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Simian Virus 40 Integration Sites in the Genome of Virus-Transformed Mouse Cells

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To gain information on the specificity of simian virus 40 (SV40) integration in the genome of transformed cells, mouse 3T3 cells were transformed by a temperature-sensitive (ts) SV40 mutant, using high multiplicity of infection (MOI). Transformed cells were superinfected with wild-type (wt) virus at high MOI. Clones were isolated and fused with permissive BSC-1 cells to promote virus rescue. All rescued viruses were of the ts type only. When the high-MOI transformants were infected with ^{3}H-labeled wt SV40, the amount of radioactivity associated with their nuclear fraction was found to be similar to that of 3T3 cells. 3T3 cells were then transformed by ts SV40 at low MOI and superinfected by wt virus at high MOI. Upon fusion with BSC-1 cells, most clones produced both ts and wt virus. These results suggest that the number of stable SV40 integration sites in the 3T3 genome is limited, since they can be saturated by transformation at high MOI. When the MOI is low, the sites are not saturated and a subsequent infection can lead to integration.

Mouse fibroblasts of the 3T3 cell line stably transformed by simian virus 40 (SV40) contain the viral genome integrated covalently into the host genome (9). These cells do not produce infectious virus but maintain the presence of the viral genome in a hereditary manner. The number of SV40 genome equivalents present in a diploid complement of DNA of transformed cells varies from 1 to 10 (2, 7). It is, however, not known whether the viral genome is integrated at a specific site(s). Weiss, using mouse × human somatic hybrid cells, attempted to identify the chromosome(s) bearing the SV40 genome in human cells. Her results suggested that integration probably occurred in several chromosomes (16). Gelb and Martin, using DNA-DNA reassociation kinetics, showed that SV40 DNA was associated with the nonrepetitive sequences of mouse DNA (6), suggesting some specificity of integration. More recently, Croce et al. obtained results supporting the hypothesis that in human cells SV40 is preferentially integrated into a specific chromosome, C7 (4).

Dubbs and Kit (5) infected mouse 3T3 cells with a mixture of two strains of SV40. Some transformed clones, after fusion with permissive cells, yielded both types of virus. These results showed that two independent viral strains could be integrated into the genome of the same cell. It is, however, not known whether the viral genomes had integrated in a contiguous manner at the same site or in different integration sites.

In this paper, we describe a series of experiments suggesting that the number of stable SV40 integration sites in the genome of mouse 3T3 cells is limited, since these sites can be saturated when the cells are transformed by using high multiplicity of infection (MOI). Since the integration sites appear to be limited, it is possible that they may also be specific.

MATERIALS AND METHODS

Cells. Mouse fibroblast cells of the 3T3 line (13) and monkey kidney cells of the BSC-1 line were used. Cells were cultured in Dulbecco modified Eagle medium containing 10% calf serum.

Virus. The wild-type strain of SV40 (wt SV40) and a derived temperature-sensitive (ts) mutant (tsB4) were obtained from P. Tegtmeyer (12). Wt and tsB4 stocks were prepared by infecting monolayer of BSC-1 cells at an MOI of 10 PFU/cell. The infected cells were grown in 2% fetal calf serum at 33 C for tsB4 and at 36.5 C for wt. Virus was purified by cesium chloride density gradient centrifugation and titered by plaque assay on BSC-1 cells. Plaques were scored after about 14 days of incubation at 39 C or 21 days at 32 C.

Transformation. 3T3 cells were transformed by tsB4 and wt viruses, respectively, as described (8). 3T3 cells were grown to semiconfluency in a 60-mm plate and infected at different MOIs. Adsorption was done at 32 C for 2 h. Cells infected with wt SV40 were incubated at 36.5 C for 24 h, whereas cells infected with tsB4 were incubated at 32 C for 24 h. After incubation, cells were diluted and plated at concentrations of 10^4, 10^3, and 10^2 cells per 100-mm plate and incubated at 36.5 C. Medium was changed every 3

days. After 3 weeks of incubation transformed colonies were scored, isolated, and subcultured. Cells of colonies that grew to high saturation density and were T-antigen positive were used for further experiments.

