Intrachromosomal Recombination Mediated by Papovavirus Large T Antigens

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To investigate the mechanism by which the large T antigen (T-Ag) of polyomavirus and simian virus 40 can promote recombination in mammalian cells, we analyzed homologous recombination events occurring between two defective copies of the polyomavirus middle T (*pmt*) oncogene lying in close proximity on the same chromosome in a rat cell line. Reconstitution of a functional *pmt* gene by spontaneous recombination occurred at a rate of about 2×10^{-7} per cell generation. Introduction of the polyomavirus large T (*plt*) oncogene into the cell line by DNA transfection promoted recombination very efficiently, with rates in the range of 10^{-1} to 10^{-2} per cell generation. Recombination was independent of any amplification of viral sequences and could even be promoted by the large T-Ag from simian virus 40, which cannot activate polyomavirus DNA replication. To explain the role of large T-Ag, we propose a novel mechanism of nonconservative recombination involving slipped-strand mispairing between the two viral repeats followed by gap repair synthesis.

Intrachromosomal recombination, that is, recombination between sister chromatids or between repeated sequences on a single chromatid, can play an important role in both gene expression and genome evolution in eucaryotes (30, 51). It is thought to involve both reciprocal and nonreciprocal modes of exchange, although rigorous proof is lacking because of the inability to recover all products of recombination (e.g., see Bollag and Liskay [6]). Gene conversion, one of the nonreciprocal modes of exchange, could be responsible for maintaining sequence homogeneity in multi-gene families and for transferring information between related genes, at least when viewed on an evolutionary time scale (3, 18). It is also the process that operates during B-cell ontogeny to generate diversity within the chicken immunoglobulin light-chain gene (24, 35, 49). In mammals, recombination in immunoglobulin genes could involve another form of exchange, such as unequal crossing over between sister chromatids (50, 52).

Despite substantial progress in understanding recombination in bacteria, little is known about the process in eucaryotes, especially about cellular factors that promote high-frequency intrachromosomal recombination. Recent studies on papovavirus DNA replication have suggested that one of the virally encoded proteins, the large T antigen (T-Ag), is associated with a DNA helicase activity and that the protein is responsible for the duplex-unwinding activity that is likely to initiate replication (13, 42). Large T-Ag is known to promote phenomena such as amplification and excision of integrated viral sequences (7, 9, 27, 31), but the mechanism leading to homologous recombination is still unclear. According to the "onionskin" model (7), initiation of replication at a given proviral locus could result in multiple rounds of DNA synthesis so as to form a localized onionskin 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 (8, 10, 12, 19, 33). To address this question, we have developed a system to analyze homologous recombination events occurring be-

tween two copies of the polyomavirus middle T (*pmt*) oncogene lying in close proximity on the same chromosome in cultured rat cells. We report here that the polyomavirus large T-Ag can promote recombination between the viral sequences very efficiently when the latter carry a functional origin of viral DNA replication. Furthermore, the simian virus 40 (SV40) large T-Ag, which does not activate replication at the polyomavirus origin, promotes recombination as efficiently as does the polyomavirus large T-Ag. We show that, contrary to the onionskin model, homologous recombination can occur independently of any amplification of the viral insert, and we suggest that the role of large T-Ag is to melt and unwind the DNA at the viral replication origin, so as to create a favorable substrate for homologous recombination.

MATERIALS AND METHODS

Plasmids and mutants. pMT97.484 (see Fig. 1) encodes two defective copies of the *pmt* gene. The first copy was obtained by producing a 30-base-pair (bp) deletion between nucleotides (nt) 1366 and 1397 in the middle T-Ag-coding sequence so as to inactivate its transformation properties (2). The second copy is a *Pst*I-*Hind*III fragment (nt 484 to 1656) lacking the polyomavirus origin of replication, the promoter, and the sequence coding for the first 104 amino acids of middle T-Ag. Both copies were separated by 3.5 kilobases (kb) of pBR322 DNA (*Hind*III-*Pst*I fragment, nt 29 to 3608). pneo-LT1 (see Fig. 1b) carries both *neo* and the *plt* gene (8). This plasmid was obtained by inserting the *Bam*HI insert of pPyLT1, the large T plasmid constructed by Zhu et al. (54), into pSV2neo (41). pneo-LT97 carries both *neo* and LT97, a *plt* mutant gene with a 30-bp deletion (nt 1367 to 1396). This mutant is deficient in the initiation of viral DNA synthesis (2). pneo-SV2 carries both *neo* and the SV40 large T gene. The sequences coding for large T-Ag were provided by mutant $\Delta 2005$, which does not produce small T-Ag. Details on this construct have been published previously (1).

