Amplification of polyomavirus DNA sequences stably integrated in rat cells

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ABSTRACT

To investigate the mechanism by which the polyomavirus large T antigen (T-Ag) promotes amplification of integrated viral sequences, we constructed a rat cell line, Hy2-ts5, carrying two different inserts of polyomavirus DNA. The first insert, designated the middle T (*pmt*) locus, was devised to analyze homologous recombination between two defective copies of *pmt* lying 3.3 kb apart on the same chromosome. Reconstitution of a functional *pmt* by spontaneous recombination occurred at a rate of about 2×10^{-7} per cell generation. The second locus contained the polyomavirus large T (*plt*) gene carrying a temperature-sensitive mutation and producing a nonfunctional large T-Ag at 39°C. A shift to the permissive temperature for as little as 24 h induced the production of a functional large T-Ag which, in turn, promoted homologous recombination in the *pmt* locus at a rate close to 1.0 per cell generation. The particularity of this system is that it allowed recombination products to be analyzed as early as a single cell doubling following the initial recombinational event. Amplification occurred by successive duplications of a discrete sequence in the viral insert. Unequal sister chromatid exchange was ruled out as the recombination mechanism promoted by large T-Ag. Instead, we proposed a model of nonconservative recombination involving mispairing between homologous sequences.

INTRODUCTION

Amplification and other forms of DNA rearrangement within the eucaryotic genome can profoundly alter the pattern of gene expression. Cellular oncogenes are often amplified in various types of human cancer, especially in highly malignant tumors, which suggests that overexpression of oncoproteins can play a major role in tumor progression (reviewed in 19, 20, 24, 25). Gene amplification can also confer resistance to a wide range of cytotoxic drugs, including pesticides in some plants and insects (13, 21) and resistance to heavy metals in *Drosophila* (14).

We have approached the analysis of gene amplification by studying the rearrangements that the polyomavirus genome can sustain following integration into the host chromosomes. Cells

transformed by polyomavirus and SV40 can undergo a high rate of amplification and excision of the integrated viral genome (3, 12, 15). Both phenomena require a functional large T-Ag, the viral replication protein, as well as the presence of homology within the integrated sequences (5). Several mechanisms leading to excision or amplification have been proposed. According to Botchan et al. (3), upon initiation of replication at a given proviral locus, multiple rounds of DNA synthesis occur to form a localized 'onion skin' of amplified sequences. This aberrant polytenic structure could then represent a favorable substrate for homologous recombination, leading to excision or amplification. Alternatively, large T-Ag could have a recombination-promoting activity per se, independent of replication (6, 16).

Previously we described a system to analyze recombination between two repeats of polyomavirus DNA sequences lying in close proximity in the genome of a rat cell line (26). Both polyomavirus and SV40 large T-Ag's promoted homologous recombination in the viral insert. However, attempts to identify the recombination mechanism promoted by polyomavirus large T-Ag were hampered by the fact that the latter induced amplification of viral sequences that contained a functional replication origin. To overcome this problem we have constructed a cell line carrying a temperature-sensitive *plt* mutant capable of promoting recombination at the permissive temperature. With this cell line, we have been able to induce transient expression of a functional large T-Ag and to analyze the amplification process as early as a single cell doubling following the initial recombinational event. We show here that amplification promoted by the polyomavirus large T-Ag in Hy2 cells occurs by successive duplications of a discrete sequence in the viral insert and we propose a new model of nonconservative recombination involving mispairing between homologous sequences.

MATERIALS AND METHODS

Cells and cultures

Details on the construction of the Hy2 cell line have been reported (26). The arrangement of the viral insert is represented in Figure 1A. It contains two incomplete copies of *pmt* lying as direct repeats stably integrated in the cellular genome. The first copy lacked the viral promoter and the sequence coding for the first 104 amino acids of middle T-Ag. The second copy was

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interrupted before nucleotide (nt) 1500 (AccI site) and lacked the SstI site at nt 1373. Both copies were separated by 3.3 kb of pAT153 DNA. Hy2 cells exhibited a normal morphology in culture but acquired the phenotype of polyomavirus transformants when their viral insert formed an intact coding region for middle T-Ag.

