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High-Level Recombination Specific to Polyomavirus Genomes Targeted to the Integration-Transformation Pathway†

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An unusually high incidence of interviral recombination was found in the process of integration of the polyomavirus genome concomitant with neoplastic transformation of nonpermissive cells. Transformants were isolated after mixed infections of Fischer rat cells with two mutants lacking restriction endonuclease sites and were analyzed for the presence of unselected integrated recombinant restriction fragments. A large fraction of the transformants isolated (38% of the 64 transformed cell lines studied) contained recombinant viral genomes that had undergone recombination in a 1.3-, 1.7-, or 3.6-kilobase-pair interval. More than 90% of these recombinant transformants showed evidence of crossovers in multiple intervals. To our knowledge, the recombination frequencies observed in these experiments represent the highest frequencies of homologous recombination reported for a mitotic mammalian system that does not involve transfection. In contrast to the elevated level of recombination in the integrated viral genomes, no evidence of recombination was obtained among the replicated unintegrated pool of viral genomes isolated from the same population of infected cells from which the recombinant transformants were derived. Either of two hypotheses can provide an explanation for the segregated recombination: either recombination occurs at elevated levels in a small, recombination-prone fraction of the population destined to become transformed, or recombination occurs only among those viral genomes which are engaged in the process of integration and thus interact with a recombinogenic host machinery (for example, the host scaffold). We favor the latter hypothesis.

During the events leading to neoplastic transformation of nonpermissive cells by polyomavirus and simian virus (SV40), the viral genome becomes integrated into the host DNA by a mechanism that is facilitated by a viral protein, large T antigen (9, 24). Usually, the integrated viral DNA is present in head-to-tail tandem copies of the viral genome (1, 2, 4), and integration can occur at multiple sites in the genome of the transformed cell (1, 4). Integration occurs by nonhomologous recombination with host DNA and is apparently random with respect to both viral and host sequences (2, 4). At the virus-host junctions, homologies of 2 to 5 base pairs (bp) have been found (5, 19, 25). Rearrangements and deletions of host DNA are usually associated with the integration events (26, 28).

Models to explain the tandem topology of integrated viral genomes have invoked a role for either replication (e.g., rolling-circle type) (7, 8) or recombination (11, 15). Basilico and collaborators have shown that the large-T-antigen function (9) and the viral origin of replication (8) are required for tandem formation, consistent with a role for viral DNA replication. A replication step has also been postulated for SV40 by Chia and Rigby (7), based on the existence of unintegrated high-molecular-weight species of viral DNA, which were assumed to represent the unintegrated precursors of the transforming viral genomes and which were predominantly (but not exclusively) nonrecombinant. Such replication models have suggested rolling-circle replication for the generation of multimeric DNA. Although support for low-level rolling-circle replication has been obtained in infections of permissive cells by polyomavirus (3), rolling circles were not seen in Fischer rat cells in preliminary experiments using the electron microscope (E. Boy de la Tour and M. M. Fluck, unpublished data). Furthermore, very high molecular weight unintegrated replication intermediates (larger than 20 kbp) were not detected in infections of Fischer rat cells during an extensive search using sucrose gradient separation (17).

Support for involvement of intervirus recombination in tandem formation comes from studies of integrated viral genomes. Previous results from our laboratory based on studies of transformants derived from mixed infections, have suggested that two parental genomes can cointegrate during the integration process (11, 15). To directly analyze and quantitate the role of intervirus recombination in the process of integration-transformation of the polyomavirus genome in nonpermissive cells, we performed mixed infections with a pair of mutants which lack restriction endonuclease sites and followed the generation of recombinant wild-type restriction fragments in the population of cells early after infection as well as in the transformed cells derived from this population. We show that integrated recombinant restriction fragments were present in a high percentage of the transformed cells. No evidence of recombination was observed, however, in the population of unintegrated viral genomes at early times after infection. The results suggest an important role for recombination in the integration pathway of polyomavirus.

MATERIALS AND METHODS

Cells and viruses. Fischer rat F-111 cells (14) were cultured as previously described (29). Before infection, the cultures were either maintained as actively dividing cells or allowed to reach confluency and held in that state for 24 h before passage for infection.

Polyomavirus mutants MOP1033 (27) and ts3 (10) were

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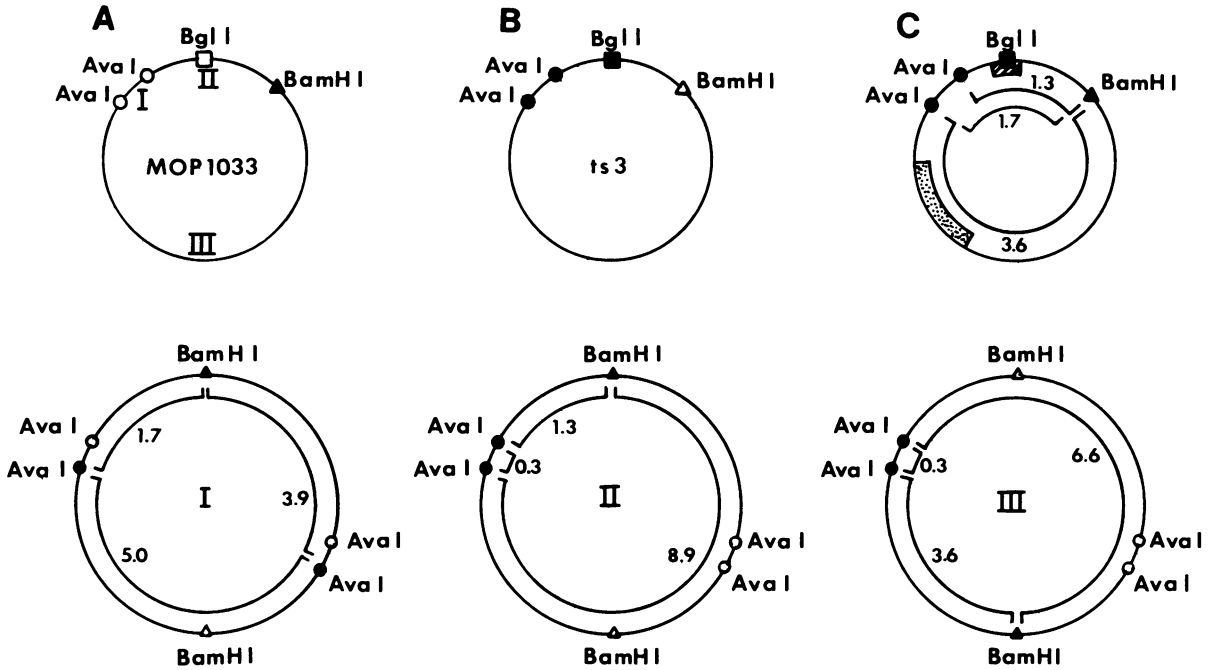

