Requirements for Excision and Amplification of Integrated Viral DNA Molecules in Polyoma Virus-Transformed Cells

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Received 1 March 1982/Accepted 19 April 1982

The integration of polyoma virus DNA into the genome of transformed rat cells generally takes place in a tandem head-to-tail arrangement. A functional viral large tumor antigen (T-Ag) renders this structure unstable, as manifested by free DNA production and excision or amplification of the integrated viral DNA. All of these phenomena involve the mobilization of precise genomic "units," suggesting that they result from intramolecular homologous recombination events occurring in the repeated viral DNA sequences within the integrated structures. We studied polyoma *ts-a*-transformed rat cell lines, which produced large T-Ag but contained less than a single copy of integrated viral DNA. In all of these lines, reversion to a normal phenotype (indicative of excision) was extremely low and independent of the presence of a functional large T-Ag. The revertants were either phenotypic or had undergone variable rearrangements of the integrated sequences that seemed to involve flanking host DNA. In two of these cell lines (*ts-a* 4A and *ts-a* 3B), we could not detect any evidence of amplification even after 2 months of propagation under conditions permissive for large T-Ag. An amplification event was detected in a small subpopulation of the *ts-a* R5-1 line after 2 months of growth at 33°C. This involved a DNA fragment of 5.1 kilobases, consisting of the left portion of the viral insertion and about 2.5 kilobases of adjacent host DNA sequences. None of these lines spontaneously produced free viral DNA, but after fusion with 3T3 mouse fibroblasts, R5-1 and 4A produced a low level of heterogeneous free DNA molecules, which contained both viral and flanking host DNA. In contrast, the *ts-a* 9 cell line, whose viral insertion consists of a partial tandem of ~1.2 viral genomes, underwent a high rate of excision or amplification when propagated at temperatures permissive for large T-Ag function. These results indicate that the high rate of excision and amplification of integrated viral genomes observed in polyoma-transformed rat cells requires the presence of regions of homology (i.e., repeats) in the integrated viral sequences. Therefore, these events occur via homologous intramolecular recombination, which is promoted directly or indirectly by the large viral T-Ag.

Cellular transformation by DNA tumor viruses is characterized by the integration of the viral genome into the host DNA.

In rat cells transformed by polyoma virus (Py), the most common type of integration is a head-to-tail tandem arrangement of viral genomes (2, 3, 5, 12, 19, 20), and an active Py large T-antigen has recently been shown to be required for the formation of such structures as well for a high efficiency of transformation (9).

The primary integration pattern is not always stable. Excision of Py genomes from the viral insertion can take place at a high rate (2, 3), together with the production of free viral DNA molecules, which originate from the integrated sequences (12, 23, 28). Also, amplification of integrated viral sequences can determine an increase in the number of viral genomes within

the original insertion (7). In cell lines transformed by the *ts-a* mutant (11) of Py, which produces a thermolabile large T-antigen (14, 16, 25), these phenomena have been observed only when cells are propagated at temperatures permissive for this viral protein (2, 7, 28). These studies, in addition to showing a requirement for a functional Py large T-antigen, also showed that both excision and amplification involved the mobilization of an integral number of genomic "units" (3, 7). This suggested the involvement of homologous intramolecular recombination events, made possible by the presence, in all of the cell lines studied, of complete or partial tandem insertions of Py molecules.

To verify this hypothesis, we studied excision, amplification, and free viral DNA production in Py *ts-a*-transformed rat cell lines carrying

single viral insertions with a complete early region and no duplication at their termini. The results reported here show that none of the cell lines studied were able to undergo excision or amplification of Py genomes with the same characteristics described before, irrespective of the presence or absence of large T-antigen function. Two cell lines showed a very low frequency of rearrangements of the viral insertion which probably involved host DNA sequences. None of the cell lines studied spontaneously produced free viral DNA. After fusion with mouse 3T3 fibroblasts, some of the cell lines produced a low amount of heterogeneous free DNA molecules which, again, contained viral and host sequences. These results suggest that, in addition to an active Py large T-antigen, the presence of regions of homology in the integrated sequences is required for excision and amplification of integrated Py genomes. In the absence of viral DNA homology, regions of partial homology at the level of host flanking sequences can probably be used and might explain the events observed at low frequency in two cell lines.

MATERIALS AND METHODS

Cells. The isolation and general properties of Swiss mouse 3T3 D cells, F2408 Fisher rat fibroblasts, and the Py-transformed rat line *ts-a* H3A have been described (2, 12, 23). The *ts-a* R5-1, *ts-a* 3B, *ts-a* 4A, and *ts-a* 9 transformed lines were obtained by transfection of F2408 cells with *ts-a* Py DNA at 39°C as previously described (9). All of the transformed cell lines were recloned once in agar medium and propagated in Dulbecco modified Eagle medium containing 10% calf serum at 39°C unless otherwise specified.

Isolation of "cured" cells. Morphological revertants were isolated as already described (2), either by 5'-fluorodeoxyuridine (FUDR) treatment or by simply plating the transformed cells at low densities. For FUDR treatment, confluent cells were incubated at 37°C in medium plus 1% calf serum. The next day, 25 µg of FUDR per ml was added without a medium change, and incubation was continued for 2 to 3 days. The cells were then washed extensively until the next medium change (ca. 4 days later).

