Malignant transformation of human cell in vitro by the SV 40 DNA and related alteration in biological activity of cell membranes

Tetsuo Kimoto[*]　　　E-iti Yokomura[†]　　　Yoriko Shimizu[‡]

Morihei Yamakawa[**]　　　Satimaru Seno[††]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama University,
[**]Okayama University,
[††]Okayama University,

Malignant transformation of human cell in vitro by the SV 40 DNA and related alteration in biological activity of cell membranes[*]

Tetsuo Kimoto, E-iti Yokomura, Yoriko Shimizu, Morihei Yamakawa, and Satimaru Seno

Abstract

In vitro cell transformation of human embryonic cells could be induced by DNA extracted from virions of SV 40 purified by density gradient centrifugation. The result shows clearly that cell transformation is in· duced by incorporation ofa part of viral DNA into the genome. In addition, for the purpose of clarifing the biological differences between the normal and transformant, the alteration of the cell membrane structures of transformants was observed from the mechanism of phagocytosis. The iron colloid particles are taken up by normal diploid fibroblasts but not by the human and hamster transformants. This fact suggests a difference in the molecular arrangement of the cell membranes between the normal and transformants. In the presence of histones, however, the transformants phagocytize the colloid particles very actively. The results show cell membranes of transformants are altered in the molecular structure responsible for the surface charge.

MALIGNANT TRANSFORMATION OF HUMAN CELL *IN VITRO* BY THE SV 40 DNA AND RELATED ALTERATION IN BIOLOGICAL ACTIVITY OF CELL MEMBRANES

Tetsuo Kimoto, E-iti Yokomura, Yoriko Shimizu, Morihei Yamakawa and Satimaru Seno

Department of Pathology, Okayama University Medical School
Okayama, Japan (Director: Prof. S. Seno)

As reported first by Abercrombie (1), the cell division of normal and non-neoplastic cell lines (fibroblasts) in culture is largely suppressed by contact with neighboring cells, i. e. contact inhibition. By the malignant transformation, however, the cells (transformants) lose much of their inhibitory effect on the neighboring cells piling up one another, eventually forming a gross lump of the transformed cells. This means the loss of normal characteristics of the cell behavior and such a change in the cell characteristics serves as the very marker for the malignant transformation of the cells, though occasionally normal cells also become slightly insensitive to the "cell contact". The outer coat of the cell membrane composed of glycolipid should be directly related to the phenomenon and the alteration in its architectural structure or molecular arrangement with some qualitative and quantative changes may be responsible for the insensitivity to contact inhibition because the changes in electric charge of the cell surface are clearly observable in relation to the malignant transformation or to the loss of contact inhibition. The changes in cell surface seem also to suggest the appearance of the specific surface antigen that is found on the cell surfaces of the transformants induced by oncogenic virus (polyoma, adeno virus 12 and SV 40). Besides these, Yokomura and others (2, 3) revealed that the phagocytic activity of the cell is closely correlated to the specificity of the cell membrane indicating that such substances absorbed on the cell surface are solely taken by the cell. This indicates that the phagocytic activity of the cell is closely connected to the specific molecular structure of the cell surface.

In this paper, dealing with human and hamster embryonic cells and their transformant induced by SV40 and its extracted DNA, it is reported that the phagocytic activity gives a good and sensitive information for the

changes in the molecular structure or arrangement of the surface of transformed cell as well as the phenomena of contact inhibition and electric charge of the cell.

MATERIALS AND METHODS

SV40 strain, its propagation, purification and biological activities

SV40 777 was supplied by Dr. Yoshiike (4, 5), National Institute of Health, Tokyo, Japan. The other strains were donated by Dr. Grace, Roswell Park Memorial Institute, Buffalo, U. S. A. For propagation of the virus, BSC-1 and Vero cell (6) lines were used. The cells were grown in $5 \times 10 \times 5$ cm bottles, containing 10 ml of Le medium (Earl's balanced salt solution containing 0.5 per cent lactalbumin hydrolysate, penicillin 100 units/ml and streptmycin 100 μg/ml) with 5 per cent bovine serum and McCoy 5 A medium (7) with 20 % calf serum. The virus was propagated on these cells by inoculating 0.5 ml of 10^8 (PFU) per bottle. Cytopathic effect was observed about one week after virus inoculation.

