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Resolution of a Polyomavirus-Mouse Hybrid Replicon: Release of Genomic Viral DNA

ALAIN PICHÉ AND PIERRE BOURGAUX*

Département de microbiologie, Faculté de médecine, Université de Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4

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RmI is a circular chimera containing 1.03 copies of polyomavirus DNA and 1,628 base pairs of mouse DNA, joined through direct and inverted repeat sequences. It is excised from the chromosome of a transformed cell via a site-specific recombination event that is dependent on the activation of the viral gene coding for large T antigen. RmI is shown here to be highly infectious for normal mouse cells. This infectivity reflects the ability of RmI to effectively yield unit-length viral DNA via intramolecular recombination. The effectiveness with which infectious viral DNA is produced from RmI is consistent with the idea that the underlying recombination event is site specific, rather than homologous or illegitimate.

Rodent cells transformed by simian virus 40 (SV40) or polyomavirus (Py) carry in their chromosomes viral DNA sequences which sometimes can be induced to excise (1–3). Such excision generally involves the expression of a viral function, presumably carried by the large T antigen polypeptide, and of one of several cellular functions constitutively expressed in permissive cells (1–3). While illegitimate, homologous, and site-specific recombination events have been invoked successively to account for it, the overall mechanism underlying excision is still largely unknown (1–3, 9, 13, 26).

We are currently studying the excision of Py DNA in mouse cells transformed at 39°C by the *ts*P155 mutant of Py, which codes for a thermosensitive large T antigen (4, 8). Transfer of these cells—designated Cyp—from 39 to 33°C results in the intracellular accumulation of low-molecular-weight DNA, including the viral origin (Ori), that is excised from the chromosome (7, 12, 26). In most Cyp cells, this DNA consists predominantly of unit-length *ts*P155 DNA, or P155 (7, 25). In one clone (C12/a1) however, a larger molecule (RmI) is systematically produced in a 20:1 ratio to P155 (25, 26). RmI is a chimera made of 1.03 copies of P155 linked to a single insertion of 1,628 base pairs (bp) of mouse DNA (Ins). Ins terminates with an imperfect inverted repeat of 7 bp and is flanked by a direct viral repeat of 182 bp, or 0.03 copies, of viral DNA (5, 24). RmI, as well as RmII, another chimera excised in a subclone from C12/a1 cells, appears to be excised from the chromosome via a site-specific recombination event (6, 12, 26).

The production of both RmI and P155 after transfer of C12/a1 cells to 33°C leads to two alternative interpretations about the generation of these molecules. Either the two species are independently excised from the chromosome (24), or RmI is first excised and then converted into P155 via intramolecular recombination. Because RmI is a molecule structurally similar to a cointegrate and is generated by site-specific recombination, the second interpretation appears particularly attractive. We report here that RmI not only replicates but also yields P155 when transfected into permissive mouse cells maintained at 33°C. Our data indicate that P155 is more effectively generated from RmI than would be expected from an intramolecular homologous recombination event occurring in a molecule with an internal homology of 182 bp. As with the production of RmI, the recombination event responsible for the resolution of RmI may be site specific.

(This work will be included in a thesis to be submitted by Alain Piché in partial fulfillment of the requirements for the M.Sc. in Microbiology from the Université de Sherbrooke.)

MATERIALS AND METHODS

Cells and viruses. We have described elsewhere the origin of the mouse 3T6 cells and *ts*P155 mutant virus used in this study, as well as the methods used to extract, purify, and characterize low-molecular-weight (MW) DNA (4, 5, 25).

DNA transfections. The assays were performed with DEAE-dextran of MW 500,000 (Pharmacia Fine Chemicals), as described by Sussman and Milman (23). Each petri dish (100 mm in diameter) was seeded with 2×10^6 cells, and the next day 1 μg of DNA was added. The plates were then incubated at 33°C, first for 1 h in 2% CO_2 and then for 3 h in 10% CO_2. After being exposed to 10% dimethyl sulfoxide for 2 min, the cultures were incubated at 33 or 39°C, and at various times thereafter, low-MW DNA was extracted by the method of Hirt (11). For plaque assays, transfections were performed on monolayers (in 60-mm dishes) that were about 70% confluent. After transfection, monolayers were overlaid with agar medium and incubated at 33°C.

