MOLECULAR AND CELLULAR BIOLOGY, Mar. 1983, p. 421–428
0270-7306/83/030421-08$02.00/0

Vol. 3, No. 3

# Relationship of Simian Virus 40 Tumor Antigens to Virus-Induced Mutagenesis

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Received 17 June 1982/Accepted 8 December 1982

We analyzed the mutation frequency to 8-azaguanine (8AZ) resistance in rat FR3T3 cells acutely infected with simian virus 40 wild type and *tsA* and early deletion mutants and in a series of temperature-sensitive (N) and temperature-insensitive (A) transformants derived from Chinese hamster lung (CHL) cells. Upon acute infection, the frequency of mutation to 8AZ resistance was raised at most by two- to eightfold over the spontaneous frequency, and it was independent of the presence of a functional 90,000-molecular-weight T antigen or 20,000-molecular-weight t antigen or both. Similarly, in the stable transformants of CHL cells, no correlation was found between functional T antigens and mutation to 8AZ resistance. It therefore seems unlikely that simian virus 40-induced transformation results from any mutagenic activity of this virus.

Simian virus 40 (SV40), in addition to its ability to transform, has been reported to induce somatic mutations in mammalian cells. SV40 DNA integrates into the host genome, apparently at random (13), and therefore in this sense must be considered to act as a mutagen. However, the frequency of this integration in transforming infections is rather low. Rarely is more than 0.6% of a cell population transformed (13), although perhaps a higher percentage of the cells contain integrated SV40 DNA which is not expressed (W. W. Brockman, personal communication). Nonetheless, if integration were completely random, then even if after exposure to the virus, every cell harbored three copies of SV40 integrated at three different sites (an overestimate of probably 100-fold), the frequency of mutation in any gene less than $2 \times 10^3$ base pairs in length would be $<3 \times 10^{-6}$ (assuming a cell contains $2 \times 10^9$ base pairs). Frequencies of mutation of this order of magnitude would be very difficult to measure since the spontaneous frequency of mutation at loci that are functionally hemizygous is considerably higher. Thus, if SV40 were a mutagen in the sense that it increased the frequency of mutation at defined loci by a measurable amount, then the mechanism of this SV40-induced mutagenic activity could not be simply a result of integration.

The extent to which any potential mutagenic activity of SV40 is related to its transforming activity is unknown. A number of studies have shown that SV40 infection of nonpermissive and semipermissive cell lines results in chromosomal changes, such as polyploidization (7, 8) and chromosomal breaks and rearrangements (8, 12, 29), and in somatic mutations at different loci (12, 24, 25, 27, 28). The observation that some cells transformed by SV40 have altered karyotypes (9, 29) led to the suggestion that polyploidization may be involved in the process of malignant transformation (9). On the other hand, polyploidization was also correlated with reversion to the nontransformed phenotype (16).

The induction of gene mutations in cultured mammalian cells infected with SV40, as well as the enhancement of the transforming activity of viruses by mutagenic agents (22), led to the hypothesis that the mutagenic activity of SV40 may play a role in the transformation process by this virus (2). This hypothesis has been tested in several laboratories (6, 23–27), but the evidence so far remains inconclusive (13). After SV40 infection, the frequency of somatic mutants increased 2- to 4-fold over the spontaneous frequency in uninfected control cells in most studies (5, 12; S. Lavi, personal communication), but an increase of almost 10-fold has been reported by one group (26, 27). Similar increases in the rates of mutation were also observed in stably transformed cells, such as a transformant of Chinese hamster cells obtained by transformation with polyoma virus, and a double transformant of the same cells obtained by superinfection with SV40 had somewhat higher rates of mutation than the parental cells (5).

We report here that whereas an increase of two- to fourfold in the spontaneous frequency of mutation to 8-azaguanine (8AZ) resistance was detected after acute infection by SV40, no signif-

icant increase in the mutation rate was found in cell lines stably transformed by SV40. In an attempt to clarify this discrepancy, we analyzed the effect of various SV40 mutants on the induction of mutation both after acute infection and in stably transformed lines.

It has been reported that a functional *A* gene is required for SV40-induced mutagenesis (25, 27). We are unable to corroborate this finding after acute infection of nontransformed cells. Furthermore, in stably transformed cell lines, the mutation rate varied from one cell line to another, but no correlation between the presence or absence of an active *A* gene and mutagenic rate was apparent. We therefore question whether the increased frequency of 8AZ-resistant colonies found upon acute infection of nontransformed cells represents true mutagenesis or merely a complex artifact having to do with the abortive transforming ability of SV40.

