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Amplification Mediated by Polyomavirus Large T Antigen Defective in Replication

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The polyomavirus large T antigen promotes homologous recombination at high rates when expressed in rat cells carrying the viral replication origin and two repeats of viral DNA sequences stably integrated into the cellular genome. Recombination consists of both reciprocal and nonreciprocal events and is promoted by mutants defective in the initiation of viral DNA synthesis (L. St-Onge, L. Bouchard, and M. Bastin, J. Virol. 67:1788–1795, 1993). We have extended our studies to a rat cell line undergoing amplification of the viral insert. We show that large T antigen promotes amplification independently of its replicative function but that its origin-specific DNA binding activity is not sufficient to promote homologous recombination.

Gene amplification is a frequently used mechanism by which somatic cells can fulfill a need for increased gene expression. It can confer resistance to a wide variety of cytotoxic drugs, and it is one of the mechanisms leading to the generation and/or progression of cancer in humans (18, 22, 31). The mechanism of gene amplification is not completely understood, but it is generally assumed that amplification of a local region is accomplished by multiple initiations within a single replicon (for example, see references 3 and 23). Several laboratories have approached the analysis of gene amplification by studying the rearrangements that the papovavirus genomes can sustain following integration into the host chromosomes. Cells transformed by polyomavirus or simian virus 40 (SV40) can undergo a high rate of amplification and excision of the integrated viral genome (3, 17, 19). Both phenomena require a functional large T antigen (T-Ag), the viral replication origin, and some homology within the integrated sequences (5). In a previous study, we described high-frequency recombination in a rat cell line, designated Hy5, that did not require the replicative function of large T-Ag (26). In this cell line, the viral insert underwent a variety of recombination events including gene conversion, inversion, and unequal sister chromatid exchange. However, amplification of the insert was never observed even in the presence of a large T-Ag active in the initiation of viral DNA synthesis. To further address the role of the replicative function of large T-Ag in amplification, we investigated the effect of polyomavirus mutants in another cell line, designated Hy2, in which reconstitution of the selection marker occurred by successive duplications of a discrete sequence in the viral insert.

Homologous recombination promoted by polyomavirus large T-Ag. The structure of the recombination substrate in Hy2 is shown in Fig. 1a. It was designed so that homologous recombination across some of the repeated sequences reconstitutes a functional middle T-Ag (*pmt*) gene and converts the cells from the normal to the transformed state. We showed previously that spontaneous transformation occurred at a rate of about 2×10^{-7} per cell generation (24). 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 (Fig. 1d). A functional *pmt* was reconstituted in the process, and Hy2 transformants expressed the 56,000-molecular-weight middle T-Ag (27).

To assess the activity of large T Ag in homologous recombination, Hy2 was transfected with the large-T-Ag gene (*plt*) linked to *neo*, and the resulting G418-resistant colonies were examined for a change in morphology. The cells had a normal phenotype at the time of G418 selection, but transformants appeared during propagation of the colonies in culture. About 20% of the colonies established by transfecting pneo-LT1 (*neo* plus wild-type *plt*) were transformed by the time they reached confluence in 15-mm-diameter Linbro microplates. Another 30% became transformed within the next 10 days. The transformants that appeared thereafter (another 20 to 30%) were not taken into account in order to exclude spontaneous transformants that could arise, although in smaller numbers, in cultures kept confluent for several weeks. Under these conditions, no transformant appeared when pSV2neo (*neo* without *plt*) was transfected into Hy2 (Table 1) or when the *plt* construct was introduced into the original FR3T3 cell line (not shown).

Effect of mutant T-Ags. To characterize the large T-Ag function implicated in recombination, we attempted to identify mutations affecting the ability of *plt* to transform Hy2 cells. First, we tested a series of mutants with in-frame deletions in the second large T exon. *dl*8, *dl*45, *dl*23, and *dl*141 are four such mutants, active in the initiation of viral DNA synthesis but affected to various degrees in the immortalization of primary cells and transactivation of viral promoters (13). These mutants triggered transformation of Hy2, although less efficiently than did wild-type *plt* (Table 1). *dl*97 and Δ300, two mutants with deletions in a large-T-Ag domain required for viral DNA replication, were inactive in recombination (Table 1).

