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Extreme Frequencies of Homologous Genetic Exchange Between Polyomavirus Chromatin in Mitotically Dividing Cells

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EXTREME FREQUENCIES OF HOMOLOGOUS GENETIC EXCHANGE BETWEEN POLYOMAVIRUS CHROMATIN IN MITOTICALLY DIVIDING CELLS

By

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ABSTRACT

EXTREME FREQUENCIES OF HOMOLOGOUS GENETIC EXCHANGE BETWEEN POLYOMAVIRUS CHROMATIN IN MITOTICALLY DIVIDING CELLS

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In this thesis, I have mapped recombination frequencies on the polyomavirus chromatin. Two variants of polyomavirus, each containing four novel unique restriction endonuclease sites, were designed and constructed in the large T antigen temperature sensitive background. The viral genome was thus divided into eight intervals that could be tested for recombination. Co-infections of non-permissive Fisher rat fibroblast cells were carried out at permissive and non-permissive temperatures. Transformed cell lines were isolated and analyzed for recombination. The results show that 19% of the transformants contain recombinant viral sequences. Crossovers occurred throughout the polyomavirus genome. On average, there are 3.4 crossovers per integrated viral sequence. The range of crossovers varied from a low of 2 to a high of 7 per integrated sequences. Two intervals, one in the early region and one in the late region, show higher than average number of crossovers. Maintenance of cell populations at permissive or non-permissive temperatures

did not affect crossover frequencies. There was, however, a dramatic reduction in the number of transformants and viral DNA replication at the non-permissive temperature.

To My Parents

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Genetic recombination is an important and ubiquitous process in the life of all organisms. Through it new combinations of the genetic material are formed. Recombination is crucial to the viability of organisms and plays an important role in evolution. It can summarily be divided into four major categories. The first of these is general recombination that occurs between homologous regions of DNA and was first described in the fruit fly by Morgan (1). Homologous recombination can be sub-divided into two categories, those which involve reciprocal exchanges of DNA and conversion events which involve non-reciprocal transfer of genetic material. This distinction, however, can only be made when both parental and progeny products can be analyzed. Transpositional recombination refers to the movement of transposable elements from one location on the DNA to another. The re-arrangement of DNA in highly specific regions is referred to as site specific recombination. Finally, there is illegitimate recombination which requires neither sequence or site specificity nor defined genetic elements. Although this classification is convenient, it is arbitrary in the sense that there are no clear boundaries between the different kinds of recombination. Homology is not only important for general recombination but also for site specific recombination.

Although there are site specific enzymes involved in catalysis of site specific recombination reactions, the interaction of enzymes with specific DNA sequences is also observed in other types of recombination. Hot spots of recombination, for instance, are likely sites of action for specific nucleases; additionally, transposable elements interact with site specific enzymes during transposition to a new site.

There are two models for the initiation of homologous recombination. These models are in agreement regarding the structure of DNA strands involved in recombination. Holliday (2-5) has described how two homologous molecules of DNA can interact in a structure known as the Holliday junction (Figure 1). This structure is formed subsequent to the juxtaposition of two molecules of DNA and a break in one of the molecules. Meselson and Radding (6, 7) have proposed that the origin of such junctions is the formation of a single stranded break in one of the parental DNA molecules involved in the recombination event. The invasion of the double stranded molecule by the single-stranded DNA displaces the resident homologous strand. The displaced strand is subsequently destroyed and the gap is repaired by DNA synthesis. Szostak and colleagues (8-10), however, propose that the origin of Holliday junctions is a double-stranded break that may become a gap. This event is then followed by repair synthesis of the gap using the homologue as a template (Figure 2).

Knowledge of the factors that may affect the probability and location of recombination events among a given set of molecules can improve our grasp of the molecular mechanisms of homologous recombination. The study of

recombination hotspots has been used in this respect. These sites may exert their effect on recombination at two points. One may be the initiation or termination of the strand exchange process and the other may involve the process through which parental molecules are brought in close proximity. The effect of these special sites may also be indirect. They may be involved in initiation of such processes as DNA replication or transcription which may influence recombination frequencies. One of the most well studied hot spots of recombination is mediated by the Chi sequence (11) in the lambda (λ) phage and *Escherichia coli* which stimulates recombination mediated by the E. coli RecBCD pathway (12-15). Phage λ contains a linear, double stranded molecule of DNA which has single stranded, unique ends. When it is injected into the *E. coli* host cell, the ends anneal and are covalently linked by the host DNA ligase. This leads to the formation of the intact cohesive site (*cos* site). Theta replication proceeds this step and results in the formation of monomeric circles that are poor substrates for packaging. Replication switches to the rolling circle mode that produces concatemeric DNA molecules suitable for packaging (16, 17). The E. coli RecBCD enzyme blocks the switch from theta to rolling circle replication (18). The λ gam gene product, however, can bind and inactivate RecBCD (19, 20). If an *E. coli* host cell which is $RecBCD^+$ is infected with λ Gam^-, the phage replication is limited to production of non-packageable monomeric circles. However, such infections do produce sufficient progeny to produce large plaques. This is as a result of homologous recombination yielding concatemeric DNA (21). The primary pathway of recombination is the RecBCD pathway that is

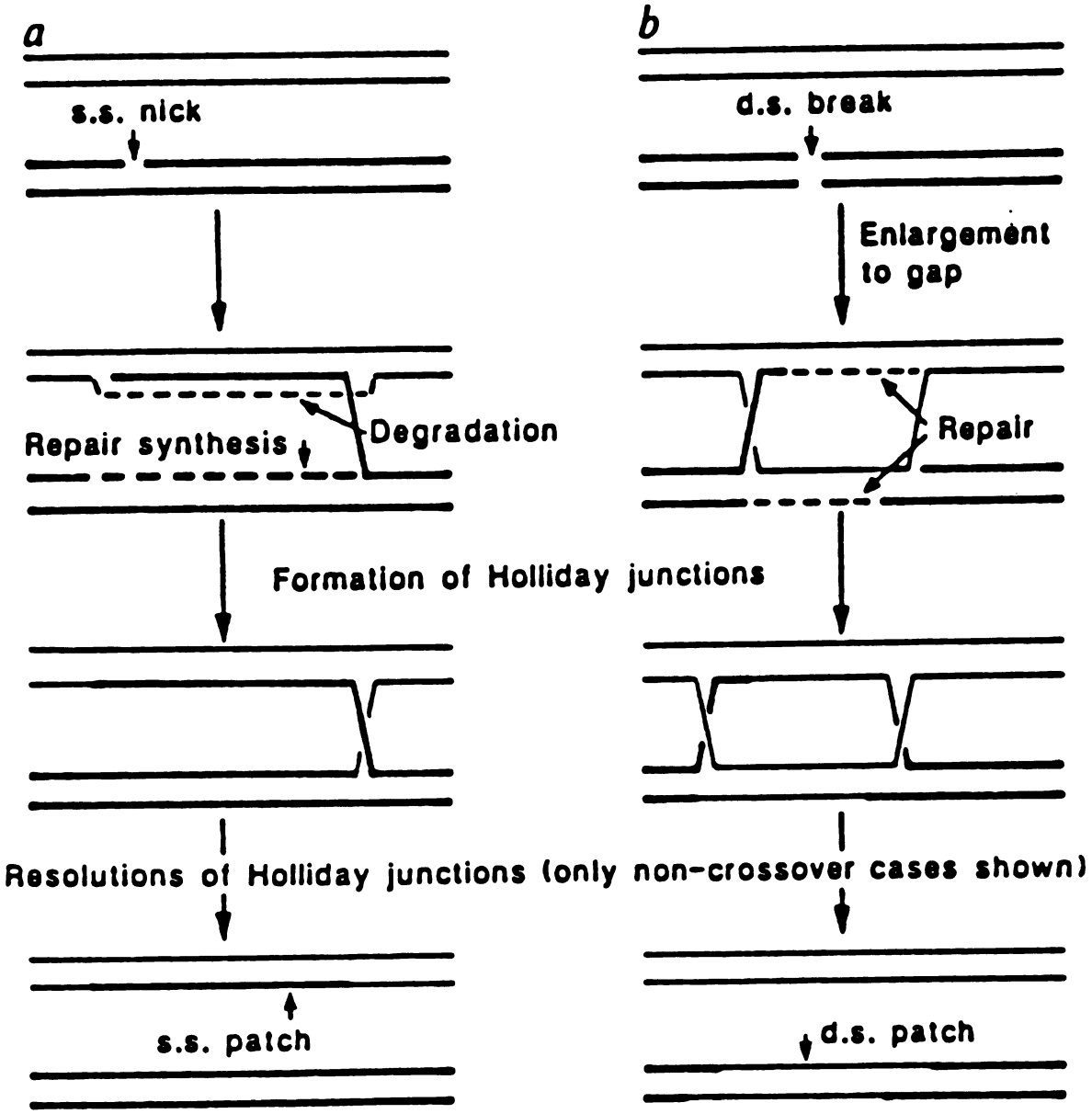
Figure 1. Holliday structure –recombination intermediate.

A structure proposed by Holliday (1964) for the strand exchange process. This structure contains heteroduplex DNA. Its resolution results in recombinant formation (1).

Figure 2. Two models of homologous recombination.

a. The Meselson-Radding model. A single stranded nick is formed followed by strand invasion, gap formation and repair synthesis. The resulting Holliday structure is resolved.

b. The double stranded break model. Recombination is initiated by a double stranded break followed by limited degradation. Repair synthesis occurs using the undamaged homologue as template. The resulting Holliday structure is resolved.


a
s.s. nick
Repair synthesis
Degradation
b
d.s. break
Enlargement to gap
Repair
Formation of Holliday junctions
Resolutions of Holliday junctions (only non-crossover cases shown)
s.s. patch
d.s. patch