Cloning in *Escherichia coli*. The methods used for cloning were those described previously (26). Recombinant plasmid pI-1 was isolated at the same time as RmII was cloned (26). Plasmids pB_1-20, pP155, and pRP155 were produced by digesting DNA from RmI, mutant *ts*P155, and revertant R[*ts*P155]A (7), respectively, with *Sal*I and ligating to *Sal*I-cleaved pAT153.

Inserts were recircularized as follows. Plasmid DNA (10 μg) was digested with *Sal*I, phenol extracted, and precipitated with alcohol. Ligation was then carried out at 16°C for 18 h in 300 μl of a buffered solution containing 0.6 mM ATP, 10 mM $MgCl_2$, 10 mM dithiothreitol, 20 mM Tris hydrochloride (pH 8), and 25 U of T4 DNA ligase. The efficacy of each enzyme reaction was monitored by gel electrophoresis of the DNA.

DNA transfer and hybridization. The procedures used have been described previously (26).

* Corresponding author.

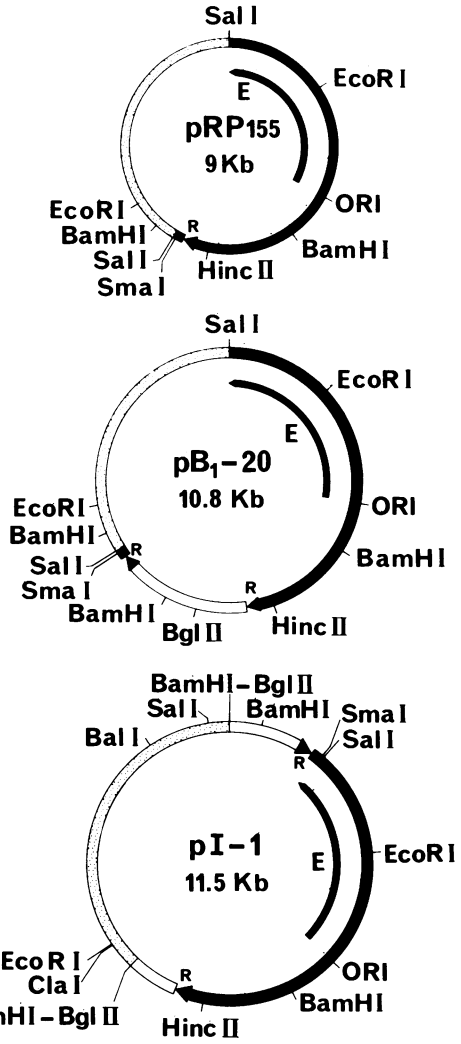

FIG. 1. Physical maps of recombinant plasmids. The vector in these recombinants (stippled area) was either pAT153 (pRP155 and pB1-20) or pBR322 (pI-1). The viral DNA and the cellular insertion (Ins) are shown as black and white areas, respectively. Both P155 and RmI contain single restriction sites for *Sal*I and *Sma*I, located immediately downstream of the polyadenylation signal for the early viral mRNAs (8). All three recombinant DNAs include a continuous Py early coding region (E) and can thus specify a full-size large T antigen polypeptide (27). The stretch of viral DNA from bp 3092 to bp 3273 (27) is shown as an arrowhead (R). It is present once in pRP155 and twice in pB1-20 and pI-1. Kb, Kilobases.

