JOURNAL OF VIROLOGY, Mar. 1982, p. 1073–1075
0022-538X/82/031073-03$02.00/0

Vol. 41, No. 3

NOTES

Small t Protein of Simian Virus 40 Is Required for Dense Focus Formation in a Rat Cell Line

SUMIO SUGANO,* NOBUO YAMAGUCHI, AND HIROTO SHIMOJO

Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-Ku, Tokyo, 108, Japan

Received 13 July 1981/Accepted 21 October 1981

Incomplete foci were formed in a Fischer rat cell line infected with simian virus 40 deletion mutants encoding defective small t antigen. The foci were small and rarely piled up. The fraction of DNA-synthesizing cells in the foci was about half of that induced by wild-type virus.

Viable mutants of simian virus 40 (SV40) having deletions between 0.54 and 0.59 map unit (*dl*54/59) code for large T antigen but fail to synthesize normal small t antigen (2, 7). The requirement of small t antigen for the maintenance of transformation has been equivocal in rat cells because the transformants induced by *dl*54/59 mutants show various degrees of transformed phenotypes (1, 5, 7–9). However, all of the reports agree that the mutants induce densely staining, transformed colonies indistinguishable from those induced by wild-type virus at the same efficiency when cells are allowed to divide several times after infection. We report here that a cell line derived from a Fischer rat embryo, 3Y1-K, a subclone of 3Y1-1-6 cells (3), failed to form dense, transformed foci after infection with *dl*54/59 mutants. The cell line formed small and flat foci which exhibited reduced DNA synthesis.

Monolayer cultures of 3Y1-K cells (about 10^6 cells per 50-mm dish) were infected with either wild-type SV40 or *dl*940, which has a deletion of about 70 base pairs long around the *Taq*I site at 0.57 map unit (4). After 3 h of adsorption, cells were suspended and replated at 10^4 cells per 50-mm dish. Eagle minimum essential medium supplemented with 10% precolostrum newborn calf serum (Mitsubishi Chemical Industries, Ltd.) was changed every 3 or 4 days. After a 6-week incubation at 37°C, cells were fixed and stained with Giemsa (Fig. 1A). The mutant, *dl*940, induced only small foci of moderate density. Foci of similar morphology were often seen in mock-infected cells. However, most of the mutant-induced foci were composed of T-antigen-positive cells (Fig. 2). Cells in these foci were smaller than normal 3Y1-K cells but exhibited only partial piling up. When we examined all T-positive foci (see below), some foci were found to be composed of cells indistinguishable from T-negative-background 3Y1-K cells. A *dl*54/59 mutant, *dl*884 (6), also induced foci in 3Y1-K cells that rarely piled up (data not shown).

Infection of actively growing cells instead of confluent monolayers or replating of infected cells at 10^3 cells per dish gave essentially the same result. The growth state at the time of infection seems to have no effect on the character of the foci. The incomplete foci remained small and flat during an additional 2-week incubation at 37°C (data not shown). The efficiency of transformation was roughly estimated by the T-antigen-positive foci, because the incomplete foci were difficult to recognize by Giemsa staining. The efficiency was 1.7×10^{-5} foci per PFU for wild-type SV40 and 2.0×10^{-5} foci per PFU for *dl*940 when the cells were infected at 100 PFU per cell.

The incomplete focus induction by *dl*940 or *dl*884 was examined in brain cells from baby Fischer rats at passage 4 or 3Y1-1-6 cells (3), a parental cell line of 3Y1-K cells. Both cells formed dense foci indistinguishable from those induced by wild-type SV40 (Fig. 1B and C). There was no difference in the efficiency of focus formation between the wild type and *dl*940 or *dl*884. These results suggest that 3Y1-K cells require small t antigen for dense focus formation. On the other hand, 3Y1-1-6 and rat brain cells formed dense foci after infection with *dl*54/59 mutants, as has been reported for other cells (1, 5, 7). All three lines of Fischer rat cells exhibited a diploid modal value of 42 chromosomes, and the chromosome numbers of most of the cells (78%) fell between 38 and 43. Both 3Y1-1-6 and 3Y1-K cell lines showed similar doubling times of about 18 h in the exponential growth phase and similar cloning efficiencies on plastic which were between 50 and 60%. These obser-

