Integration of Polyoma Virus DNA into Chromosomal DNA in Transformed Rat Cells Causes Deletion of Flanking Cell Sequences

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SUMMARY

In order to find out whether polyoma virus (Py) integration into chromosomes causes rearrangements in the cell DNA flanking the integration site, we have mapped the flanking sequences in the inducible LPT line of Py-transformed rat cells and the corresponding sequences in normal rat fibroblasts, and then compared the two maps. To carry out this study we have cloned a segment including Py DNA and flanking sequences in the bacteriophage vector AgtWES and subcloned the flanking cell DNA in a bacterial plasmid. We performed a Southern blot analysis of LPT and rat fibroblast DNA digested with various restriction enzymes and used the cloned flanking cell DNA and Py DNA as hybridization probes. Autoradiography of the LPT DNA blots revealed two sets of fragments. One set includes fragments containing both Py and cell DNA sequences; the second set consists of fragments which contain no virus DNA sequences, and are identical to the fragments observed in the corresponding normal rat DNA digests. These data indicate that LPT cells are heterozygous with respect to the Py inserts. The same data were used to map the flanking sequences in the two types of cells. A comparison between the two maps revealed that a 3.0 kb cell DNA segment, which is located next to the unoccupied integration site in the normal rat chromosomes, has been deleted from the LPT chromosome which carries Py DNA, but not from the LPT chromosome which does not carry the virus DNA. The implications for papovavirus integration are discussed.

INTRODUCTION

In cells which are permanently transformed by the small DNA tumour viruses SV40 and polyoma virus (Py), virus DNA sequences are integrated into chromosomal DNA (Sambrook et al., 1968; Shani et al., 1972; Manor et al., 1973). Studies of the pattern of integration of these viruses revealed the following characteristics. (i) Some SV40- and Py-transformed cell lines contain single virus inserts. Other transformed lines contain ≥2 inserts which map at different chromosomal sites. (ii) In independently derived transformants the cellular and the viral sites of integration map at different positions in the two genomes. No specific viral or cellular sequences occur at the virus–cell junctions. (iii) None of the inserts analysed so far consists of a single unmodified virus genome. Instead, many inserts include deletions or inversions of virus DNA sequences. Partial or complete duplications of the virus genome are also prevalent in SV40 and Py inserts. The duplications are often arranged in a direct tandem repeat (Botchan et al., 1976; Ketner & Kelly, 1976; Birg et al., 1979; Campo et al., 1979; Lania et al., 1979; Della Valle et al., 1981; Clayton & Rigby, 1981; Stringer, 1981).

One important aspect of SV40 and Py integration, which has not been thoroughly investigated as yet, is whether insertion of the virus DNA into chromosomal DNA causes rearrangements of the flanking cell sequences. This question can be examined by comparing the flanking sequences in cell lines transformed by these viruses to the corresponding sequences in the normal cells from which these lines were derived. By making such a comparison, Botchan et al. (1980) have found that in one SV40-transformed rat cell line, which contains a single SV40 insert, the sequences flanking the insert have undergone a rearrangement. They have not determined
whether the rearrangement involved deletion, inversion or translocation. Recently, Stringer (1982) has reported a cellular deletion of at least 3-0 kilobase pairs (kb) at the site of SV40 integration in another SV40-transformed rat cell line. In this article we report an attempt to determine whether Py integration causes structural alterations of cell DNA sequences flanking the integration site. The systems employed for this study were an inducible line of Py-transformed rat cells designated the LPT line, and normal rat fibroblasts.

The pattern of integration of Py DNA in the LPT line is rather unusual (Mendelsohn et al., 1982). The Py inserts in LPT cells include a variable number of whole Py genomes arranged in a direct tandem repeat and hence have variable lengths. However, all inserts map at a single chromosomal site and are therefore flanked by the same cellular DNA sequences. The Py sequences found next to the cell–virus DNA joints are also invariant.

To compare the flanking sequences in LPT cells with the corresponding sequences in normal rat fibroblasts, we carried out a Southern blot analysis of LPT and rat fibroblast DNA digested with various restriction enzymes (Southern, 1975; Botchan et al., 1976). We used as hybridization probes Py DNA and cloned flanking cell DNA. This analysis provided maps of the unoccupied and the occupied integration sites in the two types of cells. A comparison between the two maps revealed that 3-0 kb of rat cell DNA located next to the Py integration site have been deleted in the LPT chromosomes which carry Py DNA. This study also showed that LPT cells are heterozygous with respect to both the virus inserts and the cellular deletion.