T-antigen. SV40 T-antigen was identified by immunofluoroscence as previously described (8).

Rescue of SV40. Transformed cells (10^6) were mixed with BSC-1 cells (10^6) and exposed to β-propiolactone-inactivated Sendai virus by the procedure described earlier (8). Mixed cells (10^6) were cultured in 60-mm plates at 32 C for 24 h. Medium was changed and the cells were further incubated for 5 days at 32 C.

Virus uptake. Wt SV40 was grown at an MOI of 10 PFU/cell and labeled with [*methyl*-^{3}H]thymidine (10 μCi/ml, 6.7 Ci/mol) for 16 to 96 h. Virus was extracted, concentrated, and purified. Transformed cells were grown at 36.5 C and infected with radiolabeled virus at an MOI of 10 PFU/cell for 2 h. After adsorption, the cells received fresh medium and were reincubated for an additional 5 h. Cells were washed with isotonic Tris-buffered saline, trypsinized, and centrifuged. After resuspension in Tris and counting, the cells were pelleted again and suspended in reticulocyte standard buffer (0.0015 M $MgCl_2$, 0.01 M NaCl, 0.01 M Tris). An aliquot of the suspension was counted. The cells in suspension were broken in a Dounce homogenizer and then treated with Nonidet P-40 at a final concentration of 0.5%. The nuclei were centrifuged at 1,500 rpm for 4 min to separate them from the cytoplasm.

Transformed lines used. SI(L), SI(I), and SI(H) lines were produced by transforming the 3T3 cells with tsB4 at MOIs of 50, 250, and 1,000 PFU/cell, resulting in transformation frequencies of 0.12, 0.9, and 6%, respectively. A total of 10^5 cells from each of ten clones of SI(L), SI(I), and SI(H) and 10^6 cells from each of five clones of SI(L') lines were mixed. Except for the SI(I) line, the mixed cells of the rest of the lines were superinfected twice by wt virus at an MOI of 2,000 PFU/cell with an interval of 24 h of incubation at 36.5 C. Mixed cells of SI(I) line were infected by wt virus only once at an MOI of 500 PFU/cell. Thus, the following superinfected transformed lines were produced: DI(L), DI(L'), DI(H), and DI(I).

DNA-DNA reassociation kinetics. DNA-DNA hybridization kinetics and separation of single- and double-stranded DNA by hydroxyapatite chromatography were done essentially as described by Sharp et al. (11). The method of calculating the equivalents of viral DNA per diploid quantity of cell DNA was also described by Sharp et al. (11). DNA extracted from transformed and untransformed cells was incubated in 0.3 M NaOH for 7 to 10 h at room temperature, neutralized with HCl, made 0.1 M in NaCl, and precipitated with ethanol at −20 C. The precipitate was dissolved in 10 mM phosphate, 1 mM EDTA, pH 6.8, and dialyzed extensively against the same buffer. For the preparation of [^{32}P]SV40 DNA, BSC-1 cells were infected with plaque-purified SV40 virus at 40 to 60 PFU/cell. Infected cells were incubated in phosphate-free Eagle medium containing [^{32}P]orthophosphate (100 μCi/ml) for about 50 h. Viral DNA was extracted and purified by equilibrium density in cesium chloride-ethidium bromide gradients followed by sedimentation through sucrose gradients.

