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Integration of the Simian Virus 40 Genome into Cellular DNA in Temperature-Sensitive (N) and Temperature-Insensitive (A) Transformants of 3T3 Rat and Chinese Hamster Lung Cells

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We studied the pattern of integration of the simian virus 40 (SV40) genome into the cellular DNA of N-transformants (temperature sensitive) and A-transformants (temperature insensitive) derived from 3T3-Fisher rat and Chinese hamster lung cells. The SV40 DNA was covalently linked to the cellular DNA in both types of transformants. In the rat cells, most N-transformants contained SV40 sequences integrated at a single site; most A-transformants contained SV40 sequences integrated at two to five sites. In the Chinese hamster cells, no significant correlation between the number of integration sites and the phenotype of the transformant was found; one to three integration sites were observed for both the N- and A-transformants. Single copies and tandem repeats of SV40 sequences were observed in A- and N-transformants derived from rat cells. A-transformants arise neither by amplification of the SV40 genome nor by integration at a unique site.

Infection of mammalian cells by *tsA* mutants of simian virus 40 (SV40) and polyoma virus gives rise to transformants that are either temperature sensitive (N) or temperature insensitive (A) (7-10, 12, 13, 17, 20-23, 26-28, 32). The relative proportions of the two types of transformants seem to depend upon several parameters, including the growth state of the cells for the first few days postinfection (23, 26, 27) and the ability of the virus to express the small-t antigen (28). Although several models have been proposed (12, 14, 23, 26-28), the molecular mechanism(s) responsible for the difference between the two types of transformants has not been found yet. In an attempt to gain some insight into these mechanisms, we examined the integration patterns of the SV40 genome into cellular DNA in a number of N- and A-transformed rat and hamster cell lines.

It has been reported that the number of integration sites and number of copies of the SV40 genome integrated per site varied from one transformed cell line to another (5, 16). This study was undertaken to determine what, if any, correlations exist between the temperature sensitivity of the *tsA* mutant-transformed cell lines and the number of copies of the SV40 genome integrated. We found that the SV40 genome is integrated into host sequences in N-transformants as well as in A-transformants and that no special pattern of integration is associated with the difference in temperature sensitivity.

MATERIALS AND METHODS

Transformed cells. The N- and A-type *tsA209*, SV40-transformed rat cells and the *tsA209* and *tsA209* viable deletion double-mutant-transformed Chinese hamster lung (CHL) cells have been described previously (20, 27, 28).

Viral DNA. The SV40 form I DNA used for nick translation and also to obtain form II and III DNAs that were used as standards was purchased from Bethesda Research Laboratories.

Isolation of high-molecular-weight cellular DNA. Rat cells or CHL cells were grown to confluence in Dulbecco-Vogt modified Eagle medium supplemented with 10% fetal calf serum. After cells were washed with phosphate-buffered saline, they were treated with lysis buffer (10 mM Tris-hydrochloride, pH 7.9, 1 mM, EDTA, 0.5% sodium dodecyl sulfate), incubated with 0.5 mg of proteinase K (Boehringer) per ml overnight at 37°C, and extracted twice with phenol and once with chloroform-isoamyl alcohol (24:1, vol/vol). The extracts were then dialyzed, incubated with 0.1 mg of RNase A (Boehringer) per ml, incubated again with proteinase K (0.1 mg/ml), reextracted, and dialyzed, as described by Botchan et al. (4). The molecular weight of the DNA was checked by agarose gel electrophoresis.

Restriction enzymes, digestion, and agarose gels. Cellular DNA (25 μg) was digested for 2 to 5 h with restriction enzymes by using the incubation media specified by New England Biolabs or Bethesda Research Laboratories, depending on the source of the enzyme. After incubation, 20 mM EDTA and 0.1 to 0.2 M NaCl (final concentrations) were added. The samples were extracted once with phenol and once with chloroform-isoamyl alcohol (24:1). Sodium ace-

tate (pH 6.0; final concentration, 0.2 M) and 2 volumes of absolute ethanol were added, and the solutions were stored overnight at −20°C and then centrifuged in a Brinkman centrifuge for 15 min. The precipitates were suspended in a solution containing 10 mM Tris-hydrochloride (pH 7.8), 6 mM EDTA, 0.2 mg of bromophenol blue per ml, and 7% glycerol and resolved by electrophoresis in a 0.7% agarose (Marine Colloids) horizontal slab gel in a solution containing 40 mM Tris-acetate (pH 7.8), 5 mM sodium acetate, and 1 mM EDTA (31) for 16 to 18 h at 40 V at room temperature. Lambda phage DNA *Hin*dIII fragments labeled with ^{32}P at their 5′ ends (Bethesda Research Laboratories) were used as DNA molecular weight standards.

Blotting. Transfer of DNA from the gels to nitrocellulose paper was performed by the Southern technique (30) with some modifications (5, 16). Gels were stained with 2.5 μg of ethidium bromide per ml, and pictures were taken under UV illumination. The gels were then treated with 0.2 M NaOH–0.6 M NaCl for 45 to 60 min, neutralized with 1 M Tris-hydrochloride (pH 7.3)–0.6 M NaCl for 45 min, and equilibrated with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 to 45 min with gentle rotation. Blotting on nitrocellulose membrane filter paper (0.45 μm; type HAWP; Millipore Corp.) was performed downward (gel at the top) for approximately 3 h and upward for approximately 20 h, soaking the 3 MM paper bridge in 6× SSC. The nitrocellulose paper was rinsed for 15 min in 6× SSC and air dried. It was baked for 2 h at 80°C under a vacuum and kept at room temperature until it was to be used.

