatively and positively supercoiled domains which are generated in a DNA template during replication and transcription [136, 234, 89]. The participation of bacterial gyrase or eukaryotic DNA topoisomerase in nonhomologous recombination has been proposed based on in vitro and in vivo studies. For example, sequencing the integration sites of SV40 indicated that eukaryotic DNA topoisomerase I might be doing the strand transfer during viral integration [10, 39]. The eukaryotic topoisomerase I–mediated nonhomologous recombination may initiate in regions that contain single–strand gaps which can be converted to double-strand breaks by topoisomerase I [45].

Although both eukaryotic topoisomerase and lambda integrase play roles in the integration of viral or phage DNA into the host chromosome, the mechanisms are different [57, 122]. The major difference between topoisomerase and lambda integrase is that in the topoisomerase–catalyzed reactions, the same internucleotide bond broken in the first transesterification step is re–formed in the second transesterification step. However, in a lambda–type recombination catalyzed by integrase, strand switching occurs between the two steps: the 3' side of a transiently strand is joined to the 5' side of another transiently broken strand.

DNA topoisomerase might be required for the formation of recombination intermediates in which two DNA strands are wound plectonemically around each other [44, 118, 60] as well as for supercoiling of intracellular DNA. DNA topoisomerases can

also act as suppressors of recombination. In yeast, mutants with a null mutation in the *top1* gene or a temperature sensitive mutation in the *top2* gene, the frequency of mitotic recombination in the rDNA gene cluster at a semipermissive temperature is 50–200 times higher than it is in the wild type TOP^+ controls [49]. Thus the topoisomerases I and II appears to suppress mitotic recombination within the rDNA cluster. Mutations in yeast gene *top3* increase recombination between the long terminal repeats of the *Drosophila* retrotransposon Ty [219]. TOP3 is a protein homologous to bacterial topoisomerase I.

The possible mechanism invoked to explain the suppression of recombination by a topoisomerase is that the enzyme relaxes supercoiled regions of intracellular DNA and thus suppress DNA supercoiling– stimulated recombination [220]. For example, for the heavily transcribed rDNA cluster, intramolecular recombination is elevated and excision of extrachromosomal rings occurred when topoisomerase activity was insufficient to relax the supercoiled domains in a topoisomerase double mutant [116].

In conclusion, it is possible that the topoisomerase can function either positively or negatively in recombinational synapses of two complementary DNA strands from two different molecules or different regions of the same molecules. That is, recombination might normally be minimized by processes that would dissociate two inadvertently paired DNA strands, recombination would form only from structures that have escaped dissolution–by helicase [204].

1.4.3 The possible role of large T–antigen in homologous recombination

Structure and function of large T–antigen

Large T–antigen of polyomavirus is a 100 kilodalton protein with 785 amino acids. The homology between large T–antigen of polyoma and SV40 is about 90 %. The structure of SV40 large T–antigen is studied more thoroughly. SV40 large T–antigen contains an origin binding region (131–371 amino acids) [66, 109], a Zn^{2+}–finger domain (302–320 amino acids) [22, 23], a Leucine zipper domain (345–370 amino acids), a helicase activity domain (131–680 amino acids) [63, 187, 116], an ATP binding region (418–528 amino acids) [88, 206], a DNA polymerase α –binding domain(272–517 amino acids), a retinoblastoma binding region (105–114 amino acids) [64], and a p53 binding domain [123, 135], which is not present in large T–antigen of polyoma. Also, there is homology of SV40 large T–antigen and *E. coli* RecA protein in the 372 to 648 mino acids of SV40 large T–antigen ' and 36 to 352 amino acids of RecA [179].

The multifunctions of large T–antigen make it possible for it to be involved in the molecular events which are essential for both viral productive infection and for cellular neoplastic transformation. Large T–antigen plays an important role in the initiation of viral DNA replication by exerting its function of binding to the origin region, association with polymerase α–primase, helicase activity, and ATPase activity. Once the initiation of DNA replication occurs, large T–antigen is required for elongation of replication forks using its helicase and ATPase activities.

In viral transcription, large T–antigen can feedback control its own expression by

a negative regulation mechanism of blocking RNA polymerase II elongation. Large T-antigen can also act as a positive regulator of late gene expression via modification of a cellular protein rather than binding directly to the late promoter.

Regulation of large T-antigen

Large T-antigen is regulated in a very complex way such that its many functions in vitro can be carried out by one protein. One mode of regulation at the posttranslationallevel is phosphorylation since large T-antigen is known to be a phosphoprotein. For example, the the phosphorylation of large T-antigen at serine residue is faster than the turnover of the protein itself, suggesting that regulation by phosphorylation is important. Two clusters of phosphorylation sites on large T-antigen have been mapped, one near the N terminus and one near the C terminus. Each cluster contains one phosphothreonine and four phosphoserine residues [173, 165].

Dephosphorylation of serine groups on SV40 large T-antigen has been shown to activate its specific DNA binding to site II within the origin of SV40 DNA replication *in vitro*. It has been shown that the incubation of large T-antigen with alkaline phosphatase can remove all serine-bound phosphates [180], increase the binding of large T-antigen to site II at the origin of replication and stimulate its ability to support in vitro DNA replication [147].

Recently, it has been shown that phosphatase 2A (PP2A) can potentiate SV40 DNA replication *in vitro* [126]. Lawson et al. showed that the PP2A can dephosphorylate large T-antigen and lead to activation of DNA replication. Formation of stable complexes between both polyoma small T-antigen and middle T-antigen with

protein PP2A was observed. It is possible that small T-antigen plays a role in DNA replication by activating the ability of PP2A to dephosphorylate large T antigen, thereby activating large T-antigen [175, 174, 237].

Besides phosphorylation, large T-antigen undergoes other posttranslational modifications, such as oligomerization, acetylation, and glycosylation. It has been shown by Hurwitz (1990) that large T-antigen oligomerizes as two hexamers on the replication origin in the presence of ATP. Results of DNase protection and DNase protection studies showed that large T-antigen was organized into a two lobed structure at the origin . The largest complex was found by scanning transmission electron microscopy to contain 12 monomers of 12 large T-antigen.

As a higher level of regulation, large T-antigen is compartmentalized in different structural systems of the nucleus. The interaction of large T-antigen with the chromatin and the nuclear matrix is suggested to be another level of regulation. Deppert and his colleagues (1989) showed that the associations of the tsA large T antigen with both the cellular chromatin and the nuclear matrix were temperature-sensitive, while that of the SV40 wild-type large T-antigen was not [176]. Furthermore, Mann found that large T-antigen was still associated with the nuclear matrix after cells were shifted to the nonpermissive temperature for 1 hour. His results suggested that the association of SV40 large T-antigen with the nuclear matrix was DNA replication independent and origin-binding independent [140].

The role of large T–antigen in recombination

Recently, the role of large T–antigen in intrachromosomal recombination was studied by Bastin and his colleagues. They showed that the recombination between two copies of defective middle T–antigen located side–by–side was promoted from 10^{-7} to 10^{-2} per cell generation when large T–antigen was present. Recombination between the two copies of defective middle T–antigen was promoted even by the SV40 large T antigen which can not activate polyoma DNA replication. The promotion of recombination by large T–antigen was not DNA replication-dependent. They suggested that the possible role of large T–antigen in homologous recombination was to melt and unwind the DNA at the viral replication origin so as to create a favorable substrate for homologous recombination [194].

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Chapter 2

Neoplastic Transformation by Polyomavirus during the Cell Cycle

The cell cycle dependence of polyomavirus transformation was analyzed in infections of nonpermissive Fischer rat (FR3T3) cells released from G0. A 20 to 50–fold difference in relative rate of transformation was found for cells infected in early G1 phase compared to cells infected in G2 phase. The levels of early viral gene expression paralleled transformation frequencies. The difference in susceptibility was accounted only in part by a cell cycle dependence for viral adsorption (2 to 10 fold). In addition, viral gene expression showed a strong cell cycle dependence with a large synchronous burst of viral transcripts (of large heterogeneous sizes) delayed till the G1 phase of the next cell cycle post infection. Little or no viral gene expression was detected with cells infected in S or G2 phase, despite the appearance of viral genomes in the nucleus. Our results demonstrate that both adsorption and early transcription are cell cycle regulated steps in infections of G0–released FR3T3 cells. In addition, the results suggest the existence of another timed step in early processing of the viral genome; possibly nuclear transport, decapsidation, scaffold attachment, or integration which

must precede transcription but occurs later in G1 than the transcription competence step.

2.1 Introduction

Recently, the control of cell cycle has been an area of intensive study. The results have shown that at least some genes are controlled at multiple transcriptional as well as post transcriptional levels. The ability of polyomavirus and SV40 to perturb the cell cycle control has also been the focus of recent research. At least two stages of the cell cycle are affected by infection with polyomavirus [18]. The adsorption of viral particles or even empty viral capsids will induces early G1 genes, such as *c-myc* and *c-fos*. Second, the cell cycle can be further perturbed by the expression of viral early proteins. Studies with virus mutants indicate that large T-antigen alone is not sufficient, middle T-antigen is dispensable, and small T-antigen either alone or with large T-antigen may be the regulator of the second wave of induction of cellular genomes [5, 18].

However, the study of the regulation of the viral infection process during the cell cycle has received little attention [2, 11, 14]. Although it might be expected that the reliance of viruses with very limited genetic information such polyomavirus and SV40 and which therefore must rely heavily on the host cell synthetic capacity would be submitted to host regulatory processes; e.g. cell cycle.

In this report, we have analyzed the cell cycle dependence on transformation of various aspects of the infection of nonpermissive Fischer rat (FR3T3) cells by

polyomavirus. These infections are characterized by the usual landmarks of infection of nonpermissive cells by papovaviruses. About 10 % of infected cells undergo abortive transformation, and only about 1 % undergo stable transformation [16] associated with the stable integration of the viral genome into host cell DNA.

Our results show that there are large variations in transformation frequency during the cell cycle, with a maximum for cells infected early after release from G0. Virus uptake as well as another step, probably an early genome processing step may contribute to this dependence of transformation frequency. In addition, viral gene expression is also strongly cell-cycle regulated.

2.2 Materials and Methods

Virus and cells: Polyoma virus wild type A2 [12] was used. Fisher rat 3T3 (FR3T3) cells [7] were maintained in Dulbeco Modified Eagle medium (DMEM, Gibco) supplemented with 5 % calf serum (Hazleton). For synchronization, cells were grown to confluency, and changed to DMEM supplemented with 0.2 % gamma-globulin free calf serum (Gibco) on next day. Cells were allowed to stay in low serum for 1 day. To release from G0, both trypsinization and addition of fresh DMEM supplemented with 10 % normol calf serum were used. The reattachment of cells to the culture plates occurred within 3 hours.

Measurement of DNA synthesis during the cell cycle: Incorporation of tritium-labeled-thymidine (^{3}H-TdR) (1 μCi/ml, ICN) was carried out for 1 hour. After incubation, cells were washed with prewarmed phosphate buffered saline (PBS), pH

7.4, and the nucleic acids were precipitated with 10 % trichloroacetic acid (TCA) and trapped onto Whatman 2.4 cm glass fiber filters (GF/C, BMB). Incorporation of ^{3}H-TdR was determined by scintillation counting.

Measurement of transformation frequency: 1 x 10^5 FR3T3 cells were seeded per 35 mm tissue culture plate, and infected at various times after release from G0 at a multiplicity of infection (MOI) of 1 plaque forming units (pfu) per cell. Adsorption was carried out for 1 hour, after which non-attached virus was removed and DMEM supplemented with 10 % calf serum was added. Cells were fed twice a week with DMEM supplemented with 10 % calf serum. Transformation frequencies were determined by focus formation.

Isolation of nuclei: Infection was stopped 20 hours post infection prior to de novo DNA synthesis. Infected cells were washed and treated in lysis buffer containing 5 mM $MgCl_2$, 0.5 % Nonidet P40 (NP40) in 1 X PBS on ice for 5 min. The nuclei was pelleted by centrifuged at 3000 rpm at $4°C$. Cell debris was removed by washing twice with PBS. Viral DNA and was extracted as described below.

Preparation and analysis of DNA: Infected cells were washed and total DNA was extracted at 4 or 20 hours post infection. For viral DNA replication, total DNA was extracted 48 hours post infection to measure the virus uptake. Cells were lysed in a solution containing 10 mM Tris, 10 mM EDTA, 0.2 % SDS (pH 7.6) with 50 μg/ml protease K (Sigma). DNA was extracted with phenol/chloroformed once and then with chloroform twice.

DNA was digested with EcoRI (BRL) a restriction enzyme that cuts the polyoma genome once, electrophoresed on a 0.7 % agarose gel and blotted onto nitrocellulose filter paper [13]. The filter paper was then UV-cross linked, prehybridized 4 hours at $65°C$ and hybridized to a ^{32}P-radiolabeled probe containing the entire polyoma DNA for 48 to 60 hours at $65°C$ in 1X Denhardt's, 2X SSC solution and salmon testis DNA (100 μg/ml). Hybridization probes were labeled to a specific activity of 1-2 x 10^9 cpm/μg ^{32}P-dCTP (3,000 μCi/mmole, NEN) using a multiprime DNA labeling kit (Amersham). The hybridized blot was washed twice in blot washing buffer (0.1 % SDS, 0.25 M sodium phosphate, 10 mM sodium pyrophosphate) containing 2 X SSC, at room temperature for 15 min and then at $65°C$ for 30 min, with a third wash of 1 X blot washing buffer containing 0.1 X SSC at $65°C$ for 30 min. The blot was then exposed to X-ray film (Kodak) for 1 to 7 days at $-70°C$ with an intensifying screen.

Preparation and analysis of RNA: Total RNA was extracted from infected FR3T3 cells 48 hours post infection by the method of Chirping et al. (1979) [4, 6]. Briefly, cells were lysed in guanidine thiocyanate (GTC) (4.0 M GTC, 25 mM sodium citrate, pH 7.0, 0.5 % sarkosyl, 0.1 M β-mercaptoethanol) and the lysates were placed on top of a cushion of cesium chloride (5.7 M CsCl in 0.1 M EDTA, pH 7.0). The centrifugation was carried out in a Beckman ultracentrifuge using a SW41Ti rotor at 35,000 rpm at $6°C$ for a minimum of 12 hours. The supernatant was carefully removed and the RNA pellet was then washed with cold 70 % ethanol and 10 mM NaCl, resuspended in water and precipitated with 10 mM Tris (pH 7.5), 1 mM EDTA, 200 mM NaCl, and 95 % ethanol. The recovery of RNA was quantified

by spectrophotometer reading.

For Northern blot, 20 μg total RNA was electrophoresed in a 1 % (wt/vol) agarose gel containing 2.2 M formaldehyde and transferred to nytran filter paper (Schleicher and Schuell) in a 10 X SSC solution (3 M NaCl, 0.3 M trisodium citrate.2H2O, pH7) [15]. Dot blot analysis was carried out similarly with with 5 μg of total RNA.

Probes containing the sequence of the entire polyoma DNA and the sequence of glyceraldehyde dehydrogenase (GapDH) gene were prepared by multiprime labeling with ^{32}P-dCTP. The GapDH gene was used as an internal control to show the amount of RNA loaded in each lane. Two single-stranded riboprobes were made from pG3PyH3 containing fragment 3 of a HpaII polyoma digest cloned between the T7 and SP6 promoters. To detect anti-late message, a riboprobe was made from the T7 promoter of pG3PyH3 with T7 polymerase, and to detect late message, a riboprobe was made from the SP6 promoter of pG3PyH3 with SP6 polymerase.

Filters were prehybridized 2 to 4 hours and hybridized overnight at 42°C in 0.05 M sodium phosphate buffer (pH 7), 1 M NaCl, 50 % (vol/vol) formamide, 1 % (wt/vol) sodium dodecyl sulfate (SDS), 5 % dextran sulfate, and 100 μg/ml of salmon sperm DNA. The fillers was then washed at room temperature for 5 min and then at 65°C for 30 min in 2 X SSC containing 0.1 % SDS and the third wash was in 0.1 X SSC containing 0.1 % SDS at 65°C for 30 min. The blot was then exposed to Kodak X-ray film for 2 to 8 days at $-70°C$ with an intensifying screen.

DNase treatment of RNA: 10 μg total RNA was resuspended in 400 μl TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA). 20 units of RNasin (BMB Indianapolis, IN), 10

mM $MgCl_2$, and 1 ul/mg/ml of RNase-free DNase were added, and the reaction was carried out at $37°C$ for 30 min [1]. At the end of the incubation, 20 mM EDTA and 1/10 volume of 3M sodium acetate (pH 5.2) were added to stop the reaction. The RNA was then extracted with phenol: chloroform: isoamylalcohol (50: 50: 1) once and then precipitated with ethanol twice.

Protein analysis: To analyze LT-Ag, infected FR3T3 cells were lysed 48 hours post infection with sample buffer (5 % SDS, 0.03 % bromophenol blue, 29 % glycerol, 5 % β-mercaptoethanol) and boiled at $100°C$ for 5 minutes. One quarter aliquot of the lysates was electrophoresed on a 10 % polyacrylamide gel at 50 volts for 12 hours [3], and electroblotted onto nitrocellulose filter paper at 100 volts for 2 to 3 hours with running water as cooling system. 2 % bovine serum albumin (BSA) was used to block the nonspecific binding sites, and rabbit anti small T-Antigen (ST-Ag) serum was used as the primary antibody, because both polyoma LT-Ag and ST-Ag share a homology of 79 amino acids at the amino-terminus. Goat anti-rabbit IgG coupled with alkaline phosphatase was used as the secondary antibody. For detection, both bromochloroindolyl phosphate and nitroblue tetrazolium were used as the developer for the color reaction.