RESULTS

Figure 1 shows the outline of the experimental design. 3T3 cells were infected with a ts mutant of SV40 (tsB4). This mutant has a ts defect in a late function, which does not interfere with transforming ability at high temperature (12). After about 2 weeks of incubation at 37 C, typical transformed colonies were clonally isolated and cultured. After propagation, five or ten transformed clones were pooled by mixing the cells in equal number. This was done to avoid the selection of a particular cell line that might have been resistant to superinfection. After pooling, the cells were propagated for a few days and then superinfected with wt SV40 at high MOI, and the cells were plated out to allow colony formation. After the appropriate period of time, colonies were isolated. Isolation was done completely at random, and after isolation the superinfected transformed colonies were kept in anti-SV40 serum for at least 1 week. After two or three passages the cells were fused with BSC-1 monkey cells at 32 C. The rescued virus was harvested and titered at 32 and 39 C. SV40 tsB4 does not form plaques at 39 C, except for some rare revertants that occur at a frequency of about 10^{-5}. On the other hand, wt SV40 plates with approximately the same efficiency at 32 and 39 C.

It was assumed that had the SV40 integration sites been random or present in a very high number, superinfection of transformed cells would have led to integration of the superinfecting virus. On the other hand, if the number of integration sites for SV40 had been limited, the MOI used for transformation could have influenced the probability that the second infection would have led to integration. Since the first infection was performed with a ts mutant of SV40 and the second with wt virus, plating the rescued virus at 32 and 39 C would have easily distinguished between the virus used in the first and second infections.

It should be mentioned that the efficiency of transformation of tsB4 was about 10-fold lower than that of wt virus. This difference remained the same even when the transformation experiments were carried out completely at 32 C. It is likely that tsB4, in addition to the ts defect (12), also possesses a minor defect, independent of thermosensitivity, and that this defect somehow reduces the efficiency of transformation, perhaps by decreasing penetration. In both instances the frequency of transformation was dependent upon the MOI.

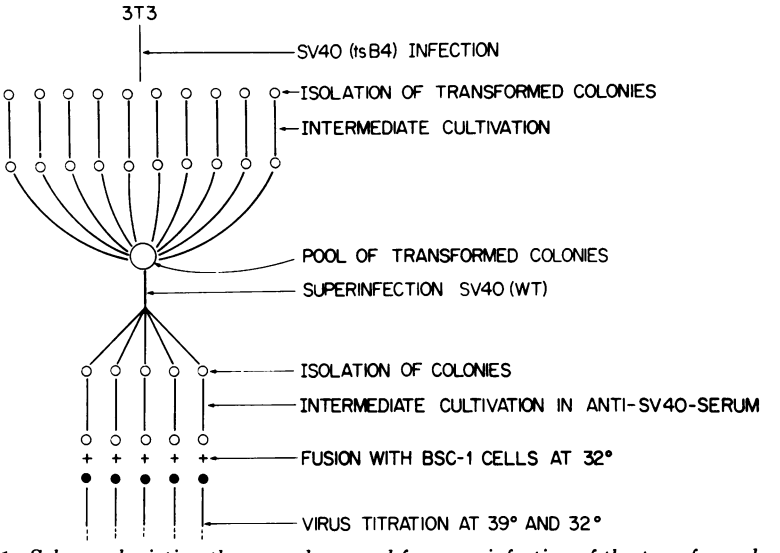

FIG. 1. *Scheme depicting the procedure used for superinfection of the transformed cells.*

Rescue of virus from the transformed cells. As expected, cells transformed by tsB4 yielded only ts virus upon fusion with BSC-1 cells. We tested cells of three clones of the SI(L) line, three clones of the SI(I) line, and one clone of the SI(H) line. After fusion, these cells yielded only ts virus. Transformed cells that had not been fused with BSC-1 did not produce any infectious virus (results not shown).

Superinfection of high-MOI transformants. Cells that had been transformed by tsB4 at an MOI of 1,000 PFU/cell [SI(H) line] were superinfected with wild-type virus. The superinfected cells were fused with BSC-1 cells to promote virus rescue (Table 1). Of 27 independent clones tested, 24 yielded only virus of the ts type but not the superinfecting wt virus. Three clones did not produce any infectious virus.

