7. I. A. Kamberi, H. P. G. Schneider, S. M. McCann, *Endocrinology*, in press; H. P. G. Schneider and S. M. McCann, *ibid.*, in press.
8. I. A. Kamberi, R. S. Mical, J. C. Porter, *Proc. Meeting Soc. Study Reprod. 2nd* (1969), p. 4.
9. J. C. Porter and R. S. Mical, *Fed. Proc.* **28**, 317 (1969).
10. J. C. Porter and K. R. Smith, *Endocrinology* **81**, 1182 (1967).
11. G. D. Niswender, A. R. Midgley, Jr., S. E. Monroe, L. E. Reichert, Jr., *Proc. Soc. Exp. Biol. Med.* **128**, 807 (1968).

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Human Diploid Cell Transformation by DNA Extracted from the Tumor Virus SV40

Abstract. *The DNA isolated from simian virus 40 (SV40) can transform human fibroblast cells in tissue culture. Clonal lines of DNA-transformed human cells have been obtained that have properties characteristic of cells transformed by whole virus. They all contain SV40 T-antigen, and infectious virus can be recovered by cocultivation. This is the first demonstration of a permanent genetic alteration produced in human cells by purified DNA.*

Simian virus 40 (SV40), a small, DNA-containing tumor virus, has been shown to induce a morphological transformation of various cell types, including human cells, in tissue culture (*1*). The virus-transformed cells are characterized by their epithelioid morphology and lack of sensitivity to contact inhibition of cell division (*2*). They contain SV40-specific messenger RNA (*3*), and infectious virus can generally be recovered (*4*). The in vitro transformed cells are tumorigenic in the appropriate animal host (*1*). SV40-infected cells produce a new antigen, the T-antigen, that can be detected in the cell nucleus (*5*). The production of this antigen persists in transformed cells and in tumor cells.

The nucleic acid extracted from SV40 was shown by Gerber to be infectious for green monkey kidney cells (*6*). The observation by McCutchan and Pagano (*7*) that the infectivity of SV40 DNA could be markedly enhanced with diethylaminoethyl-dextran led us to study the effect of SV40 DNA on human cells. The studies described below demonstrate that infectious nucleic acid can enter and permanently alter the genetic makeup of a normal human cell.

A human fibroblast strain (F.R.), obtained from a skin biopsy specimen of a clinically normal adult female, was tested. It had previously been shown to have high susceptibility to transformation by SV40 (*8*). Other cells studied included green monkey kidney cells, the mouse cell line Balb/3T3 (*9*), and a line of rat kangaroo fibroblasts. Cultures were maintained in 50-mm plastic petri dishes in Dulbecco's modification of Eagle's medium supplemented with 10 percent calf serum. For experiments with whole virus, a pool of plaque-purified SV40 small-plaque virus (SV-S) (*10*) was used.

Small-plaque SV40 DNA (*11*) was extracted with a mixture of chloroform and isoamyl alcohol in the presence of sodium dodecyl sulfate and sodium perchlorate (*12*), and the double-stranded, twisted circular DNA (DNA I) was isolated by equilibrium density centrifugation in cesium chloride containing ethidium bromide (*13*). One μg of DNA I was found to correspond to 3.0×10^4 plaque-forming units.

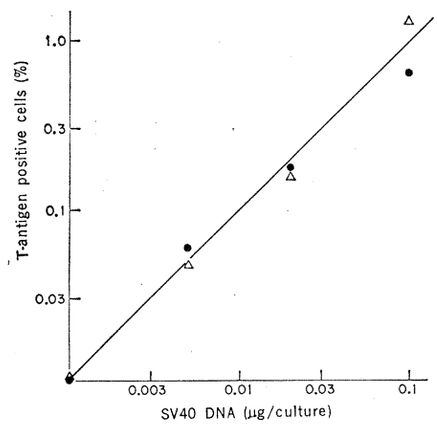

Fig. 1. Induction of SV40 T-antigen in monkey and human cells by SV40 DNA; relationship between micrograms of SV40 DNA I per infection and the fraction of T-antigen positive cells. Each point represents the data from two separate experiments; at least 20,000 cells were scored for each point. Triangles, green monkey kidney cells; dots, human diploid fibroblasts.

