Korean J. Zool. 31: 49-55, 1988

Proporties of SV40-transformed Human Cells

Kyung-Hee Choi and Seung-Hwan Hong*

*Dept. of Zoology, Seoul National University, Seoul, 151-742, Korea; *Dept. of Genetics, Albert Einstein College of Medicine, Bronx, N. Y. U. S. A.*

A few SV40-transformed human cells such as SV80 are potentially tumorigenic but rejected by athymic hosts. However, one cell line in this group (WI18/VA-2) is known to be fully tumorigenic. Two clones were obtained after the injection of WI18/VA-2, of which NW18C1-1 was tumorigenic but NW18C1-2 was not in nude mice. As examined by Southern blot analysis, NW18C1-1 appears to contain more copy number of SV40 sequences than NW18C1-2 does. However, it was unable to demonstrate that this difference elicits the tumorigenicity in NW18C1-1 but not in NW18C1-2. Therefore, the latter clone was tested if it expresses SV40 early genes to produce large T as well as small t antigens using indirect immunofluorescent assay and immunoprecipitation. In addition, mouse NIH3T3 cells were transfected with the cellular DNA of NW18C1-2 as well as that of NW18C1-1 to examine if the viral genomes in the clones can make the nontransformed cells to acquire malignant growth potential in vivo. The transformed cells expressed large T antigen and became tumorigenic. Thus, the transforming functions of NW18C1-2 cell appears to be intact. These results clearly suggest that the inability of NW18C1-2 cell to form tumor in nude mice is not because they are inherently nontumorigenic. However, the possibility that the interaction of SV40 with its host differs in these clones can not be ruled out.

KEY WORDS: SV40 transformation, DNA transfection

Malignant transformation of animal cells *in vitro* involves changes in many cellular properties, but anchorage-independent growth is the single property most consistently associated with the ability of transformed cells to form tumors in nude mice (Freedman and Shin, 1974; Shin *et al.*, 1975). Human and monkey cells transformed by SV40 are exceptionally impaired in this general correlation (Kahn *et al.*, 1983; Choi *et al.*, 1983, 1984).

One possible explanation for the inability of most SV40-transformed human cells to form tumors in nude mice is that they are inherently nontumorigenic (Stanbridge and Wilkinson, 1978), or alternatively, these cells may be potentially tumorigenic (Stanbridge *et al.*, 1982) but are prevented from growing as tumors in nude mice by tumor resistance mechanism present in the athymic host. However, some SV40-transformed human cells proved to be tumorigenic in immunologically permissive host (Kahn *et al.*, 1983 and Choi *et al.*, 1983). These reports suggest that the failure of these cells to form tumors in untreated nude mice may be due to an active rejection process mediated by cell surface antigens induced directly or indirectly by SV40 genetic information responsible for transformation (i.e. the viral early gene which codes for the large T antigen) (Abramezuk *et al.*, 1984; Choi *et al.*, 1985).

Interestingly only one cell line of SV40-transformed human cells (NW18) was reported to be fully tumorigenic in nude mice (Stanbridge *et al.*, 1982). When nude mice were injected with NW18 cells and the resulting tumors were introduced into cultures, one clone (NW18C11) was tumorigenic and the other clone (NW18C12) was nontumorigenic; nevertheless both clones showed the same degree of characteristics related to cellular transformation such as low

serum requirement, lack of density-dependent inhibition of cell division and ability to grow without anchorage to the culture plate.

It would therefore be important to focus research efforts on identifying the difference between two clones. In this paper, the integration pattern of SV40 sequences into cellular genome was examined by southern blot hybridization analysis. It was also examined the SV40 T antigen expression in both clones by immunofluorescence and immunoprecipitation analysis. Finally, NIH3T3 cells transfected with the cellular DNA from both clones to compare the transforming ability and to test the tumorigenicity of the transfectants.

