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Alterations in surface glycoproteins and level of sialyltransferase of cells transformed by a temperature-sensitive mutant of simian virus 40

(gene *A* mutant/growth regulation)

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ABSTRACT **Mouse cells transformed by a temperature-sensitive mutant of simian virus 40 belonging to complementation group *A* lost their ability to regulate cell growth when grown at the permissive temperature (35°) but showed the low saturation density of cell growth at the restrictive temperature (39.5°) that is characteristic of normal cells *in vitro*. Biochemical analysis of the membranes of cells grown under the restrictive and the permissive conditions demonstrated no qualitative temperature-dependent differences either in neutral glycolipids or in acidic glycolipids of the cells. Plasma membrane glycoproteins labeled with radioactive glucosamine showed significantly different patterns on both polyacrylamide gel electrophoresis and electrofocusing. When the levels of glycoprotein glycosyltransferases of the cells were examined, the level of sialyltransferase (CMP-*N*-acetylneuraminate:D-galactosyl-glycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1) of the cells grown at the restrictive temperature was low compared with that of cells grown at the permissive temperature. Our results indicate that the level of sialyltransferase is under the control of the gene *A* function of simian virus 40 and consequently is related to alterations in the cell surface glycoproteins.**

Biochemical analysis of virus-transformed cells and normal cells has led many to consider that the cell surface plays an important role in the regulation of cell growth (1–6).

We cannot, however, answer the following two basic questions at the present moment. First, how is the gene function of the virus involved in alterations of the structure and function of the plasma membrane, and, second, by what mechanism do the alterations in cell surface lead to the loss of cell growth regulation *in vitro*.

In recent years, there has been an avid search for temperature-sensitive (ts) mutants of tumor viruses that induce transformation under certain conditions. Successful isolations of the mutants have been reported and have provided an intriguing system enabling correlation between gene functions and transformation on the molecular level (for a review, see ref. 7).

We have isolated several clones of mouse cells transformed by a ts *A* mutant of simian virus 40 (SV40) at the permissive temperature and have compared their growth patterns at the permissive and the restrictive temperatures. The clones lost cell growth regulatory functions at the permissive temperature, whereas they reverted and appeared to possess the growth characteristics of normal cells at the restrictive temperature. Other investigators have reported similar results (8–12).

Furthermore, Brugge and Butel (10) showed that cells transformed by the ts *A* mutants exhibited the phenotype of untransformed cells at the restrictive temperature (e.g., the uptake of 2-deoxyglucose and the expression of cell surface antigen) in contrast with that of transformed cells at the permissive temperature. This finding suggests that the gene *A* function of SV40 may lead to the biochemical alterations of the cell surface.

We wish to report that the changes in glycoproteins of the plasma membrane and the level of sialyl transferase are temperature dependent in cells transformed by the ts *A* mutant.

Abbreviations: ts, temperature-sensitive; SV40, simian virus 40.

MATERIALS AND METHODS

Virus and Cells. The wild-type virus was derived from the small plaque 777 strain of SV40 by cloning and a mutant, ts*A*900, was derived from the wild-type virus after mutagenesis with UV irradiation (13). The mutant was classified into the *A* complementation group using ts*A*58, ts*B*1, and ts*B*11 as references. (The reference mutants were kindly provided by P. Tegtmeyer.) A clonal line of C3H-2K mouse cells (14), C3H-2K-C4, was established at 39.5°. The W-2K-11 line was a clonal cell line transformed by the wild-type SV40 and the 900-2K-34 line was a line transformed by the ts*A*900. Each cell line was derived from a focus produced on a monolayer of the C3H-2K-C4 cells at 35° by the method described previously (13) and recloned from a colony in soft agar medium (15). Virus rescued from each transformed cell line showed the same temperature sensitivity in terms of viral replication as the virus used for the transformation.

Radioisotopic Labeling of Glycolipids. 900-2K-34 cells and W-2K-11 cells were inoculated at a cell density of 2.5×10^4 cells per cm^2, cultured in Eagle's minimum essential medium supplemented with 10% calf serum and D-[^{14}C]galactose, 0.4 μCi/ml (specific activity 60 mCi/mmol) and harvested 4 days later.

