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Biochemistry

Amplification of integrated viral DNA sequences in polyoma virus-transformed cells

(polyoma large tumor antigen/tandem integration/restriction enzyme mapping)

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ABSTRACT **Polyoma virus (Py) transformation of rat cells requires integration of viral genomes into the host DNA, which generally occurs in a partial or full head-to-tail tandem arrangement. The instability of this structure was previously demonstrated by the high rate of loss of integrated Py genomes in the presence of viral large tumor (T) antigen. We now show that integrated Py DNA sequences can also undergo amplification. We studied two rat cell lines transformed by the ts-*a* Py mutant, which codes for a thermolabile large T antigen. In a derivative of the ts-*a* H6A cell line, we have observed loss of full-length Py DNA molecules from the integrated tandem ("curing"), accompanied by the creation of new tandem repeats of two segments of viral DNA corresponding to 38% and 10% of the viral genome, each containing the origin of DNA replication. In the ts-*a* H3A cell line, which contains an integrated partial tandem of about 1.3 viral genomes with three distinct deletions, propagation at 33°C resulted in the generation of full tandem repeats of a 94% Py DNA "unit" (including two 3% deletions), an 85% "unit" (including a 3% and the 12% deletion), or both. Amplification of integrated viral DNA was not observed in cells propagated at 39.5°C, the nonpermissive temperature for large T antigen function. Amplification of integrated Py DNA sequences thus requires an active large T antigen and can generate a full tandem of integrated viral DNA molecules long after the initial integration event.**

Transformation of rat cells by polyoma virus (Py) is characterized by integration of the viral genome into the host DNA and expression of early viral functions. Integration has been observed so far to occur exclusively in a partial or full head-to-tail tandem arrangement, and the viral DNA regions where insertion into the host DNA occurs do not seem to be specific (1–6). Therefore, tandem integration preserves the integrity of viral genes coding for transforming functions.

Py DNA integration is not totally stable. Transformed rat cells have been shown to produce free viral DNA molecules, which originate from the integrated ones by induction occurring in a minority of the cell population (7, 8). Excision of integrated viral DNA sequences can also occur (2, 5), and both production of free viral DNA (7) and excision (2) require an active viral large tumor (T) antigen, a protein known to be involved in the initiation of viral DNA replication (9). In general, excision leads to the loss of an integral number of viral DNA molecules, suggesting the involvement of an intramolecular recombination event (5).

These and other findings led us to investigate whether amplification of integrated viral DNA sequences could also occur in Py-transformed cells, and whether this process exhibited requirements similar to those observed for excision. The results presented in this paper show that, under conditions permissive for large T antigen function, integrated viral DNA sequences can undergo amplification. The amplified sequences observed so far always contain the origin of viral DNA replication. The results also show that full tandem integration can be generated at times subsequent to the initial transformation event.

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MATERIALS AND METHODS

Cells. The general properties of the F2408 Fisher rat cells transformed by Py used in these experiments have been described (7, 8). The lines used, ts-*a* H6A and ts-*a* H3A, were both transformed by the temperature-sensitive (ts)-*a* mutant (10) of Py and grown in Dulbecco's modified Eagle's medium containing 10% calf serum.

The ts-*a* H6 and H3 lines were produced by infection at 50 plaque-forming units per cell. Cells were plated in soft agar at 33°C for 5 days, then shifted to 39.5°C, and transformed colonies were isolated (2). These cell lines were recloned in agar at 39.5°C to produce subclones designated H6A and H3A. These cells were always propagated at 39.5°C unless otherwise stated.

