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THE INVOLVEMENT OF VIRAL DNA REPLICATION AND RECOMBINATION IN THE INTEGRATION PATHWAY OF POLYOMA VIRUS

By

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ABSTRACT

THE INVOLVEMENT OF VIRAL DNA REPLICATION AND
RECOMBINATION IN THE INTEGRATION PATHWAY OF POLYOMA VIRUS

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The possible contributions of viral DNA replication and recombination in the integration of viral DNA in polyoma-transformed cells were investigated. Extensive viral DNA replication was observed at early times after infection of rat cells at 33°C but not at 37°C. This was true for most strains of virus tested including wild-type A2. By ten days postinfection, the level of viral DNA was about 10-fold higher in cells infected at 33°C than in cells infected at 37°C, but the transformation frequency was only 2-3 times higher at the lower temperature. No differences were observed in the integration patterns of wild-type A2 in transformants obtained from 33°C and 37°C infections. Viral DNA replication occurred in only a small fraction of the infected cells (<0.2%). Large T-antigen expression is also higher at 33°C than at 37°C, and this viral protein is required continuously for the synthesis of viral DNA in nonpermissive cells. Since the increase in the level of viral DNA at 33°C did not dramatically increase the transformation frequency or alter the integration pattern in transformed cells, it is doubtful that this increase affects the integration pathway of polyoma. One of the viral strains (NG59RA)

which did not display an increased level of viral DNA at 33°C was investigated further. Hybrid viruses consisting of sequences from both A2 and NG59RA were constructed and analyzed for their ability to synthesize viral DNA at 33°C in nonpermissive cells. Using this approach, a mutation affecting DNA replication was mapped to large T-antigen. The fate of the viral genome in nonpermissive cells was also studied by infecting rat cells with two restriction site-minus strains. Recombination between the parental genomes was followed by the detection of wild-type restriction fragments in the population of unintegrated viral genomes at early times after infection and in transformants resulting from the mixed infections. No recombination was detected in the former, but evidence of interviral homologous recombination was observed in 38% of the transformants. These results suggest that recombination is involved in the integration pathway of polyoma virus. Since recombination was not detected at early times after infection, it may only occur in a small population of cells which ultimately become transformed.

To Mom and Dad

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Literature Review

Introduction

Polyomavirus (Py) is a DNA tumor virus of mice. It and the other members of the polyomavirus genus, the JC and BK viruses of humans, simian virus 40 (SV40) and B lymphotrophic virus of monkeys, and hamster papovavirus, are grouped with the papillomavirus genus to form the papovaviridae family (1). Papovaviruses are nonenveloped viruses with a double-stranded covalently-closed circular DNA genome which, in the case of Py, is 5,292 base pairs (bp) in length and codes for six proteins (2). The DNA is packaged in the viral capsid as a minichromosome consisting of about 24 nucleosomes (2). The icosahedral capsid has a diameter of 45 nm and is composed of three viral structural proteins, VP1, VP2, and VP3, with VP1 being the major component (2).

Py was named because of its ability to induce tumors in a wide variety of cell types after experimental infections of neonatal mice with large doses of virus. A high incidence of epithelial tumors occurs in the major and minor salivary glands, the mammary glands, the hair follicles, and the thymus (3). Additionally, mesenchymal tumors occur at numerous sites (3). The virus can infect over thirty cell types, with the kidney being the major site of viral replication and persistence (3). Although the virus is prevalent in the natural population, the incidence of tumors is actually low. The two major

factors that contribute to the protection of the natural population from tumors are passive immunity acquired during gestation and low doses of infecting virus. The mechanism of tumor protection by the immune system remains an interesting unsolved question.

The availability of tissue culture systems for the propagation of the virus in vitro has led to a detailed understanding of its molecular biology and genetics. Py is only able to grow lytically in mouse cell cultures, and these cells are therefore referred to as being permissive for virus growth. Lytic growth is initiated by the expression of the three early viral genes, an event which occurs prior to viral DNA replication. One of the early proteins, large T-antigen (LT), is required for the initiation of viral DNA synthesis and regulates the expression of the early genes. Following amplification of the viral genome, LT-stimulated expression of the three late genes encoding the viral structural proteins occurs. Virions are then assembled in the nucleus and released by cell lysis. In addition to mouse cells, Py can also infect rat and hamster cells, but cells from these two species are nonpermissive and do not support replication of the virus. Rat and hamster cells, however, are useful for the study of neoplastic transformation by the virus. In nonpermissive cells the infecting virus reaches the nucleus but only the early genes are expressed. Little viral DNA synthesis or late gene transcription is observed. A small percentage (<1%) of infected nonpermissive cells become stably transformed. This state requires the continual expression of the early genes. Maintenance of the genome is ensured by integration into the

host DNA. Growth properties of Py-transformed cells in culture include a high saturation density, a reduced serum requirement, anchorage independence, lack of contact inhibition, growth on a monolayer of normal cells, and tumor formation upon injection into susceptible animals (2). These traits, along with some other cell surface and intracellular properties, are also transiently displayed in a larger fraction of the infected nonpermissive cells. This phenomenon is termed abortive transformation and occurs at a rate that is 10-100 times greater than stable transformation. These cells do not become stably transformed because of either a failure of the viral genome to integrate or the integration occurs in such a way as to prevent early gene expression (4).

The following review will focus on the function of the early viral proteins in both permissive and nonpermissive cells, on viral DNA replication, and on integration of the viral genome in stably transformed cells. A summary of recombination in mammalian cells is also included since this topic is relevant to the understanding of the mechanism of viral integration.

Early gene expression and DNA replication in permissive cells

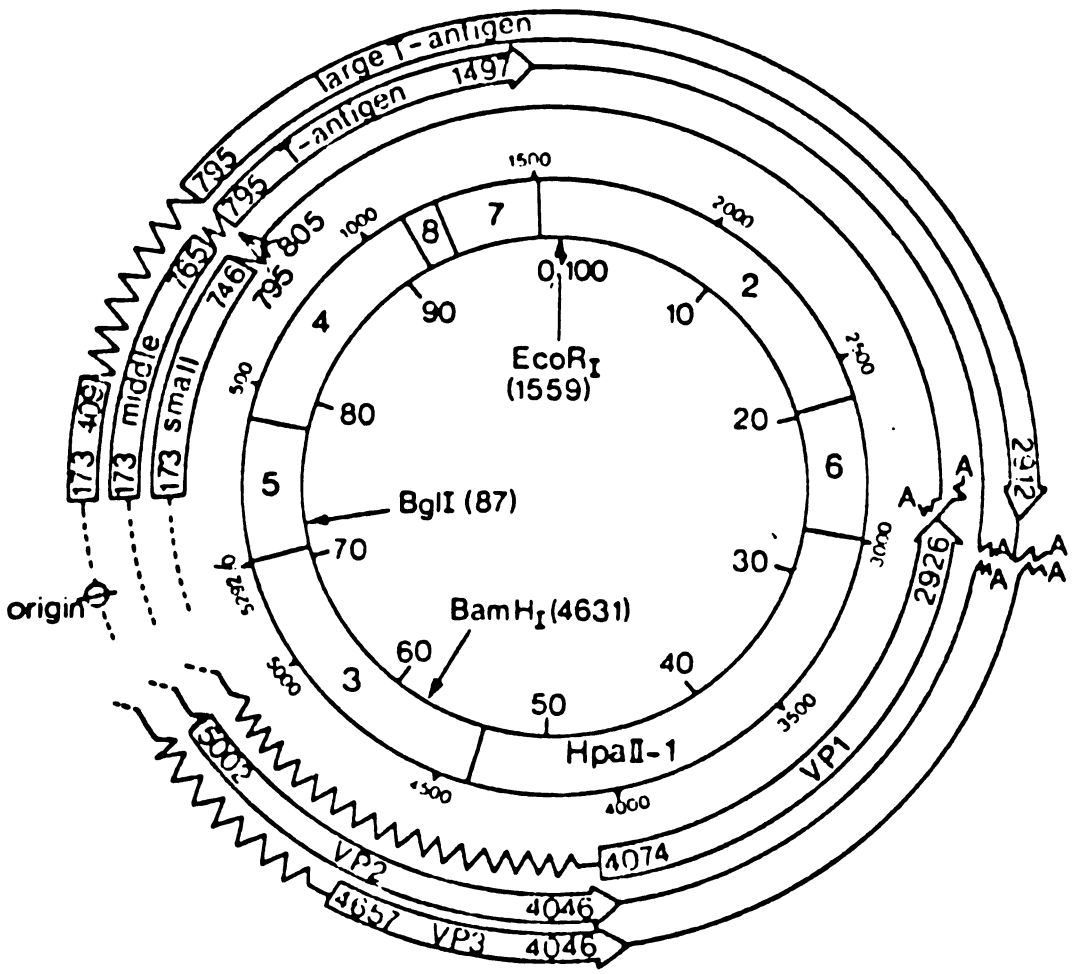
The first stage of infection of permissive cells with Py involves attachment of the virion to a cell receptor, penetration of the cell membrane, and uncoating of the viral genome. These events can be completed as early as 15 minutes after infection, but by 3 hours postinfection only about 16% of the absorbed virus has reached the nucleus (5). The capsid protein VP1 plays the major role in the

adsorption of the virus to the host cell receptor, a complex which consists of at least three membrane proteins (5,6). Recently, it has been discovered that the interaction between Py and the receptor induces the expression of two cellular proto-oncogenes, c-myc and c-fos (7). The protein products of these two cellular genes are thought to be important in the stimulation of cellular DNA synthesis. This is advantageous to the virus since it requires host enzymes for its gene expression and DNA replication. Internalization of the virus occurs via monopinocytotic vesicles which transport the virus to the nucleus where decapsidation takes place (5,8,9). Once in the nucleus the Py genome is capable of being transcribed and replicated by host enzymes. The early and late genes are transcribed in opposite directions from the 350 bp noncoding region which contains the origin of DNA replication (ori), the early and late gene promoters, and a transcriptional enhancer (Fig. 1) (10).

The three early proteins are called the tumor (T) antigens because they were first defined by antibodies present in the sera of mice bearing Py-induced tumors (11). Large T-antigen (LT) is a nuclear phosphoprotein with a molecular weight between 88 and 100 kilodaltons (kd) (12,13). Multiple forms of this protein have been detected by differences in electrophoretic mobility that can be accounted for, in part, by differences in the phosphorylation state (12,14,15). Middle T-antigen (MT) is a cytoplasmic or membrane phosphoprotein that is present in the cell as two distinct species, the 56 kd and the 58 kd forms, which differ in their phosphorylation patterns (12,13,16). The

Figure 1. Physical map of polyoma virus genome.

The inner circle represents the HpaII restriction map of polyoma virus with the numbering system of Soeda et al. (10) beginning at the junction of fragments 3 and 5. The origin of DNA replication is marked. The boxed regions represent the protein coding sequences of the early and late genes. The jagged lines represent the introns. The nucleotides of the splice junctions and of the termini of the coding regions are also shown. The figure is taken from Soeda et al. (10).


large T-antigen
1497
T-antigen
795
795
765
805
746
795
173 small
173 middle
173 4029
origin
5003
VP2
4057
VP3
4046
4046
4074
VP1
2926
2312
A
A
A
A
A
A
EcoR$_I$
(1559)
BglI (87)
BamH$_I$(4631)
HpaII-1
0
100
10
20
30
40
50
60
70
80
90
1500
2000
2500
3000
3500
4000
4500
5000
500
1000
1
2
3
4
5
6
7
8

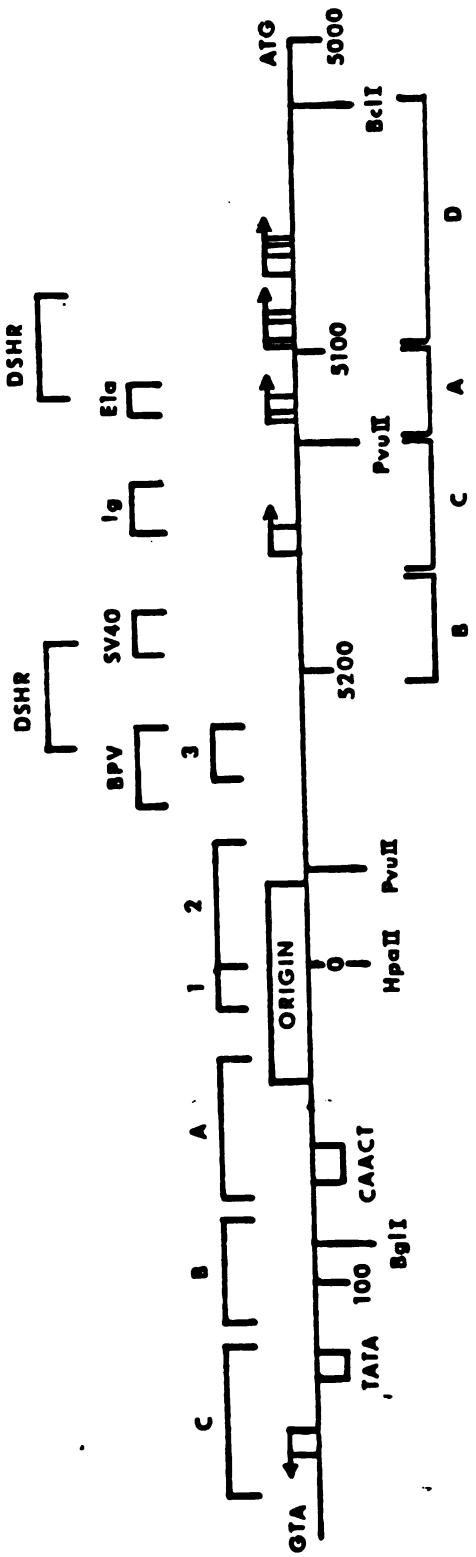
58 kd species represents less than 10% of the total MT in Py-infected cells (16). MT has a hydrophobic region of 22 amino acids at its carboxy terminus which allows it to become anchored in the plasma membrane while its amino terminus extends into the cytoplasm (10). Small T-antigen (ST) is mainly localized in the nucleus and has a molecular weight of 22 kd (12).

The T antigens are encoded by three different mRNAs transcribed from the early region (17,18). The polyadenylated mRNAs share common 5' and 3' termini with the major start sites of transcription being nucleotides 147 and 152 as determined by primer extension and S1 mapping (Fig. 2) (18,19). The translational start site for each of the proteins is located at nucleotide 173 (10). The three mRNAs encode different proteins, however, as the result of differential splicing (Fig. 1) (18,20).

The noncoding region of Py includes the promoter elements required for early and late gene transcription (19-22). The early TATA and CAAT boxes are located at nucleotides 122 and 64, respectively (Fig. 2) (10). The late promoter overlaps the transcriptional enhancer and does not contain TATA and CAAT consensus sequences. This may explain the extreme heterogeneity found at the 5' termini of late mRNAs (22). Located between the promoters is an 80 bp sequence (ori) that is required for DNA replication as determined by deletion mutagenesis (Fig. 2) (23-27). The essential nucleotides of the ori include a string of eight A:T base pairs, at least five of which are required for viral DNA replication (25), and a 34 bp sequence of dyad symmetry (25)

Figure 2. Physical map of the noncoding region of polyoma virus.

The map represents the polyoma genome from the early translation start site (left side) to the late translation start site (right side). Also shown are the early and late transcription start sites (arrows). The TATA and CAAT boxes of the early promoter and the origin of replication are boxed. The large T-antigen binding sites are shown in brackets above the genome with the major sites labeled A, B, and C and the minor sites labeled 1, 2, and 3. The four enhancer elements (A-D) are shown in brackets below the genome. The regions of homology to the bovine papilloma virus (BPV), SV40, mouse immunoglobulin, and adenovirus Ela enhancers are shown in brackets above the genome. The DNase hypersensitive regions (DHSR) are also shown. The figure is taken from Kern et al. (43).


DSHR
Ela
Ig
DSHR
SV40
BPV
3
2
1
ORIGIN
0
HpaII
PvuII
A
B
CAACT
BglI
100
TATA
C
GTA
ATG
5000
BclI
D
5100
PvuII
A
C
B
5200

which is conserved in the SV40 ori (28). The ori is bordered on the early side by three high-affinity binding sites for LT (29,30). A common feature of these sites is the pentanucleotide 5'-G(A>G)GGC-3' that is repeated 2-4 times per site at 9-11 bp intervals (29-31). Two additional minor binding sites are located within the ori and another one is found within the transcriptional enhancer (Fig. 2) (30). Sequences within the enhancer are not only required for transcription but are also required for DNA replication (25,27,32,33). This does not, however, reflect a transcriptional requirement for replication (30-32).

The enhancers from SV40 and Py were the first such transcriptional elements described (34-38). In general, enhancers act by stimulating transcription from linked promoters in an orientation- and distance-independent manner (39). The Py enhancer is located between nucleotides 5046 and 5289 (Fig. 2) (34) and is required in cis for early gene transcription (26,40-42), DNA replication (25,27,32,33), and late gene transcription (43). Deletion mutagenesis has been used to divide the Py enhancer into several structurally distinct elements that are, to an extent, functionally redundant (Fig. 2) (33,44,45). The BclI-PvuII restriction fragment contains the A and D elements (33) and is able to activate transcription from the chicken α-collagen promoter in mouse fibroblasts (44) and from the rabbit β-globin promoter in HeLa cells (34). Within the A element is a sequence which is homologous to the adenovirus Ela enhancer (46). A tandemly repeated 26-mer from the A element is sufficient to stimulate Py early gene transcription and

viral DNA replication in mouse fibroblasts (33,47). Several naturally occurring strains of Py, such as P16 and Toronto large plaque, have duplications within this region which do not affect the phenotype of the virus (48,49), but Py strains with a duplication of the A domain and a deletion of the B domain (described below) can grow on mouse PCC4 embryonal carcinoma (EC) cell lines which are nonpermissive for wild-type Py (50). The PvuII restriction fragment contains the B and C enhancer elements (Fig. 2) (33). The former shares sequence homology with the core region of the SV40 enhancer (51) while the latter contains sequences homologous to the immunoglobulin G enhancer (52). Additionally, sequence homology to the bovine papilloma virus enhancer is found between the B element and the ori (53). Py mutants selected to grow in nonpermissive mouse F9 EC cells have a single A-G transition at nucleotide 5248 within a duplicated B domain (40,47,50,54).

Cellular proteins have been shown to bind to transcriptional enhancers in order to regulate transcription in a positive or a negative manner. Several of these transcription factors have been shown to bind to the Py enhancer. Two A element-binding proteins (PEA1 and PEA2) (55) and two B element-binding proteins (PEB1 and PEB2) (56,57) have been identified in mouse 3T6 cells. Additionally, a C element-binding protein has been observed in differentiated and undifferentiated murine F9 EC cells, mouse L cells, and HeLa cells (58). PEA1 also binds to the SV40 and c-fos enhancers (55) and may be the same as activator protein 1 (AP1), a HeLa cell protein which interacts with the SV40 enhancer (59). The interaction of cellular

proteins with the Py enhancer may lead to the formation of the DNAse I hypersensitive sites localized to the late side of the A element and to the early side of the B element (Fig. 2) (60). The expression of cellular transcription factors may be tissue- or stage-specific. Therefore, the redundant enhancer elements found in the Py genome may be necessary in order for the virus to replicate in many different cell-types in the mouse.

Not only are cellular proteins important in the expression of viral genes, but LT is also a key regulatory factor. An overproduction of early mRNA occurs when cells infected with a viral mutant encoding a thermolabile LT (ts-a mutant) are shifted to the nonpermissive temperature (17). In a wild-type infection direct interaction of LT with the major binding sites near the ori results in negative autoregulation of early gene expression (31,61,62) and induction of late gene expression (43). Interestingly, LT also stimulates transcription from cellular promoters (63-65), but this effect may be indirect since the DNA binding domain of LT is not required for the stimulation (65).

Utilization of ts-a mutants has also established that LT is required for the initiation of viral DNA synthesis. When shifted to the nonpermissive temperature the ongoing round of viral DNA synthesis is completed but further initiation does not occur (66). LT may also have a mitogenic effect on host DNA synthesis (67). Phosphorylation of LT may play a significant role in its ability to perform multiple functions within the infected cell. In the comparison of infections of

quiescent and growing cells, LT is less phosphorylated in the growth-arrested cells early in infection. Following cellular DNA synthesis, though, the levels of LT phosphorylation are equivalent in the two populations of infected cells (68). These results can be interpreted to mean that dividing cells provide enzymes that are required for modifications of LT. The phosphorylation state of LT may be crucial to viral DNA synthesis, since the LT of replication-defective ts-a mutants is underphosphorylated (68).

The mechanism of papovavirus DNA synthesis is known in considerable detail. DNA synthesis begins with two replication forks moving in opposite directions from the ori (69,70). DNA synthesis from each of these forks proceeds in a semidiscontinuous manner (71). The two sites of initiation of continuous DNA synthesis have been mapped to a 16 bp region in the ori (72). The development of cell-free systems to replicate SV40 and Py DNA has led to a greater understanding of the roles of viral and cellular proteins in this process (73-76). For both Py and SV40, LT is the only viral protein required for viral DNA synthesis <u>in vitro</u> (73-76). With regards to its role in DNA synthesis it should be noted that Py LT is not only a DNA binding protein but it is also an ATPase (77) and a nucleotide binding protein (78). These two activities are structurally distinct and have been mapped to a region between the carboxy-terminus and the DNA binding domain located between amino acids 290 and 310 (79-81). Experiments reported in Chapter 3 of this thesis describe a new mutation in LT between the DNA binding region and the ATPase domain that affects DNA replication.