Preparation of Glycolipids. Cells labeled with [^{14}C]galactose were extracted with $CHCl_3$:CH_3OH (2:1, vol/vol). The lipid fraction was separated into neutral and acidic glycolipids as described below. Thin-layer chromatography of the neutral lipids and that of acidic glycolipids was carried out by one-time development with $CHCl_3$:CH_3OH:H_2O (60:40:9). Ceramide dihexoside, ceramide trihexoside, globoside, and Forssman glycolipid were used as authentic samples for the neutral lipids. Ganglioside GM_3 and ganglioside GM_1 were used for the acidic glycolipids. These authentic compounds were visualized with iodine vapor. These compounds were a gift from M. Naiki. Radioautograms were made using Kodak RP-14 x-ray film.

Differential Labeling of the Cells. The cells were cultivated with [^{14}C]glucosamine (specific activity, 50 mCi/mmol, 0.04 μCi/ml) at 35° and with [^{3}H]glucosamine (specific activity, 13.4 Ci/mmol, 1 μCi/ml) at 39.5° for 4 days.

Preparation of Plasma Membrane. Plasma membrane was prepared according to the method of Brunette and Till (16). Successful application of this method to the cells used in this experiment was reported previously (17).

Solubilization of Plasma Membrane. Fresh or frozen plasma membrane was dissolved in 10 mM Tris·HCl buffer (pH 8.0) containing 1% sodium dodecyl sulfate, 10% sucrose, 1 mM EDTA, and 1% mercaptoethanol with boiling for 2 min. The dissolved materials were applied in a 100–200 μl aliquot to a column of polyacrylamide gel. The plasma membranes for the analysis by electrofocusing were dissolved in the same buffer used for polyacrylamide gels. The solubilized membranes were dialyzed against 10 mM Tris·HCl buffer (pH 8.0) containing 1% Triton X-100.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. The electrophoresis was carried out by the method of Fairbanks *et al.* (18). Human immunoglobulin H chain (molecular weight: 160,000) and cytochrome *c* (molecular weight: 11,700) were used as markers for molecular weight estimation.

Electrofocusing. Ampholine, pH 3.5–5.0, was purchased from LKB Inc., Rockville, Md. and the electrophoresis was carried out for 72 hr at 300 V. The pH gradient was determined by measuring the pH of each fraction. The system for electrofocusing contained 1% Triton X-100 to keep membrane proteins solubilized.

Acceptors of Glycosyltransferase. Desialized fetuin prepared by the method of Grimes (19) was used as an acceptor for sialyltransferase (CMP-*N*-acetylneuraminate:D-galactosylglycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1). Pancreatic RNase was used for *N*-acetylglucosaminyl transferase (20).

Reaction Mixture. The complete enzyme system contained the following in a volume of 0.15 ml: 10 μl of 0.1 M $MnCl_2$, 10 μl of 0.1 M $MgCl_2$, 20 μl of 0.1 M phosphate-buffered saline (pH 7.2) or exogenous acceptors (450 μg of protein), 10 μl of radioactive nucleotide sugars, and 200 μg of microsomal protein. Desialized fetuin and pancreatic RNase were used as acceptor molecules for sialyl and *N*-acetylglucosaminyl transferase, respectively. The reaction mixture was incubated at 35° for 60 min and then 0.1 ml of cold 1% phosphotungstic acid in 0.5 M HCl was added and the precipitate was dissolved in ammonium hydroxide solution and radioactivity was determined in a liquid scintillation counter using dioxane scintillator. Details were described previously (17).

Counting of Double-Labeled Compounds. Each fraction from electrofocusing or gel electrophoresis was oxidized by Packard Tri-Carb sample oxidizer and ^3H and ^{14}C were recovered as ^3H_2O and $^{14}CO_2$, respectively, and their radioactivity was measured.

RESULTS AND DISCUSSION

Saturation Density. Typical growth patterns of ts-mutant-transformed cells and the wild-type-transformed cells at the permissive (35°) and the restrictive (39.5) temperature are shown in Fig. 1. At the restrictive temperature, the ts-mutant-transformed cells showed a lower saturation density than that at the permissive temperature. The wild-type virus-transformed cells did not show this phenomenon. Several clones of the ts-mutant-transformed cells were examined for their growth at both temperatures. The ratios of saturation density at the permissive temperature to that at the restrictive temperature varied from 3.7 to 6.7. The results suggested that gene-*A*-mutant transformed C3H-2K cells are unable to maintain the transformed phenotype at temperatures that do not allow the expression of the *A* gene.