RESULTS

Infectivity of cloned RmI. Most experiments were performed with two recombinant plasmids, pB_1-20 and pI-1 (Fig. 1). To construct the first recombinant, RmI was digested with the enzyme *Sal*I and ligated to *Sal*I-cleaved pAT153 DNA (see Materials and Methods). Thus, the Py coding sequence, specifically that of the major capsid protein VP1 (27), is interrupted twice in pB_1-20, once by the viral duplication and the mouse DNA insert and once by the plasmid DNA. Hence, reconstituting unit-length Py DNA from pB_1-20 would require not one but two crossovers. To construct pI-1, RmI was digested with *Bgl*II, which interrupts Ins, and inserted into the *Bam*HI site of pBR322. In pI-1, therefore, the DNA on both sides of the plasmid DNA is of mouse origin, and the viral DNA is continuous between the two elements of the direct viral repeat, so that a single crossover involving these elements would be expected to regenerate unit-length Py DNA. As a positive control in the infectivity determinations, we used pRP155, whose construction was similar to that of pB_1-20, but whose insert is genomic viral DNA (R[*ts*p155]A) isolated from Cyp cells in which the viral mutation had undergone reversion (7).

We had observed previously that RmI does not get packaged into Py capsids, presumably because of its large size (25). Thus, we knew that the ability of cloned RmI to produce virus plaques in tissue culture would be totally dependent on its ability to recombine into a smaller molecular species encoding all viral functions. Therefore, we were not surprised to observe that both pB_1-20 and pI-1 indeed were infectious, but under different conditions (Table 1). Only when the continuity of the late viral coding region was restored by treating the DNA successively with *Sal*I and ligase was pB_1-20—or pRP155—able to produce plaques on monolayers of mouse 3T6 cells. Under these conditions, the relative infectivity of pB_1-20 was about 10% of that of pRP155. This presumably indicated that only a fraction of the RmI-like DNA reconstituted from pB_1-20, hereafter called RmI_c, had recombined to yield unit-length Py DNA (see next section). In contrast with what had been observed with pB_1-20 and pRP155, the production of plaques by pI-1 required no enzymatic treatment whatsoever and was remarkably effective.

The mere fact that the infectivity of pRP155 and pB_1-20 could not be detected unless the plasmid sequences were excised with *Sal*I suggested to us that illegitimate recombination was occurring too infrequently under our conditions to account for the high relative infectivity of RmI_c and pI-1. Could such infectivity then be accounted for by homologous recombination between the two elements of the 182-bp-long

TABLE 1. Infectivity of cloned DNA at 33°C as a measure of recombination and replication

DNA used in transfection	Digestion prior to transfection	Relative infectivity[a]
pRP155	None	<1
pRP155 + *Sal*I + ligase	None	100
pB_1-20	None	<1
pB_1-20 + *Sal*I + ligase	None	10
(RmI_c)[b]	*Bgl*II	20
	*Sma*I	1
pI-1	None	42
	*Bal*I	21
	*Cla*I	31
	*Bal*I + *Cla*I	15
	*Sma*I	1

[a] Relative infectivity is the ratio of the infectivity of the particular DNA to that of pRP155 treated with *Sal*I and ligase, multiplied by 100. Three successive experiments were performed. In each, three series of three dishes were inoculated with 1, 0.1, or 0.01 μg of DNA per dish. The number of plaques was plotted against the DNA dose, and infectivity (PFU/μg) was calculated from the resulting curves. Since all curves had similar slopes, all data could be readily compiled. Absolute infectivities were usually in the range of 10^3 to 10^4 plaques per μg of DNA for the recircularized insert from pRP155 (relative infectivity, 100). Plaques were countable over a 100-fold range of DNA used, except when relative infectivity was less than 10. In a separate series of experiments, it was verified that under the conditions used here, pP155 (see Materials and Methods) was about as infectious as similarly treated pRP155. For *Sal*I- and ligase-treated pRP155 and pB_1-20, electrophoresis performed after ligation indicated that recircularization of the inserts was over 70% effective.