FIG. 1. Partial restriction maps of parental virus strains and potential recombinants. (A) Mutant MOP1033, which lacks both *Ava*I sites at nucleotides 672 and 1031 as well as the *Bgl*I site at nucleotide 102 (numbered as in reference 16). (B) Mutant ts3, which lacks the *Bam*HI site at nucleotide 4647. (C) Wild-type or double-recombinant genome. The sizes of the three possible *Ava*I-*Bam*HI fragments are given. I, II, and III refer to the three *Ava*I-*Bam*HI intervals in which recombination can occur. The results of a conservative and reciprocal crossover in each of these three intervals are shown, presented as the simplest recombination products between monomeric circles (the input DNA) which would produce multigenomic DNA (as found to be the product of the integration event). The integration event will open such dimers. Sizes of the *Ava*-*Bam*HI fragments of these recombinants are given. The map also shows the locations of *Hpa*II-5 (▨) and the *Eco*RI-*Xba*I fragment (□) used as hybridization probes. Closed and open symbols for *Bgl*I, *Bam*HI, and *Ava*I represent wild-type and mutant sites, respectively.

gifts of W. Eckhart. Stocks of these mutant strains were grown on baby mouse kidney cultures from plaque-purified virus as described previously (30). MOP1033 was derived from a wild-type strain by site-directed mutagenesis of nucleotide 1033 (29) (numbered as in reference 16). The point mutation introduced into the middle-T-antigen reading frame produced a transformation-defective virus and eliminated the *Ava*I site which starts at position 1031. The mutation at nucleotide 1033 also introduced a proline-to-leucine change in large T antigen, which still allowed normal large-T-antigen function of this mutant as judged by its normal growth pattern (27). As discussed in Results, we believe that the large-T-antigen mutation is irrelevant in the experiments described below. The *Ava*I restriction site at nucleotide 672 in the large-T-antigen intron is also absent in MOP1033 (Fig. 1A). This mutation alters the middle-T-antigen sequence without altering its transformation potential, since repair of MOP1033 at the 1033 position restores the transforming potential of this strain (27). The results presented below clearly confirm this point.

The ts3 mutant (10) was derived from a wild-type virus (strain LP, related to A2) by bisulfite mutagenesis. A mutation within the VP2 gene that prevents decapsidation at the nonpermissive temperature was obtained. Since ts3 lacks the *Bam*HI site (nucleotide 4647), also located within the VP2 coding region, it is assumed that the *Bam*HI site is the site of the decapsidation mutation, although this has not been proven (W. Eckhart, personal communication). ts3 transforms normally after a short decapsidation period at 33°C (10, 12).

Isolation of transformed cells. F-111 cells seeded at a density of 10^5 cells per 60-mm culture dish were coinfected with MOP1033 and ts3 at a 1:1 ratio or infected with either of these two mutants alone. The total multiplicity of infection for the coinfections was 200 PFU per cell except when noted otherwise. The multiplicities of the single parent infections were 100 PFU per cell except when noted otherwise. Infections were carried out at 33°C for 90 min. Cells were then fed with Dulbecco modified Eagle medium with 5% heat-inactivated calf serum and antibiotics. To ensure decapsidation of the ts3 parent, infected cells were maintained at 33°C for 24 h after infection. Some of the infected cultures were then moved to 37°C, while the others were maintained at 33°C for the duration of the experiment. Transformed foci which overgrew the monolayer were transferred to 35-mm culture dishes and grown in Dulbecco modified Eagle medium with 5% heat-inactivated calf serum. In some cases, the transformed cells isolated from foci were cloned in agar.

Preparation and analysis of DNAs. For analysis of integration sites, approximately 10^7 cells from each cell line were lysed with 0.2% sodium dodecyl sulfate, 10 mM Tris hydrochloride (pH 7.5), and 10 mM EDTA (2 to 3 ml/100-mm dish). Total cellular DNA was extracted as previously described (15). For hybridization analysis, 10 μg of total cellular DNA was digested with a combination of *Ava*I and *Bam*HI. Digested DNAs were electrophoresed on 0.7% agarose gels and transferred to nitrocellulose (23). Hybridization was carried out in 2× SSC (SSC is 0.15 M NaCl plus 0.015 sodium citrate)–1× Denhardt solution (0.1 ml/cm^2) at 65°C for 36 to 48 h, using 5×10^5 to 10×10^5 cpm/ml of hybridization solution.

For analysis of recombination at early times after infec-

tion, viral DNA was isolated from infected cells by the procedure of Hirt (20). Rat F-111 cells were plated and infected as described above. At each time point, viral DNA was extracted from one 60-mm dish. One-tenth of the DNA from each plate was digested with a combination of *Ava*I and *Bam*HI. Gel electrophoresis and DNA transfer were performed as described above. Hybridization was conducted at 65°C for 18 to 24 h, using 5×10^5 cpm/ml of hybridization solution.

For analysis of the presence of the recombinant viral genomes as integrated structures, low- and high-molecular-weight DNA from recombinant transformant M/t III-1A (see Results) was prepared as described elsewhere (20). The high-molecular-weight DNA fraction was digested with *Bgl*II and then fractionated on a 10 to 40% sucrose gradient. The sucrose solution was made up in 20 mM Tris hydrochloride (pH 8.0)–1.0 M NaCl–5 mM EDTA. Centrifugation was at 23,000 rpm for 24 h at 20°C in an SW27 rotor. Fractions of 1 ml were collected and pooled as described in Results. The pooled DNAs were digested with *Eco*RI or with a combination of *Ava*I and *Bam*HI and analyzed as described above. Hybridization was to the complete polyomavirus genome.

Hybridization probes were labeled to a specific activity of 1×10^9 to 2×10^9 cpm/μg of [^{32}P]dCTP (3,000 ci/mmol), using a multiprime DNA-labeling kit (Amersham Corp., Arlington Heights, Ill.). *Hpa*II-5 and *Eco*RI-*Xba*I restriction fragments (Fig. 1) and the *Eco*RI-digested polyomavirus genome were isolated in low-melting-point agarose (FMC Bioproducts, Philadelphia, Pa.) before labeling.

RESULTS

Description of the recombination assay. To determine the frequency of intervirus recombination in the process of integration of the polyomavirus genome in nonpermissive cells, we coinfected Fischer rat F-111 cells with two restriction site-minus mutant strains (MOP1033 [27] and ts3 [10]), a scheme previously used by Chia and Rigby for SV40 (7). MOP1033 lacks the two AvaI restriction sites located at nucleotides 672 and 1031 in the early region of the viral genome (Fig. 1A). The mutation at 672 alters the middle-T-antigen sequence without altering its transformation potential, as previously described (27) and as confirmed by the results presented here. The point mutation within the second site (at nucleotide 1033) causes premature termination of middle T antigen and therefore a defect in cell transformation. This mutation also alters the large-T-antigen sequence without altering its function in replication, since the MOP1033 mutant strain grows normally (27). The second mutant strain, ts3, lacks the *Bam*HI restriction site in the late region of the genome (nucleotide 4647) (Fig. 1) and harbors a mutation which prevents decapsidation of the virus at the nonpermissive temperature. The mutation at the *Bam*HI site may be the cause of the decapsidation defect. Transformation rates with ts3 are normal as long as the infected cells are maintained at 33°C for the decapsidation period (the first 24 h), regardless of the incubation temperature beyond this time (see Table 2) (10, 12). As argued and demonstrated below, we reason that recombination between these two strains should not generate a strain with a transformation potential greater than that of the ts3 parent and thus does not offer a selective advantage for transformation.