Thin-Layer Chromatography of Glycolipids. It is generally accepted that the incomplete elongations of the sugar moiety of glycolipids occurred in the majority of virus-transformed cells

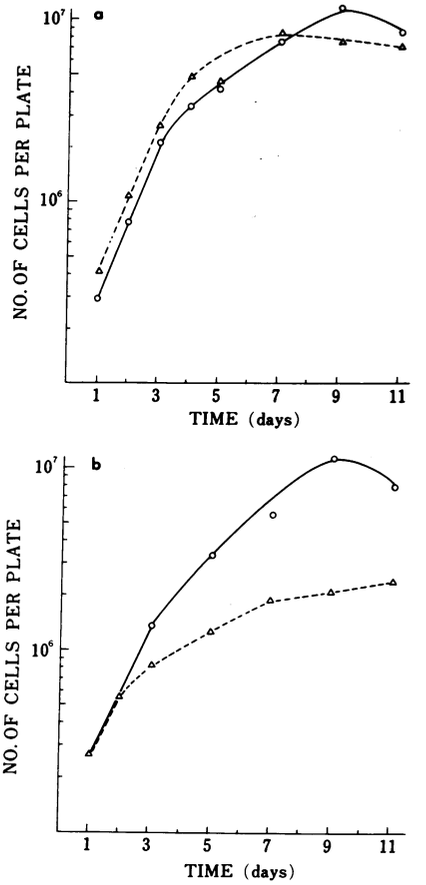

FIG. 1. Growth curves of the transformed cells plated on plastic dishes (Lux; 60 mm) at the permissive and the restrictive temperatures. (a) W-2K-11 cells, (b) 900-2K-34 cells. The growth medium was changed every day. O — O, at 35°, Δ - - - Δ, at 39.5°.

(for a review, see ref. 7). Therefore the glycolipid components of the cells grown either at the permissive or at the restrictive temperature were compared. Cells were cultured with [^{14}C] galactose for 4 days at either 35° or 39.5° and harvested. Glycolipids were extracted and isolated according to the method of Saito and Hakomori (21) and fractionated with DEAE-Sephadex according to Leeden *et al.* (22). Cells used in this experiment contained ceramide mono-, di-, and trihexosides as major constituents. Gangliosides GM_3 and GM_2 were the components of the acidic glycolipids (Fig. 2). The glycolipid components, however, were the same at both temperatures.

Polyacrylamide Gel Electrophoresis of Membrane Glycoproteins. Cells cultured with [^3H]glucosamine at the restrictive temperature and with [^{14}C]glucosamine at the permissive temperature for 4 days were harvested and plasma membrane was prepared by the method of Brunette and Till (16). Plasma membrane was solubilized as mentioned in *Materials and Methods* and analyzed on polyacrylamide gel electrophoresis. The ts-mutant-transformed cells showed significant temperature-dependent differences in electrophoretic patterns, while the wild-type-virus-transformed cells grown at the two temperatures showed very similar patterns (Fig. 3). Peaks (fraction nos. 16 and 21) in the membrane from the mutant-transformed cells grown at 39.5° were not observed in the membrane from cells grown at 35°. The electrophoretic patterns of ts-mutant-transformed cells at the nonpermissive temperature were similar to those of normal cells but not exactly the same (data not shown).

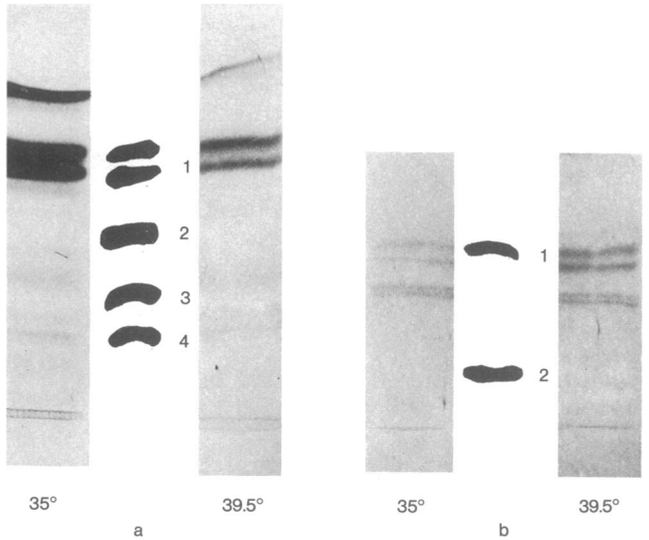

FIG. 2. Thin-layer chromatography of glycolipids. (a) Neutral glycolipids; authentic samples 1, 2, 3, and 4 are ceramide di- and trihexoside, globoside, and Forssman antigen, respectively. The band at the top is ceramide monohexoside. (b) Acid glycolipids; 1 and 2 are gangliosides GM_3 and GM_1.