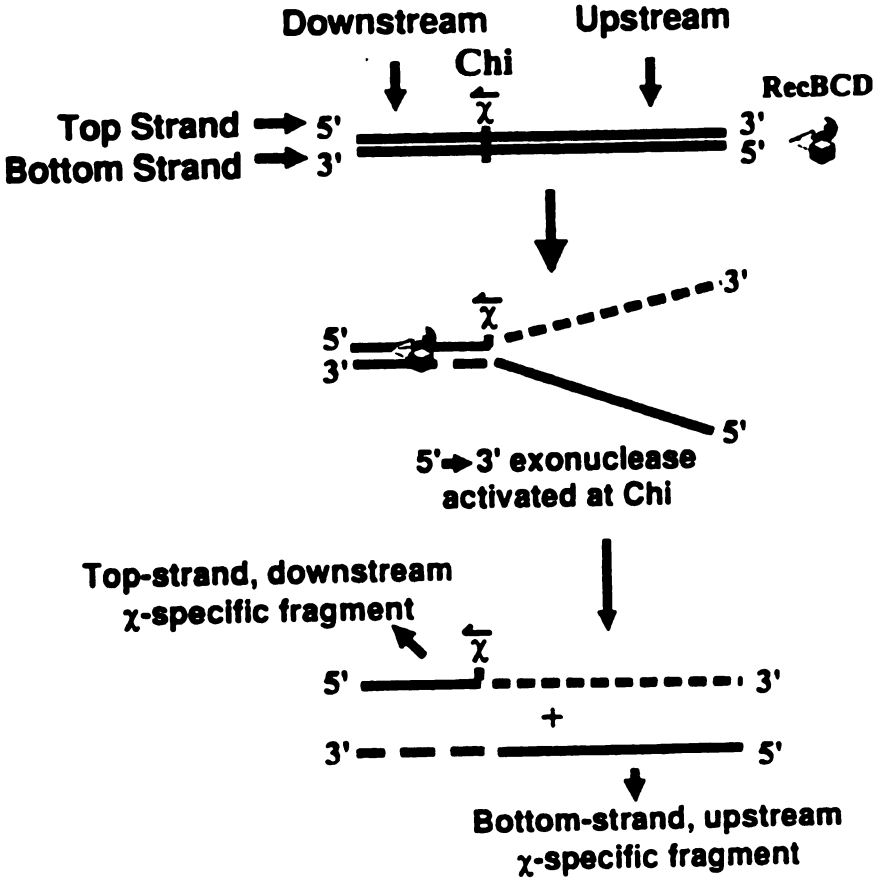
dependent upon the RecA protein and the RecBCD enzyme. Large plaque forming mutants isolated from λ Red^- Gam^- revealed four loci at which mutations had occurred (11, 22). RecBCD mediated recombination along λ was favored near these mutation sites (22-24). These observations led to the designation of these sites as Chi that were defined as enhancers of recombination. The nucleotide sequence of various Chi sties with full activity were shown to be identical. Some deviations from the 5'GCTGGTGG3' consensus sequence (25) led to partially or fully inactive Chi sties (26). In *E. coli* Chi sites occur one in every 5 kilobase pairs (Kbp) of DNA (27-29). It is believed that Chi sites reside in recombination islands of about 400 base pairs (bp) on each side of Chi. It is implicated that Chi evolved in regions with intrinsic hot spot activity and biased nucleotide composition for G and T (30). Chi is a regulatory sequence that down regulates the 3'→5' nuclease activity and activates the 5'→3' nuclease activity of RecBCD enzyme (31, 32). Subsequently, single stranded DNA which contains Chi at its 3' terminus is formed. This is a suitable substrate for the *E. coli* RecA protein and the single-strand binding (SSB) protein (33-35). Homology search and strand invasion is the subsequent step in the exchange process. Genetic exchange stimulated by Chi occurs not at the Chi site but at a distance from the 5' terminus of the sequence. The effect of Chi diminishes by a factor 2 every 2.2 Kbp (36-38). When restriction digest fragments of λ containing Chi were inverted, the hot spot activity was abolished. Re-inversion of the fragment restored the activity of the Chi site (27). This orientation dependence of Chi suggests that it acts in concert with another sequence. This sequence was

identified to be the *cos* site. The *cos* site provides a port of entry for the RecBCD enzyme (39-42) (Figure 3).

High recombination frequencies have been observed in *Schizosaccharomyces pombe*. This observation was made in one allele (M26) out of 394 alleles in *ade6* gene (43, 44). The M26 allele recombines with other ade6 alleles up to 21 times more frequently; moreover, the frequency of gene conversion at M26 is 10 times higher than at other alleles (43, 45, 46). M26 is created by a G→T transversion that introduces a nonsense codon in the open reading frame upstream of the *ade6* gene (45, 47). This transversion at the heptamer 5'-A<u>T</u>GACGT-3' creates a meiosis-specific increase of homologous recombination frequencies (43). The *ade6-M375* allele has a G →T transversion in the codon preceding the M26 allele (45, 47). This nonsense mutation, however, has no hotspot activity (43) and is used as a negative control. It has been proposed that M26 creates a site that directs an endonuclease to nick the DNA of the homologous chromatid. This chromatid is then used as an information donor for the chromatid containing the site. Alternatively, however, the nick could be made in the chromatid containing the M26 site followed by expansion of the nick to a gap by a nuclease. Subsequently, the homologue can act as a donor for information to fill the gap (43, 48). The general features of the M26 allele are similar to other meiotic hot spots of recombination. That is, in addition to imposing a recipient status on the chromosome that contains it (43), the conversion rates of markers located upstream of M26 increases as their distance from M26 decreases (43, 49). The activity of M26 depends on the

Figure 3. Schematic representation of Chi site and RecBCD enzyme.

Upon recognition of the Chi site by the RecBCD enzyme, its activity is attenuated and also the 3' $\rightarrow$ 5' exonuclease activity is switched to 5' $\rightarrow$ 3' exonuclease activity.


Downstream
Upstream
Chi
χ
RecBCD
Top Strand
Bottom Strand
5'
3'
3'
5'
3'
5'
5'
3'
5'→3' exonuclease
activated at Chi
Top-strand, downstream
χ-specific fragment
5'
3'
+
3'
5'
Bottom-strand, upstream
χ-specific fragment

chromosome context. If *ade6-M26* is translocated to another part of the genome, the hotspot activity of M26 is abolished (50, 51). Finally, M26 can enhance recombination between a plasmid and the chromosome, but only if it is located on the chromosome (50, 51). These features imply a role for chromatin structure in the M26 mechanism of action. It has been shown that any base change in the heptamer sequence abolishes hotspot activity (52). The heterodimeric protein Mts1/Mts2, which was identified as a factor binding specifically to the heptamer sequence residing on a DNA molecule (53), shows strict sequence specificity. It is not yet clear why M26 imparts a meiosis specific elevation of homologous recombination. The Mst1/Mst2 heterodimer has been shown to be present and active (in DNA binding) in both meiotic and mitotic cells (53). This ambiguity has led to the hypothesis that assumes a meiosis specific chromatin structure that contributes to M26 hotspot activity. Indeed it has been shown that the heptamer sequence alters chromatin structure by altering nucleosome positioning (54). The single base change resulting in the creating of M26 affects the phasing of nucleosomes in the region surrounding the heptamer. Evidence for influence of promoters on recombination in yeast has come from co-localization of hotspots of recombination with promoter regions that are nuclease hypersensitive (55). It has been postulated that M26 acts by enhancing an already existing site of initiation of recombination in the promoter region of *ade6* gene. Alternatively, M26 may introduce a new site for initiation of recombination (49). When the presumed promoter of *ade6* gene was deleted, hotspot activity of M26 was abolished (56). This lends support to the former hypothesis. The results,

however, are debated since it is possible that the deletion of the promoter may have introduced an alteration in the chromatin structure. Evidence for similar phenomena in other organisms has been obtained (57).

A eukaryotic mitotic, but not meiotic, recombination hot spot was identified in *Saccharomyces cerevisiae* by Roeder and co-workers (58). This hot spot is located in the ribosomal RNA (rRNA) gene cluster and stimulates both interchromosomal and intrachromosomal mitotic recombination events and was named *HOT1*. It acts on both parental sequences and thus must be present in the same side of both sequences involved. Therefore, unlike Chi and other hot spots discussed above, *HOT1* is not dominant. The required sequences for recombination hotspot activity include sequences that regulate transcription by RNA polymerase I. In fact, transcription by RNA polymerase I, initiated in the rDNA region, is responsible for the stimulation of genetic recombination. Insertion of a transcriptional terminator downstream of *HOT1*, and in the same orientation, abolishes stimulation of recombination. Two models have been proposed for the action of *HOT1*. First, *HOT1* may act to stimulate the pairing of homologous DNA molecules. RNA polymerase I action leads to a localized unwinding of the helix and thus exposes single-stranded DNA (ssDNA). The exposed ssDNA molecules may become involved in homologous pairing and thus form the first step for recombination. Alternatively, *HOT1* may influence not the frequency of initiation of recombination, rather the nature of events that occur. The latter is supported by the observation that *HOT1* stimulates cross-over events more than conversion events (59, 60).

Homologous recombination in mitotic mammalian systems is a rare event. It is estimated to be in the range of 10^{-6}-10^{-5} events/cell/generation. This has made the study of the system very difficult. The introduction of exogenous DNA molecules in mammalian cells has been used to study recombination. This system proved helpful since the frequencies of recombination observed were higher than normal. The study presented here, aims to investigate homologous recombination on the genome of polyomavirus (Py). Polyomavirus presents an ideal model for the study of homologous recombination in mitotic eukaryotic cells because of its reliance on cellular proteins for transcription and DNA replication, and the presence of such regulatory regions as an origin of replication, transcriptional enhancer and promoters.