## MATERIALS AND METHODS

**Cell lines, viruses, and media.** The cells used were the normal rat FR3T3 cells previously described by Rassoulzadegan (17) and Seif and Cuzin (19) and the N- and A-type Chinese hamster lung (CHL) cells transformed by *tsA*209 and *tsA*209 viable deletion double mutants and previously described by Martin et al. (14, 15) and Chepelinsky et al. (1). The cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum (DV10) and antibiotics. SV40 mutants *tsA*209, Δ288, and Δ292 have been described by Setlow et al. (21); Δ884 was kindly provided by T. Shenk. The virus was prepared by infection of CV-1 monkey cells at a multiplicity of infection of 0.001 PFU per cell. Virus titers of the stock solutions were determined by plaque assay.

**Infection of cells and selection of mutants.** Rat FR3T3 cells growing (~30% confluence) at 33 and 40°C or resting as confluent monolayers at 33 or 40°C were infected with approximately 10 PFU per cell as described by Seif and Martin (20) or mock infected with an equal volume of uninfected CV-1 cell lysate. After 2 h of adsorption, the infected cells either received their old medium and were incubated further for 4 to 5 days (resting cells) or received fresh medium, were reseeded after 24 h at a density of $10^4$ cells per $cm^2$ (growing cells), and were incubated until they reached near-confluence (~3 days).

Both sets of cell cultures were subsequently reseeded at a density of $10^6$ cells per 75-$cm^2$ flask and incubated overnight at 33°C for attachment, and the following day, all cultures received selective media containing 10 μg of 8AZ per ml for selection of resistant mutants. The cultures were incubated at 33 and 40°C. One set of resting-cell cultures infected with wild-type SV40 was reseeded postinfection at three different cell densities, $3 \times 10^5$, $1 \times 10^6$, and $3 \times 10^6$ cells per 150-$cm^2$ flask, and incubated at 33°C. After 1, 2, or 3 days (or until confluency was reached), quadruplicate samples from each set received the selective media and were reincubated at 33°C. The selective medium in all cultures was changed every 3 days for a period of approximately 30 days, after which the cells were fixed, stained, and scored for resistant colonies. A number of 8AZ-resistant colonies were not fixed but were isolated and maintained for further study. The efficiency of plating (EOP) was determined for each virus treatment and each temperature by plating 500 cells per 75-$cm^2$ flask and growing them in nonselective media for 2 weeks with changes every 3 days. The cultures were then fixed, stained, and scored for viable cell colonies. The mutation frequency was calculated as being equal to: mean number of resistant colonies per plate/(number of cells plated × EOP). For transformation assays, cells were seeded postinfection at densities of $10^4$ or $10^5$ cells per 25-$cm^2$ flask. After 6 to 8 weeks at 33°C, the cultures were fixed, stained, and scored for dense foci.

**HGPRTase assay.** Whole-cell extracts were prepared from several 8AZ-resistant clones and used to assay for hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) activity in vitro, as described by Gillin et al. (4).

**Fluctuation analyses.** For fluctuation analyses, cultures of N- and A-transformants were seeded with 1,000 cells per 25-$cm^2$ flask to be used for selection and 1,000 cells per 75-$cm^2$ flask to be used for determining the EOP. After incubation overnight at 37°C, half of the cultures were shifted to 33°C and half to 40°C. All media were changed every 3 days. After the cells reached confluence, they were trypsinized, counted, and diluted so that approximately $5 \times 10^5$ to $1 \times 10^6$ cells were seeded into 75-$cm^2$ flasks. They were incubated overnight at 33 and 40°C, respectively, and the following day, 8AZ (10 μg/ml) was added to each flask. All media were changed every 3 days for 3 to 4 weeks, and then the cultures were fixed, stained, and scored for resistant colonies. Dilutions of 100- and 1,000-fold of the same stock of cells were also seeded at the same time to determine the EOP. These were incubated 7 to 10 days at the appropriate temperature, fixed, and then stained and scored for viable cells. The rate of appearance of 8AZ-resistant colonies for each cell line was determined by fluctuation analysis as described by Luria and Delbrück (11).

## RESULTS

**Frequency of mutation in acutely infected FR3T3 cells.** The putative mutagenic action of SV40 and the role of the T antigens in SV40-induced mutagenesis were first examined in rat FR3T3 cells infected with SV40 wild type; *tsA*209, a mutant temperature sensitive for the large-T antigen; Δ884, a deletion mutant lacking the small-t antigen; and several *tsA*209 deletion double mutants. To determine whether there is any involvement of the 20,000-molecular-weight (20K) t antigen in this process, we used both actively growing and resting cells, since it has been found that the 20K t antigen is required for transformation of resting, but not of growing, cells (15, 20).