METHODS

Cells and virus. A clonal derivative of the LPT line designated clone 1A was used for these studies. The methods employed for propagating this clone were described previously (Manor & Neer, 1975). Growth of polyoma virus and purification of Py DNA were also carried out as described before (Manor et al., 1973; Manor & Neer, 1975). Rat secondary cultures were prepared according to Winocour & Sachs (1960).

Preparation of cell DNA. High molecular weight chromosomal DNA was prepared from LPT cells by a modified Hirt procedure (Hirt, 1967; Mendelsohn et al., 1982). The method of preparation of DNA from rat secondary cultures was also described by Mendelsohn et al. (1982).

Analysis of restriction enzyme digests by the Southern technique. The procedures employed for digestion of DNA with restriction enzymes, electrophoresis in horizontal agarose gels, blotting, DNA-DNA hybridization and autoradiography were described by Mendelsohn et al. (1982). The lengths of the fragments observed in the blots were determined by comparing their electrophoretic mobilities to those of known markers which were electrophoresed in parallel (ibid). The markers used for these experiments were bacteriophage λ DNA cleaved with EcoRI, or with both EcoRI and BamHI, and Py DNA cleaved with either HpaII, or with HincIII, or with EcoRI. DNA hybridization probes were prepared by nick translation (Rigby et al., 1977). The specific radioactivities of the probes were in the range of 2.5 × 10⁶ to 5.0 × 10⁶ ct/min/μg.

Heteroduplex mapping. DNA molecules prepared from phages λpt1 and λpt2 were dissolved in a solution containing 50% formamide, 1 mM-EDTA and 10 mM-PIPES pH 7. The final concentration of each type of DNA was 2 μg/ml. The solution was heated for 3 min at 80°C and quenched in ice. NaCl was then added to a concentration of 0.4 M and the mixture was annealed for 30 min at 37°C. Next, the DNA was spread for microscopy by the cytochrome c method (Davis et al., 1971). The grids were rotary-shadowed with platinum–palladium and were visualized in the JEM-100B electron microscope. Length measurements were carried out as described previously (Zuckermann et al., 1980).

Cloning in the plasmid pBR322. Cloning the 0.90 kb fragment into the EcoRI site of the plasmid pBR322 was carried out as described by Bolivar & Backman (1979).

Construction of a library of LPT cell DNA fragments in bacteriophage λgtWES. High molecular weight chromosomal DNA was prepared from LPT cells by the modified Hirt procedure. It was partially cleaved with the enzyme EcoRI at an enzyme/DNA ratio of 0.12 units/μg DNA for 1 h at 37°C. The DNA was centrifuged for 16 h at 20000 rev/min and 10°C in a 5 to 20% sucrose gradient in the SW27 rotor of the Spinco ultracentrifuge. Fractions sedimenting faster than 20S were collected and pooled. Bacteriophage λgtWES was grown and purified, and the phage DNA was extracted, as described by Enquist et al. (1976). The DNA was cleaved with EcoRI into three fragments (ibid). The two longer fragments which contain the genes needed for lytic growth (the λgtWES arms) were purified by agarose gel electrophoresis. These fragments were ligated with the purified LPT cell DNA fragments and packaged into λ coats according to Enquist & Sternberg (1979).

Isolation of bacteriophage clones containing Py DNA sequences from the LPT cell DNA library. The procedure described by Benton & Davis (1977) was used for screening the in vitro packaged phages for recombinants containing Py DNA sequences. The screening was carried out with no prior amplification of the library. The
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Phages were seeded on a lawn of the *Escherichia coli* strain LE392 in 24 × 24 cm square plates (Nunc). Each plate contained 50,000 to 75,000 plaques. Nitrocellulose filters were brought in direct contact with the phage plaques and the DNA was later denatured, neutralized and fixed on the filters. Plaques that gave positive signals were picked, propagated in *E. coli* LE392 and further analysed.

**RESULTS**

*Isolation and characterization of a recombinant phage containing Py and non-viral DNA from LPT cells*

High molecular weight LPT cell DNA was prepared and partially cleaved with the restriction enzyme *Eco*RI. The fragments were cloned into the bacteriophage vector λgtWES (Enquist *et al.*, 1976; see Methods). The genomic library generated by this procedure was screened by a plaque hybridization assay (Benton & Davis, 1977) for recombinant phage clones containing Py DNA sequences. Three recombinant phages out of 1.5 × 10⁶ that have been screened contained Py DNA sequences and the DNA of one of them, designated λlpt2, was also found to include non-viral sequences (Neer, 1981).