[b] The recircularized insert from pB_1-20 is designated RmI_c.

viral repeat? Others have shown that recombination between repeated sequences carried on a single plasmid can be enhanced 10-to 100-fold by introducing double-stranded breaks at appropriate positions in the construct prior to its transfection into mammalian cells (14). We thus decided to investigate the effect of various single-cut enzymes on the infectivity of RmI_c and pI-1 (Table 1; see also Fig. 1). Not unexpectedly, we found that interrupting the late viral coding region at the unique *Sma*I site strongly reduced the infectivity of both RmI_c and pI-1. Other enzymes interrupting either the mouse or the plasmid sequences, such as *Bgl*II and *Cla*I, had relatively little effect on the infectivity of these molecules. However, *Bal*I or *Bal*I and *Cla*I in combination clearly reduced the infectivity of pI-1, even though neither enzyme cut in the cloned insert. Such results may indicate that homologous recombination is already too frequent in cyclic RmI_c and pI-1 to be enhanced by linearization or alternatively that the process yielding infectious viral DNA from these molecules is not homologous recombination.

Production of P155 from RmI_c and pI-1. Whatever the mechanism responsible for the generation of infectious DNA from cloned RmI, this mechanism did not appear to be strongly dependent on the ability of the transfected DNA to either replicate or accumulate prior to recombination. Indeed, pI-1 is a molecule which, unlike RmI_c, carries plasmid sequences inhibitory for replication (15) and yet is quite effective in initiating infection (Table 1). However, our experiments thus far provided no proof that RmI_c could accumulate more effectively than pI-1 after transfection of tissue culture cells. To test this assumption, and hopefully to identify some of the recombination products, we decided to follow the fate of transfected cloned RmI.

Mouse 3T6 cells were exposed to undigested pI-1 or RmI_c and incubated at 33 or 39°C for various lengths of time. Low-MW DNA was then extracted by the Hirt method (11), purified, electrophoresed, and blotted onto nitrocellulose sheets prior to hybridization with a viral DNA probe (21). Autoradiography of the blots indicated that material migrating as RmI accumulated in cells incubated at 33°C following transfection with RmI_c (Fig. 2, left). This finding suggested that RmI could function as a replicon not only in Cyp cells (7), but also in normal mouse cells. Eight days after transfection, material migrating as P155 became detectable in addition to RmI (Fig. 2, left). As input DNA had long since disappeared by that time (see below), we felt that such unit-length viral DNA was more likely to have arisen from RmI than from RmI_c. However, the late appearance of P155 following transfection with RmI_c was not to be taken as indicating that recombination was dependent on replication. The results observed after transfection with pI-1 suggested that, quite to the contrary, recombination was prompt and effective when replication was minimal (Fig. 2, right). In that instance, P155 was readily detectable from day 6 after transfection and from then on was more abundant than its precursor. Replication of the precursor was not completely abolished, however. All of the DNA detected after 6 days or more of incubation, including the small amounts of DNA migrating as pI-1 (Fig. 2, right), was *Dpn*I-resistant and had thus replicated after transfection (not shown). This observation indicated that although it carried the "poisonous" sequences of pBR322 (15), pI-1 could be amplified to a limited extent in tissue culture cells.

The striking observation, illustrated by Fig. 2, was that pI-1 is far more readily converted into P155 than is RmI_c. One explanation for this observation would be that compared with RmI_c, pI-1 is not only a poorer substrate for replication but also, by coincidence, a better substrate for recombination. Alternatively, pI-1 could be more likely to recombine than RmI precisely because it is less able to replicate. Such would be the case if recombination depended on a limiting factor also needed for replication (18). In either case, the data clearly established that maximal accumulation of P155 was compatible with minimal accumulation of its precursor, a finding that proved to be important for understanding the recombination event under study (18).