Transformation assays were performed in parallel as a control (Table 1) using resting cells at the permissive temperature. In agreement with results reported previously, increases of at least 170-fold (there were no spontaneous transfor-

TABLE 1. Frequency of 8AZ-resistant mutants in resting and growing FR3T3 cells infected with SV40 wild type, *tsA*209, and *tsA*209 viable deletion double mutants

| Infecting virus | Antigen | | EOP | | | | Frequency of 8AZ-resistant mutants ($\times 10^{-5}$) | | | | Frequency of transformation ($\times 10^{-5}$) in resting cells at 33°C |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Resting | | Growing | | Resting cells | | Growing cells | | |
| | T | t | 33°C | 40°C | 33°C | 40°C | 33°C | 40°C | 33°C | 40°C | |
| Wild type | + | + | 0.21 | 0.07 | 0.28 | 0.16 | 7.1 | 6.9 | 0.8 | 1.1 | 170 |
| *tsA*209 | ts[a] | + | 0.20 | 0.09 | 0.37 | 0.23 | 7.2 | 4.4 | 0.2 | 0.5 | 140 |
| *tsA*209Δ287 | ts | − | ND[b] | ND | 0.48 | 0.14 | ND | ND | 0.4 | 2.6 | ND |
| *tsA*209Δ288 | ts | − | 0.19 | 0.18 | 0.45 | 0.13 | 2.4 | 4.7 | 0.8 | 2.1 | 20 |
| Δ884 | + | − | 0.13 | 0.11 | 0.51 | 0.22 | 6.4 | 6.4 | 0.1 | 2.9 | 16 |
| Mock | | | 0.24 | 0.11 | 0.40 | 0.10 | 3.3 | 3.6 | 0.1 | 0.7 | 0[c] |

[a] ts, Temperature sensitive.
[b] ND, Not determined.
[c] No foci were obtained in mock-infected cultures.

mants in the mock-infected controls) were observed in the frequency of transformation when wild-type virus was used, and much smaller increases were found when the deletion mutants were used. These transformation frequencies are comparable to those reported previously in which both resting and growing cells were analyzed (13, 19).

In both resting and growing cells, approximately a two- to eightfold increase in the frequency of 8AZ-resistant colonies was observed in wild-type-infected and deletion mutant-infected cells at both temperatures (Table 1). We do not consider the exceptions, Δ884 in growing cells at 33°C and *tsA*209Δ288 in resting cells at 33°C, to be significant since the same result was not found for the other deletion mutants or for the same mutants at 40°C. To test the possibility that a longer expression time postinfection might reveal more induced mutations, we replated resting FR3T3 cells in normal medium at different cell densities immediately after infection with wild-type SV40 and then, at different times, exposed them to the selective media. Consistent with the results shown in Table 1, the infected cells showed a sevenfold maximum increase over the mutation frequencies of identically treated mock-infected cells, regardless of the extent of cell growth allowed (Table 2). The overall mutation frequencies were likewise similar.

The HGPRTase activity of representative 8AZ-resistant clones, isolated at random, was determined by direct in vitro measurement. A total of 15 of 17 resistant clones isolated from SV40-infected cultures and 10 of 14 resistant clones isolated from mock-infected cultures had significantly reduced (less than 50% of normal), or in some cases undetectable, enzyme activity, suggesting the presence of direct mutations at the HGPRT locus (Table 3).

Taken together these results are similar to those obtained in other laboratories (23, 24; Lavi, personal communication) and suggest that SV40 may increase the mutation rate of FR3T3 cells to 8AZ resistance. However, other interpretations of these results are also possible (see below).

The apparent absence of any influence of the T antigens on the frequency of mutation is particularly striking. Only a small decrease in the frequency of 8AZ-resistant colonies was observed in *tsA*209-infected cells at the nonpermissive temperature. For the cells infected with the viable deletion double mutant *tsA*209Δ288, no significant change in the frequency of 8AZ-resistant colonies was found at either temperature in resting cells. On the other hand, in both *tsA* deletion double mutants in growing cells, a two- to eightfold increase was seen at both temperatures. The deletion mutants in both resting and growing cells gave comparable mutation rates. Although there was a clear but unexplained difference between the mutation frequencies of the resting and growing cells, the latter being generally lower, these rates were not