Radioactive labeling of SV40 DNA. Nick translation was performed by the method of Maniatis et al. (18), with modifications suggested by Shoshana Segall (personal communication). The incubation mixture contained [α-^{32}P]dATP [tetra(triethylammonium) salt] and [α-^{32}P]dCTP [tetra(triethylammonium) salt] (300 to 500 Ci/mmol [New England Nuclear Corp.] or approximately 2,000 to 3,000 Ci/mmol [Amersham Corp.] previously dried), each at a concentration of 3.6 μM; the mixture also contained 3.6 μM unlabeled dGTP, 3.6 μM TTP, 50 mM Tris-hydrochloride (pH 7.8), 3 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 μg of bovine serum albumin per ml, and 10 μg of SV40 form I DNA (Bethesda Research Laboratories) per ml. The mixture was preincubated for 15 min at 15°C. *Escherichia coli* DNA polymerase I (40 U/ml; Boehringer) and 1.2 ng of DNase I (Worthington Biochemicals Corp.) per ml were then added, and the incubation was continued for 110 to 120 min (until a plateau was reached in the incorporation of ^{32}P into trichloroacetic acid-precipitable material). A solution containing 10 mM Tris-hydrochloride (pH 7.5), 0.1 M NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate was added; the sample was extracted with phenol and passed through a Sephadex G-50 fine column equilibrated with 10 mM Tris-hydrochloride (pH 7.5)–0.1 M NaCl–1 mM EDTA, and the fractions that contained the labeled DNA were pooled. An approximate yield of 1.5×10^8 to 6×10^8 cpm/μg of DNA was obtained.

Hybridization. Blotted nitrocellulose papers were soaked in 3× SSC for approximately 45 min at room temperature. They were then preincubated for 3 h in 3× SSC–10× Denhardt solution (1× Denhardt solution contains 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone) at 68°C and later with a solution containing 3× SSC, 10× Denhardt solution, 0.1% sodium dodecyl sulfate, 10 μg of polyadenylic acid per ml, and 100 μg of sheared and denatured salmon sperm DNA per ml for 1 h at 68°C. The hybridization mixture contained 3× SSC, 10× Denhardt solution, 0.1% sodium dodecyl sulfate, 10 μg of polyadenylic acid per ml, 100 μg of sheared and denatured salmon sperm DNA per ml, and 0.9×10^7 to 1.2×10^7 cpm of denatured ^{32}P-labeled SV40 DNA (obtained by nick translation; concentration, 20 to 90 ng/ml) per ml in a total volume of 7 to 14 ml; the hybridization was in sealed bags at 68°C for approximately 40 h. After hybridization, there were four to five washings with a solution containing 3× SSC, 10× Denhardt solution, 0.1% sodium dodecyl sulfate, and 50 μg of sheared and denatured salmon sperm DNA per ml and one washing with a solution containing 0.1× SSC, 50 μg of denatured and sheared salmon sperm DNA per ml, and 0.1% sodium dodecyl sulfate at 68°C; the filter paper was then rinsed once with 3× SSC at room temperature (15). Millipore filter papers were air dried and exposed to Kodak X-Omat R film with Max I Picker screens at −70°C for 2 to 9 days.

RESULTS

Integration of the SV40 genome in N- and A-type *tsA209* and *tsA209* viable deletion double-mutant-transformed CHL cells. High-molecular-weight cellular DNA from CHL cells transformed by the *tsA209* and *tsA209* viable deletion double mutants of SV40 was digested with restriction enzymes that cut the cellular DNA but not SV40 DNA. One of the limitations of the Southern technique is that the transfer efficiency of pieces of DNA approximately 20,000 base pairs long or longer is lower than that of smaller pieces (1, 5) and may go undetected. Therefore, several restriction enzymes were used, and the highest number of bands obtained with any of them was considered the minimum number of integration sites. The enzymes used were *Bgl*II, *Sac*I, *Sst*I (isoschizomer of *Sac*I), and *Xba*I.

Figure 1 shows the patterns of integration of SV40 into the cellular DNA of several N- and A-type CHL cell lines transformed either by *tsA209* or by one of several double mutants. Since hamster cells are semipermissive for SV40, the cells were grown at 41°C in order to avoid the presence of free SV40 DNA that occurs at 33°C (19). No form I or II SV40 DNA was observed.

Table 1 shows the minimum number of integration sites and the sizes of the different bands obtained with each restriction enzyme for the *tsA209* N- and A-transformants. From the sizes of the restriction fragments it is possible to calculate the maximum number of copies of the SV40 genome possible per integration site; i.e.,

