MOLECULAR AND CELLULAR BIOLOGY, Oct. 1985, p. 2608–2612
0270-7306/85/102608-05$02.00/0

Vol. 5, No. 10

Alternative Excision Products Originating from a Single Integration of Polyomavirus DNA

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Received 22 April 1985/Accepted 10 July 1985

The Cyp cell line consists of mouse cells transformed by a thermosensitive polyomavirus (Py) genome and routinely propagated at 39°C. Cyp cells are readily induced to synthesize free Py DNA by being transferred to 33°C. In one subclone (C12/a1/S48, or S48) of this line, such induction resulted in the intracellular accumulation of three discrete species of cyclic DNA, i.e., genomic Py DNA, RmI, and RmII. RmI and RmII are Py-mouse chimeras, each of which contains a distinct set of sequences originating from the site of integration. Conceivably, genomic Py DNA, RmI, and RmII could persist at 39°C as free replicating plasmids or originate from distinct populations of cells in S48 cultures. The data indicated that all three species arise at 33°C from a genetically homogeneous cell population in which neither RmI nor RmII replicates at 39°C. Examination of the sequence at the viral-cellular junction unique to RmII indicated that this chimera is excised from the host chromosome through a recombination event involving a complex viral sequence and a simple cellular sequence. Therefore, RmII provides another example of precise recombination occurring between nonhomologous sequences in a mammalian cell, as already observed for RmI (B. S. Sylla, D. Huberdeau, D. Bourgaux-Ramoisy, and P. Bourgaux, Cell 37:661–667, 1984).

The Cyp cell line resulted from the immortalization of mouse embryo cells by the *ts*P155 mutant of polyomavirus (Py) (2). Cyp cell cultures are routinely propagated at 39°C and undergo a massive cytopathic effect within 48 h of being transferred to 33°C (2). After the temperature downshift, free viral DNA accumulates from often less than 0.1 copy to as many as 10^4 copies per cell (6). This free viral DNA has been analyzed in several clones, all derived from the same Cyp cell line. In most instances, it has been found to consist almost exclusively of genome-sized molecules of *ts*P155 DNA (P155) (6, 18). In one derivative (C12/a1) of clone C12, however, a cyclic molecule called RmI is also produced at 33°C, generally in a 20:1 ratio to P155 (18). RmI is a hybrid made of 1.03 copies of viral DNA and a 1,628-base-pair (bp) segment of mouse DNA called Ins (3, 17). Ins originates from sequences flanking the left end of the integrated viral DNA (4), which consists of a little over two copies of Py DNA arranged in a head-to-tail tandem (3, 4). RmI is the product of a recombination event occurring between the integrated viral DNA and adjacent nonhomologous cellular sequences (19).

Recloning of the C12/a1 cells yielded subclones which were all able to produce RmI and P155 after a temperature downshift (Fig. 1A). It also led to the isolation of one subclone (S48) whose induction at 33°C generated three, instead of two, species of low-molecular-weight DNA: P155, RmI, and RmII (Fig. 1B). The simultaneous production of P155, RmI, and RmII by induced S48 cells raises several questions. Firstly, do any of these molecules behave in Cyp cells as plasmids which, while maintained at a low copy number at 39°C, are readily amplified at 33°C? Secondly, is RmII produced by cells which are also capable of yielding P155 and RmI? Thirdly, what is the mechanism underlying the excision of RmII? We report here the results of experiments undertaken to examine these questions. Our data indicate that (i) P155, RmI, and RmII do not replicate at 39°C in S48 cells; (ii) most S48 cells are endowed with the ability to produce all three molecular forms; and (iii) when RmII is excised, a complex viral sequence is joined to a monotonous cellular sequence. Therefore, RmI and RmII are the products of precise, and presumably alternative, recombination events occurring between nonhomologous sequences.

(Part of this work will be included in a thesis submitted by D.H. in partial fulfillment of the requirements for an M.S. degree in Microbiology from the Université de Sherbrooke.)

MATERIALS AND METHODS

Cells and viruses. The origin of the Cyp cells and of the viruses used in this study, as well as their propagation, have already been described in detail elsewhere (2, 18). C12/a1 cells were recloned by plating 10 to 100 cells per 60-mm plastic petri dish in liquid medium and incubating the plates for 10 to 15 days at 39°C. Only those dishes containing five colonies or fewer were kept for subclone isolation. The medium was aspirated from the plate, and each colony was separately trypsinized and subcultured. Out of 65 colonies picked, 56 subclones were successfully derived.