Recombination between the two viral genomes can be assessed by analysis with restriction endonucleases. Transformation-competent recombinants must carry the normal *Ava*I site at nucleotide 1031. Digestion of those recombinants with a combination of *Bam*HI and *Ava*I will produce restriction fragments of 3,616 bp if homologous recombination has occurred in the large *Ava*I-*Bam*HI interval and fragments of 1,317 or 1,676 bp if the two parental genomes have recombined in the small *Ava*I-*Bam*HI interval or between the two *Ava*I sites, respectively (Fig. 1). Reciprocal recombination events would produce fragments of 3,971, 4,933, 6,603, and 8,908 bp (Fig. 1). The two parental genomes can also be detected, since codigestion with *Ava*I and *Bam*HI will produce a single genome-size restriction fragment (5,292 bp) from the MOP1033 genome and two fragments, 4,933 and 359 bp, from the ts3 genome. In these experiments, the 359-bp fragment was usually electrophoresed past the end of the gel. The presence of the 3,616-bp *Ava*I-*Bam*HI fragment was traced by hybridization with the polyomavirus 917-bp *Eco*RI-*Xba*I fragment (Fig. 1C), and the 1,317- and 1,676-bp *Ava*I-*Bam*HI fragments were detected by using the polyomavirus *Hpa*II-5 400-bp fragment (Fig. 1C). Reciprocal recombinant fragments, except the 3,971-bp fragment, can be detected by either probe; the 3,971-bp fragment can be detected only by the *Eco*RI-*Xba*I probe.

Absence of recombination in the population of unintegrated viral genomes. Monolayers of Fischer rat F-111 cells were infected with a combination of MOP1033 and ts3 as described in Materials and Methods. After the decapsidation period, the monolayers were maintained at either 33 or 37°C. Intervirus recombination in the population of unintegrated viral genomes (replicated or parental) was monitored from the time of infection until the time when transformed foci appeared in the monolayers. Viral DNA was extracted by the Hirt separation method (as described in Materials and Methods) and digested with a combination of *Ava*I and *Bam*HI. Results of a typical experiment using hybridization with the *Eco*RI-*Xba*I probe are shown in Fig. 2. The parental restriction fragments (5.3- and 5-kbp fragments of MOP1033 and ts3) but no recombinant *Ava*I-*Bam*HI fragment (3.6 kbp) were present at detectable levels in the population of infected cells at the times indicated. The same was true for the 1.3- and 1.7-kbp recombinant fragments detected by the *Hpa*II-5 probe (not shown). This result was obtained even when longer film exposures were used. From a reconstruction experiment, we estimate that we would be able to detect a 5% level of recombinant wild-type fragment (not shown). Viral DNA replication occurred at both 37 and 33°C, as evidenced by the increase in hybridization intensities over the input signal, and replication was higher at 33 than at 37°C (Fig. 2), a common finding in Fischer rat cells (17, 18).

High level of recombination in integrated genomes. Independent transformed foci arising from the infections described above were visible by 10 and 17 days postinfection for the 37 and 33°C cultures, respectively. After the foci were isolated, total DNA was extracted from the transformants and analyzed for integrated recombinant viral genomes as described above. The integration analysis confirmed the independent derivation of the transformed foci.

(i) Recombination in the 3.6-kbp *Ava*I-*Bam*HI interval. Recombination in the 3.6-kbp interval was analyzed by hybridizing blots of the *Ava*I-*Bam*HI digestions with the polyomavirus *Eco*RI-*Xba*I probe. The results for nine transformants are shown in Fig. 3A. Seven transformants from this set had acquired a 3.6-kbp fragment, indicating that recombination between the two had occurred. The 3.6-kbp *Ava*I-*Bam*HI restriction fragment was present in 23 (35%) of the 65 cell lines analyzed (Table 1). In addition to the recombinant fragment, either or both of the 5.0- and 5.3-kbp

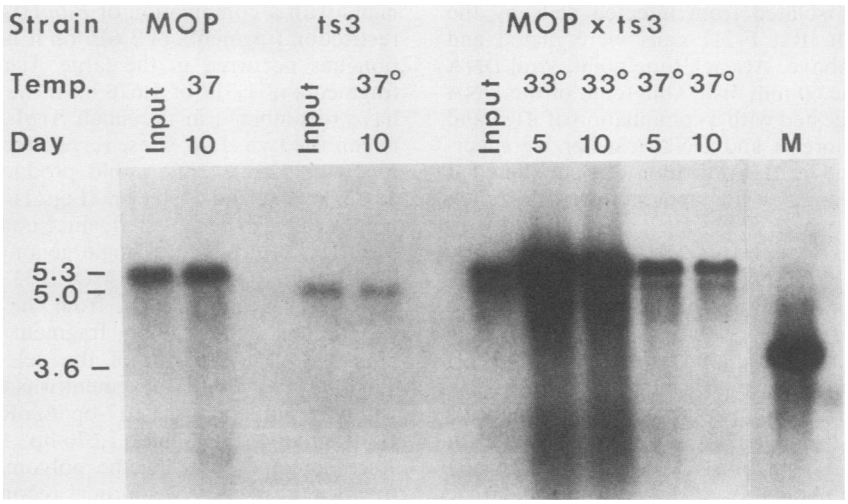

FIG. 2. Analysis of recombination in unintegrated viral genomes. F-111 cells were infected with MOP1033 or ts3 alone or in combination, using a multiplicity of 50 PFU of each per cell. The results shown are from experiment 6 (Table 1). For each time point, the viral DNA from one plate of infected cells was extracted by the Hirt procedure as described in Materials and Methods. One-tenth of each sample was digested with a combination of *Ava*I and *Bam*HI and hybridized with the *Eco*RI-*Xba*I probe. Lane M represents wild-type DNA. Relevant sizes (determined with markers) are shown: the 3.6-kbp *Ava*I-*Bam*HI wild-type fragment, the MOP1033 genome-size (5.3-kbp) fragment, and the large (5.0-kbp) ts3 fragment. The input concentration was determined at 24 h, when no replication had yet occurred.

fragments from the two parental viral genomes were also visible in many cases (Fig. 3A, lanes 1, 3, and 5). Both parental genomes were present in 14 of 23 cell lines, and a single parental genome was present in 6 of 23 cell lines. Usually, the parental genomes were present at lower levels than were the recombinant genomes.

(ii) Recombination in the 1.3- and 1.7-kbp *Ava*I-*Bam*HI intervals. Blots from the analysis described above were washed and then hybridized with *Hpa*II-5 to probe for recombination within the small *Ava*I-*Bam*HI interval or between the *Ava*I sites (Fig. 3B). In many of the cell lines, both the 1.3- and 1.7-kbp restriction fragments were present. As discussed above, the 5.0- and 5.3-kbp fragments from the two parental genomes were also visible. Either the 1.3- or the 1.7-kbp fragment was present in 24 (37%) of the 65 cell lines analyzed. In all, 25 (38%) of the 65 transformants contained at least one of the three fragments that was diagnostic of recombination between MOP1033 and ts3. In most cases, more than one of the recombinant restriction fragments was present in a given transformant. For example, all seven recombinant transformants shown in Fig. 3 contained the 3.6-kbp fragment in addition to either or both of the 1.3- and 1.7-kbp fragments.