2.3 Results

The cell cycle of FR3T3 cells: Fischer rat FR3T3 cells were brought to G0 by contact inhibition and serum starvation, and then released from G0 by trypsinization and feeding in high serum as described in Materials and Methods.

The timing of the cell cycle was determined by measuring DNA synthesis as described in Materials and Methods. As shown in Figure 2.1.A, low levels of tritium labeled thymidine (^{3}H–TdR) incorporation were observed between 0 to 9 hours post release from G0, indicating the borders of the first G1 phase. The S phase occurred between 9 and 23 hours post release from G0, as indicated by a peak of incorporation of ^{3}H–TdR at this period of time. Assaying cell division by hemacytometer counting, we found that the cell number doubled at 26 hours post G0 (Figure 2.1) indicating a short G2 phase. The uninfected cells double the cell number twice by the end of second cell cycle. In the infected cells, cell number doubled with a faster rate at the second cell cycle. The second cell cycle occurred between 26 hours and 39 hours post G0 with a much shorter G1 phase. These results also showed that FR3T3 cells were well synchronized by both contact inhibition and serum starvation, and that infection by polyomavirus did not disturb the cell cycle.

Transformation frequency during the cell cycle: The synchronized FR3T3 cells were infected with polyoma wild type strain A2 at various times post release from G0; focus formation was observed and found to reach a plateau between 10 to 14 days post infection. The number of foci was determined from duplicated plates and averaged. As shown in Figure 2.1.B, a peak of transformation frequency was observed in cells infected in early G1 phase shortly after released from G0, coinciding with the earliest possible time for cell attachment to form an intact monolayer, regardless of the multiplicity of infection used(1 or 10). A 20 to 50–fold difference was obtained between the minimal and the maximal transformation frequency. A peak of transformation

Figure 2.1: The phases of the cell cycle and determination of transformation frequencies during the cell cycle.

(A). DNA synthesis. Uninfected or infected cells released from G0 were allowed to incorporate ^{3}H-TdR (1 μCi/ml) during pulses of 1 hour. Cells were then precipitated with TCA and radioactivity was determined by scintillation counting, as described in Materials and Methods. (B). Cell number doubling. Cell numbers of both uninfected and infected cell were counted by hemacytometer counting. (C). Transformation. Cells synchronized as above were infected at the time post release from G0 shown 1 x10^{5} cells/35 mm plate with polyoma wild type A2 at an MOI of 10. Focus formation was followed for 10 to 14 days post infection.

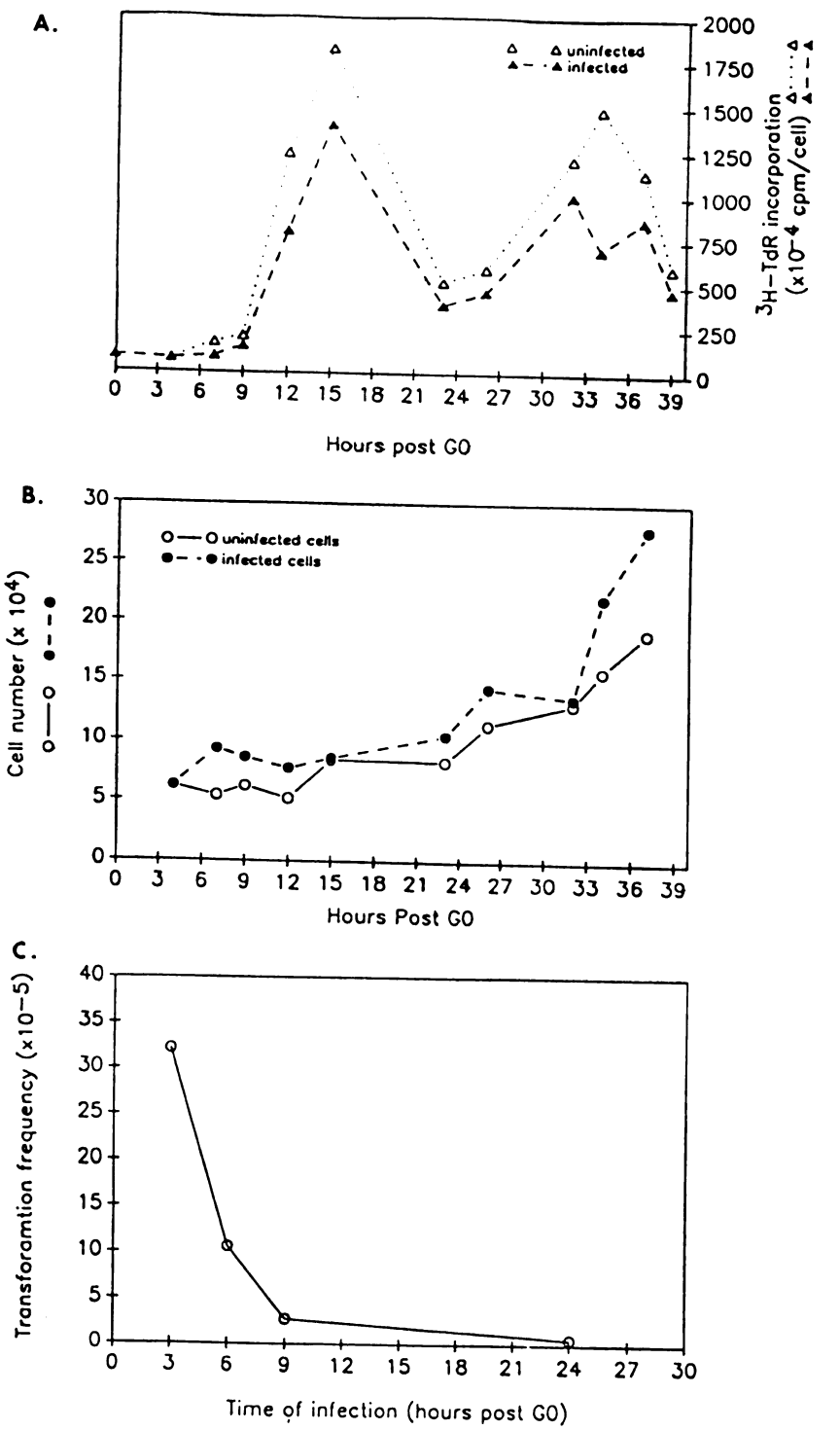

Figure 2.1: The phases of the cell cycle and determination of transformation frequencies during the cell cycle.

was also observed in cells infected in G1 phase for both infection of cells in suspension, and cells synchronized at low cell density by starvation only without the treatment of trypsin. Similar results were obtained in multiple experiments with small variations in the length of the cell cycle observed (from 24 hours to 30 hours). However, a peak of transformation at 3 hours post infection was always observed and followed a continuous decrease until 24 hours . Thus, in all subsequent experiments, time points chosen were 3, 6, 9 and 24 hours post G0. As follows below many parameters of infection were followed. In Figures 2.3, 2.5 and 2.6 which follow all parameters were assayed in the same experiments. The cell cycle dependence of each phenotype was tested in duplicated samples in at least 3 individual experiments.

Viral adsorption: To assay whether different viral uptake at different phases of the cell cycle accounts for the differences in transformation, total DNA was extracted from infected cells 20 hours post infection, prior to any de novo DNA synthesis. As shown in Figure 2.2.A, a peak in viral uptake was observed in cells infected early in the G1 phase, with a 2 to 10-fold ratio between cells infected at 3 hours and those infected at 24 hours post release from G0. We also analyzed nuclear uptake in the same experiment at 20 hours post infection. Total DNA was thus extracted from nuclei, as described in Materials and Method. As seen in Figure 2.2.B, a peak of virus signal in the nuclei was also observed in cells infected in early G1, with a 22-fold ratio between cells infected 3 hours and those infected as 24 hours post release from G0. The difference between adsorption and nuclear uptake suggests that there may be a cell cycle-dependent step between viral adsorption and entry into the nucleus.

This is also suggested by the difference between the adsorption differential and the transcription differential (see below). However, this difference relies on an accurate measure of the adsorption differential. This may be problematic for two reasons which would both lead to an underestimation: (1) only the 5.3 Kb signal was used for the quantification measurement ignoring other bands in the 3 hours sample, (2) the signal in the 24 hours sample may contain viral genomes which were associated to the cell membrane but would never enter into the cells. However, comparing the range of differences in adsorption and nuclear uptake transcription/transformation in every experiment suggests that adsorption alone truly does not account for the difference in the other parameters.

Viral gene expression: Viral gene expression was monitored in cells infected at different phases. For this purpose, FR3T3 cells were synchronized and infected at various times as described above. Total RNA was extracted 48 hours post infection, blotted and hybridized as described in Materials and Methods, and the results are shown in Figure 2.3. A 33–fold difference in viral RNA was observed in cells infected 3 hours compared to those infected 24 hours post release from G0. A probe containing glyceraldehydephosplate dehydrogenase (GapDH) sequence was used to hybridize to the same blot and shows that equal amounts of RNA were loaded in each lane.

The size of the transcripts was heterogeneous (Figure 2.3). Some of the RNA represents species of 4–5 times the unit length of polyoma genome. Most of the mature viral transcripts in infected rat cells would be expected to be early transcripts, whose sizes are 18S and 19S [8, 9]. The large size of RNA transcripts in the Northern blot

Figure 2.2: Analysis of virus uptake in intact cells as well as in nuclei.
(A). Cells were released from G0 and duplicated plates were infected at 3, 6, 9, and 24 hours post release from G0. Total DNA was extracted 20 hours post infection, digested with EcoRI, electrophoresed on a 0.7 % agarose gel. A ^{32}P-labeled probe containing the entire polyoma DNA was used for hybridization. (B). Cells were infected as described and nuclei were isolated from cells 20 hours post infection. Total DNA was extracted and analyzed as described above.

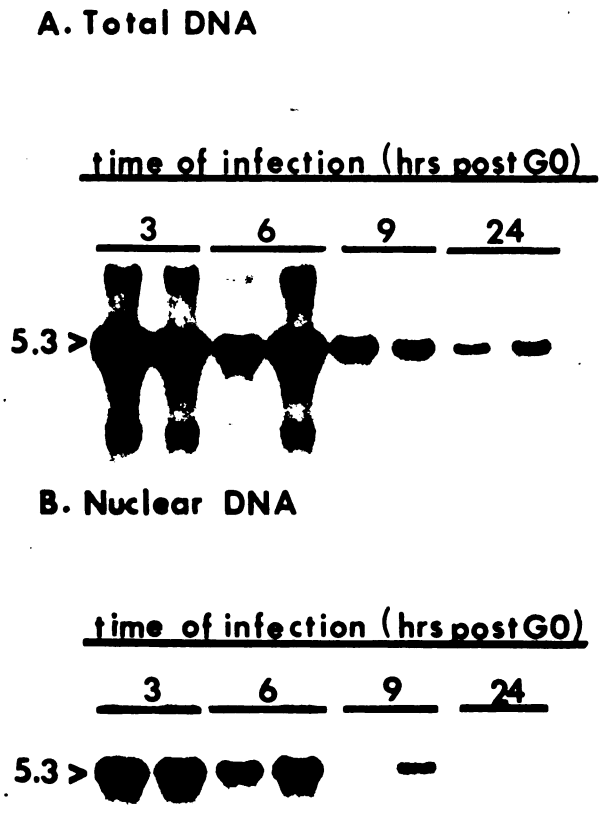

Figure 2.2: Analysis of virus uptake in intact cells as well as in nuclei.

Figure 2.3: Analysis of viral transcripts.

20 μg of total RNA, prepared from cells 48 hours post infection, was electrophoresed, blotted and hybridized with a ^{32}P-labeled probe containing either the entire polyoma DNA or the GapDH DNA, as described in Materials and methods.

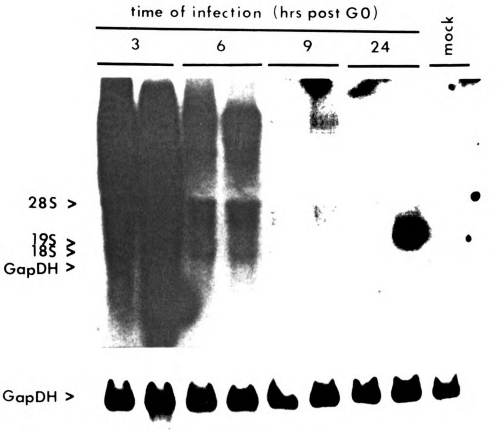

Figure 2.3: Analysis of viral transcripts.

suggests that the RNA was not degraded. The compact band GapDH 2.4 Kb message in each lane, further rules out the possibility of RNA degradation. The heterogeneity in the size of the polyoma signal was not caused by contamination with DNA, since treatment with DNase did not alter the RNA pattern while an added plasmid DNA control was completely digested (data not shown).

Another possible explanation for the high molecular weight RNA is that run through transcription of the polyoma early messages obviating the poly A addition signal, generates heterogeneous transcripts which are larger than the viral genomes. Run through transcription has been described in the case of late transcripts produced in polyoma-infected mouse cells [17]. Run through transcription of early message would require the production of anti-late message. To test this, hybridization of the same Northern blot to a single-strand riboprobe of appropriate polarity was carried out. The RNA signals detected by a riboprobe for anti-late message observed in cells infected at 9 and 24 hours post infection in figure 2.4 are somewhat strong; whereas, there was little or no RNA signal detected by a probe containing the entire polyoma DNA (Figure 2.3). The signal seen in cells infected at 9 and 24 hours in Figure 2.3 could be due to the nonspecific binding of the riboprobe, since it has been shown that riboprobe usually gives rise to high background. The high molecular weight RNA could be the precursor of rRNA. The unchanged hybridization pattern with the riboprobe (Figure 2.4) support this hypothesis. We also assayed for the presence of true late messages by hybridizing a Northern blot to the appropriate riboprobe. The results showed that little or no late message was present in the infected FR3T3 cells (data not shown). From the above results, we conclude that the heterogeneous size of

polyoma RNA transcripts represent early as well as anti-late messages derived from run-through transcription.

Viral early protein expression: As a further analysis of viral gene expression, the expression of viral proteins was analyzed 48 hours post infection by Western blotting analysis as described in Materials and Methods. For detection of large T-antigen, a polyclonal rabbit anti-polyoma small T-antigen which detects both large T-antigen and small T-antigen was used. As shown in Figure 2.5, the expression of large T-antigen followed the same time course as did RNA expression, i.e., a peak of large T-antigen expression was observed in cells infected early in G1 phase, with a 10-fold increase in cells infected 3 hours compared to 24 hours post release from G0.

Viral DNA replication: To determine whether viral DNA synthesis parallels viral gene expression, total DNA was extracted from cells 48 hours post infection. As shown in Figure 2.6, the maximal viral DNA signal was seen in cells infected in early G1 phase, with a 5-fold and a 2.6-fold increase in cells infected 3 hours compared to 9 hours and 24 hours post release from G0 respectively. There was an increase of DNA signals in cells infected 24 hours post release from G0, and this has been observed in 2 to 3 different experiments. As discussed below, it is less likely that either the viral DNA synthesis occurs prior to early message transcription, or viral DNA synthesis is mediated by cellular protein(s) which replaces the requirement of large T-antigen.

The kinetics of viral gene expression and viral DNA replication: The kinetics of both viral DNA and RNA syntheses in cells infected 3 hours post release

Figure 2.4: Detection of anti-late message.

RNA was isolated from cells 48 hours post infection as described in Materials and Methods, and hybridized to a ^{32}P-labeled strand-specific riboprobe specific for the detection of anti-late message.

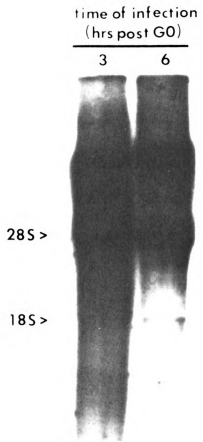

Figure 2.4: Detection of anti–late message.

Figure 2.5: Western blot analysis of large T antigen.

Cell lysates were harvested from infected cells 18 hours post infection. Aliquots of the cell lysates were analyzed in 10 % polyacrylamide gel, and electroblotted onto nitrocellulose filter paper. The filter paper was blocked with 2 % BSA, and reacted with the primary antibody: rabbit anti-small T antigen, and then reacted with the secondary antibody: goat anti-rabbit IgG.