For purification 20 ml of concentrated crude virus suspension obtained after one week's propagation was layered gently over 9 ml of saturated KBr solution and centrifuged at 25,000 rpm for 3 hours. The purified virion was obtained from full particles.

The virion preparation thus obtained was further separated into two fractions by CsCl density gradient centrifugation in an SV 39 rotor of a Spinco Model L. For the separation, the virion particles obtained from the lower band in a KBr-cushion, were suspended in CsCl solution (p=1.33) and spun in a rotor No. 40 of a Spinco Model L. at 35,000 rpm for 20 hours. Fractions were collected and 1 ml of phosphate buffered saline was added to each fraction. The biological activities of the virion was assayed by the procedures described by Uchida (8).

Viral DNA ; its extraction and biological assay

For the extraction of viral DNA the purified virions were dialysed against saline citrate (0.15 M NaCl, 0.015 M sodium citrate) and incubated at 37°C for 2 hours in the presence of 0.1 mg/ml pronase P (Kaken Kagaku), by the method of Yoshiike (4, 5). From this material DNA was isolated by extracting with phenol three times. Phenol was removed by dialysis against 0.14 M NaCl, 0.01 M phosphate buffer pH 7.3.

Biological assay of DNA was made by the procedure as described in the previous report (9, 10). The infectivity titration was performed with the cell monolayers cultured in McCoy 5 A medium (10 per cent bovine serum) by calculating TCID 50.

Cells used for transformation

Cells from human embryos (6 weeks old), golden syrian hamster embryos and their kidneys in primary cultures were used for the observation of cell transformation. The embryonic cells were grown initially in 199 medium and McCoy 5 A supplemented with 20 % calf serum respectively. Cells, grown on the glass, were infected with DNA and SV 40 complete virus particles. After 2

hours' adsorption at 37°C, the medium was discarded and then fresh medium added. Cells grown on coverslips of Leighton tubes were stained with Giemsa and hematoxylin for morphological observation. Immunofluorescent antibody reaction for tumor antigen and surface antigen were also examined by the indirect methods.

Phagocytosis of colloidal iron

For this purpose the cells were given colloidal iron particles in the form of iron chondroitin sulphate (Cs-Fe) in culture media. Cs-Fe which had been supplied by Dainippon Seiyaku Co., Osaka, Japan, was a colloidal solution, pH 7.21 and 1 ml of solution contained 4 mg of iron. The size of the iron colloidal particles estimated by electron microscopy ranged from about 30 Å in the smallest unit to about 200 Å in the aggregated group particles. The colloid particles in Hank's solution proved to be of negative charge as revealed by the paper electrophoresis in veronal-acetate buffer (pH 7.4). Iron was combined so firmly with chondroitin sulphate that the two components were hardly separated by electrophoresis.

The iron colloid solution was added to the culture media, in a 1 : 3 volume, in the Leighton tubes in whith the cells had been grown on the coverslips, and 1, 2, 3, 12, 24, 48, and 72 hrs after the addition of iron colloid, the cells were washed by Hanks' solution, dried, fixed with ethanol, stained by Perls' reaction for iron with nuclear stain by Kernechtrot and observed under light microscope.

For electron microscopy, the cells were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer at 4°C for 30 min., and post-fixed with 1 per cent OsO_4 in phosphate buffer for 30 min. After fixation, they were washed with cold water, dehydrated through ethanol series and embedded in Epon 812. The sections were stained with 5 % uranyl acetate in 7 % aqueous ethanol and observed by electron microscope, Hitachi HU-11 A.

For the observation of the effect of polycation on the phagocytic activity, histones were used. These were extracted from calf thymus by the modified method of JOHNS (11), and UI (12). Two fractions, arginine-rich and lysine-rich histones, were used. Besides these, histone type II (Sigma Co.) was also used. These were added to the culture medium at the respective concentration to observe changes in phagocytic activity of the cells for iron colloid particles.