Structure of the integrated viral genomes. The transformants in which the parental genomes had undergone recombination had the following hallmarks of normal polyomavirus transformants: head-to-tail tandem integration of the recombinant genomes, as evidenced by the presence of 5.3-kbp genome-size restriction fragments in digestions with a single cutter such as *Eco*RI or *Bam*HI (one example is shown in Fig. 4A, lane 3), multiple sites of integration, as shown by the presence of multiple restriction fragments in digests with a zero cutter such as *Bgl*II (Fig. 4B), and free viral DNA, as shown by the presence of genome-size supercoiled (form I) polyoma DNA in digests with the zero cutter *Bgl*II (Fig. 4A, lane 4). Along with recombinant viral genomes, parental genomes were also present in most of the transformants. The parental genomes were integrated as head-to-tail tandems, as evidenced by the presence of genome-size restriction fragments (5.0 and 5.3 kbp) in the products of codigestions with *Ava*I and *Bam*HI (Fig. 3).

The following set of experiments was designed to ensure that the recombinant viral sequences were present in integrated form in the host genome rather than produced in postexcision recombination events between genomes of parental type generated from integrated parental sequences in those cell lines. For this purpose, one cell line (M/t III-1A) containing all three recombinant fragments was analyzed further by first separating the DNA into high- and low-molecular-weight fractions by the Hirt procedure (20). To eliminate trapping of unintegrated low-molecular-weight viral sequences in the high-molecular-weight fraction, the latter was digested with *Bgl*II (which does not cleave polyomavirus DNA) and then fractionated by using a sucrose gradient. DNA sizes were determined by gel electrophoresis, and fractions representing DNA larger than 15 kbp and those of sizes between 4 and 15 kbp were pooled separately. The >15-kbp fraction was electrophoresed under two conditions. On a 0.7% agarose gel (short electrophoresis), this fraction contained polyomavirus sequences which migrated as a major broad band at limit entry (Fig. 4A, lane 1). This band separated into four to five bands of sizes larger than 22 kbp in a longer electrophoresis on a 0.4% agarose gel (Fig. 4B). As expected, very little genome-size (unintegrated) DNA was present in the high-molecular-weight (>15 kbp) fraction (Fig. 4A, lane 1, and 4B). The 4- to 15-kbp fraction, on the other hand, contained mostly free viral DNA, as evidenced by bands migrating at the position of linear and supercoiled polyoma DNA (Fig. 4A, lane 4). When the DNAs from the two fractions were digested with a combination of *Ava*I and *Bam*HI, identical patterns were obtained; that is, the three recombinant restriction fragments were generated (3.6, 1.7, and 1.3 kbp), as well as a fragment of polyomavirus size (Fig. 4A, lanes 2 and 5; the 1.3-kbp band is visible in lane 2 upon longer exposure). Conversion of the high-molecular-weight species of the polyomavirus genome, which was presumably integrated, into the three recombinant bands seen in lane 2 strongly suggested that the

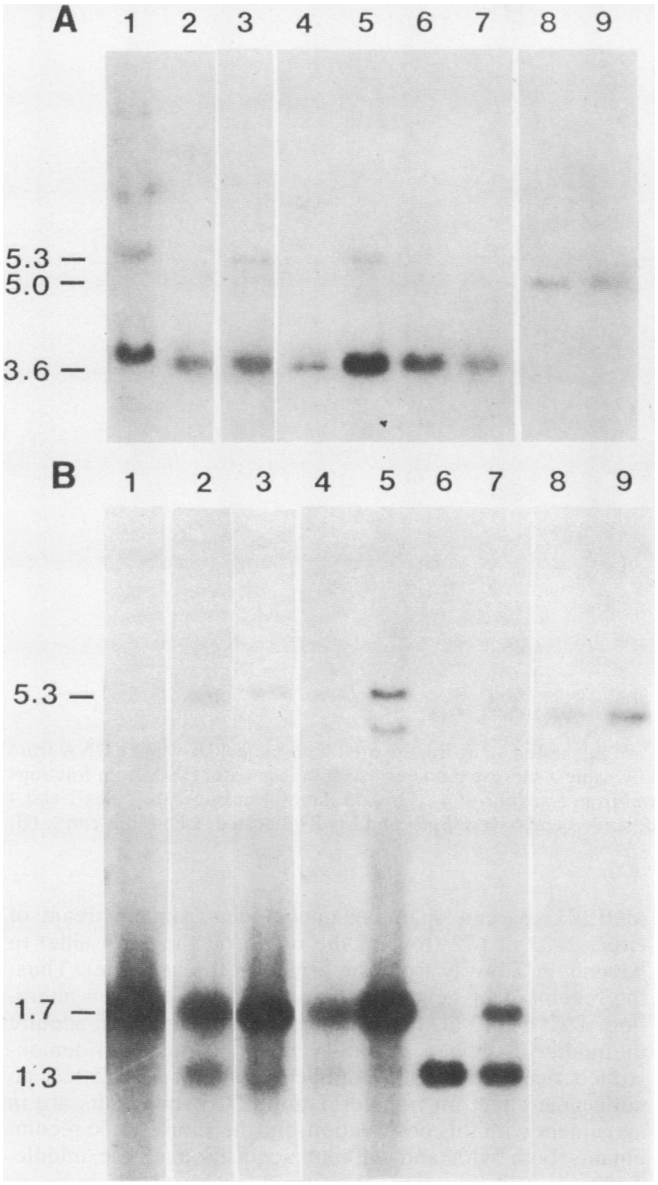

FIG. 3. Recombination in integrated viral genomes. (A) Recombination in the 3.6-kbp *Ava*I-*Bam*HI interval. Total cellular DNA (10 μg) was digested with a combination of *Ava*I and *Bam*HI. Hybridization was to the polyomavirus *Eco*RI-*Xba*I probe. The cell lines shown in the nine lanes were established from the 37°C coinfections in experiments 6 and 7 (Table 1). Locations of the 3.6-kbp *Ava*I-*Bam*HI fragment, the MOP1033 genome-size (5.3-kbp) fragment, and the large (5.0 kbp) *Ava*I fragment from ts3 are shown. (B) Recombination in the 1.3-kbp *Ava*I-*Bam*HI and 0.3-kbp *Ava*I intervals. Conditions were as described above. Locations of the 1.3- and 1.7-kbp *Ava*I-*Bam*HI fragments are shown, except that hybridization is to *Hpa*II-5.

recombinant genomes were integrated. Since this particular transformant apparently contained only one of the parents (MOP1033), the hypothesis that the recombinant genomes were indeed integrated is given further support. The analysis also demonstrates that the unintegrated viral sequences represented a faithful copy of the integrated sequence. Given the apparently high number of integration sites, it is very interesting that the recombinant sequences predominated over the parental sequences (as mentioned above, a low