Cells and cultures. All cells were grown at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The pMT97.484 construct was transfected with *hygro* (CG214 $\times$ SVHy; a gift from C. Gélinas) into monolayers of FR3T3 cells, and colonies of hygromycin-resistant

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FIG. 1. (a) Structure of the plasmid used in the construction of the Hy2 cell line. pMT97.484 contains two defective copies of the *pmt* oncogene (boxes) in the same orientation. The first copy (*pmt-dl1*) has a 30-bp deletion (*dl1*) around the *SstI* site at nt 1373. The second copy (*pmt-dl2*) lacks the 5' portion of *pmt* up to the *PstI* site at nt 484. Note the presence of the intron in *pmt-dl2*. Polyomavirus sequences (thick lines and boxes) are separated by 3.5 kb of pBR322 DNA (pBR) on one side and by 3.3 kb of pAT153 DNA (pAT) on the other side. pBR and pAT sequences are in opposite orientations in the plasmid. Homologous *pmt* sequences are represented by black boxes. (b) Structure of the *pmt* plasmid. pneo-LT1 carries both the *neo* gene and the gene coding for large T-Ag. The structure shown is inserted between the *BamHI* and *PvuII* sites of pAT153 (not shown). The position of the deleted intron in *pmt* is indicated by the triangle.

cells, designated Hy1, Hy2, Hy3, etc., were isolated. All of the cell lines isolated in this manner exhibited a normal phenotype indistinguishable from that of the parental FR3T3 cell line. After multiple passages in the absence of hygromycin, the cells retained resistance to the drug, indicating that the transgene was stably integrated into the cell genome (37). About half of the cell lines were able to reconstitute a functional *pmt* gene by homologous recombination following retransfection with *pmt*. The rate of spontaneous transformation was determined by the fluctuation test of Luria and Delbruck (23). Parallel clonal populations were grown to confluence in either 6- or 15-mm Linbro microplates, i.e., sufficiently small populations so that no transformants would be observed in a significant proportion of cultures. The mutation rate is given by the expression $\alpha = (-\ln P_0 \cdot \ln 2) / (N_f - N_i)$ 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.

To introduce large T into Hy2, we retransfected the cells with plasmids of the pneo series (e.g., pneo-LT1 and pneo-SV2) and carried out *neo* selection as described previously (8).

Analysis of middle and large T-Ags. The procedures for radiolabeling of cells with [^{35}S]methionine, T-Ag extraction, and immunoprecipitation have been published in detail previously (38). The T-Ags were released from protein A-Sepharose by boiling for 2 min in 50 μl of dissociation buffer and were 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 (40). The DNA was digested by restriction endonucleases and fractionated by agarose gel electrophore-

sis. The fragments were transferred onto nitrocellulose paper and hybridized to ^{32}P -labeled probes.

RESULTS

Strategy of detecting homologous recombination. The strategy we used to analyze homologous recombination involved the construction of rat cell lines carrying two mutated copies of the *pmt* oncogene lying as direct repeats on the same chromosome. The recombination substrate, pMT97.484, is shown in Fig. 1a. It was designed such that homologous recombinations across some of the repeated sequences reconstitute a functional transforming gene and convert the cells from the normal to the transformed state. pMT97.484 was introduced into rat FR3T3 cells by cotransfection with *hygro*, and colonies of hygromycin-resistant cells, designated Hy1, Hy2, Hy3, etc., were isolated and established into cell lines. All of the cell lines established in this manner exhibited a normal phenotype indistinguishable from that of FR3T3. Three of them, Hy2, Hy3, and Hy5, were selected for further analysis. Characterization of both Hy3 and Hy5 will be presented elsewhere (L. Bouchard, unpublished data). In Hy2, the recipient cell line used in the present study, the *pmt-dl1* copy was interrupted between the *AvaI* site (nt 1016) and the *AccI* site (nt 1500), presumably as a result of the integration of pMT97.484 into the cellular genome. However, as shown below, Hy2 was able to form an intact coding region for the polyomavirus middle T-Ag, and the particular structure of its insert facilitated the detection of homologous recombination by Southern blotting.