This point mutation causes a proline to alanine change that may alter the tertiary structure of the protein.

In addition to the activities mentioned above, SV40 LT also has a helicase activity *in vitro* (82,83). By using the cell-free systems it has been shown that LT is required prior to the initiation of DNA synthesis (84). This step also requires ATP and cellular proteins but not dNTPs (84). During preinitiation LT may function to unwind the ori to allow for the binding of DNA polymerase α/primase (83). Since SV40 LT has helicase activity it has been suggested that it is involved in elongation as well as initiation. This has been shown to be the case *in vitro* (82,83), but *in vivo* results show that LT dissociates from the viral genome after replication is 70-80% complete (85).

Both SV40 and Py have a narrow host-range with regard to productive infections. Py only replicates in mouse cells while SV40 replicates in monkey and human cells (2). The cell-free replication systems have allowed, in part, for an analysis of the cellular proteins involved in permissivity. SV40 DNA is replicated *in vitro* in the presence of LT and extracts from either human or monkey cells but not from mouse cells (86,87). Py DNA replication, on the other hand, proceeds in the presence of mouse cell extract but not human or monkey cell extracts (86). Addition of the partially purified mouse DNA pol α / primase complex to HeLa or monkey cell extracts allows for *in vitro* synthesis of Py DNA, suggesting that the DNA pol α/primase is a major factor in permissivity for papovaviruses (86). Monoclonal antibodies to SV40 LT have been shown to coprecipitate LT and DNA pol α from

SV40-infected HeLa cell extracts, suggesting that these proteins interact directly (88). It is likely that other cellular proteins besides DNA pol α/primase are responsible for the permissivity of Py DNA replication. Experiments presented in Chapter 2 of this thesis show that a considerable amount of viral DNA synthesis occurs in established rat cell lines at 33°C as compared to 37°C. Other workers have shown that replication of the integrated viral genomes in Py-transformed cell lines occur in 1-2% of the cells (89). Other lines of evidence from SV40 infections also implicate additional factors. Infection of preimplantation mouse embryos with SV40 results in the production of infectious virions (90).

Other cellular proteins known to be required for in vitro replication include topoisomerase I (91); a single-stranded DNA binding protein (91); proliferating cell nuclear antigen (PCNA), a cell-cycle regulated protein associated with cellular DNA replication (92); and topoisomerase II which is required for resolution of the two daughter DNA molecules (93).

In contrast to the case of LT, little is known about the functions of MT and ST in the lytic cycle. Hr-t mutations located in the LT intron affect both MT and ST and have been studied in detail. The minichromosome of hr-t mutants is deficient in the acetylation of histones H3 and H4 (94). Hr-t mutants also have an underphosphorylated VP1 (98), a decreased output of viral DNA (95), and an absence of induction of c-myc and c-fos expression postinfection (7) as compared to wild-type infections. They also stimulate only one round of

cellular DNA synthesis as compared to the multiple rounds induced by wild-type strains (67). These observations show that either ST, MT, or both have a role in virus assembly, viral DNA replication, and virus-host interactions involving cellular gene expression and DNA replication. When ST alone is introduced into mouse NIH-3T3 cells they grow to a higher saturation density than monolayers of the same cells in the absence of ST (96,97). This result suggests that ST has a role in growth regulation of Py-infected cells (7,96). The question remains, however, whether this function is unique to ST or if it is also a function of MT since only 4 out of 196 amino acids of ST are not shared with MT.

Neoplastic transformation by polyomavirus

As described above, Py is able to transform nonpermissive rat and hamster cells is culture and to cause tumors in newborn mice (2). Early studies utilized two groups of nontransforming mutants, the ts-a (99-101) and hr-t strains (102,103), to determine the viral proteins involved in neoplastic transformation. Mixed infections at the nonpermissive temperatures with mutants from these two complementation groups generate stable transformants (104,105). With the knowledge that the hr-t mutations map to the LT intron and affect both ST and MT (106,107) and that the ts-a mutations map to the carboxy-terminal half of LT (14,15,106,107), it was evident that stable transformation by Py required LT and either ST, MT, or both MT and ST. Subsequent experiments revealed that transformation by Py can be divided into two steps called initiation and maintainence. The initiation event

requires the transient expression of LT and results in the integration of the viral genome (99-101,108). Maintainence of the transformed state is achieved with that part of the early region encoding, ST, MT, and the amino-terminal half of LT (109-112).

More recent developments using expression vectors or reconstructed Py genomes encoding only one early protein have led to a more detailed understanding of the activities of ST, MT, and LT in transformation. A Py genome encoding only MT is able to transform established rat cell lines (113). Established or immortalized cells, as opposed to primary cells, are able to grow in long-term culture. Established cell lines are derived from rare cells within a population of primary cells that survive many passages in culture. The establishment of cells is considered to be an initial step in the pathway toward full transformation of a cell. Transformants from the experiment above are tumorigenic as demonstrated by their ability to form tumors after infection into Fischer rats (113). DNA encoding only MT is also tumorigenic in newborn hamsters but not in newborn rats (114,115). MT alone, however, cannot transform primary rat embryo fibroblasts (116). These results clearly demonstrate that MT plays the major role in the maintainence of transformation by Py. The fact that it does not cause tumors in newborn rats or transform primary rat cultures, however, demonstrates a requirement for the other early viral proteins.

In contrast to MT, LT by itself cannot transform established rat cell lines (114,115). It can, however, immortalize primary mouse and rat embryo fibroblasts (117). The immortalizing function has been

mapped to a 200 bp region in the 5'-proximal half of the LT gene (118). Immortalization of cells is not understood at the molecular level, and it is not clear how LT functions in this capacity. One phenotype of LT-immortalized cells, however, is a reduced requirement for serum growth factors (117). Since LT is a DNA binding protein its action may be a regulator of cellular gene transcription. Immortalization by LT, though, does not require the DNA binding domain of this protein (81).

The question of which viral proteins besides MT are required for tumorigenesis in newborn rats was answered by injecting DNA fragments encoding either ST or LT along with the MT gene. Surprisingly, these experiments revealed that either ST or LT could cooperate with MT to cause tumors in this host (114,115). In contrast to these results, transformation of primary rat embryo fibroblasts requires all three early proteins (115). As described above, established rat cells can be transformed by MT, bypassing the need for LT and ST. It should be noted that these transformed cells require serum growth factors to remain viable (116). The growth factors apparently alleviate the need for LT or ST. These results support the theory that tumorigenesis is a complex process that requires not one but multiple cellular events (119). Additional support for this theory comes from transfections of primary cells with combinations of proto-oncogenes and polyoma early genes. Either LT or c-myc can complement c-ras in the transformation of primary mouse embryo fibroblasts (120), while primary baby rat kidney cells are transformed by a combination of the Ela proteins of adenovirus and either MT or c-ras (121).

The mechanism by which MT acts to transform cells has been the subject of intense interest in recent years. MT from wild-type infected cells but not from hr-t infected cells is associated with a tyrosine kinase activity *in vitro* (13,122,123). It was later discovered that the kinase activity is contributed by the proto-oncogene $pp60^{c\text{-}src}$ (c-src) (124). The kinase-active MT/c-src complex is associated with the plasma membrane (16,124,125), and monoclonal antibodies to either MT or c-src will coprecipitate the two proteins from Py-infected cell extracts (126). Only a small fraction of the MT in an infected cell is associated with c-src (127), and it is the level of this complex which determine the transformation phenotype. Cells containing the MT gene linked to an inducible promoter differ in transformation state depending upon the level of kinase-active MT/c-src (128). Anchorage-independent growth and tumor formation require higher levels of the complex than are required for focus formation and morphological transformation (128). It appears that the 58 kd form of MT is the one that activates c-src. MT from the hr-t mutant, NG59, can be phosphorylated to produce the 56 kd species but not the 58 kd species (16). The 56 kd form associates with c-src but is inactive in the *in vitro* kinase assay (129). In wild-type infections phosphorylation of the 58 kd form, but not the 56 kd species, is stimulated by the tumor promoter 12-Ø-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C (130). The MT/c-src interaction increases the tyrosine kinase activity of c-src *in vitro* probably by altering the phosphorylation state of the protein

(131-133). MT-associated c-src is deficient in phosphorylation at tyrosine 527 (134). Site-specific mutagenesis of the c-src gene has shown that phosphorylation at this position decreases the tyrosine-specific kinase activity of the protein (135-137). Some points concerning the kinase activity of the c-src/MT complex are still not clear. For one thing, it is not kown if the increased kinase activity of MT/c-src seen *in vitro* is relevant to its activity *in vivo*. The level of phosphotyrosine in Py-transformed cells is not increased with respect to untransformed cells (138). This could be explained, however, if the MT/c-src complex has one or a few specific substrates whose increase in phosphorylation did not raise the level of total phosphotyrosine by a detectable amount. As yet, a specific substrate for MT/c-src has not been identified.

The c-src binding domain of MT has been localized to the amino-terminal end of the molecule. Mutations within this region prevent association with c-src (139-142). Tyrosine phosphorylation of MT may also play a role in its association with and activation of c-src. The phosphorylation of four tyrosines in the carboxy-terminal half of MT (amino acid positions 250, 297, 315, and 322) has been studied using site-directed mutagenesis and hr-t mutants. While the absence of phosphorylation of 297 and 322 has no effect on the transforming activity of MT, the absence of phosphorylation on either 250 or 315 decreases, but does not completely eliminate, the transforming ability of MT (143,144). Thus, the phosphorylation state of MT may influence its ability to interact with c-src to form a kinase-active complex.

Stable transformation of cells by Py requires the integration of the viral genome into the host DNA (2). The viral DNA is usually integrated as a head-to-tail tandem (109,145,146). The generation of integrated tandems requires LT (147) and a functional viral origin of replication (148). Recombination between the host and viral DNA appears to be nonhomologous with only a 2-5 bp homology at the viral-host join (149-152). As analyzed by hybridization of Southern blots of DNA from Py-transformed cell lines, integration is apparently random with respect to both the host and the viral DNA (145,146). Integration sites in the host genome, however, have not been mapped using in situ hybirdization of chromosomes. Integration of Py can result in deletions, duplications, and rearrangements of host DNA at the integration site by mechanisms which have not been elucidated (152,153).

Two models have been proposed for the integration of head-to-tail tandem viral genomes. The first invokes rolling-circle replication of the viral genome to produce a linear multimer that may serve as a substrate for integration. Such a model would fit with the requirements for LT and the viral origin of replication. High molecular weight forms of SV40, proposed to have arisen by rolling-circle replication and to be the precursors to the integrated tandems, have been identified in infections of nonpermissive cells (154). Py genomes that appear to be involved in rolling-circle replication have been identified in Py-infected mouse cells by electron microscopy (155). Evidence for a recombination step in the

tandemization of Py genomes comes mainly from the study of integrated viral genomes in transformed cell lines. When fused with permissive cells, transformants from mixed infections with a ts-a strain and an hr-t strain yield, in most cases, wild-type virus along with the two parents, suggesting that the two parental genomes recombined at some time during the integration process (156). Cointegration of the two parental genomes at a single site has been demonstrated in one cell line from this experiment (157). In Chapter 4 of this thesis, further evidence for a high level of recombination in the integration pathway is provided. In these experiments mixed infections with two restriction-site minus mutants of Py resulted in recombination of the two parents in about 35% of the cell lines analyzed. With regard to the role of LT in the recombination model, some evidence has emerged to suggest that this viral protein has a recombinogenic activity. Intramolecular recombination of a plasmid containing 1.Ø3 copies of the Py genome is enhanced in murine cells if LT is provided in trans (158). In this system, replication of the Py plasmid inhibited recombination, a result that may indicate that the function of LT in recombination is different than its function in DNA synthesis (159). Recombination and replication may compete for a limiting amount of LT. For tandem formation during integration, however, recombination and replication may be linked. If recombination is the favored pathway for tandem formation, then the requirement for a functional ori in this process may reflect a need for a replicating substrate. It is also possible that both rolling-circle replication and recombination are involved in

tandem formation. In this model a rolling-circle intermediate is first synthesized and then is capable of recombining with either viral DNA monomers or multimers. The reverse is also possible with recombination occurring prior to rolling-circle replication.

After integration, the Py ori remains active. The viral DNA produced from integrated genomes ("free" viral DNA) is supercoiled and when a transformed cell population is analyzed, the viral DNA is present at the level of 10-60 copies per transformed cell (160), but the free DNA is only present in a small number of the transformed cells at any one time so that the number of copies of DNA per producing cell is actually 100-1000 times higher than this figure (89). Excision of integrated viral DNA from transformed cells also occurs at a high rate to generate cells which have reverted to the normal phenotype (89,145,146). Excision requires regions of Py sequence homology (161,162), LT (146), and a functional origin of replication (163). The same requirements have been found for the amplification of integrated viral genomes (161,163).

DNA recombination in mammalian cells

DNA recombination in mammalian cells is not as well understood as in the prokaryotes and fungi, but substantial progress has been made recently due to the development of recombination assays based on the introduction of exogeneous DNA substrates into cultured cells via calcium phosphate precipitation, DEAE-dextran mediated transfection, microinjection, or viral infection. Assays have been designed to measure either homologous or nonhomologous recombination using

substrates which undergo either intra- or intermolecular recombination. The following discussion will review these methods and some of the models proposed for DNA recombination in mammalian cells.

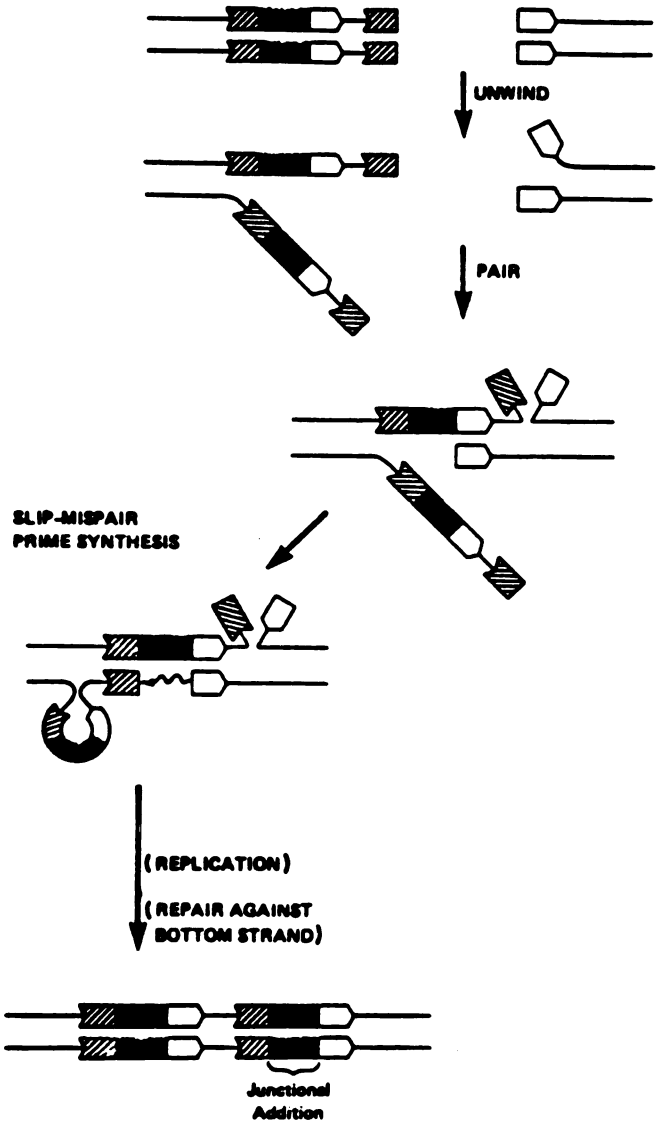
Examples of recombination events which require little or no sequence homology are prevalent in mammalian cells and include integration of papovaviruses (149-152), reciprocal chromosome translocations (164), rearrangements of antibody and T-cell receptor genes (165,166), gene amplification events (167), and the formation of processed pseudogenes (168). In general, the mechanism of nonhomologous recombination is thought to involve DNA breakage and end joining (169-172). Linear DNA introduced into mammalian cells can efficiently undergo nonhomologous recombination by end joining and thus may serve as useful assay for this recombination pathway (169-172). The mechanism of the ligation of ends in these systems is similar to that seen at the junctions found in reciprocal chromosome translocations (164) and antibody gene rearrangements (165). The common element in these events is addition of nucleotides of unknown origin at the junctions. It has been proposed that the extra nucleotides are added to free ends by terminal transferase prior to ligation (165,169,173,174). An extensive analysis of nonhomologous recombination junctions following recircularization of a linearized SV40 genome has been carried out. In this experiment the ends of the linearized SV 40 genome were mismatched with one being blunt and the other having a 5'-protruding strand (173). Sequencing of the junctions of the recircularized SV40 genomes revealed that 87% of them had arisen

by end joining as described above, while the remaining 13% belonged to a class in which the junctions contained duplications. In a model to explain the minor class of junctions it was proposed that the free ends mispair by way of short homologies (Fig. 3). The mispaired region could then serve as a template for repair synthesis restoring the duplex but leaving an unpaired region. Further repair synthesis against the strand with the unpaired region creates a duplication (173).

Homologous recombination events that occur in mammalian cells include meiotic recombination (174), mitotic recombination (175), sister chromatid exchange (176), and gene conversion (177). Homologous recombination of DNA introduced into mammalian cells has also been demonstrated (178-186). These systems usually involve the transfection of two nonfunctional copies of either a selectable gene or a viral genome into the appropriate cells. Recombination is then quantitated by assaying for a functional gene or viral genome. In these systems the frequency of recombination depends upon the length of homology (172,182,183,187,188) and is increased by the presence of double-strand breaks in or near the homologous regions (181,182,189- 191). In addition it has been observed that homologous recombination in these experiments is nonconservative or nonreciprocal (182,190,192, 193). To explain these observations, the double-strand break (194) and the single-strand annealing models (182,195) have been proposed. In the former a double-strand break or gap in one DNA molecule provides an end to initiate a strand invasion event in a region of homology on a second DNA molecule. The invading strand is used as a primer for DNA

Figure 3. Proposed model for formation of duplicate junctions following nonhomologous recombination.

Short homologies are shown with open and hatched boxes. As illustrated in the figure, the duplication occurs following mispairing of one of the 3' ends and repair synthesis. The figure was taken from Roth et al. (173).



DUPLICATION JUNCTION

synthesis with the intact copy of the homologous region being used as the template. This model requires that the double-strand break be located in a region of homology which is shared by the two molecules. Homologous recombination between DNA molecules in which the break occurs within a region of nonhomology cannot be explained by this model (182,192,193). To account for these results the single-strand annealing model was formulated (182,195). In this model the double-strand break is a substrate for either a 5' to 3' (182) or a 3' to 5' (195) exonuclease that produces complementary single-strands (Fig. 4). After reannealling, the molecule is repaired by DNA synthesis. Additionally, in vitro systems for recombination have been developed using whole cell extracts (196-198). Using this approach a recombinase from human cells has been partially-purified (199). Such advances should be extremely beneficial in the study of the mechanism and enzymology of homologous recombination in mammalian cells.

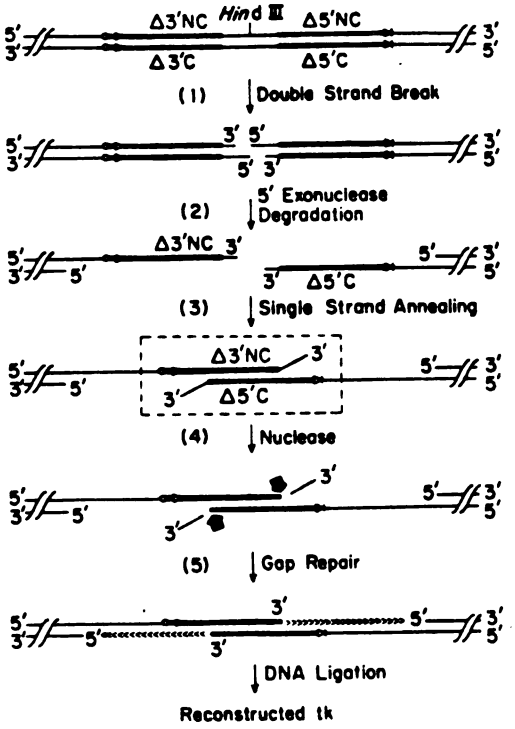
The ratio of homologous to nonhomologous recombination has been measured in several systems which utilize either intramolecular (171,172,189-200) or intermolecular recombination (187). One of the intramolecular recombination assays employed a linearized SV40 genome with a 131 bp duplication at its termini (171). A functional genome could be produced by either homologous or nonhomologous recombination. Following transfection of the DNA into monkey cells, recombination was scored by plaque assay. The subsequent sequence analysis of functional recombinant genomes indicated that nonhomologous recombination was favored by a factor of 2-3 over homologous recombination (171). One

Figure 4. Single-strand annealing model of homologous recombination.