TABLE 1. Summary of recombination events in transformed cell lines

Expt no.[a]	Transformants with *Ava*I-*Bam*HI fragment[b] at a temp of:							
	33°C				37°C			
	1.3 kbp	1.7 kbp	3.6 kbp	Total	1.3 kbp	1.7 kbp	3.6 kbp	Total
1					2/6	0/6	2/6	2/6
2					0/5	0/5	1/5	1/5
3	0/3	0/3	0/3	0/3	2/10	1/10	2/10	2/10
4	0/7	0/7	0/7	0/7	3/4	3/4	3/4	3/4
5	2/4	0/4	1/4	2/4	3/4	2/4	3/4	3/4
6	0/5	1/5	1/5	1/5	3/6	4/6	4/6	4/6
7	0/5	2/5	1/5	2/5	5/6	5/6	5/6	5/6
Total	2/24	3/24	3/24	5/24	18/41	15/41	20/41	20/41
	8 events/5 transformants				53 events/20 transformants			

[a] For experiments 1, 3, 5, and 7, the cell cultures were confluent before passage for infection. For experiments 2, 4, and 6, the cell cultures were exponentially grown before passage for infection. See Materials and Methods for details.

[b] Presence of the 1.3- and 1.7-kbp *Ava*I-*Bam*HI restriction fragments was detected by hybridization to the *Hpa*II-5 probe. Presence of the 3.6-kbp fragment was detected by hybridization to the *Eco*RI-*Xba*I probe. See Fig. 1 for identification of these fragments. Overall, experiments at 33°C gave 8 events per 5 transformants and those at 37°C gave 53 events per 20 transformants.

level of the MOP1033 parent could be detected in this cell line [Fig. 4A]). Similar results have been obtained for other cell lines in less extensive analyses.

Conditions which affect recombination. Previous results from this laboratory have shown that the level of viral DNA replication in nonpermissive cells is higher at 33 than at 37°C (17, 18). The same results were obtained in this experiment (Fig. 2). To determine whether the level of replication affects recombination, we isolated transformants from cells maintained at either 33 or 37°C after the 24-h decapsidation incubation at 33°C. Of the 24 cell lines isolated from 33°C infections, 5 (21%) were positive for one of the recombinant fragments, whereas at least one of these fragments was detected in 20 (49%) of 41 cell lines isolated from cultures switched to 37°C (Table 1), corresponding to a 2.4-fold increase in recombination in the latter group over the former. The total number of recombination events per recombination-positive transformant followed the same pattern: 8 recombination events in 5 recombinant transformants at 33°C versus 53 events in 20 transformants in the 33-to-37°C switch, i.e., a 1.7-fold increase in the latter group.

To begin analyzing which factor(s), if any, may affect recombination, cells were maintained in different growth states before infection (Table 1). For exponential cells, the cultures were maintained in a state of active cell division before passage for infection. For confluent cells, the cultures were allowed to become confluent and were maintained this way for 24 h before passage for infection. This population of cells was thus essentially synchronized at the time of infection (though not rigorously so). These two protocols did not noticeably alter the recombination frequency (Table 1). Interestingly, the transformation rates in recombination-dependent (crosses with two transformation-defective mutants) and recombination-independent (infections with wild type) transformation events peaked at the same phase of the cell cycle (H. H. Chen and M. M. Fluck, manuscript in preparation).

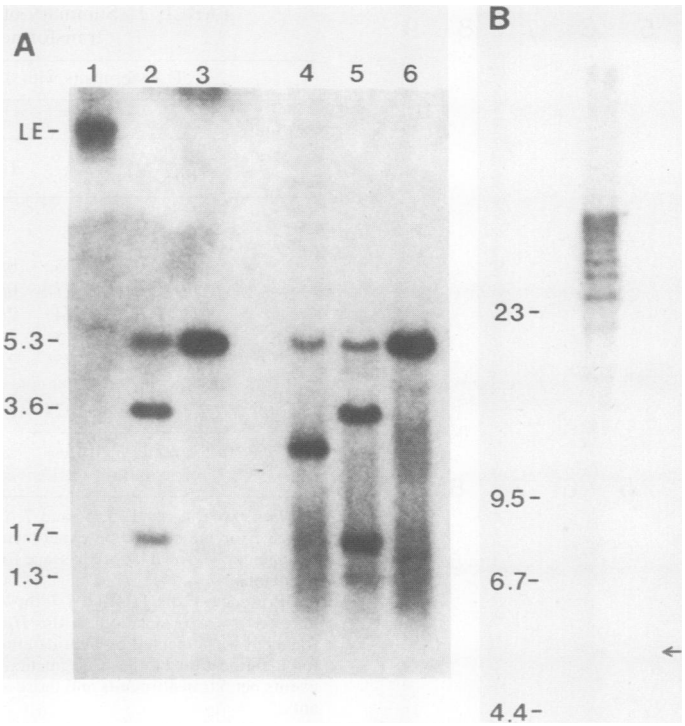

FIG. 4. Further demonstration that the recombinant viral sequences are integrated. Hirt-fractionated high-molecular-weight DNA from transformant M/t III-1A was digested with *Bgl*II and further fractionated by using a sucrose gradient. (A) 0.7% agarose; DNA from fractions containing restriction fragments larger than 15 kbp (lanes 1 through 3) and from fractions of 4 to 15 kbp (lanes 4 through 6). Lanes 1 and 4 were *Bgl*II digested; lanes 2 and 5 were *Bgl*II, *Ava*I, and *Bam*HI digested; lanes 3 and 6 were *Bgl*II and *Eco*RI digested. LE, Limit entry. (B) 0.4% agarose; *Bgl*II digested. The arrow points to the genome size.

Analysis of ts3 and MOP1033 viral stocks. Several approaches were used to ensure that the wild-type fragments observed in the transformed cell lines were indeed generated by recombination rather than due to a wild-type contamination of one of the viral stocks. First, genomic DNA from cell lines transformed by ts3 alone were analyzed by codigestion with *Ava*I and *Bam*HI. The blots were hybridized with the *Hpa*II-5 (Fig. 5) and the *Eco*RI-*Xba*I (not shown) probes. Of the 17 cell lines studied, none contained the 1.3-, 1.7-, or 3.6-kbp *Ava*I-*Bam*HI restriction fragment. Second, no transformants were ever obtained from infections with MOP1033, an indication that the stock of this nontransforming virus does not harbor a wild-type contaminant. Finally, analysis of the input viral DNA by codigestion with *Ava*I and *Bam*HI (Fig. 2) did not reveal the presence of any of the recombination-diagnostic restriction fragments.