Cells in the logarithmic phase of growth were washed twice with serum-free medium and incubated with 0.2 ml of fluid containing SV40 DNA and 300 μg/ml of diethylaminoethyl-dextran in Earle's balanced salt solution without sodium bicarbonate. After 30 minutes at room temperature with frequent gentle agitation, the cultures were washed twice and fresh medium was added. Within 24 hours the cells were transferred both to dishes containing cover slips for SV40 T-antigen studies and to dishes at appropriate dilutions for the transformation assay (*8*). Following exposure to SV40 DNA the cells were maintained in medium supplemented with 0.5 percent rabbit antiserum to SV40.

Cells containing SV40 T-antigen were stained by the indirect fluorescent antibody method (*5*). The fraction of T-antigen positive cells was scored at 72 hours, a time at which, under our conditions, the maximum number of positive cells was seen either with intact virus or with DNA. In a typical test 20,000 to 40,000 cells were counted to determine the fraction of cells that were T-antigen positive. Infectious virus was recovered from DNA-transformed clones by cocultivation with green monkey kidney cells in the presence of ultraviolet-inactivated Sendai virus (*4*).

Human cells and green monkey kidney cells were infected with serial dilutions of DNA I. Figure 1 shows that the percentage of SV40 T-antigen positive cells rose with the concentration of DNA for both monkey and human cells. The efficiency of infection was comparable in spite of the fact that whole virus is over 100 times more efficient at inducing T-antigen in monkey cells than in human cells. The same efficiency of T-antigen induction by DNA was also found with Balb/3T3 cells, and with rat kangaroo cells. In the latter case the cells were fully resistant to T-antigen induction by whole virus.

Transformed colonies were first observed in DNA-infected human cultures 2 to 3 weeks after infection. The transformation frequency was assayed at 3 to 4 weeks. In their tissue culture properties the transformed colonies that developed were typical of SV40-transformed cells. The cells were epithelioid,

Table 1. Efficiency of T-antigen induction and transformation in human cells by SV40 DNA and SV40.

SV40	Infectious units added	SV40 T-antigen (% positive)	Transformed colonies/ 5×10^5 cells*	T-antigen/ transformed colonies
DNA†	1.5×10^2	0.06	0	
DNA	6.0×10^2	.18	3	3.0×10^2
DNA	3.0×10^3	.68	21	1.6×10^2
DNA	1.5×10^4	1.2	26	2.3×10^2
DNA	5.0×10^4	1.5	21	3.6×10^2
Virus	5.0×10^4	< .01	0	
Virus	5.0×10^7	5.8	164	1.8×10^2

* Average of two separate experiments. At each concentration in each experiment 5×10^5 cells were exposed. † One microgram of DNA I contained 3.0×10^4 infectious units.

showed pronounced loss of contact inhibition of cell division, and readily formed multiple cell layers. They also showed persistent nuclear abnormalities and many giant cells.

In Table 1 virus and viral DNA were compared for their transforming activities. With the DNA I preparation transformed colonies were seen by using as little as 6.0×10^2 plaque-forming units or 0.02 μg per culture. When 5.0×10^4 infectious units were used, transformed colonies were readily seen with crude DNA or DNA I while no transformed colonies were found by using whole virus. At the lower DNA concentrations approximately 200 infectious units of DNA I were sufficient to produce one transformed human colony. At higher DNA concentrations there appeared to be a plateau, both for infectivity, as judged by the percentage of T-antigen positive cells, and for transformation. With intact SV40 small-plaque virus approximately 2.5×10^5 infectious units were required to produce one transformed colony. Viral DNA was, therefore, 1000-fold more efficient per infectious unit than whole virus. The inefficiency of whole virus was due to inefficiency at an early step in human cell infection, for example, adsorption, penetration, or uncoating, or a combination of these. Once either the virus or the viral DNA reached the stage of inducing SV40 T-antigen there was a comparable but low probability that the cell would develop into a transformed colony. The T-antigen/transformation value ranged from 1.6 to 3.6×10^2 for DNA and was 1.8×10^2 for whole virus [see also (*8*)].

Several clones of DNA-transformed human cells have been isolated and grown up to mass culture in the presence of SV40 antiserum for at least 20 to 30 cell generations. Ten transformed clones, each the product of an independent DNA-cell interaction, were stained for T-antigen. With each clone practically every cell was found to be T-antigen positive. By cocultivation with green monkey kidney cells in the presence of ultraviolet-inactivated Sendai virus, infectious virus was recovered from each clone. As with transformation by intact virus, therefore, the entire viral genome is present and recoverable from the DNA-transformed cells.