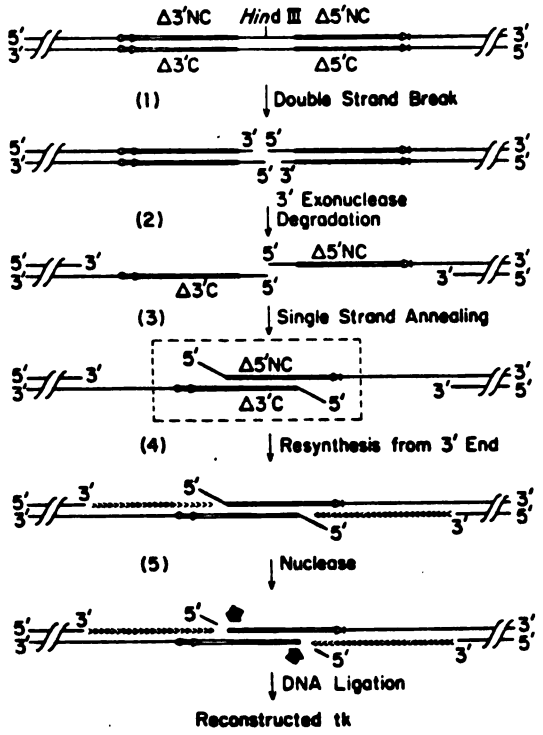
A.) Generation of single strands by 5' exonuclease. The hatched regions represent two nonfunctional copies of the herpesvirus thymidine kinase (tk) gene. A double-strand break between regions of homology is followed by exonuclease digestion of the free 5' ends to reveal single-stranded regions of homology. Homologous pairing and repair synthesis produces a functional tk gene. The figure is taken from Lin et al. (182).

B.) Generation of single strands by 3' exonuclease. Two non-functional copies of the herpesvirus tk gene are shown as black boxes. A double-strand break between the regions of homology is followed by 3' exonuclease activity. The single-stranded regions are repaired as above. The figure is from Lin et al. (195).

A.


Δ3'NC Hind III Δ5'NC
Δ3'C Δ5'C
(1) Double Strand Break
(2) 5' Exonuclease Degradation
(3) Single Strand Annealing
(4) Nuclease
(5) Gap Repair
DNA Ligation
Reconstructed tk

B.


Δ3'NC Hind III Δ5'NC
Δ3'C Δ5'C
(1) Double Strand Break
(2) 3' Exonuclease Degradation
(3) Single Strand Annealing
(4) Resynthesis from 3' End
(5) Nuclease
DNA Ligation
Reconstructed tk

intramolecular assay involved introduced of both a bacterial plasmid with the ampicillin resistance gene and a mammalian expression vector into mouse cells. The two substrates shared regions of homology. After transfection, low molecular weight DNA was extracted from the cells and used to transform E. coli. Ampicillin-resistant colonies were screened by in situ hybridization for the presence of the expresssion vector. Analysis of the recombination junctions revealed that nonhomologous recombination was favored over homologous recombination by a factor of seven (187). In comparison, nonhomologous recombination is favored over homologous recombination by a factor of 10^4 to 10^5 with regard to integration of exogenous DNA into the host DNA (201).

In general, the cellular machinery is very efficient in recombining DNA introduced into cells. This is especially true for linear DNA. In all cases examined, nonhomologous recombination is favored over homologous recombination. Recombination is also very rapid. When DNA is introduced directly into the nucleus the recombination events occur within one hour (190). Experiments are described in Chapter 4 of this thesis that show that homologous recombination between Py genomes is high in cells that have been transformed by the virus.

SUMMARY

Since the discovery of Py over thirty years ago, a large body of information has been obtained concerning its biology. Many of its interactions with permissive and nonpermissive cells are understood in

great detail. Py and SV40 have been particularly important in the discovery of RNA splicing and transcriptional enhancers. These viruses have also been beneficial in the analysis of eukaryotic DNA replication, neoplastic transformation, and control mechanisms for eukaryotic gene expression.

The experiments presented in this thesis have been briefly described in the text above. In summary, the pathway of integration of the viral genome in Py-transformed cells was investigated. Evidence for the replication of viral DNA in nonpermissive cells was obtained, but this replication could not be tied to the formation of the integrated tandems. In the course of this work a new LT mutation was discovered which affected viral DNA replication in nonpermissive cells. In another set of experiments, evidence for homologous recombination between viral genomes in the integration pathway of Py was obtained. From these results I conclude that recombination can account for a major fraction of the integrated tandems in Py-transformed cells but other mechanisms to produce tandems, such as rolling-circle replication, cannot be excluded.

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Chapter 2.

The Role of Viral DNA Synthesis in Neoplastic Transformation of Nonpermissive Rat F-111 Cells by Polyoma Virus[1]

ABSTRACT

We have investigated the role of polyoma viral DNA synthesis in the formation of the precursors to the integrated viral sequences found in cells transformed by the virus: i.e. tandem repeats of the viral genome. We show that after infection of Fischer rat F-111 cells, polyoma DNA synthesis at 37°C results in a 2-3-fold increase over the input signal. In situ hybridization shows that synthesis is restricted to a small fraction of the population. In contrast, viral DNA synthesis at 33°C is about ten times higher than at 37°C for most strains tested, including standard wild-type A2. Exceptions to this are the strains NG59RA and Py 1-12. Most of the viral DNA produced is supercoiled (form I DNA). By in situ hybridization we show that more cells are permissive for viral DNA synthesis at the lower temperature and that the level of synthesis per permissive cell is higher at 33°C. The DNA synthesis observed is large T-antigen dependent and is paralleled by an increase in the expression of this viral protein. In the absence of large T-antigen the half-life of the de novo synthesized viral DNA is less than 12 hours. The overall levels of viral DNA

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synthesis in F-111 cells may not affect the integration of the viral genome since the patterns of integration in cells transformed by wild-type A2 at 33°C and 37°C appear similar. Furthermore, although an increase in transformation frequency of A2 is observed at 33°C (frequencies 2-3 times higher than at 37°C), it does not parallel the increase in viral DNA synthesis.

INTRODUCTION

The problem of cellular permissivity in papovarirus infections has been intriguing since the discovery of these viruses. Permissivity refers to the extent of viral DNA synthesis (and virus production) in cells which display viral receptors and can support viral early gene transcription. Those host cells which are fully permissive (mouse cells for polyoma virus [Py]) undergo a cytopathic infection and are killed in the process of virus production (7,42). Nonpermissive host cells (rat and hamster cells) produce little to no viral DNA, survive the infection, and undergo abortive and stable neoplastic transformation at moderate and low frequencies, respectively (16,17,18). The existence of trans-dominant factors controlling permissivity has been suggested from the observation that hybrids between permissive and nonpermissive hosts are permissive for virus production (1,42,45). Recent evidence suggests that the permissivity factors include the host DNA polymerase α/DNA primase complex which appears to interact directly with large T-antigen (33,34,36). It has also been recently reported that increasing the level of viral early gene expression (large T-antigen) to the level observed in permissive cells does not increase viral DNA replication of SV40 in nonpermissive mouse cells (26).

The potential role of viral DNA replication in the neoplastic transformation of nonpermissive cells remains unclear. On the one hand, a substantial decrease in viral DNA synthesis and late gene expression compared to the levels observed during the lytic infection

may be required for neoplastic transformation, since completion of the lytic cycle leads to extensive cytopathic effects and cell death (42). In fact, a tendency towards the inactivation of viral DNA replication functions after transformation of permissive and nonpermissive cells has been noted (see ref. 13 for a discussion). On the other hand, a role for DNA replication in neoplastic transformation can be invoked from other observations. First, low levels of DNA synthesis have been observed in most (if not all) infections of nonpermissive cells by both polyoma and SV40 (8). This limited replication is derived from only a small percentage of infected cells which may also produce virus (16,17,18). Because the fraction of cells which support viral DNA synthesis is of the same order of magnitude as the proportion which becomes stably transformed, it has been suggested that the former may be the precursors of transformed cells (18). Second, an important role for the polyoma viral DNA replication function has been demonstrated for the initial steps of transformation (15,24) since the absence of large T-antigen in the first two days postinfection leads to a great decrease in transformation frequency (24). Finally, a role for DNA replication has also been suggested from the analysis of the integration patterns of the viral genomes in transformed cells. Typically, the rare cells which become transformed contain head-to-tail tandems of covalently integrated viral genomes (4,6,32). Requirements for the integration of tandemly repeated viral genomes include the origin of replication (9) and large T-antigen (11). These results support, but do not prove, a role for DNA replication in the

integration pathway of the viral genome, and it has been suggested that replicative intermediates from a rolling circle type of replication may be the precursors of the integrated viral genomes (11). Evidence for the production of such intermediates has been obtained in the case of infection of nonpermissive mouse cells by SV40 (8). However, none of these observations provides direct evidence for a role of viral DNA replication in the integration/transformation pathway of papovaviruses. We have recently accumulated evidence that recombination can account for the generation of a large fraction of the integrated tandem viral genomes (14,22, and Hacker and Fluck, submitted). In the present experiments, we have further characterized the roles of viral DNA replication and large T-antigen in the infection of nonpermissive cells by polyoma virus. The results are discussed in the context of the role of these functions in the integration/transformation pathway of polyoma virus.

MATERIALS AND METHODS

Cells and viruses. Fischer rat F-111 cells (21) and mouse NIH-3T3 cells (30) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated calf serum and antibiotics and maintained in a humidified incubator with 5% CO_2 at the temperature indicated in the text.

The following polyoma strains were used: standard wild-type A2 (27); small plaque (SP) (3); Pasadena large plaque (LP), the parental strain of A2 and A3 (44); ts-a (23); NG59RA, a presumptive wild-type strain derived from the hr-t mutant NG59 by marker rescue of its

transformation defect (12); and Py 1-12 and Py 3-22 which were derived from the LP strain and contain deletions around the BglI site (nucleotide number 87) of 29 and 19 bp, respectively (2).

Analysis of low molecular weight DNA. Cells plated at a density of 1-2 x 10^5 cells/60 mm dish were infected with viral strains at a multiplicity of infection (MOI) of 10 plaque-forming units (pfu)/cell. Low molecular weight DNA was prepared by the method of Hirt (29), extracted with phenol and chloroform, precipitated with isopropyl alcohol, and resuspended in 0.05 ml of 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA. For analysis on 0.7% agarose gels in 0.04 M Tris-acetate, pH 7.6, and 2 mM EDTA (TAE), 5 µl of DNA from each sample was linearized with EcoRI. Southern transfer to nitrocellulose was performed as described (37).

Analysis of total DNA from polyoma-transformed cells. Monolayers of rat F-111 cells at a density of 2 x 10^5 cells/60 mm dish were infected with A2 at an MOI of 5 pfu/cell. After 2-3 weeks, foci were isolated from the monolayers and cloned by anchorage independent selection in agar. Total DNA was prepared from the cell lines as previously described (22). The DNA was analyzed for the presence of tandem repeats of the viral genome by digesting 10 µg with EcoRI, an enzyme which cuts polyoma DNA once, followed by electrophoresis on 0.7% agarose gels in TAE. Determination of the number of integration sites per cell line was performed by digestion of 10 µg of DNA with BglII, an enzyme which does not cleave polyoma DNA followed by electrophoresis on 0.4% agarose gels in TAE.

Hybridization analysis of DNA immobilized on nitrocellulose paper. (^{32}P)-labeled pPy-1, representing the complete polyoma genome in pBR322 (a gift of Dr. F. Cuzin), was used in DNA hybridizations to Southern blots at a specific activity of 1-6 x 10^8 cpm/μg. Hybridizations were carried out in 1X Denhardt's solution/2X SSC (0.1 ml/cm^2) at 65°C for either 24 hours using 0.5 x 10^6 cpm/ml of hybridization solution for the viral DNA replication experiments or 72 hours using 2 x 10^6 cpm/ml for the analysis of total DNA from polyoma-transformed cell lines.

Immunoblot analysis of large T-antigen. Rat F-111 cells were plated at a density of 6 x 10^5 cells/100 mm dish, infected with A2 at an MOI of 10 pfu/cell, and grown at 33°C or 37°C. For each time point, cells were trypsinized, washed twice in phosphate buffered saline (PBS), and suspended in sample buffer (5% sodium dodecyl sulfate; 100 mM Tris-HCl, pH 6.8; 0.03% bromophenol blue; 20% glycerol; and 5% B-mercaptoethanol). Proteins were solubilized by heating the cell suspensions to 100°C for 5 minutes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% polyacrylamide gels as described (31). Electrophoretic transfer of proteins to nitrocellulose was carried out as described (43). Briefly, transfer was conducted for 1 hour at 1-1.5 A in 25 mM Tris, 192 mM glycine, and 20% methanol. The blot was first washed in 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 0.05% Tween 20 (Sigma Chemical Co.) (TBS/Tween 20) and then in TBS/Tween 20 with 5% fetal calf serum before being probed with rat anti-polyoma tumor ascites fluid (diluted 1:500 in TBA/Tween 20) for 4 hours. After extensive washing in

TBS/Tween 20, the blot was treated for 2 hours with alkaline phosphatase-conjugated goat anti-rat antibody (Sigma Chemical Co.) diluted 1:1000 in TBS/Tween 20. After exposure to the second antibody the blot was washed in TBS/Tween 20 and then in 0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl; and 50 mM $MgCl_2$ (AP buffer) followed by development in AP buffer containing 33 mg/100 ml nitroblue tetrazolium and 16.7 mg/100 ml bromochloroindolyl phosphate (Sigma Chemical Co.) for 15 to 20 minutes before being stopped with water.

In situ hybridization of polyoma-infected cells in culture. Rat F-111 cells were passed to 35 mm dishes each of which contained a 22 mm^2 glass coverslip that had been treated with Denhardt's solution as described (28). The cells were plated at a density of 4 x 10^4 cells/35 mm dish, infected with A2 at an MOI of 10 pfu/cell, and grown at 33°C or 37°C. At each time point, cells were washed twice with PBS and then fixed with EtOH:acetic acid (3:1) for 20 minutes. Fixed cells were treated with protease K, fixed with 5% paraformaldehyde (Sigma Chemical Co.), and acetylated as described (28). Denaturation of double-stranded DNA was accomplished by heating the coverslips at 65°C for 15 minutes in 95% formamide containing 0.1X SSC.

For a probe, the 400 base pair (bp) HpaII-5 fragment of polyoma was labeled with ^{35}S-dCTP (1200 Ci/mmole; Amersham Corp.) by random priming to a specific activity of 1-2 x 10^9 cpm/µg DNA using a kit from the Amersham Corp. Hybridizations were carried out on silanized microscope slides in a volume of 18 µl/coverslip. The hybridization solution contained 1 x 10^6 cpm/coverslip; 45% formamide; 2X SSC; 10 mM

Tris-HCl, pH 7.4; 10 mM dithiothreitol; 0.5 mg/ml salmon testes DNA; and 5% dextran sulfate. Hybridization was performed for 72 hours at 48°C. The coverslips were then washed at 65°C for 1 hour in 2X SSC, 30 minutes at in 1X SSC, and 30 minutes in 0.5X SSC. After dehydration of the coverslips in graded ethanol washes, they were mounted on microscope slides, coated with NTB-2 nuclear track emulsion (Kodak Chemical Co.) in 0.3 M ammonium acetate, and stored in the dark at 4°C for 1-3 weeks before being developed.

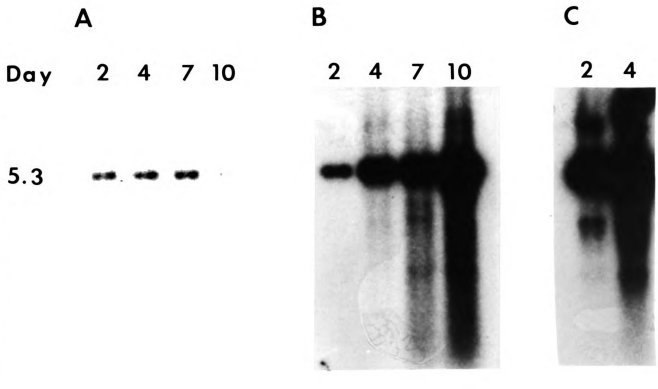
RESULTS

Quantitation and kinetic analysis of viral DNA synthesis. As reviewed in the Introduction, the role of viral DNA replication in the integration/transformation pathway of polyoma virus in nonpermissive cells is unclear. The fate of viral DNA at early times after infection was monitored in nonpermissive Fischer rat F-111 cells. Cells were infected as described in Materials and Methods, and low molecular weight DNA was isolated at several time points, digested with EcoRI which cuts polyoma DNA once to produce a 5.3 kbp linear molecule, and analyzed as described in Fig. 1. The results indicate that very little net synthesis of viral DNA occurs at 37°C (Fig. 1A). On the average, a 2 to 3-fold increase over the original signal is observed. The peak of the increase occurs between 5-7 days postinfection. Somewhat higher levels of DNA synthesis are observed in rat FR-3T3 cells (not shown).

Because transformation of F-111 cells is somewhat more efficient at 33°C than at 37°C, viral DNA synthesis was assayed at both temperatures. By comparing Fig. 1A and 1B, it is evident that by 10

Figure 1. Comparison of viral DNA synthesis in permissive and nonpermissive cells.

Rat F-111 and mouse NIH-3T3 cells were plated on 60 mm culture dishes, infected at an MOI of 10 pfu/cell, and incubated at either 37°C or 33°C as indicated. At the times designated in the figure, viral DNA was isolated. For each time point, 10% of the sample was digested with EcoRI, electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a polyoma probe. A.) F-111 cells at 37°C. B.) F-111 cells at 33°C. C.) NIH-3T3 cells at 33°C.


A
B
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Day
2
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2
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2
4

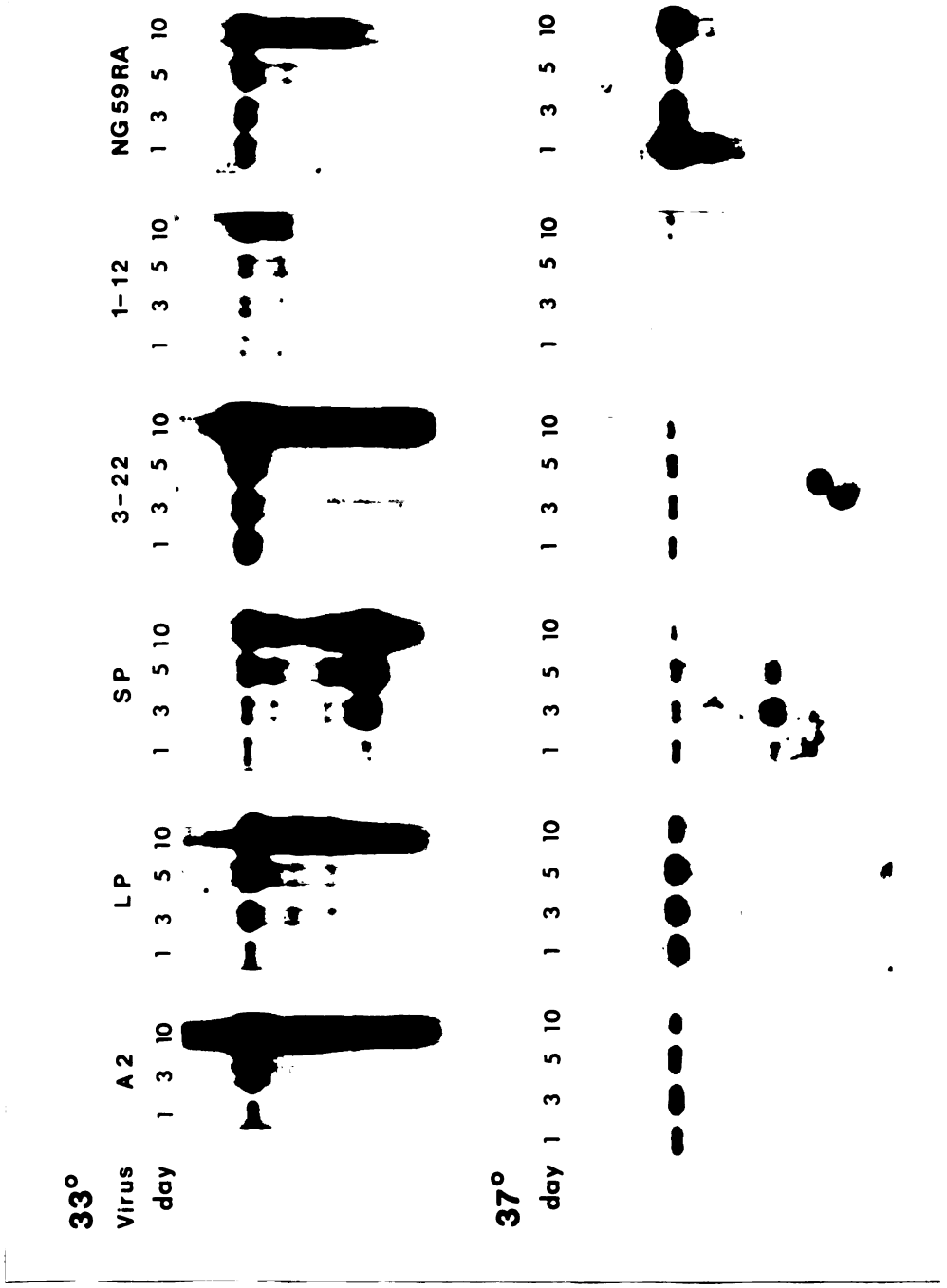
days postinfection the level of viral DNA is approximately 10-fold higher at 33°C than at 37°C. The magnitude of the increase in viral DNA at 33°C is illustrated by comparing infections of rat F-111 (Fig. 1B) cells and permissive mouse NIH-3T3 cells (Fig. 1C). By four days postinfection the mouse cells were beginning to lyse, and therefore, the viral DNA level seen at this time point approaches its maximum under these conditions. For A2-infected F-111 cells the amount of viral DNA present at 10 days is comparable to the level of 2 days in the mouse cells. The amount of viral DNA present in rat cells at 33°C continues to increase up to 15 days (data not shown). By this time, transformed foci are becoming visible in the monolayer. The potential contribution of viral DNA syntnesis in these transformed cells will be discussed later. Similar increases in the yield of viral DNA at 33°C have also been observed in rat FR-3T3 cells but not in baby hamster kidney (BHK) cells (data not shown). Decreasing the incubation temperatures below 33°C results in a pattern similar to that seen at 37°C. In the range of temperature tested (31°C to 39°C), the peak of synthesis appears to be at 33°C.