Effect of polyomavirus large T-Ag. To assess whether the

TABLE 1. Activity of recombinant plasmids

Plasmid transfected	Coding capacity	No. of transformed cultures/no. of cultures ^a (%)
pSV2neo	<i>neo</i>	0/75 (0)
pneo-LT1	<i>neo</i> + <i>plt</i>	40/77 (51.9)
pneo-LT97	<i>neo</i> + <i>plt</i> (<i>dl97</i> mutant)	0/52 (0)

^a Colonies of G418-resistant cells were picked and transferred into 15-mm Linbro microplates. The cultures were observed every 48 h for morphological changes. About half of the transformants appeared before the cultures reached 70% confluence (16 to 17 cell generations). The other half appeared within the next 10 days.

polyomavirus large T-Ag could induce recombination in the viral insert, we retransfected Hy2 with the *plt* gene linked to *neo* and examined the resulting G418-resistant colonies for morphological changes. The cells had normal morphology at the time of selection in G418. However, transformed cells appeared during propagation of the colonies in culture. About half of the lines established by transfecting pneo-LT1 (*neo* and *plt*) became transformed within 10 days following confluence in 15-mm Linbro microplates (Table 1). No spontaneous transformants appeared under the same conditions of transfection with pSV2neo (*neo* without *plt*) or pneo-LT97, a *plt* mutant defective in the initiation of viral DNA synthesis (2). Furthermore, no transformation was observed when the large T construct was introduced into the original FR3T3 cell line.

To verify that the transformants expressed middle T-Ag, we picked six foci at random, subcloned them, and analyzed them by immunoprecipitation after labeling cells with [³⁵S]methionine. The 56K middle T-Ag appeared clearly in all of the transformants (Fig. 2). The parental cell line, Hy2, produced a polypeptide of about 53,000 daltons which was recognized by the polyomavirus anti T-serum and which remained visible in the Hy2 transformants. Such a polypeptide has not been detected before in FR3T3 or FR3T3-derived cell lines. It is, presumably, a truncated form of middle T-Ag produced by the resident *pmt-dl1* copy.

FIG. 2. Analysis of middle and large T-Ags in transfected cells. Cell lines of the Hy2LT series were isolated as G418-resistant colonies by transfecting Hy2 with pneo-LT1. The positions of middle T-Ag (MT), large T-Ag (LT), and the 53K protein are indicated. Kd, Kilodaltons.

Although the six cell lines expressed G418 resistance, the 100K large T-Ag was barely detectable in only two of them. A possible explanation is that large T could reconstitute a functional *pmt* gene even when expressed at very low levels or that, in the absence of selection, the cells tended to lose the ability to express the gene. We verified that the transformants had stably integrated the transfected *plt* gene into the genomic DNA (see Fig. 3C). Furthermore, all of the representative transformants analyzed showed the properties characteristic of polyomavirus transformation, such as growth in soft agar and production of tumors in animals (data not shown).

Recombination in the viral insert. To analyze possible changes in the structure of the insert, we isolated DNA from a number of transformants that had presumably sustained recombination and examined it with Southern blots hybridized to a *pmt* probe. The DNA from transformants exhibited major changes in restriction patterns (Fig. 3B and C). The most obvious one was an amplification of the viral insert which occurred in 10 of 27 different cell lines analyzed. This amplification was not surprising because it is well known that large T-Ag can initiate DNA synthesis at the viral origin even when the DNA is integrated and when the cells are only semipermissive for viral DNA replication (7, 31). All of the transformants analyzed exhibited a 2.2-kb *Bam*HI-*Hind*III fragment which was produced as a result of homologous recombination in the insert (see map in Fig. 4). Digestion by *Sst*I yielded a 742-bp fragment which was not detected in Hy2, the parental cell line (Fig. 3C). This fragment was produced by reconstitution of the *Sst*I site in the *pmt-dl1* copy. Both 2.2-kb and 742-bp fragments were amplified in some cell lines, suggesting that amplification could occur after recombination. Some transformants had sustained recombination without any evidence of amplification (data not shown). However, one could argue that the amplified sequences were lost following homologous recombination.