Phage λlpt2 was further characterized as follows: one sample of the phage DNA was partially digested with the enzyme *Eco*RI, and another sample was digested to completion with this enzyme. The digests were analysed by gel electrophoresis. Fig. 1 (track 1) shows the electropherogram of the complete digest. It can be seen that this digest includes two fragments derived from the LPT DNA insert and the two λgtWES arms. The lengths of the LPT DNA fragments are 2.6 kb and 0.9 kb respectively, and the lengths of the λgtWES arms are 21.9 kb and 13.9 kb. Track 3 in Fig. 1 presents a blot of this gel hybridized with 3²P-labelled Py DNA. It can be seen that only the 2.6 kb fragment contains Py DNA sequences. Partial cleavage with *Eco*RI produced two additional fragments of 6.1 kb and 3.5 kb from the cloned DNA (track 2), both of which were found by blot analysis to contain Py DNA sequences (track 4). These results indicate that the length of the cloned segment in phage λlpt2 is 6.1 kb and that it consists of the 0.9 kb fragment inserted between two repeats of the 2.6 kb fragment; only this arrangement can yield a 3.5 kb fragment upon partial digestion. Further support for this presumed structure was obtained by heteroduplex analysis. In this study, phage λlpt2 DNA was annealed with DNA of another recombinant phage clone, designated λlpt1, which contains two whole Py genomes inserted in a head-to-tail configuration between the two λgtWES arms (Neer, 1981). The hybrid molecules were visualized by electron microscopy. Fig. 2(a, b) shows an electron micrograph and a trace of a typical heteroduplex, and the legend to Fig. 2 presents the average lengths of various regions of the heteroduplexes analysed in this study. This analysis has shown that the cloned LPT DNA in phage λlpt2 contains two direct repeats of a continuous 2.15 kb segment of Py DNA (regions D and G in Fig. 2b) which hybridized with the corresponding sequences in phage λlpt1. These two Py repeats are separated by a 1.15 kb segment that remained single-stranded in the heteroduplexes (region F in Fig. 2b), and apparently includes the 0.9 kb segment which was shown in Fig. 1 to consist of non-viral sequences. In addition, there is a shorter region of DNA at one end of the λlpt2 insert (region C in Fig. 2b), which is not homologous to the Py DNA sequences found in the corresponding region of phage λlpt1. The regions B, E and H in Fig. 2(b) are λlpt1 sequences which are absent from λlpt2. The left boundary of the regions B and C, and the right boundary of the regions G and H correspond to the *Eco*RI site on the Py physical map (Griffin *et al.*, 1980). Measurements of distances of the various regions from these points allow mapping of the Py DNA regions D and G.

Fig. 3 shows a physical map of phage λlpt2 based on the electron microscope data and on the *Eco*RI cleavage pattern presented in Fig. 1. The map has been corroborated by other restriction enzyme data presented elsewhere (Neer, 1981). The positions of the fragments generated by the various enzymes are also indicated in Fig. 3. It is to be noted that the 6.1 kb insert in phage λlpt2 does not correspond to any known segment of the LPT chromosomal DNA (see Discussion). However, it will be shown below that the 0.9 kb segment, whose position in the physical map of the phage is shown in Fig. 3, represents an authentic cell DNA piece which maps next to the Py integration site in LPT cells.
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Fig. 1. Analysis of EcoRI digestion products of the recombinant bacteriophage λpt2 DNA. One μg samples of phage λpt2 DNA were either partially or completely digested with the enzyme EcoRI. The complete digest (track 1), and the partial digest (track 2), were electrophoresed for 16 h at 2 V/cm in a 0.7% agarose gel. The gel was stained with ethidium bromide and visualized by illumination with u.v. light. Subsequently, the fragments were transferred on to nitrocellulose sheets and the blots were hybridized with 32P-labelled Py DNA. Tracks 3 and 4 show autoradiograms of the blots prepared from the gels shown in tracks 1 and 2 respectively. The numbers on the left designate the lengths (in kb) of the fragments observed. The procedure employed for length determinations is described in Methods.