Transformation frequencies in mixed infections with MOP1033 and ts3. To ascertain that recombination between the two parental genomes had no selective advantage over infection by the ts3 parent alone, transformation frequencies were measured in single and mixed infections (Table 2). The presence of the nontransforming virus decreased the transformation rate of the ts3 virus by 1.5- to 17-fold at 37°C. This effect (a dominant lethal effect) of a nontransforming parent in mixed infections has been previously noted (12) and is described in detail elsewhere (Chen and Fluck, in preparation). The transformation frequency at 33°C was 1.5- to 2-fold higher than at 37°C for the mixed infections, as is typical for cell transformation by polyomavirus (9, 17). In the recombination events depicted in these experiments, the ts3 parent must retain the wild-type transforming sequence at 1033 and acquires the *Ava*I-*Bam*HI sequence of the MOP1033 parent, spanning nucleotides from upstream of either 1033 or 672 (toward the origin on the early side) to beyond 4647 (away from the origin on the late side). Thus, the recombinant never acquires the large-T-antigen alteration of MOP1033 at site 1033, and it may or may not acquire the middle-T-antigen alteration at 672. It has been demonstrated that the middle-T-antigen mutation at 672 has no noticeable effect on transformation (27). Our results are in agreement with this observation. Furthermore, since recombinants both with and without acquisition of the middle-T-antigen 672 mutation have been observed, there is no reason to believe that acquisition of the mutant sequence at 672 represents a selective advantage. Similarly, acquisition of the *Bam*HI site at 4647 (which may represent the decapsidation mutation site in ts3) is not believed to present a selective advantage. By the time recombination occurs, decapsidation of ts3 must have already taken place. Furthermore, as discussed above, a short incubation at 33°C bypasses the decapsidation problem of ts3. Further evidence to support the hypothesis that the elevated level of recombination observed in the cross between MOP1033 and ts3 is not an artifact for these specific mutants or of the conditions in which these experiments were carried out is presented below.

DISCUSSION

The experiments described above were designed as an inquiry into the involvement of recombination between viral genomes in the process of the integration of the viral genome into the host genome concomitant with neoplastic transformation by polyomavirus. This inquiry originates from the

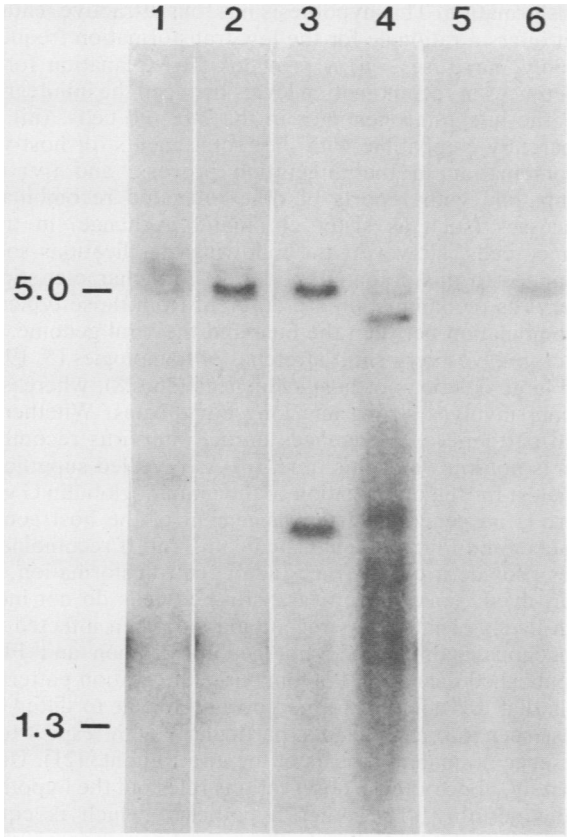

FIG. 5. Analysis of ts3-transformed cell lines. Transformants were established from infections with ts3, and total cellular DNA (10 μg) from these cell lines was digested with a combination of *Aval* and *Bam*HI. Results for six cell lines are shown. Hybridization was to the *Hpa*II-5 probe. Locations of the 1.3-kbp *Aval*-*Bam*HI fragment and the large (5.0-kbp) *Aval* fragment from ts3 are indicated.

observation that integrated genomes are organized in tandem arrays; intervirus recombination may provide a mechanism by which such tandems are formed. The design makes use of restriction endonuclease polymorphisms between different virus strains and avoids selection for intervirus recombination. One of the parental strains, ts3 (which lacks the unique *Bam*HI site), transforms normally, whereas the other, MOP1033 (which lacks the two *Aval* sites), is a transformation-defective mutant. Transformants were isolated from mixed infections and analyzed for the presence of unselected

TABLE 2. Transformation rates

Expt no.[a]	No. of transformants[b]			
	MOP1033[c]	ts3[c]	MOP1033 × ts3	
			33°C	37°C[c]
3	0	32	26	17
4	0	19	22	14
5	0	100	10	6
6	0	56	33	15
7	0	75	15	8

[a] Experiments are numbered as in Table 1.
[b] Based on infection of 10^5 cells at a multiplicity of 50 PFU per cell.
[c] Cultures were maintained at 37°C after the decapsidation period.

recombinant viral sequences. Our results can be summarized as follows.

(i) Recombinant viral genomes were observed at very high frequency among the transformants. Overall, 38% of the 64 transformants analyzed contained recombinant viral genomes. The fraction of recombinant transformants was somewhat variable from experiment to experiment and reached 83% (i.e., five of six independent transformants analyzed) in one experiment.

(ii) Recombinant viral genomes were integrated into the genome of the transformed cell lines.

(iii) The recombinant transformants contained all of the usual hallmarks of polyomavirus transformants: integration at apparently multiple sites of the host genome, integration of head-to-tail tandem arrangements of the viral genome, and production of unintegrated viral genomes.

(iv) The unintegrated population of viral genomes, presumably excised after in situ replication of the integrated genomes (4), faithfully reflected the integrated genomes.

(v) The recombinant transformants, which, as mentioned above, showed evidence for integration at multiple sites in the host genome, contained a predominance of recombinant over parental viral sequences, which suggests that most of the integration sites in these cell lines contained recombinant rather than parental genomes.

(vi) Many recombinant transformants contained evidence for three recombination events representing at least two types of recombinant genomes.

(vii) The frequency of recombinant transformants varied somewhat with the experimental conditions. Infected cells generated a 2.4-fold-higher level of recombinant transformants when shifted from 33 to 37°C past the decapsidation period than was generated upon continuous incubation at 33°C. Furthermore, the minimum number of recombination events in the 37°C recombinant transformants was also higher (by 1.7-fold) than in those isolated at 33°C.

(viii) The intervirus recombination assayed in this experiment was homologous by the criterion of generation of recombinant fragments of wild-type size and of genomes with transforming potential. Reciprocal recombination events have not been recovered in any of the recombinant transformants analyzed.

(ix) In contrast to the situation for the viral genomes integrated in transformed cells, no evidence for recombination was obtained in the pool of unintegrated viral genomes (replicated and parental) present in the same population of infected cells from which the transformants were isolated, at all times postinfection, up to the time when transformed foci started appearing in the infected monolayer.