TABLE 2. Effect of postinfection cell growth time on the frequency of 8AZ-resistant mutants of resting FR3T3 cells infected with wild-type SV40

| Time (h postinfection) of selective medium addition | Replated cell density (per flask) | Frequency of 8AZ-resistant mutants ($\times 10^{-5}$) | |
|---|---|---|---|
| | | Infected | Mock |
| 24 | $3 \times 10^5$ | 4.6 | 2.7 |
| 48 | | 7.0 | 1.0 |
| 72 | | 1.3 | 1.7 |
| 24 | $1 \times 10^6$ | 0.6 | 1.3 |
| 48 | | 3.3 | 1.0 |
| 24 | $3 \times 10^6$ | 5.9 | 1.6 |
| 48 | | 3.1 | 8.9 |

TABLE 3. HGPRTase activity of 8AZ-resistant clones of SV40-infected and mock-infected FR3T3 cells

| Clones[a] | Amt of IMP formed (pmol/min per μg of protein) |
|---|---|
| FR3T3[b] | 2.1 |
| SV2 | 0.2 |
| SV4 | 1.1 |
| SV5 | 0.5 |
| SV7 | 0.5 |
| SV8 | 0.7 |
| SV11 | 0.2 |
| SV15 | 0.0 |
| SV16 | 0.1 |
| SV17 | 0.0 |
| SV18 | 0.0 |
| SV20 | 0.0 |
| SV21 | 0.1 |
| SV30 | 1.0 |
| SV31 | 0.4 |
| SV32 | 0.3 |
| SV33 | 0.6 |
| SV34 | 0.2 |
| M4 | 1.6 |
| M5 | 2.8 |
| M7 | 1.6 |
| M15 | 0.6 |
| M20 | 1.2 |
| M21 | 0.5 |
| M22 | 0.8 |
| M23 | 0.7 |
| M26 | 0.3 |
| M27 | 0.8 |
| M28 | 0.4 |
| M29 | 0.6 |
| M30 | 0.3 |
| M31 | 0.2 |

[a] SV, SV40 infected; M, mock infected.
[b] FR3T3, parental cell line of all 8AZ-resistant clones.

affected by the absence of the small-t antigen. Thus, neither early function appeared to be involved in the putative mutagenic activity of SV40.

There are a number of variables in experiments of this kind that influence the calculation of mutation frequencies. The calculation is based on the average number of mutant colonies per plate, and that number is generally small. To obtain precise data, one would have to work with an overwhelmingly large number of cultures. Even assuming the numbers to be statistically significant, one cannot rule out the possibility that SV40 selectively stimulates those cells with a higher mutation rate to undergo abortive transformation. Under the conditions used in these experiments, approximately 25% of the cells are abortively transformed (3). Moreover, as has been discussed by Goldberg and Defendi (5), in acute viral infections of cells, there are many factors that may influence the rate of appearance of mutants, such as heterogeneous cell populations containing abortively and stably transformed as well as untransformed cells, continuous integration and excision of the viral DNA, and the induction of host DNA synthesis.

To overcome such drawbacks, we decided to analyze the mutation rates to 8AZ resistance, tubercidin resistance, and ouabain resistance in CHL cells stably transformed by SV40. We used N- (temperature-sensitive) and A- (temperature-insensitive) transformants of CHL cells induced by *tsA*209 (CHLA209L5, CHLA209L49, CHLA209L38, CHLA209L62, CHLA239L1), transformants induced by deletion mutant Δ884 (CHLΔ884M10, CHLΔ884M14), and transformants induced by double mutants containing both a deletion of the 20K t antigen and a temperature-sensitive lesion in the 90K T antigen (CHLΔ289A209L5 and CHLΔ290A209L12).

**Fluctuation analysis of mutation to 8AZ resistance.** A fluctuation analysis, as described by Luria and Delbrück (11), was performed to determine the rate of 8AZ-resistant cells arising from N- or A-type transformed cells. Twelve different transformed and three untransformed cell lines were used. For each cell line, 25 separate cultures, originating from an initial inoculum of 1,000 cells, were grown independently in tissue culture flasks. This method of calculating mutation rates is based on the average number of resistant cells per culture at the time of selection, which is a function of the spontaneous mutation rate of the culture and the rate of multiplication of the mutant cells. The number of mutant cells preexisting in the inoculating cultures, which if high, might erroneously raise the mutation rate figure, is expected to be insignificant, since those cultures contained a very small number of cells.

Table 4 shows a sample fluctuation analysis of mutation to 8AZ resistance for one of the cell lines, CHLWT24, transformed by wild-type SV40. The level of significance of the data is indicated by the very close agreement of the mutation rates obtained from experiment to experiment. Despite the variability observed in the values of some of the individual components that enter into the calculation of these data, the calculated rates agreed to within a factor of 2. In all experiments with the same cell line, the rates agreed to within a factor of 3 to 4 (cf. the values for CHL, CHLA209L5, and CHLA239L1 in Tables 5 and 6).