The 0.9 kb fragment generated by EcoRI cleavage of phage λpt2 DNA is identical to fragments produced by EcoRI digestion of LPT and normal rat DNA

The 0.9 kb fragment was isolated from an EcoRI digest of phage λpt2 DNA by preparative agarose gel electrophoresis. The purified fragment was re-electrophoresed in another gel along with EcoRI digests of LPT cell DNA and normal rat fibroblast DNA. Blots prepared from this gel were annealed with another sample of the purified 0.9 kb fragment which had been labelled with 32P. Fig. 4 (tracks 1, 2 and 3) shows autoradiograms of these blots. It can be seen that each of the blots contains a single band. Furthermore, these bands have identical electrophoretic mobilities. Track 4 in Fig. 4 shows a blot of an EcoRI digest of LPT DNA annealed with 32P-labelled Py DNA. It is evident that the Py DNA probe did not hybridize with the LPT DNA fragment observed in track 2. The fragments with which the Py DNA did hybridize have been characterized previously by Mendelsohn et al. (1982).

These data indicate that both the LPT and the normal rat genomes contain a 0.9 kb segment which is identical to the 0.9 kb segment present in phage λpt2 and is also cleavable with EcoRI. It should also be noted that the presence of a single band in the LPT and normal rat DNA digests, rather than multiple bands, is consistent with the notion that the 0.9 kb segment contains only unique rat DNA sequences. This notion is supported by quantitative assays in which known amounts of the purified 0.9 kb segment were electrophoresed in parallel with LPT DNA digests and used to estimate its abundance in the digests. The concentration of the 0.9 kb segment was found to be less than 10^-6 μg per 10 μg of LPT cell DNA (not shown).
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Fig. 2. Heteroduplex mapping of phage \( \lambda \)ipt2 DNA. DNA of phage \( \lambda \)ipt2 was annealed with DNA of phage \( \lambda \)ipt1, which contains two whole Py genomes arranged in a direct tandem repeat (see text). The hybrids were analysed as described in Methods. (a) Electron micrograph of a heteroduplex between \( \lambda \)ipt1 DNA and \( \lambda \)ipt2 DNA. (b) An interpretive trace of the micrograph shown in (a). A designates \( \lambda \) DNA. The \( \lambda \)ipt1 insert consists of the regions B + D + E + G + H; the \( \lambda \)ipt2 insert consists of the regions C + D + F + G. As indicated in the interpretive trace, the region across the loop H does not appear to be single-stranded and was probably created in this molecule by folding of the loop. This region is absent from most of the other heteroduplexes analysed in this study. The average lengths of the various regions and the standard deviations were determined in a sample of 16 heteroduplexes and are as follows: B, 0.93 ± 0.19 kb; C, 0.37 ± 0.16 kb; D, 2.19 ± 0.16 kb; E, 2.87 ± 0.39 kb; F, 1.26 ± 0.18 kb; G, 2.18 ± 0.16 kb; H, 1.84 ± 0.29 kb. Single-stranded and double-stranded circular DNA molecules of bacteriophage \( \phi \)X174 were used as standards for these length measurements. Bar marker represents 0.50 kb. \( \cdots \), Single-stranded DNA; \( \cdots \), double-stranded DNA.

Fig. 3. Physical map of the recombinant phage \( \lambda \)ipt2 based on the EcoRI cleavage pattern shown in Fig. 1 and on the electron microscope data shown in Fig. 2. It has been corroborated by cleavage of phage \( \lambda \)ipt2 DNA with other enzymes (Neer, 1981). The fragments generated by the various enzymes are designated as bars drawn above the map, and their sizes (in kb) are also indicated. The boundary regions designate limits of uncertainty for the positions of the junctions between the \( \lambda \), Py and cell DNA. Note that this map is rotated 180° relative to the heteroduplex shown in Fig. 2.
Evidence that the 0.9 kb segment maps next to the Py integration site in LPT cells

Our next goal was to determine the position of the 0.9 kb segment relative to the Py integration site in LPT cells. To this end LPT DNA was digested with restriction enzymes other than EcoRI, whose sites of cleavage within the sequences flanking the Py inserts have been previously mapped in our laboratory (Mendelsohn et al., 1982). Southern blots prepared from these digests were hybridized with Py DNA or with a recombinant pBR322 plasmid in which the 0.9 kb segment was subcloned. A similar analysis of normal rat DNA was carried out in parallel. Fig. 5 shows blots of LPT and normal rat DNA digested with the enzymes Xbal and BamHI that cut
the Py DNA once, or with both enzymes used in succession. Tracks 1 and 4 show blots of the XbaI and the BamHI digests of LPT DNA that were hybridized with Py DNA. A blot of the corresponding double-digest has been presented elsewhere (Mendelsohn et al., 1982). These blots display two linker fragments containing both Py and cell DNA sequences and also whole linear Py DNA molecules, or the double-digestion products of these molecules. These fragments have been characterized previously (ibid). In each digest one linker fragment indicated by a closed arrowhead also hybridized with the 0.9 kb segment in the recombinant plasmid probe, as shown by the blots presented in tracks 2, 5 and 7 of Fig. 5. These particular linker fragments include sequences derived from the left end of the Py inserts, and 5.7 kb or 12.2 kb of flanking cell DNA respectively (Mendelsohn et al., 1982). We conclude that the 0.9 kb segment maps less than 5.7 + 0.9 = 6.6 kb from the left cell–virus DNA joint. More precise mapping of the 0.9 kb segment is presented in another section of Results.