Several viral strains besides the widely used wild-type strain A2 were studied for their yield of viral DNA at 37°C and 33°C. These include Pasadena large plaque virus (LP), from which both A2 and A3 were isolated (44); small plaque virus (SP) (3); NG59RA, a presumptive wild-type strain derived by marker rescue of the hr-t mutant NG59 (12); and Py 3-22 and Py 1-12, which were derived from LP and contain deletions of 19 and 29 bp, respectively, within the early non-coding

region of the viral genome (2). As illustrated in Fig. 2, for cells infected with Py 3-22, LP, and SP the patterns are similar to those observed with wild-type A2 and show an increase at 33°C of approximately 10-fold over the 37°C yield. This is true even for defective viral genomes present in the stock of the SP strain. We have also observed an increase in viral DNA replication at 33°C with transformation defective hr-t mutants (not shown). On the other hand, for cells infected with Py 1-12 and NG59RA only a very slight increase in viral DNA synthesis is observed at the low as compared to the high temperature. Py 1-12 is similar to Py 3-22 in that both have a deletion between the origin of replication and the early mRNA start site. The difference is that the deletion in Py 1-12 extends further toward the transcription start site and includes the HaeIII restriction site at nucleotide number 105. No difference in viral DNA synthesis has been reported in permissive cells for Py 1-12 and Py 3-22 (2). We have not investigated Py 1-12 further. The absence of an increase with NG59RA is partially masked by the higher signal obtained with the particular virus stock used to give the same MOI as for the other strains. As mentioned above, NG59RA was derived from the hr-t mutant, NG59, by marker rescue of the hr-t phenotype (12). NG59 was selected for its transformation defect after nitrosoguanidine treatment of the SP virus. It is possible, therefore, that NG59RA carried several mutations for which a phenotype has not been described since only the defect in transformation was selected for and repaired. We have mapped

Figure 2. Comparison of various viral strains for viral DNA synthesis in rat F-111 cells at 33°C and 37°C.

The viral strains are indicated and are described in the text and in Materials and Methods. Infections, DNA extraction, digestion with EcoRI, gel electrophoresis, transfer, and hybridization were conducted as described in Fig. 1.


33°
Virus
day
A2
LP
SP
3-22
1-12
NG59RA
1 3 10
1 3 5 10
37°
day 1 3 5 10

the mutation which affects the ability of this strain to synthesize DNA at 33°C in rat cells to the central region of the large T-antigen (Hacker, et al., submitted).

The bulk of viral DNA from the Hirt extractions shown in Fig. 1 and Fig. 2 is equally divided between Form I and Forms II and III DNA. Some degraded forms are also apparent even in the A2 infections, which were carried out with a recently plaque-purified stock that does not contain any detectable defective genomes. Digestion of the DNA with HpaII, which cuts seven times within the polyoma genome, produces equal proportions of each fragment indicating that, for the most part, the complete genome is being replicated (not shown).

We have made extensive attempts to search in rat cells for replication intermediates of high molecular weight which might be the precursors to the integrated concatamers observed in transformed cells. For this purpose, we have isolated high molecular DNA from F-111 cells infected at a high MOI (60-100 pfu/cell) with various strains of polyoma. The high molecular weight DNA was separated from polyoma DNA monomers by sucrose gradient centrifugation, digested with BglII, which does not cleave polyoma DNA, and then analyzed by either agarose gel electrophoresis on 0.4% gels or by two-dimensional gel electrophoresis (46). Concatameric forms of polyoma DNA were not detected using these techniques, nor were they detected using orthogonal-field-alternation gel electrophoresis (OFAGE) (35). The absence of high molecular weight viral DNA species, however, does not eliminate the possibility of rolling circle-type replication as has been proposed for SV40 (8) and

observed in rare instances in polyoma infections of permissive cells (5,25). If rolling circle replication is occurrin , recombination and conversion to monomeric viral DNA must be very efficient.

Despite the high levels of DNA synthesis in A2-infected cells at 33°C, late gene expression as determined by indirect immunofluorescence using an anti-virion antiserum was not detected. Additionally, plaque assays of cell lysates from A2-infected rat cells were negative for infectious virions (not shown).

In situ hybridization analysis of polyoma-infected rat cells. Two possibilities could account for the difference in viral DNA synthesis at 33°C and 37°C. The temperature might affect either the number of cells that are permissive for viral DNA synthesis or the rate of synthesis per permissive cell or both. To differentiate between these possibilities, we investigated the distribution of viral DNA in single A2-infected F-111 cells using in situ hybridization. No viral DNA was detected at one day postinfection, which indicates that the input level of viral DNA (the amount corresponding to the fraction of viral DNA that is taken up by the cells following infections at 10 pfu/cell) is below the detection level of this technique (data not shown). A positive signal for viral DNA was first detected at 3 days postinfection for cells both at 33°C and 37°C (Fig. 3A). The number of DNA positive cells at both temperatures was determined for several time points. As is evident from the data presented in Table 1, more viral DNA positive cells are observed at 33°C than at 37°C for any

Figure 3. In situ hybridization of A2-infected F-111 cells.

F-111 cells were infected with A2 at an MOI of 10 pfu/cell and grown at either 33°C or 37°C. Infected cells were fixed (see Materials and Methods) at the times indicated below. The hybridization probe was the HpaII-5 fragment of polyoma virus labeled with (^{35}S)-dCTP. A.) A2-infected F-111 cells grown at 33°C or 37°C. B.) Controls. NIH-3T3 cells were infected with A2 at an MOI of 10 pfu/cell. Cells were fixed at 72 hours postinfection. Uninfected rat F-111 cells are shown in the panel marked F-111.

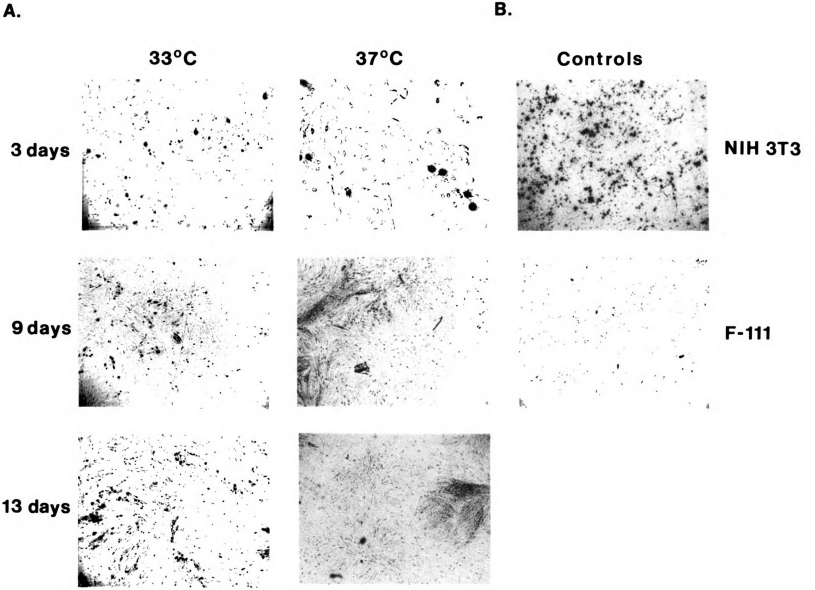

A.
B.
33°C
37°C
Controls
3 days
9 days
13 days
NIH 3T3
F-111

Table 1. Analysis of *in situ* hybridization of A2-infected F-111 cells at 33°C and 37°C

Day Post-infection	No. viral DNA positive cells[a] 33°C	37°C	33°C/37°C
3	55	14	3.9
6	58	ND[b]	-
9	119	19	6.3
11	122	29	4.2
13	196	60	3.3

[a]The number of viral DNA positive cells is based on the number of cells in 20 microscopic fields which exhibited a hybridization signal.

[b]Not done.

time point chosen up to 13 days postinfection (see Fig. 3A). For time points between 3 and 13 days, the difference in the number of DNA positive cells at the two temperatures varied between 3- and 6-fold (Table 1).

By 13 days after infection, transformed foci were visible on the coverslips at both temperatures (Fig. 3A). For the 37°C incubation, 94% of the DNA positive cells at this time were localized to the focal areas. In contrast, only 69% of the DNA positive cells were found in focal areas on the 33°C coverslips.

Throughout the time course of the experiment, it appears that a greater number of cells are supporting detectable levels of viral DNA replication at 33°C than at 37°C. The difference between the number of viral DNA replication positive cells at the two temperatures (3- to 6-fold) is slightly lower than the difference observed on Southern blots (10-fold). This may indicate that there is also an increase in the rate of synthesis per cell at the lower temperature. Both of these results could be accounted for by higher rates of initiation of viral DNA synthesis at 33°C. However, since the proportion of DNA positive cells remains small even at 33°C, it is unlikely that the increase in DNA synthesis per cell can be accounted for by statistical considerations alone (increased probability of reinitiation per cell). It is also conceivable that the same number of cells produce high levels of viral DNA at 33°C and 37°C, and that these cells are killed at 37°C before they can reach levels of DNA equivalent to those at 33°C. Considerations on the behavior of transformed cells at 33°C make this an unlikely explanation (see below).

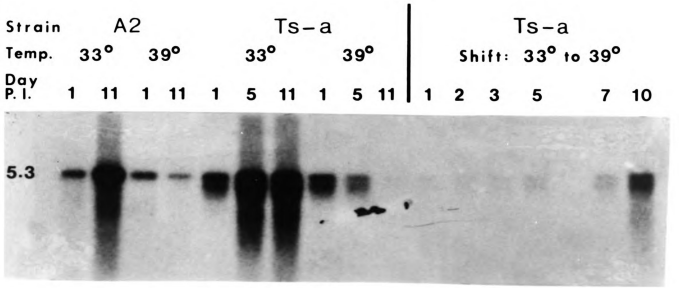
The level of detectable DNA positive cells is low (Ø.2%). The frequency of DNA positive cells is slightly higher than previous results obtained for polyoma infections of hamster BHK cells at 37°C (19). As had been noted previously in the case of infections of the BHK cell line, the proportion of DNA positive cells and the proportion of cells which become transformed are equivalent. It must be noted, however, that the in situ hybridization technique only scores cells with a relatively high level of viral DNA sequences.

Since the bulk of DNA replication appears to be contributed by a small percentage of the population, it appears that in those cells the level of DNA replication may be as high as the average level produced in permissive cells. The same conclusions have been reached for the production of unintegrated viral genomes in polyoma transformed rat cells (47).

The fate of viral DNA in the absence of large T-antigen. To investigate the role of large T-antigen in viral DNA synthesis in nonpermissive cells, F-111 cells were infected with a temperature-sensitive mutant, ts-a (23), which encodes a thermosensitive large T-antigen. The infected cells were grown at either 33°C or 39°C or shifted from the lower to the higher temperature at various times after infection. Viral DNA was extracted and analyzed as described above, and the results are presented in Fig. 4. For cells infected at 33°C with ts-a or A2, the amount of viral DNA increases throughout the time course of the experiment. At 39°C, however, little ts-a DNA remains by 11 days postinfection while the

Figure 4. The role of large T-antigen in viral DNA synthesis.

A2 and ts-a infected F-111 cells were grown at either 33°C or 39°C. Viral DNA was isolated from these conditions at the times indicated in the figure. For the ts-a infections, cells were also shifted from 33°C to 39°C at times between 1-10 days postinfection as indicated (lanes marked ts-a shift). Viral DNA from all of the 33°C to 39°C shift samples was isolated at 11 days postinfection. Gel electrophoresis and hybridization were conducted as in Fig. 1.


Strain
A2
Ts-a
Ts-a
Temp.
33°
39°
33°
39°
Shift: 33° to 39°
Day P.I.
1 11 1 11 1 5 11 1 5 11 1 2 3 5 7 10
5.3

level of viral DNA in A2-infected cells is approximately one-third of the input level. Viral DNA from cells that were shifted from 33°C to 39°C was rapidly degraded as seen in the lanes marked "shift." In ts-a mutant-infected cells shifted to 39°C at 10 days postinfection and maintained at 39°C for 24 hours, less than 25% of the level of viral DNA remains as compared to the ts-a infected cells maintained at 33°C for the entire 11 day time course. This indicates that the half-life of polyoma DNA in the absence of large T-antigen is less than 12 hours in rat F-111 cells at 39°C. It should also be pointed out that the viral DNA in the 33°C to 39°C shift experiment appears to degrade faster than the parental ts-a genomes in the cells maintained at 39°C. This may signify different stabilities at 39°C for input DNA and DNA synthesized de novo in F-111 cells. From these results we conclude that large T-antigen is required continuously for the replication of polyoma DNA in F-111 cells. Large T-antigen may also be required for the stabilization of the viral genome in nonpermissive cells.

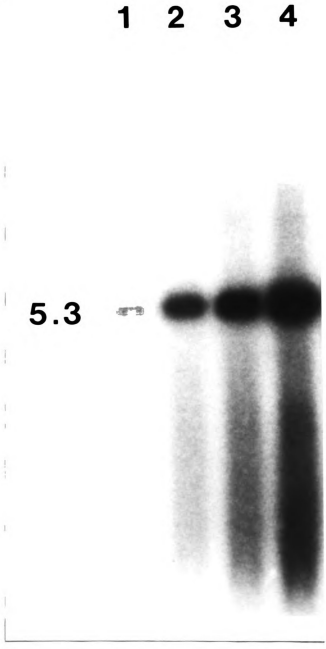
Viral DNA replication of exogenous and endogenous viral sequences in polyoma transformed cells. Multiple explanations are possible for the occurrence of a small subfraction of infected cells with a high level of viral DNA. For example, there may exist a small fraction of cells in which large T-antigen is produced at a high level, or there may be a small fraction of the population engaged in the transformation pathway. To attempt to analyze these possibilities, we tested the replication of the polyoma viral genome introduced by infection into a polyoma transformed cell line; that is, in a population of cells in

which all cells are transformed and express high levels of large T-antigen. Results from this experiment indicate that the superinfecting genome is replicated less than the endogenous viral genome at either temperature, but the replication of the superinfecting genome is greater in transformed cells than in F-111 cells (data not shown). The results do not support the hypothesis that either transformation or high levels of large T-antigen are sufficient for high levels of viral DNA replication. Similar results have been observed for SV40 (26).

Previous results have shown that integrated tandems of polyoma DNA undergo large T-antigen-mediated DNA replication in a small fraction of the cell population (47). Excision of the replication products leads to the formation of free viral DNA within the transformed cell. Since we find a large difference in DNA replication between cells infected at 33°C and 37°C, a study of the viral DNA replication in a polyoma-transformed cell line was undertaken. For this experiment we used an A2-transformed F-111 cell line, FA37-14, which contains mutliple tandem integrations of the viral genome. FA37-14 cells were grown at both 33°C and 37°C for two weeks prior to the extraction of low molecular weight DNA. The analysis of viral DNA from an equal number of cells grown at 37°C (Fig. 5, lane 1) and at 33°C (lane 4) shows that viral DNA synthesis is greater at the lower temperature. Lanes 2 and 3 represent dilutions of 1:4 and 1:2, respectively, of the DNA from the 33°C cells. By comparing the polyoma DNA concentrations in lanes 1 and 2, we conclude that viral DNA synthesis in FA37-14 is

Figure 5. Viral DNA replication in an A2-transformed cell line.

The A2-transformed cell line FA37-14 was maintained at both 33°C and 37°C for two weeks prior to viral DNA isolation. For the experiment, two plates were seeded with FA37-14 at each temperature. One of these was used to determine the cell number and the other was used for isolation of viral DNA. An aliquot of the isolated DNA from an equal number of cells from the 37°C plate (lane 1) or the 33°C plate (lane 4) was digested with EcoRI and electrophoresed, transferred, and hybridized as in Fig. 1. Also presented are 1:4 (lane 2) and 1:2 (lane 3) dilutions of the DNA aliquot in lane 4.

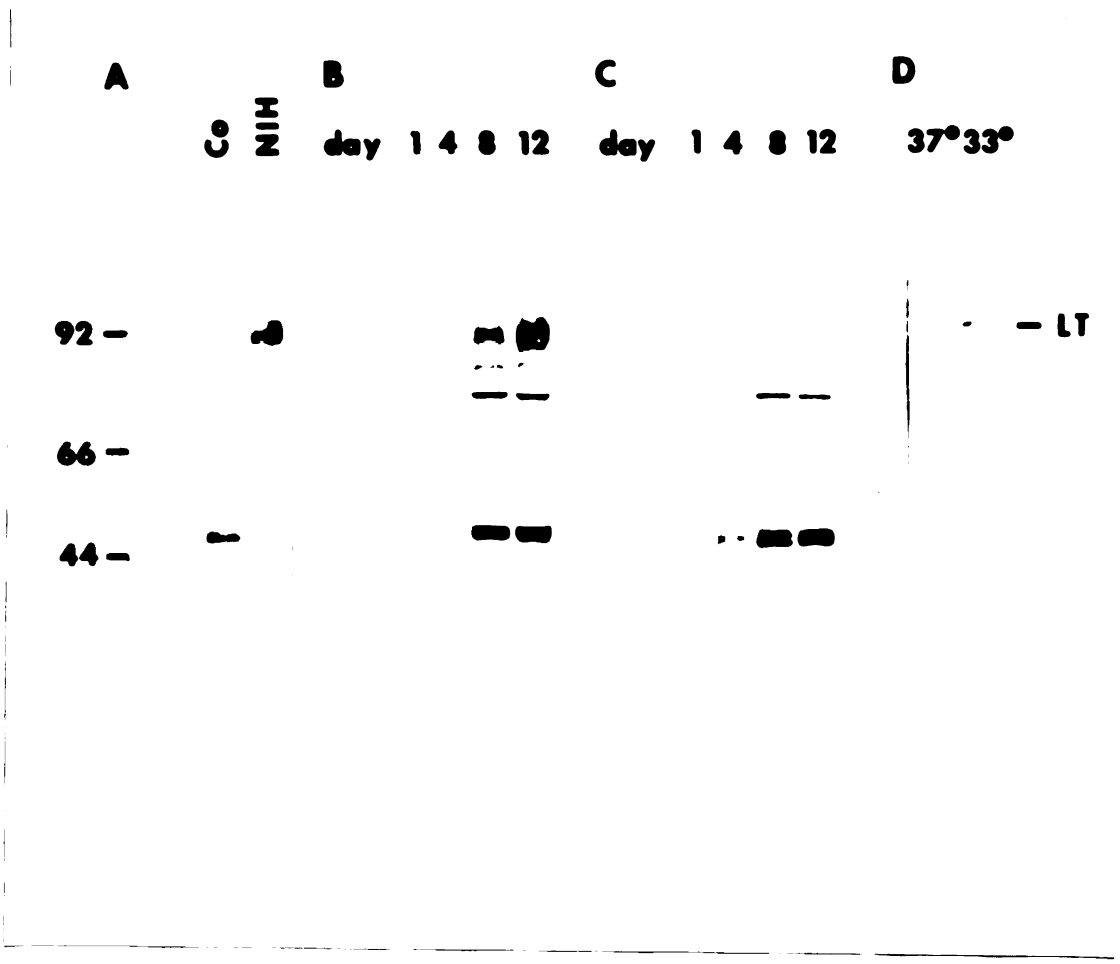

1 2 3 4
5.3

approximately 15 times greater at 33°C than at 37°C. This difference is similar to that seen in A2-infected F-111 cells by 10 days postinfection (see Fig.1).

Expression of large T-antigen at 33°C and 37°C. As shown in Fig. 4, large T-antigen is required for viral DNA synthesis in rat cells. It is conceivable that the level of expression of large T-antigen is greater at 33°C than at 37°C, and that increased levels of large T-antigen account for the increased viral DNA synthesis at 33°C. Another possibility is that the protein is more stable at the lower temperature. The accumulation of large T-antigen in A2-infected cells at early times after infection was investigated by immunoblot analysis of whole cell extracts as described in Fig. 6 and Materials and Methods. From this experiment, it is clear that the accumulation of large T-antigen by 12 days postinfection in F-111 cells is much greater at 33°C than at 37°C (compare B and C of Fig. 6). In the 37°C infection, large T-antigen can only be detected at the 12 day time point if the gel is overloaded (data not shown). By 12 days postinfection the level of large T-antigen at 33°C is approximately 10 times greater than at 37°C (data not shown). Previously, we attempted to use metabolic labeling of infected cells with ^{35}S-methionine, but we were not able to detect any large T-antigen. This result may indicate that the rate of synthesis of large T-antigen is not increased at 33°C, but that it is more stable at this temperature. Using indirect immunofluorescence large T-antigen was detected as early as three days postinfection in A2-infected F-111 cells at 33°C and 37°C.