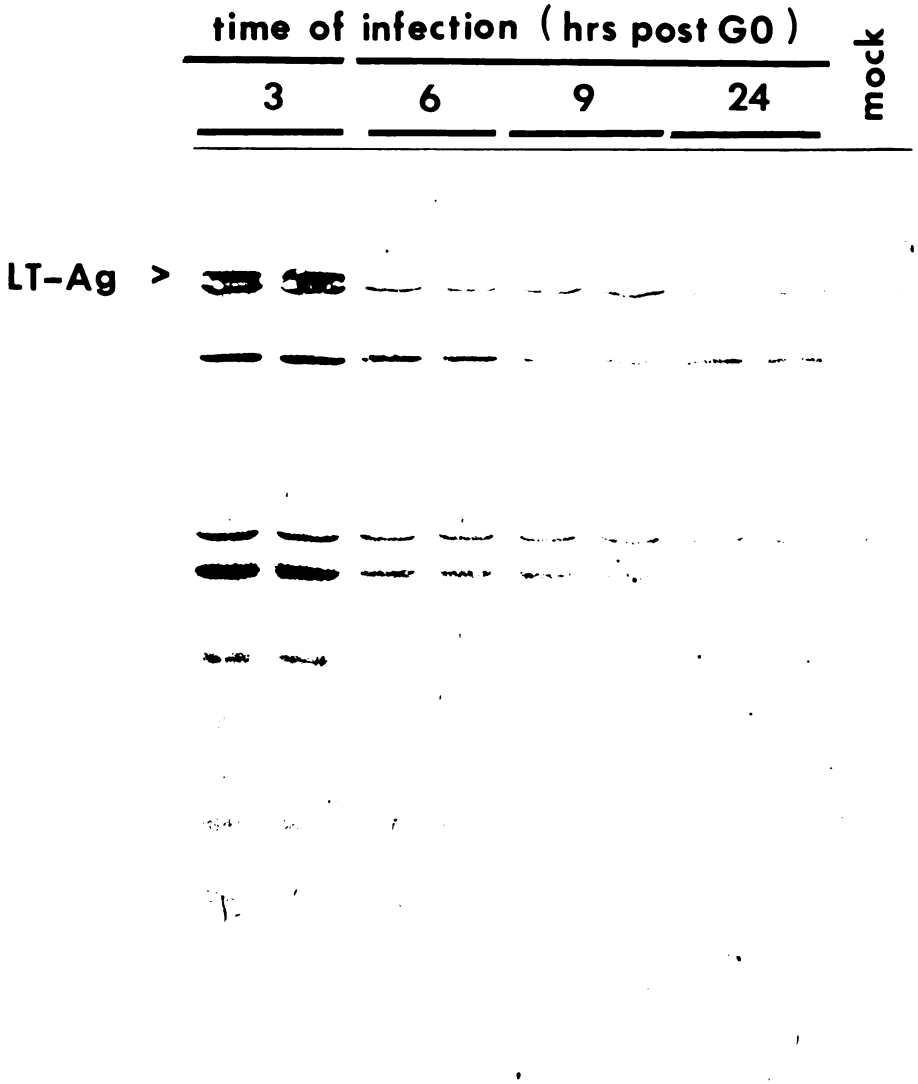

Figure 2.5: Western blot analysis of large T–antigen.

Figure 2.6: Analysis of viral DNA replication.

Cells were infected at various times, and total DNA was prepared from the infected cells 48 hours post infection. 10 μg of total DNA was digested with EcoRI and electrophoresed on a 0.7 % agarose gel, and probed to a ^{32}P- labeled probe containing the entire polyoma sequence.

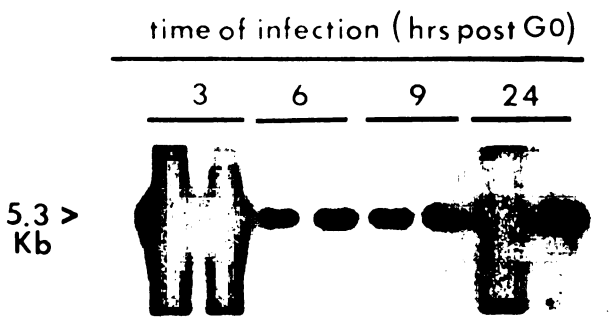

Figure 2.6: Analysis of viral DNA replication.

from G0 were studied. The timing of cell cycle in the same infection was determined by incorporation of ^{3}H-TdR and following cell multiplication. The first cell cycle was within 0 and 26 hours post release from G0, and the second cell cycle was within 27 and 40 hours post release from G0. Both total DNA and total RNA were extracted at 12, 24, 36, 48, and 60 hours post infection. As seen in Figure 2.7, both significant amount of viral transcripts and viral DNA were not observed until 36 hour post infection (Figure 3.7.A). These results showed that viral gene replication and gene expression occurred in the cell cycle subsequent to the time of infection, though virus entered the cells in early G1 of the first cell cycle. The RNA signal remained at a high level at least through 60 hours post infection.

A more detailed kinetic study of viral gene expression in cells infected 3 hours post release from G0, was carried in the intervals of every 3 hours. In the same experiment, incorporation of ^{3}H-TdR and cell number counting were carried out to determine the phases of cell cycle, and showed that cells had a cell cycle shortened to 23 hours. Results showed that viral gene expression was not detected until cells entered the G1 phase of next cell cycle (24 hours post infection), when a large synchronous burst of expression was observed (Figure 2.7.B).

Result of kinetics of viral DNA synthesis is shown in Figure 2.7.C. We found that the viral DNA synthesis occurred at 24 hours post infection, i.e., in the same cell cycle as cells were infected. Similar to what observed in Figure 2.6, cells infected at 24 hours had high level of viral DNA replication, but little or no viral DNA was synthesized in these cells (Figure 2.3). One explanation for this result is that the viral DNA synthesis can occur before the early gene transcription. However, this is least

Figure 2.7: Kinetics of viral gene expression and viral DNA replication in FR3T3 cells infected 3 hours post release from G0.

(A). Cells were infected 3 hours post release from G0. Total RNA was prepared from the infected cells 12, 24, 36, 48, and 60 hours post infection. 20 μg total RNA was analyzed in 1 % formaldehyde agarose gel, hybridized to a ^{32}P-labeled probe containing the entire polyoma DNA and GapDH, as described in Materials and Methods. (B). Cells were infected 3 hours post release from G0. Total RNA was prepared from the infected cells 12, 18, 21 and 27 hours post infection. 5 μg total RNA was analyzed on data blot, hybridized to ^{32}P-labeled probe containing the entire polyoma DNA. (C). In the same experiment, total DNA was prepared from cells 12, 24, 36, 48, and 60 hours post infection. 10 μg DNA was digested with EcoRI and analyzed as described in Materials and Methods. For hybridization, a ^{32}P-labeled probe containing the polyoma DNA was used.

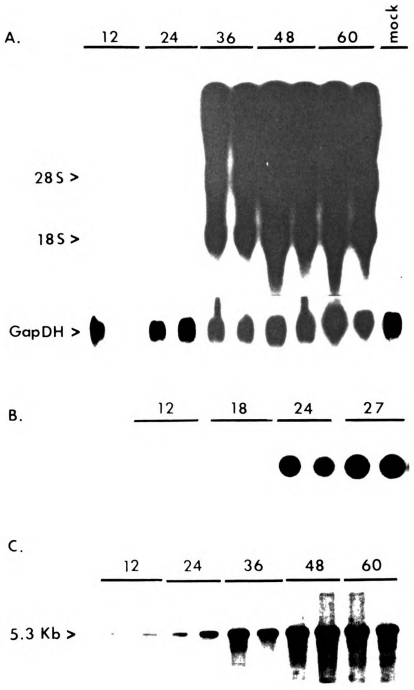

Figure 2.7: Kinetics of viral gene expression and viral DNA replication in FR3T3 cells infected 3 hours post release from G0.

likely, since it is known that large T-antigen is required for viral DNA synthesis. The other possible explanation is that cellular proteins can carry out the synthesis of viral DNA, and replace the function of large T-antigen. However, there is no evidence for the latter.

2.4 Discussion

In this paper, we present evidence for multiple levels of cell cycle control in the infectious cycle of polyomavirus in FR3T3 cells released from G0. A peak of transformation frequency was observed in cells infected early in G1 phase with a 20 to 50-fold differential between the highest and lowest transformation frequencies. To understand the factors which are responsible for the variations in transformation, we analyzed viral adsorption, viral gene expression, and viral DNA replication in cells infected at different phases, and found that (RNA, DNA, or proteins) track with transformation with maximal levels obtained in G1-infected cells.

Cell cycle regulation of transformation frequency induced by polyomavirus was first investigated by Basilico and Marin (1966) [2]. They found a 2-fold increase in neoplastic transformation for cells infected at the S/G2 boundary compared with cells infected in other phases. They suggested that the two fold increase was proportional to the number of cellular chromosomes, which doubled at the S/G2 boundary, so that there might be twice as many targets available for integration by the polyoma genomes. However, the difference in transformation frequency in our experiments was much higher (20 to 50-fold), and maximal susceptibility was observed in G1 infected

cells. Whether the results we obtained are specific for cells released from G0 or for FR3T3 cells is not clear yet.

Tamura (1983) and Matsuzaki(1989) have also studied transformation of Fisher rat 3Y1 cells by SV40 under various growth conditions [10, 14]. They showed that the frequency of transformation by SV40 and polyomavirus was reduced when cells were in the proliferating state at the time of virus inoculation as compared to cells in the quiescent state.

The 2 to 10–fold differential in viral adsorption (assayed as the total viral DNA entering into host cells) between cells infected at 3 hours compared to those infected at 24 hours post release from G0 suggests that the appearance of the polyoma receptors on the cell surface is cell cycle regulated and maximal on G1. The effect of cell cycle on SV40 viral DNA replication in nonpermissive cells was analyzed by Pages et al. (1973). They showed that the CV1 cells infected with SV40 during G1 have viral DNA replication occurring within the same mitotic cycle. But if the time of infection passed the end of G1, then these cells were unable to initiate SV40 DNA replication until the next mitotic cycle [11]. They suggested that virus had to pass through a critical stage situated near the end of G1 or the beginning of S phase in order for viral DNA replication to occur. Differential rates of viral adsorption has been described for SV40 by Pages et al. (1973) [11] as measured more directly by binding of ^{3}H-TdR–labeled virus particles to CV1 cells. Their results showed a 2 to 3–fold increase in G1 compared to G2 or M phase infection. However, Basak and Compans (1991) found that the expression of the SV40 receptor in Vero cells is increased about 3–fold in S, and G2/M phase compared to G1 phase (personal communication).

The differences in adsorption (2–10 fold) between various phases of the cycle only partially account for the differences in viral gene expression (30–40 fold) and/or in transformation (20–50 fold) in every experiment. This and other observations discussed below suggest the existence of another rate limiting step in the infection cycle between adsorption and gene expression which is also regulated during the cell cycle. This step (also maximal in differential rates for nuclear uptake were 3.7–fold larger than those for cellular uptake in the same experiment.

The kinetics of viral gene expression showed that the polyoma early promoter is very strongly cell cycle regulated in FR3T3 cells released from G0. Interestingly, viral gene expression was only detected in the cell cycle following that in which infection had occurred. Thus, it appears that the majority of viral genomes which entered cells at 3 hours into the first G1 phase of the cell cycle did not get processed in time to catch the transcription competence step and waited a full cycle to become expressed. Furthermore, if any genomes did enter cells in S, or G2 as is suggested by the adsorption results, these genomes did not become transcription competent in the next G1 phase, since very little gene expression was detected in cells infected in S or G2. As proposed above, there may be another cell cycle regulated rate limiting step in viral infection between adsorption and viral gene expression.

In conclusion, our results showed that the maximal transformation frequency induced by polyomavirus occurs in cells at G1 phase soon after release from G0. There are at least three cell cycle regulated steps in the polyoma infectious cycle: adsorption, transcription, and undefined event(s) including the processing of the polyoma genome.

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Chapter 3

The Role of Polyomavirus Large T-antigen in Interviral Recombination

Previously our laboratory has demonstrated the existence of high levels of interviral homologous recombination among the integrated polyoma viral genomes in the transformed cells. To assess whether large T-antigen plays a role in the interviral recombination, two double mutants were constructed to have both a temperature sensitive mutation of large T-antigen and mutations in restriction endonuclease sites which were as nonselective recombination markers. Coinfections of Fischer rat (FR3T3) cells with the two double mutants were carried out at both permissive $33^{o}C$ and nonpermissive ($39^{o}C$) temperatures. Viral DNA synthesis was undetectable in cells kept at $39^{o}C$. Foci of transformed cells were selected and integrated viral genomes were analyzed for the occurrence of recombination events. Homologous recombination was detected in 33 % of cells kept at the nonpermissive temperature. These results do not support the hypothesis of a role for large T-antigen in interviral recombination. If however, large T-antigen does play a role, the dosage or the domains required for recombination must be different from that required for viral DNA synthesis and other activities of T antigen must be involved.

3.1 Introduction

The head–to–tail pattern of integration of polyomavirus and SV40 into the genome of nonpermissive cells has raised the issue of the mechanism by which these are produced. In earlier studies with SV40 by Chia and Rigby (1981), it was suggested that viral DNA replication by a rolling circle mechanism generates the precursors for the integrated genomes, even though they also detected some which were formed by recombination [8].

Hacker and Fluck (1989) formed direct evidence showed that high levels of homologous recombination was found coincidental with the process of viral integration and transformation of polyomavirus [17]. They showed that in Fischer rat (F111) cells coinfected with MOP1033, a transformation–defective virus, and ts3, a temperature sensitive mutant defective in decapsidation, an elevated level of interviral recombination was observed after cells were allowed to decapsidate at $33^{o}C$ for 24 hours. This result indicates that interviral recombination plays an important role in the processes of viral integration and transformation, although it could not resolve whether the tandem structure are generated by recombination events. Interestingly, recombination was observed only among those genomes which are integrated in the genome of transformed cells, while no recombination was detected among the non–integrated viral genomes present in the population of infected cells from which the transformed cells were infected.

Results of recent experiments in our laboratory suggest that a further amplification of the viral genomes can occur by post–integration events (Syu and Fluck, unpublished

data). In an attempt to further our understanding of the integration process, we have started to address the issue of a potential role for large T-antigen in the process of interviral homologous recombination. The rationale for this experiment is that a tsa mutant, which has a thermolabile large T-antigen, has decreased transformation at $39°C$. The transformation observed at $39°C$ is either due to the leakiness of the tsa mutant or a large T-antigen-independent event.

The role of large T-antigen in viral integration has been shown by Stoker (1969). He showed that cells infected with a tsa mutant have the same level of abortive transformation at nonpermissive temperature as that of cells infected with wild type, while the stable transformation induced by tsa was reduced about 20-fold [21, 22]. These results indicate that large T-antigen is required for stable transformation. The inadequecy of stable perpetuation of the viral genome into host cell DNA causes the reduction of stable transformation induced by tsa. Della Valle et al. (1981) showed that in the absence of large T-antigen, polyoma integrated into the host chromosomes with a pattern of non-tandem repeat and with incomplete viral genomes integrated [10].

It has been shown that large T-antigen is involved in the process of viral excision in transformed cells [1, 2, 9, 19]. When duplicated regions of polyoma genomes are present after in situ replication by the "onion skin" model, homologous regions can recombine, producing complete viral molecules that can be removed and circularized as free DNA [3, 4, 5, 14, 15, 18].

Furthermore, a more direct examination of the role for large T-antigen in recombination has been addressed in a different system by Stonge et al. (1990). They

showed that the recombination between two defective copies of middle T-antigen located side-by-side in FR3T3 cells was promoted from 10^{-7} to 10^{-2} per cell generation when large T-antigen was present [23]. The promotion of recombination was also observed by SV40 large T-antigen which cannot initiate viral DNA replication of polyomavirus, suggesting that the role of large T-antigen in recombination is DNA replication-independent [23].

In the present experiments, we attempt to determine whether large T-antigen plays a role in interviral recombination which is important in the process of viral integration and transformation. To do this, we asked whether temperature sensitive mutants with a defect in large T-antigen can affect the high levels of recombination associated with the integration of the viral genome in FR3T3 cells. Our results do not support a role for large T-antigen since we observed high levels of recombination even with a temperature labile large T-antigen. However, the experiments cannot exclude the possibility of a role for large T-antigen in interviral recombination, for example, if either the dosage or the domain required for recombination is different from that for viral DNA replication.

3.2 Materials and Methods

Virus: The polyoma mutants tsa, MOP 1033, and ts3 were used to construct double mutants. The tsa mutant [13] bears a point mutation at nucleotide 2193, located within the carboxy-terminal coding sequence of large T-antigen. MOP 1033 was derived from a wild-type strain by site directed mutagenesis eliminating the AvaI

sites of nucleotides 659 and 1018 [25]. The mutation at nucleotide 659 does not have a phenotype, and the mutation at nucleotide 1018 introduced alterations into the middle T-antigen reading frame producing a transformation-defective virus. The ts3 mutant [11] harbors a a mutation within the VP2 protein which prevents decapsidation at the nonpermissive temperature. Since ts3 lacks the BamHI site (nucleotide 4634) also located within the VP2 coding region, it is assumed that the BamHI site is the site of the decapsidation mutation, although this has not been proven. ts3 can transform nonpermissive cells normally after a short decapsidation period at 33^oC. Wild type A2 virus was used in the experiments as positive controls.

Double mutants carrying either MOP 1033 or ts3 mutations with the tsa mutation were constructed by ligating the appropriate HindIII fragments from the appropriate parental strains. The phenotype of the double mutants were verified. MOP 1033 tsa (Ma) lacking the AvaI sites grows only at 33^oC in permissive cells and does not transform nonpermissive rat FR3T3 cells at any temperature. ts3 tsa (3a) lacks the BamHI site, grows at 33^oC. Similarly to tsa, 3a shows a decrease in transformation at 39^oC, even after incubation at 33^oC for 24 hours to bypass the decapsidation defect.

Infection: Fischer rat (FR3T3) [16] cells were cultured and maintained in Dulbecco's Modified Eagle medium (DMEM, Gibco) supplemented with 10 % calf serum (Hazleton). To maximize transformation, cells were grown to confluency and starved with 0.2 % gamma globulin (GG) free serum [7]. Cells were then released from G0 by trypsinization and addition of 10 % calf serum (Gibco). Coinfection of cells with Ma and 3a at a multiplicity of infection (MOI) 50 and 50 was carried out 3 hours post

release from G0, the earliest time for cell reattachment. Infections with the single parents or wild-type A2 virus served as controls. To allow decapsidation of ts3 and 3a to occur, infected cells were kept at $33°C$ for 24 hrs after infection and then half of the cultures were shifted to $39°C$. Foci of transformed cells were obtained two to three weeks post infection, and the clonal transformed cell lines were grown in DMEM supplemented with 10 % calf serum at the same temperature at which the foci were derived (at either $33°C$ or $39°C$).