DNA extraction, restriction enzyme analysis, and blot hybridization. High-molecular-weight DNA prepared from cells grown at 39°C was digested with restriction enzymes (New England Biolabs or Bethesda Research Laboratories) and fractionated on a 1% horizontal agarose slab gel. Alkali denaturation, transfer to nitrocellulose filter paper, and hybridization with denatured ³²P-labeled nick-translated (17, 24) Py probes were performed as already described (2, 8). Filters were then rinsed, dried, and exposed to XR-2 X-ray films (Kodak) with or without an intensifying screen.

Construction of a probe containing Py sequences from 52 to 82 m.u. A recombinant, *ts-a* pBR-3 (9), which contains *Bam*HI-cut Py *ts-a* DNA inserted in the *Bam*HI site of pBR322, was cleaved with *Sst*I to remove Py DNA sequences clockwise from 83 to 52 map units (m.u.). The large linear DNA fragment

containing pBR322 DNA and Py sequences from 53 to 82 m.u. was circularized by T4 DNA ligase. This molecule was used to transform HB101 to ampicillin resistance (21), and DNA was prepared by the procedure of Lacy et al. (18).

Cell fusion and extraction of low-molecular-weight DNA. An equal number of transformed cells were cocultivated overnight with 3T3 mouse fibroblasts in 100-mm dishes at a total concentration of 2×10^6 cells per plate. The cells were then treated with polyethylene glycol 1000 (22) and propagated at 33.5°C for 2 to 3 days. Low-molecular-weight DNA was then extracted according to the procedure of Hirt (13), followed by phenol extraction and ethanol precipitation. In some experiments, supercoiled form I DNA was further purified on an ethidium bromide-CsCl density gradient, containing approximately 4 µg of form I simian virus 40 DNA marker.

Immunoprecipitation. Subconfluent cultures in 60-mm plates were labeled for 4 h with 150 µCi of [³⁵S]methionine per ml (New England Nuclear Corp.; 1,000 Ci/mmol) in 2 ml of methionine-free Dulbecco modified Eagle medium at 33°C. The procedure described by Ito (15) was used to immunoprecipitate the Py T-antigens. Protein A-bearing *Staphylococcus aureus* was purchased from Calbiochem. Antipolyoma sera were obtained from Brown Norwegian rats inoculated with syngeneic Py-transformed cells.

RESULTS

Excision of integrated Py genomes in transformed cells containing partial tandem insertions. Previous data from our laboratory have shown that a high frequency of "cured" revertants can be isolated from Py-transformed rat cells under conditions permissive for viral large T-antigen (2, 3). The parental Py-transformed cells used contained full-length or defective viral genomes in head-to-tail tandem array, whereas the revertants had undergone excision of an integral number of viral DNA molecules, leaving apparently unchanged the virus-host DNA joining sequences. All cured revertants isolated from the same line displayed the same arrangement of integrated viral DNA sequences. This was not due to the repeated isolation of "sister" clones, but seemed likely to be the result of the fact that all excision events involved homologous intramolecular recombination (3).

To determine whether this hypothesis (i.e., excision involves homologous recombination) was correct, we isolated cured revertants from the *ts-a* H3A cell line. The single insertion of Py DNA in this cell line consists of a colinear partial tandem of 1.2 viral genomes containing an intact early region and two different deletions, one about 3% and the other about 12% of the Py genome within each of the repeated regions (30 to 60 m.u.) (7, 12). Propagation in the presence of an active Py large T-antigen (33°C) resulted in a high frequency of revertants (Table 1) which showed a "flat" phenotype. The organization of

TABLE 1. Frequency of revertants in *ts-a* polyoma-transformed rat lines propagated at 39 and 33°C

Cell line	Days in culture	Frequency of revertants ^a after propagation at		Frequency of revertants ^b after FUDR selection of cultures propagated at	
		39°C	33°C	39°C	33°C
<i>ts-a</i> R5-1	30	<0.1	<0.1	ND ^c	ND
	60	0.23	0.16 (0.7)	0.54	0.29
<i>ts-a</i> 4A	30	<0.1	0.26 (>2.6)	<0.16	0.73
<i>ts-a</i> 3B	30	<0.06	<0.09	— ^d	—
<i>ts-a</i> 9	30	0.06	3.3 (55)	ND	35
<i>ts-a</i> H3A	42	<0.1	10.4 (>100)	ND	ND

^a Cells were plated at 300 to 800 per 100-mm petri dish. The frequency of revertants was calculated from the proportion of morphologically normal "flat" colonies over the total number of colonies and expressed as a percent. About two thousand total colonies were examined. The numbers in parentheses are the ratios of the frequencies at 33°C to those at 39°C.

^b After propagation at the respective temperatures, cells were shifted to 39°C, incubated in 1% calf serum, and exposed to FUDR for 3 days, as described in the text. Surviving cells were plated for colony formation at 39°C, and the frequency of revertants, expressed as a percent, was determined as described in footnote *a*.

^c ND, Not determined.

^d —, Cells did not survive FUDR treatment.