Our results (Table 5) fail to show any pattern of the mutation rate in relation to the difference in the temperature sensitivity of the transformed cell lines. The rate of mutation seems to be independent of the presence of a functional 90K

TABLE 4. Sample fluctuation analysis of mutation to 8AZ resistance in a CHL cell line transformed by wild-type SV40 (CHLWT24)

| Temp (°C) | Components used in analysis[a] | | | | | | | $R^c$ | EOP | Mutation rate[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| | $c$ | $N_0$ | $N_t$ (× $10^6$) | $G^b$ (× $10^6$) | No. of cultures with the following no. of 8AZ-resistant colonies | | | | | |
| | | | | | 0 | 1–10 | 11–50 | | | |
| 33 | 25 | 730 (229)[e] | 2.7 (0.9) | 3.9 (1.1) | 21 (23) | 2 (1) | 2 (1) | 2.6 (0.76) | 0.10 (0.19) | $1.4 \times 10^{-6}$ ($1.1 \times 10^{-6}$) |
| 40 | 25 | 555 (321) | 0.8 (0.8) | 1.2 (1.1) | 18 (24) | 7 (0) | 0 (1) | 0.36 (0.84) | 0.22 (0.34) | $5.0 \times 10^{-7}$ ($7.3 \times 10^{-7}$) |

[a] $c$, Number of replicate cultures; $N_0$, initial number of cells per culture; $N_t$, final number of cells at time of selection per culture; $G$, number of cell generations.
[b] $G$ is given by $(N_t - N_0)/\ln 2$.
[c] $R$, Average number of mutants per culture.
[d] Mutation rate ($a$) was calculated by using the transcendental equation developed by Luria and Delbrück (11), $R = aG\ln(cGa)$. The equation modified to take the EOP into account becomes $R' = aG\ln(cGa)$ where $R' = R/\text{EOP}$.
[e] Numbers in parentheses represent results obtained in a separate experiment.

T antigen, since the mutation rates of the T-antigen-dependent (N) transformants at 33 and 40°C are similar to those of the T-antigen-independent (A) transformants at the two temperatures. Similarly, no difference was observed between the mutation rates of the CHL transformants induced by either the *tsA*209 viable deletion double mutants (CHLΔ289A209L5 and CHLΔ290A209L12) or the early deletion mutant Δ884 and the rest of the transformants examined. On the other hand, a clear difference was seen between the mutation rates of the different cell lines and even between those of the different sublines of the same cell line. Thus, the mutation rates of the parental untransformed CHL cell line and its two sublines, CHLSL1 and CHLSL2, differ by up to a factor of 8 at 33°C and up to a factor of 16 at 40°C. We are, therefore, forced to conclude that our data, which are comparable but considerably more extensive than those previously obtained, fail to establish any correlation between functional T antigens and mutation to 8AZ resistance in CHL transformed and nontransformed cell lines.

Furthermore, these results show that the rate of mutation to 8AZ resistance in CHL cells seems to be independent of transformation, as evidenced by comparing the rates of the untransformed to those of the transformed cell lines.

We also examined whether resistance ac-

TABLE 5. Rate of mutation of N- and A-transformants at the HGPRT locus[a]