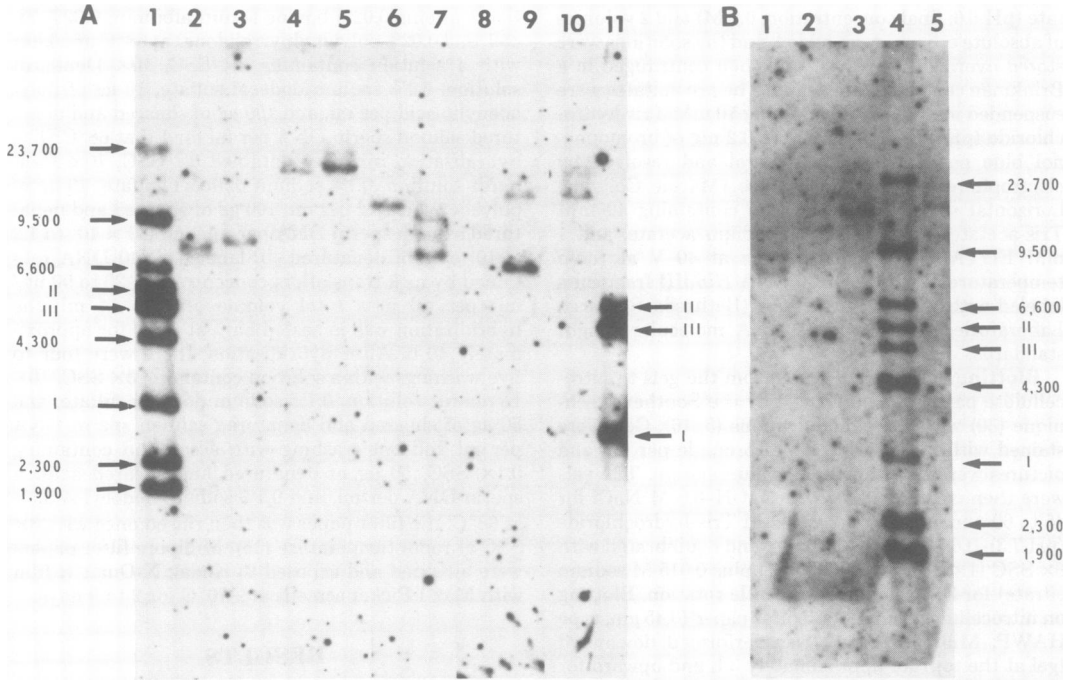

FIG. 1. *Hybridization patterns of CHL transformed cell lines with SV40 DNA no-cut enzymes. (A) Cellular DNA was digested with BglII, run in gels, and hybridized as indicated in the text. Lane 1, ^{32}P-labeled λ DNA HindIII fragments (numbers of base pairs per band are indicated by arrows; I, II, and III, SV40 form I, II, and III DNA); lane 2, CHLA209L4; lane 3, CHLA209L5; lane 4, CHLA209L38; lane 5, CHLA209L49; lane 6, CHLA209L62; lane 7, CHLA209L51; lane 8, CHLA209Δ289L5; lane 9, CHLA209Δ289L4; lane 10, CHLA209Δ288L3; lane 11, SV40 form II, III, and I DNAs. (B) XbaI digestion of CHL cellular DNA. Lane 1, CHLA209L51; lane 2, CHLA209L38; lane 3, CHLA209Δ290L4; lane 4, standards as in (A), lane 1; lane 5, CHLA209Δ292L14.*

CHLA209L4 and CHLA209L5 must have fewer than two full-size SV40 DNA copies each, whereas CHLA209L38 and CHLA209L62 must have fewer than three copies and CHLA209L49 must have fewer than four copies. One to three integration sites were observed in the different N- and A-transformed cell lines, and the patterns varied both between the N- and A-types and among the different N- and A-transformants.

N- and A-transformants of CHL cells induced by *tsA209* viable deletion double mutants (12) were also examined. The number of integration sites varied from one to three, and the maximum number of copies of the SV40 genome per integration site varied from fewer than two to fewer than six (Table 2). In cell lines CHLA209Δ292L14, CHLA209Δ287L5, and CHLA209Δ292L19 bands of similar molecular weights (inside the limits of error) were observed after digestion with *Xba*I. When these results were combined with the results with the *tsA* and double-mutant transformants, 4 of 12 N-transformants appeared to have arisen from the insertion of SV40 at a single site, and 2 of 10 A-transformants arose similarly. It may be significant that of 11 cell lines established at multiplicities of infection from 0.065 to 0.48 (Tables 1 and 2), 7 appeared to have SV40 integrated at a single site, whereas of 12 cell lines established at multiplicities of infection from 0.7 to 3, none had SV40 integrated at only a single site.

In some of the cell lines that appear to have SV40 DNA integrated at only one site, the cellular DNA was treated with restriction enzymes that cut the SV40 genome once (e.g., *Bgl*I, *Taq*I, *Bam*HI, *Eco*RI). The presence of linear, full-length SV40 DNA (form III) after this treatment would indicate a repetition of the relevant site. The flanking sequences bound to cellular DNA may or may not be detected according to their sizes and/or the size of the SV40 region attached (Table 3). No evidence for more than a single full-length insertion was found for several N-type cell lines (e.g., CHLA209L5 and CHLA209L62), whereas repetition of the *Taq*I, *Bam*HI, and *Eco*RI sequences was observed in CHLA209L49, an A-type line.

Integration of the SV40 genome in N- and

TABLE 1. *Number of SV40 genome integration sites in tsA209-transformed CHL cells*[a]

Cell line	MOI[b]	Type of transformant	No. of integration sites	No. of base pairs per band containing SV40 sequences[c]	Restriction enzyme
CHLA209L5	0.48	N	1	7,900	*BglII*
			1	ND[d]	*SstI*
CHLA209L62	0.065	N	1	11,400	*BglII*
			1	13,800	*XbaI*
CHLA209L51	0.065	N	2	8,000, 8,800	*XbaI*
			3	7,200, 8,400, 9,700	*BglII*
CHLA209L61	0.065	N	1	26,800	*XbaI*
			2	20,900, 25,100	*BglII*
CHLA209L56	0.065	N	1	15,800	*XbaI*
			1	10,100	*BglII*
CHLA209L4	0.48	N or A	1	7,600	*BglII*
			1	16,000	*XbaI*
			1	ND	*SstI*
CHLA209L49	0.065	A	1	18,100	*BglII*
			1	15,900	*XbaI*
CHLA209L38	0.15	A	1	17,400	*BglII*
			1	11,900	*XbaI*
			1	13,700	*SstI*
CHLA209L34	0.15	A	2	9,500, 11,200	*XbaI*
			3	7,900, 9,500, 13,100	*BglII*

[a] DNA from CHL *tsA209*-transformed cell lines was incubated with restriction enzymes as described in the text.