DNA Extraction and Restriction Enzyme Analysis by Blot Hybridization. Chromosomal DNA was prepared from cells grown at 39.5°C and digested with restriction endonucleases (New England BioLabs) (Fig. 1) as described (2, 6). The DNA samples were loaded on a 16 × 18 × 0.5 cm 1% agarose horizontal slab gel and run for 15–18 hr at 40 V. After alkali denaturation the DNA was transferred to nitrocellulose filter paper as described (2, 6). Hybridization with denatured ^{32}P-labeled nick-translated (11, 12) Py DNA (specific activity 5×10^7 to 1×10^9 cpm/μg) was carried out for 20–24 hr at 65°C, in the presence of 40 μg/ml of sonicated and denatured calf thymus DNA. After hybridization the filter was rinsed as described (2, 6) and exposed at −70°C to XR-2 X-ray film (Kodak) with or without an intensifying screen (Lightning-Plus, Du Pont).

RESULTS

The transformed cell lines used in this study had all been produced by infection with the ts-*a* mutant (10) of Py, which codes for a temperature-sensitive large T antigen (13–15). This allows the monitoring of the cultures under conditions permissive (33°C) or nonpermissive (39.5°C) for this viral function.

The analysis of integrated viral DNA sequences in these lines was performed by digestion of high molecular weight cellular DNA with restriction enzymes of different specificities for Py DNA (Fig. 1), followed by fractionation on agarose gels, transfer to nitrocellulose paper, and hybridization with ^{32}P-labeled Py DNA ("Southern" blot) (16).

Amplification of Viral DNA Sequences in a Derivative of the ts-*a* H6A Cell Line. The ts-*a* H6A line contains a single insertion of integrated viral DNA consisting of full length Py molecules arranged in a head-to-tail tandem repeat (2, 5). As

Abbreviations: Py, polyoma virus; T antigen, tumor antigen; kb, kilobase(s); m.u., map unit(s); ts, temperature-sensitive.

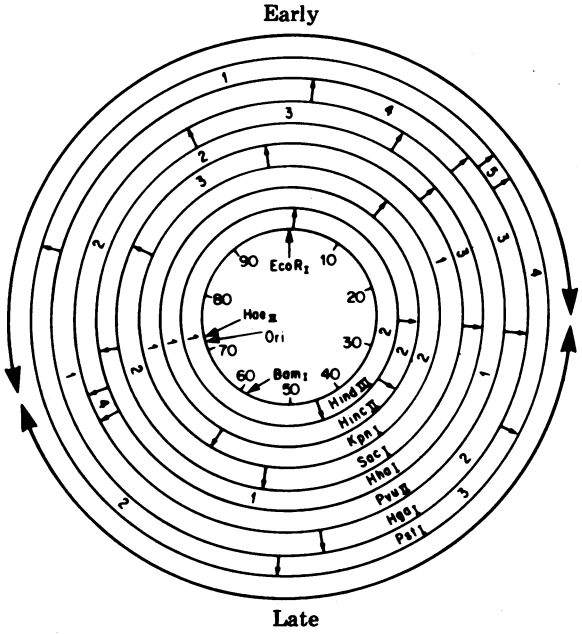

FIG. 1. Physical map of Py DNA [5.3 kilobases (kb)], showing the sites of cleavage of the restriction enzymes used in these experiments. Ori, origin of viral DNA replication.