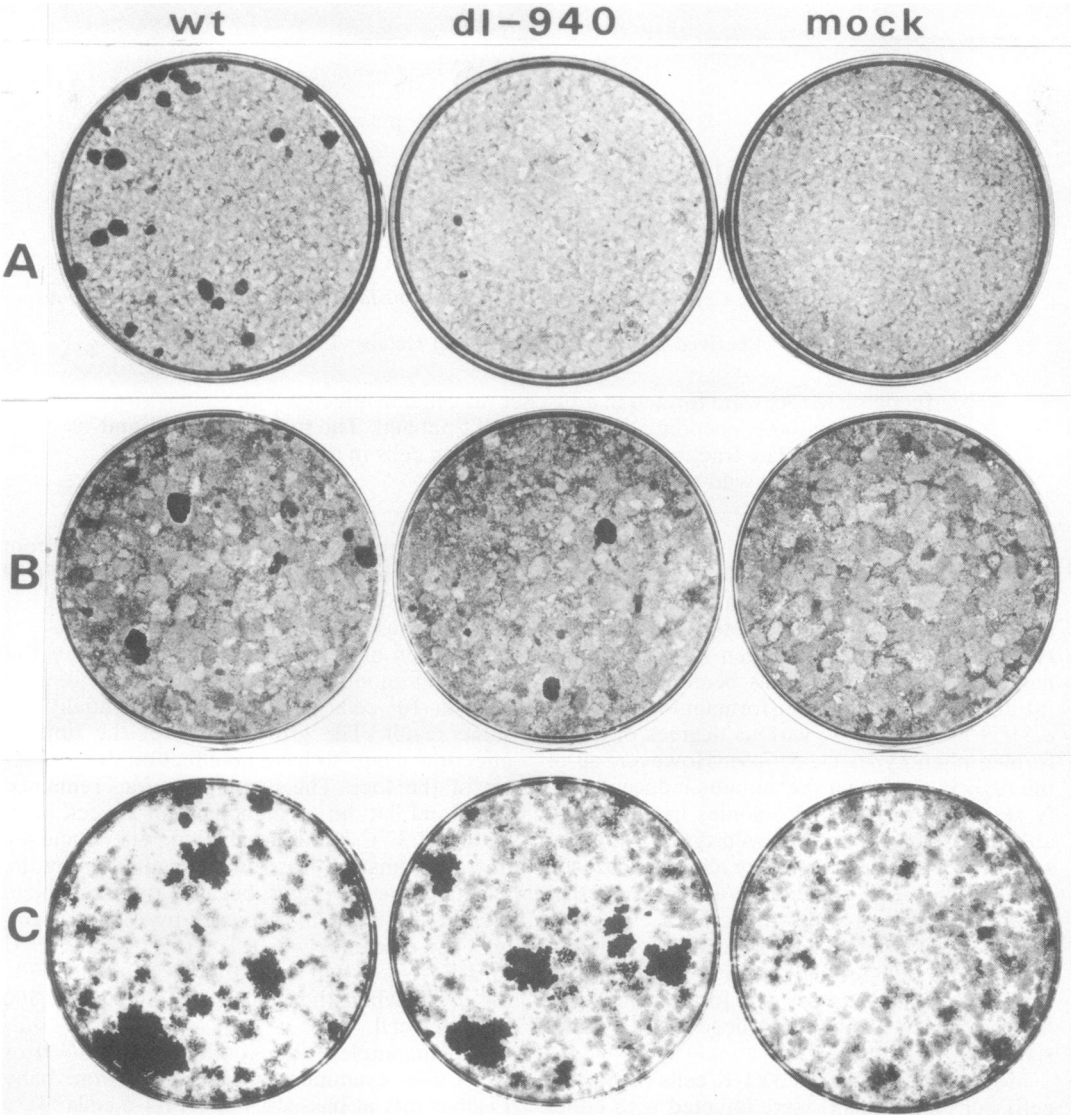

FIG. 1. Transformation of 3Y1-K (A), 3Y1-1-6 (B), and baby rat brain (C) cells by wild-type SV40 and *dl*940. Dishes were fixed with a 10% Formalin solution and stained with Giemsa after a 6-week incubation. wt, Wild-type SV40.

vations suggest that the requirement of 3Y1-K cells for small t antigen is not correlated with such properties as chromosome numbers and plating efficiencies. All of the cell lines were free of mycoplasmas. The 3Y1-K cells may have lost some small t complementing activity or more likely aquired some division-restrictive properties, which would be overcome only by the presence of both large T and small t antigens.

We examined the DNA synthesis in each focus to characterize further the incomplete foci. 3Y1-K cells were infected with wild-type SV40, *dl*940, or *dl*884 and replated onto cover slips (18 by 18 mm) at 1×10^3 to 2×10^3 cells per cover slip. After incubation for 6 weeks, the cells were labeled with [^{3}H]thymidine (3 μCi/ml, 21.5 Ci/mmol) in Eagle minimum essential medium without serum for 8 or 24 h. DNA synthesis in cells of T-positive foci was examined by autoradiography. The fraction of labeled nuclei in the foci induced by *dl*54/59 mutants was about half of that induced by wild-type SV40 after either an 8- or a 24-h labeling (Table 1). Labeled nuclei were uniformly distributed throughout the