RESULTS

The human embryonic cells in primary culture were tested for their abilities to be transformed after being exposed to the viral DNA. The DNA of 3 μg/ml was added to the media and the cells were examined during definite time intervals. Three months after the addition of the DNA, all the cells were found to contain T-antigen with some morphologic changes showing the malignant transformation. The transformed cells showed the appearance of epitheloid cells, some of which were of multi-

80   T. KIMOTO, E. YOKOMURA, Y. SHIMIZU, M. YAMAKAWA and S. SENO

nuclear giant cells (Fig. 1). In the early stage of exposure to the DNA,
viral particles were found in the nucleus of some fibroblasts by electron
microscopy, suggesting that the complete virus developed from the purified
viral DNA. Cell transformation was also observed on cell lines from
hamster embryo and kidney cells, both of which were fibroblasts in native.
Such a transformant from fibroblastic cell to epithelium-like cell should
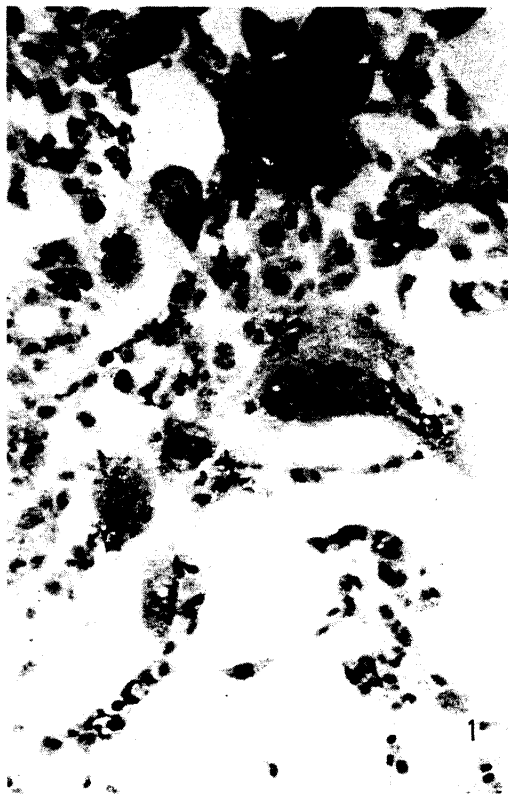be characteristic of the SV 40.

Fig. 1 *In vitro* human transformed cells induced by DNA purified
from SV40. Giemsa stain. 10×20

Immunologic tests revealed that the transformants of hamster cells
(designed as HE 12 and 13, Okayama) have the specific tumor and
surface antigens. Prior to the observation on phagocytic activity of these
cells and their transformants' toxicity of histones was checked, because
the histones were used in some experiment as the promotor of phagocytosis.
The toxicity tests were solely made on the transformed embryonic cells of
hamster. Experiments revealed that an excess of histone in the culture
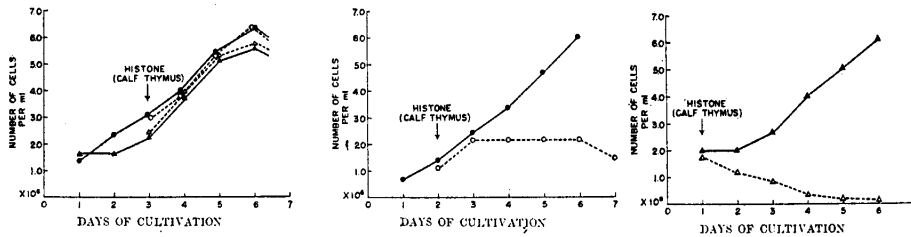
A. Rate of growth following administration of histone (2μg/ml of medium) to hamster embryonic cells and to hamster kidney cells, both of which had been transformed by SV 40.
Transformed embryonic cells (16th passage), ○: Transformed hamster kidney cells (16th passage).

B. Rate of growth following administration of histone (250 μg/ml of medium) to hamster embryonic cells which had been transformed by SV 40. 20th passage.

C. Rate of growth following administration of histone (450 μg/ml of medium) to hamster kidney cells which had been transformed by SV 40. 13th passage.

Fig. 2 Rate of growth following administration of histone to hamster embryo and kidney transformants induced by SV-40.
Unterated controls shown by the solid lines and treated cell lines by the broken lines.
(A) 2μg/ml histone. (B) 250μg/ml histone. (C) 450μg/ml histone.

medium suppressed the growth or cell division of the transformed cells (Fig. 2A, B and C); *i. e.* the arginine-rich histone in the the concentration of 2 μg to 100 μg/ml gave no inhibitory effect on the growth of the cells (Fig. 2A), but at the concentration of 250 μg/ml (Fig. 2B), the histone suppressed the growth of the transformants. And at the concentration of 450 μg/ml the division or growth of the transformant was completely stopped (Fig. 2C). Similar results were obtained on other histones.