Py DNA in the revertants was compared with that of the parental H3A by the Southern (26) blot technique, and the results obtained with two of them (T20 and T32) are shown in Fig. 1.

By digesting the revertant chromosomal DNAs with *Bgl*II (does not cut Py DNA), a band of lower molecular weight than the corresponding one in H3A DNA was visible, suggesting that these cell lines had undergone a loss of Py sequences. By using enzymes that cleave within the Py genome, two different restriction patterns, represented by the T20 and T32 revertants, were observed (Fig. 1). The *Bam*I digestion of T20 DNA showed two bands that correspond to the two virus-host DNA junction fragments in H3A, whereas the 4.6-kilobase (kb) viral fragment, present in the parental line, was absent in the revertant. The T32 DNA digested with the same enzyme showed a band which migrates like the right host-virus DNA joint fragment of H3A (1.2 kb) plus another band 0.63 kb shorter than the left junction. By restricting T20 DNA with *Hind*III, a new band of 2.4 kb was produced, in addition to the 2.7-kb segment corresponding to the left virus-host DNA junction. The *Hind*III fragment I (45 to 2 m.u., 3 kb)

and the right flanking sequence of ~4.0 kb in H3A were not present. On the other hand, only a band of 4.5 kb appeared when T32 DNA was digested with the same enzyme, indicating that *Hind*III no longer cleaved within the viral insertion. The *Eco*RI digestion produced in T20 and T32 DNAs only one band of 9 and 8.6 kb, respectively, again indicating that this restriction site was no longer present in the viral insertions.

These and other results (not shown) can be easily interpreted if one assumes that the cured lines have lost Py sequences through excision of the right Py genomic equivalent of the H3A insertion or the left genomic equivalent, which, because of the deletions present in the H3A viral DNA, correspond to a molecule of 4.6 kb (87% of the Py genome) or 5.1 kb (97% of the Py genome). Excision of the 4.6-kb molecule would produce revertants like T20, while the 5.1-kb molecule is excised in the T32 revertant (Fig. 2).

In conclusion, the characteristics of excision detected in H3A are consistent with the idea of a recombination event between homologous sequences. The fact that two types of molecules can be excised is due to the unique viral integration pattern of this line where two different deletions are contained in the repeated regions.

Excision of Py genomes in transformed cells

FIG. 1. Restriction enzyme and Southern blot analysis of Py DNA sequences integrated in H3A, T20, and T32 DNAs. A 10-μg amount of chromosomal DNA from each cell line was digested with the indicated restriction endonucleases, size fractionated on a 1% horizontal agarose slab gel, denatured, and transferred onto nitrocellulose filters. Specific Py sequences were identified by hybridization with denatured ³²P-labeled nick-translated Py DNA and visualized by autoradiography. The position and the size (in kb) of some Py DNA fragments are indicated as calculated from the mobility of phage lambda DNA fragments. m, Py DNA marker; 1, H3A high-molecular-weight DNA; 2, T20 DNA; 3, T32 DNA.

FIG. 2. Restriction maps of the viral DNA sequences integrated into the genomes of the *ts-a* H3A, T20, and T32 cell lines. Dashed lines represent the flanking host DNA, and the thin lines represent the integrated Py DNA sequences. Numbers below these lines indicate viral m.u. The cleavage sites of some of the restriction enzymes used are illustrated in the viral as well as in the host DNA sequences. V indicates the joining sequences between Py and cellular DNA. The map coordinate on the viral site represents a restriction site still present in the integrated viral sequences, while the map coordinate on the host site represents the next Py restriction site not included in the viral insertion. The triangles represent deletions of viral DNA. Symbols: ●, *EcoRI*; ○, *BamHI*; ▲, *HindIII*.

carrying single-copy insertions. The next question we addressed was whether cell lines that carry no tandem integration of Py molecules, i.e., contain less than a single genome, were able to undergo excision. These lines were obtained by transfecting rat cells with *ts-a* Py DNA at 39°C. As shown elsewhere (9), rat cells transfected by Py DNA, in conditions where large T-antigen is not active, usually contain one or more single-copy insertions. Three lines (*ts-a* R5-1, *ts-a* 3B, and *ts-a* 4A) that had a single Py integration site were studied. The map of their integrated viral DNA sequences was constructed by restriction enzyme digestion and Southern blotting as previously described (2, 8). All of the lines contained the fragments corresponding to a complete Py early region, but no repeated sequences (Fig. 3). S1 analysis (4) as well as "Northern" (1) blot hybridization showed that these lines produced all three species of polyoma early mRNAs (R. Fenton and C. Basilio, *Virology*, in press). Immunoprecipitations of [³⁵S]methionine-labeled cell extracts from *ts-a* R5-1, *ts-a* 3B, and *ts-a* 4A growing at 33°C with anti-polyoma T immune serum revealed specific bands of 100,000, 60,000, and 20,000 daltons, corresponding to full-size viral large, middle, and small T-antigens (Fig. 4).

These cell lines were propagated at 33 or 39°C and periodically examined for the presence of morphological revertants, a procedure that in the past had allowed us to identify cells which had undergone excision (2).

In these, as in the following experiments, we

used as a control the *ts-a* 9 line, which contains a partial tandem insertion of polyoma DNA, as does H3A. This cell line had been isolated in the course of the same transfection experiments used to produce R5-1, 3B, and 4A.