| Cell line | Type of transformant | Antigen | | Mutation rate at | |
|---|---|---|---|---|---|
| | | T | t | 33°C | 40°C |
| CHL | Untransformed | – | – | $4.7 \times 10^{-7}$ | $3.3 \times 10^{-7}$ |
| CHLSL1 | Untransformed | – | – | $3.6 \times 10^{-6}$ | $5.5 \times 10^{-6}$ |
| CHLSL2 | Untransformed | – | – | $1.6 \times 10^{-6}$ | $1.2 \times 10^{-6}$ |
| CHLWT23 | Wild type | + | + | $1.6 \times 10^{-6}$ | $3.8 \times 10^{-6}$ |
| CHLWT24(1) | Wild type | | | $1.4 \times 10^{-6}$ | $5.0 \times 10^{-7}$ |
| CHLWT24(2) | Wild type | + | + | $1.1 \times 10^{-6}$ | $7.3 \times 10^{-7}$ |
| CHLΔ884M10 | Wild type | + | – | $1.0 \times 10^{-7}$ | $9.5 \times 10^{-7}$ |
| CHLΔ884M14 | Wild type | + | – | $1.0 \times 10^{-6}$ | $>3.5 \times 10^{-5}$ |
| CHLA209L49 | A | ts[b] | + | $8.4 \times 10^{-7}$ | $2.4 \times 10^{-5}$ |
| CHLA209L38 | A | ts | + | $9.6 \times 10^{-6}$ | $3.8 \times 10^{-6}$ |
| CHLA209L5 | N | ts | + | $2.2 \times 10^{-6}$ | $2.6 \times 10^{-6}$ |
| CHLA209L62 | N | ts | + | $2.3 \times 10^{-5}$ | $2.0 \times 10^{-5}$ |
| CHLA239L1 (1) | N | ts | + | ND[c] | $6.4 \times 10^{-6}$ |
| CHLA239L1 (2) | | | | $8.1 \times 10^{-6}$ | $4.3 \times 10^{-6}$ |
| CHLA239L1SL2 | N | ts | + | $7.3 \times 10^{-6}$ | $1.2 \times 10^{-5}$ |
| CHLΔ289A209L5 | N | ts | – | $8.7 \times 10^{-7}$ | $5.3 \times 10^{-6}$ |
| CHLΔ290A209L12 | N | ts | – | $1.6 \times 10^{-5}$ | $1.1 \times 10^{-5}$ |

[a] The rates of mutation were calculated by using the equation presented in footnote *d* of Table 4.
[b] ts, Temperature sensitive.
[c] ND, Not determined.

TABLE 6. Comparison of rates of mutation to 8AZ resistance in cell lines mutated at a second locus

| Cell line | Type of transformant | Antigen | | EOP | | Mutation rate[a] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Oua[r] | | Tub[r] | | 8AZ[r] | |
| | | T | t | 33°C | 40°C | 33°C | 40°C | 33°C | 40°C | 33°C | 40°C |
| CHL | Untransformed | – | – | 0.51 | 0.19 | $<10^{-6}$[b] | $<3 \times 10^{-6}$ | $<10^{-6}$ | $<3 \times 10^{-6}$ | $3 \times 10^{-6}$ | $5 \times 10^{-6}$ |
| CHLA209L5 | N | ts[b] | + | 0.74 | 0.33 | $<0.7 \times 10^{-6}$ | $<1.5 \times 10^{-6}$ | $<0.7 \times 10^{-6}$ | $\sim 3 \times 10^{-6}$[c] | $0.7 \times 10^{-6}$ | $9.1 \times 10^{-6}$ |
| CHLA209L5SL3Tub[rd] | N | ts | + | 0.16 | 0.15 | | | | | $0.7 \times 10^{-6}$ | $1.1 \times 10^{-5}$ |
| CHLA239L1 | N | ts | + | 0.46 | 0.40 | $\sim 1.1 \times 10^{-6}$[c] | $<1.3 \times 10^{-6}$ | $<1.1 \times 10^{-6}$ | $<1.3 \times 10^{-6}$ | $8.8 \times 10^{-6}$ | $2.5 \times 10^{-5}$ |
| CHLA239L1SL3Oua[rd] | N | ts | + | 0.15 | 0.16 | | | | | $1.8 \times 10^{-6}$ | $1.7 \times 10^{-6}$ |

[a] Oua[r], Ouabain resistant; Tub[r], tubercidin resistant; 8AZ[r], 8AZ resistant.
[b] ts, Temperature sensitive.
[c] These rates are based on one colony each.
[d] Tub[r] and Oua[r] colonies were isolated as clones and grown in nonselective media for several passages. Fluctuation analyses were then performed as described in the text to determine the rate of mutation to 8AZ resistance. The rates of mutation were calculated as indicated in footnote *d* of Table 4.

quired at one locus affected the rates of a second mutation at a different locus. Selection for ouabain or tubercidin resistance did not appear to select cells which had higher mutation frequencies for 8AZ resistance as might be expected if mutations arose in the transformed cell lines either by transposition or by the presence of a mutator gene. Rare clones of N-transformants resistant to either tubercidin (adenosine kinase locus) or ouabain ($Na^+/K^+$ ATPase locus), which were isolated in a separate selection experiment, were tested for a second mutation to 8AZ resistance (HGPRT locus). The results in Table 6 show that the mutation rates to 8AZ resistance were the same in the parental cell line and in its daughter lines mutated to ouabain or tubercidin resistance and that both were independent of temperature.

## DISCUSSION

The hypothesis that cellular transformation after viral infection may result from the accumulation of somatic mutations has attracted the attention and efforts of many laboratories in the past several years (5, 10, 12, 23–27) and has been recently reviewed (13).