Materials and Methods

Cell Culture and DNA Transfection

Mouse NIH3T3 cells, NW18C11 and C12 were maintained in Dulbecco-Vogt modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. No antibiotics were used. Cells were periodically examined for mycoplasma by the method of Chen (1977), and were found to be free of contamination. DNA transfections were performed according to the method of Wigler *et al.* with some modification (1977). High molecular weight cellular DNA (20 μg/ml) either from NW18C11 or from NW18C12 was incubated with the cells in the presence of calcium phosphate to coprecipitate them for 10 hours. Salmon sperm DNA was used as a negative control. Transfectants were isolated after 2-3 weeks from the actively proliferating cell foci using cloning cylinders since there was no positive selection markers.

Isolation of High Molecular Weight DNA from NW18C11 and C12

Isolation of cellular DNA was performed according to the procedure of Pellicer *et al.* (1978), using Proteinase K and phenol extraction followed by treatment with RNase and reextraction with phenol and chloroform/Isoamyl alcohol mixtures.

Southern Blot Hybridization

High molecular weight DNA was digested with restriction endonucleases according to reaction conditions recommended by the manufacturers. The DNA fragments were separated on 0.7% agarose gels, then transferred from agarose gels to nitrocellulose filters by the Southern blot technique (Southern, 1975). Prehybridization and hybridization were carried as described by Maeda *et al.* (1981). DNA probes, labeled with ^{32}P, were generated by nick translation of SV40 DNA in the presence of ^{32}P-dCTP and dATP (Amersham) to a specific activity of approximately 10^8 cpm/μg. 20 ng/ml of this probe was added to a hybridization mixture. Following a 12-16 hr incubation, the filters were first washed at 65°C in 2X SSC, 0.1% SDS for 30 min and then washed in 0.1X SSC, 0.1% SDS, again at 65°C for 30 min. Kodak XAR film and Dupont intensifying screens were used for autoradiography.

Test of SV40 T Antigens

Immunofluorescence staining: The large T antigen of SV40 in the nuclei of transformed cells was visualized by indirect immunofluorescence, using a Zeiss microscope with epi-illumination. Cells of subconfluent cultures growing on glass coverslips were fixed in 2% paraformaldehyde, post-fixed with anhydrous methanol, and stained sequentially first with a monoclonal mouse antibody against SV40 large T antigen (clone 412, reactive with the C-terminal 88% of the molecule [Gurney *et al.*, 1980]) and then with fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories).

Immunoprecipitation: Exponentially growing cells were labeled by culturing for 2 hr with 100 μCi of ^{35}S-methionine (Amersham). Cells were washed in PBS and lysed in 1 ml of lysis buffer (1% Nonidet NP40, 200 mM Tris, pH 8.4, 140 mM NaCl, 1 mM DTT, and 250 μg/ml phenylmethylsulphonyl fluoride). Cell lysates were centrifuged at 48,000 $\times$ g for 1 hr. Extracts (48,000 $\times$ g supernatant) were incubated with 20 μl either of control normal hamster serum or hamster anti-SV40 tumor serum (Cappel Laboratories) at 4°C for 90 min. Immune complexes were then precipitated with protein A-sepharose CL4B (Pharmacia Fine Chemicals). The immunoprecipitated material was then analyzed by SDS-polyacrylamide electrophoresis on a 7-15% gradient gel, followed by autoradiography.

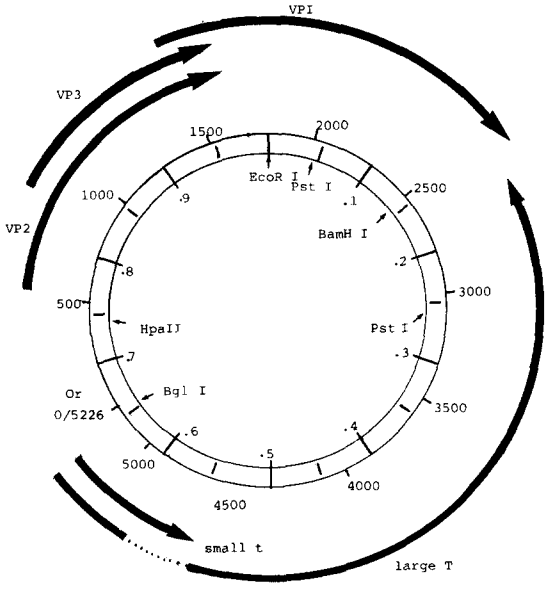

Fig. 1. Recognition sites in the SV40 map of restriction endonucleases used in the experiment. (HindIII recognition sites are located on map units of 0.32, 0.42, 0.65, 0.86, 0.94 and 0.98)