On the other hand, the electrophoresis profiles of the membrane proteins stained with Coomassie brilliant blue were essentially the same at both temperatures (data not shown). The results indicate strongly that the sugar moieties of glycoproteins were altered in the ts-mutant-transformed cells at the specific temperature.

Electrofocusing of Membrane Glycoproteins. The differences in the glycoproteins from the ts-mutant-transformed cells were confirmed by electrofocusing. Most of the membrane glycoproteins were recovered in the pH range between pH 3.0 and pH 5.0 (data not shown). Therefore the double-labeled membrane glycoproteins were compared in Ampholine, pH 3.5–5.0. There were five major peaks; peaks I, IV, and V were common to the cells grown at both temperatures. Peak II, however, was not observed in the cells grown at 35° and peak III profiles were clearly different (Fig. 4).

Changes in the Levels of Glycoprotein Glycosyltransferase. In the virus-transformed cells so far examined, the alterations in glycoproteins have been related to the changes in the levels of glycoprotein glycosyltransferases (17, 19, 23–25). The results described above prompted us to examine the possibility of related changes in the levels of glycosyltransferases. The basic requirements for the enzyme reaction and linearity of the reaction at increasing protein concentrations have already been reported (17). In addition to the conditions for the enzyme assay as described previously, a few conditions were investigated: (*a*) temperature: the glycosyltransferases were more active at 35° than 39.5°; (*b*) effect of detergent: the activity of membrane-bound enzymes (e.g., glycosyltransferase) has been reported to be influenced by the addition of detergent (26) and the addition of Triton X-100 caused a reduction in activity in our case; (*c*) stability of enzymes: since the glycosyltransferase activity, especially that of sialyltransferase in the microsomes, decayed very rapidly at −20°, the measurement was carried out with fresh microsomes.

Therefore the levels of glycosyltransferase in cells grown at either 35° or 39.5° were compared under these conditions. The ratios of sialyltransferase activity in the cells grown at 35° to that at 39.5° are shown in Table 1. The ratios in the ts-mutant-transformed cells were 2.0 and 2.7 when measured in the

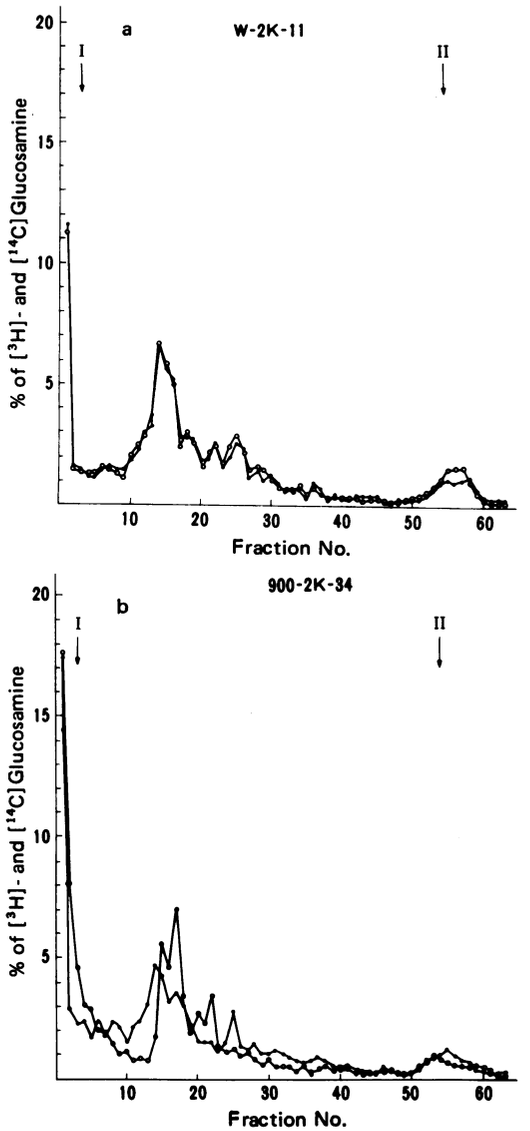

FIG. 3 Polyacrylamide gel electrophoresis patterns of the differentially labeled membrane proteins. I, Human immunoglobulin H chain; II, cytochrome *c*. 5,000 cpm each of ^3H and ^{14}C were charged on the gel. ○, [^3H]Glucosamine-labeled glycoprotein at 39.5°; ●, [^{14}C]glucosamine-labeled glycoprotein at 35°.