Effect of SV40 large T-Ag. To determine the role of large T-Ag in recombination, we attempted to obtain evidence of recombination by using a large T totally defective in viral DNA replication. *dl97*, a polyomavirus large T mutant unable to initiate viral DNA replication, was also unable to promote recombination in Hy2 (see above). However, surprising results were obtained when Hy2 was transfected with a plasmid encoding the large T-Ag from SV40. Here, selection for recombination could not be applied because the cells acquired the morphology of SV40 transformants as soon as they expressed T-Ag. Although the SV40 T-Ag does not activate the polyomavirus origin of replication (5), it recombined the Hy2 insert so efficiently that the products of recombination could be observed in the cell population without any selection. Figure 4A shows the appearance of new restriction fragments in a transfectant (SV5) grown to ca. 10⁸ cells, i.e., ca. 27 cell generations after the introduction of large T. On the basis of band intensities, we determined that the cells that had sustained recombination represented about 25% of the total cell population. This value corresponds to a recombinant rate of 1.5 × 10⁻² per cell generation (M. Bastin, unpublished data). Of eight different SV40 transfectants analyzed, five exhibited recombination in the viral insert, while three did not. Similar results were observed with *plt* (see above), since only half of the transfectants yielded foci of transformed cells. The reason for this is unclear. It is unlikely due to a lack of T-Ag expression because the SV40 transfectants expressed the transformed phenotype. We are currently investigating the possibility

FIG. 3. Southern blot analysis of Hy2 cells (top) and map of the viral insert (bottom). (A) Parental Hy2 cell line. The 1,229-bp fragment is produced by cutting the *Bam*HI site at the pAT-polyomavirus junction (nt 4632) and the *Sst*I site (nt 569) in *pmt-dl1*. The 804-bp *Sst*I fragment originates from the *pmt-dl2* copy. The presence of a *Pst*I fragment of 3.6 kb indicates that the pAT153 sequence is intact in Hy2 and that the pBR322 sequence is interrupted. The 1.9-kb fragment contains *pmt-dl2* and a portion of pAT. (B) Two of the transformants isolated as G418-resistant colonies by transfecting Hy2 with pneo-LT1. A similar analysis of Hy2 before transfection is shown in Fig. 4A. Note the presence of an amplified 2.2-kb *Bam*HI-*Hind*III fragment in both transformants. (C) Analysis of transformants by *Sst*I. Reconstitution of the *Sst*I site (nt 1373) in *pmt-dl1* by homologous recombination produces a fragment of 742 bp instead of 804 bp because *pmt-dl1* lacks the intron (see map for details). The 2,968-bp fragment comes from pneo-LT1 (nt 1373 to 4341) since it hybridizes to a specific *plt* probe (nt 1656 to 3918) (not shown). Fragment sizes are indicated in base pairs or in kilobases. Abbreviations: B, *Bam*HI; H, *Hind*III; P, *Pst*I; S, *Sst*I.

that only a subfraction of Hy2 cells carry the viral insert in a configuration that allows homologous recombination.

To determine whether the viral insert could recombine spontaneously, we used a procedure based upon the fluctuation test of Luria and Delbruck (23). Parallel clonal populations of Hy2 cells were grown in 15-mm Linbro microplates and observed for morphological transformation for up to 4 weeks following confluence. Only 1 of 96 cultures analyzed became transformed, corresponding to a recombination rate of about 2×10^{-7} per cell generation.

To determine how T-Ag rearranged the viral insert, we subcloned six different SV40 transfectants after 30 to 35 cell generations and mapped the rearranged inserts in four different subclones from two cell lines as well as in four other cell lines. In the parental cell line, *Bgl*II, a noncutting enzyme, produced a single fragment of 13 kb (Fig. 5A). After recombination, the fragment was 19 kb. The DNA was further analyzed with different restriction enzymes that cleave once (such as *Bcl*I; Fig. 5A) or several times (such as *Bam*HI and *Hind*III; Fig. 4C) within the insert, and the structure depicted in Fig. 4 was obtained for all of the recombinants analyzed. The rearranged insert contained the

entire viral sequence present in Hy2 with a duplication of the sequence between the two repeats, resulting in the addition of 5.6 kb of DNA. A striking feature of this structure is that it can be obtained by unequal sister chromatid exchange. However, unequal recombination between chromatids (Fig. 6) should yield two different products (panels b and c), each segregating into different daughter cells. In the absence of selection for a particular product, one would expect to find an insert with a deletion for every insert with a duplication. The insert with a duplication was detected in Fig. 4A. However, fragments characteristic of a recombinant with a deletion were never detected in our analyses (see the legend to Fig. 6 for details). We conclude, therefore, that reconstitution of the *pmt* gene in the Hy2 cell line did not occur by a simple mechanism such as unequal sister chromatid exchange.