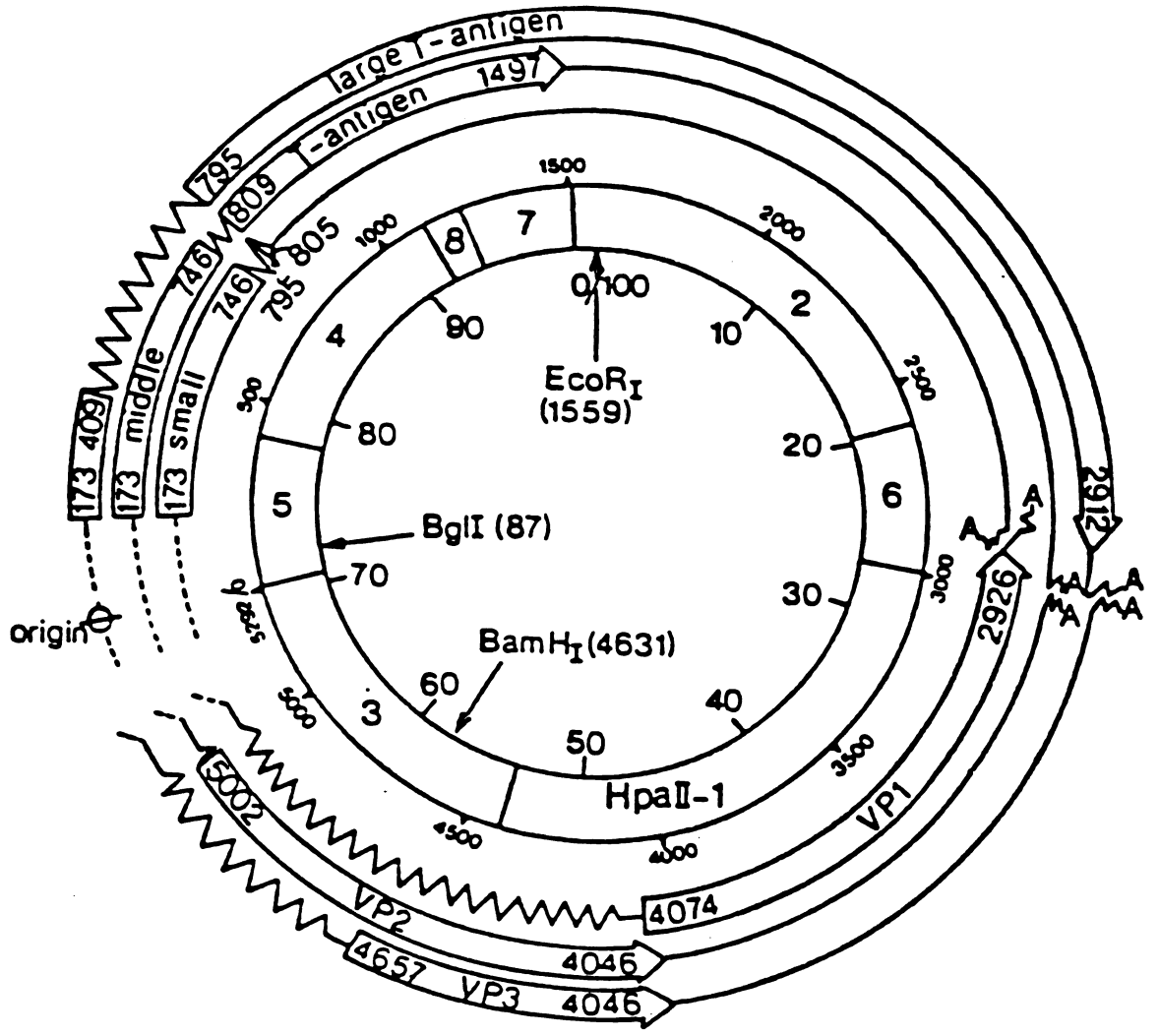
Polyomavirus (Py) is a small DNA tumor virus that belongs to the polyomavirus family (61). Its genome of about 5300 bps is packaged into chromatin and contains 21 nucleosomes. Although histone H1 is not present in the virion, it is believed that the viral DNA molecules in the host cell do contain H1. It has been demonstrated that the histones of polyomavirus are hyperacetylated and this phenomenon has been associated with the activity of the viral early protein, middle tumor antigen (mT-Ag) (62). Polyomavirus contains a non-coding, origin-enhancer region as well as two coding regions, giving rise to early functional proteins and late structural proteins. The early region is transcribed into a single precursor RNA which upon alternative splicing gives rise to messenger RNAs (mRNA) coding for the viral proteins small, middle and large tumor antigens (63). The late precursor RNA is differentially processed

to produce three mRNA molecules coding for the structural capsid proteins: VP1, VP2 and VP3 (64). The non-coding region of the viral genome controls both viral replication and transcription (Figure 4).

The outcome of Py infection of tissue culture cells depends upon the permissivity of the cells. In tissue culture systems derived from the natural hosts of the polyomaviruses, a productive infection is produced with eventual death of cells. In non-permissive (or semi-permissive) host cells little or no viral DNA replication is observed, no virus is produced and the cells survive the infection process. This appears to be dependent upon host factors that may include DNA replication proteins which interact with the viral large tumor antigen (LT-Ag) (65-67). In these cells LT-Ag and the DNA polymerase α/primase complex do not interact efficiently (67, 68). Upon infection of non-permissive cells a small fraction (0.05%-2%) of the cells become transformed (69). In these tissue culture systems, stable transformation requires integration of the viral genome into the host DNA to maintain the genome in the absence of viral DNA replication (70-76). The integration of polyomavirus genome into the host sequences appears to be through a non-homologous recombination mechanism. It is believed to be random with respect to both host and viral sequences (70, 72, 74, 77). Although patchy homology had been proposed as a model for integration of polyomavirus (78-83), its role has been questioned and discounted (84). Large deletions of host sequences accompany viral integration (79, 80, 85-88). The integrated genome is in head-to-tail tandem copies. Recent studies have suggested that integration occurs at a single site and post-integration amplification leads to

Figure 4. Physical map of the polyomavirus genome.

This schematic representation depicts the early and late coding regions of the polyomavirus genome. Each region produces a single transcript that is differentially spliced to produce three gene products. The viral T antigens, small T, middle T and large T, are encoded by the early region. The late region encodes the capsid proteins VP1, VP2 and VP3 (64).


large T-antigen
1497
T-antigen
795
809
746
805
795
746
173 middle
173 small
173 409
origin
1500
2000
2500
3000
3500
4000
4500
5000
5002
0/100
10
20
30
40
50
60
70
80
90
1000
500
EcoR$_I$
(1559)
BglI (87)
BamH$_I$(4631)
HpaII-1
1
2
3
4
5
6
7
8
2162
2926
4074
4046
4657
VP3
4046
VP2
VP1

generation of the multiple band pattern previously taken as evidence for multiple integration events (89). The majority of cells that do develop a transformed phenotype do so transiently and revert to a normal phenotype. It has been suggested that this abortive transformation phenotype (90) is due to the expression of mT-Ag from the un-integrated genomes.

Large T antigen is a nuclear phosphoprotein of 758 amino acids. It is a multi-functional protein involved in viral DNA replication, as well as integration, transformation and transcriptional regulation. LT-Ag has an autoregulatory function in regulation of early gene transcription. Its binding to sequences near the early promoter prevents binding of RNA polymerase and thus down regulates early gene expression (91-93). It also up regulates late gene expression in a concentration dependent manner (94, 95). In normal cells the product of the retinoblastoma gene, pRB, associates with E2F, a cellular transcription factor. Binding of pRB to E2F is partially responsible for regulation of E2F activity (96). Binding of LT-Ag to pRB dissociates E2F and leads to activation of E2F-responsive cellular genes (97-100). This activity of LT-Ag is thought to be the basis for its ability to alter the pattern of gene expression in the host at the G1/S border and thus drive cells into S phase (101-104). The role of LT-Ag in viral DNA replication is as an initiator. It has sequence-specific DNA binding as well as helicase and ATPase activities (105, 106). Large T antigen binds the viral origin of DNA replication as a double hexamer (107). This leads to localized melting of the double strand (108). Following this event, LT-Ag recruits cellular replication machinery to the site and acts as an ATP-dependent DNA helicase to

unwind the DNA in advance of the replication machinery (109-113). This generates bidirectional DNA replication. In the absence of the replication function of LT-Ag, the transformation frequency is reduced several fold. This suggests that LT-Ag may play a role in the initiation of the transformed phenotype (114) by promoting the integration of the viral DNA into the host genome. Temperature sensitive mutants of LT-Ag were used in temperature shift experiments. It was concluded that the presence of functional LT-Ag after infection is important in transformation efficiency (115). It has also been shown that LT-Ag temperature sensitive mutants and wild-type polyomavirus have the same frequency of abortive transformation at non-permissive temperatures. It was concluded that LT-Ag functioned in stabilization of the transformation phenotype (116) which is now equated with integration of the viral genome. The topology of the integrated viral genome is also affected by LT-Ag. That is, at non-permissive temperatures less than one genome is integrated into the host cell DNA (117).

Maintenance of the transformed phenotype is a function of the viral middle T antigen (mT-Ag) (117) which is the viral onco-protein (75, 118-120). Middle T antigen is a 57 kilodalton, membrane associated phosphoprotein (120). In its C-terminus there are 22 uncharged, hydrophobic amino acid residues which are bounded on either side by basic residues, similar to other membrane-associated proteins (120). Middle T antigen is believed to initiate a signal transduction pathway similar to that induced by platelet derived growth factor (PDGF). Upon complex formation, middle T antigen induces the tyrosine kinase activity of $pp60^{c-}$

src (121, 122). Tyrosine phosphorylation of mT-Ag provides binding sites for proteins with *src* homology domains (SH2), such as Shc (123). The mT-associated pp60$^{c\text{-}src}$ phosphorylates Shc which then interacts with Grb2 (124). Subsequently, Shc/Grb2 activates p21$^{c\text{-}ras}$ (125, 126) that functions to convert inactive Ras-GDP to Ras-GTP (127-129). The signal is transferred to c-Raf-1 serine/threonine kinase (130). Protein kinase C, which is activated by mT-Ag via association of mT-pp60$^{c\text{-}src}$ with phospholipase C gamma (PLCγ) (131), can also activate c-Raf-1 (130). Activated phospholipase Cγ will lead to the hydrolysis of phosphatidyl inositol-(4,5)bisphosphate (PIP_2) to yield diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (130). DAG activates protein kinase C (132) which eventually leads to the activation of such transcriptional activators as AP1/PEA1 and *c-ets*/PEA3 (133-135). IP_3 is a modulator of cytoplasmic calcium concentration (136). These two pathways initiated by association of mT-Ag with pp60$^{c\text{-}src}$ merge at c-Raf-1. Activated c-Raf-1 will lead to activation of mitogen activated protein (MAP) kinase kinase (137, 138) which in turn will activate MAP kinase (139). The signal is then transduced to the nucleus by activation of transcription factors such as *c-jun* (140, 141). The final result is the activation of genes under the control of such transcription factors. The mT-pp60$^{c\text{-}src}$ also associates with PI_3-kinase (142-147). The down stream targets of this pathway are not well characterized. However, it has been shown that mT-Ag mutants deficient in PI3-kinase activation have a decreased transformation capability (147, 148). It has been observed that the mT mutant viral chromatins are less acetylated than the wild type chromatin (62). This may be linked with the role of

mT-Ag in activation of the transcription factors that bind the viral enhancer. These transcription factors may then recruit histone acetylases or de-acetylases to the viral chromatin. It is important to keep this correlation in mind as chromatin structure is an important factor in recombination processes.

Small T antigen is 196 amino acids long. Its role in transformation is not clear. Small T antigen can increase transformation in cultured cells in cooperation with mT-Ag (149). Its interaction with pp2A may modulate pp2A activity. It has also been demonstrated that small T antigen can stimulate viral DNA replication.

Both Polyomavirus and SV40 have been used extensively to study mitotic recombination in eukaryotic systems. Most such studies took advantage of transfection of an exogenous molecule of DNA into an appropriate cell line. These studies contributed considerably to our understanding of homologous recombination in mammalian systems. Few studies, however, have used infection as a mode of introduction of viral DNA into the host cells. Such studies are more relevant to the research presented in this thesis.

Kit and colleagues (150) first reported inter-viral recombination between plaque morphology mutants of SV40. Large clear plaque-forming virus was isolated during mixed infections of African green monkey kidney cells (CV-1) with small clear plaque type and fuzzy plaque type mutants. The recombination frequency was reported to be 2×10^{-4}. Recombination between polyomavirus genomes was first reported by Ishikawa and Di Mayorca (151). Two temperature sensitive mutants of Py were used to infect mouse 3T3 cells. These crosses

yielded wild type Py at a calculated frequency of about 0.24%. Oligomeric forms of SV40 DNA used in infections of CV-1 cells also resulted in isolation of large clear plaque forming type SV40 (152).