Figure 6. Western blot of whole cell extracts of polyoma-infected F-111 cells.

F-111 cells were infected at an MOI of 10 pfu/cell. At the times indicated in the figure a plate of infected cells was harvested and processed as described in Materials and Methods. SDS-PAGE analysis of 1/30 of each sample was performed on a 10% acrylamide gel. A.) Control lanes. Whole cell extracts are from uninfected F-111 cells (Co) and from A2-infected mouse NIH-3T3 cells (MOI of 10 pfu/cell) at 48 hours postinfection (NIH). B.) Whole cell extracts from A2-infected F-111 cells at 33°C. Time points are 1, 4, 8, and 12 days postinfection. C.) Same as in B.) except the infection was carried out at 37°C cells. The position of large T-antigen is marked LT. D.) Whole cell extracts from A2-transformed cell line, FA37-4.


A
B
C
D
C
NIH
day 1 4 8 12
day 1 4 8 12
37°
33°
92 –
66 –
44 –
– LT

Also included in this experiment are whole cell extracts from an A2-transformed cell line, FA37-4. This cell line was maintained at both 33°C and 37°C for one week prior to the protein extraction. The results again show a higher level of large T-antigen in cells grown at 33°C compared to cells at 37°C (Fig. 6D). This difference, however, is only 3 to 4-fold compared to the 10-fold difference seen at early times after infection of rat cells.

Immunoblotting has not allowed in the detection of the other early proteins, middle and small T-antigens. Therefore, it is not known if these proteins are also expressed at higher levels in cells grown at 33°C. The other proteins visible on the blot are probably cellular proteins which react with the anti-polyoma tumor ascites fluid. In summary, an increase in large T-antigen expression corresponds to a similar increase in viral DNA synthesis. However, it cannot be determined from this experiment whether the increased DNA synthesis provides more templates for early gene transcription or if the increase in large T-antigen synthesis is required for the increase in viral DNA synthesis.

We note that no large T-antigen can be detected in infected F-111 cells at early times postinfection, a time when experiments show that large T-antigen is required for transformation. Indeed, in infections with ts-a mutants, incubation at the permissive temperature is required only during the first 2-3 days postinfection (24). Thus, if synthesis of large T-antigen is required at these times, then very low levels may be sufficient.

Effects of viral DNA synthesis on transformation. As mentioned above, we had previously noted that transformation frequencies with polyoma are somewhat higher in rat cells maintained at 33°C compared to those maintained at 37°C. The observed increase in viral DNA synthesis may cause an increase in transformation in at least two ways. One is that processes associated with viral DNA synthesis per se are required for integration (for example, unwinding or nicking of DNA during replication). The other is that the number of templates per cell is increased and consequently the integration probability is increased. Representative results from transformations at 33°C and 37°C are shown in Table 2. These results show that the large overall increase in viral DNA levels (10-fold) at 33°C compared to 37°C is not paralleled by an equivalent increase in transformation frequency (2-fold). The increase in transformation frequency at 33°C may not correlate with the increase in viral DNA synthesis since it is also observed in infections with NG59RA in which no increase in viral DNA synthesis at 33°C is observed.

Effect of the number of viral templates on integration patterns. If viral DNA replication is required for the tandem integration of viral genomes in polyoma-transformed rat cells, then the difference in viral DNA replication at 33°C and 37°C may lead to differences in the integration patterns of cells transformed at the two temperatures. The increase in the number of genomes at 33°C may also lead to an increase in the number of integration sites. These points were investigated by isolating total DNA from cell lines

Table 2. Transformation frequencies[a]

Strain	Percent transformation 33°C	37°C	33°C/37°C
A2	0.25 (r=0.22-0.29)	0.11 (r=0.07-0.15)	2.3
NG59RA	0.11 (r=0.06-0.15)	0.05 (r=0.02-0.07)	2.2

[a]F-111 cells at a density of 1 x 10^5 cells/60 mm culture dish were infected with either A2 or NG59RA at an MOI of 10 pfu/cell. After the infection the cells were passed 1:4 and grown at either 33°C or 37°C. The transformation frequencies presented are the average of three experiments for A2 and two experiments for NG59RA. The frequencies are based on the number of transformants per 1 x 10^5 cells.

transformed by A2 at the two temperatures. Digestion of DNA with EcoRI was used to determine the presence or absence of tandem repeats, and digestion with BglII was used to determine the number of integration sites per cell line. Using this approach we analyzed 19 A2-transformed cell lines. The results of representative EcoRI digests are shown in Fig. 7. Of the 19 lines analyzed only three did not have an integrated tandem repeat (see Fig. 7, lane 4; and Table 3). Two of these originated from the 33°C infection and one from the 37°C infection. Analysis of the number of integration sites in these cell lines by digestion with BglII also failed to detect differences between the 33°C and 37°C cell lines (Fig. 8). Only a few of the A2-transformed lines, 2/1Ø from 33°C and 2/9 from 37°C, contain a single integration site (see Table 3). Furthermore, the overall number of integration sites from the 33°C cell lines does not appear to be increased compared to that found in the 37°C cell lines, and the number of genomes per site as determined by the sizes of the BglII restriction fragments is not affected by temperature. Overall, the integration pattern of polyoma virus appears bimodal regardless of the temperature at which the cells were transformed and maintained: either many sites of integration with tandem repeats or a single site without tandem repeats.

DISCUSSION

We have analyzed viral DNA synthesis in nonpermissive cells during the early stages of neoplastic transformation by polyoma virus. In F-111 cells, little overall net synthesis is observed at 37°C. Higher levels of synthesis are seen in FR-3T3 and BHK cells at 33°C.

Figure 7. Presence of integrated tandem repeats in A2-transformed F-111 cells.

Foci were picked from monolayers of F-111 cells which had been infected at an MOI of 10 pfu/cell with A2 at either 33°C or 37°C. Picked foci were agar cloned at the temperature at which they were isolated. For each lane, 10 µg of total cellular DNA from 33°C (lane 1-3) and 37°C (lanes 4-6) cell lines was digested with EcoRI. Electrophoresis and transfer to nitrocellulose was the same as in Fig. 1. Hybridization was carried out for 72 hours at 65°C using (^{32}P)pPy-1 (2 x 10^6 cpm/ ml of hybridization solution).

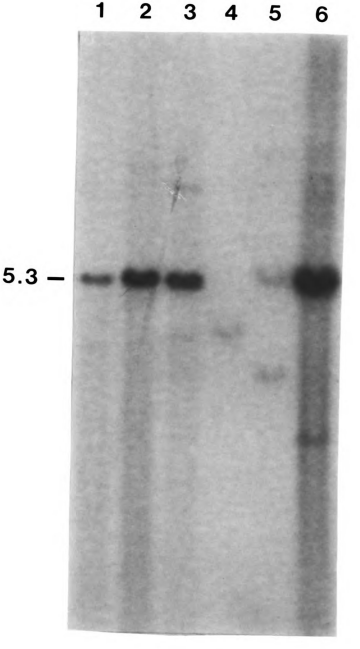

1
2
3
4
5
6
5.3

Table 3. Summary of integration analysis

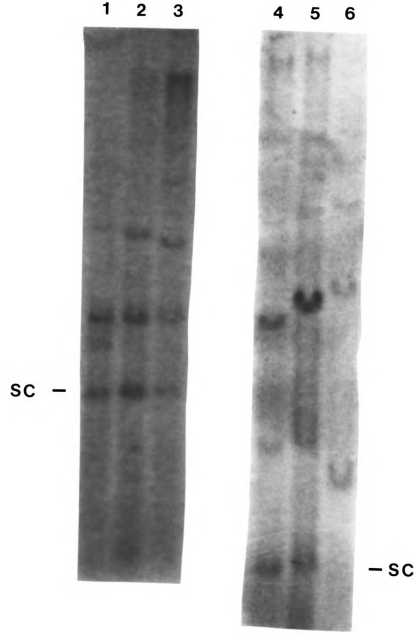
Temperature	Single site[a]	Tandem repeats[b]
33°C	2/10	8/10
37°C	2/9	8/9

[a]The number of integration sites per cell line was determined as described in Fig. 8.

[b]The presence of tandem repeats was determined as described in Fig. 7.

Figure 8. Analysis of the number of integration sites in A2-transformed cells.

The DNAs in lanes 1-3 are from cell lines isolated from a 33°C infection, and the DNAs in lanes 4-6 are from cell lines isolated from a 37°C infection. For each lane 10 μg of total cellular DNA was digested with BglII and electrophoresed on a 0.4% agarose gel. Transfer to nitrocellulose and hybridization were conducted as described in Fig. 7. The position of Form I DNA is marked SC.


1 2 3
4 5 6
SC
SC

In situ hybridization, however, demonstrates that a fraction of the infected cells are actively producing viral genomes. In contrast to the results with the 37°C infection, a large amount of viral DNA is observed at 33°C in F-111 and FR-3T3 cells. This increase is achieved by recruiting a larger number of cells to synthesize DNA and by increasing the yield of DNA per producing cell. A substantial fraction of this DNA is genome-size form I.

Similar to the requirements for viral DNA synthesis in permissive cells, synthesis in nonpermissive cells is under large T-antigen control. This is demonstrated by the existence of a non-lethal mutation in large T-antigen which greatly decreases the ability of strains carrying it to synthesize DNA at low temperature in F-111 cells (Hacker et al., submitted). Furthermore, the level of viral genomes decreases rapidly when large T-antigen is removed by shifting cells infected with a ts-a mutant to the nonpermissive temperature. This experiment indicated a half-life of about 12 hours for polyoma DNA in the absence of large T-antigen at 39°C. Finally, mutations which eliminate a large T-antigen binding site, as in Py 1-12, also decrease the viral DNA synthesis in F-111 cells.

The role of viral DNA synthesis in transformation by polyoma. The arguments for and against a role for viral DNA synthesis in neoplastic transformation have been reviewed in the Introduction. Essentially, a requirement for DNA replication has been postulated because of the integration pattern of the viral genomes and the requirement for large T-antigen in the initial steps of transformation. Although

transformation is somewhat elevated under the conditions in which more viral DNA synthesis is observed, the overall results presented here argue that viral DNA replication (i.e., de novo synthesis of viral genomes) is irrelevant to neoplastic transformation. This is supported by the fact that DNA positive cells are found in nontransformed areas of the monolayer, that transformation defective mutants also induce viral DNA synthesis, that the increase in viral DNA at low temperature is higher than the increase in transformation frequency, that transformation frequencies continue to decrease in a temperature range in which no further decrease of viral DNA synthesis is observed, and that an increase in transformation frequency is also observed at 33°C for strain NG59RA although very little increase in viral DNA, if any, is observed with this strain at that temperature. Furthermore, the requirement for large T-antigen function in polyoma transformation occurs during the first 2 days post-infection (24), a time at which no net viral DNA synthesis can be detected.

An additional argument comes from the analysis of the integration patterns. The number of viral templates present at later times postinfection apparently does not affect the frequency of tandem integration of viral genomes, the number of sites of integration in the host chromosomes, or the number of genomes integrated per site. Thus, the increase in viral DNA replication does not overtly affect the integration pattern. Overall, the integration patterns observed in these experiments and others from our lab appear bimodal: either integration occurs at multiple sites in the host with tandem repeats of

the genomes or integration of less than a single copy at a single site is observed. These two types of integration may represent different kinds of events. The absence of change in integration patterns with increased viral DNA synthesis at late times postinfection, the requirements for large T-antigen at early times postinfection, and other unpublished data from our lab all suggest that an important step in the integration/transformation event is fixed at an early time postinfection.

A final argument against an important role for net synthesis of viral genomes in transformation comes from the following observation. Viral sequences integrated in transformants derived from mixed infections between two physically marked parental genomes have undergone a high level of interviral recombination (even when recombination appears to have no selective advantage for transformation). In contrast, no recombination is observed in the population of replicated unintegrated viral molecules (Hacker and Fluck, submitted). This suggests that the population of cells which is actively replicating viral DNA is not the same as the population of cells which becomes transformed. Furthermore, these and other results from our lab suggest that a significant fraction of the tandem structures seen in transformed cells can be accounted for by recombination events. This result weakens one of the major rationales for the implication of viral DNA replication in transformation. Altogether, the results presented in this report suggest that the cells which do undergo net synthesis of viral DNA are not the precursors of the transformed cells.

The increased level of viral DNA synthesis at 33°C is intriguing, but this phenomenon may be trivial. Mammalian cells are quite sensitive to elevated temperatures. Mouse cells also produce higher yields of viral DNA and virus progeny at 33°C (unpublished results). Transformation frequencies in rat F-111 cells drop steadily as a function of temperature (Kalvonjian and Fluck, unpublished data), and at least in the range between 35°C and 40°C, this decrease is not paralleled by a decrease in viral DNA synthesis.

The mechanism of polyoma DNA synthesis in nonpermissive cells. It has often been suggested that viral DNA synthesis in nonpermissive cells might involve a rolling circle mechanism of replication (11). This has been proposed because of tandem integration of the polyoma viral genome and because of the requirements for large T-antigen (11) and a functional origin of replication in tandem formation (9). This model is appealing since it allows for the production of large yields of DNA with a single initiation event and for the production of concatameric molecules. Such a model would be consistent with a role for large T-antigen limited to the initiation of viral DNA synthesis, as suggested by experiments involving permissive cells infected with ts-a/A mutants (20,41). In vitro experiments, however, have also suggested a role for SV40 large T-antigen in chain elongation (38). The recent discovery of a helicase activity associated with the protein supports this finding (10,39). Our experiments do not help resolve this question. The absence of high molecular weight intermediates cannot be used as an argument against rolling circle replication

genome are altered (11). Since ts-a mutants are not deficient in abortive transformation (13,40), it had been proposed that the defect of ts-a mutants is an "initiation" defect such as integration. Exactly what that role is remains to be elucidated. Large T-antigen appears to be required for the formation of integrated tandem viral genome structures (11), yet at least a fraction of these appear to be created by recombination (Hacker and Fluck, submitted). What is clear from the present experiments is that the role of large T-antigen is not simply to amplify the viral genome to enhance the frequency of integration. In no way do the results exclude the possibility that a function of large T-antigen essential for DNA replication (such as the nicking or unwinding of DNA) is also essential for the integration of the viral DNA into the host genome.

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Chapter 3.

A Nonlethal Mutation in Large T-Antigen of Polyoma Virus Which Affects Viral DNA Synthesis[1]

ABSTRACT

A mutation in polyoma virus large T-antigen which affects viral DNA synthesis is described. In nonpermissive Fisher rat F-111 cells, the mutation causes a 10-20 fold decrease in the yield of viral DNA at 33°C compared to wild-type A2 infections. Differences in the integration patterns of the mutant and wild-type genomes are also observed in transformed F-111 cells. The mutation maps to a region between the HindIII and NsiI restriction sites (nucleotides 1656-1910). Sequence analysis of this region revealed a C to G transition at nucleotide 1791 which causes a proline to alanine change in the amino acid sequence of large T-antigen.

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INTRODUCTION

The role of the large T-antigens of polyoma virus and simian virus 40 (SV40) in viral DNA replication is well established (12,33). Large T-antigen is required for initiation of viral DNA synthesis (9,12,33) and may also be involved in elongation (5,31). It is the only viral protein required for viral DNA synthesis *in vitro* (24,35) as most of the proteins necessary for this process, including DNA polymerase and DNA primase, are provided by the host cell (25).

Much of our knowledge of the function(s) of the polyoma and SV40 large T-antigens has come from studies of conditional lethal mutants. These have revealed the multifunctional nature of these proteins and have led to the mapping of several domains. For polyoma large T-antigen, regions important in DNA replication (7,8,16), DNA binding (14), and cellular immortalization (26) have been defined. In addition, this viral protein has ATPase (17) and nucleotide-binding activities (3) that are important in viral DNA replication.

In the course of a study on polyoma viral DNA replication in nonpermissive rat cells, we discovered a defect in a viral strain previously assumed to be wild-type. The present report concerns the characterization and mapping of this mutation. Interestingly, the mutation maps outside of the domains of large T-antigen that were previously defined by mutations.

MATERIALS AND METHODS

Cells and viruses. Mouse NIH-3T3 (21) and two Fischer rat cell lines, F-111 (13) and FR-3T3 cells (28), were grown in Dulbecco's modidfied Eagle's medium (DMEM) with 5% heat-inactivated calf serum.

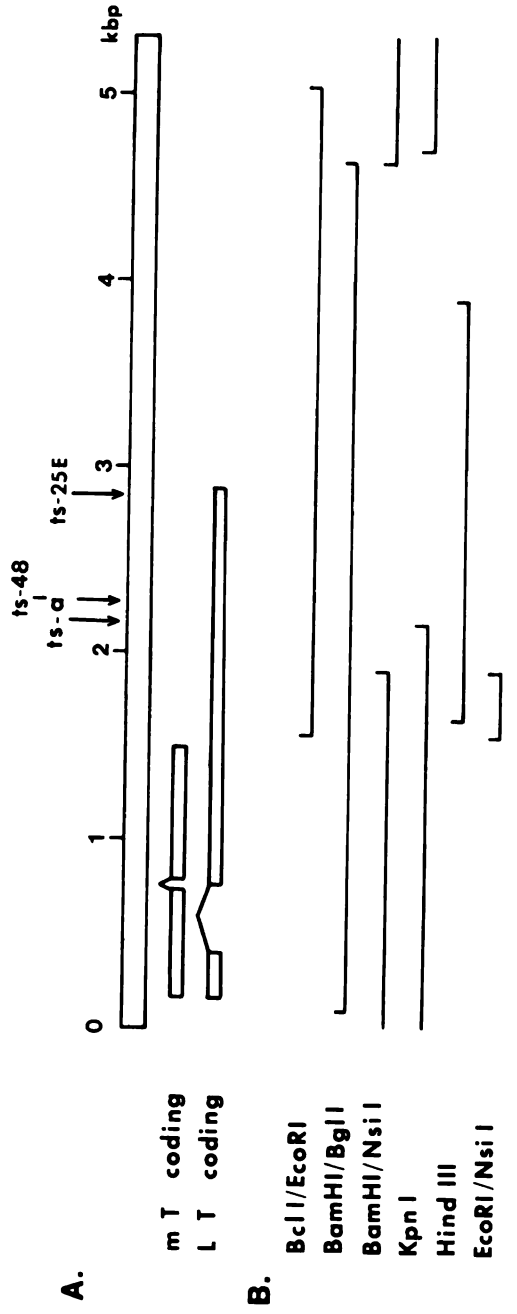
The polyoma viral strains A2 (19), NG59RA [RA] (10), and B2 (32) were grown on baby mouse kidney cultures from plaque purified viruses as described (34). The pseudo-wild-type strain RA was obtained by marker rescue of the middle T-antigen defect of the hr-t mutant NG59 (10). NG59 was derived from the Pasadena small plaque strain by nitrosoguanidine mutagenesis (1) and has been partially sequenced (2). The hr-t mutant B2 was derived from the Pasadena small plaque strain by mutagenesis with ICR-191 (32). B2 contains a deletion of nucleotides 491 through 730 (numbered as in [29]).

Viral reconstruction. Viral DNA for the construction of mutant viruses was prepared in one of two ways. Both the A2 and RA genomes were cloned at their BamHI sites into pAT153. Plasmid DNA was isolated as previously described (22). Alternatively, viral DNA from A2- and RA-infected NIH-3T3 cells was prepared by the method of Hirt (20). The restriction fragments shown in Fig. 1 were exchanged between the two parental viral genomes. The names of the resulting viruses consist of the name of the parental virus contributing the largest DNA fragment followed in parentheses by letters designating the restriction enzymes used and the name of the virus contributing the smaller fragment. For instance, A2 (E/N RA) is an A2 virus in which the 350 base pair EcoRI to NsiI fragment has been replaced with the corresponding fragment from the RA virus.

Viral DNA (500 ng) was digested with 20 units of restriction enzyme for four hours. The digested DNA was resolved by agarose gel electrophoresis, and the fragments were recovered from the gel by

Figure 1. Map of polyoma virus.

(A.) Linear map of the polyoma virus genome showing the middle (mT) and large T-antigen (LT) coding regions and the locations of several ts-a mutations. The genome is numbered as in Soeda et al. (29). (B.) The restriction fragments from A2 which confer competence for DNA replication at 33°C in F-111 cells for the RA/A2 hybrid viruses.