A particularity of *dl*8 is that its deletion removes amino acid residues 145 through 174, affecting the sequence DLXCXE, which is thought to be a binding site for the retinoblastoma gene product (8, 33). In a previous study, we attempted to restore the immortalization potential of *dl*8 by reconstituting an intact DLXCXE motif. This was achieved by inserting a tyrosine and a glutamic acid residue between Cys-144 and Glu-175. However, the new mutant, designated *dl*149, failed to immortalize primary cells (12). Further characterization of this mutant revealed that it was inactive in the initiation of viral DNA replication (25). Surprisingly,

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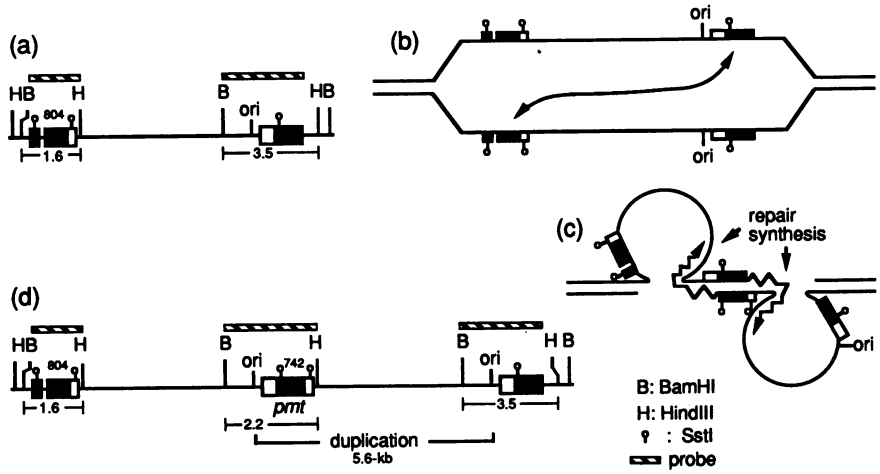

FIG. 1. Structure of the *pmt* insert in the Hy2 cell line and model explaining the role of large T-Ag in amplification. (a) Before recombination, parental cells contain two defective copies of the *pmt* oncogene (boxes) in the same orientation. The first copy lacks the 5′ portion of *pmt* up to the *Pst*I site at nucleotide 484 and contains the intron. The second copy is interrupted between the *Ava*I site (nucleotide 1016) and the *Acc*I site (nucleotide 1500). Both copies are separated by the *Bam*HI-*Hin*dIII fragment of pAT153. Homologous sequences are represented by black boxes. (b) Large T-Ag melts and unwinds the double-stranded DNA at the viral origin, allowing the repeats (black boxes) to pair with each other by slipped-strand mispairing. (c) Formation of two single-stranded loops containing one repeat and the sequence between the two repeats. Breaks occur opposite the single-stranded loops. (d) Repair synthesis produces the structure with a duplication of 5.6 kb. A functional *pmt* is reconstituted in the process. The recombination product yields a 742-bp *Sst*I fragment and a 2.2-kb *Bam*HI-plus-*Hin*dIII fragment not detected in parental Hy2 cells. The duplication comprises a repeat as well as the sequence between the two repeats.

despite this defect, *dl*149 was still active in recombination (Table 1).

Next, we tested another class of mutants, designated CR1 mutants because their lesions map in sequences homologous to conserved region 1 of adenovirus E1A (11, 34). Two of the mutants, *dl*13 and 13val, were defective in viral replication (26). Here again, the mutants transformed Hy2 cells independently of their effect on replication (Table 1). Both *dl*13 and 16val were somewhat less efficient than the wild type, but this could be due to a stability defect, because previous studies showed that these mutants were expressed at slightly lower levels in COS-1 cells (12). By contrast, 13val, which did not show any expression defect, promoted recombination with wild-type efficiency.

Analysis of recombination products. To analyze recombination products, DNA was isolated from representative transformants and examined by Southern blotting. All transformants contained sequences characteristic of the *plt* constructs (e.g., the 2.9-kb *Sst*I fragment [Fig. 2]), indicating that *plt* was stably integrated into the genomic DNA. The analysis of recombination products was hampered by the overlap between the *plt* and *pmt* sequences. Nevertheless, reconstitution of an intact *pmt* gene was observed in all transformants by the appearance of a 742-bp *Sst*I fragment. Except for 13val18, which lost the upstream *pmt* copy, all transformants had a structure compatible with the duplication observed by expressing replication-competent T-Ag in the cell line (e.g., cell line 7a2).

TABLE 1. Activity of polyomavirus large T-Ag[a]

Plasmid transfected	Coding capacity	Mutation[b]	Replication	DNA binding	No. of transformed cultures/no. of cultures (%)	
					Expt 1	Expt 2
pSV2neo	*neo*				0/53 (0)	0/60 (0)
pneo-LT1	*neo* + *plt*	None	+	+	22/40 (55)	35/87 (40.2)
pneo-LT*dl*8	*neo* + *plt-dl*8	del 145–174	+	+		19/94 (20.2)
pneo-LT*dl*45	*neo* + *plt-dl*45	del 173–195	+	+		16/46 (34.8)
pneo-LT*dl*23	*neo* + *plt-dl*23	del 195–228	+	+		7/52 (13.5)
pneo-LT*dl*141	*neo* + *plt-dl*141	del 141–146	+	ND[c]		12/42 (28.6)
pneo-LT*dl*149	*neo* + *plt-dl*149	del 149–176	−	ND		5/37 (13.5)
pneo-LT*dl*97	*neo* + *plt-dl*97	del 270–280	−	+	0/52 (0)	
pRNLTΔ300	*neo* + *plt*-Δ300	del 300–310	−	−	0/46 (0)	
pneo-LT*dl*13	*neo* + *plt-dl*13	del 13–17	−	+	22/69 (31.9)	
pneo-LT13val	*neo* + *plt*-13val	Leu-13 → Val	−	+	46/87 (52.9)	
pneo-LT16val	*neo* + *plt*-16val	Leu-16 → Val	+	+	22/64 (34.4)	