The observed frequencies of recombination have been higher when transfections were used as a method of introducing the recombination substrates into the host cells. When two restriction endonuclease-generated fragments of SV40, that shared homologous overlapping termini, were transfected into CV-1 cells, viable genome size SV40 was produced by recombination at a frequency of 0.1% (153). Wilson and colleagues (154) refined this system by designing defined oligomeric molecules of SV40. Head-to-tail partial dimers of two temperature sensitive mutants of SV40 were created. Since these molecules were not packageable due to their size, formation of packageable monomers was assayed. The results from this line of work indicated that conversion from partial dimers to packageable monomers occurred uniformly throughout the SV40 genome and that the mechanism involved was general homologous recombination. A later study focused on recombination between endogenous and introduced SV40 sequences (155). Linear enhancer-less SV40 DNA was transfected into monkey cells that have either one or multiple copies of SV40 early region stably integrated, COS1 and COS7 cells respectively. The results suggested that a double stranded break introduced into the transfected molecule in the region of homology resulted in the production of wild type virus in up to 25% of the successfully transfected cells. The double strand break-repair model was presented as an explanation; however, double crossover resulting in a

reciprocal exchange could not be excluded. In another study, a conditionally replication defective mutant of SV40 was used to rescue large T antigen function from COS cells (156). This mutant lacks the early coding region and thus can only replicate in COS cells which provide the large T function for replication in *trans*. In CV-1 cells, however, this mutant cannot replicate. Recombination was observed at a frequency of $1x10^{-6}$ as assayed by rescue of large T function and therefore replication in CV-1 cells. Others have reported recombination between integrated sequences of SV40 in monkey cells and temperature sensitive mutants of SV40 (157-159). The transformed cells, however, contained SV40 sequences with an intact origin of DNA replication making it difficult to exclude replication and excision of the resident viral sequences prior to recombination. Other studies focused on the differences between recombination of circular and linear molecules (160, 161).

One line of study of recombination using Py has focused on the possible role of the viral replication protein, large T antigen (LT-Ag), in recombination. In a detailed analysis, cell lines carrying two mutated copies of mT-Ag were established. These mutations were positioned such that functional mT-Ag could only be expressed as a result of recombination between the two sequences. The normal spontaneous rate of recombination was determined to be $1x10^{-7}$. When exogenous LT-Ag was introduced, the recombination rate increased to between $1x10^{-1}$ to $1x10^{-2}$ (162). This effect required the replication function of LT-Ag and a viral origin of replication. The model proposed involved a mechanism whereby LT-Ag functions to unwind the DNA and thus make it more recombinogenic. This

is followed by single-stranded nicks, slipped-strand mispairing between the two repeats and subsequent repair synthesis. Effects of LT-Ag mutants defective in initiation of viral DNA synthesis on homologous recombination were analyzed in an attempt to further address the role of the replication function of LT-Ag in this process (163, 164). The results indicated that LT-Ag can promote recombination independent of its replication function. The results of a later study showed that the origin specific binding activity of LT-Ag is not sufficient to promote homologous recombination. It was hypothesized that localized unwinding of the double stranded DNA is required for the process of homologous recombination promoted by LT-Ag. The elements of the Py origin of replication required for LT-Ag induced homologous recombination were determined to be identical to those required for DNA replication. Other replicative functions of LT-Ag, however, are dispensable for recombination. This is in agreement with the model that the role of LT-Ag in recombination may be localized destabilization of the double stranded DNA at the viral origin of replication to create a suitable substrate for recombination (165).

Another line of research has focused on the role of DNA sequences in the recombination of polyomavirus. This study made use of a chimeric molecule of DNA, derived from integrated Py sequences, containing Py and mouse sequences with directly repeated viral sequences. This molecule arises in permissive murine cells carrying a temperature sensitive Py virus genome that is stably integrated at elevated temperature but is excised and replicated after transfer to permissive temperatures and is called RmI (166). It is hypothesized

that a site-specific recombinase may be involved in excision of the RmI molecule. Patchy homology and palindromic sequences have been found at the junctions of this molecule with mouse DNA. Thus in the cell line repeatedly giving rise to RmI the viral genome may be integrated in a region with clustering of repetitive DNA elements (167). Such clustering has been associated with local genome instability. RmI yields genome size Py molecules by homologous intramolecular recombination. When the direct repeats (S repeats) of RmI were mutated such that the homology is limited to 40-50 bps, non-homologous recombination products were observed, unlike wild type RmI (168). It was hypothesized therefore that the initial steps in the mechanisms yielding homologous and non-homologous products could be identical. Additionally, the S repeats may contain sites that are active in recombination and are preferred crossover sites (169). Later studies revealed that deletion of the early promoter or inversion of the late promoter impaired viral DNA recombination (170, 171). This was observed under conditions which allowed for unimpaired viral DNA replication. The dependence of recombination on promoter activity and especially promoter orientation suggested that recombination may require progression of transcription through the site of crossover rather than a chromatin configuration due to transcriptional activity. More recent studies have better defined the elements in the S repeat that are responsible for enhancement of homologous recombination (172). There are three binding sites for the transcription factor Yin Yang-1 (YY1) on the polyomavirus genome (172-174). Two of these sites are on the late side of the origin and the third is within the VP1 coding region. YY1 is a zinc finger

protein (175) that has differential gene context-dependent transcriptional regulatory effects. It can initiate, enhance or repress transcription (176). The effect of YY1 on transcription is believed to be primarily through its effect on promoter structure. Mutations of the third binding site for YY1 that eliminate binding were shown to impair intramolecular homologous recombination in Rml (172). The model presented assumes that recombination occurs in a transcriptionally active viral genome, attached to the nuclear matrix by YY1.

The possibility that neoplastic transformation may in itself result in higher frequencies of recombination is of particular interest. It has been suggested that elevated levels of *c-fos* increases the frequencies of homologous recombination (177). However, no mechanistic and systematic studies are reported. This is with the exception of a system that has questioned the role of p53 in recombination. A study of the effect of p53 on homologous recombination used two mutants of SV40, which could only replicate after intermolecular homologous recombination (178). The results indicated a 10-fold increase in the rates of homologous recombination among the chromatin-associated molecules of SV40. It is hypothesized that the association of the SV40 LT-Ag with p53 relieves the inhibitory effect of p53 on homologous recombination. This was confirmed by using SV40 virions with a LT-Ag unable to bind p53 which did not increase the rate of homologous interviral recombination. In a recent study human tumor cell lines with a null mutation of p53 were used in an assay for recombination of integrated plasmid sequences (179). These cells exhibited a 10^4 fold increase in rates of homologous recombination when compared to primary cell lines. This

increase in recombination was greatest for homologous recombination. The rate of non-homologous recombination increased only 2-10 fold (as measured by the random integration of test plasmid). These studies have led to the postulation of a role in genome maintenance for p53. That is, p53 functions in this regard by inhibiting homologous recombination.

It is apparent from the body of work presented here that recombination is a complex process which in some cases may be tied with other cellular processes such as replication or transcription. The research presented in this thesis has aimed to map recombination frequencies on the Polyomavirus genome as an initial step in understanding the high frequencies of recombination observed on the Polyomavirus genome.

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Chapter 2

Extreme frequencies of homologous genetic exchange between polyomavirus chromatin in mitotically dividing cells

Abstract

In an attempt to map the frequencies of homologous recombination on the polyomavirus genome, two variants containing novel, unique restriction endonuclease sites were constructed in a temperature sensitive large T antigen background. The viral genome was divided into eight intervals that could be tested for homologous genetic exchange. Transformed cell lines from three experiments were isolated and analyzed for recombination. The results show that on the average there are 3.4 crossovers per integrated genome, with a low of 2 and a high of 7 crossovers. We have confirmed that a high percentage of transformants (19%) contain recombinant viral sequences. Although maintenance of cells at the non-permissive temperatures dramatically reduced the transformation frequency and viral DNA replication, the frequency of recombination was not affected. Analysis of interviral recombination in free viral genomes detected no recombinants. We conclude that the polyomavirus chromatin undergoes numerous crossovers in all intervals. However, two

intervals, one in the early region and one in the late region, seem to have higher numbers of crossovers.

Introduction

Because of its small size, defined chromatin structure, and almost total reliance on the host cell machinery for gene expression and DNA replication, polyomavirus (Py) has been used to study many cellular processes. Previous work from this laboratory has investigated homologous recombination on the polyomavirus genome during the process of integration of the viral genome and neoplastic transformation of non-permissive rat cells. Integration is required for the stable transformation of tissue culture cells and frequently the integrated genome is in a head-to-tail tandem arrangement. Integration is a rare event and appears to be the rate-limiting step for the process of transformation. Integration seems to be random with respect to both viral and host sequences and occurs through non-homologous recombination with the host sequences because the junction sequences are not homologous. The current understanding implicates LT-Ag in the initiation of the transformation phenotype. It is believed that LT-Ag's role as a replication protein plays a role in initiation of integration (1).

Hacker and Fluck (2) have shown that a large fraction of transformants (38%), isolated from co-infections of Fischer rat cells (FR3T3) by two polyomavirus mutants contained recombinant viral genomes. In contrast to the high level of recombination in integrated genomes, no recombination was

detected among the un-integrated viral genomes isolated from the same cells that gave rise to recombinant transformants. In these and further studies by Chen and Fluck (3) recombination frequencies along the polyomavirus genome were analyzed. The fragment of the genome studied spanned from *Bam*HI site in the late region (nucleotide 4634) to nucleotide 1387 in the early region. These experiments revealed a gradient of recombination in the region analyzed. The recombination frequency per base pair increased forty-fold in an interval between the enhancer-origin region and nucleotide 1387 in the early region. These studies, however, were carried out using polyomavirus mutants that were not isogenic and contained mutations that might have affected or selected for recombination. Additionally, only about 40% of the genome was analyzed for recombination.

The current study has sought to systematically analyze frequencies of recombination on the polyomavirus genome by introducing unique, novel restriction endonuclease sites. The genome has been divided into eight intervals and recombination in all intervals was studied in a single cross. These variants of polyomavirus were synthesized in the LT-Ag temperature sensitive (ts-a) background. This has allowed for the test of a role in recombination for the replication function of LT-Ag.

The results show that there are on the average 3.4 crossovers per integrated genome, from a low of 2 to a high of 7. This demonstrates that the frequency of recombination is high in all intervals. Crossovers occur more frequently in an interval in the early region and another in the late region. In

fourteen of the seventy-five (19%) transformed cell lines analyzed the viral sequences contained recombinant intervals. This confirms previous results that a large percentage of the transformed cell lines are recombinant. No recombinants were detected among free viral genomes derived from the same experiments. Because no differences in frequency of recombination were observed between the permissive and non-permissive temperatures, it is concluded that recombination was not contingent upon the replication function of LT-Ag.

Material and Methods

Cell lines and cell culture

The non-permissive rat fibroblast cell line, FR3T3 (4), and the permissive mouse fibroblast cell line, NIH3T3 (5), were used. These cell lines were maintained in 5% CO_2 at 37C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum and penicillin-streptomycin. Cells were passed every three days.