High frequency of intervirus recombination in the integration pathway of polyomavirus. Our experiments demonstrate clearly that during infection of nonpermissive cells, viral genomes in the process of integration-transformation undergo very elevated levels of recombination. Intervirus recombination has been observed in many other virus systems (for a review, see reference 22). Our results for polyomavirus differ from those obtained with other viruses at least in that recombination is not seen in the whole population of viral genomes but only in those which become integrated in the host genome. As discussed in Results, there appears to be no selective advantage for recombination in the transformants resulting from mixed infections in this particular cross, since (i) one of the parents (ts3) can transform by itself, (ii) the addition of the MOP1033 parent in fact depresses the transformation level, and (iii) the acquisition of sequences from the MOP1033 strain by the ts3 strain

cannot confer a selective advantage to the recombinant. The elevated level of recombination observed in crosses between these mutants is in no way unique to this situation. Although recombination levels appear to vary from experiment to experiment (for this particular cross as well as others), the results from the experiments described here can be generalized, since we have documented elevated intervirus recombination in integrated viral sequences derived from multiple crosses in which recombination does or does not present a selective advantage (C. Priehs, S. Kalvonjian, M. M. Fluck, manuscript in preparation; Hacker and Fluck, unpublished data; 11, 15).

These results indicate that a very high level of intervirus recombination generally accompanies integration of the viral genome. For example, in the case of transformants derived under usual conditions (37°C), half of those analyzed (and five of six in one experiment) contained at least one recombinant viral genome, and most contained multiple recombinant fragments. In most cases (18 of 20), recombination occurred in a short 359-bp interval (as well as in other intervals [Hacker and Fluck, manuscript in preparation]). To our knowledge, the values obtained in this cross represent the highest level of recombination reported in mitotic cells. Given that transformants represent at most 1% of the population of infected cells, it is not possible at this time to know how this factor should enter into estimates of overall recombination frequency, since we do not know the rate-limiting step(s) for transformation.

Most of the reports of elevated recombination in animal cells have come from transfection experiments. For example, Folger et al. (13), using thymidine kinase as a selectable marker, obtained 122 transformants from 10^3 microinjected cells, versus 7 per 2×10^3 injected cells when recombination between two mutants separated by 417 bp was required. In this comparison, the recombination frequency would be approximately 0.5% per polyomavirus-infected cell in an interval of 359 bp of (nucleosome-loaded) chromatin versus 0.35% per microinjected cell in an interval of 417 bp of naked DNA. This comparison is particularly interesting since it has been suggested (13) that only nucleosome-free DNA can participate in elevated recombination in the microinjection process.

Such high levels of recombination might imply that recombination is required for the integration-transformation process. The existence of apparently nonrecombinant transformants does not totally rule out this possibility, since these transformants might have arisen by recombination with self or have lost recombinant sequences in the process of integration. The observation that transformation does not depend quadratically on the multiplicity of infection in recombination-dependent as well as in wild-type transformation events (Priehs et al., in preparation) tends to make this hypothesis unlikely but again does not rule it out.

Models for integration-linked recombination. The two contrasting situations, i.e., the undetectable level of recombination in the unintegrated (replicated, parental, or both) viral genomes present in the infected cell population versus the elevated level found in the integrated genomes present in transformed cells, are open to two alternate interpretations. Either recombination occurs only in a small, recombination-prone fraction of the infected cell population, which is destined to become transformed, or recombination occurs only among those viral genomes which are engaged in the process of integration. The available data do not favor one or the other model at this stage. The former hypothesis would suggest that recombination acts like a competence factor for transformation. This hypothesis has four attractive features: (i) it offers a rationale for the low transformation frequency of polyomaviruses, (ii) it provides an explanation for the differences in recombination levels between the unintegrated and the integrated genomes in the infected cells, (iii) it is apparently compatible with the requirements for host-virus recombination in the integration process, and (iv) it is compatible with reports of other elevated recombination processes (such as sister chromatid exchange) in transformed cells. However, the following qualifications should be added to this potential model. (i) The characteristics of intervirus recombination are different from those typical of recombination between the host and the viral genome. The latter involves very short stretches of homologies (5, 19, 25) and large deletions of host sequences (26, 28), whereas the former involves strong and long homologies. Whether the viral partners lose sequences during intervirus recombination is not known at this time. (ii) As revealed superficially by a test for the organization of the immunoglobulin G gene, there is no generalized rearrangement of the host genome (Hacker and Fluck, unpublished data). (iii) If recombination does provide a competence factor for transformation, it is short-lived, since recently transformed cells do not induce high levels of intervirus recombination when infected with exogenous and marked viral genomes (Chen and Fluck, unpublished data). (iv) Polyomavirus integration patterns in cells that are not transformed do not appear to differ from those seen in transformants, particularly with respect to the presence of tandem repeats of the viral genome (21). Unfortunately, none of these observations rules out the hypothesis under scrutiny. The other hypothesis, which is equally attractive to us, is that only those viral genomes which are engaged in the process of integration interact with a highly recombinogenic machinery (for example, the host scaffold). Further experiments will be required to differentiate between these hypotheses.

Given the asymmetric distribution of the recombinant viruses to the integrated genomes of the transformed cells, the experiments described here also allow us to infer that the fraction of the infected cell population in which DNA synthesis occurs at an elevated level (determined to be less than 1% [17]) is not likely to represent the precursors of the transformed cells.

Role of intervirus recombination in the integration of head-to-tail tandem repeats of the viral genomes. The experiments described here were designed to test the role of intervirus recombination in the generation of the tandem integration topology. Some time ago, Chia and Rigby (7) showed that in the case of SV40 infections of nonpermissive BALB 3T3 cells, apparently unintegrated high-molecular-weight intermediates of the viral genomes (larger than 50 kbp) could be detected by 5 days postinfection. These were assumed to represent the unintegrated precursors of the viral genomes present in the transformants. To determine the role of intervirus recombination in their formation, use was made of mutants marked by the absence of restriction endonuclease sites. The results showed that most of the sequences present in these high-molecular-weight intermediates were of nonrecombinant type, although recombinant sequences were also present (at a calculated level of 20%). Because of the predominance of the nonrecombinant sequences, Chia and Rigby inferred (but did not confirm) that the vast majority of the transformants recovered from such mixed infections would be nonrecombinant, and they proposed that the mechanism by which integrated tandems are formed is DNA replication of a rolling-circle type. In the case of polyoma-

virus, the unintegrated viral sequences are monomeric and are of (absolute) nonrecombinant predominance; furthermore, a very high proportion of the transformants contain sequences of recombinant type. One can certainly question whether this might also have been the situation in the SV40 transformants.

It has become clear in hindsight that whereas our experiments are powerful in uncovering a strong involvement of intervirus recombination in the generation of the integrated viral genome, they fail to conclusively demonstrate at this stage that recombination is responsible for the tandemization of the viral genome. The simplest mode of production of dimeric (or multimeric) genomes by recombination from monomers would involve double-reciprocal crossovers and thus would be expected to generate reciprocal crossover fragments (i.e., fragments of 3.95, 5.0, 6.6, or 8.9 kbp; Fig. 1). The fact that these fragments (except for a 5.0-kbp fragment which may also represent a parental genome) are not recovered may reflect the fact that most of the reciprocal recombinant fragments from a dimeric recombinant (i.e., both the 6.6- and 8.9-kbp fragments as well as either the 5.0- or 4-kbp fragment) would be expected to disappear in the integration process of a dimeric reciprocal recombinant (Fig. 1). The apparent absence of reciprocal recombinant fragments may also reflect the absence of this process or, finally, the occurrence of nonconservative recombination events in which some of the parental viral sequences are digested (6). Further analysis of the integrated viral sequences after cloning may clarify this point. Furthermore, the recovery of both a 3.6-kbp fragment and either a 1.7- or 1.3-kbp fragment in most transformants leaves open the possibility that wild-type recombinants of single genome size are formed by two resolving crossovers in two adjacent intervals. To generate tandem genome integration, these would have to recombine further with other genomes or be replicated in the process of integration. However, until the integrated sequences are cloned (in progress), the recovered 3.6 kbp and 1.7- or 1.3-kbp fragments cannot be proven to be contiguous, since the transformants appear to have multiple integration sites. Since we have previously demonstrated the occurrence of integrated tandems of dual-parent origin (11, 15), it is safe to conclude that at least a fraction of the integrated tandems must owe their origin to recombination. Preliminary analysis from cloned integration sites suggests that this is the case. Thus, cloning of the integrated viral genomes should allow us to clarify what kind of recombination events generated the integrated viral genomes.