Preparation and analysis of DNA: Total DNA was extracted from infected or transformed clonal cell lines by lysing the cells in a solution of 0.2 % SDS, 10 mM Tris, pH 7.5, 10 mM EDTA, and 50 μg/ml proteinase K (Sigma) [12]. For analysis of the occurrence of recombination, 10 μg DNA was digested with endonuclease restriction enzymes (AvaI+BamHI+BglII, PvuII+AvaI+BglII, HindIII+AvaI+BamHI, EcoRI, BclI, BglI, HaeII, EcoRV, and MspI) (Figure 3.1), electrophoresed on agarose gels and transferred onto Hybond-N filter paper [20]. Prehybridization was carried out in a buffer containing 5 X SSPE, 5 X Denhardt's solution, 0.5 % (w/v) SDS and 50 μg/ml salmon testis DNA for one hour at $65°C$. Hybridization was carried out in the same buffer at $65°C$ for 48 to 60 hours. For hybridization, 1 x 10^6 cpm/ml of ^{32}P-labelled probes containing the entire polyoma DNA, or the fragments 4 or 5 of MspI-digested polyoma DNA were used for different hybridizations as mentioned in each figure legends. Probes were labeled to a specific activity of 1-2 x 10^9 cpm/μg template DNA by using a multiprime DNA labeling kit (Amersham).

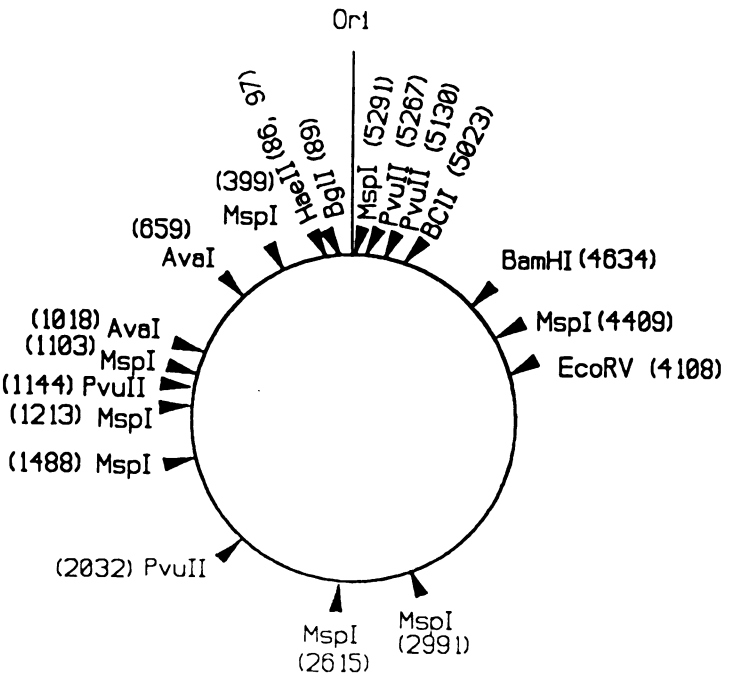

Figure 3.1: Restriction endonuclease map of polyomavirus.

Origin is located at the nucleotide 0/5292. BglI, EcoRI, EcoRV , BamHI, BclI cleave polyoma once, HaeII and AvaI cleave polyoma genome twice, PvuII cleaves polyoma genome four times, and MspI cleaves 8 times.

3.3 Results

Analysis of recombination in the cross between Ma and 3a at 39°C: To assess whether large T-antigen plays a role in the interviral recombination associated with transformation, we constructed double mutants with the tsa temperature sensitive mutation in large T-antigen and the mutations in restriction endonuclease markers of MOP 1033 and ts3 used to follow recombination [17]. The properties of the double mutants are described in Materials and Methods, and summarized in Table 3.1. There was no selection pressure for recombination in the cross between Ma and 3a since 3a can transform normally after decapsidation at $33°C$ for 21 hours. FR3T3 cells were coinfected with Ma and 3a as described in Material and Methods. Infected cells were kept at $33°C$ for 24 hours , and then half of the cultures were shifted to $39°C$. Total DNA was extracted at 4 hours post infection (hpi), at 48 and 60 hpi ($39°C$) and 60 and 90 hpi ($33°C$). Focus formation assay was used to assay transformation. A total of 49 foci of transformed cell lines were collected from 3 independent experiments carried out at $39°C$. Clonal cell lines were amplified at $39°C$and total DNA was isolated.

Viral DNA synthesis was examined to confirm that large T-antigen could not be detected at nonpermissive temperature. Total DNA was extracted 4 hours post infection (hpi) to measure the input of virus, 48 and 60 hpi at $39°C$, and 60 and 90 hpi at $33°C$. Digestion with a combination of AvaI and BamHI reveals the expected bands of a 5.3 kb fragment for Ma and a 5.0 kb fragment for 3a. In these signals as shown in Figure 3.2, there was little or no increase in viral genome copy number over the

Table 3.1: Phenotypes of tsa mutant and double mutants, Ma and 3a.

	Virus Strains		
	tsa	Ma[a]	3a[b]
replication			
33^oC	+	+	+
39^oC[d]	-	-	-
transformation			
33^oC	+	-	+
39^oC[d]	+[c] (reduced)	-	+ (reduced)
Restriction	AvaI$^+$	AvaI$^-$	AvaI$^+$
endonuclease pattern	BamHI$^+$	BamHI$^+$	BamHI$^-$

a. Ma is constructed from both MOP1033 and tsa.

b. 3a is constructed from both ts3 and tsa.

c. Transformation induced by tsa mutant observed at nonpermissive temperature is about 20–fold decreased compared to that observed at permissive temperature.

d. The first 24 hours was at 33^oC.

input signal (extraction at 4 hpi) in cells kept at 39^oC, whereas, a significant amount of synthesis was observed for cells kept at 33^oC. The viral DNAs detected were all parental viral DNA, and no recombination was evident. These results suggest that the expression of the large T-antigen defect causes the absence of DNA replication function at high temperature.

The results for the number of transformation were shown in Table 3.2. To analyze recombination, total DNA from transformants was digested with a combination of AvaI, BamHI and BglII (Figure 3.1). A 1.3 Kb fragment will be generated if recombination has occurred between the small AvaI-BamIII interval; a 1.7 Kb fragment will be generated if the recombination occurred between the two AvaI sites; and a 3.6 Kb fragment will be generated if the recombination occurred between the large AvaI-BamHI interval (Figure 3.3).

Total 13 out of 49 foci cell lines in 3 experiments showed acquisition of recombinant viral genomes. In experiment 1, recombinant viral genomes were detected in seven out of twenty-one transformed cell lines picked at 39^oC, and were analyzed in more detail. In Figure 3.3, three cell lines (# 1, 3, and 5) had only the 1.7 Kb fragment; one cell line (# 4) had both 3.6 Kb and 1.3 Kb fragments; and two cell lines (# 6 and 7) had both 3.6 Kb and 1.7 Kb fragments. One cell line transformed with wild type A2 was used as control. Cell line # 2 is not a recombinant, because no recombinant viral genome was detected. Cell line # 8 had only one 3.6 Kb fragment (data not shown).. Thus, in 4 out of 7 transformed cell lines containing recombinant viral genomes (#1, 3, 5, 8) a single recombination event had occurred; whereas viral genomes in 3 cell lines (# 4, 6, 7) had undergone 2 recombination events. Among

Figure 3.2: Lack of DNA synthesis in the double mutants, Ma and 3a, at nonpermissive temperature.

Total DNA was extracted from FR3T3 cells coinfected with Ma and 3a 48 hrs and 60 hrs post infection at either 33°C or 39°C. Infected cells were kept at 33°C for 24 hrs before half of the cultures were shifted to 39°C. DNA was isolated, digested with a combination of AvaI and BamHI, and analyzed as described in Materials and Methods. Arrows show the corresponding sizes of a 5.3 Kb fragment derived from Ma, and a 5.0 Kb fragment derived from 3a.

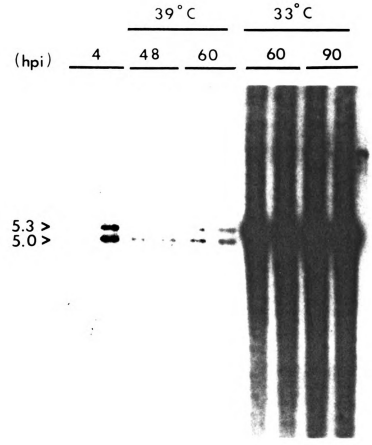

Figure 3.2: Lack of DNA synthesis in the double mutants, Ma and 3a, at nonpermissive temperature.

Table 3.2: Results of transformation and recombination from 3 independent experiments.

			temperature	
			33^oC	39^oC[g]
Experiment 1	Transformation	A2	50[a]	100[a]
		Ma	0[a]	0[a]
		3a	50[a]	94.5[a]
		Ma x 3a	17.8[b]	18.5[c]
	Recombination	Ma x 3a	7/16	7/21
Experiment 2[d]	Recombination	Ma x 3a	ND[e]	4/12
Experiment 3	Transformation	A2	50[a]	13[a]
		Ma	0[a]	0[a]
		3a	40[a]	5[a]
		Ma x 3a	20[f]	5[f]
	Recombination	Ma x 3a	ND[e]	2/16

a. Foci number averaged in 2 parallel infections.

b. Foci number averaged in 9 parallel infections.

c. Foci number averaged in 10 parallel infections.

d. The same experiment as described in Figure 3.2

e. Undetected.

f. Foci number averaged in 5 parallel infections.

g. The first 24 hours was at 33^oC.

Figure 3.3: Test for interviral recombination.

Total DNA of 8 transformed cell lines derived at $39^{\circ}C$ was digested with a combination of AvaI, BamHI, and BglII, and analyzed as described in Materials and Methods. Transformant isolated from type A2 virus infection is shown as control. Cell lines number 1, 3–7 contain recombinant viral genomes. Arrows show the sizes of 3.6 Kb, 1.7 Kb and 1.3 Kb fragments.

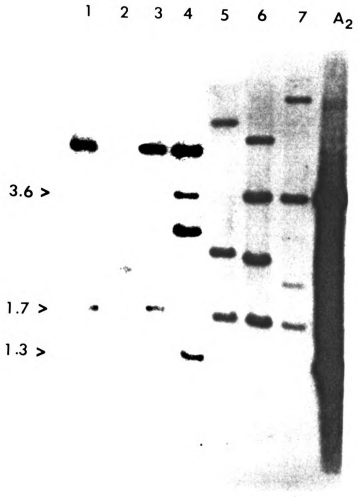

Figure 3.3: Test for interviral recombination.

these seven recombinant transformants, except # 3, complete viral genomes were detected as determined by digestion with MspI which cuts the polyoma genome eight times (data not shown). In cell line # 3, MspI fragment 3 is missing.

Among the 21 foci cell lines analyzed, 13 out of 21 had a tandem repeat of viral genome integrated, whereas 8 out of 21 had nontandem of viral genome integrated. The ratio of recombination occurred in the transformed cells with a tandem repeat of the viral genome compared to nontandem of the integrated viral genome was 4 : 1 (6/13 : 1/8).

In the six cell lines which have the complete viral genomes, the extent of of the viral repeats was further determined . Four restriction endonucleases which cut the polyoma DNA once or twice were used: BglI (cleavage site: nucleotide 87), EcoRI (nucleotide 1560), EcoRV (nucleotide 4108), and BclI (nucleotide 5023), and HaeII (nucleotides 87 and 97). Results showed that all the six cell lines possess partial head-to-tail tandem repeat (data not shown). In 5 cell lines the repeated region did not include the EcoRI site in the middle of early region, but the repeats extended through the BclI, BglI, and HaeII sites in the origin-enhancer region.

The number of viral integration sites was determined by digestion with BglII, which does not cut the polyoma genome. The results showed that 9 out of 21 cell lines have a single integration site, 6 out of 21 cell lines have 2 to 3 integration sites, 6 out of 21 have a single integration site with a ladder-like pattern of bands on top of a basal band. Examples of 6 cell lines are shown in Figure 3.4.

Figure 3.4: Integration pattern of polyoma genome in the transformed cell lines kept at $39^{o}C$.

Total DNA of was extracted from transformation cells, and digested with BglI ,electrophoresed in a 0.4 % agarose gel, and analyzed as described in Materials and Methods. Cell lines # 5, 6, and 7 are recombinants and labeled as same as shown in Figure 3.3, and cell line # 9, 10, 11 were foci picked in the same experiment but did not contain recombinant viral genome.

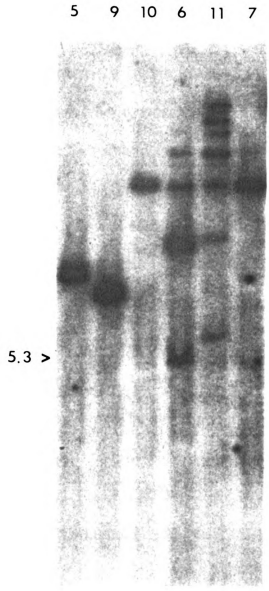

Figure 3.4: Integration pattern of polyoma genome in the transformed cell lines kept at $39^{o}C$.

Proof that the 1.3, 1.7 and 3.6 Kb fragments are recombinant: To confirm that the 1.3 Kb and 1.7 Kb fragments observed in Figure 3.2 were true recombinant fragments, DNA of cell lines with these fragment was digested with a combination of AvaI, BamHI and PvuII (Figure 3.1), and probed with fragment 5 of MspI-digested polyoma DNA. In this analysis, a 1.0 Kb fragment will be generated from a 1.7 Kb recombinant fragment; a 0.7 Kb fragment from a 1.3 Kb fragment (either from a recombinant or from the 3a parental type), and a 1.1 Kb fragment from the Ma parental genome(Figure 3.5.A). The results showed that cell lines # 1, 3, and 5 have a 1.0 fragment; cell lines # 1 and 4 has a 0.7 Kb fragment, and cell lines # 6 and 7 have both 1.0 and 1.1 Kb fragments (Figure 3.5.B). These results support the conclusion that the 1.3 Kb and 1.7 Kb fragments in the recombinant cell lines are the recombinant products.

To confirm the identity of the 3.6 Kb recombinant fragment, cell lines with the 3.6 Kb recombinant fragment were further analyzed by digestion with a combination of HindIII, AvaI, and BamHI, and hybridized to a probe containing the EcoRI-XbaI fragment of polyoma DNA (Figure 3.1). The expected 2.2 kb fragment was generated in all the cell line with a 3.6 kb fragment (data not shown).

A defective large T-antigen present in the transformants containing recombinant viral genomes: To further confirm the lack of viral DNA synthesis in cells with recombinant viral genomes, three cell lines (# 3, 6, and 7) were chosen and grown in parallel $33°C$ or $39°C$. Cell lines # 6 and 7 contain a complete integrated viral genome, whereas cell line 3 does not. Total DNA was extracted after 5 days

Figure 3.5: Confirmation of the identity of the 1.7 and 1.3 Kb recombinant fragments. (A). A 1.1 Kb, a 1.0 Kb, or a 0.7 Kb fragment will be generated after digestion with a combination of PvuII, AvaI, and BamHI, and hybridized to a probe containing the fragment 4 of MspI digested polyoma genome. (B). 10 μg DNA was digested with a combination of PvuII, AvaI, and BamHI, and analyzed as described in Materials and Methods.

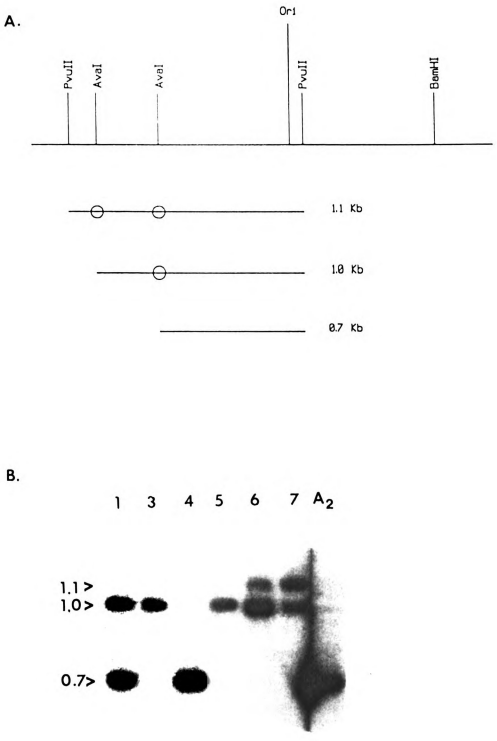

Figure 3.5: Confirmation of the identity of the 1.7 and 1.3 Kb recombinant fragments.

incubation at 39°C, or 10 days at 33°C, and analyzed by digestion with a combination of AvaI and BamHI. The results showed that a 17 to 27-fold increase of replication at 33°C in cell lines # 6 and 7 (Figure 3.6). In cell line # 3, no increase of viral signal was found at 33°C compared to at 39°C due to its incomplete viral genome. This result indicates that the transformed cells with recombinant viral genomes derived at 39°C are not able to amplify viral DNA at 39°C, (as expected for a tsa mutant), and the ability of DNA replication of large T-antigen can be restored by shifting cells to 33°C.