**Evidence that LPT cells are heterozygous with respect to the Py inserts**

Tracks 2, 5 and 7 in Fig. 5 also display, in addition to one linker fragment, a second fragment which is indicated by an open arrowhead. These fragments do not hybridize with Py DNA. On the other hand, fragments having the same mobilities are present in the corresponding digests of normal rat DNA (tracks 3, 6 and 8 in Fig. 5). These data indicate that in addition to the chromosomal site which is occupied by Py DNA, LPT cells also contain a corresponding unoccupied site. According to this interpretation, the linker fragments in tracks 2, 5 and 7 are generated from the site occupied by Py DNA and the fragments indicated by open arrowheads are generated from the corresponding unoccupied site. This interpretation is also supported by cleavage patterns of other enzymes, as reported in the next section.

The presence of an unoccupied integration site in LPT cells would be accounted for if LPT cells are heterozygous with respect to the integrated Py DNA. This hypothesis is supported by the observation that the fragments representing the unoccupied site in the normal rat DNA are more abundant than the corresponding fragments derived from LPT DNA (compare, for example, the intensities of the bands indicated by open arrowheads in tracks 2 and 3 of Fig. 5); in LPT cells only one homologous chromosome contains an unoccupied site, whereas in normal rat cells the sites present on both homologous chromosomes are not occupied.

**Detailed mapping of the 0.9 kb cell DNA segment and of sequences flanking this segment in the LPT chromosome which carries Py DNA and in the homologous normal rat chromosomes**

Figures 6, 7, 8 and 9 present additional blot analyses of various restriction enzyme digests of LPT and normal rat fibroblast DNA. The blots were hybridized with either Py DNA, or the purified 0.9 kb cell DNA segment, or the recombinant plasmid including the 0.9 kb segment. The symbols used in Fig. 5 are also employed in the rest of the blots. Thus, the linker fragments are indicated by closed arrowheads. The open arrowheads designate fragments which are observed in both the LPT and the normal rat DNA digests. Other fragments, which hybridize with Py DNA but do not hybridize with the cell DNA probe, are not marked in these photographs. These fragments were previously characterized by Mendelsohn et al. (1982).

The data presented in Fig. 4 to 9 were used to map the 0.9 kb segment and the sequences flanking this segment in LPT cells and in normal rat fibroblasts. The method is similar to that used for mapping virus inserts by the Southern technique (see Botchan et al., 1976; Mendelsohn et al., 1982). The fragments derived from the LPT chromosome which carries Py DNA can only be arranged in one way consistent with the previously derived physical map of the virus inserts (Mendelsohn et al., 1982). This unique arrangement was used to determine the position of the 0.9 kb segment in this LPT chromosome relative to that of the virus DNA, as shown in Fig. 10. Fig. 11 shows the physical map of the 0.9 kb segment and flanking sequences in normal rat fibroblasts, and the unique fragment arrangement which was used to construct this map. As discussed above, the same fragments are also present in the LPT cell DNA digests. Hence, the map shown in Fig. 11 also represents the structure of the corresponding region in the LPT chromosome which does not carry Py DNA.
Fig. 6. Analysis of $BglI$ digests of the purified 0.9 kb fragment, and of LPT and normal rat DNA. A $10^{-5}$ μg sample of the 0.9 kb fragment was digested with $BglI$ and electrophoresed on an agarose gel, as described in the legend to Fig. 4 (track 6). Another sample of this fragment was similarly electrophoresed without being digested (track 1). Other samples applied to the same gel were: LPT DNA (10 μg) digested with $BglI$ (track 3), or with $BglI + EcoRI$ (track 5), and normal rat fibroblast DNA (10 μg) digested with $BglI$ (track 2), or with $BglI + EcoRI$ (track 4). Blotting and hybridization were carried out as described in Methods. All blots were hybridized with the purified 0.9 kb cell DNA segment. The numbers on the left designate the lengths (in kb) of the fragments observed.