To introduce *plt* into Hy2, the cells were transfected with plasmids of the pneo-LT series and G418 selection was applied as previously described (4). All cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Plasmids and mutants

pneo-LT1 carries both *neo* and the *plt* gene (4). This plasmid was obtained by inserting the BamHI insert of pPyLT1, the *plt* construct (28), into pSV2neo (23). pneo-LT97 carries both *neo* and LT97, a *plt* mutant with a 30 bp deletion (nt 1367 through 1396). This mutant is deficient in the initiation of viral DNA synthesis (1). pneo-LTtsa has been described (4). It contains *neo* and the *plt* gene carrying the *ts-a* mutation (7).

Fluctuation analysis

The rate of spontaneous transformation of Hy2 was determined by the fluctuation test of Luria and Delbruck (11). Parallel clonal populations were grown to confluence in 15-mm Linbro microplates, i.e. sufficiently small populations so that no transformant would be observed in a significant proportion of cultures. The mutation rate is given by the expression $\alpha = (-\ln P_0 \ln 2) / (N_f - N_i)$ per cell generation, where P_0 is the probability that no mutation will occur and N_f and N_i are the final and initial numbers of cells respectively.

Figure 1. Structure of the *pmt* insert in the Hy2 cell line. **A.** Before recombination the insert contains 2 defective copies of the *pmt* oncogene (boxes) in the same orientation. The first copy lacks the 5' portion of *pmt* up to the PstI site at nt 484 and contains the intron. The second copy is interrupted between the Aval site (nt 1016) and the AccI site (nt 1500). Both copies are separated by the BamHI-HindIII fragment of pAT153. Homologous sequences are represented by black boxes. **B.** Reconstitution of functional *pmt* by homologous recombination yields a 742 bp SstI fragment and a 2.2 kb BamHI+HindIII fragment which are not detected in Hy2, the parental cell line. The duplication of 5.6 kb comprises a repeat as well as the sequence between the two repeats. **C.** Product with a deletion expected after unequal sister chromatid exchange.

Analysis of middle and large T-Ags

The procedures for radiolabeling cells with [³⁵S] methionine, T-Ag extraction and immunoprecipitation have been published in detail (18). The T-Ag's were released from the protein A-Sepharose by boiling for 2 min in 50 μ l of dissociation buffer and purified further by a second immunoprecipitation.

Analysis of integrated sequences

The arrangement of the plasmid sequences within the cellular DNA was analyzed by Southern blotting (22). The DNA was digested by restriction endonucleases and fractionated by agarose gel electrophoresis (1% agarose unless otherwise indicated). The fragments were transferred onto nitrocellulose and hybridized to [³²P]-labeled probes.

RESULTS

Spontaneous recombination

The structure of the viral insert in Hy2 was designed such that homologous recombination across some of the repeated sequences reconstitutes a functional transforming gene and converts the cells from the normal to the transformed state. When Hy2 cells were grown and passaged in culture, they occasionally yielded spontaneous foci that showed all of the characteristics of polyomavirus transformants. To evaluate the rate of transformation, we used a procedure based upon the fluctuation test of Luria & Delbruck (11). Parallel clonal populations were grown in 15-mm Linbro microplates and observed for morphological transformation up to 4 weeks following confluence. Only one of 96 cultures analyzed became transformed. This corresponded to a recombination rate of about 2×10^{-7} per cell generation. To determine how the *pmt* insert was rearranged, four spontaneous transformants were isolated and their inserts were mapped in detail by Southern blotting. In the parental Hy2 cell line, BglII, a noncutting enzyme, produced a single fragment of 13 kb (see below). In the four transformants, the BglII fragment