Analysis of the recombinants derived at 33°C: Sixteen foci were picked and amplified from cells coinfected with both Ma and 3a at 33°C. From the digestion with AvaI, BamHI and BglII restriction enzymes, seven out of sixteen cell lines contained recombinant fragments of 3.6 Kb, 1.3 Kb or 1.7 Kb. Among the cell lines grown at 33°C, ten cell lines showed the presence of high levels of free viral DNA (Figure 3.7). The integration site of the polyoma genome was determined by digestion with BglII. Results showed that 9 cell lines had single integration site and 6 cell lines had 2-3 integration sites (data not shown).

3.4 Discussion

We have investigated the effect of large T-antigen on viral DNA synthesis, viral integration, transformation and interviral recombination. Results of these assays in the transformed cells derived from coinfection with Ma and 3a are summarized in Table 3.3.

Figure 3.6: Lack of viral DNA synthesis in transformed cells containing the recombinant viral genomes at $39^{o}C$.

Total DNA was extracted from 4 cell lines kept at either $39^{o}C$ or after shifted to $33^{o}C$ for 10 days, and digested with a combination of AvaI and BamHI and analyzed as described in Materials and Methods. Cell lines # 3, 7, and 6 were the same cell lines as in Figure 3.3, and a cell line which was transformed by A2 was used as a control. Arrows show the sizes of the 3.6 Kb, 1.7 Kb ,and 1.3 Kb fragments.

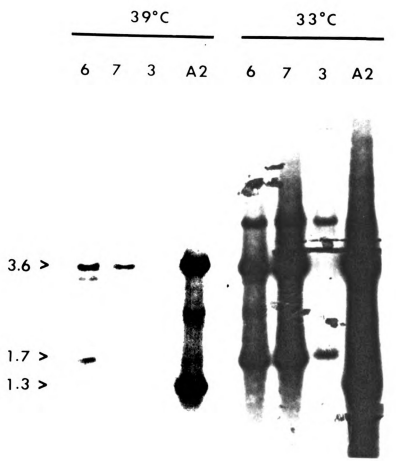

Figure 3.6: Lack of viral DNA synthesis in transformed cells containing the recombinant viral genomes at $39^{o}C$.

Figure 3.7: Analysis of the occurrence of recombination in the transformed cells derived in the cross between Ma and 3a at 33°C.

10 μg of DNA was digested with a combination of AvaI, BamHI, and BglII, and analyzed as described in Materials and Methods.

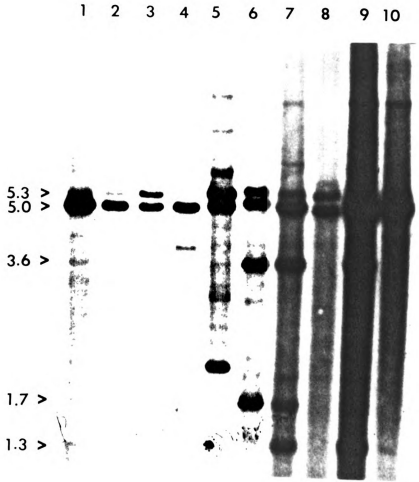

Figure 3.7: Analysis of the occurrence of recombination in the transformed cells derived in the cross between Ma and 3a at 33°C.

Table 3.3: Comparison of transformants derived in the cross between Ma and 3a at either 33°C or 39°C[a]

		temperature	
		33°C	39°C[f]
Viral DNA synthesis in transformed cells		17 X 27 X	1 X
Integration pattern[b]	complete genome	ND	13/21
	incomplete genome	ND	8/21
Viral sequence flanking to celluar DNA in cell lines with incomplete viral genome	MspI 3	ND	7/8
	MspI 1	ND	1/8
# of integration site[c]	single site	8/10	9/21
	2-3 sites	2/10	6/21
	single site w/ a ladder	0/10	6/21
Free viral DNA of both parental strains	Yes	10/16	0/21
	No	6/10	21/21
Transformation		17.8[d]	18.5[e]
Incidence of Recombination		7/16	7/21
# of events/cell	1	1/7	3/7
	2	2/7	4/7
	3	1/7	0/7
# of events occurred in viral genome	tandem	ND	6/13
	nontandem	ND	1/8
Ratio of tandem/nontandem			3.7

a. Data obtained from cells coinfected with Ma and 3a in Experiment 1.
b. Results obtained from MspI-digestion.
c. Results obtained from BglII-digestion.
d. Number averaged from 9 parallel infections.
e. Number averaged from 10 parallel infections.
f. The first 24 hours was at 33°C.

For viral DNA synthesis, little or no viral DNA synthesis in the infected cells was kept at 39°C. However, it is possible if the viral DNA synthesis occurs only in 5 % of the population of infected cells, the increase of viral signal might be underdetectable. The lack of viral DNA synthesis was also observed in transformed cells kept at 39°C. The ability of viral DNA synthesis can be restored by shifting transformed cells to permissive temperature. For example, a 17 to 27 fold increase of viral DNA synthesis was observed in cell line # 6 after shifting from 39°C to 33°C. Integration of a complete viral genome is required for the viral DNA synthesis to occur. In cell line # 3, no increase of viral DNA synthesis was observed after shifted to 33°C owing to its possession of incomplete viral genome.

The integration pattern of polyoma genome was analyzed in transformants derived from both 33°C and 39°C. The viral integration pattern observed at the nonpermissive temperature is the original integration event without further viral amplification and rearrangement. Thus, the integration pattern by tsa observed at nonpermissive temperature is the original integration event. 8 out of 21 of the viral genome transformed cell lines picked at 39°C were found with nontandem repeat integrated; whereas the other 13 cell lines had tandem repeat of polyoma genome integrated. Intriguingly, among the 8 cell lines with nontandem repeat of the viral genome , we found 7 cell lines had lost the fragment 3 of MspI -digested viral DNA, and only 1 cell line lost the fragment 1 of MspI-digested viral DNA. This result indicated that the junction of viral and host DNA resides in the MspI-fragment 3 in these 8 cell lines, suggesting that there might be a hot spot in MspI-fragment 3 for nonhomologous recombination between viral genomes and host chromosomes.

The number of integration sites was analyzed, and results showed that 9 out of 21 cell lines had a single integration site, 6 had 2–3 integration sites, and 6 cell lines had a single site integration with a ladder–like pattern of bands with unit length of 5 Kb distance observed on top of a basal band. Syu and Fluck (1991) showed that the ladder–like pattern was generated via a post–integration event which required functional large T–antigen such as in situ amplification of viral DNA [24].

As we discussed above, the large T–antigen of tsa was impaired in viral DNA synthesis of newly introduced viral genomes in infected cells as well as of integrated viral DNA in transformed cell. However, the presence of ladder–like pattern of bands suggested that the amplification of viral genome occurred and there were some levels of large T–antigen present in these cells. Thus, the leakiness of tsa mutant can facilitate the post–integration amplification of viral genome, although there is a severe defect in viral DNA synthesis with newly introduced viral genomes.

Transformation induced by tsa at nonpermissive temperature has been shown to be 20–fold decreased compared to that at permissive temperature. In our experiments, variations in transformation were observed. For example, in experiment 1, no difference of transformation frequency was observed between cells coinfected with Ma and 3a at $33°C$ and $39°C$. However, in the other experiments, the temperature effect in transformation frequency was observed. The susceptibility of transformation varied from experiment to experiment. The high susceptibility of transformation in this experiment could be due to the usage of high MOI of Ma and 3a (50 and 50). It has been known that tsa mutant is leaky in transformation when high MOI of tsa mutant is used [22].

The assessment of recombination occurred between Ma and 3a showed that there was no difference of recombination frequency either at permissive or nonpermissive temperature. The crossover events were determined in the 7 transformed cell lines containing recombinant viral genomes; 4 cell lines had a single crossover event as evidenced by the fact that they have only one recombinant viral fragment; whereas the other 3 have two crossover events with two different recombinant viral fragments. Interestingly, we found that among these 7 cell lines, 6 have tandem repeat of the polyoma genome integrated, and only 1 has nontandem viral genome integrated.

As far as we know, there was no selection pressure for recombination in the cross between Ma and 3a. The wild type polyoma sequence was the recombinant products; whereas no reciprocal recombination product was detected. The reciprocal recombination product carrying both mutations of Ma and 3a could be lost during the cell proliferation by exonuclease digestion or simply because it cannot replicate.

The interviral recombination was independent of viral DNA synthesis because high levels of interviral recombination occurred in the absence of viral DNA synthesis. The evidences of lack of viral DNA synthesis in cells coinfected with Ma and 3a in the transformed cells at nonpermissive temperature was obtained and discussed above.

These results with the tsa mutant indicated that the mutation in large T-antigen did not affect interviral recombination. Two possible interpretations of this are : (1) large T-antigen is not involved in the recombination process. (2) There are low-levels of large T-antigen present in the cells grown at $39°C$ either due to the leakiness of the tsa mutation or due to the incubation period at $33°C$. (3) The domain of large T-antigen essential for recombination resides in different region of large T-antigen from

that required for viral DNA replication. A ladder-like pattern of bands was observed in 6 out 21 transformed cell lines, suggesting there was some large T-antigen present for DNA synthesis of the integrated viral genome. The leakiness of tsa mutants may give rise to the low level of large T-antigen to carry out some functions of large T-antigen. Syu and Fluck (1991) observed that rearrangements of integrated viral sequences resulted from homologous recombination in the tsa transformed cells at $39°C$ [24]. This result also suggests that recombination can still occur with little or no large T-antigen.

Stonge et al. (1990) showed that the rate of homologous recombination was promoted from 2×10^{-7} to 10^{-2} per cell generation before and after the large T-antigen was introduced [23]. They studied the recombination between two defective copies of middle T-antigen integrated side by side in FR3T3 cells, which is very similar to the viral excision process.

A clear role for large T-antigen in homologous recombination has been demonstrated in viral excision [2, 3, 5, 9] and amplification [3, 4, 5, 21]. These processes may be linked to requirement for viral DNA replication. The present type of recombination we studied is different , i.e., the occurrence of recombination between two *de novo* introduced viral genomes rather than between two integrated viral sequences.

The evidences of the occurrence of recombination independent of viral DNA synthesis were also obtained from other groups. Stonge et al. (1990) showed that recombination between two defective middle T-antigen can be promoted by SV40 large T-antigen [23]. They suggested that the role of large T-antigen in recombination is by binding and unwinding the origin region, since SV40 large T-antigen cannot initiate

DNA synthesis from a template containing polyoma origin. Bourgaux et al. (1990) showed that viral DNA synthesis is not required for the homologous recombination, but the region near the origin is required [6].

In conclusion, the different effects of the tsa large T-antigen in different aspects of polyoma infection were obtained. On one hand, the large T-antigen of tsa mutants are defective in viral DNA replication although that our results do not exclude small levels of replication from integrated viral genomes. On the other hand, little or no effect of tsa was observed on: (1) transformation; no reduction of transformation observed, (2) integration pattern; tandem repeat of the polyoma genome found integrated in 62 % transformed cells, suggesting the presence of large T-antigen during integration, (3) appearance in "ladder pattern"; a post-integration replication event occurred, (4) recombination; high levels of interviral recombination occurred at nonpermissive temperature.

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Chapter 4

High Frequency of Homologous Recombination between Integrated Endogenous Polyomavirus Sequences and Exogenous Viral Genomes Introduced by Infection

The homologous recombination between unintegrated and integrated viral genomes was studied in a normal cell line FRLT, which are FR3T3 cells contain stably integrated polyoma large T-antigen cDNA, infected with two transformation-defective mutants, 1387 T and dl 23. Recombination between the endogenous and the exogenous polyoma sequences was assayed by scoring for transformation. Results showed that transformation occurred with a frequency at 1/34 of that observed in infection of the same cells with wild A2 virus at the same multiplicity of infection. To a high frequency, alterations of the endogenous sequences occurred resulted from the integration of the exogenous viral genomes in the same site of endogenous polyoma sequence. The acquisition of wild type sequences by the endogenous was resolved by a double crossover event between the endogenous and the exogenous polyoma sequences, which was then followed by a gene conversion.

4.1 Introduction

Recently, the targeting of foreign DNA to specific chromosomal sites by homologous recombination has provided an invaluable tool for studying gene function and for correcting genetic defects. In simple eukaryotes such as *S. cerevisiae* [16] and *Trypanosoma brucei* [1], homologous recombination can predominate over nonhomologous recombination. In organisms of slightly greater complexity, such as *Dictyostellum* [21], the ratio of homologous to nonhomologous recombination events is roughly 1 : 5.

In mammalian somatic cells, foreign DNA is introduced into cells or injected into mouse eggs to study the parameters that affect the homologous recombination within specific chromosomal loci [11, 18, 29, 31, 33]. In these experiments, the frequency of homologous recombination is about 1 homologous recombination event for every 10^3 nonhomologous integration events [12, 26]. This shift in the ratio does not represent a greater efficiency of homologous recombination in simple eukaryotes but rather an increase in the efficiency of nonhomologous or illegitimate recombination in more complex organisms. In the embryonic stem cells, DNA can be introduced either by injection or electroporation into these cells, and the frequency of homologous recombination in various loci has ranged from 10^{-2} to 10^{-5}, calculated as rate of integration at random sites to integration at homologous site, i.e., homologous recombination [3, 9, 10, 12, 19, 22, 32, 34].

In previous experiment [15], we have found unusually high rates of recombination associated with the integration of polyoma viral genomes.The interviral recombination is found concomitant with neoplastic transformation after infection of nonpermissive

rat FR3T3 cells. In the present study, we question whether and how frequently viral genomes will recombine with pre-integrated viral sequences during this process. For this purpose, a normal cell line containing stably integrated cDNA copies of the polyoma large T–antigen message was derived from the rat FR3T3 cell line and infected with transformation defective mutants with point or deletion mutations in the middle T-antigen coding region overlapping with that of large T-antigen.

Recombination between endogenous and exogenous sequences was assayed by scoring for transformation and the recombination events were analyzed by restriction endonuclease mapping. Transformation occurred at 1/34 of the frequency observed in infection of the same cells with wild type at the same multiplicity of infection. Thus in 3 % of the infected cells, an exogenous viral genome was able to recombine with endogenous viral sequences to form a wild type middle T sequence. These values are very similar to those observed for interviral recombination frequency and underscore the very high recombination rates associated with the integration of the viral genome into the host genome.

4.2 Materials and Methods

Cells and virus: Fisher rat (FR3T3) [14] and FRLT cells (described in the following) were maintained in Dulbeco Modified Eagles medium (DMEM,Gibco) supplemented with 10 % calf serum (Gibco) and antibiotics: 0.3 % pen/strep and 0.5 % fungizone for FR3T3, and 0.5 % gentamycin and 0.5 % fungizone for FRLT.

Wild type polyomavirus wild type strain A2 [27] was used in control experiments.

Middle T-antigen mutants 1387 T and dl 23 were used in recombination experiments. 1387 T has a transition point mutation at nucleotide 1387 which causes a termination of middle T-antigen [4]. dl 23 has deletion in the middle T-antigen coding region from nucleotide 1121 to 1242 [8]. Both mutants are unable to transform FR3T3 cells due to their defects in the middle T-antigen.

Construction of the FRLT cell line: Polyomavirus large T-antigen cDNA was cloned into the plasmid vector pMSG (Pharmacia) [23] downstream of the mouse mammary tumor virus (MMTV) promoter. This plasmid also contains the Xanthine-guanine phosphoribsyltransferase (*gpt*) gene linked to the SV40 promoter for hypoxanthine, aminopterin, thymidine (HAT) selection in mammalian cells, and an ampicillin resistance gene for selection in bacteria. The polyoma promoter was removed from a large T-antigen cDNA containing plasmid, by a partial digestion with BstXI which cleaves at nucleotides 167 and 1695, in the large T-antigen cDNA, and cleaves once in the vector pMSG DNA. The fragment cleaved at nucleotide 167 of large T-antigen cDNA was isolated and then digested with NheI which cleaved at the junction of plasmid vector for DNA and the large T-antigen cDNA. The ribosome initiation site was reconstructed by adding a linker. The inserted large T-antigen cDNA starts from nucleotide 167 to the end of the early message (nucleotide 2912). The constructed plasmid, pMSG/LT, was transfected into FR3T3 cells by the calcium phosphate method [13]. Stable transfectants were selected by maintaining the cultures under HAT selection. 57 clones were obtained and the expression level of large T-antigen upon dexamethasone-induction was analyzed by both immunofluorescence

and by Western blotting.

Isolation of transformants: Both FR3T3 and FRLT cells were synchronized by growing to confluency and by starvation with gamma globulin (GG)-free serum (Gibco) to obtain the maximal transformation [6]. Cells were then released from G0 by trypsinization and addition of 10 % calf serum and then seeded at a density of 1 x 10^5 cells per 35 mm tissue culture plate. Infections were carried out with A2, 1387 T, or dl 23 at various multiplicity of infection (MOI) of 10, 1 and 0.2 plaque forming units (pfu)/cell. Both focus formation over monolayer cell cultures and colony formation in soft agar were used to score for the transformation 7 to 10 days post infection. Transformed foci were selected and clonal transformed cell lines were amplified in 10 % calf serum for the analysis of recombination events.

