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Deletion of the Origin of Replication Impairs the Ability of Polyomavirus DNA to Transform Cells and to Form Tandem Insertions

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We examined the transforming properties of polyomavirus DNA molecules which can produce a functional large T-antigen but which are *cis* defective for viral DNA replication. The inability of these molecules to replicate results from the deletion of sequences comprising the viral replication origin. We found that even in the presence of a functional large T-antigen, transformation of rat cells by these viral DNAs was greatly reduced when compared with replication-competent parental DNA, and cells transformed by origin-minus mutants generally contained the integrated viral DNA in a nontandem arrangement. Therefore, polyomavirus large T-antigen promotes the establishment of transformation and tandem integration by interacting with the viral origin of DNA replication. This indicates that viral DNA synthesis is directly involved in these processes.

Cell transformation by polyomavirus (Py) results from the integration of the viral genome within the host DNA and its subsequent expression. It has been well established that the viral DNA molecules are integrated most often in a full or partial head-to-tail tandem arrangement, thus ensuring that at least one early region (responsible for maintenance of the transformed phenotype) will not be interrupted by cellular DNA (1–3, 9, 13, 14). It still remains unclear, however, by what means the tandem structure is generated. A role for Py large T-antigen (T-Ag) in integration and tandem formation has been demonstrated by the use of temperature-sensitive (*ts*) viral mutants which encode a thermolabile large-T protein. These studies showed (7) that infection of rat fibroblasts at the permissive temperature (33°C) for large-T function generates more transformants than at 39°C. In addition, transformants isolated at 33°C almost invariably contained tandem insertions of integrated viral DNA, whereas those selected from the nonpermissive temperature did not. Since large T-Ag is known to be involved in the initiation of viral DNA synthesis (20), these results suggested that the formation of integrated tandems of Py DNA was related to a replication step before the integration event. These studies did not, however, rule out the possibility that large T-Ag exerts its effect by mechanisms other than promotion of viral DNA replication, such as recombination between infecting viral genomes or induction of host recombination functions.

If Py large T-Ag promotes a high efficiency of transformation and tandem insertion of viral DNA by allowing viral DNA replication, the efficiency of transformation of molecules which are *cis* defective in replication should not be affected by the functional state of this viral protein. It has been shown that simian virus 40 DNA cloned in pBR322 is impaired in its ability to replicate in permissive cells as a result of the presence of plasmid sequences which exert their effect in *cis* (15). Removal of these "poison" sequences restores the replicative function of the simian virus 40 recombinant. *tsa* Py DNA (producing a thermolabile large T-Ag) (8, 11, 12) was cloned at the *Bam*HI site in both pBR322 and in the "poisonless" pBR derivative pML (15), and the ability of each of these recombinant plasmids to transform F2408 rat fibroblasts (17) at 33 and 39°C was tested. As previously shown (7), *tsa*-pBR showed little temperature sensitivity of transformation, and its transforming ability at 33°C was not much increased with respect to 39°C. *tsa*-pML consistently transformed at 33°C more efficiently than *tsa*-pBR, although never as well as native *tsa* DNA (data not shown).

It is worth noting that in these and other experiments, the best efficiency of transformation was always obtained with native Py DNA. Py DNA cloned in the late region in various plasmid vectors always gave a somewhat lower transformation rate, with the pBR322 constructions being the least efficient.

To study the relationship between viral DNA replication and transformation frequency more directly, we constructed a *tsa* Py variant, pB32, which contains a deletion of sequences comprising the viral DNA replication origin between base pairs 5246 and 127. We also constructed in vitro a variant of the origin-minus recombinant Py plasmid pdl3027 (21) (kindly provided by R. Kamen), which encodes a wild-type (wt) T-Ag, by replacement of the DNA sequences between the Py and the plasmid *Eco*RI sites with the corresponding *tsa*-pML fragment to make *tsa*3027 (Fig. 1). These deletion mutants are *cis* defective for DNA replication in permissive cells but are fully capable of early gene expression, as indicated by the following. (i) When transfected into rat or HeLa cells, they produce a frequency of T-Ag-positive cells comparable to that produced by control Py DNA. (ii) They can drive the replication of defective Py DNA molecules when cotransfected into mouse cells. (iii) Mouse cells transformed by these mutants express normal levels of viral early mRNAs and T-Ags and are capable of supporting the replication of origin-plus Py molecules which do not produce functional large T-Ag. Furthermore, this complementation is temperature sensitive in cells transformed by the pB32 and *tsa*3027 plasmids. (iv) The same cell lines, upon fusion with large T-Ag-negative transformed rat cells, can rescue defective Py DNA molecules integrated in the rat genome (L. Dailey and C. Basilico, manuscript in preparation).