These results suggested that the first transformation had saturated all the available SV40 integration sites and therefore the superinfecting wt virus had not been able to integrate stably into the host cell genome. It was, however, possible that these cells had been resistant to penetration by the superinfecting virus. To test this hypothesis, high-MOI transformants were superinfected with radioactively labeled wt SV40 and compared with normal 3T3 cells (Table 2). No substantial difference was found between the capacity of SV40-transformed cells and normal 3T3 cells to adsorb radioactively labeled SV40. In addition, the amount of radioactivity found in the nuclear fraction was about the same in both cases. It can be concluded that the high-MOI transformants were not resistant to penetration by the superinfecting virus.

TABLE 1. *Virus rescued from cells transformed at high MOI by tsB4 and superinfected by wt SV40*[a]

Clone no.	PFU/ml at 32 C	Virus type
DI(H-1)	2×10^2	ts
DI(H-2)	4×10^2	ts
DI(H-3)	0[b]	—
DI(H-4)	2×10^2	ts
DI(H-5)	4×10^2	ts
DI(H-6)	0	—
DI(H-9)	2×10^2	ts
DI(H-10)	3×10^2	ts
DI(H-11)	8×10^2	ts
DI(H-12)	1.5×10^2	ts
DI(H-13)	2×10^2	ts
DI(H-14)	2×10^3	ts
DI(H-15)	1×10^2	ts
DI(H-16)	4×10^2	ts
DI(H-17)	3.5×10^2	ts
DI(H-18)	3.5×10^2	ts
DI(H-19)	1.5×10^2	ts
DI(H-20)	1.3×10^2	ts
DI(H-21)	4.5×10^2	ts
DI(H-22)	0	—
DI(H-23)	6.5×10^2	ts
DI(H-24)	3×10^2	ts
DI(H-25)	1×10^3	ts
DI(H-26)	3×10^2	ts
DI(H-27)	5×10^2	ts
DI(H-28)	2×10^2	ts
DI(H-29)	1.5×10^2	ts

[a] 3T3 cells that had been transformed by SV40 tsB4 at 1,000 PFU/cell [SI(H) line] were superinfected by wt SV40 at 4,000 PFU/cell. Colonies were isolated, and virus was rescued as described and titered at 32 and 39 C. In all cases at 39 C virus yield was <2 PFU/ml.

[b] 0, <2 PFU/ml.

TABLE 2. *Uptake of tritium-labeled wt SV40 by tsB4-transformed cells*[a]

Cells	Counts/min/10^7 cells			
	Input	Cytoplasm	Nucleus	Total
SI(H-2)	8×10^4	3,618	2,464	6×10^3
SI(H-14)	8×10^4	2,754	3,724	6.4×10^3
3T3	8.5×10^4	2,722	2,865	5.5×10^3

[a] Cells were infected with [^{3}H]thymidine-labeled SV40 (1.4×10^6 counts/min per ml; 2×10^8 PFU/ml). After 2 h of virus adsorption and an additional 4 h of incubation in fresh medium, the cells were washed, counted, and fractionated into nuclear and cytoplasmic fractions as described in the text, and the radioactivity was counted.

Superinfection of low-MOI transformants. 3T3 cells were transformed by tsB4 at an MOI of 50 PFU/cell [SI(L), SI(L')]. The low-MOI transformants were superinfected by wt SV40 as described earlier. Virus was rescued from the superinfected cells [DI(L), DI(L')] and titered at 32 and 39 C (Table 3). Of the colonies tested, about 30% yielded only ts virus, whereas the rest yielded both ts and wt virus. A few of the colonies tested had been derived from cells that had been transformed at an intermediate MOI (250 PFU/cell) and then superinfected [DI(I)]. In this case, only two out of nine colonies yielded both ts and wt virus.