Determination of Cellular Tumorigenicity

Cells grown in monolayer cultures were trypsinized and resuspended in PBS at a concentration of 5×10^6 cells per 0.2 ml. 4-6 week old nude mice were then injected subcutaneously with 0.2 ml of this suspension. Mice were monitored for tumor development for at least 4 months following the injection.

Results and Discussion

Integration Pattern of SV40 Sequences in NW18 Cells

It has been previously reported that some SV40-transformed human cells such as SV80 are potentially tumorigenic but rejected by the athymic host possibly due to their expression of virus-induced transplantation antigen (Kahn *et al.*, 1983; Choi *et al.*, 1983, 1984, 1985). However, one cell line in this group (WI18/VA-2) is known to be fully tumorigenic (Stanbridge *et al.*, 1982). Two clones (NW18C11 and C12) were isolated from a tumor formed after the injection of WI18/VA-2. One (NW18C11) was tumorigenic and the other (NW18C12) was not when they were injected into nude mice. It was therefore attempted to determine why one is tumorigenic and the other is nontumorigenic despite their fully transformed phenotype *in vitro*.

Initially, the integration pattern of SV40 sequences into cellullar genome was examined if it differs in both clones. To compare the integration patterns of SV40 sequences, cellular DNA was isolated from both clones and hybridized with ^{32}P-labeled SV40 DNA by southern blot hybridization analysis. Figs. 2 and 3 are the autoradiograms of southern hybidization in NW18C11 and NW18C12, respectively. As can be seen in these blots, no major differences in band positions were detected. Compared to NW18C11 groups, there are remarkable loss of band densities in NW18C12 groups such as the second band in endonuclease EcoRI and BamHI digested group, lower two bands in HpaII treated and upper two bands in PstI treated group. It seemes likely that the overall densities in NW18C11 is higher than that of NW18C12 suggesting that NW18C11 contains more copy numbers of SV40 genomes than NW18C12 does. Since endonuclease XhoI has no recognition site in SV40 genome and has lighted up two bands in southern blots, it seems there are at least two integration sites in both NW18C11 and C12. Assuming that more than one copies of SV40 genomes are integrated at a single site as repeated manner, one can expect a band of same density in all the restiction endonuclease treated groups which has only one recognition sites in SV40 genome. Since no corresponding band was lighted up in the single cutting enzyme groups and BglI which cuts only once in the SV40 sequence produced five bands, it is likely that there are at least 3 integration sites in NW18C11 and one is missed in NW18C12 or at least some portions near BglI site were deleted. Similarly to earlier studies on the SV40 transformation of *in vitro* cultured cells (Botchan *et al.*, 1976; Steinberg *et al.*, 1978), there is no unique integraion sites in both the SV40 sequence and the cellular genome nor unique copy numbers. Also some portions of SV40 sequences can be deleted during integration into