The pB32, pdl3027, and *tsa*3027 plasmids were transfected

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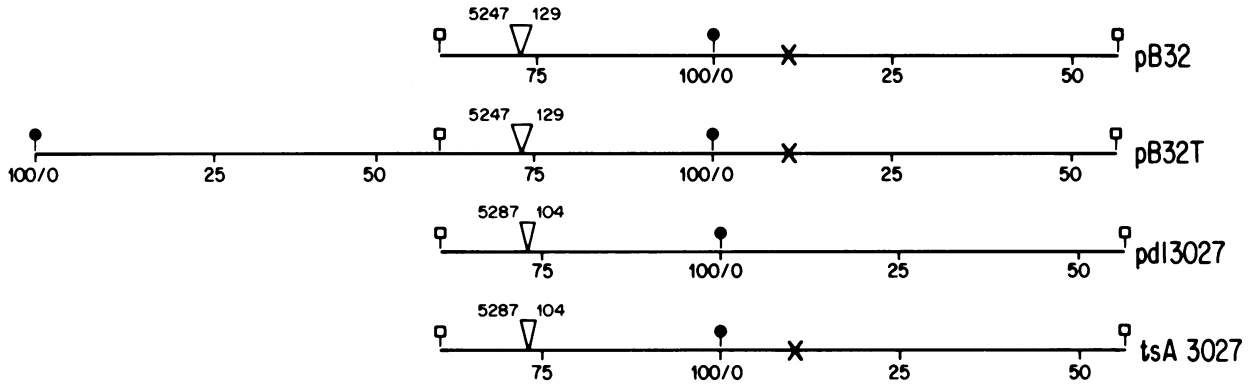

FIG. 1. Maps of mutant Py DNAs. The viral genomes of pB32, pdl3027, and *tsa*3027 are represented as linear molecules cut out of the plasmid (pML, pAT153, or pML, respectively) at the Py *Bam*HI site (nucleotide 4632) represented by ⍑ ; pB32T was constructed so as to contain a repeat of the segment of Py DNA from the *Eco*RI site (nucleotide 1560), represented by ⍑ , to the *Bam*HI site and thus is shown as a linear molecule extending from the first Py *Eco*RI site to the repeated *Bam*HI site. These sequences were cloned in pBR322 between the plasmid *Eco*RI and *Bam*Hi sites. The triangles represent deletions of Py DNA which were created by BAL 31 digestion (Dailey and Basilico, in preparation) from the viral DNA *Bgl*I site. The nucleotides comprising the boundaries of the deletions were determined by the sequencing method of Maxam and Gilbert (16). These numbers appear above the triangles and correspond to the numbering system of Soeda et al. (18). Numbers below the linearized DNAs represent Py genomic DNA map units. ×, Position of the *tsa* mutation.

into rat fibroblasts and assayed for their ability to induce colony formation in agar at 33 and 39°C. In these as in similar experiments done in the past (7), we did not compare absolute transformation frequencies directly, since it is well known that the number of variables in experiments of DNA transfection can lead to different transformation frequencies even under apparently identical experimental conditions. Rather, we preferred to compare the transformation frequencies obtained at 39 or 33°C after transfection with *tsa* DNA molecules, since this procedure allows an internal control of the results obtained. Table 1 shows that wt Py DNA transformed cells somewhat more efficiently at 33°C than at 39°C due to a temperature effect on colony formation previously observed (7). pdl3027, which is origin defective but encodes a wt large T-Ag, transformed much less than native wt Py and showed no increase at 33°C. *tsa* Py DNA and *tsa*-pML, as expected, produced transformants much more efficiently (about 50- and 10-fold, respectively) at the permissive temperature than at 39°C. Significantly, however, both pB32 and *tsa*3027 (both *tsa*) showed a mere twofold increase in colony formation at 33°C compared with 39°C, when the data are corrected for the temperature effect on colony formation observed with wt DNA.