Fig. 7. Analysis of $HindIII$, $HindIII/EcoRI$ and $HindIII/BglI$ digests of LPT and normal rat DNA. Fifteen μg samples of cell DNA were digested with restriction enzymes and electrophoresed for 16 h on a 2% agarose gel at 2 V/cm. The DNA species analysed and the enzymes used for the analysis were as follows. Tracks 1 and 2, LPT DNA, $HindIII$; track 3, normal rat DNA, $HindIII$; tracks 4 and 5, LPT DNA, $HindIII + EcoRI$; track 6, normal rat DNA, $HindIII + EcoRI$; tracks 7 and 8, LPT DNA, $HindIII + BglI$; track 9, normal rat DNA, $HindIII + BglI$. Blots prepared from this gel were hybridized with either Py DNA (track 7), or with the Py DNA fragment $HpaI$ which extends from 27-0 map units to 53-8 map units on the standard Py physical map (Griffin et al., 1980) (tracks 1 and 4), or with the purified 0.9 kb cell DNA segment (tracks 2, 3, 5, 6, 8 and 9). The lengths (in kb) of the fragments indicated by arrowheads, and of several other fragments, are shown on the left side of the photograph.

Evidence for a deletion of a cell DNA segment in the LPT chromosome which carries Py DNA

Fig. 12 shows again the physical map of the chromosomally associated Py DNA and flanking cell sequences drawn below the map of the corresponding region in the homologous normal rat chromosomes. The two maps were oriented by placing the 0.9 kb segment at equivalent positions. It can be seen that the left portions of the maps, up to and including the 0.9 kb
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Fig. 8. Analysis of BgII/BamHI, BgII/XbaI and BgII/EcoRI digests of LPT and normal rat DNA. Ten μg samples of cell DNA were digested with restriction enzymes and analysed by electrophoresis, blotting, hybridization and autoradiography, as described in the legend to Fig. 4. The recombinant plasmid containing the 0.9 kb cell DNA segment was used as a hybridization probe. The DNA species analysed and the enzymes used for the analysis were as follows. Track 1, LPT DNA, BgII + BamHI; track 2, normal rat DNA, BgII + BamHI; track 3, LPT DNA, BgII + XbaI; track 4, normal rat DNA, BgII + XbaI; track 5, LPT DNA, BgII + EcoRI; track 6, normal rat DNA, BgII + EcoRI. The lengths (in kb) of the fragments indicated by arrowheads are shown on the left side of the photograph.

Fig. 9. Analysis of HpaI, HpaI/BglII, HpaI/EcoRI, HindIII and HindIII/BglII digests of normal rat DNA. Fifteen μg samples of normal rat fibroblast DNA were digested with restriction enzymes, and were analysed by the Southern technique, as described in the legend to Fig. 7, except that all blots were hybridized with the 0.9 kb cell DNA probe. The enzymes used for digestion were as follows. Track 1, HindIII; track 2, HindIII + BglII; track 3, HpaI; track 4, HpaI + BglII; track 5, HpaI + EcoRI. The length (in kb) of the fragments indicated by arrowheads are shown on the left side of the photograph.

segment, are identical. Beyond this region to the right, there are in the top map two restriction enzyme sites, those of BgII and BglII, that are missing from the bottom map. Recently, we found that another restriction enzyme site (a KpnI site), which maps between the BglII and BgII sites in the normal rat chromosomes, is missing from the LPT chromosome which carries Py DNA (not shown in Fig. 12). Thus, it appears that a DNA segment including these three sites, and indicated on the top map, has been deleted from the region flanking the Py DNA on the right. The distance between the BglII and the BgII sites, 3-1 kb, is a minimal estimate of the length of
Fig. 10. Map position of the 0.9 kb segment relative to that of the Py inserts in LPT cells. The Py inserts and flanking sequences have been mapped by Mendelsohn et al. (1982). The black bars drawn above the map indicate the positions of linker fragments which hybridize with both Py DNA and the 0.9 kb cell DNA segment. The empty bars indicate fragments which hybridize with the 0.9 kb segment and do not hybridize with Py DNA. The 3.4 kb EcoRI fragment hybridizes only with Py DNA and is indicated by a hatched bar. The map position of the 0.9 kb segment, shown here as a rectangle, was deduced from this unique fragment arrangement. Most of the fragments were observed in the blots presented in Fig. 4 to 9. The HpaII cleavage patterns were presented in the paper by Mendelsohn et al. (1982) and the XhoI data are included in the thesis of A. Neer (1981). The boundary regions designate the uncertainty limits of the positions of the junctions between the viral and the cell DNA.