Viruses

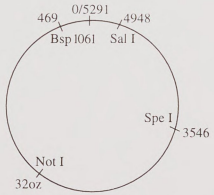
Two new variants of polyomavirus wild-type A2 (WTA2) (6) were created using in vitro site-directed mutagenesis. Each was designed to have four novel restriction endonuclease sites as well as the ts-a (temperature sensitive mutant of LT-Ag) point mutation (Figure 1). For this purpose, the WTA2 genome was scanned for sequences in which one point mutation would lead to creation of novel restriction endonuclease sites. Only silent mutations were chosen. Oligonucleotide primers encompassing the point mutations for the novel restriction endonuclease sites and the ts-a mutation were incorporated with

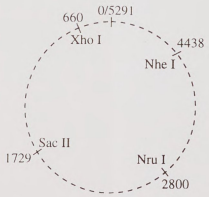
Figure 1. Maps of the newly designed Py variants.

Panel A depicts the circular maps of each variant. The position of each unique restriction endonuclease is indicated.

Panel B shows the oligonucleotides used for introduction of the unique restriction endonuclease sites in each parent. The silent point mutations leading to the introduction of new sites are highlighted. The ts-a mutation was introduced in both parent 1 and parent 2.

A





B.

Parent 1 Parent 2

	Primer	Base Change	Codon Change	Nucleotide no.	New RE
PARENT 1	5'CCTTTGGTG**C**TCATACTACCAGC3'	A→C	AGA→CGA	458→481	Bsp1O6I
	5'GACAGGCCTAGC**G**GCCGCGC3'	A→G	GCA→GCG	2291→2315	NotI
	5'CATCTGTCAC**T**AGTCCCTGG3'	A→T	CTA→CTT	3536→3555	SpeI
	5'CCGTAAGGGT**C**GACACTTCAGC3'	A→C	TCT→TCC	4938→4959	SalI

	Primer	Base Change	Codon Change	Nucleotide no.	New RE
PARENT 2	5'GGAACACCAAC**T**CGAGATGTGC3'	C→T	ACC→ACT	649→670	XhoI
	5'GTAACCGC**G**GCACCATTTCAG3'	A→G	GCA→GCG	1721→1741	SacII
	5'CATCTGTCAC**T**AGTCCCTGG3'	T→C	CGT→CGC	2791→2811	NruI
	5'CCTAGCTAG**C**CCATCCAGGAATG3'	G→C	GGC→GGG	4429→4451	NheI

Parent 1 and Parent 2		2182 → 2205
5'CCAGTGTCTTTTAA**A**GGACTTTCC3'	G→A	ts-a mutation

WTA2 cloned into pBR322 at the *EcoR*I site as template. The Transformer Site-Directed Mutagenesis kit (Clontech) was used. The presence of new restriction sites was confirmed by restriction digestion analysis. Subsequently, the recombinant plasmid was digested with *EcoR*I and electrophoresed to separate the Py genome from pBR322. The fragment corresponding to Py was cut and purified using Promega Wizard Prep Kits. The purified DNA was ligated and transfected into NIH3T3 cells using a DEAE-Dextran transfection method. The transfection lysates were used to grow large stocks of each variant. The stocks were quantitated using plaque assays. Incorporation of the ts-a mutation was confirmed by sequence analysis. Four clones of the recombinant plasmids, derived from independent synthesis reactions were sequenced over a 500bp region encompassing the ts-a mutation. No sequence variations were observed over the total 2000 bps sequenced.

Infection and Isolation of transformed cells

FR3T3 cells were arrested in G_0 by growth to confluency and serum deprivation for 48 hours. Cells were released from G_0 and were plated at a density of $4x10^5$ per 60-mm culture dish in media supplemented with 10% calf serum (7). Cells were infected with a mixture of both parents at a ratio of 1:1. The multiplicity of infection for each parent was 25 PFU per cell (total moi of 50 PFU per cell). Infections were carried out at 37°C for 45 min. Subsequently, cells were fed with Dulbecco's modified Eagle medium supplemented with 5%

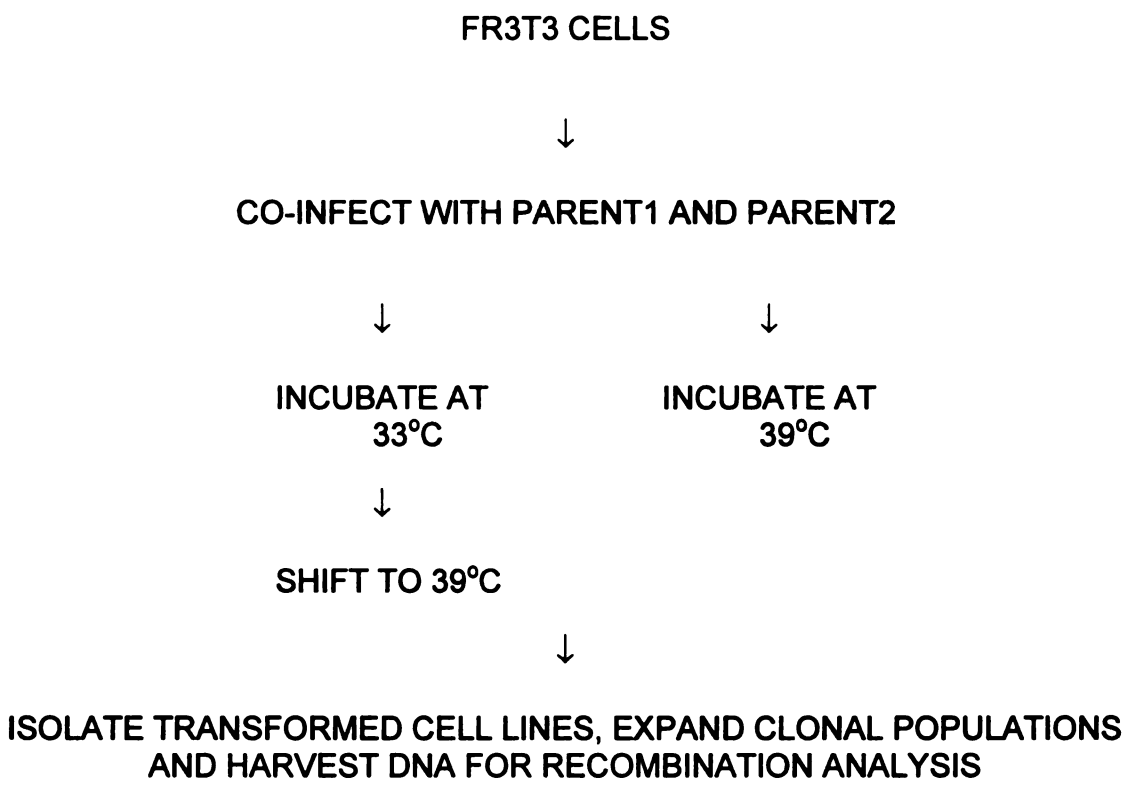
calf serum and penicillin-streptomycin. After the adsorption period, infected cells were incubated at 39°C or 33°C. As indicated in the results section, to verify equal input of each parent, total DNA was harvested 4 hours post infection. The 33°C populations were shifted to 39°C upon detection of microscopic transformed foci to prevent post integration amplification of the viral genome. Transformed cells which overgrew the monolayer were isolated and grown in 5% calf serum supplemented Dulbecco's modified Eagle medium. Each cell line was passed as necessary when confluency was reached (Figure 2).

Preparation and analysis of DNAs

For recombination analysis, approximately $4x10^7$ cells were lysed with 0.2% sodium dodecyl sulfate, 10 mM Tris hydrochloride (pH 7.5), and 10 mM EDTA (2 ml per 100-mm culture dish). Total cellular DNA was extracted. For recombination and mapping analysis, 10 μg of total DNA was digested with diagnostic restriction endonucleases, used singly or in combination, that would test all eight intervals. Digested DNAs were electrophoresed on agarose gels (1%, w/v in Tris-borate/EDTA buffer). The DNA was denatured in gel in 0.5M NaOH, 1.5M NaCl and transferred to Hybond-nylon membranes. Hybridization was carried out in 5xSSPE (0.75 M NaCl, 50 mM $NaH_2PO_4.H_2O$, 5 mM EDTA), 5x Denhardt's solution, 0.5% (w/v) SDS at 65C for 48 hours, using labeled Py genomic probes. The probes were labeled using a random multiprime DNA labeling kit (Amersham Corp.) with [α-^{32}P]dCTP (3000 Ci/mmol). The specific

Figure 2. Experimental design for isolation of transformed cells.

Two strategies were followed post infection. One set of infected cells was maintained at 33°C, to allow for normal integration of the viral genome, and shifted to 39°C when foci were observed. This prevented further amplification of the integrated viral sequences as well as helped resolve previously amplified sequences. Alternatively, cells were maintained at 39°C immediately post infection in order to abolish large T replication function.


FR3T3 CELLS
CO-INFECT WITH PARENT1 AND PARENT2
INCUBATE AT 33°C
INCUBATE AT 39°C
SHIFT TO 39°C
ISOLATE TRANSFORMED CELL LINES, EXPAND CLONAL POPULATIONS AND HARVEST DNA FOR RECOMBINATION ANALYSIS

activity of probes was in the range of $3x10^9$ to $6x10^9$ cpm/μg. Following hybridization, the membranes were washed under stringent conditions and autoradiographed and when appropriate quantitated using a beta-scanner (Ambis).

Results

Comparison of the new variants and wild-type A2 properties

The point mutations used to introduce the new restriction sites were designed to be silent mutations and were expected to have no phenotype. To test this hypothesis, the newly constructed mutants were compared to wild type A2 in a number of tests, at both 33°C and 39°C. In these tests, emphasis was placed on the transformation ability of each virus. The ability to overgrow the monolayer as well as growth in suspension media was tested. Virus production and DNA replication assays were also performed. In all of the above parameters, the mutants performed comparable to wild type A2 (Table 1).

Strategy for integration analysis

This analysis is composed of three parts. First digestion with *Bgl*II restriction endonuclease is performed. This enzyme does not recognize Py sequences and is used to enumerate integration sites as well as the extent of

Table 1. Comparison of the new variants and wild type A2 properties.

The new variants were tested along with the wild type A2 to insure that they did not exhibit different biological properties. Emphasis was placed on the transformation ability of each variant compared to the wild type. Experiments were performed in duplicate at the permissive temperature.

Panel A. The ability of each virus to transform cells was assayed as measured by the number of foci which overgrew the monolayer.

Panel B. The ability of each virus to transform cells was assayed as measured by the number of cells able to grow in suspension media.

Panel C. The potential to produce live virus particles was tested for each virus in a plaque assay.