Preparation and analysis of low molecular weight high molecular weight, and total DNA: Low molecular weight DNA was extracted by the method of Hirt , and high molecular weight DNA was extracted by a modification of the Hirt method [17]. Briefly, cells were harvested in 10 mM Tris, 10 mM EDTA, (i. e., Hirt buffer without SDS), pH 8, supplemented with 0.1 M NaCl. Both SDS was added up to 0.6 % drop-wise from a 10 % stock, and Nacl was added drop-wise from a 5 M stock to 1 M, while stirring very gently until the lysate appeared homogeneous, and kept at 4°*C* overnight. The lysate was then spun at 7000 rpm for 30 min. After removal of the supernatant, 10 ml of 10 mM Tris, and 10 mM EDTA were added, and the pellet was gently detached from the tube. Proteinase K (100 μg/ml) was

added and the mixture was incubated on a shaker at 37^oC until the pellet appeared homogeneous. The lysate was then extracted with phenol/chloroform. Total DNA was extracted by lysing the cells in a solution of 0.2 % by lysing the cells in a solution of 0.2 % SDS, 10 mM Tris, pH 7.5, 10 mM EDTA, and 50 μg/ml protease K (Sigma).

For the recombination analysis,10 μg of DNA was digested with various restriction endonucleases, electrophoresed on agarose gels and transferred onto Hybond filter paper (Amersham). Prehybridization was carried out in a buffer containing 5 X SSPE, 5 X Denhardt's solution, 0.5 % (w/v) SDS and 50 μg/ml salmon testis DNA for one hour at 65^oC. Hybridization was carried out in the same buffer for 48 to 60 hours at 65^oC, using 1 x 10^6 cpm/ml of a ^{32}P-labeled probe. Probes were labeled to a specific activity of 1–2 x 10^9 cpm/μg template DNA by using a multiprime DNA labeling kit (Amersham).

Recovery of virus from transformed cells: 30 ng low molecular weight DNA was transfected onto an NIH3T3 cell monolayer using the DEAE–dextran method [30]. DMEM supplemented with 2 % horse serum was then added. Cell lysates were harvested when 70 % cytopatic effect occurred, and tittered by plaque assay in NIH 3T3 cells. Individual plaques were picked 7 days post infection and amplified to high titer in NIH 3T3 cells.

4.3 Results

A recombination system: For study of recombination between endogenous and exogenous polyoma sequences, FRLT which are FR3T3 cells containing plasmid

pMSG/LT stably integrated into chromosomes were used. These cells were obtained as described in Materials and Methods, and originally screened for high expression of polyoma large T-antigen following induction by immunofluorescence and western blot (data not shown). The particular clone, FRLT1, used in this study was chosen among 57 clones tested. The expression of large T-antigen in the FRLT1 is at low constitutive levels and is induced to high level following treatment with dexamethasone. As shown below, FRLT cells contains multiple integrated copies of the polyoma plasmid located at a small number of site (apparently 2). Since the integrated polyoma sequences in FRLT do not contain the middle T-antigen/small T-antigen sequences located in the large T-antigen intron, including the middle T antigen splice site, the cell line does not express middle T-antigen and has a normal morphological phenotype, except for a slight increase in saturation density when large T-antigen is expressed at a high level. Transformation following infection with wild type virus was easily demonstrated by the usual assays for overgrowth of the monolayer or anchorage independence in agar. The same assays were used in experiments with transformation-defective mutants. Recombination was confirmed by restriction endonuclease analysis. This analysis was complicated by the high number of endogenous plasmid sequences.

Originally these cells were derived to study the effect of large T-antigen upon various aspects of the infection of nonpermissive rat cells by polyomavirus, including interviral recombination. However, induction of large T-antigen to high level did not appear to affect viral DNA synthesis nor transformation (data not shown). A slight decrease in the transformation potential by wild type is observed compared to the transformation frequency observed for the parental FR3T3 cell lines (Table 4.1).

Table 4.1: Transformation of FRLT and FR3T3 by wild type A2 and transformation defective mutants 1387T and dl 23.

	virus		
	A2	1387T	dl 23
MOI	1	10	10
FRLT[a]	109	156	9
FR3T3[b]	65	0	0

a. Total number for 8 parallel infections.

b. Total number for 2 parallel infection.

Two transformation-defective mutants, 1387 T and dl 23, were used in the study of homologous recombination between the endogenous integrated large T-antigen cDNA and the exogenous polyoma sequences introduced by infection, i.e., in unintegrated form.

The middle T-antigen mutations in these 2 strains are located in the region overlapping the large T-antigen sequences in the integrated cDNA sequences. In contrast, these mutants contain the large T-antigen intron sequences missing in the integrated polyoma plasmid DNA. Thus, recombination between the exogenous and endogenous polyoma sequences could produce wild type middle T-antigen sequence and hence a transforming virus. The results of infection with 1387 T are shown in Table 4.2.

Recombination frequency between endogenous and exogenous sequences was assayed by scoring transformation. Our results showed that in cells infected with 1387 T at MOI of 10, the average number of stable transformants was 19, and at MOI of 1, the number was 4. With wild type A2 at MOI of 1, the number was 27.2 (Table 4.1). Thus, the recombination frequency was estimated to be about 1/34 (i.e., 4 : 27.2). by a comparison of transformation frequency induced by 1387 T and that by wild type A2 at the same multiplicity of infection (MOI=1). Interestingly, only stable transformants were observed in the 1387 T-infected FRLT, whereas both abortive and stable transformants were observed in wild type infected cells, and the ratio of abortive transformation to stable transformation was about 4 : 1. The absence of abortive transformation was not simply due to a matter of the low dosage of recombinant wild type, since abortive transformation was observed in infection with wild type A2 even at MOI of 0.2.

Table 4.2: Transformation frequency of FRLT cells infected by wild type A2 and 1387 T.

	virus					
	A2			1387 T		
MOI	10[a]	1[b]	0.2[b]	10[a]	1[b]	0.2[b]
Total # of colonies[c]	TMTC[d]	662	52	38	4	0
# of stable transformants[e]	ND[f]	136	10	38	4	0
average number of colonies/plate	ND	27.2	2	19	0.8	0
transformation frequency(%)[g]	ND	0.9	0.066	0.6	0.026	0

a. Total number for 2 parallel infections,

b. Total number for 5 parallel infections,

c. Total number of colonies as determined under microscope,

d. Too many to count,

e. Total number of stable transformants as determined by counting with the nude eyes,

f. Undetected,

g. Transformation frequency was determined by the number of stable transformants per 3 x 10^5 cells.

Wild type sequences in transformed clonal cell lines: The crossover sites between the endogenous integrated large T-antigen cDNA and dl 23 were determined. Transformants from infection with dl 23 mutants were used for this purpose, making use of the dl 23 deletion mutation. In the case of infections with dl 23, recombination is followed easily by restriction endonuclease analysis. The enzymes used are shown in Figure 4.1. Twelve cell lines were analyzed, and results are shown for two in Figure 4.2.

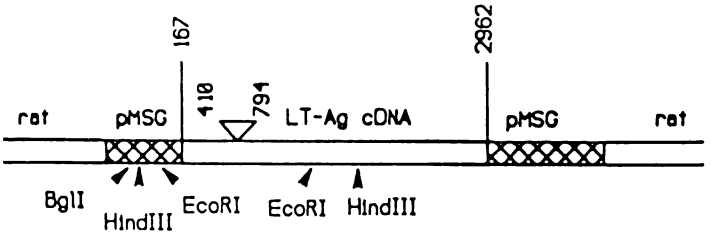
The acquisition of wild type sequences by the exogenous virus (which we will refer to as class 1 recombinants) was analyzed by digestion with BglI+HindIII, and BclI+EcoRI. Both digestions showed the appearance of new wild type size fragments in the transformed cells, not seen in the parental FRLT line. Comigration of these unique fragments with wild type sequence not present in the mutant virus is also evident. 12 transformants out of 12 analyzed showed this pattern (Figure 4.3). These results showed that wild type middle T-antigen synthesis can be controlled from the polyoma enhancer promoter which is totally contained in the BclI-EcoRI fragment. In addition to the wild type band, half of the trasnformants analyzed also contained the parental dl 23 and was similar to the case of dl23LT2 shown in Figure 4.2 and Figure 4.3.

The corollary situation, acquisition of wild type sequences by the endogenous plasmid sequence (class 2 recombinants), was analyzed by digestion with ClaI+EcoRI (Figure 4.1). Acquisition of the intron sequence would generate a unique 2.6 kb fragment which was not detected. Analysis with BglI+EcoRI also demonstrated a fact that the transformants selected and analyzed were the same type of class 1, i.e.,

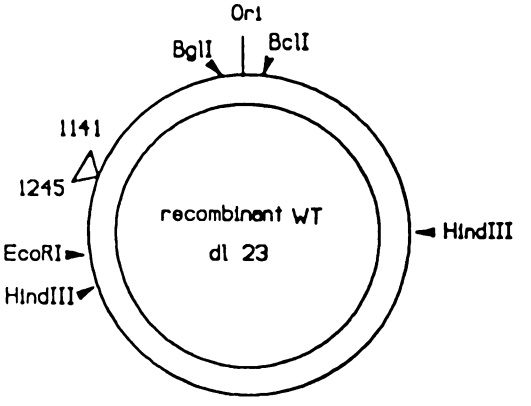
Figure 4.1: The map of restriction endonuclease sites in plasmid pMSG/LT and polyomavirus dl 23 and recombinant wild type virus, and the expected sizes of resulting fragments.

(A). A simplified map of pMSG/LT, data suggested multiple tandem copies of pMSG/LT integrated in the host chromosomes. The triangle is the deletion of the intron region. The linear line represents for pMSG/LT. The hatch-line regions are the rat DNA , the double hatched-line regions are the pMSG vector DNA, and the blank region is the large T-antigen cDNA. (B). The double circle is the polyomavirus dl 23 or the recombinant wild type sequences. Ori is the origin region, the triangle is the deletion in the dl 23. (C). The expected size of resulting fragments from digestions of three combinations of the restriction endonucleases.

A.



B.



C.

	plasmid	polyoma	
		recombinant wild type	dl 23
1. ClaI + EcoRI	2.1 Kb	5.3 Kb	5.2 Kb
2. BclI + EcoRI	5.1 Kb	1.8 Kb	1.7 Kb
3. BglI + HindIII	2.6 Kb	1.6 Kb	1.5 Kb

Figure 4.1: The map of restriction endonuclease sites in plasmid pMSG/LT and polyomavirus dl 23 and recombinant wild type virus, and the expected sizes of resulting fragments.

Figure 4.2: The presence of wild type sequences in the twelve dl 23 recombinant transformants.

10μg of DNA of dl 23 recombinant transformants was digested with BglI+HindIII, and analyzed as described in Materials and Methods.

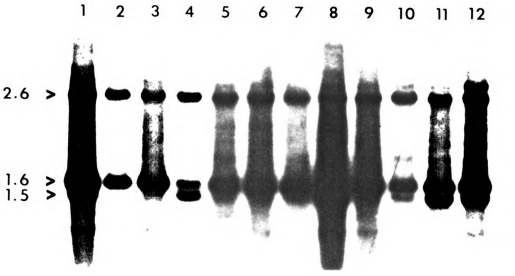


Figure 4.2: The presence of wild type sequences in the twelve dl23 recombinant transformants.

Figure 4.3: Restriction endonuclease analysis of recombination events in two dl 23 recombinant trasnformants.

10 μg total DNA of FRLT, dl23LT3, and dl23LT12 were digested with various restriction endonucleases, and analyzed as described in Materials and Methods. For hybridization, a ^{32}P-labeled probe containing the fragment 4 of MspI-digested polyoma DNA was used to limit hybridization to the regions of interest. For size markers, digests of wild type A2 and dl 23 viral DNA were electrophoresed in parallel.

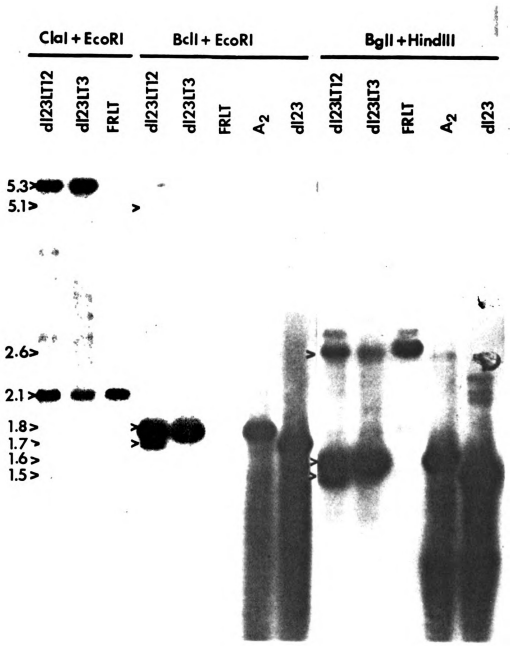


Figure 4.3: Restriction endonuclease analysis of recombination events in two dl 23 recombinant trasnformants.

they were derived from a recombination event in which the exogenous virus acquired intron sequences from the endogenous sequences. Thus, as we have observed in many other crosses, reciprocal recombination is not observed for any particular event.

The absence of class 2 recombinants might reflect the failure of generating these events or selecting them. An easy case could be found for the latter one, i.e., selecting these events. Since multiple copies of the plasmid are integrated in FRLT, it is possible that the generation of a wild type middle T-antigen copy from a single plasmid copy might not lead to sufficient middle T-antigen production even in the presence of dexamethasone for the expression of the transformed phenotype.

The recovery of wild type virus from transformed cells: As the recombination with 1387 T which has a point mutation cannot be studied by restriction endonuclease mapping, an alternative method was devised. Low molecular weight DNAs were extracted (as described in Materials and Methods) from two 1387TLT cell lines which have high levels of free viral DNA and were used to transect NIH 3T3 cells. These viruses were plaque purified and amplified to a titer of 10^8 pfu/ml. A transformation assay was performed in FR3T3 cells with the recovered virus stocks. Both viruses induced neoplastic transformation in FR3T3 cells very efficiently (data not shown). Since the original virus, 1387 T cannot transform FR3T3 cells (see Table 4.1), the transforming ability must resulted from the presence of recombinant wild type genomes in the 1387 T recombinant transformants.

Analysis of the integration patterns of both endogenous pMSG/LT polyoma and intact viral DNA in 1387TLT and dl23LT recombinant transformants: The integration patterns of the pMSG/LT plasmid in FRLT cells and the viral genomes in 1387TLT and dl23LT recombinant trasnformants were determined. Three restriction endonucleases were used to digest the total DNA extracted: (1) BclI does not cut the pMSG/LT plasmid, but cuts polyoma DNA once at nucleotide 5023; (2) BglII cuts pMSG/LT once, but does not cut polyoma DNA; (3) BstEII cuts neither DNA. The digested DNAs were resolved in a 0.4 % agarose gel and hybridized with a ^{32}P–labeled probe containing the entire polyoma DNA. The results are shown in Figure 4.3 and Figure 4.4.

As alluded above, the integration pattern of pMSG/LT in FRLT cells is complicated. When analyzed with enzymes which do cut the plasmid (BclI and BglII), two bands of very high molecular weight were seen. In BclI digestions, one band was sharp and another broad, while two broad band were obtained with BstEII. In BglII digestions, which cuts pMSG/LT once, 4 distinct strong band and 2 weaker bands were detected, and no 10 Kb fragment (the size of the plasmid). This result suggests that the pMSG/LT plasmid is integrated at 2 major sites containing multiple copies of the plasmid, and is not a simple head to tail complete tandem repeats of the plasmid.

In the fifteen 1387TLT recombinant transformants analyzed, clear alterations of the endogenous sequences were observed in both BclI and BstEII digestion. The results with BclI digestion are shown in Figure 4.3.A. In twelve dl23LT recombinant transformations, 5 cell lines showed alterations of endogenous sequences, 6 cell lines had alternations in BclI digestion, and 8 cell lines had alternations in BglII digestion.

Examples of digestions of 4 dl23LT recombinant transformants with BclI, BglI and BstEII are shown in Figure 4.3.B. The high incidence of alterations of the endogenous sequences suggested that the viral genomes had integrated into the endogenous pMSG/LT sequences.

4.4 Discussion

Our results showed that high levels of recombination frequency was observed between the endogenous large T-antigen cDNA and the exogenous polyoma viral genomes. Recombination frequency in the cross between integrated and unintegrated polyoma sequences was measured by the transformation frequency of the infected cells, since all the transformants are recombinants. In infection with 1387 T, the recombination frequency was 1/34 of that observed in cells infected with wild type A2 virus at the same multiplicity of infection.

Comparing the recombination frequency obtained from the present experiments to other systems, we found that the recombination between polyomavirus genomes in infection of cells containing pre-integrated viral sequences occurred at a high rate. For example, the frequencies of gene targeting in embryonic stem cells are: 1/300 for the *En*-2 gene [9], 1/1000 for the *hprt* gene [31], 1/400,000 for the *int*-2 gene [32], 1/117 for the $\beta_2 - microglobulin$ gene [20], 1/250 for the T-cell receptor β-subunit [24], 1/40 for the homeobox gene *Hox-3.1* [25]. In a hybrid murine-human cell line, the frequency of correction of a human β^s-globin gene was 3/126 by gene targeting [28].

Possible explanations for the high levels of recombination frequency observed in

Figure 4.4: Analysis of integration patterns of the polyoma sequences in both 1387TLT and dl23LT cells.

(A). 10 μg of DNA extracted from FRLT and fifteen 1387TLT cell lines was digested with BclI, and analyzed as described in Materials and Methods. (B). 10 μg total DNA extracted from FRLT and 4 dl23LT cell lines was digested with BclI, BglII and BstEII, and analyzed as described in Materials and Methods.