Further evidence for the existence of the deletion, and additional estimations of the length of the deleted segment, were obtained by comparing the sizes of the fragments produced by cleavage of the rat fibroblast DNA with the enzymes XbaI, BamHI, HindIII and XhoI, to the distances between the corresponding sites in the LPT chromosome containing the virus DNA. Thus, XbaI cleavage of the normal rat DNA generates one fragment of 13.7 kb which hybridizes with the 0.9 kb segment. The distance between the two corresponding XbaI sites in the chromosome containing the smallest Py insert is equal to the sum of the two XbaI linker fragments, that is 12.2 + 4.3 = 16.5 kb (Mendelsohn et al., 1982). Subtracting from this number the length of the Py insert, estimated as 5.9 kb, we obtain 16.5 - 5.9 = 10.6 kb. The difference between the two figures, 13.7 - 10.6 = 3.1 kb, is an estimate of the length of the deletion. Similar calculations based on the cleavage patterns of the enzymes HindIII, XhoI and BamHI yield estimates of 2.9 kb, 3.7 kb and 2.4 kb respectively. The average length of the deletion and the standard error, calculated from these five estimates, is 3.0 ± 0.4 kb.

**DISCUSSION**

The study of the DNA sequences flanking the Py inserts in LPT cells, and of the corresponding sequences in untransformed rat fibroblasts, was begun by attempts to clone the flanking sequences in the bacteriophage λgtWES. These attempts led to isolation of the recombinant phage λpt2 which has been mapped and found to contain a cell DNA piece
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Fig. 11. Physical map of the 0.9 kb segment and the sequences flanking this segment in normal rat fibroblasts. This map was deduced from the unique fragment arrangement drawn above it. Most of the data used here are presented in Fig. 4 to 9. The XhoI cleavage patterns are included in the thesis of A. Neer (1981).

Fig. 12. Comparison between the physical map of the sequences flanking Py inserts in LPT cells and the physical map of the corresponding region in normal rat chromosomes. The two maps shown in Fig. 10 and 11 are shown here again. The maps are oriented relative to the 0.9 kb segment. The region which has been deleted from the LPT chromosome carrying the Py DNA, and hence is absent from the bottom map, is designated as 'deleted DNA' in the top map. The boundary regions designate the uncertainty limits of the ends of the deletion and of the Py insert. The positions of the restriction sites on the right side of the bottom map have been deduced on the basis of data presented by Mendelsohn et al. (1982). The segmented arrows in the top map designate restriction sites in the normal rat DNA, whose presence was predicted but could not be demonstrated by using the 0.9 kb segment as a hybridization probe.
inserted between two direct repeats of a Py DNA segment. A continuous portion of this cloned DNA, consisting of the cell DNA piece and one Py repeat, is co-linear with an authentic LPT chromosomal segment which includes the left cell-Py DNA joint (compare Fig. 3 and 10). However, the presence of the second Py repeat and the positions of the *Eco*RI sites in the cloned Py DNA show that phage *λ*pt2 could not have been simply generated by *in vitro* ligation of a chromosomal DNA fragment with the *λ*gtWES arms. Rather, it may have been derived by *in vivo* recombination from other recombinant phages during growth of the latter in *E. coli*.

We have subsequently prepared from phage *λ*pt2 a DNA fragment containing just cellular sequences and subcloned this fragment into the plasmid pBR322. We used the purified fragment, the recombinant plasmid and Py DNA as hybridization probes in a Southern blot analysis of LPT and normal rat fibroblast DNA digested with various restriction enzymes. This analysis has led to two significant findings. First, it was shown that LPT cells contain, in addition to the chromosomal locus into which Py DNA had been inserted, an equivalent site which remained unoccupied and is therefore identical to the corresponding site in the normal rat cells. Presumably, during the infection which led to cell transformation, Py DNA has integrated into one chromosome. The other homologous chromosome remained unmodified and has been propagated ever since in all LPT cells along with the chromosome which carries the Py inserts. This interpretation is consistent with the observation that integration of Py and SV40 DNA into cellular genomes is a very low-probability event and does not involve a specific recognition of any particular chromosomal site (Botchan *et al.*, 1976; Ketner & Kelly, 1976; Birg *et al.*, 1979; Lania *et al.*, 1979). Thus, the probability of simultaneous integration of the Py DNA into two equivalent sites in two homologous chromosomes could be as small as the probability of simultaneous integration of Py DNA into two unrelated sites, that is the product of the probabilities of integration into each of the two sites. It should be noted in this connection that determinations of frequencies of reversion from the transformed phenotype of SV40-transformed rat cells have indicated that these cells are haploid with respect to the virus insert (Steinberg *et al.*, 1978). Recently, SV40-transformed rat cells were found by a Southern blot analysis to be heterozygous with respect to the SV40 inserts (Stringer, 1982).