[a] G418-resistant colonies were picked and transferred into 15-mm-diameter Linbro microplates. The cultures were observed every 48 h for morphological transformation. Transformants were scored 10 to 11 days after the cultures reached confluence.

[b] Amino acid(s) deleted (del) or substituted (arrow) in large T-Ag.

[c] ND, not done.

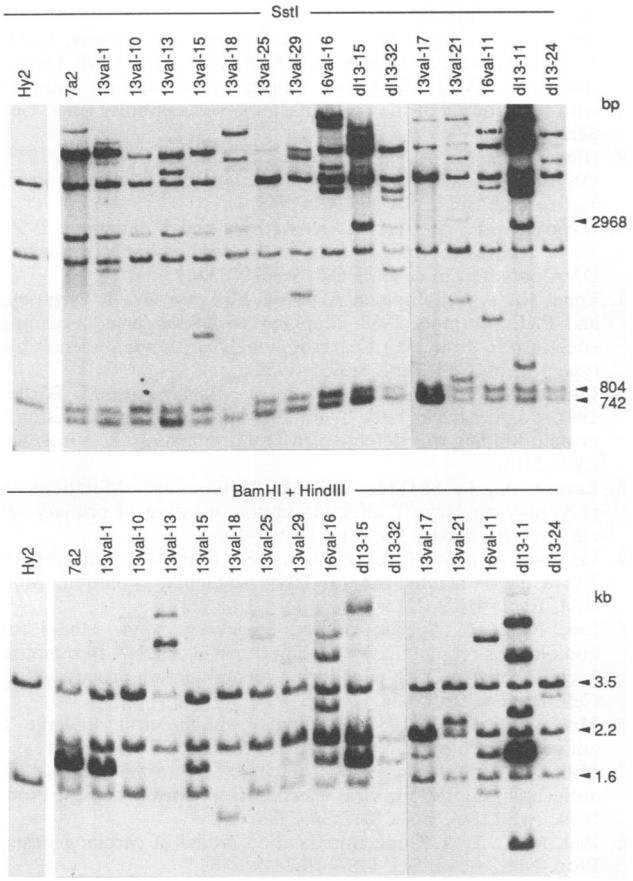

FIG. 2. Southern blot analysis of Hy2 transformants. The cell lines were isolated as G418-resistant colonies by transfecting Hy2 with plasmids of the pneo-LT series encoding *neo* and various large T-Ag mutants. The colonies became transformed within 9 days after reaching confluence in 15-mm-diameter Linbro microplates. Cells were grown to approximately 27 cell generations (10^8 cells) for DNA extraction. (Top) Analysis with *Sst*I. The 2,968-bp fragment is indicative of *plt* integration. The 742-bp fragment is produced after reconstitution of an intact *pmt*. (Bottom) Analysis with *Bam*HI plus *Hin*dIII. Reconstitution of *pmt* yields a 2.2-kb *Bam*HI-plus-*Hin*dIII fragment (see Fig. 1B for details). 7a2 is a cell line carrying a single duplication of the insert (24).

Amplification of the *pmt* insert in Hy2 was evaluated by determining the relative intensities of various fragments on Southern blots. After a single duplication, the *Sst*I fragments of 742 and 804 bp and the *Bam*HI-plus-*Hin*dIII fragments of 3.5 kb and 2.2 kb were present in equal amounts. This is illustrated in Fig. 2 with 7a2, a cell line that was shown previously to carry a single duplication of the insert (24). On the basis of band intensities, we determined that some transformants underwent only one duplication (e.g., 13val21, 16val11, *dl*13-11, *dl*13-24, 13val10, 13val15, and 16val16), while others contained more than one *pmt* copy (e.g., 13val13, 13val25, 13val29, *dl*13-15, and *dl*13-32). 13val17 contained four *pmt* copies. These results show therefore that replication-defective mutants were as efficient as wild-type T-Ag in promoting amplification of the Hy2 insert.