Focus Transformation Assay:

Polyoma Strain	Number of Foci (duplicate plates)
Parent 1	18/15
Parent 2	12/16
WT A2	20/19

Agar Transformation Assay:

Polyoma Strain	Number of Colonies (duplicate plates)
Parent 1	30/35
Parent 2	37/40
WT A2	45/47

Virus Replication Assay:

Polyoma Strain	Number of Plaques (duplicate Plates)
Parent 1	4/8
Parent 2	3/8
WT A2	3/2

viral DNA amplification. A ladder pattern is often observed in the analysis of *Bgl*II digests. This regular banding pattern has been shown by Syu and Fluck (8) to be the result of *in situ* amplification of the integrated genome at a single site followed by resolution through recombination to yield tandem repeats. The intensity of the ladder pattern indicates the degree of amplification and it is believed to be dependent on the chromosomal context and reiteration of viral sequences. The size of the smallest base band is used to determine the upper limit of the size of the integrated Py sequences. The second step in this strategy is single digestion with all eight of the new restriction endonucleases. This set of digestions allows us to determine which parent has contributed the fragment encompassing the restriction site. If the site is present only once, then the integrated genome will be cut into two fragments representing the host-virus joints. If there is reiteration of the viral sequences around those sites, then a linear 5.3 Kbp fragment will be generated as well as host-virus joints. This fragment, however, may also be generated when free circular viral DNA is present. Finally the presence of recombinant intervals must be tested. This part of the analysis involves double digestions with pair-wise combinations of the new restriction endonucleases defining the recombinant intervals. If the integrated viral genome is not a recombinant, and it has tandem repeats, it is expected that a double digestion will yield a linear fragment of 5.3 Kbp and two fragments representing the host-virus joints. If the integrated viral genome does not have a reiteration encompassing the recognition sites of restriction endonuclease used in the double digestion, then the expected pattern would be that of two host-virus

joints. If, however, a recombinant genome is integrated, a double digestion is expected to yield two diagnostic fragments defined by the restriction endonucleases used. If the integrated genome has tandem repeats, then a 5.3 Kbp fragment would also be generated.

Diagnostic tests for recombination

Transformants derived from the co-infection of FR3T3 cells with Py variants were isolated and analyzed. Two strategies were followed. Cells were maintained at 33°C until foci were detected, to allow normal integration of the viral genome, and then shifted to 39°C to prevent further amplification and help resolve previous amplification of the integrated viral genome (8). Alternatively, cells were incubated at non-permissive conditions immediately following infection. As stated later, inactivation of LT-Ag at the non-permissive temperatures led to a profound decrease in the amount of viral DNA at 72-hours post infection. Three separate experiments were performed (Table 2). In each experiment a 4-hour post infection DNA sample was collected to ensure equal input of both parents. This analysis involved double digestion with one of the newly introduced restriction endonuclease enzymes and a wild-type single cutter. Double digestion with *Nhe*I and *Xba*I was performed. *Xba*I linearizes both parents whereas *Nhe*I only cuts parent 2. In parallel, *Xba*I and *Bsp*106I double digestion was also performed. In this case parent 1 is cut with *Bsp*106I and *Xba*I, whereas parent 2 is only cut with *Xba*I. When these samples are

Table 2. Summary of results from three independent crosses.

Three independent experiments were performed. In each experiment transformed cell lines were isolated. The results are summarized in this table. The number of transformants containing recombinant viral sequences is given relative to the total number of transformants isolated in each experiment.

EXPERIMENT	33°C	39°C	TOTAL
1	0/23	0/10	0/33
2	3/13	4/13	7/26
3	ND	7/16	7/16

electrophoresed side by side, direct quantitation of linear genome-sized parents can be performed (Figure 3).

The diagnostic test for recombination in the transformed cell line used double digests with restriction enzymes that define the eight intervals. This tested for acquisition of a restriction site by either parent 1 or parent 2. Because the restriction sites defining the *Sal*I-*Bsp*106I or *Xho*I-*Sac*II reside on the same parent, a combination of double digestions was used to define the events in the above intervals. In order to test for recombination between *Xho*I and *Sac*II, the following double digestions were performed: *Bsp*106I + *Sac*II, *Xho*I + *Not*I, *Sac*II + *Not*I. To test for recombination in the *Sal*I-*Bsp*106I interval, *Nhe*I + *Bsp*106I, *Sal*I + *Xho*I and *Nhe*I + *Sal*I double digestions were used. An example of a double digestion analysis for 26 cell lines is shown in figure 4. This figure shows the results for the test for recombination in the *Bsp*106I-*Sac*II interval. The recombinant fragments are 4031 bp and 1260 bp and are marked by arrows. In all cell lines a 5.3 Kbp band is also present suggesting the presence of additional parental genomes. The variable intensity between different cell lines does not reflect unequal loading (as confirmed by ethidium bromide staining). Table 3 presents the results of the double digestion analyses, which are further summarized in table 4. It should be noted that this represents an under-estimation of the recombination events, as only acquisitions of new sites were tested. It is likely that the reciprocal events that lead to the loss of both restriction sites are also occurring.

Figure 3. Analysis of the 4-hour post infection DNA samples

To insure equal ratio of each parent for the recombination crosses, 4-hour post infection total DNA samples were harvested and analyzed in duplicate. Samples were in duplicates for the three temperatures. Lane 1 represents undigested DNA, lane 2 is the *Xba*I + *Nhe*I (a combination which linearizes parent 1 to 5.3 Kb) digest, and lane 3 is the *Xba*I + *Bsp*106I digest (a combination which linearizes parent 2). The 5.3 Kb bands are compared.

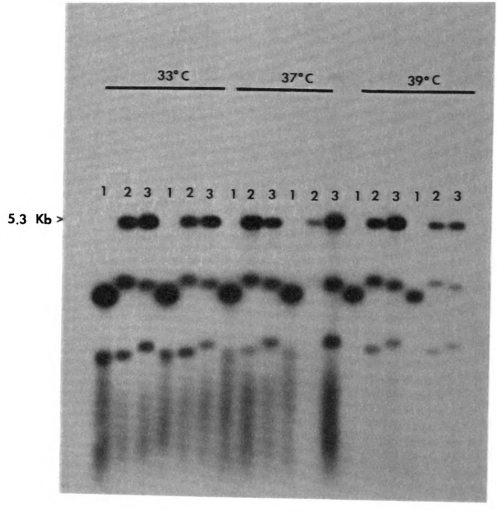

33°C
37°C
39°C
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
5.3 Kb >

Figure 4. Recombination analysis for the *Bsp*106I-*Sac*II interval.

The analyses for 26 cell lines are presented here. The cell lines were obtained from the second experiment. For each cell line 10 μg of DNA was digested with *Bsp*106I + *Sac*II under conditions specified by the manufacturer. The recombinant bands of 4 Kb and 1.2 Kb are marked by arrows. Cell lines containing these fragments are marked by "*."

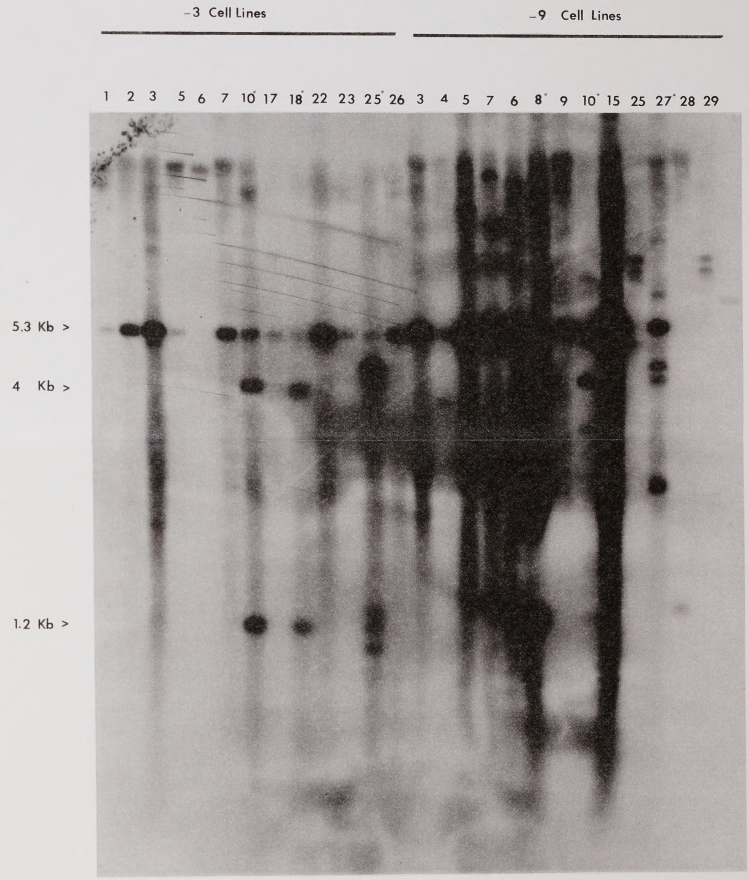

-3 Cell Lines
-9 Cell Lines
1 2 3 5 6 7 10 17 18 22 23 25 26 3 4 5 7 6 8 9 10 15 25 27 28 29
5.3 Kb >
4 Kb >
1.2 Kb >

Table 3. Summary of the double digestion analyses for all recombinant cell lines.

The data for the double digestion with the diagnostic restriction endonucleases is summarized in this table. "+/+" signs indicate the presence of the large or the small intervals defined by the novel restriction endonucleases, respectively.

	Fragment	XhoI/Bsp106I	Bsp106I/SacII	SacII/NotI	NotI/NruI	NruI/SpeI	SpeI/NheI	NheI/SalI	NheI/Bsp106I	XhoI/NotI	SalI/XhoI
	Size(bp)	5097bp/194bp	4031bp/1260bp	4712bp/579bp	4799bp/492bp	4545bp/746bp	4399bp/829bp	4781bp/510bp	3969bp/1322bp	3646bp/1645bp	4285bp/1006bp
Cell line											
10-3			+/+					+/+	+/+	+/+	+/+
18-3			+/+						+/+		
25-3			+/+					+/+	+/-		+/+
1-9			+/+	+/+	+/+	+/+	+/+	+/+	+/+		+/+
8-9			+/+	+/+		+/+					
10-9			+/+						+/-	+/+	
27-9			+/-						+/-		
III-1			+/+	+/+							
III-2			+/+								
III-3			+/+	+/+		+/+	+/+	+/+	+/+		+/+
III-4			+/+	+/+		+/+		+/+	-/+		
III-6			+/+	+/-							
III-9			+/+				+/-	+/+	+/+		+/+
III-11			+/+					+/+	+/+		+/+

Table 4. Summary of recombinant intervals.

The number of transformants containing at least one crossover in a particular recombinant interval relative to the total number of recombinant transformants is summarized in this table.