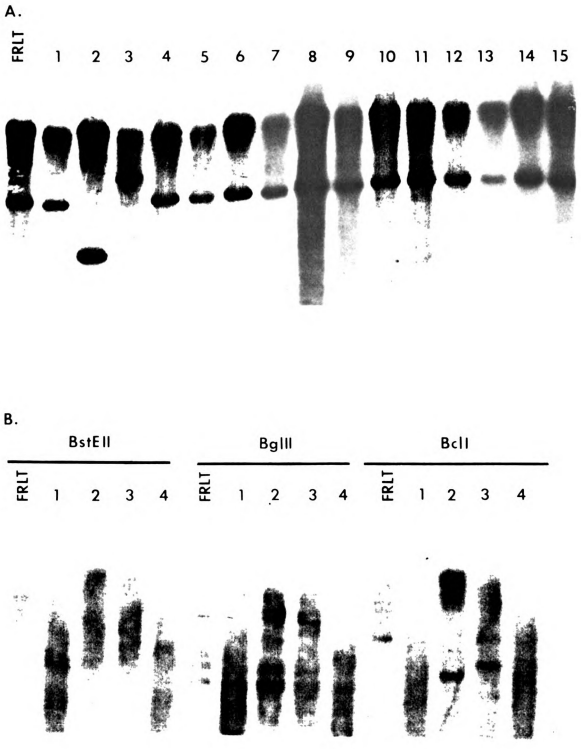


Figure 4.4: Analyses of integration patterns of the polyoma sequences in both 1387TLT and dl23LT cells.

the cross between the integrated and unintegrated viral genomes are (1) the presence of high levels of large T-antigen, (2) high copy numbers of the endogenous integrated plasmid DNA, or (3) a hot spot for recombination in the polyoma genome. The first possibility is that large T-antigen plays an important role in homologous recombination. However this can be ruled out by the fact that no significant difference in recombination frequency was observed in FRLT cells with or without dexamethasone-induction. In previous experiments, we found that high levels of interviral recombination occurred in the absence of functional large T antigen [7].

The second possibility is that high copy numbers of the endogenous plasmid pMSG/LT sequences can cause recombination to occur with a higher frequency. The pMSG/LT integrates at 2 sites of cellular chromosomes with a complicated integration pattern in FRLT cells, so that there are more templates available for recombination to occur. However, this possibility can also be ruled out by the observation that no difference of recombination frequency was observed with increasing copy number of the integrated plasmid DNA in embryonic stem cells [2]. The third possibility is that a tentative hot spot for recombination is located in the polyoma genome. The hot spot may cause high levels of recombination observed in the cross between unintegrated and pre-integrated viral genomes. A gradient of recombination along the 40 % of polyoma viral genome was observed [5]. This might indicate the presence of a hot spot which could play a role in the high frequency of the homologous recombination.

From the analysis of the integration pattern of polyoma genome in the recombinant transformants, the acquisition of the wild type polyoma sequences resulted from the recombination between the integrated polyoma sequence and exogenous vi-

ral genome. Two possible explanations for these recombination events were raised. One is that a double crossover occurs and the crossover sites are located between the intron (nucleotide 795) and the deletion mutation (nucleotides 1121 to 1245 for dl 23). The other is that a double crossover occurs downstream of nucleotide 1245, and which is then followed by a gene conversion event during DNA replication or repair (Figure 4.5). However, the regions of ClaI–EcoRI, BclI–EcoRI, and BglI–HindIII from 1the endogenous plasmid pMSG/LT sequences remain unchanged, suggesting that the crossover must have occurred downstream of the EcoRI (nucleotide 1562) or HindIII (nucleotide 1658).

The alterations of the pre–integrated polyoma sequences occurred at a high rate. These alterations arose from the integration of newly introduced viral genomes in the same sites of the integrated endogenous plasmid pMSG/LT. The fact that most (50 %) transformants, 2 viral genomes recombined at the site of integration, and in contrast, integration at more than one site with a single transformant is rare (10 %). This fact opens the possibility that the viral genomes may home to special sites on the host chromatin or scaffold; such targeting might be related to the path of entry into the nucleus.

The very high or high rates of interviral recombination occurred among the integrated genomes arising in mixed infection or in infected cells containing pre-integrated viral sequences respectively. However, on the other hand, the levels of recombination were not detected among unintegrated genomes in mixed infection. The contrast between the recombination occurred predominantly in the integrated viral genomes and not in the unintegrated viral genomes suggests that interaction with the host

chromosome or scaffold plays a major role in the recombination process. Thus, in contacting the host chromatin, viral genomes encounter a major host recombination pathway.

A comparison of the recombination frequency of the cross between unintegrated and integrated viral genomes to that of the cross between 2 parental mutant genomes in FR3T3 cells, suggests an answer to the question concerning the timing of recombination in the latter crosses. In these crosses, the recombinant viral genomes are integrated into host DNA and serve as the transforming genomes of stably transformed cells. Three major classes of events might lead to interviral recombination associated with integration. Interviral recombination may occur prior to, simultaneously with, or post integration. The first possibility appears unlikely since recombination was not observed among unintegrated viral genomes derived from the same infected cell population from which the transformants with integrated recombinant genomes were recovered. The third case was that one genome was integrated first followed by integration of the second genome into the first one by homologous recombination. However, results in the present experiments showed that the recombination frequency between unintegrated and integrated viral genomes is 10-fold lower than that observed in crosses between 2 co-infecting viral genomes. Thus, the recombination of 2 viral genomes occurs simultaneously as they integrate. Interestingly, illegitimate recombination between the viral and the host genome might occur concurrently to homologous interviral recombination.

Figure 4.5: Possible recombination events occurring in the cross between the endogenous large T-antigen cDNA and the exogenous polyoma genome.

The open triangles represent for the deletion of intron in large T-antigen cDNA, and the solid triangles are the good copy of intron in the dl 23. The open squares are the deletion of nucleotide 1140 to nucleotide 1245 in the dl 23, and the solid squares are the good copy of corresponding sequence of mutation in dl 23. Two possible events could occur: (A). A double crossover occurs between the intron and deletion mutations. (B). A double crossover occurs downstream from the deletion mutation, and followed by a gene conversion event.

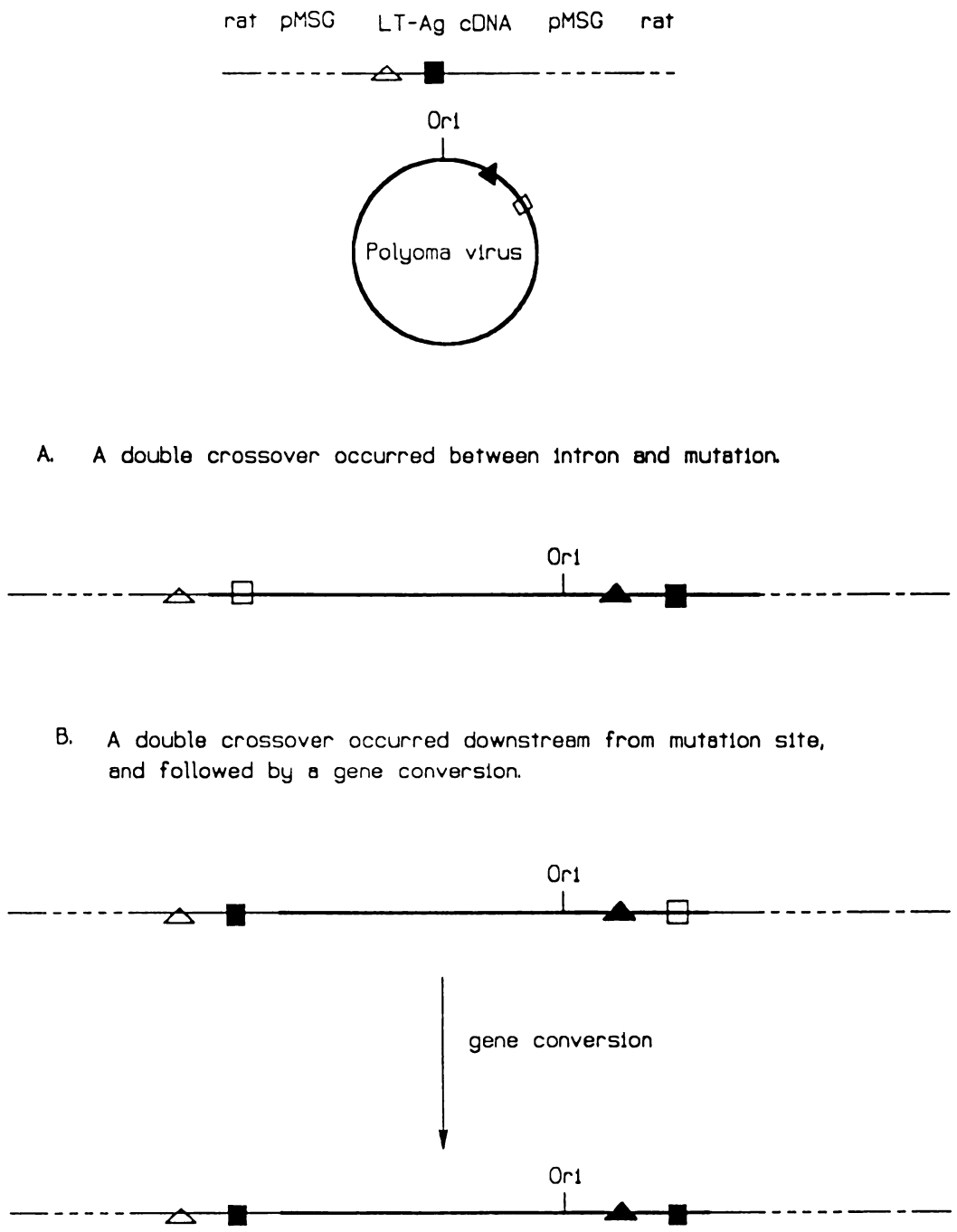


Figure 4.5: Possible recombination events occurring in the cross between the endogenous large T-antigen cDNA and the exogenous polyoma genome.

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Chapter 5

A Gradient of Recombination on the Polyomavirus Genome

We have analyzed the transformation frequency along the polyoma genome in 2 types of crosses in which we have previously described high levels of homologous recombination were obtained: (1) in infections of normal FR3T3 cells, recombinant genome are recovered integrated at the cellular genome in 33 % of stably transformed cells and, (2) in infections of FRLT cells, integrated polyoma sequences were found in 3 % on the infected cells. Our results demonstrate that the frequency of recombination is nonuniform along a segment of the polyoma genome analyzed so far between nucleotide 4634 in the late region and nucleotide 1387 in the middle of the early region, encompassing the enhancer and the origin. A gradient of recombination was seen and, by overlapping results from different crosses, a minimum was observed in the enhancer region with a continuous increase when moving towards the early region. The maximal recombination mapped so far in the early coding region between nucleotides 1245 and 1387 show a 60–fold increase over the frequency observed in the enhancer region 2 Kb away.

5.1 Introduction

Our laboratory has previously demonstrated the occurrence of high levels of homologous interviral recombination in infection of nonpermissive rat cells by polyomavirus [8]. Recombination is not detected between unintegrated viral genomes, but among the integrated genomes which serve as the transforming sequences of stably transformed cells. Recombination is also detected albeit at 10–fold lower levels between unintegrated infection viral chromatin and preintegrated polyoma sequences [5, 4]. The results suggest that viral genomes engaged in the integration process interact with a major host recombination pathway. Interestingly, the 2 phenomena appear to happen at the same time. Recombination with the host chromosome which is thought to be the mostly illegitimate recombination event occurs concommittant with homologous interviral recombination.

In the present study, we have analyzed the transformation frequency along the polyoma genome in the 2 types of crosses described above. Our results demonstrate that the frequency of recombination is nonuniform along the part of the polyoma genome analyzed so far. A gradient of recombination was seen and, by overlapping results from different crosses, a minimum was observed in the enhancer region with a continuous increase when moving towards the early region. The maximal recombination mapped so far, in the early coding region between nucleotide 1245 and 1387, show a 60–fold increase over the frequency observed in the enhancer region.

5.2 Materials and Methods

Cells and virus: Fischer rat (FR3T3) [7] and FRLT [4] cells were used in the experiments. FRLT cells are FR3T3 which contains a stably integrated plasmid with the large T-antigen cDNA sequences in multiple copies as described in chapter 4 [4]. Cells were maintained in Dulbeco Modified Eagle medium (DMEM, Gibco) and supplemented with 10 % calf serum (Hazleton). Wild type polyoma A2 [11] was used as a control in the experiments. Two double mutants, Ma and 3a, harbor both the tsa temperature sensitive mutation in large T-antigen as well as mutations in the AvaI and BamHI restriction endonuclease sites, respectively. Ma contains the MOP 1033 point mutations in the AvaI sites at nucleotides 659 and 1018, and the latter abolished the transforming ability of the virus due to a termination mutation in the middle T-antigen reading frame. 3a is temperature sensitive in decapsidation and has a point mutation at nucleotide 4634. 3a can transform normally if allowed to decapsidate at $33^{o}C$ for 24 hrs [5]. Dl 23 and dl 1015 are nontransforming mutants with deletion in the middle T-antigen/large T-antigen frame. Dl 23 has a deletion between nucleotide 1121-1245 [6]; dl 1015 has a deletion between nucleotide 1246-1275 [10]; 1387 T has a point mutation at nucleotide 1387 which caused a termination in the middle T-antigen [3].

Isolation of transformed cells: To maximize transformation, synchronized cells were used [5]. For this purpose, cells were grown to confluency. The following day, cells were transferred to low serum medium supplemented with 0.2 % garma-globulin free serum (Gibco) for 1 day. To release cells from G0, both trypsinization and adding

fresh DMEM supplemented with 10 % calf serum were used. The reattachment of the cells occurred within 3 hours.

Infections were carried out at a multiplicity of infection (MOI) of 10 plaque forming units (pfu)/cell. Both focus formation in monolayer cell culture and colony formation in soft agar were used to identify the transformed cells as described previously.

Estimation of the recombination frequency: Recombination was analyzed in the viral genome found integrated in the genome of stably transformed cells. In the cross between Ma and 3a in FR3T3 cells, no selection pressure for recombination associated with transformation was applied since the parent 3a can transform normally after decapsidation at 33° for 24 hours. The occurrence of recombination was determined by digestion with a combination of AvaI, BamHI and BglII. The cell lines containing recombinant viral genomes were further analyzed for the occurrence of recombination between the AvaI–AvaI interval (nucleotide 1018–659), the small AvaI–BamHI interval (nucleotide 659–4634), and the long AvaI–BamHI interval (nucleotide 1018–4634) (Figure 5.1).

In the cross between the integrated polyoma sequences and the exogenous unintegrated viral genomes, both viral genomes are nontransforming. In these experiments, individual transformants were derived from recombinants. We took the transformation frequency as a measurement for recombination frequency. The occurrence of recombination events in DNA intervals was analyzed, and the recombination frequency was normalized by distances between the intron and the mutations of the exogenous mutants.

Figure 5.1: Restriction endonuclease map of polyomavirus.

AvaI cleaves polyoma DNA twice at nucleotides 659 and 1018, BglI cleaves polyoma DNA once at nucleotide 89, and BamHI cleaves polyoma DNA once at nucleotide 4634.

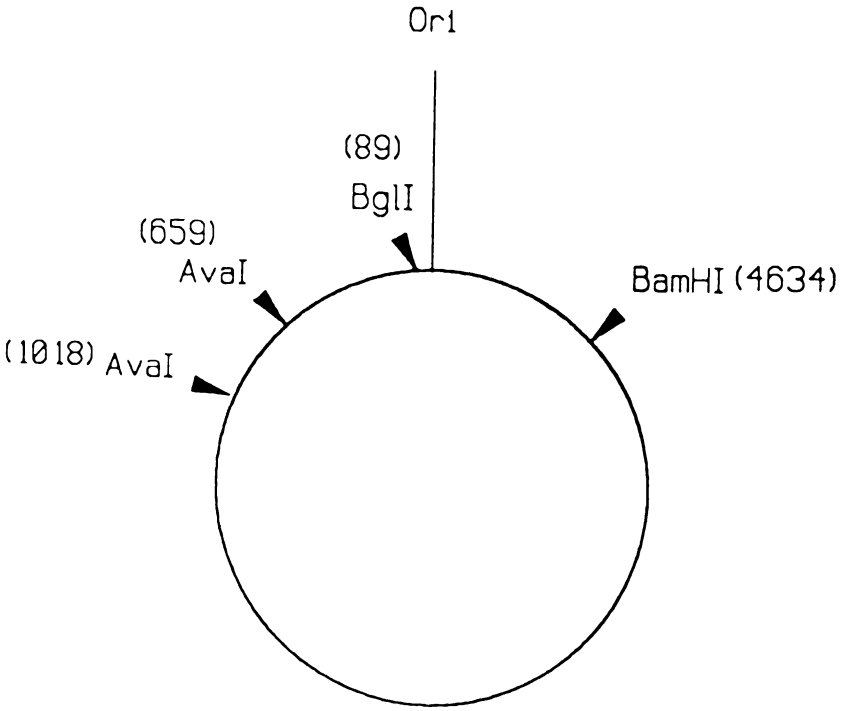

Figure 5.1: Restriction endonuclease map of polyomavirus.