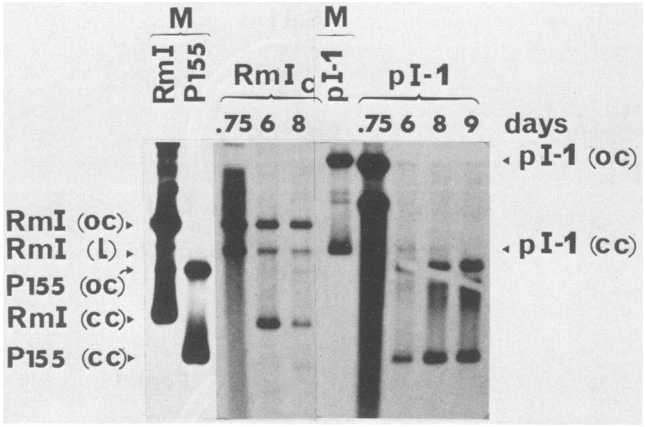

FIG. 2. Replication and recombination after transfection with RmI_c or pI-1. Monolayer cultures of mouse 3T6 cells were transfected by the DEAE-dextran procedure and incubated at 33°C for 0.75, 6, 8, or 9 days. After cell lysis, the low-MW DNA was electrophoresed for 16 h at 40 V through a 1% agarose slab gel. Following blotting and annealing with a radioactive Py DNA probe, autoradiography was performed for 5 days. Marker DNAs (lanes M) originated from either cultured mouse cells (RmI and P155) or *E. coli* (pI-1). The positions of the covalently closed circular (CC), open circular (OC), and linear (L) forms of RmI, P155, and pI-1 are indicated where appropriate. Scanning of the autoradiogram indicated that the samples collected 6 days after transfection with RmI_c and 9 days after transfection with pI-1 contained approximately equal amounts of DNA annealing with the probe. The DNA recovered 0.75 days after transfection, but not that recovered at 6, 8, or 9 days, was found to be *Dpn*I sensitive, as expected for input DNA (not shown). Note that material migrating faster than pI-1 itself was not detectable in the stock of plasmid DNA used as a marker: this is not surprising because, if it were generated by homologous recombination from pI-1, P155 could not replicate in *E. coli*. Annealing of a similar blot with a cellular DNA probe specific for Ins (18) indicated that if formed, IR, the reciprocal of P155 (see Fig. 3), does not accumulate detectably after transfection with RmI_c (not shown).

It should be pointed out here that although blots cannot be compared easily with plaque assays, the two sets of results reported above seem entirely consistent. At first, RmI_c, whose relative infectivity was only four times lower than that of pI-1 (Table 1), may appear comparatively ineffective in producing P155 (Fig. 2). However, the replication of RmI obviously delays the appearance of unit-length viral DNA and possibly reduces the yield of the first virus cycle. Yet, this could have little or no influence on the number of plaques eventually produced.

DISCUSSION

We have shown previously that RmI is not only the result of a site-specific excision event dependent on large T antigen, but also a molecule with several structural similarities to the cointegrates of bacterial transposons. In this report, we show that in permissive mouse cells, RmI is effectively