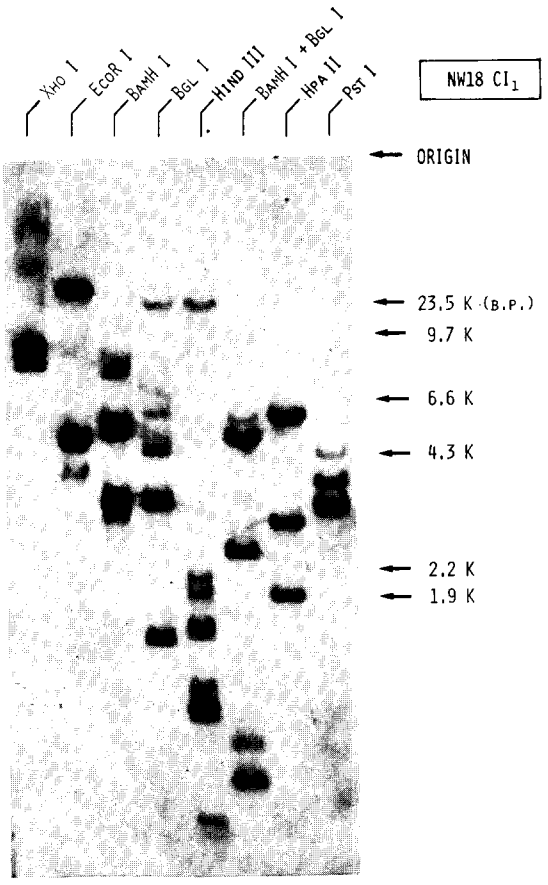

Fig. 2. Southern blot hybridization analysis of NW18Cl1.

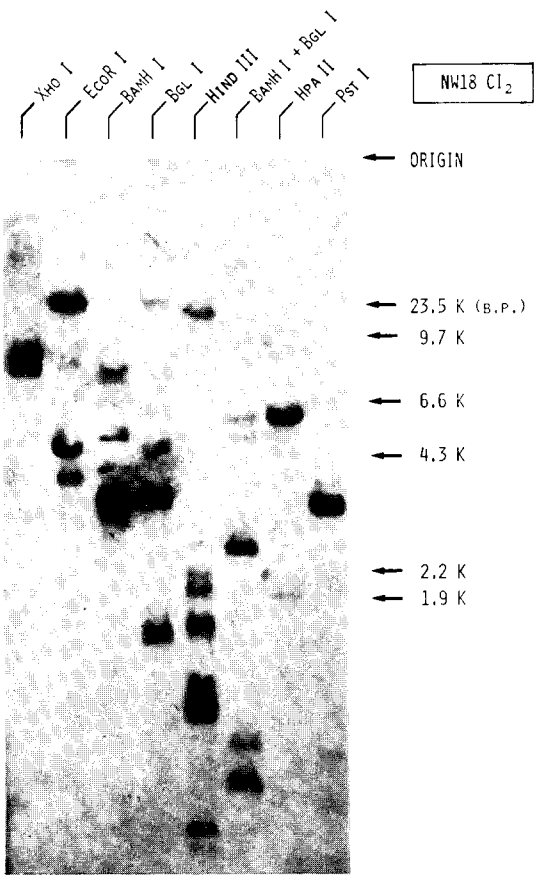

Fig. 3. Southern blot hybridization analysis of NW18Cl2.

cellular genome. So far from these results NW18C11 seemed to contain more copy numbers than NW18C12. However, it was unable to demonstrate that this difference would elicit the tumorigenicity in NW18C11 not in NW18C12. Then, it was examined whether or not NW18C12 cells are not inherently tumorigenic despite they are anchorage-independent. While it is difficult to test this hypothesis directly, the experimental approach was based on the assumption that the transformed phenotype of these cells is due to the expression of the integrated viral genome. If the cells are not inherently malignant, the virus itself must be transformation-defective. Hence, viruses recovered from such cells should therefore induce nontumorigenic phenotype in other cells which they transform.

Expression of the SV40-coded T Antigens

NW18C11 and C12 cells were examined if they express SV40 early genes to produce the large T and small t antigens. The presence of large T antigen was tested by the indirect immunofluorescent assay described in Materials and Methods. Both of these cell lines were highly positive with $> 99\%$ of the cells showing bright nuclear staining.

The presence of the large T and small t antigens was also detected directly on SDS-polyacrylamide gels following immunoprecipitation of ^{35}S-methionine-labeled cell extracts with hamster anti-SV40 tumor serum. NW18C11 and NW18C12 cells were both found to express large T and small t proteins of apparently normal molecular weight (Fig. 4).