The results of these experiments are summarized in Table 1. For *ts-a* 9, a high frequency of revertants was detected after propagation at 33°C simply by plating cells at low density and examining microscopically a large number of colonies. FUDR selection applied as described before (2) enriched the population in revertants (~10-fold) by its preferential killing of transformed cells. As in other *ts-a*-transformed lines previously examined (2, 3), reversion was much higher upon growth at 33°C than at 39°C. The *ts-a* 9 revertants displayed a totally normal "flat" phenotype, were T-antigen negative by immunofluorescence, and no longer grew in soft-agar medium. The characterization of the arrangement of their integrated viral DNA sequences with respect to that of the parental line is shown in Fig. 5 and 6.

As shown in Fig. 6, the *ts-a* 9 insertion contains a stretch of viral DNA of about 1.2 genomes spanning a complete early region. *Bgl*II digestion of the revertant DNA showed a reduction in size of the band containing viral sequences. When *Bcl*I, which cleaves in the duplicated regions, was used with *ts-a* 9 DNA, a band which migrated like a linear Py molecule (5.3 kb) plus two virus-host flanking sequences were obtained, whereas only the two junctions were present in the revertant DNA. By restricting *ts-a*

FIG. 3. Restriction maps of the integrated viral DNA of *ts-a* R5-1, *ts-a* 3B, and *ts-a* 4A. Symbols are as described in the legend to Fig. 2.

9 DNA with *Eco*RI, which cleaves once in the viral insertion, two virus-host fragments were obtained, while only a band of higher molecular

weight was obtained with the revertant DNA, indicating that the viral *Eco*RI site had been deleted. *Hinc*II digestion, while producing the small viral fragment (26 to 36 m.u.) (not visible in this blot) and two virus-host junctions in *ts-a* 9 DNA, produced only a large-molecular-weight fragment in the revertant DNA, equal to the sum of the bands obtained in the parental line, less a complete Py molecule.

It appears, therefore, that *ts-a* 9 underwent excision of complete viral genomes as did other lines previously described (Fig. 6). On the other

FIG. 4. Immunoprecipitation of polyoma T-antigens from cell extracts of the single-copy lines. Subconfluent cell cultures were incubated for 4 h with [35 S]methionine in methionine-free Dulbecco modified Eagle medium at 33°C. Immunoprecipitation was performed as described in the text, followed by fluorography. Lanes 1 and 2, Immunoprecipitation of [35 S]methionine-labeled extracts of *ts-a* R5-1 with normal (N) or anti-polyoma T (T) rat serum; lanes 3 and 4, immunoprecipitation of [35 S]methionine-labeled extracts of *ts-a* 3B with normal or anti-T serum; lanes 5 and 6, immunoprecipitation of [35 S]methionine-labeled extracts of *ts-a* 4A with normal or anti-T serum. The positions of the Py large (100,000 daltons), middle (56,000 daltons), and small (20,000 daltons) T-antigens are indicated.

FIG. 5. Restriction enzyme and Southern blot analysis of chromosomal DNA from *ts-a* 9, one of its revertants, and a culture of *ts-a* 9 cells which had been propagated at 33°C for 1 month. Methods were as described in the legend to Fig. 1 and the text. m, Py DNA marker; 9, high-molecular-weight DNA from *ts-a* 9 grown at 39°C; A, a mixed population of *ts-a* 9 cells after growth at 33°C for 4 weeks; R, a revertant of *ts-a* 9. I and II represent forms I and II, respectively, of Py DNA. kb, Kilobase pairs of DNA.

FIG. 6. Restriction maps of the integrated viral DNA of *ts-a* 9 and derivatives of *ts-a* 9 after propagation at 33°C. REV, A *ts-a* 9 revertant; AMPL, a derivative of *ts-a* 9 in which the integrated Py DNA has undergone amplification. The cross-hatched areas represent flanking host DNA. All other symbols are as described in the legend to Fig. 2.

hand, the results with the single-copy lines were quite different. *ts-a* 3B did not yield any identifiable morphological revertants after plating for colonies, and FUdR selection resulted in practically 100% cell lethality. From *ts-a* R5-1 and 4A, a few revertants were identified. Their frequency was much lower than that of revertants in *ts-a* 9 and did not appear to be dependent on the growth temperature, i.e., on the presence of a functional large T-antigen (Table 1). In addition, it became clear, after isolation and propagation, that a majority of these clones had not undergone stable phenotypic reversion and were in fact still T-antigen positive. Of four T-antigen-negative R5-1 derivatives, three showed a viral integration pattern indistinguishable from the parental line, suggesting that they owed their phenotype to undetected viral mutations or to host alterations (not shown). Restriction enzyme analysis of the integrated viral DNA in the remaining revertant revealed that while viral fragments corresponding to the sequences from approximately 92 to 30 m.u. appeared to have been deleted, considerable rearrangements of the viral integration had also occurred. The pattern observed was hard to interpret, but it seemed unlikely to have been produced by a mechanism similar to the excision process already described (data not shown).