TABLE 1. Transforming ability of origin-defective Py mutants in F2408 rat cells[a]

DNA	Transformation[b] at		39°C/33°C ratio (%)	39°C/33°C ratio corrected for wt (%)
	39°C	33°C		
Py wt native	3,770	7,700	49.0	100
Py *tsa* native	20	2,380	0.84	1.71
Py *tsa*-pML	65	1,270	5.12	10.4
pB32	132	530	24.9	50.8
*tsa*3027	130	490	26.5	54.1
pdl3027	300	550	54.5	111.2

[a] F2408 rat cells were plated at 10^6 per plate 1 day before transfection. The cells were transfected by the $CaPO_4$ method (10, 22) with 1 μg of each indicated DNA plus 19 μg of F2408 carrier DNA per 100-mm dish for 16 h at 33°C. At 20 h after the addition of the DNAs, the cells were plated in soft agar (10^5 per plate) at either 33 or 39°C. The number of colonies was counted 15 to 22 days later. The recombinant plasmids used (all cloned at the *Bam*HI site) are as diagrammed in Fig. 1. pB32 and 3027 (21) are two different origin-defective mutants of Py DNA. pB32 and *tsa*3027 produce a *ts* large T-Ag, whereas pdl3027 produces a wt large T-Ag.

[b] Colonies per 10^6 cells.

These results clearly show that in the absence of viral DNA replication, the ability of Py DNA to initiate transformation is reduced and is no longer affected by the functional state of the large-T protein. This is shown by the facts that pdl3027 transforms F2408 much less efficiently at either temperature than does wt DNA and that the origin-minus *tsa* DNAs pB32 and *tsa*3027 do not exhibit a striking difference in their transforming abilities under conditions when T-Ag is functional as compared with when T-Ag is not functional. Thus the main role that large T-Ag plays in the initiation of transformation must be dependent on viral DNA replication.

We then isolated rat cells transformed by either pdl3027, pB32, or native wt or *tsa* Py DNA and analyzed the viral DNA integration pattern by restriction enzyme digestion of high-molecular-weight DNA and Southern (19) blot hybridization. Tandem integrations were found most consistently only in cells transformed by replication-competent molecules (*tsa* DNA at 33°C or wt DNA), but not in cells transformed by replication-defective DNA (pB32 or pdl3027) even when transfected under conditions permissive for large T-Ag function (Table 2). Concordant results were obtained with cells transfected with a plasmid containing a partial tandem of the pB32 Py molecule created in vitro. In this case, tandem insertion was found in most cases, but the tandems were never longer (and usually shorter) than the transfected DNA molecule. Figure 2 shows some representative results of the restriction enzyme analysis of integrated DNA sequences in these transformed cell lines. The *tsa*B5 line had been transfected with pB32 DNA. Its integrated viral DNA pattern is representative of cell lines transformed by origin-minus DNA in that the integrated DNA sequences are not arranged in tandem. Despite three independent insertions of viral DNA, as shown by digestion with *Bgl*II, an enzyme which does not cleave Py DNA, cutting with *Bcl*I and *Eco*RI (each of which cleaves Py DNA once) did not produce viral DNA bands (lanes B) migrating as the Py DNA markers. Similarly, *Hin*dIII, which cleaves Py

TABLE 2. Frequency of tandem and single-copy integrations in rat cells transformed by origin-defective Py DNAs[a]

Transforming DNA	No. of clones analyzed	No. of clones with tandem integration	No. of clones with integration of less than a single copy
pdl3027 at 37°C	3	1	2
pB32 at 33°C	5	0	5
pB32T at 33°C	6	0	6
tsa at 33°C or wt at 37°C	10	7	3

[a] One microgram of each indicated DNA was used in the transfections as described in Table 1, footnote *a*. pB32T had been cleaved with *Bgl*I and the other DNAs had been cleaved with *Bam*HI to remove plasmid DNA. Colonies were picked from either 33 or 37°C as indicated. High-molecular-weight DNA was extracted from those transformants exhibiting large T-Ag by immunofluorescence and subjected to digestion by a number of restriction enzymes as shown in Fig. 2 and done previously (5, 7) to determine the presence of tandem viral DNA insertions. The cell lines transformed by pB32T, which consists of a tandem of Py DNA constructed in vitro (Fig. 1), are considered here as having no tandems since the viral insertions never contained a DNA molecule larger than the input partial tandem.

DNA twice, produced only the 2.3-kilobase-pair Py fragment. This is in contrast to the viral DNA pattern of cell lines such as *tsa*D which had been transfected with *tsa* Py DNA. In this case the presence of a tandem of integrated viral DNA molecules is clearly indicated by the appearance of the Py DNA monomer after *Eco*RI and *Bgl*I (another single-cut enzyme) digestion as well as by the two Py *Hin*dIII fragments which migrate as the marker.