We have analyzed the mutation frequency to 8AZ resistance in FR3T3 cells acutely infected with SV40 wild type and *tsA* mutants and in a series of temperature-sensitive (N) and temperature-insensitive (A) SV40 transformants of CHL cells. Upon acute infection, SV40 increased the frequency of 8AZ-resistant colonies at most by two- to eightfold over the spontaneous rate of approximately $10^{-5}$ as shown previously by others (5, 12, 23–28; S. Lavi, personal communication). It is not immediately apparent, however, that this increase in frequency demonstrates that SV40 behaves as a mutagen. SV40 is known to induce abortive transformation. Under the conditions used in these experiments, approximately 25% of the cells are induced to undergo multiple rounds of replication (3). Thus, although a fixed number of cells were plated after treatment with virus, if cell death does not occur immediately after replating, there may easily be two to eightfold more potential targets for mutagenesis, i.e., two- to eightfold more cells as a result of abortive transformation in the virus treated as in the untreated controls. We could think of no control to accurately measure this effect and therefore examined the behavior of *tsA* mutants which do not produce active T antigen at the nonpermissive temperature, but which nonetheless do stimulate abortive transformation.

It has been postulated that the *A* gene of SV40, which controls important steps leading to the initiation of cellular transformation (13, 18), such as viral DNA integration into the host

genome but not the initiation of one round of unscheduled cellular DNA replication under most assay conditions, may also be involved in the induction of somatic mutations (25, 27). In our data, the mutagenic activity of the virus seems to be independent of the presence of a functional *A*-gene product. Cells infected with virus temperature sensitive for the 90K T antigen had the same frequency of 8AZ-resistant mutants at both the permissive and nonpermissive temperatures. The same was true for deletion mutants lacking small-t antigen whether growing or resting cells were used.

Because we failed to detect any role for the T antigen in mutagenesis and were unable to design an adequate control to eliminate the possibility that the apparent mutagenic activity of SV40 was an artifact of abortive transformation, we turned to cell lines stably transformed by various mutants of SV40. Here there could be no such artifactual stimulation of the base line by the virus. Indeed we could find no significant increase in the mutation frequency to 8AZ resistance between nontransformed cells and cells transformed by wild-type SV40. To eliminate the possibility that one of the two early functions inhibited any mutagenic effect, we again analyzed a series of cell lines transformed by different mutants of SV40. Again we failed to find any evidence for the involvement of the 90K T antigen in somatic cell mutagenesis, since cell lines stably transformed by *tsA* mutants of SV40 had similar mutation frequencies at 33 and 40°C. These results clearly show a random fluctuation of the mutation rates to 8AZ resistance with regard to the temperature-sensitive phenotype. On the other hand, the results show a significant variability in the mutation rates from cell line to cell line indicating that mutagenicity is cell line dependent. We are therefore forced to conclude that if SV40 behaves as a mutagen, it is a very weak mutagen or is site specific for loci other than the 8AZ region. Furthermore our inability to detect a significant increase in the mutation frequency of stably transformed lines leads us to suspect that the increase observed upon acute infection is merely a complex artifact having to do with the ability of SV40 to transform abortively.

The results presented in this paper suggest that neither the 90K T antigen, whose important role in transformation is well established, nor the 20K t antigen plays a primary role in the SV40-induced increase in the frequency of 8AZ-resistant colonies. Indeed, in our results, as in those reported by other groups, the mutation frequency induced by SV40 has not been increased by more than a factor of 8 to 10 over the spontaneous frequency. Under the same conditions, we see an increase in the frequency of transformation of at least 170-fold over the spontaneous frequency (13). It seems, therefore, unlikely that any mutagenic activity of SV40 plays an important role in the induction of malignant transformation.

## ACKNOWLEDGMENTS

We thank Shaw-Shyan Wang for providing technical assistance and Nicholas Martin for invaluable assistance in the development of a computer program for the calculation of mutation rates by fluctuation analysis.