[b] MOI, Multiplicity of infection (see reference 20).

[c] The number of base pairs was determined by using ^{32}P-labeled λ DNA *Hin*dIII fragments as standards (see Fig. 1). The error in the determination of base pairs, which was based on data obtained in different experiments, was approximately 200 base pairs for fragments smaller than 9,500 base pairs; however, for fragments 10,000 to 30,000 base pairs long it was on the order of 1,000 base pairs in some cases due to the higher slope of the curve of molecular weight versus mobility in that region (2, 5).

[d] ND, Not determined.

A-type *tsA209*-transformed rat cells. The integration of the SV40 genome into the cellular DNAs of N- and A-transformed rat cells was also studied. Some of the patterns of integration obtained with the rat transformants are shown in Fig. 2. As expected, no free SV40 DNA was observed since rat cells are nonpermissive for SV40. Three of the A-type cell lines had similar integration patterns with two different restriction endonucleases that do not cut SV40 DNA. Cell lines A209-FR3T3-A30 and A209-FR3T3-A37 were obtained from the same flask; it cannot be rigorously excluded they are not derived from the same transformed cell. However, A209-FR3T3-A43 was isolated in a separate experiment and must therefore have arisen by an independent event.

Table 4 shows the results obtained with several restriction enzymes that do not cut SV40 DNA. Among the N-transformants, one to three integration sites were observed, and among the A-transformants one to five integration sites were observed. For the N-transformants there was a predominance of single-integration-site patterns (5 of 8), whereas for the A-transformants multiple integration sites predominated (9 of 10).

Insertions of sizes smaller than full-length SV40 DNA have been observed, as in A209-FR3T3-A99. In a long film exposure of A209-FR3T3-A43 digested with an SV40 DNA no-cut enzyme, a light band of 4,000 base pairs was observed, which may have been due to the presence of a small piece of SV40 DNA in that fragment.

A number of the cell lines were examined after

TABLE 2. *Number of SV40 genome integration sites in viable deletion tsA209 double-mutant-transformed CHL cells*

Cell line	MOI[a]	Type of transformant	No. of integration sites	No. of base pairs per band containing SV40 sequences[b]	Restriction enzyme
CHLA209Δ288L3	2.3	N	1	16,100	*Sst*I
			2	19,500, 23,000	*Xba*I
			2	12,900, 16,500	*Bgl*II
CHLA209Δ289L14	3	N	2	6,600, 13,200	*Bgl*II
			2	9,400, 18,000	*Xba*I
CHLA209Δ290L5	1	N	2	ND[c]	*Bgl*II
			3	7,900, 20,000, 28,100	*Sst*I
CHLA209Δ290L12	0.14	N	1	19,000	*Xba*I
			1	19,200	*Bgl*II
CHLA209Δ291L4	1.6	N	1	12,200	*Bgl*II
			1	28,600	*Xba*I
			2	10,700, 17,000	*Sst*I
CHLA209Δ292L14	2.9	N	2	ND	*Bgl*II
			3	10,400, 15,500, 21,000	*Xba*I
CHLA209Δ292L16	2.9	N	2	15,300, 19,700	*Bgl*II
			2	17,300, 25,000	*Xba*I
CHLA209Δ287L5	2	A	2	19,800, 23,000	*Bgl*II
			3	10,800, 15,500, 23,700	*Xba*I
CHLA209Δ287L6	0.9	A	2	19,500, 24,500	*Xba*I
			2	17,000, 24,000	*Bgl*II
CHLA209Δ289L5	2	A	2	9,100, 11,600	*Bgl*II
CHLA209Δ290L4	0.7	A	3	7,100, 14,500, 23,700	*Xba*I
			3	10,500, 13,800, 17,000	*Bgl*II
CHLA209Δ291L2	2.4	A	2	11,300, 21,500	*Xba*I
			3	ND	*Bgl*II
CHLA209Δ291L13	0.3	A	1	8,000	*Xba*I
			2	ND	*Bgl*II
CHLA209Δ292L19	0.9	A	2	ND	*Bgl*II
			3	10,800, 15,200, 22,900	*Xba*I

[a] MOI, Multiplicity of infection (see reference 20).
[b] See Table 1, footnote *c*.
[c] ND, Not determined.

digestion with restriction enzymes that cut SV40 DNA once. Figure 3 shows the patterns obtained after digestion of cellular DNA from some cell lines with two of these enzymes, and Table 5 summarizes all of these results. Among the N-transformants with one integration site, the following observations were made. (i) One (A209-FR3T3-N94) contained fewer than 1.5 SV40 genomic equivalents (sequence repetitions of the *Bgl*I, *Bam*HI, and *Taq*I sites were not present). (ii) Three transformants contained partial repeats of the SV40 genome, as demonstrated by the fact that some restriction enzymes that cut SV40 DNA once gave rise to full-length form III DNA, whereas others did not. And (iii) no transformant contained a complete duplication. In the only A-transformant containing one integration site (A209-FR3T3-A12) fewer than 1.4 copies of SV40 DNA were present (*Bgl*I, *Taq*I, *Hpa*II, and *Bam*HI did not release form III SV40 DNA). In the cell lines with more than one integration site, seven of nine contained at

least some partial repetition. However, we could not distinguish from which of the sites form III DNA was derived.