From the *ts-a* 4A cell line, three T-antigen-negative revertants were studied, but only one clone showed a viral insertion different from the parental line, as illustrated in Fig. 7. When this DNA was digested with *Bgl*III, a band containing Py sequences of ~4.2 kb was obtained, about 2.8 kb shorter than the corresponding fragment in 4A DNA. The *Eco*RI cleavage of the parental DNA gave two virus-host DNA fragments, whereas in the revertant DNA, only the band

corresponding to the right side of the insertion was maintained. *Hind*III digestion produced the same right junction fragment and the *Hind*III fragment 2 (2 to 45 m.u.) obtained in the 4A DNA, but not the left junction. *Pvu*II digestion showed that only the right virus-host fragment was maintained in the revertant DNA, while the *Pvu*II-2 and -3 fragments (70 to 92 and 92 to 9 m.u., respectively) were not detectable. Both sites of *Sst*I cleavage in the 4A viral sequences (82 and 96 m.u.) appeared to have been deleted in the revertant. These data suggest a deletion of host and viral DNA corresponding to the left side of the 4A viral insertion from 55 m.u. to about 0 m.u. (see Fig. 2).

FIG. 7. Restriction enzyme and Southern blot analysis of integrated Py DNA of *ts-a* 4A and of a *ts-a* 4A revertant. Methods were as described in the legend to Fig. 1 and the text. m, Py DNA marker; 4, high-molecular-weight DNA from *ts-a* 4A; R, high-molecular-weight DNA from the *ts-a* 4A revertant.

In conclusion, these results show that excision occurs at a very low rate in lines containing single-copy insertions. The few examples observed do not seem to be promoted by viral large T-antigen and suggest random deletions and rearrangements.

Amplification of Py genomes in transformed cell lines with single-copy insertions. We have previously shown that integrated Py genomes undergo amplification under conditions permissive for viral large T-antigen (7). Since precise polyoma genome equivalents were amplified, we wished to determine whether the presence of regions of homology was as important as an active A gene product in allowing amplification. The *ts-a* R5-1, *ts-a* 3B, and *ts-a* 4A cell lines, which carry less than single-copy insertions, were therefore propagated at 39 and 33°C, and, at different times, high-molecular-weight DNAs were prepared and analyzed.

As a control, we used again the *ts-a* 9 line. As expected, this line underwent a high rate of amplification upon growth at 33°C. As shown in Fig. 5, the *Bgl*III digestion of *ts-a* 9 DNA after 1 month of growth at 33°C produced bands of ~22 and 17 kb in addition to the original band of ~12 kb. When *Bcl*I was used, the pattern obtained was the same as that from the original DNA, but the intensity of the monomeric Py linear band (5.3 kb) was greatly increased. *Eco*RI digestion of the DNA from cultures at 33°C showed a band which migrated like the Py monomer and which was not present in the original insertion. A band which migrated like the viral marker fragment (36 to 26 m.u., 4.8 kb) was also obtained by restricting the DNA from the cultures at 33°C with *Hinc*II, and with both enzymes, the virus-host DNA junctions were unchanged. No changes were detected in the integration pattern of cultures propagated at 39°C. Taking into account the modality of amplification previously demonstrated in the H3A cell line (7), it appeared that amplification involved the formation of full tandem repeats of a normal-size polyoma DNA molecule (Fig. 6) and that this process required an active large T-antigen.

The results obtained with the single-copy lines showed that 3B and 4A did not undergo any change in their integration patterns even after 8 weeks of propagation at 33°C (Fig. 8). Propagation at the low temperature did, however, cause a rearrangement in the viral insertion of the R5-1 cell line (Fig. 9). The *Bgl*III digestion of the DNA from the total cell population after 2 months at 33°C revealed a new band of higher molecular weight, in addition to the one corresponding to the original viral insertion. Several clones from the population at 33°C were isolated and analyzed. *Bgl*III digestion of the DNA from one of them (R5-1A) produced a band which migrated

FIG. 8. Comparison of integrated Py DNA sequences of *ts-a* 3B and *ts-a* 4A at 39°C and after propagation at 33°C. Methods were as described in the legend to Fig. 1 and the text. m, Py DNA marker; 3, chromosomal DNA from *ts-a* 3B grown at 39°C; 4, chromosomal DNA of *ts-a* 4A grown at 39°C. Lanes A and B represent the DNA of these lines after 1 and 2 months, respectively, of propagation at 33°C. I and II represent forms I and II, respectively, of Py DNA. In this experiment, most of the Py DNA marker was nicked in the *Bgl*III digestion.

like the slower one in the total DNA from the cultures at 33°C (Fig. 9). The same DNA cleaved with *Hind*III gave the same two virus-host DNA junctions as in the parental DNA plus a band of ~5.1 kb. By digesting R5-1A DNA with *Eco*RI, in addition to the two virus-host fragments present in R5-1 DNA, a new band of about 1.5 kb was obtained (Fig. 9b). When *Sst*I and *Bcl*I were used, new bands of 2.9 and 3.4 kb, respectively, were produced (not shown). Like *Hind*III digestion, *Pst*I (Fig. 9b) and *Kpn*I (not shown) digestions produced a new band of 5.1 kb not present in the original R5-1 restriction pattern. The identification of this fragment as the viral amplification "unit," however, did not explain why *Eco*RI and *Bcl*I digestion produced new fragments of different size. The size of the new DNA fragments identified by the polyoma probe supported the hypothesis that they also contained cellular DNA sequences.