presence or absence of the acceptor molecules, respectively. However, the ratios in the wild-type-virus-transformed cells were 1.2 and 1.8 in Table 1, respectively. The true activity of sialyltransferase, in the ts-mutant-transformed cells, (a) − (b) value in Table 1, was 70% higher in the cells grown at 35° than that at 39.5°. That in the wild-type-virus-transformed cells was the same at both temperatures. Since the levels of *N*-acetylglucosaminyl- and mannosyltransferases, both in the ts-mutant-transformed and in the wild-type-virus-transformed cells, were temperature dependent (Table 1), only the change in the level of sialyltransferase seems to be due to an SV40 mutation.

To find whether the sialyltransferases in the cells grown at the two temperatures are qualitatively different, the K_m value for CMP-sialic acid was measured. The K_m value of sialyltransferase for CMP-sialic acid was found to be 23.8 μM in the cells grown at either temperature (Fig. 5). This value is comparable to that in rat liver sialyltransferase as reported pre-

Table 1. Levels of glycosyltransferases in the cells grown at 35° and 39.5°

Type of glycosyltransferase	900-2K-34 cells			W-2K-11 cells		
	35°	39.5°	Ratio 35°/39.5°	35°	39.5°	Ratio 35°/39.5°
Sialyl transferase (a) + acceptor	19,580	9,950	2.0	10,550	9,070	1.2
(b) − acceptor	7,830	2,960	2.7	3,670	1,990	1.8
(a) − (b)	11,750	6,990	1.7	6,880	7,080	1.0
N-Acetyl-glucosaminyltransferase*	65,170	44,130	1.5	33,210	17,080	1.9
Mannosyltransferase†	11,550	4,160	2.8	13,770	5,770	2.4

Enzyme activities are expressed as cpm of hexose transferred/mg of protein per hr.
* Activity was measured in the presence of an acceptor.
† Activity was measured in the absence of an acceptor.

viously by Bernacki and Bosmann (27). It is not clear, however, whether the difference in the level of sialyl transferase was due to the amounts of the enzyme or the presence of an inhibitor-like substance.

It is worth noting that the level of sialyltransferase in the wild-type-virus-transformed C3H-2K cells is 70% lower than that in the corresponding normal cells and the levels of mannosyl-, glucosyl-, galactosyl-, and fucosyltransferases are not altered as the result of transformation (17). Therefore, it was expected that the level of sialyltransferase in the cells grown at 39.5° in the ts-mutant-transformed cells would revert to the same level as in the normal C3H-2K cells. The results, however, indicated that the cells appeared normal with respect to the saturation density but the level of sialyltransferase did not revert to that of normal cells at the restrictive temperature. It is conceivable that many alterations observed in the virus-transformed cells as compared with the normal cells are the result of the integration of SV40 genome in the host chromosome and are not necessarily due to the genetic defect associated with the transformation.

Since the level of the enzyme in the ts-mutant-transformed cells, but not in the wild-type-virus-transformed cells, was altered depending upon the temperature (Table 1), it is likely that the level of sialyltransferase is under the control of the gene *A* functions of SV40 in the SV40-transformed cells directly or indirectly. It is conceivable that the ts gene product is altered in such a way that it does not function normally at the restrictive temperature, thereby leading to the suppression of sialyltransferase by an unknown mechanism. This alteration in the level of sialyl transferase may result in the observed alteration in membrane glycoproteins. The levels of sialyltransferase in the majority of the virus-transformed cells are reflected in the amount of sialic acid in the membranes (25).

Therefore there may be a link between sialic acid content and the transformed phenotype in the ts-mutant-transformed cells. It is well known that the presence or absence of sialic acid on the surface glycoproteins affects to a considerable extent the properties of the cell surface membrane such as antigenicity (28–32) and fluidity (33–35).

Since the glycolipids remained at the same level at either temperature, it appears that the glycoproteins are primarily involved in the observed temperature-dependent changes. It is therefore very possible that some alterations in the functional organization of surface membranes in the ts-mutant-transformed cells can be ascribed to the presence or absence of sialic acid in the surface glycoproteins.