The second significant finding reported in this paper is that a chromosomal DNA segment of about 3.0 kb is missing from the region flanking the Py inserts in LPT cells. The proximity of the deletion to the virus inserts indicates that there is a causal relationship between the integration and the deletion events. This view is supported by the observation that the deleted segment is present not only in the corresponding normal rat chromosomes, but also in the homologous LPT chromosome which does not carry a Py insert. Thus, the deletion could not have been caused by an inherent instability of this particular chromosomal site in LPT cells. Rather, it appears to be directly related to the integration of virus DNA at this site and to have resulted from an interaction which operated only in *cis*.

The causal relationship between the deletion and the integration events does not necessarily imply that the two events happened simultaneously; recent studies have indicated that virus integration patterns in Py- and SV40-transformed cell lines are unstable. Rearrangements of integrated Py DNA sequences were found to occur at a rather high rate in a number of Py transformants including the LPT line. These rearrangements, which include deletions and duplications of virus sequences, apparently take place by recombination between homologous segments of the virus DNA (Basilico *et al.*, 1979; Colantuoni *et al.*, 1980; Chartrand *et al.*, 1981; Mendelsohn *et al.*, 1982; Mendelsohn, 1981). Hiscott *et al.* (1980) and Sager *et al.* (1981) have observed rearrangements of integrated virus DNA sequences in subclones of SV40-transformed mouse cell lines. Sager *et al.* (1981) have reported that duplications of segments including both SV40 DNA and flanking cellular sequences also occur in these cells. In view of these findings, it is possible that the deletion occurred in LPT cells after the initial integration event. It could have been generated, for example, by a single crossover between a site within the virus insert and a site within the flanking cell DNA. However, so far, rearrangements affecting flanking host sequences have not been reported in Py-transformed cells.

On the other hand, the deletion could have occurred during the initial integration event. This possibility would be in line with current ideas on integration of papovavirus DNA. The ideas are
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Fig. 13. Double-crossover model for integration of Py DNA into chromosomal DNA. The virus precursor, of which only a part is shown here, includes a tandem array of Py monomers. Each monomer is bound by two arrowheads. The cellular DNA segment located between the two crossover points is replaced by the virus segment located between these two points.

Based on studies mentioned in Introduction which showed that in many SV40 and Py transformants, including the LPT line, the virus inserts contain partial or complete tandem duplications of the virus genome. It has also been observed that none of the inserts analysed so far consists of a single virus genome. Thus, it appears unlikely that the immediate precursor to the chromosomally associated virus DNA is a circular monomer and that the latter is integrated into the cell DNA by a single crossover event. Instead, it has been suggested that most papovavirus inserts originate from a precursor consisting of tandemly joined virus genomes and that it is integrated into the cell DNA by a mechanism involving double-crossover (Topp et al., 1980). The precursor might be a rolling circle intermediate in virus DNA replication (Topp et al., 1980; Della Valle et al., 1981), or an oligomer of tandemly joined molecules of virus DNA which is synthesized by recombination of free virus monomers (Chia & Rigby, 1981). As Fig. 13 shows, such a mechanism would generate an insert containing duplications of virus DNA and a cellular deletion. In the case of the LPT line the insert would be longer than the deletion, as indicated in Fig. 13 (see also Fig. 12). If this mechanism is general, then deletions should be found next to sites of integration in other Py- and SV40-transformed cell lines. As mentioned in Introduction, at least in one SV40-transformed rat cell line that has been characterized by an approach similar to ours, a deletion occurred at the site of SV40 integration (Stringer, 1982).

In conclusion, we have demonstrated in this article that a 3-0 kb deletion of cellular DNA occurred next to the Py inserts in LPT cells. We have also shown that LPT cells are heterozygous with respect to both the inserts and the deletion. Our data indicate that the deletion was caused by the integration event, but the relationship between these two events has not as yet been clearly defined. We have discussed two alternative ways for deletion formation.

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