For a clearer understanding of this phenomenon, DNAs from R5-1 and clone A were restricted and hybridized to a cloned ³²P-labeled Py DNA fragment extending from 52 to 82 m.u. This probe (designated as Py 52-82) recognized in R5-1 DNA only the leftmost part of the viral insertion (Fig. 3). Hybridization of restriction enzyme digests of the two DNAs to the Py 52-82 DNA fragment or to the viral genomic probe is shown in Fig. 9. When R5-1A DNA digested with *Bgl*III was hybridized to the genomic probe,

FIG. 9. Restriction enzyme and Southern blot analysis of rearrangements involving the viral DNA of the *ts-a* R5-1 line after propagation at 33°C for 2 months. Methods were as in the legend to Fig. 1 and the text. (a) 1, chromosomal DNA from R5-1 grown at 39°C; T, chromosomal DNA from the total population of R5-1 cells after 2 months at 33°C; 2, high-molecular-weight DNA of the R5-1A clone isolated from the total R5-1 T population; m, Py DNA marker. (b and c) Comparison of the integrated viral DNA of R5-1 (1) and R5-1A (2) hybridized with ^{32}P -labeled Py genomic DNA (panel b) or with a pBR322 recombinant containing Py sequences from 52 to 82 m.u. (panel c). m, Py DNA marker.

three bands were visible, a new one of 3.2 kb plus two similar to the ones obtained from R5-1 parental DNA (Fig. 9b). All three bands were recognized by the Py 52-82 probe (Fig. 9c), indicating that these sequences were present in the new band as well as in both of the virus-host junctions (Fig. 10). Digestion with *Eco*RI produced three bands in R5-1A DNA, but only one (3.7 kb) was recognized by the Py 52-82 probe and appeared more intense than in parental DNA (Fig. 9c). Thus, this band corresponds to the left DNA virus-host fragment, and the sequences from 52 to 82 m.u. were not contained in the new band of 1.5 kb present in R5-1A. The *Pst*I digest of the same DNA hybridized to the Py 52-82 probe revealed the left virus-host linker fragment and the new 5.1-kb band, which therefore contained these viral sequences. When *Hind*III-digested R5-1A DNA was hybridized to the Py 52-82 fragment, the bands of 6.7 and 5.1 kb revealed homology to the probe (not shown).

These data support the hypothesis that a tandem duplication of a composite molecule containing viral and cellular DNA has occurred in R5-1A. The host sequences involved are those adjacent to the left termini of the viral insertion and extend for about 2.5 kb (Fig. 10). The host restriction sites included in this segment explain the appearance of new bands whose sizes do not correspond to those of bona fide viral fragments. The *Hind*III and *Pst*I sites are not included in the duplicated host DNA, and they cleave, therefore, only at the viral sites that now bracket the host DNA sequences, producing a 5.1-kb

band representing the amplification unit. The amplified viral sequences (~2.6 kb) are the left sequences of the R5-1 insertion, going from 60 to about 10 m.u. (Fig. 10). It appears, therefore, that amplification of viral DNA has occurred in the R5-1 line together with amplification of adjacent cellular sequences.

The results described here confirm that amplification of complete Py genomes occurs in cells containing viral tandem integrations. In cells carrying single-copy insertions, the phenomenon did not occur or occurred at a very low rate and involved hybrid molecules containing both viral and cellular sequences. Therefore, homologous recombination between duplicated sequences appears to be required for efficient amplification.

Free viral DNA production in Py-transformed cell lines carrying single-copy insertions. Py-transformed rat cells, in addition to integrated viral genomes, generally contain unintegrated free viral DNA, whose production is under the control of the A gene function. These molecules are detected in a minority of the cell population and faithfully reflect the main species of the integrated sequences from which they are derived (5, 12, 23, 28).

We tested free viral DNA production in *ts-a* 9 and in the single-copy cell lines. As expected from the integration pattern, the low-molecular-weight DNA extracted from *ts-a* 9 at 33°C contained free polyoma molecules corresponding to a full genome (not shown). On the other hand, when the low-molecular-weight DNAs extracted

FIG. 10. Restriction maps of viral DNA integrated within the chromosomal DNA of *ts-a* R5-1 and its amplified derivative, R5-1A. R5-1A was subcloned from a population of R5-1 cells grown at 33°C for 2 months. Cross-hatched areas represent host DNA. Symbols: ○, *Bgl*II; ●, *Hind*III; △, *Eco*RI; ◇, *Sst*I; ▲, *Pst*I. All other symbols are as described in the legend to Fig. 2.

from R5-1, 3B, and 4A at 33°C were analyzed, no molecules hybridizing to polyoma DNA were detected, indicating that there was no spontaneous free viral DNA production in these cell lines (<0.5 molecules per cell; data not shown).