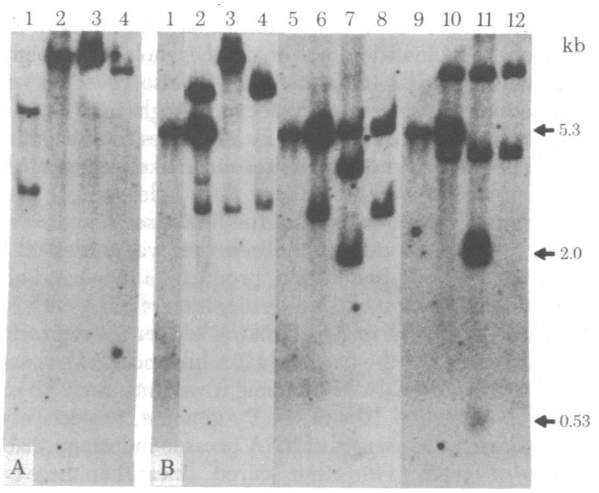

FIG. 2. Analysis of the Py DNA sequences integrated in the DNA of the SS1A cell line, the parental line ts-*a* H6A-1, and one of the H6A "cured" derivatives. About 10 μg of high molecular weight DNA per sample was digested with restriction endonucleases, fractionated through a 1% agarose slab gel, denatured, and transferred to nitrocellulose membrane filters. Specific Py sequences were detected by hybridization with denatured ^{32}P-labeled nick-translated Py DNA and visualized by autoradiography. The positions and the sizes (in kb) of some linear DNA fragments containing Py sequences are indicated. They were calculated from the mobility of λ phage DNA fragments produced by cleavage with *Bgl* II and *Eco* RI enzymes, as well as from the mobility of marker Py DNA fragments. (*A*) Lanes 1–4, *Bgl* II digestion of purified Py DNA marker, ts-*a* H6A-1 DNA, SS1A DNA, and "cured" H6A DNA. The two bands appearing in lane 1 correspond to supercoiled form I (the fastest), and relaxed form II Py DNA. (*B*) Lanes 1–4, *Eco* RI digestion of Py DNA marker, H6A-1 DNA, SS1A DNA, and "cured" H6A DNA. Lanes 5–8, *Bam* I digestion of Py DNA marker, H6A-1 DNA, SS1A DNA, and "cured" H6A DNA. Lanes 9–12, double digestion with *Bgl* II and *Hae* II of Py DNA marker, H6A-1 DNA, SS1A DNA, and "cured" H6A DNA.

previously described (2), propagation of the H6A line at the temperature permissive for large T antigen function allows the isolation of a high frequency of "cured" cells which, through excision, have lost the tandem arrangement of integrated viral molecules, while the regions of the viral DNA adjacent to the host DNA remain unchanged. These cells have undergone reversion to an "intermediate" transformed phenotype (2). The ts-*a* H6A line was recloned in agar at 39.5°C to produce a subclone designated H6A-1, whose Py integration pattern was indistinguishable from that of H6A. The ts-*a* H6A-1 line was propagated at 33°C for 2 weeks and revertants were isolated. While all of the other revertants were indistinguishable from those previously studied (2, 5), one of these, designated SS1A, had an integration pattern not previously seen.

The analysis of the integrated viral DNA sequences in these lines (Fig. 2) shows that *Eco*RI cleavage of H6A-1 DNA (Fig. 2*B*, lane 2) yields two virus–host linker sequences as well as a fragment of unit length Py DNA (5.3 kb), indicating the presence of a tandem repeat of Py genomes within the insertion (5). (The additional band seen directly below the 5.3-kb fragment is an artifact that has never appeared in other *Eco*RI digestions of this line.) Analysis of H6A-1 with *Bam* I (Fig. 2*B*, lane 6) or double digestion with *Bgl* II (which does not cleave Py DNA) plus *Hae* II (lane 10) yields a similar result, although in the former case one flanking sequence migrates just above the Py unit-length molecule and is therefore not clearly distinguishable in this blot.

All the "cured" derivatives of H6A previously studied (2, 5) showed a viral insertion of reduced molecular weight as compared to H6A, as shown in Fig. 2*A* by cleavage with *Bgl* II (which does not cut Py DNA). This reduction in molecular weight is due to loss of the tandem arrangement of Py molecules as shown by *Eco*RI (Fig. 2*B*, lane 4), *Bam* I (Fig. 2*B*, lane 8), and *Bgl* II/*Hae* II digestion (Fig. 2*B*, lane 12). The unit-length Py fragment is clearly absent. A model of the arrangement of integrated Py sequences in these lines and the parental ts-*a* H6A (5) is shown in Fig. 3.