Properties of replication-defective mutants. In view of the role of large T-Ag in viral DNA replication, it was of interest to examine the effect of replication-defective mutants on recombination. To this end, Hy2 cells were transfected with four SV40 mutants that have single-amino-acid substitutions resulting in altered specific DNA binding, ATPase, and/or

FIG. 4. Southern blot analysis of SV40 transfectants. Cell lines designated SV1, SV2, SV3, etc., were isolated as G418-resistant colonies by transfecting Hy2 with pneo-SV2, a plasmid carrying both *neo* and the SV40 large T gene (1). (A) The DNA designated SV5 was extracted from a population of ca. 10^8 cells, i.e., ca. 27 cell generations after the introduction of large T into Hy2 (cell line SV5). Note the appearance of fragments of 5.6, 5.5, and 2.2 kb which were not detected in Hy2. (B) Analysis by *Sst*I. The 742-bp fragment is barely detectable after 27 cell generations. However, after 62 cell generations, the 742- and 804-bp fragments are present in virtually equal amounts. (C) SV40 transfectants were analyzed after subcloning. Hy2, Structure of the insert in Hy2 before recombination. SV1-SV7-SV3, Structure of the insert after recombination. The duplication comprises a repeat as well as the sequence between the two repeats. In addition to the fragments shown, the locations of various sites, such as *Pst*I, *Sst*I, and *Acc*I, were confirmed on the maps (not shown).

unwinding activities of large T-Ag (25, 26, 43). The properties of the various mutants are listed in Table 2. Since the mutants were transformation competent, about half of the G418-resistant colonies had a transformed morphology and thus provided a source of Hy2 clones expressing mutant T-Ags. DNA was extracted from a total of 41 such clones grown to 25 to 26 cell generations and was subjected to Southern blot analyses similar to those shown in Fig. 4. However, none of the mutants was able to promote recombination in the viral insert (Table 2). This result indicated that, as with polyomavirus large T-Ag, recombination required an SV40 T-Ag functional in viral DNA replication.

Requirement for a functional origin of replication. To determine whether recombination in the Hy2 insert required the polyomavirus origin of replication, we constructed a plasmid similar to pMT97.484 but with a nonfunctional origin of replication. The replication defect in the construct was introduced by the deletion of 23 bp in the origin core and the addition of an *Xho*I linker at nt 37 to 60 (4). Although this mutation inactivated the replication origin, it did not affect expression from the polyomavirus early region. Six independent cell lines carrying one copy or several copies of the

construct were established by cotransfection with *hygro*. However, in no case did retransfection with polyomavirus large T and *neo* lead to the reconstitution of a functional *pmt* gene by homologous recombination (data not shown). These results indicate that the large T-Ag can promote recombination between the viral sequences only in the presence of a functional origin of replication.

DISCUSSION

Homologous recombination promoted by large T-Ag. We have devised a system to analyze intrachromosomal recombination between two copies of the *pmt* oncogene lying in close proximity on the same chromosome in cultured rat cells. The advantage of recombining *pmt* sequences instead of *neo* (36, 48) or *tk* (22) is that oncogenically transformed cells are readily detected in culture and can be isolated without selective killing by cytotoxic drugs. This system has enabled us to analyze the state of the viral insert in the whole population of transfected cells and to detect recombination events in the absence of any selection. Recombination promoted by large T-Ag occurs at very high frequencies. The

FIG. 5. (A) Analysis of SV40 transfectants by *Bgl*II (a noncutting enzyme) and *Bcl*I (a single-cut enzyme). The cell lines were subclones after 30 cell generations. (B) Arrangement of the viral insert in Hy2 as deduced from panel A. Top, Hy2 before recombination. Bottom, Hy2 after recombination. Black boxes represent the *pmt* repeats. The single *Bcl*I site is located at nt 5051 on the polyomavirus map.

appearance of new restriction fragments in the experiment shown in Fig. 4A is compatible with a recombination rate of 1.5×10^{-2} per cell generation. Considering that the rate of spontaneous recombination in the parental cell line is about 2×10^{-7} , recombination is promoted by large T-Ag by several orders of magnitude. Another study has shown that the transfer of *plt* into rat fibroblasts induces an increased frequency of homologous recombination events and the appearance of abnormal karyotypes (19). The same phenomenon has been observed with *c-myc* and functionally related oncogenes, and it has been suggested that immortalizing oncogenes may contribute to the transforming process by increasing genomic instability (10).