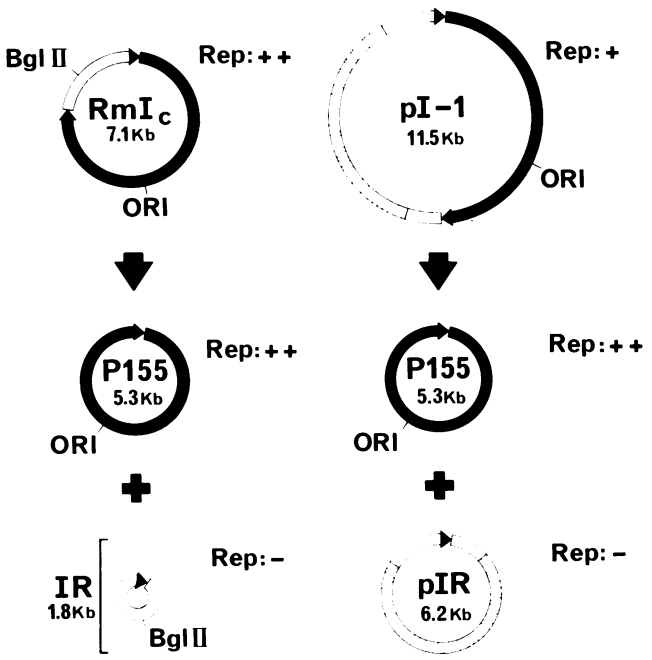

FIG. 3. Structure of RmI_c, pI-1, and their prospective recombination products. (Left) Recombination occurring in RmI_c is expected to generate P155 and possibly a small molecule comprising Ins and one element of the 182-bp viral repeat (IR). Of these three molecular species, RmI_c and P155 are expected to replicate extensively (Rep ++), whereas IR is not (Rep −). (Right) Because pI-1 was not found to replicate to a great extent (Rep +; see Fig. 2), pIR is expected to replicate even less (Rep −). Kb, Kilobases. See the legend to Fig. 1 for symbols.

converted into unit-length Py DNA detectable by its ability to form virus plaques. Besides explaining why C12/a1 cells generally produce P155 in a constant ratio to RmI (25, 26), this finding clearly raises the question of the recombination mechanism underlying such conversion. Usually, one distinguishes three types of recombination mechanisms: general or homologous recombination, nonhomologous or illegitimate recombination, and site-specific recombination. While the requirements for nonhomologous recombination are largely unknown, general recombination has been shown to display a marked dependence on homology, becoming difficult to detect when the two parent DNAs have less than a few hundred base pairs in common. Site-specific recombination generally requires the activity of one or more specific functions encoded by genes adjacent to the recombining sites, while these sites often display sequence homology (16). In view of these peculiarities, how can the mechanism responsible for the conversion of RmI into P155 be envisaged?

Subramani and Berg (22) have calculated from the infectivity of various SV40-pBR322 cyclic constructs the frequency of both homologous and nonhomologous intramolecular recombination events in transfected simian cells. From their results, it would appear that conversion of RmI into P155 occurs via a process far more effective and specific than nonhomologous recombination. This is clear not only from the infectivity of both pI-1 and RmI_c, but also from the apparent homogeneity of the product of recombination (Fig. 2). Although Subramani and Berg (22) generally observed higher frequencies for homologous than for nonhomologous recombination, it should be noted that most of the constructs used to detect the former included very large duplications. One of the constructs however, pBSVD237, was somewhat similar to pI-1, as it included a complete SV40 genome bracketed by a direct nontandem duplication of 237 bp. The specific infectivity of pBSVD237 was only 0.3% of that of wild-type SV40 DNA. Subsequently, Rubnitz and Subramani (20) showed that reducing the homology from 237 to 163 bp brought this figure down to less than 0.04%. Even if we cannot make accurate comparisons between these figures and ours, it seems obvious to us that the conversion of RmI into P155 which we observed is too effective to simply reflect homologous recombination between the two identical sets of 182 bp present in the chimera.

Linearization of the DNA was found to have a depressing rather than enhancing effect on the infectivity of the transfected DNA. This observation, while hardly surprising in view of the high infectivity of the circular DNA, may actually indicate that the recombination event under study is dependent on the DNA's assuming a circular or even supercoiled configuration, as is the case in site-specific recombination (10, 16, 28). Going back to our analogy with cointegrates, resolution of RmI via site-specific recombination could then be a conservative process yielding not one product, but two reciprocal products (16, 19) (Fig. 3). Production of either reciprocal of P155, IR or pIR, from RmI_c and pI-1, respectively, could not be detected with a probe specific for Ins (Fig. 2). This could have been because Ins lacks an origin, ensuring adequate amplification of such products. Pushing the analogy with cointegrates even further, site-specific resolution of RmI would also be expected to be dependent on the expression of a function encoded by this chimera (16). In the accompanying paper (18), we report the results of experiments, initially undertaken with the aim of assessing whether the recombination of RmI is reciprocal, which eventually demonstrated that this recombination is dependent on the supply of a factor encoded by RmI itself.

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