On the other hand, analysis of the SS1A DNA by *Bgl* II cleavage (Fig. 2*A*, lane 3) revealed a Py DNA-containing fragment of a higher molecular weight than that of the other revertants, suggesting the presence of more Py sequences within the insertion. *Eco*RI cleavage (Fig. 2*B*, lane 3) showed that although the 5.3-kb monomer was absent, one flanking sequence of SS1A migrated as did that of H6A-1 and the other "cured"

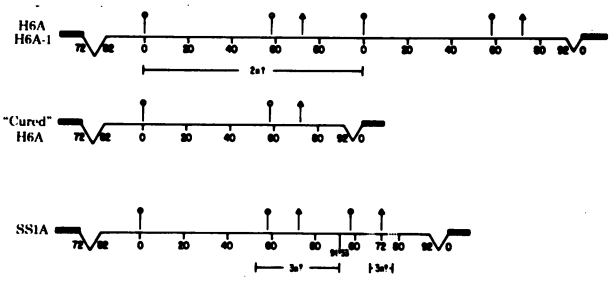

FIG. 3. Map of the viral DNA sequences integrated into the host genome in the ts-*a* H6A-1 parental line, in the SS1A cell line, and in one of the H6A revertants. The maps were constructed by analyzing the products of restriction enzyme digestion of high molecular weight DNA extracted from these lines as shown in Fig. 2. Only the cleavage sites within the viral sequences of the restriction enzymes *Eco*RI (●), *Hae* II (▲), and *Bam* I (○) are indicated in the figure. The cleavage sites of other enzymes used to construct the map are not shown. The light lines represent integrated Py DNA; the heavy lines represent flanking cellular DNA sequences. V indicates the regions of the Py map joined to the cellular DNA. The map coordinate on the viral side of the junction represents a restriction enzyme site that is still present in the integrated viral sequence, while the map coordinate on the host side of the junction represents the next restriction site of Py DNA not present in the integrated sequence. The dotted lines represent the unknown end points of the 10% duplicated fragment.

lines, but the larger flanking sequence (corresponding to the right side of the Py insertion) was of a higher molecular weight. Analysis with *Hin*dIII (data not shown) also revealed one flanking sequence of increased molecular weight in SS1A, thus pointing to the presence of "extra" Py sequences not containing the *Eco*RI or *Hin*dIII restriction sites as the likely cause of the observed molecular weight increase. *Bam* I cleavage of SS1A (Fig. 2*B*, lane 7) also showed that the linker sequence corresponding to the right side of the insertion was increased in molecular weight but, in addition, produced a new band of 2 kb (38% of the Py genome). Double digestion of SS1A with *Bgl* II and *Hae* II resulted in two flanking sequences migrating identically to those of the parental H6A line, no 5.3-kb monomer, and two new Py DNA-containing fragments, one 2 kb and one 0.53 kb (38% and 10% of the Py genome, respectively). Thus, the flanking sequences of SS1A became indistinguishable from those of H6A and the other "cured" lines when these two "extra" fragments were cut out. Because both *Bam* I and *Bgl* II/*Hae* II digestion produced the appearance of the 2-kb fragment, it was concluded that a tandem repetition of this 38% sequence was present in SS1A. The increase in molecular weight of the smaller *Bam* I flanking sequence caused by the 10% fragment (*Bam* I had cut the 38% piece from the flanking sequence) indicated that this 10% fragment was also repeated. Therefore, it was concluded that a newly formed tandem repetition of ≈3 copies of the 2-kb fragment (containing the *Bam* I and *Hae* II restriction sites) and a tandem repeat of ≈3 copies of the 0.53-kb fragment (containing the *Hae* II but not the *Bam* I site) were responsible for the difference in restriction enzyme pattern observed between SS1A and the other H6A "cured" lines. This conclusion was confirmed and the repetitions were mapped more precisely by digesting the DNA with other restriction enzymes (data not shown). A model of the arrangement of integrated Py sequences in SS1A (Fig. 3) shows that this cell line, in addition to having lost the full tandem repeat characteristic of the parental H6A, has acquired a new arrangement of head-to-tail tandem repeats of a 38% segment as well as of a 10% segment of the viral genome, both containing the Py origin of replication [map unit (m.u.) 71 (9)].