The effect of SV40 on the polyomavirus insert was unexpected. Although the large T-Ags from both polyomavirus and SV40 recognize and bind to the same DNA sequence motif in vitro (34, 39), they cannot functionally substitute for one another to promote viral DNA replication (5). It is likely that polyomavirus DNA replication requires species-specific permissive factors such as DNA polymerase α -primase from murine cells or proteins associated with this complex (28, 29). Interestingly, a recent study has shown that polyomavirus DNA replication in monkey cells is not unconditionally

FIG. 6. Diagram showing how unequal sister chromatid exchange (a) produces a chromatid with a deletion (b) and a chromatid with a duplication (c). The small vertical bars represent *Hind*III sites in the cellular DNA. Chromatid b should yield a *Hind*III fragment of 3.2 kb.

limited. DNAs containing the polyomavirus origin replicate efficiently in monkey cells which constitutively express the SV40 large T-Ag (46).

Our results are in contradiction with several reports claiming that recombination within transfected plasmid DNAs does not depend on T-Ag expression (16) or that neither DNA replication of the recombination substrate nor T-Ag is essential for recombination (44). The reasons for such discrepancies are not understood at present. They may arise from differences in the cell lines used or in the nature of the experimental designs, such as recombination within transfected plasmids versus homologous sequences stably integrated into the host genome.

The advantage of using a heterologous system is that the SV40 large T-Ag does not amplify the sequences at the polyomavirus origin of replication and the recombination products can be identified easily. All of the SV40 transfectants analyzed exhibited the same recombination product, i.e., a duplication of the repeat as well as the sequence between the two repeats. Such a duplication can be expected from an event of unequal sister chromatid exchange. However, unequal recombination between chromatids should

TABLE 2. Properties of SV40 mutants

Mutant ^a	Amino acid change	Origin binding	Helicase	Recombination ^b
pneo-SV2	None	+	+	5/8
pneo-C6-2	Asn-153 → Thr	—	+	0/4
pneo-C8A	Lys-224 → Glu	+	+	0/11
pneo-C11A	Pro-522 → Ser	+	—	0/16
pneo-T22	His-203 → Gln	—	+	0/10

^a Genomic DNA was cleaved by *Bam*HI and inserted in the *Bam*HI site of pSV2neo. pneo-SV2 carries *neo* and the wild-type SV40 large T gene.

^b Colonies of G418-resistant cells were picked and grown to 25 to 26 cell generations. The DNA was extracted and analyzed by Southern blotting as described in the legend to Fig. 4.

FIG. 7. Model explaining the role of large T-Ag in recombination. (a) Although the SV40 large T-Ag cannot initiate polyomavirus DNA replication, it recognizes some elements of the polyomavirus replication origin. (b) Large T-Ag melts and unwinds the double-stranded DNA at the origin, allowing the repeats to pair with each other by slipped-strand mispairing. (c) Two single-stranded loops are produced. (d) Breaks occur on opposite strands, resulting in large gaps of single-stranded DNA. (e) Repair synthesis produces the structure with a duplication.

yield two different products, one with a duplication and another with a deletion, each segregating into different daughter cells. Since the product with a deletion was never detected in our analyses, we conclude that recombination did not occur by unequal sister chromatid exchange.

Recombination mechanism. Several studies have reported that intramolecular recombination during transfection in mammalian cells is a nonconservative process. When molecules bearing direct repeats undergo intramolecular interactions intended to yield reciprocal exchange, the process generates only one of the two expected products (11). Similar conclusions were reached by Lin et al. (21), who proposed a model by which the removal of unpaired DNA at the junction between the paired and unpaired regions permits a gap repair process to reconstruct an intact gene. Our data are in agreement with such a nonconservative mode of homologous recombination. To account for the observed effect of SV40 large T-Ag on the recombination of the viral insert in Hy2, we propose the model shown in Fig. 7. How the SV40 protein interacts with the polyomavirus origin of replication is not clear. In vitro studies have shown that SV40 T-Ag binds to multiple discrete regions on the polyomavirus origin (34, 39). It does not unwind DNA containing the polyomavirus origin as well as it unwinds its own DNA. However, it does exhibit partial specificity for the sequence at the polyomavirus origin (E. Wang and C. Prives, personal communication). Whether the interaction is more specific in vivo remains to be seen. We propose that the function of T-Ag is to destabilize 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. Such an event would produce two single-stranded loops, each containing one of the repeats as well as the sequences lying between the two repeats. Slipped-strand mispairing has already been invoked as a mechanism that can generate small deletions or duplications in the genome (20). It can explain the generation of deletions in the *lacI* gene of *Escherichia coli* (15) and in the β -globin gene (14). 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 the 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 the cell lines after recombination.