DISCUSSION

Integration of SV40 DNA into the cell genome of transformed cells. Among the CHL and 3T3 rat transformants examined, all contained from one to five integration sites. For the CHL transformed lines, most contained SV40 DNA integrated at multiple sites. However, among those lines generated at initial multiplicities of infection of <0.48 PFU/cell, the majority had SV40 sequences integrated at a single site (7 of 11), whereas all of those generated at multiplicities of infection of >0.7 PFU/cell had SV40 sequences at more than one site (12 of 12). For the 3T3 rat transformants, which were generated at initial multiplicities of infection of from 5 to 100 PFU/cell (i.e., at multiplicities of infection higher than used with the hamster cells), most contained SV40 integrated at multiple sites (12 of 18), but a significant number had SV40 integrated at a single site. (Botchan et al. [5] also observed a predominance of multiple integration sites in SV40 rat transformants.) Thus, it appears that in rat cells single-site integrations (6 of 18) are more common at high multiplicities of infection than they are in hamster cells (0 of 12). This difference may relate to the fact that hamster cells are semipermissive and rat cells are nonpermissive. Among the cell lines that appeared to contain SV40 integrated at a single site, some may contain only a single copy of the SV40 genome (CHLA209L4, CHLA209L5, CHLA209L62, A209-FR3T3-N94, A209-FR3T3-A12), whereas others clearly contained partial repetition of the SV40 genome (A209-FR3T3-N11, A209-FR3T3-N53, A209-FR3T3-N30) and still others may contain multiple repetitions of the SV40 genome (CHLA209L49).

TABLE 3. *Presence of single copies or tandem repeats of SV40 sequences integrated in the cell genome of transformed CHL cells*

Cell line	Type of transformant	Form III SV40 DNA[a]	Restriction enzymes
CHLA209L5	N	−	*Taq*I, *Bam*HI, *Eco*RI, *Bgl*I
CHLA209L62	N	−	*Taq*I, *Bam*HI, *Eco*RI
CHLA209L4	N or A	−	*Taq*I, *Bam*HI, *Eco*RI, *Bgl*I
CHLA209L49	A	+	*Taq*I, *Bam*HI, *Eco*RI

[a] Presence (+) or absence (−) of SV40 form III DNA released after digestion with the enzymes indicated on the table.

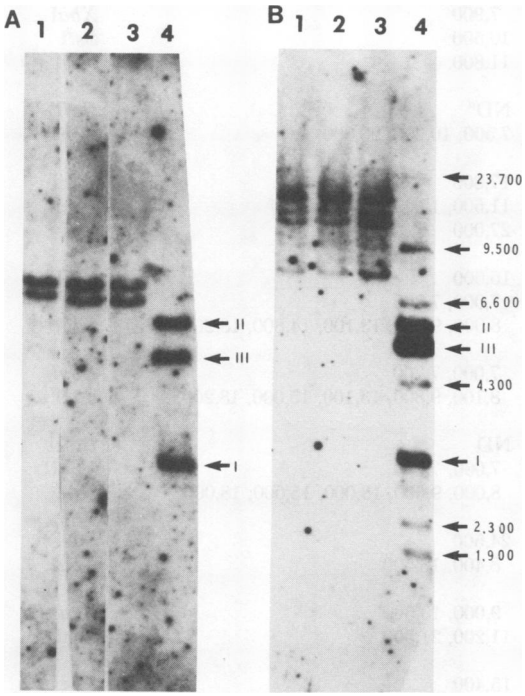

FIG. 2. *Hybridization patterns of transformed rat cell lines digested with SV40 DNA no-cut enzymes. (A) XbaI digestion of 3T3 rat transformed cells. Lane 1, A209-FR3T3-A30; lane 2, A209-FR3T3-A37; lane 3, A209-FR3T3-A43; lane 4, SV40 form II, III, and I DNAs. (B) SacI digestion of 3T3 rat transformed cells. Lane 1, A209-FR3T3-A37; lane 2, A209-FR3T3-A30; lane 3, A209-FR3T3-A43; lane 4, DNA molecular weight standards (see Fig. 1).*

Some explanation is required for the fact that transformed cell lines that contain papovavirus sequences at a single site frequently have partial repetition of the papovavirus genome. Tandem repeats of viral sequences have been observed before in polyoma virus-transformed rat cells (a semipermissive system) (2) and in SV40-transformed rat cells (a nonpermissive system) (5). We observed tandem repeats of SV40 sequences in one of four CHL lines (a semipermissive system) and in three of five rat lines (a nonpermissive system). At least one mechanism for the generation of tandem repeats is via rolling circle replication. Rolling circle replication in permissive cells has been demonstrated for polyoma virus (3) and SV40 (R. G. Martin and V. P. Setlow, Cell, in press) and suggested in nonpermissive cells for SV40 (W. Chia and P. Rigby, personal communication). We speculate that the generation of linear molecules is prerequisite to integration within the host genome and that rolling circle replication is the preferred manner for generating linear molecules. Other mechanisms for generating tandem repeats have been