Interval	Size (bp)	No. of Events
*Bsp*106I/*Xho*I	194	0/14
*Xho*I/*Sac*II	1066	13/14
*Sac*II/*Not*I	579	4/14
*Not*I/*Nru*I	492	1/14
*Nru*I/*Spe*I	746	4/14
*Spe*I/*Nhe*I	892	2/14
*Nhe*I/*Sal*I	510	7/14
*Sal*I/*Bsp*106I	812	6/14

Further mapping analysis of recombinant genomes

To inquire whether recombination events all occurred within a Py genome integrated at a single site, a restriction endonuclease that does not cut Py was used. When *Bgl*II digest is electrophoresed on high-resolution agarose gels (0.4% in Tris-acetate EDTA buffer) the number integration sites can be determined (Figure 5). From the pattern of *Bgl*II digests it is concluded that all of the recombinant cell lines analyzed in this study contain a single integration site. The *Bgl*II patterns also confirm the variability in the degree of in situ amplification of the resident viral genome. This is deduced from the intensity of the ladder pattern and the free circular genome band.

To further map the integrated genome, single digestions with all eight unique enzymes were carried out. This analysis determined what restriction sites were present in the integrated genome as well as the extent of genome contribution from each parent. These results are summarized in table 5.

Construction of maps of the recombinant genomes

A single solution that satisfies the data from the above-described analyses was found for each recombinant genome. These are presented as maps of the integrated recombinant genomes (Figure 6). Statistical analysis of the number of crossovers per interval was performed using the G-square statistics (9). Assuming that the observed numbers of crossovers are not significantly different

Figure 5. *Bgl*II analysis of thirteen cell lines.

*Bgl*II analysis of thirteen cell lines derived from the second experiment is presented. The position of the relaxed circular viral genome (form II) is indicated. This analysis determines the upper limit for the size of the viral sequences in each cell line. No variation in the *Bgl*II patterns were observed among recombinant or non-recombinant cell lines.

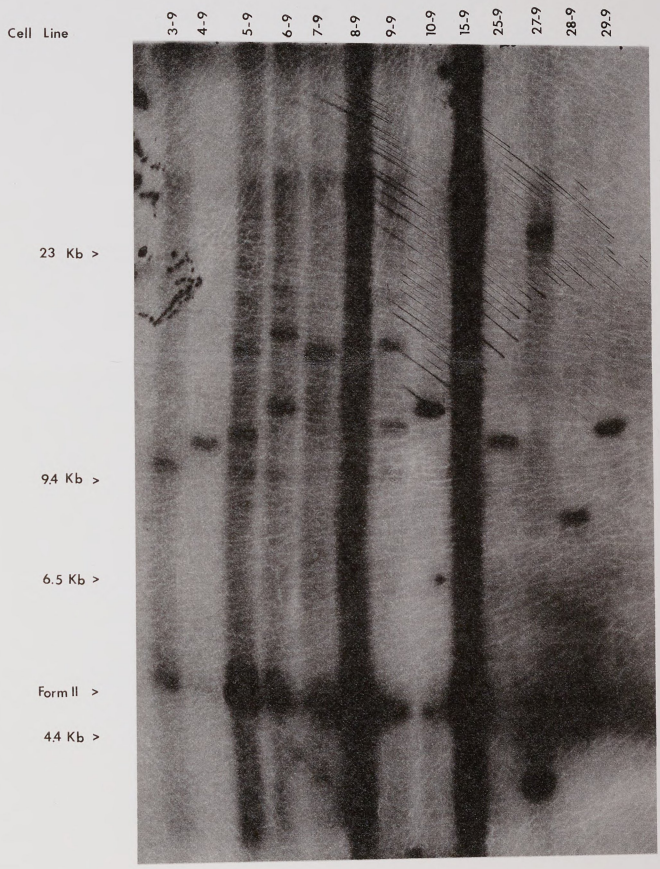

Cell Line
3-9
4-9
5-9
6-9
7-9
8-9
9-9
10-9
15-9
25-9
27-9
28-9
29-9
23 Kb >
9.4 Kb >
6.5 Kb >
Form II >
4.4 Kb >

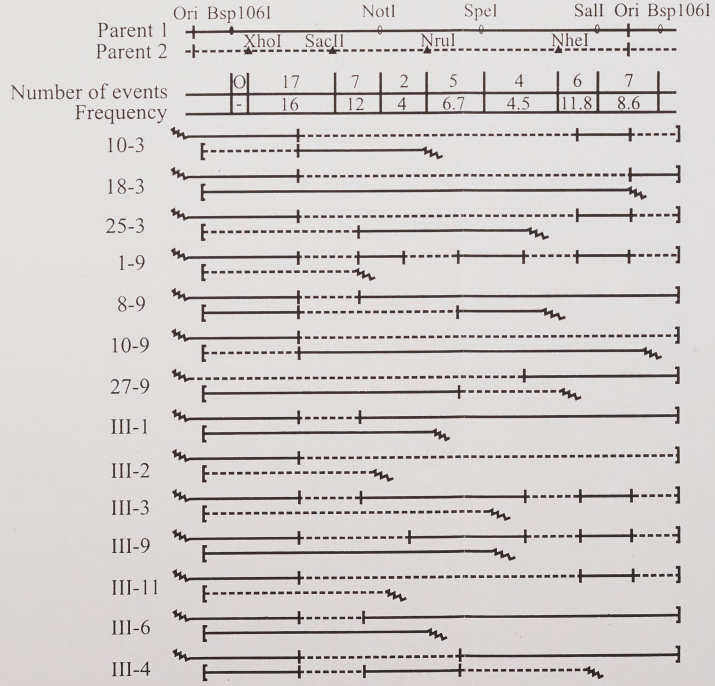
Table 5. Summary of the single digestion analysis.

The results for the single digestions with all eight unique restriction endonucleases are presented here. The generation of a genome-sized 5.3 Kb band is monitored.

	Bsp106 I	XhoI	SacII	NotI	NruI	SpeI	NheI	SalI
Cell Line								
10-3	+	+	+	+	+	+	-	+
18-3	+	+	+	-	+	-	+	-
25-3	+	+	+	-	+	+	+	+
1-9	+	-	+	+	+	+	+	+
8-9	+	-	+	+	+	+	-	+
10-9	+	+	+	+	+	+	+	+
27-9	+	+	-	+	-	+	-	+
1-III	+	-	-	+	-	+	-	+
2-III	-	+	+	-	+	-	+	-
3-III	+	+	+	+	+	+	+	+
4-III	+	+	+	+	+	+	+	+
6-III	+	-	-	+	-	+	-	+
9-III	+	+	+	-	+	+	+	+
11-III	+	+	+	-	+	-	+	+

Figure 6. Maps of the integrated, recombinant viral sequences.

The maps of all cell line are aligned. The parental genomes are depicted on the top. The sequence contribution for each parent is estimated and depicted. The table summarizes the number of crossovers in each interval and a relative number (normalized to unit length) is also given for each interval.

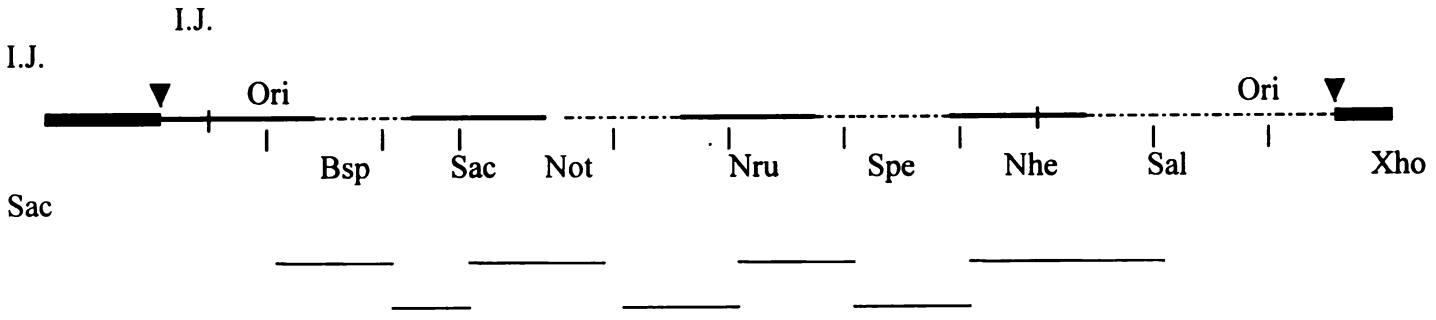

Ori Bsp106I
NotI
SpeI
SalI Ori Bsp106I
Parent 1
Parent 2
XhoI
SacII
NruI
NheI
Number of events
Frequency
O 17 7 2 5 4 6 7
- 16 12 4 6.7 4.5 11.8 8.6
10-3
18-3
25-3
1-9
8-9
10-9
27-9
III-1
III-2
III-3
III-9
III-11
III-6
III-4

with respect to the mean and each other, the probability of what is observed is 0.056. In other words, the observed number of crossovers per interval are significant with respect to the mean. This is especially true for the *Xho*I-*Sac*II interval.

Detailed analyses for two cell lines are presented here as illustrations (Figures 7 and 8). The cell line 1-9 was obtained from the second experiment and was maintained at 39°C immediately post infection. The cell line 11-III was obtained from the third experiment and also maintained at 39°C. The size of the integrated viral sequence in the 1-9 cell line was determined to be less than 10 Kbp. The level of free circular viral DNA was moderate as determined by the pattern of *Bgl*II digest. Double digestion with *Bsp*106I + *Sac*II, *Sac*II + *Not*I, *Not*I + *Nru*I, *Nru*I + *Spe*I, *Spe*I + *Nhe*I, *Nhe*I + *Sal*I, *Nhe*I + *Bsp*106I, and *Sal*I + *Xho*I resulted in the generation of recombinant sized fragments. It was therefore concluded that in this cell line recombination had occurred in the following intervals: *Bsp*106I-*Sac*II, *Sac*II-*Not*I, *Not*I-*Nru*I, *Nru*I-*Spe*I, *Spe*I-*Nhe*I, *Nhe*I-*Sal*I, and *Sal*I-*Bsp*106I. The integrated viral sequence in the 11-III cell line was estimated to be less than 11 Kbp. The level of free circular viral genomes was moderate. The cell line 11-III recombination analysis showed recombinant fragments only in double digestions with *Nhe*I + *Sal*I, *Nhe*I + *Bsp*106I, and *Sal*I + *Xho*I. These results showed that *Bsp*106I-*Sac*II, *Nhe*I-*Sal*I, and *Sal*I-*Bsp*106I intervals contained crossovers. The results of the single digestions confirmed the above results. Maps of the integrated viral sequences were drawn using the

Figure 7. Detailed analysis of cell line 1-9.

The results of single and double digestions with the diagnostic restriction endonucleases are presented. A linear map of the integrated viral sequences is drawn and the recombinant intervals are shown.