The infectivity of DNA was assessed after transfecting Swiss mouse 3T6 cells (kindly provided by Walter Eckhart) by the DEAE-dextran procedure (1).

Isolation of low-molecular-weight DNA. Subconfluent monolayers of C12/a1 cells or their derivatives were transferred from 39 to 33°C. Forty hours later, low-molecular-weight DNA was extracted by the Hirt method (8) and purified by an already described procedure (3) involving dye-buoyant density gradient centrifugation as a final step. Hence, only the covalently closed cyclic DNA was subjected to further characterization.

Endonucleolytic cleavage of DNA and preparative electrophoresis of fragments. Restriction enzymes were purchased

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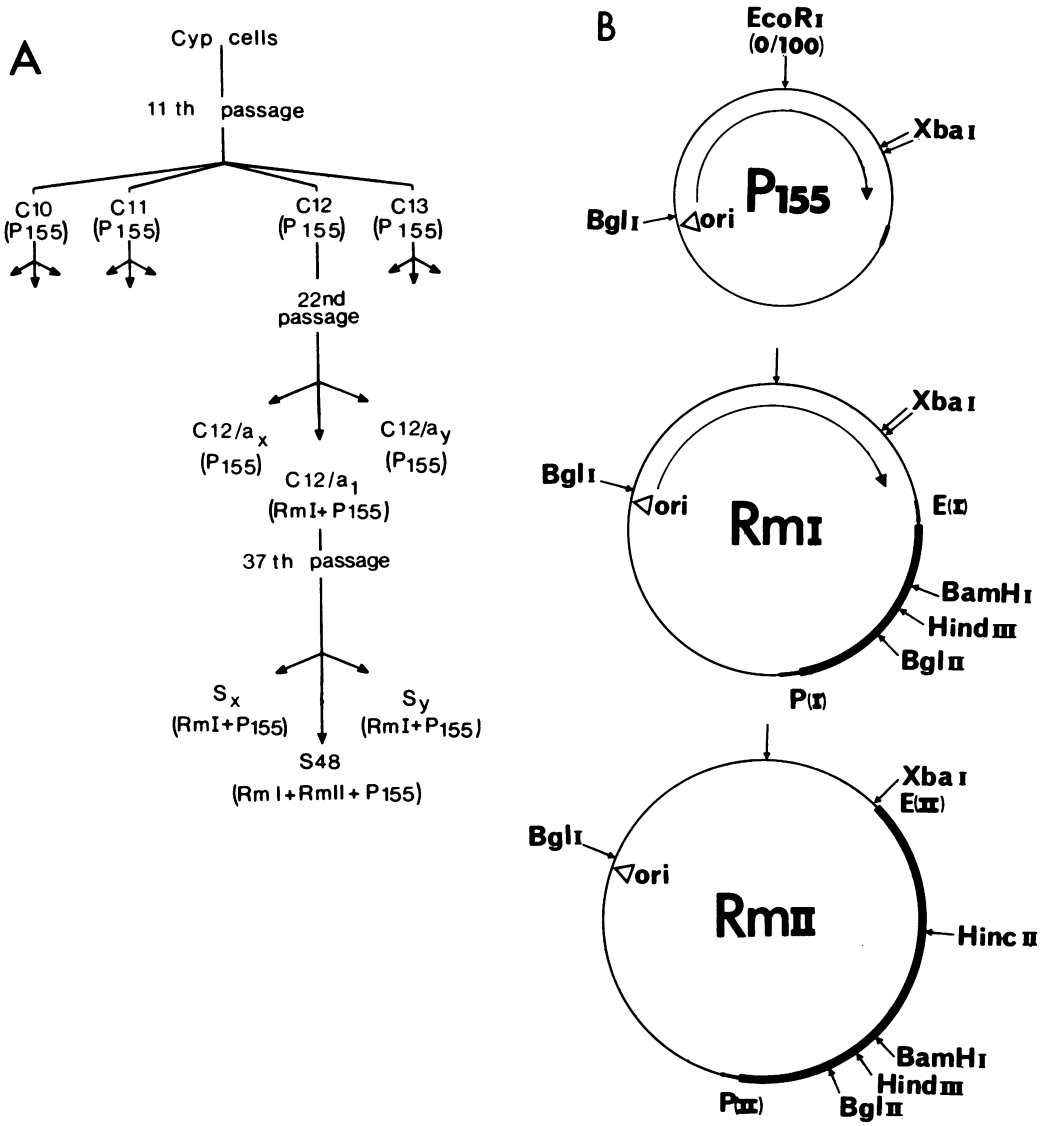