Diderholm *et al.* described transformation by SV40 DNA in mass cultures of bovine embryo cells (*14*), which were very resistant to transformation by high-titered intact virus. Black and Rowe have reported T-antigen induction by SV40 DNA in hamster BHK21 cells. These cells, too, were found to be insensitive to infection with virus (*15*). In our present report it is also shown that SV40 DNA can induce T-antigen in marsupial rat kangaroo cells in which there is a complete block to infection by whole virus. The species differences in T-antigen induction with whole virus are, therefore, probably due to differences in attachment, penetration, or uncoating, rather than to differences in the ability of the exposed viral genome to begin functioning once inside the cell. The ultimate outcome of infection, however, remains under cellular control. SV40 DNA is highly cytopathic for monkey cells, weakly cytopathic for human cells, and noncytopathic for mouse cells, behaving in the same manner as whole virus.

While transformation of human cells by DNA is more efficient than by whole virus per infectious unit, it is still highly inefficient per molecule. When 5×10^5 cells were infected with 0.1 μg of SV40 DNA I (3.0×10^3 plaque-forming units), 21 cells were transformed. If the molecular weight of SV40 DNA is 2.5×10^6 (*1*), it can be calculated that 10^9 DNA molecules are needed per transformation event. Transformation and T-antigen induction appear, at least over a limited range, to be linearly related to DNA concentration. This suggests that a single molecule of DNA is sufficient to induce SV40 T-antigen and also to permanently transform human cells.

There is considerable evidence that shows that SV40 DNA can become a permanent part of the host cell genome (*4, 16*). Most of the SV40 DNA found in transformed cells is associated with the chromosomes (*17*). Whether SV40 has an integration function similar to that of bacteriophages λ and P 22 (*18*) is not yet known. However, if the viral DNA's ability to integrate into the human cell genome can be separated from its oncogenicity, it then may be possible to use "integrating" viral DNA as a carrier to insert specific genetic information into human cells.

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References and Notes

1. P. H. Black, *Annu. Rev. Microbiol.* **22**, 391 (1968).
2. H. M. Shein and J. F. Enders, *Proc. Nat. Acad. Sci. U.S.* **48**, 1164 (1962); H. Koprowski, J. A. Ponten, F. Jensen, R. G. Ravdin, P. Moorehead, E. J. Saksela, *J. Cell. Comp. Physiol.* **59**, 281, 1962.
3. Y. Aloni, E. Winocour, L. Sachs, *J. Mol. Biol.* **31**, 415 (196 → K. Oda and R. Dulbecco, *Proc. Nat. Acad. Sci. U.S.* **60**, 525 (196 → G. Sauer and J. R. Kidwai, *ibid.* **61**, 1256 (1968).
4. P. Gerber, *Virology* **28**, 501 (196(→ H. Koprowski, F. Jensen, Z. Steplewski, *Proc. Nat. Acad. Sci. U.S.* **58**, 127 (1967).
5. J. H. Pope and W. P. Rowe, *J. Exp. Med.* **120**, 124 (1964).
6. P. Gerber, *Virology* **16**, 96 (1962).
7. J. A. McCutchan and J. S. Pagano, *J. Nat. Canc. Inst.* **41**, 351 (1968).
8. S. A. Aaronson and G. J. Todaro, *Virology* **36**, 254 (1968).
9. ———, *Science* **162**, 1024 (1968).
10. K. K. Takemoto, G. J. Todaro, K. Habel, *Virology* **35**, 1 (1968).
11. Supplied by Dr. M. A. Martin, NIH.
12. J. Marmur, *J. Mol. Biol.* **3**, 208 (1961).
13. M. A. Martin and D. Axelrod, *Science* **164**, 68 (1969).
14. H. Diderholm, B. Stenkvist, J. Ponten, T. Wesslen, *Exp. Cell Res.* **37**, 452 (1965).
15. P. H. Black and W. P. Rowe, *Virology* **27**, 436 (1965).
16. T. L. Benjamin, *J. Mol. Biol.* **16**, 359 (1966); → H. Westphal and R. Dulbecco, *Proc. Nat. Acad. Sci. U.S.* **59**, 1158 (1968).
17. → J. Sambrook, H. Westphal, P. R. Srinivasan, R. Dulbecco, *Proc. Nat. Acad. Sci. U.S.* **60**, 1288 (1968).
18. E. R. Signer, *Annu. Rev. Microbiol.* **22**, 451 (1968).
19. Supported by National Cancer Institute contract No. PH 43-66-641. We thank Dr. David Axelrod and Dr. Malcolm Martin for their advice and Elaine Rands and Lillian Killos for their excellent technical assistance.

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