A.
ts-48
ts-a
ts-25E
0
1
2
3
4
5
kbp
m T coding
L T coding
B.
Bcl I/EcoRI
BamHI/Bgl I
BamHI/Nsi I
Kpn I
Hind III
EcoRI/Nsi I

electrophoresis onto 3MM paper backed by a dialysis membrane (18). A total of 50 ng of viral DNA fragments were ligated in 10 µl of ligation buffer (66 mM Tris, pH 7.5; 10 mM $MgCl_2$; 1 mM DTT; and 1 mM ATP) for 16 hours at 16°C using T4 DNA ligase (New England Biolabs). For transfections, 10 ng of DNA was applied to 1.5 x 10^5 NIH-3T3 cells /35 mm dish using 500 µg/ml DEAE dextran at 33°C for 1 hour. The dishes were washed and overlayed with DMEM containing 0.9% agar and 5% calf serum. Plaques were picked 10 days later, and viral stocks were grown on NIH-3T3 cells.

Viral DNA replication assay. Cells were infected at a density of 1 x 10^5 cells/60 mm culture dish at a multiplicity of infection (MOI) of 10 plaque forming units (pfu)/cell. At the times indicated, viral DNA was isolated by the procedure of Hirt (20). For each time point, 10% of the extracted DNA was digested with EcoRI to linearize the viral DNA. The digested DNA was electrophoresed on a 0.7% agarose gel and then transferred to nitrocellulose (30). The blots were hybridized in 2X SSC/1X Denhardt's solution (0.1 ml/cm^2) for 18-24 hours at 65°C using a polyoma probe (1-2 x 10^9 cpm/µg; 5 x 10^5 cpm/ml of hybridization solution).

Transformation. F-111 cells were seeded at a density of 1 x 10^5 cells/60 mm culture dish and infected with either A2 or RA at an MOI of 10 pfu/cell. For the competition experiment, the cells were infected with B2 and either A2 or RA at the MOIs given in Fig. 3. Infected cells were grown at either 33°C or 37°C in DMEM containing 5% heat-inactivated calf-serum. Cells which overgrew the monolayers of

A2- and RA-infected cells were transferred to 35 mm culture dishes and grown in DMEM containing 5% heat-inactivated calf serum. The transformants were then cloned in agar.

Integration analysis. Total cellular DNA was isolated from A2- or RA-transformed cell lines as described (15). For each cell line, 10 μg of DNA was digested either with EcoRI, which cuts polyoma DNA once, or with BglII, which does not cut the viral DNA. Digested DNA was electrophoresed on 0.7% agarose gels if digested with EcoRI or on 0.4% gels if digested with BglII. After transfer to nitrocellulose (30), the blots were hybridized to a polyoma probe (1-2 x 10^9 cpm/μg). Hybridization was at 65°C for 40-48 hours in 2X SSC/1X Denhardt's solution (0.1 ml/cm^2) using 1 x 10^6 cpm of the labeled probe per ml of hybridization solution.

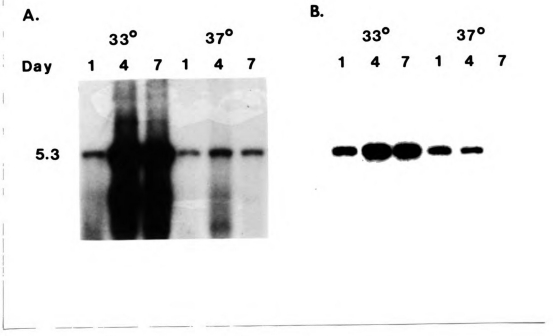
Sequencing. Sequencing was carried out according to the procedure of Maxam and Gilbert (23). The nucleotide numbering system is that of Soeda et al. (28).

RESULTS

Comparison of viral DNA synthesis between A2 and RA. Previous results from our laboratory (Hacker and Fluck, submitted) have shown that some strains of polyoma virus, including wild-type A2, undergo elevated levels of viral DNA synthesis in nonpermissive F-111 cells at 33°C, while only minimal levels are observed at 37°C (Fig. 2A). In contrast, little replication occurs at 33°C with the RA strain, so that the difference in viral DNA synthesis between the two temperatures is minimal (Fig. 2B). A similar reduction in DNA synthesis with RA was observed in Fisher rat FR-3T3 cells at 33°C (not shown).

Figure 2. Comparison of viral DNA synthesis between A2 and RA in F-111 cells.

F-111 cells were infected with A2 or RA, and viral DNA was extracted and analyzed as described in Materials and Methods. The infections were carried out at 33°C and 37°C as indicated. (A.) A-2 infected F-111 cells. (B.) RA-infected F-111 cells.

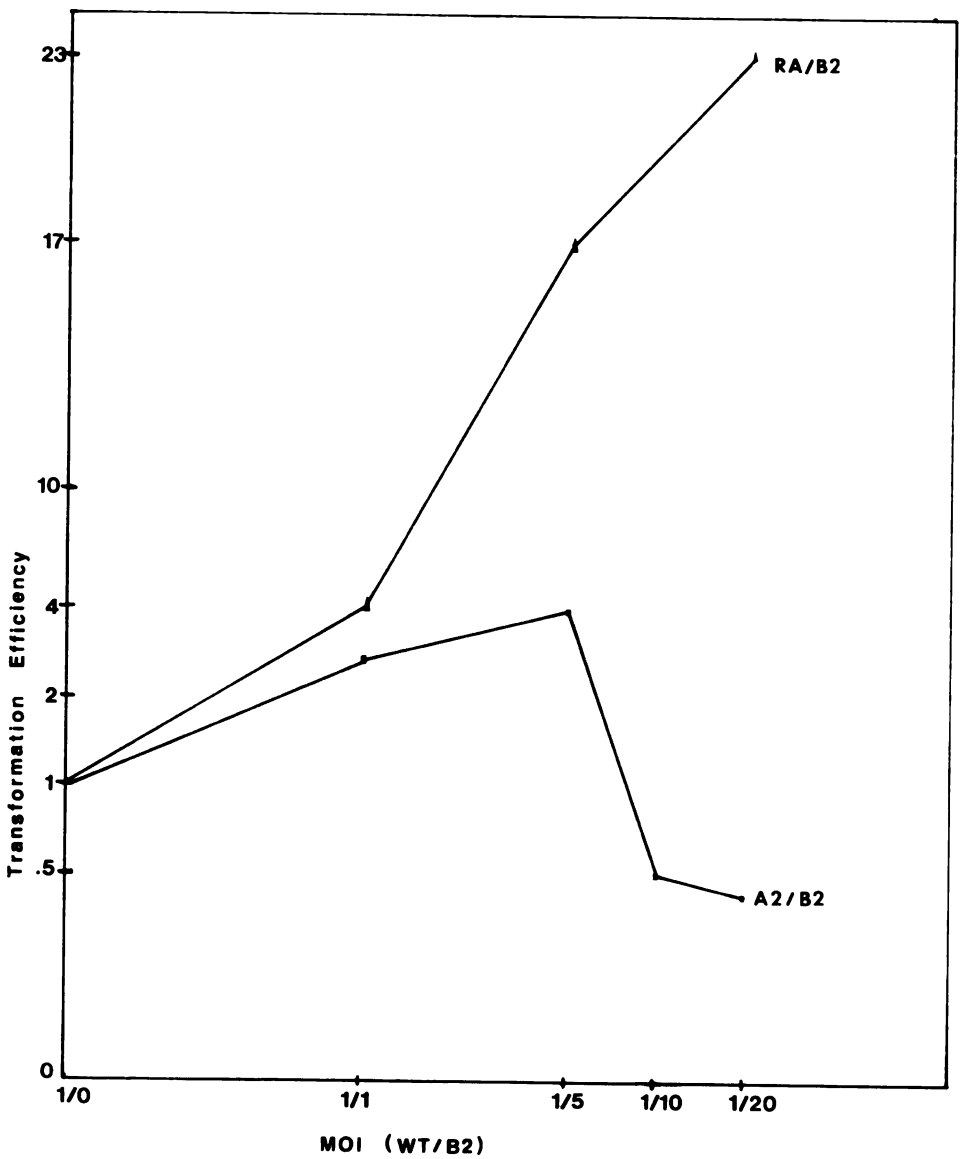

A.
33°
37°
Day
1 4 7 1 4 7
5.3
B.
33°
37°
1 4 7 1 4 7

Transformation by RA in the presence of B2. Transformation frequencies of RA and A2 were compared in rat F-111 cells at 33°C and 37°C, and the frequency of transformation by RA was two-fold less than that of A2 regardless of the temperature (not shown). Differences in the transforming behavior of these two strains were also apparent when mixed infections were conducted with a transformation defective mutant. As reported elsewhere (11), mixed infections with wild-type strains and nontransforming mutants lead to strongly depressed transformation frequencies as compared to infections with wild-type virus alone. This is evident in mixed infections with A2 and B2 (Fig. 3). No dominant lethal effect was seen, however, in mixed infections with RA as the transforming parent. As shown in Fig. 3, the yield of transformants in the mixed infection was higher than in the infection with RA alone. This observation suggests that the transformation potential of RA is slightly deficient and can be complemented in trans by a functional large T-antigen.

Analysis of integrated viral sequences in transformed cells. To analyze the potential effect of the RA mutation on integration patterns, A2- and RA-transformed cells were analyzed for the number of viral integration sites and for the presence of integrated tandem repeats of the viral genome. For this purpose total cellular DNA from each transformant was digested with BglII and EcoRI. Since BglII does not cut within the polyoma genome, the number of polyoma-specific restriction fragments observed on blots of BglII-digested DNA corresponds to the number of distinct integration

Figure 3. Transformation in mixed infections of F-111 cells.

F-111 cells were infected with mixtures of transforming (A2 or RA) and nontransforming (B2) strains and polyoma. The ratios of MOIs of A2 or RA to B2 are given. Transformation was scored by the appearance of foci over the monolayer. The transformation efficiency was determined by dividing the number of foci obtained in the mixed infection with the number of foci obtained in the infection with the transforming strain alone.


23
17
10
4
2
1
.5
0
Transformation Efficiency
RA/B2
A2/B2
1/0
1/1
1/5
1/10
1/20
MOI (WT/B2)

sites. Digestion with EcoRI, which cuts the polyoma genome once, provides a method to determine the presence of tandem repeats within a cell line.

A summary of the analysis of 19 A2- and 16 RA-transformed cell lines is shown in Table 1. A distinct difference in integration patterns was observed between A2- and RA-transformed cells. This difference was reflected in both the number of integration sites and the presence of tandem repeats. The temperature at which the infections were carried out, however, had no apparent effect on integration by either of these strains. Single integration sites were observed in 10 of the 16 RA-transformed cell lines but in only 4 out of the 19 A2-transformants. Similarly, tandem repeats are present in only 50% of the RA-transformants but in 84% of the A2-transformants. These results suggest the presence of a defect in RA which affects integration of the viral genome during neoplastic transformation of nonpermissive cells.

Viral DNA synthesis with reconstructed viruses. To locate the region of the polyoma virus genome coding for the defect in viral DNA synthesis, we constructed hybrid viruses between the RA and A2 strains. A number of reconstructions were made either by using restriction enzymes which cut at two sites within the polyoma genome or by using combinations of two enzymes, each of which cuts at a single site in the viral genome. The restriction fragments used the construction are shown in Fig. 1. Each reciprocal pair of hybrid viruses was tested for replication at 33°C in F-111 cells. When the fragments shown in Fig. 1 originated from the A2 genome, the resulting hybrid genomes

Table 1. Integration analysis.[a]

Strain	Temp.[b]	Single site[c]	Tandem repeats[d]
A2	33°C	2/10	8/9
A2	37°C	2/9	8/9
RA	33°C	4/8	3/8
RA	37°C	6/8	5/8

[a]Infections with A2 and RA were carried out at either 33°C or 37°C. Transformants from these infections were agar cloned and maintained at the same temperature as the infection.

[b]Temperature.

[c]As determined by digestion of total cellular DNA from the transformants with BglII, an enzyme which does not cleave polyoma DNA.

[d]As determined by digestion of total cellular DNA from the transformants with EcoRI, an enzyme which cuts polyoma DNA once.

replicated like A2 at 33°C. When the restriction fragments were taken from RA, the resulting hybrid genomes replicated like the RA virus at 33°C. The EcoRI/NsiI fragment from A2 (nucleotides 1560-1910) was the smallest fragment tested which conferred A2-like DNA replication on RA (Fig. 4). Since the HindIII fragment from A2 also rescued the replication defect in RA (Fig. 4), the mutation is likely to be between the HindIII site at nucleotide 1656 and the NsiI site at nucleotide 1910. Only large T-antigen is encoded by this region of the genome (Fig. 1).

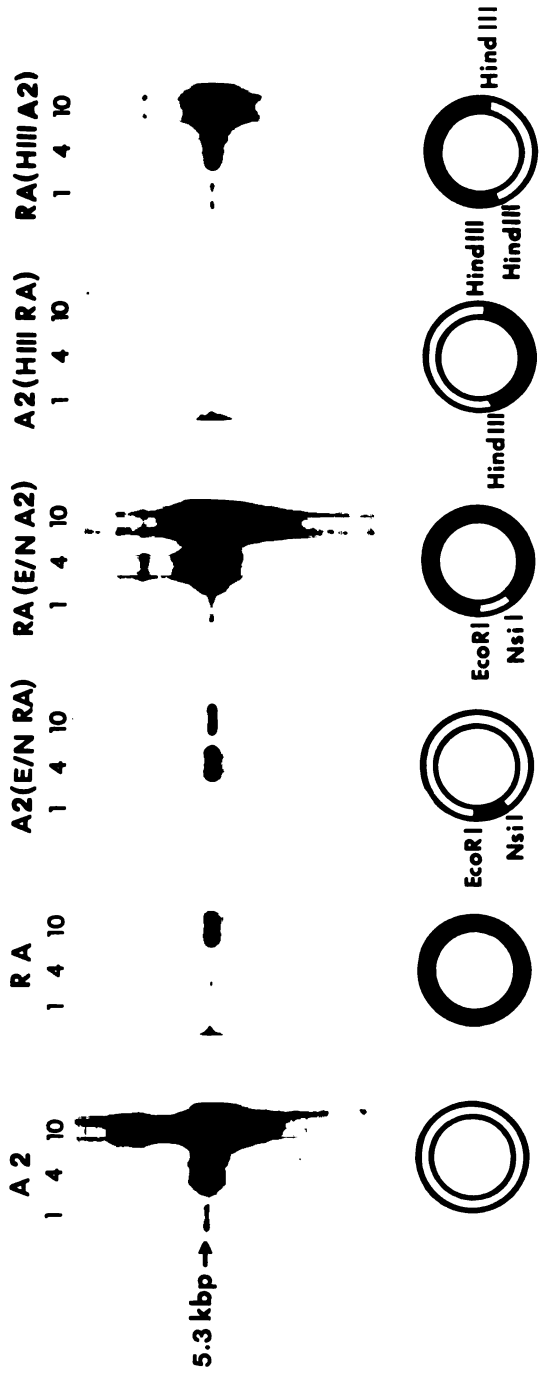
Sequence analysis of RA. A plasmid containing the RA virus was used to determine the sequence of the large T-antigen coding region downstream of the HindIII restriction site (nucleotide 1656). One sequence difference was found at nucleotide 1791, where a C to G transition has occurred in RA. This results in an amino acid change from proline to alanine at amino acide position 412 of large T-antigen. Other sequence differences in the middle T-antigen coding region between NG59, the parent strain of RA, and wild-type A2 have been observed previously (3). A valine codon which is not part of the published sequence for wild-type A2 (29), but is present in wild-type A3 (27), was discoverd near the HindIII site in both the A2 and RA strains from our laboratory.

DISCUSSION

The experiments presented in this report describe a novel mutation in large T-antigen of polyoma virus strain RA. The effect of this mutation on viral DNA replication in permissive mouse cells is not

Figure 4. Comparison of viral DNA synthesis using hybrid viruses.

F-111 cells were infected with RA, A2, or hybrid virus constructions. Infections were performed at 33°C, and viral DNA was extracted at 1, 4, and 10 days postinfection as described in Materials and Methods. The viral fragments contributed by each parent are represented in cartoon form at the bottom of the figure.


A 2
1 4 10
R A
1 4 10
A2(E/N RA)
1 4 10
RA(E/N A2)
1 4 10
A2(HIII RA)
1 4 10
RA(HIII A2)
1 4 10
5.3 kbp
EcoRI
NsiI
EcoRI
NsiI
HindIII
HindIII
HindIII
HindIII

appreciable. In nonpermissive F-111 cells, however, the mutation has a drastic effect on DNA replication at 33°C, resulting in a 10-20 fold reduction of viral DNA yield as compared to wild-type A2. Interestingly, the integration pattern of the RA genome is also different from that of wild-type A2. In RA-transformants, the frequency of tandem integrations at multiple sites in the cellular genome is reduced compared to A2-transformants. Similar patterns have been reported for integration in the absence of large T-antigen (6).

The mutation is rescued very efficiently by a small restriction fragment between the HindIII site at nucleotide 1656 and the NsiI site at nucleotide 1910. The sequence analysis of this region revealed a proline to alanine change resulting from a C to G transition at nucleotide 1791. This mutation is nonconditional and nonlethal, and it maps between the DNA-binding domain (nucleotides 1425-1487) (4) and the carboxy-terminal ATPase domain (W. Folk, personal communication) of large T-antigen, and it is upstream of all known ts-a mutants which affect viral DNA replication (Fig. 1). To our knowledge, no other mutations exist in this region of the polyoma large T-antigen gene.

The mutation in the large T-antigen of RA may not only affect DNA replication in nonpermissive cells, but it may also affect the pathogenesis of the virus in mice. Freund et al. (14) have shown that the tumor pattern of RA in mice is different from that of wild-type A2. Whereas RA induces mostly mesenchymal tumors with a long latency period, wild-type A2 induces thymic and other tumors of epithelial origin which appear with a short latency period. Reconstruction

experiments have shown that a mutation in the coding region of RA is responsible for the major difference in tumor patterns between A2 and RA (14). Although not proven, it is likely that the mutation which we have mapped and sequenced is responsible for the differences in tumor patterns. Thus, a mutation which does not have an appreciable effect on viral DNA replication in mouse fibroblast cell lines may have a profound effect at the animal level.

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Chapter 4.

The Involvement of Interviral Recombination in the Integration/Transformation Pathway of Polyoma Virus[1]

ABSTRACT

We have investigated the role of interviral recombination in the process of integration of polyoma DNA during neoplastic transformation of nonpermissive cells. Transformants were isolated after mixed infections of rat cells with two strains of polyoma virus, MOP1Ø33 and ts3, which lack restriction endonuclease sites, and then analyzed for the presence of integrated recombinant restriction fragments. Positive evidence for recombination was found in thirty-eight percent of the cell lines studied. Recombination was detected in both intervals defined by the mutations, and the viral genomes present in the recombinant transformants appeared to have undergone multiple recombination events. The structures of the integrated genomes of the recombination-positive cell lines were typical of polyoma virus: head-to-tail tandems at multiple sites in the host genome. The level of interviral recombination in the population of infected cells (i.e., in the population of unintegrated, replicated parental viral genomes), however, was below the level of detection at all time points analyzed,

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including those coinciding with the appearance of transformed foci. Since recombination does not provide a selective advantage for transformation in this particular cross, it appears that either transformants are selected from a small subfraction of the infected cell population in which a high level of recombination occurs, or that only those viral molecules which are engaged in the process of integration undergo elevated levels of recombination. These hypotheses are not mutually exclusive. These results demonstrate that interviral recombination is involved in the integration of polyoma DNA in the process of neoplastic transformation.

INTRODUCTION

During the events which lead to neoplastic transformation of nonpermissive rat cells by polyoma virus and SV40, the viral genome becomes integrated into the host DNA by a mechanism which is facilitated by a viral protein, large T-antigen (8). Usually the integrated viral DNA is present as a head-to-tail tandem copies of the viral genome (1,2,22), and integration can occur at multiple sites in the genome of the transformed cell (1,4). Integration occurs by nonhomologous recombination with host DNA and is apparently random with respect to both viral and host sequences (2,4). At the viral-host junctions, homologies of 2-5 base pairs are found (5,19,27). Rearrangements and deletions of host DNA are usually associated with the integration event (28,31).

Although the process by which the tandems originate has not been defined, models to explain their formation have been presented. These invoke either rolling circle replication (6,7) or recombination (11) as mechanisms. Basilico and coworkers have shown that the requirements for tandem formation, the large T-antigen function (8) and the viral origin of replication (7), are consistent with a role for viral DNA replication. A replication step has also been postulated by Chia and Rigby for tandem formation by SV40 based on the existence of high molecular weight species of viral DNA in nonpermissive cells (6). The high molecular weight viral DNA was mostly nonrecombinant in origin and assumed to be the unintegrated precursors of the transforming viral genomes (6). Such unintegrated intermediates have not been described

so far for polyoma virus infections of nonpermissive cells, despite an extensive search (Hacker and Fluck, submitted). Support for rolling circle replication of the polyoma genome has been obtained in permissive mouse cells (3).

Evidence for the involvement of interviral recombination in tandem formation comes mainly from the study of integrated viral genomes. Previous results have suggested that mixed infections with two nontransforming polyoma mutants produced transformants in which the two parental genomes had recombined during the integration process (11). We have also documented cointegration of two distinct viral genomes at a single site (14).