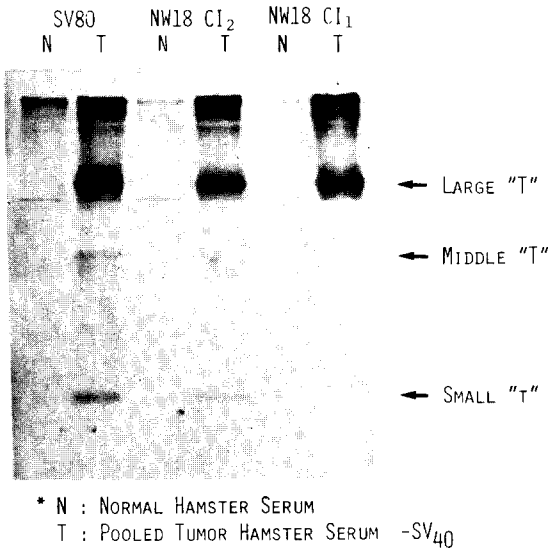

Fig. 4. Immunoprecipitation of SV40 large T and small t antigens.

Table 1. Transfection of NIH3T3 cells with restriction endonuclease digested cellular DNA.

Experimantal group	DNA source	No. of colonies formed
I	Salmon Sperm DNA only	0
II	Salmon Sperm DNA+pVBE TK (200 ng)	52
III	NW18C11 BamHI fragment	88
IV	NW18C11 XmnI fragment	125
V	NW18C11 BamHI fragment	11
VI	NW18C11 XmnI fragment	46

*Pooled from five 100 mm dishes originally started with 7×10^6 cells/dish.

Transforming Ability

The results described above indicate that the SV40 early region gene products are present in NW18C11 and NW18C12 cells, but do not address the question whether their transforming functions are altered in any way. Specifically, it was tested whether the viral genomes present in NW18 cells are capable of inducing nontransformed cells *in vitro* to acquire malignant growth potential *in vivo*. This was of particular interest with respect to NW18C12 cells, since these cells do not form tumors. In this series of experiments, cellular DNAs from NW18C11 and C12 were digested with two restriction endonucleases BamHI and XmnI both of which has only one recognition site in SV40 genome. But BamHI, recognition site of 0.14 map unit, leaves the SV40 large T portions intact whereas XmnI, recognition site of 0.26 map unit, cut out about 1/4 of the large T at the end. Mouse NIH3T3 cells were transfected with these digested cellular DNAs and tested for large T antigen expression in the transfectants by immunoprecipitation. Results are summarized in Table 1. Transfectants from both clones expressed large T antigen and many transformed phenotypes *in vitro*, including anchorage-independent growth. Upon injection into nude mice, these cells proved to be as tumorigenic. It therefore appears that the transforming functions of the NW18C12 cells are intact, despite it is nontumorigenic. From these results, it was clear that inability of NW18C12 cells to form tumors is not because they are inherently nontumorigenic. However, the possibility that the interaction of SV40 with its host differs in both clones can not be ruled out.

The other possibility which can be considered is that NW18C12 cells are also inherently tumorigenic but that nude mice actively suppress their growth. In an earlier study, evidence was presented that nude mice possess a thymus-independent mechanism which enables them to inhibit both primary tumor formation and metastatic spread by certain highly malignant cells (Reid *et al.*, 1978, 1979; Bloom *et al.*, 1980). What are the antigens present on the surfaces of SV40-transformed human cells which might cause them to be rejected by nude mice? SV40-transformed tumor cells express the SV40-coded large T and small t antigens, as well as the tumor-specific transplantation antigens (TSTA). Of these antigens, large T protein and TSTA are present on the surface of SV40-transformed cells, and evidence suggests that these are closely associated, if not identical molecules (Chang *et al.*, 1979; Pretell *et al.*, 1979; Tevethia *et al.*, 1980a, 1980b). Rejection of SV40-transformed human cells presumably depends on host recognition of one of these virus-induced surface antigens. The small t protein is localized in the cytoplasm of SV40-transformed cells (Ito *et al.*, 1977) and is probably not involved in host recognition. However, expression of large T