Creation of a Full Tandem Repeat of Integrated Viral Genomes by Amplification. To extend this conclusion and to determine whether a multiple copy insertion could be formed after the initial integration event, we used another rat cell line transformed by ts-*a* Py virus, ts-*a* H3A. This line has one viral insertion containing a stretch of Py DNA equivalent to about 1.3 genomes with three deletions (6). This unusual arrangement is shown in Fig. 6. Two of the deletions are located on the right side of the viral insertion, one corresponding to about 3% of the Py genome and mapping between 25 and 35 m.u., the other to 12% and including the *Hin*dIII site at 45 m.u. A 3% deletion mapping between 35 and 45 m.u. is present on the left side (6). We propagated this line in culture at 33°C, and at 2, 5, and 7 weeks the cells were shifted to 39.5°C, after which DNA was prepared and analyzed by blot hybridization (Fig. 4).

Digestion of the high molecular weight DNA from H3A with *Bgl* II produced only one band, indicative of a single viral insertion (6). In the DNA extracted from cells grown at 33°C for 2, 5, and 7 weeks we could detect at least three bands, one of which corresponded to that of the original line, and two others of higher molecular weight (Fig. 4*A*). Therefore, some new integration sites had been created or a portion of the cells in the population had undergone an increase in the amount of Py DNA sequences within the original insertion. We digested these DNAs with *Hin*dIII, which produces in H3A a normal size *Hin*dIII large fragment (45 to 2 m.u.) and two other fragments representing flanking sequences of viral and host DNA (6). In

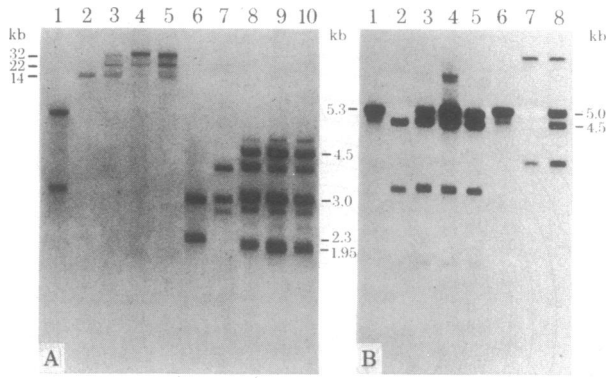

FIG. 4. Analysis of the Py DNA sequences integrated in the DNA extracted from cultures of the ts-*a* H3A cell line propagated at 33°C for 0, 2, 5, and 7 weeks. High molecular weight cell DNA was digested with restriction enzymes and analyzed as in Fig. 2. The position and the size (in kb) of some linear DNA fragments containing Py sequences are indicated. (*A*) Lanes 1–5, *Bgl* II digestion of Py DNA marker and H3A DNA after 0, 2, 5, and 7 weeks of propagation at 33°C, respectively. Lanes 6–10, *Hin*dIII digestion of Py DNA marker, and H3A DNA after 0, 2, 5, and 7 weeks at 33°C, respectively. (*B*) Lanes 1–5, *Bam* I digestion of Py DNA marker and H3A DNA after 0, 2, 5, and 7 weeks at 33°C, respectively. Lanes 6–8, *Hae* II digestion of Py DNA marker and H3A DNA after 0 or 5 weeks at 33°C, respectively.

all the 33°C samples we detected the three bands characteristic of H3A plus two major new bands (Fig. 4*A*, lanes 7–10). The faster one was smaller than the small *Hin*dIII fragment, whereas the other one corresponded to about 85% of the Py genome (4.5 kb). *Bam* I digestion of H3A DNA (Fig. 4*B*) produces a 4.5-kb viral fragment and two linker sequences. This 4.5-kb fragment is the result of cleavage at the two *Bam* I sites present in the viral insertion, which generates a viral DNA monomer shortened by the two deletions (3% and 12%) included in this segment (6). In the DNA from the cultures propagated at 33°C the same flanking sequences and the same 4.5-kb fragment were present, but a band corresponding to 94% (5.0 kb) of the Py genome appeared. *Hae* II cleaves the H3A parental insertion only once, generating two virus–host DNA fragments. In the 33°C populations at any of the time points, in addition to the same preserved joining sequences, two new bands corresponding to 94% and 85% of the Py genome appeared (Fig. 4*B*). Similar results were obtained with *Eco*RI cleavage (not shown).