Referring to the data just mentioned, the effect of the histones on the phagocytosis of the cells to iron colloid were observed at the concentration of 100–250 μg/ml. By 24 hours' cultivation with the colloid iron particles embryonic cells of human and hamster phagocytized the colloidal iron particles moderately, but the transformants showed no phagocytic activity as revealed by Perls' reaction. All the transformants of human cells (Fig. 3) induced by viral DNA and hamster cells by SV 40, were completely free of iron particles, while in the presence of histones (100–200μg/ml) the transformants cultivated with colloidal iron for 25 hrs showed a marked phagocytic activity and contained numberous colloidal particles in their cytoplasm (Fig. 4).

Electron microscopy of the transformants cultured with the iron colloid and histones revealed that the transformed cells ingested a number of iron colloid particles of about 200 Å in diameter by 24 and 48 hrs follow-

3

4

N
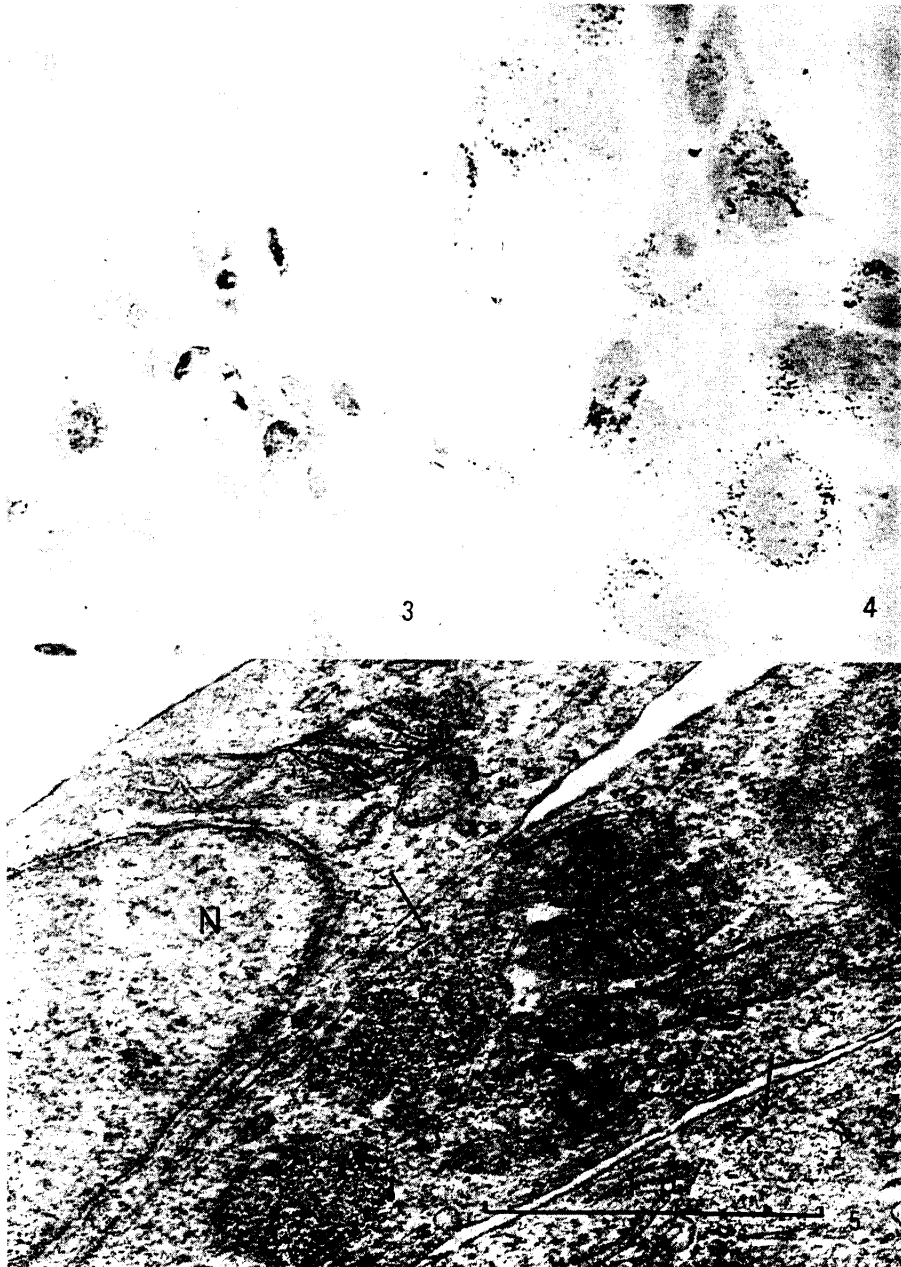
ing cultivation.   In transformants of human and hamster cells a massive accumulation of the colloid particles were seen in their phagocytic vacuoles (Fig. 5).