The conclusion that the above-mentioned lines yielded both wt and ts virus upon fusion rested on the assumption that the ratio of PFU at 39 C to PFU at 32 C would give a good indication of the proportion of wt virus among the virus progeny. In some cases, the number of plaques formed at 39 C was close to that formed at 32 C. It would have been thus possible that the rescued virus consisted of only wild type, especially because the wt SV40 used in our experiments occasionally gives a slightly lower efficiency of plating at 39 than at 32 C. To rule out this possibility, plaques produced at 32 C by virus rescued from five separate clones were isolated and individually tested for ability to grow at 39 or 32 C (Table 4). Testing of the individual plaques confirmed that the virus rescued from the superinfected low-MOI transformants consisted in most cases of a mixture of ts and wt virus. Some plaque isolates gave a 5- to 10-fold reduced number of plaques at 39 C. It is possible that those represented mixed plaques produced by both wt and ts virus.

When the yield of the colones producing both wt and ts virus upon fusion was examined, it was found that the proportion of these two types of virus in the progeny varied widely. It is impossible, however, to attach much significance to this finding, since many factors could play a role in determining the ratio of wt to ts virus in the yield at the time of harvesting; thus, we feel that this ratio may not necessarily reflect the proportion of integrated viral genomes. It is interesting, however, that the percentage of doubly transformed cells obtained in our experiment was actually higher (about 66%) than expected. In fact, wt SV40 at the multiplicity used in the superinfection experiments produced about 20% transformation of 3T3 cells.

TABLE 3. *Virus rescued from cells transformed at low MOI by tsB4 and superinfected by wt SV40*[a]

Clone no.	PFU/ml		Virus type
	39 C	32 C	
DI(L-1)	2.5×10^4	5.5×10^4	ts, wt
DI(L-2)	2.3×10^3	3.3×10^3	ts, wt
DI(L-4)	0[b]	0	—
DI(L-11)	0	2×10^3	ts
DI(L-12)	0	1×10^2	ts
DI(L-14)	7×10^3	1.5×10^4	ts, wt
DI(L'-1)	5×10^2	1×10^3	ts, wt
DI(L'-2)	3×10^2	2×10^3	ts, wt
DI(L'-3)	3×10^2	1.5×10^3	ts, wt
DI(L'-5)	4×10^2	1.7×10^3	ts, wt
DI(L'-6)	2×10^2	2.5×10^3	ts, wt
DI(L'-7)	3×10^2	4×10^3	ts, wt
DI(L'-8)	0	1×10^3	ts
DI(L'-9)	0	5×10^2	ts
DI(L'-10)	5×10^2	2×10^3	ts, wt
DI(I-1)	0[b]	4×10^2	ts
DI(I-3)	0	1.8×10^3	ts
DI(I-4)	0	1×10^3	ts
DI(I-5)	0	7×10^2	ts
DI(I-13)	0	5×10^2	ts
DI(I-14)	1×10^2	5.7×10^2	ts, wt
DI(I-17)	2×10^2	2×10^3	ts, wt
DI(I-19)	0	9×10^2	ts
DI(I-22)	0	1×10^3	ts

[a] 3T3 cells that had been transformed by SV40 tsB4 at 50 PFU/cell [SI(L), SI(L') lines] or 250 PFU/cell [SI(I) line] were superinfected by wt SV40 at 4,000 or 500 PFU/cell, respectively. Colonies were isolated, and the virus was rescued as described and titered at 39 and 32 C.

[b] 0, <2 PFU/ml.

Superinfection of hybrid cells between high-MOI transformants and untransformed 3T3 cells. If the high-MOI transformants are resistant to retransformation because of a saturation of all the available SV40 integration sites, it might be expected that "adding" chromosomes of untransformed cells would make these cells susceptible to retransformation by a

TABLE 4. *Frequency of ts and wt SV40 plaques in virus rescued from doubly transformed cell lines*[a]