TABLE 4. *Number of SV40 genome integration sites in transformed 3T3 rat cells*

Cell line	Type of transformant	No. of integration sites	No. of base pairs per band containing SV40 sequences[a]	Restriction enzyme
A209-FR3T3-N11[b]	N	1	8,900	*Xba*I
		1	23,500	*Sac*I
A209-FR3T3-N53[b]	N	1	14,500	*Sac*I
		1	17,500	*Xba*I
A209-FR3T3-N55[b]	N	1	14,800	*Xba*I
A209-FR3T3-N30[b]	N	1	8,400	*Bgl*II
		1	14,000	*Xba*I
		1	17,200	*Sac*I
A209-FR3T3-N94[c]	N	1	16,300	*Xba*I
		1	10,500	*Sac*I
		1	10,300	*Sst*I
A209-FR3T3-N82[c]	N	2	10,800, 27,000	*Xba*I
		3	15,300, 21,200, 25,500	*Sac*I
A209-FR3T3-N87[c]	N	2	8,900, 27,000	*Xba*I
		1	24,800	*Sac*I
A209-FR3T3-N91[c]	N	2	12,400, 18,800	*Bgl*II
		2	14,400, 19,800	*Sac*I
		3	9,100, 13,800, 21,000	*Xba*I
A209-FR3T3-A12[b]	A	1	7,900	*Xba*I
		1	10,500	*Sst*I
		1	11,800	*Sac*I
A209-FR3T3-A24[b]	A	1	ND[d]	*Bgl*II
		3	7,500, 10,200, 26,000	*Xba*I
A209-FR3T3-A22[b]	A	1	17,800	*Xba*I
		2	11,500, 18,000	*Sac*I
		1	27,000	*Sst*I
A209-FR3T3-A30[b]	A	1	15,000	*Bgl*II
		2	7,000, 7,700	*Xba*I
		5	8,000, 9,800, 13,100, 14,800, 18,200	*Sac*I
A209-FR3T3-A37[b]	A	2	7,000, 7,700	*Xba*I
		5	8,100, 9,800, 13,100, 15,000, 18,200	*Sac*I
A209-FR3T3-A43[b]	A	1	ND	*Bgl*II
		2	7,000, 7,700	*Xba*I
		5	8,000, 9,600, 13,000, 15,000, 18,000	*Sac*I
A209-FR3T3-A16[b]	A	1	24,500	*Sac*I
		2	8,400, 18,500	*Xba*I
A209-FR3T3-A81[c]	A	2	9,000, 10,300	*Xba*I
		2	11,200, 21,500	*Sac*I
A209-FR3T3-A84[c]	A	1	15,400	*Xba*I
		2	16,100, 25,500	*Sac*I
A209-FR3T3-A99[c]	A	2	4,100, 18,000	*Xba*I
		3	4,500, 17,200, 23,700	*Sac*I

[a] See Table 1, footnote *c*.

[b] These cell lines are the same as those described in reference 27, but with the letter L, M, or H instead of N or A before the last two digits.

[c] These cell lines were isolated as described in reference 28.

[d] ND, Not determined.

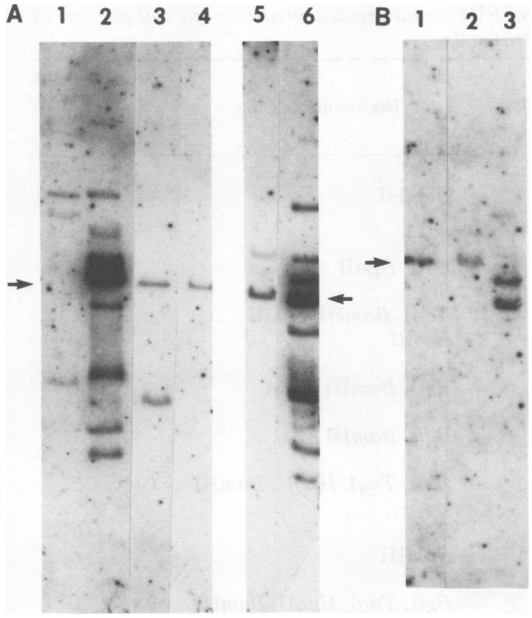

FIG. 3. *Hybridization patterns of transformed rat cell lines digested with SV40 DNA one-cut enzymes. (A) BglI and TaqI digestions of 3T3 rat cells. Lane 1, A209-FR3T3-N53 TaqI digestion; lane 2, DNA molecular weight standards (see Fig. 1); lane 3, A209-FR3T3-A22 TaqI digestion; lane 4, A209-FR3T3-N53 BglI digestion; lane 5, A209-FR3T3-A22 BglI digestion; lane 6, DNA molecular weight standards. (B) Lane 1, SV40 form III DNA; lane 2, A209-FR3T3-N30 TaqI digestion; lane 3, A209-FR3T3-A30 TaqI digestion. Arrows indicate position of form III SV40 DNA.*

proposed (2, 6).

The results obtained with the viable deletion double mutants indicate that small-t antigen is not required for integration of the SV40 genome into the cellular DNA. Furthermore, small-t antigen does not seem to affect the patterns of integration; i.e., both single-site and multiple-site integrations were found whether or not the virus was capable of expressing the small-t antigen.

The conclusion that SV40 DNA does not integrate at specific sites in the cell genome was based on the observation of different patterns of integration in different cell lines (5). However, it is important to point out that integration at specific sequences cannot be ruled out. It is possible to get different restriction patterns despite integration in the same gene if (i) different amounts of host DNA are deleted upon SV40 integration, (ii) different amounts of SV40 DNA are integrated in the same gene, or (iii) integration occurs at different portions (restriction fragments) of the same gene. Furthermore, cellular DNA contains highly repeated sequences which exist repeated in tandem or interspersed with unrelated sequences (29). The SV40 genome can recombine with these highly repeated sequences (24, 25). Different patterns of integration of the viral DNA in different cell lines may be explained by integration in a specific highly repeated sequence that is interspersed with unrelated sequences. Alternatively, if the viral DNA integrated in a sequence repeated in tandem, identical bands would be observed in different cell lines, even when integration had actually occurred at different sites. Three rat transformed cell lines (at least two of which were obtained independently) presented the same integration pattern with two different SV40 no-cut enzymes. It remains to be established whether multiple bands in a single cell line arise by independent events.