DISCUSSION

SV 40 transformed hamster fibroblasts (HE 12 and 13, Okayama) had the specific tumor antigen and surface antigen, and showed the biological characteristics of the malignant cell. Similar malignant transformation of fibroblasts was also induced by using pure DNA extracted from SV 40 (9, 10).   The present investigation demonstrated again that human cells were also transformed into malignant cell *in vitro* by SV 40 viral DNA just as in hamster cells.   These facts indicate that the oncogenicity of virus is in the viral DNA itself.   Presumablly the viral DNA is incorporated into the genome of the host cell and changes the cell characteristics.

Recently several reports showed that the DNA was responsible for oncogenicity (8, 13).

Just demonstrated, the cells transformed by viral SV 40 DNA and virus itself acquired the characteristic property of antigenicity in both cell membranes and nuclei. On the cell surfaces they had the surface antigen, and in the nucleus tumor antigen; these features are thought to be specific to the malignant cells.

It is also believed that the surface charge of the cell is altered by the malignant transformation, which should be closely correlated to the change in molecular architecture. AMBROSE (14) reported that as a result of malignant transformation the negative surface charge increased compared to non-malignant cells from which the tumor cell was derived.   The changes should proceed gradually, less negative in the early stage of tumor development and highly negative with the increase in malignancy.

Recently, SENO, YOKOMURA and co-workers (2, 3) have revealed that the phagocytic activity of the cell was related to the charge of the cell

Fig. 3 Human SV40 transformed cells (13 passage) cultivated with colloidal iron in culture media for 20 hrs. All transformants are completely free of iron particles. Perls' reaction and Kernechtrot stain.   10×20

Fig. 4 Human SV40 DNA transformed cells (10 passage) cultivated with colloidal iron and histones (100 μg/ml) in culture media for 24 hours. Transformants show a marked phagocytic activity containing numerous colloidal particles in their cytoplasm. Perls' reaction and Kernechtrot stain.   10×20

Fig. 5 Electron microscopy of human SV40 DNA transformants cultivated with colloidal iron and histones (100 μg/ml) for 24 hours. A massive accumulation of colloidal particles are seen in phagocytic vacuoles (P) M: Mitochondria N: Nucleus

surface, demonstrating that the adsorption of a particle to the cell surface was the first and essential step of phagocytosis. They believed the engulfing of the cytoplasm at the site of the adsorption of the particle should be formed by the local lysis of molecular archtecture as understood from a cooperative phenomena of high molecular physics. Thus they have demonstrated that Ehrlich ascites tumor cells, which do not phagocytize the negatively charged metal colloid particles, show a striking phagocytic activity of the colloidal particles in the presence of histone and other polycations. On the other hand, macrophages phagocytized the metal colloid particles very actively. They adsorbed colloid particles and took them by engulfing. But it was noted that each iron colloid particle adhered to the cell surface with a certain distance but not covering the whole surface area. Macrophage should have positively changed the specific groups at a certain distance on the cell surface to adsorb negatively charged colloid particles, though the charge of the cell was negative as a whole. Thus the differences in phagocytotic activity between macrophages and tumor cells directly indicate the difference in molecular structural arrangement in the surfaces of these two kinds of cells.

In the present experiments, a similar phenomenon as in macrophage and Ehrlich tumor cell was observed in normal diploid fibroblasts and their transformants induced by viral DNA and SV 40 itself. By 20 hours' or 24 hours' incubation with the iron colloid particles the fibroblast phagocytized the iron particles but the human and hamster transformant did not. In the presence of arginine-rich histone, 100 or 200 μg/ml, which gave no suppressing effect on cell growth, however, the transformants phagocytized the colloid particles very actively (Fig. 4). These facts indicate that definite changes occurred in the molecular structure of the cell surface by the transformation.