Figure 2. Analysis of recombination products by Southern blotting. **A.** Spontaneous transformants isolated from Hy2. Reconstitution of functional *pmt* is detected by the appearance of a 2.2 kb BamHI+HindIII fragment and by a 5.6 kb BclI fragment. **B.** Hy2-ts5 cells exposed to the permissive temperature for various cell doublings. (a, b). Cultures were grown to 50% confluence and (a) shifted to 33°C and allowed to reach confluence (one cell doubling) before DNA extraction or (b) maintained at 39°C. (c, d) Hy2-ts5 cells exposed to the permissive temperature for 3 to 4 cell doublings. Cultures were shifted to 33°C at 20% confluence and allowed to grow for 7 days (3 to 4 cell doublings) before DNA extraction. (c) 20 μ g DNA. (d) 4 μ g DNA. The DNA was cleaved by HindIII and analyzed with the *pmt* probe. **C.** The blot shown in B was hybridized to an actine probe.

was 19 kb. The DNA was further analyzed with different restriction enzymes that cleave once (such as BclI) or several times (such as BamHI and HindIII) within the insert (Figure 2A), and the structure depicted in Figure 1B (see also Figure 5A) was obtained for all four spontaneous transformants. The rearranged insert contained the entire viral sequence present in Hy2 with a duplication of the sequence between the two repeats which resulted in the addition of 5.6 kb of DNA. We also verified by immunoprecipitation that the spontaneous transformants expressed the 56K middle T-Ag (not shown).

Effect of large T-Ag

Hy2 cells were transfected with the *plt* gene linked to *neo*, and the resulting G418-resistant colonies were examined for a change in morphology. The cells had a normal morphology characteristic of untransformed cells at the time of selection in G418. However, transformants appeared during propagation of the colonies in culture. The time-course of phenotypic changes is illustrated in Figure 3A. About 20% of the clones established by transfecting pneo-LT1 (*neo* + *plt*) were transformed by the time they reached confluence in 15-mm Linbro microplates. Another 50% became transformed within the next 15 days. Figure 3A compares the time of appearance of spontaneous transformants with pSV2neo (*neo* without *plt*) and pneo-LT97, a *plt* mutant defective in the initiation of viral DNA synthesis (1).

Construction of LTtsa cell lines

We also transfected the *plt* gene carrying the *ts-a* mutation (7) and thus encoding a nonfunctional form of large T-Ag at high temperature. As expected, transfection of this recombinant into

Hy2 cells at 33°C, the permissive temperature for the *ts-a* mutation, yielded foci of transformed cells (Figure 3B). However, when the transfected cells were cultured at the nonpermissive temperature of 39°C, transformation was reduced by more than 90%. The clones isolated at 39°C were screened for an inducible cell line capable of promoting recombination when shifted to 33°C. Two clones were isolated, from a total of about 50, that produced foci at 33°C while remaining untransformed at 39°C. These clones were grown in the presence of G418 for 2–3 months and eventually yielded cell lines, designated Hy2-ts1 and Hy2-ts5, with a flat phenotype and a stable insert. Hy2-ts1 carried multiple copies of the transgene (not shown), whereas Hy2-ts5 carried a relatively simple insert (see below).

We then attempted to trigger recombination in the *pmt* locus by exposing cells to a functional large T-Ag for various periods. When cultures of both Hy2-ts1 and Hy2-ts5 cells were shifted to 33°C for as little as 24 h, they subsequently produced multiple foci of transformed cells that overgrew the flat monolayers. By contrast, the cultures that were not exposed to 33°C remained flat. When untransformed Hy2 cells were similarly shifted to 33°C, no change in morphology was detected. This indicated that recombination was triggered by the large T-Ag rather than the temperature shift.

To analyze expression of the viral proteins, the cells were labeled with [³⁵S]methionine and the T-Ag's were extracted, immunoprecipitated by polyomavirus anti-T serum and separated on SDS-polyacrylamide gels. At 39°C, Hy2-ts5 cells produced the 100K large T-Ag as well as another polypeptide of about 53K (Figure 4). This polypeptide which was also present in the parental Hy2 cell line was identified as a truncated middle T-Ag expressed from the *pmt* locus (26). The 56K middle T-Ag was clearly produced in four foci picked at random following the temperature shift, indicating that exposure to functional large T-Ag triggered recombination in the *pmt* locus so as to reconstitute a complete and fully active *pmt* oncogene.