Cells from which virus was rescued	Incubation temp (C)	Plating efficiency (PFU/ml × 10^{-3}) of:									
		P-1[b]	P-2	P-3	P-4	P-5	P-6	P-7	P-8	P-9	P-10
DI(L-2)	39	12	0[c]	5	14	3	ND[d]	ND	4	24	6
	32	15	9	9	16	4	ND	ND	3.8	80	6
DI(L-1)	39	50	40	45	40	55	50	30	33	0	50
	32	105	94	68	85	90	112	60	80	91	98
DI(L'-5)	39	13	0	8	15	0	0	0	0	23	ND
	32	70	25	4	70	17	18	33	64	27	ND
DI(L'-2)	39	0	0	0	4	0	ND	3	2	0	0
	32	15	10	40	50	20	ND	30	7	10	35
DI(L-14)	39	0	0	0	0	7	3	0	0	3	4
	32	30	23	32	48	44	45	42	33	36	37

[a] Virus rescued from the cell lines indicated was plated at 32 C. Individual plaques were picked and suspended in 1 ml of medium. The plaque isolates were then titered at 39 and 32 C.
[b] Plaque isolate.
[c] 0, <3 PFU/ml.
[d] ND, Not done.

superinfecting virus. To test this prediction, we isolated somatic hybrid cells between untransformed 3T3 cells and a line of high-MOI transformants that, after superinfection, still yielded only ts virus. These hybrid lines were all T-antigen positive and produced ts SV40 upon fusion with BSC-1 cells. Their chromosome number was in the range of 120. The results obtained after superinfection of one of those hybrids with wt SV40 are seen in Table 5. Approximately half of the clones from the superinfected population yielded both ts and wt virus after fusion with BSC-1 cells.

Integration of SV40 DNA in the high- or low-MOI transformants. It was considered important to test whether ts virus-transformed

TABLE 5. *Virus rescued from hybrids between a high-MOI transformant and untransformed 3T3 cells after superinfection by wt SV40*[a]

Cells	PFU/ml		Virus type
	39 C	32 C	
DI(H-1) (parent)	0[b]	2×10^3	ts
3T3-THO (parent)	0	0	—
TD1-1 hybrid	0	1.4×10^3	ts
Superinfected hybrid clones			
1	0[b]	85	ts
2	75	2.7×10^3	ts, wt
3	20	10^3	ts, wt
4	0	1.1×10^3	ts
5	0	15	ts
6	0	15	ts
7	0	9×10^3	ts
8	6×10^3	2.5×10^5	ts, wt
9	3.5×10^3	1.5×10^5	ts, wt
10	0	0	—
11	50	10^3	ts, wt
12	5×10^3	5×10^3	ts, wt
13	2×10^3	1.5×10^3	ts, wt
14	10^3	2.5×10^3	ts, wt
15	0	35	ts

[a] 10^6 cells of the DI(H-1) transformed line and 10^6 cells of the 3T3-THO line (hypoxanthine, guanine phosphoribosyl transferase$^-$, and Ouabain), kindly provided by H. Ozer, were fused by means of inactivated Sendai virus. Hybrid cells were isolated in hypoxanthine-aminopterin-thymidine medium containing 2 mM Ouabain (1). One cloncal hybrid line (TD1-1) was then superinfected by wt SV40 at 4,000 PFU/cell. Clones were isolated and virus was rescued and titered by procedure described earlier. As a control, uninfected hybrid cells and the parent DI(H-1) cells were used for virus rescue.
[b] <2 PFU/ml.

cell lines that, upon superinfection, yielded both ts and wt virus showed an increase in the number of integrated SV40 genome equivalents per cell, and, conversely, whether transformed cells that, after superinfection, yielded only the first virus had maintained the same number of SV40 genomes. A clone of the SI(L) line and a superinfected clone derived from it, and which yielded both ts and wt virus upon fusion, were compared for their content of SV40 DNA-specific sequences. We used DNA-DNA reassociation kinetics, using whole SV40 DNA labeled with ^{32}P as a probe. The number of genome equivalents per diploid complement of cell DNA rose from 5 to 7 (Fig. 2). Similarly, a clone of the SI(H) line and one of its superinfected subclones, which still yielded only ts virus, were compared. The number of integrated genomes had remained the same (Fig. 2).