Integration of the SV40 genome into the cellular DNA of N- and A-transformants. There was some suggestion that N-transformants were more likely than A-transformants to contain SV40 at a single insertion site. Taken together, 9 of 20 N-transformants and 3 of 20 A-transformants contained SV40 DNA integrated at a single site. For the CHL cells 4 of 12 N-transformants and 2 of 10 A-transformants contained single-site insertions; for the rat cells 5 of 8 N-transformants and 1 of 10 A-transformants contained single-site insertions.

Among the lines with only one integration site we found A-transformants with fewer than 1.4 copies (A209-FR3T3-A12) and with more than 1.5 copies (CHLA209L49) and N-transformants with fewer than 1.5 copies (CHLA209L5, CHLA209L62, A209-FR3T3-N94) and with more than 1.7 copies but fewer than 2 copies (A209-FR3T3-N30). The maximum and minimum numbers are based on the assumption of tandem repetition of the integrated sequences and come from the ability or failure of the SV40 DNA one-cut enzyme to release full-length SV40 DNA. No correlation between copy number and phenotype is apparent, since of those tested two CHL N-transformants have fewer than two copies and one A-transformant may have more than two copies, whereas for the rat cells three N-transformants have fewer than two copies and one may have a single copy, but the one A-transformant also may have a single copy.

Several conclusions can be drawn concerning the relationship between SV40 genome copy number and the temperature sensitivity of the resulting transformants. (i) N-transformants do not arise from the failure of SV40 to become integrated. (ii) A-transformants do not arise by gene amplification or by a simple gene dosage effect. And (iii) neither A- nor N-transformants

TABLE 5. *Presence of single copies or tandem repeats of SV40 sequences integrated in the cell genome of transformed rat cells*

Cell line	Type of transformant	Form III SV40 DNA[a]	Restriction enzyme	Minimum no. of integration sites
A209-FR3T3-N11	N	+ −	*Bgl*I *Bam*HI	1
A209-FR3T3-N53	N	− +	*Taq*I *Bgl*I, *Hpa*II	1
A209-FR3T3-N30	N	+ −	*Taq*I, *Bam*HI, *Hpa*II *Eco*RI	1
A209-FR3T3-N94	N	−	*Bgl*I, *Bam*HI, *Taq*I	1
A209-FR3T3-N82	N	+	*Bgl*I, *Bam*HI	3
A209-FR3T3-N87	N	+	*Bgl*I, *Taq*I, *Hpa*II, *Bam*HI	2
A209-FR3T3-N91	N	+ −	*Bgl*I *Bam*HI	3
A209-FR3T3-A12	A	−	*Bgl*I, *Taq*I, *Hpa*II, *Bam*HI	1
A209-FR3T3-A22	A	+	*Taq*I, *Bgl*I	2
A209-FR3T3-A30	A	− +	*Taq*I, *Bam*HI *Eco*RI, *Hpa*II	5
A209-FR3T3-A16	A	−	*Bgl*I, *Bam*HI, *Taq*I	2
A209-FR3T3-A81	A	−	*Bgl*I, *Bam*HI	2
A209-FR3T3-A84	A	+ −	*Bgl*I, *Bam*HI *Hpa*II	2
A209-FR3T3-A99	A	+	*Bgl*I, *Bam*HI	3

[a] See Table 3 and Fig. 3.

have unique integration patterns.

One possible mechanism to explain the N-phenotype was that SV40 replicated as a plasmid in N-transformants. This possibility is eliminated by the finding that all N-transformants contained integrated SV40 sequences.

A second possible explanation for the temperature-insensitive phenotype of the A-transformants is that T-antigen is required for the maintenance of transformation in all transformed cells but that high levels of the thermolabile T-antigen result in "leaky" expression of the transformed phenotype at the restrictive temperature (7, 11, 33). It is still possible that in A-transformants T-antigen accumulates more than in N-transformants. However, we have eliminated the possibility that such leakiness invariably results from the integration of multiple copies of the SV40 genome. It cannot, for example, be argued that all A-transformants contain multiple repeats of the early region of SV40 and that even those A-transformants with SV40 sequences at a single site contain multiple repeats of the SV40 genome. Cell line A209-FR3T3-A12 contains fewer than two copies of the SV40 early region, and cell lines A209-FR3T3-A16 and A209-FR3T3-A81 contain a maximum of two functional early regions. Except for CHLA209L49, we have not found an A-transformant that released unit-length form III SV40 DNA with all of the SV40 DNA one-cut restriction enzymes assayed. This allows us to rule out the possibility of more than five or six copies of the SV40 genome tandemly integrated (gone undetected by SV40 DNA no-cut restriction enzymes due to the limitations of the Southern technique). We conclude that gene dosage alone cannot explain the A-transformants.

Another possible explanation for the A-transformants is that they all result from mutation of a growth control gene by integration of the viral genome into it. T-antigen would not be required for the maintenance of transformation in these cells, in contrast to N-transformants, in which

the role of integration of the SV40 genome is to permanently provide large T-antigen (27, 28). Certainly with this approach we could discern no unique pattern of integration in A-transformants which distinguished them from N-transformants. Furthermore, there was no readily discernable common element to either the A-type or N-type integration pattern. We have not eliminated the possibility that A-transformants result from integration of the viral genome at any of a small number of control genes. Rat transformants with a single integration site were frequent among N-transformants but rare among A-transformants. If growth control genes represent a minority of the cellular genome, (i) A-transformants would arise preferentially from cells in which the viral genome was able to integrate at several sites, and (ii) N-transformants would arise more frequently from cells in which the SV40 genome was able to integrate at only one site.