To further analyze the role of interviral recombination in the process of integration of the polyoma genome in nonpermissive cells, we have performed mixed infections with a pair of mutants which lack restriction endonuclease sites. The present experiments were designed to follow the fates of the two viral genomes during the integration/transformation pathway. By analyzing the generation of wild-type restriction fragments both in transformed cells and in a population of cells early after infection, we show that integrated recombinant restriction fragments are present in a high percentage of the transformed cells. No evidence for recombination is observed, however, in the population of unintegrated viral genomes at early times after infection. The results suggest an important role for recombination in the integration pathway of polyoma virus.

MATERIALS AND METHODS

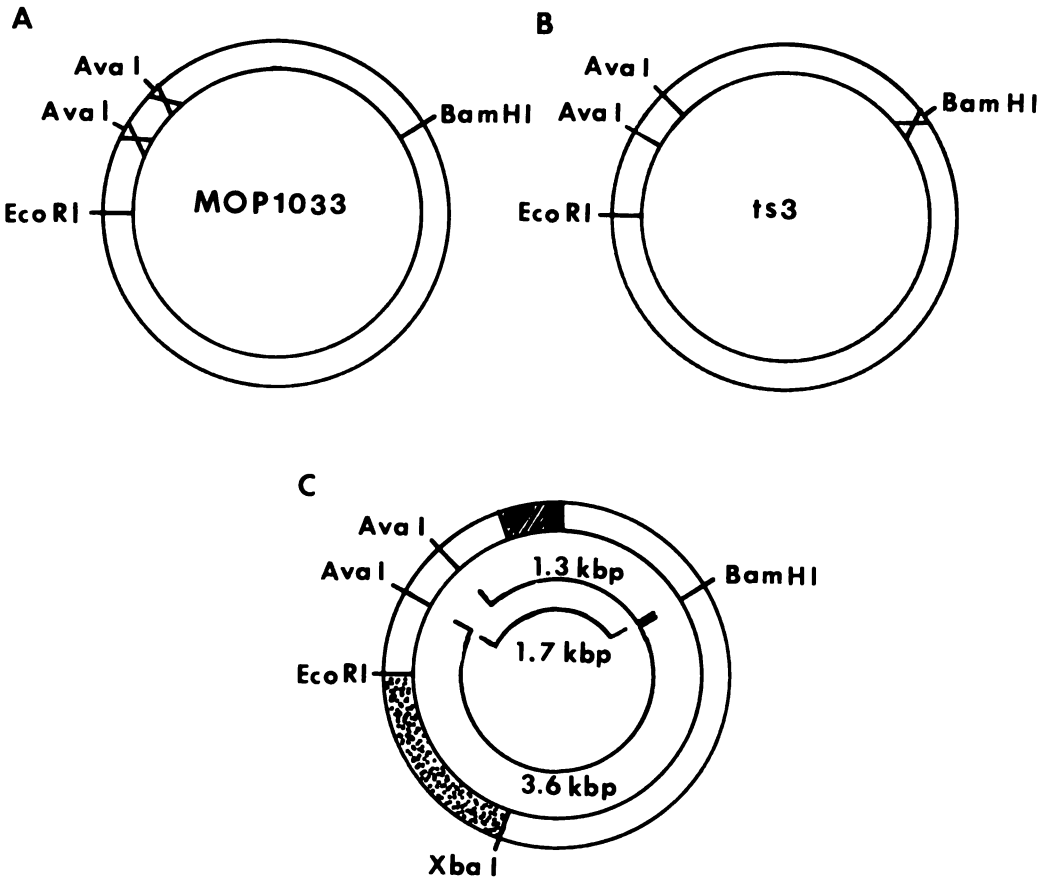
Cells and viruses. Fischer rat F-111 cells (13) were maintained as previously described (32). Prior to infection the cultures were either maintained as actively dividing cells or allowed to reach confluency and maintained in that state for 24 hours prior to passage for infection.

The polyoma strains MOP1Ø33 and ts3 were gifts of Dr. W. Eckhart. Stocks of these strains were grown on baby mouse kidney cultures from plaque-purified virus as described (33). MOP1Ø33 was derived from a wild-type strain by site-directed mutagenesis of nucleotide 1Ø33 (number as in [16]). The point mutation introduced into the middle T-antigen reading frame produced a transformation-defective virus and eliminated the AvaI site at position 1Ø31. The AvaI restriction site at nucleotide 672 is also absent in MOP1Ø33 (Fig. 1A). The ts-3 strain was derived from a wild-type virus by bisulfite mutagenesis. A mutation within the VP2 gene was obtained which prevents decapsidation at the nonpermissive temperature. Since ts-3 lacks the BamHI site (nucleotide 4647 [16]) 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 (W. Eckhart, personal communication). The ts-3 strain transforms normally after a short decapsidation period at 33°C.

Isolation of transformed cells. F-111 cells seeded at a density of 1×10^5 cells per 6Ø mm culture dish were coinfected with MOP1Ø33 and ts-3 at a 1:1 ratio or infected with either of these two strains by itself. The total multiplicity of infection (MOI) for the

Figure 1. Partial restiction maps of polyoma strains.

A.) MOP1Ø33. The Xs mark the location of the AvaI sites at nucleotides 672 and 1Ø31 (numbered as in [16]) that are absent in the MOP1Ø33 genome. B.) ts3. The X marks the location of the BamHI site at nucleotide 4647 (16) that is absent in ts3. C.) Wild-type polyoma. The sizes of the three AvaI/BamHI fragements are given. The map also shows the location of the HpaII-5 fragment (black box) and the EcoRI/XbaI fragment (stippled box) which are used as hybridization probes.


A
Ava I
Ava I
BamHI
EcoRI
MOP1033
B
Ava I
Ava I
BamHI
EcoRI
ts3
C
Ava I
Ava I
1.3 kbp
BamHI
1.7 kbp
EcoRI
3.6 kbp
Xba I

coinfections was 200 plaque forming units (pfu) per cell except when noted. The MOIs of the single parent infections were 100 pfu/cell except when noted. Infections were carried out at 33°C for 90 minutes. Cells were then fed with Dulbecco's modified Eagle's medium (DMEM) with 5% heat-inactivated calf serum and antibiotics. To insure decapsidation of the ts3 parent, infected cells were maintained at 33°C for 24 hours after infection. Some of the infected cultures were then moved to 37°C while the others were maintained at 33°C for the duration of the experiment. Transformed foci which overgrew the monolayer were transferred to 35 mm culture dishes and grown in DMEM with 5% heat-inactivated calf serum. In some cases, the transformed cells were cloned in agar.

Preparation and analysis of DNAs. For the analysis of integration sites approximately 10^7 cells from each cell line were lysed with 0.2% SDS; 10 mM Tris-HCl, pH 7.5; and 10 mM EDTA (2-3 ml/100 mm dish). Total cellular DNA was extracted as previously described (14). For hybridization analysis 10 ug of total cellular DNA was digested with a combination of AvaI and BamHI. Digested DNAs were electrophoresed on 0.7% agarose gels and transferred to nitrocellulose (26). Hybridization was carried out in 2X SSC/1X Denhardt's solution (0.1 ml/cm^2) at 65°C for 36-48 hours, using 5-10 x 10^5 cpm/ml of hybridization solution.

For the analysis of recombination at early times after infection viral DNA was isolated from infected cells by the procedure of Hirt (20). Rat F-111 cells were plated and infected as described above. At

each time point, viral DNA was extracted from one 60 mm dish. One-tenth of the DNA from each plate was digested with a combination of AvaI and BamHI. Gel electrophoresis and DNA transfer were performed as described above. Hybridization was conducted at 65°C for 18-24 hours using 5 x 10^5 cpm/ml of hybrization solution.

Hybridization probes were labeled to a specific activity of 1-2 x 10^9 cpm/ug [^{32}P]-dCTP (3,000 Ci/mmole) using a multiprime DNA labeling kit (Amersham Corp.). The polyoma HpaII-5 and EcoRI/XbaI restriction fragments (Fig. 1C) were isolated in low melting point agarose (FMC Bioproducts).

RESULTS

Description of the recombination assay. To determine the importance of interviral recombination in the process of integration of the polyoma genome in nonpermissive cells, we coinfected Fischer rat F-111 cells with two restriction site-minus strains, MOP1033 (29) and ts3 (10). MOP1033 lacks the two AvaI restriction sites located at nucleotides 672 and 1031 in the early region of the viral genome (Fig. 1A). The point mutation within the second site (nucleotide 1033) causes premature termination of the middle T-antigen, and therefore, this strain is defective in cell transformation. The second strain, ts3, lacks the BamHI restriction site (nucleotide 4647) in the late region of the genome (Fig. 1B) and harbors a mutation which prevents decapsidation of the virus at the nonpermissive temperature. The mutation at the BamHI site may be the cause of the decapsidation defect. Infections with this strain were carried out at 33°C, and the

infected cells were maintained at this temperature for 24 hours to allow for decapsidation before shift-up to 37°C or further incubation at 33°C. When this schedule is followed, the transformation rate with ts3 is normal (10; see Table 2).

Homologous recombination between the two viral genomes can be followed by analysis with restriction endonucleases. Digestion with a combination of BamHI and AvaI will produce restriction fragments of either 3,616 base pairs (bp) if recombination has occurred in the large AvaI/BamHI interval or 1,317 or 1,676 bp if the two parental genomes recombined in the small AvaI/BamHI interval or between the two AvaI sites, respectively (Fig. 1C). The two parental genomes may also be detected. AvaI/BamHI digests will produce a single genome-size restriction fragment (5,292 bp) from the MOP1033 genome and two fragments, 4,933 and 359 bp, from the ts3 genome. The 359 bp is usually not detected because it is electrophoresed past the end of the gel. Thus, recombination can be detected in the unintegrated viral DNA at early times after infection as well as in the integrated viral genomes in transformed cells. The presence of the large AvaI/BamHI fragment is detected by hybridization to the 917 bp EcoRI/XbaI restriction fragment of polyoma (Fig. 1C). The 1,317 bp and the 1,676 bp AvaI/BamHI fragments are detected using the polyoma HpaII-5 restriction fragment (400 bp) as a probe (Fig. 1C).

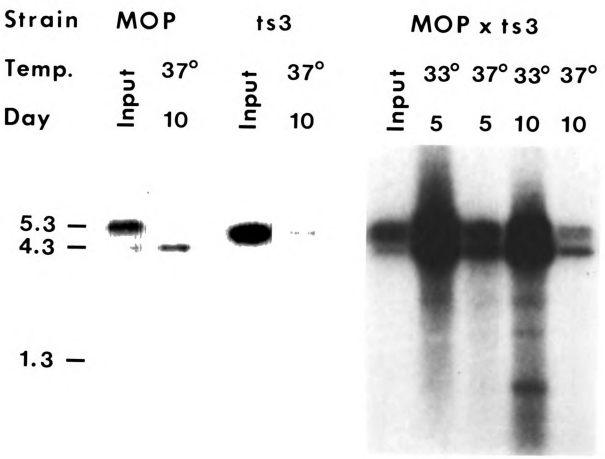
Absence of recombination in the population of unintegrated viral genomes. Monolayers of F-111 cells were infected with a combination of MOP1033 and ts3 as described in Materials and Methods. After infection

the cells were maintained at either 33°C or 37°C, since previous experiments had shown that viral DNA replication is increased at the lower temperature (Hacker and Fluck, submitted). Interviral recombination in the population of unintegrated viral genomes was monitored from the time of infection until the time when transformed foci appeared. For this purpose, viral DNA was extracted from the infected cells at times between 1 and 10 days postinfection as described in Materials and Methods. The DNAs were digested with a combination of AvaI and BamHI. After transfer to nitrocellulose, the blot was hybridized with the HpaII-5 probe (Fig. 2). Neither the 1.3 kbp nor the 1.7 kbp AvaI/BamHI restriction fragment is present at detectable levels in the population of infected cells at the times indicated. This is true even when longer film exposures are used. From a reconstruction experiment, we estimate that the wild-type would have been detected if present in more than 5% of the total viral genomes (not shown). Fig. 2 also shows that viral DNA replication is higher at 33°C than at 37°C, a common finding in F-111 cells (Hacker and Fluck, submitted).

Viral replication intermediates of higher molecular weight may exist in the infected cells. We presume that these would also appear in the Hirt supernatants and be processed through the analysis if present in detectable amounts. This is based on the fact that DNA species larger than viral size (presumably host DNA) are present in these preparations as detected by ethidium bromide straining of the gels, even though the Hirt procedure enriches for viral genome-size molecules.

Figure 2. Analysis of recombination in the unintegrated viral genomes.

F-111 cells were infected with MOP1Ø33 or ts-3 alone at an MOI of 5Ø pfu/cell. Coinfections with MOP1Ø33 and ts3 (1:1 ratio) were performed at a total MOI of 1ØØ pfu/cell. These infections are from Exp. No. 6 (Table 1). For each time point, the viral DNA from one plate of infected cells was extracted as described in Materials and Methods. One-tenth of each sample was digested with a combination of AvaI and BamHI. Hybridization was to the HpaII-5 probe. The positions of the 1.3 and 1.7 kbp AvaI/BamHI fragments and the MOP1Ø33 genome-size fragment (5.3 kbp) are marked.


Strain
MOP
ts3
MOP x ts3
Temp.
Input
37°
Input
37°
Input
33°
37°
33°
37°
Day
10
10
5
5
10
10
5.3 —
4.3 —
1.3 —

The MOP1Ø33 stock contains a defective viral genome as evidenced by the presence of an AvaI/BamHI restriction fragment which migrates at 4.3 kbp (Fig. 2). This fragment does not interfere with the detection of recombination since it does not migrate close to the 1.3, 1.7, and 3.6 kbp AvaI/BamHI restriction fragments. Another prominent restriction fragment of about 1.Ø kbp appears at 1Ø days postinfection. Longer exposures show that this fragment is present in the ts3 stock.

High level of recombination in integrated genomes. Transformed foci arising from the infections described above were visible by 1Ø and 17 days postinfection for the 37°C and the 33°C cultures, respectively. These were isolated, and total DNA was extracted from the transformants and analyzed for integrated recombinant viral genomes as described in Materials and Methods.

a.) Recombination in the 3.6 kbp AvaI/BamHI interval. Recombination in this interval was analyzed by hybridizing blots of the AvaI/BamHI digestions with the polyoma EcoRI/XbaI probe (Fig. 3). In 23 of the 65 (35%) cell lines analyzed, the 3.6 kbp AvaI/BamHI restriction fragment is present, indicating that recombination between the two parents has occurred (Table 1). In addition to the recombinant fragments, the 5.Ø and 5.3 kbp fragments from the two parental viral genomes are also visible in many cases. Both parental genomes are present in 14 out of 23 cell lines, while a single parental genome is present in 6 out of 23 cells lines.

b.) Recombination in the 1.3 kbp and the 1.7 kbp AvaI/BamHI intervals. The blots from the analysis above were washed

Figure 3. Recombination in the 3.6 kbp AvaI/BamHI interval.

Total cellular DNA (10 μg) was digested with a combination of AvaI and BamHI. Hybridication was to the polyoma EcoRI/XbaI probe. The cell lines represented here were established from the 37°C coinfections in Exp. Nos. 6 and 7 (Table 1). The locations of the 3.6 kpb AvaI/BamHI fragment, the MOP1033 genome-size fragment (5.3 kbp), and the large AvaI fragment from ts3 (5.0 kbp) are shown.

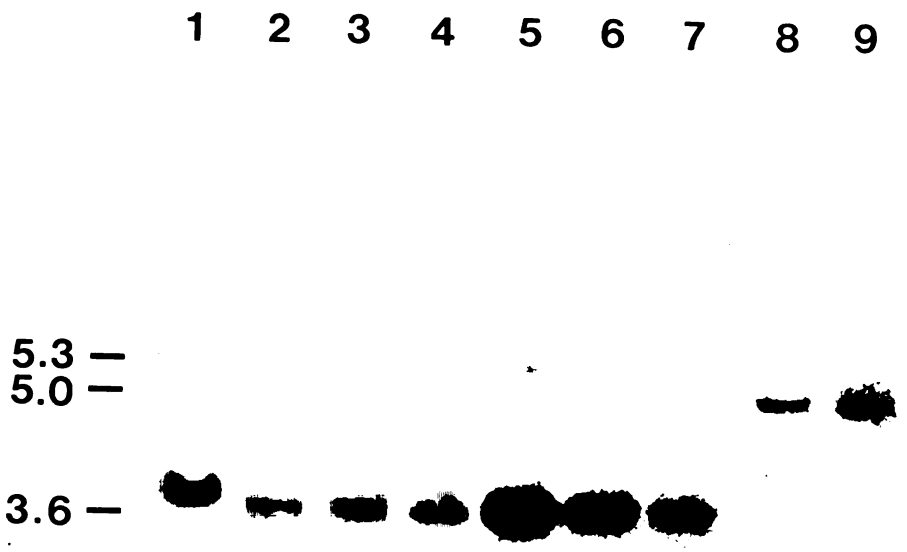

1 2 3 4 5 6 7 8 9
5.3
5.0
3.6

Table 1. Summary of recombination in transformed cell lines

Temperature		33°C				37°C			
AvaI/BamHI Fragment[a]		1.3kbp	1.7kbp	3.6kbp	Total # positive	1.3kbp	1.7kbp	3.6kbp	Total # positive
Exp. No.[b]	1	--	--	--	--	2/6	0/6	2/6	2/6
	2	--	--	--	--	0/5	0/5	1/5	1/5
	3	0/3	0/3	0/3	0/3	2/10	1/10	2/10	2/10
	4	0/7	0/7	0/7	0/7	3/4	3/4	3/4	3/4
	5	2/4	0/4	1/4	2/4	3/4	2/4	3/4	3/4
	6	0/5	1/5	1/5	1/5	3/6	4/6	4/6	4/6
	7	0/5	2/5	1/5	2/5	5/6	5/6	5/6	5/6
Total		2/24	3/24	3/24	5/24	18/41	15/41	20/41	20/41

[a]The presence of the 1.3 and 1.7 kbp AvaI/BamHI restriction fragments was detected by hybridization to the HpaII-5 probe. The presence of the 3.6 kbp AvaI/BamHI fragment was detected by hybridization to the EcoRI/XbaI probe. Refer to Fig. 1 for the identification of these fragments.

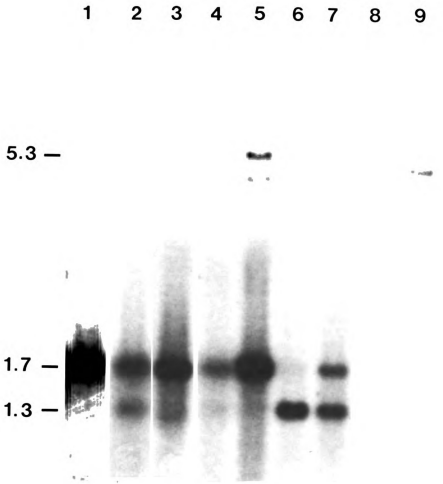
[b]For Experiment Numbers 1, 3, 5 and 7, the cell cultures were confluent prior to passage for infection. For Experiment Numbers 2, 4 and 6, the cell cultures were exponentially grown prior to passage for infection. See Materials and Methods for details.

and then hybridized with the HpaII-5 probe to detect evidence of recombination within the small AvaI/BamHI interval or between the AvaI sites (Fig. 4). In many of the cell lines, both the 1.3 and the 1.7 kbp restriction fragments are present. As before, the 5.0 and 5.3 kbp fragments from the two parental genomes are also visible. The presence of either the 1.3 or the 1.7 kbp fragment is evidence that recombination between the MOP1Ø33 and ts3 genomes has occurred during the integration/transformation process. Either the 1.3 or the 1.7 kbp fragment is present in 24 of the 65 (37%) cell lines analyzed. In all, 25 of the 65 (38%) contain at least one of the three fragments that is diagnostic of recombination between MOP1Ø33 and ts3. In most cases, more than one of the recombinant restriction fragments is present. Fourteen of the recombination-positive transformants have all three of the fragments, five have the 1.3 kbp and the 3.6 kbp fragments, three have the 1.7 kbp and the 3.6 kbp fragments, and three have only one of the fragments.

The structure of the integrated viral genomes. The transformants in which the parental genomes have undergone recombination have the following hallmarks of normal polyoma transformants: head-to-tail tandem integration of the recombinant genomes as evidenced by the presence of 5.3 kbp genome-size restriction fragments in digestions with a single-cutter such as EcoRI (not shown), multiple restriction fragments in digests with a zero-cutter such as BglII, and free viral DNA as shown by the presence of form I polyoma

Figure 4. Recombination in the 1.3 and 1.7 kbp AvaI/BamHI intervals.

Total cellular DNA (1Ø µg) from transformants established from coinfections with MOP1Ø33 and ts3 was digested with a combination of AvaI and BamHI. Hybridization was to the HpaII-5 probe. The DNAs in lanes 1-9 are from transformants established from the 37°C coinfections in Exp. Nos. 6 and 7. The locations of the 1.3 and the 1.7 kbp AvaI/BamHI fragments are shown.


1 2 3 4 5 6 7 8 9
5.3 —
1.7 —
1.3 —

DNA in digests with the zero-cutter BglII (not shown). Along with recombinant viral genomes, parental genomes are also present in most of the transformants. The parental genomes are integrated as head-to-tail tandems as evidence by the presence of genome-size restriction fragments (5.0 and 5.3 kbp) in the products of AvaI/BamHI codigestions (see Figs. 3 and 4).