Rearrangements in the viral inserts

The Hy2-ts5 cell line was studied in more detail because the arrangement of its insert was relatively simple. Cellular DNA was cleaved by various restriction enzymes and analyzed by Southern blotting. For simplicity's sake, only the results of BclI

Figure 3. Percentage of cultures yielding foci of transformed cells as a function of time. A. Hy2 cells were transfected with pSV2neo, pneo-LT1 or pneo-LT97, and selected for G418 resistance. B. Hy2 cells were transfected with pneo-LTtsa and grown at the temperature indicated. At confluence, the cultures of untransformed cells contained about 112,000 cells in 15-mm Linbro microplates.

Figure 4. Analysis of middle and large T-Ag's in Hy2-ts5 transformants. Hy2-ts5: untransformed cells maintained at 39°C. 7a4, 14c2, 21b4, 28c4: foci obtained by exposing Hy2-ts5 cells to 33°C for 7, 14, 21 and 28 days, respectively. The positions of middle T-Ag (MT), large T-Ag (LT) and the 53K protein are indicated.

digests are presented in Figure 5C. In addition to the BclI fragments of 15 and 4.5 kb from the *pmt* locus, Hy2-ts5 yielded three more fragments of 10.5, 8.1 and 2.5 kb. These fragments are arranged as shown in Figure 5B (top) and constitute the *plt* locus of Hy2-ts5. There is a single copy of *neo* followed by two copies of *plt* tandemly integrated. A single day's exposure at 33°C was sufficient to promote recombination in the *pmt* locus. All of the transformants analyzed contained the 5.6 kb fragment characteristic of the duplication already noted in spontaneous transformants. When Hy2-ts5 cells were transferred to 33°C for longer periods of 7, 14 and 21 days, the following observations were made. First, some of the clones lost one of the *plt* copies (21a4, 21b3, 21b4, 14c2 and 14c4, Figure 5C). This was likely to happen because the *plt* insert contained a functional replication origin, and excision occurred in the presence of a functional large T-Ag. In some cases, the restriction pattern was compatible with a simple crossing-over between the two *plt* repeats. Other clones (21a4 and 14c4) produced fragments indicative of a more complex mechanism. In one of the subclones (14c5), some of the *pmt* insert was deleted as well. Some cells exposed to a functional large T-Ag for several days (21b5, 14b1, 14c2 and 14c4) sustained an amplification of the *pmt* insert (5.6 kb fragment). Amplification of the 8.1 kb fragment in the *plt* locus was also detected in some clones (1b3, 21b5 and 7c2). The intensity of the 5.6 kb fragment from the *pmt* locus suggested that amplification occurred by successive duplications of the *pmt* insert. To verify this possibility, the cell lines were analyzed further by BglII, a noncutting enzyme for both loci (Figure 6). BglII produced a 13 kb fragment in the *pmt* insert and a 10 to 11 kb fragment in the *plt* locus. After spontaneous recombination, the *pmt* insert increased by 5.6 kb, yielding a BglII fragment of about 19 kb. The 19 kb fragment was clearly visible in Hy2-sp4, a spontaneous transformant, as

well as in several *plt*-induced transformants (14a3 and 7c2). This suggested that large T-Ag promoted recombination in the *pmt* insert by a mechanism similar to that occurring spontaneously. Other subclones contained a BglII fragment of about 25 kb (7b2, 14c1, 7c3 and 14c2), presumably as a result of two successive duplications of the 5.6 kb sequence. Fragments containing 3, 4, 6 and more duplications were also observed, suggesting that amplification occurred by successive duplications of a discrete fragment of 5.6 kb in the *pmt* locus.

Recombination mechanism

A striking feature of the recombination product in Hy2 is that it can be obtained by unequal sister chromatid exchange. However, such an exchange should yield two different products, one with a deletion and another with a duplication, each segregating into different daughter cells. Since there is selection for reconstitution of an intact *pmt* in spontaneous transformants, the product with a duplication is always seen while the reciprocal product is not. By contrast, recombination at high rates in the absence of selection should yield both products in equal amounts. To determine if recombination occurred by unequal sister chromatid exchange, we used the Hy2-ts5 cell line to search for the product with a deletion under conditions such that cells carrying an intact *pmt* did not overgrow the rest of the population. In a first experiment, cultures of Hy2-ts5 cells were grown to 50% confluence at 39°C and shifted to 33°C for 4 days so as to allow a single cell doubling. Under these conditions, there was no selection for recombination and yet, the 5.5 kb HindIII fragment characteristic of the duplicated insert was already visible on Southern blots (Figure 2B, lane a). On the basis of band intensities, we determined that the cells that had sustained recombination represented about 50% of the total cell population.