In comparing these results with the arrangement of integrated viral DNA in the parental H3A line (Fig. 6), it was obvious that a duplication of the left Py "unit" of the insertion (such as from m.u. 30 to 30) or the right "unit" (from 60 to 60), having occurred in some of the cells in the population, could explain the observed results. Duplication of the left "unit" would produce two *Eco*RI and two *Hae* II sites spanning a 94% monomer, while duplication of the right unit would produce two *Eco*RI and *Hae* II sites, spanning an 85% segment. *Hin*dIII digestion would be expected to produce in the first instance a new *Hin*dIII small fragment shortened by about 6%, and in the second an 85% (4.5-kb) fragment, because the 45-m.u. *Hin*dIII site on the right side of the insertion is missing due to the large deletion (6). This interpretation was in agreement with the hypothesis that the major changes in the 33°C populations had been variable amplifications of the viral DNA insertion.

We thus expected the 33°C population to be a mixture of cells containing either the original insertion or newly amplified integrated sequences. Therefore, cells that had been grown at 33°C were plated at 39.5°C, single colonies were isolated, and their DNAs were analyzed by Southern blotting. Fig. 5 shows

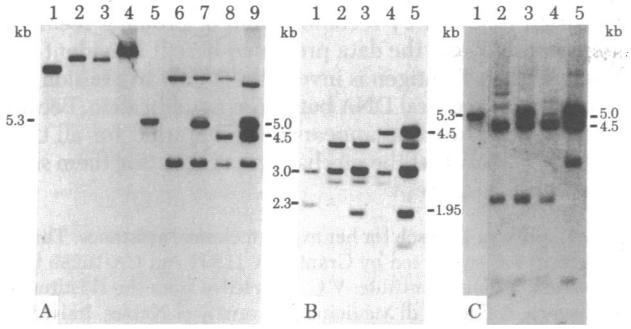

FIG. 5. Analysis of the Py DNA sequences integrated in the DNA of subclones of the H3A line isolated after propagation at 33°C. The products of DNA digestion with restriction enzymes were analyzed as described for Fig. 2. The positions and the sizes (in kb) of linear DNA fragments containing Py DNA sequences are indicated. (*A*) Lanes 1–4, *Bgl* II digestion of H3A-7 DNA, H3A-5 DNA, H3A-8 DNA, and H3A-10 DNA. Lanes 5–9, *Eco*RI digestion of Py DNA marker, H3A-7 DNA, H3A-5 DNA, H3A-8 DNA, and H3A-10 DNA. (*B*) Lanes 1–5, *Hin*dIII digestion of Py DNA marker, H3A-7 DNA, H3A-5, DNA, H3A-8 DNA, and H3A-10 DNA. (*C*) Lanes 1–5, *Bam* I digestion of Py DNA marker, H3A-7 DNA, H3A-5, DNA, H3A-8 DNA, and H3A-10 DNA. The faint bands visible in the *Bam* I blots are due to incomplete digestion.

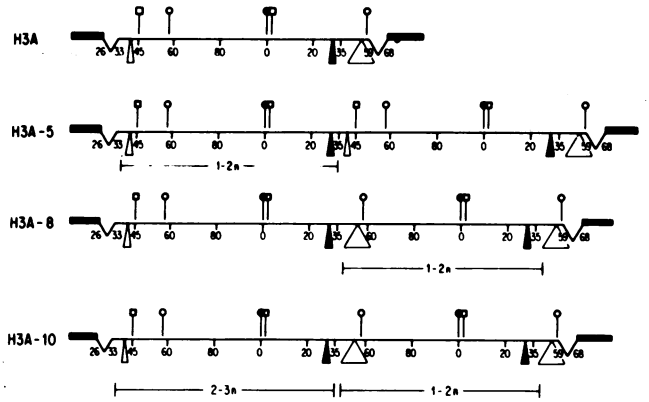

FIG. 6. Maps of the viral DNA sequences integrated into the host genome of the amplified clones derived from the H3A cell line. The maps were constructed as described for Fig. 3. The cleavage sites within the viral sequences of the restriction enzymes *Eco*RI (●), *Bam* I (○), and *Hin*dIII (□) are indicated in the figure. The cleavage sites of other enzymes used to construct the maps are not shown. The light lines represent integrated Py DNA; the heavy lines represent flanking cellular DNA sequences. △ and ▲ indicate deletions. Other symbols are as in Fig. 3.