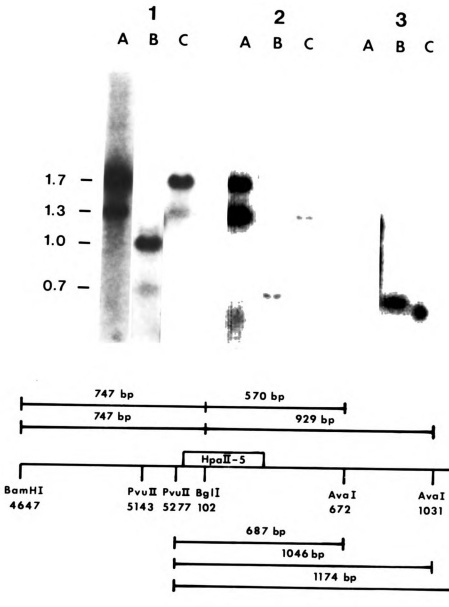
The presence of free viral DNA does not affect the interpretation concerning the presence of integrated recombinant viral genomes since the free DNA has been shown to be excised from integrated copies (1). The existence of integrated recombinant genomes is based on the following facts. First, three of the transformants contain only a recombinant genome with no obvious sign of the complete parental genomes (not shown). Second, the intensity of the hybridization signal from the recombinant fragments compared to the genome-size fragments suggests that they exist as integrated copies rather than being generated post-excision. Third, the recombinant restriction fragments are generally absent in cell lines generated from the 33°C infections. Furthermore, recombination has also been detected at similar high frequency under circumstances in which an integrated recombinant genome is required for transformation (Kalvonjian et al., submitted). Finally, the DNA from one cell line containing all three recombinant fragments was extracted by the Hirt procedure and isolated as low and high molecular weight fractions. AvaI/BamHI digestion of this high molecular weight DNA revealed that the recombinant fragments are present in this fraction (not shown).

Further analysis of the 1.3 and 1.7 kbp AvaI/BamHI restriction fragments. The presence of the 1.3 and 1.7 kbp fragments in the same cell lines is especially interesting since these may represent different recombination events. Conceivably, the 1.7 kbp AvaI/BamHI fragment may arise by incomplete digestion at the first AvaI site (nucleotide 672). To further analyze the origin of the 1.3 and 1.7 kbp restriction fragments, the DNAs from fourteen of the recombination-positive cell lines were digested with a combination of restriction enzymes: AvaI, BamHI, and either PvuII or BglI. The restriction sites in and around the small AvaI/BamHI interval for these two additional enzymes are shown in Fig. 5. Digestion with PvuII, BamHI, and AvaI generates restriction fragments of 687, 1,046, and 1,174 bp that hybridize to the HpaII-5 probe (Fig. 5). The presence of these fragments depends upon the presence or absence of the AvaI sites. The 1,174 bp fragment is generated by digestion of the MOP1033 genome, and the 687 bp fragment can originate from either the ts3 genome or a recombinant genome. Digestion of the 1.3 and 1.7 kbp AvaI/BamHI fragments with BglI generates restriction fragments of 747 and 570 bp and 747 and 929 bp, respectively, that hybridize to the HpaII-5 probe (Fig. 5). It should be noted, however, that the BglI site is absent in the MOP1033 genome. Therefore, the 1.3 and 1.7 kbp AvaI/BamHI fragments will be digested with BglI only when this restriction site is contributed by the ts3 genome.

Of the fourteen cell lines analyzed, ten contained both the 1.3 and the 1.7 kbp fragments, three contained only the 1.3 kbp fragment,

Figure 5. Analysis of the 1.3 and 1.7 kbp AvaI/BamHI restriction fragments.

Total cellular DNA (10 μg) from recombination-positive cell lines was digested with AvaI and BamHI (A.); AvaI, BamHI, and PvuII (B.); and AvaI, BamHI, and BglI (C.). The locations of the restriction sites for these enzymes within and around the wild-type 1.3 AvaI/BamHI interval are shown along with the sizes of the expected restriction fragments for (B.) and (C.) above. Hybridization was to the HpaII-5 probe.


1
2
3
A B C A B C A B C
1.7
1.3
1.0
0.7
747 bp
570 bp
747 bp
929 bp
HpaII-5
BamHI
4647
PvuII PvuII BglI
5143 5277 102
AvaI
672
AvaI PvuII
1031 1159
687 bp
1046 bp
1174 bp

and one contained only the 1.7 kbp fragment. As expected, both the 1.3 and the 1.7 kbp AvaI/BamHI fragments (Fig. 5, lanes A) were further digested by PvuII in all of the cell lines examined (Fig. 5, lanes B). Addition of BglI to the AvaI/BamHI reactions led to digestion of the 1.3 kbp AvaI/BamHI fragment in 4 out of the 14 cell lines analyzed (Fig. 5, lanes C). The 1.7 kbp AvaI/BamHI fragment, present in eleven of the cell lines, was not digested with BglI (Fig. 5, lanes C). In the ten cell lines with both the 1.3 and the 1.7 kbp fragments, the 1.3 kbp fragment was digested with BglI in two cases. These results can be interpreted to mean that the 1.7 kbp AvaI/BamHI fragment is generated by a recombination event in which the MOP1Ø33 and ts3 genomes recombined between nucleotides 672 and 1Ø16 (interval C, see Fig. 7). If this is the case, then the region between the BamHI site and the AvaI site at position 672 (interval A) is contributed by MOP1Ø33. On the other hand, the 1.3 kbp fragment is occasionally digested by BglI (4 of 14 cell lines), indicating that this restriction site can be donated by the ts3 genome if the recombination event occurs between the BamHI site and the AvaI site at nucleotide 672 (interval B).

Conditions which affect recombination. Previous results from this laboratory have shown that viral DNA replication in nonpermissive cells is higher at 33°C than at 37°C for many strains of polyoma (Hacker and Fluck, submitted). The same is true for MOP1Ø33 and ts3 (see Fig. 2). To determine if the level of replication affects recombination, we isolated transformants from cells that had been infected with MOP1Ø33 and ts3 and then maintained at either 33°C or 37°C following the 24

hour incubation at 33°C to allow for decapsidation. The presence of the 3.6 kbp AvaI/BamHI restriction fragment was detected in only 3 of 24 (12%) cell lines isolated from cultures maintained at 33°C (Table 1). On the other hand, 20 of 41 (49%) of the cell lines from cultures maintained at 37°C were positive for the presence of the 3.6 kbp fragment (Table 1). Similar results were observed when the cell lines were analyzed for the presence of either the 1.3 or the 1.7 kbp AvaI/BamHI restriction fragment. Of the 24 cell lines isolated from 33°C infections, only 5 (21%) were positive for either one of these fragments, while at least one of these fragments was detected in 20 of 41 (49%) cell lines isolated from cultures maintained at 37°C (Table 1).

To begin analyzing which cellular factor(s) may affect recombination, cells were maintained in different growth states prior to infection with MOP1033 and ts3 (Table 1). For "exponential cells", the cultures were maintained in a state of active cell division prior to passage for infection. For "confluent cells," the cultures were allowed to become confluent and were maintained this way for 24 hours prior to passage for infection. This population of cells was essentially synchronized at the time of infection. The two protocols for growing the cells did not noticeably affect the recombination frequency (Table 1).

Analysis of ts3 and MOP1033 viral stocks. Several approaches were taken to address the possibility that the wild-type fragments observed in the transformed cell lines were not generated by recombination but

rather were due to a wild-type contamination of one of the viral stocks. First, genomic DNA from cell lines transformed by ts3 alone were analyzed by codigestion with AvaI and BamHI. The blots were hybridized with the HpaII-5 (Fig. 6) and the EcoRI/XbaI (not shown) probes. Of the 17 cell lines studied, none contained the 1.3, 1.7, or 3.6 kbp AvaI/BamHI restriction fragment. Second, no transformants were ever obtained from infections with MOPlØ33: an indication that the stock of this nontransforming virus does not harbor a wild-type containment. Finally, analysis of the input viral DNA by AvaI/BamHI digestion (Fig. 2) did not reveal the presence of the 1.3 kbp or the 1.7 kbp restriction fragment.

Transformation frequencies in mixed infections with MOPlØ33 and ts3. To ascertain that recombination between the two parental genomes has no selective advantage over infection by the ts3 parent alone, transforming frequencies were measured in single and mixed infections (Table 2). The presence of the nontransforming virus decreases the transformation rate of the ts3 virus by 1.5 to 17-fold at 37°C. This effect (the dominant lethal effect) of a nontransforming parent in mixed infections has been previously noted (12) and is described in detail elsewhere (Oh et al., submitted). Transformation at 33°C was 1.5 to 2-fold higher than at 37°C for the mixed infections as is typical for cell transformation by polyoma (8; and Hacker and Fluck, submitted). The higher transformation rate and the lower recombination rate at 33°C suggests that steps other than homologous recombination affect the integration/transformation pathway.

Figure 6. Analysis of ts3-transformed cell lines.

Transformants were established from infections with ts3, and total cellular DNA (10 μg) from these cell lines was digested with a combination of AvaI and BamHI. Hybridization was to the HpaII-5 probe. The locations of the 1.3 kbp AvaI/BamHI fragment and the large AvaI fragment (5.0 kbp) from ts3 are shown.

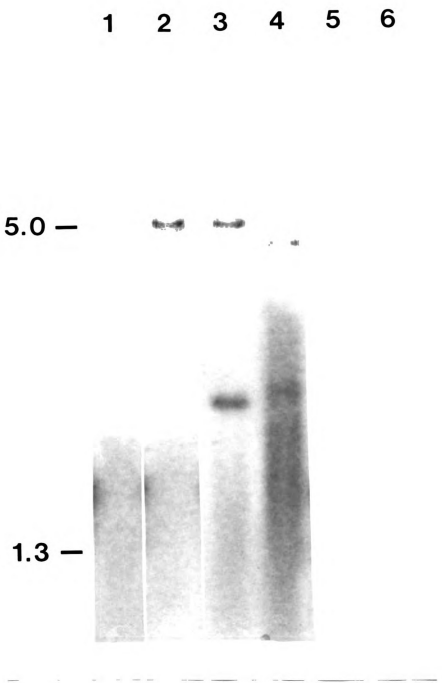

1 2 3 4 5 6
5.0 —
1.3 —

Table 2. Transformation rates.

Exp. No.[a]	No. Transformants[b]			
	MOP1Ø33[c]	ts3[c]	MOP1Ø33 x ts3 33°C	37°C
3	Ø	32	26	17
4	Ø	19	22	14
5	Ø	1ØØ	1Ø	6
6	Ø	56	33	15
7	Ø	75	15	8

[a]The experiments are numbered the same as in Table 1.

[b]Based on the infection of 1 x $1Ø^5$ cells at an MOI of 1Ø pfu/cell.

[c]Cultures were maintained at 37°C after the decapsidation period.

DISCUSSION

The experiments presented above were designed to determine the level of recombination between viral genomes in the process of transformation by polyoma virus. There was no selective advantage for recombination between viral genomes since one of parents, MOPlØ33, is a nontransforming strain. Unusually high but variable levels of recombination were observed in the transformants. Of the 65 cell lines analyzed, 25 had integrated viral genomes which had been generated by recombination. The maximal level observed corresponds to a recombination frequency of sixty-five percent in a 1.3 kbp interval of the viral genome (see Table 1, Experiment 5). These values are minimal estimates since we have not analyzed recombination events leading to the double-mutant recombinant (which would generate fragments larger than genome-size) and since recombination of two molecules of the same genotype cannot be scored. In the recombination-positive transformants (designated recombinant transformants), recombination in the 1.3 or 1.7 kbp AvaI/BamHI interval is accompanied in eighty-eight percent of the cases by recombination in the large AvaI/BamHI interval. The recombination events are homologous as judged by the size of the restriction fragments. The integration patterns in recombinant transformants are typical of polyoma-transformed cells: integration of head-to-tail tandems at multiple sites of the host genome with production of some free viral DNA. Parental genomes were also recovered in all but three of the recombinant transformants.

As shown in Table 1, recombination frequencies varied from experiment to experiment. It is surprising, however, that higher

levels of recombination were observed at 37°C than at 33°C. For instance, the 3.6 kbp AvaI/BamHI fragment was present in 49% of the 37°C cell lines but only 12% of the 33°C cell lines. We have shown elsewhere that DNA replication is substantially increased in nonpermissive cells at 33°C compared to 37°C (Hacker and Fluck, submitted). However, the apparent antagonism between recombination and replication in our experiments may be fortuitous. First, data from other experiments show that the integration/transformation linked recombination occurs prior to the detection of increased viral DNA synthesis (15; Friderici and Fluck, unpublished data). Second, it appears that cells in the process of synthesizing viral DNA are not the precursors of transformed cells (Hacker and Fluck, submitted).

Since large T-antigen is required for the formation of tandems (8) and a fraction of the tandems may be assigned to recombination events, it appears that the role of large T-antigen in the formation of tandems may not be viral DNA replication per se. Recently, results from an intramolecular recombination assay involving a polyoma-mouse hybrid replicon containing 1.Ø3 copies of the polyoma genome suggest that large T-antigen may have a recombinogenic activity (24). It is not known, however, if this is a direct effect of large T-antigen binding to the viral genome.

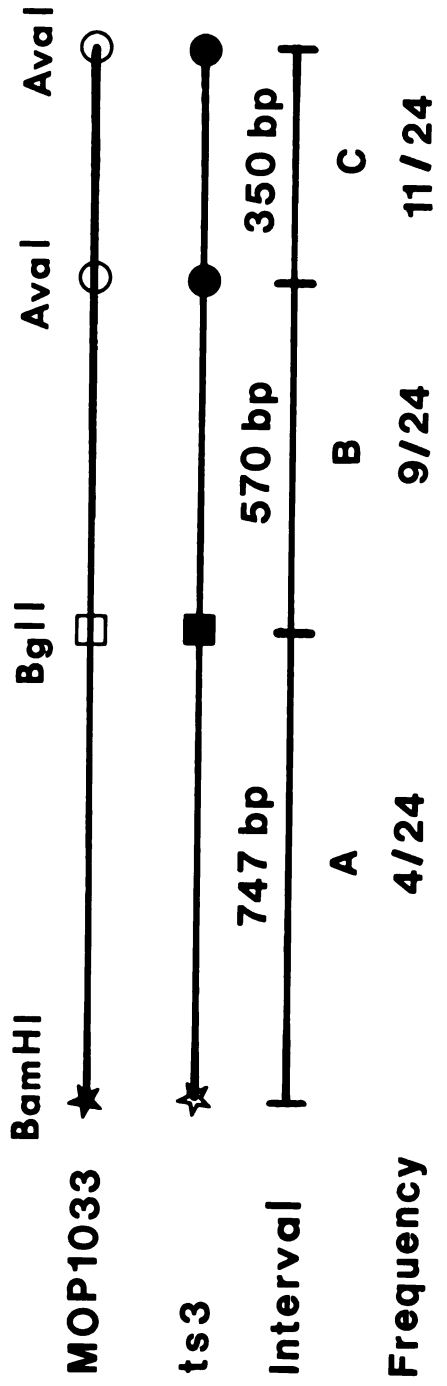
The results of the analysis of the 1.3 and 1.7 kbp AvaI/BamHI fragments proved to be very interesting. For the 1.7 kbp fragment, the absence of the BglI site and the AvaI site at nucleotide 672 illustrates that this fragment is generated by a recombination event in

interval C (Fig. 7). The 1.3 kbp fragment results from a recombination event either in interval A (Fig. 7) or in interval B (Fig. 7). The number of recombination events which occurred within these three intervals in the fourteen cell lines that were analyzed is tabulated in Fig. 7. In this group of recombinant transformants the highest number of events occurred in the smallest region, the 359 bp interval C, while the lowest number of recombination events occurred in the largest region, the 747 bp interval A. This gradient of recombination suggests the existence of a site on the viral genome near the AvaI sites that enhances recombination. Downstream from the AvaI site at nucleotide 1031 is an eight base-pair sequence 5'-GCTGGTCT-3' (nucleotides 1162-1155 [16]) in which six of the eight base pairs match the consensus sequence of the lambdaphage Chi site (5'-GCTGGTGG-3'), a nucleotide sequence which stimulates recombination by the RecA-RecBC pathways in E. coli (23). It is not known whether this sequence in the polyoma genomes has an effect on recombination in lambdaphage. Chi sites have previously been identified in the mouse immunoglobulin genes (21).

We have attempted to determine the level of recombination among unintegrated viral genomes present in the population of infected cells at early times after infection (0-10 days). Interestingly, we did not detect the recombinant genomes in this population. The presence of the wild-type restriction fragments should have been detectable at a level of 5% of the total viral DNA as determined by reconstruction experiments. The absence of recombinant fragments in the population of

Figure 7. Gradient of recombination in the 1.3 and 1.7 kbp AvaI/BamHI intervals.

Partial restriction maps of the small AvaI/BamI intervals from MOPlØ33 and ts3 are shown. The open and closed symbols represent restriction endonuclease sites which are absent or present, respectively. The number of recombination events which occurred in each of the intervals (A, B, and C) in the fourteen recombination-positive cell lines are tabulated.


BamHI
BglI
AvaI
AvaI
MOP1033
ts3
747 bp
570 bp
350 bp
Interval
A
B
C
Frequency
4/24
9/24
11/24

unintegrated viral genomes is remarkable considering the percentage of cell lines which were found positive for recombination. These results can be interpreted in two ways. The first is that the cells destined to become transformed are selected from a small pool of cells (possibly in a restricted window of the cell cycle) in which recombination is occurring at a high level. In these cells integration of the viral genomes and fortuitous recombination between viral genomes are both enhanced. The second is that only the viral molecules which are involved in the integration pathway (i.e., which interact with the host chromosome) undergo interviral recombination. These alternatives are not mutually exclusive. The present experiments do not exclude the possibility (which we consider unlikely at this time) that one genome integrates and then the second parent recombines with it at a high frequency.

The experiments described in this report were initiated as an attempt to identify the pathway that leads to formation of head-to-tail tandem integrants of the viral genome in polyoma transformants. Although interviral recombination was not selected for in these experiments, a significant portion of the integrated viral genomes in the transformants studied have undergone interviral recombination. The recombinant transformants have the typical integration pattern of polyoma transformants. Thus, the present experiments suggest that homologous recombination may account for the formation of a large fraction of the integrated head-to-tail tandems. We cannot exclude the possibility that viral DNA replication plays a role in tandem

formation, but it appears that the bulk of the cell population in which extensive viral DNA replication is occurring does not contribute to the population of transformants (Hacker and Fluck, submitted).

To our knowledge, the recombination frequencies observed in these crosses represent the highest recombination rates reported in a mitotic mammalian system, including recombination studies involving plasmid DNA and transfection procedures (9,17,18,25,30). The mechanisms underlying such elevated recombination rates remain to be elucidated.

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SUMMARY AND CONCLUSIONS

The roles of viral DNA replication and recombination in the integration of the polyoma virus genome into the host chromosome of nonpermissive cells was investigated. The experiments described in Chapter 4 establish that interviral recombination is involved in integration. Although recombinant genomes are undetectable in the population of unintegrated genomes during the early steps of nonpermissive infection, the level of recombinant genomes in transformants is high and can account for a large fraction of the integrated viral genomes. The role of viral DNA replication in integration is not as clear. The experiments described in Chapter 2 indicate that a high level of viral DNA synthesis occurs in a subpopulation of the infected nonpermissive cells at 33°C. This increase in the number of viral genomes does not dramatically affect the transformation frequency at 33°C nor the integration pattern in transformants obtained from 33°C infections. The experiments in Chapters 2 and 3 show that large T-antigen has a role in viral DNA synthesis in nonpermissive cells. Experiments from another laboratory demonstrated a requirement for large T-antigen in the integration of tandem viral genomes. It is not known if this requirement for large T-antigen involves viral DNA replication.

Unintegrated high molecular weight species of viral DNA have been hypothesized to be the precursors to the integrated tandem copies of the viral genome. The failure to detect these can be explained in several ways. It is possible that such intermediates do not exist. If

they do, they may either be present in low abundance or be short-lived due to resolution to genome-size by intramolecular recombination.

The results presented here suggest other areas of research to pursue. One of these concerns the nature of permissivity of cells to infection by polyoma. Only a small percentage of nonpermissive cells can support synthesis of viral DNA. In these cells, the block in virus production appears to be in late gene expression. Therefore, at least two stages of inhibition of viral replication occur after the virus enters the nonpermissive cell. Experiments from other laboratories have implicated the cellular DNA polymerase α/DNA primase as a major factor in permissivity with regards to viral DNA replication, but other host factors are probably involved as well. It is likely that the block in late gene expression involves host factors important in positive or negative regulation of gene transcription.

The second major area of interest developed from these experiments involves the mechanims of DNA recombination in mammalian cells. The results in Chapter 4 suggest that a high level of recombination occurs in a small population of cells. It may be that the viral genomes in these cells interact in a specific way with the host chromosomes to allow for both a high level of interviral homologous recombination and nonhomologous recombination between cellular and viral DNA. It is possible that a specific interaction with the nuclear matrix makes the viral DNA more accessible to the cellular recombination machinery. The recombination system described here may be useful in elucidating the viral-host cell interactions involved in the recombination events described in Chapter 4.