Figure 5. Arrangement of the *pmt* (A) and *plt* (B) inserts in Hy2-ts5. C. Analysis of Hy2-ts5 transformants by BclI, a single-cut enzyme for both the *pmt* and *plt* loci. Transformants are designated by a number corresponding to the time of exposure to the permissive temperature. * fragments from the *plt* insert.

This value corresponded to a recombination rate of 1.0 per cell generation (not shown). Surprisingly, the insert with a duplication was the only product being observed in the cell population. The reciprocal product with a deletion, which should yield a 3.2 kb HindIII fragment (Figure 1C), was not detected. To confirm this

result, we performed another experiment in which Hy2-ts5 cells were shifted to the permissive temperature at only 20% confluence. Under these conditions, the cultures became confluent after 3 to 4 cell doublings and still, did not show any sign of transformation. As shown in Figure 2B, (lanes c & d) the 5.5

Figure 6. Analysis of Hy2-ts5 transformants by BglII, a noncutting enzyme for both the *pmt* and *plt* loci. Hy2-sp4: spontaneous transformant from Hy2 showing the position of the BglII fragment containing a single duplication of 5.6 kb. Electrophoresis was performed in 0.4% agarose gels at 4°C.

Figure 7. Model explaining the role of large T-Ag in homologous recombination and amplification. (a) Structure of the *pmt* insert before recombination. (b) Large T-Ag melts and unwinds the double-stranded DNA at the origin, allowing the repeats (black boxes) to pair with each other by slipped-strand mispairing. (c) Breaks occur opposite the single-stranded loops. Repair synthesis produces the structure with a duplication as shown in d. (e) Alternatively, large T-Ag promotes replication at the viral origin, producing two nascent chromatids. (f) Mismatching between homologous sequences initiates unequal sister chromatid exchange. (g) The product with a duplication is conserved. (h) The product with a deletion is lost at mitosis. (d) Structure of the *pmt* insert after recombination.

kb HindIII fragment appearing as a result of recombination was already amplified. It had an intensity compatible with an amplification of 5–6 copies per cell, i.e. the average amplification observed in Hy2-ts5 transformants. Here again, the insert with a duplication was the only product detected in the cell population. The DNA was further analyzed by BclI, BamHI and BamHI + HindIII. However, fragments characteristic of the reciprocal product were never detected (not shown). Therefore, recombination in the *pmt* insert did not occur by a simple mechanism of unequal sister chromatid exchange.

DISCUSSION

We have constructed an inducible rat cell line, Hy2-ts5, carrying two separate inserts of polyomavirus sequences. The first one, the *pmt* locus, has been devised to analyze intrachromosomal recombination between two copies of the *pmt* oncogene lying in close proximity on the same chromosome. The advantage of recombining *pmt* sequences instead of other markers such as *neo* (17, 27) or *tk* (10) is that oncogenically transformed cells are readily detected in culture and can be isolated without selective killing by cytotoxic drugs. The second locus contains the *plt* gene carrying a *ts* mutation. This insert produces a nonfunctional large T-Ag at 39°C. However, a shift to the permissive temperature for as little as 24 h induces the production of a functional large T-Ag which, in turn, promotes homologous recombination. The *pmt* insert is amplified by successive repetitions of a discrete 5.6 kb sequence, whereas the *plt* insert can be deleted or amplified. On the basis of band intensities (Figure 2C), virtually the whole population of Hy2-ts5 cells sustains recombination within 3–4 cell generations. This is indicative of very high recombination rates.