that four cell types were found. Digestion of DNA from each clone with *Bgl* II (Fig. 5*A*) gave a single band corresponding approximately to the fastest (clone H3A-7), to the intermediate (clones H3A-5 and H3A-8), and to the slowest one (clone H3A-10) of those observed in the mixed population. *Eco*RI cleavage (Fig. 5*A*) showed that clone H3A-7 had the same arrangement as H3A (not shown), as expected from the *Bgl* II pattern. Clone H3A-5 showed the same linker sequences, but also a band of 5.0 kb (94% of the Py genome). In clone H3A-8 a 4.5-kb fragment (85% of the Py genome) appeared in addition to the fragments present in H3A. In H3A-10 DNA both the 94% and the 85% Py fragments are detectable. This suggested that the H3A subclones had the original integration pattern, amplification of the 94% segment containing the two small deletions, amplification of the 85% segment containing one small and the large deletion, or amplification of both. The *Hin*dIII digestion confirmed this interpretation (Fig. 5*B*). When compared to H3A or H3A-7, clone H3A-5 shows an additional band corresponding to the small *Hin*dIII fragment shortened by the two 3% deletions (1.95 kb). H3A-8 shows an additional viral band of 4.5 kb. Finally, H3A-10 DNA digestion produced both the 1.95- and the 4.5-kb bands. The *Bam* I digestion (Fig. 5*C*) showed that one additional viral band of 5.0 kb was present in H3A-5. H3A-8 showed the same pattern as H3A, but the 4.5-kb band was very intense, suggesting that this segment, already present in the parental line, had been duplicated. H3A-10 DNA digestion, finally, produced the 5.0- and the 4.5-kb bands. These results clearly showed that the H3A line had undergone amplification of the integrated sequences. Out of 11 clones examined, 6 had the original H3A integration pattern, 4 had amplified either the right or the left viral "unit," and one had amplified both.

It should be mentioned here that in the H3A-10 clone one of the virus–host linker sequences appears to have undergone changes. Fig. 5 shows that the larger linker fragment in the *Eco*RI digest and the smaller linker fragment in the *Hin*dIII digest are decreased in size, whereas the size of one *Bam* I digestion linker is increased. Because the fragments of altered size all correspond to the left side of the viral insertion (6), the most likely explanation of this phenomenon is a deletion in the host sequences adjacent to the viral DNA, which has removed the original host *Bam* I site (the closest to the viral insertion) and decreased the distance between the host *Eco*RI and *Hin*dIII sites and the next viral sites. A model of the arrangement of integrated viral DNA in H3A and three of its most representative amplified subclones is shown in Fig. 6.

Amplification requires an active *A* gene product (large T antigen), because the changes in the arrangement of the integrated Py molecules occur only at 33°C, the permissive temperature for the ts-*a* mutation. No rearrangements were observed in the DNA restriction pattern of the ts-*a* H3A line even after 3 months in culture at 39.5°C, the nonpermissive temperature for large T function.

DISCUSSION

We have analyzed two rat cell lines transformed by the ts-*a* mutant of Py virus and we have found that under conditions permissive for the *A* gene function, which is necessary for viral DNA replication (9), amplification of integrated viral DNA sequences can occur. The results obtained with the ts-*a* H3A cell line show quite clearly that this phenomenon occurs soon after exposure to conditions permissive for T antigen function. While this probably implies that cells having amplified Py sequences have some growth advantage in culture with respect to the other cells (probably as a result of a higher dosage of transforming-gene products), it also shows that amplification can occur at a rather high rate.