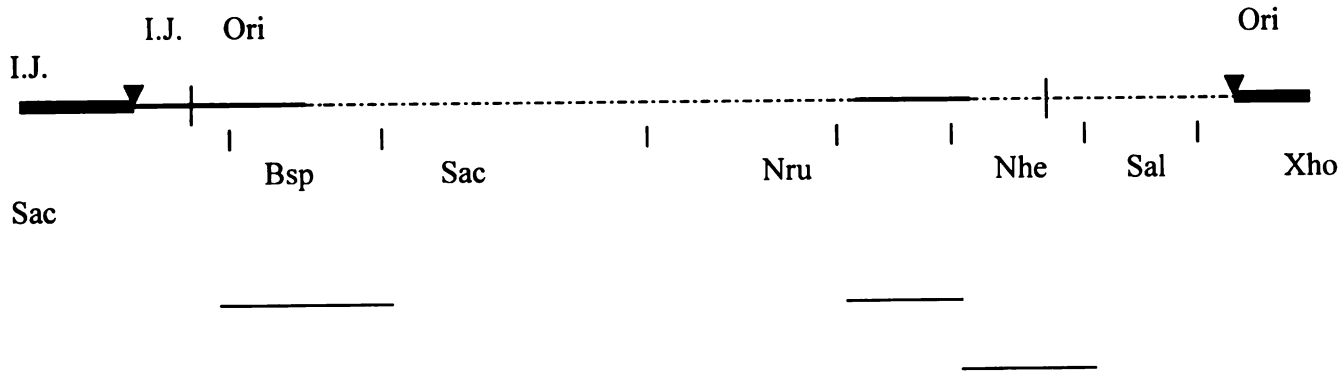


Double Digest	Recombinant
XhoI/Bsp106I	NO
Bsp106I/SacII	Yes
SacII/NotI	Yes
NotI/NruI	Yes
NruI/SpeI	Yes
SpeI/NheI	Yes
NheI/SalI	Yes
NheI/Bsp106I	Yes
SalI/XhoI	Yes
XhoI/NotI	Yes

Single Digest	Linear 5.3Kbp
XhoI	Yes
Bsp106I	Yes
SacII	Yes
NotI	Yes
NruI	Yes
SpeI	Yes
NheI	Yes
SalI	Yes

Figure 8. Detailed analysis of cell line 11-III

The results of single and double digestions with the diagnostic restriction endonucleases are presented. A linear map of the integrated viral sequences is depicted and the recombinant intervals are marked.



Double Digest	Recombinant
XhoI/Bsp106I	No
Bsp106I/SacII	Yes
SacII/NotI	No
NotI/NruI	No
NruI/SpeI	No
SpeI/NheI	No
NheI/SalI	Yes
NheI/Bsp106I	Yes
SalI/XhoI	Yes
XhoI/NotI	No

Single Digest	Linear 5.3Kbp
XhoI	Yes
Bsp106I	Yes
SacII	Yes
NotI	No
NruI	Yes
SpeI	No
NheI	Yes
SalI	Yes

available data and keeping in mind that an integrated genome in a transformed cell line must include a complete enhancer through early coding region.

Analysis of interviral recombination among unintegrated genomes

Recombinants could be formed prior to integration, at the same time as integration or post integration. In the first case un-integrated recombinant genomes may be detectable. To answer this question total DNA from FR3T3 cells coinfected with the parental Py variants, at permissive and non-permissive temperatures, was isolated and analyzed for recombination. This was obtained from the same experiments that led to high frequencies of recombination among the transformed cell lines. At 72-hour post infection the level of viral genomes in the 39°C samples were 1% of those of the 33°C samples. This was due to the temperature sensitive mutation of LT-Ag, ts-a. The double digests with the diagnostic restriction endonucleases showed no recombinant. This is, however, limited by the level of detection of this system. In order to determine the level of sensitivity of the hybridization technique, a reconstruction experiment was performed. The limit of detection of a recombinant band of about 4000 bps was established at 1% of the total DNA. An attempt to increase the detection sensitivity using a PCR assay was thwarted by the artifactual generation of recombinant fragments during the assay.

The level of recombination in total population samples was also analyzed. This analysis was also negative for the presence of recombinant intervals. This

may be in part due to the variability in focus growth. That is, different transformed foci appear at different times and therefore contribute differentially to the total number of cells on a plate. Additionally, at 33°C the replication of input parental genomes may have masked the presence of the recombinant genomes.

Analysis of recombination in permissive NIH3T3 cells at 33°C and 39°C did not show any recombination within our detection level.

Discussion

The genome of polyomavirus provides a useful substrate to study homologous recombination in mitotically dividing cells. This is an ideal system because the viral genome is packaged into chromatin and encodes a single protein, large T antigen, that could potentially affect recombination. In this report we have examined interviral recombination among the integrated genomes of polyomavirus in transformants derived from mixed infections of nonpermissive Fischer rat fibroblasts. The findings derived from the experiments are: (i) a high percentage of transformants isolated contained recombinant viral sequences (called recombinant transformants), (ii) the number of crossovers at each integration site was high averaging 3.4 crossovers, (iii) number of crossover events and the frequency of recombinant transformants did not depend on the replication function of large T antigen, and (iv) no recombination was observed in

the pool of un-integrated viral genomes. These results confirm and extend previous results which showed high frequencies of recombinant transformants.

In order to examine the role of large T antigen, temperature sensitive mutants of large T antigen were used in our experiments. Co-infections were carried out at both permissive and nonpermissive temperatures. The results suggests that the dramatic decrease in the replication function of large T antigen and transformation frequencies, at permissive and nonpermissive temperatures, did not change the frequency with which recombinant transformants were obtained or the frequency of crossover events in the integrated viral sequences. These experiments do not eliminate the involvement of large T antigen in recombination because residual functions of large T, other than its replication function, may be important. It is conceivable that although the large T antigen temperature sensitive mutant used in our experiments is incapable of supporting significant viral DNA replication, it can bind at the origin and induce localized melting of the double strand, thus providing a suitable substrate for homologous recombination. Other reports have implicated functions of large T antigen, other than its replication function, in homologous recombination (10-12).

Our experiments make a point about the generation of tandem repeats of the integrated viral sequences. We have noted that most of the recombinant cell lines contained tandem repeats of the viral sequences, from 1.2 genomes to about 1.8 genomes. This has been observed in the integration patterns of both SV40 and polyomavirus and was attributed to large T antigen mediated replication of the viral sequences (13, 14). The integrated sequences in some of

the recombinant cell lines analyzed encompass large pieces of each parent. These composites may have arisen from a reciprocal exchange between two monomeric parents to yield a dimeric molecule which is subsequently integrated. This suggests that homologous recombination, perhaps in addition to large T mediated replication, plays a role in the formation of tandem repeats during integration of polyomavirus genome. This conclusion is also supported by the fact that such integrants arise both in the presence and absence of large T replication function.

Recombination between the viral sequences could occur before, concomitant with, or after integration. To address this question, pools of un-integrated viral genomes were tested for the presence of recombinant intervals. No recombinants were detected. Such results are consistent with the hypothesis that recombination occurs either in concert with integration or post integration. However, it should be noted that the limit of recombination may be below the detection limit of our experiments. If the transformation frequency is at best 1% of the total cells, recombination occurs, on the average, in 20% of these cells, thus there is a maximum of 0.2% of cells supporting recombination events between the viral sequences. This falls short of the established detection limits of our experiments.

Integration of polyomavirus into pre-integrated viral sequences has been shown (3). An interesting observation in this regard has been that those recombinant cell lines with extensive amplification of the viral sequences did not undergo higher than average crossovers. This suggests that once a final

recombinant pattern is established in each cell line, no further recombination occurred within the time frame of the experiments.

The high frequency with which recombinant transformants were isolated may be explained using two hypotheses. First is the possibility that the experimental design of isolation of transformants selects for a subset of cells which are recombination proficient. To the best of our knowledge such phenomenon has not been documented. Furthermore, similar results have been obtained with synchronized and non-synchronized cells (2, 3). This reduces the probability that a fraction of cells in a particular phase of the cell cycle are more recombination proficient. Alternatively, it is possible that recombination and integration are interconnected. A recombination intermediate may provide a more suitable substrate for integration. Furthermore, integration of the viral sequences may occur preferentially in areas of the host genome which are more prone to recombination activity.

We can envisage two models for the interaction of viral sequences leading to genetic exchange. First, multiple crossover events between two parental viral DNA molecules followed by resolution of these structures may have resulted in the generation of the recombinant genomes. Second, the interaction may be between more than two viral DNA molecules. This final point can be tested experimentally in tri-parental crosses. It should be noted that these models are not mutually exclusive in that multiple pathways may be involved in generation of recombinant sequences.

We have noted a disparity in the percentage of recombinant transformants obtained in the three experiments performed. This observation was also made by Hacker and Fluck and Chen and Fluck. At the present time the factors that may contribute to such disparity are not clear. We hypothesize that variations in the multiplicity of infection among the experiments may account for some of the observed differences. If, indeed, recombination occurs through interactions between more than two parents, then it is conceivable that small variations in the multiplicities of infection can change recombination frequencies. Experiments are underway to test the dependence of both frequencies of recombination and the number of genetic exchanges upon multiplicity of infection.

The most intriguing result of this study is the extremely high number of crossovers in the integrated sequences of polyomavirus as well as the high frequency of recombinant transformants. The significance of these results become more apparent when compared to the reported rates of homologous recombination in mitotically dividing cells which have been estimated to be between 10^{-8}-10^{-6}. We attribute the high number of exchanges to some property of the viral genome or a viral protein. This may be either a specific sequence or the chromatin structure of the viral genome or both. Interestingly, the polyomavirus enhancer has been shown to induce chromatin accessibility, in a cis-acting manner, when placed in the HPRT gene (15). This feature of the enhancer may make the viral sequences more accessible to recombination enzymes thus allowing for such high frequencies of recombination. It is also noteworthy that the histones associated with the polyomavirus chromatin are

hyperacetylated relative to cellular histones (16). Acetylation of histones is an important modulator of chromatin accessibility. In yeast, regions of chromatin that are transcriptionally silent are hypoacetylated and also have lower frequencies of homologous recombination (17). The potential for role of the viral oncogene, middle T antigen, in recombination arises from observations that frequencies of homologous recombination are higher than normal in transformed cells. Inactivation of p53 (18, 19) and overexpression of *fos* (20) have been implicated in such instances. Interestingly, middle T antigen has been implicated in acetylation of the viral histones on the polyomavirus chromatin (16). In addition there may be a classical hot spot of recombination on the polyomavirus genome. Our experiments were designed to test this hypothesis. The viral genome was divided into eight intervals and recombination in each interval was tested. The eight intervals had variable crossover events and this variability was determined to be statistically significant. The total number of crossovers in the *Xho*I-*Sac*II interval was significant with respect to both the average number of crossovers and the observed number of crossovers for each interval. This interval may indeed be a hot spot for initiation of recombination events. That is, a single event in this interval followed by the formation of a long heteroduplex molecule and subsequent resolution and mismatch repair, or gene conversion, can also result in generation of multiple crossover events. These hypotheses are not mutually exclusive and can indeed work in concert and result in the high frequencies of genetic exchange observed.

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