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., R. Dulbecco, M. Fried, and R. Kamen.** 1979. State and organization of polyoma virus 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., G. McKenna, and P. A. Sharp.** 1973. Cleavage of mouse DNA by a restriction enzyme as a clue to the arrangement of genes. Cold Spring Harbor Symp. Quant. Biol. **38**:383–395.
5. **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.
6. **Botchan, M., W. Topp, and J. Sambrook.** 1978. Studies on simian virus 40 excision from cellular chromosomes. Cold Spring Harbor Symp. Quant. Biol. **43**:709–719.
7. **Brockman, W. W.** 1978. Transformation of BALB/c-3T3 cells by *tsA* mutants of simian virus 40: temperature sensitivity of the transformed phenotype and retransformation by wild-type virus. J. Virol. **25**:860–870.
8. **Brugge, J. S., and J. S. Butel.** 1975. Role of simian virus 40 gene A function in maintenance of transformation. J. Virol. **15**:619–635.
9. **Di Mayorca, G., J. Callender, G. Marin, and R. Giordano.** 1969. Temperature-sensitive mutants of polyoma virus. Virology **38**:126–133.
10. **Eckhart, W.** 1969. Complementation and transformation by temperature-sensitive mutants of polyoma virus. Virology **38**:120–125.
11. **Edwards, C. A. F., G. Khoury, and R. G. Martin.** 1979. Phosphorylation of T-antigen and control of T-antigen expression in cells transformed by wild-type and *tsA* mutants of simian virus 40. J. Virol. **29**:753–762.
12. **Fluck, M. M., and T. L. Benjamin.** 1979. Comparisons of two early gene functions essential for transformation in polyoma virus and SV40. Virology **96**:205–228.
13. **Fried, M.** 1965. Cell transformation ability of a temperature-sensitive mutant of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **53**:486–491.
14. **Gaudray, P., M. Rassoulzadegan, and F. Cuzin.** 1978. Expression of simian virus 40 early genes in transformed rat cells is correlated with maintenance of the transformed phenotype. Proc. Natl. Acad. Sci. U.S.A. **75**: 4987–4991.
15. **Jeffreys, A. J., and R. A. Flavell.** 1977. A physical map of the DNA regions flanking the rabbit β-globin gene. Cell **12**:429–439.
16. **Ketner, G., and T. J. Kelly, Jr.** 1976. Integrated simian virus 40 sequences in transformed cell DNA: analysis using restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. **73**:1102–1106.
17. **Kimura, G., and A. Itagaki.** 1975. Initiation and maintenance of cell transformation by simian virus 40: a viral genetic property. Proc. Natl. Acad. Sci. U.S.A. **72**:673–677.
18. **Maniatis, T., S. G. Kee, A. Efstratiadis, and F. C. Kafatos.** 1976. Amplification and characterization of a β-globin gene synthesized in vitro. Cell **8**:163–182.
19. **Martin, R. G., and J. Y. Chou.** 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. **15**:599–612.
20. **Martin, R. G., V. P. Setlow, and C. A. F. Edwards.** 1979. Roles of simian virus 40 tumor antigens in transformation of Chinese hamster lung cells: studies with simian virus 40 double mutants. J. Virol. **31**:596–607.
21. **Osborn, M., and K. Weber.** 1975. Simian virus 40 gene A function and maintenance of transformation. J. Virol. **15**:636–644.
22. **Rassoulzadegan, M., B. Perbal, and F. Cuzin.** 1978. Growth control in simian virus 40-transformed rat cells: temperature-independent expression of the transformed phenotype in *tsA* transformants derived by agar selection. J. Virol. **28**:1–5.
23. **Rassoulzadegan, M., R. Seif, and F. Cuzin.** 1978. Conditions leading to the establishment of the N (*a* gene-dependent) and A (*a* gene-independent) transformed states after polyoma virus infection of rat fibroblasts. J. Virol. **28**:421–426.
24. **Rosenberg, M., S. Segal, E. L. Kuff, and M. F. Singer.** 1977. The nucleotide sequence of repetitive monkey DNA found in defective simian virus 40. Cell **11**:845–857.
25. **Rosenberg, H., M. Singer, and M. Rosenberg.** 1978. Highly reiterated sequences of simiansimiansimian simiansimian. Science **200**:394–402.
26. **Seif, R., and F. Cuzin.** 1977. Temperature-sensitive growth regulation in one type of transformed rat cells induced by the *tsa* mutant of polyoma virus. J. Virol. **24**:721–728.
27. **Seif, R., and R. G. Martin.** 1979. Growth state of the cell early after infection with simian virus 40 determines whether the maintenance of transformation will be A-gene dependent or independent. J. Virol. **31**:350–359.
28. **Seif, R., and R. G. Martin.** 1979. Simian virus 40 small t-antigen is not required for the maintenance of transformation but may act as a promoter (cocarcinogen) during establishment of transformation in resting rat cells. J. Virol. **32**:979–988.
29. **Singer, D. S.** 1979. Arrangement of a highly repeated DNA sequence in the genome and chromatin of the African green monkey. J. Biol. Chem. **254**:5506–5514.
30. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
31. **Sugden, B., B. Detroy, R. J. Roberts, and J. Sambrook.** 1975. Agarose slab-gel electrophoresis equipment. Anal. Biochem. **68**:36–46.
32. **Tegtmeyer, P.** 1975. Functions of simian virus 40 gene A in transforming infection. J. Virol. **15**:613–618.
33. **Tenen, D. G., R. G. Martin, J. Anderson, and D. M. Livingston.** 1977. Biological and biochemical studies of cells transformed by simian virus 40 temperature-sensitive gene A mutants and A mutant revertants. J. Virol. **22**:210–218.