Such a phenomenon should be induced by the disappearance of the positively charged group or by the increase in negatively charged group, which may keep the negatively charged particles away, or both. The changes should be correlated to the changes in genetic information possibly induced viral DNA by incorporation into the host cell nuclei. Thus evidence has been presented to support the observation that phagocytic activity gives important information for the changes in charge of the cell membranes or their molecular architecture in connection with the malignant transformation.

SUMMARY

In vitro cell transformation of human embryonic cells could be induced by DNA extracted from virions of SV 40 purified by density gradient centrifugation. The result shows clearly that cell transformation is induced by incorporation of a part of viral DNA into the genome. In addition, for the purpose of clarifing the biological differences between the normal and transformant, the alteration of the cell membrane structures of transformants was observed from the mechanism of phagocytosis. The iron colloid particles are taken up by normal diploid fibroblasts but not by the human and hamster transformants. This fact suggests a difference in the molecular arrangement of the cell membranes between the normal and transformants. In the presence of histones, however, the transformants phagocytize the colloid particles very actively. The results show cell membranes of transformants are altered in the molecular structure responsible for the surface charge.

ACKNOWLEDGEMENT

REFERENCES

1. ABERCROMBIE, M.: Contact-dependent behavior of normal cells and the possible significance of surface changes in virus-induced transformation. Cold Spring Harbor Symposia on Quantitative Biology. Vol. XXVII. 427—431, 1962
2. YOKOMURA, E., SENO, S., SOGABE, K., NAKATSUKA, A. and KUBO, T.: Studies on the mechanism of phagocytosis. I. Effect of metabolic inhibitors on the phagocytosis of iron colloid particles by ascites macrophages. *Acta. Med. Okayama* 29, 93, 1967
3. SENO, S., YOKOMURA, E., KIMOTO, T., SOGABE, K. and ITOH, N.: Uptake of Metal Colloid Particles by Ehrlich Ascites Tumor Cell Induced by Histone. Proc. Int. Haemorheology. ed. Copley. Pergamon Press. Oxford & New York. 565—568, 1968
4. YOSHIIKE, K.: Studies on DNA from low-density particles of SV 40. I. Heterogeneous defective virions produced by successive undiluted passages. *Virology* 34, 391, 1968
5. YOSHIIKE, K: Studies on DNA from low-density particles of SV 40. II. Noninfection virions associated with a large plaque variant. *Virology* 34, 402, 1968
6. ITO, H., MORIMOTO, Y., DOI, Y. and SANPE, T.: Viral susceptibility of an African green monkey kidney cell line-Vero. *Virus* (The Society of Japanese Virologists) 18, 214, 1968
7. HSU, J. C. and KELLOGY, D. S.: Primary cultivation and continuous propagation *in Vitro* of tissues from small biopsy. *J. Nat. Cancer. Inst.* 25, 221, 1960
8. UCHIDA, S. and WATANABE, S.: Tumorgenicity of the antigen-forming defective virions of simian virus 40. *Virology* 35, 166, 1968
9. KIMOTO, T. and GRACE, J. T.: Oncogenic properties of nucleic acid derived from SV 40 virus. *Acta Med. Okayama* 20, 1, 1966

86   T. Kimoto, E. Yokomura, Y. Shimizu, M. Yamakawa and S. Seno

10. Kimoto, T. and Grace, J. T.: Oncogenic Properties of DNA Derived from SV 40. Nucleic Acid Metabolism Cell Differentiation and Cancer Growth. Ed. E. V. Cowdry & Seno, S. Pergamon Press. Oxford & New York. 421—431, 1969

11. Johns, E. W.: Studies on histones: Preparative methods for histone fractions from calf thymus. *Biochem. J.* **92**, 55, 1964

12. Ui, N.: Preparation, fractionation and properties of calf thymus histone. *Biochim, Biophys. Acta* **25**, 439, 1959

13. Green, M. and Fujinaga, K.: Cenetic Relatedness of Viral Specific RNAs in Tumor Cells Induced by Highly and Weakly Oncogenic Human Adenoviruses. Subviral Carcinogenesis. Ed. Ito, Y. Ist Int. Symposium on Tumor Viruses. Nagoya, 82—95, 1966

14. Ambrose, E. J.: The Surface Properties of Tumor Cells. The Biology of Cancer. Ed. Ambrose, E. J and Roe, F. J. C. Van Nostrand. 65—77, 1966