FIG. 1. (A) Pedigree of C12/a1/S48 cells. Cyp cells were derived from a colony of mouse embryo cells immortalized by *ts*P155 (2). Passage 11 of the colony yielded a series of clones (C10, C11, C12, and C13) which all generate genomic viral DNA (P155) upon induction (6, 18). Only two subclones of C12 (one of which is C12/a1) produce both Rm1 and P155 (19). A subclone of C12/a1 (C12/a1/S48) produces Rm1, RmII, and P155 (19). (B) Physical maps of P155, RmI, and RmII. All three maps have been aligned at the *Eco*RI site, which defines the 0/100 map unit position on Py DNA (20). When present, the single *Bgl*I and the two *Xba*I sites characteristic of the viral DNA (thin line) have also been positioned. A viral DNA segment base pair (3126 to base pair 3307 [12]) which is found once each in P155 and Rm11 and twice in Rm1 is shown as a hatched box (otherwise, RmI contains one unrearranged copy of P155). Rm11 lacks a portion of the viral DNA which includes one of its two *Xba*I sites (from base pair 2533 to base pair 3125; Fig. 4). The cellular DNA insertion (solid box) in Rm11 is larger than that in RmI by about 1,500 base pairs. The "proviral" junctions P(I) P(II) are identical to one another and to the left junction between mouse chromosomal DNA and integrated viral DNA in Cyp cells (4, 19). The E(I) and E(II) junctions are formed when RmI and RmII are excised (19). ori, Origin of replication.

from New England BioLabs, Inc., or Bethesda Research Laboratories and used as recommended by the supplier. Digested DNA was subjected to electrophoresis through 1.5% low-melting-point agarose (Bethesda Research Laboratories) horizontal slab gels. The desired DNA fragments were then recovered from gel slices as previously described (3). Alternatively, digested DNA was subjected to electrophoresis through regular agarose, and the fragments were electroeluted from the gel slices as described by Chouikh et al. (5).

DNA transfer and hybridization. After electrophoresis (3), DNA was transferred onto nitrocellulose sheets by the procedure of Southern (15), as modified by Jeffreys and Flavell (9).

^{32}P-labeled DNA probes were synthesized by nick translation by a procedure modified from that of Maniatis et al. (10) and described in detail elsewhere (3). Hybridization was performed, as described elsewhere (4, 7).

Recombinant DNA. Cloning in *Escherichia coli* HB101 with pBR322 as the vector was performed as described previously (3, 19).

Sequencing. The method used was that of Maxam and Gilbert (11), and the source of DNA was recombinant pRH-II-1 (19). In this recombinant, the *Hin*dIII fragment of

RmII that includes the E(II) junction is inserted at the unique *Hin*dIII site of pBR322. The sequence across the junction was determined from the viral *Ava*II site at base pair 2426 (12).

RESULTS

Homogeneity of C12/a1/S48 populations. In earlier experiments, we had observed that all cultures obtained after subcloning C12/a1 cells yielded both RmI and P155 upon transfer from 39 to 33°C (3, 19). One subclone (S48), however, generated RmII in addition to RmI and P155 (19). To investigate whether P155, RmI, and RmII are all produced by cells of the same type, we recloned subclone S48 at 39°C and isolated 25 new clones. These clones were examined for the synthesis of low-molecular-weight viral DNA at 39 and 33°C. In all instances, the three molecular forms were simultaneously produced at 33°C, whereas little free viral DNA was synthesized at 39°C (Fig. 2). This suggests that populations of S48 cells are genetically homogeneous and consist of cells which all have the ability to produce P155, RmI, and RmII.

Alternative patterns of low-molecular-weight DNA synthesis in C12/a1/S48 cells. After being transferred to 33°C, S48 cells generally produced P155, RmI, and RmII in the same relative amounts, RmI being the most abundant and P155 being the least abundant (Fig. 2). In cells maintained at 39°C, these free forms were usually too scarce to be detectable (Fig. 2). However, it was still conceivable that all or some of these molecular species were continuously being excised at a low rate at 39°C and simply amplified by autonomous replication at 33°C. The patterns of low-molecular-weight DNA synthesis which we observed on various occasions are inconsistent with this interpretation. In Fig. 3 we demonstrate the nature of the low-molecular-weight viral DNA synthesized in cultures of S48 cells analyzed simultaneously two passages after being thawed but originating from three different stocks of cells. For cells from stock 1 (Fig. 3, lanes A and B), we noted patterns similar to those already demonstrated in Fig.