The Hy2-ts5 cell line reproduces the conditions that lead to excision and amplification of polyomavirus or SV40 DNA sequences that are often seen in cells expressing a functional T-Ag. Both phenomena require a functional replication origin as well as the presence of homology within the integrated sequences such as complete or partial tandem insertions of viral DNA (5). According to the 'onion skin' model (3), initiation of replication at a given viral locus results in multiple rounds of DNA synthesis so as to form a localized onion-skin of amplified sequences. This aberrant polytomic structure could then represent a favorable substrate for homologous recombination, leading to excision or amplification. In agreement with this model we find that LT97, a replication-defective mutant, does not promote recombination in the Hy2 insert. Nevertheless, the role of the replicative function of large T-Ag is intriguing. The SV40 large T-Ag, which does not activate replication at the polyomavirus origin (2), promotes recombination very efficiently (26). If the function of T-Ag is simply to melt and unwind the DNA at the replication origin, one would expect SV40 replication-deficient mutants with helicase activity to promote recombination. We have previously shown that they do not (26). It is possible that large T-Ag is implicated by its replicative function in the activation of polymerase β or other enzymes involved in repair synthesis leading to the duplication of the viral insert.

An important feature of Hy2-ts5 is the ability to arrest the effect of large T-Ag by a temperature shift and analyze the early amplification process. 24 h exposure to the permissive temperature yields recombinants with a single duplication of 5.6 kb. Exposure for longer periods results in further increase of the insert by multiples of 5.6 kb. This shows that amplification proceeds by successive repetitions of the 5.6 kb sequence.

Another important feature of this system is that its high recombination rate permits the analysis of recombination products in the absence of any selection after just a few cell doublings. Under these conditions, the products of a reciprocal exchange should be detected in equal amounts in the cell population. Since the product with a deletion is never seen, one can argue that homologous recombination promoted by large T-Ag does not occur by unequal sister chromatid exchange.

To explain the role of large T-Ag in amplification we propose the models shown in Figure 7. The large T-Ag destabilizes the double-stranded DNA at the viral replication origin so as to create a favorable substrate for recombination. Once the two strands are separated, the repeats are free to pair with each other by slipped-strand mispairing (Figure 7b). Such an event would produce two single-stranded loops (Figure 7c), each containing one of the repeats as well as the sequences lying between the repeats. Slipped-strand mispairing has already been invoked as a mechanism that can generate small deletions or duplications in the genome (9). A deletion is produced when the single-stranded loop is recognized by DNA repair enzymes which excise the loop and rejoin the ends of the broken DNA strand. However, breaks occurring on opposite strands would produce a structure with large gaps corresponding to the single-stranded loops. Filling of the gaps by repair synthesis would generate the structure observed in Hy2 after recombination (Figure 7d).

A variation of this model which takes into account the requirement for the large T-Ag replicative function is shown in Figure 7 (e–h). Upon initiation of viral DNA synthesis, a replication bubble is formed at the origin. Unequal exchange between nascent chromatids does not yield reciprocal products because the structure is mitotically unstable. Instead, the product with a duplication is conserved while the product with a deletion is lost at mitosis. Mechanistically, the models are very similar. They involve mispairing between the two repeats and differ essentially in the nature of the enzymes implicated in gap filling. In the second model, the large T-Ag not only destabilizes the double-stranded DNA but also initiates unscheduled replication at the viral origin. Our results do not support the model that amplification occurs by resolution of an onion-skin structure since amplified inserts are observed mostly in cells that have been exposed to large T-Ag for long periods. It seems more likely that amplification is the result of successive duplications of the insert occurring sporadically in cells producing large T-Ag. At this point we cannot tell whether spontaneous recombination in Hy2 occurs by one of the mechanisms proposed here or by unequal sister chromatid exchange.

Although slipped-strand mispairing and unequal sister chromatid exchange are mechanisms that can both generate deletions or duplications in the genome, deletions have never been observed in the Hy2 insert. The reasons for this are unclear. Deletions are expected when the loops shown in Figure 7 (c and f) are excised following pairing between homologous sequences. By contrast, deletions as well as amplifications have been observed in the *plt* insert of the same cell line. It is possible that the way some homologous sequences will recombine is determined by their structure and that some arrangements can sustain different recombination mechanisms.

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