Unequal sister chromatid exchange has been directly documented in yeast (32, 45) and *Drosophila* species (47). In mammals, it has been implicated in the evolution of multigene families (3) and in the rearrangement of immunoglobulin genes (51, 52). However, cells carrying the putative reciprocal products cannot always be isolated (e.g., see Tilley and Birshtein [50] and Wabl et al. [53]). In one instance, it has been shown that the switch rearrangement in the mouse heavy-chain locus occurs by a mechanism of looping out and deletion, thus excluding the model of unequal sister chromatid exchange (17). Whether the mechanism proposed here for the large T-Ag applies to other recombinational events in the cellular genome remains to be investigated.

Spontaneous recombination occurs in the Hy2 insert at a rate of about 2×10^{-7} per cell generation. Other investigators have devised similar constructs that recombine at a rate on the order of 10^{-6} per cell generation (22, 36). It is unclear why some of these constructs, which include a functional origin of SV40 DNA replication, do not recombine at higher frequencies when they are introduced into COS cells that express the T-Ag and why the type of recombination product described here has never been observed in these cell lines. Experiments are presently under way to determine whether the SV40 origin can substitute for that of polyomavirus in pMT97.484. However, it seems that the structure or configuration of the insert is very critical in determining the mechanism by which homologous sequences will recombine in a given cell line. Since the inception of this work, we have constructed and characterized five different cell lines carrying tandem repeats of polyomavirus DNA. They all recombine under the action of polyomavirus large T-Ag, although not necessarily at the same rate and by the same mechanism. In one of them, the insert sustains deletions either spontaneously or in response to large T-Ag (8). In another line, which differs from Hy2 by the position of the polyomavirus origin with respect to the two repeats, the rearrangements involve both reciprocal (inversion) and nonreciprocal (gene conversion) recombinational events (Bouchard et al., unpublished data). The most striking feature of this line is that recombination can be promoted by SV40 replication-deficient mutants. It seems, therefore, that different functions of large T-Ag may be involved in recombination in different cell lines.

Role of large T-Ag. The model we propose may shed some light on the role of large T-Ag in phenomena such as

amplification and excision of integrated viral sequences from chromosomes. Cells transformed by polyomavirus and SV40 can undergo a high rate of amplification or excision of the integrated genome, and both phenomena require a functional large T-Ag as well as the presence of homology within integrated sequences such as complete or partial tandem insertions of viral DNA (7, 27). Several mechanisms leading to excision or amplification have been proposed. Currently, the prevailing model is that proposed by Botchan et al. (7). Upon initiation of replication at a given proviral locus, multiple rounds of DNA synthesis occur to form a localized onionskin of amplified sequences. This aberrant, polytenic structure could represent a favorable substrate for homologous recombination, leading to excision or amplification. In agreement with these studies, we find that recombination in Hy2 requires a large T-Ag functional in the activation of viral DNA synthesis as well as a functional origin. However, in contrast to the onionskin model, recombination can occur in our cell lines without any detectable amplification of sequences. The relationship between recombination in Hy2 and the replicative function of large T-Ag is of special interest. If the function of T-Ag is simply to melt and unwind the DNA at the replication origin, one would expect replication-deficient mutants with helicase activity (such as C6-2, C8A, and T22) to promote recombination. Our data show that they do not. Large T could also be implicated, by its replicative function, in the repair synthesis leading to the duplication of the viral insert (Fig. 7e). If repair is achieved through the interaction between T-Ag and polymerase α , it is not clear why the SV40 T-Ag is inactive in extrachromosomal polyomavirus DNA replication and why it fails to amplify the viral insert as does its polyomavirus counterpart. An interesting possibility is that a functional T-Ag is required for the repair of large gaps and that its role is to activate polymerase β or other enzymes involved in repair synthesis. To the best of our knowledge, this possibility has not yet been investigated.

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