Origin-specific DNA binding. Polyomavirus large T-Ag binds to several sites within the noncoding regulatory region of its viral genome (6, 9). Each binding site is characterized

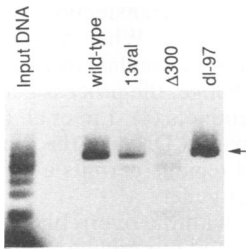

FIG. 3. Origin-specific DNA binding by polyomavirus large T-Ag. The mutations were transferred into p91023LT (7). Wild-type and mutant T-Ags were expressed in COS-1 cells and immunoprecipitated by treating nuclear extracts with a polyomavirus anti-T-Ag serum (a gift from B. Schaffhausen). Immunoprecipitates were then mixed with 10 ng of a ^{32}P-labeled *Hin*fI digest of a plasmid containing polyomavirus DNA. This *Hin*fI digest contains about 20 fragments (input DNA), one of which, 604 bp in length (arrow), contains the region of polyomavirus DNA (nucleotide 5073 to 5296 and 1 to 385) to which large T-Ag binds. Bound DNA was released from the large T-Ag–antibody complex with sodium dodecyl sulfate (1%) and fractionated on a 2% agarose gel.

by the presence of two to four copies of the pentanucleotide sequence 5′-G(A/G)GGC-3′ (6, 7). The DNA binding domain of large T-Ag has been localized to a 116-amino-acid region between residues 282 and 398 (28). Since the two recombination-defective mutants, Δ300 and *dl*97, had lesions mapping within or just outside this domain, we undertook an analysis of DNA binding with the view that it may be fundamental to the recombination-promoting activity of large T-Ag. DNA binding assays were performed by the McKay procedure (16) as modified by Cowie and Kamen (7) and Sunstrom et al. (28). Specific binding by large T-Ag selectively precipitates a 604-bp *Hin*fI fragment containing all of the large T-Ag binding sites (Fig. 3). As expected, Δ300 did not bind to the origin fragment, but *dl*97 did, as well as wild-type T-Ag. Also, binding was not affected by CR1 mutations. These results show, therefore, that origin-specific DNA binding by large T-Ag is not sufficient to promote homologous recombination.

In somatic mammalian cells, the frequency of gene amplification ranges from 10^{-4} to 10^{-6} (22) and can be increased dramatically by various means such as treatment with the tumor promoter tetradecanoyl phorbol acetate (31), UV radiation, carcinogens (29), or growth-promoting hormones (1). Thus, amplification is facilitated by agents that affect DNA synthesis and/or introduce reversible damage into DNA. Varshavski (32) has proposed that a variety of treatments can result in misfiring of replication, resulting in the generation of amplified DNA sequences. In keeping with this, polyomavirus large T-Ag could promote amplification of integrated viral sequences by initiating overreplication at the origin. Variant genomes containing tandem repetitions of a basic DNA segment have been isolated from serially passaged stocks of both SV40 and polyomavirus (30). Most of these variants have reiterations of a DNA segment which includes the viral replication origin. The overreplication hypothesis does not necessarily require the formation of an onion skin structure. Initiation of DNA synthesis by large T-Ag can produce a replication bubble at the viral origin, allowing unequal exchange between nascent chromatids. Such an exchange would not yield reciprocal products, since only the product with a duplication will be conserved while the product with a deletion will be lost at mitosis. This model is in agreement with other studies showing that intramolec-

ular 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 (4). Lin et al. (15) have proposed that removal of unpaired DNA at the junction between the paired and unpaired regions permits a gap repair process to reconstruct an intact gene.

A major difficulty with the overreplication model is that it cannot take into account our finding that amplification of the Hy2 insert is promoted by polyomavirus large T-Ag mutants defective in the initiation of viral DNA synthesis. One could surmise that if these mutants had retained some undetectable activity, they could occasionally activate replication at the viral origin and yield recombination products detectable by selection. One expects, however, any significant impairment in replication activity to translate into some reduction in recombination rates, yet both 13val and *dl*13 promote recombination at wild-type rates. Furthermore, we have previously observed recombination in Hy2 cells transformed by SV40 without selecting for polyomavirus transformants (27). Although the large T-Ags from both polyomavirus and SV40 recognize and bind to the same DNA sequence motif in vitro (20, 21), they cannot functionally substitute for one another to promote viral DNA replication (2).

The nature of the replication defect in 13val and *dl*13 is not yet understood. By analogy with SV40 (10), it is possible that they fail to interact with DNA polymerase α-primase. To explain their effect in recombination, we propose that despite their inability to initiate replication, both 13val and *dl*13 are able to destabilize the double-stranded DNA at the viral replication origin so as to create a favorable substrate for homologous recombination (Fig. 1b). *dl*97, on the other hand, has lost the ability to unwind the DNA, even though it can recognize and bind specifically to the origin region. Melting of the DNA would allow the repeats to pair with each other by slipped-strand mispairing (Fig. 1c). Such a mechanism has already been invoked to explain the generation of small deletions or duplications in the genome (14).

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