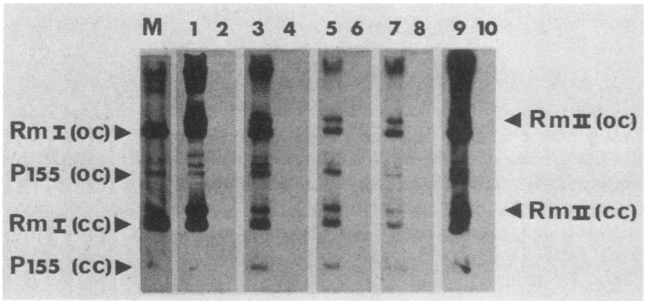

FIG. 2. Low-molecular-weight DNA synthesis in subcloned C12/a1/S48 cells. DNA extracted by the Hirt procedure (8) from monolayer cultures of the C12/a1 clone (lane M) and of five derivatives of the S48 subclone was purified, electrophoresed through a 1% agarose gel, and blotted. An autoradiogram obtained after annealing with ^{32}P-labeled viral DNA and exposing for 1 day is shown. In all instances, the DNA was extracted 40 h after the cells had been first seeded and then incubated at either 39°C (even-numbered lanes) or 33°C (M and odd-numbered lanes). The positions of the bands produced by the covalently closed (CC) and open circular (OC) forms of P155, RmI, and RmII are indicated. Lane M was run with 10^{-2} μg of RmI; all other lanes were run with the DNA from about 10^6 cells. The intensity of the signal in lane 9 is that which one would expect if every cell produced over 10^4 copies of RmI or RmII. Longer exposures of the same blot indicated that cultures maintained at 39°C contained, on the average, less than one copy of free viral DNA (RmI, RmII, or P155) per cell.

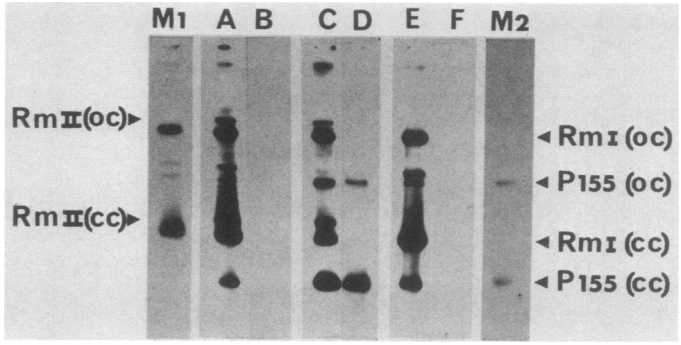

FIG. 3. Alternative patterns of low-molecular-weight DNA synthesis in C12/a1/S48 cells. Samples of frozen cells were thawed, put into cultures and, after two passages, examined for low-molecular-weight DNA synthesis as in Fig. 2. Three stocks of cells frozen at different times, stock 1 (lanes A and B), stock 2 (lanes C and D), and stock 3 (lanes E and F), were thus characterized. The incubation temperature during the 40 h before DNA extraction was either 33°C (lanes A, C, and E) or 39°C (lanes B, D, and F). The autoradiograms shown were obtained after a 2-day (lanes A, C, and E) or a 4-day (lanes B, D, and F) exposure. The positions of the covalently closed (CC) and open circular (OC) forms of P155, RmI, and RmII are indicated. Marker DNAs were extracted from C12/a1 cells (lane M1) and from mouse 3T6 cells productively infected with *ts*P155 virus (lane M2).

2: P155, RmI, and RmII were all produced at 33°C but remained undetectable at 39°C. For cells from stock 2, we again detected all three forms at 33°C, the DNA comigrating with P155 being unusually prominent, however (Fig. 3, lane C); surprisingly, this DNA, but not RmI or RmII, was also produced in large amounts at 39°C (cf. Fig. 3, lanes C and D). We used the DNA analyzed in Fig. 3, lane D, to transfect monolayer cultures of 3T6 cells and scored these cultures for the formation of viral plaques at 33 or 39°C; such experiments indicated that plaque formation by this DNA was not temperature sensitive (data not shown). This result is not surprising, as we have already observed that reversion of the *ts*P155 mutation sometimes occurs at a high frequency in Cyp cells propagated at 39°C (6). What the present experiment shows is that reversion results in the accumulation of essentially one molecular species, genome-sized Py DNA; however, a temperature downshift of the cultures in which reversion is taking place still triggers the production of RmI and RmII. Finally, cells from stock 3 also showed an unexpected pattern of DNA synthesis: although P155 and RmI were clearly produced, RmII remained virtually undetectable at 33°C (Fig. 3, lane E). Results similar to those shown in Fig. 3 were regularly obtained when cells from the same stocks were examined within two passages after being thawed. After four to six passages at 39°C, cells from stocks 2 and 3 both drifted back to patterns such as those shown for cells from stock 1. Altogether, these findings indicate that the kind of free viral DNA synthesized by S48 cells depends on how and when the restriction imposed by the temperature-sensitive viral mutation is removed. Therefore, induction by a temperature downshift is unlikely to cause the accumulation of RmI, RmII, and P155 by simply promoting the accumulation of preexisting free copies of these molecules.