Taken together with the first set of results, we can conclude the following. (i) Py large T-Ag increases the frequency of viral transformation by a mechanism which involves replication of the viral DNA and not simply recombination. (ii) Because only replication-competent viral molecules could generate tandem integration and since previous results (6) have indicated that the tandem array of Py DNA is probably formed before or during the initial integration event, the data presented here support the hypothesis (4, 7) that replication results in the formation of oligomers of viral DNA molecules (possibly by a rolling-circle mechanism) which are then integrated into the host DNA. Our results cannot rule out the possibility that oligomers of viral DNA are formed by recombination, but clearly, if this is the case, recombination must be dependent on viral DNA replication. As discussed previously (7), we do not know why tandem formation should result in a greatly increased transformation frequency; one possible explanation is that oligomers not only preserve the integrity of the viral transforming region upon integration, but also may actually have a higher probability of recombining with host DNA than do single-copy molecules.

Lastly, the slight but reproducible increase in transformation efficiency of the two origin-minus *tsa* mutants pB32 and *tsa*3027 at 33°C (Table 1) may indicate another minor role of large T-Ag in transformation initiation. This might include, for example, some way in which large T-Ag might interact with or activate host proteins or promote recombination processes. However, the major role of T-Ag in efficient transformation initiation of continuous cells lines by Py appears to be its replicative one.

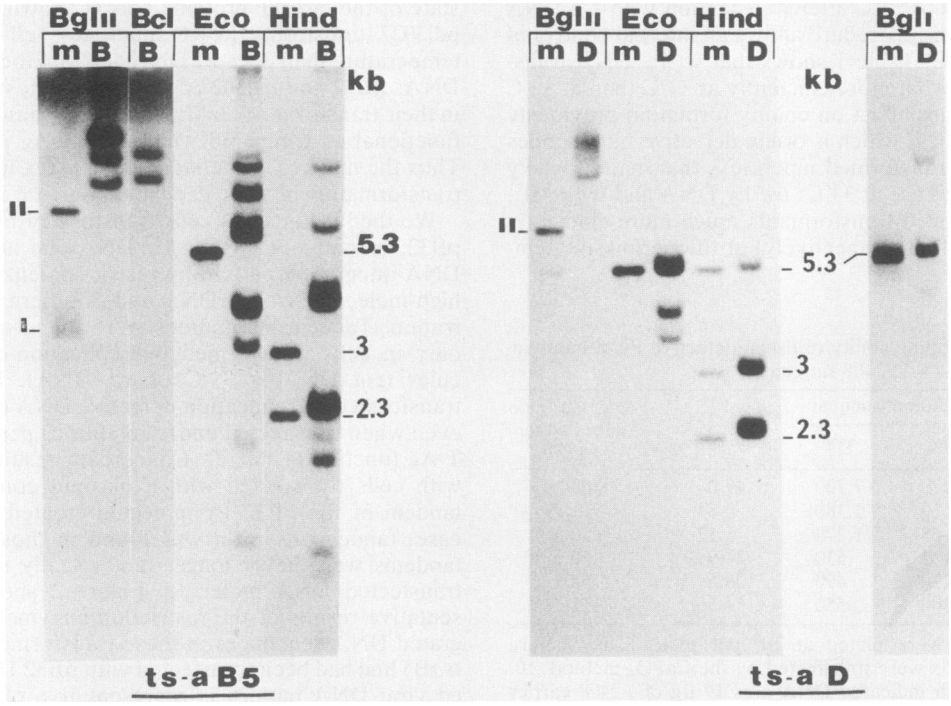

FIG. 2. Southern blot analysis of cell lines transformed with either origin-minus or origin-containing *tsa* Py DNA (*tsa*B5 and *tsa*D, respectively). F2408 cells were transfected with 1 μg of viral DNA and incubated at 33°C for 20 h. Cells were plated in agar at 33°C for ca. 2 to 3 weeks. Transformed colonies were isolated and grown at 39°C. The high-molecular-weight DNA of these cell lines was extracted, cleaved with restriction enzymes, and run on agarose gels. Southern blotting was performed as described previously (7, 19), and the Py DNA-containing bands were visualized by using a ^{32}P-labeled Py DNA probe. kb, Kilobase pairs of DNA; I and II, forms I and II, respectively, of Py DNA; Bcl, *Bcl*I; Eco, *Eco*RI; Hind, *Hin*dIII; m, Py DNA markers; B, high-molecular-weight DNA from *tsa*B5; D, high-molecular-weight DNA from *tsa*D.

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