We fused R5-1, 3B, and 4A cells with 3T3 mouse fibroblasts at 33°C, a process which has been shown to increase the amount of free molecules produced (12). The low-molecular-weight DNA collected 3 days after fusion was analyzed by restriction enzyme digestion and blot hybridization (Fig. 11). While 3B yielded

practically no material hybridizing to polyoma DNA (not shown), the low-molecular-weight DNA fraction from R5-1 and 4A contained a very heterogeneous mixture of DNA molecules containing viral sequences. These were resolved into more discrete fragments by the use of restriction enzymes, including *Bgl*II, which does not cleave polyoma DNA (Fig. 11a). It became apparent that the main bands produced by restriction enzyme digestion of the low-molecular-weight DNA from these fusions were identical to the viral fragments as well as to the virus-host

FIG. 11. Restriction enzyme and Southern blot analysis of low-molecular-weight DNA of *ts-a* R5-1 and *ts-a* 4A after polyethylene glycol-induced fusion with 3T3 cells. Either R5-1 or 4A cells were cocultivated overnight with an equal number of 3T3 cells followed by polyethylene glycol 1000 treatment as described in the text. After 2 to 3 days at 33°C, low-molecular-weight DNA was extracted, and the DNA preparations were analyzed by restriction enzymes and Southern blotting as described in the text. (a) Low-molecular-weight DNA of *ts-a* 4A after fusion with 3T3 cells. Lane: 1, Py DNA marker digested with *Bgl*II; 2, low-molecular-weight DNA from 4A fused with 3T3 cells, no enzyme; 3, low-molecular-weight DNA from 4A fused with 3T3 cells and digested with *Bgl*II; 4, Py DNA marker digested with *Hind*III; 5, low-molecular-weight DNA from 4A fused with 3T3 cells and digested with *Hind*III. (b) Py DNA (m), low-molecular-weight DNA from *ts-a* R5-1 fused with 3T3 cells (1), or 3 µg of *ts-a* R5-1 high-molecular-weight DNA (2). No enzyme was used for DNAs of the first two lanes. I and II indicate the positions of form I and form II Py DNA, respectively.

linker fragments detected in the restriction enzyme analysis of integrated viral DNA in these lines (compare with Fig. 8 and 9). To demonstrate that this finding was not due to contamination of low-molecular-weight DNA with the higher-molecular-weight fraction, form I free DNAs were purified through ethidium bromide-CsCl density gradients. In addition, we ran on the same gel an amount of high-molecular-weight DNA from these lines equivalent to a 25% contamination of the low-molecular-weight Hirt fraction with high-molecular-weight DNA (contamination is generally on the order of 5%). The viral DNA-containing bands produced by this amount of high-molecular-weight DNA were almost undetectable, showing that the fragments obtained in the low-molecular-weight fraction were indeed derived from free viral forms (Fig. 11b).

The most likely interpretation of these results is that the heterogeneous free viral DNA molecules produced by R5-1 and 4A upon fusion with mouse cells contain host sequences corresponding to those flanking the viral insertion, which explains not only the similarity of the free DNA restriction pattern with that of integrated DNA, but also the fact that these forms are cleaved by *Bgl*II, an enzyme specific for host sequences (Fig. 11a).

DISCUSSION

The studies presented in this manuscript on excision and amplification in polyoma-transformed cell lines indicate, in addition to a requirement for large T-antigen function previously demonstrated (2, 3, 7), a strict requirement for intramolecular homology (i.e., repeats) in the integrated viral DNA sequences both for excision and amplification.

Excision. We previously showed that in cells containing full tandem repeats of integrated viral DNA molecules, excision consisted of the removal of an integral number of viral genomes, leaving unchanged the host-virus DNA junctions (3).

This conclusion was strengthened by the results obtained with the *ts-a* H3A and *ts-a* 9 lines. In H3A, two different molecules of 5.1 or 4.6 kb can be excised because of the presence of two different deletions in the regions of homology of the integrated viral sequences. This can be explained if homologous recombination occurred within the viral sequences, between 45 and 60 m.u. or 30 and 40 m.u., respectively (see Fig. 2). In *ts-a* 9, where no deletions are present in the viral sequences, recombination can produce and excise only one type of molecule, i.e., a full-length genome.

On the other hand, in transformed cell lines containing viral insertions corresponding to less

than one Py genome, no excision comparable to the one described above can be detected. The frequency of revertants isolated (indicative of excision) was extremely low and independent of the presence or absence of a functional large T-antigen. Moreover, a large majority of the revertants isolated showed no apparent changes in the integrated viral sequences, suggesting that they owed their phenotypic change to undetected mutations in the viral or host DNA. A few revertants showed a change in the integration pattern which was difficult to interpret and suggested rearrangements of the integrated sequences rather than excision as previously defined.

It could be argued that the single-copy lines we studied do not synthesize a functional large T-antigen because of undetected mutations, deletions, etc. This is unlikely for the following reasons. (i) A full-size large T-antigen can be immunoprecipitated (15) from extracts prepared from these lines at 33°C. (ii) These cell lines exhibit autoregulation of early transcription by large T-antigen (Fenton and Basilico, *Virology*, in press), a process that would obviously require some T-antigen function. (iii) In two of the cell lines, fusion with 3T3 cells at 33°C (but not at 39°C) results in the production of free viral DNA molecules. These molecules must have undergone some replication to be detected, a process which again implies the presence of a functional large T-protein.