An intriguing role for large T antigen in tandem formation has been previously demonstrated (9); at this point, we cannot prove whether T antigen is involved in the recombination process, a replication process, or both; however, some of our results are compatible with a role for large T antigen in the intervirus recombination process (Kalvonjian and Fluck, in preparation).

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LITERATURE CITED

1. **Basilico, C., S. Gattoni, D. Zouzias, and G. Della Valle.** 1979. Loss of integrated viral DNA sequences in polyoma-transformed cells is associated with an active viral A function. Cell **17**:645–659.
2. **Birg, F., M. Dulbecco, M. Fried, and R. Kamen.** 1979. State and organization of polyomavirus DNA sequences in transformed rat cell lines. J. Virol. **29**:633–648.
3. **Bjursell, G.** 1978. Effects of 2′-deoxy-2′-azidocytidine on polyoma virus DNA replication: evidence for rolling circle-type mechanism. J. Virol. **26**:136–142.
4. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell **9**:269–287.
5. **Bullock, P., W. Forrester, and M. Botchan.** 1984. DNA sequence studies of simian virus 40 chromosomal excision and integration in rat cells. J. Mol. Biol. **174**:55–84.
6. **Chakrabarti, S., and M. M. Seidman.** 1986. Intramolecular recombination between transfected repeated sequences in mammalian cells is nonconservative. Mol. Cell. Biol. **6**:2520–2526.
7. **Chia, W., and P. W. J. Rigby.** 1981. Fate of viral DNA in nonpermissive cells infected with simian virus 40. Proc. Natl. Acad. Sci. USA **78**:6638–6642.
8. **Dailey, L., S. Pellegrini, and C. Basilico.** 1984. Deletion of the origin of replication impairs the ability of polyoma virus DNA to transform cells and to form tandem insertions. J. Virol. **49**: 984–987.
9. **Della Valle, G., R. G. Fenton, and C. Basilico.** 1981. Polyoma large T-antigen regulates the integration of viral DNA sequences into the genome of transformed cells. Cell **23**:347–355.
10. **Dulbecco, R., and W. Eckhart.** 1970. Temperature-dependent properties of cells transformed by a thermosensitive mutant of polyoma virus. Proc. Natl. Acad. Sci. USA **67**:1775–1781.
11. **Fluck, M. M., R. Shaikh, and T. L. Benjamin.** 1983. An analysis of transformed clones obtained by coinfections with Hr-t and Ts-a mutants of polyoma virus. Virology **130**:29–43.
12. **Fluck, M. M., R. J. Staneloni, and T. L. Benjamin.** 1977. Hr-t and ts-a: two early gene functions of polyoma virus. Virology **77**:589–597.
13. **Folger, K. R., K. Thomas, and M. R. Capecchi.** 1985. Nonreciprocal exchanges of information between DNA duplexes coinfected into mammalian cell nuclei. Mol. Cell. Biol. **5**:59–69.
14. **Freeman, A. E., R. V. Gilden, M. L. Vernon, R. G. Woldford, P. E. Hugunin, and R. J. Huebner.** 1973. 5-Bromo-2-deoxyuridine potentiation of transformation of rat embryo cells induced *in vitro* by 3-methyl-cholanthrene: induction of rat leukemia virus gs antigen in transformed cells. Proc. Natl. Acad. Sci. USA **70**:2415–2419.
15. **Friderici, K., S. Y. Oh, R. Ellis, V. Guacci, and M. M. Fluck.** 1984. Recombination induces tandem repeats of integrated viral sequences in polyoma-transformed cells. Virology **137**:67–73.
16. **Friedman, T., A. Esty, P. LaPorte, and D. Deininger.** 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid homology with SV40. Cell **17**:715–724.
17. **Hacker, D. L., and M. M. Fluck.** 1989. Viral DNA synthesis in nonpermissive rat F-111 cells and its role in neoplastic transformation by polyomavirus. Mol. Cell. Biol. **9**:648–658.
18. **Hacker, D. L., K. H. Friderici, C. Priehs, S. Kalvonjian, and M. M. Fluck.** 1988. Viral DNA synthesis and recombination in the transformation pathway of polyomavirus, p. 173–181. *In* R. E. Moses and W. C. Summers (ed.), DNA replication and mutagenesis. American Society for Microbiology, Washington, D.C.
19. **Hayday, A., H. E. Ruley, and M. Fried.** 1982. Structural and biological analysis of integrated polyoma virus DNA and its adjacent host sequences cloned from transformed rat cells. J. Virol. **44**:67–77.
20. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
21. **Lania, L., M. Griffiths, B. Cooke, Y. Ito, and M. Fried.** 1979. Untransformed rat cells containing free and integrated DNA of a polyoma nontransforming (Hr-t) mutant. Cell **18**:793–802.
22. **Ramig, R. F.** 1985. Principles of animal virus genetics, p. 101–128. *In* B. N. Fields and D. M. Knipe (ed.), Fundamental virology. Raven Press, New York.
23. **Southern, E. M.** 1975. Detection of specific sequences among

DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
24. **Stoker, M., and R. Dulbecco.** 1968. Abortive transformation by the tsa mutant of polyoma virus. Nature (London) **223**:397–398.
25. **Stringer, J.** 1981. Integrated simian virus 40 DNA: nucleotide sequences at cell-virus recombinant junctions. J. Virol. **38**: 671–679.
26. **Stringer, J.** 1982. DNA sequence homology and chromosomal deletion at a site of SV40 integration. Nature (London) **296**: 363–366.
27. **Templeton, D., and W. Eckhart.** 1982. Mutation causing premature termination of the polyoma virus medium T antigen blocks cell transformation. J. Virol. **41**:1014–1024.
28. **Williams, T. J., and M. Fried.** 1986. Inverted duplication-transposition event in mammalian cells at an illegitimate recombination joint. Mol. Cell. Biol. **6**:2179–2184.
29. **Winberry, L., C. Priehs, K. Friderici, M. Thompson, and M. M. Fluck.** 1985. Expression of proto-oncogenes in normal and papovavirus-transformed or -infected rat fibroblasts. Virology **147**:154–168.
30. **Winocour, E.** 1963. Purification of polyoma virus. Virology **19**:158–168.