The H3A cell line has an unusual arrangement of integrated viral DNA sequences, consisting of a partial tandem of 1.3 viral genomes, in which the repeated portions of the integrated viral DNA (≈m.u. 30–60) contain two different size deletions (3% and 12%). An additional deletion (≈3%) is present in the unique region of the integrated viral DNA. The analysis of the integrated DNA from the amplified clones reveals that two different sequences can be amplified: one corresponding to 94% and the other to 85% of the Py genome, i.e. the left Py "unit" containing the 3% deletion between m.u. 35 and 45 and the unique 3% deletion, or the right "unit" containing the unique 3% deletion and the 12% deletion (Fig. 6). These two species correspond exactly to the families of free viral DNA produced by the H3A cell line (6). A functional large T antigen is required for both free viral DNA production and amplification. Thus newly synthesized free viral DNA molecules could reintegrate at the level of the homologous sequences, leading to an amplification of the integrated species.

On the other hand, in SS1A we do not find reduplication of complete molecules, but only of a segment extending from 53 to 91 m.u. and of another extending approximately from 67 to 77 m.u. (Fig. 3). The amplified segments symmetrically bracket the origin of viral DNA replication and do not correspond to the major species of free viral DNA produced by the parental H6A line, which consists of full-size viral genomes (6). If the amplified segments of SS1A are not considered, its arrangement is the same as that of the H6A "cured" lines originated by excision (2, 5). In both cases a complete early region coding for large T antigen is missing. Because an active A-gene product is required for amplification of the integrated sequences, we conclude that this process did not take place in a "cured" cell, but probably before or during excision.

Gene duplication is a common event in bacteria, usually not limited to a single gene, but involving as much as 20–25% of the entire bacterial chromosome (17). This does not seem to be the case in our system, because this mechanism would not generate the pattern of tandem integration of viral DNA molecules that we observed. Examples of duplication of specific genes in eukaryotic cells are the bar and the bobbed loci of *Drosophila*, in which tandem expansion of these genes might occur by unequal crossing-over between the same genes present on homologous chromosomes or by an unequal exchange between sister chromatids (18–20). Other examples are the amplification of the genes for dehydrofolate reductase and for the multifunctional protein having carbamoyl-phosphate synthetase, aspartate transcarbamoylase, and dihydro-orotase activities detected under selective pressure in cultured mammalian cells (21, 22). On the other hand, the amplification of the ribosomal genes in amphibian oocytes involves the generation of extrachromosomal copies of the genes, which replicate and then probably reintegrate at the same place from which they originated (23, 24). As discussed above, the requirement for large T antigen for integrated Py DNA amplification in our system could be explained by a similar mechanism. This mechanism, however, seems unlikely to have occurred in the SS1A cell line, and it is difficult to explain why the free viral DNA does not also reintegrate in other regions of the host genome, generating new viral insertions. Although we cannot exclude that this may happen in some cases, our results show clearly that this process is not a common event. Thus, it is possible that amplification of integrated polyoma DNA sequences occurs by "in situ" replication (triggered by large T antigen), and that the newly formed viral DNA molecules do not physically separate from the parental strands and reintegrate by homologous recombination, generating new tandem repeats. This mechanism could explain the results with both the ts-*a* H3A and the SS1A cell lines if we assume that in the latter viral DNA replication was interrupted shortly after initiation.

We have shown previously that Py DNA molecules can be excised from their tandem integration in the host chromosome, leading to the formation of "cured" derivatives in which an integral number of genomes are deleted from the integration site and the flanking sequences are preserved (2, 5). We have also shown that this process is under the control of a functional large T antigen, which could either induce rounds of viral DNA replication followed by recombination or promote recombination per se. From the data presented here it is evident that an active large T antigen is involved not only in excision and production of free viral DNA but also in amplification. Because the same viral function appears to be required for all these events, it is likely that the mechanisms originating them share one or more basic steps.

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