After these experiments we tested the number of genome equivalents present in the low- and high-MOI transformants before superinfection. If transformation at high MOI produces transformed cells that have more integration sites occupied than cells transformed at low MOI, then the high-MOI transformants might have shown, on the average, a higher content of SV40 DNA sequences. Surprisingly, that was not the case. We tested three clones of the SI(H) line and three clones of the SI(L) line. The numbers of SV40 genome equivalents per diploid cell in the high-MOI transformants were found to be about 3.2, 2, and 3, respectively, and in the low-MOI clones 5, 6, and 5.5, respectively. It must be kept in mind, however, that this experiment was performed by using whole SV40 DNA as a probe. If the high-MOI transformants contained a high percentage of defective SV40 molecules or fragments of molecules, this determination could have yielded values that are not representative of the number of SV40 fragments integrated at different host locations (3).

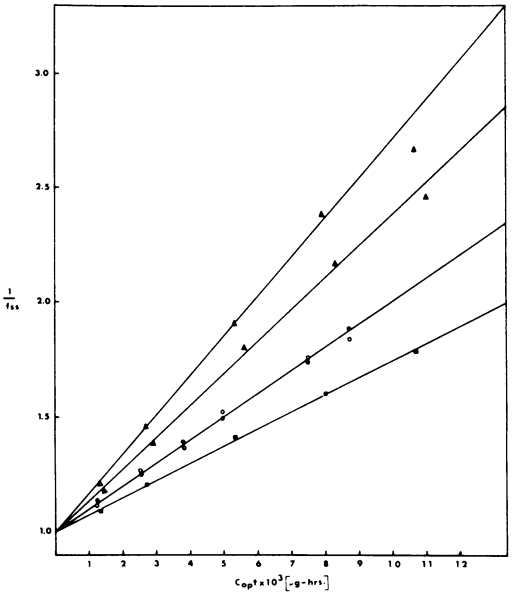

FIG. 2. *Renaturation of [^{32}P]SV40 DNA in the presence of unlabeled DNAs from SV40-transformed cells. Each reaction mixture contained 500 μg of cellular DNA per ml and 2.5 × 10^{-3} to 2.7 × 10^{-3} μg of [^{32}P]SV40 DNA per ml (specific activity 2.1 × 10^{6} counts/min per μg). The hybridizations were carried out at 68 C essentially as in reference 11. Before hybridization, cellular DNA was fragmented by sonication and subsequently boiled together with the probe for 10 min in 0.3 M NaOH. Samples were removed from the mixtures at intervals and the fraction of ^{32}P-labeled single-stranded DNA (fss) was determined by chromatography on hydroxyapatite. The data were plotted as 1/fss versus C_{op} × t, where C_{op} represents the input of probe in micrograms and t the time of sampling in hours. Symbols: (■) Renaturation of [^{32}P]SV40 DNA in the presence of DNA from normal 3T3 cells (control); (●) DNA from the high-MOI transformant SI(H-2); (○) DNA from a superinfected subclone of SI(H-2); (▲) DNA from the low-MOI transformant SI(L-1); (△) DNA from a superinfected subclone of SI(L-1) which yeilds both ts and wt SV40 upon fusion.*

DISCUSSION

The results presented in this paper show that superinfection of SV40-transformed 3T3 cells leads to integration of the superinfecting viral DNA only when the cells used have been transformed by using low MOI. The simplest interpretation of these results seems to be the following. The number of stable SV40 integration sites in the 3T3 cell genome is limited. Therefore, when cells are transformed by using high MOI, all the available sites have a high probability of being occupied and a second infection cannot lead to stable integration of SV40 DNA molecules. Transformation at low MOI, on the other hand, leaves some sites unoccupied, and those can be reached by superinfecting viral DNA molecules.