Sequence across the E(II) junction. It is obvious from Fig. 2 and 3 that RmII consists of a seemingly homogeneous population of cyclic molecules generally produced in S48 cells in only slightly smaller amounts than RmI. As we had already assumed that site-specific recombination accounted

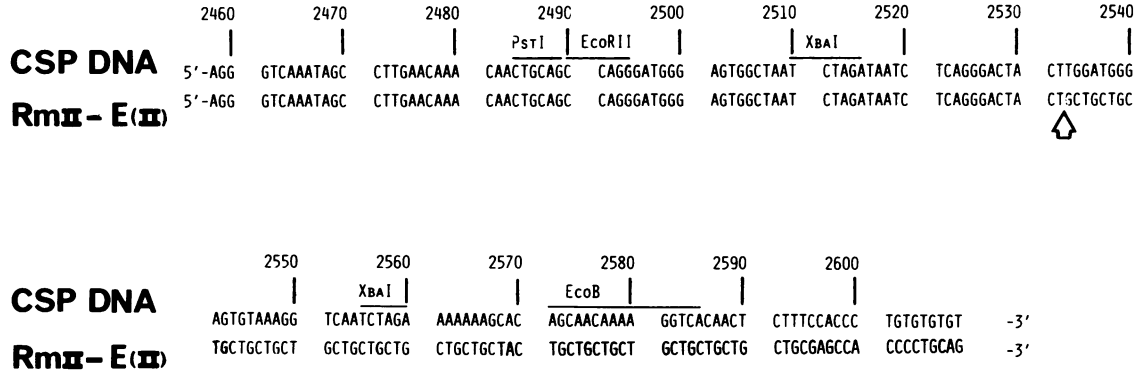

FIG. 4. E(II) junction in RmII. The top line shows part of the sequence of the early strand (20) of the DNA of the Crawford small-plaque (CSP) variant of Py (12), to which mutant *ts*P155 appears to be closely related (L. Delbecchi and P. Bourgaux, unpublished observations). The bottom line shows the sequence of the strand of RmII which has the same polarity as the viral early strand across the E(II) junction (Fig. 1B). Notice that the two sequences are identical on the 5' side of the arrow and different on the 3' side of the arrow. Coordinates (in base pairs) and restriction enzyme sites refer to the viral DNA (12).

for the excision of RmI (19), our observations on RmII clearly raised the question of the mechanism underlying its production.

In an attempt to clarify this situation, we decided to determine the sequence across the E(II) junction (Fig. 1B), which is formed when RmII is excised (19). A recombinant plasmid containing this junction had already been obtained by transformation of *E. coli* and subjected to physical mapping (19). This recombinant DNA was further analyzed by the method of Maxam and Gilbert (11). Thus, we found the E(II) junction to represent a sharp transition from a typically viral sequence to a monotonous cellular sequence in which the triplet 5'-CTG-3' was repeated 21 times (Fig. 4). This finding may or may not indicate that the recombination event giving rise to RmII is driven, at least in part, by a homology between a CT dinucleotide in the integrated viral DNA and one of many, almost adjacent, such doublets in the flanking cellular DNA. Because we have sequenced only one E(II) junction at the moment, it is unclear whether the 21 triplets mentioned above are characteristic of RmII or not. As to the position of the junction on the viral DNA, we noted that the viral sequence was interrupted in RmII after the 5'-CT-3' at coordinates 2531 and 2532 on the viral DNA map, very near the *Xba*I site at coordinate 2510 (Fig. 4). Recent data indicate that this site is invariably retained in RmII (data not shown).