Preparation and analysis of total DNA: Total DNA was extracted from transformed cells for the analysis of the occurrence of recombination. 10 μg of DNA was digested with a combination of AvaI, BamHI, and BglII, electrophoresed on a 1 % agarose gel, transferred onto Hybond-N filter paper, and hybridized to a ^{32}P-labeled probe containing the entire polyoma DNA. Probes were prepared by multiprime labeling kit (Amersham) to a specific activity of 1-2 x 10^9 cpm/μg template DNA.

5.3 Results

A gradient of recombination frequency in the cross of Ma and 3a: In chapter 3, we have described the recombination between Ma and 3a in FR3T3 at 39°C. There was no selection for recombination in the cross between Ma and 3a, because 3a can transform after decapsidation at 33°C for 24 hours. 65 transformants were picked from cells coinfected with Ma and 3a. 20 out of 65 transformed cell lines were found containing recombinant viral genomes. The occurrence of recombination events among these recombinant transformants was further studied among three DNA intervals: from the first to the second AvaI site (nucleotide 659-1018), the first AvaI to the BglI site (nucleotide 659-89), the small AvaI-BamHI interval (nucleotide 659-4634), and the long interval of the AvaI to the BamHI site (nucleotide 1018-4638). Digestions with a combination of AvaI, BamHI, and BglII were carried out for this analysis. If the recombination occurs within the AvaI-AvaI interval, a 1.7 Kb fragment would be detected; if the recombination occurs within the small AvaI-BamHI interval, a 1.3 Kb fragment would be detected; or if the recombination occurs with

the long AvaI–BamHI interval, a 3.6 Kb fragment would be detected. In the digestion with a combination of AvaI, BamHI and BglII, results showed that 7 out of 20 cell lines had the 1.7 Kb fragment, 15 had the 3.6 Kb fragment, and only 6 had the 1.3 Kb fragment. After normalization with size of DNA interval, a ratio of 4.7 : 1.1 : 1 was found in intervals of the 2 AvaI sites, the small AvaI–BamHI interval, the large AvaI–BamHI interval. If the recombination occurs between the AvaI–AvaI interval, a 1.7 Kb fragment will be observed; if the recombination occurs between the first AvaI–BglI interval, a 1.3 Kb fragment as well as a 374 bp fragment will be observed; or if the recombination occurred between the BglI–BamHI interval, fragments of 747 bp, 570 bp, and 374 bp will be observed. In order to normalize the distance effect on recombination frequency, the number of recombinants was divided by the length of the DNA interval.

In the digestion with a combination of AvaI, BamHI, and BglI, we found that recombination occurred within the interval of two AvaI sites in 5 cell lines, and only 1 cell line showed occurrence of recombination between BglI–BamHI interval by the fact that it has 744 bp, 570 bp, 374 bp fragments (Figure 5.2). Combining the results of the cross between MOP 1033 and ts3, carried out by D. Hacker, we found that 15 events, 9 events, and 5 events occurred between the two AvaI sites, the AvaI–BglI interval, and the BglI–BamHI interval, respectively. After normalizing the incidence of recombination occurred by the size of each DNA interval, we found a 6.7 : 2.4 : 1 ratio of recombination frequency between the two AvaI sites, the AvaI–BglI interval, and the BglI–BamHI interval (Table 5.1).

Taking the above results together, we estimated the recombination frequency along

Table 5.1: Recombination occurred between Ma and 3a in the regions of AvaI-AvaI, the AvaI-BglI interval and the BglI-BamHI interval.

	DNA interval		
	AvaI-AvaI[a]	AvaI-BglI[b]	BglI-BamHI[c]
Size of the DNA intervals (bp)	359	570	748
Incidence of recombination[d]	16	9	5
Incidence of recombination/bp	16/359	9/570	5/748
Ratio of recombination frequency	6.7	2.4	1

a. Between nucleotide 659 and 1018,

b. Between nucleotide 659 and 89,

c. Between nucleotide 89 and 4634,

d. Recombination events occurred among 20 recombinant transformants derived from a cross between Ma and 3a, and a cross between MOP 1033 and ts3.

polyoma genome and found a 40 : 20 : 6.7 : 2.4 : 1 ratio of recombination frequency in the intervals between nucleotide 1387–1245, 1245–1140, 1140–795, 795–89, and 89–4634 (the BglI–BamHI interval) (Figure 5.4).

Recombination between endogenous large T–antigen cDNA and exogenous viral genome: The other type of recombination studied was recombination between an integrated polyoma plasmid containing large T–antigen cDNA and exogenous sequences, i.e., viral genomes introduced by infection. In these experiments, FRLT cells, which have the large T–antigen cDNA stably integrated into the cellular chromosomes, were infected by three transformation–defective viruses. These cells can become transformed only if recombination occurs between the integrated large T–antigen cDNA and the unintegrated viral genomes. FRLT cells infected with wild type polyoma A2 were used as positive control.

The homologous recombination in these crosses was determined. The number of foci were 156 in 1387 T–infected FRLT cells, 47 in dl 1015–infected cells, and 9 in dl 23–infected cells. In A2–infected FRLT cells, the foci number was 109 (Table 5.2). For negative controls, FR3T3 were infected with 1387 T, dl 1015, and dl23 individually. Our results showed no transformation detected in these infected cells (Table 5.2).

We normalized the relative recombination frequency by the distances between the intron of T–antigen DNA and the site of mutations in the viral genome. These distances are 347 bp for dl 23 (nucleotide 795–1140), 452 bp for dl 1015 (nucleotide 795–1245), and 539 bp for 1387 T (nucleotide 795–1387). Our results showed a 10 : 4 : 1 ratio of recombination frequency in the infection of FRLT cells with 1387

Figure 5.2: Analysis of the occurrence of recombination in the regions of AvaI-AvaI, AvaI–BglI, and BglI–BamHI.

10 μg of DNA extracted from cell lines 1, and 3–7 (same labeling as in Figure 3.2) was digested with a combination of AvaI, BamHI, and BglI, and analyzed as described in Material and Methods. As size markers, viral DNA of A2 strain was digested with the same restriction endonucleases.

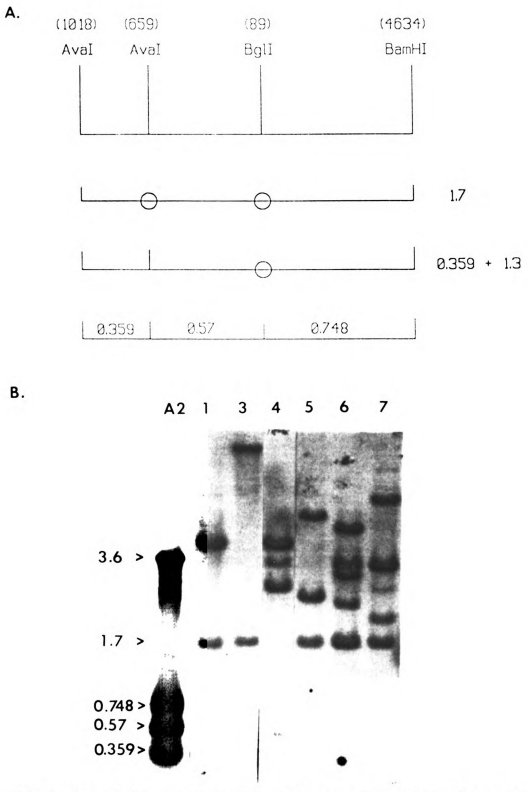

Figure 5.2: Analysis of the occurrence of recombination in the regions of AvaI–AvaI, AvaI–BglI, and BglI–BamHI.

Table 5.2: Recombination frequency between the endogenous polyoma sequences and the exogenous transformation-defective polyomavirus.

	virus			
	A2	1387 T	dl 1015	dl 23
MOI	1	10	10	10
# of foci[a]	109	156	47	9
distance between intron and mutation sites (bp)	ND[b]	593	452	347
# of foci/bp	ND	156/593	47/452	9/347
ratio of recombination frequency	ND	10	4	1
# of foci in infected FR3T3 cells	65	0	0	0

a. Total number for 8 parallel infections,

b. Undetected,

c. Total number for 2 parallel infections.

T, dl 1015, and dl 23 (Table 5.2).

A subtraction suggests that 6 times more recombination events/per nucleotide could be assigned to interval between 1245–1387 than in the interval 795–1140 (Figure 5.3).

5.4 Discussion

We have compared recombination frequencies along the polyoma genome between the BamHI site located at nucleotide 4634 and nucleotide 1387, an interval representing about 40 % of the viral genome, which encompasses some late coding sequence, the enhancer, the origin, and approximately half of the early region. These studies were carried out in two types of crosses using nonpermissive rat FR3T3 cells, a system in which we have previously reported elevated interviral recombination.

In one case, infections were carried out with 2 parental genome marked with defects in specific restriction endonuclease sites which allow the assignment of the crossover site to a specific interval between 2 restriction redonuclease sites. In these experiments, there was no selection for recombination. Mixed infections were carried out and transformed cells were selected. Moreover, the viral genome integrated in these cells were screened for recombination in a 1.7 Kb interval spanning from the BamHI site to the AvaI site at 1018. Recombination was also scored in the corresponding 3.6 Kb AvaI–BamHI interval region were further analyzed for the presence of restriction redoclease sites within the 1.7 Kb interval and crossover sites were thus assigned to either of 3 intervals.

Figure 5.3: Recombination frequency derived in the crosses between the endogenous polyoma sequences and the exogenous viral genomes.

The mutations of the endogenous polyoma sequences, or in the dl 23 and dl 1015 mutation are shown. Deletions are represented by doted lines, while a point mutation is represented by a simple dot. The hatched boxes show the distances involved in the 3 crosses between the intron mutation and the mutations in dl 23, dl 1015, and 1387 T mutations. Deductively, a ratio of 1 : 3 : 6 of recombination frequency was obtained for the three intervals 795–1140, 1140–1245, and 1245–1387, respectively.

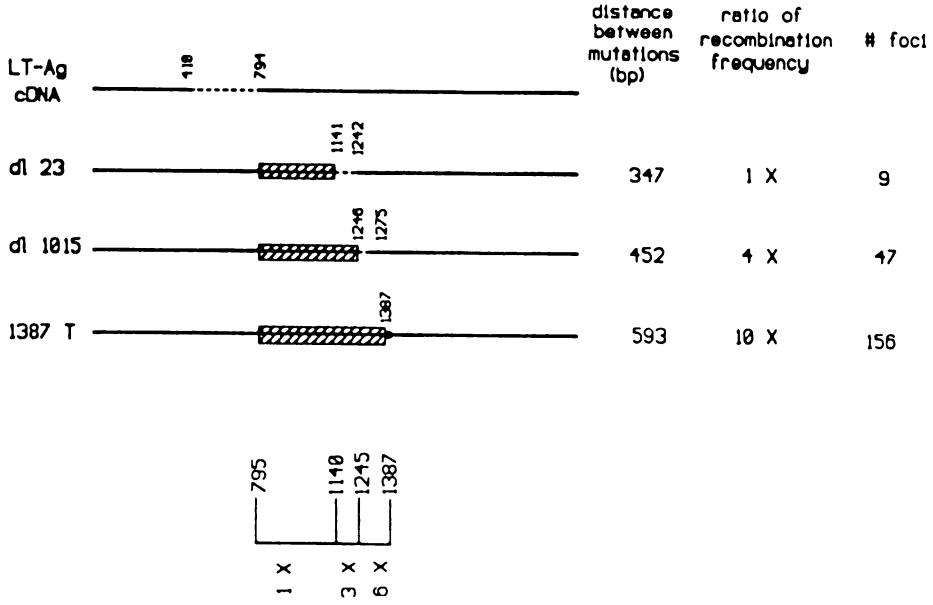

Figure 5.3: Recombination frequency derived in the crosses between the endogenous polyoma sequences and the exogenous viral genomes.

Figure 5.4: A gradient of recombination frequency was detected in the regions between nucleotides 1387 and 4634 (the BamHI site).

After chromosomal walking on the polyoma genome, a 40 : 20 : 6.7 : 2.4 : 1 ratio of recombination frequency was observed in the intervals between nucleotides 1387–1245, 1245–1140, 1140–795,795–89 and 89–4634 (the BglI–BamHI interval).

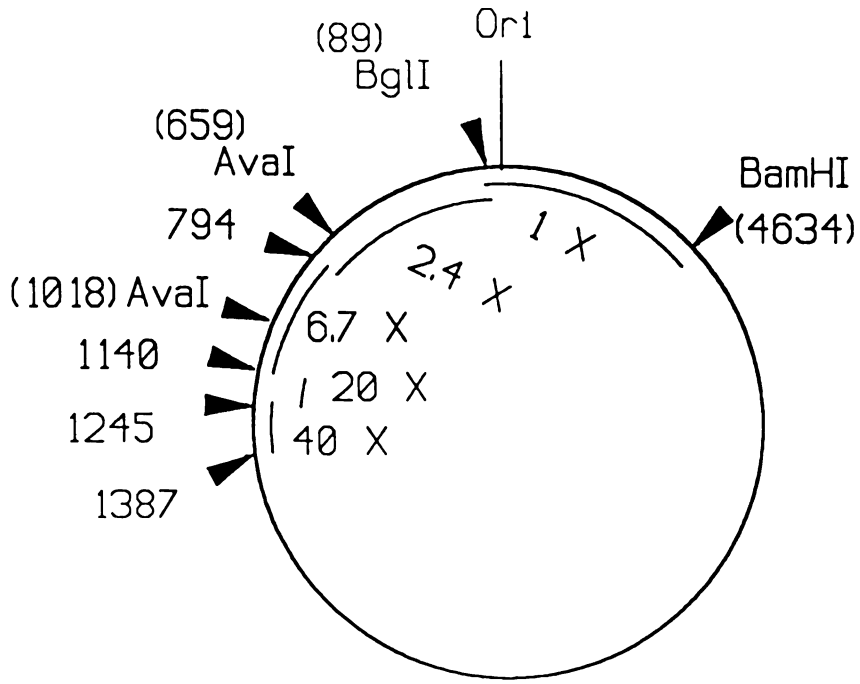

Figure 5.4: A gradient of recombination frequency was detected in the regions between nucleotide 1387 and 4634 (the BamHI site).

An analysis of 20 recombinants derived from 4 crosses showed that the majority of the recombination events had occurred in a short interval of 374 nucleotides (the small AvaI interval between nucleotide 659 and nucleotide 1018). Comparison of the number of events in each interval normalized as recombination events per unit length revealed a 6–10 fold ratio in the BglI–BamHI interval which encompasses the origin–enhancer region. This increase in recombination in the small AvaI interval compared to the BamHI–BglI interval was observed in transformants derived from interaction with wild type large T-antigen [8], or those with a temperature sensitive large T-antigen [8] carried out at $33°C$ as well as those carried out at the nonpermissive temperature.

In the other case, infections were carried out with a cell line, derived from FR3T3, carrying multiple copies of a plasmid with polyoma sequences using transformation defective mutants with either point or deletion mutations. Recombination was selected for by selecting for transformation. Three infections were analyzed using 3 different mutants at the same multiplicity of infection and the transformation frequencies in the 3 infections were compared to each other, and assumed to faithfully reflect recombination frequencies in the 3 overlapping intervals defined in these infections. By subtraction, a relative recombination frequency for 3 adjacent intervals was obtained and revealed a gradient of recombination as well, that is increasing when moving away from the origin towards the 3'end of the early region.

Since the intervals studied in the 2 types of infection are overlapping, a case can be made for a continuous gradient of recombination increasing from a low value in the enhancer region to high levels in the early region. The steepness of the gradient

(60-fold increase over a 2 Kb interval) revealed in these studies are very elevated compared to other systems.

Because of the lack of markers in the other half of the genome in the present experiments, this region was not studied. However, it appears that elevated recombination is also observed in that region. in that region. In most of the recombination events studied in the crosses between the AvaI$^-$ and the BamHI$^-$ mutants, double recombinants were observed with a recombination event in the small AvaI interval and a second one in the 3.6 Kb large AvaI–BamHI interval. Further experiments with mutants marked in multiple regions will be required to map the whole genome in a single cross. A site in the late region (between nucleotide 3092 and 32713) has been previously suggested to represent a hot spot of recombination [1, 2].

The fact that recombination appears to occur rarely in the enhancer region is somewhat surprising since the viral chromatin in this region is relatively devoid of nucleosome and it has been previously suggested that nucleosome free DNA (as that introduced in transfection experiments) is much more recombinogenic than chromatin [16].

The existence of recombination gradients has been associated with the presence of a hot spot of recombination. The best studied system is the *E. coli* Chi system [12, 14, 15] which is encoded by the sequence 5'–GCTGGTGG–3' [13] and which mediates generalized homologous recombination in *E. Coli* catalyzed by the enzymes of the Rec BCD pathway [9]. Whether a similar system is active here is of course a question quite far from reach at the present time. A few points are worth noting in this context are that the rate of homologous recombination undergone by the polyoma

viral chromatin are unusually high; recombination is seen in as high as 50 % of transformation cells derived from experiments carried out at moderate multiplicities (10–50 plaque forming units/per cell). Experiments also suggest that only those viral molecules which are interacting with the host chromosome (those actually engaged in the process of integration) are undergoing recombination. Thus it is likely that these are in contact with the host recombination machinery. Interestingly, nonhomologous recombination between the viral and the host genome appears to occur simultaneously with homologous recombination of 2 viral chromatin. The only viral protein which might be invoked in this process is large T–antigen. However, no strong case of the requirement of large T–antigen in this process is available to date. Thus this system should be a very helpful model system to study some of the parameters of homologous recombination in mammalian cells.

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