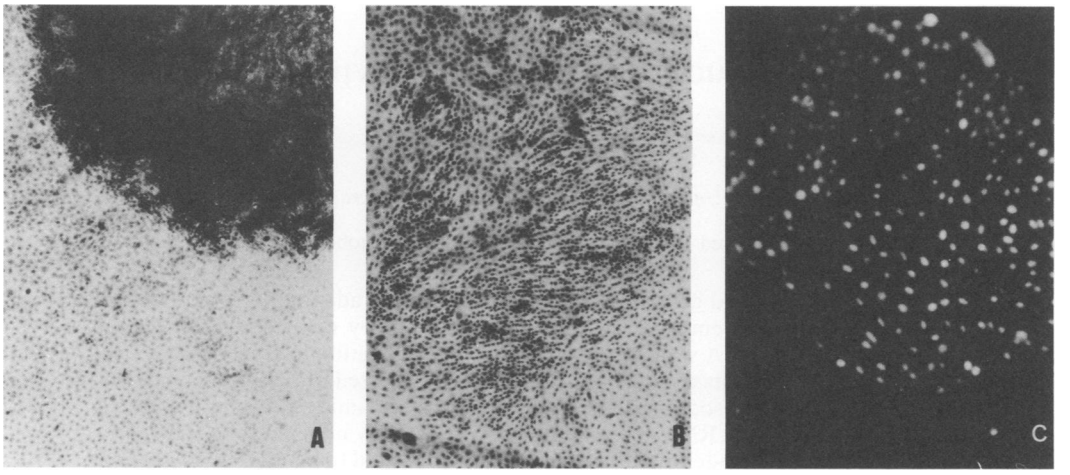

FIG. 2. Microscopic pictures of a transformed focus of 3Y1-K cells induced by either wild-type SV40 (A) or *dl*940 (B) in Fig. 1A and the presence of T antigen in a *dl*940-transformed incomplete focus (C). For immunofluorescent staining, infected 3Y1-K cells were plated at 10^3 cells per cover slip (18 by 18 mm), cultured for 6 weeks, and fixed with acetone for 10 min at room temperature.

foci. Although the percentage of labeled nuclei in the foci induced by the mutants was about half of that transformed by wild-type SV40, the value was much higher than that of T-negative 3Y1-K monolayers. This DNA synthesis might have led the cells to pile up during prolonged incubation periods. However, the incomplete foci remained small and rarely piled up. This suggests that in incomplete foci, a loss of cells occurs at a comparable rate to DNA synthesis. One hypothesis for this is that DNA synthesis is induced by the function of large T antigen in *dl*54/59-transformed 3Y1-K cells, but the cells eventually die because cell division is restricted in confluence in the absence of the function of small t antigen. Another hypothesis is that newly divided *dl*54/59-transformed 3Y1-K cells fail to spread onto the cell monolayer and detach easily. The unique phenotype of *dl*54/59-transformed 3Y1-K cells may provide a useful system for elucidating the function of small t antigen.

TABLE 1. DNA synthesis in transformed foci[a]

Virus	% of labeled nuclei[b]	
	8-h labeling	24-h labeling
Wild type	30 ± 16.8	68 ± 10.7
*dl*940	16 ± 3.6	37 ± 6.0
*dl*884	17 ± 7.0	37 ± 3.4
Mock	0.6[c]	1.5

[a] Cells were labeled with [^{3}H]thymidine for 8 or 24 h. Foci of T-antigen-positive cells were selected and examined for DNA synthesis by autoradiography. After the cells were stained with Giemsa, the center of each focus was photographed, and the labeled and nonlabeled nuclei were counted.

[b] Data represent an average ± one standard deviation of five to eight foci.

[c] A total of 700 nuclei were counted.

We thank Genki Kimura for the 3Y1 clones, Thomas E. Shenk for *dl*884, Hiroshi Chosa for the examination of mycoplasmas, and Kinichiro Oda for critical reading of the manuscript and constant encouragement.

This research was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan and by the Princess Takamatsu Fund for Cancer Research.

LITERATURE CITED

1. **Bouck, N., N. Beales, T. Shenk, P. Berg, and G. di Mayorca.** 1978. New region of simian virus 40 genome required for efficient viral transformation. Proc. Natl. Acad. Sci. U.S.A. **75**:2473–2477.
2. **Crawford, L. V., C. N. Cole, A. E. Smith, E. Paucha, P. Tegtmeyer, K. Rundell, and P. Berg.** 1978. Organization and expression of early genes of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **75**:117–121.
3. **Kimura, G., A. Itagaki, and J. Summers.** 1975. Rat cell line 3Y1 and its virogenic polyma- and SV40-transformed derivatives. Int. J. Cancer **15**:694–706.
4. **Segawa, M., S. Sugano, and N. Yamaguchi.** 1980. Association of simian virus 40 T antigen with replicating nucleoprotein complexes of simian virus 40. J. Virol. **35**:320–330.
5. **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.
6. **Shenk, T. E., J. Carbon, and P. Berg.** 1976. Construction and analysis of viable deletion mutants of simian virus 40. J. Virol. **18**:664–671.
7. **Sleigh, M. J., W. C. Topp, R. Hanich, and J. F. Sambrook.** 1978. Mutants of SV40 with an altered small-t protein are reduced in their ability to transform cells. Cell **14**:79–88.
8. **Steinberg, B. M., and R. Pollack.** 1979. Anchorage independence: analysis of factors affecting the growth and colony formation of wild-type and *dl*54/59 mutants SV40-transformed lines. Virology **99**:302–311.
9. **Topp, W. C., and D. B. Rifkin.** 1980. The small-t protein of SV40 is required for loss of actin cable networks and plasminogen activator synthesis in transformed rat cells. Virology **106**:282–291.