In conclusion, excision of integrated Py molecules occurs at a high rate in transformed rat cells carrying fully or partially duplicated copies of viral genomes, whereas no such event has been observed in single-copy Py-transformed cell lines. This implies that excision requires homologous recombination and that this event is somehow triggered by Py large T-antigen.

Amplification. The results obtained with the *ts-a* 9 cell line, which contains a partial tandem of integrated viral DNA sequences, are very similar to those previously reported for the H3A cell line (7). Amplification occurs at a high rate and is internal and dependent on the presence of a functional large T-antigen.

On the other hand, no amplification was detected in the single-copy lines 3B and 4A even after 8 weeks of propagation at 33°C. The propagation of R5-1 at the low temperature for the same period caused the appearance in a small portion of the cell population of a different integration pattern. A detailed analysis of the DNA from these cells has shown that a duplication had occurred on the left side of the insertion, likely to involve both viral and host DNA. The viral part of the duplicated fragment (~60 to 10 Py m.u.) contains the viral origin of replication, while the cellular part includes DNA se-

quences adjacent to the left border of the viral insertion. The phenomenon observed can probably be ascribed to a recombination event involving regions of partial homology existing between the viral and the adjacent host DNA sequences.

Free viral DNA production. The production of nonintegrated viral molecules has been tested in both the *ts-a* 9 line and the single-copy lines. As expected, the former one produces free DNA corresponding to full-length Py genomes. The latter lines, on the other hand, are not able to spontaneously produce any form of free DNA. After fusion of these cells with mouse 3T3 fibroblasts, a heterogeneous population of free molecules containing viral sequences becomes detectable, and these molecules appear to contain host as well as viral DNA sequences.

The lack of spontaneous free DNA production in R5-1, 3B, and 4A in the presence of a functional large T-antigen indicates that this protein is necessary, but not sufficient, in determining this event. Fusion with 3T3 cells could promote either the replication (and therefore the detection) of a very small number of free molecules spontaneously produced or recombination *per se*. Either of these processes would allow the detection of rare recombination events which could take advantage of partial homology between viral and flanking host sequences.

Mechanism of excision and amplification. The data presented on excision, amplification, and free viral DNA production indicate that in addition to an active Py large T-antigen, full or partial tandem integrations are required for these phenomena to take place. This supports the hypothesis that homologous recombination plays a decisive role in these events that could be initiated by any of several mechanisms.

It is possible that "permissive" factors provided by the cells can activate Py large T-antigen, which then binds the integrated viral origin of replication, inducing replication *in situ* (6). When duplicated regions of Py genomes are present, homologous sequences can recombine, producing complete viral molecules that can be removed and circularized as free DNA. If the parental strands are involved in this recombination event, this would result in physical excision of integrated viral DNA and generate "cured" cells. Finally, the newly replicated molecules could recombine with homologous sequences of the parental strands, leading to an increase in the copy number of integrated viral genomes. The process of replication itself may allow the "exposure" to recombination of existing homologous sequences by introducing transient nicks and gaps in these regions. In single-copy lines, even after initiation of DNA replication, the lack of homologous sequences in the original viral insertion would drastically decrease the proba-

bility of recombination and thus the occurrence of all subsequent events. Alternatively, recombination could take place between the viral DNA and adjacent cellular DNA that shows partial homology with Py sequences, resulting in a low and variable rate of excision or amplification.

It is also possible that recombination occurs without replication, implying that Py large T-antigen can have a recombination-promoting activity, similar, for example, to that of the Rec A protein, independent of its role in viral DNA replication. In this case, the regions of homology would determine the size of the DNA segment that will become the "unit" involved in excision or, after replication, in amplification. The composite free viral molecules containing viral and host DNA, produced in the single-copy lines, might be explained on the basis of partially homologous sequences existing between viral and cellular DNA that would determine the size of the molecules to be excised or amplified.

Another possible mechanism for the generation of excision and amplification (but not free viral DNA production) in polyoma-transformed rat cells with tandem viral integrations could be that of unequal sister chromatid exchange (27), resulting from the out-of-register pairing of the repeated regions of the viral DNA insertions. This mechanism, however, predicts not only that large T-antigen promotes sister chromatid exchange (6a), but also that for any excision event taking place, a parallel amplification event should occur. This is not supported by our data on the rate of appearance of cured and amplified cells in our transformed lines (unpublished data).

It has recently been shown that recombination between 12-base pair sequences of perfect homology is enough to generate tandem duplications of the AmpC gene in *Escherichia coli* and that this event is promoted by the Rec A gene product (10). However, the necessary extent of homology as well as the effect of chromatin configuration or other cellular factors or both on appropriate pairing in our system is unknown. Cloning of the viral insertion and sequencing the fragments of adjacent cellular DNA would provide the basis to search for possible homology. Indeed, the results obtained by Bullock and Botchan (6b), who studied the heterogeneous free viral DNA molecules produced by a simian virus 40-transformed rat line upon fusion with permissive monkey cells, suggest that the usage of partial virus-host homology can well explain the generation of these molecules.

ACKNOWLEDGMENTS

We thank E. Deutsch, P. Santanello, and N. Porzio for skilled technical assistance.

This investigation was supported by Public Health Service grants CA11893 and CA16239 from the National Cancer Institute.

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