and/or TSTA by transformed cells is not sufficient to elicit their recognition, since SV40-transformed rodent cells, which express both of these antigens are usually tumorigenic in nude mice. Thus, the cellular background in which the viral antigens are displayed appears to be critical in determining whether the nude mouse will reject the cells. It is possible that nude mice recognize virus-induced antigens in association with one or more cellular antigens present on the surface of NW18C12 cells but absent from NW18C11 cells. Alternatively, the viral antigens recognized by the nude mouse on NW18C12 cells may be identical to those on NW18C11 cells, except that NW18C12 cells express these antigens in greater amounts.

References

Abramczuk, J., S. Pan, G. Maul, and B. B. Knowles, 1984. Tumor induction by simian virus 40 in the mouse in controlled by long term persistence of the viral genome and the response of the host. *J. Virol.* **49**:540-548.

Bloom, B., N. Minato, A. Neighbor, L. Reid, and D. Marcus, 1980. Interferon and NK cells in resistance to persistently virus-infected cells and tumors. *In:* Natural Cell-Mediated Immunity Against Tumors (ed. R. Herberman, R. ed.), Academic Press, New York. pp 505-524.

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.

Chang, C., R. G. Martin, D. M. Livingston, S. W. Luborsky, C. P. Hsu and P. T. Mor, 1979. Relationship between T-antigen and tumor-specific transplantation antigen in simian virus 40-transformed cells. *J. Virol.* **29**:69-75.

Chen, T. R., 1977. *In situ* detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res.* **104**:255-263.

Choi, K. H. and S. Shin, 1984. Selective rejection of SV40-transformed human cells by nude mice. *Exp. Cell Biol.* **52**:209-213.

Choi, K. H. and S. Shin, 1985. Differential rejection of human tumor cells expressing a complete or partial SV40 T antigen by athymic nude mice. *Korean J. Genetics* **7**:163-176.

Choi, K. H., S. S. Tevethia, and S. Shin, 1983. Tumor formation by SV40-transformed human cells in nude mice: The role of SV40 T antigens. *Cytogenetics and Cell genetics* **36**:366-640.

Freedman, V. H. and S. Shin, 1974. Cellular tumorigenicity in nude mice: Correlation with cell growth in semi-solid medium. *Cell* **3**:355-359.

Gurney, E. G., R. O. Harrison, and J. Fenno, 1980. Monoclonal antibodies against simian virus 40 T antigens. Evidence for distinct subclasses of large T antigen and for similarities among nonviral T antigens. *J. Virol.* **34**:752-763.

Ito, Y., J. R. Brocklehurst, and R. Dulbecco, 1977. Virus-specific proteins in the plasma membrane of cells lytically infected or transformed by polyoma virus. *Proc. Natl. Acad. Sci. USA* **74**:4666-4670.

Kahn, P., W. C. Topp, and S. Shin, 1983. Tumorigenicity of SV40 transformed human and monkey cells in immunodeficient mice. *Virology* **26**:348-360.

Maeda, S., M. Gross, and S. Pestka, 1981. Annealing of oligo(dG)-tailed plasmid and transformation of E. coli × 1776 with recombinant DNA. *Methods Enzymol.* **79**:613-618.

Pellicer, A., M. Wigler, R. Axel, and S. Silverstein, 1978. The transfer and stable integration of the HSV thymidine Kinase Gene into mouse cells. *Cell* **14**:133-141.

Pretell, J., R. S. Greenfield, and S. S. Tevethia, 1979. Biology of simian virus 40 (SV40) transplantation rejection antigen (TrAg). V. *In vitro* demonstration of SV40 TrAg in SV40 infected nonpermissive mouse cells by the lymphocyte mediated cytotoxicity assay. *Virology* **97**:32-41.

Reid, L. M., C. Jones, and J. Holland, 1979. Virus carrier state suppressess tumorigenicity of tumor cells in athymic nude mice. *J. Gen. Virol.* **42**:609-614.