DISCUSSION

The synthesis of cyclic DNA molecules induced after a temperature downshift of C12/a1 or S48 cell cultures is remarkable in several respects (3, 18, 19). For instance, the predominant molecular species produced are Py-mouse chimeras (3, 19), and the viral structure integrated into the host chromosome is made up of a little over two unrearranged copies of Py DNA (4). We have shown previously that the excision of RmI requires the recombination of essentially nonhomologous viral and cellular sequences (19). This conclusion also applies to RmII, in view of the results described here (Fig. 4) and of others yet to be reported (manuscript in preparation). Indeed, characterization of the DNA flanking the integrated viral structure in the Cyp cell chromosome indicates that the cellular sequence which participates in the formation of the E(II) junction includes a stretch of 50 rather than 20 5'-CTG-3' triplets (J. Ash and P. Chartrand, personal communication). One interpretation of the dominance of RmI and RmII over P155 in induced S48 cells would be that the excision of integrated Py DNA occurs more readily via nonhomologous recombination than via homologous recombination. This interpretation, however, offers no direct explanation for the apparent homogeneity of RmI and RmII populations. Such homogeneity could then be accounted for in one of two ways. RmI and RmII could be "plasmids" which are maintained at a low copy number at 39°C and are simply amplified after being transferred to 33°C. Alternatively, RmI and RmII could be produced by recombination events triggered by a temperature downshift. Given the effectiveness of induction and the apparent homogeneity of RmI and RmII populations, excision would then result from a site-specific recombination event.

Several findings reported here are inconsistent with the "plasmid hypothesis." Firstly, we have not succeeded in isolating subclones of S48 cells unable to produce either RmI or RmII, even though S48 cells carry less than one copy of either molecule at 39°C. Secondly, we have noted that RmII sometimes is not produced at 33°C, although it again becomes detectable in subsequent passages of the same cells. Finally, we have observed a relationship between the nature of the free viral DNA and the conditions responsible for its accumulation. When high-frequency reversion occurs at 39°C, genome-sized Py DNA is the species predominantly found in S48 cells. Although it is too early to interpret this quite reproducible finding, the most trivial interpretation can already be excluded. The culture medium used to grow S48 cells contains anti-Py serum, which we know to be very effective in suppressing the spreading of viral infections (2). Thus, the accumulation of genome-sized revertant Py DNA which we have noted is not the result of a chronic infection. If not magnified by a process of reinfection, then the reversion shown in Fig. 4 is also remarkable for its high frequency. This may well be another manifestation of the high "instability" of the Cyp cell chromosome at the viral integration site (Fig. 1A): C12/a1, which produces P155 and RmI, originates from C12, in which only P155 is produced; C12/a1 in turn gives rise to S48, which yields P155, RmI, and RmII. These features of the Cyp system are reminiscent of those of some mouse cells that have been transformed by simian virus 40 and that show a very high rate of genomic rearrangements at the integration site (13).

The recombination event generating RmII involves a complex viral sequence and a monotonous cellular sequence. From our results, it would appear that viral and cellular DNAs are joined at a site at which homology is

limited to two nucleotides, 5′-CT-3′. Homologies involving few nucleotides are often found between sites which are joined during recombination of mammalian DNA (see reference 9 for a review). However, the significance of the homology at the junction site is particularly unclear in this instance. Indeed, the repeat in the mouse DNA does not consist of 5′-CT-3′ doublets but of 5′-CTG-3′ triplets, and such a triplet is present in the viral DNA within the *Pst*I site at coordinate 2484. Joining the two DNAs at this site would eliminate a viral *Xba*I site which is retained by all RmII molecules (see Results and Fig. 4). These considerations suggest that the recombination event which generates RmII is not one which depends on the extent of homology at the joining site. Such a conclusion would also apply to the recombination event which generates RmI (19). Interestingly, the recombination of viral DNA with monotonous-sequence cellular DNA has already been described in the context of simian virus 40 DNA integration (16). In that particular instance, it was hypothesized that simple-sequence DNA may be a target for specialized enzymes involved in gene conversion (14, 16). This interpretation is of special interest to us, as it implies that providing limited sequence homology may not be the main contribution of simple-sequence DNA to RmII excision. Having already been assumed in the case of RmI (19), the participation of specialized recombination enzymes in excision may thus explain why both RmI and RmII are produced in S48 cells.

ACKNOWLEDGMENTS

We are grateful to Robert A. Weinberg, Pierre Chartrand, and Louis Delbecchi for discussions and suggestions. We thank Daniel Gendron for assistance and help with the illustrations.

This work was supported by a grant from the Medical Research Council of Canada.

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