## LITERATURE CITED

1. **Chepelinsky, A. B., R. Seif, and R. G. Martin.** 1980. 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. J. Virol. **35**:184–193.
2. **Dulbecco, R.** 1976. From the molecular biology of oncogenic DNA viruses to cancer. Science **192**:437–440.
3. **Fluck, M. M., and T. L. Benjamin.** 1979. Comparison of two early gene functions essential for transformation in polyoma virus and SV40. Virology **96**:205–228.
4. **Gillin, F. D., D. J. Roufa, A. L. Beaudet, and C. T. Caskey.** 1972. 8-Azaguanine resistance in mammalian cells. I. Hypoxanthine-guanine phosphoribosyltransferase. Genetics **72**:239–252.
5. **Goldberg, S., and V. Defendi.** 1979. Increased mutation rates in doubly viral transformed Chinese hamster cells. Somatic Cell Genet. **5**:887–895.
6. **Hirai, K., and V. Defendi.** 1976. The effects of serum concentration on the level of integration of simian virus (SV40) genome and the transformation frequency in SV40-infected Chinese hamster cells. Virology **69**:229–236.
7. **Horan, P. K., J. H. Jett, A. Romero, and J. M. Lehman.** 1974. Flow microfluorometry analysis of DNA content in Chinese hamster cells following infection with simian virus 40. Int. J. Cancer **14**:514–521.
8. **Lehman, J. M.** 1974. Early chromosome changes in diploid Chinese hamster cells after infection with simian virus 40. Int. J. Cancer **13**:164–172.
9. **Lehman, J. M., and V. Defendi.** 1970. Changes in DNA synthesis and regulation in Chinese hamster cells infected with simian virus 40. J. Virol. **6**:738–749.
10. **Lukash, L. L., T. I. Buzhievskaya, N. B. Varshaver, and N. I. Shapiro.** 1981. Oncogenic adenovirus as mutagen for Chinese hamster cells in vitro. Somatic Cell Genet. **7**:133–146.
11. **Luria, S. E., and M. Delbrück.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
12. **Marshak, M. I., N. B. Varshaver, and N. I. Shapiro.** 1975. Induction of gene mutations and chromosomal abberrations by simian virus 40 in cultured mammalian cells. Mutat. Res. **30**:383–396.
13. **Martin, R. G.** 1981. The transformation of cell growth and transmogrification of DNA synthesis of simian virus 40. Adv. Cancer Res. **34**:1–67.
14. **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.
15. **Martin, R. G., V. P. Setlow, C. A. F. Edwards, and D. Vembu.** 1979. The roles of the simian virus 40 tumor antigens in transformation of Chinese hamster lung cells. Cell **17**:635–643.
16. **Pollack, R., and A. Vogel.** 1973. Isolation and characterization of revertant cell lines. II. Growth control of a polyploid revertant line derived from SV40-transformed 3T3 mouse cells. J. Cell. Physiol. **82**:93–100.
17. **Rassoulzadegan, M., R. Seif, and F. Cuzin.** 1978. Condi-

tions 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.
18. **Rigby, P.** 1979. The transforming genes of SV40 and polyoma. Nature (London) **282**:781–784.
19. **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.
20. **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.
21. **Setlow, V. P., M. Persico-DiLauro, C. A. F. Edwards, and R. G. Martin.** 1980. The isolation of SV40 tsA/deletion, double mutants, and the induction of host DNA synthesis. Virology **101**:250–260.
22. **Stich, H. F., and R. H. San.** 1970. DNA repair and chromatid anomalies in mammalian cells exposed to 4-nitroquinoline-1-oxide. Mutat. Res. **10**:389–404.
23. **Theile, M., and H. Krause.** 1979. The combined mutagenic action of simian virus 40 and other carcinogens in Chinese hamster cells. Stud. Biophys. **76**:45–46.
24. **Theile, M., S. Scherneck, and E. Geissler.** 1976. Mutagenesis by simian virus 40. I. Detection of mutations in Chinese hamster cell lines using different resistance markers. Mutat. Res. **37**:111–124.
25. **Theile, M., S. Scherneck, and E. Geissler.** 1980. DNA of simian virus 40 mutates Chinese hamster cells. Arch. Virol. **65**:293–309.
26. **Theile, M., and M. Strauss.** 1977. Mutagenesis by simian virus 40. II. Changes in substrate affinities in mutant hypoxanthine-guanine phosphoribosyl transferase enzymes at different pH values. Mutat. Res. **45**:111–123.
27. **Theile, M., M. Strauss, L. Lübbe, S. Scherneck, H. Krause, and E. Geissler.** 1979. SV40-induced somatic mutations: possible relevance to viral transformation. Cold Spring Harbor Symp. Quant. Biol. **44**:377–382.
28. **Wolman, S. R., K. Hirschorn, and G. J. Todaro.** 1964. Early chromosomal changes in SV40-infected human fibroblast cultures. Cytogenetics **3**:45–61.
29. **Zuna, R., and J. M. Lehman.** 1977. Heterogeneity of karyotype and growth potential in simian virus 40-transformed Chinese hamster clones. J. Natl. Cancer Inst. **58**:1463–1471.