Reid, L. M. and S. Shin, 1978. Transplantation of heterologous endocrine tumor cells in nude mice. *In:* The nude Mouse in experimental and Clinical Research (Fogh, J. and B. C. Giovanella eds.), Academic Press, New York. pp. 313-352.

Shin, S., V. H. Freedman, R. Risser, and R. Pollack, 1975. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage-independent growth *in vitro*. *Proc. Natl. Acad. Sci.* **72**:4435-4439.

Southern, E., 1975. Detection of specific sequences among DNA fragment separated by gel electrophoresis. *J. Mol. Biol.*, **98**:503-518.

Stanbridge, E. J., C. J. Der, C. J. Doersen, R. Y. Nishimi, D. M. Peehl, B. E. Weissam, and J. Wilkinson, 1982. Human cell hybrids: Analysis of transformation and tumorigenicity. *Science* **215**:252-259.

Stanbridge, E. J. and J. Wilkinson, 1978. Analysis of malignancy in human cells: malignant and transformed phenotypes are under separate genetic control. *Proc. Natl. Acad. Sci. USA* **75**:1466-1469.

Steinberg, B., R. Pollack, W. Topp, and M. Botchan, 1978. Isolation and characterization of T antigen negative revertants from a line of transformed rat cells containing one cpoy of the SV40 genome. *Cell* **13**:19-32.

Tevethia, S. S., D. C. Flyer, and R. Tijan, 1980a, Biology of simianvirus 40 (SV40) transplantation antigen (TrAg). Mechanism of induction of SV40 transplantation immunity in mice by purified SV40 T antigen (D2 protein). *Virology* **107**:13-23.

Tevethia, S. S., R. S. Greenfield, D. C. Flyer, and M. J. Tevethia, 1980b, SV40 transplantation antigen: relationship to SV40-specific proteins. *Cold Spring Harbor Symp. Quant. Biol.* **44**:235-242.

Wigler, M., S. Silverstein, L. S. Lee, A. Pellicer, Y. C. Cheng, and R. Axel, 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* **11**:223-232.

(Accepted December 22, 1987)

SV40 바이러스로 형질전환된 사람종양세포의 특성

최경희 · 홍승환*(서울대학교 자연대 동물학과; *알버트 아인슈타인의과대학 유전학과)

SV80와 같은 SV40로 형질전환된 사람세포는 종양을 일으킬 수 있는 능력은 가지고 있으나 면역기구인 흉선이 없는 누드마우스에서는 거부반응을 일으켜 종양을 일으키지 않는다. 그러나, 예외적으로 WI18/VA-2세포는 누드마우스에서 종양을 일으키며 이에서 얻은 두클론중 NW18Cl1은 종양을 일으키나 NW18Cl2는 종양을 일으키지 않는다. 본 실험에서는 이들 두 클론의 차이점들을 조사하였다. 실험결과, NW18Cl1은 NW18Cl2보다 더 많은수의 SV40 sequence를 포함하고 있음을 southern blot방법을 통해 확인하였으며 또한 immunofluorescence와 immunoprecipitation방법을 사용하여 두 클론 모두 정상크기의 SV40유전자산물인 large T와 small t 단백질을 생성함을 확인하였다. 한편 두 클론내에 포함되어 있는 바이러스유전자가 비형질전환세포로 하여금 생체내에서 악성종양 형성능력을 획득하도록 형질전환시킬수 있는지 확인하기위해 두 클론의 DNA를 추출하여 마우스 NIH3T3세포에 주입시켜 형질전환된 세포를 선별하였다. 이세포들은 모두 large T단백질을 생성하였으며 누드마우스에서 종양을 일으켰다. 이들 결과로써 NW18Cl2세포의 형질전환능은 완전하며, 이세포가 누드마우스에서 종양을 일으키지 않는것은 유전적으로 종양을 일으킬 수 없기 때문이 아니라 누드마우스에서 거부반응에 기인하는 것으로 생각된다.