3T3 cells transformed at high or low MOI do not exhibit any significant difference in their phenotype. All cells we examined were T-antigen positive and exhibited the typical SV40-transformed phenotype. Superinfection did not lead to any detectable morphological alteration, and in fact the isolation of the superinfected colonies was done completely at random.

Although the high- or low-MOI transformants

did not show any detectable phenotypic difference, they behaved differently when challenged with a second infection. Low-MOI transformants became "doubly" transformed with a high probability, whereas high-MOI transformants did not. We were able to show that the latter case was not due to a failure of the superinfecting virus to penetrate and reach the nucleus of these cells. In addition, when these cells were hybridized with untransformed 3T3 cells, superinfection of the resulting hybrids could lead to integration.

It must be pointed out that the use of the terms "high MOI" and "low MOI" is purely relative. In terms of physical particles per cell, both these input MOIs were high. However, in terms of efficiency of transformation, the probability of every cell being transformed was about 30-fold lower when infected at low as compared with high MOI.

It is possible that a differential degree of immunity to superinfection exists between cells transformed at low or high MOI. This hypothesis, however, seems unlikely, since the available evidence suggests that SV40-transformed cells do not possess immunity to superinfection (reviewed in 14). In addition, such an immunity should at least decrease the probability of integration also in the low-MOI transformants. As we discussed under Results, this was found instead to be higher than what was expected on the basis of the normal efficiency of transformation of SV40 in 3T3.

It is also possible that transformation at low or high MOI recruits a different cell population. At low MOI the more susceptible cells could be selected, and such cells could be cells containing more integration sites. At higher MOI, cells with a lower number of integration sites would also be transformed. The different responses of the two cell populations to superinfection would then result from a preexisting difference, which has been selected for by the modality of transformation. Although our 3T3 cell population was cloned shortly before the experiment, it is difficult to rule out such a hypothesis. It should be pointed out, however, that even if this were true, it would still imply that the SV40 integration sites in the 3T3 genome are limited in number.

It is pertinent to mention here that Scher et al. (10) tested a number of SV40-transformed cells for stable association of new viral DNA molecules after superinfection in a way essentially similar to the one we described. They reported that in no case could they rescue virus from the second infection from these cells. Since the origin of the transformed cells they used was not controlled, it is likely that their results reflect the fact that those cells had been transformed at high MOI or for some other reason had no available integration sites.

The results presented above support the hypothesis that SV40 does not integrate at random sites in the mouse cell genome. There seems to be a limited number of such sites (and it must be kept in mind that 3T3 cells are almost tetraploid) unless some interference excludes the coexistence of a high number of integrated SV40 molecules in the same cell. The determination of the number of SV40 genome equivalents present in the high- or low-MOI transformants, however, showed that, if anything, the low-MOI transformants contained more "doses" of SV40 than the high-MOI transformants. As pointed out under Results, however, this quantitation rests on the assumption that all sequences of the labeled DNA probe are present at equal frequencies in all the lines tested. As shown by Botchan et al. (3), this assumption may be unjustified. In our case it is not unlikely that the high-MOI transformants contained more fragments of SV40 molecules than the low-MOI transformants because of the modality of infection with virus, which we suspect now of having contained an appreciable percentage of defective molecules. At high MOI the probability of cells having been exposed to defective viral molecules would be higher than in cells infected at low MOI. In addition, complementation between defective molecules would be less likely to occur in cells infected at low MOI. In this case, the technique used may not yield values representative of the number of SV40 fragments integrated in the host genome (3). This matter will have to be investigated further.

A final point of interest can be derived from our work. A number of phenotypic "revertants" of virally transformed cells have been found to be resistant to "retransformation" by the same virus (8, 15). This is generally interpreted as consistent with the hypothesis that phenotypic reversion is due to a cellular mutation. If there are constraints to integration of a large number of SV40 genomes, such interpretation of the experiments would be justified only in the case of cells in which further integration of viral genomes can be demonstrated.

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