This paper and others have demonstrated that the gene *A* function is involved in the maintenance of the transformed phenotype (8–12). The pleiotropic function proposed for gene *A* of SV40 is very attractive (36, 37). However, the proposal cannot explain all the properties of the ts-mutant-transformed cells in the restrictive condition and the function of the gene *A* products still remains to be clarified in the transformed cells.

On the other hand, the selected markers used for the char-

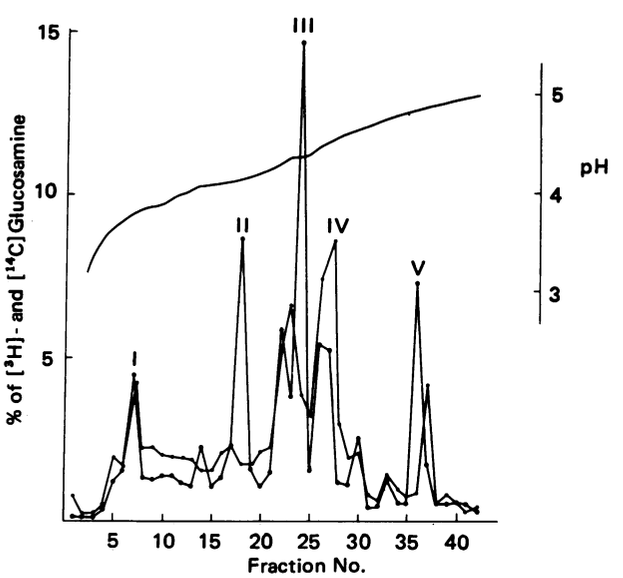

FIG. 4. Electrofocusing patterns of the differentially labeled membrane proteins from 900-2K-34 cells. ○, [³H]Glucosamine-labeled glycoprotein at 39.5°. ●, [¹⁴C]glucosamine-labeled glycoprotein at 35°. 1.2 × 10⁵ cpm each of ³H and ¹⁴C were charged into the tube.

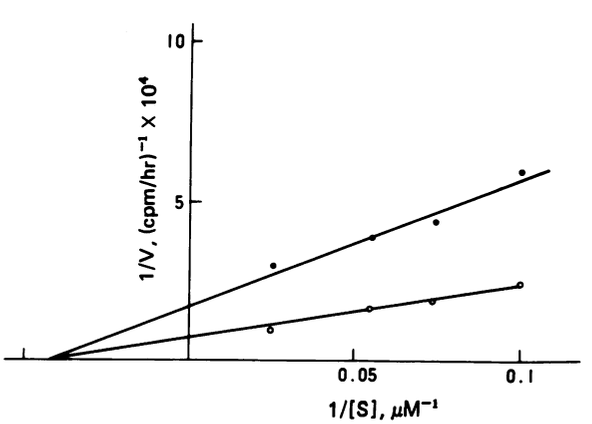

FIG. 5. K_m value of sialyltransferase for CMP-sialic acid. Sialyltransferase of 900-2K-34 cells grown at either 35° (○) or 39.5° (●).

acterization or the selection of the transformed cells seemed to be related to the functional organization of cell membrane, e.g., the loss of density-dependent growth regulation, cell growth in soft agar medium, agglutinability by plant lectin, and serum requirement for cell growth. Taking the properties of the revertant of the virus-transformed cells (38, 39), and those of the ts-mutant-transformed cells in the restrictive condition into consideration, it is conceivable that the complementation of cell membrane function with respect to cell growth regulation may occur on the structural level at the cell surface, even though the viral genome remains to be integrated at the chromosome sites.

Therefore we feel that the gene *A* product affects directly or indirectly the level of glycosyltransferase. The altered level of this enzyme system may then lead to the alteration of surface glycoproteins, the function of which may be critical for the cell growth regulation. The mechanism is not clear at his moment.

The changes in the level of glycosyltransferases in the ts-mutant-transformed cells by Rous sarcoma virus were also temperature dependent (40). The membrane biology of this system has been described but the basic questions as outlined in the introduction still remain to be answered (41).

The functional organization of the cell surface membrane in the ts-mutant-transformed cells seems to be involved in the expression of the transformed phenotype. The study of this functional organization is expected to shed some light on the involvement of the cell membrane in the regulation of cell growth.

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